Global anthropogenic threats in Portuguese streams: ecological effects on aquatic macroinvertebrates assessed at different levels of biological organization

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Publications

The following manuscripts were prepared under the scope of this Thesis and were submitted for publication in international peer-reviewed scientific journals or are in a final phase of preparation:

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Gama M., Canhoto C. and Guilhermino L. **Comparison of three shredders response to acute stress induced by eucalyptus leaf leachates and copper: single and combined exposure at two distinct temperatures**.

MANUSCRIPTS IN PREPARATION

Gama M., Canhoto C. and Guilhermino L. **Effects of eucalyptus leachates and copper (single and in mixture) on cholinesterase activity and oxidative stress parameters of three shredder species assessed at two temperatures.** *In preparation*

Gama M., Guilhermino L. and Canhoto C. **Chronic toxicity of eucalyptus leaf leachates and copper to *Schizopelex festiva* and *Echinogammarus meridionalis* assessed at two distinct temperatures.** *In preparation*

Gama M., Guilhermino L. and Canhoto C. **Effects of food quality on the stoichiometrical composition and selected biomarkers of *Echinogammarus meridionalis* assessed at two temperatures.** *In preparation*

Abstract

In the present context of global climate changes and exponential increase of the human population, environmental contamination by chemicals (anthropogenic/natural sources) and temperature are most relevant stressors in aquatic ecosystems. Forested streams are particularly vulnerable because of their relatively low water volume and dependence on the riparian vegetation (major source of organic matter). This organic matter is processed by the detritivorous community, especially by shredders, which have a central role in nutrient cycling. The litter and leafs provided by particular riparian species (e.g. *Eucalyptus globulus*) may have toxins and a relatively low nutricional quality when compared to native species (e.g. *Alnus glutinosa*). In South Europe streams, considerable changes of water temperature are common in some periods of the year and the simultaneous exposure of shredders to eucalyptus toxins and other environmental contaminants, such as metals (e.g. copper), is likely to occur. However there is a lack of knowledge on the effects induced by multi-stressors, particularly on shredders. Therefore, the main goal of the present Thesis was to investigate the single and combined effects of copper, an ubiquous environmental contaminant, and eucalyptus leaf leachates (EL), planted in monocultures and widespread in Portugal, on three shredder species found in South Europe streams (the shrimp *Atyaephyra desmarestii*, the amphipod *Echinogammarus meridionalis* and the insect *Schizopelex festiva*) at two ecologically relevant temperatures (10 and 20°C).

In the first study (Chapter II), the acute toxic effects of EL and copper on *A. desmarestii*, *E. meridionalis* and *S. festiva* were investigated in 96h toxicity bioassays using mortality as effect criterium. The median lethal concentrations (LC_{50}) of EL to the tested species ranged from 81.1 to 567.8 mg/L at 10°C; and from 34.4 to 482.1 mg/L at 20°C (concentration of tannic acid). The LC_{50}s of copper ranged from 0.22 to 9.0 mg/L at 10°C, and from 0.04 to 7.3 mg/L at 20°C. Comparison of the toxicity curves indicated that the most tolerant species was *S. festiva*. The relative sensitivity of *A. desmarestii* and *E. meridionalis* was chemically and termally dependent, with *E. meridionalis* being particularly sensive to copper at 20°C. Interactions between EL and copper (antagonism in *A. desmarestii* and synergism in *E. meridionalis*) and an increase of the toxicity with temperature raise were found.

These findings raised several questions on the molecular/physiological mechanisms that may contribute to the differences of sensitivity found. Therefore, the acute effects of EL, copper and their mixtures were further investigated (Chapter III) by analyzing the activity of the enzymes cholinesterases (ChE), involved in neurotransmission, and glutathione S-transferases (GST), involved in biotransformation and lipid peroxidation prevention, and the levels of lipid peroxidation (LPO), as indicative of oxidative damage. Results suggest that these shredders have different sensitivities to chemically-induced acute stress and that some of the mechanisms of toxicity and detoxication involved are modulated by temperature. At increased temperature, in control organisms, the ChE activity in *S. festiva*, and the GST activity in *S. festiva* and *E. meridionalis* were reduced, while LPO levels increased in *S. festiva* and *A. desmarestii*.

In the wild, the most common situation is the long-term exposure to relatively low concentrations of environmental contaminants. Thus, in Chapter IV, the single or combined exposure to EL and copper on the most tolerant (*S. festiva*)

and the most sensitive species (*E. meridionalis*) were assessed at 10 and 20°C using growth, food consumption and survival rates as effect criteria. Elemental body composition and selected biomarkers (ChE, GST and LPO) were evaluated in *S. festiva*. Temperature significantly accelerated growth rates of *S. festiva* while the presence of the chemical agents determined lower intrinsic growth rates (at both temperatures) and no effects on survival were detected. For *E. meridionalis* the presence of low concentrations of chemical agents increased growth rates at 10°C. Survival was negatively affected by increased temperature and stressors exposure. *S. festiva* is unable to remain homeostatic and the ability to retain phosphorus is compromised (at higher temperature and increased copper concentrations). Increasing oxidative damage at 20°C was also observed. Results suggest that, even low amounts of chemical agents in chronic exposures, are able to affect life history parameters of both *S. festiva* and *E. meridionalis* with possible negative consequences for stream macroinvertebrates.

Deterrent effects of afforestations namely the replacement of native trees (e.g. Alnus glutinosa) by monocultures of *E. globulus* in riparian areas may affect shredders that may feed on these particular leaves. Therefore, the effects of consuming high (alder) or low (eucalyptus) quality leaves combined with the influence of temperature (10 and 20°C) were tested in *E. meridionalis* (Chapter V). Elemental body composition and selected biomarkers (ChE, GST and LPO) were determined. Results suggest that invertebrates are homeostatic even when fed low quality food (eucalyptus). At 20°C increased oxidative damage in shredders fed with eucalyptus was observed.

In summary, the results indicated: (i) differences of sensitivity to acute and chronic stress induced by copper and EL at ecologically relevant concentrations; (ii) that temperature change interacted with the toxicity of both stressors in a species-specific manner probably due to differences at physiological/molecular levels; (iii) that exposure to stressors influences the stoichiometrical composition of the invertebrates. These findings suggest that EL and/or copper contamination may modulate the structure and dynamics of the shredder's community in streams and exposure to increased temperature may influence the process. Alterations in the shredders community may cause throphic imbalances, alterations of ecosystem functioning and reduction of ecosystem services.

Keywords: *Atyaephyra desmarestii*; *Echinogammarus meridionalis*; *Schizopelex festiva*; shredders; copper; eucalyptus; temperature; acute and chronic toxicity; biomarkers; elemental composition.

Resumo

No atual contexto de mudanças climáticas globais e aumento exponencial da população humana, a contaminação ambiental por produtos químicos de origem antropogénica e natural e a temperatura são dos factores de stress mais relevantes em ecossistemas aquáticos devido aos seus potenciais impactos adversos. Ribeiros em áreas florestais são especialmente vulneráveis a estes impactos devido ao seu volume de água reduzido, e dependência da vegetação ripícola que é a principal fonte de matéria orgânica nestes ecossistemas. Esta matéria orgânica é processada pela comunidade de detritívoros, nomeadamente pelos trituradores que têm um papel fulcral no ciclo dos nutrientes. Esta matéria orgânica fornecida por determinadas espécies (e.g- *Eucalyptus globulus* - eucalipto) pode conter toxinas e uma qualidade nutricional relativamente baixa quando comparadas com as espécies nativas (e.g. *Alnus glutinosa* – amieiro). Em ribeiros do Sul da Europa, onde variações consideráveis da temperatura da água são comuns em alguns períodos do ano, a exposição simultânea dos trituradores a toxinas de eucalipto e outros contaminantes ambientais, tais como os metais (e.g. cobre), é de provável ocorrência. Apesar da investigação que tem vindo a ser desenvolvida nos últimos anos, o conhecimento científico sobre os efeitos induzidos simultaneamente por múltiplos factores de stress é ainda bastante limitado, especialmente nas comunidades de detritívoros. Assim, o objetivo central do presente estudo foi investigar os efeitos isolados e combinados da exposição ao cobre, um dos contaminantes ambientais mais comuns e, a lixiviados de folhas de eucalipto (EL), comum em Portugal e frequentemente plantado em regime de monocultura, em três espécies trituradoras encontradas em ribeiros do Sul da Europa (o camarão *Atyaephyra desmarestii*, o anfípode *Echinogammarus meridionalis* e o insecto *Schizopelex festiva*) a duas temperaturas ecologicamente relevantes (10 e 20°C).

No primeiro estudo realizado (Capítulo II), foram investigados os efeitos tóxicos agudos de EL e do cobre em *A. desmarestii, E. meridionalis* e *S. festiva* em bioensaios de toxicidade de 96h baseados nos efeitos letais induzidos. Nos bioensaios com os agentes isolados, as concentrações letais medianas (LC50) de EL, para as espécies testadas variaram entre 81,1 e 567,8 mg/L a 10°C e entre 34,4 e 482,1 mg/L a 20°C (concentração de ácido tânico). Os LC50 para o cobre variaram entre 0,22 e 9,0 mg/L a 10°C e entre 0,04 e 7,3 mg/L a 20°C. A comparação das curvas de toxicidade e a análise geral dos dados indicaram que a espécie mais resistente foi *S. festiva*. A sensibilidade relativa das outras duas espécies parece ser dependente da temperatura e do agente testado, sendo *E. meridionalis* particularmente sensível ao stress induzido pelo cobre à temperatura mais elevada (20°C). Nos bioensaios de misturas, foram observadas interacções toxicológicas entre EL e cobre (antagonismo em *A. desmarestii* e sinergismo em *E. meridionalis*), tendo-se ainda verificado um aumento da toxicidade com o aumento da temperatura.

Os resultados dos ensaios agudos levantaram várias questões interessantes, sobre os mecanismos moleculares/fisiológicos que podem contribuir para as diferenças de sensibilidade observadas entre as três espécies. Assim, no Capítulo III foram investigados os efeitos agudos do EL e do cobre, isolados e em mistura na atividade das enzimas colinesterases (ChE), envolvidas na neurotransmissão colinérgica, e glutationa *S*- transferases (GST), envolvidas na biotransformação e prevenção da peroxidação lipídica, e os níveis de peroxidação lipídica (LPO),

indicativo de dano oxidativo nos lípidos. Nos organismos controlo, verificou-se uma diminuição da atividade das ChE em *S. festiva* , das GST em *S. festiva e E. meridionalis* e aumento dos níveis de LPO em *S. festiva e A. desmarestii* com o aumento da temperatura. Os resultados obtidos sugerem que as espécies testadas possuem diferenças a nível molecular e/ou fisológico, as quais podem contribuir para as diferenças de sensibilidade observadas, indicando ainda a influência da alteração da temperatura em alguns dos processos.

Na natureza, a situação mais comum é a exposição a longo prazo a concentrações relativamente baixas de vários contaminantes ambientais. Assim, no capítulo IV, o efeito da exposição isolada ou combinada de EL e de cobre nas taxas de crescimento, consumo e sobrevivência dos detritívoros *S. festiva* (mais resistente) e *E. meridionalis* (mais sensível) foram avaliadas a duas temperaturas (10 e 20°C). A composição corporal elementar e os biomarcadores (ChE, GST e LPO) também foram avaliados em *S. festiva*. Na ausência de stress químico, o aumento da temperatura acelerou de forma significativa as taxas de crescimento de *S. festiva* e, a presença de substâncias tóxicas, determinou taxas de crescimento intrínsecas menores (a ambas as temperaturas), não sendo detectados efeitos sobre a sobrevivência. Para *E. meridionalis* a presença de pequenas quantidades de tóxicos aumentou as taxas de crescimento a 10°C. A sobrevivência foi afetada negativamente pelo aumento da temperatura e exposição a tóxicos (isolados e em combinação), especialmente na temperatura mais alta. A manutenção da homeostasia em *S. festiva* é comprometida nomeadamente a capacidade de retenção de fósforo na presença de cobre a 20°C. Um aumento dos danos oxidativos ocorreu a 20°C. Os resultados sugerem que, mesmo pequenas quantidades de agentes químicos em exposições crónicas, são capazes de afetar parâmetros funcionais de ambos os invertebrados com possíveis consequências negativas para estas comunidades.

A alteração da vegetação ripícola, nomeadamente a substituição de espécies nativas por eucalipto pode afetar os trituradores que se alimentam destas folhas. Por conseguinte, os efeitos do consumo de alimento de elevada (amieiro) ou de baixa qualidade (eucalipto) assim como a influência da temperatura foram testados na espécie *E. meridionalis* (Capítulo V), determinando ainda a composição elementar e biomarcadores. Os resultados obtidos sugerem que os invertebrados são homeostáticos independentemente da temperatura, mesmo quando alimentados com alimento de baixa qualidade (eucalipto). No entanto a 20°C, um maior dano oxidativo foi detectado em trituradores alimentados com folhas de eucalipto.

Em resumo, os resultados obtidos no âmbito da presente Tese indicam que: (i) existem diferenças de sensibilidade entre as espécies testadas ao stress agudo e crónico induzido por cobre e EL a concentrações de exposição ecologicamente relevantes; (ii) a temperatura interage com a toxicidade dos dois agentes químicos de modo diferente em espécies distintas, provavelmente porque eles têm diferenças ao nível fisiológico/molecular; (iii) que a presença de cobre e de EL pode influenciar a composição elementar das espécies testadas (*S. festiva*).

Portanto, estes resultados sugerem que a contaminação de ribeiros por EL e/ou cobre podem modular a estrutura e dinâmica das comunidades de trituradores e que a variação de temperatura pode influenciar o processo. Alterações na comunidade de trituradores podem causar desequilíbrios tróficos,

alterações no funcionamento e redução dos serviços prestados por estes ecossistemas.

palavras-chave: *Atyaephyra desmarestii; Echinogammarus meridionalis; Schizopelex festiva;* detritívoros; cobre; eucalipto; temperatura; toxicidade aguda e crónica; biomarcadores; composição elementar.

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Acronyms and abbreviations

AChE -	Acetylcholinesterase enzyme
ATSDR -	Agency for Toxic Substances and Disease Registery, U.S. Department of Health and Human Services, Atlanta, Georgia
C -	Carbon
CaCO3 -	Calcium carbonate (indicative of water hardness)
Cd -	Cadmium
CDNB -	1 chloro-2,4-dinitrobenzene
ChE -	Cholinesterases enzymes
CPOM -	Coarse particulate organic matter
D.O. -	Water dissolved oxygen
DTNB -	5.5' -Dithio-bis - (2-nitrobenzoic acid)
EL -	Eucalyptus leaf leachates
FPOM -	Fine particulate organic matter
GST -	Glutathione *S*-transferases enzymes
H_2O_2 -	Hydrogen peroxide
IPCC -	Intergovernmental Panel on Climate Change
K^+ -	Potassium ion
LC_{10} -	Concentration of the test substance(s) that induced 10% of mortality in the population of the tested species in the experimental conditions used .
LC_{20} -	Concentration of the test substance(s) that induced 20% of mortality in the population of the tested species in the experimental conditions used.

LC_{50} -	Concentration of the test substance(s) that induced 50% of mortality in the population of the tested species in the experimental conditions used.
LPO -	Lipid peroxidation
MIX -	Mixture
N -	Nitrogen
Na^+ -	Sodium ion
NADH -	Nicotinamide adenine dinucleotide reduced
NADP+ -	Nicotinamide adenine dinucleotide phosphate
NADPH -	Nicotinamide adenine dinucleotide phosphate reduced
O_2^- -	Superoxide anion radical
OH^- -	Hydroxyl radical
P -	Phosphorus
Pb -	Lead
PMS -	Post-mitochondrial supernatant fraction
PO_4^{3-} -	Phosphate ion
ROS -	Reactive oxygen species
S.E.M. -	Standart error of the mean
SD -	Sample standard deviation
SOD -	Superoxide dismutase
TBARS -	Thiobarbituric acid reactive species
Tris -	Tris(hydroxymethyl)-aminomethane

Chapter I

General introduction

In the present context of climate changes and human population growing exponentially, an increasing demand of chemicals are required to support higher needs of food, industrialization, prevention and treatment of human and animal diseases. Such higher demands increase the production of more residues increasing environmental contamination namely that of aquatic ecosystems (Woodward *et al*., 2010), the most impaired systems of the world (Malmqvist and Rundle, 2002; Dudgeon *et al*., 2006). Several of these ecosystems are also contaminated by natural toxins (e.g. cyanotoxins, plant toxins) whose occurrence have been increased and geographically spread as a result of anthropogenic activities and/or abiotic alterations promoted by global climate changes (Sokolova and Lannig, 2008; Ormerod *et al*., 2010).

Studies indicate that the exposure to elevated concentrations of environmental contaminants may impair ecosystem function compromising ecosystem services (Leslie *et al*., 1999; Camargo and Alonso, 2006, Guilpart *et al*., 2013). In spite of that, the knowledge on the effects of multiple stressors is still limited, especially when considering scenarios of temperature changes. Temperature is a main stressor *per si* because several biological processes are temperature-dependent (Woods *et al*., 2003). In addition, temperature has been shown to interact with the toxicity of some environmental contaminants to several aquatic species (Prato *et al*., 2009; Kopecka-Pilarczyk, 2010). This knowledge is urgently needed to improve the scientific basis for ecological risk assessment of both environmental contaminants and climate changes (Laskowski *et al*., 2010; Spurgeon *et al.,* 2010).

1.1. Stream ecosystems

A stream used to be considered simply as a body of water with a defined current and contained within a narrow channel and banks (Langbein and Iseri, 1995). Nowadays growing importance has been attributed to the study of not only this confined channel but also of its drainage basin and its composition, namely the composition of the riparian areas. It is though very important to assess the effects of disturbances not only on the river channel but also in its drainage basin. First through third order streams are also called headwater streams and these are located at the head of the fluvial continuum, constituting up to 85% of the total length of a lotic system (Anderson and Sedell, 1979). Forested headwaters are heterotrophic and instream processes are mainly dependent on the organic matter supplied by the surrounding riparian areas. Here, the light input is low due to the presence of an extent canopy covering the stream. Primary productivity is very low; therefore, litterfall (allochthonous organic matter supplied by the riparian vegetation), constitutes the main energy source for the stream food webs (Fisher and Likens, 1973). The amount and type of detritus present in the streams depends on the geography, characteristics of the riparian forest but also on morphological characteristics of the streams (that may enable in different ways and extents the retention of these detritus for longer time periods). In temperate regions, up to 73% of the total leaf litter from the surrounding deciduous forests ends up on streams banks or channels in autumn (Abelho and Graça, 1998). These allochtonous organic inputs are mainly constituted by leaves (although depending on the type of forest present, woody debris may also be important) that, after entering the streams, are rapidly trapped and further decomposed. The

breakdown of these leaves occur in more or less defined phases that may overlap: leaching, conditioning and fragmentation. The duration and intensity of each one depend on leaf intrinsic properties (chemistry), stream biota (Hieber and Gessner, 2002, Gulis *et al.,* 2006) and environmental factors such as temperature (Spänhoff and Meyer, 2004), current velocity (Ferreira and Graça, 2006), water chemistry (Sridhar and Bärlocher, 2000; Gulis *et al*., 2006), among others. Leaves decomposition is a key ecosysytem-level process integrating biological and abiotic features of a stream; its sensitivity to environmental stressors allows its use as a functional tool for the assessment of river ecosystem health (Young *et al.,* 2008).

Leaching is the initial phase of leaves decomposition and starts immediately after leaves immersion in the stream. A rapid loss of soluble inorganic and organic compounds (Suberkropp *et al*., 1976) occurs usually in the first 24 to 48h determining an important mass loss of the leaf (*e.g.* Canhoto and Graça, 1996; Gessner *et al.,* 1999). Microbial colonization typically follows leaching. Leaves are colonized by fungi (mainly Aquatic Hyphomycetes) that dominate in early phases of decomposition and bacteria in a process usually designated as *conditioning* (Boling *et al*., 1975; Gessner *et al*., 1999). Conditioned leaves are more palatable for invertebrates (Graça, 2001), because the increase in microbial (fungal) biomass and fungal activities make leaves softer and increase their nutritional value. In fact, fungi are known to be able to enzymatically digest recalcitrant leaf polymers and to promote nutrient immobilization making leaves more palatable for invertebrates (Gessner *et al.,* 1999; Krauss *et al.*, 2011). *Fragmentation* may occur due to physical abrasion promoted by water turbulence, discharge or invertebrate's biological activity (Hieber and Gessner, 2002). Invertebrates that

feed on coarse particulate organic material (CPOM) such as leaves or woody debris are known as shredders (Cummins, 1973). It is generally accepted that shredders accelerate decomposition (*e.g*. Mckie and Malmqvist, 2009) and that they prefer conditioned over unconditioned leaves, specific fungi or leaf/fungi associations (Lecerf *et al.*, 2005). Such feeding behavior is frequently species-specific (Kominoski *et al*., 2012) depends on invertebrates' development stage, and has important repercursions in their survival, growth and reproduction (Canhoto and Graça, 2006).

Shredders functional feeding group plays a crucial role transforming CPOM into fine particulate organic matter (FPOM) by their actions of ingestion, digestion, egestion and excretion (Eggert and Wallace, 2007; Villanueva *et al*., 2012). These invertebrates usually present high feeding rates and low assimilation efficiencies (Wallace *et al*., 1982) contributing to the nutrient cycling (Vanni, 2002). In temperate areas, mixed deciduous riparian forests supply low order streams with leaf litter material presenting large differences between their nutrient content and those from the consumers. Imbalances between this CPOM and invertebrates (Sterner and Elser, 2002) are normally counteracted by the leaf conditioning process and may dependent on the type of leaf present, colonizers and colonization status, and the taxonomic characteristics of the invertebrates (Vanni *et al*., 2002; Frost *et al*., 2006). Based on the ecological stoichiometry (ES) theory, invertebrates tend to maintain their elemental composition (carbon (C), nitrogen (N) and phosphorus (P) content) at a fairly constant rate (Homeostasis), regardless of their feeding habits, presenting lower C:nutrient ratios and high N and P contents than detritus or living plants (Sterner and Elser, 2002; Cross *et al*., 2005;

Evans-White *et al*., 2005). It is though generaly accepted that invertebrates may use different physiological and behavioral strategies in order to compensate imbalances in the elemental composition of dead leaf material and their bodies, controlling feeding, egestion and/or excretion rates (Balseiro and Albariño, 2006, Villanueva *et al.,* 2012). To understand how invertebrates deal with differences between the elemental composition of resources and their ecological necessities, and the way stressors presence may modify these relationships with effects on ecosystem processes, are actually important lines of inquiry.

1.2. Environmental stressors in streams

Streams, important spots of diversity (Dudgeon *et al.,* 2006), are particularly vulnerable to environmental stressors mainly due to their close dependence on terrestrial subsidies, and low water volume which minimizes its buffering abilities against physical (e.g. temperature, D.O., pH), or chemical (e.g. metals) stressors.

Eucalyptus. In Mediterranean areas, low order streams are frequently submitted to seasonal droughts. Such hydrological pattern is expected to be amplified in frequency, time length and intensity due to global warming (IPCC, 2007) and anthropogenic activities such as changes in forestry practices. In fact, permanent streams running through mixed native forests have been referred to become intermittent when the riparian cover was replaced by eucalyptus (*Eucalyptus globulus* Labill.) monocultures (Canhoto *et al*., 2013). Such reduction in riparian diversity (Kominosky *et al.*, 2013) and quality (Pozo *et al*., 1998) along with low flow/drought events (Canhoto and Laranjeira, 2007) are already known to promote deleterious consequences on the structure and function (Abelho and

Graca, 1996; 1998; Canhoto and Graca, 1996; 1999) of these stream ecosystems. However, the specific reasons for such consequences are still far from completely understood.

Eucalyptus plantations are economically important in several countries of the world (Diaz-Balteiro *et al.,* 2009; Huu-Dung and Yeo-Chang, 2012), Portugal included (eucalyptus represent over 812000 acres in Portuguese forest; IFN6, 2013). Trees are mainly used to get timber for the papermill industry and also for pharmaceutical uses. Eucalyptus essential oils, mainly gathered from the leaves, are important due to their antiseptic, analgesic, antibacterial (Cimanga *et al*., 2002, Hendry *et al*., 2009, Bendaoud *et al*., 2009, Patrone *et al*., 2010) and antioxidant properties (Lee and Shibamoto, 2001; Eyles *et al*., 2004; Naceur Ben Marzoug *et al*., 2011). The importance of these compounds in terrestrial, riparian and aquatic systems have been tested with recognised deleterious direct and indirect effects on the ecology of the edafic and lotic fauna (Canhoto and Graça, 2006; Canhoto and Laranjeira, 2007; Larrañaga *et al*., 2009; Canhoto *et al*., 2013; Martins *et al.*, 2013). In streams with eucalyptus riparian cover, considerable inputs of biologically active substances may enter into the water through leaves and other litter components as most eucalyptus leaf defenses remain active after senescence and immersion (Canhoto and Graça, 1999; Canhoto *et al.,* 2002, Canhoto and Laranjeira, 2007). Some of these substances, their degradation products and/or metabolites may be toxic to shredders (Canhoto and Graça, 2006; Canhoto and Laranjeira, 2007, Larrañaga *et al*., 2009) although little is known on their mechanisms of toxicity. Invertebrates may be exposed to these toxins through feeding and/or by exposure to leachates mainly released to the water during the

leaching phase. Responses to such stressors seem to be species-specific and are clearly modulated by environmental factors (Larrañaga *et al*., 2009; Canhoto *et al.,* 2013).

Most studies on the effects of *E. globulus* afforestations on stream biota occurred in periods of permanent and high flow and when water temperatures are lower. Approaches dealing with harsh periods are scarce (e.g. Otermin *et al.,* 2002) but needed to fully understand the observed empoverishment of the biota of the eucalyptus stream when compared with their deciduous counterparts (Abelho and Graca, 1996; 1998; Bärlocher and Graça, 2002; Canhoto and Graca, 1996; 1999): low flow/drought events promotes deterioration of water quality through the concentration of leachates, increased temperature in leachates pools and low oxygen conditions, that may impact shredders (Canhoto and Laranjeira, 2007).

Copper. Heavy metals have been increasing in several European countries as a direct result of human activities, for example, agriculture, minning, industry (Sonmez *et al*., 2006). These anthropogenic activities contribute to mobilize and diffuse metals in the environment faster than that expected for natural occurring processes. At elevated environmental concentrations, they are toxic to aquatic organisms, including shredders (Vargas *et al*., 2001), crucial players in the transfer of energy to higher trophic levels in the detritus based food chains.

Among metals, copper is of special interest because it is an element essential for a high number of species, is one of the metals commonly found in aquatic ecosystems (Schintu *et al*., 2008), including streams, it can be accumulated by several species (Martins *et al.,* 2011; Rainbow *et al.*, 2012), and may induce adverse effects at different levels of biological organization at ecologically relevant

concentrations. Copper is very important for organisms as a functional part of the respiratory protein hemocyanin found in most invertebrates. All invertebrate animals have the universal copper cellular proteins: cytochromes, iron-sulfur proteins, superoxide dismutase, cytochrome oxidase. As trace metals copper have an affinity for nitrogen or sulphur, and can be easily bound to proteins inside invertebrate's organism. Copper intake by aquatic organisms is in proportion to the dissolved concentration of the metal (Rainbow, 1995). Deterrent effects of copper in excess on organisms are responsible for increased mortality (Gerhardt and Palmer, 1998; Brown *et al*., 2004a), neurological impairment (Vieira *et al*., 2009), increased oxidative stress (Vutukuru *et al*., 2006), egg loss and hatching impairment (Brix *et al*., 2006). Sub lethal exposure resulted in growth inhibition, reproduction impairment and affected embryo hatching on snails (Real *et al*., 2003).

Temperature. Stream water temperature varies with factors like latitude and altitude (Flenner *et al*., 2010) or riparian cover (Rutherford *et al.,* 2004). The effects of the stream's thermal regime in the aquatic biota may be direct (Brown *et al.,* 2004*b*) and indirect through changes in oxygen solubility, standing stock of organic matter, the hydrological regime or intensity of anthropogenic stress (Buzby and Perry, 2000; Berezen *et al.,* 2005; Ormerod, 2009; Woodward *et al.*, 2010; Chinnayakanahalli *et al.*, 2011). It is generally accepted that temperature variation interacts with chemical agents (Laskowski *et al*., 2010) and higher/increases in temperature may amplify the toxicity of heavy-metals to organisms (Khan *et al*., 2006; Prato *et al*., 2009; Kopecka-Pilarczyk, 2010, Lapointe *et al.,* 2011).

In the same ecosystem daily and seasonal variations are registered. Such oscillations, with distinct degrees of amplitude, are often considered to require adaptation from the biota (Flenner *et al*., 2010). The range of tolerance to temperature may be different among distinct invertebrate species and thus temperature is a key driver of the structure and dynamics of shredders communities (Imholt *et al*., 2009, Dallas and Ketley, 2011). Native shredder communities are in general well adapted to a certain degree of temperature variation but variations outside the optimal range may be tolerated up to a certain degree requiring increased demands of energy that needs to be allocated from other functions. Increasing temperature may be directly responsible for effects on reproduction and decreased survival (Hofmann and Todgham, 2010) and functional parameters such as growth rates may also be affected (Sutcliffe *et al*., 1981). Several studies indicate that this key environmental factor may affect invertebrate's growth directly by influencing food ingestion rates, assimilation and metabolism efficiency (Sweeney *et al*., 1986) and, indirectly, by affecting microbial colonization on leaf conditioning and faecal content and consequently consumption by shredders or filter feeders (Rowe *et al.,* 1996).

With rising importance on the understanding of predicted changes in stream's water temperature under the influence of global warming scenarios, the study of the isolated or combined effects of temperature and other stressors (e.g. heavy metals, biotoxins) on macroinvertebrate communities is much needed in order to fully protect shredders communities and minimize the possible negative ecological effects.

1.3. Assessment of stressors effects in stream shredders

The effects of environmental contaminants and other stressors such as temperature on the biota may be assessed directly in real scenarios or in laboratory conditions (or using both approaches). Common assessments of the effects in the field include biomonitoring studies in natural populations (e.g. Conti *et al*., 2011; Guimarães *et al*., 2012) and *in situ* bioassays (Faria *et al*., 2007, Correia *et al*., 2013). Laboratorial assessments include several types of assays, from which laboratorial toxicity bioassays (hereafter indicated as bioassays) are of special relevance. Field assessments have a very high ecological relevance and thus they are strongly recommended when this aspect is determinant for the objectives of the study. However, in general the control of environmental conditions other than the factor(s) under study is difficult and this is a limitation to their use for specific purposes. On the contrary, laboratory bioassays offer the possibility of controlling in a more effective way the experimental conditions, while the need of extrapolation from laboratory to field (that may be particularly difficult in some cases) may cause some limitations to their use for in several situations.

In general, in laboratory bioassays, standard species are used (e.g. OCDE, 2011; 2012*a,b*). However, the sensitivity of these species may considerable differ from native species and therefore the extrapolation from the evaluations made to real scenarios may be challenging. Thus, it is of interest to assess the effects of environmental stressors using native species (Pestana *et al.,* 2007; Macedo-Sousa *et al*., 2008). In low order streams the use of shredder species in laboratorial/field tests seem particularly relevant due to their pivotal role in nutrient cycling in the detritus-based systems.

Common used effect criteria in ecotoxicity bioassays are mortality, growth and reproduction because they have a direct impact on population growth rate and thus they have a high ecological relevance (Gravato and Guilhermino, 2009; Oliveira *et al*., 2012). However, because the first effects of stressors are in general induced at the molecular level and may result in a cascade of successive alterations along the increasing biological organization levels, in the last decades employment of other effect criteria at individual and sub-individual levels (e.g. molecular, cellular, organs, physiological functions), generally known as environmental biomarkers, have been used (Depledge and Fossi, 1994; Lagadic *et al*., 1994). In the last years researchers have assessed different "biomarkers" to evaluate the extent of the damage provoked by the contact of these toxicants with organisms. A biomarker is a biological response to an environmental chemical which gives a measure of exposure, and sometimes also of toxic effects (Peakall and Walker, 1994). It could be the measurement of a certain enzymatic rate or the increase in a selected compound in the organisms for example. Cholinesterases (ChEs) are a family of enzymes, which includes acetylcholinesterase (AChE) and pseudocholinesterase (PChE) and are amongst the most used biomarkers. AChE plays an important role in neurotransmission of both vertebrates and invertebrates (Guilhermino *et al*., 1998), being responsible for the degradation of the neurotransmitter acetylcholine in cholinergic synapses. ChE inhibition disrupts nervous system leading to overstimulation of the central and peripheral nervous systems, and may cause adverse effects on several functions including respiration, feeding and behavior eventually leading to death. These enzymes have been widely used as biomarkers for environmental contaminants such as

organophosphates and carbamate pesticides (e.g. Varò *et al.*, 2003, Xuereb *et al.*, 2009). More recently, ChEs of some species have been also found to be inhibited by some heavy metals (Diamantino *et al.*, 2003; Guilhermino *et al.*, 1998; Frasco *et al.*, 2005).

Several contaminants (such as nitroaromatic compounds or metals *e.g.* Winston and Di Giulio, 1991; Stohs and Bagghi, 1995; Orbea *et al.*, 2002) induce the production of reactive oxygen species (ROS) causing oxidative stress. To minimize oxidative damage to cellular components, organisms have developed antioxidant defense mechanisms. Enzymes such as glutathione *S*-transferases (GST) are involved in both detoxification and lipid peroxidation prevention. Soluble glutathione-*S*-transferases (GST) belong to a supergene family of proteins that catalyse the conjugation of glutathione (GSH) with endogenous substances and xenobiotic compounds (Ketterer *et al.*, 1983), increasing their water solubility and facilitating their elimination, and play an important role preventing oxidative damage. These enzymes can respond to the presence of several environmental contaminants (Frasco and Guilhermino, 2002; Vieira *et al.*, 2009).

Organisms can adapt to increasing ROS production by regulating antioxidant defenses, regulating the activities of antioxidant enzymes (Livingstone, 2003). Failure of these antioxidant defenses to detoxify excess ROS production can lead to significant oxidative damage. In particular, lipid peroxidation is considered to be one of the major mechanisms contributing to tissue damage as a result of oxyradicals action, leading to alterations in physicochemical properties of cell membranes and consequently impaired cellular function which in turn may disrupt vital functions.

In recent years, researchers have been particularly interested in the relationship between sub organism level measurements and impairment at population and ecosystem level in order to develop successful indicators to predict the effects of stressors. Therefore the use of biomarkers may enable us to identify early effects of stressor exposure and relate them to ecological or functional parameters, contributing to forecast stressors effects.

2. Thesis aims

The main goal of the present thesis was to investigate the single and combined effects of copper and eucalypt leaf leachates (EL) on three shredder species found in Portuguese streams at two ecologically relevant temperatures (10 and 20°C). This is particularly relevant because the existing knowledge on the effects of multi-stressors on shredders is limited, and this information is most important to improve the basis for ecological risk assessments of environmental contaminants and climate changes in freshwater ecosystems, particularly in South Europe streams.

The invertebrates *Atyaephyra desmarestii* (Millet, 1831) (Crustacea, Decapoda), *Echinogammarus meridionalis* (Pinkster, 1973) (Crustacea, Amphipoda) and *Schizopelex festiva* (Rambur, 1842) (Insecta, Trichoptera) were selected as test organisms for this study because they are common in central Portuguese streams, where they may appear in large numbers and play an important role in leaf processing. These invertebrates present distinct feeding approaches and therefore they might be exposed to stressors by different ways.

A. desmaresti is one of the few freshwater shrimps in Europe, omnivorous, tolerant to temperature and salinity variations (Fidalgo and Gerhardt, 2003). Also, toxicity tests with freshwater gammarids from the genus *Echinogammarus* are scarce but we can find information regarding lethal toxicity of pollutants with *Echinogammarus tibaldii* (Pantani *et al.,* 1997). *S. festiva* is an insect species present in our small streams in Central Portugal, phylogenetically related to other important sericostomatidae: *Sericostoma vittattum,* responsible for the processing of leaf litter inputs in low order streams and, that we know of, never have been utilized in toxicity assays.

EL and copper were selected as test substances for the present study because they are ecological relevant contaminants of streams water in several regions of South Europe, Portugal included, as previously indicated.

10°C is a common water temperature in headwater streams during autumn/winter seasons or day/night dichotomy and 20°C may be observable in these same reaches particularly those that run through eucalyptus plantations during summer or in leachates pools (Canhoto and Laranjeira, 2007).

2.1. Thesis objectives

To attain this general goal, **five specific objectives** were considered to:

1) assess the acute toxic effects of EL and copper, single and in mixture, to *A. desmarestii*, *E. meridionalis* and *S. festiva*, at 10 and 20°C. This was considered important to understand the relative sensitivity of the tested species to EL and copper, the possible toxicological interactions potentially resulting from the

simultaneous exposure to these agents that is likely to occur in real scenarios, and the potential influence of temperature on the process.

2) investigate selected mechanisms of toxicity potentially contributing to the effects observed at individual level. This was considered relevant to understand any potential differences of sensitivity among species to the acute stress induced by the environmental contaminants and the influence of temperature. In addition it could help on the selection of endpoints to be used in further experiments.
3) evaluate the effects of long-term chronic exposure to lower concentrations of eucalyptus leaf leachates, copper and their equitoxic mixtures to invertebrate species by evaluating different growth-developmental parameters (growth, consumption and mortality rates). As contaminants often occur in small concentrations, and organisms are often exposed for long time periods, it was important to assess the extent of low chronic exposure effects on functional parameters.
4) evaluate the extent of neurotoxic and oxidative damage after long-term chronic exposure to sub-lethal concentrations of eucalyptus leaf leachates, copper and their equitoxic mixtures as well as the effects on the elemental composition and selected biomarkers (ChE, GST and LPO).
5) understand if different exposure routes to eucalyptus toxicity (via ingestion of eucalyptus leaves) influences invertebrates by determining elemental body composition and quantifying biomarkers expression (ChE, GST and LPO) after consuming high (alder) and low (eucalyptus) quality leaves and considering the influence of temperature on this process.

2.2. Thesis outline

This thesis is organized in seven chapters: a general introduction (Chapter I), four chapters where the specific objectives were addressed (Chapters II to V), a general discussion (Chapter VI) where the main findings and their contribution to the advance of the knowledge are discussed in an integrated way, and a final Chapter (VII) with the list of the references that supported all the previous Chapters.

The 1st chapter corresponds to a general introduction where the threats to freshwater systems, in particular small water courses, are identified and a general view on the structure, function and sensitivity of these small water courses, especially shredders communities, to main stressors is presented. The importance of temperature in modulating biological processes is discussed and the importance of detecting effects at individual level by evaluating stoichiometrical composition and biomarker expression of shredders is also focused.

In Chapter II, the hypotheses that chemical contaminants (eucalyptus leaf leachates and copper) present in streams water at high concentrations for short periods of time are able to disrupt the balance among different populations of shredders due to distinct sensitivities to the chemicals present, and that temperature is able to modulate chemicals' toxicity were tested. In laboratory acute toxicity bioassays (96h) carried out at 10 and 20°C, the sensitivity of *A. desmarestii*, *E. meridionalis* and *S. festiva*, to copper and eucalyptus leaf leachates (as single substances and in mixture) were compared. The results obtained indicated that *S. festiva*, *A. desmarestii* and *E. meridionalis* have differences of sensitivity to stressors in single and combined exposures with *S. festiva* being the

less sensitive species at both 10 and 20°C. *A. desmarestii* and *E. meridionalis* relative sensitivity to chemical exposure seems to be chemical and temperature dependent and the simultaneous exposure to copper and eucalyptus extracts resulted in toxicological interactions.

The chapter III investigated the neurotoxic and oxidative stress effects of eucalyptus leaf leachates (EL) and copper, single and in equitoxic mixtures, in three shredder species: *S. festiva*, *A. desmarestii* and *E. meridionalis* by determining effects on three different biomarkers (AChE, GST and LPO); these were used as tools to determine the effects at sub-individual levels as a response to combined effects of toxics and temperature. Both copper and EL were found to be able to induce neurotoxicity and oxidative damage with different responses between species. Temperature raise was able to influence the pattern of response to chemical stress and/or its intensity.

In Chapter IV it was investigated the effects, of long-term chronic eucalyptus leaf leachates and copper toxicity exposure to two of the invertebrates *S. festiva* (the least sensitive) and *E. meridionalis* (the most sensitive species) (assessed in Chapter II and III) by evaluating growth rates, mortality and consumption rates at two selected temperatures (10 and 20°C). After chronic exposure to toxicants, stoichiometrical composition and biomarkers were determined at both temperatures in *S. festiva* to evaluate if, despite lower mortality rates, exposing this species to toxics triggers any effects at sub individual level. Toxic exposure and increased temperature negatively affected growth rates. *E. meridionalis* survival was also negatively affected by exposure to toxics and increased temperature. Elemental body composition indicated that invertebrate's ability to retain

phosphorus may be compromised upon exposure to higher temperature and increased copper concentrations. Biomarker determination suggested that increased temperature and the presence of high amount of eucalyptus leachates or the combination of both toxics leads to ChE inhibition with possible decreased responses in neurological mechanisms. Increasing oxidative damage (increased LPO levels) especially at 20°C that was not prevented by GST (as inhibition occurs) was also detected.

Eucalyptus monocultures may affect stream invertebrates through waterborne toxicity or by the ingestion of their leaves. Chapter V included an additional approach to assess, at sub-individual level, the potential effect of eucalyptus leaves toxicity to *E. meridionalis* (the most sensitive species). Invertebrates were fed with leaves of distinct quality (*Alnus glutinosa* or *Eucalyptus globulus*) and maintained under two thermal scenarios: 10 and 20°C and elemental composition and selected biomarkers (ChE, GST and LPO) were investigated after short exposure. Low quality food did not affect the elemental composition of the shredder. No neurotoxic effects were detected but a significant increase of LPO levels, indicative of oxidative damage, was found in invertebrates fed with eucalyptus leaves, at 20°C. The combination of increased temperatures and low food quality may have adverse effects on stream invertebrate's physiology.

The chapter VI is composed by a general discussion and final remarks. Here, the relative sensitivity of the three species in this study is discussed and also the importance of temperature in modulating relationships in the environment as well as the overall influence on toxicity of eucalyptus leaf leachates and copper. Also the

importance of further studies on the effects of eucalyptus leaf leachates toxicity for freshwater invertebrates is highlighted.

The chapter VII is the last chapter of the thesis and is composed by the bibliographical references used in this thesis.

Chapter II

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

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

ABSTRACT

The objectives of this study were to compare the sensitivity of three shredder species (*Atyaephyra desmarestii, Echinogammarus meridionalis* and *Schizopelex festiva*) to acute stress induced by eucalyptus leaf extracts and copper, single 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 96h 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 less 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. The simultaneous exposure of *A. desmarestii* and *E. meridionalis* to copper and eucalyptus extracts resulted in toxicological interactions. The type of interaction was synergism in *E. meridionalis*, and antagonism in *A. desmarestii*. Overall, these findings suggest that single and combined chemical stress may modulate the biodiversity of stream shredders communities due to differential sensitivity of individual species and that the process may be influenced by temperature, highlighting the need for more

knowledge on this matter as well as on the molecular mechanisms responsible for chemicals toxicity.

Keywords: chemical acute toxicity, combined effects of stressors, *Atyaephyra desmarestii, Echinogammarus meridionalis, Schizopelex festiva*

1. *INTRODUCTION*

Small forested streams depend on their riparian areas as a source of energy and nutrients (Canhoto and Graça, 2006). 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 2010; Woodward *et al*., 2010). Quantitatively they are very important, as headwater streams may constitute up to 85% 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 protagonized by fungi, namely aquatic hyphomycetes, and shredders (Cummins 1973), a functional feeding group of detritivorous invertebrates. The abundance and richness of these shredder communities mainly depend on water physicochemical characteristics and are 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 (Liess and Beketov, 2011; Brix *et al*., 2011; Peters *et al*., 2011). Alterations in these communities potentially resulting from the influence of single or multiple stressors, may have reflexes at

higher levels of biological organization and on ecosystem functioning (McMahon *et al*., 2012; Sanpéra-Calbet *et al*., 2009).

Forestry practices constitute one of the main threats to stream ecosystems (Lecerf and Richardson, 2010). Among these, exotic eucalyptus afforestations are now one of the main causes of streams impairment in several regions (e.g. Molinero and Pozo, 2004). Eucalyptus 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 with eucalyptus leaf litter low quality and toxicity (Canhoto and Graça, 1999; Canhoto *et al*., 2013). Whether these effects are generalized across the shredders guild or modulated by temperature is still not known but they are 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 they have been found to induce adverse effects on shredders as a result of long-term exposure to low 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 exposure to high levels of contamination (Dédourge-Géffard *et al*., 2009; Macedo-Sousa *et al*., 2008). Because the combined exposure of shredders to both leaf toxins and metals is likely to

occur in streams and toxicological interactions may occur, it is particularly important to investigate the combined effects of these environmental contaminants on these organisms. In the last years, some studies showed that the presence of metals in streams may affect leaf conditioning processes and consequently shredders consumption and growth rates with possible ecologically relevant impacts (Batista *et al*., 2012; Pradhan *et al*., 2012). However, a lack of knowledge on the potential toxicological interactions between leaf toxins and metal contamination in shredders still exists.

Temperature has a major influence on 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 favor the increased concentration of chemical contaminants in the water (Chatzinikolaou, 2006). Considering the warming scenarios expected for several regions as a result of global climate changes (IPCC, 2007), the expected increase of chemicals use by a growing human population (Dudgeon *et al*., 2006), and the key ecological role of shredders communities on stream ecosystems (Graça, 2006), it is most important to investigate the combined effects of chemical and thermal stress on these organisms.

Therefore, the objective of this study was to compare the sensitivity of three shredder species (*Atyaephyra desmarestii, Echinogammarus meridionalis* and

Schizopelex festiva) to acute stress induced by eucalyptus leaf extracts and a common metal, copper, single and in mixture. Considering the ability of temperature to influence the chemicals' toxicity (Boeckman and Bidwell, 2006; Prato *et al*., 2009), tests were performed at both 10 and 20°C that are common temperatures in colder and warmer seasons, respectively, in the streams from where the invertebrates came from (Canhoto and Laranjeira, 2007; Ferreira *et al*., 2010). *A. desmarestii, E. meridionalis* and *S. festiva* were chosen as they play a key role in leaves decomposition in low order streams, are abundant and easily maintained in the laboratory; their distinct morphological and physiological characteristics may allow evaluating distinct sensibilities to stress. Furthermore, *A. desmarestii* and *E. meridionalis* have been 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).

2. MATERIAL AND METHODS

2.1 - Collection and acclimation of invertebrates

Organisms were collected in the wild (*A. desmarestii*: 40°10.248'N, 8°18.101'W; *E. meridonalis:* 39°58.726'N, 8°34.393'W; *S. festiva*: 40°32'01''N, 8°09'15''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 and maintained in 5L aquaria filled with aerated ASTM hard water (ASTM 1980), which had a layer of (10cm) sterile sediment, under a 12h light (L): 12h dark (D) photoperiod. Specimens were fed *ad libitum* for one 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 each species organisms 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 the autumn of 2009, just after abscission, and were air-dried and stored until needed. Leaves were weighed in batches of 4.5–5g, moistened, enclosed in coarse mesh bags (10 mm mesh size) and colonized for 3 weeks in natural conditions (Ribeira de S. João 40°11'N; 8° 25'E).

2.2- Bioassays with eucalyptus leachates

Eucalyptus leaf leachates were prepared from senescent eucalyptus leaves (*E. globulus*) collected, just after abscission, between September and October 2009. Leaves were transported to the labõratory, air dried in paper boxes at room temperature and stored in the dark until needed. Leaf leaching was obtained from 28 g/L of dried eucalyptus leaves immersed in the artificial reconstituted ASTM hard water (ASTM 1980) (alkalinity 110 to 120 mg/L as $CaCO_3$ and hardness 160 to 180 mg/L as $CaCO_3$;) hereafter indicated as ASTM, for 7 days, under continuous moderate aeration (15°C; photoperiod 12h light: 12h dark). The leachate was decanted and stored at 4°C until further use. Before the experiments, the leachate was analysed for total polyphenols (Graça *et al*. 2005), pH (JENWAY 3310, Essex, UK), conductivity (WTW LF 330, Weilheim, Germany), dissolved organic carbon (Elementar Analysensysteme Gmbh LiquiTOC, Hanau, Germany) and dissolved oxygen (WTW ProfiLine Oxi 3210, Weilheim, Germany). The characterization of the leachate is indicated in Table 1.

Table 1 - Physico-chemical characteristics of eucalyptus leachates. Analysis was performed in a mixed sample of leachate solutions (28g eucalyptus leaf litter/L; n=3).

Parameters	Mean (±S.E.M.)
pH	3.90 (0.115)
Conductivity (µS/cm)	1116 (54.354)
Tanic acid equivalents (mg/L)	465 (41.8)
Oxygen (mg/L)	1.70 (0.231)
DOC (µg/L)	2.538 (0.055)

The following range of dilutions, prepared from the original leachate solution, were used: 465 (T1), 232.50 (T2), 116.25 (T3), 58.10 (T4), 29.10 (T5), 14.50 (T6), and 7.30 (T7) mg/L tannic acid equivalents corresponding to 100%, 50%, 25%, 12.5%, 6.25%, 3.13% and 1.56% of the original eucalyptus leachate. An additional treatment (ASTM only) was used as control (T8). *For S. festiva*, a second bioassay with tannic acid concentrations between 349, 412 and 465 mg/L corresponding to 75, 89 and 100% of eucalyptus leachates at 10°C and 279, 325 and 434 mg/L corresponding to 60, 70, 93 % of eucalyptus leachates at 20°C was conducted. The pH values of test solutions measured at the beginning of the eucalyptus leachates bioassay carried out at 10°C were (mean±SE): 3.9±0.00 (T1); 4.5±0.04 (T2); 7.3±0.01 (T3); 7.6±0.04 (T4); 7.6±0.04 (T5); 7.7±0.02 (T6); 7.7±0.05 (T7); 7.7±0.02 (T8). The corresponding pH values in the 20°C bioassay were: 3.9±0.01 (T1); 4.6±0.07 (T2); 7.3±0.05 (T3); 7.6±0.01 (T4); 7.6±0.03 (T5); 7.7±0.00 (T6); 7.7±0.08 (T7); 7.7±0.02 (T8).

The bioassays were carried out under laboratory conditions (12:12-hour light/dark photoperiod; 10±1°C or 20±1°C). A total of 160 organisms per species (n=80 for each temperature) were used in the bioassays, with the following ranges of dry weight (mean dry weight (d.w.) ±S.E.M.) 2.67±0.024mg for *A. desmarestii*, 1.11±0.008mg for *E. meridionalis* and 0.02±0.0002mg for *S. festiva*. In each bioassay, 10 individuals were randomly distributed per each of the 8 different treatments (7 eucalyptus leachates concentrations and 1 ASTM control). Organisms were individually exposed in plastic test chambers filled with 200 ml of the test solution (hereafter indicated as eucalyptus leachates) with continuous aeration. Feeding was stopped 24h before the starting of the assays and no food was provided during the exposure period (96h). Effect criteria was mortality, recognized by immobility after stimulating, by a gentle touch, the invertebrates with a plastic pipette (or when found outside the case for *S. festiva*). Water temperature, conductivity, pH, dissolved oxygen (D.O.), and invertebrate's mortality were monitored and recorded at 24 h intervals.

2.3 - Bioassays with copper

Copper 96h-bioassays were also carried out with single species at both temperatures in equal laboratorial conditions (12:12-hour light/dark photoperiod; 10±1°C or 20±1°C). For each bioassay, a stock solution of copper sulfate pentahydrate (CAS no. 7758-99-8 purchased from Merck KGaA , Darmstadt, Germany) was prepared in ultra pure water (conductivity <5 µS/cm; Seralpur PRO 90 CN, Seral, Ransbach-Baumbach, Germany). The concentration of the stock solution was 25.5 mg/L (ionic Cu). 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, 0.03 mg/L for *A. desmarestii* at both temperatures; 0.81, 0.41, 0.20, 0.10, 0.05, 0.03 mg/L for *E. meridionalis* at 10°C and 0.41, 0.20, 0.10, 0.05, 0.03, 0.01 and 0.006 mg/L for *E. meridionalis* at 20°C; and 8.14, 4.07, 2.04, 1.02, 0.51 and 0.25 mg/L Cu for *S. festiva* at both temperatures.

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 dry weight (d.w.)±S.E.M.): 2.90mg±0.01 for *A. desmarestii*, 1.12mg±0.002, for *E. meridionalis* and 0.017mg±0.0002 for *S. festiva*. Ten individuals were randomly distributed per treatment as described in section 2.1.

2.4 - 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 LC10, LC20 and LC50 obtained in the bioassays with single substances (Table 2), except in the case of *S. festiva* where the concentration of tannic acid in the 100% eucalyptus leachates (465 mg/L) was tested, because the estimated LC50 of tannic acid was higher than the maximal tannic acid concentration present in the eucalyptus leaf leachates prepared (Table 1). Briefly, for each mixture bioassay and temperature, four treatments were considered: control (ASTM only), copper LC10 + eucalyptus leachates LC10 (Cu-LC10+EL-LC10); Cu LC20 + eucalyptus leachates LC20 (Cu-LC20+EL-LC20); and Cu

LC50 + eucalyptus leachates LC50 (Cu-LC50+EL-LC50). Mixture test solutions were prepared by diluting stock solutions of copper and eucalyptus leachates (prepared as described in previous sections) in ASTM hard water. Bioassays were carried out in conditions similar to those described in sections 2.1 and 2.2. The ranges of dry weight (d.w.) of the tested organisms were: 2.68mg±0.010 at 10°C and 2.67mg±0.011 at 20°C for *Atyaephyra desmarestii*, 1.12mg±0.003 at 10°C and 1.12mg±0.004 at 20°C for *Echinogammarus meridionalis* and 0.017mg±0.0002 at 10°C and 0.015mg±0.0004 at 20°C for *Schizopelex festiva*.

2.5 - Statistical analysis

The concentrations inducing 10% (LC10), 20% (LC20) and 50% (LC50) of mortality were determined from the log concentration *vs* 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 *à posteriori* LSD tests whenever necessary.

Preliminary check of ANCOVA assumptions was done. To investigate the type of toxicological interactions of copper and eucalyptus leachates in the studied species, the Toxic Units (TU) based approach (Sprague and Ramsay, 1965; Wang *et al*., 2011) was used:

$$TU=\sum_{i=1}^{n}\frac{ci}{ECxi}$$

where: n is the number of mixture components (i); ECx_i is the concentration of the mixture component i that induces x% of effect in single exposure bioassay ; ci is the concentration of the mixture component i in the mixture. If TU = 1 the interaction is addition, if TU < 1 the interaction is synergism, and if TU > 1 the interaction is antagonism. Analyses were performed using the SPSS 17.0® software package. The significance level was 0.05.

3. RESULTS

3.1- Species sensitivity to single substances and temperature effects

In all the bioassays carried out with eucalyptus leachates, the mortality in control treatments was always equal or inferior to 10% except in the test with *E. meridionalis,* at 20°C, where a 20% of mortality in the control group was recorded. In each test chamber, the water temperature variation was less than 1°C, the water dissolved oxygen was always higher than 9.12 mg/L; the maximum pH variation was 0.91; and the conductivity variation was always lower than 71 $\mu S.cm^{-1}$. The mortality recorded is indicated in Appendix 1, while the 96h LC10, LC20 and LC50 calculated from the toxicity curves (Figure 1) are indicated in Table 2. For *S. festiva*, the calculation of LCx 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 LC10, LC20 and LC50 was possible but without confidence limits; also, the estimated LC50 of tannic acid exceeded its concentration in 100% of eucalyptus leachates. Therefore, due to these

constraints, this species was not included in further statistical analysis. The LC50s obtained for the other species in single bioassays ranged from 34.4 to 141.6 mg/L of tannic acid equivalents (Table 3). 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.

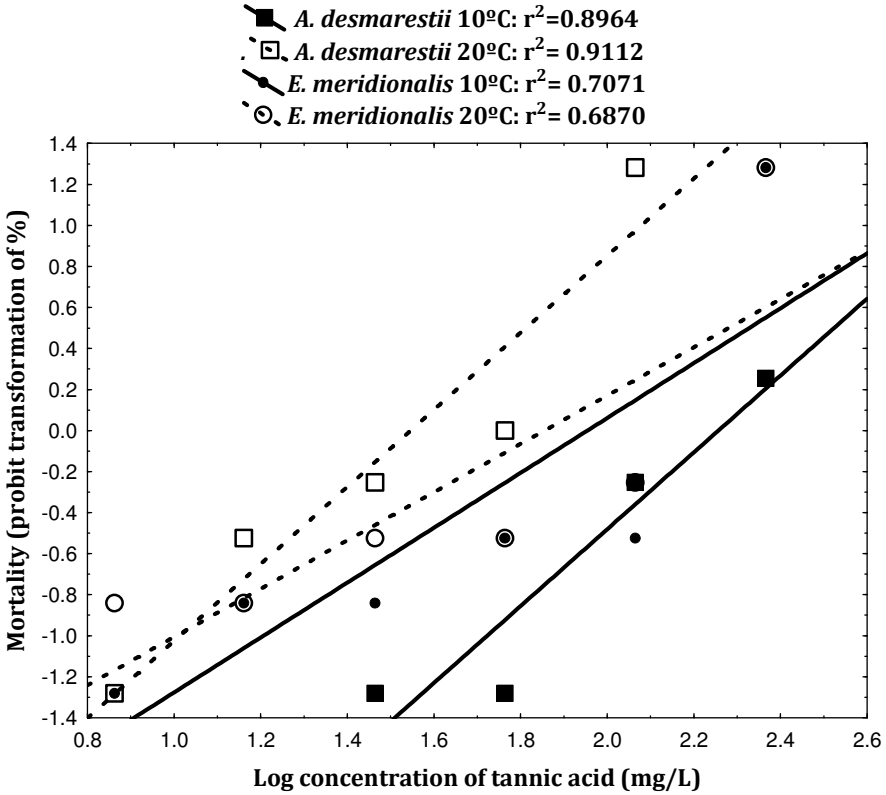

Fig. 1- Acute toxicity of eucalyptus leachates to *Atyaephyra desmarestii* and *Echinogammarus meridionalis* after 96h of exposure at 10°C and 20°C. Lines represent linear regression, r square for the two organisms and temperatures are displayed in the graphic.

The comparison of *A. desmarestii* and *E. meridionalis* toxicity curves (Figure 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<0.05) explaining 40.3% of the 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

raise of temperature from 10°C to 20°C, significantly increased the toxicity of eucalyptus leachates to *A. desmarestii* (ANCOVA: $F_{(1,8)}$=36, p<0.05) but not to to *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 less than 1°C; water dissolved oxygen was always higher than 9.09 mg/L; the maximum of pH variation was 0.6; and the conductivity variation was always lower than 62 µS cm^{-1}. The mortality recorded in copper bioassays is indicated in Appendix 1, while the LC10, LC20 and LC50s are shown in Table 2. The LC50s of copper ranged from 0.036 to 9.0 mg/L, with LC50 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 (Figure 2) by 2-way ANCOVA, indicated significant differences among species ($F_{(2,22)}$=162.1, p<0.05) contributing for 94% of the variance; significant effects of temperature ($F_{(1,22)}$=51.2, p<0.05) explaining 70% of the variance and the interaction between species and temperature was also significant ($F_{(1,22)}$=25.0, p<0.05) explaining 69% of the variance. Comparing now the three species at 10°C, significant differences of sensitivity were found (ANCOVA $F_{(1,11)}$=68.87, p<0.05) with *S. festiva* being less sensitive (Table 3) than *E. meridionalis* (*S. festiva* vs *A. desmaresti*: p<0.05; *S. festiva* vs *E. meridionalis*: p<0.05), while 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 others were found (*S. festiva* vs *A. desmaresti*: p<0.05; *S. festiva* vs *E.*

meridionalis: p<0.05), with *S. festiva* being less sensitive that the other species; significant differences between *A. desmarestii* and *E. meridionalis* were also found (p<0.05). Temperature significantly increased the toxicity of copper for *A .desmarestii* and *E. meridionalis* (ANCOVA $F_{(1,8)}$=6.5, p<0.05 for *A. desmarestii* and $F_{(1,8)}$=53.6, p<0.05 for *E. meridionalis*) with more pronounced effects in *E. meridionalis*. No significant differences between temperatures were found for *S. festiva* (ANCOVA $F_{(1,3)}$=4.3, p=0.13).

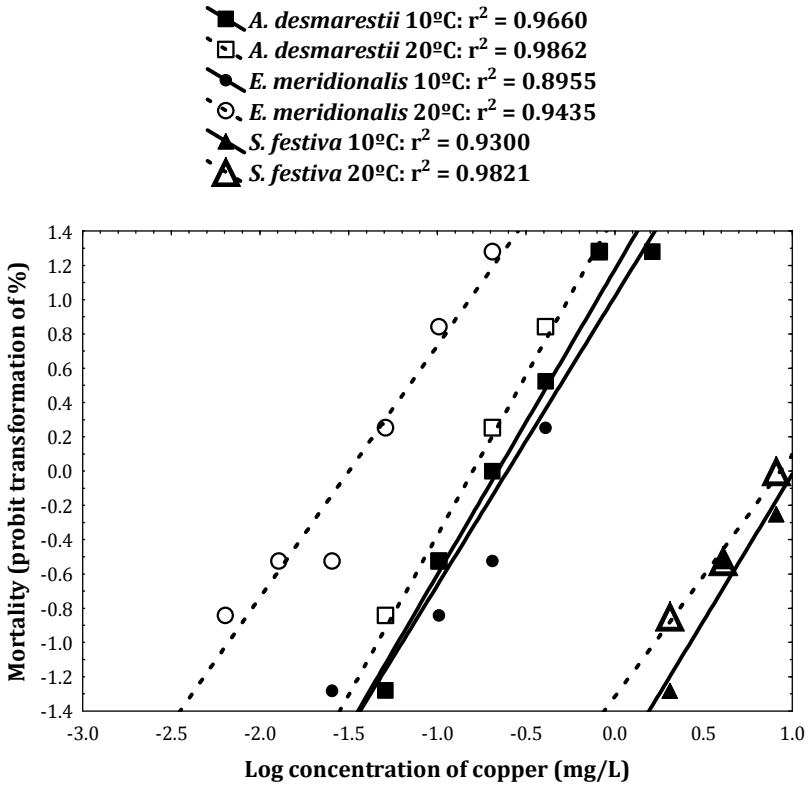

Fig. 2 - Acute toxicity of copper to *Atyaephyra desmarestii*, *Echinogammarus meridionalis*, and *Schizopelex festiva* after 96h exposure at 10°C and 20°C. Lines represent linear regression, r square for the three organisms and temperatures are displayed in the graphic.

Table 2 - Eucalyptus leachates (EL) and copper (Cu) concentrations causing 10% (LC10), 20% (LC20) and 50% (LC50) 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***		
		LC10	LC20	**LC50**	LC10	LC20	**LC50**	LC10	LC20	**LC50**
EL (mg/L)	**10°C**	46.24 (19.398-71.369)	67.89 (35.748-99.193)	**141.58** (96.638-221.824)	12.91 (3.557-24.340)	24.26 (9.639-41.080)	**81.13** (48.887-148.447)	348.48 -	412.05 -	**567.78** -
	20°C	8.30 (2.653-14.561)	13.51 (5.710-21.633)	**34.35** (21.408-53.331)	7.93 (1.466-16.918)	16.35 (4.938 -30.095)	**65.28** (36.679-124.597)	273.84 -	332.52 -	**482.10** -
Cu (mg/L)	**10°C**	0.05 (0.018-0.087)	0.08 (0.038-0.133)	**0.22** (0.139-0.343)	0.05 (0.012-0.088)	0.09 (0.034-0.144)	**0.27** (0.161-0.541)	2.30 (0.372-3.868)	3.68 (1.508-7.084)	**9.00** (5.209-94.989)
	20°C	0.04 (0.016-0.073)	0.07 (0.032-0.106)	**0.17** (0.106-0.251)	0.01 (0.001-0.011)	0.01 (0.003-0.019)	**0.04** (0.020-0.062)	1.84 (0.397-3.055)	2.95 (1.244-5.008)	**7.27** (4.394-32.498)

3.2. *Mixture bioassays*

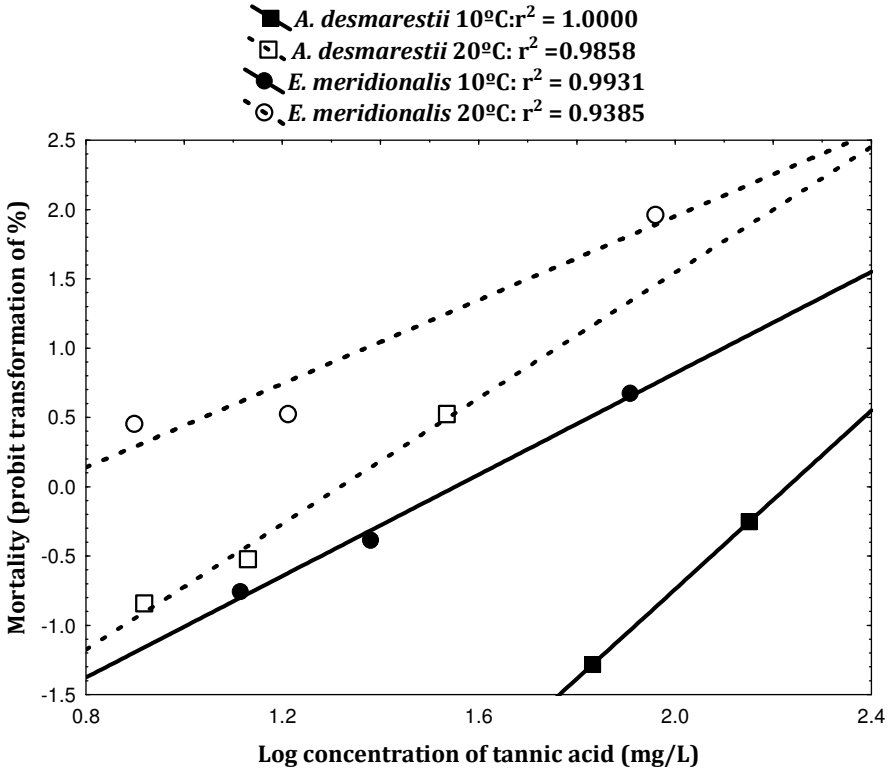

Fig. 3 - Acute toxicity after 96h of simultaneous exposure to copper and eucalyptus leachates to *Atyaephyra desmarestii* and *Echinogammarus meridionalis* at 10°C and 20°C. Lines represent linear regression, r square for the two organisms and temperatures are displayed in the graphic.

The results of the mixture (eucalyptus leachates and copper) bioassays, expressed as percentage of mortality are indicated in Appendix 1. Because the low mortality of *S. festiva* even at the highest exposure concentrations tested in the eucalyptus leachates bioassays, and the lack of relevance of testing eucalyptus leachates concentrations higher than 100%, this species was not included in further statistical analysis of mixture bioassays. The LC50s for the mixture are shown in Table 4 and the type of interactions based on the TU approach is presented in Table 5. The comparison of the mixture toxicity curves based on the concentration of tannic acid (Figure 3) indicates significant differences between species (2-way ANCOVA: $F_{(1,6)}$=92.04, $p<0.05$), explaining 94% of the variability,

significant differences between temperatures (2-way ANCOVA $F_{(1,6)}$=108.45, p<0.05) explaining 95% of variability, and no significant interaction between species and temperature (2-way ANCOVA: $F_{(1,6)}$=3.95, p=0.09).

Table 3 - Acute toxicity after 96h of simultaneous exposure to eucalyptus leachates and copper for *Atyaephyra desmarestii*, and E*chinogammarus meridionalis* at 10°C and 20°C. LC50- 50% lethal concentrations calculated from the toxicity curves (log concentrations vs probit transformation of mortality %). Cu – copper; EL – eucalyptus leachates concentrations expressed in mg of tannic acid/L. 95% confidence limits for the estimates are given within brackets.

Temperature (°C)	Species	LC50 (mg/L)
10	*A. desmarestii*	0.26 Cu + 160.87 EL (0.161-17.151;111.564-3782.292)
	E. meridionalis	0.12 Cu + 35.90 EL (0.093-0.178;26.409-52.589)
20	*A. desmarestii*	0.10 Cu + 20.87 EL (0.061-0.515;12.055-114.242)
	E. meridionalis	0.003 Cu + 4.97 EL (n.a.;n.a.)

Table 4 - Type of toxicological interaction between copper (Cu) and eucalyptus leachates (EL) in *Atyaephyra desmarestii*, and E*chinogammarus meridionalis* at 10°C and 20°C. Temp. – temperature. Conc. Mix – concentration in the mixture causing 50% of mortality; 96h-LC50 – 50% lethal concentrations of the single toxicants after 96 hours of exposure. TU – toxic units. 95% confidence limits of LC50s within brackets.

Species	Temp (°C)		Conc. Cu mixt	Cu LC single (mg/L)	TU (Cu)	Conc. EL mixt	EL LC single (mg/L)	TU (EL)	ΣTU	Type of interaction
A. desmarestii	10°C	LC50	0.26 (0.161-17.151)	0.22	1.18	160.9 (111.564-3782.292)	141.6	1.14	2.32	*Antagonism*
	20°C	LC50	0.10 (0.061-0.515)	0.17	0.59	20.9 (12.055-114.242)	34.4	0.61	1.20	*Antagonism*
E. meridionalis	10°C	LC50	0.12 (0.093-0.178)	0.27	0.44	35.9 (26.409-52.589)	81.1	0.44	0.88	*Synergism*

4. DISCUSSION

4.1- Bioassays with single substances and temperature effects

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 is less sensitive to chemical stress and temperature changes than *A. desmarestii* and *E. meridionalis*. Several causes may contribute to this relative tolerance to chemicals, including toxicant avoidance or any other mechanisms able to decrease stress exposure, general mechanisms decreasing toxicant uptake and/or increasing toxicants elimination, specific mechanisms of tolerance to copper and eucalyptus toxins, low sensitivity of molecular targets, among other possibilities. Toxicant avoidance or reduced exposure to the tested substances is likely to have occurred during our experiments because *S. festiva* has a case and the test period was relatively short (96h); in these conditions, the starvation pressure may have not been high enough to force the organisms to go out of the case to search for food and thus they were able to avoid (or reduce) the exposure to environmental contaminants; this could explain also at least in part the lack of influence of temperature on chemicals' toxicity in this species. The finding that this species was the less sensitive to both eucalyptus leachates and copper may also suggest the presence of general mechanisms decreasing the uptake of environmental contaminants or increasing their elimination from the body, which do not exclude the potential protective effects of the invertebrate's case. Mechanisms of tolerance to metals are present in other Trichopteran larvae (Darlington and Gower, 1990) as well as differential patterns of metal accumulation (Cain and Luoma, 1998; Rainbow, 2002, 2007; Rainbow *et al*., 2012)

so they may also exist in *S. festiva*. The lower sensitivity of this species to chemical 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), suggesting a ability common to this family. Independently of the mechanisms involved that will be very interesting to investigate, the relatively low sensitivity of this species should be considered when planning monitoring studies with shredders.

The results of Figure 1 and their statistical analysis indicate that *A. desmarestii* and *E. meridionalis* have no significant differences of sensitivity to eucalyptus leachates not to copper at 10°C. However, temperature raise from 10 to 20°C significantly increased the toxicity of eucalyptus leachates to *A. desmarestii* (but not to *E. meridionalis*) and of copper for both species with more strong effects on *E. meridionalis* (Figure 2). Therefore, the relative sensitivity of the species to chemical stress is dependent of the substance/substances and temperature. Thus, in real scenarios the potential pre-existing equilibrium between populations of *A. desmarestii* and *E. meridionalis* is not expected to be disrupted after exposure to copper or eucalyptus leachates in the range of concentrations tested at temperatures around 10°C but the outcome may be considerable different at higher temperatures.

From the conclusions above, at least two interesting questions emerge: (i) why temperature raise increases the toxicity of copper (both species) and eucalyptus leachates (one species)? Why the temperature effect is different in the two species? Copper is a well known oxidative stress inducer in both vertebrates

(e.g. Olivari *et al*., 2008; Vieira *et al*., 2009; Roy *et al*., 2009; Boveris *et al*., 2012) and invertebrates (Gomes *et al*., 2012; Maria and Bebianno, 2011), including shredders (Bouskill *et al*., 2006; Sroda and Cassu-Leguiller, 2011), a mechanism of toxicity that may lead to death (Brinkman and Johnston, 2008; Tollet *et al.,* 2009). Thus, damage in crucial molecules (e.g. proteins, lipids, DNA), resulting from oxidative stress, may contribute significantly for its toxic effects. Temperature is also known to increase the formation of radical oxygen species (ROS) (Abele *et al.,* 1998, 2002; Heise *et al*., 2003) and therefore the expected result is an increase of copper toxicity at higher temperatures. Temperature is also able to modulate several other processes and thus it may influence cooper uptake, its disposition, interaction with molecular targets and elimination. Thus, the increase of copper toxicity at higher temperatures, as well as the differences among species found may be due to differences in these processes. Eucalyptus oils and extracts contain several valuable components, such as anti-oxidant agents (Amakura *et al*., 2002), as well as toxins (e.g. tannic acid, Table 1). The extracts are complex mixtures of substances and some of them may undergo biotransformation (Pass *et al*., 1999; Liapis *et al*., 2000), therefore, differences between species may have additional causes such as differences in biotransformation and distinct sensitivities of targets of the substances present in the extracts. These are very interesting issues deserving further investigation.

4.2- Mixture bioassays

Toxicological interactions between copper and eucalyptus leachates were found in both *A. desmarestii* and *E. meridionalis* (Table 4). In *A. desmarestii*, the exposure to the mixture resulted in antagonistic effects at both 10 and 20°C. Because copper is a well know oxidative stress inducer (Bouskill *et al*., 2006; Sroda and Cassu-Leguiller, 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 antagonism found. Interestingly, in *E. meridionalis*, synergistic effects were found indicating a distinct response to mixture exposure, suggesting differences between the species in the mechanisms of toxicity and biotransformation, among others, as the results from single substances bioassays also suggested. 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, while 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 that *S. festiva*, *A. desmarestii* and *E. meridionalis* have differences of sensitivity to copper, eucalyptus leaf extracts and their mixture, with *S. festiva* being the less sensitive species at both 10 and 20°C and the relative sensitivity of *A. desmarestii* and *E. meridionalis* being

chemical and temperature dependent. Overall, these findings suggest that single and combined chemical stress may modulate the biodiversity of stream shredders communities due to differential sensitivity of individual species and that the process may be influenced by temperature, highlighting the need of more knowledge on the subject as well as on the molecular mechanisms responsible for chemicals toxicity.

Acknowledgments

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Chapter III

Effects of eucalyptus leachates and copper (single and in mixture) on cholinesterase activity and oxidative stress parameters of three shredder species assessed at two temperatures

Effects of eucalyptus leachates and copper (single and in mixture) on cholinesterase activity and oxidative stress parameters of three shredder species assessed at two temperatures

ABSTRACT

In the present study, the effects of eucalyptus leachates (EL) and copper (single and in mixture) on cholinesterase activity and oxidative stress parameters of three shredder species (*Atyaephyra desmarestii, Echinogammarus meridionalis* and *Schizopelex festiva*) were investigated at two different temperatures (10 and 20°C). For each species, 96h laboratory bioassays were conducted, by exposing groups of organisms to control conditions, LC10, LC20 and LC50 of EL, copper and their equitoxic mixtures previously determined, at both temperatures. At the end of the bioassays, the following biomarkers were determined: the activity of the cholinesterases (ChE) enzymes as a neurotoxicity biomarker; the activity of glutathione S-transferases (GST) enzymes which are involved in biotransformation and oxidative stress prevention mechanisms; and lipid peroxidation levels (LPO) as indicative of oxidative damage. The results indicate that temperature raise from 10 to 20°C significantly reduced ChE activity in *S. festiva*, GST activity in *S. festiva* and *E. meridionalis*, and increased LPO levels in *S. festiva* and *A. desmarestii* (control organisms). Thus, these effects should be taken in consideration when using these parameters as biomarkers under temperature variation scenarios. In *S. festiva*, no significant anti-cholinesterase effects were observed, and lipid oxidative damage was only found at one mixture concentration, possibly due to a scavenger

effect of GST. *A. desmarestii* ChE were significantly increased by exposure to the highest mixture concentration, and both EL and copper (single and in mixture) were able to significantly increase LPO levels at 10°C but not at 20°C. The highest concentration of EL and the mixture caused a significant inhibition of ChE in *E. meridionalis* at 20°C but not at 10°C, and significant oxidative damage in the mixture at the lowest temperature but not at the highest one. Overall, these findings indicate that the tested shredder species have different sensitivities to chemically-induced acute stress and at least some of the mechanisms of toxicity and detoxication involved are modulated by temperature. Therefore, more studies on the combined effects of chemical and temperature stress on these species should be carried out to improve the basis for ecological risk assessment of both environmental contaminants and climate changes, especially on freshwater shredders community.

Keywords: *Atyaephyra desmarestii*, *Echinogammarus meridionalis*, *Schizopelex festiva*, temperature and chemical stress, mixture toxicity, biomarkers

1. INTRODUCTION

Shredders play a crucial role in small streams ecosystems supporting the transfer of trophic energy and organic matter from the surrounding forests to lower stream sections as a consequence of their feeding/egestion activities (Eggert and Wallace, 2007). These ecosystems experience daily or seasonal temperature fluctuations and are particularly vulnerable to chemical contamination either from anthropogenic activities (e.g. mining, fertilizers and pesticides from surrounding crops) or from natural sources, such as toxins from riparian vegetation (Chatzinikolaou, 2006; Borgmann *et al.,* 2007; Maltby and Hills, 2008).

Distinct invertebrate species may have different sensitivities to stressors and the presence of environmental contaminants may change shredder communities with potential impacts at ecosystem level (Schulz *et al.,* 2002, Liess and Von der Ohe, 2005). Differences of sensitivity to chemical stressors among species in response to metal exposure namely copper have been reported (Boeckman and Bidwell, 2006; Roman *et al*., 2007). Different invertebrates may cope distinctly with the presence of metals. Decapods regulate concentrations of essential elements (e.g. Cu) to approximately constant levels, excreting the excess metal (Rainbow, 2007) and amphipods are net accumulators of essential metals (Rainbow and White, 1989; Rainbow, 1998). Some species of Trichoptera are able to accumulate copper (Solá and Prat, 2006) and exhibit differential patterns of metal accumulation (Cain and Luoma, 1998; Rainbow, 2002, 2007; Rainbow *et al*., 2012). And mechanisms of behavioural avoidance after stressor exposure have also been observed for several invertebrates (Gerhardt and Palmer, 1998; Amorim *et al*., 2005; 2008).

Eucalyptus (*Eucalyptus globulus*) is now the predominant tree in the Portuguese forest (IFN6, 2013). Afforestations with this species are known to have serious impacts on streams ecosystems (Abelho and Graça, 1996; Larrañaga *et al*., 2006) namely on shredders (Larrañaga *et al*., 2009). Negative impacts of eucalyptus leachates on streams ecosystems have been detected (Canhoto and Laranjeira, 2007; Canhoto *et al*., 2013) although further research is needed to accurately predict and mitigate impacts on macroinvertebrate communities. Therefore the objective of the present study was to investigate the effects of eucalyptus leachates (EL) and copper (single and in mixture) on cholinesterase activity and oxidative stress parameters of three shredder species (*Atyaephyra desmarestii, Echinogammarus meridionalis* and *Schizopelex festiva*) at two different temperatures. These particular effects were selected because copper was found to inhibit the activity of ChE of several species (Garcia *et al*., 2000; Brown *et al*., 2004a; Vieira *et al*., 2009) and is a well known oxidative stress inducer (Mosleh *et al*., 2006; Maria and Bebbiano, 2011; Gomes *et al*., 2012), while eucalyptus toxins have been showing anti-oxidant properties (Bendaoud *et al*., 2009; Naceur Ben-Marzoug *et al*., 2011). Ten and 20°C were selected to represent natural temperatures observed in the invertebrate's streams of origin during colder and warmer seasons (Canhoto and Laranjeira, 2007; Ferreira *et al*., 2010).

2. MATERIAL AND METHODS

2.1 - Collection and acclimation of organisms

The organisms were collected between May 2010 and February 2011 in three streams of Central Portugal: *A. desmarestii* in Varandas do Ceira, Condeixa, central Portugal (40°10.248' N, 8°18.101'W); *E. meridionalis* in Redinha, Pombal, central Portugal (39°58.726'N, 8°34.393'W) and *S. festiva* in Múceres, Caramulo, central Portugal (40°32'01''N, 8°09'15''W). They were transported to the laboratory in containers with water from the original streams. In the laboratory, organisms were maintained in photoperiod (12h light: 12h dark) and temperature (10 or 20°C according their further use) controlled rooms, in aerated aquariums (5L of capacity, with a 5cm layer of sterile sediment) with 4 L of ASTM hard water (ASTM, 1980) for one week prior to the start of the tests. During this time period, invertebrates were fed *ad libitum* with conditioned alder leaves. Twenty-four hours prior to the start of the test food was retrieved to allow gut clearance.

2.2 - Preparation of stock and test solutions

ASTM was used in all tests (alkalinity 110 to 120 mg/L as $CaCO_3$; hardness 160 to 180 mg/L as $CaCO_3$). A stock solution of copper sulfate was prepared with copper sulfate pentahydrate (CAS no. 7758-99-8, from Merck KGaA, Darmstadt, Germany) in ultra pure water, corresponding to 25.5 mg/L of ionic copper. EL were prepared as indicated in Canhoto and Laranjeira (2007) by adding 28g of dry eucalyptus leaves per L of ASTM and maintaining the solution in continuous moderate aeration for 7 days in controlled conditions (15°C; photoperiod 12h light: 12h dark). After this time period, the leachates solution was decanted and

stored at 4°C. Prior to the beginning of the bioassays this solution was analyzed for polyphenols (Graça *et al.,* 2005), dissolved organic carbon (DOC) (Elementar Analysensysteme Gmbh LiquiTOC, Hanau, Germany) dissolved oxygen (WTW OXI 92 oxygen meter), pH (Wissenschaftlich Technische Werkstätten 537 pH meter, WTW, Weilheim, Germany), and conductivity (WTW LF 92 conductivity meter). The concentration of the polyphenolic compounds in the solution, expressed as tannic acid equivalents, was extremely high (465±42.0 mg/L). This solution presented high dissolved organic carbon (DOC) levels (2.48±0.05 µg/L) and conductivity (1127±322 $\mu S.cm^{-1}$), low oxygen concentrations (1.88±0.355 mg/ml) and acidic pH (3.96±0.731).

Test solutions were prepared by dilution of these stock solutions (copper and EL) in ASTM. Mixture test solutions were prepared by adding the correspondent copper and/or leachates stock solutions and adjusted to the final intended volume with ASTM hard water.

2.3 - Experimental design and exposure conditions

Bioassays were carried out for 96h in a temperature (10±1°C or 20±1°C) and photoperiod (12h light: 12h dark) controlled room. In previous bioassays the LC10, LC20 and LC50 of copper and eucalyptus leachates to *A. desmarestii*, *E. meridionalis* and *S. festiva* were determined at both 10 and 20°C. Concentrations of EL (expressed as mg/L of tannic acid) and copper (ionic concentrations in mg/L) where tested single and in mixture (LC10 EL + LC10 Cu; LC20 EL + LC20 Cu; LC50 EL + LC50 Cu) at both 10 and 20°C as indicated below (Table 5)

In each bioassay, a control with ASTM only was included in the experimental design. Organisms were exposed individually in test chambers (plastic cups) filled with 200 ml of each solution and no food was provided during the assays. Mortality, dissolved oxygen, conductivity and temperature were monitored at each 24h intervals. Organisms that died during the assays were immediately removed and frozen at -80°C. At the end of the bioassays, organisms were collected and frozen at -80°C until being used for biomarkers determination.

Table 5 - Concentrations of eucalyptus leachates (EL) and copper (Cu), single and in mixture, used in the bioassays with *Atyaephyra desmarestii*, *Echinogammarus meridionalis* and *Schizopelex festiva* carried out at 10°C and 20°C. These were the estimated concentrations causing 10%, 20% and 50% of mortality in previous bioassays. EL concentrations are expressed in mg/L of tannic acid and copper concentrations correspond to Cu(II).

Species	***Temperature***	***Treatment***	***Stressor concentration (mg/L)***		
			EL	***Cu***	***Mixture***
A. desmarestii	10°C	LC10	46	0.05	46EL+0.05Cu
		LC20	68	0.08	68EL+0.08Cu
		LC50	142	0.22	142EL+0.22Cu
	20°C	LC10	8	0.04	8EL+0.04Cu
		LC20	14	0.07	14EL+0.07Cu
		LC50	34	0.17	34EL+0.17Cu
E. meridionalis	10°C	LC10	13	0.05	13EL+0.05Cu
		LC20	24	0.09	24EL+0.09Cu
		LC50	81	0.27	81EL+0.27Cu
	20°C	LC10	8	0.005	8EL+0.005Cu
		LC20	16	0.01	16EL+0.01Cu
		LC50	65	0.036	65EL+0.036Cu
S. festiva	10°C	LC10	349	2	349EL+2Cu
		LC20	412	4	412EL+4Cu
		LC50	465	9	465EL+9Cu
	20°C	LC10	279	2	279EL+2Cu
		LC20	325	3	325EL+3Cu
		LC50	434	7	434EL+7Cu

2.4 - Biomarkers

Biomarkers analysis were done individually in *A. desmarestii* and *S. festiva*, thus in a total of 10 replicates (individual organisms) per treatment. In the case of *E. meridionalis*, because of their small size and the relatively low enzymatic activities recorded in preliminary assays, 4 replicates (pooled samples of 10 animals each) were used per treatment. Heads were isolated on ice and used for ChE enzymatic determinations, while the remaining bodies were isolated on ice and used for GST activity and LPO determinations. Heads samples were put in 0.5 ml of ice cold phosphate buffer (0.1 M, pH 7.4), homogenized (Ystral GmbH d-7801 Dottingen homogeniser) for 30s on ice, and centrifuged at 3300×g for 3min at 4 °C (SIGMA 3 K 30). The supernatants were carefully collected and used to determine ChE activity after standardization of protein content (1 mg/mL) by the Ellman's technique (Ellman *et al*., 1961) adapted to microplate (Guilhermino *et al.,* 1996). Briefly, at each 0.05 ml of sample it was added 0. 250 ml of the reaction solution (30 ml of K-phosphate buffer (pH=7.2, 0.1M), 1 ml of a dithiobisnitrobenzoate 10 mM solution (20 mM of acid dithiobisnitrobenzoate and 18 mM of sodium hydrogen carbonate in 0.1 M K-phosphate buffer, pH 7.2), and 0.2 mL of acetylcholine iodide in u.p. water (75 mM)). The increase of absorbance was measured at 412 nm in a microplate reader (Bio Tek Power Wave 340) at 25 °C. In all the determinations, acetyltiocholine was used as substrate. As no previous characterization of the enzymes present in the head of the tested species was made, the cholinesterase activity measured will be hereafter indicated as the activity of all the ChE enzymes present in the samples.

Body samples were also homogeneized (Ystral GmbH d-7801 Dottingen homogeniser) in 1ml of ice cold phosphate buffer (0.1 M, pH 7.4) for 30s on ice and then half of this homogenized (0.5ml) was centrifuged (SIGMA 3 K 30) at 10000×g for 20 min at 4 °C. GST activity was quantified by the method of Habig *et al.* (1974) with adaptations (Frasco and Guilhermino, 2002) through the conjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB). To 0.250 mL of the reaction solution (75 mL of phosphate buffer 0.2 M pH 6.5, 2.34 mL of 1-chloro-2,4 dinitrobenzene in ethanol (60mM) and 13.5 mL of a 10 mM GSH solution in ultra-pure water), 0.05 mL of sample (previously diluted in homogenization buffer in order to have a final protein concentration of 1 mg/mL), was added in the microplate well. Absorbance was measured at 340 nm in a microplate reader (Bio Tek Power Wave 340) for 5 min at 25°C.

ChE and GST activities were expressed per Units (U) per concentration of protein; one U corresponds to 1 nano mole of substrate hydrolyzed per min per mg of protein. The concentration of protein in the samples was determined according to Bradford *et al*. (1976) adapted to microplate (Frasco and Guilermino, 2002) using bovine γ -globulin as protein standard.

The thiobarbituric acid reactive species (TBARS) were measured to determine LPO, following the methods described in Ohkawa *et al*., 1979 and Bird and Draper (1984), with some modifications (Torres *et al*., 2002; to prevent artifactual lipid oxidation, by adding 0.2 mM butylhydroxytoluene). To each sample, 1 mL of 12% trichloroacetic acid, 0.8 mL of 60 mM Tris–HCl solution (pH 7.4) with 0.1 mM diethylenetriaminepentaacetic acid (DTPA) and 1 mL of 0.73% thiobarbituric acid (TBA) were added. Samples were incubated at 100°C for 60

min. Then, centrifuged at 12000×g for 5 min at 4°C, and the resultant supernatant was used to determine LPO levels at 535 nm. Results were expressed in nano moles of TBARS per gram of wet weight. A Jasco V-630 spectrophotometer was used.

2.5 - Data analysis

Data were checked for normality of distribution using the Kolmogorov–Smirnov test, and for homoscedasticity using the Levene's test. Appropriated data transformations (log (x+1)) were made when necessary (Zar 1999). For each biomarker, species and temperature, different treatments were compared by one-way analysis of variance (ANOVA). When significant differences among treatments were found by ANOVA, the Tukey's test was used to identify significantly different treatments. When the ANOVA assumptions could not be achieved, the Kruskal-Wallis analysis was used to compare different treatments and the multiple comparisons test was used to identify significantly different treatments. The controls at 10 and 20°C were compared with a Student t-test. StatSoft\Statistica 8® software package was used for all the analysis. In all cases, the significance level was 0.05.

3. RESULTS AND DISCUSSION

3.1 - Effects of stressors on S. festiva

No significant effects of EL, copper and their mixtures on *S. festiva* ChE activity were found (Figure 4), either at 10°C ($F_{(9,89)}$= 1.11, p>0.05) or at 20°C ($F_{(9,90)}$= 1.15, p>0.05). These results indicate that in the range of concentrations

tested and in the experimental conditions used, the environmental contaminants have no anti-cholinesterase effects in this species. They also indicate that the mortality induced by EL and copper single and in mixture on *S. festiva* is not due to effects on the cholinergic system.

At 10°C, significant effects of EL, copper and their mixtures on *S. festiva* GST activity ($H_{(9,88)}$=48.68, $p<0.05$) and LPO levels ($F_{(9,90)}$= 3.90, $p<0.05$) were found. In exposures with single chemicals, *S. festiva* GST activity was reduced by both EL and copper in a dose dependent manner (Figure 5A), reaching about 74% and 80% of decrease at the highest concentrations of EL (465 mg/L) and copper (9mg/L). Under simultaneously exposure to EL and copper, *S. festiva* GST activity was reduced by about 80% at all the concentrations tested and no dose-response was observed. The decrease of GST activity may be due to the binding of the enzyme to the environmental contaminants to decrease their free amount and thus their toxic effects. This is a well known role of this enzyme (Klaassen, 2008). Inhibition of GST under exposure to copper was also observed in the aquatic worm *Tubifex tubifex* (Mosleh *et al.,* 2005) and in the gastropod *Nucella lapillus* (Cunha *et al.,* 2007). The lack of significant increase of LPO levels in organisms exposed to the most part of copper and EL treatments seems to support this hypothesis (Figure 6A). At the highest concentration of the mixture tested, a significant increase of LPO levels was observed (Figure 6A). This finding and the GST response pattern in mixture treatments suggests that anti-oxidant defenses were not able to cope with the oxidative stress and thus lipid damage occurred. Because lipids are important components of cell membranes, lipid damage may have a wide range of severe effects (Klaassen, 2008). These effects may have contributed to the mortality

observed as suggested by the higher mortality recorded at the highest mixture concentration (33 %) than in the remaining ones (0% and 10%, respectively) (Chapter II).

The raise of temperature from 10 to 20°C (Figures 4B, 5B and 6B), significantly reduced the activity of ChE ($T=6.03$; $p<0.05$) and GST ($T=-2.72$; $p<0.05$) enzymes, and increased LPO levels ($T=-4.37$; $p<0.05$) in control treatments. These effects should be considered when using the ChE activity of this species as a biomarker under scenarios of temperature variation. At 20°C, significant differences in GST activity ($F_{(9,90)}= 8.12$, $p<0.05$) and LPO levels ($F_{(9,90)}= 3.46$, $p<0.05$) among treatments were also found but with some differences in the pattern of response relatively to 10°C (Figures 5B and 6B). Regarding GST and relatively to the control group: a significant reduction of activity was observed under EL exposure but only at the intermediate concentration with the activity becoming not significantly different from the control group at the highest concentration tested; and no significant effects of copper nor of the mixture treatments were found. These findings suggest that the raise of temperature modifies the pattern of some *S. festiva* responses to chemical stress. In addition, they also indicate that *S. festiva* is relatively resistant to the oxidative stress induced by copper that is a well known oxidative stressor (Boveris *et al.,* 2012).

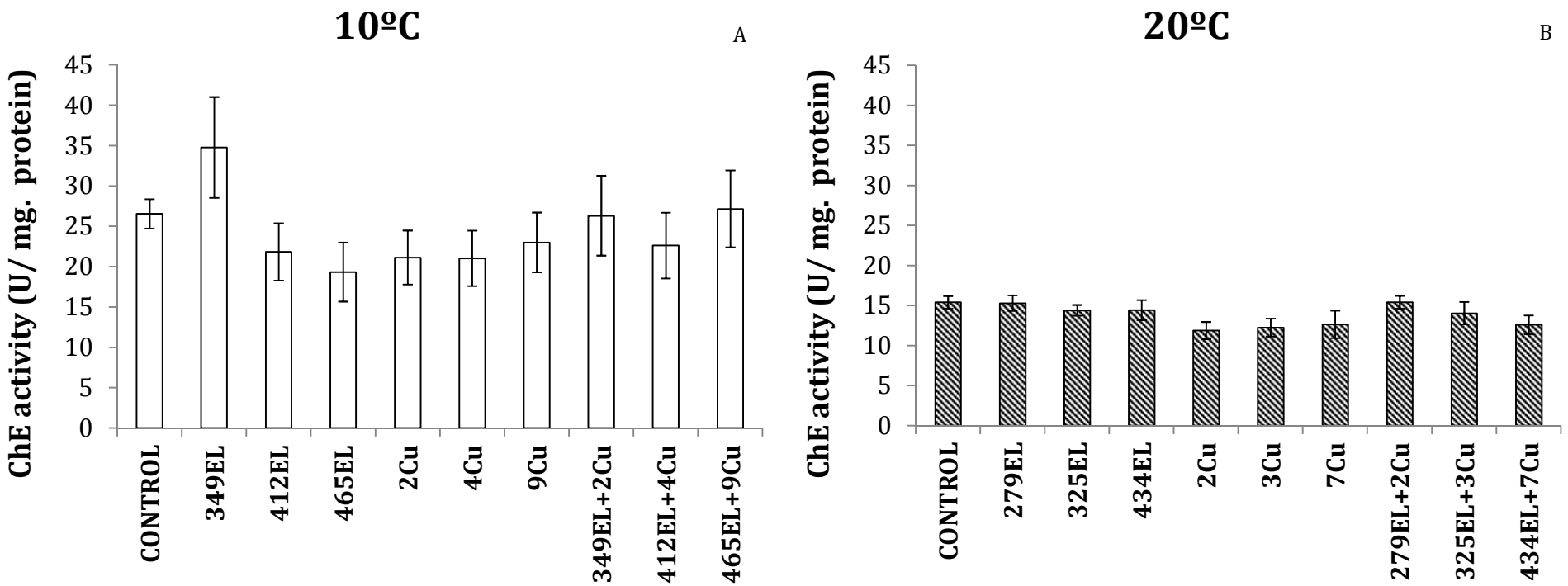

Fig. 4 - Effects of eucalyptus leachates (EL), copper (Cu), and their equitoxic mixtures (mg/L) to *Schizopelex festiva* after 96h of exposure at 10°C (A) and 20°C (B). ChE - head cholinesterase activity. Enzymatic activities are expressed in Units (U) per concentration of protein, one U corresponding to 1 nano mole of substrate hydrolised per minute per mg of protein. Values are the means±S.E.M.; n=10. Enzymatic activities (mean±S.E.M.) in control groups at 10°C: 26.53 U/mg protein±1.82; Corresponding values at 20°C: 15.40 U/mg protein±0.78.

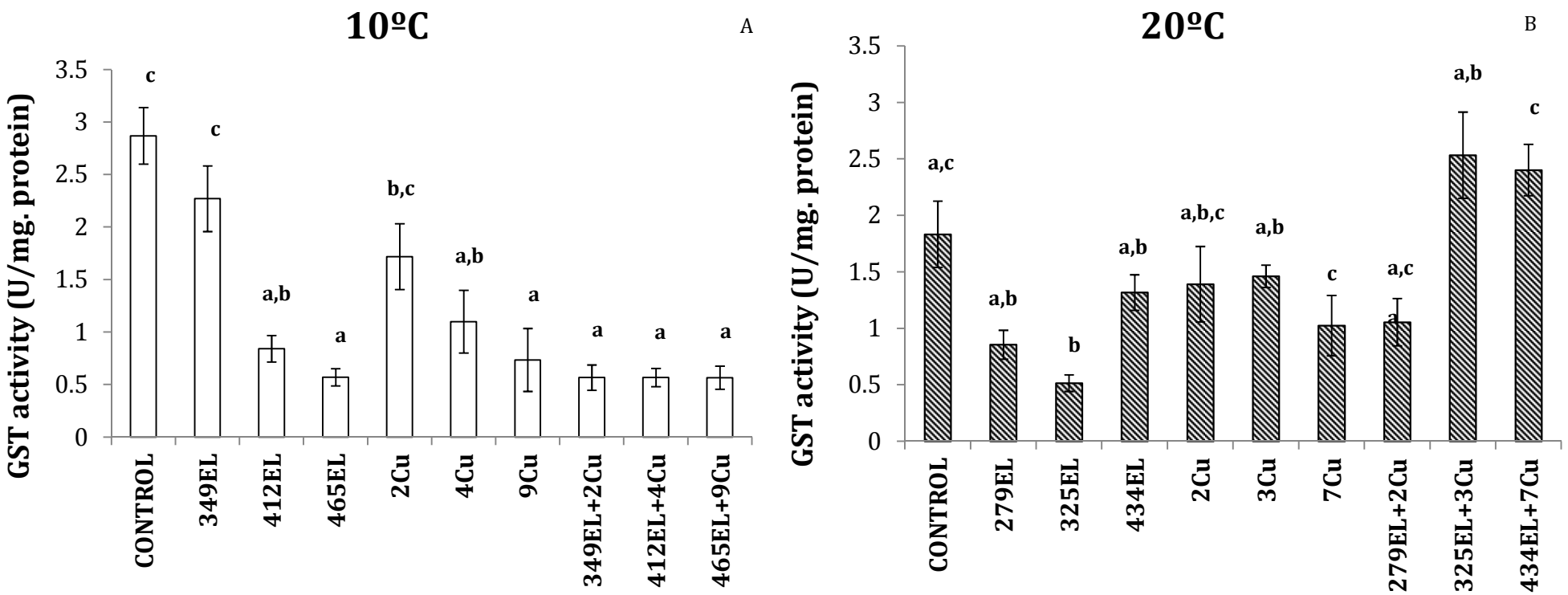

Fig. 5 - Effects of eucalyptus leachates (EL), copper (Cu), and their equitoxic mixtures (mg/L) to *Schizopelex festiva* after 96h of exposure at 10°C (A) and 20°C (B). GST – body without head glutathione *S transferases* activity. Enzymatic activities are expressed in Units (U) per concentration of protein, one U corresponding to 1 nano mole of substrate hydrolised per minute per mg of protein. Values are the means±S.E.M.; n=10. Different letters indicate statistically significant differences at 0.05 (ANOVA and Tukey's test or non-parametric equivalent). Enzymatic activities (mean±S.E.M.) in control groups at 10°C: 2.87 U/mg protein±0.0.30; Corresponding values at 20°C: 1.83 U/mg protein±0.29.

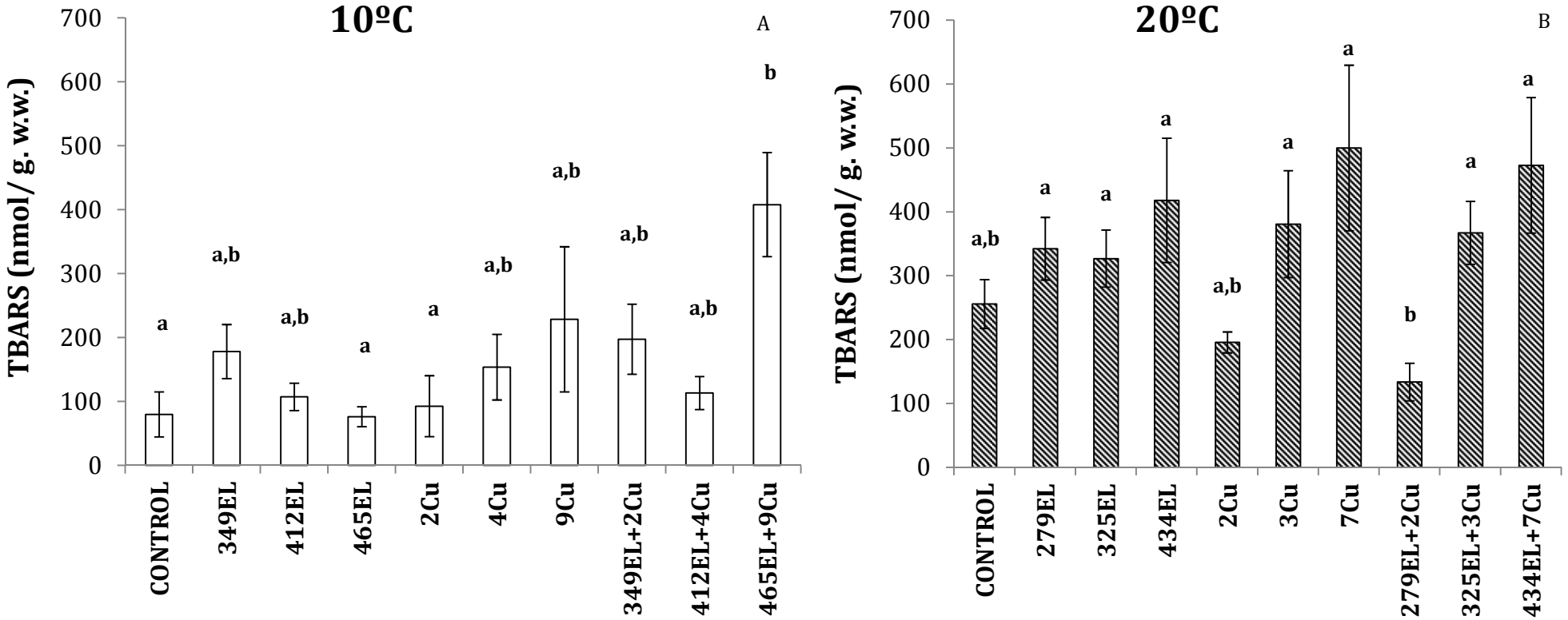

Fig. 6 - Effects of eucalyptus leachates (EL), copper (Cu), and their equitoxic mixtures (mg/L) to *Schizopelex festiva* after 96h of exposure at 10 (A) and 20°C (B). LPO - body (without head) lipid peroxidation levels. Values are the means±S.E.M.; n=10. Different letters indicate statistically significant differences at 0.05 (ANOVA and Tukey's test or non-parametric equivalent). Lipid peroxidation levels (mean±S.E.M.) in control groups at 10°C: 79.53 nmol/g. w.w.±35.24; Corresponding values at 20°C: 255.54 nmol/g. w.w.±38.29.

3.2 - Effects of stressors on Atyaephyra desmarestii.

In *A. desmarestii* bioassays, significant differences in ChE activity among treatments were found (Figure 7) at both 10°C ($H_{(9,90)}$=23.89, $p<0.05$) and 20°C ($F_{(9,89)}$= 5.21, $p<0.05$). In single exposures, EL and copper did not caused significant changes in the enzymatic activity, but a significant increase in the groups exposed to the highest concentration of the mixture was observed at both 10°C (112%) and 20°C (170%). The increase of ChE observed in the present study carried out in vivo may be due to several mechanisms including: (i) an increased release of acetylcholine to the synaptic cleft potentially induced somehow by the highest concentration of the mixture and the induction of ChE activity in an attempt of degrading the excess of the neurotransmitter avoiding the overstimulation of post-synaptic receptors; and (ii) a general increase of ChE activities to bind or hydrolyse the toxic substances in circulation and decreasing their toxic effects, a function of these enzymes that is especially important towards some compounds (Johnson and Moore, 2012; Taylor *et al*., 2013). Unfortunately our experiment design does not allow going further in this interesting question. No significant differences in ChE activity between controls of bioassays carried out at 10 and 20°C were found ($T=-0.77$; $p>0.05$) suggesting that *A. desmarestii* ChE activity is not affected by temperature variation in the 10-20°C range.

At 10°C, no significant differences in GST activity were found among treatments ($F_{(9,90)}$= 1.69, $p>0.05$), suggesting no involvement of this enzyme in the biotransformation of the tested substances at this temperature (Figure 8). The raise of temperature from 10 to 20°C (Figure 8B) did not significantly increased the GST activity in the control group ($T=0.78$; $p>0.05$). However, at 20°C,

significant differences in GST activity among treatments were found ($F_{(9,90)}$= 1.69, p>0.05), with the highest concentration of the mixture causing a reduction of the enzymatic activity (≈54%). This suggests that GST may be binding to the toxicants or their metabolites resulting in the decrease of its activity.

At 10°C, exposure to EL, copper and their mixtures significantly ($F_{(9,90)}$= 4.98, p<0.05) increased LPO levels (Figure 9A) suggesting that lipid peroxidation damage occurred in *A. desmarestii*. The raise of temperature from 10 to 20°C significantly increased the levels of LPO in controls (T=-7.81; p>0.05) (Figure 9B). However, despite the overall increase of LPO levels, no significant differences among treatments were found ($H_{(9,90)}$= 13.15, p>0.05).

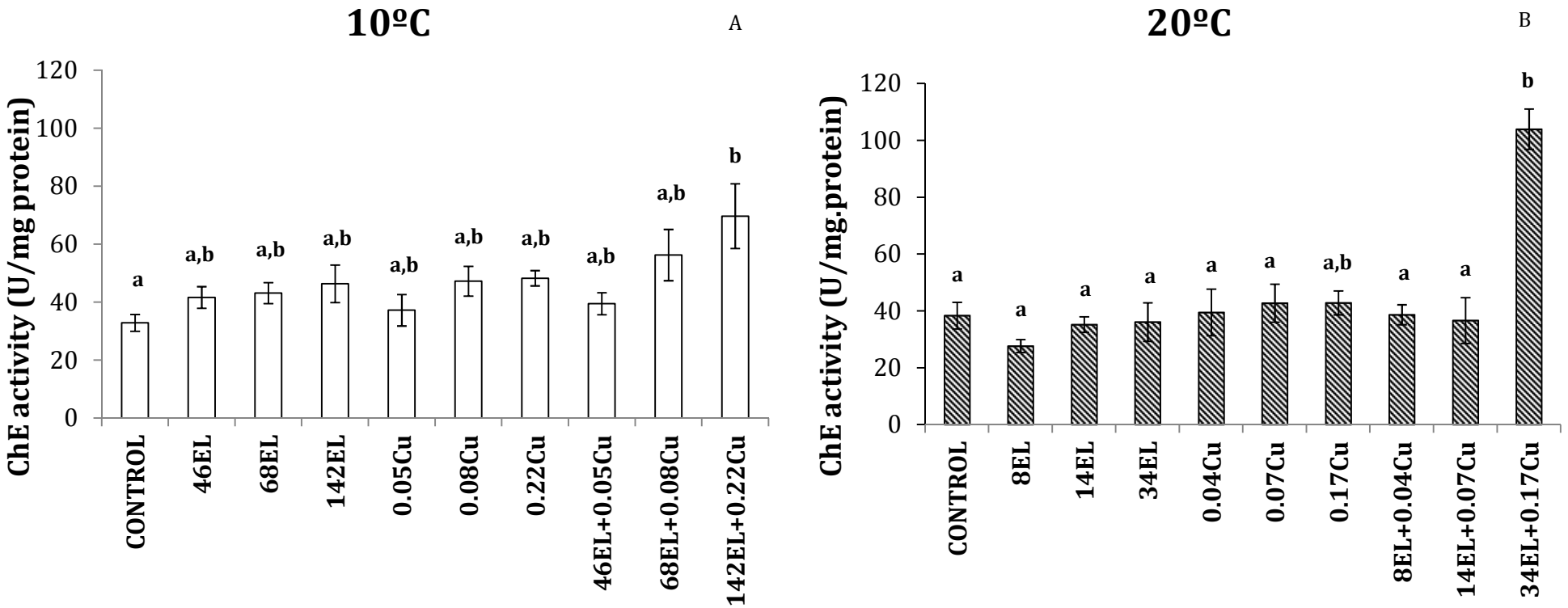

Fig. 7 - Effects of eucalyptus leachates (EL), copper (Cu), and their equitoxic mixtures (mg/L) to *Atyaephyra desmarestii* after 96h of exposure at 10 (A) and 20°C (B). ChE - head cholinesterase activity. Enzymatic activities are expressed in Units (U) per concentration of protein, one U corresponding to 1 nano mole of substrate hydrolised per minute per mg of protein. Values are the means±S.E.M.; n=10. Different letters indicate statistically significant differences at 0.05 (ANOVA and Tukey's test or non-parametric equivalent). Enzymatic activities (mean±S.E.M.) in control groups at 10°C: 26.53 U/mg protein±1.82; Corresponding values at 20°C: 15.40 U/mg protein±0.78.

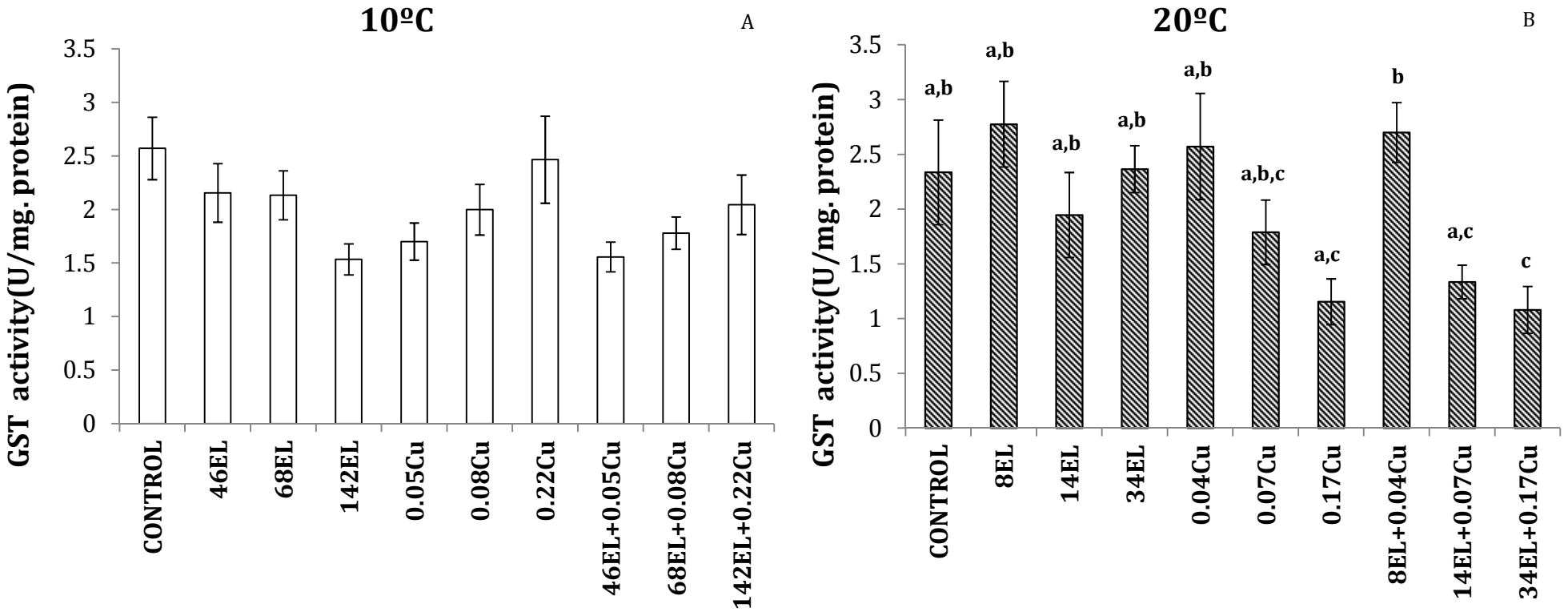

Fig. 8 - Effects of eucalyptus leachates (EL), copper (Cu), and their equitoxic mixtures (mg/L) to *Atyaephyra desmarestii* after 96h of exposure at 10 (A) and 20°C (B). GST – body without head glutathione *S transferases* activity. Enzymatic activities are expressed in Units (U) per concentration of protein, one U corresponding to 1 nano mole of substrate hydrolised per minute per mg of protein. Values are the means±S.E.M.; n=10. Different letters indicate statistically significant differences at 0.05 (ANOVA and Tukey's test or non-parametric equivalent). Enzymatic activities (mean±S.E.M.) in control groups at 10°C: 2.57 nmol/min/mg protein±0.29; Corresponding values at 20°C: 2.34 nmol/min/mg protein±0.48.

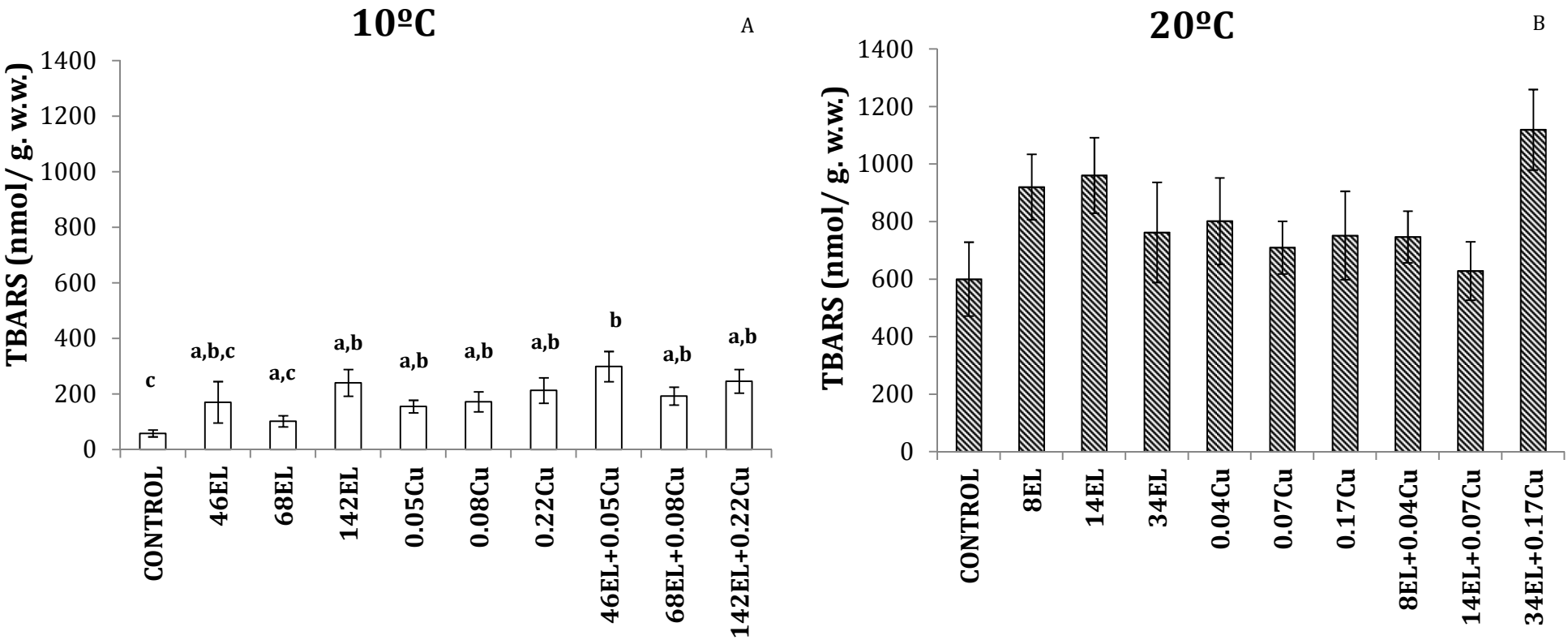

Fig. 9 - Effects of eucalyptus leachates (EL), copper (Cu), and their equitoxic mixtures (mg/L) to *Atyaephyra desmarestii* after 96h of exposure at 10 (A) and 20°C (B). LPO - body (without head) lipid peroxidation levels. Values are the means±S.E.M.; n=10. Different letters indicate statistically significant differences at 0.05 (ANOVA and Tukey's test or non-parametric equivalent). Lipid peroxidation levels (mean±S.E.M.) in control groups at 10°C: 58.35 nmol/g. w.w.±12.25; Corresponding values at 20°C: 600.06 nmol/g. w.w.±128.48.

3.3 - Effects of stressors on Echinogammarus meridionalis

At 10°C (Figure 10A), no significant differences in ChE activity were found ($H_{(9,30)}$=14.74, p>0.05) indicating that at this temperature the environmental contaminants tested, either as single agents or in mixture, have no anti-cholinesterase effects. The raise of temperature from 10 to 20°C did not significantly altered ChE activity in controls (T=-0.82; p>0.05). However, at 20°C (Figure 10B), significant differences among treatments were found ($H_{(9,30)}$=28.22, p<0.05), with the highest concentration of EL and of the mixture causing a significant reduction of ChE activity (69% and 72%, respectively). These results suggest an interaction between temperature and EL effects.

Significant differences in GST activity among treatments were found both at 10°C ($F_{(9,30)}$= 6.10, p<0.05) and 20°C ($F_{(9,30)}$=2.65, p<0.05), and in LPO levels at 10°C ($F_{(9,30)}$= 4.596, p<0.05) but not at 20°C ($F_{(9,30)}$=1.70, p>0.05). At 10°C (Figure 11A), EL when tested alone significantly decreased GST activity by about 78% at all the concentrations tested. A significant decrease of GST relatively to control values was also induced by the lowest concentration of copper (0.05 mg/L) and the lowest concentrations of the mixture but a recovery of activity was observed at higher concentrations. Because a significant increase of LPO levels in organisms exposed to the highest concentration of the mixture (Figure 12A) and a slightly increase in those exposed to the highest concentration of copper, it is possible that the GST activity increase was an attempt to cope with increased levels of oxidative stress. Temperature increase reduced *E. meridionalis* GST activity in the control group (T=2.58; p<0.05) and had no significant effects on LPO levels (T=-1.89; p>0.05). At 20°C, only the highest concentration of the mixture induced a

significant change of GST activity (≈ 70 % reduction) from the control group. Thus, these findings suggest that temperature change was able to modify the response to oxidative stress.

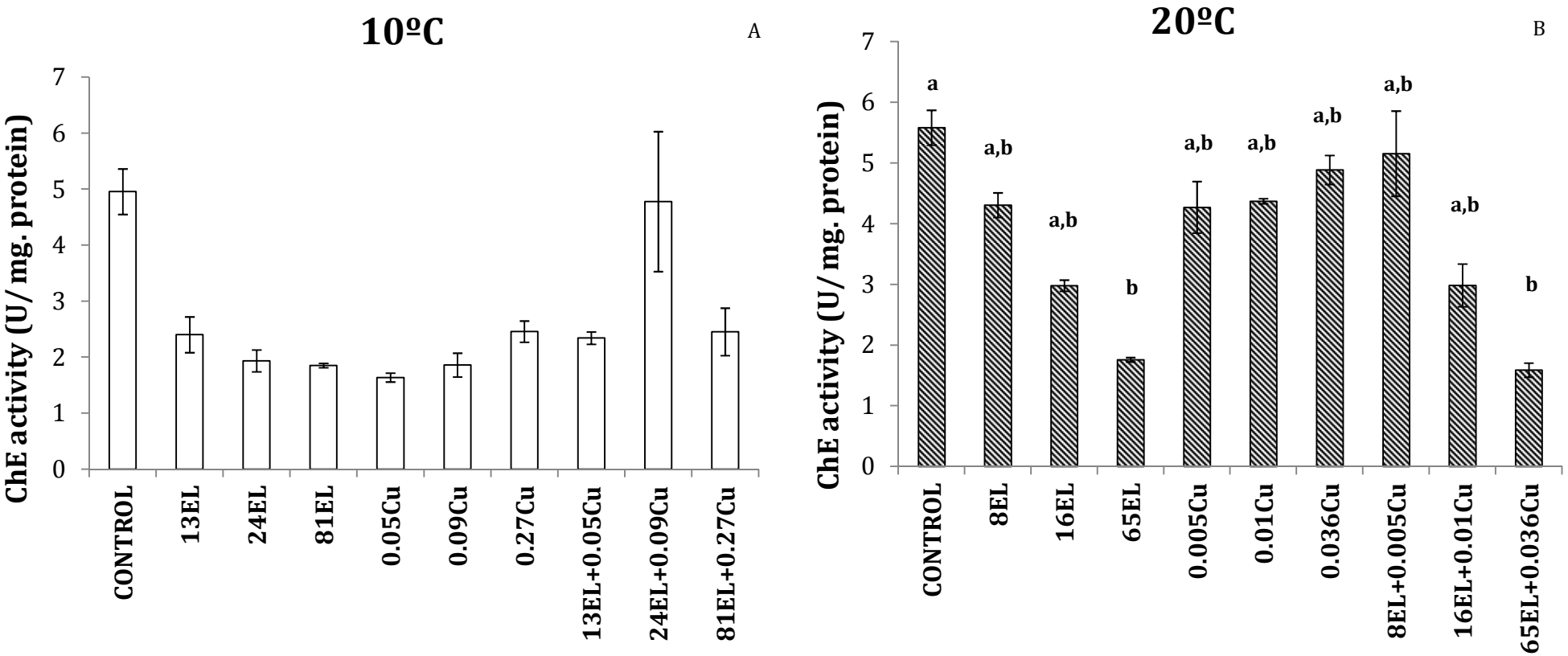

Fig. 10 - Effects of eucalyptus leachates (EL), copper (Cu), and their equitoxic mixtures (mg/L) to *Echinogammarus meridionalis* after 96h of exposure at 10 (A) and 20°C (B). ChE - head cholinesterase activity. Enzymatic activities are expressed in Units (U) per concentration of protein, one U corresponding to 1 nano mole of substrate hydrolised per minute per mg of protein. Values are the means±S.E.M.; n=10. Different letters indicate statistically significant differences at 0.05 (ANOVA and Tukey's test or non-parametric equivalent). Enzymatic activities (mean±S.E.M.) in control groups at 10°C: 4.95 U/mg protein±0.41; Corresponding values at 20°C: 5.58 U/mg protein±0.29.

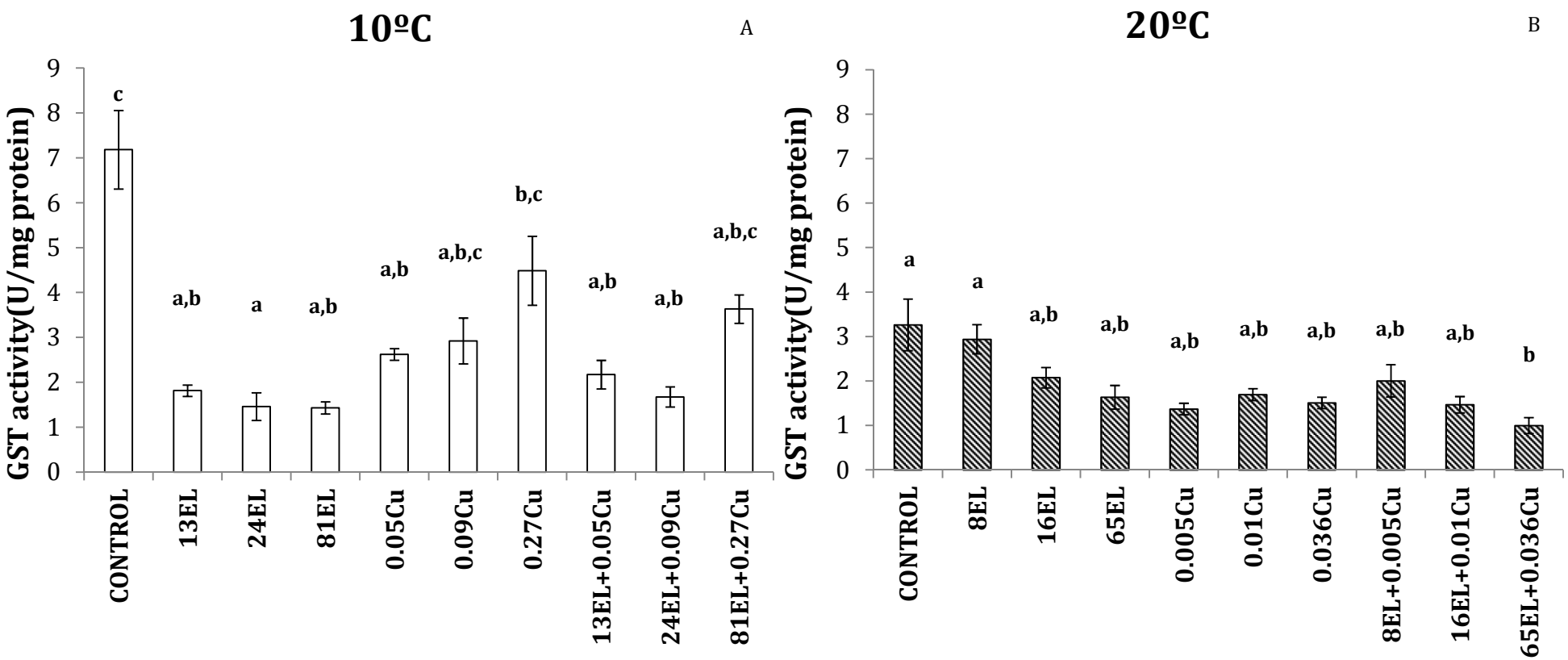

Fig. 11 - Effects of eucalyptus leachates (EL), copper (Cu), and their equitoxic mixtures (mg/L) to *Echinogammarus meridionalis* after 96h of exposure at 10 (A) and 20°C (B). GST – body without head glutathione *S transferases* activity. Enzymatic activities are expressed in Units (U) per concentration of protein, one U corresponding to 1 nano mole of substrate hydrolised per minute per mg of protein. Values are the means±S.E.M.; n=10. Different letters indicate statistically significant differences at 0.05 (ANOVA and Tukey's test or non-parametric equivalent). Enzymatic activities (mean±S.E.M.) in control groups at 10°C: 7.18 U/mg protein±0.87; Corresponding values at 20°C: 3.26 U/mg protein±0.58.

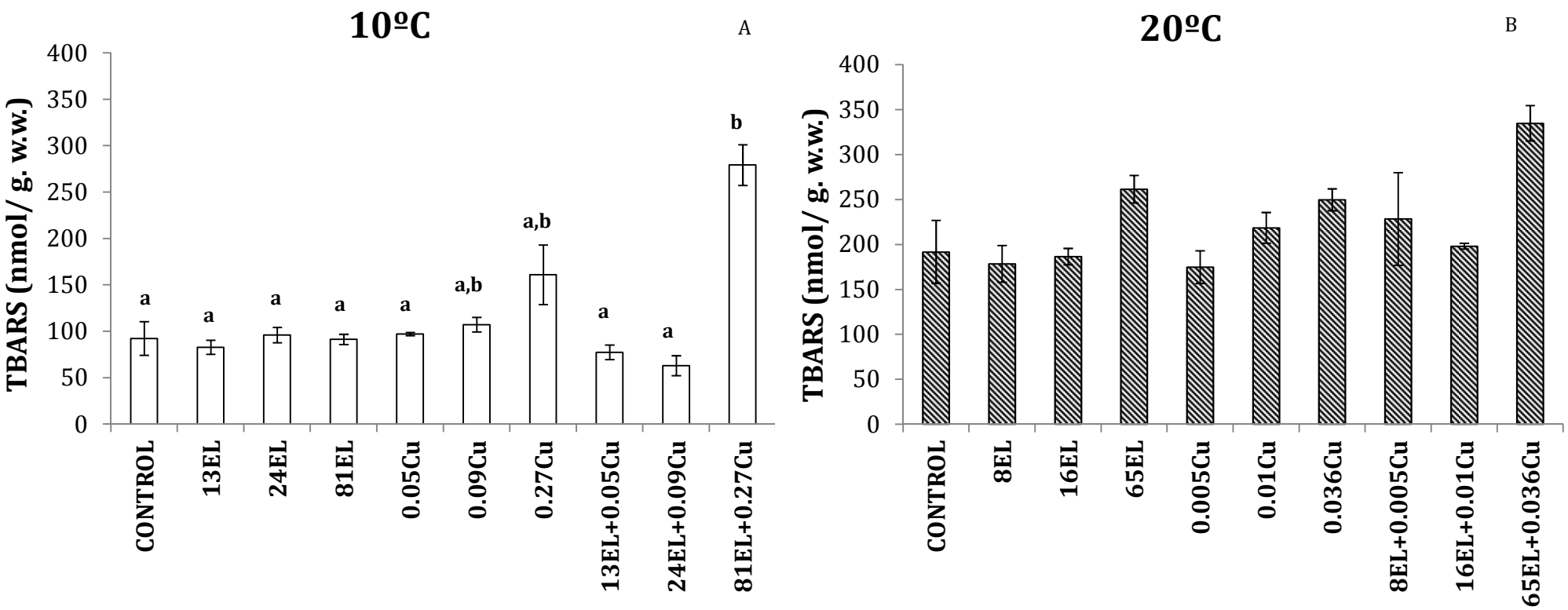

Fig. 12 - Effects of eucalyptus leachates (EL), copper (Cu), and their equitoxic mixtures (mg/L) to *Echinogammarus meridionalis* after 96h of exposure at 10 (A) and 20°C (B). LPO - body (without head) lipid peroxidation levels. Values are the means±S.E.M.; n=10. Different letters indicate statistically significant differences at 0.05 (ANOVA and Tukey's test or non-parametric equivalent). Lipid peroxidation levels (mean±S.E.M.) in control groups at 10°C: 92.27 nmol/g. w.w.±18.13; Corresponding values at 20°C: 191.64 nmol/g. w.w.±34.94.

Conclusions

In summary, the results of the present study indicate that temperature raise from 10 to 20°C significantly reduced ChE activity in *S. festiva*, GST activity in *S. festiva* and *E. meridionalis*, and increased LPO levels in *S. festiva* and *A. desmarestii* (control organisms). Thus, these effects should be taken in consideration when using these parameters as biomarkers under temperature variation scenarios.

The environmental contaminants tested here were not able to induce significant anti-cholinesterase effects on *S. festiva*; the lipid oxidative damage was only found at one mixture concentration and GST seems to have an effective role in protecting against oxidative stress possibly by binding to the toxicants or their metabolites. *A. desmarestii* ChE were significantly increased by exposure to the highest mixture concentration at both 10 and 20°C. Both EL and copper (single and in mixture) were able to significantly increase LPO levels at 10°C but not at 20°C.

This suggest that this species is more sensitive to chemical stress at the lowest temperature tested, possible because at higher temperatures GST may play an important role in preventing oxidative damage. The highest concentration of EL and of the mixture caused a significant inhibition of ChE in *E. meridionalis* at 20°C but not at 10°C, and significant oxidative damage in the mixture, at the lowest temperature but not at the highest one. Overall, these findings indicate that the tested shredder species have different sensitivities to chemically-induced acute stress and at least some of the mechanisms of toxicity and detoxication involved are modulate by temperature.

Acknowledgments:

We thank to Dr. C. Gravato all the technical support provided to M. Gama during the initial phase of the work.

Chapter IV

Chronic toxicity of eucalyptus leaf leachates and copper to *Schizopelex festiva* and *Echinogammarus meridionalis* assessed at two distinct temperatures

Chronic toxicity of eucalyptus leaf leachates and copper to *Echinogammarus meridionalis* and *Schizopelex festiva* assessed at two distinct temperatures

Abstract

In this study, the single or combined exposure to eucalyptus leaf leachates and copper were assessed on growth, consumption and survival rates of the shredders *Schizopelex festiva* and *Echinogammarus meridionalis,* at two different temperatures (10 and 20°C). Elemental body composition and selected biomarkers (ChE, GST and LPO) were also evaluated on *S. festiva*. Temperature significantly accelerated growth rates for *S. festiva* and the presence of toxics determined lower intrinsic growth rates (at both temperatures) and lower final invertebrate's size especially at 10°C. No effects on survival were detected. For *E. meridionalis* the presence of low amounts of toxics increased growth rates only at 10°C. The increase in temperature accelerates growth rates in the absence of the toxics. Survival was negatively affected by increased temperature. Stressors exposure (single and in combination) negatively affected survival rates especially at the highest temperature. Elemental body composition indicates that *S. festiva* ability to retain phosphorus may be compromised upon exposure to higher temperature in treatments with increased copper concentrations. Biomarker determination suggests that exposure to increased temperature and the presence of high amount of leachates or the combination of both toxics leads to ChE inhibition. Increasing oxidative damage (increased LPO levels), especially at 20°C, and GST inhibition (in

the presence of single copper treatments) was also detected. Overall results suggest that long term contamination of freshwaters by eucalyptus leaf leachates and copper may result in distinct responses by the invertebrate species present and may lead to imbalances in invertebrate's physiological mechanisms, with possible ecological relevant consequences in species abundance or presence. To this it is important to highlight the negative impact of increased temperatures on these processes.

Keywords: eucalyptus, copper, shredders, growth, stoichiometry, biomarkers

1. INTRODUCTION

Due to increased industrialization, urbanization, overexploitation of resources and increased human activities, contamination of inland waters by heavy metals such as copper has rapidly spread (Malmqvist and Rundle, 2004; Valavanidis and Vlachogianni, 2010). Copper is an essential trace metal but can become a severe pollutant depending on the dosage. Being one of the most used chemicals in the world in agricultural fields (as herbicides/fungicides) (ATSDR, 1990), acute copper toxicity to freshwater organisms has been broadly studied (Van der Geest *et al.,* 2000; Milam *et al*, 2005; Evans *et al*., 2006; Solá and Prat, 2006, Vutukuru *et al*., 2006; Knakievicz and Ferreira, 2008). Nonetheless the effects of long-term, chronic Cu exposure are comparatively less reported in freshwater environments (e.g. Roman *et al*., 2007; Brix *et al*., 2011). Increased afforestations with *Eucalyptus globulus* have expanded throughout the Mediterranean area, being this tree species currently widespread in continental Portugal (IFN6, 2013) with monocultures lining headwaters or forming more or less dense riparian corridors in streams draining agricultural areas or under the influence of copper contamination. Several studies have reported deleterious impacts of eucalyptus plantations on shredders (Canhoto and Graça, 1999; Canhoto and Laranjeira, 2007; Larrañaga *et al*., 2009; Villanueva *et al*, 2011), namely on the negative effects of exposure to eucalyptus leaf leachates (Canhoto *et al*., 2013) but a lack of knowledge on the effects of long term chronic toxic exposure to these invertebrates still exists.

Invertebrates tend to present a homeostatic elemental body composition (Sterner and Elser, 2002; Karimi and Folt, 2006) inspite of recognised imbalances

between these consumers and their resources (Cross *et al*., 2003). Studies with shedders tend to corroborate that theory (Balseiro and Albariño, 2006). Nevertheless little is known on this ability to cope with such imbalances when facing multiple stressors, with temperature possibly having an important influence in the process (Persson *et al*., 2011; Wojewodzic *et al*., 2011; Villanueva *et al*., 2011).

In recent years, growing importance of evaluating stressor exposure effects between different levels of biological organization lead to the use of different endpoints. In this scenario, combining the determination of functional parameters and the measurement of biochemical endpoints such as biomarkers or the understanding of stoichiometrical relationships may aid in understanding the effects of these stressors. Therefore it seems relevant to establish the relationship between ecological relevant parameters (such as growth, consumption rates and mortality), biomarkers and invertebrates homeostatic capacity in the presence of multiple stressors (copper and eucalypt leachates) and relevant thermal scenarios. In the last few years studies have positively demonstrated the relationship between ecologically relevant parameters and biomarkers (Moreira *et al*. 2006; Gravato and Guilhermino, 2009) focussing the attention to the importance in exploiting this relationships.

The purpose of this study was to determine the effects of long-term, chronic eucalyptus leaf leachates and copper sub-lethal exposures on two shredders (*Schizopelex festiva* and *Echinogammarus meridionalis*). Survival, growth, and feeding rates of *E. meridionalis* and *S. festiva* were determined at two common temperatures, 10 and 20°C, usually reported in South Europe streams in autumn

and summer, respectively. The impacts of these stressors on the biological state of the individuals assessed through their elemental body composition (Carbon (C), Nitrogen (N) and Phosphorus (P)) and three selected biomarkers were also determined for *S. festiva*. Biomarkers determined where: Cholinesterases (ChE), which are involved in neurotransmission in vertebrates and invertebrates (Guilhermino *et al*., 1998); the activity of glutathione *S*-transferases (GST) involved in biotransformation and prevention of lipid peroxidation; and lipid peroxidation levels (LPO) to assess the extent of oxidative damage.

2. MATERIAL AND METHODS

2.1 - Collection and acclimation of organisms

Schizopelex festiva Rambur (Trichoptera) and *Echinogammarus meridionalis* Pinkster (Amphipoda) individuals were collected in the wild in Ribeira de Múceres (Caramulo, central Portugal; 40°32'01''N, 8°09'15''W) and Ribeira da Redinha (Pombal, central Portugal; 39°58.726'N, 8°34.393'W), respectively. Both shredder specimens were handpicked and brought back to the laboratory in containers filled with stream's water. In the laboratory, invertebrates were transferred to 5L aquariums filled with reconstituted hard water (alkalinity 110 to 120 mg/L as $CaCO_3$ and hardness 160 to 180 mg/L as $CaCO_3$; ASTM 1980), in order to minimize potential stress to invertebrates. During acclimation to assay conditions (12h light: 12h dark photoperiod; 10 or 20°C) organisms were maintained in continuous aeration, with a layer of 5cm of ashed sediment (550°C for 8h) and fed *ad libitum* with alder leaves conditioned for 3 weeks in Ribeira de S. João (Lousã, central Portugal; 40°11'N; 8° 25'E) in order to assure optimal fungal colonization.

2.2 - Preparation of stock and test solutions

Eucalyptus leaf leachates were obtained from senescent eucalyptus leaves collected from the same stand of trees in late summer of 2009, dried and stored in the dark until needed. To prepare leaf extracts, a mass of 28g of dried eucalyptus leaves were used per liter of ASTM (Canhoto and Laranjeira, 2007); the water was continuously aerated for 7 days under laboratory conditions. The leachate solution was then decanted and stored at 4°C in closed bottles, in the dark. Dissolved organic carbon (DOC) (Elementar Analysensysteme Gmbh LiquiTOC, Hanau, Germany), tannic acid equivalents (Graça *et al.,* 2005), dissolved oxygen (WTW OXI 92 oxygen meter), pH (Wissenschaftlich Technische Werkstätten 537 pH meter, WTW, Weilheim, Germany), and conductivity (WTW LF 92 conductivity meter) of the leachates were determined prior to the beginning of the bioassays. Test solutions of eucalyptus leachates were further prepared by serial dilutions of the leachates stock solution with ASTM.

To obtain copper solutions a stock solution of copper sulfate pentahydrate (CAS no. 7758-99-8 purchased from Merck KGaA , Darmstadt, Germany) was prepared in nano pure water (conductivity <5 µS/cm; Seralpur PRO 90 CN, Seral, Ransbach-Baumbach, Germany) with a concentration of 25.5 mg/L (ionic Cu). Test solutions of copper were prepared by serial dilutions of the stock solution in ASTM. Mixture test solutions of eucalyptus leachates and copper were also prepared by adding the correspondent copper stock solution to the respective leachates stock solution adjusted for final intended volume with ASTM.

2.3 - Experimental design and exposure conditions

In chronic tests, eucalyptus leaf leachates and copper concentrations used are reported in Table 6 and were determined based on LC10 values previously determined for individual substances (chapter II). Invertebrates of each species were exposed to 2 distinct concentrations of single copper or eucalyptus leachates (with concentrations equivalent to previously determined LC10 concentrations, and half of that concentration), one treatment with both toxics in mixture (with half the concentration of each toxic) and one treatment with only ASTM which acted as a control. Hereafter these treatments are designated as control; ½Cu; Cu; ½EL, EL and MIX (½EL+½Cu). Tests were run at 10 and 20°C simultaneously. Treatments with EL were not corrected for pH in order to mimic natural conditions.

A total of 180 invertebrates with the exact same size (*S. festiva* and *E. meridionalis* mean dry weight±S.E.M.: 3.54mg±0.000 and 0.14mg±0.000, respectively), randomly distributed into 6 groups (control; ½Cu; Cu; ½EL, EL and MIX) of 30 animals were exposed individually in test chambers (plastic cups) filled with 200 ml of each solution at 10 and 20°C. Each individual was provided with 2 leaf disks (Ø=14mm) of conditioned alder. Leaf disks were pre-weighed (105°C; 24h) to the nearest 0.01mg and rehydrated with distilled water before being offered to the invertebrates. Food, water and sediment were changed weekly, when animals were measured. Survivorship was evaluated on a daily basis and registered once a week. *S. festiva* dry mass (DM, mg) was obtained by measuring the anterior case opening (CO, mm) and calculated from the expression: $DM = 0.5177e^{(0.0961 * CO)}$ previously obtained (r^2=0.95; n=50).The maximum body length

of *E. meridionalis* live individuals (BL; mm) was used to determine the dry mass (DM; mg) according to the expression previously obtained: DM=0.1633BL – 0.02606 (r^2=0.95; n=18). The experiment ended when the first *S. festiva* in each temperature was found in a pupal stage and when *E. meridionalis* individuals reached the final size class (size class 7, from 12 to 14 mm total body length).

Growth rate of individual larvae was taken as the slope of exponential regressions of size (dry mass) against time (k). No values after maximum growth rate were used for this purpose. Individual consumption (mg) was estimated as the difference between the initial and final dry mass of the leaves (105°C; 24h), corrected from controls. Consumption was evaluated during week 7 and expressed per mg dry mass of individual, per day. At the end of the growth experiments one third of the 30 speciments of *S. festiva* were preserved at -80°C and another third (10 specimens) dried (at 60°C for 48h) and stored, in order to further determine selected biomarkers and stoichiometrical composition. Specimens of *E. meridionalis* were excluded from these determinations because, as each replicate had only one invertebrate, this did not allow the enough protein content or animal content (after desiccation) necessary to further analyses (biomarkers and stoichiometry).

Table 6 - Eucalyptus leachates (EL) and copper (Cu) concentrations used in the chronic toxicity experiments with *Schizopelex festiva* and *Echinogammarus meridionalis* at 10°C or 20°C. Concentrations expressed in mg /L.

Temperature	Stressor concentration (mg/L)	*S. festiva*	*E. meridionalis*
10°C	**½ EL**	174.24	6.45
	EL	348.48	12.91
	½ Cu	1.15	0.02
	Cu	2.30	0.05
	MIX	174.24 +1.15	6.45 +0.02
20°C	**½ EL**	136.92	3.97
	EL	273.84	7.93
	½ Cu	0.92	0.003
	Cu	1.84	0.005
	MIX	136.92 +0.92	3.97 +0.003

2.3 - Elemental composition and biomarker determination

2.3.1 - Elemental composition

At the end of the growth experiments organisms were dried (24h at 105°C), milled and further analysed for total N and C (Flash EA 1112 for IRMS Delta V Advantage ThermoIRMS). Total P was evaluated according to Graça *et al*. (2005). Results were expressed as percentage and mass ratios (C:N; C:P and N:P) calculated for each combination temperature/treatment.

2.3.2 - Biomarker determination

In order to determine ChE activity, invertebrate's heads were used; the remaining bodies were used for the evaluation of GST activity and LPO levels. For ChE, samples were placed in 0.5 ml of ice cold phosphate buffer (0.1 M, pH 7.4), homogenized for 30s (Ystral GmbH d-7801 Dottingen homogeniser) on ice, and centrifuged at 3300×g for 3min at 4°C (SIGMA 3 K 30). The supernatants were collected and ChE activity was determined after standardization of protein content (1 mg/mL) following the methodology of Ellman's (Ellman *et al*., 1961) adapted to microplate (Guilhermino *et al.,* 1996). Therefore to each 0.050ml of the supernatant homogenate, 0.250 ml of the reaction solution was added (30ml of phosphate buffer (pH=7.2, 0.1M), 1 ml of a dithiobisnitrobenzoate 10 mM solution (20 mM of acid dithiobisnitrobenzoate and 18 mM of sodium hydrogen carbonate in 0.1 M K-phosphate buffer, pH 7.2), and 0.2 mL of acetylcholine iodide in u.p. water (75 mM)). Increased absorbance was measured at 412 nm in a microplate reader (Bio Tek Power Wave 340) at 25°C. Acetyltiocholine was used in all determinations and the activity determined corresponds to the substrate hydrolysis made by all the ChE enzymes.

The remaining body (i.e. without the heads) samples were homogeneized for 30s on ice (Ystral GmbH d-7801 Dottingen homogeniser) in 1ml of ice cold phosphate buffer (0.1 M, pH 7.4) and further centrifuged (0.8 ml) (SIGMA 3 K 30) at 10000× g for 20 min at 4 °C. GST activity was quantified by the method of Habig *et al.* (1974) with adaptations (Frasco and Guilhermino, 2002) by the conjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB). To 0.250 mL of the reaction solution (75 mL of phosphate buffer 0.2 M pH 6.5, 2.34 mL of 1-

chloro-2,4 dinitrobenzene in ethanol and 13.5 mL of a 10 mM GSH solution in ultra-pure water) was added 0.05ml of whole body PMS previously diluted in homogenization buffer in order to have a final protein concentration of 1 mg/mL. Absorbance was measured in a microplate reader (Bio Tek Power Wave 340) at 340 nm for 5min at 25°C.

Enzimatic activities (ChE and GST) were expressed per Units (U) per concentration of protein, one U corresponding to 1 nano mole of substrate hydrolised per min per mg of protein determined according Bradford *et al*. (1976) adapted to microplate (Frasco *et al*., 2002) using bovine γ-globulin as protein standard.

The remaining body homogenate (0.2ml) was used to determine the extent of endogenous LPO by measuring the thiobarbituric acid reactive species (TBARS) following the method described by Ohkawa *et al*. (1979) and Bird and Draper (1984), with some modifications proposed by Torres *et al*. (2002). Artifactual lipid oxidation was prevented by adding 0.2 mM butylhydroxytoluene (BHT) to the homogenate. To each sample (0.2ml), 1 mL of 12% trichloroacetic acid, 0.8 mL of 60 mM Tris–HCl solution (pH 7.4) with 0.1 mM diethylenetriaminepentaacetic acid (DTPA) and 1 mL of 0.73% thiobarbituric acid (TBA) were added. Samples were incubated in a water bath at 100°C for 60 min. Then, centrifuged at 12000×g for 5 min at 4°C and the resultant supernatant was used to determine LPO levels at 535 nm (Jasco V-630 spectrophotometer) and expressed in nmol TBARS per gram of wet weight.

2.4 - Data analysis

All data was previously checked for normality, using the Kolmogorov–Smirnov test, and for homoscedasticity, using the Levene's test, and appropriated data transformations (log x+1) were made whenever necessary (Zar, 1999). Data was always corrected for mortality. Larval growth, consumption rates, stoichiometrical composition and biomarkers among treatments were compared by one-way analysis of variance (ANOVA) and the Tukey's test was used to identify significantly different treatments. When the ANOVA assumptions could not be achieved, the Kruskal-Wallis analysis was peformed and the multiple comparisons test was used to identify significantly different treatments. Survivorship of the sets of individuals was compared using ANCOVA with temperature and treatment as categorical factors and time as covariable. Invertebrates in treatment control were compared between temperatures using t-tests. StatSoft\Statistica 8® software package was used for all the analysis. In all cases, the significance level was 0.05.

3. RESULTS AND DISCUSSION

3.1 - Chronic toxicity of eucalyptus leaf leachates and copper to S. festiva and E. meridionalis

At 10°C *S. festiva* growth rates (Figure 13) were significantly different between treatments (ANOVA, $F_{(5,174)}$=6.48, p<0.05). The presence of the toxicants determined lower growth rates when compared to control (Tukey's test p<0.05). At 20°C there were also significant differences between treatments (ANOVA, $F_{(5,174)}$=10.97, p<0.05). Invertebrates in control had significantly higher growth rates than in treatments ½Cu, Cu and ½EL (Tukey's test p<0.05), growth rates of invertebrates in treatment Cu were also significantly different from EL (Tukey's test p<0.05) that had higher growth rates. Treatment ½EL was significantly different from EL and MIX (Tukey's test <0.05). Daily growth rates were, at 10°C, (mean±Se) in each treatment (control, ½Cu, Cu, ½EL, EL and MIX): 0.240±0.009, 0.194±0.013, 0.210±0.014, 0.177±0.010, 0.191±0.007, 0.194±0.010 mgDM gained/day. Corresponding values at 20°C where: 0.347±0.023, 0.247±0.006, 0.279±0.008, 0.245±0.007, 0.308±0.009 and 0.357±0.018 mgDM gained/day.

The increase in temperature significantly affected growth rates of invertebrates in control treatment (t-test p<0.05) with fastest growth rates at 20°C probably as a consequence of increased metabolic rates due to increased rearing temperature (Brown *et al.*, 2004b). At low temperatures the presence of toxics seems to decrease intrinsic growth rates as all treatments have lower growth rates than control. Invertebrate's final size at 10°C was significantly different between treatments (Kruskal-Wallis test H=18.81, p<0.05).

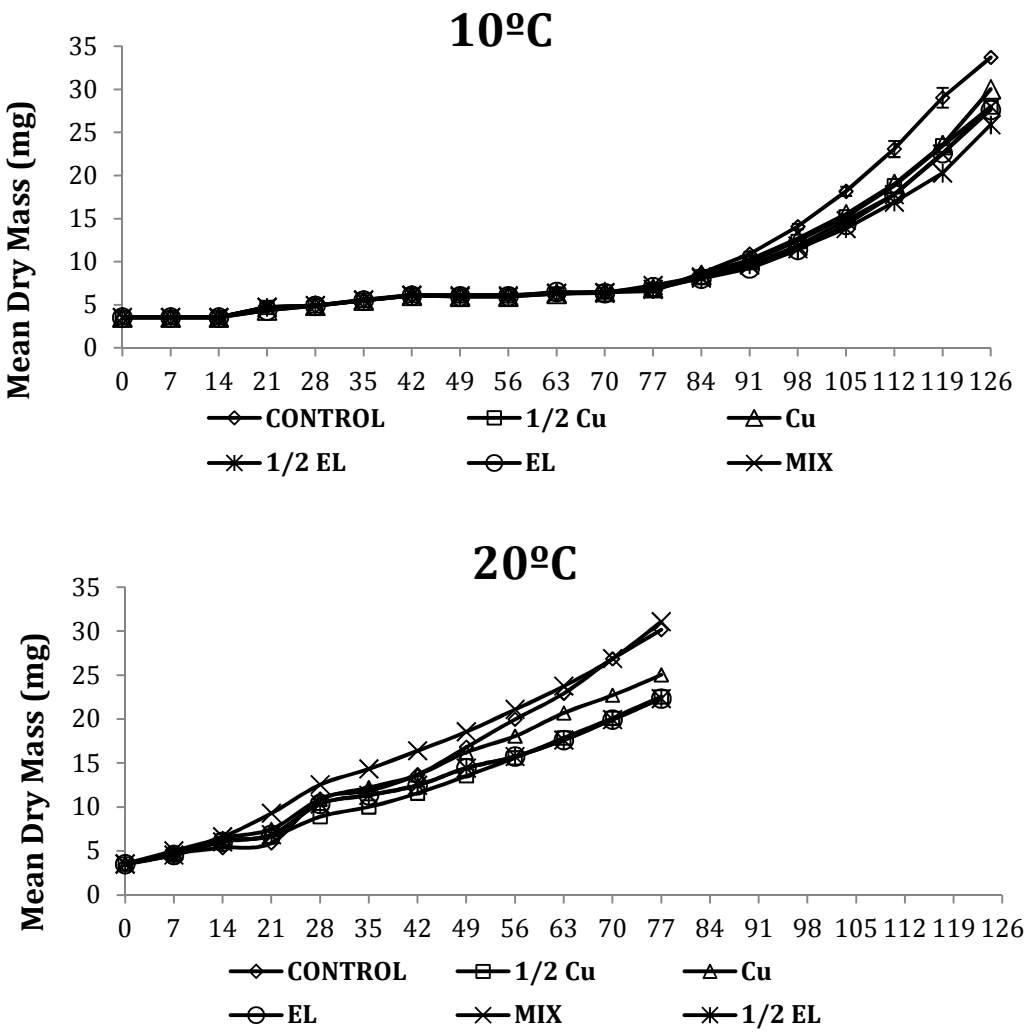

Fig. 13 - Mean dry mass of *Schizopelex festiva* (n=180; corrected for mortality) at two different temperatures 10 and 20°C, for a maximum period of 126 days. Values are means±S.E.M. Legend for the different treatments is displayed in the graphics..

Final dry mass in invertebrates in control treatment was significantly higher than in treatments ½Cu, ½EL, EL and MIX ($p<0.05$) which suggest that invertebrates besides growing at lower rates in the presence of the toxics achieve a final smaller size. At 20°C significant differences were also observed (Kruskal-Wallis test $H=77.67$, $p<0.05$) with invertebrates in treatments ½Cu, Cu and ½EL having significantly lower final mass than shredders in control ($p<0.05$) although, when both stressors were combined, these differences were not significant. The

different patterns of coping with the presence of the tested stressors, at high or low temperatures, may have lead to differences in growth rates and final invertebrate sizes with potential consequences at community level. Such effect is probably due to the distinct consumption observed in the presence of the toxicants alone or in mixtures. Significant differences occur both at 10°C (Kruskal-Wallis test $H=31.78$, $p<0.05$) and 20°C (Kruskal-Wallis test $H=16.67$, $p<0.05$) in consumption rates (Figure 14). At 10°C consumption rates in control and MIX were significantly higher than in ½EL ($p<0.05$); invertebrates maintained in Cu also had significantly lower consumption rates than the ones maintained in ½Cu and MIX ($p<0.05$). At 20°C consumption rates in MIX were significantly lower than ½Cu ($p<0.05$). When different temperatures are compared differences occur in invertebrates exposed to control (t-test $p<0.05$) with a decrease in consumption rates at 20°C. In fact, it was surprising to notice that an increase in temperature determined a general decrease in the consumption rates of the Trichoptera. This may be due to the fact that at both temperatures consumption was measured at week 7 when invertebrates at 20°C had twice the dry mass as the correspondent shredders at 10°C (Figure 13) therefore being more close to the last instar and pupal stage (that was observed for the first time at 20°C 21 days after the measurement of consumption rates) where a decrease in consumption is normally detected (Wagner, 1990).

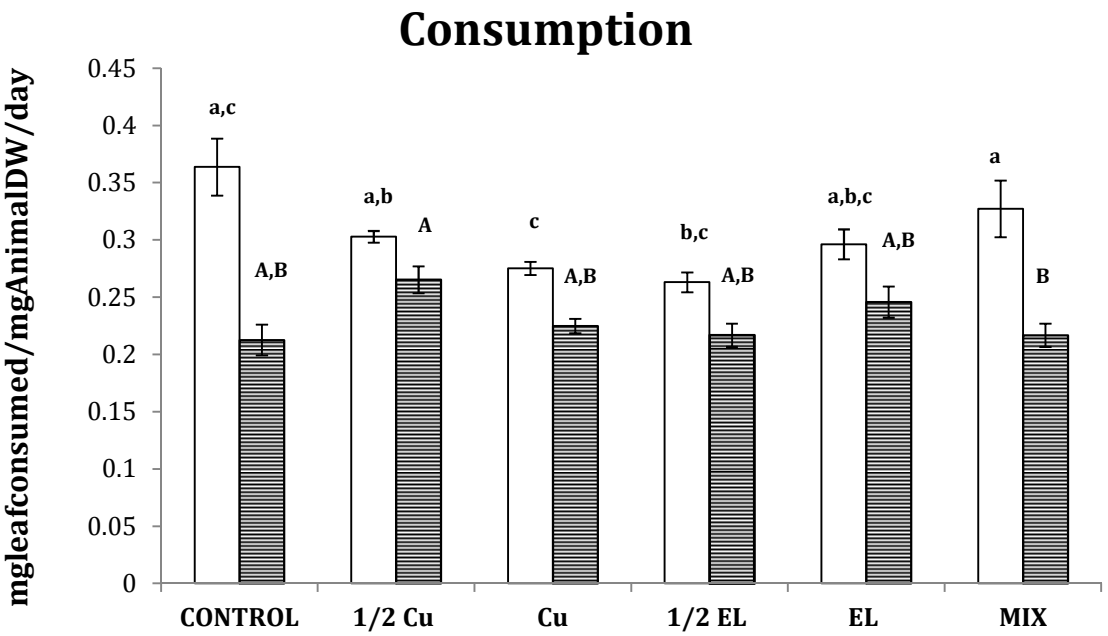

Fig. 14 - Consumption rates of *Schizopelex festiva* expressed as mg leafconsumed/mgAnimalDW/day at two different temperatures - 10°C (white bars) and 20°C (patterned bars). Values are means±S.E.M. Legend for the different treatments is displayed in the graphics. Different letters indicate statistically significant differences at 0.05 (ANOVA and Tukey's test or non-parametric equivalent) at 10°C (lowercase letters) and 20°C (uppercase letters).

The first invertebrate to reach a pupa stage belonged to treatment MIX (2 invertebrates at once) in day 77 at 20°C and at 10°C in treatment Cu in day 126. Invertebrates exposed to the highest tested temperature reached pupal stage 49 days before invertebrates reared at 10°C. This result is in accordance with those of Wagner (1990) in which larval instar duration decreased with increasing temperature for *C. villosa* (Trichoptera). Several studies suggest that increases in temperature are able to modify growth patterns in shredders, altering emergence patterns and egg hatching (Imholt *et al.*, 2009; Li *et al.*, 2011) as the water temperature threshold, contributing to the conclusion of a certain developmental stage or generational time, may be achieved earlier with consequences for community composition as suggested by Haiddeker and Hering (2008) and Li *et al.* (2009). Results in this study suggest that the fact that invertebrates reach pupal

stage earlier in treatments with stressors presence maybe due to an "earlier escape" strategy accelerated by increased temperature.

No mortality occurred in any of the treatments at 10°C. At 20°C no differences between treatments were observed (ANCOVA $F_{(5,59)}$=1.93, p>0.05). In this case, mortality occurred only after day 63 in treatments ½EL and EL and after day 70 for MIX treatments. Survival rates in these treatments were 87%, 70% and 97%, respectively. Trichopthera is a very widespread group (Cummins, 1973; Vieira Lanero, 2000) which can in part explain the resistance to increased toxics and temperature. Despite the fact that at 10°C mortality has not occurred and at 20°C no significant differences occur between treatments an increase in mortality in the highest toxics treatments is detectable in the presence of eucalyptus leachates after 63 days. Our personal observations indicate that the continuous exposure to eucalyptus leachates may determine the formation of a mucilaneous and greasy matrix that gradualy obstructs the invertebrate's case opening eventually preventing the invertebrates of actively getting out probably inhibiting consumption and O_2 acquisition (eventually contributing to their death, namely at higher temperatures). Previous studies indicate that in the presence of high leachates concentrations, *Sericostoma vittatum* growth rates and survival was negatively affected especially in unaerated conditions (Canhoto and Laranjeira, 2007; Canhoto *et al*., 2013). Although the presence of essencial oils or derivatives have been frequently pointed out as one of the reasons for waterborne toxicity, promoted by exposition of the invertebrates to the eucalyptus leachates (Canhoto and Laranjeira, 2007), the present mortality results do not support this point of

view. It seems likely that, under the used concentrations here this effect was negligible.

Growth, consumption and mortality rates were also assessed for *E. meridionalis* specimens exposed at 10 and 20°C. At 10°C *E. meridionalis* growth rates (Figure 15), were significantly different between treatments (Kruskal-Wallis test $H=22.70$, $p<0.05$): treatments ½Cu, ½EL and MIX had significantly higher growth rates when compared to control ($p<0.05$). At 20°C there were no significant differences between treatments (Kruskal-Wallis test $H=8.29$, $p>0.05$). Daily growth rates were (mean±se) in each treatment (control, ½ Cu, Cu, ½EL, EL and MIX) at 10°C: 0.023±0.000, 0.025±0.001, 0.025±0.000, 0.025±0.000, 0.024±0.000 and 0.025±0.000. Corresponding values at 20°C where: 0.028±0.000, 0.027±0.000, 0.029±0.000, 0.029±0.000, 0.029±0.000 and 0.029±0.000.

At 10°C treatments with low concentrations of toxics seem to lead to increased growth rates while at 20°C the presence of toxics did not influence growth rates. This may represent also an "escape strategy" used by the invertebrates in the presence of lower stressors concentrations, nonetheless that approach does not occur with increasing temperature or chemical concentration. When both temperatures are compared growth rates of invertebrates in control are significantly different between 10 and 20°C with higher growth rates at the highest temperature. Invertebrates reared at 20°C reached the final size class 14 days before invertebrates reared at 10°C which may be the result of high metabolic rates in the presence of high temperatures.

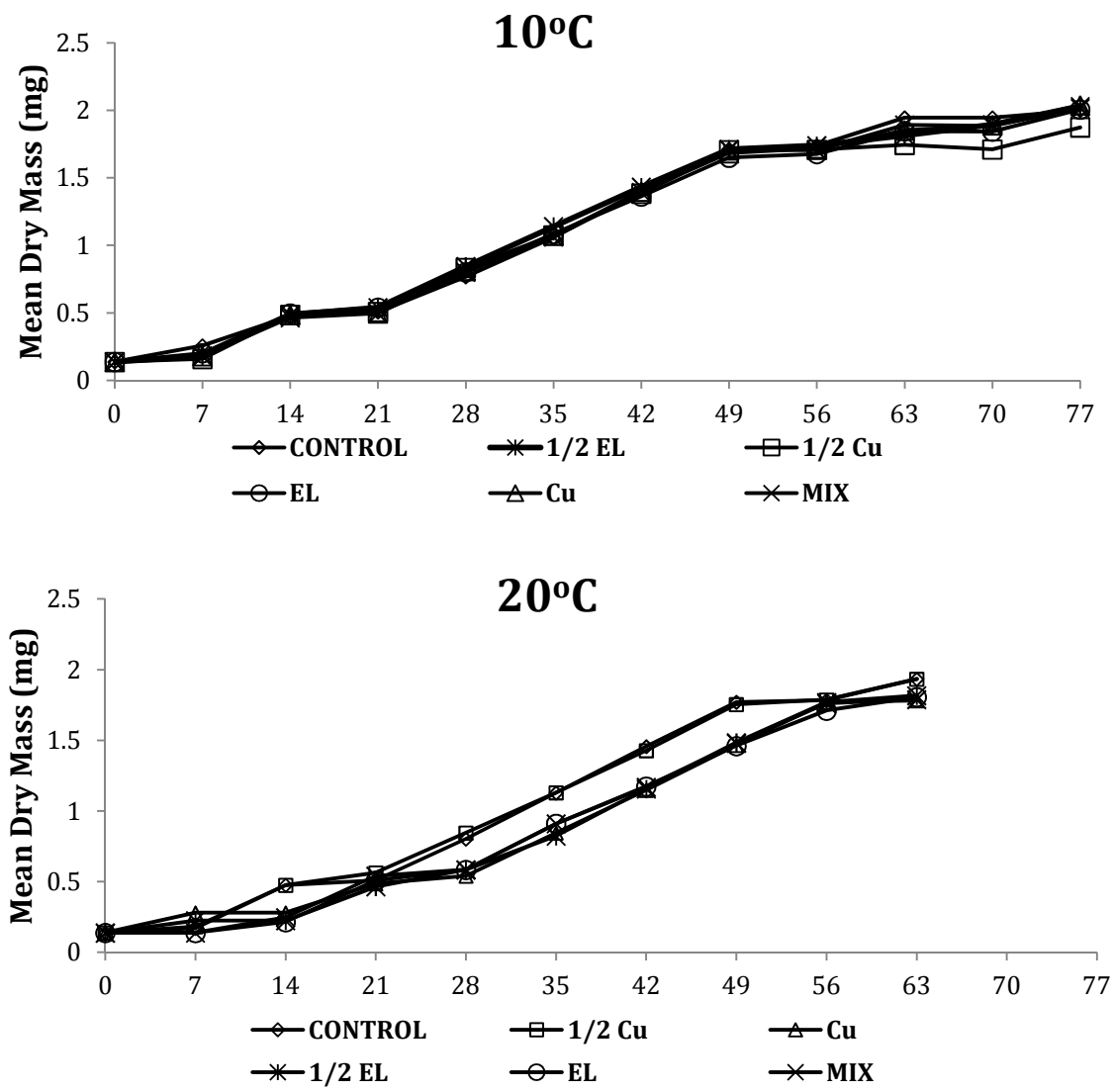

Fig. 15 - Growth of *Echinogammarus meridionalis* (n=180; corrected for mortality) at two different temperatures - 10 and 20°C, for a maximum period of 77 days. Values are means±S.E.M. Legend for the different treatments is displayed in the graphics.

When consumption rates (Figure 16) are compared at 10°C significant differences occur (Kruskal-Wallis test H=15.931, $p<0.05$) with decreased consumption rates in MIX treatment (≈-32%) compared to ½Cu and ½EL ($p<0.05$). In this case, the presence of both chemicals in combination, lead to decreased growth rates when compared with single exposures. At 20°C significant differences between treatments occurred (Kruskal-Wallis test H=18.49, $p<0.05$). Consumption rates in control (0.0015 mgleafconsumed/mgAnimalDW/day±0.0001; mean±S.E.M) were significantly lower than ½EL (0.0022 mgleafconsumed/mgAnimalDW/day±0.0001; mean±S.E.M) ($p<0.05$). Considering the global results, it seems possible that a physiological stress promoted by the contaminants may determine an erratic foraging behaviour rather than a consistent change in consumption rates.

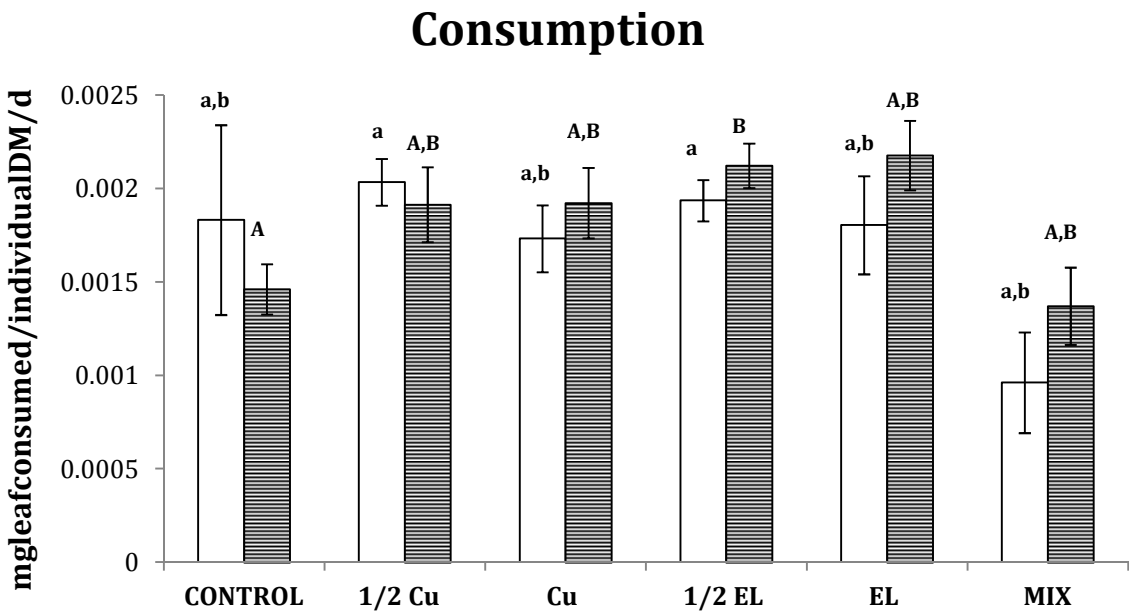

Fig. 16 - Consumption rates of *Echinogammarus meridionalis* expressed in mg leafconsumed/mgAnimalDW/day at 2 different temperatures 10°C (white bars) and 20°C (patterned bars). Values are means±S.E.M. Legend for the different treatments is displayed in the graphics. Different letters indicate statistically significant differences at 0.05 (ANOVA and Tukey test or non-parametric equivalent) at 10°C (lowercase letters) and 20°C (uppercase letters).

At both 10 and 20°C survival rates (Figure 17) were affected by stressor presence (ANCOVA $F_{(5,59)}$=6.103, $p<0.05$ and $F_{(5,47)}$=4.578, $p<0.05$, respectively). Invertebrates in treatments ½Cu, EL and MIX had significantly lower survival rates than control at 10°C (Tukey's test, $p<0.05$). At 20°C invertebrates reared in treatments Cu, EL and MIX had significantly lower survival rates than control (Tukey's test $p<0.05$). Results suggest that the presence of eucalyptus leachates (in the highest concentration tested) may be responsible for increased mortality. Known the fact that eucalyptus plantations supply an almost continuous amount of leaves to the river channel (as opposite to deciduous forests) and in situations of low flow or leachates pools formation (e.g. in summer) invertebrates may not be able to cope with an elongated presence of these leachates even in lower concentrations. At 10°C high mortality was detected at low copper concentrations while at 20°C, on the contrary, it was observed for treatments with high copper. It is well known that copper is an oxidative stress inducer in invertebrates namely in shredders (Bouskill *et al*., 2006; Sroda and Cassu-Leguiller, 2011) that may interfere with survival (Brinkman and Johnston, 2008; Tollet *et al*., 2009). Survival data suggest that increased mortality occurs after 4 weeks – (28 days) at 20°C and 5 weeks (35 days) at 10°C even when invertebrates were continuously fed with alder leaves, a high quality food suggesting that no additional nutritional stress was present. This may suggest that invertebrates up to that date may be able to cope with small amounts of both toxics single or in mixture even when well fed, but prolonged exposure may have deterrent consequences in community composition and population dynamics may be affected in these ecosystems.

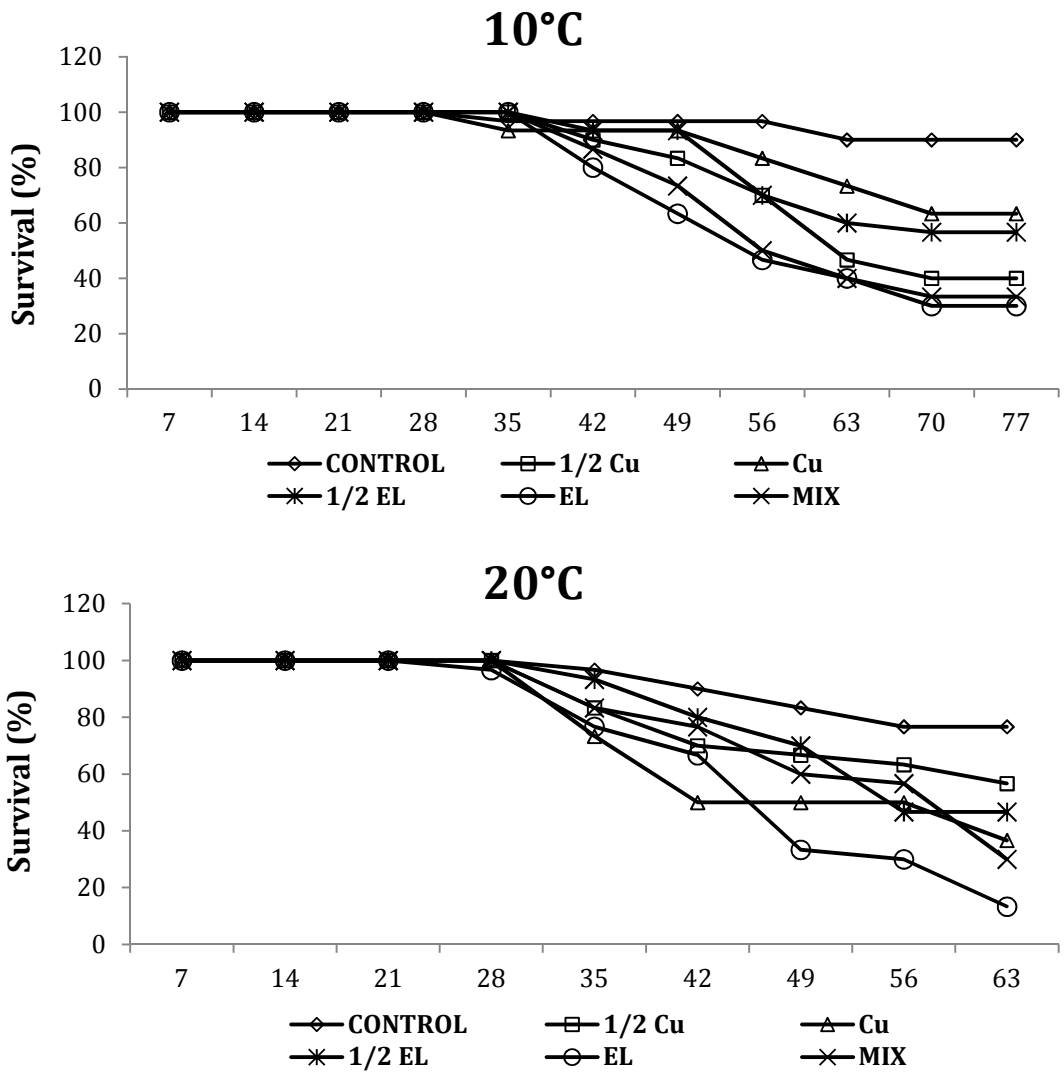

Fig. 17 - Survival (%) of *Echinogammarus meridionalis* (n=180), at 2 different temperatures (10 and 20°C), for a maximum period of 126 days. Legend for the different treatments is displayed in the graphics.

3.3 - Elemental body composition and biomarker determination in S. festiva.

S. festiva appears to be resistant to both acute and chronic exposure to eucalyptus leaf leachates and copper with mortality rates being comparatively lower than the other two species in this study (as corroborated by results in Chapter II and this study). Invertebrate's elemental composition and effects on

three selected biomarkers were assessed to search for sub lethal effects in response to chronic exposure to the selected toxics.

At 10°C, both C:N and N:P ratios (Figure 18) did not differ between treatments (ANOVA, $F_{(5,54)}$=2.326, p>0.05 and $F_{(5,54)}$=1.849, p>0.05 respectively) in opposition to C:P ratios (ANOVA, $F_{(5,54)}$=3.409, p<0.05); treatments Cu and EL presented significantly lower ratios than control (Tukey's test, p<0.05). At 20°C, ratios were significantly different between treatments (ANOVA, $F_{(5,54)}$=2.49, p<0.05, $F_{(5,54)}$=5.979, p<0.05 and $F_{(5,54)}$=5.076, p<0.05 for C:N, C:P and N:P, respectively). However, Tukey's test did not detect which treatments were different for C:N. For both C:P and NP treatment Cu had significantly higher ratio than treatments control, ½Cu, ½ EL and EL (Tukey's test p<0.05). When both temperatures are compared C:P ratios are significantly different in control (t-test p<0.05). In our experiment shredders were fed with a continuous supply of conditioned alder leaves, which are known to have high N content (Canhoto and Graça, 1995), being a high quality food source for invertebrates (Azevedo-Pereira *et al*., 2006) and this increased N composition may have aided in the maintenance of homeostasis in what concerns N balances. Our results suggest that in terms of C:N and N:P ratios, *S. festiva* is homeostatic at 10°C with their stoichiometrical composition remaining constant and this did not happen with C:P. This may sugest that these invertebrates are not strictly homeostatic in what concerns P.

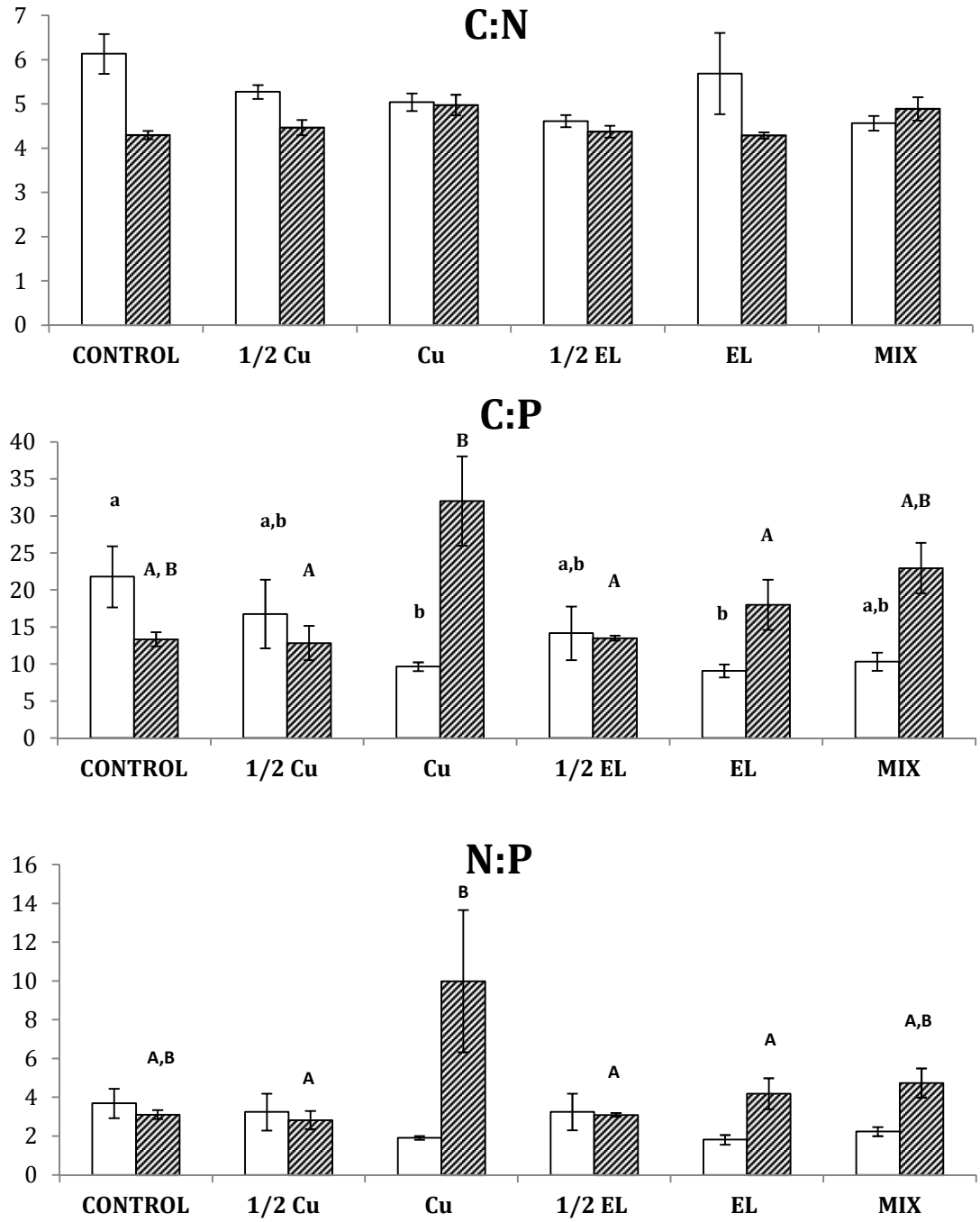

Fig. 18 - C:N, C:P and N:P body elemental ratios of *Schizopelex festiva* exposed to 10°C (white bars) and 20°C (patterned bars). Values are means±S.E.M. Different letters indicate statistically significant differences at 0.05 (ANOVA and Tukey's test or non-parametric equivalent) at 10°C (lowercase letters) and at 20°C (uppercase letters).

Rheosthasis, as suggested by Liess and Hillebrand (2005), states that a species can exhibit a non strictly homeostatic status, and may show slightly changes in elemental composition when submitted to different stressors, in this case, copper, leachates and temperature. In fact with increasing temperature all ratios (C:N, C:P and N:P) are significantly different between treatments and despite the fact that further statistical analysis was not able to detect differences in C:N ratios, results suggest that the increase in temperature accentuates the difficulty of the invertebrates to maintain homeostasis. A reduction of P retention in streams exposed to copper contamination is expected with increasing temperature, as C:P ratios of invertebrates exposed to treatments Cu at 20°C were significantly higher suggesting the presence of lower amounts of P in the invertebrate's body.

C:P ratios were below the values published for other benthic invertebrates including insects (between 60 and 263 , Bowman *et al*., 2005; Evans-White *et al*., 2005; Ferreira *et al*., 2010). Invertebrates exposed to the highest copper concentration had significantly lower %P and lower growth rates than invertebrates in control suggesting that higher temperatures (20°C) may result in lower phosphorus retention and, consequently, lower growth rates. According to the growth rate-hypothesis (Sterner and Elser, 2002), a positive relationship is observed between P body contents (and rRNA) and growth (Sterner and Elser, 2002; Vrede *et al*., 2004, Persson *et al.,* 2011). Rapid growth rates should depend of whole-body increased P concentrations due to RNA dependent protein synthesis demands (Elser *et al*., 2000; 2003) with results suggesting that increased temperature may have a determinant effect on the process (Wojewodzic *et al*., 2011).

Table 7 - Chemical composition and body elemental ratios of *Schizopelex festiva* bodies (n=10) exposed at 10 and 20°C to copper (Cu) and eucalyptus leachates (EL). Values are mean±S.E.M.. Note that C, N and P, are expressed as elemental mass ratios. P, phosphorus; N, nitrogen; C, carbon.

	%C		%N		%P		C:N		C:P		N:P	
	10°C	20°C	10°C	20°C	10°C	20°C	10°C	20°C	10°C	20°C	10°C	20°C
Control	51.36	47.79	8.66	11.17	3.37	3.75	6.13	4.30	21.80	13.36	3.69	3.12
	±1.64	±0.28	±0.47	±0.27	±0.66	±0.28	±0.45	±0.10	±4.12	±0.95	±0.76	±0.23
½ Cu	49.06	44.11	9.38	9.96	3.78	4.44	5.27	4.47	16.76	12.86	3.25	2.83
	±0.54	±2.59	±0.29	±0.67	±0.37	±0.66	±0.16	±0.17	±4.63	±2.33	±0.95	±0.47
Cu	45.53	41.10	9.14	8.33	4.86	1.57	5.04	4.98	9.65	32.03	1.92	10.00
	±1.06	±1.77	±0.36	±0.34	±0.28	±0.34	±0.20	±0.23	±0.59	±6.04	±0.09	±3.67
½ EL	45.30	45.58	9.91	10.49	4.38	3.39	4.61	4.38	14.16	13.52	3.25	3.10
	±1.80	±0.59	±0.49	±0.30	±0.59	±0.10	±0.14	±0.13	±3.63	±0.34	±0.95	±0.09
EL	48.42	52.15	9.77	12.11	5.82	3.36	5.69	4.29	9.08	18.03	1.82	4.20
	±4.15	±3.98	±1.25	±0.84	±0.72	±0.37	±0.92	±0.07	±0.88	±3.39	±0.25	±0.80
MIX	34.33	66.64	7.50	13.63	3.44	3.35	4.57	4.89	10.33	22.97	2.24	4.75
	±3.27	±7.09	±0.65	±1.26	±0.18	±0.48	±0.17	±0.26	±1.22	±3.40	0.23	±0.75

3.3.2- Biomarkers.

No significant differences were observed between treatments (ANOVA $F_{(5,54)}$= 0.717, p>0.05) on *S. festiva* ChE activity (Figure 19) at 10°C. At 20°C, differences between treatments were significant (1-ANOVA $F_{(5,54)}$=7.265, p<0.05) with treatments control, ½Cu and Cu having significantly higher enzymatic activity than EL and MIX (Tukey's test p<0.05). MIX was also significantly different from ½EL (Tukey's test p<0.05). Values reported in this study are lower than the ones previous found in acute exposure (Chapter III). Nonetheless at the lowest temperature results are similar to acute exposure bioassays (where treatments at both 10 and 20°C were similar); however, at 20°C, an inhibition in ChE happens after exposure to the highest leachate concentration or in the MIX treatment. These were two of the treatments were mortality occurred and the lower ChE activity observed may have contributed to this. Our results seem to suggest that continuous exposure to eucalyptus leachates may inhibit ChE activity with possibly deleterious effects on neurological mechanisms, eventually promoted by cineole or derivatives that may act on the nervous systems (Klaassen, 2008) and may have contributed to increased mortality.

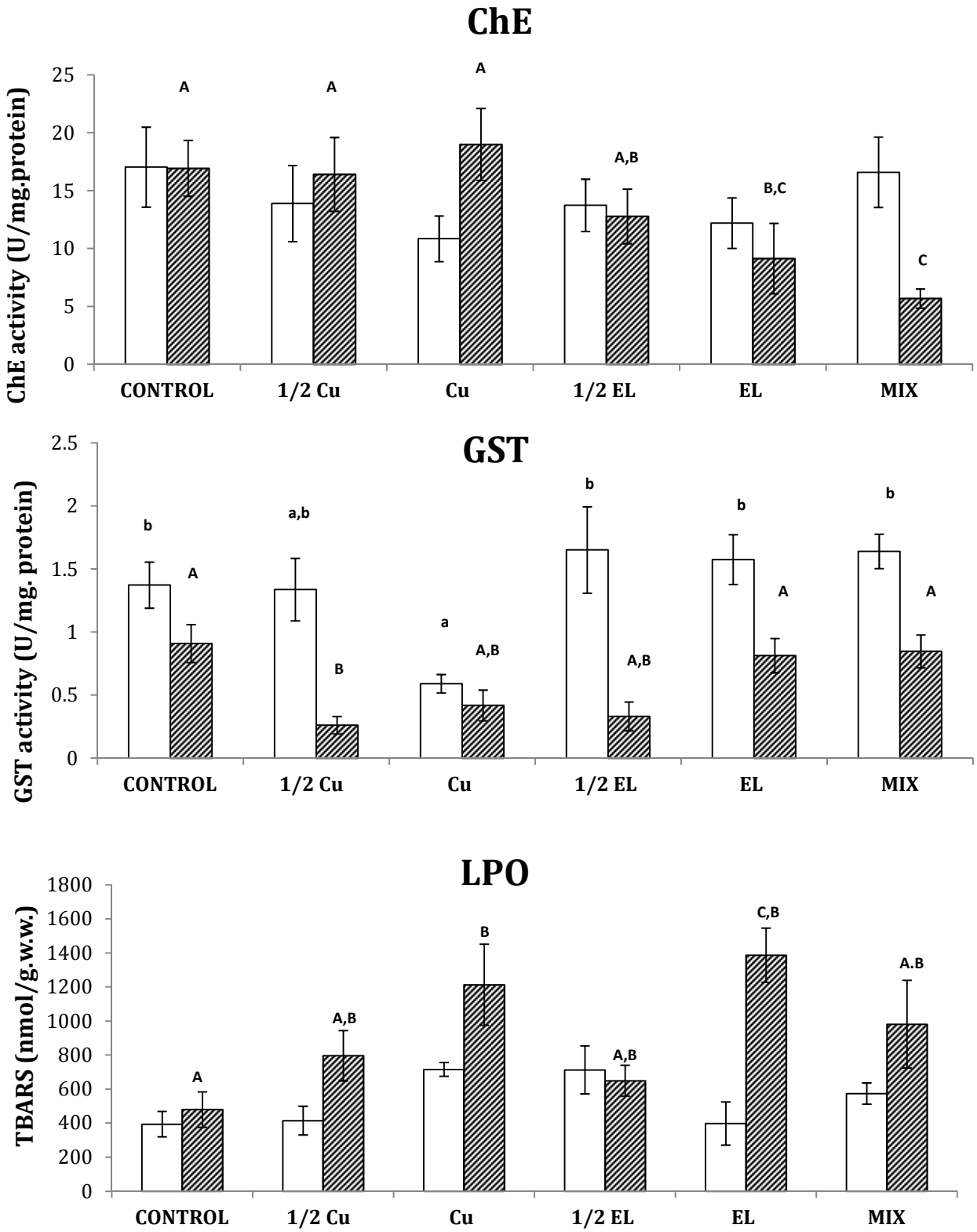

Fig. 19 - Values of head cholinesterase (ChE), and total body homogenates glutathione *S* transferase (GST) and lipid peroxidation levels (LPO) in *Schizopelex festiva* at 10°C (white bars) and 20°C (patterned bars) in the selected treatments. Values represent the mean of 10 samples (one organism per sample) and the correspondent standard error bars. Significant differences between treatments (Tukey's test) at 10°C (lowercase letters) and 20°C (uppercase letters) and are displayed in the graphic.

At 10°C, GST activity (Figure 19) in invertebrates was significant different between treatments (ANOVA $F_{(5,54)}$=4.926, p<0.05). Invertebrates in treatment Cu had significantly lower enzymatic activity than treatments control, ½EL, EL and MIX (Tukey's test p<0.05). At 20°C differences between treatments were also significant (ANOVA $F_{(5,54)}$=54.505, p<0.05) with invertebrates in treatments control, EL and MIX having significantly higher enzymatic activity than Cu ½ (Tukey's test p<0.05). Also GST activity in this study was slightly lower than previous acute toxicity results (Chapter III). At 10°C, a clear inhibition occured for the highest copper treatment a result similar to those found for acute exposure where, at this temperature, increased copper concentrations were also responsible for GST inhibition (Chapter III). At the highest temperature apparently only ½Cu showed a clear inhibition. GST multifunctional enzyme system serves to conjugate endogenous glutathione with electrophiles, forming more polar compounds that are further easily excreted/metabolized and, also conjugates breakdown products of lipid peroxides to glutathione (Ketterer *et al*., 1983) preventing lipid peroxidation. The inhibition of this system may increase lipid peroxidation values and may compromise invertebrate's mechanism of defense against xenobiotics. GST inhibition as a response to Cu has been reported in invertebrates (Mosleh *et al.,* 2005, Cunha *et al.,* 2007).

Lipid peroxidation levels (Figure 19) were significant different for invertebrates exposed to different treatments at both 10 and 20°C (ANOVA $F_{(5,54)}$=2.603, p<0.05 and $F_{(5,54)}$=6.538, p<0.05, respectively), but following Tukey's test was not able to detect where these differences occurred at 10°C. At 20°C invertebrates in treatments Cu and EL had significantly higher LPO levels than

control (Tukey's test $p<0.05$) suggesting increased lipid oxidative damage in these treatments that may explain the higher mortality observed in EL treatments at 20°C. Treatment EL is also significantly different from ½EL (Tukey's test $p<0.05$). On the contrary (compared to ChE and GST levels), here an increase in LPO values were observed, when compared to those of previous acute exposure (Chapter III) which may suggest that chronic exposure to toxics may lead to elevated lipid oxidative damage *per se*. Results are in accordance with previous GST quantification where in increased temperature and copper treatments GST inhibition was observed being this enzymatic system unable to prevent lipid damage. LPO may disrupt cell membranes, affecting their structure and function possibly leading to an increase in citotoxicity and this may in part contribute to the higher mortality observed at 20°C in treatments with high LPO levels (EL and MIX).

Conclusions

Despite the fact that *S. festiva* survival rates seem to be less affected by the stressor presence (which is in accordance with previous acute toxicity results) further analysis suggest that these chemical agents influence their elemental composition. At higher temperatures (20°C), the invertebrates presented an increased difficulty in retaining P when exposed to higher copper concentrations. This may justify the observed lower growth rates with potential important ecological consequences in invertebrate's fitness. Also the exposure to both stressors lead to changes in biomarker activity in *S. festiva*, with an increase in lipid oxidative damage. *E. meridionalis* is very sensitive to both the presence of toxics and increased temperature as increased mortality suggests. Distinct sensitivity of both invertebrates to chronic copper and eucalypt leachates exposure may contribute to distinct community composition in streams impacted by these stressors and temperature may contribute to modulate the extent of these relationships.

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Chapter V

Effects of food quality on the stoichiometrical composition and selected biomarkers of *Echinogammarus meridionalis* assessed at two temperatures

Effects of food quality on the stoichiometrical composition and selected biomarkers of *Echinogammarus meridionalis* assessed at two temperatures

Abstract

In this study, the effects of food quality on the stoichiometrical composition and selected biomarkers of the shredder *Echinogammarus meridionalis* were investigated at two distinct temperatures (10 and 20°C). In 14-day bioassays carried out at both temperatures, *E. meridionalis* specimens were feed with alder (*Alnus glutinosa*) or eucalyptus (*Eucalyptus globulus*) leaves, considered high and low quality food, respectively, to this species. After 14-days, the stoichiometrical composition (C, N and P) and selected biomarkers, namely the activity of cholinesterase (ChE) and glutathione S-transferases (GST) enzymes, and the lipid peroxidation (LPO) levels were determined. No significant differences in the elemental composition of *E. meridionalis* fed with different food types were found at any temperature. No significant differences in ChE and GST activities between alder and eucalyptus fed organisms were found at any temperature, or in LPO levels at 10°C. However, at 20°C, higher LPO levels were found in organisms fed with eucalyptus leaves, suggesting increased oxidative damage relatively to those fed with alder leaves. These results indicate that feeding on eucalyptus leaves under high temperatures increases the oxidative stress and lipid oxidative damage in this species, an effect that may decrease the individual fitness. Thus, consumption of low quality food as eucalyptus leaves combined with high

temperatures may have adverse effects on stream invertebrate's ecophysiology, with possible effects for higher levels of biological organization.

Keywords: *Echinogammarus meridionalis*, temperature, stoichiometry, biomarkers, eucalyptus, alder.

1. INTRODUCTION

Stream water temperature and available resources are key interacting factors (Sweeney and Vannote, 1986) able to affect invertebrate's life history parameters (Braune *et al*., 2008; Moline and Poff, 2008), the structure and also the functioning of low order streams (Abelho and Graça, 1996; Mckee and Atkinson, 2000, Alexander and Palmer, 2002, Perkins *et al.,* 2010). Both factors, acting *per s*e or simultaneously, are known to have a crucial influence on shredders rates of ingestion (Motomori *et al*., 2001, Leberfinger and Bohman, 2010), growth dynamics (Fenoglio *et al*, 2005, Moline and Poff, 2008, Graça and Cressa, 2010,), survivorship (Durance and Ormerod, 2007), and/or abundance (Burgmer *et al.,* 2007; Larrañaga *et al.,* 2009). Considering the critical role of this functional feeding role in leaves decomposition, any changes in food quality or in the thermal regime of the aquatic system may affect stream's secondary production and the transfer of energy to higher trophic levels. Changes in riparian areas composition, as the ones promoted by reforestations or riparian harvesting practices, may change dramatically the phenology and quality of litter inputs (Gessner *et al*., 2010; Lecerf and Richardson, 2010) to streams as well as light incidence contributing to changes in water temperature (Imholt *et al*., 2009). Plantations of the exotic evergreen *Eucalyptus globulus* Labill are now common in the Iberian Peninsula replacing the native forest (IEFN6, 2013) and determining the impoverishment of the invertebrate communities (Canhoto and Graça, 1996; Graça *et al.*, 2002) due to changes in the hydrology (Molinero and Pozo, 2004), seasonality, quantity, quality (Molinero *et* and Pozo*,* 2003) of the monospecific allochthonous inputs. A number of field (Pozo *et al*., 1998; Sampaio *et al*., 2001) and laboratorial (Canhoto and

Graça, 1999) studies even argue the unimportance of invertebrates, mainly shredders, in eucalyptus leaves decomposition (Sampaio *et al*., 2004; Gama *et al*., 2007). Whether or why this is true is still not completely clarified as shredders are not completely lacking in most streams lined by eucalyptus (Larrañaga *et al*., 2009); some apparently cope with the high contents of phenols, oils and a the thick cuticle of the eucalyptus leaves (Canhoto and Graça, 1999). The permanent supply of eucalyptus leaves to the ground and water is enhanced in summer (Abelho and Graça, 1996) when the stream water temperature is higher, flows is reduced and water quality may be deteriorated (Chatzinikolaou *et al*., 2006; Lillebö *et al*., 2007). In order to survive, shredders must possess inherent mechanisms to total or partially cope with these stressors low food quality and waterborne toxicity, originated by the compounds released by the leaves, and high temperatures.

According to the Ecological stoichiometry (ES) theory, shredders maintain their stoichiometrical composition (C, N and P content) constant (homeostasis) independently on resource composition. But decomposing leaves and other detritus usually present lower N and P content and higher C amount (Sterner and Elser, 2002; Cross *et al*., 2005; Evans-White *et al*., 2005). Therefore invertebrates in order to cope with changes in riparian vegetation should use different physiological strategies to compensate for low quality food (Frost *et al*., 2005). The type and intensity of this response has been refered to be influenced by temperature (Villanueva *et al*., 2008; Wojewodzic *et al*., 2011).

In the last decades, biomarkers have been widely used as early warning tools to assess the adverse effects of chemical environmental contaminants and other stressors, in invertebrates, including in gammarids (e.g Timofeyev *et al*.,

2006 *a,b*; Xuereb *et al*., 2007, 2009). The activity of cholinesterase (ChE), glutathione S-transferases (GST) enzymes as well has the lipid peroxidation (LPO) levels have been used to assess the extent of oxidative stress parameters and neurological impairment. Combined with other ecological approaches, such as stoichiometrical composition determination, these tools may provide most important insides on the mechanisms leading to changes observed at individual and population levels in low order streams. This seems particularly useful in streams lined by eucalyptus afforestations where the biota has been extensively referred as impoverished (Pozo *et al.,* 1998; Sampaio *et al.,* 2001) and with physiological limitations (Larrañaga *et al.*, 2009) that may lead to difficultion in the digestion (Canhoto and Graça 2006) of the available recalcitrant food itens.

In this study, the effects of food quality on the stoichiometrical composition and selected biomarkers of the shredder *Echinogammarus meridionalis* were investigated at two temperatures (10 and 20°C) common in autumn and summer in streams draining eucalyptus plantations. The individual and combined effects of leaf litter quality and temperature were addressed by evaluating the elemental composition and biomarker activities o*f Echinogammarus meridionalis* specimens fed *Alnus glutinosa* or *Eucalyptus globulus* during a 14-day bioassay. Considering that eucalyptus is a low quality leaf, with the presence of chemical compounds that can be negative to the invertebrates (Canhoto and Laranjeira, 2007) it will be interesting to assess if consumption of these particular leaves may lead to changes in stoichiometrical composition of the invertebrates or if any negative effects in biomarker activity are observed.

2. MATERIAL AND METHODS

2.1 - Collection and acclimation of organisms and leaf litter

Test organisms were *Echinogammarus meridonalis* Pinkster (Crustacea: Amphipoda). Organisms and water were collected in the spring 2009 in Ribeira da Redinha, Pombal, central Portugal (39°58.726' N, 8°34.393'W) brought to the laboratory and acclimatized to laboratory conditions (12h light: 12h dark photoperiod). They were maintained at 10±1°C or 20±1°C, one week before the beginning of the experiments, and fed *ad libitum* according to the leaf/temperature treatments they would have been assigned.

Leaves of *Alnus glutinosa* L. and *Eucalyptus globulus* Labill. were collected just after abscission, in autumn and summer, respectively. All leaves were air dried at room temperature and stored until needed. Prior to being used as food, the leaves of both species were individually assembled into groups of ±4g, enclosed in 0.5mm mesh bags (10 x 14 cm) and conditioned, for 3 weeks, at Ribeira de S. João, in the Lousã mountain (40°05'59"N, 8°14'02"W), a reference stream in central Portugal to ensure optimal fungal colonization.

2.2 - Experimental design and exposure conditions

A total of 240 individuals were used in the experiments: 120 for stoichiometry determinations (60 for each temperature), and 120 for biomarker analysis (60 for each temperature). For both stoichiometry and biomarkers analysis, the experimental design was: 2 groups of animals (30), one feed with eucalyptus leaves (3 replicates with 10 invertebrates each) and the other fed with alder leaves (3 replicates with 10 invertebrates each), at 10 or 20°C. In all tests, organisms were maintained in 500 ml

erlenmeyers filled with filtered aerated stream water (Glass fibber filters; Millipore APFF) in photoperiod (12h light : 12h dark) and temperature controlled rooms (one at 10°C and the other at 20°C).

2.2.1 – Stoichiometry

Two groups of 60 similar size *E. meridionalis* (1.27mg±0.076; mean±SE), acclimatized to 10 or 20 °C, were distributed in groups of 10 by 500 ml sterile Erlenmeyer flasks filled with aerated filtered stream water. Specimens of each treatment (A, E) were assigned based on the leaf species they had been fed during the previous days. In each group of 6 replicates (with 10 invertebrates each) the invertebrates were fed *ad libitum* for 14 days with conditioned alder (A) – 3 replicates - or eucalyptus (E) – 3 replicates. At the end of the experiment leaves were dried (60°C; 48h) and a mixed sample (n=3) analysed for total N and C using a Flash EA 1112 for IRMS Delta V Advantage ThermoIRMS mass spectrometer for the simultaneous analysis of the isotope ratios and for total P. Determinations were done according to Graça *et al*. (2005).

2.2.2 - Biomarker determination

The second group of 120 (60 for each temperature) intermediate size *E. meridionalis* (1.27mg±0.076, mean±SE), was randomly distributed in groups of 10 by 500 ml Erlenmeyer flasks filled with aerated filtered stream water at 10 or 20 °C. Each group of 3 replicates was fed conditioned alder (A) or eucalyptus (E) (at 10°C or 20°C). Specimens of each treatment (A, E) at each temperature were assigned as

above in stoichiometrical composition determinations during the same time period (14 days).

At the end of the test, invertebrates from each replicate (10 organisms) were sacrificed, their heads separated from the remaining body and stored in eppendorfs at -80°C for posterior biomarker determination. Heads and the remaining body were homogenized (Ystral GmbH d-7801 Dottingen homogeniser) in cold phosphate buffer (0.1 M, pH 7.4) for 30s on ice. Animal heads were centrifuged at 3300×g for 3min at 4°C (SIGMA 3 K 30), and resulting brain supernatants were used to measure ChE by the Ellman's technique (Ellman *et al*.,1961) adapted to microplate (Guilhermino *et al*., 1996). To 0.250 mL of the reaction buffer prepared with 30 mL of K-phosphate buffer (pH 7.2, 0.1 M), 1 mL of dithiobisnitrobenzoate (DTNB) 10 mM (20 mM acid dithiobisnitrobenzoate and 18 mM sodium hydrogen carbonate in 0.1 M K-phosphate buffer, pH 7.2) and 0.2 mL of acetylcholine iodide 75 mM, was added 0.05 mL of the supernatant (invertebrate's head homogenate), previously diluted in homogenization buffer to standardize protein content at 1 mg/mL, in a 96 well microplate (four replicates per sample). The increase in absorbance was determined at 412 nm in a microplate reader (Bio Tek Power Wave 340) for 5 min at 25 °C. Acetylthiocholine was used as substrate in all the assays and no distinction was made between different forms of ChE that might be present.

A part of the remaining body homogenate (0.800ml) was centrifuged at 10000×g for 20 min at 4°C to isolate the post mitochondrial supernatant (PMS). PMS was used for determination of GST activity which was quantified by the conjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm (Habig *et al*., 1974) with some adaptations (Frasco and Guilhermino, 2002). In brief,

0.250 mL of the reaction solution (75 mL of phosphate buffer 0.2 M pH 6.5, 2.34 mL of 1-chloro-2,4 dinitrobenzene in ethanol and 13.5 mL of a 10 mM GSH solution in ultra-pure water) was added to 0.05 mL of sample previously diluted in homogenization buffer in order to have a final protein concentration of 1 mg/mL (four replicates per sample). The optical density was measured at 340 nm in a microplate reader (Bio Tek Power Wave 340) for 5 min at 25 °C.

ChE and GST activities were expressed in Units (U) per concentration of protein, one U corresponding to 1 nano mole of substrate hydrolised per min per mg of protein. The concentration of protein in the samples was determined according to Bradford *et al*. (1976) adapted to microplate (Frasco and Guilhermino, 2002) using bovine γ -globulins as protein standard.

LPO was determined by measuring the thiobarbituric acid reactive species (TBARS) (Ohkawa *et al*., 1979; Bird and Draper, 1984). To 0.200 mL of the remaining whole body homogenates, 0.2 mM butylhydroxytoluene (BHT) (Torres *et al*., 2002) was added to prevent artificial lipid oxidation. To each sample 1 mL of 12% trichloroacetic acid, 0.8 mL of 60 mM Tris–HCl solution (pH 7.4) with 0.1 mM diethylenetriaminepentaacetic acid (DTPA) and 1 mL of 0.73% thiobarbituric acid (TBA) were added. After an incubation bath for 60 min at 100°C, the solution was centrifuged at 12000×g for 5 min at 4°C and LPO levels were determined in the resultant supernatant at 535 nm in a Jasco V-630 spectrophotometer and expressed in nmol TBARS per g of wet weight.

2.4 - Data Analysis

Data were previously tested for normality (Kolmogorov–Smirnov normality test) and homogeneity of variance (Levene's test) (Zar, 1999). Departures from normality and homoscedasticity were corrected (log (x + 1) transformation) whenever necessary. Leaves chemical composition was compared using 1-way Analysis of Variance (ANOVA). Elemental ratios (C:N, C:P, N:P) of the body and biomarker determination were compared by 2 way Analysis of Variance (2-ANOVA) using leaf type and temperature as categorical factors. Leaf elemental composition was compared with body ratios with 1-way Analysis of Variance (ANOVA). The Tukey*'s* test for *post-hoc* multicomparisons was used when significant differences were found. Analyses were performed with STATISTICA 8 ® software.

3. RESULTS AND DISCUSSION

3.1 – Stoichiometry.

The results of the statistical analysis to the ratios of different elements in alder, eucalyptus leaves and *E. meridionalis* body, at both 10 and 20°C are shown in Table 8 while the ratios composition is shown in Table 9. Alder leaves had significantly lower C:N and C:P ratios than those of eucalyptus (ANOVA $F_{(1,4)=}354.94$ $p<0.05$ and $F_{(1,4)=}27.144$ $p<0.05$, respectively) while no significant differences in the N:P ratio (ANOVA $F_{(1,4)=}6.41$ $p>0.05$) between both leaf species were found (Table 8). Our results suggest that alder leaves are comparatively richer in N and P than eucalyptus. In our case the conditioning process appeared to have favoured alder leaves probably due to differential fungal colonization patterns (Haapala *et al*., 2001; Sampaio *et al*., 2001) increasing their nutrient content when compared with eucalypt leaves

(Bärlocher and Graça, 2002). The presence of oil vesicules and leaf intrinsic characteristics is known to influence the fungal communities present during decomposition.

In *E. meridionalis* body composition, no significant effects of food quality nor of temperature were found for C:N, C:P and N:P ratios (2-ANOVA $F_{(1,20)}$= 0.28, p>0.05 and $F_{(1,20)}$= 1.62, p>0.05 for C:N; $F_{(1,20)}$=0.04, p>0.05 and $F_{(1,20)}$= 0.50, p>0.05 for C:P and $F_{(1,20)}$= 0.01, p>0.05 and $F_{(1,20)}$= 1.13, p>0.05 for N:P, respectively) and no interactions between factors were found (2-ANOVA $F_{(1,20)}$= 0.00, p>0.05 for C:N; $F_{(1,20)}$=1.17, p>0.05 for C:P and $F_{(1,20)}$=1.26, p>0.05 for N:P). These results suggest that invertebrates are homeostatic regardless of the food type present or the rearing temperature. Both N and P are limiting elements in lotic systems, but structural and functionally important to detritivore's growth (Woods *et al*., 2003; Vrede *et al.,* 2004).

When leaves elemental composition and invertebrates body composition is compared it is observed that alder leaves have higher C:N ratios (ANOVA $F_{(2,12)}$= 380.85, p<0.05) than animal´s body at both temperatures (Tukey's test p>0.05) and that C:P and N:P ratios were significantly lower (ANOVA $F_{(2,12)}$= 39.46, p<0.05 and $F_{(2,12)}$= 16.77, p<0.05 respectively) at both 10 and 20°C (Tukey's test p>0.05). This may indicate that conditioned alder is a high quality food source for these invertebrates (not just *per se*; Cross *et al*., 2003).

Table 8 - ANOVA results (F and *p* values) of leaves and body ratios of *Echinogammarus meridionali*s analysis. Leaves ratios were compared by 1-way Analysis of variance (ANOVA) and body or faecal ratios with 2-way Analysis of variance (2-ANOVA) using leaf type and temperature as categorical factors.

			C:N	C:P	N:P
Leaves (ANOVA)		***F value***	5.67	8.73	5.84
		p value	<0.05	<0.05	>0.05
Body (2-ANOVA)	*temperature*	***F value***	1.62	0.50	1.12
		p value	>0.05	>0.05	>0.05
	leaf type	***F value***	0.28	0.04	0.01
		p value	>0.05	>0.05	>0.05
	interaction	***F value***	0.00	1.17	1.26
		p value	>0.05	>0.05	>0.05

Eucalyptus leaves had significantly higher C:N and C:P ratios (ANOVA $F_{(2,12)}$= 279.66 $p<0.05$ for C:N and $F_{(2,12)}$= 97.12 $p<0.05$ for C:P) than invertebrate's bodies at 10°C or 20°C (Tukey's test $p<0.05$) while N:P ratios were significantly lower in eucalyptus leaves (ANOVA $F_{(1,20)}$= 194.01, $p<0.05$) at both temperatures (Tukey's test $p<0.05$) suggesting that elemental imbalances between resources and consumers occur and that this leaf type is of poor quality for the invertebrates.

Table 9 - Chemical composition of leaves (n=3) and consumers (n=30). Values are mean±S.E.M. Note that C, N and P, are expressed as elemental mass ratios. E- Eucalyptus, A- Alder. Elemental imbalance is calculated as the elemental difference between the food resource and its consumer.

			%C	%N	%P	C/N	C/P	N/P
Leaf		**E**	57.06 ±0.54	1.74 ±0.07	4.24 ±0.83	**32.77** ±0.94	**14.40** ±2.40	**0.44** ±0.07
		A	49.74 0.01	3.06 ±0.07	15.26 ±3.03	**16.25** ±0.38	**3.54** ±0.73	**0.22** ±0.05
Body	**10°C**	**E**	37.26 ±0.97	7.29 ±0.27	3.93 ±0.26	**5.13** ±0.19	**9.69** ±0.71	**1.88** ±0.08
		A	38.36 ±0.43	7.69 ±0.42	3.61 ±0.13	**5.04** ±0.22	**10.70** ±0.48	**2.16** ±0.19
	20°C	**E**	39.84 ±1.60	7.47 ±0.47	4.08 ±0.45	**5.38** ±0.19	**10.35** ±1.12	**1.93** ±0.22
		A	36.84 ±0.99	7.01 ±0.23	4.86 ±1.27	**5.27** ±0.17	**9.18** ±1.26	**1.74** ±0.23

		Elemental imbalance		
		C/N	**C/P**	**N/P**
10°C	**Eucalyptus**	27.64	4.71	-1.44
	Alder	11.21	-7.16	-1.94
20°C	**Eucalyptus**	27.39	4.06	-1.49
	Alder	10.98	-5.63	-1.52

Invertebrates are usually assumed to have higher nutrient contents than detritus (Sterner *et al*., 1998) and here leaf C:N ratios were significantly higher when compared with invertebrate´s body composition. Despite this fact, alder leaves present a relatively higher % N when compared with eucalyptus leaves therefore being a more nutritive resource for the invertebrates. In fact, alder leaves (even unconditioned) are generally considered a nutritious food source for most

invertebrates allowing the best performances in comparison with other native and exotic leaves (e.g. Canhoto and Graça, 1995, 1999; Graça and Cressa, 2010).

It is also possible that the eucalyptus conditioning process may compensate low N leaf contents and that a preferential consumption of fungal hyphae may smooth the differences in the N contents of these leaf detritus and the shredder bodies. This exotic leaf has high phenolic compounds and oils that may affect, along with a tough cuticle, not only leaves consumption, but also N assimilation through leaf chemical or body physiological constraints (see Canhoto and Graça, 1999) and this can be potentiated with a raise in stream's temperature. Whether these amphipods have the capacity to preferentially acquire (eventually by scraping) or digest the fungal mycelium is not known, but likely. If so the leaf conditioning status should be a key factor to determine a selective feeding behaviour by the invertebrates in order to fulfill their N and P requirements. In fact Graça *et al*. (1993), in a study with crustaceans, also showed the importance of fungi (*vs*. leaf) in the diet and performance of *Asellus aquaticus* and it has been stated that extreme imbalances in C:nutrient ratios may stimulate feeding specialization in insect herbivores (Elser *et al*., 2000).

The body ratios (C:P) were far below to those published for benthic invertebrates (>63; Bowman *et al*., 2005), stream insects (263±113; mean±SD) or crustaceans (898±15) (Evans-White *et al*., 2005). However, these values seem to depend on macroinvertebrate species and environmental conditions as nutrients (*e.g.* James *et al.,* 2007) and temperature (Kyle *et al*., 2006; this study).

3.2 – Biomarkers

The results of the biomarkers determined in *E. meridionalis* and the corresponding statistical analysis are shown in Table 10. In a previous study with similar sized *E. meridionalis* (Chapter III) means of 2.78 U/mg protein±0.251 and 3.44 U/mg protein±0.315 for ChE at 10 and 20°C, respectively, and corresponding values of 1.89 U/mg protein±0.177 and 2.94 U/mg protein±0.447 for GST activity, and 114.72 nmol/g. w.w.±15.818 and 222.14 nmol/g. w.w.±12.350 for LPO levels were found for the different treatments (Chapter III). These values are in the same range than the ones determined here. No significant effects of food quality and temperature on ChE and GST activities were found and the interaction between the two factors was also not significant for both biomarkers. These results indicate the robustness of *E. meridionalis* ChE and GST in relation to chronic exposure to low food quality at both 10 and 20°C, and suggest that these are suitable biomarkers for use in real scenarios. In a previous study (Chapter III) with *E. meridionalis* submitted to 96h of fasting, no significant differences in ChE activity were found between organisms maintained at 10 and 20°C, thus in good agreement with the present findings. These results are consistent with those from Xuereb *et al. (*2009) witch observed that no water temperature effect was observed on the *Gammarus fossarum* AChE basal enzymatic levels when exposed to temperatures ranging from 6°C to 24°C. The mean AChE activity values in that study are similar to the ones observed here (from 8.7±0.4 to 9.5±0.9 $nmolmin^{-1}$).

Table 10 - Biomarkers in *Echinogammarus meridionalis* fed with eucalyptus or alder leaves at 10°C and 20°C after a 14-day bioassay (+1 week for acclimation). ChE - head cholinesterase activity; GST – body without head glutathione *S transferases* activity; LPO - body (without head) lipid peroxidation levels. ChE and GST activity are expressed in Units (U) per concentration of protein, one U corresponding to 1 nano mole of substrate hydrolised per min per mg of protein. Lipid peroxidation values are expressed in TBARS (nmol/ g. w.w). Values are the means±S.E.M.; n=30. ANOVA results (F and *p* values) for the effects of temperature and leaf type are also shown. Different letters indicate statistically significant differences at 0.05 (Tukey's test).

		ChE		GST		LPO	
10°C	***Eucalyptus***	4.47	±0.446	1.19	±0.209	191.57 **a**	±14.673
	Alder	4.31	±0.734	0.94	±0.231	138.62 **a,b**	±11.730
20°C	***Eucalyptus***	5.52	±0.702	1.12	±0.145	229.56 **a**	±19.845
	Alder	4.13	±0.217	1.52	±0.304	119.85 **b**	±13.190
Temperature	**F value**	0.59		0.004		0.24	
	***p* value**	>0.05		>0.05		>0.05	
Leaf type	**F value**	1.87		1.37		17.61	
	***p* value**	>0.05		>0.05		<0.05	
Interaction	**F value**	1.16		1.91		3.24	
	***p* value**	>0.05		>0.05		>0.05	

However, in the previous study (Chapter III), a significant reduction of GST activity was found in organisms maintained at 20°C relatively to those maintained at 10°C, thus in contrast to the results shown in Table 10. These differences may be due to the fact that invertebrates where here exposed to eucalypt toxins via ingestion and not by direct contact with the eucalypt leachates as in the previous experiments and this difference may be due to specific feeding effects or the duration of the bioassay among other factors. Significant differences in LPO levels were found between organisms fed with alder and eucalyptus leaves in *E. meridionalis* maintained at 20°C (Table 10). These results indicate that low quality food increases oxidative stress and

damage in this species at elevated temperatures. This effect of temperature and food on LPO should be taken into consideration when using LPO determined in this species as a biomarker in the wild.

Conclusions

In summary, our results tend to support the validity of the stoichiometric theory for stream macroinvertebrates in a detritus-based ecosystem – that invertebrates are homeostatic independently of the leaf species elemental composition they fed on. The influence of temperature in this feeding process should not be discarded as for *E. meridionali*s the combination of low quality food/elevated temperature was responsible for increased oxidative stress. Results suggest that both stoichiometry and biomarker determination are good tools to assess the effects of low quality food or increased temperature in the case of *E. meridionalis* and may be usefull to help explaining the differences on invertebrates' biodiversity and community's structure observed on native *vs* exotic streams.

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Chapter VI

General Discussion

Relative sensitivity of the three shredders species

Shredders are a key component in small stream's communities. With their feeding and egestion/excretion activities they transfer the energy from the leaves and logs that reach the stream to higher trophic levels and to downstream ecosystems (Graça, 2006; Wipfly *et al.,* 2007; Eggert and Wallace, 2007). As shredder communities may be composed by invertebrates with distinct sensitivities to toxics these communities may suffer significant changes in their composition depending on the type and the intensity of stressor pressure as well as different temperature regimes (e.g. Forrow and Maltby, 2000). Consequently it is very important to study detritivorous communities in order to understand what may be the effects at ecosystem level of increasing stressor pressure by evaluating effects on different single species.

Results of the present Thesis suggest that *S. festiva* is the most resistant species of the three shredders studied to both the influence of chemical stress as a result of the exposure to eucalyptus leaf leachates and copper, and the combined exposure to increased temperature. This apparent increased tolerance of the Trichoptera may be due to the presence of a protective case that prevents the continuous exposure to the toxics especially in acute bioassays where the duration of the tests is relatively short. Despite being deprived of food in short acute toxicity tests the starvation pressure may not be enough to force the invertebrates to abandon the protective environment of the case in order to search for food. Nonetheless in chronic exposures mortality for these invertebrates remained still very low which may suggest that they also have mechanisms to avoid toxicant exposure or are able to decrease toxicant uptake or to increase the rate of toxic

elimination or storage (e.g. Cain *et al*., 2004). Mechanisms of tolerance to metals are present in other Trichopteran larvae (Darlington and Gower, 1990). Similar results are in good agreement with previous findings in the caddisfly *Sericostoma vittatum* exposed to aerated eucalyptus leachates (Canhoto and Laranjeira, 2007; Canhoto *et al.*, 2013). Nonetheless if good oxygenation conditions are not verified, the presence of these leachates may be responsible for increased mortality, being this a very important issue in streams running trough eucalyptus plantations especially in low flow conditions, usually associated with the formation of small pools were oxygen availability is lower.

Despite the very low mortality observed in acute and chronic toxicity tests one must pay attention to further results that may suggest that this species may be affected by the exposure to several toxics concentrations in long and short exposure periods with deterrent consequences other than mortality. Oxidative damage as a result of increased lipid peroxidation values was detected in short and long therm exposure to the stressors especially in the highest temperature. LPO may disrupt cell membranes, affecting their structure and function, possibly leading to an increase in citotoxicity. In fact when exposed to stressors these invertebrates were not able to remain completely homeostatic especially in what concerns phosphorus balances. At the lower temperature a increase in the presence of P in invertebrate's body composition is detected for treatments with high toxic agents concentration while at 20°C invertebrates exposed to the highest copper concentrations had significantly less P amount than control. This inability to remain completely homeostatic at different temperatures in the presence of stressors may influence the presence or absence of this particular species for

example when stream temperature is higher (due to seasonal variations or to different canopy cover as observed for example in streams running through eucalyptus plantantions). Results suggest that the increase in temperature accentuated the difficulty in retaining phosphorus, which may suggest that the interaction between toxic and increased temperature which result in lower phosphorus retention capacity may influence nutrient cycling patterns in small streams. Little attention has been paid to the importance of resource stoichiometry and consumer driven nutrient recycling patterns as a structural function able to modulate community responses (e.g. Danger *et al.,* 2008) but the extent of these relationships and the ability of these communities to adjust should be taken into consideration, especially in the presence of stressors, such as increased contaminantion by chemical agents (e.g. copper) or alterations in riparian land use or environmental factors such as temperature (Mehler *et al.,* 2013).

The decapod *Atyaephyra desmarestii* (Millet, 1831) and the amphipod *Echinogammarus meridionalis* (Pinkster, 1973) are two benthic freshwater crustaceans that occupy an important position in the food chain, since they are important food items for several species of fish. Moreover, they both feed on coarse particulate organic matter, playing an important role in detritus processing and nutrient cycling in Portuguese streams. Relative toxicity for these two invertebrates is both chemical and temperature dependent. As a consequence of acute exposure, results indicate that at 10°C both species have similar relative sensitivity to both stressors and a temperature raise from 10 to 20°C has found to significantly increase the toxicity of copper. At 20°C an increase in the toxicity of

eucalyptus leachates to *A. desmarestii* and of copper for both species with more strong effects on *E. meridionalis* is observed. These results suggest that exposure to these two stressors may be the result of different mechanisms of toxicity and biotransformation. In fact simultaneous exposure of *A. desmarestii* and *E. meridionalis* to copper and eucalyptus leachates resulted in toxicological interactions, synergism in *E. meridionalis* and antagonism in *A. desmarestii*. One possible explanation is, in the case of *A. desmarestii*, that eucalyptus extracts may have anti-oxidant properties (Sacchetti *et al*., 2005; Singh *et al*., 2012), probably counteracting deterrent effects of copper exposure as an oxidative stress inducer (Bouskill *et al*., 2006; Sroda and Cossu-Leguille, 2011) explaining the antagonism found. On the contrary synergistic effects were observed in *E. meridionalis*, which suggested that the combination of both stressors resulted in a more negative output. A few studies suggest that amphipods may be extremely sensitive to low pH values affecting osmoregulation (Felten *et al*., 2008) and also respond negatively to the presence of metals (Dédourge-Geffard, 2009), therefore the combination of both stressors (as eucalyptus leachates solution is characterized by low ph values) may be responsible for the increased mortality observed here. Crustacea are known to be quite tolerant to copper, an essential element used in their hemolymph (Gerhardt, 1995) but it has been reported different patterns in metal acumulation between decapoda and amphipoda (Rainbow and White, 1989; Rainbow, 1998), that may result in different metal sensitivity, as results seem to suggest here. Also, it is imperative to highlight the importance of rearing temperature in determining the relative toxicity of stressors to these related species as results suggest that an increase in temperature may alter the relative

sensitivity of these species. Therefore the possible balance between populations of *A. desmarestii* and *E. meridionalis* coexisting may be altered after exposure to copper or eucalyptus leachates depending on the temperature. If this distinct sensitivity to stressor's presence is extendable to other shredder species in the benthic community, longitudinal (upstream to downstream) diversity may be altered (Clements *et al.,* 2002). Between the two crustacean, *A. desmarestii* appears to be slighty more resistant which is concomitant with its wide geographic range (Anastasiadou *et al*., 2004) nonetheless it may be affected by the presence of both stressors with increasing temperature.

Stressors in a changing climate: the importance of temperature.

Temperature affects all biological processes and is predicted to exponentially increase metabolic rates (Brown *et al*., 2004*b*), with effects at individual, population and community levels (Savage *et al*., 2004). Temperature increase due to seasonal variations may be exacerbated by the projected increase in global temperature (IPCC, 2007). As invertebrates aren't able to regulate internal body temperature, increasing temperatures may have negative effects on their ecophysiology (Woods *et al*., 2003). Our results seem to suggest that despite its inherent toxicity, the exposure to either eucalyptus leachates or copper and the consumption of eucalyptus leaves, may be exacerbated by increased temperature. Increased toxicity with increased temperature has been reported in several invertebrates exposed to different toxics (Rathore and Khangaroth, 2002; Boeckman and Bidwell, 2005; Prato *et al*., 2009). Several explanations have aroused to explain this, for example increased metabolic rates at higher

temperature that may lead to increased respiration rates which enhances toxic exposure. Increased accumulation rates of metals have also been detected in invertebrates with increasing temperature (Serafim *et al.*, 2002; Kopecka-Pilarczyk, 2010). Also, enzymatic activity is strongly influenced by temperature with changes on enzyme's physical structure, catalytic efficiency or binding capacity which can negatively influence by itself invertebrate's ability to deal with toxic presence. In fact increased temperature has significantly increased the toxicity of eucalyptus leachates to *A. desmarestii* and of copper to both *A. desmarestii* and *E. meridionalis* in acute exposures. Following acute exposure, biomarker determination suggest that an increase in temperature is able to modulate biomarkers activity for all the three invertebrates which may indicate that it is an important stressor factor by itself. The increase in rearing temperature is able to significantly modulate survival patterns for the invertebrates (*S. festiva* and *E. meridionalis*). Stoichiometrical analysis suggest that for *S. festiva* an increase in 10°C in rearing temperature combined with the presence of high copper concentrations is responsible for a nutrient deficiency (P) that may affect ribosome synthesis, with a possible reduction in protein synthesis and consequently may affect somatic growth (Elser *et al.* 2000). Similar results have been observed for other species (Persson *et al.*, 2011; Wojewodzic *et al.*, 2011).

Eucalyptus leaf extracts: are we neglecting the importance of leachates toxicity?

The effects of eucalyptus plantations on streams ecosystems have been intensively studied, nonetheless attention paid to the effects of eucalyptus leaf leachates toxicity on shredders remains scarce. In this work, results suggest, that the presence of these leachates is able to trigger several physiological responses in shredders. Increased mortality was observed in *E. meridionalis* and *A. desmarestii* in acute exposures and in *E. meridionalis* after chronic exposures and to a lesser extent to *S. Festiva*. As we know, eucalyptus leaves inputs occur throughout the year (as opposed to deciduous forests which occur mainly in autumn) so a continuous supply of leaves occurs. In autumn/winter, high flows may mitigate negative consequences of these leachates but in low flow seasons this can become a serious ecological problem. Eucalyptus leachates may present very low pH values and high phenolic and tannin content and reduced oxygen is frequently observed in leachates pools, which may contribute to their increased toxicity. As observed in this study (Chapter IV), even small amounts of these leachates may trigger alterations in biomarkers activities as occurred with *S. Festiva*. When exposed to eucalyptus leaf leachates invertebrates weren't able to remain completely homeostatic at the lower temperature. Growth, consumption and survival of *Sericostoma vittatum* larvae were also negatively affected by the presence of eucalyptus leachates (Canhoto and Laranjeira, 2007). Eucalyptus leaves possess oil vesicules, with cineol and pinene as major components, that are toxic for invertebrates (Canhoto and Graça, 1999). Also the presence of phenolic compounds in the environment that may adsorb to the surface of other leaves may

contribute to overall impacts observed in these communities. Nonetheless, this is a subject that is poorly understood and in my opinion deserves further thought, so efforts should be made to best understand the impacts of eucalyptus leaf leachates on individual shredders.

Final remarks and future research

Monitoring of freshwaters has been based on physical and chemical parameters and through invertebrate community structure measures as biomonitoring tools. This approach, focused on benthic macroinvertebrates and biotic indices, may have some inconveniences due to the difficulty in applying several biotic indices in Mediterranean rivers, with specific hydrological regimes (Coimbra and Graca, 1996). Another methodology may be the use of single species responses to stressors in order to complement and enrich macroinvertebrate surveys and physico-chemical characterization. The development of toxicity tests that take into account the presence of chemical agents (such as eucalyptus toxins and copper) are needed to accurately and realistically assess the effects of contamination on freshwater ecosystems. In real scenarios toxics agents seldom occur isolated thus in order to realistic evaluate environmental contamination one must pay attention to the importance of evaluating mixtures of these environmental contaminants. The species used in this study allowed the comparison of representatives of major aquatic groups (Insecta and Crustacea) and also of different microhabitats and the results of this study suggest that the three selected species have distinct sensitivities to the stressors studied here. Further studies with different shredders, where the traditional ecotoxicological

bioassays (with endpoints such as mortality) are used must be combined with biochemical approaches (evaluation of distinct biomarkers activity), or the determination of organism's elemental composition and evaluated at different levels of biological organization in order to successfully protect stream's communities. Results of this study suggest that the combination of distinct assays is useful in order to establish impacts of chemical contamination to shredders communities and that distinct invertebrates may have distinct sensitivity to stressors although, and especially concerning the effects of eucalyptus leachates exposure more research must yet be done.

Chapter VII

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Appendix

Appendix 1- Mortality (%) of *Atyaephyra desmarestii*, *Echinogammarus meridionalis* and *Schizopelex festiva* recorded in bioassays carried out at both 10 and 20°C with single eucalyptus leachates (EL), single copper (Cu) and with both stressors

		A. desmarestii		***E. meridionalis***		***S. festiva (c)***				
						1st bioassay		**2nd bioassay**		
	Concentration	**10°C**	**20°C**	**10°C**	**20°C**	**10°C**	**20°C**	**Conc.**	**10°**	**20°C**
EL (mg/L)	0	0%	0%	0%	20%	0%	0%	0	0%	0%
	7	0%	10%	10%	20%	0%	0%	279	-	10%
	15	0%	30%	20%	20%	0%	0%	325	-	20%
	29	10%	40%	20%	30%	0%	0%	349	10%	-
	58	10%	50%	30%	30%	0%	0%	412	20%	-
	116	40%	90%	30%	40%	0%	0%	434		40%
	233	60%	100%	90%	90%	0%	0%	465	30%	-
	465	100%	100%	100%	100%	30%	60%			

Table 2 cont.				***A. desmarestii***		***E. meridionalis***		***S. festiva (c)***	
	Concentration			**10°C**	**20°C**	**10°C**	**20°C**	**10°C**	**20°C**
	(a)	(b)	(c)						
Cu (mg/L)	0	0	0	0%	0%	0%	20%	0%	0%
	0.03	0.006	0.25	10%	20%	-	20%	0%	0%
	0.05	0.01	0.51	30%	30%	-	30%	0%	0%
	0.10	0.03	1.02	50%	60%	10%	30%	0%	0%
	0.20	0.05	2.04	70%	80%	10%	40%	10%	20%
	0.41	0.10	4.07	90%	90%	20%	80%	30%	30%
	0.81	0.20	8.14	90%	100%	30%	90%	40%	50%
	3.36	0.41		100%	100%	60%	100%		
		0.81				90%	-		

Table 2 cont.		***A. desmarestii***		***E. meridionalis***		***S. festiva* (c)**	
	Concentration	**10°C**	**20°C**	**10°C**	**20°C**	**10°C**	**20°C**
	EL 0 + CU 0 (control)	0%	0%	15%	20%	0%	0%
Mixtures	EL-LC10 + Cu-LC10	0%	20%	22.5%	67.5%	0%	20%
EL+CU	EL-LC20 + Cu-LC20	10%	20%	35%	47.5%	10%	40%
	EL-LC50 + Cu-LC50	40%	70%	75%	97.5%	30%	60%