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# Biodiversity and activity of microbial decomposers of leaf litter in streams under anthropogenic stress

Ph. D. Thesis in Sciences

Work Supervised By
Prof. Dr. Fernanda Cássio

June 2008

É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA TESE APENAS PARA EFEITOS DE INVESTIGAÇÃO MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO QUE A TAL SE COMPROMETE

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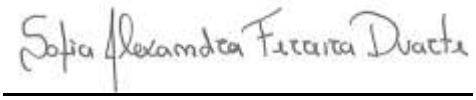

(Sofia Alexandra Ferreira Duarte)

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## Abstract

Human activities are threatening biodiversity in freshwaters leading to irreversible alterations in ecosystem processes. One of the most important processes for the functioning of small-forested streams is the decomposition of allochthonous plant litter, which constitutes the major source of nutrients and energy for freshwater food-webs. Microbial decomposers, namely fungi and bacteria, play a critical role in this process degrading leaf material and increasing leaf palatability for invertebrate shredders. An obvious question that arises is in what extent pollution can affect the diversity of microbial decomposers altering the functions they perform in freshwater ecosystems.

In a microcosm experiment, we showed that the loss of aquatic fungal species affected fungal biomass and reproduction, but not leaf mass loss. Complementarity effects appeared to occur between fungal species because multicultures had higher performances than those expected from individual performances in monocultures. Moreover, lower fungal biomass and leaf mass loss were found in the absence of *Articulospora tetracladia* and species identity affected all measured parameters.

In a transplant experiment, we investigated how a community of microbial decomposers adapted to a reference site responds to a sudden decrease in the water quality. The transfer of leaves colonized at a reference site to a site with high concentration of nutrients and heavy metals in the stream water reduced fungal diversity and sporulation, but not fungal biomass and leaf decomposition. This suggests that high diversity of fungi may mitigated the impact of anthropogenic stress in streams.

Most studies addressing microbial diversity on decomposing leaves rely on the microscopic identification of fungal conidia and on the number of bacterial morphotypes. However, the production of conidia by fungi varies with the species and it is affected by several environmental factors. On the other hand, bacteria have few morphological differences, making it difficult to accurately assess microbial diversity. In our work, DNA fingerprinting techniques were successfully used for assessing fungal and bacterial diversity. Denaturing gradient gel electrophoresis (DGGE) showed a more diverse microbial community on decomposing leaves than microscopic techniques. Moreover, DGGE allowed detecting shifts in microbial communities during leaf decomposition and under different stress conditions (eutrophication and metal pollution).

The structure of fungal and bacterial communities on decomposing leaves changed along a gradient of inorganic nitrogen and phosphorus in streams, as indicated by canonical correspondence analysis based on the morphology of fungal conidia and on DNA fingerprinting. Sporulation was depressed in the most eutrophic streams, while bacterial biomass appeared to be stimulated, except in the presence of high nitrites and ammonium concentrations. Leaf decomposition rate was stimulated at only one site with moderate eutrophication.

The exposure of naturally colonized leaves to environmentally realistic concentrations of copper and zinc alone or in mixtures showed that metal exposure altered the structure of fungal and bacterial communities on decomposing leaves. Exposure to metal mixtures or to the highest Cu concentration significantly reduced leaf decomposition rates and fungal reproduction, but not fungal biomass. Bacterial biomass was strongly inhibited by all metal treatments. Moreover, the combined effects of Cu and Zn on microbial decomposition of leaf litter were mostly additive, because observed effects did not differ from those expected as the sum of single metal effects. However, antagonistic effects on bacterial biomass were found in all metal combinations and on fungal reproduction in metal combinations with the highest Cu concentrations, particularly at longer exposure times.

Moreover, the sequence by which metals were added to microcosms affected fungal biomass and sporulation, but not bacterial biomass, probably because microbial sensitivities to the metals were different. The resistance of microbial decomposers to Cu did not increase when communities were previously acclimated to Zn and *vice-versa*. Microbial decomposers could be expending considerable energy to maintain their functions under the stress imposed by the first metal and if so, species resistance might be diminished when the second metal was added. After release from metals, the structure of fungal communities became similar to that of control, as indicated by the principal response curves of sporulating species and also by the DGGE analyses. A recovery of the microbial activity seemed also to occur, as shown by the lack of differences in leaf mass loss, bacterial biomass and fungal reproduction between control and metal treatments.

# Resumo

As actividades humanas estão a ameaçar a biodiversidade dos ecossistemas aquáticos, conduzindo a alterações irreversíveis no seu funcionamento. Um dos processos mais importantes para o funcionamento dos ecossistemas de rios de baixa ordem é a decomposição de detritos vegetais alóctones, que constituem a principal fonte de carbono e energia para as cadeias alimentares nesses sistemas de água doce. Os microrganismos decompositores aquáticos, nomeadamente os fungos e as bactérias, desempenham um papel fundamental na decomposição dos detritos vegetais e aumentam a sua palatabilidade para os invertebrados trituradores. Uma questão relevante no âmbito da Ecologia actual é a de saber se a poluição afecta a biodiversidade e quais os impactos para o funcionamento dos ecossistemas aquáticos.

Numa experiência em microcosmos, mostrámos que a perda de espécies de fungos aquáticos afectava a biomassa e a reprodução dos fungos, mas não a decomposição de folhada. As espécies de fungos pareceram exibir efeitos de complementaridade uma vez que as culturas mistas tiveram desempenhos superiores ao esperado a partir dos desempenhos em cultura pura. Contudo, a identidade das espécies afectou todos os parâmetros analisados. Além disso, a biomassa dos fungos e a perda de massa foliar foram menores na ausência de *Articulospora tetracladia*.

Numa experiência de transplante de folhas entre rios, investigámos como uma comunidade de microrganismos decompositores adaptados a um local de referência responde a um declínio abrupto na qualidade da água. A transferência de folhas colonizadas num local de referência para um local com concentrações elevadas de nutrientes e metais pesados na água reduziu a diversidade e a esporulação dos fungos, mas não a sua biomassa e a decomposição foliar. Isto sugere que uma elevada diversidade de fungos pode contribuir para atenuar o impacto de stressores antropogénicos nos rios.

A maioria dos estudos efectuados tem analisado a diversidade de microrganismos associados a folhas em decomposição com base na identificação microscópica das conídias libertadas pelos fungos e nos tipos morfológicos de bactérias. No entanto, a produção de conídias pelos fungos varia com a espécie e é afectada por vários factores ambientais. Por outro lado, as bactérias possuem poucas diferenças morfológicas entre si, tornando difícil avaliar com exactidão a diversidade microbiana. No nosso trabalho, a electroforese em gradiente desnaturante (DGGE) do DNA microbiano mostrou uma comunidade de fungos e de bactérias mais diversa em folhas em decomposição do que as técnicas de microscopia. O DGGE permitiu detectar alterações na estrutura das comunidades durante a decomposição foliar e em diferentes condições de stresse (eutrofização e poluição por metais).

A estrutura das comunidades de fungos e de bactérias nas folhas em decomposição sofreu alterações ao longo de um gradiente de azoto e de fósforo nos rios, como indicado

pelas análises de correspondência canónica baseadas na morfologia das conídias dos fungos e no perfil de DGGE. A esporulação diminuiu nos rios mais eutrofizados, enquanto que a biomassa de bactérias pareceu ser estimulada, excepto na presença de concentrações elevadas de nitritos e amónia. A taxa de decomposição foliar foi estimulada apenas num dos locais com eutrofização moderada.

A exposição de folhas colonizadas naturalmente a concentrações de cobre e de zinco, ambientalmente realísticas, alterou a estrutura das comunidades de fungos e de bactérias nas folhas em decomposição. A exposição às misturas de metais ou à concentração de Cu mais elevada reduziu significativamente a taxa de decomposição foliar e a reprodução dos fungos, mas não inibiu a biomassa dos fungos. A biomassa bacteriana foi inibida em todos os tratamentos com metais. Além disso, os efeitos combinados do Cu e do Zn na decomposição microbiana da folhada foram maioritariamente aditivos, uma vez que os efeitos observados não diferiram dos esperados a partir da soma dos efeitos de cada metal isolado. No entanto, foram observados efeitos antagonísticos na biomassa de bactérias, em todas as combinações de metais, e na reprodução de fungos nas combinações contendo Cu na concentração mais elevada, particularmente em tempos mais longos de exposição.

A sequência pela qual os metais foram adicionados aos microcosmos afectou a biomassa e a esporulação dos fungos, mas não a biomassa das bactérias provavelmente porque a sensibilidade dos microrganismos aos dois metais era diferente. A resistência dos microrganismos decompositores ao Cu não aumentou quando as comunidades foram previamente aclimatadas ao Zn e *vice-versa*. Os microrganismos poderiam estar a consumir uma fracção de energia considerável para manter as suas funções na presença do primeiro metal e, por este motivo, a sua resistência poderia estar diminuída quando o segundo metal foi adicionado. Após libertação do stresse metálico, a estrutura das comunidades de fungos tornou-se semelhante à das comunidades controlo, como indicado pelas curvas de resposta principal das espécies identificadas a partir dos esporos e pela análise de DGGE. Além disso, a actividade microbiana pareceu recuperar após a libertação do stresse metálico, como sugerido pela ausência de diferenças na perda de massa foliar, na biomassa das bactérias e na reprodução dos fungos entre o controlo e os tratamentos com metais.

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# Chapter 1

*General introduction*

## 1.1. The importance of biodiversity for the functioning of ecosystems

### *1.1.1. Hypotheses explaining the relationship between biodiversity and ecosystem functioning*

The loss of species and alterations in the composition of biotic communities, as result of human activities, increased the concern on the effects of biodiversity on ecosystem functioning, particularly during the last two decades (Hooper *et al.* 2005). Most studies showed a positive relationship between increased species diversity and ecosystem functioning (Naeem *et al.* 1994, 1996, Tilman and Downing 1994, Tilman *et al.* 1997), while others did not find any relationship (Huston *et al.* 2000, Schwartz *et al.* 2000).

Several hypotheses have been proposed to explain the effect of species loss on ecosystem functioning (reviewed in Johnson *et al.* 1996): the stability-productivity hypothesis (MacArthur 1955), the rivet hypothesis (Erlich and Erlich 1981), the redundancy hypothesis (Walker 1992) and the idiosyncratic hypothesis (Lawton 1994). The stability-productivity hypothesis, predicts a linear, positive relationship between species richness and ecosystem functioning, with no function with no species and the highest function at the maximum species richness. Thus, the increase in species number within a community would increase the productivity, and the ability of the community to recover from disturbances. The rivet hypothesis predicts a positive non-linear relationship between species diversity and ecosystem functioning. This hypothesis assumes that species make up important parts of an ecosystem like rivets holding and engine together. The effect of losing parts is always slightly negative, but when reaching a critical number of losses, a large negative effect on the functioning will be produced. On the other hand, the redundancy hypothesis predicts a positive, asymptotic relationship where the lost of species is of little consequence to ecosystem functioning, as long as all functional groups are well represented. Many species have the same function, and the loss of one species can therefore be offset by other species. The idiosyncratic hypothesis predicts the possibility of a null or indeterminate relationship between species richness and ecosystem function, depending on the environmental context and the traits of lost species.

It is still not clear which of these hypotheses best describes the relationship between biodiversity and ecosystem functioning. The experiments concerning biodiversity effects on ecosystem functioning gave rise to results that support one or

more of these hypotheses and thus a single and general relationship between species diversity and rates of ecological processes does not possibly exist (Johnson *et al.* 1996, Cardinale *et al.* 2000). Moreover, the form and cause of these relationships may change with the environmental context and thus the relationship between species diversity and ecosystem functioning are expected to be dynamic and changing over time and space (Cardinale *et al.* 2000).

### *1.1.2. Mechanisms underlying biodiversity effects*

There are several experimental and theoretical studies that deal with the mechanisms involved in the positive effects of biodiversity on ecosystem functioning (Tilman *et al.* 1997, Loreau 1998, Loreau and Hector 2001, Cardinale *et al.* 2002). However, the proposed mechanisms can produce very similar results, which make the clarification of the underlying mechanisms responsible for the biodiversity effects a very difficult task. Pioneer experiments with terrestrial plant assemblages explained the positive effects of biodiversity through two main processes: sampling effect and niche complementarity (Tilman *et al.* 1997, Huston 1997, Aaersen 1997, Hector *et al.* 1999). The sampling effect implies that the probability of encountering dominant species, which tend to be those with the greatest impact on ecosystem functioning, is higher in highly diverse communities (e.g. Huston 1997, Hector *et al.* 1999). It is a very used explanation for diversity effects (Aarsen 1997, Huston 1997, Tilman *et al.* 1997) but there is still a debate if this is a confounding effect in experimental studies (Huston 1997) or an important property of natural communities (e.g. Tilman *et al.* 1997).

More recently, the selection effect, a mechanism related to the sampling effect, was also proposed (Loreau 2000). The selection effect takes into account selective pressures determining extreme trait values in a community. Depending on which traits within the community are favoured, effects of species richness on ecosystem processes may be negative or positive.

Niche complementarity is often difficult to distinguish from sampling effect, and they may be viewed as concomitant processes (Naeem 2002). In a community of species with complementary niches, positive interactions predominate (facilitation) and the differences between species (niche differentiation), which are higher in more diversified communities, lead to a better use of resources (Loreau and Hector 2001).

### *1.1.3. Biodiversity effects in fluctuating environments*

All ecosystems are exposed to environmental changes and an important aspect in ecosystem functioning is stability, defined as the system ability to avoid displacement following a perturbation (resistance) and to return to its previous state after disturbance (resilience) (McCann 2000).

The insurance hypothesis (Walker 1992) was proposed to explain how processes are affected by the loss of species in an environment under fluctuations. This hypothesis states that increasing biodiversity insures ecosystems against declines in their functions caused by environmental changes. Yachi and Loreau (1999), proposed a stochastic model of the insurance hypothesis to assess the effects of species richness within a trophic level or functional group on the expected temporal mean and variance of ecosystem productivity based on individual species responses to environmental fluctuations. This model was based on two ideas: 1) increasing diversity increases the chance that at least some species will respond differently to variable conditions and perturbations and 2) greater diversity increases the chance that an ecosystem will contain species that are capable of replacing functionally keystone species.

The underlying theory is the functional redundancy among species. Since different species are able to fulfil the same function, changes in the ecosystem functioning will be prevented (Walker 1992, Gitay *et al.* 1996). Thus, in a system with high diversity a disturbance may remove some redundant species without loss of function, but in a system with low diversity a decrease of species richness could more easily lead to a loss of function.

### *1.1.4. The role of biodiversity in the functioning of freshwater ecosystems*

Freshwaters are among the most threatened ecosystems on Earth (Dudgeon *et al.* 2006), however studies on the effects of species loss on stream processes only started to emerge five-eight years ago (Jonsson and Malmqvist 2000, Jonsson *et al.* 2002, Cardinale *et al.* 2002, Covich *et al.* 2004). Aquatic systems have particular features which are probably large enough to render different results about the consequences of species loss to the functioning of ecosystems (Giller *et al.* 2004, Gessner *et al*. 2004, Humbert and Dorigo 2005). The physical nature of water and hydrological processes, the weakness of barriers and the strong interconnection between habitat types are the main features of aquatic systems. This allows higher

propagule dispersion, faster rate of transfer across membranes, greater biogeochemical dynamics and a rapid turnover of primary producers (Warwick and Clarke 2001). The capacity of recolonization by new recruits after disturbance and extinction events is also higher in aquatic systems than in terrestrial ones (Giller *et al.* 2004).

Much of the research on the relationships between aquatic biodiversity and ecosystem functioning was done with macroinvertebrates and decomposition process in streams (Jonsson and Malmqvist 2000, 2003a,b, Jonsson *et al.* 2002, Cardinale *et al.* 2002). The major conclusions were that species richness matters for process rates among stream macroinvertebrates in a variety of ways depending on the type of organisms, their interactions with other species and the type and resources they feed (Jonsson and Malmqvist 2000, 2003a,b, Jonsson *et al.* 2002, Cardinale *et al.* 2002). Positive interactions found on several studies were mainly attributed to complementarity through facilitation among freshwater invertebrates (Cardinale *et al.* 2002, Jonsson and Malmqvist 2000, 2003a). Other studies with macrophyte species (Engelhardt and Ritchie 2001, Zedler *et al.* 2001) attributed the positive effects of biodiversity (increase of total biomass and reduction of phosphorus loss) to the sampling effect.

Important evidences on the role of aquatic diversity in ecosystem functioning may also be provided by microorganisms since they drive ecosystem processes to a considerable extent. Naeem *et al.* (2000), in a microcosm experiment manipulating the diversity of both producers (green algae) and decomposers (heterotrophic bacteria), found that variations in algal production were strongly influenced by the co-dependency between algae and bacteria for carbon use, rather than being explained by either algal or bacterial diversity alone.

Some studies also indicated that fungal diversity appears to be important for plant-litter decomposition in freshwaters, although some controversial results were found (Bärlocher and Corkum 2003, Treton *et al.* 2004, Dang *et al.* 2005, Raviraja *et al.* 2006, see Pascoal and Cássio 2008, for a review). Positive effects were mainly attributed to complementarity (resource partitioning among fungal species), but also to the sampling effect (Bärlocher and Corkum 2003, Treton *et al.* 2004, Raviraja *et al.* 2006).

## 1.2. Plant-litter decomposition in freshwater ecosystems

The allochtonous input of coarse particulate organic matter (CPOM) from surrounding vegetation is the major source of nutrients and energy for food webs in small-forested streams (Cummins 1974). Generally, these systems have little primary production due to shading from the surrounding canopies, low levels of inorganic nutrients and low temperatures (Benfield 1996). Coarse particulate organic matter is mainly constituted by leaves that enter the streams and can be used by stream consumers and decomposers or stored or transported downstream, depending on the retentiveness of the stream reach (Larrañaga *et al.* 2003, Elosegi 2005).

It is assumed that during breakdown, leaves release solutes and are colonized by microorganisms and invertebrates, and the original leaf is transformed into several products including microbial and invertebrate biomass, fine particulate organic matter (FPOM), dissolved organic matter (DOM), inorganic nutrients and carbon dioxide (Gessner *et al.* 1999); this occurs during three distinct stages: leaching, conditioning and fragmentation (for a review *see* Gessner *et al.* 1999, Abelho 2001). Although these stages tend to occur sequentially, leaf decomposition is a complex process and some of the events can occur simultaneously (Gessner *et al.* 1999).The first stage, leaching, is characterized by a substantial abiotic loss (up to 30%) of soluble substances, such as phenolics, carbohydrates and amino acids within 24 hours to up to 7 days after leaf immersion (Graça and Pereira 1995, Canhoto and Graça 1996, Casas and Gessner 1999). Conditioning is characterized by the colonization and growth of microorganisms, mainly fungi and bacteria, on leaf litter that enhance leaf palatability to invertebrate shredders (Suberkropp 1998b, Graça 2001). Finally, the last stage occurs by means of physical and biotic fragmentation and usually follows some period of tissue softening by the microbial enzymes. Physical fragmentation occurs as a result of the abrasion exerted by the flowing water, while biotic fragmentation is mainly generated through the feeding and digestive activities of invertebrate shredders that significantly contribute for the production of FPOM (Cummins 1974). Microorganisms, in particular fungi, also contribute for biotic fragmentation, since they produce enzymes that break structural plant polymers promoting leaf mass loss (Cummins and Klug 1979, Suberkropp 1998b, Gessner *et al.* 2007).

Plant-litter decomposition in streams can be affected by several internal and external factors that affect the biotic assemblages involved in the process, altering

its velocity (Abelho 2001). Internal factors include the chemical and physical characteristics of the leaves, and also its state, e.g., senescent leaves loose their compounds faster than fresh leaves (Bärlocher 1997). External factors include e.g., stream water chemistry (pH, alkalinity and nutrient concentrations), climate and hydrologic fluctuations (reviewed in Gessner *et al.* 1999, Abelho 2001).

## 1.3. Aquatic biota involved in plant-litter decomposition

### *1.3.1. Microorganisms: fungi and bacteria*

Leaves shed from riparian vegetation can be colonized by a variety of terrestrial fungi (Bärlocher and Kendrick 1974, Suberkropp and Klug 1976), but little is known about the activity of these fungi after the entrance of leaves in streams, and a predominant role in leaf conditioning by fungi has been attributed to aquatic hyphomycetes (Suberkropp 1998b, Bärlocher 2005a, Gessner *et al.* 2007). Aquatic hyphomycetes, also named Ingoldian fungi, are an ecological group of fungi whose sexual stage of most species has not been described yet (Shearer *et al.* 2007). Most evidences from the anamorph/teleomorph relationships show that most species belong to Ascomycota and some to Basidiomycota (Marvanová 1997, Nikolcheva and Bärlocher 2002, Baschien 2003, Belliveau and Bärlocher 2005, Bärlocher 2007, Shearer *et al.* 2007).

Aquatic hyphomycetes are commonly found growing on a wide range of substrates, such as leaves and wood, in flowing waters worldwide (Bärlocher 2005a, Gessner *et al.* 2007). The success of these fungi as substrate colonizers is mainly attributed to morphological and physiological adaptations to fast flowing waters (Suberkropp 1998b, Bärlocher 2005a). Morphological adaptations include the high production rates of tetraradiate- or sigmoid-shaped conidia, which allow an efficient attachment to substrata also enhanced by the production of mucilages at the ends of conidial arms (Read *et al.* 1992). Physiological adaptations are related to their ability of producing a variety of extracellular enzymes, with cellulolytic, pectinolytic and proteolytic activity (Suberkropp and Klug 1980, Chamier 1985, Suberkropp 1992), which are able to break the major plant polysaccharides. Moreover, aquatic hyphomycetes can grow and reproduce at relatively low temperatures (Suberkropp 1984) common in temperate climates during autumn fall.

In addition to filamentous fungi, yeasts and yeast-like organisms are commonly found in aquatic environments (Spencer *et al.* 1970), with saprophytic

yeasts being common on the surfaces of leaves (Sampaio *et al.* 2001, 2004, 2007). Although only few yeast species are able to degrade cellulose (Dennis 1972) and xylan (Biely *et al.* 1978, Jiménez *et al.* 1991), several species have been found associated with decomposing plant litter and they differ among leaf types and during time of leaf decomposition (Sampaio *et al.* 2001, 2004, 2007).

Bacteria are also able to produce enzymes that degrade the polysaccharides of plant litter (Burns 1982), but its contribution to plant litter decomposition in streams appears to be lower than that of fungi, as assessed from microbial biomass and productivity (Baldy *et al.* 1995, 2002, Hieber and Gessner 2002, Pascoal and Cássio 2004, Pascoal *et al.* 2005a,b). However, in submerged marsh plant litter, bacteria appear to have a greater contribution than fungi to total microbial production (Buesing and Gessner 2006). The lower contribution of bacteria to leaf decomposition can be related to the lack of invasive ability, which confines bacteria to leaf surfaces, with the exception of tunneling bacteria (Porter *et al.* 1989).

Recently, Das *et al.* (2007) showed that actinomycetes could also play a role in leaf-litter decomposition in streams, which is consistent with previous studies showing that actinomycetes can grow on recalcitrant compounds such as lignocellulose (Adhi *et al.* 1989) and lignin (Moran and Hodson 1989). On the other hand, DNA levels of Archaebacteria were consistently low during litter breakdown, suggesting a minor contribution of this group of bacteria to the process (Manerkar *et al.* 2008).

Fungi and bacteria are reported to have both synergistic (Wohl and McArthur 2001, Romaní *et al.* 2006) and antagonistic (Gulis and Suberkropp 2003c, Mille-Lindblom and Tranvik 2003, Romaní *et al.* 2006, Wohl and McArthur 2001) interactions during leaf decomposition. Bacteria can utilize organic compounds (namely FPOM and DOM) released from the degradation of plant litter due to fungal and invertebrate activities (Sinsabaugh and Findlay 1995) and from the lysis of dead fungal mycelia (Gulis and Suberkropp 2003b). In addition, bacteria are reported to grow better together with fungi than alone and to have low enzymatic activities in the absence of fungi (Romaní *et al.* 2006). However, both groups of microorganisms may also compete for resources. Aquatic fungi are reported to produce antibiotics that inhibit the growth of bacteria (Gulis and Stephanovich 1999) and a suppression of fungal growth was reported in the presence of bacteria (Wohl and McArthur 2001, Romaní *et al.* 2006), probably due to the production of fungicides or chitinolytic enzymes.

### *1.3.2. Macroinvertebrate detritivores*

Cummins (1973) classified macroinvertebrates into the major categories of shredders, grazers or scrapers, collectors (both gatherers and filterers), and predators. Both shredders and collectors constitute the macroinvertebrates detritivores, feeding on CPOM and FPOM, respectively (Graça 2001). However, a major role on litter breakdown in streams is attributed to shredders, since they have mouthparts adapted for the maceration of CPOM particles, which are physically converted to FPOM (Graça 2001, Alan and Castillo 2007). Additionally, FPOM is also produced by shredders in the form of fecal pellets.

Several experiments show that shredders preferentially feed on conditioned leaves, revealing the importance of microbes, particularly fungi, to the improvement of the nutritional quality of leaves (Chergui and Pattee 1991, Graça *et al.* 1993a,b, Graça 2001). Invertebrates may benefit from the microbial biomass on leaves by two distinct ways. Firstly, by directly feeding on microorganisms, since nutrient content per unit mass can be several folds greater than that of the leaf substrate (Alan and Castillo 2007), and secondly, by eating the modified plant substrates due to the enzymatic microbial action over the structural carbohydrates (cellulose, hemicellulose, and pectin) (Bärlocher 1985). This includes both partial digestion of the plant substrates into subunits that detritivores are capable of assimilate and the activity of the exoenzymes produced by microorganisms that remain active even after invertebrate ingestion (Alan and Castillo 2007). Furthermore, consumption of leaves by shredders appears to be affected by the type of fungal species colonising leaves (Bärlocher and Kendrick 1973, Arsuffi and Suberkropp 1985, Graça *et al.* 1993a,b, Lecerf *et al.* 2005).

## 1.4. Methods to assess plant-litter decomposition in streams

### *1.4.1. Leaf decomposition rates*

Rates of plant litter decomposition are typically determined by placing known amounts of leaves in packs or bags in streams and measuring at several time intervals the amount of organic matter remaining (Suberkropp 1998b, Bärlocher 2005b). To partition the contribution of shredders and microorganisms to the process, breakdown of leaf litter enclosed in fine-mesh bags (ca. 0.5 mm mesh

size), which avoid the entrance of the animals, is compared to that of leaves placed in coarse-mesh bags (Boulton and Boon 1991).

Leaf litter breakdown in streams is usually characterized by the negative exponential model $m_t = m_0 \cdot e^{-kt}$, where $m_t$ is the leaf mass remaining at time $t$, $m_0$ is the initial leaf mass, $k$ is the rate of leaf decomposition and $e$ is the base of natural logarithm (Webster and Benfield 1986). Based on the decomposition rate, leaves can be classified as having fast (k>0.01 $d^{-1}$), medium (k=0.005-0.01 $d^{-1}$) and slow (k<0.005 $d^{-1}$) processing rates (Petersen and Cummins 1974). The wide variation in the decomposition rates among leaf species is well documented, with non-woody generally decomposing faster than woody plant leaves (Allan and Castillo 2007).

The negative exponential model for litter decomposition has been subject of several criticisms and revealed to be inappropriate for some situations, such as when leaves consist of two different components that decay at different rates (Bärlocher 2005b). In these situations, other mathematical equations describing the data more precisely or providing more realism were developed. However, the negative exponential model has been able to describe rather accurately the process of leaf breakdown in streams (Webster and Benfield 1986). Some of the alternative models are even derivations of the negative exponential model, such as those that account for the effect of temperature in breakdown rates (e.g. Rowe *et al.* 1996).

### *1.4.2. Microbial biomass and activity*

Fungal hyphae besides growing on leaves are able to penetrate leaf substrates, making it difficult to separate them from leaves (Newell 1992, Gessner and Newell 2002). As a result, the quantification of fungal biomass has been done by measuring cellular constituents specific of fungi that occur at constant amounts in the mycelium.

Ergosterol, which is a major component of fungal membranes, has been widely used to determine fungal biomass associated with decomposing leaf litter (Gessner and Newell 2002, Gessner 2005). The use of this membrane component combines a number of advantages: ergosterol is the major sterol in the majority of eumycotic fungi (Zygomycota, Ascomycota, Basidiomycota and Deuteromycota) and it is absent from vascular plants, metazoan and most other organisms; its structure allows to be well distinguished spectrophotometrically from plant sterols such as sitosterol and campesterol; and it is likely to be rapidly degraded upon cell death

suggesting that it can be considered an indicator of living fungal biomass (reviewed in Gessner and Newell 2002). Ergosterol quantification usually combines sterol extraction and saponification followed by the purification of the crude lipid extract, by high performance liquid cromatography (HPLC). Ergosterol can then be converted to fungal biomass by using a conversion factor of 5.5 mg ergosterol per g fungal dry mass, which was determined using several fungal isolates, allowing rough estimates of fungal biomass in natural systems (Gessner and Chauvet 1993).

Other alternatives for fungal biomass quantification are the determination of chitin, which is a cell-wall component but also determines dead fungal mass (Newell 1992), and the quantification of ATP, which is not specific for fungi since it occurs in all living cells (Suberkropp *et al.* 1993).

For bacteria, cell counting in combination with data from bacterial cell volumes (Bratbak 1993) are the most used approach for determining bacterial biomass on decomposing plant litter (Baldy *et al.* 1995, Buesing and Gessner 2002, Hieber and Gessner 2002, Pascoal *et al.* 2005b). Bacterial cells are dislodged from plant material, usually by ultrasonic probes or in ultrasonic baths, and the cells collected on a filter are stained with a fluorescent dye, usually DAPI (4',6-diamidino-2-phenylindone) (Buesing 2005). The detached and stained bacterial cells are counted in an epifluorescence microscope and assigned to size and shape classes. Usually, bacterial biovolumes are determined as $V = (\pi / 4) \cdot W^2 \cdot (L - W / 3)$, where $W$ is the cell width and $L$ is the cell length (Bratbak 1993) and converted into bacterial carbon according to the allomeric model $C = 89.6 \cdot V^{0.59}$, where $V$ is the bacterial volume in $\mu m^3$ and $C$ is the bacterial carbon in *f*g (determined using bacterial assemblages from seawater, Simon and Azam 1989). Another used approach is the constant biomass model, which assumes that bacterial carbon per cell is constant corresponding to a mean value of 20 *f*g (determined from natural bacterioplankton assemblages, Lee and Fuhrman 1987, Norland 1993).

The advantages of this technique are that microscopic methods are direct and the underlying assumptions for estimating biomass from biovolume are few and relatively simple (Bratbak 1993). The disadvantages include the fact that it is not possible to distinguish live from dead bacteria, it is a labor intensive method and the limited resolution of light microscopes makes accurate size measurements of bacteria difficult (Bratbak 1993). Additionally, an efficient detachment of bacterial cells is crucial for accurately assess bacterial abundances and biomasses, and special care should be taken when choosing the detachment procedure (Buesing

and Gessner 2002). Nowadays it is also possible to use flow cytometry to reduce the time required for the analysis (del Giorgio *et al.* 1996). However, for bacteria associated with plant detritus this can be a problem due to the presence of detrital particles, which may be difficult to discriminate from the bacteria.

Microbial respiration has been widely used to estimate microbial activity associated with decomposing leaves (Graça and Abelho 2005). However, it is difficult to distinguish the contribution of leaf-associated bacteria from that of fungi to total respiration rates, even with the addition of selective inhibitors (Padgett 1993). Better approaches to measure fungal and bacterial activities are provided by measuring the incorporation rates of radiolabeled compounds (acetate for fungi and thymidine or leucine for bacteria) into biomolecules (ergosterol for fungi and DNA or protein for bacteria) (Suberkropp and Weyers 1996, Pascoal and Cássio 2004, Suberkropp and Gessner 2005, Buesing and Gessner 2005). In these methods, it is assumed that the rate of synthesis of these biomolecules is directly proportional to cell growth. However, in studies in which losses of fungal mass are negligible over the period of interest, growth rate and productivity can be inferred from measured differences in biomass (Newell 1992). Since a substantial proportion of fungal production on decomposing leaves is invested in the production of propagules (Bärlocher 2005c), namely asexual spores, sporulation rates can also be used as a measure of fungal activity. Moreover, high correlations have been established between fungal sporulation and biomass (Suberkropp 1991, Maharning and Bärlocher 1996). However, high sporulation rates of a species do not imply a high mycelial biomass (Bermingham *et al.* 1997), and it is active mycelium but not the conidia that decomposes leaves.

### *1.4.3. Microbial diversity: traditional versus molecular approaches*

Much of the current knowledge on aquatic hyphomycete diversity on decomposing leaves in streams has been acquired by the identification of their characteristic conidial shapes (Gessner *et al.* 2003, Bärlocher 2005a,c). Leaves colonized in streams are brought to the laboratory and aerated in microcosms containing filtered stream water, for approximately two days, and the released conidia are trapped in a filter, stained and identified under a light microscope (Gessner *et al.* 2003, Bärlocher 2005c). However, the contribution of each fungal species based on their reproductive ability can miss fungal taxa that are not sporulating (Nikolcheva *et al.* 2003, Nikolcheva *et al.* 2005). Indeed, other taxa than

aquatic hyphomycetes, belonging to Ascomycota, Basidiomycota, Chytridiomycota, Zygomycota and Oomycota, were found (Nikolcheva and Bärlocher 2004, Shearer *et al.* 2007). Moreover, since sporulation is often more sensitive than biomass to environmental stressors, the true diversity on leaves may be underestimated when taxon identification only rely on analysis of the reproductive structures (Niyogi *et al.* 2002).

For leaf-associated bacteria, diversity studies have been scarce and limited to the analysis of cultivable genera or the number of different morphotypes, after staining with a fluorescent dye (Suberkropp and Klug 1976, Baldy *et al.* 1995, Hieber and Gessner 2002). Suberkropp and Klug (1976) isolated bacteria on decomposing leaves, belonging to the genera *Flexibacter*, *Achromobacter*, *Flavobacteria*, *Pseudomonas*, and *Cytophaga*, but few of those were able to degrade structural polymers, such as cellulose.

Due to these limitations, traditional microbiological techniques and conventional microscopy can be insufficient to study the composition of microbial communities and the activity of their individual members on decomposing plant litter. A first attempt of circumventing this limitation for aquatic hyphomycetes was the use of monoclonal antibodies, which allowed the localization and quantification of four species in substrates through immunofluorescence (Bermingham *et al.* 1995, 1997, 2001). However, due to its high specificity, antibodies will be needed for identification of each species within a community, which limited further uses in ecological investigation. Other approach was provided by fluorescent *in situ* hibridization (FISH), where the target cells were fixed, hybridized with fluorescent-labeled oligonucleotide probes and quantified by epifluorescence microscopy (McArthur *et al.* 2001, Baschien *et al.* 2001). However, there are several disadvantages related to this technique, such as the autofluorescence of hyphae and/or substrates, which make it difficult to recognize and quantify the signal due to hydridization between the probe and the mycelia of the specific species (McArthur *et al.* 2001, Bärlocher 2007). FISH with probes targeting to the Domain Bacteria and to α-, β- and γ-*Proteobacteria*, indicated that α-*Proteobacteria* made up most bacteria on the surface of decomposing maple leaves (McNamara and Leff 2004).

Community fingerprinting techniques, such as terminal restriction fragment length polymorfism (T-RFLP) and denaturing gradient gel electrophoresis (DGGE), applied to 18S and to internal transcribed spacer (ITS) regions in fungi and 16S rRNA genes in bacteria, have been widely used to assess fungal and bacterial diversity on environmental samples (Muyzer *et al.* 2003, Muyzer *et al.* 2004,

Kolwalchuk and Smit 2004). In both techniques, DNA is extracted from mixed populations and primers are used to amplify the sequences of a specific group of organisms, via polymerase chain reaction (PCR).

In T-RFLP, DNA amplification is done with one or both primers fluorescently labelled at the 5' end, the PCR products are digested with a restriction enzyme and the labelled terminal fragments are then separated and detected in a DNA sequencer (Liu *et al.* 1997). The number of different sequence sizes gives an estimate of strains present in the community (Liu *et al.* 1997, Kim and Marsh 2004).

In DGGE, the amplicons of the same length but with different sequences are separated in a denaturing gradient gel of polyacrylamide, based on their differential denaturation profile (Fischer and Lerman 1983, Muyzer *et al.* 1993). The denaturing conditions are provided by urea and formamide (100% of denaturant solution consists of 7M urea and 40% formamide). Low and high denaturing solutions are prepared, mixed with the acrylamide solution and poured in a gel casting using a gradient former to generate a linear denaturing gradient (Muyzer *et al.* 2004). During denaturation, the two strands of a DNA molecule separate or melt at a specific denaturant concentration, and the sequence stops its migration in the gel. However, the optimal resolution of DGGE is obtained when the molecules do not completely denature, because if total denaturation is achieved the PCR products would continue run through the gel as single stranded DNA. To prevent this, a so-called GC clamp (a stretch of DNA of 40-60 nucleotides composed by guanine and cytosine) is attached to the 5' end of one of the PCR primers, resulting in a product with one end having a very high melting domain (Muyzer *et al.* 1993). Therefore, a PCR product containing the GC clamp when running through the gel will partially denature. The fragment will form a Y-shaped piece of DNA that will stick firmly at its position on the gel. The number of bands on the gel will be indicative of the genetic diversity of the original sample (Muyzer *et al.* 1993, 2004).

T-RFLP and DGGE were useful to asses fungal and bacterial diversity on decomposing leaf litter in streams (Nikolcheva *et al.* 2003, Nikolcheva *et al.* 2005, Nikolcheva and Bärlocher 2005, Das *et al.* 2007), in salt marshes (Buchan *et al.* 2003) and in lakes (Mille-Lindblom *et al.* 2006). In addition, DGGE was valuable to assess the diversity of aquatic hyphomycete conidia in the stream water (Raviraja *et al.* 2005). Both techniques showed that dominant bands on decomposing leaves in streams appear to belong to aquatic hyphomycetes and that species richness on decomposing leaves is higher than that suggested through the microscopic identification of released conidia (Nikolcheva and Bärlocher 2005, Nikolcheva *et al.*

2003, 2005). DGGE was also useful in detecting shifts in both fungal and bacterial populations during leaf decomposition in streams (Das *et al.* 2007, Nikolcheva and Bärlocher 2005, Nikolcheva *et al.* 2003, 2005) and to discriminate members of different fungal phyla using taxon specific primers (Nikolcheva and Bärlocher 2004). A great advantage of DGGE over T-RFLP is that it is possible to obtain taxonomic information since bands can be excised, re-amplified and sequenced, and specific bands can also be hybridized with specific oligonucleotides probes (Heuer *et al.* 1999, Riemann and Widing 2001). Moreover the intensity of each band may provide an estimate of the abundance of specific taxa (Nübel *et al.* 1999, Nikolcheva *et al.* 2003).

The main disadvantages of using DNA fingerprinting techniques include PCR biases (amplification errors, formation of chimeric and heteroduplex molecules and preferential amplification) (Wintzingerode *et al.* 1997), variable DNA extraction efficiencies (Theron and Cloete 2000), and introduction of DNA contaminations during DNA isolation and PCR, thus special caution is needed during these stages (Muyzer *et al.* 2004). Additionally, in DGGE, the fact of only small fragments (up to 500 bp) can be separated may limit sequence information, and minor populations can be below the detection limit (>1% of target) and different DNA sequences may have similar motilities due to identical GC contents (Muyzer *et al.* 2004). Therefore, one band may not necessarily represent one species (Gelsomino *et al.* 1999) and possible intra-specific or intra-isolate heterogeneity of rRNA genes can give rise to multiple banding patterns for one species (Nakatsu *et al.* 2000, Michaelsen *et al.* 2006). Moreover, the specificity of the coverage of the analysis is a function of the quality of the primers chosen (Kowalchuk and Smit 2004).

A new developed technique is the quantitative real time PCR (Q-RT-PCR), which allows estimating copy numbers of specific genes in environmental samples (Smith 2005). It was recently applied to quantify fungal and bacterial (both Bacteria and Archaea) biomasses on decomposing leaves, using specific primers for the regions ITS and 16S rDNA, respectively (Manerkar *et al.* 2008). With this technique, it was shown that fungal DNA exceeded that of bacteria and DNA levels of Archaea were low. A great potential of Q-RT-PCR is the use of specific probes for smaller groups at the level of phyla, genera or even species, making the analysis of the relative contributions of each taxonomic group or species to leaf-litter decomposition possible (Suzuki *et al.* 2000, Manerkar *et al.* 2008). However, in the case of fungi, the uncertainty of the number of copies of rRNA operons per fungal cell for the majority of the species can complicate further quantification (Manerkar *et al.* 2008).

The use of clone libraries can also be useful for assessing both fungal and bacterial diversity on decomposing leaves, as applied to fungi and bacteria on decaying salt marshes (Buchan *et al.* 2003) or to aquatic fungi in the hyporheic zone (Bärlocher *et al.* 2007) and on decomposing leaves (Seena *et al.* 2008). However, such approaches are expensive and time consuming and also suffer from biases introduced during nucleic acids extraction, amplification and cloning steps (von Wintzingerode *et al.* 1997). The lack of reference sequences for many isolates, may also limit its use.

## 1.5. Effects of pollution on plant-litter decomposition

### *1.5.1. Nutrient enrichment*

Human activities have greatly contributed to the high nutrient loads in streams, affecting biotic activities and ecosystem processes. Nutrient enrichment is mainly caused by nonpoint sources of pollution, such as agriculture due to the use of fertilizers and animal wastes (Allan and Castillo 2007). Phosphorus (P) reaches streams mainly from sewage inputs and soil erosion, while nitrogen (N) is disposed in the environment mainly through the manufacture of fertilizers and burning of fossil fuels (production of nitrogen oxides). Urban areas can also be significant nutrient sources due to municipal wastes and fertilizers (Allan and Castillo 2007).

Generally, elevated concentrations of N and P in both field and microcosm experiments are reported to enhance the activity of fungi (biomass, sporulation or production) (Suberkropp 1998a, Sridhar and Bärlocher 2000, Grattan and Suberkropp 2001, Gulis and Suberkropp 2003a,b, Pascoal and Cássio 2004, Ferreira *et al.* 2006, Gulis *et al.* 2006) and bacteria (biomass or production) (Gulis and Suberkropp 2003a,b, Pascoal and Cássio 2004, Baldy *et al.* 2007) on decomposing leaves leading to faster leaf decomposition in nutrient-enriched environments (Sridhar and Bärlocher 2000, Grattan and Suberkropp 2001, Pascoal *et al.* 2001, Pascoal *et al.* 2003, 2005a, Gulis and Suberkropp 2003a,b). Fungal diversity was also stimulated in streams enriched with N and P (Gulis and Suberkropp 2003b, 2004, Artigas *et al.* 2008).

Nutrient enrichment in streams is reported to stimulate macroinvertebrate density (Robinson and Gessner 2000, Pascoal *et al.* 2003, 2005a, Greenwood *et al.* 2007) and leaf breakdown rates, but not invertebrate diversity (Pascoal *et al.* 2003, 2005a). On the other hand, nitrate addition did not affect abundance or richness of

leaf-associated invertebrates (Ferreira *et al.* 2006). Since nutrient enrichment increase the rates of detritus processing though the increase of microbial activity (Pascoal *et al.* 2003, 2005a, Ferreira *et al.* 2006), this may result in food limitation for some longer-lived detrital consumers (Greenwood *et al.* 2007). Indeed, an increase in leaf breakdown rates and a decline in invertebrate shredders at sites affected by sewage effluents was attributed to a possible stimulation of microbial activity due to nutrient enrichment (Pascoal *et al.* 2001).

Organic pollution, is reported to reduce fungal sporulation and diversity but not leaf decomposition rates (Au *et al.* 1992 a,b, Raviraja *et al.* 1998), probably due to a redundancy among microbial decomposers (Raviraja *et al.* 1998). Additionally, microbial activity and rates of leaf decomposition are reported to be depressed in some nutrient enriched sites with low oxygen concentration and sedimentation, supporting that positive effects of nutrients may be counteracted by other factors (Pascoal and Cássio 2004, Pascoal *et al.* 2005a, Mesquita *et al.* 2007).

### *1.5.2. Metals*

Metal pollution in streams is of major concern due to the non-degradability of metals and because of their adverse effects on aquatic life. Although heavy metals occur naturally in the composition of earth and can be released into the environment through weathering of rocks, the main source that makes them toxicants is of anthropogenic origin (Ayres 1992). The industrial processes, such as mining, smelting, finishing and plating of metals and dye manufacture (Rand *et al.* 1995), are the major sources of metal contamination in aquatic environments. Essential metals, such as zinc (Zn) and copper (Cu) are common in enzymatic systems in living organisms and those that readily form two different oxidation states (e.g. Cu) are often involved in redox reactions. Other metals, such as cadmium (Cd), do not have any apparent biological function (Gadd 1993). However, above certain threshold concentrations, both essential and non-essential metals can be toxic (Gadd 1993).

High concentrations of metals in stream water are reported to strongly inhibit leaf decomposition rates (Bermingham *et al.* 1996a, Sridhar *et al.* 2001, 2005, Niyogi *et al.* 2001, 2002, Baudoin *et al.* 2007), microbial activity (Bermingham *et al.* 1996a, Niyogi *et al.* 2002, measured as respiration), fungal diversity (Bermingham *et al.* 1996a, Sridhar *et al.* 2001, Niyogi *et al.* 2002, Baudoin *et al.* 2007) and to alter the structure of microbial communities on leaf litter (Maltby and Booth 1991,

Bermingham *et al.* 1996a, Baudoin *et al.* 2007). Aquatic hyphomycete reproduction is often found to be more sensitive than biomass to metal stress (Niyogi *et al.* 2002, Baudoin *et al.* 2007), but in streams with a long history of metal pollution both parameters are reported to be severally depressed (Sridhar *et al.* 2001). The greatest impacts of metal contamination to aquatic fungal communities on leaves were found during the initial stages of leaf colonization (Sridhar *et al.* 2005).

Laboratory experiments showed that metals inhibit the growth (Miersch *et al.* 1997, Guimarães-Soares 2005, Azevedo 2007) and sporulation of several aquatic hyphomycete species (Abel and Bärlocher 1984, Azevedo 2007) and sporulation was more sensitive than growth (Abel and Bärlocher 1984, Bermingham *et al.* 1996b, Azevedo 2007). Zinc (up to 150 µM) depressed leaf decomposition rates, fungal reproduction, production and diversity of fungal communities (Duarte *et al.* 2004), but did not affect fungal biomass. On the other hand, copper (up to 1.2 µM) did not inhibit fungal biomass and sporulation but reduced leaf decomposition rates and the abundance of leaf-associated invertebrates (Roussel *et al.* 2007). Reduced biomass of shredding invertebrates is also reported in streams polluted with metals (Niyogi *et al.* 2001, Carlisle and Clements 2005). Conversely, bacterial abundance (Lemke and Leff 1999) was not much affected by high levels of metals in the stream water but bacterial production and nitrification were affected either directly or indirectly in streams receiving mine drainage (Niyogi *et al.* 2003).

### *1.5.3. Multiple stressors*

The effects of environmental stressors are usually tested individually, however in nature organisms are often exposed to several stressors simultaneously, which can interact producing combined impacts on biodiversity and ecosystem functioning (Folt *et al.* 1999, Vinebrooke *et al.* 2004).

Several models have been used to study the effects of multiple stressors on organisms (single comparative effects, additive effects and multiplicative effects), but since in the majority of studies one do not know *a priori* the mechanisms of action of each stressor, more than one model should be tested (Folt *et al.* 1999). However, summing the individual effects of each stressor has been by far the most common used model to test the effects of multiple stressors (Folt *et al.* 1999). The joint action of two stressors with similar mode of action, which do not interact, should be additive and the combined effect predicted from single stressor studies. However, if interactions occur, effects can be larger (more-than-additive or

synergism) or smaller (less-than-additive or antagonism) than predicted from the sum of individual responses to each toxicant (Norwood *et al.* 2003).

Since metals are released to the environment in mixtures that are threatening aquatic ecosystems, it has been an increasing number of studies on the effects of metal mixtures on several aquatic organisms, including algae, bacteria, invertebrates, fish, protozoans and aquatic macrophytes (reviewed in Norwood *et al.* 2003). These studies revealed that although a general tendency towards the antagonistic effects was found, the responses in mixtures were variable. This should be expected since the nature of the interaction can depend on the species of organism being exposed and on the number, types and concentrations of metals in a mixture (Franklin *et al.* 2002).

Although several experiments with multiple stressors have been done, studies on microbial decomposers of leaf litter in streams are practically inexistent. The negative effects of Cd on the growth of five aquatic fungal species were alleviated in the presence of Zn (Abel and Bärlocher 1984). However, most studies of multiple stressors on aquatic organisms examined metal interactions on single species (reviewed in Norwood *et al.* 2003), and this can be insufficient to predict the effects at the community level.

Furthermore, there are evidences suggesting that the co-occurrence of metals with other stressors such as nutrients (Fernandes *et al. submitted*) and hydrocarbons (Moreirinha 2007) appear to potentiate their individual effects on aquatic decomposer fungal communities and leaf-litter breakdown in streams.

## 1.6. Microcosms has a tool to investigate plant-litter decomposition in freshwaters

Microcosms and mesocosms are basically small ecosystems in containers (Fraser 1999) and have a long history in ecology, providing an easy way to manipulate a high range of communities including microorganisms (Griffiths *et al.* 2004, Duarte *et al.* 2004), protists (Warren *et al.* 2003), plants (Naeem *et al.* 1994, Hector *et al.* 1999) and animals (macroinvertebrates: Jonsson and Malmqvist 2000, Cardinale *et al.* 2002; nematodes: Mikola and Setälä 1998), from marine, streams and terrestrial habitats.

The greatest advantages of using microcosms are the limitation of the number and type of components, ease of replication and the precise control over environmental factors as well as the ability to manipulate and control spatial

heterogeneity at a relatively low cost (Drake *et al.* 1996, Fraser 1999). The major criticisms derive precisely from the benefits of experimental microcosms including the simplification, control of scale and environmental effects, and a reduction in spatial heterogeneity (Carpenter 1996, Fraser 1999). The complex array of interactions found in natural ecosystems cannot always be simulated and microcosms may also contain intrinsic artefacts (e.g. wall effects) which may confound extrapolation of results from controlled experiments to conditions in natural ecosystems (reviewed in Carpenter 1996).

Despite these disadvantages, microcosms have greatly contributed for increasing the information about the role of microbial decomposers on leaf-litter decomposition in streams. Additionally, they are particularly useful when studying the impact of harmful compounds, since *in situ* additions are not always possible because of their toxicity to the biota (e.g. Abel and Bärlocher 1984, Duarte *et al.* 2004, Roussel *et al.* 2007).

Much of the knowledge on how fungal and bacterial growth rates, fungal reproductive outputs and activities respond to variations in temperature (Chauvet and Suberkropp 1998), nutrient enrichment (Suberkropp 1998a, Sridhar and Bärlocher 2000, Gulis and Suberkropp 2003b, Bärlocher and Corkum 2003) and metal pollution (Abel and Bärlocher 1984, Duarte *et al.* 2004, Roussel *et al.* 2007) was acquired using microcosm experiments. Microcosm studies lend important information about the relationships between fungal growth and sporulation (Suberkropp 1991), the interactions between fungi and bacteria on leaf-litter decomposition (Gulis and Suberkropp 2003c, Mille-Lindblom and Tranvik 2003, Romaní *et al.* 2006), and the role of fungal diversity in ecosystem functioning (Bärlocher and Corkum 2003, Treton *et al.* 2004, Dang *et al.* 2005, Raviraja *et al.* 2006).

## 1.7. Aim and outline of the thesis

Plant-litter decomposition is an important ecosystem process in low-order streams that links microbial and invertebrate activity, riparian vegetation and environmental factors. Microbial decomposers, namely fungi and bacteria, are critical mediators in the transference of energy and carbon for invertebrate consumption. Anthropogenic stressors are threatening freshwater ecosystems and can negatively affect microbial decomposers potentially compromising their ecological functions. Nutrient enrichment and metal contamination are among the

major threats in streams all over the world, including those in the Northwest of Portugal. In this study, the effects of anthropogenic stressors from high levels of nutrients or metals in the stream water were assessed on leaf-litter decomposition and the associated fungi and bacteria. Since microbial diversity is often found to be lower in streams under stress, the effect of fungal species loss on leaf decomposition was also investigated.

Chapter 1 provides information on the role of biodiversity in ecosystem processes and the mechanisms underlying diversity effects. The impacts of anthropogenic stressors, namely metals and nutrients, in freshwaters are also addressed. Particular attention is given to plant-litter decomposition in streams and the biota that governs this process. The methods more frequently used to study leaf-litter decomposition and the associated communities are also described.

In chapter 2 we conducted a microcosm experiment with monocultures and all possible combinations of four aquatic hyphomycete species to examine the potential effect of species diversity on three aspects of stream ecosystem functioning: leaf mass loss, overall fungal biomass and reproductive effort. In chapter 3, leaves colonized at a reference site, harboring a highly diverse community, were transplanted to a site polluted with nutrients and metals to assess how aquatic microbial decomposers react to a sudden alteration in their habitat condition. In chapter 4, a DNA-based technique (denaturing gradient gel electrophoresis - DGGE) was used to study the diversity of leaf-associated fungi and bacteria during leaf-litter decomposition in a low-order stream. Since DGGE strongly relies on polymerase chain reaction, four different primer pairs targeting different regions of fungal and bacterial ribosomal DNA were tested. In chapter 5, leaf-litter decomposition and the associated fungi and bacteria were monitored in 5 streams along a gradient of nitrogen and phosphorus, to test whether changes in nutrient concentrations in the stream water would lead to changes in the structure of microbial communities and ecosystem processes. In chapter 6 and 7, the effects of environmentally realistic levels of copper and zinc were tested, either alone or in combination, on diversity and functions of leaf-associated microbial communities. The effects of metal mixtures on the activity of microbial decomposers were compared to those expected from each metal alone, to test whether fungal and bacterial functions shift in a predictable manner when exposed to metal mixtures (chapter 6). Moreover, the sequence of metal addition and the ability of microbial decomposers to recover after release from metal stress was investigated (chapter

7). In chapter 8 the main conclusions are presented in order to provide a global perspective of the work and possible lines of future research.

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# Chapter 2

## *Aquatic hyphomycete diversity and identity affect leaf-litter decomposition in microcosms*

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

## Abstract

We conducted a microcosm experiment with monocultures and all possible combinations of four aquatic hyphomycete species, *Articulospora tetracladia*, *Flagellospora curta*, *Geniculospora grandis* and *Heliscus submersus*, to examine the potential effects of species richness on three functional aspects: leaf litter decomposition (leaf mass loss), fungal production (ergosterol buildup) and reproductive effort (released spores). Both species richness and identity significantly affected fungal biomass and conidial production (number and biomass of released spores), whereas only species identity had a significant effect on leaf mass loss. In mixed cultures, all measures of fungal functions were greater than expected from the weighted performances of participating species in monoculture. Mixed cultures outperformed the most active monoculture for biomass accumulation but not for leaf mass loss and conidial production. The three examined aspects of aquatic hyphomycete activity tend to increase with species richness, and a complementary effect was unequivocally demonstrated for fungal biomass. Our results also suggest that specific traits of certain species may have a greater influence on ecosystem functioning than species number.

## 2.1. Introduction

Many ecosystems are suffering dramatic alterations due to human activities (Vitousek *et al.* 1997). This has focussed attention on how and to what extent ecosystem functioning may be affected by changes in biological communities. Several hypotheses have been proposed concerning the relationship between biodiversity and ecosystem functioning (Johnson *et al.* 1996). MacArthur (1955) predicted a positive, linear relationship between species numbers and both productivity and the ability of ecosystems to recover from disturbances. However, a linear correlation has rarely been observed (Tilman *et al.* 1996, Hooper and Vitousek 1997). The rivet hypothesis (Ehrlich and Ehrlich 1981) describes a positive, non-linear relationship, where species loss has a slight negative effect on ecosystem functions until a critical diversity level, beyond which further losses will seriously compromise ecosystem integrity. Similarly, the redundancy hypothesis (Walker 1992) predicts a positive and asymptotic relationship, where the loss of species is of little consequence as long as all functional groups are well represented. Finally, the idiosyncratic hypothesis (Lawton 1994) assumes that the relationship between species richness and ecosystem functioning does not follow a consistent pattern, but depends crucially on the identity of species and on the order in which they are lost from ecosystems.

There is no agreement as to which of these hypotheses best describes the relationship between biodiversity and ecosystem functions, but generally diversity has been positively associated with primary productivity or plant biomass (Naeem *et al.* 1996, Tilman *et al.* 1996), other ecosystem processes, such as detritus processing (Heneghan *et al.* 1999, Jonsson and Malmqvist 2000), and ecosystem stability and resilience (Naeem *et al.* 1996, Tilman *et al.* 1996, Hector *et al.* 1999).

In a community of species with complementary niches, positive interactions predominate (e.g., facilitation among freshwater invertebrates, Cardinale *et al.* 2002, and combination of facilitation and niche differentiation among grassland producers, Loreau and Hector 2001), leading to a greater overall efficiency of resource use. This raises community performance above the level expected from the sum of performances by individual species. On the other hand, the probability of encountering dominant species, which tend to be the most productive species, will increase with the number of species present in a community (sampling effect, Huston 1997, Tilman *et al.* 1997). The performance of a diverse community will therefore largely be determined by the inclusion of the species with the highest

performance in monoculture (Huston 1997, Tilman *et al.* 1997). It is often difficult to distinguish between complementarity and sampling effects, and they may occur simultaneously, but if sampling effects predominate, the performance of multicultures asymptotically approaches an upper limit defined by the top performing monoculture (Loreau and Hector 2001). If complementary or facilitation interactions dominate, ecological function will increase monotonously with diversity, and multicultures will outperform the top monoculture.

Most studies have addressed the functional consequences of biodiversity in terrestrial ecosystems (Kinzig *et al.* 2001, Loreau *et al.* 2002). The comparatively few studies in aquatic systems did not reveal a consistent relationship between diversity and ecosystem functioning (Covich *et al.* 2004, Giller *et al.* 2004).

Aquatic hyphomycetes are the main microbial decomposers of leaf litter in streams (Bärlocher 1992). There are numerous aspects to their ecological function (Bärlocher 2005, Suberkropp 1998): aquatic hyphomycetes affect autotrophs (by releasing inorganic nutrients), other heterotrophic microorganisms (competing for or sharing organic nutrients) and invertebrates (improving leaf palatability to shredders, releasing fine particulates consumed by collectors). Several field studies have examined aquatic hyphomycete diversity and leaf decomposition in streams from different geographic areas (e.g., tropics, Mathuriau and Chauvet 2002, temperate climate, Hieber and Gessner 2002) and under different environmental conditions (e.g., nutrient load, Gulis and Suberkropp 2003, Pascoal and Cássio 2004; riparian vegetation, Bärlocher and Graça 2002; heavy-metal pollution, Sridhar *et al.* 2001). However, the only two controlled laboratory studies on how aquatic hyphomycete species diversity affects their function pointed to a positive relationship between diversity and leaf mass loss (Bärlocher and Corkum 2003, Treton *et al.* 2004), but not for the reproductive investment (Treton *et al.* 2004).

In this study we used monocultures and all possible combinations of four species of aquatic hyphomycetes to examine the potential effect of species richness on three aspects of stream ecosystem functioning: leaf mass loss, overall fungal production (ergosterol buildup) and reproductive effort (both number and biomass of released spores).

## 2.2. Materials and Methods

### *2.2.1. Microcosms*

In September 2002, 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 22 mm diameter disks. Sets of 20 disks were placed in 250 mL Erlenmeyer flasks and autoclaved for 20 min. To each Erlenmeyer flask, 100 mL of a mineral solution (0.01 g $MgSO_4.7H_2O$, 0.01 g $CaCl_2.2H_2O$, 0.01 g $KNO_3$, 0.01 g $K_2HPO_4$, and 0.5 g 2-[N-morpholino] ethanesulfonic acid per 1 L, pH 6.0) were added aseptically.

The aquatic hyphomycetes *Articulospora tetracladia* Ingold, *Flagellospora curta* J. Webster, *Geniculospora grandis* (Greath.) Sv. Nilsson and Nolan, and *Heliscus submersus* H. J. Huds. were isolated from single spores collected in the Este River (NW Portugal), and grown on 7 mL of 1% Malt Extract Agar. Microcosms (250 mL Erlenmeyer flasks) were inoculated with agar plugs collected from the edge of 18 days-old colonies of the four fungi as follows: monocultures of the four species (4 treatments x 3 replicates), all combinations of two species (6 treatments x 3 replicates), all combinations of three species (4 treatments x 3 replicates) and all species combined (1 treatment x 3 replicates). 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.

Throughout the 27 days of the experiment, microcosms were aerated aseptically with aquarium pumps, and kept at 18 ºC under artificial light. Mineral solutions were replaced every 3 days. Discarded solutions were sampled to establish conidial production, as described below. At the end of the experiment, leaf disks were used to determine remaining dry mass and fungal biomass.

### *2.2.2. Leaf dry mass*

Sets of 15 leaf disks from each replicate microcosm were dried at 50 ºC to a constant mass (72 ± 24 h) and weighed (± 0.001 g). Sets of leaf disks before fungal inoculation were used to estimate initial dry mass.

### 2.2.3. Fungal biomass

Metabolically active fungal biomass was estimated as ergosterol content (Gessner and Newell 2002). Sets of 5 leaf disks from each replicate microcosm were refluxed in KOH-methanol for lipid extraction. Ergosterol was purified by solid phase extraction and quantified by high performance liquid chromatography (Gessner and Schmitt 1996). Ergosterol was converted to fungal biomass using a conversion factor of 5.5 mg ergosterol $g^{-1}$ fungal dry mass (Gessner and Chauvet 1993).

### 2.2.4. Conidial production

Conidial suspensions were mixed with 250 µL Tween 80 (0.5%) and 10 mL aliquots of serial decimal dilutions were passed through 5 µm membrane filters. The retained conidia were stained with 0.1% cotton blue in lactic acid, and the filters were scanned under a microscope (400x, Leica Biomed) for conidial types and numbers.

Conidial numbers were converted to biomass based on published values of conidium mass for *A. tetracladia* (Gessner and Chauvet 1993, Chauvet and Suberkropp 1998) or estimated ($M = -0.058\ V^2 + 641\ V$, where M is the conidial mass in *f*g and V is the conidial volume in $\mu m^3$; Baldy *et al.* 2002) for the other species. Conidial volumes were calculated according to Bärlocher and Schweizer (1983).

### 2.2.5. Data analyses

Nested ANOVA was used to test the effects of species number and species identity (nested within species number) on leaf mass loss, fungal biomass and total conidial production (as number and biomass) (see Jonsson and Malmqvist 2000). In a second analysis, the expected leaf mass loss, fungal biomass and total conidial production 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 (residuals) were tested against the null hypothesis that the average difference equalled 0 (t-test), and a linear regression was used to test if the differences varied with species number in mixed cultures (Bärlocher and Corkum 2003). Leaf mass loss, fungal biomass and total conidial

production of the most active monoculture were compared with those in mixed cultures containing that species (t-test). To achieve normal distribution, the percentage of leaf mass loss was arcsine square root transformed, and fungal biomass and total conidial production (as number and biomass) were ln-transformed (Zar 1996). Statistical analyses were performed with Statistica 6.0 for Windows (StatSoft, Inc.).

## 2.3. Results

### *2.3.1. Leaf mass loss, fungal biomass and conidial production*

After 27 days in microcosms, mass loss of alder leaves inoculated with 1 to 4 aquatic hyphomycete species varied between 14.8% and 59.6% for monocultures of *H. submersus* and *A. tetracladia*, respectively (Fig. 2.1A).

Higher mass losses were generally obtained whenever *A. tetracladia* was present. Species identity nested within species number, but not species number, significantly affected leaf mass loss (Table 2.1).

Leaf mass losses obtained in mixed cultures were significantly higher than expected from the sum of mass losses in single cultures taking into account the relative proportions of the inoculum ($t=2.14$, $P=0.04$) (Fig. 2.2A). However, the difference between observed and expected mass losses did not increase significantly with species number ($Y=-0.0554+0.9446X$, $P=0.58$). Mass loss in the microcosm containing the most active species alone (*A. tetracladia*) was significantly higher than mass losses in mixed cultures with this species ($t=-9.45$, $P<0.0001$).

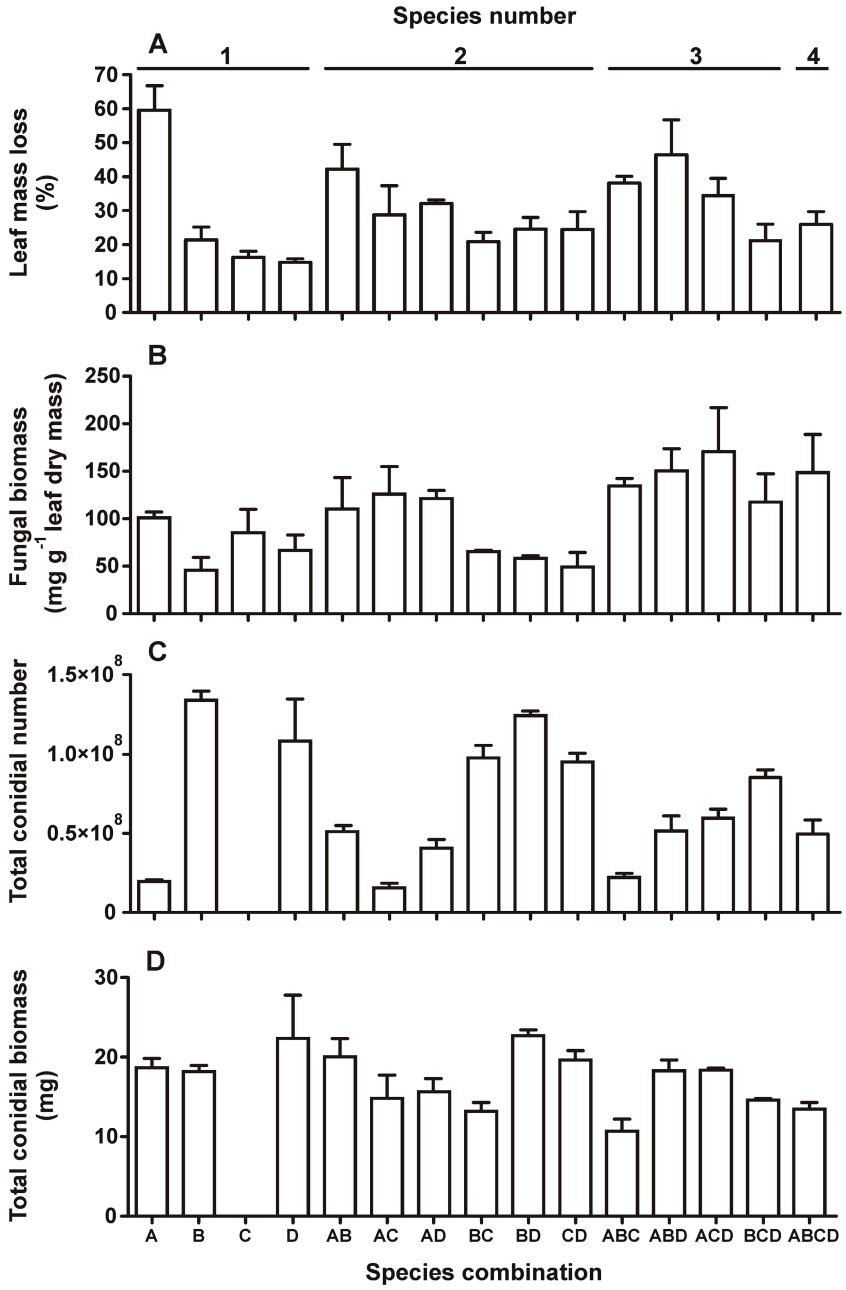


**Figure 2.1.** Mass losses (A), fungal biomasses (B), total conidial numbers (C) and total conidial biomasses (D) of alder leaf disks inoculated with one to four aquatic hyphomycete species after 27 days of incubation. Mean ± SEM, n=3. A *Articulospora tetracladia*, B *Flagellospora curta*, C *Geniculospora grandis*, and D *Heliscus submersus*.

**Table 2.1.** Nested ANOVAs of the effects of species number and species identity nested within species number on leaf mass loss, fungal biomass, total conidial number, and total conidial biomass.

| Parameter | Effect | d.f. | SS | MS | F | P |
|---|---|---|---|---|---|---|
| Leaf mass loss | Identity {species number} | 11 | 2306 | 210 | 6.6 | <0.0001 |
| | Species number | 3 | 181 | 60 | 1.9 | 0.15 |
| | Error | 30 | 955 | 32 | | |
| Fungal biomass | Identity {species number} | 11 | 4.0 | 0.37 | 2.4 | 0.029 |
| | Species number | 3 | 3.8 | 1.28 | 8.4 | 0.0004 |
| | Error | 28 | 4.3 | 0.15 | | |
| Conidial number | Identity {species number} | 11 | 343 | 31 | 240.8 | <0.0001 |
| | Species number | 3 | 69.5 | 23.2 | 178.8 | <0.0001 |
| | Error | 30 | 3.9 | 0.13 | | |
| Conidial biomass | Identity {species number} | 11 | 21.5 | 1.96 | 64.4 | <0.0001 |
| | Species number | 3 | 3.2 | 1.05 | 34.5 | <0.0001 |
| | Error | 30 | 0.9 | 0.03 | | |

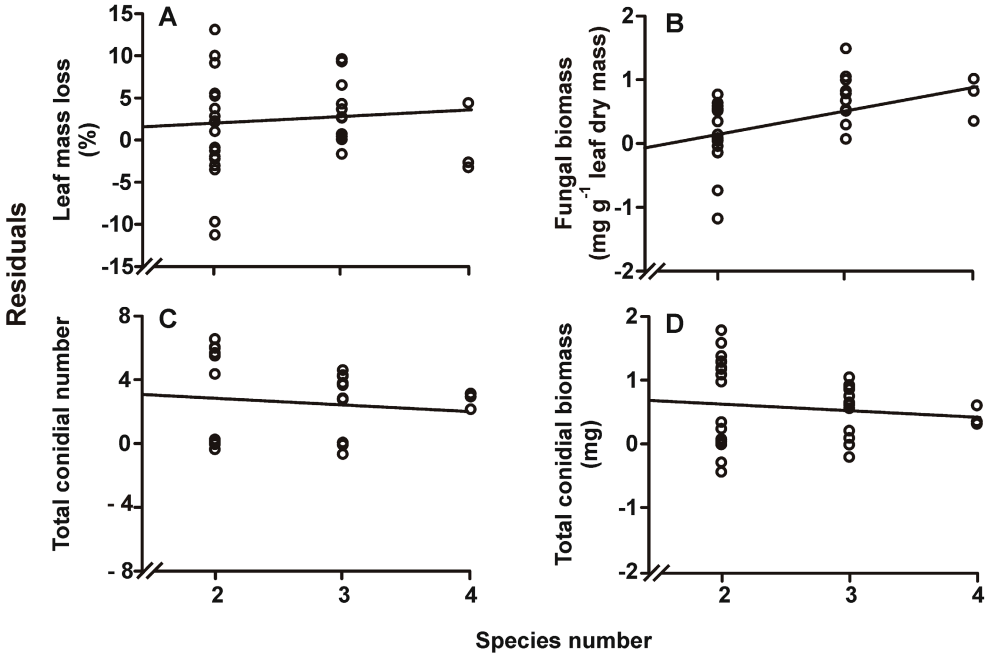


**Figure 2.2.** Linear regression of residuals, which are the differences between observed and expected alder leaf mass losses (A), fungal biomasses (B), total conidial numbers (C) and total conidial biomasses (D), in mixed cultures against species number. All residuals were significantly higher than 0 (P<0.05).

At the end of the experiment, fungal biomass on leaves varied between 46 and 171 mg per g leaf dry mass for the monoculture of *F. curta* and the mixed culture of *A. tetracladia* + *G. grandis* + *H. submersus*, respectively (Fig. 2.1B). Both species number and species identity nested within species number had significant effects on fungal biomass (Table 2.1). Higher values for fungal biomass were again obtained in microcosms where *A. tetracladia* was present.

Fungal biomasses in mixed cultures were significantly higher than predicted from the sum of fungal biomasses in microcosms with single cultures (t=4.22, P=0.0002) (Fig. 2.2B). In contrast to leaf mass loss, the differences between observed and expected fungal biomasses increased significantly with species number (Y=-0.6499+0.4086X, P=0.003). In addition, the most productive species (*A. tetracladia*) produced significantly less biomass by itself than multicultures containing it (t=3.96, P=0.0009).

Total number of released conidia ranged between 554 and $1.3x10^8$ conidia for monocultures of *G. grandis* and *F. curta*, respectively (Fig. 2.1C). Total conidial numbers were higher in the five microcosms where lower fungal biomasses were found (*F. curta*, *H. submersus*, *F. curta* + *H. submersus*, *F. curta* + *G. grandis* and *G. grandis* + *H. submersus*). Microcosms inoculated with species with smaller conidia (*F. curta* and *H. submersus*) produced more conidia than those inoculated with species with larger conidia (*A. tetracladia* and *G. grandis*). Both species number and species identity had a significant effect on total number of released conidia (Table 2.1).

When inoculum was supplied by several species, total conidial numbers were significantly higher than expected from the weighted sum of conidial numbers obtained with single species (t=6.53, P<0.0001) (Fig. 2.2C). The difference between observed and expected conidial numbers did not change significantly with species number (Y=3.1146-0.1212 X, P=0.86). Moreover, significantly higher total conidial production was obtained in microcosms inoculated with the most active species alone (*F. curta*) than in mixtures containing that species (t=-6.72, P<0.0001).

Total conidial biomass ranged between $9.2 \times 10^{-4}$ mg (*G. grandis*) and 22.7 mg (*F. curta* + *H. submersus*) (Fig. 2.1D). Both species number and identity significantly affected total conidial biomass (Table 2.1).

Total conidial biomasses obtained in mixed cultures were significantly higher than expected from the weighted sum of biomasses obtained with single species (t=5.74, P<0.0001) (Fig. 2.2D). The difference between observed and expected total conidial biomasses was not correlated with species number (Y=0.8835-0.1197X,

P=0.44). Significantly higher total conidial biomass was obtained in monocultures with the most active species (*H. submersus*) than in mixed cultures containing that species (t=-4.75, P=0.0001).

The species *A. tetracladia* and *H. submersus* generally increased their contributions to total conidial production in mixtures over values expected from monocultures (5 or 4 out of 7 combinations, respectively, Table 2.2). Conversely, *F. curta* made lower contributions in mixtures than expected from monocultures. Even *A. tetracladia* increased its contribution to the total conidial production in mixtures with *F. curta* and/or *H. submersus*, although these two small-spored species always produced larger numbers of conidia.

**Table 2.2.** Percentage contribution of each aquatic hyphomycete species to the total conidial production in combinations of two, three and four species.

| Combination | Species | Contribution in mixed culture (%) | |
|---|---|---|---|
| | | Expected | Observed |
| 2 species | | | |
| A x B | A | 13 | 32 |
| | B | 87 | 68 |
| A x C | A | 100 | 100 |
| | C | 0 | 0 |
| A x D | A | 17 | 23 |
| | D | 83 | 77 |
| B x C | B | 100 | 100 |
| | C | 0 | 0 |
| B x D | B | 57 | 33 |
| | D | 43 | 67 |
| C x D | C | 0 | 0 |
| | D | 100 | 100 |
| 3 species | | | |
| A x B x C | A | 13 | 41 |
| | B | 87 | 59 |
| | C | 0 | 0 |
| A x B x D | A | 8 | 27 |
| | B | 52 | 44 |
| | D | 40 | 29 |
| A x C x D | A | 17 | 15 |
| | C | 0 | 0 |
| | D | 83 | 85 |
| B x C x D | B | 57 | 48 |
| | C | 0 | 0 |
| | D | 43 | 52 |
| 4 species | | | |
| A x B x C x D | A | 8 | 15 |
| | B | 52 | 17 |
| | C | 0 | 0 |
| | D | 40 | 68 |

### *2.3.2. Effect size of species richness*

Figure 2.3 shows effect sizes of species number on leaf mass loss, fungal biomass, and conidial number and biomass. The most consistent and clearly positive effect was found for fungal biomass. Effects on leaf mass loss and on reproductive output (both conidial number) were less pronounced and without a clear trend.

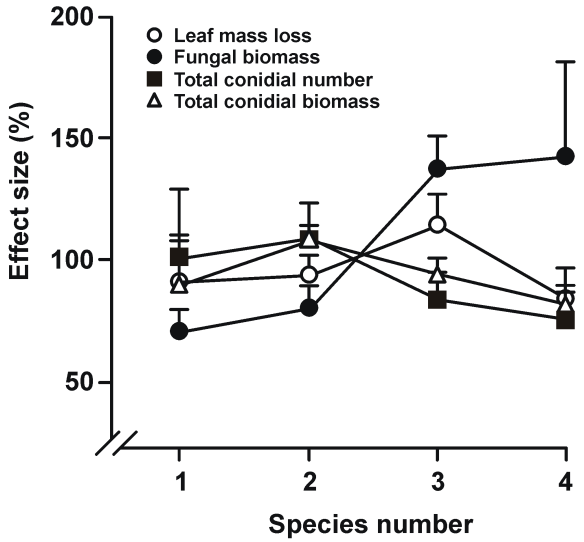


**Figure 2.3.** Effect sizes of species number on mass loss of alder leaf, fungal biomass, total conidial number, and total conidial biomass expressed as percentage of deviation from grand mean (grand mean = 100%).

## 2.4. Discussion

In the current study, alder leaf mass loss was affected by species identity, but not by species number. The decisive factor appeared to be the presence of *A. tetracladia*, the species causing the greatest loss in monoculture. On average, mass losses in multicultures were greater than expected from the sum of individual contributions by component species (weighted by initial inoculum), indicating that mixed cultures degraded alder leaves more efficiently. The same result was found in an earlier study with 5 species (Bärlocher and Corkum 2003). This suggests complementary effects. However, since we could not track the biomasses of individual species through the experiment and mixed cultures did not outperform the single most active species, we cannot exclude a sampling effect (Hector 1998).

Accumulation of fungal biomass, as measured by ergosterol, was affected by both species number and identity. Again, *A. tetracladia* was the most active species in monoculture, and fungal biomass in mixed cultures exceeded the values expected from monocultures. In several mixtures, fungal biomass was higher than that of the most productive species, clearly indicating a complementary effect, which significantly increased with species number. Combined with the mass loss data, our results suggest that multiple cultures were more efficient in producing fungal biomass per unit mass loss of alder leaf.

Both conidial number and biomass were significantly influenced by species number and identity, but mixed cultures again did not outperform the most active monoculture, and we cannot distinguish between complementarity and sampling effects. *F. curta* was the most prolific conidial producer, followed by *H. submersus* and *A. tetracladia*, but when converted to conidial biomass, the reproductive efforts of the three species were similar. This inverse relationship between spore number and biomass confirms earlier observations (Chauvet and Suberkropp 1998, Treton *et al.* 2004).

Overall, all aspects of fungal functions we examined were influenced by species identity, and all but leaf decomposition were affected by species number. Effect sizes, however, were small (20-30%) except for fungal biomass (close to 70%). Taking into account two previous studies (Bärlocher and Corkum 2003, Treton *et al.* 2004), we conclude that various aspects of aquatic hyphomycete activity tend to increase with species number, at least at low levels of diversity (up to 5 species). Our results also suggest that specific traits of certain species may have a greater influence on ecosystem processes, lending support to the idiosyncratic hypothesis (Lawton 1994). Comparable observations have been reported from other biological communities (Hooper and Vitousek 1997, Cox *et al.* 2001, Engelhardt and Ritchie 2001). For example, in serpentine grassland, differences in plant composition explained more the variation in production and nitrogen dynamics than the number of functional groups per se (Hooper and Vitousek 1997). Mass loss of *Pinus sylvestris* needles inoculated with one of two fungal species, commonly associated with decomposing pine needles, was greater than that obtained with a more complex fungal community in the field (Cox *et al.* 2001). Similarly, in mesocosms simulating wetlands ecosystems, a positive correlation between macrophyte biomass or phosphorus retention and species diversity was attributed primarily to the presence of a dominant species (Engelhardt and Ritchie 2001).

To date, an unequivocal complementary effect has been demonstrated in two studies of aquatic hyphomycetes (mass loss: Treton *et al.* 2004, biomass buildup: current study), and has been proposed for fungi in soil microcosms (mass loss, Setälä and McLean 2004). Such complementary effects might be expected to be common among aquatic hyphomycetes: different species have variable patterns of exoenzymes active against a range of plant polymers (Suberkropp *et al.* 1983). Combining enzymes from several species might therefore have a synergistic effect on leaf decay rates. The predominance of cooperative (or at least neutral) interactions between aquatic hyphomycetes is also suggested by extensive

intermingling of hyphae from several species on leaves decaying in streams (Shearer and Lane 1983). On the other hand, most interspecific interactions on artificial media resulted in inhibition of one or both members of a pair (Shearer and Zare-Maivan 1988). Simultaneous inoculation of *Tetrachaetum elegans* and *Flagellospora curvula* reduced the reproductive effort (conidial production) of both species (Treton *et al.* 2004), and surfaces of newly introduced leaves quickly become less hospitable for the settling and germination of conidia, possibly due to interference by earlier arrivals (Nikolcheva *et al.* 2005). These observations suggest the possibility of antagonistic interactions, which, at least in some cases, may lower the collective function. In the current study, some species (e.g., *F. curta*) had a reduced reproductive output in mixed cultures, but this was compensated for by increased success of others (*A. tetracladia* and *H. submersus*). Treton *et al.* (2004) reported reduced conidial production when *T. elegans* and *F. curvula* were paired; nevertheless, leaf mass loss in the mixed culture exceeded mass losses in the two monocultures.

A closer look reveals considerable variation in all three measured functions: in several combinations, increasing species number appears to have had no or a negative effect. In streams, leaves are typically colonized by 10-20 fungal species (e.g., up to 26 species on alder leaves in Portuguese streams, Bärlocher *et al.* 1995, Pascoal and Cássio 2004, Pascoal *et al.* 2005a). Even a lower number of 10 yields 45 distinct species pairs and 120 distinct species triplets. While on average species combinations may increase ecological functions, it seems unlikely that all of them do, and detailed analyses may reveal more such cases.

Laboratory experiments have clearly demonstrated a positive correlation between fungal functions and diversity; to date, no confirmatory evidence has been found in field studies. The impoverishment of fungal communities in streams bordered by exotic vegetation (Bärlocher and Graça 2002) or affected by pollution (Raviraja *et al.* 1998, Pascoal *et al.* 2005a) was not accompanied by decreased leaf decomposition, suggesting that functional redundancy may occur among fungal decomposers in streams. A similar discrepancy between laboratory and field studies was described for invertebrate grazer-collector interactions by Heard and Buchanan (2004). They attributed it to additional mechanisms absent in the laboratory and to much greater variability in the field (environmental noise), which can easily overwhelm diversity effects. In laboratory experiments with aquatic hyphomycetes, inorganic nutrients (nitrogen and phosphorus) had a much greater impact on leaf decomposition than species number (Bärlocher and Corkum 2003). Decay rates in

streams were also more strongly related to dissolved nitrogen and phosphorus in the water than to fungal diversity (Chauvet *et al.* 1997).

Our study demonstrated the occurrence of diversity effects in aquatic hyphomycete communities. It is conceivable that the probability of retaining dominant species diminishes in impacted streams, where a decrease in fungal diversity has been reported (organic pollution, Raviraja *et al.* 1998, heavy metals, Sridhar *et al.* 2000, organic and inorganic loading, Pascoal *et al.* 2005a,b). But the available evidence suggests that there is usually sufficient redundancy in the remaining species to prevent measurable losses in function. In addition, other fungal taxa (Nikolcheva and Bärlocher 2004) as well as bacteria occur on decaying leaves (Suberkropp 1998). The contribution of other fungi is largely unknown and may increase when aquatic hyphomycetes decline. Contribution of bacteria for litter decomposition is often found to be minor (Gulis and Suberkropp 2003, Pascoal and Cássio 2004), although antagonistic effect between fungi and bacteria has been reported (Mille-Lindblom and Tranvik 2003). The situation may be different with wood-colonizing fungi. Wong *et al.* (1998) reported that only few species of freshwater fungi (6-10 of 37-42 species identified on wood) produced enzymes that degrade wood lignocellulose. Under these circumstances, keystone species are more likely to occur. Identifying them and determining the sequence of their disappearance in impacted ecosystems will be crucial when trying to predict the consequences of species loss (Jonsson *et al.* 2002, Dangles *et al.* 2004).

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# Chapter 3

## *High diversity of fungi may mitigate the impact of pollution on plant-litter decomposition in streams*

S Duarte, C Pascoal, F Cássio (2008)
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## Abstract

We investigated how a community of microbial decomposers adapted to a reference site responds to a sudden decrease in the water quality. For that, we assessed the diversity and activity of fungi and bacteria on decomposing leaves that were transplanted from a reference (E1) to a polluted site (E2), and results were compared to those from decomposing leaves either at E1 or E2. The two sites had contrasting concentrations of organic and inorganic nutrients and heavy metals in the stream water. At E2, leaf decomposition rates, fungal biomass and sporulation were reduced, while bacterial biomass was stimulated. Fungal diversity was 4-times lower at the polluted site. The structure of fungal community on leaves decomposing at E2 significantly differed from that decomposing at E1, as indicated by the Principal Response Curves analysis. *Articulospora tetracladia*, *Anguillospora filiformis* and *Lunulospora curvula* were dominant species on leaves decomposing at E1 and were the most negatively affected by the transfer to the polluted site. The transfer of leaves colonized at the reference site to the polluted site reduced fungal diversity and sporulation, but not fungal biomass and leaf decomposition. Overall, results suggest that the high diversity on leaves from the upstream site might have mitigated the impact of anthropogenic stress on microbial decomposition of leaves transplanted to the polluted site.

## 3.1. Introduction

Human activities have greatly altered freshwater ecosystems worldwide. Among such alterations water pollution, including excessive levels of nutrients and other chemicals, leads to irreversible changes in aquatic biodiversity (Dudgeon *et al.* 2006). Therefore, the interest in addressing the importance of biodiversity to aquatic ecosystem processes and properties has been growing, particularly over the last decade (Dudgeon *et al.* 2006, Giller *et al.* 2004). Several studies point out a positive relationship between biodiversity and ecosystem functioning (MacGrady-Steed *et al.* 1997, Jonsson and Malmqvist 2000, Cardinale *et al.* 2002, Emmerson *et al.* 2001, chapter 2) or ecosystem stability (Yachi and Loreau 1999, Dang *et al.* 2005). Yachi and Loreau (1999) suggest that biodiversity helps to buffer environmental variability and to maintain ecosystem processes, because greater diversity will increase the probability of a community to contain species that respond differently to environmental stress, leading to functional compensations among species (Loreau *et al.* 2002).

Microbial decomposers, namely fungi and bacteria, play an active role in leaf litter decomposition through the production of enzymes, which transform leaf material into a more suitable food source for invertebrates in streams (Suberkropp 1998, Bärlocher 2005). Fungi are the major microbial decomposers of leaf litter (Baldy *et al.* 2002, Baldy *et al.* 2007), although the relative contribution of bacteria to overall microbial decomposition may increase in eutrophic streams (Pascoal and Cássio 2004, Pascoal *et al.* 2005a).

There is considerable body of evidence that leaf litter decomposition responds differently to anthropogenic stressors, such as eutrophication (Pascoal *et al.* 2001, Pascoal *et al.* 2003, Pascoal *et al.* 2005a, Ferreira *et al.* 2006, Gulis *et al.* 2006, Lecerf *et al.* 2006, Mesquita *et al.* 2007) and metal pollution (Niyogi *et al.* 2001, Niyogi *et al.* 2002b, Sridhar *et al.* 2001, 2005). High levels of inorganic nutrients in the stream water are reported to accelerate leaf decomposition through stimulation of microbial activity (Pascoal *et al.* 2003, Pascoal and Cássio 2004), while an opposite effect is generally attributed to metal contamination (Duarte *et al.* 2004, Niyogi *et al.* 2002b, Sridhar *et al.* 2001). Although studies on the impacts of anthropogenic stressors on microbial decomposers have increased over the last years, the knowledge on how microbial communities drive ecosystem processes in streams under stress is still very limited.

The transfer of colonized leaf material from one stream to another with contrasting water chemistry is a useful approach to measure how aquatic decomposers can react to sudden alterations in their habitat condition. Transplantation experiments, with colonized leaves by particular fungal species or assemblages gave variable results that appear to depend on water chemistry and the initial fungal inoculum (Suberkropp and Chauvet 1995, Sridhar *et al.* 2005). In this study, we assessed the activity and diversity of fungi and bacteria on decomposing leaves transplanted from a reference site (E1) to a polluted site (E2), and results were compared to those from leaves decomposing either at E1 or E2. The two sites had contrasting concentrations of organic and inorganic nutrients and heavy metals in the stream water. We hypothesized that the polluted site had lower microbial diversity and biomass on leaves than the reference site, leading to retarded leaf decomposition. We also hypothesized that a well-established and diverse community was able to maintain leaf-litter decomposition under stress conditions, at least for a certain period of time.

## 3.2. Materials and Methods

### *3.2.1. Study area*

The sampling sites were in the Este River, which is located in the Northwest of Portugal in an agricultural area with high population density. The Este River flows through the town of Braga and its Industrial Park. We selected two sites with contrasting nutrient concentrations in the stream water. The reference site (E1) is at the spring of the Este River and is about 0.2-0.3 m deep and 0.3-0.5 m wide with the bottom consisting of granitic rocks, pebbles and gravel, and the riparian vegetation constituted by *Eucalyptus globulus* Labill., *Pinus pinaster* Aiton, *Pteridium aquilinum* Khun and *Juncus* sp. The polluted site (E2) is 8 km downstream of the spring and near the Industrial Park. At E2, the stream is about 0.5-1 m deep and 1-2 m wide, the bottom consists mainly of gravel and mud, and the riparian vegetation is dominated by *Alnus glutinosa* (L.) Gaertn., *Salix* sp., *Populus nigra* L., *Quercus robur* L. and *Rubus ulmifolius* Schott.

### 3.2.2. Field procedures and transfer experiment

Leaves of *Alnus glutinosa*, collected from trees in October 2003, were dried at room temperature and placed into 72 leaf bags (0.5 mm mesh size; 15 x 15 cm). Forty four and 28 leaf bags were immersed at E1 and E2, respectively, and after 13 days, 16 bags were transplanted from E1 to E2 (E1-2). Four replicate leaf bags were collected from each site after 0, 5, 13, 20, 29, 40 and 54 days of leaf immersion, between 12 November 2003 and 6 January 2004. At each sampling date, alder leaves from each bag were washed with deionised water to remove sediments, and cut into 22 mm-diameter disks. Sets of leaf disks were used to estimate leaf mass loss, fungal sporulation, fungal biomass and bacterial biomass.

### 3.2.3. Physical, chemical and microbial analyses of the stream water

At each sampling date, pH, redox potential, temperature, conductivity and oxygen concentration in the stream water were measured in situ with field probes (Multiline F/Set-3 no. 400327 WTW). Stream water samples were collected to determine the concentrations of ammonia (HACH kit, program 385), nitrate (HACH kit, program 355), and phosphate (HACH kit, program 480), and to quantify chemical oxygen demand (HACH kit, program 435) and densities of total and fecal coliforms (membrane filter technique) (APHA 1998).

### 3.2.4. Fungal biomass and sporulation

Sets of 5 leaf disks were preserved in KOH/methanol at -20 ºC until processed. Leaf-associated fungal biomass was estimated as ergosterol content, according to Gessner (Gessner 2005). Ergosterol was purified and quantified by High-Performance Liquid Chromatography, after solid-phase extraction. Ergosterol was converted to fungal biomass using a conversion factor of 5.5 mg ergosterol $g^{-1}$ dry mass (Gessner and Chauvet 1993).

Sets of 10 leaf disks were placed into 150 mL Erlenmeyer flasks with 60 mL of filtered stream water (1.2 µm pore size, Whatman GF/C) under aeration (48 h, 18 ºC) to induce sporulation. After 48 h, conidial suspensions were mixed with 250 µL of 0.5% Tween 80, filtered (5 µm pore size, Millipore), and stained with 0.05% cotton blue in lactic acid. At least 300 conidia were identified and counted per replicate (400x, Leica Biomed).

### *3.2.5. Bacterial biomass*

Bacterial cells, preserved in phosphate buffered formalin (3.7% final concentration), were dislodged from each set of 5 leaf disks by sonication for 4 min (Branson 2510, sonication bath, Danbury, CT, USA). To minimize cell disruption, samples were put on ice each 1 min of sonication. Aliquots of 2 mL of appropriate dilutions of bacterial suspensions were stained with 40 µL of 0.1 mg $mL^{-1}$ 4',6-diamidino-2-phenylindole (DAPI, Molecular Probes) for 10 min in the dark, and filtered through black polycarbonate filters (0.2-µm pore size, GTTP, Millipore). Filters were mounted between two drops of immersion oil, and at least 300 bacterial cells were counted per filter under a fluorescence microscope (1000x, Leitz Laborlux S). To determine bacterial biovolumes, cells were assigned to classes defined by bacterial size and shape. Bacterial biovolumes were calculated as $V = (\pi / 4) \cdot W^2 \cdot (L - W / 3)$, where $W$ is the cell width and $L$ is the cell length (Bratbak 1993). The average biomass of each bacterial class was converted in bacterial biovolume according to $C = 89.6 \cdot V^{0.59}$, where $V$ is the bacterial volume in $\mu m^3$ and $C$ is the bacterial carbon in *f*g (Simon and Azam 1989), assuming a 50% of carbon in bacterial dry mass.

### *3.2.6. Leaf mass loss*

Sets of 40 disks of alder leaves of each replicate were dried at 60 ºC to constant mass (72 ± 24 h), and weighed to the nearest 0.01 mg. Sets of leaves immersed 15 min at each site of the Este River were used to determine the initial mass of leaves. Ash free dry mass (AFDM) remaining was determined after combustion of leaf disks at 550 ºC for 6 h.

### *3.2.7. Data analyses*

Ash-free dry mass remaining was fit to the exponential model $m_t = m_0 \cdot e^{-kt}$, where $m_t$ is the AFDM remaining at time $t$, $m_0$ is the initial AFDM and $k$ is the rate of leaf decomposition. Regression lines of ln-transformed values of AFDM were compared by ANCOVA followed by Tukey´s tests (Zar 1996).

Shannon index (H) was used to assess the diversity of aquatic hyphomycetes as: $H = -\sum_{i=1}^{s} P_i (lnP_i)$, where *Pi* is the relative abundance of conidia of taxon *i* and *S* is the total number of sporulating taxa (Legendre and Legendre 1998).

Differences in stream water parameters between E1 and E2 were compared by a paired t-test (Zar 1996). Differences in fungal sporulation, diversity and biomass, and bacterial biomass on decomposing leaves were compared by repeated measures (mixed model) ANOVA with matched observations for time (Zar 1996). Data were ln(x+1) transformed to achieve normal distribution.

Differences in the structure of aquatic hyphomycete communities on leaves were analysed by Principal Response Curves (PRC) based on the redundancy analysis, which is a constrained form of the Principal Component Analysis (Van den Brink and Ter Braak 1999). The statistical model for the PRC is: $Y_{d(j)tk} = Y_{0tk} + b_k C_{dt} + \varepsilon_{d(j)tk}$, where $Y_{d(j)tk}$ is the ln abundance of species *k* in replicate *j* of site *d* at time *t*, $Y_{0tk}$ is the mean abundance of species *k* on date *t* at the reference site (*d*=0), $C_{dt}$ is the response pattern for every site *d* and time *t*, $b_k$ is the weight of each species with this response pattern, and $\varepsilon_{d(j)tk}$ is an error term with mean zero and variance $\sigma_k^2$. By definition, $C_{0t}$ = 0 for every *t*. Specifically, in our experiment, when the coefficients $C_{dt}$ are plotted against time t, the resulting PRC diagram displays a curve for each site that can be interpreted as the PRC of the community (Pardal *et al.* 2004, Van den Brink and Ter Braak 1999). The PRC diagram was complemented with a diagram with the species weights $b_k$ that measures the affinity of a particular species to the community response pattern. The significance of the PRC diagram was tested by Monte Carlo permutation tests (Van den Brink and Ter Braak 1999).

Variance analyses and PRC analyses were done with Statistica 6.0 (StatSoft, Inc.) and CANOCO 4.5 (Microcomputer Power, NY, USA), respectively.

## 3.3. Results

### *3.3.1. Stream water characteristics*

Analysis of stream water parameters showed that pH (6.0 vs 7.1), temperature (11.0 vs 12.4 ºC), conductivity (39.7 vs 194.9 µS $cm^{-1}$) and chemical oxygen

demand (1.8 vs 48.8 mg $O_2$ $L^{-1}$) were lower at the spring of the Este River (E1) than at the downstream-polluted site (E2) (Table 3.1; t-test, P<0.01). A similar trend was found for inorganic nutrients, namely ammonia (0.006 vs 7.8 mg $L^{-1}$ $N\text{-}NH_4^+$), nitrates (1.6 vs 4.5 mg $L^{-1}$ $N\text{-}NO_3^-$) and phosphates (0.06 vs 1.3 mg $L^{-1}$ $P\text{-}PO_4^{3-}$) (Table 3.1; t-test, P<0.01). Density of total and fecal coliforms was 2 and 3 orders of magnitude lower at E1 than at E2, respectively. On the contrary, concentration of dissolved oxygen (10.9 vs 8.3 mg $L^{-1}$) and redox potential (65.2 vs 23.0 mV) were higher at the spring of the Este River (t-test, P<0.001).

**Table 3.1.** Physical, chemical and microbial characteristics of the stream water at the sampling sites (E1 and E2) of the Este River.

| Parameter | E1 | E2 | n |
|---|---|---|---|
| pH | 6.0 ± 0.1 | 7.1 ± 0.3 | 12 |
| Temperature (ºC) | 11.0 ± 0.3 | 12.4 ± 0.4 | 12 |
| Oxygen (mg $L^{-1}$) | 10.9 ± 0.6 | 8.3 ± 0.3 | 12 |
| Redox potential (mV) | 65.2 ± 5.0 | 23.0 ± 4.0 | 10 |
| Conductivity (µS $cm^{-1}$) | 39.7 ± 2.4 | 194.9 ± 30.3 | 10 |
| $N\text{-}NH_4^+$ (mg $L^{-1}$) | 0.006 ± 0.002 | 7.8 ± 3.2 | 5 |
| $N\text{-}NO_3^-$ (mg $L^{-1}$) | 1.6 ± 0.2 | 4.5 ± 0.6 | 5 |
| $P\text{-}PO_4^{3-}$ (mg $L^{-1}$) | 0.06 ± 0.04 | 1.3 ± 0.3 | 5 |
| Chemical oxygen demand (mg $O_2$ $L^{-1}$) | 1.8 ± 1.3 | 48.8 ± 17.6 | 5 |
| Total coliforms (CFU $mL^{-1}$) | 8.5 ± 6.4 | 9600 ± 2100 | 5 |
| Fecal coliforms (CFU $mL^{-1}$) | 0.2 ± 0.1 | 2900 ± 451 | 5 |

Mean ± SEM; n, number of samples.

CFU, colony forming units.

### *3.3.2. Fungal diversity on decomposing leaves*

Over the entire study, a total of 27 aquatic hyphomycete species were found on leaves decomposing at the reference site, while only 7 species were found at the downstream-polluted site (not shown). Species number (Fig. 3.1A) and the Shannon index (Fig. 3.1B) of aquatic hyphomycetes were significantly higher at E1 than at E2 (ANOVA, P<0.001, for both comparisons). Both time and interactions between sites and time significantly affected species number (ANOVA, P<0.05), while the Shannon index was only affected by the interaction between the two factors (ANOVA, P=0.01). The transfer of leaves from E1 to E2 led to a decrease in diversity, assessed by species number and Shannon index (Tukey, P<0.05),

although transplanted leaves harbored higher fungal diversity than those at E2 (Tukey, P<0.001) (Fig. 3.1A and 3.1B).

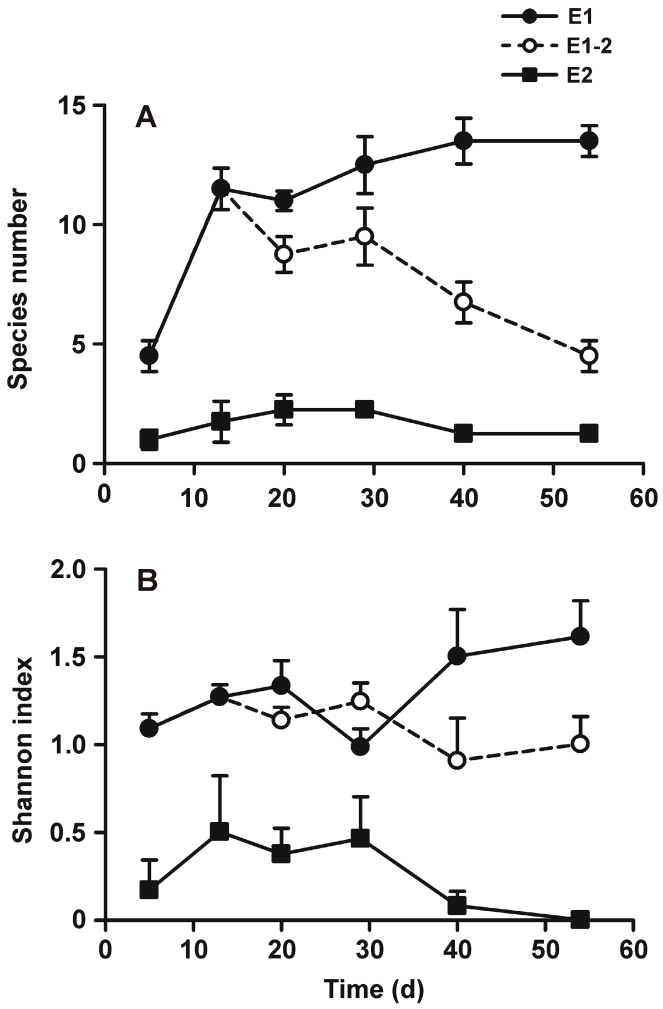


**Figure 3.1.** Species richness (A) and Shannon index (B) of aquatic hyphomycetes on decomposing alder leaves at the reference site (E1), the polluted site (E2) and transplanted from E1 to E2 (E1-2). Mean ± SEM, n=4.

The PRC diagram (Fig. 3.2A) shows that the structure of aquatic hyphomycete communities on leaves decomposing at the reference differed from that at the polluted site. The transfer of leaves from E1 to E2 led to shifts in the community structure, with fungal communities deviating from control further over time. Of the total variance, 26% is explained by time (x-axis), 60% is explained by differences between sites, and the remaining 14% can be attributed to differences between replicates. The differences in fungal communities between sites were significant (Monte Carlo, P=0.002), with the y-axis of the PRC diagram explaining 53% of the variance attributed to sites (Fig. 3.2A).

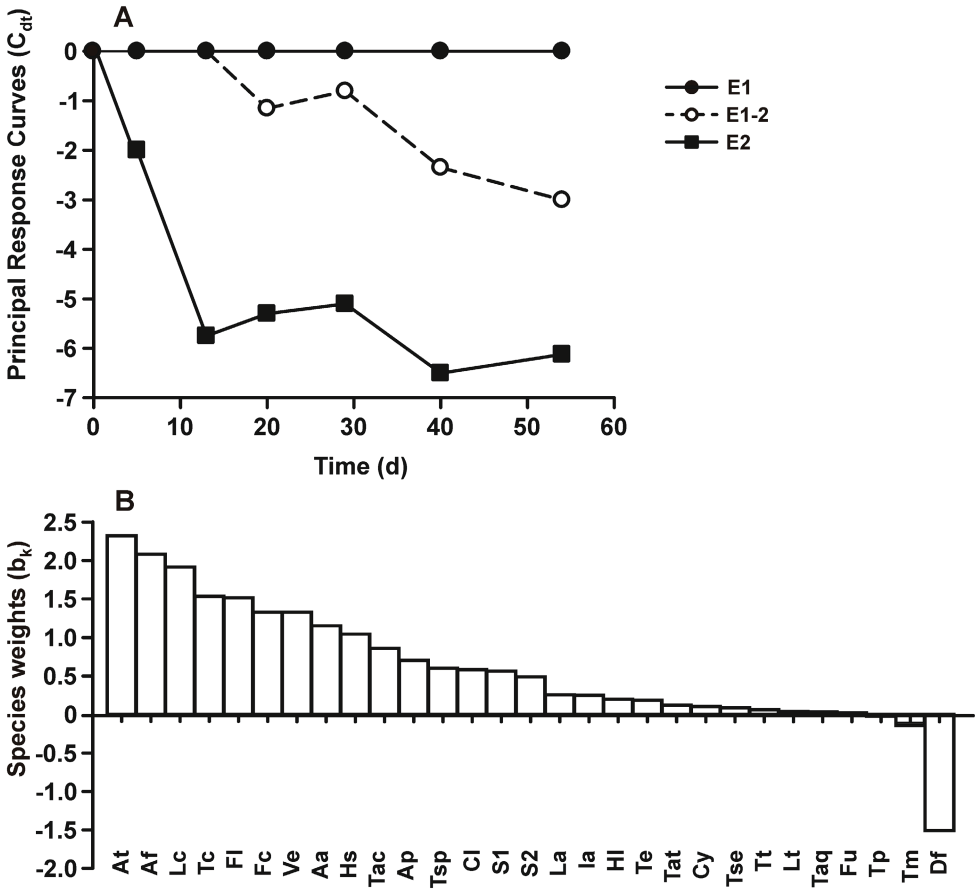


**Figure 3.2.** Principal response curves (A) showing the response of the aquatic hyphomycete communities on decomposing alder leaves at the reference site (E1), the polluted site (E2) and transplanted from E1 to E2 (E1-2). Species weights (B) indicate the relative contribution of each species to the community response pattern. Fungal taxa: At, *Articulospora tetracladia* Ingold; Af, *Anguillospora filiformis* Greath.; Lc, *Lunulospora curvula* Ingold; Tc, *Tricladium chaetocladium* Ingold; Fl, *Flagellospora* sp.; Fc, *Flagellospora curta* J. Webster; Ve, *Varicosporium elodeae* W. Kegel; Aa, *Alatospora acuminata* Ingold; Hs, *Heliscella stellata* (Ingold and V. J. Cox) Marvanová; Tac, *Triscelophorus* cf. *acuminatus* Nawawi; Ap, *Alatospora pulchella* Marvanová; Tsp, *Tricladium splendens* Ingold; Cl, *Clavatospora longibrachiata* (Ingold) Sv. Nilsson ex Marvanová & Sv. Nilsson; S1, Sigmoid 1 (70-125/0.8-1.7 µm); S2, Sigmoid 2 (75-80/2.5-3.5 µm); La, *Lemonniera aquatica* De Wild.; Ia, *Infundibura adhaerens* Nag Raj and W. B. Kendr.; Hl, *Heliscus lugdunensis* Sacc. and Therry; Te, *Tetrachaetum elegans* Ingold; Tat, *Tricladium attenuatum* S. H. Iqbal; Cy, *Cylindrocarpon* sp.; Tse, *Tetracladium setigerum* (Grove) Ingold; Tt, *Tricladium terrestre* D. Park; Lt, *Lemonniera terrestris* Tubaki; Taq, *Tumularia aquatica* (Ingold) Descals & Marvanová; Fu, *Fusarium* sp.; Tp, *Tripospermum prolongatum* R. C. Sinclair and Morgan-Jones; Tm, *Tripospermum myrti* (Lind) S. Hughes; Df, *Dimorphospora foliicola* Tubaki.

The taxa with the highest positive weights were *Articulospora tetracladia*, *Anguillospora filiformis* and *Lunulospora curvula* (Fig. 3.2B), suggesting that they were negatively affected by pollution. Conversely, *Dimorphospora foliicola* had the highest negative weight, which agrees with its higher contribution for spore production on leaves decomposing at E2.

### 3.3.3. Microbial activity on decomposing leaves

Sporulation rates of aquatic hyphomycetes on decomposing leaves were significantly higher at E1 than at E2 and were affected by time of leaf immersion and by the interaction between time and sites (ANOVA, P<0.0001, for all factors) (Fig. 3.3). Peaks of fungal sporulation occurred earlier at E1 (13 days, 1.2 x $10^6$ conidia $g^{-1}$ AFDM $d^{-1}$) than at E2 (54 days, 1.1 x $10^6$ conidia $g^{-1}$ AFDM $d^{-1}$). Leaf transfer from the reference to the polluted site (E1-2) led to a significant decrease in overall fungal sporulation (Tukey, P=0.01).

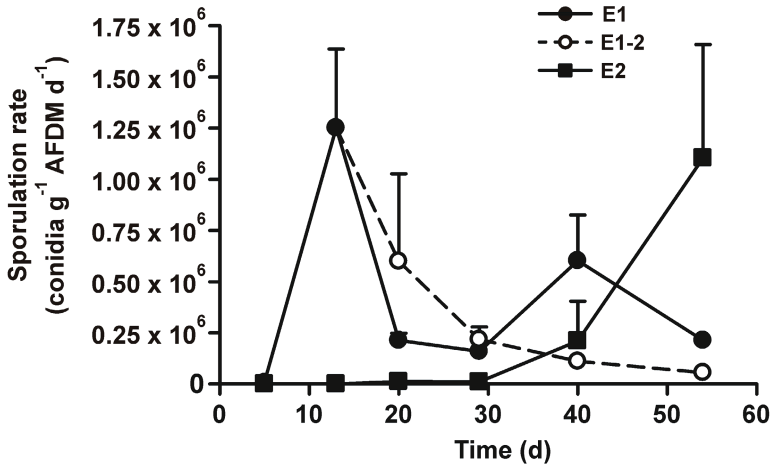


**Figure 3.3.** Sporulation rates of aquatic hyphomycetes on decomposing alder leaves at the reference site (E1), the polluted site (E2) and transplanted from E1 to E2 (E1-2). Mean ± SEM, n=4.

Leaf-associated fungal biomass differed between the two sites of the Este River (ANOVA, P=0.00002) (Fig. 3.4A), with lower values at the polluted site. Peak fungal biomass was 5-times higher at the spring of the Este River (E1, 92.4 mg $g^{-1}$ AFDM) than at the polluted site (E2, 18.1 mg $g^{-1}$ AFDM). The transfer of leaves from the reference to the polluted site (E1-2) did not affect overall fungal biomass (Tukey, P=0.8), which was higher than that found on leaves at E2 (Tukey, P=0.0002).

Bacterial biomass increased significantly with time (ANOVA, P<0.0000001), attaining 0.22 mg $g^{-1}$ AFDM after 54 days of leaf immersion at both sites. Interaction between time and sites was also significant (ANOVA, P=0.005). Overall bacterial biomass was significantly lower at E1, intermediate at E2 and higher on leaves transplanted from E1 to E2 (Tukey, P<0.05) (Fig. 3.4B).

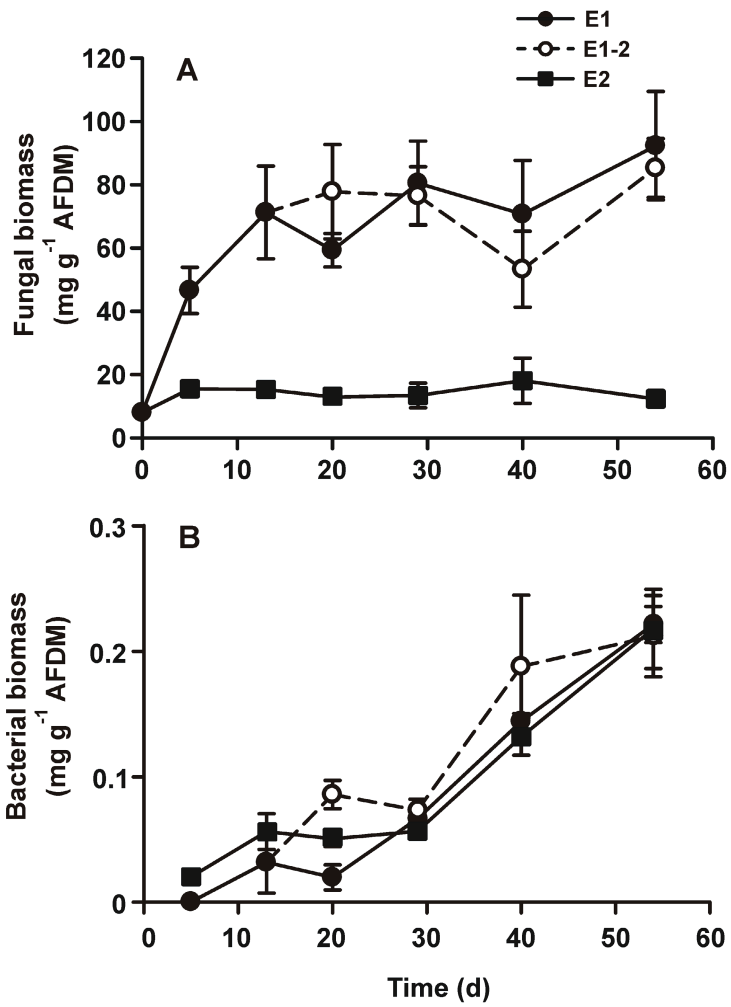


**Figure 3.4.** Biomass of fungi (A) and bacteria (B) on decomposing alder leaves at the reference site (E1), the polluted site (E2) and transplanted from E1 to E2 (E1-2). Mean ± SEM, n=4.

Mass loss of alder leaves followed an exponential decay model along 54 days of leaf immersion (Fig. 3.5), and leaf decomposition was significantly faster at the reference (E1, k=0.0107 $d^{-1}$) than at the polluted site (E2, k=0.0066 $d^{-1}$) (Table 3.2) (ANCOVA, $P<0.05$). The transfer of decomposing leaves from E1 to E2 (E1-2) did not significantly decrease leaf decomposition rate (Fig. 3.5, Table 3.2) (ANCOVA, $P>0.05$).

**Table 3.2.** Decomposition rates (k) of alder leaves immersed at the reference site (E1), the polluted site (E2) and transplanted from E1 to E2 (E1-2).

| Site | k ($d^{-1}$) ± SE | $W_0$ (%) | $r^2$ |
|---|---|---|---|
| E1 | 0.0107 ± 0.0011[a] | 92.2 | 0.77 |
| E2 | 0.0066 ± 0.0008[b] | 101.6 | 0.74 |

| | | | |
|---|---|---|---|
| E1-2 | 0.0092 ± 0.0011[a,b] | 90.3 | 0.73 |

SE standard error; $W_0$ intercept; $r^2$ coefficient of determination. Identical superscript letters indicate no significant differences (ANCOVA, Tukey´s test P>0.05); n=28.

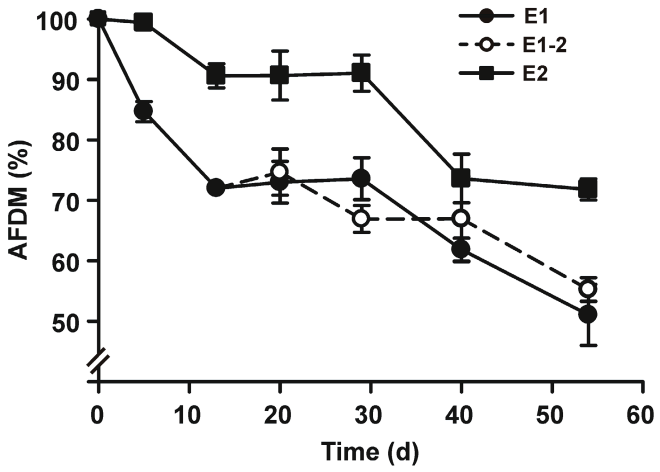


**Figure 3.5.** Ash-free dry mass (AFDM) remaining of decomposing alder leaves at the reference site (E1), the polluted site (E2) and transplanted from E1 to E2 (E1-2).

## 3.4. Discussion

One of the consequences of species loss may be the alteration or loss of certain ecosystem processes and, therefore, understanding how changes in biodiversity affect the cycling of nutrients and carbon is a priority in ecological research (Covich *et al.* 2004). Most research on the role of biodiversity in ecosystem functioning has focused on the general relationship between species richness and some ecological processes, regardless of how environmental factors or disturbances affect species richness per se (but see Niyogi *et al.* 2002a, Norberg 2004).

In this work, we investigated how a community of microbial decomposers adapted to a reference site responds to a sudden decrease in the water quality by transplanting colonized leaves from a reference to a polluted site. We found slower leaf decomposition at the polluted than at the reference site that corresponded to the lower fungal diversity, biomass and sporulation. The upstream reference community had high number of fungal species (27 species) when compared to other communities in streams of the Iberian Peninsula (Bärlocher *et al.* 1995, Chauvet *et al.* 1997, Pascoal *et al.* 2003, Ferreira *et al.* 2006), probably as a consequence of the moderate nutrient concentrations found in the stream water at the spring of the Este River. Also, peaks of fungal biomass and sporulation were high but within the

range reported for alder leaves in streams of the Northwest Portugal (Duarte *et al.* 2004, Pascoal *et al.* 2005a). The high fungal activity probably helps to explain the rapid leaf decomposition found at the reference site.

The differences in fungal diversity between the two studied sites were remarkable taking into account that the polluted site is only ca. 8 km downstream the reference site and that the recruitment of new species is conceivable to occur. However, a decline in the number of aquatic hyphomycete species has been found in streams of the Northwest Portugal impacted by urbanization, agricultural and industrial activities (Pascoal *et al.* 2005a,b). The impoverishment of decomposer communities due to anthropogenic stress has shown variable effects on leaf litter decomposition. Leaf decomposition is reported to be less affected by both inorganic and organic pollution than biodiversity (Raviraja *et al.* 1998, Niyogi *et al.* 2002b, Duarte *et al.* 2004, Pascoal *et al.* 2005a), but in streams of central Germany with a long history of mining activities both leaf decomposition and fungal diversity are severally reduced (Sridhar *et al.* 2001, 2005).

The transfer of leaves colonized at the reference to the polluted site did not affect leaf decomposition or fungal biomass, but reduced fungal sporulation and diversity on leaves. The reduction in fungal diversity associated with the absence of significant effects on functions (biomass build-up and decomposition) suggests that aquatic fungi might be functionally redundant, with the loss of more sensitive species being compensated by more tolerant ones (Walker 1992, Raviraja *et al.* 1998, Pascoal *et al.* 2005a). However, as fungal diversity on leaves was assessed from released spores, and sporulation was strongly inhibited at the downstream-polluted site, we cannot rule out the hypothesis that some fungal species just stopped sporulating while their mycelia were still growing on leaves. But, if some species fail to reproduce, they will tend to disappear as time goes by with possible effects on ecosystem functions.

On the other hand, bacterial biomass was higher on leaves exposed to the polluted site or transplanted from the reference site. Bacteria on leaves are reported to increase with the increase in nutrient loading, although its contribution to leaf decomposition is considerably lower when compared to that of fungi (Gulis and Suberkropp 2003a, Pascoal and Cássio 2004). Moreover, in our work, maximum bacterial biomass on leaves remained at the lower limit reported in literature (Hieber and Gessner 2002, Gulis and Suberkropp 2003a,b, Pascoal *et al.* 2005a). Thus, it is unlikely that the augmented bacterial biomass had contributed markedly to leaf

decomposition at the study sites, pointing to fungi as the main drivers of leaf decomposition.

It is often argued that ecosystem functioning is likely to be less affected by the loss of species in species-rich communities than in species-poor communities, because highly diverse communities have greater probability of containing very productive species or functionally redundant species (Hooper *et al.* 2005). Indeed, in our work, the loss of fungal species (9 species) on transplanted leaves did not lead to a decrease in ecosystem performance, probably because initial fungal diversity on those leaves was high that mitigated the impact of pollution on overall activity of the community after some species were lost. However, given that recent theoretical studies emphasize that spatial and temporal scales may change the response patterns and the mechanisms explaining biodiversity effects (Cardinale *et al.* 2004, Loreau *et al.* 2003), larger spatial and longer temporal scales have to be considered to fully understand the role of biodiversity in ecosystem functioning and stability.

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# Chapter 4

*Assessing diversity of fungi and bacteria during leaf decomposition in a low-order stream by traditional and molecular techniques*

## Abstract

Microorganisms play an important role during decomposition of leaf detritus in streams; however, the relative contribution of aquatic fungi and bacteria to this ecosystem process is not fully understood, mainly because of difficulties to accurately access their diversity. In this work, we addressed this question by monitoring diversity and activity of fungi and bacteria associated with decomposing alder leaves in a low-order stream (Este River, NW Portugal) during 56 days. Decomposition of alder leaves was fast, and fungal biomass as ergosterol concentration was much higher than bacterial biomass, from bacterial counts after DAPI staining. Based on conidial shape, a total of 22 aquatic hyphomycete sporulating taxa were found on leaves, being *Heliscus lugdunensis*, *Articulospora tetracladia* and *Flagellospora* sp. the most contributors for total conidial production. Denaturing gradient gel electrophoresis of fungal and bacterial DNA pointed to a higher diverse microbial community on decomposing leaves than assessed by microscopic-based techniques. Although higher number of bands was obtained with the primer pair targeting the ITS2 region than with the one targeting the 18S region of rDNA, both primers revealed a succession of fungal taxa during leaf decomposition time. Concerning bacteria, primer pairs targeting the V3 region and the V6-V8 regions revealed similar number of bands.

## 4.1. Introduction

Allochthonous plant-litter is the main source of nutrients and energy in low-order forested streams (Suberkropp 1998, Bärlocher 2005). Microbial decomposers, namely fungi and bacteria, play a crucial role in plant litter decomposition because they produce an extent array of enzymes that transform plant litter into a more suitable food source for stream detritivores (Suberkropp 1998, Bärlocher 2005). In a wide range of streams with contrasting water properties, fungi, particularly aquatic hyphomycetes, have been found to contribute more than bacteria to plant-litter decomposition at least in the earlier stages of the process, but bacteria appear to increase their contribution at later times (e.g. Baldy *et al.* 1995, Hieber and Gessner 2002, Gulis and Suberkropp 2003a, Pascoal and Cássio 2004, Pascoal *et al.* 2005b). Most studies addressing the role of microbial decomposers associated with plant-litter in streams are based on measurements of fungal and bacterial biomasses (Baldy *et al.* 1995, 2002, 2007, Hieber and Gessner 2002, Gulis and Suberkropp 2003a,b, Pascoal and Cássio 2004, Pascoal *et al.* 2005a,b). In addition to this, the structure of microbial decomposer communities have been often examined, particularly that of fungi (Hieber and Gessner 2002, Pascoal *et al.* 2005a,b,c, Gulis and Suberkropp 2003a,b).

The traditional approach to assess the contribution of each aquatic fungal species to leaf-litter decomposition in streams is based on the microscopic identification and counting of conidia either on leaf surfaces (Suberkropp and Klug 1976) or released from leaves (Bärlocher 1982). However, fungal species with high sporulation rates are not always the ones that produce more biomass (Bermingham *et al.* 1997, chapter 2) and it is the active mycelium and not the conidia that decompose the leaves. Consequently, we may not be able to detect nonsporulating mycelia and to accurately assess the contribution of fungal species to leaf-litter decomposition. Diversity studies on leaf-litter associated bacteria are scarce; they are mostly limited to analysis of the number of different cellular shapes (Baldy *et al.* 1995, Hieber and Gessner 2002) or to studies on cultivable genera (Suberkropp and Klug 1976), without any information on accurate taxon diversity. Additionally, only 1 to 15% of bacteria have been reported to be cultivable and identifiable by traditional methods (Torsvik *et al.* 1990, Ward *et al.* 1990, Amann *et al.* 1995).

Techniques of DNA fingerprinting, such as denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorfism (T-RFLP), have been successfully used to study fungal communities on decomposing

leaf litter (Nikolcheva *et al.* 2003, Nikolcheva and Bärlocher 2005, Nikolcheva *et al.* 2005, Das *et al.* 2007). The application of these techniques revealed a high number of fungal taxa after a short period of leaf immersion in streams, contrasting to the low number of sporulating species generally found (Nikolcheva and Bärlocher 2005, Nikolcheva *et al.* 2005). Moreover, DGGE has been valuable in detecting seasonal shifts of fungal taxa in different leaf species and wood material (Nikolcheva and Bärlocher 2005) and changes in fungal and bacterial communities during leaf decomposition in streams (Das *et al.* 2007).

Since DNA fingerprinting techniques strongly rely on polymerase chain reaction (PCR), the choice of adequate primers for accurate characterization of microbial communities is critical (Schmalenberger *et al.* 2001). Several primer sets have been developed to analyse fungal (Kowalchuk and Smit 2004) and bacterial communities (Schmalenberger *et al.* 2001, Muyzer *et al.* 2004). The primer pairs ITS3GC/ITS4, targeting the internal transcribed spacer region 2 (ITS2) and NS1/GCfung, targeting a portion of the 5' end of the 18S rDNA, have been used to characterize fungal communities on decomposing leaves and the diversity of conidia in streams using DGGE (Nikolcheva *et al.* 2003, Das *et al.* 2007 for NS1/GCfung and Nikolcheva and Bärlocher 2005, Nikolcheva *et al.* 2005, Raviraja *et al.* 2005 for ITS3GC/ITS4).

For bacteria, several primer pairs have been developed for different variable regions of the bacterial 16S rDNA (Schmalenberger *et al.* 2001, Muyzer *et al.* 2004). Primer pairs targeting the V3 region (e.g. 338GC/518) and the V6-V8 regions (e.g. 984GC/1378) have been widely used in a number of studies to characterize bacterial communities from different habitats (Øvreas *et al.* 1997, Nakatsu *et al.* 2000, Ibekwe *et al.* 2001, 2002, Assigbetse *et al.* 2005, Moura *et al.* 2007) including bacteria on decomposing leaves in aquatic systems (Das *et al.* 2007).

In the current study we followed microbial colonization of alder leaves in a low-order stream by measuring the diversity and activity of leaf-associated fungi and bacteria. For that, we immersed alder leaves at the spring of the Ester River and we assessed leaf mass loss and the associated microbial biomass and fungal sporulation over 56 days. Bacterial and fungal diversity on decomposing alder leaves was assessed by microscopy-based techniques and by DGGE fingerprinting. Since microbial communities on decomposing leaves are still poorly characterized, two different primer pairs targeting different regions of the ribosomal DNA of fungi and bacteria were used to better analyse the shifts of fungal and bacterial communities along leaf decomposition.

## 4.2. Materials and methods

### *4.2.1. Study site*

The Este River is located in the Northwest of Portugal and flows through the town of Braga. At the spring, the Este River has about 30 cm deep and 50 cm wide with a bottom mainly constituted by rocks, pebbles and gravel. The riparian vegetation consists of *Eucalyptus globulus* Labill., *Pinus pinaster* Aiton, *Pteridium aquilinum* Khun, *Juncus* sp., *Alnus glutinosa* (L.) Gaerthner and *Rubus ulmifolius* Schott.

Temperature, pH, redox potencial, oxygen concentration and conductivity of the stream water was measured *in situ* with field probes (Multiline F/set 3 no 400327, WTW) and water samples were periodically collected during the study for determination of nitrate (HACH kit, program 355), phosphate (HACH kit, program 480) and ammonia concentrations (HACH kit, program 385) using a HACH DR/2000 photometer (Hach company, Loveland, CO, USA) and also for the determination of total and fecal coliforms (membrane filtration technique) (APHA 1998).

### *4.2.2. Field experiment*

Leaves of *A. glutinosa*, collected in October 2004 just prior to abscission and dried at room temperature, were distributed by 21 fine-mesh bags (0.5 mm mesh size; 15 x 15 cm). Leaf bags were immersed at the spring of the Este River on 2 February 2005. On each sampling date (5, 10, 16, 25, 41 and 56 days), three leaf bags were randomly collected from the stream. At the beginning of the experiment, three leaf bags were immersed in the stream water at the study site for 15 min and used to estimate the initial mass of the leaves and the associated microbial parameters.

### *4.2.3. Leaf bag processing*

At the laboratory, leaves from each bag were rinsed with deionized water to remove sediments and invertebrates, when present, and cut into disks. Sets of leaf disks from each replicate bag were used for ergosterol quantification (5 disks), DNA extraction (3 disks), sporulation experiments (8 disks), quantification of bacterial biomass (3 disks) and isolation of bacteria (2 disks). For ergosterol quantification

and DNA extraction leaf disks were kept freeze-dried until used. The remaining leaves were dried for 48 hours at 80 ºC and weighed to the nearest 0.01 mg. The dried samples were ashed at 550 ºC for 6 hours and weighed to determine the organic matter content (ash-free dry mass, AFDM).

### *4.2.4. Fungal biomass*

Fungal biomass associated with leaf disks was estimated from ergosterol concentration. Lipids were extracted from leaves by heating in 0.8% of KOH/methanol (80 ºC, 30 min) and the extract was purified by solid-phase extraction according to Gessner (2005). Ergosterol was quantified by HPLC using a LiChrospher RP18 column (250 x 4 mm, Merck), connected to a Beckmann Gold liquid chromatographic system. The system was run isocratically with HPLC-grade methanol (Riedel de-Haën) at 1.4 mL $min^{-1}$ and column temperature at 33 ºC, and ergosterol was detected at 282 nm. Standard series of ergosterol (Fluka) in isopropanol were used to estimate the ergosterol concentration in the samples. Ergosterol was converted to fungal biomass by using a factor of 5.5 mg ergosterol $g^{-1}$ fungal dry mass (Gessner and Chauvet 1993).

### *4.2.5. Fungal sporulation rates*

Eight leaf disks from each replicate bag were transferred to 150 mL Erlenmeyer flasks containing 60 mL of sterilized stream water (120 ºC, 1 atm, 20 min). The flasks were kept at 120 rpm, 15 ºC during 48 h. After that, conidial suspensions were mixed with 200 µL of 0.5% of Tween 80 and filtered (5 µm pore size, Millipore); the retained conidia were stained with 0.05% cotton blue in lactic acid (Fluka). Conidia were identified and counted under a light microscope (400x, Leica Biomed), and at least a total of 300 conidia were scanned per filter.

### *4.2.6. Bacterial biomass*

A set of 5 leaf disks from each replicate bag was kept in tubes containing 10 mL phosphate buffered formalin (3.7% final concentration) at 4 ºC until processed. Bacterial cells were dislodged from leaf disks in a sonication bath (5 min, Branson 2510, Danbury, CT, USA). A final volume of 2 mL of each bacterial

suspension diluted appropriately was stained with 40 µL of 0.1 mg $mL^{-1}$ of DAPI (4', 6-diamidino-2-phenylindone, Sigma) for 10 min in the dark and filtered through black polycarbonate filters (0.2 µm pore size, GTTP, Millipore). Each filter was mounted in one slide between two drops of mineral oil and bacteria were counted using an epifluorescence microscope (1000x, Leitz Laborlux S). To determine bacterial biovolumes, cells were assigned to classes defined by bacterial size and shape. Bacterial biovolumes were calculated as $V = (\pi / 4) \cdot W^2 \cdot (L - W / 3)$, where $W$ is the cell width and $L$ is the cell length (Bratbak 1993). The average biomass of each bacterial class was converted in bacterial biovolume according to $C = 89.6 \cdot V^{0.59}$, where $V$ is the bacterial volume in $\mu m^3$ and $C$ is the bacterial carbon in *f*g (Simon and Azam 1989), assuming a 50% of carbon in bacterial dry mass.

### *4.2.7. DNA sources*

The fungal species *Alatospora acuminata* Ingold (UMB 173.01), *Anguillospora filiformis* Greath. (UMB 225.02), *Articulospora tetracladia* Ingold (UMB 22.01), *Dimorphospora foliicola* Tubaki (UMB 30.01), *Flagellospora curta* J. Webster (UMB 39.01), *Heliscus lugdunensis* Sacc. & Therry (UMB 159.01), *Lunulospora curvula* Ingold (UMB 123.01), *Tricladium chaetocladium* Ingold (UMB 163.01), *Tricladium splendens* Ingold (UMB 54.01) and *Varicosporium elodeae* W. Kegel (UMB 20.01) were grown on 1% malt extract agar and two plugs of mycelia with 5 mm diameter each were used for DNA extraction, as described in the next section.

Pure cultures of leaf-associated bacteria were obtained as follows: 2 leaf disks, kept in 10 mL of filtered stream water (0.2 µm pore size, Sarstedt) at 4 ºC, were placed in a sonication bath to release the bacteria as described above. Serial dilutions of bacterial suspensions were prepared and 0.1 mL of each was inoculated in PYG (peptone 0.1%, yeast extract 0.02%, glucose 0.1%, Suberkropp and Klug 1976) and $R_2A$ agar plates and incubated at 15 ºC. A total of 60 bacterial isolates were obtained and 18 were differentiated based on the color and morphology of the colony, cellular morphology, Gram staining, presence/absence of catalase activity and the ability to grow on Endo agar.

### *4.2.8. DNA extraction and amplification*

DNA was extracted from a total of 4 halves of 4 different freeze-dried leaf disks, from 2 plugs of mycelia or from 2 colonies of bacteria grown on $R_2A$ agar with FastDNA SPIN kit for soil in conjunction with the FastPrep FP120 Instrument (Qbiogene, Heidelberg, Germany).

Fungal diversity was assessed with the primer pairs ITS3GC/ITS4 and NS1/GCfung, which amplifies the ITS2 region of fungal rDNA and the 5' end of the 18S rDNA, respectively (Table 4.1). Bacterial diversity was examined with the primer pairs 338FGC/518R and 984FGC/1378R, which targets the V3 and V6-V8 regions of bacterial 16S ribosomal DNA (Table 4.1). The forward primers had a 40-bp GC tail at the 5' end, which ensures separation on DGGE gels (Muyzer *et al.* 1993, Nübel *et al.* 1996).

For PCR reactions 1x of Taq buffer (mixture of KCl:$(NH_4)_2SO_4$ buffers), 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. All PCR reagents were purchased from MBI Fermentas excepting the primers that were from MWG-Biotech AG. PCRs were carried out in an iCycler Thermal Cycler (BioRad Laboratories, Hercules, CA, USA). The amplification program of fungal and bacterial DNA started with an initial 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 and Bärlocher 2005, Nikolcheva *et al.* 2005).

**Table 4.1.** Primers used in the PCRs.

| Microorganism and primer | Primer sequence (5' – 3') | Reference |
|---|---|---|
| Bacteria | | |
| F338GC | CG-ACTCCTACGGGAGGCAGCAG | Lane 1991 |
| R518 | ATTACCGCGGCTGCTGG | Muyzer *et al.* 1993 |
| F984GC | GC-AACGCGAAGAACCTTAC | Nübel *et al.* 1996 |
| R1378 | CGGTGTGTACAAGGCCCGGAACG | Heuer *et al.* 1997 |
| Fungi | | |
| NS1 | GTAGTCATATGCTTGTCTC | White *et al.* 1990 |
| GCfung | GC-ATTCCCCGTTACCCGTTG | May *et al.* 2001 |
| ITS3GC | GC-GCATCGATGAAGAACGCAGC | White *et al.* 1990 |
| ITS4 | TCCTCCGCTTATTGATATGC | White *et al.* 1990 |
| GC (bacteria) | CGCCCGCCGCGCGCGGCGGGCGGG GCGGGGGCACGGGGGG | Nübel *et al.* 1996 |
| GC (fungi) | CGCCCGCCGCGCCCCGCGCCCGGC CCGCCGCCCCCGCCCC | Muyzer *et al.* 1993 |

F, Forward primer; R, reverse primer; GC, G+C rich sequence attached to 5' end of the sequence.

### *4.2.9. Denaturing gradient gel electrophoresis*

DGGE analyses were performed using a DCode™ Universal Mutation Detection System (BioRad Laboratories, Hercules, CA, USA). DNA samples with a final concentration of 750 ng of fungal DNA (pooled from the three replicates) from the amplification products of 380-400 bp (ITS3GC/ITS4) or of 370 bp (NS1/GCfung) were loaded on 8% (w/v) polyacrylamide gels in 1x TAE with denaturing gradients from 30 to 70% or 20 to 55%, respectively (100% denaturant corresponds to 40% formamide and 7 M urea). Samples of bacterial DNA with 750 ng (pooled from the three replicates) from the amplification products of 200 bp (338GC/518) or of 433 bp (984GC/1378) were loaded on 8% or 6% (w/v) polyacrylamide gels in 1x TAE with a denaturing gradient from 35 to 80% or 52.5 to 75%, respectively. The gels were run at 55 V, 56 ºC for 16 h and stained with 1 µg $mL^{-1}$ of ethidium bromide (BioRad) for 5 min. The gel images were captured under UV light in a transiluminator Eagle eye II (Stratagene, La Jolla, CA, USA).

### *4.2.10. Statistical analyses*

Ash-free dry mass of remaining leaves was fit to the exponential model $m_t = m_0 \cdot e^{-kt}$, where $m_t$ is leaf dry mass remaining at time *t*, $m_0$ the initial leaf dry mass and *k* the decomposition rate. The rate of leaf decomposition was determined from, the linear regression of ln-transformed values of ash-free dry mass of remaining leaves against time (Zar 1996) using GraphPad Prism 5.0 for windows.

The structure of microbial communities at each sampling date was assessed by UPGMA-cluster analysis based on the Jaccard coefficient of similarity. The dendogram for fungal sporulating communities was constructed with the Primer v6 software package. Dendograms for fungal and bacterial DGGE banding patterns were constructed with the GelCompar II program (Applied Maths, Belgium).

## 4.3. Results

### *4.3.1. Characterization of stream water, leaf decomposition and microbial activity*

At the spring of the Este River, the stream water had an average pH of 7 and a conductivity of 33 µS $cm^{-1}$ (Table 4.2). Average stream water temperature was 8 ºC and oxygen concentrations were above saturation. Mean values for concentration of inorganic nutrients in the stream water were: 0.9 mg $L^{-1}$ of $N\text{-}NO_3^-$, 0.2 mg $L^{-1}$ of $P\text{-}PO_4^{3-}$ and 0.01 mg $L^{-1}$ of $N\text{-}NH_4^+$ (Table 4.2). Fecal coliforms were not detected in the stream water during the study, while densitiy of total coliforms corresponded to 23 CFU $mL^{-1}$.

**Table 4.2.** Characteristics of the stream water at the spring of the Este River.

| Parameter | Mean (range) | n |
|---|---|---|
| pH | 6.9 (5.9-7.5) | 7 |
| Temperature (ºC) | 9.2 (5.8-12.9) | 7 |
| Conductivity (µS $cm^{-1}$) | 42.1 (30-52.3) | 7 |
| Oxygen (%) | 110.5 (88.7-135.5) | 7 |
| $N\text{-}NO_3^-$ (mg $L^{-1}$) | 0.9 (0.6-1.7) | 7 |
| $P\text{-}PO_4^{3-}$ (mg $L^{-1}$) | 0.2 (0-0.8) | 7 |
| $N\text{-}NH_4^+$ (mg $L^{-1}$) | 0.01 (0-0.04) | 7 |
| Total coliforms (CFU $mL^{-1}$) | 23 | 1 |
| Fecal coliforms (CFU $mL^{-1}$) | 0 | 1 |

CFU, colony forming units.

After 56 days of leaf immersion at the spring of the Este River alder leaves lost about 90% of their initial mass. Leaf decomposition was fast, with a decomposition rate of 0.045 $d^{-1}$ ($r^2$=0.86; intercept, 114.8%) (Fig. 4.1A).

Fungal sporulation rate increased till 26 days of leaf immersion, reaching a maximum of 1.4 x $10^6$ conidia $g^{-1}$ AFDM $d^{-1}$, but then sharply declined till 41 days of leaf immersion (Fig. 4.1B). Fungal biomass increased till the end of the experiment, attaining 58 mg $g^{-1}$ AFDM (Fig. 4.1C), while bacterial biomass showed a peak of 0.83 mg $g^{-1}$ AFDM after 41 days of leaf immersion and then declined (Fig. 4.1D). During the study period, fungal biomass largely exceeded that of bacteria, contributing with more than 98% to the total microbial biomass on decomposing leaves.

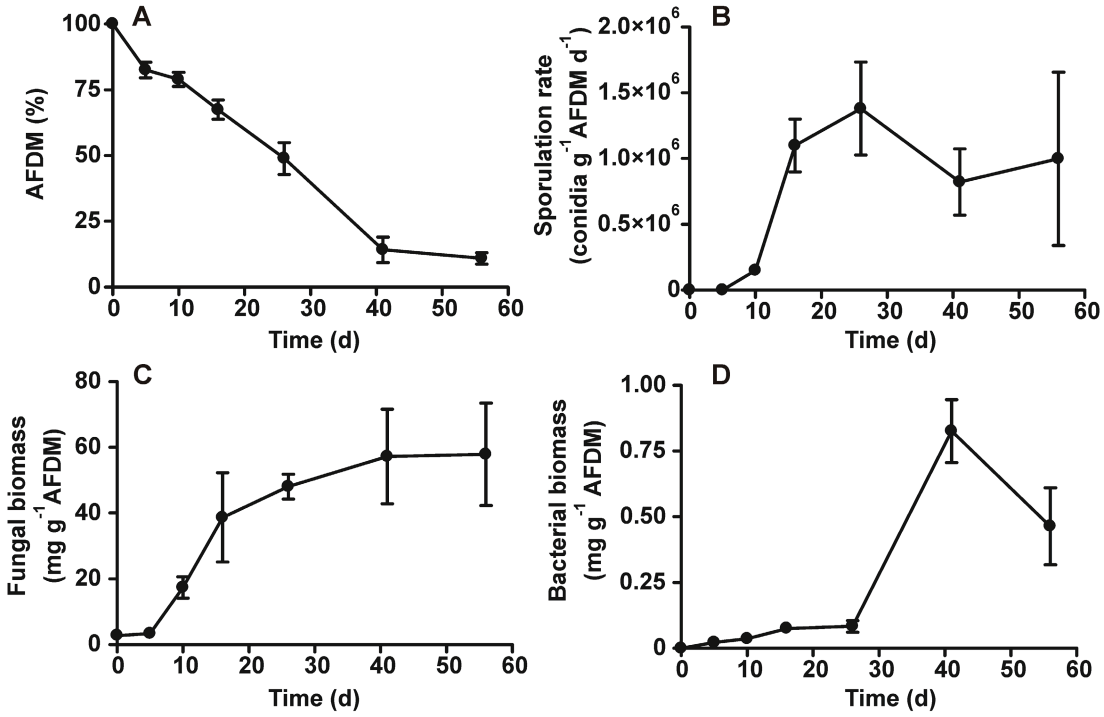


**Figure 4.1.** Ash-free dry mass remaining (AFDM) (A), fungal sporulation (B), fungal biomass (C) and bacterial biomass (D) associated with decomposing leaves at the spring of the Este River. Mean ± SEM, n=3.

### *4.3.2. Fungal diversity*

From conidial identification and counting, 22 aquatic fungal taxa were found on decomposing alder leaves during the whole study, with a maximum of 19 taxa after 41 days of leaf immersion (Table 4.3). DNA amplification of the environmental

samples with the primer pair ITS3GC/ITS4 yielded a higher number of different bands than with the primer pair NS1/GCfung (35 and 26 bands, respectively) (Fig. 4.3, Table 4.4). Additionally, DGGE analysis of DNA of the aquatic hyphomycete pure cultures indicated that from a total of 10 species, the primer pair ITS3GC/ITS4 allowed the discrimination of 9 bands while the primer pair NS1/GCfung discriminated only 4 bands (Fig. 4.4).

Cluster analyses of both sporulating fungal communities and DGGE banding patterns, with fungal DNA amplified by the two primer pairs, revealed differences in the microbial community at different stages of leaf immersion, indicating a succession of fungal taxa throughout decomposition (Fig. 4.2 and 4.3).

**Table 4.3.** Percentage contribution of aquatic hyphomycete species for the total conidial production on decomposing leaves at the spring of the Este River, n=3.

| Species | Time (d) | | | | | |
|---|---|---|---|---|---|---|
| | 5 | 10 | 16 | 26 | 41 | 56 |
| *Alatospora acuminata* Ingold | | | | 0.2 | 0.5 | 0.7 |
| *Alatospora* cf. *flagellata* (J. Gönczöl) Marvanová | | | | | <0.1 | |
| *Alatospora pulchella* Marvanová | | | | 0.4 | 0.1 | 0.3 |
| *Anguillospora filiformis* Greath. | | 0.4 | 11.1 | 2.8 | 3.0 | 0.1 |
| *Articulospora tetracladia* Ingold | | 38.2 | 44.0 | 58.4 | 6.6 | 5.6 |
| *Clavatospora longibrachiata* (Ingold) Sv. Nilsson ex Marvanová & Sv. Nilsson | | | 0.4 | 0.1 | 0.6 | 1.7 |
| *Cylindrocarpon* sp. | | | 0.1 | | <0.1 | 0.2 |
| *Dimorphospora foliicola* Tubaki | | 1.4 | 0.4 | 0.6 | 3.4 | 12.1 |
| *Flagellospora curta* J. Webster | | | | 0.2 | 0.3 | 0.2 |
| *Flagellospor*a sp. | | 7.6 | 18.7 | 27.4 | 66.2 | 48.7 |
| *Fusarium* sp. | | | | 0.5 | | 0.2 |
| *Heliscella stellata* (Ingold & V.J. Cox) Marvanová | | | | | 0.6 | 2.4 |
| *Heliscus lugdunensis* Sacc. & Therry | | 50.9 | 19.3 | 7.7 | 3.5 | 0.5 |
| *Infundibura adhaerens* Nag Raj & W. B. Kendr. | | | | | 2.1 | 1.2 |
| *Lunulospora curvula* Ingold | | 0.4 | 0.6 | 0.1 | 3.3 | 7.9 |
| *Tricladium chaetocladium* Ingold | | 1.1 | 4.1 | 0.5 | 7.2 | 10.4 |
| *Tricladium splendens* Ingold | | | 0.1 | <0.1 | 2.0 | 7.1 |
| *Tripospermum myrti* (Lind) S. Hughes | 100.0 | | 0.1 | | | |
| *Triscelophorus* cf. *acuminatus* Nawawi | | | | | <0.1 | |
| *Varicosporium elodeae* W. Kegel | | | 0.8 | 0.9 | 0.5 | 0.4 |
| Sigmoid 1 (70-125/0.8-1.7 µm) | | | 0.1 | 0.1 | | |
| Sigmoid 2 (75-80/2.5-3.5 µm) | | | | | 0.2 | 0.2 |
| Nº of species | 1 | 7 | 13 | 15 | 19 | 18 |

For fungal sporulating communities two main divisions were found: one comprising communities corresponding to 5 days of leaf immersion and the other including the communities from later times. UPGMA analysis of the DGGE fingerprints showed two major clusters for both primer pairs; fungal communities on leaves immersed for 5 and 10 days deviated from those of the later dates (Fig. 4.3).

After 10 days of leaf immersion, *Heliscus lugdunensis* was the species that most contributed to the total conidial production, but after 16 and 26 days it was surpassed by *Articulospora tetracladia*. At later times (41 and 56 days) *Flagellospora* sp. dominated spore production.

The analysis of DGGE gels showed that some of the bands that appeared when leaves were immersed in the stream disappeared at later dates, while the opposite occurred with bands that matched with those from aquatic hyphomycetes in pure cultures (Fig. 4.3). For example, using ITS3GC/ITS4 primer pair, the bands

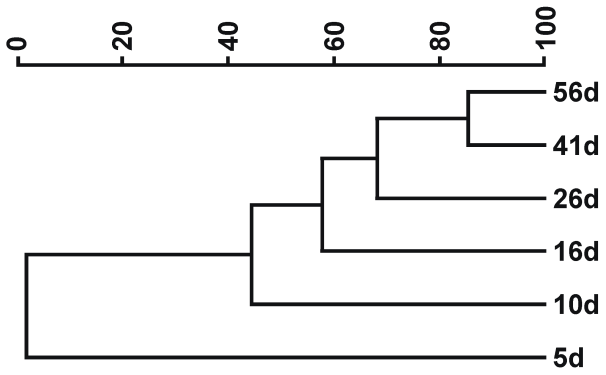


**Figure 4.2.** Dendogram from UPGMA cluster analysis based on the Jaccard coefficient of similarity from the aquatic hyphomycete sporulating communities associated with decomposing leaves at the spring of the Este River.

**Table 4.4.** Fungal and bacterial diversity from DGGE bands and conidial morphotypes associated with decomposing leaves at the spring of the Este River.

| Time (d) | Conidial morphotypes | DGGE bands | | | |
|---|---|---|---|---|---|
| | | NS1/GCfung | ITS3GC/ITS4 | 338GC/518 | 984GC/1378 |
| 0 | n.d. | 10 | 10 | 10 | 10 |
| 5 | 1 | 11 | 18 | 15 | 13 |
| 10 | 7 | 11 | 20 | 23 | 20 |
| 16 | 13 | 15 | 18 | 24 | 23 |
| 26 | 15 | 10 | 20 | 29 | 22 |
| 41 | 19 | 13 | 22 | 22 | 21 |
| 56 | 18 | 13 | 22 | 21 | 20 |

n.d. not determined

that matched with those produced by pure cultures of *A. tetracladia* and *V. elodeae* appeared between the 16 and 56 days of leaf immersion, but did not appear at the two first sampling dates (Fig. 4.3, Table 4.3). Using NS1/GCfung primer pair, a similar trend was found for the band that matched with that of the pure culture of *Tricladium splendens*.

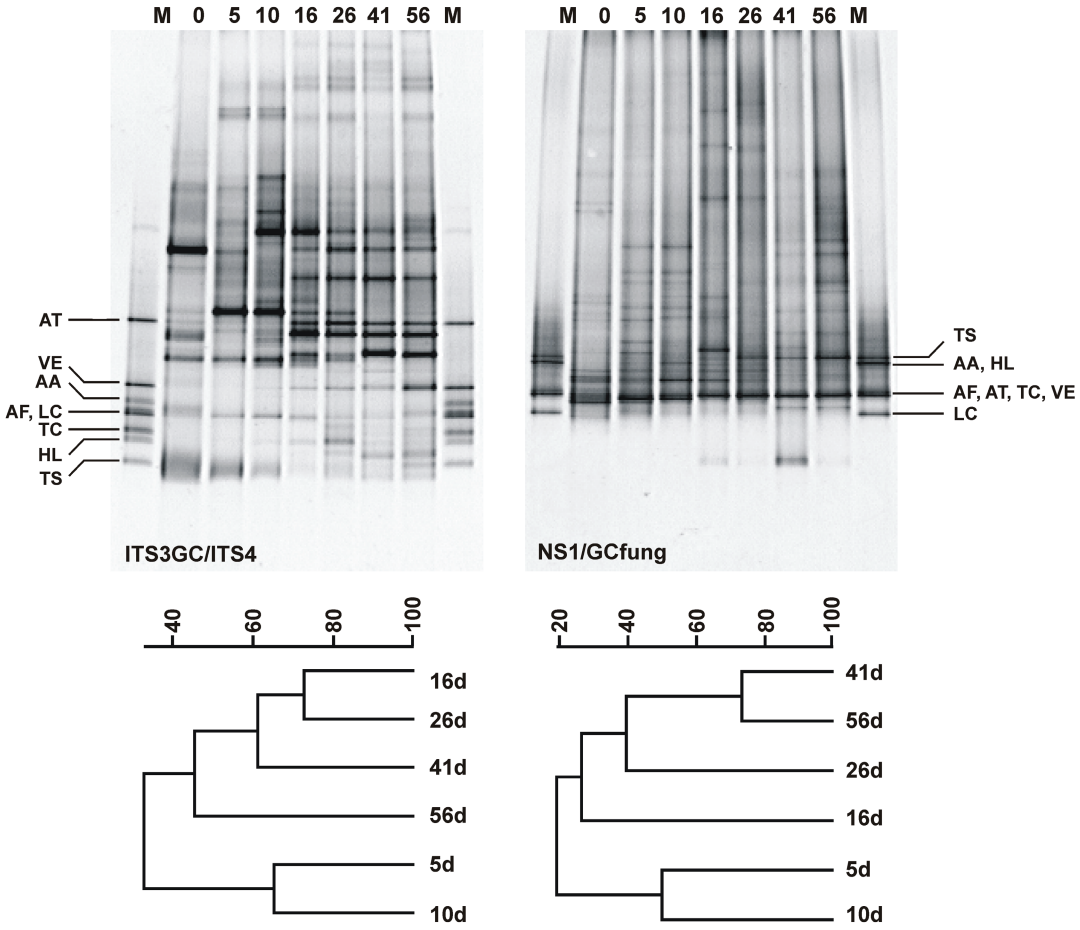


**Figure 4.3.** DGGE patterns of the DNA of fungal communities on decomposing alder leaves in the Este River amplified with the primer pairs ITS3GC/ITS4 and NS1/GCfung. Dendograms were constructed from UPGMA analysis based on the Jaccard coefficient of similarity. M, Mixture of DNA of 8 fungal species.

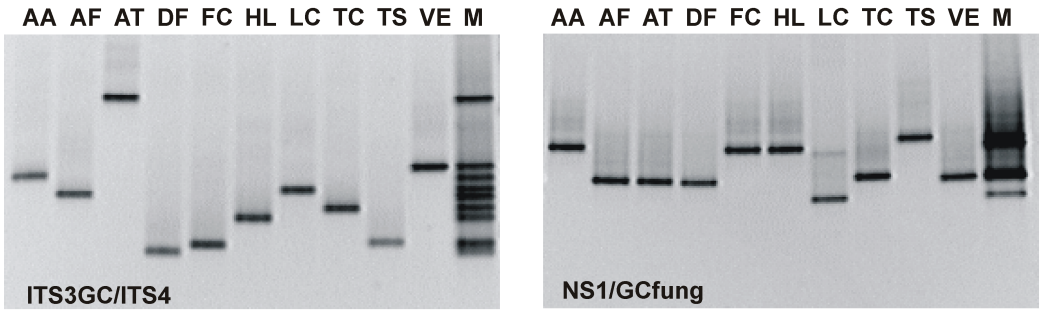


**Figure 4.4.** DGGE patterns of DNA of the aquatic hyphomycete pure cultures amplified with primer pairs ITS3GC/ITS4 and NS1/GCfung. AA, *Alatospora acuminata*; AF, *Anguillospora filiformis*; AT, *Articulospora tetracladia*; DF, *Dimorphospora foliicola*; FC, *Flagellospora curta*; HL, *Heliscus lugdunensis*; LC, *Lunulospora curvula*; TC, *Tricladium chaetocladium*; TS, *Tricladium splendens*; VE, *Varicosporium elodeae*. M, Mixture of DNA of the 10 fungal species.

### *4.3.3. Bacterial diversity*

The microscopic survey of bacterial communities on decomposing leaves showed a total of 13 morphotypes of bacteria. The bacterial rod morphology was dominant at all sampling dates and contributed from 40% to 70% to the total bacterial biomass (not shown). A total of 60 isolates with colonies with different colours, namely yellow, white, orange and violet, were obtained from decomposing leaves. Most of them were Gram negative and catalase positive (not shown). Only 5 isolates were able to grow on Endo agar. From 18 selected isolates, 10 produced only one DGGE band while the remaining 8 yielded more than one band with the primer pair 338GC/518 (data not shown). The primer pair 984GC/1378 did not discriminate well the 18 bacterial isolates and most of them produced smeared faint bands (not shown). However, the amplification of bacterial DNA from environmental samples yielded similar numbers of DGGE bands with both primer pairs (33 and 35 bands for 338GC/518 and 984GC/1378, respectively) (Fig. 4.5).

Cluster analysis of bacterial communities from DGGE fingerprints using both primer pairs showed a succession of the bacterial taxa along time of leaf decomposition in the stream: the primer pair 338GC/518 separated bacterial communities of 5 days and the 984GC/1378 primer pair separated communities of 5 and 10 days from those of the remaining dates. Moreover, both primer pairs discriminated bacterial communities at later times (41 and 56 days) from those of intermediate times (16 and 26 days) (Fig. 4.5).

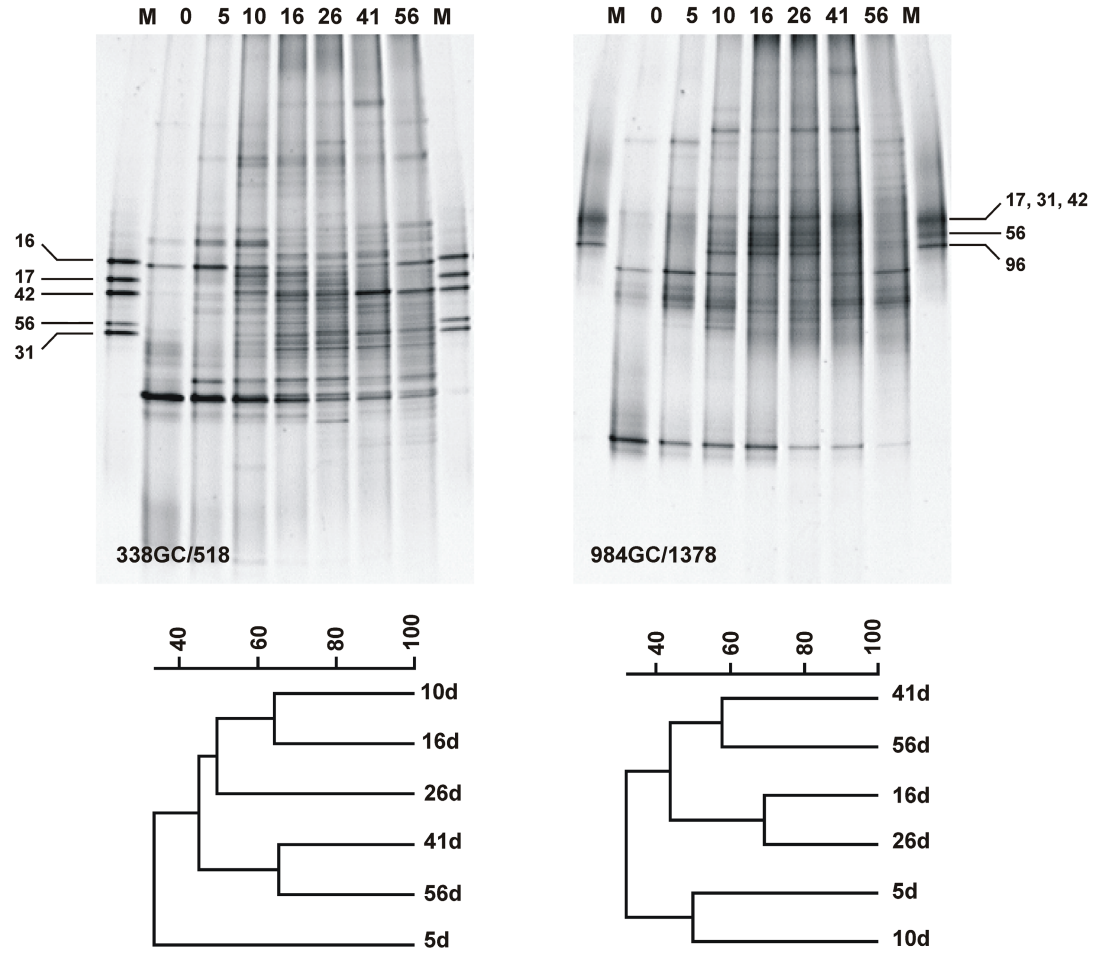


**Figure 4.5.** DGGE patterns of the DNA of bacterial communities on decomposing alder leaves in the Este River amplified with the primer pairs 338GC/518 and 984GC/1378. Dendograms were constructed from UPGMA analysis based on the Jaccard coefficient of similarity. M, Mixture of DNA of 5 bacterial isolates.

## 4.4. Discussion

Leaf decomposition at the spring of the Este River was fast, which could be attributed to the moderate nutrient concentrations usually found at this site (Duarte *et al*. 2004, Pascoal *et al.* 2005b, chapter 3). The higher values for fungal and bacterial biomasses and fungal sporulation rates were associated with the greater leaf mass losses, e.g., between 16 and 41 days of leaf immersion; corroborating that microbial decomposers are in fact important mediators of leaf-litter breakdown in streams (Hieber and Gessner 2002, Pascoal and Cássio 2004, Pascoal *et al.* 2005b). The contribution of fungi to the total microbial biomass was higher than 98% at all sampling dates, supporting once more the greater role of fungi than bacteria

during decomposition of leaf litter (Baldy and Gessner 1997, Gulis and Suberkropp 2003a, Pascoal and Cássio 2004, Pascoal *et al.* 2005b).

In the current study, two different primer pairs were used to characterize the fungal communities on decomposing leaves by DGGE. The primer pair ITS3GC/ITS4 revealed a higher diversity based on DGGE bands than the primer pair NS1/GCfung. This is consistent with data obtained from 10 pure cultures of aquatic hyphomycetes, in which the primer pair NS1/GCfung was only able to show 4 DGGE bands. This is not surprising taking into account the differences between the two targeted regions. The 18S rRNA gene, or portions of it, may not contain the necessary variability to distinguish between closely related taxa. For example, several Ascomycete genera, with a wide range of differences in morphological and physiological characteristics, possess almost identical 18S rRNA sequences (Berbee *et al.* 1996). This was probably the main reason for the low fungal diversity on decomposing leaves revealed by the primer pair NS1/GCfung, particularly after 16 days of leaf immersion. On the contrary, the highly variable ribosomal ITS regions may represent a more suitable target for a higher level of discrimination (Gardes and Bruns 1993, Larena *et al.* 1999, Viaud *et al.* 2000, Buchan *et al.* 2002). However, the sequence variation between rRNA operon copies within single species may lead to overestimations of fungal diversity (Kowalchuck and Smit 2004) when using ITS regions, which can sometimes result in complex patterns (more than one band per species). A recent study showed that the primer pair ITS3GC/ITS4 yielded multiple banding patterns for several fungal isolates from paper material, while a primer pair for the ITS1 region (ITS1GC/ITS2) yielded reproducible single bands (Michaelsen *et al.* 2006). In the current study, the primer pair ITS3GC/ITS4 produced two bands (one with very low density) only in 1 out of 10 aquatic hyphomycete species selected among the most frequent ones in the studied stream. Conversely, Nikolcheva *et al.* (2003) were able to discriminate 10 DGGE bands from 12 pure cultures using NS1/GCfung. Since the primer selection depend on the biological system, the environmental conditions where the samples came from and the level of discrimination needed, one should test different primer sets to check which one will more completely fulfil all the criteria desired for the ideal fungal-specific PCR-DGGE analysis (Kowalchuk and Smit 2004).

Differences in fungal diversity from DGGE bands and spore counting were more pronounced at the earlier stages of leaf decomposition than at later times (after 41 and 56 days), which is in accordance with previous findings (Nikolcheva *et al.* 2003, Nikolcheva and Bärlocher 2005). Nevertheless, cluster analyses of both

conidia and DGGE data with both primer pairs revealed a temporal succession of the fungal taxa on alder leaves throughout decomposition. In the DGGE gel some of the bands that appeared at the day of leaf immersion disappeared at later dates, while the opposite occurred with bands that matched with those from aquatic hyphomycete pure cultures. This result is expected since before the immersion in the stream leaves are expected to carry terrestrial fungi (Bärlocher and Kendrick 1974, Dix and Webster 1995, Nikolcheva *et al.* 2005), which in subsequent stages are displaced by aquatic species that successfully colonize leaves due to their morphological and physiological adaptations to running waters (Suberkropp *et al.* 1998, Bärlocher 2005).

In the current study, the two tested primer pairs for the variable regions V3 and V6-V8 of the 16S rDNA of bacteria yielded approximately the same number of DGGE bands, but generally the number of bands at each sampling date was higher using the primer pair 338GC/518 (for V3 region). Schmalenberger *et al.* (2001) also found that primers targeting for the V2-V3 and V6-V8 regions result in a similar diversity of bacteria from rizhospheres of field-grown maize, when applying the molecular fingerprinting technique single-strand-conformation polymorfism (SCCP). Several of the 18 selected pure cultures of bacteria, isolated from decomposing leaves at the spring of Este River, produced more than one band using the primer pair targeting V3 region. Nakatsu *et al.* (2000) also found that several bacterial pure cultures amplified with primers targeting for this region produced more than one PCR product, when separated by DGGE, due to heterogeneities between rRNA operon copies.

Overall, DNA fingerprinting detected a higher diversity of fungi and bacteria than that found from the traditional microscopy-based method. Both primer pairs for fungi and bacteria proved useful in detecting shifts in microbial communities on decomposing leaves during the time of leaf immersion. For fungi, primers targeting the ITS2 region yielded a higher number of DGGE bands and were able to discriminate a higher number of aquatic hyphomycete species. However, a fully characterization of microbial decomposers of leaf litter should include the construction and sequencing of ribosomal gene libraries, as done for bacterial and fungal communities on decomposing salt marsh grasses (Buchan *et al.* 2003) and for fungi in the hyporheic region or on leaves in streams (Bärlocher *et al.* 2007, Seena *et al. in press*). Moreover, the sequencing of known cultivable aquatic fungal isolates should be done to fulfil the scarcity of fungal sequences in databases. The traditional microscopy-based methods for assessing diversity should also be

considered, at least for fungi, since they give important information about their reproductive ability, which is a very important measure of fungal dispersion in streams.

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# Chapter 5

*Effects of eutrophication on microbial decomposers of submerged leaf litter*

## Abstract

The anthropogenic release of inorganic nutrients into aquatic systems is reported to affect the diversity and activity of biological communities with consequences to ecosystem processes. In this work, the effects of nutrient enrichment on microbial decomposers of leaf litter were investigated by monitoring leaf mass loss, bacterial biomass and diversity, fungal biomass, reproduction and diversity during 78 days of leaf immersion in 5 streams in the Northwest of Portugal, with contrasting concentrations in nitrogen and phosphorus. The nutrient concentration ranges were: 0.02-0.07 mg $L^{-1}$ for $P\text{-}PO_4^{3-}$; 0.3-5.4 mg $L^{-1}$ for $N\text{-}NO_3^-$; 0.002-0.06 mg $L^{-1}$ for $N\text{-}NO_2^-$, and <0.004-7.1 mg $L^{-1}$ for $N\text{-}NH_4^+$. The structure of fungal and bacterial communities on decomposing leaves changed along the gradient of nutrients, as indicated by CCA analysis based on the morphology of fungal conidia and the DNA fingerprints from DGGE of fungal and bacterial communities. Fungal biomass and sporulation were depressed in the most eutrophic streams, while bacterial biomass appeared to be stimulated, with the exception of the site with the highest $N\text{-}NO_2^-$ and $N\text{-}NH_4^+$ concentrations. Leaf decomposition rate was stimulated at only one site with moderate eutrophication.

## 5.1. Introduction

In small-forested streams with low autotrophic production leaf-litter decomposition is a very important process since it provides the carbon and energy for the functioning of aquatic food-webs (Suberkropp 1998b, Bärlocher 2005). In these food-webs, the microbial decomposers, as fungi and bacteria, have an important role in increasing the palatability of leaf litter for stream invertebrates (Suberkropp 1998b, Bärlocher 2005, Gessner *et al.*2007).

During the last decades, freshwater ecosystems are experiencing several pressures, including those from nutrient enrichment, with a great impact over aquatic diversity and ecosystem processes (Sala 2000, Dudgeon *et al.* 2006). Urbanization, the increase in animal wastes and the use of agricultural fertilizers are the main sources of nutrient enrichment in streams (Alan and Castillo 2007). Generally, high concentrations of nitrates and phosphorus are reported to accelerate leaf decomposition (Pascoal *et al.* 2003, Pascoal *et al.* 2005a, Gulis and Suberkropp 2003a,b, Ferreira *et al.* 2006, Castela *et al.* 2008) and to enhance microbial respiration (Gulis and Suberkropp 2003a,b, Baldy *et al.* 2007) and other fungal (biomass, sporulation or production) (Suberkropp 1998a, Gulis and Suberkropp 2003a,b, Pascoal and Cássio 2004, Ferreira *et al.* 2006, Castela *et al.* 2008) and bacterial (biomass or production) activities (Gulis and Suberkropp 2003a,b, Pascoal and Cássio 2004, Baldy *et al.* 2007). The increase of nitrates and phosphorus in streams is also reported to enhance fungal diversity (Gulis and Suberkropp 2004) and to alter community structure (Gulis and Suberkropp 2004, Pascoal *et al.* 2005c, Artigas *et al.* 2008, Castela *et al.* 2008).

Although microbial biomass and diversity can be stimulated by an increase of phosphorus and nitrates in streams with low to moderate eutrophication (Ferreira *et al.* 2006, Gulis *et al.* 2006), in hypertrophic streams receiving various anthropogenic inputs, either the concentration or the type of N and P may differentially affect the response of microbial communities (Baldy *et al.* 2007). Additionally, fungal diversity and sporulation were reduced in a stream of the Northwest of Portugal with high nutrient loads and heavy metals (Pascoal *et al.* 2005b). Other studies reported depressed rates of leaf decomposition in some nutrient-enriched sites with low oxygen concentrations and sedimentation, indicating that the positive effects of nutrients can be counteracted by other factors (Pascoal and Cássio 2004, Pascoal *et al.* 2005a, Mesquita *et al.* 2007).

In the current study we selected five stream sites in the Northwest of Portugal with contrasting concentrations of dissolved inorganic nitrogen ($N\text{-}NO_3^-$, $N\text{-}NO_2^-$, and $N\text{-}NH_4^+$) and phosphorus ($P\text{-}PO_4^{3-}$) and we examined leaf mass loss, bacterial and fungal biomass, and fungal reproduction on decomposing alder leaves during 78 days. Additionally, the structure and diversity of microbial communities were assessed using both traditional (conidial morphology for fungi) and molecular approaches (denaturing gradient gel electrophoresis – DGGE for both fungi and bacteria). We expected that microbial activity and diversity on decomposing leaves would be stimulated at moderately nutrient-enriched sites but declined at the most eutrophic sites.

## 5.2. Materials and Methods

### *5.2.1. Study areas*

Field studies were conducted at five sites in streams of the Ave and Cávado River basins in the Northwest of Portugal where granitic rocks dominate the geological substratum. Three sampling sites Este 2, Este 3 and Souto are in the Ave River basin in an area with high population density and intensive agricultural and industrial activities. The other two sampling sites Algeriz and Maceira belong to the Cávado River basin (Fig. 5.1).

The site Este 2 is located about 1 Km from the spring of the Este River and Este 3 is about 10 Km downstream, near the Industrial Park of the town of Braga. The dominant riparian vegetation at Este 2 is *Eucalyptus globulus* Labill, *Juncus* sp., *Pinus pinaster* Aiton, *Pteridium aquilinum* Khun and *Alnus glutinosa* (L.) Gaertn., while at Este 3 the riparian vegetation is constituted by *A. glutinosa*, *Platanus hybrida* Brot., *Populus tremula* L., *Quercus robur* L. and *Salix* sp. Souto site is located near the town of Guimarães, and the surrounding vegetation is mainly constituted by *A. glutinosa*, *Q. robur* and *P. aquilinum*. At Algeriz the dominant riparian vegetation is *Q. robur*, *P. aquilinum* and *E. globulus*. Maceira is located at the Peneda-Gerês National Park and is bordered by *Q. pyrenaica* Willd., *Q. robur*, *Chamaecyparis* sp. and *Ilex aquifolium* L. At Algeriz, Este 2 and Maceira, the dominant substrates are boulders, pebbles and sand, while at Este 3 and Souto are gravel and mud.

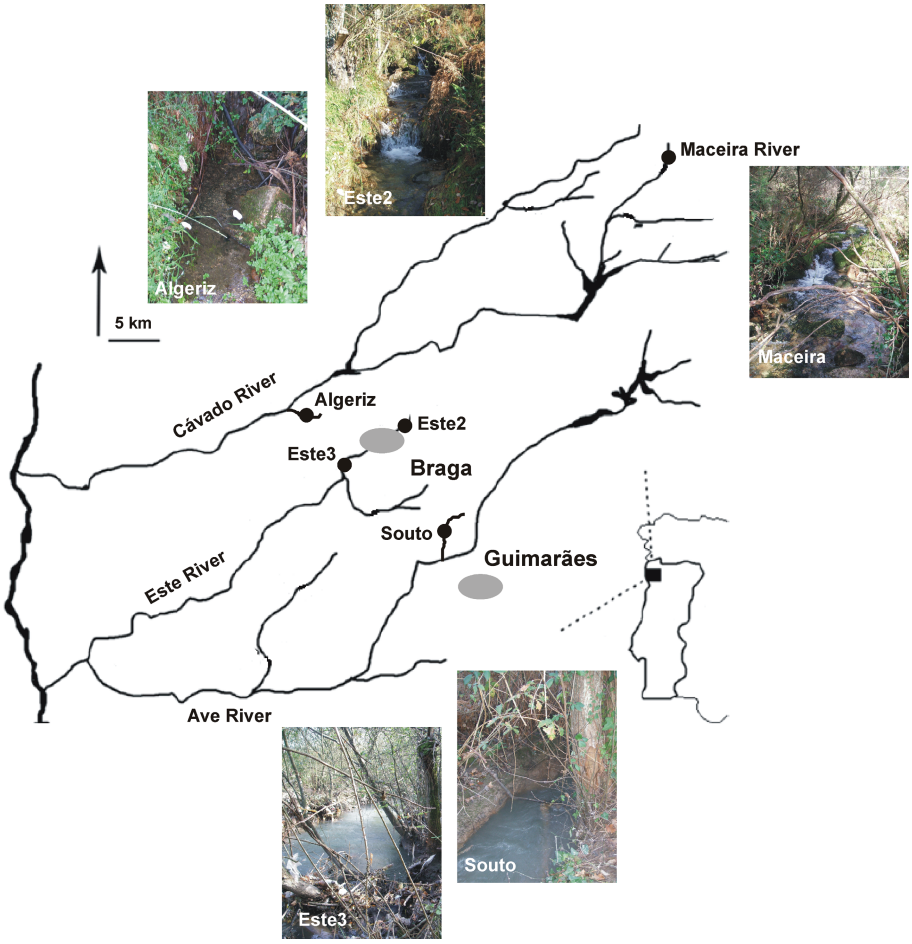


**Figure 5.1.** Location of the sampling sites in the Ave River (Este 2, Este 3 and Souto) and Cávado River (Algeriz and Maceira) basins in the Northwest of Portugal.

### *5.2.2. Experimental field setup*

In October 2006, freshly fallen *A. glutinosa* leaves were collected from trees at a riparian zone and dried for one week at room temperature. One hundred and forty sets of 4 ± 0.05 g of alder leaves were immersed for 5 min in deionised water (to prevent break-up) and introduced into 0.5-mm mesh bags (20 x 20 cm). Twenty-eight leaf bags were immersed at each sampling site on 21st November 2006. After 8, 15, 22, 28, 43, 57 and 78 days, four replicate leaf bags were retrieved from each stream site. At each sampling date, leaf bags were transferred into zip-lock plastic bags containing stream water and transported in a cool box to the laboratory. For estimating initial parameters, four additional leaf bags of approximately 4 g were immersed in filtered deionised water (0.2 µm pore size, Sarstedt) and processed as described below.

### 5.2.3. Physical, chemical and microbial analyses of the stream water

On each sampling date, the pH, dissolved oxygen, conductivity and temperature were measured *in situ* with field probes (Multiline 340i, WTW, Weilheim, Germany). The current velocity was determined with a flow meter (model 2030R, General Oceanics Inc, Miami, Florida). Stream water samples were collected in sterile glass bottles, transported on ice and analysed within 24 h. A HACH DR/2000 photometer (Hach company, Loveland, CO, USA) was used to quantify chemical oxygen demand (COD) by dichromate digestion (HACH kit, program 435), nitrate concentration by cadmium reduction (HACH kit, program 355), nitrite concentration by the diazotization method (HACH kit, program 371), ammonia concentration by the salicylate method (HACH kit, program 385) and reactive phosphorus by the ascorbic acid method (HACH kit, program 490). An aliquot of stream water (0.45 µm membrane pore size, Millipore) was acidified and stored at 4 ºC before for determination of Ca and Mg concentrations by inductively coupled plasma atomic emission spectrometry (ICP-AES spectrometer, Philips PU 7000, Philips, Holland). Total and fecal coliforms were determined by the membrane filter technique (APHA 1998).

### 5.2.4. Leaf bag processing and leaf mass loss

In the laboratory, leaves were gently rinsed with deionised water to remove sediments. From each replicate bag, leaf disks were cut with a 1.2 cm cork borer: four leaf disks were stored in 10 mL of a 2% formaldehyde solution at 4 ºC for bacterial counts; eight disks were freeze-dried for ergosterol quantification; two sets of four disks were also freeze-dried for DNA extraction, and eight disks were used for sporulation experiments. The rest of leaves were dried at 80 ºC for 3 days and weighed to the nearest 0.01 g. Portions of about 1 g of dry leaves were ground, ashed at 550 ºC for 6 hours and weighed to determine ash-free dry mass (AFDM).

### 5.2.5. Fungal biomass and sporulation

Sets of eight freeze-dried leaf disks were used to determine ergosterol concentration on leaves, as a measure of fungal biomass according to Gessner (2005). Briefly, lipids were extracted from leaf disks by heating (80 ºC for 30 min) in 0.8% (w/v) KOH/methanol, purified by solid-phase extraction and quantified by high

performance liquid cromatrography (HPLC). Ergosterol was converted to fungal biomass using a factor of 5.5 mg ergosterol $g^{-1}$ fungal dry mass (Gessner and Chauvet 1993).

To induce sporulation, sets of eight leaf disks from each replicate bag were incubated at 14 ºC under shaking (140 rpm) in 100 mL Erlenmeyer flasks with 40 mL of filtered (0.45 µm membrane pore size, Sarstedt) stream water from each respective site. After 48 hours conidial suspensions were mixed with 35 µL of 15% (w/v) Triton X-100 and 4 mL of 37% formaldehyde in 50 mL polypropylene tubes (Sarstedt). For conidial identification and counting, samples of conidial suspensions were filtered on cellulose nitrate membranes (5 µm pore size, Millipore) and stained with 0.05% (w/v) cotton blue in lactic acid. At least 300 conidia were identified and counted per replicate (400x, Leica Biomed).

### *5.2.6. Bacterial biomass*

Bacterial cells were detached from four leaf disks by a 12.7 mm flat tip ultrasonic probe connected to a digital Sonifier 250 (Branson Ultrasonics, Danbury, CT, USA), operating continuously for 1 min at a 50% amplitude (Buesing and Gessner 2002, Baldy *et al.* 2007). The bacterial suspension was re-suspended, allowed to settle for 10 s, and a 200 µL sample was taken about 5 mm below the surface and mixed with filtered (0.2 µm pore size membrane) deionised water to a final volume of 2 mL. Appropriate dilutions of the bacterial suspensions were stained by adding 288 µL of 40 mg $L^{-1}$ 4', 6-diamidino-2-phenylindone (DAPI) (Sigma) for 10 min. Bacterial numbers were determined by epifluorescence microscopy at a 1000x magnification. At least 300 cells were counted per filter in a total of 10 fields. Bacterial numbers were converted to bacterial carbon using a conversion factor of 20 *f*g bacterial carbon per cell (Norland 1993) and to bacterial biomass assuming that carbon was 50% of bacterial dry mass.

### *5.2.7. Molecular analyses*

DNA was extracted from four halves of four freeze-dried leaf disks with a soil DNA extraction kit (MoBio Laboratories, Solana Beach, California), according to the manufacturer instructions. The ITS2 region of fungal ribosomal DNA was amplified with the primer pair ITS3GC and ITS4 (White *et al.* 1990). For PCR of fungal DNA,

1x of Taq buffer (KCl:$(NH_4)_2SO_4$), 3 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of each primer, 1.5 U of DNA Taq polymerase (Fermentas) and 1 µL of DNA were used in a final volume of 50 µL.

The V6 to V8 region of bacterial ribosomal DNA was amplified with the primer pair 984GC (Nübel *et al.* 1996) and 1378 (Heuer *et al.* 1997). For PCR of bacterial DNA, 30 µL of reaction mix containing 1x colorless GoTaq$^R$ Flexi Buffer (Promega Corporation), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.8 µM of each primer and 2.5 U of DNA Taq polymerase were added to 20 µL DNA.

Fungal PCRs were carried out in a MyCycler Thermal Cycler (BioRad Laboratories, Hercules, CA, USA) using the following program: initial denaturation at 95 ºC of 2 min; followed by 36 cycles of denaturation at 95 ºC for 30 s, primer annealing at 55 ºC for 30 s and extension at 72 ºC for 1 min. Final extension was at 72 ºC for 5 min (Nikolcheva and Bärlocher 2005, Nikolcheva *et al.* 2005). For bacteria, PCR was carried out in a MJ Research PTC-200 thermocycler (Global Medical Instrumentation, Inc., MN, USA) using the following program: initial denaturation at 94 ºC for 5 min; 10 touchdown cycles of denaturation at 94 ºC for 1 min, annealing at 65–55 ºC for 1 min (decreasing 1 ºC each cycle) and extension at 72 ºC for 3 min; 20 standard cycles of denaturation at 94 ºC for 1 min, annealing at 55 ºC for 1 min and extension at 72 ºC for 3 min. Final extension was at 72 ºC for 5 min. Fungal and bacterial DNA from 4 replicates was amplified and the PCR amplicons from each replicate pooled for DGGE analysis.

DGGE analysis was performed using a DCode$^{TM}$ Universal Mutation Detection System (BioRad Laboratories, Hercules, CA, USA). For fungal DNA, 700 ng samples from the amplification products of 380-400 bp were loaded on 8% (w/v) polyacrylamide gel in 1x TAE with a denaturing gradient from 25 to 70% (100% denaturant corresponds to 40% formamide and 7 M urea). The gels were run at 55 V, 56 ºC for 16 h and stained with 1 µg $mL^{-1}$ of ethidium bromide (BioRad) for 5 min. The gel images were captured under UV light in a molecular imager ChemiDoc$^{TM}$ XRS system (BioRad Laboratories, Hercules, CA, USA). In each DGGE gel for fungal fingerprinting a DNA mixture of the taxa *Anguillospora* sp., *Anguillospora filiformis* Greath. UMB 225.02, *Articulospora tetracladia* Ingold UMB 22.01, *Clavariopsis aquatica* De Wild. UMB 19.99, *Dimorphospora foliicola* Tubaki 30.01, *Lemonniera aquatica* De Wild. UMB 143.01, *Tetracladium marchalianum* De Wild. UMB 94.01, *Tricladium chaetocladium* Ingold UMB 163.01 and *Varicosporium elodeae* W. Kegel UMB 20.01, was used as standard to calibrate the gels in further

analyses. For bacterial DNA fingerprinting, one of the samples (Este 2, 22d) was also included in all DGGE gels to allow calibration in further analyses.

For bacterial DNA, 700 ng samples from the amplification products of 433 bp were loaded on 6% (w/v) polyacrylamide gel in 1x TAE with a denaturing gradient from 30 to 60%. The gels were run at 100 V, 60 ºC for 16 h and stained with SYBR Green (Sigma-Aldrich) diluted 2000x for 45 min. The gel images were captured by a CCD camera under UV light using the Vision-Capt software (Vilber Lourmat, Marne-la-Vallée, France).

### *5.2.8. Data analyses*

Ash-free dry mass remaining of alder leaves was fit to the exponential model $m_t = m_0 \cdot e^{-kt}$, where $m_t$ is the AFDM remaining at time $t$, $m_0$ is the initial AFDM and $k$ is the rate of leaf decomposition. Regression lines of ln-transformed values of AFDM were compared by ANCOVA followed by Tukey´s tests (Zar 1996).

Two-way analyses of variance (Two-way ANOVA) were used to test if stream site and time significantly affected fungal and bacterial biomass and fungal sporulation (Zar 1996). To achieve normal distribution, data from fungal biomass were ln transformed while data from fungal sporulation and bacterial biomass were ln(x+1) transformed (Zar 1996). Differences in stream water parameters between sites were compared by one-way ANOVA followed by Tukey's tests (Zar 1996).

DGGE gels were aligned and normalized, and the relative intensities of bands in the fingerprints were analyzed in Gelcompar II (Applied Maths, Belgium).

Shannon's diversity (*H'*) and Pielou's equitability (*J'*) indices were used to assess the diversity of aquatic fungi and bacteria as: $H' = -\sum_{i=1}^{s} P_i (lnP_i)$ and $J' = H' / lnS$ where $P_i$ is the relative abundance of conidia or relative surface intensity of taxon *i* or band *i* and *S* is the total number of sporulating taxa or bands (Legendre and Legendre 1998).

Spearman rank correlation was used to examine the relationship between stream water variables and leaf breakdown rates, fungal and bacterial biomasses, and fungal sporulation to identify environmental variables that better explain the differences among the five stream sites.

A Principal Component Analysis (PCA) was used to ordinate sites according to stream water variables, after standardization. Canonical correspondence analysis

(CCA) was used to determine the relationships between stream water variables and fungal sporulating species or DNA fingerprints of fungi and bacteria. This is a direct gradient ordination technique that uses regression procedures to relate species and environmental data assuming that species have unimodal (or Gaussian) responses to environmental variables (Ter Braak and Verdonschot 1995). Monte Carlo permutation tests based on 499 permutations were used to test the null hypothesis that communities of fungal sporulating taxa and DGGE fingerprints were unrelated to environmental variables (Lepš and Šmilauer 2003). The resulting ordination biplots approximated the weighed average of each species (abundance of conidia and relative intensity of each DGGE band) with respect to each environmental variable that are represented by arrows. The length of these arrows indicated the relative importance of that variable in explaining variation in sporulating taxa or DNA fingerprints, while the angle between the arrows indicates the degree to which they are correlated. ANCOVA, ANOVA and Spearman rank correlations were done in Statistica 6.0 for Windows (Statsoft, Inc.) and Shannon's and Pielou's indices were calculated with Primer v6 software package. PCA and CCA ordinations were performed with CANOCO version 4.5 for windows (Microcomputer Power, Ithaca, New York).

## 5.3. Results

### *5.3.1. Stream water characteristics*

Stream water temperature and pH were similar at the study sites (Table 5.1) with the exception of Maceira, whose high altitude may have contributed to a temperature of 2-4 ºC below than that of the other sites. Oxygen concentration in the stream water ranged from 9.4 to 12.4 mg $L^{-1}$. Maceira, Algeriz and Este 2 had the lowest values for conductivity and concentrations of Ca, Mg, N and P in the stream water. No differences were found in $P\text{-}PO_4^{3-}$ concentration between Souto and Este 3 (0.06-0.07 mg $L^{-1}$, respectively) (one-way ANOVA, Tukey's, $P>0.05$). Concentration of $N\text{-}NO_3^-$ was higher at Este 3 (5.4 mg $L^{-1}$) than at Souto (one-way ANOVA, Tukey's, $P<0.05$), but Souto had higher conductivity (295 µS $cm^{-1}$), COD (210 mg $L^{-1}$) and concentrations of $N\text{-}NO_2^-$ (0.06 mg $L^{-1}$) and $N\text{-}NH_4^+$ (7.1 mg $L^{-1}$) (one-way ANOVA, Tukey's, $P<0.05$). The highest numbers of total and fecal coliforms were found at Este 3 and no fecal coliforms were found in the stream water at Maceira, Algeriz and Este 2 (Table 5.1).

PCA ordination of the five stream sites according to the stream water variables (Fig. 5.2) showed that axes 1 and 2 explained 59% of the total variance. The first PC axis discriminated Souto and Este 3 from Algeriz, Este 2 and Maceira, while the second axis separated Souto from Este 3.

**Table 5.1.** Characteristics of the stream water at the five sampling sites.

| Parameter | Maceira | Algeriz | Este 2 | Este 3 | Souto | n |
|---|---|---|---|---|---|---|
| Longitude N | 41º46'07.64'' | 41º35'24.56'' | 41º35'05.50'' | 41º31'36.83'' | 41º31'34.43'' | - |
| Latitude W | 8º08'49.07'' | 8º22'36.96'' | 8º21'05.93'' | 8º26'08.50'' | 8º17'16.03'' | - |
| Elevation (m) | 857 | 220 | 406 | 148 | 138 | - |
| pH | 7.0 ± 0.3 | 7.1 ± 0.04 | 6.7 ± 0.06 | 7.0 ± 0.06 | 7.1 ± 0.1 | 7 |
| Water temperature (ºC) | 9 ± 0.4 | 13 ± 0.3 | 13 ± 0.3 | 13 ± 0.5 | 11 ± 0.7 | 7 |
| Conductivity ($\mu S\ cm^{-1}$) | 14 ± 0.5 | 42 ± 0.9 | 44 ± 0.7 | 161 ± 2.3 | 295 ± 85 | 7 |
| Oxygen ($mg\ L^{-1}$) | 10.7 ± 0.5 | 12.4 ± 0.6 | 11 ± 0.3 | 9.4 ± 0.6 | 9.9 ± 0.7 | 7 |
| $N\text{-}NO_3^-$ ($mg\ L^{-1}$) | 0.3 ± 0.04 | 0.6 ± 0.1 | 0.9 ± 0.1 | 5.4 ± 0.2 | 3.3 ± 0.5 | 7 |
| $N\text{-}NO_2^-$ ($mg\ L^{-1}$) | 0.002 ± 0.0003 | 0.003 ± 0.0009 | 0.002 ± 0.0002 | 0.02 ± 0.003 | 0.06 ± 0.01 | 7 |
| $N\text{-}NH_4^+$ ($mg\ L^{-1}$) | <0.004 | 0.004 ± 0.002 | 0.004 ± 0.003 | 0.7 ± 0.09 | 7.1 ± 4.2 | 7 |
| $P\text{-}PO_4^{3-}$ ($mg\ L^{-1}$) | 0.02 ± 0.007 | 0.04 ± 0.01 | 0.02 ± 0.005 | 0.07 ± 0.02 | 0.06 ± 0.02 | 6 |
| Ca ($mg\ L^{-1}$) | 0.6 ± 0.1 | 0.8 ± 0.1 | 1.6 ± 0.2 | 10.5 ± 0.1 | 6.4 ± 1.5 | 3 |
| Mg ($mg\ L^{-1}$) | 0.2 ± 0.003 | 0.6 ± 0.05 | 0.6 ± 0.01 | 2.5 ± 0.09 | 2.1 ± 0.6 | 3 |
| Chemical oxygen demand ($mg\ L^{-1}$) | 4 ± 2.5 | 9 ± 3.8 | 4.3 ± 1.7 | 25 ± 5.3 | 210 ± 98 | 3 |
| Current velocity ($cm\ s^{-1}$) | 31 ± 9.7 | 37 ± 7.9 | 28 ± 11 | 44 ± 4.3 | 43 ± 4.4 | 6 |
| Total coliforms ($CFU\ mL^{-1}$) | 1.1 ± 0.7 | 2.6 ± 0.2 | 0.9 ± 0.09 | 2,300 ± 907 | 296 ± 272 | 3 |
| Fecal coliforms ($CFU\ mL^{-1}$) | <1 | <1 | <1 | 360 ± 75 | 0.7 ± 0.6 | 3 |

Mean ± SEM, with the exception of longitude, latitude and elevation, n=nº of samples.

CFU, colony forming units.

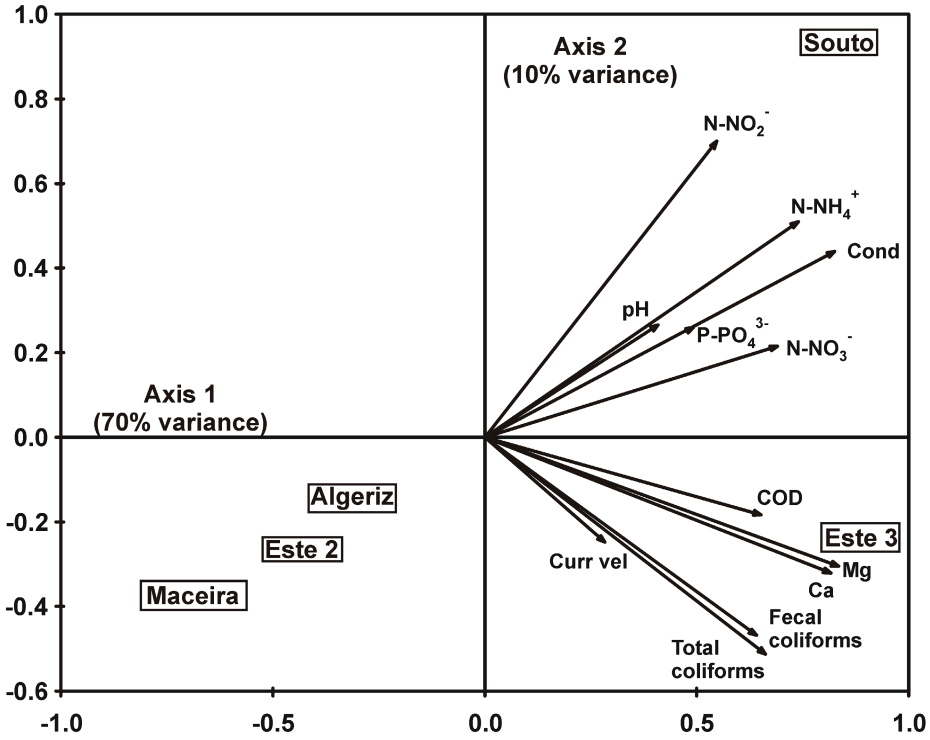


**Figure 5.2.** Principal component analysis (PCA) of the chemical, physical and microbial stream water parameters at the five stream sites, Maceira, Algeriz, Este 2, Este 3 and Souto. The direction of the arrows reflects the maximum variation of each variable.

### *5.3.2. Microbial diversity on decomposing leaves*

During the study, a total of 35 aquatic fungal taxa were found sporulating on leaves decomposing at the five sites (Table 5.2). The highest values for fungal species richness (28 taxa), and Shannon's (H´=1.77) and Pielou's (J´=0.53) indices were found at Este 2 and the lowest ones at Souto (8 taxa, H´=0.17 and J´=0.082) (Table 5.2). *Articulospora tetracladia* had the highest contribution for the total conidium production on decomposing leaves at Este 2, Maceira and Algeriz, followed by *Anguillospora filiformis* at the two former sites, and by *Clavatospora longibrachiata* at Algeriz. *Dimorphospora foliicola* was the dominant species at Este 3 and Souto, contributing more than 90% to the total conidium production (Table 5.2). The species *Fontanospora fusiramosa*, *F. eccentrica*, *Mycocentrospora acerina* and *Dendrospora tenella* appeared only on leaves decomposing at Maceira, while the species *Clavariopsis aquatica* appeared only on leaves decomposing at Algeriz and Este 3 (Table 5.2).

**Table 5.2.** Mean percentage of aquatic fungal species sporulating on decomposing alder leaves at the five stream sites. Abb, species abbreviation.

| Abb | Species | Maceira | Algeriz | Este 2 | Este 3 | Souto |
|---|---|---|---|---|---|---|
| AA | *Alatospora acuminata* Ingold | <0.1 | 0.3 | 2.3 | | |
| AFl | *Alatospora* cf. *flagellata* (J. Gönczol) Marvanová | | <0.1 | 0.1 | | |
| AP | *Alatospora pulchella* Marvanová | <0.1 | 3.1 | 0.6 | | |
| AFi | *Anguillospora filiformis* Greath. | 10.4 | 1.8 | 23.7 | 2.3 | 2.1 |
| AT | *Articulospora tetracladia* Ingold | 71.0 | 56.3 | 48.5 | <0.1 | 0.2 |
| CA | *Clavariopsis aquatica* De Wild. | | 0.3 | | 2.5 | |
| CL | *Clavatospora longibrachiata* (Ingold) Sv. Nilsson ex Marvanová & Sv. Nilsson | | 15.7 | 2.3 | 0.1 | |
| DT | *Dendrospora tenella* Descals & J. Webster | <0.1 | | | | |
| DF | *Dimorphospora foliicola* Tubaki | <0.1 | 5.9 | 7.4 | 93.9 | 97 |
| FCt | *Flagellospora curta* J. Webster | | | 0.9 | <0.1 | 0.1 |
| FCv | *Flagellospora curvula* Ingold | 0.2 | | | | |
| Flsp | *Flagellospora* sp. | 0.4 | | 0.3 | | |
| FE | *Fontanospora eccentrica* (R.H. Petersen) Dyko | 1.9 | | | | |
| FF | *Fontanospora fusiramosa* Marvanová, Peter J. Fisher & Descals | 3.1 | | | | |
| Fusp | *Fusarium* sp. | 0.6 | 1.1 | 0.7 | 0.2 | 0.2 |
| HS | *Heliscella stellata* (Ingold & V.J. Cox) Marvanová | | 0.9 | 2.5 | | |
| HL | *Heliscus lugdunensis* Sacc. & Therry | 0.6 | 0.2 | <0.1 | <0.1 | |
| LAl | *Lemonniera* cf. *alabamensis* R.C. Sinclair & Morgan-Jones | | 4.4 | | | |
| LAq | *Lemonniera aquatica* De Wild. | | 1.1 | 0.3 | | |
| LC | *Lunulospora curvula* Ingold | <0.1 | <0.1 | 1.7 | <0.1 | |
| MA | *Mycocentrospora acerina* (R. Hartig) Deighton | <0.1 | | | | |
| MC | *Mycofalcella calcarata* Marvanová, Om-Kalth. & J. Webster | | | 0.2 | | |
| TE | *Tetrachaetum elegans* Ingold | | 0.94 | 0.8 | 0.6 | |
| TB | *Tetracladium breve* A. Roldán | | | 0.1 | | |
| TSe | *Tetracladium setigerum* (Grove) Ingold | | | <0.1 | | |
| TAt | *Tricladium attenuatum* S. H. Iqbal | 0.1 | 0.1 | <0.1 | | |
| TC | *Tricladium chaetocladium* Ingold | <0.1 | 2.5 | 2.1 | 0.1 | |
| TSp | *Tricladium splendens* Ingold | | 0.4 | 0.4 | | |
| TAc | *Triscelophorus* cf. *acuminatus* Nawawi | | 0.7 | 0.6 | | 0.2 |
| VE | *Varicosporium elodeae* W. Kegel | 2.7 | 0.3 | 1.3 | | |
| S1 | Sigmoid 1 (10-20/2-3 µm) | | 0.1 | <0.1 | <0.1 | <0.1 |
| S2 | Sigmoid 2 (5-10/0.5-1 µm) | <0.1 | | 0.3 | | |
| S3 | Sigmoid 3 (70-80/2-3 µm) | <0.1 | <0.1 | <0.1 | <0.1 | |
| S4 | Sigmoid 4 (80-120/1-2 µm) | <0.1 | 0.1 | 0.7 | | 0.1 |
| S5 | Sigmoid 5 (20-25/2.5-3 µm) | 8.8 | 3.4 | 1.7 | | |
| Nº of species | | 21 | 24 | 28 | 13 | 8 |
| Shannon's diversity H' | | 1.10 | 1.66 | 1.77 | 0.32 | 0.17 |
| Pielou's equitability J' | | 0.36 | 0.52 | 0.53 | 0.12 | 0.082 |

Analysis of fungal communities on decomposing leaves assessed from DNA fingerprints showed that Algeriz had the highest diversity (37 DGGE bands) and Este 3 had the lowest one (28 DGGE bands) (Fig. 5.3). The highest bacterial diversity was also found at Algeriz (50 DGGE bands) and the lowest diversity at Este 3 (44 DGGE bands) (Fig. 5.4). A strong band in the fungal DNA fingerprints matched with that produced by the pure culture of *D. foliicola* at Este 3 (22 and 57 days) and Souto (43 and 57 days); it was the species that most contributed to the total conidium production at those sites. Also, DGGE bands matching with those produced by pure cultures of *A. tetracladia* and *A. filiformis* were present in the fungal DNA fingerprints from Algeriz, Este 2 and Maceira (Fig. 5.3).

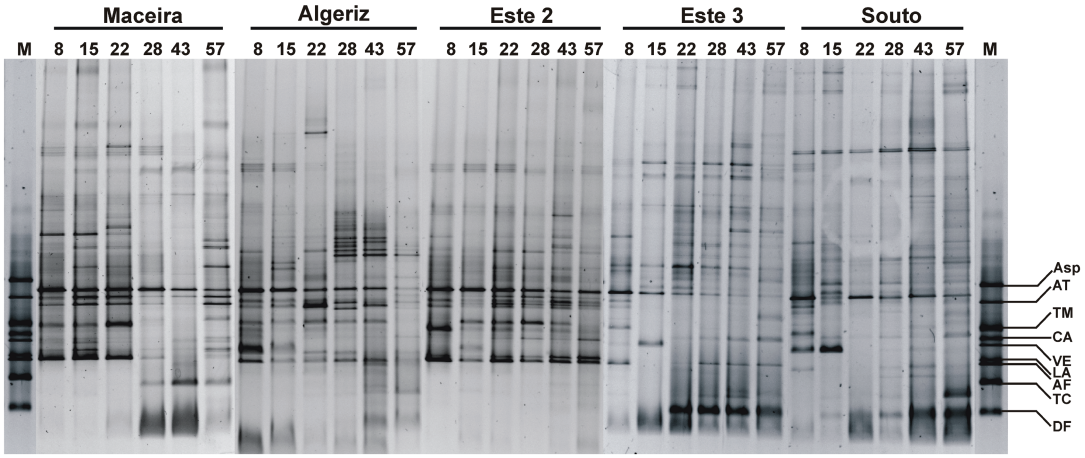


**Figure 5.3.** DGGE fingerprints of the ITS2 region of rDNA of fungal communities on decomposing leaves after 8, 15, 22, 28, 43 and 57 days of leaf immersion at the fiver stream sites. M, mixture of DNA from pure cultures. Asp, *Anguillospora* sp.; AT, *Articulospora tetracladia*; TM, *Tetracladium marchalianum*; CA, *Clavariopsis aquatica*; VE, *Varicosporium elodeae*; LA, *Lemonniera aquatica*; AF, *Anguillospora filiformis*; TC, *Tricladium chaetocladium*; DF, *Dimorphospora foliicola*.

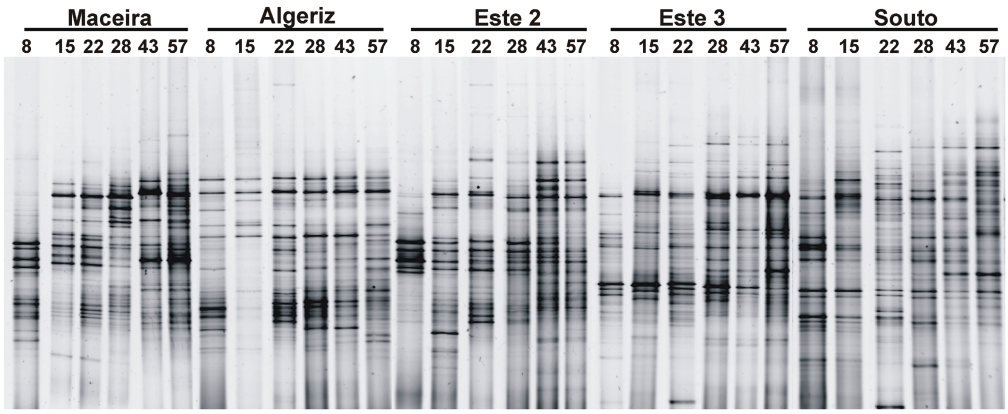


**Figure 5.4.** DGGE fingerprints of the V6 to V8 region of 16S rDNA of bacterial communities on decomposing leaves at the five stream sites.

CCA ordinations show the overall relationships between the stream water variables and fungal communities based on conidial morphology (Fig. 5.5A) or DNA fingerprints (Fig. 5.5B). For conidial morphology data, the first and the second axes explained 64% of the total variance that can be attributed to the environmental variables (Fig. 5.5A). The profile of fungal communities and the stream water variables were highly correlated with axes 1 and 2 ($r>0.86$) indicating that changes in sporulating fungal communities strongly corresponded to differences in stream water variables between the sampling sites. Additionally, Monte Carlo permutation test indicated that the first axis explained a significant part of variance within the community ($P=0.002$). Three groups of sites were discriminated by the CCA ordination: 1) Maceira, 2) Algeriz and Este 2, and 3) Este 3 and Souto. The stream water variables that most contributed to the ordination of fungal species by sites were $N\text{-}NO_3^-$, $N\text{-}NH_4^+$ and conductivity ($P<0.05$), which accounted for 63% of the variance explained by all variables.

CCA ordination of fungal communities based on DGGE fingerprints and environmental variables shows that the two first axes explained 42% of all variance (Fig. 5.5B). Ordination based on DGGE fingerprints discriminated the same three groups of sites as based on sporulating species. The two first axes were highly correlated with the environmental variables ($r>0.94$) and Monte Carlo permutation test confirmed that the first axis explained significant part of variation within the DGGE fingerprints ($P=0.002$). The most significant variables explaining the community structure were $N\text{-}NO_3^-$ and $N\text{-}NO_2^-$ ($P<0.05$), which accounted for 34% of all variance explained by the stream water variables.

CCA ordination of bacterial communities based on DGGE fingerprints and environmental variables (Fig. 5.6) shows that the two first axes explained 42% of the total variance and were highly correlated with the stream water variables ($r>0.87$). Monte Carlo permutation tests indicated that the first axis explained a significant part of all variance within bacterial community ($P=0.01$). The environmental variable that was most correlated with the community structure was $N\text{-}NO_3^-$ ($P<0.05$), accounting for 18% of the variance explained by the stream water variables.

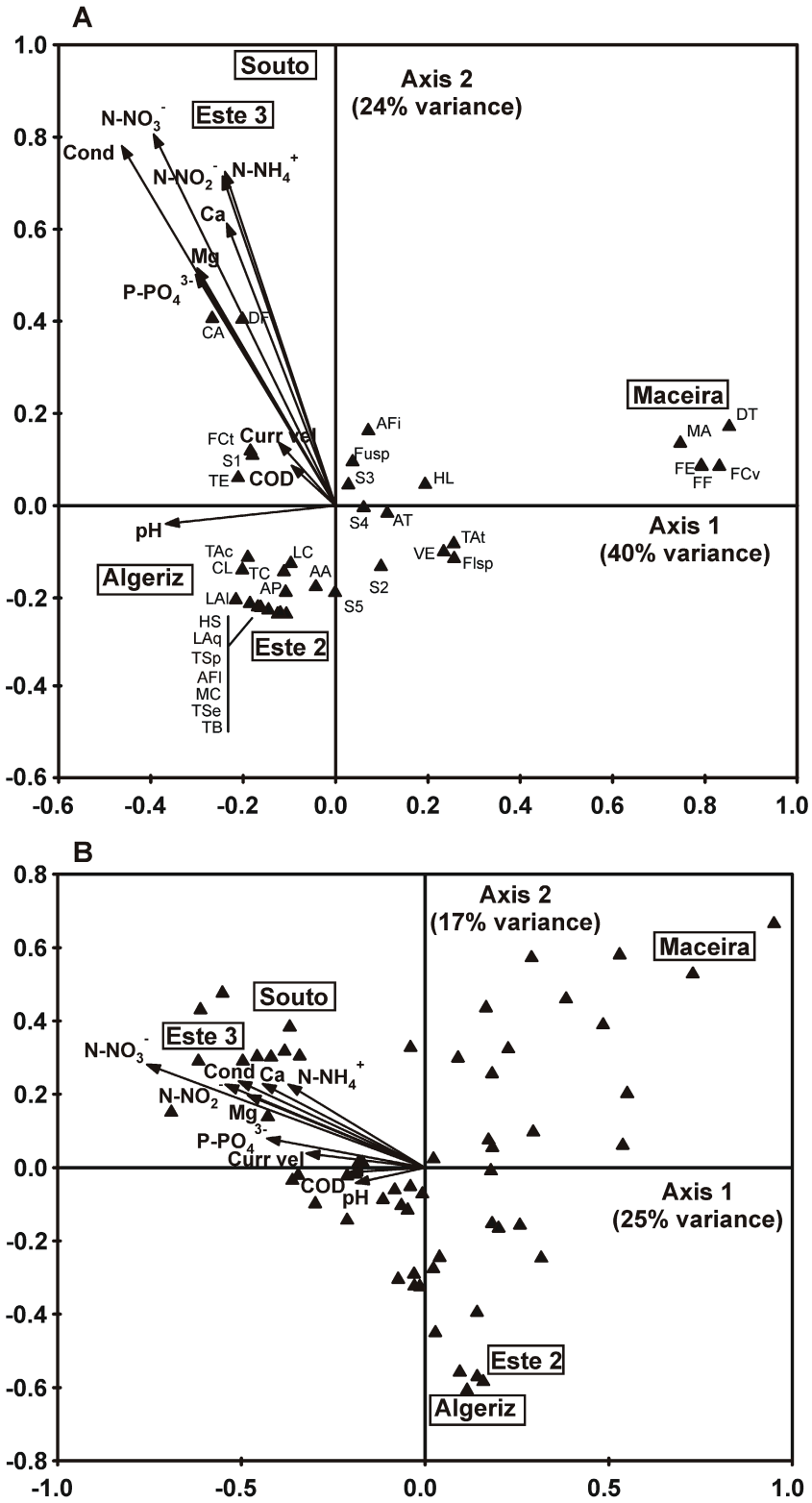


**Figure 5.5.** Canonical Correspondence Analysis (CCA) diagram for ordination of stream water variables and fungal taxa on decomposing leaves at the five stream sites based on conidial morphology (A) and DGGE fingerprints (B). See Table 5.2 for species names and abbreviations in A.

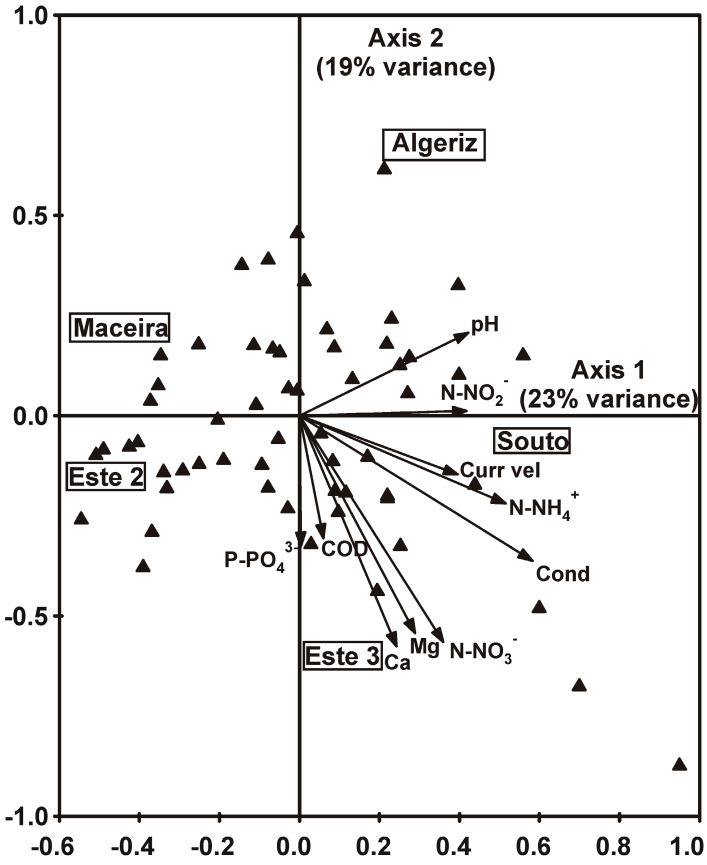


**Figure 5.6.** Canonical Correspondence Analysis (CCA) diagram for ordination of stream water variables and bacterial taxa based on DGGE fingerprints.

### *5.3.3. Microbial activity on decomposing leaves*

Fungal biomass on leaves peaked at 43 days of immersion at Algeriz and Este 2 (71.8 and 69.2 mg $g^{-1}$ AFDM, respectively), while for the remaining streams maximum values were reached after 57 days (81.7, 49.7 and 46.3 mg $g^{-1}$ AFDM at Maceira, Souto and Este 3, respectively) (Fig. 5.7A). Fungal biomass differed between sites and along time and interactions between sites and time were significant (Two-way ANOVA, $P<0.0000001$). Fungal biomass was significantly lower on leaves decomposing at Este 3 and Souto than at the other sites (Tukey's, $P<0.05$). Significant negative correlations were found between fungal biomass and conductivity ($r=-0.45$, $P=0.007$) or concentrations of $N\text{-}NO_3^-$ ($r=-0.57$, $P=0.0005$), $N\text{-}NO_2^-$ ($r=-0.50$, $P=0.002$), $N\text{-}NH_4^+$ ($r=-0.53$, $P=0.001$) and Ca ($r=-0.56$, $P=0.03$) in the stream water.

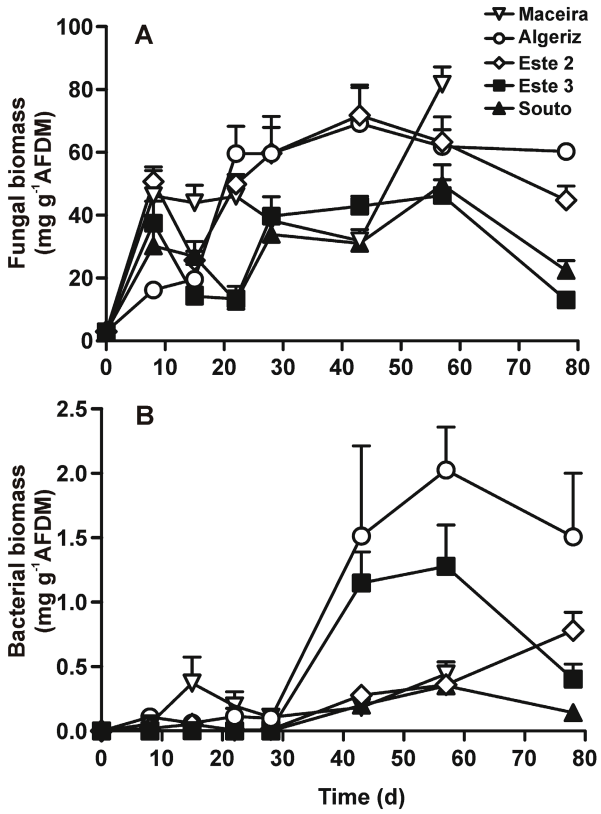


**Figure 5.7.** Biomass of fungi (A) and bacteria (B) on leaves decomposing at the five stream sites. Mean ± SEM; n=4.

Apart from Este 2, bacterial biomass on decomposing leaves peaked at 57 days with the highest value at Algeriz (2.0 mg $g^{-1}$ AFDM) and the lowest one at Souto (0.3 mg $g^{-1}$ AFDM). At Este 2 peak bacterial biomass (0.9 mg $g^{-1}$AFDM) was attained after 78 days (Fig. 5.7B). Time of leaf immersion, stream site and the interaction between both significantly affected bacterial biomass (Two-way ANOVA, $P<0.0000001$). Leaf-associated bacterial biomass was significantly higher at Algeriz than at the other sites (Tukey's test, $P<0.05$). Bacterial biomasses were also higher on leaves decomposing at Este 3 than at Souto or Este 2 (Tukey's test, $P<0.05$). Significant negative correlations were found between bacterial biomass and conductivity ($r=-0.41$, $P=0.02$) or $N\text{-}NO_3^-$ concentration ($r=-0.49$, $P=0.003$) in the stream water.

Fungal sporulation rates associated with decomposing leaves peaked after 22 days at Este 2 and Maceira ($9.8 \times 10^5$ and $5.8 \times 10^5$ conidia $g^{-1}$ AFDM $d^{-1}$, respectively), after 28 days at Algeriz and Este 3 ($1.2 \times 10^6$ and $9.3 \times 10^5$ conidia $g^{-1}$ AFDM $d^{-1}$, respectively) and only after 43 days at Souto ($1.1 \times 10^5$ conidia $g^{-1}$ AFDM $d^{-1}$) (Fig. 5.8). Fungal sporulation differed between sites and along time, and the interaction between the two factors was also significant (Two-way ANOVA,

P<0.0001). As for fungal biomass, sporulation rates were significantly lower on leaves decomposing at Este 3 and Souto than on leaves decomposing at the other sites (Tukey's test, P<0.01). Significant negative correlations were found between fungal sporulation rates and conductivity (r=-0.50, P=0.003), COD (r=-0.81, P=0.004) or concentrations of $N\text{-}NO_3^-$ (r=-0.46, P=0.007), $N\text{-}NO_2^-$ (r=-0.47, P=0.005) and $N\text{-}NH_4^+$ (r=-0.56, P=0.0006) in the stream water.

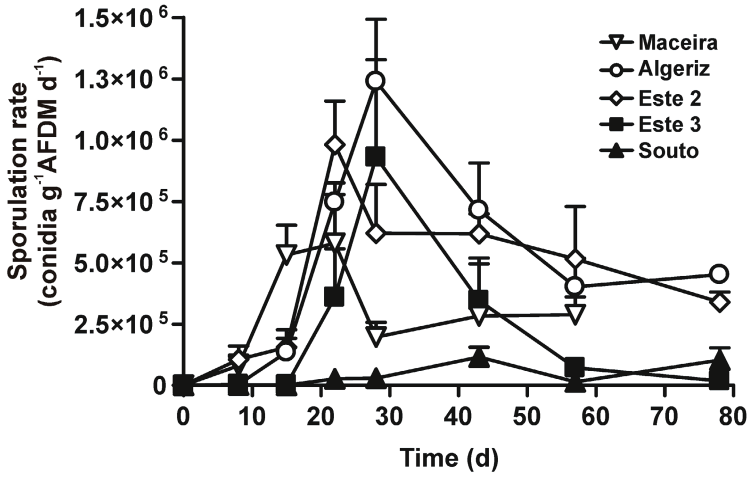


**Figure 5.8.** Sporulation rates of aquatic fungi on leaves decomposing at the five stream sites. Mean ± SEM; n=4.

Rates of leaf decomposition differed significantly between sites (ANCOVA, P<0.000001) (Table 5.3, Fig. 5.9). Leaf breakdown rates were higher at Algeriz (k= -0.0155 $d^{-1}$), intermediate at Este 2 (k= -0.0121 $d^{-1}$) and lower at all the other sites (Tukey's, P<0.0001). No significant correlations were found between leaf breakdown rates and any of the measured physical and chemical parameters of the stream water.

**Table 5.3.** Decomposition rates (k) of alder leaves immersed at the five stream sites.

| Site | k ($d^{-1}$) ± SE | $W_0$ (%) | $r^2$ |
|---|---|---|---|
| Maceira | -0.0090 ± 0.0010$^b$ | 88.1 | 0.75 |
| Algeriz | -0.0155 ± 0.0008$^a$ | 93.2 | 0.93 |
| Este 2 | -0.0121 ± 0.0016$^{a,b}$ | 81.2 | 0.69 |
| Este 3 | -0.0091 ± 0.0011$^b$ | 85.2 | 0.72 |
| Souto | -0.0088 ± 0.0011$^b$ | 83.7 | 0.73 |

SE standard error; $W_0$ intercept; $r^2$ coefficient of determination. Identical superscript letters indicate no significant differences (ANCOVA, Tukey´s test P>0.05); n=28.

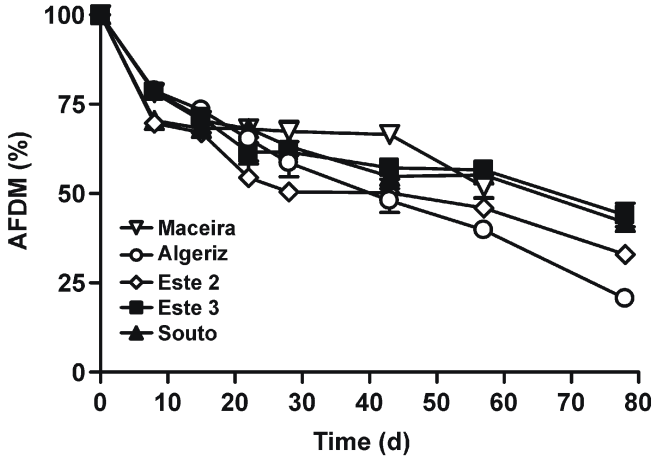


**Figure 5.9.** Ash free dry mass (AFDM) remaining of alder leaves decomposing at the five stream sites. Mean ± SEM; n=4.

## 5.4. Discussion

In the current study, the five selected stream sites had contrasting concentrations of dissolved inorganic nitrogen ($N\text{-}NO_3^-$, $N\text{-}NO_2^-$ and $N\text{-}NH_4^+$), being Maceira the less eutrophic site, Algeriz and Este 2 moderately eutrophic, and Este 3 and Souto the most eutrophic sites. These differences were highly correlated with changes in the structure of fungal communities between sites, as indicated by CCA ordination of both sporulating species and DNA fingerprint. Remarkable differences were found in the sporulating fungal taxa, even between the two sites from the same stream. At Este 3, sporulation was dominated by *D. foliicola*, while *A. tetracladia* was the dominant species on leaves decomposing at Este 2. This was expected since water chemistry has been reported as a major factor affecting the structure of fungal communities in streams (Pascoal and Cássio 2004, Solé *et al.* 2008, Castela *et al.* 2008). Indeed, 20 to 35 times higher concentrations of dissolved inorganic nitrogen concentrations ($N\text{-}NO_3^-$ + $N\text{-}NO_2^-$ + $N\text{-}NH_4^+$) were found between the most oligotrophic (Maceira) and the most eutrophic stream sites (Este 3 and Souto, respectively).

Although a strong decrease of fungal diversity, as species number and Shannon's index of sporulating taxa, was found on leaves decomposing at the most eutrophic sites, the number of DGGE bands did not differ much between sites. Fungal sporulation is often the most sensitive parameter to eutrophication (Pascoal *et al.* 2003, 2005a,b, Ferreira *et al.* 2006) and contamination by organic compounds (Au *et al.* 1992, Raviraja *et al.* 1998) or heavy metals (Niyogi *et al.* 2002 Sridhar *et al.* 2001, 2005). Fungal diversity based on conidial morphology has been also

reported to be negatively affected by high nutrient concentrations in the stream water (Pascoal *et al.* 2005b,c, Solé *et al.* 2008), as observed in the current study at the most eutrophic sites. In these cases the use of molecular techniques, such as DGGE that do not rely on fungal reproduction, appears to be advantageous to detect the actual diversity on leaves. However, both methods were sensitive to changes in the structure of fungal communities on leaves. The dominance of *D. foliicola* among sporulating fungi from leaves decomposing at Este 3 and Souto was complemented in the respective DGGE fingerprints by a strong band that migrated to the same position of the DNA of a pure culture of this species, isolated from a polluted site in the Este River (Pascoal *et al.* 2005c). *D. foliicola*, has been consistently found in polluted streams of the Northwest Portugal (Pascoal *et al.* 2003, 2005a,b,c chapter 3) and its contribution to the total conidium production is reported to increase at nutrient-enriched sites (Gulis and Suberkropp 2003a). This suggests that this species may tolerate or have some specific adaptation to high nutrient concentrations in the stream water that allow it to prevail over other species that are probably more sensitive. Additionally, this species was found at Algeriz and Este 2 (moderate nitrogen concentrations), but was almost undetectable at Maceira (lowest nitrogen concentrations). Previous works, mentioned the potential application of aquatic hyphomycetes as indicators of water pollution (Solé *et al.* 2008, Pascoal *et al.* 2005b, Gulis *et al.* 2006). However, aquatic hyphomycetes undergo seasonal fluctuations on decomposing leaves (Nikolcheva and Bärlocher 2005) that may limit its use as bioindicators.

Fungal biomass was also lowered at the most eutrophic sites, and negatively correlated with dissolved inorganic nitrogen ($N\text{-}NO_3^-$, $N\text{-}NO_2^-$ and $N\text{-}NH_4^+$) in the stream water. Nitrate concentrations similar to those found in the current study were reported to have no effect (Pascoal *et al.* 2005b) or to stimulate (Baldy *et al.* 2007) fungal biomass. However, the high concentrations of other compounds, such as nitrite and ammonia at our hypertrophic sites (Este 3 and Souto) were probably responsible for the reduction in fungal biomass, as previously suggested (Baldy *et al.* 2007). Moreover, contamination by heavy metals has been reported at sites of the Este River near the Industrial Park of Braga (Gonçalves 2001), which might negatively affect microbial decomposers (Sridhar *et al.* 2001, 2005, Solé *et al.* 2008), counteracting the positive effects of nutrients.

Bacterial taxa are much unknown than fungal taxa, but bacterial numbers, biomass and production on decomposing leaves are often reported to be stimulated by increased nutrient concentrations in the stream water (Pascoal and Cássio 2004,

Pascoal *et al.* 2005a,b, Baldy *et al*. 2007, chapter 3). Differences in bacterial community structure were also found between the five stream sites, and bacterial biomass appear to be stimulated with the increasing gradient of eutrophication from Maceira to Este 3, but declined at Souto. Previous studies indicated that bacterial plankton community composition differed among five sites in a lake and differences were attributed to the variability in water chemistry, including the nutrient levels in the different lake areas (Yan *et al.* 2007).

Although no significant correlations were found between leaf decomposition rates and the chemical parameters of the stream water, leaf decomposition was faster at Algeriz, which had moderate concentrations of inorganic N and P, and slower at sites with higher or lower N and P concentrations. When no confounding factors are present, leaf decomposition rates are expected to fit to linear or Michaelis-Menten models as a function of phosphorus (Gulis *et al.* 2006) or nitrate (Ferreira *et al.* 2006) concentrations in stream water. However, in the nature such conditions are rarely found, and in the present study, other factors, namely the high concentrations of ammonia and nitrites may have counteracted the positive effects of phosphorus on leaf decomposition rates and microbial biomass at the most eutrophic sites. Although there are no studies that attributed negative effects of ammonia to microbial decomposers, unionized ammonia is reported to be the most toxic form of inorganic nitrogenous compounds for aquatic animals (Camargo and Alonso 2006). Indeed, a decrease in leaf decomposition rates and shredder abundance and diversity was attributed to the deleterious effects of ammonium and its associated products, ammonia and nitrites, but microbial breakdown of leaf litter was not affected (Lecerf *et al.* 2006). However, in the current study concentrations of ammonium are 2 to 18 times higher than those found in the study of Lecerf *et al.* (2006) or in previous studies conducted in streams of the Northwest of Portugal (Pascoal *et al.* 2003, Pascoal and Cássio 2004), and thus this might be toxic for microbial decomposers. Additionally, unionized ammonia was also found to be very toxic to bacteria involved in the nitrification process (Russo 1985).

Overall, our results indicated that the increase in the concentrations of inorganic nitrogen was responsible for the differences in microbial community structure among the five stream sites. The most oligotrophic (Maceira) and the two moderate eutrophic sites (Algeriz and Este 2) exhibited higher fungal biomasses and sporulation, than the most eutrophic sites (Este 3 and Souto). However, diversity assessed by DGGE did not differ much between the five stream sites and higher leaf breakdown rates were only found at one of the moderately eutrophic sites. In

stressed soils, both fungal and bacterial communities are also reported to be dominated by a stable pool of organisms determined from rDNA (Torsvik *et al.* 1996, Felske and Akkermans 1998). However, many organisms can be inactive and community profiles derived from rRNA, which are indicative of active communities in soils (Girvan *et al.* 2004), may provide higher resolution for the shifts of microbial communities in future studies on impacted streams. Our results also suggest that microbial parameters were more sensitive to water quality than decomposition rates, as previously found (Pascoal *et al.* 2005b, Castela *et al.* 2008) and that anthropogenic nutrient enrichment can result in profound changes in the composition of aquatic food webs and to a lesser extent in ecosystem functioning.

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# Chapter 6

## *Copper and zinc mixtures induce shifts in microbial communities and reduce leaf-litter decomposition in streams*

S Duarte, C Pascoal, A Alves, A Correia, F Cássio (2008)
Copper and zinc mixtures induce shifts in microbial communities and reduce leaf litter decomposition in streams.
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## Abstract

To assess the impact of metal mixtures on microbial decomposition of leaf litter, we exposed leaves previously immersed in a stream to environmentally realistic concentrations of Cu and Zn (three levels), alone and in all possible combinations. The response of the microbial community was monitored after 10, 25 and 40 days of metal exposure by examining leaf mass loss, fungal and bacterial biomass, fungal reproduction, and fungal and bacterial diversity. Analysis of microbial diversity, assessed by Denaturing Gradient Gel Electrophoresis (DGGE) and identification of fungal spores, indicated that metal exposure altered the structure of fungal and bacterial communities on decomposing leaves. Exposure to metal mixtures or to the highest Cu concentration significantly reduced leaf decomposition rates and fungal reproduction, but not fungal biomass. Bacterial biomass was strongly inhibited by all metal treatments. The effects of Cu and Zn mixtures on microbial decomposition of leaf litter were mostly additive, because observed effects did not differ from those expected as the sum of single metal effects. However, antagonistic effects were found on bacterial biomass in all metal combinations and on fungal reproduction in metal combinations with the highest Cu concentrations, particularly at longer exposure times.

## 6.1. Introduction

Anthropogenic changes are threatening biodiversity and functional integrity of ecosystems (Vitousek *et al.* 1997). Freshwater ecosystems include some of the most impaired on Earth, with the highest rates of species extinction, raising the significance of addressing whether the biota is able to maintain ecological functions in impacted systems (Dudgeon *et al.* 2006). Pollution by metals is of major concern due to their toxicity to living organisms and their persistence in the environment. Metal impacts in freshwaters have been the focus of much research (Rand *et al.* 1995), but their combined effects on the biota remain difficult to predict.

The interactions between metals in mixtures can be determined by comparing the observed toxic effect in the mixture with that expected based on single toxic effects (Norwood *et al.* 2003). In a review on the effects of metal mixtures in aquatic biota that includes several groups of organisms, such as bacteria, algae, invertebrates, fish and macrophytes, Norwood *et al.* (2003) showed that metal interactions fall into three categories: synergistic, if the observed toxic effect is greater than expected; additive, if the observed effect is similar to that expected; and antagonistic, if the observed effect is less than expected. Although there is a tendency toward antagonistic effects (Norwood *et al.* 2003), metal interactions may depend on species traits (Braek *et al.* 1980), the parameter measured (Starodub *et al.* 1987), the combination of metals and their concentration (Prevot and Soyer-Gobillard 1986, Franklin *et al.* 2002), and various other environmental factors (Starodub *et al.* 1987). However, those studies are based on individual responses to toxic metals, and are clearly insufficient to predict the impacts on whole community. Therefore, studies at the community level are of high priority if we want to elucidate the combined effects of metal on ecosystem functioning.

In streams, bacteria and fungi play a key role in organic matter decomposition and by converting plant litter from the surrounding vegetation into a more suitable food source for invertebrates (Bärlocher 2005). Fungi, mainly aquatic hyphomycetes, are often dominant over bacteria during earlier stages of leaf litter decomposition, even though the contribution of fungi and bacteria to organic matter turnover may change in polluted streams (Pascoal and Cássio 2004, Baldy *et al*. 2007). Several studies report that metal pollution depresses plant litter decomposition, fungal activity and diversity of aquatic hyphomycetes (Bermingham *et al.* 1996, Sridhar *et al.* 2001, Niyogi *et al.* 2002). Conversely, bacterial abundance (Lemke and Leff 1999) and productivity (Niyogi *et al.* 2003) do not seem to be

severely affected by high levels of metals in the stream water. However, those studies were carried out in streams where multiple stressors with possible confounding effects occur making it difficult to understand the effects of metals on microbial decomposers.

In this study, we assessed the impact of metals on diversity and function of microbial communities associated with decomposing leaf litter, and we tested whether fungal and bacterial functions shift in a predictable manner when exposed to metal mixtures. We conducted a microcosm experiment with leaves previously exposed in a stream to examine the effects of Cu and Zn and their possible interactions. This approach combines realism with simplicity of manipulation and it is a feasible way to study the impact of harmful compounds, such as metals. Firstly, we examined the effects of metals on microbial abundance, diversity and activity on decomposing leaves. Secondly, we compared the effects of metal mixtures with those expected from each metal alone.

## 6.2. Materials and Methods

### *6.2.1. Sampling site and microcosm setup*

The sampling site is located at the spring of the Este River, a low-order stream in the Northwest of Portugal. At the spring, the stream is about 0.2 m deep and 0.5 m wide and the substratum consists of granitic rocks, pebbles and gravel. The dominant riparian vegetation is *Eucalyptus globulus* Labill., *Pinus pinaster* Aiton, *Pteridium aquilinum* Khun and *Juncus* sp. Previous analytical studies of the stream water indicated trace levels of metals at the spring of the Este River (Gonçalves 2001). About 10 km downstream, the Este River passes through the town of Braga and its Industrial Park, which contribute to the high levels of metals in the stream water, such as Cu (up to 150 µM) and Zn (up to 80 µM) (Gonçalves 2001).

Leaves of *Alnus glutinosa* (L.) Gaertn., collected in October 2004, were dried at room temperature until used. The leaves were cut into 22 mm diameter disks and sets of 80 disks were placed into each of 84 fine-mesh bags (0.5 mm pore size to prevent invertebrate colonization). On the 2 February 2005, leaf bags were immersed at the spring of the Este River for 16 days to allow microbial colonization. Additionally, water samples were collected from the spring for use in microcosm experiments.

In the laboratory, the contents of each leaf bag were rinsed in deionised water and put into 250 mL Erlenmeyer flasks with 150 mL of sterilized stream water (120 ºC, 20 min). The stream water contained 930 (600 - 1700) µg $L^{-1}$ $N\text{-}NO_3^-$, 170 (0 - 830) µg $L^{-1}$ $P\text{-}PO_4^{3-}$, and 10 (0 - 40) µg $L^{-1}$ $N\text{-}NH_4^+$ The microcosms were supplemented with Cu or Zn (added as chlorides, Sigma) at two concentrations, corresponding to 20 µM (Cu1 and Zn1) and 100 µM (Cu2 and Zn2), added alone or in mixtures at all possible combinations (three replicates of each). Microcosms without added metals were used as controls (Cu0 and Zn0). The microcosms were incubated on a shaker (120 rpm, Certomat BS 3) at 15 ºC under permanent artificial light, and solutions were changed every 5 days. After 10, 25 and 40 days of metal exposure, 27 microcosms were sacrificed to assess fungal biomass, sporulation and diversity, bacterial biomass and diversity and leaf mass loss as described below.

### *6.2.2. Fungal biomass*

A subset of five freeze-dried leaf disks from each microcosm was 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). Ergosterol was converted to fungal biomass by using a factor of 5.5 mg ergosterol $g^{-1}$ fungal dry mass (Gessner and Chauvet 1993).

### *6.2.3. Fungal sporulation*

A subset of eight leaf disks from each microcosm was transferred to 150 mL Erlenmeyer flasks with 50 mL of sterilized stream water either with no metal supplementation (for microcosm controls) or supplemented with Zn and/or Cu at concentrations indicated above. Sporulation was induced by shaking (120 rpm) for 48 h at 15 ºC. Conidial suspensions were mixed with 200 µL of 0.5% Tween 80, filtered (5-µm pore size, Millipore) and the conidia retained were stained with 0.05% cotton blue in lactic acid. At least 300 conidia per replicate were identified and counted under a light microscope at 400x magnification (Leica Biomed).

### *6.2.4. Bacterial biomass*

A subset of five leaf disks from each microcosm was placed into screw–top tubes with 10 mL of phosphate buffered formalin (3.7% final concentration) and kept at 4 ºC until processed. Bacterial cells were dislodged from leaf disks by 5 min sonication (Branson 2510 sonication bath, Danbury, CT, USA). Aliquots of 2 mL of appropriate dilutions of bacterial suspensions were incubated with 40 µL of 0.1 mg $mL^{-1}$ of 4',6-diamidino-2-phenylindole (DAPI, Sigma) for 10 min in the dark and filtered through black polycarbonate filters (0.2 µm pore size, GTBP, Millipore). Each filter was mounted in a slide between two drops of mineral oil and bacteria were counted using an epifluorescence microscope (Leitz, Laborlux) at a magnification of 1000x. Bacterial numbers were converted to bacterial carbon using a conversion factor of 20 *f*g bacterial carbon per cell (Norland 1993) and to bacterial biomass assuming that carbon was 50% of bacterial dry mass.

### *6.2.5. DNA analysis*

DNA was extracted from freeze-dried leaves (four halves of four leaf discs) with FastDNA SPIN kit for soil, using a FastPrep FP120 device (Qbiogene, Heidelberg, Germany) according to the manufacturer instructions.

The ITS2 region of fungal rDNA was amplified with the primer pair ITS3GC and ITS4 (White *et al.* 1990) and the V3 region of bacterial 16S rDNA was amplified with the primer pair 338F_GC and 518R (Muyzer *et al.* 1993). The forward primers had an additional 40-bp GC tail on the 5' end, which ensures separation on DGGE gels (Muyzer *et al.* 1993). For PCR reactions 1x 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. All PCR reagents were from MBI Fermentas except primers that were from MWG-Biotech AG. PCR was carried out in an iCycler Thermal Cycler (BioRad Laboratories, Hercules, CA, USA). 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 and Bärlocher 2005, Nikolcheva *et al.* 2005). For bacterial DNA, the amplification started with a denaturation of 9 min at 94 ºC, followed by 36 cycles of denaturation for 30 s at 94 ºC, primer annealing for 30 s at 55 ºC and extension for

30 s at 72 ºC. Final extension was at 72 ºC for 7 min (adapted from Nakatsu *et al.* 2000).

DGGE analysis was performed using a DCode™ Universal Mutation Detection System (BioRad Laboratories, Hercules, CA, USA). For fungal DNA, 20 µL samples from the amplification products of 380-400 bp were loaded on 8% (w/v) polyacrylamide gel in 1x TAE with a denaturing gradient from 30 to 70% (100% denaturant corresponds to 40% formamide and 7 M urea). For bacterial DNA, 45 µL samples of 200 bp amplified products were loaded on 8% (w/v) polyacrylamide gels in 1x TAE with a denaturing gradient from 35 to 80%. The gels were run at 55 V, 56 ºC for 16 h and stained with 1 µg $mL^{-1}$ of ethidium bromide (BioRad) for 5 min. The gel images were captured under UV light in a transiluminator Eagle eye II (Stratagene, La Jolla, CA, USA). In each fungal DGGE gel mixtures of DNA of the fungal species *Articulospora tetracladia* Ingold UMB 22.01, *Anguillospora filiformis* Greath. UMB 225.02, *Heliscus lugdunensis* Sacc. & Therry UMB 159.01, *Tricladium splendens* Ingold UMB 54.01 and *Varicosporium elodeae* W. Kegel UMB 20.01, were included to calibrate the gels in further analyses. In a similar way, in each bacterial DGGE gel, mixtures of DNA of the isolates 16, 17, 31, 42 and 56 (see chapter 4), were included.

### *6.2.6. Leaf mass loss*

A subset of 57 leaf disks from each microcosm was dried to constant mass (60 ºC, 72 ± 24 h) and weighted to the nearest 0.001 mg. Initial dry mass of leaf disks was estimated by immersing three identical bags containing 80 leaf disks for 30 min at the spring of the Este River. Leaf disks were subsequently re-dried and weighted as above.

### *6.2.7. Data analyses*

To test the effects of Cu concentration (three levels), Zn concentration (three levels) and exposure time (three levels) on fungal biomass and sporulation, and bacterial biomass a three-way ANOVA was used (Zar 1996). To achieve normal distribution, data were ln-transformed (Zar 1996). Leaf decomposition rate (k) was determined by linear regression after ln-transformation as follows: $\ln(m_t / m_0) = -kt + b$ , where $m_t$ is the leaf dry mass remaining at time $t$, $m_0$ is the initial

dry mass of leaves, *t* is the time in days and *b* is the *Y* intercept. Differences in decomposition rates were tested by a two-way ANCOVA, with Cu and Zn concentrations as factors (Zar 1996). To test for differences between control and metal supplemented microcosms, a Dunnett's post-hoc test was done. Differences were considered significant at $P<0.05$ (Zar 1996).

For each measured parameter, inhibition effects of metal mixtures were predicted as the sum of the inhibition effects observed in microcosms containing each metal alone at the corresponding concentration (response addition model, Norwood *et al.* 2003). The differences obtained were tested against zero by a t-test (Zar 1996) to check the occurrence of additive, synergistic or antagonistic effects.

Metal effects on aquatic hyphomycete diversity, estimated from conidial counts, were analysed by the Principal Response Curves method (PRC), based on the Redundancy Analysis ordination technique, which is a constrained form of Principal Component Analysis (Van den Brink and Ter Braak 1999). The statistical model for the PRC is: $Y_{d(j)tk} = Y_{0tk} + b_k C_{dt} + \varepsilon_{d(j)tk}$, where $Y_{d(j)tk}$ is the ln abundance of species *k* in replicate *j* of treatment *d* at time *t*, $Y_{0tk}$ is the mean abundance of species *k* on date *t* at the control (d=0), $C_{dt}$ is the response pattern for every treatment *d* and time *t*, $b_k$ is the weight of each species with this response pattern, and $\varepsilon_{d(j)tk}$ is an error term with mean zero and variance $\sigma_k^2$. By definition, $C_{0t} = 0$ for every *t*. When the coefficients $C_{dt}$ are plotted against time *t*, the resulting PRC diagram displays a curve for each treatment that can be interpreted as the PRC of the community (Van den Brink and Ter Braak 1999). The species weight $b_k$ indicates how closely the response of each individual taxon matches the overall community response as displayed in the PRC diagram. Monte Carlo permutation tests were used to test if the PRC diagram displayed a significant part of treatment variance. To discriminate treatments that significantly differed from the control, the "No Observed Effect Concentration" at the community level (NOECcommunity) was estimated by a one-way ANOVA, followed by a Dunnet's post-hoc test applied to the scores of the first principal component for each sampling date.

Univariate analyses were done with Statistica 6.0 (StatSoft, Inc.) and PRC analysis was done with CANOCO 4.5 (Microcomputer Power, NY, USA). Cluster analysis of DGGE banding patterns for fungal and bacterial communities was carried out by unweighed pairgroup method average (UPGMA) using the Dice

coefficient of similarity. DGGE fingerprints were analysed with the GelCompar II program (Applied Maths, Belgium).

## 6.3. Results

### *6.3.1. Effects of copper and zinc on the structure of microbial decomposer communities*

The fungal community consisted of 13 sporulating species before metal exposure. Fungal species richness peaked after 10 days in microcosms and tended to decrease with metal exposure (Table 6.1).

**Table 6.1.** Metal effects on fungal diversity assessed by the number of bands in DGGE analysis and number of species identified from conidial counts associated with decomposing leaves in microcosms. Metal levels: Cu0 or Zn0, without metal addition; Cu1 or Zn1, 20 µM; and Cu2 or Zn2, 100 µM. Initial communities had 13 sporulating species and 18 DGGE bands.

| Treatment | 10 d | | 40 d | |
|---|---|---|---|---|
| | Conidia | DGGE | Conidia | DGGE |
| Cu0Zn0 | 16 | 17 | 11 | 15 |
| Cu0Zn1 | 14 | 18 | 10 | 18 |
| Cu0Zn2 | 15 | 22 | 6 | 20 |
| Cu1Zn0 | 18 | 17 | 13 | 18 |
| Cu1Zn1 | 12 | 21 | 9 | 15 |
| Cu1Zn2 | 9 | 20 | 8 | 19 |
| Cu2Zn0 | 6 | 23 | 3 | 15 |
| Cu2Zn1 | 5 | 16 | 3 | 17 |
| Cu2Zn2 | 8 | 19 | 1 | 16 |

The PRC diagram of the aquatic hyphomycete community, assessed as conidial identification and counts, clearly shows that fungi were affected by Cu and Zn (Fig. 6.1A), with metal treatment explaining a significant part of the variance (Monte Carlo, $P<0.01$). Of the total variance, 28% was explained by the exposure time (x-axis), 37% was explained by metal treatment and the remaining 35% can be attributed to differences between replicates. Metal treatment explained a significant part of the total variance, of which a significant part (55%) is displayed on the y-axis of the PRC (Monte Carlo, $P<0.01$).

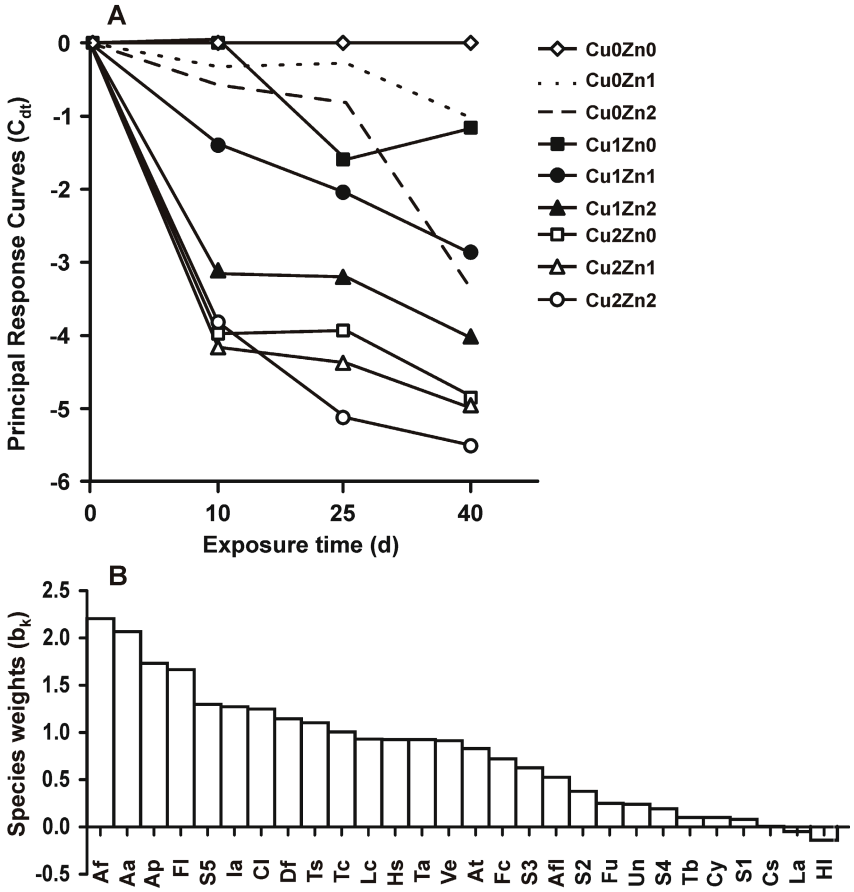


**Figure 6.1.** Principal Response Curves of metal effects (A) on the aquatic hyphomycete community, assessed as conidial identification and counts. Species weights (B) showing the relative contribution of individual fungal species to the community response. The lines represent the effect of metal treatments along time. Metal levels: Cu0 or Zn0, without metal addition; Cu1 or Zn1, 20 µM; and Cu2 or Zn2, 100 µM. Fungal taxa: Af, *Anguillospora filiformis* Greath.; Aa, *Alatospora acuminata* Ingold; Ap, *Alatospora pulchella* Marvanová; Fl, *Flagellospora* sp.; S5, Sigmoid 5 (25-35/0.5-1.0 µm); Ia, *Infundibura adhaerens* Nag Raj & W. B. Kendr.; Cl, *Clavatospora longibrachiata* (Ingold) Sv. Nilsson ex Marvanová & Sv. Nilsson; Df, *Dimorphospora foliicola* Tubaki; Ts, *Tricladium splendens* Ingold; Tc, *Tricladium chaetocladium* Ingold; Lc, *Lunulospora curvula* Ingold; Hs, *Heliscella stellata* (Ingold & V. J. Cox) Marvanová; Ta, *Triscelophorus* cf. *acuminatus* Nawawi; Ve, *Varicosporium elodeae* W. Kegel; At, *Articulospora tetracladia* Ingold; Fc, *Flagellospora curta* J. Webster; S3, Sigmoid 3 (10-20/1-1.5 µm); Afl, *Alatospora flagellata* (J. Gönczöl) Marvanová; S2, Sigmoid 2 (70-90/1.5-2 µm); Fu, *Fusarium* sp.; Un, Unknown sp.; S4, Sigmoid 4 (65-80/2.5-3.5 µm); Tb, *Tetracladium breve* A. Roldán; Cy, *Cylindrocarpon* sp.; S1, Sigmoid 1 (80-95/2.5-3.5 µm); Cs, *Casaresia sphagnorum* Gonz. Frag.; La, *Lemonniera aquatica* De Wild.; Hl, *Heliscus lugdunensis* Sacc. & Therry.

The largest deviations from the control community were observed at the highest Cu concentration and when both metals were added together. Moreover, $NOEC_{community}$ changed over time (one-way ANOVA, Dunnet´s test, $P<0.05$). After 10 days, significant differences were found for Cu1Zn2, Cu2Zn0, Cu2Zn1 and Cu2Zn2 treatments with the $NOEC_{community}$ corresponding to Cu1Zn1. After 25 and 40 days, significant differences were found for all treatments, except for those without Cu

(Cu0Zn1 and Cu0Zn2) after 25 days and for Cu0Zn1 after 40 days. The $NOEC_{community}$ was Cu0Zn2 and Cu0Zn1 for 25 and 40 days, respectively. Most fungal taxa were negatively affected by metal treatments as indicated by their positive weights in the PRC (Fig. 6.1B). The strongest inhibition effects were observed on *Anguillospora filiformis* and *Alatospora acuminata*. Other species, such as *Heliscus lugdunensis* and *Lemonniera aquatica*, had a weight near zero, indicative of no response to metal exposure or a non-related response to the general pattern of the community.

DGGE analysis of fungal communities showed 18 bands before metal exposure, and minor changes in the number of bands occurred after metal treatments (Table 6.1; Fig. 6.2). Generally, higher fungal diversity was found from DGGE analysis than from conidial counts, and disparities were more pronounced in metal treatments at later exposure times (e.g., one sporulating species in contrast to 16 DGGE bands in the Cu2Zn2 treatment, Table 6.1). Cluster analysis of DGGE fingerprints showed that fungal communities exposed for 10 days to metal mixtures and to the highest Cu concentration grouped together, and were clearly separated from all the others (Fig. 6.2A). At this time (10 d), bacterial communities of control and Cu0Zn1 treatment were separated from the remaining metal-exposed communities (Fig. 6.2B). At the longest exposure time (40 days), fungal and bacterial control communities were separated from all metal treatments.

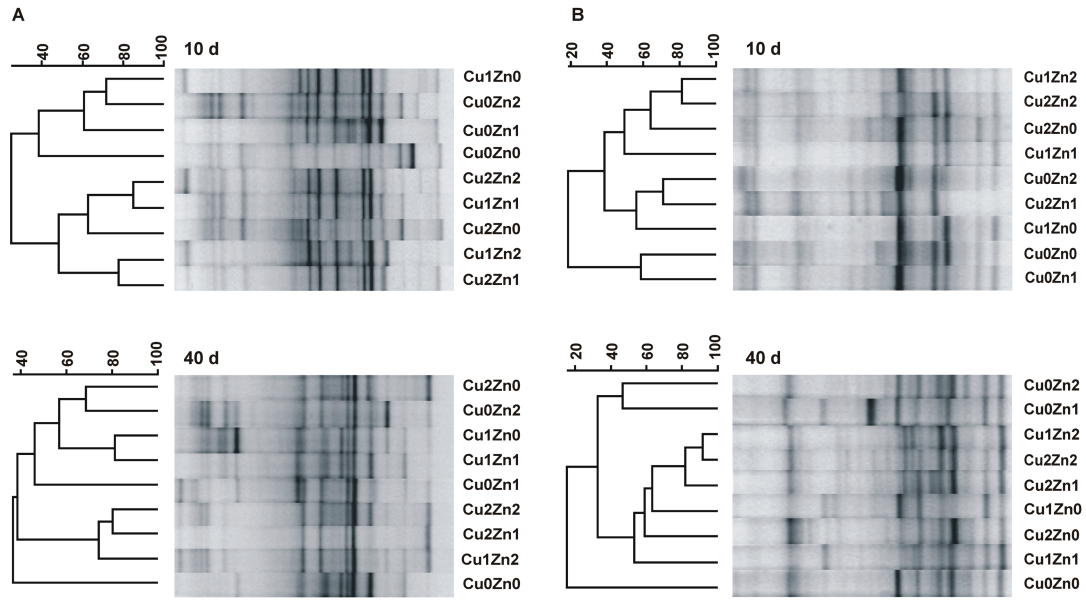


**Figure 6.2.** DGGE fingerprints and cluster dendogram of fungal (A) and bacterial (B) communities exposed to Cu and/or Zn for 10 and 40 days in microcosms. Dendograms were constructed from UPGMA analysis based on the Dice coefficient of similarity. Metal levels: Cu0 or Zn0, without metal addition; Cu1 or Zn1, 20 µM; Cu2 or Zn2, 100 µM.

### *6.3.2. Effects of copper and zinc on the activity of microbial decomposers*

The leaf mass loss increased with time and decomposition rates varied from 0.0079 to 0.0269 $d^{-1}$ for Cu2Zn2 and the control, respectively (Table 6.2; Fig. 6.3A). Decomposition rate was affected by both Cu and Zn (two-way ANOVA, P<0.05), but not by an interaction between Cu and Zn. Leaf decomposition was depressed in treatments with the highest Cu concentration and in metal mixtures, except for Cu1Zn1 (Dunnett's test, P<0.05).

**Table 6.2.** Metal effects on breakdown rates (k) of alder leaves in microcosms (n=15). Metal levels: Cu0 or Zn0, without metal addition; Zn1 or Cu1, 20 µM; Zn2 or Cu2, 100 µM. $d^{-1}$, $day^{-1}$; SE, standard error; $r^2$, coefficient of determination.

| Treatment | k ($d^{-1}$) ± SE | $r^2$ |
|---|---|---|
| Cu0Zn0 | 0.0269 ± 0.0031 | 0.85 |
| Cu0Zn1 | 0.0227 ± 0.0028 | 0.84 |
| Cu0Zn2 | 0.0224 ± 0.0036 | 0.74 |
| Cu1Zn0 | 0.0240 ± 0.0028 | 0.85 |
| Cu1Zn1 | 0.0209 ± 0.0023 | 0.86 |
| Cu1Zn2 | 0.0160 ± 0.0018* | 0.86 |
| Cu2Zn0 | 0.0134 ± 0.0010* | 0.93 |
| Cu2Zn1 | 0.0138 ± 0.0015* | 0.86 |
| Cu2Zn2 | 0.0079 ± 0.0018* | 0.61 |

*, treatments where breakdown rates were significantly different from control (Dunnet's test, P<0.05).

Copper, Zn and exposure time had a significant effect on fungal biomass, bacterial biomass and fungal sporulation (three-way ANOVA, P<0.05), and significant interactions were found between time and metals, and between metals for fungal biomass and fungal sporulation, but not for bacterial biomass (Table 6.3).

**Table 6.3.** Effects of exposure time, Cu and Zn concentrations and their interactions on fungal biomass, bacterial biomass and fungal sporulation.

| | Fungal biomass | | | Bacterial biomass | | | Fungal sporulation | | |
|---|---|---|---|---|---|---|---|---|---|
| | d.f. | F | P | d.f. | F | P | d.f. | F | P |
| Time | 2 | 11.6 | <0.001 | 2 | 6.7 | 0.002 | 2 | 126.6 | <0.001 |
| Cu | 2 | 8.2 | <0.001 | 2 | 9.6 | 0.0002 | 2 | 147.4 | <0.001 |
| Zn | 2 | 3.6 | 0.03 | 2 | 14.8 | <0.001 | 2 | 22.8 | <0.001 |
| Time x Cu | 4 | 12.2 | <0.001 | 4 | 1.8 | 0.1 | 4 | 2.6 | 0.04 |
| Time x Zn | 4 | 5.3 | 0.001 | 4 | 0.4 | 0.8 | 4 | 5.6 | 0.0006 |
| Cu x Zn | 4 | 3.6 | 0.01 | 4 | 2.2 | 0.07 | 4 | 4.6 | 0.003 |

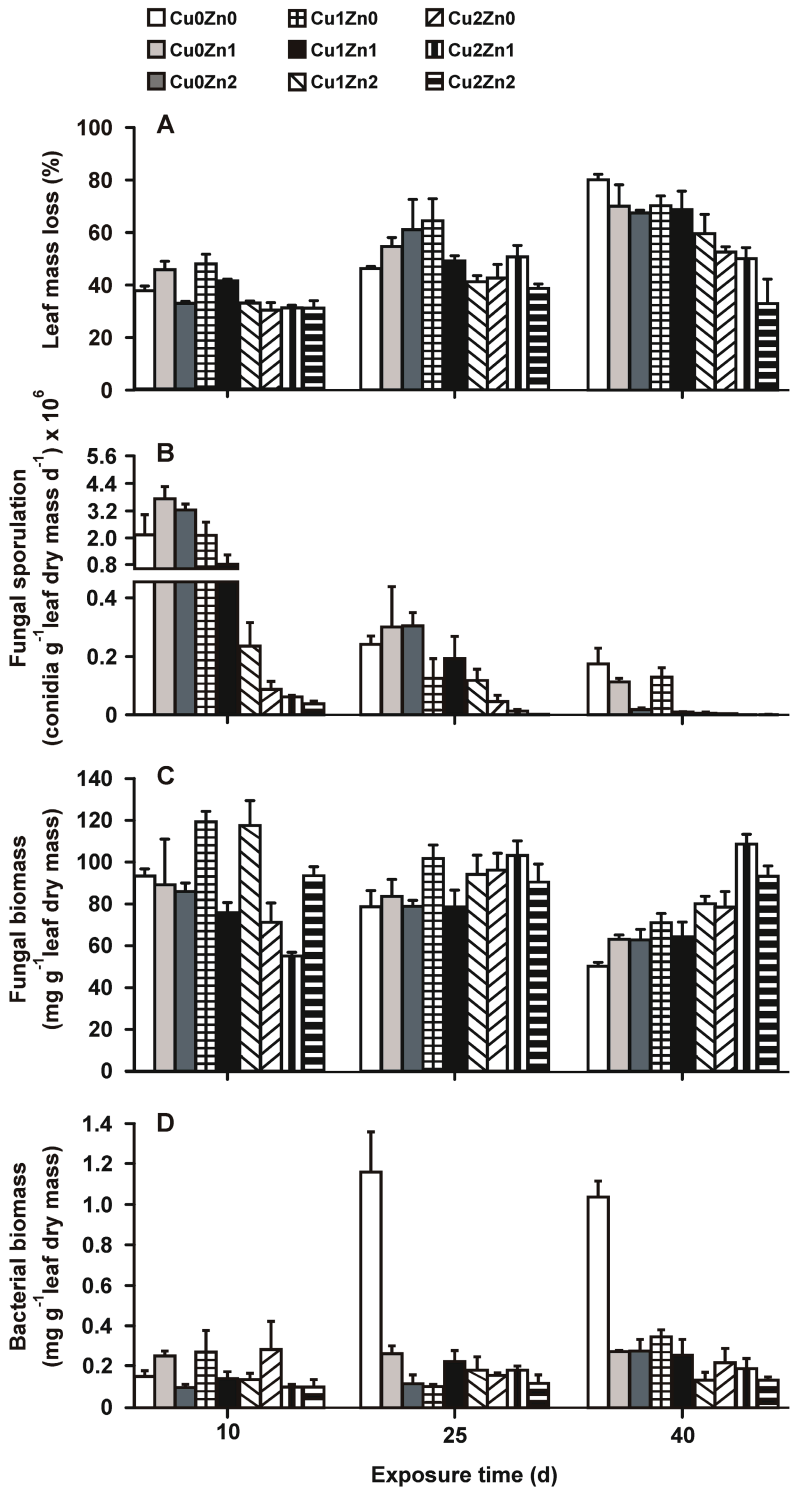


**Figure 6.3.** Effects of Cu and/or Zn on (A) leaf mass loss, (B) fungal sporulation, (C) fungal biomass and (D) bacterial biomass after 10, 25 and 40 d of exposure to metals in microcosm, after 16 days of microbial colonization at the spring of the Este River. Mean ± SEM, n=3. Metal levels: Cu0 or Zn0, without metal addition; Cu1 or Zn1, 20 µM; and Cu2 or Zn2, 100 µM.

Fungal sporulation decreased along time and ranged from 1.6 x $10^2$ to 2.0 x $10^5$ conidia $g^{-1}$ leaf dry mass $d^{-1}$ at the end of the experiment for Cu2Zn2 and control, respectively (Fig. 6.3B). The strongest inhibition of sporulation was found in metal mixtures and at the highest Cu concentration (Dunnett's test, $P<0.05$). Overall fungal biomass was either unaffected or stimulated by metals alone or in mixtures, although differences were only significant for Cu1Zn0, Cu1Zn2 and Cu2Zn2 treatments (Dunnett's test, $P<0.05$). At the end of the experiment, fungal biomass ranged from 50 to 109 mg $g^{-1}$ leaf dry mass for control and Cu1Zn2, respectively (Fig. 6.3C). Bacterial biomass greatly increased with time, but it was reduced at least four-times by exposure to metals alone or in mixtures (Dunnett's test, $P<0.05$; Fig. 6.3D).

Copper and Zn had additive effects on leaf mass loss and fungal biomass, regardless of concentration or exposure time, because observed effects in mixtures were similar to those predicted from the sum of observed effects of each metal at each concentration (t-test, $P>0.05$; Fig. 6.4). Additive effects were also found for fungal sporulation, with the exception for mixtures with the highest Cu concentration, which had an antagonistic effect at the longest exposure time (t-test, $P<0.05$). For bacterial biomass, an additive effect was obtained after 10 days of metal exposure (t-test, $P>0.05$), while an antagonistic effect was found for all mixtures at later exposure times (t-test, $P<0.05$).

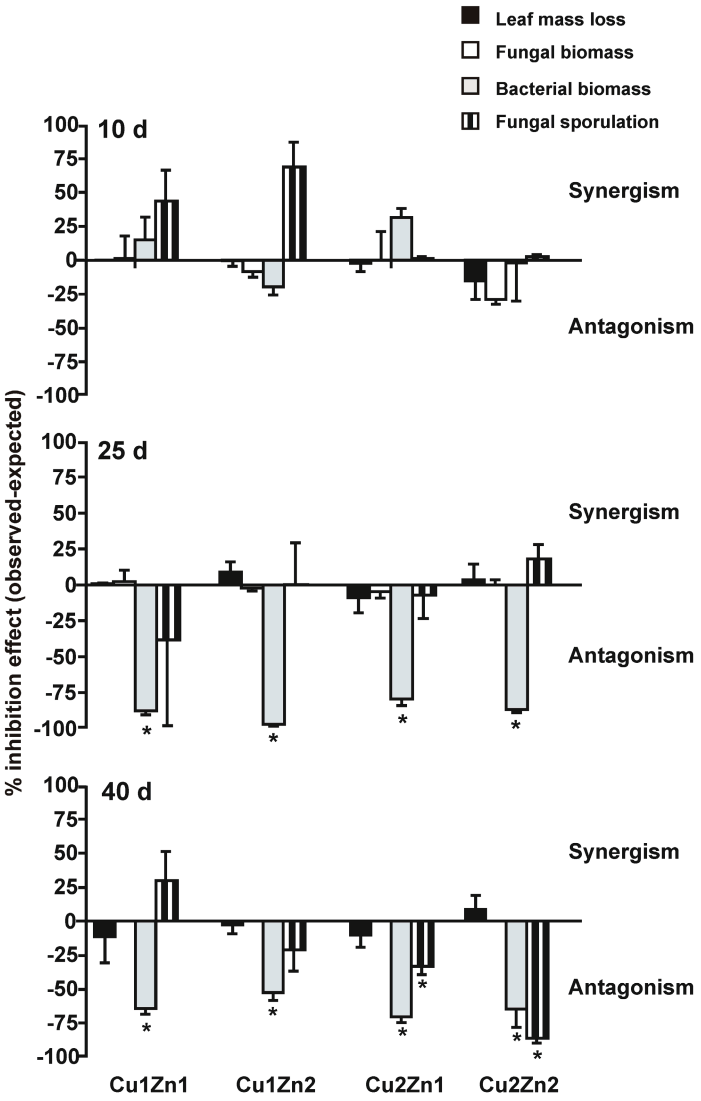


**Figure 6.4.** Differences between observed and expected inhibition effects (%) of metal mixtures on leaf mass loss, fungal biomass, bacterial biomass and fungal sporulation, after 10, 25 and 40 days of exposure. Metal levels: Cu1 or Zn1, 20 µM; and Cu2 or Zn2, 100 µM. Mean ± SEM, n=3. Differences were tested against zero by a t-test; *, P<0.05.

## 6.4. Discussion

In this work, we used a Principal Response Curve approach to compact the complexity of time-dependent community-level effects of metal stress into a simple diagram. This analysis clearly shows that metal exposure affected the structure of aquatic hyphomycete-sporulating communities on decomposing leaves, with stronger effects in metal mixtures and at longer exposure times. In addition, PRC analysis allowed the interpretation of metal effects at the level of individual species of the community, showing that *Anguillospora filiformis* and *Alatospora acuminata* were the most sensitive species to metal stress. By contrast, *Heliscus lugdunensis*

did not seem to be affected by metals, consistent with its presence in impoverished-fungal communities in metal-polluted streams (Sridhar *et al.* 2001, Sridhar *et al*. 2005).

Shifts in the structure of fungal communities with metal exposure were also detected by cluster analysis based on DGGE fingerprint. Metal effects were stronger at the longest exposure time, when control communities of fungi and bacteria were clearly separated from those exposed to all metal treatments. This is in agreement with that reported from metal-contaminated soils, where both fungal (Nordgren *et al.* 1983) and bacterial (Torsvik *et al.* 1998, Müller *et al.* 2001) communities differed from those in non-contaminated soils.

DNA-based techniques, such as DGGE, have proved useful for assessing the diversity of aquatic fungi on decomposing leaves and offer the advantage of detecting species from non-sporulating mycelia (Nikolcheva *et al.* 2003; Nikolcheva and Bärlocher 2005, Nikolcheva *et al.* 2005). In our work, metal exposure did not apparently alter microbial diversity as assessed by the number of DGGE bands. This finding can be due to: i) the number of fungal species was not actually affected or ii) some species were affected, decreasing competitive interactions and allowing species with initial low biomass (probably not detected by DGGE) to take advantage. On the other hand, the exposure to metals severely reduced the number of aquatic hyphomycete species sporulating on leaves (e.g., one versus 11 species in control and Cu2Zn2 mixture, at the end of the experiment), probably because fungal reproduction was seriously compromised by metal exposure. Although the assessment of fungal diversity based on spore production may underestimate the number of species, it focuses on reproductive species that are able to disperse, increasing the chance of colonizing new substrata (see Nikolcheva and Bärlocher 2005). This is relevant for predicting the long-term effects of metals on ecosystem functioning.

In the present work, metal exposure depressed leaf decomposition, which can be attributed to a reduction in fungal and/or bacterial decomposing activities. Bacterial biomass was severely reduced even at low metal levels. However, bacteria appear to contribute less to leaf decomposition than fungi (Pascoal and Cássio 2004), as supported by the 3,000 times lower biomass of bacteria in our control microcosms. Therefore, it is conceivable that the inhibition of leaf decomposition was mainly due to a reduction in fungal activity. Although viable fungal biomass was not depressed under metal stress, fungal sporulation was strongly inhibited, suggesting that less leaf carbon is being channelled to fungal reproduction. This

finding could account for the lower leaf mass loss under metal stress, and supports the hypothesis that fungal decomposition was compromised. In addition, our results are in alignment with those reporting that fungal biomass is less sensitive to metals than fungal reproduction (Niyogi *et al.* 2002; Duarte *et al.* 2004, present work) and productivity, based on incorporation rates of radiolabeled acetate into ergosterol (Duarte *et al.* 2004).

Copper has been reported to be more toxic than Zn to several species of aquatic hyphomycetes (Azevedo *et al.* 2007, Guimarães-Soares, Pascoal and Cássio 2007), but to our knowledge this is the first study at the community level addressing Cu effects alone and in mixtures. Metal effects on leaf decomposition were stronger when microbial communities were exposed to Cu and Zn mixtures or to the highest Cu concentration. The effects of metal mixtures on fungal and bacterial parameters varied from antagonistic to additive. Additive effects were observed for fungal biomass and leaf mass loss, while antagonistic effects were found for bacterial biomass and fungal sporulation at longer exposure times. Because there were shifts in the structure of bacterial and fungal communities during decomposition, it is conceivable that the effects of metal mixtures change across time. Indeed, effects varied with the exposure time and metal concentrations, consistently with that found in marine algae (Prevot and Soyer-Gobillard 1986).

A previous study that analysed the combined effects of two metals on aquatic fungi shows that Zn alleviates the effect of Cd toxicity on the growth of five aquatic hyphomycete species (Abel and Bärlocher 1984). In a review on the effects of Cu and Zn mixtures in several aquatic organisms, Norwood *et al.* (2003) point out that, from a total of 21 studies, 52% fall in the category of antagonistic and 43% correspond to synergistic effects. However, those studies analysed the effects of metal mixtures at the species level. Since the current experiment focuses on the effects of Cu and Zn mixtures at the community level, the observed effect is expected to be the sum of the individual effects of each constituent species. If the effect of each metal differs between species within a community, as found for algae (Braek *et al.* 1980), the overall effect is difficult to predict. In our work, we found mostly additive effects, particularly on fungal biomass and leaf decomposition, suggesting no interaction between Cu and Zn or that the magnitude of synergistic effects in some species was offset by antagonistic effects in others.

In summary, concentrations of Cu and Zn, within the range found downstream of the site from where microbial communities were collected, depressed leaf decomposition. This reduction was attributed to changes in the structure and activity

of microbial communities that are governing leaf-litter decomposition. Our results also suggest that, at least for a highly diverse community, metal mixtures may have mostly additive effects on microbial decomposition of leaf litter in streams. However, caution is needed when extrapolating conclusions to whole ecosystem, because metal impacts may vary with environmental factors, duration and type of other co-occurring stressors, and composition of microbial communities.

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# Chapter 7

*Functional stability of microbial decomposers under copper and zinc stress in streams*

## Abstract

It is recognized that resistance and resilience are key components of ecosystem stability. In this study we investigated the effects of Cu and Zn on leaf-litter decomposition and the associated microbial decomposers in streams by examining leaf mass loss, fungal reproduction and bacterial and fungal biomass, and diversity. Alder leaves, colonized in a reference stream were exposed in microcosms to Cu or Zn (one level) alone or in mixtures. After 10 days, microcosms exposed to one metal were subjected to the other metal, and after 20 days systems were released from the stressors. The resistance and resilience of stressed microcosms were compared to non-stressed ones. Generally, leaf mass loss and fungal reproduction were reduced in metal treatments and the structure of both fungal and bacterial communities was altered. Metals did not affect fungal biomass, but bacterial biomass was reduced. When microcosms were released from metal stress, the structure of fungal communities became similar to that of control, and a recovery of the microbial activity seemed to occur as shown by the lack of differences in leaf mass loss, bacterial biomass and fungal reproduction between control and metal treatments.

## 7.1. Introduction

In the last decades, an increasing number of studies have focused on the relationship between biodiversity and ecosystem functioning (Loreau *et al.* 2001). Current knowledge, mostly developed with terrestrial communities, indicates that there is a positive relationship between diversity and important ecosystem functions such as productivity (Naeem *et al.* 1994, Knops *et al.* 1999, Setäla and McLean 2004).

Aquatic ecosystems have particular features that differ from those of terrestrial ones (Giller *et al.* 2004, Gessner *et al.* 2004) and thus studies focusing on the relationship between aquatic biodiversity and ecosystem processes are of major importance. Since aquatic systems suffer from multiple stressors and stressors usually do not operate independently (Vinebrooke *et al.* 2004, Giller *et al.* 2004), it is important to assess their combined impacts on biodiversity and ecosystem processes. Moreover, stressors are frequently released in aquatic systems at different times and thus it is important to investigate whether exposure of communities to one stressor might increase, have no effect or decrease their resistance to additional stressors (Vinebrooke *et al.* 2004). The exposure to a stressor may allow that some species within a community acclimate to that change or develop adaptation increasing the tolerance to other stressors (Vinebrooke *et al.* 2004). Indeed, communities of marine periphyton chronically exposed to toxins were reported to be less sensitive to exposure to other pollutants (Blanck and Wängberg 1988).

Among anthropogenic stressors, metals are of major concern in aquatic systems due to their toxicity to organisms and persistence in the environment after a contamination event. It is also known that different metals may have different targets in cells and thus different toxicities (Gadd 1993). Consequently, the sequence by which an aquatic organism or community is exposed to different metals will probably determine the response to the latest metal and will differently affect ecosystem processes.

Aquatic microbial decomposers, namely fungi and bacteria, play a key role in converting leaf litter into a more nutritious food source for stream invertebrates (Suberkropp 1998, Bärlocher 2005). Due to their importance for many aquatic food webs, the resistance and the resilience of these organisms after a disturbance, as metal exposure, became a crucial subject of study. In this work, we assessed the functional stability of aquatic microbial decomposers as the resistance of fungal and

bacterial assemblages to metal stress and their ability to recover after release from the stressors. For that, alder leaves were colonized in a reference stream for one week and subsequently exposed to Cu and Zn, alone or in mixtures, added together or sequentially for 30 days in microcosms. After 20 days of metal exposure, a set of microcosms was released from metal stress to assess the recovery of the system during the following 10 days. The microbial community structure was assessed by DNA fingerprinting of leaf-associated fungi and bacteria and also by analysing the asexual reproductive structures of fungi. The resistance of the system was examined by measuring leaf decomposition, fungal biomass, and sporulation in stressed microcosms; the resilience of the system was assessed by measuring the same parameters in microcosms released from metals. We expect that the sequence of metal addition will affect leaf decomposition due to different susceptibilities of species within the community to the two metals. We also expect that if the microbial communities are able to acclimate to metals an increased resistance to a second metal can be found. Finally, we expect a recovery of the process if the initial inoculum is sufficiently diverse and redundant to sustain function after release from metal stress.

## 7.2. Materials and methods

### *7.2.1. Microcosm setup*

Leaves of *Alnus glutinosa* (L.) Gaertn. were collected in October 2005 and dried at room temperature. The leaves were lixiviated during 28 hours, cut into 22 cm diameter disks and sets of 30 disks were distributed by 57 fine-mesh bags. Leaf bags were immersed in the stream of Santa Maria (NW Portugal) on 26 May 2006 to allow microbial colonization of the leaves. At the study site, the stream had about 50 cm deep and 100 cm wide with a bottom mainly constituted by rocks, pebbles and gravel and the riparian vegetation mainly constituted by *A. glutinosa*. During leaf colonization, stream water had on average a temperature of 13.2 ºC (± 0.2), pH of 5.8 (± 0.02), dissolved oxygen concentration of 9.7 mg $L^{-1}$ (± 0.1) and conductivity of 54 µS $cm^{-1}$ (± 2.2) measured *in situ* with field probes (Multiline F/set 3 nº 400327, WTW). Stream water used in microcosms had 1.9 mg $L^{-1}$ (± 0.2) of $N\text{-}NO_3^-$, 0.001 mg $L^{-1}$ (± 0.0009) of $N\text{-}NO_2^-$, 0.01 mg $L^{-1}$ (± 0.005) of $N\text{-}NH_4^+$ and 0.08 mg $L^{-1}$ (± 0.04) of $P\text{-}PO_4^{3-}$, measured with a HACH DR/2000 photometer (Hach company, Loveland, CO, USA).

After 7 days of leaf immersion the bags were brought to the laboratory and the content of each bag was placed in fifty-four 250 mL Erlenmeyer flasks containing 150 mL of sterilized stream water (120 ºC, 20 min). The microcosms were supplemented with 50 µM of Cu or Zn (as chlorides, Sigma) or with the two metals together (3 replicates each x 2 for testing metal effects and recovery). Metals were added at day 0 (1Cu, 1Zn, 1Cu1Zn) or after 10 days (2Cu, 2Zn, 1Cu2Zn, 1Zn2Cu, 2Cu2Zn) in microcosms.

To test the recovery (R) of the system, after 20 days of metal exposure, half of the microcosms were released from the metals, and at the end of the experiment results were compared to those that were not released from the stress.

Microcosms were incubated on a shaker (120 rpm, Certomat BS 3), at 17 ºC, under permanent artificial light, and solutions were changed each 5 day. After 30 days of experiment, all microcosms were sacrificed for determination of leaf mass loss, fungal sporulation, and fungal and bacterial biomass and diversity.

### *7.2.2. Diversity of microbial decomposers*

Each 5 days, aliquots of conidial suspensions collected from each microcosm were mixed with 200 µL of 15% of Triton X-100 and filtered (5 µm pore size membranes, Millipore). The conidia retained on the filters were stained with 0.05% cotton blue in lactic acid and identified under a light microscope at 400x magnification (Leica Biomed). At least a total of 300 conidia were counted per filter.

DNA from three freeze-dried leaf disks was extracted with the soil DNA extraction kit (MoBio Laboratories, Solana Beach, California), according to the manufacturer instructions.

The ITS2 region of fungal DNA was amplified with the primer pair ITS3GC and ITS4 (White *et al.* 1990) and the V3 region of bacterial 16S rDNA was amplified with the primer pair 338F_GC and 518R (Muyzer *et al.* 1993). For PCR reactions 1x 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. PCR was carried out in an iCycler Thermal Cycler (BioRad Laboratories, Hercules, CA, USA). Both fungal and bacterial 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 and Bärlocher 2005, Nikolcheva *et al.* 2005).

DGGE analysis was performed using a DCode$^{TM}$ Universal Mutation Detection System (BioRad Laboratories, Hercules, CA, USA). For both fungal and bacterial DNA, 750 ng samples of the amplification products of the expected size were loaded on 8% (w/v) polyacrylamide gels in 1x TAE with a denaturing gradient from 30 to 70% (100% denaturant corresponds to 40% formamide and 7 M urea) for fungal DNA or from 35 to 80% for bacterial DNA. The gels were run at 55 V, 56 ºC for 16 h and stained with 1 µg mL$^{-1}$ of ethidium bromide (BioRad) for 5 min. The gel images were captured under UV light in a transiluminator Eagle eye II (Stratagene, La Jolla, CA, USA). In each fungal DGGE gel a DNA mixture of the fungal species *Articulospora tetracladia* Ingold UMB 22.01, *Varicosporium elodeae* W. Kegel UMB 20.01, *Lemonniera aquatica* De Wild. UMB 143.01, *Anguillospora filiformis* Greath. UMB 225.02 and *Tricladium chaetocladium* Ingold UMB 163.01 was loaded, while for bacterial DGGE gels a mixture of DNA of the isolates 16, 17, 31, 42 and 56 (see chapter 4) was used to calibrate the gels in further analyses.

### *7.2.3. Microbial activity*

A subset of 5 freeze-dried leaf disks from each microcosm was used to determine fungal biomass, as ergosterol concentration. 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). Ergosterol was converted to fungal biomass by using a factor of 5.5 mg ergosterol g$^{-1}$ fungal dry mass (Gessner and Chauvet, 1993).

A subset of 3 leaf disks were preserved in phosphate buffered formalin (3.7% final concentration) at 4 ºC to determine bacterial biomass. Bacterial cells were dislodged from leaf disks in a sonication bath (5 min, Branson 2510, Danbury, CT, USA) and 2 mL of appropriate dilutions of each bacterial suspension were stained with 40 µL of 0.1 mg mL$^{-1}$ of DAPI (4', 6-diamidino-2-phenylindole, Sigma) for 10 min in the dark before filtered through black polycarbonate filters (0.2 µm pore size, GTTP, Millipore). Each filter was mounted in one slide between two drops of mineral oil and bacteria were counted using an epifluorescence microscope (Leitz, Laborlux S) at a magnification of 1000x. Bacterial numbers were converted to bacterial biomass, using a conversion factor of 20 *f*g bacterial carbon per cell (Norland 1993) and assuming 50% of cell carbon content.

Fungal sporulation was determined by counting the total number of conidia in each microcosm suspension, as described in the previous section.

A subset of 17 leaf disks from each sacrificed microcosm was dried at 80 ºC to a constant mass (48 h) and weighted to the nearest 0.01 mg to determine leaf mass loss. Sets of leaf bags immersed 5 min at the study site were used to estimate the initial dry mass of alder leaves.

### *7.2.4. Statistical analyses*

For testing the significance of the factors Cu, Zn, and sequence of metal addition on leaf mass loss, fungal and bacterial biomass and fungal sporulation, a three-way nested ANOVA, with the sequence of metal addition nested in Cu and Zn, was used. Microcosms that were released from metals were compared with a similar nested design separately. To test for differences between control microcosms and metal supplemented microcosms, a Dunnett's post-test was done. Differences were considered significant for $P<0.05$ (Zar 1996). To achieve normal distribution, data from bacterial and fungal biomass and sporulation were ln-transformed and percentages of leaf mass losses were arcsine square-root transformed (Zar 1996).

The effects of metal treatments on aquatic hyphomycete diversity, assessed from conidial identification and counts, were analysed by the Principal Response Curves method (PRC), which is based on the Redundancy Analysis ordination technique, a constrained form of the Principal Component Analysis (Van den Brink and Ter Braak 1999). A Monte Carlo permutation test was performed to test if the PRC diagram displayed a significant part of the treatment variance. To know which treatments significantly differed from the control, a one-way ANOVA followed by a Dunnett's post-test was applied to the scores of the first principal component at each exposure time.

Variance analyses were performed with Statistica 6.0 (Statsoft, Inc.) and PRC analyses were performed with CANOCO 4.5 (Microcomputer Power, NY, USA), both for Windows. Cluster analyses of the DGGE banding patterns of fungal and bacterial communities were done by Unweighed Pair-Group Method Average (UPGMA) using the Dice coefficient of similarity. DGGE fingerprints and cluster analyses were done with GelCompar II program (Applied Maths, Belgium).

## 7.3. Results

### *7.3.1. Microbial community structure*

After 30 days of metal exposure the number of fungal sporulating species on leaves was reduced from two- to four-times in treatments with Cu alone or in mixtures with Zn, and the release of metal stress increased the number of sporulating species (Table 7.1).

**Table 7.1.** Metal effects on microbial diversity associated with decomposing leaves. Fungal diversity was assessed by the number of species identified from conidial counts and by the number of bands detected by DGGE. Bacterial diversity was assessed from the number of bands detected by DGGE. Sequence of metal addition: 1 – added at 0 d, and 2 – added after 10 d. R microcosms released from metals after 20 days of treatment.

| | Fungi | | Bacteria |
|---|---|---|---|
| | Conidial morphotypes | Number of bands | Number of bands |
| Control | 14 | 13 | 15 |
| 1Cu | 7 | 16 | 12 |
| 2Cu | 8 | 14 | 15 |
| 1Zn | 11 | 13 | 14 |
| 2Zn | 13 | 16 | 13 |
| 1Cu1Zn | 6 | 12 | 15 |
| 1Cu2Zn | 4 | 11 | 14 |
| 1Zn2Cu | 7 | 12 | 14 |
| 2Cu2Zn | 6 | 13 | 16 |
| 1CuR | 11 | 11 | 15 |
| 2CuR | 9 | 15 | 12 |
| 1ZnR | 11 | 10 | 13 |
| 2ZnR | 11 | 11 | 17 |
| 1Cu1ZnR | 10 | 10 | 16 |
| 1Cu2ZnR | 11 | 12 | 12 |
| 1Zn2CuR | 10 | 11 | 16 |
| 2Cu2ZnR | 10 | 12 | 16 |

The PRC diagram of the aquatic fungal community clearly showed that sporulating fungal species were affected by metal exposure (Fig. 7.1), with treatments explaining a significant part of the variance (Monte Carlo test, P=0.002). The largest deviations from the control community occurred in Cu treatments or in metal mixtures, with Cu and Zn added either together or sequentially in time (Fig. 7.1A). Of the total variance, 35% was explained by exposure time (x-axis of the

PRC), 36% was explained by metal treatment (y-axis of the PRC) and 30% was attributed to differences among replicates.

The structure of fungal communities was significantly affected by metal exposure at all dates (one-way ANOVA, P≤0.00005). Until 10 days, fungal communities were negatively affected by Cu alone or in mixtures (Dunnett's test, P≤0.0005), and communities were still affected by those treatments after 15 days (Dunnett's test, P≤0.002). After 20 days, only metal mixtures affected the aquatic fungi (Dunnett's test, P≤0.02), while at later times communities were affected by all metal treatments, except by Zn (Dunnett's test, P≤0.03).

Fungal communities exposed to 1Cu, 1Cu1Zn and 1Zn2Cu were not able to recover after metal release at day 25 (one-way ANOVA, P=0.0009, Dunnett's tests, P≤0.02) (Fig. 7.1B). However, at day 30 no significant differences were found between control and treatments (one-way ANOVA, P=0.08).

*Anguillospora filiformis* had the highest positive weights followed by *Flagellospora curvula and Tricladium splendens* indicating that these species were the most negatively affected by the metals (Fig. 7.1C). Conversely, species as *Flagellospora curta* and *Lemonniera aquatica* had negative weights indicating that their sporulation ability increased after metal exposure.

At the end of the experiment, fungal diversity as number of DGGE bands appeared to be less affected by metal exposure than when assessed from sporulating species (Table 7.1). Also, bacterial diversity from DGGE fingerprint did not differ much among metal treatments (Table 7.1). However, cluster analyses of DNA fingerprints revealed that both fungal and bacterial communities were affected by metal exposure and a recovery appeared to occur after metal release (Fig. 7.2). Both fungal and bacterial communities were separated in two main clusters, one comprising metal treatments and the other including the communities released from metals. For fungi, control communities grouped with those that were released from metals, and these communities were separated from those that were under metal stress (Fig. 7.2A). Bacterial communities released from metals were separated from the remaining treatments, while control communities grouped with those exposed to metals but were separated into a different branch at 60% of similarity (Fig. 7.2B).

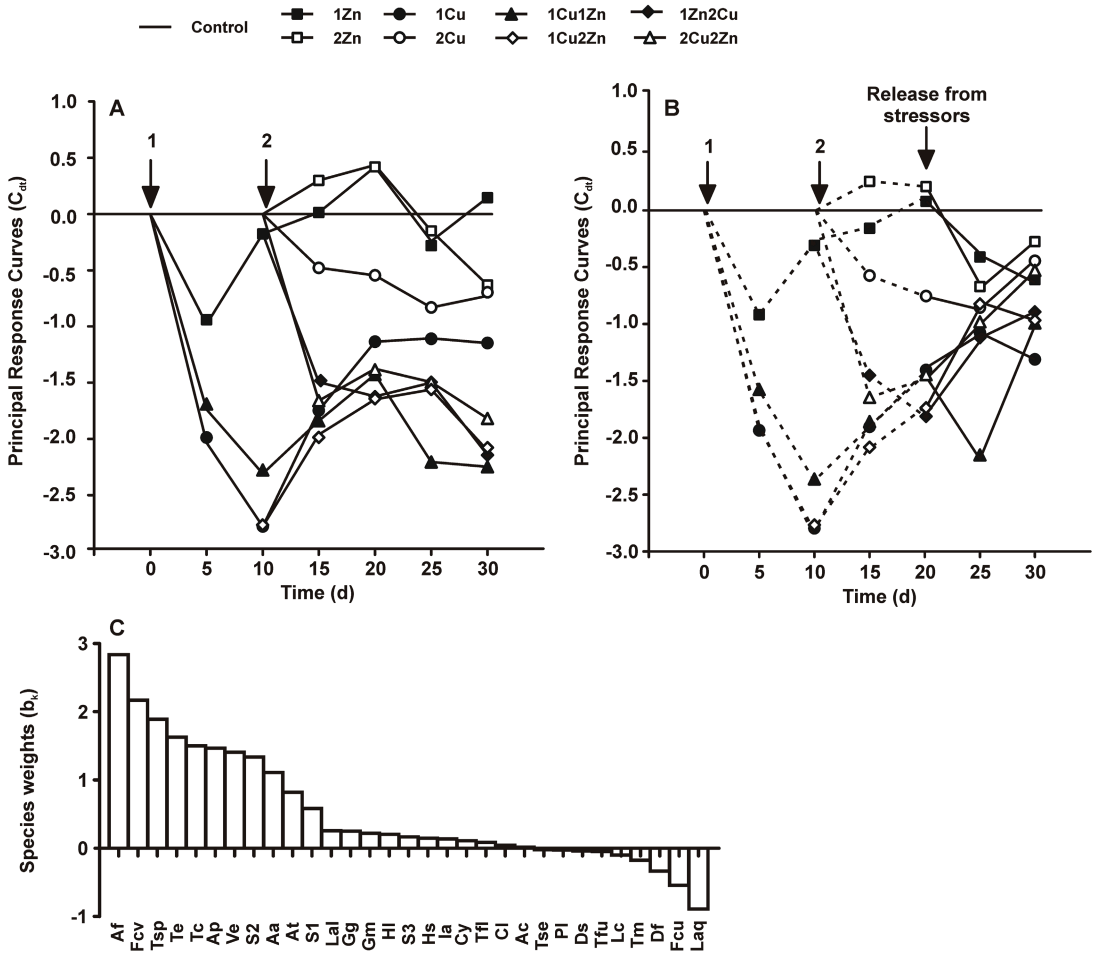


**Figure 7.1.** Principal Response Curves of Cu and/or Zn effects on the aquatic hyphomycete community, as conidial identification and counts. Communities were (A) not released from metals and (B) released from metals. Species weights (C) is a measure of the affinity of each taxon to the community response pattern observed under metal stress. The lines represent the effect of metal treatments along time. Fungal taxa: Af, *Anguillospora filiformis* Greath.; Fcv, *Flagellospora curvula* Ingold; Ts, *Tricladium splendens* Ingold; Te, *Tetrachaetum elegans* Ingold; Tc, *Tricladium chaetocladium* Ingold; Ap, *Alatospora pulchella* Marvanová; Ve, *Varicosporium elodeae* W. Kegel; S2, Sigmoid 2 (50-100/2-3 µm); Aa, *Alatospora acuminata* Ingold; At, *Articulospora tetracladia* Ingold; S1, Sigmoid 1 (30-50/2-4 µm); Lal, *Lemonniera* cf. *alabamensis* R.C. Sinclair & Morgan-Jones; Gg, *Geniculospora grandis* (Greath.) Sv. Nilsson & Nolan; Gm, *Goniopila monticola* (Dyko) Marvanová & Descals; Hl, *Heliscus lugdunensis* Sacc. & Therry.; S3, Sigmoid 3 (75-80/3-4 µm); Hs, *Heliscella stellata* (Ingold & V. J. Cox) Marvanová; Ia, *Infundibura adhaerens* Nag Raj & W. B. Kendr.; Cy, *Cylindrocarpon* sp.; Tfl, *Tricladiopsis flagelliformis* Descals; Cl, *Clavatospora longibrachiata* (Ingold) Sv. Nilsson ex Marvanová & Sv. Nilsson; Ac, *Anguillospora crassa* Ingold; Tse, *Tetracladium setigerum* (Grove) Ingold; Pl, *Pleuropedium* sp.; Ds, *Diplocladiella scalaroides* G. Arnaud & M.B. Ellis; Tf, *Tetracladium furcatum* Descals; Lc, *Lunulospora curvula* Ingold; Tm, *Tripospermum myrti* (Lind) S. Hughes; Df, *Dimorphospora foliicola* Tubaki; Fcu, *Flagellospora curta* J. Webster; Laq, *Lemonniera aquatica* De Wild. Metals were added together or sequentially in time. Sequence of metal addition: 1 – added at 0 d, and 2 – added after 10 d. The arrows indicate the time of metal additions. Dashed lines in B indicate the response of communities before metal release.

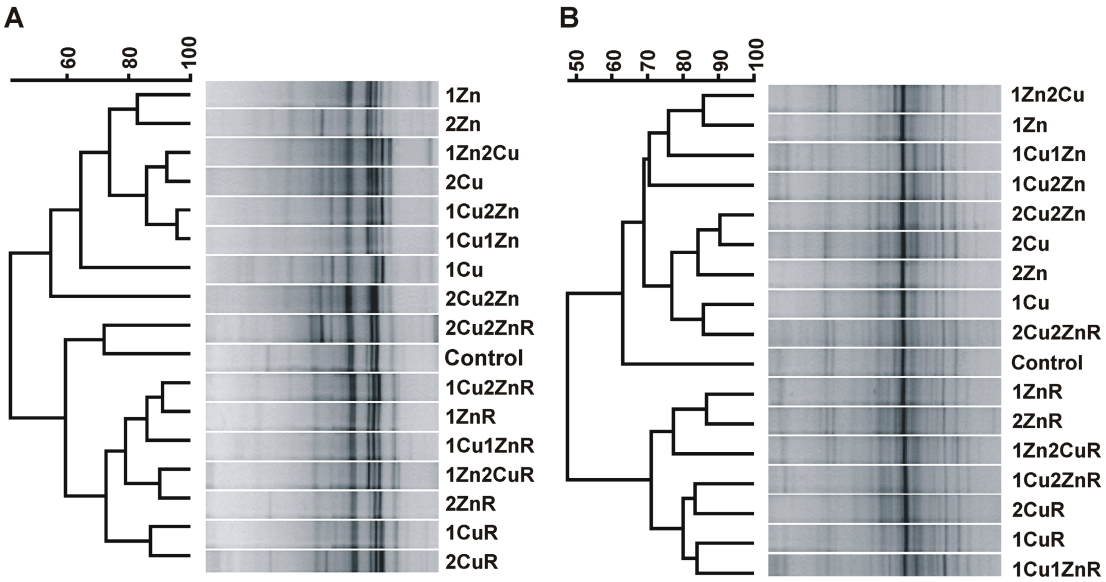


**Figure 7.2.** DGGE fingerprints and cluster dendograms of fungal (A) and bacterial (B) communities exposed to Cu and/or Zn for 30 days in microcosms. Dendograms were constructed from UPGMA analysis based on the Dice coefficient of similarity. Metals were added together or sequentially in time. Sequence of metal addition: 1 – added at 0 d, and 2 – added after 10 d, R microcosms released from metals.

### *7.3.2. Functional stability*

Copper, Zn and metal interactions affected leaf mass loss, bacterial biomass and fungal sporulation rate, with stronger effects in microcosms with metal mixtures (nested ANOVA, $P \leq 0.01$), while the sequence of metal addition significantly affected fungal biomass and sporulation (nested ANOVA, $P < 0.05$) (Fig. 7.3, black bars).

Leaf mass loss ranged from 52% (1Cu1Zn) to 78% (control) and was significantly reduced by metal exposure (Dunnett's test, $P < 0.05$), except for 2Zn and 2Cu (Dunnett's test, $P > 0.05$), (Fig. 7.3A). In control microcosms fungal sporulation rate was 1.1 x $10^5$ conidia $g^{-1}$ leaf dry mass $d^{-1}$ and it was 6 to 16-times decreased in metal mixtures (Dunnett's test, $P < 0.05$), except for 1Cu1Zn (Dunnett's test, $P > 0.05$) (Fig. 7.3B). Fungal biomass varied from 6.2 (2Cu2Zn) to 35.4 mg $g^{-1}$ leaf dry mass (1Cu2Zn), with lower values in the 2Cu2Zn treatment (Dunnett's test, $P = 0.007$) (Fig. 7.3C). Bacterial biomass was lower in microcosms supplemented with 1Cu, 1Cu1Zn, 1Zn2Cu and 1Cu2Zn (Dunnett's test, $P < 0.05$), with values ranging from 0.04 (1Cu1Zn) to 0.5 mg $g^{-1}$ leaf dry mass (control) (Fig. 7.3D).

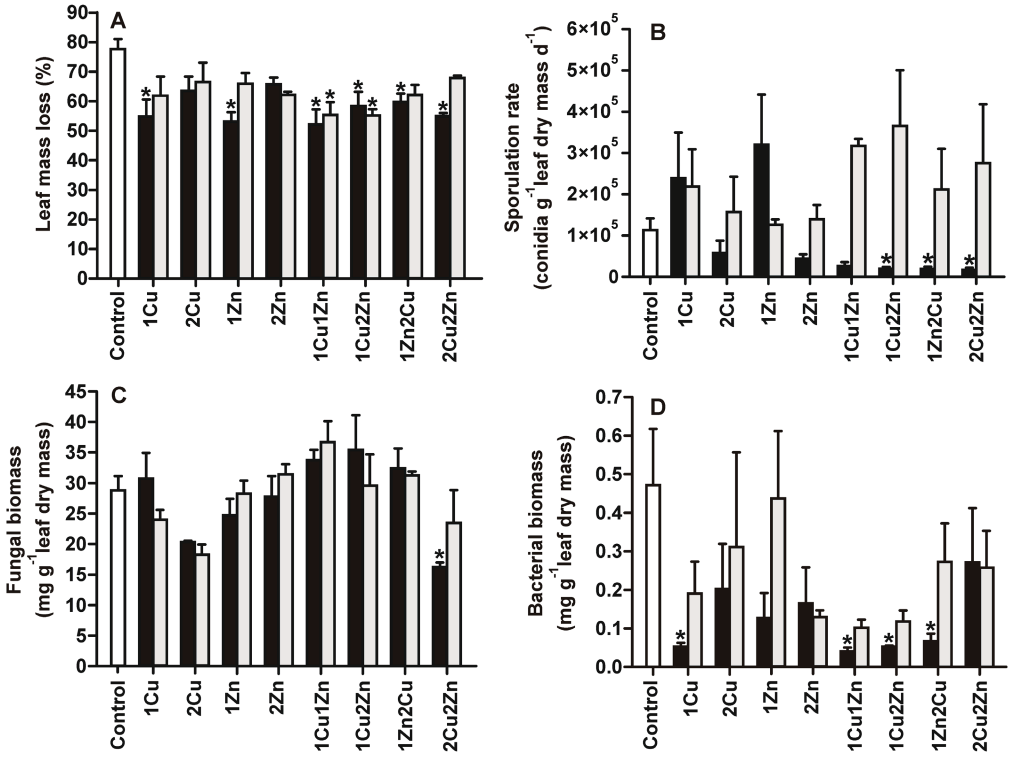


**Figure 7.3.** Effects of Cu and/or Zn on leaf mass loss (A), fungal sporulation (B), fungal biomass (C) and bacterial biomass (D). Metals were added together or sequentially in time. Sequence of metal addition: 1 – added at 0 d, and 2 – added after 10 d in microcosms. Black bars indicate metal treatments, grey bars indicate microcosms released from metals. Mean ± SEM, n=3; *, treatments different from control (P<0.05).

Generally, the release from stressors after 20 days of metal exposure led to an increase in leaf mass loss, bacterial biomass and fungal sporulation (Fig. 7.3, black bars *versus* grey bars). Leaf mass loss (Fig. 7.3A) varied between 55.2% (1Cu2ZnR) and 67.8% (2Cu2ZnR), but treatments with metal mixtures in which Cu was added at the beginning of the study (1Cu2ZnR and 1Cu1ZnR) did not attain leaf mass loss of control microcosms (78%) (Dunnett's test, P=0.006, for both comparisons). Fungal sporulation (Fig. 7.3B) varied between 1.2 x $10^6$ (1ZnR) and 3.7 x $10^6$ conidia $g^{-1}$ leaf dry mass $d^{-1}$ (1Cu2ZnR) in microcosms under recovery and no significant differences were found comparing with control (1.1 x $10^6$ conidia $g^{-1}$ leaf dry mass $d^{-1}$) (Dunnett's test, P>0.05). Fungal biomass (Fig. 7.3C) varied from 18 (2CuR) to 36.7 mg $g^{-1}$ leaf dry mass (1Cu1ZnR), while bacterial biomass (Fig. 7.3D) varied from 0.1 (1Cu2ZnR) to 0.4 mg $g^{-1}$ leaf dry mass (1ZnR), and no differences were also found relatively to control microcosms (28.7 mg $g^{-1}$ leaf dry mass and 0.5 mg $g^{-1}$ leaf dry mass, for fungal and bacterial biomass, respectively) (Dunnett's test, P>0.05).

## 7.4. Discussion

In human-impacted ecosystems, loss of biodiversity and/or alterations in community structure are expected to occur but the consequences for ecosystem functioning are not fully understood yet (Hooper *et al.* 2005). In the current study, DNA fingerprints of microbial communities indicated that more than diversity, the structure of fungal and bacterial communities was affected by metal exposure. Great similarity was found between metal-exposed communities which differed from control communities. Similar evidences were found in aquatic systems (chapter 6), while in soils, metal pollution did not markedly alter the pattern of the bacterial DGGE profiles (Kandeler *et al.* 2000). However, long-term exposure to metals was reported to alter both community structure and diversity of bacteria in soils (Müller *et al.* 2001, Li *et al.* 2006).

On the contrary, the number of fungal sporulating species was severally reduced after exposure to metal mixtures probably because its reproductive activity was very sensitive to metal stress, as previously found (Sridhar *et al.* 2001, 2005, Niyogi *et al.* 2002, Duarte *et al.* 2004, chapter 6). Since species that have their ability of dispersion compromised will tend to disappear, the consequences for ecosystem functions may be dramatic at longer times. In our study, *Anguillospora filiformis* was the most affected species, while *Lemmoniera aquatica* and *Flagellospora curta* increased their contribution in some metal treatments. *F. curta* has been found in polluted streams of the Northwest of Portugal (Pascoal *et al.* 2005a,b) and an isolate of this species was tolerant to concentrations of Cu and Zn higher than those used in this study (see Guimarães-Soares *et al.* 2006, 2007).

When aquatic organisms are exposed to multiple stressors their combined effects may vary with the type and sequence by which they are released to the environment (Giller *et al.* 2004, Vinebrooke *et al.* 2004). In the current experiment, both Cu and Zn affected all microbial parameters with the exception of fungal biomass, but stronger effects were found in the presence of Cu. In addition, the sequence by which metals were added to microcosms affected fungal biomass and sporulation. Copper has been reported to be more toxic than Zn to several species of aquatic hyphomycetes (Azevedo *et al.* 2007, Guimarães-Soares *et al.* 2007) and to microbial communities on decomposing leaves (chapter 6). It is conceivable that if Cu is more toxic than Zn and if it is added for longer times, it will have a more deleterious effect than if the less toxic metal was added first and this might be the reason why the sequence of metal addition affected fungal parameters.

In our study, the acclimation to a first metal did not seem to increase the resistance of the microbial community to the second metal. Microbial decomposers could be expending considerable energy to maintain their functions under the stress imposed by the first metal and, if so, species resistance might be diminished when the second metal was added. In soils, the functional performance of bacteria was generally unaltered by a transient disturbance, but it was lowered by an additional disturbance in the presence of a permanent one (Müller *et al.* 2002). An increase in system resistance may also not be found if the different stressors antagonistically affect different sets of species (Giller *et al.* 2004).

After release from metals, the structure of fungal communities became similar to that of control, as indicated by the principal response curves of sporulating species and also by the DGGE analyses. Fungal sporulating diversity also increased to levels similar to those of control. These findings give support to results from DGGE analysis, suggesting that the original number of fungal species was not much affected by metal treatments. This encourages the use of molecular techniques for assessing fungal diversity since they do not rely on the ability of fungi to sporulate.

A shift in the structure of bacterial communities was also found after metal release but communities still differ from the control community. This might be due to the high sensitivity of bacterial decomposers to metals as indicated by the strong inhibition of biomass production in this and previous studies in both aquatic (chapter 6) and terrestrial systems (Kandeler *et al.* 2000, Müller *et al.* 2001, Khan and Sculion 2002).

A recovery in leaf mass loss, bacterial biomass, fungal biomass and sporulation seemed to occur, as suggested by the lack of differences between control and metal-released microcosms. Leaf mass loss did not recover in only two metal combinations, in which fungal sporulating diversity was reduced and the community structure strongly affected. We should point out that the recovery period was only of ten days and if we have extended this period, a more pronounced recovery might have been noticed. However, studies conducted in soils indicated that changes in the structure of microbial communities due to Cu exposure led to losses of functional stability (Griffiths *et al.* 2004), suggesting that species composition influences community function and stability at the same extent as species diversity. In addition, bacterial diversity in soils was able to attain its original value following a disturbance (tylosin treatment), but changes in community structure were permanent (Westergaard *et al.* 2001). However, the effect of a disturbance on microbial community function depends on its duration and specificity.

Theoretically, after a transient disturbance, system function may eventually return to its former state, whereas permanent disturbance will result in a new altered state (Rykiel 1985). In soils, some experimental evidence revealed this tendency (Griffiths *et al.* 2000). No resilience was observed in soils chronically exposed to Cu, but soils exposed to a transient heat stress showed a clear trend of resilience (Griffiths *et al.* 2000).

Overall, results indicate that metals decreased microbial activity on decomposing leaves, with stronger effects observed for Cu alone or in mixtures. The resistance of microbial decomposers to Cu did not increase when communities were previously acclimated to Zn and vice-versa. However, the sequence of metal exposure matters, probably because microbial sensitivities to the two metals were different. It is conceivable that some species are more affected by metals than others, explaining the shifts in species composition and dominance. It is also possible that microbial decomposers were directing energy for defending themselves from the injurious effects of metals, rather than using it for reproduction or leaf decomposition. However, a recovery of microbial activity occurs after metal release. Further studies should be conducted to clarify the effects of sequential exposure to stressors, including the interactions of Cu or Zn with other metals or other stressors often found in aquatic systems.

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# Chapter 8

*General discussion and future perspectives*

## General discussion and future perspectives

Human activities are greatly threatening biodiversity and altering ecosystems at a worldwide scale (Vitousek *et al.* 1997). Although freshwaters are of the most endangered ecosystems in the world (Dudgeon *et al.* 2006), studies concerning the relationship between aquatic biodiversity and ecosystem functioning started to emerge only five to eight years ago (Covich *et al.* 2004). Microorganisms drive ecosystem processes to a considerable extent; for instance, microbial decomposers, namely bacteria and fungi, are recognized to play an important ecological role in organic matter turn-over in streams improving plant-litter palatability for invertebrate consumption. Nevertheless, the impacts of loosing microbial decomposer species are poorly documented in aquatic systems.

A tractable system to study the effects of microbial species loss on stream processes, as leaf-litter decomposition, can be provided by manipulating species of aquatic fungi (Bärlocher and Corkum 2003, Dang *et al.* 2005, Treton *et al.* 2006, Raviraja *et al.* 2006). The studies conducted so far gave some controversial results (see Pascoal and Cássio 2008 for a review); however, fungal diversity seemed to be important for litter decomposition in freshwaters (Bärlocher and Corkum 2003, Treton *et al.* 2004, Raviraja *et al.* 2006) and the process is less variable when diversity is high (Dang *et al.* 2005). Evidences also confirm that diversity effects among aquatic fungi are highly context dependent (Bärlocher and Corkum 2003, Raviraja *et al.* 2006).

In this work, by manipulating monocultures and all possible combinations of 1 to 4 aquatic hyphomycete species common in streams of the Northwest of Portugal, we found that fungal biomass and reproduction were affected by species diversity (chapter 2). Observed performances in multicultures were also higher than those expected from the sum of individual performances, suggesting complementarity effects among fungal species for biomass buildup and leaf decomposition. This was expected since different fungal species probably colonize different parts of the leaves (resource partitioning) and produce different arrays of extracellular enzymes (Suberkropp *et al.* 1983) leading to a better exploitation of the resources. However, since it was not possible to date to measure the individual species contribution to the decomposition process, a sampling effect cannot be excluded when explaining these positive effects (Hector 1998). Moreover, the current study provided evidences for the importance of keystone species, since all measured parameters

were affected by species identity. Indeed, fungal biomass and leaf mass loss were lower in the absence of *Articulospora tetracladia*.

Another important benefit of biodiversity is that it can help to buffer environmental variability and to maintain ecosystem processes, because greater diversity will increase the probability of a community to contain species that respond differently to stress (Yachi and Loreau 1999, Loreau *et al.* 2002). Although the loss of fungal species has been associated with several anthropogenic disturbances, it was not always accompanied by changes in leaf decomposition rates (e.g. Au *et al.* 1992a,b, Raviraja *et al.* 1998, Lecerf *et al.* 2005). In this work, we found slower leaf decomposition, fungal biomass, sporulation and diversity at an impacted site of the Este River, with high levels of nutrients and metals, than at a reference site of the same stream (chapter 3). The transference of leaves colonized at the reference site to the polluted site did not alter leaf decomposition rate or fungal biomass, but affected the structure of fungal community and reduced diversity and sporulation on decomposing leaves (chapter 3). On the other hand, bacterial biomass was higher at the downstream-polluted site and was enhanced after transplant, although never surpassing fungal biomass. These findings reinforce that fungi are the main microbial decomposers (Pascoal and Cássio 2004, Pascoal *et al.* 2005a) and that some functional redundancy may occur between aquatic fungi (Pascoal *et al.* 2005b). If so, the loss of sensitive species would be compensated by the more tolerant ones after a disturbance event (Walker 1992).

Fungal sporulation is often found to be very sensitive to pollution (Au *et al.* 1992b, Sridhar *et al.* 2001, 2005, Niyogi *et al.* 2002, chapter 3), therefore, the observed low fungal diversity on decomposing leaves in stressed streams can be a consequence of assessing diversity by identifying spores released from leaves (Suberkropp 1998, Bärlocher 2005). Bacterial diversity has been assessed by identifying cellular morphotypes (Suberkropp and Klug 1976, Baldy *et al.* 1995, 2002) or cultivable genera isolated from leaves (Suberkropp and Klug 1976). Since only 1 to 15% of bacteria are reported to be cultivable and identifiable by traditional methods (Torsvik *et al.* 1990, Amann *et al.* 1995), such approaches do not give enough information on the actual diversity. DNA fingerprinting of microbial communities, such as from denaturing gradient gel electrophoresis (DGGE) (Nikolcheva *et al.* 2003, 2005, Nikolcheva and Bärlocher 2005) or terminal restriction fragment length polymorfism (T-RFLP) (Nikolcheva *et al.* 2003); have been used to better characterize microbial communities. However, these techniques strongly rely on PCR and the choice of the primers is a critical step. In this work (chapter 4),

DGGE fingerprints of DNA, amplified with two different primer pairs proved to be useful to detect shifts of both bacterial and fungal communities during leaf decomposition. However, a high diverse fungal community was detected with the primer pair ITS3GC/ITS4, targeting the ITS2 region of rDNA, than with the primer pair NS1/GCfung targeting the 18S rDNA. This was expected since ITS region is reported to have higher level of variation than 18S rDNA or portions of it, which may not contain the necessary variation to distinguish between closely related taxa (Kowalchuk and Smit 2004). Previous results with soil bacterial communities gave comparable number of bands when typing bacterial communities with two different primer pairs (one targeting the V2-V3 and the other the V6-V8 regions of 16S rDNA, Schmalanberger *et al.* 2001).

In the current study, we examined leaf decomposition and the associated-microbial decomposers along a gradient of inorganic phosphorus and nitrogen at five stream sites in the Northwest Portugal (chapter 5). Results from both microscopic identification of fungal conidia and DGGE of fungal and bacterial DNA showed that the structure of microbial communities was altered and differences in inorganic nitrogen concentrations between the sites were responsible for such shifts. Fungal sporulating diversity was clearly depressed at the most eutrophic sites (Este 3 and Souto), probably as a direct consequence of sporulation being reduced. Fungal biomass was also reduced at the most polluted sites and bacterial biomass appeared to be stimulated. However, bacterial biomass was depressed at the most polluted site (Souto), probably because of high levels of ammonium and nitrites, which may counteracted the positive effects of nitrate and phosphorus. Although several studies have reported positive effects of inorganic nutrients on microbial diversity and activity on decomposing leaves (Pascoal *et al.* 2003, 2005a,b, Gulis and Suberkropp 2003a,b, 2004, Ferreira *et al.* 2006, Gulis *et al.* 2006, Castela *et al.* 2008), some evidences suggest that the presence of other stresses, such as low oxygen concentrations and sedimentation (Pascoal *et al.* 2005a, Mesquita *et al.* 2007), can surpass the positive effects of nitrate and phosphorus. Moreover, higher concentrations of ammonium in the stream water reduced decomposition rates through inhibition of invertebrates when no limiting concentrations of nitrate and phosphorus were present in the stream water (Lecerf *et al.* 2006, Baldy *et al.* 2007).

Other pollutants, in particular metals, are of major concern mainly because their non-degradability and persistence in the environment (Gadd 1993). High concentrations of metals have been found in some streams of the Northwest of Portugal (Gonçalves 2001). Metal pollution in the stream water is reported to

strongly inhibit leaf decomposition (Bermingham *et al.* 1996, Sridhar *et al.* 2001, 2005, Baudoin *et al.* 2008), fungal diversity and reproduction (Maltby and Booth 1991, Sridhar *et al.* 2001, 2005, Bermingham *et al.* 1996, Baudoin *et al.* 2008), while fungal biomass has not always been affected (Niyogi *et al.* 2002, Baudoin *et al.* 2008). In laboratory experiments, high zinc concentrations (up to 150 µM) also inhibited leaf decomposition, fungal reproduction and diversity (Duarte *et al.* 2004), while copper (up to 1.2 µM) reduced leaf decomposition, but not sporulation of aquatic hyphomycetes (Roussel *et al.* 2007). In both experiments, fungal biomass was not negatively affected by metal exposure (Duarte *et al.* 2004, Roussel *et al.* 2007).

Even though metals in streams are frequently found in mixtures, the effects of metal mixtures on aquatic microbial decomposers and plant-litter decomposition are practically inexistent. In this work, we studied the effects of Cu and Zn mixtures on aquatic microbial decomposers and leaf-litter decomposition in microcosms. In chapter 6, we show that metals (up to 100 µM), especially in mixtures, strongly inhibited leaf decomposition and fungal reproduction, and led to shifts in both fungal and bacterial communities assessed from conidial counts and DNA fingerprinting. Bacterial biomass was severally reduced by metal exposure, but fungal biomass remained unaltered or even increased as found by others (Niyogi *et al.* 2002, Duarte *et al.* 2004, Baudoin *et al.* 2008). The effects of Cu and Zn were mostly additive for microbial decomposition of leaf litter, while antagonistic effects were found on bacterial biomass in all metal combinations and on fungal sporulation in metal combinations with the highest Cu concentrations.

It has been reported that aquatic hyphomycetes show different sensitivities to metals, and Cu appear to be more toxic than Zn (Azevedo *et al.* 2007, Guimarães-Soares *et al.* 2006, 2007). Accordingly, we found that Cu was more toxic than Zn to microbial decomposer communities and leaf-litter decomposition (chapter 6 and chapter 7). Moreover, the sequence of metal addition affected microbial communities, probably because the susceptibility of microbial species within the community to the two metals might be different. Moreover, we did not find increased resistance to a second metal addition when the communities were previously acclimated to a first metal (chapter 7). Probably, microbial decomposers were expending considerable energy to face the stress imposed by the first metal and thus had their resistance diminished when the second metal was added. Indeed, in several aquatic fungal species, the exposure to metals led to an increase in the production of thiol-compounds able to sequester toxic metals (Miersch *et al.* 1997,

2001, 2005, Guimarães-Soares *et al.* 2006, 2007, Jaeckel *et al.* 2005, Braha *et al.* 2007) and in the activity of antioxidant enzymes able to deal with metal-induced oxidative stress (Azevedo *et al.* 2007, Braha *et al.* 2007).

The release of microbial communities from metal stress led to the reestablishment of functions, with some few exceptions, and the structure of fungal and bacterial communities became similar to that of control (chapter 7). Metal stress decreased fungal reproduction much more than diversity, as shown by DNA fingerprinting and a recovery in fungal diversity after metal release was found. Metal exposure induced shifts in the structure of both fungal and bacterial communities, which appeared to decrease the activity of microbial decomposers (chapters 6 and 7). Previous studies conducted in soils also point that rather than diversity, shifts in the community structure due to a disturbance can affect ecosystem functioning to a great extent (Griffiths *et al.* 2000, 2004).

Overall, our results suggest that fungal diversity matters for leaf decomposition in streams, with species identity being of major importance (chapter 2). However, it is also known that there are shifts between aquatic fungal species during litter decomposition (Suberkropp 1998, Bärlocher 2005) and that season and available resources can influence species composition on leaves (Nikolcheva and Bärlocher 2005). Moreover, microbial activity and leaf decomposition are strongly dependent on external factors such as pH, temperature, nutrient availability and anthropogenic stressors in the stream water (Alan and Castillo 2008). Having this in mind, different spatial and temporal scales should be analysed since the relationship between biodiversity and ecosystem functioning is expected to be dynamic and to change over space and time (Cardinale *et al.* 2000). Thus, future research should focus on studies across different spatial and temporal scales and simulations of species loss should also follow the sequences in which they are predicted to disappear in response to anthropogenic disturbances (Jonsson *et al.* 2002). The interaction with higher trophic levels should also be tested since fungal species identity is reported to strongly affect invertebrate consumption of leaf litter (Graça *et al.* 1993a,b, Lecerf *et al.* 2005). Molecular techniques such as *in situ* hibridization (Baschien *et al.* 2001, McArthur *et al*. 2001) and quantitative real time PCR (Manerkar *et al.* 2008) using specific probes at the species level may allow the quantification of DNA of specific species and this might help to better understand the role of particular fungal species during plant-litter decomposition in streams.

Both types of pollution (nutrient enrichment and metals) appeared to lead to great changes in the structure of microbial communities and leaf decomposition

(chapters 3, 5, 6 and 7), highlighting that, besides diversity, community composition can also be very important for the functioning of aquatic ecosystems. Molecular techniques, that do not rely on the ability of fungi to sporulate or of bacteria to grow on culture media, also confirm that shifts in the structure of microbial communities occurred during time of decomposition in streams under stress (chapter 5) and especially after metal exposure (chapter 6 and 7). Although data from DNA fingerprints suggest that both fungal and bacterial diversity was not much affected by eutrophication and metals (chapter 5, 6 and 7), we did not evaluate whether all species were active. Therefore, community profiles derived from reverse transcription-PCR of portions of 16rRNA or 18S rRNA (Torsvik *et al.* 1996, Felske and Akkermans 1998, Girvan *et al.* 2004) should be conducted to better understand the relationships between diversity of microbial decomposers and their functions in freshwater ecosystem.

Since microbial diversity on decomposing plant litter in streams is still much uncharacterized, some efforts should be done to overcome this limitation. The sequencing of aquatic bacterial and fungal decomposers and the construction of clone libraries (Bärlocher *et al.* 2007, Seena *et al. in press*) have been recently used to fulfil some lacunas, namely in the succession of microorganisms during decomposition and for detecting shifts in species composition after disturbances. These tools provide only estimations of the species present in the communities and little information about cell metabolism or ecosystem functioning (Dorigo *et al.* 2005). The development of microarrays, which allow simultaneous detection of thousands of genes in small samples and the analysis of their expression/repression under different environmental contexts, will greatly contribute to a better understanding of the role of microbes in freshwaters. Since genome sequences will have to be known *a priori*, a lot of work is still needed. However, microarrays have been successfully applied in the detection of microorganisms (Small *et al.* 2001) and in monitoring gene expression patterns in mixed microbial communities in wastewaters (Dennis *et al.* 2003)

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