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Editor's choice

Comparison of three shredders response to acute stress induced by eucalyptus leaf leachates and copper: single and combined exposure at two distinct temperatures

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Abstract – The objectives of this study were to compare the sensitivity of three freshwater macroinvertebrate shredder species (*Atyaephyra desmarestii*, *Echinogammarus meridionalis* and *Schizopelex festiva*) to acute stress induced by eucalyptus leaf extracts and copper, independently and in mixtures, and the ability of temperature to influence the chemicals' toxicity. Laboratory bioassays based on mortality with single substances and mixtures were carried out with the three species at 10 and 20 °C. After 96 h of exposure, *S. festiva*, *A. desmarestii* and *E. meridionalis* were found to have differences of sensitivity to copper, eucalyptus leaf extracts and their mixtures, with *S. festiva* being the least sensitive species at both 10 and 20 °C. The relative sensitivity of *A. desmarestii* and *E. meridionalis* to chemical exposure seems to be chemical and temperature dependent. Overall, these findings suggest that chemical stress may modulate the biodiversity of stream shredders communities due to differential sensitivity of individual species to environmental contaminants, and that temperature may influence the process. Thus, more knowledge on the combined effects of multi-stressors is needed, particularly on temperature and chemicals' interactions and on the molecular mechanisms underlying the responses observed at individual level.

Key words: Chemical acute toxicity / combined effects of stressors / *Atyaephyra desmarestii* / *Echinogammarus meridionalis* / *Schizopelex festiva*

Introduction

Small forested streams depend on their riparian areas as source of energy and nutrients (Vannote *et al.*, 1980; Richardson and Danehy, 2007). Their strong interactions with the catchment and their relative low water volume make them particularly vulnerable to changes caused by natural factors and/or anthropogenic activities (Malmqvist and Rundle, 2002; Ormerod *et al.*, 2010; Woodward *et al.*, 2010). Headwater streams may constitute up to 80% of the total length of the fluvial net (Allan and Castillo, 2007). These heterotrophic systems play a pivotal role as hot spots of biodiversity and are key organic matter suppliers to higher-order streams (Perkins *et al.*, 2010). Thus, efforts should be devoted to the preservation of their good ecological status.

Detritus processing is a key process for the stream heterotrophic production that is primarily undertaken by fungi, namely aquatic hyphomycetes, and shredders (Cummins, 1973; Gessner *et al.*, 2010), a functional feeding group of detritivorous invertebrates. The abundance and richness of these shredder communities mainly depend on water physicochemical characteristics and is closely linked with the type, amount, spatial and temporal distribution of leaves in the watercourse (Gessner *et al.*, 2010). The importance of this group, as a link between detritus and higher trophic levels, and the distinct sensitivities of macrobenthos to environmental factors make them important tools to assess the effects of environmental stressors on stream ecosystems (Brix *et al.*, 2011; Liess and Beketov, 2011; Peters *et al.*, 2011). Alterations in these macroinvertebrate communities potentially resulting from the influence of single or multiple stressors, may have consequences at higher levels of biological organization

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and ecosystem functioning (Sanpéra-Calbet *et al.*, 2009; McMahon *et al.*, 2012).

Forestry practices constitute one of the main threats to streams (Lecerf and Richardson, 2010). Among these, exotic eucalyptus afforestations are now one of the main causes of stream's impairment in several regions (*e.g.* Santos, 1997; Molinero and Pozo, 2004; Kominoski *et al.*, 2013). Eucalyptus (namely *Eucalyptus globulus*) monocultures are frequently associated with altered flow regimes, modified food webs and distinct dynamics of organic matter, where shredder's role as leaf processors has been reduced (Graça *et al.*, 2002; Larrañaga *et al.*, 2009). This has been related to low eucalyptus leaf litter quality and direct toxicity (Canhoto & Graça, 1999). Whether these effects are generalized across the shredders guild or modulated by temperature is still not known but particularly important in an era where air and water thermal conditions are changing (Morrill *et al.*, 2005; IPCC, 2007).

In addition to natural toxins, other environmental contaminants may be present in stream's water and sediments. Among these, ubiquitous contaminants such as metals (*e.g.* copper) are of special interest because they are introduced at considerable amounts in aquatic ecosystems as result of several anthropogenic activities (*e.g.* different industries, mining activities, agriculture, veterinary and human medicine) and natural processes (*e.g.* volcanic eruptions). Metals have been found to induce adverse effects on shredders ecology as a result of long-term exposure to low environmental contamination levels (Farag *et al.*, 1998; Leslie *et al.*, 1999; Forrow and Maltby, 2000; Macedo-Sousa *et al.*, 2007; Faria *et al.*, 2007, 2008; Hogsden and Harding, 2012), or to punctual or pulses exposure to high levels (Macedo-Sousa *et al.*, 2008; Dédourge-Géffard *et al.*, 2009). In the last years, some studies showed that the presence of metals in streams may affect leaf conditioning processes and consequently shredders performance (Batista *et al.*, 2012; Pradhan *et al.*, 2012). However, a lack of knowledge on the combined effects of leaf toxins and metal contamination to shredders still exists.

Temperature has a major influence in biological and ecological processes: it may be a stressor by itself (Ferreira *et al.*, 2010; McFeeters and Frost, 2011; Wojewodzic *et al.*, 2011), it may modify the toxicity of chemicals (Prato *et al.*, 2009; Lapointe *et al.*, 2011; Vieira and Guilhermino, 2012), and may change abiotic conditions (*e.g.* water eutrophication and oxygen depletion) (Woodward *et al.*, 2010). Furthermore, warmer temperatures are usually coupled with low flow events (Malmqvist and Rundle, 2002; Woodward *et al.*, 2010; Canhoto *et al.*, 2013) that favour the increased concentration of chemical contaminants in the water (Chatzinikolaou, 2006). Considering the expected warming scenarios resulting of global climate changes (IPCC, 2007), and the predictable increase of chemicals use by a growing human population (Dudgeon *et al.*, 2006), it is most important to investigate the combined effects of chemical and thermal stress on the protagonists of key stream ecosystem-level processes (*i.e.* leaf litter decomposition) such as shredders.

The objective of this study was to compare the sensitivity of three shredder species [*Atyaephyra desmarestii* Millet (1981), *Echinogammarus meridionalis* Pinkster (1973) and *Schizopelex festiva* Rambur (1842)] to acute stress induced by eucalyptus leaf leachates and a copper, independently and in mixture. Considering the ability of temperature to influence chemicals' toxicity (Boeckman and Bidwell, 2006; Prato *et al.*, 2009), tests were performed at 10 and 20 °C. These are common temperatures in colder and warmer seasons, respectively, in the streams where the invertebrates were collected (Canhoto and Laranjeira, 2007; Ferreira *et al.*, 2010). *A. desmarestii*, *E. meridionalis* and *S. festiva* were selected for this study because they play a key role in leaves decomposition in low-order streams where they occur, are abundant and easily maintained in the laboratory. Furthermore, *A. desmarestii* and *E. meridionalis* have been widely used as model species in ecology and ecotoxicology (Pantani *et al.*, 1997; Gerhardt *et al.*, 2004; Pestana *et al.*, 2007; Macedo-Sousa *et al.*, 2007, 2008).

Material and methods

Collection and acclimatization of invertebrates

Organisms were collected in the wild (*A. desmarestii*: 40°10'N, 8°18'W; *E. meridonalis:* 39°59'N, 8°34'W; *S. festiva*: 40°32'N, 8°09'W) from January 2010 to February 2011, including those used in preliminary assays. Individuals were brought to the laboratory in coolers filled with stream water. In the laboratory, they were maintained in 5 L aquaria filled with aerated ASTM hard water (ASTM, 1980), with a layer of (10 cm) sterile sediment, under a 12 h light (L): 12 h dark (D) photoperiod. Specimens were fed *ad libitum* for 1 week prior to the start of the test, with conditioned alder leaves (as described below), with medium renewal every other day until the beginning of the experiments. During this period, half of the invertebrates from each species were maintained at 10 ± 1 °C and the other half at 20 ± 1 °C according to their further use in bioassays. Alder leaves were collected from the same stands of trees in autumn 2009, just after abscission, and were air-dried and stored until needed. Leaves were weighed in batches of 4.5–5 g, moistened, enclosed in coarse mesh bags (10 mm mesh size) and colonized, for 3 weeks, in Ribeira de S. João, Portugal (40°11'N; 8°25'W).

Bioassays with eucalyptus leachates

An original eucalyptus leaf leachate, hereafter indicated as stock-eucalypt leachates (EL), was prepared from senescent eucalyptus leaves (*E. globulus*) collected just after abscission between September and October 2009 in Pinhal de Marrocos, Coimbra, Portugal (40°11'N; 8°24'W). Leaves were transported to the laboratory, air dried in paper boxes at room temperature in the dark, and

Table 1. Physico-chemical characteristics of the original eucalyptus leachate solution (stock-EL). For each parameter, the values are the means of three determinations in the stock-EL with corresponding standard deviation.

Parameters	Average (± SD)
pH	3.90 (0.115)
Conductivity ($\mu S.cm^{-1}$)	1116 (54.354)
Tannic acid equivalents ($mg.L^{-1}$)	465 (41.8)
Oxygen ($mg.L^{-1}$)	1.70 (0.231)
DOC ($\mu g.L^{-1}$)	2.53 (0.055)

stored until needed. Leaf leaching was obtained from 28 $g.L^{-1}$ of dried eucalyptus leaves immersed in the ASTM hard water (ASTM, 1980) (alkalinity 110–120 $mg.L^{-1}$ as $CaCO_3$ and hardness 160–180 $mg.L^{-1}$ as $CaCO_3$), here after indicated as ASTM, for 7 days, under continuous moderate aeration (15 °C ± 1; photoperiod 12 h light: 12 h dark) according to Canhoto and Laranjeira (2007). The leachate was decanted and stored at 4 °C until further use. Before the experiments, the stock-EL was analysed for total polyphenols (Graça *et al.*, 2005), pH (JENWAY 3310, Essex, UK), conductivity (WTW LF 330, Weilheim, Germany), dissolved organic carbon (DOC) (Elementar Analysensysteme Gmbh LiquiTOC, Hanau, Germany) and dissolved oxygen (D.O.) (WTW ProfiLine Oxi 3210, Weilheim, Germany). The characterization of the stock-EL is indicated in Table 1.

From the stock-EL, dilutions (1:2 (v/v)) in ASTM were prepared to obtain the following test concentrations: 100% (no dilution), 50, 25, 12.5, 6.25, 3.13 and 1.56%. These solutions were used as different treatments in the bioassays with EL and contained the following concentrations of tannic acid: 465, 232.5, 116.3, 58.1, 29.1, 14.5 and 7.3 $mg.L^{-1}$. Hereafter, the different treatments will be indicated in relation to their tannic acid concentrations. An additional treatment (ASTM only) was used as control. Because of a low response of *S. festiva* to the lowest concentrations tested in the first bioassays, two other bioassays were carried out with this species with the following concentrations of tannic acid: 349, 412 and 465 $mg.L^{-1}$ (corresponding to 75, 89 and 100% of the stock-EL) at 10 °C; 279, 325 and 434 $mg.L^{-1}$ (corresponding to 60, 70 and 93% of the stock-EL) at 20 °C. The pH of test solutions, measured at the beginning of the bioassays is indicated in supplementary material (Appendix 1). The bioassays were carried out under laboratory conditions (12:12-h light/dark photoperiod; 10 ± 1 °C or 20 ± 1 °C). A total of 160 organisms per species ($n = 80$ for each temperature) were used, with the following ranges of dry weight (mean dry weight (d.w.) ± standard deviation): 2.667 ± 0.024 mg for *A. desmarestii*, 1.109 ± 0.008 mg for *E. meridionalis* and 0.0159 ± 0.0002 mg for *S. festiva*. In each bioassay, ten individuals were randomly distributed per treatment in eight different treatments corresponding to seven EL treatments and one control (ASTM). Organisms were individually exposed in plastic beakers filled with 200 ml of the test solution with continuous aeration. Feeding was stopped 24 h before the starting of the assays and no food was provided during the exposure period (96 h). Effect criterion was mortality, recognized by the immobility after stimulation by a gentle touch with a plastic pipette or when found outside the case for *S. festiva*. Water temperature, conductivity, pH, D.O. and mortality were monitored at beginning of the bioassays and at each 24 h intervals.

Bioassays with copper

Copper 96 h-bioassays were carried out with single species at both temperatures under laboratorial conditions similar to those described for EL bioassays (12:12-h light/dark photoperiod; 10 ± 1 or 20 ± 1 °C). For each bioassay, a stock solution of copper sulphate pentahydrate (CAS no. 7758–99–8, ≥ 98% purity, purchased from Merck KGaA, Darmstadt, Germany) was prepared in ultra-pure water (conductivity < 5 $\mu S.cm^{-1}$; Seralpur PRO 90 CN, Seral, Ransbach-Baumbach, Germany). The concentration of the stock solution was 25.5 $mg.L^{-1}$ (Cu concentration). Test solutions were obtained by serial dilution of the stock solution in ASTM (ASTM, 1980). The following Cu concentrations were tested (selection based on preliminary bioassays): 3.26, 1.63, 0.81, 0.41, 0.20, 0.10, 0.05 and 0.03 $mg.L^{-1}$ for *A. desmarestii* at both temperatures; 0.81, 0.41, 0.20, 0.10, 0.05 and 0.03 $mg.L^{-1}$ for *E. meridionalis* at 10 °C and 0.41, 0.20, 0.10, 0.05, 0.03, 0.01 and 0.006 $mg.L^{-1}$ for *E. meridionalis* at 20 °C; and 8.14, 4.07, 2.04, 1.02, 0.51 and 0.25 $mg.L^{-1}$ Cu for *S. festiva* at both temperatures. The pH of test solutions, measured at the beginning of the bioassays is indicated in the supplementary material (Appendix 1).

To assess the lethal toxicity of copper, 180 specimens of *A. desmarestii* ($n = 90$ for each temperature), and 160 *E. meridionalis* and *S. festiva* were used ($n = 80$ for each temperature) with the following ranges of dry weight (mean d.w. ± SD): 2.90 mg ± 0.01 for *A. desmarestii*, 1.12 mg ± 0.002, for *E. meridionalis* and 0.017 mg ± 0.0002 for *S. festiva*. Ten individuals were randomly distributed per treatment as described in the section 'Bioassays with eucalyptus leachates'.

Combined effects of eucalyptus leachates and copper, and temperature effects

The experimental design for mixture bioassays, carried out at 10 and 20 °C with the three species, was based on the LC_{10}, LC_{20} and LC_{50} obtained in the bioassays with single substances (Table 2), except in the case of *S. festiva*. For this species, the concentration of tannic acid in the 100% eucalyptus leachates treatment (465 $mg.L^{-1}$) was tested, because the LC_{50} of tannic acid calculated from data of the bioassay with EL alone was higher than the tannic acid concentration present in the EL stock solution (Table 1). Briefly, for each mixture bioassay and for each temperature, four treatments were considered: control (ASTM only), copper LC_{10} + eucalyptus leachates LC_{10}

Table 2. Eucalyptus leachates (EL) and copper (Cu) concentrations causing 10% (LC_{10}), 20% (LC_{20}) and 50% (LC_{50}) of mortality on *Atyaephyra desmarestii*, *Echinogammarus meridionalis* and *Schizopelex festiva* at 10 or 20 °C. 95% confidence intervals are shown within brackets.

	A. desmarestii			*E. meridionalis*			*S. festiva*		
	LC_{10}	LC_{20}	LC_{50}	LC_{10}	LC_{20}	LC_{50}	LC_{10}	LC_{20}	LC_{50}
EL ($mg.L^{-1}$)									
10 °C	46.238 (19.398–71.369)	67.894 (35.748–99.193)	141.577 (96.638–221.824)	12.906 (3.557–24.340)	24.258 (9.639–41.080)	81.128 (48.887–148.447)	348.475 –	412.049 –	567.779 –
20 °C	8.296 (2.653–14.561)	13.510 (5.710–21.633)	34.346 (21.408–53.331)	7.929 (1.466–16.918)	16.349 (4.938–30.095)	65.276 (36.679–124.597)	273.837 –	332.519 –	482.099 –
Cu ($mg.L^{-1}$)									
10 °C	0.05 (0.018–0.087)	0.83 (0.038–0.133)	0.221 (0.139–0.343)	0.048 (0.012–0.088)	0.086 (0.034–0.144)	0.266 (0.161–0.541)	2.304 (0.372–3.868)	3.678 (1.508–7.084)	9.0 (5.209–94.989)
20 °C	0.043 (0.016–0.073)	0.068 (0.032–0.106)	0.165 (0.106–0.251)	0.005 (0.001–0.011)	0.010 (0.003–0.019)	0.036 (0.020–0.062)	1.840 (0.397–3.055)	2.949 (1.244–5.008)	7.274 (4.394–32.498)

(Cu-LC_{10} + EL-LC_{10}); copper LC_{20} + eucalyptus leachates LC_{20} (Cu-LC_{20} + EL-LC_{20}); and copper LC_{50} + eucalyptus leachates LC_{50} (Cu-LC_{50} + EL-LC_{50}). Mixture test solutions were prepared by diluting stock solutions of copper and EL (prepared as described in previous sections) in ASTM hard water, and mixing them. It should be noted that stock-EL has high concentration of DOC (Table 1). Because no standardization of DOC content among mixture test solutions was made, different treatments have distinct DOC contents. This is important because DOC is likely to bind to copper reducing its bioavailability in test media. Therefore, the toxicological interactions assessed in the present study are those expected to occur in real scenarios under simultaneous contamination of water with EL and copper, considering the overall effects on organisms and not those resulting from individual chemicals. The pH values of test solutions, measured at the beginning of the bioassays are indicated in Table 3, and they were always higher than 7.13 pH units. Bioassays were carried out in conditions similar to those described in sections 'Collection and acclimatization of invertebrates' and 'Bioassays with eucalyptus leachates'. The ranges of d.w. of the tested organisms were: 2.68 mg ± 0.010 at 10 °C and 2.67 mg ± 0.011 at 20 °C for *A. desmarestii*, 1.12 mg ± 0.003 at 10 °C and 1.12 mg ± 0.004 at 20 °C for *E. meridionalis* and 0.017 mg ± 0.0002 at 10 °C and 0.015 mg ± 0.0004 at 20 °C for *S. festiva*.

Statistical analysis

The concentrations inducing 10% (LC_{10}), 20% (LC_{20}) and 50% (LC_{50}) of mortality were determined from the log concentration *versus* response (probit transformation of mortality percentages) toxicity curves. To compare the sensitivity of different species at distinct temperatures, a two-way analysis of covariance (2 way-ANCOVA) was used. The probit transformed % of mortality was used as dependent variable; temperature and species as independent variables (fixed factors) and the $\log_{10}$ of the chemical concentration as covariate; when significant differences were found, one-way analysis of covariance (ANCOVA) was used to identify their potential causes, followed by *a posteriori* LSD tests whenever necessary. In the mixture bioassays, the results are expressed as the concentrations of tannic acid only; the concentrations of copper are not indicated to avoid bias resulting from the potential binding of copper to DOC and thus from the differences between nominal and actual copper concentrations. Binary comparisons of the toxicity curves obtained for each species and temperature in the bioassays with EL alone and in the mixture with copper where compared using ANCOVA. Preliminary check of ANCOVA assumptions was done in all cases. In the mixture bioassays, no mixture models were used to investigate the types of interactions because of the potential bias in the bioavailability of copper due to differences in DOC content in distinct treatments.

Table 3. Values of pH (in pH units) measured at the beginning of the bioassays with eucalyptus leachates (EL) and copper (Cu) mixtures carried out at 10 and 20 °C.

Temp. (°C)	Mixture bioassay		
	Treat.	0 h	Variat.
		A. desmarestii	
10	EL-LC_{10} + CuLC_{10}	7.67 ± 0.00	0.58
	EL-LC_{20} + CuLC_{20}	7.32 ± 0.01	0.28
	EL-LC_{50} + CuLC_{50}	7.32 ± 0.01	0.61
20	EL-LC_{10} + CuLC_{10}	7.23 ± 0.01	0.49
	EL-LC_{20} + CuLC_{20}	7.22 ± 0.00	0.82
	EL-LC_{50} + CuLC_{50}	7.13 ± 0.01	0.48
		E. meridionalis	
10	EL-LC_{10} + CuLC_{10}	7.66 ± 0.00	0.59
	EL-LC_{20} + CuLC_{20}	7.58 ± 0.01	0.42
	EL-LC_{50} + CuLC_{50}	7.33 ± 0.00	0.38
20	EL-LC_{10} + CuLC_{10}	7.68 ± 0.01	0.41
	EL-LC_{20} + CuLC_{20}	7.36 ± 0.00	0.22
	EL-LC_{50} + CuLC_{50}	7.29 ± 0.02	0.32

The values indicated are the mean of the pH values determined in ten replicates per treatment with the corresponding standard errors. Temp., temperature; Treat., treatment; Variat., pH variation during the assay (pH at time 0 – pH at 96 h). pH units in control treatment: 7.82 ± 0.05; Variat = 0.63.

Results

Species sensitivity to single substances and temperature effects

In all the bioassays carried out with eucalyptus leachates, the mortality in control treatments was 0%, except in the test with *E. meridionalis* at 20 °C where 20% of mortality in the control group was recorded. In each test beaker, the water temperature variation was less than 1 °C, D.O. was always higher than 9.12 $mg.L^{-1}$; the pH of test solutions at the beginning of the bioassays was always higher than 3.9 pH units (Appendix 1), the maximal pH variation during the assays was 0.91; and the conductivity variation was always lower than 71 $\mu S.cm^{-1}$. Mortality recorded is provided in supplementary material (Appendix 2), whereas the 96 h LC_{10}, LC_{20} and LC_{50} calculated from the toxicity curves (Fig. 1) are indicated in Table 2. For *S. festiva*, the calculation of LC_x values from the results of the first bioassay was not possible because mortality was only observed at the highest concentration tested. In the second bioassay, the calculation of LC_{10}, LC_{20} and LC_{50} was possible but without confidence limits; also, the estimated LC_{50} of tannic acid exceeds its concentration in 100% of eucalyptus leachates. Therefore, due to these constraints, this species was not included in further statistical analysis. The LC_{50} obtained for *A. desmarestii and E. meridionalis* in single bioassays ranged from 34.346 to 141.577 $mg.L^{-1}$ of tannic acid (Table 2). The lowest values were obtained at 20 °C for both species, being about 4.1- and 1.2-folds lower than the corresponding values obtained at 10 °C for *A. desmarestii* and *E. meridionalis*, respectively. The comparison of *A. desmarestii* and *E. meridionalis* toxicity curves (Fig. 1) by 2 way-ANCOVA indicated no significant differences between species ($F_{(1,16)} = 0.5$, $P > 0.05$); a significant effect of temperature ($F_{(1,16)} = 10.8$, $P < 0.01$) explaining 40.3% of total variance, and a significant interaction between species and temperature ($F_{(1,16)} = 5.9$, $P < 0.05$) explaining 26.9% of the variance. The increase of temperature from 10 to 20 °C, significantly increased the toxicity of

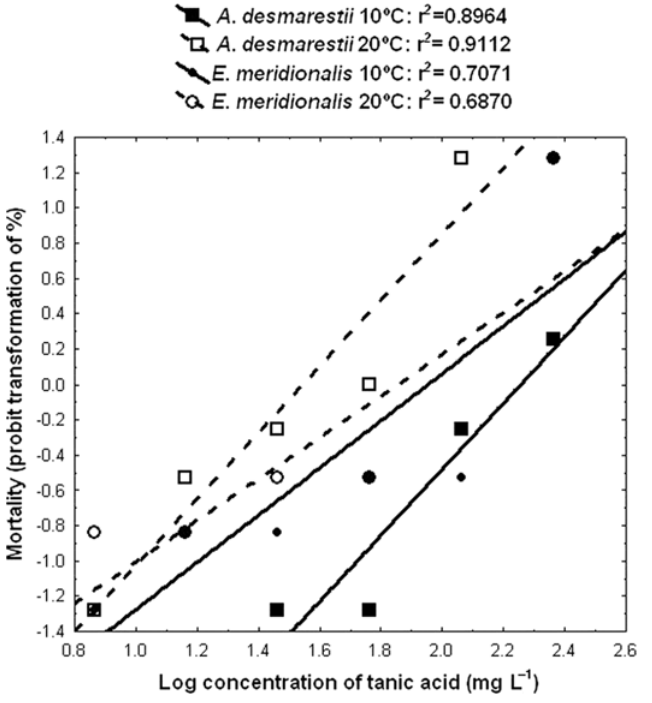

Fig. 1. Acute toxicity of eucalyptus leachates to *A. desmarestii* and *E. meridionalis* at 10 and 20 °C. Lines represent linear regression, R^2 for the two organisms and temperatures are displayed in the graphic.

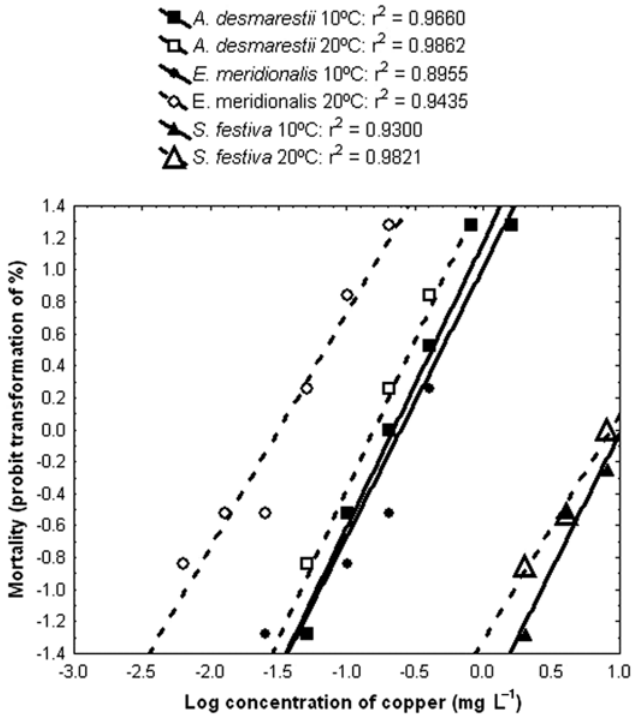

Fig. 2. Acute toxicity of copper to *A. desmarestii*, *E. meridionalis* and *S. festiva* at 10 and 20 °C. Lines represent linear regression, R^2 square for the two organisms and temperatures are displayed in the graphic.

eucalyptus leachates to *A. desmarestii* (ANCOVA: $F_{(1,8)} = 36$, $P < 0.01$) but no significant differences between the toxicity curves at 10 and 20 °C were found for *E. meridionalis* (ANCOVA: $F_{(1,8)} = 0.4$, $P > 0.05$).

In all the bioassays carried out with copper, the mortality in control treatments was always equal or lower than 10%, the water temperature variation was < 1 °C; D.O. was always higher than 9.09 mg.L^{-1}; the pH of test solutions at the beginning of the bioassays was always higher than 7.5 pH units (Appendix 1), the maximal pH variation during the assays was 0.82; and the conductivity variation was always lower than 62 µS.cm^{-1}. The mortality recorded in copper bioassays is provided in the supplementary material (Appendix 2), whereas the LC_{10}, LC_{20} and LC_{50} are shown in Table 2. The LC_{50} of copper ranged from 0.036 to 9.0 mg.L^{-1}, with LC_{50} values being higher at 10 °C than at 20 °C (about 1.3-folds for both *S. festiva* and *A. desmarestii,* and 7.4-folds for *E. meridiona*lis, respectively) (Table 2). The comparison of the toxicity curves (Fig. 2) by 2-way ANCOVA, indicated the significant differences among species ($F_{(2,22)} = 162.1$, $P < 0.01$); significant effects of temperature ($F_{(1,22)} = 51.2$, $P < 0.01$); and the interaction between species and temperature was also significant ($F_{(1,22)} = 25.0$, $P < 0.01$). Comparing now the three species at 10 °C, significant differences of sensitivity were found (ANCOVA: $F_{(1,11)} = 68.87$, $P < 0.01$) with *S. festiva* being less sensitive (Table 2) than *A. desmarestii* and *E. meridionalis* (*S. festiva* versus *A. desmaresti*: $P < 0.01$; *S. festiva* versus *E. meridionalis*: $P < 0.01$), whereas no significant differences of sensitivity between *A. desmarestii* and *E. meridionalis* were found ($P > 0.05$). At 20 °C, significant differences of sensitivity between *S. festiva* and each of the other species were found (*S. festiva* versus *A. desmaresti*: $P < 0.01$; *S. festiva* versus *E. meridionalis*: $P < 0.01$), with *S. festiva* being less sensitive that the other two species; significant differences between *A. desmarestii* and *E. meridionalis* were also registered ($P < 0.01$). Temperature significantly increased the toxicity of copper to *A. desmarestii* and E. *meridionalis* (ANCOVA: $F_{(1,8)} = 6.5$, $P < 0.05$ for *A. desmarestii* and $F_{(1,8)} = 53.6$, $P < 0.01$ for *E. meridionalis*) with more pronounced effects in *E. meridionalis*. No significant differences between temperatures were observed for *S. festiva* (ANCOVA $F_{(1,3)} = 4.3$, $P > 0.05$).

Mixture bioassays

In all the mixtures bioassays, the water temperature variation was less than 1 °C; D.O. was always higher than 9.32 mg.L^{-1}; the pH of test solutions at the beginning of the bioassays was always higher than 7.13 pH units (Table 3); the maximal pH variation during the assays was 0.82; and the conductivity variation was always lower than 67 µS.cm^{-1}. The results of the mixtures bioassays, expressed as percentage of mortality are detailed in the supplementary material (Appendix 2). Because of the low mortality of *S. festiva* in the eucalyptus leachates solutions, even at the highest exposure concentrations tested, and the lack of relevance of testing eucalyptus leachate concentrations higher than 100%, this species was not included in further statistical analysis of mixture bioassays. The comparison of the mixture toxicity curves based on the concentration of tannic acid (Fig. 3) indicates significant differences between species (2-way ANCOVA: $F_{(1,6)} = 92.04$, $P < 0.01$), significant differences between temperatures (2-way ANCOVA: $F_{(1,6)} = 108.45$, $P < 0.01$), and no significant interaction between species and temperature (2-way ANCOVA: $F_{(1,6)} = 3.95$, $P > 0.05$). The LC_{50} for the mixture are shown in Table 4, based on the concentrations of tannic acid. Significant differences between the toxicity curves of EL alone and of the mixture with copper were found for *E. meridionalis* at 20 °C (ANCOVA: $F_{(1,6)} = 25.10$, $P < 0.01$), whereas at 10 °C no significant differences were found (ANCOVA: $F_{(1,6)} = 2.49$, $P > 0.05$). For *A. desmarestii* at both temperatures no significant differences between the toxicity curves of EL alone and of the mixture were found (ANCOVA: $F_{(1,3)} = 1.12$, $P > 0.05$ and $F_{(1,5)} = 3.76$, $P > 0.05$, for 10 and 20 °C, respectively).

Discussion

Bioassays with single substances and temperature effects

In the bioassays with EL, no correction of pH or DOC concentration were made to simulate the overall processes

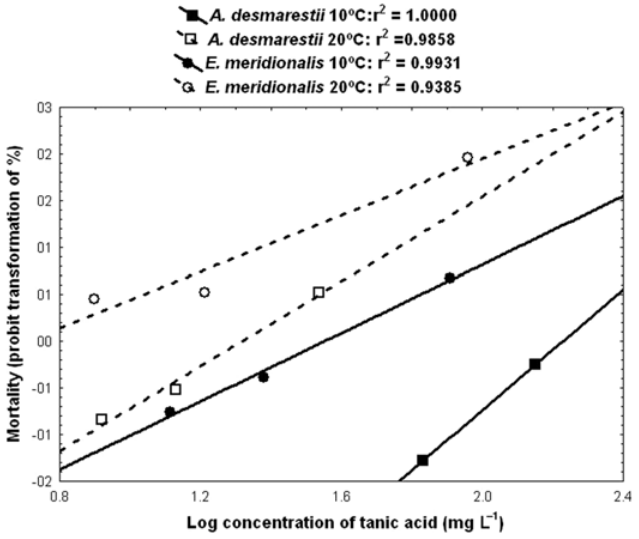

Fig. 3. Acute combined toxicity of eucalyptus leachates to *A. desmarestii* and *E. meridionalis* at 10 and 20 °C. Lines represent linear regression, R^2 for the two organisms and temperatures are displayed in the graphic.

likely occurring in real scenarios. Thus, the toxic effects assessed include those induced by eucalyptus leaf toxins and pH effects under different DOC concentrations. This is important because low pH values such as those found in the two highest EL treatments (Appendix 1) may have adverse effects on the organisms *per se* (Felten and Guérold, 2006; Janssens de Bisthoven *et al.*, 2006), and distinct species may have different sensitivities to both eucalyptus leaf toxins and pH variation.

The low mortality of *S. festiva* in the bioassays with single substances and the lack of significant influence of temperature on the toxicity of the chemicals tested indicate that this species was less sensitive to chemical/pH stress and temperature changes than *A. desmarestii* and *E. meridionalis*. *S. festiva* has a case and the test period was relatively short (96 h); in these conditions, the starvation pressure may have not been high enough to force the organisms to go out of the case in order to search for food. That behaviour probably allowed the larvae to avoid (or reduce) the exposure to environmental contaminants and low pH values, as suggested by the low mortality recorded in the two highest EL treatments with pH values of 3.9 and 4.5 (supplementary material); this could also explain, at least in part, the lack of influence of temperature on chemicals' toxicity in this species. The finding that this species was the least sensitive to both EL and copper-induced stress may also suggest the presence of general mechanisms decreasing the uptake of environmental contaminants, increasing their elimination, and/or an efficient ionic regulation. In fact, mechanisms of tolerance to metals (Darlington and Gower, 1990) and differential patterns of metal accumulation were reported for other Trichopteran larvae (Cain and Luoma, 1998; Rainbow, 2002, 2007; Rainbow *et al.*, 2012). Furthermore, the lower sensitivity of *S. festiva* to chemical and/or pH stress relatively to other shredder species found in the present study, is in good agreement with previous findings in the caddisfly *Sericostoma vittatum* exposed to aerated eucalyptus leachates (Canhoto and Laranjeira, 2007; Canhoto *et al.*, 2013).

Table 4. Acute combined (96 h) toxicity of eucalyptus leachates and copper for *Atyaephyra desmarestii*, and *Echinogammarus meridionalis* at 10 and 20 °C. LC_{50}- 50% lethal concentrations calculated from the toxicity curves (log concentrations versus probit transformation of mortality %)

Temp. (°C)	Species	LC_{50} (mg.L^{-1})	
10	*A. desmarestii*	160.869 EL	(111.564–3782.292)
	E. meridionalis	35.898 EL	(26.409–52.589)
20	*A. desmarestii*	20.874 EL	(12.055–114.242)
	E. meridionalis	4.972 EL	(n.a.)

EL, eucalyptus leachates concentrations expressed in mg of tannic acid.L^{-1}. 95% confidence limits for the estimates are given within brackets.

In the present study, no significant differences of sensitivity to copper-induced stress between *A. desmarestii* and *E. meridionalis* were found at 10 °C (Fig. 2, Table 2). The main mechanism of acute copper toxicity in aquatic organisms is the disruption of ionoregulatory processes, particularly through its influence on Na^+ transport mechanisms, with effects on the internal concentrations and transport of several other ions (Santore *et al.*, 2001; Grosell *et al.*, 2002, Paquin *et al.*, 2002). Thus, our study suggests a similar action of copper on these mechanisms in both *A. desmarestii* and *E. meridionalis* at 10 °C. Additionally, copper is a well-known oxidative stress inducer in both vertebrates (*e.g.* Olivari *et al.*, 2008; Roy *et al.*, 2009; Vieira *et al.*, 2009; Boveris *et al.*, 2012) and invertebrates (Maria and Bebianno, 2011; Gomes *et al.*, 2012), including shredders (Bouskill *et al.*, 2006; Sroda and Cossu-Leguille, 2011). Thus, damage in crucial molecules (*e.g.* proteins, lipids and DNA) as a result of oxidative stress, may also have contributed to its toxic effects. If so, the findings of the present study, suggest an overall similar effect in both species at 10 °C. The raise of temperature from 10 to 20 °C increased the toxicity of copper to both species, with more pronounced effects in *E. meridionalis* (Fig. 2, Table 2). Ionoregulatory processes in ectotherms are temperature dependent, in general with higher turnovers at higher temperatures (Huang *et al.*, 2009; Tattersall *et al.*, 2012). Thus, the increase of copper toxicity at 20 °C relatively to 10 °C may be due to an increased ion turnover at the highest temperature. Also, the raise of temperature in general increases the production of reactive oxygen species (Abele *et al.*, 1998, 2002; Heise *et al.*, 2003) potentially increasing oxidative stress effects and damage. Therefore, the differential effect of temperature on the copper toxicity to *E. meridionalis* and *A. desmarestii* suggests that ion turnover and/or oxidative stress targets and defenses are affected by temperature in a different way in the two species. This is a most interesting topic deserving further investigation.

Previous studies indicate that *A. desmarestii* may show a pH-tolerance (pH > 5) when facing short-term exposure to acidified water at temperatures about 18 °C (Janssens

de Bisthoven *et al.*, 2006). Nonetheless with pH values between 3.3 and 4.4 (similar to the ones found in our study for the highest EL treatments) mortality increased and behavioural alterations were also detected and this negative effect may have been amplified by increasing temperature. Amphipods have been referred as extremely sensitive to low pH with effects on osmoregulation processes: Felten *et al.* (2006, 2008) and Felten and Guérold (2006) detected a depletion of haemolymph Na^+ and Cl^- in *Gammarus fossarum* when exposed to acidic conditions with observed increased mortality. The lack of significant differences on EL toxicity between *A. desmarestii* and *E. meridionalis* at 10 °C (Fig. 1, Table 2) suggests a comparable sensitivity to eucalyptus leaf toxins and/or low pH values at this temperature. This similar low pH-induced stress in the highest EL treatments suggests a comparable ability of regulating H^+ ions thus supporting the hypothesis raised above on the similarity of ionoregulatory processes between the two species. However, the raise of temperature increased the toxicity of EL to *A. desmarestii* but not to *E. meridionalis* suggesting that other mechanisms are involved in the toxicity and or biotransformation of EL, which are modulated by high temperatures in a distinct way in the two species. Unfortunately, our experimental design does not allow going further on the mechanisms potentially involved.

Mixture bioassays

In the mixture bioassays with EL and copper, low pH and DOC may act as important confounding factors: pH variation may change the speciation of copper with implications for its toxicity, and low pH causes toxicity *per se* (Felten and Guérold, 2006; Janssens de Bisthoven *et al.*, 2006); DOC binds copper molecules decreasing its bioavailability (De Schamphelaere and Janssen, 2004). In our experimental conditions, the pH of all test solutions was similar (Table 3) and the maximal pH variation in test beakers was 0.82. Thus, no significant stress due to low pH values or pH variation occurred. To mimic processes likely happening in real stream scenarios, the DOC concentrations were not standardized across treatments, resulting in a different DOC content in different EL treatments. Therefore, the overall effects of EL and copper in mixture are likely those occurring in real scenarios, where DOC concentrations may change with implications for copper bioavailability and toxicity. The LC_{50} values of the mixture bioassays (Table 4) are expressed in concentrations of tannic acid because the concentrations of bioavailable copper in test solutions may be different from the nominal concentrations due to binding to DOC. LC_{50} values for *A. desmarestii* were higher than the ones obtained for *E. meridionalis*, which can indicate a low sensitivity of this species to EL in the presence of copper. Additionally, the LC_{50} obtained for the mixture at 10 °C was higher than the one previously obtained in single exposures (Tables 2 and 4). Because copper is a well-known oxidative stress inducer (Bouskill *et al.*, 2006; Sroda and Cossu-Leguille, 2011) and eucalyptus components present in oils and extracts have anti-oxidant properties (Sacchetti *et al.*, 2005; Singh *et al.*, 2012), this may contribute, at least in part, for the higher LC_{50} of the mixture relatively to LC_{50} of the chemicals alone in *A. desmarestii* (Tables 2 and 4). Interestingly, in *E. meridionalis* the toxicity of the mixture was higher than the toxicities induced by EL and copper individually, especially at 20 °C (Tables 2 and 4, Figs. 1–3). Thus, these results also suggest a distinct effect of temperature raise between *A. desmarestii* and *E. meridionalis*.

Stressors' exposure may be a most important pressure driving the composition and dynamics of shredders communities, mainly because different species may have distinct sensitivities to them (Liess and Schulz, 1999; Woodcock and Huryn, 2005; Maltby and Hills, 2008). Thus, under stress exposure, the populations of the most sensitive species are expected to decline and even disappear, whereas the most tolerant ones may overdevelop due to the lack of competition, possibly occupying the ecological niches of the extinct ones.

Conclusions

In summary, the results of this study indicated differences of sensitivity to the stress induced by copper, eucalyptus leaf leachates (EL) and their mixtures among *S. festiva*, *A. desmarestii* and *E. meridionalis*. *S. festiva* was the least sensitive species at both 10 and 20 °C. The relative sensitivity of *A. desmarestii* and *E. meridionalis* was dependent of the chemicals tested, and of the environmental temperature. Overall, the findings of the present study suggest that single and combined chemical stress induced by copper and EL, when present alone or in mixture in stream's water, is able to modulate the biodiversity of stream shredders communities due to differential sensitivity of individual species to environmental contaminants, and that temperature influences the process. Therefore, more knowledge on the effects of multi-stressors effects on shredder communities is needed.

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