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Original Articles

Assessment of metal exposure (uranium and copper) by the response of a set of integrated biomarkers in a stream shredder

Marina Tagliaferro[a,*], Ana M.M. Gonçalves[b,c], Melissa Bergman[b], Olímpia Sobral[b], Manuel A.S. Graça[b]

[a] Instituto de Ecología y Desarrollo Sustentable (INEDES – CONICET), Universidad Nacional de Luján, Ruta 5 y 7, Luján, 6700, Buenos Aires, Argentina
[b] MARE – Marine and Environmental Sciences Centre, Department of Life Sciences, University of Coimbra, 3001-456, Coimbra, Portugal
[c] Department of Biology and CESAM, University of Aveiro, 3810-193, Aveiro, Portugal

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ABSTRACT

Environmental pollution with toxic trace metals is of great concern for the environment and for public health. Here we assess the response of the shredder caddisfly *Calamoceras marsupus* to sub-lethal exposures to copper and uranium. As endpoints we used growth, feeding and growth efficiency, and a set of enzyme biomarkers (oxidative stress: glutathione-S-transferase and catalase; respiration: lactate dehydrogenase; and the activities of acetylcholinesterase and Na^+/K^+-adenosine triphosphatase). We found that survival, growth, feeding and growth efficiency were not affected by any of the copper (0, 35 and 70 μg L^{-1}) or uranium (0, 25 and 50 μg L^{-1}) conditions. However, catalase activity increased with increased copper concentration, from 0.20 to 0.85 nmol min^{-1} mg^{-1} protein (mean ± SE; $p < 0.0001$). Na^+/K^+ ATPase activity decreased with increased U concentration (from 0.16 ± 0.01 to 0.11 ± 0.01; mean ± SE; $p < 0.001$). The activities of LDH, GST and AChE enzymes did not differ across treatments. We concluded that CAT and Na^+/K^+-ATPase were the most sensitive biomarkers for copper and uranium respectively, at concentrations below levels that would affect growth and feeding.

1. Introduction

Each year, 730 million tons of pollutants including pharmaceutical drugs, pesticides and metals are released into aquatic environments (UNESCO, 2015), harming the environment and water quality for humans. At high concentrations, pollutants impact the survival of some organisms and induce changes in diversity, taxa distribution and ecosystem functions (Borowitzka, 1972; de Beeck et al., 2014; Schwarzenbach et al., 2010). Therefore, changes in community structure can be used as indicators of environmental stress.

At low concentrations, pollutants may cause sublethal effects including decreases in growth, food ingestion, reproduction and recruitment (Naylor et al., 1989; Sheehan, 1984), or subtle changes in metabolism, osmoregulation, photosynthesis and enzyme activities (Castro et al., 2004; Ramsden et al., 2009). Changes in enzyme activities have been used as biomarkers to assess the stress induced by chemicals and other environmental changes. It has been stated that biochemical markers are highly sensitive and respond quickly to stress (Carlisle and Clements, 2005; Colin et al., 2015; Gonçalves et al., 2017; Martinez-Haro et al., 2015). Therefore, biomarkers can be considered early warning signals of pollutants and global changes (Amiard-Triquet et al., 2015; Colin et al., 2015; Gonçalves et al., 2017), and allow pollutant effects to be quantified (Martinez-Haro et al., 2015).

Some common biomarkers include (1) biotransformation enzymes of organic compounds, e.g. glutathione S-transferase (GST); (2) nerve-function enzymes such as acetylcholinesterase (AChE); (3) antioxidant defenses against environmental pro-oxidants, e.g. superoxide dismutase (SOD), peroxidases such as catalase (CAT) and glutathione-peroxidase (GPx), and lactate dehydrogenase (LDH); (4) osmoregulation enzymes such as sodium-potassium ATPase (Na^+/K^+-ATPase); and (5) multi-xenobiotic resistance that protects against toxins in fuel oil, i.e. poly-aromatic hydrocarbons (PAHs).

Glutathione-S-transferases are a family of enzymes involved in phase II of xenobiotic metabolism, catalyzing the conjugation reaction of reduced glutathione (GSH) with xenobiotic metabolites containing electrophilic centers (nitro-compounds, organophosphates, organo-chlorines), which increases their water solubility and facilitates their excretion (Domingues et al., 2010; Hyne and Maher, 2003; Sherratt and Hayes, 2002; Vranković and Slavić, 2015). Acetylcholinesterase plays an important role in the neural system of both vertebrates and

* Corresponding author.
E-mail address: azulmarinita@gmail.com (M. Tagliaferro).

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invertebrates (Frasco et al., 2005), and is responsible for the degradation of acetylcholine in cholinergic synapses (Phyu and Tangpong, 2014). Initially used as a specific biomarker for organophosphate and carbamate pesticides, AChE activity is also affected by other environmental toxicants such as metals (Sherratt and Hayes, 2002). AChE inhibition disrupts the nervous system, leading to overstimulation of the central and peripheral nervous systems, and may affect several functions including respiration, feeding and behavior, eventually leading to death (Domingues et al., 2010). Na^+/K^+-ATPase is a transmembrane enzyme that functions as an ATP-powered pump, transporting Na^+ and K^+ ions against their concentration gradients. Inhibition of Na^+/K^+-ATPase caused by environmental pollutants can affect cell osmolarity, inducing physiological stress at the organism level (Bonga and Lock, 2008; Monserrat et al., 2007; Vasić et al., 2008). Catalase is an antioxidant enzyme that reduces H_2O_2 to H_2O and O_2, and is used by cells to maintain their oxidative status (Chelikani et al., 2004; Sinha, 1972; Winston and Di Giulio, 1991). LDH is responsible for catalyzing the conversion of lactate to pyruvate, with concomitant conversion of NADH to NAD^+, and acts as an indicator of the metabolic state of the organism (Adams et al., 1973; Bessa et al., 2016; Market, 1984).

Here, we used copper and uranium as toxic models for sublethal exposure. Both metals are of environmental concern, because of mining. Copper is an essential element required by a variety of enzymes involved in critical areas of metabolism (e.g. cytochrome c-oxidase, the terminal enzyme in the electron transport chain; and Cu/Zn superoxide dismutase (SOD), involved in antioxidant defense) (Lutsenko and Petris, 2003; Tchounwou et al., 2014). In arthropods and molluscs, copper is also involved in the respiratory protein hemocyanin (Arce Funck, 2014; Markl, 2013). At high concentrations, copper causes free-radical induction, through the accumulation of reactive oxygen species, which produce metabolic alterations and damage DNA and mitochondria (Gaetke et al., 2014; Haidari et al., 2001; Lippard, 1999; Pinho et al., 2007). Copper exposure causes decreases in food intake, recruitment and/or survival (Othman and Pascoe, 2002; Rocha et al., 2016). This compound is one of the most widespread metal contaminant in domestic, industrial and municipal wastes, antifouling paints, and agriculture (as a pesticide) (De Oliveira-Filho et al., 2004; Meinrath et al., 1996; Parkhurst et al., 1984).

Uranium is an ubiquitous metal occurring in small amounts in soil and water (Ribera et al., 1996). As it is not used by living organisms, it becomes toxic only at high concentrations (Liber et al., 2011; Thiébault et al., 2007). In the organism, uranium is concentrated by lysosomes, removing supplies of antioxidant agents such as glutathione, SOD, and CAT, allowing free radicals to run uncontrolled through tissues and organs, damaging DNA and mitochondria (Tasat et al., 2012). Uranium may also compete with Na^+ in ion transport, occupying Na^+ sites on ATPase (Nechay et al., 1980). Toxic effects include increased mitochondrial respiration in fish (Lerebours et al., 2010), and decreased carbon assimilation in daphniids (Massarin et al., 2010). Uranium exposure decreased food intake and growth of aquatic insects (Gonçalves et al., 2011), caused tail deformities in the Perez's frog *Rana perezi* (Marques et al., 2008), and decreased survival and mouthpart deformities in larvae of the midge *Chironomus riparius* (Dias et al., 2008).

Leaf litter is a major energy supply in low-order streams, where the litter consumed by a key functional feeding group known as shredders (Abelho, 2001; Elosegi and Pozo, 2016). *Calamoceras marsupus* (Brauer, 1865; Trichoptera) is a widely distributed shredder, locally abundant, with an important role in energy transfer from leaf litter to stream food webs. Here we determined the responses of *C. marsupus* larvae to exposures to uranium and copper, by the use of a set of biomarkers (GST, AChE, LDH, CAT and Na^+/K^+-ATPase). We also measured survival, growth (GR) and feeding (FR) rates and growth efficiency (GE, defined as the ratio of the increase in body mass: mass of the ingested food). A final goal of this study was to identify potential biomarkers as early warning signals in ecotoxicological studies. We hypothesized that (1) growth, feeding rates and growth efficiency would decrease with increasing concentration of stressors; and (2) these responses at the individual levels would preceded by physiological changes, which can be studied through biomarkers, which might be more sensitive than growth and feeding parameters.

2. Materials and methods

2.1. Invertebrate and litter conditioning

Specimens of *Calamoceras marsupus* (Brauer, 1865; Trichoptera) were collected in early September 2016 from a stream at Póvoa de Luzianes, Portugal (40°30′42.22″N; 07°49′1.54″W; 15.1 °C, 9 mg L^{-1} oxygen - 92% saturation, pH 7.4, and electrical conductivity 318 μS cm^{-1}). Specimens were acclimated for 1 week to laboratory conditions (transition from natural stream water to synthetic culture medium, alder leaves previously stream-conditioned as food, constant temperature 15.7 ± 0.6 °C, 12:12 h light/dark cycle).

Senescent leaves of black alder (*Alnus glutinosa*) were enclosed in 500 μm-mesh bags, and conditioned in the São João stream (Lousã, Portugal; 40°5′59.12″N; 008°14′2.42″W) for periods of 7 days during September and October 2016. Specimens were individually fed with 1.8 mm leaf discs, cut from leaves with a cork borer. Discs were cut in pairs; one of each pair was dried to constant mass (3 days, 60 °C) and assumed to be equal to the mass of the second disc exposed to the consumers. Every 2 days for 35 days, exposed leaf discs were retrieved, dried and weighed. Retrieved discs were replaced by new ones.

2.2. Experimental conditions

Calamoceras marsupus were reared individually in 7 cm-diameter cups containing 150 mL of water, according to the USEPA protocol for standard moderately hard synthetic freshwater (Lewis et al., 1994) and a final concentration of 92 mg L^{-1} $NaHCO_3$, 60 mg L^{-1} $CaSO_4{\cdot}2H_2O$, 120 mg L^{-1} $MgSO_4$, and 4 mg L^{-1} KCl. We tested three concentrations of copper (0, 35 and 70 μg L^{-1}) and three of uranium (0, 25 and 50 μg L^{-1}) (6 treatments, 10 replicates per treatment). The highest copper level was based on the range of LC_{50} values reported in the literature, mainly for aquatic insects (van der Geest et al., 2000), and below the values reported to cause a 50% reduction in the richness of Ephemeroptera, Plecoptera and Trichoptera (Iwasaki and Ormerod, 2012). Uranium levels were below the EC_{50} range reported for growth and larval emergence or survival of the midge *Chironomus tentans* (Muscatello and Liber, 2010) and the amphipod *Hyalella azteca* (Liber et al., 2011). The copper and uranium solutions were prepared from $CuSO_4{\cdot}5H_2O$ and $UO_2(NO_3)_2{\cdot}6H_2O$, respectively. Laboratory bioassays were performed under constant temperature 15.7 ± 0.6 °C and photoperiod (12:12 h light/dark) for 35 days. Water and food were replaced every 2 days to correct for metal adsorption (APHA, 2005); food was offered *ad libitum*. A thin layer (1 spoonful) of autoclaved river sand was used as the substrate; gentle aeration was also provided via pipette tips connected to an air pump.

2.3. Feeding, growth and assimilation

The sizes (lengths) of specimens were determined from regressions between leg parts (femur, tibia, tarsus or trochanter from the 2nd or 3rd pairs of legs) and dry mass from selected specimens covering a wide size range (in all cases $R^2 > 0.7$; Table A.1, Supplementary material). Since more than one part of the caddisfly body was used to estimate dry mass, we used the mean values of all measurements. Dry mass was measured in a Kern 870 precision balance (± 0.1 mg; Kern & Sohn, Balingen, Germany), and lengths were measured under a stereoscopic microscope (Leica M80, Wetzlar, Germany) using LAS software.

Feeding rates (FR) were calculated as the difference in the mass (mg) of exposed (Lm_e) and unexposed (Lm_u) pairs of litter discs, divided by the mean larval mass (m) in mg (see below) and the elapsed time (t) in days following Graça et al. (2005).

$$FR = \frac{Lm_u - Lm_e}{m \times t}$$

Growth rates (GR) were estimated over the 35-days period, as the difference between the final (m_f) and initial (m_i) invertebrate mass divided by the mean mass and the elapsed time (t) in days following Graça et al. (2005).

$$GR = \frac{m_f - m_i}{((m_f - m_i)/2) \times t}$$

We computed a growth efficiency index, as the ratio of mass gained divided by the litter mass ingested.

2.4. Biomarkers

We measured the activity of five enzymes in specimens exposed to all treatments: glutathione-S-transferases (GST; EC 2.5.1.18), acetylcholinesterase (AChE; EC 3.1.1.7), lactate dehydrogenase (LDH; EC 1.1.1.2), catalase (CAT; EC 1.11.1.6), and sodium-potassium adenosine triphosphatase (Na^+/K^+-ATPase; EC 3.6.3.9). However, in this case, specimens were reared groups of 10 in 1.5 L (3 replicates). After exposure, the caddisflies were uncased and individually transferred to microtubes with 180 µL of buffer solution specific for each biomarker (see below), and manually homogenized with a pestle, centrifuged (5000 rpm; 15 min; 5 °C), and the supernatant was frozen at −80 °C until spectrophotometric analysis (Jenway 6715 UV/VIS spectrophotometer, Bibby Scientific, Stone, UK).

A 100 mM phosphate buffer was used for AChE and GST (pH = 7.2 and 6.5, respectively), Tris-NaCl (pH = 7.2) buffer for LDH, 50 mM phosphate buffer with 10% Triton for catalase (pH = 7.0), and a 320 mM sucrose – 10 mM HEPES-Tris (pH = 7.4) buffer for Na^+/K^+-ATPase. Prior to the enzyme assays, the sample supernatant was diluted in the respective buffer, to obtain a protein concentration of approximately 1.0 ± 0.2 mg L^{-1}.

Protein content in the samples was determined as described by Bradford (1976). A 1:10 supernatant dilution was used for GST and Na^+/K^+-ATPase, and 1:20 dilution for LDH, catalase, and AChE. Enzyme activity was determined in three biological replicates per treatment, with three technical replicates. The measured values were related to protein content. All enzyme activities were expressed as nmol · min^{-1} mg^{-1} of protein, but Na^+/K^+-ATPase which was expressed as nmol of inorganic phosphate (Pi) min^{-1} mg^{-1} of protein.

The soluble GST activity was determined according to Habig et al. (1974). Briefly, 1-chloro-2,4-dinitrobenzene (CDNB) was used as the substrate, which when conjugated with glutathione (GSH) generates a thioester with absorbance at 340 nm in a reaction solution containing 156 µL of 1 mM CDNB, 900 µL of 1 mM GSH, and 4950 µL of 100 mM phosphate buffer (pH 7.5). Spectrophotometric readings were done in disposable microcuvettes with a mixture of 167 µL of sample and 333 µL of reaction solution. The increase in absorbance was measured for 4 min at 25 °C. The enzyme activity per min was estimated as the slope of the absorbance over time. The enzyme activity value was corrected by the molar extinction coefficient (ε) for GSH-CDNB conjugate (ε = 9.6 mM^{-1} cm^{-1}), and related to the volume of the sample and the protein content (Habig et al., 1974).

The AChE activity was determined according to Ellman et al. (1961). The method is based on acetylcholine degradation by AChE to acetate and thiocholine. Thiocholine generates a complex with dithiobisnitrobenzoic acid (DTNB), a yellow compound (ε = 1.36 × 10^4 M^{-1} cm^{-1}) with absorbance at 412 nm. Absorbance was measured during the first 30 min, starting at min 10, and every 5 min thereafter, in a solution containing 75 µL of sample and 375 µL of reaction solution (1 mL of 10 mM DTNB, 30 mL of 100 mM phosphate buffer and 0.2 mL of 0.075 M acetylcholine). The enzyme activity per min was estimated as the slope of the absorbance over 5 min, of the most stable period (between minutes 15 and 20 of the enzyme reaction).

LDH activity was measured by monitoring NADH oxidation every 20 s for 5 min through the reduction of absorbance at 339 nm, according to Vassault (1983). A 60.6 µL sample was mixed with the reaction solution containing 378.8 µL of β-NADH (0.24 mM), and 60.6 µL of pyruvate (12.15 mM) in 0.1 M Tris–NaCl buffer (pH = 7.2). The enzyme activity per min was estimated as the slope of the absorbance over time, corrected by the molar extinction coefficient (ε = 6.3 mM^{-1} cm^{-1}; Bergmeyer, 1974), and related to the volume of the sample and the protein content.

Catalase activity (CAT) was determined in diluted (1:20) supernatant using 50 mM phosphate buffer and 10% Triton X-100 (pH = 7). CAT activity was measured as the decrease in absorbance at 240 nm (ε = 40 mM^{-1} cm^{-1}) due to the consumption of H_2O_2, as substrate, according to Beutler (1982), every 30 s for 2 min. This activity was measured in a reaction solution of 0.1% H_2O_2 in 50 mM of phosphate buffer (pH = 7). The decrease in enzyme activity per minute was also estimated as the slope of the absorbance over time and was related to the sample volume and protein content.

Na^+/K^+-ATPase activity was calculated as ADP production, indirectly measured at 660 nm through inorganic phosphate production (Holman, 1943; Graham and Rickwood, 1997; Schwartz et al., 1972). The sample (supernatant) was diluted 1:10 in 320 mM sucrose and 10 mM HEPES-Tris (pH = 7.4), and a sample of 50 µL was mixed with 447.5 µL of reaction solution (100 mL contained 128 mM NaCl, 5 mM KCl, 3 mM MgCl, 0.1 mM EGTA, and 10 mM HEPES-Na; pH 7.4). The enzyme reaction was triggered by the addition of 7.5 µL of 200 mM ATP-Mg to the sample, in a 35 °C bath for 5 min. The reaction was terminated with 125 µL of TCA 20%, with test tubes in ice. The amount of inorganic phosphate generated was computed by measuring the absorbance at 660 nm. A calibration curve was obtained from inorganic phosphate previously estimated by the reaction of 50 mM phosphate buffer and Fe-molybdate (Holman, 1943; Murphy and Riley, 1962).

2.5. Statistical analysis

Growth, feeding rates, and assimilation efficiency were compared among treatments using a one-way ANOVA with copper and uranium concentrations as factors (Infostat software; Córdoba, Argentina). Feeding rates values were Ln-transformed to achieve normality. Each enzyme activity across treatments (factor copper with 3 levels: 0, 35, 70 µg L^{-1}; factor uranium with 3 levels: 0, 25, 50 µg L^{-1}) were analyzed by a one-way ANOVA under absolute values (Na^+/K^+-ATPase), Ln-transformed (LDH, GST, AChE, and CAT). The null hypothesis for each enzyme was that individual expose to different concentration of copper or uranium would have the same enzymatic activity as control.

3. Results

3.1. Survival, growth, feeding and growth efficiency

Invertebrate survival was above 80% for all treatments. The initial weight of specimens was 1.7 ± 0.1 mg (mean ± SE); during 35 days, a few specimens decreased in size, while most of them increased, up to ~8 fold. Growth rates ranged from −0.013 to 0.045 mg mg^{-1} d^{-1}; however, there were no differences across treatments (F = 1.5, df = 2, 23, p = 0.24 for copper, and F = 0.24, df = 2, 23, p = 0.79 for uranium; Fig. 1). Feeding rates ranged from 0.01 to 2.96 mg leaf mg^{-1} d^{-1} with no differences across treatments (F = 2.09, df = 2, 22, p = 0.15 for copper, and F = 0.39, df = 2, p = 0.68 for uranium; Fig. 1). However, feeding rates (FR) tended to decrease with increased copper concentration (mean ± SE: $FR_{Cu=0}$ = 1.30 ± 0.29, $FR_{Cu=35}$ = 0.89 ± 0.20, $FR_{Cu=70}$ = 0.70 ± 0.10). Finally, growth efficiency ranged from 0.001 to 0.040 with no difference across treatments (F = 1.59, df = 2, 22, p = 0.23 for copper, and F = 0.28, df = 2, 22, p = 0.76 for uranium). A tendency to increase growth efficiency with copper and uranium concentration was observed.

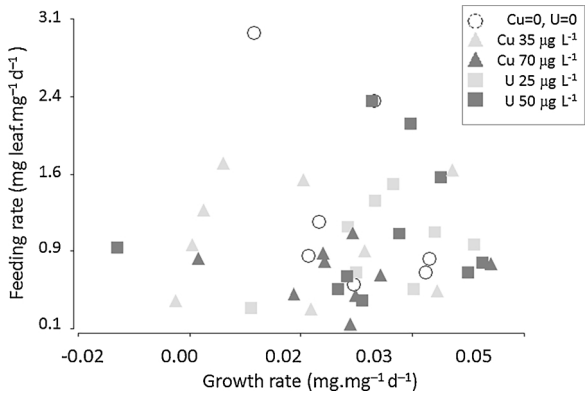

Fig. 1. Feeding rate and growth rate among the copper and uranium treatments. Circles, triangles, and square indicate treatments with uranium concentrations of 0, 25 and 50 µg L^{-1}, respectively. White, light grey, and dark grey indicate treatments with copper concentrations of 0, 35 and 70 µg L^{-1}, respectively.

3.2. Biomarkers

No significant differences were observed in the activities of LDH, GST and AChE among the uranium or copper treatments (Table 1). Catalase activity significantly increased with the increase in copper concentration ($F = 12.7$, df = 2, 8 $p = 0.003$; Tukey post-hoc test $p < 0.05$; Table 1; Fig. 2) from 0.20 ± 0.04 to 1.10 ± 0.21 nmol min^{-1} mg^{-1} protein (mean ± SE). Na^+/K^+-ATPase activity was affected by uranium, with lower activity (0.11 ± 0.02 nM Pi min^{-1} mg^{-1} protein) at high U concentrations (50 mg U L^{-1}) and higher activity (0.15 ± 0.02 nM Pi min^{-1} mg^{-1} protein) at lower concentrations (Control and 25 mg U L^{-1}; ANOVA – $F = 5.49$, df = 2, 8, $p = 0.029$; $p < 0.05$, Tukey post-hoc test among concentrations; Table 1; Fig. 3).

4. Discussion

We found that Na^+/K^+-ATPase and CAT were sensitive to uranium and copper stress conditions, respectively, at concentrations that were not high enough to cause detectable changes in growth, feeding rates or growth efficiency. Although the findings do not support our first hypothesis (decreases in feeding, growth and growth efficiency with increased stress conditions), they support our second hypothesis that physiological changes affecting enzyme activities precede other changes at the organism-response level.

Testing concentrations were based on previously published data on effective doses. However, differences between our findings and the reported ones can be explained by differences in taxa sensitivity and laboratory conditions. For instance, concentrations of 1,500 µg L^{-1} of copper reduced midge larvae survival by 50% (LC_{50}–96 h) (Nekeber et al., 1984); 700–1,350 µg L^{-1} reduce feeding of caddisfly larvae by 50% (van der Geest et al., 1999), and 212 µg L^{-1} was enough to reduce feeding and growth in amphipods (Othman and Pascoe, 2002; Silva Santos et al., 2000) (Table 2). The concentration levels in our experiment, 35–70 µg L^{-1}, were within the range of levels that cause a decrease in growth, feeding or reproductive output in midges, mayflies, caddisflies and amphipods (Table 2).

The uranium concentrations that cause measurable toxic effects range from a few µg L^{-1} to several thousand mg L^{-1} (Table 3). Detectable reductions in body length were observed at 2.99 mg L^{-1} uranium for *Hyalella azteca* and 1.97 mg L^{-1} for the cladoceran *Ceriodaphnia dubia* (Kuhne et al., 2002; Liber et al., 2011) and concentrations as low as 22–28 µg L^{-1} reduced growth and feeding in *Daphnia* spp. and in caddisflies (Gonçalves et al., 2011; Massarin et al., 2010; Plaire et al., 2013; Zeman et al., 2008). Although the concentrations of uranium used here (25 and 50 µg L^{-1}) affected growth and feeding in the trichopteran *Sericostoma vittatum* (Gonçalves et al., 2011), no differences were found for *C. marsupus*.

Under stress conditions, organisms may redirect energy to maintenance, with costs in terms of growth (Gonçalves et al., 2011; Liber et al., 2011; Zubrod et al., 2015). Metabolism and food intake may also be affected under stress conditions (Lerebours et al., 2010; McGeer et al., 2000). Some researchers have combined changes in food intake and energy respired in a single measurement, "scope for growth" to assess stress conditions (including metals) in mussels and gammaridean amphipods (Amiard-Triquet et al., 2015; González-Fernández et al., 2016; Mubiana and Blust, 2007).

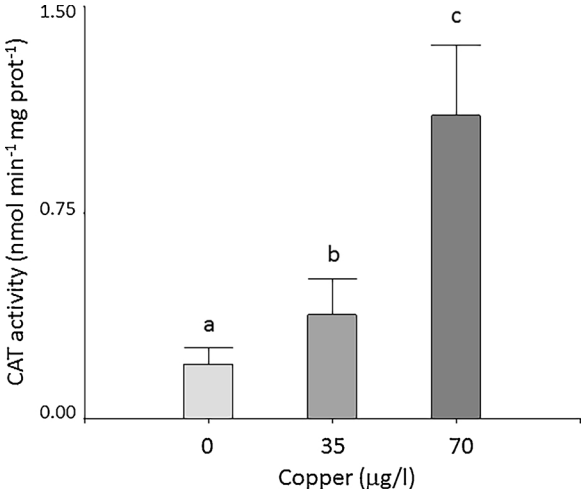

Fig. 2. Catalase activity along the three treatments. Bars indicate the standard error. Significant differences were found among the three copper treatments ($p = 0.003$). Mean with a different letter are significantly different ($p < 0.05$).

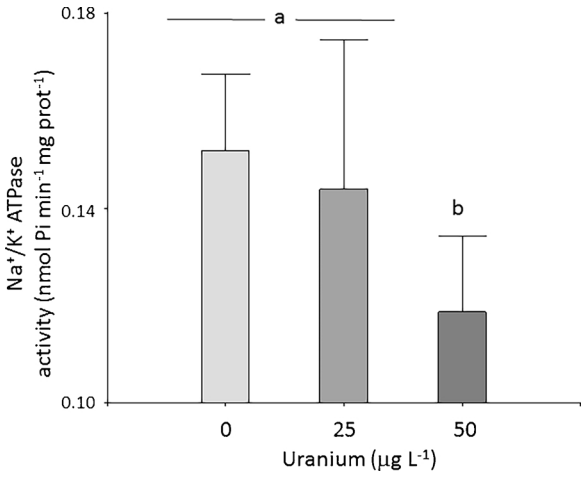

Fig. 3. Na^+/K^+ ATPase activity along the three treatments. Bars indicate the standard error. Significant differences were found among the uranium treatments ($p = 0.029$; differences between U = 0 µg L^{-1} and U = 25 µg L^{-1} with U = 50 µg L^{-1}). Mean with a different letter are significantly different ($p < 0.05$). Pi means inorganic phosphate.

Table 1
Statistical analysis of sublethal effects of copper and uranium on *Calamoceras marsupus*. Significant differences indicated in **bold**. F = ANOVA F parameter; GST = Glutathione S-transferase; AChE = Acetylcholinesterase; LDH = Lactate dehydrogenase; CAT = Catalase activity; Na^+/K^+-ATPase = Na^+/K^+-adenosine triphosphatase.

Variable	Statistic	p value
GST	$F_{Cu,2,8} = 0.24$; $F_{U,\ 2,8} = 0.02$	$p_{Cu} = 0.79$; $p_U = 0.98$
AChE	$F_{Cu,2,8} = 0.87$; $F_{U,\ 2,8} = 0.46$	$p_{Cu} = 0.45$; $p_U = 0.64$
LDH	$F_{Cu,\ 2,8} = 0.21$; $F_{U,\ 2,8} = 0.35$	$p_{Cu} = 0.81$; $p_U = 0.72$
CAT	$F_{Cu,\ 2,8} = 12.7$; $F_{U,\ 2,8} = 0.58$	$\mathbf{p_{Cu} = 0.003}$; $p_U = 0.58$
Na^+/K^+-ATPase	$F_{Cu,\ 2,8} = 0.87$; $F_{U,\ 2,8} = 5.49$	$p_{Cu} = 0.45$; $\mathbf{p_U = 0.029}$

Table 2
Copper (Cu) toxicity effects: survival (S), growth rate (GR), glutathione disulfuric (GSSG), 50% of function affected (EC_{50}), glutathione peroxidase (GPx), lethal dose 50% (LC_{50}), emergence (E), reproduction (R), reactive oxygen species (ROS), glutathione S-transferase (GST), lysosomal membrane stability (LMS), catalase activity (CAT), Na^+/K^+-ATPase activity (Na^+/K^+-ATPase), feeding rate (FR), superoxide dismutase (SOD), where " > " and " < " indicate higher and lower values respectively. Copper concentrations are expressed in μg L^{-1}. The symbol * indicates that the value was estimated from the graph.

[Cu μg L^{-1}]	Effect	Species	Major group	Reference
2	< GPx, > CAT in hepatopancreas	*Lymnaea stagnalis*	Gastropoda	Atli and Grosell, 2016
5	< S, < GR	*Asellus aquaticus*	Isopoda	De Nicola Giudici et al., 1988
5	< lipid content	*Perna viridis*	Mytiloida	Yeung et al., 2016
13–43	EC_{50}–48 h immobilization	*Daphnia similis*	Cladocera	De Oliveira-Filho et al., 2004
16	< GPx	*Echinogammarus meridionalis*	Amphipoda	Quintaneiro et al., 2015
17	EC_{50}–48 h ventilation	*Hydropsyche angustipennis*	Trichoptera	van der Geest et al., 1999
20	LC_{50}–96 h	*Gammarus pseudolimnaeus*	Amphipoda	Arthur and Leonard, 1970
> 20	< S, < E, < R	*Clistoronia magnifica*	Trichoptera	Nekeber et al., 1984
25	< lipid content	*Gammarus fossarum*	Amphipoda	Zubrod et al., 2015
25	Cell damage	*Pseudokirchneriella subcapitata*	Chlorophyceae	Soto et al., 2011
25	> ammonia excretion	*Perna perna*	Bivalvia	Boudjema et al., 2016
28–80	LC_{50}–96 h	*Gammarus pulex*	Amphipoda	Bat et al., 2000
30	< swimming velocity (after 9 h)	*Daphnia magna*	Cladocera	Untersteiner et al., 2003
32	< body length (ind < 2 mm)	*Hyalella azteca*	Amphipoda	Othman and Pascoe, 2002
39	LC_{50}–96 h	*Physa integra*	Gastropoda	Arthur and Leonard, 1970
40	< S, < Leaf consumption	*Gammarus fossarum*	Amphipoda	Rosenfeldt et al., 2015
55*	LC_{50}–5 d	*Tubifex* spp.	Oligochaeta	Hunting et al., 2013
> 55	< S, < body length (ind > 2 mm)	*Hyalella azteca*	Amphipoda	Othman and Pascoe, 2002
58.8	LC_{50}–96 h	*Oreochromis niloticus*	Perciformes	Ezeonyejiaku et al., 2011
62.5	ROS; > GST and DNA damage	*Laeonereis acuta*	Polychaeta	Ferreira-Cravo et al., 2009
63–714	LC_{50}–48 h	*Danio rerio*	Cypriniformes	De Oliveira-Filho et al., 2004
63.5	< LMS, > Lipid content, < CAT	*Hediste diversicolor*	Polychaeta	Bouraoui et al., 2016
70.1	LC_{50}–96 h	*Clarias gariepinus*	Siluriformes	Ezeonyejiaku et al., 2011
71–137	EC_{50}–96 h Growth inhibition	*Raphidocelis subcapitata*	Chlorophyceae	De Oliveira-Filho et al., 2004
75	> oxygen consumption, > Na^+/K^+ ATPase	*Oncorhynchus mykiss*	Salmoniformes	McGeer et al., 2000
77	LC_{50}–96 h	*Ephoron virgo*	Ephemeroptera	van der Geest et al., 2000
90	> SOD mantle, muscle 48 h	*Lymnaea stagnalis*	Gastropoda	Atli and Grosell, 2016
95 ± 7	EC_{50} reproduction	*Nitocra spinipes*	Copepoda	Perez-Landa and Simpson, 2011
100*	LC_{50}–5 d	*Asellus aquaticus*	Isopoda	Hunting et al., 2013
100	> CAT	*Pseudokirchneriella subcapitata*	Chlorophyceae	Soto et al., 2011
100	< GPx	*Atyaephyra desmarestii*	Decapoda	Quintaneiro et al., 2015
100	< lipid peroxidation	*Asellus aquaticus*	Isopoda	Bouskill et al., 2006
100	< lipid peroxidation	*Dreissena polymorpha*	Bivalvia	Bouskill et al., 2006
106	LC_{50}–96 h	*Labeo rohita*	Cypriniformes	Khounnavongsa et al., 2015
160	EC_{50}–48 h inactivity	*Hydropsyche angustipennis*	Trichoptera	van der Geest et al., 1999
163	LC_{50}	*Hydropsyche augustipennis*	Trichoptera	van der Geest et al., 2000
179–854	LC_{50}–48 h	*Biomphalaria glabrata*	Gastropoda	De Oliveira-Filho et al., 2004
180	LC_{50}–96 h	*Adenophlebia auriculata*	Ephemeroptera	Gerhardt and Palmer, 1998
183	LC_{50}–96 h	*Chironomus ramosus*	Diptera	Majumdar and Gupta, 2012
200	< GST	*Atyaephyra desmarestii*	Decapoda	Quintaneiro et al., 2015
212	< FR	*Farfantepenaeus paulensis*	Decapoda	Silva Santos et al., 2000
257–478	LC_{50}–96 h	*Hydropsyche angustipennis*	Trichoptera	van der Geest et al., 1999
320	LC_{50}–96 h	*Ephemerella subvaria*	Ephemeroptera	AQUA/INFO, 1994
355	LC_{50}	*Cyrnus trimaculatus*	Trichoptera	van der Geest et al., 2000
360	LC_{50}–72 h	*Burnupia stenochorias*	Gastropoda	Gerhardt and Palmer, 1998
500	< CAT	*Procambarus clarkii*	Decapoda	Wei and Yang, 2016
540	LC_{50}–96 h	*Chironomus tentans*	Diptera	AQUA/INFO, 1994
630	LC_{50}-48h	*Daphnia magna*	Cladocera	Lu et al., 2017
704–1,355	EC_{50} gut content	*Hydropsyche angustipennis*	Trichoptera	van der Geest et al., 1999
742	LC_{50}–96 h	*Chironomus javanus*	Diptera	Lyly et al., 2015
759	LC_{50}–96 h	*Cyrnus trimaculatus*	Trichoptera	van der Geest et al., 2000
853	LC_{50}–96 h	*Oreochromis niloticus*	Perciformes	Lyly et al., 2015
1×10^3*	< CAT	*Allogamus ligonifer*	Trichoptera	Pradhan et al., 2016
1.7×10^3	LC_{50}–96 h	*Campeloma decisum*	Gastropoda	Arthur and Leonard, 1970
4.6×10^3	LC_{50}–96 h	Zygoptera spp.	Odonata	AQUA/INFO, 1994
4×10^3	> SOD 48–72 h	*Procambarus clarkii*	Decapoda	Wei and Yang, 2016
5×10^3*	> GST	*Allogamus ligonifer*	Trichoptera	Pradhan et al., 2016
$5–25 \times 10^3$	LC_{50}–96 h	*Adenophlebia auriculata*	Ephemeroptera	Gerhardt and Palmer, 1998
5.5×10^3	LC_{50}–96 h, < CAT, < SOD	*Esomus danricus*	Cypriniformes	Vutukuru et al., 2006

Catalase activity increased with increased copper concentrations. This finding agrees with other studies that used different organisms (e.g. insects, mussels and fish) and stressors (e.g. organic load and temperature) (Gnatyshyna et al., 2012; Lavarías et al., 2017; Madeira et al., 2016). However, some researchers have proposed a bell-shaped dose-response relationship, in which an increasing stress first induces an increase in activity (overcompensating for ROS production), but at higher stressor concentrations, the enzyme can be depleted due to severe damage (Amiard et al., 2006; Chen et al., 2016a, 2016b; Dagnino et al., 2007; Oliveira et al., 2012; Sadauskas-Henrique et al., 2017). If we assume a bell-shaped dose-response, we can conclude that in the present study, the copper concentration used was low enough to generate a stimulatory response without causing severe damage.

The uranium concentrations used (up to 50 μg L^{-1}) were not high enough to generate a significant difference in CAT activity, similarly to the response found for tadpoles with concentrations up to 254.9 μg L^{-1}

Table 3
Uranium (U) toxicity effects: reproduction (R), respiration rate (ResR), growth rate (GR), growth (G), feeding rate (FR), glutathione peroxidase (GPx), lipid peroxidation (LPO), malondialdehyde (MDA), oxygen consumption (OC), glutathione disulfuric (GSSG), 50% of function affected (EC_50), mean lethal dose 50% (LC_{50}), lowest observed effect (LOEC), catalase activity (CAT), Na^+K^+ ATPase activity (Na^+/K^+-ATPase), Phenoloxidase-like activity (PO) where " > " and " < " indicate higher and lower values respectively. The symbol * indicates that the value was estimated from the graph.

[U µg L^{-1}]	Effect	Species	Major group	Reference
> 3.5*	< CAT	*Danio rerio*	Cypriniformes	Geng et al., 2012
9.9	DNA alterations	*Daphnia magna*	Cladocera	Plaire et al., 2013
20	> induction oxidative response genes, deregulation expresión gene ATPase	*Danio rerio*	Cypriniformes	Armant et al., 2017
20	< PO	*Danio rerio*	Cypriniformes	Gagnaire et al., 2013
20	Cell mortality leucocyte	*Danio rerio*	Cypriniformes	Gagnaire et al., 2014
22.2	< body length	*Daphnia magna*	Cladocera	Plaire et al., 2013
25	< body dried mass, < R, > ResR	*Daphnia magna*	Cladocera	Massarin et al., 2010; Zeman et al., 2008
25–75	< carbon assimilation	*Daphnia magna*	Cladocera	Massarin et al., 2010
27.8	< GR, < FR	*Sericostoma vittatum*	Trichoptera	Gonçalves et al., 2011
30	< GST, < GPx	Mitochondria of *Procambarus clarkii*	Decapoda	Kaddissi et al., 2012
30	> ResR, > cytochrome c oxidase	*Danio rerio*	Cypriniformes	Lerebours et al., 2010
44	EC_{50}–48 h G pH = 6.5	*Chlorella* sp.	Algae	Franklin et al., 2000
50	< fecundity	*Daphnia magna*	Cladocera	Plaire et al., 2013
50	G (EC_{50}–7 d), < R	*Daphnia magna*	Cladocera	Zeman et al., 2008
75	< FR	*Daphnia magna*	Cladocera	Zeman et al., 2008
78	EC_{50}–48 h G pH = 5.7	*Chlorella* sp.	Algae	Franklin et al., 2000
100	< R (10% reduction)	*Daphnia magna*	Cladocera	Zeman et al., 2008
100	> ROS	*Danio rerio*	Cypriniformes	Gagnaire et al., 2013
110	< R (50% reduction)	*Ceriodaphnia dubia*	Cladocera	Goulet et al., 2015
126–146	G (EC_{50}–96 h) low water hardness	*Ceratophyllum demersum*	Algae	Markich, 2013
160	LC_{50}	*Ceriodaphnia dubia*	Cladocera	Goulet et al., 2015
> 250	< AChE	*Corbicula* sp.	Bivalvia	Labrot et al., 1996
254.9	> GPx, > LPO	*Pelophylax perezi*	Anura	Marques et al., 2013
300	> somatic growth (9 d exposure)	*Chironomus tentans*	Diptera	Muscatello and Liber, 2010
340	LC_{50}	*Hyalella azteca*	Amphipoda	Goulet et al., 2015
390	LC_{50}–48 h pH = 7	*Daphnia magna*	Cladocera	Zeman et al., 2008
480	G (LOEC)	*Chironomus dilutus*	Diptera	Liber et al., 2011
> 500	< R	*Daphnia magna*	Cladocera	Poston et al., 1984
560	> MDA	*Brachydanio rerio*	Cypriniformes	Labrot et al., 1996
571.9	< LPO, < GPx, < G	*Pelophylax perezi*	Anura	Marques et al., 2013
640	EC_{50} larval development	*Oncorhynchus mykiss*	Salmoniformes	Goulet et al., 2015
758	< GR	*Lemna aequinoctialis*	Lemnaceae	Charles et al., 2006
1,380	< AChE, < LDH, > GST	*Carassius auratus*	Cypriniformes	Bessa et al., 2016
1,404	50.4% immobility	*Daphnia magna*	Cladocera	Antunes et al., 2007
1,404	28.4% immobility	*Daphnia longispina*	Cladocera	Antunes et al., 2007
1.52×10^3	LC_{50}–14 d	*Hyalella azteca*	Amphipoda	Kuhne et al., 2002
1.97×10^3	< body length	*Ceriodaphnia dubia*	Cladocera	Kuhne et al., 2002
2.1×10^3	LC_{50}–4 d	*Pimephales promelas*	Cypriniformes	Goulet et al., 2015
2.05×10^3	LC_{50}–10 d	*Hyalella azteca*	Amphipoda	Liber et al., 2011
$> 2.5 \times 10^3$	< AChE	*Eisenia fetida*	Oligochaeta	Labrot et al., 1996
2.99×10^3	G (LOEC) concentrations	*Hyalella azteca*	Amphipoda	Liber et al., 2011
3.91×10^3	< R	*Ceriodaphnia dubia*	Cladocera	Kuhne et al., 2002
4.2×10^3	LC_{50} (fry)	*Oncorhynchus mykiss*	Salmoniformes	Goulet et al., 2015
5.5×10^3	LC_{50}–96 h in soft water	*Salvelinus fontinalis*	Salmoniformes	Parkhurst et al., 1984
7.8×10^3	LC_{50}–48 h pH = 8	*Daphnia magna*	Cladocera	Zeman et al., 2008
8.2×10^3	LC_{50}–96 h	*Hyalella azteca*	Amphipoda	Liber et al., 2011
10.5×10^3	LC_{50}–96 h	*Ceriodaphnia dubia*	Cladocera	Kuhne et al., 2002
16.4×10^3	< G	*Lemna minor*	Lemnaceae	Goulet et al., 2015
23×10^3	LC_{50}–96 h in hard water	*Salvelinus fontinalis*	Salmoniformes	Parkhurst et al., 1984
$> 25 \times 10^3$	< AChE	*Brachydanio rerio*	Cypriniformes	Labrot et al., 1996
33.5×10^3	LC_{50}–96 h	*Chironomus dilutus*	Diptera	Liber et al., 2011
37	> LPO, DNA damage	*Pangasius sutchi*	Siluriformes	Annamalai and Arunachalam, 2017
78	> SOD	*Pangasius sutchi*	Siluriformes	Annamalai and Arunachalam, 2017
137.5×10^3	> CAT	*Corbicula* sp.	Bivalvia	Labrot et al., 1996
148	LC_{50}–96 h	*Pangasius sutchi*	Siluriformes	Annamalai and Arunachalam, 2017
275×10^3	< CAT	*Eisenia fetida*	Oligochaeta	Labrot et al., 1996
275×10^3	< CAT	*Brachydanio rerio*	Cypriniformes	Labrot et al., 1996
500×10^3	< GPx	*Brachydanio rerio*	Cypriniformes	Labrot et al., 1996
2800×10^3	> MDA	*Eisenia fetida*	Oligochaeta	Labrot et al., 1996
2800×10^3	> MDA	*Corbicula* sp.	Bivalvia	Labrot et al., 1996
5000×10^3	< GPx	*Corbicula* sp.	Bivalvia	Labrot et al., 1996

(Marques et al., 2013). However, concentrations of 314 µg L^{-1} and 750 µg L^{-1} (i.e. 6–15-fold higher than in our study) caused a decrease in CAT activity in the red earthworm *Eisenia fetida* and the zebrafish *Danio rerio* (Geng et al., 2012; Labrot et al., 1996).

The other enzyme affected by metals in our study was Na^+/K^+-ATPase. This enzyme is involved in Na^+/K^+ transport across the cell membrane and is therefore involved in osmoregulation (Monserrat et al., 2007; Vasić et al., 2008). Na^+/K^+-ATPase activity decreased at 50 µg L^{-1} of uranium, but was not affected by copper (up to 70 µg L^{-1}). Na^+/K^+-ATPase activity was reported to be inhibited by

tributyltin in the gills of juvenile carp *Cyprinus carpio* (Li et al., 2016), and high nutrient loads in the ten-spotted live-bearer fish *Cnesterodon decemmaculatus* (de la Torre et al., 2007). Consistently with our study, Na^+/K^+-ATPase activity decreased in the gills, liver and gonad when zebrafish were exposed to uranium mill tailings (6 μg L^{-1} of uranium in the presence of other metals in a leaching solution) (Geng et al., 2012). Although the Na^+/K^+-ATPase activity of *Calamoceras marsupus* was not affected by copper (up to 70 μg L^{-1}) in our study, concentrations as low as 10 μg L^{-1} in freshwater amphipods (Brooks and Mills, 2003), and 12 μg L^{-1} in freshwater mussels (Giacomin et al., 2013), as well as 100 μg L^{-1} in copepods (Pinho et al., 2007) were enough to affect Na^+/K^+-ATPase activity.

Even though AChE and LDH activities were reduced by uranium in previous studies (Bessa et al., 2016; Gagnaire et al., 2014), the concentrations tested here were below the concentrations reported to cause an effect: 2.5 mg L^{-1} in clams (Labrot et al., 1996), 1.38 mg L^{-1} in fish (Bessa et al., 2016), and 2.5 mg L^{-1} in oligochaetes (Labrot et al., 1996). On the other hand, concentrations up to 2 mg L^{-1} waterborne uranium did not show evidence in Cholinesterase in studies on *Daphnia magna* (crustacean), *Corbicula fluminea* (mollusc), and *Carassius auratus* (fish) (Nunes et al., 2017).

Pollutants in general and metals in particular typically co-occur, with potential synergistic or antagonistic interactions (Sheehan, 1984). Even though we did not evaluate the interaction effects for copper and uranium exposure, it worth to remark that copper-uranium antagonism was reported for lesser duckweed *Lemna aequinoctialis,* causing reductions in growth (Charles et al., 2006). Moreover, other factors besides the presence of other metals, like pH and water hardness might change metal toxicity, accumulation, and adsorption of uranium and copper (Crawford et al., 2016; Franklin et al., 2000; Khounnavongsa et al., 2015).

Enzyme biomarkers are considered useful for detection of sublethal concentration effects (Livingstone, 1993). However, their responses seem to be affected by sex, reproductive status, age and diet (Hyne and Maher, 2001), and vary among taxonomic groups (Domingues et al., 2010; Ippolito et al., 2017; Sadauskas-Henrique et al., 2017), among tissues of the same species (Madeira et al., 2016), exposure times to the pollutant (Chen et al., 2016a, 2016b; Sadauskas-Henrique et al., 2017), and stressor. For this reason, many researchers suggested the use of a set of biomarkers to assess biological impacts of pollutants (e.g. Cazenave et al., 2009; Colin et al., 2015). Our findings support this suggestion; here, Na^+/K^+-ATPase and CAT proved to be the most sensitive biomarkers for sublethal concentrations of uranium and copper, respectively.

Finally, species of Trichoptera are assumed to have an intermediate to low tolerance to pollutants in general and to copper in particular (Birge et al., 2000; Bonada et al., 2004) (Table 2); in other studies, shredder and caddisfly abundances decreased with increased metal concentrations under field conditions (Clements et al., 2000). Since the metal concentrations used here caused no significant decreases in growth, feeding and growth efficiency, and considering the wide distribution of *C. marsupus* among reference (with low metal concentrations) and polluted rivers (Feio et al., 2005), this species can be considered tolerant.

5. Conclusions

CAT and Na^+/K^+-ATPase seem to be promising biomarkers for use as ecotoxicological endpoints for monitoring stress conditions in freshwaters. They are responsive at concentrations below the doses that cause mortality or changes in feeding, growth or growth efficiency.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ecolind.2017.10.065.

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