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Extreme radio-resistance of the bdelloid rotifer Adineta vaga

Investigating DNA repair using NHEJ/HR inhibitors and protective effect of lysates on human A549 cells

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Faculté de Médecine

EXTREME RADIO-RESISTANCE OF THE BDELLOID ROTIFER *ADINETA VAGA*: INVESTIGATING DNA REPAIR USING NHEJ/HR INHIBITORS AND PROTECTIVE EFFECT OF LYSATES ON HUMAN A549 CELLS

Mémoire présenté pour l'obtention

du grade académique de master en sciences biomédicales

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Studying extreme radio-resistance of the bdelloid rotifer *Adineta vaga*: Investigating DNA repair using NHEJ/HR inhibitors and protective effect of lysates on human A549 cells

PETIT Laura

Abstract

Background. Bdelloid rotifers are microscopic aquatic invertebrates present ubiquitously. Since their discovery, they have been considered an evolutionary scandal because of their strict parthenogenetic mode of reproduction and their ability to enter dormancy at any stage of their life-cycle, both uncommon features in the metazoan kingdom. Bdelloid rotifers are able to withstand high doses of ionizing radiation and frequent desiccation events, which lead to initiation of free radical based reactions and a consequent DNA damage. Such chemical insults are usually cytotoxic but bdelloid rotifers possess efficient protective mechanisms and notably, an antioxidant arsenal to protect themselves.
Aims. Two aspects of bdelloid rotifers were investigated in this work: DNA repair pathways occurring after a desiccation or irradiation episode and secondly, the potential protective effect of bdelloid rotifer lysate supplementation on irradiated human A549 cells.
Materials and methods. To better understand the DNA repair pathways, different specific inhibitors of key actors (B02, DDRI-18, Mirin and SCR7) involved in DNA DSBs repair were added in the culture media. Biological impact on the rotifer's ability to recreate a population was assessed by following the population dynamics with time as well as their ability to repair a broken genome by Pulsed-Field Gel Electrophoresis (PFGE). During the second part, human A549 cells were cultured with bdelloid rotifer lysate prior to X-ray exposure. Cell viability was assessed through Trypan Blue assay and cell proliferation through colony-formation assay.
Results. By combining fertility results and repair results, bdelloid rotifers are impaired in their reproduction but no evidence show that inhibitors really reach their target and act precisely at this level. Moreover, rotifer lysate applied on human cells did not show consistent results concerning cell viability and proliferation after X-rays exposure.
Conclusion. DNA repair inhibitors experiments do not allow to clarify the DNA repair pathways occurring in bdelloid rotifers. However, they could constitute a good tool to understand DNA repair pathways by adopting *in vitro* approaches. Secondly, the protective effect of rotifer lysate on human cells could neither be assessed but the protocol should be improved.

Keywords

Bdelloid rotifers, DNA repair, DNA repair inhibitors, human A549 non-small cell lung cancer cells, oxidative stress.

Mémoire de master en sciences biomédicales
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Thesis Supervisors: Karine Van Doninck / Boris Hespeels, Rohan Arora

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Finally, thanks to my parents and my family for their strong support and their trust in me. I certainly would not have reached the end without them.

Table of contents

List of abbreviations

aa	Amino acid
A. vaga	*Adineta vaga*
C. elegans	*Caenorhabditis elegans*
D. radiodurans	*Deinococcus radiodurans*
DNA	Deoxyribonucleic acid
DNA-PKcs	DNA-Protein Kinase catalytic subunit
DSB	Double Strand Break
Fe	Iron
GLM	Generalized Linear Model
Gy	Gray
HGT	Horizontal Gene Transfer
HR	Homologous Recombination
HSP	Heat Shock Proteins
ICP-MS	Inductively Coupled Plasma-Mass Spectrometry
IR	Ionizing Radiation
LEA	Late Embryogenesis Abundant
L. plantarum	*Lactobacillus plantarum*
M	Molar
ml	Milliliter
mM	Millimolar
mm	Millimeter
Mn	Manganese
NHEJ	Non-homologous end-joining
nm	Nanometer
PBS	Phosphate Buffer Saline
PFGE	Pulsed-Field Gel Electrophoresis
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic acid

ROS	Reactive Oxygen Specie
S. cerevisiae	*Saccharomyces cerevisae*
TB	Trypan Blue
TSP	Trehalose-6-Phosphate Synthase
µl	Microliter
µM	Micromolar
µmol	Micromole
WB	Western Blot

1. Introduction

"Sleeping beauties", "evolutionary scandals", "single ladies, etc., different nicknames to designate the same microscopic aquatic animal: the bdelloid rotifer. Since their discovery in 1702 by Antony Van Leeuwenhoek, these animals have never ceased to amaze scientists: their mode of reproduction and their resistance to multiple stresses unbearable for most organisms make them particularly intriguing to study [Tunnacliffe A. and Lapinski J., 2003]. Despite all the technologies currently available, they still remain largely misunderstood.

The *Rotifera* phylum

The *Rotifera* phylum (from Latin *rota* "wheel" and *-fer* "bearing") is divided into 3 classes: the *Seisonidea*, the *Monogononta* and the *Bdelloidea* [Segers H., 2007]. All are characterized by the presence of an apical ciliated organ called the corona, allowing them to swim and to filter their environment for feeding. However, they diverge at different levels: the *Seisonidea* live in marine water where they reproduce sexually and only 3 species have been described, whereas *Monogonota* is a species-rich clade living in marine and freshwater environments, being part of the zooplankton and showing facultative parthenogenesis. *Bdelloidea* live in freshwater semi-terrestrial habitats and appear to be obligate asexual [Hespeels B., 2016; Fontaneto D. and De Smet W.H., 2015].

The *Bdelloidea* class

Bdelloid rotifers are invertebrates measuring less than one millimeter (some species, as *Rotaria neptunia*, can reach two millimeters but remain exceptions), which makes them one of the smallest animals on Earth [Örstan A. and Plewka M., 2017]. Their body is divided into three regions (Figure 1): the head bearing the corona, the trunk and the foot ending with toes and adhesive glands allowing their fixation to substrates. Despite their apparent morphological simplicity, bdelloids possess complete systems: nervous, muscular, digestive and excretory systems as well as complex paired gonads [Ricci C. and Fontaneto D., 2009].

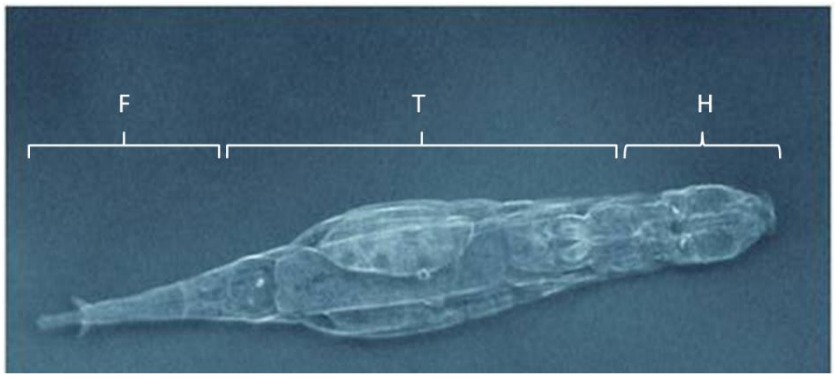


Figure 1: Picture of Adineta vaga. H, head; T, trunk; F, foot. Given by Hespeels B.

These gonads are composed of oocytes and nurse cells. Most bdelloids lay unfertilized eggs in a one-cell stage that then goes through mitotic divisions to reach approximately 1,000 cells after two days at room temperature [Hespeels B., 2016]. The development is then completed and the eggs hatch. Juveniles will then grow into adults without any new cell divisions,

bdelloid rotifers are eutelic [Ricci C. and Fontaneto D., 2009]. A few species are viviparous and give birth to juveniles directly [Hespeels B., 2016]. Their clonal mode of reproduction is parthenogenetic since new females develop from a gamete without fertilization. Whether the final gamete is formed mitotically or through a modified meiosis remains unknown. Other evidence of their asexuality is the absence of males, vestigial male structures or hermaphrodites [Fontaneto D. and De Smet W.H., 2015]. Fossil evidence suggests that they are evolving in this trend for at least 60 million years [Tang C.Q. *et al*, 2014].

The *Adineta vaga* species

Adineta vaga, the study model of this work, is around 200 micrometers (µm) long. In contrast with the other genera, the *Adineta* individuals possess a corona disposed ventrally. Below the corona, the mouth scrapes the surface to find bacteria, algae, etc. to feed on. At 20°C, they live 16.2 days on average and lay 21.26 eggs per individual lifetime [Hespeels B., 2016; Latta L. *et al*, 2019].

Single ladies, an evolutionary scandal

As mentioned previously, bdelloid rotifers appear to reproduce asexually for millions of years [Tang C.Q. *et al*, 2014]. In 2013, Flot J.-F. *et al* published the first bdelloid rotifer genome, from *A. vaga*, confirming that the genome structure seemed incompatible with conventional meiosis. The pairing of homologous chromosomes, a key step of meiosis, cannot be achieved given that allelic pairs were rearranged with a loss of colinearity. However, recent analyses using long read sequencing did not highlight the same atypical feature, instead, 6 pairs of homologous chromosomes were discovered. Research in the LEGE laboratory is finalizing the assembly to shed light on this enigmatic genome and on their reproductive mode [Narayan J. *et al*, in prep].

Bdelloid rotifers have diversified into more than 460 morphospecies asexually. Since asexual reproduction is prone to deleterious mutation accumulation among others, bdelloid rotifers were referred to as an evolutionary scandal [Segers H., 2007; Hespeels B., 2016]. On the other hand, sexual reproduction intermixes allelic pools from distinct individuals, thereby yielding heterogenous genotypes which do not experience the negative effect of mutational accumulation [Danchin E. *et al*, 2010].

Scientists have therefore wondered “how have bdelloids escaped the negative effects of their asexual reproduction?’’ [Ricci C., 2017]. One of the possible mechanisms highlights horizontal gene transfers (HGT) where genes and alleles are shared among different organisms and/or conspecifics. Currently, 8 to 10% of the *A. vaga* genome is composed of genes of putative non-metazoan origin: the HGT genes acquired appear to be from bacteria, fungi and plants. The possibility that bdelloid individuals exchange genetic material is thus probable but the exact mechanisms are uncertain [Hespeels B., 2016; Debortoli N. *et al*, 2016; Ricci C., 2017]. Secondly, gene conversion phenomenon can also prevent accumulation of deleterious mutations by overwriting a deleterious allele by a beneficial or neutral one, when homologous recombination is performed with the homologous chromosome as a template, rather than the sister chromatid. This is however a double-edged mechanism where mutations can be removed but also added to the chromosome, impacting the fitness of the individuals in one direction or the other. Abundant evidences of gene conversion were observed in the genome of *A. vaga* [Flot J.-F. *et al*, 2013].

Sleeping beauties (in their own manner)

Most bdelloids inhabit semi-terrestrial environments such as ponds, interstices of soil and around the moist environment surrounding mosses and lichens. These habitats are unpredictable and submitted to frequent drying events. In the absence of water, bdelloid rotifers contract their muscles, retract their head and foot into the trunk to adopt the typical compact "tun" shape leading to a volume decrease of 60%, having entered dormancy [Ricci C. and Fontaneto D., 2009]. In the tun state, rotifers have the capacity to survive harsh periods of dryness, while being easily dispersed by the wind to colonize hydrated habitats [Wilson C. and Sherman P., 2010].

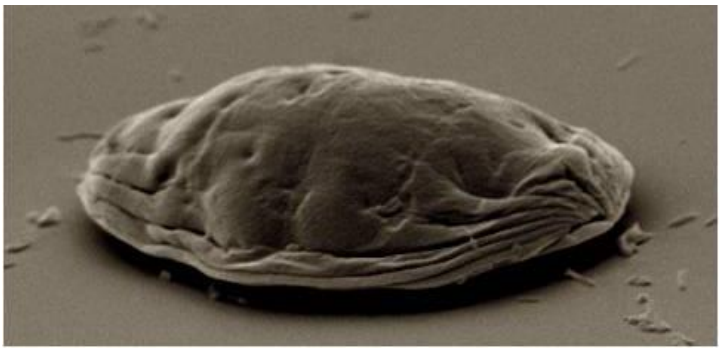


Figure 2: The typical "tun" shape adopted by bdelloid rotifers in absence of water. Given by Hespeels B.

Their capacity to enter dormancy and to suspend their ageing at any stage of their life when submitted to desiccation (also called anhydrobiosis) is the origin of their nickname "sleeping beauties". Indeed, the time spent in a desiccated state does not matter, this is a pause in their lifespan. Desiccation means a decrease in their cellular water content of 90% or more. The percentage of water remaining in the body is so low that the monolayer usually present around the macromolecules disappears, preventing enzymatic reactions and metabolism as confirmed by calorimetry approach [Alpert P., 2005; Ricci C., 2017]. Desiccation must not be confused with drought tolerance which is "a reduction in water availability in the environment for long or short time" [Alpert P., 2005]. If drought tolerance is widespread among living organisms, desiccation tolerance is quite rare in the metazoans and is a characteristic of only few metazoans such as tardigrades, a few nematodes species and bdelloid rotifers [Hespeels B., 2016]. Once water becomes available in their environment, rotifers resume their life-cycle: they rehydrate, regain their elongated form and start moving, feeding and reproducing [Ricci C. and Fontaneto D., 2009]. Until now, the longest desiccation period that they survived was 9 years [Guidetti R. and Jönsson K., 2002].

If bdelloid rotifers are able to resume their life-cycle after a desiccation episode, their biological processes are nevertheless impacted. Dehydration of the cells increases the oxidative state of cells, triggering the production of reactive oxygen species (ROS) creating an oxidative stress [França M.B. *et al*, 2007]. ROS are known to have a critical impact on intracellular macromolecules, notably leading to a loss of genomic integrity by the creation of multiple DNA double-strand breaks (DSB), one of the most cytotoxic damages for cells. As shown by Hespeels B. *et al* in 2014, desiccation impacts genomic integrity, probably through ROS production. Indeed, the genomic profiles of desiccated *A. vaga* were analyzed by Pulsed-Field Gel Electrophoresis (PFGE). The increase in DNA DSBs was observed after seven days spent in a desiccated state and accumulated with time (see Figure 3a). Interestingly, this

damage reduced gradually upon rehydration, suggesting an efficient DNA repair mechanism (see Figure 3b) [Hespeels B. *et al*, 2014].

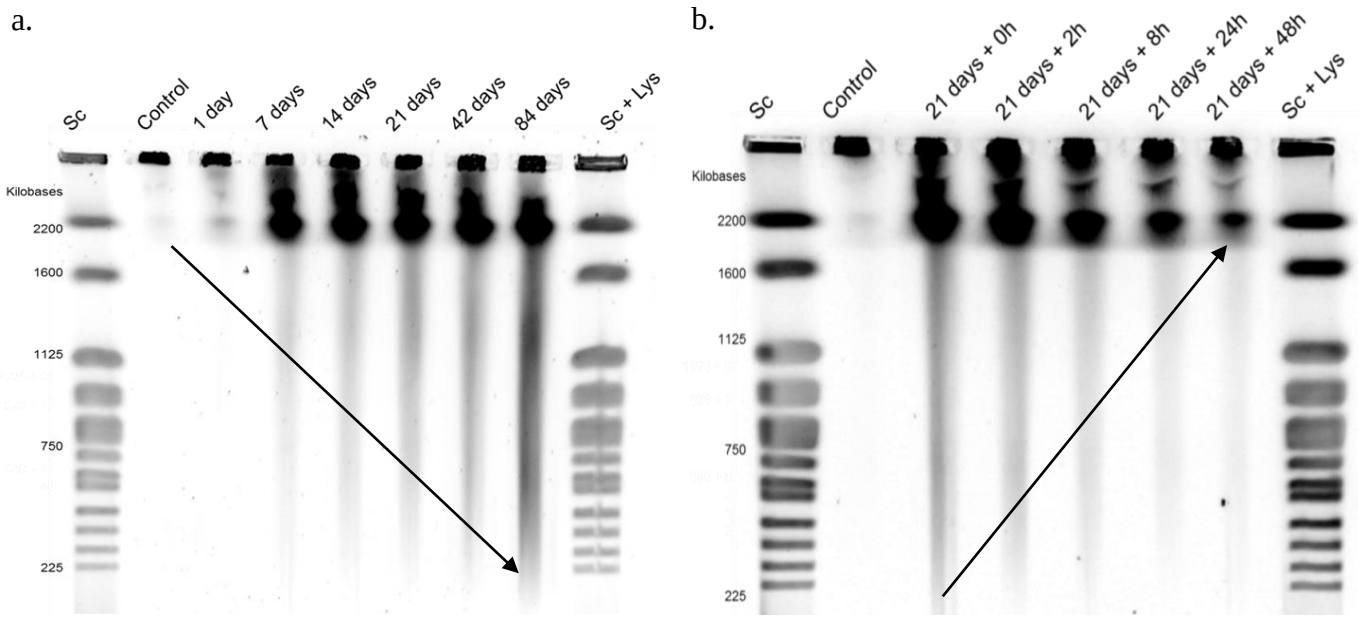
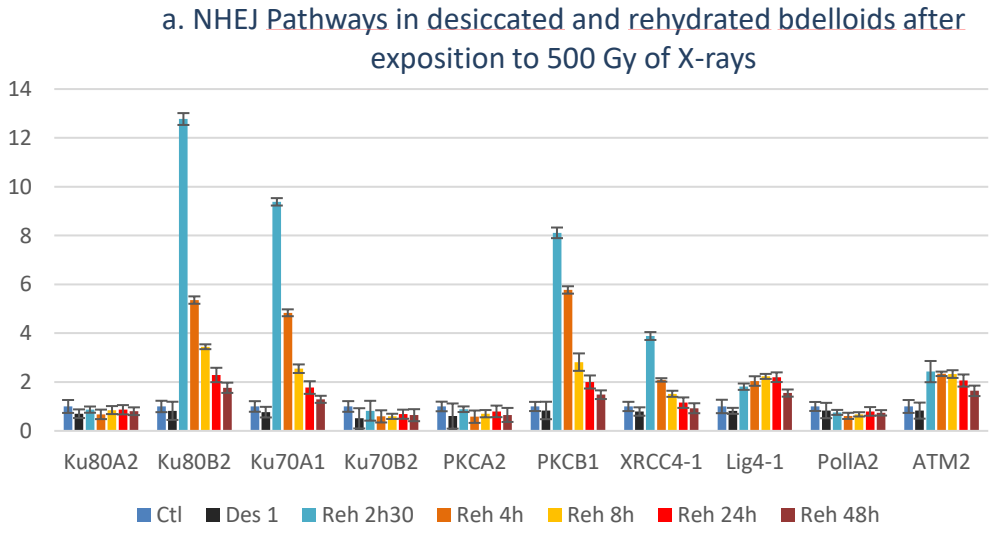


Figure 3: Genomic integrity of desiccated *A. vaga* as observed through pulsed-field gel electrophoresis. The first and last lanes correspond to *S. cerevisiae* chromosomes used as ladders, treated or not with a lysis solution. The picture "a." shows the loss of genomic integrity with the time spent in a desiccated state. The picture "b." shows the DNA repair kinetic of *A. vaga* desiccated for 21 days and rehydrated for different durations. In both cases, controls are 1,000 hydrated individuals [Hespeels B. *et al*, 2014].

DNA DSBs repair

Bdelloid rotifers are able to repair DNA DSBs efficiently. However, the exact nature of their DNA repair mechanisms allowing them to process their damaged genome is not yet known. Genomic data confirmed that bdelloid rotifers possess genes belonging to the two common DNA DSBs repair pathways: homologous recombination (HR) and non-homologous end-joining (NHEJ) [Flot J.-F. *et al*, 2013; Hecox-Lea BJ. and Mark Welch DB., 2018]. Unpublished RNA transcriptomic data (qPCR analysis and transcriptome sequencing) shows that the mRNA of the actors involved in NHEJ are over-expressed during rehydration after exposure to desiccation and 500 Gy ionizing radiation, while the genes of HR are not more expressed than in the control (see Figure 4).



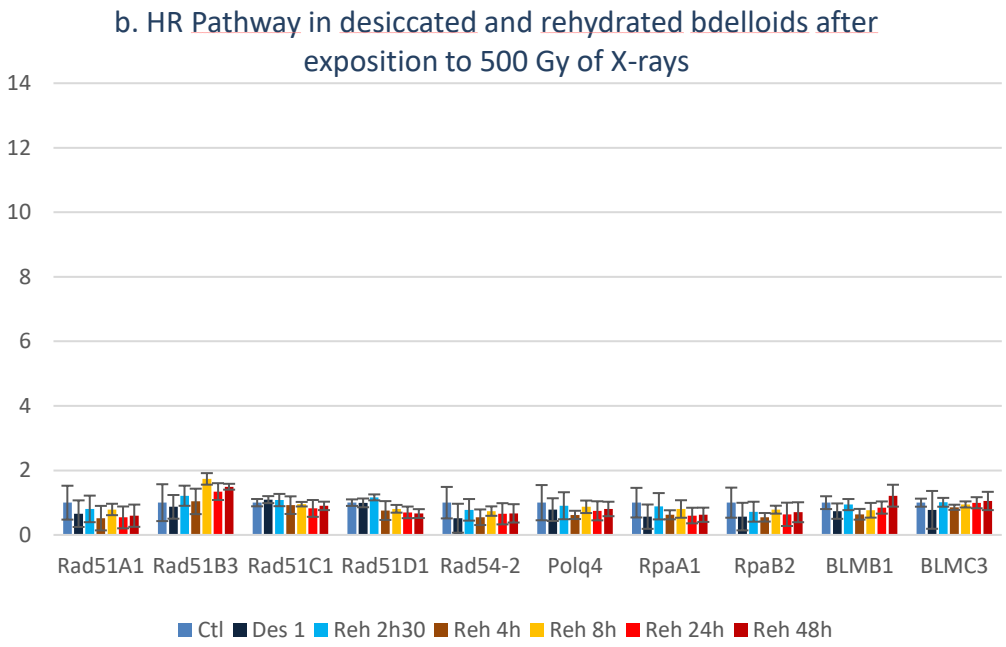
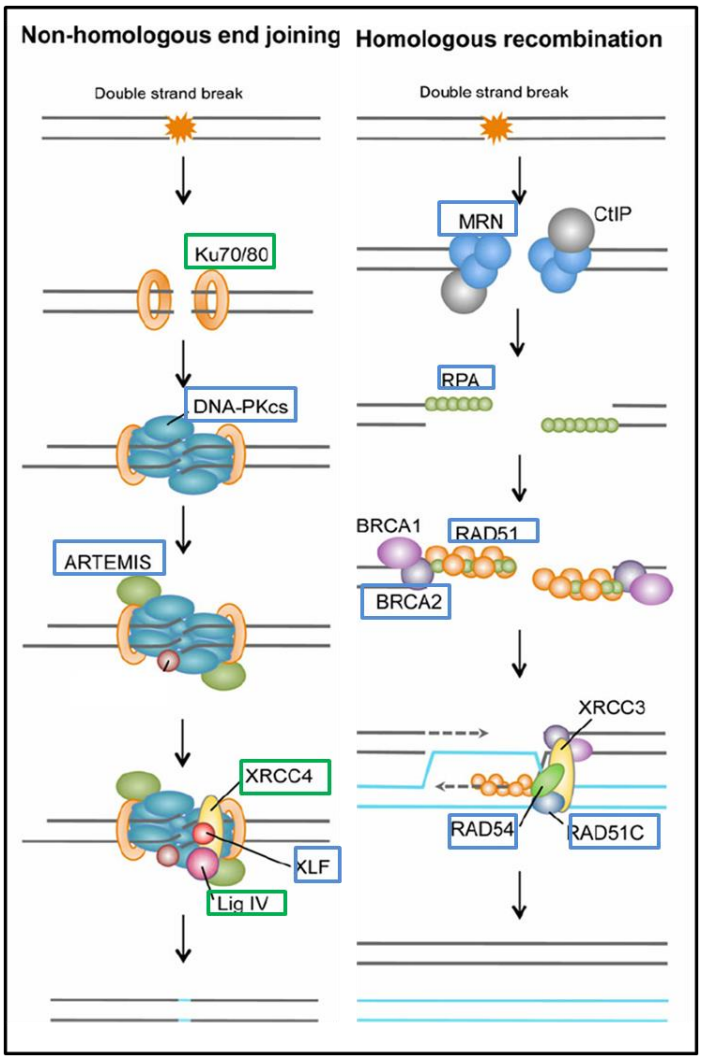


Figure 4: NHEJ and HR actors expression after desiccation and exposition to 500 Gy of X-rays. These two graphs show the relative expression of different key proteins involved in NHEJ (a.) and HR pathways (b.). The actors are represented on the x-axis, the different columns correspond to the expression in a hydrated control state; desiccated state after exposition to 500 Gy of X-Rays and desiccated and rehydrated for different durations after exposition to 500 Gy. Data were obtained after RNA extraction and qPCR of complementary DNA [Hespeels B. *et al*, In Prep].

NHEJ consists in a ligation process of the two overhangs surrounding the DNA breaks to recreate continuity in the DNA strand. NHEJ could result in base removal and therefore not repair the DNA faithfully to the original sequence [Jekimovs C. *et al*, 2014]. The first actor to intervene in this pathway is the ring-like heterodimer Ku70/80 which recognizes and binds the DNA ends (see Figure 5). As a scaffold, it recruits the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs), a serine-threonine kinase. Together, they are able to recruit and activate by phosphorylation Artemis, which trims the single-stranded DNA ends and makes them blunt. Finally, the ligase IV is recruited to the nascent DNA chain by XRCC4 and aids in sealing breaks. XLF was recently discovered as a third partner of the ligase IV-XRCC4 complex and thus regulates the complex activity [Burma S. *et al*, 2006].

HR is a high-fidelity DNA repair mechanism using the intact sister chromatid or homologous strand as a template to repair the broken DNA. This pathway can only occur when a sister chromatid is available, in S and G2 phases. HR is organized around key proteins (see Figure 5): first, hSSB1, the DSB sensor recruits the MRN complex (composed of Mre11/Rad50/NBS1, this last subunit is not present in the *A. vaga* genome [Hecox-Lea BJ. and Mark Welch DB., 2018]) to the damaged site to resect DNA around the break in a 5'-3' direction, stimulated by CtIP. Following this resection, single-stranded DNA (ssDNA) is exposed and prone to form secondary structures or is degraded by nucleases. To prevent this degradation and allow the DNA repair to continue, RPA intervenes and binds the ssDNA. BRCA1 plays a role by retaining RPA. The recombinase Rad51, helped by BRCA2, displaces RPA to form a filament along the strand and leads to strand invasion of the sister chromatid [Jekimovs C. *et al*, 2014]. Rad54 physically interacts with Rad51 to increase its strand



exchange activity [Mazin AV. *et al*, 2010], XRCC3 and Rad51C are two RAD51 paralogs and seem to help RAD51 in its activity [Brenneman *et al*, 2002].

Figure 5: Two ways to repair DNA DSBs: NHEJ (left) versus HR (right). NHEJ and HR pathways are mapped, blue frames correspond to genes constantly expressed in the transcriptome of hydrated, desiccated and rehydrated *A.vaga* individuals. Green frames represent genes upregulated during the rehydration of *A.vaga* submitted to two weeks of desiccation [Hecox-Lea BJ. and Mark Welch DB., 2018; Hespeels B. *et al*, in prep].

Extreme radioresistance

Another peculiar characteristic of these animals is their ability to withstand huge doses of ionizing radiation (IR). In 2008, Gladyshev and Meselson demonstrated that hydrated bdelloid rotifers were able to survive up to 1,200 Gy of gamma radiation without any change in their behavior. A survival rate of 99% was demonstrated in desiccated individuals after receiving 800 Gy of proton radiation. Their fecundity was only affected tardily: 1,000 Gy were necessary to decrease the fecundity of 90% while about 150 Gy and 200 Gy were sufficient to produce the same effect in *Caenorhabditis elegans* and the radiosensitive monogonont rotifer *Euchlanis dilatata* respectively [Hespeels B. et al, 2014; Gladyshev E. and Meselson M., 2008]. In comparison, “a whole-body exposure of just 10 Gy is lethal to most vertebrate animals, including humans” [Daly M., 2009].

IR are characterized by an energy deposition pattern sufficient to remove electrons from molecules and cause their ionization. Among them, X-rays are photons penetrating tissues and interacting with electrons of the matter. When it happens, these electrons are ejected from their orbital via the photoelectric or the Compton effect, producing ions which can then interact with other molecules [Hespeels B. *et al*, Submitted; Cox M. and Battista J., 2005].

In organisms, proteins, carbohydrates, lipids and DNA can be damaged by IR by two different ways. First, the "direct damage" which is the effect of radiation itself on a target, affecting mainly DNA and secondly, the "indirect action" happens when macromolecules are damaged by intermediate products such as ROS. This second mode of action is responsible for the majority of cell lesions. By ejecting electrons of water molecules (which represent up to 70% of cell mass), IR creates ROS as hydroxyl radicals, hydrogen peroxide and superoxide [Daly M., 2009; Von Sonntag C., 1987]. In Box 1, the major reactions happening in cells are summarized. By sharing similar reagents and products, reactions contribute to maintain and increase ROS production.

Water radiolysis: $H_2O \rightarrow OH\bullet$ (hydroxyl radical), $H\bullet$ (hydrogen radical), e^-_{aq} (hydrated electron), H_2(dihydrogen), H_2O_2 (hydrogen peroxide), $HO_2\bullet$ (hydroperoxyl radical)

In presence of oxygen: $H\bullet + O_2 \rightarrow HO_2\bullet$
$O_2 + 4\ e^- \rightarrow O_2\bullet^-$ (superoxide)
$O_2 + 2\ e^- + 2H+ \rightarrow H_2O_2$

In presence of transition metal cations (mainly iron) in the cell:

$Fe^{3+} + O_2\bullet^- \rightarrow Fe^{2+} + O_2$
$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\bullet + HO^-$ Fenton reaction

$H_2O_2 + O_2\bullet^- \rightarrow OH\bullet + HO^- + O_2$ Haber-Weiss reaction

Box 1: Water radiolysis products and main reactions producing ROS. ROS are indicated in red [Daly M., 2009].

The Fenton reaction is a process forming hydroxyl radicals from ferrous iron and hydrogen peroxide. This process produces large quantities of hydroxyl radicals in cells, one of the most reactive radicals [Hespeels B. *et al*, Submitted].

Given that bdelloids are never exposed to IR in their natural environment, what makes them so resistant to IR? By analogy with the extreme desiccation and radiation resistant bacteria *Deinococcus radiodurans*, a hypothesis suggests that mechanisms underlying IR resistance in bdelloid rotifers could be an adaptation to desiccation resistance. Indeed, damage resulting from desiccation and IR exposure is similar: DNA DSBs production of ROS leading to macromolecule damage [Gladyshev E. and Meselson M., 2008].

Biological damage

What are the main consequences at a biological level, caused by IR via a direct effect or via the production of ROS and caused by desiccation via ROS only?

DNA, as the largest molecule in the cell, is the most likely to suffer from a direct effect of IR leading mainly to the creation of single/double-strand breaks in the sugar phosphate backbone and to base damage [Von Sonntag C., 1987]. DNA DSBs are known to be the most cytotoxic damage a cell can undergo and if not correctly repaired, trigger cell death [Jekimovs C. *et al*, 2014]. On the other hand, ROS-induced DNA modifications are numerous in number including DNA breaks, base and deoxyribose modifications and DNA-protein crosslinkings [Azzam E. *et al*, 2012].

RNA can undergo the same injuries as DNA, given their similarities which could involve RNA breaks, base damages and crosslinks RNA/RNA or RNA/proteins [Kong Q. and Lin C.-L., 2010].

Lipids are important targets of free radical attacks and contribute to the formation of new free radicals and therefore, to the diffusion of oxidative injuries. This often leads to membrane fluidity, culminating in cell lysis [Hespeels B., 2016].

Proteins, being the most abundant intracellular macromolecules, have the maximum propensity to be affected by oxidants, whereby they can lose their structural 3D conformation, notably through fragmentation and aggregation, and therefore their function. Damaged proteins can accumulate in tissues generating insoluble aggregates and disturb the normal cell functions [Hespeels B., 2016; Kong Q. and Lin C.-L., 2010]. The most common, severe and irreversible damage at protein level is the carbonylation of different residues leading to the degradation of the proteins or disrupting their functional state. Protein carbonylation is recognized as a universal marker of oxidative stress and can lead to severe disorders. This modification can be easily measured in organisms by different approaches [Fedorova M. *et al*, 2014]. In the "Death by Protein Damage model", the death of irradiated cells is stated to be "a direct result of the progressive accumulation of oxidative damage to the proteome", by notably inactivating enzymes responsible for DNA repair [Daly M., 2012].

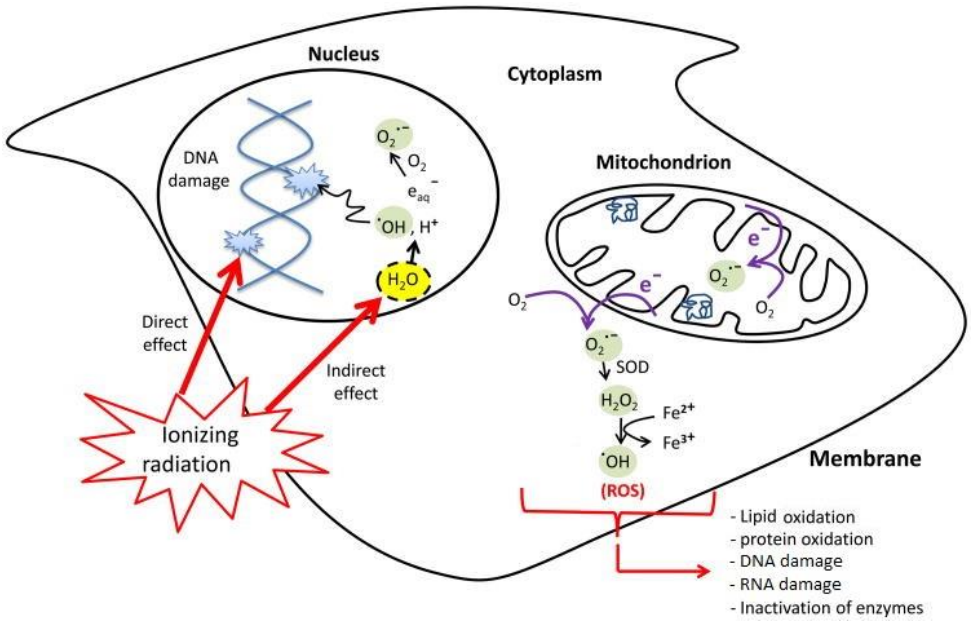


Figure 6: Summary of IR impacts on cells. IR damage cells via a direct action by interacting directly with the macromolecules and via an indirect action by the creation of ROS after interaction with water molecules. Modified from [Azzam E. *et al*, 2012].

ROS scavenging

If ROS formation can be a consequence of desiccation and IR exposure, they are also physiologically produced in organisms through respiration in mitochondria and "play a regulatory role in cellular metabolic processes via activation of various enzyme cascades and several transcriptional factors" [Fedorova M. et al, 2014]. However, when the balance

between ROS production and ROS elimination is disturbed in favor of ROS, cells are in "oxidative stress" condition and exposed to the dangers mentioned previously. Cells therefore possess enzymatic and non-enzymatic mechanisms neutralizing them and preventing their accumulation. Superoxide dismutases disproportionate $O_2^{\bullet -}$ into H_2O_2. Then, H_2O_2 molecules are converted in H_20 and 0_2 by catalases or alternatively reduced either by glutathione peroxidases or by peroxiredoxins in H_20 [Hespeels B., 2016; Pizzino G. *et al*, 2017].

Moreover, several alternatives and independent pathways act against ROS. Vitamin E, encompassing a series of lipophilic molecules, is described to modulate oxidative stress induced pathways. Vitamins A and C as well as flavonoids, carotenoids and glutathione scavenge ROS and are implied in their elimination [Hespeels B., 2016; Pizzino G. *et al*, 2017].

Desiccation and IR resistance: mechanisms

Desiccation and IR resistance is acquired through specific and independent mechanisms and molecules. One actor involved in desiccation resistance are non-reducing disaccharides such as trehalose, in metazoans, bacteria, fungi and some plants, and sucrose in higher plants. These sugars are important stabilizers of nucleic acids, enzymes, proteins and membranes via the water replacement hypothesis. Indeed, phospholipids in membranes are normally hydrogen-bound to water molecules. When water is absent, van der Waals' interactions between hydrocarbon chains increase and as a result, the phase transition temperature increases too. At room temperature, dry lipids pass from a liquid to a gel phase. Upon subsequent rehydration, membranes would be impacted and less functional. Trehalose intervenes to decrease this gel-to-liquid transition phase and to vitrify the cytoplasm, protecting the membranes and the cellular content from vesicles fusion and osmotic stress. Increasing amounts of trehalose and sucrose are correlated, in most cases, with high survival to desiccation [Potts M., 1999]. Surprisingly, trehalose has never been detected in bdelloids. However, they possess genes involved in trehalose metabolism: the trehalose-6-phosphate synthase (TPS) and the trehalase genes, respectively involved in the synthesis of the trehalose precursor and its hydrolysis. Interestingly, the enzymes genes found in the bdelloid rotifer *A. vaga* had a plant-fungal and a bacterial origin. Expression of both genes are upregulated upon desiccation. On the contrary, the third enzyme, the trehalose-6-phosphate phosphatase, which produces trehalose from its precursor, was not detected in bdelloids. Growing evidence suggest that precursors and enzymes (such as TPS) involved in the trehalose pathway act as signaling molecules and messengers during dehydration [Hespeels B. *et al*, 2015].

Another family of proteins involved in desiccation tolerance are the Late Embryogenesis Abundant (LEA) proteins. These proteins, classified into 7 groups, are present in a wide range of organisms including bacteria, plants, nematodes and bdelloid rotifers. They possess a reversible structure: because of their high hydrophilicity they are unfolded in hydrated cells, but become structured when water decreases, acquiring a secondary structure (mainly α-helices). Under this state, LEA proteins play different roles: some are chaperones reducing protein aggregation during water stress and therefore, preventing their inactivation; others are proposed to act as non-reducing disaccharides by interacting with the membranes and stabilizing them or by vitrifying the cytoplasm. LEA proteins are constitutively expressed in bdelloid rotifers, suggesting that they appear to be always present in the cells to deal with a desiccation episode and react quickly [Hespeels B., 2016; Tripathi R. *et al*, 2012].

Heat Shock Proteins (HSP) may also contribute to the desiccation tolerance in bdelloid rotifers. As chaperones, they act at different levels: biosynthesis, folding, assembly, secretion and degradation of proteins. These capacities are important to prevent protein denaturation and to refold damaged proteins. Involvement in desiccation tolerance is already suggested in *C. elegans* and tardigrades among others [Hespeels B., 2016; Jönsson K.I. and Schill R.O., 2007].

A series of molecular adaptations are common to all desiccation resistant organisms facing a dryness episode, these are grouped together under the term "desiccome". LEA and HSP proteins are part of the desiccome, as well as antioxidants. In the *A. vaga* genome, 213 antioxidant genes were identified, belonging to different enzyme families such as superoxide dismutase and glutathione peroxidase, sometimes acquired from non-metazoans through HGT. These genes are more numerous in bdelloid rotifers than any other studied organism. Most of them appear constitutively expressed and up-regulated upon desiccation. Therefore, antioxidants may have "the capacity to neutralize ROS production during dehydration, limiting its accumulation during dryness and activating the detoxification process at the onset of rehydration" [Hespeels B., 2016; Flot J.-F. *et al*, 2013].

As mentioned previously, IR resistance in bdelloid rotifers is thought to be evolutionary related to their desiccation resistance. Indeed, both are harmful to the organisms by creating an oxidative stress, itself leading to a wide range of damage. Since the consequences of both conditions share similarities, some protective mechanisms can be ubiquitous [Gladyshev E. and Meselson M., 2008]. One of the first goals in radiobiology is to explain why most organisms are so sensitive to radiation [Daly M., 2012]. In the 1950s, DNA, lipids and proteins were proposed as major IR targets. Because genomic integrity is essential for cell survival, researchers mainly focused on DNA damage [Daly M., 2009]. However, a recent hypothesis called "death by protein damage" supported by different studies, states that "radiation toxicity is determined mainly by the level of protein oxidation caused during irradiation rather than the amount of DNA damage" [Daly M., 2012]. Indeed, DNA DSBs are found in the same narrow range in radiosensitive and radiotolerant organisms: 0.002-0.006 DSB/Gy/Mb for a haploid genome. On the contrary, protein oxidation rate can be up to 100 times higher in radiosensitive organisms than in resistant ones. The cornerstone of radio-resistance seems not to reside in the susceptibility of DNA to damage but rather in protein protection against oxidative modifications. Death by IR would mainly depend on the susceptibility of DNA repair proteins, which, if inactivated, render each DNA damage event lethal [Daly M. *et al*, 2010].

Manganese (Mn) involvement in extreme IR resistance is also highlighted. Efforts made during the last decade identified Mn-based antioxidants as an important candidate to prevent IR-induced protein oxidation [Daly M., 2009]. How is Mn working? Iron (Fe), present in the cells attached to proteins or acting as enzyme cofactors, catalyzes Fenton reactions and thus, the production of high amounts of ROS. Mn however shields these proteins and replaces Fe as cofactors to prevent the Fe-dependent ROS formation. Besides limiting Fenton reactions, Mn acts as an antioxidant by neutralizing superoxides and peroxides. Such effects are usually brought about by its binding with either small molecules like phosphate, lactate, amino acid, etc. or proteins like superoxide dismutase [Daly M., 2009; Daly M., 2012].

In extreme-tolerant organisms in which Fe participates in the production of pro-oxidants through Fe-S cluster proteins and through Fenton reactions, Fe is only mildly down-regulated while Mn is observed to be highly up-regulated [Daly M. *et al*, 2010].

Studies performed on Mn protective potential against ROS are promising. Two examples to outline here: *in vitro* reconstituted mixtures of *D. radiodurans* extract were able to preserve 50% of the glutamine synthetase enzyme activity until 50 kGy, which is normally inactivated with 150 Gy; preparations from *D. radiodurans* were able to fully protect human cells up to 16 Gy and preserve *Escherichia coli* viability [Daly M., 201; Daly M. *et al*, 2010].

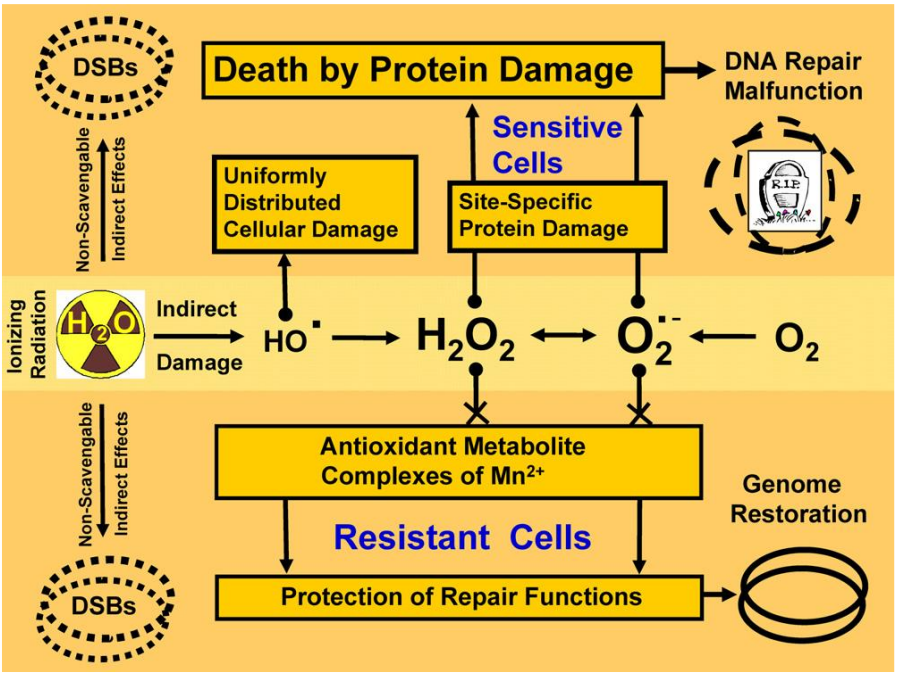


Figure 7: Radiosensitive versus radioresistant cells. IR mainly impacts the cells via the ROS production. Protein damage, and especially at the DNA repair proteins level, are mainly responsible of cell death. However, Mn intervention can protect them [Daly M., 2012].

2. Objectives

The Laboratory of Evolutionary Genetics and Ecology (LEGE) headed by Pr. Karine Van Doninck, studies different aspects of the asexual and extremely resistant bdelloid rotifers including their genome evolution and diversification, the mechanisms of extreme desiccation and radiation resistance with a specific focus on DNA DSBs repair and oxidative stress responses. The bdelloid rotifer model species in the LEGE laboratory is *Adineta vaga*, whose genome was assembled at chromosome level (Narayan J. *et al*., in prep). The aim of this master thesis was to integrate the ongoing research focusing on DNA DSBs repair and oxidative stress whilst investigating the extreme resistance of bdelloid rotifers to IR.

The two major questions addressed were:

1) HR versus NHEJ DNA DSBs repair: what are the consequences for the bdelloid rotifer *Adineta vaga* if these repair pathways are inhibited?

A hypothesis based on experimental results obtained by Terwagne M. *et al* (in prep.) in the LEGE laboratory is that somatic cells of *A. vaga* (being eutelic) are in G0/G1 phase once the development is completed. In the absence of sister chromatid, NHEJ is often the privileged DNA DSBs repair mechanism. On the other side, germ line oocytes in *A. vaga* are in G2 phase and may preferentially perform HR using the sister chromatid as template during DNA DSBs repair. A high-fidelity repair in the germ line could contribute to the resistance of bdelloid rotifers to DNA damage by ensuring genomic fidelity in the subsequent generations [Terwagne M. *et al*, in prep] as in *Caenorhabditis elegans*, where NHEJ plays no role in the germ line whereas HR is favored to ensure genomic integrity [Clejan I. *et al*, 2006].

In this Master thesis, a new *in vivo* approach was adopted to better understand the DNA DSBs repair mechanisms of *A. vaga*. Proteins involved in the two DNA DSBs repair pathways present in bdelloid rotifers can be targeted and inhibited by different drugs to study their activity during DNA DSBs repair. These drug molecules are studied to prevent the repair in human cancer cells where the prevalence of DNA DSBs is high due to the unlimited growth [Jekimovs C. *et al*, 2014]. Four inhibitors were investigated: SCR7 and DDRI-18 known to target the non-homologous end-joining pathway, more specifically SCR7 inhibits the ligase IV by interfering with its DNA binding site while the inhibition action of DDRI-18 remains unknown [Jekimovs C. *et al*, 2014], [Jun DW *et al*, 2012]. Two homologous recombination inhibitors were also tested: B02 and Mirin, the first inhibits Rad51 by disrupting its binding to DNA and nucleoprotein filament formation and the second inhibits the 3'-5' exonuclease activity of Mre11, subunit of the MRN complex [Jekimovs C. *et al*, 2014].

First, the impact of these drugs was tested on the reproductive capacity of irradiated *A. vaga*, where HR inhibitors were expected to decrease the number of descendants by negatively interacting with the repair in the germ line following IR. Second, the effect of the inhibitors was assessed by studying through PFGE the genome repair kinetic of *A. vaga* following IR. Here, we specifically investigated the repair in somatic cells, where we expected to observe a DNA repair impairment by the NHEJ inhibitors.

2) Does the *Adineta vaga* lysate provide any protection to irradiated human A549 lung carcinoma cells against oxidative stress?

Human and mammals in general cannot withstand stresses such as desiccation and IR exposure. Both are harmful to the organisms, mainly by creating huge amounts of ROS. Survival of A549 non-small cell lung cancer cells exposed to 2 and 10 Gy of X-rays is respectively equal to 65 and less than 1% [Wéra A.-C. *et al*, 2012]. The majority of cells die mainly through mitotic catastrophe [Riquier H. *et al*, 2013], *i.e.* a delayed type of cell death occurring during or as a result of an aberrant mitosis [Eriksson D. and Stigbrand T., 2010]. Strikingly, some bacteria like *Deinococcus radiodurans, Lactobacillus plantarum* and invertebrates such as bdelloid rotifers, tardigrades and nematodes are extremely resistant to desiccation and IR and form interesting model systems to study their radiation resistance [Hespeels B., 2016].

Besides the numerous enzymes involved in ROS protection, Mn is also part of the antioxidant arsenal of *D. radiodurans* and *L. plantarum*. In *A.vaga* many antioxidant genes were characterized and are found to be constitutively expressed. In 2019, Mn/Fe ratios were measured in *A. vaga* through Inductively Coupled Plasma Mass Spectrometry (ICP-MS) and showed to be high as in *D. radiodurans* and *L. plantarum*. Researches performed with *D. radiodurans* demonstrated that protein-free extracts of these bacteria, sharing extreme features with bdelloid rotifers, applied before IR exposure on Jurkat T cells were able to fully preserve their viability when exposed up to 16 Gy of γ-rays [Daly M. *et al*, 2010]. The lysate of *A. vaga* could therefore contain an efficient mixture of antioxidant protection, as shown for *D. radiodurans*.

In this Master Thesis, the role of *A. vaga* lysate in protecting X-rays induced damage in human A549 lung carcinoma cells was investigated. Human cells culture media were supplemented with rotifer lysate prior to their irradiation. Survival of cells was measured with Trypan Blue staining and MTT assay and the proliferative capacity of cells was observed through colony formation assay.

3. Materials and Methods

3.1. DNA repair study using NHEJ/HR inhibitors

Bdelloid rotifer culture

To perform the experiments, the specie *Adineta vaga* was used. All specimens are isogenic clones descending from one individual from the Meselson laboratory of Harvard University [Van Doninck K. *et al*, 2009]. *A. vaga* were cultured in 145x20 mm Petri dishes placed at 21°C, maintained hydrated with Spa® water and fed with autoclaved lettuce juice three times a week. Debris were removed by rinsing the plates with Spa® water when needed.

Exposition to inhibitors

Four different DNA repair inhibitors were tested individually (Sigma-Aldrich, USA): B02, SCR7, DDRI-18 and Mirin (Lucie Bruneau mainly performed the experiments with the two last drugs). Initially in powder form, the drugs were resuspended in DMSO following company's instructions.

The Table 1 summarizes the important parameters of DNA repair experiments which are detailed below.

Table 1: Recapitulative table of experiments performed with DNA repair inhibitors

<table>
<tr><th rowspan="2">Drug</th><th rowspan="2">Target</th><th rowspan="2">Path-way</th><th rowspan="2">Initial concentration (millimolar (mM))</th><th colspan="2">Fertility</th><th colspan="2">DNA repair kinetic</th><th colspan="2">DNA repair kinetic 7 days incubation</th></tr>
<tr><th>Final concentration (micromolar (µM))</th><th>Population check</th><th>Final concentration (µM)</th><th>Fixation time point</th><th>Final concentration (µM)</th><th>Fixation time point</th></tr>
<tr><td>B02</td><td>Rad51</td><td>HR</td><td>73.66</td><td>0, 10, 50, 100, 150</td><td>Days 4, 7, 11</td><td rowspan="3">150</td><td rowspan="3">Hours 4, 8, 24, 48</td><td rowspan="3">100</td><td rowspan="3">Hours 2.5, 8, 24, 48</td></tr>
<tr><td>Mirin</td><td>MRN complex</td><td>HR</td><td>45.40</td><td>0, 10, 50, 100, 150, 200</td><td>Days 4, 7, 12</td></tr>
<tr><td>SCR7</td><td>Ligase IV</td><td>NHEJ</td><td>30.09</td><td>0, 50, 100, 150, 200</td><td>Days 4, 7, 12</td></tr>
<tr><td>DDRI -18</td><td>Un-known</td><td>NHEJ</td><td>24.04</td><td>0, 10, 50, 100, 150, 200</td><td>Days 4, 7, 11</td><td colspan="2">1,000 individuals per time point</td><td colspan="2">1,000 individual per time point</td></tr>
<tr><td colspan="4"></td><td colspan="2">Initially, 1 individual per concentration (in 12 replicates)</td><td colspan="4"></td></tr>
</table>

Evaluation of the reproductive capacity in presence of a DNA repair inhibitor

A. vaga cultures were washed with Spa® water to remove debris and dead animals. 12 rotifers per concentration to test were randomly isolated from a culture plate in a 5-ul droplet and

each individual was deposited in a well of a 96-well plate. 150 microliters (µl) of culture media were poured in each well. Media were prepared as follows: stock solution of each drug was diluted in water with 2% of lettuce juice to reach the different concentrations to test (see Table 1). Resuspension and dilution steps were done at 37°C to prevent precipitation of the drugs. In parallel, control solutions were prepared by replacing the volume of drug resuspended in DMSO by an equal volume of DMSO alone.: *e.g.* to reach a concentration of 100 µM of SCR7, 0.5 µl of the drug were diluted in 3 µl of lettuce juice and 146.5 µl of Spa® water. Control condition was performed as follows: 0.5 µl of DMSO in 149.5 µl of lettuce juice and Spa® water.

The complete handling was repeated with *A. vaga* individuals which were first irradiated at 500 Gy of X-rays (X-RAD 225XL, Precision X-Ray, UK) prior to the addition of culture media (with or without drug). All the plates were then incubated at 21°C. The steps are schematized in the Figure 8.

Fertility was defined as the ability of one *A. vaga* individual to recreate a population by laying fertile eggs. After 4, 7 and 11 (or 12) days, the number of alive adults and the number of eggs were counted in each well.

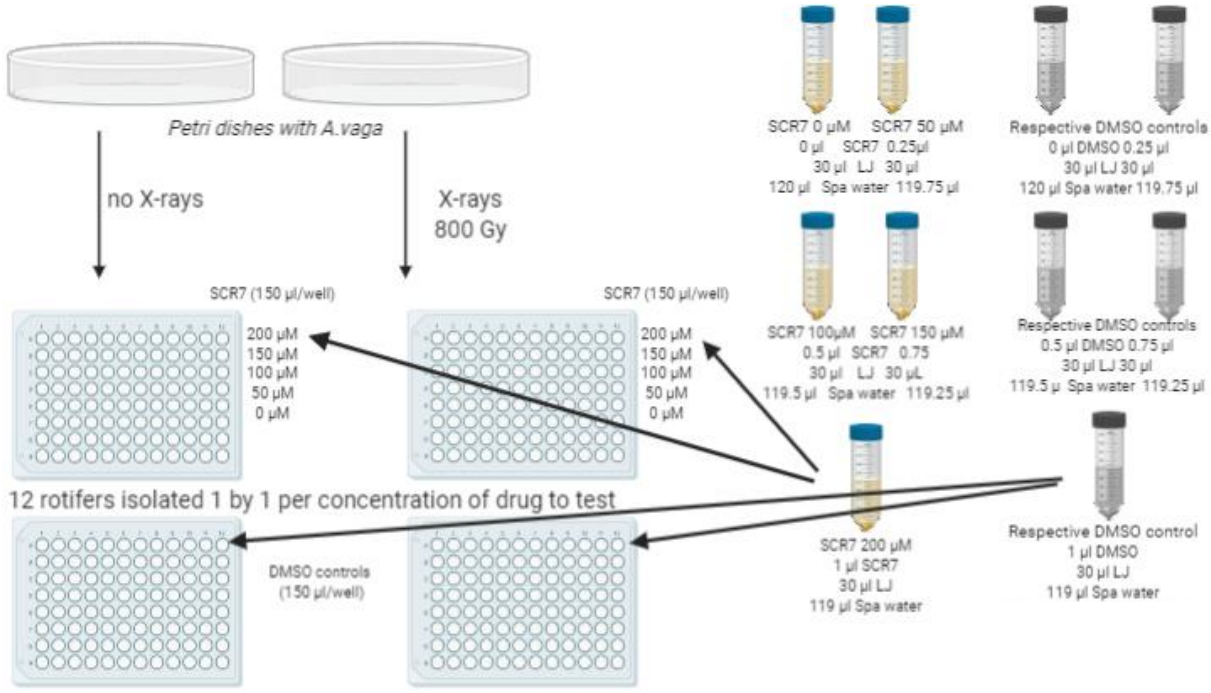


Figure 8: Schematic figure of the experiment. SCR7 was taken as example. "LJ": lettuce juice.

Statistical analysis

To highlight statistically significant effects of the different factors (drugs and X-Rays) on fertility, the data set of the 7th day were analyzed by statistical tests.

First, condition by condition, adults and eggs were summed and considered as a whole population. A generalized linear model (GLM) was applied to the concentration factor for the irradiated and non-irradiated data separately with a Gaussian distribution.

A Shapiro-Wilk test was performed to assess the normality of the data. If the p-value was lower than 0.05, normality was rejected and a Kruskal-Wallis test was realized. Finally, a post-hoc pairwise Wilcoxon test with a Bonferroni correction was done. However, if the p-value was higher than 0.05, the normality was assessed and an ANOVA test performed on the GLM model.

DNA repair kinetic in presence of a DNA repair inhibitor

To assess the consequences of the inhibitors on the repair of artificially induced damage, DNA repair kinetics were performed by PFGE.

A. vaga cultures were rinsed with 15 milliliters (ml) of Spa® water to remove debris and dead animals. Individuals were harvested by adding 450 µl of NaCl 5 molars (M) and quickly vortexing the dishes. The media were poured in 15-ml Falcon tubes. After 15 minutes of centrifugation at 4,000 rpm, pellets were pooled together and resuspended in Spa® water. Then, hydrated rotifers were transferred in a Petri dish to undergo the irradiation at 1,000 Gy of X-rays (X-RAD 225XL, Precision X-Ray) on ice in a final volume of 10 ml to generate multiple DNA DSBs in their genome.

At the end of the irradiation, 8,000 *A. vaga* individuals were collected and placed in a well of a 24-well plate with 500 µL of the experimental mix (drug 150 µM, lettuce juice 5% and water). In parallel, in the control wells, 8,000 individuals were placed in 500 µL of the mix lettuce juice 5% and water. The plate was incubated at 21°C.

To study a DNA repair kinetic, rotifers were fixed at different times: one well and its control were fixed in Solution A (EDTA 50 mM pH 8 and Tris HCl 10 mM pH 8) after 4, 8, 24 and 48 hours. At each time point, 15 µL of NaCl 5M were poured in the wells to collect. Rotifers were transferred in Eppendorf tubes and completed with 750 µL of Spa® water was added to rinse the wells. After 1 minute of centrifugation at 14,800 rpm, supernatants are removed and washed with water. Two washes were performed with solution A before the final fixation in solution A. This solution was especially formulated to fix rotifers and preserve them in their state. Samples were then frozen at -80°C.

An irradiated control – rotifers irradiated with 1,000 Gy of X-rays - was performed by fixing rotifers in solution A soon after the irradiation. These individuals show the extent of DNA breaks at the end of the irradiation process and constitute a reference for the experiment.

Hydrated control –non-irradiated rotifers- were also prepared as a reference to study genomic integrity. Supplemental cultures were collected as described previously and individuals were placed in Eppendorf tubes and directly fixed in solution A. Both controls were frozen at -80°C until the PFGE start.

PFGE

The genomic integrity of the following samples was assessed using PFGE: hydrated animals; irradiated rotifers; rotifers fixed after 4 hours in presence of the drug, then its control; rotifers fixed after 8, 24 and 48 hours. The samples, conserved at -80°C (see before), were thawed, 1,000 rotifers were taken out from each Eppendorf tube and placed in a new one. These were centrifuged and the supernatants removed to reach a total volume of 31µL, then 19 µL of 2% CleanCut Agarose (BioRad, USA) at 56°C were added, the whole was well mixed and casted

in a plug mould. After 15 min of polymerization at 4°C, plugs were individually transferred into 500 µL of digestion buffer (100 mM EDTA, 50 mM Tris pH8, 5.2% of proteinase K (ThermoFisher Scientific, USA) and 3.33% N-Lauroylsarcosine sodium solution), placed 1 hour at 4 °C and incubated 18 hours at 56 °C. Plugs were then rinsed with 0.5x Tris Borate EDTA (TBE) and kept for 3 hours in 0.5x TBE at 4 °C. They were stored at 4°C for further use in 500µL 0.5M EDTA pH 8 after rinse with 0.5x TBE.

Plugs were loaded in a 0.8% agarose gel (Lonza, USA) along *Saccharomyces cerevisiae* chromosomes as ladder (BioRad, USA). Migration was performed in a BioRad CHEF-DR III system (parameters: migration time = 22 hours, temperature = 14 °C, Volts/cm = 5.5, switch angle = 120°, switch times = 60 to 185 seconds with a linear ramp). Finally, the gel was stained with SYBR Gold (Invitrogen, USA), analyzed by a BioRad Chemidoc XRS camera and the images were processed by the software ImageLab 3.0.

In order to resolve larger fragments of DNA (225- approximately 7000 kb), the following parameters were followed: migration time = 96 hours, temperature = 14 °C, Volts/cm = 1.5, switch angle = 106°, switch times = 600 to 4800 seconds with a linear ramp. The plugs were loaded in a 0.7% agarose gel (Lonza, USA). Two different ladders were used: *S. cerevisiae* and *Hansenula wingei* chromosomes (BioRad, USA).

DNA repair kinetic after 7 days of incubation with SCR7

Consequence of a prolonged contact of the inhibitors (7 days) on the DNA repair pathways occurring in bdelloids was assessed by PFGE. This experiment was only done with SCR7.

A. vaga cultures were rinsed with 15 ml of Spa® water to remove debris and dead animals. Individuals were harvested by adding 450 µl of NaCl 5 M and quickly vortexing the dishes. The media were poured in 15-ml Falcon tubes. After 15 minutes of centrifugation at 4000 rpm, pellets were pooled together and resuspended in Spa® water. Then, hydrated rotifers were transferred in a 12-well plate and cultured for 7 days at 21°C in 3 ml of Spa® water containing 5% of lettuce juice and 100 µM of SCR7. Controls were performed in parallel without SCR7. Media were changed after 3 days and replaced by new ones with the same composition.

After exposure to SCR7 for 7 days, individuals were irradiated at 1,000 Gy of X-rays on ice to generate DNA DSBs in their genome. At the end of the irradiation, media were renewed and replaced by new media containing 100 µM of SCR7.

To study the repair kinetic, rotifers were fixed at different times: one SCR7 well and its control were fixed in Solution A directly after the irradiation, then, 2.5, 8, 24 and 48 hours post-irradiation. Two supplementary conditions were performed: non-irradiated rotifers incubated for 7 days with SCR7and rotifers irradiated with 1,000 Gy of X-rays. Fixation and PFGE protocols are described previously.

3.2. Effect of bdelloid rotifer lysate on human cells

Bdelloid rotifer culture

See 3.1. Bdelloid rotifer culture

A549 non-small cell lung adenocarcinoma cell culture

A549 cells were cultured in Glutamax Minimal Essential Medium (Gibco, USA) supplemented with 0.5% Penicillin-Streptomycin (Sigma-Aldrich, USA) and 10% fetal bovine serum (Gibco, USA). Cells were grown at 37°C with 5% of CO_2.

Bdelloid rotifers protein extraction

A. vaga cultures were rinsed with 15 ml of Spa® water to remove debris and dead animals. 300,000 individuals were collected by scraping the dishes and poured in 15-ml Falcon tubes. After 15 minutes of centrifugation at 4,000 rpm, pellets were pooled together and resuspended in Spa® water. Then, hydrated rotifers were transferred in a 94x16 mm Petri dish with Penicillin-Streptomycin (Gibco, USA) at a dilution 1:1000 and placed for 16 to 18 hours at 21°C.

Individuals were then pooled in a 15-ml Falcon tube and centrifugated for 15 minutes at 4,000 rpm. Supernatant was entirely removed and the pellet was transferred to a 2-ml dounce with 300 µL of suspension buffer prepared at 4°C (Phosphate buffer 50 mM pH 7.4 and one tablet of Protease Inhibitor Cocktail/ 10 ml, cOmplete™ ULTRA Tablets, Mini, EDTA-free, *EASYpack*, Roche, Switzerland). On ice, 1,000 mechanical ruptures were performed and the sample was centrifuged at 12,000 rpm for 15 minutes at 4°C. The supernatant was transferred to a new Eppendorf and left on ice. The protein extraction process was repeated twice by using the pellet obtained from the previous protein extraction process with a suspension buffer volume of 200 µl and 150 µl respectively. Fractions were pooled and frozen at -20°C until use.

A549 cells protein extraction

Cells at confluence were trypsinised and centrifugated at 1,000 rpm for 5 minutes. The pellet was rinsed twice with PBS and transferred to a 2-ml dounce and lysed following the process described previously.

Protein quantification

Protein concentrations of rotifer lysate and A549 cell lysate were quantified through a Pierce Protein Assay (Pierce 660 nm Protein Assay Reagent, ThermoFisher Scientific, USA). A standard curve was prepared with a range of bovine serum albumin concentrations (BioRad, USA), which were then incubated with the Pierce reagent for 5 minutes and then read on a plate reader. Absorbance was measured at 660 nm. The experimental samples were prepared in a similar manner and protein concentrations were estimated with an extrapolation to the standard curve.

Western Blot

Samples composed of proteins extracts and 1x Bolt LDS Sample Buffer (ThermoFisher Scientific, USA) were incubated for 5 minutes at 95°C to denature proteins. They were loaded on Bolt™ 4-12% Bis-Tris Plus Gel (10-well, ThermoFisher Scientific, USA) along the ladder (SeeBlue™ Plus2 Pre-stained Protein Standard, ThermoFisher Scientific, USA) and the positive control (rotifers lysed with a buffer composed of Tris-HCl pH 8.0 10 mM, EDTA 1

mM, DTT 5 mM, PMSF 1 mM, Kcl 1 M, CHAPS 1% and one tablet of Protease Inhibitor Cocktail/ 10 ml). The buffer used for the positive control was developed by Anaïs Boutsen during her master thesis and was optimized to isolate the cytoplasmic and nuclear protein fraction of bdelloid rotifers. SDS page was electrophoresed at 120 volts for one hour in 1x Bolt MES SDS Running buffer (ThermoFisher Scientific, USA). After the electrophoresis, gel was transferred on a PVDF membrane (Amersham Hybond P 0.45 PVDF blotting membrane, GE healthcare, USA) for 1 hour at 20 volts in 1x Bolt Transfer buffer with Bolt antioxidant (ThermoFisher Scientific, USA) and 20% methanol.

After 1 hour of blocking in 5% non-fat dry milk diluted in 1x TBST buffer (Tris HCl pH 7.6 2 mM, NaCl 15 mM, 0.1% Tween 20), the PVDF membrane was incubated 1 hour with the primary antibody. The primary antibody used was: mouse monoclonal antibody anti-α-tubulin (Sigma-Aldrich, USA) and rabbit polyclonal anti-histone H3 antibody (Bethyl Laboratories) at a dilution of 1:1000 in TBST supplemented with 1% non-fat milk. The membrane was then washed in TBST and incubated with the secondary antibody conjugated to horseradish peroxidase (Goat anti-rabbit HRP, Goat anti-mouse HRP, ThermoFisher Scientific, USA), diluted at 1:5000 in TBST and non-fat milk 1% for 1 hour. After 3 more washes with TBST, the chemiluminescent horseradish peroxidase substrate (SuperSignalTM West Pico PLUS Chemiluminescent Substrate, ThermoFisher Scientific, USA) was put in contact with the membrane for 10 minutes prior capturing the results with BioRad Molecular Imager ChemiDoc XRS+.

Coomassie Blue staining

Preparation of samples and migration were performed similarly to the Western Blot protocol. Once the migration was done, the gel was fixed in the fixing solution composed of 50% methanol and 10% glacial acetic acid for 1 hour. Then, it was stained in the staining solution (0.1% Coomassie Brilliant Blue R-250, 50% methanol, 10% glacial acetic acid) for 20 min and finally de-stained in the de-staining solution (40% methanol, 10% glacial acetic acid). This solution was changed twice or thrice to ensure proper de-staining of the background of the gel. These steps were performed with gentle agitation.

A549 cells X-rays irradiation

Irradiations were performed 24-26 hours after plating at 225 keV (X-RAD 225XL, Precision X-Ray, UK) and at room temperature. Day of irradiation was considered as day 0; if no X-rays exposure was planned, day 0 was assumed to be the day after the plating.

Cell viability assay

Cell viability assays were performed first, without and then, with rotifer lysate and its appropriate controls. Cells were seeded in 24-well plates. In the experiments where rotifer lysate was added on cells, media were renewed 5 to 6 hours later and replaced by 500 µl of new media supplemented with rotifer lysate. 3 controls were undertaken composed of fresh media, media supplemented with A549 cell lysate and media supplemented with suspension buffer. These solutions were prepared as described previously and filtered through a 0.20 µm filter prior use. Volume of suspension buffer used as control was always the same than the volume of rotifers proteins added to reach a precise concentration. Plates were exposed to different X-rays doses: 5, 10 or 20 Gy. Control plates were also prepared and underwent the same manipulations without irradiation. Cell viability was measured after 1, 2 and 3 days or 3,

5 and 7 post-irradiation through a MTT assay and/or a Trypan Blue (TB) staining. Concerning the MTT assay, cell culture media were removed and MTT solution 1 mg/ml was added (Thiazolyl Blue Tetrazolium Bromide, Sigma-Aldrich, USA) and incubated with the cells for 2h30 to 3h. Finally, MTT was discarded and replaced by DMSO prior to reading the absorbance results with a spectrophotometer at 570 nm. On the other side, viable cells were manually counted after staining with Trypan Blue (Trypan Blue staining 0.4%, Invitrogen, USA) after trypsinisation. The different experiments undertaken with rotifer lysate are listed in the Table 2.

Table 2: List of experiments performed to study rotifer lysate effect on A549 human cells

	Number of cells / well	Type of plate	Total volume of media / well	Conditions tested after media refreshing	X-Rays	Viability assessment
1.	10,000	24-well plate	500 µl	- Media supplemented with 45 µg of rotifers proteins - Media supplemented with suspension buffer	- 0 Gy -10 Gy	Analyzing cell viability on the 1st, 2nd and 3rd days post-irradiation with Trypan Blue
2.	10,000	24-well plate	500 µl	- Media only - Media supplemented with 100 µg of rotifers proteins - Media supplemented with suspension buffer - Media supplemented with 100 µg of A549 cells proteins	- 0 Gy	Analyzing cell viability on the 1st, 2nd and 3rd days with Trypan Blue
3.	10,000	24-well plate	500 µl	- Media only - Media supplemented with 500, 250, 125, 62.5, 31.25 and 15.625 µg of rotifers proteins respectively - Media supplemented with lysis buffer - Media supplemented with 500, 250, 125, 62.5, 31.25 and 15.625 µg of A549 cells proteins respectively	- 0 Gy	Analyzing cell viability on the 3rd day with Trypan Blue

Colony-forming assay

Colony-forming assays were performed first, without and then, with rotifer lysate and its appropriate controls. One hundred or two hundred A549 cells were seeded in 12-well plates. In the experiments where rotifer lysate was added on cells, 5 to 6 hours after plating, media were removed from each well and replaced by 1 ml of fresh culture media alone, supplemented by rotifer lysate, A549 cells lysate or suspension buffer. Control plates underwent the same manipulations but were not exposed to X-rays while the other plates were exposed to 2 and 5 Gy. Cells were incubated at 37 °C and 5% CO_2 for 12 days. Colonies (clumps of at least 50 cells) were counted after staining with crystal violet. The surviving fraction was obtained by calculating the ratio of the colony number for the irradiated cells to the colony number for control cells. The different experiments undertaken with rotifer lysate are listed in the Table 3.

Tableau 3: List of experiments performed to study rotifer lysate effect on A549 human cells

	Number of cells / well	Type of plate	Total volume of media / well	Conditions tested after media refreshing	X-Rays	Colonies counting
1.	100	12-well plate	1 ml	- Media only - Media supplemented with 100 µg of rotifers proteins - Media supplemented with suspension buffer	- 0 Gy - 2 Gy - 5 Gy	After crystal violet staining
2.	200	12-well plate	1 ml	- Media only - Media supplemented with 100 µg of rotifers proteins - Media supplemented with suspension buffer - Media supplemented with 100 µg of A549 cells proteins	- 0 Gy	

Preparation of samples for ICP-MS analysis

ICP-MS is an analytical technique measuring the content of one or several elements simultaneously in an original sample by using standards, here Fe and Mn. The liquid sample is first nebulized in a central tube, itself heated by an argon plasma which can reach up to 8,000°C. At that temperature, molecules contained in the droplets are atomized and ionized. Ions are then separated according to amass-to-charge ratio, which are then identified and quantified. Initial concentration of each element can thus be calculated by comparison to corresponding calibration standards [Pénicault B. *et al*, 2006].

Dilutions of Fe and Mn (1000 mg/L, Roth, Deutschland) were done in order to prepare standard curves, respectively 1:1,000, 1:3,000, 1:9,000, 1:27,000, 1:81,000, 1:243,000 and 1:9,000, 1:27,000, 1:81,000, 1:243,000 and 1:729,000.

100,000 individuals were collected by scraping the dishes. Mixture of Spa® water and collected rotifers was poured in 15-ml Falcon tubes. After 15 minutes of centrifugation at 4000 rpm, pellet was resuspended in Spa® water and rinsed 3 times with distillated water. The pellet is then transferred in an Eppendorf tube, recovered with 250 µL of distillated water and placed in a dry bath at 98°C with the lid open to allow water evaporation. After 2 hours, 500 µl of nitric acid (20%) are finally added in the Eppendorf tube for 20 hours. Appropriate negative controls were prepared too.

Fe and Mn samples, samples to test and the negative controls were provided to the PC2 platform of UNamur and analyzed by the person responsible of the ICP-MS machine. Equipment used for element analyses is a Shimadzu AA-7000F Atomic Absorption Spectrophotometer (Shimadzy, Japan).

4. Results

The bdelloid rotifer *Adineta vaga* is an organism resistant to desiccation and IR. Theses stresses cause, among other things, DNA DSBs. Those breaks are highly cytotoxic and represent thus a serious threat to survival. DNA DSBs were demonstrated to be efficiently repaired over time in *A. vaga*, suggesting that bdelloid rotifers possess DNA repair mechanisms which are still active after exposure to such stresses. According to numerous studies, Mn, found in high quantities in radioresistant organisms, is involved in the preservation and thus the non-inactivation of proteins by acting as an antioxidant. In this work, the bdelloid rotifers DNA repair pathways were investigated, Mn amounts were quantified as well as the potential protective effect of rotifer lysate on irradiated human cells.

The first part focused on the use of DNA repair inhibitors to study the NHEJ and HR repair pathway activity in bdelloid rotifers. The chosen inhibitors are described in the literature as effective DNA repair inhibitors, being promising therapeutic drugs leading to cell death. Drugs were especially chosen because the genes of the proteins they target are present in rotifers: Rad51 and the MRN complex in the HR pathway (human MRN complex is composed of Mre11/Rad50/NBS1, NBS1 presence was not attested in the *A. vaga* genome) and the ligase IV in the NHEJ pathway [Hecox-Lea BJ. and Mark Welch D., 2018]. However, inhibitors are designed with a high specificity to the human targets, homologies were thus checked by blasting *A. vaga* amino acids (aa) sequences to the human ones on the NCBI website. Results are shown in Figure 9.

Figure 9: Screenshot of homology results. *A. vaga* and human sequences of inhibitors targets were compared using BLAST program on NCBI website. Rad51, MRE11 and ligase IV are respectively inhibited by B02, Mirin and SCR7. No result is available for DDRI-18 which targets a still unknown actor of the NHEJ pathway.

"Identities" refer to the percentage of aa which are identical between both sequences and "Positives" correspond to the percentage of aa that are either identical between the query and the subject sequence or have similar chemical properties. "Positives" values are respectively of 88, 62 and 55% for Rad51, MRE11 and the ligase IV. Sequences of human and bdelloid targets are thus not equal but share similarities. Comparison of target sequences could be reduced to the region where the inhibitor acts, in other words, to the aa sequence recognized and bound by the inhibitor. Similarity of these sites could be more informative than the similarity of the entire proteins. However, this is not easy given that inhibitors act at different levels which are sometimes not yet identified. Moreover, this information was not found in the literature. SCR7 remains an exception: different articles tested its efficacy in CRISPR-Cas9. It turns out that SCR7 inhibits the binding of the ligase IV to the DNA. The putative enzyme sequence targeted by SCR7 in humans was identified and compared to the corresponding region in the *A. vaga* sequence: 100% of correspondence was found between both sequences and is composed as follows: aspartate – leucine – lysine – leucine – glycine – valine – serine – glutamine – glutamine (DLKLGVSQQ) [Srivastava M. *et al*, 2012]. As a consequence, even if the ligase IV sequence only showed 55% of positives aa, the targeted site is entirely similar.

4.1. Fertility of *A. vaga* individuals cultured in HR and NHEJ inhibitors

The potential effect and toxicity of DNA repair inhibitors was first characterized on the reproductive capacity of *A.vaga*. In this first experiment, isolated rotifers were cultured in a medium containing increasing concentration of inhibitors. The impact of DMSO, used to dissolve each drug, was assessed in parallel. This study was performed on hydrated *A. vaga* and on hydrated and irradiated *A. vaga* to see if the inhibitor effect was specific to the presence of DNA DSBs. The ability of isolated adults to produce fertile eggs was assessed by counting adults and eggs after 4, 7 and 11 (or 12) days post exposure to the drug (see Supplemental data 1).

For each drug, statistical analyzes were performed on the data set of day 7 (see Figure 11). Data collected after 11 days was more contrasting between conditions (see Supplemental data 1). Since it was not always possible to report the correct number of individuals in each well due to population growth (> 20 rotifers per well results in a wrong counting of individuals), we missed interpreting the differences at day 11. This limitation could have been avoided by fixing the animals before counting.

First, the control populations (adults + eggs) reported from each of the four tests (*i.e.* grown in absence of drugs and DMSO) were pooled to study the effect of exposure to X-rays (500 Gy) on the generation of new populations 7 days after the initiation of the assays. We reported a significant difference between population size in hydrated *A. vaga* versus hydrated *A. vaga* exposed to 500 Gy of X-rays (p-value < 0.001). The exposure to X-rays is thus correlated with a decrease of population growth reported 7 days post exposure in absence of DMSO or drugs (see Figure 10).

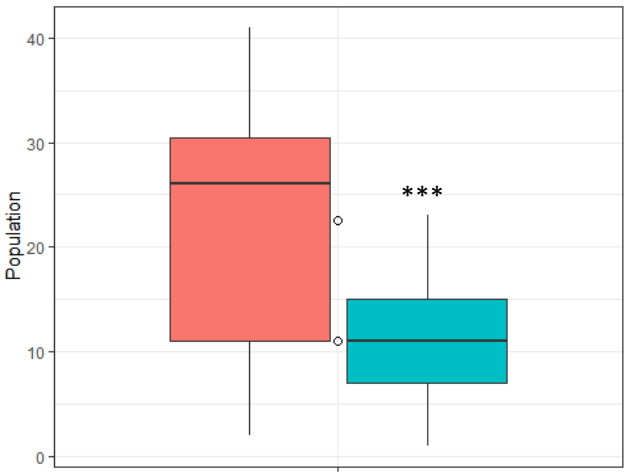


Figure 10: Average population in presence or in absence of X-rays. Boxplots show the average population (alive adults and eggs) at the 7th day. Pink color corresponds to the non-irradiated population and the blue color to the individuals exposed to 500 Gy. Stars correspond to the significance level: * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001.

Second, the potential effect of increasing concentrations of each inhibitor on the *A. vaga* population was assessed separately for irradiated and non-irradiated populations and the impact of DMSO was also studied (see Figure 11).

This assay focused on the ability of one individual to create a new population by laying fertile eggs. HR inhibitors were hypothesized to interfere with DNA repair in germ line cells after exposure to X-rays and the creation of DNA DSBs. If no repair in the germ line occurred due to the inhibition of the HR repair pathway, the laying or hatching of new individuals could have been prevented. For the NHEJ inhibitors, we expected few effects at the fertility level if the NHEJ pathway was not playing a major role at the germ line level.

Results are shown in Table 4 and Figure 11. B02 and Mirin (HR inhibitors) both negatively affected *A. vaga* population growth in the presence of X-rays but also without exposure, from concentrations ranging between 50 µM and 100 µM. SCR7 significantly impaired reproduction at a concentration of 50 µM or higher after X-ray exposure. In the case of DDRI-18, effects appeared from 50 and 10 µM with and without X-ray exposure.

DMSO impacted fertility in some cases only, suggesting that this effect is very low. This effect could be linked to the individuals isolated at the beginning of each assay: if more juveniles or more adults were isolated, the population growth will not be the same between the conditions. Therefore, we could conclude that population decrease in presence of inhibitors can only be assigned to the presence of the inhibitors in the media and not to a cocktail effect DMSO-inhibitors since DMSO alone did not play a major role.

Table 4: Recapitulative table of fertility results in presence of a DNA repair inhibitor. Inhibitors are indicated in the first column, their target in the second one. Effect of the drugs and DMSO are summarized in the third and the last column.

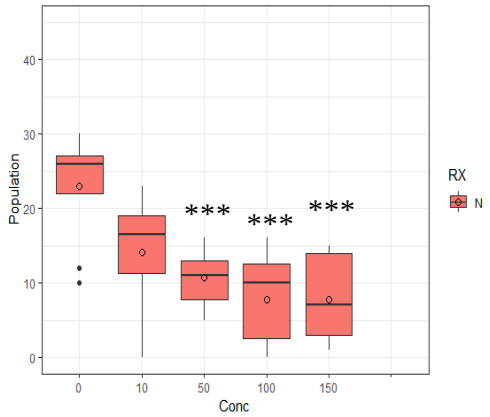
HR inhibitors			
B02	Rad51	Fertility reduced from a concentration of 50 µM (p-value <0.001) in presence and absence of X-rays	No DMSO impact
Mirin	MRN complex	Fertility reduced from a concentration of 100 µM in presence (p-value < 0.01) and absence of X-rays (p-value < 0.001)	No DMSO impact
NHEJ inhibitors			
SCR7	Ligase IV	Fertility reduced from a concentration of 50 µM in presence of X-rays (p-value <0.01), no decrease in absence of X-rays	DMSO effect at the 2 highest concentrations
DDRI-18	Unknown	Fertility reduced from a concentration of 50 µM without X-rays (p-value < 0.001) and from 10 µM in presence of X-rays	Variable DMSO effect

Concerning the statistical tests, the significant effect of the inhibitor/DMSO can only be compared to the control without drug or DMSO of each group. More specifically, the irradiated group (X-rays) and non-irradiated group of the same inhibitor cannot be compared given that X-rays alone negatively impact the population growth. A ratio between irradiated and non-irradiated individuals could eventually be established but nothing consistent was found here. Second, one non-parametric test was performed on data related to DDRI-18 + X-rays exposure given that normality of the residuals was not attested by the Shapiro-Wilk test. The 150 and 200 µM values were removed from the statistical analysis: the low level of observations and the very small variability of the data (only one adult in each well, no reproduction over time) disturbed the test which showed non-relevant biological results.

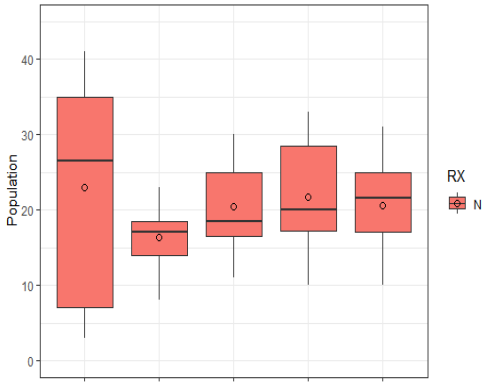
To summarize, both HR inhibitors B02 and Mirin decreased the fertility of *A. vaga* populations at higher concentrations with or without X-rays, suggesting that the inhibitor is lethal to *A. vaga* at a certain concentration. The same observation can be done for the NHEJ inhibitor DDRI-18. The second NHEJ inhibitor SCR7, on the other side, impaired reproduction specifically after X-ray exposure.

HR pathways inhibitors

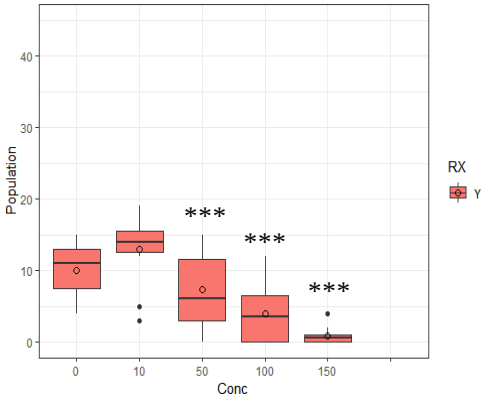
a. B02, no X-Rays



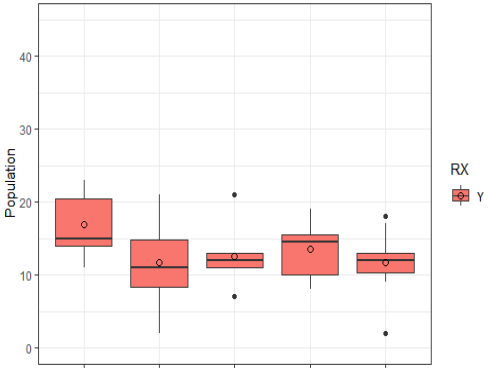
b. B02 DMSO, no X-Rays



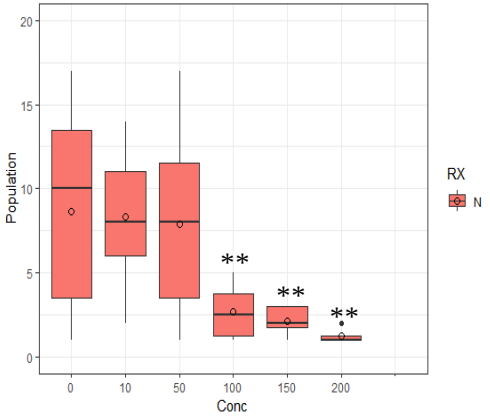
c. B02, post X-Rays



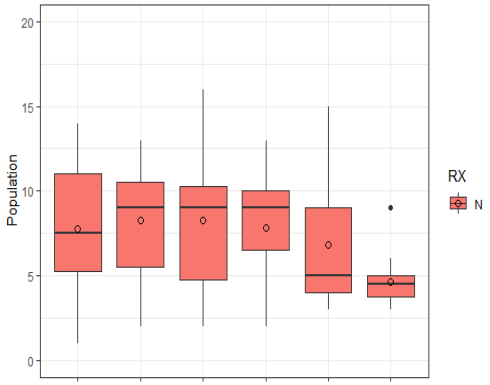
d. B02 DMSO, post X-Rays



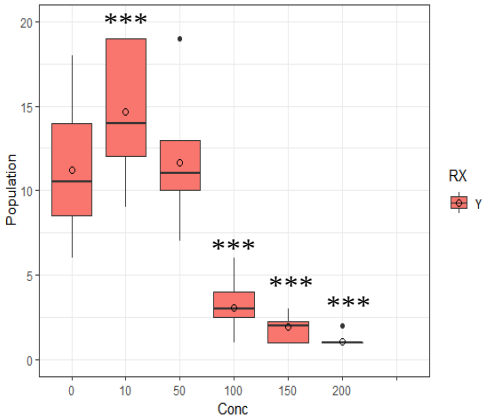
e. Mirin, no X-Rays



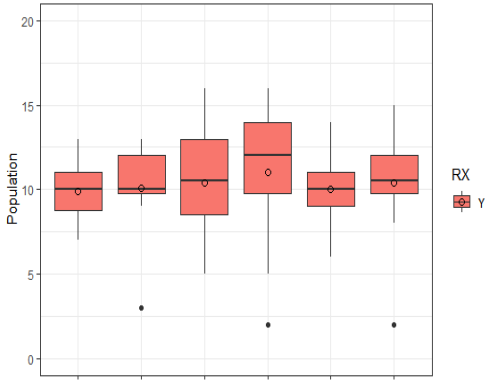
f. Mirin DMSO, no X-Rays



g. Mirin, post X-Rays

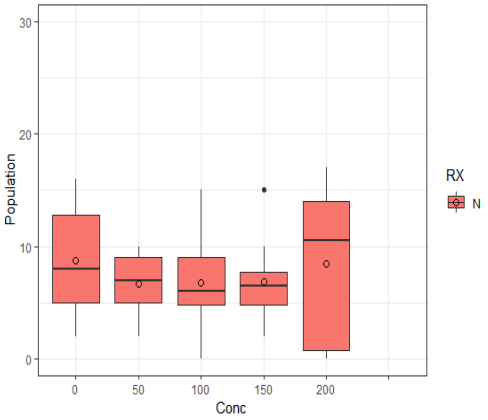


h. Mirin DMSO, post X-Rays

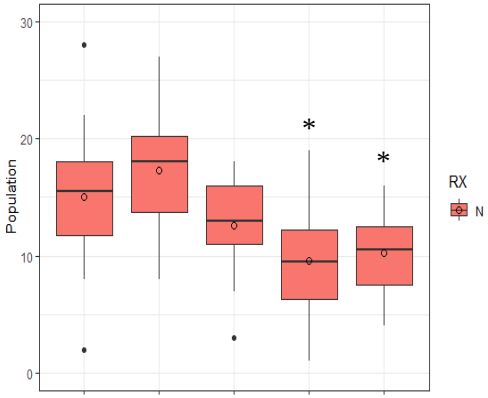


NHEJ pathway inhibitors

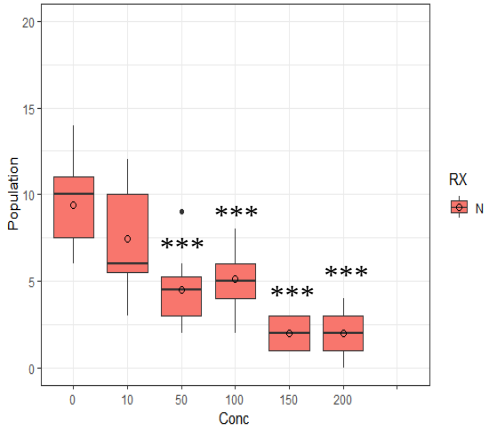
i. SCR7, no X-Rays



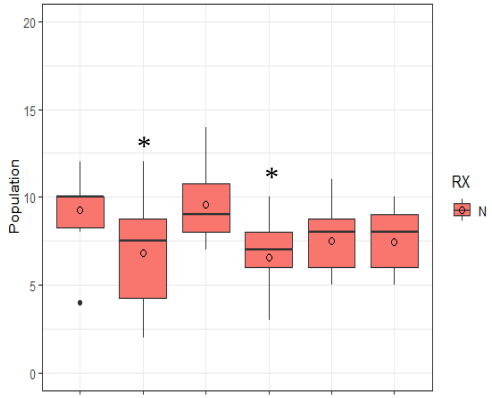
j. SCR7 DMSO, no X-Rays



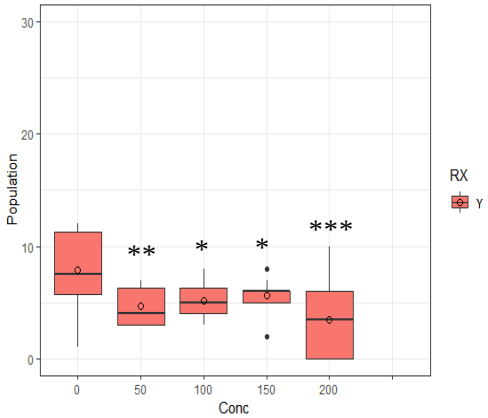
m. DDRI-18, no X-Rays



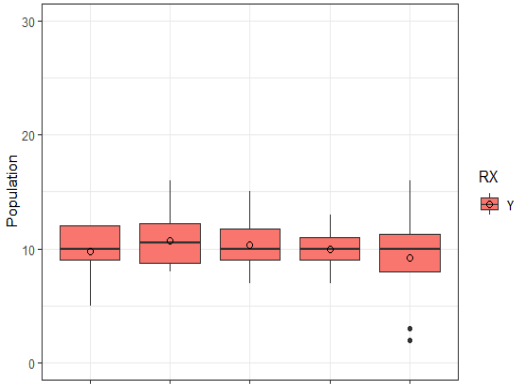
n. DDRI-18 DMSO, no X-Rays



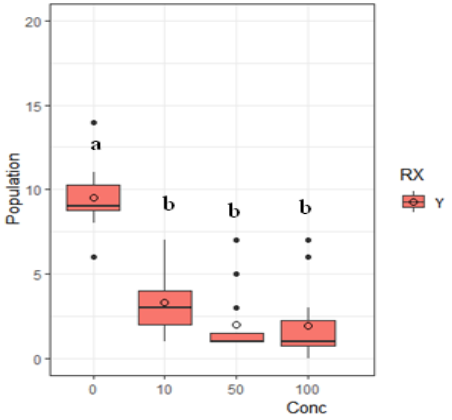
k. SCR7, post X-Rays



l. SCR7 DMSO, post X-Rays



o. DDRI-18, post X-Rays



p. DDRI-18 DMSO, post X-Rays

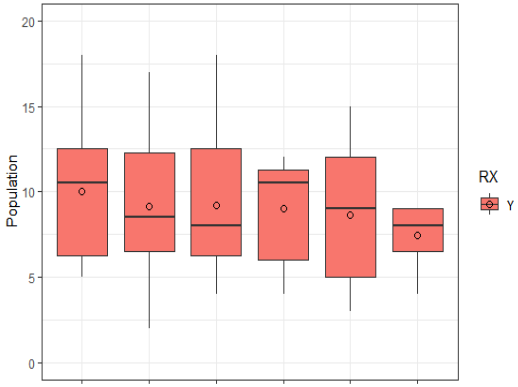


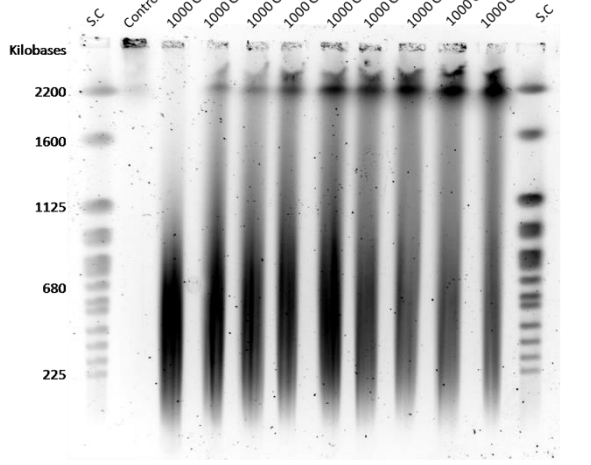
Figure 11: Average population grown in presence of inhibitors (B02 and Mirin for the HR pathway and SCR7 and DDRI-18 for the NHEJ pathway) exposed or not to X-Rays. Boxplots show the average population (alive adults and eggs) at the 7th day. Concentrations are represented on the x-axis. Stars correspond to the significance level: * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001. Letters are indicated if the normality was not assessed. Groups sharing the same letter are not significantly different. The x-axis of the DMSO graphs don't represent the concentration of DMSO but the volume of DMSO used in the control plates to replace the volume of drugs resuspended in DMSO in the experimental plates.

4.2. DNA repair kinetic of somatic cells in presence of inhibitors

The second part of our DNA repair inhibitor screening aimed to study the putative consequences of NHEJ inhibitors on the DNA repair kinetic in somatic cells in irradiated *A. vaga*. Hydrated rotifers were first irradiated to break their genome in multiple fragments. Then, *A. vaga* individuals were put in contact with a drug (experimental condition) or with water (control condition) to assess the potential impact of the condition on the DNA repair. Drugs tested were Scr7 and DDRI-18 at a concentration of 150 µM at which fertility is decreased (HR inhibitors were also tested, results are available in Supplemental data 2). Rotifers were fixed after different exposure periods and genomic integrity was screened using PFGE in order to study DNA repair kinetic in somatic cells with time. PFGE can be used to assess DNA DSBs by separating big fragments up to 15 mega bp thanks to an electric field varying between spatially distinct pairs of electrodes [Cedervall B., and Källman P., 1994; Sharma-Kuinkel, BK. *et al*, 2016].

Results are shown in Figure 12a. SCR7 and b. DDRI-18. First and last lanes (S.C) correspond to the karyotype of *Saccharomyces cerevisiae*. The intact genome of *A. vaga* is composed of 12 chromosomes of approximately 20 Mb and does not match the PFGE resolution (225-2200 kb). Control DNA therefore remained in the wells as observed in the "Control" lanes, with a weak signal around 2,000 kb. In the 1,000 Gy lane, (*i.e.* rotifers fixed directly after the irradiation), a smear appeared reflecting the presence of DNA fragments ranging from 225 to 1125 kb. The maximal intensity is around 680 kb (corresponding to approximately 29 DSBs per chromosome). After 4 hours of repair and at the following time points, smear intensity decreased with the progressive reappearance of bigger fragments of 2,200 kb. No difference in terms of DNA repair can be observed between irradiated rotifers cultured in presence of the inhibitors (experimental condition) or with water (control condition). Similar results were reported for the analysis focusing on larger fragments ranging from 2-7 Mb (see Supplemental data 3).

a.



b.

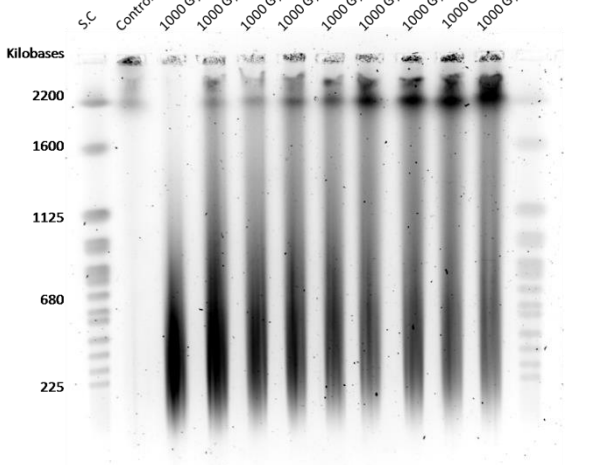


Figure 12: Genomic integrity of *A. vaga*. PFGE after 22 hours of migration. First and last lanes (S.C) correspond to *S. cerevisiae* chromosomes. The "Control" lane corresponds to 1,000 hydrated *A. vaga* individuals. Other lanes correspond to 1,000 *A. vaga* submitted to 1,000 Gy and put in contact with a DNA repair inhibitor: (a) Effect of SCR7 (150 µM) on the DNA repair after 4, 8, 24 and 48 hours of contact. (b) Effect of DDRI-18 (150 µM). Next to each experimental condition is placed the control condition.

Present data suggest no impact of the inhibitors on the DNA repair processes and teach us that rotifers have the capacity to repair their DNA, as shown by PFGE, despite the drug presence.

4.3. DNA repair kinetic of somatic cells after a long incubation with SCR7

In the scientific studies, inhibitors are usually injected in mice, tested on cell cultures or on cell-free extracts but not on entire organisms [Srivastava M *et al*, 2012]. In the repair kinetic experiment, it is therefore possible that the drugs did not access their target inside rotifer cells. We increased thus the exposure time between *A. vaga* individuals and SCR7: the drug was added at a concentration of 100µM to the rotifer cultures during 7 days prior X-rays exposure. Media with drugs were refreshed after 3 days and after irradiation to limit biases due to inhibitor degradation through time or by X-rays. The repair kinetic was again studied by PFGE (see Figure 13).

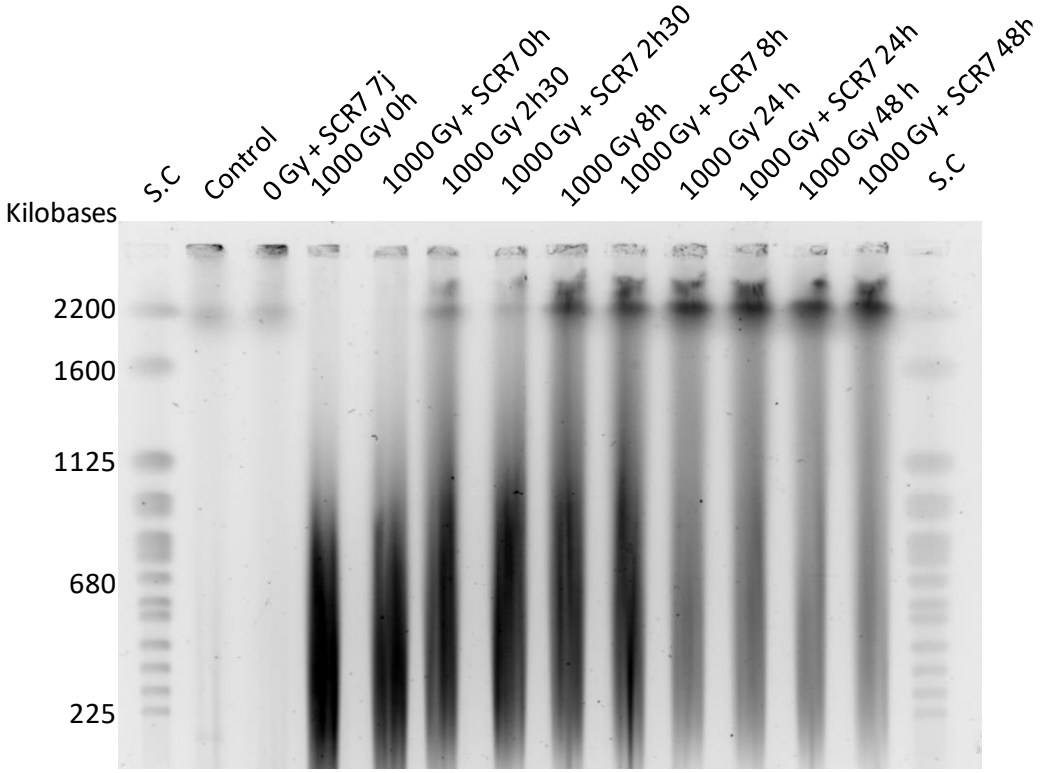


Figure 13: Genomic integrity of *A. vaga*. PFGE after 22 hours of migration. First and last lanes (S.C) correspond to *S. cerevisiae* chromosomes. The "Control" lane corresponds to 1,000 hydrated *A. vaga* individuals. "O Gy + SCR7 7d" lane represents individuals cultured in SCR7 for 7 days and fixed prior the irradiation. Other lanes correspond to 1,000 *A. vaga* submitted to 1,000 Gy and put in contact with SCR7 for 7 days prior irradiation. They were fixed at different time points post X-rays (0h, 2h30, 8h, 24h and 48h). Next to each experimental condition is placed the control condition.

First and last lanes (S.C) correspond to the karyotype of *S. cerevisiae*. The "Control" lane corresponds to 1,000 hydrated *A. vaga* individuals with an intact genome: the DNA band remained mainly within the well with a weak signal around 2,000 kb. At 0 Gy and 7 days SCR7 exposure, the DNA integrity of 1,000 *A. vaga* individuals was not affected and similar to the control. When exposed to 1,000 Gy of X-rays with rotifers being fixed directly after

irradiation, a smear appeared reflecting the presence of DNA fragments ranging between 225 and 1125 kb. The same result was observed when the drug SCR7 is present. At 2h30, 8h, 24h and 48h post-irradiation, smear intensity decreased with the progressive reappearance of bigger fragments of 2,200 kb. No difference in terms of DNA repair can be observed between irradiated rotifers cultured in presence of SCR7 inhibitor (experimental condition) or with water (control condition). These results clearly show that increasing time of contact between the inhibitors and *A. vaga* does not influence the DNA repair capacity of bdelloids, suggesting no effect of the inhibitors on the repair pathways of *A. vaga*.

The second part of this master thesis investigated the role of *Adineta vaga* lysate in the protection of irradiated human A549 lung carcinoma cells. Indeed, humans and most living organisms rapidly suffer from X-rays damage and subsequent ROS production affecting the macromolecules and resulting in cell death. Bdelloid rotifers are one of the few metazoans able to survive more than 1,000 Gy of X-rays. We investigated here if bdelloid lysate could provide protection against IR damage, as studied for *D. radiodurans* [Daly M. *et al*, 2010]. Moreover, since Mn is involved in the antioxidant arsenal of radioresistant organisms, we quantified Mn/Fe ratios in bdelloid rotifers through ICP-MS.

4.4. ICP-MS results

ICP-MS is an analytical technique measuring the content of one or several elements simultaneously in an original sample by atomizing, ionizing and separating them according to a mass-to-charge ratio. Replicates of 300,000 *A. vaga* individuals were prepared in order to measure their content in Mn and Fe. To this end, bdelloid rotifers were pooled and digested overnight by nitric acid (20%) and samples were analyzed in the PC2 platform at UNamur.

Results are presented below and expressed in micromoles (µmol):

	Manganese	Iron	Mn/Fe
For 300,000 *A. vaga*	0.0037 µmol	0.0042 µmol	0.88

The ratio Mn/Fe is equal to 0.88, which is quite similar to the ratio obtained by the Daly's group for the radioresistant bacteria *D. radiodurans* [Daly M. *et al*, 2010]. This high ratio was encouraging for the following experiments testing the effect of rotifer lysate.

4.5. Effect of X-rays on the survival and proliferation of A549 cells

In order to evaluate the effect of rotifer lysate on irradiated A549 lung carcinoma cells, preliminary experiments were first performed with human cells to optimize their cell density, the range of X-ray irradiation used and their cell survival and proliferation post-irradiation using Trypan blue staining, MTT assay and crystal violet staining. The first experiment involved seeding 5,000 and 10,000 cells, followed by X-ray exposure with 0, 5, 10 and 20 Gy, 24 hours after seeding. Survival was measured on 1st, 2nd and 3rdday post-irradiation by Trypan Blue (TB) staining and via MTT assays (see Figure 15). The same experiment was repeated in order to understand the effect of X-rays after a longer period, namely after the 3rd, 5th and 7thday post-irradiation (data not shown). The experiments highlighted a morphological change in irradiated cells: they appeared enlarged as compared to the controls, a sign of

mitotic catastrophe, considered as one of the major type of cell death induced by IR (see Figure 14) [Riquier H. *et al*, 2013].

This preliminary experiment highlighted an optimal growth curve for cells seeded with an initial density of 10,000 cells, which was used in subsequent experiments. The X-ray doses had a deleterious effect on cell survival: cells exposed to 5 Gy of X-rays were observed to have a low proliferation during the three days post irradiation, while cells exposed to 10 Gy of irradiation were not observed to proliferate. Cells exposed to 20 Gy of X-ray irradiation had extremely low cell survival and therefore nothing significant could be interpreted. Experiments performed with the MTT assay highlighted an increased amount of metabolic activity post-irradiation (Day 1 to 3 and 5 to 7) and therefore, effects on cells post-irradiation could not be established. Indeed, MTT assay measures the cell survival by monitoring their metabolic activity. After X-ray exposure, cells died and their metabolic activity should decrease. We observed the opposite here with an increasing metabolic activity with time, which was not biologically relevant.

a. b.

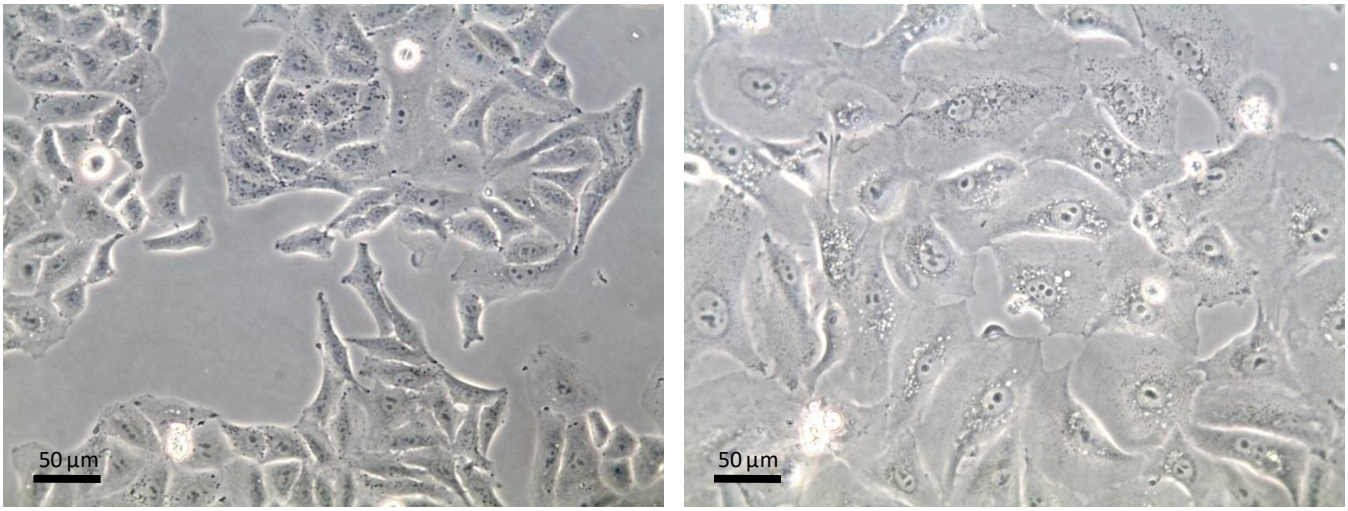


Figure 14: A549 cells exposed to 0 Gy (a) and 20 Gy (b) 3 days post X-rays exposure. Cells exposed to X-rays appear bigger than control cells because they underwent mitotic catastrophe. 200x magnification.

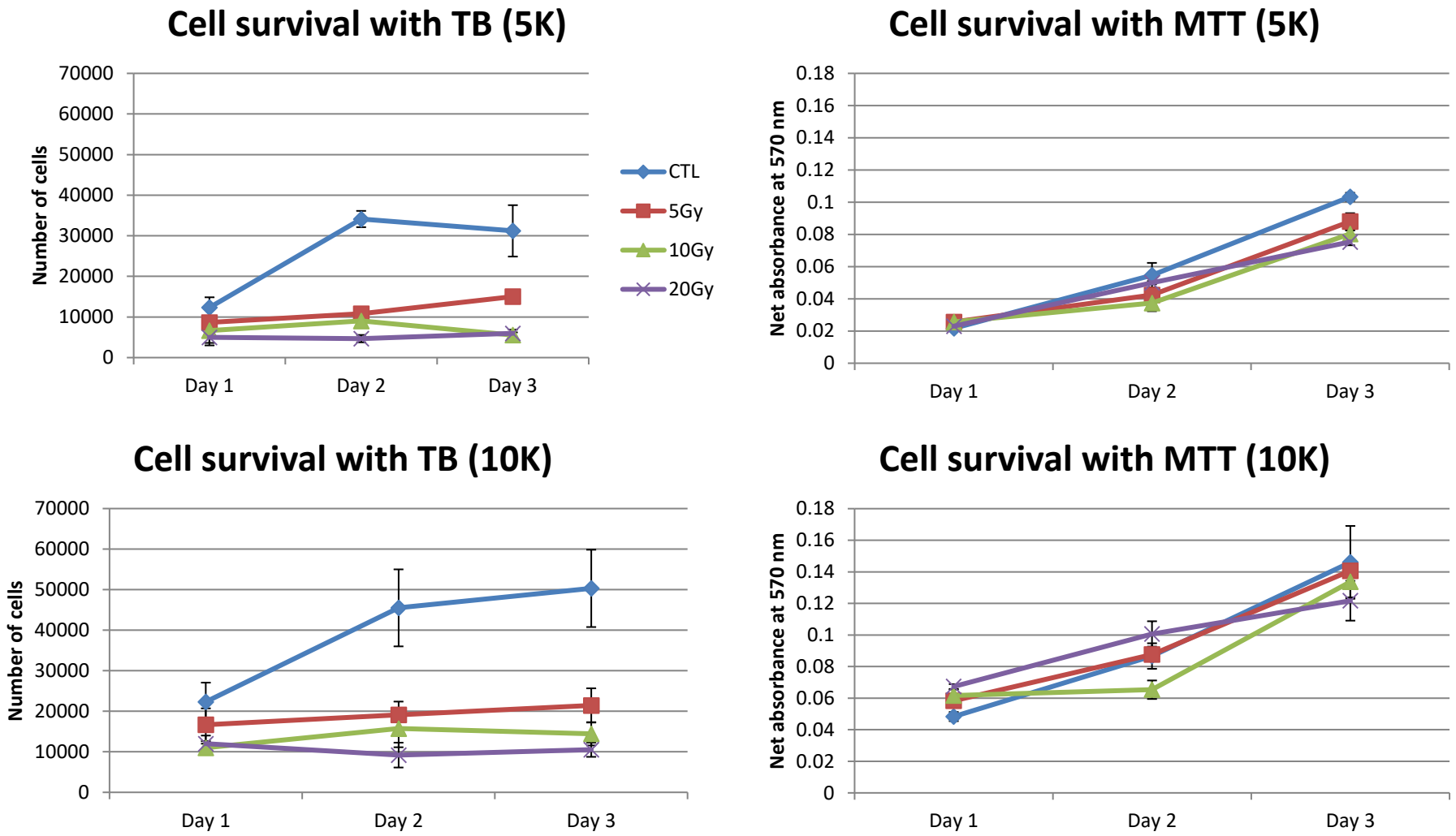


Figure 15: Graphs showing the cell survival 1, 2 and 3 days after irradiation with 0 (CTL), 5, 10 and 20 Gy of X-rays. Cell survival was measured through Trypan Blue (TB) staining and MTT assay. 2 cell densities were tested: 5,000 (5K) and 10,000 (10K) cells. Time is represented on the x-axis and on the y-axis, the number of cells or the net absorbance at 570 nm according to the technique. Standard deviations are indicated on the graphs.

To understand the effect of X-ray irradiation on cell proliferation, clonogenic assay was performed. This is based on the principle of seeding isolated cells in a multi-well plate and following the proliferation of each cell to form a colony of at least 50 cells in 10 to 12 days after X-ray irradiation. First, cell density optimization was performed by seeding 100 and 200 cells and irradiating them at 0, 2 and 5 Gy of X-ray irradiation. 12 days post- irradiation, colonies were counted using crystal violet staining and the survival percentages were calculated and plotted. Survival percentage represents the ratio of colonies counted in the irradiated conditions to the colonies counted in the control group. For both groups (100 and 200 cells), it remained quite similar with 70% of cell being proliferative after exposure to 2 Gy, while 5 Gy was observed to have about 30-35% of cells actively proliferating (see Figure 16).

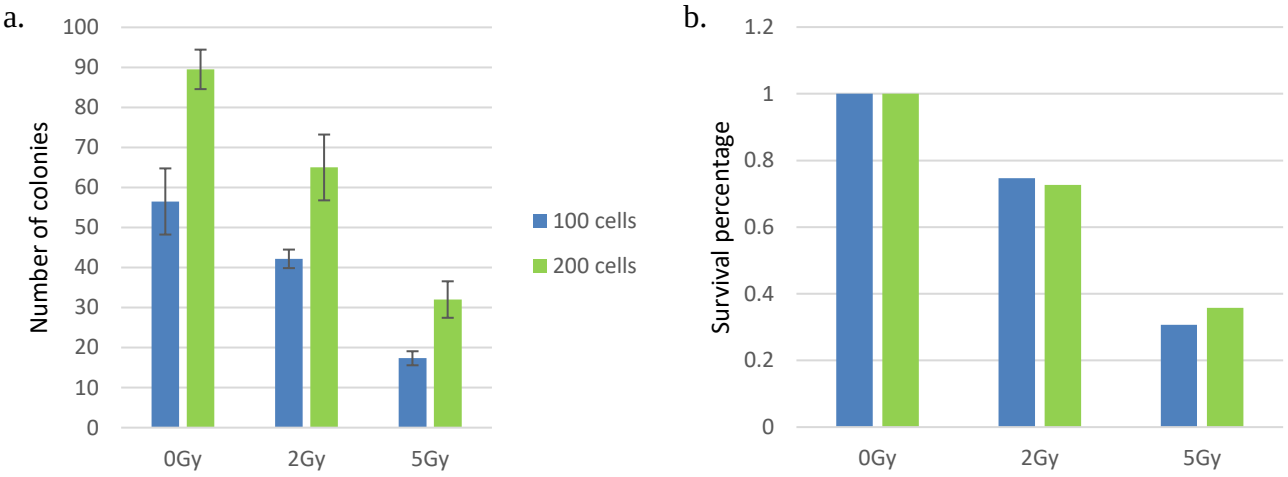


Figure 16: Graphs showing the number of colonies (a) and the survival percentage (b). X-ray doses are represented on the x-axis, the y-axis represents the number of colonies 12 days post irradiation or the survival percentage. Blue color corresponds to the initial seeding of 100 cells and the green color to the seeding of 200 cells. Standard deviations are represented.

4.6. Protein extraction from hydrated *A. vaga*

In order to prepare *A. vaga* lysate suitable with human cell culture, individuals were pooled, suspended in a phosphate buffer and lysed in a dounce by performing 1,000 mechanical ruptures. The sample was then centrifugated to separate the pellet, composed of membrane debris, from the supernatant containing proteins. The supernatant was kept and stored for further experiments. In a second step, the pellet was re-lysed in buffer using the dounce and re-centrifuged to maximize the protein extraction. The lysis-centrifugation step was repeated 3 times and 3 protein fractions were obtained. The extraction protocol relies thus only on the mechanical rupture of rotifer cells.

10 µg of proteins of the two first fractions were loaded on SDS gels in order to perform a Coomassie blue staining to obtain a proteome overview. Western blots (WB) using antibodies directed against histone H3 (nuclear protein) and α-tubulin (cytoplasmic protein) were also done to observe if the nuclei and the cytoplasmic region were reached by the extraction protocol (see Figure 17). As depicted by the Coomassie blue stained gel, proteins from a high to a low molecular weight are present in both fractions. On the WB, an α-tubulin signal (~50 kDa) is clearly visible in both fractions meaning that cells were well lysed and fractions were enriched in cytoplasmic regions. However, the nuclear marker histone H3 was not detected although a very weak signal could be observed around 15 kDa in the second fraction.

a.

b.

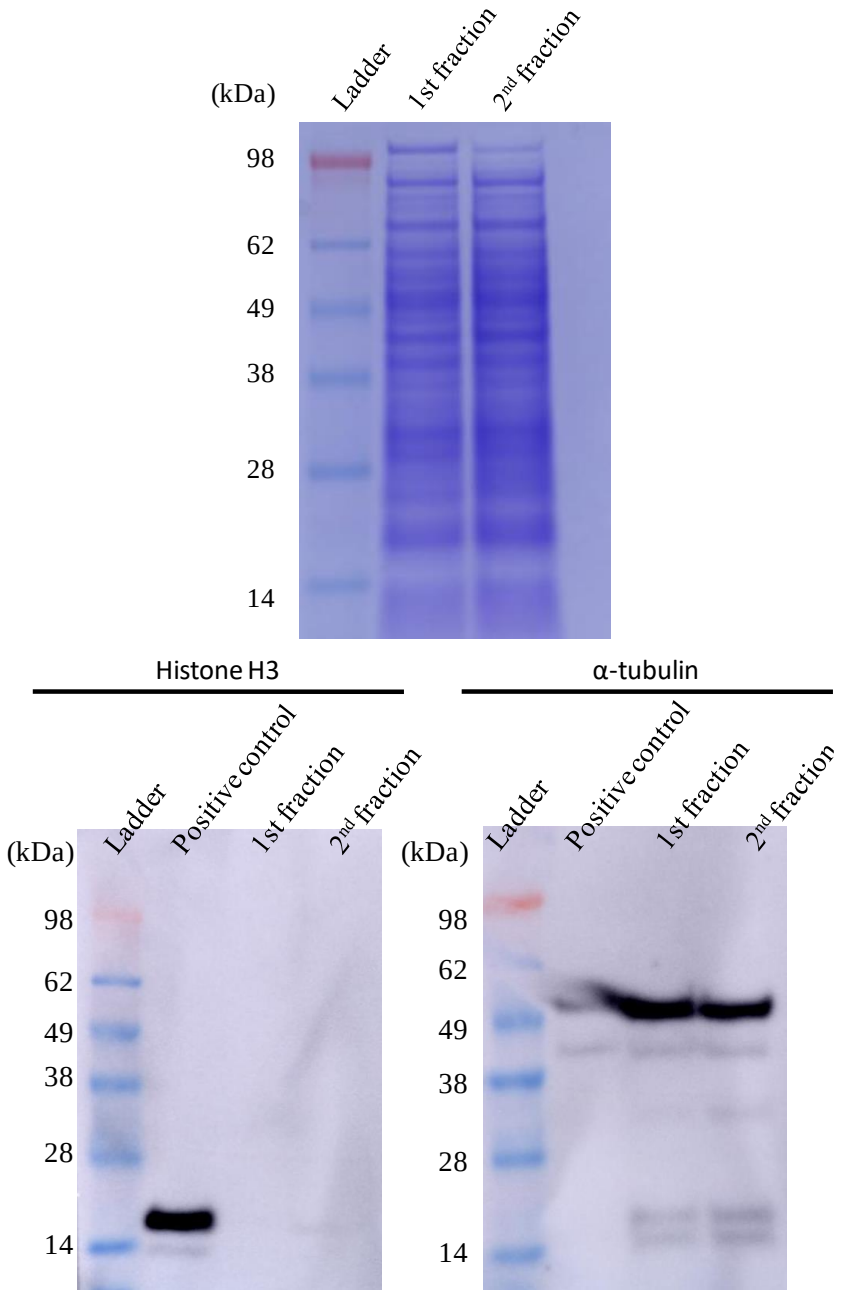


Figure 17: Coomassie blue stained gel (a) and western blots (b) charged with 10 µg of proteins. Proteins were extracted by mechanical ruptures of pooled *A. vaga*. The two first fractions were loaded on the gels. The Coomassie blue stained gel shows the proteome profile. For the Western blots, a positive control was added: a bdelloid rotifers extract containing cytoplasmic and nuclei proteins. Antibodies used were directed against α-tubulin and histone H3.

4.7. A549 cells supplemented with *A. vaga* lysate

The potential protective effect of supplementing human A549 cells with rotifer lysate was investigated here. First, cells were supplemented with 45 µg of *A. vaga* proteins 24 hours prior to X-ray irradiation at 10 Gy. Controls were prepared by replacing the volume of rotifer lysate by the same volume of suspension buffer. Viable human cells were counted using TB staining the 1st, 2nd and 3rd days post-irradiation (Figure 18).

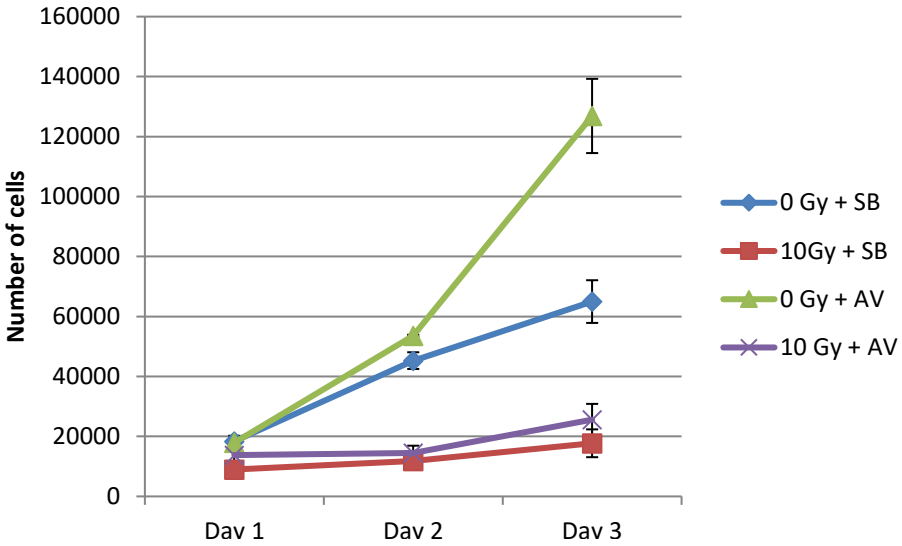


Figure 18: Number of A549 cells supplemented with 45 µg of rotifer lysate (AV) or with suspension buffer (SB). Cells exposed to 0 and 10 Gy of X-rays were counted with TB staining the 1st, 2nd and 3rd days after the irradiation. Days are represented on the x-axis and the number of cells on the y-axis. Standard deviations along the mean are also represented.

Data show that human cells incubated with rotifer lysate (AV) had a higher proliferation as compared to the ones that are incubated with suspension buffer (SB). Such a proliferative advantage after rotifer lysate incubation was observed in the control group where cells were not irradiated: there was a 2-fold increase of cell density 3 days post-irradiation (see Figure 18). In the experimental group, cells that were incubated with rotifer lysate and irradiated with 10 Gy of X-rays also showed a higher proliferation than cells incubated with only suspension buffer, but only a mere 1.5-fold difference.

To investigate more in detail this proliferative effect, 10,000 human cells were seeded and supplemented with their culture media only, with media suspended with 100 µg of rotifer lysate, media suspended with equal volume of suspension buffer and media supplemented with 100 µg of proteins extracted from A549 human cells. This last condition was added as a negative control to observe the protection effect by proteins of A549 culture. Day 0 corresponded to the day of X-ray exposure, and in the condition without irradiation, day 0 was considered as the day following the plating. Number of cells were counted on the 1st, 2nd and 3rd days (see Figure 19). The results outlined no significant proliferative advantage from any extract, although cells incubated with rotifer lysate and culture media seemed to have more cell density on the 3rd day.

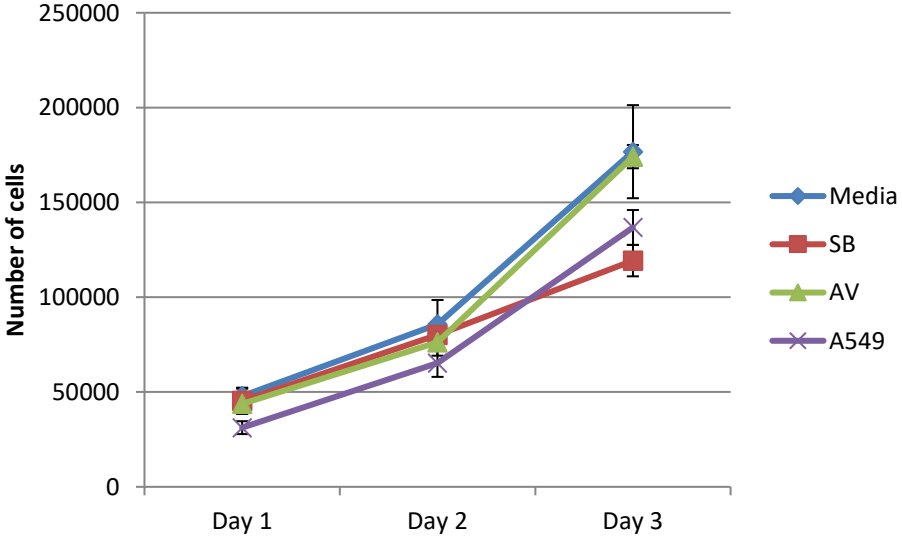


Figure 19: Number of A549 cells supplemented with media only (MD), with 100 µg of rotifer lysate (AV), with suspension buffer (SB) and 100 µg of A549 lysate (A549). Day 0 is considered as the day following the plating, cells were then counted on Day 1, 2 and 3. Days are represented on the x-axis and the number of cells on the y-axis. Standard deviations are also represented.

The following experiment investigated the highest quantity of rotifer lysate and human cell lysate that could be supplemented to A549 human cells without any toxic effect. To this end, a range of rotifer and human protein concentrations (serially diluted from 500 to 15.624 µg) were tested. Higher concentrations of proteins were expected to exert a toxic effect. This experiment showed that 100 – 125 ug of protein lysate was the optimal concentration for both extracts. Higher protein concentrations had a negative effect on cell density, probably because of the absence or reduced availability of fetal bovine serum in the growth medium. The suspension buffer, when present in high quantities, negatively impacted cells too.

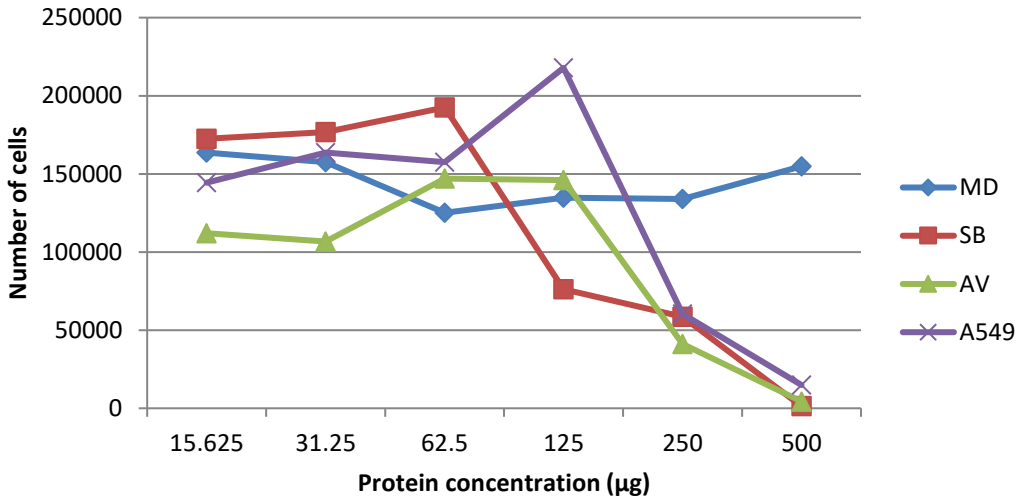
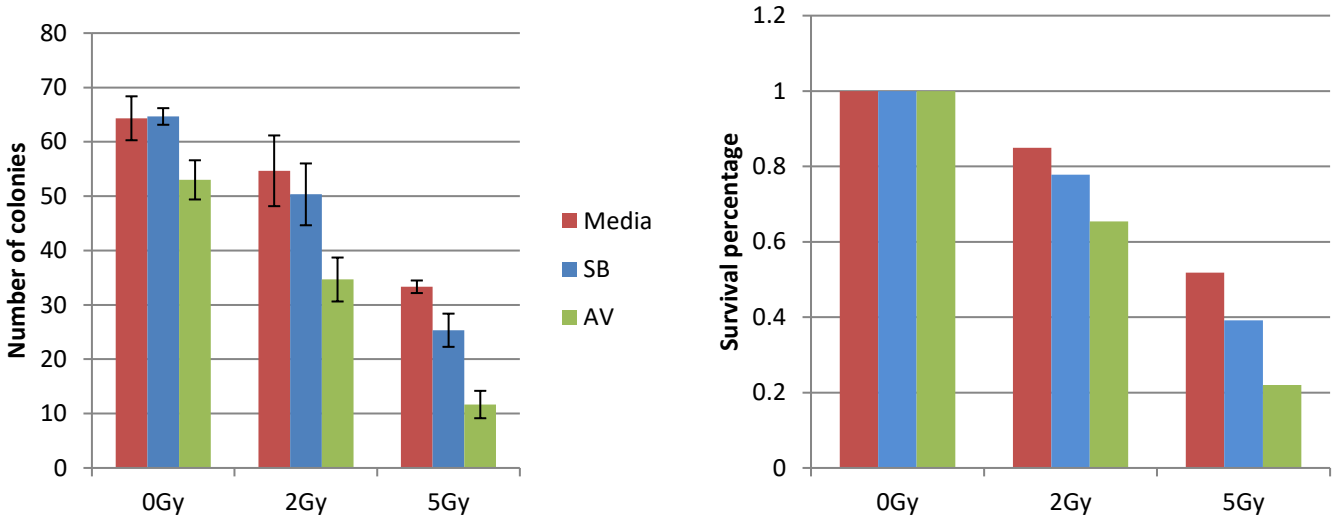


Figure 20: Number of A549 cells in presence of different protein concentrations of rotifers and human cells lysate. "MD" means media only, "SB": suspension buffer, "AV": rotifer lysate and "A549": human A549 cell lysate. Quantities of proteins are represented on the x-axis and the number of cells on the y-axis.

Last, two clonogenic assays were performed where a few hundred human cells were seeded at the beginning, and their ability to create colonies of at least 50 cells was assessed after 10-12 days. If rotifer lysate effectively stimulatd cell division, colonies were expected to grow faster in the presence of rotifer lysate.

In the first clonogenic assay (Figure 21a.), 100 cells were seeded and the media changed 6-7 hours after seeding with media not supplemented or supplemented with 100 µg of rotifers lysate or suspension buffer. After 12 days, colonies were counted using the crystal violet staining. During these experiments, human cells were contaminated (see Figure 21b.) in the conditions where rotifer lysate were added and hence nothing could be interpreted. Repeating these experiments was impossible due to lack of time. Contaminations were seen in different experiments which were not included in this work. It appeared after at least 2 or 3 days of incubation and mostly in the wells where cells were supplemented with rotifer and human proteins. Bacteria appeared to be the source of contamination with human cells appearing stressed, a "whitening" of the media and sometimes, bacteria visible in the supernatant.

a.



b.

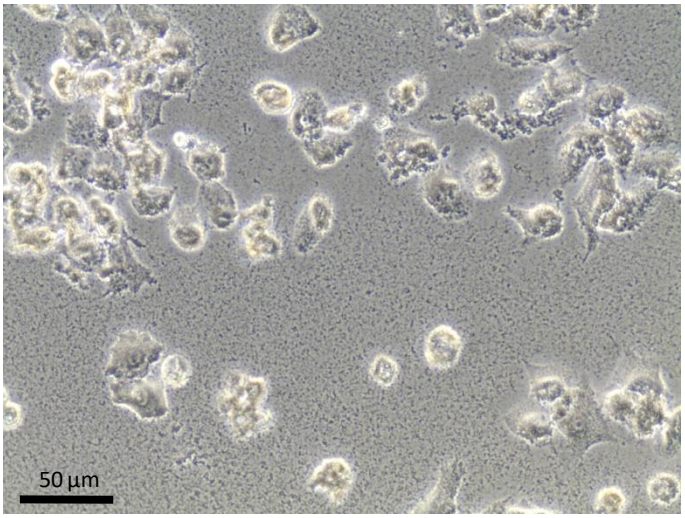


Figure 21: Results of the first clonogenic assay in presence of 100 µg of rotifer proteins. a. Number of colonies and survival percentage. X-ray doses are represented on the x-axis, the y-axis shows the number of colonies or the survival percentage. "SB": suspension buffer, "AV": rotifer lysate. b. Appearance of cells supplemented with rotifer lysate. 200x magnification.

The second experiment which consisted of seeding 200 cells and supplement them with culture media, suspension buffer, 100 µg of rotifer proteins or human proteins showed, after 10 days of development, similar number of colonies throughout all conditions (between 147 and 163) (see Figure 22).

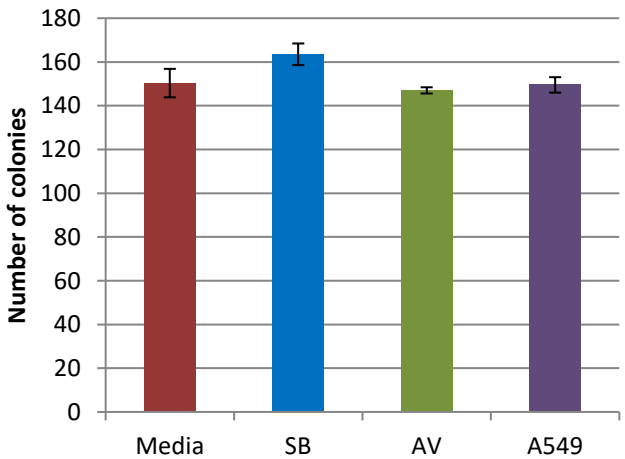


Figure 22: Number of colonies 10 days after seeding of 200 cells in presence of 100 µg of rotifer or human proteins. Cells were supplemented with "SB": suspension buffer, "AV": rotifer lysate, "A549": human A549 proteins. Conditions are on the x-axis, number of colonies are on the y-axis.

5. Discussion and perspectives

If the bdelloid rotifer *Adineta vaga* is resistant to desiccation and IR, it nonetheless undergoes the consequences of these two conditions: the DNA DSBs. As hypothesized by Daly's group, the ability to resist and survive seems to reside in protein protection as opposed to DNA protection, since these extreme-tolerant organisms suffer from similar levels of DNA DSBs than sensitive organisms [Daly M., 2009]. However, the exact nature of the DNA DSB repair mechanisms active in bdelloid rotifers as well as the antioxidant system protecting against damage are not elucidated.

Within this Master thesis, we studied their DNA DSBs repair system using known NHEJ and HR inhibitors to test if it was possible to inhibit these two DNA repair pathways occurring in bdelloid rotifers. Increasing concentrations of NHEJ and HR inhibitors were added in the culture media of hydrated and irradiated *A. vaga* to follow their ability to recreate a population. If their reproduction was impaired following X-ray radiation, the number of individuals was expected to stay the same or decrease, suggesting that the drugs may have inhibited the repair machinery in the germ line cells. Since germ line cells are in a G2/S state and probably preferentially use the HR pathway, HR inhibitors were particularly of interest in this fertility assay [Terwagne M. *et al*, in prep].

First, we observed that bdelloid rotifer reproduction decreased when exposed to 1,000 Gy of X-rays. It is consistent with the results presented by Latta L. *et al* (2019) where bdelloid rotifers exposed to IR, facing an oxidative stress, were investing their efforts in somatic maintenance rather than reproduction. When adding NHEJ and HR inhibitors, *A. vaga* reproduction was affected at higher concentrations and the population size decreased, independent of X-rays radiation, except for SCR7 where effects appeared only after X-ray exposure.

Second, we did experiments that focused on the impact of inhibitors on the DNA repair kinetic of bdelloid rotifers through PFGE. Here, differences between control and experimental conditions were expected only for the NHEJ inhibitors. Indeed, PFGE shows a signal emitted by somatic cells, constituting the largest part of the bdelloid body, and which are in a G0/G1 state. In absence of sister chromatids, NHEJ is the preferred repair pathway suggested to occur [Terwagne M. *et al*, in prep]. However, differences in the repair were not observed: PFGE signal was identical in the presence or absence of drugs and *A. vaga* repaired their DNA with the same efficiency in both cases.

We hypothesized that the main problem was the penetration of the inhibitors inside the cells and their interaction with their target. To eventually solve the penetration problem and in order to confirm the potential observed effect of SCR7, we decided to re-do the repair kinetic assay with SCR7 by leaving the *A. vaga* culture for 7 days with 100 µM SCR7 prior to irradiation. We hypothesized that eggs, by going through different stages in their development, would be more permeable at some points and allowed drugs to enter inside the cells or adults might have integrated with time the inhibitor in their cells. Media were refreshed several times to prevent problems linked to the degradation of the inhibitor with time or during X-ray exposure. No differences in the repair kinetic were observed as compared to the control condition.

By combining the fertility and repair kinetic results during inhibitor exposure, we concluded that *A. vaga* individuals were impaired in their reproduction but without evidence showing that the inhibitors reached their target and acted at this level. We hypothesized that the deleterious effect (*i.e.* an impaired development of the population) was linked to a non-specific mechanism, since effects were only observed at high inhibitor concentrations and independently of X-rays exposure, preventing any conclusion regarding the specific action of the drugs tested. In the literature, B02 concentration used to efficiently inhibit human Rad51 is 10 µM [Alagpulinsa D. *et al*, 2014] and 25-50 µM for Mirin directed against the MRN complex [Dupré A. *et al*, 2008]. For SCR7, the concentration used on human cells is 50-100 µM [Vartak S. *et al*, 2018], at this concentration we observed a significant impairing of *A. vaga* reproduction after X-ray exposure only. However, this result was not confirmed when *A.vaga* were exposed a second time to 100 µM SCR7 during 7 days. In brief, inhibitors are suggested to have an off-target effect in *A. vaga* and appear to create an environmental toxicity, preventing rotifers to reproduce. Moreover, the BLAST results comparing the human targets to the *A. vaga* targets showed a decrease of similarity of each inhibitor target. To conclude, the penetration or the recognition of the targets appears problematic when using human drugs.

However, an experiment studying the DNA repair kinetic following inhibitor exposure should be done with desiccated *A. vaga*. Our tests with the inhibitors were done only on hydrated individuals which already began to repair their DNA while the irradiation was not yet finished. By adding the drug just after X-rays exposure, we may have missed the critical time point of blocking the target before it already started repairing the DNA DSBs. Since desiccated individuals are "in pause" (*i.e.* no active metabolism, [Ricci C., 2017]), the repair will only start upon rehydration and this could be done in a medium containing the inhibitors. Moreover, desiccated *A. vaga* could have more permeable membranes, increasing the chance of drug penetration inside the cells.

This inhibitor assay was only one of the different research experiments ongoing in the LEGE laboratory to discover the DNA DSBs repair mechanisms of *A. vaga*. If *in vivo* results are not convincing, probably because of permeability problems (or target issue), *in vitro* approaches could be considered. *In vitro* ligation assays are currently being optimized in the LEGE team and they could use the inhibitors. More specifically, rotifer lysate is applied on purified small DNA fragments. If the *A. vaga* DNA repair machinery is active, we may see a repair and a ligation of these fragments. This repair can then be monitored by gel electrophoresis. Once this first step is validated, inhibitors could be added with the rotifer lysate. If the repair is impacted, the target was effectively inhibited and thus, its expression in *A. vaga* is attested.

The second part of this Master thesis focused on the antioxidant arsenal of *A. vaga*. Since bdelloid rotifers are able to withstand huge doses of IR, we could naturally wonder if the effect could be reproduced *in vitro* by incubating rotifer lysate with human cells. Besides possessing numerous genes coding for enzymes such as superoxide dismutases and glutathione peroxidases to neutralize the huge production of ROS, the involvement of Mn-based antioxidants was highlighted. One common trait of radioresistant organisms which differentiates them of radiosensitive organisms is not only containing huge amounts of Mn, but rather a high Mn/Fe ratio. The first step here was to measure this ratio in *Adineta vaga* through ICP-MS and, it was observed to be equal to 0.88 which is of similar magnitude as observed by Daly's group for the bacteria *D. radiodurans* [Daly M. *et al*, 2010]. It is planned in the future experiments of the LEGE group to go further and perform the measurements on

various bdelloid species, each one being characterized by its own resistance level to desiccation and IR to eventually highlight differences.

Daly M. *et al* demonstrated in 2010 that *D. radiodurans* ultrafiltrates, free of proteins but enriched in Mn-low molecular weight complexes were able to fully protect Jurkat T cells until 16 Gy of IR exposure. Given that bdelloid rotifers share similar high resistance properties, we tried to reproduce the experiment to observe if *A. vaga* lysate can provide protection when added to human cells exposed to IR. This experiment was totally exploratory within this Master thesis since it was never conducted before. Whole rotifer lysates were obtained by douncing the individuals and were added on human cells. Unfortunately, results obtained were highly variable. A proliferative effect of human cells when supplemented with rotifer lysate was once highlighted, in presence or not of X-rays, but could not be replicated. A protective effect against IR damage was neither demonstrated. Indeed, cell viability was, at best, similar to the respective control condition just containing media and even sometimes lower. This lowering effect was sometimes linked to the appearance of contaminations despite the use of antibiotics and the filtration of lysates prior use. This problem mainly occurred in the wells where cells were supplemented with rotifer lysate and human protein lysate whereas the other wells of the same plate were not affected, excluding a whole contamination due to experimental errors. We thought that the contaminations in human A549 lysates were the consequence of the fact that we used the same dounce than for rotifer lysates, without any sterilization step in between. We therefore performed the same lysis protocol by using an autoclaved dounce but the same contamination problem still reappeared. This first problem led us to think about an alternative protein extraction protocol which would chemically lyse bdelloid rotifers and the bacteria present in their culture media, without damaging proteins neither inactivating them. Indeed, the extraction protocol performed until now just relied on the mechanical lysis of rotifers with a dounce, probably keeping bacteria intact in the lysate. After chemical lysis, a dialysis would be done to remove the buffer, probably incompatible with a future application on human cells, and replace it with a phosphate buffer. An additional step would be probably requested to concentrate the proteins. Moreover, by this second extraction protocol, rotifer lysate would be enriched in cytoplasmic fractions and in nuclear fractions, which is not currently the case and maybe deprives lysates from important nuclear components. Feasability of this second extraction protocol will be tested in the future.

When no contamination was visible with our classic extraction protocol, variability between the tests appeared problematic. This should be reduced by using all the lysate concentrations (*i.e.* 1 mg/ml or 0.5 mg/ml). Between the different experiments we performed, the same quantity of lysates was not always added to reach a certain amount, for example 100 µg of proteins, and was depending on the initial concentration of the lysate.

What is currently lacking in our experiments is a positive control, for example, *D. radiodurans* ultrafiltrate or *in vitro* reconstituted mixtures of Mn, peptides, orthophosphates, etc. as described in Daly M. *et al*, 2010 with concentrations approximating those in the *D. radiodurans* cytosol. This reconstituted mixture protected large and multimeric enzymes until 50 kGy [Daly M. *et al*, 2010]. We could check if A549 cells incorporate the components added in the media and are protected against X-rays damage. Indeed, we don’t see any protective effect of rotifer lysate: is it a problem concerning the rotifer lysate or the inability of cells to take profit of it?

The aim of this rotifer lysate experiment is to study whether rotifer extracts protect human cells from oxidative stress damage and in a second step, shed light on which components

exert the protective effect against X-rays damage: is it the Mn bound to small complexes or the interaction of Mn with the numerous enzymes involved in the ROS neutralization? An ultra-centrifugation step could thus be added after the lysis protocol to separate components such as proteins and on the other side, Mn and low-molecular weight complexes.

6. Conclusion

Bdelloid rotifers *Adineta vaga* are microscopic metazoans with peculiar features: their ancient asexuality and their ability to resist desiccation and IR are surprising. Basis of this resistance seems to reside in their ability to protect their proteins from ROS damage, and thus, preserve their DNA repair machinery. Their important antioxidant arsenal comprising different enzyme based and non-enzymatic systems is suspected to shield the proteome from oxidative insults. However, different questions come to mind: which DNA repair mechanism(s) do they use? Can the molecules providing extreme stress tolerance be isolated? What is the molecular basis of a high Mn uptake in bdelloid rotifers?

In this master thesis, we tried to better understand their DNA repair machinery by using different NHEJ and HR inhibitors. Moreover, we tried to characterize the possibility of a rotifer lysate to protect human cells against X-rays damage and we quantified the amount of manganese and iron in complete rotifer extracts.

The NHEJ/HR inhibitors approach to better understand the DNA DSBs repair pathways was not conclusive and unfortunately, we were thus unable to highlight the specific action of inhibitors on their targets and thus the occurrence of NHEJ or HR in bdelloid rotifers. However, other approaches in the LEGE laboratory have already demonstrated the expression of transcripts of genes coding for the NHEJ and HR pathways. Some transcripts seem to be constitutively present and stresses such as desiccation and IR impacted their level of expression. This could explain why bdelloid rotifers react quickly and are very efficient in their DNA repair. *In vitro* ligation assays which are currently optimized will bring information concerning the proteins and their expression level.

Second, we were not able to reproduce the interesting results obtained by the preliminary experiment performed by incubating A549 cells with rotifer lysate. However, this experiment, being at its beginning, still needs to be improved and optimized. With the huge similarity in the antioxidant arsenal of the radioresistant bacteria and bdelloid rotifers, we hope to be able to show similar results with *A. vaga* lysate. The cornerstone of their radioresistance seems to reside in their proteome protection thanks to numerous enzymes but also the accumulation of Mn. The correct knowledge of these mechanisms would be of great importance in the management of oxidative stress, opens new perspectives and potential applications for the daily life: developing new radioprotectants in radiotherapy treatment, new skin care products preventing aging of the skin (the so-called fountain of youth), etc. [Jin M. *et al*, 2019].

If the secrets allowing bdelloid rotifers to live and prosper around the world, even in harsh conditions are well kept, the new technologies and approaches used by scientists in the LEGE team highlight progressively interesting elements of bdelloids physiology. The new genome of *A. vaga* is currently being finalized and shows the presence of homologous chromosomes; HGT seem to be implied in the bdelloid rotifers survival without sex since million years as well as their desiccation resistance which, coupled with their effective DNA repair mechanisms, allow them to repair their DNA and the potential deleterious mutations after creation of DNA DSBs; the sequence of steps involved in eggs development which was a total black box until recently but shows promising preliminary results in the ongoing research, among other things. Perspectives for further researches are thus numerous and diverse.

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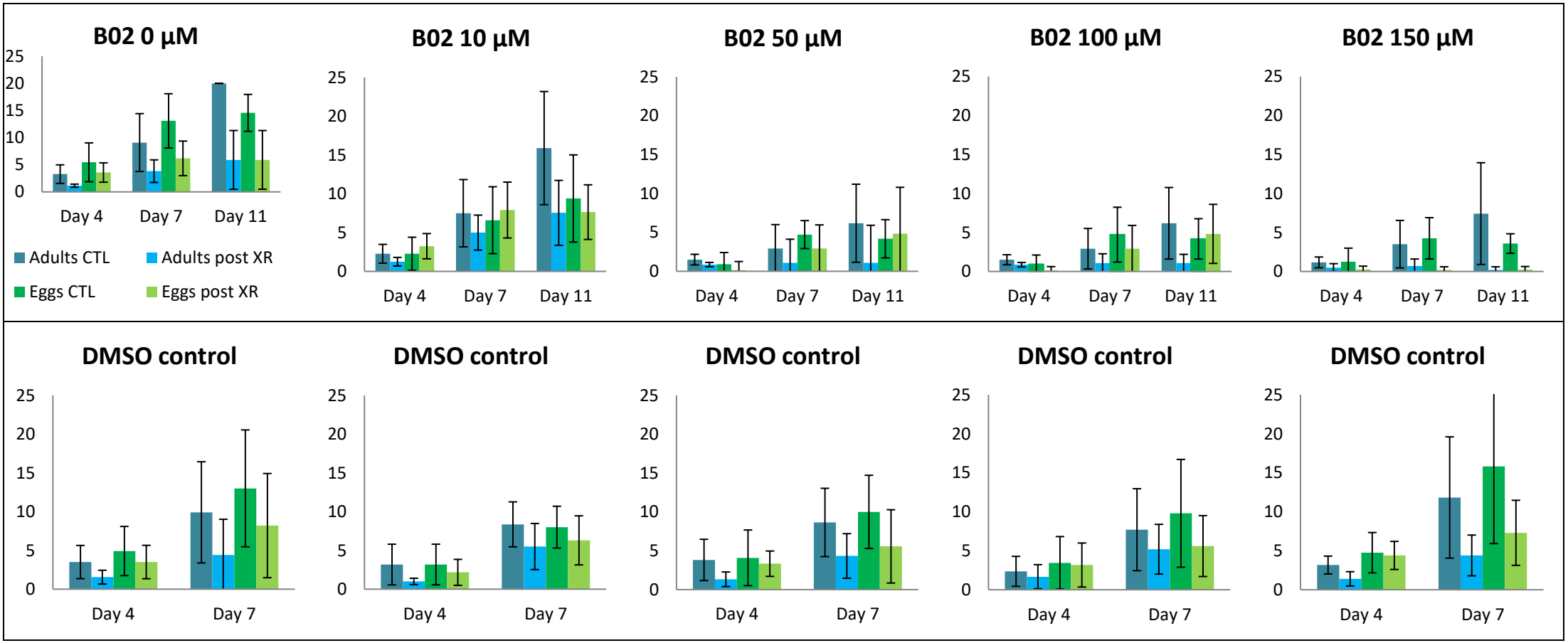
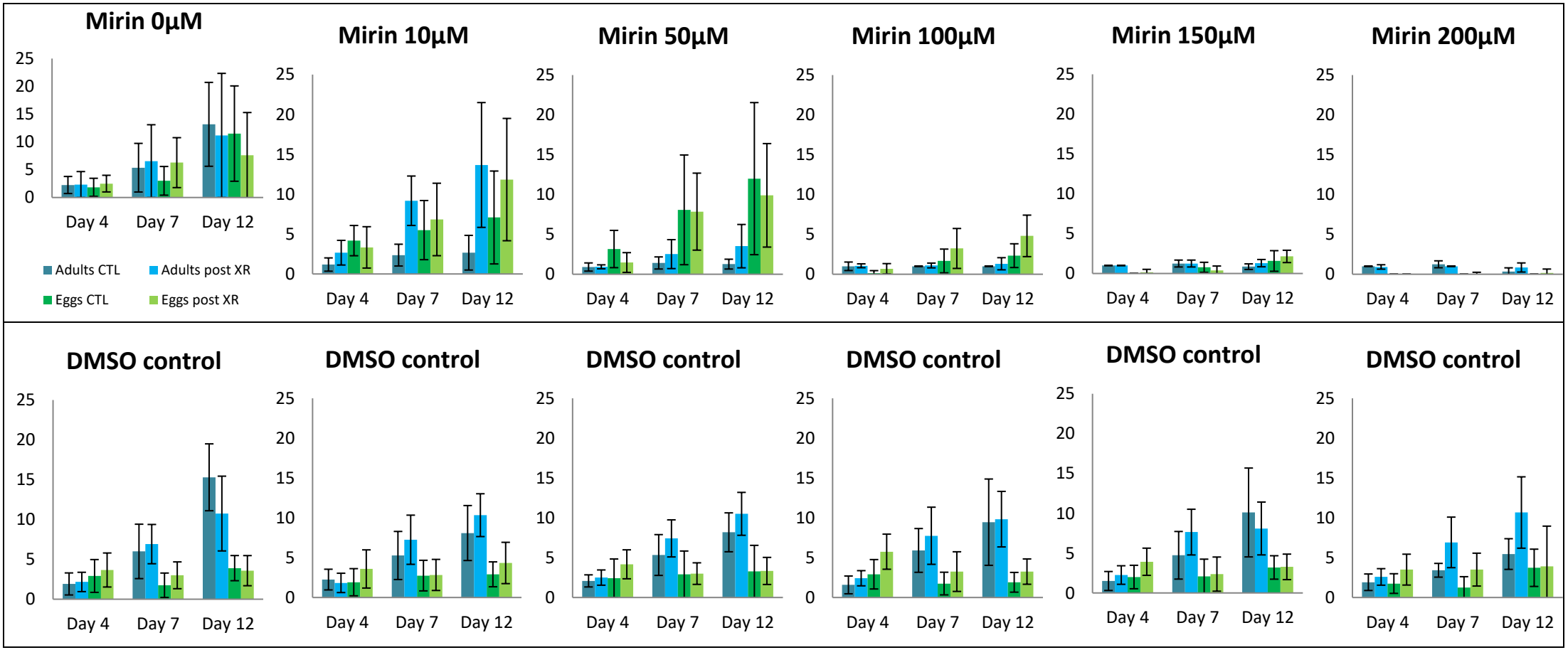
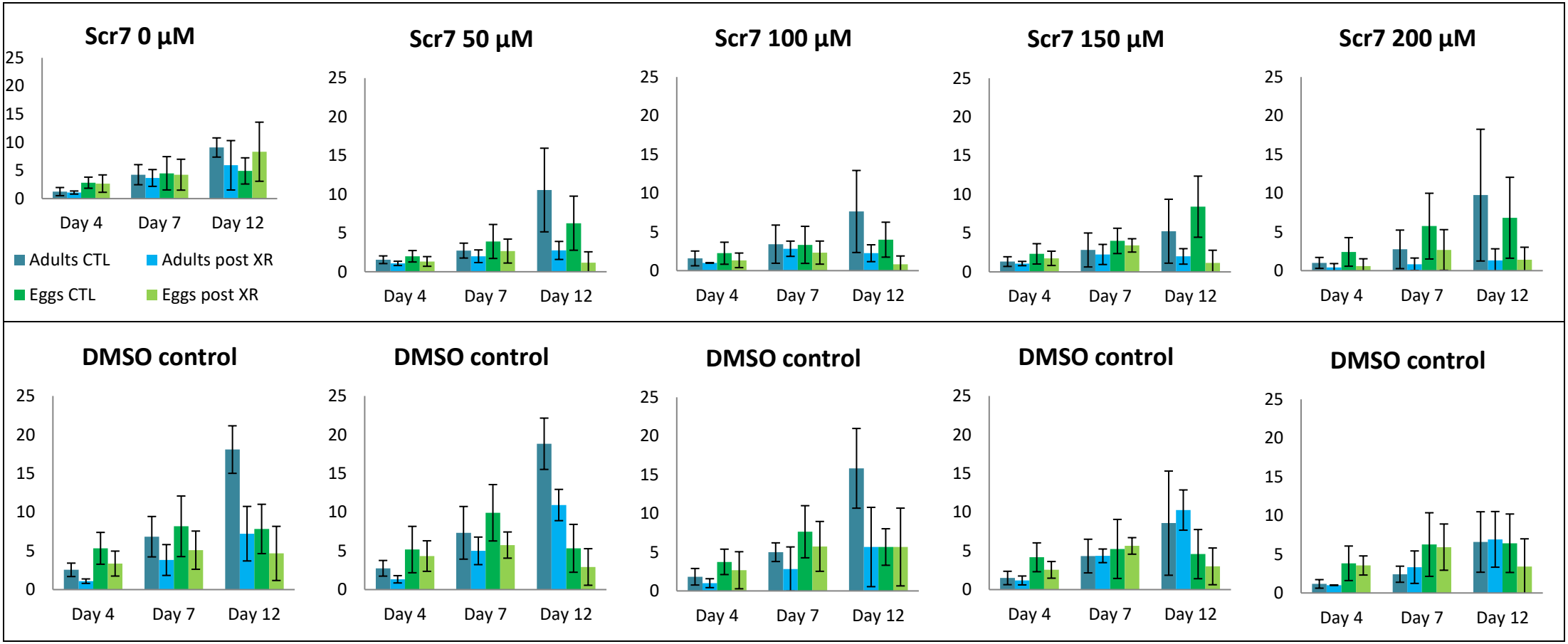
8. Supplemental data

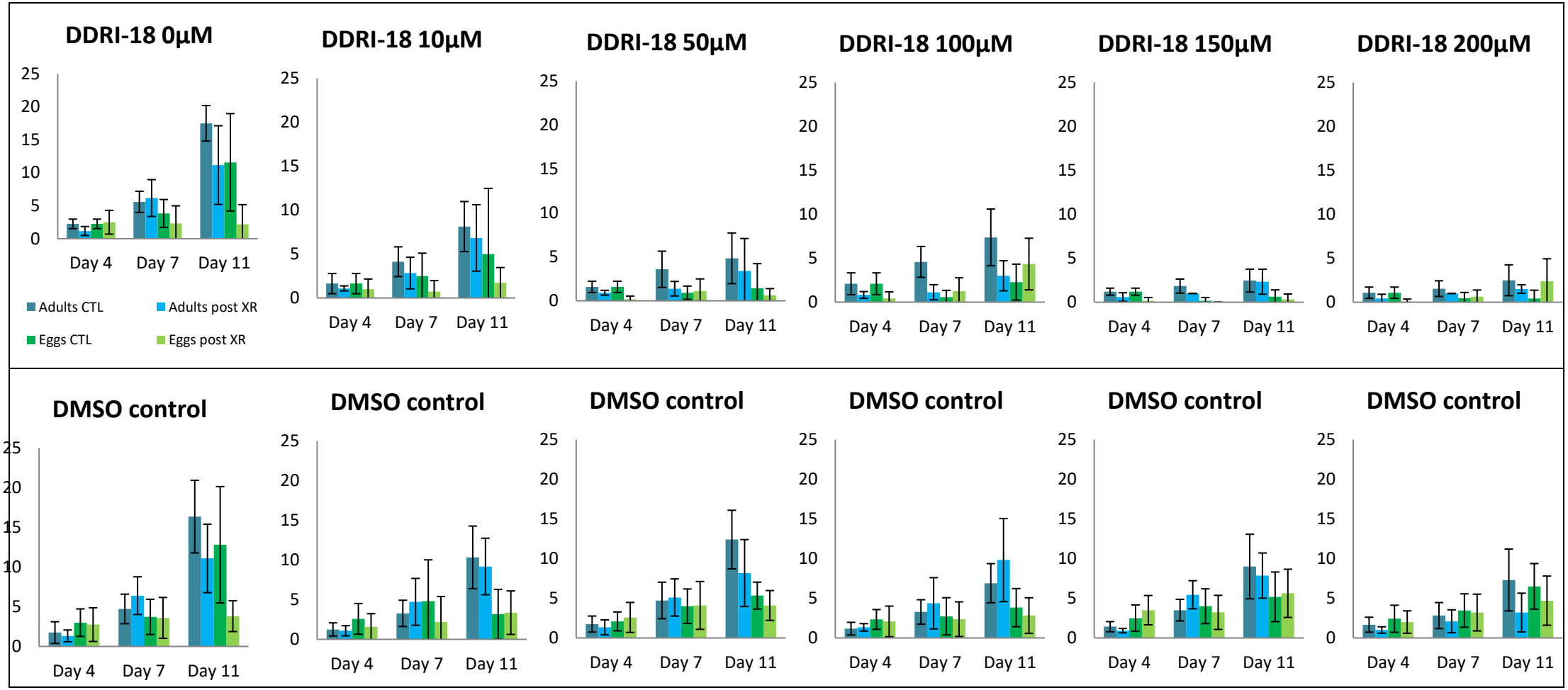
8.1. Supplemental data 1: Evolution of the population over time in fertility studies, in control and experimental conditions

These graphs show the evolution of the population over time in control and experimental conditions. Days are represented on the x-axis, the different columns correspond to the average number of alive adults and eggs exposed ("post XR") or not (CTL) to X-Rays. Standard deviations are also represented. DMSO controls lead in parallel of B02 are incomplete: at day 11, wells were totally evaporated due to a wrong placement in the incubator and countings could not be done.




Mirin 0µM
Mirin 10µM
Mirin 50µM
Mirin 100µM
Mirin 150µM
Mirin 200µM
DMSO control
DMSO control
DMSO control
DMSO control
DMSO control
DMSO control
Day 4
Day 7
Day 12
Adults CTL
Adults post XR
Eggs CTL
Eggs post XR

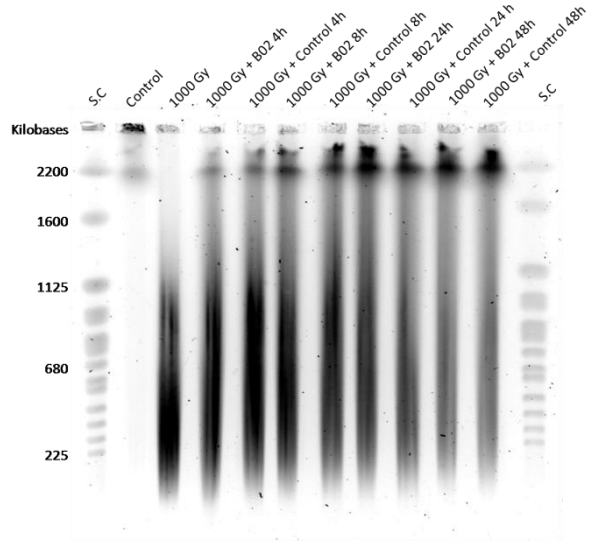

Scr7 0 µM
Scr7 50 µM
Scr7 100 µM
Scr7 150 µM
Scr7 200 µM
DMSO control
DMSO control
DMSO control
DMSO control
DMSO control
25
20
15
10
5
0
Day 4
Day 7
Day 12
Adults CTL
Adults post XR
Eggs CTL
Eggs post XR


DDRI-18 0µM
DDRI-18 10µM
DDRI-18 50µM
DDRI-18 100µM
DDRI-18 150µM
DDRI-18 200µM
DMSO control
DMSO control
DMSO control
DMSO control
DMSO control
DMSO control
25
20
15
10
5
0
Day 4
Day 7
Day 11
Adults CTL
Adults post XR
Eggs CTL
Eggs post XR

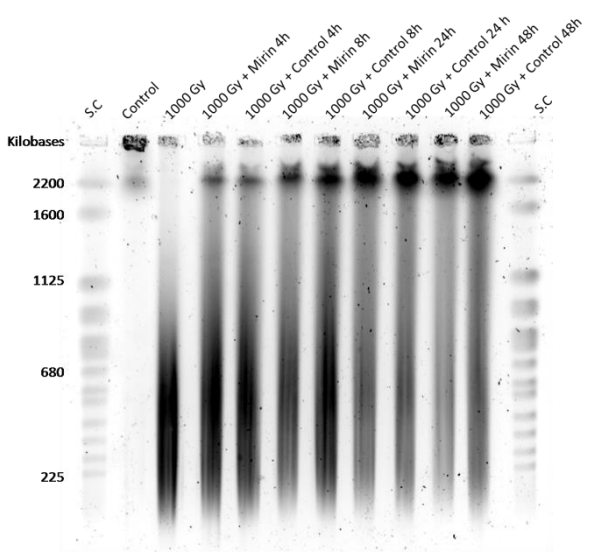
8.2. Supplemental data 2: Genomic integrity of *A. vaga* after incubation with NHEJ inhibitors

PFGE after 22 hours of migration. First and last lanes (S.C) correspond to *S. cerevisiae* chromosomes. The "Control" lane corresponds to 1,000 hydrated *A. vaga* individuals. The intact genome of *A. vaga* is composed of 12 chromosomes of approximately 20 Mb and does not match the PFGE resolution (225-2200 kb). Control DNA therefore remained in the wells as observed in the "Control" lanes, with a weak signal around 2,000 kb. In the 1,000 Gy lane, (*i.e.* rotifers fixed directly after the irradiation), a smear appeared reflecting the presence of DNA fragments ranging from 225 to 1125 kb. Other lanes correspond to 1,000 *A. vaga* submitted to 1,000 Gy and put in contact with a DNA repair inhibitor. (a) Effect of B02 (150 µM) on the DNA repair after 4, 8, 24 and 48 hours of contact. (b) Effect of Mirin (150 µM). Next to each experimental condition was placed the control condition. After 4 hours of repair, a smear was visible between 225 and 1125 kb. For the following time points, smear intensity decreaseed with the progressive reappearance of bigger fragments of 2,200 kb, suggesting a repair of the genome. None difference can be seen between the experimental and control conditions at any time point.

a.



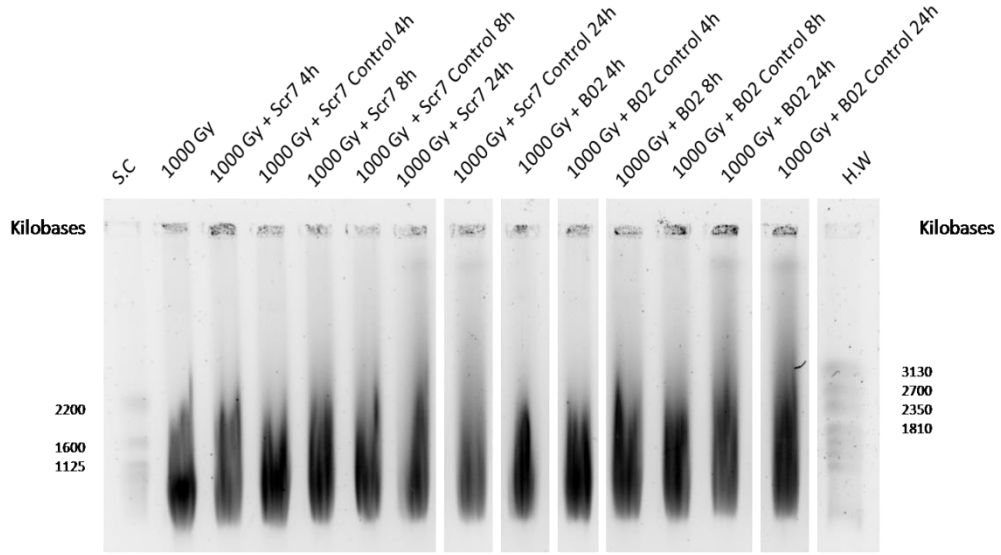
b.



8.3. Supplemental data 3: Genomic integrity of *A. vaga* in presence of NHEJ/HR inhibitors after 96 hours of migration

PFGE showing the genomic integrity of *A. vaga* after 96 hours of migration. First and last lanes correspond to *Saccharomyces cerevisiae* chromosomes (S.C) and *Hansenula wingei* (H.W) chromosomes. The "Control" lane corresponds to 1,000 hydrated *A. vaga* individuals. The lanes "1,000 Gy" are 1,000 *A. vaga* individuals directly fixed after the irradiation, a smear was visible between 2200 kb and the end of the gel. Other lanes correspond to 1,000 *A. vaga* submitted to 1,000 Gy and put in contact with a DNA repair inhibitor **(**experimental condition) or with water (control condition). (a) Effect of Scr7 and B02 (150 µM) on the DNA repair after 4, 8, 24 and 48 hours of contact. Due to manipulation error, this picture was modified in order to ease the reading of the results. (b) Effect of DDRI-18 and Mirin (150 µM). Next to each experimental condition was placed the control condition. None difference can be seen between the experimental and control conditions at any time point.

a.



b.