UNIVERSIDADE D
COIMBRA

Melissa Bergmann

URANIUM BIOACCUMULATION, POTENTIAL BIOMAGNIFICATION AND DISPERSION BY FRESHWATER INSECTS

Tese no âmbito do Doutoramento em Biociências, ramo de especialização em Ecologia, orientada pelo Professor Doutor Manuel Augusto Simões Graça e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

Maio de 2019

Departamento de Ciências da Vida

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Abstract

Uranium is found naturally on Earth as uranium oxides (UO_2, U_2O_5), but mining activities may release this and other metals and metalloids in soils and natural waters, potentially affecting the aquatic biota. Although uranium impairments in stream dwelling invertebrates and leaf-litter decomposition by aquatic hyphomycetes have been studied, uranium trophic transfer and biodispersion have not. The aim of this thesis was to investigate how uranium affects the physiological processes of benthic macroinvertebrates and aquatic hyphomycetes, and whether it is transferable across trophic levels.

In Chapter I, I tested the survivorship (96-h acute toxicity test) of three species of macroinvertebrates at increasing uranium concentrations (0-262 $mg \cdot L^{-1}$): *Schizopelex festiva*, *Proasellus* sp. and *Theodoxus fluviatilis*, and performed sublethal toxicity assays at 25 $\mu g \cdot L^{-1}$ (feeding, growth, respiration rates, source of bioaccumulation) in caddisflies larvae. *S. festiva* survived in all concentrations, whereas LC50-96 h for the other two species was above 24 $mg \cdot L^{-1}$ in the acute assays. In sublethal assays, *S. festiva* had 47% of reduction in growth at 25 $\mu g \cdot L^{-1}$, a concentration found in some polluted streams; consumption and respiration rates did not differ between treatments, and the uranium accumulation in caddisflies resulted from both water and ingested food.

In Chapter II, I investigated how environmental and sublethal uranium concentrations up to 100 $\mu g \cdot L^{-1}$ affected the enzyme activities and growth of *S. festiva*. Activities of acetylcholinesterase (AChE), glutathione-S transferase (GST) and catalase (CAT) were reduced with time under laboratory conditions (from 24 h to 32 d). Na^+K^+ATPase activity diminished after 32 d in specimens exposed to 100 $\mu g \cdot L^{-1}$. None of the enzymes GST, CAT or AChE enzymes changed their activities as a response to uranium exposure. The growth rates of *S. festiva* under laboratory conditions did not differ among uranium treatments.

In Chapter III, I analysed uranium effects on fungal growth, sporulation, biomass and leaf- litter decomposition using four aquatic hyphomycete species (*Articulospora tetracladia, Heliscus lugdunensis, Varicosporium elodeae, Tricladia splendens*) and six strains of *Heliscus lugdunensis* sampled from polluted and unpolluted sites. Mycelial growth on solid media differed among species, strains and uranium concentrations. The growth inhibition of *H. lugdunensis* strains in solid media at concentrations up to 262 $mg \cdot L^{-1}$ was independent from uranium concentrations in streams where fungi were isolated. In microcosms, sporulation was the most sensitive parameter with inhibition from 1 $mg \cdot L^{-1}$ of uranium, compared with litter decomposition and biomass, which were inhibited from 16 $mg \cdot L^{-1}$.

Finally, in Chapter IV I investigated how uranium in the environment affects the abundance, richness, and composition of aquatic macroinvertebrates and whether uranium flows along trophic levels and is biodispersed to land by invertebrate emergence. I sampled aquatic and terrestrial organisms (aerial stages of aquatic macroinvertebrates and spiders), water and sediment at a reference point and downstream (four locations) of recovered uranium mines. The macroinvertebrate assemblage composition differed among stream sites. Macroinvertebrate diversity in the stream site with the highest levels of uranium in the water and sediments was lower than the less impacted site, but not different from the reference. Uranium concentrations in all organisms increased linearly with uranium concentration in the stream water. The highest uranium concentration among the invertebrates was found in scrapers, shredders and predators, whereas in adult insects and spiders it did not differ among sites. Uranium accumulation in the organisms decreased with the increase in the $\delta^{15}N$ values, suggesting that uranium was not biomagnified along trophic levels, nor biodispersed by the emergence of insects in the aerial stage.

Overall, environmental and sublethal concentrations of uranium (25 – 50 $\mu g \cdot L^{-1}$) may cause reductions in growth and alterations in the cell-membrane-associated enzyme activities in freshwater invertebrates. Aquatic hyphomycetes were more tolerant to uranium than invertebrates, and the bioaccumulation in aquatic organisms was correlated to water and sediment contamination. Despite this, uranium was not biomagnified along trophic levels.

KEYWORDS: environmental perturbations, macroinvertebrates, biomarkers, aquatic hyphomycetes, stable isotopes, food chain.

Resumo

Urânio é um elemento naturalmente encontrado na crosta terrestre como óxidos de urânio, tais como UO_2, U_2O_5. No entanto, atividades de mineração liberam este e outros metais e semimetais nos solos e nas águas superficiais, podendo afetar a biota aquática. Alterações na fisiologia de macroinvertebrados e na decomposição do folhiço pelos fungos aquáticos devido à contaminação por metais têm sido estudadas, mas os efeitos do urânio nos organismos e sua transferência trófica e dispersão não foi completamente compreendida. O objetivo desta tese foi verificar se o urânio afeta a fisiologia de macroinvertebrados, as atividades de fungos aquáticos e se ocorre transferência entre os níveis tróficos.

No Capítulo I, eu testei a sobrevivência (teste de toxicidade aguda de 96 h) de três espécies de macroinvertebrados sob crescentes concentrações de urânio (0-262 $mg \cdot L^{-1}$): Schizopelex festiva, Proasellus sp. e Theodoxus fluviatilis, e testei os efeitos crônicos deste contaminante (25 $\mu g \cdot L^{-1}$) nas taxas de consumo, crescimento e respiração, bem como as vias de bioacumulação nas larvas de tricópteros da espécie S. festiva. Os espécimens de S. festiva sobreviveram a todas as concentrações testadas, enquanto que os valores de CL50-96 h para as outras duas espécies estiveram acima de 24 $mg \cdot L^{-1}$ nos testes de toxicidade aguda. Nos ensaios de toxicidade crônica, S. festiva teve seu crescimento reduzido em 47% sob 25 $\mu g \cdot L^{-1}$, concentração esta encontrada em rios poluídos; as taxas de consumo e respiração não diferiram entre os tratamentos, e a bioacumulação de urânio nos tricópteros foi resultado da água contaminada e do alimento ingerido.

No Capítulo II, eu investiguei como as atividades enzimáticas e o crescimento de S. festiva são afetados por concentrações ambientais e subletais de urânio (0 - 100 $\mu g \cdot L^{-1}$). As atividades da acetilcolinesterase (AChE), glutationa-S transferase (GST) e catalase (CAT) foram reduzidas ao longo do tempo (de 24 h para 32 d) sob condições de laboratório. Os espécimens expostos à concentração de 100 $\mu g \cdot L^{-1}$ apresentaram uma diminuição da atividade da Na^+K^+ATPase após 32 d. As enzimas GST, CAT e AChE não alteraram as suas atividades em função da exposição ao urânio. As taxas de crescimento de S. festiva não diferiram entre os tratamentos testados.

No Capítulo III, eu analisei os efeitos do urânio no crescimento, esporulação, biomassa e decomposição de discos foliares por quatro espécies de hifomicetos aquáticos (Articulospora tetracladia, Heliscus lugdunensis, Varicosporium elodeae, Tricladia splendens) e seis estirpes de H. lugdunensis amostradas de locais poluídos e não-poluídos. O crescimento micelial em meio sólido diferiu entre espécies, estirpes e concentrações de urânio. A inibição do crescimento das estirpes de H. lugdunensis sob concentrações acima de 262 $mg \cdot L^{-1}$ foi independente das concentrações de urânio nos ribeiros de onde elas foram isoladas. Nos microcosmos, a

esporulação foi o parâmetro mais afetado, com inibição a partir de 1 mg·L^{-1}, em comparação com a decomposição dos discos foliares e biomassa, os quais foram inibidos a partir de 16 mg·L^{-1}.

No Capítulo IV, eu investiguei como o urânio presente no ambiente afeta a abundância, riqueza e composição dos macroinvertebrados e se sua concentração aumenta ao longo dos níveis tróficos e se dispersa para o ambiente terrestre através da emergência dos insetos aquáticos. Eu amostrei organismos aquáticos e terrestres (insetos em estágio aéreo e aranhas), água e sedimento em locais de referência e à jusante (quatro pontos) de minas de urânio em vias de recuperação ambiental. A composição dos macroinvertebrados diferiu entre os locais amostrados. A diversidade destes no local com maior concentração de urânio na água e no sedimento foi mais baixa que a do local menos contaminado, mas não diferiu do local de referência. As concentrações de urânio em todos os organismos aumentaram linearmente com a concentração de urânio nas águas do ribeiro. Fragmentadores, raspadores e predadores acumularam as maiores concentrações de urânio, enquanto que a acumulação nos insetos terrestres e aranhas não diferiu entre os locais. A acumulação de urânio nos organismos diminuiu com o aumento de δ^{15}N*, indicando que não houve biomagnificação ao longo dos níveis tróficos nem dispersão pela emergência de insetos com estágio aéreo.*

Em geral, concentrações subletais e ambientais de urânio (25 – 50 µg·L-1) provocam redução no crescimento de invertebrados aquáticos e alterações no funcionamento de enzimas associadas com as membranas celulares (Na^+K^+*ATPase). Os hifomicetos foram mais tolerantes ao urânio do que os invertebrados, e a bioacumulação nos organismos aquáticos foi relacionada com a contaminação da água e do sedimento nos ribeiros amostrados. No entanto, o urânio não apresentou indícios de biomagnificação ao longo dos níveis tróficos.*

PALAVRAS-CHAVE: *alterações ambientais, macroinvertebrados, biomarcadores, hifomicetos aquáticos, isótopos estáveis, cadeia alimentar*.

Índice

General Introduction

General Introduction

Uranium in the environment

Uranium occurs in the environment in natural or geogenic conditions, mining, industrial activities, and from fertilizers used in agriculture. Agricultural activities can enhance the basal low-levels of uranium in soils and groundwater (Liesch et al. 2015). Uranium can be sorbed in deeper soil and rock layers, leached or eroded (Bigalke et al. 2018). Uranium mining may results in Pb, Cd, Zn, Cu, Mn and radionuclides (such as uranium) run off to the soil surface and groundwater (Pereira et al. 2008). The main techniques for uranium exploitation are open pit and underground mining (Woods 2016). I*n situ* leaching in underground mining and heap leaching of the poorest ore at the surface are carried out through the addition of sulphuric acid, and acid mine drainage effluents persist even after the cessation of exploitation (Pereira et al. 2008; Carvalho et al. 2009).

Whatever the origin of uranium in the environment, its speciation is related to geochemical factors such as Eh, pH, the presence of complexing agents (inorganic carbon, phosphorous, calcium, dissolved organic matter) and microbial activities (Novotnik et al. 2018). In oxygenated environments, uranium is present predominantly as U (VI), or (UO_2^{2+}, +VI oxidation state), which is highly soluble and mobile. In anoxic environments, uranium is present in its reduced state, less mobile U (IV), or (UO_2) (Novotnik et al. 2018), although it can be found in oxidising environments with low pH (Campbell et al. 2014).

In oxic natural waters containing Ca^{2+}, $CO_{2(g)}$, SO_4^-, Na^+, Cl^-, aqueous U (VI)-sulphate complexes are predominant at low pH (1.5-5), whereas in neutral to basic pH, U (VI) species form complexes predominantly with carbonates and calcium (Campbell et al. 2014). Sediments and water-logged soils can be natural reducing environments, where U (VI) is predominantly reduced to sorbed U (IV) species (Seder-Colomina et al. 2018). Nevertheless, U (IV) species can be oxidised into aqueous U (VI) carbonate complexes under anoxic conditions (Seder-Colomina et al. 2018). Sorbed U (IV), also known as non-crystalline, monomeric or amorphous species, are more labile and susceptible to re-oxidation than U (IV) crystalline (uraninite) species (Fu et al. 2018). Sorbed U (IV) species are predominant in wetlands soils (Wang et al. 2014), lacustrine sediments (Stetten et al. 2018) and aquifer sediments (Campbell et al. 2012). Uranium interacts with the solid phases and increases its accumulation in depositional sediments downstream of uranium mines (Crawford et al. 2018). It has a pronounced ability to adsorb to Fe and Mn oxides in sediments (Alam & Cheng 2014; Crawford et al. 2018). In wetlands, U (IV) species can be

associated with Fe (II) and organic matter colloids in porewater, increasing uranium mobility in this system (Wang et al. 2014). On the other hand, poor mineral and lower Fe (II) content in porewater colloids can reduce the uranium mobility in the wetlands. In marine and estuary sediments, diagenetic reactions of Mn and Fe oxides were linked to the geochemical cycling of alkaline elements (Ra and Ba) and redox sensitive elements (U) (Hong et al. 2018). As in freshwater sediments, U (VI) is reduced to U (IV) when the redox potential is low in marine porewater (Hong et al. 2018). Figure 1 summarises the uranium movement in the environment in oxic and anoxic conditions.

Uranium also binds to dissolved organic carbon (DOC), especially at pH (6-7), but in higher pH the organic carbon becomes less important for binding the metal due to greater competition between carbonate complexes and DOC (Crawford et al. 2018). In natural waters and sediments, humic acids (organic macromolecules) are soluble and can form soluble humic acids-U complexes through the presence of functional groups such as carboxylic groups, alkyl, phenolic groups (Mibus et al. 2007; Yang et al. 2013). The mobility of uranium can be increased by the formation of humic acids-U complexes and in the presence of mineral colloids (Geng et al. 2012; Chen et al. 2018). An example is the leaching of soluble compounds of leaf-litter release dissolved organic carbon, such as sugar and amino acids, followed by humic and fulvic acids which contribute to the remobilisation of uranium from sediments (Schaller et al. 2011).

Microbial activities in uranium mobilisation

Uranium (VI) can be the main electron acceptor in microbial biofilms, for instance for Bacteroidia, Clostridia, Epsilon-proteobacteria, which can reduce U (VI) (Ontiveros-Valencia et al. 2017). The immobilisation of uranium by indigenous microbial communities is a strategy for the in situ bioremediation of environments contaminated by uranium (Cologgi et al. 2014). The presence of bacterial biofilms can be more effective for the faster immobilisation of the uranium than abiotic pathways, with accumulation of the non-crystalline U (IV) species (Stylo et al. 2015) (Figure 1). Lower pH and higher levels of contaminants, including nitric acid, radionuclides (uranium) and other metals, are factors for selective pressure on the microbial communities (Akob et al. 2007), with lower total species diversity in acidic pH sediments. Bacteria capable of reducing U (VI) to U (IV) can dominate the biofilm community (Ontiveros-Valencia et al. 2017). These dissimilatory metal-reducing bacteria can gain energy for growth from the oxidation of electron donors coupled with a reduction of uranyl cations (Cologgi et al. 2014). Due to higher nitrate

concentrations in groundwater contaminated by uranium, denitrifying bacteria that are also uranium-reducing can be favored in acidic high-nitrate environments (Spain & Krumholz 2012).

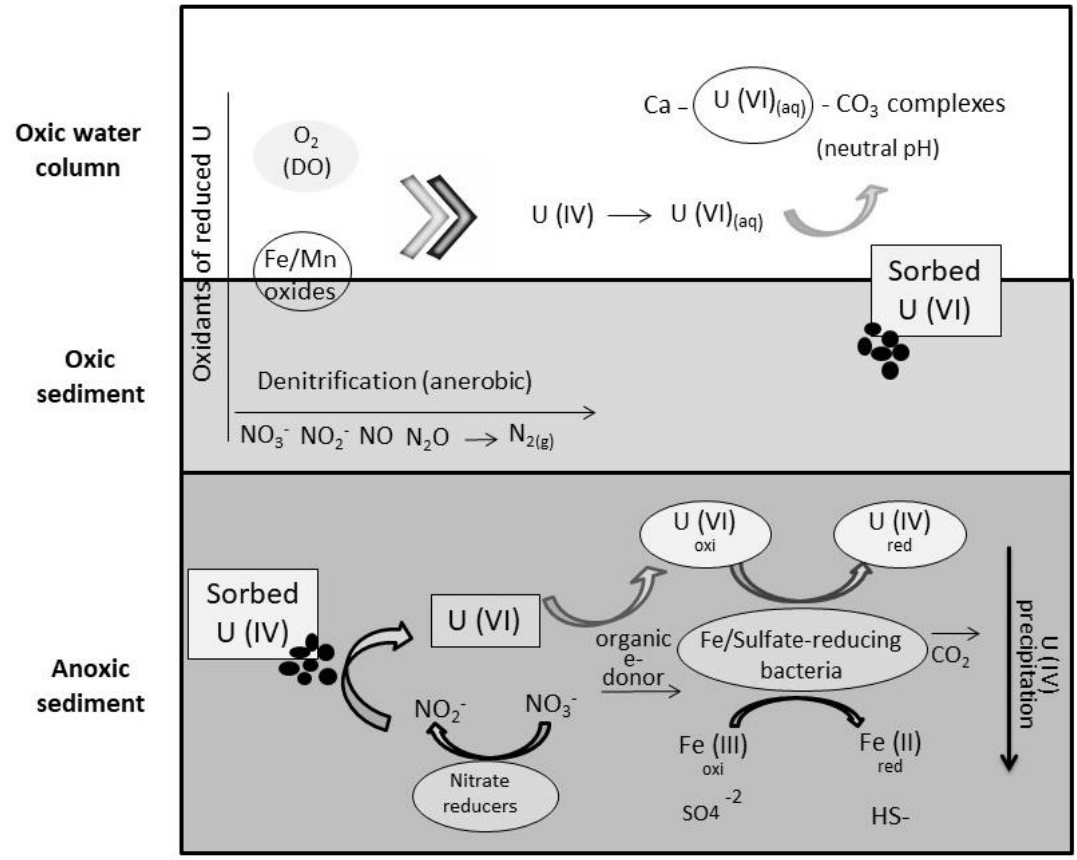


Figure 1. Uranium in the environment in oxic and anoxic conditions. DO = dissolved oxygen (Adapted from Bi & Hayes 2014; Geng et al. 2012; Chen et al. 2017; Yabusaki et al. 2017).

The rhizosphere of wetland plants can also act in the immobilisation of uranium when Fe (II) is provided for the stimulation of iron-reducing bacteria (e.g. *Geobacter*), and consequently a reduction of U (VI) (Chang et al. 2014). The conversion of Fe (III) to Fe (II) by metal-reducing bacteria provides electron donation for U (VI) reduction (Campbell et al. 2014). At low metal concentrations in water, algae and fungi have the ability to prevent the entry of metals into the cytoplasm through the formation of metal complexes outside cells (Das et al. 2009). Fungi can accumulate uranium into biomass through the production of biomineralised uranyl-phosphates complexes, and consequently the immobilisation of the metal (Fomina et al. 2007).

As in sediments and groundwater, microorganisms at low uranium concentrations in soils differ from soils with higher levels. Soil bacterial phyla have mechanisms to convert uranium to less soluble forms through biosorption, bioaccumulation and binding

to the metal (Mumtaz et al. 2018). Furthermore, the organic matter in the soils contributes to the prevalence of more insoluble and immobile U (IV) rather than U (VI) (Davies et al. 2018), due to the strongest affinity of the humic acids with U (IV) (Mitchell et al. 2013). The mobility of U (VI) in soils depends, however, on the formation of dissolved inorganic substances (CO_3^{2-}, HPO_4^{2-}, SO_4^{2-}), with high affinity for dissolved carbonates (Mitchell et al. 2013). The UO_2^{2+}, uranyl-carbonate complexes and $UO_2PO_4^-$ are the main species of uranium taken up by the roots of plants (Vandenhove et al. 2007).

Bioavailability of uranium and bioaccumulation by aquatic organisms

The higher affinity of uranium to organic matter and biosorption to biofilms contributes to its bioavailability to diverse invertebrate functional groups such as leaf-shredders, grazers and scrapers (Bergmann et al. 2018; Scheibener et al. 2017). The uranium mobilised from sediments (Figure 1) can bind to particulate organic matter and become available for collector-gathering invertebrates (Crawford et al. 2018). Metal contaminated sediments can eliminate sensitive benthic macroinvertebrates feeding on deeper compartments (Mocq & Hare 2018). The bioaccumulation of uranium has been linearly related to uranium concentrations in sediments, porewater and overlying water for amphipods (Goulet & Thompson 2018) and Chironomidae (overlying water and sediment porewater) (Crawford & Liber 2015), because the accumulation is mediated by a desorption of uranium from sediment into porewater and then it is taken by these organisms. The bioavailability of uranium in sediments depends on certain factors, however, such as clay minerals and organic matter (with higher DOC), which have been demonstrated to reduce uranium bioaccumulation in Chironomidae larvae at increasing contents (Crawford & Liber 2015).

Nevertheless, metal speciation in the environment is one of the multiple explanations for the bioaccumulation and toxicity to aquatic organisms. Metal uptake from solution based on its dissolved speciation was initially developed in the 1980s (Free ion activity model – FIAM) (Wang & Rainbow 2008). It has been used to explain the interactions between metals and aquatic organisms, where metal uptake and toxicity are regulated by free metal ions (Brown & Markich 2000). The biotic ligand model (BLM) evolved basically from the free ion activity model and from the gill surface interaction model and refers to a free metal ion uptake in a biological site of action (biotic ligand)

(Niyogi & Wood 2004). Another explanation for toxicity and bioaccumulation is the subcellular partitioning model (SPM), which associates metal accumulation by aquatic organisms with subcellular compartments, such as granules, cellular debris and organelles (Wang & Rainbow 2006). It takes into account the internal complexity of the organisms, which can affect the toxicity and trophic transfer of the metals (Figure 2).

A more recent model of bioaccumulation refers to metal taken up from solutions and metal taken up from food, less metal that has been excreted (Rainbow 2007). Metals in this model are accumulated by animals in both dissolved and dietary phases, and the uptake from both pathways is considered additive (Rainbow 2007). Bioaccumulation based on toxicokinetic-toxicodynamic models simulate and predict toxicity over time, linking metal accumulation to toxicity (Wang & Rainbow 2008).

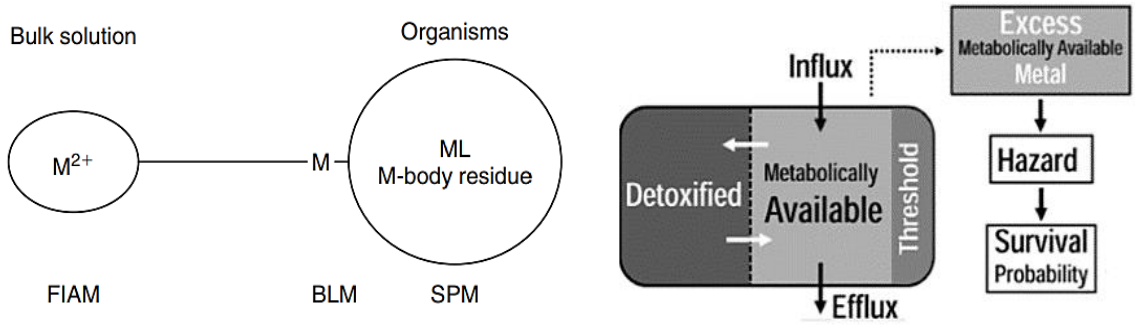


Figure 2. Models for bioaccumulation and toxicity of metals to aquatic organisms. Left: FIAM = Free ion activity model; BLM = Biotic ligand model; SPM = Subcellular partitioning model; ML = metals binding with the subcellular compartments. Right: Toxicokinetic- Toxicodynamic model with two compartments: metabolically available (essential metals can play an essential role), the excess of essential and non-essential metals is accumulated in a detoxified compartment. When the metal exceeds the threshold concentration, internal damages and the probability of mortality increase. Adapted from: Wang & Rainbow 2006 and Tan & Wang 2012.

Uranium mining

Uranium is mined primarily for fuelling nuclear power plants. A small proportion of mined uranium is used for isotopes production for medicinal use such as radioisotopes employed for radiotherapy (World Nuclear Association 2017). Radioisotopes are also employed for food preservation, and for the detection and study of movements of pollutants in the environment. Uranium was used from the 1930s for military purposes, where bombs and naval reactors used highly-enriched uranium (> 90% U^{235}). Since the 1990s, however, military uranium has been used for electricity production, through the dilution of highly-

enriched uranium with depleted uranium (U^{238} 99.9% U^{235} 0.2%; U^{234} 0.001%) (World Nuclear Association 2017). The largest uranium mines are located in Canada, Kazakhstan, Niger and Australia, accounting for 53% of the production of the world, and 31.46 tons of uranium (World Nuclear Association 2017).

Electricity generation makes a significant contribution to greenhouse gas emissions, at ~41% of total emissions globally (Norgate et al. 2014). Global greenhouse gas emissions (for instance CO_2 emissions) will need to be reduced at an accelerating rate to reach the aims of the Paris Agreement (IAEA 2016). As a global response to the threat of climate change, the Paris Agreement aims to hold "the increase in the global average temperature to well below 2 °C above pre-industrial levels and to pursue efforts to limit the temperature increase to 1.5 °C above pre-industrial levels" (United Nations 2015). An alternative to reducing carbon dioxide emissions is the use of low-carbon clean energy, such as nuclear power, hydro power and renewable energy. Greenhouse gas emissions for nuclear power are less than that from coal and natural gas-based electricity (Norgate et al. 2014).

In Brazil, the main reserves of uranium are located in Bahia, Ceará, Paraná and Minas Gerais. Uranium production in Brazil began in 1982 in Poços de Caldas, Minas Gerais, and lasted for 13 years, generating a total of 1,242 tons of U_3O_8. Although mining activities have ceased in Poços de Caldas, the chemical plant for the liquid effluent treatment is still active (Ferrari et al. 2017). In Ceará State, the deposit of uranium in Santa Quiteria is associated with phosphate rock (INB 2016). The Santa Quiteria Project estimates uranium production as about 1,600 t U/year (deposit of uranium with 80,000 t U_3O_8). The only uranium mine currently operating in Brazil is located in Caetité, Bahia State, where uranium mineralisation consists of uraninite (INB 2016). In the town of Caetité, the *Indústrias Nucleares do Brasil* (INB) performs the two first stages of nuclear fuel cycle: mining and uranium processing (1700 ha). A nuclear fuel factory is located in Resende, Rio de Janeiro State, where four stages of the nuclear fuel cycle take place: uranium enrichment, conversion of UF_6 to UO_2, pellets production and fuel fabrication for energy generation in nuclear power plants (INB 2016). In 2016, only 3% of the electricity of Brazil came from nuclear power, whereas 66% was from hydro, but uranium production is expected to increase from 150 tons in 2017 to 250 t/year from 2018 (World Nuclear Association 2017).

Although nuclear power is considered a low-carbon energy source, uranium mining can be responsible for environmental impacts such as waste rock; acid mine drainage and

tailings with high concentrations of hazardous contaminants that may lead to the acidification and contamination of surface- and groundwater; the atmospheric release of radon from underground mines and surface mining, emissions of radionuclides, NOx and SOx from milling operations; and ecosystem impact due to increased concentrations of uranium and radioactive material in flora, fauna and food chains (Dudar et al. 2015). We thus need to understand the environmental effects of uranium in order to implement management strategies for mine site rehabilitation, re-establishing nutrient cycling and plant and animal communities in the affected ecosystems.

Objectives and thesis outline

Streams and rivers can be affected by mining activities due to the release of metals, metalloids and radionuclides. Increase metal concentrations have been associated with a decrease in benthic taxon richness (Kilgour et al. 2018), changes in fungal communities (Solé et al. 2008) and a reduction in leaf decomposition (Fernandes et al. 2009). Uranium can also be transferred to aquatic invertebrates through food (Simon et al. 2013), increasing the potential for it to be transferred along food chains.

In this thesis, I have investigated how uranium affects aquatic organisms, particularly those involved in organic matter decomposition (i.e. aquatic hyphomycetes and shredders). I also investigated whether uranium is transferred along food webs and if it is biodispersed by emergent aquatic insects. Four questions were asked to achieve this main objective:

- Does uranium affect the biological functions of the leaf-shredder freshwater organisms playing a key role in the organic matter cycle in low order streams? To address this question, I used the caddisfly *Schizopelex festiva* as a model organism. I performed assays to assess survivorship (acute test with 0 to 262 $mg \cdot L^{-1}$), feeding, growth and respiration rates under laboratory conditions and environmental concentrations (25 $\mu g\ L^{-1}$) – Chapter I;

- Can uranium exposure cause enzymatic alterations in aquatic invertebrates before any visible physiological alteration? To address this question, a) the activities of four enzymes (AChE, Na^+K^+ATPase, GST and Catalase) of the caddisfly *Schizopelex festiva* were assessed at 24 h and 32 d, under increasing environmental uranium concentrations (0 to 100 $\mu g \cdot L^{-1}$) and b) a growth assay was performed at the same concentrations – Chapter II;

- How sensitive to uranium are aquatic hyphomycetes, a group of freshwater fungi involved in leaf-litter degradation? To address this question, four assays were performed to investigate growth, spore production, biomass and litter decomposition at increasing uranium concentrations in microcosms (0 to 262 $mg \cdot L^{-1}$), using four species of aquatic hyphomycetes and six strains of one aquatic hyphomycete species – Chapter III;

- Does uranium in streams affect invertebrate community structure? Is uranium transferred along food webs? To answer this question, I sampled stream sites differing in uranium

concentration and assessed invertebrate diversity and bioaccumulation in different trophic levels– Chapter IV.

Publications

This thesis is based on following published or submitted manuscripts:

Chapter I

Bergmann, M., Sobral, O., Pratas, J., Graça, M.A.S., 2018. Uranium toxicity to aquatic invertebrates: A laboratory assay. Environmental Pollution. 239: 359-366.

Chapter 2

Bergmann, M., Sobral, O., Graça, M.A.S. Activities of oxidative stress- and cell-membrane-associated enzymes in a freshwater leaf-shredder exposed to uranium. (Submitted).

Chapter III

Bergmann, M., Graça, M.A.S. Uranium affects growth, sporulation, biomass and leaf-litter decomposition by aquatic hyphomycetes. (Submitted).

Chapter IV

Bergmann, M., Graça, M.A.S. Bioaccumulation and dispersion of uranium by freshwater organisms. (Submitted).

CHAPTER I

Uranium toxicity to aquatic invertebrates: a laboratory assay

Uranium toxicity to aquatic invertebrates: a laboratory assay

Abstract

Uranium mining is an environmental concern because of runoff and the potential for toxic effects on the biota. To investigate the uranium toxicity to freshwater invertebrates, we conducted a 96-h acute toxicity test to determine lethal concentrations (testing concentrations up to 262 $mg \cdot L^{-1}$) for three stream invertebrates: a shredder caddisfly, *Schizopelex festiva* Rambur (Trichoptera, Sericostomatidae); a detritivorous isopod, *Proasellus* sp. (Isopoda, Asellidae); and a scraper gastropod, *Theodoxus fluviatilis* (Gastropoda, Neritidae). Next, we ran a chronic-toxicity test with the most tolerant species (*S. festiva*) to assess if uranium concentrations found in some local streams (up to 25 $\mu g \cdot L^{-1}$) affect feeding, growth and respiration rates. Finally, we investigated whether *S. festiva* takes up uranium from the water and/or from ingested food. In the acute test, *S. festiva* survived in all uranium concentrations tested. LC_{50}-96-h for *Proasellus* sp and *T. fluviatilis* were 142 $mg \cdot L^{-1}$ and 24 $mg \cdot L^{-1}$, respectively. Specimens of *S. festiva* exposed to 25 $\mu g \cdot L^{-1}$ had 47% reduced growth compared with specimens under control conditions (21.5 ± 2.9 vs. 40.6 ± 4.9 µg of mass increase·day^{-1}). Respiration rates (0.40 ± 0.03 µg $O_2 \cdot h^{-1} \cdot$mg $animal^{-1}$) and consumption rates (0.54 ± 0.05 µg·µg $animal^{-1} \cdot day^{-1}$; means ± SE) did not differ between treatments. Under laboratory conditions *S. festiva* accumulated uranium from both the water and the ingested food. Our results indicate that uranium can be less toxic than other metals or metalloids produced by mining activities. However, even at the low concentrations observed in streams affected by abandoned mines, uranium can impair physiological processes, is bioaccumulated, and is potentially transferred through food webs.

KEYWORDS*:* shredders, sublethal effects, bioaccumulation, mining

Introduction

The world energy demand is expected to increase by 48% from 2012 to 2040, with non-fossil renewable energy and nuclear power expected to increase by 2% per year during

the same period (EIA 2016). Currently, nuclear power contributes about 7% of the global energy demand (Asif & Muneer 2007), with a tendency to increase. The nuclear energy demand involves mining of more than 100,000 tons of uranium during the next 15 years (Dittmar 2012), with consequent production of mining wastes.

Extensive information is available on the environmental effects of several metals and metalloids, but comparatively little is known about the effects of uranium. Although uranium appears to be toxic at high concentrations, it is a non-essential metal that accumulates on aquatic biota even in low levels and concerns to protect it must be considered. For the protection of freshwater life under chronic (long-term) exposure, Canadian Water Quality Guidelines for Uranium (CWQG) recommends a maximum of 15µg·L^{-1}, and 33 µg·L^{-1} for short-term exposure during transient events (CEQG 2011).

Exposure of aquatic invertebrates to uranium may cause induction of reactive oxygen species (ROS), leading to DNA damage (Simon et al. 2011) Uranyl ions bind to nucleotides through phosphoric groups (ATP-UO_2^{2+}), compete with calcium and magnesium ions and inhibit ATPase activity and ATP production (De Stefano et al. 2005). Uranium also indirectly affects the heme group of oxyhemoglobin, interfering with O_2 binding (Kumar et al. 2016). The U-hemoglobin complex can be a pathway for uranium to enter animal organs that are not directly exposed (Bucher et al. 2016). Tagliaferro et al. (2018) found that exposure of the caddisfly shredder *Calamoceras marsupus* to 50 µg·L^{-1} of uranium decreased Na^+/K^+ ATPase activity, an enzyme related to signal transduction and regulation of cell growth (Xie & Askari 2002). Regarding to physiological effects, studies have reported that invertebrate exposure to uranium results in malformations, reduction on development time and survival (Dias et al. 2008; Lagauzère et al. 2009), and reduced growth and reproduction (Beaudouin et al. 2012; Mooney et al. 2016). For instance, decreased growth at 300 µg·L^{-1} in water was reported in *Chironomus tentans* for 9 days (Muscatello & Liber 2010), and at 27 µg·L^{-1} in *Sericostoma vittatum* for 60 days (Gonçalves et al. 2011), while the inhibitory concentration for growth (IC_{50}, 10 days) for *Chironomus dilutus* was 0.91 mg·L^{-1} of uranium (Liber et al. 2011).

Although these studies reported some physiological effects at sublethal concentrations of uranium, we still need to know whether environmentally realistic levels found in streams are high enough to affect biological processes, and whether uranium is accumulated by stream-dwelling consumers. To address these questions, we first conducted a 96-h acute toxicity assay to determine lethal uranium concentrations for three stream-

dwelling invertebrates, the shredder *Schizopelex festiva* Rambur, 1842 (Trichoptera, Sericostomatidae), the detritivore *Proasellus* sp. (Isopoda, Asellidae), and the scraper *Theodoxus fluviatilis* Linnaeus, 1758 (Gastropoda, Neritidae). We then exposed the most tolerant species, *S. festiva*, for 37 days to uranium concentrations similar to those found in streams affected by abandoned uranium mines in Portugal, to test for effects on survivorship, feeding, growth and respiration rates. Finally, we investigated if *S. festiva* accumulates uranium, and if so, whether it is taken up from the water or from the ingested food. We selected the *S. festiva* species because Sericostomatidae are common leaf-shredders in Europe, playing an important role on organic matter cycling in low order streams (Feio & Graça 2000). If the most tolerant species is affected by environmental realistic concentrations of uranium, them all other species will likely be affected as well. *S. festiva*.

Material and Methods

Invertebrates and water

Specimens of *S. festiva* were collected from sandy substrates in a reference stream at Múceres (N 40° 32' 01"; W 008° 09' 15", pH 6.9, 0.35 ± 0.10 $\mu g \cdot L^{-1}$ of uranium in the water and 1.40 ± 0.50 $mg \cdot kg^{-1}$ in the sediments). *Proasellus* sp. were collected from a stream in Póvoa de Luzianes (N 40° 30' 43", W 007° 49' 02", pH 7.0, conductivity: 156.50 ± 17.70 µS/cm, 2.20 ± 1.60 $\mu g \cdot L^{-1}$ of uranium in the water and 24.40 ± 7.10 $mg \cdot kg^{-1}$ in the sediments). *T. fluviatilis* were sampled at the Anços River source (N 39° 58' 43" W 008° 34' 23", pH 7.3, conductivity: 539 µS/cm, 0.25 ± 0.08 $\mu g \cdot L^{-1}$ of uranium in the water). The specimens, leaf-litter, and some stones with periphytic algae (in the case of *T. fluviatilis*) were transported in stream water to the laboratory and acclimated for 5 days with aeration, 18 ± 1 °C and photoperiod of 14:10 h L:D. On Day 3, half of the stream water was replaced by standard water to be used in the test. The standard water was based on the USEPA international recommendations for moderately hard water (Lewis et al.1994): $MgSO_4$ (60 $mg \cdot L^{-1}$), $NaHCO_3$ (96 $mg \cdot L^{-1}$), KCl (4 $mg \cdot L^{-1}$), and $CaSO_4.2H_2O$ (60 $mg \cdot L^{-1}$). During acclimation, invertebrates were fed with alder leaves (*Alnus glutinosa*) conditioned in the laboratory (as described below). At the end of Day 5, the invertebrates were transferred to cups containing the test solutions. Uranyl nitrate hexahydrate ($N_2O_8U.6H_2O$) was used as source of uranium for the acute and sublethal tests.

Leaf conditioning

To feed the invertebrates in all chronic tests and for biosorption of uranium assay, air-dried *A. glutinosa* leaves from Mondego River Park (Coimbra, Portugal) were microbially conditioned in the laboratory. This was done by inoculating a ~ 6 L aquaria with stream water and litter at different decomposition stages (from Múceres, Central Portugal) as inoculum. Leaves were enclosed into 500 µm-mesh bags (19.50 x 13 cm), ~ 30 g/bag and conditioned for 7 days under strong aerations. Stream water was changed every two days to prevent accumulation of litter leachates.

Acute (96-hour) toxicity test

Ten uranium concentrations and 4 replicates of 5 specimens each were tested. We selected invertebrates with similar sizes in terms of case, body size and shell length for *S. festiva*, *Proasellus* sp., and *T. fluviatilis*, respectively. We used 200 specimens of each species, with biomasses of 2.30 ± 0.08 mg (mean ± SE; dry weight) in the case of *S. festiva*, 0.75 ± 0.035 mg (length 5.35 ± 0.09 mm) in the case of *Proasellus* sp., and 2.50 ± 0.46 mg (5.0 ± 0.62 mm shell length) in the case of *T. fluviatilis*. The test vessels were plastic cups, 10 cm high, 5.5 cm diameter, containing 150 mL test solution (see below). For *S. festiva*, we also added 10 g of stream sand, previously sieved through a 1-mm mesh, incinerated at 450 °C for 8 h and washed in distilled water.

Uranium treatments for *S. festiva* and *Proasellus* sp. ranged from 0 to 262 $mg \cdot L^{-1}$. Nominal concentrations were 0.004 $mg \cdot L^{-1}$ and multiples of 4 up to 262 $mg \cdot L^{-1}$ (9 concentrations plus control). However, at the end of the experiments the final concentrations in water were in average 0.0025, 0.014, 0.044, 0.121, 0.634, 2.67, 10.77, 38.20, and 254.50 $mg \cdot L^{-1}$ (reduction of 32 ± 15 %). Uranium concentrations in test with *T. fluviatilis* ranged from 10.0 $mg \cdot L^{-1}$ to 75.9 $mg \cdot L^{-1}$ (1.5 × increases) based on a preliminary assay in which no mortality occurred below 10.0 $mg \cdot L^{-1}$ and 100% mortality occurred above 75.9 $mg \cdot L^{-1}$. Survivorship was measured every 24 h. Mortality (%) was calculated as the number of dead organisms divided by initial number of individuals, multiplied by 100 (n = 20 specimens/concentration). The pH, dissolved oxygen and conductivity were documented daily in a random set of cups. At the end of the test, the water from each microcosm was acidified with 65% HNO_3 to pH = 2 (v/v) and stored at 4 °C until analysis of uranium.

Sublethal tests – Growth, consumption and respiration rates

To test for sublethal uranium toxicity, we performed three assays to measure growth, food consumption and respiration rates by *S. festiva*, the most tolerant species from the previous experiment. To test the environmentally realistic concentrations, we measured the uranium concentrations in several streams affected by abandoned uranium mines in Central Portugal and obtained values from the literature for rivers in the region (Table 1; Table A1). Since the mean levels of uranium from polluted streams ranged from 1.98 to 35.45 $\mu g \cdot L^{-1}$, *S. festiva* was exposed to 25 $\mu g \cdot L^{-1}$ and 0 $\mu g \cdot L^{-1}$.

Table 1. Concentrations of uranium in streams below mines in Portugal. NF = not found; NR = not reported.

	U ($\mu g \cdot L^{-1}$)			
Region	Minimum	Maximum	Mean	Reference
Northwest (Horta da Vilariça)	0.61	5.56	1.98	Cordeiro et al. 2016
Central (Beiras)	0.40	113	12.6	Favas et al. 2016
Central (Tábua, Nelas, Oliveira do Hospital)	0.40	1220	35.45	Pratas et al. 2012
Central (Tábua)	NF	13	NF	Favas & Pratas 2012
Canas de Senhorim (Ribeira da Pantanha)	NR	NR	27.80	Gonçalves et al. 2011

For growth and consumption assays, *S. festiva* specimens were individually allocated to cups (150 mL test water, replaced every 2 days), 20 replicates for each concentration, 25 $\mu g \cdot L^{-1}$ and 0 $\mu g \cdot L^{-1}$ of uranium. To measure size, we regressed the case opening (CO, mm) on body dry mass (W, mg): W = (8.27 x CO) – 15.366; n = 42, R^2 = 0.90, $p < 0.001$). Case opening was measured in a stereoscopic microscopic (ocular with graduated scales) at 16 x. To feed the specimens, we conditioned (see above) and soaked alder leaves for 48 h in the testing uranium concentrations. The initial body mass was estimated by regression from a set of specimens not used in the tests, and the final dry mass was obtained by weighing the tested specimens after 37 days. Growth rate ($\mu g \cdot day^{-1}$) was determined as:

$$GR = \frac{\ln(Wf/Wi)}{t}$$

Wf and Wi are the final and the initial dry mass, and *t* is the time in days (Olson & Hawkins 2017).

Consumption rates were measured during the growth assay and for a period of 10 days. This was done by replacing the alder leaves with two pre-weighed leaf discs (∅ 12 mm, wet weight), from leaves conditioned in laboratory. Leaf discs were replaced every 2 days. The retrieved discs were oven-dried (45 °C; 72 h) and weighed to the nearest 0.005 g. Consumption (*C*) was measured as the difference between the initial (Wli) and final (Wlf) dry mass of the leaf discs divided by the shredder mean dry mass (W_{sh} = Wi+Wf/2) and time (t) in days

$$C = \frac{\mathrm{Wli} - \mathrm{Wlf}}{\mathrm{Wsh\ x\ t}}$$

Since the offered discs were wet-weighed, a set of 32 leaf discs (~ 46.14 mg wet mass) were oven-dried for 72 h at 45 °C and reweighed to obtain a factor (0.2426) to convert wet mass to dry mass.

Respiration rates were measured after 20 days of exposure to both treatments of 0 and 25 µg·L^{-1} of uranium (n = 13 + 13; invertebrates from the growth assay). Specimens were individually allocated to sealed 3-mL chambers in which an oxygen electrode was inserted (Strathkelvin 929 6-Channel Oxygen System, North Lanarkshire, UK). A magnetic stirrer inside the chamber (but separated from the test specimens by a nylon net) provided turbulence and homogenized the oxygen content in the chambers. The temperature was regulated at 18 °C by a water bath in which the chambers were inserted. Oxygen concentration was measured continuously for 20 min. Respiration (R) was calculated as the difference in oxygen concentration in each chamber (specimens individually reared), corrected for control variability (chambers with no organisms), the chamber volume in liters (0.003), the time in hours (60/20) and the specimen mean dry mass (W_{sh}).

$$R = \frac{[(\text{initial } O_2 - \text{final } O_2) - (\text{initial } O_2 \text{ control} - \text{final } O_2 \text{ control})] \times 0.003 \times 60/20}{\mathrm{Wsh}}$$

Biosorption and elimination of uranium by alder leaves

To investigate the dynamics of uranium accumulation and release by leaves, leaf-discs (12-mm diameter) from conditioned leaves were cut with a cork borer. Groups of 26 discs were transferred to 250-mL cups containing 150 mL of 25 $\mu g \cdot L^{-1}$ uranium solution (10 replicates). The uranium solution was replaced every 2 days for 15 days. Two leaf discs were retrieved from each cup before the uranium addition, and 2 more discs after 24 h of exposure and subsequently every 2 days. After 15 days in the uranium solution, the remaining leaf discs were transferred to distilled water and additional leaf discs were retrieved from this group every 2 days for 10 days. To investigate whether uranium adsorption was affected by microbial colonization, 15 microbially and 15 non-colonized leaf discs (5 cups/replicates for each group) were subjected to water with 25 $\mu g \cdot L^{-1}$ uranium. Three leaf-discs were retrieved after 24 h, 2; 4; 6 and 8 d and the adsorbed uranium measured.

Pathways of uranium bioaccumulation: Diet and water exposure

To investigate if consumers accumulate uranium, and if uranium is taken up from the water or from the ingested food (or both), *S. festiva* was exposed to four treatments: (i) uncontaminated water and leaves, (ii) leaf discs previously exposed to a nominal concentration of 25 $\mu g \cdot L^{-1}$ for 48 h and clean water (indirect pathway), (iii) specimens exposed to contaminated water (25 $\mu g \cdot L^{-1}$) and uncontaminated leaf discs (direct pathway), (iv) specimens exposed to both contaminated water and contaminated leaf discs (25 $\mu g \cdot L^{-1}$, combined effect). Water and food were replaced every 2 days; at the end of 8 days, the specimens were dried (45 °C, 72 h), and their uranium content measured as indicated below.

Uranium measurements

The uranium concentration in the water (from acute test: N= 20, growth assay: N = 66, and pathways of uranium accumulation: N = 12), organisms and alder leaves (from growth assay, Table 2), and leaf discs (from uranium biosorption assays: N = 180) was measured by fluorometry, through determination of the fluorescence of uranyl ions at λ = 530 nm (Van Loon & Barefoot 1989). Organisms and leaves were oven-dried at 45 °C for at least 72 h, weighed, incinerated in a muffle furnace at 450 °C for 8 h, and weighed again to determine the ash-free dry mass (AFDM). The ash was transferred to 50-mL test tubes

and dissolved in 8 mL of 2.5 M nitric acid in a boiling-water bath for 1 h. After cooling, 10 mL of ethyl acetate was added to the samples, and the inorganic solvent incinerated. The residue was dissolved in 7 mL of 0.005% nitric acid. The sample fluorescence was measured in a Fluorat 02-2M (Lumex), and compared with a standard curve (2; 10; 100 and 1000 $\mu g \cdot L^{-1}$) (Van Loon and Barefoot, 1989). Uranium in the water was measured in a 0.5 mL sample in a polyethylene vial, after adding 5.0 mL of distilled water and 0.50 mL of the polysilicate solution. The fluorescence was measured as described above.

Statistical analysis

Prior to any statistical test, data were checked for normality and homoscedasticity. When assumptions for parametric tests were violated, data were transformed (square-root or log transformations) or used equivalent nonparametric tests (Kruskal-Wallis one- way Analysis of Variance). In the 96-h acute toxicity test, one-way analysis of variance was used followed by multiple-comparison test (Tukey´s post hoc) to determine differences between treatments in terms of conductivity, pH and dissolved oxygen. The median lethal concentration (LC_{50}, 95% confidence limit) was calculated for each species by the probit method. The two treatments (0 $\mu g \cdot L^{-1}$ and 25 $\mu g \cdot L^{-1}$) for growth, consumption and respiration rates were compared by *t-test*. To assess the role of microbial conditioning on leaves, uranium accumulation over time in the conditioned group was compared with the non-conditioned one by two-way repeated measures ANOVA, followed by a pairwise multiple comparison test (Tukey´s post hoc). Data for pathways of uranium accumulation in *S. festiva* were log-transformed and assessed by two-way ANOVA. All analyses were conducted with the software Statistica 7.0 (StatSoft, USA).

Results

Acute (96-hour) toxicity test

Of the three species tested, *S. festiva* was the most tolerant, with only 4% random mortality (non-significant) in all treatments, and with a 100% survival rate in the highest (262 $mg \cdot L^{-1}$) uranium concentration. For *Proasellus* sp. and *T. fluviatilis*, LC_{50}-96-h values were 142 $mg \cdot L^{-1}$ (CI 139.11–144.9) and 24 $mg \cdot L^{-1}$ (CI 15.38–32.86), 95% confidence limits, respectively (Figure1).

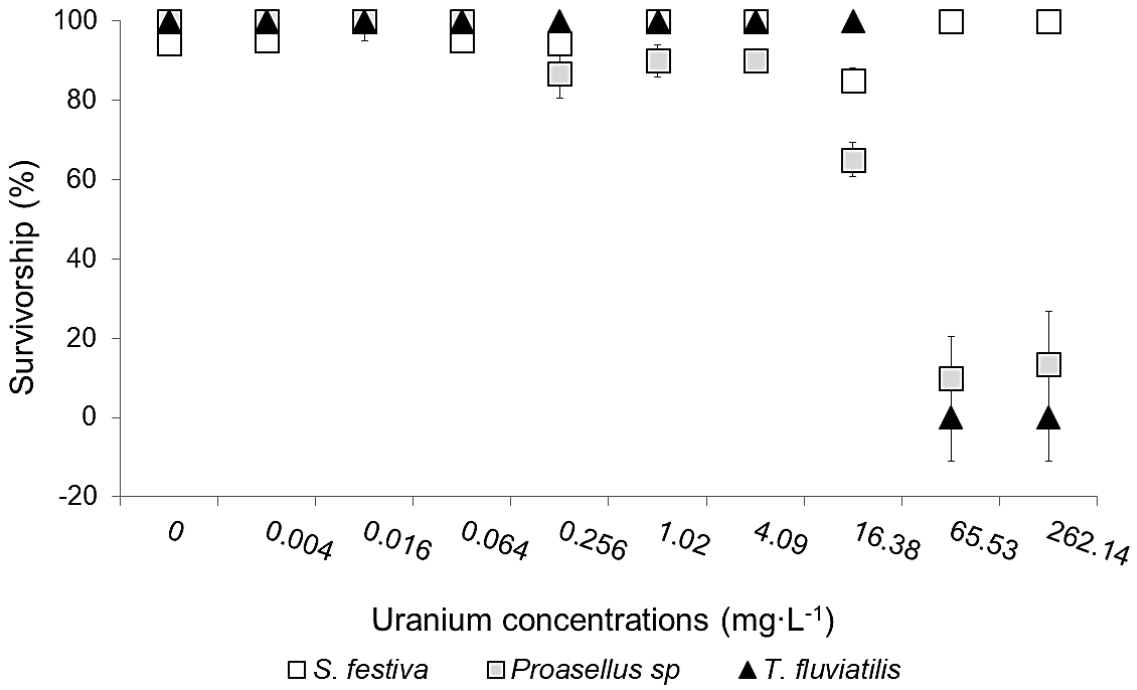


Figure 1. Survivorship rates of the 3 species of aquatic invertebrates from acute toxicity test at 10 nominal uranium concentrations: 0; 0.004; 0.016; 0.064; 0.256; 1.02; 4.09; 16.38; 65.53 and 262.14 mg· L^{-1}.

During the experiment, dissolved oxygen content was 9.7 ± 0.1 mg·L^{-1} (means ± SD) in all treatments and the electrical conductivity was 417 ± 22 µS·cm^{-1} in the highest uranium concentration, and 301 ± 4 µS·cm^{-1} in all other concentrations (Table 2). The differences in conductivity were significant only for the highest uranium concentration (ANOVA: $p < 0.001$, $F= 61.86$, $df = 9$; Tukey´s pairwise: $p < 0.001$, $df = 160$). Similarly, pH was 4.8 ± 0.1 in 262 mg·L^{-1} of uranium, and 8.0 ± 0.0 in all other concentrations (Kruskal-Wallis test: $p < 0.001$; post-hoc Mann-Whitney comparison test: $p < 0.001$).

Growth, consumption and respiration rates

S. festiva specimens under control conditions grew at 40.6 ± 4.9 (mean ± SE) µg·day^{-1}, while specimens exposed to uranium had a 47% reduction in growth (21.5 ± 2.9 µg·day^{-1}, mean ± SE), and this difference was statistically significant (*t-test*: $p = 0.006$, $df = 32$, Figure 2).

Table 2. Mean ± SD of water parameters and uranium concentrations measured in initial (freshly prepared), 2-day-old water (prior to renewal) in the growth experiment, and conditioned leaves after consumption for 2 days. The leaves were soaked for 2 days in uranium solution (25 µg·L^{-1}).

Samples	U (25 µg·L^{-1})	Control (0 µg·L^{-1})
pH (*n* = 9)	007.63 ± 0.16	007.08 ± 0.30
Conductivity (µS/cm; *n* = 9)	313.71 ± 4.57	306.77 ± 8.94
Dissolved oxygen (mg·L^{-1}; *n* = 9)	09.75 ± 0.23	9.82 ± 0.35
Initial Water (µg·L^{-1}, *n* =10)	27.12 ± 2.25	0.90 ± 0.11
2-day-old water (µg·L^{-1}, *n* =56)	18.71 ± 4.17	1.36 ± 0.13
Leaves (µg·g^{-1}, *n* =10)	16.00 ± 8 .00	0.70 ± 0.53
Invertebrates (µg·g^{-1}, *n* =11)	19.00 ± 4.19	1.69 ± 0.30

Exposure of *S. festiva* and leaves to 25 µg·L^{-1} in the growth assay resulted in uranium accumulation up to 19.0 ± 4.2 and 16 ± 8 µg·g^{-1} respectively. These values were significantly higher than the control conditions (*t-test*: $p < 0.001$, df = 17, Table 2). There were no significant differences in food consumption between *S. festiva* specimens exposed to 25 µg·L^{-1} of uranium (0.53 ± 0.05 µg·µg animal^{-1}·day^{-1}) and the control specimens (0.55 ± 0.08 µg·µg animal^{-1}·day^{-1}, mean ± SE) (*t-test*: $p = 0.67$, df = 31).

Similarly, respiration rates were 0.41 ± 0.06 µg O_2·h^{-1}·µg animal^{-1} for control and 0.39 ± 0.03 µg O_2·h^{-1}·µg animal^{-1} for animals exposed to uranium (mean ± SE) with no significant differences between treatments (*t-test*: $p = 0.19$, df = 24).

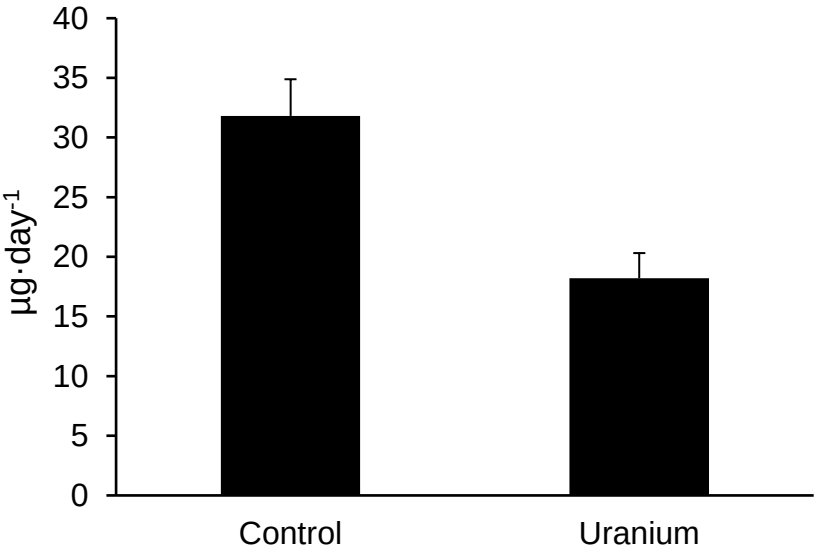


Figure 2. Growth rates of *Schizopelex festiva* specimens in control (no uranium) and in water containing 25 µg·L^{-1} for 5 weeks (mean ± SE).

Biosorption and elimination of uranium by alder leaves

Conditioned alder leaves accumulated uranium when exposed to a concentration of 25 µg·L^{-1}. After one day of exposure, uranium in leaves reached 9.8 ± 3.5 µg·g^{-1} and at day 15, 84 ± 4.8 µg·g^{-1}. When placed in clean water, the contaminated leaves released uranium at a slower rate than they accumulated it. After 12 days in clean water, the leaves still retained 60% of the incorporated uranium (Figure 3). When exposed to uranium, conditioned and non-conditioned leaf discs biosorbed uranium similarly (two-way repeated measures ANOVA, df =1; F =12, p (groups) = 0.01; df = 4, F = 47, p (time) < 0.001; interaction (time x groups): df = 4, F = 1.34, p = 0.28). Biosorption for the colonized group of leaves was higher only in the first 48 h (Tukey´s test, p < 0.001).

Pathways of uranium bioaccumulation in *S. festiva*: Diet and water exposure

Specimens of *S. festiva* accumulated uranium from the water (10.64 ± 1.13 µg·g^{-1}), from the ingested food (6.90 ± 0.51 µg·g^{-1}) and from the combined pathway (13.14 ± 2.22 µg·g^{-1}; Figure 4). Both pathways (water and food) were important to uranium accumulation by shredders and there was significant interaction of pathways (ANOVA two-way, Water: df = 1; F = 113.51; p < 0.001; Food: df =1; F = 60.3; p < 0.001; Water x Food: df = 1; F = 42.9; p < 0.001).

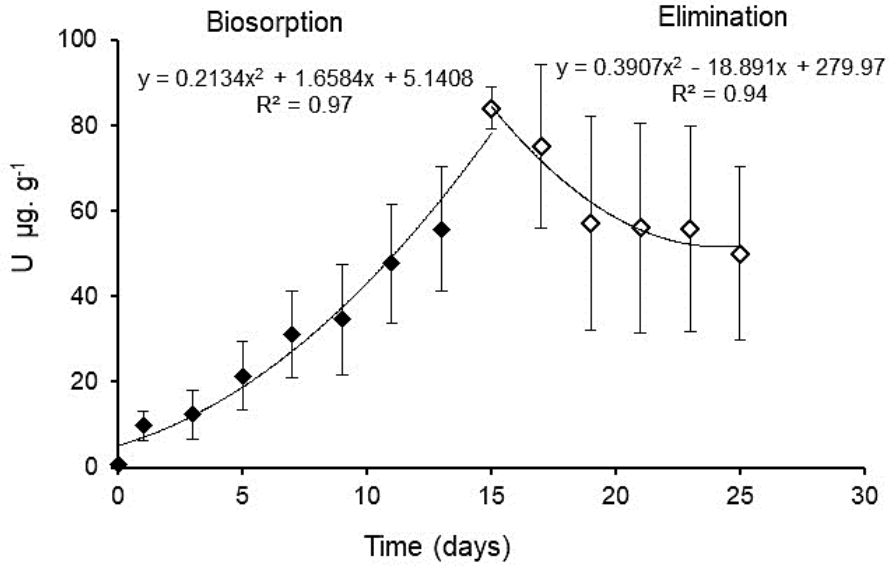


Figure 3. Uranium accumulation and elimination by alder leaves exposed for 15 d to 25 µg·L^{-1} and then transferred to clean water (mean ± SD; *n*=10).

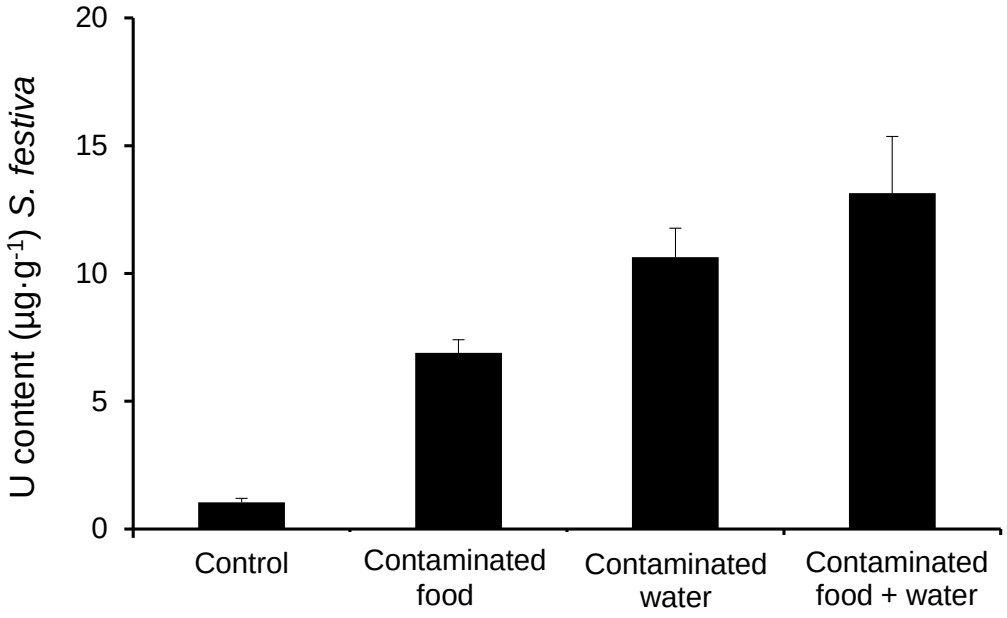


Figure 4. Uranium content in specimens of *Schizopelex festiva* exposed for 8 d to four conditions (mean ± SE; n=10 in each category).

Discussion

Acute (96-hour) toxicity test

Uranium toxicity differed widely among the three species tested. While mortality for *S. festiva* was zero at 262 mg·L^{-1}, for *Proasellus* sp. and *T. fluviatilis* the lethal uranium concentrations for 50% of the test populations (LC_{50}) were 142 and 24 mg·L^{-1} respectively. The LC_{50} values in the present study were higher than those reported for other freshwater invertebrates, e.g., 0.14–0.20 mg·L^{-1} for *Hyallela azteca* (Kuhne et al. 2002; Goulet et al. 2015); 0.39–10.50 mg·L^{-1} for daphnids (Semaan et al. 2001; Kuhne et al. 2002; Zeman et al. 2008); 8.0 mg·L^{-1} for crayfish (Al Kaddissi et al. 2011), and 0.06 mg·L^{-1} for *Ceriodaphnia dubia* (Pickett et al. 1993). An exceptionally high LC_{50}-96-h of 1872 mg·L^{-1} was reported for the bivalve *Corbicula fluminea* (Labrot et al. 1999). Although *S. festiva*, *Proasellus* sp. and *T. fluviatilis* were highly tolerant to uranium in terms of mortality, comparisons to the other organisms must be made with caution.

Differences in tolerance can be explained by several factors. (1) We used field-caught invertebrates, collected from reference streams rather than model organisms. Field-collected organisms are expected to show higher genetic variability than laboratory strains. Invertebrate sensitivity to metals may also be influenced by the phenology and season of

the collection (Clements et al. 2013). (2) The individuals tested were selected for similar body length, assuming that for each species, their developmental and physiological stages were driven identically by environmental conditions (temperature, food availability, and water physical and chemical parameters). (3) Factors such as the chemical composition of the test medium (Sorvari & Sillanpää 1996; Guilhermino et al. 1997; Nakajima et al. 2012) and water hardness (Charles et al. 2002; Antunes et al. 2007; Alam & Cheng 2014), alkalinity and pH (Goulet et al. 2015) are known to influence metal toxicity as well as uranium speciation (Markich 2013).

In our experiment, as moderately hard water was used according to USEPA guidelines, with an alkalinity of 57–64 $mg \cdot L^{-1}$ of $CaCO_3$ and a pH of 8, except for 262 $mg \cdot L^{-1}$ that became acidic, uranyl-carbonate/hydroxide complexes were the predominant soluble forms (Alam & Cheng 2014). For molluscs, the bioavailability of dissolved uranium is closely related to uranium-carbonate complexes ($UO_2 (CO_3)_2^{-2}$) (Croteau et al. 2016). In our 96-h acute toxicity experiment, most *T. fluviatilis* mortality occurred in the first 48 h (LC_{50} 30.63 $mg \cdot L^{-1}$), probably due to absorption through the integument and the ability of uranium-carbonate complexes to cross cellular membranes through anionic channels (Croteau et al. 2016). This could explain the high sensitivity of *T. fluviatilis* compared to the other species tested, since absorption through the integument is less effective in arthropods (Lillywhite & Maderson 1988). The remarkably high tolerance of *S. festiva* to uranium may be related to an accumulation/elimination dynamics, according to toxicodynamic-toxicokinetic models (e.g., Jager et al. 2011). *S. festiva* may have accumulated large amount of uranium in granules until reaching the capacity for storage and then excreted it (Rainbow 2002; Bednarska et al. 2017). Differently of the metals, for other compounds such as hydrophobic organic chemicals lipids are the main compartment of storage, and the loss depends on decay rate and biotransformation of the chemical in the organism (Ashauer et al. 2010). This possibility requires further investigation, since the mechanisms that allow *S. festiva* to survive in such high uranium concentrations are unknown.

Sublethal effects: Growth, consumption and respiration rates

S. festiva grew 47% less than control animals at 25 $\mu g \cdot L^{-1}$ of uranium, without changes in feeding and respiration rates. This suggests that under stressing conditions, *S. festiva* is diverting energy from growth to maintenance (repair of damages at the cellular

and subcellular levels or for accumulation and excretion, as discussed below). Energy uptake can be constant or increased (with no effects on assimilation efficiency), but the energy available for somatic growth can be reduced under metal exposure (e. g. Alonzo et al. 2008; Yang et al. 2015). An alternative explanation for the decreased growth could be a decrease in energy assimilation under uranium exposure, caused by damages in the gut cells (Augustine et al. 2012; Goussen et al. 2015).

In comparable experiments, growth was also reduced in the midge larvae *C. tentans* (> 157 $\mu g \cdot L^{-1}$) and in the cladoceran *C. dubia* (> 1.97 $mg \cdot L^{-1}$) (Kuhne et al. 2002; Muscatello & Liber 2009; Gonçalves et al. 2011). For *S. vittatum* (at 27 $\mu g \cdot L^{-1}$) and *Daphnia magna* (at 25 $\mu g \cdot L^{-1}$) a decreased growth was accompanied by a reduction in feeding or carbon assimilation (Zeman et al. 2008; Massarin et al. 2010; Gonçalves et al. 2011).

In our experiment, consumption of alder leaves was similar to other caddisflies (see Pradhan et al. 2012). Although we did not measure the quality of the leaves (in terms of N, C: N or toughness) offered to invertebrates, their feeding rates were similar in both treatments (with and without uranium), indicating that uranium did not interfere directly on consumption rates at the tested concentration. Since shredders prefer soft leaves from the microbial conditioning over unconditioned leaves (Graça et al. 2001), and that they can avoid leaf-litter metal-enriched (Gonçalves et al. 2011), we expected a decrease in feeding rates given the stressing physiological condition (Amiard-Tricket et al. 2015).

As with feeding, respiration rates may decrease or increase with stressing conditions. Respiration rates increased at uranium exposure (25 $\mu g \cdot L^{-1}$) in daphnids (Zeman et al. 2008), and in shrimps at cadmium exposure (Lerebours et al. 2010; (Chandurvelan et al. 2017). Decreased respiration rates were observed for crustaceans and mussels exposed to several metals, probably due to damages to invertebrates gill epithelia (Chinni et al. 2002; Wu & Chen 2004; Barbieri 2007; Chandurvelan et al. 2012; Zhang et al. 2014; Blewett & Wood 2015). In our experiment, respiration rates of *S. festiva* exposed to sublethal (chronic) uranium concentration remained unchanged.

Pathways of uranium bioaccumulation

In our experiments, leaves used as food by *S. festiva* took up (biosorbed) uranium from the water, as previously reported (Antunes 2012). Adsorption was independent of microbial colonization of leaf discs. Although we did not measure fungal biomass of the

conditioned leaves, other studies reported that incubation of leaf-litter under the same laboratory conditions was enough for the development of fungal assemblages in leaves (Biasi et al. 2017).

At high concentrations, uranium adsorption occurs within a few minutes or hours after leaves fall into contaminated water (Aydin et al. 2012). In our assays, the low concentration allowed a gradual uranium biosorption just after 24 h and over 8-15 days. The desorption was a slower process when leaves were exposed to unpolluted water, probably due to formation of uranyl oxalates complexes that immobilized metallic ions as a result of the biosorption to cell walls of the microbes and plant tissues (Mkandawire et al. 2006; Gadd 2010). This has an important ecological significance: at neutral conditions (pH 6-8) litter can rapidly take up large amount of uranium, rapidly mobilize it from the water to consumers, and release it slowly. Due to this, feeding on contaminated leaf-litter could be a pathway for bioaccumulation and extended exposure to invertebrates.

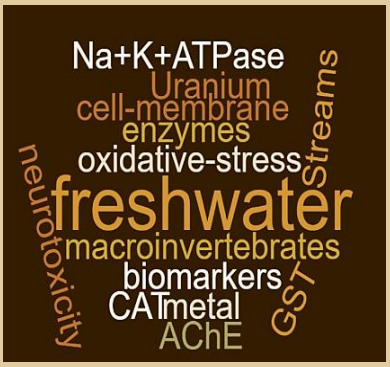
Indeed, we found that uranium accumulation in *S. festiva* resulted from exposure to contaminated food (active uptake) and to contaminated water (passive uptake). The accumulation was in average 35% higher in water than food alone, whereas both pathways in combination resulted in 19% more accumulation than water and 47% more than food exposure only. This demonstrate that in combination both pathways were higher in average than each separately, but the effect on uranium bioaccumulation was less than additive. Under both pathways in combination, *S. festiva* was exposed to greater amount of uranium, which may have reached storage capacity more quickly and started the excretion of the excess of the metal (Rainbow 2002). Uranium from water may have been slowly incorporated into tissues, remaining in the gills or haemolymph (Muscatello & Liber 2010), whereas the metal from the diet may have been rapidly assimilated.

Nevertheless, the importance of direct (water) and indirect (food) pathways can be metal-specific. For instance, Hepp et al. (2017) found that the caddisfly shredder *S. vittatum* accumulated arsenic almost entirely from food ingestion. In contrast, Zubrod et al. (2015) found that gammarids take up copper mainly from the water, presumably by adsorption to the cuticle. We did not investigate where *S. festiva* retains the accumulated uranium for a long period, but desorption experiments with gammarids demonstrated that most of the uranium was fixed to the surface of the body when exposed to contaminated stream water (Schaller et al. 2011). Hepp et al. (2017) demonstrated that although stream invertebrates accumulated arsenic from a polluted stream, non-significant levels were found in emerging

adults, presumably because the metalloid was trapped in the exoskeleton and was eliminated during molting. Uranium and other metals and metalloids may also accumulate in the gills, epithelium and hepatopancreas (Al Kaddissi et al. 2011; Bucher et al. 2016). Finally, here we only dealt with water and food as contamination sources, but we must keep in mind that sediments can also be a source of contamination for stream-dwelling invertebrates (Lagauzère et al. 2009; Franz et al. 2013).

CHAPTER II:

Activities of oxidative stress- and cell membrane- related enzymes in a freshwater leaf-shredder exposed to uranium



CHAPTER II: Activities of oxidative stress- and cell membrane-related enzymes in a freshwater leaf-shredder exposed to uranium

Abstract

Rivers are prone to contamination from agricultural activities, industrial waste and mining, with effects on the biota ranging from the scale of biochemical processes to that of ecosystems. Ongoing climate change requires the replacement of carbon energy sources with alternative energies, and nuclear power is one option. Uranium mining may result in run-off and the contamination of water courses. We investigated the effects of uranium on enzyme activities (acetylcholinesterase (AChE), Na^+K^+-ATPase, glutathione S-transferase (GST) and catalase (CAT)) in a freshwater caddisfly *Schizopelex festiva* exposed to uranium concentrations up to 100 µg·L^{-1}. Enzyme activities and uranium accumulation were determined at 24 h and 32 d of exposure. We also measured growth rates (after 32 d) and calculated bioconcentration factors, as the ratio of uranium in the whole body of the invertebrates to uranium concentration in the test water. Enzyme activity from 24 h to 32 d was reduced for AChE (~52%), GST (~44%) and CAT (78%). No changes were observed for Na^+K^+ATPase activities. Enzyme activities across uranium concentrations were reduced for Na^+K^+ATPase after 32 d in the highest concentration. For GST activities, there were differences in enzyme activities across concentrations, although no differences among concentrations and control were observed for 24 h and 32 d. Activities did not change significantly across concentrations for CAT and AChE. The growth rates of *S. festiva* under laboratory conditions averaged 6.98 ± 0.64 µg·µg $animal^{-1}$ d^{-1}, with no significant differences between treatments. The ratio of uranium in the invertebrate body to concentrations in the water ranged from 211 to 1663, increasing with time and decreasing with the concentration of uranium in the water. The exposure of *S. festiva* to uranium resulted in its accumulation, and triggered changes in the activities of some enzymes, but the species was in general tolerant to realistic values observed in the field. If our findings can be extrapolated to other consumers, then large amounts of uranium would be needed to cause measureable biological changes.

KEYWORDS*:* Streams, metal, freshwater macroinvertebrates, neurotoxicity, oxidative-stress biomarkers, cell membrane enzymes.

Introduction

Uranium occurs naturally in the earth's crust and is used mainly for military purposes and for generating nuclear energy. Mining for uranium may result in runoff to streams and rivers and potentially cause environmental problems. Soluble uranium salts can cross biological membranes, reaching internal organs (Hinck et al. 2010). Uranium can accumulate in the soft (e.g. gills, gonads, kidney) and mineralised (e.g. bones and scales) tissues of fish (Cooley & Klaverkamp 2000).

The small number of studies with aquatic invertebrates exposed to uranium have described sublethal effects on growth (*Chironomus riparius, Daphnia magna*), reproduction (*Ceriodaphnia dubia*), accumulation in gills and internal organs (*Corbicula fluminea, Orconectes limosus*), disorders in enzyme activity (*Procambarus clarkii*, *Calamoceras marsupus*), and downregulation of the mitochondrial genes *cox1* and *sod* (Mn) (*P. clarkii*) (Kuhne et al. 2002; Simon & Garnier-Laplace 2004; Antunes et al. 2007; Muscatello & Liber 2010; Al Kaddissi et al. 2011; Tagliaferro et al. 2018). Uranium also decreased the reproductive output of *Danio rerio* (Simon et al. 2011) and caused subcellular disturbances in hepatic and blood cells of this same species (Barillet et al. 2011).

Metal toxicity depends on the affinity to nucleotides, mainly due to the stability of bonding with phosphate groups. When uranyl ions bind to adenosine 5'-triphosphate (ATP-UO_2^{2+} complex), they will compete with calcium and magnesium ions, causing intracellular imbalances and compromising ATPase activity and ATP production (De Stefano et al. 2005). Another pathway for uranium toxicity is through oxyhaemoglobin, when uranium joins the heme group, interfering with oxygen bonding (Kumar et al. 2016).

A biomarker is defined as a measurable sublethal change at the cellular, biochemical, physiological or behavioural levels, resulting from the exposure of organisms to xenobiotics (Hyne & Maher 2003). Biomarkers are early signals of stressors because they precede visible effects such as slowed growth and/or reproduction, and death (Colin et al. 2015). They are related to the impairment of individual fitness, such as swimming

behaviour, cognitive aspects, growth, larval emergence in insects, and adult size (Luís & Guilhermino 2012; Ren et al. 2015; Rodrigues et al. 2015). Biomarkers that are widely used to assess stress induced by metal exposure include the activities of the enzymes catalase (CAT), glutathione S-transferase (GST), acetylcholinesterase (AChE), and Na^+K^+-ATPase (Vieira et al. 2009; Geng et al. 2012).

Despite the energy and evolutionary advantages of aerobic metabolisms to living organisms, metabolic products, known as reactive oxygen species (ROS), are potentially cytotoxic at high levels. Superoxide dismutases (SOD) convert superoxide anions ($-O_2^{\cdot -2}$) into hydrogen peroxides (H_2O_2), which are detoxified into water and molecular oxygen by catalase or peroxidases (DeJong et al. 2007). Hydrogen peroxides can become highly reactive hydroxyl radicals (OH) when they react with reduced metal ions (DeJong et al. 2007). Catalase is thus involved in cell antioxidant defences, reducing ROS levels (Jemec et al. 2010). Glutathione S-transferase (GST) plays an important role in detoxification. GST catalyses the conjugation of glutathione (GSH) with xenobiotics through the bonding of electrophilic nuclei with the –SH group of glutathione, producing less toxic and more water-soluble compounds that are easier to remove from cells (Habig et al. 1974; Lee et al. 1988). Changes in GSTs activity have been identified in insects exposed to metals and to insecticides (Xu et al. 2015; Vojoudi et al. 2017). In shrimp, metals inhibited GST activity by changing the binding site of GSH (Salazar-Medina et al. 2010).

Na^+K^+-ATPase (sodium potassium pump) is a P-type ATPase required to maintain potential electrochemical differences in cells through the movement of Na^+ and K^+ across cell membranes, and is essential for cell signaling and secondary active transport (Nelson & Cox 2004). Na^+K^+-ATPase can be activated at low concentrations of metals and inhibited at higher concentrations (Watson & Benson 1987; de la Torre et al. 2007; Mosher et al. 2010).

Acetylcholinesterase (AChE) is a serine enzyme hydrolysing the neurotransmitter acetylcholine. Contaminants may react with serine at the catalytic site, disabling the AChE function, which results in the accumulation of acetylcholine in the synapse (Domingues et al. 2007). In insects, the post-synaptic potential continues to be stimulated even when AChE is inhibited, causing unusual behaviours, changes in feeding rates, larval emergence, and eventually death (Miao et al. 2016).

In a previous study, we found that uranium is moderately toxic to the stream-dwelling *Theodoxus fluviatilis* (Gastropoda) and *Proasellus* sp. (Isopoda) (LC_{50} 24 and 142

mg·L^{-1}), while the trichopteran *S. festiva* was able to survive at concentrations as high as 262 mg·L^{-1}, but concentrations of 0.025 mg·L^{-1} inhibited growth (Bergmann et al. 2018). We also found that uranium under realistic environmental concentrations, as observed in polluted mining sites (50 µg·L^{-1}), caused a decrease in Na^+K^+-ATPase activity in the caddisfly shredder *C. marsupus* (Tagliaferro et al. 2018). We have continued to investigate uranium effects on stream consumers, searching for changes in a set of biomarkers (AChE, Na^+K^+-ATPase, GST, CAT) and in growth. We used the freshwater caddisfly shredder *S. festiva* Rambur (Trichoptera, Sericostomatidae) as a test organism. This species occurs in high abundances and is functionally important in the energy transference from litter to the food web. We predicted that key enzyme activities would be affected by uranium exposure at concentrations below those causing a decrease in growth.

Material and Methods

Invertebrates and leaf-litter conditioning

Specimens of the shredder *S. festiva* were sampled from a reference stream (Múceres, central Portugal; 40°32'01'' N; 008°09'15'' W; pH 6.89, [U] in water: 0.35 µg·L^{-1}, [U] in sediments: 1.0 mg kg^{-1}, n = 3). Caddisflies were collected with a 500 µm-mesh net and transported to the laboratory in an insulated box with stream water. They were acclimated for five days in the laboratory, in aerated water in 3-L boxes at 18 ± 1 °C, 14-h light: 10-h dark. Synthetic moderately hard water was reconstituted according to USEPA protocol (Lewis et al. 1994) and final concentrations of 96 mg·L^{-1} $NaHCO_3$, 60 mg·L^{-1} $MgSO_4$, 60 mg·L^{-1} $CaSO_4$. $2H_2O$ and 4 mg·L^{-1} KCl.

Alder leaves [*Alnus glutinosa* (L.) Gaertn.] were used to feed *S. festiva*. Freshly fallen senescent leaves were collected from a single tree stand in the Mondego River Park (Coimbra, Portugal) in autumn, air-dried and stored until use. Each week, batches of 10 leaves were exposed to a mixture of stream water and leaf-litter from a reference stream (Múceres, central Portugal), in an aquarium with strong aeration to allow fungal colonisation. According to previous studies, the incubation of leaves for one week with stream water and litter is sufficient to allow microbial colonization under laboratory conditions (Biasi et al. 2017).

Experimental design

We investigated the effects of uranium on the activities of four enzymes, AChE, Na^+K^+-ATPase, GST, and CAT, and on the growth rates of *S. festiva* for 32 days at six uranium concentrations (0, 6.25, 12.50, 25, 50 and 100 $\mu g \cdot L^{-1}$). These concentrations were selected based on uranium concentrations found in a previous survey at 213 stream sites in the vicinity of abandoned mines in Portugal (< 10 $\mu g \cdot L^{-1}$), measurable effects in growth of *S. festiva* (Bergmann et al. 2018) and on biomarkers in aquatic organisms (Labrot et al. 1996). The activity of the enzymes and uranium uptake were measured at the end of 24 h and 32 d of exposure. Uranyl nitrate ($N_2O_8U.6H_2O$) (Panreac Química SL, Spain) was used as the uranium source. Test solutions were prepared by diluting a stock solution (1000 $mg \cdot L^{-1}$) to the final nominal concentrations listed above.

Before they were offered to the caddisflies, conditioned leaves were further incubated for 48 h at the respective uranium concentrations used in the test. Organisms with similar weight (3.30 ± 0.05 mg; see below) were allocated to containers 5 cm high × 9 cm wide × 13 cm long, with 250 mL of test solution and a thin layer of autoclaved stream sand. We prepared 18 boxes for each time (24 h and 32 d), each containing 12 specimens, totalling 36 containers and 432 specimens. At the end of 24 h and 32 d, three replicate boxes from each concentration were removed and 8 individuals were used for biomarker determinations and 4 for uranium analysis (Figure 1). The solution test and food were changed every two days for the group of 32 d. The pH, electrical conductivity, and dissolved oxygen were measured weekly (N = 5 per concentration) with a multi-parameter probe (340i/SET), WTW GmbH, Germany.

Growth rates

We estimated the growth rates of *S. festiva* exposed to increased concentrations of uranium as the difference between the final (DMf) and initial mass (DMi) divided by the mean mass (DMm) and the elapsed time in days (t) (Tagliaferro et al. 2018).

$$GR = \frac{\mathrm{DMf} - \mathrm{DMi}}{\mathrm{DMm\ x\ t}}$$

Caddisfly mass was estimated from a regression between the case-opening diameter (CO, in mm; measured under a stereoscopic microscope Leica M80, LAS software) and dry mass (DM, in mg). The equation was obtained from a set of 38 individuals, which were measured, uncased, dried (60 °C, 72 h) and weighed (DM = 0.0032 × CO –0.0044, n = 38, $r^2 = 0.98$, $p < 0.001$).

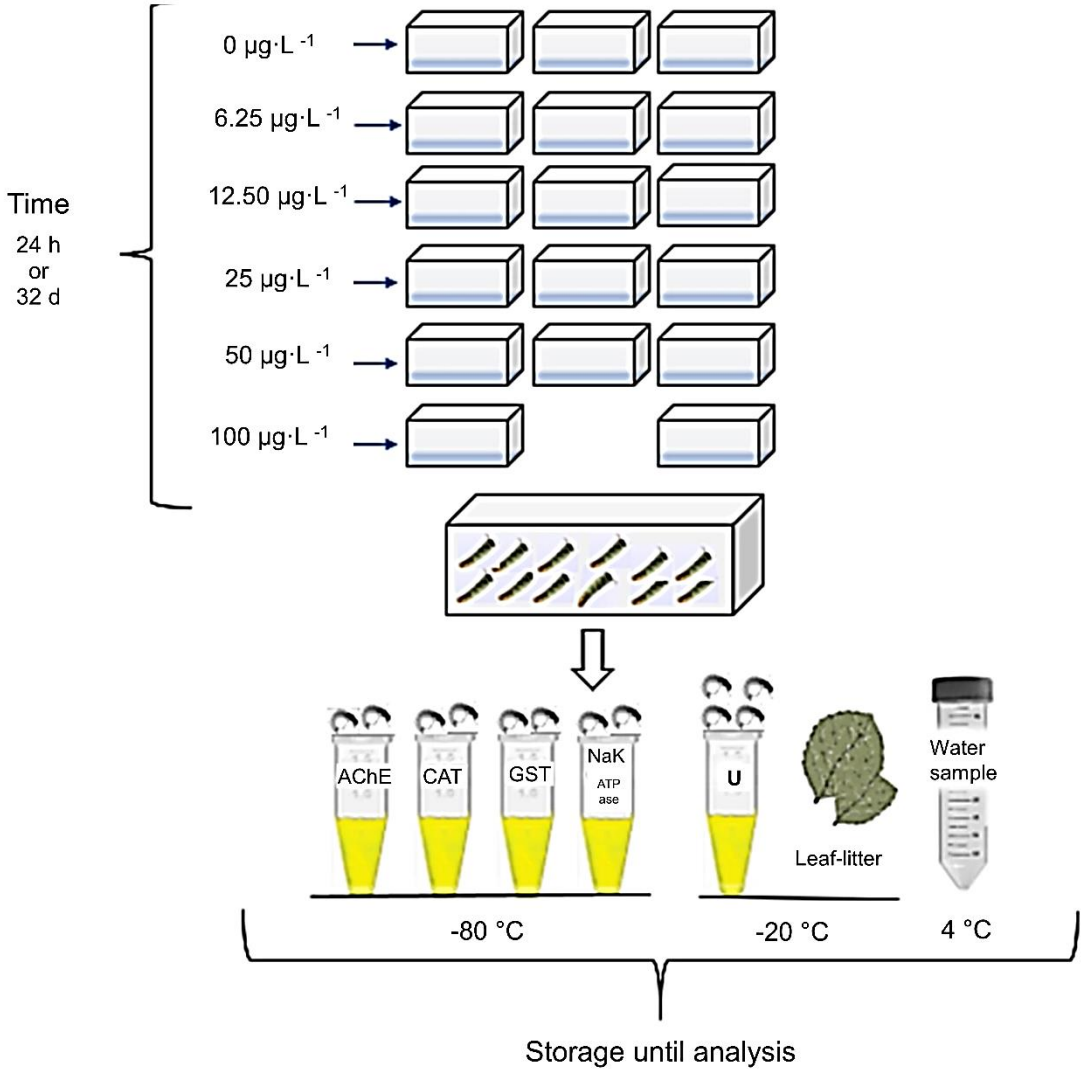


Figure 1. Experimental design for biomarkers and uranium determinations in 24 h and 32 d.

Determination of uranium and bioconcentration factors

Uranium in the water, leaves, and in the caddisflies (at 24 h and 32 d exposure; n = 36) was measured by fluorometry (λ = 530 nm). Leaves and caddisflies were stored at -20

°C until analysed. Leaves and invertebrates were oven-dried (45 °C, 72 h), weighed and ignited (450 °C, 8 h) to determine ash-free dry mass (AFDM). We added 8 mL of 2.5 M nitric acid (in 50-mL test tubes) to the ash in a boiling-water bath for 1 h, followed by the addition of 10 mL ethyl acetate after cooling and ignition of the inorganic solvent. The residue was dissolved in 7 mL of 0.005% nitric acid and fluorescence was compared with the standard curve (2, 10, 100, and 1000 $\mu g \cdot L^{-1}$), according to Van Loon & Barefoot (1989). We also calculated bioconcentration factors (BCF) for each concentration tested, as the ratio of uranium in the whole body of the invertebrates (mg kg^{-1} dry weight) to uranium concentration in the test water ($mg \cdot L^{-1}$) (Miller et al. 2016).

Enzyme activities

For enzyme activity assays, caddisflies were uncased and manually homogenised with a pestle in microtubes with 180 µL of specific buffer solutions (2 organisms/tube) for each enzyme (see below), and centrifuged at 5000 rpm for 15 min at 4 °C. The supernatant was removed and stored at –80 °C until further analysis. For AChE and GST determinations, 100 mM phosphate buffer was used at pH 7.2 and 6.5, respectively. Catalase buffer solution was 50 mM phosphate with 10% Triton X-100 (final concentration 1%; pH = 7.0), and for Na^+K^+-ATPase buffer was sucrose 320 mM Hepes-Tris 10 mM (pH = 7.4). The pH was adjusted with solutions of 0.1 M sodium hydroxide (NaOH) or 37% hydrochloric acid (HCl).

Prior to sample preparation for biochemical determinations, the supernatant was diluted to a concentration of 1.0 $mg \cdot L^{-1}$ of protein with the corresponding (above) buffer solution. Protein was quantified by the Bradford method (according to Elumalai et al. 2007) using bovine γ-globulin as standard. We used three biological replicates per treatment and three technical replicates for each. 500-µL microcuvettes were used for enzyme determinations.

AChE activity was determined using the colorimetric method described by Ellman et al. (1961), using acetylthiocholine iodide as substrate and dithiobisnitrobenzoic acid (DTNB) as reagent. The yellow product of the hydrolysis of acetylthiocholine iodide ($\varepsilon = 1.36 \times 10^4\ M^{-1}cm^{-1}$) was measured at 412 nm during the first 10 and 15 min of reaction. The solution for enzyme determination consisted of 75 µL of sample and 375 µL of reaction

solution (1 mL DTNB 10 mM, 30 mL phosphate buffer 100 mM, 0.2 mL acetylcholine 0.075 M). Activity was expressed as nmol acetylthiocholine min^{-1} mg^{-1} protein.

For Na^+K^+-ATPase, 50 µL of sample was mixed with 447.50 µL of reaction solution (128 mM NaCl, 5 mM KCl, 3 mM MgCl, 0.1 mM EGTA, 10 mM HEPES-Na, pH 7.4). To start the enzyme reaction, 7.5 µL of 200 mM ATP-Mg was added to each sample and kept in a water bath for 5 min at 35 °C. The reaction was interrupted with 125 µL of 20% trichloroacetic acid (TCA) and the tubes transferred to ice. Absorbance was measured at 660 nm and compared with the calibration curve of inorganic phosphate, obtained by the reaction of 50 mM phosphate buffer with Fe-molybdate to estimate the quantity of inorganic phosphate (Holman 1943). Activity was expressed as nmol Pi min^{-1} mg $protein^{-1}$.

Glutathione-S-transferase was determined using CDNB (1-chloro-2,4-dinitrobenzene) as a substrate that conjugates with glutathione (GSH), forming a thioether that was measured at 340 nm (Habig et al. 1974). The reaction solution consisted of 156 µL of 1 mM CDNB, 900 µL of 1 mM GSH, and 4,950 µL of 100 mM phosphate buffer. A mixture of 167 µL of the homogenate and 333 µL of reaction solution was used for the spectrophotometric readings. The absorbance increase was measured for 5 min, and the enzyme activity was corrected using the molar extinction coefficient for GSH-CDNB conjugate ($\varepsilon = 9.6$ mM^{-1} cm^{-1}). The activity was expressed as nmol GSH conjugated min^{-1} mg^{-1} protein. Catalase activity was measured by reacting 333 µL of the sample with 167 µL of 0.1% H_2O_2 (30% H_2O_2 in 50 mM of phosphate buffer). The absorbance was measured at 240 nm ($\varepsilon 240 = 40$ mM^{-1} cm^{-1}) (Peric et al. 2017) for 2 min every 30 s. Activity was expressed as nmol min^{-1} mg^{-1} protein.

Data analysis

Enzyme activities were analysed between 24 h and 32 d by paired t-tests on log transformed data (AChE) or absolute values. Enzyme activities across uranium concentrations were analysed individually by one-way ANOVA or Rank ANOVA followed by a Holm-Sidak post-test for multiple comparison with control (no uranium). The relationship between the uranium content in the caddisflies, water and leaves was investigated using linear regression. Differences in growth rates among the uranium treatments were determined by one-way ANOVA. The relationship between uranium

concentration in water and the uranium concentration in caddisflies and leaves was assessed by simple linear regression analysis. The analyses were performed using Statistica Statsoft 7.0 and Sigma Stat 3.5 software.

Results

Growth rates of *S. festiva* and uranium uptake

The mean pH was 7.75 ± 0.21, electrical conductivity was 299 ± 2.44 µS/cm, and dissolved-oxygen content was 7.56 ± 0.85 mg·L^{-1} (mean ± SD) across all concentrations tested. The uranium content in the water after 2 d in contact with sediment, leaves and animals was lower than nominal concentrations (Table 1). In contrast, leaves and caddisflies had higher uranium content than the water, mainly at the lower and medium uranium concentrations. The bioconcentration factors (BCF) for caddisflies increased from 24 h to 32 d, and were highest in *S. festiva* exposed to the lowest uranium concentrations (Table 1). The uranium content in the caddisflies bodies and in leaves was strongly related to the uranium concentrations in the water (caddisflies: $F_{(1,5)} = 37.26$, $p < 0.01$, $r^2 = 0.96$; leaves: $F_{(1,5)} = 454.38$, $p < 0.001$, $r^2 = 0.97$). Uranium concentrations in the caddisflies were also related to uranium content in the leaves: $F_{(1,5)} = 43.91$, $p < 0.01$, $r^2 = 0.91$ (Figure 2). After the 32 d of exposure, all specimens combined had grown 6.98 ± 0.64 µg·µg $animal^{-1}$ d^{-1} (mean ± SE). Growth rates did not differ between treatments (ANOVA, F = 0.59; df = 5, 154; p = 0.70).

Table 1. Uranium concentrations in water, caddisflies, and leaves used to feed the animals during the biomarker experiment, and bioconcentration factors (BCFs) for the five uranium concentrations; values calculated from 24 h and 32 d (mean ± SE). *Value below of the chronic Predicted No Effect Concentration (PNEC) (0.3 µg·L^{-1}; INERIS 2008).

	Nominal uranium concentrations in water (µg·L^{-1})					
	0	6.25	12.50	25	50	100
Water-test (µg·L^{-1})	0.27 ± 0.03*	3.88 ± 0.28	9.73 ± 0.75	17.97 ± 0.80	34.29 ± 2.60	70.45 ± 2.83
Caddisflies (µg·g^{-1})	1.32 ± 0.18	5.27 ± 1.14	5.74 ± 0.72	9.71 ± 3.75	16.68 ± 5.18	24.35 ± 7.74
Leaves (µg·g^{-1})	0.80 ± 0.12	9.65 ± 2.63	15.98 ± 0.17	17.43 ± 0.23	30.9 ± 3.31	84.51 ± 2.16
BCF (24 h)	NC	1143 ± 57	558 ± 41	386 ± 8.6	311 ± 28	211 ± 18
BCF (32 d)	NC	1663 ± 159	875 ± 70	728 ± 49	445 ± 50	291 ± 12

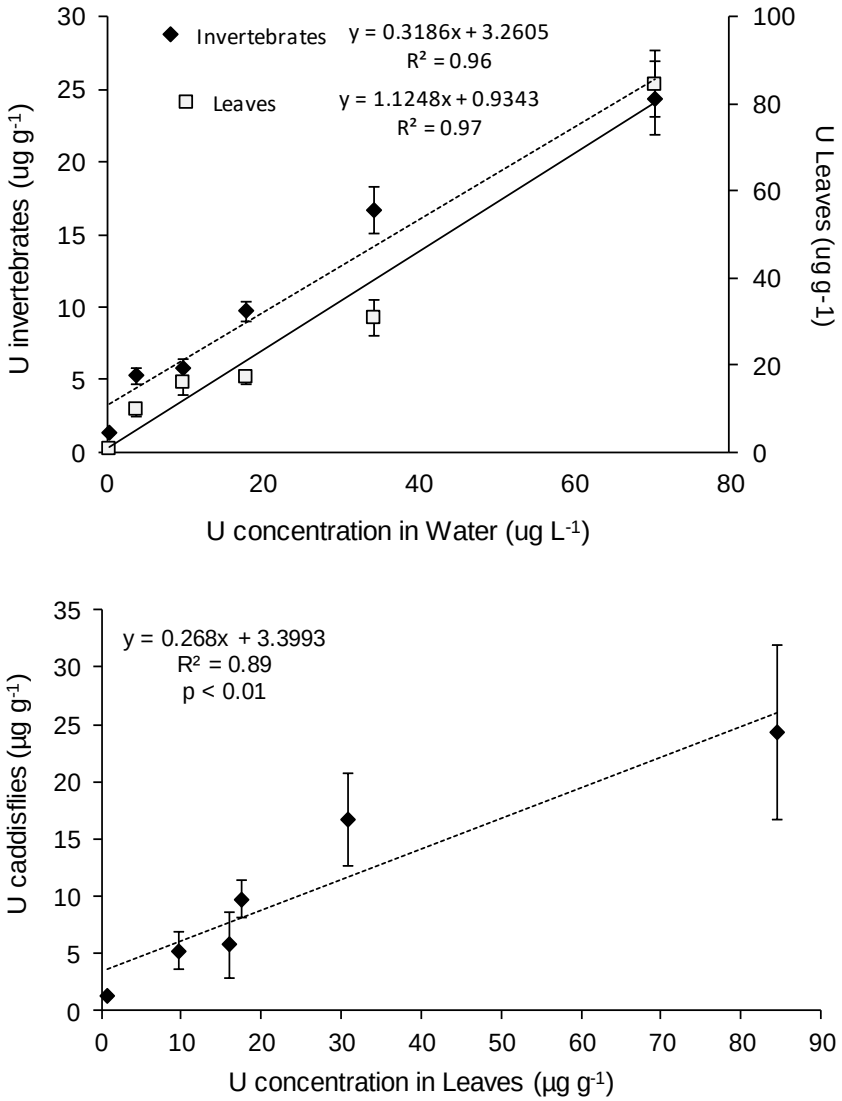


Figure 2. Relationships of accumulated uranium in caddisflies, leaves and water in 24 h and 32 d of exposure to the metal (mean ± SE).

Enzyme activities

Enzyme activities were reduced after 32 d under laboratory conditions for CAT (t = 3.606, df = 5, p = 0.015), GST (t = 6.770, df = 5, p = 0.001) and AChE (t = 5.228, df = 5, p = 0.003). The activity did not change for Na^+K^+ATPase (t = 0.0692, df = 5, p = 0.948). Na^+K^+ATPase activity was reduced only in 100 µg·L^{-1} of uranium (F = 4.772, p = 0.012) by Day 32. GST activity differed across uranium concentrations (F = 3.321, p = 0.041), although there were no differences between the control and any of the testing

concentrations. CAT and AChE activities did not change significantly across concentrations (Figure3).

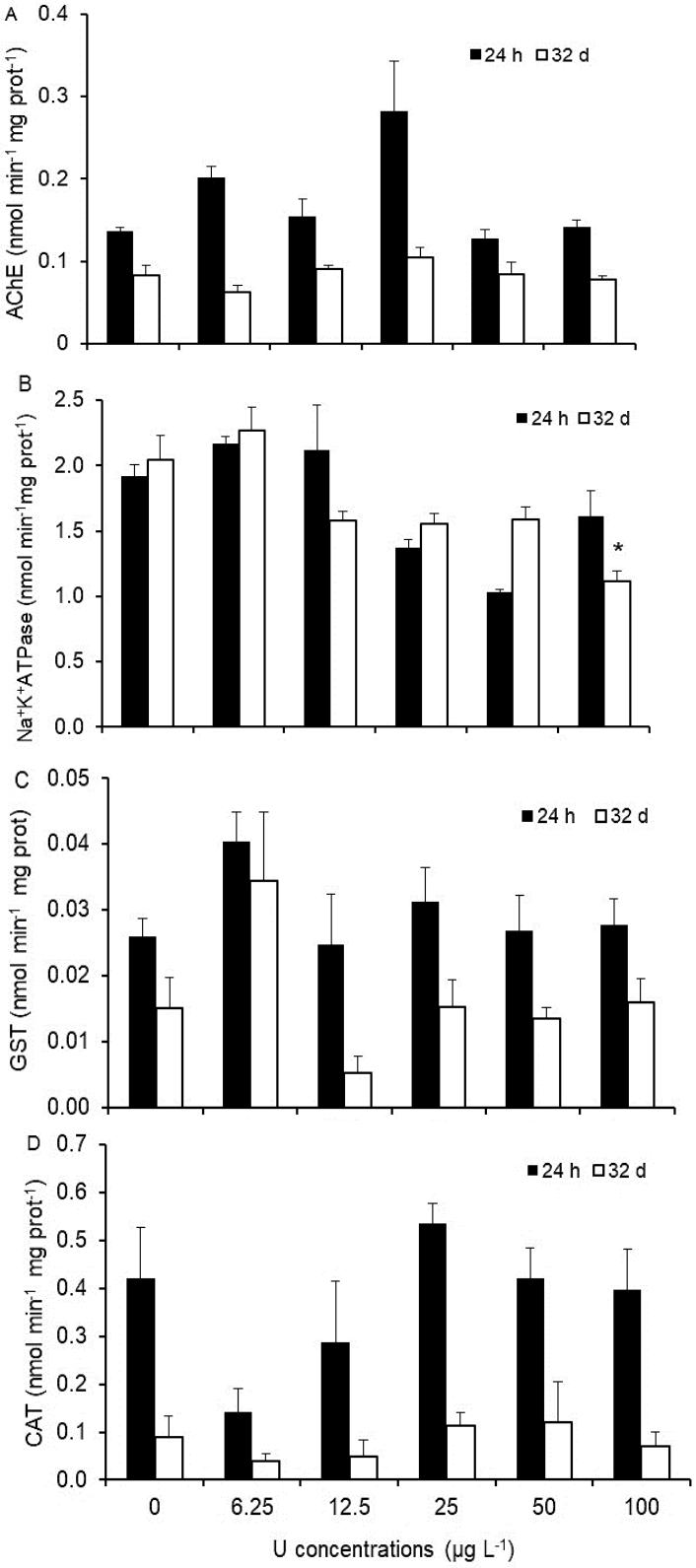


Figure 3. AChE (A), Na^+K^+-ATPase (B), GST (C), and catalase (D), activities after 24 h (dark bar) and 32 d (light bar) exposure of *Schizopelex festiva* to uranium (mean ± SE). * = $p < 0.05$ against control (0 $\mu g \cdot L^{-1}$).

Discussion

Biomarkers have been used as a convenient tool to evaluate physiological disturbances, in order to extrapolate the factors causing ecological imbalances of populations or higher levels (Lagadic et al. 1994), but studies on freshwater macroinvertebrates are relatively uncommon. Leaf-shredding freshwater invertebrates are ecologically important organisms in streams, since they contribute to energy transfer from leaf-litter to higher trophic levels. Factors affecting the performance of shredders may therefore potentially affect the carbon cycle at local scales. The effects of pollutants on invertebrate biomarker activity can vary according to the development stage, tissues or organs of invertebrates, and the form of enzyme analysed (Hyne & Maher 2003).

Here, we found that *S. festiva* grew equally well in both control and uranium-contaminated water (up to 100 $\mu g \cdot L^{-1}$). These findings contrast with previous experiments in which growth was reduced by 47% in specimens exposed to 25 $\mu g \cdot L^{-1}$ (Bergmann et al. 2018). There are several possible reasons for the contrasting results. First, we reared specimens in groups, and although food was provided *ad libitum*, we should not underestimate the potential interactions among consumers, with costs in the food ingested. Secondly, while our experimental specimens weighed 3.30 ± 0.05 mg, in the previous experiment the specimens weighed 5.73 ± 0.24 mg. Feeding and growth rates are related to the individual mass, and the total mass gained per day may increase with the individual mass, but mass gained per organism body mass may decrease with size (González & Graça 2003; Feio & Graça 2000). Third, differences in body size can influence metal accumulation and sensitivity to contaminants (Muscatello & Liber 2009; Cid et al. 2010; Ruppert et al. 2016); shredders generally shift their diet from fine (early instars) to coarse detritus (late instars) (Basaguren et al. 2002), which may affect metal ingestion, stress conditions, and therefore growth. Fourth, the degree of leaf conditioning could also have differed in the two experiments, affecting the feeding rates (e.g., Graça et al. 2001). Fifth and finally, while in the previous experiment the specimens were collected in February (winter), here we used specimens collected in October (autumn), and they may have been in a different physiological stage, affecting growth under stress conditions.

Whatever the source of variation, the growth rates (11.60 $\mu g \cdot mg\ animal^{-1}\ d^{-1}$) were in average lower here than those reported in previous experiment (Bergmann et al. 2018).

These growth rates are within the range reported for other stream shredders, however, 0.5 to 56 µg mg animal^{-1} d^{-1} (Azevedo-Pereira et al. 2006; Pradhan et al. 2012; Balibrea et al. 2017). Impaired growth under uranium exposure has been observed in other freshwater invertebrates: *C. dubia* (Kuhne et al. 2002), *Chironomus tentans* (Muscatello & Liber 2009), and *S. vittatum* (Gonçalves et al. 2011).

In our experiments, uranium did not affect growth, but *S. festiva* specimens accumulated uranium according to the concentration in both the water and the food (leaf-litter). We evaluated the potential for the uranium concentration in *S. festiva* to exceed the concentration in the water, using bioconcentration factors (BCF), a simple model that estimates partitioning between the environment and the organism. Usually, higher BCF values are related to higher toxicity levels, because this model was developed based on Fick's Law for neutral organic toxicants that diffuse through the membranes (Arnot & Gobas 2006). However, metals are subject to ion-charge interactions with biomembranes and specific transport mechanisms (uptake and elimination). In our experiment, BCF values were highest at the lowest uranium concentration: uranium accumulates more rapidly at low concentrations. BCF may therefore not be a good indicator of metal toxicity because of its inverse relationship with the metal concentration in the solution (higher BCF values at lower exposure concentration) (McGeer et al. 2003; Regoli et al. 2012). Moreover, most of the uranium uptake (up to 90%) occurred in the first 24 h of exposure. Uranium uptake from the solution could be rapidly incorporated into insect haemolymph (Muscatello & Liber 2010) and stored in a detoxified and insoluble form in tissues, permanently or until the uranium is excreted (Rainbow 2002; Barillet et al. 2011).

The reduction of uranium in the test solution after 2 days of exposure may be due to adsorption to (1) the test vessel, (2) leaf-litter, (3) *S. festiva* body (~11–93% higher than in water), (4) their cases (~22%), or some combination of these. Uranium in leaves was proportional to the uranium content in the water; in contrast, less uranium was accumulated in *S. festiva* than in leaves. This difference can be explained by the ability of the fungi to biosorption of uranium to polysaccharides, proteins, and lipids from their cell walls (He & Chen 2014), while the caddisflies use physiological mechanisms, such as glutathione S-transferase, to sequester and eliminate metals (Merritt & Bewick 2017).

Some enzyme activities were affected by uranium in our experiment. Although we observed no change in *S. festiva* growth within the range of tested concentrations, we found changes in Na^+K^+-ATPase and GST activities. Na^+K^+-ATPase activity decreased at 100

µg·L^{-1} in relation to control in 32 d. In similar experiments with the shredder caddis *C. marsupus*, Na^+K^+-ATPase activity was also reduced at 50 µg·L^{-1} (Tagliaferro et al. 2018). Cell membranes are the first cell defensive barrier for control of entrance and exit of substances; the membranes depend on the functional integrity of ATPases to maintain active transport of molecular compounds (Jorgensen et al. 2003). The use of Na^+K^+-ATPase as an early indicator of uranium toxicity should be further explored as a biomarker for freshwater invertebrates (Jorgensen et al. 2003). Uranyl nitrate functions as an inhibitor of both Na^+K^+-ATPase and Mg^{2+}-ATPase activities, and binds to the Na^+ site on the enzyme (Nechay et al. 1980), blocking K^+ transport across the cell membrane (efflux). Inhibition of Na^+K^+-ATPase was also reported for lixiviates from uranium-mill tailings (Geng et al. 2012; Xie et al. 2016). The activity of this enzyme in the gills and kidney of fish increased in the presence of metals such as Cu and Cd (Canli et al. 2016), probably due to upregulation to restore Na^+ reabsorption and osmolality (Moyson et al. 2016).

GST was sensitive to uranium in our experiment, but the variability was high. Similar reports of GST variability across series of metal concentrations were described for the common goby *Pomatoschistus microps* exposed to Hg and the mussel *Ruditapes decussatus* exposed to Ag nanoparticles (Vieira et al. 2009; Hidouri et al. 2017). It is plausible that these responses could be due to different GST isoforms, which can be increased or decreased as a tissue-specific function at different metal concentrations and exposure times (Espinoza et al. 2012; Vidal-Liñán et al. 2016). A still greater effect on GST activity was expected, because the enzymes involved in NADH production are potential targets for uranium (Eb-Levadoux et al. 2017). Beside GST, other GSH associated enzymes such as GSH reductase, GSH peroxidases (GPx) and other ROS-scavenging enzymes (superoxide dismutase-SOD, catalase) cumulatively protect aquatic organisms from ROS (Srikanth et al. 2013). Uranium induced increase in GSH activities after 12.5 to 50 µM (2 mg·L^{-1}) in *Arabidopsis thaliana* but not at 75 µM, probably due to a reduction in biosynthesis of GSH. Overall, uranium induces global decreases in total glutathione content (due to conversion of GSH into its oxidized form – GSSG) showing an impairment in the intracellular redox state (Barillet et al. 2011; Srikanth et al. 2013).

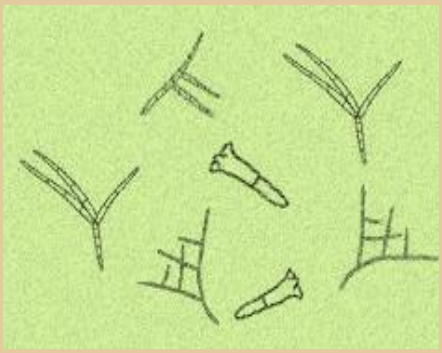
Although AChE activity did not change with uranium increasing concentrations, it was reduced by ~40 to ~70% after 32 d of exposure for all uranium concentrations. These differences between 24 h and 32 d can be related to the normalization of the enzyme as a

compensatory response after short exposure to metals (Richetti et al. 2011). Uranium also did not cause significant alterations in cholinesterases activities of the exposed invertebrates *C. fluminea* and *D. magna* (Nunes et al. 2017). Variations in time and levels of exposure to Hg^{2+} and Pb^{2+} were observed for AChE in zebrafish, with a recovery of the activity after chronic exposure for 30 days (Richetti et al. 2011). Under metal pollution, other studies have reported a decrease (Oliveira et al. 2016; Peric et al. 2017) (Al, Fe, Cu, Zn) or increase in AChE activities (Kalantzi et al. 2016) (Cu, Cd, Pb). Barillet et al. (2011) found a ~30% decrease in AChE activity in the brain tissue of *D. rerio* after 3 days of uranium exposure, an increase after 5 days, followed by a decrease by the 10^{th} day of exposure. Although we used organisms of similar size in our experiments, individual differences could explain the high variability observed in some treatments. Further experiments with organisms in different larval stages or physiological status are needed to better understand how uranium affects AChE activity.

Catalase activity was not sensitive to uranium exposure, but its activity was reduced by ~ 80% from 24 h to 32 d. Decrease in catalase activities were observed for zebrafish until five days (20 - 500 $\mu g \cdot L^{-1}$), but no significant effects were recorded after 10 days whatever uranium concentration in water (Barillet et al. 2011). A similar result was reported for the caddis shredder *C. marsupus* (Tagliaferro et al. 2018) and tadpoles (Marques et al. 2013). This was surprising, because uranium is able to induce structural damages in contaminated organs, leading to a decline in the number of mitochondria and consequently activation of mitochondrial antioxidant defenses (Al Kaddissi et al. 2011, 2012). Cells can adopt compensatory mechanisms, with an increase in the number of mitochondria when exposed to uranium (Al Kaddissi et al. 2012). However, catalase and other enzyme activities involved in antioxidant defenses can decrease with time in uranium exposure, perhaps due to interference in the levels of antioxidants. For aquatic macrophytes, CAT activity decreased at 20 and 100 $mg \cdot L^{-1}$ of uranium (Srivastava et al. 2010). These examples show that catalase is sensitive to uranium. However, because our biological samples came from whole-organism homogenates and not from individual tissues, catalase, as well GST and AChE sensitivity to uranium may have been attenuated.

CHAPTER III

Uranium affects growth, sporulation, biomass and leaf-litter decomposition by aquatic hyphomycetes



CHAPTER III: Uranium affects growth, sporulation, biomass and leaf-litter decomposition by aquatic hyphomycetes

Abstract

Contamination by uranium mining activity may lead to harmful effects on freshwater biota, and can affect the reproduction, activity and diversity of aquatic fungi. Here we investigated uranium inhibition on fungal growth in solid medium, using (1) four species of aquatic hyphomycetes and (2) six strains of *Heliscus lugdunensis*. We also measured (3) fungal sporulation, (4) fungal biomass and (5) leaf-litter decomposition in laboratory microcosms exposed to uranium. The uranium concentration causing 50% growth inhibition (EC_{50}) ranged from 12.5 to 45 $mg \cdot L^{-1}$, with *Articulospora tetracladia* the most sensitive and *Varicosporium elodeae* the most tolerant species. Strains sampled from reference and uranium polluted waters differed in their tolerance, but the tolerance was independent of the uranium concentration in the streams where fungi were isolated. The EC_{50} for the six strains ranged from 9 to 25 $mg \cdot L^{-1}$. Sporulation was inhibited in microcosms at uranium concentrations $\geq$ 1 $mg \cdot L^{-1}$, and the minimum concentration inhibiting litter decomposition and biomass standing crop over 24 days was 16 $mg \cdot L^{-1}$. Leaf-litter exposed to uranium accumulated the metal up to 89 $mg\ kg^{-1}$ (in 262 $mg \cdot L^{-1}$). Overall, the amount of uranium in many streams receiving discharges from abandoned or recovered mining sites is high enough to impair the fitness of some aquatic hyphomycete species.

KEYWORDS*:* metal pollution tolerance; fungal reproduction; mining effects

Introduction

Uranium is naturally found on Earth mainly as uranium oxides UO_2, U_2O_5. Mining may release uranium and other metals in soil and waters, potentially affecting the aquatic biota. Uranium mining in Portugal was an important activity until the end of the 1990s and there are nowadays ~60 abandoned uranium mines. Although many mines were subjected to requalification, which significantly reduced the uranium leaching into freshwaters, there are still low uranium contamination levels in these sites (Pereira et al. 2014).

Given the high energy demands for a growing human population and the need to cut C emission to meet the Paris Agreement goals (United Nations 2015), it is likely that mining for uranium will increase worldwide in the upcoming decades. This may result in water contamination from uranium and deteriorations in environmental quality as suggested by evidence from field and laboratory studies. For instance, stream dwelling invertebrates exposed to uranium had a decreased growth (Muscatello & Liber 2009; Bergmann et al. 2018), food ingestion (Gonçalves et al. 2011) and altered fundamental enzymatic activities (Hyne et al. 1993; Tagliaferro et al. 2018).

Although information on uranium toxicity for aquatic invertebrates is available, comparatively little is known about its effects on aquatic hyphomycetes, despite their functional key role in streams. Aquatic hyphomycetes colonise and decompose plant debris playing an important role in the energy transfer from dead organic matter to higher trophic levels in streams (Bärlocher 1992; Maharning & Bärlocher 1996). They also improve the nutrient content and palatability of the leaves for consumers (Chung & Suberkropp 2009; Suberkropp & Arsuffi 1984).

Some species of aquatic hyphomycetes can tolerate and are able to sporulate in metal polluted streams (Krauss et al. 2001). However, metal exposure may depress sporulation at concentrations below the threshold causing reductions in fungal biomass and their functional role in litter decomposition (Duarte et al. 2004; Medeiros et al. 2008, 2010). Processes such as the complexation of –SH compounds with metals, biosorption, bioaccumulation, precipitation and biomineralisation or genetic adaptation may be involved fungal resistance to metals (Baldrian & Gabriel 2002; Braha et al. 2007; Fomina et al. 2007; Miersch et al. 1997).

Intraspecific differences in metal tolerance have been reported. Such differences in strains isolated from polluted or non-polluted streams include strain-specific accumulation and conidia size variations (Braha et al. 2007; Pradhan et al. 2014, 2015). The adaptation of the strains to contaminants can allow fungal assemblages to retain the capability to perform their functional role in litter decomposition, but fungal metal biosorption may decrease leaf-litter quality for consumers (Ferreira et al. 2010; Gonçalves et al. 2011).

Here, we investigated the tolerance of aquatic hyphomycetes to uranium and asked three questions: (a) Do aquatic hyphomycetes differ in their tolerance to uranium? (2) Are

there intraspecific differences in aquatic hyphomycete species in their tolerance to uranium pollution? and (3) Which biological/functional fungal parameters are more sensitive to uranium? To address these questions, we performed assays measuring increased uranium concentration effects on fungal growth, sporulation, biomass and litter decomposition. We used four species of aquatic hyphomycetes sampled from uncontaminated stream and six strains of *Heliscus lugdunensis* sampled from the reference and uranium contaminated streams.

Material and Methods

Fungi isolation

We used four aquatic hyphomycete species isolated from reference streams in Central Portugal (Múceres N 40° 32' 01"; W 08° 09' 15", pH 6.9, 0.35 $\mu g \cdot L^{-1}$ of uranium in water and 1.0 mg kg^{-1} in sediments) as testing organisms. The isolated species were *Articulospora tetracladia* Ingold, *Tricladia splendens* Ingold, *Varicosporium elodeae* Kegel, *Heliscus lugdunensis* Saac. We also used six strains of an ubiquitous aquatic hyphomycete *H. lugdunensis* Sacc, five of which were sampled from uranium contaminated sites in Central Portugal (courtesy of Seena Sahadevan; Table 1).

Fungi were isolated from leaf-litter collected at a reference site (Múceres). The litter was maintained in plastic vessels in the laboratory with stream water (~10 L) under strong aeration for 10 days. Samples of water taken from the vessels were observed under a binocular microscope and the suspended spores were picked up with an eyelash mounted in a needle and transferred to an agar-based medium (see below) for germination (Descals 2005).

Mycelium formed from colonies developed in the agar plates were individually cut and transferred to new media in plates (pure culture plates). Cuts from pure cultures were transferred to Erlenmeyer flasks with distilled water on an orbital shaker (48 h) to allow sporulation and confirmation of identification. Pure fungal cultures were maintained in malt extract agar (1.5%; distilled water) in petri dishes at 15 ± 1 ºC and 12-h light/12-h dark conditions for ~ 15 days before use in the assays.

Fungal growth inhibition

Two experiments were run. The first measured growth inhibition in four species of aquatic hyphomycetes exposed to six concentrations of uranyl nitrate solution ($UO_2(NO_3)_2$) in malt extract agar (MEA, 1.5%): 0; 0.004; 0.064; 1.024; 16.384 and 262 $mg \cdot L^{-1}$. The final design consisted of 72 petri dishes (4 fungal species × 6 uranium concentrations × 3 replicates). In a second experiment, growth inhibition was measured in six strains of *H. lugdunensis*. In this case, the final design consisted of 108 petri dishes (6 strains × 6 uranium concentrations × 3 replicates). Plugs (5 mm) of pure fungal cultures were placed on the centre of each petri dish. The colony average diameter (mm) was measured every 2 days for 20 days (n = 10 measurements). The individual growth was expressed in daily area (mm^2) increase. The colony morphology (colour, size, boundary shape) in each treatment was also recorded.

Table 1. Sampling locations for six *H. lugdunensis* strains and uranium concentration in the water (n = 3; mean ± SD).

Strain	Geographical coordinates	Location	Mining status	U in the water ($\mu g \cdot L^{-1}$)
A	40° 36'59.46"N; 7° 40'45.63"W	Ribeira de Ludares (Pinhal do Souto mine)	Abandoned	2.68 ± 0.69
B	40° 03'56.07"N; 8° 06'18.10"W	Ribeira de Sinhel (Escádia Grande mine)	Abandoned	3.30 ± 0.24
C	40° 04'2.98"N; 8° 06'22.38"W	Ribeira de Sinhel (Escádia Grande mine)	Abandoned	1.89 ± 1.05
D	40° 30'42.05"N; 7° 49'2.27"W	Ribeira do Castelo (Quinta do Bispo mine)	Abandoned	6.35 ± 2.24
E	40° 03'54.18"N; 8° 06'9.81"W	Ribeira de Sinhel (Escádia Grande mine)	Abandoned	2.75 ± 1.42
F	40° 32' 01.0"N; 8° 09'15.0"W	Ribeira de Múceres	Reference	0.35 ± 0.8

Fungal sporulation, fungal biomass in leaves and leaf-litter decomposition

We used the four fungal species isolated from the reference stream for this assay. Leaf discs were cut with a cork-borer (12 mm diameter) from the senescent leaves of *Alnus glutinosa* collected in Mondego River Park (Coimbra, Portugal) in autumn, 2016. Sets of seven discs were autoclaved (120º C, 20 min), oven-dried (105º C, 48 h), and weighed (±

0.1 mg) to obtain initial dry mass (DMi). They were placed in 250-mL Erlenmeyer flasks containing 30 mL of sterile distilled water (18 flaks for each species and 18 flaks as controls, with no inoculation; 90 flasks in total). Five 5-mm plugs from pure cultures were used as inoculum. The flasks were maintained for six days on an orbital shaker, with a change of water by Day 3. On Day 6, the plugs and the water were removed, and uranium solutions were added (from 0 to 262 $mg \cdot L^{-1}$ as above; 3 replicates/treatment). The media was changed every three days. The pH was measured at the beginning and at the end of the three days. On Day 24 the leaf discs were removed, freeze-dried weighed and stored at -20º C.

The spore suspensions from the microcosms retrieved every three days were transferred to 500-mL plastic bottles with 2 mL of 37% formalin for preservation. On Day 25, 100 µl of 0.5% Triton X-100 solution were added to the spore suspension and stirred. An aliquot (10 – 100 mL) was filtered in a 25 mm Ø, 5-µm pore size membrane (Sartorius Stedim Biotech GmbH, Göttingen Germany) and stained with 0.05% cotton blue in 60% acid lactic. Spores were counted under a microscope at 200× in a total of 425 microscope fields. Sporulation was expressed as the numbers of spores released initial mg^{-1} leaf DM (e.g. Bärlocher 2005):

$$C = \frac{n \times A}{a \times f} \times \frac{\frac{V}{v}}{DM}$$

where C = conidia/mg Leaf dry mass; n = number of spores counted; A = filter area where spores are retained (489 mm^2); a = area of the field of view (0.95 mm^2), f = number of fields counted; V = total suspension volume; v = suspension volume filtered; DM = discs dry mass (mg).

Ergosterol (as a proxy of fungal biomass) was extracted from five of the seven lyophilised discs from each microcosm (Gessner 2005). Discs were weighed (± 0.01 mg), transferred to 30-mL glass tubes for ergosterol extraction and saponification with 10 mL KOH/methanol (8 $g\ L^{-1}$) at 80 ºC for 30 min. The extract was purified (Waters Sep-Pak © Vac RC tC18 cartridges; Waters Corp., Milford, MA, USA) and analysed by liquid chromatography at 282 nm (HPLC; Dionex DX-120, Sunnyvale, CA, USA). The HPLC system was prepared for 100% methanol in the mobile phase, flow rate 1 mL min^{-1} and

temperature of 33 ºC. A factor of 5.5 mg ergosterol g^{-1} mycelial dry mass was used to convert ergosterol to fungal biomass (Gessner & Chauvet 1993). The results were expressed as mg fungal DM g^{-1} leaf DM.

Leaf-litter decomposition was computed as the difference between initial and final leaf disc dry mass and expressed as dry mass loss (%) after 24 days. Eighteen microcosms without fungal inoculation on leaf discs were kept in pure distilled water and were used as a control for leaf mass loss related to factors other than fungal decomposition (*e.g.* fragmentation by shaking).

Uranium adsorption by leaf discs

Two discs from each microcosm were retrieved for uranium determinations. The leaf discs were weighed (± 0.01 mg), ignited in a muffle (450 ºC, 8 h) for ash free dry mass (AFDM) determination, and transferred to falcon tubes with 8 mL of 2.5 M nitric acid and heated to 60 ºC. After 1 h, I added 10 mL acetic acid and stirred for 2 min; 5 mL of this solution was transferred to porcelain crucibles for inorganic solvent ignition. The residual was suspended in 7 mL of 0.005% nitric acid. Uranium was measured by fluorescence (Bergmann et al. 2018); 0.50 mL of sample was diluted in 5.0 ml of distilled water and 0.50 ml of the polysilicate solution. Fluorescence was compared to standard curves (2; 10; 100 and 1000 $\mu g \cdot L^{-1}$ (Van Loon & Barefoot 1989) at λ = 530 nm (Fluorat 02-2M, Lumex).

Data analysis

Fungal growth inhibition in 10% (EC_{10}), 20% (EC_{20}) and 50% (EC_{50}) on agar plates at increased uranium concentrations was estimated using probit analysis (log-transformed concentrations). It was not possible to compute EC_{10} values for some species and *H. lugdunensis* strains because of large variability (inaccurate confidence intervals). To obtain an indication of the response to metal stress, a tolerance index (TI) was calculated, given by "Growth at a given [U] × 100 / Growth in the Control" (Fazli et al. 2015); when no inhibition occurs, TI = 100; values above 100 indicate stimulation. Differences in growth between species and between *H. lugdunensis* strains across uranium concentrations were assessed using analysis of variance (two-way ANOVA), after assessing for

homoscedasticity (Bartlett test) and normality (Shapiro-Wilk test). We used the Tukey´s post hoc test for multiple comparison.

Differences in sporulation, biomass, and litter decomposition were assessed using one-way ANOVA among concentrations for each species followed by a Holm-Sidak test for comparison with control groups. When data did not conform to normal distribution, the differences were assessed by Rank ANOVA followed by Dunn´s Method for comparison with control groups. Analyses were performed using the Statistica Statsoft 7 and SigmaStat 3.5 software.

Results

Interspecific differences in growth under uranium exposure

A. tetracladia grew faster (2.29 mm^2 d^{-1}) under control conditions, while *V. elodeae* grew slowest (0.88 mm^2 d^{-1}; Figure 1) among the four species of aquatic hyphomycetes. Mycelial growth differed between species ($F_{3,15}$ = 97.28; p < 0.001), and uranium concentrations ($F_{5,15}$ = 409.07; p < 0.001), Table A2. The uranium concentration causing 50% mycelial growth inhibition (EC_{50}) ranged from 12.5 to 45 $mg \cdot L^{-1}$ (Table 2), with *A. tetracladia* the most sensitive and *V. elodeae* the most tolerant species. A uranium concentration of 0.85 $mg \cdot L^{-1}$ was enough to cause 20% reduction in growth in *A. tetracladia*, while the same 20% inhibition in *H. lugdunensis* occurred at 3 $mg \cdot L^{-1}$. At the lower uranium concentrations (< 1 $mg \cdot L^{-1}$), *T. splendens* and *H. lugdunensis* grew up to 12% faster than the control (Table 3).

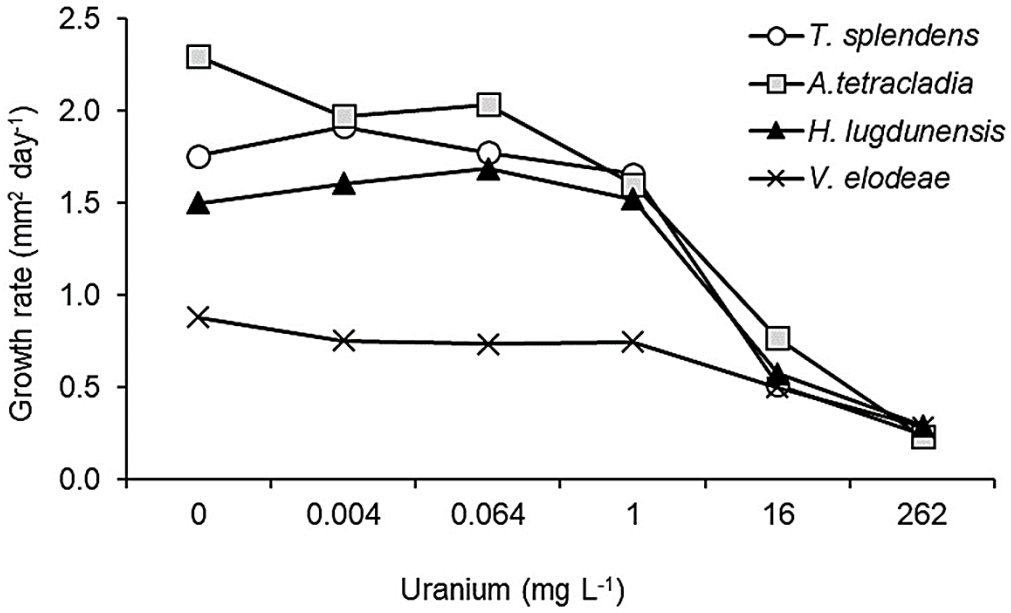


Figure 1. Growth (mm^2 day^{-1}) of four hyphomycetes species in agar plates under six uranium concentrations (mean ± SE; n = 3).

Table 2. Uranium concentrations ($mg \cdot L^{-1}$) inhibiting mycelial growth in 10% (EC_{10}), 20% (EC_{20}) and 50% (EC_{50}). NC: Not calculated. CI: 95% Confidence interval.

Species	**EC_{10} (CI)**	**EC_{20} (CI)**	**EC_{50} (CI)**
T. splendens	1.2 (0.8 – 1.8)	2.9 (2.2 – 4.1)	21.5 (16.0 - 30.0)
A. tetracladia	NC	0.9 (0.7 – 1.0)	12.5 (11.3 – 13.8)
H. lugdunensis	1.1 (0.7 – 1.6)	3.0 (2.2 – 4.0)	25.0 (18.5 - 33.0)
V. elodeae	NC	1.2 (1.0 – 1.5)	45.0 (36.3 – 46.8)

Table 3. Tolerance Index of the four fungal species growing in agar plates at increasing uranium concentrations. TI > 100% = stimulation; TI < 100% = inhibition.

	U ($mg \cdot L^{-1}$)				
TI	0.004	0.064	1	16	262
A. tetracladia	86	89	70	33	10
T. splendens	109	101	94	29	14
H. lugdunensis	197	112	101	38	19
V. elodeae	85	84	85	57	32

Differences between strains of *H. lugdunensis*

Mycelial growth differed between strains ($F_{5,25}$ = 88.93; p < 0.001), and uranium concentrations ($F_{5,25}$ = 879.57; p < 0.001), Table A3. Under control conditions (no uranium) growth in agar plates ranged from 1.26 mm^2 d^{-1} (Strain D, from the most contaminated site) to 1.89 mm^2 d^{-1} (Strain A) (Figure 2). Inhibition was unrelated to the origin of the strains (Table 4). Under increasing uranium concentrations, the strain from the most polluted site (D) grew more slowly than the others and was one of the less tolerant strains. The strain from the reference site (F) was more tolerant than the others. The most sensitive strain (C), determined by its low ECs and TI values was sampled from a site with comparatively low uranium concentration.

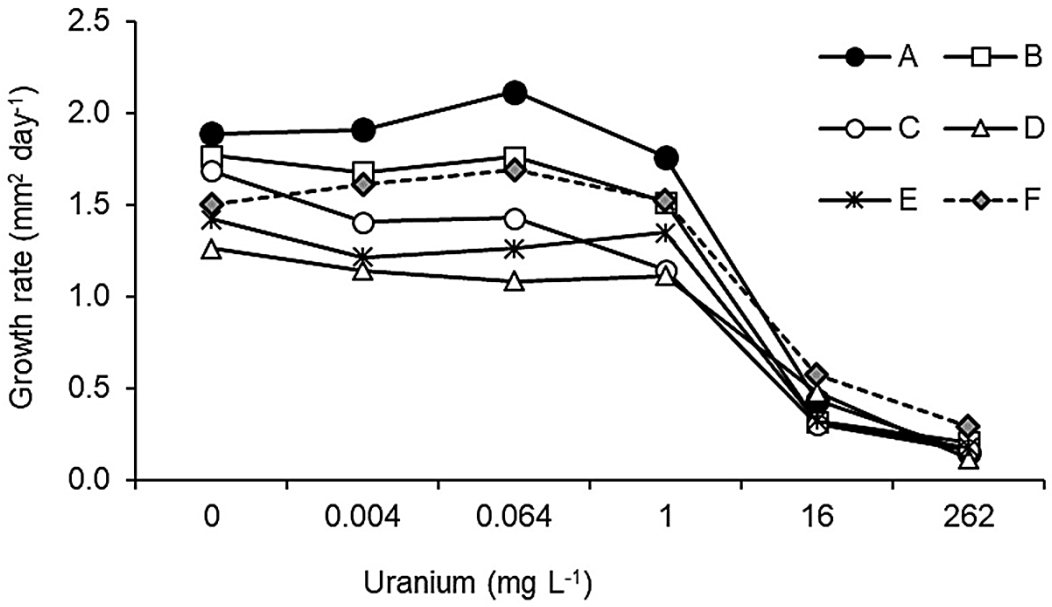


Figure 2. Growth (mm^2 day^{-1}) of the six *Heliscus lugdunensis* strains in agar plates under increasing uranium concentrations (mean ± SE; n = 3).

Colony morphology

Colony morphology differed among strains and concentrations. The colonies grew with a regular boundary for all strains and uranium concentrations < 1 $mg \cdot L^{-1}$. At the highest concentrations, boundaries became whitish and irregular. Strain colonies were brown-orange under low uranium concentrations but acquired irregular boundaries under 16 and 262 $mg \cdot L^{-1}$ (Figure 3).

Table 4. Uranium concentrations ($mg \cdot L^{-1}$) inhibiting mycelial growth in 10% (EC_{10}), 20% (EC_{20}) and 50% (EC_{50}) in six strains of *H. lugdunensis* growing in increasing concentrations of uranium. NC: Not calculated; CI: 95% Confidence interval. Dark grey, light grey and white bands represent respectively the strains more sensitives, with intermediate sensitivity and the most tolerant strain.

Strain	U water ($\mu g \cdot L^{-1}$)	EC_{10} (CI)	EC_{20} (CI)	EC_{50} (CI)
A	2.68 ± 0.69	0.7 (0.5 – 1.0)	2.9 (1.6 - 2.4)	15.0 (12.5 – 17.5)
B	3.30 ± 0.24	NC	1.5 (1.3 – 1.9)	13.5 (12.0 – 15.5)
C	1.89 ± 1.05	NC	0.4 (0.2 – 0.7)	09.0 (07.2 – 10.0)
D	6.35 ± 2.24	NC	1.3 (1.1 – 1.5)	14.3 (13.3 – 15.5)
E	2.75 ± 1.42	NC	1.2 (1.0 – 1.4)	13.5 (12.2 – 15.0)
F	0.35 ± 0.8	1.1 (0.7 – 1.6)	3.0 (2.2 – 4.0)	25.0 (18.5 – 33.0)

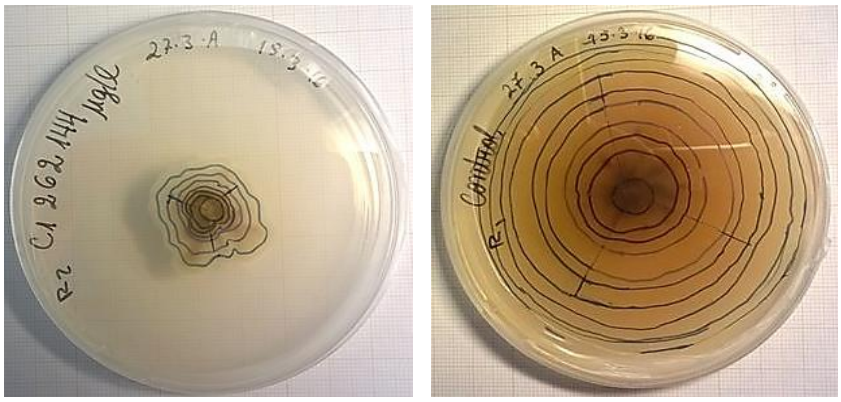


Figure 3. Colony morphology of *Heliscus lugdunensis* (strain A) grown under control (no uranium) (left) and under 262 $mg \cdot L^{-1}$ (right). At high uranium concentration the colony edge becomes irregular and has a different color (lines indicate the weekly increases in area).

Sporulation rates, fungal biomass and leaf-litter decomposition in microcosms

The pH in the microcosms averaged 7.3 ± 0.7 except for a uranium concentration of 262 $mg \cdot L^{-1}$, where the pH was 4.8 ± 0.3. Under control conditions, average spore production ranged from 86 (*A. tetracladia*) to 3,138 spores per mg dry mass (*H. lugdunensis*) (Figure 4). Under low uranium concentrations (up to 0.064 $mg \cdot L^{-1}$), sporulation rates were stimulated in some species, but inhibited at concentrations above 1

mg·L^{-1} (*V. elodeae*) and 262 mg·L^{-1} (*H. lugdunensis*). At 262 mg·L^{-1}, *T. splendens* and *A. tetracladia* demonstrated 64% and 100% inhibition in sporulation when compared to control, respectively (Figure 4). Fungal biomass in leaf discs under control conditions ranged from 18 (*H. lugdunensis*) to 23 (*V. elodeae*) mg g^{-1} DM. Fungal biomass was reduced by uranium at concentrations of 16 mg·L^{-1} (*T. splendens* and *H. lugdunensis*) and 262 mg·L^{-1} (*A. tetracladia*) (Figure 5). Leaf-litter decomposition was reduced by 16 mg·L^{-1} for all species, except for *A. tetracladia* (Figure 6).

Average litter decomposition ranged from 15.2 ± 0.6% (*T. splendens*) to 23.9 ± 0.1 % (*H. lugdunensis*) for control conditions (no uranium); and 6.3 ± 2.0 % (*T. splendens*) to 8.9 ± 6.1% (*V. elodeae*) for the highest uranium concentration (262 mg·L^{-1}). After 24 days in the microcosms, the leaf-discs had increased levels of uranium according to concentrations in the solutions tests (Figure 7), up to 89 mg kg^{-1} in 262 mg·L^{-1}.

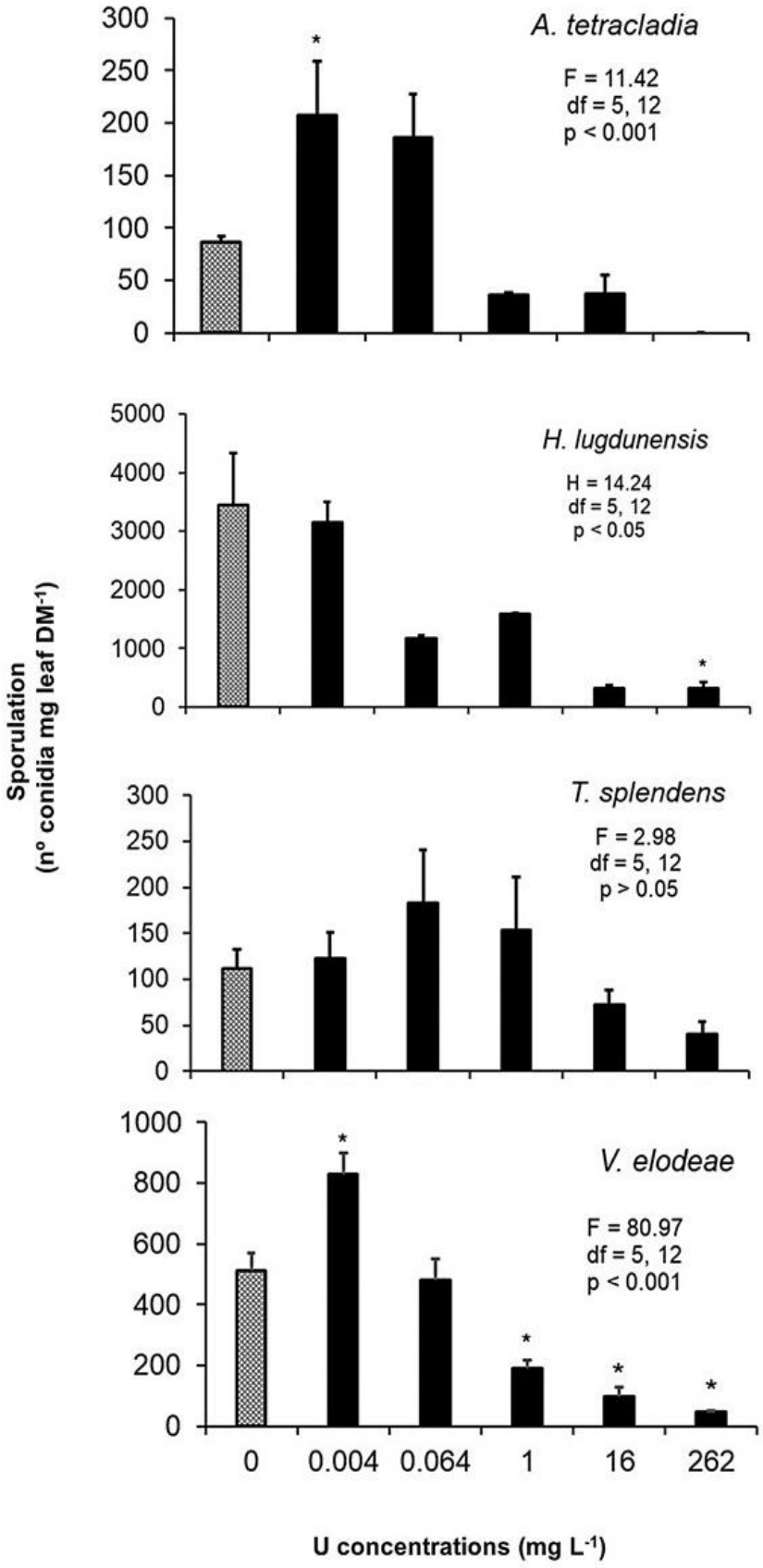


Figure 4. Cumulative conidia production of four aquatic hyphomycetes species (mean ± SE) growing in leaves and exposed to increased uranium concentrations for 24 days; *n* = 3; one-way ANOVA followed by Holm-Sidak test against the control, or Rank ANOVA followed by Dunn´s Method; * = p < 0.05.

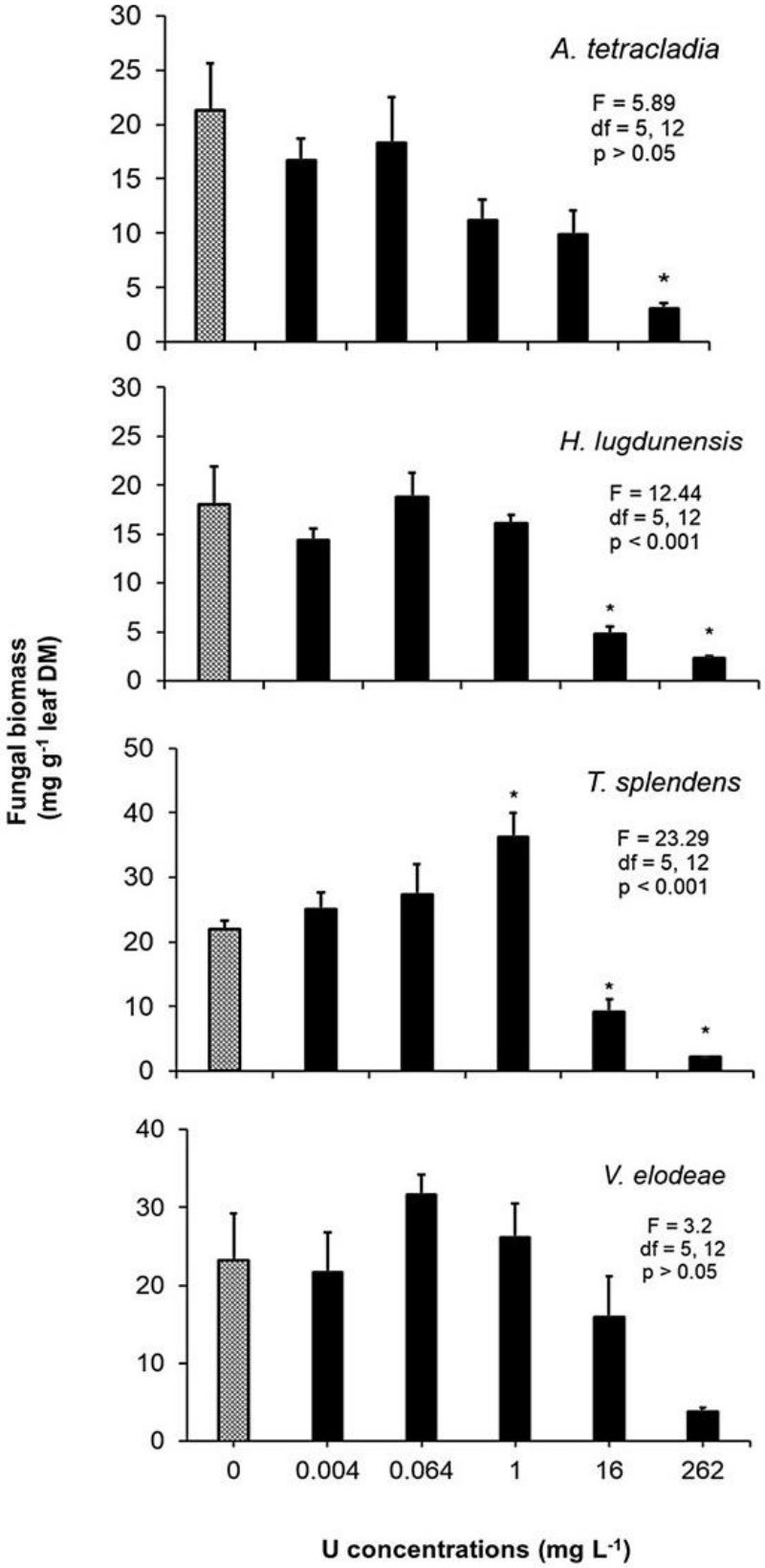


Figure 5. Fungal biomass of four aquatic hyphomycetes species (mean ± SE) growing in leaves and exposed to increased uranium concentrations for 24 days; *n* = 3; one-way ANOVA followed by Holm-Sidak test against the control; * = $p < 0.05$.

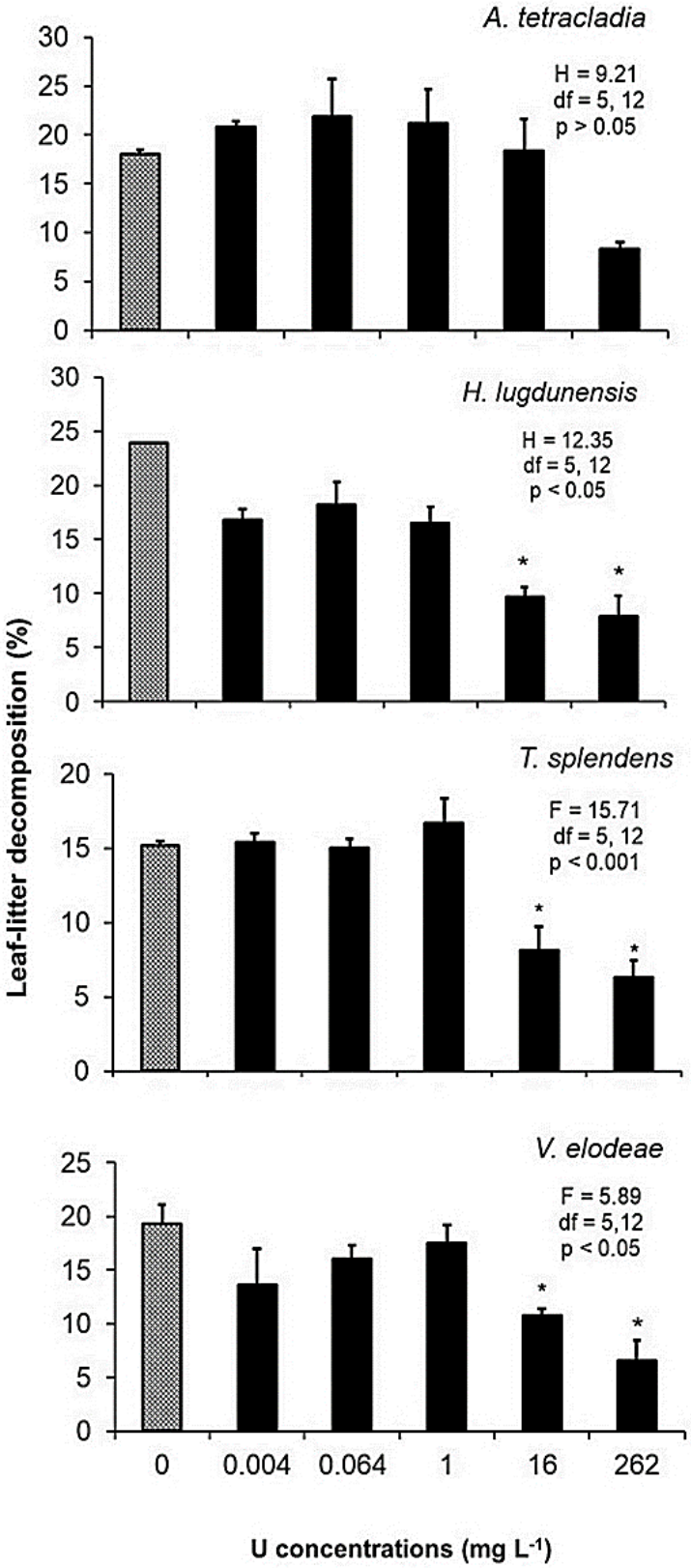


Figure 6. Leaf-litter decomposition for the four aquatic hyphomycetes species (mean ± SE) growing in leaves and exposed to increased uranium concentrations for 24 days; *n* = 3; one-way ANOVA followed by Holm-Sidak test against the control, or Rank ANOVA followed by Dunn´s Method; * = $p < 0.05$.

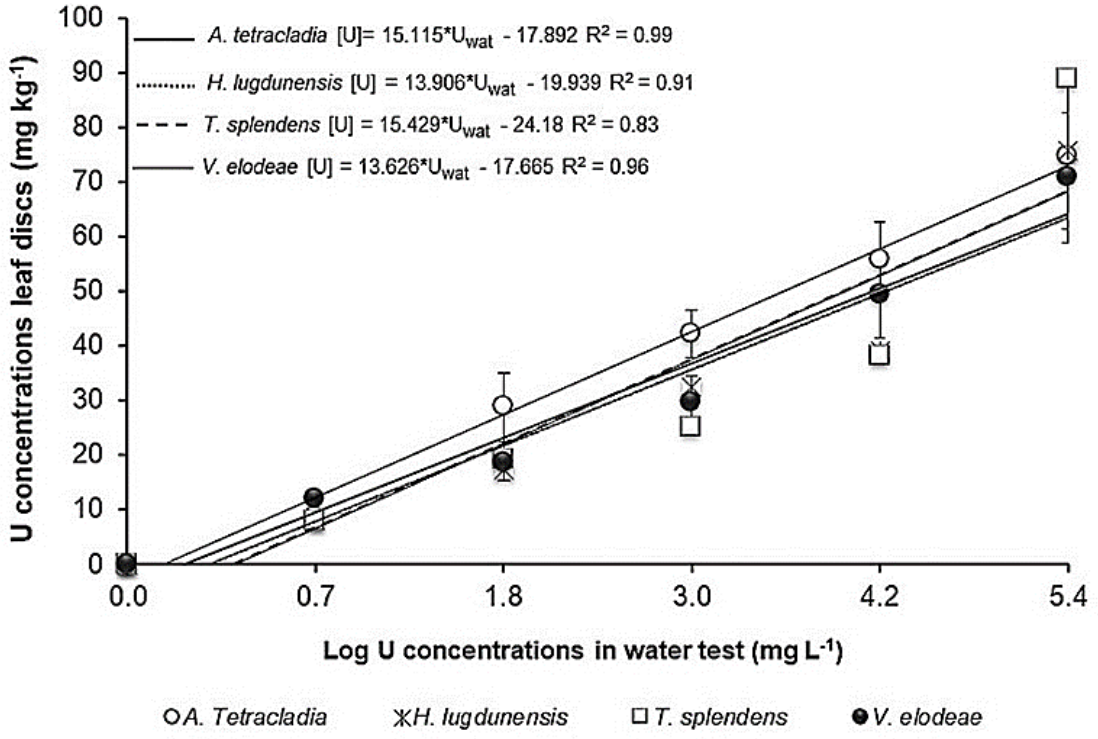


Figure 7. Uranium in alder leaves exposed to increased concentrations (mean ± SE), $n = 3$

Discussion

Uranium concentrations below 1 mg·L^{-1} caused no significant inhibition on fungal growth, sporulation, biomass or litter decomposition. Inhibition was observed at uranium concentration ranging from 1 to 262 mg·L^{-1}, depending on the species. According to these data, some of the sampled streams from which *H. lugdunensis* strains were obtained are slightly polluted (0.35 – 6.35 µg·L^{-1}). Bergmann et al. (2018) reported that 95% of the 298 sites receiving waters from deactivated uranium mines in Portugal had < 10 µg·L^{-1}, while 1.3% had > 100 µg·L^{-1}, and 0.33% > 1000 µg·L^{-1}. Our findings indicate that some of those streams may exhibit ecological impairment.

In our experiments, uranium concentrations of 0.4 – 9 mg·L^{-1} inhibited mycelial growth in 20% to 50% (Table 2). It is apparent that aquatic hyphomycetes are less tolerant to uranium than other fungi such as *Aspergillus niger* and *Paecilomyces javanicus*, whose tolerance indices ranged from 20 to 28% in 10 mM uranium (400 mg·L^{-1}) (Liang et al. 2015), or *Rhizopus arrihizus* which grows normally in 200 mg·L^{-1} (Wang et al. 2010).

Aquatic hyphomycetes were more tolerant that other aquatic organisms such as the algae *Chlorella* sp, however, whose growth was inhibited by 50% at 78 µg·L^{-1} (Franklin et al. 2000), while 200 µM (8 mg·L^{-1}) inhibited growth of the bacterial strains from subsurface sediments (Brzoska & Bollmann 2016). Compared with aquatic invertebrates, values similar to the lowest inhibition rates in this study (~ 1 mg·L^{-1}, EC10) caused 50% immobility for *D. magna* (1.4 mg·L^{-1}) (Antunes et al. 2007) or 50% mortality in *Hyalella azteca* (LC50; 1.52 mg·L^{-1}) (Kuhne et al. 2002).

Fungal reproduction is one of the parameters most sensitive to metal contamination (Gessner & Chauvet 1997; Duarte et al. 2004). Fungal biomass was significantly reduced here at 262 mg·L^{-1} for *A. tetracladia*, whereas sporulation was inhibited for this same species and for *V. elodeae* (from 1 mg·L^{-1}) (Figure 4).

Conidia production has been correlated to aquatic hyphomycete decomposition activity (Duarte et al. 2004). Aquatic hyphomycetes can allocate ~ 50% of their total production to conidia with a proportional average of the total leaf-litter mass loss (Gessner & Chauvet 1997). As with sporulation and biomass, leaf- litter decomposition was also reduced (~ 46 - 58%) in the 16 and/or 262 mg·L^{-1} of uranium for three of the four species in relation to control/no uranium exposure. The mechanism causing inhibition is not known, but it is possible that aquatic hyphomycetes enzymes become nonfunctional under metal exposure, interfering with degradative activities and consequently also leaf-litter mass loss (Sridhar et al. 2001).

The increased accumulation/biosorption of uranium in leaf-discs across concentrations may have limited the availability of oxygen and inorganic compounds for the mycelia (Sridhar et al. 2001). The reduction in sporulation and litter decomposition in our experiment indicated a pronounced decrease in the allocation of resources (energy and material) for conidia production at higher uranium exposure (Duarte et al. 2008). Under higher uranium concentration, however, pH was also low due to the larger amount of uranyl ions (UO_2^{2+}) (Alam & Cheng 2014). It is difficult to predict how acidity could have affected sporulation, especially for *A. tetracladia*, *H. lugdunensis* and *V. elodeae*, although the latter had already decreased spore production in concentrations of 16 mg·L^{-1}. Low pH (<5) has been shown to decrease litter decomposition in acidic streams (Cornut et al. 2012; Ferreira & Guérold 2017), by reducing the abundance and biomass of the sensitive shredders (Dangles & Guérold 2001), a reduction of microbial activity (Dangles et al. 2004), and a

decrease in aquatic hyphomycetes biomasses and species (Baudoin et al. 2008). Not all these factors for the reduction in leaf mass loss under acidic conditions were present in the laboratory but is possible that cell wall components degradation was inhibited by acidification (Kok et al. 1992).

Low uranium concentrations stimulated fungal growth, biomass and sporulation by aquatic hyphomycetes in some species. This observation is consistent with a large body of literature relating pollutants to biological processes and is known as hormesis. Hormesis occurs when a stressor causes noxious effects in a biological system at high concentration, and the enhancement of a biological parameter at low quantity (Calabrese & Baldwin 2001). As in the mycelial growth assay for some species, conidia production was also stimulated under low uranium concentrations in *A. tetracladia* and *V. elodeae*. The faster growth for the lower tested concentrations, however, may reveal the capacity of fungi to deal with metals. Fungal biomass may biosorb up to 600 mg g^{-1} dry weight for uranium from aqueous solutions (Ogar et al. 2014). Uranium adsorption by *A. tetracladia* mycelium was 140 mg g^{-1} dry weight at 2000 $\mu g \cdot L^{-1}$, indicating that native hyphomycetes can retrieve the metal from the streams waters (Ferreira et al. 2010). Due to the biosorption kinetics, fungal hyphae can excrete organic acids that bind to uranium particles and extracellular uranium can be precipitated (Fomina et al. 2008; Krauss et al. 2011). It is also possible that uranium was accumulated into vacuoles in the older cells of fungal hyphae, allowing tip cells differentiation and growth even in a metal contaminated media (Isaure et al. 2017).

High uranium concentrations caused an inhibition of fungal growth, biomass, sporulation and litter decomposition. The growth inhibition observed at the highest uranium concentrations indicates toxicity. High uranium concentration potentially changes the surface of fungal cells (Zheng et al. 2017), which may explain the changed colonies morphology and decreasing growth. Uranyl ions bind to cell walls, precipitate with phosphates and can accumulate intracellularly (Strandberg et al. 1981). The low pH observed in our experiment for 262 $mg \cdot L^{-1}$ might have favored uranium biosorption by fungi (Bayramoğlu et al. 2006).

Overall, the uranium tolerance of *H. lugdunensis* strains was found to be independent of the level of pollution at the site of origin. This was unexpected, since stressing conditions should select genotypes capable of copping with new conditions (here, metal pollution), and the most sensitive genotypes should be eliminated. Aquatic hyphomycetes produce a large number of spores which are transported downstream by the

current and may attach to organic matter further downstream where conditions are different from those of the site where they are produced (~1.8 km; Fabre 1997). A polluted site may thus be continuously being colonised by genotypes produced upstream (and not exposed) of the contamination. In this same way, conidia produced at a polluted site may be transported away from the polluted site.

CHAPTER IV

Bioaccumulation and dispersion of uranium by freshwater organisms

Illustrative image: Water Strider / Pond Skater (*Gerris lacustris*) with mosquito prey, retrieved from https://www.naturepl.com/search/page-2/hemiptera+aquatic.

CHAPTER IV: Bioaccumulation and dispersion of uranium by freshwater organisms

Abstract

Uranium is the heaviest naturally-occurring element on Earth. Uranium mining may result in ground and surface water contamination with potential bioaccumulation and dispersion by aquatic invertebrates with aerial stages. We investigated the effects of uranium contamination at community level in terms of abundance, richness, the composition of communities and functional traits. We also investigated uranium mobility across aquatic food webs and its transfer to land via the emergence of aquatic insects. We sampled water, sediment, biofilm, macrophytes, aquatic invertebrates, adult insects and spiders in the riparian zone across sites with a gradient of uranium concentrations in stream water (from 2.1 to 4.7 $\mu g \cdot L^{-1}$) and sediments (from 10.4 to 41.8 $\mu g \cdot g^{-1}$). Macroinvertebrate assemblages differed between sites with a higher diversity and predominance of Nemouridae and Baetidae at the reference site and low diversity and predominance of Chironomidae in sites with the highest uranium concentration. Uranium concentrations in producers and consumers increased linearly with uranium concentration in stream water and sediment ($p < 0.05$). The highest accumulation was found in litter (83.76 ± 5.42 $\mu g \cdot g^{-1}$) and macrophytes (47.58 ± 6.93 $\mu g \cdot g^{-1}$) in the most contaminated site. Uranium was highest in scrapers (14.30 ± 0.98 $\mu g \cdot g^{-1}$), followed by shredders (12.96 ± 0.81 $\mu g \cdot g^{-1}$) and engulfer predators (7.01 ± 1.3 $\mu g \cdot g^{-1}$). Uranium in adult insects in the riparian zone of all sites ranged from 0.25 to 2.90 $\mu g \cdot g^{-1}$, while in spiders it ranged from 0.96 to 1.73 $\mu g \cdot g^{-1}$, with no significant differences between sites ($p > 0.05$). Overall, there was a negative relationship between $\delta^{15}N$ and uranium, suggesting there is no biomagnification along food webs. We conclude that uranium is accumulated by producers and consumers, but not biomagnified nor dispersed to land with the emergence of aquatic insects.

KEYWORDS: metal, bioaccumulation, food chain, macroinvertebrates, stable isotopes.

Introduction

Uranium is the heaviest natural element, found as a mixture of the isotopes ^{238}U, ^{235}U and ^{234}U (Bleise et al. 2003). It can be released into the environment via the nuclear

fuel cycle, military uses, in phosphate-containing fertilisers, and mining. Uranium in freshwater typically ranges from 0.03 to 2.1 $\mu g \cdot L^{-1}$ (Bleise et al. 2003), but values of 12 $\mu g \cdot L^{-1}$ to 2 $mg \cdot L^{-1}$ have been reported for polluted systems (Simon et al. 2013). In sediments, natural geochemical background level of uranium is on average 10 $\mu g \cdot g^{-1}$ dry mass (Lagauzère et al. 2014), and toxic effects can be detected from ~ 100 mg kg^{-1} (Sheppard et al. 2005). Benthic macroinvertebrates feeding in sediments are particularly sensitive to metal contamination (Mocq & Hare 2018).

Overall, pollution by metals and metalloids can cause changes in the abundances and species composition of invertebrates (Solà et al. 2004; Ali et al. 2018) and in periphytic algae (Carlisle & Clements 2003). Life-history parameters may also change along metal-disturbed gradients, including feeding, size, life cycle duration and motility (Piló et al. 2016; Mocq & Hare 2018).

Uranium can be taken up from water columns and sediment through the gills and skin (Frelon et al. 2013), or diet (Fisher & Hook 2002). Organic matter, fungi and algae have a high metal-binding abilities (Gadd 2010), thus increasing in this way uranium intake by consumers. Shredders and scrapers feeding on leaf-litter detritus and biofilms accumulate uranium from food (Scheibener et al. 2017; Bergmann et al. 2018) and contribute to increasing the surface area of litter particles, leading to more space for biosorption and complexation of the metals (Schaller et al. 2011). Uranium mobilised from sediment can bind to particulate organic matter (POM), and become available to collector-gathering invertebrates (Crawford et al. 2018). Different patterns in ingestion rates, assimilation and elimination efficencies dictate differences in bioaccumulation among organisms (Cid et al. 2010; Kraemer & Evans 2012). Some metals and other pollutants may be biomagnified along the food webs (Einoder et al. 2018; Punshon et al. 2003), but this is not always the case (e.g. Cui et al. 2011; Hepp et al. 2017).

The movement of emergent insects from contaminated freshwaters may disperse the pollutants to land (Mogren et al. 2012). Emergent insects can be consumed by birds, bats (Naidoo et al. 2013; Howie et al. 2018), and other arthropods (Paetzold et al. 2005), particularly ground-dwelling carnivorous beetles, rove beetles, spiders and ants (Wood et al. 2007). The potential biomagnification and transfer of the metals from streams to land can be assessed by nitrogen (^{15}N) stable isotopes, in combination with the metal concentrations in the organisms (Hepp et al. 2017; Einoder et al. 2018). Nitrogen isotopes

provide information about the trophic position of consumers since there is a 2.3 – 3.4 ‰ ^{15}N enrichment per trophic level (Cremona et al. 2009; Li et al. 2018 b).

In a previous study, we found that leaf-litter consumers accumulate uranium from water and ingested food, and that uranium biosorted to litter can lead to the continuous metal exposure of aquatic invertebrates (Bergmann et al. 2018). Uranium concentrations of 50 $\mu g \cdot L^{-1}$, a value found in several uranium polluted streams, can affect important enzymes involved in electrochemical potential differences in cells (such as Na^+K^+ATPase) (Tagliaferro et al. 2018). Here we ask whether the effects observed under laboratory conditions result in community changes in terms of abundance, richness and functional traits. We also investigated uranium mobility across aquatic food webs and its transfer to the terrestrial environment with the emergence of aquatic insects with aerial stages.

Materials and Methods

Study area

We sampled four test sites (TS) in the Ribeira do Castelo stream, a tributary of the Mondego River receiving drainage from the recovered mines Cunha Baixa and Quinta do Bispo: TS1 40º 34´12.17”N, 7º46´12.67”W; TS2 40º 33´32.50”N, 7º47´17.85”W; TS3 40º32´04.08”N, 7º48´59.21”W; TS4 40°30′42.22″N; 7°49′1.54″W), and a nearby reference stream (RF) 40º30´11.39”N, 7º48´35.72”W, Figure 1.

Sampling

To test whether (1) the uranium levels in the water and sediments affect abundance, richness and composition of the community of aquatic invertebrates; (2) uranium is bioaccumulated and (3) whether it is biodispersed to land, we took biological and environmental samples on four occasions: spring, summer and autumn (2016) and winter (2017). Macroinvertebrates were sampled with a 500-µm mesh hand net (3 cumulative samples, covering major macro-habitats). Additional specimens attached to submerged stones were manually retrieved. Live samples were taken to the laboratory in cooler boxes, where they were separated, and identified at genus or family/sub-family. We allocated invertebrates into functional feeding groups as scrapers (Baetidae, Siphlonuridae, Physidae), shredders (Calamoceratidae, Tipulidae), and predators to

assess uranium bioaccumulation from water and sediment. Predators were classified into engulfers (Odonata: Cordulegasteridae), and piercers (Hemiptera: Notonectidae, Nepidae and Belostomatidae) (Cole & Weihe 2016; Tachet et al. 2000). On each sampling occasion we also collected leaf-litter from the stream bed, aquatic macrophytes (*Oenanthe crocata* – leaves, *Lemna* sp) and biofilm. Biofilm was scraped from natural submerged stones from a 20 cm^2 area (delimited by a petri dish) (n = 3 /site) with a toothbrush, and transported in 20-mL plastic flasks to the laboratory in a cooler box.

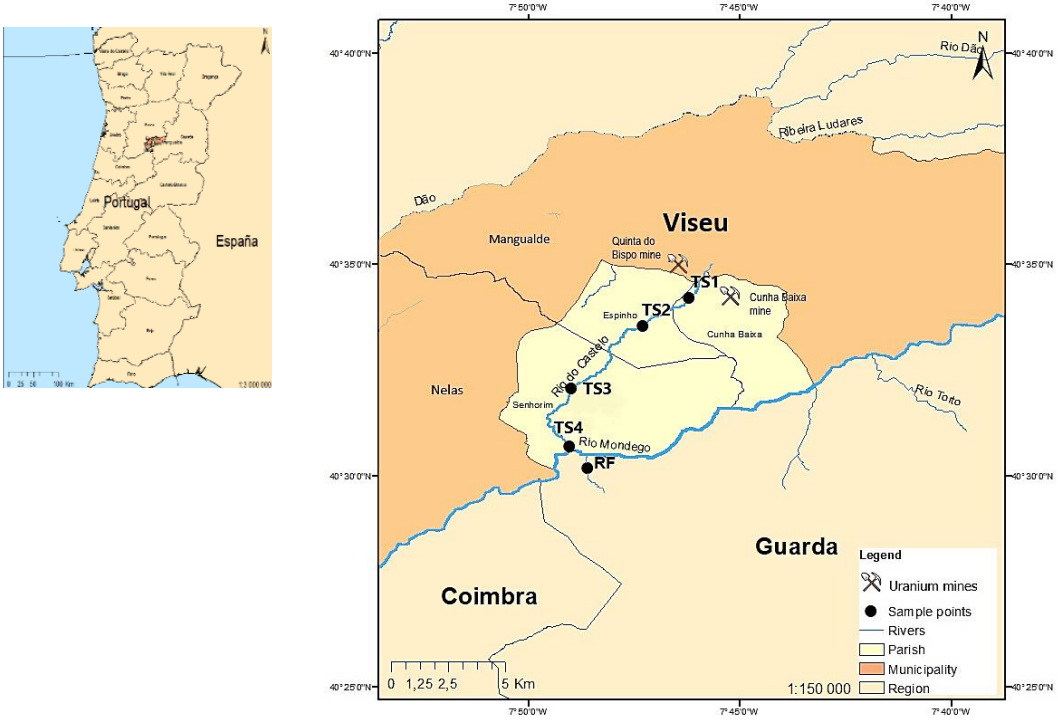


Figure 1. Location of Cunha Baixa and Quinta do Bispo Mines and sampling sites.

Uranium trophic transfer along the food webs was assessed by measuring uranium content in producers and consumers from the most contaminated test site (TS1), as indicated above. Our samples included bryophytes (*Fontinalis* sp) in spring and collector-gathering (Chironomidae) in the winter. Uranium transfer to terrestrial consumers was assessed by sampling the adults of Chironomidae, Trichoptera and Odonata with a hand net from shrubs and branches at the stream margins (~ 5 m from edges). Spiders were collected with tweezers from riparian vegetation (< 1m from the edges), including branches hanging over water. At least 12 spiders were collected at each sampling site.

Some specimens were preserved in ethanol 80% for identification (Nentwig et al. 2019) and the remainder were frozen for further analyses. All biological samples (except biofilm) were allocated into two groups, one for stable isotope analysis and the other for uranium determination (see below).

General chemical analysis

On each sampling occasion we measured *in situ* water temperature, pH, conductivity and dissolved oxygen (Multiparameter 340i/SET, WTW Germany), and collected water samples for chemical analysis (phosphates, nitrates, alkalinity and uranium). Water samples were filtered through 0.45-µm Millipore filter, cooled to 4 ºC and analysed within 24 h by ion chromatography (phosphates and nitrates, Dionex DX-120, Sunnyvale, California, USA). Alkalinity was determined by titration to an endpoint of pH 4.5, and soluble reactive phosphorus (SRP) was determined using the ascorbic acid method (APHA 1995). Uranium was analysed from filtered and acidified water to pH < 2 with 65% nitric acid (V/V). A composite 3 kg sample of stream sediment was collected at multiple places in each site at depths of 5-10 cm avoiding gravel and vegetation (Pinto et al. 2004). Sediment samples were transported in plastic bags in a cool box to the laboratory, oven dried (45 ºC, 72 h) and sieved (1 mm, 0.5 mm and 180 µm sieve column).

Uranium analysis

Uranium in stream waters, sediments, leaf-litter, macrophytes, biofilm and in the invertebrates was measured using fluorometry (λ = 530 nm, Fluorat Lumex 2M). Biological samples were oven dried (45º C, 72 h), weighed (± 0.5 mg) and ignited (450° C, 8 h) to determine ash free dry mass (AFDM). Samples of biofilm were transferred to pre-weighed porcelain crucibles, dried (45 ºC, 72 h), weighed and analysed for uranium. Ashed samples were transferred to 50-mL testing tubes and digested in 8 ml of 2.5 M nitric acid for 1 hour in a boiling-water bath. After cooling, 10 mL ethyl acetate was added and mixed for ~ 5 min, and 5 mL of the supernatant was retrieved to porcelain crucibles for ignition of the inorganic solvent. The residue was dissolved in 7 ml of 0.005% nitric acid; 0.5 mL of this sample was diluted in 5 mL of distilled water with 0.5 mL of polysilicate. The samples were measured in the fluorometer, and the fluorescence was compared to the standard curve (0; 2; 10; 100; and 1000 $\mu g \cdot L^{-1}$), according to Van Loon

& Barefoot (1989). Portions of the 0.5 g of sediments retained by the 180 µm sieve were transferred to 50-mL testing tubes and uranium extracted in 10 mL of nitric acid (HNO_3, 65%) and 10 mL of hydrochloric acid (HCl 35%). After 72 h, the supernatant was filtered, and analysed as water samples.

Isotope analysis

We measured the $\delta^{15}N$ / $\delta^{14}N$ of invertebrates to assess their trophic position. Oven dried samples were manually macerated, weighed in a microbalance (Mettler Toledo UMX2) to approximately 0.4 mg for animals and 0.5 mg for macrophytes and leaf-litter, and placed in tin capsules. Nitrogen isotopes $\delta^{15}N$ / $\delta^{14}N$ were analysed in a Flash Analyser EA 1112 Series for IRMS Delta VS Thermo.

Data analysis

Macroinvertebrate richness and Shannon-Wiener (H´) diversity were compared across sites by one-way ANOVA and Tukey´s multiple comparison test. Macroinvertebrate assemblages at different sites were compared by non-metric multidimensional scaling (NMDS), based on the Bray-Curtis similarity index of log(x+1) transformed abundance data and by cluster analysis (Primer 7). Differences were assessed using permutational multivariate analysis of variance (PerManova).

The most abundant 30 taxa were classified in five functional traits potentially responding to metal contamination, based on Piló et al. (2016): feeding mode, life span, body size, motility, and aquatic stage. The modalities of each trait were gathered from Tachet et al. (2000); (Table 1).

A score between 0 and 3 was attributed for each trait modality, according to the affinities of the given species with that modality. “0” indicates no affinity, “1” and “2” express partial affinity, and “3” indicates high or exclusive affinity. For example, *Tipula* sp is a shredder (3), and may consume fine sediments (2) and other animal preys (2). The relative abundance of each trait is given by the sum of scores in the modalities, dividing each score by the sum, and multiplying the results by the total number of individuals of the respective genera. The number of individuals of a given species was then multiplied by the final score. The relative abundances were given in % at each site. Differences in trait composition and modalities between sites was assessed using PerManova (traits and respective modalities based on abundances in each site).

The relationship between uranium in the water and biota (litter, macrophytes, biofilm, shredders, scrapers and predators) was investigated using a simple linear regression on log transformed uranium concentrations. Uranium accumulation in aquatic and terrestrial (adult insects and spiders) organisms was compared by one-way ANOVA using nominal or log transformed (macrophytes) values, followed by a Tukey HSD multiple comparison test. The mobility of uranium across trophic levels was assessed by linear regression of bioaccumulated uranium vs. $\delta^{15}N$, separated analysis by season. Statistical analyses were performed with Primer 7 software (PerManova + add) and Statsoft Statistica 7.

Table 1. Macroinvertebrate functional traits and respective modalities.

Functional traits	**Trait modalities**
Feeding mode	Shredders, Filter feeders, Fine sediment feeders, Scrapers, Grazers, Predators
Life span	≤ 1 year, > 1 year
Body size (mm)	2.5-5.0, 5.0-10, 10-20, 20-40, 40-80
Motility	Swimmer, Burrower, Crawling, Temporary fixation, Flight
Aquatic stage	Larvae, Nymph, Adult

Results

Uranium in the environment

Uranium concentrations in water at the site closest to the mine (TS1) ranged from 1.65 to 9.80 $\mu g \cdot L^{-1}$, and in the reference site it ranged from 1.48 to 3.74 $\mu g \cdot L^{-1}$; however, there were no statistical differences across sites (Table 2). Uranium in sediments was significantly higher in TS1 (41.8 ± 2.8 $\mu g \cdot g^{-1}$) than in the reference site (10.4 ± 0.9 $\mu g \cdot g^{-1}$; $F=14.92$; $p= 0.001$).

Table 2. Physical-chemical variables in four testing sites located mines (TS) and a reference site (RF) sampled 4 times in a year (mean ± SE, n = 4 or *n =3). Comparisons among sites were made with ANOVA or Kruskal-Wallis. Different letters indicate significant differences among stream sites (Tukey´s test or Dunn´s post hoc, $p < 0.05$).

Variables	RF	TS1	TS2	TS3	TS4	*F*- or H-values*	*p* value
Uranium ($\mu g \cdot L^{-1}$)	$^{a}2.11 \pm 0.5$	$^{a}4.7 \pm 1.8$	$^{a}3.5 \pm 0.9$	$^{a}2.24 \pm 0.8$	$^{a}2.15 \pm 0.8$	1.01	0.432
U sediment ($\mu g \cdot g^{-1}$)	$^{a}10.4 \pm 0.9$	$^{b}41.8 \pm 2.8$	$^{b}27.8 \pm 3.7$	$^{b}33.1 \pm 4.1$	$^{b}32.4 \pm 5.1$	14.92	0.001
Water temperature (ºC)	$^{a}13.9 \pm 1.6$	$^{a}13.9 \pm 1.7$	$^{a}13.4 \pm 1.6$	$^{a}14.3 \pm 1.9$	$^{a}15.7 \pm 1.7$	0.23	0.915
pH	$^{a}6.9 \pm 0.07$	$^{ab}7.3 \pm 0.2$	$^{b}7.6 \pm 0.05$	$^{ab}7.5 \pm 0.14$	$^{ab}7.15 \pm 0.15$	3.45	0.036
Conductivity ($\mu S\ cm^{-1}$)	$^{a}137 \pm 11$	$^{a}209 \pm 20$	$^{a}186 \pm 17$	$^{a}220 \pm 28$	$^{a}210 \pm 38$	1.83	0.174
Dissolved Oxygen ($mg \cdot L^{-1}$)	$^{a}9.6 \pm 0.6$	$^{a}7.5 \pm 1.2$	$^{a}9.8 \pm 0.5$	$^{a}9.6 \pm 0.9$	$^{a}9.5 \pm 0.7$	1.30	0.313
Nitrate ($mg \cdot L^{-1}$)*	$^{a}0.35 \pm 0.08$	$^{b}0.83 \pm 0.14$	$^{a}0.38 \pm 0.05$	$^{a}0.32 \pm 0.07$	$^{a}0.33 \pm 0.05$	15.36	0.001
Phosphate ($mg \cdot L^{-1}$)*	$^{a}0.08 \pm 0.005$	$^{b}0.40 \pm 0.07$	$^{ab}0.12 \pm 0.06$	$^{a}0.08 \pm 0.01$	$^{ab}0.09 \pm 0.006$	9.61*	0.045
Alkalinity ($mgCaCO_3\ L^{-1}$)*	$^{a}34.7 \pm 3.3$	$^{b}131.33 \pm 36.3$	$^{a}41 \pm 4$	$^{a}32.7 \pm 3.1$	$^{a}22.9 \pm 1.4$	43.82	0.001

Invertebrate communities and traits

Macroinvertebrate diversity differed across sites (ANOVA, $F_{(4,15)} = 6.91$, $p < 0.01$; Tukey´s test $p < 0.05$), being the lowest in TS1 (the site with highest uranium content in sediments) and highest in TS2 and TS4 (Figure 2). In the reference site, the most common taxa were Nemouridae (29%) and Baetidae (22%), and in the other sites the most abundant taxa were *Chironomus* sp (46%, TS1), Orthocladinae (24%, TS4), *Lymnae* sp and *Physa* sp. (20%, TS3), and *Habrophlebia* sp (11%, TS2). Macroinvertebrate assemblage composition differed between stream sites, including RF (PerManova, $F_{(4,19)} = 22.8$, $p = 0.001$; Figure 3). Life span was the only trait that was different across sites (PerManova, $F_{(4,85)} = 2.26$, $p = 0.033$; Figure 4). The TS4 stream site was represented by more individuals with life span ≤ 1 year in relation to reference (*t-test* pairwise $p = 0.003$).

A total of 87 adult Chironomidae, 38 Trichoptera and 10 Odonata was sampled from all sites across the sampling occasions, with a higher abundance in the spring (69 specimens) and lower in winter (44 specimens) (Table A4).

We collected 244 specimens of Araneae in the riparian zone, distributed among 19 families: Tetragnathidae (*Tetragnatha* sp., *Metellina* sp), Araneidae (*Larinioides* sp.), Clubionidae (*Clubiona* sp.) and Linyphiidae (*Microlinyphia* sp., *Tenuiphantes* sp, *Lepthyphantes* sp.) were the dominant groups (Table 3).

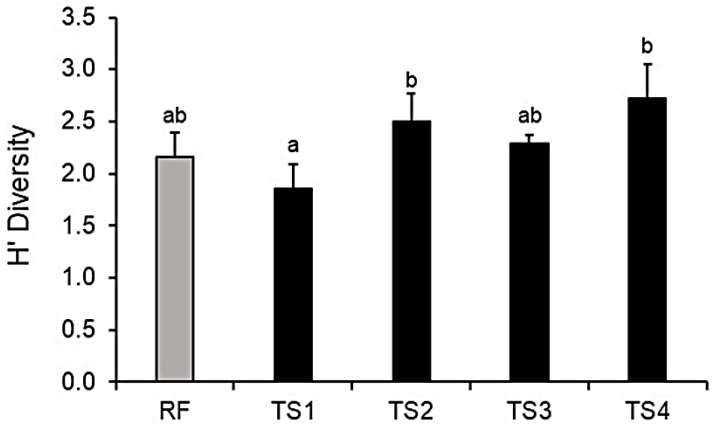


Figure 2. Macroinvertebrate diversity (Shannon-Wiener index) in four sites downstream of recovered uranium mines and a reference site in Central Portugal sites ($n = 4$; mean ± SE).

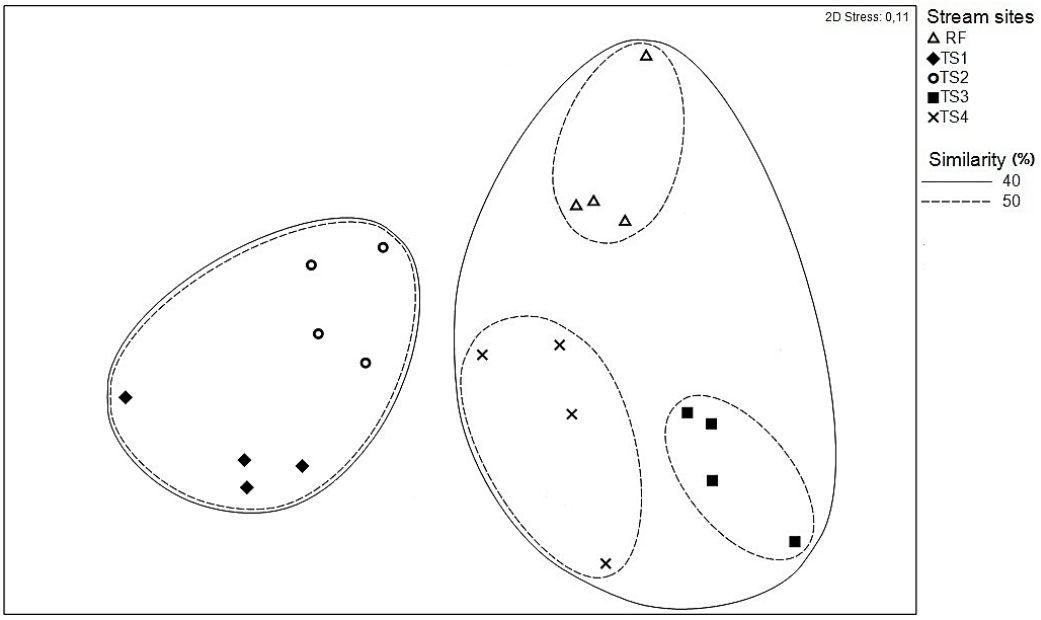


Figure 3. Non-metric Multidimensional Scaling (NMDS) with overlay of correspondent cluster analysis of samples from five stream-sites (RF, TS1, TS2, TS3, TS4) in spring, summer, autumn and winter, based on Bray-Curtis similarity matrix (log(x+1)).

Table 3. Spiders sampled in the riparian zone (<1 m from the water edge) at the five sites. NI = not identified.

Genus/Species	Number of individuals				
	RF	TS1	TS2	TS3	TS4
Pachygnatha sundevalli Senglet 1973 (Tetragnathidae)				8	
Clubiona aducta Simon 1932 (Clubionidae)				9	
Tetragnatha sp. Latreille 1804 (Tetragnathidae)	8			4	8
Pulchellodromus sp. (Philodromidae)		5	6		4
Episinus sp. (Theridiidae)			9		
Metellina sp. (Tetragnathidae)	4		10	7	
Eratigena sp.(Agelenidae)			7		
Lepthyphantes minutus Blackwall 1833 (Linyphiidae)			8		
Clubiona sp. (Clubionidae)	10		3	5	4
Larinioides sp. (Araneidae)	6	3	5		4
Dictyna sp. (Dictynidae)	8				
Nigma sp.(Dictynidae)	5				9
Metellina mengei Blackwall 1869 (Tetragnathidae)	7			8	
Tenuiphantes tenuis Blackwall 1852 (Linyphiidae)		3			11
Anyphaena sp. (Anyphaenidae)		12			
Pisaura sp. (Pisauridae)		11		7	
Microlinyphia pusilla Sundevall 1830 (Linyphiidae)		8			
Enoplognatha sp. (Theridiidae)			3		7
Araneidae (NI)		6			2

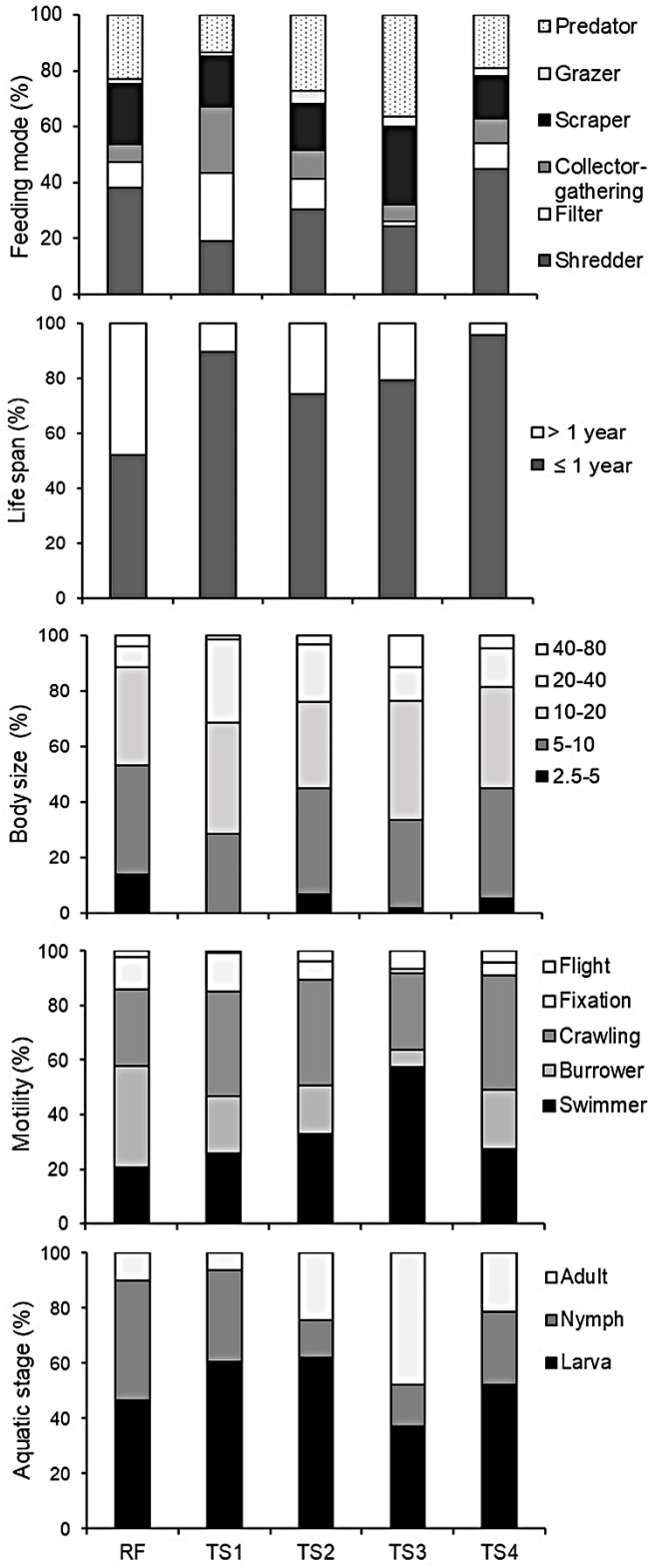


Figure 4. Relative abundances of the five traits modalities in reference (RF) and the four sites downstream recovered uranium mines (TS).

Relationship between uranium concentrations in stream water, sediments and biota, and their transfer along trophic levels

Although the range of uranium concentrations in the water and sediments was relatively low in all sites, (water: range 1.48 – 9.8 $\mu g \cdot L^{-1}$; sediments: 8.5 – 47.94 $\mu g \cdot g^{-1}$), uranium concentrations differed among almost all organisms ($F > 3.6$; $p < 0.05$; Table A5). Uranium content in the organisms was better explained by stream water than by sediment, and increased linearly mainly in litter, macrophytes, shredders and scrapers (Table 4; Figure 5). Among macroinvertebrates, scrapers had the highest amounts of uranium (14.30 ± 0.98 $\mu g \cdot g^{-1}$, TS1), followed by shredders (12.96 ± 0.81 $\mu g \cdot g^{-1}$, TS1) and engulfer predators (7.01 ± 1.3 $\mu g \cdot g^{-1}$, TS1) (Figure 6). Organic matter and producers also accumulated uranium, being highest in litter (83.76 ± 5.42 $\mu g \cdot g^{-1}$, TS1) and macrophytes (47.58 ± 6.93 $\mu g \cdot g^{-1}$, TS1) and lowest in biofilm (19.17 ± 2.75 $\mu g \cdot g^{-1}$, TS1).

Table 4. Regression analysis for aquatic organisms and uranium concentrations in water and sediments (n = 4) for each group.

Organisms	Water	Sediment
Litter	$U_{Lit} = -42.23 + 26.104 * U_{wat}$ $R^2 = 0.82$, $p < 0.001$	$U_{Lit} = -26.468 + 2.1027 * U_{wat}$ $R^2 = 0.55$, $p < 0.001$
Macrophytes	$U_{Mac} = -26.772 + 14.98 * U_{wat}$ $R^2 = 0.81$, $p < 0.001$	$U_{Mac} = -15.053 + 1.1145 * U_{wat}$ $R^2 = 0.46$, $p < 0.001$
Biofilm	$U_{Biof} = -2.8619 + 4.313 * U_{wat}$ $R^2 = 0.59$, $p < 0.001$	$U_{Biof} = -1.5243 + 0.3911 * U_{wat}$ $R^2 = 0.50$, $p < 0.01$
Shredders	$U_{Shr} = -4.4783 + 3.971 * U_{wat}$ $R^2 = 0.78$, $p < 0.001$	$U_{Shr} = -0.2554 + 0.257 * U_{wat}$ $R^2 = 0.33$, $p < 0.05$
Scrapers	$U_{Scr} = -3.4695 + 4.019 * U_{wat}$ $R^2 = 0.75$, $p < 0.001$	$U_{Scr} = 0.1401 + 0.2826 * U_{wat}$ $R^2 = 0.38$, $p < 0.01$
Predators (engulfers)	$U_{Engulfers} = 2.119 + 1.086 * U_{wat}$ $R^2 = 0.21$, $p < 0.05$	$U_{Engulfers} = 1.4503 + 0.1332 * U_{wat}$ $R^2 = 0.33$, $p < 0.01$
Predators (piercers)	$U_{Piercers} = 1.4431 + 0.486 * U_{wat}$ $R^2 = 0.18$, $p > 0.05$	$U_{Piercers} = 0.9175 + 0.0674 * U_{wat}$ $R^2 = 0.35$, $p < 0.05$

Uranium in adult Chironomidae in the riparian zone of all sites ranged from 0.29 to 2.90 $\mu g \cdot g^{-1}$, in Trichoptera from 0.25 to 0.89 and Odonata from 0.90 to 1.80 $\mu g \cdot g^{-1}$, with no significant differences between sites (Table A5). Uranium in spiders in the riparian zone ranged on average from 0.96 to 1.73 $\mu g \cdot g^{-1}$, but did not differ between sites (Figure 6). The relationship between uranium and $\delta^{15}N$ in the biological tissues varied across seasons, being stronger in autumn and weakest in summer (Figure 7, Table A6).

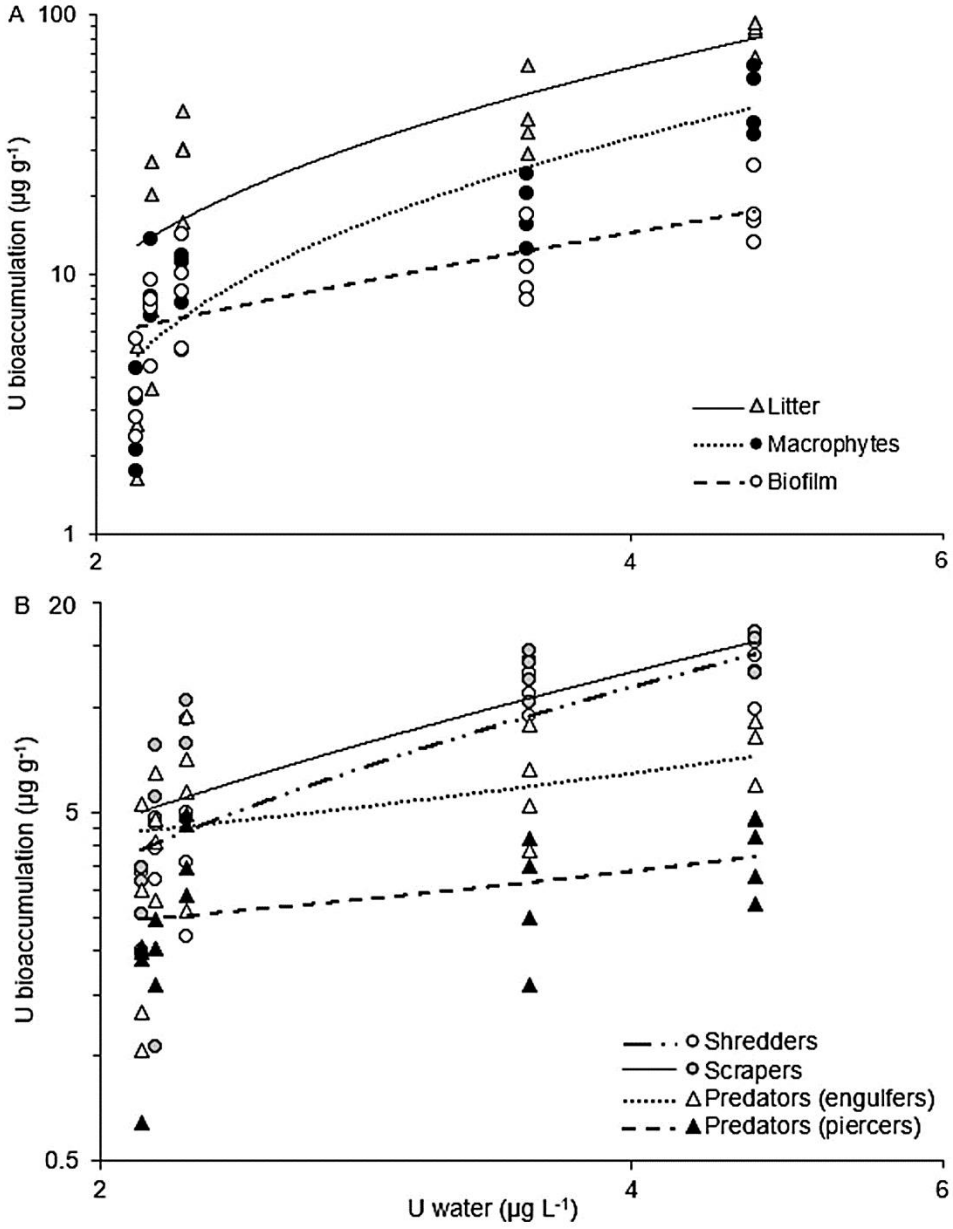


Figure 5. Relationship between uranium in aquatic biota and stream water: (A) producers, (B) consumers.

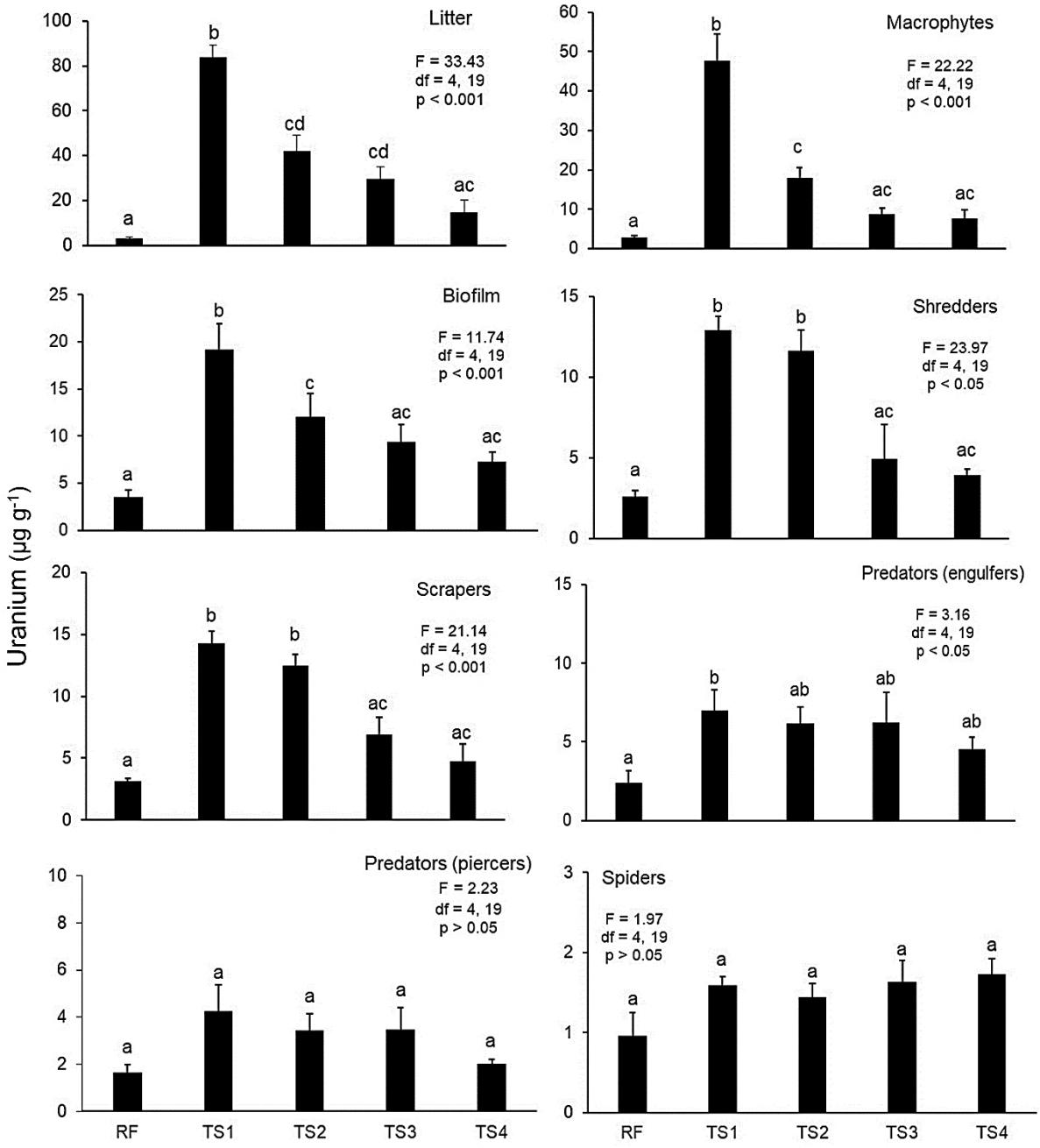


Figure 6. Uranium in litter, macrophytes (*Oenanthe crocata*; *Lemna* sp), shredders (*Calamoceras marsupus*; *Tipula* sp), scrapers (Baetidae, *Siphlonurus* sp, *Physa* sp.), predators (engulfers, Odonata: *Cordulegaster* sp), predators (piercers, Hemiptera: *Notonecta* sp, *Nepa* sp, Belostomatidae) and spiders sampled in four sites downstream a recovered uranium mine and a reference site (mean ± SE).

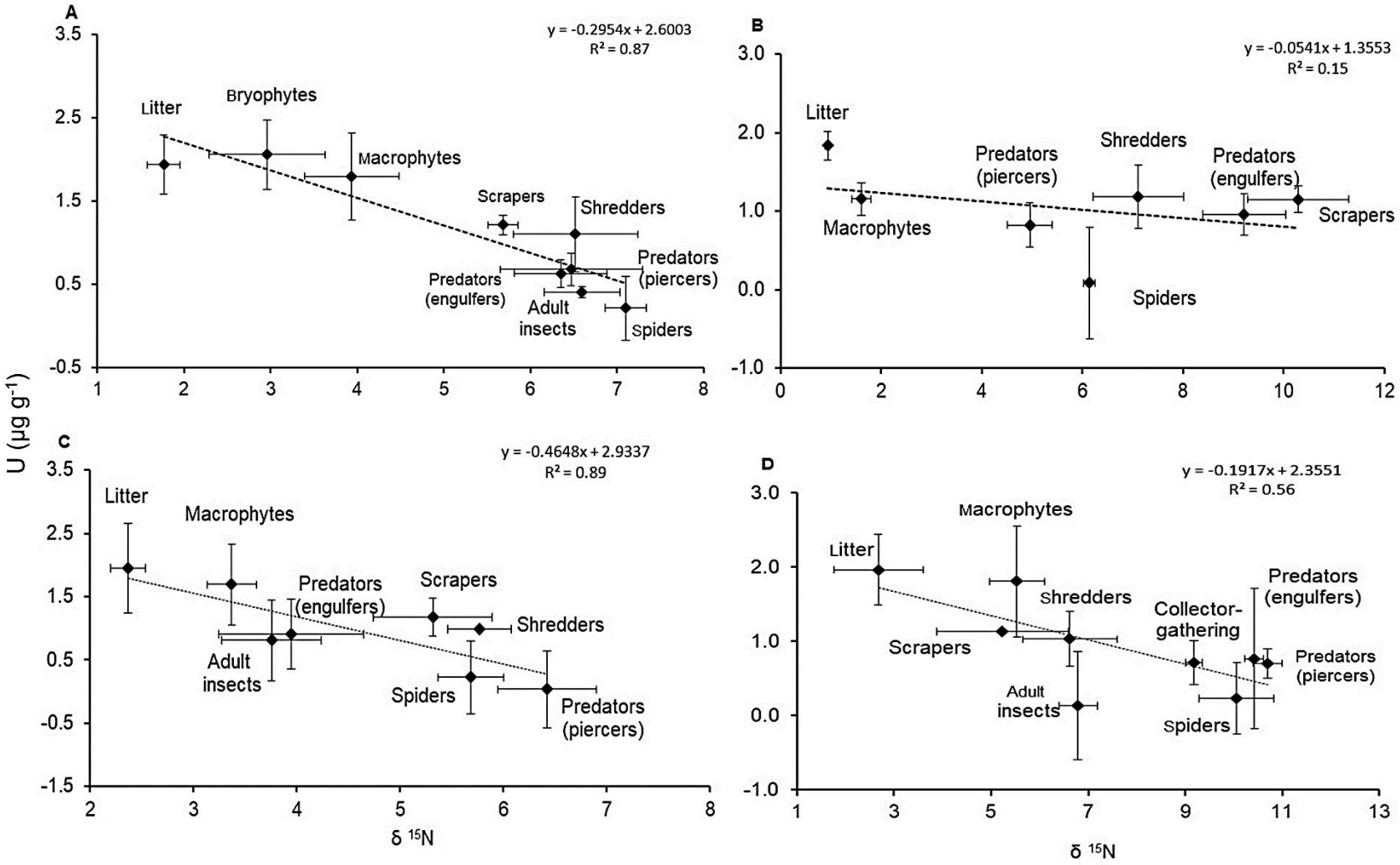


Figure 7. Relationship between uranium bioaccumulation in aquatic and terrestrial biota and nitrogen signatures (^{15}N) in the most contaminated stream site (TS1) in different seasons: (A) spring, (B) summer, (C) autumn and (D) winter. * Adult insects = aerial stages of Chironomidae, Trichoptera, Odonata.

Discussion

We asked whether stream biota takes up uranium from water and sediments, and if uranium is biomagnified along food webs and biodispersed by emerging aquatic invertebrates. Uranium concentrations in stream waters in three (out of five) sites were below the "no observed effect concentration" (NOEC) for invertebrates (2.7 $\mu g \cdot L^{-1}$) (INERIS 2008) and in only two sites was the uranium above this value (i.e., up to 4.7 $\mu g \cdot L^{-1}$). The reference site was the only site with uranium in sediments close to natural geochemical background levels (~10 $\mu g \cdot g^{-1}$ dry weight) (Lagauzère et al. 2014), but all sites had concentrations below those considered toxic to aquatic organisms (< 100 $\mu g \cdot g^{-1}$) (Sheppard et al. 2005). We can conclude that uranium levels in our system were very low. The main form of uranium in the freshwaters systems and under oxic conditions is the hexavalent U (VI), which can be found as aqueous uranyl ion (UO_2^{2+}), responsible for toxicity to aquatic organisms (Crawford et al. 2018). Uranyl ions have a strong affinity to bind to natural organic matter and sediments (Kaplan et al. 2017).

Invertebrate communities and traits

The reference site differed from the others in terms of uranium in the sediments and macroinvertebrate assemblage, however, we cannot fully impute the difference in community structure to uranium. In general there were no differences for most modalities of the traits analysed. Invertebrates with small body sizes and short life cycles may have advantages in polluted and degraded habitats, while in less disturbed areas large body sizes and long life spans are more advantageous (McKie et al. 2018; Piló et al. 2016). Accordingly, we found that the most contaminated site (TS1) had 56% of the fine sediment feeders (Chironomidae), with life spans ≤ 1 year and intermediate body size (10-20 mm).

Relationship between uranium in sediments, water and biota

Uranium concentration in the biota was correlated with uranium in stream water and sediments, indicating that organisms adsorb uranium from the environment and/or from ingested food. In previous experiments we found that the caddisfly *S. festiva* incorporated uranium from both paths, water and food, however, the prominent path may

be pollutant- specific. For example *Sericostoma vittatum* incorporated arsenic predominantly from food (Hepp et al. 2017) while *Chironomus tentans* incorporated uranium mainly from water (Muscatello & Liber 2009).

Litter and macrophytes had higher uranium content than biofilms and invertebrates. Macrophytes and algae can precipitate uranyl ions directly from solution (Jha et al. 2016), through adsorption of the hexavalent uranium by the extracellular polymeric substances (EPS) (Li et al. 2018) or bound to phosphate minerals (Nie et al. 2017). Biofilms (attached communities of algae and bacteria) can also meadiate the reduction of U (VI), leading to its intracellular immmobilisation (Krawczyk-Bärsch et al. 2012). Uranium in the organic matter (litter) was at higher or similar levels to that from macrophytes. Rooted macrophytes (for instance *Oenanthe crocata*) are able to uptake trace elements from water columns and from sediment or interstitial water, but water columns are often the predominant source of uranium (Overall & Parry 2004). Nevertheless, roots may accumulate higher quantitities of uranium from sediments and soils (Favas et al. 2016), and this could explain lower accumulation in the leaves of the macrophytes compared with dead organic matter from the most contaminated site (TS1).

Uranium transfer across trophic level

Shredders and scrapers had higher uranium than predators (~51% in TS1). Scrapers acquire uranium predominatly by food (algae and biofilm), which, with the increase of uranium in the water, also increases adsorption and intracellular accumulation, as discussed above. Scheibener et al. (2017) found that higher concentrations of uranium in solution caused higher uranium accumulation in periphyton, and that consequently the content of the metal in mayflies larvae feed on that periphyton was higher than that in the control larvae. The higher metal accumulation by shredders (here represented by Calamoceratidae and Tipulidae) in the most contaminated site can be explained by the ingestion of large quantities of leaves with high uranium content, and the close contact with sediment (with ~ 42 $\mu g \cdot g^{-1}$). This is consistent with the literature reporting that the accumulation of cadmium and copper in benthic invertebrates also increased with the concentration of the metals in sediments (Amiard et al. 2007).

Engulfers (Odonata: Cordulegasteridae) from the most contaminated site had higher uranium content than specimens from the reference site, however, this was not the

case for piercer (Hemiptera) predators. Whereas engulfers consume their prey whole and are exposed to contaminants by the exoskeleton and internal tissues, piercer predators will acquire contaminants only via internal tissues since they suck out the internal fluids of their prey (Brooks et al. 2009). Uranium bioaccumulation increased in long-lived dragonfly predators (Cordulegasteridae) from winter to summer, problably due to the consumption of larger prey by the older instars (Cremona et al. 2008). The important observation here is that uranium in predators was lower than in their prey, suggesting no bioaccumulation. Edwards et al. (2014) reported lower uranium concentrations in dragonfly larvae and tadpoles than in biofilms and detritus.

Our conclusions about the functional feeding groups were consistent with the consumers' $\delta^{15}N$ enrichment: uranium is not biomagnified along the trophic chains. Other metals such as arsenic also decrease with the increase of trophic levels in freshwater food chains (Rahman et al. 2012), while cadmium concentrations may increase within epiphyte-based food webs (Croteau et al. 2005). Metal species of methyl mercury (MeHg) may biomagnify more efficiently than total Hg, but dilutions across trophic levels are possible due to growth rates or the biomass of the organisms (Lavoie et al. 2013).

Low values for uranium in the adult insects from the riparian zone of the most contaminated site (TS1) suggest the elimination of uranium during metamorphosis. For instance, losses up to 50% of uranium were recorded for Chironomidae during metamorphosis to the adult stage, compared with the larval stage (Muscatello & Liber 2009). Uranium may also be lost through the excretion of metal-containing granules in adult stages (Kraus et al. 2014). In contrast, losses during metamorphosis were not accompanied by the elimination of persistent organic pollutants such as PCBs, which can contribute to biomagnification and the aquatic to terrestrial transfer of pollutants (Daley et al. 2011).

If uranium is not biomagnified, then it is not a surprise that spiders had similar uranium content across sites. If we assume that spiders derive a substantial proportion of their diet from aquatic emergent insects (e.g. (Nakano & Murakami 2001) and judging by the $\delta^{15}N$ enrichment in consumers, we conclude that uranium is not dispersed to land by emergence. Similarly, Lycosidae (Araneae predators) had high nitrogen signatures and the lowest uranium concentrations (0.50 $\mu g \cdot g^{-1}$), with no evidence of transfer of metals (U, Ni, Hg and Ti) from soil to the invertebrates (O'Quinn 2005). Spiders can also

accumulate lower levels or to prevent assimilation of non-essential metals when they fed on contaminated preys with lead, zinc and cadmium, (Hopkin & Martin 1985; Rybak et al. 2019).

Overall, the relationship between the trophic levels of aquatic invertebrates and metal concentrations may depend on the metal, such as a decline for Fe (biodilution), an increase for Zn (biomagnification) or no effect of metal levels on trophic position (for instance Cu) (Quinn et al. 2003).

General Conclusion

General Conclusion

Metals are taken up by aquatic invertebrates from their dissolved phase and from food (algae, detritus, sediment) (Wang & Fisher 1999). Metal transfer to higher trophic levels depends on different routes of exposure (e.g. metals accumulated from water on exoskeletons may be less bioavailable), ingestion rates, assimilation efficiency, and weight specific metabolic rates (Wang & Fisher 1999). Non-essential metals can be accumulated without excretion (in detoxified form), or with some excretion (metals are excreted in detoxified form) (Rainbow 2002). Nevertheless, there is a major problem when metals are available metabolically and bind to essential proteins, leading to a reduction in growth, exhaustion and subsequent death (Goulet & Thompson 2018). The shredder caddisfly *S. festiva* was tolerant to high concentrations of uranium in acute toxicity tests, but an impairment of growth was observed at environmentally realistic uranium concentrations (Chapter I). The results of this study also suggest that contaminated leaf-litter can be a continuous source of uranium for stream dwelling macroinvertebrates due to the rapid accumulation and slower release of the metal. Both pathways (water and food) should therefore be considered for ecological risk assessment.

The impairment of growth (and overall fitness) can be achieved at subtle levels of contaminants, while still surviving (Chapter I). It is important to be able to predict chronic or subtle effects in organism and ecosystem health and function before losses occur (Hook et al. 2014). Detectable biochemical and tissue-level changes can be used as biomarkers to identify early responses to contaminants. The results of Chapter II revealed measurable enzymatic effects on organisms exposed to uranium contamination at concentrations below the lethal dose, and below the concentrations causing reductions in growth. The changes in enzyme activities did not conform to a dose-response model (except for Na^+K^+ATPase), however, and therefore the responses are difficult to use as direct indicators of environmental stress caused by uranium. This information can be combined with other indicators when evaluating the physiological effects of the exposure of aquatic organisms to stressors.

One of the objectives of this thesis was to measure the effects of uranium on the dynamics of growth, reproduction and leaf-litter decomposition by aquatic hyphomycetes (Chapter III). This is a polyphyletic group of true fungi found on decaying plant detritus

(leaf, wood, decaying roots) in running waters (Bärlocher 2016; Sridhar 2017). The activities of the aquatic hyphomycetes (community shifts, biomass production, allocation of resources for reproduction) have been associated with rates of decomposition of organic matter and nutrient dynamics (Gessner et al. 2007). Global warming and metal contamination may affect fungal substrates (changes in riparian forests), however, and aquatic hyphomycetes resistance/tolerance (Bärlocher & Marvanová 2010). Growth and reproductive activities can be depressed for strains isolated from metal-contaminated streams (Ferreira et al. 2012), but in this thesis the performance of the fungal strains was found to be independent of their origin (uranium-contaminated or unpolluted streams) (Chapter III). The EC50 values calculated for growth assays suggested that aquatic fungi were more tolerant to uranium than aquatic invertebrates (environmental concentrations such as 25 $\mu g \cdot L^{-1}$ depressed growth, Chapter I).

Macroinvertebrates and leaf-litter accumulated uranium under laboratory (Chapters I, II and III) and field conditions, but it was not biomagnified across trophic levels (Chapter IV). Uranium bioaccumulation was higher among aquatic organisms at the most contaminated stream site, showing a strong correlation between water and sediment contamination and concentrations in biota. In laboratory assays, caddisfly body concentrations were also increased over exposure concentrations, but bioconcentration factors (BCFs) diminished from lower to higher uranium concentrations in water tests (Chapter II). This reduction in BCFs with an increase in uranium concentrations could indicate internal regulation and control in metal accumulation (McGeer et al. 2003), although higher uranium concentrations reduced the activities of the membrane associated enzymes (Chapter II).

The excretion rates of metals in aquatic organisms, physiological biodynamics, habitats and food web structure should also be considered in the bioaccumulation and biomagnification processes (Croteau et al. 2005). There was less uranium accumulation at higher trophic levels (predators, Chapter IV), than at the lower levels, probably as a result of the higher excretion rates in these organisms (e.g. Ahsanullah & Williams 1989) and lower trophic transfer of this metal (Simon & Garnier-Laplace 2005). Uranium uptake from water can be higher than that from diet (excess uranium from food can be excreted) (Ahsanullah & Williams 1989; Simon et al. 2019), and this could explain the higher accumulation by consumers at the most contaminated stream sites (Chapter IV).

Although the biomagnification of uranium is rare (Kraemer & Evans 2012), metal enrichment can be found along food webs (e.g. cadmium, Croteau et al. 2005, Dietz et al. 2000), and non-metal trace elements can be transferred intraspecifically (adults to eggs; (Conley et al. 2009). The lack of biomagnification, however, does not mean an absence of exposure or no concern about thophic transfer (European Comission 2011). Metal transfer may exhibit different patterns in specific communities (for instance, a benthic food web can differ from mollusc-crabs communities regarding biomagnification) (Zeng et al. 2013). The uranium accumulation in sediments and on biota, as found in this thesis, showed that this element was present in the analysed ecosystems, and that environmental concentrations may have subtle or physiological effects on aquatic organisms, as indicated by laboratory assays.

Final remarks

In studies for remediation of uranium contaminated areas, the determination of the local/regional food webs structures is important to predict dietary preferences and to distinguish species and habitat-specific bioaccumulation (Ofukany et al. 2014). Species-specific bioaccumulation can be distinguished among pelagic and benthic species, which will accumulate uranium from the water and food, or from the water, food and sediment, respectively. Biokinetics parameters to estimate relative contributions of diet, water and physiological conditions (e.g. growth) to metal uptake by aquatic organisms should be also considered in bioaccumulation determinations. Because the physicochemical properties of water and sediment are modifiers of the bioavailability of uranium, mechanistic and empirical models may be used to assess it across different sites/habitats (Crawford et al. 2018).

The co-occurrence of uranium and other metals leading to indirect (i.e. food webs) effects of toxicity to aquatic organisms is another point to be considered. As an example, growth stunting in fish was found in metal-polluted lakes, probably due to the reduction in communities of benthic invertebrates associated to contaminated sediment and consequently limited prey choices by the consumers (Sherwood et al. 2002). Molecular techniques (e.g. environmental DNA) may be used as a complement to the classical methods for biodiversity assessment in river catchments and even across the land-water

interface (Deiner et al. 2016), as a response of species and communities to environmental stressors.

More studies are needed to understand the effects of uranium in the aquatic and terrestrial ecosystems, as well its interaction with other metals and pollutants, to improve remediation technologies and monitoring techniques. The present thesis aimed to contribute to the understanding of this pollutant in the freshwater ecosystems.

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Appendix

Supplementary Table A1. Uranium concentrations found in streams in Portugal. (*n* = 212 locations; U values obtained by fluorometric method). Source: João Pratas, Department of Earth Sciences, University of Coimbra, Portugal.

Latitude (N)	Longitude (W)	U ($\mu g \cdot L^{-1}$)
40°28'49.1"	008°03'18.0"	0.81
40°29'12.8"	007°51'01.6"	1.22
40°29'17.7"	007°51'56.1"	18.60
40°29'40.9"	007°51'04.1"	0.52
40°28'27.0"	008°02'19.4"	0.63
40°27'44.2"	007°58'19.5"	1.84
40°28'04.0"	008°02'03.4"	0.68
40°28'13.7"	007°50'15.7"	0.54
40°20'38.6"	008°04'32.3"	1.08
40°18'30.2"	008°06'56.2"	0.39
40°19'36.8"	008°05'30.2"	1.44
40°20'16.8"	008°05'16.1"	0.74
40°22'16.3"	007°56'53.5"	0.48
40°22'33.8"	007°58'32.0"	0.47
40°20'38.1"	008°06'14.7"	113.00
40°20'38.0"	008°06'12.9"	34.67
40°29'16.7"	007°50'13.7"	1.02
40°29'12.7"	007°50'07.8"	1.18
40°29'16.3"	007°50'55.8"	1.20
40°29'07.2"	007°52'23.3"	1.73
40°29'10.2"	007°52'25.2"	1.75
40°21'15.8"	008°07'17.4"	0.50
40°22'52.1"	008°00'31.9"	3.34
40°20'41.3"	008°04'37.4"	1.20
40°22'54.9"	008°00'38.1"	14.27
40°22'57.8"	008°00'37.5"	1.02
40°32'20.2"	007°49'00.0"	3.02
40°32'04.6"	007°48'58.0"	3.19
40°32'24.5"	007°48'23.3"	3.43
40°32'13.6"	007°44'31.7"	3.01
40°33'32.0"	007°47'18.5"	3.51
40°34'37.4"	007°48'01.4"	3.65

40°30'42.8"	007°49'02.2"	8.24
40°29'52.9"	007°50'02.6"	3.09
40°29'19.4"	007°52'07.3"	56.12
40°30'05.4"	007°52'49.9"	179.00
40°19'02.3"	007°53'41.6"	2.49
40°21'17.2"	007°51'59.9"	3.76
40°19'40.5"	007°54'40.8"	3.58
40°19'35.1"	007°54'26.1"	2.98
40°19'49.7"	007°54'34.7"	3.05
40°20'14.8"	007°53'59.2"	12.38
40°20'34.4"	007°54'44.4"	6.24
40°21'31.0"	007°52'57.8"	2.59
40°21'28.8"	007°56'26.4"	2.40
40°21'08.8"	007°55'08.0"	5.46
40°21'30.3"	007°53'36.2"	2.76
40°20'52.4"	007°55'46.6"	2.59
40°22'12.9"	007°54'22.3"	2.71
40°22'28.6"	007°55'19.1"	2.89
40°22'14.7"	007°55'26.4"	3.23
40°22'23.0"	007°52'56.7"	3.15
40°23'22.6"	007°50'50.9"	2.89
40°23'30.4"	007°52'29.3"	3.06
40°34'17.2"	007°45'12.6"	1220.40
40°34'07.0"	007°46'29.4"	331.00
40°34'16.9"	007°45'19.8"	4.84
40°20'31.1"	008°06'05.1"	3.26
40°30'56.0"	007°53'15.5"	9.39
40°34'05.9"	007°46'33.3"	16.96
40°34'01.6"	007°46'23.9"	37.84
40°34'32.3"	007°46'00.3"	9.35
40°35'07.8"	007°45'23.2"	2.69
40°35'33.4"	007°48'01.1"	0.81
40°35'02.5"	007°49'23.2"	0.94
40°35'15.7"	007°49'54.8"	1.13
40°34'23.6"	007°51'34.1"	1.11
40°36'23.4"	007°52'07.3"	0.89
40°37'49.6"	007°54'08.7"	0.81

40°39'19.2"	007°47'47.4"	1.10
40°40'09.1"	007°48'35.5"	0.63
40°38'05.2"	007°49'11.5"	0.83
40°38'04.2"	007°44'14.6"	1.35
40°38'01.0"	007°41'25.0"	1.36
40°37'11.5"	007°42'22.8"	2.19
40°38'17.9"	007°38'48.6"	1.13
40°36'45.8"	007°38'53.9"	1.38
40°36'28.1"	007°41'13.4"	1.22
40°34'57.4"	007°42'20.0"	0.72
40°34'19.0"	007°43'55.3"	0.69
40°33'17.1"	007°44'05.5"	0.60
40°33'48.1"	007°41'22.2"	1.30
40°31'51.0"	007°40'50.0"	0.78
40°29'34.7"	007°37'42.2"	0.66
40°31'14.9"	007°36'40.4"	0.79
40°32'17.4"	007°37'33.6"	2.51
40°33'47.7"	007°36'19.6"	0.87
40°35'08.4"	007°32'24.8"	1.23
40°32'58.4"	007°31'41.4"	0.95
40°33'00.5"	007°31'39.6"	1.04
40°34'12.0"	007°34'47.5"	0.85
40°35'08.1"	007°34'01.4"	1.38
40°40'36.8"	007°44'23.6"	0.98
40°40'36.1"	007°43'39.8"	0.79
40°39'57.3"	007°40'52.4"	1.26
40°41'37.8"	007°41'52.1"	1.20
40°46'05.9"	007°44'19.6"	0.70
40°47'27.1"	007°44'36.8"	0.60
40°42'12.3"	007°34'29.7"	1.29
40°40'58.9"	007°31'56.8"	1.00
40°42'05.3"	007°31'04.5"	1.14
40°41'17.2"	007°24'04.8"	1.86
40°41'48.5"	007°27'02.4"	2.11
40°44'01.7"	007°27'23.4"	1.73
40°46'44.2"	007°30'22.3"	1.81
40°48'26.3"	007°31'39.3"	2.36

40°48'09.8"	007°30'00.3"	1.91
40°49'23.4"	007°28'18.6"	1.87
40°49'15.9"	007°27'48.1"	2.16
40°49'43.6"	007°28'01.8"	2.45
40°48'37.4"	007°26'54.2"	2.31
40°48'51.2"	007°25'55.0"	2.43
40°50'05.6"	007°25'02.1"	3.70
40°47'53.4"	007°26'00.4"	2.33
40°51'06.9"	007°21'01.1"	1.94
40°43'14.7"	007°33'23.2"	2.01
40°46'40.5"	007°34'35.2"	1.94
40°43'45.5"	007°37'52.6"	2.07
40°45'05.2"	007°35'50.0"	1.89
40°52'37.7"	007°30'44.3"	1.63
40°51'02.8"	007°29'47.5"	2.35
40°55'02.9"	007°30'55.5"	1.87
40°54'53.3"	007°30'48.2"	2.24
40°55'16.1"	007°32'05.7"	0.74
40°52'10.7"	007°26'45.4"	1.02
40°53'49.5"	007°22'53.7"	0.95
40°37'57.3"	007°25'59.0"	0.93
40°42'19.6"	007°19'45.2"	1.00
40°49'13.2"	007°15'43.7"	1.12
40°49'26.6"	007°13'22.4"	1.38
40°48'18.9"	007°12'07.6"	1.28
40°48'40.6"	007°11'44.8"	1.39
40°52'57.7"	007°16'44.3"	1.70
40°53'36.0"	007°16'45.0"	5.06
40°53'55.8"	007°15'01.7"	2.64
40°54'21.9"	007°13'55.2"	1.90
40°54'38.8"	007°11'15.1"	1.27
40°52'31.5"	007°10'18.1"	1.50
40°46'06.2"	007°05'11.6"	1.77
40°45'25.3"	007°04'43.7"	1.22
40°45'56.4"	007°03'08.4"	1.19
40°43'52.2"	007°01'12.0"	1.34
40°44'03.5"	007°04'28.4"	1.17

40°39'59.8"	007°05'06.2"	0.84
40°39'07.0"	007°01'38.4"	1.28
40°41'37.9"	007°00'16.3"	0.91
40°42'07.7"	007°00'17.8"	1.47
40°37'23.2"	007°00'24.5"	0.87
40°37'28.8"	007°01'40.2"	1.31
40°37'46.7"	007°02'36.9"	1.32
40°37'30.2"	007°07'02.5"	1.84
40°38'47.0"	007°08'00.1"	0.90
40°34'38.0"	007°11'18.5"	1.20
40°35'52.3"	007°12'17.8"	0.23
40°36'09.7"	007°02'45.3"	1.41
40°36'07.6"	007°01'58.1"	4.23
40°30'43.8"	007°02'47.3"	1.57
40°29'21.3"	007°02'40.2"	0.76
40°27'39.0"	007°02'26.6"	0.70
40°27'46.7"	007°01'04.2"	1.86
40°26'38.8"	007°02'34.0"	0.52
40°25'12.4"	007°02'08.8"	0.69
40°24'46.2"	007°08'31.2"	1.41
40°25'21.2"	007°07'23.0"	0.95
40°25'42.0"	007°08'38.3"	0.99
40°32'17.9"	007°07'39.4"	1.12
40°31'09.8"	007°12'54.6"	4.01
40°31'00.4"	007°14'27.6"	2.18
40°30'59.7"	007°19'48.8"	0.82
40°35'17.9"	007°17'26.6"	1.91
40°26'25.0"	007°16'08.0"	0.75
40°25'46.6"	007°17'58.0"	0.65
40°25'55.4"	007°18'08.3"	0.65
40°24'15.9"	007°20'10.9"	0.92
40°22'41.6"	007°20'52.8"	0.68
40°19'48.0"	007°19'26.6"	0.57
40°19'40.8"	007°19'50.4"	0.86
40°20'13.4"	007°17'29.4"	0.95
40°17'59.8"	007°13'23.6"	1.41
40°20'00.3"	007°13'35.7"	2.47

40°20'49.8"	007°14'10.9"	2.20
40°21'39.3"	007°11'13.0"	3.12
40°21'45.6"	007°15'51.1"	11.32
40°23'19.1"	007°15'17.1"	0.95
39°25'51.3"	007°43'18.7"	59.42
39°25'54.6"	007°43'14.6"	2.03
39°24'49.5"	007°38'50.9"	2.28
39°31'14.5"	007°36'29.7"	2.82
39°30'54.9"	007°34'56.1"	1.28
39°28'48.8"	007°33'17.3"	1.51
39°32'04.6"	007°36'32.7"	1.96
39°33'56.9"	007°33'18.8"	0.82
39°28'47.3"	007°38'12.6"	1.85
39°26'02.7"	007°32'11.7"	3.77
39°24'46.6"	007°47'58.8"	1.09
39°30'59.1"	007°46'09.4"	1.58
41°13'52.1"	007°07'29.8"	111.00
41°14'25.7"	007°07'57.0"	2.00
41°13'57.4"	007°08'16.0"	1.34
41°14'00.3"	007°08'16.3"	2.06
41°14'55.9"	007°09'05.2"	1.00
41°15'15.7"	007°08'46.2"	1.34
41°14'37.3"	007°08'13.4"	1.52
41°14'13.3"	007°07'28.0"	1.54
41°14'45.3"	007°07'39.0"	1.48
41°14'27.2"	007°06'40.6"	0.61
41°14'16.5"	007°05'41.6"	0.93
41°14'09.5"	007°05'52.6"	1.56
41°14'09.1"	007°06'17.5"	1.26
41°13'28.0"	007°06'20.7"	5.56
41°12'52.5"	007°07'02.1"	1.82
41°12'27.4"	007°06'04.4"	4.54
41°13'00.5"	007°05'43.1"	3.18

Table A2. Two-way ANOVA and Tukey HSD multiple comparison test (n = 3) of mycelial growth among fungal species and uranium concentrations.

	SS	df	MS	F	p
Species	0.21	3	0.07	97.28	< 0.001
Concentration	1.48	5	0.29	409.07	< 0.001
Species*Concentration	0.12	15	0.008	10.73	< 0.001
Total	0.03	48	0.00072		

Main effect: species	Tukey HSD multiple comparison test
V. elodeae ≠ *T. splendens*/*A. tetracladia*/*H. lugdunensis*	p < 0.001
Main effect: concentration	
16 and 262 mg L^{-1} reduced growth for all species	p < 0.001
Species*Concentration	
Control/0.004/0.064/1 mg L^{-1} *V. elodeae* < *T. splendens*/*A. tetracladia*/*H. lugdunensis*	p < 0.001
16 mg L^{-1} *A. tetracladia* > *T. splendens*	p < 0.001

Table A3. Two-way ANOVA and Tukey HSD multiple comparison test (n = 3) of mycelial growth among fungal strains and uranium concentrations.

	SS	df	MS	F	p
Strain	1.09	5	0.22	88.93	< 0.001
Concentration	10.83	5	2.16	879.57	< 0.001
Strain*Concentration	1.09	25	0.04	17.75	< 0.001
Total	0.18	72	0.0025		

Main effect: strain	Tukey HSD multiple comparison test
Strain B ≠ C/D/E/F	p < 0.01
Strains C/D/E ≠ A/B/F	p < 0.01
Strain F ≠ B/C/D/E	p < 0.01
Main effect: concentration	
1 mg L^{-1} reduced growth for strain C, and 16 mg L^{-1} for the other strains	p < 0.001
Strain*Concentration	
Control (no U exposure): A > D	p = 0.02
0.064 mg L^{-1} (F > D)	p = 0.004
16 mg L^{-1} (F > C/D/E)	p < 0.01
262 mg L^{-1} (F > A/C/D/E)	p < 0.01

Table A4. Number of adult insects collected in all sampling seasons (spring, summer, autumn and winter) and sites. NC = not collected.

Adult insects	RF	TS1	TS2	TS3	TS4
Chironomidae	16	24	22	10	15
Trichoptera	8	7	10	6	7
Odonata	3	3	NC	NC	4

Table A5. One-way Anova of uranium accumulated in groups of aquatic organisms, adult insects and spiders sampled at one reference and four stream sites downstream uranium mines.

Aquatic organisms	SS	df	MS	*F*- value	*p*
Litter	15563	4, 19	3890	33.43	< 0.001
Macrophytes (Apiaceae, Lemnaceae)	3.49	4, 19	0.87	22.22	< 0.001
Biofilm	539.59	4, 19	134.89	11.74	< 0.001
Shredders (Tipulidae, Calamoceratidae)	364.68	4, 19	91.17	23.97	< 0.001
Scrapers (Baetidae, Siphlonuridae, Physidae)	378.19	4, 19	94.54	21.14	< 0.001
Predators (engulfers, Cordulegasteridae)	53.80	4, 19	13.45	3.16	< 0.05
Predators (piercers, Nepidae, Notonectidae, Belostomatidae)	19.19	4, 19	4.79	2.23	> 0.05
Adult insects	0.95	4, 19	0.24	0.58	> 0.05
Spiders	1.49	4, 19	0.37	1.97	> 0.05

Table A6. Uranium concentrations (μg g^{-1}) and respective nitrogen signatures ($\delta^{15}N$ ‰) for aquatic, adult insects and terrestrial predators (spiders) sampled in the four seasons at the most contaminated stream site (mean ± SE). ND = not determined.

Organisms	Summer		Spring		Autumn		Winter	
	$\delta^{15}N$	U	$\delta^{15}N$	U	$\delta^{15}N$	U	$\delta^{15}N$	U
Litter	0.9 ± 0.04	67.9 ± 1.8	1.3 ± 0.2	86.26 ± 2.3	2.4 ± 0.2	88.26 ± 5.0	27.09 ± 0.9	92.54 ± 3.0
Macrophytes	1.6 ± 0.2	14.2 ± 1.1	3.4 ± 0.5	62.8 ± 3.3	3.4 ± 0.2	49.01 ± 0.2	5.5 ± 0.6	64.24 ± 5.0
Bryophytes (*Fontinalis* sp)	ND	ND	2.5 ± 0.7	114 ± 0.4	ND	ND	ND	ND
Shredders (Calamoceratidae, Tipulidae)	7.0 ± 0.9	15.3 ± 0.4	6.0 ± 1.1	12.6 ± 2.8	5.8 ± 0.3	9.7 ± 1.1	7 ± 0.1	11 ± 2
Scrapers (Ephemeroptera, Physidae)	10 ± 0.9	14.2 ± 1.5	5.2 ± 0.2	16 ± 0.8	5.3 ± 0.6	14.9 ± 2.0	6.2 ± 1.3	13.6 ± 1.0
Collector-gathering (Chironomidae)	ND	ND	ND	ND	ND	ND	9 ± 0.2	5 ± 2
Predators (engulfers: Cordulegasteridae)	9.2 ± 0.8	9.1 ± 0.5	6.0 ± 0.8	4.7 ± 1.6	3.9 ± 0.7	8.2 ± 0.3	10 ± 0.2	5.9 ± 0.1
Predators (piercers: Belostomatidae, Nepidae)	4.9 ± 0.4	6.7 ± 0.5	5.8 ± 0.5	4.3 ± 1.5	6.9 ± 0.5	1.0 ± 0.1	10.7 ± 0.3	5.1 ± 0.6
Adult insects (Chironomidae, Trichoptera, Odonata)	ND	0.59	6.0 ± 0.4	2.5 ± 1.6	3.7 ± 0.4	0.6 ± 0.2	6.8 ± 0.4	1.4 ± 0.2
Spiders	6.1 ± 0.1	1.2 ± 0.2	6.6 ± 0.2	1.6 ± 0.9	5.7 ± 0.3	1.8 ± 0.4	10 ± 0.8	1.7 ± 0.3