Universidade de Aveiro Departamento de Biologia
Ano 2012

CARLA PATRÍCIA QUINTANEIRO ANTUNES

AVALIAÇÃO DE TOXICIDADE DE METAIS ESSENCIAIS EM DETRITÍVOROS AQUÁTICOS

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Prof. Doutor António José Arsénia Nogueira, Professor Associado com Agregação do Departamento de Biologia da Universidade de Aveiro e do Prof. Doutor James Francis Ranville, Professor Associado, Colorado School of Mines, EUA.

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Dedico este trabalho ao Kurcudiluh.

o júri

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palavras-chave

Metais essenciais; detritívoros; Ecossistemas de água doce; *Atyaephyra desmarestii*; *Echinogammarus meridionalis*; inibição alimentar; biomarcadores

resumo

Os contaminantes provenientes quer de fontes naturais quer como consequência da atividade humana, têm contribuído para a degradação dos ecossistemas aquáticos. Entre estes encontram-se os metais que podem, ou não ser essenciais mediante o papel que desempenham no metabolismo dos organismos. O cobre e o zinco são exemplos de metais essenciais, contudo quando atingem concentrações elevadas podem tornar-se tóxicos. Os detritívoros aquáticos desempenham um papel fundamental na decomposição da matéria orgânica, alimentando-se de carcaças e partes de plantas que caem nos cursos de água. Assim, estes organismos permitem que o ciclo dos nutrientes se complete e servem como elo de ligação entre todos os grupos funcionais do ecossistema mantendo o seu equilíbrio estrutural e funcional. Sendo a matéria orgânica a sua principal fonte de energia estão sujeitos à contaminação existente no meio, pelo que é de todo o interesse proceder-se à avaliação dos efeitos da toxicidade de metais nestes organismos. Uma vez que as diferenças comportamentais consequentes desta exposição podem originar variações na densidade e diversidade, o que se refletirá a nível das comunidades, originando alterações na estrutura e funcionamento do ecossistema. Tendo em vista a avaliação dos efeitos da contaminação por metais em detritívoros, o principal objetivo deste trabalho foi comparar a sensibilidade a metais essenciais de dois detritivoros aquáticos, o camarão *Atyaephyra desmarestii* e o anfípode *Echinogammarus meridionalis*. Para tal, avaliaram-se os efeitos do cobre e do zinco a diferentes níveis de organização biológica. Primeiro, foram determinadas as preferências alimentares de *A. desmarestii* e *E. meridionalis* considerando tanto a área das folhas como a contaminação por metais das folhas. Em seguida, avaliaram-se os efeitos do cobre e do zinco na sobrevivência e inibição alimentar de ambas as espécies. Finalmente, avaliaram-se os efeitos destes mesmos metais a nível bioquímico utilizando uma bateria de biomarcadores que incluiu enzimas de stresse oxidativo, o sistema de defesa antioxidante e as colinesterases. Ambos os organismos não mostraram preferência em relação a folhas de área diferente. A presença de uma maior ou menor concentração de metais essenciais no alimento não teve qualquer influência na sua escolha pelo alimento (contaminado ou não). Os ensaios agudos de cobre e zinco mostraram que o cobre é mais tóxico para ambas as espécies do que o zinco. O camarão demonstrou ser mais sensível ao zinco que o anfípode, tendo este sido mais sensível ao cobre (CL50 do cobre para *A. desmarestii* foi de 0,128 $mg.l^{-1}$ e o de *E. meridionalis* foi de 0,050 $mg.l^{-1}$; os valores correspondentes para o zinco foram 7,951 e 11,860 $mg.l^{-1}$, respectivamente. Em relação aos efeitos sub-letais, o cobre teve efeitos notórios na taxa de alimentação de *E. meridionalis*, mas não afectou a de *A. desmarestii*. No que diz respeito à exposição ao zinco, ambas as espécies parecem apresentar tendência para inibir a

alimentação. A caracterização das colinesterases revelou que a principal forma presente em ambas as espécies é a acetilcolinesterase, a qual que não foi afetada pela presença dos metais, no caso do camarão, mas parece ser inibida pelo zinco no caso do anfípode. O cobre inibiu o sistema de defesa enzimático de ambas as espécies, sem sinais de danos lipídicos. Para além disto, inibiu uma das enzimas antioxidantes (GPx) do anfípode. Apesar de não ter ocorrido dano lipídico após exposição ao cobre, observou-se um ligeiro aumento dos níveis das LPO, o que pode ser indicativo de uma potencial existência de dano oxidativo, como resultado da falha do sistema de defesa antioxidante. Por outro lado, o zinco induziu o sistema de defesa em *E. meriodionalis* prevenindo o dano lipídico. Enquanto em *A. desmarestii* o sistema enzimático antioxidante não respondeu, tendo ocorrido dano celular oxidativo considerando-se, assim, que o sistema de defesa antioxidante do camarão pode ser comprometido por exposição a metais. Ainda que os danos celulares oxidativos tivessem ocorrido a baixas concentrações de zinco. A exposição a este metal também induziu a actividade da GST de *E. meriodionalis*. Considerando que a taxa de alimentação foi severamente reduzida no caso deste organismo, o zinco parece ser o metal cuja concentração no ecossistema requer maior atenção. Integrando as respostas dos biomarcadores parece também evidente que *A. desmarestii* responde de uma maneira geral a maiores concentrações dos dois metais, enquanto a resposta de *E. meridionalis* ocorre a concentrações inferiores. Pelo que, *E. meridionalis* parece ser mais sensível ao nível bioquímico. Neste trabalho, os dois detritívoros, com ligeiras diferenças no modo como utilizam a matéria orgânica disponível, apresentam diferenças na sensibilidade aos metais essenciais a vários níveis de organização biológica, sendo o zinco o metal que poderá causar maior preocupação a nível bioquímico, enquanto o cobre parece ser o mais tóxico ao nível do organismo, causando mortalidade a concentrações mais baixas.

keywords

Essential metals; detritivores; freshwater ecosystems; *Atyaephyra desmarestii*; *Echinogammarus meridionalis*; feeding inhibition; biomarkers

abstract

The constant input of contaminants to freshwater ecosystems, from either anthropogenic or natural sources, has contributed over the years to ecosystem degradation. Among them are metals, which can be divided into non essential and essential catagories by means of their role in organisms metabolism. Copper and zinc are essential metals. However, despite their importance for organisms, when high concentrations are achieved they can be toxic. Aquatic detritivore feeding, namely on carcasses and plant leaves that fall into watercourses, have a fundamental role in organic matter decomposition allowing the accomplishment of nutrient cycles. Therefore, these organims act as a link for all ecosystem functional groups, allowing the maintenance of ecosystem structural and functional equilibrium. As organic matter is their main source of energy, they are exposed to environment contamination and therefore toxicity of metals to detritivores must be assessed. Alterations in their behaviour can lead to variations in the diversity and density of communities and consequently induce deleterious effects to the equilibrium of aquatic ecosystems. The main goal of the present work is to assess the effects of metal contamination on detritivores. To achieve this goal we evaluated the sensitivity of two aquatic detritivores, the shrimp *Atyaephyra desmarestii* and the amphipod *Echinogammarus meridionalis* to two essential metals, copper and zinc. Different sets of experiments at different organization levels were performed. Firstly, we assessed the feeding preferences of *A. desmarestii* and *E. meridionalis* considering both leaf area and metal contamination. Secondly, we evaluated the effects of copper and zinc on survival and ingestion rates of both species. Finally, the effects at the biochemical level were assessed using a battery of biomarkers, including oxidative stress enzymes, the defence antioxidant system and cholinesterases. In general, the organisms did not show any preference regarding leaf area and the presence of copper or zinc did not influence their feeding choice. The acute assays with copper and zinc showed that copper is more toxic to both species than zinc. the shrimp species is more sensitive to zinc than the amphipod which is more sensitive to copper. The LC50 values for copper were 0.128 $mg.l^{-1}$ and *A. desmarestii* and 0.050 $mg.l^{-1}$ for *E. meridionalis*. The correspondent values for zinc were 7.951 and 11.860 $mg.l^{-1}$, respectively. Considering sub-lethal effects, copper caused delaterious effects on the feeding rate of *E. meridionalis* but did not affect *A. desmarestii*. On the other hand, after exposure to zinc for both species showed some tendency for feeding inhibition. The main form of cholinesterase present in both species is acetylcholinesterase. This enzyme was not affected by the presence of copper and zinc in the case of the shrimp, but was apparently inhibited by zinc in the amphipod species. The enzymatic defence system of both species was inhibited by the exposure to copper, without any signs of oxidative damage. Furthermore, copper inhibited one of the anti-oxidants enzymes (GPx) of the amphipod *E. meridionalis.* Despite the fact that no lipidic damage occurred after exposure to copper, there was a slight increase in LPO levels. This might be indicative of a potential occurrence of oxidative damage, as a result of a failure of the antioxidant defence system. On the other hand, zinc induced the antioxidant defence system in *E. meriodionalis*, which prevented the occurrence of lipidic damage. In *A. desmarestii*, the antioxidant

enzymatic system did not respond and oxidative cellular damage occurred and thus it appears that the antioxidant defence system of the shrimp species could be compromised by metal exposure. Furthermore, the oxidative cellular damage was observed for low concentrations of zinc. Exposure to zinc also induced the activity of the amphipod GST. Considering that the feeding rate was also severely reduced in *E. Meridionalis,* zinc seems to be a metal of major concern. From analysis of the integrated biomarker response it also seems evident that *A. desmarestii* responds in general to higher concentrations of both metals while *E. meridonalis* to lower concentrations. Thus, *E. meridonalis* is more sensitive at biochemical level. In this work, the detritivores, with slightly differences in available organic matter use, presented differences in sensitivity to metals at several biological organisation levels. Moreover, zinc seems to be a metal of major concern at the biochemical level, more so than copper, which however seems to be the most toxic at the organism level, leading to mortality at lower concentrations.

TABLE OF CONTENTS

FIGURES

TABLES

CHAPTER 1. General Introduction

1. General Introduction

1.1. Freshwater ecosystems

Freshwater ecosystems play a key role in the biosphere, as they support unique and complex ecological communities, conduct water and nutrients from continental areas to the sea and are often responsible for defining the structure and functioning of the surrounding terrestrial ecosystems (Bailey et al., 2004). Furthermore, they are essential to sustain human existence, as they are an important source of fishery, crops irrigation, drinking water and hydroelectric power; they serve also as a recreational area, as well as for other activities such as related with industry and transportation. Therefore, individuals and societies have settled preferentially close to water bodies (Revenga et al., 2005). Thus, assessment, protection and restoration of "health", "status" or "condition" of freshwater ecosystems is a priority since humans, through their activities, are primarily responsible for the degradation of these ecosystems.

Freshwater ecosystems are complex and dynamic and are subjected to a wide range of natural variation, under disturbance, to maintain viability or resilience (Baron et al., 2002). These systems consist of groups of species, from different trophic levels, interacting with the abiotic component of the ecosystems being affected by physical, biogeochemical, and ecological processes that act on and among them. Hence, alterations in these attributes and processes will ultimately contribute to variable and dynamic responses. Trophic interactions provide the fundamental linkages among species (Newman and Clements, 2008). The structure of freshwater communities is determined by these interactions (Figure 1.1) where producers are responsible for photosynthesis and constitute sources for the first consumers (herbivores) that serve as prey for the second consumers (predators) which are in turn prey for higher predators. Organisms in all groups produce detritus whether in the form of faeces or dead organisms (Newman and

Clements, 2008). These detritus are the main source of energy for another trophic group, the detritivores and decomposers. They are responsible for the degradation and transformation of this organic matter into inorganic nutrients that are needed by producers. Thus, the flow of energy and transport of materials through ecosystems are intimately linked. It is well established that flow of energy through biological systems is inefficient with only 10% of energy being transferred from one trophic level to another. On other hand, the abiotic materials, such as nutrients and carbon, are cycled through ecosystems, and the amount of these materials increases with trophic level.

In freshwater ecosystems, sediments are often a "sink" for contaminants from natural or anthropogenic origins. In this sense, some of freshwater invertebrates, because of their preference for benthic habitats, can be confronted with environments with high levels of contamination, which can ultimately lead to stress situations (Dallinger and Rainbow, 1993).

1.1.1. Detritivores

Detritivores are essential to preserve the structure and functioning of freshwater ecosystems by feeding on dead plants and animals and their detritus. They also have an important role in litter decomposition (Allan and Castillo, 2007) and the recycling of nutrients for producers (Covich et al., 1999). Furthermore, they are also a food source for several predatory species (Forrow and Maltby, 2000; MacNeil et al., 2000; Alonso et al., 2009). Therefore, detritivores serve as linkages to all functional groups of freshwater ecosystems (Figure 1.1).

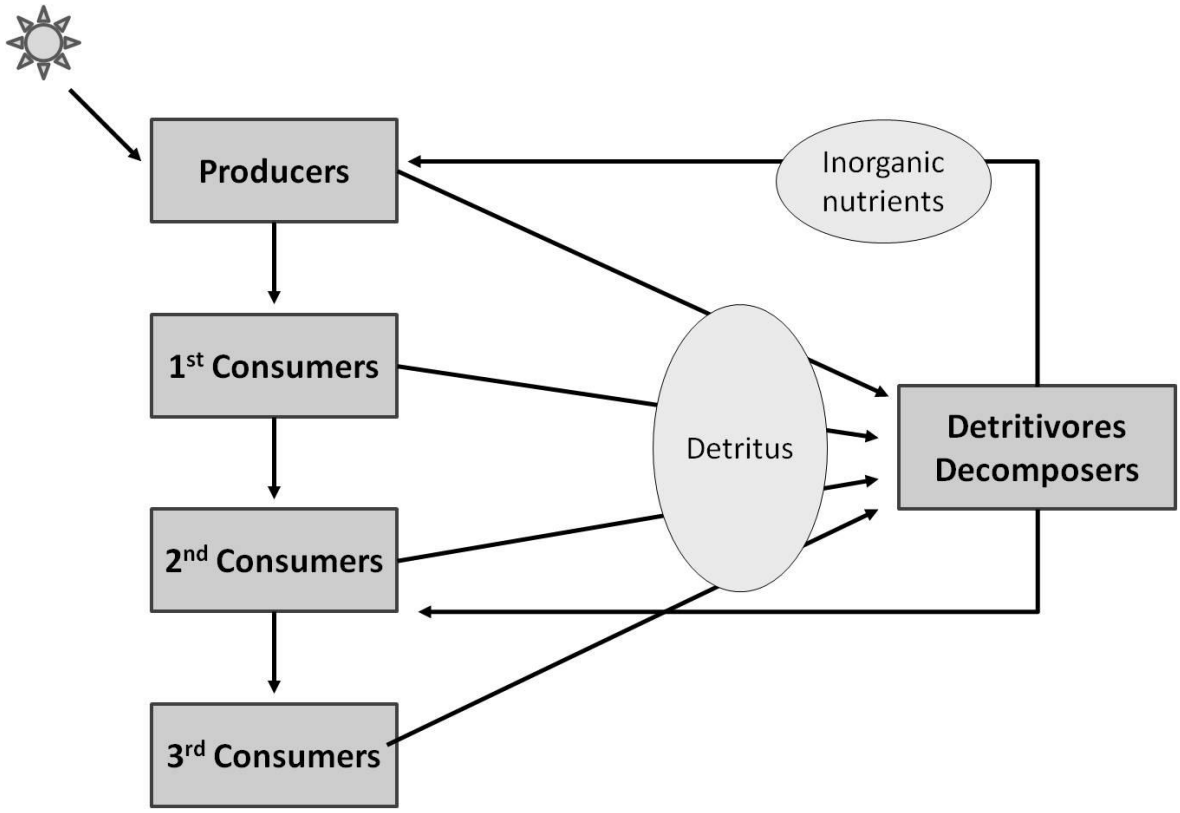

Figure 1.1: Simplified representation of interactions of principal functional groups in the freshwater ecosystem structure.

Detritivores can be exposed to chemical substances through food (Wilding and Maltby, 2006), and other routes. The decaying leaf material and detritus in the natural environments can accumulate chemical substances, like metals, present in the water column, introduced from the subjacent terrestrial ecosystems or from direct sources. Once in the water column, metals can be incorporated or adsorbed to organic matter in high amounts (Maltby and Crane, 1994; Sridhar et al., 2008). Therefore, organic matter, which allows a good functioning of metabolic processes by inputting essential metals (Dobson et al., 2004) can also be a source of toxic effects when the concentrations exceed organism needs and the equilibrium of physiological/detoxification processes is broken. Therefore, the effects of metals or other chemical substances on organisms of this group can lead to alterations of their normal breakdown detritus behaviour, which can have

impacts on litter decomposition and consequently on ecosystems processes (Bjelke and Herrmann, 2005).

Atyaephyra desmarestii Millet and *Echinogammarus meridionalis* Pinkster, two species that belong to the functional group of detritivores, were selected for this work. *A. desmarestii* is a freshwater decapoda that occurs in the Mediterranean area and is distributed throughout the Middle-East and European freshwater ecosystems, with the exception of the Britain Islands. This caridean inhabits slowly running waters (lentic systems) associated with aquatic plant communities. It is characterized by a high tolerance to temperature and salinity variations (Janssens de Bisthoven et al., 2006), and is an omnivore species that feeds on fine particulate organic matter and lives attached to periphyton (Callisto, 2006). Despite feeding on coarse organic matter (COMP, <1mm) (Pestana et al., 2007), this shrimp prefers fine organic matter particles (FMOP, <0.5mm) obtained from scraping the surface and breaking the leaves (Meurisse-Genin et al., 1985). *A. desmarestii* also serves as food for several predator species of fishes (Fidalgo and Gerhardt, 2002). Thus, it is an important link in food-webs, and can be a potential model organism for ecotoxicological studies. A few studies were performed with this species in relation to contaminant exposure, including the assessment of sub-lethal level endpoints as behaviour and feeding rates after metal exposure (Janssens de Bisthoven et al., 2006; Pestana et al., 2007), and mortality after exposure to bleached kraft mill (Ferreira et al., 2002) and textile effluents (Casimiro and Fidalgo, 2008).

Like *A. desmarestii*, the amphipod *E. meridionalis* also lives in slowly running waters with associated aquatic plant communities, and occasionally with some degree of pollution resulting from domestic effluents (Macedo-Sousa et al., 2007). So far, the biology of this species is not well known. However, as detritivore, it feeds primarily on detritus, thus, it can also be exposed to

contaminants, associated with water and detritus, in freshwater aquatic ecosystems. As an amphipod, a group that is widely used in ecotoxicology, it also appears to have some potential utility in the assessment of effects of metals on freshwater ecosystems, as a sentinel species. This amphipod is a fragmenter that feeds primarily on coarse organic matter particles, it has an important role in detritus processing, and consequently in freshwater ecosystem structure and functioning (Macedo-Sousa et al., 2007). A limited amount of studies were performed with this species in relation to exposure to contaminants. For example, its feeding rate was assessed after exposure to metals (Pestana et al., 2007) and effects of an acid mine drainage effluent on locomotion, feeding behaviour and ventilation were also evaluated (Macedo-Sousa et al., 2007).

1.2. Freshwater ecotoxicology

The toxicity screening of polluted environments is done usually by standardized methods that can involve acute and chronic responses of a sensitive bioindicator. Usually, the mortality, feeding and behaviour, as well alterations in reproduction are used as endpoints for acute and chronic toxicity, respectively. Despite these methods being fundamental, the information obtained about the mechanisms of the observed response itself is scarce and limited when using them alone. Moreover, the evaluation of effects at more ecological relevant higher levels of biological organisation (e.g. community, population) is very complex due to an amalgam of factors that can contribute to the observed stress response and to the time needed to assess them (Figure 1.2). While, in lower biological organisation levels (e.g. sub-individual, molecular) despite the lower ecological relevance, the complexity and time of analysis decreases and more information on the mechanisms of toxicity may be obtained (Figure 1.2). This could be important if remediation and prediction actions need to be implemented. Thus, the use of lower levels of biological organisation with the further extrapolation to higher levels have been suggested for ecotoxicological programs and ecological

risk assessment (ERA), highlighting the so called biomarkers as a "early warning tools" (Stegeman et al., 1992).

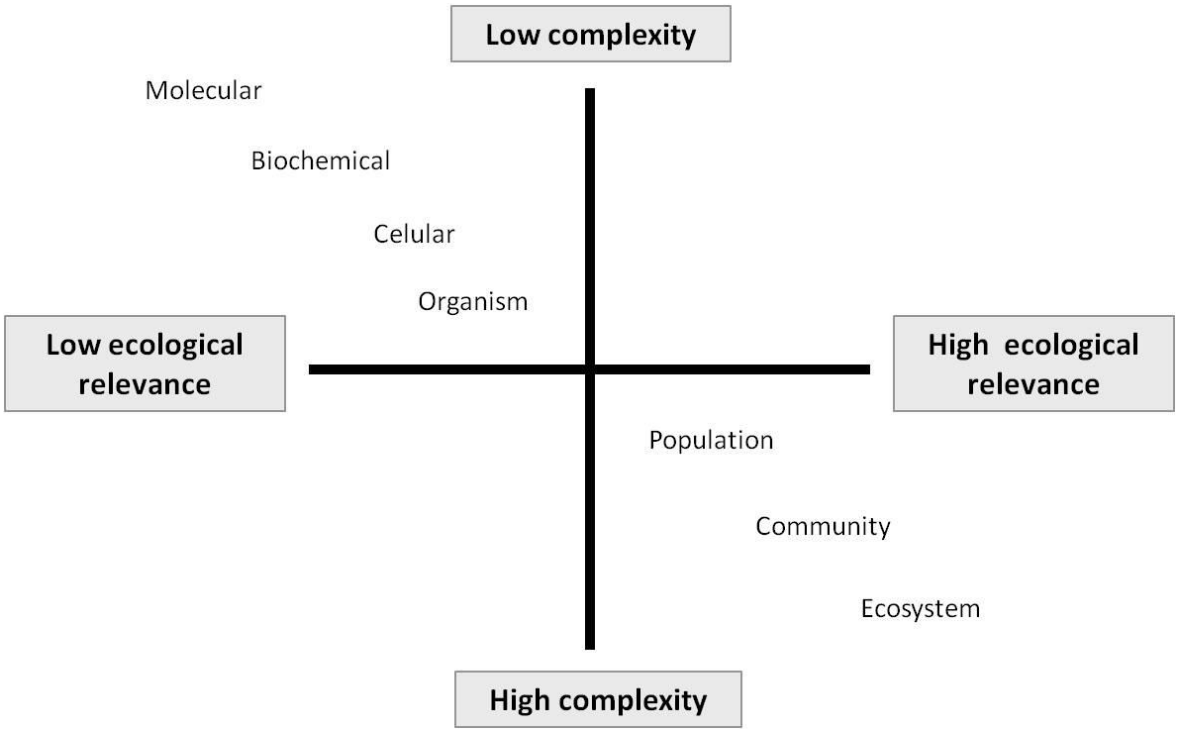

Figure 1.2: Representation of the levels of biological organisation in relation to the complexity and ecologic relevance.

The term biomarkers has been used as a designation for the biochemical alterations that are the reflex of the interaction between the biological system and the potential risk (Peakall, 1992). The establishment of the link between exposure and effect is due to the ability to measure a characteristic response to a chemical (Peakall, 1992). Van Gestel and Van Brummelen (1996) suggested that a biomarker is any response at sub-individual level to xenobiotics, quantified in the organism itself or in its products, indicating a deviation from the normal response of non exposed organisms. The use of a battery of biomarkers and specially the system biology approach with the integration of responses at different levels of biological organisation is nowadays one great challenge for ecotoxicologists.

1.3. Essential metals

Some potentially toxic compounds, such as metals, are released into the aquatic environment from natural processes such as volcanic activity and the weathering of rocks, but also through human activities, such as industrial processes, which have greatly increased the concentrations of many toxic compounds in the environment. Organisms can be exposed to metals from different sources (e.g. water, sediment, food), and their potential to cause adverse effects depends of their bioavailability, that is influenced by several factors like chemical composition of water and sediments, and size of food and sediment particles (Rainbow, 1997). At high concentrations, metals can induce harmful effects either at physiological or biochemical levels (Sunda and Hanson, 1987). These deleterious effects can after be reflected in population densities or community composition and ultimately lead to the extinction of an entire population affecting ecosystem structure.

Metals are commonly divided into non-essential metals (e.g. cadmium, mercury), which have no known function in organisms; and essential metals (e.g. copper, zinc) that have important roles in metabolic functions (Rainbow, 1997, 2002). Organisms can have different sensitivities to high levels of essential metals. For example, some have the capability to bioaccumulate and store them, while others are able to regulate them. The accumulation of essential metals requires mechanisms of detoxification, usually binding to high affinity sites of inorganic granules or to proteins with detoxification roles, including metallothionines or ferritins (Rainbow, 2007). The organisms which regulate the internal concentration of essential metals are able to excrete the excess metal so the balance between uptake/excretion is maintained and the internal concentration remains the same (Rainbow, 2007). Therefore, toxicity does not depend of the total metal concentration accumulated, but is related to the internal metabolically available threshold, and occurs when the rate of metal uptake exceeds the rates of detoxification and excretion, and the available metal

molecules interact with molecular targets. This may interfere with the normal functioning of cells and systems (Rainbow, 2007).

Copper and zinc are essential metals for several organisms, including crustaceans. Copper, is a strong oxidizing essential metal widely distributed in the environment and is involved in heamocyanin transport in molluscs and crustaceans, being also an important of some enzymes (Reddy et al., 2006; Rainbow, 2007). Deficiency in copper can decrease several processes of the antioxidant system of organisms, changing their cellular redox status (Uriu-Adams et al., 2005), while its excess can induce cellular toxicity (Viarengo et al., 1990; Livingstone, 2001).

Zinc is also an essential metal widely distributed in the environment that is involved in the functioning of more than 200 enzymes (Muyssen and Janssen, 2002). Some authors believe that zinc has an important function as an antioxidant and is responsible for the stabilization of cell membranes as well as having some function at the cell proliferation level (Milos, 1973; Bray and Bettger, 1990; MacDonald, 2000; Voie and Mariussen, 2010). Deficiency of zinc in organisms may result in growth delay and reduction of food intake (MacDonald, 2000).

1.4. Organism level endpoints

The standard measured effects of contaminants can be of short duration (acute tests) or long-term (chronic tests). Acute tests are mainly used as a “screening tools” and use mainly mortality as endpoint. Usually, in this type of tests, the concentrations are far from the concentrations found in the environment, being for this reason quite unrealistic tests. On the other hand, long-term tests are often applied to evaluate effects of long-term exposures to low sub-lethal concentrations of chemicals.

As food is the main input of energy in crustaceans (Santos et al., 2000), sub-lethal effects of metals in the feeding behaviour is a valuable endpoint of. In this sense, feeding tests were developed, using feeding rate as an indicator of stress, which

is sensitive to environmental changes. Feeding rate is also an ecologically relevant parameter since it can be linked to growth, reproduction and survival of organisms, allowing the extrapolation of individual effects to higher levels of biological organisation (Maltby, 1999; Slijkerman et al., 2004; Pestana et al., 2007). Several works were done in order to understand how metals inhibit the feeding rate of organisms during and after the exposure periods (Santos et al., 2000; Wilding and Maltby, 2006; Macedo-Sousa et al., 2007; Pestana et al., 2007; Satapornvanit et al., 2009). However, to the best of our knowledge, only few studies have been done to assess the recovery of feeding in organisms after exposure to metals.

1.5. Biochemical endpoints

Going down in organisation level, markers at the biochemical level have being considered as "early warning tools" for the assessment of environmental quality and are nowadays, routinely used (Peakall, 1992). Among them, the activity of the enzymes cholinesterases, anti-oxidant enzymes and other parameters of the anti-oxidant system have been widely used in studies with vertebrates and invertebrates.

1.5.1. Cholinesterases

Cholinesterases (ChE), have been wdely used to assess the effects of contaminants on freshwater organisms, especially in relation to organosphosphates and carbamates insecticides (Peakall, 1992; Fulton and Key, 2001; Key et al., 2003). However, recent studies suggest that ChE can also be used to monitor freshwater environments apparently not contaminated with pesticides as the normal level of ChE activity can be altered by a wide range of contaminants, especially complex mixtures of pesticides and metals (Payne et al., 1996; Hamza-Chaffai et al., 1998; Forget et al., 1999; Diamantino et al., 2000;

Frasco et al., 2005; Frasco et al., 2007). In animal tissues, including crustaceans, ChE are present in several forms that can have different sensitivities to anticholinergic agents, which can be a source of variation in the results of toxicity tests (Bocquene et al., 1990; Garcia et al., 2000).

Acetylcholinesterase (AChE) and pseudo cholinesterase (PChE), are the two main types of ChE and have the ability to hydrolyse the neurotransmitter acetylcholine at cholinergic synapses (Huggett et al., 1992; Darvesh et al., 2003). The role of AChE is well established, it degrades acetylcholine into choline and acetic acid in cholinergic synapses, being important in the normal function of nervous system (Huggett et al., 1992). Therefore, the inhibition of this enzyme leads to an overstimulation of the central and peripheral nervous system, by increasing acetylcholine levels and resulting in the disruption of the nerve function, which can lead to a deleterious effects for the organism and eventually death (Garric et al., 2008). On the other hand, the role of butyrylcholinesterase (BChE), a PChE, remains unclear (Mesulam et al., 2002). Mack and Robitzki (2000) suggested they are involved in the regulation of cell proliferation and in the differentiation of early neuronal stages. It has also been proposed that AChE and BChE have a complementary function depending on the concentration of acetylcholine (Minic et al., 2003). The interest in which ChE type is present in invertebrates has been increasing over the past years, due to the use of these organisms in biomonitoring programs. Thus, several authors have characterised the type of ChE present in different species of this type of organisms (Forget and Bocquene, 1999; Frasco et al., 2006; Quintaneiro et al., 2006; Xuereb et al., 2007; Gagnaire et al., 2008; Bonacci et al., 2009), among others.

1.5.2. Oxidative stress

Some metals are able to induce oxidative stress in organisms with the subsequent production of reactive oxygen species (ROS), potentially associated with alterations at DNA level, proteins and membranes (Vieira et al., 2011). ROS are

oxygen free radicals that result from the reduction of one, two or three electrons of molecular oxygen (O_2), namely the superoxide anion ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) (Huggett et al., 1992). ROS are among the most reactive compounds produced during metal stress (Dazy et al., 2009) and are produced in a continuum by organisms in their metabolic processes, for example in respiration and biotransformation processes (Huggett et al., 1992; van der Oost et al., 2003) even if not exposed to metals. To cope with them, healthy cells activate their antioxidant defence system (van der Oost et al., 2003), which is composed by several enzymes that directly scavenge ROS (e.g. catalase, CAT), internal lipid peroxidation products (e.g. glutathione peroxidise, GPx), and xenobiotics metabolites (e.g. glutathione-S-transferase, GST). The large production of ROS may exceed the capability of defences to deal with them, resulting in situations of oxidative stress and damage (Lushchak, 2011).

Lipid peroxidation (LPO), oxidation of polyunsaturated fatty acids, is one of the major mechanisms though which ROS can damage tissues leading to impaired cellular functions and alterations of membrane properties of the cells. This process may cause the disruption of functions vital for the organism. Several works have demonstrated that exposure to contaminants, including metals, results in enhancements of lipid peroxidation of several tissues (Huggett et al., 1992; Barata et al., 2005; Bouskill et al., 2006).

The biotransformation of xenobiotics is composed by three mains phases, namely oxidation (phase I), conjugation (phase II) and elimination (phase III) (Figure 1.3).

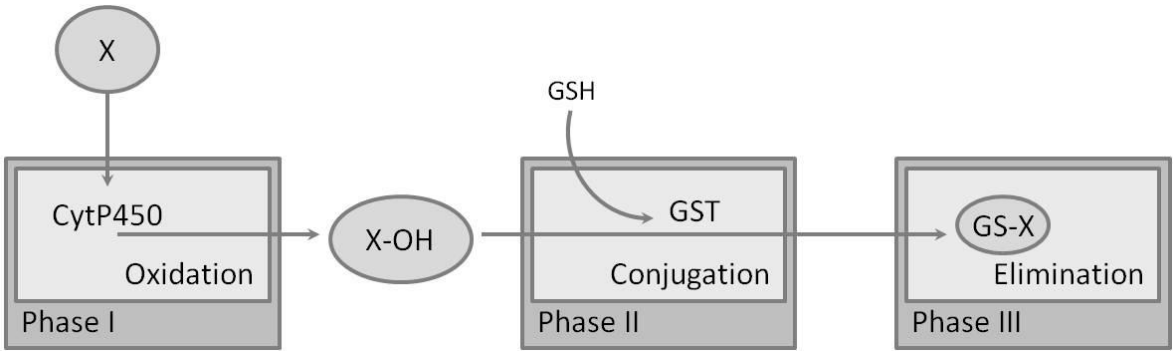

Figure 1.3: Representation of the biotransformation process of a xenobiotic (X).

Several toxicants are oxidised in the first phase of the process by the enzymes associated to the cytochrome P450. In this process, an hydroxyl group is introduced, after that subsequent reactions of conjugation were preformed to link metabolites to several water-soluble endogenous compounds (phase II) in order to increase their solubility and facilitate the elimination (phase III) decreasing its toxicity (Huggett et al., 1992). Glutathione-S-transferases (GST) are an important family of enzymes involved in the phase II of xenobiotic biotransformation with the conjugation of xenobiotics or their metabolites with the tripeptide glutathione as its main role (van der Oost et al., 2003). In addition, they have an important role in the detoxification of exogenous and endogenous compounds. Their activity is induced by the exposure to several compounds, including pesticides, PCBs, PAHs and metals (Setegman et al., 1992; Pedrajas et al., 1995; van der Oost et al., 2003; Gravato et al., 2006; Liu et al., 2006). The activity of the enzymes can also be inhibited by xenobiotics (Setegman et al., 1992; van der Oost et al., 2003). In this process of biotransformation, the release of ROS originates from redox cycle. ROS are subsequently detoxified by the antioxidant defence system.

Superoxide dismutase (SOD) is the first line of defence in oxidative stress, as it catalyses the reaction by which $O_2^{\bullet}$ is transformed into H_2O_2 and O_2. It is then followed by the activation of catalase (CAT), the primordial enzyme responsible

for scavenging H_2O_2, which catalyses the transformation of this radical into water and oxygen, facilitating its removal. The interpretation of CAT activity alterations could be difficult as this enzyme is localised mainly in peroxissomes of most cells and is involved in fatty acid metabolism (Huggett et al., 1992).

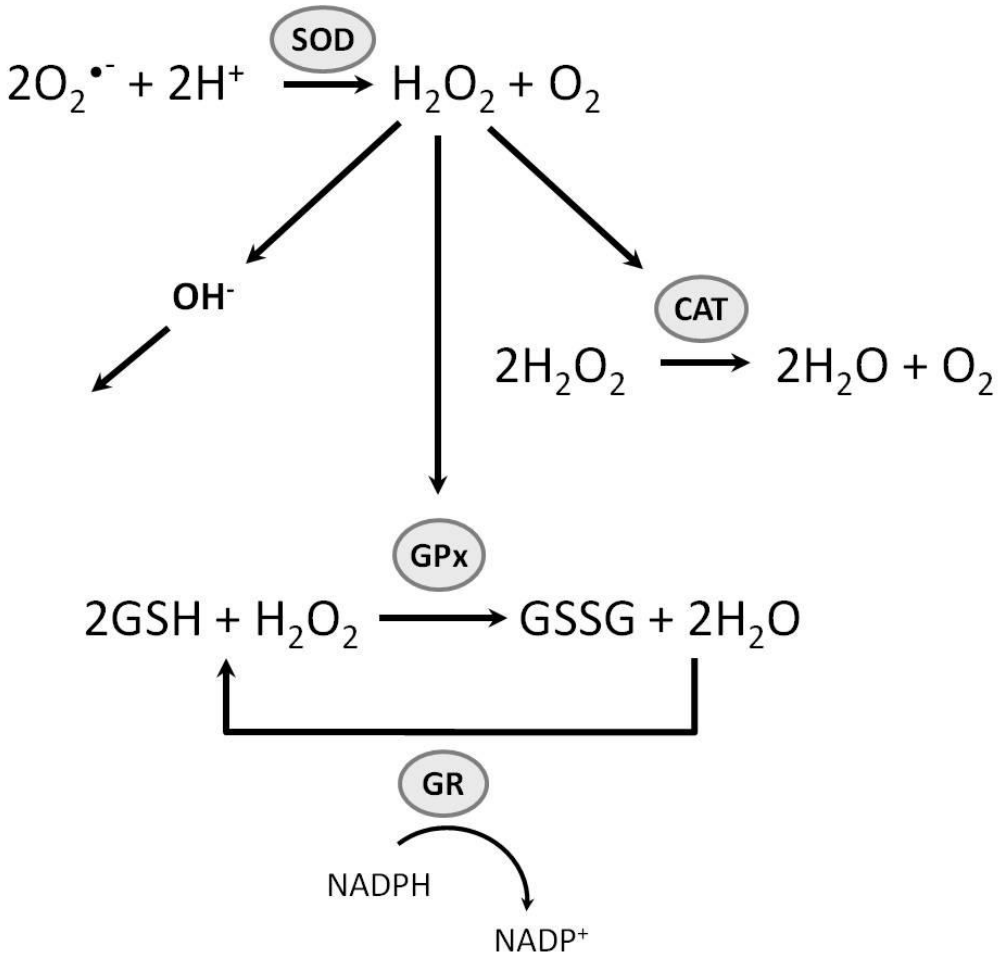

Figure 1.4: Representation of the antioxidant defence system, adapted from (Howcroft et al., 2009).

Glutathione, especially in its reduced form (GSH) has a key role in the defence thowards oxidative stress as it can be used as a substrate for glutathione peroxidase (GPx), for GST and can be linked directly to pro-oxidant compounds serving as nonenzymatic scavengers of oxyradicals (Meister, 1995). In "unstressed" conditions, the reduced forms of glutathione (GSH) possess a protective role. Increased levels of oxyradicals could change the GSH status and/or the metabolism in several ways, namely decreasing the ratio of reduced

glutathione to oxidised glutathione (GSSG), which could also occur due to the reduction in availability of NADPH.

Gluthathione redutase (GR), although not being primary in the antioxidant defence system as CAT and SOD enzymes, merits attention because it is responsible for the maintenance of the GSH:GSSG homeostasis under oxidative stress conditions (Winston and Digiulio, 1991). GR catalyse the reaction of reduction of the GSSG to GSH with the concomitant oxidation of NADPH to $NADP^+$. In its turn, GSSG (oxidised glutathione) accumulation could inactivate thiol-containing enzymes through the formation of mixed disulfides, and could inhibit the synthesis of proteins (Huggett et al., 1992). Some contaminants, directly affect the ratio GSH:GSSG decreasing it, due either to the increase of peroxidase activity or direct radical scavenging. Nevertheless, GSSG inhibits protein synthesis, and an accumulation of it may therefore result in deleterious effects on developmental responses or in those stress resistance processes involving induction mechanisms (Huggett et al., 1992).

1.6. Aim and outline of the thesis

The main goal of this thesis was to compare if the sensitivity of two species of freshwater detritivores (*A. desmarestii* and *E. meridionalis*) to essential metals. To achieve that, the effects of copper and zinc were assessed at two different levels of biological organisation: biochemical biomarkers and individual endpoints.

This Thesis is divided in six chapters: the first one is the general introduction (Chapter I) and is followed by four chapters that describe the experiments done to achieve the main goal of this work (Chapters II, III, IV and V). Finally, a last chapter making a general discussion and some concluding remarks are presented (Chapter VI).

In more detail:

(i) in the **Chapter II** entitled *"Feeding preferences of two detritivores: the crustaceans* Atyaephyra desmarestii *Millet and* Echinogammarus meridionalis *Pinkster"*, the feeding preferences of the two species were assessed comparatively considering both leaf area and metal contaminations;

(ii) in the **Chapter III** entitled *"Physiological effects of essential metals in two detritivores:* Atyaephyra desmaresii *Millet and* Echinogammarus meridionalis *Pinkster"*, the effects of copper and zinc at a physiological level of both species were assessed on survival and ingestion rates;

(iii) in the **Chapter IV** entitled *"Cholinesterase activity on Echinogammarus meridionalis Pinkster and* Atyaephyra desmare*stii Millet: characterisation and effects of essential metals"*, submitted to Chemosphere, the value of cholinesterase activities as a biomarker of metal stress was evaluated for both species;

(iv) in the **Chapter V** entitled *"Effects of essential metals in two freshwater detritivores species: biochemical approach", the activity and levels of antioxidant defences o*f both species were determined to assess copper and zinc oxidative stress potential and were related with effects at the organism level, namely feeding behaviour;

(v) in the **Chapter VI** entitled *"General discussion and concluding remarks"*, the results and findings of the preceding chapters are discussed.

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CHAPTER 2. Feeding preferences of two detritivores: the crustaceans *Atyaephyra desmarestii* (Millet) and *Echinogammarus meridionalis* (Pinkster)

2. Feeding preferences of two detritivores: the crustaceans *Atyaephyra desmarestii* (Millet) and *Echinogammarus meridionalis* (Pinkster)

Quintaneiro, C., Ranville, J., Nogueira, A.J.A

2.1. Abstract

The equilibrium of the structure and functioning of freshwater ecosystems is dependent of detritivores that link all the other functional groups. The decapod *Atyaephyra desmarestii* and the amphipod *Echinogammarus meridionalis* were the selected detritivores to evaluate the feeding preferences for particle size (leaves with different areas) and metals contamination (leaves with several concentrations of the essential metals copper and zinc). Several no-choice and multi-choice assays were done to determinate which of the different leaf areas the amphipod and the decapod would eat when they had, and when they did not have, alternatives available. These experiments included a set of dual-choice assays with contaminated and uncontaminated food. No significant preference was shown by either organism in relation to the area of leaves, either in no-choice or multi-choice assays. The presence of essential metals on food didn't have any influence on feeding choice of these organisms, over the concentration range studied. Both have no preference in ingesting food spiked with these essential metals, except *E. meridionalis* for leaves with 2.19µg.l^{-1} of copper, since they prefer ingesting these instead of uncontaminated leaves.

Keywords: *Atyaephyra desmarestii*; *Echinogammarus meridionalis*; multi-choice; no-choice; food preference; copper; zinc.

2.2. Introduction

Detritivores are essential to preserving the structure and the functioning of freshwater ecosystems. By feeding on dead plants and animals or on their detritus, they play an important role in the decomposition of litter (Allan and Castillo, 2007), recycling the nutrients which became available by producers (Covich et al., 1999). They can also serve as prey for many predators (Forrow and Maltby, 2000; MacNeil et al., 2000; Alonso et al., 2009). Thus, detritivores are very important as they link all the functional groups of food webs. However compared with consumers or producers few ecotoxicological studies were done with them.

The selected organisms, *Atyaephyra desmarestii* Millet and *Echinogammarus meridionalis* Pinkster, as detritivores, contribute to the equilibrium of the freshwater ecosystems. They feed essentially on particulate organic matter (Meurisse-Genin et al., 1985; Fidalgo and Gerhardt, 2002; Pestana et al., 2007), being responsible for degradation and fragmentation of leaves and other detritus that falls into the rivers. *E. meridionalis* is mostly a fragmentor, feeding on coarse organic matter particles (COMP, >1mm). *A. desmarestii* also feeds on COMP (Pestana et al., 2007), but instead of breaking the leafs into little fragments, this decapod scrapes leaf surfaces (personal observation). It also feeds on fine organic matter particles (FOMP, >0.5mm <1mm) (Meurisse-Genin et al., 1985) and presented cannibalistic characteristics, but only on dead organisms (Fidalgo, 1985). Slightly differences in ability to break down detritus might have impacts on litter decomposition and consequently on ecosystem processes (Bjelke and Herrmann, 2005).

In natural environments, decaying leaf material may accumulate toxicants, including metals present in the water column. Thus, detritivores can be exposed to them by food (Wilding and Maltby, 2006). Metals are divided in non-essential, when have no function in organisms and essential when have an important role in metabolic functions (Rainbow, 1997; Güven et al., 1999). However, at high

concentrations, even the essential ones can produce deleterious effects either at physiological or biochemical levels (Santos et al., 2000; Rainbow, 2007), which can leads to community and populations density alterations and consequently result in the breakage of ecosystem equilibrium.

Copper and zinc are essential metals for several organisms, including crustaceans. Several species and even various organisms of the same species can have different sensitivities to high levels of metals, some have the capability for metals bioaccumulation and storage, and others have the capability to regulate them (Rainbow, 2007). Metals are potentially very toxic if the internal available concentrations exceeds the capacity of physiological/detoxification processes (Sunda and Hanson, 1987; Santos et al., 2000; Rainbow, 2002, 2007). Copper is widely distributed in environments, and have an essential role in the transport of heamocyanin in molluscs and crustaceans, and it is also important for the functioning of some enzymes (Ali, 2000; Rainbow, 2002, 2007). Zinc is involved in the functioning of more than 200 enzymes (Muyssen and Janssen, 2002; Rainbow, 2002), however in high concentrations, can cause several problems and result in lacks of organism stability (Muyssen and Janssen, 2002).

Metals are available to organisms from several sources and their potential effect on organisms depends of bioavailability, which is influenced by water and sediment chemical characteristics and size of sediment and food particles (Dallinger and Rainbow, 1993; Rainbow, 1997). They may be incorporated or adsorbed on organic matter in high amounts (Maltby and Crane, 1994; Sridhar et al., 2008). Being that organic matter is the main food source for detritivores, it is also an important input of essential metals permitting the good functioning of metabolic processes (Dobson et al., 2004).

The central objective of this study was to evaluate if *A. desmarestii* and *E. meridionalis* reveal some preference for feeding on leaves with different areas and if they have preference for leafs contaminated with essential metals. To achieve these, no-choice assays were done to determinate which of the different leaf areas the amphipod and the decapods would eat when they had no

alternatives available. Also multi-choice assays to evaluate relative preferences for the different areas, and a dual-choice preference assay with contaminated and uncontaminated food were performed.

2.3. Material and Methods

2.3.1. Sampling and acclimation of organisms

Adults of *A. desmarestii* were collected at Rio Ceira near Coimbra, Portugal (40°10'13.21''N 8°23'26.28''W) with a kick-sampling net, and were transported to the laboratory in local water. Adults of *E. meridionalis* were collected in a reference site at the Lena River, near Leiria, Portugal (38°35'28.3''N 8°40'30.2'W) and transported in local water. Acclimation was performed in the laboratory where the organisms were maintained for at least one week in aerated artificial pond water (APW), at 20°C and with a photoperiod of 16h:8h (light:dark) before experiments. Dried alder leaves (*Alnus aglutinosa*) were given *ad libitum* during the acclimation period.

2.3.2. Size food preference tests

To evaluate the size of particle food that *A. desmarestii* and *E. meridionalis* prefer two simple assays were performed. One where organisms were given the choice of selection among several different sizes of pre-weighed alder leaf discs (multi-choice) and other where the shrimp only have one available size of pre-weighed discs to eat (no-choice). Organisms for these tests were not allowed to feed 48h prior to the beginning of the test.

The exposure chambers (Figure 2.1) are made of two plastic beakers assembled together: the top one, where the organism was exposed, have a net in the bottom to prevent the contact of the organism with detritus that fall into the underlying beaker; the top beaker was divided in half with a net to allow the evaluation of autogenic changes of the leafs discs in the absence of organism.

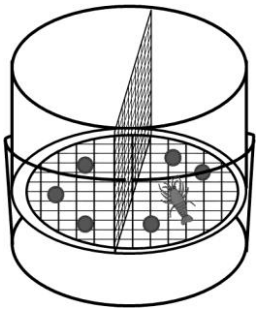

Figure 2.1: Test chamber scheme.

In multi-choice tests, one shrimp or two amphipods were used in each of the ten test chambers with the same area of leafs. Food was offered in four different sizes of discs and one more with irregular form were used. In the no-choice test the chambers had only one size of leaf discs and the same number of organisms used in the previous test. Ten replicates per treatment were used. During the 96h of tests, the temperature, dissolved oxygen, conductivity, pH, presence of moult and mortality was recorded daily. At the end, leafs discs were dried at 60ºC until stable weight in order to determine the feeding rate.

Ingestion rate (Ir) were determined by

$$Ir = \frac{\Delta Lw}{Lwi} \times \frac{1}{d \times Ow}$$

where, ΔLw, variation of the leaves weight (initial minus final weight); Lwi, leaves initial weight; d, number of days; and Ow, organism weight. Ir is given in $\mu g.mg^{-1}.day^{-1}$.

2.3.3. Contaminated *vs.* uncontaminated food tests

To evaluate if *A. desmarestii* and *E. meridionalis* reveal some preference between contaminated or uncontaminated food, two simple tests of dual-choice were performed with each metal (copper and zinc). Organisms for these tests were not allowed to feed 48h prior to the beginning of the test. In each chamber test with clean APW, one shrimp or two amphipods had the opportunity to select between

contaminated and non-contaminated pre-weighed leaf discs on tests with either copper or zinc. For each metal, five concentrations (2.0 to 130.0µg.l^{-1} and 1.0 to 500.0µg.l^{-1} of copper and 0.5 to 15mg.l^{-1} and 0.5 to 10.0mg.l^{-1} of zinc for *A. desmarestii* and *E. meridionalis*, respectively) plus control, with ten replicates each, were used. At the end of the 96h exposure period leafs were dried until a stable weight was obtained. Chemical analysis of the organisms was also performed. Every day the physical-chemical parameters, mortality and presence of molt were checked. The contaminated leaf discs were prepared placing them for 48h in metal solutions with the different concentrations, with constant stirring.

Food selectivity (Fs) was calculated by

$$Fs = \frac{iLnc}{iLc}$$

where $iLnc$ represents the ingestion of uncontaminated leaves, and iLc represents the ingestion of contaminated leaves; if Fs is lower than 1, organisms prefer contaminated leaves, if its higher than one organisms avoid them, if its 1, no preference is observed between contaminated or non-contaminated leaves.

The concentrations of metals in leaves contamination solutions and in organisms at the end of the assay were analyzed by ICP-MS. Using standard referencd material the percentage of recovery was found and is presented in Table 2.1.

Table 2.1: Concentration of reference material and percentage of recovery in ICP-MS analysis.

	Reference material (µg.l^{-1})	***ICP-MS (µg.l^{-1})***	***% Recovery***
TORT-2			
copper	106.0±10.0	97.87±3.90	92.3±3.7
zinc	180.0±6.0	164.95±7.59	91.6±4.2

2.3.4. Statistical analysis

The statistical analysis of the preference tests were performed with SigmaPlot v11.0. To determine what size of leaf discs was preferred by *A. desmarestii* and *E. meridionalis*, data were analyzed by one-way ANOVA (for no-choice assays) and by GLM (for multi-choice). For the total ingestion of contaminated *vs.* uncontaminated food, the differences were analyzed by one-way ANOVA. Data of metal contents in organisms were analyzed by one-way ANOVA followed by a Holm-Sidak post-hoc test in order to identify significant differences. All data were square-root or logarithmic transformed previously, if necessary, to achieve data normality and homocedasticity.

2.4. Results

2.4.1. Size food preference tests

The physical-chemical parameters measured during the *A. desmarestii* no-choice assay were: temperature 18.5±0.5°C, conductivity 604±50.3µS.cm^{-1}, pH 7.4±0.1, dissolved oxygen 7.9±0.2mg.l^{-1} and percentage of oxygen saturation 84.7±1.8%. In this assay the mortality was 10 organisms at the end of the test period. The ingestion rates of the *A. desmarestii* for the no-choice assay for the different areas of leaves are presented on Figure 2.2. No significant differences (ANOVA, $F_{4,31}$=1.667; p=0.187) were found for the different sizes of leaves, however when no other leaf sizes were offered to the decapods, leafs with area of 0.950cm^2 seems to be the ones that were ingested in higher quantity (3.65±1.480µg.mg^{-1}.day^{-1}). Smallest leaves were the least ingested ones (0.56±0.161µg.mg^{-1}.day^{-1}).

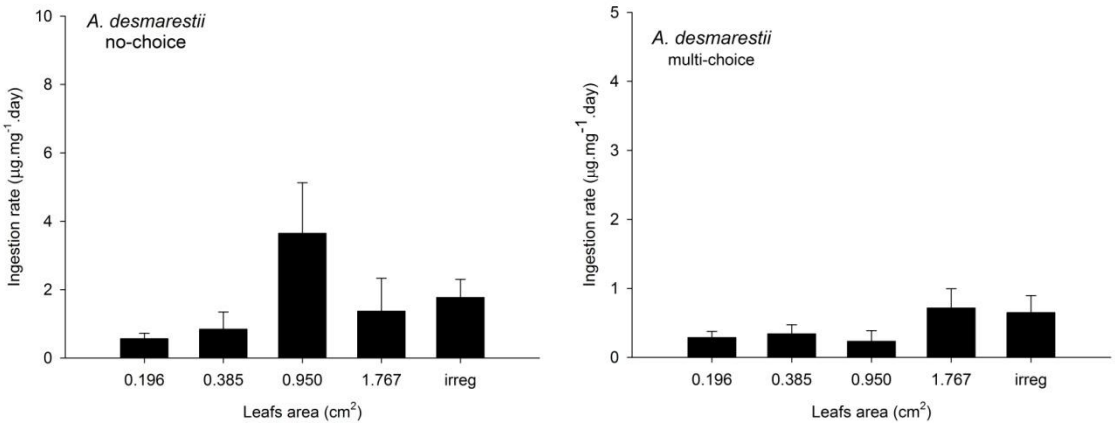

Figure 2.2: Results of ingestion rates (µg.mg^{-1}.day^{-1}) of *A. desmarestii* on no/multi-choice assays. Results expressed as mean ± SE (n=10).

During the *A. desmarestii* multi-choice assay no mortality was registered. The physical-chemical parameters measured were: temperature 18.5±0.5°C, conductivity 679±14.3 µS.cm^{-1}, pH 7.8±0.1, dissolved oxygen 8.3±0.3mg.l^{-1} and percentage of oxygen saturation 88.4±2.7%. Results are presented in Figure 2.2. Furthermore, in this case, no significant differences (GLM, $F_{9,49}$=1.107, P=0.383; $F_{4,49}$=1.203, p=0.326) were found between different areas of leaves. All particles sizes were ingested in similar quantities. The leaves with 0.950cm^2 of area were ingested in lower amount (0.23±0.154µg.mg^{-1}.day^{-1}).

Physical-chemical parameters registered on *E. meridionalis* no-choice assay were: temperature 18.9±0.8°C, conductivity 715±41.4µS.cm^{-1}, pH 7.6±0.2, dissolved oxygen 7.8±0.2mg.l^{-1} and percentage of oxygen saturation 83.5±1.7%. In this assay the mortality was 21 organisms at the end of the test period distributed among the different sizes of leaves. Figure 2.3 shows the ingestion rate of the *E. meridionalis* in the no-choice assay for the different areas of leaves. Although no significant differences (ANOVA, $F_{4,34}$=1.307; p=0.290) were found, ingestion rates were higher (18.40±7.700µg.mg^{-1}.day^{-1}) on larger leafs (1.767cm^2) than on others.

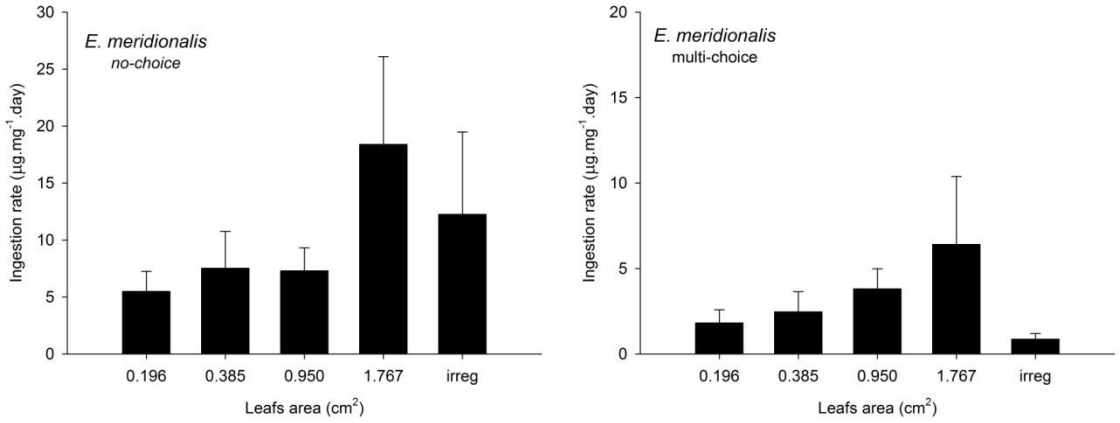

Figure 2.3: Results of ingestion rates (µg.mg^{-1}.day^{-1}) of *E. meridionalis* on no/multi-choice assays. Results expressed as mean ± SE (n=20).

In *E. meridionalis* multi-choice assay no dead organisms was registered. The physical-chemical parameters registered were: temperature 19.1±0.7°C, conductivity 695±16.3µS.cm^{-1}, pH 7.6±0.2, dissolved oxygen 8.1±0.3mg.l^{-1} and percentage of oxygen saturation 87.2±2.7%. Results for multi-choice assay are presented in Figure 2.3. When different areas of leaves are available for the amphipod, the leaves with higher ingestion rate (6.41±3.980µg.mg^{-1}.day^{-1}) was also the larger ones, nevertheless, no significant differences were found (GLM, $F_{8,44}$=2.229, p=0.051; $F_{4,44}$=1.883, p=0.138).

When comparing results for both species, amphipods present higher ingestion rates than decapods.

2.4.2. Contaminated *vs.* uncontaminated food assays

The only mortality in the *A. desmarestii* assays with/without contaminated leaves was a single one in tests on leaves with 10.6µg.l^{-1} of copper. The physical-chemical parameters for copper assay registered were: temperature 20.0±0.2°C, conductivity 590.7±9.9µS.cm^{-1}, pH 7.9±0.0, dissolved oxygen 7.6±0.2mg.l^{-1} and percentage of oxygen saturation 83.7±2.4%. For zinc assay were: temperature 20.1±0.4°C, conductivity 600.5±13.1µS.cm^{-1}, pH 7.9±0.1, dissolved oxygen 7.7±0.2mg.l^{-1} and percentage of oxygen saturation 83.9±3.6%. The mortalities for

zinc were one at leaves with the first concentration and two organisms in 24.6µg.l^{-1} and 15.0µg.l^{-1} of zinc.

No significant differences were observed (ANOVA, $F_{6,29}$=0.701; p=0.651) for total ingestion in copper assay for *A. desmarestii* (Figure 2.4). However, regarding the results, the first two concentrations registered lower total ingestion than the control (2.82±0.458µg.mg^{-1}.day^{-1}) and the third concentration showed the highest total ingestion (4.03±2.006 µg.mg^{-1}.day^{-1}).

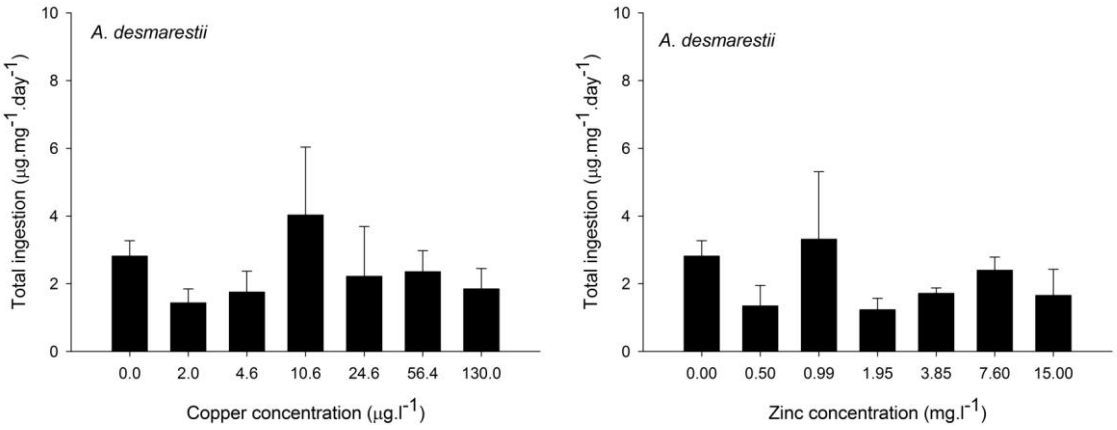

Figure 2.4: Results of total ingestion (µg.mg^{-1}.day^{-1}) of *A. desmarestii* on dual-choice assays with nominal concentrations of copper and zinc contaminated/uncontaminated leaves. Results expressed as mean ± SE (n=10).

The total ingestion of zinc contaminated leaves by *A. desmarestii* is shown in Figure 2.4 where no significant differences were registered (ANOVA, $F_{6,27}$=1.034; p=0.431). Control leaves presented a total ingestion of 2.82±0.458µg.mg^{-1}.day^{-1}.

Figure 2.5 shows the preference of ingestion of uncontaminated leaves compared with copper and zinc contaminated leaves. The highest concentration of copper tested show a ratio between contaminated and uncontaminated leaves higher than 1, while the third and fourth concentrations (10.6µg.l^{-1} and 24.6µg.l^{-1}, respectively) the ratio is lower than 1, revealing preference for copper spiked leaves. Regarding zinc spiked leaves, the ratio was higher than 1 with leaves spiked with the third concentration (1.95mg.l^{-1}) revealing the preference for non-spiked food, the first concentration the ratio was zero, no uncontaminated food was ingested.

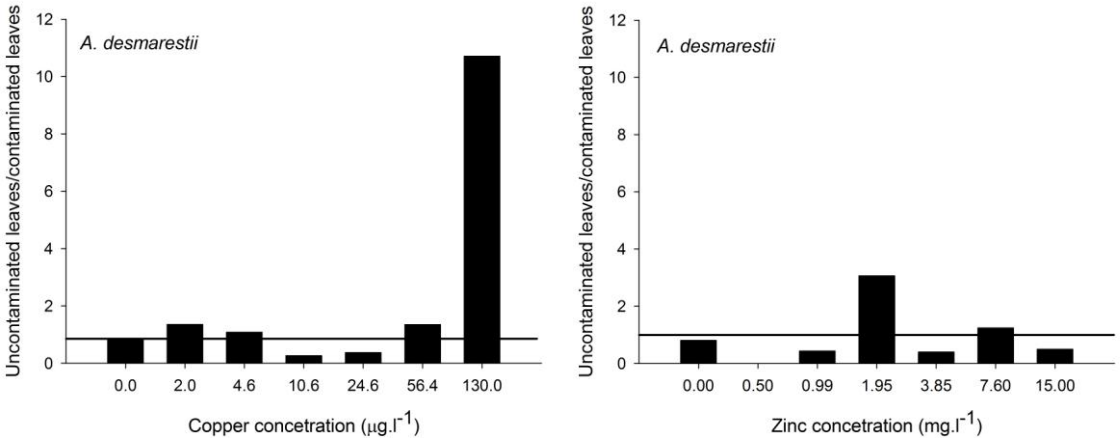

Figure 2.5: Results from *A. desmarestii* preference of ingestion leaves uncontaminated/contaminated with nominal concentrations of copper or zinc.

Percentages of ingested contaminated leaves in relation to uncontaminated leaves are presented in

Figure 2.6. The percentage of ingested contaminated leaves in relation to uncontaminated leaves was higher for the third concentration of copper 10.6 µgl^{-1} with high variability, but above this concentration there seems to be a decrease in ingestion of contaminated leaves over the uncontaminated, with a higher percentage (91.46%) for the last concentration (130.0µg.l^{-1}).

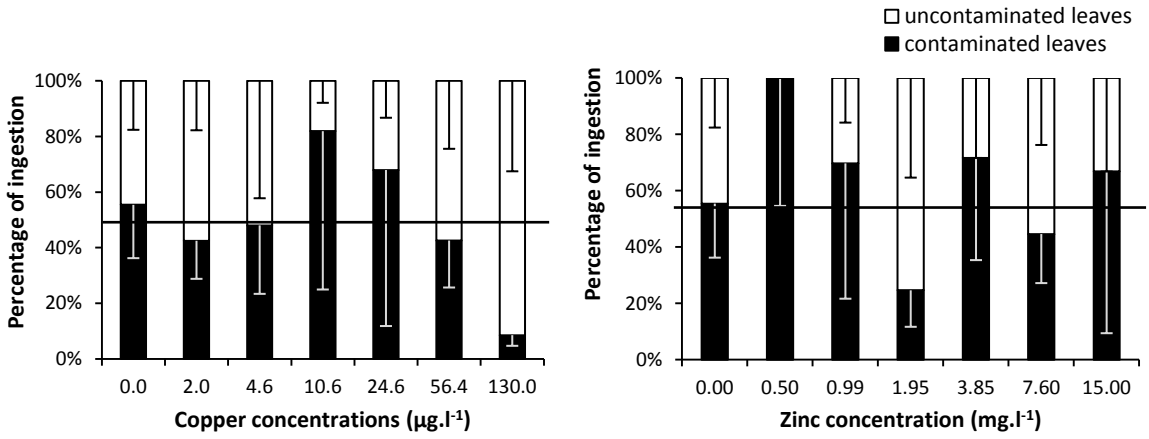

Figure 2.6: Percentage of ingested copper and zinc contaminated (nominal concentrations) leaves in relation to uncontaminated leaves to *A. desmarestii*.

With zinc, at the treatment of 0.5mg.l^{-1} shrimps only ingested the spiked leaves instead uncontaminated leaves. Decapods ingested uncontaminated leaves at higher percentage (75.36%) over the spiked leaves (24.64%) with 1.95mg.l^{-1} of zinc.

Table 2.2: *A. desmarestii* metals body-burdens. * indicates statistically significant differences in relation to control group, Holm-Sidak p<0.05.

Copper		***Zinc***	
Spike solution (µg.l^{-1})	*Shrimp (µg.g^{-1})*	*Spike solution (mg.l^{-1})*	*Shrimp (µg.g^{-1})*
0.0	117.1±7.57	*0.0*	73.8±2.84
2.0	94.3±4.26	*0.50*	72.7±3.07
4.6	111.6±5.52	*0.99*	70.1±4.10
10.6	91.6±5.81	*1.95*	77.6±2.72
24.6	90.8±7.91	*3.85*	88.1±3.37
56.4	110.5±9.49	*7.60*	93.7±6.51*
130.0	79.0±10.29*	*15.00*	90.8±5.23

Metals contents in the whole-body of shrimp are shown in Table 2.2. At the treatment with leaves spiked with the highest concentration of copper (130.0µg.l^{-1}) organisms presented significantly lower levels of copper (ANOVA, $F_{6,33}$=3.358; p<0.05) than control organisms. There was an effect of diet in the amount of zinc in the shrimp (ANOVA, $F_{6,29}$=5.045; p<0.005), this effect was seen with the leaves spiked with 7.60mg.l^{-1} of zinc, being the only organisms with higher zinc content, and that showed differences from the control.

The mortality of the *E. meridionalis* assays with/without contaminated leaves was less than 10% (one amphipod) in the control and two amphipods in leaves with 4.780µg.l^{-1} of copper. The physical-chemical parameters for copper assay registered were: temperature 19.0±0.4°C, conductivity 679±24.9µS.cm^{-1}, pH

7.6±0.2, dissolved oxygen 7.8±0.2mg.l^{-1} and percentage of oxygen saturation 84.3±1.7%. For zinc assay were: temperature 18.8±0.7°C, conductivity 689±29.1µS.cm^{-1}, pH 7.5±0.2, dissolved oxygen 7.7±0.2mg.l^{-1} and percentage of oxygen saturation 83.1±1.8%, and the mortality was one amphipod for leaves with 0.910µg.l^{-1} and 3.020µg.l^{-1} of zinc and two amphipods for leaves with 1.660µg.l^{-1} of zinc.

No significant differences in total ingestion (Figure 2.7) were found (ANOVA, $F_{6,30}$=0.961; p=0.472) when leaves were spiked with copper although at 4.78µg.l^{-1} copper, the total ingestion rate was the highest, 20.42±9.405µg.mg^{-1}.day^{-1}. Controls presented a total ingestion of 6.60±1.929µg.mg^{-1}.day^{-1}.

For the zinc spiked leaves (Figure 2.7), the total ingestion of the control was 10.31±4.679µg.mg^{-1}.day^{-1}, the highest ingestion rate (29.99±19.236µg.mg^{-1}.day^{-1}) was verified for the last concentration (10.0mg.l^{-1}), however no significant differences were found (ANOVA, $F_{6,29}$=0.351; p=0.902).

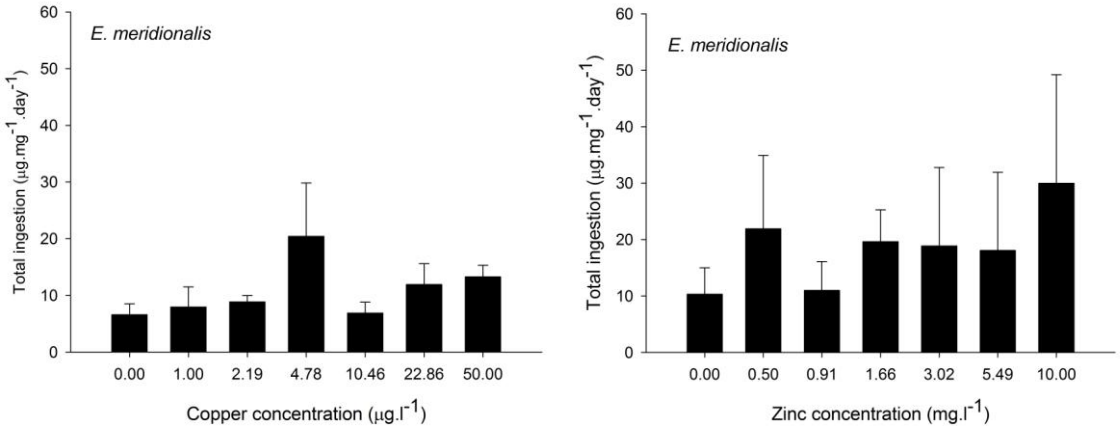

Figure 2.7: Results of total ingestion (µg.mg^{-1}.day^{-1}) of *E. meridionalis* on dual-choice assays with nominal concentrations of copper and zinc contaminated/uncontaminated leaves. Results expressed as mean ± SE (n=20).

Figure 2.8 shows the preference of ingestion of uncontaminated leaves compared with copper and zinc contaminated leaves. For all copper concentrations, except with 10.46µg.l^{-1}, which presented a ratio higher than 1 (3.08), the ratio between uncontaminated and contaminated leaves is lower than 1. Regarding the

preference for the zinc spiked leaves over uncontaminated the ratio was always higher than 1, except for 0.5mg.l^{-1}, the first concentration of zinc (0.41).

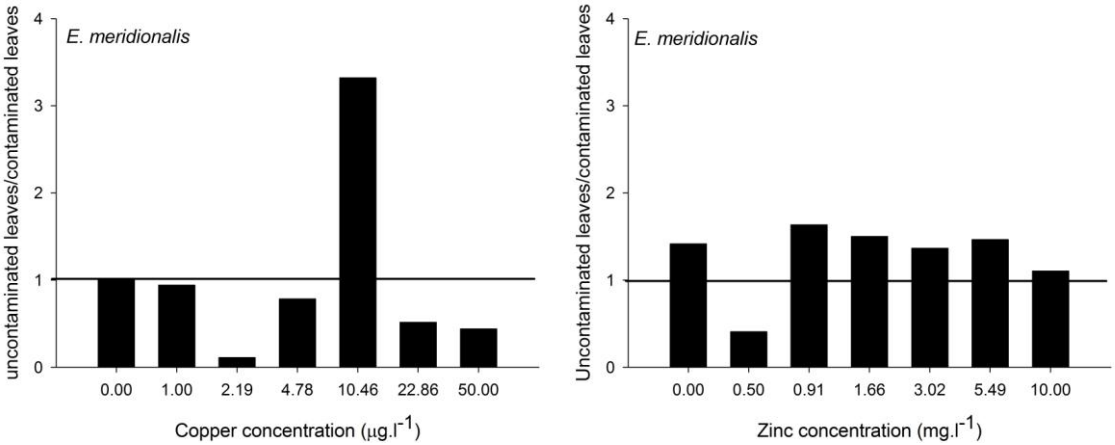

Figure 2.8: Results from *E. meridionalis* preference of ingestion leaves uncontaminated/contaminated with copper or zinc nominal concentrations.

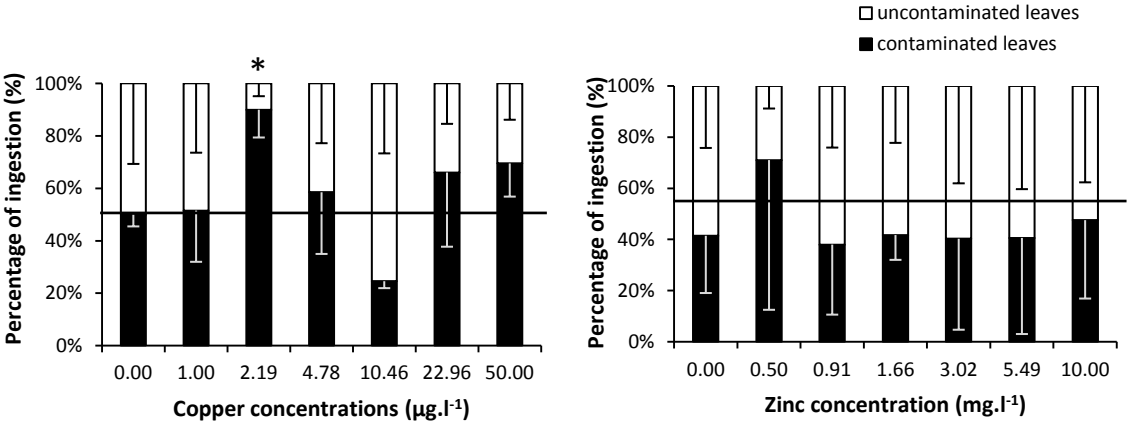

Figure 2.9: Percentage of ingested copper and zinc contaminated leaves (nominal concentrations) in relation to uncontaminated leaves to *E. meridionalis*. * indicates statistically significant differences between contaminated and uncontaminated leaves, GLM $p<0.05$.

Regarding the percentage of ingested copper contaminated leaves in relation to uncontaminated leaves (Figure 2.9), the first ones to be ingested in higher percentage (89.95%) occured when the leaves were spiked with 2.19µg.l^{-1} of copper. When the spiked concentration was 10.46µg.l^{-1} the uncontaminated leaves were ingested in higher percentage (75.47%). With zinc-contaminated leaves (Figure 2.9), the percentage of uncontaminated leaves was always higher,

except for the first spiked concentration (0.5mg.l^{-1}), however a high variability was found.

Table 2.3: *E. meridionalis* metals body-burdens. * indicates statistically significant differences in relation to control group, Holm-Sidak p<0.05.

Copper		***Zinc***	
Spike solution ($\mu g.l^{-1}$)	*Amphipod* ($\mu g.g^{-1}$)	*Spike solution* ($mg.l^{-1}$)	*Amphipod* ($\mu g.g^{-1}$)
0.0	78.95±11.215	*0.0*	32.46±4.807
1.0	78.59±4.603	*0.5*	20.88±7.733
2.19	96.88±3.413	*0.91*	30.90±8.516
4.78	81.73±8.844	*1.66*	39.62±7.781
10.46	75.19±11.055	*3.02*	29.20±3.162
22.86	93.52±22.781	*5.49*	26.74±5.430
50.00	77.834±16.639	*10.00*	32.84±2.007

Metals content in whole-bodies of amphipods is shown in

Table 2.3. Organisms fed with copper spiked leaves presented similar levels of copper (ANOVA, $F_{6,20}$=0.432; p=0.845) for all treatments. No significant differences were also registered in the zinc content of the amphipod with all concentrations of spiked leaves (Kruskal-Wallis ANOVA, $H_{6,21}$= 4.321 ; p=0.633).

2.5. Discussion and Conclusions

Feeding assays with organisms should be developed with diets providing them all nutritional requirements, and it is important to know the optimum size range of dietary particles and the quantity at which they should be fed. Clearly, dietary particles should be of a size that maximizes ingestion and at a density that allows maximal ingestion but minimizes waste (Zeng et al., 2004), which could result in poor water quality. Thus, the purpose of this work was to determine if the *A.*

desmarestii and *E. meridionalis* have some preference for feeding on leaves with different areas. This is in order to assess the dietary particles size that maximize the ingestion for the further feeding studies. These organisms usually feed on COMP, but the decapod scrapes leaf surfaces, so probably the preferences of the leaves size was expected to be different from the ones preferred by the amphipod, which is a fragmenter.

In alternative food assays, the organisms have the possibility to choose from different sizes of leaves, which is the real scenario, as in rivers there exist a panoply of types and food sizes. However, in laboratory assays, in order to minimize the number of variables, only one type of food was provided, so the no alternative choice food tests give the capacity of organisms to ingest only one size of leaves.

A. desmarestii presented lower ingestion rates than *E. meridionalis* in all preformed assays. When several sizes of food were offered, *A. desmarestii* did not present any significant preference for any size, the ingestion rates being very similar, however a lower area of food particles (0.950cm^2) resulted in a slightly lower ingestion rate. In the no alternative assay, where only one size of food was offered to *A. desmarestii*, the leaves with 0.950cm^2 of area was the ones that presented higher ingestion rates, nevertheless no preference was clearly observed.

Distribution of food was also important, for the same amount of area available for ingestion, little leaves permits a homogeneous distribution of leaves on test chambers, which can be an essential issue in searching for food by organisms as they can dispend lower energy to find it, and instead channel this energy to other processes, namely growth, reproduction, etc. Leaves with smaller area imply a lower scrapping surface and being that *A. desmarestii* is a scraper (personal observation), the surface available to scrape was also important. So the selection of the leaf area for future feeding assays must be the result of a compromise between ingestion, distribution and surface area of leaves.

The leaves with 0.950cm^2 of area, despite presenting higher ingestion rates for the no alternative food assays, have a less regular distribution in the exposure chamber as only two leaves discs were put in it, but they presented a considerable surface for scraping. However, when an alternative was given to the organisms (more similar to real scenario), these leaves was less ingested. On other hand, leaves with 0.385cm^2 of area, despite the slightly lower ingestion rates, have a better distribution as four discs were put in chambers. From the results of this work it seems reasonable that for *A. desmarestii* the selected food for future feeding tests can be the leaves with 0.385cm^2 of area, given that no clear preference regarding the ingestion rates was seen and that this seems to be a size with a homogeneous distribution.

E. meridionalis, in both assays, with/no alternative food, did not present a clear preference for any size of discs, but in both there was registered an increase in ingestion rates with the increase of discs area. As this amphipod is mostly a fragmentor feeding on COMP, the size of discs it is an important issue to take into account. Little discs, despite the better distribution in chambers, provide less area to fragment and become more difficult to handle. Since the leaves with 1.767cm^2 of area were ingested at higher rates, it seems reasonable that this area was the most appropriate to use in future feeding assays.

Food is one of the sources of entrance of contaminants for several aquatic organisms (Wang, 2002; Rainbow, 2007). As detritivores, *A. desmarestii* and *E. meridionalis* feeding on dead plants and animals or on their detritus, are exposed to metals present in food. Additionally, there is growing evidence that diet-borne metal toxicity is important in aquatic ecosystems, apart from waterborne metal toxicity (De Schamphelaere et al., 2007). Given the fact that some metals are essential for metabolic processes, it becomes interesting to evaluate if these specie have some selectivity to choose food with certain concentrations of these metals, in order to provide more availability of metals in order to accomplish metabolic processes.

Regarding results from assays where contaminated food and uncontaminated food was offered to shrimps, the increasing concentration of copper or zinc on leaves have no effect on total ingestion of food.

For copper, the total ingestion of *A. desmarestii* seems to be slightly lower at lower concentrations and regarding the percentage of ingested contaminated and uncontaminated leaves at these concentrations, it seems that the presence of contaminated leaves didn´t have any effect on ingestion, as no clear preference or avoidance for contaminated leaves was registered. The total ingestion was slightly higher when leaves were spiked with 10.6µg.l^{-1} of Cu, and despite the high variability the percentage of ingestion of contaminated leaves was higher than uncontaminated. Several authors have the opinion that crustaceans absorb copper from food so it should be included in the diet (Bryan, 1968; White and Rainbow, 1982). Deshimaru and Yone (1978) opined that copper may not be required in diet of shrimps. De Schamphelaere et al. (2007) found lower, or no effects on growth of *Daphnia magna*, with diets contaminated by copper and zinc respectively. Ali (2000) showed that with the increasing of copper in diet, the shrimp *Penaeus indicus* didn´t register an increase in growth, but with increase of zinc until 23.6mg.l^{-1} in food, the growth was improved. The slight increase found at this concentration of copper (10.6µg.l^{-1}) can be the result of detection of leaves with essential metal. Despite no preference for any type of leaves was verified, the leaves discs spiked with the higher concentration of copper (130µg.l^{-1}) seems to be avoided by organisms that eat preferentially uncontaminated leaves. This was observed as the percentage of ingested contaminated leaves was below 10% and the ratio uncontaminated/contaminated leaves was more than 10. This avoidance of contaminated food suggest that at this level of metal, the needs for metabolic processes were fulfilled and the entrance of more metal can cause a break in metal internal regulation, which can lead to damaging effects for the shrimp including the reduction in ingestion in order to decrease the metal uptake. As it is known, decapods have the capacity to regulate essential metals including copper (Marsden and Rainbow, 2004) but when the metal level in diet

has high, there was an inhibition of feeding (Weeks, 1993; Wilding and Maltby, 2006). However in this work, due to the high variability observed in ingestion rates, no clear preference or avoidance can be attributed, as no significant differences between the contaminated and uncontaminated leaves were registered within the treatments.

For assays with zinc spiked leaves, the increase of concentration of this metal in food seems to have no affect on the total ingestion by *A. desmarestii*, which suggests that the presence of the essential metal on food had no influence on feeding behaviour of this species. Unlike zinc, copper is an essential metal that, according to several authors (Davis et al., 1993; Ali, 2000; Cuzon et al., 2004; Shiau and Jiang, 2006) should be introduced in diets to improve growth of several marine crustaceans, and thus it seems reasonable that it should also be included in diets of freshwater crustaceans. No preference or avoidance was also registered for zinc contaminated food, and once again a great amount of variability was observed in ingestions rates. The absence of ingestion of uncontaminated leaves when leaves with 0.5mg.l^{-1} of zinc were present cannot be attributed to the preference for zinc contaminated leaves as no significant differences were found between the types of leaves and great variability in ingestion was observed. Unlike for the copper assay, with zinc no pattern can be observed on the percentages of ingestion of certain type of leaves in relation to the total ingestion. Taking into account the variability observed, at all concentrations, the percentage of the contaminated an uncontaminated leaves fall around the line of 50%, which indicates that no preference of one or other type of leaves exists. This is also verified statistically as no significant differences were found, neither between concentrations, nor types of food, or interactions between them.

Copper body burden of *A. desmarestii* was high when fed on leaves spiked with lower concentrations. This contradicts the results from the work of De Schamphelaere et al. (2007) who found an increase in copper and zinc *D. magna* body burdens with the increase of copper and zinc in diet. Also, Ali (2000) found

an enhancement of *P. indicus* body burdens for these two metals with the increase in food. Lee and Shiau (2002) indicate the increase in whole-body copper concentration of *P. monodon* as dietary copper supplementation increased. Zidar et al. (2003) described an increase in copper body burdens for the isopod *Porcellius scaber* with the increase of copper concentrations in food. However, according to White and Rainbow (1982, 1984b) and Rainbow (2007), they concluded that decapods have mechanisms to regulate essential metals, in this work the body concentrations of copper in shrimps are apparently regulated and maintained at certain concentration.

Regarding zinc results, an increase of zinc body burden concentration was registered, being significantly higher with leaves spiked with 7.60mg.l^{-1}, after which they decrease. This decrease suggests that until this concentration shrimps accumulate metal and after this concentration a mechanism of regulation begins in order to maintain the levels of zinc. This might include increases in the excretion of the metal through the faeces, moult, etc. This is also in accordance to White and Rainbow (1984a) who was concluded that *Palaemon elegans* had the capacity to regulate body zinc concentrations when exposed to 100.0 µg.l^{-1} of zinc. *A. desmarestii* as a decapod has the capacity to regulate essential metals like zinc. The increase registered in body burdens of zinc are in good agreement with Shiau and Jiang (2006) that showed whole-body zinc concentration in *P. monodon* increases with the zinc supplementation in diet.

Considering the results of the dual choice assays for the amphipod *E. meridionalis*, the total ingestion of food seems to be unalterable by the presence of food having increasing concentrations of zinc. And like for *A. desmarestii*, the presence of the essentials metals, copper and zinc, in food seems didn't influence the feeding behaviour of this amphipod. For copper, the total ingestion mean was always similar to control except at the third concentration (4.78mg.l^{-1} of copper) which is slightly higher, for zinc the total ingestion mean in control beakers was slightly lower than in other concentrations. Regarding the two types of leaves

offered to amphipod (contaminated and uncontaminated), a preference of contaminated leaves relative to uncontaminated leaves was verified for 2.19µg.l^{-1} of copper, which suggests that organisms prefer feeding on leaves with metal instead leaves without metal, which can be due to the metabolic needs of amphipods to accomplish the internal processes. This supports the idea that for maintaining this type of organisms, the diet offered has to have some copper in order to improve the functioning of metabolic processes to lead to better growth. Some authors had documented the idea that the presence of copper in diets of crustaceans can improve their growth (Davis et al., 1993; Ali, 2000; Lee and Shiau, 2002; De Schamphelaere et al., 2007). The result obtained for the ratio uncontaminated/contaminated leaves reflects this preference, as at concentration of 2.19µg.l^{-1} of copper the ratio was below 1, and more than 80% of the total ingested leaves were contaminated leaves. The opposite was verified for 10.46µg.l^{-1} of copper, where the organisms seems to avoid the contaminated leaves as only about 20% of the total leaves ingested were contaminated. However as the variability of the ingested uncontaminated leaves was high no significant differences can be found, thus this avoidance cannot be considered. On the other hand, in assays with zinc leaves, no preference between leaves was registered for any concentration of zinc, which suggests that amphipods didn't use food as a source of uptake of zinc for their metabolic needs. Even with high variability in percentage of ingestion of leaves, the mean percentage was always between the 40% and 50% for contaminated leaves except for the first concentration, which was higher.

No significant differences were found for the body burdens of both metals in *E. meridionalis*. This was unexpected since, according to Marsden and Rainbow (2004), amphipods are net accumulators of trace metals both from solution and diet being indicative of the uptake over a period of time (Rainbow, 1997). However, as the organisms have the choice to feed on uncontaminated leaves and as the percentage of both types of leaves were similar, maybe the ingested of uncontaminated leaves have some influence on the metal content of

amphipods. Moreover, it is interesting that despite no significantly higher content, the higher levels of copper was registered in amphipods from the treatment with highest percentage of ingested contaminated leaves (2.19µg.l^{-1}). This suggests that if amphipods selected spiked leaves, the metal content in whole-body increases. In the case of zinc no clear relation can be attributed, as no significant preference of the contaminated leaves was registered, however at spike concentration of 1.66mg.l^{-1} of zinc, which is the concentration with lower variation, the amphipods presented the higher level of zinc, despite not being significantly higher.

In summary, the results of this work lead to the conclusion that both *A. desmarestii* and *E. meridionalis* didn't present any ingestion preference for a certain area of leaves either when no-alternative or an alternative was offered, although the areas of leaves more appropriate for future works are 0.385cm^2 and 1.767cm^2 for *A. desmarestii* and *E. meridionalis*, respectively. Only the amphipod presented preference for contaminated leaves instead uncontaminated when the leaves had 2.19µg.l^{-1} of copper, which suggests that to maintain this organisms health, the diet should include some percentage of this metal in order to accomplish the metabolic needs of organism. Nevertheless, as the variability was high, further studies will be needed in order to corroborate these results.

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CHAPTER 3. Physiological effects of essential metals in two detritivores: *Atyaephyra desmarestii* (Millet) and *Echinogammarus meridionalis* (Pinkster)

3. Physiological effects of essential metals in two detritivores: *Atyaephyra desmarestii* (Millet) and *Echinogammarus meridionalis* (Pinkster)

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3.1. Abstract

Freshwater ecosystems are essential for humans, however it is man himself who has, through his activities caused deleterious effects on them. Thus, is urgent to assess the health of these ecosystems in order to allow actions for prevention and remediation. Essential metals, like copper and zinc, despite the important role in organisms, become toxic when high environmental concentrations are attained. In order to evaluate the physiological effects of these two essential metals for two freshwater detritivores, *Atyaephyra desmarestii* and *Echinogammarus meridionalis*, acute tests were performed. Forty-eight hour LC50 values were estimated for copper and zinc in these species using static bioassays. Sub-lethal assays for both metals with several phases were also done to evaluate the effects on feeding behavior. The LC50 values of copper for *A. desmarestii* and *E. meridionalis* were 0.128 and 0.050 $mg.l^{-1}$, respectively, and for zinc were 7.951 and 11.860 $mg.l^{-1}$, respectively. The results indicated that copper is more toxic to the species and that the shrimp is more sensitive to zinc than the amphipod which is more sensitive to copper. Only *E. meridionalis* showed deleterious effects of copper on feeding rate. Zinc showed some tendency for feeding inhibition in both species.

Keywords: *Atyaephyra desmarestii*; *Echinogammarus meridionalis*; copper; zinc; feeding.

3.2. Introduction

Freshwater ecosystems have been, throughout the years, exposed to human activities, which sometimes can result in deleterious effects. Freshwater is essential for humans being important to fisheries, crops irrigation, drinking, personal hygiene, washing, cooking, and other activities related with industry and transportation (Sarukhán and Whyte, 2005). It has become essential to evaluate the health of these ecosystems. The sediments act as a sink for trace metals which results from natural or anthropogenic sources. Some freshwater invertebrates can, due to their preference for benthic habitats, be confronted with environments with high levels of metals, which can lead to stress situations (Dallinger and Rainbow, 1993).

Copper is an essential trace metal required in small doses by organisms for metabolic functions and is important to oxygen transport, but it is also potentially very toxic if the internal available concentrations exceeds the capacity of physiological/detoxification processes (Sunda and Hanson, 1987; Guillaume et al., 1999). Zinc is involved in the functioning of more than 200 enzymes (Guillaume et al., 1999; Muyssen and Janssen, 2002), however in high concentrations zinc can cause several problems and result in lack of organism stability (Muyssen and Janssen, 2002).

Organisms could be exposed to metals directly by water and/or indirectly by sediment ingested as a food source (Dallinger and Rainbow, 1993). The transfer of energy throughout food web allows the establishment of the structure and the equilibrium between the communities of ecosystems. Considering food ingestion as the major input of energy (Santos et al., 2000), sub-lethal effects of metals should be assessed. Feeding rate seems be a good indicator of general stress, being sensitive to environmental changes. It is also an ecological relevant parameter since, along with the other energy budget components, it can be linked with growth, reproduction and survival of organisms. Measurement of feeding rate can allow the assessment of contamination at higher levels of

biological organisation (Maltby, 1999; Slijkerman et al., 2004; Pestana et al., 2007).

Several studies were performed in order to understand how metals inhibit the feeding rate of freshwater crustaceans during or post exposure period (Wilding and Maltby, 2006; Macedo-Sousa et al., 2007; Pestana et al., 2007; Satapornvanit et al., 2009; Agostinho et al., 2012), however the recovery of the feeding behaviour after a period of exposure and a subsequent period of depuration has received less attention.

Atyaephyra desmarestii Millet is freshwater decapoda that lives in slowly running waters associated with aquatic plants, and feeds on fine particulate organic matter and attaches to periphyton (Callisto, 2006). Firstly, this caridean inhabits freshwaters from the Mediterranean area and is also widely distributed throughout Europe inlands waters, with exception of the British Isles (Fidalgo and Gerhardt, 2002; Callisto, 2006), and the Middle East (Anastasiadou and Leonardos, 2008). *A. desmarestii* is an omnivore species, ingesting algae, mud and faecal pellets and is quite tolerant to temperature and salinity variations (Janssens de Bisthoven et al., 2006). It is also predated by several species of fishes (Fidalgo and Gerhardt, 2002), so it is an important link in the food-web, which leads to it being a potential organism for ecotoxicological studies. Several works were performed with this species exposed to several contaminants, some of them included metals, with sub-lethal endpoints including behaviour and feeding rate (Janssens de Bisthoven et al., 2006; Pestana et al., 2007). Other studies examined bleached kraft mill effluent (Ferreira et al., 2002) and textile effluents (Casimiro and Fidalgo, 2008) with mortality as endpoints.

The amphipods *Echinogammarus meridionalis* Pinkster, like *A. desmarestii*, also lives in slowly running waters, sometimes with some pollution from domestic effluents. Being a detritivore, it feeds primarily on detritus, mainly on coarse organic matter, which confers an important role in detritus processing and consequently in structure and function of freshwater ecosystems (Macedo-Sousa et al., 2007). Recently, a few studies were done with this species exposed to

metals, with feeding rate after exposure as the main endpoint (Pestana et al., 2007; Agostinho et al., 2012) and to an acid mine drainage with behaviour and feeding rate as the sub-lethal endpoints (Macedo-Sousa et al., 2007).

The main objective of this work was to evaluate the effects at the physiological level of the essential metals, copper and zinc in *A. desmarestii* and *E. meridionalis*. To achieve this goal, two acute assays for each species were done in order to determine the LC50 for each metal, and a set of assays, for both species, with sub-lethal concentrations of the each metal where feeding inhibition was assessed during several phases.

3.3. Material and Methods

3.3.1. Sampling and acclimation of organims

Adults of *A. desmarestii* were collected at Rio Ceira near Coimbra, Portugal (40°10'13.21''N 8°23'26.28''W) with a kick-sampling net, and adults of *E. meridionalis* were collected at Rio Lena near Leiria, Portugal (38°35'28.3''N 8°40'30.2'W). Both organisms were transported to the laboratory in local waters. During an acclimation period in laboratory, the organisms were maintained for at least two weeks in aerated artificial pond water (APW) (Table 3.1), at 20°C and with a photoperiod of 16h-8h (light, dark) before experiments. Alder leaves were given *ad libitum* during the acclimation period.

Table 3.1: Composition of the artificial pond water (APW).

Stock Solution (S)	$g.l^{-1}$	$ml\ S.l^{-1}$ distilled water
1. $CaCl_2.H_2O$	58.80	5
2. $MgSO_4.7H_2O$	24.65	5
3. $NaHCO_3$	12.95	5
4. KCl	1.15	5

3.3.2. Metal solutions

Test solutions were prepared by dissolving copper ($CuCl_2.2H_2O$) and zinc ($ZnCl_2$) stock solutions in APW. The metal concentration in the samples was analyzed by ICP-MS (inductively coupled plasma-mass spectroscopy).

3.3.3. Acute tests

In order to evaluate the sensitivity of *A. desmarestii* and *E. meridionalis* to copper and zinc, and establish a range of sub-lethal concentrations of these metals for the feeding assays, lethality test were performed. The organisms were exposed for 48h to different concentrations of copper and zinc in plastic beakers with 300ml of test solution. For each concentration, one organism was added per beaker and 10 beakers were used. Copper nominal concentrations were 25.0 to 250.0 $\mu g.l^{-1}$ for shrimps and 10.0 to 350.0 $\mu g.l^{-1}$ for amphipods plus a no metal added control, and for zinc 2.5 to 15.0 $mg.l^{-1}$ plus a control was used for both species. No food was given during the exposure period. Every day the physical-chemical parameters and mortality were registered. At the end of test, the organisms were dried until a stable weight was obtained and the metal concentration of contamination solutions and in the body was measured with ICP-MS. Use of a standard tissue allowed for the determination of the percent recovery, presented on table 2.1 in chapter 2.

3.3.4. Sub-lethal feeding tests

In order to evaluate sub-lethal effects of copper and zinc on feeding behaviour of *A. desmarestii* and *E. meridionalis*, one test with four phases of 48h each was done. The range of copper and zinc nominal concentrations for shrimps were 0.0 to 130.0 $\mu g.l^{-1}$ and 0.0 to 15.0 $mg.l^{-1}$, respectively. For amphipods assays were 0.0 to 50.0 $\mu g.l^{-1}$ and 0.0 to 10.0 $mg.l^{-1}$, for copper and zinc correspondingly. For each metal and each concentration five replicates were done. One side of the chamber

had only pre-weighed leaf discs to register autogenic changes while on the other side there were leafs discs and one shrimps or two amphipods. During the first phase (acclimation), the organisms were placed in clean APW with uncontaminated leaf discs. During the second phase (exposure) they were exposed to contaminated APW and they were allowed to feed contaminated food, while on the third phase (depuration) they were put back into clean APW with clean food and finally one more phase again with clean APW and food (recovery). Every day the physical-chemical parameters, mortality and presence of moult were registered. At the end of each period, leaf discs and organisms were dried until a stable weight in order to determine the ingestion rate.

Ingestion rate (Ir) was determined by

$$Ir = \frac{\Delta Lw}{Lwi} \times \frac{1}{d \times ind}$$

where, ΔLw, variation of the leaves weight (initial minus final weight); Lwi, leaves initial weight; d, number of days; and ind, number of organisms. Ir is in $\mu g.ind^{-1}.day^{-1}$.

3.3.5. Statistical analysis

Mortality of *A. desmarestii* and *E. meridionalis* were recorded during the exposure period and the LC50 for both metals were determined with a probit analysis with the software PriProbit ver.1.63. Metal content in organisms were analysed by one-way ANOVA or nonparametric Kruskal-Wallis when no normality was achieved. Significantly different treatments were identified using Dunn's method. The effects of metals on feeding behaviour of both species were analyzed by the GLM analysis with mean weight of organisms used as a covariate and to found the differences the Dunnet post-hoc test was performed.

All statistical analysis was performed using SigmaPlot for Windows, version 11.0 (Systat Software Inc., California, USA) and Minitab 15.0.

3.4. Results

3.4.1. Acute tests

Physical-chemical parameters registered for the copper *A. desmarestii* LC50 assay were: temperature 20.09±0.018°C, conductivity 576.05±2.035µS.cm^{-1}, pH 7.92±0.018, dissolved oxygen 7.82±0.039mg.l^{-1} and percentage of oxygen saturation 85.85±0.413%. For the LC50 assay with zinc these were: temperature 19.73±0.023°C, conductivity 577.75±1.602µS.cm^{-1}, pH 7.79±0.008, dissolved oxygen 7.44±0.036mg.l^{-1} and percentage of oxygen saturation 81.99±0.398%.

On assays with the amphipods *E. meridionalis*, physical-chemical parameters registered for the copper LC50 assay were: temperature 19.13±0.069°C, conductivity 651.62±2.897µS.cm^{-1}, pH 7.59±0.012, dissolved oxygen 7.87±0.017mg.l^{-1} and percentage of oxygen saturation 85.20±0.150%. For the zinc LC50 assay these were: temperature 18.83±0.059°C, conductivity 709.74±2.516µS.cm^{-1}, pH 7.33±0.010, dissolved oxygen 8.06±0.017mg.l^{-1} and percentage of oxygen saturation 86.03±0.144%.

Real concentrations from both assays were above the 90% of the nominal concentrations.

Table 3.2: The 48-hour LC50 values with 95% confidence interval (CI) for shrimps and amphipods exposed to copper and zinc.

	Cu (µg.l^{-1}) LC50 (95% CI)	**Zn (mg-l^{-1})** LC50 (95% CI)
A. desmarestii	128.3 (90.35-212.38)	7.95 (5.795-11.975)
E. meridionalis	50.0 (36.0-72.0)	11.86 (8.409-35.560)

Metals content in the whole-body of shrimps is shown in Figure 3.1. Organisms exposed to copper presented no significantly higher levels of copper from the control group (ANOVA, $F_{5,56}$=0.644; p=0.667). However shrimps exposed to zinc presented a significant increase on zinc whole-body content related to the increase of zinc in solution (Kruskal-Wallis, $H_{5,56}$=35.449 ; p<0.05).

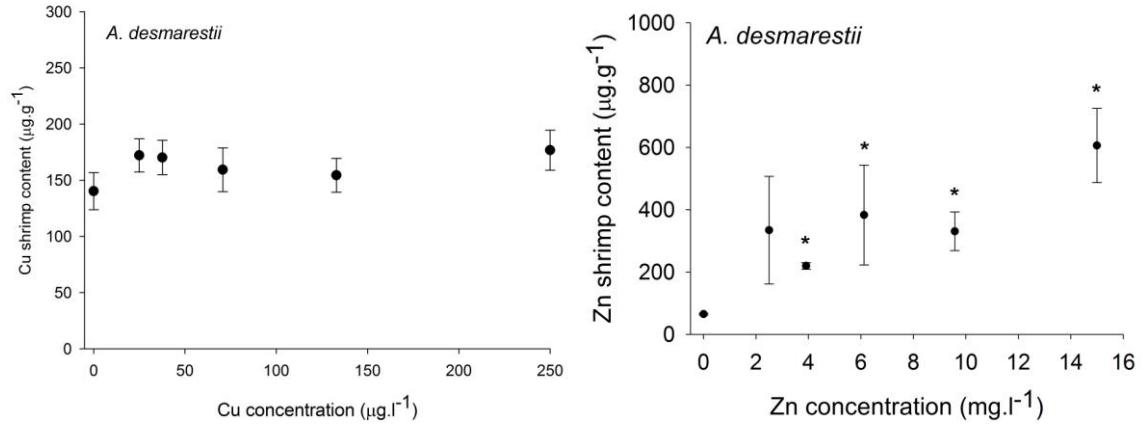

Figure 3.1: Metals content in whole shrimps *A. desmarestii* at different nominal concentrations treatment. Results expressed as mean ± SE (n=10). * indicates statistically significant differences, Kruskal-Wallis p<0.05.

Metals content in the whole-body of amphipods is presented in Figure 3.2. No significant differences were found in content of both metals in amphipods after exposure to all concentrations of copper and zinc (ANOVA, $F_{5,16}$=0.882; p=0.525 and $F_{5,17}$=2.816; p=0.066, respectively).

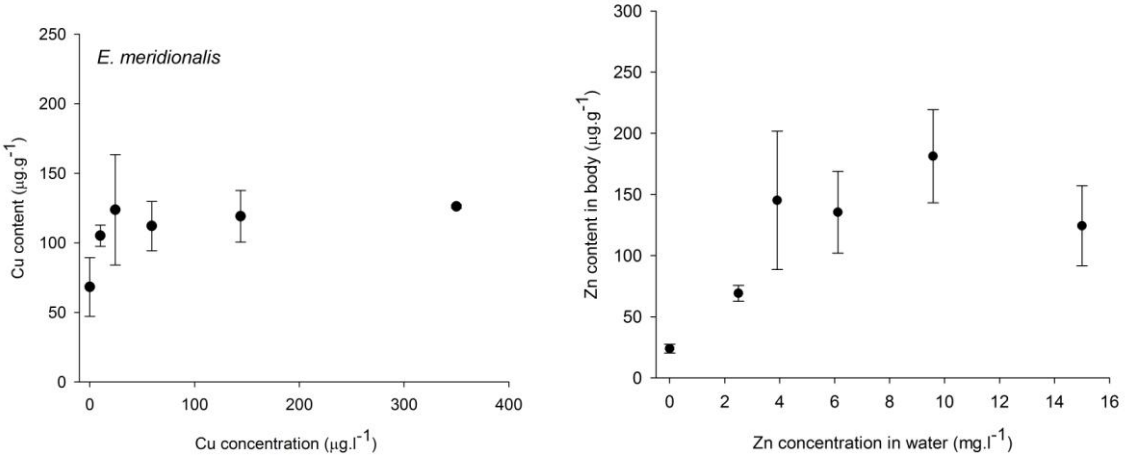

Figure 3.2: Metals content in whole amphipods *E. meridionalis* at different nominal concentrations treatment. Results expressed as mean ± SE (n=3).

3.4.1. Sub-lethal feeding tests

Physical-chemical parameters registered for the *A. desmarestii* feeding assay with copper were: temperature 18.30±0.039°C, conductivity 584.99±1.259µS.cm-

^{1}, pH 7.61±0.016, dissolved oxygen 8.2±0.021mg.l^{-1} and percentage of oxygen saturation 85.63±0.204%. No mortality was observed in this assay.

In the zinc assay, the physical-chemical parameters registered were: temperature 18.76±0.027°C, conductivity 571.24±1.401µS.cm^{-1}, pH 7.69±0.013, dissolved oxygen 8.15±0.010mg.l^{-1} and percentage of oxygen saturation 86.60±0.082%.

Table 3.3 and Table 3.4 present the cumulative percentage of mortality for the two metals assays with both species.

Table 3.3: Cumulative percentage of *A. desmarestii* mortality during the 192h of the experiment.

		Cumulative percentage of *A. desmarestii* mortality (%)			
	n	Acclimatation	Exposure	Depuration	Recovery
Copper (µg.l^{-1})					
0.0	10	0.0	0.0	0.0	0.0
2.0	10	0.0	0.0	0.0	0.0
4.6	10	0.0	0.0	0.0	0.0
10.6	10	0.0	0.0	0.0	0.0
24.5	10	0.0	0.0	0.0	0.0
56.4	10	0.0	0.0	0.0	0.0
130.0	10	0.0	0.0	0.0	0.0
Zinc (mg.l^{-1})					
0.00	10	0.0	0.0	0.0	0.0
0.50	10	0.0	0.0	0.0	0.0
0.99	10	0.0	0.0	10.0	10.0
1.95	10	0.0	0.0	0.0	0.0
3.85	10	0.0	10.0	10.0	10.0
7.60	10	0.0	20.0	20.0	20.0
15.00	10	0.0	30.0	30.0	30.0

Table 3.4: Cumulative percentage of *E. meridionalis* mortality during the 192h of the experiment.

		Cumulative percentage of *E. meridionalis* mortality (%)			
	n	Acclimatation	Exposure	Depuration	Recovery
Copper (µg.l^{-1})					
0.0	20	0.0	0.0	0.0	0.0
1.0	20	0.0	0.0	10.0	20.0
2.2	20	0.0	10.0	15.0	25.0
4. 8	20	0.0	0.0	15.0	20.0
10.5	20	5.0	15.0	15.0	30.0
22.9	20	0.0	20.0	20.0	25.0
50.0	20	5.0	40.0	70.0	70.0
Zinc (mg.l^{-1})					
0.00	20	0.0	0.0	0.0	0.0
0.50	20	0.0	5.0	20.0	20.0
0.91	20	0.0	0.0	0.0	10.0
1.66	20	0.0	5.0	5.0	10.0
3.02	20	0.0	0.0	5.0	15.0
5.49	20	0.0	10.0	25.0	50.0
10.00	20	0.0	20.0	50.0	60.0

The

Figure 3.3 shows *A. desmarestii* daily mean of ingestion rate during the 4 phases and with the different concentrations of copper.

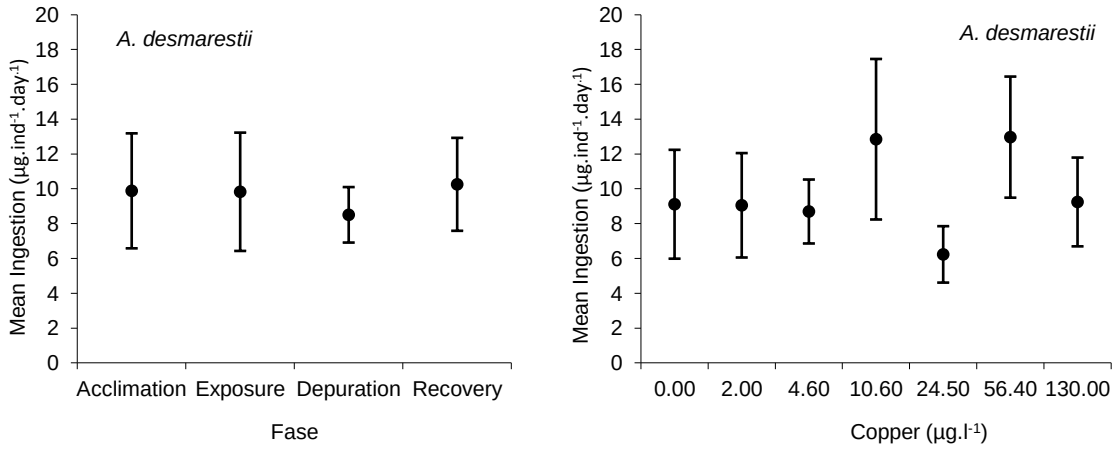

Figure 3.3: Daily mean of ingestion of *A. desmarestii* during the different phases (n=28) and in different nominal concentrations of copper (n=5). Results expressed as mean ± SE.

The average daily of ingestion rates of *A. desmarestii* during the 4 phases and with the different concentrations of zinc are presented in Figure 3.4.

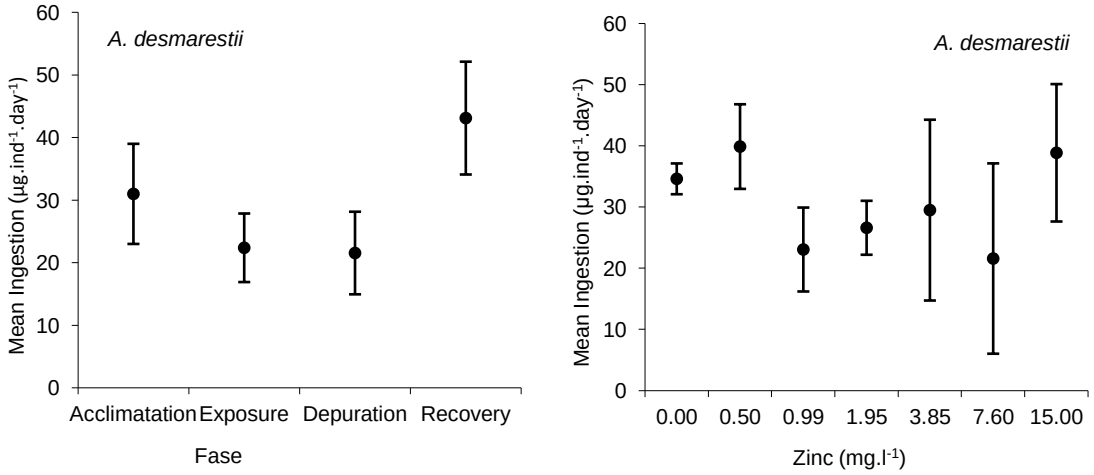

Figure 3.4: Daily mean of ingestion of *A. desmarestii* during the different phases (n=28) and in different nominal concentrations of zinc (n=5). Results expressed as mean ± SE.

The daily average of ingestion rates of *E. meridionalis* during the 4 phases and with the different concentrations of copper are showed in Figure 3.5.

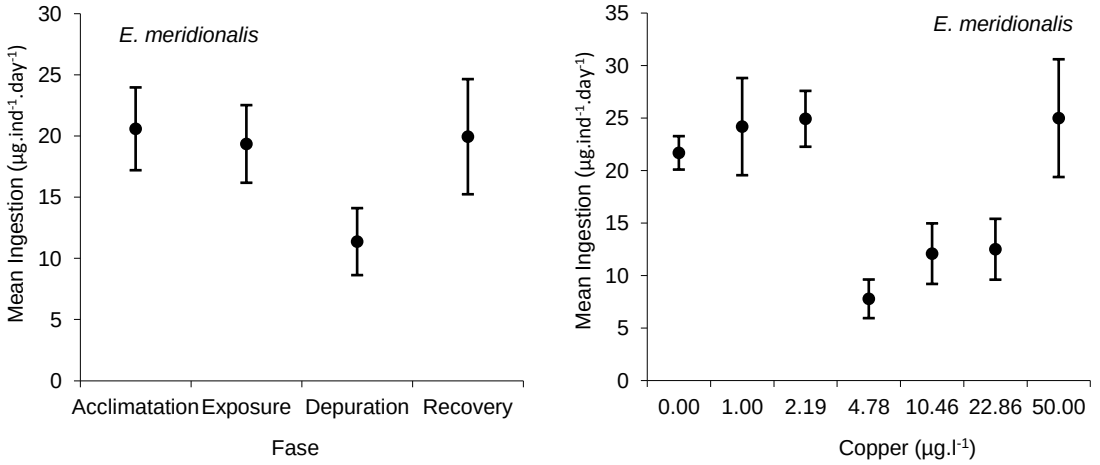

Figure 3.5: Daily mean of ingestion of *E. meridionalis* during the different phases (n=28) and in different nominal concentrations of copper (n=5). Results expressed as mean ± SE. *,** indicates statistically significant differences, GLM $p<0.05$, $p<0.01$, respectively.

In Figure 3.6 are presented the *E. meridionalis* daily mean of ingestion rate during the 4 phases (left side) and with the different concentrations of zinc (right side).

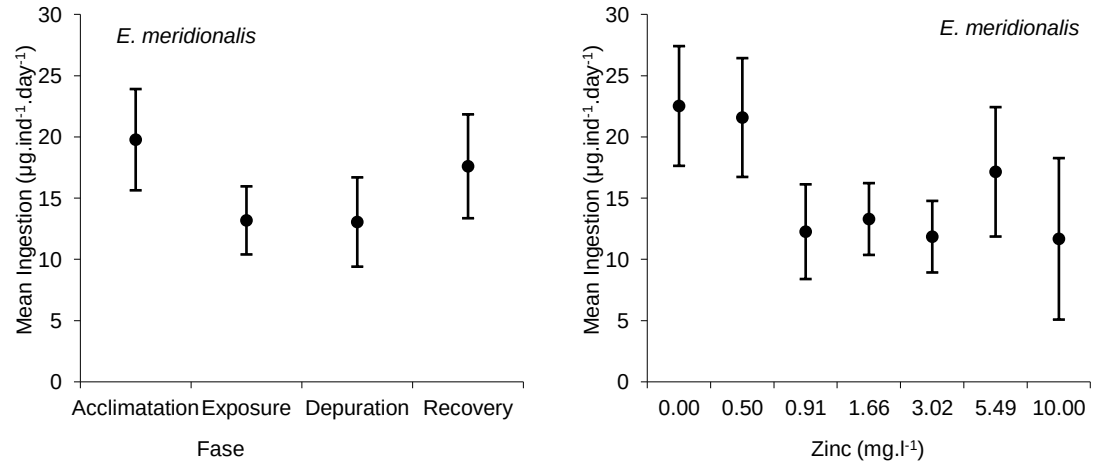

Figure 3.6: Daily mean of ingestion of *E. meridionalis* during the different phases (n=28) and in different nominal concentrations of zinc (n=5). Results expressed as mean ± SE.

In relation to the metals contents on whole-body of organisms at the end of the experimental procedure (Table 3.5), only the highest concentration of copper in case of the shrimp registered significantly higher values than control (ANOVA, $F_{6,32}=2.487$; $p<0.05$).

Table 3.5: *A. desmarestii* and *E. meridionalis* body-burdens. * indicates statistically significant differences in relation to control group, Holm-Sidak, p<0.05.

A. desmarestii				***E. meridionalis***			
Cu ($\mu g.l^{-1}$)	*Cu content* ($\mu g.g^{-1}$)	*Zn* ($mg.l^{-1}$)	*Zn content* ($\mu g.g^{-1}$)	*Cu* ($\mu g.l^{-1}$)	*Cu content* ($\mu g.g^{-1}$)	*Zn* ($mg.l^{-1}$)	*Zn content* ($\mu g.g^{-1}$)
0.0	151,2±11.58	0.0	97,9±20.82	0.0	79,4±4.40	0.0	59,7±12.47
2.0	165,0±9.88	0.5	118,6±9.88	1.0	76,1±5.85	0.5	42,2±0.92
4.6	186,2±5.80	1.0	89,0±3.50	2.2	79,0±7.22	0.9	35,1±20.23
10.6	183,8±12.72	1.0	100,4±16.52	4.8	79,6	1.7	20,4
24.5	192,2±9.67	3.9	113,6±15.16	10.5	93,4±8.84	3.0	58,0±21.64
56.4	191,6±9.37	7.6	115,2±8.48	22.9	73,4±14.86	5.5	----
130.0	203,9±16.54 *	15.0	135.8±41.18	50.0	----	10.0	----

3.5. Discussion and Conclusions

Freshwater ecosystems are essential for humans; however it is man himself who, through his activities, has been responsible for their degradation. Thus, there is urgency to assess their health in order to allow actions of prevention and remediation. Essential metals, like copper and zinc, despite their importance for organisms, become toxic when high environmental concentrations are attained. Thus, it seems crucial to determine what effects these metals have at physiological level of *A. desmarestii* and *E. meridionalis*.

Changes in feeding are a sensitive endpoint and one of the first responses to environmental changes (Maltby and Crane, 1994; McLoughlin et al., 2000). Since feeding is related to the growth, reproduction and survival of organisms, alterations of this physiological parameter can have repercussions at higher organisational levels, like populations or even ecosystems. It has been observed that, the reduction in feeding of *Gammarus pulex* resulted in a decrease of its abundance and consequently a reduction of detritus processing in ecosystem (Maltby et al., 2002).

Little is known about toxicity of several contaminants on physiological state of the two selected species. Existing studies documented the accumulation of metals in *A. desmarestii* (Abdennour et al., 2000) and the sensitivity of this shrimps to acid mine drainage (Gerhardt et al., 2004) while another study demonstrated the effects of cadmium and zinc in feeding rates of the both species (Pestana et al., 2007). One study documented the effect of acid mine drainage in behaviour and feeding of this species (Macedo-Sousa et al., 2007; Macedo-Sousa et al., 2008) while another study focused on the sensitivity and the effects of copper on feeding rate of *E. meridionalis* (Agostinho et al., 2012).

The sensitivity of the two species to a specific metal is different. Furthermore, the same species can be affected differently by the exposure to different metals. The 48h LC50 values for copper (0.128mg.l^{-1}) and zinc (7.95mg.l^{-1}) in *A. desmarestii*, found in this work, were within the range reported for other freshwater shrimps species. Murti and Shukle (1984) determined 0.338 and 10.702mg.l^{-1} as 96h LC50 value for the freshwater shrimps *Macrobrachium lamarei* exposed to copper and zinc sulphate, respectively. The copper and zinc LC50 for *M. carcinus*, a freshwater shrimp, were 0.1 and 0.2mg.l^{-1}, respectively (Correa, 1987). Lodhi et al. (2006) have recently, determined LC50 for *M. lamarei* and *M. dayanum* exposed of 48h to copper sulphate (0.361 and 0.988mg.l^{-1}, respectively). Usually, the sensitivity to the same metal differ inter- and intra-specie, and is affected by an increase of exposure period. In case of zinc, the results compared with the value found by Pestana et al. (2007) for 96h for *A. desmarestii* (7.81mg.l^{-1}). These results are in accordance with that expected, once it is recognized that in general, with an increase in the exposure period, the sensitivity of the organisms also tends to increase, decreasing the LC50 value. The same effect was verified by these authors for the amphipods where the zinc 48h LC50 was 13.68mg.l^{-1}, which is higher than 96h LC50 (6.67mg.l^{-1}). Results from this work showed the sensitivity of the amphipods to copper and zinc was 0.05mg.l^{-1} and 11.86mg.l^{-1}, respectively. The value of LC50 from organisms exposed to copper was lower than the LC50 found by Agostinho et al. (2012)

(0.198mg.l^{-1}) for the same species with 48 h exposures to copper. This discrepancy in results can be due to the salt of copper used in this work (copper chloride) being different from the used from the authors work (copper sulphate), or by the fact that the natural populations used are from different rivers. However, other authors determined lower values for other freshwater amphipods, like *G. pulex* with 48h LC50 of 0.17mg.l^{-1} (Güven et al., 1999). Lower zinc 96h LC50 values were determined also for *Hyalella azteca* (between 0.200 and 0.436mg.l^{-1}) by (Eisenhauer et al., 1999) and (Collyard et al., 1994). This fact shows that even similar species have different sensitivities to the same metal. The results indicated that copper is more toxic for both species, and the shrimp seems to be more sensitive to zinc than the amphipod which is more sensitive to copper. Several authors showed also that for freshwater crustaceans, in general copper was more toxic than zinc (Crane and Maltby, 1991; Wong, 1993; Phipps et al., 1995; Wong and Pak, 2004).

Regarding the accumulation of metals in organisms, the similar copper content of *A. desmarestii* with the increasing concentrations of metal in solution found in this work, was also found to other freshwater decapods. As it is known, decapods have the capacity to regulate essential metals including copper (Marsden and Rainbow, 2004). Copper body burden of *A. desmarestii* were not dependent on concentrations in solution, since the concentration of copper in whole-organism was similar to that for control group organisms. Apparently body concentrations of copper are regulated, which is in accordance with the suggestion of some authors (White and Rainbow, 1982, 1984; Rainbow, 2007). No significant alterations on total body copper and zinc concentrations in *Palaemon elegans* with the increased of dissolved metal concentration (White and Rainbow, 1982; Rainbow and White, 1989). However, according to Vijayram and Geraldine (1996) the freshwater decapod *M. malcolmsonii* is not capable of regulating copper, once an internal increase occurs from an increase in exposure concentration, but the period of exposure (22 days) was longer than the exposure in this work (48h).

The concentration of zinc in whole-body of shrimp increased significantly above 3.91mg.l^{-1} of zinc in solution. After this concentration, the uptake is probably higher than excretion. This suggests that, either zinc can be stored in the organism as a mechanism of detoxification, or that no regulation of zinc is performed by *A. desmarestii*. The latter is not very likely since, according to Marsden and Rainbow (2004), decapods should have the ability to regulate essential metals. Or if no mechanism of detoxification is activated this increase in zinc content can lead to a toxic situation. The increase of zinc concentrations in whole-body were also been verified for several other crustaceans, like *P. elegans*, *H. azteca*¸ *E. pirloti* (White and Rainbow, 1982; Rainbow and White, 1989; Borgmann et al., 1993).

Considering the results of the contents of metals in amphipods, both metals presented no significant differences from exposed organisms from that of control groups. Total copper concentration in *H. azteca* was similar with the chronic exposure (ten weeks) to increasing concentrations of metal, however in short-term exposures (one week) the internal concentration increases, the authors suggest that despite the fact that *H. azteca* have the capacity to regulate copper, the regulation process is not instantaneous (Borgmann et al., 1993). Also *E. pirloti* presented net accumulation and no evidence of copper regulation (Vijayram and Geraldine, 1996). Results from copper in this work were opposite to what was expected. The exposure period might have been responsible for the observed differences since the period of exposure was shorter than the exposure periods used by previous authors. The case of zinc seems to show a clearly increasing pattern with the increase of concentration in solution. Moreover, despite no significant differences being verified by the statistical analysis, we believe that with an increase in the number of samples, this difference will appear. So, probably *E. meridionalis* could be a net accumulator as was suggested for amphipods (Rainbow, 1997; Marsden and Rainbow, 2004).

Since food ingestion is the major input of energy in crustaceans (Santos et al., 2000), the effects of sub-lethal copper and zinc concentrations on this parameter

should be assessed. Thus, attending to metal effects on feeding behaviour of both species, after statistical analysis, no differences were found between either phase or the different concentrations of copper and zinc for *A. desmarestii*.

The results for shrimp showed no effects of different phases. These organisms seem to ingest the same amount of leaves during all test periods even when they were exposed and feed leaves with different concentrations of copper. As no effects of phase were found, the analysis of the mean ingestion in relation to copper concentrations was performed, but no significant differences were verified. Organisms under stressed conditions have to cope with an increased energy demand (Maltby, 1999). Thus, to compensate for this, shrimp may feel the need to maintain feeding activity, at least for shorter periods, even if food is contaminated. Furthermore, some authors revealed that essential metals are needed in the diet to accomplish metabolic needs (Piedad-Pascual, 1989; Davis et al., 1993; Guillaume et al., 1999; Mukhopadhyay et al., 2003). However, as copper is an essential metal and is nutritionally important for several crustaceans that obtain it not only from the diet but also from the water, it may have positive effects on metabolism, tissue mineralization, growth and food consumption (Wu and Chen, 2005).

No mortality was observed in this assay even with the highest concentration which approached the LC50 value, which can be the result of the use of different natural populations in the tests. These tests were performed with field collected organisms at different seasons of year (LC50s were assessed with spring populations and feeding with winter populations). In the sub-lethal assay with the input of food contaminated during an exposure period, an increase of copper whole-body at the highest concentration was registered. This fact suggest that if the exposure period is increased it could be possible to find effect in ingestion rate, as the increase in accumulation of copper can lead to a decline in feeding, to decrease the uptake of metal and maintain the copper regulation.

Regarding the results of the zinc assay, although no differences were found between the different phases, the mean ingestion decreased during the exposure

and depuration in relation to the acclimation and after that increased in the recovery phase. This suggests that probably the shrimps when detecting some levels of zinc, decrease the feeding rate as response to adverse conditions, and take some time to recover even when the stress condition disappear, as the case of depuration phase. Decrease in feeding activity was also observed in other crustaceans species exposed to metals, like *E. meridionalis* and *A. desmarestii* (Pestana et al., 2007), *G. pulex* (Maltby and Crane, 1994), *Macrobrachium rosenbergii* (Satapornvanit et al., 2009) and the marine *Farfantepenaeus paulensis* postlarvae (Santos et al., 2000). However the energy demand prevents it from no feeding at all, as the food is the main source of energy and the shrimp needs energy to metabolic processes, and as expected the increase of ingestion occurs in recovery phase, which organisms are in clean APW and fed no contaminated food.

Regarding the effects of concentration of zinc on the ingestion of leaves by the shrimp, although no significant differences were found, the mean ingestion decrease for shrimp at the second concentration reveals that they eat less quantity of leaves. For the last three concentrations, the variability found was maybe due to the mortality registered after the exposure period. The results suggest that if the exposure period was longer, with the lower concentrations of zinc, probably the mean ingestion will be lower and differences will be found.

Sub-lethal effects of copper on feeding behaviour of amphipods revealed a significantly lower ingestion of leaves during depuration when compared to the acclimation period. This suggested that organisms decrease feeding on leaves during this period. This decrease appears only during the depuration period after the exposure to stress conditions, maybe as a result of the need to maintain the metabolic processes in order to deal with adverse conditions in exposure period. These are in according with the results found for *G. pulex*, which revealed feeding inhibition with exposure to metal contaminated water and food, which persists even after the contaminant has been removed (Wilding and Maltby, 2006). Nonetheless, mean ingestion rate starts to recover at values similar to the

acclimation period, after some time without contaminant in recovery phase. Regarding the effects of the metal concentration during all periods of the test, the third concentration presented significantly lower mean ingestion of leaves for amphipods as compared to the control group. Inhibition of ingestion by exposure to copper was registered for other species of crustaceans (Taylor et al., 1993; Dedourge-Geffard et al., 2009). The ingestion at all concentrations, except the highest, was also lower than the verified for control and first two concentrations groups. The increase of the ingestion on the highest concentration of copper can be the result of a high mortality of this concentration (70% after depuration) and consequently the survival amphipods were more tolerant and fed more to cope with stress conditions.

The effects of zinc are not as obvious as for copper. Although no significant differences were observed for the different phases, or concentrations, some patterns can be addressed. A decrease in mean ingestion occurred during exposure and only during recovery did the mean ingestion start to rise. This suggests that amphipods, like shrimp, seem to detect the zinc during exposure and responding with a decrease in ingestion to cope with stress conditions and to prevent the abnormal uptake of zinc from contaminated leaves, and maintain the regulation of internal concentration. Amphipods from groups at different concentrations of zinc, although not significantly different for ingestion, showed a decrease above the second concentration. This can support the idea that after this concentration they need to decrease the consumption of contaminated food even with increase of energy requirements. This will prevent the uptake of higher quantities of metal that can be deleterious to metabolic processes and for organism. A feeding decrease was found for the freshwater prawn *M. rosenbergii*, *E. meridionalis* and *A. desmarestii* after exposure to a range of zinc concentrations (Pestana et al., 2007; Satapornvanit et al., 2009). Once again the higher mortality observed at higher concentrations revealed that amphipods are sensitive to higher concentrations of this metal and after a certain threshold no

strategy or mechanism to regulate the zinc or the stored at a detoxified form was efficient and metal starst to be toxic, leading to the death.

In conclusion, the copper was the most toxic metal to both species, however, the amphipods revealed higher sensitivity to copper in relation to the shrimp that was more sensitive to zinc. For *A. desmarestii*, no effective physiological effects were observed at the feeding rate level. However, for zinc some alterations started to be apparent. The *E. meridionalis* feeding rate seems to be sensitive to the presence of sub-lethal concentrations of copper, expressing lower feeding rates after exposure to this metal. The zinc effects for amphipods were not so clear as copper.

3.6. References

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CHAPTER 4. Cholinesterase activity on *Echinogammarus meridionalis* (Pinkster) and *Athyaephyra desmarestii* (Millet): characterisation and effects of essential metals

4. Cholinesterase activity on *Echinogammarus meridionalis* (Pinkster) and *Athyaephyra desmarestii* (Millet): characterisation and effects of essential metals

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4.1. Abstract

Throughout the years, metals were released into freshwater ecosystems from natural and anthropogenic sources, which compromise their structural and functional equilibrium. As early warning tools, cholinesterases (ChEs) are usually used to assess the effects of organophosphate and carbamate pesticides but they are also known to be inhibited in organisms exposed to metals. Thus, in order to use the ChEs of the shrimp *Atyaephyra desmarestii* (Millet) and the amphipod *Echinogammarus meridionalis* (Pinkster) as a biomarker, the characterisation of ChEs forms present in these organisms was performed. The effects of two essential metals, copper and zinc, on ChE activity were determined by *in vivo* 48h exposures of both species to a range of metals concentrations. The cholinesterase form present in cephalotorax of *A. desmarestii* and in whole-body of *E. meridionalis* was acetylcholinesterase (AChE) since both revealed preference for the AcSCh substrate, total inhibition was verified using BW284C51 and eserine, and both species showedinsensitivity to iso-OMPA. Exposures to copper and zinc seems have no effects on the ChEs of the shrimp. A decrease on the activity of this enzyme in amphipods was registered by exposure to zinc, however is not affected by copper.

Keywords: cholinesterases; *Echinogammarus meridionalis*; *Atyaephyra desmarestii*; copper; zinc.

4.2. Introduction

Biochemical markers have been proposed as early warning tools for the assessment of environmental quality (Peakal, 1992). Cholinesterases (ChEs) are among the most used biomarkers to evaluate the effects of pollution in aquatic systems, especially of organophosphates and carbamates insecticides (Fulton and Key, 2001; Key et al., 2003). However, recent studies have found alterations to the normal level of ChE activity can be due to a wide range of contaminants, and are observed under both *in vitro* and *in vivo* conditions. These alterations were observed especially in complex mixtures (Forget and Bocquene, 1999; Guilhermino et al., 2007) and in the presence of metals (Hamza-Chaffai et al., 1998; Diamantino et al., 2000; Elumalai et al., 2002; Frasco et al., 2005; Cunha et al., 2007; Frasco et al., 2007; Guilhermino et al., 2007). Furthermore, it has been shown that ChE can be used to monitor freshwater environments contaminated not only by pesticides but also by metals or others inorganic compounds (Payne et al., 1996).

Cholinesterases are estereases capable of hydrolysing choline, a carboxylic ester, and are inhibited by eserine (Sanchez-Hernandez, 2006). At cholinergic synapses, two enzymes have the capability to hydrolyse the acetylcholine, the acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). Although the role of AChE is well established, being important to the normal function of the nervous system (Peakal, 1992), as it degrades the neurotransmitter acetylcholine in cholinergic synapses, the role of BChE remains unclear. Mack and Robitzki (2000) suggested that BChE is involved in regulation of cell proliferation and in differentiation of early neuronal stages. Also have been proposed that AChE and BChE have a complementary function depending on acetylcholine concentration (Minic et al., 2003). AChE is more active at lower acetylcholine concentrations and BChE at higher concentrations of acetylcholine or acts as a backup when the AChE is not present or when it is inhibited (Minic et al., 2003; Masson and Lockridge, 2010).

The inhibition of AChE leads to the overstimulation of the central and peripheral nervous system, increasing the acetylcholine levels, resulting in the disruption of the nerve function, which leads to deleterious effects for the organism and eventually death (Garric et al., 2008).

As the different types of ChEs have diverse hydrolytic capabilities and show different sensitivities to the same anti-cholinergic agent, it is important to characterise the enzymatic form present in the study organism. Through an approach based on the properties of the mammalian ChEs, the type present in organisms has been studied by their responses to selective inhibitors and their substrate preference (Frasco et al., 2006).

A great diversity of studies on the characterisation of ChEs has been done with several organisms, the most studied are the vertebrates, and some of them have mainly the two forms of ChEs (Chuiko, 2000; Garcia et al., 2000; Monteiro et al., 2005; Arufe et al., 2007). Several authors have determined both the type and tissue distribution of the ChEs present in different organisms (Key and Fulton, 2002; Varo et al., 2002; Diamantino et al., 2003; Cunha et al., 2005; Frasco et al., 2006; Xuereb et al., 2007). Some authors have described ChEs as having atypical properties, including atypical responses to selective inhibitors and the overlap of substrate preference, each of which make difficult the classification of ChEs as AChE or BChE (Varo et al., 2002; Diamantino et al., 2003; Cunha et al., 2005). In recent years, interest in knowing the ChE types present in invertebrates is increasing, as the use of these organisms as biomonitors. Therefore, several authors have characterised the ChEs presents in crustaceans, molluscs and arthropods (Forget and Bocquene, 1999; Key and Fulton, 2002; Frasco et al., 2006; Quintaneiro et al., 2006; Xuereb et al., 2007; Gagnaire et al., 2008; Bonacci et al., 2009; Ferreira et al., 2010).

Sediments act as a “sink” for contaminants, especially metals, and freshwater invertebrates can be confronted with high levels of contamination, due to their preference for benthic habitats, that can lead to stress situations. *Echinogammarus meridionalis* (Pinkster) and *Atyaephyra desmarestii* (Millet), as

detritivores, have an important role on the structure and function of freshwater ecosystems. They have been used as a model in ecotoxicological studies (Macedo-Sousa et al., 2007; Pestana et al., 2007; Macedo-Sousa et al., 2008). This work aimed to determine the value of ChE activities as biomarkers of metal stress in these aquatic detritivores species. To achieve this, ChE activities were characterised using different substrates and selective inhibitors and the effects of copper and zinc on ChE activity were investigated, in *E. meridionalis* and *A. desmaresti*.

4.3. Material and Methods

4.3.1. Chemicals

Acetylthiocholine iodide (AcSCh), butyrylthiocholine iodide (BuSCh), propionylthiocholine iodide (PrSCh), eserine sulfate, 1,5-bis(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide (BW284C51) and tetra-(monoisopropyl)pyrophosphor-tetra-mide (iso-OMPA) were obtained from Sigma (The Netherlands) and Bradford reagent was purchased from Bio-Rad (Germany). All other chemicals were from Merck (Germany).

4.3.2. Biological material

The amphipods were collected in a reference site at the Lena River, Porto-de-Mós, Leiria, Portugal (38°35'28.3''N 8°40'30.2'W) and decapods at Ceira River, Ceira, Coimbra, Portugal (40°10'13.21''N 8°23'26.28''W). The organisms were transported alive to the laboratory in local water and were randomly divided in two groups. One group was used to the characterisation of ChE and sacrificed by immersion in liquid nitrogen (case of the amphipods where the entire organisms was used due to it little size), or by decapitation (case of decapods where only the cephalothorax was used since this is an enzyme of the nervous system). The amphipods and the cephalothorax of shrimps were stored at -80°C until further

processing. The other group of organisms of each species was used for metal exposure tests. Organisms were acclimated at least one week under laboratory conditions in artificial pond water (APW) and fed with alder leaves (*Alnus glutinosa* (L.)) prior to usage in tests.

4.3.3. ChE determination

The biologic material was homogenized in 1ml of potassium phosphate buffer (0.1M, pH 7.2). The homogenates were centrifuged (4°C, 6000 rpm, 3 min), the supernatants obtained were removed and used as an enzyme extract for ChE activity determinations, which were performed in triplicate according to Ellman et al. (1961) adapted to microplate by Guilhermino et al. (1996). The enzymatic activity was expressed in $U.mg^{-1}$ of protein (1U is a nmol of substrate hydrolyzed per minute).

4.3.4. Protein assays

The Bradford method (Bradford, 1976) adapted to microplate was used, in triplicate, to determine the protein content in samples, using *γ*-bovine globulins as standard and a wavelength of 595nm. A Labsystem Multiskan EX microplate reader was used in all assays.

4.3.5. Cholinesterase characterization

The characterisation of the ChE forms was performed using different substrates and selective inhibitors using the supernatants of the homogenates.

Concentrations of 0.005 to 20.480 mM of AcSCh, BuSCh and PrSCh were used in substrate assays.

Eserine, BW284C51 and iso-OMPA were selected as inhibitors of ChE, AChE and BChE, respectively. Stock solutions were prepared in mili-Q water or in ethanol,

as appropriate. The concentrations were 6.25 to 200.00 µM for eserine and BW284C51 and 0.25 to 8.00 mM for iso-OMPA. For each experiment, 5 µl of the stock solution were incubated with 495µl of sample supernatant for 30 min at 25°C before ChE determination. Controls were incubated with 5µl of mili-Q and additional controls with ethanol when appropriate.

4.3.6. *In vivo* exposure to metals

The effects of copper and zinc on ChE activity of *E. meridionalis* and *A. desmarestii* were evaluated.

For amphipods, groups of 10 organisms each were exposed in plastic beakers to zinc and copper nominal concentrations of 0.0 to 3.00 $mg.l^{-1}$ and 0.0 to 0.05 $mg.l^{-1}$, respectively. For decapods, groups of 3 organisms each were exposed to zinc and copper nominal concentrations of 0.0 to 3.00 $mg.l^{-1}$ and 0.0 to 0.20 $mg.l^{-1}$, respectively. Ten replicates were used for both species. The duration of exposure was 48h, in a 20°C temperature controlled room, with a photoperiod of 16h:8h (light:dark) with aeration and food supply. The conductivity, dissolved oxygen, pH and temperature were measured daily. At the end of the experiment, one organism of each group was used to perform chemical analysis and the others were used in enzymatic assays.

4.3.7. Statistical analysis

The statistical analysis of ChE characterisation and metal exposure data was performed using one-way analysis of variance (ANOVA), when the criteria of normality and equality of variance were satisfied after $\log_{10}$ transformation (if necessary), or using the nonparametric Kruskal–Wallis test in the remaining cases. Significantly different treatments were identified using Dunnet's test.

All statistical analysis was performed using SigmaPlot for Windows, version 11.0 (Systat Software Inc., California, USA) for a significance level of 0.05.

4.4. Results

4.4.1. Cholinesterase characterisation

In figure 4.1 are presented the results from the specific inhibitor eserine. Both species demonstrated a significant inhibition of enzymatic activity ($p<0.001$) at the lowest concentration of eserine used (6.25µM), where the inhibition was about 96.7% and 95.8%, for *E. meridionalis* and *A. desmartestii*, respectively. For the highest concentration was 98 and 99% respectively.

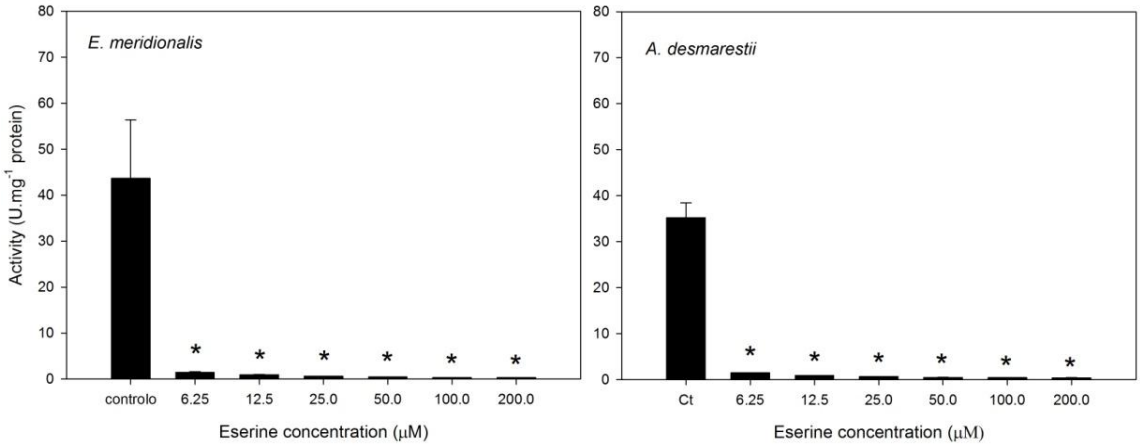

Figure 4.1: Effect of eserine on ChE activity in the *E. meridionalis* and in the cephalotorax of *A. desmarestii*, using AcSCh as substrate. Results are expressed as the mean±SE (n=3); *significantly different from control ($p<0.001$).

In order to investigate the substrate preference of the ChEs present in *E. meridionalis* and in the cephalotorax of *A. desmarestii*, the enzyme activity was determined in three independent assays with increasing concentrations of the substrates. In the amphipod the substrate cleaved at the highest rate, with the highest activity being obtained at 5.12mM (49.98±1.764 $U.mg^{-1}$ protein) was AcSCh, whereas BuSCh was minimally hydrolysed (figure 4.2). Except for BuSCh, the substrates presented a reduction of ChE activity at the highest concentration tested. In the decapod, AcSCh was also the substrate cleaved at the highest rate and with the highest activity registered (57.32±0.187 $U.mg^{-1}$ protein) at 20.48mM, but at higher concentrations the BuSCh became to be slightly hydrolysed (figure 4.2).

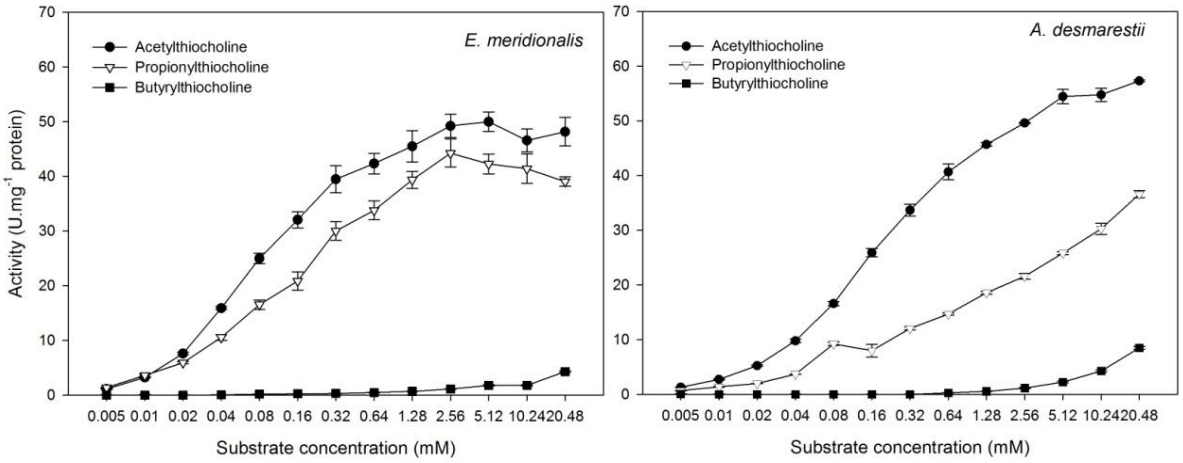

Figure 4.2: ChE activity on *E. meridionalis* and cephalotorax of *A. desmarestii* at increasing concentrations of the substrates acetylthiocholine iodide (AcSCh), propionylthiocholine iodide (PrSCh) and S-butyrylthiocholine iodide (BuSCh). Results are expressed as the mean±SE (n=3).

The results from the *in vitro* assay with BW284C51, the selective inhibitor for AChE, for both species are presented in figure 4.3. The ChE activity was significantly inhibited ($p<0.05$) in *E. meridionalis* even at the lowest concentration tested (6.25µM) of BW284C51 (96.2% inhibition). Although at the lowest concentration tested the inhibition displayed by BW284C51 was about 96.4%, *A. desmarestii* only presented significant differences ($p<0.05$) for the three highest concentrations of inhibitor used.

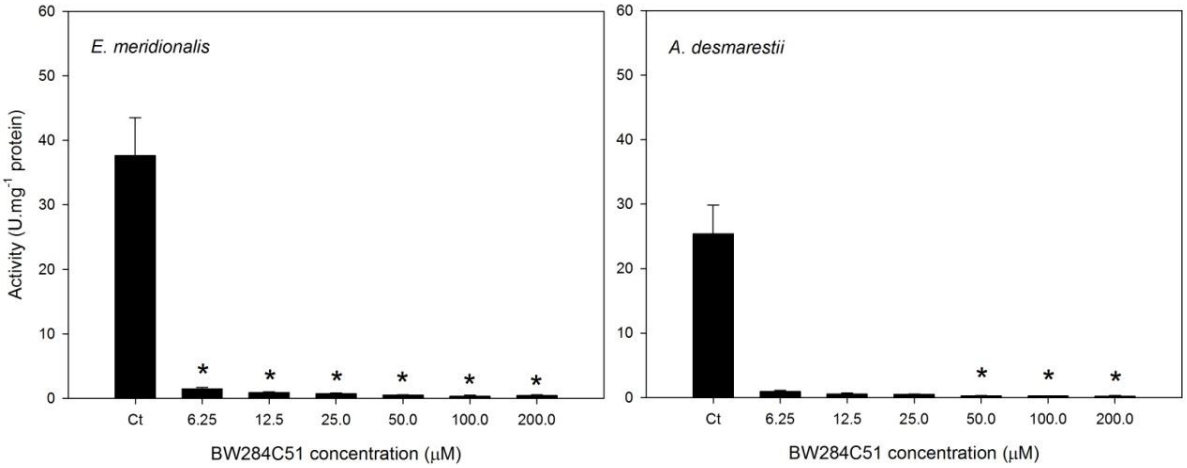

Figure 4.3: Effect of BW284C51 on ChE activity in the *E. meridionalis* and in the cephalotorax of *A. desmarestii*, using AcSCh as substrate. Results are expressed as the mean±SE (n=3); *significantly different from control ($p<0.05$).

The effects of iso-OMPA, the selective inhibitor for BChE, are presented in figure 4.4. Both species seems to be insensitive to iso-OMPA as the ChE activity remain unaffected with all concentrations tested.

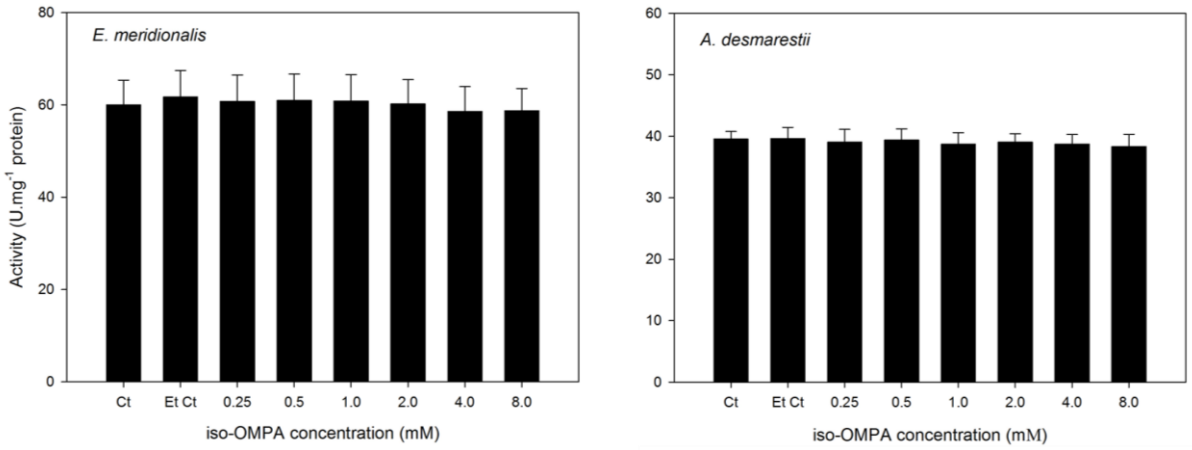

Figure 4.4: Effect of iso-OMPA on ChE activity of *E. meridionalis* and *A. desmarestii* cephalotorax using AcSCh as a substrate. Results are expressed as the mean±SE (n=3).

4.4.2. Exposure to metals

The effects of copper, on ChE activity for both species are presented in figure 4.5. The levels of ChE activity in exposed amphipods *E. meridionalis* and decapods *A. desmarestii* were not significantly different from the control organisms.

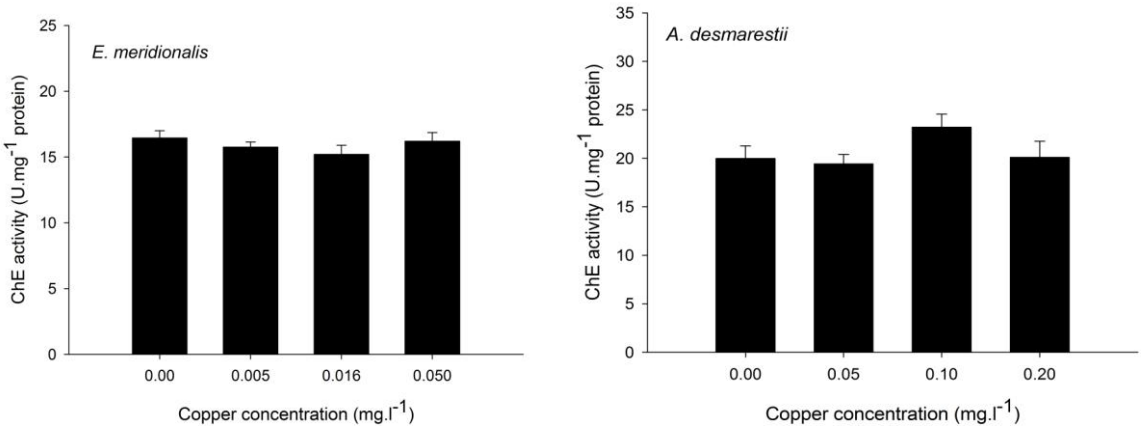

Figure 4.5: Effects of copper on ChE activity of *E. meridionalis* and *A. desmarestii*. Results are expressed as the mean±SE (n=10); *significantly different from the control ($p<0.05$).

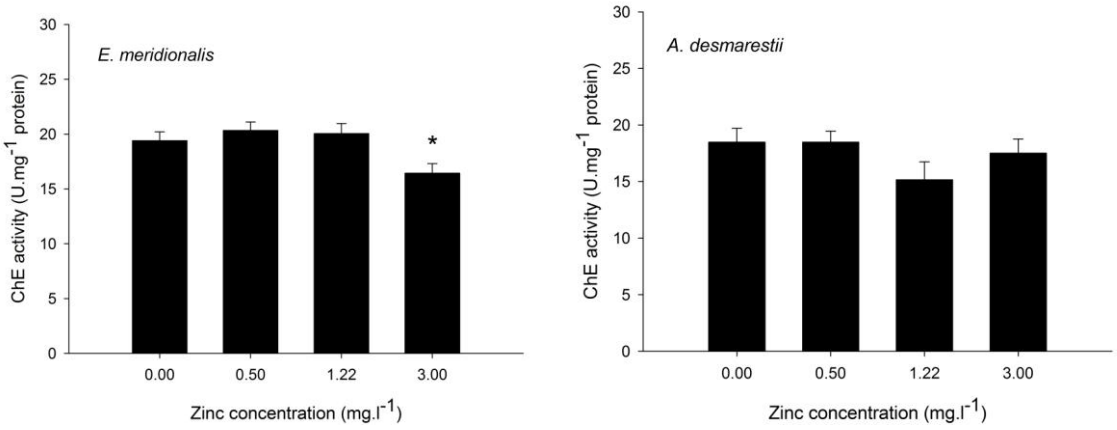

Figure 4.6: Effects of zinc on ChE activity of *E. meridionalis* and *A. desmarestii*. Results are expressed as the mean±SE (n=10); *significantly different from the control ($p<0.05$).

The effects of zinc on ChE activity of the both species are presented in figure 4.6. A significantly decrease in ChE activity levels occurred on *E. meridionalis* exposed to the highest concentration of zinc (3.00 $mg.l^{-1}$), the activity of ChE of *A. desmarestii* was not affected by zinc exposure.

4.5. Discussion and Conclusions

To characterise the ChEs present in *E. meridionalis* and in the cephalotorax of *A. desmarestii* it is necessary to distinguish the ChE from nonspecific esterases, since different tissues may contain significant amounts of other esterases, which could contribute to the measured activity, and present different sensitivities toward anti-cholinergic agents (Garcia et al., 2000). Eserine is a specific inhibitor for ChE at low concentrations (Eto, 1974). In the present study, for both species, when eserine was used the enzymatic activity was highly inhibited, which is considered typical for ChE. Thus, the results suggest that the measured activity in both species was mainly from ChE(s) and not other type of esterases. Several authors found similar results in other crustacean species. Quintaneiro et al. (2006) found a significant inhibition of eserine on ChE activity in the cephalotorax of the estuarine decapod *Crangon crangon* (L.), similar results were seen by

Frasco et al. (2006) in *Palaemon serratus* (Pennant) eyes; Varó et al. (2002) in two species of *Artemia* sp.; and Xuereb et al. (2007) in the amphipod *Gammarus pulex* (L.). This is also a typical result found in saltwater and freshwater fishes. For instance, Monteiro et al. (2005) found an inhibition of enzymatic activity to low concentrations of the compound eserine sulphate for the different tissues of estuarine fish *Pomatochistus microps* (Krøyer). Rodrigues et al. (2011) also found an inhibition with eserine in *Lepomis gibbosus* (L.).

Typically, in vertebrate species, AChE hydrolyses AcSCh more rapidly than PrSCh or BuSCh and is inhibited by higher concentrations of substrate (Eto, 1974). Results from substrates assays with the amphipod species clearly suggest the presence of a AChE form with typical characteristics. Preference for the substrate AcSCh was also registered in the cephalotorax of the decapod species, indicating that the enzyme form should also be AChE, however no inhibition was registered at the highest concentrations of substrates used. Similar preferences for the substrate AcSCh were obtained by other authors with other decapods (Frasco et al., 2006; Quintaneiro et al., 2006) and amphipods (Xuereb et al., 2007) and to other crustaceans and fishes (Forget and Bocquene, 1999; Monteiro et al., 2005; Rodrigues et al., 2011).

Vertebrate cholinesterases can be additionally differentiated by the selective inhibitor of AChE, BW284C51, and by the inhibitor of BChE, iso-OMPA (Sanchez-Hernandez, 2006; Arufe et al., 2007). In this study, the amphipod presented a significant inhibition of ChE activity by BW284C51 that indicates the presence of AChE, once this is a selective inhibitor for this enzyme form. In shrimp, only the three higher concentrations showed a significant inhibition of ChE activity, however, even in lower concentrations the inhibition was already higher (more than 96.4% from control), thus seems that this result can be due to the no normalization of the data, which leads to the use of non-parametric tests. Nevertheless, regarding the percentage of inhibition, it seems reasonable to consider that AChE is the form of ChE present in the cephalotorax of this organism. In addition, both species seem to be insensitive to all concentrations of

iso-OMPA, suggesting that BChE is not present. These results are indicative of the presence of only one form of ChEs in both species. Fact that seems to be in accordance to several studies that indicate that AChE was the only form present in all body or specific tissue of other crustaceans species (Monteiro et al., 2005; Frasco et al., 2006; Quintaneiro et al., 2006; Xuereb et al., 2007; Rodrigues et al., 2011).

Summarizing all the results from the substrate and inhibitors assays, AChE is the ChE form present in both species, since presented a perceptible affinity to choline esters and high sensitivity to eserine sulphate, an inactivity with BuSCh, which is consistent with the insensitivity to iso-OMPA, and an high sensitivity to BW284C51.

It is well known that carbamates and organophosphates inhibit AChE activity, however other types of contaminants, like metals, can also inhibit this enzyme. On present work both, *A. desmarestii* and *E. meridionalis* exposed to copper, revealed similar ChE activities to the organisms in control group. Different responses of AChE activity in organisms exposed to this metal have been reported, some registered inhibitions, other found no alterations and even some registered an increase in activity of this enzyme. Hamza-Chaffai et al. (1998) and Vieira et al. (2009) found inhibition on AChE activity by the clam *Ruditapes decussatus* (L.) and in the estuarine fish *P. microps* after exposure to 75.0 $\mu g.l^{-1}$ and 25.0 $\mu g.l^{-1}$ of copper, respectively. In crustaceans, Brown et al. (2004) also found an inhibition of *Carcinus maenas* (L.) AChE activity with 68.1$\mu g.l^{-1}$ of copper. Cunha et al. (2007) did not register any significant effects of this metal on *in vivo* assays with *Monodonta lineate* (Micallef) and *Nucella lapillus* (L.). Lehtonen and Leiniö (2003) found no alterations of AChE activity of *Macoma balthica* (L.) or for *Mytilus edulis* (L.) at low-doses of copper (40$\mu g.l^{-1}$), but did find a decrease with 200$\mu g.l^{-1}$ of copper for *M. edulis*. AChE activity of *M. edulis*, according to Brown et al. (2004) was not affected by copper. Romani et al. (2003) and Brown et al. (2004) registered induction of AChE activity with exposure to

copper for fish and molluscs, respectively. The results of the present work suggests that copper had no affect on the activity of AChE, both in the decapod and the amphipod, which lead to the suggestion that this enzyme is not the proper tool to monitor copper contamination using this species.

The exposure to zinc revealed different results to each of the species. *A. desmarestii* showed similar ChE activities as presented by organisms of control group, which suggests no effect of this metal on this enzyme activity. *E. meridionalis* when exposed to high concentrations of this metal showed a significantly lower AChE activity than in organisms of control group, suggesting that high concentrations of this essential metal can inhibit the enzymatic activity. As in copper, the effect of zinc on AChE activity is not consensual. Frasco et al. (2005) performed a study to investigate the potential of five metal ions (nickel, copper, zinc, cadmium and mercury) to inhibit AChE activity *in vitro* and concluded that all of them, except nickel, have the capacity to inhibit the AChE activity. Suresh et al. (1992) found suppression in AChE activity in all organs of the carp *Cyprinus carpio* (L.) after exposure to zinc. Elumalai et al. (2002) found a decrease on *C. maenas* with an EC50 of 14.68mg.l^{-1} of zinc. On the other hand, McLoughlin et al. (2000) didn't observe any effects on ChE activity of the amphipod *Gammarus pulex* (L.) after exposure of 24h and 48h to 8.0mg.l^{-1} zinc. Ibrahim et al. (1998) has documented no consistent negative effects on *in vivo* AChE activity of the chironomid *Chironommus riparius* (Meigen) apparent when exposed to zinc chloride, the mean activity remaining within 1.3% of the controls even at high concentrations (80.0mg.l^{-1}). In addition, Locatello et al. (2009) and Lionetto et al. (2003) have related the decrease in AChE activity with the presence of metals in urban effluents without agricultural influence. Despite the results for the amphipod when exposed to higher concentrations of zinc, the ChE activity of these organisms seems to be mostly unaffected by these metals, which leads to the conclusion that the ChE activity may not be the best tool to metal contamination assessment with these detritivores.

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CHAPTER 5. Effects of essential metals in two freshwater detritivores species: biochemical approach

5. Effects of essential metals in two freshwater detritivores species: biochemical approach

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5.1. Abstract

Biochemical biomarkers may provide early detection of contaminant exposure, and an early indication of potential effects at higher levels of organisation. Metals induce oxidative stress in organisms through production of reactive oxygen species. To evaluate the effects of a 48h of exposure to copper and zinc on *Atyaephyra desmarestii* (Millet) and *Echinogammarus meridionalis* (Pinkster) a battery of several biomarkers of oxidative stress were used together with alterations to ingestion rates. Results showed that responses of biomarkers were different among both the species and the metal. Copper inhibited the enzymatic defence system of both species, without signs of oxidative damage. On the other hand, zinc induced the defence system in *E. meriodionalis* which prevented the occurrence of oxidative damage. In *A. desmarestii* the enzymatic system did not prevent oxidative damage. In addition, zinc also significantly affected the ingestion rate of *E. meridionalis*. The integrated biomarkers response showed that *A. desmarestii* responds in general to high concentrations of metals and *E. meridonalis* to lower concentrations.

Keywords: detritivores; copper; zinc; oxidative stress; acetylcholinesterase.

5.2. Introduction

In freshwater ecosystems, the level of trace metals can be enhanced as a consequence of natural or industrial activities. In addition to water quality degradation, the increase of metal levels can lead to biological alterations in organisms and plants. Trace metals can be transferred along food chains resulting in health consequences for humans (Rainbow, 1997). Despite not always resulting in biota lethality, metal exposure may produce sub-lethal effects that can compromise organism biochemistry, physiology, and reproductive success and eventually affect the long-term survival of populations (MacFarlane et al., 2006).

Biochemical biomarkers are considered to be one of the most promising tools for ecotoxicological applications (Huggett et al., 1992; Peakall, 1992); as they may provide early detection of contaminant exposure, and an early indication of potential effects at higher levels of organisation, like population and ecosystem levels (McLoughlin et al., 2000). Metals are known inducers of oxidative stress in organisms with the subsequent production of reactive oxygen species (ROS). Oxygen free radicals, like hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet -}$) and hydroxyl (OH^-) radicals, are among the most reactive compounds produced during heavy metal stress (Dazy et al., 2009). These are generally associated with alterations at the level of DNA, proteins and membranes, and can result in cellular damage (Huggett et al., 1992; Vieira et al., 2011). Organisms are able to cope with ROS, through an antioxidant defence system, and only its failure results in oxidative stress and subsequent cellular damage (Livingstone, 2001; Lushchak, 2011).

Copper and zinc are essential metals for aquatic crustaceans, however they can produce deleterious effects if internal concentrations exceed the organism´s requirements and its detoxification capability (Viarengo et al., 1990; Correia et al., 2002). On the one hand, copper deficiency decreases the enzymatic activity of several enzymes implicated in oxidative defence systems and changes the cellular content of reactive oxygen species (ROS) scavengers (Uriu-Adams et al., 2005).

On the other hand, the excess of this metal induces the formation of ROS via the formation of oxidizing radicals, and ultimately leads to cellular toxicity (Viarengo et al., 1990; Livingstone, 2001; Gaetke and Chow, 2003). Waterborne and dietary exposure to high concentrations of copper induced oxidative stress (Lushchak, 2011). Zinc has several physiological/biochemical roles in organisms, and is involved in the functioning of more than 200 enzymes (Muyssen and Janssen, 2002; Geret and Bebianno, 2004). Several authors have proposed the redox inert metal zinc as an antioxidant (Powell, 2000; Valko et al., 2005). However, others support that an excess of zinc is related to ROS generation or the decline of antioxidant enzymes in several organisms including invertebrates (Geret and Bebianno, 2004).

The major mechanism through which ROS can cause tissue injury is lipid peroxidation (LPO) that can result in impaired cellular function, alterations in membrane properties and consequently disrupt vital functions (Rikans and Hornbrook, 1997). To reduce the negative effects of ROS, organisms have a protective and effective antioxidant defence system (van der Oost et al., 2003; Timofeyev, 2006; Woo et al., 2009; Novais et al., 2011; Vieira et al., 2011). This system is constituted of several enzymes divided in three principal groups that directly scavenge ROS: as catalase (CAT); internal lipid peroxidation products, namely glutathione peroxidase (GPX); and secondary oxidation radical products, like glutathione-S-transferase (GST). Catalase is the major enzyme involved in the scavenging of H_2O_2, catalyzing H_2O_2 to water and oxygen. Several recent works that evaluated the effects of metals on this enzyme focused on the analysis of activity alterations (Barata et al., 2005; Gravato et al., 2006; Liu et al., 2006; Vieira et al., 2011; Oliva et al., 2012). Glutathione peroxidase (GPx) also detoxifies H_2O_2 and organic hydroperoxides by, for example, lipid peroxidation (LPO) (Correia et al., 2003). Several authors found alterations in GPx enzyme activity in different organisms as a result of heavy metal exposure (Ahmad et al., 2005; Ahmad et al., 2006; Gravato et al., 2006; Liu et al., 2006). GST catalyses the conjugation of glutathione (GSH) with xenobiotics including metals, playing an

important role in their detoxification (Huggett et al., 1992; Jemec et al., 2010). Several authors have found alterations in the normal activity of GST by the presence of metals and products of oxidative stress (Hayes and Pulford, 1995; van der Oost et al., 2003). Liu et al. (2006) observed in liver of the goldfish an induction of GST activity by copper exposure. While, Gravato et al. (2006) found an increase on GST activity of liver tissue of eels after an exposure to copper.

Glutathione (GSH), also has an important role in defence of cellular damage by being a substrate for glutathione peroxidade (GPx), a cofactor for glutathione-S-transferase (GST) and directly linked to pro-oxidants, namely transition metals (Meister, 1995a, b; Saint-Denis et al., 1998; Lushchak, 2011). While, glutathione redutase (GR) has an important function in cell protection, it catalyzes the reduction of GSSG (glutathione oxidized form) into GSH (reduced and active form) (Huggett et al., 1992; Saint-Denis et al., 1998; Novais et al., 2011). Gravato et al. (2006) found a slight decrease on this enzyme activity, in the liver of European eel, after exposure to copper.

The central aim of this work is evaluate the biochemical effects of two essential metals, copper and zinc, in two detritivores species, the shrimp *Atyaephyra desmarestii* (Millet) and the amphipod *Echinogammarus meridionalis* (Pinkster), on enzymes of oxidative stress, and their value as biomarkers of metal exposure. In addition, the study relates biochemical effects with those on organism level, like feeding behaviour.

5.3. Material and Methods

5.3.1. Sampling and acclimation of organims

Adults of both species were collected with a kick-sampling or an handling net in Rio Ceira near Coimbra, Portugal (40°10’13.21’’N 8°23’26.28’’W, *A. desmarestii*) and in Rio Lena near Leiria, Portugal (38°35’28.3’’N 8°40’30.2’W, *E. meridionalis*), and transported to the laboratory in local water. Organisms were maintained for

at least one week in aerated artificial pond water (APW, see table 3.1 of chapter 3), at 20°C and with a photoperiod of 16h-8h (light, dark) for acclimation purposes. Alder leaves were given *ad libitum* during this period.

5.3.2. Metal solutions

Test solutions were prepared by dissolving copper ($CuCl_2.2H_2O$) and zinc ($ZnCl_2$) stock solutions in APW. The metal concentration on samples was analyzed by ICP-MS (inductively coupled plasma-mass spectroscopy).

5.3.3. Exposure assays

In order to evaluate the effects of copper and zinc on feeding and biochemical biomarkers of the shrimp *A. desmarestii* and the amphipod *E. meridionalis*, the organisms were exposed for 48h to different concentrations of each metal in plastic beakers with 300ml of test solution. Ten replicas were used with three shrimp or ten amphipods per beaker. Copper nominal concentrations for shrimp were 0.0, 0.05, 0.1 and 0.2mg.l^{-1} and 0.0, 0.005, 0.016 and 0.05mg.l^{-1} for amphipod. Zinc nominal concentrations for both species were 0.0, 0.5, 1.22 and 3.0mg.l^{-1}. The duration of exposure was 48h, in a 20°C temperature controlled room, with a photoperiod of 16h:8h (light:dark) with aeration and food supply (pre-weight leaf discs). Conductivity, dissolved oxygen, pH and temperature were measured daily. At the end of the experiment, one organism per replicate was used to perform chemical analysis and the others were used for enzymatic assays. The leaf discs of each group were dried at stable weight in order to determine ingestion rates following:

$$Ir = \frac{\Delta Lw}{Lwi} \times \frac{1}{d \times Ow}$$

where, ΔLw, is the variation in leaves weight (initial minus final weight); Lwi, is the leaves initial weight; d, is the number of days; and Ow, is the organism weight. Ir is given in $\mu g.mg^{-1}.day^{-1}$.

5.3.4. Enzymatic assays

The abdomen of shrimp and the amphipods of each replicate were sonicated in (1:15, 100mg of tissue in 1500ml of buffer) in 0.1MK-phosphate buffer (pH 7.4). Part of the homogenate was used for LPO determinations and the other was centrifuged for 20 min at 10000 g, at 4°C, to obtain the post-mitochondrial supernatant (PMS), which was used to determine protein and GST, CAT, GPx, GSSG and TG.

1) Protein assays

The Bradford method (Bradford, 1976) adapted to microplate (Lab system Multiskan EX microplate) was used, in triplicate, to determine the protein content in samples, using *γ*-bovine globulins as standard and a wavelength of 595nm.

2) Lipid peroxidation determination

The Ohkawa et al. (1979) and Bird and Draper (1984) methods adapted by Wilhelm et al. (2001) and Torres et al. (2002) were used to determine LPO by measuring the thiobarbituric acid reactive substances (TBARS) at 535nm and 25°C. LPO is expressed as nmol TBARS hydrolyzed per g of wet weight, using a molar extinction coefficient of $1.56x10^{-5}M.cm^{-1}$.

3) Catalase determination

Catalase activity was determined following the method of Clairborne (1985), that measures substrate (H_2O_2) consumption by the decrease in absorbance at 240 nm at 25°C. Enzymatic activity is expressed as unit (U) per mg of protein. A U

corresponds to one µmol of substrate hydrolyzed per minute, using a molar extinction coefficient of 40 $M^{-1}.cm^{-1}$.

4) Glutathione-S-Transferase determination

Glutathione-S-transferase activity was measured following the conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm and 25°C, as described by Habig and Jakoby (1981), adapted to microplate by Frasco and Guilhermino (2002). Enzymatic activity is expressed as Units (U) per mg of protein (1 U is one nmol of substrate hydrolysed per minute), using a molar extinction coefficient of $9.6x10^{3}M.cm^{-1}$.

5) Glutathione peroxidase determination

Glutathione peroxidase was determined according to Mohandas et al. (1984) by measuring the decrease in NADPH at 340nm and 25°C, using H_2O_2 as a substrate. Enzymatic activity is expressed as Units (U) per mg of protein (1 U is one nmol of substrate hydrolysed per minute), using a molar extinction coefficient of $6.22x10^{3}M.cm^{-1}$.

6) Glutathione reductase determination

Glutathione reductase activity was determined according to Cribb et al. (1989) measuring the decrease in NADPH levels at 340nm and 25°C. Enzymatic activity is expressed as Units (U) per mg of protein (1 U is one nmol of substrate hydrolysed per minute), using a molar extinction coefficient of $6.22x10^{3}M.cm^{-1}$.

7) Total glutathione, glutathione oxidized and reduced glutathione determination

Total glutathione content (TG) and GSSG were determined at 412 nm and 25°C measuring the recycling reaction of GSH with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in the presence of excess GR (Tietze, 1969; Baker et al., 1990). 2-Vinylpyridine was conjugated with GSH for GSSG determination in order to

prevent oxidation (Griffith, 1980). GSH was calculated by subtracting GSSG from the TG levels. The levels of substrate are expressed in nmol of substrate hydrolysed per minute per mg of protein, using a molar extinction coefficient of $14.1x10^{3}M.cm^{-1}$.

5.3.5. Integrated Biomarker Response

All measured parameters were combined into a general "stress index" - "Integrated Biomarkers Response", according to Beliaeff and Burgeot (2002), in order to integrate the impact of selected metals on both species. In the present study, the following biomarkers were considered: ingestion rate, AChE (data from chapter 4), GST, GR, GPx, GSH, GSSG, TG, TBARS and CAT. For the zinc exposure with the amphipod species, the results from CAT were removed in the final analysis, as the response was so intense that it masked the response of the other biomarkers.

The standardized value of each biomarker Y_i is determined by:

$$Y_i = \frac{(X_i - \bar{X})}{s_i}$$

where, X_i is the mean value of biomarker of each treatment, $\bar{X}$ is the general mean value for the biomarker and s_i is the standard deviations of each treatment. The min value of Y_i, Y_{min} is determined and the score, S_i is calculated by:

$$S_i = Y_i + |Y_{min}|$$

Finally, the IBR for each treatment is calculated by the formula:

$$IBR = \sum_{i=1}^{n} \left[\left(\frac{(S_i \times S_{i+1})}{2} \right) \div n \right]$$

The final IBR value is divided by the number of biomarkers according to Broeg and Lehtonen (2006).

5.3.6. Statistical analysis

The effects of metals on enzymatic activities, gluthathiones and feeding rate were analysed using one-way analysis of variance (ANOVA), when the criteria of normality and equality of variance were satisfied after log transformation (if necessary), or using the nonparametric Kruskal-Wallis test in the remaining cases. Significantly different treatments were identified using Holm-Sidak post-hoc test.

All statistical analyses were performed using SigmaPlot for Windows, version 11.0 (Systat Software Inc., California, USA) for a significance level of 0.05.

5.4. Results

5.4.1. Effects of metals on biochemical biomarkers

1) *Effects of copper on the shrimp* Atyaephyra desmarestii

The effects of copper on several enzymatic biomarkers of *A. desmarestii* are presented in Figure 5.1. No significant alterations were observed in CAT activity levels with exposure to copper at all concentrations in relation to the control group. A significant decrease in GST activity levels occurred with exposure to the highest concentration of copper (0.20mg.l^{-1}). GPx activity decreased in the first two concentrations and was significantly lower than in the control at a copper exposure of 0.10mg.l^{-1}. However, in the highest concentration (0.2mg.l^{-1}) there was a slight increase, however activity remained lower than in the control.

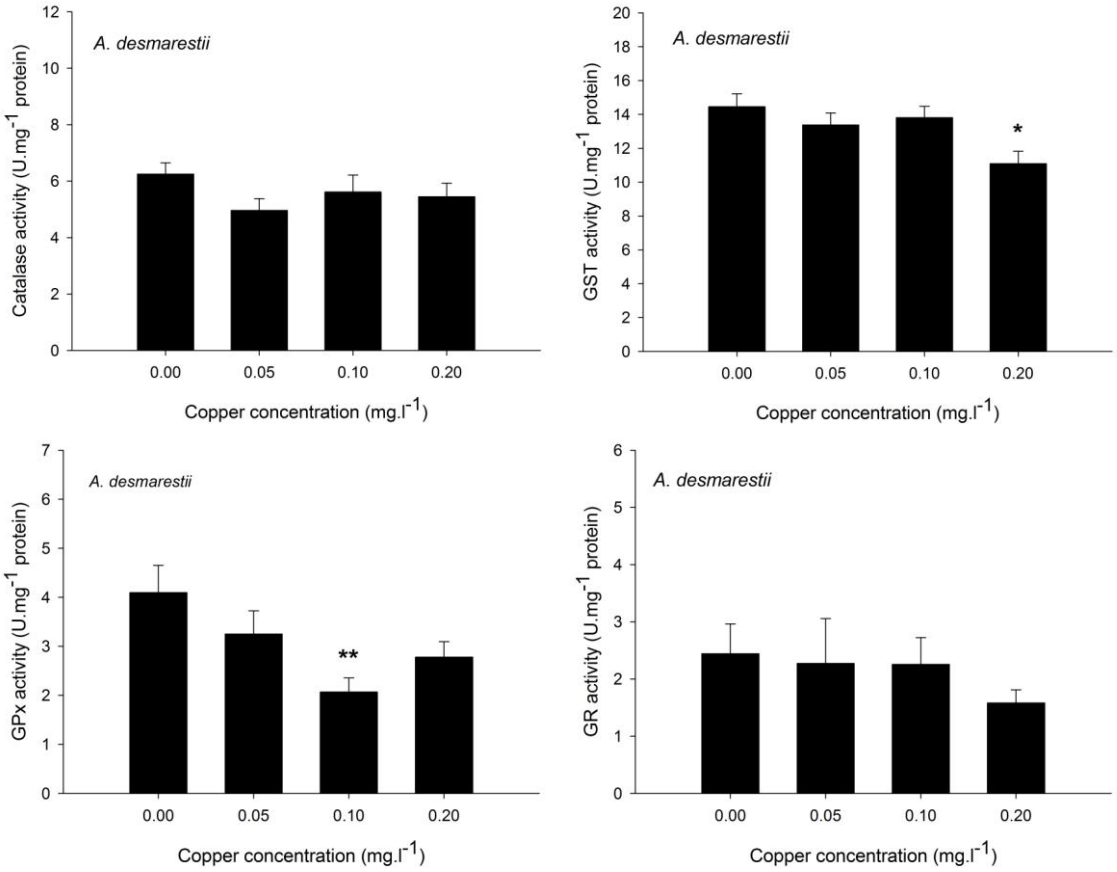

Figure 5.1: Results of detoxification (GST) and antioxidant defenses (CAT, GPx and GR) of *A. desmarestii* exposed to three concentrations of copper (mg.l^{-1}) plus control. Results expressed as mean ± SE (n=10). *,** indicates statistically significant differences between exposed groups and control, Holm-Sidak $p<0.05$, $p<0.01$, respectively.

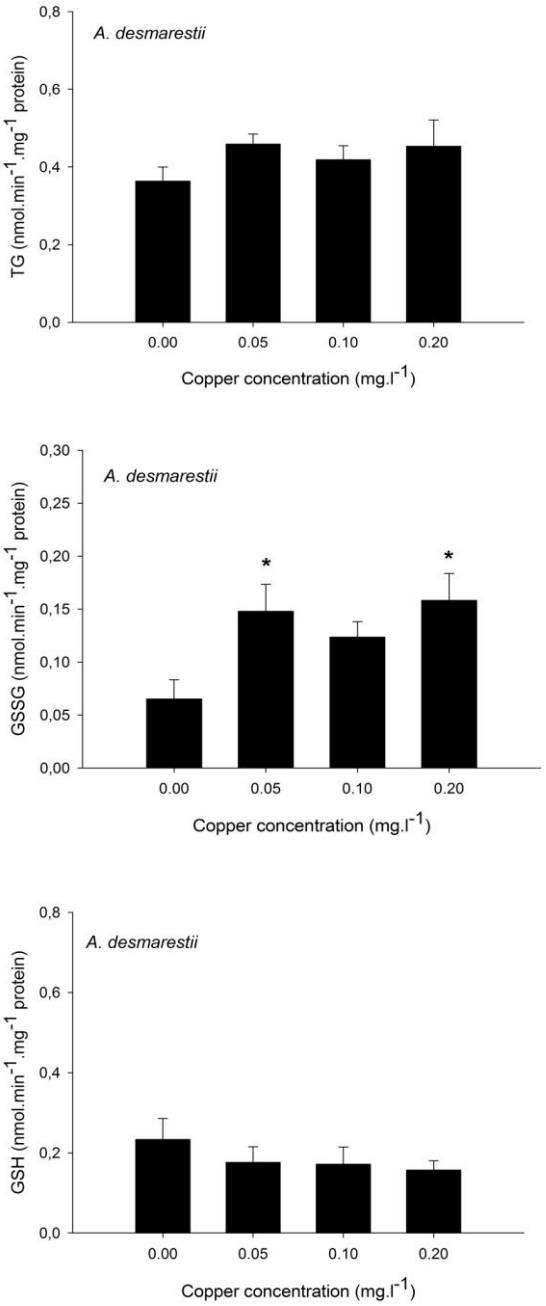

Figure 5.2: Results of the non enzymatic antioxidant defenses (TG, GSSG and GSH) of *A. desmarestii* exposed to 3 concentrations of copper ($mg.l^{-1}$) plus control. Results expressed as mean ± SE (n=10). * indicates statistically significant differences between exposed groups and control, Holm-Sidak $p<0.05$.

The effects of copper exposure on glutathiones concentrations are shown in Figure 5.2. TG and GSH levels did not vary with copper exposure. However, there was a significant increase on the levels of GSSG in the lowest ($0.005mg.l^{-1}$) and highest concentration ($0.2mg.l^{-1}$) when compared to the control group.

Despite, the increase pattern on LPO levels with the rise of exposure concentration, no significant differences were found for LPO in *A. desmarestii* (Figure 5.3).

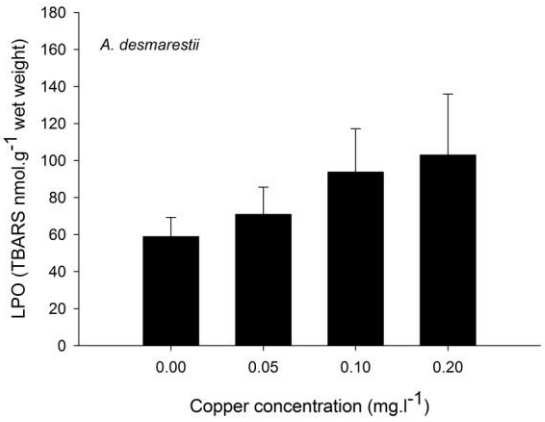

Figure 5.3: Results of LPO levels of *A. desmarestii* exposed to 3 concentrations of copper (mg.l^{-1}) plus control. Results expressed as mean ± SE (n=10).

2) *Effects of copper on the amphipod* Echinogammarus meridionalis

No significant alterations were registered for CAT and GST activity levels of *E. meridionalis* exposed to copper (Figure 5.4). GPx presented significantly lower activity in organisms exposed to 0.016mg.l^{-1} of copper. For GR, no significant alterations were registered. Nonetheless, a slight decrease was observed with the increase of exposure concentration.

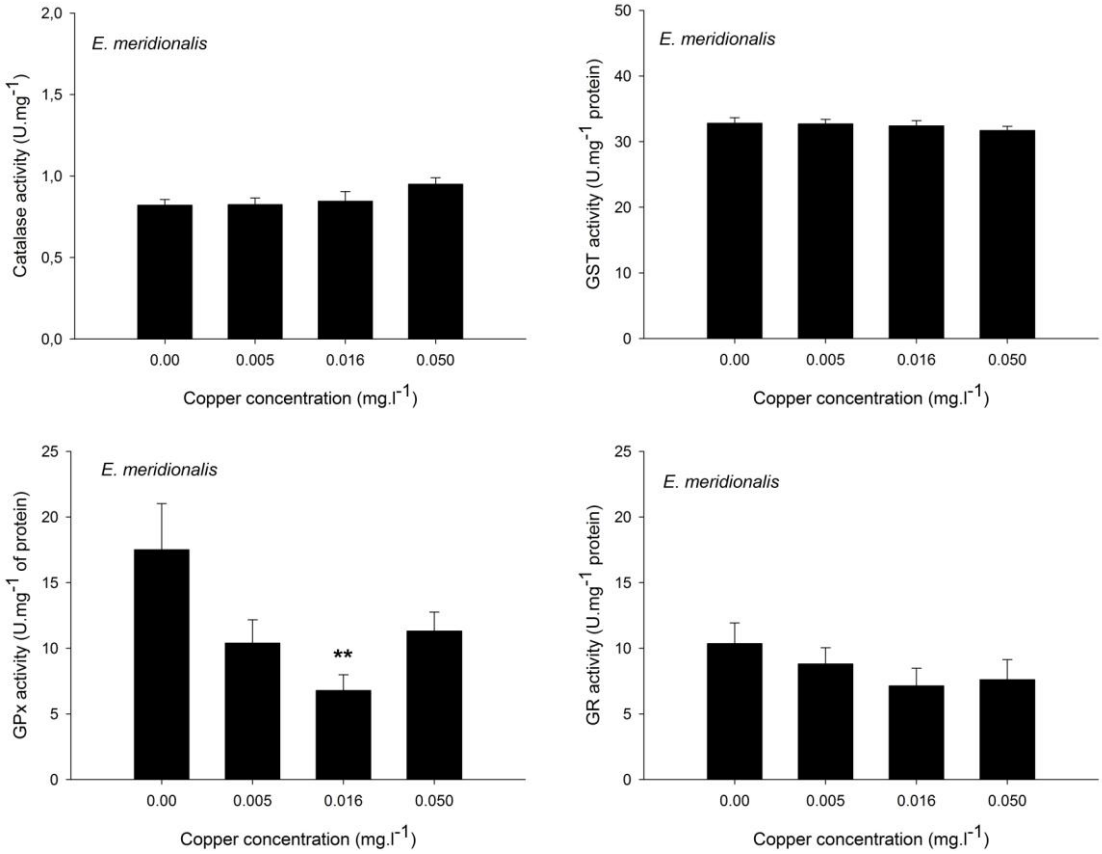

Figure 5.4: Results of detoxification (GST) and antioxidant defenses (CAT, GPx and GR) of *E. meridionalis* exposed to 3 concentrations of copper ($mg.l^{-1}$) plus control. Results expressed as mean ± SE (n=10). ** indicates statistically significant differences between exposed groups and control, Holm-Sidak $p<0.01$.

Regarding the results for glutathione levels, presented in Figure 5.5, no significant differences were registered either for TG, or for GSSG or GSH. Nevertheless, a slight depletion of GSSG levels with the increase in concentration were observed concomitantly with a slight increase on GSH levels.

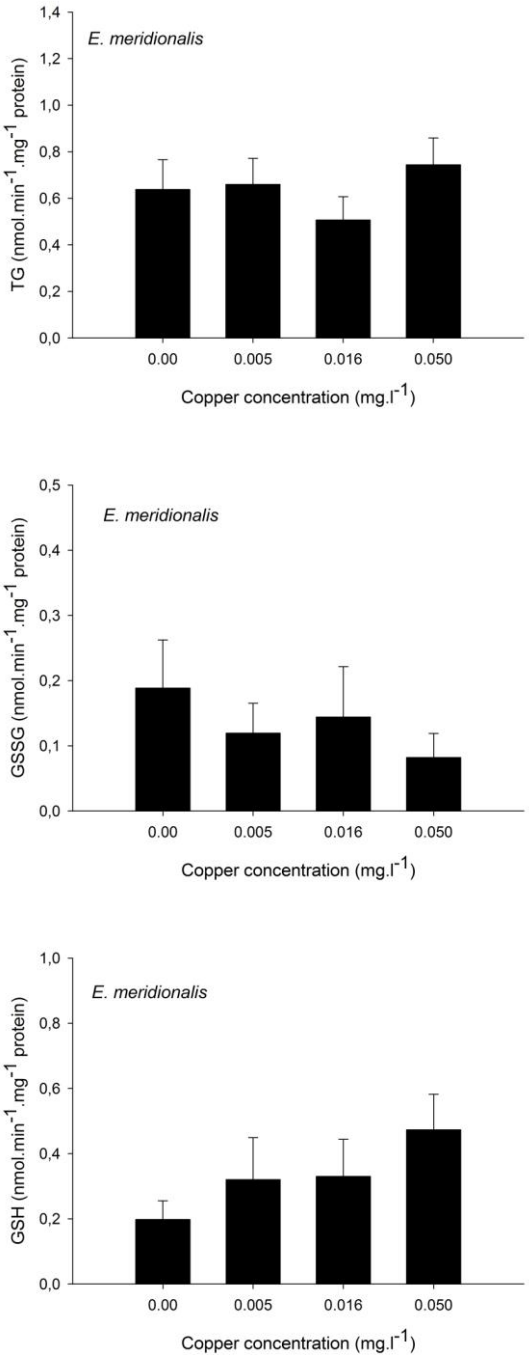

Figure 5.5: Results of the non enzymatic antioxidant defenses (TG, GSSG and GSH) of *E. meridionalis* exposed to 3 concentrations of copper ($mg.l^{-1}$) plus control. Results expressed as mean ± SE (n=10).

No oxidative damaged was observed since no significant differences were registered for LPO at all copper concentrations (Figure 5.6).

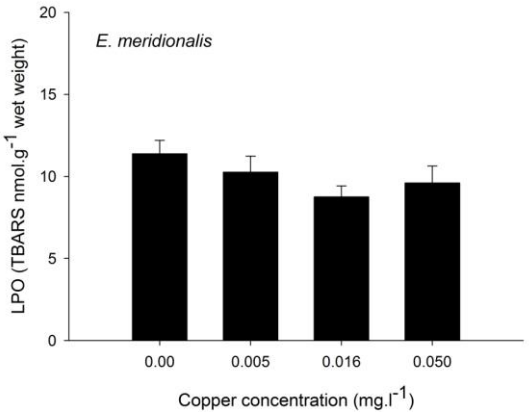

Figure 5.6: Results of LPO levels of *E. meridionalis* exposed to 3 concentrations of copper (mg.l^{-1}) plus control. Results expressed as mean ± SE (n=10).

3) *Effects of zinc on the shrimp* Atyaephyra desmarestii

The effects of zinc on the enzymatic antioxidant defence system (CAT, GPx, GR) and detoxification (GST) of *A. desmarestii* are shown in Figure 5.7. Only GPx presented an increase of activity levels with the increase of exposure concentration, being significantly different in the higher exposure of 3.0mg.l^{-1}. CAT, GR and GST were not significantly affected by exposure to these concentrations of zinc.

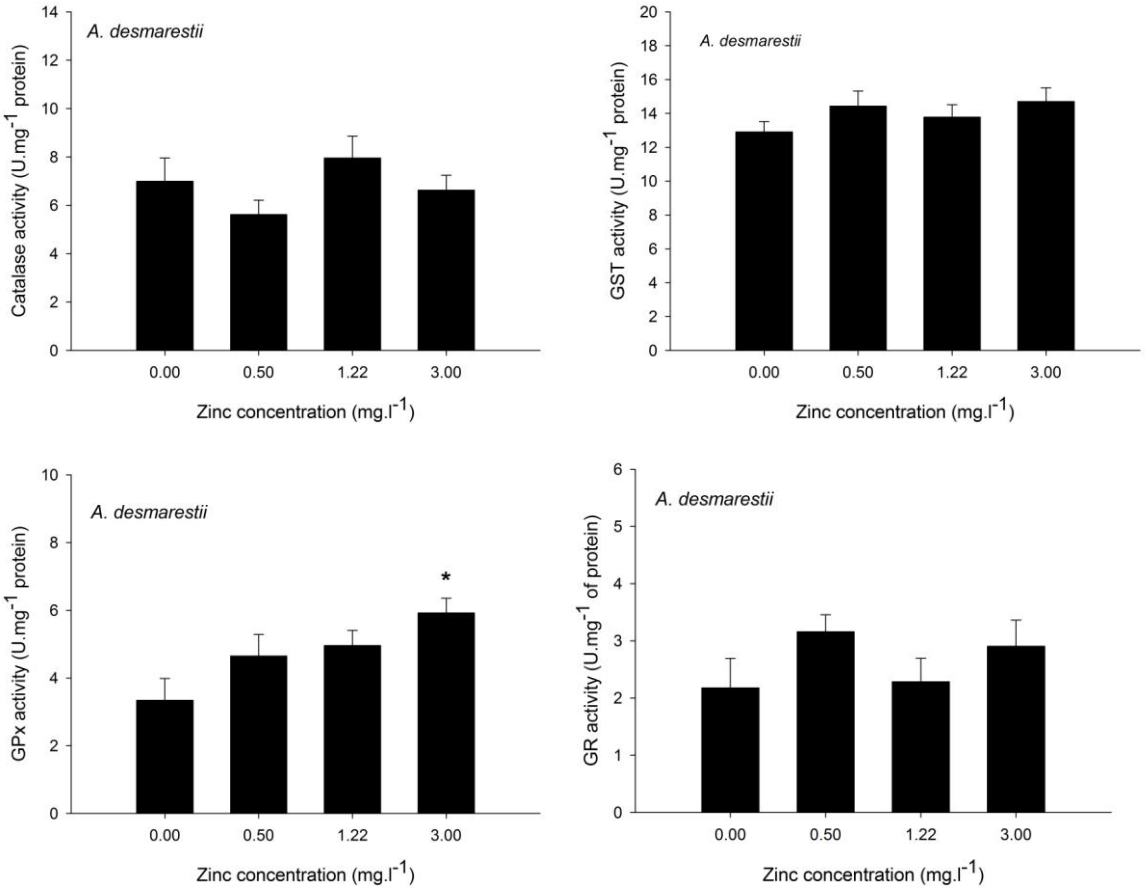

Figure 5.7: Results of detoxification (GST) and antioxidant defenses (CAT, GPx and GR) of *A. desmarestii* exposed to 3 concentrations of zinc (mg.l^{-1}) plus control. Results expressed as mean ± SE (n=10). * indicates statistically significant differences between exposed groups and control, Holm-Sidak $p<0.05$.

No significant alterations in TG levels were observed because of zinc exposure in the shrimp species (Figure 5.8). Furthermore, the level of the oxidized form of glutathione (GSSG) was similar at all exposure concentrations, and the reduced form (GSH), although not significant, was like TG, higher in the highest concentration.

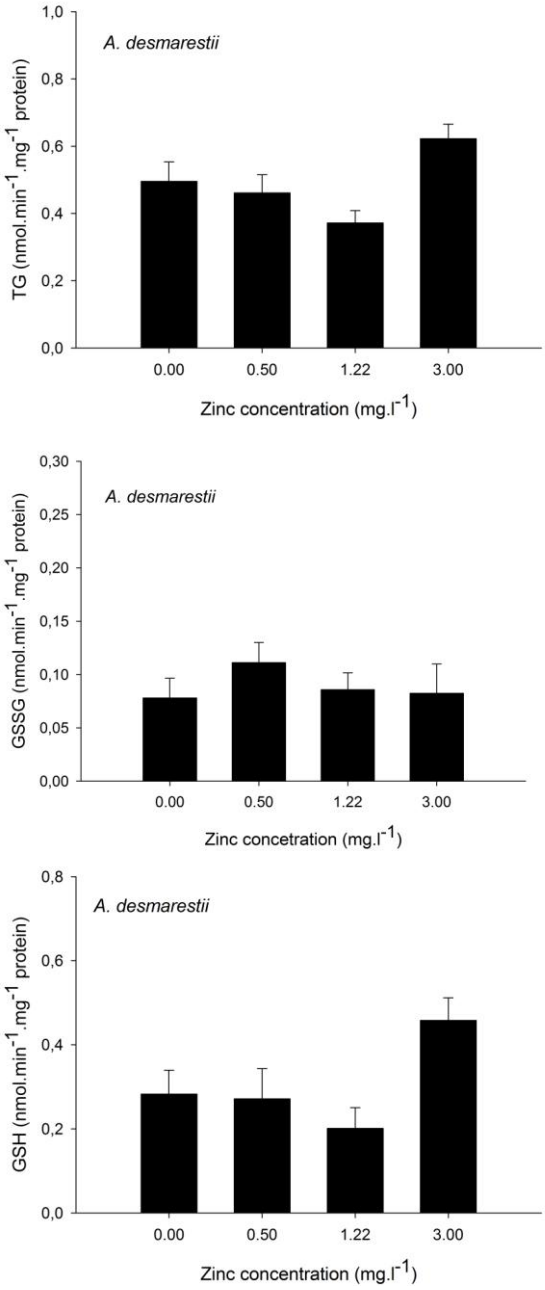

Figure 5.8: Results of the non enzymatic antioxidant defenses (TG, GSSG and GSH) of *A. desmarestii* exposed to 3 concentrations of zinc ($mg.l^{-1}$) plus control. Results expressed as mean ± SE (n=10).

Oxidative damage was observed in all zinc exposure concentrations (0.5, 1.22 and 3.00$mg.l^{-1}$) since LPO levels were significantly higher than LPO level from control group (Figure 5.9).

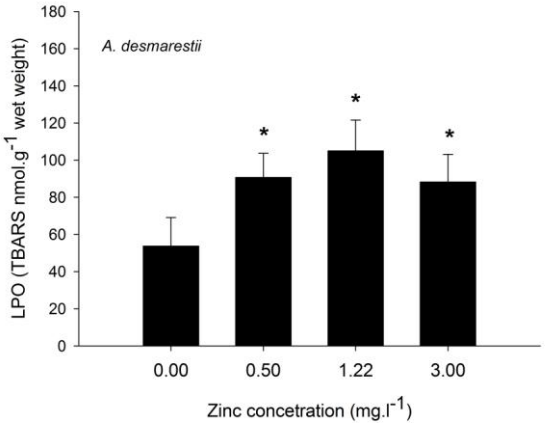

Figure 5.9: Results of LPO levels of *A. desmarestii* exposed to 3 concentrations of zinc (mg.l^{-1}) plus control. Results expressed as mean ± SE (n=10). * indicates statistically significant differences between exposed groups and control, Holm-Sidak p<0.05.

4) Effects of zinc on the amphipod Echinogammarus meridionalis

5)

The effects of zinc on the enzymatic antioxidant defence system (CAT, GPx, GR) and detoxification (GST) of *E. meridionalis* are shown in Figure 5.10. A significant depletion was registered for CAT activity levels, in relation to the control, at higher concentrations (1.22mg.l^{-1}, and 3.00mg.l^{-1}). GST activity was significantly higher at 3.00 mg.l^{-1} of zinc. No significant differences were found on GPx and GR activity in amphipods, however a slight decrease with 3.00mg.l^{-1} can be observed in GR.

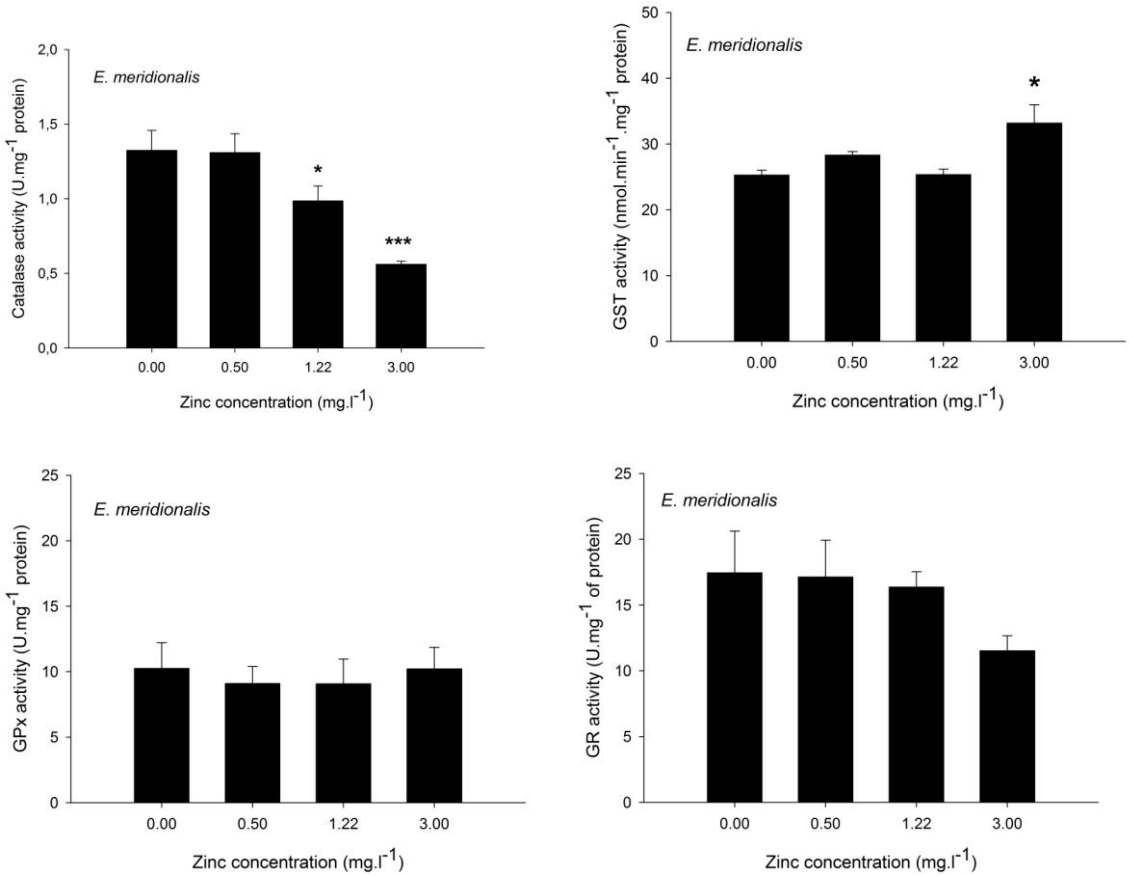

Figure 5.10: Results of detoxification (GST) and antioxidant defenses (CAT, GPx and GR) of *E. meridionalis* exposed to 3 concentrations of zinc (mg.l^{-1}) plus control. Results expressed as mean ± SE (n=10). *, *** indicates statistically significant differences between exposed groups and control, Holm-Sidak $p<0.05$, $p<0.001$, respectively.

Similar concentrations of TG levels showed a slight decrease with increase in concentrations, and the GSSG and GSH concentrations were also similar, none the less, higher GSH correspond to lower GSSG (Figure 5.11).

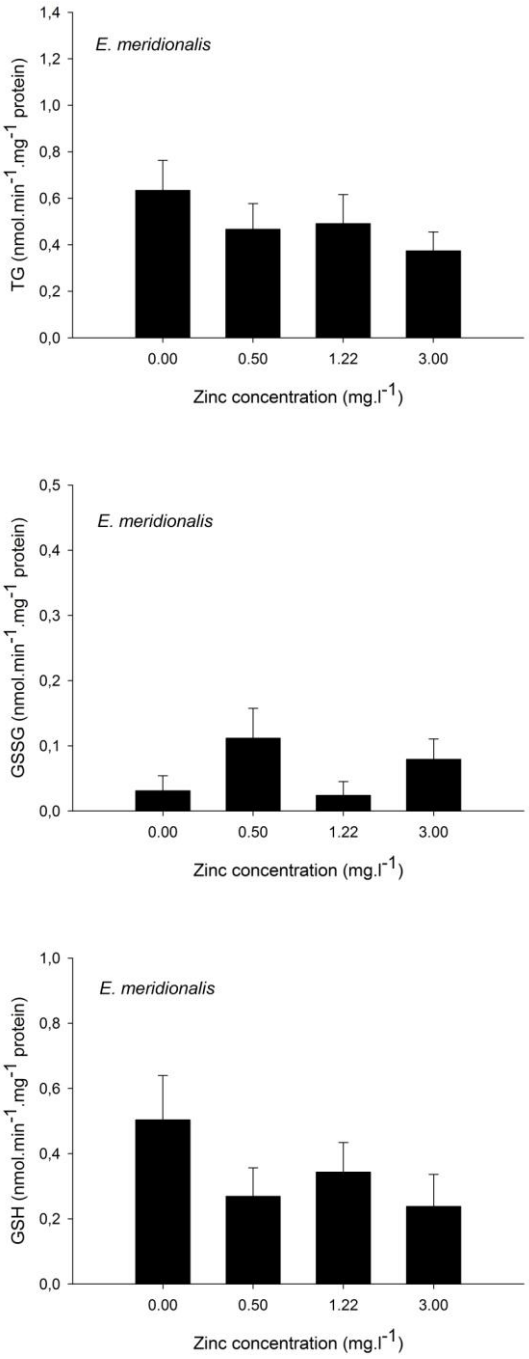

Figure 5.11: Results of the non enzymatic antioxidant defenses (TG, GSSG and GSH) of *E. meridionalis* exposed to 3 concentrations of zinc ($mg.l^{-1}$) plus control. Results expressed as mean ± SE (n=10).

Despite the pattern of increase in LPO levels with the rise of exposure concentration, no significant differences were found for LPO in *E. meridionalis* (Figure 5.12).

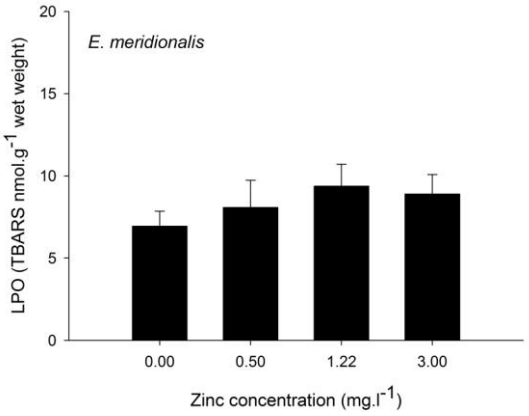

Figure 5.12: Results of LPO levels of *E. meridionalis* exposed to 3 concentrations of zinc (mg.l^{-1}) plus control. Results expressed as mean ± SE (n=10).

5.4.2. Effects of metals on feeding

Alterations in the normal feeding rate of *A. desmarestii* and *E. meridionalis*, with exposure to both metals, copper and zinc, are presented on Figure 5.13. No differences were found regarding ingestion rates of shrimp for both metals. However, as the power of the statistic test was below the desired value, the differences were less likely to be detected, thus these data should be interpreted cautiously.

In amphipods, copper did not affect ingestion rates, however, like with the other species the power of the test was below the desired value, so the interpretation should be careful. On other hand, zinc exposure significantly decreased ingestion rates.

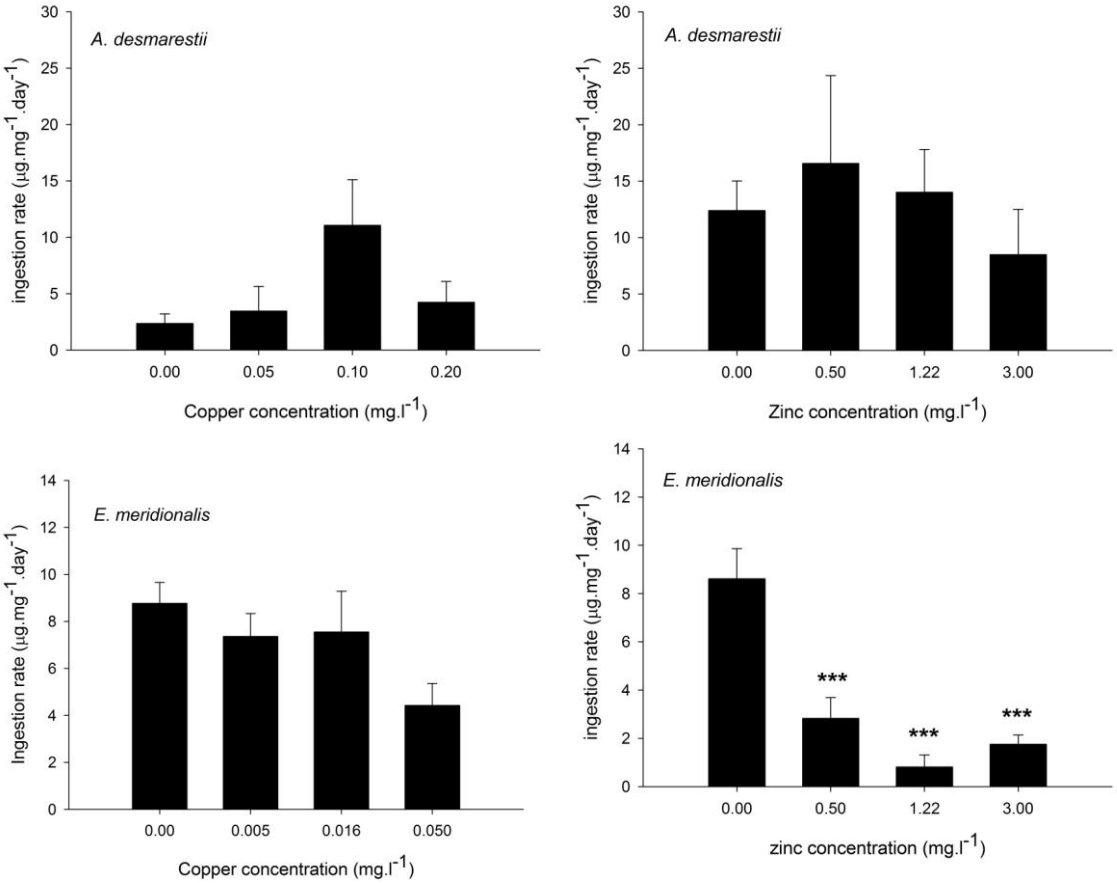

Figure 5.13: Feeding activity of *A. desmarestii* and *E. meridionalis* with exposure to metals, copper and zinc. Results are expressed as mean of ingestion rate ± SE (n=10). *** indicates statistically significant differences between exposed groups and control, Holm-Sidak p<0.001.

5.4.3. Integrated Biomarker Response

Figure 5.14 shows the scores of the different biomarkers after exposure to copper and zinc, for both species. The copper exposure of shrimp revealed a distribution of the response of different biomarkers for all concentrations. With zinc exposure, a higher response was observed with the third concentration, and the main biomarkers that responded were TBARS, TG, GSH and GPx. Like shrimp, the exposure of copper to amphipods revealed responses distributed for all biomarkers at all concentrations. The exposure to zinc, the response of CAT was so intense that it masked all the biomarkers response, so it was retired from the

IBR analysis and thus the first concentration was the one that presented a high response for more biomarkers, including ingestion rate, AChE, GST, GR.

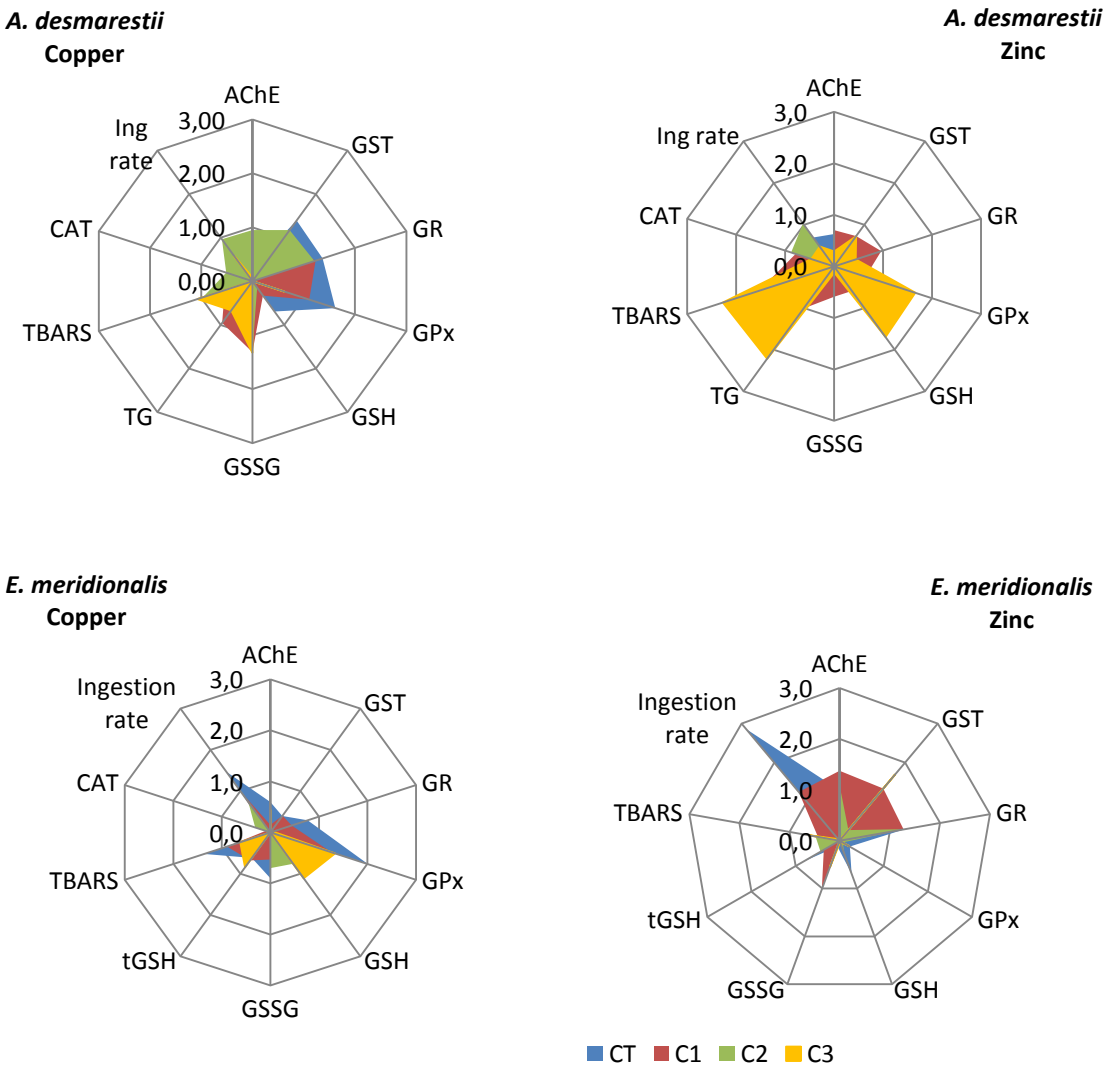

Figure 5.14: Integrated biomarker response of *A. desmarestii* and *E. meridionalis* with exposure to metals, copper and zinc. Concentrations CT, C1, C2 and C3 correspond to the nominal concentrations of the respective assays.

5.5. Discussion and Conclusions

Copper and zinc, despite being essential for freshwater organisms, can cause deleterious effects if there is a high level of exposure that, ultimately, will have repercussions in ecosystems. It is known that metals induce oxidative stress by

the production of ROS and a strong antioxidant defence system is crucial to neutralize their impact (Pandey et al., 2008). Thus, the evaluation of the effects of these metals at biochemical level becomes important. The main aim of this work was the investigation of the responses of several biomarkers of oxidative stress in the two selected detritivores with exposure to copper and zinc via the water column and their potential use as biomarkers of metals toxicity in integrated ecotoxicological studies.

1) *Effects of copper on shrimp* Atyaephyra desmarestii *and on amphipod* Echinogammarus meridionalis

Copper exposure does not seem to affect CAT activity in either of the species. Roche and Boge (1993) suggested that the enhancement of CAT activity could compensate for the decline in activity of other enzymes affected by oxiradicals however, no alterations on CAT activity could be explained by the increase of other antioxidant enzymes, such as GPx and/or non-enzymatic mechanisms, like GSH and metalothionines (Huggett et al., 1992).

Regarding the other antioxidant enzymes, GR activity of both species was not affected by copper. Alterations on GR activity were difficult to find, as this enzyme seems to be poorly responsive to contamination, and its activity depends on the organ where it is measured (van der Oost et al., 2003). Matos et al. (2007) suggested that an increase in GR activity in an oxidative stress situation would be normal. Such increase could be related with the activation of the machinery to convert GSSG into GSH, as it is less expensive energetically than synthesizing new GSH (Pena-Llopis et al., 2001; Oliveira et al., 2009).

GPx was significantly inhibited by copper exposures for both species. The inhibition of GPx can have implications for the cell, as it can lead to an oxidative damage situation. The balance of the cellular redox status could be compromised, more even if GR activity does not change, as GPx have an important role to

maintain the ration GSH/GSSG. Atli and Canli (2010) observed GPx inhibition at acute metal exposure and increases in activity for chronic exposures.

In the present study, exposure to copper resulted in an inhibition of GST activity in the shrimp species, but the GST activity of the amphipod was not affected. Barata et al. (2005) always found an induction in *Daphnia magna* (Straus) after exposures to several compounds, including copper. While, a decrease in GST activity was observed in the hepatopancreas of the freshwater prawn *Macrobranchium rosenbergii* (De Man) after seven days of exposure to low copper concentrations ($0.01 mg.l^{-1}$), rising at higher concentrations (Li et al., 2008). The authors suggested that the depletion of the enzyme could be the result of negative effects of copper contamination on its structure and function. Other authors also found inhibitions of GST as a consequence of exposure to different xenobiotics, including metals (Mosleh et al., 2006; Cunha et al., 2007; Novais et al., 2011). Various explanations were advanced in an attempt to explain this inhibition. It may have occurred through direct action of the metal on the enzyme, leading to inactive complexes thus facilitating compound elimination. Or it can be inhibited indirectly by the production of ROS, which will interact directly with the enzyme, or can decrease due to depletions of the substrate (GSH), and/or by down regulation of GST genes through different mechanisms (Cunha et al., 2007; Novais et al., 2011).

Copper exposures affected the non-enzymatic mechanisms of shrimp, and no affected significantly in case of amphipod. The concentration of shrimp GSSG increased. However, these were not accompanied by the increase in GSH or TG levels. Moreover, GSH levels were slightly lower in the exposed shrimps. Despite no significant alterations on the ratio GSH/GSSG, responsible for the balance of cellular resox status of the organism, a slightly decrease can be observed. This breakdown concomitant with the depletion of shrimp GPx and GST could result in an accumulation of ROS that can compromise the integrity of the cellular components. Similar observations were reported by Novais et al. (2011). The depletion on GSH content could be attributed to metal accumulation in cells,

which results to a decrease in availability of GSH once it forms GS-metal complexes (Pandey et al., 2008), these finds are in agreement with the depletion of GPx, GST and GR, which are GSH dependent enzymes.

Copper exposures not resulted in oxidative damage for both species, as LPO levels were similar. However, in case of shrimps, can be observed a trend of higher TBARS levels that suggests that oxidative damage was starting to occur, and cells were bound to suffer injuries caused by inability of the antioxidant defence system and the non-enzymatic mechanisms to deal with the ROS production. Moreover, even the observed inhibition on GST of shrimp after copper exposure was no sufficient to cause oxidative damage. In case of the amphipod species, despite the GPx inhibition and no alteration on GR, no oxidative damage was observed; the increase tendency of GSH simultaneous with the decrease tendency of GSSG concentrations in order to maintain the redox status of cell could be an explanation for this.

2) *Effects of zinc on shrimp* Atyaephyra desmarestii *and on amphipod* Echinogammarus meridionalis

Exposures to zinc have no affected the CAT activity of the shrimp, but inhibited on amphipod. This inhibition observed for the amphipod could be due to its inactivation by superoxide radical or due to the decrease in rate of the reaction as a result of production of high amounts of H_2O_2 (Vutukuru et al., 2006). Rapid inactivation of this enzyme at high H_2O_2 concentrations could result from the conversion of active enzyme compounds into inactive compounds (Atli et al., 2006). CAT has been shown to be either induced or inhibited by metals, depending on the dose, the species and/or the route of exposure (Sanchez et al., 2005). Inhibition of CAT was also observed in gills of the freshwater fish *Channa punctata* (Bloch) with an exposure a mixture of metals throughout exposure period (Pandey et al., 2008).

For both species the activity of GR was not affected by zinc exposure, as well was observed for the copper exposure. Nevertheless, the amphipod GR with the highs concentrations showed slightly lower activity, which could be the result of lower GSSG production throughout GPx action and consequently lower conversion of GSSG into GSH needed to maintain the ratio GSH/GSSG.

Furthermore, amphipod GPx activity was not affected by zinc, but was induced in the shrimp, suggesting, in this case, the activation of the antioxidant system. Although, GPx is more costly energetically that CAT, CAT is used to detoxify mainly endogenous ROS, like those resulting from respiration, which can explain why CAT did not respond (Anto et al., 2009). This is in good agreement with the results from this work, once an induction of GPx was observed and no alterations were found on CAT activity.

The GST activity was not affected by zinc in case of shrimp, but was induced in the amphipod. Gravato et al. (2006) observed an induction of GST activity of *Anguilla anguilla* (L.) after exposure to high concentration of copper. An induction on GST activity enzyme suggests that the phase II of biotransformation was activated, in order to conjugate the metal or the oxidative stress products with the substrate GSH in order to detoxify it. The fact of GST need GSH to play its role could be one of the explanations to no alterations found on the other GSH dependent enzymes of the amphipod, as GPx and GR.

The non-enzymatic mechanisms were not affected by zinc for both species. However, in case of shrimp, as GPx catalyze the conversion of H_2O_2 in to GSSG and H_2O, higher amounts of GSSG were produced, thus as GR activity was not changed, become important to maintain the balance between GSH/GSSG so the total amount of glutathione (TG) have to increase. This can be observed on this work, as although no significant differences were found, the shrimp TG concentrations presented a slightly increase at higher concentrations of zinc. Concomitant with this increase, the shrimp GSH concentration also was a slightly increase, which allow the maintenance of the GSH/GSSG ratio being similar at all exposure concentrations, once in spite of the GPx induction, the GSSG remain

similar. In case of amphipod, the low activity of GR and the induction of GST could be the explanation for the observed decrease pattern on levels of GSH, as it is a substrate used by GST and a product of GR activity. Like GSH, despite no significant alterations, a decrease tendency of TG levels was observed.

The shrimp specie was the one that presented oxidative damage even at the low concentration of zinc, in contrast with the amphipod where no oxidative damage was observed. The increase of TBARS contents in shrimp suggests the situation of oxidative damage, as injuries to cell components, like proteins, lipids and DNA probably occurred as a result of inefficient capability of shrimp antioxidant defence system lead with the production of ROS resultants from zinc exposure. Even with the activation of antioxidant defence system with the induction of GPx was not sufficient to prevent the lipid peroxidation. In case of amphipod, despite the antioxidant defence system seems to be affected by zinc, the induction of GST was sufficient to prevent a situation of lipid peroxidation, as this enzyme have a crucial role in detoxification, and a defence system against oxidative stress.

3) Effects of both metals on ingestion rate

The effects of these essential metals on ingestion rate of both species were also analysed. No clearly effects were registered for *A. desmarestii* with exposure to copper, however ingestion seems to be higher at 0.10mg.l^{-1} and after that decrease again. Only zinc was a pronounced effect inhibiting the ingestion of *E. meridionalis* even at lower concentration (0.5mg.l^{-1}). Several authors were found decrease on feeding rates of several organisms exposed to metals. Pestana et al. (2007) observed a significantly reduction in ingestion rates of the shrimp *A. desmarestii* and of the amphipod *E. meridionalis* after exposure to sub-lethal concentrations of zinc and cadmium. The feeding rate of the freshwater prawn *Macrobranchium rosenbergii* (De Man) was lower after exposure to zinc (Satapornvanit et al., 2009). Reductions on feeding rates of *Gammarus pulex* (L.)

were also observed by Maltby and Crane (1994) after exposures to metals. Results from this work suggest that the feeding behaviour of the amphipod was more sensitive to metal exposure, mainly to zinc than shrimp. Nevertheless, the zinc had decreased slightly the feeding rates of the shrimp, as the copper did with the amphipod.

4) Integrated biomarker response

Regarding integrated biomarker response, results showed that for *A. desmarestii* exposed to copper, the biomarkers LPO (TBARS) and GSSG maybe were associated with the highest concentration. The second concentration presented higher responses at ingestion rate level and at the enzyme AChE. With zinc exposure, highest concentration revealed higher expression at oxidative damage (LPO), but also was associated with the non-enzymatic mechanisms (TG and GSH) and with the GPx, enzyme of oxidative defence system. Once again, the ingestion rate was associated with the second concentration. In case of the amphipod exposed to copper, once LPO are associated with the highest concentration as GSH and GPx and the second concentration with the non-enzymatic mechanisms. For the zinc exposure, the lower concentration was associated with alterations to AChE, GST GR and GSSG biomarkers, and the second with AChE, GR and also with the LPO. These findings suggest that for assessment of copper contaminations, several biomarkers at biochemical level should be used, namely LPO, GSSG, GSH, AChE, GPx but also at individual level, as the ingestion rate. For zinc contamination assessment, LPO, GPx AChE, GR, non-enzymatic mechanisms and also an ingestion rate.

In summary, this work suggested that the oxidative defence system of *A. desmarestii* exposed to copper could start to be compromised, which implies that shrimp would be vulnerable to challenge with this metal. Copper exposures

despite having inhibited one of the anti-oxidants enzymes of the amphipod *E. meridionalis* was not enough to cause oxidative damage, probably due to other anti-oxidative enzymes and to non-enzymatic mechanisms that perhaps were able to maintain the cellular redox status and preventing against oxidative stress.

Zinc exposures caused oxidative damage in the shrimp species even after the induction of the GPx, which was revealed to be not enough to cope with the oxidative stress. In other hand, the GST induction observed in the amphipod case revealed to be enough to prevent the oxidative damage in this species. The feeding rate of amphipod was severely reduced with zinc exposures, which lead to the suggestion that longer exposures to this metal can have severe effects at higher biological organisation levels, but also at biochemical level. The energy input will be lower, thus the metabolic processes could be compromising, as well the defence system against oxidative stress. All the results from this work suggest that to assess the environmental contamination, as in ecosystems was present an amalgam of contaminants, several biomarkers should be used at different biological organisation levels, as well several organisms, as they respond different to diverse contaminants.

5.6. References

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CHAPTER 6. General Discussion and Conclusions

6. General Discussion and Conclusions

The main goal of this thesis was to evaluate the effects of essential metals on organisms that belong to the same functional group with slight differences in organic matter usage. To achieve this goal, several experiments were conducted in order to determine the effects of copper and zinc on different endpoints, namely survival, feeding rates, antioxidant defences and cholinesterases. The two organisms used to perform this work were the freshwater shrimp *Atyaephyra desmarestii* and the amphipod *Echinogammarus meridionalis*, which belongs to the functional group detritivores.

Neither species showed feeding preference for a particular size of leaves (Chapter II). Moreover, in the case of shrimp no preference, for the ingestion of leaves spiked with different concentrations of essential metal, was found, but leaves spiked with high concentrations of copper seemed to be avoided by this species. The amphipod, on the other hand, revealed a preference for leaves with some copper content, which can suggest that these organisms need diets with some percentage of this metal in order to accomplish the metabolic needs of organism.

The traditional ecotoxicological bioassays to evaluate the effects of copper and zinc on survival of the shrimp and the amphipod (chapter III) showed that copper is the most toxic metal for both species. Moreover, the shrimp species revealed more sensitivity to zinc than the amphipod species, and to copper. These results indicate also that despite being essential metals, copper and zinc at high concentrations can lead to the death of organisms.

The effects of the two metals at low concentrations on feeding behaviour of these two detritivores (chapter III) were evaluated through alterations on ingestion rates during different phases. The *E. meridionalis* feeding rate seems to

be sensitive to the presence of sub-lethal concentrations of copper, expressing lower feeding rates after exposure to this metal. This inhibition of ingestion could lead to the decrease in energy input, which can have effects on the metabolic processes of the organism, like growth and reproduction. This can have repercussions to the population densities. These changes in density can lead to changes in rate and quantity of litter degradation, resulting in alterations to the cycle of nutrients and energy leading eventually to breakdown of ecosystem equilibrium. Effects of zinc were not so clear. In the case of *A. desmarestii*, despite no clear observable alterations in the feeding rate for either of the metals, results from zinc indicate that some effects on feeding can be starting to occur.

On the biological organisation level (chapter IV and V), results from the characterization of cholinesterases (ChE), neurotransmission enzyme, revealed first of all, that the main form of ChE present in the amphipod and in the cephalothorax of the shrimp is the acetylcholinesterase (AChE), as it has preference for the acetylcholine substrate, is inhibited by eserine and BW284C51 and is insensitive to iso-OMPA. Only higher concentrations of zinc seem to inhibit the activity of this enzyme in the amphipod, while copper did not affect AChE. In the case of *A. desmarestii* neither of these metals seem to affect AChE activity. These results suggest that this enzyme may not be the best tool to assess metal contamination with this shrimp species, but the AChE of the amphipod give some clues about metal contamination, however other parameters should be included in the evaluation such as, chemical analysis, other enzymes, for example the methalothionines, or stress oxidative enzymes.

Results from the antioxidant defences study (chapter V) showed that in case of the shrimp, the defence system could start to be compromised after metal exposure, thus it would be a vulnerable species to environmental metal contamination. Oxidative cellular damage was observed for low zinc exposures, which is a clue for the supposed failure of the antioxidant defence system. With

copper, despite the inhibition of one of the antioxidant enzymes, no lipidic damage occurred, probably due to the other antioxidant enzymes and to the non-enzymatic mechanisms that were eventually able to maintain the redox status of the cells. However the slight increase of TBARS levels is indicative of the potential occurrence of oxidative damage as result of a failure of antioxidant defence system.

Copper exposures, despite having inhibited one of the anti-oxidants enzymes of the amphipod *E. meridionalis,* showed no oxidative damage, probably due to other anti-oxidative enzymes and to non-enzymatic mechanisms that maintained the cellular redox status preventing against oxidative stress. With zinc exposures, results showed that zinc induced the activity of the amphipod GST, an enzyme of the phase II of biotransformation, and consequently the amphipod had the capability to cope with this metal and prevent the oxidative damage at the tested concentrations. Moreover, the feeding rate was severely reduced, suggesting that longer exposures to this metal can have severe effects not only at biochemical level, but also at higher biological organisation levels, as organism, population or ecosystem. When exposed to environments with metals, the amphipod can reduce the ingestion of food in order to reduce the input of metal into body, once food is one of the sources of metals. Reducing feeding, the energy input will be also lower, thus the metabolic processes could become compromised, as well the defence system against oxidative stress.

In real scenarios a large variety of contaminants can be present. Thus, in order to evaluate the environmental contamination using a biochemical approach, several biomarkers should be used at different biological organisation levels, as well as several test organisms, as they might respond differently to metal contaminants. And if possible, include other endpoints, like feeding rates, which will also give a good contribution to understanding how the contamination could affect the ecosystem. The endpoints at biochemical level, despite being considered as early warning tools, can lead to the conclusion that the organisms are able to cope

with the environmental contamination. However, as can be observed in this work sometimes endpoints like feeding are already affected with consequences to ecosystems structure and function.