**Universidade do Minho**
Escola de Ciências

Maria Manuel da Silva Azevedo

# Toxicity of metals in aquatic hyphomycetes: cellular targets and defense mechanisms

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Ph.D. Thesis in
Sciences

Work Supervised By
Prof. Dr. Fernanda Cássio

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I compare this phase of my life to a big and agitated boat trip where a lot of things have happened, however this journey is over, I have resisted and the final balance is highly positive.

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## Abstract

Human activities contribute to a high release of heavy metals to the environment at rates and concentrations sufficient to make them pollutants. Certain metals, such as Cu and Zn, are needed for the growth and metabolism of organisms, while others, as Cd, have no recognized biological functions. However, above critical levels, both essential and non-essential metals became toxic to living organisms.

Aquatic hyphomycetes are a polyphyletic group of fungi that play a key role in plant-litter decomposition in streams. They produce an array of exoenzymes able to degrade plant cell-wall polymers and increase plant-litter palatability for invertebrate detritivores. Even though these fungi occur in metal-polluted streams, the mechanisms underlying their resistance/tolerance to metals are poorly documented.

In this study, the exposure to metals inhibited reproduction, as sporulation rates, of the aquatic hyphomycetes. Moreover, fungal reproduction was more sensitive to metals than growth. The sensitivity of aquatic hyphomycetes to metals, assessed as the metal concentration inhibiting biomass production in 50% ($EC_{50}$), showed that *Ypsilina graminea* and *Varicosporium elodeae* were the most resistant species to Zn, while *Heliscus submersus* was the most resistant to Cu. The $EC_{50}$ values were about 20-times higher in solid medium than in liquid medium. However, the patterns of species resistance to metals in either liquid or solid medium, with similar composition, were identical. Generally, Ni or Cd were more toxic than Zn or Cu to fungi. *H. submersus* and *V. elodeae* had remarkable ability to adsorb Cu and Zn, respectively. Because these fungal species were highly tolerant to each metal, biosorption may be a relevant mechanism to avoid unrestrained uptake of metals.

We demonstrated that the generation/accumulation of reactive oxygen species (ROS) contributed noticeably to metal toxicity in aquatic hyphomycetes, particularly under Cu stress, as indicated by a recovery in biomass production by the presence of an antioxidant agent. Our results showed that plasma membrane integrity of *V. elodeae* and *H. submersus* was more affected by Cu than Zn, pointing to this cellular structure as a potentially vulnerable target of Cu. At short-term (10 min), Cu completely inhibited the activity of the plasma membrane $H^+$-ATPase of *H. submersus* and *V. elodeae*, while Zn only led to a similar effect on that of *H. submersus*. However, a recovery of plasma membrane integrity was observed after 150 min of metal exposure. A strong stimulation

of the proton pump was found in the most tolerant species (i.e. when *H. submersus* was exposed to Cu and *V. elodeae* was exposed to Zn) at longer times (8 days). The activation of $H^+$-ATPase may contribute to counteract metal-induced dissipation of the electrochemical gradient of protons across the plasma membrane, suggesting that $H^+$-ATPase may be involved in aquatic hyphomycete acclimation to metals.

Our studies on antioxidant defenses showed that catalase had a greater role in alleviating the stress induced by Cu and Zn than superoxide dismutase. In addition, the increased activity of glucose-6-phosphate dehydrogenase, after long-term exposure to metals (8 days), points to the involvement of the pentose phosphate pathway in metal acclimation. Before metal exposure, *H. submersus* and *Flagellospora curta* isolated from a metal-polluted stream had higher levels of thiol compounds than *V. elodeae*, isolated from a clean stream. However, the latter species rapidly increased the levels of thiols after metal exposure. These findings are in agreement with the recognized role of thiol compounds as metal sequesters and/or ROS scavengers.

Finally, we showed that Cu and Zn are able to induce programmed cell death (PCD) in aquatic hyphomycetes, a process in which cells actively participate in their own death. The exposure to Cu promoted ROS production and caspase activation in *H. submersus* and *F. curta*. Conversely, under Zn stress, aquatic hyphomycetes showed high number of cells with nuclear morphological alterations and/or DNA strand-breaks. The different pattern of PCD markers suggests that the triggering cell death signal is most probably related to different cellular targets for Cu and Zn in aquatic hyphomycetes.

## Resumo

As actividades humanas contribuem para o aumento da libertação de metais pesados para o ambiente, a taxas e a concentrações que os tornam poluentes. Alguns metais, como o Cu e o Zn, são necessários para o crescimento e metabolismo dos organismos, enquanto que outros, como o Cd, não lhes é atribuída qualquer função biológica. Contudo, acima de certas concentrações, os metais, quer os essenciais quer os não essenciais, tornam-se tóxicos para os organismos vivos.

Os hifomicetos aquáticos são um grupo de fungos filogeneticamente heterogéneo que desempenham um papel chave na decomposição dos detritos vegetais nos rios. Estes fungos produzem um conjunto de enzimas extracelulares capazes de degradar os polímeros das paredes das células vegetais aumentando a palatabilidade dos detritos vegetais para os invertebrados detritívoros. Apesar dos hifomicetos aquáticos estarem presentes quer em rios de referência quer em rios poluídos com metais pesados, os mecanismos subjacentes à sua resistência/tolerância aos metais são pouco conhecidos.

Neste estudo, a reprodução dos fungos, avaliada pela taxa de esporulação, foi inibida pela exposição aos metais. Além disso, a reprodução dos fungos foi mais sensível aos efeitos negativos dos metais do que o seu crescimento. A sensibilidade dos hifomicetos aquáticos aos metais, avaliada pela concentração de metal capaz de inibir a produção de biomassa em 50% ($EC_{50}$), mostrou que *Ypsilina graminea* e *Varicosporium elodeae* foram as espécies mais resistentes ao Zn, enquanto que *Heliscus submersus* foi a mais resistente ao Cu. Os valores de $EC_{50}$ foram cerca de 20 vezes mais elevados em meio sólido do que em meio líquido. Porém, os padrões de resistência aos metais exibidos pelos hifomicetos aquáticos foram semelhantes em meio sólido ou em meioo líquido com idêntica composição química. Geralmente, o Ni ou o Cd foram mais tóxicos do que o Zn ou o Cu. *H. submersus* e *V. elodeae* exibiram capacidade elevada para adsorver, respectivamente, Cu e Zn. Dado que estes fungos foram muito tolerantes a esses metais, a bioadsorção pode constituir um mecanismo relevante para controlar a entrada dos metais nas células.

Neste trabalho demonstrámos que a produção de espécies reactivas de oxigénio (ROS) contribuiu notavelmente para a toxicidade dos metais, sobretudo no caso do Cu, como indicado pela recuperação da biomassa produzida pelos fungos na presença de um agente antioxidante. A integridade da membrana plasmática de *V. elodeae* e de *H.*

*submersus* foi mais afectada pelo Cu do que pelo Zn, sugerindo que esta estrutura celular pode ser um alvo potencial para o Cu. A tempos curtos de exposição (10 min), o Cu bloqueou a actividade da $H^+$-ATPase da membrana plasmática de *H. submersus* e de *V. elodeae*, enquanto que o Zn só promoveu um efeito semelhante em *H. submersus*. Contudo, uma recuperação da integridade da membrana plasmática foi observada a tempos mais longos (150 min). Após 8 dias de exposição, um estímulo forte da bomba de protões foi encontrado nas espécies mais tolerantes, i.e. em *H. submersus* exposto a Cu e em *V. elodeae* exposto a Zn. A activação da $H^+$-ATPase pode contribuir para contrabalançar a dissipação do gradiente electroquímico de protões induzida pelo metal, sugerindo o envolvimento desta bomba na aclimatação dos fungos ao stress metálico.

Os nossos estudos sobre as defesas antioxidantes mostraram que a catalase teve um papel mais importante do que a superóxido dismutase na mitigação do stress induzido pelo Cu e pelo Zn. Além disso, o estímulo da actividade da glucose-6-fosfato desidrogenase após 8 dias de exposição aos metais, sugere o envolvimento da via das pentoses na aclimatação dos fungos aos metais. As espécies *H. submersus* e *Flagellospora curta*, isoladas de um rio poluído com metais, tinham níveis mais elevados de compostos ricos em grupos tiol do que a espécie *V. elodeae*, isolada de um rio de referência. Contudo, esta última espécie aumentou rapidamente o seu conteúdo em compostos tiólicos após a exposição ao metal. Estes resultados estão de acordo com o reconhecido papel dos compostos tiólicos na sequestração de metal e/ou ROS nas células.

Finalmente, os nossos resultados mostraram que o Cu e o Zn foram capazes de induzir morte celular programada (PCD) em hifomicetos aquáticos, um processo de morte celular activa. O stress induzido pelo Cu estimulou sobretudo a produção de ROS e a actividade das caspases em *H. submersus* e em *F. curta*. Por outro lado, os hifomicetos aquáticos expostos a Zn mostraram um elevado número de células com alterações morfológicas no núcleo e/ou quebras na cadeia de DNA. O diferente padrão de resposta dos marcadores de PCD sugere que o sinal de morte celular pode estar relacionado com diferentes alvos celulares do Cu e do Zn em hifomicetos aquáticos.

## TABLE OF CONTENTS

**Chapter 1**
**General introduction**
1.1. Aquatic hyphomycetes in streams 3
1.2. Metal pollution in aquatic life 4
1.3. Cellular mechanisms involved in metal detoxification and tolerance 4
1.3.1. Extracellular complexation 4
1.3.2. Cellular barriers against metal stress 5
1.3.2.1. Cell wall 5
1.3.2.2. Plasma membrane 6
1.3.2.3. The role of $H^+$-ATPase 7
1.3.2.4. The role of efflux pumps and vacuole 9
1.3.2.5. Cu and Zn transport and its transcriptional regulation 9
1.3.3. Intracellular complexation of heavy metals: the role of thiol compounds 10
1.3.4. Oxidative stress induced by metals 12
1.3.4.1. Enzymatic and non-enzymatic defenses against oxidative stress 12
1.3.4.2. Gene expression in response to oxidative stress 14
1.3.4.3. DNA damage induced by ROS and programmed cell death 17
1.4. Aim and outline of the thesis 19
References 20

**Chapter 2**
**Effects of metals on growth and sporulation of aquatic hyphomycetes**
Abstract 35
2.1. Introduction 36
2.2. Materials and methods 37
2.2.1. Fungal species and culture maintenance 37
2.2.2. Growth experiments 37
2.2.2.1. Effects of metals on fungal growth in solid medium 37
2.2.2.2. Effects of metals on fungal growth in liquid medium 37
2.2.3. Effects of metals on fungal sporulation 38
2.2.4. Data analysis 39
2.3. Results 40
2.3.1. Effects of metals on fungal growth 40
2.3.1.1. Fungal growth in solid medium 40
2.3.1.2. Fungal growth in liquid medium 44
2.3.2. Effects of Zn and Cd on fungal sporulation 47
2.4. Discussion 49
References 52

**Chapter 3**
**Biochemical responses to Cu and Zn stress in aquatic fungi: the major role of antioxidant defenses**
Abstract 57
3.1. Introduction 58
3.2. Materials and methods 59
3.2.1. Fungal species, growth conditions and metal exposure 59
3.2.2. Scanning electron microscopy 60
3.2.3. Plasma membrane integrity 60
3.2.4. Reactive oxygen species production 61
3.2.5. Preparation of cell-free extracts and determination of enzymatic activities 61
3.2.6. Statistical analysis 62

3.3. Results 62
3.3.1. Comparison of metal sensitivity 62
3.3.2. Biochemical responses associated with cellular barriers against metal stress 64
3.3.3. Oxidative stress induced by Cu and Zn 66
3.3.4. Antioxidant defenses triggered by Cu and Zn exposure 67
3.3.5. Effects of Cu and Zn in mixtures 69
3.4. Discussion 70
References 74

**Chapter 4**
**Copper and Zn affect the activity of plasma membrane $H^+$-ATPase and thiol content in aquatic fungi**
Abstract 79
4.1. Introduction 80
4.2. Materials and methods 82
4.2.1. Fungi and culture maintenance 82
4.2.2. Growth conditions and metal exposure 82
4.2.3. Assessment of $H^+$-ATPase activity 83
4.2.4. Preparation of cell free-extracts and quantification of thiol compounds 83
4.2.5. Metal adsorption and accumulation 84
4.2.6. Data analysis 84
4.3. Results 85
4.3.1. Metal toxicity, adsorption and accumulation 85
4.3.2. Effects of Cu and Zn on $H^+$-ATPase activity 86
4.3.3. Effects of Cu and Zn on the production of thiol compounds 88
4.4. Discussion 90
References 93

**Chapter 5**
**Metal stress induces programmed cell death in aquatic fungi**
Abstract 99
5.1. Introduction 100
5.2. Materials and methods 102
5.2.1. Fungal species and conditions of maintenance 102
5.2.2. Growth conditions and preparation of fungal mycelium suspensions 102
5.2.3. Production of reactive oxygen species 103
5.2.4. Activity of caspases 103
5.2.5. Nuclear morphological alterations 103
5.2.6. TUNEL and propidium iodide staining 104
5.3. Results 104
5.3.1. Cu and Zn induce reactive oxygen species production 104
5.3.2. Cu and Zn induce caspase-like activity 106
5.3.3. Cu and Zn induce nuclear morphological alterations revealed by DAPI staining 106
5.3.4. Cu and Zn induce DNA strand-breaks revealed by TUNEL assay 107
5.4. Discussion 108
References 110

**Chaper 6**
**General discussion** 117
References 121

# Chapter 1

*General introduction*

## 1.1. Aquatic hyphomycetes in streams

Aquatic hyphomycetes are a group of freshwater fungi, also named Ingoldian fungi in honour to Prof. Ingold the pioneer of the study of its taxonomy. Althought the anamorphic stages of aquatic hyphomycetes, characterized by the production of asexual spores or conidia, have been extensively studied, less is known about their teleomorphic stage (Shearer *et al*., 2007). Until now, a small number of aquatic hyphomycetes are known to have sexual states, and the described anamorph/teleomorphic connections show links mainly to *Ascomycota*, lesser to *Basidiomycota* (Shearer *et al*., 2007). These results are in agreement with studies of phylogenetic relationships based on homologies in rDNA sequences (Campbell *et al*., 2002). The degree to which the sexual stage is relevant in the natural life cycle of this group of aquatic fungi remains largely unknown (Shearer *et al*., 2007). Conidia of these fungi are generally large and exhibit several distinctive shapes, such as sigmoid or tetraradiate, which allow their identification. These structures can be found in foams, dispersed in the water, floating on the water surface or associated with decomposing organic substrates as leaf litter and twigs (Suberkropp, 1998).

Nowadays, about 300 species of aquatic hyphomycetes are described with a worldwide distribution (Shearer *et al*., 2007). They can be found in relatively clean and well-aerated running waters (Bärlocher, 1992) and are the major fungal decomposers in either clean or polluted streams (Pascoal and Cássio, 2004; Pascoal *et al*., 2005a). Aquatic hyphomycetes play an important role as intermediaries between plant detritus and invertebrates in streams (Bärlocher, 1992), mainly because they are able to degrade the major polysaccharides of plant cell walls (Suberkropp, 1998). Morphological and physiological adaptations, such as the production of a variety of extracellular degradative enzymes, the capacity to grow at low temperatures and the efficient attachment of conidia to substrata, may be responsible for the success of aquatic hyphomycetes as decomposers in freshwaters (Suberkropp, 1998).

Several studies have demonstrated that the distribution and activity of aquatic hyphomycetes are affected by the physical and chemical characteristics of the stream water (Pascoal *et al*., 2005a,b) and the riparian vegetation (Graça *et al*., 2002).

## 1.2. Metal pollution in aquatic life

In recent years, toxic effects of heavy metals to living organisms, mainly as a result of their continuing anthropogenic mobilization in the environment, have attracted considerable worldwide attention. Metals are common in urban aquatic ecosystems and, in contrast to most pollutants, they are not biodegradable and thus persistent in the environment. Aquatic organisms can incorporate these elements directly or indirectly through the food chain. Metals like Fe, Cu, Zn and Ni are essential to the organism's maintenance; many others have no apparent essential function such as Al, Cd, Hg and Pb (Gadd, 1993). However, both essential and non-essential metals can be toxic when present above certain threshold concentrations (Gadd, 1993). Metal toxicity varies among organisms, with the physico-chemical properties of each metal and environmental factores (Gadd, 1993). In aquatic environments, organisms may be exposed not only to a single chemical but also to a mixture of different substances at the same or nearly the same time, and this can affect biotic communities and ecological processes in a non-predictable way (Duarte *et al*., 2008)

Heavy metals are known to inhibit the growth (Miersch *et al*., 2001; Guimarães-Soares, 2005) and reproduction (Abel and Bärlocher, 1984; Rodrigues, 2002) of aquatic hyphomycetes. Additionally, metals can decrease aquatic hyphomycete diversity (Pascoal *et al*., 2005; Krauss *et al*., 2005). It has been shown that Zn and/or Cu slow down leaf decomposition due to alterations in the structure and activity of aquatic hyphomycete communities (Duarte *et al*., 2004, 2008). Furthermore, it has been observed that acute metal contamination has the greatest impact to aquatic hyphomycete communities during the initial stages of leaf colonization (Sridhar *et al*., 2005).

## 1.3. Cellular mechanisms involved in metal detoxification and tolerance

### *1.3.1. Extracellular complexation*

Complexation of metals by organic molecules is a significant process in which organic acids play fundamental roles in the environmental mobilization of metals (Gadd, 1999). Wood-rotting fungi can overexcrete organic acids (oxalic and citric acids)

with strong metal-chelating properties, suggesting that a ligand-promoted mechanism is the main mechanism of metal dissolution (Jarosz-Wilkolazka and Gadd, 2003). Also, *Aspergillus Niger* produces metal oxalates in the presence of a wide range of metal compounds, including insoluble metal phosphates, for example of Co, Zn, Cu and Mn (Sayer and Gadd, 1997). On the contrary, organic acid production was not detected in aquatic hyphomycetes exposed to metals, despite the observed decrease in the pH of extracellular medium (Guimarães-Soares, 2005).

### *1.3.2. Cellular barriers against metal stress*

#### *1.3.2.1. Cell wall*

Cell walls act as the first physical barrier restricting solute uptake. Chitin, chitosan, glycoproteins and melanins, among others, are the main components of fungal cell walls, and may confer some protection against metal ions (Gadd, 1993).

Metals can rapidly bind to fungal cell walls by non-metabolic processes such as ion exchange, adsorption, complexation, precipitation and cristalization (Gadd, 1993; Cervantes and Gutierrez-Corona, 1994; Blaudez *et al*., 2000). Surface biosorption may be the most significant mechanism in controlling metal uptake, being implicated in metal resistance of microrganisms (Podgorskii *et al*., 2004). Metal sequestration during biosorption occurs by means of complex mechanisms that include mainly ionic interactions and formation of complexes between metal cations and ligands in the structure of the cell wall, as well as precipitation on the cell wall matrix (Schiewer and Volesky, 1996).

It has been reported that metal-tolerant fungal species exhibit higher metal biosorption rates to cell walls than less tolerant ones (Gardea-Torresdey *et al*., 1997). A research with wood-rotting fungi exposed to Cu found that 38% to 77% of metal could be biosorbed (Gabriel *et al*., 2001). Studies on biosorption of Pb by filamentous fungi show that *Aspergillus niger* and *Mucor rouxii* had an exceptionally high Pb biosorption capacity, and curiously both contain chitin and chitosan in the cell wall, which appear to provide prominent metal adsorption ability. Biosorption of metals by filamentous fungal biomass is strongly affected by pH, initial metal ion concentration, medium composition and exposure time (Waihung *et al*., 1999). In *Mucor rouxii*, a decreased biosorption capacity with a simultaneous decrease in pH suggests that metal cations and protons compete for the same binding sites in the cell wall (Waihung *et al*., 1999).

In yeasts, the role of cell walls in confering metal protection is controversial; *Saccharomyces cerevisiae* treated with Cu did not show metal bound to the cell wall, but localized intracellularly (Sarais *et al*., 1994); however, Podgorskii and co-workers (2004) found that yeasts of the genera *Saccharomyces*, *Pichia* and *Candida* can efficiently promote biosorption of metals.

In aquatic hyphomycetes, the involvement of the cell wall in heavy-metal resistance is less documented. In *Heliscus lugdunensis*, Cd and Zn biosorption increased with the increase of metal concentration (Jaeckel *et al*., 2005). However, Cd and Cu biosorption rates in two strains of *H. lugdunensis* isolated from sites with different degree of metal pollution were not correlated to metal tolerance (Braha *et al*., 2007).

#### *1.3.2.2. Plasma membrane*

Toxic effects of metals include disruption of cellular membrane integrity. In *S. cerevisiae*, it has been extensively reported that Cd and Cu induce plasma membrane permeabilization with cellular $K^+$ efflux (Gadd, 1993; Avery *et al*., 1996). Similar effects were reported in higher organisms and have been attributed to the redox-active nature of Cu and its ability to catalyze the generation of free radicals, promoting lipid peroxidation (Stohs and Bachi, 1995). For *S. cerevisiae*, Cu was more toxic than Cd; however, Cu exposure resulted in less $K^+$ release than the observed for Cd (Howlett and Avery, 1997). In this situation, toxicity of Cu was attributed to the direct interaction with nucleic acids or misincorporation into metalothioneins (Cervantes and Guttierrez-Corona, 1994).

Physical properties of cell membranes are largely determined by their lipid composition, especially the degree of fatty acid unsaturation, which may be an important characteristic determining the differential susceptibility of individual microorganisms to Cu toxicity (Avery *et al*., 1996). Primary targets of free radicals in biological membranes are the polyunsaturated fatty acids (PUFA) and the enrichment of *S. cerevisiae* membranes with the PUFA linoleate markedly enhanced the susceptibility to Cu (Avery *et al*., 1996). The increased susceptibility of PUFA-enriched *S. cerevisiae* to Cd and Cu induced plasma membrane perturbation, and toxicity was correlated with elevated lipid peroxidation (Howlett and Avery, 1997). During normal cellular metabolism, the formation of high levels of thiobarbituric acid reactive substances (TBARS) can be disallowed by glutathione peroxidase activity (GPx). Glutathione

peroxidase converts lipid hidroperoxides to their corresponding hidroxy fatty acids (Davies, 1995). Since reduced glutathione (GSH) is a principal cellular target or sequestration site of Cd (Stohs and Bachi, 1995), the higher levels of TBARS in Cd-exposed cells may reflect GSH depletion and a reduced capacity of cells to repair lipid peroxidation (Howlett and Avery, 1997).

In *Scenedesmus sp.* increased lipid peroxidation was found in the presence of both Cu and Zn, although more severely with Cu (Tripathi *et al*., 2006). Copper and Zn induced less oxidative stress in adapted than in non-adapted cells. In the latter situation, the ability of Cu or Zn to generate lipid peroxidation was significantly lower (Tripathi *et al*., 2006). However, lipid peroxidation was not found in aquatic hyphomycetes grown in the presence of Cu, Zn or Cd, but all metals induced loss of plasma membrane integrity (Guimarães-Soares, 2005).

#### *1.3.2.3. The role of $H^+$ -ATPase*

The chemical and electrical gradients across membranes is one of the main requirements of living cells, and transport proteins embedded in plasma membrane are responsible for the maintenance of these gradients (Wolfgand, 1997). A key component of fungal plasma membrane responsible for the maintenance of the electrochemical gradient of protons is the plasma membrane $H^+$-ATPase (Serrano, 1988).

Proton ATPases belong to the P-type ATPase family, which are proton pumps driven by ATP hydrolysis. Proton extrusion provides energy for the transport of ions and nutrients in and out of cells and contributes for the maintenance of intracellular pH (Gancedo and Serrano, 1989). Specific conditions are required for the $H^+$-ATPase activity such as: i) stability of plasma membrane, ii) stability of $H^+$-ATPase enzyme and iii) presence of sufficient ATP (Karamushka and Gadd, 1994). This enzyme is the major protein in plasma membrane of *S. cerevisiae* and was estimated to consume between 10 and 15% of the total ATP during yeast growth (Gancedo and Serrano, 1989).

In yeasts, the major plasma membrane $H^+$-ATPase is encoded by *PMA1* (Ghislain and Goffeau, 1991). In addition, a second gene, *PMA2*, encoding $H^+$-ATPase and showing high homology with *PMA1* was found (Ghislain and Goffeau, 1991). Nevertheless, Pma2p is expressed at very low levels (Supply *et al*., 1993).

Various authors have shown that the $H^+$-ATPase activation in cells under stress constitutes a response that presumably helps the cells to counteract the stress-induced

dissipation of proton motive force across the plasma membrane and the decrease of intracellular pH (Alexandre *et al*., 1996; Fernandes and Sá-Correia, 2000). A more active plasma membrane $H^+$-ATPase was observed in *S. cerevisiae* in the presence of Cu rather than in cells grown the absence of this metal. This activation is not due to increased expression of *PMA1*, whose expression is normally low under Cu stress (Fernandes and Sá-Correia, 1999). Under these conditions, a slightly lower content of ATPase protein was detected in the plasma membrane of cells grown in the presence of Cu.

Copper is a potent depolarizer of cell electrical potential and can inhibit the $H^+$-ATPase at relatively low concentrations (Karamushka and Gadd, 1994). Tallineau and co-workers (1984) suggested that the formation of Cu-ATP complexes might directly inhibit $H^+$-ATPase. Moreover, the disruption of membrane permeability leads to a considerable reduction of ATP in cells limiting proton extrusion (Serrano, 1980). Cells of *S. cerevisiae* adapted to intermediate Cu stress exhibited a more active plasma membrane ATPase, which decreases at higher Cu concentrations. Under high Cu stress, the capacity of the yeast cells to cope with the deleterious effects of Cu was exceeded and plasma membrane $H^+$-ATPase activity drastically declined (Fernandes *et al*., 2000).

Copper induced drastic alterations in plasma membrane lipid organization, probably due to higher levels of lipid peroxidation, which may affect the performance of $H^+$-ATPase and plasma membrane function as a barrier. $H^+$-ATPase activation may be the result of differences in plasma membrane physical properties and/or lipid composition of cells growing under Cu-induced stress (Howlett and Avery, 1997). This kind of explanation was also suggested for ATPase activation by decanoic-acid (Alexandre *et al*., 1996).

The proton pump activity is also modulated by other metals. For instance, increasing Zn concentrations led to a decrease in $H^+$ pumping activity in *S. cerevisiae* (Karamushka and Gadd, 1994), and Cd inhibited the activity of this enzyme in rice roots (Ros *et al*., 1992). Furtheremore, a depolarization of transmembrane electrical potential after Cd and Al exposure was observed in roots of maize (Pavlovkin *et al*., 2006) and *Arabidopsis* (Illéš *et al*., 2006).

#### *1.3.2.4. The role of efflux pumps and vacuole*

The involvement of active efflux pumps in mechanisms of drug resistance has been described in different cell types (Hirata *et al*., 1994b; Ramage, *et al*., 2002). The human P-glycoprotein, an integral membrane protein that functions as an ATP-dependent efflux pump, has been described to be important to reduce intracellular drug accumulation in resistant cells (Scarborough, 1995). Gray and co-workers (2003) isolated a gene from the fungus *Paracoccidioides brasiliensis*, which encodes a half-ABC transporter, designated as *Pfr1*, which shares high identity with members of the ABC-superfamily involved in multidrug resistance.

In the freshwater protozoa, *Euglena gracilis*, an efflux pump similar to the multidrug resistance P-glycoprotein was found to be involved in Cd resistance (Einicker-Lamas *et al*, 2003). Also, a multixenobiotic resistance protein was induced by several metals in *Corbicula fluminea*, a freshwater clam (Achard *et al*., 2004).

The control of metal concentrations within cells may also depend on metal transport to organelles, such as vacuoles. In plants and fungi, metals are sequestered into the vacuole, and ABC-type transporters may play a major role in metal detoxification pathways (Ortiz *et al*., 1995). Several studies show that Zn accumulation in vacuoles of plants is decisive for Zn homeostasis (Küpper *et al*., 1999; Kobae *et al*., 2004). Vacuoles can store Zn for later use under deficient conditions, and acts as a buffer when rapid changes in intracellular Zn levels occur (MacDiarmid *et al*., 2002). Experiments in vacuolar-defective mutants of *S. cerevisiae* confirmed the essential role of the vacuole in Zn, Co, Mn and Ni detoxification, but not in compartmentalization of Cu and Cd (Ramsay and Gadd, 1997). However, the accumulation of Cd in the vacuole of *Paxillus involutus* appears to be essential for metal detoxification (Blaudez *et al*., 2000).

#### *1.3.2.5. Cu and Zn transport and its transcriptional regulation*

Copper uptake occurs by high- and low-affinity transport systems. This metal exists in two different valence states; the lower valence form is the substrate for both the high- and low-affinity transport systems (Hassett and Kosman, 1995). In *S. cerevisiae*, reduction of the most commonly occurring $Cu^{2+}$ is achieved by two plasma membrane reductases encoded by *FRE1* and *FRE2* genes (Georgatsou *et al*., 1997). Transcription of *FRE1* is regulated by intracellular Cu concentration through the copper-dependent

transcription factor Mac1p (Georgatsou *et al*., 1997; Hassett and Kosman, 1995). The high-affinity Cu uptake is mediated by two plasma membrane transporters encoded by *CTR1* and *CTR3* (Knight *et al*., 1996). Under Cu-limiting conditions, *CTR1*, *CTR3* and *FRE1* are highly expressed, whereas under Cu-replete conditions these genes are downregulated (Martins *et al*., 1998). Under high concentrations of Cu ions, the otherwise stably present Mac1p is rapidly degraded, preventing the expression of the Cu transport genes (Zhu *et al*., 1998).

In *S. cerevisiae*, Zn uptake is carried out by one of two transport systems: a high-affinity system, encoded by *ZRT1* that is induced by low Zn concentrations (Zhao and Eide, 1996a) and a low-affinity system, encoded by *ZRT2* that is active in Zn-replete cells (Zhao and Eide, 1996b). Expression studies show that the Zrt1p is specific for Zn and does not transport other metals (van Ho *et al*., 2002). However, uptake assays showed that $Cu^{+}$ and $Fe^{+}$ inhibited Zn uptake by the low affinity system, suggesting that they can be substrates for the transporter (van Ho *et al*., 2002). Evidence for transcriptional regulation is based on the fact that *ZRT1* mRNA levels are regulated in response to cellular Zn levels; Zn-depleted cells had 10-times more *ZRT1* mRNA than Zn-replete cells. Mutants of *Neurospora crassa* showed increased Zn resistance due to lower uptake of this metal, suggesting a partial block of Zn uptake (Rama Rao *et al*., 1997).

### *1.3.3. Intracellular complexation of heavy metals: the role of thiol compounds*

Thiol compounds include nonproteinaceos glutathione (GSH), phytochelatins (PCs) and the metallothionein proteins of families 8-13 (fungi I-VI MTs), which can sequester metal ions (Cervantes and Gutierrez-Corona, 1994; Cobbett and Goldsbrough, 2002). Biosynthesis of GSH occurs in two consecutive ATP-dependent steps; in the first step, γ-glutamylcysteine synthetase catalyzes the synthesis of γ-glutamylcysteine from L-glutamate and L-cysteine; in the second step, catalyzed by glutathione synthetase, glycine is added to the C-terminal site of γ-glutamylcysteine to yield the GSH tripeptide (Meister, 1988).

Metals, such as Zn and Cu, led to a decrease in GSH (Nagalakshmi and Prasad, 2001; Geret and Bebianno, 2004) Additionally, GSH play an important role in Cd detoxification in *S. cerevisiae* (Li *et al*., 1997). In *S. pombe* (Al-Lahham *et al*., 1999), *N. crassa* (Kneer *et al*., 1992), *Mucor racemosus* (Miersch *et al*., 2001) and *Paxillus*

*involutus* (Courbot *et al*., 2004), Cd exposure led to an increase in nonprotein thiol compounds. In aquatic hyphomycetes, a linear increase in GSH levels with increasing Cd concentrations was found (Miersch *et al.*, 2001).

Phytochelatins are a family of small cysteine-rich peptides enzymatically synthesized from GSH. Its general structure is $(\gamma\text{-Glu Cys})_n\text{-Gly}$, where n=2-11 (Cobbet and Goldsbrough, 2002). Zinc and Cu induce the production of phytochelatins and/or phytochelatin-related peptides in *S. cerevisiae* (Kneer *et al*., 1992) and *Schizosaccharomyces pombe* (Hayashi and Mutoh, 1994). In the latter species, detoxification of Cd can occur by synthesis of PCs, which mediate Cd sequestration into the vacuole (Vande Weghe and Ow, 2001). On the other hand, in the aquatic hyphomycete *Articulospora tetracladia* diminished levels of GSH after Cu-exposure were not accompainied by the synthesis of PCs (Miersch *et al*., 2001).

Metallothioneins are cysteine-rich molecules with low molecular weight (6.5 kDa) that in their reduced state provide thiols for sulphur-seeking metals (Gadd, 1993; Miersch *et al*., 2001). These compounds are important for metal detoxification either as metal-chelating agents or ROS scavengers (Kiningham and Kasarskis, 1998). Metallothioneins are produced after Zn exposure in *S. pombe* (Borrelly *et al*., 2002) and after Cu exposure in *S. cerevisiae* (Gadd, 1993; Macreadie *et al*., 1994), *Candida glabrata* (Mehra *et al.,* 1989) and *Neurospora crassa* (Münger *et al*., 1987). In ectomycorrhizal fungi, tolerance to Cd was also associated with the presence of MTs which probably protect the host plant in metal-polluted sites (Courbot *et al*., 2004). Some strains of aquatic hyphomycetes are known to increase the production of MTs under metal stress (Miersch *et al.*, 2001; Jaeckel *et al*., 2005; Guimarães-Soares *et al*., 2006).

Gluthatione and MTs have cooperative protection role against Cd toxicity, as an initial defence for the former and a second-stage defence for the latter. In fact, although the main role in metal detoxification can be attributed to MTs, induction of MTs by metal cations is relatively slow, and considerable toxic effects can occur before the establishment of effective levels of MTs (Ochi *et al*., 1988).

### *1.3.4. Oxidative stress induced by heavy metals*

#### *1.3.4.1. Enzymatic and non-enzymatic defenses against oxidative stress*

Molecular oxygen ($O_2$) is essential for aerobic organisms, as terminal electron acceptor in mitochondrial respiration, where it is ultimately reduced to water during oxidative phosphorylation. However, the reduction of $O_2$ to water requires four electrons and this reduction precedes sequentially through the one-, two-, and three-electron products, namely superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide ($H_2O_2$) and hydroxyl radical ($^{\cdot}OH$) respectively (Di Guilio *et al*., 1995). Superoxide anion and $^{\cdot}OH$ are potent oxidants and $^{\cdot}OH$ is extremely reactive, attacking non-specifically biomolecules, such as proteins and nucleic acids (Bai *et al*., 2003). Although not considered a free radical, $H_2O_2$ is also reactive, and via the Waber-Weiss reaction with $O_2^{\cdot-}$ serves as an important precursor to $^{\cdot}OH$. Superoxide anion, $O_2^{\cdot-}$, is not a particularly damaging species, but it can generate $H_2O_2$ and $^{\cdot}OH$. When transition metals are involved ($Fe^{2+}$ or $Cu^{+}$) a higher production of $^{\cdot}OH$ can occur via Fenton reaction (Bai *et al*., 2003).

$$H_2O_2+Fe^{2+}\ (Cu^{+}) \longrightarrow Fe^{3+}(Cu^{2+})+\ ^{\cdot}OH+OH^{-}$$

To keep $O_2^{\cdot-}$, $H_2O_2$ and transition metals, such as Cu and Fe, under control, cells developed sophisticated strategies (Bai *et al*., 2003). Under normal physiological conditions, antioxidant defense mechanisms are almost certainly adequate to maintain ROS at basal unharmful levels and to repair cellular damages.

Adaptative responses to oxidative stress include increased activities of antioxidant enzymes and/or concentrations of non-enzymatic antioxidant components (Gaetke and Chow, 2003; Fujs *et al*., 2005). Important antioxidant enzymes are: i) superoxide dismutases (SODs), which include a group of metalloproteins, namely Fe-SOD, Mn-SOD and Cu/Zn-SOD, with a major role in the $O_2^{\cdot-}$ detoxification; ii) catalase (CAT), an ubiquitous enzyme which detoxifies $H_2O_2$; iii) glutathione peroxidases (GPx), which catalyse the reduction of $H_2O_2$ and other peroxides, using GSH as the electron donor; iv) glutathione reductase (GR) responsible for the reduction of oxidized GSH and maintenance of the GSH:GSSH ratio in cells; and v) glucose-6-phosphate dehydrogenase (G6-PDH), the first and rate-limiting enzyme of the pentose phosphate pathway, important for the generation of NADPH essential to maintain the cellular redox balance (Pócsi *et al*., 2004).

In algae, an increase in CAT and SOD activities under Cu and Zn stress was observed (Tripathi *et al*., 2006). In *Scenedesmus* sp. both Cu and Zn affected GR activity (Nagalakshmi and Prasad, 2001; Tripathi *et al*., 2006), through metal binding to SH-groups at the active site of the enzyme (Nagalakshmi and Prasad, 2001). Catalase activity was enhanced by Zn exposure in mussels (Geret and Bebianno, 2004). Different yeast strains under Cu and Zn stress also showed SOD and CAT activation (Lapinskas *et al*., 1993; Fujs *et al*., 2005). In aquatic hyphomycetes, it was found an increase in CAT and G6-PDH activities in presence of Cu, Zn and Cd in *Fontanospora fusiramosa* and an increase in CAT activity in *F. curta* after Cu exposure (Guimarães-Soares, 2005). Accordingly, G6PDH-deficient cells of *S. cerevisiae* are more susceptible and unable to adapt to oxidative stress (Izawa *et al*., 1998). In addition, an increase in peroxidase activity under Cd stress and a decrease in GR activity after Cd and Cu exposure were found in strains of the aquatic hyphomycete *H. lugdunensis* (Braha *et al*., 2007).

Non-enzymatic defense systems consist of small molecules present in aqueous or lipidic environments that remove oxidants from solution, acting as free radical scavengers (Jamieson, 1998). Important non-enzymatic antioxidants in microorganisms are GSH, trehalose, carotenoids, L-ascorbic acid and tocopherols (Bai *et al.,* 2003). Gluthatione can directly scavenge radicals and/or provide reducing equivalents for the reduction of peroxides by GPx (Di Giulio *et al*., 1995). In *S. cerevisiae*, the γ-glutamylcysteine synthetase (*gsh1*) mutants, deficient in GSH synthesis, are hypersensitive to $H_2O_2$ and $O_2^{\cdot-}$ (Stephen and Jamieson, 1996). Under peroxide stress, imposed by $H_2O_2$ and tert-butyl hydroperoxide, *Penicillium chrysogenum* showed remarkable resistance to this hyperoxidant environment, and both GSH and GSH-dependent enzymes were involved in $H_2O_2$ elimination (Bai *et al*., 2003). In lichens, elevated Cu concentrations caused a significant decrease in GSH, possibly due to metal-induced oxidation of GSH to GSSH (Bačkor *et al*., 2006). In contrast, induction of GSH synthesis was detected in *Paxillus involutus* and in *H. lugdunensis* under Cd stress (Courbot *et al*., 2004; Jaeckel *et al*., 2005).

Trehalose, a non-reducing disaccharide, is found in a wide variety of microorganisms and its involvement in the resistance to heat-shock and oxidative stress has been reported (Fillinger *et al*., 2001; Bai *et al*., 2003). Several studies in yeasts

showed trehalose accumulation during exposure to $H_2O_2$, $CuSO_4$, or 4-hydroxy-2-nonenal (a product of lipid peroxidation) (Wonisch *et al*., 1997; Pedreno *et al*., 2002).

Ascorbic acid is important especially for higher eukaryotes and can complex Cd or redox metal ions displaced by Cd, preventing lipid peroxidation (Stohs and Bagchi, 1995). High intakes of ascorbic acid and Zn may provide protection against Cu toxicity preventing excess of Cu uptake (Gaetke and Chow, 2003). Tocopherols are also important for the inhibition of lipid peroxidation in membranes as demonstrated by the protective effect of vitamin E against lipid peroxidation induced by Cr (Valko *et al*., 2005).

#### *1.3.4.2. Gene expression in response to oxidative stress*

Several of the genes that participate in cellular defense against oxidative stress are known to display increased expression under oxidative stress conditions (Moradas-Ferreira *et al*., 1996). Much of the regulation of the antioxidant responses in *S. cerevisiae* is at the transcription level. Several transcription factors regulate gene expression in response to oxidants (Jamieson, 1998). From these, Yap1p, Yap2p and Gcn4p have been extensively studied and all play a crucial role protecting yeast cells against stress (Fernandes *et al*., 1997). The role of Yap1p in the regulation of antioxidant enzymes was first suggested when *Yap1* mutants of *S. cerevisiae* were found to be hypersensitive to oxidants (Schnell *al*., 1992). These mutants showed reduced activities of SOD and G6-PDH, and their adaptive responses to $H_2O_2$ were severely affected, showing that Yap1p affects the transcription of genes involved in such responses (Stephen *et al*., 1995). However, the *Yap1* mutant retained a small $H_2O_2$-adaptative stress response, implicating additional factors in this process. Expression of *YAP1* in high copy number resulted in a modest increase of GSH levels and activity of SOD and G6-PDH. *YAP2* was identified by its ability, when overexpressed, to confer resistance to Cd, and by the hypersensitivity of *Yap2* null mutants to oxidants (Hirata *et al*., 1994a). Yap2p plays an important role in the regulation of the $H_2O_2$ adaptive stress response, since induction of this response was diminished in *Yap2* null mutant (Stephen *et al*., 1995). Furthermore, *YAP1* and *YAP2* overexpression can both enhance Cd resistance (Hirata *et al*., 1994a).

Hap1p is responsible for the regulation of the expression of both *CYC1* (iso-1-cytochrome) and *CYC7* (iso-2-cytochrome) genes in response to oxygen and heme

(Zitomer and Lowry, 1992). For this reason, Hap1p has consequently been involved in the regulation of a variety of genes encoding hemeo-proteins, such as *CTT1* and *CTA1* (the cytosolic and peroxisomal catalases) and components of the mitochondrial respiratory chain as *SOD2* (mitochondrial manganese superoxide dismutase) (Gralla and Kosman, 1992). Cta1p and Ctt1p are hypersensitive to $H_2O_2$ and both single and double catalase yeast mutants are unable to display an adaptative stress response to $H_2O_2$ (Izawa *et al*., 1995).

Copper is an important co-factor for Cu/Zn-SOD, stimulating both SOD mRNA accumulation and enzyme activity *in vitro*. The cytoplasmatic SOD, which is coded by *SOD1*, appears to be a key enzyme involved in the regulation of intracellular levels of ROS, protecting cells from exogenous toxicity of oxidant agents (Jamieson, 1998). Previous studies demonstrated that Ace1p is the transcription factor responsible for Cu induction of *SOD1* expression (Gralla *et al*., 1991). In *S. cerevisiae*, the integrity of only one *SOD* gene is enough to confer resistance to oxidative conditions originated by $H_2O_2$ (Pereira *et al*., 2001). However, the defense against oxygen toxicity involves both Cu/Zn-SOD and Mn-SOD (Longo *et al*., 1996). *S. cerevisiae* null mutants of SOD have several biochemical defects, indicating that *SOD* genes may protect numerous metabolic enzymes against oxygen-induced damage (Gralla and Valentine, 1991).

Other genes of oxidative-stress protection are known to be regulated by metal-responsive transcription factors and sometimes more than one system may be operative (Moradas-Ferreira *et al*., 1996). *CTT1*, can be activated by both $H_2O_2$ and heat shock through the general stress-response element (Schuller *et al*., 1994).

Concerning GSH, their recycling is dependent on the maintenance of an intracellular pool of NADPH mainly via the pentose phosphate pathway, in which the reaction catalyzed by the G6-PDH is the rate-limiting step (Jamieson, 1998). Mutations in *ZWF1*, the gene which encodes G6-PDH, make cells hypersensitive to oxidants such as $H_2O_2$ (Juhnke *et al*., 1996). In contrast, overexpression of *ZWF1* gene in G6-PDH deficient cells restored the ability to induce adaptation to $H_2O_2$ stress (Izawa *et al*., 1998).

The genes *GSH1* and *GSH2*, encoding enzymes of GSH biosynthesis, were identified in *S. cerevisiae* (Ohtake and Yabuuchi, 1991; Inoue *et al*., 1998). In this species, the expression of *GSH1* is induced by Cd (Stephen and Jamieson, 1997) and is controlled by the Yap1 transcription factor (Wheeler *et al*., 2003). Both *gsh1* and *yap1*

mutants show hypersensitivity to Cd (Wu *et al*., 1993). The *gsh1* mutants are also sensitive to oxidative stress imposed by $H_2O_2$ and t-butyl hydroperoxide (Izawa *et al*., 1995). Deletion of *GSH2* does not affect resistance to $H_2O_2$ and t-butyl hydroperoxide, when appropriate concentrations of the dipeptide γ-glutamylcysteine are present, protecting cells against oxidative injury (Grant *et al*., 1997). However, expression of *GSH1* and *GSH2* in the wild-type strain was induced by $H_2O_2$ and t-butyl hydroperoxide, and was under control of Yap1p (Sugiyama *et al*., 2000).

There are a number of mechanisms by which the PCs biosynthetic pathway may be regulated; the first is likely the regulation of GSH biosynthesis. Zhu and collaborators (1999) demonstrated that when expression of the enzymes of the GSH biosynthetic pathway increased, the PCs biosynthesis and Cd tolerance also increased. According to these results, regulation of GSH biosynthesis is a plausible endogenous mechanism by which PC expression may be modulated. Exposure of *Arabidopsis* to Cd and Cu led to an increase in transcript levels of *GSH1* and *GSH2* (Xiang and Oliver, 1998). The PC synthase catalyzes the transpeptidation reaction of the γ-Glu-Cys moiety of a GSH molecule onto another GSH molecule, forming $(\gamma\text{-Glu-Cys})_2$-Gly or onto another $(\gamma\text{-Glu-Cys})_n$-Gly molecule, forming the n+1 oligomer (Grill *et al*., 1989).

Metal detoxification also occurs via-metal mediated transcriptional activation of MT genes by increasing synthesis of MTs (Zhang *et al*., 2001). Transcriptional induction of MT genes is mediated by the metal-responsive transcription factor 1 (MTF-1), an essential Zn finger protein that binds to specific DNA motifs termed metal-response elements. Transcriptional induction of MTs genes by Zn can be achieved by elevated Zn concentration alone, but induction by Cd or Cu requires the presence of a Zn-satured metallothioneins (Zhang *et al*., 2003). This is explained by the preferential binding of Cd or Cu to MTs, with the concomitant release of Zn, which in turn, leads to the activation of the transcription factor MTF-1. The release of Zn from cellular components, including MTs, and the sequestration of Zn by newly produced apometalothioneins may be a basic mechanism to regulate MTF-1 activity upon cellular stress. Metallothioneins are not only passive targets of MTF-1, but may rather contribute to the regulation of its activity (Zhang *et al*., 2003).

In response to Cu toxicity, *CUP1* gene is transcriptionally activated in *S. cerevisiae* and MT are formed (Thiele, 1992). Studies with *SOD1* mutants in presence of Cu also suggest that *CUP1*-encoded MT can function as an antioxidant (Liu and

Thiele, 1997). A second *S. cerevisiae* MT is encoded by *CRS5* gene and is also induced by Cu, even though to a less extent than *CUP1* (Culotta *et al*., 1994). The gene *CRS5* was transcriptionally repressed by oxygen (Cullota *et al*., 1994). Both *CUP1* and *CRS5*, are transcriptionally activated by Ace1-transcription factor responsible for Cu induction of *SOD1* expression (Gralla *et al*., 1991). Since Cu catalyses reactions that generate free radicals from $O_2^{\cdot -}$, MTs and Cu/Zn-SOD remove these components. The presence of Cu bound to the Mac1p and Ace1p suggests that these transcription factors may be redox active and, therefore, may play a role in the ability of cells to sense oxidants (Jungmann *et al*., 1993).

#### *1.3.4.3. DNA damage induced by ROS and programmed cell death*

Several types of enzymatic repair processes developed during evolution are essential to maintain the fidelity and integrity of genetic information. DNA is the only molecule with capacity of self-repair, replacing damaged segments; therefore, if a mutagen induces a DNA lesion and the lesion is repaired before "fixation", there may be no effect on DNA. This is especially true after low-level of mutagen exposure, where excision repair enzymes are not satured by a significant number of DNA damaged sites. Exposure of an organism to genotoxic chemicals may include a cascade of DNA-damaging events; initially, structural alterations are formed, then DNA damage is processed and subsequently expressed in mutant gene products (Shugart, 1995).

DNA plays an important role in life and reproduction of each organism; in light of this fact it is of extreme importance to study the effect of oxidative stress and metal induced-oxidative stress on DNA damage. Reactive oxygen species produced *in vivo*, at levels that cannot be dealt conveniently by endogenous antioxidant systems, can lead to damage of lipids, proteins, carbohydrates and nucleic acids (Bai *et al*., 2003). These reactive species may affect all cellular functions, since they are non-specific in their action, although DNA oxidative damage is the most critical target. The non-radical singlet oxygen $^1O_2$ and the radical $^{\cdot}OH$ are the major damaging oxidative species. These species can be generated inside cells during normal aerobic metabolism and fulfill essential prerequisites to be genotoxic agents. Moreover $H_2O_2$, and $O_2^{\cdot -}$ also induce a spectrum of DNA lesions, such as single-strand breaks, double-strand breaks, crosslinking of DNA and damages to bases (Jornot *et al*., 1998).

Furtheremore, ROS can be assumed as signalling molecules which activate crucial components of programmed cell death (PCD) or in alternative can act indirectly by modifying the cellular redox potential, which regulates key regulatory proteins involved in PCD (Madeo *et al*., 1999). Low external doses of $H_2O_2$ or depletion of glutathione triggers *S. cerevisiae* into PCD, whereas depletion of ROS prevents PCD (Madeo *et al*., 1999). A recent study reported that ROS accumulation is apparent in almost every apoptotic scenario (Ludovico *et al*., 2005). In filamentous fungi, such as *Aspergillus nidulans* and *A. fumigatus*, the involvement of ROS in PCD has also been reported (Semighini *et al*., 2006; Mousavi and Robson, 2003). Furtheremore, the ability of some antioxidant enzymes, such as catalase, to block PCD argues for the central role for oxidative stress in PCD (Buttke and Sandstrom, 1994).

Metal effects in fungi associated with PCD processes are poorly documented. However, exposure to Cd, Cu, Zn and Pb in plants (Gichner *et al*., 2006), to Cd, Zn, Cu in mammalian cells (Wätjen *et al*., 2002; Wolfe *et al*., 1994) or to Zn in HEP-2 cells (Rudolf *et al*., 2005) may induce phenotypical alterations characteristics of PCD processes.

Programmed cell death is characterized by phenotypical alterations, such as chromatin condensation (Clifford *et al*., 1996), DNA fragmentation, formation of membrane-enclosed cell fragments (apoptotic bodies) and caspase activation (Tsujimoto, 1997). However, cells under PCD do not always harbour all cardinal features of this cell death type (Schulze-Osthoff *et al*., 1994).

Caspases have been considered important mediators of apoptosis, playing a critical role in the downstream execution of the PCD pathway in higher eukaryotes (Earnshaw *et al*., 1999). Caspase activity is responsible directly or indirectly for cleavage of several intracellular proteins, including proteins of the nucleous, endoplasmic reticulum and cytosol, which are characteristically proteolysed during PCD (Rosse *et al*., 1998). The genome of *S. cerevisiae* encodes a single metacaspase, Yca1p (Madeo *et al*., 2002). The yeast apoptotic responses often dependent on Yca1p (Silva *et al*., 2005; Madeo *et al*., 2002), though not always (Wissing *et al*., 2004), indicating a non-exclusive role for metacaspase in PCD in response to toxics. Disruption of *YCA1* in *S. cerevisiae* rescue yeasts from PCD, confirming its functional role as an executor of PCD (Vanovska and Hardwick, 2005). Two metacaspases have been found in *A. fumigatus* (Mousavi and Robson, 2003), and two caspases-like

(caspase 3 and caspase 8) activities have been identified in *A. nidulans* during sporulation (Thrane *et al*., 2004).

Programmed cell death allows the rapid removal of unwanted or damaged cells that could otherwise inflame the surrounding cells with their cytoplasmic contents (Madeo, 1997). This process is considered an altruistic mechanism, since spares energy sources for the undamaged cells, and may constitute an evolutionary advantage.

Several investigations revealed that oxidation of thiols other than GSH can mediate induction of PCD, suggesting that the intracellular thiol redox status would be the key factor of the cell death signalling pathways (Sato *et al*., 1995). In fact, PCD can be induced by growing a *gsh1* delection mutant in the absence of GSH (Madeo *et al*., 1999) or by the oxidation of cellular sulfhydryl groups (Sato *et al*., 1995). Thiols have been proposed to play a protective role in oxidative DNA damage by quenching radical species in solution and repairing deoxyribose and nucleo-base radicals. In fact, in the presence of Zn or Cu, MTs can act as effective antioxidants preventing apoptotic mechanisms (Santon *et al*., 2004). Moreover, an active pentose phosphate pathway is required for double-strand-break rejoining in mammalian cells exposed to a mild thiol oxidant (Ayene *et al*., 2000).

## 1.4. Aim and outline of the thesis

In this study, we assessed the effects of heavy metals in aquatic hyphomycetes by examining several cellular targets and putative defense mechanisms against metal stress to better understand the ability of these group of fungi to survive in metal-polluted environments.

Chapter 1 provides information on the role of aquatic hyphomycetes in freshwater ecosystems and focuses on the negative effects of metal pollution to biota. Particular attention is given to the cellular mechanisms involved in metal detoxification and tolerance. In Chapter 2, the effects of metals, such as Cu, Zn, Cd and Ni, on growth and sporulation of several aquatic hyphomycete species are evaluated. This allowed the selection of fungal species with different sensitivities to metals, to further investigate the interations between aquatic hyphomycetes and metals. Chapther 3 focuses on the role of antioxidant defenses against Cu and/or Zn stress in *Varicosporium elodeae* and *Heliscus submersus*. Firstly, we assessed the ability of metals to induce reactive oxygen

species (ROS) and plasma membrane disruption. Subsequently, we evaluated the role of catalase, superoxide dismutase and glucose-6-phosphate dehydrogenase to deal with acute- and chronic-metal stress. In Chapter 4, we examined the ability of three aquatic hyphomycete species (*Varicosporium elodeae*, *Heliscus submersus* and *Flagellospora curta*) to adsorb and accumulate Cu or Zn. Then, we assessed the effects of these metals on $H^+$-ATPase activity and on the levels of thiol-containing compounds. In Chapter 5, we tested whether Cu and Zn stress is able to induce programmed cell death in aquatic hyphomycetes through the evaluation of typical apoptotic markers, namely ROS production, caspase activation, alterations in nuclear morphology and the occurrence of DNA strand-breaks. Finally, in Chapter 6, the main conclusions are presented to provide a global perspective of this work.

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# Chapter 2

*Effects of metals on growth and sporulation of aquatic hyphomycetes*

## Abstract

In this work, we investigated the effects of Zn, Cu, Ni and Cd on the growth and sporulation of several aquatic hyphomycete species. Effects of metals on growth were assessed in solid and liquid media with different composition (1% malt extract- ME and a mineral medium supplemented with vitamins and 2% glucose- MK), and fungal sensitivity to metals was compared. The exposure to Zn or Cd inhibited sporulation of *Heliscus submersus* and *Tricladium chaetocladium*, being the effects stronger in the latter species. In solid medium, mycelial growth was linear and, in most cases, metals negatively affected fungal growth. The sensitivity of aquatic hyphomycetes to metals, assessed as the metal concentration inhibiting biomass production in 50% ($EC_{50}$), showed that *Ypsilina graminea* and *Varicosporium elodeae* were the most resistant species to Zn, while *Alatospora acuminata*, *H. submersus* and *Flagellospora curta* appeared to be the most sensitive species to this metal. On the contrary, *H. submersus* was the most resistant fungus to Cu. Generally, lower toxicity of Zn or Cu than Ni or Cd was found. Moreover, the patterns of species resistance to metals in either liquid or solid medium with similar composition were identical. However, $EC_{50}$ values were about 20-times higher in solid medium than in liquid medium. Changes in nutrient supplies to fungi affected metal toxicity, as shown by higher $EC_{50}$ values in MK than in ME. In addition, fungal tolerance to metals varied with fungal species and metal type, and the tolerance to one metal did not confer tolerance to all metals, suggesting that different mechanisms and /or cellular targets might be implicated in fungal tolerance to different metals.

## 2.1. Introduction

Heavy metals can be released to the environment from natural processes, but mainly from human activities, such as agriculture, mining and industry. Metal pollution constitutes a serious environmental danger (Ayres, 1992), because metals are not biodegraded, and can be accumulated in living organisms, passing through food chains. Essential metals, such as Zn, Cu and Ni, and non-essential, as Cd, can exert toxicity when present above certain threshold concentrations. Freshwaters are frequently the destination of metals released into the environment. The functioning of these ecosystems depends on recycling of nutrients and energy from allochthonous plant detritus. In this process, a group of fungi known as aquatic hyphomycetes play a critical role. These fungi produce extracellular enzymes able to degrade plant detritus and transform them into a more suitable food source for invertebrate detritivores (Suberkropp, 1998).

Several studies demonstrated that metals can negatively affect growth and reproduction of aquatic hyphomycetes. A decrease in fungal radial growth after exposure to Cd (≥ 50 µM), Cu (≥ 50 µM), Zn (≥ 150 µM) (Miersch *et al*., 1997) or Ni (>200 µM) (Rodrigues, 2002) has been observed. In addition, Cd (> 0.9 µM; Abel and Bärlocher 1984) and Zn (25 µM Rodrigues, 2002; 150 µM; Duarte *et al*., 2004) inhibit conidial production of aquatic hyphomycete species. Moreover, data from literature pointed to a higher toxicity of metals to conidial production than to micelial growth (Abel and Bärlocher, 1984; Bermingham *et al.,* 1996; Rodrigues, 2002). However, metal toxicity depends on the fungal species, metal type and several environmental factors, including pH and nutrient availability, which are expected to affect fungal activity and metal bioavailability (Gadd, 1993).

In this work, we investigated the effects of Zn, Cu, Ni and Cd on the growth and sporulation of several aquatic hyphomycete species. Effects of metals on growth were assessed in solid and liquid media and fungal sensitivity were compared by determining metal inhibition parameters, namely the concentration inhibiting growth in 50% ($EC_{50}$), the no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC).

## 2.2. Materials and methods

### *2.2.1. Fungal species and culture maintenance*

The aquatic hyphomycetes *Flagellospora curta* J. Webster (UMB-39.01), *Heliscus submersus* H. J. Huds (UMB-135.01), *Tricladium chaetocladium* Ingold (UMB-86-01), *Varicosporium elodeae* W. Kegel (UMB-142.01), *Ypsilina graminea* (Ingold, P. J. Mc Dougall and Dann) Descals, J. Webster and Marvanová (UMB-111-01) and *Alatospora acuminata* Ingold (UMB-173-01) were used in this study. *F. curta*, *H. submersus*, *T. chaetocladium* and *Y. graminea* were isolated from the Este River at the industrial park of the town of Braga, where metal concentrations in the stream water attained 80 µM for Zn, 150 µM for Cu, 52 µM for Ni and 0.53 µM for Cd (Gonçalves, 2001). *A. acuminata* was isolated from a clean site located at the source of the same river and *V. elodeae* was isolated from a clean stream in the Peneda-Gerês National Park. *H. submersus*, *F. curta* and *T. chaetocladium* were isolated from leaves, while *Y. graminea*, *V. elodeae* and *A. acuminata* were isolated from foams. All species were isolated from single spores. Details on fungal species and characterization of water chemistry of their origin sites are in Pascoal *et al*. (2005).

In the laboratory, fungi were maintained on solid medium containing 2% (w/v) (ME) and 1.5 % (w/v) agar, at 18º C under permanent artificial light.

### *2.2.2. Growth experiments*

#### *2.2.2.1. Effects of metals on fungal growth in solid medium*

The effects of Zn, Cu, Ni and Cd on fungal growth were evaluated in 5 species of aquatic hyphomycetes: *H. submersus, T. chaetocladium, V. elodeae*, *Y. graminea* and *A. acuminata*. Fungi were grown on 1% ME, 1.5% agar at pH 5.0, supplemented or not with different concentrations of metals. Solid media were inoculated with a 7 mm agar plug (2 week-old cultures) placed centrally on Petri dishes and incubated at 18ºC. Every 3 days, mycelium radial expansion was measured in four replicates.

#### *2.2.2.2. Effects of metals on fungal growth in liquid medium*

Effects of metals on fungal growth were evaluated in two different liquid media, namely 1% ME, and mineral medium with vitamins and 2% glucose (MK).

Fifty ml Erlenmeyer flasks containing 20 ml of culture medium were inoculated with conidial suspensions (final concentration, 6 conidia $ml^{-1}$) of *V. elodeae*, *Y. graminea*, *H. submersus* and *F curta*. The effect of Zn, Cd and Cu on fungal growth was determined in 1% ME supplemented or not with metals at different concentrations for *V. elodeae*, *Y. graminea* and *H. submersus*. The effect of Zn and Cu in mineral medium was evaluated for *V. elodeae*, *H. submersus*, *F. curta* and *Y. graminea*. In 1% ME fungal growth of *V. elodeae* and *Y. graminea* in presence of Cd and Zn was followed during 8 days (18ºC and 160 rpm), while for the other species in this medium and in medium with glucose the biomass was quantified at a fixed day ($8^{th}$ day). After grown, mycelia were harvested by filtration, washed twice with deionised water, dried at 85ºC to constant mass and weighed.

MK was composed of a base medium (0.5% (w/v) $(NH_4)$ $SO_4$; 0.5% (w/v) $KH_2PO_4$; 0.05% (w/v) $MgSO_4$ $7H_2O$; 0.013 (w/v) $CaCl_2.2H_2O$ and deionised water, q.b), supplemented with 2% (w/v) glucose and 0.05% (v/v) of each of the three solutions: oligoelement solution A (1% (v/v) $H_3BO_3$; 0.2% (v/v) KI; 0.4% (v/v) $NaMoO_4.2H_2O$ and deionised water q.b); oligoelement solution B (0.08% (w/v) Cu $SO_4.5H_2O$; 0.4% (w/v) $FeCl_3.6H_2O$; 0.8% (w/v) $MnSO_4.4H_2O$; 0.8% (w/v) $ZnSO_4.7H_2O$; 0.8% (v/v) HCl $10^{-3}$ N and deionised water q.b); and vitamin solution (0.001% (w/v) biotin; 0.08% (w/v) calcium pantothenate; 4% (w/v) myoinositol; 0.16% (w/v) niacin; 0.16% (w/v) piridoxin hydrochloride; 0.16% (w/v) thiamine hydrochloride, deionised water q.b).

The malt extract and the base media were autoclaved (1 atm, 20 min) while oligoelement solutions, vitamins, and metal solutions were sterilized by filtration.

### *2.2.3. Effects of metals on fungal sporulation*

To test the effects of metals on fungal sporulation, fungi were grown in solid medium with 2% ME at pH 5.0 and 18ºC during 20 days. Sporulation experiments were carried out in 250 ml Erlenmeyer flasks containing 100 ml of 10 mM $KH_2PO_4$ (pH 5.0), supplemented or not with different concentrations of Zn and Cd, as indicated in the results. Four agar plugs (Ø 5 mm) of each fungal culture were inoculated in each flask, which was kept aseptically under continuously aeration, with aquarium pumps, for 10 days. Every 2 days, all inocula were transferred to fresh solutions with the same concentration of Zn or Cd. To estimate fungal sporulation, conidial suspensions were

mixed with Tween 80 (0.8%) to release the conidia adhered to the glass, and adequate volumes of solutions were filtered (pore size, 5 μm; Millipore). Conidia on filters were stained with 0.1% cotton blue in lactic acid and counted (144 $mm^2$) under a microscope (magnification 100 or 400 X).

Phosphate solutions were autoclaved and metal solutions were sterilized by filtration (0.22 μm pore size membrane). Three independent experiments were performed.

### *2.2.4. Data analysis*

The rate of fungal radial growth (kr) was estimated by linear regression of mycelial radial growth along time. Metal concentrations inhibiting fungal growth in 50% ($EC_{50}$) were estimated by the Probit Method. The values of $EC_{50}$ values were compared by t-tests, at 2 levels of comparisons, or by one-way ANOVA when more than 2 levels were considered (Zar, 1996). After one-way ANOVA, a Tukey test was used to identify where the significant effects occur. To determine values of NOEC (No Observed Effect Concentration) and LOEC (Lowest Observed Effect Concentration) at a fixed time, values of biomass production were compared by one-way ANOVA, followed by a Dunnett´s post-test to identify significant effects ($p<0.05$; Zar, 1996). Data were Ln-transformed to achieve normal distribution and homocedasticity.

To determine metal effects on fungal sporulation, conidial production was converted in percentage of the control (100%), divided by 1000 and normalized by arcsine square root transformation. Data were analyzed by two-way ANOVA, with exposure time and metal concentration as factors. Bonferroni post-test were used to discriminate significant differences ($p<0.05$; Zar, 1996).

Statistic analysis was done using Prism4 for Windows (GraphPad Software Inc., San Diego).

## 2.3. Results

### *2.3.1. Effects of metals on fungal growth*

#### *2.3.1.1. Fungal growth in solid medium*

In the absence of metals, rates of radial growth (kr) of the aquatic hyphomycetes on 1% ME varied from 1.23 to 0.44 mm $d^{-1}$ (Table 2.1), being the highest value observed for *V. elodeae* and the lowest one for *A. acuminata*.

Table 2.1. Rates of radial growth (kr) of aquatic hyphomycetes on 1% ME

| Fungal species | kr ± SE (mm $d^{-1}$) | $r^2$ |
|---|---|---|
| *Varicosporium elodeae* | 1.23 ± 0.024 | 0.99 |
| *Ypsilina graminea* | 0.95 ± 0.017 | 0.99 |
| *Tricladium chaetocladium* | 0.80 ± 0.017 | 0.99 |
| *Heliscus submersus* | 0.60 ± 0.040 | 0.87 |
| *Alatospora acuminata* | 0.44 ± 0.011 | 0.98 |

$r^2$, coefficient of determination; values are mean ± SE, n=80.

In general, the presence of Zn in the culture medium led to a reduction in the kr for all fungal species (Figure 2.1). *A. acuminata* was the most sensitive species, showing a 95% reduction in the kr at Zn concentration of 1500 µM, while a similar inhibition on kr of the other species was only attained with metal concentrations of about 5-times higher. Moreover, Zn till concentrations of 1500 µM increased the kr of *T. chaetocladium* (Figure 2.1).

Metal concentration inhibiting mycelial growth in 50% ($EC_{50}$) for Zn varied from 4258 to 670 µM, with the highest value observed in *Y. graminea* and the lowest one in *H. submersus* (Table 2.2). The analysis of no observed effect concentration (NOEC) and low observed effect concentration (LOEC) values for Zn corroborated the highest sensitivity of *H. submersus* to this metal (Table 2.3).

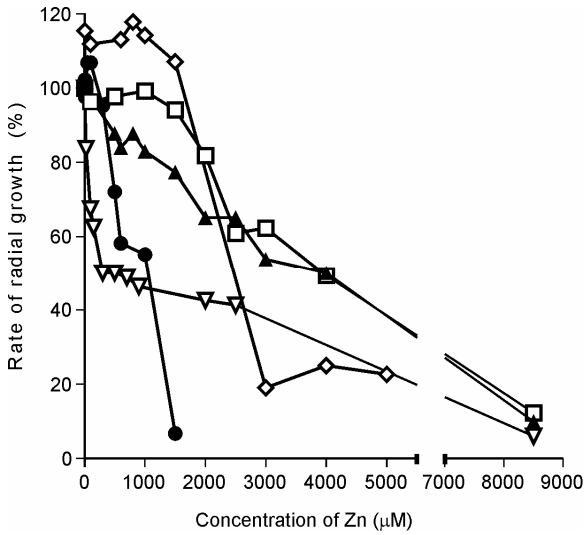


Figure 2.1. Effect of Zn on the rates of radial growth (kr) of *V. elodeae* (□), *Y. graminea* (▲), *H. submersus* (∇), *T. chaetocladium* (◊) and *A. acuminata* (●), in 1% malt extract, at pH 5.0 and 18ºC. Results are mean ± SE of four replicates.

Table 2.2. Metal concentrations (μM) inhibiting mycelial growth in 50% ($EC_{50}$) for Zn, Cu, Ni and Cd in *V. elodeae, Y. graminea, H. submersus, T. chaetocladium* and *A. acuminata* after 10 days of metal exposure.

| Fungal species | Zn | Cu | Ni | Cd |
|---|---|---|---|---|
| | $EC_{50}$ | $EC_{50}$ | $EC_{50}$ | $EC_{50}$ |
| *V. elodeae* | 4134±132 | 1189±49 | 101±9 | 120±3 |
| *Y. graminea* | 4258±245 | 1049±35 | 106±5 | 77±5 |
| *H. submersus* | 670±78 | 2236±126 | 844±29 | 360±16 |
| *T. chaetocladium* | 1963±446 | 1406±13 | 24±3 | 222±4 |
| *A. acuminata* | 1192±466 | 495±5 | 215±71 | 66±3 |

Values are means ± SE.

Table 2.3. Values of NOEC and LOEC obtained in solid medium in presence of Zn, Cu, Ni and Cd in *V. elodeae, Y. graminea, H. submersus, T. chaetocladium and A. acuminata* after 10 days of metal exposure (µM).

| Fungal species | Zn | | Cu | | Ni | | Cd | |
|---|---|---|---|---|---|---|---|---|
| | NOEC | LOEC | NOEC | LOEC | NOEC | LOEC | NOEC | LOEC |
| *V. elodeae* | 1000 | 1500 | 300 | 700 | - | 50 | - | 81 |
| *Y. graminea* | - | 500 | 200 | 250 | 25 | 50 | - | 81 |
| *H. submersus* | 150 | 300 | 900 | 1500 | 300 | 600 | 364 | 405 |
| *T. chaetocladium* | 1500 | 3000 | 700 | 900 | - | 100 | 138 | 162 |
| *A. acuminata* | 500 | 600 | - | 40 | 100 | 150 | - | 41 |

The effects of Cu on the kr of these aquatic hyphomycetes indicated that *H. submersus* and *V. elodeae* were the most resistant species to Cu and *A. acuminata* the most sensitive one (Figure 2.2). Copper at concentrations until 500 µM and 200 µM stimulated the kr of *H. submersus* and *T. chaetocladium*, respectively.

Values of $EC_{50}$ for Cu ranged from 2236 to 495 µM, with the highest value for *H. submersus* and the lowest for *A. acuminata* (Table 2.2). Analysis of LOEC also supported that *A. acuminata* was the most sensitive species and *H. submersus* the most resistant one (Table 2.3).

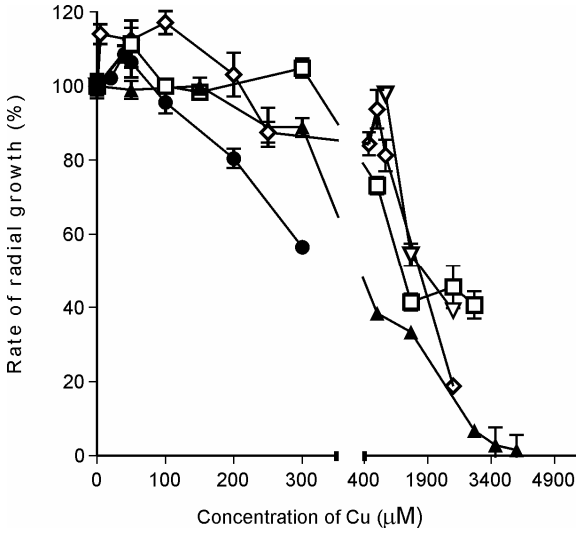


Figure 2.2. Effect of Cu on the rates of radial growth (kr) of *V. elodeae* (□), *Y. graminea* (▲), *H. submersus* (∇), *T. chaetocladium* (◊) and *A. acuminata* (●), in 1% malt extract, at pH 5.0 and 18ºC. Results are mean ± SE of four replicates.

Analysis of Figure 2.3 shows that the highest kr inhibition for Ni was found in *Y. graminea* and *T. chaetocladium*. In the presence of 200 µM of Ni, an inhibition in kr of at least 90% was found for these species. The lowest kr inhibition by Ni was found in *H. submersus* which was the only species whose kr was stimulated by Ni (Figure 2.3).Values of $EC_{50}$ ranged from 844 to 24 µM with the highest values for *H. submersus* and the lowest for *T. chaetocladium* (Table 2.2). Analysis of NOEC and LOEC values also pointed to *H. submersus* as the most resistant aquatic hyphomycete species to Ni (Table 2.3).

Effects of Cd on kr indicated that *H. submersus* was the most resistant species while *A. acuminata* was the most sensitive species to this metal (Figure 2.4). The concentration needed to inhibit the kr in 50% was 4 times higher in *H. submersus* than for *A. acuminata*. Analysis of Figure 2.4 also shows that the tested Cd concentrations significantly decreased the kr of all species, excluding concentrations till 138 µM for *T. chaetocladium.* $EC_{50}$ values for Cd varied from 360 to 66 µM, with the highest value for *H. submersus* and the lowest for *A. acuminata* (Table 2.2). Analysis of LOEC confirmed the highest resistance of *H. submersus* to Cd and the highest sensitivity of *A. acuminata* (Table 2.3).

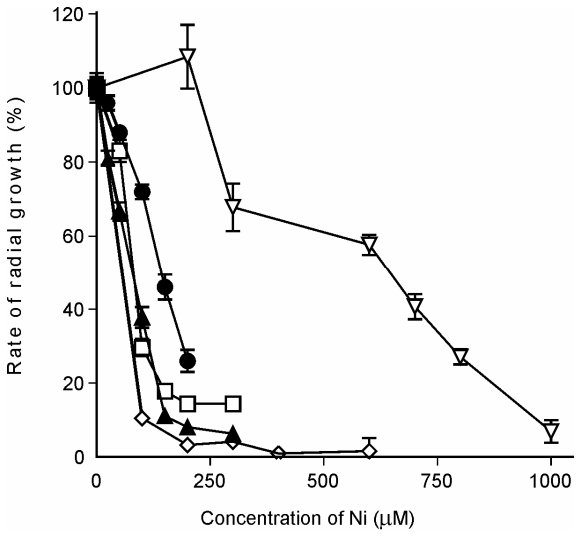


Figure 2.3. Effect of Ni on the rates of radial growth (kr) of *V. elodeae* (□), *Y. graminea* (▲), *H. submersus* (∇), *T. chaetocladium* (◊) and *A. acuminata* (●), in 1% malt extract, at pH 5.0 and 18ºC. Results are mean ± SE of four replicates.

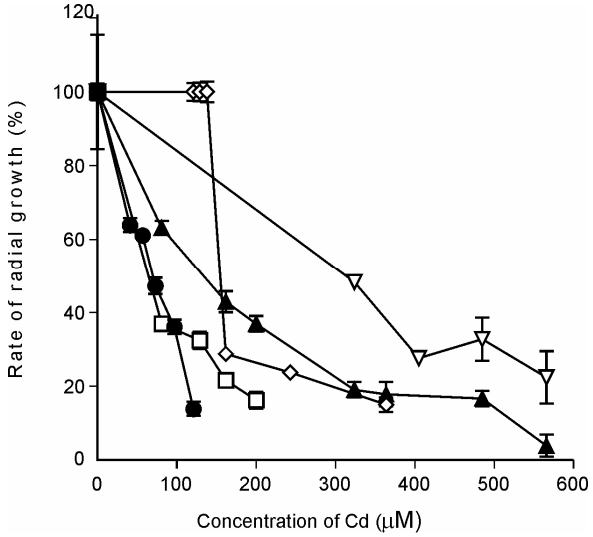


Figure 2.4. Effect of Cd on the rates of radial growth (kr) of *V. elodeae* (□), *Y. graminea* (▲), *H. submersus* (∇), *T. chaetocladium* (◊) and *A. acuminata* (●), in 1% malt extract, at pH 5.0 and 18ºC. Results are mean ± SE of four replicates.

When analyzing the degree of toxicity of Zn, Cu, Ni and Cd for each fungal species in terms of $EC_{50}$ values, the following toxicity patterns can be established: *V. elodeae* Ni≥ Cd>Cu>Zn; *Y. graminea* Cd≥ Ni>Cu>Zn; *H. submersus* Cd≥ Zn≥ Ni>Cu; *A. acuminata* Cd>Ni≥ Cu>Zn; *T. chaetocladium* Ni≥ Cd>Cu≥ Zn. This indicates higher sensitivity of aquatic hyphomycetes to Cd and Ni than to other metals, except for *H. submersus* (Table 2.2).

### *2.3.1.2. Fungal growth in liquid medium*

Two liquid media were used to test the effects of metals on the growth of aquatic hyphomycete species, namely 1% malt extract (ME) and mineral medium with vitamins and 2% glucose (MK).

When *V. elodeae* and *Y. graminea* were grown in 1% ME in the absence of metal, visible growth occurred after 2 days in the former species (Figure 2.5A) and after 4 days in the latter species (Figure 2.5B). After 8 days of growth *V. elodeae* produced more biomass (0.76 mg dry mass $ml^{-1}$) than *Y. graminea* (0.35 mg dry mass $ml^{-1}$) (Figure 2. 5). Cadmium exposure led to a decrease in biomass production in *V. elodeae* and *Y. graminea*, and the effects were more pronounced with increasing Cd concentrations (Figure 2.5). After 8 days, the highest Cd concentration (28 μM) led to an inhibition of biomass production in 55 and 86% in *V. elodeae* and *Y. graminea,* respectively (Figure

2.5). Furthermore, an increase in the growth lag phase with increasing Cd concentrations was observed for *Y. graminea* (Figure 2.5 B).

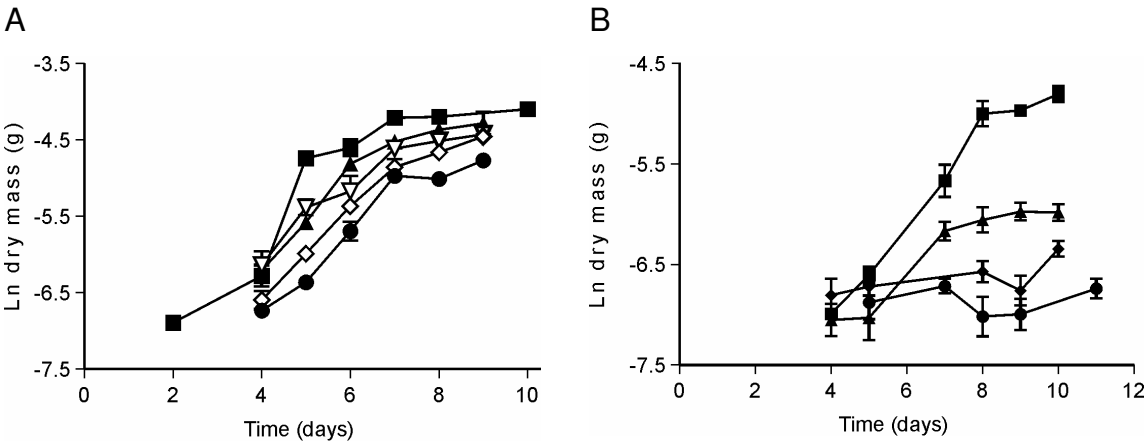


Figure 2.5. Effects of Cd on biomass production by *V. elodeae* (A) and *Y. graminea* (B) in 1% ME. Symbols are: ■, control cultures; ▲, 8 µM; Δ, 16 µM; ◊, 24 µM; ●, 28 µM in graph A and ■, control cultures; ▲, 4 µM; ♦, 20 µM; ●, 28 µM in graph B. Results are mean ± SEM of three replicates.

Figure 2.6 show the effects of Zn on the growth of *V. elodeae* and *Y. graminea* in 1% ME. Exposure to concentrations of Zn higher than 50 µM led to a decrease in biomass production in *V. elodeae*. For *Y. graminea* all the tested Zn concentrations inhibited biomass production (Figure 2.6 B). An increase in the growth lag phase for the highest Zn concentrations was observed for *Y. graminea*.

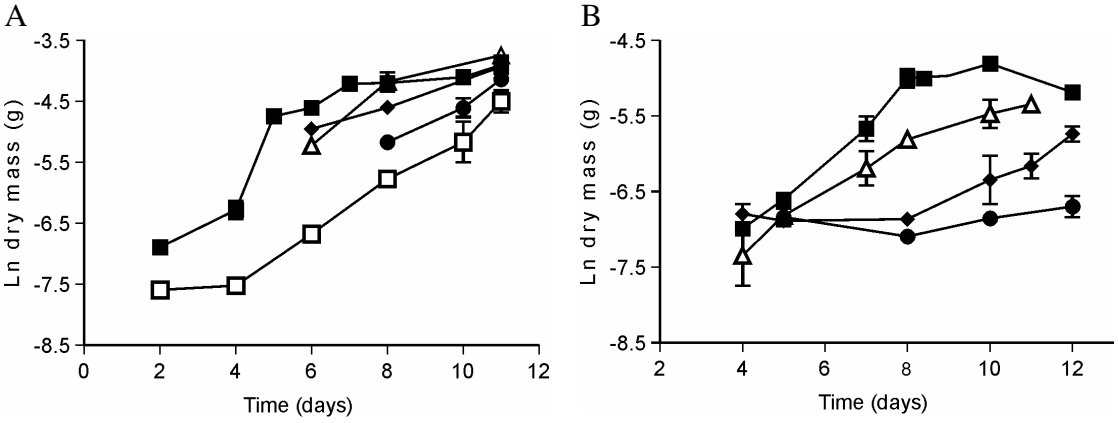


Figure 2.6. Effects of Zn on biomass production by *V. elodeae* (A) and *Y. graminea* (B) in 1% ME. Symbols are: ■, control cultures; Δ, 50 µM; ♦, 100 µM; ●, 150 µM; □, 175 µM in graph A and ■, control cultures; Δ, 50 µM; ♦, 250 µM; ●, 300 µM in graph B. Results are mean ± SEM of three replicates.

Growth inhibition parameters, namely $EC_{50}$, LOEC and NOEC, determined after 8 days exposure to metals, showed that *V. elodeae*, *Y. graminea* and *F. curta* were more sensitive to Cd than Zn. Moreover *Y. graminea* was the more resistant species to Zn and *F. curta* the most sensitive one (Table 2.4). Concerning Cu effect *H. submersus* was the more resistant species and *V. elodeae* the more sensitive one (Table 2.4). Values of LOEC and NOEC confirmed that *Y. graminea*, *H. submersus* and *F. curta* were the most resistant species respectively from Zn, Cu and Cd (Table 2.4).

The effects of Zn and Cu on biomass production by *V. elodeae*, *H. submersus*, *F. curta* and *Y. graminea* were also assessed in MK and results are shown in table 2.5. Analysis of $EC_{50}$ values showed that *V. elodeae* was the most resistant species to Zn, with an $EC_{50}$ value 16-times higher than that of *H. submersus*, which was the most sensitive species. However, *H. submersus* was the most resistant species to Cu, with an $EC_{50}$ 8-times higher than that of *F. curta*, the most sensitive species to this metal (Table 2.5). Consistently, the highest NOEC and LOEC values were obtained for Zn and Cu in *V. elodeae* and in *H. submersus*. Moreover, the most sensitive species to Zn (*H. submersus*) and to Cu (*F. curta*) showed the lowest LOEC values (Table 2.5).

Furthermore, results showed that higher concentrations of Zn than Cu were necessary to promote 50% of biomass inhibition for all species, excluding *H. submersus* (Table 2.5).

Table 2.4. Concentrations (µM) inhibiting biomass production in 50% ($EC_{50}$), concentrations that had no effect on biomass production (NOEC) and the lowest concentrations that induced effect on biomass production (LOEC) for Zn, Cu and Cd in *V. elodeae*, *Y. graminea*, *H. submersus* an *F. curta* grown 8 days in 1% ME.

| Fungal species | Zn | | | Cu | | | Cd | | |
|---|---|---|---|---|---|---|---|---|---|
| | $EC_{50}$ | NOEC | LOEC | $EC_{50}$ | NOEC | LOEC | $EC_{50}$ | NOEC | LOEC |
| *V. elodeae* | 152±4.9 | 100 | 150 | 54±4.7 | 20 | 50 | 12.3±1.3 | 8 | 16 |
| *Y. graminea* | 194±5.5 | 150 | 250 | - | - | - | 8.5±0.6 | - | - |
| *H. submersus* | 103±1.6 | - | 50 | 102±1.7 | 30 | 60 | - | - | - |
| *F. curta** | 78.2 | - | - | 90.4 | 15 | 30 | 19.3 | 12 | 20 |

Values are mean ± SE (-) not determined, * values from Guimarães-Soares (2005).

Table 2.5. Concentration (µM) inhibiting biomass production in 50% ($EC_{50}$), concentration that had no effect on biomass production (NOEC) and the lowest concentration that induced effect on biomass production (LOEC) for Zn and Cu in *V. elodeae*, *Y. graminea*, *H. submersus* and *F. curta* grown 8 days in MK.

| Fungal species | Zn | | | Cu | | |
|---|---|---|---|---|---|---|
| | $EC_{50}$ | NOEC | LOEC | $EC_{50}$ | NOEC | LOEC |
| *V. elodeae* | 7315±305.7 | 7000 | 7500 | 457±58.8 | - | 500 |
| *Y. graminea* | 3740±120.9 | 1200 | 2000 | 776±94.9 | - | 600 |
| *H. submersus* | 465±83.7 | - | 800 | 1510±19 | 1000 | 1500 |
| *F. curta* | 1304±54.9 | 1000 | 2000 | 183±7.0 | 70 | 180 |

Values are mean ± SE.

### *2.3.2. Effects of Zn and Cd on fungal sporulation*

Figure 2.7 show conidial production along time by *H. submersus* and *T. chaetocladium*. In the absence of Zn, a peak of sporulation was observed at the 4$^{th}$ day of the experiment, corresponding to 21643 conidia ml$^{-1}$ for *H. submersus* (Figure 2.7A) and 3528 conidia ml$^{-1}$ for *T. chaetocladium* (Figure 2.7B). The sporulation capacity of *H. submersus* was 6-times higher than that of *T. chaetocladium*.

Metal effects on sporulation of *H. submersus* were evaluated in concentrations between 0.25 and 25 µM of Zn. A significant decrease in conidial production was found for all treatments and times ($p$<0.001; Figure 2.7A). In *T. chaetocladium* a significant decrease in conidial production ($p$<0.001) was observed at all tested Zn concentrations Figure 2.7B. Exposure time significantly affected sporulation of *T. chaetocladium* ($p$<0.001; Figure 2.7B). Exposure to concentrations of 0.025 µM Zn led to an earlier peak of sporulation (2$^{nd}$ day) while lower Zn concentrations, delayed the peak of sporulation (6$^{th}$ day) (Figure 2.7B).

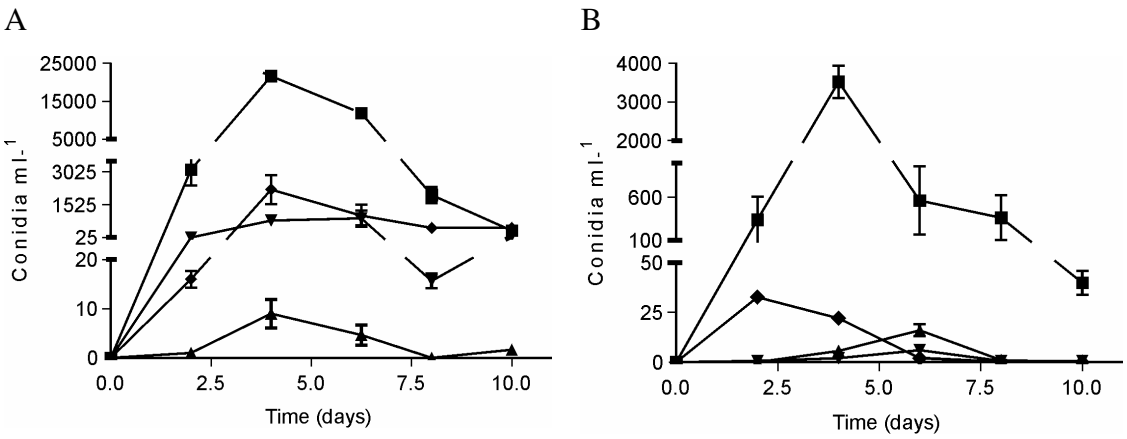


Figure 2.7. Effects of Zn on sporulation by *H. submersus* (A) and *T. chaetocladium* (B). Symbols are: ■, control; ♦, 0.25 µM; ▼, 2.5 µM; ▲, 25 µM in graph A; ■, control; ▼, 0.00025 µM; ▲, 0.0025 µM; ♦, 0.025 µM in graph B. Results represent the mean ± SEM of three replicates.

Cumulative conidial production by *H. submersus* exposed to Zn for 10 days significantly decreased, particularly at higher Zn concentrations (Figure 2.8). In *T. chaetocladium*, Zn at all concentrations drastically inhibited cumulative conidial production, however no significant differences were found between the treatments (Figure 2.8).

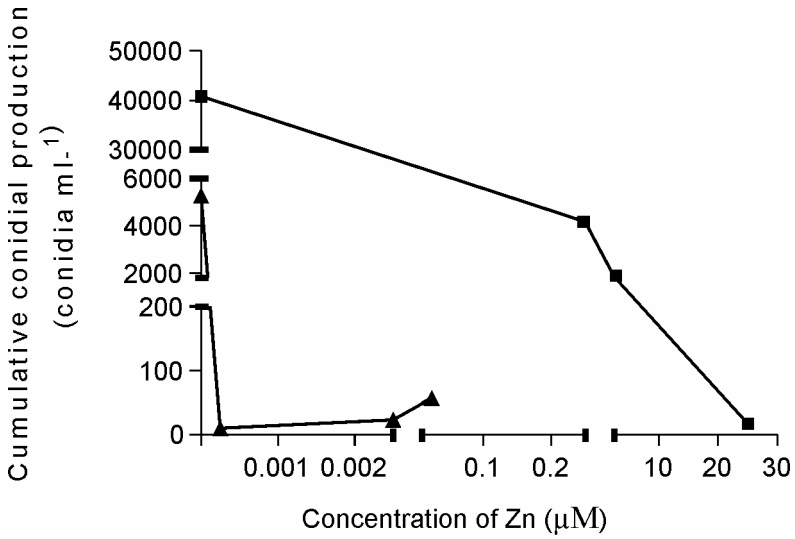


Figure 2.8. Cumulative production of conidia by *H. submersus* (■) and *T. chaetocladium* (▲) in the presence of Zn, after 10 days exposure.

Effects of Cd for *H. submersus* were evaluated in concentrations between 1 and 100 µM along 10 days. The exposure to low Cd concentration did not change the magnitude and position of sporulation peak ($p$>0.05; Figure 2.9A), although significantly lower conidial production was found ($p$<0.001; Figure 2.9A). For 10 µM of Cd, conidial production was very low, with 2 and 1 conidia $ml^{-1}$ produced after 4 and 6 days, respectively (Figure 2.9A). Concentrations higher than 10 µM completely inhibited sporulation.

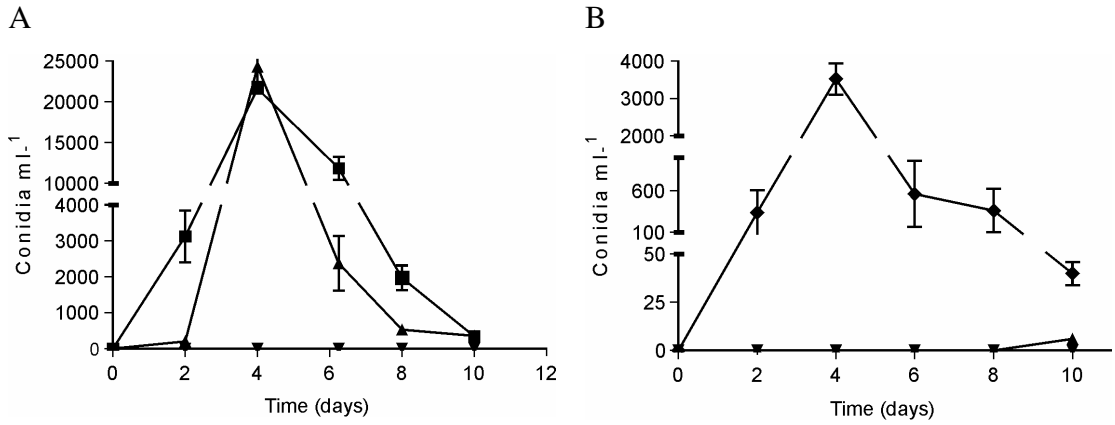


Figure 2.9. Effect of Cd on sporulation of *H. submersus* (A) and *T. chaetocladium* (B). Symbols are: ■, control; ▲, 1 µM; ▼, 10 µM in graph A; ♦, control; ▼, 0.005 µM in graph B. Results represent the mean ± SEM of three replicates.

In *T. chaetocladium*, the effects of Cd were tested in concentrations between 0.005 µM and 50 µM. Conidial production by this fungal species was only found after 10 days of exposure to the lowest Cd concentration (0.005 µM Cd), with 6 conidia produced per ml of sporulation medium (Figure 2.9B).

## 2.4. Discussion

In natural environments, fungi rarely encounter conditions that allow optimal growth, since they are conditioned by abiotic factors, nutrient availability and pollutants (Gadd *et al*., 2001). Metals are common in urban aquatic ecosystems since they are not biodegradable and can persist in the environment. Pollution by metals is reported to

decrease fungal diversity in streams (Sridhar *et al*., 2000; Niyogi *et al*., 2002; Pascoal *et al.,* 2005). Also, fungal activity, as reproduction and biomass buildup, can be negatively affected by high levels of metals in the stream water (Bermingham *et al*., 1996; Miersch *et al*., 1997; Duarte *et al*., 2004, 2008). Nevertheless, fungi are ubiquitous and can occur in metal-polluted habitats (Sridhar *et al*., 2000). In the present work, we assessed the effects of Zn, Cu, Ni and Cd on the growth and sporulation of several aquatic hyphomycete species to understand their ability to survive in metal-polluted streams.

In the absence of metals, conidial production of *H. submersus* was 6-times higher than that of *T. chaetocladium,* which may be due to the marked differences in their conidial size. Indeed, conidia of the former species are, at least, three-times smaller than that of *T. chaetocladium*. This observation is in agreement with previous studies reporting different life strategies of aquatic fungi; generally, fungi with small-size conidia appear to invest in reproduction more than in growth (Chauvet and Suberkropp, 1998; Trenton *et al*., 2004; Duarte *et al*., 2006). The exposure to Zn or Cd inhibited sporulation of both fungal species. Conidial production of *T. chaetocladium* was more inhibited by metals than that of *H. submersus*. In addition, Cd was more toxic than Zn to fungal reproduction, which is consistent with the general higher toxicity of Cd than Zn found by other authors in aquatic hyphomycetes (Rodrigues, 2002).

In solid medium, mycelial growth was linear and, in most cases, metals negatively affected fungal growth. Nevertheless, our results showed that low concentrations of Zn, Cu and Ni were able to stimulate the rate of radial growth of some aquatic hyphomycete species, such as *T. chaetocladium*, *H. submersus* and *A. acuminata,* which is consistent with the role of these metals as micronutrients (Gadd, 1993). Indeed, Zn and Cu are essential components of enzymes, such as cytochrome oxidase and Cu/Zn superoxide dismutase (Walker *et al*., 1996) and Ni is an essential micronutrient for many microorganisms serving as enzyme cofactor that catalyzes a diverse array of reactions (Hausinger and Zamble, 2007). The general lower toxicity of Zn and Cu than other metals found in this work is corroborated by previous reports (Gadd, 1993; Miersch *et al*., 1997; Blaudez *et al*., 2000; Colpaert *et al*., 2000; Rodrigues, 2002; Guimarães-Soares, 2005). The sensitivity of aquatic hyphomycetes to metals, assessed as $EC_{50}$ values, showed that *Y. graminea* and *V. elodeae* were the most resistant species to Zn, while *A. acuminata*, *H. submersus* and *F. curta* appeared to be the most sensitive species to this metal. On the contrary, *H. submersus* was the most resistant species to

Cu. Moreover, the patterns of species resistance to metals found either in liquid or solid medium with similar composition were identical. However, $EC_{50}$ values were about 20-times higher in solid medium than in liquid medium, probably because agar might decrease metal bioavailability to fungi (Gadd, 1993).

Changes in nutrient supplies to fungi are expected to change metal toxicity. Indeed, maximum $EC_{50}$ values in MK were at least of one order of magnitude higher than in ME. Gadd *et al*. (2001) showed that metal toxicity decreased if the amount of carbon source (glucose) increased in the culture medium; MK has higher glucose content than ME, which probably favoured the aquatic hyphomycete nutritional status, allowing them to tolerate higher metal stress.

The high sensitivity of *A. acuminata* to all metals may be related to the fact that this species was isolated from decomposing leaves collected in a clean stream. Consistently, *H. submersus* and *F. curta* isolated from a metal-polluted stream showed high tolerance to the most toxic metals (Cu, Ni and Cd). These findings suggest that fungi adapted to metal-polluted environments tolerate higher metal concentrations. However, this was not always the case. For example, *Y. graminea* isolated from the same metal-polluted stream was tolerant to Zn but not to Cd. Also, *V. elodeae* a species isolated from a clean site was able to tolerate high levels of Zn but not of Cu. This may indicate that fungal tolerance to metals can vary with fungal species and metal type. Blaudez *et al*. (2000) using 39 isolates (21 of which from contaminated sites) of 5 different species of ectomycorrhizal fungi showed that $EC_{50}$ values for isolates of polluted sites did not differ from those of non-contaminated sites. Further studies using isolates adapted to different metal-stress conditions may help to clarify whether metal tolerance of aquatic hyphomycetes is more dependent on fungal species or strain.

Our results also indicate that fungal tolerance to one metal does not confer tolerance to all metals, suggesting that different mechanisms and/or cellular targets might be implicated in fungal tolerance to different metals. These aspects will be investigated in the next chapters of this thesis, aiming to better understand the interactions between aquatic hyphomycetes and toxic metals.

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# Chapter 3

## *Biochemical responses to Cu and Zn stress in aquatic fungi: the major role of antioxidant defenses*

Azevedo M-M, Carvalho A, Pascoal C, Rodrigues F and Cássio F. 2007. Responses of antioxidant defenses to Cu and Zn stress in two aquatic fungi. *Science of the Total Environment* 377: 233-243.

## Abstract

Aquatic hyphomycetes are fungi that play a key role in plant litter decomposition in streams. Even though these fungi occur in metal-polluted streams, the mechanisms underlying their tolerance to metals are poorly documented. We addressed the effects of Zn and Cu in *Varicosporium elodeae* and *Heliscus submersus* by examining metal adsorption to cell walls, plasma membrane integrity and production of reactive oxygen species at metal concentrations inhibiting biomass production in 50% or 80%. The activity of the enzymes catalase, superoxide dismutase and glucose-6-phosphate dehydrogenase was measured to elucidate their role in coping with oxidative stress induced by metals at short- (14 hours) and long- (8 days) term exposure. Results show that *V. elodeae* was more susceptible to the toxic effects induced by Cu and Zn than *H. submersus*, as indicated by more extensive inhibition of biomass production. Both metals, particularly Cu, induced oxidative stress in the two fungal species, as shown by the noticeable recovery of biomass production in the presence of an antioxidant agent. In both fungi, Cu induced a more severe disruption of plasma membrane integrity than Zn. Our studies on antioxidant defenses showed that catalase had a greater role alleviating stress induced by Zn and Cu than superoxide dismutase. Chronic metal stress also stimulated the production of NADPH, via the pentose phosphate pathway by increasing the activity of glucose-6-phosphate dehydrogenase. Our results suggest that the tolerance of aquatic hyphomycetes to Cu and Zn is associated with the ability of these fungi to initiate an efficient antioxidant defense system.

## 3.1. Introduction

Freshwater pollution by heavy metals is a worldwide problem with serious environmental consequences. Heavy metals can be introduced into ecosystems through industrial effluents and wastes, agricultural fungicide runoff, domestic garbage dumps and mining activities (Merian, 1991). The non-degradability of metals, their accumulation in biota, and biomagnification along aquatic food chains (Spacie *et al*., 1995) contribute to the importance of studying metal effects in biological systems.

Metals, such as Cu and Zn, are essential for living organisms, including fungi, although elevated concentrations of metals can result in growth inhibition and toxicity. The ability of organisms to survive in environments with high levels of metals depends on their capacity to regulate intracellular concentration of metal ions. In fungi, metal tolerance has been attributed to several mechanisms, including trapping of metal by cell wall components, altered metal uptake, extracellular chelation or precipitation by secreted metabolites, and intracellular complexation by metallothioneins (Gadd, 1993).

The toxicity of metals can be the result of the generation of reactive oxygen species (ROS) that may cause wide-ranging damage to proteins, nucleic acids and lipids, eventually leading to cell death (Moradas-Ferreira *et al*., 1996; Bai *et al*., 2003). In *Saccharomyces cerevisiae*, the primary mechanism of Cu toxicity is the disruption of cellular and organellar membranes, resulting in a loss of membrane integrity and impairment of membrane function (Ohsumi *et al*., 1998). This effect has been attributed to the redox active nature of Cu and its ability to generate free radicals that promote lipid peroxidation (Stohs and Bagchi, 1995). On the other hand, non-redox active metals like Zn can deplete free-radical scavengers, such as thiol-containing compounds, resulting in ROS production (Dietz *et al*., 1999).

Tolerance of the yeast *Candida intermedia* to different metals has been associated with its ability to deal with ROS generation (Fujs *et al*., 2005). Fungi display several antioxidant enzymes against ROS, including catalase (CAT), superoxide dismutases (SOD), glutathione peroxidase and glutathione reductase, capable of removing oxygen radicals and their products and/or repairing oxidative damage (Jamieson, 1998; Bai *et al*., 2003). In addition, molecules such as glutathione, besides playing an important role in cellular protection during oxidative stress, may complex metals in cells (Penninckx

and Elskens, 1993). Glutathione recycling is dependent on the maintenance of an intracellular pool of NADPH mainly via the pentose phosphate pathway, in which the reaction catalyzed by glucose-6-phosphate dehydrogenase (G6PDH) is the rate-limiting step (Jamieson, 1998).

Aquatic hyphomycetes are a phylogenetically heterogenous group of fungi that play a crucial role in plant litter decomposition in streams, mediating carbon and energy transfer to higher trophic levels (Bärlocher, 1992). Metals, such as Cd, Cu and Zn, are known to inhibit the growth and reproduction of aquatic hyphomycetes in both axenic cultures (Abel and Bärlocher, 1984; Miersch *et al*., 1997) and natural mixed assemblages (Sridhar *et al*., 2001; Duarte *et al*., 2004). Metals are also reported to decrease fungal diversity in freshwaters (Sridhar *et al*., 2000). However, several aquatic hyphomycete species have been found in severely metal-polluted streams (Sridhar *et al*., 2000; Krauss *et al*., 2001), increasing the interest of elucidating the mechanisms underlying the resistance/tolerance of these fungi to metal stress.

In this study, we investigated the response mechanisms to Cu and Zn exposure in two aquatic hyphomycete species, *Varicosporium elodeae* and *Heliscus submersus*. In a first approach, biochemical responses associated with cellular barriers against metal stress, like metal ion adsorption to cell walls and plasma membrane integrity, were evaluated. Since cell damages by metals may occur through the generation of ROS, we can expect changes in the enzymatic antioxidant defenses to deal with metal-induced oxidative stress. Therefore, the activities of CAT, SOD and G6PDH were examined under acute and chronic stress induced by Cu and/or Zn.

## 3.2. Materials and methods

### *3.2.1. Fungal species, growth conditions and metal exposure*

The aquatic hyphomycetes were isolated from single spores collected in streams in the Northwest of Portugal. *Varicosporium elodeae* W. Kegel (UMB-142.01) was isolated from foam sampled in a clean stream at the Peneda-Gerês National Park, while *Heliscus submersus* H. J. Huds. (UMB-135.01) was isolated from leaves retrieved in the Este River at the industrial park of the town of Braga, where Zn and Cu concentrations in the water column attained 80 µM and 150 µM, respectively. Details on fungal species and characterization of water chemistry of their origin sites are in Pascoal *et al*. (2005).

The fungi were grown in 1% malt extract (pH 5.0), with or without addition of Cu or Zn, with shaking (160 rpm; Certomat BS 3, B. Braun Biotech International) at 18ºC under permanent artificial light, using spores as inoculum (final concentration, 6 conidia $ml^{-1}$).

Growth medium was autoclaved and solutions of copper ($CuCl_2$) and zinc ($ZnCl_2$) were sterilized by filtration (0.22 µm pore size membrane), before aseptic addition to the medium. Final metal concentrations ranged from 10 to 150 µM for Cu and from 50 to 200 µM for Zn. For long term-exposure, fungi were grown in media with or without metal addition for 8 days. For short-term exposure, mycelia grown 8 days without metal were transferred to fresh media with or without added Cu, Zn, or a mixture of the two metals for periods from 30 min to 14 hours. The pH of cultures was measured at the end of experiments.

To determine the contribution of metal-induced ROS to biomass inhibition, fungi were grown 8 days in the absence or presence of Cu or Zn, at concentrations inhibiting biomass production by 50% ($EC_{50}$) or 80% ($EC_{80}$), with or without the antioxidant butylated hydroxytoluene (BHT; final concentration, 1.13 µM).

To quantify fungal biomass, mycelia were dried at 85ºC to constant mass and weighed to the nearest 0.001 g.

### *3.2.2. Scanning electron microscopy*

Scanning electron microscopy was used to examine the surface of mycelia and to evaluate Cu and Zn adsorption to cell walls, after short- (14 hours) and long-term (8 days) exposure to metals at $EC_{50}$ and $EC_{80}$, under the conditions indicated above. Mycelia were harvested by filtration, washed twice with deionized water, dissociated into small pieces and fixed in 3% (v/v) glutaraldehyde for 22 hours. Subsequently, mycelia were dehydrated in ethanol (v/v) as follows: 30%, 5 hours; 60%, 2 hours; and 100%, 1 hour. Mycelia were then glued onto 20-mm diameter metal mounts, coated with gold under vacuum and scanned with scanning electron microscopy (Leica Cambridge S 360) coupled to an energy dispersive X-ray microanalysis setup (20 KeV).

### *3.2.3. Plasma membrane integrity*

Plasma membrane integrity was assessed by a membrane impermeable dye, propidium iodide (PI; Molecular Probes, Eugene, OR), which enters the cells and binds

to nucleic acids when plasma membrane disruption occurs. Mycelia were dissociated into small pieces in phosphate buffer (1x PBS, pH 7.4) and incubated with PI (final concentration, 0.005 μg $\mu l^{-1}$) for 15 min at room temperature. Subsequently, mycelia were exposed to $EC_{50}$ concentrations of Cu or Zn during 150 min and scanned each 30 min under an epifluorescence microscope (Zeiss Axioskop connected to an AxioCam HRc camera).

### *3.2.4. Reactive oxygen species production*

ROS production was monitored with the MitoTracker Red CM-$H_2$XRos (Molecular Probes, Eugene, OR). The reduced form of this dye does not fluoresce until entering an actively respiring cell, where it is oxidized by ROS to a red fluorescent compound, which is sequestered in mitochondria. Mycelium suspensions, prepared as above, were passed through a syringe, and incubated with CM-$H_2$XRos (final concentration, 3.3 μg $ml^{-1}$) for 15 min at room temperature. Mycelia were then exposed to $EC_{50}$ and $EC_{80}$ concentrations of Cu or Zn for 30 and 90 min and scanned under an epifluorescence microscope.

### *3.2.5. Preparation of cell-free extracts and determination of enzymatic activities*

Fungal mycelia were harvested by filtration, washed twice with deionized water, and pressed between two layers of filter paper to remove the excess of water. Mycelia were mixed with purified sea sand (2 g $g^{-1}$ mycelium wet mass) and ground in liquid nitrogen in a cooled mortar for 4 min. The mixture was suspended in a buffer solution (20 mM Tris, 1 mM EDTA; pH 7.5), and cell-free extracts were obtained in 2 steps of centrifugation (6200 *g* for 10 min; 18000 *g* for 50 min) at 4ºC.

Superoxide dismutase (SOD) activity was determined according to McCord and Fridovich (1969). One unit of SOD is the amount of enzyme able to inhibit the reduction of cytochrome c by 50%. The reaction mixture consisted of: 800 μl 50 mM potassium phosphate, 0.1 mM EDTA (pH 7.8); 50 μl 0.2 mM cytochrome c; 50 μl 1 mM xanthine in 1 M sodium hydroxide; 50 μl xanthine oxidase (5 units); 45 μl buffer solution 20 mM Tris 1 mM EDTA (pH 7.5); and 5 μl sample.

Catalase (CAT) activity was determined by measuring the decrease in absorbance at 240 nm due to $H_2O_2$ consumption according to Beers and Sizer (1952). The reaction

mixture consisted of: 657 µl 50 mM phosphate buffer pH 7.0; 333 µl 30 mM $H_2O_2$; and 10 µl sample.

Glucose-6-phosphate dehydrogenase (G6PDH) activity was based on the increase in absorbance at 340 nm, resulting from NADP reduction according to Postma *et al*. (1989). The reaction mixture consisted of: 870 µl $H_2O$ desionized; 50 µl 1 M Tris-HCl pH 8.0; 10 µl $NADP^+$ (disodium); 10 µl $MgCl_2.6H_2O$; 50 µl glucose-6-phosphate; and 10 µl sample.

Enzymatic activities were measured after short- (14 h) and long-term (8 days) exposure to metals, and were expressed as U $mg^{-1}$ of total protein.

Protein concentration was determined according to Lowry *et al*. (1951) using bovine serum albumin (BSA) as standard.

### *3.2.6. Statistical analysis*

Data of Cu and Zn effects on enzymatic activities were expressed as percentage of control. Values were divided by 1000 and arcsine square root transformed to achieve normal distribution and homocedasticity (Zar, 1996). For each metal and fungal species, enzymatic activities were compared by one-way ANOVA, followed by a Dunnett's test to identify significant effects ($p < 0.05$; Zar, 1996).

Metal concentration corresponding to $EC_{50}$ and $EC_{80}$ of biomass inhibition were determined by non-linear regression. Inhibition rates of biomass production ($k_i$) were compared by an F-test using non-transformed data (Motulsky and Christopoulos, 2003).

Statistical analysis was done using Prism 4 for Macintosh (GraphPad software Inc., San Diego).

## 3.3. Results

### *3.3.1. Comparison of metal sensitivity*

To characterize the sensitivity of aquatic hyphomycetes to Cu and Zn, we determined the effects of these metals in biomass production after 8 days of growth on a concentration-dependent basis (Figure 3.1). The analysis of growth inhibition parameters, namely metal concentration inhibiting biomass production by 50% ($EC_{50}$)

and 80% ($EC_{80}$), and inhibition rate of biomass production ($k_i$), showed that the two species had different levels of resistance to metal stress (Table 3.1). *H. submersus* was more resistant to Cu than *V. elodeae*, even though the effect of Cu on the $k_i$ did not differ significantly between species. The exposure to Zn did not inhibit biomass production in *V. elodeae* until 100 µM, a concentration corresponding to $EC_{50}$ in *H. submersus* (Figure 3.1). However, *H. submersus* had a significantly lower $k_i$ than *V. elodeae* when exposed to Zn, suggesting higher tolerance of the former species to this metal (Table 3.1). Higher concentrations of Zn than Cu were necessary to promote identical toxicity effects in both fungi.

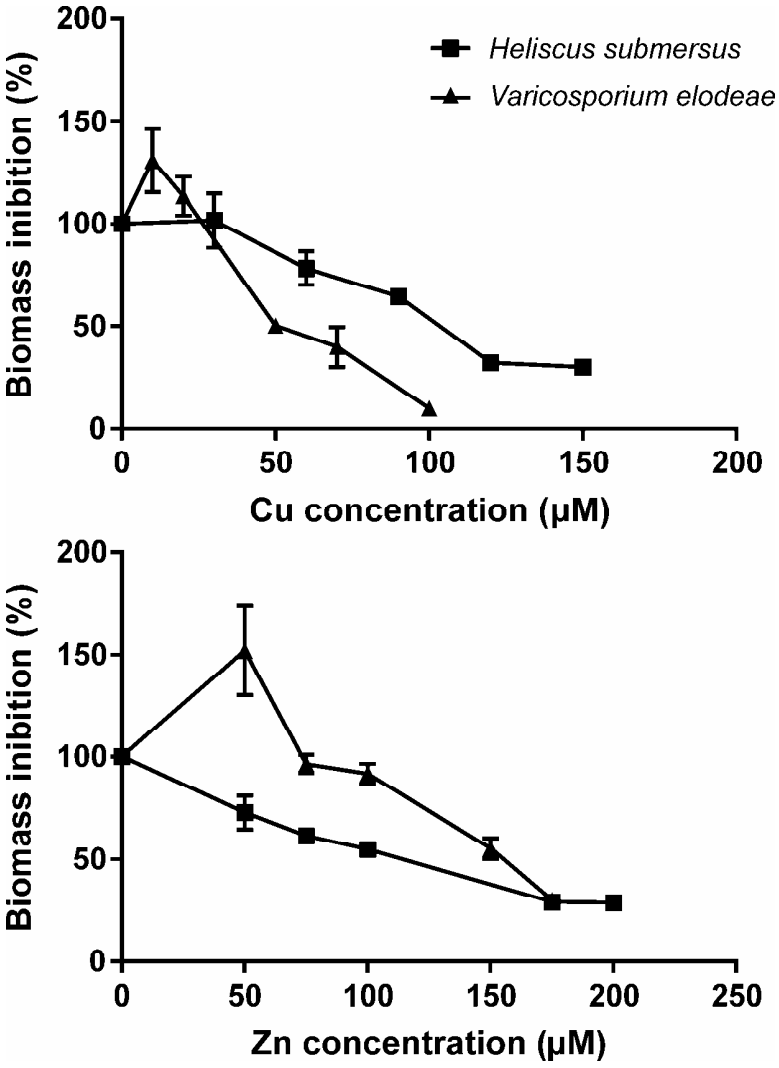


Figure 3.1. Biomass production by the aquatic hyphomycetes *H. submersus* and *V. elodeae* exposed for 8 days to Cu and Zn. Mean ± SEM, $n = 3$.

Table 3.1. Concentrations inhibiting biomass production in 50% ($EC_{50}$) and 80% ($EC_{80}$), and biomass production inhibition rate ($k_i$) for Cu and Zn in the aquatic hyphomycetes *Varicosporium elodeae* and *Heliscus submersus*

| Fungal species | Cu | | | Zn | | |
|---|---|---|---|---|---|---|
| | $EC_{50}$ (µM) | $EC_{80}$ (µM) | $k_i$ ($\mu M^{-1}$) | $EC_{50}$ (µM) | $EC_{80}$ (µM) | $k_i$ ($\mu M^{-1}$) |
| *V. elodeae* | 54 ± 4.7 | 85 ± 2.6 | 3.3 ± 0.8 [a] | 152 ± 4.9 | 189 ± 4.2 | 5.7 ± 0.8 [b] |
| *H. submersus* | 102 ± 1.7 | 174 ± 3.6 | 2.8 ± 0.5 [a] | 103 ± 1.6 | 267 ± 5.1 | 1.5 ± 0.1 [c] |

Values are means ± SE. Similar letters indicate no significant differences ($p > 0.05$) between $k_i$ (F test)

### *3.3.2. Biochemical responses associated with cellular barriers against metal stress*

To assess the biochemical responses associated with cellular barriers against Cu and Zn, metal adsorption to cell walls and plasma membrane integrity were evaluated. In addition, we followed changes in the pH of the medium, because some fungi are able to release organic acids that can bind metal ions (Gadd, 1993). In both aquatic hyphomycete species metals did not elicit medium acidification (not shown).

Scanning electron microscopy of mycelia of *V. elodeae* and *H. submersus*, exposed to Cu or Zn for 14 hours or 8 days at $EC_{50}$ and $EC_{80}$, showed some morphological alterations such as cell shrinkage, particularly after short-term exposure to either metal or long-term exposure to the highest Cu concentration (see Figure 3.2, for *H. submersus* exposed to Cu). However, metal adsorption onto cell walls was not detected under these conditions.

To elucidate whether plasma membrane could be a primary target of metal induced stress, we assessed cellular permeabilization to propidium iodide (PI) of mycelia exposed for short-term (30 - 150 min) to Cu or Zn. Exposure of *V. elodeae* or *H. submersus* to Cu resulted in severe disruption of plasma membrane integrity, particularly in the former species, while Zn elicited a much less pronounced response (see Figure 3.3-I, for *V. elodeae*). A recovery of plasma membrane integrity was detected after 150 min of exposure to Cu in *H. submersus*, but not in *V. elodeae* (not shown).

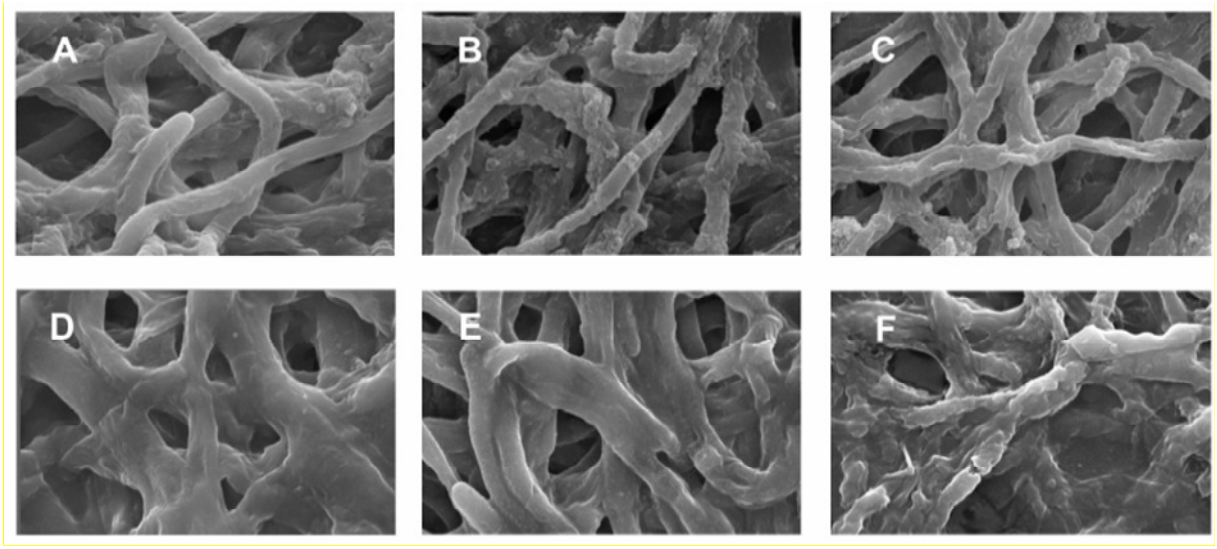


Figure 3.2. Scanning electron microscopy of *H. submersus* mycelia exposed for short- (B, C) or long-term (E, F) to Cu at concentrations of $EC_{50}$ (B, E) or $EC_{80}$ (C, F). Control mycelia for short- (A) and long-term (D) experiments; magnification, X5,000.

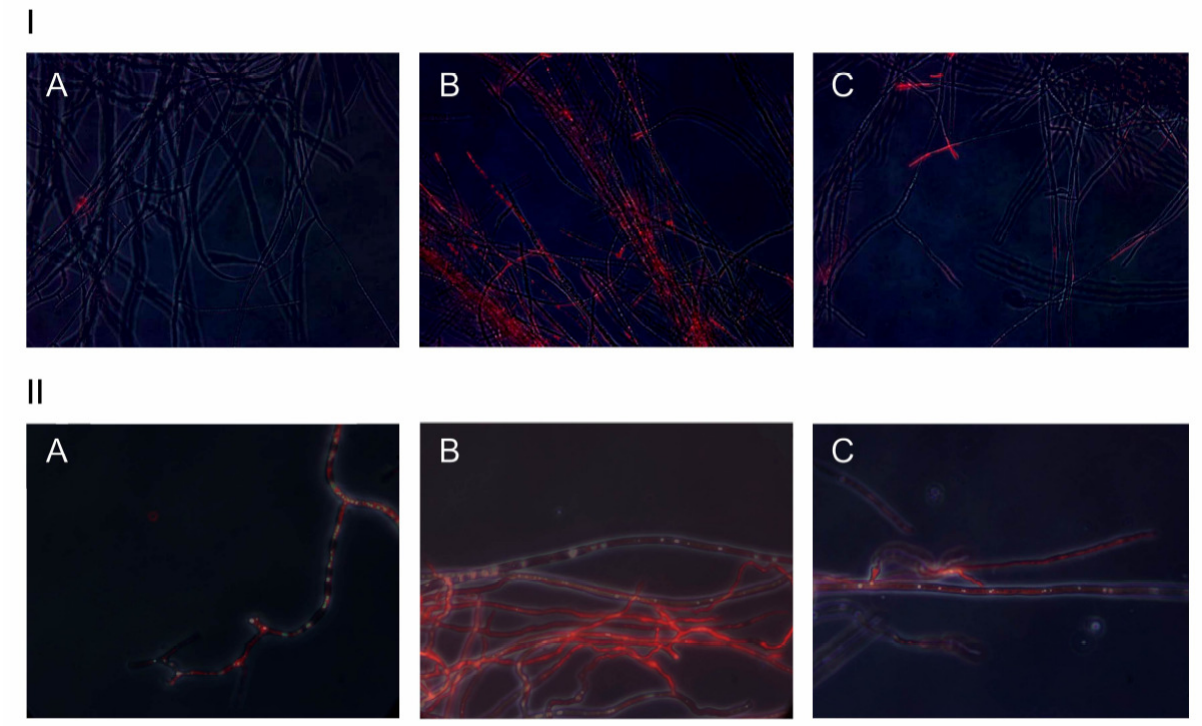


Figure 3.3. Fluorescence microscopy images of *V. elodeae* mycelia. I, Plasma membrane integrity assessed by propidium iodide in mycelia unexposed (A) or exposed for 30 min to $EC_{50}$ of Cu (B) or Zn (C); magnification, X400. II, ROS production assessed by CM-$H_2$XRos in mycelia unexposed (A) or exposed for 30 min to $EC_{50}$ of Cu (B) or Zn (C); magnification, X1000.

### *3.3.3. Oxidative stress induced by Cu and Zn*

The exposure of *H. submersus* and *V. elodeae* to Cu or Zn induced ROS generation as shown by MitoTracker Red CM-$H_2$XRos staining (see Figure 3.3-II, for *V. elodeae*). Higher levels of ROS were detected under Cu exposure in both fungal species. Moreover, these free radicals increased in *H. submersus* mycelia in a time- and concentration-dependent manner, while in *V. elodeae* only a concentration-dependent effect was observed (not shown).

To determine the effects of ROS induced by metals in the inhibition of biomass production, the antioxidant butylated hydroxytoluene (BHT) was included in the culture medium. The presence of this antioxidant agent resulted in an increase in biomass production in both species (Figure 3.4), particularly in the case of Cu. In cultures without metal addition, biomass production did not differ significantly in the presence or absence of BHT (not shown).

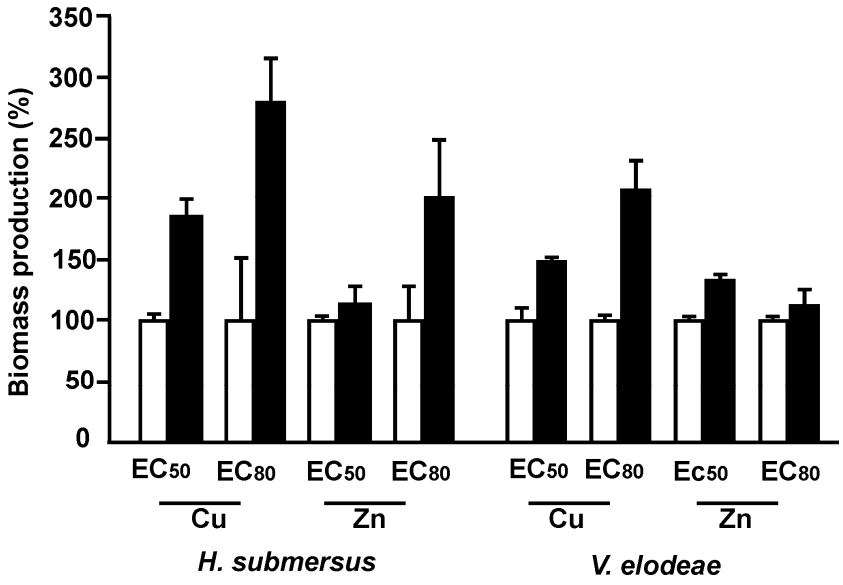


Figure 3.4. Contribution of metal-induced ROS to biomass production by *H. submersus* and *V. elodeae* exposed to $EC_{50}$ or $EC_{80}$ of Zn or Cu in the absence (white) or presence of the antioxidant BHT (black). Biomass production in the absence of BHT was equaled to 100%. Mean + SEM, $n = 3$.

### *3.3.4. Antioxidant defenses triggered by Cu and Zn exposure*

The specific activities of the enzymes CAT, SOD and G6PDH in mycelia of aquatic hyphomycetes grown in media without addition of metals were higher in *V. elodeae* than in *H. submersus* (Table 3.2). Short-term exposure (14 hours) of *H. submersus* to Cu led to a general increase in the SOD and CAT activities (Figure 3.5A, 3.5C). CAT activity was also increased after long-term exposure (8 days) to Cu, particularly at the highest concentration, in which CAT appeared to replace SOD as the major antioxidant defense (Figure 3.5A, 3.5C). In the case of *V. elodeae*, SOD activity remained unaltered (Figure 3.5B), while an increase in CAT activity was observed under short-term exposure to Cu (Figure 3.5D). In addition, we found that the activity of G6PDH was stimulated after long-term exposure of *V. elodeae* to the lowest Cu concentration (Figure 3.5F).

Long-term exposure to Zn enhanced the activity of G6PDH (Figure 3.6E, 3.6F) and CAT (Figure 3.6C, 3.6D) in *H. submersus* and *V. elodeae*. CAT also seemed to be an important antioxidant defense in *H. submersus* under acute Zn stress, because its activity increased at short exposure time (Figure 3.6C). Conversely, SOD did not appear to be involved in Zn stress (Figure 3.6A, 6B).

Table 3.2. Specific activities of catalase (CAT), superoxide dismutase (SOD) and glucose-6-phosphate dehydrogenase (G6PDH) in *H. submersus* and *V. elodeae* grown 8 days without addition of metals.

| Fungal species | CAT (U mg protein$^{-1}$) | SOD (U mg protein$^{-1}$) | G6PDH (U mg protein$^{-1}$) |
|---|---|---|---|
| *V. elodeae* | $21 \times 10^{-6} \pm 4.9 \times 10^{-6}$ | $0.10 \pm 0.036$ | $20 \times 10^{-5} \pm 4.3 \times 10^{-5}$ |
| *H. submersus* | $11 \times 10^{-6} \pm 4.7 \times 10^{-6}$ | $0.08 \pm 0.009$ | $15 \times 10^{-5} \pm 2.4 \times 10^{-5}$ |

Values are means ± SE, n = 3.

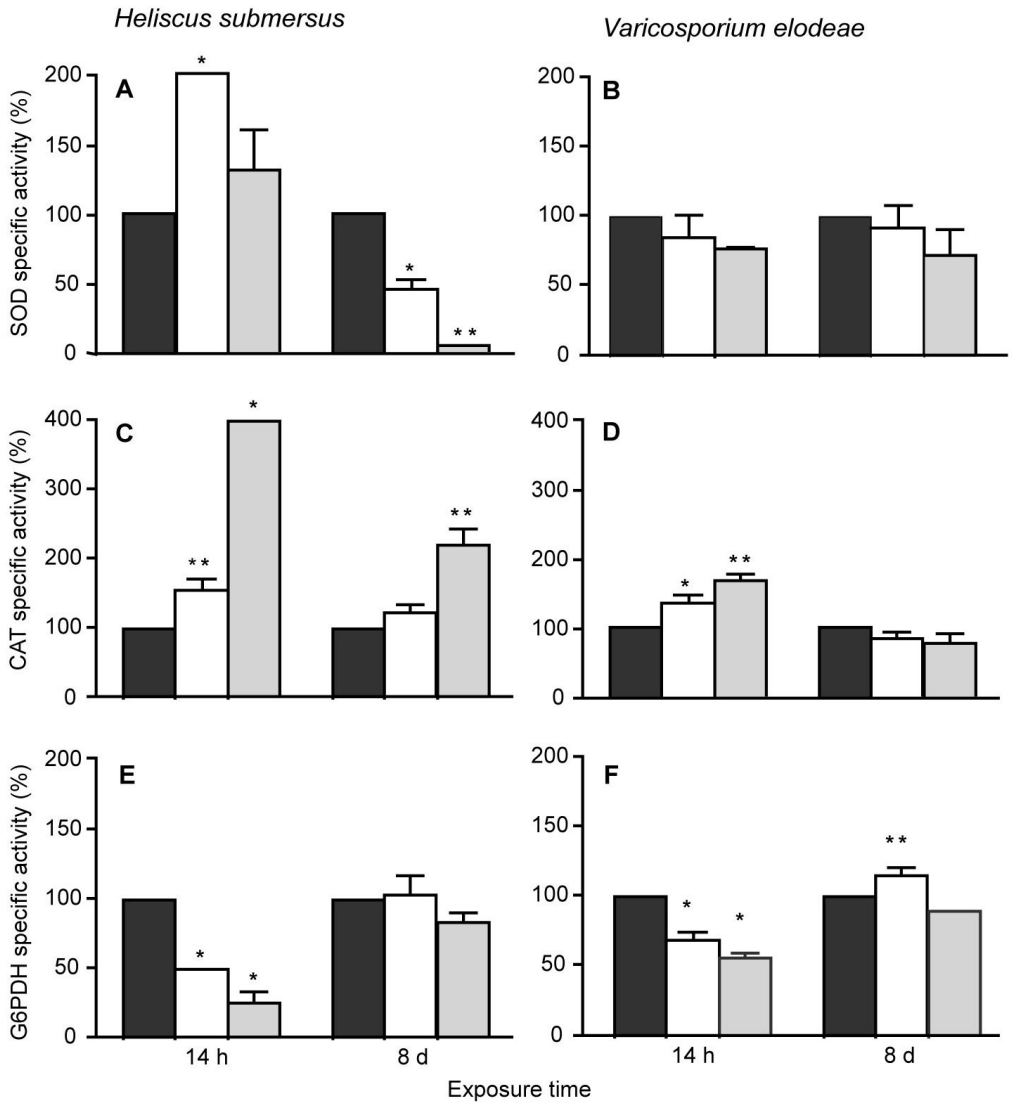


Figure 3.5. Activity of the enzymes SOD (A, B), CAT (C, D), and G6PDH (E, F) in *H. submersus* and *V. elodeae* unexposed (black) or exposed for short- (14 hours) and long-term (8 days) to Cu at concentrations of $EC_{50}$ (white) or $EC_{80}$ (grey). Mean + SEM, $n = 3$. Significant differences: *, $p < 0.05$ or **, $p < 0.01$.

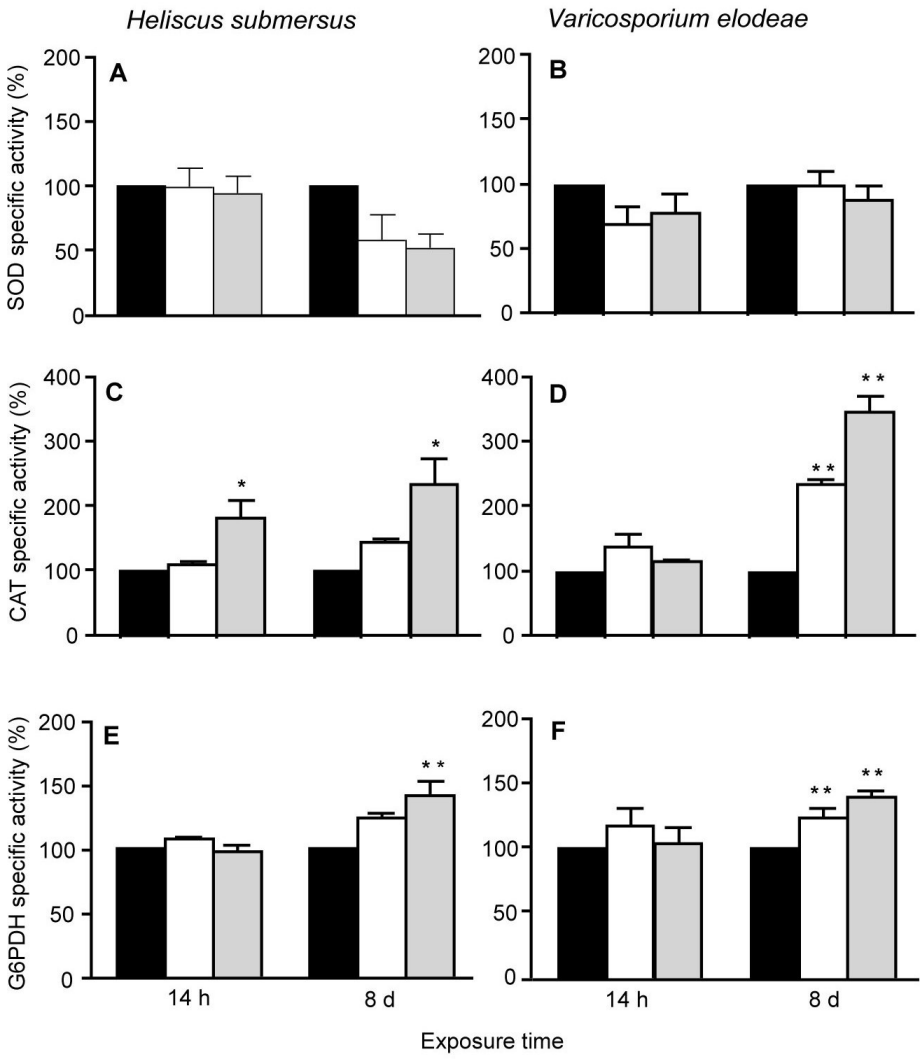


Figure 3.6. Activity of the enzymes SOD (A, B), CAT (C, D) and G6PDH (E, F) in *H. submersus* and *V. elodeae* unexposed (black) or exposed for short- (14 hours) and long-term (8 days) to Zn at concentrations of $EC_{50}$ (white) or $EC_{80}$ (grey). Mean + SEM, $n = 3$. Significant differences: *, $p < 0.05$ or **, $p < 0.01$.

### *3.3.5. Effects of Cu and Zn in mixtures*

The response pattern of the enzymes SOD, CAT and G6PDH in *H. submersus* and *V. elodeae* after short-term exposure (14 hours) to equitoxic mixtures of metals ($EC_{50}$ or $EC_{80}$) was similar to that of single Cu exposure (Figure 3.5-3.7), except for CAT whose activity was severely inhibited in the former species (Figure 3.7C). In addition, metal effects on enzymatic activities were generally more pronounced after exposure to mixtures than to Cu alone.

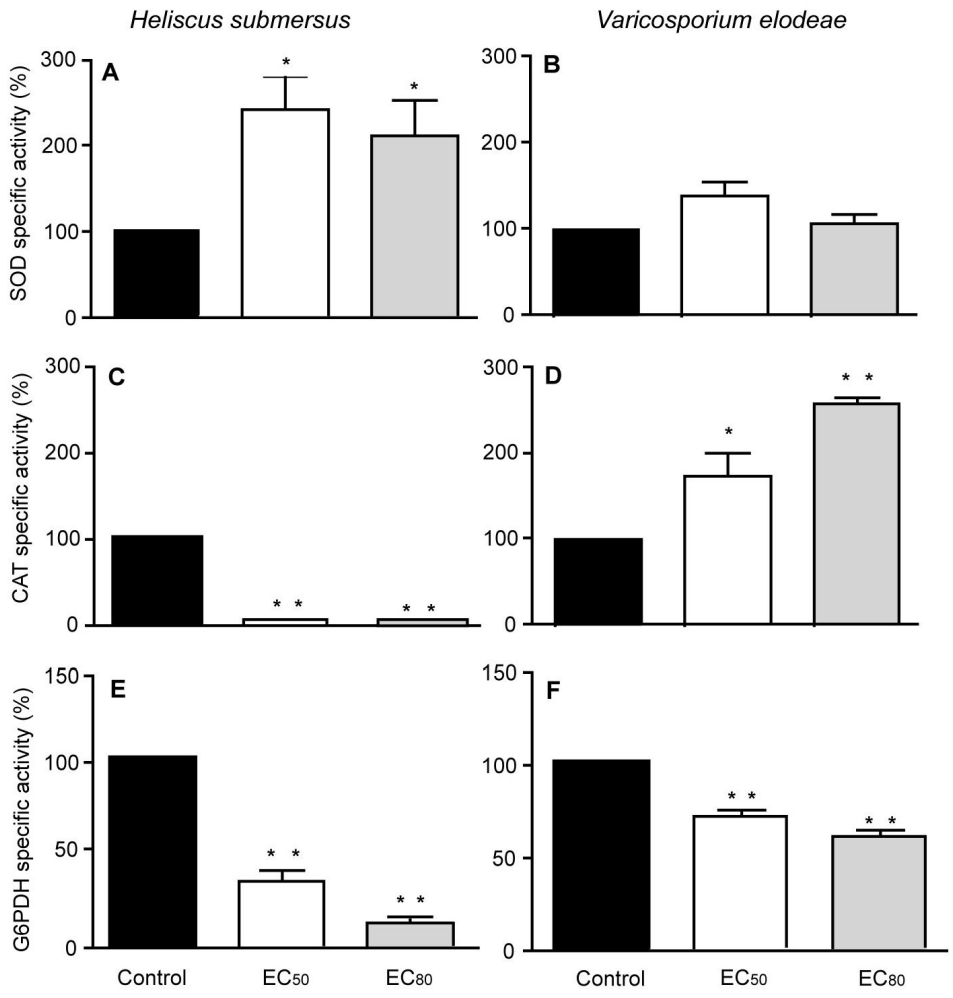


Figure 3.7. Activity of the enzymes SOD (A, B), CAT (C, D), and G6PDH (E, F) in *H. submersus* and *V. elodeae* unexposed or exposed for 14 h to equitoxic mixtures of Cu and Zn corresponding to $EC_{50}$ or $EC_{80}$. Mean + SEM, $n = 3$. Significant differences: *, $p < 0.05$.

## 3.4. Discussion

Because of the crucial role of aquatic hyphomycetes in organic matter turnover in freshwater ecosystems (Bärlocher, 1992) and their ability to survive in metal-polluted environments (Krauss *et al.*, 2001), it is clearly of interest to elucidate the cellular mechanisms underlying metal tolerance of this group of fungi. In this study, tested concentrations of metals (Zn up to 200 µM; Cu up to 150 µM) are environmentally realistic because they are within the range reported in metal-polluted streams (e.g., mining district in Central Germany: up to 19076 µM Zn and 93.8 µM Cu, Sridhar *et al.*,

2000; Krauss *et al*., 2001; industrial park of Braga, Northwest Portugal: 80 µM Zn and 150 µM Cu, Gonçalves, 2001; Pascoal *et al*., 2005).

Our study showed that Cu and Zn induced alterations in cell wall morphology of *H. submersus* and *V. elodeae* as shown by scanning electron microscopy. Even so, no noticeable adsorption of these metals to cell walls was found, minimizing the protective role of this structure in the internalization of Cu or Zn ions. The adsorption of metals by filamentous fungi has been reported to be affected by pH, initial metal ion concentration, medium composition and exposure time (Lo *et al*., 1999); therefore, the ability of the cell walls of these aquatic hyphomycetes to bind metals cannot be excluded in conditions differing from those of our study.

Metal effects on biomass production by aquatic hyphomycetes indicated higher toxicity of Cu than Zn, which agrees with reports for several other organisms (e.g., microorganisms, Gadd, 1993; Miersch *et al*., 1997; algae, Collén *et al*., 2003; invertebrates, Kobayashi and Okamura, 2005). Moreover, the similarity in the magnitude of inhibition rates of biomass production after Cu exposure suggests that Cu may have identical cellular targets in *V. elodeae* and *H. submersus*. It has been reported that Cu induces plasma membrane disruption in fungi (Ohsumi *et al*., 1988; Stohs and Bagchi, 1995). In agreement, our work showed that plasma membrane integrity of *V. elodeae* and *H. submersus* was more affected by Cu than Zn, pointing to this cellular structure as a potentially vulnerable target of Cu. Loss of membrane integrity has been attributed to the formation of ROS (Stohs and Bagchi, 1995). We clearly demonstrated that generation of ROS contributed noticeably to metal toxicity, with a particularly strong effect under Cu stress, as indicated by the increase of biomass production in the presence of an antioxidant agent.

Fungi, like all aerobic organisms, have a set of defense mechanisms to deal with oxidative stress (Moradas-Fereira *et al*., 1996; Bai *et al*., 2003). Enzymes, such as SOD, CAT and G6PDH, have been reported to be activated against ROS in several organisms under Cu and/or Zn stress (yeasts, Romandini *et al*., 1992; algae, Collén *et al*., 2003; Tripathi *et al*., 2006; mussels, Geret and Bebiano, 2004). The first two enzymes are crucial for cellular detoxification, controlling the levels of superoxide anion radical and hydrogen peroxide (Penninckx and Elskens, 1993; Bai *et al*., 2003); G6PDH is essential for the replenishment of NADPH intracellular pool to maintain cellular redox balance (Penninckx and Elskens, 1993). In our study, control cultures of *V. elodeae* had higher

activities of CAT, SOD and G6PDH than those of *H. submersus* (Table 3.2). Although *V. elodeae* had been isolated from a clean stream, it is distributed worldwide (e.g., Portugal, Pascoal *et al*., 2005; France, Chauvet, 1991; Canada, Nikolcheva and Bärlocher, 2005) and it may have antioxidant defenses against environmental stressors, including metals. Consistently, metal exposure did not inhibit the growth of *V. elodeae* until a threshold concentration of 100 µM Zn or 20 µM Cu, above which the biomass production was inhibited. CAT appeared to have a primary defense role against acute Cu stress, but not under chronic stress. In addition, Cu seemed to change the cellular redox status in *V. elodeae*, as suggested by the inhibition of G6PDH activity under acute stress followed by its stimulation under chronic stress. Copper and Zn diminished the activity of glutathione reductase (GR) in *Scenedesmus* sp. (Nagalakshmi and Prasad, 2001; Tripathi *et al*., 2006) through metal binding to SH-groups at the active site of the enzyme (Nagalakshmi and Prasad, 2001). If inactivation of GR had also occurred in our study, it might explain the inhibition of G6PDH after short-term exposure to Cu in *V. elodeae* and *H. submersus*, avoiding a futile production of NADPH. Overall, our results point to a possible role of G6PDH in aquatic hyphomycete acclimation to Cu or Zn. In this connection, Izawa *et al*. (1998) reported that G6PDH-deficient cells of *S. cerevisiae* were more susceptible and unable to adapt to oxidative stress. Thus, in aquatic hyphomycetes, it is conceivable that NADPH could be used for glutathione recycling needed for metal detoxification during acclimation. This hypothesis is supported by previous observations pointing to a major role of glutathione and phytochelatins in Cu and Zn binding in aquatic hyphomycetes under chronic stress (Guimarães-Soares *et al*., 2006).

In *H. submersus* both SOD and CAT were stimulated under acute-stress by Cu, and CAT activity increased with the increasing of Cu concentration. Also the magnitude of the increase in the CAT activity was much more pronounced in *H. submersus* than in *V. elodeae*, probably contributing to the higher tolerance of *H. submersus* to Cu and to its ability to survive in metal-polluted streams as at the site from which the fungus was originally isolated (industrial park of Braga; metal concentrations are above). The activity of CAT remained high during chronic exposure to Cu, suggesting that CAT also plays an important role in the acclimation of *H. submersus* to Cu stress. In this fungus the inhibition of SOD activity under chronic stress could be related to its potential function as a metallothionein (Culotta *et al*., 1995), which is important for cellular

detoxification either as metal-chelating agent or ROS scavenger (Kiningham and Kasarskis, 1998). Overall, our findings point to SOD and CAT enzymes as having an effective role in protecting *H. submersus* against ROS induced by Cu, which agrees with the less pronounced effects of this metal on the plasma membrane of *H. submersus* than that of *V. elodeae*.

An important role of Zn in living organisms is related to its antioxidant properties (Powell, 2000). However, in our study, excess of Zn caused severe effects on biomass production, and oxidative stress was evident although to a lesser extent than that induced by Cu. In contrast to reports on *Phaseolus vulgaris* (Weckx and Clijsters, 1997), CAT activity was stimulated in both *H. submersus* and *V. elodeae* highlighting the importance of this enzyme as a major antioxidant defense in aquatic hyphomycetes.

In metal-polluted streams aquatic hyphomycetes are commonly exposed to mixtures of metals, but so far no data are available on the effects of metal mixtures in this group of fungi. It is reported that *Pleurotus ostreatus* exposed to Cu and Zn mixtures, accumulates more Cu than Zn (Baldrian, 2003), probably causing an increased production of ROS. Similarly, Franklin *et al*. (2002) reported that in equitoxic mixtures, Cu reduced the binding and cellular uptake of Zn by *Chlorella* sp., but Zn had no appreciable effect on the uptake of Cu. This suggests that effects of Cu are dominant over Zn in eliciting cell responses when a mixture of Cu and Zn is applied. In our study, the antioxidant defenses displayed by the aquatic hyphomycetes exposed to Cu and Zn mixtures were similar to those of Cu. In addition, the responses of SOD in *H. submersus* and of CAT in *V. elodeae* were stronger under exposure to Cu plus Zn mixtures than to Cu alone, suggesting that metal mixtures induced higher oxidative stress than the individual metals.

In summary, both Zn and Cu induced oxidative stress in aquatic hyphomycetes. CAT appeared to play a greater role alleviating the stress induced by metals. In addition, the increased activity of G6PDH after long-term exposure to metals points to the involvement of the penthose phosphate pathway in metal acclimation. Our results suggest that the ability of aquatic hyphomycetes to cope with metal stress is related to their ability to mount an efficient defense against oxidative stress. These findings may contribute to a better understanding of the response mechanisms of aquatic hyphomycetes to metal stress and to gain insights into metal-microbe interactions in natural environments.

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# Chapter 4

*Copper and Zn affect the activity of plasma membrane $H^+$-ATPase and thiol content in aquatic fungi*

## Abstract

Aquatic hyphomycetes are the major microbial decomposers of plant litter in streams. In this work, we selected three aquatic hyphomycete species with different ability to tolerate, adsorb and accumulate Cu and Zn, and we investigated the effects of these metals on the $H^+$-ATPase activity and on the levels of thiol-containing compounds. Before metal exposure, the species isolated from a metal-polluted stream (*Heliscus submersus* and *Flagellospora curta*) had higher levels of thiol compounds than the species isolated from a clean stream (*Varicosporium elodeae*). However, *V. elodeae* rapidly increased the levels of thiols after metal exposure, emphasizing the importance of thiol coumpounds in fungal survival under metal stress. The highest amounts of metals adsorbed to fungal mycelia were found in the most tolerant species to each metal i.e., *H. submersus* exposed to Cu and *V. elodeae* exposed to Zn. Short-term (10 min) exposure to Cu completely inhibited the activity of $H^+$-ATPase of *H. submersus* and *V. elodeae,* while Zn only led to a similar effect on that of *H. submersus*. However, at longer exposure times (8 days) the most metal-tolerant species exhibited increased $H^+$-ATPase activities, suggesting that the plasma membrane proton pump may be involved in the acclimation of aquatic hyphomycetes to metals.

## 4.1. Introduction

In current days, it is hard to find pristine habitats due to the increased anthropogenic pressure on the environment. For instance, human activities from urbanization, agriculture and industry have greatly contributed to the increase of metals in aquatic ecosystems, leading to negative effects on the biota and ecological processes they govern.

Fungi play a critical role in organic matter turnover in streams. Among fungi, aquatic hyphomycetes appear to have the greatest ecological role as decomposers of plant detritus in streams (Baldy *et al*., 2002; Pascoal and Cássio, 2004). Even though metal pollution lowers biodiversity and activity of aquatic hyphomycetes, the occurrence of these fungi have been consistently reported in metal-polluted streams (Sridhar *et al*., 2005; Pascoal *et al.*, 2005a). This probably explains the increased number of studies focusing on the responses of aquatic fungi to metal stress over the last few years.

All metals are toxic above certain concentrations but some, like Zn and Cu, are required as micronutrients for the metabolism and growth of cells (Gadd, 1993). Therefore, living organisms, including fungi, have to tightly regulate intracellular metal concentration in such a way that a safe uptake of metal ions needed in their cytosol and organelles can occur without cellular damage due to metal toxicity (Kneer *et al*., 1992). Metal tolerance in fungi can be achieved by several complex mechanisms, including extracellular precipitation, biosorption, controlled uptake and intracellular sequestration and/or compartmentation, whose relative contributions for metal detoxification can vary with metal type and fungal species (Gadd, 1993). Therefore, we are still far from fully understanding the mechanisms underlying metal tolerance/resistance in fungi, despite the large amount of information on metal effects in living organisms.

Plasma membrane is the foremost barrier between cytoplasm and the environment and it constitutes the first functional site of contact between metal ions and cells. In fungi, the proton-pump ATPase ($H^+$-ATPase) is the major protein component of the plasma membrane, attaining 15 to 20% of the total plasma membrane proteins (Ambesi *et al*., 2000). It couples ATP hydrolysis to the extrusion of protons generating an electrochemical gradient (Serrano, 1988). This proton pump plays a key role in cell physiology because it controls essential cellular functions, such as nutrient uptake and intracellular pH regulation (Serrano, 1988; Portillo, 2000). It has been reported that $H^+$-

ATPase activity influences fungal tolerance to several environmental stressors, including heat (Piper, 1993), ethanol (Rosa and Sá-Correia, 1992), weak acids (Holyoak *et al*., 1996; Viegas *et al*., 1998) and metals (Karamushka and Gadd, 1994; Fernandes *et al*., 1998).

Metal toxicity to fungi may result from direct interaction between metal ions and biomolecules or from mechanisms related to the ability of metals to generate reactive oxygen species (ROS) (Stohs and Bagchi 1995; Azevedo *et al*., 2007). Transition metals, as Cu and Fe, greatly enlarge ROS production through the Fenton reaction (Bai *et al*., 2003). However, even non-redox active metals, such as Zn, can lead to ROS production by depleting free radical scavengers, like thiol (SH) compounds (Dietz *et al*., 1999). Among cellular macromolecules, the polyunsaturated fatty acids of biological membranes are preferential targets for ROS attack (Howlett and Avery, 1997). In fungi, lipid peroxidation induced by Cu leads to a decline in plasma membrane lipid order (Howlett and Avery, 1997) and a subsequent increase in the non-specific permeability of this membrane (Ohsumi *et al*., 1988). Therefore, Cu readily permeates the plasma membrane acting as a potent depolarizer of cell electrical potential (Kennedy and Gonsalves, 1987). In *Saccharomyces cerevisiae*, mild Cu stress stimulates $H^+$-ATPase, probably for the re-establishment of the cellular electrochemical gradient, but the activity of this pump declines at maximal Cu concentration that allows yeast growth (Fernandes *et al*., 1998).

Stadler *et al.* (2003) showed that specific-Cys residues of the $H^+$-ATPase are targets for Fe- and Cu-Fenton reagents leading to the enzyme inactivation. Consistently, reduced-thiol groups revealed to be essential for maintaining $H^+$-ATPase activity under Fe stress in wheat-root plasma membranes (Yang *et al*., 2003). In aquatic hyphomycetes, metal tolerance has been associated with increased levels of SH compounds in fungal cells (Miersch *et al*., 2001; Braha *et al*., 2007; Guimarães-Soares *et al*., 2007) and recent evidences point to glutathione, phytochelatins and metallothioneins as putative metal sequesters or ROS scavengers (Jaeckel *et al*., 2005; Guimarães-Soares *et al*., 2006).

In the present work, we selected three aquatic hyphomycete species with different sensitivities to Cu and Zn and we examined their ability to adsorb and accumulate these metals. Subsequently, we assessed effects of Cu and Zn on $H^+$-ATPase activity and on the levels of SH-containing compounds. We expect that more tolerant species to metals

have higher ability to adsorb metal ions, minimizing their uptake, and/or higher intracellular levels of SH-containing compounds to deal with metal accumulation within cells. In addition, an increased activity of the plasma membrane proton pump is expected to occur to counteract metal-induced dissipation of the electrochemical gradient, which is essential for fungal survival.

## 4.2. Materials and Methods

### *4.2.1. Fungi and culture maintenance*

The aquatic hypmomycetes *Heliscus submersus* H. J. Huds. (UMB-135.01), *Flagellospora curta* J. Webster (UMB-39.01) and *Varicosporium elodeae* W. Kegel (UMB-142.01) were isolated from single spores collected in streams in the Northwest of Portugal. The two former species were isolated from leaves collected in the Este River, downstream the industrial park of Braga, at a site with high nutrient loading (Pascoal *et al.*, 2005b) and heavy metals in the stream water (Gonçalves, 2001). The latter species was isolated from foams collect in a clean stream in the Peneda-Gerês National Park (Pascoal *et al.*, 2005b).

In the laboratory, fungi were maintained on 2% (w/v) malt extract and 1.5 % (w/v) agar, at 18 ºC under artificial light.

### *4.2.2. Growth conditions and metal exposure*

Conidial suspensions (6 conidia $ml^{-1}$, final concentration) of each aquatic hyphomycete species were placed into Erlenmeyer flaks containing mineral medium with vitamins and 2% (w/v) glucose (van Uden, 1967) at pH 5.0, with or without addition of Cu ($CuCl_2$) or Zn ($ZnCl_2$). Metal concentrations ranged from 50 to 2000 µM for Cu and from 250 to 10000 µM for Zn. The flasks were kept for 8 days at 18ºC, 160 rpm (Certomat BS 3, B. Braun Biotech International) under artificial light. At the harvest time, fungal cultures were at the end of exponential growth phase (not shown).

To assess long-term effects of metals on the $H^+$-ATPase activity and thiol-compound production, fungal mycelia were exposed for 8 days to Cu or Zn concentrations inhibiting biomass production in 50% ($EC_{50}$). To assess short-term effects, mycelia grown 8 days without metal addition were transferred to fresh medium

and exposed to $EC_{50}$ of Cu or Zn for 10 min in the case of $H^+$-ATPase assay or for 14 and 62 h to estimate concentration of thiol compounds in fungal mycelia.

To quantify fungal biomass, mycelia were harvested by filtration, washed twice with deionised water and dried at 85ºC to constant mass, before weighed (0.001 g).

### *4.2.3. Assessment of $H^+$-ATPase activity*

The $H^+$-ATPase activity was evaluated by measuring the rate of proton efflux after addition of 0.2 % glucose to suspensions of fungal mycelium. Fungal suspensions were prepared by homogenizing mycelia in deionised water with a dounce homogenizer. Rate of proton efflux was measured by recording proton movements with a standard pH meter (PHM 92 Lab pH Meter) connected to a recorder (Kipp e Zonen 024). The pH electrode was immersed in a water-jacketed chamber magnetically stirred. One millilitre of the mycelium suspension was diluted in water to a final volume of 5 ml, and the pH of the mixture was adjusted to 5.0 with NaOH (1M to 10 mM) or HCl (1M to 10 mM) prior to the addition of glucose. The slope at the initial part of the acidification curve allowed the determination of initial proton movements. Changes in pH were converted in nmol of $H^+$ $s^{-1}$ $mg^{-1}$ dry weight by comparing the acidification curve after glucose addition to cell suspensions with that of the addition of known amounts of 10 mM HCl.

### *4.2.4. Preparation of cell free-extracts and quantification of thiol compounds*

Fungal mycelia were harvested by filtration, washed twice with deionised water and pressed between two layers of filtering-paper to remove water surplus. Then, mycelia were mixed with purified sea sand (2 g $g^{-1}$ mycelium wet mass) and ground in liquid nitrogen in a cooled mortar for 4 min. The mixture was suspended in 20 mM Tris, 1 mM EDTA, pH 7.5, and cell-free extracts were obtained by 2 centrifugation steps (6200 g for 10 min; 18000 g for 50 min) at 4ºC.

The concentration of total thiols (T-SH) and nonprotein thiols (NP-SH) was determined according to Sedlak and Lindsay (1968), with 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB). To determine T-SH compounds, 50 µl of the cell-free extracts were mixed with 150 µl of 0.2 M Tris, pH 8.2, and 10 µl of 0.01 M DTNB in a final volume of 1.0 ml of absolute methanol. After 15 min, the mixtures were centrifuged (3000 g, 15 min) and the absorbance measured at 412 nm (Perkin Elmer Lambda 2 UV/VIS

Spectrometer) against a blank (without sample), using reduced glutathione (Sigma) as standard.

To determine the concentration of NP-SH, 500 µl of the cell-free extracts were mixed with 400 µl of milli-Q water and 100 µl of 50% trichloroacetic acid. The mixtures were shaken during 12 min before centrifuged (3000 g, 15 min). Then, 200 µl of the supernatant was mixed with 400 µl of 0.4 M Tris, pH 8.9, and 10 µl of 0.01 M DTNB, and absorbance was measured within 3 min. The concentration of protein-bound thiols (PB-SH) was calculated by subtracting the NP-SH to T-SH concentrations. All buffers and solutions were previously gassed 1-2 min with a nitrogen stream.

### *4.2.5. Metal adsorption and accumulation*

Fungal mycelia were harvested by filtration, washed with distilled water (100 ml), washed 3 times with 100 ml of 20 mM $NiCl_2$ and again with distilled water (100 ml) to remove metals adsorbed to fungi. Fifty milligrams of the mycelium were digested with 4 ml of 65% (v/v) $HNO_3$ and 2 ml of 30% (v/v) $H_2O_2$ in a water-bath at 100 ºC during 40 min.

Concentrations of Cu or Zn in the $NiCl_2$ washings (for adsorption) and in the digested mycelium (for accumulation) were determined by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES; PU 7000 ICP- Philips).

### *4.2.6. Data analysis*

Data on the effects of Cu and Zn on the $H^+$-ATPase activity and production of thiol compounds were expressed in percentage of control. Values were divided by 1000 and arcsine square root transformed to achieve normal distribution and homoscedasticity (Zar, 1996). For each metal and fungal species, $H^+$-ATPase activity and production of thiol compounds were compared by one-way ANOVA, followed by Dunnett's test to identify treatments that differ significantly from control (Zar, 1996).

Values of $EC_{50}$ for Cu and Zn were estimated by the Probit method (Wardlaw, 1985) and compared by one-way ANOVA, followed by Tukey´s test to identify significant differences (Zar, 1996).

Statistic analysis was done using Prism4 for Windows (GraphPad Software Inc., San Diego).

## 4.3. Results

### *4.3.1. Metal toxicity, adsorption and accumulation*

The sensitivity of the aquatic hyphomycetes *H. submersus*, *V. elodeae* and *F. curta* to Cu and Zn was assessed by comparing metal concentrations inhibiting biomass production in 50% ($EC_{50}$) after 8 days of growth (Table 4.1). *H. submersus*, with an $EC_{50}$ of 1510 µM for Cu, was the most resistant species to this metal, followed by *V. elodeae* and *F. curta.* The most resistant species to Zn was *V. elodeae* ($EC_{50}$ = 7315 µM), while *H. submersus* ($EC_{50}$ = 465 µM) was the most sensitive one.

The mycelium of *H. submersus* showed higher ability to adsorb Cu (70.5 µmol $g^{-1}$) accumulating 2.6-times more Cu (13.5 µmol $g^{-1}$ dry mass) than the less accumulative species (*V. elodeae*) (Table 4.2). Differences in metal adsorption and accumulation between fungal species were more pronounced for Zn than for Cu (Table 4.2). *V. elodeae* had the highest adsorption ability for Zn, being 8.5- and 32-times higher than that of *F. curta* and *H. submersus*, respectively. *F. curta* was able to accumulate 5- and 159-times more Zn in the mycelium (175.1 µmol $g^{-1}$ dry mass) than *V. elodeae* and *H. submersus*, respectively (Table 4.2).

Table 4.1. Concentrations of Cu and Zn inhibiting biomass production by aquatic hyphomycetes in 25% ($EC_{25}$) and 50% ($EC_{50}$). Fungi were grown in mineral medium supplemented with vitamins and 2% glucose during 8 days with or without metal addition.

| | Cu (µM) | | Zn (µM) | |
|---|---|---|---|---|
| Fungal species | $EC_{25}$ | $EC_{50}$ | $EC_{25}$ | $EC_{50}$ |
| *H. submersus* | 1013 ± 12 [a] | 1510 ± 19 [a] | 51 ± 9 [a] | 465 ± 83.7 [a] |
| *V. elodeae* | 323 ± 38 [b] | 457 ± 48 [b] | 5721 ± 203 [b] | 7315 ± 306 [b] |
| *F. curta* | 61 ± 2 [c] | 183 ± 7 [c] | 728 ± 30 [c] | 1304 ± 54.9 [c] |

Values are means ± SEM. In each column, different letters indicate significant differences (Tukey´s test, $p < 0.05$)

Table 4.2. Metal adsorption and accumulation in fungal mycelia. Fungi were grown in mineral medium supplemented with vitamins and 2% glucose without metal during 8 days and exposed 14 h to $EC_{50}$ of Cu and Zn.

| Fungal species | Cu | | Zn | |
|---|---|---|---|---|
| | Adsorption ($\mu mol\ g^{-1}$) | Accumulation ($\mu mol\ g^{-1}$) | Adsorption ($\mu mol\ g^{-1}$) | Accumulation ($\mu mol\ g^{-1}$) |
| *H. submersus* | 70.5 | 13.5 | 11.2 | 1.1 |
| *V. elodeae* | 34.1 | 5.2 | 354 | 36.2 |
| *F. curta* | 8.3 | 9.0 | 41.7 | 175.1 |

Values are the mean of two independent experiments

### *4.3.2. Effects of Cu and Zn on $H^+$-ATPase activity*

The $H^+$-ATPase activity was evaluated by measuring the rate of proton efflux after addition of glucose to suspensions of fungal mycelium. The addition of glucose to fungal suspensions led to an efflux of protons, as exemplified in Figure 4.1 for *F. curta*. Proton efflux varied from 0.023 ± 0.006 to 0.061 ± 0.012 nmol $H^+\ s^{-1}\ mg^{-1}$ dry weight, with the lowest value found in *V. elodeae*, the intermediate in *H. submersus* and the highest in *F. curta* (Table 4.3).

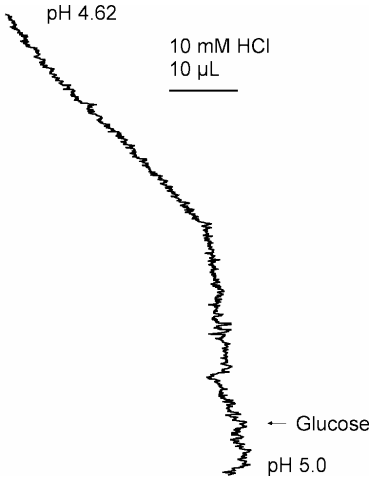


Figure 4.1. Typical acidification curve obtained after the addition of 0.2% glucose to mycelial suspensions of *F. curta* grown 8 days with $EC_{50}$ concentration of Cu.

Long-term exposure (8 days) to $EC_{50}$ of Cu increased significantly the $H^+$ efflux associated with the addition of glucose in *H. submersus* (2.3-times augment) and in *F. curta* (1.7-times augment), but did not affect the activity of this proton pump in *V. elodeae* (Table 4.3). Long-term exposure to $EC_{50}$ of Zn led to a 2.7-times increase in the $H^+$-ATPase activity in *V. elodeae* (Table 4.3). On the contrary, Zn had no significant effect on the $H^+$ efflux in *H. submersus* and *F. curta* (Table 4.3).

Table 4.3. Activity of the $H^+$-ATPase in aquatic hyphomycetes exposed or not for 8 days to $EC_{50}$ of Cu or Zn. The proton efflux was measured upon addition of 0.2% glucose, at pH 5.0 and 20 ºC.

| Fungal species | $H^+$-ATPase (nmol $H^+$ $s^{-1}$ $mg^{-1}$ dry weight) | | |
|---|---|---|---|
| | Control | Cu | Zn |
| *H. submersus* | 0.030 ± 0.006 | 0.070 ± 0.019* | 0.024 ± 0.001 |
| *V. elodeae* | 0.023 ± 0.006 | 0.025 ± 0.004 | 0.062 ± 0.007* |
| *F. curta* | 0.061 ± 0.012 | 0.106 ± 0.009* | 0.051 ± 0.001 |

Values are Mean ± SEM, n=3, *Dunnett´s test, $p < 0.05$

Short-term exposure (10 min) to $EC_{25}$ or $EC_{50}$ of Cu led to a total inhibition of the $H^+$ efflux in *H. submersus* and *V. elodeae* (Table 4.4). In *F. curta*, the exposure to $EC_{25}$ of Cu had no significant effect on the $H^+$-ATPase activity, but a 1.5-times stimulation was found by exposure to $EC_{50}$ of this metal (Table 4.4). The $H^+$-ATPase activity was not affected by short-term exposure to $EC_{25}$ of Zn in any fungal species. The exposure to $EC_{50}$ of Zn totally inhibited the proton pump of *H. submersus*, had no effect on that of *V. elodeae* and stimulated that of *F. curta* (Table 4.4).

Table 4.4. Effects of short-term exposure to Cu or Zn on the $H^+$-ATPase activity in aquatic hyphomycetes. Assays were carried out at pH 5.0 and 20 ºC. Actual values of the $H^+$-ATPase activity in the absence of metals are in Table 4.3.

| Fungal species | $H^+$-ATPase (% control) | | | |
|---|---|---|---|---|
| | Cu | | Zn | |
| | $EC_{25}$ | $EC_{50}$ | $EC_{25}$ | $EC_{50}$ |
| *H. submersus* | - | - | 81.1±4.2 | - |
| *V. elodeae* | - | - | 119.7±8.1 | 83.4 ± 1.7 |
| *F. curta* | 83.5±1.9 | 146.6 ± 13.3* | 80.0±1.9 | 146.6 ± 9.3* |

Mean ± SEM, n=3, - total inhibition, * Dunnett´s test, $p < 0.05$

### *4.3.3. Effects of Cu and Zn on the production of thiol compounds*

The levels of thiol compounds were evaluated in 8 days-old mycelia of the aquatic hyphomycetes *H. submersus, F. curta* and *V. elodeae* exposed or not to $EC_{50}$ concentrations of Cu or Zn. *H. submersus* grown without added metals had the highest level of total thiol compounds (T-SH), whereas *V. elodeae* showed the lowest level (Table 4.5). The contribution of non-protein (NP-SH) and protein-bound (PB-SH) thiols to the T-SH compounds varied among the species, with NP-SH thiols being 80%, 65% and 50% of the T-SH in *H. submersus, F. curta* and *V. elodeae*, respectively.

Table 4.5. Concentration of total (T-SH), nonprotein (NP-SH) and protein-bound (PB-SH) thiols in the mycelia of aquatic hyphomycetes grown 8 days with no addition of metals and transferred to fresh medium for different incubation periods.

| Fungal species | Incubation period (h) | Thiol-compounds (μmol $g^{-1}$ dry mass) | | |
|---|---|---|---|---|
| | | T-SH | NP-SH | PB-SH |
| *H. submersus* | 0 | 6.32±1.22 | 5.06±0.91 | 1.25±0.69 |
| | 14 | 4.93±0.60 | 2.33±1.67 | 2.60±1.07 |
| | 62 | 4.36±0.62 | 2.03±0.76 | 2.33±1.22 |
| *V. elodeae* | 0 | 2.59±0.32 | 1.28±0.18 | 1.3±0.13 |
| | 14 | 2.55±0.12 | 1.06±0.10 | 1.49±0.21 |
| | 62 | 5.35±0.34 | 2.9±0.18 | 2.44±0.5 |
| *F. curta* | 0 | 5.41±1.11 | 3.51±0.14 | 1.89±0.98 |
| | 14 | 5.19±2.07 | 2.25±0.78 | 2.95±1.42 |
| | 62 | 7.54±1.67 | 4.75±0.24 | 2.79±1.44 |

Mean ±SEM of at least 3 independent experiments

Exposure to $EC_{50}$ of Cu induced a significant decrease in the T-SH and NP-SH compounds in *H. submersus* at all times (Figure 4.2A). Short-term exposure (14 or 62 h) of *V. elodeae* to Cu led to a significant increase in the levels of all types of thiol compounds, while long-term exposure (8 days) to Cu only led to a significant increase in PB-SH (Figure 4.2B). Moreover, in all fungal species the levels of NP-SH significantly decreased after 8 days of exposure to Cu (Figure 4.2).

Short-term exposure (14 h) to Zn increased the levels of T-SH, by increasing both NP-SH and PB-SH (Figure 4.2B) in *V. elodeae*, and of NP-SH in *F. curta* (Figure 4.2C). Long-term exposure (8 days) to Zn, significantly increased PB-SH level in *H. submersus* (Figure 4.3A) and no other significant effects were found.

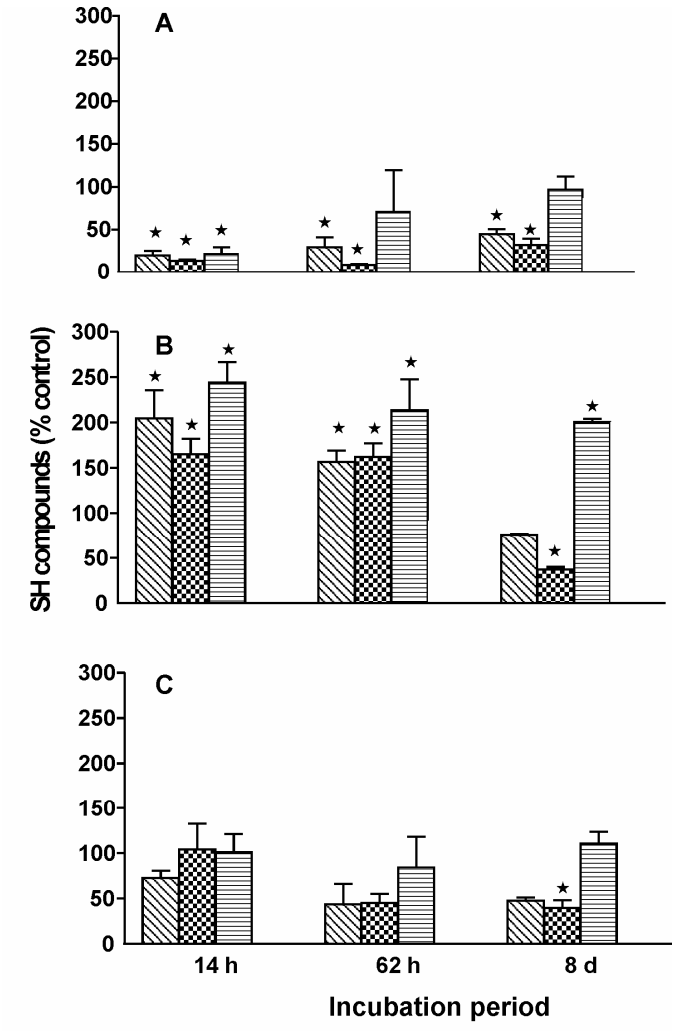


Figure 4.2. Concentration of total (diagonal lines), nonprotein thiols (checkers) and protein-bound thiols (horizontal lines) after short-term (14 and 62 h) and long-term (8 d) exposure to $EC_{50}$ of Cu in *H. submersus (*A), *V. elodeae* (B) and *F. curta* (C). Values are % of control. Mean ± SEM, n=3. *Dunnet´s test, $p < 0.05$. Actual values of controls are in Table 4.5.

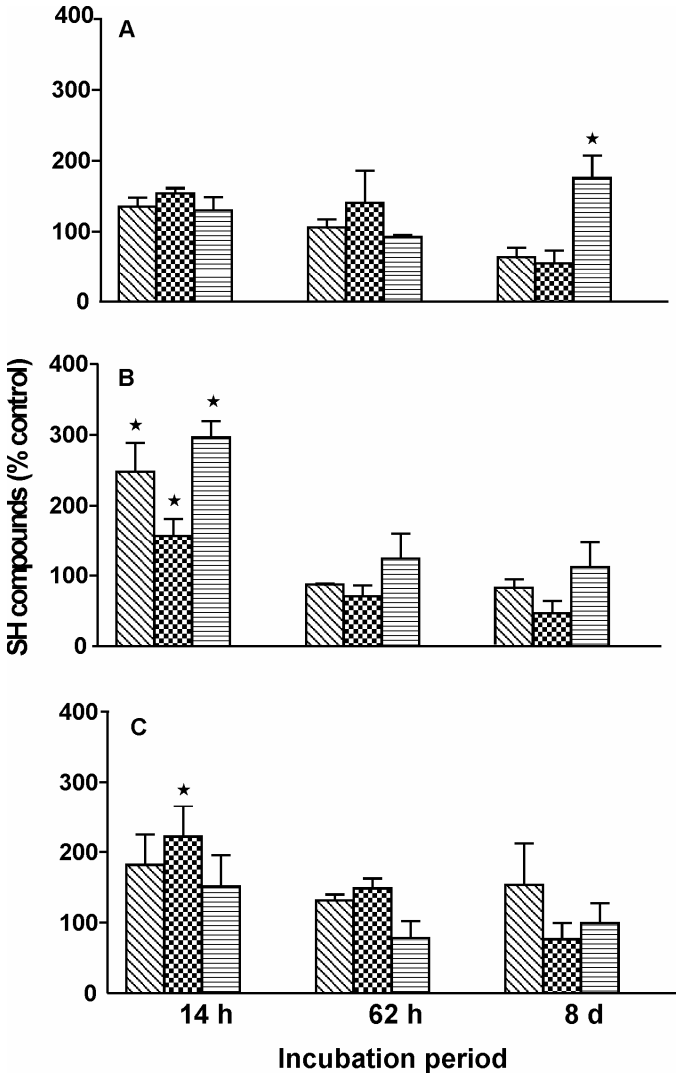


Figure 4.3. Concentration of total (diagonal lines), nonprotein thiols (checkers) and protein-bound thiols (horizontal lines) after short-term (14 and 62 h) and long-term (8 d) exposure to $EC_{50}$ of Zn in *H. submersus (*A), *V. elodeae* (B) and *F. curta* (C). Values are % of control. Mean ± SEM, n=3. * Dunnet´s test, $p < 0.05$. Actual values of controls are in Table 4.5.

## 4.4. Discussion

In previous studies, we demonstrated that the generation of reactive oxygen species (ROS) contribute noticeably to Cu and Zn toxicity in aquatic hyphomycetes (Guimarães-Soares, 2005; Azevedo *et al.*, 2007). The interaction of ROS with biological membranes results in a variety of functional alterations due to either direct interaction with the molecular cell machinery and/or oxidative modification of biological macromolecules (Stark, 2005). Moreover, metals can directly interact with

biomolecules, such as enzymes and transport proteins of essential nutrients and ions, compromising their biological functions (Gadd, 1993). A severe disruption of plasma membrane integrity in several species of aquatic hyphomycetes is reported to occur after short-term (30 min) exposure to Cu and Zn, particularly to the former metal (Azevedo *et al*., 2007); this could compromise the activity of plasma membrane ATPase. In this work, we found that short-term exposure to Cu completely inhibited the activity of the $H^+$-ATPase of *H. submersus* and *V. elodeae*, while Zn only led to a similar effect on that of *H. submersus*. In *S. cerevisiae*, damage in plasma membrane caused by Cu affected the functioning of the $H^+$-ATPase (Fernandes *et al*., 1998). The reduced ATPase activity under Cu stress may be attributed either to the drastic decrease in plasma membrane organization due to Cu-induced lipid peroxidation (Howlett and Avery 1997) and/or formation of Cu-ATP complexes (Tallineau *et al*., 1984). However, since a recovery of plasma membrane integrity was observed after 150 min of exposure to Cu in *H. submersus* (Azevedo *et al*., 2007), a functional restoration of the $H^+$-ATPase is expected to occur at longer times. In the present work, 8 days of metal exposure led to strong stimulations of the proton pump in the most tolerant species, i.e. when *H. submersus* was exposed to Cu and *V. elodeae* was exposed to Zn. The activation of $H^+$-ATPase by metal exposure may be related to its ability to counteract metal-induced dissipation of the electrochemical gradient of protons across the plasma membrane (Serrano, 1988; Fernandes *et al*., 1998), suggesting that $H^+$-ATPase may be involved in aquatic hyphomycete acclimation to metals. Moreover, the acidification of the extracellular medium by the proton pump activation may lead to an increased competition of cations for cellular binding sites, reducing potential interactions between metals and cells (Gadd, 1993), even when the decrease of pH increase metal bioavailability (Douglas and Wiener, 1991). Accordingly, metal uptake in fungi is often reduced by the decrease of extracellular pH (Cu, Gadd and White, 1985; Zn, Ross, 1994).

In this work, the highest amounts of Cu and Zn adsorbed to fungal mycelia were found in *H. submersus* and in *V. elodeae*, respectively. Because these fungi were the most tolerant species to each metal, biosorption appears to be a relevant mechanism to avoid unrestrained uptake of metals minimizing their deleterious effects. Nevertheless, these fungal species accumulated metals in their mycelia, although at much lower amounts comparing to that adsorbed. The ability of aquatic hyphomycetes to take up

and store large quantities of metals makes them potential candidates for bioremediation. In this context, *V. elodeae* and *F. curta* had remarkable ability to adsorb and accumulate Zn (a total of 390 and 217 µmol per gram of dry mycelium during only 14 h of metal exposure) comparing with values reported for aquatic fungi (Guimarães-Soares, 2005; Jaeckel *et al*., 2005) Also, *H. submersus* was able to retain ca. 7-times more Cu than metal tolerant strains of *H. lugdunensis* (Braha *et al*., 2006). However, adsorption of metals by filamentous fungi depends on several factors, including pH, initial metal concentration and medium composition (Gardea-Torresdey *et al*., 1997; Lo *et al*., 1999), probably explaining why no noticeable metal adsorption was previously found in *V. elodeae* and *H. submersus* (Azevedo *et al.,* 2007).

In aquatic fungi, metal tolerance has been also associated with the synthesis of SH-enriched compounds, which are able to bind metals within cells (Miersch *et al*., 1997; Miersch *et al*., 2001; Guimarães-Soares *et al*., 2006, 2007). In the present work, *H. submersus* and *F. curta*, the two species isolated from a metal-polluted stream, had higher levels of NP-SH- and PB-SH compounds before metal exposure comparing to *V. elodeae*, the species isolated from a clean stream. Short-term exposure to Cu led to a decrease in both types of thiol compounds in the most resistant species (*H. submersus*), but not in the most sensitive one (*F. curta*). The decrease in reduced thiol compounds might be due to their oxidation during metal sequestration (Cobbett and Goldsbrough, 2002; Guimarães-Soares *et al*., 2006) or ROS scavenge (Bai *et al*., 2003), suggesting that the high constitutive levels of thiols might have helped *H. submersus* to deal with Cu stress. In addition, the decrease in the NP-SH compounds in all fungal species after long-term exposure to Cu is consistent with the ability of peptides with very low molecular weight, most likely glutathione and phytochelatins, to bind Cu in aquatic hyphomycetes (Guimarães-Soares *et al*., 2006). *V. elodeae*, that had the lowest constitutive thiol levels, responded to metal exposure with a rapid increase in the levels of NP-SH and PB-SH. These findings reinforce previous observations reporting that high constitutive levels of thiols or the rapid increase of their production may help aquatic hyphomycetes to deal with metal stress (Guimarães-Soares *et al*., 2006, 2007).

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# Chapter 5

*Metal stress induces programmed cell death in aquatic fungi*

## Abstract

Aquatic hyphomycetes are a phylogenetically heterogeneous group of fungi that occur in clean and metal-polluted streams. We examined the ability of Cu and Zn to induce programmed cell death (PCD) in three aquatic hyphomycete species through the evaluation of typical apoptotic markers, namely production of reactive oxygen species (ROS), caspase activation, nuclear morphological alterations and the occurrence of DNA strand-breaks, evaluated by TUNEL assay. In *Heliscus submersus* and *Flagellospora curta*, Cu exposure resulted in a high number of cells with ROS production and caspase activation, but a low number of fungal cells showed nuclear morphological alterations and DNA strand-breaks. Conversely, under Zn stress, aquatic hyphomycetes showed high number of cells with nuclear morphological alterations or DNA strand-breaks. In *Varicosporium elodeae*, Cu induced caspase activation, nuclear morphological alterations and DNA strand-breaks, but no ROS production. These results may indicate that *V. elodeae* developed a PCD process independent of ROS production. In addition, under Zn stress, *F. curta* appeared to develop a PCD process independent of caspase activity.

## 5.1. Introduction

Human activities contribute to a high release of metals to the environment at rates and concentrations sufficient to make them pollutants (Brown *et al*., 1999). Certain metals, such as Cu and Zn, are needed for the growth and metabolism of microorganisms (Gadd, 1993); however, above critical levels, they are known to inhibit a variety of metabolic activities affecting cellular processes (Cobbet and Goldsbrough, 2002).

The toxicity of metals can result from the generation of reactive oxygen species (ROS) that may cause damage to proteins, nucleic acids and lipids, eventually leading to cell death (Stohs and Bagchi, 1995). Madeo *et al*. (1999) showed that programmed cell death (PCD) could be induced in yeasts by oxidative stress triggered by exposure to $H_2O_2$. ROS can also act indirectly by modifying the cellular redox potential, which modulates key regulatory proteins involved in PCD (Mignotte *et al*., 1998). In fact, PCD can be induced by growing a *gsh1* yeast mutant in the absence of GSH (Madeo *et al*., 1999) or by the oxidation of cellular sulfhydryl (SH) compounds (Sato *et al*., 1995). Moreover, the ability of some antioxidant enzymes, such as catalase, to block apoptotic-PCD argues for the central role of oxidative stress in cell death processes (Buttke and Sandstrom, 1994). In spite of noteworthy evidences pointing to ROS as crucial PCD mediators, it was recently reported an active cell death process independent of ROS in yeasts (Almeida *et al*., 2007).

Programmed cell death, in which cells actively participate in their own death, is characterized by phenotypical alterations, such as chromatin condensation (Clifford *et al*., 1996), DNA fragmentation, formation of membrane-enclosed cell fragments (apoptotic bodies) (Kerr *et al*., 1972) and caspase activation (Earnshaw *et al*., 1999). Metals are reported to induce PCD processes in various cell systems. For instance, DNA damage was caused by exposure to complexes of 1,10-phenanthroline and metals in yeast and mammalian cells (Barry *et al*., 2004), by exposure to Cd, Cu, Zn and Pb in tobacco and potato plants (Gichner *et al*., 2006) or to Cu in rat thymocytes (Wolfe *et al*., 1994). Also, caspase activation was observed after exposure of HEP-2 cancer cells to Zn (Rudolf *et al*., 2005).

In contrast to the increasing number of studies that are beginning to unravel the PCD pathways in yeast and mammalian cells, relatively little work has been done in

filamentous fungi (Robson, 2006). Genomes of filamentous fungi contain the complement of genes involved in PCD in *Saccharomyces cerevisiae* (Fedorova *et al*., 2005; Glass and Dementhon, 2006), although Aspergilli have many putative components of the mammalian apoptotic machinery, including several proteins not found in *S. cerevisiae* (Fedorova *et al*., 2005).

Processes of PCD involving DNA degradation were associated with heterokaryon incompatibility in *Neurospora crassa* (Marek *et al*., 2003) and caspase-like activities were described during cell death at the stationary-phase of *Aspergillus fumigatus* (Mousavi and Robson, 2003) and during asexual sporulation of *A. nidulans* (Thrane *et al*., 2004). Consistently, searches in *A. nidulans* genoma revealed two genes encoding metacaspase proteins (Thrane *et al*., 2004). Despite the evidences that in *A. fumigatus* the loss of cell viability and death caused by toxic levels of $H_2O_2$ are associated with phenotypic characteristics of apoptosis, no significant activity against caspase substrates was found (Mousavi and Robson, 2004).

Studies examining whether exposure to environmental stressors triggers the development of apoptotic phenotypes are scarce in filamentous fungi (Ramsdale, 2006; Robson, 2006) and virtually unknown in freshwater fungi. Among these, aquatic hyphomycetes are an ecologically relevant group of fungi that play an important role as intermediaries between plant detritus and invertebrates in either clean or metal-polluted streams (Sridhar *et al*., 2001; Bärlocher, 2005; Pascoal *et al*., 2005 b). Previous reports showed that the exposure of aquatic hyphomycetes to metals led to an intracellular ROS accumulation (Guimarães-Soares, 2005; Azevedo *et al*., 2007) and to shifts in the levels of GSH or protein-bound SH compounds (Miersch *et al*., 2001; Jaeckel *et al*., 2005; Guimarães-Soares, 2006, 2007; Braha *et al*., 2007). To test whether Cu and Zn stress is able to induce PCD in aquatic hyphomycetes, we characterized cell death processes in three fungal species through the evaluation of typical apoptotic markers, namely ROS production/accumulation, caspase activation, alterations in nuclei morphology, and the occurrence of DNA strand-breaks. The identification of PCD in aquatic hyphomycetes under metal stress will improve our understanding on the mechanisms of cell death helping to explain the survival of fungi in metal-polluted streams.

## 5.2. Materials and Methods

### *5.2.1. Fungal species and conditions of maintenance*

The aquatic hyphomycetes *Heliscus submersus* H. J. Huds. UMB-135.01, *Flagellospora curta* J. Webster UMB-39.01 and *Varicosporium elodeae* W. Kegel UMB-142.01 were isolated from single spores collected from streams in the Northwest of Portugal. The first two species were isolated from leaves collected in the Este River, at a site with high nutrient loading (4.968 mg $L^{-1}$ $N\text{-}NO_3^-$; 0.249 mg $L^{-1}$ $N\text{-}NH_4^+$; and 0.176 mg $L^{-1}$ $P\text{-}PO_4^{3-}$, Pascoal *et al*., 2005a) and heavy metals in the stream water (5.87 mg $L^{-1}$ Cu; 2.02 mg $L^{-1}$ Zn, Gonçalves, 2001) due to urbanization, intensive agriculture and industrial activities. *V. elodeae* was isolated from foams collected in a clean stream (0.099 mg $L^{-1}$ $N\text{-}NO_3^-$; <0.008 mg $L^{-1}$ $N\text{-}NH_4^+$; and 0.010 mg $L^{-1}$ $P\text{-}PO_4^{3-}$, Pascoal *et al*., 2005a) at the Peneda-Gerês National Park.

Fungi were maintained on solid medium containing 2% (w/v) malt extract and 1.5 % (w/v) agar, at 18º C under artificial light.

### *5.2.2. Growth conditions and preparation of fungal mycelium suspensions*

Fungal spores (final concentration of 6 conidia $ml^{-1}$) were inoculated in Erlenmeyer flasks containing mineral medium with vitamins and 2% (w/v) glucose (van Uden, 1967), at pH 5.0, with or without addition of Cu or Zn. The flasks were incubated on a shaker (160 rpm; Certomat BS 3, B. Braun Biotech International) at 18ºC under permanent artificial light, during 8 days. At this time fungal cultures were at the end of exponential growth phase (not shown).

Stock solutions of copper ($CuCl_2$) and zinc ($ZnCl_2$) were added to the growth medium at concentrations that inhibited biomass production in 50% ($EC_{50}$). Metal concentrations were: 1510 µM Cu and 465 µM Zn for *H. submersus*; 183 µM Cu and 1304 µM Zn for *F. curta*; 457 µM Cu and 7315 µM Zn for *V. elodeae*.

Fungal mycelia were harvested by filtration and homogenized in PBS buffer (0.12% (w/v) $Na_2HPO_4$ anhydrous, 0.02% (w/v) $KH_2PO_4$ anhydrous, 0.8% (w/v) NaCl and 0.02% (w/v) KCl). Mycelium suspensions were washed twice with cold PBS buffer before the assays.

### *5.2.3. Production of reactive oxygen species*

Reactive oxygen species (ROS) production was monitored with MitoTracker Red CM-$H_2$XRos (Molecular Probes, Eugene, OR). The reduced form of this dye does not fluoresce until entering an actively respiring cell, where it is oxidized by ROS to a red fluorescent compound, which is sequestered in mitochondria. Mycelium suspensions, prepared as above, were incubated with 0.25 µg µl$^{-1}$ MitoTracker Red CM-$H_2$XRos for 15 min at room temperature and then scanned under an epifluorescence microscopy (BX 61 Olympus, magnification 1000 X).

### *5.2.4. Activity of caspases*

The fluorochrome-labeled inhibitor of caspases (FITC-VAD-FMK) was used to detect active caspases *in situ*. Because this compound has affinity to the active centre of caspases, its binding to apoptotic cells can indicate caspase activation (Pozawowski *et al*., 2003). Since in yeast cells unspecific binding of FITC-VAD-FMK to propidium iodide-positive cells has been reported (Wysocki and Kron, 2004), caspase activity was monitored only in propidium iodide negative cells.

Mycelium suspensions prepared as above were ressuspended in 200 µl of staining solution (50 µM FITC-VAD-FMK and 5 µg ml$^{-1}$ propidium iodide) and incubated 40 min at 25ºC in the dark. After this, mycelia were washed twice by centrifugation (6200 g, 10 min) ressuspended in 20 µl of PBS and scanned by epifluorescence microscopy.

### *5.2.5. Nuclear morphological alterations*

The morphology of cell nuclei was assessed with 4',6-diamidino-2-phenylindole (DAPI). This compound is known to form fluorescent complexes with double-stranded DNA and thus localizes nuclei. Nuclei are considered to have the normal phenotype when is bright and homogenous. Apoptotic nuclei can be identified by the condensed chromatin at the periphery of nuclear membranes or by the appearance of nuclear bodies.

Suspensions of fungal mycelium were fixed in ethanol 70% (v/v) during 30 min at 4ºC. Then, mycelium was centrifuged during 4 min at 11500 g (Bifuge-Pico-Heraeus) and the ethanol was discarded. After that, mycelium was incubated 20 min with 0.1 mg ml$^{-1}$ of DAPI (Sigma) under dark at room temperature. Subsequently, mycelia were

washed twice, ressuspended in 20 µl of PBS buffer and scanned by epifluorescence microscopy.

### *5.2.6. TUNEL and propidium iodide staining*

DNA strand breaks were visualized by terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL) and propidium iodide staining with the *In situ* Cell Death Detection Kit, Fluorescein (Boehringer Mannheim). This technique labels free 3'-OH termini with FITC-labelled deoxyuridine triphosphate (dUTP), which was detected by epifluorescence microscopy.

Fungal mycelium was fixed with 3.7% (v/v) formaldehyde and cell walls digested with zymoliase during 2 h at 37ºC and 150 rpm (Med Line SI-600R). Then, mycelium suspensions were prepared as described above and centrifuged (3000 g for 3 minutes). Subsequently, cytospins of cell suspensions were done using a Shandon Cytospin 2 cytocentrifuge at 1000 rpm for 5 min. Slides were incubated in a permeabilization solution (0.1% (v/v) Triton X-100 in 0.1% sodium citrate (w/v)) during 10 min, rinsed twice in PBS buffer and incubated with the TUNEL reaction mixture. Slides were incubated in a humidified atmosphere in the dark (1 h; 37ºC). Ten microlitres of a mixture containing 100 µl of the antifading agent Vectashield, 2 µl of propidium iodide (PI; 50 µg $ml^{-1}$), to co-localize DNA, and 2 µl RNase (0.5 µg $ml^{-1}$) was added to each slide. Positive controls were prepared by incubating slides with 30 µl DNase (30 min and 37ºC), before incubation with the TUNEL reaction mixture. Mycelia were scanned by epifluorescence microscopy.

## 5.3. Results

### *5.3.1. Cu and Zn induce reactive oxygen species production*

Exposure to metals led to an intracellular ROS accumulation in mycelia of *H. submersus*, *F. curta* and *V. elodeae*, as shown by red fluorescence after staining with MitoTracker Red CM-$H_2$XRos (Figure 5.1 and Table 5.1). Zinc exposure induced intracellular ROS production in a low number of fungal cells in all species (Figure 5.1 and Table 5.1). The response to Cu was more heterogeneous; *H. submersus* showed higher number of cells with ROS accumulation, *F. curta* showed low levels of

intracellular ROS, and *V. elodeae* had no noticeable ROS production (Figure 5.1 and Table 5.1).

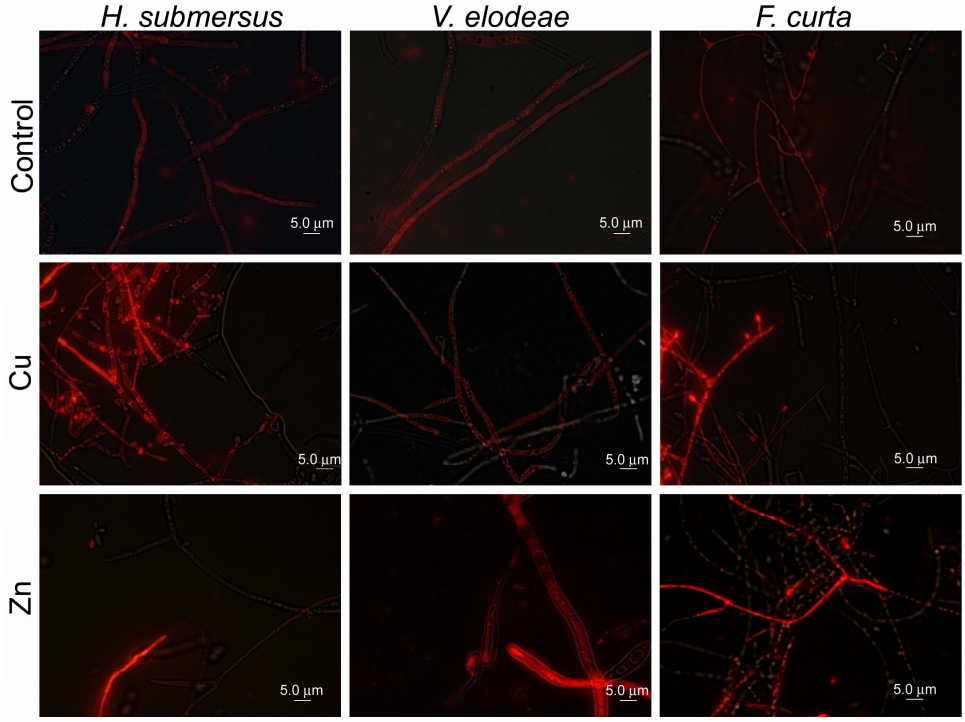


Figure 5.1. ROS production, assessed by MitoTracker Red CM-$H_2$XRos staining, in mycelia of *H. submersus*, *V. elodeae* and *F. curta* non-exposed or exposed to $EC_{50}$ of Cu or Zn. ROS-positive cells show red fluorescence.

Table 5.1. Qualitative analysis of ROS production, caspase activation, nuclear morphological alterations (NMA) and DNA strand-breaks induced by exposure of aquatic hyphomycetes to $EC_{50}$ of Cu or Zn.

| Fungal species | Cu | | | | Zn | | | |
|---|---|---|---|---|---|---|---|---|
| | ROS | Caspases | NMA | DNA strand-breaks | ROS | Caspases | NMA | DNA strand-breaks |
| *H. submersus* | ++ | ++ | + | + | + | + | + | ++ |
| *V. elodeae* | - | + | ++ | + | + | ++ | ++ | + |
| *F. curta* | + | ++ | + | - | + | - | + | ++ |

(- No effect; + Low effect; ++ High effect)

### *5.3.2. Cu and Zn induce caspase-like activity*

Caspase activation was not found in control mycelia of all tested aquatic hyphomycete species, as shown by the absence of green fluorescence after FITC-VAD-FMK staining (Figure 5.2). Metal exposure led to caspase activation, except in *F. curta* mycelia exposed to Zn (Figure 5.2 and Table 5.1). Zinc exposure promoted higher number of caspase-positive cells in *V. elodeae* than in *H. submersus*. On the contrary, Cu induced higher proportion of caspase-positive cells in mycelia of *H. submersus* and *F. curta* than in *V. elodeae* (Figure 5.2 and Table 5.1). In general, Cu induced higher levels of caspase activity than Zn except for *V. elodeae* (Figure 5.2 and Table 5.1).

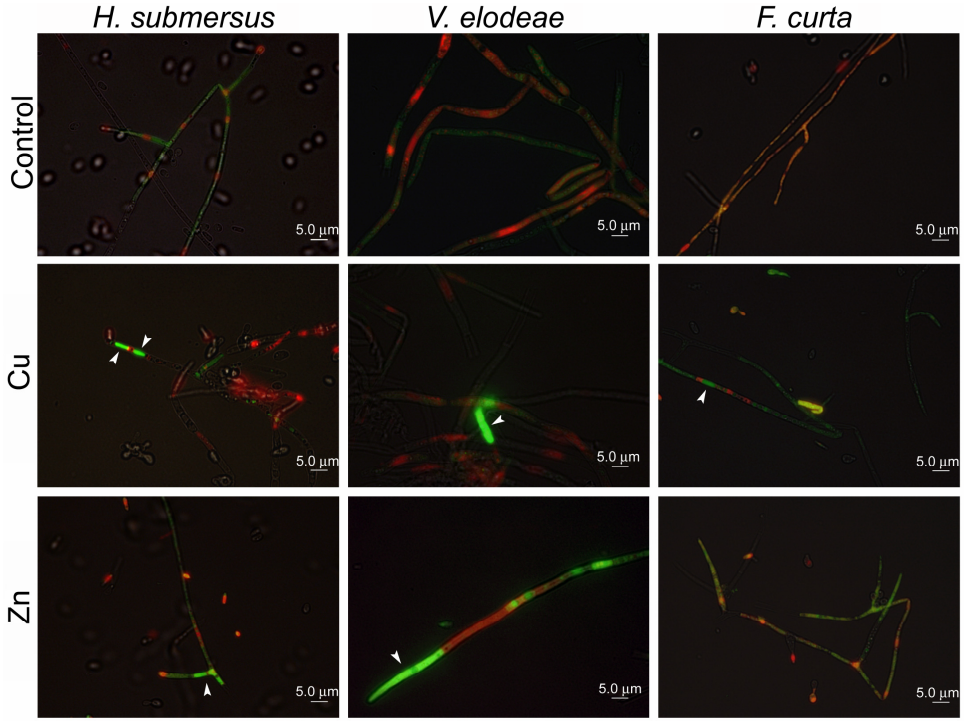


Figure 5.2. Caspase activity, assessed by FITC-VAD-FMK, in mycelia of *H. submersus*, *V. elodeae* and *F. curta* non-exposed or exposed to $EC_{50}$ of Cu or Zn. Caspase-positive cells show green fluorescence.

### *5.3.3. Cu and Zn induce nuclear morphological alterations revealed by DAPI staining*

Nuclei of control mycelia of the aquatic hyphomycetes appeared as single round spots when stained by DAPI (Figure 5.3). The exposure to Cu and Zn induced nuclear alterations in all aquatic hyphomycete species, as arrangements in half-rings or nuclear fragments randomly distributed (Figure 5.3 and Table 5.1). These alterations were

found in lower percentage in mycelia of *H. submersus* and *F. curta* than in *V. elodeae* (Figure 5.3 and Table 5.1).

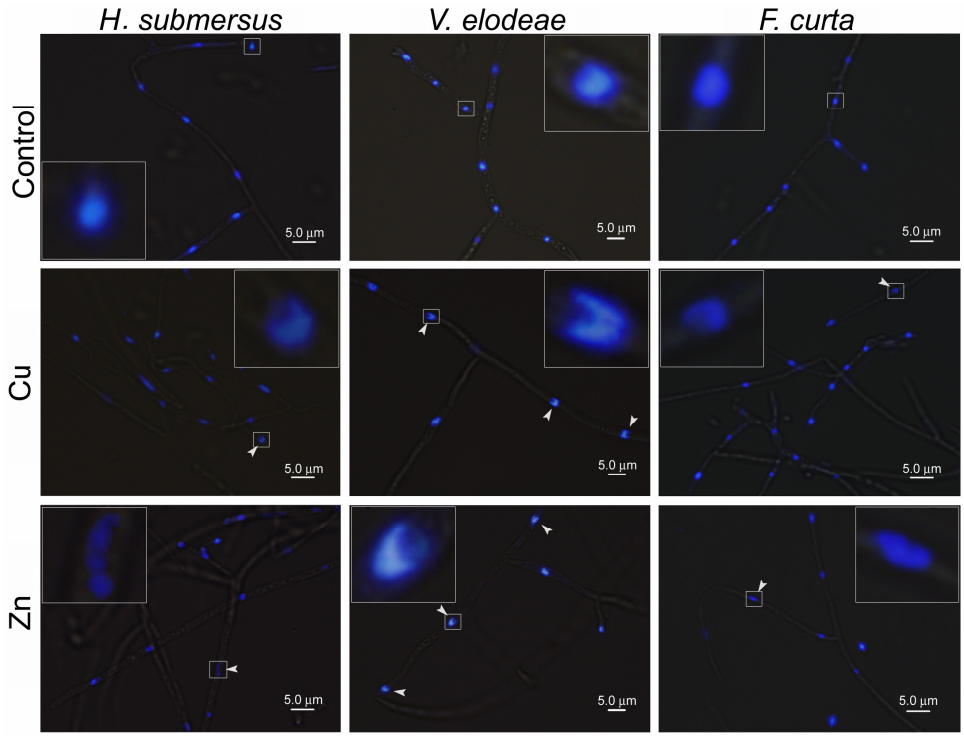


Figure 5.3. Morphology of nuclei revealed by DAPI staining in *H. submersus*, *V. elodeae* and *F. curta* non-exposed or exposed to $EC_{50}$ of Cu or Zn. Arrows indicate nuclei with altered morphology. Inserts show detailed nuclear morphology, with nucleous alterations as half-ring arrangements or nuclear fragments.

### *5.3.4. Cu and Zn induce DNA strand-breaks revealed by TUNEL assay*

No detectable TUNEL-positive phenotype was observed in control mycelia of the three aquatic hyphomycete species (Figure 5.4). Metal exposure led to DNA cleavage, as shown by the yellow nuclear fluorescence as the result of superimposition of green and red fluorescence due to simultaneous staining with TUNEL and PI. Zinc induced a greater number of cells displaying DNA strand-breaks in *H. submersus* and *F. curta* than in *V. elodeae*, as shown by higher number of cells with TUNEL-positive phenotype in the two former species (Figure 5.4 and Table 5.1). Exposure to Cu resulted in a small number of cells with a TUNEL-positive phenotype in *H. submersus* and *V. elodeae*, while no DNA cleavage was detected in *F. curta* (Figure 5.4 and Table 5.1).

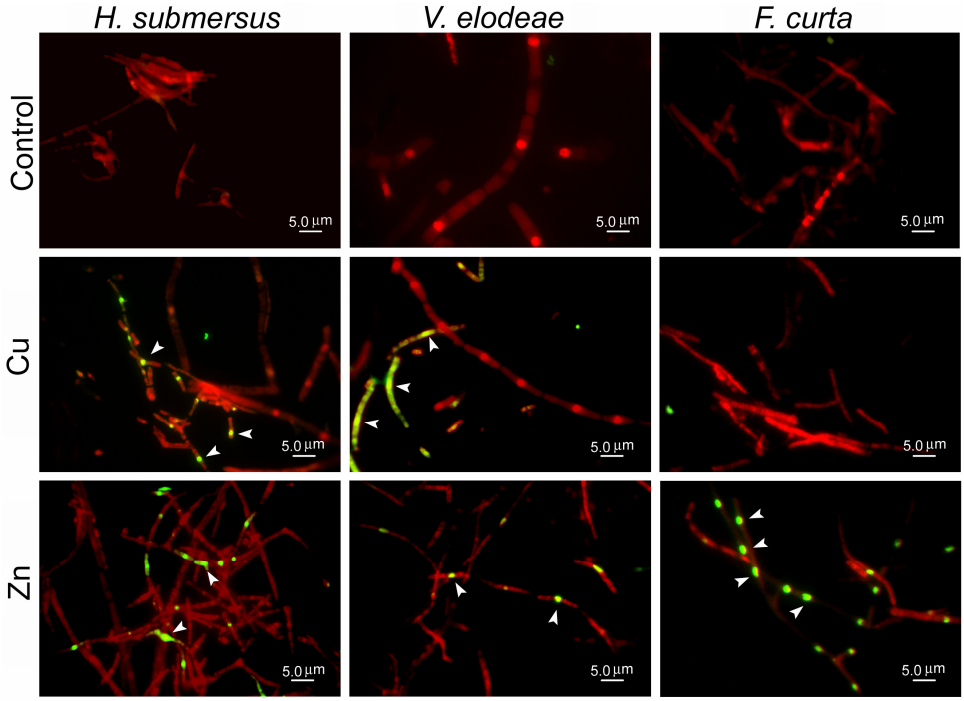


Figure 5.4. DNA strand-breaks visualized by TUNEL assay in *H. submersus*, *V. elodeae* and *F. curta* non-exposed or exposed to $EC_{50}$ of Cu or Zn. TUNEL-positive cells are shown by yellow nuclear fluorescence, as the result of superimposition of green (FITC-labelled nucleotides) and red fluorescence due to simultaneous staining with TUNEL and PI.

## 5.4. Discussion

The maintenance of cellular homeostasis is dependent on the ability of cells to respond to diverse environmental stressors. Metals can cause, directly or indirectly, an increase in ROS production in cells (Stohs and Bagchi, 1995). ROS production by damaging lipids, proteins and nucleic acids (Bai *et al.*, 2003) can affect cellular functions and subsequently may induce PCD (Madeo *et al.*, 1999). Moreover, it has been demonstrated that ROS production during PCD can occur upstream of other apoptotic events, such as activation of caspases (Buttke and Sandstrom, 1994) and nuclear fragmentation (Masato *et al.*, 1998). However, cells under PCD do not always harbour all cardinal features of this cell death type (Schulze-Osthoff *et al.*, 1994), being the most characteristic traits the fragmentation of nucleus with condensed chromatin, extensive membrane blebbing and DNA strand-breaks (Mignotte *et al.*, 1998).

We previously demonstrated that metal-induced ROS production contributes noticeably to Cu and Zn toxicity in aquatic hyphomycetes (Azevedo *et al*., 2007). However, to our knowledge, this is the first study linking metal-induced oxidative stress to PCD processes in aquatic fungi. In our study, *H. submersus* and *F. curta* displayed high ROS production/accumulation and caspase activation under Cu stress, but a low number of cells had nuclear morphological alterations and DNA strand-breaks. The exposure of *V. elodeae* to Cu did not induce ROS production/accumulation. However, nuclear morphological alterations, caspase activation and DNA strand-breaks were evident in this fungal species. This is in agreement with data linking PCD to an increase in the proportion of TUNEL-positive nuclei in filamentous fungi under stress (early stationary-phase, Mousavi and Robson, 2003; exposure to sphingoid long-chain bases, Cheng *et al*., 2003). In addition, the absence of detectable ROS in *V. elodeae* under Cu stress suggests that pro-oxidative conditions are not a general prerequisite for PCD in aquatic hyphomycetes, similarly to that found in *S. cerevisiae* treated with an antifungal agent (Almeida *et al*., 2007) or aspirin (Balzan *et al*., 2004).

The exposure of *H. submersus* and *F. curta* to Zn led to more DNA strand-breaks than ROS production and caspase activation. This is the opposite to that happened under Cu stress. Moreover, although no active caspases were found after exposure of *F. curta* to Zn, we cannot discard that a different PCD pathway might occur in this fungal species. Treatments of *A. fumigatus* with $H_2O_2$ or amphotericin B induced a strong apoptotic phenotype independent of caspase activity (Mousavi and Robson, 2004). Also, the two major sphingoid bases of fungi with fungicidal activity against *A. nidulans* induced a PCD not dependent on metacaspase activity (Cheng *et al*., 2003). This suggests that a caspase-independent pathway may operate in filamentous fungi as described in mammalian systems (Cheng *et al*., 2003).

It has been proposed that yeast cells can commit altruistic suicide to provide nutrients for others, probably younger and fitter cells (Herker *et al*., 2004). In filamentous fungi PCD can occur after treatments with various stress agents (Cheng *et al.,* 2003; Mousavi and Robson, 2004) and during developmental processes (Mousavi and Robson, 2003; Thrane *et al*., 2004). Therefore, the occurrence of a tightly regulated death pathway, such as PCD, in aquatic hyphomycetes under metal stress might constitute an advantageous way of fungal acclimation in metal-polluted streams, because it would allow the sacrifice of certain cells for the benefit of whole mycelium

(Richie *et al*., 2007). For the first time, we provided evidences that Cu and Zn can trigger apoptotic-PCD in aquatic hyphomycetes. The most tolerant species either to Zn (*V. elodeae*, $EC_{50}$ 7315 µM) or Cu (*H. submersus*, $EC_{50}$1510 µM) exhibited the higher levels of PCD markers. Moreover, different combinations of apoptotic markers were found suggesting the triggering of different cell death pathways in aquatic hyphomycetes. This may be linked to fungal resistance/tolerance to Cu and Zn and are worthy of further studies.

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# Chapter 6

*General discussion*

In recent years, freshwater pollution by heavy metals has attracted considerable attention because it is a worldwide problem with serious environmental consequences. Heavy metals released in the environment result from natural processes, but mainly from human activities, such as agriculture, mining and industry (Ayres, 1992). The non-degradability of metals, their accumulation in biota and biomagnification along aquatic food chains (Spacie *et al*., 1995) contribute to the importance of studying metal effects on biological systems. Essential metals, such as Cu, Zn and Ni are needed for the growth and metabolism of organisms. However, both essential and non-essential metals (e.g., Cd) can be toxic when present above certain threshold concentrations.

In freshwater ecosystems, fungi, particularly aquatic hyphomycetes, have been recognized as playing a dominant role in microbial decomposition of leaf litter (Baldy *et al*., 1995; Pascoal and Cássio, 2004; Pascoal *et al*., 2005a), by degrading plant cell-wall polymers and increasing leaf palatability for invertebrate consumption (Bärlocher, 2005). Even though aquatic hyphomycetes are primarily associated with clean and well-aerated freshwaters, they also occur in metal-polluted streams (Sridhar *et al*., 2000; Pascoal *et al*., 2005b). However, pollution by metals is reported to decrease aquatic hyphomycete diversity (Sridhar *et al*., 2000; Pascoal *et al*., 2005b) and activity, as reproduction (Rodrigues, 2002; Duarte *et al*., 2004, 2008) and growth (Miersch *et al*., 1997; Rodrigues, 2002).

In this work, the exposure to metals inhibited reproduction, as sporulation rates, of the tested aquatic hyphomycetes (Chapter 2). Additionally, we found that fungal reproduction was more sensitive to metals than growth, which is in accordance with several reports (Abel and Bärlocher, 1984; Bermingham *et al*., 1996; Duarte *et al*., 2004, 2008). The sensitivity of aquatic hyphomycetes to metals, assessed as the metal concentration inhibiting biomass production in 50% ($EC_{50}$), showed that *Ypsilina graminea* and *Varicosporium elodeae* were the most resistant species to Zn, while *Heliscus submersus* was the most resistant fungus to Cu (Chapter 2). Generally, Ni or Cd were more toxic to fungi then Zn or Cu, which is corroborated by previous reports (Gadd, 1993; Miersch *et al*., 1997; Rodrigues, 2002; Guimarães-Soares, 2005). Moreover, the patterns of species resistance to metals found in either liquid or solid medium with similar composition were identical (Chapter 2). However, $EC_{50}$ values were about 20-times higher in solid medium than in liquid medium, probably because agar may decrease metal bioavailability to fungi (Gadd, 1993). Changes in nutrient

supplies to fungi affected metal toxicity, as shown by higher $EC_{50}$ values found in mineral medium supplemented with vitamins and glucose than in malt extract (Chapter 2). This is in agreement with the decrease in metal toxicity with increased concentration of the carbon source in the culture medium (Gadd *et al.,* 2001).

Data from literature indicate higher metal tolerance in fungi isolated from metal-contaminated sites (e.g., Miersch *et al*., 1997; Colpaert *et al*., 2000), but this is not always the case (Miersch *et al*., 1997; Blaudez *et al*., 2000; Colpaert *et al*., 2000). In the present work, *Alatospora acuminata*, isolated from decomposing leaves collected in a clean stream, was very sensitive to all metals (Chapter 2). Consistently, *H. submersus* and *Flagellospora curta* isolated from a metal-polluted stream showed high tolerance to the most toxic metals (Cu, Ni and Cd). These findings suggest that fungi adapted to metal-polluted environments tolerate higher metal concentrations. However, *Y. graminea* isolated from a metal-polluted site was tolerant to Zn but not to Cd. Also, *V. elodeae* a species isolated from a clean site was able to tolerate high levels of Zn but not of Cu. This indicates that fungal tolerance to metals can vary with fungal species and metal type, suggesting that different mechanisms or cellular targets may be involved in fungal tolerance to different metals. Therefore, we selected three aquatic hyphomycete species, namely *H. submersus*, *V. elodeae* and *F. curta*, with different sensitivities to Cu and Zn to investigate the mechanisms underlying metal tolerance in aquatic hyphomycetes.

Copper and Zn induced alterations in cell-wall morphology of the tested aquatic hyphomycete species, as shown by scanning electron microscopy (Chapter 3). The highest amounts of Cu and Zn adsorbed to fungal mycelia were found in *H. submersus* and *V. elodeae*, respectively (Chapter 4). Because these fungi were the most tolerant species to each metal, biosorption may be a relevant mechanism to avoid unrestrained uptake of metals minimizing their deleterious effects. Nevertheless, these fungal species accumulated metals in their mycelia, although at much lower amounts comparing to that adsorbed. The ability of aquatic hyphomycetes to take up and store large quantities of metals makes them potential candidates for bioremediation. In this context, *V. elodeae* and *F. curta* had remarkable ability to adsorb and accumulate Zn comparing with values reported for aquatic fungi (Guimarães-Soares, 2005; Jaeckel *et al*., 2005) Also, *H. submersus* was able to retain ca. 7-times more Cu than metal-tolerant strains of *H. lugdunensis* (Braha *et al*., 2007). However, adsorption of metals by filamentous fungi

depends on several factors, including pH, initial metal concentration and medium composition (Gardea-Torresdey *et al*., 1997; Lo *et al*., 1999), probably explaining why no noticeable metal adsorption was found in *V. elodeae* and *H. submersus* under different environmental conditions (Chapter 3).

The primary mechanism of Cu toxicity in fungi is the disruption of cellular or organellar membranes (Ohsumi *et al*., 1988). In agreement, our results showed that plasma membrane integrity of *V. elodeae* and *H. submersus* was more affected by Cu than Zn, pointing to this cellular structure as a potentially vulnerable target of Cu (Chapter 3). This effect can be attributed to the redox-active nature of Cu and its ability to generate free radicals that promote lipid peroxidation (Stohs and Bagchi, 1995). On the other hand, non-redox active metals, like Zn, can deplete free-radical scavengers, such as thiol-containing compounds, resulting is ROS production (Dietz *et al*., 1999). In this study, we clearly demonstrated that generation of ROS contributed noticeably to metal toxicity in aquatic hyphomycetes, particularly under Cu stress, as indicated by the increase of biomass production in the presence of an antioxidant agent (Chapter 3). Moreover, metals can directly interact with biomolecules, such as transport proteins of essential nutrients and ions, compromising their biological functions (Gadd, 1993). In this work, we found that short-term exposure (10 min) to Cu completely inhibited the activity of the $H^+$-ATPase in *H. submersus* and *V. elodeae*, while Zn only led to a similar effect on that of *H. submersus* (Chapter 4). However, since a recovery of plasma membrane integrity was observed after 150 min of exposure to Cu *H. submersus* (Chapter 3), a functional restoration of the $H^+$-ATPase is expected to occur at longer times. Indeed, 8 days of metal exposure led to strong stimulations of the proton pump in the most tolerant species, i.e. when *H. submersus* was exposed to Cu and *V. elodeae* was exposed to Zn (Chapter 4). The activation of $H^+$-ATPase by metal exposure may be related to its ability to counteract metal-induced dissipation of the electrochemical gradient of protons across the plasma membrane (Serrano, 1988; Fernandes *et al*., 1998), suggesting that $H^+$-ATPase may be involved in aquatic hyphomycete acclimation to metals.

In aquatic fungi, metal tolerance has been also associated with the synthesis of thiol (SH)-enriched compounds, which are able to scavenge ROS or bind metals within cells (Miersch *et al*., 1997; Miersch *et al*., 2001; Guimarães-Soares *et al*., 2006, 2007). In the present work, *H. submersus* and *F. curta*, the two species isolated from a metal-

polluted stream, had higher levels of non-protein (NP-SH) and protein-bound (PB-SH) thiols before metal exposure comparing to *V. elodeae*, the species isolated from a clean stream (Chapter 4). The latter species, that had the lowest constitutive thiol levels, rapidly increased NP-SH and PB-SH levels under exposure to Cu or Zn. These findings reinforce previous observations that high constitutive levels of thiols or the rapid increase of their production may help aquatic hyphomycetes to deal with metal stress (Guimarães-Soares *et al*., 2006, 2007). In addition, the decrease in the NP-SH containing compounds in all fungal species after long-term exposure (8 days) to Cu is consistent with the ability of peptides with very low molecular weight, most likely glutathione and phytochelatins, to bind Cu in aquatic hyphomycetes (Guimarães-Soares *et al*., 2006).

All organisms, including fungi, have a set of enzymatic defenses to deal with oxidative stress (Bai *et al*., 2003). Enzymes, such as superoxide dismutase (SOD), catalase (CAT) and glucose-6-phosphate dehydrogenase (G6PDH), have been reported to be activated against ROS in several organisms under Cu and/or Zn stress (yeasts, Romandini *et al*., 1992; algae, Collén *et al*., 2003; Tripathi *et al*., 2006; mussels, Geret and Bebiano, 2004). The first two enzymes are crucial for cellular detoxification, controlling the levels of superoxide anion radical and hydrogen peroxide (Penninckx and Elskens, 1993; Bai *et al*., 2003); G6PDH is essential for the replenishment of NADPH intracellular pool to maintain cellular redox balance (Penninckx and Elskens, 1993). Our studies on antioxidant defenses showed that CAT had a greater role alleviating the stress induced by Zn and Cu than SOD (Chapter 3). In addition, the increased activity of G6PDH after long-term exposure to metals, points to the involvement of the pentose phosphate pathway in metal acclimation.

In this work, we also tested whether Cu and Zn are able to induce programmed cell death (PCD), a process in which cells actively participate in their own death (Robson, 2006). For that, we examined typical apoptotic markers, namely ROS production, caspase activation, alterations in nuclear morphology and the occurrence of DNA strand-breaks (Kerr *et al*., 1972; Clifford *et al*., 1996; Earnshaw *et al*., 1999; Madeo *et al*., 1999). However, cells under PCD do not always harbour all cardinal features of this cell death type (Schulze-Osthoff *et al*., 1994), being the most characteristic traits the fragmentation of nucleus with condensed chromatin, extensive membrane blebbing and DNA strand-breaks (Mignotte *et al*., 1998). In our study, *H.*

*submersus* and *F. curta* displayed high ROS production and caspase activation under Cu stress, but a low number of cells had nuclear morphological alterations and DNA strand-breaks (Chapter 5). In *V. elodeae*, Cu induced caspase activation, nuclear morphological alterations and DNA strand-breaks, but did not increase ROS production (Chapter 5). These results may indicate that *V. elodeae* developed a PCD process independent of ROS production, similarly to that recently described in yeasts (Almeida *et al*., 2007). The exposure to Zn led to more DNA strand-breaks or nuclear morphological alterations than ROS production and caspase activation in the tested aquatic hyphomycetes (Chapter 5). For the first time, we provided evidences that Cu and Zn can promote PCD in aquatic hyphomycetes. The occurrence of a tightly regulated death pathway, such as PCD, in aquatic hyphomycetes under metal stress may constitute an advantageous way of fungal acclimation in metal-polluted streams, because it would allow the sacrifice of certain cells for the benefit of whole mycelium (Richie *et al*., 2007). However, further experiments are needed to better understand the role of PCD in aquatic hyphomycete homeostasis under metal stress.

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