

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

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ABSTRACT

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

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 investigate uranium inhibition of 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) litter decomposition in laboratory microcosms exposed to uranium. The uranium concentration causing 50 % growth inhibition (EC₅₀) ranged from 12.5 to 45 mg/l, 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 EC₅₀ for the six strains ranged from 9 to 25 mg/l. Sporulation was inhibited in microcosms at uranium concentrations ≥ 1 mg/l, and the minimum concentration inhibiting litter decomposition and biomass standing crop over 24 days was 16 mg/l. Leaf-litter exposed to uranium accumulated the metal up to 89 mg/kg (in 262 mg/l of U). Overall, the amount of uranium in many streams receiving discharges from abandoned or recovered mining sites is high enough to impair fitness of some aquatic hyphomycete species.

Key words: metal pollution tolerance; fungal reproduction; mining effects

RESUMO

Urânio afeta o crescimento, esporulação, biomassa e decomposição de detritos foliares por hifomicetos aquáticos

A contaminação das águas superficiais pela mineração de urânio pode levar a efeitos nocivos à biota aquática, interferindo na reprodução, na atividade e na diversidade dos fungos aquáticos. Os efeitos do urânio no crescimento dos hifomicetos aquáticos em meio sólido foram investigados em (1) quatro espécies de fungos e (2) seis estirpes de *Heliscus lugdunensis*. Também foram investigados os efeitos na (3) esporulação, (4) biomassa e (5) decomposição de detritos foliares em microcosmos contaminados com urânio. As concentrações de urânio que causaram 50 % de inibição do crescimento (CE₅₀) estiveram entre 12.5 e 45 mg/l, sendo *Articulospora tetracladia* a espécie mais sensível e *Varicosporium elodeae* a mais tolerante. As estirpes amostradas em locais poluídos e de referência diferiram em seus graus de tolerância, mas esta foi independente da concentração de urânio nos locais onde os fungos foram isolados. A concentração de efeito (CE₅₀) para as seis estirpes variou de 9 para 25 mg/l. Nos microcosmos, a esporulação foi inibida em concentrações de urânio ≥ 1 mg/l, enquanto que a concentração mínima inibitória da decomposição dos detritos foliares e da biomassa durante 24 dias foi de 16 mg/l. Os detritos foliares expostos a urânio acumularam até 89 mg/kg na concentração de 262 mg/l. A quantidade de urânio em águas que recebem metais de minas abandonadas ou em recuperação pode ser suficientemente elevada para afetar as atividades biológicas e o desempenho dos hifomicetos aquáticos nos ecossistemas fluviais.

Palavras chave: tolerância a metais, reprodução fúngica, mineração

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 decrease 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 enzymatic activities (Hyne *et al.*, 1993; Tagliaferro *et al.*, 2018).

Although information on uranium toxicity to 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, 2012; 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 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 biominerali-

sation or genetic adaptation may be involved in 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 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 measured increased uranium concentration effects on fungal growth, sporulation, biomass and leaf-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.

MATERIALS AND METHODS

Fungal 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 $\mu\text{g/l}$ U in water and 1.0 mg/kg U in sediments). The isolated species were *Articulospora tetracladia* Ingold, *Tricladia splendens* Ingold, *Varicosporium elodeae* Kegel, *Heliscus lugdunensis* Saac. We also used six strains of a ubiquitous aquatic hyphomycete *H. lugdunensis* Sacc, five of which were sampled from uranium contaminated sites in Central Portugal (courtesy of Seena Sahadevan; Table S1, available at <http://www.limnetica.net/en/limnetica>).

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 to confirm the identification. Pure fungal cultures were maintained in malt extract agar (1.5 %; distilled water) in Petri dishes at 15 ± 1 °C 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 we measured growth inhibition in four species of aquatic hyphomycetes exposed to six concentrations of uranyl nitrate solution ($\text{UO}_2(\text{NO}_3)_2$) in malt extract agar (MEA, 1.5 %): 0; 0.004; 0.064; 1.024; 16.384 and 262 mg/l. The final design consisted of 72 Petri dishes (4 fungal species \times 6 U concentrations \times 3 replicates). In a second experiment we measured growth inhibition in six strains of *H. lugdunensis*. In this case, the final design consisted of 108 Petri dishes (6 strains \times 6 U concentrations \times 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.

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 (120 °C, 20 min), oven-dried (105 °C, 48 h), and weighed (± 0.1 mg) to obtain initial dry mass (DMi). Leaf discs were placed in 250-mL Erlenmeyer flasks containing 30 mL of sterile distilled water (18 flasks for each species and 18 flasks 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 three. By Day six, the plugs and the water were removed, and uranium solutions were added (from 0 to 262 mg/l U 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. By Day 24 the leaf discs were removed, freeze-dried weighed and stored at -20 °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. By 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 \varnothing , 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 \times in a total of 425 microscope fields. Sporulation was expressed as the number of spores/mg 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).

We extracted ergosterol (as a proxy of fungal biomass) from five of the seven lyophilised discs from each microcosm (Gessner, 2005). Discs were weighed (± 0.01 mg) and transferred to

30-mL glass tubes for ergosterol extraction and saponification with 10 mL KOH/methanol (8 g/l) at 80 °C 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 and temperature of 33 °C. 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 leaf DM.

Leaf-litter decomposition was computed as the difference between initial and final leaf discs 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 discs were weighed (± 0.01 mg), ignited in a muffle (450 °C, 8 h) for ash free dry mass (AFDM) determi-

nation, and transferred to falcon tubes with 8 mL of 2.5 M nitric acid and heated to 60 °C. After 1 h, we 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 $\mu\text{g/l}$ (Van Loon & Barefoot, 1989) at $\lambda = 530$ nm (Fluorat 02-2M, Lumex).

Data analysis

Fungal growth inhibition in 10 % (EC₁₀), 20 % (EC₂₀) and 50 % (EC₅₀) on agar plates at increasing uranium concentrations was estimated using probit analysis (log-transformed concentrations) (OECD, 2003). It was not possible to compute EC₁₀ values for some fungal species and *H. lugdunensis* strains because of large variability (inaccurate confidence intervals). To obtain an indication of the response to metal stress we calculated a tolerance index TI, given by “Growth at a given [U] \times 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 leaf-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 software Statistica Statsoft 7 and SigmaStat 3.5 software.

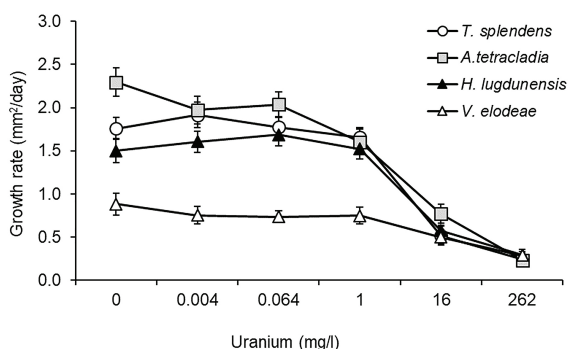


Figure 1. Growth (mm²/day) of four hyphomycetes species in agar plates under six uranium concentrations (mean \pm standard deviation; n = 3). *Crescimento (mm²/day) de quatro espécies de hifomicetos aquáticos em placas de Petri sob seis concentrações de urânio (média \pm desvio padrão; n = 3).*

Table 1. Uranium concentrations (mg/l) inhibiting mycelial growth in 10 % (EC₁₀), 20 % (EC₂₀) and 50 % (EC₅₀). NC: Not calculated. CI: 95 % Confidence interval. *Concentrações de urânio (mg/l) que causaram inibição do crescimento micelial em 10 % (CE₁₀), 20 % (CE₂₀) e 50 % (CE₅₀). NC: Não calculado. CI: Intervalo de confiança (95 %).*

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

Table 2. Uranium concentrations (mg/l) inhibiting mycelial growth in 10 % (EC₁₀), 20 % (EC₂₀) and 50 % (EC₅₀) in six strains of *H. lugdunensis* growing in increasing concentrations. 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. *Concentrações de urânio (mg/l) que causaram inibição do crescimento micelial em 10 % (CE₁₀), 20 % (CE₂₀) e 50 % (CE₅₀) nas seis estirpes de H. lugdunensis. NC: Não calculado; CI: Intervalo de confiança (95 %). As bandas em cinza escuro, cinza claro e branco representam as estirpes mais sensíveis, com sensibilidade intermediária e as mais tolerantes, respectivamente.*

Strain	U water (µg/l)	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)	9.0 (7.2 – 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)

RESULTS

Interspecific differences in growth under uranium exposure

A. tetracladia grew faster (2.29 mm²/d) under control conditions, while *V. elodeae* grew slowest (0.88 mm²/d; Fig. 1) among the four species of aquatic hyphomycetes. Mycelial growth differed among species ($F_{3,15} = 97.28$; $p < 0.001$), and uranium concentrations ($F_{5,15} = 409.07$; $p < 0.001$), Table S2, available at <http://www.limnetica.net/en/limnetica>. The uranium concentration causing 50 % mycelial growth inhibition (EC₅₀) ranged from 12.5 to 45 mg/l (Table 1), with *A. tetracladia* the most sensitive and *V. elodeae* the

most tolerant species. A uranium concentration of 0.85 mg/l was enough to cause a 20 % reduction in growth in *A. tetracladia*, while the same 20 % inhibition in *H. lugdunensis* occurred at 3 mg/l. At the lower uranium concentrations (< 1 mg/l), *T. splendens* and *H. lugdunensis* grew up to 12 % faster than the control (Table S3, available at <http://www.limnetica.net/en/limnetica>).

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 S4, available at <http://www.limnetica.net/en/limnetica>. Under control conditions (no uranium) growth in agar

plates ranged from 1.26 mm²/d (Strain D, from the most contaminated site) to 1.89 mm²/d (Strain A) (Fig. 2). Inhibition was unrelated to the origin of the strains (Tables 2 and S5, available at <http://www.limnetica.net/en/limnetica>). 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.

Colony morphology

Colony morphology differed among strains and concentrations. The colonies grew with a regular boundary for all strains and uranium concentrations < 1 mg/l. 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/l (Fig. 3).

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

The pH in the microcosms averaged 7.3 ± 0.7 except for uranium concentration of 262 mg/l, where the pH was 4.8 ± 0.3 . Under control condi-

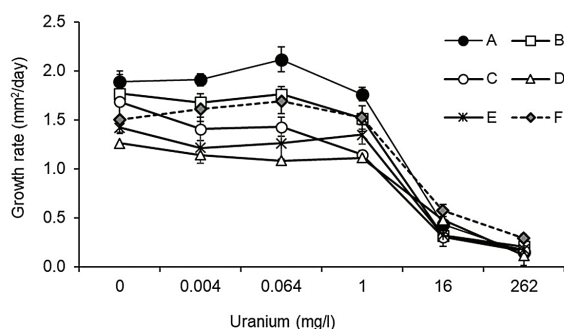


Figure 2. Growth (mm²/day) of six *Heliscus lugdunensis* strains in agar plates under six uranium concentrations (mean \pm standard deviation; $n = 3$). *Crescimento (mm²/day) de seis estirpes de Heliscus lugdunensis em placas de Petri sob seis concentrações de urânio (média \pm desvio padrão; $n = 3$).*

tions, average spore production ranged from 86 (*A. tetracladia*) to 3,138 spores per mg dry mass (*H. lugdunensis*) (Fig. 4). Under low uranium concentration (up to 0.064 mg/l), sporulation rates were stimulated in some species, but inhibited at concentrations above 1 mg/l (*V. elodeae*) and 262 mg/l (*H. lugdunensis*). At 262 mg/l, *T. splendens* and *A. tetracladia* demonstrated 64 % and 100 % of inhibition in sporulation when compared to control, respectively (Fig. 4).

Fungal biomass in leaf discs under control conditions ranged from 18 (*H. lugdunensis*) to 23 (*V. elodeae*) mg/g DM. Fungal biomass was reduced by uranium at concentrations of 16 mg/l (*T. splendens* and *H. lugdunensis*) and 262 mg/l (*A. tetracladia*) (Fig. 5). Leaf-litter decomposition was reduced by 16 mg/l for all species, except for *A. tetracladia* (Fig. 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). After 24 days in the microcosms, the leaf discs had increased levels of uranium according to concentrations in the solutions tests (Fig. 7), up to 89 mg/kg in 262 mg/l.

DISCUSSION

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

In our experiments, uranium concentrations of 0.4 – 9 mg/l inhibited mycelial growth in 20 % to 50 % (Table 2). It is apparent that aquatic hyphomycetes are less tolerant to uranium than

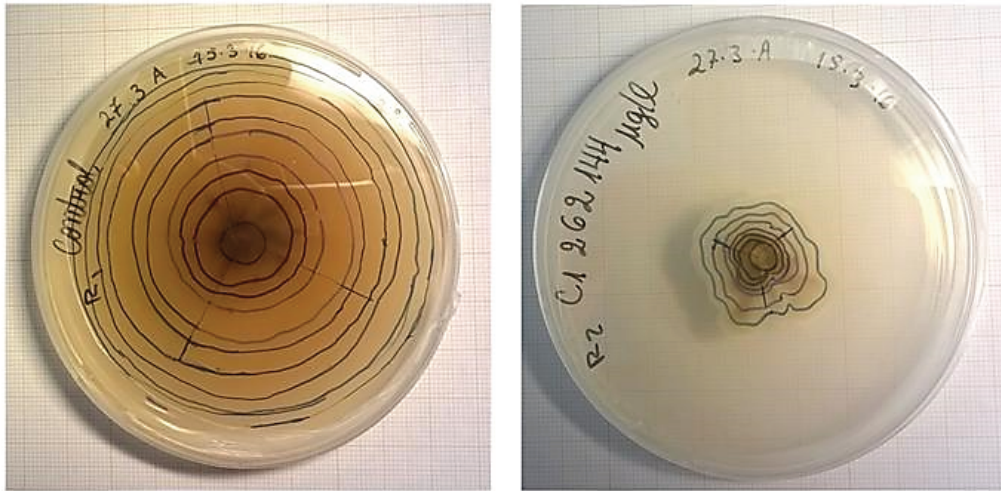


Figure 3. Colony morphology of *Heliscus lugdunensis* (strain A) grown under control (no uranium) (left) and under 262 mg/l of uranium (right). At high uranium concentration the colony edge becomes irregular and has a different color (lines indicate the weekly increases in area). *Morfologia da colônia de Heliscus lugdunensis (estirpe A) crescida sem adição de urânio (controle, esquerda) e sob concentração de 262 mg/l (direita). Em concentrações elevadas de urânio, as margens das colônias eram mais irregulares e com diferente coloração (as linhas indicam o crescimento semanal em área).*

other fungi such as *Aspergillus niger* and *Paecilomyces javanicus* whose tolerance indices ranged from 20 to 28 % in 10 mM uranium (400 mg/l) (Liang *et al.*, 2015). Another example is the growth ability of *Rhizopus arrhizus* in 200 mg/l that reflects its maximum biosorption capacity and removal of uranyl ions under acidic pH (Wang *et al.*, 2010). On the other hand, aquatic hyphomycetes were more tolerant than the algae *Chlorella* sp whose growth was inhibited by 50 % at 78 μ g/l due to greater binding of uranium to the cell surface and its uptake into the cell (Franklin *et al.*, 2000). Higher uranium concentrations such as 200 μ M (8 mg/l) inhibited growth of the bacterial strains from subsurface sediments under experimental conditions (Brzoska & Bollmann, 2016). These bacterial strains can survive well in the natural environment, however, even in the presence of metals contaminants probably due to their ability to denitrify and consequently to be less susceptible to toxic effects of uranium (Brzoska & Bollmann, 2016). Compared with aquatic invertebrates, values similar to the lowest inhibition rates in this study (\sim 1 mg/l, EC10) caused 50 % immobility for *D. magna* (1.4 mg/l) (Antunes *et al.*, 2007) or 50%

mortality in *Hyaella azteca* (LC50; 1.52 mg/l) (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 for *A. tetracladia*, whereas sporulation was inhibited for this same species and for *V. elodeae* from 1 mg/l (Fig. 4).

Conidia production has been correlated to aquatic hyphomycete decomposition activity (Duarte *et al.*, 2004). Aquatic hyphomycetes can allocate \sim 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 (\sim 46 - 58 %) in the 16 and/or 262 mg/l 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 with leaf-litter mass loss (Sridhar *et al.*, 2001).

The increased accumulation/biosorption of

uranium in leaf discs across concentrations may change the physical structure of leaf discs tissues (Ding *et al.*, 2012) and may have limited the availability of oxygen and inorganic compounds for the mycelia (Sridhar *et al.*, 2001). The reduction in

sporulation and leaf-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

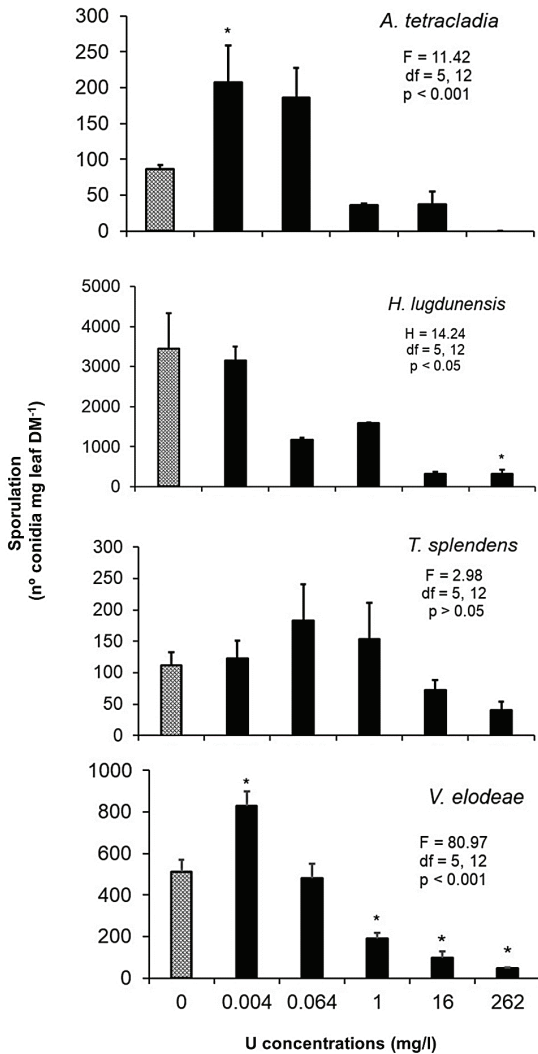


Figure 4. Cumulative conidia production of four aquatic hyphomycetes species (mean \pm standard error) growing in leaves exposed to increased uranium concentrations for 24 days; $n = 3$; one-way ANOVA followed by Holm-Sidak test against the control, or Rank ANOVA followed by Dunn's Method; * = $p < 0.05$. *Produção cumulativa de esporos por quatro espécies de hifomicetos aquáticos (média \pm erro padrão) em discos foliares expostos a concentrações crescentes de urânio durante 24 dias; $n = 3$; ANOVA de uma via e análise post-hoc contra o grupo controle (Holm-Sidak), ou Rank ANOVA e teste de Dunn: * = $p < 0.05$.*

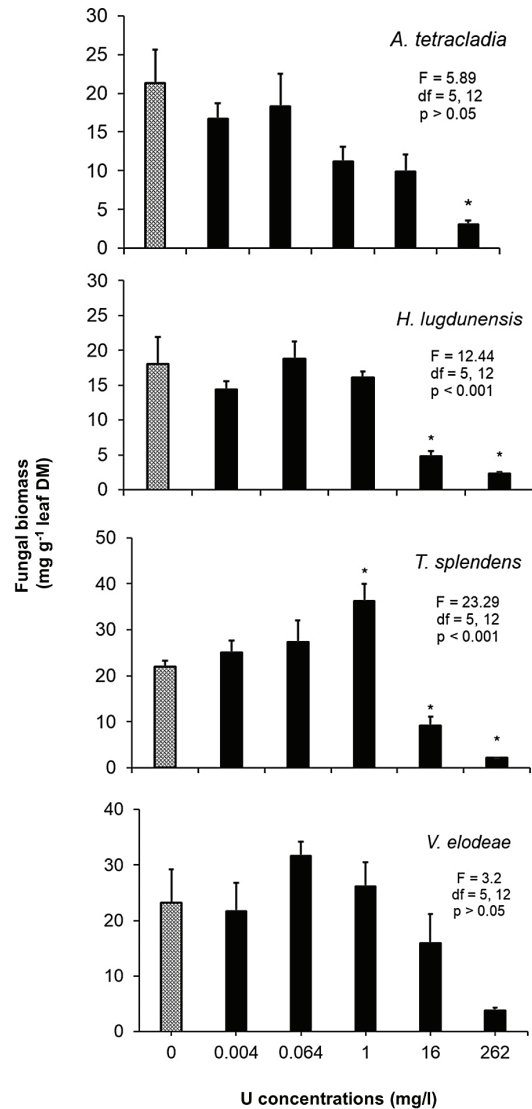


Figure 5. Fungal biomass of four aquatic hyphomycetes species (mean \pm standard error) growing in leaves exposed to increased uranium concentrations for 24 days; $n = 3$; one-way ANOVA followed by Holm-Sidak test against the control; * = $p < 0.05$. *Biomassa de quatro espécies de hifomicetos aquáticos (média \pm erro padrão) em discos foliares expostos a concentrações crescentes de urânio durante 24 dias; $n = 3$; ANOVA de uma via e análise post-hoc contra o grupo controle (Holm-Sidak); * = $p < 0.05$.*

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 concentration of 16 mg/l. Low pH (< 5) has been shown to decrease litter decomposition in acidic streams (Cornut *et al.*, 2012; Ferreira & Guérol, 2017), by reducing the abundance and biomass of the sensitive shredders (Dangles & Guérol, 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, but it causes 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 at low uranium concentrations in *A. tetracladia* and *V. elodeae* (Fig. 4). The faster growth for the lower uranium concentrations tested, however, may reveal the capacity of fungi to deal with metals. Fungal biomass may biosorb up to 600 mg/g dry weight for uranium from aqueous solutions (Ogar *et al.*, 2014). Uranium adsorption by *A. tetracladia* mycelium was 140 mg/g dry weight at 2000 $\mu\text{g/l}$, indicating that native hyphomycetes can retrieve the metal from the stream water (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 leaf-litter decomposition. The growth inhibition observed at the highest U concentrations indicates toxicity. High uranium concentration

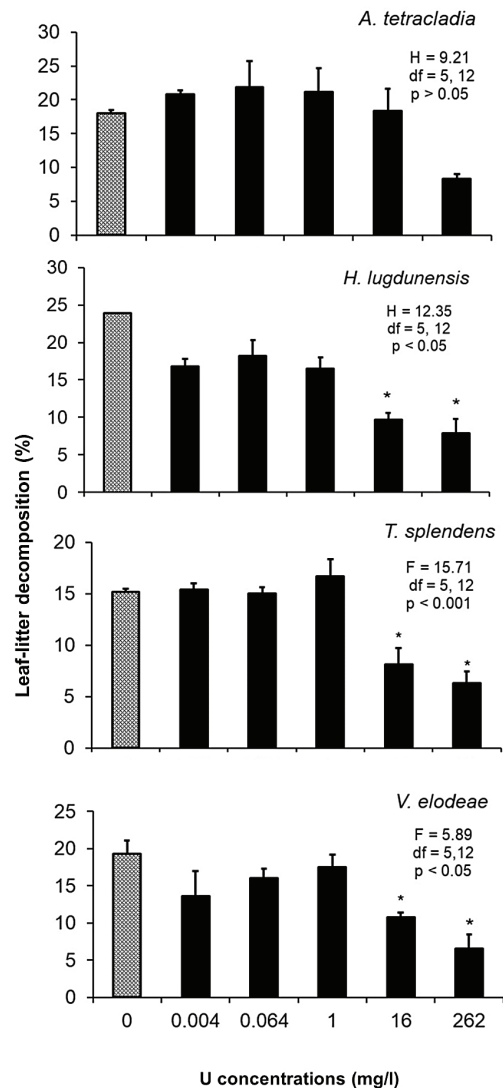


Figure 6. Leaf-litter decomposition for the four aquatic hyphomycetes species (mean \pm standard error) growing in leaves exposed to increased uranium concentrations for 24 days; $n = 3$; one-way ANOVA followed by Holm-Sidak test against the control, or Rank ANOVA followed by Dunn's Method; * = $p < 0.05$. *Decomposição foliar para quatro espécies de hifomicetos aquáticos (média \pm erro padrão) em discos foliares expostos a crescentes concentrações de urânio durante 24 dias; $n = 3$; ANOVA de uma via e análise post-hoc contra o grupo controle (Holm-Sidak), ou Rank ANOVA e teste de Dunn: * = $p < 0.05$.*

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/l might have favoured 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 coping 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 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 contamina-

tion. In this same way, conidia produced at a polluted site may be transported away from the polluted site.

CONCLUSION

Growth, sporulation, biomass and leaf mass loss were significant at uranium concentrations > 1 mg/l. There are interspecific differences in fungal sensitivity to uranium. Finally, the performance of *H. lugdunensis* strains was independent of pollution levels at the site of origin, suggesting differences in aquatic hyphomycetes sensitivity.

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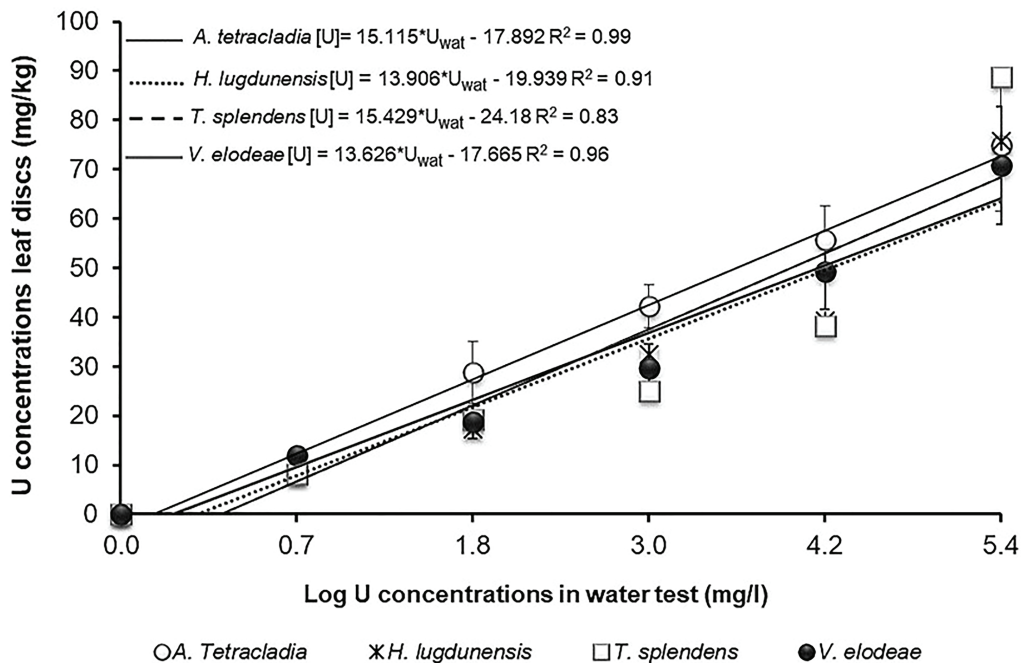


Figure 7. Amounts of uranium in alder leaves exposed to increased concentrations of the metal (mean \pm standard error), $n = 3$. *Quantidade de urânio em folhas de amieiro expostas a crescentes concentrações do metal (média \pm erro padrão); $n = 3$.*

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REFERENCES

- ALAM, M. S. & T. CHENG. 2014. Uranium release from sediment to groundwater: Influence of water chemistry and insights into release mechanisms. *Journal of Contaminant Hydrology*, 164: 72–87. DOI: 10.1016/j.jconhyd.2014.06.001
- ANTUNES, S. C., D. R. DE FIGUEIREDO, S. M., MARQUES, B. B. CASTRO, R. PEREIRA & F. GONÇALVES. 2007. Evaluation of water column and sediment toxicity from an abandoned uranium mine using a battery of bioassays. *Science of the Total Environment*, 374: 252–259. DOI: 10.1016/j.scitotenv.2006.11.025
- BALDRIAN, P. & J. GABRIEL. 2002. Intraspecific variability in growth response to cadmium of the wood-rotting fungus *Piptoporus betulinus*. *Mycologia*, 94: 428. DOI: 10.2307/3761777
- BÄRLOCHER, F. 2005. Sporulation by aquatic hyphomycetes, In: *Methods to Study Litter Decomposition: A Practical Guide*. M.A.S. Graça, F. Bärlocher & M.O. Gessner (eds.): 185 - 188, Springer Netherlands.
- BÄRLOCHER, F. (ed.). 2012. *The ecology of aquatic hyphomycetes*. Springer-Verlag Berlin Heidelberg. DOI: 10.1007/978-3-642-76855-2
- BAUDOIN, J. M., F. GUÉROLD, V. FELTEN, E. CHAUVET, P. WAGNER & P. ROUSSELLE. 2008. Elevated aluminium concentration in acidified headwater streams lowers aquatic hyphomycete diversity and impairs leaf-litter breakdown. *Microbial Ecology*, 56: 260–269. DOI: 10.1007/s00248-007-9344-9
- BAYRAMOĞLU, G., G. ÇELİK, G. & M. Y. ARICA. 2006. Studies on accumulation of uranium by fungus *Lentinus sajor-caju*. *Journal of Hazardous Materials*, 136: 345–353. DOI: 10.1016/j.jhazmat.2005.12.027.
- BERGMANN, M., O. SOBRAL, J. PRATAS & M. A. S. GRAÇA. 2018. Uranium toxicity to aquatic invertebrates: A laboratory assay. *Environmental Pollution*, 239: 359–366. DOI: 10.1016/j.envpol.2018.04.007
- BRAHA, B., H. TINTEMANN, G. KRAUSS, J. EHRMAN, F. BÄRLOCHER & G. J. KRAUSS. 2007. Stress response in two strains of the aquatic hyphomycete *Heliscus lugdunensis* after exposure to cadmium and copper ions. *BioMetals*, 20: 93–105. DOI: 10.1007/s10534-006-9018-y
- BRZOSKA, R. M. & A. BOLLMANN. 2016. The long-term effect of uranium and pH on the community composition of an artificial consortium. *FEMS Microbiology Ecology*, 92: 1–8. DOI: 10.1093/femsec/fiv158
- CALABRESE, E. J. & L. A. BALDWIN, L. A. 2001. Hormesis: A generalizable and unifying hypothesis. *Critical Reviews in Toxicology*, 31: 353–424.
- CHUNG, N. & K. SUBERKROPP. 2009. Contribution of fungal biomass to the growth of the shredder, *Pycnopsyche gentilis* (Trichoptera: Limnephilidae). *Freshwater Biology*, 54: 2212–2224. DOI: 10.1111/j.1365-2427.2009.02260.x
- CORNUT, J., H. CLIVOT, E. CHAUVET, A. ELGER, C. PAGNOUT & F. GUÉROLD. 2012. Effect of acidification on leaf litter decomposition in benthic and hyporheic zones of woodland streams. *Water Research*, 46: 6430–6444. DOI: 10.1016/j.watres.2012.09.023
- DANGLES, O. & F. GUÉROLD. 2001. Linking shredders and leaf litter processing: Insights from an acidic stream study. *International Review of Hydrobiology*, 86: 395–406. DOI: 10.1002/1522-2632(200107)86:4/5
- DANGLES, O., M. O. GESSNER, F. GUÉROLD & E. CHAUVET. 2004. Impacts of stream acidification for assessing ecosystem functioning implications. *Journal of Applied Ecology*, 41: 365–378.
- DESCALS, E. 2005. Techniques for handling ingoldian fungi. In: *Methods to Study Litter Decomposition: A Practical Guide*. M.A.S. Graça, F. Bärlocher & M.O. Gessner (eds.): 129 - 141. Springer Netherlands.
- DING, D. X., X. T. LIU, N. HU, G. Y. LI & Y. D. WANG. 2012. Removal and recovery of

- uranium from aqueous solution by tea waste. *Journal of Radioanalytical and Nuclear Chemistry*, 293: 735–741. DOI: 10.1007/s10967-012-1866-z
- DUARTE, S., C. PASCOAL & F. CÁSSIO. 2004. Effects of zinc on leaf decomposition by fungi in streams: Studies in microcosms. *Microbial Ecology*, 48: 366–374. DOI: 10.1007/s00248-003-2032-5
- DUARTE, S., C. PASCOAL, A. ALVES, A. CORREIA & F. CÁSSIO. 2008. Copper and zinc mixtures induce shifts in microbial communities and reduce leaf litter decomposition in streams. *Freshwater Biology*, 53: 91–101. DOI: 10.1111/j.1365-2427.2007.01869.x
- FABRE, E. 1997. Changes in concentration of aquatic hyphomycete conidia in water passing through a concrete pipe. *Mycological Research*, 101: 908–910. DOI: 10.1017/S0953756297003948
- FERREIRA, V., A.L. GONÇALVES, J. PRATAS & C. CANHOTO. 2010. Contamination by uranium mine drainages affects fungal growth and interactions between fungal species and strains. *Mycologia*, 102: 1004–1011. DOI: 10.3852/09-248
- FERREIRA, V. & F. GUÉROLD. 2017. Leaf litter decomposition as a bioassessment tool of acidification effects in streams: Evidence from a field study and meta-analysis. *Ecological Indicators*, 79: 382–390. DOI: 10.1016/j.ecolind.2017.04.044
- FOMINA, M., J. M. CHARNOCK, S. HILLIER, R. ALVAREZ & G. M. GADD. 2007. Fungal transformations of uranium oxides. *Environmental Microbiology*, 9: 1696–1710. DOI: 10.1111/j.1462-2920.2007.01288.x
- FOMINA, M., J. M. CHARNOCK, S. HILLIER, R. ALVAREZ, F. LIVENS & G. M. GADD. 2008. Role of fungi in the biogeochemical fate of depleted uranium. *Current Biology*, 18: 375–377. DOI: 10.1016/j.cub.2008.03.011
- FRANKLIN, N. M., J. L. STAUBER, S. J. MARKICH & R. P. LIM. 2000. pH-dependent toxicity of copper and uranium to a tropical freshwater alga (*Chlorella* sp.). *Aquatic Toxicology*, 48: 275–289. DOI: 10.1016/S0166-445X(99)00042-9
- GESSNER, M. O. & E. CHAUVET. 1997. Growth and production of aquatic hyphomycetes in decomposing leaf litter. *Limnology and Oceanography*, 42: 496–505. DOI: 10.4319/lo.1997.42.3.0496
- GESSNER, M. O. 2005. Ergosterol as a measure of fungal biomass. In: *Methods to Study Litter Decomposition: A Practical Guide*. M.A.S. Graça, F. Bärlocher & M.O. Gessner (eds.): 189 - 195, Springer Netherlands.
- GESSNER, M. O., V. GULIS, K. A. KUEHN, E. CHAUVET & K. SUBERKROPP. 2007. Fungal decomposers of plant litter in aquatic ecosystems. In: *The Mycota - Environmental and Microbial Relationships*. C.P. Kubicek, I.S. Druzhinina (ed.): 301–324. Springer, Berlin Heidelberg.
- GESSNER, M. O. & E. CHAUVET. 1993. Ergosterol-to -biomass conversion factors for aquatic hyphomycetes. *Applied and Environmental Microbiology*, 59: 502–507. <https://aem.asm.org/content/aem/59/2/502.full.pdf>
- GONÇALVES, A. L., A.V. LÍRIO, J. PRATAS & C. CANHOTO. 2011. Uranium contaminated water does not affect microbial activity but decreases feeding by the shredder *Sericostoma vittatum*. *Fundamental and Applied Limnology/ Arch. für Hydrobiologie*, 179: 17–25. DOI: 10.1127/1863-9135/2011/0179-0017
- HYNE, R. V., G. D. RIPPON & G. ELLENDER. 1993. Investigation of Uranium-induced toxicity in freshwater Hydra. In: *Ecotoxicology of Metals in Invertebrates*. R. Dallinger, P. Rainbow (eds.). Lewis Publishers.
- ISAURE, M. P., B. LEYH, M. SALOMÉ, G. J. KRAUSS, D. SCHAUMLÖFFEL & D. DOBRITZSCH. 2017. The aquatic hyphomycete *Heliscus lugdunensis* protects its hyphae tip cells from cadmium: A micro X-ray fluorescence and X-ray absorption near edge structure spectroscopy study. *Spectrochimica Acta - Part B Atomic Spectroscopy*, 137: 85–92. DOI: 10.1016/j.sab.2017.09.012
- KOK, C. J., W. HAVERKAMP & H. A. VAN DER AA. 1992. Influence of pH on the growth and leaf-maceration ability of fungi involved in the decomposition of floating leaves of *Nymphaea alba* in an acid water. *Journal of General Microbiology*, 138: 103–108. DOI: 10.1099/00221287-138-1-103

- KRAUSS, G., F. BÄRLOCHER, P. SCHRECK, R. WENNRICH, W. GLÄSSER & G. J. KRAUSS. 2001. Aquatic hyphomycetes occur in hyperpolluted waters in Central Germany. *Nova Hedwigia*, 72: 419–428. DOI: 10.1127/NOVA.HEDWIGIA/72/2001/419
- KRAUSS, G. J., M. SOLÉ, G. KRAUSS, D. SCHLOSSER, D. WESENBERG & F. BÄRLOCHER. 2011. Fungi in freshwaters: Ecology, physiology and biochemical potential. *FEMS Microbiology Reviews*, 35: 620–651. DOI: 10.1111/j.1574-6976.2011.00266.x
- KUHNE, W. W., C. A. CALDWELL, W. R. GOULD, P. R. FRESQUEZ & S. FINGER. 2002. Effects of depleted uranium on the health and survival of *Ceriodaphnia dubia* and *Hyalella azteca*. *Environmental Toxicology and Chemistry*, 21: 2198–2203. DOI: 10.1002/etc.5620211025
- LIANG, X., S. HILLIER, H. PENDLOWSKI, N. GRAY, A. CECI & G. M. GADD. 2015. Uranium phosphate biomineralization by fungi. *Environmental Microbiology*, 17: 2064–2075. DOI: 10.1111/1462-2920.12771
- MAHARNING, A. R. & F. BÄRLOCHER. 1996. Growth and Reproduction in Aquatic Hyphomycetes. *Mycologia*, 88: 80–88.
- MEDEIROS, A. O., P. ROCHA, C. A. ROSA & M. A. S. GRAÇA. 2008. Litter breakdown in a stream affected by drainage from a gold mine. *Fundamental and Applied Limnology / Arch. für Hydrobiologie*, 172: 59–70. DOI: 10.1127/1863-9135/2008/0172-0059
- MEDEIROS, A., S. DUARTE, C. PASCOAL, F. CÁSSIO & M. A. S. GRAÇA. 2010. Effects of Zn, Fe and Mn on leaf litter breakdown by aquatic fungi: A microcosm study. *International Review of Hydrobiology*, 95: 12–26. DOI: 10.1002/iroh.200911182
- MIERSCH, J., F. BÄRLOCHER, I. BRUNS & G. J. KRAUSS. 1997. Effects of cadmium, copper, and zinc on growth and thiol content of aquatic hyphomycetes. *Hydrobiologia*, 346: 77–84. DOI: 10.1023/A:1002957830704
- MUSCATELLO, J. R. & K. LIBER. 2009. Accumulation and chronic toxicity of uranium over different life stages of the aquatic invertebrate *Chironomus tentans*. *Archives of Environmental Contamination and Toxicology*, 57: 531–539. DOI: 10.1007/s00244-009-9283-1
- OECD, 2003. *Draft guidance document for on the statistical analysis of ecotoxicity data* (Technical Report). Environment Directorate Organization for Economic Co-operation and Development, Paris.
- OGAR, A., A. GRANDIN, V. SJÖBERG, K. TURNAU & S. KARLSSON. 2014. Stabilization of Uranium(VI) at low pH by fungal metabolites: Applications in environmental biotechnology. *APCBEE Procedia*, 10: 142–148. DOI: 10.1016/j.apcbee.2014.10.032
- PEREIRA, R., S. BARBOSA & F. P. CARVALHO. 2014. Uranium mining in Portugal: A review of the environmental legacies of the largest mines and environmental and human health impacts. *Environmental Geochemistry and Health*, 36: 285–301. DOI: 10.1007/s10653-013-9563-6
- PRADHAN, A., S. SEENA, D. DOBRITZSCH, S. HELM, K. GERTH, M. DOBRITZSCH, G. J. KRAUSS, D. SCHLOSSER, C. PASCOAL & F. CÁSSIO. 2014. Physiological responses to nanoCuO in fungi from non-polluted and metal-polluted streams. *Science of the Total Environment*, 466–467: 556–563. DOI: 10.1016/j.scitotenv.2013.07.073
- PRADHAN, A., S. SEENA, D. SCHLOSSER, K. GERTH, S. HELM, M. DOBRITZSCH, G. J. KRAUSS, D. DOBRITZSCH, C. PASCOAL & F. CÁSSIO. 2015. Fungi from metal-polluted streams may have high ability to cope with the oxidative stress induced by copper oxide nanoparticles. *Environmental Toxicology and Chemistry*, 34: 923–930. DOI: 10.1002/etc.2879
- SRIDHAR, K. R., G. KRAUSS, F. BÄRLOCHER, N. S. RAVIRAJA, R. WENNRICH, R. BAUMBACH & G. J. KRAUSS. 2001. Decomposition of alder leaves in two heavy metal-polluted streams in central Germany. *Aquatic Microbial Ecology*, 26: 73–80. DOI: 10.3354/ame026073
- STRANDBERG, G. W., S. E. SHUMATE & J. R. PARROT. 1981. Microbial Cells as Biosorbents for Heavy Metals: Accumulation of uranium by *Saccharomyces cerevisiae* and *Pseudomonas aeruginosa*. *Applied Environmental Microbiology*, 41: 237–245.
- SUBERKROPP, K. & T. L. ARSUFFI. 1984.

- Degradation, growth, and changes in palatability of leaves colonized by six aquatic hyphomycete species. *Mycologia*, 76: 398–407.
- TAGLIAFERRO, M., A. M. M. GONÇALVES, M. BERGMAN, O. SOBRAL, O. & M. A. S. GRAÇA. 2018. Assessment of metal exposure (uranium and copper) by the response of a set of integrated biomarkers in a stream shredder. *Ecological Indicators*, 95: 991-1000 DOI: 10.1016/j.ecolind.2017.10.065
- UNITED NATIONS. 2015. *Adoption of the Paris Agreement*, 21st Conference of the Parties (Report).
- VAN LOON, J. C. & R. R. BAREFOOT. 1989. *Analytical methods for geochemical exploration*. San Diego: Academic Press.
- WANG, J., X. HU, J. WANG, Z. BAO, S. XIE & Y. JINHUI. 2010. The tolerance of *Rhizopus arrhizus* to U(VI) and biosorption behavior of U(VI) onto *R. arrhizus*. *Biochemical Engineering Journal*, 51: 19–23. DOI: 10.1016/j.bej.2010.04.010
- ZHENG, X. Y., X. Y. WANG, Y. H. SHEN, X. LU, T. S. WANG. 2017. Biosorption and biomineralization of uranium(VI) by *Saccharomyces cerevisiae*—Crystal formation of chernikovite. *Chemosphere*, 175: 161–169. DOI: 10.1016/j.chemosphere.2017.02.035

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