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Departamento de Biologia

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia e Ecotoxicolgia, realizada sob a orientação científica da Doutora Ana Marta Gonçalves, Investigadora do MARE (Centro de Ciências do Mar e do Ambiente), Departamento de Ciências da Vida, Universidade de Coimbra e do Departamento de Biologia & CESAM da Universidade de Aveiro e do Doutor Fernando José Mende Gonçalves, Professor Associado com agregação, Departamento de Biologia, CESAM, da Universidade de Aveiro

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Dedico este trabalho à estrelinha que mais brilha no meu céu! À minha avó Luíza.

**o júri**

presidente

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**Palavras-chave**

Sulfato de cobre; *Cerastoderma edule; Scrobicularia plana*; Ensaios de Toxicidade; Ácidos Gordos; Atividade Enzimática; Peroxidação Lipídica

**resumo**

Nos últimos anos tem-se registado um uso intensivo de fertilizantes e de pesticidas, principalmente na região do Mediterrâneo, devido à crescente produção agrícola necessária para aumentar a produção de alimentos de modo a responder ao crescimento exponencial da população humana mundial. O cobre, com várias aplicações em atividades industriais e agrícolas, é, muitas vezes, arrastado por escorrência para sistemas aquáticos, podendo representar uma ameaça para os ecossistemas e para as comunidades aquáticas que habitam nesses sistemas. O sulfato de cobre está presente em formulações de pesticidas e fertilizantes e é frequentemente utilizado em práticas agrícolas para controlo de pragas. Estes produtos químicos, uma vez libertados para o ambiente, podem ter diferentes destinos e comportamentos. Devido aos efeitos adversos que o cobre pode ter em espécies aquáticas e nos sistemas aquáticos, é fundamental avaliar os efeitos deste produto químico em espécies não-alvo. Assim, o presente estudo pretende determinar os impactos tóxicos e bioquímicos do sulfato de cobre em duas espécies de bivalves com importante valor comercial, *Cerastoderma edule* e *Scrobicularia plana*, e em duas classes de tamanho. Os organismos foram recolhidos no estuário do Mondego (Figueira da Foz, Portugal) e transportados para o laboratório em malas térmicas, que no interior continham água do estuário. Após um período de depuração no laboratório, os organismos foram expostos a uma gama de concentrações de sulfato de cobre (0,6 mg / L; 0,9 mg / L, 1,2 mg / L, 1,5 mg / L, 1,8 mg / L e 2,1 mg / L para ambas as classes de tamanho de *C.edule,* e 1,0 mg / L, 1,5 mg / L, 2,0 mg / L, 2,5 mg / L; 3,0 mg / L; 3,5 mg / L; 4,0 mg / L para ambas as classes de tamanho de *S. plana*) sob condições de temperatura e fotoperíodo controladas. Posteriormente avaliaram-se alterações no perfil de ácidos gordos e na atividade das enzimas Glutationa redutase (GR), Glutationa S-transferase (GST) e Glutationa peroxidase (GPx), bem como a ocorrência de peroxidação lipídica, pela medição das espécies reativas ao ácido tiobarbitúrico (TBARS), para ambas as classes de tamanho em ambas as espécies de bivalves. As análises bioquímicas foram realizadas em: 1) organismos recolhidos no campo, 2) organismos após um período de depuração no laboratório, e 3) organismos expostos a uma gama de concentrações de sulfato de cobre. Os resultados mostraram que *C. edule* é mais sensível à ação do químico (LC50 = 0,818 (0,595- 0,987) mg / L; 1,129 (0,968 - 1,289) mg / L, para organismos grandes e pequenos, respetivamente) do que *S. plana* (LC50 = 2,563 (2,229 – 2,903) mg / L; 4,705 (3,540 -12,292) mg / L, para organismos grandes e pequenos, respetivamente), com a classe de tamanho pequeno a apresentar maior tolerância ao composto do que a classe de tamanho grande em ambas as espécies. Ao nível bioquímico, *S. plana* também apresentou um valor nutritivo maior do que *C. edule. S. plana* apresentou maior abundância e variedade em FA e ácidos gordos essenciais (EFA), nomeadamente DHA e EPA, do que *C. edule*. O comportamento e as atividades enzimáticas também foram afetados pelas concentrações de sulfato de cobre. No entanto, *C. edule* exibiu uma resposta mais constante à exposição ao químico do que *S. plana,* com *C. edule* a revelar ser a espécie mais sensível. Em conclusão, apesar dos efeitos observados nas vias bioquímicas e moleculares de *S. plana* serem menos acentuados, *C. edule* apresentou um padrão nutricional mais coerente no teor em ácidos gordos e na atividade enzimática. Assim, *C. edule* pode ser considerado um bom bioindicador em estudos ecotoxicológicos para detetar a presença de sulfato de cobre em sistemas aquáticos, através da medição do perfil em ácidos gordos e da atividade enzimática que revelaram ser bons biomarcadores para detetar a presença deste químico. O músculo (pé) revelou ser um tecido adequado na determinação do efeito de poluentes no sistema de defesa anti-oxidante de bivalves aquáticos, para além da glândula digestiva e das brânquias.

**keywords**

Copper Sulphate; *Cerastoderma edule; Scrobicularia plana*; Toxicity Assays; Fatty Acids; Enzymatic Activity; Lipid Peroxidation.

**abstract**

In the past years, an intensive usage of fertilizers and pesticides has been reported, mainly in the Mediterranean region, due to the wide agriculture production required to increase food production to respond to world exponential growth of human population. Copper, with several applications at industrial and agricultural activities is often discharged in the aquatic systems and this input can lead to damages to the ecosystems and its communities. Copper sulphate, is found in pesticides and fertilizers formulations, frequently used in the agricultural practice to control the pests. These chemicals once released into the environmental may have different fates and behaviour. Due to the adverse effects copper may have on aquatic species and to the aquatic systems, it becomes pivotal to evaluate the effects of this chemical in non-target species. Thus, the present study proposes to address the toxic and biochemical impacts of copper sulphate in two important commercial bivalve species, *Cerastoderma edule* and *Scrobicularia plana*, and two size classes, big (B) and small (S). Organisms were collected at the Mondego estuary (Figueira da Foz, Portugal) and transported to the laboratory in cold boxes with water from the estuary. After a depuration period, organisms from different size classes and both species were exposed to a range of concentrations of copper sulphate (0.6 mg/L; 0.9 mg/L; 1.2 mg/L; 1.5 mg/L; 1.8 mg/L; and 2.1 mg/L to both size classes of *C.edule*, and 1.0 mg/L; 1.5 mg/L; 2.0 mg/L; 2.5 mg/L; 3.0 mg/L; 3.5 mg/L; 4.0 mg/L to both size classes of *S. plana*) under laboratorial conditions. Changes in the fatty acid profiles and at the activity of the enzymes Glutathione reductase (GR), Glutathione S-transferase (GST) and Glutathione peroxidase (GPx) and the occurrence of lipid peroxidation, by the measurement of the thiobarbituric acid reactive species (TBARS) levels, were then determined to both size classes of the two bivalve species Biochemical analyses were conducted to: 1) organisms collected in the field, 2) organisms collected at the field and under a depuration period in the lab, and 3) organisms exposed to a range of copper sulphate concentrations under laboratory conditions. The results showed *C. edule* is more sensitive to the chemical (LC50= 0.818 (0.595-0.987) mg/L; 1.129 (0.968 - 1.289) mg/L, to big and small organisms, respectively) action than *S. plana* (LC50= 2.563 (2.229 - 2.903) mg/L; 4.705 (3.540 -12.292) mg/L, to big and small organisms, correspondingly), with the small size exhibiting higher tolerance to the compound than the big size class in both bivalve species. At the biochemical level, *S. plana* also present a higher nutritive value than *C. edule. S. plana* show greater abundance and variety of FA and essential fatty acids (EFA), namely DHA and EPA, rates than *C. edule*. The behaviour and enzymatic activities were also affected by the copper sulphate concentrations, although *C. edule* exhibited a more consistent response to the chemical exposure than *S. plana* with *C. edule* being more affected. In conclusion, this study highlights Although the lower effects in biochemical and molecular pathways of *S. plana*, *C. edule* exhibits a more the coherent behavioural pattern in terms of fatty acid content and enzymatic of *C. edule* suggesting the usage of this species as a good bio-indicator in ecotoxicological studies to detect the presence of copper sulphate in aquatic systems, and the determination of fatty acid profile and enzymatic activity as good biomarkers to this chemical. The muscle (foot) proved to be a suitable tissue to determine the effect of pollutants in the antioxidant defence system of bivalve species, in addition to the digestive gland and gills.

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# Chapter I: General Introduction

# General Introduction

## 1.1 Estuarine Ecosystems

Coastal marine areas represent approximately 4% of Earth's total land surface. These systems host about half of the world's human population and play a key contribution to global economic profits and ecological services (Barbier, 2012; Mancinelli, 2015; Selleslagh et al., 2012). There are important resources and processes provided by these natural ecosystems with benefits to human beings, mainly to the local population, defined as ecosystem services. Some of the benefits are in the food sources provided by the system (e.g. crustaceans and fisheries production) or the water to irrigate the land used to agriculture activities, that have been greatly exploited at the past 50 years, due to the deeply needs of the humanity (Roberts, 2009). However, on the last decades, at a global scale, has been observed an intense degradation and loss of coastal areas (Duarte et al., 2015). The effects of coastal degradation (e.g. loss of habitats and biodiversity, fishery decline, and a consequent decrease in the life quality of local populations) are usually perceptible; conversely, the causes of degradation may be multiple and simultaneous, as several anthropogenic pressures such as, overfishing, urban and industrial pollution, overlap their effects on ecosystems (Boesch et al., 2001; Lotze et al., 2006). The hydrological and ecological complexity of estuarine and coastal systems makes them extremely susceptible to stressors, such as to global changes and extreme climate events, whose effects may combine with other direct and indirect factors. The increase in the frequency and magnitude of flooding events is expected to increase the flow of nutrients and chemicals into aquatic systems, which may have severe repercussions to the aquatic communities and, at a worst scenario, to the trophic food web (Mancinelli and Vizzini 2014).

Estuaries are located at the interface between the continental and marine domains and represent a complex mosaic of different habitats. These systems are characterized by a semi-closed water body with free connection to the open sea, where occurs seawater dilutions, contributing to a salinity gradient along the estuarine system. Estuaries are characteristically dynamics exhibiting a high degree of temporal and spatial variability in environmental conditions. Indeed, estuaries are subject to multiple environmental stressors and a major site for problems associated with inorganic and organic

contaminants. A wide biological variety characterizes these ecosystems, with a high primary production as well as conditions more favorable to the biological development, when compared to rivers and the oceans.

Due to favorable conditions of the estuarine ecosystems, there are an intensification of the coastal activity and therefore an increase of the stress on these ecosystems. With the intensification of the coastal activity, we observe an intense urbanization of coastal areas, and an intensive agricultural activity of the fields in the surrounding areas, with an overuse of pollutants and fertilizers (McCarthy et al., 2007; Smalling et al., 2013). In response to the behaviour set of the maritime and fluvial forces, estuaries are exposed to a wide variety of compounds, such as pesticides, metals, oils, pollutants from industries and navigation ports (Macedo et al. 2005). Estuarine ecosystems are very important and productive areas, supporting a great variety of life resources, however these resources are extremely sensitive to the adverse effects of the several pollutants, from the affluent rivers and drainage of the surrounding farms, discharged to the estuaries (McCarthy et al., 2007; Smalling et al., 2013). The contaminants, mainly the persistent, tend to accumulate on the environmental and on the organisms, inducing exchanges in water quality and biological communities (Macedo et al. 2005; Cardoso et al. 2004).

### 1.2 Study area: The Mondego Estuary

The study area of the present work is the Mondego estuary (Figure 1). The estuarine system is in a Mediterranean region, on the Atlantic coast of Portugal (40°08'N, 8°50'W), near Figueira da Foz city, Portugal. It is a small estuary extended for about 8 km and covers an area of approximately 3.4 $km^2$.

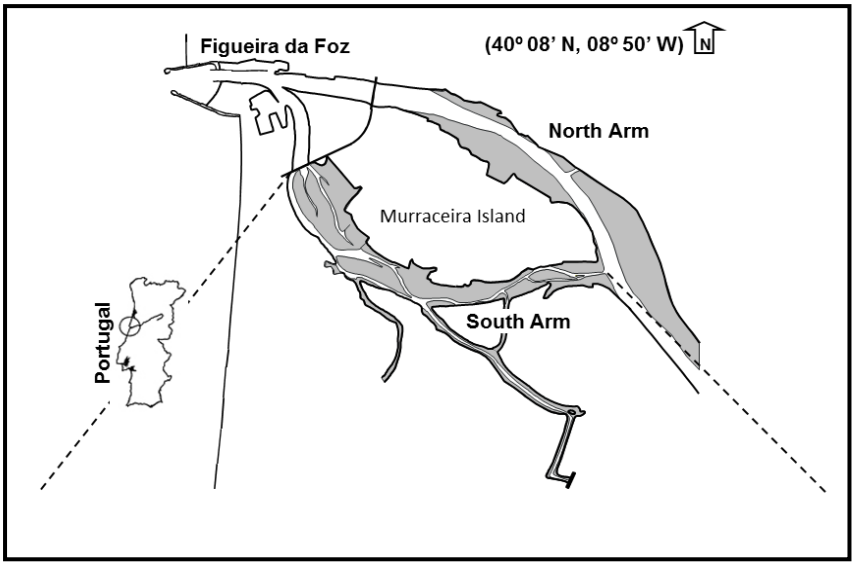


**Figure 1:** Map of the Mondego estuary, located near Figueira da Foz city, Portugal.

The estuary consists of two arms, north and south, separated by the Morraceira island. The north arm is deeper (4–10 m during high tide, tidal range 1–3 m), highly hydrodynamic and the main navigation channel and consequently the location of the Figueira da Foz harbour. The south arm is shallower (2–4 m during high tide, tidal range 1–2 m) and is characterized by large areas of exposed intertidal flats during low tide. Until 1998, the south arm was almost silted up in the innermost areas, and the river outflow occurred mainly via the north arm. Then, water circulation was at this time mostly dependent on tides and on the freshwater input from the Pranto River, a small tributary with a flow controlled by a sluice, which was regulated per the water level of rice fields in the Mondego Valley.

Mondego estuary, as other estuaries, is quite affected by a strong anthropogenic pressure. Besides the beach, port and industrial activities and the exploitation of marine resources, the intensive agricultural practice, mainly the rice and corn cultures, in the Mondego valley, lead to a significant input of nutrients and pesticides on estuarine waters, by leaching of surrounding agricultural farms, culminating on the ecosystem eutrophication (Neto et al., 2007; Verdelhos et al., 2005). Thus, has been observed a decrease of the global environmental quality of the estuary, as well as the degradation of water quality and turbidity increase, since the life quality and populations support depend on the conservation of the natural conditions of aquatic ecosystems and mitigation of negative impacts from utilization of water resources like receptors of point

(industrial and domestic) and diffuse (agriculture and aquiculture) discharges, responsible for the progressive eutrophication of fluvial and estuarine ecosystems and consequent exchange of the trophic structure (Cardoso et al., 2004; Verdelhos et al., 2005). Pesticides used in agriculture practices have been found both on surface and ground waters. This contamination may have (eco)toxicological effects to the aquatic flora and fauna and consequently to the human health (Cerejeira et al., 2003), with also impacts in molecular and biochemical levels (Filimonova et al., 2016a, b; Gonçalves et al., 2016).

### 1.3 Fertilizers and Pesticides Utilization

The rise of human population growth since the mid-20$^{th}$ century conducted to an excessive production of food and energy, being it partially responsible for an increase of anthropogenic usage of pesticides and fertilizers (Bednarek et al., 2014). Nowadays, commercial fertilizers are responsible for 40% to 60% of food production in the world (Roberts, 2009). It is expected that the human population reach to 9 billion in 2050, more 30% than currently. Consequently, the food production must answer to the needs of the human-being growth, and so the energy consumption and the contaminants emission from the agriculture activity will raise (Bednarek *et al.* 2014).

The chemical compounds are often used on the fight against pests like snails or weeds, to optimize the food production (Roberts, 2009). However, their action affect others species (non-target species), leading to damages at the toxicological and biochemical level (Al-Malki and Moselhy, 2011; Filimonova et al., 2016a, b, Gonçalves et al., 2016).

Mondego estuary is a recover system of eutrophication process mainly due to the wide and indiscriminate usage of fertilizers and pesticides in the surronding fields, with the implementation in 1998 of mitigating programs, similarly of what has been conducted in other coastal and estuarine systems of the Mediterrean region surrounded by agricultue fields (Galhano et al., 2011). Into the main pesticides that affect this aquatic ecosystem are Primextra® Gold TZ, used in the corn farms and VIPER, applicated in the rice farms (information disponibilized by the Mondego valley coorporatives).

Primextra® Gold TZ is a systemic herbicide suitable to control weeds mostly grasses and *Cyperus esculentus*. The herbicide is absorbed through the leaves and roots on

development, preventing the formation of weeds. However, the ecotoxicological effects of this herbicide used in agriculture affect several biological organization levels, from molecular to ecosystem level, may affecting early from germination to growth of the plant, leading to alteration in biochemical, physiological and different enzymatic and non-enzymatic antioxidants, resulting in residues in plant, vegetables, fruit and different non-target organisms (Parween et al. 2014). On the other hand, VIPER (DOW AgroSciences) is a post-emergent systemic herbicide applied in rice fields, for the control of annual grasses, sedges, and broadleaf weeds (Roberts et al., 2003). The active ingredient of VIPER is penoxsulam, a triazolopyrimidine sulfonamide compound, which acts as an acetohydroxyacid synthase (AHAS) inhibitor. The main target of AHAS is the biosynthesis of branch-chained amino acids (valine, leucine, and isoleucine), a metabolic pathway present in fungi, microorganisms, and plants, but not in animals (Roberts et al., 2003). Some studies have observed deleterious effects on the feeding activity, survival, growth and fecundity success on *Daphnia magna* and *Daphnia longispina* (Marques et al., 2012) and effects on the crustaceans movement (Cattaert et al., 2002), being this impacts suggested as a consequence of the methanol present on VIPER formulation, and reported to induced neurotoxic effects probably associated to the physicochemical action, with impacts to the membrane fluidity (van Wezel et al., 1997). Copper is one of the main constituents of several pesticides formulations (Stevens et al., 2014), highlighting the importance in nowadays to study the response of non-target species to these toxicants.

### 1.4 Copper and Copper Sulphate: characterization

Metals can be divided into essential and non-essential elements to the organisms. Some metals become toxic at high concentrations, because they are biologically essential. However, other metals are toxic to the organisms even at low concentrations (Bae and Lim, 2012).

Copper is an essential element to the living organisms, necessary to some cellular functions (Mayor et al., 2013). It may act as enzyme cofactor and a key element in many metabolic pathways (Ritter et al., 2008). For example, copper is a component of superoxide dismutase, an antioxidant enzyme which helps the organism against reactive oxygen species (ROS) (Barman, 1974), although it can be toxic, causing mortality or

sub-lethal stress in high concentrations or chronic exposure to low concentrations (Mazon et al., 2002; McBride et al., 1997; Pourahmad and O'Brien, 2000).

Copper salts are commonly used in the agricultural practices mainly as fungicide or pesticides' constituent. Copper Sulphate, also called Cupric Sulphate ($CuSO_4$), is a chemical derivative of copper forming blue crystals, which are soluble in water and methanol (León et al., 2014). Copper sulphate can be present on pentahydrate ($CuSO_4 \cdot 5H_2O$) or anhydrous ($CuSO_4$) form. Pentahydrate form is soluble in water (230.5 g/kg to 25ºC), with a molar mass of 249.69 g/mol (Sigma Aldrich, 2016).

### 1.5 Copper sulphate applications and effects on non-target species

This compound is used in industrial activities, but also it is used in pesticides formulations (as herbicide, fungicide, molluscide or algicide), with application in agricultural practices, namely in rice farms to control pests like *Isidorella newcombi* (Stevens et al., 2014).

Cereals may be highly affected by fungi during the entire process of growth in the field, passing by the storage up to the processing (Stoloff, 1976). The fungal metabolites or mycotoxins (Hamilton, 1987) may reduce the nutritive value (by use of thiamin or other nutrient) and digestibility in feed barn-door (Fritz et al., 1973), leading to a decrease of body weight, serum protein (Huff et al., 1992), feed deficiency and increasing mortality. Thus, the application of chemical methods appear to be the most efficient to inhibit the fungal growth and thus, avoid changes, caused by fungi, in the metabolism of the organisms that feed on these agriculture products. Various chemical compounds are used as fungal inhibitors, such as copper sulphate (Paster, 1979; Romoser et al., 1979).

Copper sulphate, a fungicide, is one copper-based product. It has been widely used to control diseases in various fruits, vegetables, nuts, and field crops. It is used in combination with lime and water as a protective fungicide, designed as Bordeaux mixture. It is applied on plant leaf and for seed treatment. It can also be used as an algicide, herbicide or as a molluscicide (Olu-Owolabi et al., 2012). This fungicide has been banned due its toxicity but it is still being applied in some countries. In addition, the heavy metals are being associated with human and animal health failures, which are aggravated by their long-term persistence in the environment and their non-degradable nature (Yoon et al., 2006). Since copper is introduced into the environment, it cannot be

degraded biologically. However, this chemical can move into the different fractions of the soil based on the prevailing biogeochemistry of the environment, once this is one of the mobile metals (Ramirez et al., 2005).

The cupric ions' mode of action may be explained by three main processes: i) the affinity of Cu (II) with thiol-, imidazole-, and carboxyl-groups of amino acid leads to their interactions and consequently to protein inactivation; ii) Interactions between Cu (II) and deoxidants, with production of Cu (I), take part in Fenton's reaction as a catalyzing agent for formation of hydroxyl radicals, which belongs to the group of reactive oxygen species (ROS); iii) ions of copper displace essential cations from specific binding sites. Therefore, at cellular level, copper ions interfere in the metabolism of fatty acids and proteins, and may inhibit respiration and nitrogen fixation processes in photosynthetic organisms (Maazouzi et al., 2008; Ritter et al., 2014). Many substances affect the aquatic ecosystem, among them, metals, acids and pesticides. Metal contaminants may not affect directly the organisms when entering at the marine aquatic ecosystem at low concentrations, however, they can be accumulated into marine aquatic organisms by different processes and effects, such as bioaccumulation, bioconcentration, and by the food chain process, becoming toxic (Al-Malki and Moselhy, 2011; Filimonova et al., 2016a).

Copper sulphate ($CuSO_4$) can contaminate aquatic ecosystems indirectly during the spraying of crops due to entrainment caused by rain and, naturally, by soil erosion. The copper ion also reaches aquatic environments directly when industrial effluents are released into water bodies and when copper sulphate is used to control algae in reservoirs and at irrigation equipment (Boyd, 2015; WHO, 1998).

In aquatic ecosystems, copper can be adsorbed to the sediment by ion complexation reactions and influence the bioavailability, transport and migration of metallic cations (Bezerra et al., 2009). Moreover, the toxicity of dissolved copper depends on the pH and temperature of water, increasing in the coming years with the seawater pH decreases and temperature increases (Mayor *et al.*, 2013). An excess of this metal may lead to detrimental effects on photosynthesis, chlorophyll synthesis, fatty acid metabolism, carbohydrate synthesis (Ritter *et al.*, 2008), as well as on cellular respiration process, ATP production, pigment synthesis and inhibition in cell division (Sibi et al., 2014).

Copper, due its toxicity, at high concentration may cause the impairment of feeding mechanisms, and increase the susceptibility to sickness, development of histopathological abnormalities, and consequently a decrease of growth rates and reproduction (Al-Subiai et al., 2011). Moreover, copper may cause several biochemical effects (Filimonova et al., 2016a; Gaetke and Chow, 2003; Viarengo, 1985), such as the transition of metals, that catalyses the generation of reactive oxygen species (ROS) causing oxidative damage on various biomolecules (Sies, 1986). For instance, fatty acids generate by-products such as malondialdehyde (MDA) (Gutteridge, 1984; Brown and Kelly, 1996), affecting the DNA expression and the population level; 7,8-dihydro-8-oxodeoxyguanine (8-oxodG) is one of the multiple oxidative causes of damage induced in DNA by hydroxyl radical and this DNA damage can lead to necrosis, apoptosis, or heritable mutations and, therefore, has the potential to impact individuals as well as populations (Kuchino et al., 1987; Halliwell, 1993). According to the literature, there are effects in lipid metabolism of some organisms after copper exposure (Engle and Spears, 2000; Engle et al., 2000; Ward and Spears, 1997). It is not clear the mechanisms about how copper affects the fatty acids profile but it may include effects on esterification, desaturation and mobilization from triacylglycerols (Engle et al., 2001).

Studies evaluating the effect of a pollutant on the aquatic population or community are less common, but from the results obtained at the organism level we may predict about the impact of these substances in the ecosystem. As indicated above, there are many species that may be affected by copper sulphate action, belonging to different trophic levels. Moreover, some chemicals may become lethal due to their ability to make stable compounds, which may stay along the trophic chain, altering metabolic pathways, so compromising the structure and physicochemical properties of the membrane, with potential damage to the cells, tissues and organs. A long term exposure may have severe consequences such as a decrease of the growth and reproduction rates and an increase of the mortality rate, with changes on community structure and diversity (Bae and Lim, 2012; Gabryelak et al., 2000; Mazon et al., 2002; Pelgrom et al., 1994).

## 1.6 Biomarkers

### *1.6.1 Antioxidant defence system and lipid peroxidation*

Oxidative stress, often described by reactive oxygen species (ROS) production, is a deleterious process that can be an important mediator of damage to the cell structure, including lipids and membranes, proteins and DNA (Valko et al., 2007). Damage on nucleic acids, proteins and lipids may affect the health and cellular viability or induce many responses by the generation of secondary reactive species, at the last time leading to cell death by necrosis or apoptosis (Flora, 2009; Valko et al., 2007). Moreover, lipid peroxidation induced by oxidative stress may led to changes at the biological proprieties of the membrane, such as degree of fluidity, inactivation of the binding membrane-receptors or enzyme, that can impair the cellular membrane function and increase the tissues permeability (Kurutas, 2016). Furthermore, the products from the lipid peroxidation, such as thiobarbituric acid reactive species (TBARS), have been often used as biomarkers of oxidative stress because the generation of oxidized products from the lipid peroxidation may contribute to or amplify the cell damage (Carini et al., 2004; Cracowski et al., 2002). To contrariwise the ROS action and to prevent the oxidative stress, the organisms have complex systems of defence (Almeida et al., 2010; Antunes et al., 2013). Antioxidant enzymes, such as glutathione S-transferase (GST), glutathione reductase (GR), Glutathione peroxidase (GPx) are included at the defence system, which the first aim is the ROS detoxification and prevent the cellular damages (Marques et al., 2011). Thus, these antioxidants components have been commonly used as molecular biomarkers to evaluate the health status and the exposure to stress conditions of the organisms. GST catalyzes the detoxification of many xenobiotics, it is the principal primary enzymes at the antioxidant system, acting directly to neutralize the free radicals and the main regulators of its activity are the health status, environmental conditions and the nutritional diet (Gonçalves et al., 2017). In the cells, GR catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH), using NAPH as cofactor. The GPx activity depends on the GSH concentration, and in physiological conditions, the glutathione is present mainly in the reduced form. GPx may be found in two forms, GPx selenium-dependent and GPx selenium-independent. The forms differ at the subunits number, in the bound to selenium in the active center

and in the catalytic mechanism. The efficiency of this enzyme is based at the elimination of peroxides (potential substrates to Fenton reactions). Moreover, selenols react faster than thiols, helping at the prevention against superoxide generation from the oxygen molecules. GPx degrades the peroxides into water or alcohols and at the same time oxidizes the GSH, using often the hydrogen peroxide as substrate (Duracková, 2008).

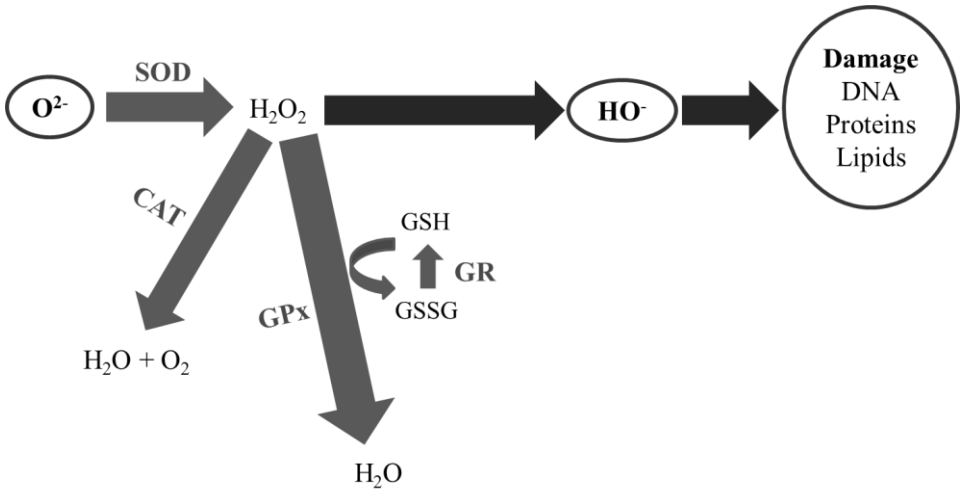


**Figure 2:** Graphical explanation of the operating mode of enzymatic antioxidant defense system.

### *1.6.2 Fatty Acids*

Fatty acids (FA) are one of the main constituents of the cell membrane, occurring in great concentrations in neural system. Fatty acids play a key role at the biological level, being one of the most important molecules transferred across the plant-animal interface in aquatic food webs. These compounds are involved in several biochemical pathways, are an important source of energy and constituent of cell membranes acting on membrane permeability, influencing the traffic of cell compounds and the activity of membrane proteins and signals (Ibarguren and López, 2014; Liu et al., 2015). FAs are claimed to be a good bio-indicator of ecosystem health and bio-indicators of stress due to their sensitiveness to chemical and environmental stress conditions (Gonçalves et al., 2016, 2017). Metal ions may interact with several cell structures, being dangerous at the biochemical level, from the enzymatic activity inhibition to damages on cell membrane structure and functions (Viarengo et al., 1990). The membranes are the first constituent

to be affected by the stressors, with several of them causing physiological changes on the lipid membrane, with consequences in the dynamic and fluidity of the membrane. Moreover, alterations on the lipid composition are one protective strategy of the cells under stress (Bertoli et al., 2001). Metal ions are direct and indirectly involved in lipid peroxidation reactions and promote the free radicals and other reactive species generation (Company et al., 2004; Geret et al., 2002; Valavanidis et al., 2006; Valko et al., 2005). Furthermore, the lipid peroxidation products accumulate on the membrane bilayer, with alterations in the membrane structure and functions, and changes on the membrane-bound proteins activity (Girotti, 1998). Since, metal ions cause disturbs on the lipid metabolism, reflecting this on lipid and fatty acids composition, these molecules may be used as biomarkers of toxic effects on aquatic organisms like marine bivalves (Fokina et al., 2013). Proteins and polyunsaturated fatty acids (PUFA) has been reported as the first target of the free radicals action (Girotti, 2001; Gabryelak et al., 2000). PUFA are a family of lipids that contains some subgroups identified by the position of the last double bond in their structure and include many important compounds, such as essential fatty acids (EFA). Although the terms "PUFA" and "EFA" are not synonymous, they are often used interchangeably since many biological functions of EFAs are exerted by EFA–derived PUFAs (Breet and Muller-Navarra, 1997), pointed out that PUFA are almost exclusively synthesized by plants, with animals being able of converting PUFA by elongation or desaturation, and only a few could synthesize this type of fatty acids. PUFA play an important role in the organism, regulating cell membranes properties, serving as precursors of important hormones and being essential to the organisms (Neves et al., 2015). Highly unsaturated fatty acids (HUFA) (e.g. eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6(n-3), DHA)), are linked to the species growth, reproductive success, and neural development, playing a key role in the health and function of all animals at all trophic levels, including plankton invertebrates, benthos, fish and humans. HUFA are essential metabolites that cannot be synthesized *de novo*, or at least not in sufficient amounts, being taken up via food sources (Ladhar et al., 2014). Indeed, some groups of organisms feed in high amounts of HUFA present higher growth rate, which strength the importance of fatty acids as ecophysiological indicators. Thus it is pivotal to determine the impacts of organic and inorganic pollutants in the fatty acid profile of aquatic

organisms mainly those directly exposed to different sources of chemical pollution, with repercussions to the trophic web.

### 1.7 Bivalves Species: *Cerastoderma edule* and *Scrobicularia plana*

Bivalves play a key role at the trophic web since they act as a link between primary producers and consumers, being the major prey to crustaceans, fish and wading birds. These organisms have the capacity to filtrate organic material, clean the freshwater column and influences the food available and energy flow in the entire community, playing a key role on the trophic structure of the ecosystems. Moreover, bivalves can accumulate pollutants and parasite species (Verdelhos et al., 2014), being used as standard species in biomonitoring programs and ecotoxicological studies, due to their sensitivity to pollutants, once they absorb organic and inorganic particles from the water and with bioaccumulation capacity, coupled with relatively low metabolic detoxification rates, sessile life style and easy handling and collection (Bolognesi et al., 2004; Colakoglu et al., 2012). Thus, bivalves have been considered good bio-indicators of environmental and chemical stressor conditions in the environment, with species showing different tolerances to the same contaminant (Almeida et al., 2013; Fernández-Tajes et al., 2011). Physiological and biochemical responses may be used as earlier warning indicators of potential ecosystem damages, caused by pollutants such as metals and organic contaminants (Filimonova et al., 2016a,b; Gonçalves et al., 2016; Nilin et al., 2012). Furthermore, these species have a great economic value, since they are extremely appreciated as food source (Paul-Pont et al. 2010a). In this study, it is used two bivalve species, *Cerastoderma edule* (Fig. 3a) and *Scrobicularia plana* (Fig. 3b), abundant in the Mondego estuary and very appreciated as food source by human beings.

*Cerastoderma edule* is a bivalve mollusc, from the family *Cardiidae*, being one of the most abundant shellfish in tidal flats, bays and estuaries in Northern Europe and Western Europe. It is widely distributed, it is found from North Africa to Northern Norway and Murmansk in the Arctic, presented on the east coast of the Atlantic, but not being found in the Mediterranean and Baltic seas (Freitas et al., 2014). It is a infaunal suspension-feeder living on intertidal shallow areas, burrowing just below the sediment surface, plays a key role as a link between primary producers (phytoplankton) and the consumers (Verdelhos et al., 2015), such as crabs, shrimps, fish and birds. Due to their

large filtration capacity and ability to accumulate a large amount of environmental pollutants *C. edule* is widely used as environmental bio-indicator (Cardoso et al., 2013; Freitas et al., 2014; Nilin et al., 2012; Paul-Pont et al., 2010b). *Scrobicularia plana* is also a bivalve mollusc, belongs to the *Semelidae* family, typical from brackish waters, it is a dominant species of intertidal soft-substrate in estuaries, lagoons and bays along NE Atlantic seaboard communities, from Norway to the Mediterranean and West Africa. *S. plana* is a deposit filter feeder, inhabiting intertidal and subtidal areas, burrowing on mud to muddy sand sediments to a depth of 25 cm (Verdelhos et al., 2015). Like other bivalves *S. plana* has a large capacity to accumulate and filtrate pollutants that accumulates in the digestive gland (Freitas et al., 2014; Paul-Pont et al., 2010a, 2010b).

*Cerastoderma edule* and *Scrobicularia plana* are two of the most important bivalve species from estuarine benthic communities worldwide, considering abundance, biomass and infaunal production (Dolbeth et al., 2007; Mistri et al., 2001).

A B

**Figure 3:** Studied species – *Cerastoderma edule* and *Scrobicularia plana*

### 1.8 Main Aims

The input of contaminants in estuarine ecosystems constitutes an important force affecting trophic chain patterns and may lead to biodiversity losses. Despite the extensive literature under anthropogenic pressures issues, a more functional approach to trace changes in trophic food webs due to the physicochemical forcing factors and relate it with changes on species' biochemical composition is scarce. These changes may have repercussions on the food quality and may play a key role to determine how polluted is an aquatic system. Feeding behaviour is the basic process in the food web for the transfer of energy and material from lower to higher trophic levels. Nutrients, mainly

lipids, are involved in many vital functions of aquatic individuals, constituting some of them useful trophic markers and be essential on physiological functions and on the metabolism of all animals and on the prevention of diseases (Dalsgaard et al. 2003). Thus, it becomes pivotal to determine ecotoxicological and biochemical changes on aquatic species. This work investigates toxic and biochemical (namely fatty acids profiles and antioxidant system defence) effects of copper sulphate in two marine bivalve species - *Cerastoderma edule* and *Scrobicularia plana*. This study also investigates the nutritional value of both bivalve species under different conditions (field, depuration, laboratory bioassay conditions) and infer about repercussions in the trophic web. Regarding toxic effects, were determined the lethal concentration and the activity index of both species (including two size classes: big and small organisms). In a second point, to evaluate the biochemical effects, were compared the fatty acid (FA) contents of the organisms from the field and depuration with those under laboratory conditions. Moreover, the enzymatic activity was determined, to both species and size classes, considering antioxidant enzymes (Glutathione Peroxidase and Glutathione Reductase), biotransformation enzymes (Glutathione S-transferase) and the lipid peroxidation was evaluated by the measurement of thiobarbituric acid reactive species (TBARS).

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# Chapter II: The impact of copper sulphate on the fatty acids profiles of the bivalves *Cerastoderma edule* and *Scrobicularia plana*

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# The impact of copper sulphate on the fatty acids profiles of the bivalves *Cerastoderma edule* and *Scrobicularia plana*


## Abstract

At the past 30 years were recorded an intensive practice in the use of fertilizers and pesticides, mainly in the European Mediterranean region, that, in particular cases, exceeded the limits of regular legislations established by the European Union. The widespread use of these chemicals compounds and the pressure over agricultural fields near valuable ecologically coastal areas conducted to the implementation of monitoring plans to the recovering of aquatic ecosystems. Since the 80´s Mondego estuary (Figueira da Foz, Portugal) has been harassed by anthropogenic pressures, which triggered the implementation of mitigation monitoring programs at the last 18 years, allowing its recovery. Copper sulphate is used in industrial activities, but also it is much used in pesticides formulations, with application in agricultural activities, namely in rice farms to control pests. Studies reported that copper may affect biochemical processes, such lipid metabolism of some organisms, although specific changes in fatty acid (FA) profiles are still unknown. Nowadays, bivalve species are used in ecotoxicological bioassays due some particular characteristics, such as the wide distribution, ecological relevance, the capacity to filter and ingest large volumes of sediment particles and water and ease handling in the field and in the laboratory. Therefore, this work aims to assess the biochemical effect of copper sulphate, namely on FA profiles, in the two marine bivalve species *Cerastoderma edule* and *Scrobicularia plana,* considering small (medium body size = 1.97 cm and 3.47 cm, respectively) and big (medium body size = 2.45 cm and 4.20 cm, respectively) size classes. In a first phase organisms were exposed under laboratorial conditions to copper sulphate to determine lethal concentration; at a second phase, it was compared the FA profile and the nutritive quality of both species and size classes at the field and in the lab. Our results state *C. edule* is more sensitive to copper sulphate ($LC_{50}$= 0.818 (0.595- 0.987) mg/L; 1.129 (0.968 - 1.289) mg/L, to big and small organisms, respectively) than *S. plana* $LC_{50}$= 2.563 (2.229 - 2.903) mg/L; 4.705 (3.540 -12.292) mg/L, to big and small organisms, respectively), furthermore the last one presents greater abundance and variety of FA and essential fatty acids (EFA), namely DHA and EPA, rates than *C. edule*. Still, big size class of both bivalve species is the most affected by the contaminant.

**Keywords:** Marine bivalves; Fatty acids content; Nutritious quality; Lethal concentrations; Copper sulphate effects.

## 1. Introduction

Estuaries are highly subjected to metals (e.g., Cd, Cr, Pb, Hg, Ni, Cu) that are consider an important group of pollutants in these systems affecting the physiological and biochemical integrity of aquatic organisms. These stressors may be from natural (volcanic activity, vegetation decrease) or anthropogenic sources (industrial processes, agricultural activities or use of antifouling paints). At the last decades the production of copper has increased and consequently also the pollution by this metal. Its concentration achieved, in some cases, high concentrations, mainly in agriculture fields due to an overuse of pollutants where copper is one of the main constituents (Lencioni et al., 2016; Parry and Pipe, 2004).

Copper sulphate is used on industrial activities, but also in pesticides formulations (as herbicide, fungicide, molluscide or algicide), with application in agricultural activities, namely in rice farms to control pests like *Isidorella newcombi* (Stevens et al., 2014). Copper is an essential element to the normal performance of the organisms, acting as cofactor of many enzymes, i.e., it is a component of superoxide dismutase, an enzyme defending living organisms against reactive oxygen species (Barman, 1974), however in high concentrations or chronic exposure to low concentration, it may become toxic. Copper can cause the commitment of reproduction and growth rates, feeding mechanisms, and also the increase and development of histopathological anomalies and sickness (Al-Subiai et al., 2011). Moreover, copper may cause several biochemical effects (Filimonova et al., 2016a, b; Gaetke and Chow, 2003; Lencioni et al., 2016; Viarengo, 1985), such as the transition metals, that catalyses the production of reactive oxygen species (ROS) causing oxidative stress on several biomolecules (Sies, 1986).

Therefore, it is necessary and highly relevant to investigate the influence of these pollutants on non-target species. The application of biomarkers to determine the effects of different stressors on biochemical pathways that regulate the organism's health and fitness will provide higher detailed information than indirect measurements, being used as sensitive early warning bio-indicator of stress (Filimonova et al., 2016a, b; Gonçalves et al., 2012, 2016; Neves et al., 2015; Vieira et al., 2009).

Fatty acids molecules are important compounds due to its presence in neural system and at several biochemical pathways, being a source of energy and one of the main constituents of cell membranes developing several functions at the activity of membrane proteins and signals, at the traffic of cell compounds and on the action of membrane permeability (Ibarguren et al., 2014; Liu et al., 2015). Polyunsaturated fatty acids (PUFA), where are included some of the essential fatty acids (EFA) and some omega 6 and omega 3, are a family of lipids that contains some subgroups identified by the position of the last double bond in their structure. The terms "PUFA" and "EFA" are not synonymous, although they are always used indistinctly as most of the biological functions of EFAs are exerted by EFA–derived PUFAs. Animals may convert PUFA by desaturation or elongation, still a few are able to synthetize this group of molecules, with the ratio between the amount produced and the energy spent not worthwhile, with animals obtaining PUFA mainly by food sources (Brett and Müller-Navarra, 1997). This group of FA serves as precursors of important hormones and are essential to the regulation of cell membranes (Neves et al., 2015). Highly unsaturated fatty acids (HUFA) like eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), are related to reproductive and growth rates success, neural development, presenting a key role at the functional and health status of all animals from all trophic levels. HUFA are essential metabolites that cannot be synthesized *de novo*, or at least not in sufficient quantities, being taken up via food sources (Ladhar et al., 2014). HUFA are nutritional key constituents of bivalves diet and are the algae nutritional value for bivalves, determined by EFA (Hendriks et al., 2003).

Although the consumption of nutrients by bivalves become from many material particles, like resuspended benthic microalgae, phytoplankton, detritus from many sources, bacterial and microheterotrophos, they are classified as herbivorous with phytoplankton being their primary food source (Pernet et al., 2012). At this work two important commercial bivalve species, *Cerastoderma edule* and *Scrobicularia plana,* were investigated.

*Cerastoderma edule* presents a widely geographical distribution, from North Africa to Northern Norway and Murmansk in the Arctic, present also on the east coast of the Atlantic (Freitas et al., 2014). It is a suspension-feeder living on intertidal shallow areas, with an important role between primary producers and consumers (Verdelhos et al., 2015). Due to the large filtration ability of *C. edule* to accumulate high quantities of pollutants this species is widely applied as bio-indicator in ecological studies (Paul-Pont et al., 2010;

Freitas et al., 2014; Paul-Pont et al., 2010; Cardoso et al., 2013; Nilin et al., 2012). *Scrobicularia plana* also shows a wide distribution from Norway to the Mediterranean and West Africa. *S. plana* is a deposit filter feeder, inhabiting intertidal and subtidal areas, burrowing on mud to muddy sand sediments to a depth of 25 cm (Verdelhos et al., 2015). This species has a great ability to accumulate and filtrate pollutants that accumulates in the digestive gland (Paul-Pont et al., 2010; Paul-Pont et al., 2010).

Bivalves are considered standard species in ecotoxicology due to their sessile life style, easy sampling collection, maintenance, handling and sensitivity to chemicals (Gonçalves et al., 2016). The molecular and physiological responses of these species to organic and inorganic compounds may be used as earlier indicators of potential ecosystem damages (Nilin et al., 2012). Moreover, the relevance of these organisms is also related to their socio-economic importance, since they are extremely appreciated as food source. By all this, ecotoxicological studies with bivalve species are crucial to determine the effect of contaminants in these organisms, mainly in biochemical pathways. Thus, in the present study are investigated: 1) the toxic effects of copper sulphate on *Cerastoderma edule* and *Scrobicularia plana*, 2) the effects on FA profiles of *C. edule* and *S. plana* when exposed to the contaminant and 3) the changes on the nutritious quality of both marine bivalves species, and size classes, before (at the field) and after the exposure to the toxic.

## 2. Materials and Methods

### *2.1 Study site and field sampling*

The Mondego estuary is located in a Mediterranean region, on the Atlantic coast of Portugal (40°08'N, 8°50'W), near Figueira da Foz city, Portugal (Figure 1). It is a small estuary extended for about 8 km and covers an area of approximately 3.4 $km^2$. The estuary contains two arms, north and south, separated by the Murraceira Island. The sampling of bivalves (*Cerastoderma edule* and *Scrobicularia plana*) was conducted in the north and south arms. The organisms were captured using a dredge and transported in cold boxes with brackish water.

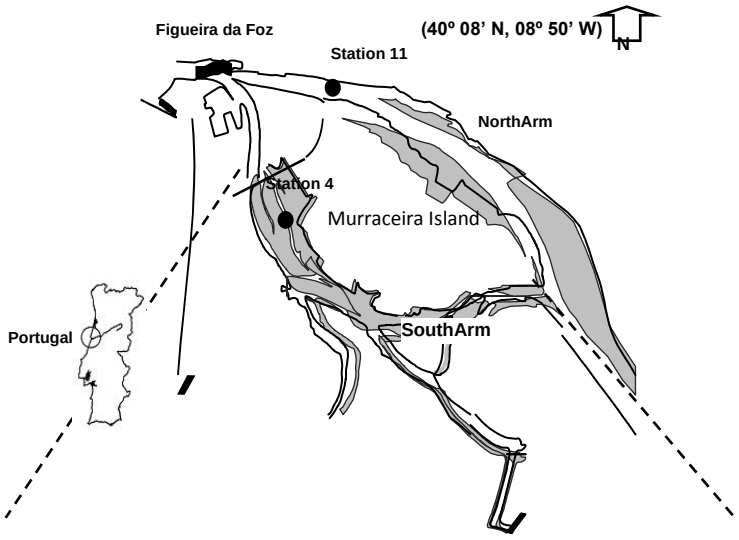


**Figure 1:** Location of the Mondego estuary and the sampling sites within the estuary.

### *2.2 Laboratory procedures and bivalves bioassays*

In the lab, organisms were divided in aquaria by species and size classes (B=big size (medium body size =2.45 cm to *C.edule* and 4.20 cm to *S.plana*); S=small size (medium body size =1.97 cm to *C.edule* and 3.47 cm to *S.plana*)). In order to calculate the condition indices, the shell length, the total weight, the tissue weight and the weight of the foot of 10 larger and 10 smaller individuals, from the field and not exposed to any laboratorial procedure, were assessed. After these measurements, the muscle (foot) of each individual was removed and stored at -80ºC for fatty acid analysis. The remaining collected individuals were maintained over 48 hours in filtrated seawater at salinity of 20, without food, for depuration.

After the depuration time, both size classes of *C. edule* were submitted to 7 treatments: one negative control plus six concentrations of copper sulphate (0.6 mg/L; 0.9 mg/L; 1.2 mg/L; 1.5 mg/L; 1.8 mg/L; and 2.1 mg/L). The both size classes of *S. plana* were submitted to 8 treatments: one negative control plus seven concentrations of copper sulphate (1.0 mg/L; 1.5 mg/L; 2.0 mg/L; 2.5 mg/L; 3.0 mg/L; 3.5 mg/L; 4.0 mg/L). Each treatment consisted in ten replicates, with each vial filled with 1000 mL of test medium to big size organisms and 500 mL to small size organisms. Bivalves were fed daily with a commercial mixture of rotifers and microalgae and transferred to new prepared test solutions every other day. Bivalves were checked every day for mortality and behavioural conditions (to evaluate the

activity of the siphon, conditions of the valves and the organism behaviour during feeding). Bioassays were conducted under a $12h^{L}$:$12h^{D}$ photoperiod, under control temperature (20±2ºC), with filtrated sea water medium at a salinity of 20, during 96 h. After the exposure period, all survival organisms passed by a set of phases (dissection, measurement of the weight and the body length and evaluation of their condition indices).

### *2.3 Fatty acids analysis*

The muscle tissue of each marine species, and ten organisms of each size class, was isolated and frozen at −80ºC in three steps: 1) after field collection 2) after depuration time 3) at the end of bioassays.

The total lipids of bivalves were extracted and methylation to FA methyl esters (FAMEs) was conducted following the methodology described by Gonçalves et al. (2012). The addition of C19 on our samples allowed to a later quantification by using this FA as an internal standard (Fluka 74208). Further information about the apparatus procedures followed at this section can be consulted in Gonçalves et al. (2016).

### *2.4 FA trophic markers (FATMS)*

In order to ascertain the maintenance of bacterial, algae or animal ratios at lipids extracts of both marine species and size classes, FA ratios were determined and applied based on Ezgeta-Balić et al. (2012). Food sources are rich in different FA, with bivalves typically feeding on animals diets showing a raise at the quantities of oleic acid (C18:1n9), linoleic acid (C18:2n6) and docosahexaenoic acid (DHA), occurring these fatty acids in zooplankton organisms (Zhukova and Kharlamenko, 1999). Another ratio to determine the food preferences of bivalve is DHA/EPA (Mansour et al., 1999; Dalsgaard et al., 2003). DHA is an omega 3 HUFA extremely important throughout life, with enormous benefits to the health of all organisms, with this ratio reflecting the proportion of zooplankton and diatoms/dinoflagellates in the bivalves diet. DHA is often dominant in zooplankton and dinoflagellates (Kharlamenko et al., 2001; Zhukova and Kharlamenko, 1999; Budge and Parrish, 1998; Mansour et al., 1999; Dalsgaard et al., 2003), whereas EPA is found mainly in diatoms (Budge and Parrish, 1998; Dunstan et al., 1994; Dalsgaard et al., 2003). High proportion of C15:0 and C17:0 denote the presence of bacterial on bivalves diet (Mayzaud

et al., 1989; Najdek et al., 2002), since bacteria biosynthesis large amounts of iso and ante-iso branched chains containing 15-17 carbons (Gonçalves et al., 2012).

### *2.5 Statistical analysis*

Probit analysis (Finney, 1971) was applied to determine the $LC_{10}$, $LC_{20}$, and $LC_{50}$ with corresponding 95% confidence intervals for each species and size class. The FA profiles were found by determining total FA concentrations (mg/ind).

Multivariate statistical analyses were carried out using PRIMER-6 software (Clarke and Gorley, 2006) to examine the variation in FA composition through non-metric multidimensional scaling (n-MDS) plots. In addition, a dendrogram, obtained by a hierarchical clustering, using the data converted into similarity triangular matrices using Bray-Curtis resemblance measures (Clarke and Warwick, 2001) and a cluster mode based in group average distance linkage, and a variant by which a larger cluster is down weighted (Kindt and Coe, 2005), was used to assess the degree of similarity between FA samples. One-way analysis of similarity (ANOSIM) was used to test differences in FA profiles across species and size classes. The contribution of individual FAs to similarities and dissimilarities within and between sample groups was tested using the similarity percentage (SIMPER) analysis routine. The PCA analysis, non-transformed data, samples center and standardize and species center by species, was used to highlight size or interspecific patterns in the bivalve species' diets using CANOCO version 4.5 (Ter Braak and Smilauer, 1998).

## 3. Results

### *3.1 Exposure to copper sulphate*

The LC values for both species showed *C. edule* is more sensitive than *S. plana* to copper sulphate, in regard of both size classes ($LC50_B$= 0.818 mg/L (0.595-0.987), $LC50_S$= 1.129 mg/L (0.968 -1.289) and $LC50_B$= 2.563 mg/L (2.229-2.903), $LC50_{S=}$ 4.705 mg/L (3.540-12.292), correspondingly). The results also indicated the bigger organisms are more sensitive than the smaller ones, to both bivalve species (*S. plana:* $LC50_B$= 2.563 mg/L (2.229-2.903), $LC50_{S=}$ 4.705 mg/L (3.540-12.292); *C. edule:* $LC50_B$= 0.818 mg/L (0.595-0.987), $LC50_S$= 1.129 mg/L (0.968 -1.289)) (Figure 2).

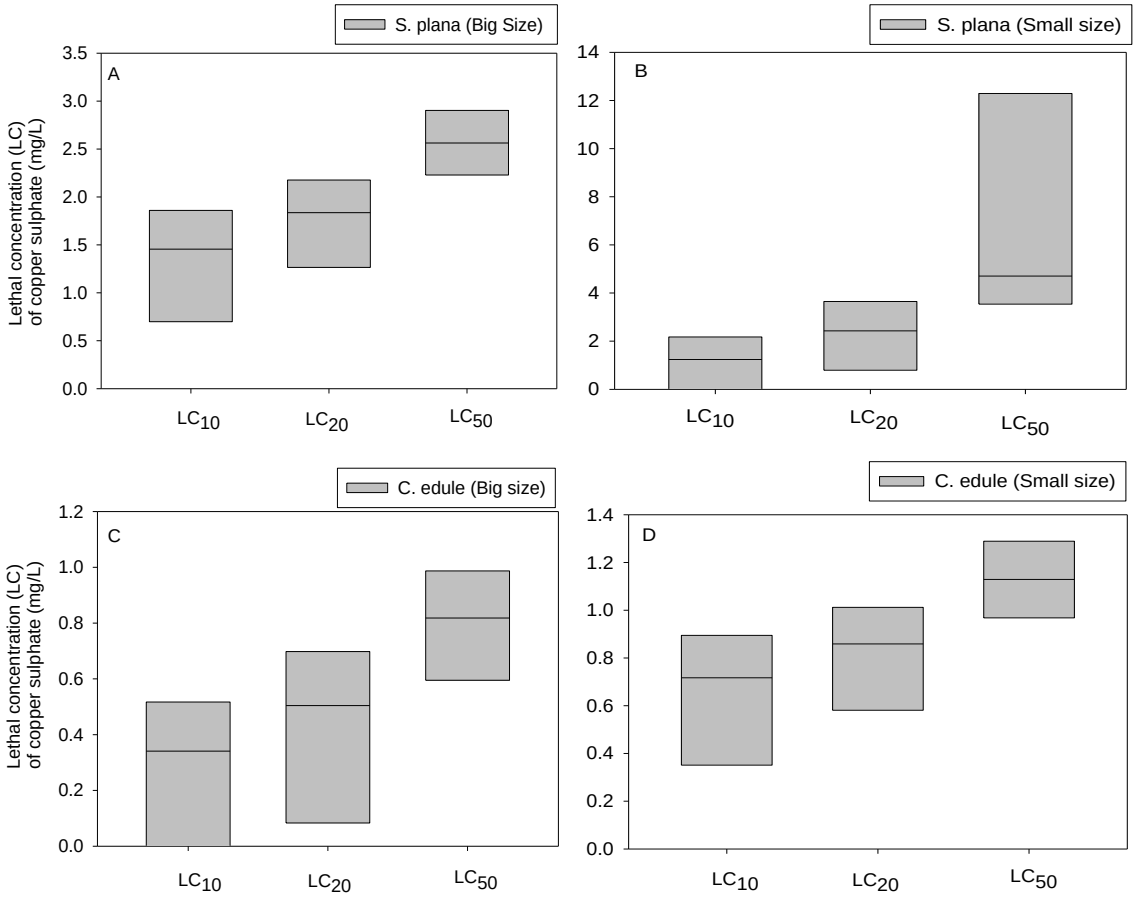


**Figure 2:** Selected LC (10, 20 and 50) values (mg $L^{-1}$) estimated for the four biological models studied. The bottom and top of the box represent the lower and upper 95% confidence limits.

### *3.2 Bivalves fatty acid profiles*

In general, over the range of concentrations of copper sulphate, saturated fatty acids (SFA) and unsaturated fatty acids (UFA) decreased on both bivalve species of the small size class (Tables 1 and 2). Considering the small organisms maintained in laboratory under depuration period, both species exhibit an increase of SFA (22.55% to *S. plana* and 10.60% to *C. edule* ) and MUFA (8.35% to *S. plana* and 6.42% to *C. edule*) and a decrease of PUFA (13.54% on *S. plana* and 2.68% on *C. edule*) and HUFA (17.36% on *S. plana* and 14.34% on *C. edule*). Regarding to the organisms exposed to the range of copper sulphate, small size classes, of both species, was observed a decrease of SFA (until to 27.14% at concentration of 2.5 mg/L on *S. plana*; and up to 15.43% at concentration of 1.2 mg/L on *C. edule*), and MUFA (up to around of 13% at concentrations of 1.5, 2.0 and 3.0 mg/L on *S. plana*; and 11.71% at concentration of 1.2 mg/L on *C. edule*). Both bivalve

species also presented a reduction of PUFA, had been noticeable a reduction around 3.00% and 4.69% to *C. edule* at concentrations of 0.9 mg/L and 1.2 mg/L, , respectively; and around 1.08% and 6.62%, on *S. plana* organisms, at concentration of 1.5 mg/L and 2.0 mg/L, respectively. Still, the latter one with a raise at the remaining concentrations, up to 20.75%. HUFA percentage increased on both species, up to 31.82% and 24.94%, on *Cerastoderma edule* (at concentration of 1.2 mg/L) and *Scrobicularia plana* (at concentration of 2.5 mg/L), respectively. The big size class of organisms of both species, exhibited a different trend, with the organisms sampled in the field and maintained in laboratory under depuration period presenting a decrease of SFA (2.98 %; and 8.10%, respectively) and PUFA (24.36% on *C. edule*; and 1.25 % on *S. plana*), and an increase of MUFA (6.09 %; and 6.29%, respectively) and HUFA (21.26% on *C. edule*; and 3.06% on *S. plana*). On the other hand, the results regarding the exposure show SFA increased on both species of big size class (31.78% from control (0.0 mg/L) to 1.2 mg/L – on *Cerastoderma edule*; 16.98% from control to 3.0 mg/L on *Scrobicularia plana*, while UFA's abundance maintained in *S. plana*, but PUFA increased in *C. edule* (10. 60% from control to third concentration), whereas MUFA and HUFA decreased (4.65% and 37.73%, respectively). In general, after the exposure to the pollutant, *S. plana* showed higher FA content than *C. edule* to both size classes. For the two marine bivalves species, the small size class of organisms presented higher changes in FA composition than the larger organisms after exposure to the compound. However, HUFA (mostly EPA and DHA) occupied the greatest part of the fatty acids content, keeping these essential fatty acids dominant at the profile. In general, the small size class organisms of both marine bivalve species (at the study area and after the exposure to the metal) exhibited higher FA content than the organisms of bigger size class (Tables 1 and 2).

**Table 1:** Quantification of FA profile (SFA, MUFA, PUFA e HUFA, in µg/ind) of *C. edule* (both size classes – big and small) determined in organisms, after field collection (Field), after depuration (Depuration) and after exposure to copper sulphate(CTL,C1, C2, C3).

| ***Cerastoderma edule*** | | | | | | |
|---|---|---|---|---|---|---|
| **Big Size** | | | | | | |
| FA | Field | Depuration | CTL | C1 (0.6mg/L) | C2 (0.9 mg/L) | C3 (1.2 mg/L) |
| C6:0 | | 258.14 | | | 987.72 | |
| C8:0 | 214.34 | 437.48 | | | | 123.18 |
| C10:0 | | 334.63 | | | | 547.58 |
| C11:0 | | 518.55 | | | | |
| C17: | | 113.85 | | | | 178.26 |
| C20:0 | | | | | 848.78 | |
| C21:0 | 137.13 | | | 579.17 | 114.72 | 293.82 |
| C22:0 | | | | | 365.58 | |
| C23:0 | | | | | | 361.85 |
| C24:0 | | | 697.20 | | | |
| **TOTAL SFA** | **351.47** | **1662.65** | **697.20** | **579.17** | **2316.80** | **1504.70** |
| C14:1 | 991.85 | 422.47 | | | | 171.51 |
| C15:1 | | | 969.45 | 967.15 | 789.52 | 142.77 |
| C16:1 | | | 441.63 | | | 274.64 |
| C17:1 | 573.48 | | 113.41 | 999.76 | 347.34 | |
| **TOTAL MUFA** | **1565.33** | **422.47** | **1524.49** | **1966.91** | **1136.86** | **588.92** |
| C20:1n9 | 239.15 | | 873.64 | 884.52 | 248.34 | |
| C24:1n9 | | | | 223.98 | | |
| C18:2n6t | | 132.94 | | | | 228.73 |
| C18:3n6 | | | | 713.33 | | 489.27 |
| C18:3n3 | 324.62 | | | 168.82 | | |
| C20:2 | 559.64 | 174.29 | 268.53 | 154.12 | 413.83 | 322.36 |
| C20:3n3 | 195.41 | | | 0.00 | | 452.76 |
| C20:4n6 | 453.66 | | 151.90 | 357.60 | 297.38 | 965.27 |
| C22:2 | | | | 764.87 | 152.41 | 234.19 |
| **TOTAL PUFA** | **1772.48** | **307.24** | **1294.07** | **3267.25** | **1111.96** | **2692.58** |
| EPA | 844.45 | 279.32 | 423.62 | 942.12 | 735.59 | 343.16 |
| DHA | 319.62 | 442.58 | 412.95 | 645.42 | 746.87 | 441.72 |
| **TOTAL HUFA** | **1164.07** | **721.90** | **836.57** | **1587.54** | **1482.47** | **784.89** |
| **N** | **11** | **10** | **9** | **13** | **12** | **16** |

| *Cerastoderma edule* | | | | | | |
|---|---|---|---|---|---|---|
| **Small Size** | | | | | | |
| FA | Field | Depuration | CTL | C1 (0.6 mg/L) | C2 (0.9 mg/L) | C3 (1.2 mg/L) |
| C18:0 | 0.06 | | | | | |
| C20:0 | | | | | | |
| C21:0 | 10.67 | 20.64 | 25.74 | 16.67 | 4.49 | |
| C22:0 | | 27.15 | 32.76 | | | |
| C23:0 | | 62.78 | 22.91 | | 13.89 | |
| C24:0 | | | | | | 19.75 |
| **TOTAL SFA** | **10.73** | **110.57** | **81.40** | **16.67** | **18.39** | **19.75** |
| C15:1 | | 44.75 | 13.94 | | | |
| C16:1 | | 23.30 | | | | |
| C17:1 | 0.30 | 16.98 | 7.21 | | 10.97 | |
| C20:1n9 | 0.92 | | 21.16 | 10.66 | | 23.38 |
| **TOTAL MUFA** | **1.23** | **85.04** | **42.31** | **10.66** | **10.97** | **23.38** |
| C18:3 | | 51.47 | | | | |
| C18:3n3 | | | 16.23 | 34.36 | 17.03 | 34.65 |
| C20:2 | 30.83 | 52.18 | 71.03 | 35.49 | 45.75 | 58.93 |
| C20:3n3 | | | | 25.19 | | 36.67 |
| C20:4n6 | 26.66 | 53.54 | 36.93 | 24.26 | 18.60 | 28.82 |
| C22:2 | | | 33.03 | 11.63 | 17.76 | 35.34 |
| **TOTAL PUFA** | **57.49** | **157.19** | **157.22** | **130.93** | **99.13** | **194.41** |
| EPA | 40.24 | 45.99 | 46.03 | 53.73 | 83.28 | 31.81 |
| DHA | 41.87 | 66.62 | 111.15 | 78.08 | 80.03 | 57.21 |
| **TOTAL HUFA** | **82.11** | **112.61** | **157.18** | **131.81** | **163.31** | **89.01** |
| **N** | **8** | **11** | **12** | **9** | **9** | **9** |

**Table 2:** Quantification of fatty acids profile (SFA, MUFA, PUFA e HUFA, in µg/ind) of *S. plana* (both size classes – big and small) determined in organisms, after field collection (Field) after depuration (Depuration) and after exposure to copper sulphate (CTL, C1-C5 – SpB; C1-C7 –SpS).

| *Scrobicularia plana* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Big Size** | | | | | | | |
| FA | Field | Depuration | CTL | C1 (1.0 mg/L) | C2 (1.5 mg/L) | C3 (2.0 mg/L) | C4 (2.5 mg/L) | C5 (3.0 mg/L) |
| C8:0 | 4.25 | | | | | | | |
| C10:0 | 2.24 | | | | | | | |
| C18:0 | | | | | 13.83 | | | 11.80 |
| C21:0 | 10.63 | 8.82 | 9.56 | 10.72 | | 24.73 | 11.86 | |
| C23:0 | 24.29 | | | 51.41 | 56.99 | 46.29 | 25.50 | 66.98 |
| **TOTAL SFA** | **41.41** | **8.82** | **9.56** | **62.13** | **70.81** | **71.02** | **37.36** | **78.78** |
| C14:1 | 1.08 | | | | | | | |
| C15:1 | 20.93 | 32.00 | 24.62 | 39.28 | 27.86 | | 28.35 | 21.92 |
| C17:1 | 17.91 | 26.46 | 29.22 | 29.20 | 32.99 | 25.21 | 14.27 | 38.98 |
| C20:1n9 | | | 16.05 | | | | 15.29 | |
| **TOTAL MUFA** | **39.92** | **58.46** | **69.89** | **68.49** | **60.84** | **25.21** | **57.91** | **60.89** |
| C18:2n6t | 19.10 | 19.72 | 21.77 | 21.57 | 34.59 | 31.85 | 38.16 | 38.66 |
| C18:3 | 30.26 | 65.43 | 54.40 | 30.17 | 63.40 | 28.98 | 72.98 | 67.81 |
| C18:3n3 | 15.93 | 17.50 | | 10.21 | 15.34 | 13.35 | | |
| C20:2 | 48.91 | 50.71 | 79.00 | 58.87 | 34.41 | 59.63 | 79.59 | 57.67 |
| C20:3n3 | | | | | | | 60.38 | |
| C20:4n6 | 24.78 | 70.08 | 23.51 | 31.18 | 38.23 | 41.36 | 77.69 | |
| C22:2 | | | | | | | 20.44 | |
| **TOTAL PUFA** | **138.98** | **223.44** | **178.69** | **152.00** | **185.97** | **175.17** | **349.23** | **164.14** |
| EPA | 49.98 | 61.24 | 66.26 | 51.64 | 44.50 | 75.93 | 66.72 | 41.05 |
| DHA | 85.54 | 81.49 | 130.54 | 83.77 | 68.43 | 78.25 | 106.56 | 64.75 |
| **TOTAL HUFA** | **135.51** | **142.73** | **196.79** | **135.41** | **112.94** | **154.18** | **173.29** | **105.79** |
| **N** | **14** | **10** | **10** | **11** | **11** | **10** | **13** | **9** |

| *Scrobicularia plana* | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Small Size** | | | | | | | | | | |
| FA | Field | Depuration | CTL | C1 (1.0 mg/L) | C2 (1.5 mg/L) | C3 (2.0 mg/L) | C4 (2.5 mg/L) | C5 (3.0 mg/L) | C6 (3.5 mg/L) | C7 (4.0 mg/L) |
| C18:0 | | | | | | | | | 7.41 | 8.65 |
| C21:0 | 13.58 | 12.46 | 8.70 | | 10.92 | 10.19 | | 9.27 | 17.98 | 10.66 |
| C23:0 | | | 127.18 | 51.08 | 95.05 | 99.62 | | 55.10 | | 47.39 |
| **TOTAL SFA** | **13.58** | **12.46** | **135.88** | **51.08** | **105.97** | **109.82** | **0.00** | **64.36** | **25.39** | **66.70** |
| C15:1 | | | 25.96 | | | | | | | 13.73 |
| C17:1 | | | 41.48 | 28.25 | | | | | 21.45 | 8.42 |
| C20:1n9 | 13.61 | | | | | | 8.83 | | 7.03 | |
| C24:1n9 | 9.29 | | | | | | | | | |
| **TOTAL MUFA** | **22.90** | **0.00** | **67.44** | **28.25** | **0.00** | **0.00** | **8.83** | **0.00** | **28.48** | **22.16** |
| C18:2n6t | | | 18.41 | | | | | | 14.60 | 15.91 |
| C18:3 | | 23.03 | 72.08 | 46.80 | 57.66 | 48.07 | 42.03 | 49.86 | 33.68 | 33.06 |
| C18:3n3 | 58.18 | 22.66 | | 14.13 | | | | | 11.24 | 8.94 |
| C20:2 | 74.86 | 68.63 | 56.47 | 53.07 | 41.85 | 67.65 | 70.36 | 45.38 | 38.47 | 35.71 |
| C20:3n3 | 16.58 | | | | | | 24.51 | 6.28 | | |
| C20:4n6 | 23.49 | 59.04 | 9.36 | | | | 56.23 | 8.07 | 91.98 | 25.84 |
| **TOTAL PUFA** | **173.11** | **173.35** | **156.31** | **114.00** | **99.51** | **115.72** | **193.13** | **109.59** | **189.97** | **119.47** |
| EPA | 91.87 | 64.21 | 62.30 | 76.00 | 56.86 | 74.87 | 105.85 | 38.87 | 42.22 | 77.76 |
| DHA | 84.64 | 64.78 | 79.72 | 71.67 | 69.38 | 148.57 | 88.61 | 56.26 | 82.17 | 67.12 |
| **TOTAL HUFA** | **176.50** | **128.99** | **142.03** | **147.67** | **126.23** | **223.43** | **194.47** | **95.13** | **124.39** | **144.88** |
| **N** | **9** | **7** | **10** | **7** | **6** | **6** | **7** | **8** | **11** | **12** |

### *3.3 Multivariate analyses*

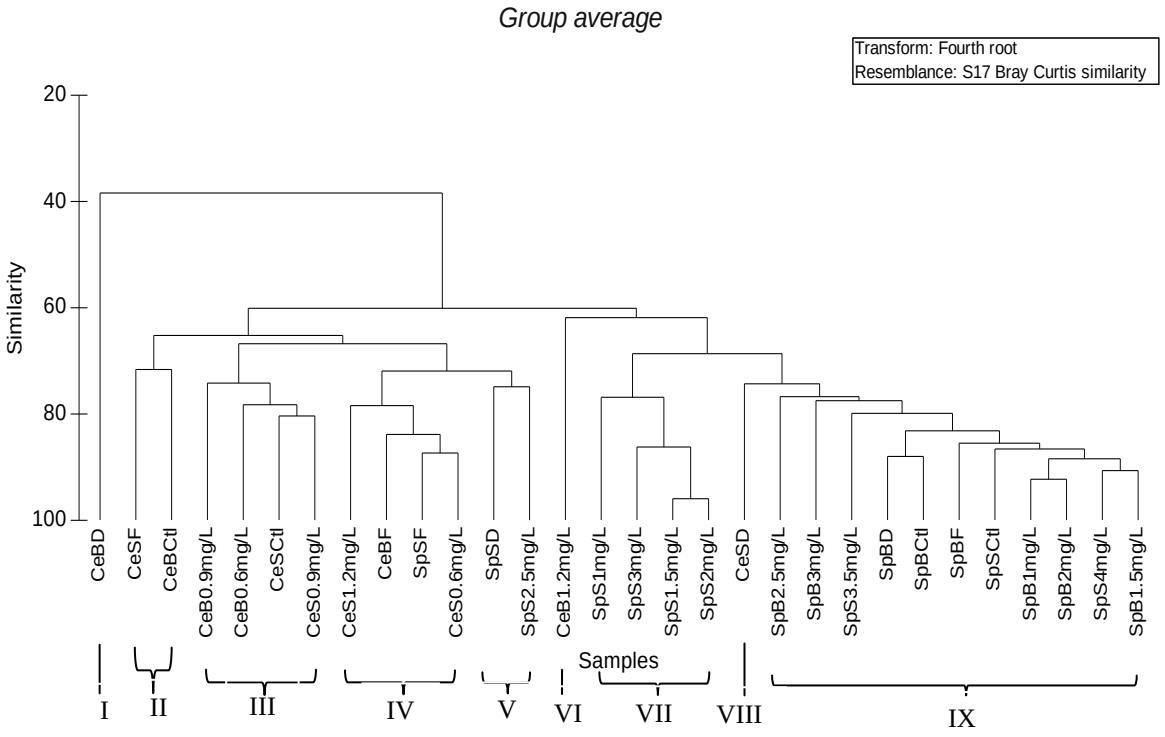


**Figure 3:** Cladogram (cluster analysis) grouping both bivalve species (Ce and Sp) and both classes of sizes (Big - B and Small - S) related with FA content after field collection (F), depuration (D), exposure to copper sulphate (Ctl, 1.0 - 3.0 mg/L to *C. edule* (B & S) and to *S. plana* (B); 1.0 - 4.0 mg/L to *S. plana* (S)).

Following the cluster analysis, the FA samples, determined after field collection (F), after depuration (D) and at the end of exposure to copper sulphate (Ctl, 1.0 - 3.0 mg/L, 1.0 - 4.0 mg/L), of the two species (*C. edule* and *S. plana*) and to both classes of size (S and B), were spread by 9 groups (I to IX) according to the abundance and diversity on FA samples (Figure 3). A distinct separation amongst both marine bivalves organisms was noticeable, with *C. edule* samples occupying the first groups (from I to IV, and VI and VIII), with the exception of SpSF (see group IV), whereas *S. plana* samples were distributed in the last groups (V, VII and IX). Furthermore, for *S. plana*, the both size classes presented a well-defined pattern, separated in distinct groups, with small size in groups V and VII and big size class in group IX. However, regarding this group, three samples of small size organisms were recorded: SpSCtl, SpS4mg/L and SpS3.5mg/L.

Regarding *C. edule*, the analysis by size classes was not well defined as for *S. plana*. In this case, it was observed six groups (from I to VI) of samples presenting larger and smaller size classes mixed.

Figure 4 showed organisms and their class sizes distributed according to the FA contents (stress= 0.17). At group A is presented the two classes of size (big and small) of *C. edule*, whereas at the group B is shown the small size classes of organisms of *S. plana.* After the depuration period, the large size classes of *C. edule,* were positioned outside both groups, in an independent position revealing a distinct content of FA profiles.

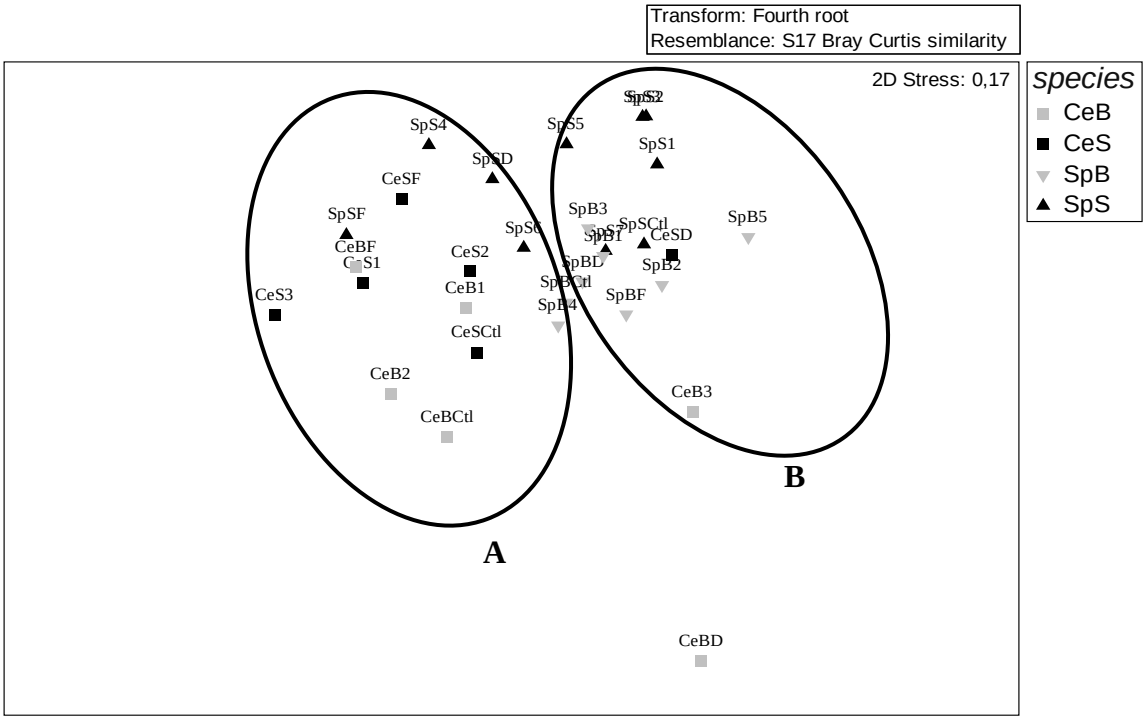


**Figure 4:** Two-dimensional non-metric MDS ordination plot of FA content of both *C. edule* and *S. plana* and class sizes. Light grey symbols represent the big size class of the tow marine bivalve species and dark symbols represent the small size class to both species determined after the field collection, after depuration and after the exposure to the pollutant under laboratory conditions, respectively. A and B represent distinct groups at the MDS plot. CeB – *C. edule* big size class; CeS – *C. edule* small size class; SpB – *S. plana* big size class; SpS – *S. plana* small size class; F – samples determined after the field collection; D – samples determined after depuration time; 1, 2, 3, 4 and 5 - are the different concentrations tested (Ce1- 0.6 mg/L; Ce2- 0.9 mg/L; Ce3- 1.2 mg/L; Sp1- 1.0 mg/L; Sp2- 1.5 mg/L; Sp3- 2.0 mg/L; Sp4- 2.5 mg/L; Sp5- 3.0 mg/L).

Analysis of ANOSIM showed a distinction of both distinct groups (R= 0.368; p= 0.001). All sizes classes presented significant differences ($p < 0.05$) but presented low R value, showing low segregation between almost all groups, unless to CeB / CeS that showed no significant differences and presented a low segregation (R= 0.019; p= 0.535).

At table 3 are shown three main fatty acids (DHA, EPA and C20:2) that contributed to a large similarity inside each group described by the abbreviation of the species' name (Ce, Sp) and size class (B, S). Distinct variations on FA composition and abundance state dissimilarities between groups. FA that main contributed for the dissimilarities among groups are C18:3n6; C23:0 and C18:2n6t.

**Table 3:** SIMPER analyses showing average dissimilarity among groups of samples, related with *n*-MDS analysis.

| Similarity | Fatty Acids | Av.Abund | | Av.Sim | Sim/SD | Contrib% | Cum.% |
|---|---|---|---|---|---|---|---|
| 66.83 | C22:6n3 (DHA) | 0.51 | | 12.68 | 8.66 | 18.97 | 18.97 |
| | C20:5n3 (EPA) | 0.47 | | 11.65 | 6.62 | 17.43 | 36.40 |
| | C20:2 | 0.47 | | 11.53 | 8.76 | 17.26 | 53.66 |
| 54.62 | C20:5n3 (EPA) | 0.48 | | 11.22 | 7.02 | 20.54 | 20.54 |
| | C22:6n3 (DHA) | 0.47 | | 11.19 | 7.86 | 20.49 | 41.03 |
| | C20:2 | 0.41 | | 9.57 | 7.94 | 17.52 | 58.55 |
| 70.30 | C22:6n3 (DHA) | 0.53 | | 14.35 | 8.45 | 20.41 | 20.41 |
| | C20:5n3 (EPA) | 0.51 | | 13.63 | 7.18 | 19.39 | 39.80 |
| | C20:2 | 0.48 | | 13.02 | 6.67 | 18.53 | 58.33 |
| 81.26 | C22:6n3 (DHA) | 0.54 | | 11.14 | 15.91 | 13.71 | 13.71 |
| | C20:5n3 (EPA) | 0.49 | | 10.03 | 13.97 | 12.35 | 26.06 |
| | C20:2 | 0.49 | | 10.02 | 10.87 | 12.33 | 38.39 |
| **Dissimilarity** | | | | **Av.Diss.** | **Diss/SD** | | |
| 39.18 | C18:3n3 | 0.26 | 0.13 | 2.90 | 1.15 | 7.41 | 7.41 |
| | C22:2 | 0.26 | 0.17 | 2.68 | 1.15 | 6.84 | 14.25 |
| | C23:0 | 0.21 | 0.07 | 2.55 | 1.01 | 6.52 | 20.76 |
| 41.74 | C18:3n6 | 0.47 | 0.13 | 4.18 | 1.86 | 10.01 | 10.01 |
| | C18:2n6t | 0.41 | 0.09 | 3.64 | 2.11 | 8.71 | 18.72 |
| | C23:0 | 0.34 | 0.07 | 3.60 | 1.41 | 8.62 | 27.33 |
| 44.12 | C18:3n6 | 0.41 | 0.13 | 4.34 | 1.58 | 9.85 | 9.85 |
| | C23:0 | 0.31 | 0.07 | 4.04 | 1.15 | 9.15 | 19.00 |
| | C20:1n9 | 0.09 | 0.24 | 2.88 | 1.23 | 6.53 | 25.53 |
| 37.17 | C18:3n6 | 0.47 | 0.08 | 4.79 | 2.25 | 12.88 | 12.88 |
| | C18:2n6t | 0.41 | 0.00 | 4.78 | 7.10 | 12.86 | 25.73 |
| | C15:1 | 0.36 | 0.13 | 3.29 | 1.45 | 8.86 | 34.60 |
| 36.71 | C18:3n6 | 0.41 | 0.08 | 4.95 | 1.77 | 13.47 | 13.47 |
| | C23:0 | 0.31 | 0.21 | 3.80 | 1.18 | 10.35 | 23.82 |
| | C22:2 | 0.00 | 0.26 | 3.41 | 1.37 | 9.30 | 33.12 |
| 29.26 | C18:2n6t | 0.11 | 0.41 | 3.77 | 1.73 | 12.87 | 12.87 |
| | C15:1 | 0.07 | 0.36 | 3.71 | 1.70 | 12.69 | 25.56 |
| | C17:1 | 0.15 | 0.40 | 3.29 | 1.42 | 11.23 | 36.79 |

### *3.4 FATMS*

At figure 5 is presented the FA composition of rotifers and microalgae that was used to daily feed the both class sizes of *C. edule* and *S. plana*, during the exposure period to the contaminant. The composition of microalgae was mostly constituted by PUFA (≈ 86.5%) but with little amount of SFA (≈7.7%), HUFA (3.0%) and MUFA (≈3.0%). Rotifers food

was mostly composed by SFA (≈62.7%) and MUFA (23.0%) but present low quantity of PUFA (9.0%) and HUFA (≈5.4%). At tables 1 and 2, the samples of the different treatments of the bioassays showed an increase of total HUFA, and a decrease of total SFA at big size class organisms, whereas small size class organisms of both species presented an increase of total SFA, and a decrease of total HUFA. In all cases was shown an increase of total MUFA and a decrease of total PUFA.

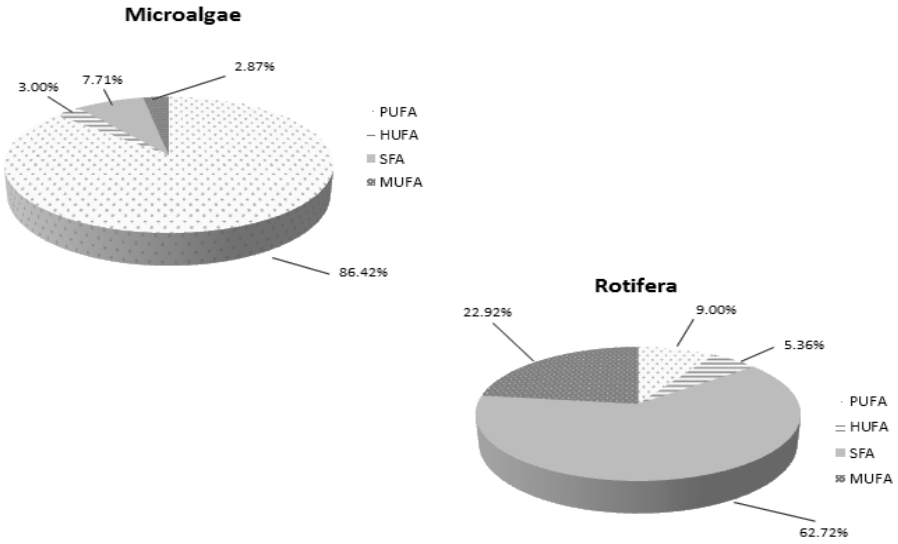


**Figure 5:** FA constitution of microalgae and rotifers bivalves' feeding solution during the experiments.

At figure 6 PCA analysis showed that *C. edule* presented higher amounts of SFA (C6:0, C12:0), MUFA (C20:1n9) and PUFA (C18:3n3), whereas *S. plana* showed also higher quantity of PUFA (C20:2, C18:3n6, C20:4n6) and SFA (C23:0), but also exhibit HUFA (DHA and EPA), contrarily to *C. edule*. Based on FATMs (Table 4), the composition on fatty acids of both bivalve species may indicate a dietary food source based mainly on phytoplankton. Still, FATMs also showed the ingestion of zooplankton, so suggesting an omnivorous diet, to both species, at the field and at under laboratory conditions. It is also showed a higher consumption of diatoms and bacteria by *C. edule* (big size), mainly in the samples of organisms collected in the field.

**Table 4:** Comparison of FATMs of small and big sizes classes of *C. edule* and *S. plana* after sampling in the field and after copper sulphate exposure.

| | *Scrobicularia plana* | | | | *Cerastoderma edule* | | | |
|---|---|---|---|---|---|---|---|---|
| **Size** | **Big** | | **Small** | | **Big** | | **Small** | |
| **Origin** | **Field** | **Bioassays** | **Field** | **Bioassays** | **Field** | **Bioassays** | **Field** | **Bioassays** |
| DHA/EPA | 1.711 | 1.970 | 0.921 | 1.280 | 0.378 | 1.584 | 1.040 | 2.415 |
| EPA | 0.050 | 0.066 | 0.092 | 0.062 | 0.844 | 0.279 | 0.040 | 0.046 |
| 16:1 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.442 | 0.000 | 0.000 |
| DHA | 0.086 | 0.131 | 0.085 | 0.080 | 0.320 | 0.413 | 0.042 | 0.111 |
| 18:2n6 | 0.019 | 0.022 | 0.000 | 0.018 | 0.000 | 0.000 | 0.000 | 0.000 |
| ∑iso and anteiso-C15 and C17 | 0.039 | 0.054 | 0.000 | 0.067 | 0.573 | 1.083 | 0.000 | 0.021 |

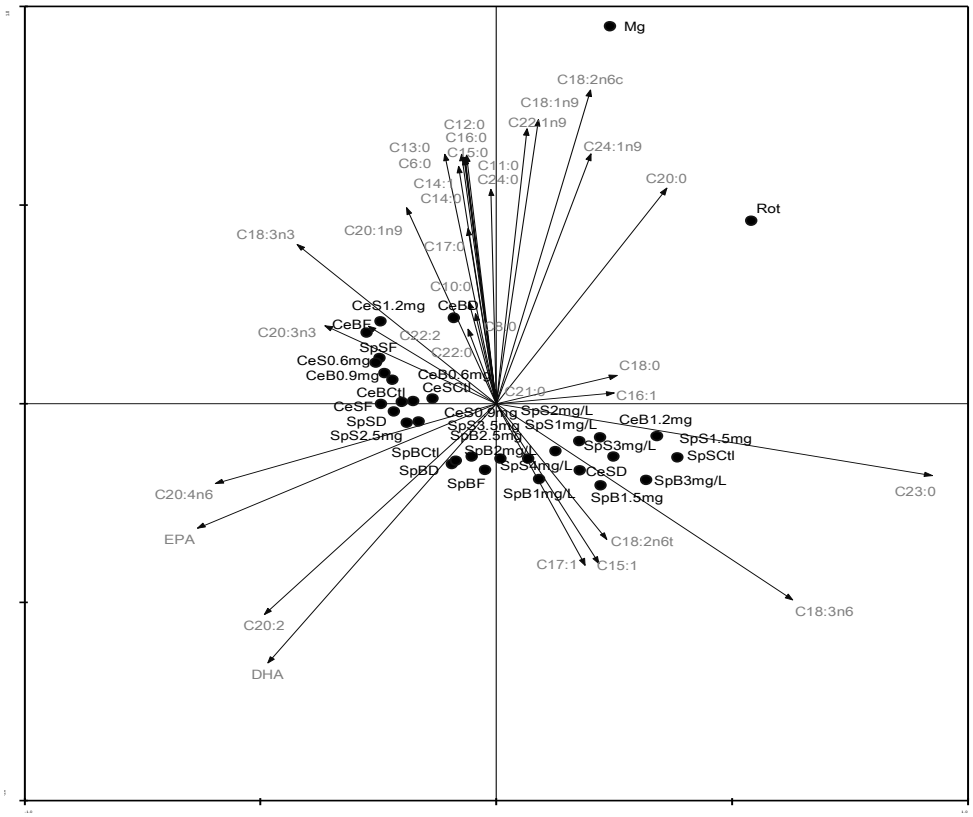


**Figure 6:** Principal component analysis (PCA) showing the relation between FA contents and FATMS determined to *S. plana* and *C. edule* (both classes of size – S and B) after field sampling and after exposure to copper sulphate treatments.

## 4. Discussion

This work highlights toxic and biochemical (namely fatty acids) effects of copper sulphate in two important commercial bivalve species. A 100% of mortality is achieved at higher concentrations to both species with the smaller size of organisms being more tolerant than the big size organisms, except $LC_{10}$ of *S. plana*. Furthermore, both size classes of *C. edule* show a higher sensitiveness to the metal than *S. plana*. According to the literature, few authors report copper sulphate and copper toxicities on bivalve species. For instance, Satyaparameshwar et al. (2006) report a $LC_{50}$ to *Lamellidens marginalis* of 3.990 mg/L, after 72 hours of exposure to copper sulphate. Comparing with our studied organisms, *L. marginalis* is more tolerant to copper sulphate action than *Cerastoderma edule* (LC50 = 1.129 (0.968; 1.289) mg/L; LC50 = 0.818 (0.595; 0.987) mg/L, small and big size classes, respectively, as well as the organisms of big size class of *Scrobicularia plana* (LC50= 2.563 (2.229; 2.903) mg/L). Kováts et al. (2010) report copper toxicity to *Anodonta anatina* that exhibit a $LC_{50}$ of 18.9 (10.0–31.1) µg/L, *Pseudanodonta complanata* a $LC_{50}$ of 29.3 (25.4–34.0) µg/L and *Unio tumidus* a $LC_{50}$ of 19.0 (16.2–22.0) µg/L. Sousa et al. (2005) report copper $LC_{50}$ value of 58.84 (35.45–82.29) µg/L to the gastropod species *Nassarius reticulatus*, after 96 hours of exposure. Making an estimate of the percentage of copper at copper sulphate, at the different treatments of our study, we may conclude that *S. plana* and *C. edule* are more tolerant to copper action than the bivalves and gastropod species reported at other studies. Guilhermino et al. (2000) report a $LC_{50}$ = 0.0826 (0.0823; 0.0829) mg/L to *Daphnia magna*, a standard species on ecotoxicological studies, after an exposure time of 48 hours. This result evidence that the cladoceran species is more sensitive to copper sulphate action than the bivalve species reported in our study. A recent work by Filimonova et al. (2016b) show the toxicity of copper sulphate is higher to the diatom *Thalassiosira weissflogii* and the copepod *Acartia tonsa* than to the bivalves species here studied. The brine shrimp *Artemia franciscana* studied by Filimonova et al. (2016b) showed to be the planktonic species the most tolerant to the pollutant, and also less sensitive than the cockle and the peppery furrow shell here studied. Other studies reported a toxicity of copper sulphate and copper to fish species. Gharedaashi et al. (2013) showed a $LC_{50}$ value of 2.310 ppm (2.165; 2.463) after 96 hours of exposure of *Rutilus frisiikutum* to copper sulphate. *Rasbora sumatrana* presented a $LC_{50}$ value of 5.6 µg/L after 96 hours of exposure to copper, while for *Poecilia reticulata* the $LC_{50}$ value was 37.9 µg/L (Shuhaimi-

Othman et al., 2010). Nekoubin et al. (2012) reported a $LC_{50}$ of 1.717 mg/L to *Ctenopharyngodon idella* after exposure to copper sulphate, being more tolerant than *C. edule.* All other studies show a higher sensitiveness of the fish species to the metal than the bivalve species studied on the present work.

According to the literature, copper may affect the lipid metabolism of some organisms (*Rhodococcus erythropolis*: De Carvalho (2012), *Pseudomonas putida:* Popova et al. (2008), *Gracilaria tenuistipitata:* Pinto et al. (2011), *Acartia tonsa, Artemia franciscana* and *Thalassiosira weissfloggi:* Filimonova et al. (2016b)). Alterations on the FA profile are expected given the action mode of copper, since under metal stress, decrease of lipid content and alterations on fatty acids profile may occur (alterations on the levels and composition of glycolipids, neutral lipids and phospholipids; lipid peroxidation) (Filimonova et al., 2016a; Ouariti et al., 1997; Rocchetta et al., 2006). The mechanisms about copper affecting the fatty acids profile are not clear but may include effects on desaturation, esterification, and mobilization from triacylglycerols (Engle et al., 2001). These changes may have several biochemical effects, such as at the transition of metals that catalyze the generation of ROS, causing oxidative damage.

In a general perspective, the composition of FA profile change between species and within species according to the size class. The bigger bivalve organisms exhibit more FA diversity still lower abundance, than the smaller ones, with *S. plana* exhibiting a higher nutritious value than *C. edule.* Both size classes of organisms show a distinct FA content among them, creating two main groups that emphasize distinct FA content among both sizes. An isolated group is performed by the big size class of *C. edule*, after depuration, that presents a distinct FA content, not related to any other group of organisms, being characterized by a great increase of SFA and a decrease of MUFA. EPA, DHA and C20:2 are the main FA that most contribute for the major similarities within size classes, whereas C18:3n6; C23:0 and C18:2n6t are the main FA that contribute to the most differences between size classes. These differences on FA profiles are justified by food preferences, but also by the chemical stress caused to the organism and its performance to synthesize and assimilate the nutrients. Indeed *C. edule* presented higher amounts of SFA (C6:0, C12:0), MUFA (C20:1n9) and PUFA (C18:3n3) and *S. plana* also showed higher quantity of PUFA (C20:2, C18:3n6, C20:4n6) and SFA (C23:0), but also exhibited HUFA (DHA and EPA), in opposite to *C. edule.* Most of these compounds are vital nutrients playing a key role in

organisms' health and functioning. For instance, *n-3* PUFA deficiency in animals is associated with visual and central nervous function defects (Salem et al., 2001). C18:3n3 (α LNA) serve as substrate at the process of elongation and desaturation in mammals to synthetize EPA and DHA (Givens, 2008). EPA and DHA are associated with the fetal development and with neurological, cognitive and cardiovascular functions. DHA is the predominant FA present in the eye and brain tissues. C18:2n6, C20:4n6 and C18:3n6 are substrates involved in the biosynthesis of long chain PUFA (Givens, 2008). EPA and ARA serve as precursors of eicosanoids that are responsible for many immune and inflammatory responses, neurologic and reproductive functions and enhance the adaption of organisms to environmental and anthropogenic stressors (Fokina et al., 2013). EFA are mainly obtained through organisms' diets, being the FA content a closely related with food consumption ("you are what you eat"). According to Delaporte et al. (2005), the FA content of bivalve species is affected by the diet quality. Some species (e.g., *Crassostrea gigas*, *Ostrea edulis*, *Ruditapes philippinarum*, *Ruditapes decussatus*) present a selective behavior to particles form, dimension, nutritious quality or chemical composition, which can contribute to distinct dietary food sources among species and class sizes, and thus distinct fatty acids contents (Gonçalves et al., 2016). Furthermore, the food available and the temperature are crucial parameters to regulate the growth of marine invertebrates like molluscs bivalves. Changes on these molecules of lipids may also be related to the rate of filtration and seasonal food availability (Ezgeta-Balić et al., 2012). Some works (e.g., Filimonova et al., 2016a) also report changes in FA profile of marine organisms from different trophic levels when exposed to chemicals. Filimonova et al. (2016b) report a significant reduction on EFA on three marine planktonic species (*T. weissflogii*, *A. tonsa* and *A. franciscana*) when exposed to copper sulphate.

As reported for two marine bivalve species, the presence of chemical stressors (e.g., copper sulphate) in the aquatic systems may disrupt the good health status of the ecosystems and thus the entire trophic food web. Therefore, it is of high importance to understand their impacts in the traits and on the biochemical pathways of aquatic species in order to act at the prevention of potentially stressful situations and on the management and implementation of mitigation measurements.

## 5. Conclusions

Our results stated changes in FA profile of commercial marine bivalve species, with slightly higher impacts at the larger sizes, caused by the presence of an inorganic chemical stressor (copper sulphate). These alterations may also cause changes in the nutritious value of the organisms and thus on the trophic food chain. This work highlights the importance of FA biomarkers as a relevant tool and endpoint in ecotoxicological studies that may be used as early-warning indicators on the detection of contaminants in aquatic systems. Therefore, current research may be considered by environmental agencies and stakeholders to be used on the guidance and establishment of regulation and legislation to these chemicals (e.g. copper and copper sulphate).

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# Chapter III: The effect of copper sulphate on the antioxidant defence system of the bivalves *Cerastoderma edule* and *Scrobicularia plana*

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# The effect of copper sulphate on the antioxidant defence system of the bivalves *Cerastoderma edule* and *Scrobicularia plana*


## Abstract

Anthropogenic activities, such as agriculture or industrial activities, are a main source of pollution contributing for the degradation of water quality and thus affecting the living organisms of the aquatic systems. Copper is one of the major constituents of pesticides formulations and widely used in industrial activities and on antifouling paints being often released into the aquatic systems, and may cause negative effects in the ecosystems and its communities. Copper sulphate is a copper-based formulation, widely used in the agriculture practices to control pests. At this study is proposed to determine the effects of copper in the antioxidant defence system of two important commercial bivalve species, *Cerastoderma edule* and *Scrobicularia plana*, and two size classes. At this work was observed the behaviour activity of the organisms during the exposure time to copper sulphate and subsequently it was determined the enzymatic activities of glutathione-S-transferases (GSTs), glutathione reductase (GR), and glutathione peroxidase (GPx; both selenium-dependent (SeGPx) and total (tGPx)) in the muscle tissue (foot). Moreover, lipid peroxidation was evaluated through thiobarbituric acid reactive substances (TBARS) measurement in the muscle tissue. The results showed changes in the behaviour and enzymatic activity at the different copper sulphate concentrations. Furthermore *C. edule* exhibited a more consistent molecular response to the chemical exposure than *S. plana*. Moreover, according to TBARS levels, lipid peroxidation possibly occurred on small size class of *S. plana* and on big size class of *C. edule. C. edule* revealed to be a good bioindicator and the antioxidant enzymes good biomarkers to be used in ecotoxicological studies to detect the presence of copper sulphate in aquatic systems. The muscle tissue (foot) showed to be also a good tissue to use in biochemical analysis to detect the presence of toxicants.



**Keywords:** Estuarine bivalve species; Copper sulphate; Biomarkers; Behaviour activity; Enzymatic activity.

## 1. Introduction

Metals' discharges in the aquatic environment are one of the major concerns in the world. Metal pollution, may be from natural (e.g. volcanic activities) or anthropogenic (leaching of agricultural fields) sources (Ferreira-Cravo et al., 2008), leading to serious impacts in the surrounding aquatic systems and their communities. Some metals may be toxic only at great concentrations, since they are biologically essential and natural constituents of the aquatic ecosystems, being designated essential elements (e.g. copper), whereas others are toxic even at very low concentrations, being designated non-essential elements (e.g. mercury) (Bae and Lim, 2012).

Copper is a well-known aquatic contaminant, due to the intense use on antifouling paints and pesticides formulations (Maria and Bebianno, 2011). In spite of copper be an essential element, necessary to the maintenance of cellular functions (Mayor et al., 2013), acting as enzymatic cofactor and essential element of several metabolic pathways (Ritter et al., 2008), this metal may become toxic at greater concentrations and its toxicity depends on the pH and water temperature, which has increased in the recent years, with the pH decrease and temperature increase in the aquatic systems (Mayor et al., 2013). Copper toxicity can be explained by distinct mechanisms, (a) it can act establishing redox cycles, then take part in Fenton's reaction, as catalyser agent, and consequently producing highly unstable reactive oxygen species (ROS), such as $O^{-}$ and $HO^{-}$;(b) the metal can interact with functional groups (e.g. carboxyl, hydroxyl, sulfhydryl) and therefore lead to its inactivation or (c) it can replace essential cofactors to the enzyme function (Filimonova et al., 2016a; Maazouzi et al., 2008; Ritter et al., 2014; Zhang et al., 2010). Several studies revealed that the increase of the ROS production may affect many metabolic pathways, such as glycolysis, protein, fatty acids and amino acids metabolism. Furthermore the ROS also have the ability to induce lipid peroxidation and DNA damage (Galaris and Evangelou, 2002) and ultimately can lead to the cell death. Considering the consequences in molecular processes of organisms exposed to high metal concentrations, mainly in the aquatic systems (Antunes et al., 2007; Marques et al., 2008), biomarkers are important tools and endpoints in ecotoxicology studies widely used as earlier-warning indicators of contamination. Therefore, the evaluation of a set of biomarkers involved in the antioxidant responses, can give an indication about the antioxidant defence systems of the organisms and can be used to determinate potential oxidative effects of the toxic (Stohs and Bagchi,

1995; Valavanidis et al., 2006). Although there are numerous studies evaluating the effect of chemicals in the enzymatic activity of several organisms, none of them has described the effects of copper sulphate, in *C. edule* and *S. plana*, with a great economic interest. These species have an important ecological role, acting as link between primary producers and consumers, like fish, crustaceans or wading birds. *C. edule* is a suspension-feeder, that habit on intertidal shallow areas. At the other hand *S. plana* is a deposit filter feeder, living on intertidal and subtidal areas (Verdelhos et al., 2015). These bivalves species, similar to other bivalves species, are widely used in ecological and toxicological studies, since they have a large filtration ability and may accumulate high quantities of pollutants in their tissues (Cardoso et al., 2013; Freitas et al., 2014; Nilin et al., 2012; Paul-Pont et al., 2010a, 2010b). Moreover their sessile life style, the ease of sampling collection, maintenance in laboratory and sensitivity to chemicals make them good standard species, being also considered good bioindicators (Gonçalves et al., 2016).

By this, this work aims to determine: a) the oxidative stress response of Glutathione S-Transferases (GSTs), Glutathione Reductase (GR) and Glutathione Peroxidase (GPx); b) the thiobarbituric acid reactive species (TBARS) levels, to verify the occurrence or not of lipid peroxidation (LPO) , in the muscle tissue (foot) of two size classes of *C. edule* and *S. plana* after exposure to copper sulphate.

## 2. Materials and Methods

### *2.1. Study site and field sampling*

The Mondego estuary located in the Portuguese Western coast, near Figueira da Foz (40º08'N, 8º50'W), is a small estuary, extended for about 8 km and cover an area of 3.4 $km^2$, approximately. The estuary is composed by two arms (north and south), that represent two distinct subsystems with different hydrodynamic characteristics, and divided by the Morraceira island. Mondego estuary has an important socio-economic role, given the variety of resources and services provided to the population. Consequently, this system is under an intensive anthropogenic pressure, with eutrophication stress mainly due to the excessive nutrients inputs from the Pranto River. The organisms (*Cerastoderma edule* and *Scrobicularia plana*) were collected in the two arms (Figure 1), with dredge support and transported to the lab in cold boxes with field water.

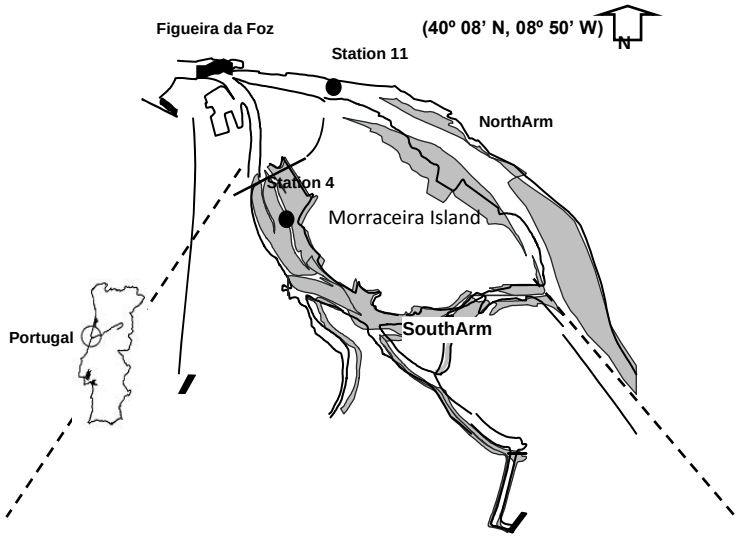


**Figure 1:** Location of the Mondego estuary and the sampling sites within the estuary.

### *2.2. Laboratory procedures and bivalve bioassays*

Arrived to the laboratory, was selected 10 bigger and 10 smaller organisms from each species, not exposed to any laboratory procedure and were measured some parameters (the shell length, the total weight, the tissue weight and the foot weight) to determine the condition indices. For biomarker analysis, the muscle tissue of each organism was removed and stored at -80ºC.

The other sampled organisms were subjected to a depuration period, over 48 hours, without food, in filtrated seawater at salinity of 20. After this time, the individuals were divided in aquaria according to the species and size class (B=big size (medium body size =2.45 (2.24; 2.72) cm to *C. edule* and 4.20 (3.82; 4.87) cm to *S. plana*); S=small size (medium body size =1.97 (1.64; 2.37) cm to *C. edule* and 3.47 (2.77; 4.07) cm to *S. plana*)).

With the purpose of evaluate the effect of copper sulphate on the two bivalve species, the both size classes of *S. plana* and *C.edule* were submitted to 8 treatments: one negative control plus seven concentrations of copper sulphate (1.0 mg/L; 1.5 mg/L; 2.0 mg/L; 2.5 mg/L; 3.0 mg/L; 3.5 mg/L; 4.0 mg/L) and (0.35 mg/L; 0.6 mg/L; 0.9 mg/L; 1.2 mg/L; 1.5 mg/L; 1.8 mg/L; and 2.1 mg/L), respectively. The effective copper concentration at seawater was determined using flame atomic absorption spectrophotometry (ICE3000

Series AA Spectometer– Thermo Scientific), being the limit of quantification for undiluted samples of 10 µg/L.

Each treatment was composed by ten replicates, with each vial filled with 1000 mL of test medium to big size organisms and 500 mL to small size organisms, to both species. Bivalves were fed every day with a commercial mixture of microalgae and rotifers, and the medium changed every two days. Bioassays occurred during 96 hours, under a $12h^{L}$:$12h^{D}$ photoperiod, under controlled temperature (20±2ºC), with filtrated sea water medium at a salinity of 20 psu. At the end of the exposure period, all survival organisms passed by a set of processes (dissection, measurement of the weight and the body length and evaluation of their condition indices).

### *2.3. Mortality and Behaviour Activity*

Bivalves were observed daily to analyse mortality and behavioural conditions, being evaluated the siphon activity, valves condition and the organism behaviour during feeding. Mortality (inability to close the valves upon mechanical stimulus) was observed and registered every 12h, and dead individuals removed from the flask.

Behaviour activity was determined considering three main behavioural characters which were recorded as 0/1 corresponding to its absence/presence. It was considered siphon activity, related to feeding and excretion activities (0 means no siphon activity and 1 means observed or signs of siphon activity); valve activity related to the ability of the valves to close (0 means no ability and 1 means ability to close); and reaction to mechanical stimulus or perturbation e.g. siphon, valves, foot (0 means no or slow reaction and 1 means instant reaction) (Gosling, 2003).

Each behavioural character was estimated through the quotient between the organisms that exhibited activity and the total organisms (e.g. Siphon activity= *n* Observed siphon activity / *n* Total individuals), where "*n*" means the number of individuals in each treatment; range from 0 to 1. Additionally, the activity in each treatment was determined as the sum of the three traits (Siphon activity, Valves activity and Reaction), range from 0 to 3. Lastly, an activity index for the entire assay was estimated as:

$$\text{Total Activity} = \frac{\text{Activity (24h)+Activity (48h)+Activity (72h)+Activity(96h)}}{4}$$

according to Verdelhos et al. (2015).

### *2.4. Biomarkers Analysis*

The oxidative stress responses were evaluated in the muscle tissue (foot) by the determination of GPx (total and selenium dependent), GR and GSTs activity and the lipid peroxidation by the TBARS measurement. Muscle tissue was homogenized in ice-cold phosphate buffer (50 mM, pH=7.0 with 0.1% Triton X-100). Afterwards, the homogenized was centrifuged at 15000 G during 10 minutes and the supernatants divided into five aliquots (one for each determination – GSTs, GR, GPx, TBARs) and one to protein quantification. The aliquots were stored to -80ºC for subsequent determination. At the end, all biomarkers were expressed in function of the protein content of each corresponding sample. Moreover to statistical aims and to reduce the biochemical determination variability, it was considered the average biomarker value of six organisms by treatment.

#### *2.4.1. GSTs*

GSTs (EC 2.5.1.18) activity was evaluated through spectrometry, according to Habig et al. (1974). GST catalyzes the conjugation of substrate 1-chloro-2,4-dinitobenzene (CDNB) with the reduced glutathione (GSH), originating a thioeter (molecular extinction coefficient of 9.6 $mM^{-1}cm^{-1}$), that can be monitored by the absorbance increase at 340 nm. Enzymatic activity was determined in quadruplicate and the results were expressed in nmol of substrate hydrolyzed per minute per mg of sample protein.

#### *2.4.2. GR*

GR (EC 1.8.1.7) activity was assayed by spectrometry, according to protocols of Carlberg and Mannervik (1985). GR involved in the NADPH oxidation was following at a wavelength of 340 nm (molecular extinction coefficient of 6.22 $mM^{-1}cm^{-1}$). Enzymatic activity was determined in quadruplicate and the results were expressed in nmol of NADPH oxidized per min per mg of sample protein.

#### *2.4.3 GPx*

GPx (EC 1.11.1.9) activity was determined according to Flohé and Günzler, (1984), following the NADPH oxidation at 340 nm (molecular extinction coefficient of 6.22 $mM^{-1}cm^{-1}$), when oxidized glutathione (GSSG) is reduced back to the reduced form by the reductase glutathione. The GPx activity was evaluated using two independent substrates,

the hydrogen peroxide (0.255 mM) to the glutathione peroxidase selenium-dependent and the cumene hydroperoxide (0.7 mM) to the total GPx measurement. Enzymatic activity was determined in quadruplicate and the results were expressed in nmol of substrate hydrolyzed per minute per mg of sample protein.

### *2.4.4. TBARS*

The lipid peroxidation was evaluated by the TBARS quantification, according to Buege and Aust, (1978). The TBARS levels were measured by spectrometry, was determioned in duplicate at 535 nm (molecular extinction coefficient of $1.56 \times 10^5$ $mM^{-1}cm^{-1}$), based on the reaction of lipid peroxidation by-products, with 2-thiobarbituric acid (TBA), therefore the results were expressed in nmol per mg of protein.

### *2.4.5. Total Protein Concentrations*

The total concentration of protein of each sample was determined by spectrometry at 595 nm, according Bradford method (Bradford, 1976) adapted to the microplate. The Bradford method depend on the link of the dye Coomassie Blue G-250 to proteins, and the protein concentration can be estimated by comparison with a standard solution of γ-bovine globulin.

## *2.5. Statistical Analysis*

The statistical analysis was performed using the SPSS Statistics 20 software. To evaluate the existence of statistical significant differences between the organisms exposed at different copper concentrations, regarding to antioxidant enzymes and TBARs levels, the analysis of variance (ANOVA) assumptions was checked and an one-way ANOVA was carried out, followed by a Tukey's test to compare all treatments (including the organisms exposed to copper sulphate at the bioassays, and the organisms from the field and after depuration period). To reject de null hypothesis was considered a level of significance less than 0.05.

## 3. Results

### 3.1. Exposure to copper sulphate

#### *3.1.1. Mortality*

Considering the LC values for both species, *C. edule* exhibited less tolerance than *S. plana* to copper sulphate to both size classes ($LC50_B$= 0.818 mg/L (0.595-0.987), $LC50_S$= 1.129 mg/L (0.968 -1.289) and $LC50_B$= 2.563 mg/L (2.229-2.903), $LC50_{S=}$ 4.705 mg/L(3.540-12.292), correspondingly). Moreover, the bigger organisms showed be more sensitive than the smaller ones, regarding to both bivalve species (Table 1).

**Table 1:** Values of lethal concentration (LC) of copper sulphate for *S. plana* and *C. edule* (big and small sizes classes). In brackets are indicated the 95% confidence limits.

| | | Big size (mg/L) | Small size (mg/L) |
|---|---|---|---|
| *Scrobicularia plana* | $LC_{10}$ | 1.456 (0.699; 1.860) | 1.238 (0.000; 2.175) |
| | $LC_{20}$ | 1.836 (1.265; 2.177) | 2.428 (0.794; 3.648) |
| | $LC_{50}$ | 2.563 (2.229; 2.903) | 4.705 (3.540; 12.292) |
| | | Big size (mg/L) | Small size (mg/L) |
| *Cerastoderma edule* | $LC_{10}$ | 0.341 (0.000; 0.571) | 0.717 (0.351; 0.895) |
| | $LC_{20}$ | 0.504 (0.083; 0.698) | 0.859 (0.581; 1.012) |
| | $LC_{50}$ | 0.818 (0.595; 0.987) | 1.129 (0.968; 1.289) |

#### *3.1.2. Behaviour Activity*

Behaviour was evaluated observing the organisms' activity during the experiment. The absence/presence of 3 behavioural characters (siphon activity, valves activity and reaction) was registered and integrated in an activity index to both species and size classes. In the case of *C. edule*, it was observed a gradual decrease of the activity index with the increase of the copper sulphate concentrations to both size classes and the small size class organisms exhibited a bigger activity index than the big size class of organisms (Figure 2). However in the case of *S. plana*, it was not possible to observe a so defined pattern. Regarding to big size class of organisms, it was observed a decrease on the total activity index at the four first concentrations of copper sulphate, with an increase of total activity index at the fifth and sixth concentration and a new decrease at the last concentration. In

the other hand, at the small size class of organisms, it was observed a decrease of the total activity index at the three first concentrations of the compound, with an increase at the fourth concentration, followed by a decrease at the next two concentrations and an increase at the last concentration of the chemical. The larger organisms exhibited almost always a greater total activity index than the smaller organisms, except to the concentrations of 2.5 mg/L, 3.0 mg/L and 4.0 mg/L of copper sulphate (Figure 2).

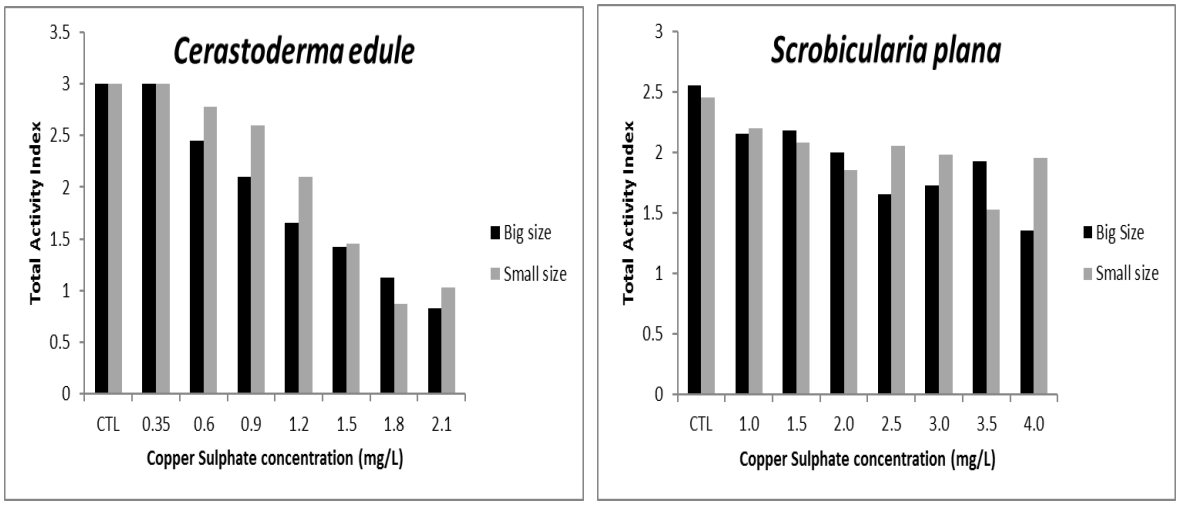


**Figure 2:** Behavioural activity, measured through the total activity index, along copper sulphate concentrations to *Cerastoderma edule* and *Scrobicularia plana*, to both size classes.

### *3.2. Biomarkers*

The analysis of enzymatic responses was performed only to the organisms from the field, depuration time and exposed to concentrations, when the mortality rate was lower than 50% (*C.edule*$_{Big}$: CTL, 0.35 mg/L and 0.60 mg/L; *C.edule*$_{Small}$: CTL, 0.35 mg/L, 0.60 mg/L and 0.90 mg/L; *S.plana*$_{Big}$: CTL, 1.00 mg/L, 1.50 mg/L, 2.00 mg/L; *S.plana*$_{Small}$: CTL, 1.00 mg/L, 1.50 mg/L, 2.00 mg/L, 2.50 mg/L, 3.00 mg/L and 4.00 mg/L).

A Tukey test was carry out to compare the enzymatic responses between the organisms from the field, the organisms submitted to a depuration period and the organisms exposed to copper sulphate concentrations and the control treatment under laboratory conditions. The significant differences between all conditions and treatments were marked by different letters.

Biomarkers analysis showed significant differences to both species and size classes with after the exposure to copper sulphate. In the case of *C. edule* (Figure 3), regarding to big

size class, at GSTs analysis were not observed significant differences among the treatments, only it was observed a little activity increase at 0.35 mg/L of copper sulphate, but no statistically significant. GR only exhibited significant differences at the first concentration (0.35 mg/L) of the chemical. Moreover, it was observed a gradual increase of the selenium dependent GPx activity, with significant differences at 0.6 mg/L of copper sulphate, with total GPx also presenting an increase at the activity with the concentration gradient, being observed significant differences at all conditions, except to the organisms under depuration period, when compared to the control treatment. TBARs levels were also evaluated and the organisms exposed to 0.35 mg/L of copper sulphate showed significant differences, revealing more lipid peroxidation on the organisms exposed at 0.35 mg/L of copper sulphate. Regarding to small size class, the organisms from the field and exposed to the higher concentration of copper sulphate (0.9 mg/L) exhibited significant differences in the GSTs activity. Additionally it was possible to observe a decrease of the GSTs activity with the increase of copper sulphate concentration, GR activity presented significant differences at the organisms from the field, under depuration period and exposed at the first concentration of copper sulphate, observing inhibition of the activity. Furthermore, at total GPx were not observed significant differences at any treatment, nevertheless selenium dependent GPx revealed significant differences only to the organisms exposed to 0.35 mg/L of copper sulphate, where it was exhibited a raise in the activity. Still, it was not observed significant differences at the TBARS.

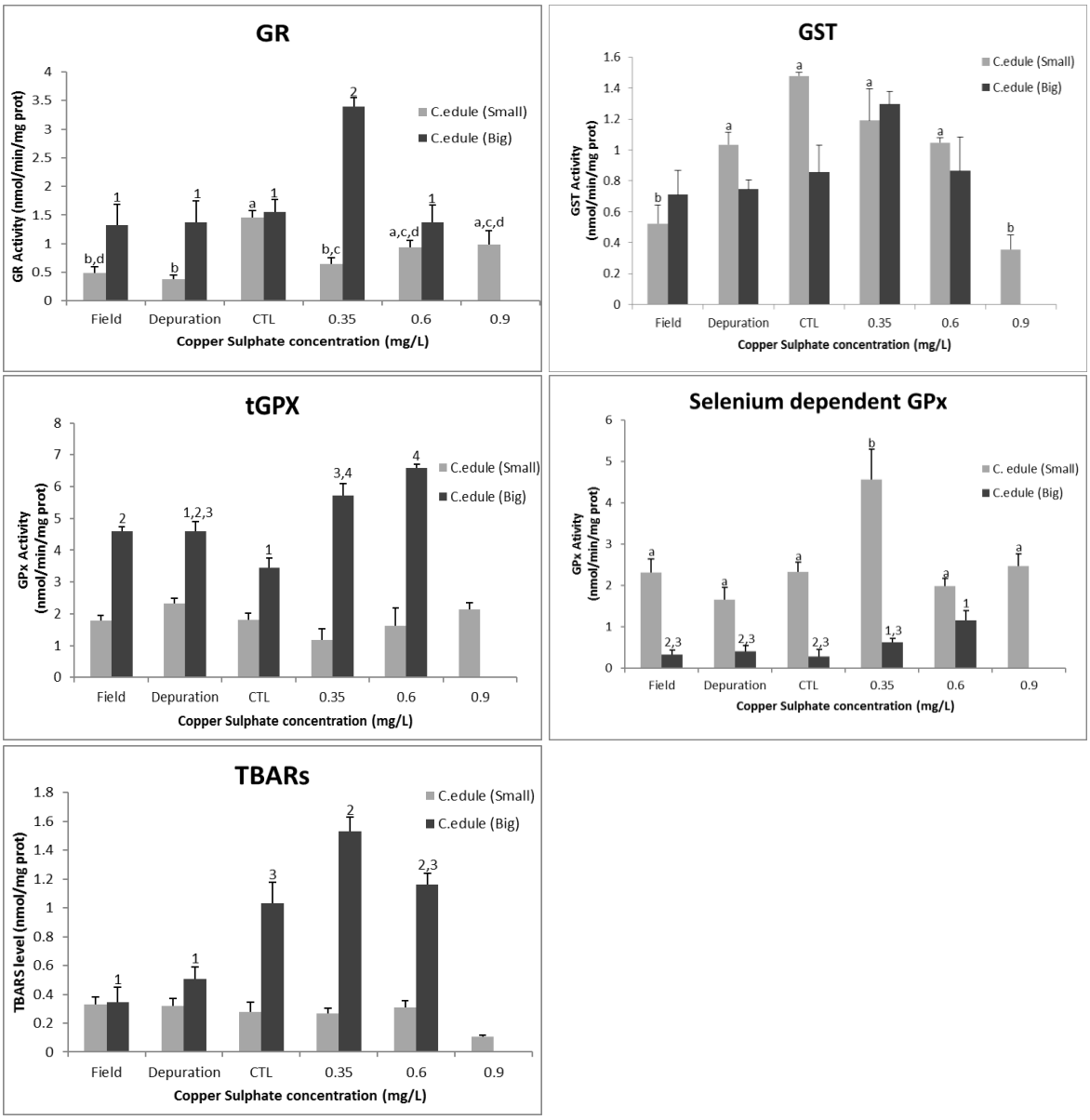


**Figure 3:** Glutathione S –transferase (GST), glutathione reductase (GR); total-glutathione peroxidase (tGPx), selenium dependent- glutathione peroxidase (SeGPx) and Tiobarbituric reactive species (TBARS), in the foot of *Cerastoderma edule* (big and small size classes) from the field, after depuration period, control and copper sulphate treatments under laboratory conditions. Error bars represent standard error and the similar enzymatic activity between the conditions (statistical significant difference to p value < 0.05) are express by equal letters (small size class) and numbers (big size class).

On other hand, considering *S. plana* (Figure 4), organisms of big size class showed a significant increase of the GST activity at the concentration of 1.0 mg/L of copper sulphate. At GR activity, at the concentration of 1.5 mg/L of the compound, it was also observed significant differences to the total GPx activity at the organisms from the field, under depuration period and exposed to 2.0 mg/L of copper sulphate. Moreover, it was noticeable an increase of the selenium dependent GPx activity and TBARS levels, suggesting lipid peroxidation. The small size class of organisms did not show significant differences to GSTs activity at any treatments, only showing a little inhibition up to the fourth concentration of the toxic; GR and selenium dependent GPx activities were affected only at the organisms from the field and sunder depuration period; nonetheless total GPx activity presented significant inhibition to all conditions, whereas the organisms exposed to 2 mg/L of copper sulphate showed a significant increase at TBARs levels.

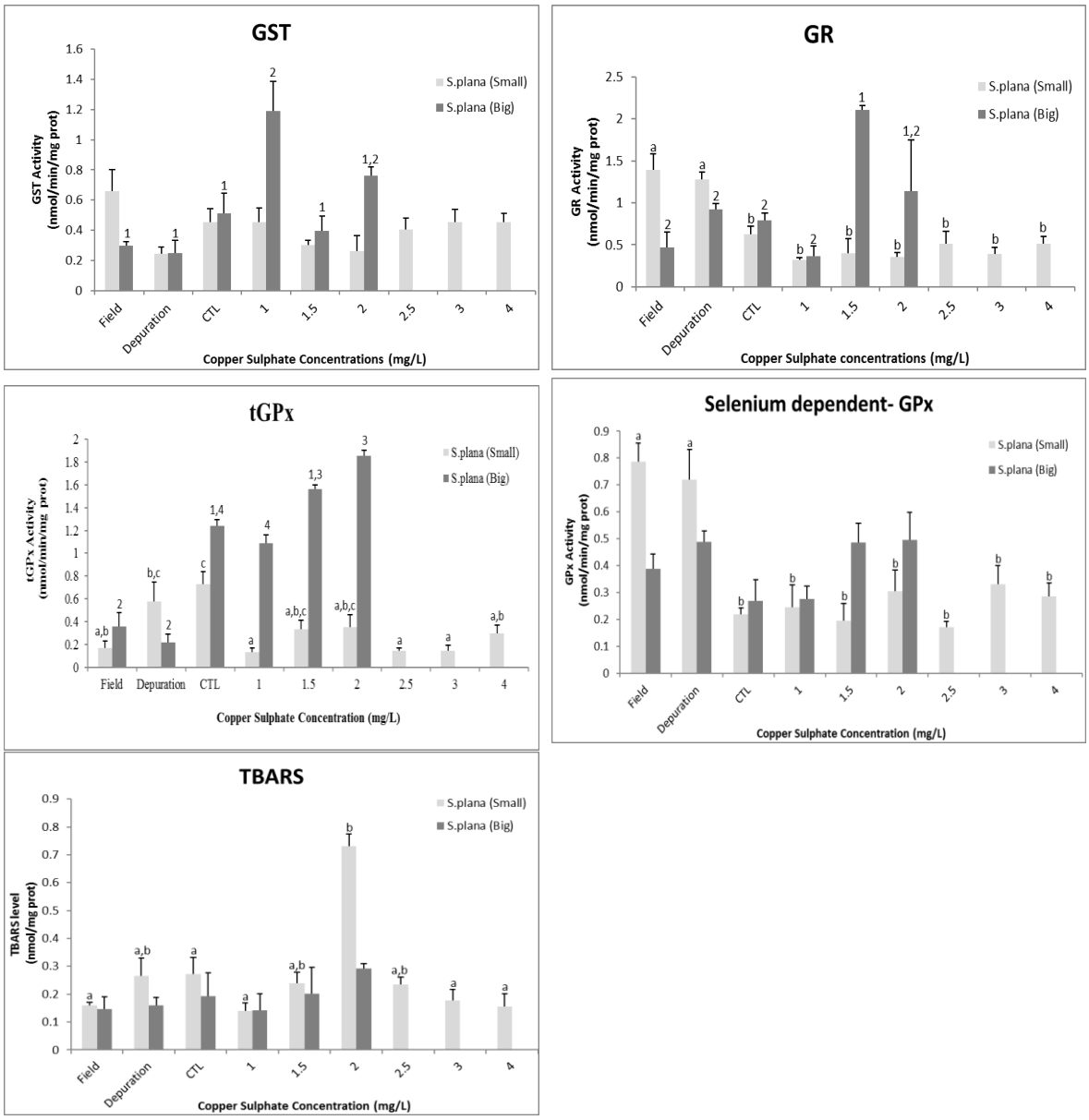


**Figure 4:** Glutathione S –transferase (GST), glutathione reductase (GR); total-glutathione peroxidase (tGPx), selenium dependent- glutathione peroxidase (SeGPx) and Tiobarbituric reactive species (TBARS), in the foot of *Scrobicularia plana* (big and small size classes) from the field, after depuration period, control and copper sulphate treatments under laboratory conditions. Error bars represent standard error and the similar enzymatic activity between the conditions (statistical significant difference to p value < 0.05) are express by equal letters (small size class) and numbers (big size class).

## 4. Discussion

This work highlights ecotoxicological and biochemical effects of copper sulphate in two bivalve species with small size class of both species showing a higher tolerance to the pollutant than the big size class. Furthermore, both size classes of *C. edule* exhibited a less tolerance to the compound than *S. plana*.

Many studies report the toxic effects of copper sulphate and copper on bivalve species. Satyaparameshwar et al. (2006) show a $LC_{50}$ to *Lamellidens marginalis* of 3.990 mg/L, after 72 hours of exposure to copper sulphate. Comparing with our studied organisms, *L. marginalis* show more tolerance to copper sulphate action than *Cerastoderma edule* (LC50 = 1.129 (0.968; 1.289) mg/L; LC50 = 0.818 (0.595; 0.987) mg/L, small and big size classes, respectively, as well as the organisms of big size class of *Scrobicularia plana* (LC50= 2.563 (2.229; 2.903) mg/L). Kováts et al. (2010) report a $LC_{50}$ of 18.9 (10.0–31.1) µg/L to *Anodonta anatina*, a $LC_{50}$ of 29.3 (25.4–34.0) µg/L to *Pseudanodonta complanata* and a $LC_{50}$ of 19.0 (16.2–22.0) µg/L to *Unio tumidus*, when exposed to copper. Sousa et al. (2005) exposed the gastropod *Nassarius reticulatus* to copper during 96 hours and determined a copper $LC_{50}$ value of 58.84 (35.45–82.29) µg/L. So, estimating the percentage of copper at copper sulphate, at the different treatments of our study, we may conclude that *S. plana* and *C. edule* are more tolerant to copper action than the bivalves and gastropod species reported at other studies. *Daphnia magna*, a standard species on ecotoxicological studies, was exposed by 96 hours to copper sulphate and exhibited a $LC_{50}$ = 0.0826 (0.0823; 0.0829) mg/L (Guilhermino et al., 2000), showing to be more sensitive to the compound action than the species here studied.

The behavioural activity results showed a decrease of the activity index along the copper sulphate concentrations to both size classes of *C. edule*, with the bigger organisms exhibiting often major activity index than the smaller organisms, except to the chemical concentrations 0.35 mg/L and 1.8 mg/L and the control treatment. Regarding to *S. plana*, this pattern was not observed. Moreover the small size class organisms exhibited greater activity index than the bigger ones at the copper sulphate concentrations of 1.00 mg/L, 2.50 mg/L, 3.00 mg/L and 4.00 mg/L.

Several studies showed that the exposure to the metal lead to the increase of reactive oxygen species (ROS) production in the cells (Novelli et al., 1998; Stohs and Bagchi, 1995) that can cause several cellular damages. Accordingly, copper toxicity may be due to

generation of ROS via Fenton or Haber-Weiss reactions (Letelier et al., 2005; Sun et al., 2009). So, regarding to the big size classes of both bivalve species, it was observed the activation of the enzymatic antioxidant defence system, suggesting an attempt of detoxification. The simultaneous increase of GR, GSTs and GPx activity may suggest the GSH increase, which in the presence of metals can be synthesized, helping to detoxification. However, the detoxification process did not prove effective, how is suggested by the increase of TBARS level, revealing the possible increase in lipid peroxidation on *C. edule* and *S. plana* organisms at 0.35 mg/L and 2.0 mg/L, respectively. So, once again *S. plana* seems to display more tolerance to copper sulphate than *C. edule*, since the lipid peroxidation occurs to a higher concentration. In the other hand, regarding to the small size classes, at *C. edule* was verified a statistically significant inhibition of GR activity and a decline trend of the tGPx activity, although not statistically significant. Moreover, the decrease of the GST activity along the concentrations, being this inhibition statistically significant at the highest concentration, suggests the reduction of the GSH availability. The SeGPx is part of a second defence line on the antioxidant system (Faria et al., 2009) and was not inhibited, contrariwise it increased significantly at the concentration of 0.35 mg/L, which may explain the efficiency in the fight against the lipid peroxidation, once it was not observed significant changes at the TBARS levels. *S. plana* only presented a statistically significant inhibition to tGPx activity to all concentrations, with the others antioxidants enzymes do not exhibiting significant changes compared to the control situation. Then the antioxidant defence system showed inefficient to protect the organisms against ROS action, suggested by the increase of lipid peroxidation at the concentration of 2.0 mg/L of copper sulphate, given the increase of TBARS levels at this concentration.

Although different responses were observed between the control and the organisms from the field and after the depuration period, these differences may be not be due only to the action of copper sulphate, but also to other stress factors not controlled in the field and at during the depuration period. In the field, the organisms are under the action of several contaminants, since in the Mondego estuary was reported the presence of Hg, Cu, Cd, Cr, and Zn, that may become from anthropogenic activities, such as diffuse sources and discharges of the Pranto tributary and higher levels of Fe, maybe associated to precipitation of Fe oxides (Pereira et al., 2005), which also affect the antioxidant defence system of aquatic organisms (Filimonova et al., 2016). Furthermore, a recent study by Gonçalves et

al. (2017), evaluating the effect of salinity on the antioxidant system of the two studied bivalve species *C. edule* and *S. plana*, showed alterations at the enzymatic activity level with the salinity changes. Similar results were observed by others authors to others species, such as to *Venerupis corrugata* (Carregosa et al., 2014), *Litopenaeus vannamei* (Li et al., 2008), and also to *S. plana* (Tankoua et al., 2011). On the other hand, the depuration process, often performed in sterile sea water, during 24 to 72 hours (Ramos et al., 2005) is also pointed out as able to induce traumatic and physiologic stress, leading to debility and death of the organisms (Ruano et al., 1998). Ruano et al. (2012) observed that depuration process, effectively induces stress at biochemical level on the bivalves' species, with consequences on the condition index and nutritional level.

Several studies evaluated the effects of copper on the antioxidant system of bivalve species, being found different and transitory responses on mussels exposed to metals in the field and under laboratory conditions (Regoli and Principato, 1995). Buffet et al. (2011) reported to *S. plana*, exposed to nanoparticules of copper oxid (CuONP) during 16 days, a significant increase of GST activity, trend also observed in this work to the big size class of both bivalve species, suggesting oxidative stress endured by the organisms, although were not observed changes at the TBARS levels under laboratorial conditions (10 µg Cu/L). Regarding to other bivalve species, Zhang et al. (2010), did not report significant changes on the GST and GPx activities to *Chlamys farreri* exposed to 3 µg Cu /L, during 96 hours. However, according De Almeida et al. (2004), when *Perna perna* was exposed to 40 µg/L of copper sulphate, during 120 hours, after 72 hours of exposure was observed a depletion of GSH levels, despite GPx and GST not exhibiting significant changes. Moreover, Canesi et al. (1998) also reported a decrease of the GSH levels on *Mytilus galloprovincialis* exposed to 40 µg Cu/L after 72 hours. According the above cited studies, it is often observed a decrease of GSH levels, indicating a possible effect of the metal exposure. Despite the antioxidant enzymes do not exhibit significant changes, this absence of significant effects may be due to the low concentrations used at previous studies. Moreover, different tissues may exhibit different responses to metal exposure (Ahmad et al., 2011; Maria and Bebianno, 2011). Considering longer exposure times, Maria and Bebianno (2011) observed significant increase of LPO on *Mytilus galloprovincialis* up to 200% in the gills. The lipid peroxidation in *M. galloprovincialis* was also observed after 6 days at concentration of 40 µg Cu/L (Viarengo et al., 1996, 1990). Furthermore, to

*Ruditapes decussatus* exposed to 2.5 µg Cu/L after 28 days was also reported an increase of LPO (Geret et al., 2002).

## 5. Conclusions

The present work highlights the use of *C. edule* as a good bio-indicator species and the antioxidant enzymatic activities as good biomarkers to detect the presence of copper sulphate in aquatic systems. Furthermore, this study also reveals muscle (foot) as a good tissue to determine changes at the activity of antioxidant enzymes and the occurrence of lipid peroxidation. Although it is not usual the use of tissue to evaluate the effects on the antioxidant system, with the gills and the digestive gland being the tissues commonly used at this analysis due to the higher activity of the antioxidant enzymes at these tissues, the muscle may store greater lipid content. Thus, it may be established a closer relationship between the changes of the antioxidant enzymes activity, lipid peroxidation and alterations on the lipid profile in the muscle tissue to assess the biochemical effects of pollutants in bivalve species.

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# Chapter IV: General Discussion and Conclusions

## General Discussion and Conclusions

This study highlights both size classes of *S. plana* are more tolerant to copper sulphate action than *C. edule*, although *S. plana* exhibit a nutritional and behavioural activity patterns less coherent than *C. edule* in the presence of the pollutant. Furthermore, considering the results obtained in this work, big size class organisms of *C. edule* show a faster response to copper sulphate than the remaining classes and species here considered. Moreover, the analyses of fatty acid profile and the determination of the enzymatic activity revealed to be good biomarkers to stress conditions, namely to the toxic. Furthermore, since *C. edule* and *S. plana* are species with commercial interest and greatly appreciated as food source, it is quite important to determine and assess the potential effects of chemicals daily discharged in the ecosystem in the organisms and determine possible changes in their nutritive value due to the presence of toxicants in concentrations equal or above those where effects may occur. Ecotoxicological studies are of great importance to determine the effects of toxicants, with a tendency to be more often this works due to the higher usage at the past years of more pesticides mainly in agriculture practices, with copper at the based formulations. Copper sulphate helps to control pests and so the food production, with extreme importance due to the growth of human population. Still, this compound comprises negative implications to the environment, mainly to the aquatic ecosystems, becoming crucial to develop new tools, environmental friendly, to avoid the widely usage, sometimes in an indiscriminate way, of chemicals at the intensive agriculture practices (Roberts, 2009). Several authors have studied the effects of toxicants on species from different trophic levels and it has been reported a great damage at biochemical (De Carvalho, 2012; Filimonova et al., 2016a, b; Maria and Bebianno, 2011; Pinto et al., 2011; Rocchetta et al., 2006) and ecotoxicological levels of the individual and also at the communities (Guilhermino et al., 2000; Satyaparameshwar et al., 2006). Copper sulphate, a based copper chemical, exhibited negative effects to the species here studied at lethal and sub-lethal levels. At a sub-lethal level was observed a decrease of the activity index on *C. edule* and *S. plana,* to both size classes, when exposed to copper sulphate. Regarding to the biochemical effects, namely the changes on the fatty acids profile, *S. plana* showed to be more tolerant to the compound action than *C. edule,* and the big size class of the organisms was more affected than the small size class. A similar pattern was observed to the enzymatic activity. Copper sulphate showed to induce changes at the fatty acids profile on

the two bivalve species, being observed a decrease of the saturated (SFA) and unsaturated fatty acids (UFA). Regarding the big size class of both bivalve species, was observed a distinct trend with a decrease of SFA and monounsaturated fatty acid (MUFA); considering the small size class, were observed an increase of SFA and MUFA and a decrease of polyunsaturated fatty acids (PUFA) and highly unsaturated fatty acids (HUFA), with implications at their nutritional value and with repercussions to the trophic food web (as demonstrated at chapter 2). Moreover several fatty acids are associated to performance and reproductive success, so their deficiency may cause many disturbs (Filimonova et al., 2016 a,b; Gonçalves et al., 2017). According to the literature, C14:0, C16:0, C16:1n7, C18:1n7 and C18:1n9 are reported as fatty acids related to the embryonic development of the bivalve eggs, as well as, C18:3n3, C18:4n3, C20:1n9 and C20:2n6 are involved in the oocytes maturation of bivalves species (Baptista et al., 2014; Ojea et al., 2004). In the present study was observed a decrease of the FAs related to the embryonic development and the oocyte maturation on both species and size classes. Regarding *C. edule* (big size class), it was observed a decrease of C20:1n9 and C20:2n6 along the range of concentrations, whereas C18:3n3 was present only on the organisms from the field; considering the small size class, it was observed a decrease of C20:1n9 and C20:2n6 and an increase of C18:3n3 with the raise of copper sulphate concentrations. In the other hand, regarding *S. plana* (big size class), C20:1n9 was observed only at the control treatment, while a decrease of C20:2n6 was detected along the compound concentrations. Moreover, the small size class presents a decrease of C18:3n3, whereas C20:2n6 was observed only in the organisms of the field and C20:1n9 only in the organisms of the field and at the highest concentrations of the toxicant (2.5 mg/L and 3.5 mg/L). So, *S. plana* seems to be more affected than *C. edule* to the presence of the chemical, with regard to the reproductive success, since the abundance and diversity of the main FA related to this parameter are lower in *S. plana* than in *C. edule*. Still, HUFA (where are included the essential fatty acids – DHA and EPA) maintained dominant on the profile along the range of toxic concentrations to both bivalve species and size classes. Considering the results here presented, it may be inferred that *S. plana* may keep their survival and health at the cost of the reproductive failures, contrary to *C. edule*. In addition, and as expected, the antioxidant system defence was also affected by copper sulphate exposure, as verified at chapter 3 with changes on the activity of some antioxidant enzymes, such as GST, GR,

GPx, and the occurrence of lipid peroxidation (observed across the increase of TBARS levels). Regarding to *C. edule* was observed an increase of the tGPx activity, an antioxidant enzyme that hydrolyses $H_2O_2$ into $H_2O$, by the reduction of the oxidized glutathione. It was also observed an increase of the GR activity, and of the TBARS levels (at the first concentration) to the big size class, with the small size class just showing a gradual inhibition of the GST activity. In the other hand, *S. plana* showed an increase only on the GR and GST activities to the big size class. In this case the antioxidant system showed to be efficient, since was not observed an increase of the TBARS levels. Still, in the small size class was only observed an increase at the TBARS levels at the concentration of 2.0 mg/L, without alterations on the antioxidant enzymes' activity, which may reveal an incapacity of the organisms to recognize or to act against the xenobiotic, and detoxify. In summary, metal ions are able to interact with several cell structures, changing their properties, and in case the antioxidant system defence be inefficient, the lipid peroxidation may occur by the generation of free radicals and others reactive species. So, metal ions may cause negative effects at the metabolism of lipids, with consequent alterations at the lipid and fatty acid composition. The fails at the antioxidant system defence, and the consequent fatty acid changes, may led to death, as observed at the higher concentrations, reaching a mortality rate of 100% to both size classes of *C. edule*.

The biomarkers here determined showed be good endpoints in ecotoxicological studies to determine and evaluate the effect of copper sulphate on the bivalve species. Moreover, at chapter 3, was used the muscle tissue (foot) to determine the activity of antioxidant enzymes and the occurrence of lipid peroxidation. The usage of this tissue to evaluate the effects on the antioxidant system is uncommon, often are used the gills and digestive gland, where exist more activity of antioxidant enzymes, however, once we observed that may be detected changes on the antioxidant enzymes activity on the muscle tissue, and this tissue may store greater lipid content, it is possible to establish a closer relationship between the changes of the antioxidant enzymes activity, lipid peroxidation and alterations on the lipid profile. Moreover, the usage of lower quantity of biomass to biomarkers analysis may allow a more cost-effective methodology with less quantities of reagents. More research studies to evaluate the effects of chemical compounds in aquatic ecosystems and at the biological communities should be conducted, namely with species with higher socioeconomic value such as mollusc, crustacean or fish species that may be used as food

source of human beings, to reduce and limit the overuse of these chemicals and so the impacts to the ecological status of these ecosystems and repercussions along the trophic web and to the health of human beings.

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