Universidade do Minho
Escola de Ciências

Isabel Rodrigues Fernandes

Effects of fungal diversity and cadmium on leaf litter decomposition in streams: studies in microcosms

July 2008

Universidade do Minho
Escola de Ciências

Isabel Rodrigues Fernandes

Effects of fungal diversity and cadmium on leaf litter decomposition in streams: studies in microcosms

Master Thesis
Molecular Genetics

Work Supervised By
Prof. Dr. Fernanda Cássio
Prof. Dr. Cláudia Pascoal

July 2008

Universidade do Minho, Julho de 2008

(Isabel Rodrigues Fernandes)

Acknowledgements

There are some persons that helped me during this work and to whom I would like to express my gratitude:

To the supervisors of my work Prof. Fernanda Cássio and Prof. Cláudia Pascoal for providing me the opportunity of develop this work, for all the support, encouragement, and scientific knowledge share.

To the persons that welcomed and/or shared the lab with me: Né, Sofia and more recently, Begüm and Paulo. To Né and Sofia a special thank you for all your help, patience and friendship. You became truly friends. To Sofia, I can not thank you enough.

To all the persons in the Department of Biology, always ready to give me a hand, thank you.

To lab technicians from the Department of Biology. Amaro and Manuela for their sympathy, corridor talks (always very funny) and help with anything. To Magda, for her huge help with the sequences analyses. To D. Isabel, Liliana and Sr. Adelino, for their sympathy and for their help with those “tons” of stream water and flasks.

To the persons who initiate this journey with me, during my first year of Master, for their sympathy and friendship. A special thank to Nadia.

To João, Luís, Vítor, Humberto and Cristina for their friendship, support and for listening my not always funny jokes.

To Sara and Jorge, for their unconditional friendship, for always listening my lamentations and utopian dreams, for all our N moments (you know what I mean). Thank you, thank you, thank you!

To Ju, my best, best friend! Thank you for all your support and friendship even at 2000 Km of distance. You’re always in my heart!

To my parents António and Gracinda, and my sister Xana for all their love, support and for all the missed moments.

To Nelson, your love lights up my life…

Abstract

Biodiversity loss caused by anthropogenic activities has been observed, but the consequences to ecosystem functioning are not fully understood yet. Freshwater environments are among the most endangered in the world, being metal pollution of great concern due to metal non-degradability and biomagnification along food webs. Aquatic hyphomycetes play a key role in plant litter decomposition in freshwater ecosystems, and a positive relationship has been established between fungal diversity and this ecosystem-level process. This increases the interest in better understanding the relationship between fungal diversity and leaf decomposition under metal stress.

Three aquatic hyphomycete species (*Articulospora tetracladia*, *Tricladium splendens* TS100 and *Flagellospora curta* FC39) and two strains of the same species with different background (*A. tetracladia* AT72 and AT61, isolated from a clean and metal-polluted stream, respectively) were selected. Monocultures and multicultures of all possible combinations of 2 and 3 species (never mixing the two strains from the same species) were used to evaluate the effects of species diversity, as species number and identity, on leaf decomposition, fungal biomass and sporulation in the absence or presence of cadmium (Cd). For each fungal isolate, ergosterol-to-biomass conversion factors were calculated and Real-Time Polimerase Chain Reaction assays were developed to discriminate the contribution of each species to total biomass production in multicultures.

The ergosterol content varied with growth phase and fungal isolate but not with metal exposure. The isolates FC39 and AT61 were more tolerant to Cd than TS100 and AT72, suggesting that metal tolerance can be not only species-specific but also strain-specific. Cadmium exposure and species identity affected leaf mass loss, fungal biomass and sporulation, while species number only affected the last two parameters. In control conditions, fungal biomass was higher in multicultures than that expected from the sum of biomasses in monocultures, mainly as a result of complementarity between species. Under Cd exposure, fungal biomasses were lower than that expected from the sum of monocultures and competitive interactions between fungal species appeared to occur. The presence of a key-species was suggested by the increased fungal biomasses in multicultures with *A. tetracladia* and the lower fungal biomasses, sporulations and leaf mass losses when this species was absent. Moreover, fungal performances and Cd tolerance were higher in treatments containing the strain of *A. tetracladia* isolated from the polluted site comparing to treatments with the strain isolated from the clean site. These findings emphasize the importance of strain traits for leaf decomposition and suggest that fungal populations adapted to metals may have better performances under metal stress than non-adapted ones, helping to maintain ecosystem processes.

Resumo

As actividades antropogénicas têm levado a uma perda da biodiversidade não se conhecendo ainda na totalidade as consequências dessa perda para o funcionamento dos ecossistemas. Os ambientes de água doce estão entre os mais afectados do mundo, sendo a poluição por metais uma grande preocupação devido à sua não degradação e bioacumulação ao longo das cadeias alimentares. Os hifomicetos aquáticos desempenham um papel importante no processo de decomposição da folhada em águas doces e a sua diversidade está positivamente relacionada com este processo. Assim, torna-se importante perceber melhor a relação entre a diversidade de fungos e a decomposição da folhada em condições de stress provocado pela exposição a metais.

Neste trabalho, seleccionámos três espécies de hifomicetos aquáticos (*Articulospora tetracladia*, *Tricladium splendens* TS100 e *Flagellospora curta* FC39) e duas estirpes da mesma espécie (*A. tetracladia* AT72 e AT61 isoladas, respectivamente, de um rio limpo e poluído por metais). Monoculturas e culturas mistas de todas as combinações possíveis de 2 e de 3 espécies, nunca misturando as duas estirpes da mesma espécie, foram usadas para avaliar o efeito do número e da identidade das espécies na decomposição da folhada e na biomassa e taxas de esporulação dos fungos na presença ou ausência de cádmio (Cd). Foram calculados os factores de conversão ergosterol-biomassa e desenvolvidos ensaios de PCR em tempo real, utilizando a região ITS2 do rDNA, para avaliar a contribuição de cada espécie para a produção total de biomassa fúngica.

O conteúdo em ergosterol variou com a fase de crescimento e com o isolado do fungo, mas não com a exposição ao metal. Os isolados FC39 e AT61 foram mais tolerantes ao Cd que os TS100 e AT72 sugerindo que, a tolerância a metal, para além de ser específica da espécie, pode ser específica da estirpe. A exposição ao Cd e a identidade das espécies afectaram a perda de massa da folha e a biomassa e a taxa de esporulação dos fungos, enquanto que o número de espécies apenas afectou os últimos dois parâmetros. Na ausência de Cd, a biomassa de fungo foi maior nas culturas mistas do que o esperado pela soma das biomassas nas monoculturas, devido essencialmente a efeitos de complementaridade entre as espécies. Sob exposição ao Cd, a biomassa de fungo nas misturas foi menor do que a esperada, provavelmente devido a competição entre as espécies de fungos. *A. tetracladia* parece ser uma espécie-chave dado que a biomassa de fungo, a perda de massa de folha e as taxas de esporulação foram menores nas culturas mistas em que esta espécie estava ausente. Além disso, a estirpe de *A. tetracladia* isolada do local poluído mostrou uma maior tolerância ao Cd comparativamente à estirpe isolada do local limpo. Estes resultados salientam a importância dos traços das estirpes para o processo de decomposição de folhada, sugerindo que populações de fungos adaptadas à presença de metais podem ter melhor desempenho sob stress metálico do que populações não adaptadas, o que pode ajudar a manter os processos do ecossistema.

Table of contents

List of Figures

List of Tables

Chapter 1

General Introduction

1.1. The role of biodiversity in ecosystem processes

The extinction of a high number of species as a consequence of the increasing human impacts on our planet has been observed, particularly over the last decades (Chapin et al. 2000). This led to an exponential increase in the number of studies relating biodiversity and ecosystem processes.

Several hypotheses have been proposed to explain the relationships between biodiversity and ecosystem functioning. The rivet hypothesis (Ehrlich and Ehrlich 1981) states that ecosystem functions can be maintained until a critical diversity level, below which further losses will compromise ecosystem integrity, like a machine that have lost too many rivets. The redundancy hypothesis (Walker 1992) predicts a positive and asymptotic relationship, as long as the loss of some species is compensated by other species within the same functional group. The idiosyncratic hypothesis (Lawton 1994) assumes an inconsistent pattern between species richness and ecosystem functioning praising the species traits, the order by which species are lost from ecosystems (Vitousek and Hooper 1993, Lawton 1994) and the environmental context (Cardinale et al. 2000).

It was observed that exposure to stress tends to increase positive interactions of species within communities (Mulder et al. 2001, Callaway et al. 2002). In a study using bryophyte communities, no relationship between species richness and biomass was found, but under drought conditions communities increased their biomass with species richness probably due to facilitative interactions between species (Mulder et al. 2001). Also, in subalpine and alpine plant community competition generally dominated species interactions at lower elevations (less stressful conditions), but at high elevations (high stress) the interactions among plants were predominantly positive (Callaway et al. 2002).

The increased ecosystem processes with diversity can result from complementary relationships between species, due to facilitative interactions or niche differentiation, leading to greater performances than that expected from individual performances (Hughes and Petchey 2001). However, communities with higher diversity will also have an increased probability of containing highly productive species (selection effect) (Loreau and Hector 2001). In microcosm experiments, fungal species number had positive effects on leaf decomposition, but it was not possible to discriminate if they resulted from complementarity or selection effect (Bärlocher and Corkum 2003, Duarte et al. 2006) because individual species performances within the community are difficult to assess. Moreover,

complementary and selection effects can operate in combination explaining the positive effects of biodiversity on ecological processes (Loreau and Hector 2001).

1.2. Leaf decomposition in freshwater ecosystems

In low-order forested streams, the abundant riparian vegetation reduces primary production by decreasing light availability (Benfield 1996). As a consequence, the main energy and nutrient source in food webs is the allochthonous input of organic matter coming from breakdown of leaf litter from riparian vegetation (Webster and Benfield 1986). When leaves enter the streams they undergo chemical and physical alterations that include leaching of soluble compounds, microbial conditioning, and physical and biotic fragmentation (for a review see Gessner et al. 1999). The rapid loss of leaf compounds, including soluble sugars, polyphenols and tannins, facilitates the colonization by microorganisms, mainly fungi and bacteria, improving leaf palatability to invertebrate shredders (Graça 2001). Shredders can take benefit from fungal degradative activity on leaves and/or feed on fungi directly (Graça 2001). Leaf fragmentation occurs as a result of feeding activities of shredders and flowing water abrasion (Cummins 1974). Although leaf decomposition events tend to occur sequentially in time, some stages can occur simultaneously (Gessner et al. 1999). During this process, microorganisms and invertebrates convert leaf litter into biomass, fine particulate organic matter, dissolved organic matter and CO_2 (Webster and Benfield 1986, Gessner et al. 1999).

Higher shredder abundance and biomass has been associated with faster leaf breakdown (Robinson and Gessner 2000, Graça 2001) and their exclusion in streams by application of an insecticide led to a decrease in leaf breakdown rates (Wallace et al. 1996). However, a minor role of shredders in litter breakdown has been found in large rivers (Chauvet et al. 1993), tropical streams (Mathuriau and Chauvet 2002) and polluted streams (Pascoal et al. 2005a). In these conditions, microorganisms may have a major contribution to the leaf decomposition process.

Among microbial decomposers, fungi appear to play a major role in leaf litter decomposition constituting more than 90% of the total microbial biomass (Baldy et al. 1995, Pascoal and Cássio 2004). Aquatic hyphomycetes are a phylogenetically heterogeneous group of fungi that dominates at earlier stages of plant-litter decomposition in streams (Bärlocher 1992, Gessner et al. 2003, Gessner et al. 2007). These fungi have morphological and physiological adaptations to lotic environments that dictate their success as colonizers and decomposers of leaf litter (Suberkropp 1998, Bärlocher 2005). They produce a wide range of extracellular

enzymes that attack plant cell-wall polysaccharides contributing to their decomposition (Suberkropp 1998). Moreover, aquatic hyphomycetes are able to remain active at relatively low temperatures typical of cold seasons in temperate climates (Read et al. 1992). Furthermore, high conidial production and germination rates, the conidial shapes (tetraradiate or sigmoid) and the mucilage produced by conidial arms allow an efficient dispersion and attachment to new substrata (Read et al. 1992).

The activity of decomposers on leaf litter can be influenced by several stream water variables, such as pH (Dangles et al. 2004), nutrient concentrations (Gulis and Suberkropp 2003) and temperature (Suberkropp and Weyers 1996, Abdel-Raheem 1997). Moreover, microbial decomposing activity on leaves can be also affected by leaf litter quality (Sampaio et al. 2001) or its state of senescence (Bärlocher 1997).

1.3. Relationships between fungal diversity and leaf litter decomposition in streams

Some authors propose that fungal diversity affects leaf-litter decomposition in freshwaters (Bärlocher and Corkum 2003, Duarte et al. 2006). Studies in which aquatic hyphomycete species were manipulated point to a positive relationship between fungal diversity and leaf decomposition (Bärlocher and Corkum 2003, Treton et al. 2004, Duarte et al. 2006, Raviraja et al. 2006). However, Dang et al. (2005) failed to detect effects of fungal diversity on leaf mass loss and fungal reproduction, but showed that higher diversity decreases the variability of process rates, probably increasing ecosystem stability (portfolio effect or statistical average effect, see Doak et al. 1998).

In field studies, patterns explaining diversity-ecosystem functioning relationships are even more difficult to detect, probably due to great environmental variability. In a polluted stream with high nutrient load and heavy metals, leaf decomposition did not decline with decreasing aquatic hyphomycete diversity (Pascoal et al. 2005b). Organic pollution, which was reported to reduce fungal sporulation and diversity, did not decrease leaf decomposition rates (Raviraja et al. 1998). Also, increasing riparian plant species richness led to higher aquatic hyphomycete diversity, but no differences in leaf decomposition rates were observed (Lecerf et al. 2005). However, both fungal diversity and their ecological functions were reduced in severely polluted streams (Sridhar et al. 2001). These findings suggest that some redundancy among aquatic hyphomycete species occur because ecosystem functions can be maintained under stressful conditions capable of decreasing fungal diversity.

However, when the stress exceeds the species tolerance threshold, the ecosystem functions can no longer be maintained. This reflects the dependence of the relationship between biodiversity and ecosystems functioning on the traits of species lost, as well as the type and severity of stressors and their interactive effects.

1.4. Fungal diversity and activity on decomposing leaves in streams under metal stress

Freshwaters are among the most endangered ecosystems in the world (Ricciardi and Rasmussen 1999, Dudgeon et al. 2006). Pollution by metals is of great concern due to its non-degradability, accumulation in the biota and biomagnification along aquatic food webs. Metals can reach the streams through several human activities, including mine drainage, industrial emissions, garbage disposal and agricultural fertilizers (Merian 1991). Some metals, like zinc (Zn) and copper (Cu), are involved in the organism growth, metabolism and differentiation (Rand et al. 1995, Mitra and Rensing 2007); others, like cadmium (Cd), do not appear to have any biological function (but see the Cd containing carbonic anhydrase from the marine diatom, Lane et al. 2005).

Several studies demonstrate that both essential and non-essential metals can negatively affect the performance of aquatic hyphomycetes. A decrease in fungal growth after exposure to different metals, such as Cd, Cu and Zn has been observed under controlled conditions (Miersch et al. 1997, Guimarães-Soares 2005, Jaeckel et al. 2005, Braha et al. 2007, Azevedo et al. 2007, Miersch and Grancharov 2008). Also, the ability of aquatic hyphomycetes to produce conidia is generally compromised by metal exposure (Rodrigues 2002, Azevedo 2007, Abel and Bärlocher 1984).

In metal-polluted streams, the diversity in aquatic hyphomycete species is commonly low (Bermingham et al. 1996a, Niyogi et al. 2002, Pascoal et al. 2005b). This has been associated with a reduction in fungal sporulation, biomass and leaf decomposition (Bermingham et al. 1996a, Sridhar et al. 2001, Niyogi et al. 2001). However, most studies indicate that fungal diversity and reproduction seem to be more affected by metals than fungal biomass or leaf decomposition (Niyogi et al. 2002, Duarte et al. 2004), pointing once more to functional redundancy among fungal species as an important way to maintain ecological functions in streams under stress (Niyogi et al. 2002, Pascoal et al. 2005b).

1.5. Contribution of molecular techniques for assessing fungal diversity and activity on decomposing leaves

Aquatic hyphomycetes are identified on the basis of the shape of asexually produced spores, typically tetraradiate or sigmoid conidia (Webster 1959, Webster and Davey 1984). Aquatic hyphomycete communities in streams have been characterized by counting and identifying conidia suspended in foam (Chauvet 1992, Pascoal et al. 2005c), stream water (Shearer and Webster 1985, Bärlocher 2000) or released from leaves decomposing in streams (Pascoal and Cássio 2004, Pascoal et al. 2005b). The obvious shortcoming of these techniques is that the absence of conidia may be due to the absence of species or to the presence of non-sporulating mycelium (Nikolvecha et al. 2003). Moreover, sporulation ability may vary with the species and several environmental conditions like the nutrient concentration in water (Ramos 2003, Gulis and Suberkropp 2004, Treton et al. 2004). Also, the majority of fungal biomass on decaying leaves consists of hyphae that cannot easily be identified by microscopy (Nikolvecha et al. 2003).

Molecular approaches based on nucleic acids, that are present in all stages of the fungal life cycle, might help to circumvent problems associated with the microscopy-based techniques to assess fungal diversity (Nikolvecha et al. 2003). Two methods have been used to study fungal communities on decomposing plant detritus: terminal restriction fragment length polymorphism (T-RFLP) analysis and denaturing gradient gel electrophoresis (DGGE) (Nikolcheva et al. 2003, Nikolcheva and Bärlocher 2004, Das et al. 2007, Duarte et al. 2008). Species richness estimates based on DGGE exceeded those based on spore identification (Nikolcheva et al. 2003, Duarte et al. 2008). Also, cluster analysis of DGGE fingerprint allowed detecting shifts in the structure of fungal communities with metal exposure (Duarte et al. 2008). Fungal diversity estimated by T-RFLP analysis and conidium identification was similar at early times, but the number of T-RFLP peaks declined at later times (Nikolcheva et al. 2003).

DGGE analyses have some limitations: the limit of resolution is about 1% of the community population (Muyzer et al. 1993), one band in the DGGE gel can represent more than one species (Nikolcheva et al. 2003) and one species can give more than one band (Michaelsen et al. 2006). T-RFLP is also limited in the resolution among aquatic hyphomycetes since analysis yielded only 8 distinct fragments for 12 species investigated (Nikolcheva et al. 2003).

Even though the advances on the application of molecular techniques, like the DGGE or T-RFLP, to assess aquatic hyphomycete diversity, none can undoubtedly

identify all species on decomposing leaves and/or discriminate the contribution of each species to overall community function.

Traditionally, fungal activity has been assessed by measuring biomass build-up and reproduction associated with decomposing leaves. Fungal biomass on leaves is difficult to assess since hyphae penetrate inside the substrate they are decomposing. To overcome this problem, ergosterol, a fungal membrane constituent (not present in bacteria or plant material) that suffers rapid oxidation after cell death, has been used as a measure of living fungal biomass on leaves (Gessner and Newell 2002). However, some results showed that ergosterol can be present at appreciable concentrations and for considerable time in the absence of living fungi (Mille-Lindblom et al. 2004). Moreover, the ergosterol content can vary with the species (Gessner and Chauvet 1993), their nutritional status (Charcosset and Chauvet 2001), growth phase (Barajas-Aceves et al. 2002) and/or the presence of stressors (e.g. fungicide zineb, Barajas-Aceves et al. 2002).

A more dynamic measure of fungal activity can be given by estimating fungal productivity, from incorporation rates of radioactive labelled acetate into ergosterol (Baldy et al. 1995, Pascoal and Cássio 2004, Suberkropp and Gessner 2005), that reflect the specific fungal growth rate on leaf litter. Nevertheless, neither productivity nor ergosterol quantification can discriminate the performances of individual fungal species within a community.

Monoclonal antibodies (Mabs) were used in a number of assays, such as the enzyme-linked immunosorbent assay (ELISA), which allows the identification and quantification of mycelium, and immunofluorescence (IMF) which allows visualization of mycelium on leaf material (Bermingham et al. 1995, 2001). As far as we know, these techniques allowed the identification and biomass quantification of four species of aquatic hyphomycetes on leaves: *Anguillospora longissima*, *Alatospora acuminata*, *Tetracladium marchalianum* and *Heliscus lugdunensis* (Bermingham et al. 1995, 1996b, 1997, 2001). However, this approach may not discriminate species from the same genus (Bermingham et al. 1995).

Fluorescent in situ hybridization (FISH) can be an alternative to Mabs. It uses short DNA sequences provided with a fluorescent tag that are complementary to the *taxon* of interest. This technique was applied with some success to detect, identify and quantified aquatic fungi by epifluorescence microscopy (Baschien et al. 2001, McArthur et al. 2001). However, the autofluorescence of hyphae and colonized substrates, and the weak probe-conferred signals due to low probe permeabilization may lead to confounding results (Baschien 2003).

The relative intensity of each band (phylotype) in DGGE gels can give semi-quantitative estimates of the relative biomass of each fungal species within the community (Nikolcheva and Bärlocher 2005, Borges et al. 2006). However, caution is needed since in molecular techniques relying in polymerase chain reaction (PCR), like DGGE, bias such as preferential amplification can occur (Reysenbach et al. 1992, Kanagawa 2003). PCR can be limited by inhibitors of the polymerase reaction, reagent limitation, or accumulation of pyrophosphate molecules, being the reaction no longer at an exponential rate and so generating more products in some reactions than in others (Ginzinger 2002).

The Real-Time Polimerase Chain Reaction (Real-Time PCR) technique allows measurements of the amount of PCR product when the reaction is still at the exponential phase, by determining a fluorescence signal threshold at which all samples can be compared (Ginzinger 2002). The fractional number of PCR cycles required to generate enough fluorescent signal to reach the threshold (Ct value) is directly proportionate to the amount of starting template (Ginzinger 2002), allowing the correct quantification of species DNA.

In Real-Time PCR, primers, oligonucleotide probes or amplicons labelled with molecules capable of fluorescing are used to monitor the amplicon accumulation (reviewed by Mackay 2004). SYBR Green I dye (Inglis and Kalischuk 2004, Chutinimitkul et al. 2005, Mangold et al. 2005) and Taqman probe (Layton et al. 2006, Zhang et al. 2006, Kennedy et al. 2007) are examples of commonly used Real-Time PCR chemistries. SYBR Green I binds to double stranded DNA, but its specificity is dependent on the specificity of the primers (Bustin and Mueller 2005). Taqman probes hybridize with its complementary target, introducing an additional level of specificity (Bustin and Mueller 2005).

In Real-Time PCR the amount of template in a sample can be described either relatively or absolutely (see Freeman et al. 1999). In relative quantitation the amount of a target sequence is compared to the levels of a reference control, while the absolute quantitation gives the exact number of nucleic acid targets in relation to a specific unit (Freeman et al. 1999).

Since its first documentation in 1993 by Higuchi and collaborators, Real-Time PCR has been widely applied. In soil ecology, it allowed the quantification of ammonia-oxidizing bacterial populations (Okano et al. 2004) or the study of competitive interactions between two ectomycorrhizal species, *Rhizopogon occidentalis* and *R. salebrosus*, on *Pinus muricata* seedlings (Kennedy et al. 2007). Real-Time PCR was also applied in aquatic ecosystems to assess the abundance of populations of *Pseudoalteromonas* (Skovhus et al. 2004) or of harmful algal (Coyne

et al. 2005). Therefore, Real-Time PCR is a promising technique to analyse diversity and activity of aquatic hyphomycetes on decomposing leaves, and to provide a better understanding on the contribution of each fungal species to the overall process. This may give further insights on the relationships between fungal biodiversity and leaf litter decomposition in freshwater ecosystems.

1.6. Aim and outline of the thesis

To better understand the relationships between biodiversity and ecosystem functions, the effects of fungal species number and traits on leaf litter decomposition in streams were investigated in the absence or presence of Cd.

Chapter 1 provides an overview on the role of biodiversity in ecosystem processes, focusing on plant-litter decomposition in streams and the associated biota. The impacts of anthropogenic activities, as metal pollution, in plant-litter decomposition and fungal diversity are also addressed. The methodologies used to study the diversity and activity of leaf-associated fungal communities are also described.

In Chapter 2 we assessed the sensitivity of aquatic hyphomycetes to Cd by using three different fungal species and two strains of the same species isolated from clean and metal-polluted streams. The ergosterol content in each fungus was quantified in the absence and presence of the metal to establish ergosterol-to-biomass conversion factors. Also, DNA from pure cultures of each fungus was quantified by Real-Time PCR to establish standard curves and further estimate the biomass of each species when fungi were grown together. In chapter 3, a microcosm experiment was carried out to examine the effects of species diversity, identity and Cd exposure on leaf-litter decomposition. We inoculated alder leaves with three different fungal species and two strains of the same species with different backgrounds (clean *versus* metal-polluted sites), either alone or in all possible combinations of three species. The following functional parameters were examined: mass loss, fungal biomass (from both ergosterol and DNA content) and sporulation.

In chapter 4 the main conclusions are presented to provide a global perspective of the work and possible lines for future research.

References

Abdel-Raheem AM (1997) Colonization pattern of aquatic hyphomycetes on leaf packs in subtropical stream. *Mycopathologia*, 138:163-171.

Abel TH, Bärlocher F (1984) Effects of cadmium on aquatic hyphomycetes. *Appl Environ Microbiol*, 48:245-251.

Azevedo M-M (2007) *Toxicity of metals in aquatic hyphomycetes: cellular targets and defense mechanisms*. PhD thesis, University of Minho, Braga, Portugal.

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

Baldy V, Gessner MO, Chauvet E (1995) Bacteria, fungi and the breakdown of leaf litter in a large river. *Oikos*, 74:93-102.

Barajas-Aceves M, Hassan M, Tinoco R, Vazquez-Duhalt R (2002) Effect of pollutants on the ergosterol content as indicator of fungal biomass. *J Microbiol Methods*, 50:227-236.

Bärlocher F (1997) Pitfalls of traditional techniques when studying decomposition of vascular plant remains in aquatic habitats. *Limnetica*, 13:1-11.

Bärlocher F (2000) Water-borne conidia of aquatic hyphomycetes: seasonal and yearly patterns in Catamaran Brook, New Brunswick, Canada. *Can J Bot*, 78:157-167.

Bärlocher F (2005) Freshwater fungal communities. In, Dighton J, Oudemans P, White J (eds) *The fungal community*. 3rd ed, CRC Press, Boca Raton.

Bärlocher F (ed) (1992) *The ecology of aquatic hyphomycetes*. Springer-Verlag, Berlin, Germany.

Bärlocher F, Corkum M (2003) Nutrient enrichment overwhelms diversity effects in leaf decomposition by stream fungi. *Oikos*, 101:247-252.

Baschien C (2003) *Development an evaluation of rRNA targeted in situ probes and phylogenetic relationships of freshwater fungi*. PhD thesis, Technischen Universität Berlin, Berlin.

Baschien C, Manz W, Neu TR, Szwezyk U (2001) Fluorescence in situ hybridization of freshwater fungi. *Int Rev Hydrobiol*, 86:371-381.

Benfield EF (1996) Leaf breakdown in stream ecosystems. In, Hauer FR, Lamberti GA (eds) *Methods in stream ecology*. Academic Press, San Diego, California, 579-589.

Bermingham S, Dewey FM, Fisher PJ, Maltby L (2001) Use of a monoclonal antibody-based immunoassay for the detection and quantification of *Heliscus lugdunensis* colonizing alder leaves and roots. *Microb Ecol*, 42:506-512.

Bermingham S, Dewey FM, Maltby L (1995) Development of a monoclonal antibody-based immunoassay for the detection and quantification of *Anguillospora longissima* colonizing leaf material. *Appl Environ Microbiol*, 61:2606-2613.

Bermingham S, Maltby L, Cooke RC (1996a) Effects of a coal mine effluent on aquatic hyphomycetes. I. Field Study. *J Appl Ecol*, 33:1311-1321.

Bermingham S, Maltby L, Dewey FM (1996b) Monoclonal antibodies as tools to quantify mycelium of aquatic hyphomycetes. *New Phytol*, 132:593-601.

Bermingham S, Maltby L, Dewey FM (1997) Use of immunoassays for the study of natural assemblages of aquatic hyphomycetes. *Microb Ecol*, 33:223-229.

Borges F, Duarte S, Pascoal C, Cássio F (2006) Assessment of fungal biomass based on DGGE band intensities. *11th International Symposium on Microbial Ecology*, Vienna, Austria.

Braha B, Tintemann H, Krauss G, Ehrman J, Bärlocher F, Krauss G-J (2007) Stress response in two strains of the aquatic hyphomycete *Heliscus lugdunensis* after exposure to cadmium and copper ions. *BioMetals*, 20:93-105.

Bustin SA, Mueller R (2005) Real-time reverse transcription PCR (qRT-PCR) and its potential use in clinical diagnosis. *Clin Sci*, 109:365-379.

Callaway RM, Brooker RW, Choler P, Kikvidze Z, Lortiek CJ, Michalet R, Paolini L, Pugnaire FI, Newingham B, Aschehoug ET, Armas C, Kikodze D, Cook BJ (2002) Positive interactions among alpine plants increase with stress. *Nature*, 417:844-848.

Cardinale BJ, Nelson K, Palmer MA (2000) Linking species diversity to the functioning ecosystems: on the importance of environmental context. *Oikos*, 91:175-183.

Chapin III FS, Zavaleta ES, Eviner VT, Naylor RL, Vitousek PM, Reynolds HL, Hooper DU, Lavorel S, Sala OE, Hobbie SE, Mack MC, Díaz S (2000) Consequences of changing biodiversity. *Nature*, 405:234-242.

Charcosset J-Y, Chauvet E (2001) Effect of culture conditions on ergosterol as an indicator of biomass in the aquatic hyphomycetes. *Appl Environ Microbiol*, 67:2051-2055.

Chauvet E (1992) Dynamique saisonnière des spores d'hyphomycètes aquatiques de quatre rivières. *Nova Hedwigia*, 54:379-395.

Chauvet E, Giani N, Gessner MO (1993) Breakdown and invertebrate colonization of leaf litter in two contrasting streams: significance of oligochaetes in a large river. *Can J Fish Aquat Sci*, 50:488-495.

Chutinimitkul S, Payungporn S, Theamboonlers A, Poovorawan Y (2005) Dengue typing assay based on real-time PCR using SYBR Green I. *J Virol Methods*, 129:8-15.

Coyne KJ, Handy SM, Demir E, Whereat EB, Hutchins DA, Portune KJ, Doblin MA, Cary SC (2005) Improved quantitative real-time PCR assays for enumeration

of harmful algal species in field samples using an exogenous DNA reference standard. *Limnol Oceanogr: Methods*, 3:381-391.

Cummins KW (1974) Structure and function of stream ecosystems. *BioScience*, 24:61-64.

Dang CK, Chauvet E, Gessner MO (2005) Magnitude and variability of process rates in fungal diversity-litter decomposition relationships. *Ecol Lett*, 8:1129-1137.

Dangles O, Gessner MO, Guerold F, Chauvet E (2004) Impacts of stream acidification on litter breakdown: implications for assessing ecosystem functioning. *J Appl Ecol*, 41:365-378.

Das M, Royer TV, Leff LG (2007) Diversity of fungi, bacteria, and actinomycetes on leaves decomposing in a stream. *Appl Environ Microbiol*, 73:756-767.

Doak DF, Bigger D, Harding EK, Marvier MA, O'Malley RE, Thomson D (1998) The statistical inevitability of stability-diversity relationships in community ecology. *Am Nat*, 151:264-276.

Duarte S, Pascoal C, Alves A, Correia A, Cássio F (2008) Copper and zinc mixtures induce shifts in microbial communities and reduce leaf litter decomposition in streams. *Freshw Biol*, 53:91-101.

Duarte S, Pascoal C, Cássio F (2004) Effects of zinc on leaf decomposition by fungi in streams: studies in microcosms. *Microb Ecol*, 48:366-374.

Duarte S, Pascoal C, Cássio F, Bärlocher F (2006) Aquatic hyphomycete diversity and identity affect leaf litter decomposition in microcosms. *Oecologia*, 147:658-666.

Dudgeon D, Arthington AH, Gessner MO, Kawabata Z-I, Knowler DJ, Lévêque C, Naiman RJ, Prieur-Richard A-H, Soto D, Stiassny MLJ, Sullivan CA (2006) Freshwater biodiversity: importance, threats, status and conservation challenges. *Biol Rev*, 81:163-182.

Ehrlich PR, Ehrlich AH (1981) *Extinction. The causes and consequences of the disappearance of species*. Random House, New York.

Freeman WM, Walker SJ, Vrana KE (1999) Quantitative RT-PCR: pitfalls and potential. *Biotechniques*, 26:112-125.

Gessner MO, Bärlocher F, Chauvet E (2003) Qualitative and quantitative analyses of aquatic hyphomycetes in streams. *Fungal Divers Res Ser*, 10:127-157.

Gessner MO, Chauvet E (1993) Ergosterol-to-biomass conversion factors for aquatic hyphomycetes. *Appl Environ Microbiol*, 59:502-507.

Gessner MO, Chauvet E, Dobson M (1999) A perspective on leaf litter breakdown in streams. *Oikos* 85: 377-384.

Gessner MO, Gulis V, Kuehn KA, Chauvet E, Suberkropp K (2007) Fungal decomposers of plant litter in aquatic ecosystems. In, Kubicek CP, Druzhinina IS (eds) *The Mycota: environmental and microbial relationships*. Vol IV, 2nd ed, Springer, Berlin, 301-321.

Gessner MO, Newell SY (2002) Biomass, growth rate, and production of filamentous fungi in plant litter. In, Hurst CJ, Crawford RL, Knudsen G, McInerney M, Stetzenbach LD (eds) *Manual of environmental microbiology*. 2nd ed, ASM Press, Washington, DC, USA, 390-408.

Ginzinger DG (2002) Gene quantification using real-time quantitative PCR: An emerging technology hits the mainstream. *Exp Hematol*, 30:503-512.

Graça MAS (2001) The role of invertebrates on leaf litter decomposition in streams – a review. *Int Rev Hydrobiol*, 86:383-393.

Guimarães-Soares L (2005) *Biochemical and physiological responses of the aquatic fungi* Fontanospora fusiramosa *and* Flagellospora curta *to cadmium, copper and zinc*. PhD thesis, University of Minho, Braga, Portugal.

Gulis V, Suberkropp K (2003) Leaf litter decomposition and microbial activity in nutrient-enriched and unaltered reaches of a headwater stream. *Freshw Biol*, 48:123-134.

Gulis V, Suberkropp K (2004) Effects of whole-stream nutrient enrichment on the concentration and abundance of aquatic hyphomycete conidia in transport. *Mycologia*, 96:57-65.

Higuchi R, Fockler C, Dollinger G, Watson R (1993) Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Biotechnol*, 11:1026-1030.

Hughes JB, Petchey OL (2001) Merging perspectives on biodiversity and ecosystem functioning. *Trends Ecol Evol*, 16:222-223.

Inglis GD, Kalischuk LD (2004) Direct quantification of *Campylobacter jejuni* and *Campylobacter lanienae* in feces of cattle by real-time quantitative PCR. *Appl Environ Microbiol*, 70:2296-2306.

Jaeckel P, Krauss G-J, Krauss G (2005) Cadmium and zinc response of the fungi *Heliscus lugdunensis* and *Verticillium* cf. *alboatrum* isolated from highly polluted water. *Sci Total Environ*, 346:274-279.

Kanagawa T (2003) Bias and artifacts in multitemplate polymerase chain reactions (PCR). *J Biosci Bioeng*, 96:317-323.

Kennedy PG, Bergemann SE, Hortal S, Bruns TD (2007) Determining the outcome of field-based competition between two *Rhizopogon* species using real-time PCR. *Mol Ecol*, 16:881-890.

Lane TW, Saito MA, George GN, Pickering IJ, Prince RC, Morel FMM (2005) Biochemistry: a cadmium enzyme from a marine diatom. *Nature*, 435:42.

Lawton JH (1994) What do species do in ecosystems? *Oikos*, 71:367-374.

Layton A, McKay L, Williams D, Garrett V, Gentry R, Sayler G (2006) Development of bacteroides 16S rRNA gene TaqMan-based Real-Time PCR assays for estimation of total, human, and bovine fecal pollution in water. *Appl Environ Microbiol*, 72:4214-4224.

Lecerf A, Dobson M, Dang CK, Chauvet E (2005) Riparian plant species loss alters trophic dynamics in detritus-based stream ecosystems. *Oecologia*, 164:432-442.

Loreau M, Hector A (2001) Partitioning selection and complementarity in biodiversity experiments. *Nature*, 412:72-76.

Mackay IM (2004) Real-time PCR in the microbiology laboratory. *Clin Microbiol Infect*, 10:190-212.

Mangold KA, Manson RU, Koay ESC, Stephens L, Regner M, Thomson Jr RB, Peterson LR, Kaul KL (2005) Real-Time PCR for detection and identification of *Plasmodium* spp. *J Clin Microbiol*, 43:2435-2440.

Mathuriau C, Chauvet E (2002) Breakdown of leaf litter in a neotropical stream. *J N Am Benthol Soc*, 21:384-396.

McArthur FA, Baerlocher MO, Maclean NAB, Hiltz MD, Bärlocher F (2001) Asking probing questions: can fluorescent in situ hybridization identify and localise aquatic hyphomycetes on leaf litter. *Int Rev Hydrobiol*, 86:429-438.

Merian E (ed) (1991) *Metals and their compounds in the environment*. VCH-Verlag, Weinheim, Germany.

Michaelsen A, Pinzari F, Ripka K, Lubitz W, Piñar G (2006) Application of molecular techniques for identification of fungal communities colonising paper material. *Int Biodeterior Biodegrad*, 58:133-141.

Miersch J, Bärlocher F, Bruns I, Krauss G-J (1997) Effects of cadmium, copper, and zinc on growth and thiol content of aquatic hyphomycetes. *Hydrobiologia*, 346:77-84.

Miersch J, Grancharov K (2008) Cadmium and heat response of the fungus *Heliscus lugdunensis* isolated from highly polluted and unpolluted areas. *Amino Acids*, 34:271-277.

Mille-Lindblom C, von Wachenfeldt E, Tranvik LJ (2004) Ergosterol as a measure of living fungal biomass: persistence in environmental samples after fungal death. *J Microbiol Methods*, 59:253-262.

Mitra B, Rensing C (2007) Zinc, cadmium and lead resistance and homeostasis. In, Nies DH, Silver S (eds) *Molecular Microbiology of heavy metals*. Springer Verlag, Heidelberg. 321-341.

Mulder CPH, Uliassi DD, Doak DF (2001) Physical stress and diversity-productivity relationships: the role of positive species interactions. *Proc Natl Acad Sci USA*, 98:6704-6708.

Muyzer G, de Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol*, 59:695-700.

Nikolcheva LG, Bärlocher F (2004) Taxon-specific fungal primers reveal unexpectedly high diversity during leaf decomposition in a stream. *Mycol Prog*, 3:41-49.

Nikolcheva LG, Bärlocher F (2005) Seasonal and substrate preferences of fungi colonizing leaves in streams: traditional versus molecular evidence. *Environ Microbiol*, 7:270-280.

Nikolcheva LG, Cockshutt AM, Bärlocher F (2003) Determining diversity of freshwater fungi on decaying leaves: comparison of traditional and molecular approaches. *Appl Environ Microbiol*, 69:2548-2554.

Niyogi DK, Lewis Jr WM, McKnight DM (2001) Litter breakdown in mountain streams affected by mine drainage: biotic mediation of abiotic controls. *Ecol Appl*, 11:506-516.

Niyogi DK, McKnight DM, Lewis Jr WM (2002) Fungal communities and biomass in mountain streams affected by mine drainage. *Arch Hydrobiol*, 155:255-271.

Okano Y, Hristova KR, Leutenegger CM, Jackson LE, Denison RF, Gebreyesus B, Lebauer D, Scow KM (2004) Application of Real-Time PCR to study effects of ammonium on population size of ammonia-oxidizing bacteria in soil. *Appl Environ Microbiol*, 70:1008-1016.

Pascoal C, Cássio F (2004) Contribution of fungi and bacteria to leaf litter decomposition in a polluted river. *Appl Environ Microbiol*, 70:5266-5273.

Pascoal C, Cássio F, Marcotegui A, Sanz B, Gomes P (2005a) Role of fungi, bacteria, and invertebrates in leaf litter breakdown in a polluted river. *J N Am Benthol Soc*, 24:784-797.

Pascoal C, Cássio F, Marvanová L (2005b) Anthropogenic stress may affect aquatic hyphomycete diversity more than leaf decomposition in a low-order stream. *Arch Hydrobiol*, 162:481-496.

Pascoal C, Marvanová L, Cássio F (2005c) Aquatic hyphomycete diversity in streams of Northwest Portugal. *Fungal Divers*, 19:109-128.

Ramos F (2003) *Prospecção de hifomicetos aquáticos no Rio Ave e optimização das condições de crescimento e esporulação de* Heliscus submersus*,* Heliscus lugdunensis *e* Tricladium splendens. MSc thesis, University of Minho, Braga, Portugal.

Rand GM, Wells PG, McCarty LS (1995) Introduction to aquatic toxicology: effects, environmental fate, and risk assessment. In, Rand GM (ed) *Fundamentals of Aquatic Toxicology*. 2nd ed, Taylor and Francis, London, 3-66.

Raviraja NS, Nikolcheva LG, Bärlocher F (2006) Fungal growth and leaf decompostion are affected by amount and type of inoculum and by external nutrients. *Sydowia*, 58:91-104.

Raviraja NS, Sridhar KR, Bärlocher F (1998) Breakdown of *Ficus* and *Eucalyptus* leaves in an organically polluted river in India: fungal diversity and ecological functions. *Freshw Biol*, 39:537-545.

Read SJ, Moss ST, Jones EBG (1992) Attachment and germination of conidia. In, Bärlocher F (ed) *The ecology of aquatic hyphomycetes*. Springer-Verlag, Berlin, Germany, 135-151.

Reysenbach A-L, Giver LJ, Wickham GS, Pace NR (1992) Differential amplification of rRNA genes by Polymerase Chain Reaction. *Appl Environ Microbiol*, 58:3417-3418.

Ricciardi A, Rasmussen JB (1999) Extinction rates of North American freshwater fauna. *Conserv Biol*, 13:220-222.

Robinson CT, Gessner MO (2000) Nutrient addition accelerates leaf breakdown in an alpine springbrook. *Oecologia*, 122:258-263.

Rodrigues A (2002) *Efeito do zinco, cobre, níquel e cádmio no crescimento e na esporulação de hifomicetos aquáticos* Tricladium splendens *e* Heliscus submersus. Graduation thesis, University of Minho, Braga, Portugal.

Sampaio A, Cortes R, Leão C (2001) Invertebrate and microbial colonisation in native and exotic leaf litter species in a mountain stream. *Int Rev Hydrobiol*, 86:527-540.

Shearer CA, Webster J (1985) Aquatic hyphomycete communities in the River Teign. I. Longitudinal distribution patterns. *Trans Br Mycol Soc*, 84:489-501.

Skovhus TL, Ramsing NB, Holmström C, Kjelleberg S, Dahllöf I (2004) Real-Time quantitative PCR for assessment of abundance of *Pseudoalteromonas* species in marine samples. *Appl Environ Microbiol*, 70:2373-2382.

Sridhar KR, Krauss G, Bärlocher F, Raviraja NS, Wennrich R, Baumbach R, Krauss G-J (2001) Decompostion of alder leaves in two heavy metal–polluted streams in central Germany. *Aquat Microb Ecol*, 26:73-80.

Suberkropp K (1998) Microorganisms and organic matter decomposition. In, Naiman RJ, Bilby RE (eds) *River ecology and management: lessons from the Pacific Coastal Ecoregion*. Springer, Berlin Heidelberg New York, 120-143.

Suberkropp K, Gessner MO (2005) Acetate incorporation into ergosterol to determine fungal growth rates and production. In, Graça MAS, Bärlocher F, Gessner MO (eds) *Methods to study litter decomposition: a practical guide*. Springer, Dordrecht, The Netherlands, 197-202.

Suberkropp K, Weyers H (1996) Application of fungal and bacterial production methodologies to decomposing leaves in streams. *Appl Environ Microbiol*, 62:1610-1615.

Treton C, Chauvet E, Charcosset J-Y (2004) Competitive interaction between two aquatic hyphomycete species and increase in leaf litter breakdown. *Microb Ecol*, 48:439-446.

Vitousek PM, Hooper DU (1993) Biological diversity and terrestrial ecosystem biogeochemistry. In, Schulze E-D, Mooney HA (eds) *Biodiversity and Ecosystem Function*. Springer-Verlag, Berlin. 3-14.

Walker BH (1992) Biodiversity and ecological redundancy. *Conserv Biol*, 6:18-23.

Wallace JB, Grubaugh JW, Whiles MR (1996) Biotic indices and stream ecosystem processes: results from an experimental study. *Ecol Appl*, 61:140-151.

Webster J (1959) Experiments with spores of aquatic hyphomycetes. I. Sedimentation, and impaction on smooth surfaces. *Ann Bot*, 23:595-611.

Webster J, Davey RA (1984) Sigmoid conidial shape in aquatic fungi. *Trans Br Mycol Soc*, 83:43-52.

Webster JR, Benfield EF (1986) Vascular plant breakdown in freshwater ecosystems. *Annu Rev Ecol Syst*, 17:567-594.

Zhang L, Liu X, Zhu S, Chen S (2006) Detection of the nematophagous fungus *Hirsutella rhossiliensis* in soil by real-time PCR and parasitism bioassay. *Biol Control*, 36:316-323.

Chapter 2

Fungal biomass assessed from ergosterol and DNA contents

2.1. Introduction

Aquatic hyphomycetes are a phylogenetically heterogeneous group of filamentous fungi that play a key role in plant litter decomposition in freshwater ecosystems (Bärlocher 1992, Gessner et al. 2003). These fungi enhance leaf palatability for shredding invertebrates (Graça 2001) by producing extracellular enzymes that attack structural plant polysaccharides (Suberkropp 1998).

Several studies have shown that aquatic hyphomycetes are distributed worldwide (Bärlocher 1992) and its occurrence is reported in clean and polluted streams (Pascoal et al. 2005b). In streams polluted by metals, a decrease in aquatic hyphomycete diversity and activity has been observed (Sridhar et al. 2001, Niyogi et al. 2001, 2002). Several studies demonstrated that, above a certain threshold, both essential and non-essential metals can negatively affect the growth of aquatic hyphomycetes (Miersch et al. 1997, Jaeckel et al. 2005, Azevedo et al. 2007, Braha et al. 2007, Guimarães-Soares et al. 2007, Miersch and Grancharov 2008).

In streams, the role of fungi in leaf litter decomposition has been assessed by measuring fungal biomass (Gessner and Chauvet 1993), productivity (Weyers and Suberkropp 1996) and sporulation (Pascoal et al. 2005a). Using these approaches, it was found that peaks of fungal biomass and sporulation correlate with rates of leaf litter decomposition (Maharning and Bärlocher 1996). During leaf litter decomposition, fungal hyphae penetrate into substrates making it difficult to estimate microbial biomass by the usual methods. Ergosterol is a constituent of fungal membranes that is absent in vascular plants, metazoan animals and most other organisms, making it suitable for quantifying fungal biomass (see Gessner and Newell 2002). In addition, ergosterol suffers rapid oxidation after cell death being a measure of living fungal biomass (Newell 1992). However, ergosterol quantification cannot discriminate the biomass of individual fungal species within communities, making it difficult to assess the contribution of each species to overall community performance. Moreover, several authors point out that ergosterol content may change with fungal species (Gessner and Chauvet 1993), its nutritional status (Charcosset and Chauvet 2001), growth phase (Barajas-Aceves et al. 2002) and/or the presence of stressors (e.g. fungicide zineb, Barajas-Aceves et al. 2002).

Real-Time Polymerase Chain Reaction (Real-Time PCR) has been used for detection and quantification of organisms at different taxonomic levels (species level, Galluzzi et al. 2004, Kennedy et al. 2007; domain level, Manerkar et al. 2008). Because PCR products can be measured while the reaction is still at the exponential phase they are directly proportionate to the amount of starting template (Ginzinger

2002), allowing its correct quantification. This technique was able to discriminate the role of fungi, bacteria and archaea in leaf litter decomposition in streams (Manerkar et al. 2008).

In this work, four aquatic hyphomycetes isolated from clean and metal-polluted streams were used to assess fungal biomass as ergosterol and DNA contents. Because ergosterol content varies with fungal species and environmental conditions, the ergosterol concentration in each fungus was quantified in the absence and presence of cadmium (Cd) to estimate ergosterol-to-biomass conversion factors. In addition, DNA from pure cultures of each fungal isolate was quantified by Real-Time PCR to establish standard curves to further estimate fungal biomasses of each species when fungi were grown together on leaves (Chapter 3). Moreover, fungal sensitivity to Cd was evaluated.

2.2. Materials and methods

2.2.1. Fungal isolates

Four aquatic hyphomycete isolates were used in this work: *Articulospora tetracladia* Ingold (UMB-61.01 and UMB-72.01), *Flagellospora curta* J. Webster (UMB-39.01) and *Tricladium splendens* Ingold (UMB-100.01), from now on called AT61, AT72, FC39 and TS100, respectively. Fungi were isolated from streams of Northwest Portugal. The strain AT72 was isolated from foams in the Maceira River, a non-polluted stream at the Peneda-Gerês National Park. All the other fungi were isolated from decomposing leaves in the Este River, near the town of Braga, at a site polluted by metals and eutrophication (Soares et al. 1999, Gonçalves 2001, Pascoal et al. 2005a).

2.2.2. Fungal sensitivity to cadmium in solid medium

The aquatic hyphomycetes were grown on 2% malt extract agar without or with Cd at the following concentrations: 5, 10, 20, 30 and 60 mg L^{-1} (added as $CdCl_2$, Sigma). Solid medium was inoculated with a 6-mm-diameter plug of mycelium taken from the edge of a colony of each fungus and incubation occurred at room temperature. Radial growth was measured every two or three days, during 19 days. Six replicates per treatment were done.

2.2.3. Biomass quantification in liquid medium

The aquatic hyphomycetes were grown in 250-mL Erlenmeyer flasks with 50 mL of 1% malt extract without or with 1.5 mg L^{-1} of Cd. The flasks were inoculated with a 6-mm-diameter plug of mycelium taken from the edge of a colony of the fungus and the culture incubated at 16 ºC and 140 rpm. After 5 days, the plugs were removed. Sets of 3 replicates were harvested through filtration (0.45-µm pore size, Millipore) and washed with deionized water, at each sampling time, except for Cd treatment, in which mycelium was harvested after 23 days. All samples were lyophilized and frozen at -80ºC until used.

2.2.4. Ergosterol quantification in fungal mycelium

Fungal biomass collected from each culture in liquid medium (section 2.2.3) was used to determine the ergosterol concentration in each isolate, according to Gessner (2005). Lipids were extracted from ca. 5 mg of fungal culture by heating (80ºC, 30 min) in 0.8% of KOH/methanol, purified by solid-phase extraction and quantified by high performance liquid chromatography.

2.2.5. DNA quantification by Real-Time PCR

For sequencing, DNA was extracted from 6-mm-diameter agar plug, collected from the edge of 18-days-old colonies of the four fungi, using the UltraClean Soil DNA kit (MoBio Laboratories, Solana Beach, CA, USA). The ITS2 region of fungal rDNA was amplified with the primer pair ITS3 and ITS4 (Table 1). For PCR reactions 0.5x of Taq buffer (KCl : $(NH_4)_2SO_4$), 3 mM of $MgCl_2$, 0.2 mM of dNTPs, 0.4 µM of each primer, 1.5 U of DNA Taq polymerase and 1 µL of DNA were used in a final volume of 50 µL. Fungal DNA amplification started with a denaturation of 2 min at 95 ºC, followed by 36 cycles of denaturation for 30 s at 95 ºC, primer annealing for 30 s at 55 ºC and extension for 1 min at 72 ºC. Final extension was at 72 ºC for 5 min (Nikolcheva et al. 2005). PCR was carried out in an iCycler Thermal Cycler (BioRad Laboratories, Hercules, CA, USA). All PCR reagents were from MBI Fermentas except primers that were from Bonsai Technologies.

PCR products were purified with GenElute™ PCR Clean-Up Kit (Sigma) according to the manufacturer instructions and sequenced at the Centro de Biologia Molecular e Ambiental, University of Minho, using an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems).

Table 2.1. Primer pairs used for PCR.

Primer	Sequence (5'-3')	Amplified region	Reference
ITS3F	GCA TCG ATG AAG AAC GCA GC	ITS2	White *et al*. 1990
ITS4R	TCC TCC GCT TAT TGA TAT GC		

The sequenced ITS2 rDNA region of each fungal isolate was used to design species-specific primer pairs, for further use in Real-Time PCR assays, using Primer Express 2.0 software (Applied Biosystems) (Table 2.2). DNA was isolated from fungal cultures grown in liquid medium during 20 days with the UltraClean Soil DNA kit (MoBio Laboratories, Solana Beach, CA, USA). Each 10 µL reaction contained 1 µL of sample DNA, 5 µL of 2x LightCycler® 480 SYBR Green I Master mix (Roche), each primer at 250 nM and the required amount of nuclease-free water. DNA amplification started with initial denaturation at 95 ºC for 10 min, followed by 45 cycles of denaturation at 95 ºC for 10 s, primer hybridization at 60 °C during 20 s and extension at 72 ºC during 15 s.

Table 2.2. Primer pairs used for Real-Time PCR. AT72, *Articulospora tetracladia* (UMB-72.01); AT61, *Articulospora tetracladia* (UMB-61.01); FC39, *Flagellospora curta* (UMB-39.01); TS100, *Tricladium splendens* (UMB-100.01).

Species	Primer pairs (5'-3')	
AT72 and AT61	Forward	AGCTCCCGCTTGGTATTGG
	Reverse	CACTGATTTAGAGGCTGCGAAA
FC39	Forward	AAATTTAGTGGCGGCCCG
	Reverse	CGAGGTTAAGTTACTACGCAGAGGTC
TS100	Forward	TGGCGGTACGGCCG
	Reverse	GGGACCCTGTAGCGAGAAGATT

Independent regressions between known DNA amounts and Ct values (cycle number at which the fluorescence value exceeds the background fluorescence and crosses the threshold) were done for each fungal isolate (standard curves). Standard samples were diluted at tenfold intervals from 10 ng to 1 pg. Duplicates of all dilutions were done and negative controls were performed by testing all primers against all isolates. Real-Time PCR was done in a LightCycler®480 System (Roche) at the Unidad de Genómica "Toñi Martín Gallardo", Parque Científico de Madrid, Spain.

2.2.6. Data analyses

The rates of radial growth for each fungal isolate were estimated by linear regression of radial growth versus the incubation time. The effect of Cd concentration on radial growth rates was analysed by ANCOVA (Zar 1996).

Changes in the ratio ergosterol/biomass over incubation time were analyzed by linear regression. The differences in ergosterol content between the four fungal isolates were compared by one-way ANOVA (Zar 1996). The effect of Cd exposure in the ergosterol content of each fungus was compared by a t-test (Zar 1996).

Linear regressions were done to establish the standard curves between log (DNA amount) and Ct values. ANCOVA were performed to assess the differences of standard curves between isolates.

Statistical analyses were done with Prism 4.0 for Windows (GraphPad software Inc., San Diego).

2.3. Results

2.3.1. Fungal sensitivity to cadmium

A linear relationship between radial growth and incubation time either in the absence or presence of Cd was found for the four aquatic hyphomycete isolates (Fig. 2.1). Radial growth rates of the aquatic hyphomycetes in the absence of Cd ranged from 0.945 to 1.053 mm d^{-1} for *Flagellospora curta* (FC39) and *Articulospora tetracladia* isolated from the contaminated site (AT61), respectively (Table 2.3). The exposure to the highest Cd concentration (60 mg L^{-1}) led to a decline of about 90% in growth rates of *A. tetracladia* from the clean site (AT72) and *Tricladium splendens* (TS100), while a reduction of 25-30% was observed for the other two isolates (Fig. 2.2). The lowest observed effect concentration (LOEC) of Cd was 5 mg L^{-1} for all fungi (ANCOVA, $p < 0.05$).

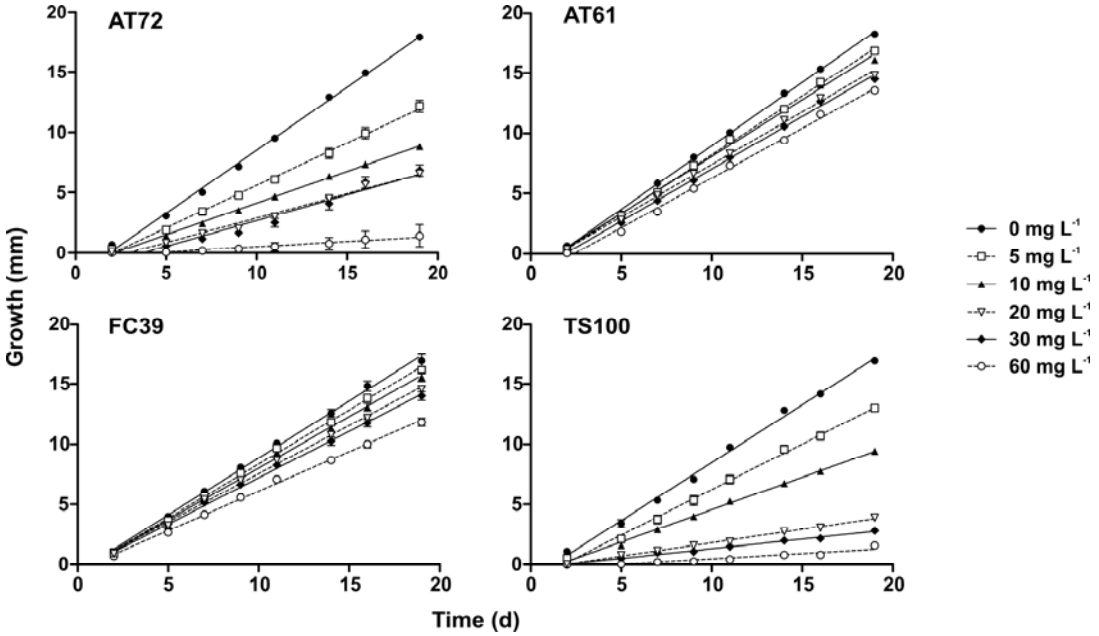

Figure 2.1. Radial growth (mm) of aquatic hyphomycetes grown on malt extract agar 2% (w/v) without or with Cd during 19 days. AT72, *Articulospora tetracladia* (UMB-72.01); AT61, *Articulospora tetracladia* (UMB-61.01); FC39, *Flagellospora curta* (UMB-39.01); TS100, *Tricladium splendens* (UMB-100.01); M ± SEM; n = 6.

Table 2.3. Radial growth rates (mm d^{-1}) of aquatic hyphomycetes grown on malt extract agar 2% (w/v) without or with Cd. AT72, *Articulospora tetracladia* (UMB-72.01); AT61, *Articulospora tetracladia* (UMB-61.01); FC39, *Flagellospora curta* (UMB-39.01); TS100, *Tricladium splendens* (UMB-100.01).

Cadmium (mg L^{-1})	Radial growth rates (mm d^{-1})			
	AT72	AT61	FC39	TS100
0	1.045 (r^2 = 0.99)	1.053 (r^2 = 1.00)	0.945 (r^2 = 0.98)	0.961 (r^2 = 0.99)
5	0.705 (r^2 = 0.96)	0.979 (r^2 = 0.99)	0.898 (r^2 = 0.99)	0.756 (r^2 = 0.97)
10	0.526 (r^2 = 0.97)	0.944 (r^2 = 0.99)	0.858 (r^2 = 0.99)	0.537 (r^2 = 0.99)
20	0.405 (r^2 = 0.96)	0.872 (r^2 = 0.99)	0.800 (r^2 = 0.99)	0.222 (r^2 = 0.96)
30	0.427 (r^2 = 0.87)	0.869 (r^2 = 0.99)	0.770 (r^2 = 0.98)	0.160 (r^2 = 0.96)
60	0.087 (r^2 = 0.16)	0.826 (r^2 = 0.99)	0.655 (r^2 = 0.98)	0.088 (r^2 = 0.71)

r^2 – Coefficient of determination

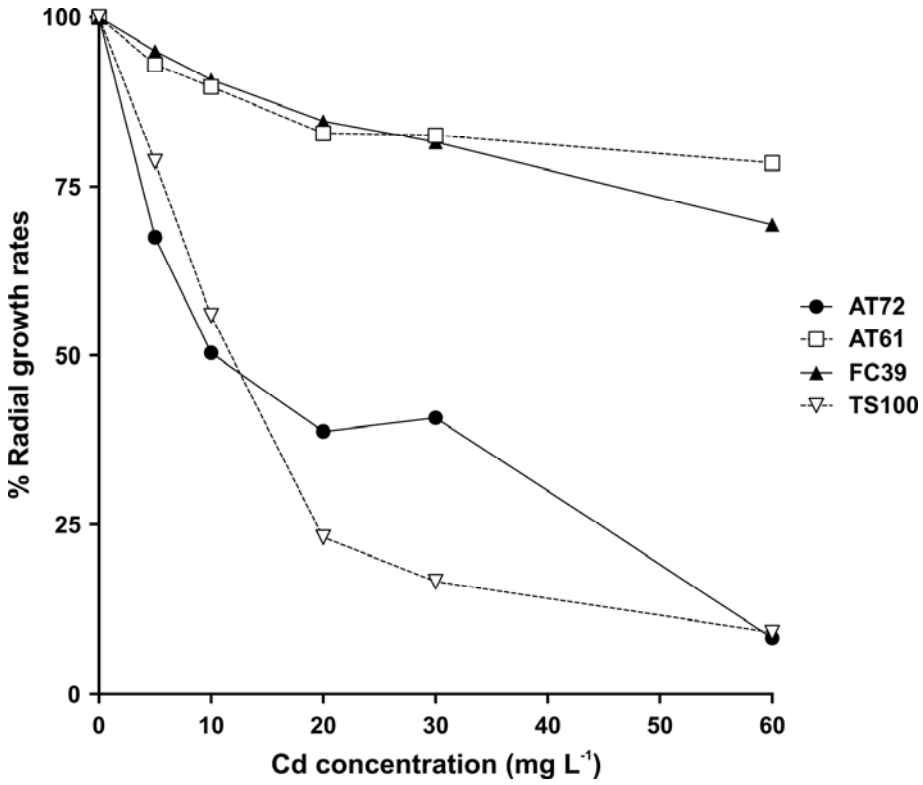

Figure 2.2. Percentage of radial growth rates of aquatic hyphomycetes grown on malt extract agar 2% (w/v) without or with Cd addition. AT72, *Articulospora tetracladia* (UMB-72.01); AT61, *Articulospora tetracladia* (UMB-61.01); FC39, *Flagellospora curta* (UMB-39.01); TS100, *Tricladium splendens* (UMB-100.01).

2.3.2. Biomass quantification

All aquatic hyphomycete isolates showed a similar pattern of growth in liquid medium, except *F. curta* (FC39), which started to grow earlier and attained higher biomass than all the others (Fig. 2.3). Maximum fungal biomass varied from 35 to 55 mg dry mass for the strain AT72 of *A. tetracladia* and for *F. curta*, respectively.

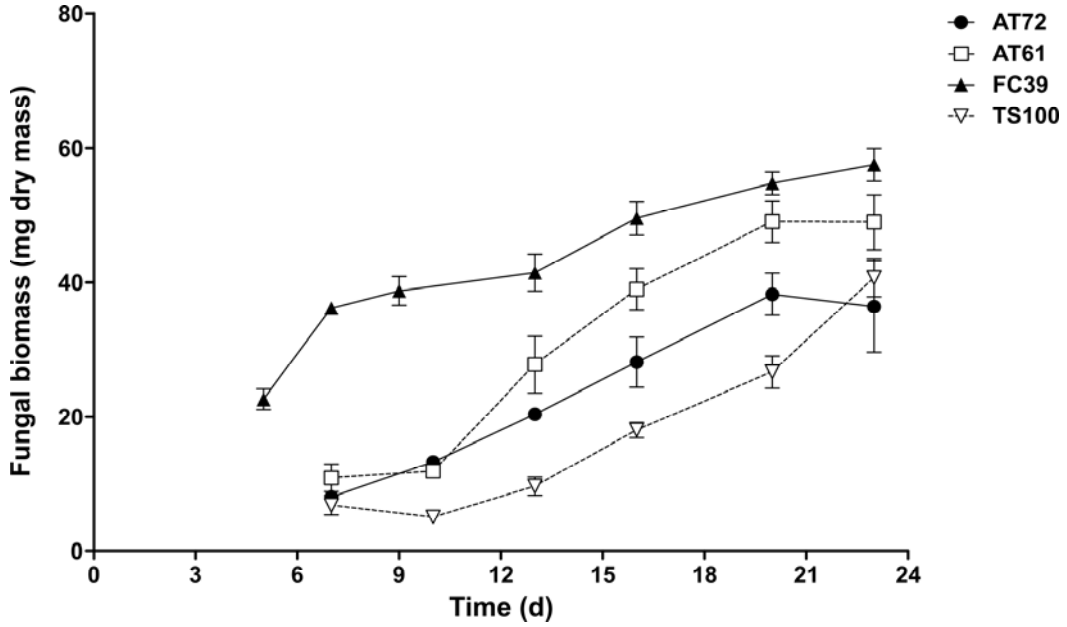

Figure 2.3. Growth curves of four aquatic hyphomycetes in malt extract 1% (w/v) at 16 ºC under shaking. AT72, *Articulospora tetracladia* (UMB-72.01); AT61, *Articulospora tetracladia* (UMB-61.01); FC39, *Flagellospora curta* (UMB-39.01); TS100, *Tricladium splendens* (UMB-100.01); M ± SEM; n = 3.

2.3.3. Ergosterol quantification

Ergosterol concentration was determined in the fungal biomass collected from liquid cultures along time (Fig. 2.4). A significant increase in ergosterol concentration per unit of fungal dry mass was observed with time for the two strains of *A. tetracladia* (AT72 and AT61) and for *T. splendens* (TS100) (linear regression, $p < 0.05$; Fig. 2.4), but not for *F. curta* (FC39). Based on these data, mean values for ergosterol-to-biomass conversion factors were 3.1, 3.4, 3.7 and 10.3 µg ergosterol mg^{-1} fungal dry mass for TS100, AT72, AT61 and FC39, respectively (Table 2.4). The ergosterol content was significantly higher in *F. curta* than in the other isolates (one-way ANOVA, $p < 0.05$, Table 2.4).

The presence of 1.5 mg L^{-1} of Cd in the growth medium did not change the ergosterol content in *F. curta* and in one of the strains of *A. tetracladia* (AT61) (t-test, $p > 0.05$; Fig. 2.5). The other strain of *A. tetracladia* (AT72) and *T. splendens* did not grow in liquid medium with 1.5 mg L^{-1} of Cd.

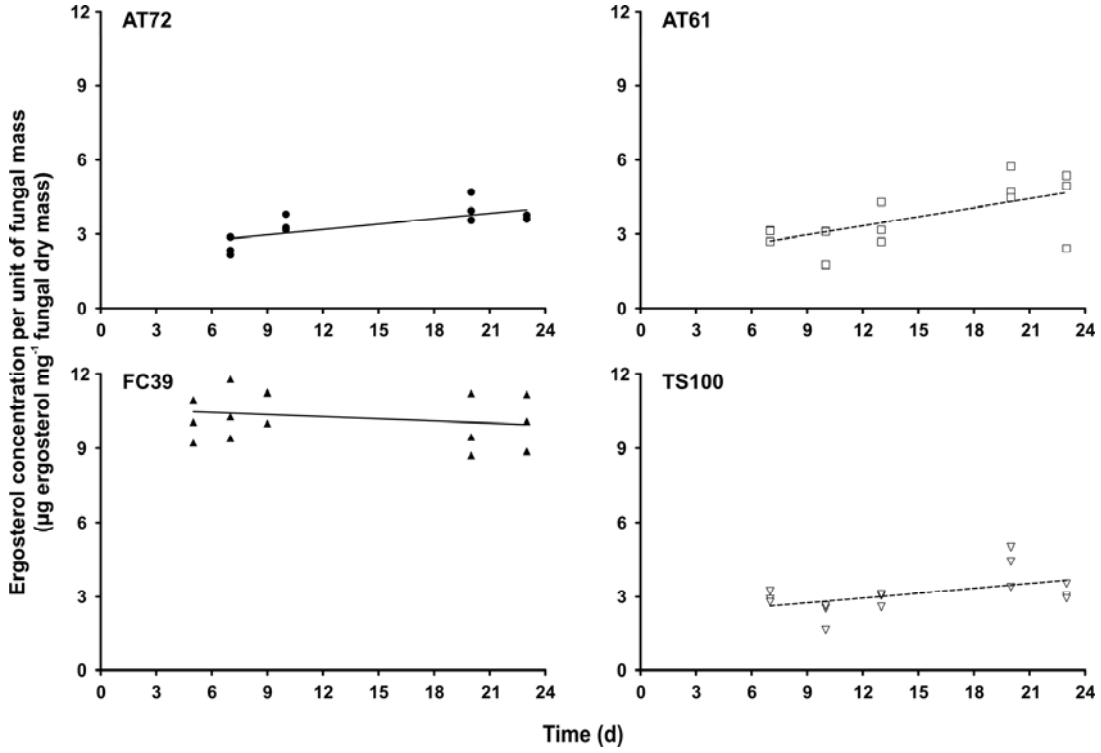

Figure 2.4. Ergosterol content in the four aquatic hyphomycetes grown in malt extract 1% (w/v). AT72, *Articulospora tetracladia* (UMB-72.01); AT61, *Articulospora tetracladia* (UMB-61.01); FC39, *Flagellospora curta* (UMB-39.01); TS100, *Tricladium splendens* (UMB-100.01).

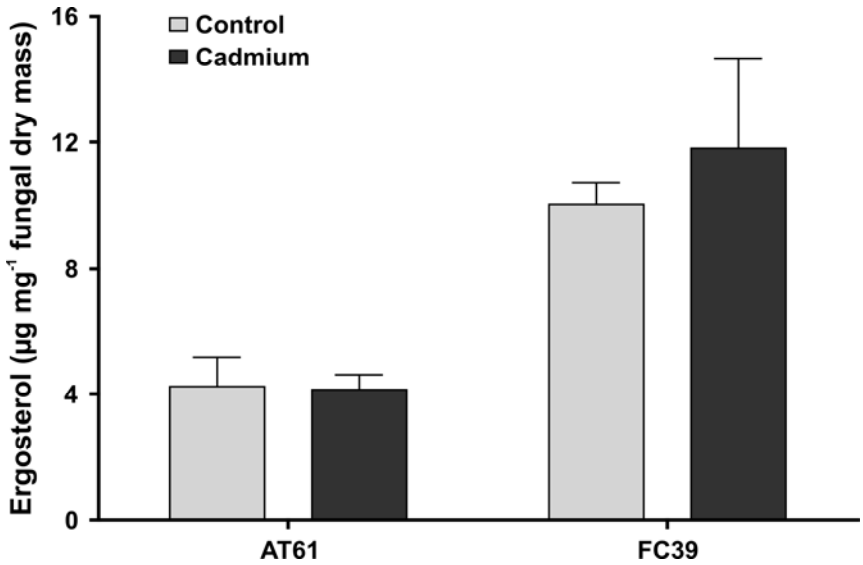

Figure 2.5. Ergosterol content in *Articulospora tetracladia* (AT61) and *Flagellospora curta* (FC39) grown 23 days in malt extract 1% without or with 1.5 mg L^{-1} of Cd.

Table 2.4. Ergosterol-to-biomass conversion factors for aquatic hyphomycetes grown in malt extract 1% (w/v). AT72, *Articulospora tetracladia* (UMB-72.01); AT61, *Articulospora tetracladia* (UMB-61.01); FC39, *Flagellospora curta* (UMB-39.01); TS100, *Tricladium splendens* (UMB-100.01). M ± SEM, n = 15. (one-way ANOVA; *, $p < 0.05$).

Fungal species	Ergosterol conversion factor ($\mu g\ mg^{-1}$ fungal dry mass)
AT72	3.4 ± 0.3
AT61	3.7 ± 0.4
FC39	10.3 ± 0.2*
TS100	3.1 ± 0.3

2.3.4. DNA quantification by Real-Time PCR

Slopes of the standard curves of DNA amount differed between isolates (ANCOVA, $p < 0.05$), except for AT61 *versus* FC39 and AT61 *versus* AT72 (Fig. 2.6; Table 2.5). For the isolates whose slopes did not differ, different intercept values were observed ($p < 0.05$). The coefficient of determination of all fits was > 0.99 (Table 2.5). Higher intercepts of the standard curves were observed for the species *A. tetracladia* (AT72 and AT61 strains) comparing to *F. curta* (FC39) and *T. splendens* (TS100) (Table 2.5). PCR efficiencies ranged from 1.759 to 1.983 for AT61 and TS100, respectively (Table 2.5). No fluorescence of non-target template was observed in single-species treatments (data not shown).

Table 2.5. Standard curves assessed by Real-Time PCR for the four aquatic hyphomycetes. Slope was calculated from Ct values at each of the five tenfold dilutions (10 ng to 1 pg). PCR efficiency was calculated as 10^(-1/[slope]). AT72, *Articulospora tetracladia* (UMB-72.01); AT61, *Articulospora tetracladia* (UMB-61.01); FC39, *Flagellospora curta* (UMB-39.01); TS100, *Tricladium splendens* (UMB-100.01).

Species	Slope	Intercept	r^2	PCR efficiency
AT72	-3.796[a]	34.16	0.997	1.841
AT61	-3.643[a,b]	32.70	0.991	1.759
FC39	-3.473[b]	29.54	0.999	1.935
TS100	-3.087[c]	27.35	0.996	1.983

Similar letters indicate no significant differences between slopes (ANCOVA, $p > 0.05$).

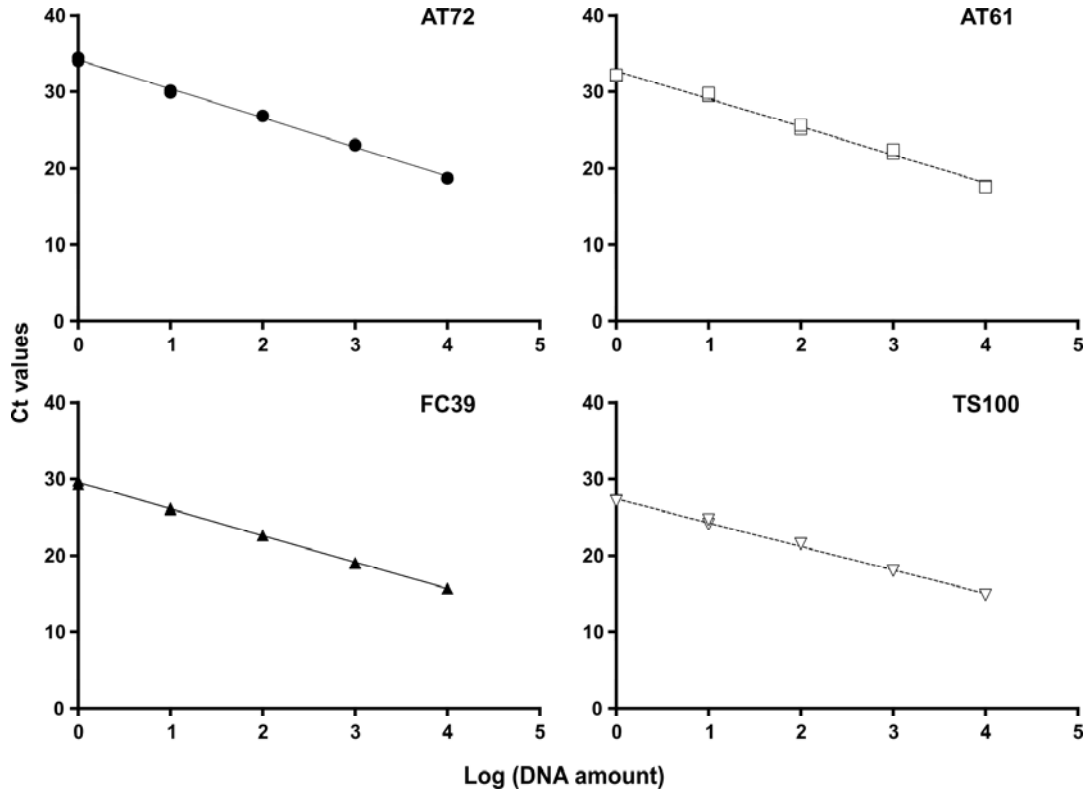

Figure 2.6. Standard curves assessed by Real-Time PCR for the four aquatic hyphomycetes. AT72, *Articulospora tetracladia* (UMB-72.01); AT61, *Articulospora tetracladia* (UMB-61.01); FC39, *Flagellospora curta* (UMB-39.01); TS100, *Tricladium splendens* (UMB-100.01).

2.4. Discussion

In the absence of Cd, radial growth rates were similar for all fungi. However, in the presence of Cd, *Articulospora tetracladia* isolated from the clean site (AT72) and *Tricladium splendens* (TS100) showed ca 3- to 3.6-times higher growth rates inhibitions than *Flagellospora curta* (FC39) and the strain of *A. tetracladia* isolated from the polluted site (AT61). The higher sensitivity of *A. tetracladia* AT72 than *A. tetracladia* AT61 to Cd might be related to the fact that the first strain was isolated from a reference site while the second one was isolated from a metal-polluted site (0.06 mg L^{-1}, maximum Cd concentration in the water column and 0.14 g kg^{-1} of Cd in the < 0.63-µm fraction of the sediments, Gonçalves 2001 and Soares et al. 1999, respectively). This suggests that populations adapted to metal-polluted environments may be able to tolerate higher metal concentrations. However, *T. splendens* isolated from the same metal-polluted stream did not show high Cd tolerance. It was observed that tolerance to metals can vary with fungal species and metal type (Azevedo 2007). Our results are in agreement with those reporting that,

besides species-specific, metal tolerance in aquatic hyphomycetes can be strain-specific (Braha et al. 2007, Miersch and Grancharov 2008).

The ergosterol content in fungal mycelium ranged between 3.4 and 10.3 µg mg^{-1} fungal dry mass for *A. tetracladia* (AT72) and *F. curta* (FC39), respectively, and it was in the range of ergosterol content previously found in aquatic hyphomycetes (2.3 to 11.5 mg^{-1} g dry mass, Gessner and Chauvet 1993). Differences in ergosterol content between the stationary- and the exponential-growth phase were found in one out of four aquatic hyphomycete species (Gessner and Chauvet 1993) and also in two white-rot fungal strains (Barajas-Aceves et al. 2002). In our work, a significant increase in ergosterol content with growth phase was also observed in three of the four tested isolates. This supports previous findings regarding the variability of ergosterol content with growth phase and fungal species (Gessner and Chauvet 1993, Barajas-Aceves et al. 2002).

In this work, the exposure to 1.5 mg L^{-1} of Cd did not alter the ergosterol content in the tested aquatic hyphomycetes (AT61 and FC39). Similarly, heavy-metals did not affect ergosterol content in the biomass of a white-root fungus (Barajas-Aceves et al. 2002), supporting that ergosterol can be a suitable indicator of fungal biomass under metal stress. However, other Cd concentrations and fungal species should be tested to confirm these findings.

The ITS2 region has been used to distinguish aquatic hyphomycete species (Nikolcheva et al. 2003, Raviraja et al. 2005, Duarte et al. 2008). Its high inter-specific variation allowed differentiating species within a genus (Kennedy et al. 2007). In this work, using species-specific primer pairs, standard curves between DNA amount and Ct values for each of the four aquatic hyphomycete isolates were done based on Real-Time PCR. No fluorescence of non-target template in all samples of the single-species treatments was observed, indicating no cross reactivity between the primers of the other isolates and so attesting their specificity. The intercepts of standard curves were not equal in all fungi, suggesting a different ITS2 copy numbers per species (Hibbett 1992) or different nucleus numbers per biomass.

Using this Real-Time PCR approach, we might be able to discriminate the individual DNA amount of each fungal species when fungi are growing together in mixtures. This might help to clarify the effects of fungal species number and identity on leaf litter decomposition to better understand the relationships between biodiversity and ecological functions.

References

Azevedo M-M (2007) *Toxicity of metals in aquatic hyphomycetes: cellular targets and defense mechanisms*. PhD thesis, University of Minho, Braga, Portugal.

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

Barajas-Aceves M, Hassan M, Tinoco R, Vazquez-Duhalt R (2002) Effect of pollutants on the ergosterol content as indicator of fungal biomass. *J Microbiol Methods*, 50:227-236.

Bärlocher F (ed) (1992) *The ecology of aquatic hyphomycetes*. Springer-Verlag, Berlin, Germany.

Braha B, Tintemann H, Krauss G, Ehrman J, Bärlocher F, Krauss G-J (2007) Stress response in two strains of the aquatic hyphomycetes *Heliscus lugdunensis* after exposure to cadmium and copper ions. *BioMetals*, 20:93-105.

Charcosset J-Y, Chauvet E (2001) Effect of culture conditions on ergosterol as an indicator of biomass in the aquatic hyphomycetes. *Appl Environ Microbiol*, 67:2051-2055.

Duarte S, Pascoal C, Alves A, Correia A, Cássio F (2008) Copper and zinc mixtures induce shifts in microbial communities and reduce leaf litter decomposition in streams. *Freshw Biol*, 53:91-101.

Galluzzi L, Penna A, Bertozzini E, Vila M, Garcés E, Magnani M (2004) Development of a Real-Time PCR assay for rapid detection and quantification of *Alexandrium minutum* (a Dinoflagellate). *Appl Environ Microbiol*, 70:1199-1206.

Gessner MO (2005) Ergosterol as a measure of fungal biomass. In, Graça MAS, Bärlocher F, Gessner MO (eds) *Methods to study litter decomposition: a practical guide*. Springer, Dordrecht, Netherlands, 189-196.

Gessner MO, Bärlocher F, Chauvet E (2003) Qualitative and quantitative analyses of aquatic hyphomycetes in streams. *Fungal Divers Res Ser*, 10:127-157.

Gessner MO, Chauvet E (1993) Ergosterol-to-biomass conversion factors for aquatic hyphomycetes. *Appl Environ Microbiol*, 59:502-507.

Gessner MO, Newell SY (2002) Biomass, growth rate, and production of filamentous fungi in plant litter. In, Hurst CJ, Crawford RL, Knudsen G, McInerney M, Stetzenbach LD (eds) *Manual of environmental microbiology*. 2nd ed, ASM Press, Washington, DC, USA, 390-408.

Ginzinger DG (2002) Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. *Exp Hematol*, 30:503-512.

Gonçalves MAP (2001) *Determinação de metais pesados em águas superficiais recolhidas no rio Este*. MSc thesis, University of Minho, Braga, Portugal.

Graça MAS (2001) The role of invertebrates on leaf litter decomposition in streams – a review. *Internat Rev Hydrobiol*, 86:383-393.

Guimarães-Soares L, Pascoal C, Cássio F (2007) Effects of heavy metals on the production of thiol compounds by the aquatic fungi *Fontanospora fusiramosa* and *Flagellospora curta*. *Ecotoxicol Environ Saf*, 66:36-43.

Hibbett DS (1992) Ribosomal RNA and fungal systematics. *Trans Mycol Soc Jpn*, 33:533-556.

Jaeckel P, Krauss G-J, Krauss G (2005) Cadmium and zinc response of the fungi *Heliscus lugdunensis* and *Verticillium* cf. *alboatrum* isolated from highly polluted water. *Sci Total Environ*, 346:274-279.

Kennedy PG, Bergemann SE, Hortal S, Bruns TD (2007) Determining the outcome of field-based competition between two *Rhizopogon* species using real-time PCR. *Mol Ecol*, 16:881-890.

Maharning AR, Bärlocher F (1996) Growth and reproduction in aquatic hyphomycetes. *Mycologia*, 88:80-88.

Manerkar MA, Seena S, Bärlocher F (2008) Q-RT-PCR for assessing Archaea, Bacteria, and Fungi during leaf decomposition in a stream. *Microb Ecol*, DOI 10.1007/s00248-008-9365-z.

Miersch J, Bärlocher F, Bruns I, Krauss G-J (1997) Effects of cadmium, copper, and zinc on growth and thiol content of aquatic hyphomycetes. *Hydrobiologia*, 346:77-84.

Miersch J, Grancharov K (2008) Cadmium and heat response of the fungus *Heliscus lugdunensis* isolated from highly polluted and unpolluted areas. *Amino Acids*, 34:271-277.

Newell SY (1992) Estimating fungal biomass and productivity in decomposing litter. In, Carroll GC, Wicklow DT (eds) *The fungal community. Its organization and the role in the ecosystem*. 2nd ed, Marcel Dekker, Inc., New York, USA, 521-561.

Nikolcheva LG, Bourque T, Bärlocher F (2005) Fungal diversity during initial stages of leaf decomposition in a stream. *Mycol Res*, 109:246-253.

Nikolcheva LG, Cockshutt AM, Bärlocher F (2003) Determining diversity of freshwater fungi on decaying leaves: comparison of traditional and molecular approaches. *Appl Environ Microbiol*, 69:2548-2554.

Niyogi DK, Lewis Jr WM, McKnight DM (2001) Litter breakdown in mountain streams affected by mine drainage: biotic mediation of abiotic controls. *Ecol Appl*, 11:506-516.

Niyogi DK, McKnight DM, Lewis Jr WM (2002) Fungal communities and biomass in mountain streams affected by mine drainage. *Arch Hydrobiol*, 155:255-271.

Pascoal C, Cássio F, Marvanová L (2005a) Anthropogenic stress may affect aquatic hyphomycete diversity more than leaf decomposition in a low-order stream. *Arch Hydrobiol*, 162:481-496.

Pascoal C, Marvanová L, Cássio F (2005b) Aquatic hyphomycete diversity in streams of Northwest Portugal. *Fungal Divers*, 19:109-128.

Raviraja NS, Nikolcheva LG, Bärlocher F (2005) Diversity of conidia of aquatic hyphomycetes assessed by microscopy and by DGGE. *Microb Ecol*, 49:301-307.

Soares HMVM, Boaventura RAR, Machado AASC, Esteves da Silva JCG (1999) Sediments as monitors of heavy metal contamination in the Ave river basin (Portugal): multivariate analysis of data. *Environ Pollut*, 105:311-323.

Sridhar KR, Krauss G, Bärlocher F, Raviraja NS, Wennrich R, Baumbach R, Krauss G-J (2001) Decompostion of alder leaves in two heavy metal–polluted streams in central Germany. *Aquat Microb Ecol*, 26:73-80.

Suberkropp K (1998) Microorganisms and organic matter decomposition. In, Naiman RJ, Bilby RE (eds) *River ecology and management: lessons from the Pacific coastal ecoregion*. Springer, New York, 120-143.

Weyers HS, Suberkropp K (1996) Fungal and bacterial production during the breakdown of yellow poplar leaves in 2 streams. *J N Am Benthol Soc*, 15:408-420.

Zar JH (1996) *Biostatistical analysis*. 3rd ed, Prentice-Hall, Englewood Cliffs.

Chapter 3

Effects of fungal diversity on leaf litter decomposition under cadmium stress: species number and traits

3.1. Introduction

In the last decades, biodiversity loss as a consequence of the increasing anthropogenic impact on Earth has been observed (Chapin et al. 2000). This led to an increase of studies relating biodiversity and ecosystem processes (reviewed by Hooper et al. 2005). Freshwaters are one of the most impacted environments due to human activities but the consequences to ecosystems functioning are not fully understood (Covich et al. 2004).

Metals can reach streams by mine drainage, industrial emissions, garbage disposal and by-products of agricultural fertilizers (Rand et al. 1995). Metal pollution in aquatic ecosystems is of major concern because of the non-degradability of metals and their accumulation and biomagnification along food webs (Croteau et al. 2005, Marie et al. 2006).

Aquatic hyphomycetes are a polyphyletic group of fungi that dominates in earlier stages of plant-litter decomposition in freshwater ecosystems (Bärlocher 2005). Their enzymatic skills ensure the degradation of the structural plant cell-wall polysaccharides (Suberkropp 1998), improving leaf palatability to higher trophic levels, primarily to macroinvertebrate detritivores (Suberkropp 1998, Bärlocher 2005).

Although aquatic hyphomycetes are mainly documented in clean streams (Bärlocher 1992), they have also been found in streams affected by metal pollution (Sridhar et al. 2001). Studies on the ability of aquatic hyphomycetes to survive under metal stress, carried out with fungal species isolated from clean and polluted sites, showed that the exposure to one metal does not confer tolerance to all metals, and that tolerance to metals can vary with fungal species and metal type (Guimarães-Soares 2005, Azevedo 2007). Also, strains from the same aquatic hyphomycete species isolated from clean and metal-polluted streams (Miersch and Grancharov 2008) or from sites with different degrees of metal pollution (Braha et al. 2007) responded differently to cadmium (Cd) exposure suggesting that metal tolerance can be strain-specific.

Metal stress seemed to have a strong effect on the structure of aquatic fungal communities (Duarte et al. 2004, 2008), suggesting that some species are more tolerant to metals than others. Although diversity was not strongly affected by metals, leaf decomposition was decreased under metal stress (Duarte et al. 2004, 2008), indicating that shifts in species composition may lead to changes in community functions. However, in severely metal-polluted streams, a reduction in

both fungal diversity and activity was found (Sridhar et al. 2001, Niyogi et al. 2001, 2002).

In laboratory experiments, where aquatic hyphomycete diversity was manipulated, a positive relationship between fungal diversity and leaf decomposition was observed (Bärlocher and Corkum 2003, Duarte et al. 2006). This positive relationship can result from both complementary interactions among species and/or the presence of a dominant species, leading to increased community performances (Loreau and Hector 2001). However, it is difficult to distinguish between complementarity and selection effects (Bärlocher and Corkum 2003, Duarte et al. 2006), since most of the methodologies used to assess fungal activity (e.g. biomass build-up from ergosterol quantification, Gessner 2005; fungal productivity from incorporation rates of radiolabeled acetate into ergosterol, Suberkropp and Gessner 2005) cannot discriminate the contribution of each species to the overall performance of the community. Real-Time Polymerase Chain Reaction (Real-Time PCR) is a molecular tool that emerged in the last two decades and seems promising to overcome this problem since it accurately quantifies the amount of the starting DNA template (Ginzinger 2002). This allowed, for instance, the quantification of soil ammonia-oxidizing bacterial populations (Okano et al. 2004), the study of competitive interactions between two ectomycorrhizal species on *Pinus muricata* seedlings (Kennedy et al. 2007), or to assess population abundances of *Pseudoalteromonas* (Skovhus et al. 2004) and harmful algae (Coyne et al. 2005). Real-Time PCR was only applied once to leaf decomposition in streams to discriminate the contribution of archaea, bacteria and fungi to this process (Manerkar et al. 2008).

In this work, to better understand the relationships between biodiversity and ecosystem functions, the effects of fungal species number and traits on leaf litter decomposition were investigated in the absence or presence of Cd. Three different species of aquatic hyphomycetes and two strains of the same species, with different background (clean versus metal-polluted streams), were used either alone or in all possible combinations of the three species. The measured endpoints were leaf mass loss, fungal biomass as ergosterol, fungal DNA content and fungal reproduction.

3.2. Materials and methods

3.2.1. Microcosms

In October 2007, leaves of *Alnus glutinosa* (L.) Gaertn. were collected immediately before abscission and dried at room temperature. The leaves were leached in deionised water for 2 days and cut into 12 mm diameter disks. Sets of 25 disks were placed in 150-mL Erlenmeyer flasks and autoclaved for 20 min. To each Erlenmeyer flask, 70 mL of filtered and autoclaved (120 ºC, 20 min) stream water was added aseptically. The microcosms were supplemented with 1.5 mg L^{-1} Cd (added as chlorides, Sigma). Microcosms without added metals were used as controls.

On 11 December 2007, stream water samples were collected from the Algeriz stream for microcosm experiments. Temperature, pH, conductivity and oxygen dissolved on the stream water were measured *in situ* with field probes (Multiline F/set 3 no. 400327, WTW). Stream water was collected into sterile glass bottles, transported in a cold box (4 ºC), and used within 24 h for chemical analyses. Concentration of nitrate (HACH kit, program 355), nitrite (HACH kit, program 371), ammonia (HACH kit, program 385) and phosphate (HACH kit, program 480) were determined. Data from physical and chemical analysis of the stream water indicated circumneutral pH, low conductivity, moderate concentration of nitrate and low concentration of other inorganic nutrients (Table 3.1).

The aquatic hyphomycetes *Articulospora tetracladia* Ingold (UMB-61.01 and UMB-72.01), *Flagellospora curta* J. Webster (UMB-39.01) and *Tricladium splendens* Ingold (UMB-100.01) were grown on 8 mL of malt extract agar 2% (w/v).

Table 3.1. Physical and chemical characteristics of the stream water of the Algeriz River on 11 December 2007.

Parameter	Value
pH [a]	6.34
Conductivity (µS cm^{-1}) [a]	40
Oxygen dissolved (mg L^{-1})	10.8
Temperature (ºC) [a]	8.5
$N\text{-}NO_3^-$ (mg L^{-1})	0.3
$N\text{-}NO_2^-$ (mg L^{-1})	0.002
$N\text{-}NH_3$ (mg L^{-1})	<0.01
$P\text{-}PO_4^{3-}$ (mg L^{-1})	0.05

[a] Measurements were done *in situ* at 9 a.m.

Microcosms (150-mL Erlenmeyer flasks) were inoculated with agar plugs collected from the edge of 19-days-old colonies of the four fungi as follows: monocultures of the four strains (4 treatments x 4 replicates), all combinations of two species (5 treatments x 4 replicates) and all combinations of three species (2 treatments x 4 replicates), never mixing the strains from the same species. Inoculation of single species microcosms was done with a 6-mm-diameter plug. For multiple-species microcosms, the total inoculum size was maintained and divided equally among all species.

The microcosms were incubated for 35 days on a shaker (120 rpm, Certomat BS 3, Melsungen, Germany) at 18 ºC, under permanent artificial light and stream water was changed every 5 days. Discarded solutions were scanned to assess conidial production, as described below. At the end of the experiment, leaf disks were used to determine leaf mass loss, ergosterol concentration and fungal DNA content.

3.2.2. Leaf dry mass

Sets of 11 leaf disks from each replicate microcosm were lyophilized to constant mass (72 ± 24 h) and weighed (± 0.001 g). Sets of leaf disks before fungal inoculation were used to estimate initial dry mass.

3.2.3. Fungal biomass

Sets of 6 freeze-dried leaf disks from each microcosm were used to determine ergosterol concentration as a measure of fungal biomass on leaves. Lipids were extracted from leaf disks by heating (80 ºC, 30 min) in 0.8% of KOH/methanol, purified by solid-phase extraction and quantified by HPLC, according to Gessner (2005).

3.2.4. Conidial production

Conidial suspensions were mixed with 200 µL of 0.5% Tween 80, appropriate volumes were filtered (0.45-µm pore size, Millipore) and the retained conidia were stained with 0.05% cotton blue in lactic acid. Approximately 300 conidia per filter were identified and counted under a light microscope (400x magnification).

3.2.5. Real-time PCR assays

DNA was isolated from 4 leaf disks (1 from each replicate) with the UltraClean Soil DNA kit (MoBio Laboratories, Solana Beach, CA, USA).

Real-time PCR assays were run on LightCycler®480 System (Roche). Each 10 µL reaction contained 1 µL of sample DNA, 5 µL of 2x LightCycler® 480 SYBR Green I Master mix (Roche), each primer at 250 nM and the required amount of nuclease-free water.

PCR conditions were the following: initial denaturation at 95 ºC for 10 min, followed by 45 cycles of denaturation at 95 ºC for 10 s, primer hybridization at 60 °C during 20 s and extension at 72 ºC during 15 s. Each reaction was performed in duplicate. Negative controls were performed by testing all primers against all samples and no fluorescence of non-target template in all samples was observed (data not shown).

3.2.6. Data analyses

Nested ANOVAs were used to test the effects of Cd, species number and species identity (nested within species number) on leaf mass loss, ergosterol concentration and fungal sporulation. In a second analysis, the expected leaf mass loss, ergosterol concentration and fungal sporulation in mixed cultures were estimated as the sum of each monoculture performance (see Bärlocher and Corkum 2003). The differences between the observed and the expected performances (net diversity effect) were tested against the null hypothesis that the average difference equalled 0 (t-test) (Bärlocher and Corkum 2003). Leaf mass loss, ergosterol concentration and sporulation rate of the most active monoculture were compared with those in mixed cultures containing that species (t-test).

The percentage of leaf mass loss was arcsine square root transformed and fungal biomass and sporulation were ln-transformed to achieve normal distribution (Zar 1996).

The statistical analyses were performed with Statistica 6.0 for Windows.

Using the standard curves presented in Table 2.5 (Chapter 2), the Ct values of each strain obtained by Real-Time PCR were converted to DNA and were used to calculate the percentage of each strain in multicultures as follows:

$$P_i = \frac{DNA_i}{\sum_{i=1}^{S} DNA_i},$$

where P_i is the percentage of species i in multicultures, S is the number of species and DNA_i is the DNA amounts of species i assessed by Real-Time PCR. Ergosterol-to-biomass conversion factors (Table 2.4, Chapter 2) where used to convert ergosterol into fungal biomass as:

$$B_t = \frac{E_t}{\sum_{i=1}^{S}(P_i \times F_i)},$$

where B_t is the total fungal biomass, E_t is the total ergosterol content, P_i is the percentage of species *i* in multiculture containing *S* species (assessed by Real-Time PCR) and F_i is the ergosterol-to-biomass conversion factor of species *i.* The partioning model (Loreau and Hector 2001) was applied to fungal biomass, assuming that net diversity effect were the sum of complementarity and selection effects.

3.3. Results

3.3.1. Leaf mass loss

After 35 days in microcosms, leaf mass loss of alder leaves inoculated with one to three aquatic hyphomycete species, without Cd addition, varied between 35% in the multiculture with *Articulospora tetracladia* from the clean site (AT72), *Flagellospora curta* (FC39) and *Tricladium splendens* (TS100), and 17% in the monoculture with FC39 (Fig. 3.1A). Cadmium and species identity, but not species number, significantly affected leaf mass loss (three-way nested ANOVA, $p < 0.05$; Table 3.2). A significant decrease in leaf mass loss was observed after Cd addition, especially in treatments with AT72 and TS100. The strain of *A. tetracladia* (AT61) isolated from a polluted site seemed to be less sensitive to Cd than AT72, alone or in mixtures.

In both control or Cd treatments, no significant differences were obtained in leaf mass loss between the monoculture with the most active species (AT61) and mixed cultures containing it (t-tests, $p > 0.05$). In the absence of Cd, leaf mass losses in multicultures with two species were higher than that expected from the sum of monoculture performances (t-test, $p < 0.05$; Fig. 3.2A). However, in the presence of Cd no significant net diversity effects (differences between expected and observed values) on leaf mass losses were found (t-test, $p > 0.05$; Fig. 3.2A).

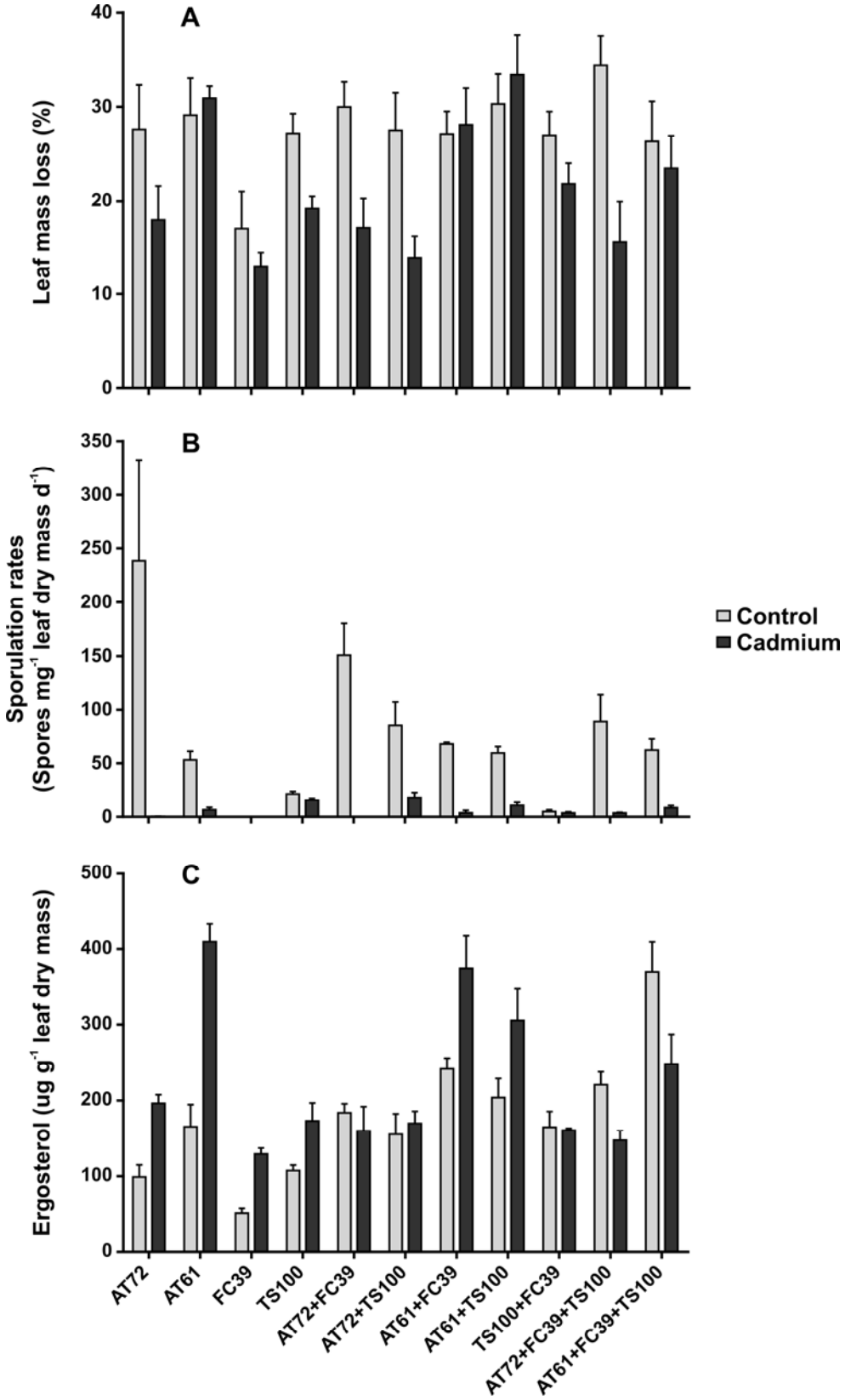

Figure 3.1. Leaf mass loss (A), fungal sporulation rates (B) and ergosterol concentration (C) on decomposing alder leaves after 35 days in microcosms non-exposed or exposed to 1.5 mg L^{-1} Cd. M + SEM; n = 4. AT72, *Articulospora tetracladia* (UMB-72.01); AT61, *Articulospora tetracladia* (UMB-61.01); FC39, *Flagellospora curta* (UMB-39.01); TS100, *Tricladium splendens* (UMB-100.01).

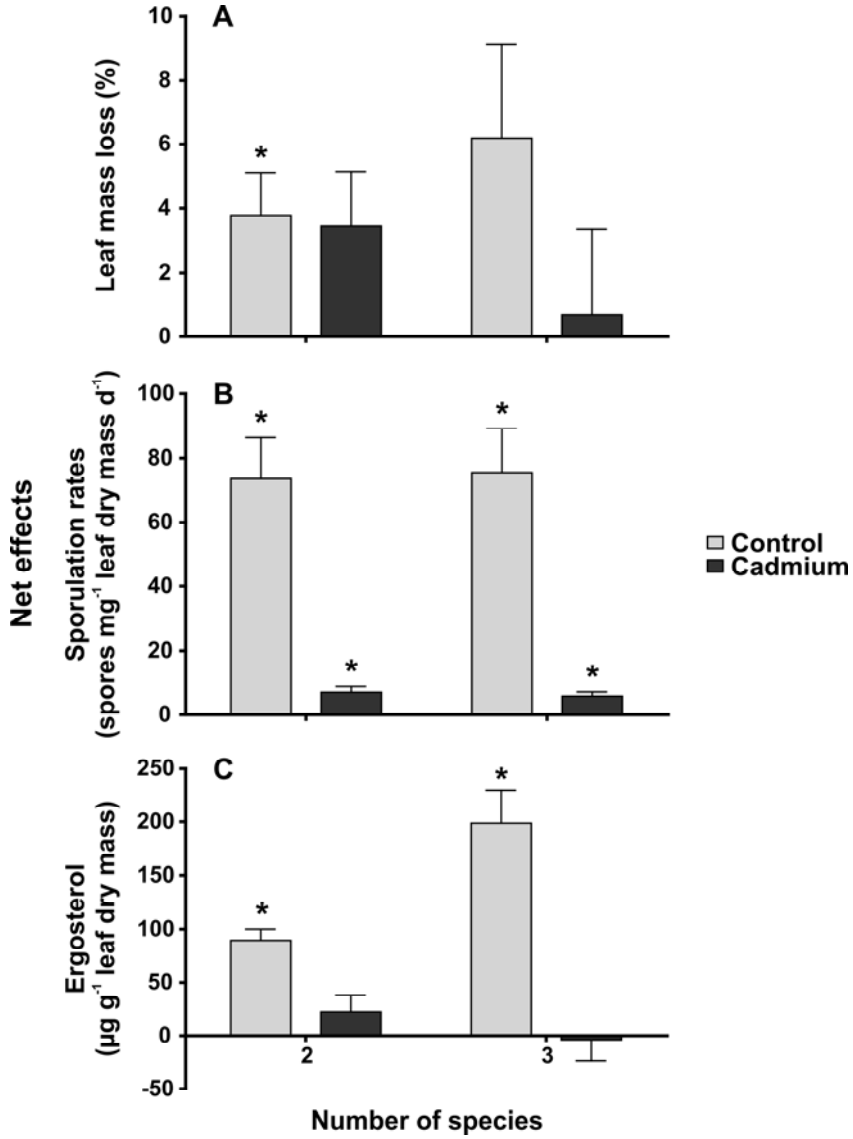

Figure 3.2. Net effects are the differences between observed and expected values of leaf mass losses (A), sporulation rates (B) and ergosterol concentration (C), in mixed cultures with 2 and 3 species, non-exposed or exposed to 1.5 mg L^{-1} Cd. *, significant differences from zero (t-tests, $p < 0.05$).

3.3.2. Sporulation rates

After 35 days in microcosms, fungal sporulation rates on alder leaves inoculated with one to three aquatic hyphomycete species, without Cd addition, varied between 0.013 and 240 spores mg^{-1} leaf dry mass d^{-1} in monocultures of *F. curta* (FC39) and *A. tetracladia* (AT72), respectively (Fig. 3.1B). Cadmium, species identity and species number significantly affected sporulation rates (three-way nested ANOVA, $p < 0.05$; Table 3.2). Treatments with the strain of *A. tetracladia* from the clean site (AT72) showed higher sporulation than those with *A. tetracladia* from the polluted site (AT61). Cadmium significantly reduced sporulation rates, especially in

treatments with strain AT72. In the absence of Cd, no significant difference was obtained in the monoculture with the most active species (AT72) comparing with mixed cultures containing that species (t-test, $p > 0.05$). In the presence of Cd, sporulation rates were higher in microcosms with the most active species alone (TS100) than in mixtures containing it (t-test, $p < 0.05$).

Net biodiversity effects were positive for sporulation in the multicultures because obtained values were higher than that expected from the sum of monocultures either in the absence or presence of Cd (t-test, $p < 0.05$; Fig. 3.2B).

Table 3.2. Nested ANOVAs of the effects of Cd, species number and species identity, nested within species number, on leaf mass loss, fungal sporulation and ergosterol concentration.

	Effect	df	SS	MS	F	p
Leaf mass loss	Identity {species number}	8	692.8	86.6	3.5	0.002
	Species number	2	80.4	40.2	1.6	0.206
	Cd	1	479.9	479.9	19.3	<0.001
	Species number * Cd	2	46.8	23.4	0.9	0.395
	Error	73	1816.1	24.9		
Sporulation	Identity {species number}	8	557.5	69.7	17.7	<0.001
	Species number	2	68.4	34.2	8.7	<0.001
	Cd	1	176.7	176.7	44.8	<0.001
	Species number * Cd	3	12.6	6.3	1.6	0.210
	Error	73	288.0	3.9		
Ergosterol	Identity {species number}	8	9.1	1.1	19.0	<0.001
	Species number	2	3.5	1.7	29.2	<0.001
	Cd	1	0.5	0.5	9.0	0.004
	Species number * Cd	2	4.0	2.0	33.3	<0.001
	Error	74	4.4	0.1		

3.3.3. Fungal biomass as ergosterol and DNA contents

After 35 days in control microcosms, ergosterol concentration on alder leaves inoculated with one to three aquatic hyphomycete species varied between 50 µg ergosterol g^{-1} leaf dry mass in monocultures of *Flagellospora curta* (FC39) and 370 µg ergosterol g^{-1} leaf dry mass in multicultures containing *Articulospora tetracladia* from the polluted site (AT61), *Flagellospora curta* (FC39) and *Tricladium splendens* (TS100) (Fig. 3.1C). Cadmium, species identity, species number and the interaction between Cd and species number significantly affected ergosterol concentration (three-way nested ANOVA, $p < 0.05$; Table 3.2). Cadmium led to increased

ergosterol concentrations in all treatments, except in multicultures of three species. The highest ergosterol concentrations corresponded to treatments with the isolate AT61 with or without Cd addition. In controls, ergosterol concentration increased with the number of species, while in the presence of Cd this tendency was not found. In the absence of Cd, the most productive isolate in monocultures (AT61) produced significantly less ergosterol than in multicultures containing it (t-test, $p < 0.05$), but this was not found under Cd exposure (t-test, $p > 0.05$).

In the absence of Cd, ergosterol concentration in multicultures was significantly higher than that expected from the sum of ergosterol concentration in monocultures (t-test, $p < 0.05$; Fig. 3.2C). No significant net diversity effects were found in Cd-exposed microcosms (t-test, $p > 0.05$).

Under control conditions, DNA data from Real-Time PCR showed a clear dominance of *A. tetracladia* (both AT72 and AT61; >85%) in all multicultures containing this species (Fig. 3.3). Cadmium exposure led to a decrease of at least 60% in the contribution of AT72 in multicultures with either 2 or 3 species, while increased the contribution of FC39 to more than 70%. The isolate AT61 maintained its contribution above 90% even in the presence of Cd in all multicultures. In the absence of Cd, TS100 and FC39 in mixtures had similar contributions to total fungal DNA but the presence of Cd increased the contribution of the latter species in more than 90%.

Fungal biomass on decomposing leaves (Fig. 3.4) were estimated from ergosterol concentrations (using the ergosterol-to-biomass conversion factor for each isolate, Table 2.4, Chapter 2) and taking into account the relative DNA content of each fungus in multicultures (Fig. 3.3). Fungal biomass showed a pattern of fungal performances on leaves similar to the one obtained from ergosterol measurements alone (Fig. 3.1C *versus* Fig. 3.4). However, *F. curta* (FC39) showed lower biomasses because it had higher concentrations of ergosterol in mycelium than the other isolates. The strain of *A. tetracladia* isolated from the polluted site (AT61) had consistently higher biomasses than the corresponding strain from the clean site (AT72) in the absence or presence of Cd. In control, the biomass of the most active strain (AT61) was lower than in the multicultures containing it. *A. tetracladia* from the polluted site (AT61) and *F. curta* (FC39) appeared to be the most tolerant fungi to Cd. However, FC39 was the only isolate that increased its biomass in all treatments with Cd.

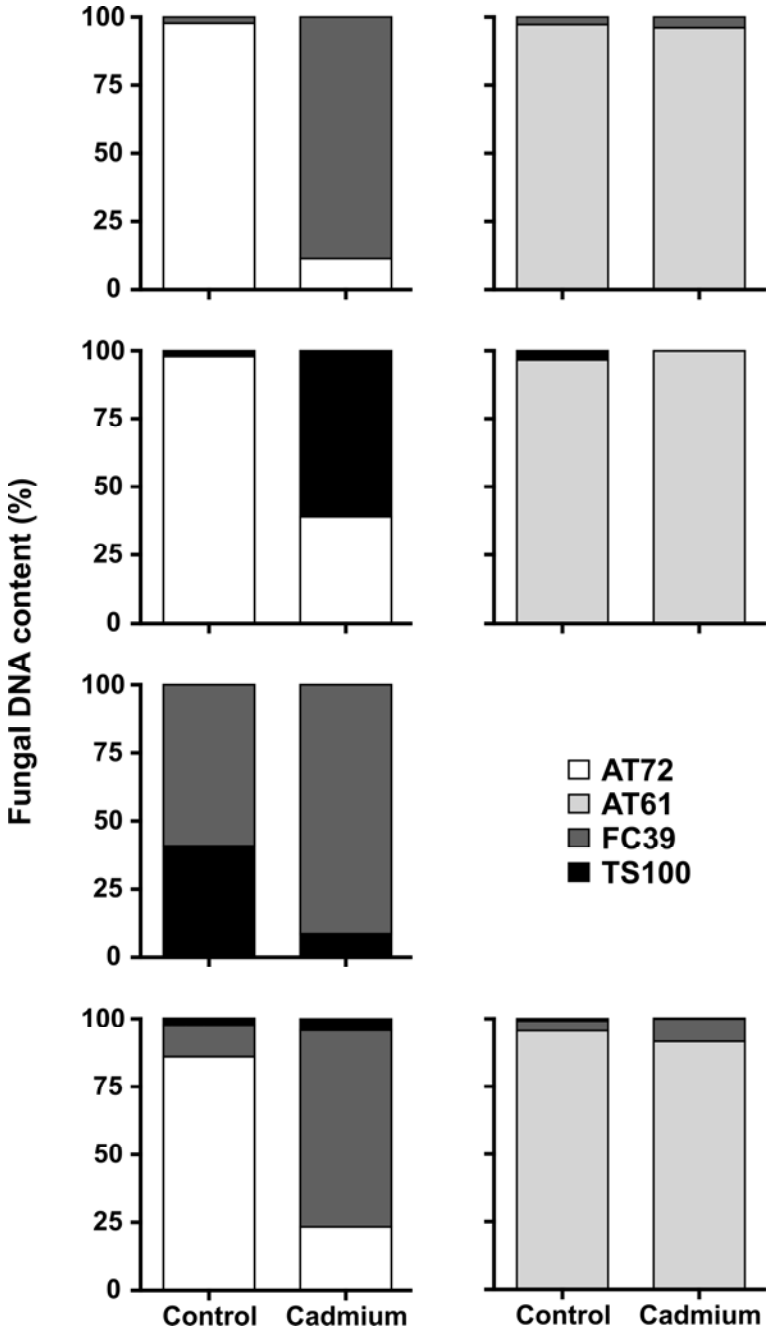

Figure 3.3. Percentage of contribution of each fungal isolate to total DNA content assessed by Real-Time PCR. AT72, *Articulospora tetracladia* (UMB-72.01); AT61, *Articulospora tetracladia* (UMB-61.01); FC39, *Flagellospora curta* (UMB-39.01); TS100, *Tricladium splendens* (UMB-100.01).

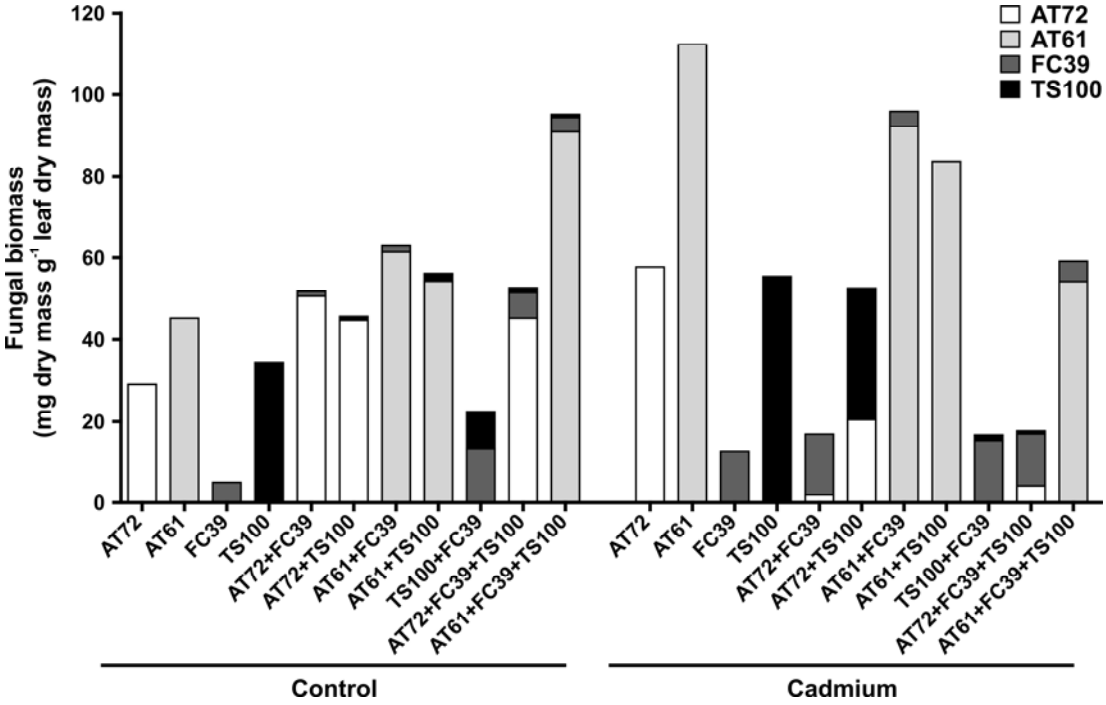

Figure 3.4. Fungal biomass on decomposing alder leaves non-exposed or exposed to 1.5 mg L^{-1} Cd during 35 days. Biomass was estimated from ergosterol concentrations using the conversion factors calculated in Chapter 2 and taking into account the contribution of each isolate from Real-Time PCR assay. AT72, *Articulospora tetracladia* (UMB-72.01); AT61, *Articulospora tetracladia* (UMB-61.01); FC39, *Flagellospora curta* (UMB-39.01); TS100, *Tricladium splendens* (UMB-100.01).

In control, a positive net diversity effect on fungal biomass was observed in multicultures, meaning that the biomass of multicultures was higher than expected from the sum of biomasses in monocultures (Fig. 3.5). Also, increased net effects were observed with increasing species number. These net diversity effects on biomass resulted mainly from species complementarity. Contrarily, in the presence of Cd the net diversity effects were negative. Moreover, the lower biomasses observed in multicultures comparing to that expected from monocultures were mainly a result of selection effects.

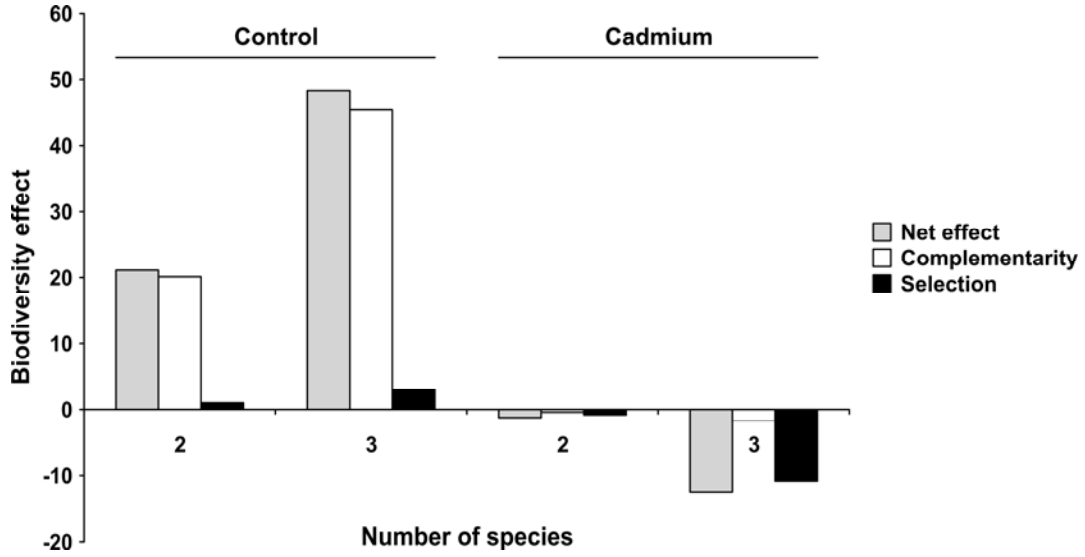

Figure 3.5. Biodiversity effects on fungal biomass for multicultures non-exposed or exposed to 1.5 mg L^{-1} Cd. Net effect, complementarity and selection were estimated according to the partioning model (Loreau and Hector 2001).

3.4. Discussion

Biodiversity and aquatic ecological processes are known to be affected by metal stress. In streams, leaf decomposition and aquatic hyphomycete diversity tends to be reduced by exposure to metals (Bermingham et al. 1996a, Sridhar et al. 2001, Niyogi et al. 2002). Also, in microcosms, the exposure to metals decreased leaf decomposition, fungal diversity and altered the structure of fungal communities on decomposing leaves (Duarte et al. 2004, 2008). Studies manipulating aquatic hyphomycete species pointed to a positive relationship between fungal diversity and leaf decomposition (Bärlocher and Corkum 2003, Treton et al. 2004, Duarte et al. 2006, Raviraja et al. 2006). In addition, species identity appeared to be more important than species number for leaf decomposition (Duarte et al. 2006). In this study, both Cd and species identity, but not species number, significantly affected leaf mass loss. Moreover, not only fungal species but also strain traits were important since the strain of *A. tetracladia* isolated from the polluted site (AT61) degraded more efficiently the leaves and was less sensitive to Cd than the strain isolated from the clean site (AT72). In the absence of Cd, positive net diversity effects on leaf mass loss were found because mass losses in multicultures were greater than that expected from the sum of individual monocultures. This suggests complementary effects. However, performances in multicultures containing the most active species did not differ from those of monocultures with it, and so a selection effect cannot be excluded (Hector 1998, Loreau and Hector 2001).

It is known that fungal diversity and reproduction tends to be more negatively affected by metals than biomass (Abel and Bärlocher 1984, Bermingham et al. 1996b, Niyogi et al. 2002, Duarte et al. 2004, Azevedo 2007). Sporulation rates of aquatic hyphomycetes on decomposing leaves were significantly affected by metals alone or in mixtures (Duarte et al. 2004, 2008). In this work, Cd exposure, species identity and species number significantly affected sporulation rates. In the absence of Cd, the highest sporulating fungus was *A. tetracladia* isolated from the clean site (AT72), while under Cd stress, this strain suffered a severe decrease, becoming *T. splendens* (TS100) the most sporulating species. This suggests a higher tolerance of TS100 isolate to Cd exposure, regarding sporulation. Again, the decrease in sporulation upon Cd exposure was more pronounced for AT72 than for AT61, suggesting higher tolerance of the latter strain to this metal. Decreases in sporulation rates were observed in multicultures of two species comparing to the expected performances of each species alone (Treton et al. 2004). This contrast to data from the present work, in which overall sporulation in multicultures was greater than that expected from the sum of individual monocultures indicating that mixed cultures reproduced more actively and suggesting complementary effects also for reproduction.

In previous studies, metal concentrations able to reduce leaf decomposition did not affect fungal biomass (Duarte et al. 2004, 2008). However, fungal biomass on decomposing leaves is reported to be low in severely metal-polluted streams (Sridhar et al. 2001, Niyogi et al. 2002). In the present work, Cd exposure, species identity and species number significantly affected fungal biomass (as ergosterol concentration). Under control conditions, a clear dominance of both *A. tetracladia* strains to total fungal biomass was observed in all multicultures containing this species. Treatments in which *A. tetracladia* was absent showed lower fungal biomass, sporulation and leaf decomposition, pointing to *A. tetracladia* as a key-species. These data are in agreement with the higher contributions of *A. tetracladia* to the total conidial production in reference streams of Northwest Portugal (Duarte et al. 2004, Pascoal et al. 2005a). Fungal biomasses were higher in all monocultures with Cd, comparing to control, being AT61 the most active species. Since all fungi, except the strain AT72, were isolated from a metal-polluted site (Pascoal et al. 2005b) they might be adapted to Cd stress. Indeed, Cd led to a decrease in the contribution of AT72 in multicultures, while the strain AT61 was less affected, suggesting once more a higher tolerance of the latter strain to this metal. Although *F. curta* had always lower biomasses comparing to *A. tetracladia* of the polluted site

(AT61), it was the only isolate that increased its biomass in all treatments with Cd, corroborating the high tolerance of *F. curta* to this metal (Guimarães-Soares 2005).

The net diversity effects can result from mechanisms of complementarity and/or selection, whose relative contributions can be quantified if individual species performances in multicultures are determined (partioning model, Loreau and Hector 2001). In this study, we used Real-Time PCR to track the biomass of individual species within fungal communities. In the absence of Cd, net diversity effects on fungal biomass were positive and they resulted mainly from species complementarity. This can be a result of resource partitioning or facilitative interactions among species (Loreau and Hector 2001). Actually, it was demonstrated that aquatic hyphomycete species preferentially colonize different patches of leaves (Bermingham et al. 1995, Bermingham et al. 1996c), probably leading to a better resource use. Also, these positive net effects increased with increasing species number, supporting higher fungal biomasses in more diverse communities (Duarte et al. 2006). An increase of positive interactions between species within communities was observed by exposure to stressors (Mulder et al. 2001, Callaway et al. 2002). In contrast, a perturbation weakened the positive diversity-biomass relationship, showing that communities with more species had greater biomass reduction than the ones with fewer species (Zhang and Zhang 2006). In this work, Cd exposure led to negative net diversity effects on fungal biomass that increased with increasing species number, suggesting some negative interactions among fungal species, probably due to interspecific competition (Loreau and Hector 2001).

In this work, Real-Time PCR assays provided new insights regarding the contribution of each species to the total biomass production, helping the elucidation of the mechanisms explaining diversity effects on ecosystem functions. However, only percentages of contribution of each species were achieved. But, with the development of appropriate normalizations, absolute biomass quantifications might be assessed, making it possible to discriminate the biomass of each species within a community with no need of further techniques, like ergosterol quantification. It is known that ergosterol concentration in mycelium can vary with species, resulting in pitfalls in biomass quantification; it was the case of *F. curta* (FC39) in which the higher ergosterol content in mycelia suggested higher biomass for this species than it actually has.

Overall, species number affected fungal sporulation and biomass. Fungal performances were generally higher in multicultures than that expected from the sum of monocultures, suggesting complementary effects between species.

However, when exposed to Cd lower fungal biomasses were observed with increasing species number suggesting the occurrence of some interspecific competition. *A. tetracladia* strain from the polluted site (AT61) appeared to be more tolerant to Cd, because it produced more biomass and decomposed leaves more efficiently than the corresponding strain from the clean site (AT72). These findings emphasize that besides the importance of species identity, strain traits matter for leaf decomposition. Results also suggest that fungal populations adapted to metals may have better performances under metal stress than non-adapted ones helping to maintain ecosystem processes.

References

Abel TH, Bärlocher F (1984) Effects of cadmium on aquatic hyphomycetes. *Appl Environ Microbiol*, 48:245-251.

Azevedo M-M (2007) *Toxicity of metals in aquatic hyphomycetes: cellular targets and defense mechanisms*. PhD thesis, University of Minho, Braga, Portugal.

Bärlocher F (2005) Freshwater fungal communities. In, Dighton J, Oudemans P, White J (eds) *The fungal community*. 3rd ed, CRC Press, Boca Raton.

Bärlocher F (ed) (1992) *The ecology of aquatic hyphomycetes*. Springer-Verlag, Berlin, Germany.

Bärlocher F, Corkum M (2003) Nutrient enrichment overwhelms diversity effects in leaf decomposition by stream fungi. *Oikos*, 101:247-252.

Bermingham S, Dewey FM, Maltby L (1995) Development of a monoclonal antibody-based immunoassay for the detection and quantification of *Anguillospora longissima* colonizing leaf material. *Appl Environ Microbiol*, 61:2606-2613.

Bermingham S, Maltby L, Cooke RC (1996a) Effects of a coal mine effluent on aquatic hyphomycetes. I. Field Study. *J Appl Ecol*, 33:1311-1321.

Bermingham S, Maltby L, Cooke RC (1996b) Effects of a coal mine effluent on aquatic hyphomycetes. II. Laboratory toxicity experiments. *J Appl Ecol*, 33:1322-1328.

Bermingham S, Maltby L, Dewey FM (1996c) Monoclonal antibodies as tools to quantify mycelium of aquatic hyphomycetes. *New Phytol*, 132:593-601.

Braha B, Tintemann H, Krauss G, Ehrman J, Bärlocher F, Krauss G-J (2007) Stress response in two strains of the aquatic hyphomycete *Heliscus lugdunensis* after exposure to cadmium and copper ions. *BioMetals*, 20:93-105.

Callaway RM, Brooker RW, Choler P, Kikvidze Z, Lortiek CJ, Michalet R, Paolini L, Pugnaire FI, Newingham B, Aschehoug ET, Armas C, Kikodze D, Cook BJ

(2002) Positive interactions among alpine plants increase with stress. *Nature*, 417:844-848.

Chapin III FS, Zavaleta ES, Eviner VT, Naylor RL, Vitousek PM, Reynolds HL, Hooper DU, Lavorel S, Sala OE, Hobbie SE, Mack MC, Díaz S (2000) Consequences of changing biodiversity. *Nature*, 405:234-242.

Covich AP, Austen MC, Bärlocher F, Chauvet E, Cardinale BJ, Biles CL, Inchausti P, Dangles O, Solan M, Gessner MO, Statzner B, Moss B (2004) The role of biodiversity in the functioning of freshwater and marine benthic ecosystems. *BioScience*, 54:767-775.

Coyne KJ, Handy SM, Demir E, Whereat EB, Hutchins DA, Portune KJ, Doblin MA, Cary SC (2005) Improved quantitative real-time PCR assays for enumeration of harmful algal species in field samples using an exogenous DNA reference standard. *Limnol Oceanogr: Methods*, 3:381-391.

Croteau M-N, Luoma SN, Stewart AR (2005) Trophic transfer of metals along freshwater food webs: evidence of cadmium biomagnification in nature. *Limnol Oceanogr*, 50:1511-1519.

Duarte S, Pascoal C, Alves A, Correia A, Cássio F (2008) Copper and zinc mixtures induce shifts in microbial communities and reduce leaf litter decomposition in streams. *Freshw Biol*, 53:91-101.

Duarte S, Pascoal C, Cássio F (2004) Effects of zinc on leaf decomposition by fungi in streams: studies in microcosms. *Microb Ecol*, 48:366-374.

Duarte S, Pascoal C, Cássio F, Bärlocher F (2006) Aquatic hyphomycete diversity and identity affect leaf litter decomposition in microcosms. *Oecologia*, 147:658-666.

Gessner MO (2005) Ergosterol as a measure of fungal biomass. In, Graça MAS, Bärlocher F, Gessner MO (eds) *Methods to study litter decomposition: a practical guide*. Springer, Dordrecht, Netherlands, 189-196.

Ginzinger DG (2002) Gene quantification using real-time quantitative PCR: An emerging technology hits the mainstream. *Exp Hematol*, 30:503-512.

Guimarães-Soares L (2005) *Biochemical and physiological responses of the aquatic fungi* Fontanospora fusiramosa *and* Flagellospora curta *to cadmium, copper and zinc*. PhD thesis, University of Minho, Braga, Portugal.

Hector A (1998) The effect of diversity on productivity: detecting the role of species complementarity. *Oikos*, 82:597-599.

Hooper DU, Chapin III FS, Ewel JJ, Hector A, Inchausti P, Lavorel S, Lawton JH, Lodge DM, Loreau M, Naeem S, Schmid B, Setälä H, Symstad AJ,

Vandermeer J, Wardle DA (2005) Effects of biodiversity on ecosystem functioning: a consensus of current knowledge. *Ecol Monogr*, 75:3-35.

Kennedy PG, Bergemann SE, Hortal S, Bruns TD (2007) Determining the outcome of field-based competition between two *Rhizopogon* species using real-time PCR. *Mol Ecol*, 16: 881–890.

Loreau M, Hector A (2001) Partitioning selection and complementarity in biodiversity experiments. *Nature*, 412:72-76.

Manerkar MA, Seena S, Bärlocher F (2008) Q-RT-PCR for assessing Archaea, Bacteria, and Fungi during leaf decomposition in a stream. *Microb Ecol*, DOI 10.1007/s00248-008-9365-z.

Marie V, Baudrimont M, Boudou A (2006) Cadmium and zinc bioaccumulation and metallothionein response in two freshwater bivalves (*Corbicula fluminea* and *Dreissena polymorpha*) transplanted along a polymetallic gradient. *Chemosphere*, 65:609-617.

Miersch J, Grancharov K (2008) Cadmium and heat response of the fungus *Heliscus lugdunensis* isolated from highly polluted and unpolluted areas. *Amino Acids*, 34:271-277.

Mulder CPH, Uliassi DD, Doak DF (2001) Physical stress and diversity-productivity relationships: The role of positive interactions. *Proc Natl Acad Sci USA*, 98:6704-6708.

Niyogi DK, Lewis Jr WM, McKnight DM (2001) Litter breakdown in mountain streams affected by mine drainage: biotic mediation of abiotic controls. *Ecol Appl*, 11:506-516.

Niyogi DK, McKnight DM, Lewis Jr WM (2002) Fungal communities and biomass in mountain streams affected by mine drainage. *Arch Hydrobiol*, 155:255-271.

Okano Y, Hristova KR, Leutenegger CM, Jackson LE, Denison RF, Gebreyesus B, Lebauer D, Scow KM (2004) Application of Real-Time PCR to study effects of ammonium on population size of ammonia-oxidizing bacteria in soil. *Appl Environ Microbiol*, 70:1008-1016.

Pascoal C, Cássio F, Marvanová L (2005a) Anthropogenic stress may affect aquatic hyphomycete diversity more than leaf decomposition in a low-order stream. *Arch Hydrobiol*, 162:481-496.

Pascoal C, Marvanová L, Cássio F (2005b) Aquatic hyphomycete diversity in streams of Northwest Portugal. *Fungal Divers*, 19:109-128.

Rand GM, Wells PG, McCarty LS (1995) Introduction to aquatic toxicology: effects, environmental fate, and risk assessment. In, Rand GM (ed) *Fundamentals of Aquatic Toxicology*. 2nd ed, Taylor and Francis, London, 3-66.

Raviraja NS, Nikolcheva LG, Bärlocher F (2006) Fungal growth and leaf decomposition are affected by amount and type of inoculum and by external nutrients. *Sydowia*, 58:91-104.

Skovhus TL, Ramsing NB, Holmström C, Kjelleberg S, Dahllöf I (2004) Real-Time quantitative PCR for assessment of abundance of *Pseudoalteromonas* species in marine samples. *Appl Environ Microbiol*, 70:2373-2382.

Sridhar KR, Krauss G, Bärlocher F, Raviraja NS, Wennrich R, Baumbach R, Krauss G-J (2001) Decompostion of alder leaves in two heavy metal–polluted streams in central Germany. *Aquat Microb Ecol*, 26:73-80.

Suberkropp K (1998) Microorganisms and organic matter decomposition. In, Naiman RJ, Bilby RE (eds) *River ecology and management: lessons from the Pacific Coastal Ecoregion*. Springer, New York, USA, 120-143.

Suberkropp K, Gessner MO (2005) Acetate incorporation into ergosterol to determine fungal growth rates and production. In Graça MAS, Bärlocher F, Gessner MO (eds) *Methods to study litter decomposition: a practical guide*. Springer, Dordrecht, The Netherlands, 197-202.

Treton C, Chauvet E, Charcosset J-Y (2004) Competitive interaction between two aquatic hyphomycete species and increase in leaf litter breakdown. *Microb Ecol*, 48:439-446.

Zar JH (1996) *Biostatistical analysis*. 3rd ed, Prentice-Hall, Englewood Cliffs.

Zhang Q-G, Zhang D-Y (2006) Species richness destabilizes ecosystem functioning in experimental aquatic microcosms. *Oikos*, 112:218-226.

Chapter 4

General discussion and future perspectives

General discussion and future perspectives

Leaf-litter from riparian vegetation constitutes the major source of energy in low-order forested streams (Webster and Benfield 1986). The decomposition of this material is a key process in freshwater ecosystems and it is mainly accomplished by microbial decomposers and invertebrate shredders (Gessner et al. 1999). Among microbial decomposers, fungi, particularly aquatic hyphomycetes, seem to have a major role in leaf decomposition (Baldy et al. 2002, Pascoal and Cássio 2004) and enhance leaf nutritional value to shredder consumption (Graça 2001).

A positive relationship between fungal diversity and leaf decomposition has been found (Bärlocher and Corkum 2003, Treton et al. 2004, Duarte et al. 2006, Raviraja et al. 2006, but see Dang et al. 2005). In this work, four isolates of aquatic hyphomycetes, comprising three species and two strains with different background (isolated from clean and metal-polluted sites) were manipulated to assess the effect of fungal diversity on leaf decomposition. Overall, both species number and identity affected fungal activity on decomposing leaves (Chapter 3). Moreover, fungal performances in multicultures were higher than those expected from the sum of monoculture performances, which is in agreement with previous findings (Bärlocher and Corkum 2003, Duarte et al. 2006). This suggests complementarity effects among fungal species for biomass buildup, fungal reproduction and leaf decomposition. However, in previous works, it was not possible to assess the contribution of individual fungal species within communities to overall performances, making it difficult to confirm if the positive effects of fungal diversity on leaf decomposition resulted from complementarity or selection effects. The contributions of species in multicultures, calculated from Real-Time Polymerase Chain Reaction (Real-Time PCR) assays using specific primers for each fungal species (Chapter 3), together with the ergosterol-to-biomass conversion factors calculated for each isolate (Chapter 2), permitted to assess the contribution of each fungal species to the total biomass in multicultures. In the absence of stress, the higher performances of fungal species in multicultures resulted mainly from complementarity effects, suggesting the existence of resource partitioning and/or facilitation among fungal species (Loreau and Hector 2001).

Freshwaters are among the most endangered ecosystems in the world (Dudgeon et al. 2006) being metal pollution of great concern due to metal non-degradability, accumulation in the biota and biomagnification along aquatic food webs (Croteau et al. 2005, Marie et al. 2006). In this work, fungal biomass was affected by cadmium (Cd) exposure either when fungi were grown in culture medium (Chapter 2) or on

decomposing leaves (Chapter 3). The strain of *Articulospora tetracladia* (AT61) and *Flagellospora curta* (FC39) both isolated from a metal-polluted site were more tolerant to metal stress than the other fungal isolates. However, *Tricladium splendens* (TS100), which was also isolated from a polluted site, did not show an increased tolerance to Cd suggesting, as previously observed, that metal tolerance can be species-specific (Guimarães-Soares 2005, Azevedo 2007). Also, the strain of *A. tetacladia* (AT72) isolated from a clean site showed lower tolerance to Cd than the correspondent strain from the polluted site (AT61) corroborating that metal tolerance can be strain-specific (Braha et al. 2007, Miersch and Grancharov 2008). It is also interesting to note that the pattern of species tolerance to Cd was similar across different substrates (either when fungi were growing on culture medium or on leaves), as previously observed (Azevedo 2007).

In metal-polluted streams, fungal biomass and leaf decomposition seem to be less affected by metals than fungal diversity and/or reproduction (Niyogi et al. 2002, Duarte et al. 2004). However, in severely metal-polluted streams both fungal diversity and ecological functions were reduced (Sridhar et al. 2001). In our study, leaf mass loss and fungal biomass and sporulation were affected by Cd (Chapter 3). Fungal biomass was lower in multicultures than expected from the sum of monocultures suggesting interspecific competition among fungal species (Loreau and Hector 2001). Still, microcosms in which *A. tetracladia*, isolated from the polluted site (AT61), was present showed similar leaf decomposition in the presence or absence of Cd, suggesting that some populations can acclimate and/or adapt to stressful conditions and maintain ecological functions (Niyogi et al. 2002, Pascoal et al 2005). Treatments in which *A. tetracladia* was absent showed lower leaf decomposition, and fungal biomass and sporulation. These findings together with the high percentages of contribution to total conidia production shown by *A. tetracladia* in reference streams of Northwest Portugal (Duarte et al. 2004, Pascoal et al. 2005) suggests that it can be a key-species. Moreover, the importance of specific strain traits was evident in this work since the strain of *A. tetracladia* isolated from the polluted site showed higher leaf decomposition and biomass performances, than the correspondent strain from the clean site. Thus, if stress is severe enough to inhibit this species activity, decomposition process in freshwater ecosystems might be compromised.

Real-Time PCR assay by discriminating the relative percentages of each fungal species in multicultures helped to explain the effects of species number and identity on fungal activity. Additionally, it would be interesting to develop a Real-Time PCR assay to quantify the absolute fungal biomass of each species within multicultures,

to achieve fungal contribution to total biomass. Also, an effort should be driven to increase our knowledge about the genome of aquatic fungi in order to facilitate the application of molecular techniques like Real-Time PCR to a larger number of species.

As a whole, fungal species number and identity seemed to have an important role in leaf decomposition process. Fungal species identity can influence the palatability of leaf litter for invertebrate consumption (Suberkropp and Arsuffi 1984). Also, increased invertebrate mortality was reported after consumption of fungal biomass grown with metals (Abel and Bärlocher 1988). Therefore, it would be interesting to study the effects of fungal species diversity and identify on invertebrates feeding preferences under metal stress, to assess the potential consequences of metal pollution to higher trophic levels.

References

Abel T, Bärlocher F (1988) Uptake of cadmium by *Gammarus fossarum* (Amphipoda) from food and water. *J Appl Ecol*, 25:223-231.

Azevedo M-M (2007) *Toxicity of metals in aquatic hyphomycetes: cellular targets and defense mechanisms*. PhD thesis, University of Minho, Braga, Portugal.

Baldy V, Chauvet E, Charcosset J-Y, Gessner MO (2002) Microbial dynamics associated with leaves decomposing in the mainstem and floodplain pond of a large river. *Aquat Microb Ecol*, 28:25-36.

Bärlocher F, Corkum M (2003) Nutrient enrichment overwhelms diversity effects in leaf decomposition by stream fungi. *Oikos*, 101:247-252.

Braha B, Tintemann H, Krauss G, Ehrman J, Bärlocher F, Krauss G-J (2007) Stress response in two strains of the aquatic hyphomycete *Heliscus lugdunensis* after exposure to cadmium and copper ions. *BioMetals*, 20:93-105.

Croteau M-N, Luoma SN, Stewart AR (2005) Trophic transfer of metals along freshwater food webs: Evidence of cadmium biomagnification in nature. *Limnol Oceanogr*, 50:1511-1519.

Dang CK, Chauvet E, Gessner MO (2005) Magnitude and variability of process rates in fungal diversity-litter decomposition relationships. *Ecol Lett*, 8:1129-1137.

Duarte S, Pascoal C, Cássio F (2004) Effects of zinc on leaf decomposition by fungi in streams: studies in microcosms. *Microb Ecol*, 48:366-374.

Duarte S, Pascoal C, Cássio F, Bärlocher F (2006) Aquatic hyphomycete diversity and identity affect leaf litter decomposition in microcosms. *Oecologia*, 147:658-666.

Dudgeon D, Arthington AH, Gessner MO, Kawabata Z-I, Knowler DJ, Lévêque C, Naiman RJ, Prieur-Richard A-H, Soto D, Stiassny MLJ, Sullivan CA (2006) Freshwater biodiversity: importance, threats, status and conservation challenges. *Biol Rev*, 81:163-182.

Gessner MO, Chauvet E, Dobson M (1999) A perspective on leaf litter breakdown in streams. *Oikos*, 85:377-384.

Graça MAS (2001) The role of invertebrates on leaf litter decomposition in streams – a review. *Int Rev Hydrobiol*, 86:383-393.

Guimarães-Soares L (2005) *Biochemical and physiological responses of the aquatic fungi* Fontanospora fusiramosa *and* Flagellospora curta *to cadmium, copper and zinc.* PhD thesis, University of Minho, Braga, Portugal.

Loreau M, Hector A (2001) Partitioning selection and complementarity in biodiversity experiments. *Nature*, 412:72-76.

Marie V, Baudrimont M, Boudou A (2006) Cadmium and zinc bioaccumulation and metallothionein response in two freshwater bivalves (*Corbicula fluminea* and *Dreissena polymorpha*) transplanted along a polymetallic gradient. *Chemosphere*, 65:609-617.

Miersch J, Grancharov K (2008) Cadmium and heat response of the fungus *Heliscus lugdunensis* isolated from highly polluted and unpolluted areas. *Amino Acids*, 34:271-277.

Niyogi DK, McKnight DM, Lewis Jr WM (2002) Fungal communities and biomass in mountain streams affected by mine drainage. *Arch Hydrobiol*, 155:255-271.

Pascoal C, Cássio F (2004) Contribution of fungi and bacteria to leaf litter decomposition in a polluted river. *Appl Environ Microbiol*, 70:5266-5273.

Pascoal C, Cássio F, Marvanová L (2005) Anthropogenic stress may affect aquatic hyphomycete diversity more than leaf decomposition in a low-order stream. *Arch Hydrobiol*, 162:481-496.

Raviraja NS, Nikolcheva LG, Bärlocher F (2006) Fungal growth and leaf decomposition are affected by amount and type of inoculum and by external nutrients. *Sydowia*, 58:91-104.

Sridhar KR, Krauss G, Bärlocher F, Raviraja NS, Wennrich R, Baumbach R, Krauss G-J (2001) Decompostion of alder leaves in two heavy metal–polluted streams in central Germany. *Aquat Microb Ecol*, 26:73-80.

Suberkropp K, Arsuffi TL (1984) Degradation, growth and changes in palatability of leaves colonized by six aquatic hyphomycete species. *Mycologia*, 76:398-407.

Treton C, Chauvet E, Charcosset J-Y (2004) Competitive interaction between two aquatic hyphomycete species and increase in leaf litter breakdown. *Microb Ecol*, 48:439-446.

Webster JR, Benfield EF (1986) Vascular plant breakdown in freshwater ecosystems. *Annu Rev Ecol Syst*, 17:567-594.