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

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ABSTRACT

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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 an option. Uranium mining may result in run-off and the contamination of water courses. We investigated the effects of uranium on enzyme activities (cholinesterases (ChEs), Na^+K^+-ATPase, glutathione S-transferase (GST) and catalase (CAT)) in a freshwater caddisfly shredder *Schizopelex festiva* exposed to six U concentrations up to 100 µg/l. Enzyme activities and U accumulation were determined at 24 h and 32 d of exposure. We also measured growth rates (32 d) and calculated bioconcentration factors, as the ratio of U in the whole body of the invertebrates to U concentration in the test water. Enzyme activity from 24 h to 32 d was reduced for ChEs (~52 %), GST (~44 %) and CAT (78 %). No change was observed for Na^+K^+ATPase activities. Enzyme activities for Na^+K^+ATPase decreased after 32 d only at the highest U concentration. For GST activities, there were significant differences among concentrations for each time, but the treatments did not differ from the control groups. The growth rates of *S. festiva* under laboratory conditions averaged 6.23 ± 0.66 µg g^{-1} animal d^{-1}, with no significant differences between treatments and control groups. The ratio of U in the invertebrate body to U concentrations in the water ranged from 211 to 1663, increasing with time and decreasing with the concentration of U in the water. The exposure of *S. festiva* to U 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 larger amounts of U would be needed to cause measurable biological changes.

Key words: streams, metal, freshwater macroinvertebrates, neurotoxicity, oxidative-stress biomarkers, cell-membrane enzymes

RESUMO

Atividades de enzimas relacionadas ao estresse oxidativo e membranas celulares em um macroinvertebrado fragmentador exposto a urânio

Os rios são propensos a receber poluentes provenientes das atividades agrícolas, industriais e de mineração, com efeitos na biota que vão desde processos bioquímicos até os ecossistemas. A substituição das fontes de energia do carbono por energias alternativas faz com que a energia nuclear possa ser uma opção frente às alterações climáticas. A mineração do urânio pode resultar no escoamento de metais e contaminação das águas superficiais. Os efeitos do urânio nas atividades enzimáticas das colinesterases (ChEs), Na^+K^+-ATPase, glutationa S-transferase (GST) e catalase (CAT) foram investigados, usando como organismo-teste o tricóptero Schizopelex festiva*, expostos a seis concentrações de até 100 µg/l. Fatores de bioconcentração (relação entre U nos invertebrados e U na água) e taxas de crescimento também foram calculados. As atividades das enzimas e a acumulação de U foram determinadas em 24 h e 32 d. As atividades enzimáticas foram reduzidas de 24 h para 32 d para ChEs (~52 %), GST (~44 %) e CAT (~78 %), enquanto que para a Na^+K^+ATPase não se observaram diferenças entre os tempos de exposição. As atividades enzimáticas da Na^+K^+ATPase reduziram após 32 d, mas só na mais alta concentração de*

U. Para GST, houve diferenças nas atividades enzimáticas entre concentrações de U entre períodos de exposição, mas não se observaram diferenças entre controle (0 µg/l) e as restantes concentrações. As taxas de crescimento de S. festiva *foram em média de 6.23 ± 0.66 µg* g^{-1} *animal* d^{-1}*, sem diferenças significativas entre tratamentos. Os fatores de bioconcentração variaram entre 211 e 1663, aumentando com o tempo de exposição e diminuindo com a concentração de U na água. A exposição de* S. festiva *ao U resultou na sua acumulação e provocou alterações nas atividades de algumas enzimas, mas esta espécie foi em geral tolerante a valores encontrados nas águas superficiais. Se nossos resultados puderem ser estendidos a outros consumidores, quantidades de U superiores às testadas seriam necessárias para causar alterações biológicas mensuráveis.*

Palavras chave: *rios, metais, macroinvertebrados, neurotoxicidade, biomarcadores de estresse oxidativo, enzimas da membrana celular*

INTRODUCTION

Uranium (U) 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 bond to adenosine 5'-triphosphate, forming the complex ATP-UO_2^{2+}, 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 U 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), cholinesterases (ChEs), 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-}) into hydrogen peroxides (H_2O_2), which are detoxified into water and molecular oxygen by catalase or peroxidases (DeJong *et al.*, 2006). Hydrogen peroxides can become highly reactive hydroxyl radicals (OH) when they react with reduced metal ions (DeJong *et al.*, 2006). 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 activity of GSTs 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, 2013). 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).

Cholinesterases (ChEs) are a group of esterases that hydrolyse mainly choline esters, and can be differentiated by specific substrates such as acetylcholine (brain and nervous systems as the main source), butyrylthiocholine and propionylthiocholine (Pestana *et al.*, 2014). The neurotransmitter acetylcholine is hydrolysed by the serine enzyme acetylcholinesterase (AChE). Contaminants may react with serine at the catalytic site, disabling the cholinesterasic 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), while the trichopteran *Schizopelex festiva* was able to survive at concentrations as high as 262 mg/l, but concentrations of 0.025 mg/l inhibited growth (Bergmann *et al.*, 2018). We also found that uranium under realistic environmental concentrations, as observed in polluted mining sites (50 µg/l), caused a decrease in Na^+K^+-ATPase activity in the caddisfly shredder *Calamoceras marsupus* (Tagliaferro *et al.*, 2018). We have continued to investigate uranium effects on stream consumers, searching for changes in a set of biomarkers (ChEs, 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 numbers in some streams of the Iberian Peninsula and is functionally important in the energy transference from litter to the food webs. We predicted that key enzyme activities would be affected by uranium exposure at concentrations below those causing a decrease in growth.

MATERIALS 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; 08° 09' 15" W; pH 6.89, [U] in water: 0.35 µg/l, [U] in sediments: 1.0 mg/kg, n = 3). Caddisfly larvae 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 ASTM international recommendations: 96 mg/l $NaHCO_3$, 60 mg/l $MgSO_4$, 60 mg/l $CaSO_4$. $2H_2O$ and KCl 4 mg/l.

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 colonisation under laboratory conditions (Biasi *et al.*, 2017).

Experimental design

We investigated the effects of uranium on the activities of four enzymes, ChEs, Na^+K^+-ATPase, GST, and CAT, and on the growth rates of

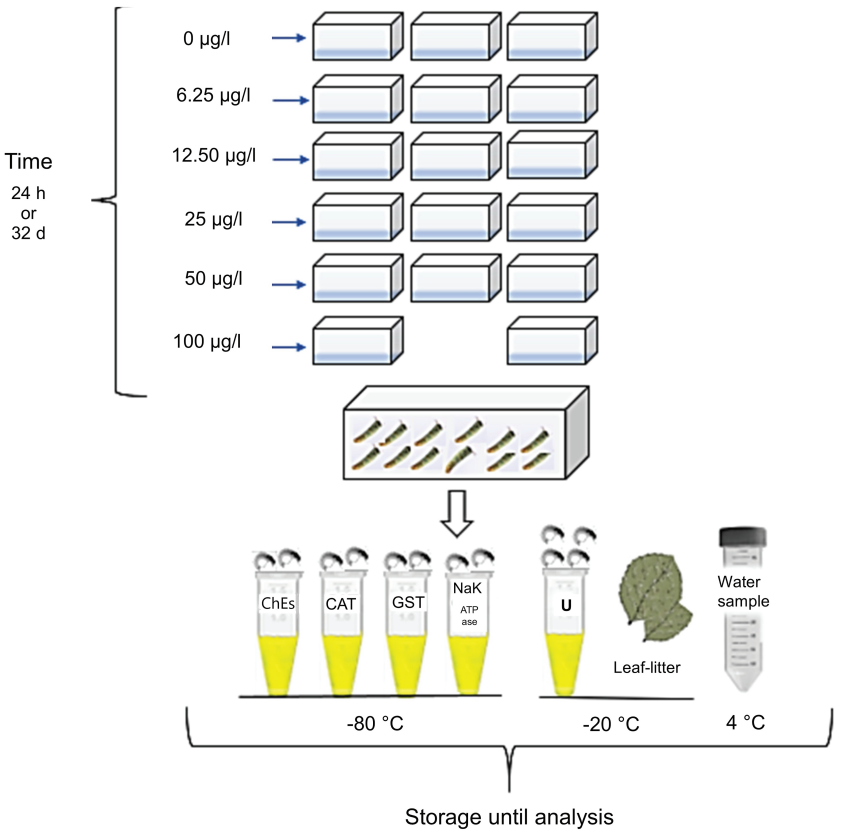

Figure 1. Experimental design for biomarkers and uranium determinations at 24 h and 32 d. *Esquema experimental para determinação dos biomarcadores e urânio em 24 h e 32 d.*

S. festiva for 32 days at six U concentrations (0, 6.25, 12.50, 25, 50 and 100 µg/l). 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 µg/l), on 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 U 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 U source. Test solutions were prepared by diluting a stock solution (1000 mg/l) to the final nominal concentrations listed above.

Before they were offered to the caddisfly larvae, 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 36 boxes, each containing 12 specimens, totaling 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 U analysis (Fig. 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 U 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 U as the

difference between the final (DMf) and initial mass (DMi) divided by the mean body size initial mass (DMm) and the elapsed time in days (t) (Arsuffi & Suberkrop, 1986)

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

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, $r^2 = 0.98$, $p < 0.001$).

Determination of uranium and bioconcentration factors

Uranium in the water, leaves and in the caddisfly larvae (at 24 h and 32 d exposure; n = 36) was measured by fluorometry (λ = 530 nm). Leaves and caddisfly larvae 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 (0, 2, 10, 100, and 1000 µg/l), according to Van Loon & Barefoot (1989). We also calculated bioconcentration factors (BCF) for each concentration tested, as the ratio of U in the whole body of the invertebrates (mg/kg dry weight) to U concentration in the test water (mg/l) (Miller *et al.*, 2016).

Enzyme activities

For enzyme activity assays, two caddisfly larvae were uncased and manually homogenised with a pestle in microtubes with 180 µl of specific buffer solutions for each enzyme replicate (6 larvae for each enzyme x 2 times) (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 ChEs 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/l 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. For enzyme determinations, 500-µl microcuvettes were used.

ChEs activities were 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 $MgCl_2$, 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 (ε = 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 (ε240 = 40 mM^{-1} cm^{-1}) (Perić *et al.*, 2017) for 2 min every 30 s. Activity was expressed as nmol min^{-1} mg^{-1} protein.

Data analysis

Enzyme activities at 24 h and 32 d were compared by paired t-tests on log transformed data (ChEs) 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 the control (no uranium). The relationship between the uranium content in the caddisfly larvae, water and leaves was investigated using linear regression. Differences in growth rates between the uranium treatments were determined by one-way ANOVA. The relationship between uranium concentration in water and the uranium concentration in caddisfly larvae 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

Uranium uptake and growth rates of *S. festiva*

The mean pH was 7.75 ± 0.21, electrical conductivity was 299 ± 2 µS/cm, and dissolved-oxygen content was 7.56 ± 0.85 mg/l (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 caddisfly larvae had higher U contents than the water, mainly at the lower and medium uranium concentrations. The bioconcentration factors (BCF) for caddisfly larvae increased from 24 h to 32 d, and were highest in *S. festiva* exposed to the lowest U concentrations (Table 1). The U content in the caddisfly larvae body and in leaves was strongly related to the uranium concentrations in the water (caddisfly larvae: $F_{(1,5)} = 37.26$, $p < 0.01$, $r^2 = 0.96$; leaves: $F_{(1,5)} = 454.38$, $p < 0.001$, $r^2 = 0.97$). Uranium concentrations in the caddisfly larvae were also related to U content in the leaves: $F_{(1,5)} = 43.91$, $p < 0.01$, $r^2 = 0.91$ (Fig. 2). After the 32 d of exposure, all specimens combined had grown 6.98 ± 0.64 µg g^{-1} animal

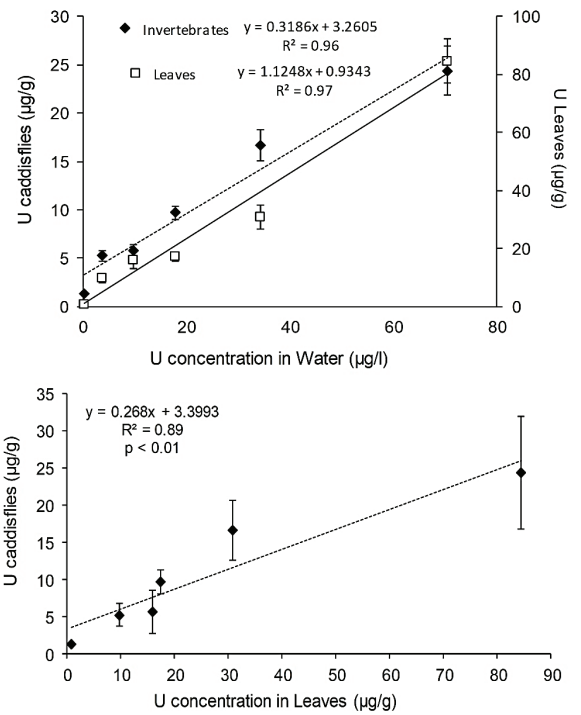

Figure 2. Relationships of accumulated uranium in caddisfly larvae, leaves and water from 24 h and 32 d of the metal exposure (mean ± standard error). *Relações entre o urânio acumulado nas larvas de tricópetros, folhas e água, em 24 h e 32 d da exposição ao metal (média ± erro padrão).*

Table 1. Uranium concentrations in water, caddisfly larvae, and leaves used to feed the animals during 32 d, and bioconcentration factors (BCFs) calculated from 24 h and 32 d (mean ± standard error). *Value below of the chronic Predicted No Effect Concentration (PNEC) (0.3 µg/l; INERIS, 2008). *Concentrações de urânio na água, invertebrados e folhas usadas para alimentar os animais durante 32 dias, e fatores de bioconcentração (BCFs); calculados em 24 h e 32 d (média ± erro padrão). *Valores abaixo de Predicted No Effect Concentration (PNEC) (0.3 µg/l; INERIS, 2008).*

	Nominal U concentrations in water (µg/l)					
	0	6.25	12.50	25	50	100
U in water (µg/l)	0.27 ± 0.03*	3.88 ± 0.28	9.73 ± 0.75	17.97 ± 0.80	34.29 ± 2.60	70.45 ± 2.83
Caddisfly larvae (µg/g)	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)	0.80 ± 0.12	9.65 ± 2.63	15.98 ± 0.17	17.43 ± 0.23	30.9 ± 3.31	84.51 ± 22.16
BCF (24 h)	NC	1143 ± 57.13	558.46 ± 41.24	386.50 ± 8.57	310.71 ± 27.91	211.42 ± 18.27
BCF (32 d)	NC	1663 ± 159.15	875 ± 70.20	728 ± 49.05	445 ± 50.4	291 ± 11.66

d^{-1} (mean ± SE). Growth rates did not differ among treatments (ANOVA, F = 0.59; df = 5, 154; $p = 0.70$).

Enzyme activities

The enzyme differed in their activities at 24 h: ChEs (0.07 – 0.16 nmol acetylthiocholine min^{-1} mg^{-1} protein), GST (0.01 – 0.05 nmol GSH conjugated min^{-1} mg^{-1} protein), Na^+K^+ATPase (0.98 – 3.18 nmol Pi min^{-1} mg $protein^{-1}$), and CAT (0.05 – 0.98 nmol min^{-1} mg^{-1} protein). These 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 ChEs (t = 5.228, df = 5, $p = 0.003$). Na^+K^+-ATPase activity was reduced at 100 µg/l (F = 4.772, $p = 0.012$) by Day 32. GST activity differed across U concentrations (F = 3.321, $p = 0.041$), although there were no differences between the control and any of the testing U concentrations. CAT and ChEs activities did not change significantly across concentrations (Fig. 3).

DISCUSSION

Here, we found that *S. festiva* grew equally well in both control and U-contaminated water (up to 100 µg/l). These findings contrast with previous experiments in which growth was reduced by 47 % in specimens exposed to 25 µg/l (Bergmann *et al.*, 2018). There are several possible reasons for the contrasting results. First, here we reared specimens in groups, and although they were provided food 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 were larger, weighing 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, growth rates

(11.60 µg mg animal^{-1} d^{-1}) were lower here than the 40.6 µg mg animal^{-1} d^{-1} reported under control conditions and the 21.5 µg mg animal^{-1} d^{-1} in specimens exposed to U (Bergmann *et al.*, 2018). These growth rates are within the range reported for other stream shredders, i.e., 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 U 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, U did not affect growth, but *S. festiva* specimens accumulated U according to the concentration in both the water and the food (leaf litter). We evaluated the potential for the U 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; i.e., U 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 U 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

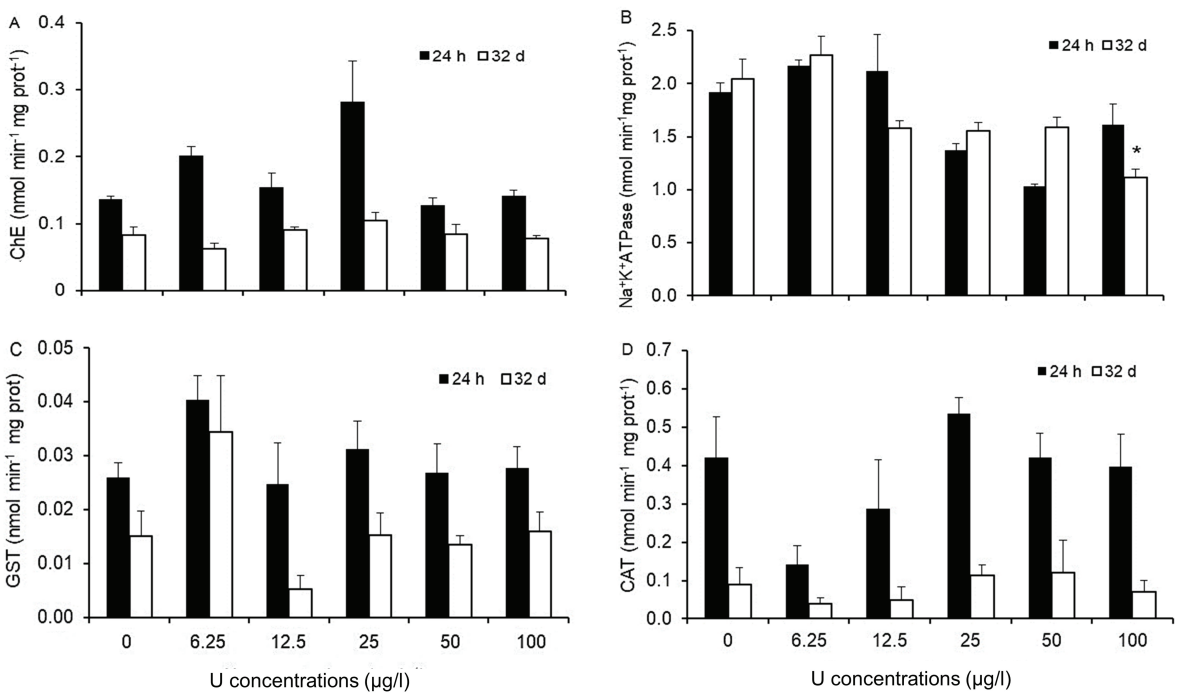

Figure 3. ChEs (a), Na^+K^+-ATPase (b), GST (c), and catalase (d), activities after 24 h (dark bar) and 32 d (light bar) exposure of *Schizopelex festiva* to uranium (mean ± standard error). * = $p < 0.05$ against control (0 µg/l). *Atividades da ChEs (a), Na^+K^+-ATPase (b), GST (c) e catalase (d) após 24 h (barra escura) e 32 d (barra clara) da exposição de* Schizopelex festiva *ao urânio (média ± erro padrão).* * = p < *0.05 em relação ao controle (0 µg/l).*

tissues, permanently or until the U is excreted (Rainbow, 2002; Barilett *et al.*, 2011).

The published information of the chronic predicted no effect concentration (PNEC) for U found in literature ranges from 0.3 µg/l (generic value including geochemical background) or 3.2 µg/l for freshwater ecosystems (INERIS, 2008; Mathews *et al.*, 2009), to 8 200 µg/l (chronic exposure) and 270 000 µg/l (acute exposure) or 100 mg/kg in sediment (Sheppard *et al.*, 2005; Beaugelin-Seiller *et al.*, 2011). NOEC/EC10, values of 2.7 µg/l of U were defined to freshwater invertebrates (INERIS, 2008), and 11.2 µg/l was the most sensitive endpoint (dry weight) for chronic toxicity data found for the midge *Chironomus tentans* (Herwijnen & Verbruggen, 2014).

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. U in leaves was proportional to the U content in the water; in contrast, less U accumulated in *S. festiva* than in leaves (Table 1). This difference can be explained by the ability of the fungi in the leaves for biosorption of U to polysaccharides, proteins, and lipids from their cell walls (He & Chen, 2014), while the caddisfly larvae use physiological mechanisms, such as glutathione S-transferase, to sequester and eliminate metals (Merritt & Bewick, 2017).

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 of biological organisation (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).

Some enzyme activities were affected by U 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 in relation to control in 32 d (Fig. 3B). In similar experiments with the shredder caddis *C. marsupus*, Na^+K^+-ATPase activity was also reduced at 50 µg/l (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 U in our experiment, but the variability was high (Fig. 3C). 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) 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).

Cholinesterase activities were unspecific in this study, but ChEs in the caddisfly larvae Sericostomatidae seem to be composed mainly of AChE (Pestana *et al.*, 2014). In addition, since we used the acetylthiocholine as a substrate for ChEs measurements, it is likely that acetylcholinesterase (AChE) was the main form of ChEs determined in this study. Although ChEs activity did not change with uranium increasing concentrations, it was reduced by ~40 to ~70 % after 32 d of exposure for all U concentrations. These differences between 24 h and 32 d can be related to the normalization of the enzymes 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 ChEs (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; Perić *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 U exposure, an increase after 5 days, followed by a decrease by the 10th 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 U affects ChEs activity.

Catalase activity was not sensitive to U exposure, but its activity was reduced by ~80 % from 24 h to 32 d (Fig. 3D). Decrease in catalase activities were observed for zebrafish until five days (20 - 500 µg/l), 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 U (Al Kaddissi *et al.*, 2012). However, catalase and other enzyme activities involved in antioxidant defenses can decrease with time in U exposure, perhaps due to interference in the levels of antioxidants. For aquatic macrophytes, CAT activity decreased at 20 and 100 mg/l of U (Srivastava *et al.*, 2010). These examples show that catalase is sensitive to U. However, because our biological samples came from whole-organism homogenates and not from individual tissues, catalase, as well GST and ChEs sensitivity to U may have been attenuated.

CONCLUSIONS

Dissolved U to a concentration up to 100 µg/l did not affect growth in the shredder *S. festiva*. Na^+K^+-ATPase was sensitive to U (100 µg/l) after long exposition (32 d). Taken together, our results revealed some measurable physiological effects on organisms exposed to U contamination at concentrations below the lethal dose, and below the concentration causing reductions in growth. Except for Na^+K^+-ATPase, the changes in enzyme activities did not conform to a dose-response model, 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 physiological effects of exposure of aquatic organisms to stressors.

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