



Uranium mining wastes: Bystander and transgenerational effects in *Daphnia magna*

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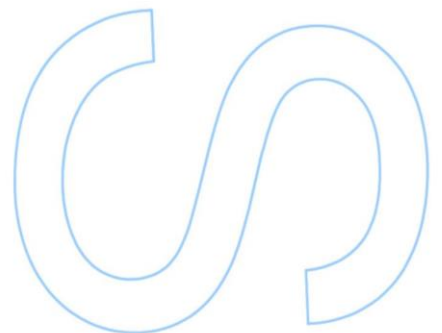
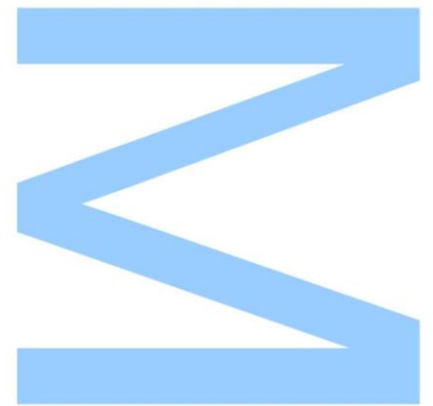
Mestrado em Biologia e Gestão da Qualidade da Água
Departamento de Biologia
2017

Orientador

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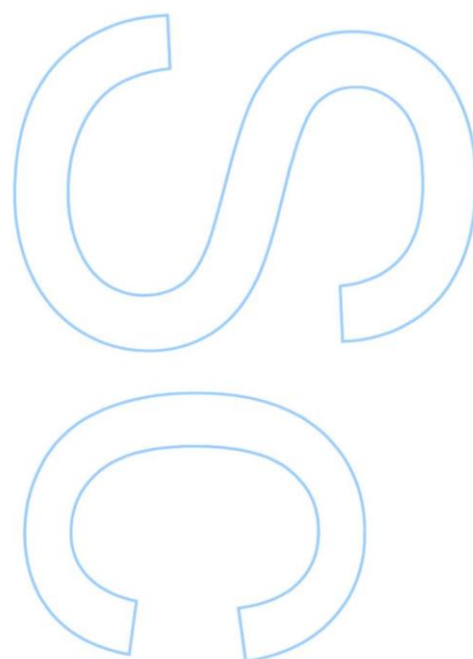
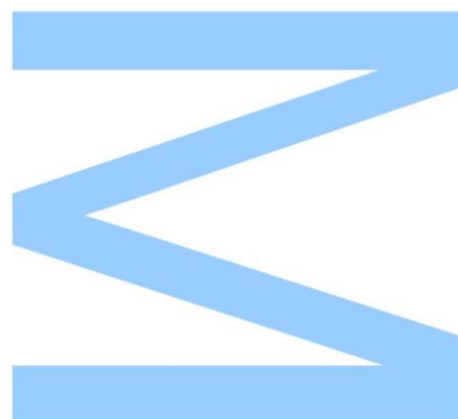
Coorientador

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Todas as correções determinadas
pelo júri, e só essas, foram efetuadas.
O Presidente do Júri,

Porto, ____/____/____



Dissertação submetida à Faculdade de Ciências da Universidade do Porto, para a obtenção do grau de mestre em Biologia e Gestão da Qualidade da Água, da responsabilidade do Departamento de Biologia.

A presente tese foi desenvolvida sob a orientação científica da Doutora Ruth Maria de Oliveira Pereira, Professora Auxiliar do Departamento de Biologia da FCUP; e coorientação científica da Doutora Joana Isabel do Vale Lourenço, Investigadora Post-doc do CESAM, Universidade de Aveiro

“Os homens já tanto conquistaram.

Vejam! Até asas tomaram-

Artes, ciências,

mil exigências.

E apenas do sopro do vento

O corpo tem conhecimento.”

Henry David Thoreau in *Walden*

Agradecimentos

Detesto agradecimentos generalistas. São ociosos. Sobre qualquer sentimento de verdadeira gratidão porventura neles contidos actua o factor de diluição da generalização que os banaliza. Por isso me perdoem se me alongar. Eu não quero agradecer a meio mundo, mas aqueles a que devo um Obrigado merecem bem mais que todo o mundo e por consequência algumas linhas individuais nesta folha.

O meu primeiro Obrigado pertence indubitavelmente à Professora Doutora Ruth Pereira, porque se não fosse a sua sempre lúcida e atenciosa orientação, todas as páginas se seguem neste livro estariam em branco. Por isso em todas as frases do mesmo, está latente um reconhecido e sincero agradecimento à confiança que desde o início depositou em mim, a todas as suas palavras de incentivo e mais do que isso, a toda a outrora adormecida paixão pela ciência da vida e da natureza que despertou em mim com o seu exemplo ímpar enquanto profissional entusiasta e de garra insaciável em tudo que faz.

E porque sou um rapaz de sorte, não tive apenas uma muito boa orientadora, mas sim duas. Agradeço assim à Doutora Joana Lourenço, que escamoteando qualquer papel secundário que o prefixo “co-“ pudesse eventualmente conter, assumiu também um papel principal no decorrer de todo este meu ano de trabalho. Por todas as suas sugestões, por todos os métodos laboratoriais que me ensinou e por toda a simpatia com que me abriu as portas (metafórica e literalmente, também) da Universidade de Aveiro, eu lhe dirijo um muito Obrigado.

Não posso também deixar de dar a minha palavra de apreço à professora Doutora Natividade Vieira, que para além de Directora do meu mestrado, é também, para mim, uma amiga. Assim como não posso também deixar de referir a preciosa participação do professor Doutor Fernando Carvalho e da sua equipa, na realização das análises químicas ao efluente mineiro, bem como deixar uma palavra de estima e consideração à professora Doutora Sónia Mendo pela minha integração no seu laboratório do CESAM bem como por todo o material que gentilmente me facilitou.

E se a minha odisséia com o fim último desta tese, foi uma jornada incrível e inesquecível, há alguns amigos cientistas aos quais devo tal. Ana Gavina, foste o primeiro sorriso que eu recebi, quando a medo entrei pela porta do LABRISK, e isso bem como todos os ensinamentos e conversas da treta, eu nunca esquecerei. Inês Nogueira, obrigado por toda a galhofa, por todos os sorrisos que diariamente colocaste neste teu rambo com gostos musicais do outro mundo. E agora, ao meu noivo Professor

Doutor Saul Simão, tenho que mandar o maior abraço do mundo, pois foste muito mais que o melhor companheiro de trabalho da UJr do mundo. És um grande companheiro de conversas, um comparsa de bebedeiras, és um bom amigo. Por fim, deixar uma menção honrosa de amigo cientista, ao Andres Rodriguez, o meu Doutor Minhoca de Ouro. És espectacular e acredita que deixaste saudades nesta Daphnia lusitanica, seu galego.

E se apenas do sopro do vento o corpo tem conhecimento e nenhum sopro aquece e dá mais sentido ao viver, do que a aragem do amor, quero agradecer a todos aqueles que assopram uma brisa de calor gostoso ao meu coração e me fazem acreditar que viver ainda vale a pena, e muito.

Ao meu camarada Miguel Basto, um obrigado por todas as conversas, por todo o companheirismo, por me fazeres sonhar com uma amizade que dure até à velhice. E eu sei que há-de durar.

À Brel, por todos aqueles cigarros de conversa, por todos aqueles almoços que de prazer inebriam os relógios. A sua lucidez de pensamento, a sua cultura, e todos esses mundos fascinantes que se escondem no seu cérebro e apenas a alguns olhares sortudos se revelam, cativam-me por completo. Admiro-a muito!

Ao meu Militar e à sua Dulcineia Joana, a vossa amizade não tem preço e acredito que validade também não.

Ao João Paulo, a minha Mascote (no bom sentido, claro), tu és um diamante em bruto. Tens um valor inestimável e sei que vais longe, acredita. E eu tenho muito a agradecer-te; não só todas as palavras de encorajamento, e risadas parvas que me fazes soltar, mas também aquele reavivar um pouco de mim, daquele meu lado curioso-estupido-fascinado por tudo que nos rodeia. És um grande amigo!

Ao Alfredo das Camionetas, que sei que do alto de todos os castelos e monumentos históricos deste Portugal à beira mar, contempla com um ronco de choro entalado na garganta, este seu neto zarpar numa fragata ainda sem rumo conhecido, mas com alguma terra não menor que Vera Cruz no horizonte. Obrigado, por todos os ventos que invisivelmente sopras de feição.

À minha avó Carrolas, a velha mais nova que existe, pelas risadas contagiantes e histórias levadas da breca, por todo aquele amor de avó babada, pelas mãos que sei que sempre me ampararão.

À minha mãe, por me ensinares a andar (não é literal, é bem mais!), por me ensinares a amar, por estares sempre mas sempre lá quando eu chamava por ti. És a minha origem e razão de ser e eu tenho tanto orgulho em ti! És a mulher mais bela, uma beleza de uma força ímpar, e parafraseando Herman Melville *“a verdadeira força em nada altera a beleza ou a harmonia, muito pelo contrário, antes a reproduz, e em tudo que é imponentemente belo, a força tem muito a haver com magia.”* E tu és a mulher mais imponentemente bela deste mundo, minha mãezoca.

Ao meu pai, por me ensinares a “tratar de ser feliz” todos os dias, por seres este picantezinho gostoso que tempera a vida, por seres o meu maior exemplo, porque muito mais do que uma inspiração, tu és a minha maior aspiração. Quero um dia conseguir ser parte do Homem que tu és. Tu és tão grande, meu paizão.

Ao meu Salvador, o meu pestinha, o meu herói-guerreiro! Obrigado por me salvars! Obrigado por me mostrares que os sonhos por vezes realizam-se! Obrigado por me ensinares o prazer e a responsabilidade de ser um herói! Obrigado por existires, meu maninho, por todos os dias me fazeres despertar! Nasceste e contigo nasceu o Sol, nasceu a certeza de que viver é bom, é magnífico e vale tanto a pena.

E se com o Sol, nasci, sem uma Lua não havia forma de viver. E eis, que te encontrei, minha Sete-Luas! Revolucionaste o meu mundo! Agigantaste-o! O meu mundo tornou-se aquele véu estrelado sobre o qual eu achei um amor maior. Tornando todo o resto pequeno, tornaste isto do viver em algo maior. Contigo achei aquilo que procurei toda a vida: um amor maior do que aquele que retractam os livros. E quando me perguntam *“Até quando julga o senhor que podemos continuar neste ir e vir dum caralho?”*, eu, navegando a teu lado por este rio com margens de cólera, que é a vida, a teu lado respondo *“Toda uma vida!”*.

Abstract

Uranium is a highly dense metal with radioactive proprieties (α -particle emitter), which make it of special commercial interest, due to its applications, especially in the energy sector. Despite nuclear fallouts of the past and the occidental commitment to invest in renewable energy, the fast growing world's energy demand will increase investment on nuclear energy and consequently increase uranium exploration, especially in new developing countries (e.g. China and India). Uranium is a ubiquitous naturally occurring element in the Earth's crust (2.8 ppm) with background range values in aquatic systems in the order of μg per liter. However, due to uranium mining activities, some water basins can reach values up to 2 mg U L^{-1} , along with high concentrations of associated radionuclides. As so, it is vital to truly assess the impacts of uranium and uranium mining effluents on nearby aquatic ecosystems, to secure the long-term health and sustainability of ecosystem services.

Uranium toxicity is not linear and encompasses not only its chemical toxicity, but also its radiotoxicity, which despite usually regarded as of least concern, should not be overlooked. Therefore, both properties have to be integrated to perform a correct assessment of uranium-rich waste impacts in ecosystems. Its effects on organisms largely vary according to the organism's group, route of exposure, dose and species of uranium. Uranium exposure can cause a severity of genotoxic and damaging effects to the cells compounds, through interaction with proteins, lipids and DNA molecules. It is able to promote DNA damage, causing single and/or double strand breaks, and loss of bases from the DNA molecule, affect mitochondrial processes, DNA repair mechanisms and gene expression, induce apoptosis, the formation of free radicals and oxidative stress. All that may lead to the reduction of individual fitness and affect population's parameters such as growth and development, as well as be transmitted to the offspring. A correct understanding of uranium mining impacts gets even more complex, if we take into account that low doses of α -radiation induce genetic damages in the cell nuclei of non-irradiated cells. These non-targeted-effects (NTEs) of ionizing radiation (IR), occur only in the low dose range of IR and encompass the radiation induced genomic instability (RIGI) and radiation induced bystander effect (RIBE). RIGI is the phenomenon in which progeny cells of irradiated ones, display damages that result from parental exposure to IR; and RIBE is the induction of IR responses in non-irradiated cell that share the same medium as irradiated ones. All that may propagate the effects of IR, which is not necessarily bad, once the responses in bystander cells differ and encompass injuries such as cell death, DNA damage and neoplastic transformations, but can also benefit the bystander population by inducing radio-adaptive responses (RAR). Curiously, in the

last years, this phenomenon have also been reported at an inter-organism level; i.e., damage responses were detected in non-irradiated organisms that were housed together or shared the same medium of organisms previously exposed to low radiation.

Taking into account, all the above mentioned, this thesis was conceptually designed to complement the existing data regarding the double-toxicity of uranium as well as the real mixture effect scenario of effluents discharges (which contain several metals and radionuclides), in order to contribute for a more truthful environmental risk assessment of radioactive wastes and wastewaters. In order to do that, two major genotoxicity assays were performed in *Daphnia magna* after short-term exposures to both a highly diluted uranium mine effluent (UME) containing a complex mixture of metals and radionuclides (from a deactivated uranium mine located in the Center region of Portugal) and a matching dose of waterborne uranium (WU). The first assay intends to address the transgenerational effects caused by short-term exposures, i.e., to perceive if the genotoxic effects were perceived in the offspring, and if and how it affects its life history traits. The second, regards the detection of bystander effects at an inter-organismic level and its possible impact in the field of environmental risk assessment. These experiments were performed to try to fulfill some of the current gap of knowledge regarding this kind of effects in invertebrates, as well as in radioactive environmental samples.

From our data, it was evident the induction of DNA damage in daphnids after a single short-term exposure to low doses concentrations of WU and highly diluted UME. However that was not translated in significant damaging effects on the life history traits of *D. magna* populations in a long-term scenario. Our data also revealed the occurrence of RIBE at an inter-organismic level in both exposures. However, it tends to diminish with time and was less pronounced in UME. Despite some exposure-age-time-dependent variability in the impacts of exposure, and different recovery rates of genetic damage, our data indicates that *D. magna* populations are able to tolerate some UME contamination if they are exposed at low doses, spaced in time. Nevertheless, further studies would be need to allow us to state a non-hazardous scenario for aquatic ecosystems subject to this intermitent and low doses discharges of uraniferous effluents, especially for benthic organisms.

All the data obtained with these studies bring some valuable new points for the discussion of environmental risk assessment of radionuclide's rich-wastewaters, that should in the future be taken further and complemented with benthic organisms and microcosms studies, as well as mimic different time rates and doses of intermittent uranium mining discharges.

Resumo

O urânio é um metal denso com propriedades radioactivas (emite partículas α), o que o torna um metal de interesse comercial, devido as suas múltiplas aplicações, especialmente no sector energético. Apesar dos desastres nucleares do passado e de um compromisso de investimento nas energias renováveis por parte dos países ocidentais, o crescimento exponencial da procura energética torna expectável um aumento significativo do investimento na energia nuclear, e por consequência na exploração mineira de urânio, especialmente nos países em desenvolvimento (ex. China e Índia). Urânio é um elemento natural ubíquo na crosta terrestre (2.8 ppm) com valores de ocorrência nos ecossistemas aquáticos na ordem da unidade da μg por litro. Contudo, devido à exploração mineira do mesmo, algumas bacias hidrográficas podem apresentar concentrações mais elevadas (até as 2 mg U L^{-1}), assim como elevado conteúdo em radionuclídeos associados. Como tal, é vital aferir com veracidade os impactos do urânio assim como dos efluentes que resultam da sua exploração mineira nos corpos aquáticos adjacentes da mesma, para assim assegurar o bem-estar e sustentabilidade ambiental dos serviços de ecossistema dos mesmos.

Toxicidade do urânio não é linear e engloba não só a sua toxicidade enquanto elemento químico mas também a sua radiotoxicidade, a qual apesar de ser normalmente encarada em segundo plano, não deve ser subestimada. Como tal, ambas as propriedades tem de ser integradas para uma correcta aferição dos impactos ecológicos dos efluentes ricos em urânio nos ecossistemas. Os seus efeitos nos organismos variam bastante conforme o filo do organismo, via e dose de exposição, bem como especiação do urânio. Exposições a urânio podem resultar numa série de efeitos genotóxicos e danos nos componentes das células, através de interacção do mesmo com proteínas, lípidos e moléculas de DNA. Urânio é capaz de induzir danos genéticos, através de quebras simples ou duplas da cadeia de DNA, perda de bases nas moléculas DNA, interferência nos processos mitocondriais, mecanismos de reparação do DNA, assim como induzir apoptose, formação de radicais livres e stress oxidativo. Tudo isto, pode levar a uma redução da aptidão individual dos organismos expostos, assim como afectar parâmetros populacionais, tais como crescimento e desenvolvimento, podendo ainda tais efeitos ser transmitidos à descendência. Uma compreensão holística dos impactos ambientais dos efluentes mineiros, torna-se ainda mais complexa, se tivermos em conta que baixas doses de radiação α induzem dano genético no núcleo de células não irradiadas. Estes efeitos não-alvo (NTEs) da radiação ionizante (IR) ocorrem apenas na gama das baixas doses da IR e englobam a indução de instabilidade genómica (RIGI) assim como o efeito bystander (RIBE). RIGI é o fenómeno no qual células descendentes

de células irradiadas, apresentam danos que resultam da exposição parental a IR; ao passo que o RIBE é a indução de respostas à IR em células que, não sendo expostas, apenas partilharam o mesmo meio que as células irradiadas. Tudo isto pode potencialmente propagar os efeitos da IR, o que não é necessariamente mau, visto que as respostas em células bystander variam e englobam efeitos danosos, tais como morte celular, dano genético e transformações neoplásticas dos compostos celulares, mas também podem beneficiar a população bystander através de respostas adaptativas (RAR). Curiosamente, nos últimos anos, este fenómeno também tem sido reportado a um nível inter-organismo; isto é, efeitos danosos têm sido detectados em organismos não expostos que coabitaram ou partilharam o mesmo meio com organismos previamente expostos a baixas doses de radiação.

Tendo em conta, tudo que até ao momento foi mencionado, esta tese foi conceptualmente desenhada para complementar os dados existentes acerca da dupla-toxicidade do urânio assim como o efeito mistura de efluentes que resultam da sua exploração mineira (os quais contêm diversos metais e radionuclídeos), para assim contribuir para uma mais veraz aferição dos riscos ambientais de descargas de efluentes radioactivos. Com tal finalidade, foram realizados dois ensaios genotóxicos de envergadura considerável em *Daphnia magna* após a sua exposição de curta duração a uma elevada diluição de um efluente uranífero (UME), contendo uma complexa mistura de metais e radionuclídeos (de uma mina de urânio actualmente desactiva, localizada na região centro de Portugal) e uma dose similar de urânio aquoso (WU). O primeiro estudo pretende aferir os efeitos os transgeracionais causados por uma curta e pontual exposição, isto é, almeja perceber se os danos genotóxicos são transmitidos à descendência, e como e se, esses efeitos se repercutem no percurso de vida dos organismos. O segundo ensaio, foca-se na detecção do fenómeno bystander a um nível inter-organismo e as suas possíveis implicações para a aferição dos riscos ambientais em ecossistemas sujeitos a descargas pontuais de efluentes uraníferos. Estas experiências foram concebidas para tentar colmatar algumas das lacunas do conhecimento acerca deste tipo de efeitos em invertebrados aquáticos, assim como em amostras ambientais de efluentes radioactivos.

Os dados obtidos, evidenciam uma clara indução de dano no DNA dos dafnídeos após uma curta exposição a baixas concentrações de WU e elevada diluição de UME. Contudo, tais perdas de integridade genética não se repercutiram em danos significativos no percurso de vida das populações de *D. magna* num cenário de longo prazo. Os nossos dados também revelaram a ocorrência de RIBE a um nível inter-organismo em ambos as exposições. Apesar de alguma variabilidade dos dados

dependente da exposição, idade e tempo, assim como diferentes taxas de reparação dos danos genéticos, os resultados obtidos indiciam que as populações de *D. magna* são capazes de tolerar alguma contaminação de UME se a mesma for em baixas concentrações e espaçadas no tempo. Contudo, seriam necessários estudos a posteriori para nos permitir concluir uma ausência de risco ambiental para os ecossistemas aquáticos sujeitos a descargas intermitentes e de baixa doses de efluentes uraníferos, especialmente no que se refere à fauna bêntica.

A reunião de todos os dados que deste estudo derivaram, oferecem pontos bastante válidos e úteis de ter em conta para a discussão científica da aferição dos riscos ambientais de efluente ricos em radionuclídeos, os quais deverão ainda ser complementados no futuro com organismos bênticos e estudos de microcosmos, assim como devem mimicar diferentes espaçamentos temporais e concentrações de descargas advindas da exploração mineira do urânio.

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Chapter III

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Abbreviations

ERA – Environmental Risk Assessment

HRM – High Resolution Melt

iNOS – Inducible Nitric Oxide Species

IR – Ionizing Radiation

ISL – In Situ Leaching

LET – Linear Energy Transference

NO – Nitric Oxide

NTEs – Non-Target Effects

RAR – Radiation-induced Adaptive Response

RBE – Relative Biological Effectiveness

RFLP – Restriction Fragment Length Polymorphism

RIBE – Radiation Induced Bystander Effects

RNS – Reactive Nitrogen Species

ROS – Reactive Oxygen Species

TGF- β 1 – Transforming Growth Factor Beta

TNF- α – Tumor Necrosis Factor Alpha

UME – Uranium Mine Effluent

WNA – World Nuclear Association

WU – Waterborne Uranium

Chapter I

General Introduction

1. Uranium

Uranium is a high density metal (19.05 kg/dm^3) of the actinide family, i.e., on the periodic table is on the third group (transition metals) and seventh period (atomic number of 92 and a molar mass of 238.032) [1].

Uranium has an average concentration of 2.8 ppm in the earth's crust, as so, it can be found in a wide range of rocks but with local distribution mean values varying according to the type of rock (e.g. 300 ppm in phosphate rock, 3.8 ppm in granites, 3.7 ppm in shists and 0.3 ppm in basaltic rocks) [2]. Due to its geochemical cycle, uranium is also present in the aquatic system ranging from 0.02 to $6 \mu\text{g L}^{-1}$ in freshwater environments and $3.3 \mu\text{g L}^{-1}$ in marine medium [3]. Therefore, it can affect biota depending on its bioavailability (detailed in chapter 3). Nevertheless, depending on the characteristics of the soils and the presence of anthropogenic activities (mining and milling of uranium ore and nuclear power facilities) the value of uranium in water basins can be raised up to 2 mg L^{-1} [4].

1.1. Main applications and current market trends

In nature, uranium is usually a mixture of three isotopes (variants of an element that differ in the neutron number): ^{238}U , ^{235}U , and ^{234}U , with a relative abundance of 99.284%, 0.711% and 0.005%, respectively [1], being that ^{235}U radioisotope, is the only fissile isotope in nature whose chain reaction can release huge amounts of energy, making this metal a resource of extreme commercial interest with multiple applications [5]. The vast majority of uranium is used in the energy sector, as low-enriched uranium (3-5% of ^{235}U), to fulfill the nuclear power stations requirements; but it can be highly enriched for applications in naval propulsion and production of nuclear weapons (enriched to 97% of ^{235}U) [6,7]. The utilities of this element also encompass medical purposes, due to its isotopes (e.g. from decay of ^{233}U), and aviation industry, where depleted uranium (almost exclusively ^{238}U) is used in counterbalances for helicopter rotors, gyrocompasses, armor-piercing ammunition and radiation shielding [7,8].

The mineral market tends to have cyclical fluctuations along the years, related in most cases to demand/offers and perceptions of scarcity. However, throughout time these

prices fluctuations have been relying exclusively on production cost at the mines. The uranium market is an exception to that, with extreme and irregular price fluctuations along the years (Fig.1). Those fluctuations are related with political scenarios, rather than effective demand and supply (increasing during the Cold War and decreasing with gradual disarming at the end of that tension period) and perceptions of the general public relative to nuclear power plants, as a consequence of the nuclear disasters of Chernobyl and Fukushima.

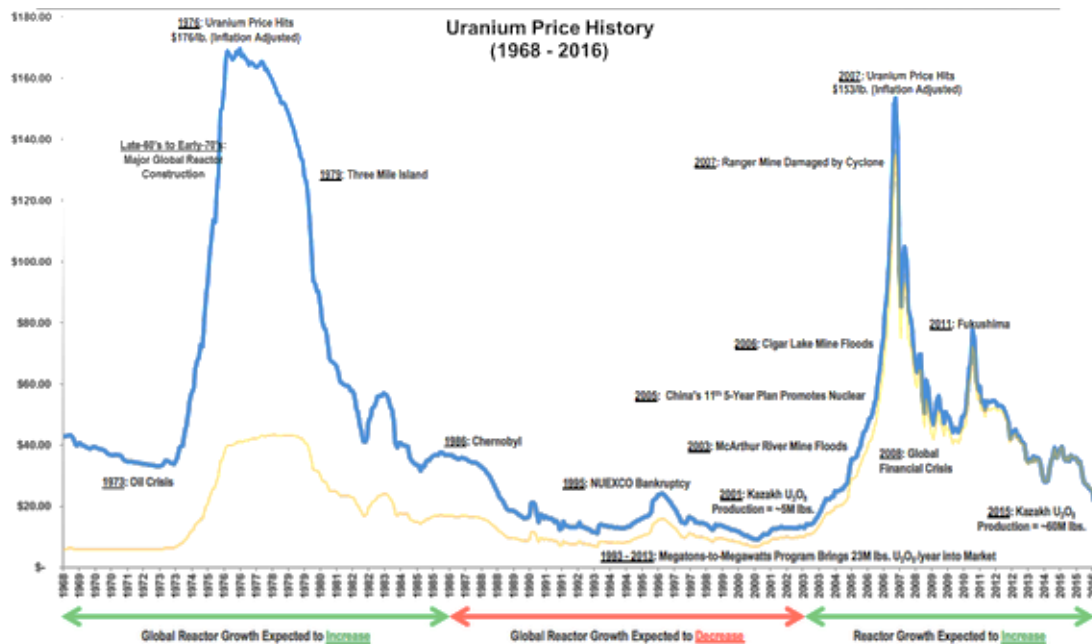


Figure 1 - A long-term look (1968-2016) at the history of uranium prices conjugated with events and macroeconomic factors. Source: <https://get.whotrades.com/u5/photo8ECE/20632148024-0/blogpost.jpeg>

At the present moment, uranium is negotiated at cheapest prices (between 20-30 US\$/lb. U_3O_8), but before we focus on the analysis of the markets evolution in the past and try to anticipate the more expected scenarios for the future, it may be worth to insight at which point we are presently, relatively to supply and demand.

Uranium is a quite common metal in the earth's continental crust, but economically relevant concentrations (i.e., 100 ppm) are not found so frequently in nature [8]. However, three cycles of exploration efforts (1945-1958, driven by military purposes; 1974-1983 and 2003-2006 due to civil nuclear power demand) resulted in the world's known economic viable uranium supply of 5.9 Mt U_3O_8 , which at the present rate of consumption, would last for about 90 years [9]. This prediction does not account for further exploration, improvements in nuclear power stations technology and other secondary sources beyond uranium mining that would increase that amount. These would include: a) mining wastes rich in uranium as by product (e.g.

phosphate/phosphorite deposits (up to 22 Mt U); b) stockpiles of depleted uranium left over after enrichment of uranium for nuclear warheads; c) dilution of highly enriched uranium used for nuclear bombs, with depleted uranium d) recycled uranium from reprocessing used fuel [8,9].

Uranium extraction is performed in about 20 countries and market concentration is noticeable. Only three countries (Kazakhstan, Canada and Australia) account for more than two-thirds of the world's uranium mine production, and 89% of the uranium mines are owned by only 11 companies, with the major four hold 65% of the total. Currently, the uranium provided by mines accounts for 84% of annual nuclear power station requirements with the remaining coming from the secondary sources described above [8].

All markets work based on supply and demand, and as above seen, we have a significant supply, but we also have a noteworthy global demand for uranium, that is currently about 67,000 tU/yr, which equals to 74,000 tonnes U_3O_8 /yr [8]. Most part is destined to civil power demand (Fig.2) to fuel the 445 nuclear reactors existing worldwide, with combined capacity of over 390 Gwe [8].

So, it may be worth to look at the evolution of demand and supply of uranium over the last decades (Fig. 2). By looking at Fig. 2, we note that until now, and independently of socio-political scenarios, the global demand has been growing consistently. That was not accompanied by the uranium production from mines. A two decade's gap between uranium production in mines and global demand for this metal (purple arrow in Fig.2), as a result of the decommissioning of nuclear warheads with the end of Cold War, dropped the prices during that period, leading to the closure of many mines [9,10].

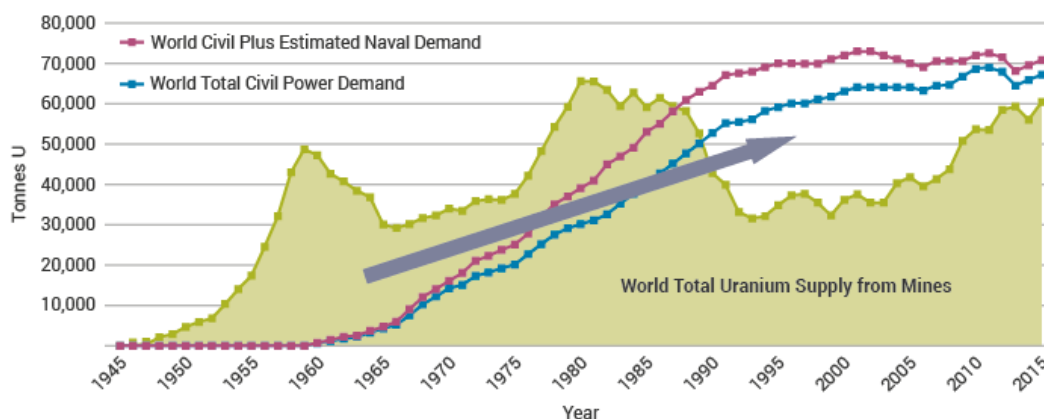


Figure 2 – World Uranium Production and Demand (1945-2015).

Source: <http://www.world-nuclear.org/getmedia/45af6b62-0e32-4845-8b77-b1dff656e704/world-uranium-production-and-demand-2015.png.aspx>

That gap started to shorten around 2003, with the opening of new mines, as result of investor's interest in the nuclear energy industry, to respond to the global rise of fossil fuel prices and the foreseeable growth of the world's population and demand for energy [11]. Despite the 2008 global crisis and the 2011 Fukushima accident, which strongly affected uranium prices, the demand for this metal and investments in nuclear energy as an efficient and greener solution for the world's energy demand, allows the foreseeing of an expansive future in the uranium mining industry [8,11].

In a world concerned with limiting carbon emissions and at the same time an expected increase in electricity demand by 70%, from 2013 to 2040 [8], nuclear energy is considered by many, as a greener energy solution, since one pound of fully fissioned uranium yields the same amount of energy as burning 1,500 tons of coal [12]. The establishment of nuclear energy as a solution for the future is notorious (although not consensual), when we take into account that at the moment, there are 66 new nuclear reactors under construction worldwide (two-thirds expected to be operating in the next three years) [8].

Therefore, in accordance with the World Nuclear Association [13]2017 Nuclear Fuel Report, the demand for uranium is expected to increase by 26% until 2025. This predicted growth could be higher according to, the forecasted approvals of lifetime extensions of older reactors. It's newsworthy that 86 % of this expected growing demand, lies on new developing countries (e.g. China and India). At the same time this overall growing is counterbalanced with the political agenda of some European countries (e.g. France and Germany) that are abandoning the nuclear power in exchange for a commitment to renewable energy [13].

To answer this expectable global demand for uranium, for nuclear power plants fuel fabrication, there are projects for opening new mines worldwide, with some of them already expected to start production in the next years (e.g. Salamanca (Spain in 2017); Mulga Rock e Wiluna (Australia); Canyon (USA); Arrow (Canada)) [8].

2. Uranium mining

Despite the fact that uranium could be found in almost any type of soil and rock, its economically viable concentrations are mainly found in phosphate rock, lignite and monazite sands [12]. Like every metals, uranium is always found combined with other elements. As such, feasible uranium mining consists in finding a geological deposit of

ore grade sufficient to allow an economically profitable extraction, and then detach and purify the uranium containing compounds from the raw ore. The prospection of uraniferous areas is usually easier than for other mineral resources, once the radiological proprieties of uranium and its decay products allow deposits to be mapped from the air by aeroradiometry [14].

At the present, most of uranium mines exploit ore grades of 1000 ppm on average, but this value is variable, once there are some mines that can be economically self-sufficient with uranium deposits of 200 ppm [9]. In contrast there are also some Canadian mines that exploit ores up to 20% U grade (200000 ppm) [8]. But not all uranium ores are the same; in fact, there are more than one hundred uranium ores, based on type, porosity and mineralogy of host rocks, structural setting and uranium species [6].

Uranium ore minerals (table 1, describes some of the dominants) are in general divided into primary and secondary, in accordance with their reduction-oxidation potential; primary uranium ores incorporate reduced uranium, i.e., as U^{4+} , and secondary ones integrate oxidized species, i.e., uranium as U^{6+} , and are therefore known as weathered uranium ores [14].

Table 1. Main uranium ores, their composition and respective description.

	Ores	Composition	Description	Notes
Primary Ores	Uraninite/ Pitchblende	$UO_2 + UO_3$	A steel-, velvet-, to brownish-black in color; pitchblende it is the same as Uranininite but with an amorphous instead of a crystalline structure	It is by far, the principal ore for mining industry
	Brannerite	$U(TiFe)_2O_2$	A black, brownish, olive greenish ore	Present in granitic deposits, associated with uraninite
	Carnotite	$K_2O \cdot 2U_2O_3 \cdot V_2O_5 \cdot 3 H_2O$	Bright-, lemon-, or greenish-yellow mineral	Can be found in sandstone, associated with tyuyamunite and U–V oxides

	Coffinite	$U(SiO_4)_{1-x}(OH)_{4x}$	A black or pale-to-dark brown mineral in sandstone	Present in sandstone associated with uraninite
Secondary Ores	Autunite	$Ca(UO_2)_2(PO_4)_2 \cdot 10 H_2O$	Yellow-to-greenish mineral, formed under oxidizing conditions	It is a common secondary uranium mineral
	Torbenite	$Cu(UO_2)_2(PO_4)_2 \cdot 10 H_2O$	An emerald-, grassy-, to apple-green mineral	Appears associated with uraninite and autunite
	Tyuyamunit e	$Ca(UO_2)_2(VO_4)_2 \cdot (5-8) H_2O$	A canary-, lemon-, to greenish-yellow mineral	It can be found in limestone associated with carnotite
	Uranophan e	$Ca(UO_2)_2(HSiO_4)_2 \cdot 5 H_2O$	It is slightly lighter in color than autunite	The origin and occurrence are very similar to that described for autunite and torbernite.

Sources: [1,14–16]

Despite its properties as a radiological element, uranium mining is not very different from other kinds of metal exploration. Presently, 42% of the uranium extraction is done in conventional mines (open pit or underground), 51% by in situ leach and 7% recovered as a by-product [8].

The choice of the mining method depends upon several factors related to the ore, such as grade, size, shape, thickness and permeability, as well as to the proximity of groundwater reservoirs, surface topography and ground conditions, e.g., soil aggregation [8].

Starting from the most conventional techniques, we have the open pit and underground mining techniques that differ essentially by the depth of the uranium-containing rock. In both methods, the ore is extracted through mechanical means (e.g. blasting, drilling, shoveling) and transported to the surface. Open pit mining consists in the removal of superficial rocks to get to the uranium ore, while underground mining, due to deepness

of the ore, involves the construction of access shafts and tunnels. The latter process, results in less waste rocks, and therefore it has less environmental impact, at least at first glance [17].

Once the ore is at the surface, it has to be crushed, grinded and watered to create slurry with about 50% of solids, which is then leached. Then, uranium oxides are stripped from the extraction solvent and precipitated as yellowcake, predominantly U_3O_8 [5].

Usually, the ore slurry that results from both underground and open pit mining, are leached using one, out of two types of processes: conventional agitation leaching and heap leaching.

Uranium ores above 1000 ppm, usually follow conventional agitation leaching, i.e., the slurry is forwarded to a sequence of tanks (Fig. 3) where it is first mixed with a leaching solution (acid or alkaline) and an oxidant (e.g., oxygen, sodium chlorate, hydrogen peroxide, or manganese dioxide) in a controlled pressure and temperature tank (50°C-60°C and 90-95°C for acid or alkaline leaching, respectively), in order to strip uranium from the ore and dissolve it [17].

Sulphuric acid or carbonate are the most common acid and alkaline leaching solutions, respectively; with the choice of the solution and oxidant being dependent on the composition of the host rocks [17].

Despite not very common, some low-grade uranium mines employ microorganisms (e.g., *Acidithiobacillus ferrooxidans* or *Leptospirillum ferrooxidans*) as leaching catalysts, to improve the recovery of uranium. This process is named bioleaching, and the enhanced uranium recovery is due to the increase in the availability ferric ions promoted by microorganisms, which avoid using oxidants further than oxygen. The presence of microorganisms makes this process cheaper and with lower environmental impact [18].

The liquid solution containing uranium needs later to be separated from the remaining solids, as so, the ore slurry is washed and decanted in countercurrent (with acidified or clearing water, depending on the leach solution used upstream), and then filtered (e.g. by horizontal belt and drum filters). As a result of this process, some tailings are produced, i.e., the washed leftover solids. The uranium liquor, is then purified by means of ion exchange or solvent extraction. From the concentrated uranium solution (75-85% of uranium content), known as “pregnant solution”, a U_3O_8 powder, usually called “yellowcake”, is obtained through precipitation (using e.g., hydrogen peroxide,

magnesium oxide, sodium hydroxide), compression and dehydration. This overall process, usually allows to extract 95-98% of uranium from the host rock [8,17].

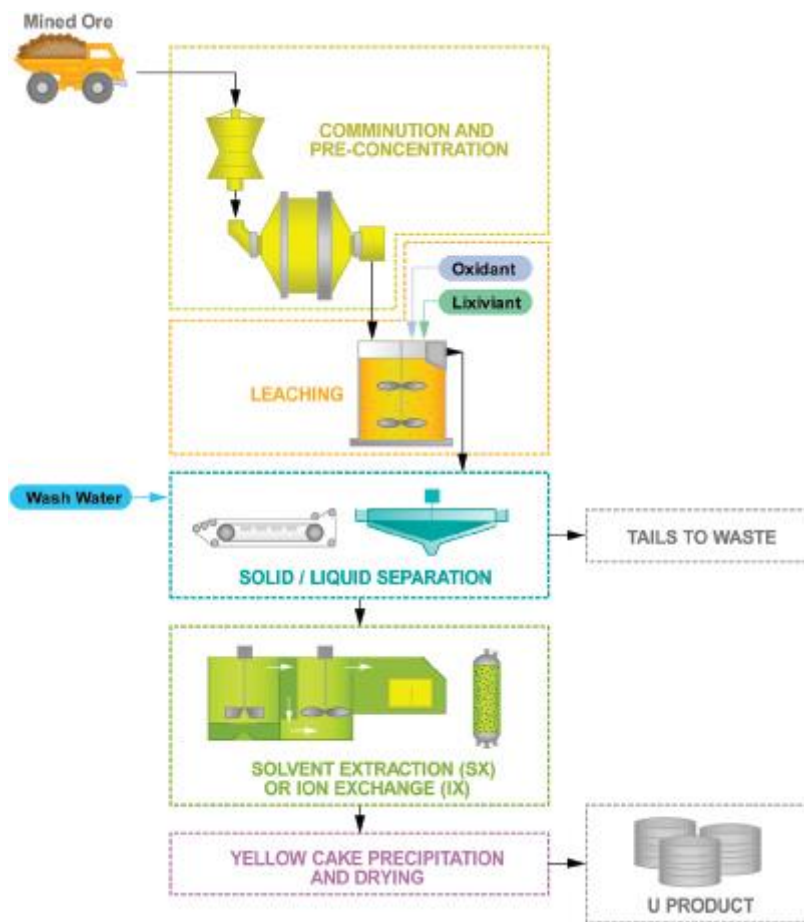


Figure 3- Conventional agitation leaching process

Source: <https://www.nap.edu/openbook/13266/xhtml/images/p113.jpg>

When the ore resulting from underground or open pit mining is very low-grade, it is usually treated by heap leaching. In this process (represented in Fig.4) the leaching process does not take place in tanks. Instead, the broken ore is piled in heaps up to 30 meters on an impermeable surface and irrigated at the top with the leaching solution, over many weeks. The resulting pregnant liquor is collected in a basin at the bottom of the pile and sent to a processing plant for the extraction of the solvent, following the same processes above described for the conventional agitation leaching. The rate of uranium recovery in this process is generally lower (50-80%) and once the piled ore ceases to yield a liquor significantly uranium-enriched it is removed and replaced by new ore [8,17].

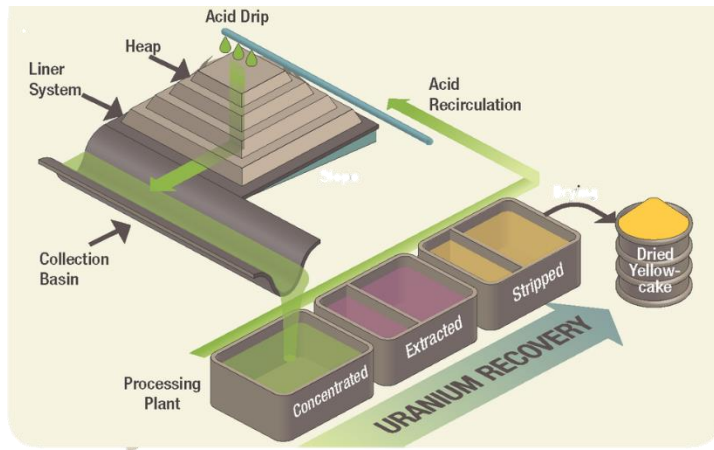


Figure 4. Diagram representing the heap leaching process for uranium recovery from poor ore.

Source: <https://www.nrc.gov/images/materials/uranium-recovery/extraction-methods/heap-leach-recovery.jpg>

In addition to conventional mining techniques, a significant percentage of uranium is extracted by in situ leaching (ISL). This process (figured in Fig. 5) does not imply the removal of rock from the ground, once the leaching/removal of uranium from the host rock is done underground. ISL can only be performed on uranium ore bodies laying on unconsolidated/loose material, such as gravel or sandstone uranium deposits confined vertically and ideally horizontally between two impermeable layers (e.g., clay). The process consists in slowly injecting the leaching solution through a well in the ore body, followed by pumping to the surface, through a recovery well. The uranium-pregnant liquor, is then forwarded to a processing plant to undergo the same treatment described for the conventional agitation leaching. Additional wells are opened/used to monitor the stability of layers and eventual run-offs of the leaching solutions [17].

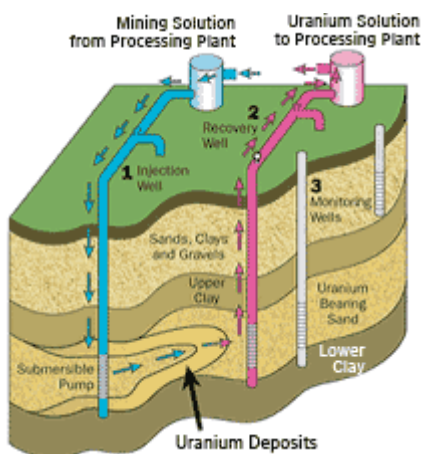


Figure 5. Diagram representing the situ leaching (ISL) mining of uranium ore.

Source: https://www.earthworksaction.org/images/uploads/insitu-leach-diagram_NRC_273x225.gif

Despite less expressively, uranium can also be recovered as a by-product from other mining activities such as exploration of phosphates, but also gold, nickel or copper. In this case, the recovery of uranium can also be undertaken for environmental reasons or to guarantee the purity of the product of interest (e.g., in the production of phosphoric acid fertilizer) [17].

After extraction and purification, the resulting dried yellowcake is then refined and enriched (e.g., by gaseous diffusion, gas centrifuge separation, thermal separation, and more recently by laser separation) and converted in UF_6 or ceramic uranium dioxide (UO_2), i.e., enriched uranium to be used for example, as fuel for nuclear power stations. The leftover of the process is depleted uranium, which can be used for example, as counterbalances for helicopter rotors as previously described [7].

To sum up, uranium mining activities are not very different from other metal exploitations, except that the radiological proprieties of the uranium implies more concerns respecting workers, local inhabitants [19] and environment.

2.1. Characterization and associated risks of uranium mine effluents

All mining industries, and uranium mining is not an exception, generate waste rocks, i.e., host rock that is valueless, and other wastes resulting from the extraction and purification of the mineral of interest, such as tailings and wastewaters.

To gain a perspective on the amount of the waste material resulting from uranium mining, it may be interesting to perceive that during a year, a single standard nuclear reactor (loading factor: 80%; thermal conversion rate: 33%; daily burn-up: 40000 MW) requires the extraction and smelt of more than 130 000 tons of ore (assuming an ore grade of 2000 ppm and a uranium recovery rate of 93%; ^{235}U content of 0.3%) [20].

Mine wastes can be divided into waste rock, tailings and wastewaters. Waste rock can be defined as the material that was removed to gain access to the ore, and usually has a relatively low concentration of uranium). Its main impact is on site instability and on landscape visual amenity. Nevertheless, piles of waste rock may contain elevated concentrations of radionuclides with long half-time life (some of them, more radioactive than uranium itself) compared to rocks of non-uraniferous areas, and be subject to acid drainages (as discussed below).

Tailings are the waste that results from grinding and chemical process of uranium extraction, i.e., it is a slurry of sands, leftovers from crushing process plus residual elements from the chemical procedures. Therefore, it contains several metals and other

contaminants, as well as uranium and its progeny [5,21]. Currently, this material is usually stored into confined sedimentation lagoons, i.e., a ground hollow enclosed by barriers where the tailings are placed, to prevent the seepage of this material into soil and groundwater [22]. However, in the past, tailings were stacked in unconfined open piles or used as construction material, in concrete buildings and roads [23].

One of the major environmental problems of these two types of wastes, are potential acid drainages, i.e., the outflow of acidic waters containing uranium, daughter radionuclides, and other metals and metalloids in solution. Acid drainages occur through natural weathering of waste rocks and tailings, containing sulfide minerals (e.g., pyrite (FeS_2)). These drainages are of special concern, once the dissolution in acidic water of the toxic elements (metals and radionuclides) increases their mobility and bioavailability. The availability of oxygen and bacteria induces the production of sulfuric acid inside the pile, resulting in an endless production of acid leachates, as so, an eternal source of contamination of groundwater [17].

Beyond acid leachates, there are a plenty of liquid effluents that result from uranium mining industry; in fact, all the mineral processing steps (e.g., ore extraction, crushing and grinding), metal recovery phases (e.g., leaching, solvent extraction and precipitation), as well as equipment cooling and dust control, require a significant amount of water [24]. The wastewaters from this industry can be classified upon mine, mining, milling and process water and leachates, which can be all joined in the same ponds and named as mine effluents if discharged into surface or groundwater, often after undergoing a treatment process. The potential toxicity of these wastewaters rely on a several number of factors: 1) type of ore (e.g. ores tends to be more toxic according with its content on metals, metalloids and substances such as sulphides that promote the solubility and mobility of contaminants); 2) chemicals used in the mineral processing and metal extraction; 3) climate (arid regions tends to have a higher degree of soil and waters contamination, in part due to a lower water availability/lower dilution power); 4) life stage of the mine, management practices and environment policies enforced [24].

Once identified the sources of wastewaters and the main factors governing their characteristics, it may be useful to analyze a typical uranium mining effluent. Despite its heterogeneity, it presents high electrical conductivity (above $1000 \mu\text{S}/\text{cm}$) due to high concentration of dissolved salts. Its most likely contaminants can be broadly categorized as: organic chemicals (oils, grease, detergents, dyes and phenolic compounds), inorganic chemicals (metals, acids, alkalis and dissolved cations and anions), biological (some bacteria and viruses) and radiological (uranium and its progeny) [25].

Uranium mine effluents are in general not very different from those originated by other mining industries, with the exception of the significant presence of radionuclides. Besides radioactive contaminants, a diversity of non-radioactive metals and salts, such as iron, copper, vanadium, nickel, arsenic, manganese, magnesium, molybdenum, selenium, fluorides, sulphates, chlorides, carbonates, nitrates and organic solvents, are usually found in uranium mine effluents (depending on the ore body, gangue mineralogy and the processing techniques used) [26]. The presence of this panoply of contaminants can exacerbate or mask the availability of the radionuclides on the wastewaters and can have harmful effects on human and non-human biota. Therefore, there is more to raise concerns about than radiological risks associated to uranium mine effluents (e.g. the chemical toxicity of the radionuclides, metals, metallic and non-metallic compounds present in the ore or introduced during mining processes; increased acidity, salinity and turbidity).

The radiotoxicity of uranium and other radionuclides, as well as uranium chemotoxicity will be extensively discussed below. But to have a better overview of the complex mixture of contaminants that may contribute to the toxicity of uranium mine effluents, it may be useful to analyse the table 2, which summarizes some of the potential harmful effects for aquatic biota caused by non-radioactive contaminants usually found in uranium mine wastewaters.

Table 2- Some of the most common non-radioactive contaminants found in uranium mining wastewaters and their known potential effects in aquatic biota.

Source	Contaminant	Some notes of potential harmful effects
Waste rock or tailings	Aluminum	Main harmful effects are related with its ability to affect some enzyme systems that are important for the uptake of nutrients. In acidic waters, can induce impaired gas exchange in some organisms, especially in embryo stages [27]. It is also neurotoxic [28].
	Iron	Precipitates of ferric hydroxide and of iron-organic matter can affect the metabolism and osmoregulation mechanisms of organisms and may cause a decrease in the diversity and abundance of some benthic species by changes on their habitats. Beyond that, it can also acidify the water when ferric irons hydrolyze [29].

	Copper	Even at very low doses, it can compromise photosynthesis and growth in algae and present teratogenic effects in some aquatic species. At higher concentrations it may reduce survival of many macroinvertebrate species. Also have neurotoxic effects on fish [30].
	Vanadium	Even at low concentrations it may cause neurotoxic and hepatotoxic effects as well as, reproduction and breathing disorders [17].
Chemicals used in uranium processing	Sulfuric acid	Acidification of wastewaters, which in turn promotes dissolution and major bioavailability of other toxic compounds such as uranium, aluminum and iron [17].
	Sodium hydroxide	Not toxic by itself but in large amounts may cause the raise of pH level to limits that may affect some aquatic species [17].
	Carbonate and bicarbonate	It can affect aquatic ecosystems by raising the alkalinity of water [24].
	Ammonia	Under alkaline conditions it can affect aquatic organisms, leading to increased heart and respiratory rates in fish, as well as reduced hatching success and growth. It can also cause damage in several organs, such as liver and kidneys [24].
	Dodecanol	It results from lubricants, surfactants and solvents used in mining and its main target organs are the lung and liver. It can also be bioaccumulated. It is more toxic to saltwater rather than to freshwater organisms [17].
	Kerosene	Some of the compounds of kerosene (e.g., benzene, toluene, and xylene) are persistent and may be bioaccumulated. They can affect and cause chronic effects in a great variety of systems, such as respiratory, immunological, reproductive, hepatic, and circulatory. They can also have teratogenic and genotoxic effects [31].

Taking in account all the above, it is clear that the uranium mining industry can negatively affect the quality of the surrounding ecosystems (both water resources and soils) with

direct impact in the species richness and communities structure and functioning [32–34]. It can also impact the human health by means of contamination of surface and/or groundwater resources [19].

Regarding the nuclear fuel cycle, there is a lot of literature on the consequences of radionuclides exposure or uptake by humans. However, in the field of environmental risk assessment, most of studies only focus on fallouts or accidents in nuclear power facilities and leave aside the nefarious impacts to the biosphere that are triggered by uranium mining effluents. This anthropogenic focus paradigm is gradually shifting, not only due to some environmental education programs and public awareness for this subject, but also due to scientific evidences on the deterioration of fishery areas in the surrounding of uranium mines [35]; the perception of radionuclides uptake by plants and respective bioaccumulation and bioamplification [36], reports on long-term availability of radionuclides in aquatic sediments to bottom feeders [37]. However, the main driver for adoption of environmental policies in relation to uranium mines, is the accumulate of evidences that low radiation doses may impact human health in a long-term scenario. As so, it's important to truly understand the extent of all the effects of uranium mining effluents in the ecosystems, mainly in aquatic ones, in order to be able to draw more effective environmental risk assessments in uraniferous areas.

2.2. Treatment of uranium mining effluents and mine rehabilitation

Liquid effluents are the main source by which the uranium mining industry negatively impacts the environment. As such, all mines in countries with some kind of environmental legislation, have the obligation of removing some of the contaminants from the effluents before its environmental discharge. To help on decontamination and also to seek for the most cost-efficiency remediation process, several treatments (which can be applied in a single or combined way) have been developed [38].

The treatments can be broadly separated into: a) active/conventional treatments, usually applied during the operation period of the mine and for larger volumes ($>50 \text{ m}^3/\text{h}^{-1}$); b) passive treatments, prevailing during decommissioning and long-term monitoring of the mines, as well as for smaller volumes, once it is cheaper and requires low-maintenance. However, both types of treatment systems may be applied simultaneously [38,39].

The table below (table 3) compiles some of the most common treatments of uranium mine effluents with a brief description of the method, its efficiency/advantages and disadvantages.

Table 3. Some of the most common chemical and biological treatments applied to uranium mine effluents.

	Treatment	Description	Advantages	Disadvantages
Active treatment systems	Lime neutralization	It is usually used for acidic effluents. An amount of calcium hydroxide (15-20%) sufficient to raise pH to 10 is added to the effluent in a reactor, and then decanted for solids stabilization and sludge deposition.	low cost; co-precipitates most of metals (uranium is precipitated as calcium diuranite) as well as sulfates and hydroxides.	High volume of sludge produced (2 to 15% of solid content, depending on the amount of process cycles).
	Ferric chloride precipitation	It is usually a complement of lime neutralization process. Ferric chloride (FeCl_3) is added to the slurry resulting from neutralization to precipitate arsenic in a very low solubility form as well as to adsorb some metals and radionuclides, which are then decanted or settled by gravity.	Efficient removal of arsenic (down to $<0.1 \text{ mg L}^{-1}$); some additional removal of metals and radionuclides; small amount of chemicals required.	High volume of sludge produced; may need previous adjustment of pH.
	Barium chloride precipitation	It is often used in association with ferric chloride precipitation and lime neutralization. Barium chloride (BaCl_2) is added to the effluent to co-precipitate radium ($\text{Ba}(\text{Ra})\text{SO}_4$) as well as other radionuclides, which are then decanted or settled by gravity. When used as complement of	Efficient removal of radium (down to $<0.3 \text{ BqL}^{-1}$); removal of other radionuclides; small amount of barium chloride required (30-60 mg L^{-1}).	High volume of sludge produced (depending on sludge recirculation); may need a previous adjustment of pH.

		lime neutralization as a sludge thickener.		
	Ion exchange	It is generally used only in specific scenarios: to achieve high water quality standard or to remove a specific contaminant for further use or economic valuation. It is a process based on the exchange of dissolved ions of the same electric charge, i.e., a synthetic polymeric resin loaded with specific charged ions which will be exchanged with others depending on the pK values of the functional groups of the resin, removing them from the effluent. When the resins are full/spent, they need to be regenerated by backwashing them with different solutions, depending on the type of resin.	Highly efficient for a variety of contaminants (however the contaminant removed depend upon the resin that is used).	Expensive process due to the cost of regeneration resins.
	Ion adsorption	It is relatively similar to ion exchange, once it is “contaminant-specific”. It consists in removing a specific contaminant or group of contaminants from the effluent by adsorption to the surface of a specific adsorbent. The synthetic adsorbent can be a fixed structure or can be added to the effluent, where its reactive surfaces adsorb the respective contaminant, forming a complex, which then can be precipitated and removed by filtration or precipitation.	Highly efficient for a variety of contaminants (however demand a specific synthetic adsorbent for each contaminant).	Expensive. However presents higher cost benefits than ion exchange (if the contaminant is not of economic value).

Passive treatment systems	Constructed wetlands	It is the built of wetland ecosystems specially designed to optimize naturally processes of plants such as the uptake and adsorption of metals; i.e., the effluent is in contact with hyperaccumulator/hypertolerant plants and/or plant roots, for different retention times, which will remove the contaminants from the effluents trapping them on their tissues. This system can be superficial, subsuperficial or underground flux.	Removal of metals and radionuclides.	Requires larger areas; only applicable to effluents with low content of contaminants, i.e., is a complement to other treatments; plants once used must be treated as residues; retention time is a limiting factor for the effectiveness of this treatment.
	Anoxic limestone drains	It is essentially an anoxic underground limestone bed, through which the effluent flows by gravity. In the process, limestone is dissolved, adding CaCO_3 to the effluent and raising its alkalinity. Then, the effluent goes to an aeration pond or aerobic wetland to oxidize and remove the precipitated metals.	Efficient raising of pH level (as it can add up to 300 mg L^{-1} of CaCO_3 to wastewater), which may allow metal oxidation, hydrolysis and precipitation to occur.	Clogging of pores with precipitated iron and aluminum hydroxides, shorten the efficiency and longevity of the method; only efficient in effluents with low level of ferric iron, aluminum and dissolved oxygen
	Permeable Reactive Barriers	It is an <i>in situ</i> permeable treatment barrier, i.e., a buried barrier of a reactive material (e.g. limestone, zeolites, activated carbon and apatite)	Removal of trace metals and radionuclides; low maintenance	The passive and slow rate of the method, may require several years or

		that intercepts the underground flow of the effluent removing some of its contaminants.	costs; suitable in the context of long-term site remediation.	decades for an efficient remediation; mineral precipitation and biofouling may clog the barrier.
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Sources: [38–40]

Note: From a physicochemical point of view it is difficult to distinguish precipitation from co-precipitation and adsorption, once that all three processes may be responsible together – to a varying degree – for the removal of ions from solution at any time.

The choice of the treatment besides the singularities of each effluent depends upon the receiving waters and the final effluent quality objectives, the type and specificities of the mine (e.g. flow rates of effluents as well as its variability during decommissioning and remediation) and the costs of each treatment. As a consequence, the strategy applied may vary over time. For example, during the production period of a uranium mine and immediately after its closure (when the volume of effluent is higher) the choice of active treatment processes is recommended. However with the decrease of the effluent volume through the years and the corresponding load of contaminants to receiving systems, the treatment paradigm may switch to a passive strategy (as illustrated in Fig.6) [41].

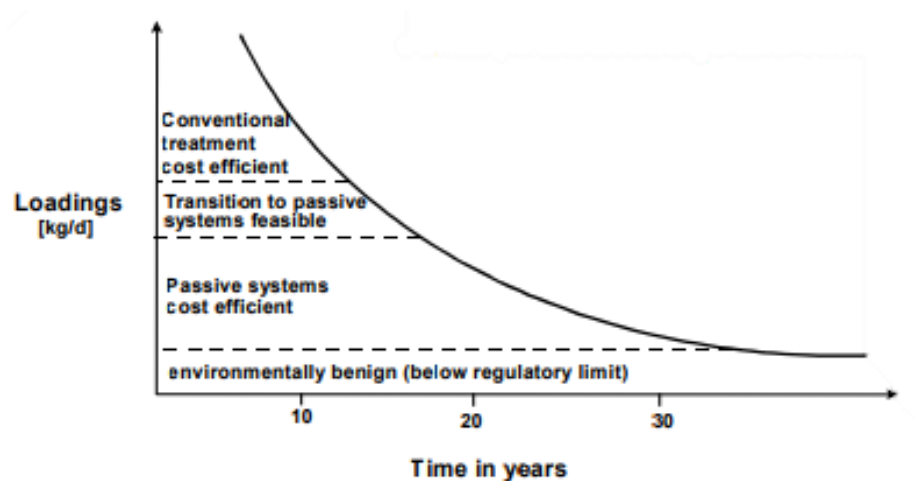


Figure 6. Selection of cost efficient water treatment strategy as a function of contaminant loadings and time. Source: [41]

Uranium mines that ceased their activity recently or in the past have to undergo environmental remediation works to minimize its environmental legacy. The main goal of rehabilitation of uranium mining sites is to recover the land for safe future uses. Whenever that is not possible, the goal is to restrict the access to the affected area [42].

In a general and simplistic way, this encompasses removal of all former mining structure; remediation of affected water and soils; management of any resulting wastes/leftovers and preparation of the site, taking into account the future planned uses [42]. Although theoretically simple, the multiplicity and variability of the environmental risks associated with uranium mines do not allow the development of a standard remediation approach applicable to all mines. Thus, environmental site-specific risk assessment and risk management (Fig.7) is required at all stages of the process. A decision-making process on the remediation strategy and targets for a specific uranium-mining legacy needs to be supported on a truthful characterization of the risk of the site. That “description” of the risk encompasses a series of qualitative and quantitative ecotoxicological information and can only be drawn by risk assessment, which by its turn is supported on scientific research [43].

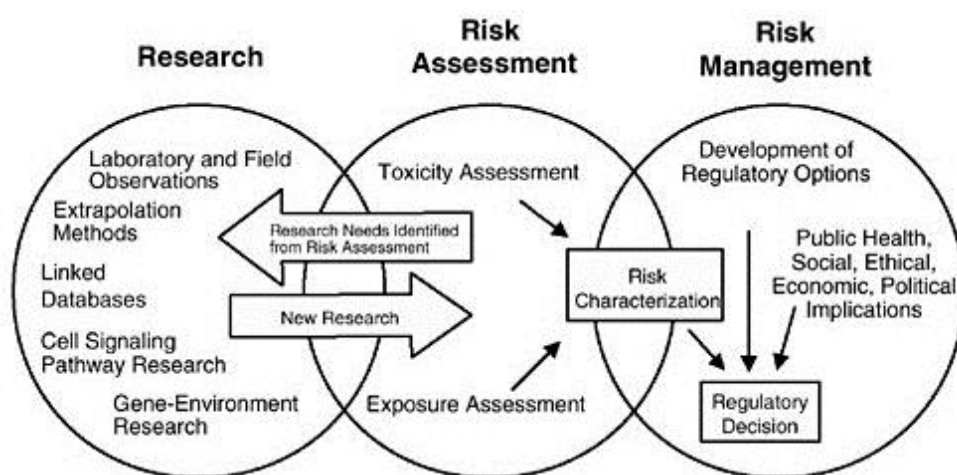


Figure 7. Risk assessment and risk management paradigm.

Source: [43]

2.3. Legal framework for the discharge of uranium mine’s wastewaters

Taking into account the potential effects (as previously elucidated) of discharges of radioactive effluents, not only to the exposed nearby humans inhabitants, but also to the receiving ecosystems, most of countries with uranium mining activities (at the moment or in the past) draw laws and regulation programs to restrict and control the doses of radioactive material that are discharged in water courses, as well as deposited in soils (in some countries) [8,40].

The authorized limits of radionuclides in uraniferous discharges (usually quantified at or near the source) are variable (or may not exist), since each country can define its own laws. However, in theory, each country tries to draw the lowest limits of discharge

parameters economically and reasonably possible [40]. Some of the countries with these mining activities also take part in some conventions relevant in the field of radioactive waste management and draw programs for the environmental monitoring of effluents [40].

Despite the ephemerality of the permissible values for effluent discharges from uranium mines (given the constant change of laws due not only due to governments of each country but also due to the evolution of the scientific knowledge and public pressure on environmental issues), some examples of parameters limits in uranium mine effluents in some countries, for the year 2002, are displayed in the table below (table 4).

Table 4. Concentration limits of some trace elements and parameters present in uranium effluents or uranium plants for different countries in 2002 [40]

Country	U	²²⁸ Ra	Pb	Zn	Cu	Ni	As	SO ₄	Cl	pH
	mg/l	Bq/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	-
Czech Republic	0,1	0,3	0,1	0,2	0,1	0,15	0,1	300	350	6-9
France	1,8	0,37					0,1	2000		6,5-9
Germany	0,2-0,5	0,2-0,4					0,1-0,3	2500 - 7500	1000 - 1500	6,5-8,5
Spain			0,2	3	0,2	2	0,5	2000	2000	5,5-9,5
United States		0,18	0,015	5	1,		0,05	250	250	6,5-8,5
Portugal			1		1	2	1	2000		6-9

In the above table, the values relatively to uranium are depicted in mg/L. However, currently, some countries choose to define uraniferous effluent limits in terms of radioactivity content [42].

In Portugal, at the moment, there is no regulation for uranium mining discharges in particular, once they are regulated as the general residual waters (as so, these discharges are only subjected to some simplistic boundaries, like chemical oxygen demand (COD), concentration of some metals, pH and conductivity), which is in part explained by the absence of uranium mines in activity. However, for drinking water, the 2015 actualization of european directive on water for human consumption, was translated to national legislation on Law by Decree nº23/2016 [44] and implement limit benchmarkes of radioactivity content of α and β particles of 0.1 and 1.0 bq/L, respectively and if these limits are exceeded, some specific radionuclides (e.g., U²³⁸, U²³⁴, Ra²²⁶ and Po²¹⁰) need to be measured and must be below specific defined thresholds.

3. Speciation and bioavailability

In order to understand the environmental distribution of uranium and how it can affect biota, it is first necessary to understand its speciation, i.e., uranium chemical forms or species, and its characteristics [45]. Only then, uranium's bioavailability, i.e., the degree to which a chemical compound in a potential source is available/free for uptake [46], can be assessed.

Unlike organic compounds, metals (e.g. uranium) do not degrade. Instead, they circulate in the environment in various forms or species [3]. Chemically, uranium has four possible valences (+III to +VI), being valences IV and VI the more frequent states in the structure of uranium minerals and in aqueous solution media [3]. The state in which uranium presents itself is related with environmental oxidizing-reduction conditions and has an enormous impact in its solubility and mobility in aquatic ecosystems [47]. In low redox potential environments (e.g. anoxic waters), a significant part of uranium occurs in the IV state (U^{4+}) and has a tendency to precipitate (e.g. in solid uraninite UO_2) [47]. On the other hand, in waters that have high oxygen levels, uranium tends to appear in state VI, which results in the formation of the uranyl ion (UO_2^{2+}) [47]. This is the main form of uranium available for organisms as a free ion, but may also form quite soluble and mobile complexes (e.g. uranyl-carbonates and uranyl-sulphates). Besides the redox potential, the speciation of the uranyl ion on freshwater (and by consequence uranium's bioavailability to aquatic biota) also depend upon: 1) alkalinity due to the formation of uranyl ion complexes with carbonates, which then may not be available for assimilation by organisms surface cells due to its molecular size; 2) content of humic substances, which reduces bioavailability of uranium by forming stable uranyl complexes [47]. Water hardness lowers the bioavailability of uranium due to the competition between uranium, calcium and magnesium for the binding surface of organism's cells [47]. It is worth highlighting that there is some interconnection between the above-mentioned factors, once for example, a raise in hardness results in a higher alkalinity of the medium.

4. Natural radionuclides: uranium decay chain and ionizing radiation

There are more than 16 radioisotopes of uranium (most of them only occur in nuclear reactors).[1]. Since part of the economic interest, as well as of the toxicity of uranium is due to its radioactivity, in this chapter we will discuss not only the concept of ionizing radiation (IR) but also some of the natural radionuclides, which are usually found in mining effluents, as they belong to the decay series of uranium forms found in nature (^{238}U , ^{235}U , and ^{234}U).

There are several natural radionuclides, which can be theoretically divided in those who occur solely and those who are part of one of the three natural radioactive chains (^{238}U ; ^{235}U ; ^{232}Th) [48]. The two chains that encompass all the three natural occurring uranium radionuclides are depicted in figure 8. A decay chain refers to the different unstable products and to the energy (ionizing radiation) released by each parental radionuclide when it is decaying to a more stable and less energetic form. The time rate at which these transformations/decays occur follow a decay constant, that is the fingerprint of each specific radioisotope, generally named half-life (the time that it takes to half of the initial population of a parental radionuclide have decayed to their daughter isotopes), which can vary from some seconds to millions of years (e.g. half-life of $^{238}\text{U} \approx 4.47 \times 10^9$ years) [49].

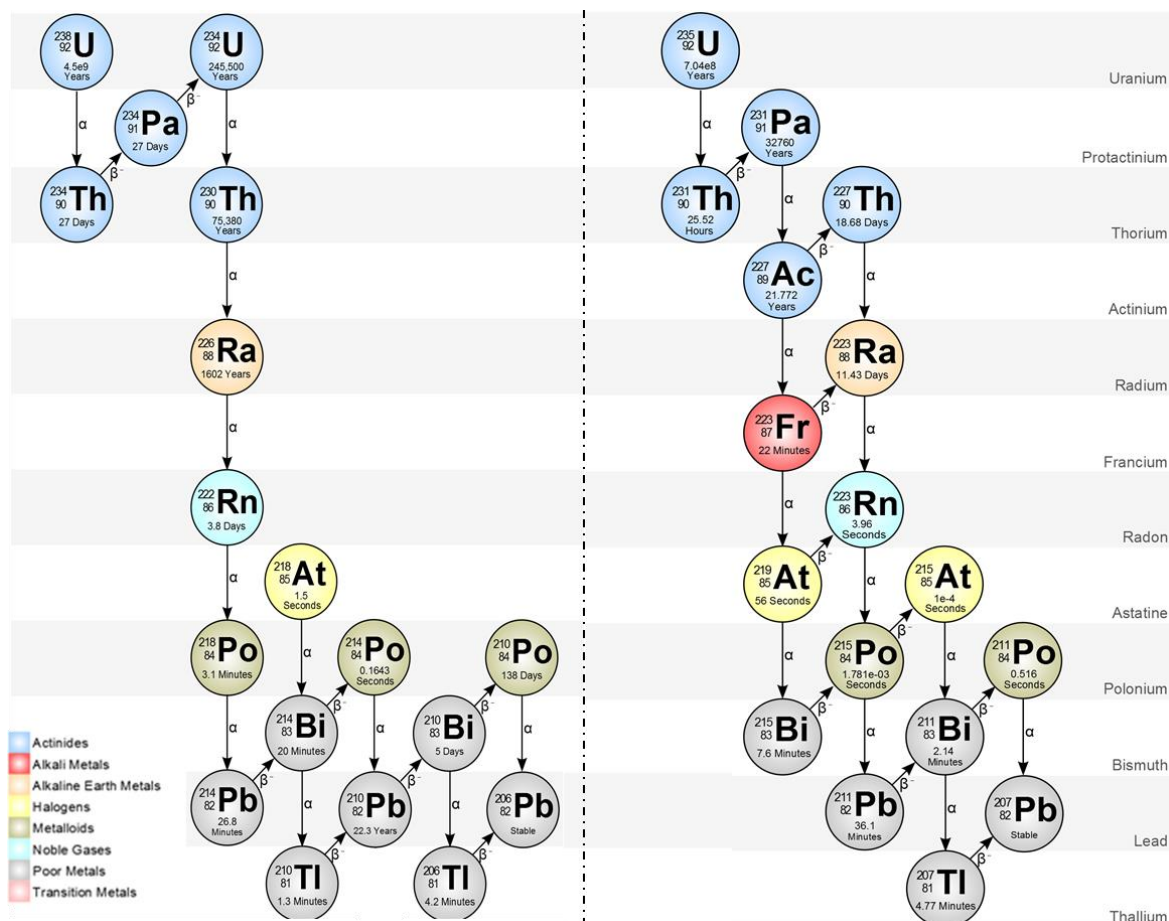


Figure 8. Uranium decay chains showing all the decay products, its half-lives as well type of IR released: Left- ^{238}U decay chain (contains radionuclide ^{234}U). Right- ^{235}U decay chain
Source: <http://metadata.berkeley.edu/nuclear-forensics/Decay%20Chains.html>

Figure 8 shows all the natural radionuclides present, which belong to two different decay chains (one starting in ^{238}U and the other on ^{235}U) and also the types of IR that each radionuclide releases when undergoing decay and their half life's. It is possible to see that most of them releases α or β particles, however, some of them release residual γ -rays, which are not represented in the figure (e.g. ^{235}U also beyond α -particles, also release γ -rays). The radioactive decay of uranium, results in the formation of lead (^{206}Pb), which is stable and does not emit ionizing radiation [50].

Ionizing radiation is the energy, in the form of particles (α and β) and/or rays (x and γ), which is released from radioactive materials. When interacting with atoms and molecules it displaces electrons from higher-level orbitals, giving rise to ions [51]. A radioactive atom seeks to gain stability by altering the number of protons in the nucleus, and to that end, it can transform a neutron into a proton, thereby releasing a β^- (electron) particle or by converting a proton into a neutron with β^+ (positron) release [51]. However, certain higher mass atoms (e.g. uranium), release α instead of β particles. Alpha particles are

similar to the nucleus of a helium atom, with two protons and two neutrons. These particles, due to their 2+ charge, have the ability to "steal" up to two electrons, ionizing all the molecules along their path. On the contrary, γ and X rays are electromagnetic radiation (do not have mass) that may deposit a huge amount of energy in a small volume. Therefore, all types of IR have the ability to damage biological tissues [51]. That damage can be direct, i.e., by directly interacting with the molecule and destabilizing its structure potentially, affecting critical molecules like enzymes, DNA, RNA; or indirect, i.e., when the molecules (e.g. H_2O) upon which radiation strikes by losing their atoms, form free radicals which may recombine with other molecules forming toxic compounds, such as hydrogen peroxide (H_2O_2). It is important to note that interaction between IR and biological tissues depend on the type of radiation, i.e., on its different characteristics such as LET (linear energy transference), path length and RBE (relative biological effectiveness).

Based on the amount of energy it transfers per unit path length it travels, IR may be classified as either high or low LET, which will seriously influence its interaction with molecules. As illustrated in figure 9, a higher LET means high energy transfer to the molecules in its path causing a high number of ionizing events/collisions in a very short distance, therefore interacting strongly with the material it traverses (e.g. DNA molecule). The high number of ionization/collision events in conjunction with the particles size lowers its speed. which means that high LET particles have low penetrating power..

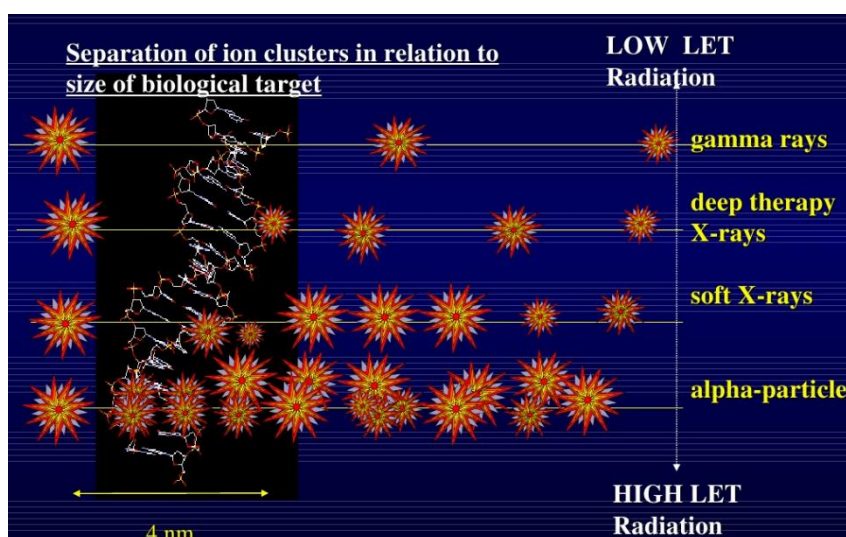


Figure 9. Relationship between LET, spatial distribution of ionizing events and size of a target DNA molecule.

Source: <https://www.slideshare.net/wfrrt1360/05-linear-energy-transfer>

For example, α particles have high LET, i.e., transfers/releases a high amount of energy per unit of path length that travels, however its penetration power is very low (25-80 μm), which means that it cannot penetrate on most of organisms after being emitted from an external radiation source [51], as such it will only be able to impact cells only if ingested. Organisms can be exposed to both external and internal IR doses. External IR comes from sources outside the body, such as medical radioactive procedures (X-rays or CT-scans), cosmic radiation, background levels of terrestrial radiation, as well from nuclear fallout [51]. Due to its ability in irradiating from distant sources and highly penetrating power, the γ rays are the one of most concern in this type of exposure, since the α and β have a low penetrating power. Internal radiation doses concerns the ionizing radiation that a radioactive material gives off during the time it is inside the organisms body. In this scenario, the α particles due to its high LET are of special concern [52]. The pathways to each a radionuclide enters and is redistributed throughout the body relies on the sources, on the chemical speciation and bioavailability (discussed in sub-chapter 3) and do not depend on its radioactive properties.

To conjugate some of these factors underlying a specific IR impact, it can be useful to quantify its relative biological effectiveness (RBE). While LET is related to physical characteristics, RBE is based on biological damage caused, i.e., it is the ratio between a dose of a reference radiation (usually low let x-rays(200 keV)) divided by the dose of radiation in question to produce a given level of damage [53]. RBE is expected to raise with increasing LET. The type of damage induced by high LET radiation may be irreparable or very difficult to repair when comparing with the type of damage induced by low LET radiation [54].

4.1. Toxicity: chemical and radiotoxic effects

As already referred above, uranium is a radioactive non-essential metal that presents dual-toxicity as it is a chemo- and a radiotoxic element. Despite the general assumption that natural uranium chemical toxicity is of greater concern than its radiotoxicity [55], its radioactive impacts should not be overlooked [56], and both have to be integrated to perform a correct assessment of uranium mining waste impacts in ecosystems.

In section 3, the factors that influence the bioavailability of uranium were exposed. However, in order to perceive how uranium can exert its toxicity, we have to first understand how it is assimilated by exposed organisms and reaches its target sites. In

other words, we have to perceive the toxicokinetics of uranium, i.e, its mechanisms of absorption, distribution, metabolism, and elimination [45].

Organisms can uptake uranium by skin sorption or by means of ingestion, or through indirect dietary exposure. Once inside the organism, it is distributed heterogeneously among the different tissues depending upon the different affinities of biomolecules and cell membranes to uranium. Then, once in contact with a cell's surface, uranium can be transported to the cytoplasm, by facilitated transport mechanisms, as well as by passive diffusion [57], where it can exert its toxicity.

Uranium is a non-essential metal, as such, in the presence of uranium, cell's metabolism change its oxidation state with the aim of facilitating its excretion. As so, uranium concentration within organisms is a result of the balance between its uptake and its elimination and relies on many different factors. Uranium uptake may depend upon: a) organism size (smaller organisms uptake more due to surface area/volume ratio); b) anatomic characteristics (hard-shelled animals can accumulate metals during growth, whereas soft-organisms quickly equals the internal concentration with the external media); c) feeding activity pattern and d) metabolism of metals [45]. On the other hand, elimination of uranium strongly varies within organism's complexity and may include uptake inhibition, detoxification and storage, and/or transformation for excretion.

It is when assimilation rate of a metal overcomes its elimination, that a metal starts to accumulate within an organism (e.g. for human exposure to uranium, bone and liver are the tissues where most of the accumulation occurs) and may start causing damage if the concentration of metal overcomes the threshold dose per time which an organism can deal with [58].

As such, uranium can be bioaccumulated in organisms, and be uptaken through ingestion. Therefore, uranium can be transferred through the food chain. However, due to a low metal assimilation level, there is no bioamplification. As such, uranium concentrations are higher among lower trophic level species [59].

The effects of uranium exposure largely depend on several factors such as species and their intrinsic characteristics, behavior and ecology, the route of exposure, and the speciation of uranium. For example, vertebrates can be exposed through inhalation, oral and dermal pathways and organs such as kidney, lung, liver, nervous and cardiovascular system can be affected [60–62], mainly due to its chemotoxicity. The radiotoxicity of uranium is related with an internalization of α -emitter radionuclides [51]. Several studies addressing different routes of exposure and endpoints were finely reviewed by the U.S.

Agency for Toxic Substances and Disease report on Toxicological Profile For Uranium [1] and Ionizing Radiation [51].

An important mechanism of toxicity of uranium and its associated IR, is the interaction with proteins, lipids and DNA molecules [51,63,64]. It is able to promote DNA damage, causing single and/or double strand breaks, and loss of bases from the DNA molecule [64], and it can also affect mitochondrial processes, DNA repair mechanisms, gene expression, induction of apoptosis, formation of free radicals and induction of oxidative stress [51,52]. These damaging effects may lead to the reduction of individual fitness and affect populations parameters such as growth and development [65–67], as well as be possibly transmitted to the offspring [68,69].

Taken into account the broad context of the master degree in which this dissertation is inserted, i.e, the quality of aquatic ecosystems, and to give the reader an overview of the range of uranium doses that impact different aquatic species, this subchapter finishes with the predicted no-effect (PNEC) values, proposed by Sheppard et al. [55] delivered for aquatic organisms, namely: 5 $\mu\text{g U L}^{-1}$ - freshwater invertebrates; 100 mg U $\text{Kg}^{-1}_{\text{dry}}$ sediments - freshwater benthos; 0.4-23 mg U L^{-1} - freshwater fish.

4.2. Non targeted effects of ionizing radiation

As previously described, part of uranium's toxicity is related with its ability to release IR, which can cause a severity of genotoxic and other cellular damages, through direct (e.g. disrupting of the DNA structure due to direct deposition of ionizing energy) and indirect (e.g. formation of free radicals) pathways [70,71]. Those effects can be acute, i.e, high dose exposures that lead to cell's death, or chronic, i.e., related with long-term exposures that leads to detrimental effects [70]. Furthermore, and for many years, it was thought that all effects occurred only in the cells that were directly target by IR (classic target theory). However, a plethora of findings in the radiobiology field [72–76] that cannot be perceived at the light of the classic target theory, have been accumulating in the last decades. The first evidence of that, are the results from a study published in 1992 [73] on sister chromatid exchanges in irradiated Chinese hamster ovary cells, which reported that low doses of α -radiation induce genetic damages in the cell nuclei of non-irradiated cells. These effects out-of-field of IR, i.e., in non-irradiated cells, which displayed injury responses/effects similar to those observed in irradiated cells, were named as non-targeted-effects (NTEs).

NTEs were found to occur only at low doses of IR and encompass the radiation induced genomic instability (RIGI) and radiation induced bystander effect (RIBE) [77]. RIGI is the phenomenon in which the progeny of irradiated cells, display damages that result from parental exposition to IR [77]. RIBE is the induction of IR responses (that may be or not harmful) in non-irradiated cells that share the same medium as irradiated ones [78], and in that way propagate the effects of IR. That is not necessarily bad, once the responses in bystander cells differ and encompass injuries such as cell death, DNA damage and neoplastic transformations, but can also benefit the bystander population by inducing radio-adaptive responses (RAR), i.e., the protection of cells and whole organisms against endogenous damage or damage due to a subsequent dose of radiation [79].

RIBE was detected in several *in vitro* studies at a cell-to-cell level and its induction occurs apparently through gap-junction intercellular communication [80,81]. However, *in vivo* assays also reported RIBE at tissue, organ, and organism level [82,83]. Curiously, in the last years, this phenomenon have also been reported at an inter-organism level; i.e., damage responses were detected in non-irradiated organisms that were housed together or shared the same medium of organisms previously exposed to low radiation doses [78,84–86]. All these *in vivo* assays suggested the involvement of soluble molecules/factors as vectors for the transmission of IR effects. There is a large number of potential mediators of bystander signals secreted by the irradiated cells, which include cytokines [87], TNF- α ; TGF- β 1; [88] and mainly ROS and RNS (reactive oxygen and nitrogen species) [89]. Special emphasis must be given to NO (nitric oxide) that was mentioned in several studies as being involved in RIBE [72,90–92]. NO is generated from arginine through activity of inducible nitric oxide (iNOS) synthase, being the main source of RNS formation. However, its role as a stress signal is not yet fully understood, once it can be both an antioxidant and a pro-oxidant, i.e., radio-protector and a radio-sensitizer [93]. NTE of ionizing radiation (IR) may also be epigenetically mediated [94,95], once DNA demethylation has been reported in bystander cells [74]. Also, changes in microRNA expression were detected in bystander tissues [95].

It is noteworthy that several endpoints have been used to report the RIBE (e.g. DNA induction of mutations [96], micronuclei formation [97], sister chromatid exchanges [73], chromosomal instability [98], cell death or apoptosis [75], altered gene expression [88], and alteration in the microRNAs profile [99]) but all of these encompass DNA damage.

To sum up, we can perceive that the assessment of ecological impacts associated with uranium mine wastes is not linear. It encompasses not only its direct chemical effects as a non-essential high dense metal, but also its radioactive properties and consequently

its target and non-target effects. All that multiplicity of radionuclides and their toxicity-factors, along with the above mentioned possible transmission of the induced genetic damage to the offspring, results in serious difficulties to predict the real impacts of uranium mine effluents on the aquatic ecosystems. Most of the studies regarding this matter tend to address these effluent impacts on biota by directly exposing organisms to a single stressor (uranium) and by evaluating endpoints at an individual (organism) level. However, the plethora of stress factors in the effluents, as well as the multiplicity of toxicity-factors may lead to misleading results that underestimate the real environmental risks. As such, for a more realistic evaluation of radionuclide's rich-wastewater impacts on freshwater biota, valuable data may be provided by the evaluation of sub-lethal effects at a population level on a multigenerational scenario after a single short-term exposure to a real effluent sample (mimicking the reality of punctual radioactive discharges in water basins). The occurrence of phenomena that may amplify the impacts of this contamination (e.g. non-target effects and transmission of genetic damage to non-exposed offspring) are also worth evaluating for a more truthful environmental risk assessment.

5. Research purposes

Taking into account that aquatic ecosystems are one of the most significant sinks of metals and radionuclides, which can reach high concentrations due to anthropogenic activities such as uranium mining, this thesis was designed to complement the existing data regarding the double-toxicity of uranium single and also in complex mixtures with its decay radionuclides and metals. All that, with the aim of contribute for a more truthful environmental risk assessment of radioactive wastes and wastewaters (which frequently attain freshwater resources, through intermittent point discharges, even after the cessation of mining exploration).

As so, in order to bring new insights about the potential risks for the aquatic ecosystems posed by punctual discharges of uraniferous effluents, two major assays were performed with *Daphnia magna* short-term exposures to both a highly diluted uranium mine effluent and a matching dose of waterborne uranium. Firstly, in chapter II, it is reported a transgenerational study, whose major objectives were to perceive if the genotoxic effects caused by the short-term exposures, were transmitted to the offspring of *D. magna*, and how it affects its life history traits. Then, in chapter III, we approach the bystander effects

at an inter-organismic level and its possible impact in the field of environmental risk assessment, to try to fulfill some of the current gap of knowledge regarding this phenomenon in invertebrates as well as in radioactive environmental samples.

Both chapter II and III of this thesis are presented in the form of articles which were submitted to international peer-reviewed journals and are currently under review.

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Chapter II

Life history traits and genotoxic effects on *Daphnia magna* exposed to low doses of waterborne uranium and a uranium mine effluent - a transgenerational study

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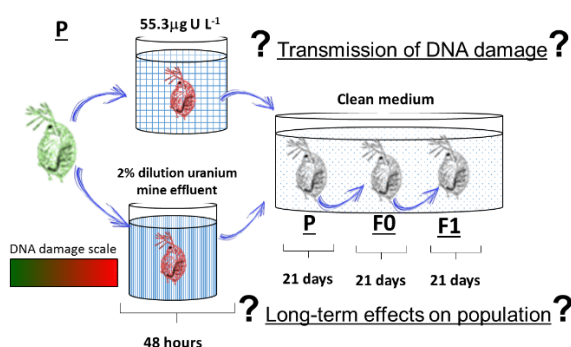
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Submitted to: Aquatic Toxicology

Abstract

The assessment of the impact of uranium mine industry in the nearby aquatic ecosystems is vital to secure the long-term health and sustainability of ecosystem services. As such, we designed a transgenerational study on *Daphnia magna*, in order to perceive at which point intermittently discharges of uranium mine effluents on watercourses may impact the DNA integrity and life history traits of cladocerans. Organisms were exposed for 48h to a 2% dilution of complex uranium mine effluent (UME), as well as to a matching dose of solely waterborne uranium (WU) that according to our preliminary data would induce significant DNA damage. After that daphnids were transferred to a clean medium, where three successive generations were monitored for genotoxicity and individual and population effects. Despite some variance between WU and UME data, our results revealed that the negative impacts of that short-term exposure gradually fade out in a clean medium. These results suggest that under these intermittent stresses, daphnids are able to recover DNA integrity, which after a short period (at time of the 3rd brood release) is no longer transmitted to the offspring, as so, does not significantly impact the offspring's life traits. Although our results indicate that populations of *D. magna* are not affected by intermittent and highly diluted uranium mining discharges, they should not be seen as a hazardous-free scenario. Future studies in this field that take into account not only radionuclides in the water column but also their accumulation in the sediments, as well as multiple life stages, are recommended.

Graphical abstract



Keywords: Transgenerational effects; DNA integrity; Waterborne uranium; Uranium mine effluent; *Daphnia magna*

1. Introduction

Uranium is an ubiquitous naturally occurring radioactive metal from the actinide series with average values of abundance in the Earth's crust of 2.8 ppm [1] and background values in aquatic systems that range from 0.02 to 6 $\mu\text{g L}^{-1}$ in freshwater environments and of 3.3 $\mu\text{g L}^{-1}$ in marine medium [2]. However, in some watercourses due to the composition of the surrounding soils and anthropogenic activities such as, uranium mining and milling and the nuclear power industry, it can reach values up to 2 mg L^{-1} [3].

For a better understanding of the problematic of environmental discharges of uranium mining activities, we may look for example to the environmental legacy of former uranium mines around the world. Taking for example, the deactivated uranium mines located in the Center region of Portugal, significant concentrations of uranium as well as other contaminants are still being detected in wastewaters, generated by the uprising of aquifers or by the precipitation on solid wastes, even after limestone neutralization and barium chloride precipitation (e.g. 1380 $\mu\text{g U L}^{-1}$ [4]; 3143 $\mu\text{g U L}^{-1}$ [5]. These effluents are intermittently discharged on the nearby watercourses, where despite underlying dilution when discharged on watercourses, may still affect aquatic ecosystems [6–8].

The double toxicological hazard of uranium (chemical and radiological [9]), the high number of active and derelict uranium mining areas and the world's growing demand for nuclear energy [10], increased the urgency of assessing the real impacts of uranium mine effluents on the biota, in more common and ecologically significant scenarios to better predict the risks of environmental radioactive discharges.

Exposure to low levels of uranium, its associated ore metals and radionuclides, can affect organisms at many levels. Metals and radionuclides are known to promote DNA damage, causing single and/or double strand breaks, and loss of bases from the DNA molecule [11]. They can also affect DNA repair, gene expression, and cause apoptosis and the formation of free radicals [11,12].

As aquatic ecosystems are one of the most significant sinks of metals and radionuclides, it is of all importance to accurately assess the toxicological effects of double-hazard substances in order to establish accurate safety parameters. Despite the fact that uranium's chemotoxicity overcomes its radiotoxicity, the effects of the ionizing radiation released by its daughter radionuclides should not be ignored [13].

A possible parental transmission of DNA damage induced by uranium was already the subject of a transgenerational study by Plaire [14] in 2013. An additional study following three successive generations undertaken in 2015 [15] also observed an increasing sensitivity to low doses of external gamma radiation (0.007 mGy h^{-1}). However, we consider that parental transmission of DNA damage to offspring needs to be addressed under more ecological relevant conditions, and by addressing the consequences at the population level. If confirmed it will bring new insights on the risk of environmental exposures to radioactive wastes.

Our decision on which endpoints and aquatic organisms to use was supported by an extensive review on studies performed on radioactively contaminated areas [16]. This review highlighted the assessment of genotoxicity endpoints in bioindicator species as the most accurate and useful way to assess risks associated to radioactive contaminated wastes, due to the high correlation between genotoxic responses in human and non-human biota and the responsiveness of DNA endpoints to this type of contamination. As such, since the objective of this study was to provide new insights about the potential risks posed by exposures to low doses of uranium and daughter radionuclides present in environmental matrices, it is of all sense to monitor the most sensitive endpoints in crustaceans exposed to radioactive compounds (genotoxic and reproductive effects [17]) in such a relevant model species for aquatic systems as *Daphnia magna* [18]. Therefore, in this study we exposed *D. magna* to low doses of waterborne uranium and of a uranium mine effluent (with a complex mixture of metals and alpha-emitting radionuclides) for a short period of time, after which, three successive generations, of exposed individuals were monitored in a clean medium. A short single exposure of parental individuals to a low dose of waterborne uranium as well as to a high dilution of the uranium mine effluent was performed to mimic point discharges of treated uranium mine effluents, occurring in the uranium mining areas, taking into account the dilution power of the receiving systems. This approach was also followed because conclusions from ecotoxicological studies using a single compound can overestimate or underestimate the effects occurring under real scenarios of exposure to complex mixtures. Thus the concentrations of uranium tested in this study were selected to match those found in a highly diluted effluent.

Besides DNA damage, also population growth parameters (e.g., survival, age and rate of reproduction) were assessed and the intrinsic rate of population increase growth calculated. It was also evaluated parameters such as dry weight and body length of daphnids.

In summary, the following questions were addressed in this study: (1) Is the DNA damage caused by the exposure to waterborne uranium (WU) or to the uranium mine effluent (UME) transmitted to the offspring?; (2) Are the genotoxic effects caused by short-term exposures repaired when the exposure ceases?; (3) Can a single-event exposure to low concentrations of WU or of UME, negatively affect the life history traits of *D. magna*? (4) Are DNA damages recorded translated into population growth rate and individual fitness parameters across at least three generations of *D. magna*, after a short-term exposure of parental organisms to low-concentrations of either WU or UME?

In order to select the concentrations and exposure duration for the transgenerational study, a preliminary study was performed previously to obtain answers for the following questions: (1) Which are the minimum concentrations (WU and UME) and exposure times to induce significant DNA damage in *D. magna*?; (2) Is DNA damage more responsive to the exposure duration or to different concentrations of WU or UME?; (3) Is *D. magna* equally sensitive to WU and UME?

2. Material and methods

2.1. Culture conditions

Daphnia magna were maintained in 800 mL flasks at a density of 30 individuals per bottle in ASTM artificial freshwater medium [19], at 20°C ($\pm 1^\circ\text{C}$), with a natural photoperiod ($\approx 16\text{L}:8\text{D}$). The medium was renewed three times a week and the animals fed with suspensions of green algae *Raphidocelis subcapitata* (3×10^5 cells/mL), supplemented with an organic seaweed extract of *Ascophyllum nodosum* (Algae Fert Solid).

The health status of the cultures was previously confirmed through the fulfilling of the OECD criteria on survival and reproduction rates [20]. All the experimental assays started with newly released neonates (less than 24 hours) from the third brood.

2.2. Preliminary exposure conditions

Three important criteria were used for the choice of the concentration ranges to be preliminarily tested: 1) the ecological relevance of the study; 2) values below immobilisation EC_{10} [21] and in the range of concentrations of other studies that addressed effects of uranium in *D. magna*, in different multigenerational approaches

[22,14]; 3) the awareness that on exposures to UME above a threshold concentration, the effects caused by metals and radionuclides may be mislead with pH effects [5].

Thus, *D. magna* was exposed to low doses of waterborne uranium ($53.3 \mu\text{g L}^{-1}$; $80 \mu\text{g L}^{-1}$; $120 \mu\text{g L}^{-1}$) and to two dilutions (2% and 4%) of an uranium mine effluent from Quinta do Bispo (Mangualde, Portugal), that matched the waterborne uranium concentrations ($53.3 \mu\text{g L}^{-1} \approx 2\%$ dilution; $120 \mu\text{g L}^{-1} \approx 4\%$ dilution) for three exposure times (24h, 48h, 72h). For each experimental condition (uranium concentration and effluent dilution), three replicates, each one with 20 organisms, were evaluated in terms of DNA damage. All replicates consisted in four sub-replicates of 5 neonates each at a density of 5ml medium daphnia⁻¹.day⁻¹, renewed daily [23]. No food source was provided throughout the test.

Uranium was obtained from Panreac as uranyl nitrate hexahydrate ($\text{UO}_2(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$). To ensure that nitrate (NO_3^-) was not toxic by itself to the organisms, a nitrate-control (containing the same amount of NO_3^- as that in the larger concentration of uranium tested) was also performed, besides the negative control without WBU/UME. The pH was also not a confounding factor, since it was similar across all exposure conditions (maximum range: 7.53 – 8.07, not associating with treatment ranges).

2.3. Transgenerational exposure design

The transgenerational assay began by exposing daphnids (called the parental (P) generation, which was collected from a 3rd brood and with less than 24h) to a $53.3 \mu\text{g L}^{-1}$ concentration of WU and to a UME dilution of 2% during 48h, which allowed obtaining significant genotoxic effects in *D. magna*, according to, the results observed in the preliminary study. The exposure followed the same scheme of the preliminary study, after which the organisms were passed to clean ASTM medium, where three successive generations (P, F0; F1; F2) were monitored, in a 21 days OCDE chronic test [20]. Neonates from the 3rd brood of each generation, with less than 24h, were the starters of the following one. For better understanding, the experimental design of the study it is illustrated in Fig.1.

Each chronic test condition consisted of 12 replicates, each one holding one *D. magna* per glass beaker containing 50mL of ASTM medium. Test units were monitored daily for parameters: survival, number of neonates released and genotoxic effects in neonates of the 2nd, 3rd and 4th brood of each generation. The 1st brood was always discarded as recommended by the OECD guidelines [20], since it was proven that the effort per

offspring increases with maternal age, with consequences in terms of the life-history traits of individuals [24]. DNA damage evaluation was also performed at the end of 48h exposure to the tested substances in P daphnids. Each generation was maintained for 21 days. The medium was renewed and the organisms fed every other day with green algae *Raphidocelis subcapitata* (3×10^5 cells/mL) [25] and, the medium supplemented with an organic seaweed extract of *Ascophyllum nodosum* (Algae Fert Solid). After 21 days, the individual dry weight and body length (from the top of its head to the base of its tail spine) of each *D. magna* female alive was assessed.

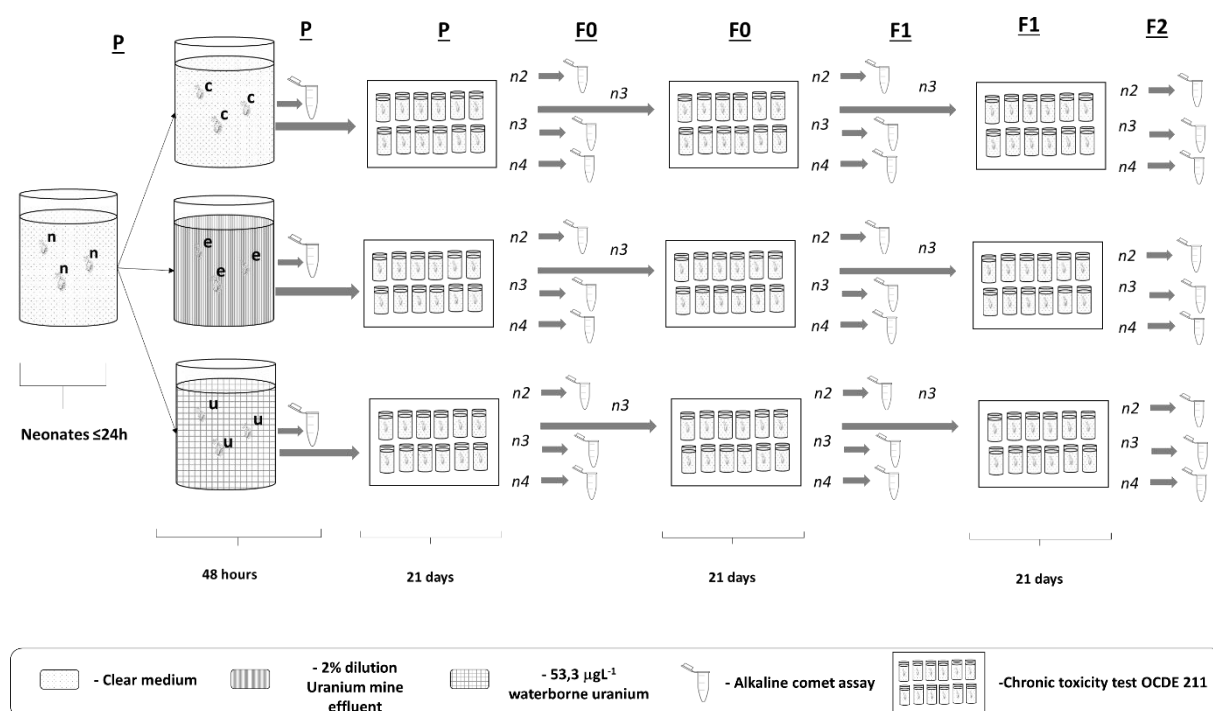


Figure 1- Schematic representation of the transgenerational experimental design. **n** - newly released neonates (less than 24 hours old); **c** – Control - daphnids exposed to clean ASTM medium for 48 hours; **e** – daphnids exposed to a 2% dilution of a uranium mine effluent for 48 hours; **u** – daphnids exposed to waterborne uranium at a concentration of $53.3 \mu\text{g U L}^{-1}$ for 48 hours.

2.3. DNA damage evaluation

DNA damage was evaluated through the alkaline comet assay. Three replicates were used for each of the defined broods (2nd, 3rd, 4th) for each treatment (as well as for daphnids after 48h exposure) and the control, each one containing a pool of twenty neonates. The organisms were placed in a 1.5 mL microtube containing 800 μL of a solution consisting in phosphate-buffered saline (PBS), 10% (v/v) dimethyl sulfoxide (DMSO) and 20 μM ethylene diamine tetra-acetic acid disodium salt (Na_2EDTA). Organisms were then gently macerated using a pestle, in order to release the cells.

Microtubes were then centrifuged at 200 g, for 10 min at 4°C and the supernatant almost completely removed leaving about 50 µL to resuspend the pellet. Then 15 µL of the cell suspension was mixed with low melting point agarose (0.5% (w/v)) at 37°C and placed on top of pre-coated slides (1% normal melting point agarose). Slides were then placed in a lysing solution (2.5M NaCl + 100 mM EDTA + 10 mM Tris-HCl + 1% DMSO + 10% TritonX-100) for at least one hour at 4°C, protected from the light. After lysis, slides were subjected to denaturation, in an alkaline buffer (0.3 M NaOH and 1 mM EDTA, pH 13) for 15 min and, electrophoresis for 10 min at 0.7 V/cm, 300 mA. Slides were then neutralized in Tris-HCL (0.4M), and after that, submerged for a few seconds in absolute ethanol, left to dry for at least 24 h and stored in the dark until observation. The assay was conducted under yellow light, to prevent UV-induced DNA damage.

Before observation, slides were stained with ethidium bromide (20 µg mL⁻¹) and scored using a fluorescent microscope (amplification 400X). To avoid bias on the results, the observations were blind, i.e., scored without any previous knowledge of the origin of the slide, and always by the same person. Visual scoring of cellular DNA on each slide was based on the categorization of 100 cells random. The comet like formations were visually graded into 5 classes, depending on DNA damages, and classified as illustrated in figure 2: class 0 - no visible tail; class 1 - tail with low fluorescence and head still round and bright; class 2 - head and tail equally bright; class 3 - long and bright tail; class 4 - long tail with no round head. From the visualization of 100 random cells, it result a value in arbitrary units of DNA integrity of daphnids by sum of the value of each class multiplied by the number of cells in each category, divided by total cells viewed.

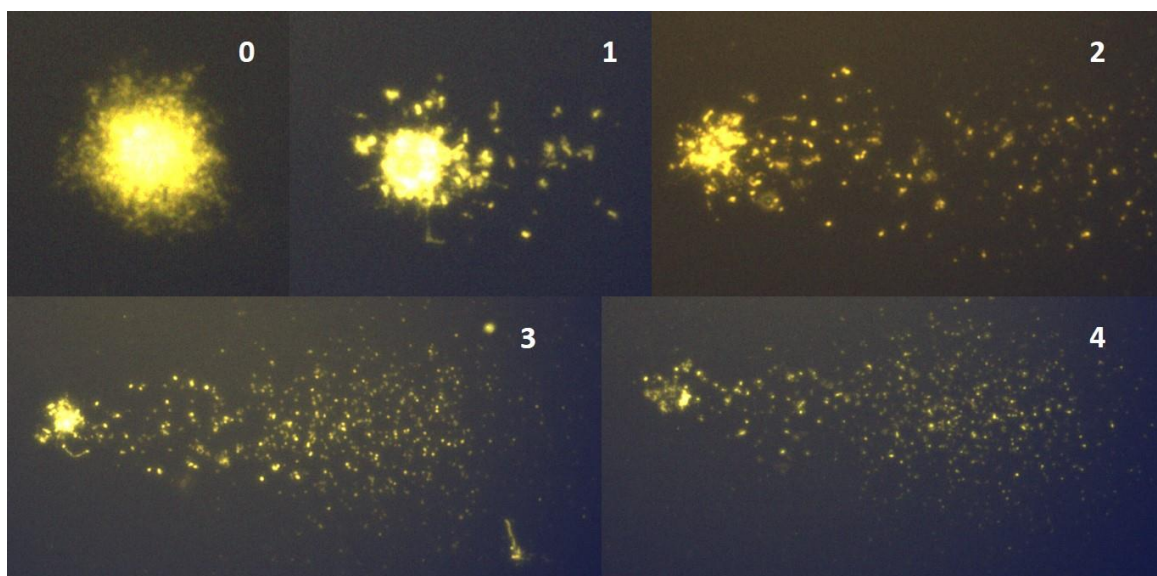


Figure 2- Alkaline comet assay: visual scoring of DNA damage in *Daphnia magna*, from 0 to 4 according to comet appearance. (Amplification: 400X)

2.4.1 Individual parameters

Each female (P, F0, and F1), at the end of 21 days of exposure [20], was measured (maximum body length (from the top of the head to the base of the tail spine)) using a microscope equipped with a micrometer computer system. After that, each female was transferred to a pre-weighted aluminum plate and left to dry for 24 h, at 60°C, which were then left to cold in a desiccator and immediately weighted in a microbalance MXA21/1 with precision of 1µg.

2.4.2. Population growth parameters

The data (survival, fecundity and time of broods release) from 21 days *D. magna* reproduction test [20], which were collected by daily monitoring individuals survival and neonates release, were all integrated and used to calculate the intrinsic population growth rate. That allowed us to have a clear understanding of the effects of the single event exposure to low concentrations of WU or UME dilutions on population dynamics of *D. magna*, as well as to perceive to which extent can effects in the DNA be translated into population effects. This parameter was calculated with the Euler-Lotka equation (Eq.1) [26] and the standard deviation derived iteratively from Jackknife method [27].

$$\sum (l_x \cdot M_x \cdot e^{-Rx}) = 1$$

Equation 1- Euler-Lotka equation where l_x : the proportion of individuals surviving at the age x (days); M_x : not cumulative fecundity at the age x (days); R : intrinsic rate.

2.5. Chemical analysis of the effluent

2.5.1. Determination of radionuclides and trace metals

The measurement of radionuclides and trace metals in the selected effluent followed exactly the same method and procedure as described in Lourenço et al. [5].

2.5.2. Estimation of radiation exposure dose

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

2.6. Statistical analyses

Both in the preliminary and transgenerational exposure scheme, the detection of significant differences to treatment-controls were performed through one-way ANOVA complemented with Holm-Sidak post hoc test. Two levels of significance were considered: $p < 0.05$ and $p \leq 0.01$. Data from the preliminary exposure scheme were also treated for both WBU and UME in a two-way ANOVA regarding exposure duration and concentration. All the statistical analyses were performed on IBM SPSS Statistics v17.0 software. Although all the statistical treatments were performed on raw data, for a more straightforwardly analysis, data for both individual parameters and population growth parameters in the transgenerational exposure scheme, were converted into percentage relative to the control (with control response reflected as 100%) for graphical representation purposes.

3. Results

3.1. Effluent characterization

The results of the chemical analyses of the effluent (which is the same sample tested on another study of our research group (chapter III)), revealed a complex mixture of metals and radionuclides (Tab.1). Some of the metals (e.g. Al and Mn) were above the “emission limit values” established for wastewater discharges, in receptor media, according to the Portuguese Law by Decree 236/98 [28]. Regarding the radionuclides, no comparison with wastewaters discharges legislation can be made, once they are absent in that regulation. Still in the radionuclides context, a point should be made: despite the focus on the percentage that is in solution during the study, the readers should take into account that the majority of the radionuclides is in the particulate form ($\geq 45\mu\text{m}$) and daphnids are exposed to both fractions as they are filter feeding organisms, being that they are able to feed from some particles on that range [29].

Table 1- Chemical characterization of the uranium mine effluent from Quinta do Bispo (Mangualde, Portugal)

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

3.2. Estimated radiation doses

The estimated radiation doses received by the daphnids exposed to a 2% dilution of the uranium mine effluent (Tab.2), were below the limit value of 1.00E-02 Gy·d⁻¹ for radiation risk exposure of biota [31], even when all the radionuclides were summed up.

Table 2- Dose estimates (Gy·d⁻¹) received by neonates of *D. magna* exposed to 2% dilution of the UME. Data of radiation doses are discriminated by radionuclide and also summed as total

Nuclides	²³⁸ U	²³⁵ U	²³⁴ U	²³⁰ Th	²²⁶ Ra	²¹⁰ Po	²³² Th
Gy·d ⁻¹ discriminated	8.78E-04	4.15E-05	8.34E-04	2.10E-07	9.49E-04	1.04E-05	6.28E-06
Gy·d ⁻¹ summed	2.72E-03						

3.3. Preliminary exposure

All the results obtained in the preliminary assay are shown in Fig.2 (and in table.S1 of Annex section).

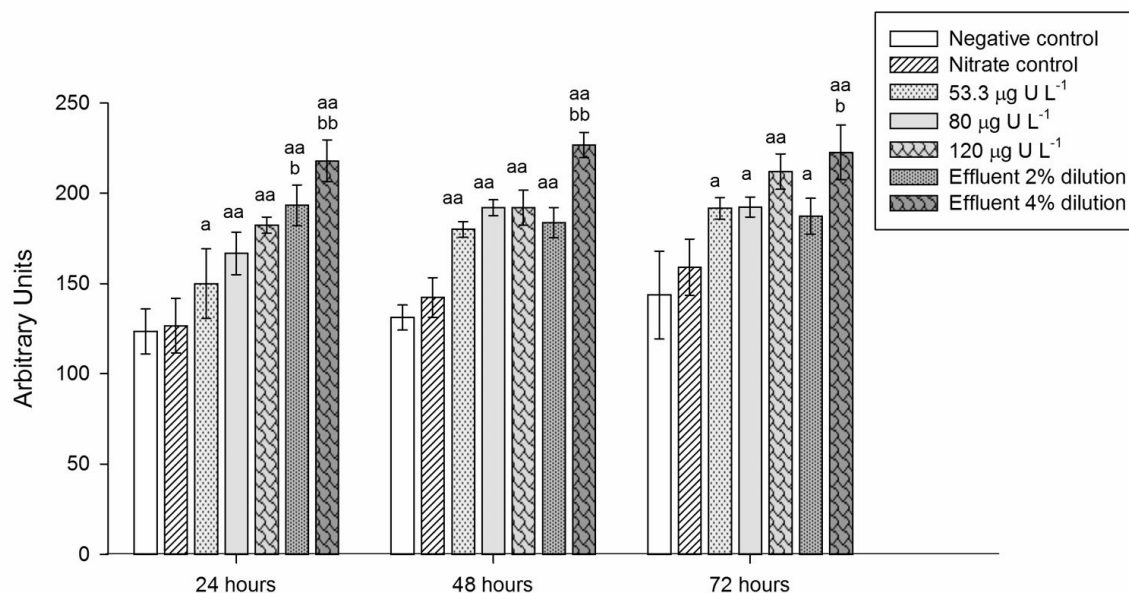


Fig.2 - Weighted average of the DNA damage (arbitrary units) in the Comet Assay in relation to three exposure periods (24 h, 48 h, 72 h) to uranium mine effluent concentration (dilutions of 2% and 4%) and waterborne uranium concentration (53.3µg L⁻¹, 80µg L⁻¹ and 120µg L⁻¹). Letters indicate similarities and statistical differences among treatments: A- comparative to respective control; B- relatively to matching WU concentration. One lowercase- p: ≤0.05; two lowercases- p≤0.01. Error bars represents standard deviation

DNA damage resulting from the exposure of *D. magna* to uranyl hexahydrate nitrate (UO₂(NO₃)₂·6H₂O) was due to the action of the uranyl ion (UO₂²⁺), because on both controls (negative and nitrate) the DNA damage was basal.

Loss of DNA integrity was detected in all conditions and periods tested, however, with some variance among them (e.g. in the lowest concentration of WU only in ≥48h exposures the DNA damage reaches the same level of that observed in the lowest dilution of UME). In a general way, the integrity of DNA decreases with the increase of exposure duration. However, there were differences between WU and UME, which can be better understood in Table 3.

Table 3. Results of the two-way ANOVA performed on preliminary exposure data to assess the effect of time and WU and UME concentration on the severity of DNA damage on daphnids

	WU			UME		
	F	df	p	F	df	p
Concentration	55.397	35;24	<0.001	113.719	26;18	<0.001
Time	15.111	35;24	<0.001	0.186	26;18	0.832
Concentration x Time	1.144	35;24	0.368	0.585	26;18	0.678

From the two-way analysis it was possible to perceive that for WU, both factors significantly influenced DNA damage occurrence on exposed organisms. However, that was not observed for the effluent, where the level of DNA damage was significantly influenced by the percentage of dilution but not by the exposure time. Further, no significant interaction between both factors was recorded in any case.

Considering the results obtained in this preliminary test (i.e., loss of DNA integrity in all concentrations and periods tested), they provided the basis for the selection of exposure periods and concentrations for the transgenerational study and helped to attain one of the objectives of this study that was the ecological relevance of the same. As so, a concentration of $53.3 \mu\text{g U L}^{-1}$, a 2% dilution of the effluent and an exposure time of 48 hours were selected for the transgenerational assay, which were the minimum concentrations and exposure time needed to record significant and uniform (between WU and UME) DNA damages in *D. magna*.

3.4. Transgenerational follow up of exposed parents

3.4.1. Genotoxicity analysis

All the data regarding evaluation of DNA damage through alkaline comet assay are depicted in Fig.3 (and in table.S2 of Annex section). After 48 hours of P daphnids exposure to waterborne uranium and uranium mine effluent, the alkaline comet assay revealed a significant level of DNA damage in the organisms, in comparison to the control (CTL). Furthermore, the neonates of the 2nd brood of F0 released by P in clean medium also showed a significant increase in the level of DNA damage relatively to CTL. Such damage was more evident in the exposure to WU. The organisms from the remaining

broods of P (i.e. belonging to F0) and from the next generations did not reveal a level of DNA damage significantly different from the control, in both exposure to WU and UME.

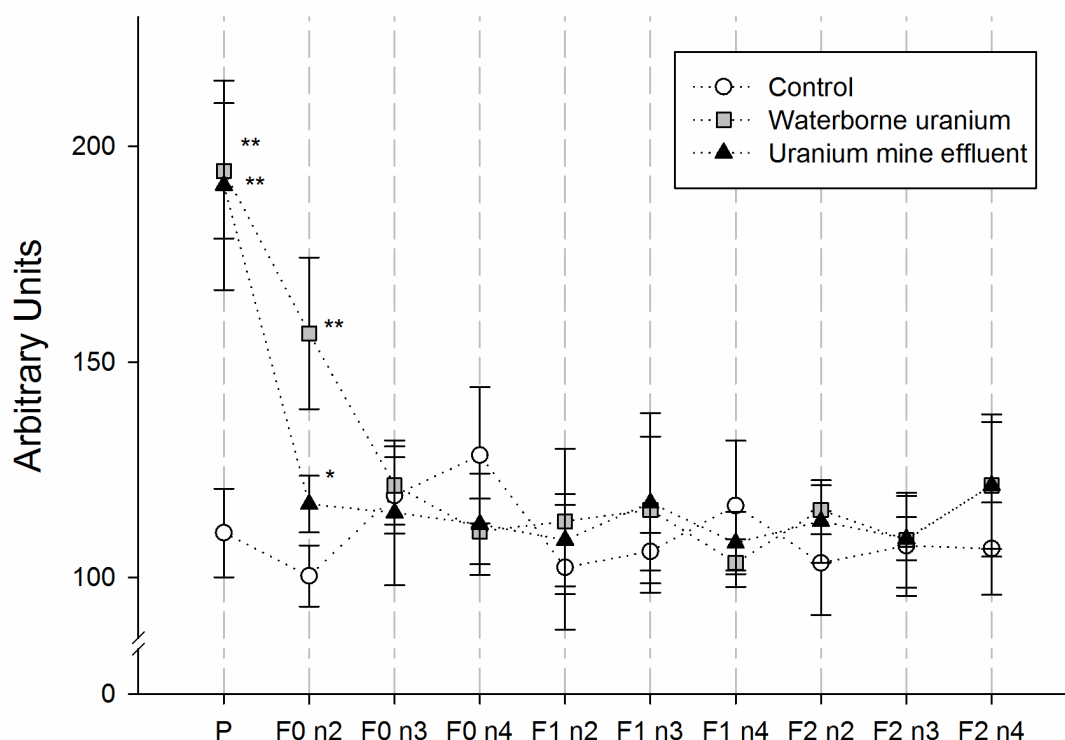


Fig.3 - Weighted average of the DNA damage (arbitrary units) in the Comet Assay of P organisms after 48h of exposure and from neonates from 2nd, 3rd and 4th brood of generation F0, F1 and F2 in the two treatments and the control. Single-factor ANOVA followed by a multiple comparison test (Holm-Sidák post hoc): Differences from the respective negative control *p<0.05// **p<0.01.

3.4.2. Effects on individual parameters

All the population growth parameters assessed for the three generations of daphnids are depicted in Fig.4 (and in table.S3 of Annex section)

Both exposures (WU and UME) resulted in a significant reduction on the body maximum length of the organisms in the exposed generation (P), however in the following generations, no significant effects were recorded, being that in F1 of WU exposure, the daphnids were even larger than the control.

Concerning dry mass, the level of variance between replicates (i.e. standard deviation) was in general larger than in body length, which may be in part responsible for the lack of significant differences. However, it was evident some decrease in body mass in both treatments in the P generation, and in the descendants (F0 and F1) of exposed to UME while neonates.

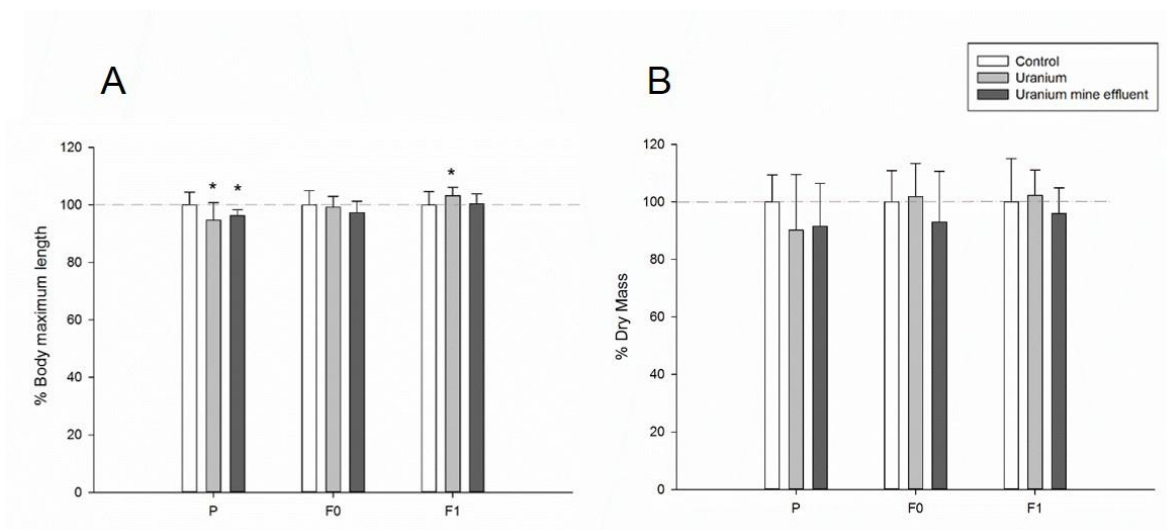


Fig.4- Individual fitness relative to the control. **A-** Body maximum length at the end of OCDE 21-days chronic test; **B-** Body dry mass at the end of OCDE 21-days chronic test. Each bar and line represents the average and standard deviation of 12 replicates. Differences relative to respective control: * $p \leq 0.05$ (one-way ANOVA with Holm-Sidak post-hoc). The dotted line indicates the response of the control.

3.4.3. Effects on population growth parameters

All the population growth parameters assessed for the three generations of daphnids are depicted in Fig.5 (and in table.S4 of Annex section).

There were no significant differences for all the generations of organisms exposed to both WU and UME. However, for some parameters it is clear a larger assortment between replicates in the treatments relatively to the control at least for parameters A, B and C, which seems to have some meaning, although not allowing significant differences between treatments to be detected (with exception for F0 offspring size of WU).

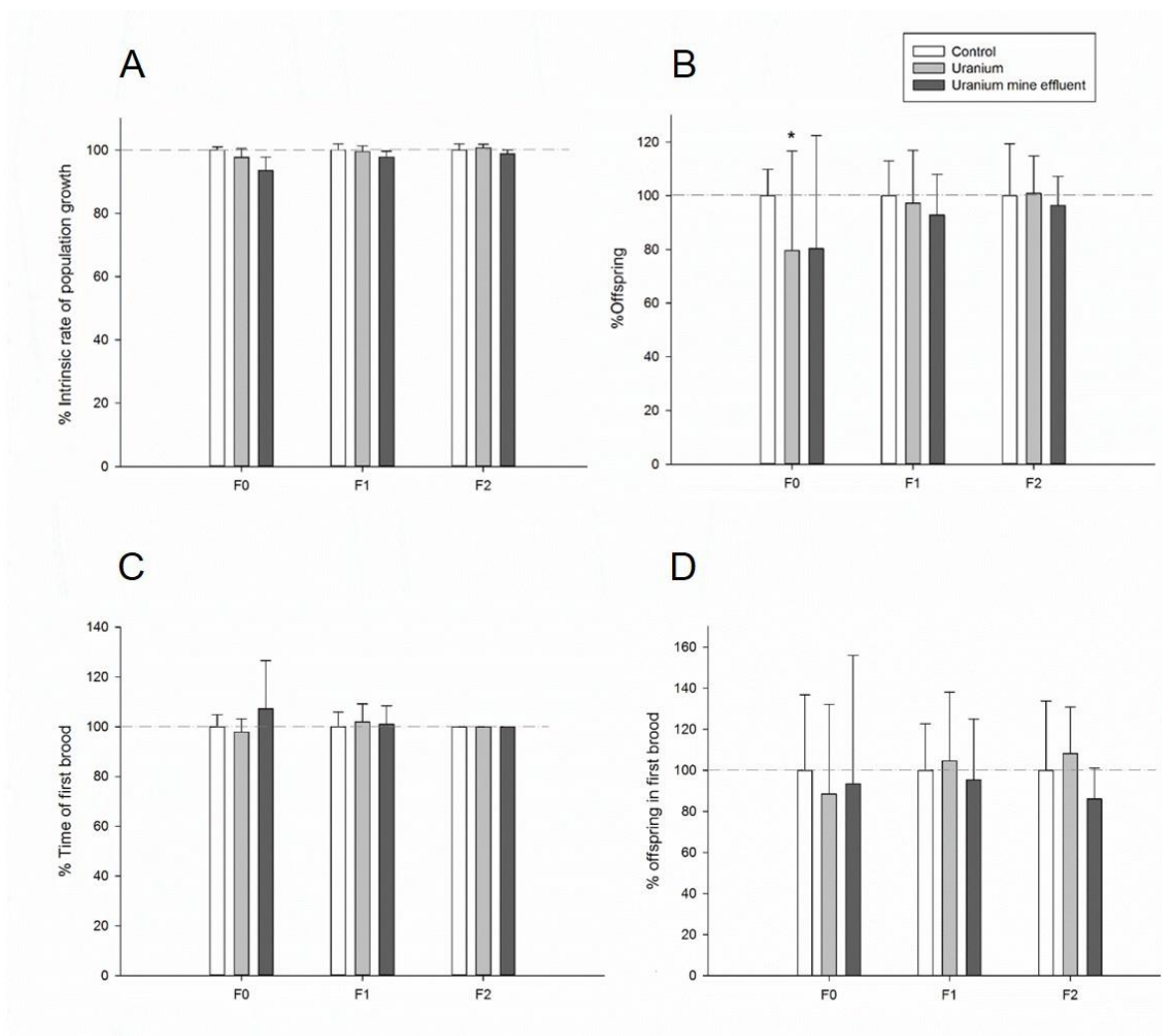


Fig.5- Population growth parameters relative to the control. **A-** Intrinsic rate of population growth; **B-** Rate of offspring number; **C-** Rate of time to first brood; **D-** Rate of offspring number in first brood. Each bar and line represents the average $\pm$ standard deviation of 12 replicates. Differences relative to respective control: * $p \leq 0.05$ (one-way ANOVA with Holm-Sidak post-hoc). The dotted line indicates the response of control.

4. Discussion

4.1. Transmission of DNA damage across generations after a single-event exposure

Our study started by observing that when post-hatching daphnids are exposed to a highly diluted uranium mine effluent (dilution factor of 50) or to a matching uranium concentration ($55.3 \mu\text{g U L}^{-1}$) it requires only short periods of time (24-48h) to induce a degree of genotoxic effect that is significantly detectable by alkaline comet assay. This is not the first time that loss of DNA integrity is assessed in *D. magna* to such a range of

low doses of uranium and IR-emitters [14,15]. However, it is the first time that it is analyzed in exposed daphnids after such a short exposure period and that such data are complemented and verified matching uranium concentration, in a mixture of radionuclides as part of a multi-stressor environmental sample, and in a matching WU concentration.

Regarding the transmission of DNA damage, several studies reported an indubitable occurrence of that phenomenon under low doses of IR and WU exposures in *D. magna* [14,15], as well as in other organisms (e.g. mice implanted with depleted uranium [31]). However, that conclusion is not as straightforward and pronounced in our study, which may be due to different exposure schemes. Our results also revealed a loss of DNA integrity in offspring of exposed daphnids, however, that only occurred in the first broods of the parents' generation after exposure ends, and it is less pronounced in the UME than in WU. That may be related with the faster recovery of daphnids in the UME, as illustrated and discussed in chapter III of this thesis. The plethora of stressors present in the UME, besides uranium, do not allow us to interpret all the possible synergistic, and above all, antagonistic interactions between stressors. However, some essential metal ions present in UME, may act as cofactors in DNA repair mechanisms [32]. Also, a lower bioavailability of radionuclides due to the chemical characteristics of the effluent [33] may have occurred.

Contrarily to previous studies that assessed the transmission of DNA damage in similar low ranges of WU, in our study, we can state that in a more ecologically realistic/relevant exposure, the DNA damage in daphnids, after a short period (at time of 3rd brood release) is no longer transmitted to offspring, once organisms were able to quickly recover DNA integrity. It is noteworthy that in a realistic scenario, multiple life-stages and not only neonates, would be exposed to the radioactive contaminants. However, although youngest organisms are more susceptible to genotoxic damage [34], in chapter III of this thesis, we observe that older daphnids presented higher DNA damage than neonates after a 48 h exposure to both WU and UME. As so, we could infer that in this study we approached the worst case scenario regarding life-stages, which gives more robustness and confidence to the conclusion that when low doses of radioactive discharges are intermittent, the transmission of genotoxic damage to offspring is not a reason for high concern for cladocerans.

At this point, we can already answer the two first questions proposed for this study in the introduction: (1) Yes, the DNA damages recorded in *D. magna*, after a short-time exposure to both highly diluted UME and a low concentration of WU, loss of DNA integrity

is assessed in the offspring; (2) However, that loss of DNA integrity lost significance at the time of 3rd brood of F0, which may be related to the activity of efficient DNA recovery mechanisms on daphnids, once exposure ceases.

4.2. Influence/effects of short-term exposure to uranium and mine effluent on life history traits of *D. magna*

The not so long lasting transmission of genotoxic effects would allow us to anticipate lower effects on the life history traits of *D. magna* populations, and that was exactly what happened. Only the individual fitness and population parameters of the exposed organisms revealed some nefarious effects, which were similar for all parameters with exception of the intrinsic population growth rate, where organisms exposed to UME, probably due to the complexity of multiple stressors, displayed slightly more damage than WU ones. The reasons already pointed out for the quick recovery of genetic damage, are probably the main reasons for the absence of transgenerational effects in a long-term scenario, i.e., despite damage effects in some individuals, which do not compromise the long-term sustainability of *D. magna* population. Curiously, regarding individual fitness more specifically maximum body length, the offspring (F2) from short-term exposed parents (P) to WU, displayed a larger size than control organisms. This kind of size compensatory mechanism / delayed hormesis [35], observed in the second offspring generation of organisms exposed to a low WU concentration, is similar to the observed growth increase in larvae of *Rana perezi*, during a 96 h recovery assay after acute exposure to a uranium mine effluent [36].

Similarly to what was observed for the transmission of DNA damage, our data also contrast with studies of different multigenerational schemes with longer exposures to low doses of uranium and IR [14,15,22,37]. Those studies observed an increase in effects severity along generations, which is justified by the fact that they have performed continuous or multiple intermittent exposures along the assay, i.e., both parents and offspring were exposed. But similarly to those studies, regarding individual fitness, we also note more differences in body maximum length than in dry mass [14]. Regarding the size of the offspring released by exposed organisms, that reduction is concordant with previous studies of low doses of WU [14,21,22].

In literature, reported effects of this non-essential metal, as well as, of internal alpha radiation in *D. magna* comprise several endpoints. Regarding the ecotoxicological data available for this species, 48 h LC₅₀ of waterborne uranium ranges from 390 µg L⁻¹ to

51,900 $\mu\text{g L}^{-1}$, depending on pH and hardness of the test medium [21,38,39]. Such variation is mainly explained by the fact that U(VI), being the most soluble form of uranium, gives rise to uranyl ions (UO_2^{2+}) once in aqueous media [40]. The toxicity of UO_2^{2+} varies as a function of hardness [40], since it can form complexes with soluble carbonates and compete with calcium and magnesium for surface binding sites in cells [41]. Negative effects on life-history traits, as decrease in reproduction rates, early mortality, reduced carbon assimilation and reduced eggs dry mass were assessed in exposures to ecologically relevant doses of Am-241 (an alpha-emitting radionuclide [37]) and waterborne uranium [22]. The sensitivity of all of these endpoints increased across generations [14,22,37]. In our study, notwithstanding the short-term exposure, in both exposed generation (P) (and only in that organisms), nefarious effects were observed (despite not all statistically significant) at the individual level (endpoints: body maximum length and dry mass), as well as at the population level (endpoint: fecundity and intrinsic growth rate for UME exposure).

Comparing our study with data from chronic exposure to Am-241 [37] we can corroborate the general assumption that chemical toxicity of uranium overlap its radiotoxicity [13,39]. When we try to correlate the studies and diminish its differences by looking only to the data that are comparable (in terms of exposure design), we verify that at two fold-higher radiation doses of the ones used in the current study, no effects (in terms of fecundity, dry mass and body length) were detected in the exposed generation, which contrast with our effects (even taking into account the fact that our exposure was short-term (48h) and not continuous).

With all the data gathered and above discussed, we are now able to answer the 3rd and 4th main questions proposed in the introduction: (3) No, the life history traits of *D. magna* populations are not affected by a single-event exposure to both low concentrations of WU or highly diluted UME; (4) No, the loss of DNA integrity induced by both WU and UME in exposed organisms, only results in damaging effects at the individual and population level in the exposed daphnids (P), i.e., DNA damage effects does not translate into problems in population growth rate and individual fitness in a long-term scenario (across at least three generations).

5. Conclusions

The data gathered allows us to state that a single short-term exposure to low concentrations of WU and highly diluted UME is able to induce loss of DNA integrity in the exposed daphnids. That DNA damage are detected on the first broods of exposed generation, after which, DNA damage is no longer detected. Overall, that is not enough to significantly affect the life history traits of *D. magna* populations in a long-term scenario.

Although our results indicate that in a long-term scenario, the populations of *D. magna* are able to recover and tolerate some radioactive contamination of the medium by uranium mine discharges at low doses and intermittent, this should not be translated as a non-hazardous scenario for this type of discharges. Future studies in this field, taking into account not only radionuclides in the water column but also in the sediments [39], as well as multiple life stages, are recommended.

Acknowledgments

FCT, through National Funds, provided financial support to Joana Lourenço through Post-Doc grant (SFRH/BPD/92554/2013). This research was also partially supported by the Strategic Funding UID/ Multi/04423/2013 and UID/AMB/50017/2013 through COMPETE and national funds provided by FCT and ERDF (PT2020). The authors would like to thanks to EDM for the collaboration given for this work.

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Annex

Table S1. Results of one-way ANOVA performed to analyse the results of preliminary exposure assay

	Nitrate control			55.3 µg U L-1			80 µg U L-1			120 µg U L-1			2% dilution UME			4% dilution UME		
	F	df	p	F	df	p	F	df	p	F	df	p	F	df	p	F	df	p
24h	0.114	8;7	0.745	6.47	8;7	0.038	24.841	8;7	0.002	59.374	8;7	<0.001	66.403	8;7	<0.001	120.042	8;7	<0.001
48h	3.057	8;7	0.124	55.099	8;7	<0.001	81.943	8;7	<0.001	68.577	8;7	<0.001	55.394	8;7	<0.001	175.315	8;7	<0.001
72h	0.958	8;7	0.36	10.695	8;7	0.014	11.023	8;7	0.013	20.81	8;7	0.003	8.496	8;7	0.023	25.698	8;7	0.001

Table S2. Results of one-way ANOVA performed to analyse the level of DNA damage on the trasgenerational exposure scheme

	P						F0						F1						F2					
	WU			UME			WU			UME			WU			UME			WU			UME		
	F	df	p	F	df	p	F	df	p	F	df	p	F	df	p	F	df	p	F	df	p	F	df	p
after 48h	60.193	5;4	0.001	28.156	5;4	0.006	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
n2	---	---	---	---	---	---	26.396	5;4	0.007	8.929	5;4	0.04	0.692	5;4	0.452	0.371	5;4	0.576	2.568	5;4	0.184	1.186	5;4	0.337
n3	---	---	---	---	---	---	0.101	5;4	0.766	0.133	5;4	0.734	0.906	5;4	0.395	0.854	5;4	0.408	0.0209	5;4	0.892	0.0523	5;4	0.83
n4	---	---	---	---	---	---	3.043	5;4	0.156	1.981	5;4	0.232	2.078	5;4	0.223	0.81	5;4	0.419	1.946	5;4	0.236	1.669	5;4	0.266

Table S3. Results of one-way Anova performed on the data from trasgenerational exposure scheme to analyse the individual fitness of daphnids. A- Body maximum length; B- Body dry mass

	P						F0						F1					
	WU			UME			WU			UME			WU			UME		
	F	df	p	F	df	p	F	df	p	F	df	p	F	df	p	F	df	p
A	5.319	21;20	0.032	5.02	20;19	0.037	0.171	23;22	0.684	1.86	22;21	0.187	3.759	23;22	0.065	0.0492	22;21	0.827
B	2.568	21;20	0.125	2.533	20;19	0.128	0.148	23;22	0.704	1.348	22;21	0.259	0.193	23;22	0.665	0.562	22;21	0.462

Table S4. Results of one-way Anova performed on the data from trasgenerational exposure scheme to analyse the four population growth parameters. A- Intrinsic growth rate of population; B- Size of offspring; C- Time of the realese of first brood; D- Size of offspring on first brood

	F0						F1						F2					
	WU			UME			WU			UME			WU			UME		
	F	df	p	F	df	p	F	df	p	F	df	p	F	df	p	F	df	p
A	0.638	23;22	0.433	2.433	23;22	0.133	0.0333	23;22	0.857	0.709	23;22	0.409	0.116	23;22	0.737	0.272	23;22	0.607
B	4.76	23;22	0.04	3.451	23;22	0.077	0.155	23;22	0.698	1.546	23;22	0.227	0.0159	23;22	0.901	0.3	23;22	0.59
C	1.005	22;21	0.327	1.24	22;21	0.278	0.478	23;22	0.496	0.116	23;22	0.737	1	23;22	1	1	23;22	1
D	0.495	22;21	0.489	0.098	22;21	0.757	0.133	23;22	0.719	0.177	23;22	0.678	0.428	23;22	0.52	1.629	23;22	0.215

Chapter III

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

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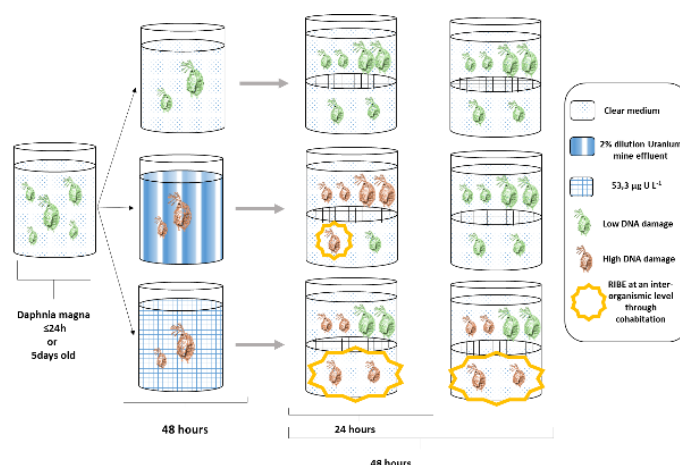
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Submitted to: Journal of Hazardous Materials

Abstract

The induction of radiation induced bystander effect (RIBE) is a non-target effect of low radiation doses that was already been verified at an inter-organismic level in fish and small mammal species. Although its possible theoretical impact in the field of environmental risk assessment (ERA) there is a gap of knowledge regarding this phenomenon in invertebrate group as well as in environmental samples with radionuclide content. As such, to perceive at which extent does RIBE should be taken into account for ERA of radionuclide's rich-wastewaters, we exposed *Daphnia magna* (<24h and 5 d old) for 48h to a 2% dilution of a uranium mine effluent and a matching dose of waterborne uranium ($55.3 \mu\text{g L}^{-1}$), which then cohabitated (24 and 48 h) in a clean medium with non-exposed neonates. Although there may be some exposure-age-time-dependent variability, the assessment of DNA integrity (Comet assay) clearly reveals the occurrence of this phenomenon in *Daphnia magna*, however less pronounced in a ecological relevant uranium mine effluent scenario than just for uranium. The data gathered bring some valuable new worthwhile points for the discussion of RIBE relevance for environmental risk assessment.

Graphical abstract



Keywords: Bystander effect, *Daphnia magna*; Uranium mine effluent; Waterborne uranium; Environmental risk assessment; Radiobiology; DNA integrity

1. Introduction

Ionizing radiation (IR) is the energy released in the form of particles or rays from radioactive materials, which has ionizing capacity [1]. Therefore, when interacting with molecules it has the ability of expelling electrons from their atoms [1]. Concerns with exposures to IR are mainly linked with nuclear tests, nuclear power plant accidents (Chernobyl and Fukushima) or with diagnostic or radiation medical treatments. However, most radiation exposures are low dose rate exposures that can come from many sources as cosmic radiation, soil background radiation, nuclear power plants normal activity and hazardous wastes/wastewaters that are mainly discarded in dumping sites or waterbodies [1].

When IR interacts with cells it can damage DNA, but also proteins and lipids. It can affect gene expression, mitochondrial processes, DNA repair mechanisms, induce apoptosis and form free radicals [2–5]. Regarding genotoxic effects, these are mainly due to the ability of IR to promote single and/or double strand breaks, and loss of bases on the DNA molecule [6].

For many years, all the above mentioned damages were thought to be induced only in irradiated cells. However, this erstwhile dogma, has been challenged since Nagasawa and Little [7], conducting a study on sister chromatid exchanges in irradiated Chinese hamster ovary cells, reported that low doses of α -radiation may induce genetic damages in the cell nuclei of non-irradiated cells. Since then, hundreds of studies demonstrated that there are similar injury responses in neighbor cells, non targeted by IR, as the ones directly exposed [8–10]. Those out-of-field effects, were named non-targeted-effects (NTE) and encompass the well-established radiation induced genomic instability (RIGI) and radiation induced bystander effect (RIBE) [11]. In this paper we will focus on the last one.

RIBE represents a one-way stress communication, where non-irradiated cells/organisms display responses that are assumed to result of the exposure of others to IR [12]. The responses in bystander cells vary and encompass injuries such as cell death, DNA damage and neoplastic transformations, but can also benefit the bystander population by inducing radio-adaptive responses (RAR) and hormesis [13]. Radiation induced bystander effects seem more prominent and relevant at very low doses of radiation (< 5

mGy). They increase proportionally to the number of irradiated cells, with a dose-response usually saturated with the increase of the IR dose [14,15].

In vitro studies stated bystander effects as an ubiquitous consequence of radiation exposure, whose effects may or may not be harmful to the non-irradiated cells [9,16]. The plethora of *in vitro* studies concluded that the mechanisms underlying RIBE encompass both the transmission of signals by physical cell to cell contacts, through gap-junction intercellular communication [17,18], and the release by the irradiated cells of soluble molecules/factors into the medium [19].

However, such phenomenon does not necessarily occur only at a cellular level, and *in vivo* assays have reported that bystander effects also happens at tissue and organism levels [20,21]. Curiously, in the last decade it has been reported that RIBE can also occur at an inter-organism level; i.e., damage responses were detected in non-irradiated organisms that were housed together or shared the same medium of organisms previously exposed to low radiation doses [12,22,23]. However, it should be noted that the test species used in those studies were exclusively vertebrates. All that studies supported the idea that bystander signals are water soluble stable molecules [15,24]. Results became more ecologically relevant because it was reported that RIBE can be communicated through a waterborne route between organisms of different species, but also through diet, namely by ingestion of irradiated *Lumbriculus variegatus* by rainbow trout [25].

Besides the studies mentioned above, there is an extreme paucity of data regarding this phenomenon in invertebrates and at low doses of high LET radiation. Only few studies reported bystander effects induced by alpha particles. The ones that did, were performed on zebrafish embryos [23,26]. Radio-adaptive responses were reported in bullfrog tadpoles (*Rana catesbeiana*) housed for one week with tadpoles that had been previously exposed to tritiated water [27]. Despite all the studies performed so far, the Committee of US government on the Biological Effects of Ionizing Radiation in its last report on low levels of IR [28], concluded that it was too early to assess whether non targeted effects of IR, including bystander effects, had any relevance for risk assessment. That may be due to the paucity of studies *in vivo*, and of studies addressing the relevance of these effects in more realistic environmental scenarios.

All these studies, made us realize the actual gap of knowledge regarding the induction of this phenomenon by low doses of natural α -emitting radionuclides and complex natural-occurring mixtures containing radionuclides and other metals or stressors (exceptions made for co-exposures to IR and copper and aluminum [29–32]).

Nevertheless, we consider that it is of high relevance to study RIBE not only under exposure to high LET emitting radioisotopes such as uranium, but also to complex uranium mine effluents. This will give a great contribution for the environmental risk assessment of radioactive wastes and wastewaters, which frequently attain freshwater resources, through intermittent point discharges.

In this context, this study intends to answer the following questions: 1) Can RIBE at inter-organism-level be detected in *Daphnia magna*? 2) In what way, factors such as time of cohabitation or the age of irradiated organisms modulate RIBE in *D. magna*? 3) To what extent bystander effects between organisms change the current paradigm used in predicting the risks of radionuclide's rich-wastewaters? Furthermore, the following questions will also be addressed: a) Will the DNA damage induced by exposure to both uranium/ uranium mine effluent be repaired during the period of cohabitation in clean medium? b) Is the age at which the organisms were exposed, a factor of influence in that recovery?

To answer these questions, an experiment was performed where *D. magna* with two different ages (less than 24h and 5d old) were exposed to low doses of waterborne uranium and to an uranium mine effluent and then allowed to share the same clean medium with non-exposed organisms (with less than 24h). The genotoxic effects of IR were assessed through alkaline comet assay, since this technique proved to be consistent with other methods available for evaluating DNA damage in several freshwater organisms exposed to genotoxicants [33] and have been successfully used in bystander assays [34].

D. magna was selected because it is a model species in aquatic ecotoxicology and risk assessment. Moreover, this species in particular, and cladocerans in general, are very important in freshwater food webs and there is a lack of studies, addressing bystander effects in aquatic invertebrates. Furthermore, once chemical signaling in this species is documented [35], it was expected that bystander signals (probably excreted molecules) could also be exchanged and perceived between exposed and non-exposed daphnids.

2. Material and methods

2.1.Culture conditions

D. magna are maintained, in our laboratory, in 800mL flasks at a density of 30 individuals per flask in ASTM artificial freshwater medium [36], at 20°C ($\pm 1^\circ\text{C}$) with a natural

photoperiod ($\approx 16\text{L}:8\text{D}$). Every two days the medium is renewed and the animals fed with green algae *Raphidocelis subcapitata* (3×10^5 cells/mL) and supplemented with an organic seaweed extract of *Ascophyllum nodosum* (Algea Fert Solid).

The health status of the cultures was previously confirmed through the fulfilling of the OECD criteria on survival and reproduction rates [37]. All the experimental assays started with newly released neonates (less than 24h) from the third brood.

2.2. Experimental design

In a previous study of our research group (chapter II of this thesis) it was recorded that both a concentration of $55.3 \mu\text{g U L}^{-1}$, and a 2% dilution of an uranium mine effluent with ASTM hardwater medium, and an exposure time of 48h were able to cause significant DNA damage in *D. magna*. Thus, these were the concentrations and the exposure time considered in this study.

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

Part A) - Newly released neonates, with less than 24h were exposed for 48h to defined concentrations of uranium (u), uranium mine effluent (e) and also to clean ASTM medium (c-negative control). At the end of the exposure the integrity of DNA was analyzed in some random exposed organisms. The remaining organisms were placed cohabiting in clean ASTM medium with neonates (less than 24h old) for 24 and 48h at a density of 2:1 in flasks specifically prepared for this experiment (Fig.1 in Appendix). Each flask was composed by two continuous compartments separated by a nylon net ($170 \mu\text{m}$ mesh openings). Three replicates for each cohabitation condition were performed, each one with 40 previously exposed daphnids at the top and 20 unexposed daphnids at the bottom part of the flask. At all steps, daphnids were kept in starvation and at a density of 2.5 mL of medium per daphnia per day, fulfilling the criteria of the OCDE protocol for acute chemical tests in *D. magna* [37]. After 24 and 48h of cohabitation, exposed and bystander organisms were collected for DNA damage evaluation.

Part B) The second part of the experimental design followed the same approach described in part A, with a slight difference: instead of newly released neonates, five days old daphnids were used.

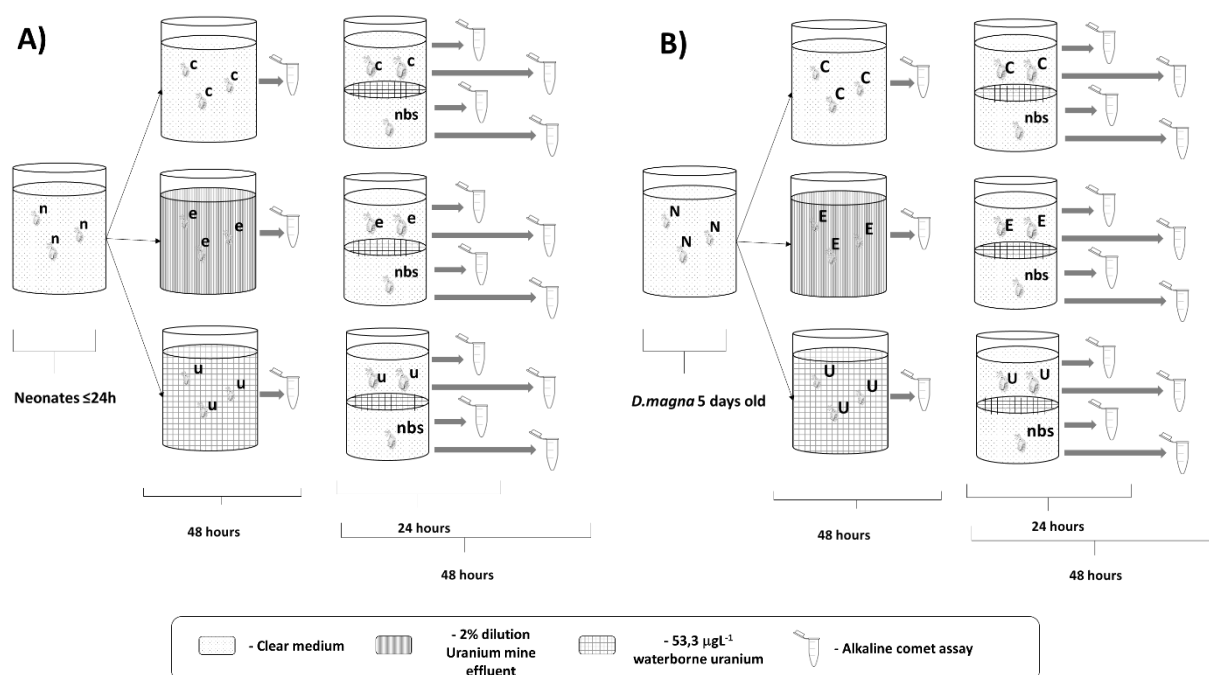


Figure 1- Schematic representation of the experimental design (part A and B). **n** - newly released neonates (less than 24h old); **c** - Control - daphnids exposed to clean ASTM medium for 48h; **e** - daphnids exposed to a 2% dilution of a uranium mine effluent for 48h; **u** - daphnids exposed to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

2.3. DNA damage evaluation

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

electrophoresis for 10 min at 0.7 V/cm, 300 mA. Slides were then neutralized in Tris-HCL (0.4 M), and after that, submerged for a few seconds in absolute ethanol, left to dry for at least 24h and stored in the dark until observation. The assay was conducted under yellow light, to prevent UV-induced DNA damage.

Before observation, slides were stained with 100 μ L of ethidium bromide (20 μ g L⁻¹) and scored using a fluorescent microscope (amplification 400X). To avoid bias, the observations were blind, i.e., scored without any previous knowledge of the origin of the slide, and always by the same person, and proceeded as follows: Visual scoring of cellular DNA on each slide was based on the categorization of 100 cells randomly selected. The comet like formations were visually graded into 5 classes (Fig.2), depending on DNA damage, and scored as described by Garcia [38]. From the visualization of 100 random cells, it result a value in arbitrary units of DNA integrity of daphnids by sum of the value of each class multiplied by the number of cells in each category, divided by total cells viewed.

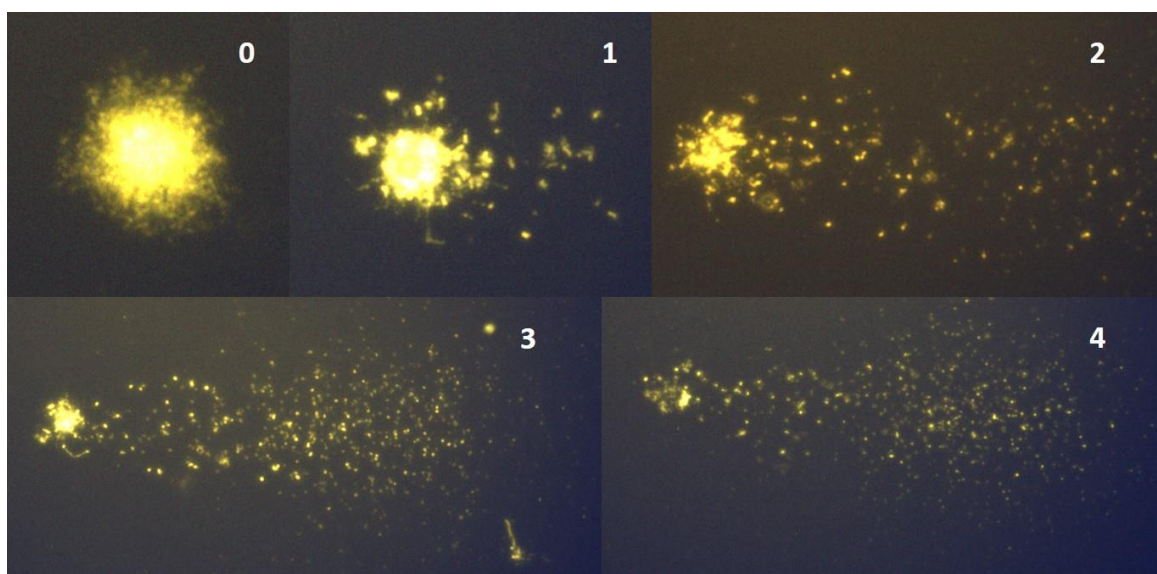


Figure 2- Alkaline comet assay: visual scoring of DNA damage in *Daphnia magna*, from 0 to 4 according to comet appearance. (Amplification: 400X)

2.4. Chemical analysis of the effluent

2.4.1. Determination of radionuclides and trace metals

The method of analysis used in the measurement of radionuclides and trace metals in the selected effluent followed exactly the same method and procedure described in the paper of Lourenco et al. [39].

2.4.2. Estimation of radiation dose exposure

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

2.5. Statistical analyses

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

3. Results

3.1. Effluent characterization

The chemical analyzes to the effluent (which is of the same sample tested on chapter II) reveal a complex mixture with a plethora of metals and radionuclides (Tab.1). Some of the metals (e.g. Al and Mn) were measured in concentrations above the limit values in waste water discharges, according to the Portuguese Law by Decree 236/98 [40]. Some of the radionuclides values were so high that exceed national legislation on water for human consumption (Law by Decree 23/2016) [41] by more than ten and three times (^{238}U , ^{234}U and ^{210}Po , ^{226}Ra , respectively). In Portugal there are no legal limits specified for radionuclides, with exception of water for human consumption.

Table 1- Chemical characterization of uranium mine effluent from Quinta do Bispo (Mangualde, Portugal)

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

3.2. Estimated radiation doses

The estimated radiation doses received by the daphnids exposed to a 2% dilution of the uranium mine effluent (Tab.2), was below the dose limit of $1.00\text{E-}02 \text{ Gy}\cdot\text{d}^{-1}$ which is the limit value for biological risk criteria [42].

Table 2- Dose estimates ($\text{Gy}\cdot\text{d}^{-1}$) received by neonates of *D. magna* exposed to 2% dilution of the uranium mine effluent. Data of radiation doses are discriminated by radionuclide and also summed as total

Nuclides	^{238}U	^{235}U	^{234}U	^{230}Th	^{226}Ra	^{210}Po	^{232}Th
$\text{Gy}\cdot\text{d}^{-1}$ discriminated	$8.78\text{E-}04$	$4.15\text{E-}05$	$8.34\text{E-}04$	$2.10\text{E-}07$	$9.49\text{E-}04$	$1.04\text{E-}05$	$6.28\text{E-}06$
$\text{Gy}\cdot\text{d}^{-1}$ summed	$2.72\text{E-}03$						

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

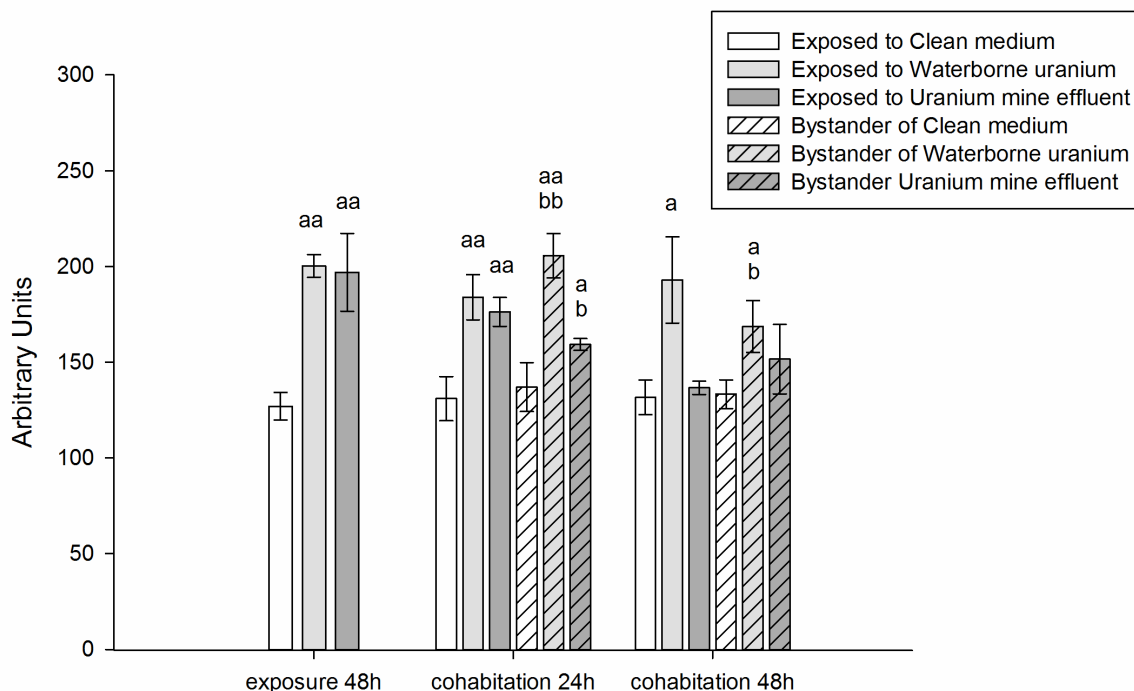


Figure 3 - Weighted average of the DNA damage (arbitrary units) in part A of experimental design. Letters indicate significant differences among treatments: One lowercase- $p \leq 0.05$; two lowercases- $p \leq 0.01$. Error bars represent standard deviation.

Fig.3 represents the results of the comet assay from part A of the experimental design. The absence of mortality in all conditions, the basal damage in all controls and the significant level of DNA damage recorded in both 48h exposure to waterborne uranium and uranium mine effluent, was in accordance with previous data of our research group (chapter II of this thesis), allowing us to validate the assay.

Regarding the exposed organisms: In waterborne uranium exposed organisms (**u** in Fig.1) no DNA damage recovery was registered after both 24 and 48h in clean medium under cohabitation treatments, and the DNA damage detected in comet assay remained significantly high (Fig.3). However, in uranium mine effluent exposed organisms (**e** in Fig.1), the scenario was different, after 48h in clean medium, there was an almost completely recovery of DNA damage, as DNA integrity increased to levels similar to the control and differed significantly from the same organisms immediately after the 48h exposure (Fig. 3).

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

bystander organisms, reached the highest level after 24h of cohabitation, and slightly attenuated after 48h. In the uranium mine effluent bystander organisms, the RIBE was not so remarkable, as in waterborne uranium, however, there was a significant induction of DNA damage after 24h of cohabitation, which completely lost its expression after 48h of cohabitation.

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

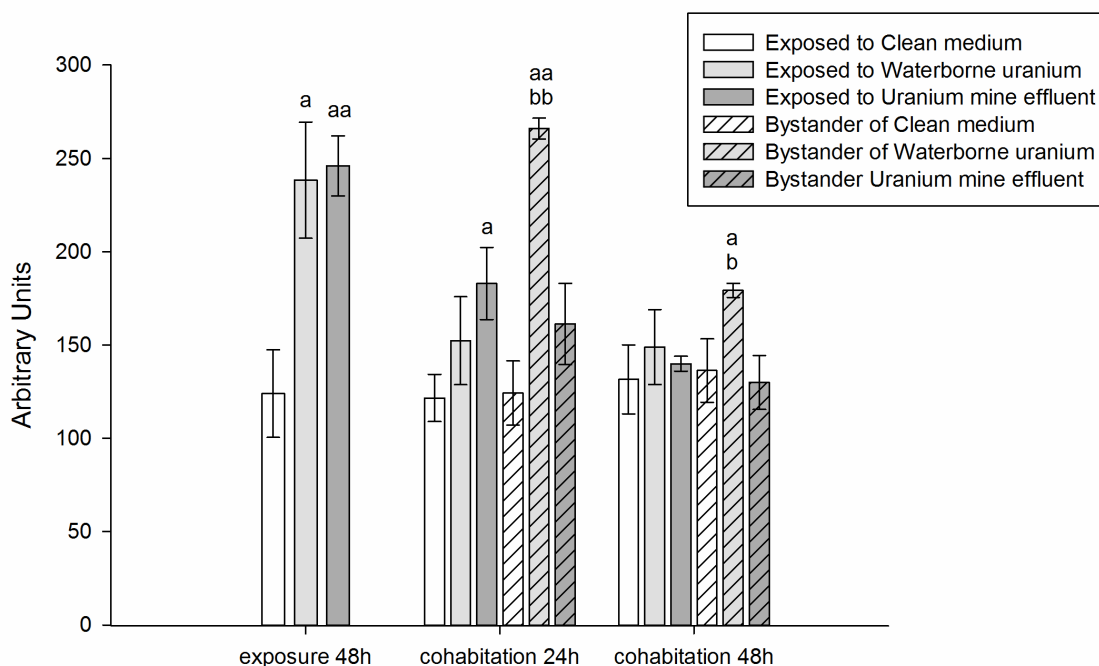


Figure 4 - Weighted average of the DNA damage (arbitrary units) in part B of experimental design. Letters indicate significant differences among treatments: One lowercase- $p \leq 0.05$; two lowercases- $p \leq 0.01$ Error bars represent standard deviation.

The assay performed with 5d old daphnids (Part B) (Fig.4) displayed similar results with some differences that are referred below:

In the case of organisms exposed to waterborne uranium: there was a more pronounced bystander response, especially in the first 24h of cohabitation and unlike the exposed neonates (with less than 24h) from part A, there was a clear recovery of DNA damage in the exposed organisms, after 24h in the clean medium (Fig.4).

The exposure to uranium mine effluent in the part B only differ from part A, when considering the induction of genotoxic effects in the bystander organisms, where despite we can perceive a bystander response, the high standard deviation does not allow to statistically confirm that bystander signals induced DNA damage (Fig.4).

It is also interesting to denote that, especially for the effluent exposure, the level of genotoxic damage observed after 48 hours of exposure was higher in 5d old daphnids rather than in newly released neonates.

4. Discussion

The data from this paper clearly demonstrated the induction of RIBE in neonates of *D. magna* housed together with previously exposed daphnids to both waterborne uranium as well as a uranium mine effluent. Despite some differences in the bystander effect observed for the two exposure scenarios, which will be further discussed, it is clear that this paper contributes for new insights about RIBE at the inter-organism level, for key species playing an important role in food-chains, as primary consumer of freshwater systems, and at a more ecological relevant scenario.

Before the analysis of the bystander mechanism and responses, it is important to focus on the genotoxic effects on daphnids directly exposed to low doses of radiation from natural radionuclides. Genotoxicity is known to be the most sensitive endpoint in crustaceans exposed to radioactive compounds [43] and was already observed in *D. magna* exposed to concentrations as low as $22.2 \mu\text{g L}^{-1} \text{ U}$ [44]. Therefore, it was expected that a significant level of DNA damage would be recorded in our study after 48h of exposure of daphnids of both ages. It should be noticed that significant levels of DNA damage were recorded in a very high dilution in effluent exposure. Also, the daily value of radiation to which daphnids were exposed ($2.72\text{E-}03 \text{ Gy}\cdot\text{d}^{-1}$) was below the limit value for biological risk criteria [42]. However, as already addressed by Lourenço et al. [39], in a scenario of multiple stressors, genotoxic effects may occur even if the organisms are exposed to radiation doses below the predicted biological risk limit. Our study also suggest that, at least when exposed to the effluent, neonates evidenced less DNA damage, but this may be due to the fact that older daphnids (5 d) uptake more from the medium [45] than 24h neonates, thus being more exposed to stressors or to the fact that the molting moments which occur at that age, are sensitivity times. Age differential damage in effluent exposure may also be related with metal stressors, once Hoang and Klaine [46] in a study addressing the age-sensitivity (age range: 3h to 10d old) of *D. magna* to some metals (Cu, Zn, Se and As) reported a peak of sensitivity in 3-4 days old organisms instead of <24h old neonates. Despite age related differences, it should be noticed that inside the same life stage, after 48h of exposure, both uranium and effluent triggered a similar level of damage in daphnids. Moreover, Atlantic salmon

(*Salmo salar*) also displayed similar levels of damage effects when exposed to low levels of ionizing radiation (IR) and under co-exposures of IR and aluminum (which is the most abundant metal in our effluent) [29].

Regarding the ability to repair DNA damage, neonates exposed to waterborne uranium once in the clean medium, were not capable of repairing damage, contrasting with the significant recovery of 5d old organisms. The complexity of the damages resulting from the chemical and radiobiological action of uranium, coupled with different rates of cell proliferation is a plausible justification for that disparities, since neonates have a faster growth rate and a higher metabolic activity, which gives less time for cells to repair damage. The disparities in our data related to the age of exposed organisms are concordant with David et al. [47]. These authors observed the response of two life-stages of *D. magna* (adults and neonates) to genotoxicants and recorded a higher number of transcripts encoding genes involved in the response to DNA damage in adult daphnids. That could reflect a higher level of genotoxic damage in adults, and also their greater capacity to respond and repair damage, as they also detected more mRNA for DNA repair genes in this life stage.

Unlike the observed DNA damage recovery age-differential, already discussed for waterborne uranium exposure, in the organisms exposed to uranium mine effluent, both states of maturity, presented high efficiency of DNA repair mechanisms, which may be partly explained by the presence in the effluent of some metal ions which may be important modulators of biological responses and act as important cofactors in DNA repair mechanisms [48]. An alternative explanation may be the lower bioavailability of metal and radionuclides, or minor sensitivity of the daphnids to toxicants due to the physico-chemical properties of the effluent (an example of how chemical properties of the medium may play a role in the responsiveness of organisms to contaminants is the medium hardness in a uranium solution which can lower the responsiveness of the organisms to uranium, since uranyl ion competes with calcium and magnesium for the binding sites at the cell surface [47,48]). However, given the complexity of the effluent, it would be naïve to state that the higher DNA repair ability in effluent was only due to a single chemical proprieties, e.g. hardness (ASTM is also a hard-medium and the effluent was highly diluted), as so, the only verdict we can state is that due to its complexity we can not predict the synergistic and, above all, antagonistic effects that may occur between contaminants.

In relation to recovery of genotoxic damage, there is a point that should be made, taking into account the assessment method used in this study. Comet assay allows us to

measure breaks in the DNA molecule and not mutations that could have occurred during the repair process. As so, future works on this subject, should be complemented with different methods of genotoxicity assessment as HRM (High Resolution Melt) or RFLP (Restriction Fragment Length Polymorphism) analyses, once our DNA integrity evaluation does not guarantee that there were no mutations that could be transmitted to offspring with long-term effects on populations.

At this point we can already answer the secondary questions (a and b) proposed in the introduction: a) Yes; for both uranium and effluent, the organisms exposed repair damages during cohabitation in clean medium, however this recovery was more pronounced in the mine effluent. b) Yes; at least in waterborne uranium exposure, the recovery after exposure was age-dependent (neonates didn't repair so well the DNA damage).

Focusing now on RIBE at an inter-organismic level, our results were in accordance with previous studies, taking into account the literature reports about chemo-signaling in *D. magna* [35,49] and previous evidences of this phenomenon in other species. For example, Surinov et al., [22] reported that after the exposure to volatile compounds of the urine of rats irradiated with low doses of X-rays, the non-exposed ones exhibited the same injury responses (decreased thymus dependent humoral immune response) than exposed mice. RIBE between species of fish, i.e. rainbow trout (*Oncorhynchus mykiss*) [15,50], zebrafish (*Danio rerio*) [24] and Medaka (*Orzias latipes*) [51] were also detected when non-exposed fish were housed together or swim in medium previously used by organisms irradiated with low doses of X-rays (0.5 Gy). As so, although this study did not intent to investigate the signals/mechanisms underlying RIBE between organisms, we can corroborate that water is the conducting element of the chemical signal(s) responsible for induction of the bystander effect; i.e., the signal is water-soluble.

Mechanisms aside, this study clearly demonstrated that low doses of radiation, from natural α -emitting radionuclides, such as uranium, are able to induce genotoxic effects in non-exposed organisms as a bystander effect. In all waterborne uranium treatments, it was observed a genotoxic bystander response in neonates, however, the strength of the response differ, especially between times of cohabitation. In both assays (A and B) the level of DNA damage in bystander organisms reached a significant level after 24h of cohabitation, but decreased after 48h of cohabitation. There are several mechanisms/processes that concomitantly are likely responsible for these observations: (a) the chemical signal is not stable and starts to degrade with time; (b) the emission of the bystander signal end's as exposed organisms are able to repair damage; (c)

bystander organisms quickly repair the DNA damage that result from cohabitation with exposed organisms. It may be interesting to note that Mothersill et al [24], in a study of interorganism RIBE using *Oncorhynchus mykiss* irradiated with low doses of X-ray, denoted that 6h after irradiation, the bystander signals emitted by irradiated fish lost the strength/ability to induce a response in partner rainbow trout. Regarding the different life stages exposed to waterborne uranium, our data evidence higher levels of DNA damage in bystander organisms, that cohabitated with 5 day's old daphnids, which it's probably a result of the size of the daphnids that released to the medium a higher amount of signals, than neonates. As already mentioned, this is the first time that a study of inter-organismic RIBE is performed with cladocerans, as so, we are not able to compare with other studies. However, our data seem to differ from Mothersill et al [50], which showed that for rainbow the magnitude of induction bystander effect was not dependent on the life stage at which irradiation occurred.

The results obtained with the highly diluted uranium mine effluent suggest than in environmental scenarios, the RIBE phenomenon also occurs, however at a lower degree. The assays exposing organisms to such complex mixtures, as uranium mine effluents, frequently give rise to data difficult to scrutinize [39,52]. For example, in this case, despite metals being recognized for their ability to affect cell signaling pathways and promote the formation of ROS [53], DNA damage tended to be lower than in waterborne uranium treatments. Probably there was some kind of complexation, lower bioavailability or inhibition of the bystander signal by the chemical components of the effluent. It may be difficult to discuss this, since bystander signaling mechanisms are not fully understood, however, taking into account that NO (nitric oxide) is pointed out in many studies as being a key element of RIBE [10,54,55], we may hypothesize that some of the metals present in effluent (e.g. Zn, Co and Ni) may be the responsible for lowering the production of bystander signals, once it is known that some divalent transition metals, (e.g. Cu, Zn, Co, Ni) inhibit NO synthase catalysis [56].

With the information coming from the data discussed above, we can now answer to the main questions of this study: 1) Yes, in *D. magna*, radiation induced bystander effects can be detected at an interorganismic level for both uranium and uranium mine effluent exposures; however, this phenomenon was less pronounced in the effluent exposure; 2) Inter-organismic RIBE in *D. magna* was influenced by cohabitation time, since bystander DNA damage reached a peak after 24h of cohabitation, but that damage decreased with time; regarding the age of organisms from both exposures, the 5d daphnids were able to induce more bystander damage than <24h old neonates.

After answering all the other questions posed during this work, we can now answer the overlooking question of this paper: To what extent bystander effects between organisms change the current paradigm used in predicting the risks of radionuclide's rich-wastewaters?

The relevance of non-targeted effects (including the bystander effect) of IR is a not an easy and unanimous issue that had already been addressed for example for human health risk assessment (e.g.[57]) and more recently in terms of environmental radiation protection [58] suggesting that they may be worth taking into account due to their relevance at population level. Although we do not intend with this study to make any definitive statements regarding this issue, there are some points that are worth highlighting.

First, we would like to emphasize the progress to the discussion of this issue brought by our option in addressing RIBE using natural α -emitting radionuclides such as uranium and a uranium mine effluent. This approach allowed to complement studies that use only IR or radiation mixed with few metals, giving us a more realistic overview of the possible relevance and non-linearity/complexity of bystander effects.

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

Despite the conservation of this physiological trait i.e. the capacity of irradiated organisms to induce bystander effects, the fast disappearance of such effects, especially in the uranium mine effluent (but also in uranium) legitimizes the doubts about the ecological relevance of this trait, in a multigenerational scenario, for the organisms that are subjected to bystander signals.

5. Conclusions

D. magna single-event exposure to both uranium mine effluent and waterborne uranium is able to induce bystander effects in non-exposed daphnids through cohabitation. This is the first observation of this phenomenon in invertebrates and it complements similar data for vertebrates. Despite a mild bystander effect, in an ecologically relevant exposure scenario, and some variance depending on exposure (waterborne uranium versus effluent), time of cohabitation and age of exposed daphnids, this paper brings new insights to the discussion of the relevance of RIBE to environmental risk assessment. Although it is thought to be evolutionarily desirable [58], we still do not understand the true evolutionary background and purpose of this trait and how it contributes to the fitness of populations.

Acknowledgments

FCT, through National Funds, provided financial support to Joana Lourenço through Post-Doc grant (SFRH/BPD/92554/2013). This research was also partially supported by the Strategic Funding UID/ Multi/04423/2013 and UID/AMB/50017/2013 through COMPETE and national funds provided by FCT and ERDF (PT2020). The authors would like to thank EDM for the collaboration given for this work.

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Annex

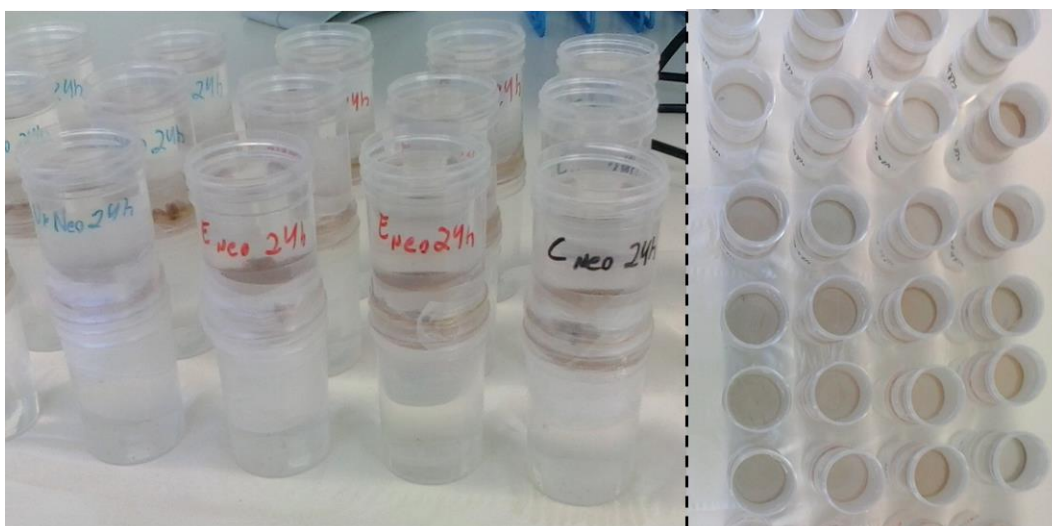


Fig.S1 – Photos (side and top side) of the flasks specifically prepared for this experiment.

Table S1. Results of one-way Anovas performed to analyse the results of bystander assays from Part A and B of the experimental design, and to assess the effect of age on the severity of DNA damage on daphnids.

		after 48h						cohabitation 24h						cohabitation 48h					
		WU			UME			WU			UME			WU			UME		
		F	df	p	F	df	p	F	df	p	F	df	p	F	df	p	F	df	p
part A	Exposed bystander	186.873	5;4	<0.001	31.681	5;4	0.005	30.982	5;4	0.005	32.563	5;4	0.005	19.182	5;4	0.012	0.801	5;4	0.421
		---	---	---	---	---	---	48	5;4	0.002	8.683	5;4	0.042	15.562	5;4	0.017	2,615	5;4	0.181
part B	Exposed bystander	26	5;4	0.007	55	5;4	0.002	4	5;4	0.118	21	5;4	0.01	1.209	5;4	0.333	0.581	5;4	0.488
		---	---	---	---	---	---	183.936	5;4	<0.001	5.343	5;4	0.082	18.267	5;4	0.013	0.242	5;4	0.649
AGE	Exposed	4.316	5;4	0.106	10.735	5;4	0.031												

Chapter IV

Concluding Remarks

The work that constitutes the present thesis, by approaching the subject of environmental impacts of radionuclide's rich-wastewaters through the performing of assays in a freshwater invertebrate with a real UME sample (as also a comparable dose of WU) that mimic the intermittent low doses discharges of radioactive effluents in freshwater ecosystems, brings some valuable points for discussion of ERA of uranium mining activity and legacy.

The multiplicity of factors (radionuclides chemiotoxicity and radiotoxicity, which by its turn encompass target but also non-target effects, as well as a plethora of multi-stressors, as e.g., metals) that should be taken into account in the evaluation of radionuclides impacts on aquatic systems, turns the accurate evaluation of uraniferous effluents impacts on aquatic ecosystems, pretty challenging. Nevertheless, this thesis allow us to conclude that the lose of DNA integrity, as well as the occurrence of RIBE phenomenon at an inter-organismic level, after short-term exposures to low doses of WU and UME are evident. However, that genetic damage is not transmitted to offspring and do not significantly impact the life history traits of *D. magna* populations on a long term scenario.

So, despite we can conclude that in this case scenario, daphnids populations are able to tolerate spaced time discharges of low doses of UME, it would be required more studies, namely with benthic organisms and microcosmos assays, before we can state a non-hazardous scenario for aquatic ecosystems subject to this intermittent and low doses discharges of uraniferous effluents.