Universidade de Aveiro Departamento de Biologia

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Metallothionein functions: metal chelation and antioxidant activity

Funções das Metalotioninas: quelação metálica e atividade antioxidante

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada – ramo Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Etelvina Figueira (Professora auxiliar do Departamento de Biologia da Universidade de Aveiro) e da Doutora Rosa Freitas (Investigadora Auxiliar do Departamento de Biologia e CESAM, Universidade de Aveiro).

Dedico este trabalho aos meus pais que me proporcionaram esta oportunidade.

Para ser grande, sê inteiro: nada
Teu exagera ou exclui.

Sê todo em cada coisa. Põe quanto és
No mínimo que fazes.

Assim em cada lago a lua toda
Brilha, porque alta vive.

Ricardo Reis

o júri

presidente

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key words ***Cerastoderma edule*, Cadmium, Hydrogen Peroxide, Metallothioneins, Oxidative Stress, Metal detoxification**

abstract

It is generally accepted that the principal roles of metallothioneins (MTs) lie in the detoxification of toxic metals and regulation of the metabolism of essential trace metals. However, there is increasing evidence that it can act as a free radical scavenger. Although the great number of studies on the antioxidant activity of MTs, the effective physiological role of this protein is still unclear. In order to understand the role of MTs in the protection against metal contamination and oxidative stress, the bivalve *Cerastoderma edule* was used to evaluate the response of MTs in both situations. Cadmium, a widely reported MT inducer, was used to simulate metal contamination whereas H_2O_2, an oxidizing compound, was used to simulate oxidative stress. In the first approach, cockles were exposed to a range of Cd and H_2O_2 concentrations and MTs and TBARS were quantified. Results showed that both treatments induced MT synthesis, confirming the involvement of MTs in metal contamination and oxidative stress. Indeed, the use of MTs as biomarkers for metal pollution was questioned due to the similar synthesis of MT in the two highest concentration used. At last, one concentration of Cd (10 µM) and of H_2O_2 (20 µM) were selected and cockles were exposed again. TBARS concentration and the intracellular amount of H_2O_2 were determined. Metal-MT complexes in the two conditions and control were isolated by size exclusion chromatography, and the binding of Zn and Cd to MTs and other cytosolic proteins was evaluated. Furthermore, MTs were quantified and their content in each treatment and control was compared to the amount of Zn associated to them. Results showed that the >H2O2 treatment induced high levels of oxidative stress, demonstrated by the high lipid peroxidation and intracellular concentration of H_2O_2. Data also indicated that Cd was mainly associated with MTs pool in the Cd treatment, confirming that the protective role of MTs in metal contamination in this bivalve species was due to the binding of MTs to Cd ions. Additionally, the percentage of Zn bound to MTs decreased in the H_2O_2 treatment, indicating Zn release in oxidative stress. Also, MTs molecules were not as metalated as in the control, confirming Zn release from MTs in oxidative stress and indicating that MTs were needed for demands other than Zn distribution. Further studies on the redox status of MTs are needed to determine the redox status of MTs in the oxidative stress, and understand if, in this bivalve, MTs are acting as ROS scavengers.

palavras-chave ***Cerastoderma edule*, Cádmio, Peróxido de Hidrogénio, Metalotioninas, Stresse Oxidativo, Desintoxicação de Metais**

resumo

Os papéis geralmente associados às metalotioninas (MTs) resumem-se á desintoxicação de metais tóxicos e à regulação do metabolismo dos metais essenciais. No entanto, existem evidências cada vez mais acentuadas de que as MTs atuam na proteção contra o stresse oxidativo. Apesar do grande número de estudos que se focam na actividade antioxidante das MTs, o papel fisiológico efetivo destas proteínas não foi ainda clarificado. A fim de compreender o papel das MTs no stress oxidativo e na proteção contra o efeito dos metais, o bivalve *Cerastoderma edule* foi selecionado neste estudo para avaliar a resposta das MTs em ambas as situações. O cádmio, um forte indutor das MTs foi usado para causar contaminação metálica enquanto o H_2O_2, sendo um composto oxidante, foi usado para provocar stresse oxidativo. Numa primeira abordagem, os berbigões foram expostos a uma gama de concentrações de Cd e H_2O_2 e as MTs e os TBARS foram quantificados. Os resultados mostraram que apenas o H_2O_2 provocou peroxidação lipídica no berbigão e que ambos os tratamentos induziram a síntese de MTs, confirmando o envolvimento destas na contaminação metálica e no stresse oxidativo. De facto, a utilização das MTs como biomarcadores de poluição metálica foi neste estudo questionada devido á síntese de quantidades semelhantes de MTs nas duas concentrações mais elevadas de Cd e H_2O_2. Numa segunda abordagem, os berbigões foram novamente expostos a uma concentração selecionada para cada tratamento (10 µM de Cd e 20 µM de H_2O_2). A concentração dos TBARS e a quantidade intracelular de H_2O_2 foram determinados. Os complexos metal-MT em ambas as condições e no controlo foram isolados por cromatografia de exclusão molecular e a ligação entre os iões de Zn e Cd e as MTs e outras proteínas citosólicas foi avaliada. Para além disso, As MTs foram quantificadas em cada tratamento e no controlo, sendo o seu conteúdo comparado com a quantidade de Zn ligado. Os dados indicaram que o tratamento com H_2O_2 induziu elevados níveis de stresse oxidativo, demonstrado pela elevada peroxidação lipídica e pela grande concentração intracelular de H_2O_2. Relativamente aos resultados da cromatografia, os iões de Cd estavam principalmente ligados às MTs no tratamento com Cd, confirmando o efeito protector das MTs na contaminação metálica nesta espécie de bivalve. Adicionalmente, a percentagem de Zn ligado às MTs diminuiu no tratamento com H_2O_2, indicando que o stresse oxidativo impõe a libertação de Zn por parte das MTs. Em jeito de confirmação, as MTs estavam menos metaladas no tratamento com H_2O_2 do que no controlo. Seriam necessários estudos complementares para perceber se neste bivalve as MTs actuam como eliminadoras de ROS.

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CHAPTER 1

1. Introduction

1.1 Contextualization

Facing chemical stress, each organism has a set of processes that makes possible to maintain their physiological homeostasis. The excess of metals and reactive oxygen species (ROS) are factors that disturb the normal functioning of organisms. Essential metals like Zn and Cu are involved in numerous important physiological processes, whereas non-essential metals such as Cd generally have no known biological function. Nevertheless, both types of metals can be toxic when concentrations surpass a critical level. Similarly, when the production of ROS is above a certain limit, cells can be damaged by oxidative stress (Manduzio *et al*., 2005; Wang and Rainbow, 2010). These two situations have been linked to the existence of specific metal-binding proteins termed as metallothioneins (MTs). MTs, first reported five decades ago (Margoshes and Vallee, 1957), are cysteine rich, low molecular weight, ubiquitous intracellular proteins with high affinity for metals. They are phylogenetically widespread, being found in bacteria, fungi, plants and animal species (Coyle *et al*., 2002). Some of the hypotheses advanced for the role of MTs are that these proteins, (1) participate in Zn and Cu homeostasis; (2) are involved in the detoxification of toxic metals such as Hg and Cd; and (3) may protect against ROS and reactive nitrogen species (RNS) (Sutherland and Stillman, 2011).

This work was designed to clarify the physiological role of MTs as metal chelators and as oxidative stress protectors. Cadmium, as one of the most dangerous pollutant, was used to create metal contamination whereas hydrogen peroxide (H_2O_2), being an oxidizing compound, was used to impose oxidative stress. *Cerastoderma edule* was selected for this study because its use as a sentinel organism had been suggested in environmental pollution biomonitoring studies (Lobo *et al*., 2010). Furthermore, the induction of MTs by metals and by oxidative stress must be evaluated in order to ascertain if the use of MTs as metal-specific biomarkers is correct. Additionally, if MTs are induced by oxidative stress, it is important to know their role in this condition. As MTs have high affinity for metals, we proposed to evaluate the role of MTs in respect to the binding of Zn (the most important essential metal in the redox homeostasis) and Cd. This procedure allows to understand if, in

oxidative stress, the role of MTs is to bind Zn and behave as a metal chaperone, or is to react directly with ROS and neutralize them.

To facilitate the reading, this master thesis is structured into four chapters. A general introduction is given in the Chapter 1, where the characteristics, functions, structure, genes and application as biomarker are described. The importance of choosing *C. edule* as the experiment organism is also focused in this chapter. The investigation was performed in two stages that correspond in this thesis to two chapters: In the first stage (Chapter 2) MTs synthesis in organisms submitted to different levels of Cd and H_2O_2 was determined and the level of oxidative stress imposed by each condition was also evaluated. The results obtained in this stage provided the information needed to select Cd and H_2O_2 concentrations to proceed to the second stage (Chapter 3). The objective of this chapter was to understand the role of MTs in the protection against Cd and oxidative stress, through the quantification of the Zn and Cd bound to MTs in each condition: metal or oxidative stress. Finally in Chapter 4 general conclusions and considerations about the investigation are presented, showing the limitations of the work and future perspectives.

1.2 Metallothioneins

1.2.1 General Features

Metallothioneins (MTs) are a superfamily of intracellular metalloproteins with remarkable characteristic features. They are low-molecular weight (6-7 kDa), cysteine-rich, metal-binding proteins with heat stability and no aromatic amino acids or histidine. It is generally accepted that all MTs have a set of features in common, which differentiate them from other proteins (Table 1). The cysteinyl residues constitute about one-third of the constituting amino acids and their thiol groups (-SH) serve as ligands for metal chelation, being these characteristics the reasons for the choice of their name (Sato and Kondoh, 2002; Amiard *et al.*, 2006; Sutherland and Stillman, 2011). Their synthesis can be induced by a wide variety of metal ions, such us Cd, Cu, Zn, Hg, Co, Ni, Bi, and Ag. These unique biomolecules have captured the attention of biologists and chemists due to their remarkable chemical structure that confers a degree of specificity, stability and dynamic behaviour (Coyle *et al*., 2002). Besides of being present in the cytosol of cells, in mammals MTs can also occur in extracellular media such as blood plasma and cerebrospinal fluid (Blindauer and Leszczyszyn, 2010). MTs are the major Zn binding intracellular thiol and in many cases represent the single most abundant intracellular protein thiol (Miles *et al*., 2000). These metalloproteins occur not only throughout the all animal kingdom, but also in other eukaryotes such as plants, yeasts and phylamentous fungi and in prokaryotic microorganisms like cyanobacteria (Kägi, 1993).

The alignment of Cys-Cys, Cys-X-Cys and Cys-X-Y-Cys sequences, where X and Y are amino acids other than cysteine, is characteristic of all MTs, constituting the criterion that allows the distinction between different MTs families and that leads to many different isoforms of the same protein (Miles *et al.*, 2000; Amiard *et al.*, 2006).

Table 1. Characteristic features of metallothioneins (adapted from Blindauer and Leszczyszyn (2010) and Nordberg (1998))

1	Low molecular weight (<10 kDa)
2	High metal and sulfur content (>10 %)
3	Absence or scarcity of aromatic amino acids
4	Spectroscopic features typical of metal-S bonds: absorption at 250, 225 and/or 275 nm (for Cd, Zn and Cu, respectively)
5	Heat stability (70ºC)
6	Unique amino acid sequence
7	Apo-MT form lack secondary structural elements because the tri-dimensional structure is dictated by binding to metals

1.2.2 Historical review

Until now, more than 2500 references to MTs or MT-like proteins from very different organisms were submitted to GenBank database (http://ncbi.nlm.nih.gov/) but the scientific history of these proteins started at almost 55 years ago when Margoshes and Vallee (1957) found a Cd binding protein in equine horse kidney tissues. Few years later, the same authors reported for the first time the name "metallothionein" due to the extremely high content of sulphur, Cd and Zn (Kägi and Vallee, 1960). Studies on the physico-chemical properties of MTs were performed during the next years by several groups, which estimated the molecular weight, the amino acid composition, the sedimentation and diffusion constants, the partial specific volume, the friction ratio and the metal content (Nordberg, 1998). The first health studies on animals gave support to the idea that MTs induction is a mechanism that fights against Cd toxicity (Klaassen *et al*., 1999). In 1982, a feature of the tertiary structure was described: MTs were constituted by two metal clusters, α and β-domains with four and three Cd ions, in the C- and N-terminals, respectively (Winge and Miklossy, 1982). After that, the investigation on MTs expanded for other metals such as Cu and Mg. Yet in the 90's, MTs started to be related to tumours due to its

expression in cancer cells, and since there, they were identified in human tumours of the kidney, breast, lung, nasopharynx, salivary gland, ovary, testes, urinary bladder, leukemia, and non-Hodgkin's lymphoma (Cherian *et al*., 2003). Currently, according with a research done by Capdevila *et al*. (2011), 12350 publications exist. Furthermore, there have been four international meeting focused on MTs, excellent books and book chapters appeared, special issues in scientific journals and more than 100 reviews were written on this subject, demonstrating the great interest that MTs have attracted since their discovery. The same authors compared the number of publications in each decade since the 50's and concluded that, after a stationary phase during 15 years (starting in the 90's) when the number of published papers was maintained similar, there was an increased interest on MTs between 2009 and 2010 with a average of about 600 articles per year. Mammalian MTs have been receiving the most attention, while less work have been carried on MTs from other organisms (Capdevila *et al.*, 2011).

1.2.3 Nomenclature of MTs

Many of the proteins isolated from non-mammalian vertebrates and invertebrates are different from the typical mammalian MTs, the first to be isolated. When the wide variation in chemical structure became apparent, MTs were subdivided into three classes, in a "vertebrate-centric" viewpoint (Fowler *et al*., 1987). According to the recommendations of this first attempt of classification, Class I includes all proteinaceous molecules related in primary structure to equine renal MT, i.e., the location of cysteine residues should be close similar to mammalian MT-1 and -2; Class II comprises forms displaying none, or only a very distant correspondence to the mammalian forms, such as the MTs from the sea urchin and yeast; finally, Class III consists in non-proteinaceous MTs now recognized as Phytochelatins, which are enzymatically synthesized oligopeptides constituted by γ-glutamyl-cysteinyl units that had been isolated from plants (Grill *et al*., 1985), microorganisms such as *Euglena gracilis* (Weber *et al*., 1988), *Schizosaccharomyces pombe* (Reese *et al*., 1988) and algae (Gekeler *et al*., 1988).

Almost 15 years later, given the increasing number of MTs sequences, this kind of nomenclature became inappropriate. The length of MTs sequence, their amino acid composition and the number and repetitions of the cysteine residues showed to be very

variable and should not be kept as criteria of classification. Additionally, with the number of gene sequences that have become available, the functional properties of MTs can be previewed, providing new classification criterion. Thus, with the aim to better differentiate MTs, a new classification based on sequence similarities and phylogenetic relationships was established. Next to Binz and Kagi (1999), all MT proteins (the older class I and II) are a super-family that is subdivided into families, subfamilies, sub-groups, and isoforms. The MT super-family is composed by all polypeptides which resemble equine renal MT, i.e., those that have a low-molecular weight, a high metal content, a high cysteine content and none or few aromatic amino acids residues, an unique amino acid sequence with characteristic distribution of cysteines and spectroscopic manifest cations characteristic of metal thiolate clusters. A MTs family includes MTs sharing a specific amino acid sequence that are able to be aligned and that are thought to be evolutionary related. A MTs subfamily corresponds to MTs inside one family that posses more restricted features. Currently, this careful classification system is not used in the literature or in databases, which use the later system to refer MTs isoforms. Another criterion based on stoichiometric, spectroscopic and spectrometric features was proposed (Valls *et al.*, 2001) differentiating two categories: Zn-thioneins and Cu-thioneins, those MTs that strongly bind Zn or Cu, respectively.

1.2.4 MTs functions

The primary biological function of MTs remains an enigma, being their biological functions a subject of controversy. However, given their metal-binding capacity, MTs are undoubtedly involved in processes associated with metal metabolism, which importance depends on specific requirements of the organism (Stegeman *et al.*, 1992; Coyle *et al.*, 2002). Other evidence that shows how important MTs are, is their ubiquity and conservation, which suggests that they participate in essential processes in the cell (Amiard *et al.*, 2006). Mason and Jekins (1995) proposed two roles for MTs: first they comprise a non-toxic Zn and Cu reservoir, available for the synthesis of metalloenzymes, fulfilling enzymatic demands and allowing the homeostasis of many cellular processes; and second, they can reduce the non-specific binding of non-essential metals within cells, alleviating their toxic potential. Resuming, MTs play central roles in metal detoxification, regulation

of Zn and Cu, and donation of metals to metalloproteins (Stegeman *et al*., 1992). Later, other roles for MTs had been demonstrated by several authors. In fact, the analysis of their promoter sequences, which contain response elements for other stimuli besides metals, for example, glucocorticoids, ROS, and cytokines, confirming the involvement of MTs in more than one cellular process (Davis and Cousins, 2000). For instance, Cai *et al*. (1999) suggested a protective activity against ionizing radiation and other authors demonstrated a more general antioxidant role for MTs (Viarengo *et al.*, 1999; Cavaletto *et al.*, 2002; Correia *et al*., 2002; Gagné *et al*., 2008).

In mammals MTs have been considered to have anti-inflammatory, anti-apoptotic, antioxidant, proliferative, and angiogenic activities, and to be also involved with resistance to chemotherapy. Additionally, the role of MTs in Zn and Cu homeostasis is crucial. Copper is implicated in a variety of neurodegenerative diseases, and Zn, because is involved in the activity of more than 300 enzymes and in the regulation of gene expression, has an important role in immunity, signalling, cancer, and aging. So Cu and Zn dysregulation has been identified as a component of several types of injury. Thus, considering that metal metabolism is altered and ROS are produced during most pathological disorders, including neurodegenerative diseases and senescence, it is not surprising that MTs have been associated with several diseases and conditions (Carpenè *et al.*, 2007; Blindauer and Leszczyszyn, 2010).

Regarding organisms from groups distant from mammals, for example in gastropods possessing haemocyanin as respiratory oxygen Cu transporter, a MT isoform is responsible for Cu homeostasis in cells that synthesize this respiratory protein (Dallinger *et al*., 2005).

Knock-out organism models have been demonstrated that MTs absence is not lethal, nor greatly deleterious in normal conditions. However, in stressing conditions, the lack of MTs genes resulted in reduced metal tolerance, higher sensitivity to oxidative stress and a tendency to worsen support inflammatory and infection (Coyle *et al*., 2002).

At last, a very recent review elegantly resumes the facts about MTs functions(Capdevila *et al.*, 2011). In true, scientific community has been trying to find a biological role for MTs. The problem is that there is no single biological role for MTs because they are implicated in tens of physiological processes, and their functions vary from organism to organism and even between isoforms. According to these authors, the

unique way we should look at this subject is: MTs have two precise molecular functions that are involved in a dozen of different biological processes. These two molecular functions are metal binding and redox activity (Capdevila *et al*., 2011).

1.2.5 Structure of MTs

The general structure of all metallothioneins is resumed to the formation of metal-thiolate clusters involving terminal and bridging cysteinyl thiolate groups. The arrangement of the cysteine residues in the chain and the coordination specificities with the metals are the base for the tri-dimensional structure of the protein. The metal-free protein, also known as thionein or apoMT, appears to possess a predominantly disordered structure, or better, a random coil with no secondary structural features. However, upon the binding of metal ions to apoMTs, a well defined protein fold develops (Romero-Isart and Vašák, 2002). Mammalian MTs are single-chain polypeptides of 61 to 68 amino acid residues, comprising 20 cysteine residues (Figure 1) that interact with metals (monovalent group 11 and divalent group 12) to form thiolates (Coyle *et al*., 2002).

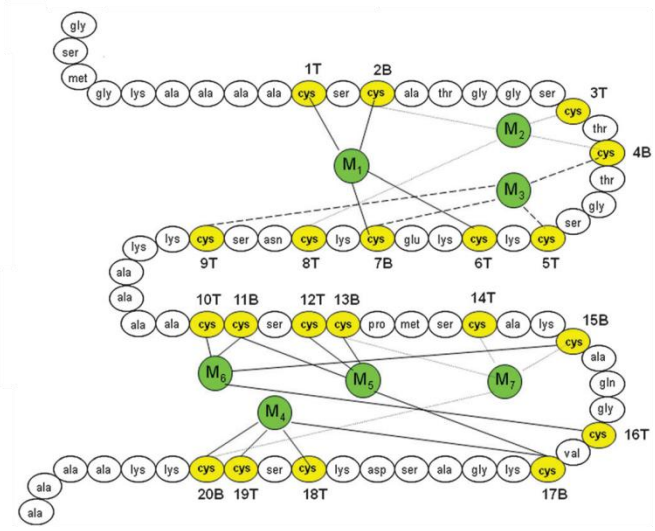

Figure 1. Primary structure (amino acid sequence) of human MT-1. Cysteine residues are represented in yellow and the two characteristic β and α domains are at the top and at the bottom, respectively. The diagram shows how each one of the seven metals (represented by M) is bound to four cysteine residues, giving place to the tetrahedral arrangement. Bridging and terminal cysteine thiolates are indicated by a B or T, respectively. Adapted from Sutherland and Stillman (2011).

The first elucidated tri-dimensional MT structure was determined from the mammalian MT-2 by NMR and X-ray crystallographic methods. Robbins *et al*. (1991) determined the X-ray structure of MT-2 isolated from rat liver overloaded with Cd (Figure 2). The protein did not contain aromatic amino acids and formed a monomeric pseudosymmetrical, dumbbell-shaped molecule, composed by two independent domains. The β-domain located in the N-terminal of the molecule was composed by 9 cysteine residues which bind 3 metal ions and the α-domain, in the C-terminal comprised 11 cysteine residues bound to 4 metals. The two domains are connected by a flexible hinge region composed by a conserved Lys-Lys segment named linker. Zangger *et al*. (1999) reported that the structure of MT-1 from recombinant mouse was highly similar to that of MT-2, although having a slight difference in the β-domain flexibility. Contrarily to the divalent Cd or Zn containing MTs, in the monovalent Cu containing MT-1 and -2, the metal is coordinated by two or three cysteine ligands, originating digonal and trigonal geometries (Stillman, 1995).

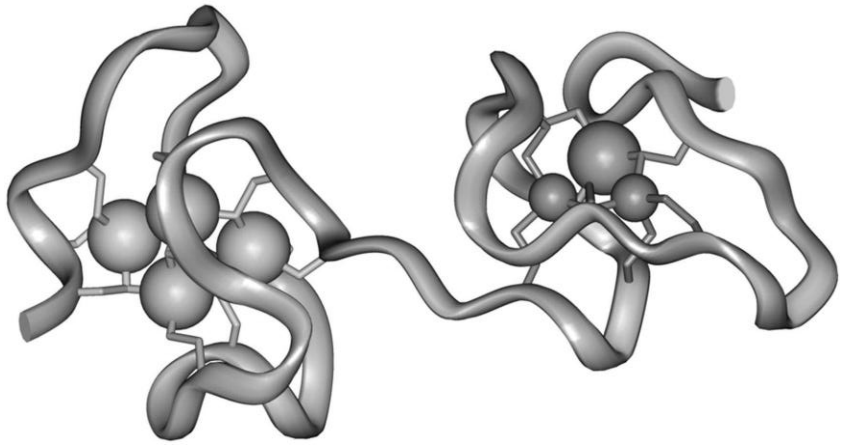

Figure 2. Crystal Structure of Cd_5, Zn_2-MT-2 from the rat liver. The MT is composed by two domains, the N-terminal β domain (in the right) and the C-terminal α domain (in the left). The two domains enfold, in this case, ions of Cd and Zn. The β domain binds two atoms of Zn and one atom of Cd with nine cystein residues while the α domain binds four atoms of Cd with eleven cysteine residues. Adapted from Romero-Isart and Vašák (2002).

Invertebrate MTs tri-dimensional structures have also been described. For this, the first tri-dimensional structures of invertebrate MTs were determined using the crustacean blue crab, *Callinectes sapidus* (Narula *et al*., 1995) and the equinoderm sea urchin, *Strongylocentrotus purpuratus* (Riek *et al*., 1999) both with Cd-containing MT-1. These structures resemble mammalian MTs because they are monomeric proteins with two globular domains that posses a metal-thiolate cluster each. Crustacean MT-1 contains two thiolate-clusters with three Cd ions each. Instead, the sea urchin protein structure is composed by one four-metal cluster and one three-metal cluster in a reverse orientation when compared to mammalian MTs, i.e., the cluster composed by 11 cysteine residues bound to four Cd ions located in the N-terminal domain, and the cluster composed by 9 cysteine bound to three Cd ions is located in the C-terminal of the protein (Figure 3). In this protein, unlike mammalian and crustacean MTs, the cysteine-metal coordination bonds have different connectivity pattern, the backbone of the polypeptide has unique local folds and the protein has an aromatic amino acid in the C-terminal domain.

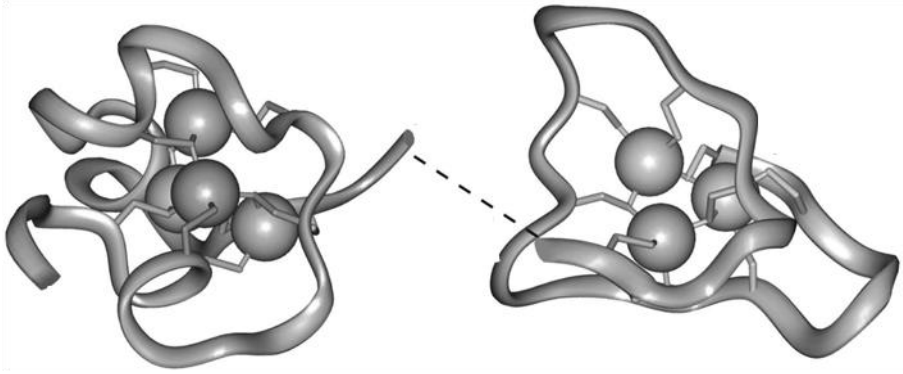

Figure 3. NMR structure of sea urchin Cd-MTA. The N-terminal α-domain enfold a cluster composed by 4 Cd atoms bound to 11 cysteine residues. The C-terminal β-domain enfold a cluster composed by 3 Cd atoms bound to 9 cysteine residues. The discontinuous line represents a discontinuity in the structure between the two domains because of the lack of information about their mutual orientation. Adapted from Romero-Isart and Vašák (2002).

The MTs from the fungus *Neurospora crassa* is constituted by only 25 amino acids, but the tri-dimensional structure of the molecule was not yet determined. However, it seems that a single domain with six Cu atoms is present (Malikayil *et al*., 1989). Bacterial MTs are the only ones that contain some secondary elements: a β-bridge, a β-hairpin, and a α-

helix that form a treble-clef Zn finger fold and, besides cysteine residues, it also uses histidine residues to bind metals (Blindauer and Leszczyszyn, 2010).

The mammalian MTs structure does not have a rigid nature, but instead posses a certain level of mobility. The polypeptide loops between cysteines exhibit an extraordinary flexibility without disrupting the geometry of the metal clusters. Furthermore, the metal-thiolated clusters of some MTs structures can have dynamic processes within it, being possible inter and/or intramolecular metal exchanges in the β- and α-domains and between domains (but much slower for the last two). Non-mammalian MTs have been also subject for structural dynamics studies, namely for yeast and equinodermal MTs, and it was concluded that a variety of cluster arrangements are possible due to the cysteine arrangements that allow many different topologies of the clusters (Romero-Isart and Vašák, 2002).

Until recent years, the metal-free state of the MTs did not arouse interest due to the lack of an organized structure and the short lifetime in the cell (Miles *et al*., 2000). However, it was reported the existence, in the rat liver, brain, and kidneys, of a stable apo-MT in quantities equal to those of the metalated protein (Yang *et al*., 2001). These data might indicate a potential role for apo-MTs *in vivo* in the absence of metals, and structural features may be also possible, contrarily to the previews assumptions.

1.2.6 MTs gene, expression and regulation

The location of MTs genes varies depending on species. In mice, MTs genes reside in a 60-Kb region on chromosome 8 (Haq *et al*., 2003), whereas in humans and other primates, MTs genes (at least 16) are clustered within the q13 region on chromosome 16 (West *et al*., 1990). The various isoforms are encoded in several alleles. In mammals, four isoforms of MTs have been described. The MT-1 and MT-2 are expressed at all stages of development and in virtually all cell types of most organs, mainly in the liver, being very inducible by a variety of agents and conditions including metals, hormones, cytokines and stress signals. The expression of MT-3 and -4 is constitutive and more restricted to certain cells and tissues (Palmiter, 1998; Haq *et al.*, 2003). MT-3 is expressed mainly in the brain, particularly in neurons and glia, and less in other organs such as male reproductive organs

(Coyle *et al*., 2002), whereas MT-4 is only expressed in stratified squamous epithelial cells in skin and tonge (Quaife *et al*., 1994).

The promoter region of the MTs genes is composed by metal regulatory elements (MREs), present in multiple copies, by an antioxidant regulatory element (ARE) also called electrophile response element, and by the classical TATA box that recruits the transcription factors that compose the pre-initiation complex for transcription (Figure 4) (Andrews, 2000).

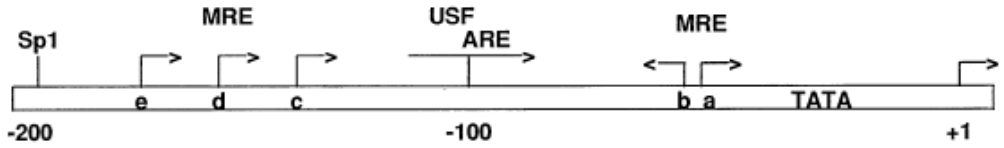

Figure 4. Representative scheme of the mouse MT-1 promoter elements. The known responsive elements are MRE, metal regulatory element (from a to e); Sp1-binding site; USF/ARE, combination of upstream stimulatory element and antioxidant response element; and the traditional TATA Box where the transcription initiation complex is assembled. Adapted from Andrews (2000).

MREs are required for induction of MTs genes by metals (Zn and Cd), but is also necessary for the basal gene expression and for oxidative stress response (Andrews, 2000; Haq *et al*., 2003). MREs act in conjugation with MTF-1, which is a Zn-dependent transcription factor. So, MREs, MTF-1, and the essential Zn ions are the players for both basal and inducible MTs expression. Figure 5 shows a model that explains how MTs gene expression is induced. MTF-1 is always present in cells and, upon metal exposure or stress, it is transported from the cytoplasm to the nucleus, where it binds to the MREs in the promoter of MTs. Then, MTF-1 interacts with components of the transcriptional machinery, regulating MTs transcription. The binding of MTF-1 to MRE only happens after the alosteric binding of Zn the MTF-1 Zn-fingers. This fact gives rise to the question: "if Zn is the only MTF-1 activator, how other metals such as Cd induces MTs transcription?". The answer is that when, for instance, Cd enters the cell, it will displace Zn from MTs and other intracellular proteins increasing the pool of free Zn. Consequently, Zn ions will became available to activate MTF-1. The result is the induction of MTs expression (Koropatnick, 2000).

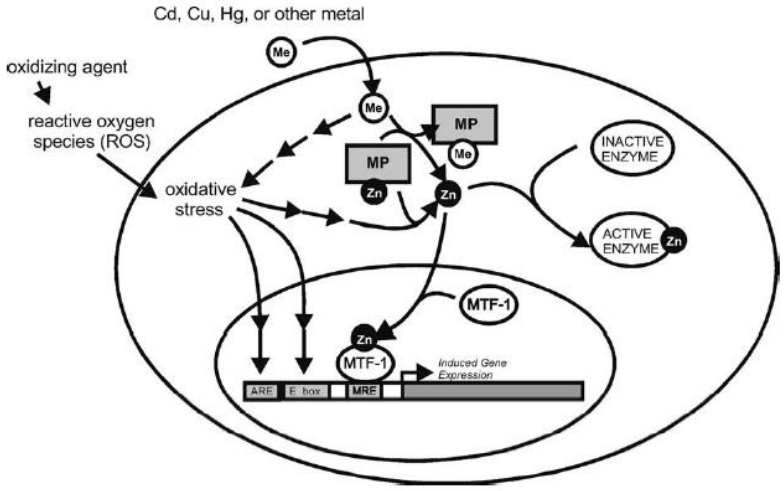

Figure 5. Model for the induction mechanism of MTs gene expression. There is a cell with the plasma membrane and the nucleus where the MTs gene is represented. MP represents a metalloregulatory protein that senses intracellular availability of metal ions. MRE is a metal regulatory element that interacts with MTF-1, a Zn-dependent protein that stimulates the transcription of MTs gene. ARE is an antioxidant regulatory element that responds to oxidative stress signals and E box is another *cis* element associated with USF (upstream stimulatory factor). Adapted from Haq *et al.* (2003).

Another proposal for MTs gene activation by Cd says that Cd activates MTs gene expression by mechanisms distinct from Zn, interacting additionally with AREs, because Cd induces oxidative stress (Andrews, 2000). However, MREs do not associate only with MTF-1, but also with other proteins, namely C'BP-1 and C'BP-2, which are transcription factors (Haq *et al*., 2003). AREs govern the expression of free radical (such as H_2O_2) responsive genes such as those encoding detoxification enzymes, though the interaction with Nrf-2 (NF-E2 related factor 2), which works as a positive regulation (Kensler, 2005). The activity of AREs can be repressed by the binding to Fos and Fra-1 transcription factors. Another constituent of the MTs gene promoters is the CG box, which interact with Zn finger transcription factors such as Sp1, contributing for the basal MTs transcription (Haq *et al*., 2003).

Besides the regulation via transcription, which is the "first line" regulation, MTs genes expression can also be regulated at the epigenetic level, i.e., DNA methylation and chromatin structural conformation changes can repress or activate gene expression, depending on the situation. In addition, it was observed that the transcription of MTs

mRNA was not always correlated with the level of protein synthesis, so post-transcriptional events seem to be important to determine MTs protein levels (Haq *et al*., 2003).

1.2.7 Biodegradation of MTs

The rates of biodegradation of MTs are dependent on the metal that is bound to the protein and on the organism species and its age (Klaassen *et al*., 1999). MTs induced in the liver by Cu, Zn, or Cd have a estimated half time of 17, 20, and 80 h, respectively, although the rates of degradation varying between animal species (Richards, 1989). MTs, like other proteins, are expelled as cellular wastes from the cytosol and are stored in the lysosomes where degradation takes place via proteolytic action of the enzymes cathepsins B and/or L. Some MTs are degraded also in the cytosol by proteases. Metalated MTs have longer half-lives than apo-MTs and MT-1 degrades more rapidly than MT-2 (Miles *et al*., 2000). Metals bound with MTs may be transported to lysosomes for deposition into insoluble residual bodies or metal-rich granules, potentially with subsequent excretion (Wang and Rainbow, 2010).

1.3 Cadmium toxicity and MTs

Between the Top 20 Hazardous Substances Priority List (ATSDR, 1999) is the environmental pollutant Cd, ranked in the eighth position. It is naturally present in the environment in trace amounts, being the weathering of sulfide minerals, volcanic activity and forest fires the main natural sources. Human activities, particularly mining and industrial usage of Cd (Cd-Ni battery, electroplating and paint pigments), has markedly increased the release of Cd into the environment (Ravera, 1984; ATSDR, 1999). Food is the major source of Cd exposure for the general population, and cigarette smoking significantly increases this exposure (Klaassen *et al*., 1999). Although some forms of life adjust to the presence of this metal (Morel, 2008), most biological effects of Cd are deleterious, particularly to mammals. Cd exposure is toxic to a number of tissues, producing hepatic, testicular and pulmonary injury, kidney and bone damage and increases the risk of cancer and mortality (Klaassen *et al.*, 1999; Järup and Åkesson, 2009).

MTs were first reported as Cd detoxifying agents by Margoshes and Vallee (1957), being metal detoxification their first assumed biological function. Because Cd has a high affinity binding for MTs, upon their entry in the cells, Cd ions are sequestered by MTs, preventing non-specific binding of Cd to important macromolecules. Additionally, the protection of MTs against Cd may also include the maintenance of the essential metal (Zn) homeostasis, the scavenging of reactive oxygen species, the regulation of gene expression and tissue regeneration (Klaassen *et al*., 2009).

Thus when intracellular metal concentrations exceed those necessary for metabolic functions, MTs play a key role in metal detoxification. In mammals, the disruption of the MT-1 and MT-2 genes and abolishment of MTs synthesis resulted in a loss of tolerance to Cd (Masters *et al.*, 1994; Liu *et al.*, 1999b); pretreatment with small doses of metals such as Zn, Cu, Hg or Cd induces MTs synthesis and results in reduced lethality after administration of high doses of Cd (Goering and Klaassen, 1983; Conrad *et al.*, 1997); transgenic mice overexpressing MTs show decreased susceptibility to Cd-induced lethality when compared to the wild-type mice (Liu *et al*., 1995), and a higher tolerance to very high Cd concentrations *in vitro* (Lee *et al*., 1996); disorders resulted from chronic poisoning of Cd administration appear more markedly in MT-null mice than in wild type (Habeebu *et al*., 2000); the MTs concentration in liver, kidney and intestine is increased after exposure

to various metals (Miles *et al*., 2000), and cell lines that are very resistant to Cd showed high levels of MTs and an increased resistance to other metals (Durnam and Palmiter, 1984).

MTs handle metal detoxification activity not only in mammals or vertebrates. Invertebrate species also synthesize higher levels of MTs in the presence of excess metal concentrations (Dallinger, 1996; Amiard *et al.*, 2006). Even after a short-term exposure to Cd, the concentration of MTs rapidly increases in the midgut gland of snails (Dallinger and Berger, 1993); oyster exposed to Cd showed that new MTs synthesis in implicated in acquired tolerance to Cd toxicity (Unger and Roesijadi, 1996); mussels exposed to Cd showed an increase in MTs mRNA levels (Soazig and Marc, 2003); the production of MTs enable spiderling populations to persist in ecosystems polluted with Cd (Eraly *et al*., 2010); in crabs, MTs expression levels were correlated to duration time and increased with Cd concentrations in water (Gao *et al*., 2010). Metals bound to MTs are possibly transported to lysosomes for degradation into insoluble residual bodies, with subsequent excretion (Mason and Jekins, 1995).

1.4 Oxidative stress and MTs

Oxygen is the base of several biological reactions and processes, and thus is an essential molecule to aerobic organisms but, due to its great oxidizing capacities, it can become potentially dangerous for biomolecules (Manduzio *et al*., 2005). Oxidative stress can be defined as the loss of balance between the production of ROS and the cellular antioxidant defense system (Colangelo *et al*., 2004). ROS are atoms or molecules that, due to their unpaired electron(s), are highly unstable and potentially reactive (Manduzio *et al*., 2005). All the reactions involving the use of oxygen generate ROS, being persistently produced in cells by, mainly, the action of the mitochondrial electron transport system. Although they are normally produced with a regulatory purpose, when their concentration is excessive, ROS must be rapidly eliminated to prevent cell damage (Storey, 1996). Several cellular components are susceptible to the attack by ROS: the amino acids of proteins can be modified and sulfhydryl groups oxidized leading to conformational changes, altered enzymatic activity, peptide bond cleavage; DNA strands can be broken and bases modified; membranes can be disrupted due to the changes in the fluidity caused by the production of lipid hydroperoxides (Manduzio *et al*., 2005). Therefore, cells must possess mechanisms to control the excess and the damaging effects of ROS. These systems are composed by non-enzymatic antioxidants and antioxidative enzymes, which include enzymes that inactivate ROS, i.e. superoxide dismutase (SOD) for dismutation of O_2• (superoxide anion), catalase and glutathione peroxidase for H_2O_2 detoxification in peroxisomes and mitochondria, and cytosol, respectively. There is, however, no specific defense mechanism against OH•, which is the most dangerous ROS (Colangelo *et al*., 2004).

Although it was first believed that the biological function of MTs was the protection of organisms against the toxicity of metals, other roles have been suggested for these ubiquitous proteins (Coyle *et al*., 2002). *In vivo*, MTs do not exist only in the metalated form, but also partially metalated, oxidized and even in the apo form, i.e., without metals (Maret, 2008). Furthermore, MTs can be present in different redox states due to the formation of disulfide bonds between cysteine residues in the same molecule or between different molecules. Also, MTs are very inducible by chemicals and substances that cause oxidative stress (Sato and Bremner, 1993). These reasons support the hypothesis of an

oxidative stress protecting activity for MTs (Blindauer and Leszczyszyn, 2010). Several lines of evidence sustain the protective role of MTs in the oxidative stress. The first suggestion of an antioxidative function came from a study examining the effects of MTs on the radiosensivity of human epithelial and mouse fibroblast cells (Bakka *et al.*, 1982). The first report on the scavenging activity of MTs was published by Thronalley and Vasak (1985), showing that purified MTs were a more potent hydroxyl radical scavenger *in vitro* than glutathione. After this article, other authors confirmed this property. For instance, Anderson *et al*. (1999) demonstrated that in oysters MTs do not only bind metals but also have the ability to scavenge ROS. Atif *et al*. (2006) showed that MTs are capable of scavenge superoxide anion and nitric oxide in fish. In addition, other line of studies showed that MTs are capable to protect various biomolecules such as lipids and DNA from oxidative damage. Chubatsu and Meneghini (1993) demonstrated that hamster cells depleted of MTs became more susceptible to the DNA-damaging action of H_2O_2 than cells enriched with MTs.

On the other hand, *in vivo*, MTs do not have an antioxidant activity. Lohrer and Robson (1989) concluded that cell lines overexpressing MTs were as sensitive to the lethal effect of oxidative stress as control cells. Additionally, Liu *et al*. (1999b) reported that transgenic mice with higher tissue levels of MTs had similar sensitivity to γ-radiation than controls. Similarly, Ono *et al*. (1998) reported that transgenic mice with 9-fold higher levels of MTs in the brain showed similar levels of lipid peroxidation as control mice after exposure to γ-radiation.

Considering these and other articles, the effective physiological function of MTs in the protection against oxidative stress is not yet clarified, but there are some clues about the mechanisms underlying this function: 1) direct interception of free radicals by sulfhydryl nucleophility; and 2) complexation of metal ions capable to induce oxidative stress (Ercal *et al*., 2001), for example those responsible for the depletion of thiol-containing antioxidants and enzymes or for radical generation. Additionally, MTs may exert their antioxidant function indirectly by affecting two important metals: Zn and Cu. Zn and Cu *per se* may increase the activity of antioxidant enzymes, such as superoxide dismutase (Lazo *et al*., 1998).

Upon reacting with free radicals, the metal thiolate clusters of MTs get oxidized releasing metals from the protein (Kang, 2006). Stefanidou *et al*. (2006) refer in their review that MTs, being a potent ROS scavenger, protect biological structures and DNA from oxidative damage, by distributing Zn, since this metal undergoes rapid inter- and intra-cluster exchange, maintaining the MTs tissue concentrations. Indeed Zn has an important role in protection against oxidative stress because the long-term scarcity of Zn makes the organism more susceptible to injury induced by oxidative stress; more specifically, Zn deficiency increases the level of lipid peroxidation in membranes. Zinc exerts its effects as an antioxidant indirectly by stabilizing the cell membrane structure and DNA; being an essential component of SOD; being a protective agent for thiols; preventing the interaction between chemical groups with iron to form free radicals; and maintaining the adequate MTs tissue concentrations, which *per se* is a ROS scavenger (Tapiero and Tew, 2003).

1.5 MTs as Biomarkers of metal exposure

Since MTs can be induced by the exposure to metal ions such as Cd, Hg, Zn, Cu, Ag, the use of MTs as biomarkers of environmental metal pollution has been proposed using different organisms, including both vertebrates and invertebrates (Amiard *et al*., 2006). A biomarker was defined as "A biochemical, cellular, physiological or behavioral variation that can be measured in tissue or body fluid samples or at the level of whole organisms that provides evidence of exposure to and/or effects of, one or more chemical pollutants (and/or radiations)" (Depledge, 1993). Biomarkers such as cytochrome P4501A, acetylcholinesterase, DNA integrity, lipid peroxidation, protein oxidation, antioxidant enzymes and MTs synthesis are of great importance for biomonitoring of ecotoxicological impact of different pollutants (Sarkar *et al.*, 2006; Monserrat *et al.*, 2007). For a protein to be used as a biomarker, the following properties and information should be available: 1) the assay should be sensitive, reliable, and easy; 2) baseline data for the concentration/activity of the biomarker should be known to distinguish between natural variability and contaminant-induced stress; 3) the basic biology/physiology of the test organism should be known so that sources of uncontrolled variation can be minimized; 4) all the factors that affect the biomarker should be known; 5) it should be established whether changes in biomarker concentration are due to physiological acclimation or to genetic adaptation; and finally 6) increased levels of the biomarker should be correlated with the "health" or "fitness" of the organism (Stegeman *et al*., 1992).

Nowadays, MTs are recognized as part of the "core biomarkers" in the European framework and they are also part of a suite of biomarkers in the Mediterranean Action Plan of the Unite Nations Environment Program for the monitoring of marine environment (Amiard *et al*., 2006). Nonetheless, the induction of MTs is affected by several factors other than the concentration, the duration of exposure and the metal type. At least in mammals, MTs can be induced by hormones, cytokines, oxidants and stress signals (Haq *et al*., 2003). In the case of aquatic organisms, MTs synthesis can be induced also by factors unrelated to metal contamination, such as handling, starvation, anoxia, freezing, and the presence of antibiotics, vitamins or herbicides and even the processes of growth, nutritional status, reproduction, tissue regeneration and infection (Stegeman *et al*., 1992). Furthermore, any factor able to interfere with protein metabolism, metal uptake and metal accumulation

will influence directly MTs concentrations (Amiard *et al*., 2006). Thus, the relative influence of natural (temperature, salinity, size, moult stage, season) and contamination factors limits the use of MTs as a biomarker of metal exposure. Still, besides the fact that MTs genes can be induced by other factors than metals, there have been other type of obstacles in respect to the usefulness of MTs as biomarkers for metal contamination. Some authors reported that some species did not show increased MTs concentrations in areas where metals were bioavailable at high concentrations (Pedersen and Lundebye, 1996; Geffard *et al.*, 2003), and also bivalves transplanted from clean to contaminated sites needed many months to an induction of MTs levels could be seen (Couillard *et al*., 1995a; Geffard *et al*., 2001) in opposition to the short period in laboratory experiments (Viarengo *et al.*, 1985; Del Ramo *et al.*, 1989; Martinez *et al.*, 1996; Barka *et al*., 2001).

Nonetheless, these proteins have much to offer as a possible biomarker of excess exposure to some common toxic metals in aquatic environments. Assay procedures for these proteins are sensitive and evidence of induction at relatively low metal dosage levels. Application of metal composition studies to analyses of this protein also confers a degree of chemical specificity with regard to the probable inductive agent. Additionally, the analysis of MTs offers an advantage over the simple measure of total tissue concentration of metals – the knowledge of intracellular metal partitioning provides important information about mechanisms that defend cell from metal injury (Stegeman *et al*., 1992).

For a better use of MTs as metal biomarkers, the interpretation of their values has to be improved because sometimes high values may be overestimated due to the natural and metal-independent changes in MTs concentrations. The determination of the physiological role of MTs, and the understanding of the effect of all factors that influence MTs are very important to prevent errors in the interpretation. In this context, it is very important to understand if the MTs had been well-applied in monitoring studies of environmental metal contamination using in sentinel organisms such as bivalves. When the aquatic environment is contaminated with a pollutant other than metals which induces the synthesis of MTs, the evaluation will be wrong. The enhanced level of MTs will be assumed as metal contamination, which is incorrect. So, the evaluation about the synthesis of MTs in the presence of other stimuli than metals is of crucial importance to understand their correct use.

1.6 The bivalve molluscs and their importance as sentinel organisms - *Cerastoderma edule*

Human wastes have been introduced in the aquatic systems leading to an increasing concern about the threat that pollutants show to environmental health. The dominant sources of marine pollution are industrial, agricultural, and household wastes. Between the most concerning pollutants are toxic metals such as Cd, Hg, Ag, Co, Cr, Ni, and Pb (Jorgensen, 1990).

Due to the introduction of pollutants into the environment and because they are bioaccumulated and biomagnified throughout the food chain, monitoring programmes became crucial for environmental impact studies. Costly and laborious direct analysis showed to be inappropriate. Therefore, the analysis of the organisms exposed to the pollutants has been applied. The first programme to be created was named "Mussel Watch" which used *Mytilus edulis* and related species to monitor contaminant levels in water (Jorgensen, 1990). Molluscs are highly sensitive to changes in their environment and for this reason they have been seen as good indicators of the pollution level. In particular, bivalves gather a set of features that allow their use as bioindicators. They are wide geographically distributed and have abundant and stable populations, live in a sedentary style, can accumulate high levels of pollutants and still be tolerant to environmental alterations and contaminants. Also, they have a reasonable long life and an adequate size, being sturdy enough to survive in laboratory and field studies. Finally, they occupy an important position in the food chain and in the human diet. Lots of bivalves have been adopted in the biomonitoring of metal pollution in aquatic ecosystem (Zhou *et al*., 2008). Bivalves are considered as the best choice as bioindicators for environmental biomonitoring programmes due to their fast response to environmental changes. Being filter-feeding organisms, they assimilate and concentrate xenobiotics present in water or sediments providing information about local contamination and its effects to biota (Solé *et al*., 2000).

In the biomonitoring of aquatic pollution, specifically toxic metal contamination, several methods can be performed: quantification of metal content in the organism, measurement biochemical alterations and histopathological observations (Sarkar *et al*., 2006). The quantification of MTs levels is one of the biochemical parameters used to assess

metal contamination. Bivalves have been intensively studied for the determination of MTs (Bebianno and Langston, 1991; Couillard *et al.*, 1993; Couillard *et al.*, 1995; Geffard *et al.*, 2001; Mourgaud *et al.*, 2002; Simes *et al.*, 2003; Soazig and Marc, 2003; Dragun *et al.*, 2004; Lecoeur *et al.*, 2004) and increased expression of MTs in response to harmful levels of metal has been demonstrated, giving credibility to the quantification of MTs levels in bivalves to evaluate metal contamination.

Cerastoderma edule (Bivalvia: Cardiidae), known as the common cockle, is an edible infaunal filter-feeding bivalve that lives buried in the upper few centimetres of the sediment (Paul-Pont *et al*., 2012). Cockles live in sandy tidal flat areas along the northwest European coasts in marine and estuarine environments (Kang *et al*., 1999). They are extensively exploited as seafood, being one of the most abundant bivalve in Ria de Aveiro Portugal (Rodrigues *et al*., 2011). Because they are very tolerant to environmental variations of physical and chemical parameters such as sediment grain size and salinity, they can be employed as an indicator organism along an estuarine gradient (Lobo *et al*., 2010).

C. edule organisms were recently subject in studies concerning the monitoring of metals and other pollutants. Lobo *et al*. (2010) performed a study in which cockles were analyzed at biochemical and histopathological levels to evaluate their potential to be used as bioindicators of estuarine sediment contamination. In this work, the MTs induction and the morphology of digestive glands showed that this species were suitable for biomonitoring. Freitas *et al*. (2012) used *C. edule* to look for suitable biomarkers and reported that cockles synthesized high levels of MTs, reflecting the level of metal contamination. Pereira *et al*. (2011) compared *C. edule* with *M. galloprovinciallis* for the assessment of pollution levels and, due to the greater sensibility of cockles and proposed the use other species coupled with the most used mussels for biomonitoring polluted marine environments.

1.7 General Objectives

Since MTs are metal detoxifying agents but also oxidative stress protectors, the main objective of this thesis was to understand the dual role of MTs in metal detoxification and oxidative stress protection in widely used contamination indicators and whether they can be applied as biomarkers in environmental monitoring of metal pollution. We also aimed to understand if the role of MTs, in the case of oxidative stress, is related to Zn distribution or possibly just to ROS scavenging in this species.

To accomplish this goal, a comparison between the two conditions was made using the common-cockle *Cerastoderma edule*. Metal stress was generated by Cd exposure whereas oxidative stress was induced by exposure to H_2O_2. The study was divided in two parts, whose individual objectives were to:

- Determine the level of MTs induction and oxidative stress by a range of Cd and H_2O_2 concentrations in *C. edule* (Chapter 2);
- Choose one concentration of each treatment (Cd and H_2O_2) that fulfill the ideal conditions to compare the role of MTs in metal contamination and oxidative stress (Chapter 2);
- Analyze the amount of Cd, Zn and Cu ions bound to MTs in each situation and compare them (Chapter 3);
- Evaluate the physiological role of MTs in oxidative stress, comparing it to control and Cd contamination. The proposal was to try to ascertain if in oxidative stress MTs are protecting cells through the sequestration of Zn or directly via the scavenging of ROS (Chapter 3).

CHAPTER 2

2. Effect of Cd and H_2O_2 in oxidative damage and metallothionein synthesis

2.1 Background

Metallothioneins (MTs) are cytosolic, small, heat-stable, cysteine-rich proteins. They have attracted attention due to their high metal content and to their wide occurrence among organisms (Stillman, 1995). In physiological conditions they generally bind Zn and Cu ions through the thiol groups of the cysteine residues, forming two characteristic metal-thiolate clusters. MTs are not all identical, occurring in the organism as different isoforms that differ by slightly amino acid positions, and by isoelectric points and hydrophobicity (Kägi and Schaeffer, 1988).

The function of these metalloproteins is still under discussion. However, due to their nature, MTs had been considered to have two indisputable roles focused in the regulation of essential trace metals (Cu and Zn, for instance) and in the detoxification of essential and non-essential metals (such as Cd, Hg, As). Concerning on the ability of MTs to bind toxic metals and protect organisms from their effects, they have been used as molecular biomarkers of metal exposure (Amiard *et al*., 2006).

Cadmium is one of the most toxic pollutants due to its persistence, toxicity and potential for bioaccumulation. The deleterious effects of Cd contamination in organisms result from its accumulation within specific tissues (Järup and Åkesson, 2009). Most of Cd in the organism is bound to MTs, implying a role in Cd distribution and detoxification by these proteins (Klaassen *et al*., 1999). A number of studies in mammals have been gathering evidences pointing for an important role of MTs in Cd detoxification. Cells that contain an excess amount of MTs are resistant to Cd toxicity (Karin *et al*., 1983), whereas cell lines that cannot synthesize any MTs are sensitive to Cd (Enger *et al*., 1986). The exposure of increasing Cd doses revealed an acquired tolerance to Cd lethality in wild-type mice, with a 7-fold difference in LD50 values, while such tolerance did not happen in the MT-null mice (Park *et al*., 2001).

In aquatic invertebrates, MTs have also been related to detoxification processes due to their involvement in metal accumulation and tolerance. Stuhlbacher and Maltby (1992) observed that the rise in MTs concentration can be linked to the decrease in the sensitivity to metals in excess in the crustacean species *Gammarus pulex*. Also, higher MTs concentrations were found in two crustacean populations that live in metal polluted medium (Ross *et al*., 2002). Pre-exposure to metals in early stages of the oyster *Crassostrea virginica* resulted in a strong MTs induction that provided an advantage against Cd toxicity (Roesijadi *et al*., 1997). Similarly, the less Cd- and Zn-sensitive developmental stages of *Mytilus galloprovincialis* had the highest MTs concentrations (Pavičić *et al*., 1994). The work of Unger and Roesijadi (1996) in the oyster *C. virginica* showed that Cd exposure enhanced the levels of MTs mRNAs. Leung and Furness (1999) concluded that MTs amounts increased in Cd exposed dogwhelks *Nucella lapillus*. These and other works proposed that MTs can be a suitable and promising biomarker, indicating the levels of bioavailable metals in the organism and reflecting the levels of metal contamination in the environment.

Furthermore, other roles were proposed for MTs, such as the intracellular scavenging of free radicals, as a way to protect cells against oxidative stress (Sato and Bremner, 1993). There are several studies on mammals which demonstrated the oxidative stress protection role of MTs. For instance, administration of agents that stimulate the production of reactive oxygen species (ROS) caused MTs levels to increase 20 to 30-fold in mice (Bauman *et al*., 1991); Thornalley and Vasák (1985) found that MTs had scavenging activity *in vitro* for hydroxyl radicals; Kumari *et al*. (1998) suggested a remarkable *in vitro* scavenging activity against free-radical species; mice cells overexpressing MTs are resistant to oxidative stress (Schwarz *et al*., 1994), whereas mice cells MT-deficient are sensitive to oxidants (Lazo *et al*., 1995).

Regarding aquatic invertebrates, Viarengo *et al*. (1999) used the mussel *M. galloprovincialis* to test MTs antioxidant properties, revealing that MTs induction (in this work achieved by pre-exposure to Cd) was responsible for the protection of cells and whole organisms from oxidative stress (induced by Fe exposure). Another study on aquatic molluscs, carried out by Leung and Furness (2001) in *N. lapillus*, showed that H_2O_2 can induce MT-like proteins, questioning the use of total MTs as metal-specific biomarker.

However, despite the encouraging data for the antioxidant activity of MTs, some observations pointed out to confounding results and the effective physiological role of MTs in oxidative stress has not yet been clarified.

Because bivalves are considered as good sentinel organisms, being widely used to monitor the presence of toxic compounds in marine environments (Amiard *et al*., 2006), and because cockles have been recently proposed to be included in the group of species useful for biomonitoring (Machreki-Ajmi and Hamza-Chaffai, 2008; Machreki-Ajmi *et al.*, 2008; Lobo *et al.*, 2010; Fernández-Tajes *et al.*, 2011; Pereira *et al.*, 2011), and additionally because they showed a good association between MTs synthesis and metal contamination levels (Freitas *et al.*, 2012), including Cd (Paul-Pont *et al.*, 2010), *C. edule* was selected for this study.

2.2 Objectives

The main objective of this chapter is to present the work developed regarding the evaluation of the response of MTs in respect to the detoxification of toxic metals (Cd) and oxidative stress protection (H_2O_2) in *C. edule* organisms exposed to a range of Cd and H_2O_2 concentrations. The work pretended to answer the question:

- Are MTs synthesized in response of other stimuli besides metals, for example, by compounds able to impose oxidative stress? If yes, is the level of synthesis lower, higher or identical? They can be used as metal-specific biomarkers?

For this, the following procedures were performed:

- Exposure of *C. edule* organisms to a range of Cd and H_2O_2 concentrations (independently) and to a control (no metal and no H_2O_2);
- Quantification of the MTs concentration in the exposed and control organisms in each concentration used;
- Determination of the level of oxidative stress in all the conditions tested through the quantification of TBARS (thiobarbituric acid reactive species);

2.3 Materials and Methods

2.3.1 Study area

The present study was conducted with cockles collected in the Ria de Aveiro (40º380' N, 8º440' W), a shallow coastal estuary situated on the north west Atlantic coast of Portugal, with an area of approximately 47 km^2. This estuary comprises a complex system of bays and channels, with a maximum length of 45 km and width of 8.5 km wide and is separated from sea by a sand bar. The Ria de Aveiro has an irregular and complex geometry, where four main channels can be identified: Canal de Ílhavo, Canal de Mira, Canal de Ovar and Canal do Espinheiro. Vouga and Antuã rivers, which have average flows of 50 and 5 $m^3 s^{-1}$ respectively, account for most of the freshwater discharge into this ecosystem (Dias and Lopes, 2006).

2.3.2 Sampling and exposure experiment

C. edule specimens were collected in October 2010 in a low-contaminated area of Ria de Aveiro (Portugal). Cockles (about 300 individuals) were maintained in laboratory at 20 ± 1ºC with a photoperiod 12:12h, using artificial light sources for one week period prior to the experiments. *C. edule* organisms were fed with a concentrated algae culture (*Pseudokirchneriella subcapitata*) every day during both acclimatization and exposure experiments. The culture of microalgae was carried out at the laboratory under controlled conditions allowing us to obtain an axenic culture. Experimental procedure was conducted in the laboratory using plastic containers of 11 cm x 12 cm x 14 cm with 1 L of clean sea water (salinity = 30) aerated by a diffuser system. A 3-cm layer of clean sand was used to create conditions similar to the environment, allowing the cockles to bury. After the acclimatization period, cockles were submitted to two different exposure conditions during 5 days under the conditions mentioned above. The concentrations used in the experiments were 0.01, 0.1, 1, 5 and 10 µM of Cd (added as $CdCl_2$) and 0.02, 0.2, 2, 10 and 20 µM of H_2O_2 (for a better understanding see figure 6). Four replicates were used for each Cd and H_2O_2 concentration with 7 cockles per replicate. A control condition (no metal or H_2O_2) was also performed using 3 replicates with 7 cockles each. In all experiments, the water

was renewed every day to maintain the Cd and H_2O_2 levels during the experiment. At the end of the exposure experiment, cockles were frozen at -20ºC before the TBARS and MTs quantification assays.

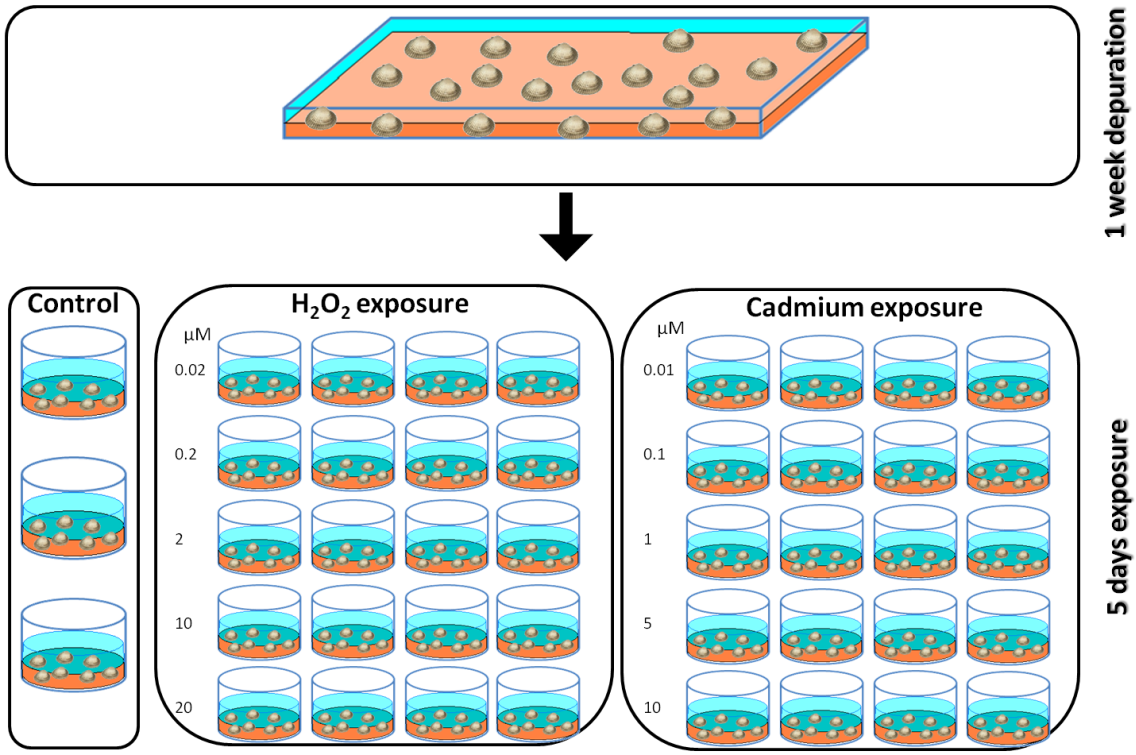

Figure 6. Schematic representation of the experimental procedure. After the acclimatization period, cockles were exposed to a range of Cd (0.01, 0.1, 1, 5 and 10 µM) and H_2O_2 (0.02, 0.2, 2, 10 and 20 µM) concentrations and sea water (control) during 5 days. The blue cylinders represent the plastic containers used in the experiment and the light orange bands correspond to the 3-cm sand layer deposited in the bottom of plastic containers.

2.3.3 TBARS quantification

Three cockles per exposure concentration plus control were weighed and homogenized individually in ice-cold phosphate buffer (50 mM, pH = 7.0 with 0.1% TRITON X-100). Homogenates were centrifuged at 10000 x *g* for 10 min at 4 ºC and supernatants were divided in three aliquots, one for TBARS quantification, other for protein determination and the last for MTs analysis. TBARS quantification is a measure of lipid peroxidation, providing specific information about the degree of oxidative stress

experienced by cells. This methodology was based on the reaction of lipid peroxidation by-products, such as malondialdehyde (MDA), with 2-thiobarbituric acid (TBA) and was performed according to Buege and Aust (1978). The amount of TBARS was measured spectrophotometrically, at the wavelength of 535 nm (molar extinction coefficient of 1.569105 M^{-1} cm^{-1}), and results were expressed as nmol of MDA equivalents per mg of protein sample.

2.3.4 Protein quantification

Protein concentration of each sample was determined in triplicate, according to the spectrophotometric (wavelength = 595 nm) method of Bradford (1976) adapted to microplate.

2.3.5 MTs quantification

2.3.5.1 Sample preparation and derivatization

Metallothioneins were determined according to Alhama *et al*. (2006), with some adaptations. Supernatants (300 μl) from 2.3.3 section were reduced and denatured for 10 min at 75 ºC and centrifuged at 12000 x *g* for 10 min at 4 ºC to precipitate denatured proteins. The resulting supernatants (200 μl) were neutralized with 0.1 M NaOH, after the addition of 200 μl of 0.1 M Tris–HCl buffer (pH 8), 25 μl of 2 mM DTE (dithioerythritol) and 50 μl of 0.1 M EDTA (ethylenediamine tetraacetic acid). The mixture was incubated during 30 min at room temperature and 75 μl of 10 mM mBBr (monobromobimane - Calbiochem) were added. Derivatization was performed in the dark for 40 minutes at 35ºC. The reaction was stopped by the addition of acetic acid 5% (v/v) up to a total volume of 1.5 mL. Samples were stored at 4 ºC until RP-HPLC (Reversed-Phase High Liquid Chromatography) analysis.

2.3.5.2 HPLC analysis

Derivatized proteins were separated by Reversed Phase High Performance Liquid Chromatography (RP-HPLC) (Gilson Liquid Chromatograph, model 306). Samples (20 μl)

were injected on a RP C18 column (250mm × 4.6mm i.d., 5 µm, Gilson). The column was equilibrated with previously degassed eluant A (0.01% aqueous trifluoroacetic acid (TFA) (v/v)) and developed by a linear gradient of 0–70% eluant B (90% acetonitrile, in 0.01% aqueous TFA) during the first 15 min, followed by an isocratic elution of 70% eluant B for the next 10 min. The complete analysis was performed in 25 min. After each run, the column was washed by raising the concentration of eluant B to 100% and re-equilibrated with eluant A. Thiols were resolved and eluted at a flow rate of 1 mL/min and detected by fluorescence (Jasco 821-FP Intelligent Spectrofluorometer) with excitation at 380 nm and emission at 480 nm. Thiol identification was based on the purified rabbit liver MT-1 standards. MTs were expressed per fresh weight rather than a protein basis, since the first is physiologically more relevant to the organism.

2.3.6 Statistical analysis

Data were submitted to hypothesis testing using the PERMANOVA routine (permutational multivariate analysis of variance) from PRIMER v6 (Clarke and Gorley, 2006; Anderson *et al.*, 2008), following the calculation of Euclidean distance matrices among samples. The pseudo-F values in the PERMANOVA main tests were evaluated in terms of significance among different treatments. When the main test revealed statistical significant differences, pairwise comparisons between the treatments and control were performed. The t-statistic in the pair-wise comparisons was evaluated in terms of significance among treatments. Values lower than 0.05 were considered as significantly different. The null hypotheses tested were: for survival percentages: no significant differences exist between the exposure concentrations and control at the end of the experiment; for TBARS and MTs values: no significant differences exist between treatment concentrations and control.

2.4 Results

2.4.1 Survival of *C. edule* to Cd and H_2O_2 exposures

The survival percentages of *C. edule* exposed to Cd and H_2O_2 are shown in Figure 7. Under our experimental conditions, different survival percentages between the two treatments and between the concentrations used were observed. Cockles exposed to Cd had the highest survival rates, being the minimum percentage of survival at the end of the experiment was 71% at the highest concentration (10 μM). Any Cd concentration showed a significant lower survival percentage than control ($P > 0.1$). In the H_2O_2 treatment, mortality was higher, reaching percentages of almost 80% in the highest concentration (20 μM), i.e., 80% of the individuals died at the end of the exposure period. In this treatment, 3 concentrations (2, 10 and 20 μM) showed to have significant lower survival percentages from control ($P < 0.001$) at the end of the exposure period. The concentration 2 μM of H_2O_2, without a reasonable explanation, did not allow the survival of cockles and, even after the repetition of this condition several times, there was no survivors.

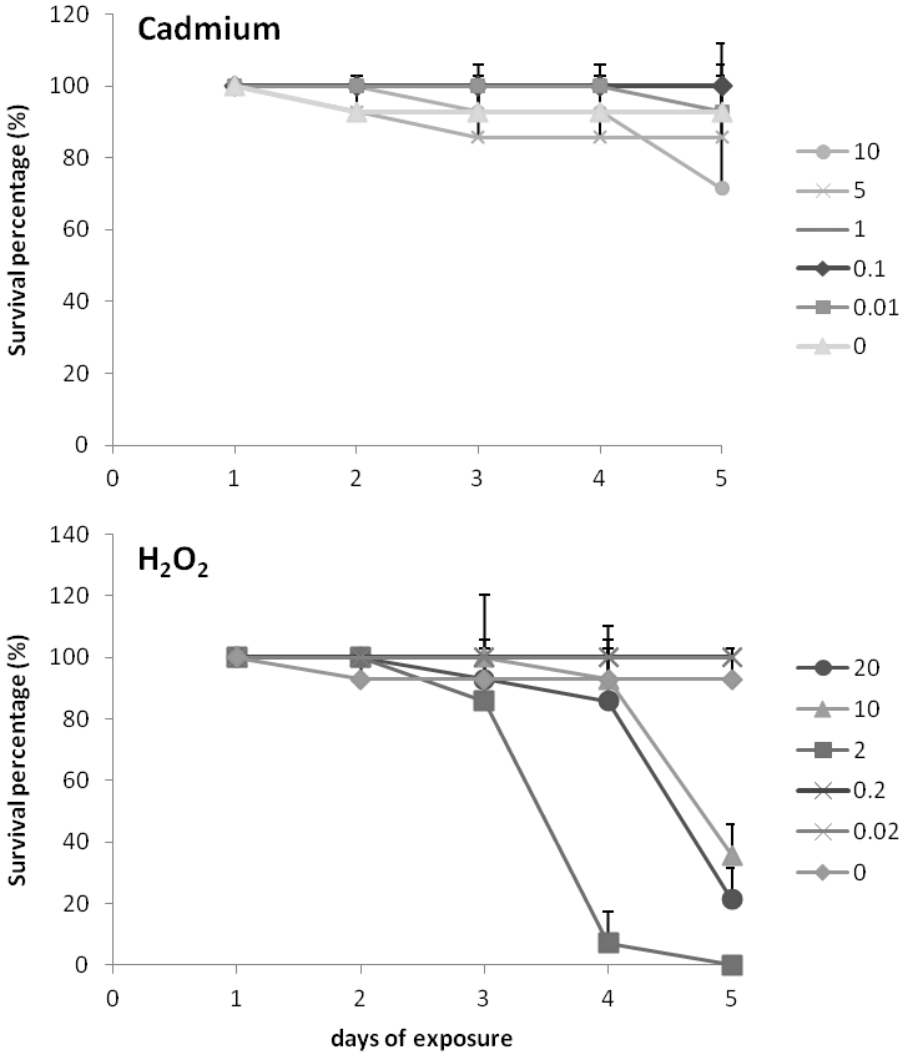

Figure 7. Survival percentage of *C. edule* exposed a range of concentrations of Cd (0, 0.01, 0.1, 1, 5 and 10 µM) and H_2O_2 (0, 0.02, 0.2, 2, 10 and 20 µM) during 5 days. The values are the mean percentage of three replicates and SD.

2.4.2 Metallothioneins response upon Cd and H_2O_2 exposures

In order to determine the level of MTs synthesis in *C. edule* upon Cd and H_2O_2 exposures, the amount of MTs in each sample was quantified by RP-HPLC. The results showed that MTs were induced by both treatments, although at different levels (Figure 8A and B). At lower concentrations (0.01 to 2 µM), *C. edule* produced 3 to 4 times less MTs

when exposed to H_2O_2 than to Cd, but at the highest concentrations (20 µM for H_2O_2 and 10 µM for Cd) both treatments induced similar amounts of MTs.

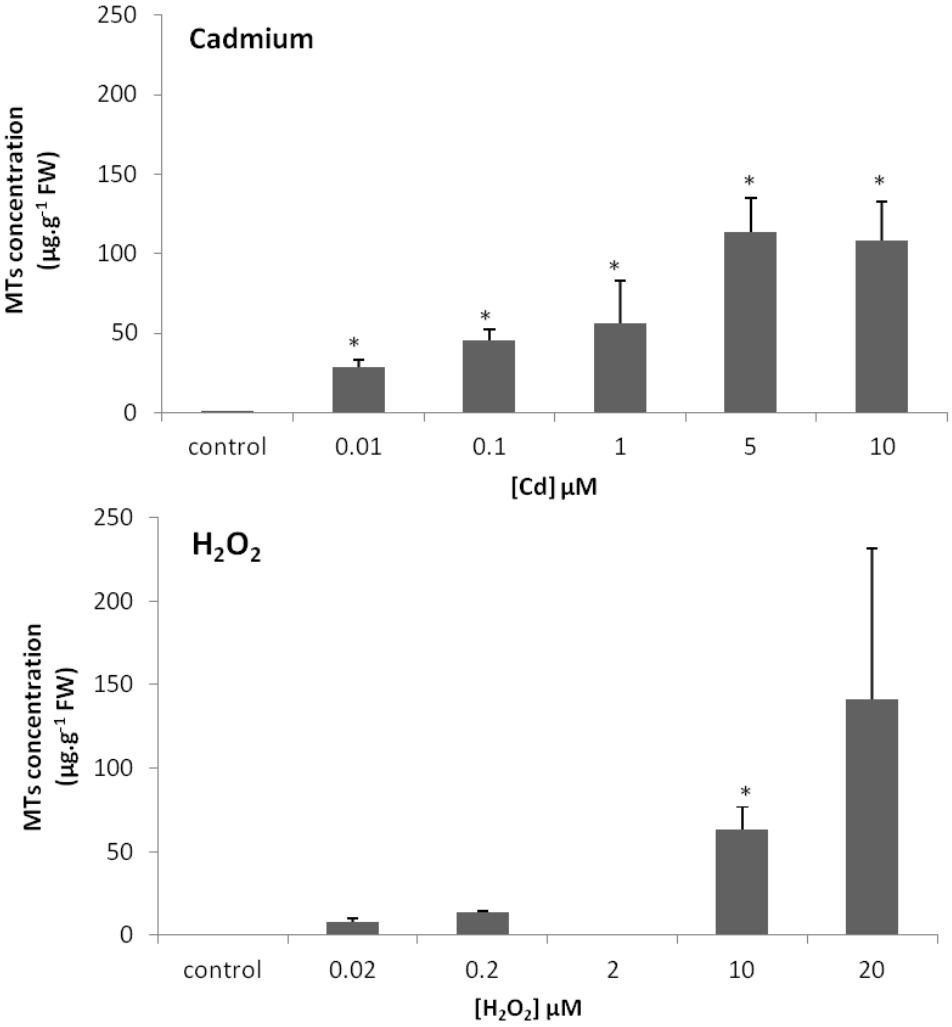

Figure 8. Metallothioneins concentrations in whole soft tissues of *C. edule* organisms exposed during 5 days to 0, 0.01, 0.1, 1, 5 and 10 µM of Cd and 0, 0.02, 0.2, 2, 10 and 20 µM of H_2O_2. Values represent the mean of 3 replicates with SD. * indicates significance at the $P< 0.05$ level compared to control.

2.4.3 Lipid peroxidation levels in Cd and H_2O_2 exposures

TBARS amounts were quantified in the cockles exposed to a range of Cd and H_2O_2 concentrations (Figure 9A and B, respectively). The quantification of TBARS provided a measure of the lipid peroxidation of the cells. Knowing that lipid peroxidation is a consequence of oxidative stress that cells are experiencing, the results of these work indicated that, at least in the tested concentrations, H_2O_2 imposed significant increased levels of oxidative stress ($P < 0.05$), while Cd did not. Results showed that TBARS content in *C. edule* individuals exposed to Cd concentrations was not significant different from the control individuals ($P > 0.05$). Contrarily, TBARs content in the organisms exposed to H_2O_2 concentrations (particularly 0.2, 10 and 20 µM) showed significantly different values compared to the control ($P < 0.05$).

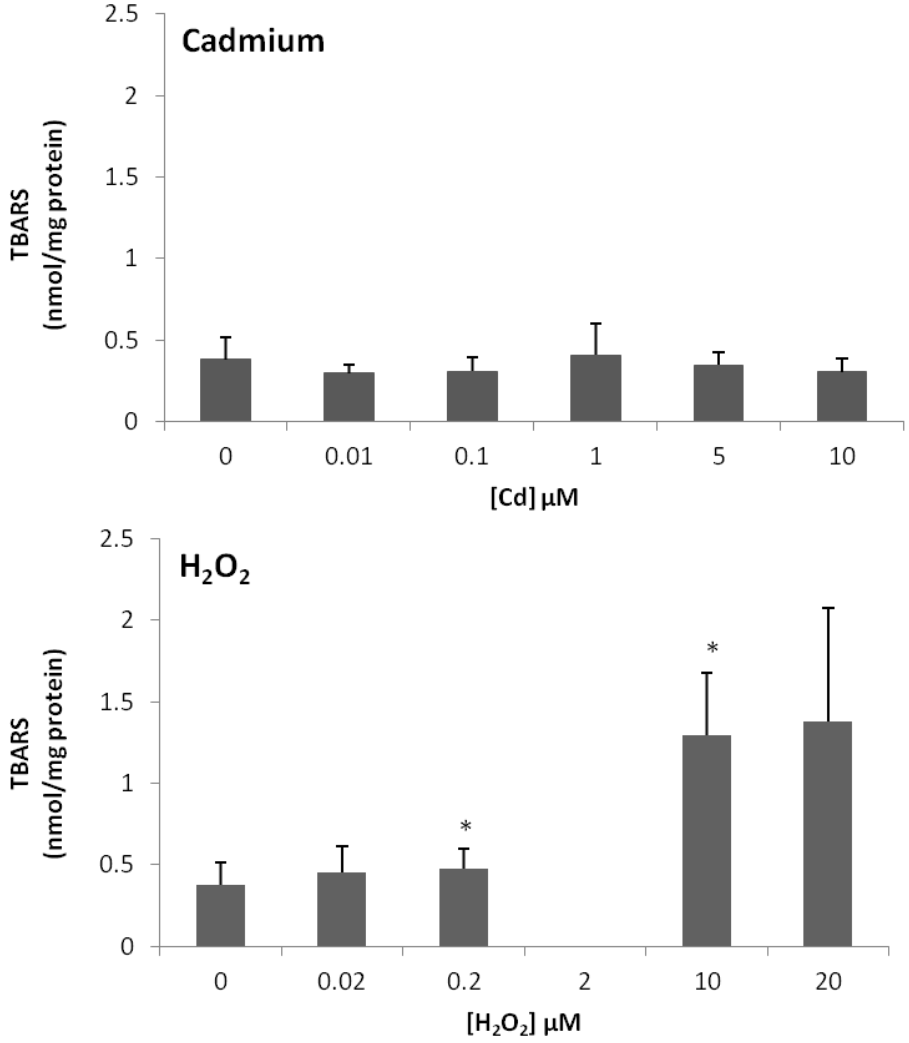

Figure 9. Lipid peroxidation levels determined by the measure of the concentration of thiobarbituric acid reactive species (TBARS), are expressed as nmoles of malondialdehyde (MDA) per mg of protein. Values represent the mean of 3 replicates with SD. The quantification was made in the whole soft tissues of *C. edule* organisms exposed to 0, 0.01, 0.1, 1, 5 and 10 µM of Cd and 0, 0.02, 0.2, 2, 10 and 20 µM of H_2O_2 for 5 days. * indicates significance at the $P< 0.05$ level compared to control.

2.5 Discussion

2.5.1 The effect of Cd and H_2O_2 in the survival of *C. edule*

In this study, *C. edule* organisms were exposed to a range of Cd and H_2O_2 concentrations during 5 days. Figure 7 shows the percentage of living organisms along the 5 days of exposure to H_2O_2 and Cd concentrations. Results show that the concentrations of H_2O_2 used provoked a higher mortality than Cd concentrations. Leung and Furness (2001) showed that the exposure to high levels of H_2O_2 (2 and 1000 ppm, equivalent 0.05 and 29 mM) provoked a significant decrease in the weight of the dogwhelk *N. lapillus* (reflected by the calculation of condition index), but no mortality. Nevertheless, even though *N. lapillus* showed a higher tolerance to H_2O_2 exposure than *C. edule*, the effects of H_2O_2 were also noticed and, according to Leung and Furness (2001), could be a consequence of the higher metabolic cost implied by detoxification mechanisms that are taking place. In regard to Cd exposure, the same work showed that the decrease in the condition index of *N. lapillus* in the presence of 0.5 ppm of Cd (4.45 μM) was less evident than in 1000 ppm of H_2O_2. This result indicated that Cd (although at lower levels) had lower damaging effects than H_2O_2, which was also observed in the present study by the higher survival percentages of organisms when submitted to Cd than to H_2O_2 treatment. Cavalleto *et al*. (2002) tested the effect of H_2O_2 (20 μM) in the digestive gland of *M. galloprovincialis* and observed the destabilisation of lysosome membrane during the 7 days of exposure, indicating that H_2O_2 had a harmful effect in the mussel physiology, and explaining the lower survival rates of *C. edule* to H_2O_2 in this study. However, the same authors obtained no mortality during their experiment, unlike the present study, suggesting that mussels may be more tolerant than cockles to the effects of H_2O_2.

3.5.2 Metallothioneins response upon Cd and H_2O_2 exposures

Since non-essential metals, such as Cd, enter the cell, a competition between them and existing essential metals for intracellular ligands will indubitably take place. The non-specific binding of Cd or the substitution between Cd and essential metals in proteins with sulfhydryl groups and metalloenzymes is one of the reasons why Cd exerts dangerous

damage to organisms (Moulis, 2010). However, organisms possess defense mechanisms to respond to the excess of metals. One of these defenses is the sequestration of metals by MTs (Amiard *et al*., 2006).

MTs induction and concentration is dependent on the organism species, the tissue where it is quantified, the metal, the concentration and time of exposure (Amiard *et al*., 2006). In this study, Cd was used as the contamination metal and the period of exposure was defined to 5 days in all experiments. The only variable was the level of exposure. Cadmium treatment induced significant increases in MTs synthesis compared to control and had a dose-dependent effect until the 5 μM concentration ($P < 0.05$).

A set of works in individual organs or the whole body of bivalve species have been showing the ability of MTs to be induced by Cd both in laboratory and in field experiments (Roesijadi and Klerks, 1989; Bebianno and Langston, 1991; Couillard *et al.*, 1993; Couillard *et al.*, 1995; Chan *et al.*, 2002; Lecoeur *et al.*, 2004). Most of the works reported increases in the MTs synthesis towards Cd exposure, but less works analyzed in laboratory conditions the effect of increasing Cd concentrations on the synthesis of MTs. Among them is the study performed of Barka *et al*. (2001) that also observed dose dependent significant increases in MTLP concentrations in the crustacean *Tigriopus brevicornis* after 1 day of exposure to three Cd concentrations (0.47, 2.35 and 4.7 $\mu g.L^{-1}$). Martinez *et al*. (1996) also showed that exposure to a range of Cd concentrations (100 to 1000 $\mu g.L^{-1}$) produced MTs in a dose dependent manner during 1 day in the amphipod *Echinogammarus echinosetosus.* In respect to bivalves, but at the transcriptomic level, MT mRNA levels increased in a dose-dependent manner in oysters (*C. gigas*) exposed to 10, 50, or 100 $\mu g.L^{-1}$ Cd for 11 days (Choi *et al*., 2008). Similarly, the clams *Meretrix lusoria* exposed to 50, 200, or 1000 $\mu g.L^{-1}$ Cd for 7 days also showed an increase in MT mRNA which depended on the metal concentration (Chang *et al*., 2007). Yet, the amphipod (Martinez *et al*., 1996) synthesized lower levels of MTs (9 $\mu g.g^{-1}$) than *C. edule*, indicating that bivalve species such as cockles are much more suitable for the monitoring of Cd contamination than crustacean species, in the case of using MTs as a biomarker. Cockles, which showed to have a stronger MTs synthesis in the presence of low Cd levels, will sense the presence of Cd even in a lower contaminated site. Freitas *et al*. (2012) observed that the amount of MTs in *C. edule* was higher than in *Diopatra neapolitana,* being both species collected from the same

contaminated areas, also indicating that cockles produce higher levels of MTs than other species.

MTs concentration did not increase from 5 to 10 μM of Cd exposure. This was in agreement with other studies that showed a reduction of MTs concentration at high metal exposures, possibly due to the toxic effects that are preventing detoxification processes from being fully functional (Martinez *et al.*, 1996; Roesijadi *et al.*, 1997; Mouneyrac *et al.*, 2002). In our study, the decrease of MTs concentration was not actually observed but the maintenance of the values seemed to corroborate these results.

In the present study, the H_2O_2 concentrations increased the synthesis of MTs. Although the statistical analysis did not show significant differences in all concentrations, it can be observed that the mean values of MTs increased throughout the concentrations used. Indeed, this result indicated that MTs were involved in the oxidative stress protection in bivalves as suggested by some authors (Anderson *et al.*, 1999; Viarengo *et al.*, 1999; Cavaletto *et al.*, 2002; Gagné *et al.*, 2008).

In the lower H_2O_2 exposures (0.02 and 0.2), organisms produced less MTs than in the lower Cd exposures (0.01, 0.1 and 1 μM). This is in agreement with Kagi (1993) which stated that, despite MTs synthesis can be induced by other factors than metals, the level of induction is usually lower than that caused by metals. Nevertheless, it is crucial to consider the importance of other inducers upon the use of MTs as a metal-specific biomarker.

Leung and Furness (2001) studied the effect of H_2O_2, Cd and both on the induction of MTs in dogwhelk (*N. lapillus*) and, in general, concentrations of MTs in the leiblein gland were higher than in the control. Although H_2O_2 alone induced MTs synthesis, these authors concluded that Cd or Cd+H_2O_2 together promoted a higher induction of MTs than H_2O_2, which are in agreement with our results which showed that MTs were induced by both stressors, although in our work we tested them individually, and also we observed a higher synthesis in response to Cd than to H_2O_2 in the lower concentrations.

In fact, MTs responded to H_2O_2 and Cd because the promoter regions of their genes are composed by MREs (metal regulatory elements) and also by AREs (antioxidative regulatory elements) (Andrews, 2000). In the case of Cd, upon the entrance of Cd ions in the cell, Zn is released from MTs, activating MTF-1. This zinc-dependent factor will then bind to MREs and activate the transcription of MTs genes (Haq *et al.*, 2003). MTs induction

observed in H_2O_2 treatment could be due to the ARE which activate transcription in response to the increase of ROS concentrations in cell (Andrews, 2000). The role of MTs in the metal stress protection is very clear: MTs bind metals preventing their non-specific binding to important biomolecules in the cell (Roesijadi, 1992). However, the role of MTs in the protection against oxidative stress still unclear (Sato and Bremner, 1993). MTs can scavenge ROS and/or distribute Zn, since this metal is very importance in the protection against oxidative damage.

3.5.3 Oxidative damage in *C. edule* upon Cd and H_2O_2 exposures

When H_2O_2 enters the cells, it is detoxified by the antioxidant system but the excess may alter cell physiology because Cu and Fe ions in the cytosol react with H_2O_2 producing hydroxyl radicals which can react with biomolecules in the cytosol and with membrane lipids (Cavaletto *et al*., 2002). In this work we evaluated lipid peroxidation levels imposed by a range of Cd and H_2O_2 concentrations in cockles exposed during 5 days. It was observed that only H_2O_2 concentrations provoked an increase in the levels of lipid peroxidation, indicating that cells were under oxidative stress.

The fact that Cd does not impose high levels of lipid peroxidation has been observed by other works. The work performed by Viarengo *et al*. (1999) with *M. galloprovincialis* did not find significant differences between organisms exposed during 7 days to Cd (200 $\mu g.L^{-1}$) and controls, indicating that Cd did not impose oxidative stress in this bivalve, as observed for *C. edule* in the present study. *M. galloprovincialis* also showed a markedly synthesis of MTs upon Cd exposure, in a 10-fold increase compared to control, a value that can be explained by the higher level of Cd exposure that were used in the study. The high level of Cd exposure (200 $\mu g.L^{-1}$) was expected to induce damage in cells, namely oxidative injury (Cuypers *et al*., 2010), but the strong MTs synthesis was possibly the responsible for the absence of lipid peroxidation in this mussel, showing that this species is very tolerant to Cd contamination. A study on Cd contamination in the clam *Ruditapes decussatus* showed an increase in MTs after 7 days of exposure, while the MDA increase was visible only after 14 days of Cd exposure (Geret *et al*., 2002), indicating that, at least in some bivalve species, for Cd to exert oxidative damage, the concentration must be high or the period of exposure very extended.

Exposure to H_2O_2 is expected to induce high levels of oxidative stress and hence increase lipid peroxidation levels. Cavalleto *et al*. (2002) also determined the MDA concentrations in *M. galloprovincialis* and they observed significant increases in the digestive glands at the 4^{th} day of exposure to H_2O_2 (20 µM). Our results are in agreement with the results of Cavalleto *et al.* (2002) which showed that concentrations higher than 0.2 µM of H_2O_2 were able to induce oxidative stress and promote the synthesis of MTs. In fact, according to the data presented in this work and by other authors, namely Geret *et al*. (2002) in the gills of *R. decussatus* and Couillard *et al*. (1995), in the bivalve *Pygadon grandis*, there is a relationship between lipid peroxidation and MTs values, indicating that MTs are involved in the protection against oxidative stress.

Therefore, this work originated another question: Which is the role of MTs in oxidative stress? To answer this question, further work was performed (Chapter 3). One Cd and H_2O_2 concentrations were selected: the highest concentrations (20 µM for H_2O_2 and 10 µM for Cd) both treatments induced similar amounts of MTs, thus indicating that these concentrations are adequate for the subsequent study.

2.5 Conclusions

Because bivalve molluscs are regarded as macroconcentrators and bioindicators of metal contamination in the environment and MTs are used as metal-specific biomarkers in this sentinel species (Cajaraville *et al*., 2000), it was interesting to clarify the effect of Cd and H_2O_2 in the synthesis of MTs in *C. edule*.

In this study, MTs were synthesized in response to Cd and high concentrations of H_2O_2. As Cd did not impose oxidative stress, evidenced by no increases in lipid peroxidation levels, and H_2O_2 did, our results confirmed the involvement of MTs in two conditions, as proposed earlier: metal detoxification and oxidative stress. The use of total MTs as metal-specific biomarkers for environmental contamination is questioned due to their responsiveness to oxidative compounds such as H_2O_2. Contrarily to the conclusions of Cavalleto *et al*. (2002) which encouraged the use of MTs as a biomarkers more strictly related to metal pollution, this work showed that, although the lowest exposure levels indicated that Cd had a stronger effect than H_2O_2 in MTs synthesis, at higher levels, MTs synthesis was similar in metal (10 μM) and H_2O_2 (20 μM) exposures. In the environment, some situations such as oil pollution and sewage disposal are able to release substantial amounts of oxidative substances which may induce MTs and overestimate the level of metal contamination.

One propose of this experiment was to select a Cd and a H_2O_2 concentration inducing the same amount of MTs, but where the organisms were not in oxidative stress when exposed to Cd, in order to differentiate the effect of metals and oxidative stress in the induction of MTs. The selected concentrations were 10 and 20 μM for Cd and H_2O_2, respectively. These concentrations induced similar amounts of MTs, providing an identical MTs background for the subsequent work, and the two concentrations impose different oxidative scenarios: Cd imposes metal contamination but no oxidative stress, and H_2O_2 causes oxidative stress. Thus, we will be able to achieve two different responses, providing the basis to understand the two different roles of MTs separately: metal detoxification and oxidative stress protection.

CHAPTER 3

3. The double role of metallothioneins:

Metal chelator and oxidative stress protector

3.1 Background

As it has been described in the present study, the function of metallothioneins (MTs) and their isoforms is still a matter under discussion. Since their discovery in the 50's (Margoshes and Vallee, 1957), it has been considered that MTs are traditionally involved in the regulation of essential trace metals, such as Cu and Zn and in the detoxification of essential and non-essential metals (Roesijadi, 1996). Later, since the classical work of Thornalley and Vasák (1985) on the scavenging activity of MTs towards free hydroxyl and superoxide radicals, much more evidences have been accumulated on the antioxidant activity of MTs *in vivo* and *in vitro* (Sato and Bremner, 1993).

The metal-thiolate clusters are readily oxidized *in vitro*, thus they could scavenge deleterious reactive oxygen species (ROS), by neutralizing hydroxyl radicals (Palmiter, 1998). Studies at three different levels have been demonstrating this antioxidative function of MTs: (1) Studies on MTs induction revealed that MTs are induced by compounds able to cause oxidative stress, as well as by agents involved in inflammatory processes, suggesting that MTs may protect against ROS and reactive nitrogen species (RNS). Bauman group (Bauman *et al*., 1991; Bauman *et al*., 1992) and Cai *et al*. (1999) showed that the cellular levels of MTs can be increased by eleven different oxidant chemicals and by radiation exposure; (2) Studies on the use of MT-null animals or cells investigating the consequences of the lack of MTs. As example, cultured cells from transgenic MT-null mice showed a higher sensitivity to oxidative stress (Lazo *et al*., 1995; Zheng *et al*., 1996); (3) Studies on the overexpression of MTs genes in cells or animals. Schwarz *et al*. (1994), for instance, observed that cultured cells (fibroblast) overexpressing MTs can resist better to oxidative stress caused by tert-butyl hydroperoxide. Regarding *in vivo* studies, Tamai *et al*. (1993) suggested that there was a functional substitution of MTs for the Cu, Zn-SOD (superoxide dismutase), protecting yeast cells from oxidative damage; (4) Investigations on the scavenger activity of MTs and, according to Atif *et al*. (2006), MTs from fish have the

ability to sequester ROS and NOS *in vitro*. Kumari *et al*. (1998) showed that isoforms I and II of hepatic MTs are able to scavenge free radicals. In addition, Miura *et al*. (1997) estimated that the peroxyl radical scavenger activity of MTs is 100 times greater than that of reduced glutathione (GSH).

However, some authors do not defend this idea, and provide doubts about the protective effect of MTs in the oxidative stress. For instance, the high MTs tissue concentration and the overexpression of MTs in transgenic-mice did not confer protection against the toxic effects produced by oxidative stress inducers (Liu *et al.*, 1999a; Conrad *et al.*, 2000).

The multiple cysteine residues of MTs can be oxidized by oxidizing agents, and the metals are released from the thiol groups of the protein, which facilitate their role as first defence against oxidative stress (Kang, 2006). So, the metal binding and antioxidant activity are two linked functions of MTs, because, on one hand, the binding of Cu ions to MTs prevents their toxic effects and, on the other hand, the release of Zn from MTs is involved in the redox activity of MTs (Capdevila *et al*., 2011). Moreover, the release of Zn has been proposed to be important in the protection against oxidative damage (Maret, 2000). Under normal physiological conditions, MTs exist in three different states: the metal associated form, the oxidized apoprotein (thionin) and the reduced apoprotein (thionein) (Achard-Joris *et al*., 2007). The role of MTs as an oxidative stress protector can be achieved in two ways: (1) by direct reacting with ROS, as demonstrated in some *in vitro* studies (Thornalley and Vašák, 1985; Abel and de Ruiter, 1989; Cai *et al.*, 2000); (2) or by distributing Zn, as this metal plays a role in protecting the cells from oxidative stress by several ways: protecting against lipid peroxidation, maintaining the adequate level of MTs, being an essential component of SOD, protecting thiol groups, preventing the free radical formation caused by the interaction between chemical groups with iron and stabilizing DNA (Chvapil *et al.*, 1972; Thomas *et al.*, 1986; Maret, 2008). Another implication of MTs in oxidative stress protection is that the involvement of MTs redox cycle is coupled to the GSH/GSSG (glutathione disulfide) metabolism, so it is indirectly related in the antioxidant activity of GSH as an intracellular ROS scavenger (Kang, 2006).

Despite the large body of literature on this topic, the exact functions and physiological roles involving MTs and the protection against oxidative stress remain

unsettled (Conrad *et al*., 2000; Davis *et al*., 2001). Although it is known that MTs are really involved in the protection of oxidative stress, it is not known their functioning. In this study we used the edible cockle *C. edule*, to investigate the role of MTs in oxidative stress. Few works have focused in the antioxidant activity of MTs in aquatic invertebrates. Nevertheless, Viarengo *et al*. (1999) suggested an antioxidant role for MTs in the mussel *M. galloprovincialis*, which seemed to occur through oxyradical scavenging. The work of Leung and Furness (2001) in dogwhelks (*N. lapillus)* showed that MTs were induced by metals and also by oxidative stress.

3.2 Objectives

In the previous chapter the evaluation of the MTs synthesis and lipid peroxidation levels in *C. edule* exposed to Cd and H_2O_2 showed that MTs are synthesized in response to metal contamination and to oxidative stress. The question that remained in the previous chapter was:

- Which is the role of MTs in oxidative stress? It is a Zn distributer or not?

Then, the objective of the work described in this chapter was to understand the role of MTs in oxidative stress, comparing the differences in the binding of MTs to Zn in the two situations. Furthermore, the evaluation of Cd amount bound to MTs was evaluated in the case of metal contamination, in order to understand how MTs protect against Cd effects in this bivalve.

To answer this question and achieve the goals, the following approaches were performed:

- Exposure of *C. edule* organisms to control conditions, 10 μM of Cd and 20 μM of H_2O_2;
- Determination of the oxidative stress level through the quantification of TBARS in the Cd and H_2O_2 exposed and control organisms;
- Isolation of the metal-metallothionein complexes by size exclusion chromatography to obtain a protein profile corresponding to each treatment and control;
- Quantification of Zn and Cd concentrations in the eluted fractions in each treatment and control;
- Comparison of the protein profiles with metal content between the treatments and the control to identify the distribution of metals among the different eluted proteins (Higher proteins, MTs and smaller molecules);

- Quantification of MTs in the control condition, Cd and H_2O_2 treatments.

3.3 Materials and Methods

3.3.1 Sampling and exposure experiment

C. edule specimens were collected in November 2010 in a low-contaminated branch of Ria de Aveiro (Portugal). Cockles (about 100 individuals) were maintained in laboratory at 20 ± 1 ºC with a photoperiod of 12:12h, using artificial light sources for one week period prior to the experiments. *C. edule* were fed with a concentrated algae culture (*P. subcapitata*) every day during both acclimatization and exposure experiments. The culture of microalgae was carried out at the laboratory under controlled conditions allowing us to obtain an axenic culture without trace metal exposure. Experiments were conducted in the laboratory using plastic containers of 11 cm x 12 cm x 14 cm with 1 L of clean sea water (salinity = 30) aerated by a diffuser system. A 3-cm layer of clean sediment was used to maintain similar environmental conditions, allowing the cockles to bury. After the acclimatization period, one group of cockles was exposed to 10 µM of Cd (as $CdCl_2$) and another group to 20 µM of H_2O_2 during 5 days with the conditions mentioned above. Five replicates for each treatment were used with 7 cockles per replicate. Three replicates with 7 cockles were also used to perform the control conditions (no metal or H_2O_2). The water was renewed every day to maintain the Cd and H_2O_2 levels during the experiment. In the end of the 5^{th} day, cockles were frozen at -20 ºC before the TBARS quantification and the extraction for the size exclusion chromatography.

3.3.2 TBARS quantification

Five cockles per treatment (control, 10 µM of Cd and 20 µM of H_2O_2) were weighed and homogenized individually in ice-cold phosphate buffer (50 mM, pH = 7.0 with 0.1% TRITON X-100). Homogenates were centrifuged at 10 000 x *g* for 10 min and supernatants were divided in three aliquots, one for TBARS quantification, another for protein quantification and the last for H_2O_2 quantification. TBARS were quantified by the procedure described in the chapter 2.

3.3.3 Hydrogen peroxide quantification

The H_2O_2 assay was based on horseradish peroxidase-dependent oxidation of phenolsulfonphthalein (phenol red) by H_2O_2 according to Pick and Keisari (1980) with some modifications. After the extraction, the supernatants were added to the previous prepared solution which consisted of phosphate (50 mM, pH 7.4) buffer with phenol red and horseradish peroxidase to a final concentration of 0.28 mM and 8.5 U/mL, respectively. The mixture was incubated at 25°C for 10 min with agitation. After the reaction was stopped by adding 20 µl of 1 M NaOH, the absorbance was measured at 610 nm against a blank containing phenol red, horseradish peroxidase, NaOH, and assay buffer at the appropriate concentrations. H_2O_2 concentrations were calculated from a standard curve made from dilutions of 30% H_2O_2.

3.3.4 Protein quantification

Protein concentration of each sample was determined by the procedure described in the chapter 2.

3.3.5 Metal-Metallothionein complexes isolation

3.3.5.1 Extraction procedure

Three cockles from each replicate were joined and constituted one replicate. At least three replicates for each treatment (Cd and H_2O_2) were homogenized with liquid nitrogen using a mortar and a pestle. The buffer used was 100 mM (pH 8.6) HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 1 mM phenylmethylsulfonyl fluoride and 0.2% Tween 20 (v/v). The extract was centrifuged at 10000 x *g* during 10 min at 4 ºC and the supernatant collected. The cell debris was washed 2 more times with the same buffer, followed by centrifugations at 10000 x *g* during 10 min at 4 ºC. The supernatants were pooled, constituting the material for the separation of metallothionein complexes. Two sub-samples of the extract were made for the total protein determination and for size exclusion chromatography.

3.3.5.2 Size exclusion chromatography

The extracts were fractionated by gel filtration, according to Rauser (2000) in a Sephacryl S-100 (25 x 0.5 cm i.d., Amershan Biosciences) column. The column was equilibrated with degassed elution buffer 10 mM HEPES and 300 mM KCl. Samples (500 µl) were eluted at a flow rate of 1 $mL.min^{-1}$ at room temperature. The absorbance was monitored at 254 nm and the fractions collected every 3 min intervals. All fractions were sub-sampled for subsequent Cd and Zn quantification and for analysis of MTs by HPLC. For each treatment and control, at least three replicates were performed.

3.3.6 Metal quantification

Cd and Zn present in the fractions from the size exclusion chromatography were quantified by ICP-MS (Inductively Coupled Plasma Mass Spectrometry).

3.3.7 MTs quantification

3.3.7.1 Sample preparation and derivatization

Metallothioneins were determined according to Alhama *et al*. (2006), with some adaptations. The pooled fractions from MT peak (300 µl) were reduced, denatured and derivatized by the procedure described in the chapter 2.

3.3.7.2 HPLC analysis

Derivatized proteins were separated by RP-HPLC by the procedure described in chapter 2.

3.3.8 Statistical analysis

Data were submitted to hypothesis testing using the PERMANOVA routine (permutational multivariate analysis of variance) from PRIMERv6 (Clarke and Gorley, 2006; Anderson *et al.*, 2008) following the calculation of Euclidean distance matrices among samples. The pseudo-F values in the PERMANOVA main tests were evaluated in

terms of significance among different treatments. When the main test revealed statistical significant differences, pairwise comparisons between the treatments and control were performed. The t-statistic in the pair-wise comparisons was evaluated in terms of significance among treatments. Values lower than 0.05 were considered as significantly different. The null hypotheses tested were: for each MTs, TBARS, H_2O_2 and Zn values: no significant differences exist between treatments and control.

3.4 Results

3.4.1 Lipid peroxidation levels in *C. edule* after Cd and H_2O_2 exposures

The quantification of TBARS was performed in order to guarantee that the selected concentrations provided similar results of those in chapter 2. TBARS values (Figure 10) indicated that both treatments induced significant levels of lipid peroxidation, but the H_2O_2 treatment showed a higher significance than Cd treatment (P = 0.0011 for Cd and P = 0.0009 for H_2O_2). The level of lipid peroxidation in this experiment showed to be higher than the level obtained in the early experiments for both treatments and also for control organisms. Nevertheless, in the H_2O_2 treatment lipid peroxidation was 2.7 times higher than in the Cd treatment.

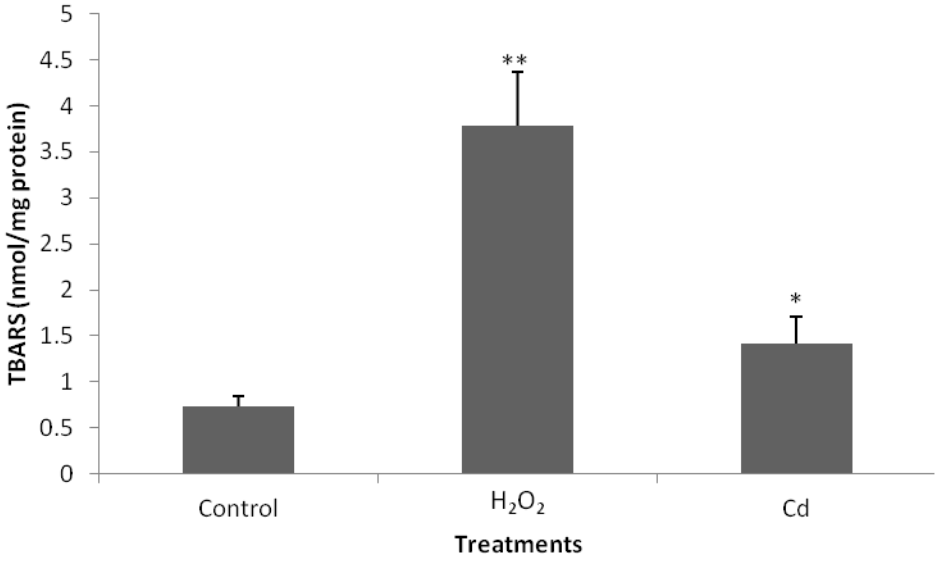

Figure 10. Lipid peroxidation levels estimated by measuring the concentration of thiobarbituric acid reactive species (TBARS) in the whole soft tissues of *C. edule* organisms exposed to control, H_2O_2 (20 µM) and Cd (10 µM) for 5 days, expressed as nmoles of malondialdehyde (MDA) per mg of protein. Values represent the mean of 3 replicates with SE. * and ** indicates significance at the $P < 0.01$ and $P < 0.001$ level, respectively.

3.4.2 Hydrogen peroxide levels in *C. edule* exposed to Cd and H_2O_2

The amount of H_2O_2 in the cytosol was determined in order to ascertain if both treatments (H_2O_2 and Cd) were generating reactive oxygen species. Data presented in Figure 11 showed that exposure to H_2O_2 strongly enhanced the intracellular concentration of hydrogen peroxide ($P = 0.0006$ for H_2O_2). The H_2O_2 amount in H_2O_2 treated cockles was 7.5 times higher than Cd treated cockles and 53.7 times higher than the control. Since H_2O_2 is a ROS, its presence in cells is reflects their redox status. In this case, data showed that organisms exposed to H_2O_2 are in a higher level of oxidative stress than Cd exposed cockles, which sowed higher H_2O_2 levels than the control but the significance was very low ($P = 0.0138$).

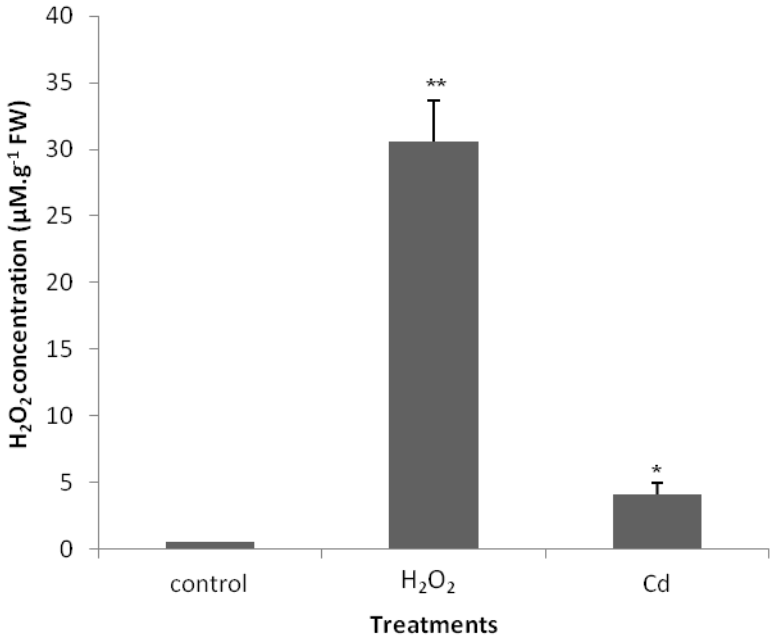

Figure 11. Hydrogen peroxide levels estimated in the whole soft tissues of *C. edule* exposed to H_2O_2 (20 µM), Cd (10 µM) and control during 5 days. Values represent the mean and standard error of 3 replicates with SE. * and ** indicates significance at the $P < 0.05$ and $P < 0.001$ level, respectively.

3.4.3 Metal-Metallothionein complexes in Cd and H_2O_2 exposures

Size exclusion chromatography separated proteins by their molecular weight. Our procedure conditions, namely the buffer used to extract the cytosolic proteins, prevented the

dissociation of metals from proteins. By this way, the isolation of metal-MTs complexes was possible. The chromatographic profiles (Figure 12) show the elution pattern in the treatments and control of the proteins. The metals, Zn and Cd, associated to each chromatographic fraction are also presented.

Concerning the high absorbance at 254 nm, which is very characteristic of MTs (Bebianno and Langston, 1991; Serra *et al*., 1995; Simes *et al*., 2003), and the posterior quantification (in the section 3.4.4), we assumed that fractions between 9 and 16 corresponded to a MTs peak. Fractions before 9 were classified as higher molecular weight (HMW) proteins while fractions after 16 were named smaller molecular weight (SMW) proteins. For each treatment (Figure 12B and C) and control (Figure 12A) a typical chromatographic profile is shown. Similar chromatograms were obtained for H_2O_2 and Cd treated cockles, while for control cockles a different profile was obtained.

The distribution of metals among fractions was also different between treatments (Figure 12 and Table 2). The pattern of Zn distribution was similar in the control and Cd treated organisms, although the percentages of Zn in HWM and MTs pool showed to be different. Zinc was mainly associated with the MTs fractions in control (53.2 %) but in both treatments (H_2O_2 and Cd) the percentage decreased (45 and 31.5 %, respectively). In the Cd treatment, the percentage of Zn was higher in the HMW fractions (68.4 %). The percentage of Zn in the HMW fractions was similar in the H_2O_2 treatment and in the control organisms (32.7 and 36.8 %, respectively).

Regarding soluble Cd distribution between the chromatographic fractions, Cd was absent in control and H_2O_2 treatments but was present in the Cd treated organisms. The major part of intracellular soluble Cd was bound to the MTs fractions (79.2 %) and a small portion to the HMW fractions (20.8 %), indicating that MTs was important to prevent the non-specific binding of soluble Cd ions to biomolecules such as enzymes.

The presence of both Cd and Zn ions in the MTs fractions of Cd-treated organisms suggested that MTs were capable to bind the two metals, although the amount of Zn bound to MTs was higher than Cd.

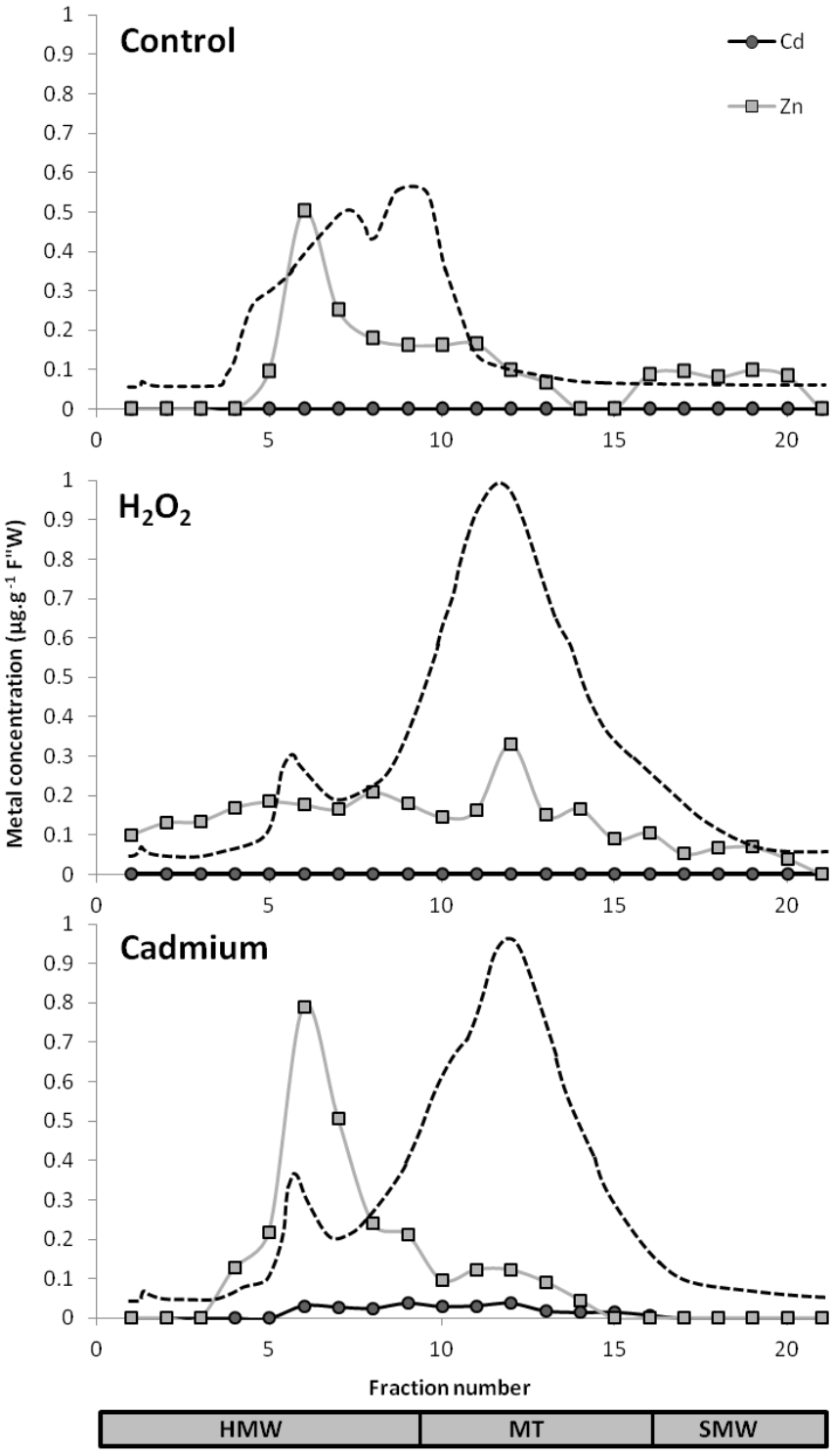

Figure 12. Chromatographic Sephacryl S-100 profile of soluble cytosolic proteins of *C. edule* after 5 days of exposure to control, 20 µM H_2O_2 and 10 µM Cd. The elution was made with 10 mM HEPES, 300 mM KCl, pH 8.6 buffer. Each fraction was analyzed for Cd (circles) and Zn (squares). The dashed line indicates the absorbance at 254 nm (protein content).

Table 2. Percentages of zinc and cadmium in the higher molecular weight (HMW) proteins pool and in the metallothioneins (MTs) pool resulted from size exclusion chromatography in Cd (10 μM) and H_2O_2 (20 μM) treatments and control.

	Zinc	
Treatments	HMW proteins	MTs pool
Control	32.7 %	53.2 %
H_2O_2	36.8 %	45.0 %
Cd	68.4 %	31.5 %
	Cadmium	
Cd	20.8 %	79.2 %

3.4.4 Metallothioneins response upon Cd and H_2O_2 exposures

The fractions belonging to MTs peak were pooled and analyzed by RP-HPLC in order to quantify MTs concentration in each treatment and control (Figure 13A). Data confirmed the results obtained in the preliminary experiment, which were very similar and indicated that the Cd and H_2O_2 selected concentrations (10 μM and 20 μM, respectively) induced the synthesis of similar amounts of MTs ($P > 0.05$). The control showed clearly higher values when compared to the previous experiment (0.0061 $\mu g.g^{-1}$), but the level of MTs synthesis still maintained significantly lower compared to the treatments ($P < 0.01$). Additionally, we confirmed that the fractions between 9 and 16 corresponded to the MT peak.

Analyzing the amount of MTs molecules in each treatment and comparing it with the amount of Zn associated with MTs (Figure 13B), we observed that the amount of MTs synthesized in the H_2O_2 treatment increased about 4.2 times compared to control while the amount of Zn associated with MTs in the H_2O_2 treatment increased only approximately 2.7 times when compared to the control. The increase of MTs synthesis was not followed by a similar increase in the amount of Zn associated with MTs molecules, indicating that MTs molecules in H_2O_2 treatment (oxidative stress) were not as metalated as the control MTs.

This confirmed the release of Zn from MTs and suggested that MTs could not only be required for distribution of Zn but also for other demands of cells in oxidative conditions.

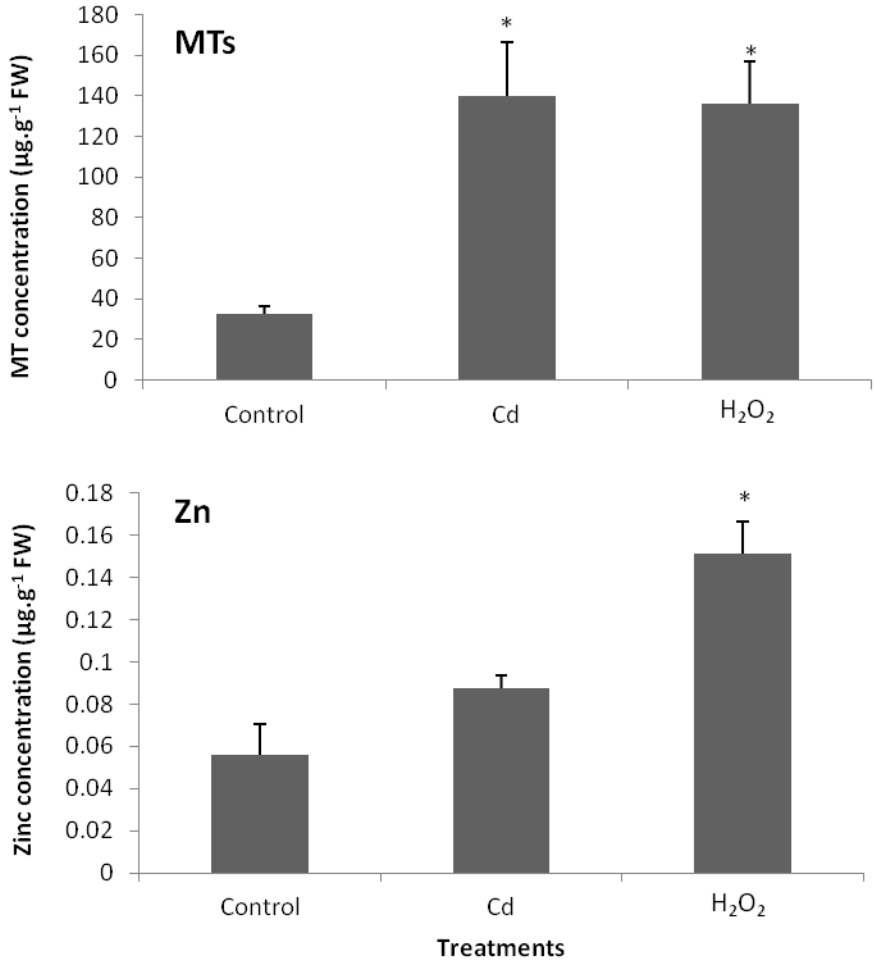

Figure 13. Metallothioneins and Zn concentration (µg.g^{-1} FW) in the pooled fractions belonging to the second peak (MTs pool) resulted from the size exclusion chromatography in *C. edule* whole soft tissues exposed to Cd (10 µM) and H_2O_2 (20 µM) and in the control condition during 5 days. Values are the mean of 3 replicates with SE. * indicates significance at the $P < 0.01$ level compared to control.

3.5 Discussion

In the present study we attempted to achieve the MTs antioxidant response using cockles as the experimental organism. This bivalve species, besides being used as a bioindicator of contamination, inclusively with the quantification of MTs as metal-specific biomarker, is also easy to manipulate and possesses a MTs that is similar to mammalian MTs, which are the most well-known MTs (Amiard *et al*., 2006). Furthermore, cockle MTs showed to be induced by the two simulated situations, as demonstrated by the experiment described in Chapter 2. In this work we exposed cockles to Cd and H_2O_2 to simulate metal contamination and oxidative stress. Metal contamination is the condition best studied for MTs response, so this situation was used, together with control, not only to analyze the MTs response as a metal chelator but also to compare the MTs antioxidative response.

Firstly (in the Chapter 2), we selected one concentration for Cd and H_2O_2 which provided the base for the study of the role of MTs in oxidative stress and Cd detoxification. These concentrations were then used in the subsequent work (in the present chapter) in order to isolate metal-MT complexes and analyze the distribution of metals between the different cytosolic molecules (proteins and enzymes). By this way, it was possible to verify the percentages of Zn and Cd bound to MTs when organisms were under oxidative stress and exposed to Cd. With this procedure, we tried to understand the physiological roles of MTs in the protection against metals and oxidative stress.

Indeed, the selected concentrations (10 μM of Cd and 20 μM of H_2O_2) provided two different conditions: Oxidative stress imposed by H_2O_2 and Cd contamination with very low levels of lipid peroxidation. Cockles exposed to H_2O_2 had much higher amounts of lipid peroxidation and H_2O_2 in their cytosol than Cd exposed organisms, indicating again that these organisms were in presence of a high concentration of this reactive oxygen species. When H_2O_2 enters the cells, it is normally transformed into H_2O and O_2 by the antioxidant enzymes catalase and peroxidases (Storey, 1996). In this case, H_2O_2 concentration in the H_2O_2 treatment was more than 50 times higher than in the control, possibly due to the failure of the antioxidant defence system, namely catalase activity. In fact, the results from Cavalleto *et al*. (2002) showed that the activity of antioxidant enzymes decreased to the same level of controls after 7 days of exposure to 20 μM of H_2O_2

in *M. galloprovincialis*. This can result in the higher presence of H_2O_2 molecules free to react with metals like Fe and Cu, originating hydroxyl radicals, as indicated by the results of Viarengo *et al*. (1999), which observed an increased production of hydroxyl radicals after exposure of *M. galloprovincialis* to 20 μM of H_2O_2. This increased production of hydroxyl radicals are extremely toxic to biomolecules, and interfere with the normal physiology of cells (Stohs and Bagchi, 1995). Thus, both lipid peroxidation and H_2O_2 quantification indicated that H_2O_2 induced a much higher level of oxidative stress than Cd, possibly by the higher production of hydroxyl radicals. This was very important to clarify because metal ions are known to induce also oxidative stress (Cuypers *et al*., 2010), so we guaranteed that Cd concentration used did not impose the same effect that H_2O_2 treatment. Furthermore, the levels of lipid peroxidation obtained in this Chapter were higher than those obtained in the Chapter 2.

MTs response upon Cd exposure is currently well-documented through size exclusion chromatography. The occurrence of a peak corresponding to MTs with a strong absorbance at 254 nm and no absorbance at 280 nm, was always reported in these chromatographic profiles. For example, Simes *et al*. (2003) obtained a peak at the 13700 Da position in the clam *R. decussatus* exposed to Cd (100 $\mu g.L^{-1}$) during 20 days. Serra *et al*. (1995) investigated the Cd accumulation and its sequestration mechanisms in the cytosol in the bivalve *Scapharca inaequivalvis* after exposure to 0.5 $\mu g.mL^{-1}$ of Cd during 28 days. They also performed size exclusion chromatography and obtained a Cd-containing peak with a molecular weight of 10 kDa, showing that Cd was mostly associated to MT-like proteins.

However, the bulk of reports that performed size exclusion chromatography in Cd exposed organisms aimed to evaluate the presence of MTs, explaining Cd accumulation in tissues. The present study, by the other hand, aimed to evaluate the synthesis of MTs on bivalve response to oxidative stress. Using the same criteria that Giguère *et al*. (2003) did in their article, the protein profile obtained from the size exclusion chromatography was divided into three different cytosolic pools for a better interpretation of the results: HMW (higher molecular weight) fractions, which according to these authors corresponded to the pool of enzymes of higher molecular weight; the MTs fractions, which comprises the molecular weight corresponding to the characteristics of MTs (18-6 kDa); and the SMW

(smaller molecular weight) fractions, which can include amino acids and very small peptides. The Cd treatment showed an expected chromatographic profile, being the majority of soluble intracellular Cd associated with the MT peak (79.2 %). The exposure of *P. grandis* (mussel) to metals (including Cd) in field resulted in 80.6 % of cytosolic Cd bound to the MTs pool, whereas 6.6 % was associated with the LMW fraction and 12.9 % was bound to the HMW pool (Giguère *et al*., 2003). The percentage of Cd bound to the MTs pool was similar to the present study, highlighting the role of MTs in Cd detoxification. In oyster *C. gigas* MTs bound 67% of cytosolic Cd but only after 3 months of exposure to a high Cd contaminated area (Mouneyrac *et al*., 2002). Berthet *et al*. (2003) showed that the percentage of cytosolic Cd bound to MTLPs varied between 9-47% for Cd, in the worm *Hediste diversicolor* collected from Cd contaminated sites (ranging from 0.05 to 2.76 $\mu g.g^{-1}$ of sediment). The amount of Cd bound to MTs was higher in the present work, possibly due to the exposure of lower amounts of Cd in our experiment.

Relatively to Zn percentage, the study of Berthet *et al*. (2003) showed that 14 to 21 % of Zn was bound to the MTLPs fractions, which were also under the results from the present study in *C. edule* (31.5 %). When analyzing the percentage of Zn associated with the MTs pool in control and Cd treatment (53.2 and 31.5 %, respectively), we observed that some Zn ions were displaced from MTs. This displacement of Zn from MTs could be indicative of two situations. Since the binding affinity of Cd for MTs is several orders of magnitude higher than Zn, it displaces Zn ions from the cysteine residues of MTs molecules. Therefore, Cd renders inoffensive to other biomolecules in the cell, not perturbing the metabolism and not leading to cellular damage. Furthermore, Cd-MT complexes are transported to lysosomes and Cd ions are stored as metal-rich granules that can be excreted from the cell. The other consequence that arrives from Zn displacement from MTs is the higher availability of free Zn to activate MTF-1 and induce the synthesis of more MTs molecules, which in turn will help the cell to overcome the excess of toxic metals. Although the majority of cytosolic Cd was bound to MTs pool, some of the Cd ions were found associated to the HMW fractions, which can mean that some Cd atoms were capable of non-specifically bind other proteins such as enzymes. These binding of Cd ions explained the higher level (but less significant than H_2O_2 treatment) of lipid peroxidation in the Cd treatment compared to control.

Taking into account the very high *in vitro* reactivity of MTs with the hydroxyl radical ·OH (Thornalley and Vašák, 1985), it has been speculated that the antioxidant role of MTs could mainly consist in a scavenging activity of this dangerous ROS, a view also reinforced by the fact that no specific enzyme for hydroxyl radical inactivation is known (Stohs and Bagchi, 1995). Although MTs can act as an oxyradical scavenger, little is known about the mechanisms by which MTs antioxidant effects can be accomplished.

Many studies actually indicated and antioxidant role of MTs, but some did not, contributing to the complex picture about the role of MTs in the cellular antioxidant defense (Sato and Bremner, 1993). The majority of the studies was performed *in vitro*, but it is questionable if it is trustworthy to transpose these findings to *in vivo* systems. Our work does not analyze the reactivity of MTs to ROS, but evaluates the antioxidant response of MTs in the whole organism, trying to enrich the knowledge about MTs protection in real systems when exposed to oxidative conditions.

In our work, size exclusion chromatography showed that Zn distribution among cytosolic proteins and enzymes was altered during oxidative stress. Zinc, which in the control was mainly bound to the MTs pool, in the H_2O_2 treatment the percentage of Zn associated to MTs decreased, meaning that MTs had a preponderant role in Zn distribution in oxidative stress. This result was reasonable if we have in mind that Zn plays a role as an antioxidant due to its involvement in the stabilization of the cell membrane and DNA structure, in the contribution for the activity of SOD (as a cofactor), in the prevention of the interaction between chemical groups with iron (which otherwise could form free radicals) and also in the maintenance of the level of MTs concentration in cells (Stefanidou *et al*., 2006). Thus, MTs seemed to behave as a metallochaperone, donating or binding Zn according to the needs of the cells, which is substantiated by our work. This is possible due to the existence of a range of different affinities of Zn for MTs binding sites which allows the rapid metalation and demetalation of Zn in MTs (Krężel and Maret, 2007).

In fact, both reduced (metalated) and oxidized forms of MTs were detected *in vitro* and *in vivo* studies, suggesting that MTs coexists in different states in cells (Maret, 2008). Because the mechanism of metalation in MTs is non-cooperative, MTs are not always saturated by ions, as though earlier. Instead, MTs can exist in a form partially metalated and there is also the possibility to have a MT that is partially oxidized, maintaining some of

metals bound (Sutherland and Stillman, 2011). The oxidation of the metallic MTs releases Zn in the intracellular environment under oxidizing conditions (Formigari *et al*., 2007). In this work, results showed a decrease in the percentage of Zn associated with MTs compared to control, suggesting that MTs were having a place in the distribution of Zn in the cell. In fact, when analyzing the amount of MTs that were synthesized in H_2O_2 treatment, we observe that H_2O_2 induced 4.2 times more MTs than control. The increase in the amount of Zn associated to MTs in H_2O_2 treatment was not proportional to the increase of MTs synthesis, meaning that MTs were less metalated in H_2O_2 treatment than in the control. This can implicate that MTs, besides of being produced to modulate Zn distribution, were also used to other demands, namely to scavenger ROS.

Gagné *et al.* (2008) suggested that Zn was efficiently released from MTs in the presence of superoxides and was sequestered by MTs in the presence of reducing agents such as glutathione and NADH. So, metal release is a mechanism that allows MTs to be available to react with ROS in the case of oxidative stress. Indeed, it was observed that MTs were capable of forming disulfide bonds after the release of Zn (Feng *et al*., 2006). The number of disulfide bonds increases in oxidative stress conditions and can be formed between cysteine residues from the same molecule (between α and β domains) and also between two MTs molecules, originating dimeric proteins (Romero-Isart and Vašák, 2002). The work of Gagné *et al*. (2008) showed that about 46% of Zn-MTs were oxidized in the control and this oxidized form increased to 55% in the presence of superoxide radicals and from to 60% in the presence of H_2O_2, corroborating the mechanism of release of Zn from MTs in oxidative stress. The present study suggested that MTs were involved in the distribution of Zn and also in the scavenging of reactive oxygen species in oxidative stress conditions, which was indicated by the percentage of Zn associated to MTs and by the comparison between Zn ions and MTs amount in the H_2O_2 treatment.

3.6 Conclusions

The objective of this work was to understand the effective role of MTs in metal contamination, and especially in oxidative stress. Both conditions were successfully accomplished given that Cd contamination and H_2O_2 exposure both similarly induced MTs and only H_2O_2 treatment induced very significant levels of oxidative stress.

In the Cd treatment, Zn was mainly bound to high molecular weight cytosolic proteins. This demonstrated the role of MTs in Cd contamination due to the fact that displacement of some of Zn ions from MTs prevented a high level of non-specific binding of Cd ions to important enzymes and proteins. So, lipid peroxidation was low in the Cd treatment due, in part, to this protective role of MTs

The chromatographic profile suggested a different behaviour in the distribution of Zn in both treatments and control. The percentage of Zn associated to the MTs pool in the oxidative stress (H_2O_2 treatment) was lower than in the control. This suggested that MTs and Zn are both involved in oxidative stress conditions. The lower percentage of Zn in the MTs pool indicated that Zn ions were released from MTs, leaving the molecules to be available for their antioxidative functions.

The quantification of MTs content in the MTs fractions revealed that MTs molecules bound less Zn ions in the H_2O_2 treatment than in the control. We suggested that MTs were less metalated in H_2O_2 treatment, possibly because their cysteines were used to scavenge ROS. This Zn release may be a mechanism that acts to donate Zn-dependent proteins and enzymes that are important in oxidative stress conditions and also allows enough Zn ions available to induce the synthesis of more MTs molecules. Further studies would provide the answers that remained opened in this study: Is Zn released from MTs to act itself in the protection against oxidative damage or is Zn released because MTs are needed for ROS elimination?

CHAPTER 4

4. General conclusions and considerations

The main propose of this work was to obtain a better understanding about the role of MTs in oxidative stress. In the 54 years of MTs's history, a lot of work has been done concerning the roles of this unique protein. Nevertheless, until now their main and primary role was not yet clarified. Indeed, a lot of researchers believe that it is a waste of time to try to find a specific and unique role for MTs because they are "multipurpose proteins" (Coyle *et al*., 2002) that are involved in numerous biological processes. However, there is no doubt that their biological functions are supported by their capacity to bind metals and by their redox activity (Capdevila *et al*., 2011).

Due to the wide aquatic contamination of nowadays, the monitoring of pollution levels is of great importance to assess the health status of the environments. Some metals may become persistent metallic compounds, which can be bioaccumulated in the organisms, be transferred along the food chain and reach human health (Zhou *et al*., 2008). Concerning this problem, in recent years biomarkers have been extensively studied and validated in the monitoring of environmental contamination, including metal pollution (Sarkar *et al*., 2006). MTs, as proteins that are inducible by metals, are recognized among the suite of biomarkers of metal pollution (Amiard *et al*., 2006). However, they are also induced by other type of stimuli such as ROS, protecting cell components from oxidative stress (Capdevila *et al*., 2011). Consequently, their use as metal specific biomarkers could be questioned. The present work suggested that MTs were induced by Cd and H_2O_2. Although MTs synthesis upon Cd exposure was stronger than H_2O_2 exposure in the lower concentrations, the level of MTs synthesis was similar in the highest Cd and H_2O_2 concentrations, meaning that the role of MTs in the redox state of cells limits their use as a metal-specific biomarker. In situations where organisms are exposed to other contaminants able to cause oxidative stress, the determination of the total MTs concentration in organisms can lead to an overestimation of the metal contamination level. Furthermore, it has been suggested that the different MTs isoforms play different functions in organisms (Miles *et al*., 2000). In this perspective, in a future work, the study of the response of the

different MTs isoforms in Cd and H_2O_2 exposure would be of interest in order to evaluate the role of each isoform in each situation.

The role of MTs in metal chelation was underlined in this investigation since the major part of the soluble intracellular Cd was bound to MTs, preventing the harmful effects of non-specific binding of Cd ions to important biomolecules. The protecting effect of MTs in this situation was evidenced by the high survival percentages of cockles and the lower lipid peroxidation levels when compared to H_2O_2 exposure. However, for a further understanding of MTs protection of Cd effects, a more global analysis of the antioxidant defence system of the organism would be evaluated in a future work. It would be interesting to find out if the antioxidative enzymes (SOD, CAT and peroxidases) or glutathione were active in the protection of Cd effects, namely helping with the possible increased production of ROS caused by Cd ions. By this way it may be discussed if MTs activity was the preponderant defence mechanism or if it was only one more protein helping between the set of antioxidant protectors.

Because Zn is one of the most important trace metals in the organism, being involved in immune responses, apoptosis, ageing and, oxidative stress (Stefanidou *et al*., 2006), in the present work we analyzed the distribution of Zn between the different cytosolic proteins in order to evaluate the role of MTs in oxidative stress. The involvement of MTs with Zn homeostasis is clear given that under physiological conditions, Zn-MT is the predominant form of the metal-binding protein (Miles *et al*., 2000). Our results suggested that in oxidative stress the percentage of Zn associated with MTs was lower than in the control condition. Furthermore, MTs molecules in the H_2O_2 treatment were not as metalated as in the control. Thus, we were not able to identify a separated physiological role for MTs in oxidative stress. Instead, we suggested that MTs satisfied two cellular needs in this bivalve: due to their affinity to metals, they constituted a Zn reservoir to respond to the Zn cellular demands; and, due to their high thiol content, they were possibly used to scavenger ROS. The evaluation of the redox status of MTs would be appropriated to determine the proportion of MTs that was oxidized in the case of oxidative stress, which would indicate if they were acting mainly as a ROS scavenger. Furthermore, the evaluation of Zn-MT complexes should be performed at the beginning of H_2O_2 exposure to observe if the binding or release of Zn to or from MTs occurred immediately after cells were

experiencing oxidative stress signals or if MTs were readily used as ROS scavenger as a first defense line.

5. References

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