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# The importance of fungal richness on leaf decomposition in salt-enriched streams: a microcosm study

Janine Pereira da Silva

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# The importance of fungal richness on leaf decomposition in salt-enriched streams: a microcosm study

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia, realizada sob a orientação científica da Professora Doutora Cristina Maria M. Monteiro Leal Canhoto (Universidade de Coimbra) e co-orientação do Professor Doutor Felix Bärlocher (Mount Allison University, Canada).

Janine Pereira da Silva

2015

*Ao meu pai-tio,*

Foi e deixou o silêncio. No ar, no balcão da cozinha, no gume da faca com que me ensinou a picar cebola, nas tralhas perdidas no fundo das gavetas, a ladear os móveis e as pinturas e a refletir-se nas paredes.

Deixou o vazio. A preencher o lugar à mesa, o banco do condutor, os sapatos debaixo da cama e as camisas no armário, as alianças de casamento e bodas de prata e um pedaço do meu coração.

Deixou espaço a mais. Como se fosse suposto abrir um novo mar, onde não chove senão lágrimas. Como se nós as tivéssemos que chorar todas antes de a tristeza acabar e não houvesse forma da maré por fim encher.

O relógio da cabeceira está parado. O tempo ficou assim, diferente, passa de uma forma estranha. Às vezes não sei se passaram dias ou meses, se sonhei enquanto dormia ou se estava acordada, se já foi mesmo ou se ainda aqui está. É então que se ergue a imagem dos estranhos homens de gravata preta dentro da nossa casa, os que o levaram suspenso em quatro tábuas. Os vizinhos no *hall*, a vê-lo sair. É então que sinto a sua testa gélida contra os meus lábios e me lembro que nunca voltará.

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## RESUMO

A relação entre a diversidade e a função dos ecossistemas é, cada vez mais e pela sua controvérsia, objeto de discussão entre a comunidade científica. Particularmente em ecossistemas ribeirinhos, alvo da ação das mais diversas ameaças, o reconhecimento da sua relevância é ainda escasso e inconclusivo. Neste trabalho, foi investigada a importância da biodiversidade de hifomicetes aquáticos no processo de decomposição da folhada em meios contaminados com sal (NaCl). Para o efeito, e utilizando microcosmos, foi avaliada a decomposição foliar por comunidades fúngicas com 3 níveis de riqueza específica (1, 4 e 8 espécies) sob o efeito de 3 níveis de salinidade (0, 8 e 16 g/L), sendo que diferentes réplicas eram compostas por comunidades distintas, com vista a anular o efeito da identidade.

A salinidade afectou as taxas de decomposição foliar, havendo um decréscimo significativo na perda de massa com o aumento da concentração de NaCl. A riqueza específica não teve efeito na perda de massa. Considerando os parâmetros que descrevem a atividade fúngica, a respiração (consumo de $O_2$) foi influenciada pela salinidade, mas não pela riqueza em espécies, contrariamente à produção de biomassa que não foi afetada por nenhum dos dois fatores. Relativamente à reprodução, a produção de conidia foi inibida pela salinidade.

A ausência de um expectável efeito positivo da diversidade na decomposição poderá dever-se a uma redundância funcional entre espécies - os resultados mostram que um número reduzido de espécies (entre 1 e 4) seria suficiente para manter a função do ecossistema.

As consequências dos processos de salinização, cada vez mais frequentes em ecossistemas ribeirinhos como resultado de alterações climáticas e acções antropogénicas, parecem resultar numa perda de biodiversidade fúngica, comprometendo a bioreciclagem de nutrientes e o funcionamento dos ecossistemas ribeirinhos.

Contudo, devemos proceder com precaução ao concluir acerca do ecossistema como um todo. Os efeitos da salinidade podem alterar-se com fatores ambientais, outros factores de stresse que atuem simultaneamente, ou até mesmo a composição ou riqueza específica das comunidades fungicas.

## ABSTRACT

The diversity and ecosystem functioning relationship has been progressively appearing as subject of discussion and controversy among the scientific community. Particularly in freshwater ecosystems, which are one of the most important targets to several environmental threats, the knowledge on this matter is yet lacking or inconclusive. The current study evaluated the importance of aquatic hyphomycetes diversity on leaf litter decomposition process, when subjected to salinization. In order to understand that, in a microcosm study, we analysed leaf litter decomposition by fungal communities with 3 levels of species richness (1, 4 and 8 species) under the effect of 3 levels of salinity (0, 8 and 16 g/L), in which each replicate was composed by different communities, to eliminate identity effects.

Salinity affected leaf litter decomposition rates, for there was a significant decrease in mass loss with increasing NaCl concentration. Species richness did not show any effect in mass loss. Considering fungal activity descriptors, respiration ($O_2$ consumption) was influenced by salinity, but not by fungal richness, contrarily to biomass production which was not affected by neither one of the two factors. Regarding reproduction, conidial production was inhibited by salinity.

The inability to find a positive effect of diversity on decomposition may be related with species functional redundancy – results show that a few species (between 1 to 4) would be enough to keep ecosystem functioning.

The consequences of salinization processes, increasingly happening in freshwater ecosystems as a result of climate and anthropogenic actions, may

result in the loss of fungal diversity, compromising nutrient recycling and the functioning of freshwater ecosystems.

Nevertheless, we should not carelessly extrapolate conclusions to the whole ecosystem. Salinity effects may vary according to environmental variables, other types of stress factors acting at the same time and even microbial communities richness and composition.

# INDEX

# CHAPTER 1

# - Introduction -

# 1. INTRODUCTION

## 1.1. Freshwater ecosystems

A river system, in which about 85% of the total length of the running water are low order streams, is a large network of smaller tributaries coalescing into larger rivers (Anderson & Sedell 1979). Here we focus on small streams ecosystems, where the ratio of shoreline to stream bottom area is high and the close canopy creates a heterotrophic environment. Allochthonous organic matter incoming from the surrounding terrestrial area constitutes the main form of reduced carbon compounds in these systems (Fisher & Likens 1972; Webster & Benfield 1986). Thus, there is a crucial linkage between terrestrial and aquatic environments to maintain stream productivity (Anderson & Sedell 1979; Abelho 2001; Canhoto & Graça 2006).

The organic matter from the riparian zone acts as the main fuel to the aquatic food webs. In temperate climates the peak of the organic matter input occurs mostly during autumn (73% of annual input; Abelho 2001). This litterfall includes several plant parts such as leaves and leaf fragments, floral components, bark, wood (branches and twigs), cones, nuts and fruits. However, leaves comprise the largest component – 41 to 98% of total litterfall. Small temperate streams are characteristically retentive - 60 to 70% of the annual inputs are retained long enough to allow colonization by stream microorganisms and consumption by macroinvertebrates (Anderson & Sedell 1979; Abelho 2001; Canhoto & Graça 2006).

As soon as the leaf litter reaches the stream water, the aquatic decomposition process begins. Organic matter is incorporated in the biomass of heterotrophs or metabolized to $CO_2$, and in that process coarse particle organic matter (CPOM; > 1 mm diameter) is transformed into dissolved organic matter (DOM; < 0.45 μm) and fine particle organic matter (FPOM; 0.45 μm – 1 mm (Abelho 2001; Hieber & Gessner 2002; Canhoto & Graça 2006).

Leaf litter degradation is a key ecosystem-level process consisting of three different phases: leaching, conditioning and (physical and biological) fragmentation that, acting as a whole, explain the overall leaf mass loss in small lotic systems (Gessner *et al.* 1999).

Leaching corresponds to the release of soluble compounds, which include nitrogen, phosphorous, magnesium, potassium and polyphenols (Abelho 2001). This leads to a mass loss of up to 4 – 42% within the first 24 to 48 hours after leaves' immersion (Gessner *et al.* 1999; Abelho 2001). Subsequently, the leaf litter nutritional quality is enhanced by the accumulation of microbial biomass (fungal mycelia and bacteria) in a process named conditioning (Suberkropp *et al.* 1976; Gessner 1999). Aquatic hyphomycetes, a particular group of fungi, assume the major role in leaf litter breakdown, especially in the early stages of decomposition (Hieber & Gessner 2002). Fungal hyphae have a high nutritional value - 2 to 4 times as many digestible nutrients per unit mass than unconditioned leaf material - and a high surface-to-volume ratio that favours nutrient immobilization, namely nitrates, by the increased contact with the environment (Canhoto & Graça 2008). Plant tissue is converted into microbial biomass with consequent chemical modifications of leaf material mostly by fungal enzymes activity that cleaves plant polymers,

which makes leaves softer and facilitates leaf-consuming invertebrates, i.e. shredders (Cummins 1974), feeding activity (Bärlocher & Kendrich 1974; Suberkropp *et al.* 1976; Gessner 1999; Krauss *et al.* 2011). Fungal exoenzymes keep active in the digestive tract of most invertebrates, in order to help the breakdown of structural compounds rich in energy but difficult to digest (i.e. cellulose). For those reasons, shredders prefer to consume partly decomposed over freshly fallen leaves (Canhoto & Graça 2008). Mycelium growth may also add micronutrients, specific carbohydrates and lipids to the leaf; these compounds may be necessary for metamorphosis and reproduction of some invertebrates (Canhoto & Graça 2008).

Therefore, conditioned leaves include unchanged leaf material, partially digested leaf structural compounds, microbial biomass and its enzymes (Bärlocher & Kendrich 1974). It can be said that a leaf is fully conditioned when fungal biomass and activity peak, which can take several weeks to months (Boling *et al.* 1975). This whole process depends on the nature of the substrate, on the fungal assemblages, and on the extent of fungal colonization (Gessner *et al.* 1999; Gessner *et al.* 2010). Environmental conditions such as temperature (Chauvet & Suberkropp 1998), nutrients (Gulis *et al.* 2006), pH (Schlief & Mutz 2006) and velocity current (Ferreira *et al.* 2006b) modulate the decomposition process.

Fungi are the “driving force behind the decomposition of leaves”. Fungal biomass represents 63% to more than 99% of the total microbial biomass on decomposing leaves in streams (Canhoto & Graça 2006; Canhoto & Graça 2008; Krauss *et al*. 2011). As time goes by bacteria gain a significant role, complementing fungi but not replacing it in the decomposition process. As the

size of the particles gets smaller, bacteria become more important, but fungi assume a greater role in this process (Canhoto & Graça 2006; Abelho 2011). Aquatic hyphomycetes are a polyphyletic group of true fungi clearly adapted to life in freshwater, namely with the ability to sporulate under water. They produce large, multiradiate or sigmoid spores whose tips may be covered with sticky mucilage, which facilitates the attachment of the spore to the substrate (Suberkropp *et al.* 1976; Wong *et al.* 1998). Colonization of leaves occurs through different pathways, either by direct contact as a result of hyphal outgrowth from a previously colonized leaf touching another leaf or by detached hyphal fragments or asexual spores called conidia that land on the leaf surface. Conidia are released into the water and carried away from a few hundred meters to a few kilometres, maintaining their ability to germinate for several days and this appears to be the predominant mechanism of colonization (Sridhar & Bärlocher 1997). Conidia germinate and develop one or more germ tubes that form appressoria on the substratum, allowing a solid adherence and a subsequent penetration of the plant tissues (Dang *et al.* 2007). It is estimated that approximately 50% of total fungal production is channelled into conidia (Bärlocher & Brendelberger 2004). In average, one decomposing leaf is colonized by 5 to 12 species in natural streams (Bärlocher 1992; Dang *et al.* 2005). Estimating fungal diversity is possible either by traditional methods, including examination of sporulation structures on leaves (Bärlocher & Kendrick 1974), or on conidia released from leaves (Bärlocher 1982) or by molecular methods such as DNA extraction analysis (Das *et al.* 2008).

This group of fungi is affected by a wide range of factors that determine their abundance, development and activity. The quantity and identity of fungal

mycelia have an intrinsic relationship with the local fungal species pool, leaf litter quality and quantity, external environment characteristics (Krauss *et al.* 2011) and stream biota (Abelho 2011). Macroinvertebrate and fungal communities are strongly linked. In one hand, fungal assemblages in leaves influence the feeding behaviour and performance of some shredders. On the other hand, macroinvertebrates are able to modify the fungal communities and their function either by selective consumption of leaf patches, competition for the leaf resources or stimulation of microbial degradation trough the disruption of physical barriers of leaves and changes of basal resources (Canhoto & Graça 1999; Canhoto & Graça 2008; Gonçalves *et al.* 2013).

## 1.2. Biodiversity and Stream Ecosystem Functioning

The concept of biodiversity and the relationship between biodiversity and ecosystem functioning has been subject of discussion and controversy for the past two decades among the scientific community (Loreau *et al.* 2002).

Biodiversity is important since it determines how a species assemblage intervenes in the ecosystem processes (Reiss *et al.* 2011). Its value is related with a direct economic contribution to productivity, an insurance for future unexpected events and because it represents a genetic information storehouse for the prevalence of the ecosystem services (Dudgeon *et al.* 2005). Therefore, the currently concern related with the accelerated loss of biodiversity is due to the harmful effect it can have on the ecosystem functions and services (Yachi & Loreau 1999; Bärlocher & Corkum 2003; Krauss *et al.* 2011). In the loss of biodiversity we include all the changes that reduce or simplify biological

heterogeneity (Walker 1992). Nowadays, this is mainly caused by the action of stressors, which in nature rarely act alone. However, is not yet known whether few or many species are required to bear ecosystem functioning under the action of one or multiple stressors (Perkins *et al*. 2014).

Performed research points to a positive effect of biodiversity in the functioning of the ecosystem, across a wide spectrum of organisms and systems (Gessner *et al* 2010; Reiss *et al.* 2011; Cardinale *et al.* 2012). It is assumed that a higher biodiversity will ensure a more stable supply of ecosystem goods and services as variability increases due to environmental fluctuations (Pascoal *et al.* 2010; Yachi & Loreau 1999). Several non-exclusive hypotheses are now trying to clarify this crucial relationship: initially, it was thought that if the number of species in a community increases, the stability also increases, linearly, through an improvement in the flow of energy in the food web, which is described by the *Linear or diversity-stability hypothesis* (MacArthur 1995). Other authors consider that this relationship is positive but not linear. So, the system is somewhat negatively affected by species loss until a certain point, where it may even crumbles (Ehrlich & Ehrlich 1981). The *redundancy hypothesis* proposes that in a community where the functional groups are well represented, the removal of species will be compensated by the remaining redundant species. Therefore, there is a positive relationship between species richness and function (Walker 1992). However, this hypothesis becomes more complex when a group of species with the same function responds differently to environmental conditions. Consequently, it is difficult to predict whether the loss of species has a negative effect on the ecosystem function, because all depends on the environmental fluctuations, which gave

rise to the *Idiosyncratic hypothesis* (Walker 1992; Lawton 1994). Lastly, the Redundancy hypothesis and the Idiosyncratic hypothesis were coupled and the resulting *Insurance hypothesis* states that species richness may buffer the negative effects of species disappearance and enhance the ecosystem performance when facing changing environmental conditions because the probability of existence of redundant functional species increases. The productivity will be maintained to its maximum value as long as species richness is high enough for redundancy (Yachi & Loreau 1999; O'Connor & Crowe 2005).

Several studies have been performed on the influence of biodiversity on decomposition rates in streams, concerning both fungal and macroinvertebrate communities, but, at the moment, the overall outcomes have been ambiguous, although there is a propensity in believing that a species richer community has positive effects on the performance of the ecosystem (Dang *et al.* 2005; Gessner *et al.* 2010; Geraldes *et al.* 2012). An increase in the decomposition rates of leaf litter when in the presence of a richer aquatic hyphomycete assemblage was observed in numerous laboratory experiments (Bärlocher & Corkum 2003; Duarte *et al.* 2006; Pascoal *et al.* 2010; Fernandes *et al.* 2011). The positive effect has been explained by a complementarity effect, which leads to an increase in total resource use because community performance is higher than expected from the performance of individual species, either by facilitation effects or niche partitioning (Cardinale *et al.* 2002; Loreau & Hector 2011). However, on the other hand, some other studies were not able to find significant differences among diversity levels, which suggests that there is enough functional redundancy among aquatic hyphomycete species to dodge the

possible harmful effect of biodiversity loss. Fungal species identity and therefore their traits may be more important to the ecosystem than species number *per se* (Dang *et al.* 2005; Fernandes *et* al. 2011; Krauss et al. 2011). Plus, traits that determine how the ecosystem is affected by that species, that is how a species is defined in terms of its ecological role, may be different from the traits that control how that species respond to environmental fluctuations (Fernandes *et al.* 2011). Moreover, the ecosystem functioning may differ drastically depending on the identity of the species lost (O'Connor & Crowe 2005). Performed studies claimed that only species identity had an effect on leaf mass loss (Duarte *et al.* 2006). As a matter of fact, diversity showed a small impact in decomposition, when compared with nutrient availability (Bärlocher & Corkum 2003) and several studies even verified a greater impact of biodiversity on fungal activity – such as reproduction – than on decomposition (Duarte *et al.* 2006; Pascoal *et al.* 2010; Fernandes *et al.* 2011; Geraldes *et al.* 2012). This inability to find significant impacts in decomposition when aquatic hyphomycete diversity is lower may also be caused by the experimental approaches, where the conditions are highly controlled and factors such as the substrata characteristics, the number of species and its identity and even other environmental factors are not considered (Bärlocher & Corkum 2003).

Nevertheless, we must not forget that, at the lights of the insurance hypothesis, environmental fluctuations can bring traits of some species that would be redundant under constant conditions (Fernandes *et al*. 2011).

Freshwater is a really small part of the world's water (0.01%) but it shelters almost 6% of the total species numbers, where the populations are

suffering major and troubling declines. Small streams are among the most vulnerable ecosystems in the world and, due to their position in the landscape, pose a major target to several environmental stressors (Dudgeon *et al*. 2006; Meyer *et al.* 2007).

## 1.3. The streams and the salt (NaCl) as a stressor

### 1.3.1. Salinity and its impacts

A great part of these freshwater ecosystems are subjected to a salinization process, a worldwide growing threat to biodiversity (*Freshwater Ecosystem Biodiversity management issues*, 2001; *Milleninym Ecosystem Assessment*, 2005; Piscart *et al.* 2006c; Cañedo-Argüelles *et al.* 2013; Staudt *et al.* 2013; Szocs *et al.* 2013). The term salinity refers to the total concentration of dissolved inorganic ions in the water or soil (Ziemann & Schulz 2010; Cañedo-Argüelles *et al.* 2013). The accumulation of salt may occur through natural or human influence (Munns 2013). The first, natural or primary salinity, takes long periods of time to occur and results from natural processes as weathering of rock materials with soluble salts or deposition of oceanic salt carried by the wind or rain. Secondary or human-induced salinization results from human activities and may reach freshwater ecosystems through clearing of native vegetation, agricultural irrigation, rising groundwater, mining activity, industrial discharge and by using salt (particularly NaCl) as a chemical deicing and anti-icing agent

in roads (*Freshwater Ecosystem Biodiversity management issues*, 2001; Cañedo-Argüelles *et al.* 2013; Munns 2013; Szocs *et al*. 2013).

### 1.3.2. Freshwater organisms facing salinity

Salinity may have direct and indirect effects on freshwater ecosystems, but it is not yet well known how it affects the freshwater communities (Szocs *et al.* 2013). Shredders have been the centre of investigations (e.g. Piscart *et al.* 2006) and only two studies evaluated the effects of chronic (Schäfer *et al.* 2012) and short-term pulses of salinity (Cañedo *et al.* 2014) on litter decomposition. Salinity can change the natural riparian flora that may be replaced by salt tolerant species (*Water facts* 2000). This will have an indirect effect in the whole system, as it will change the shading in the river, as well as the organic matter input, which will modify the freshwater trophic food web (*Water facts* 2000; *Biodiversity management issues* 2001). Secondary salinization also seems to lead to structural changes both on the ecosystem and the freshwater communities concerning density, species richness and functional aspects as well as alterations in physiological responses with consequences on goods and services provided to men (*Freshwater Ecosystems Biodiversity management issues*, 2001; Millennium Ecosystem Assessment, 2005; Cañedo-Argüelles *et al.* 2013; Szocs *et al.* 2013).

Generally, diversity is low in salinized streams. High concentrations of salt reduce diatom, macroinvertebrate, amphibian and fish species density (Hart *et al.* 1991; Sangiorno *et al.* 2007; Cañedo-Argüelles *et al.* 2013). Even though freshwater organisms seem resistant to a wide range of salt concentrations (Piscart *et al.* 2006c), salinities of 1 to 3 g/L have been shown to modify

macroinvertebrate assemblages with a reduction of diversity and abundance (Hart *et al.* 1991; Piscart *et al*. 2005). Even though different organisms may have a different response to salinity, most freshwater species are osmoregulators who have a metabolic cost to react to an increase in salinity. Therefore, if the salt concentration becomes too high, the osmoregulatory mechanisms will collapse because the organism went beyond his capacity of viability or resilience (Cañedo-Argüelles *et al.* 2013).

Considering fungal communities, especially aquatic hyphomycetes, little is known about the effects of salinity on the physiology, function or community structure. It is possible that, if salinity increases beyond a threshold of resilience, the osmoregulatory mechanisms collapse causing cellular damage and even death. However, there is evidence that there are differences between the fungal communities in freshwater ecosystems, when compared with sea communities or even transitional systems as estuaries (Jones 2000; Tsui & Hyde 2003). This suggests that salt concentration may contribute to change the community composition and it even may favour the presence of salt-tolerant specialized taxa (Mohamed 2011).

What is already known is that, if salinity affects the microbial community, it may have implications in its functional role – leaf litter decomposition (Hart *et al.* 1991). Thus, as salinity increases, the species richness generally decreases and the breakdown of allochthonous organic matter can be reduced (Hart *et al.* 1991; Sangiorno *et al.* 2007; Cañedo-Argüelles *et al.* 2013). Even though freshwater organisms seem resistant to a wide range of salt concentrations, freshwater aquatic hyphomycetes seem to

have their reproduction and spore germination inhibited at salinities between 7 to 14 g/L (Byrne & Jones 1975).

Freshwater organisms have varying tolerance to salinity depending on other environmental factors such as temperature, water hardness or pH (Cañedo-Argüelles *et al.* 2013). This leads us to another issue concerning the action of multiple stress factors combined on the ecosystem and its biodiversity. Given the fact that biodiversity is particularly important in stress conditions and aquatic hyphomycetes are susceptible organisms with a key role in decomposition, it would be advantageous to understand the impact of salinity on the ecology of this group.

Here we try to clarify the effect of aquatic hyphomycete diversity (specific species richness) in the presence of a stressor (salinity); we expect that a higher richness may help to buffer its potential deleterious effects on fungal communities ecology and function (Yachi & Loreau 1999).

### 1.4. Objectives

In this study, a microcosm experiment was conducted to evaluate the importance of the aquatic hyphomycetes richness (3 levels) on leaf litter (*Populus nigra* L.) mass loss, fungal biomass production (ergosterol buildup), reproduction (number of produced spores) and respiration (oxygen consumption) under an ecologically relevant gradient (3 levels) of NaCl. Monocultures and random combinations of 4 or 8 species gathered from a pool of 12 common aquatic hyphomycetes species will be used in order to eliminate the effect of identity in the assemblages. It is hypothesized that species rich

fungal assemblages are functionally more buffered against high levels of salinity (NaCl) than species poor assemblages independently of the identity of the formed assemblages.

# CHAPTER 2

## – Materials and Methods –

## 2. MATERIALS AND METHODS

Experiments were carried out in the laboratory using microcosms designed to mimic leaf decomposition by fungi in streams. Microcosms consist of 100 mL Erlenmeyer flasks incubated on an orbital shaker (120 rpm) under a 12 h light: 12 h dark photoperiod.

Senescent poplar (*Populus nigra* L.) leaves were collected immediately after abscission, air-dried and kept in the dark at room temperature until needed. The initial phosphorus (Graça et al., 2005), carbon and nitrogen (IRMS Thermo Delta V advantage with a Flash EA 1112 series), total phenolic concentrations and toughness (Graça et al., 2005) of poplar leaves were determined to allow the characterization of initial leaf quality (Tab. 1).

Poplar leaf discs (12-mm-diameter) were punched out with a cork borer and oven-dried before being preweighted in sets of 20. Those sets were placed in different Erlenmeyer flasks and autoclaved (20 min, 121º C) with 20 mL of distilled water to induce leaching. The distilled water was substituted by 40 mL of a mineral salt solution (containing per litre 100 mg $CaCl_2.2H_20$, 10 mg $MgSO_4.7H_2O$, 0.5 morpholino propane sulfonic acid, 100 mg $KNO_3$ and 5.5 mg $K_2HPO_4$, pH adjusted to 7.0; Dang *et al.* 2005), supplemented with three levels of salinity (0, 8 and 16 g/L NaCl), added aseptically, and the leaching process kept for another 24 h. After this period, 8 leaching control microcosms were sacrificed to determine a mass loss correction factor due to handling and leaching.

**Table 1.** Initial physic-chemical composition of *Populus nigra* leaves (mean ± SE; n=4).

| | **Poplar** |
|---|---|
| **% C** | 42.97 ± 2.98 |
| **% N** | 1.79 ± 0.44 |
| **% P** | 64.61 ± 1.65 |
| **% Phenols** | 12.95 ± 0.02 |
| **Toughness (g)** | 61.61 ± 2.73 |

Twelve aquatic hyphomycete species commonly found in temperate streams were used in the experiments: *Tricladium splendens* Ingold, *Tetracladium marchalianum* de Wild, *Tetrachaetum elegans* Ingold, *Flagellospora curta* Webster, *Tricladium chaetocladium* Ingold, *Lemonniera aquatica* de Wildeman, *Varicosporium elodeae* Kegel, *Clavariopsis aquatica* de Wildeman, *Lemonniera pseudofloscula* Dyko *et al.*, *Anguillospora filiformis* Greath, *Articulospora tetracladia* Ingold, *Heliscus lugdunensis* Sacc. & Thérry. Microcosm were inoculated with agar plugs collected from the edge of 14-day-old colonies of the twelve fungi (previously obtained and grown on 2% malt extract agar) as follows: single species (3 replicates x 12 species x 3 salinity levels), a random combination of four fungal species (3 replicates x 3 salinity levels) and a random combination of eight species (3 replicates x 3 salinity levels), which makes a total of 126 microcosms. To eliminate identity effects

and therefore the specific trait effects of some species, the community composition within the same species richness level differed among replicates *per* salinity concentration. In the total, three different communities were obtained at random for each fungal richness level. Single species microcosms were inoculated with a 12-mm-diameter plug and for multispecies microcosms, the total inoculum size was maintained and divided equally among all species (Fig.1).

The mineral solution was replaced after 6 days and then every 2 days. After the 42 days of the experiment, the discs from each microcosm were freeze dried (to fungal biomass determination) or oven-dried (105 $^{o}$C for 48 h) and weighted to determine dry mass loss (% DM) as the difference between initial and final mass of each set of discs.

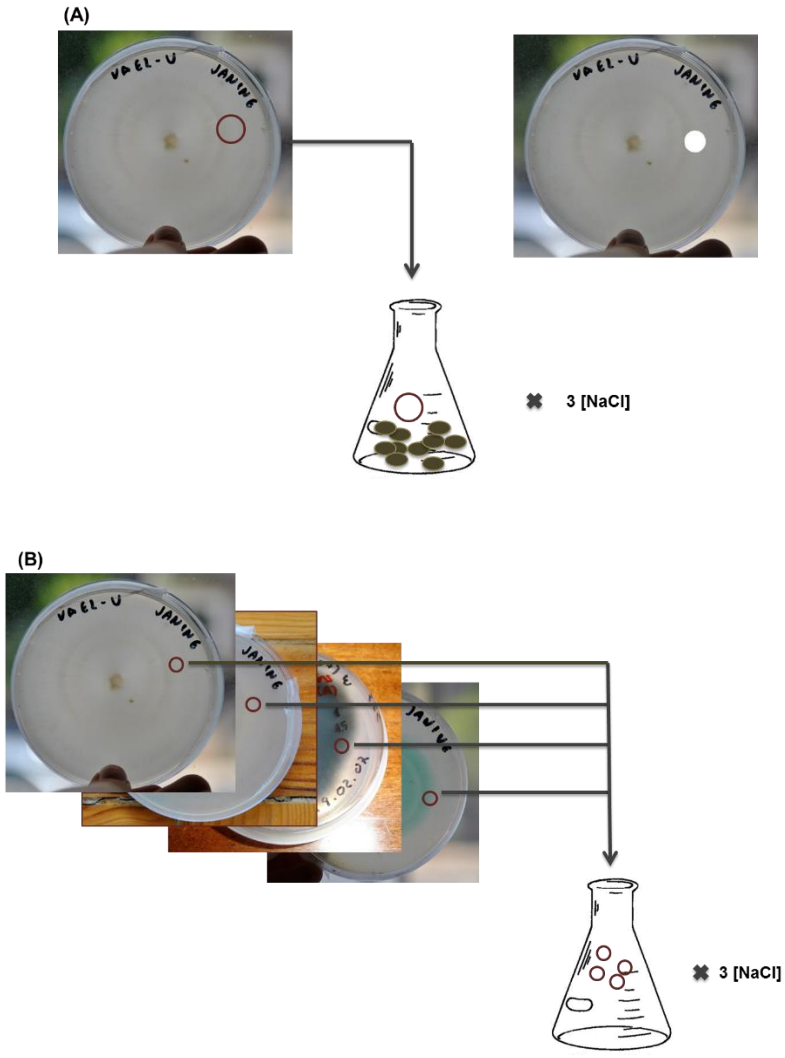


**Figure 1.** – Schematic representation of the inoculation of agar plugs, collected from previously obtained fungal colonies, in microcosms with (A) representing single species (12-mm-diameter plug) and (B) an example of a four species community microcosm (6-mm-diameter plug).

*Fungal biomass*

To determine ergosterol concentration as a measure of fungal biomass (Gessner & Chauvet, 1993; Young, 1995), a subset of 3 leaf discs of each microcosm was freeze dried, weighted, suspended in 2 ml of methanol and treated with 0.5 ml of 2 M aqueous sodium hydroxide before being heated in a

microwave oven (three times 20 seconds, with 3 minutes breaks, in a total of 1 min; 2 450 MHz and 750 W). The ergosterol was extracted with pentane (ca. 6 ml), which was evaporated to dryness in a sand bath, at 55 $^{o}$C, and then was re-dissolved in 1 ml of methanol. Ergosterol was quantified by high performance liquid chromatography (HPLC) using a Merck LiChroCART 250-4 (LiChrospher 100) RP-18 column by measuring absorbance at 282 nm (Young, 1995). The concentration of ergosterol was converted into fungal biomass using a conversion factor of 5.5 μg ergosterol $mg^{-1}$ fungal dry mass (Gessner & Chauvet, 1993) and the results were expressed as mg fungal biomass $g^{-1}$ DM.

*Microbial respiration*

A subset of 5 leaf discs from each microcosm was used to calculate leaf associated microbial respiration. The 5 discs were incubated in falcon tubes with 20 ml of the previously used standard media saturated with oxygen. The initial oxygen concentration (mg $O_2$) was measured with an oxygen sensor (Jenway 9200, UK) and the tubes were covered with aluminium foil and kept in the dark for 24h. The oxygen concentration was measured again and the oxygen consumption (as a measure of microbial respiration) was calculated as the difference between the initial and the final concentration and expressed in mg $O_2$ mg dry $mass^{-1}$ $h^{-1}$.

*Sporulation rate*

Based on the literature, we predicted that the aquatic hyphomycetes sporulation should be peaked between 2 and 3 weeks after inoculation in stream (Graça *et al.* 2005). Here, 15 days after the beginning of the experiment the media was collected to Falcon tubes. To fix the conidia, formalin was added (37%; 2 ml) and the conidial suspensions were mixed with 100µL of Triton X – 100 solution (0.5%). An aliquot of conidial suspension was filtered (Millipore SMWP filters, 5 μm pore size) and retained spores were stained with 0.05% cotton blue in lactic acid (60%). Conidia were counted and identified with the assistance of a compound microscope at 250x (Graça *et al*. 2005). Results were expressed as the total number of conidia mg leaf dry mass$^{-1}$ day$^{-1}$.

*Statistical Analysis*

Two-way Analysis of Variance (ANOVA) was performed to compare the poplar leaf mass loss among treatments, with fungal richness and salinity concentration as categorical factors. When significant statistical differences were found ($P < 0.05$), planned comparisons were used to identify the significant effects of one factor within the other and followed by Tukey's HSD test or Unnequal HSD test when necessary. The statistical procedure was the same to all variables, including microbial respiration, fungal biomass and sporulation rates.

The data were transformed when necessary to ensure that the assumptions of normality and homocedasticity of variances were fulfilled. Means (± standard error) presented in the text and figures were calculated

using non-transformed data. All statistical analyses were performed using STATISTICA 7 software (StatSoft, Tulsa, Oklahoma).

# CHAPTER 3

# – Results –

## 3. RESULTS

*Leaf decomposition*

Leaf mass loss was influenced by salinity concentration (2-way ANOVA: $F_{2,112}$ = *11.83,* p<0.001), but species richness *(*2-way ANOVA: $F_{2,112}$ = 2.96, P = 0.056) and the interaction between the two categorical factors (2-way ANOVA: $F_{4,112}$ = 1.18*,* P = 0.324) were not significant. There were significant differences between salinity levels on leaf mass loss (Tukey's test: P < 0.001), except between the intermediate and highest levels (Tukey's test: P = 0.19) (Fig. 2A).

*Microbial Respiration*

Both salinity concentration and species richness have a significant effect on microbial respiration (2-way ANOVA: respectably, $F_{2,117}$ = 3.943, P = 0.022 and $F_{2,117}$ = 3.609, P = 0.030), but no interaction was found between the two factors. Even though microbial respiration is significantly affected by all levels of salinity (Tukey's test: P < 0.041), species richness does not determine any significant differences in oxygen consumption (Tukey's: P > 0.127*;* Fig.2B). Tukey's test did not distinguish differences between treatments show by ANOVA (Fig. 2B).

*Fugal Biomass*

Fungal biomass varied between 609 to 1212 mg $g^{-1}$ leaf dry mass for all single species and communities treated with 0 g/L salinity; 614 to 1993 mg $g^{-1}$ leaf dry mass in the intermediate level of salinity (8 g/L) and 447 to 1198 mg $g^{-1}$ leaf dry mass in the highest level of salinity (16 g/L). Neither salinity nor species

richness significantly affected fungal biomass (2-way ANOVA, respectively, $F_{2,113}$ = 2.45, P = 0.09 and $F_{2,113}$ = 0.22, P = 0.80; Fig. 2C), as did not the interaction between the two factors.

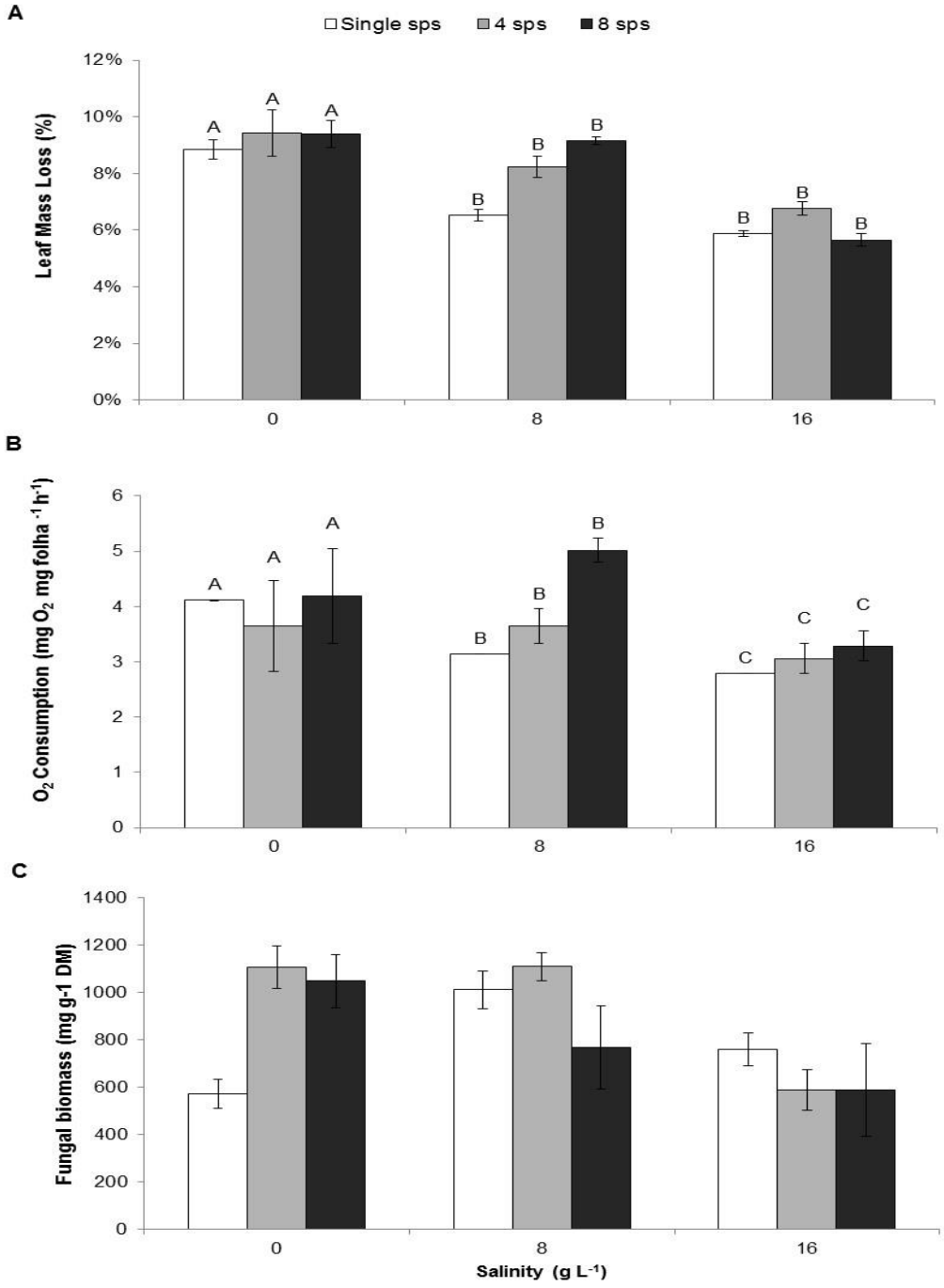


**Figure 2.** Leaf mass loss of poplar leaf discs **(A),** microbial respiration **(B)** and fungal biomass **(C)** in microcosms, incubated with three fungal diversity levels (single species – white, four species – light

grey, eight species – dark grey) of aquatic hyphomycetes species and subjected to three levels of salinity. Different letters indicate significant differences among treatments referring to pooled single, poor and rich replicates (capital letters) within each salinity level.

*Sporulation Rates*

Sporulation rates were the most sensitive variable to salinity. Conidia were not produced when salinity concentration was highest, and both salinity and species richness significantly influenced sporulation (respectively, 2-way ANOVA: $F_{2,117} = 73.58$, $P < 0.001$ and 2-way ANOVA: $F_{2,117} = 7.66$, $P = 0.001$), as well as the interaction between the two factors (2-way ANOVA: $F_{4,117} = 2.83$, $P = 0.028$). For all the three levels of species richness, significant differences were found among salinity concentrations (Planned comparisons: $F_{2,117} = 20.37$, $P < 0.001$). Concerning the effect of species richness within each level of salinity, no significant differences were found in the lower and higher salinity concentrations (respectively, planned comparisons: $F_{2,117} = 2.12$, $P > 0.125$ and planned comparisons: $F_{2,117} = 0.00$, $P > 1.000$). However, within the intermediate level of salinity, species richness showed a significant effect on sporulation rates (Planned comparisons: $F_{2,117} = 11.21$, $P < 0.001$), but the post-hoc test was not able to discriminate the differences (Tukey's: $P > 0.184$) (Fig. 3).

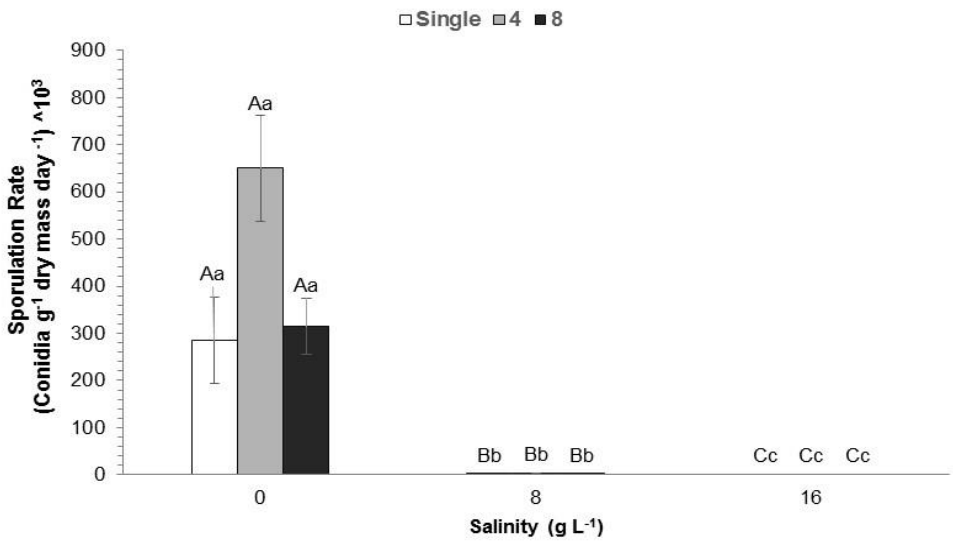


**Figure 3.** Sporulation rates of aquatic hyphomycetes associated with poplar leaf discs incubated in microcosms with three fungal diversity levels (single species – white, four species – light grey, eight species – dark grey) and subjected to three levels of salinity. Different letters indicate significant differences, when tested within each salinity concentration (capital letters) and species richness (lowercase letters).

# CHAPTER 4

# – Discussion –

## 2. DISCUSSION

In our study, leaf litter decomposition and microbial respiration were affected by salinity but not by fungal diversity.

Even though the insurance hypothesis (Yachi & Loreau 1999) states that richer fungal assemblages may be more able to buffer the consequences of stress conditions, under the effect of salinity, there was no detectable positive relationship of aquatic hyphomycete richness on leaf decomposition. However, even though we were not able to find statistically significant differences, it seems that richer communities tend to perform better than single species, which is graphically evident especially in the case of intermediate concentrations. No other studies exist on the effects of richness or diversity in salt-rich streams. Yet, the importance of fungal diversity in the decomposition process has been tested in the presence of other stress factors such as temperature (Geraldes *et al.* 2012; Gonçalves *et al.* 2015), heavy metal pollution (Pascoal *et al.* 2005b; Pascoal *et al.* 2010; Fernandes *et al.* 2011), nutrient supply (Dang *et al.* 2005; Ferreira *et al.* 2006b) and alterations in the riparian vegetation (Bärlocher & Graça 2002). Positive (Bärlocher and Corkum 2003; Duarte *et al.* 2006; Pascoal *et al*. 2010; Fernandes *et al.* 2011) and no effects (Pascoal *et al.* 2005b; Ferreira *et al*. 2006b; Bärlocher & Graça 2002; Dang *et al.* 2005; Pascoal *et al.* 2010; Fernandes *et al.* 2011; Geraldes *et al.* 2012; Gonçalves *et al.* 2015; present study) have been observed.

We suggest that diversity effects depend on the environmental context, as the interactions between species are altered by the gradient of

environmental harshness (Bärlocher & Corkum 2003; Pascoal *et al.* 2010; Fernandes *et al.* 2011; Geraldes *et al.* 2012), in this case, of NaCl. Functional redundancy may have masked richness effects, since it is possible that a few species are enough to maintain decomposition (Gessner *et al.* 2010) and our study suggests that that number might be somewhere between 1 to 4 species. Other studies state that 3 to 5 species of aquatic hyphomycetes are enough to maintain decomposition rates (Pascoal *et al.* 2010; Geraldes *et al.* 2012; Gonçalves *et al.* 2015).

We must also consider the fact that different species composing our assemblages may have different responses to salinity, for some species might be more salt-tolerant than others with highly variable EC50 for growth rates (7.8 – 40.86 g/L NaCl; Simões *et al*. submitted). The absence of a richness effect may result from the establishment of dominant species within the assemblages, as it is the rule in natural communities (Reiss *et al.* 2011) or, more likely, to the fact that our random communities might have been largely composed of tolerant species. If that was the case, those dominant species' performance was enough to sustain decomposition (Gonçalves *et al.* submitted).

Salinity proved to have an important effect on fungal mediated decomposition in streams, independently of the fungal community richness. The salt concentrations tested in our study are ecologically relevant (0-16 g/L NaCl), since, naturally, salinity varies from 10's of mg/L to a few 100's of g/L in inland waters (Kefford *et al.* 2012) and salinized rivers commonly showed NaCl concentrations ranging from 7 to 15 g/L (Crowther & Hynes 1977). Parallel effects on leaf decomposition were observed by Schäfer *et al.* (2012) in an experiment performed in a salted stream and by Cañedo *et al.* (2014) in artificial

flow-through streams with repeated salt pulses, in the presence of diatom and stream invertebrate communities. The presence of salt also depressed microbial respiration. Connoly *et al.* (2014) showed similar results for microbial respiration, when evaluating salinity effects on *phragmites* decomposition dynamics in freshwater wetlands.

Most studies show a positive relationship between the increase in fungal biomass and decay rates (Sridhar & Bärlocher 2000; Pascoal & Cássio 2004; Gonçalves *et al.* 2014). However, the opposite was observed in the presence of NaCl, once fungal biomass production was not affected by salinity. It seems possible that trade-offs between enzymatic production and mycelium metabolism must occur in salt-rich media in order to keep the mycelium integrity and osmolality (Cañedo *et al.* 2013).

It is likely that, in the presence of high concentrations of salt, aquatic hyphomycetes invest more on mycelium build-up than on respiration or reproduction. This was also observed by Byrne & Jones (1975), where aquatic hyphomycete growth was preserved even at 35 g/L, although sporulation and spore germination were inhibited at salinities between 7 to 14 g/L. The present study is in accordance with these results, once sporulation was totally inhibited by salinity. There is a sharp decline in sporulation rates as salinity increases, from $3\times10^5$ to $9\times10^2$ conidia $g^{-1}$ dry mass $^{-1}$ $day^{-1}$ in intermediate levels, to the total absence of conidia to salinities of 16 g/L. Our study corroborates the idea that sporulation rates are much more sensitive to stress, than all the other fungal descriptors (Pascoal *et al.* 2010). With this parameter, salinity proves itself to be of great risk to the functioning of freshwater ecosystems. Aquatic hyphomycetes release conidia in order to reproduce and therefore complete

their life cycle, for if there is no conidial production, the successful colonization of new substrata is compromised and the survival of the species becomes threatened (Geraldes *et al.* 2012). Considering the crucial role of aquatic hyphomycete as links between litter and invertebrates, cascading effects through the food chain may be expected.

As an overall conclusion of the present study, salinity seems to have a greater impact than species richness on the decomposition process in streams. Its effect is severe and may compromise nutrient cycling and therefore ecosystem functioning. Microbial activity is reduced by salinity, except for fungal biomass, but that might not be enough to maintain the viability of species, once reproduction is truly compromised and this may result in the loss of fungal diversity.

This leaves a great deal of questions unanswered, once salinity effects may change when considering other environmental variables and stress factors acting together, as well as microbial communities richness and composition.

# CHAPTER 5

# – Final Remarks –

## 3. FINAL REMARKS

For more than 20 years now, the relationship between biodiversity and ecosystem functioning has been subject of great concern, not only because of the impact the loss of species may have on nature itself, but also because it affects the services ecosystems provide to society (Cardinale *et al.* 2012).

Initial losses in biodiversity may not have a great impact on ecosystem functioning, but this will probably change if species continue to disappear through time (Cardinale *et al*. 2012). Shor-term and small scale studies may not show an accurate impact of species loss in functioning, because time fluctuations and environmental heterogeneity are essential not to overestimate this relationship (Cardinale *et al*. 2012). Caution is needed to proceed extrapolating results from laboratory experiments with microcosms. Even though microcosms are a very useful tool to isolate and test the specific effect of a single stress factor, as it was here, there is no environmental noise. In natural ecosystems it is probable that stressors do not operate alone, but as a set of different perturbation acting at the same time and space. We must not forget that the effects of diversity may largely depend on the harshness of the environmental contexts (Pascoal *et al*. 2010). On the other hand, the effect of salinity cannot be dissociated from other relevant stressor for aquatic hyphomyctes as decrease in water $O_2$. Therefore, we suggest a thinner and wider scale of the number of aquatic hyphomycetes species tested, in order to tackle the real number of species necessary to maintain ecosystem processes (if any) in the presence of multistressors, as well as field experiments which

include other variables. For example, it is known that global warming is expected to increase salinity (Cañedo-Argüelles *et al.* 2013), like so, it might be significant to explore a whole new chapter on these two stress factors acting together in biodiversity and freshwater ecosystem functioning.

Freshwater ecosystems are of extreme importance to society, not only for recreational purposes, but also because they provide us unique services as no other ecosystem does. Salinity proved itself to be a major threat to these ecosystems, but these were only the first steps to understand a small part of how nature responds to a constantly changing world, for a long path still lies ahead.

# CHAPTER 6

# - References -

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