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Effects of stream salinization on fungal-mediated leaf decomposition: a microcosm approach

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A Coimbra,

A minha segunda casa

E à minha mãe,

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RESUMO

A salinização de ribeiros é um processo natural definido por um aumento de iões inorgânicos na água. Este processo pode, no entanto, apresentar sérias consequências quando atividades antropogénicas interferem com a origem desses iões ou a taxa a que os mesmos são depositados. Neste caso, o processo é denominado por salinização secundária, o qual representa uma ameaça ao nível das comunidades, com possíveis consequências a ocorrerem desde o mais baixo nível ecológico.

Em zonas temperadas, os ribeiros de baixa ordem são caracteristicamente heterotróficos, obtendo a sua energia por parte da área ripícola, maioritariamente folhas. No processo de decomposição foliar, a atividade dos decompositores (particularmente hifomicetes aquáticos) é de crucial importância, uma vez que iniciam o processo que fornece matéria orgânica a todo o sistema aquático.

Para concluir acerca dos efeitos biológicos da salinização secundária em hifomicetes aquáticos e sua atividade decompositora, 14 espécies comuns foram expostas a concentrações sucessivamente maiores de NaCl (0 – 20 g/L). Para cada uma das espécies foi medida a taxa de crescimento e referente EC50, e a taxa de esporulação; para diferentes comunidades, compostas pelas espécies cuja atividade reprodutiva foi tolerante a determinada concentração de NaCl, mediu-se a capacidade decompositora de folhas de carvalho, taxas de respirometria e esporulação total e concentração de ácidos nucleicos.

O crescimento das espécies de hifomicetes aquáticos foi significativamente reduzido a concentrações > 4 g/L, sendo que os valores de EC50 variaram de 7,8 g/L to 40,86 g/L. Observaram-se apenas cinco espécies com capacidade de esporulação a 2 g/L NaCl, uma a 4 e 8 g/L, e nenhuma a concentrações superiores. As comunidades compostas por estas espécies sobreviventes demonstraram uma significante redução na sua capacidade decompositora e na produção de conídios apenas a partir da concentração de 4 g/L, o que demonstra a presença de alguma redundância funcional a mais baixos níveis de stress. No entanto, as taxas de respirometria, que medem a atividade biológica, sofreram uma imediata redução na mais baixa concentração de NaCl, enquanto o rácio RNA:DNA aumentou com o aumento da salinidade. Estes resultados permitem-nos concluir que mesmo a baixos níveis de salinidade a atividade das comunidades fúngicas é afetada, e que com o aumento da salinidade a energia disponível é investida maioritariamente no crescimento celular; no

entanto, sem capacidade reprodutora, a colonização das folhas é largamente inibida, com sérias consequências para o sistema aquático.

ABSTRACT

Stream salinization is a process that occurs whenever there is an increase in inorganic ions on aquatic systems. This natural occurring process can, however, present serious consequences when anthropogenic activities interfere with the nature of those ions or the rate at which those are disposed. In this case, the process is called secondary salinization and represent a community-level threat, with possible consequences starting at the lowest ecological level.

Temperate headwaters are characteristically heterotrophic, obtaining its energy from riparian inputs, mainly leaves. In the leaf-decomposing process, the activity of decomposers (primarily aquatic hyphomycetes) is of crucial importance, since they start the process that provides organic matter to the entire watershed.

To infer about the biological effects of secondary salinization on aquatic hyphomycetes and its decomposing activity, 14 common species were exposed to increasing NaCl concentrations (0 – 20 g/L). Growth rates and respective EC50, as well as sporulation rates of single species were measured. At each NaCl concentrations, the different salt-tolerant communities were evaluated in its oak-leaves decomposing capacity, respiration and total sporulation rates, and nucleic acids concentration.

Growth rate of aquatic hyphomycetes species was significantly reduced at > 4 g/L NaCl; EC50 values varied from 7.80 g/L to 40.86 g/L. Only five species were able to sporulate at 2 g/L; one at 4 and 8 g/L, and none at higher concentrations. Communities composed of those surviving species showed reduced decomposing capacity and conidia production only beyond 4 g/L, which shows evidence of some functional redundancy at lower stress levels. However, respiration rates – the measure of biological activity – were immediately reduced at the lowest NaCl treatment, while RNA:DNA increased with the increase of NaCl. These results allow us to conclude that, even at low levels of salinity, the fungal communities' activity is affected, and that the increase in NaCl causes the available energy to be invested in cell growth; however, without reproductive capacity, the leaf colonization is largely inhibited, with serious consequences for the aquatic system.

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CHAPTER 1

GENERAL INTRODUCTION

In December 2003, it was proclaimed by the United Nations General Assembly that the period 2005-2015 would be the International Decade for Action 'Water for Life', growing awareness for the importance of water in all aspects of humans' life. It started on March 22, 2005, with the primary goal of promoting efforts for a sustainable management of water resources; it is known that, with the continuing population growth, there will be an increase in water demands, as well as more waste and pollution affecting this resource.

Humans have always revolved around water; the first settlements were determined by the location of water sources, and the development of the biggest cities has always taken place near the ocean or big rivers. Nowadays, we depend on water for domestic, industrial and agricultural purposes, as well as a source of energy, a way of transportation and for leisure activities. It is interesting to notice that, as before, even today our biggest quest is to find water; if not in our own planet, then somewhere else in the universe.

In every aspect of our life, water plays a crucial role, and this omnipresence can lead us to think that it will always be there for our use; after all, more than 70% of Earth's surface is covered by water. However, from this, only 0.8% constitutes fresh water (Geist, 2011) and these habitats' biodiversity has been declining at a faster rate than the most affected terrestrial ecosystems (Décamps, 2011). Despite being a tiny fraction of the total amount of water in our planet, freshwater habitats support at least 100 000 species (Dudgeon et al., 2006), and confine as much as 35% of all vertebrate species (Christian et al., 2011). Running waters are unique systems: they undergo an intimate, dependent relation with surrounding terrestrial ecosystems, with a dominant linear form and unidirectional flow. All these characteristics contribute to define their unique biota (Malmqvist & Rundle, 2002).

Freshwater systems experience multiple threats, from overexploitation, water pollution, flow modification, destruction or degradation of habitat, and invasion by exotic species (Dudgeon et al., 2006); although seas and oceans are also affected by all of these factors, their volume has the capacity to dilute contaminants and mitigate negative impacts (Dudgeon et al., 2006), to a higher extent. Concerning water pollution, alterations of the water chemistry – from acidification to nutrient addition, metal contamination and salinization – are global threats that can have a severe impact on these systems (Malmqvist & Rundle, 2002). From the entire watershed, headwater streams can be seen as the beginning of the continuum, which will feed all downstream areas, until reaching the sea; any threat we cause upon those systems will have consequences for the entire aquatic ecosystem.

Headwater streams

Headwater streams are located at the head of the watershed; they are very abundant, comprising up to 80% of the length of the drainage area (Richardson & Danehy, 2007). Headwaters are characterized by strong interactions with the surrounding terrestrial ecosystem (Lowe & Likens, 2005), acting as sources of sediment, water, nutrients and organic matter (Gomi et al., 2002; Wipfli et al., 2007; Clarke et al., 2008) to downstream areas. This shows their crucial role for the entire river network: they sustain the structure, function and productivity of the aquatic ecosystem (Wipfli et al., 2007). Also, they provide an array of habitats for many organisms (Meyer et al., 2007), they support genetically isolated species (Gomi et al., 2002; Wipfli et al., 2007) and provide refuge for many species (Meyer & Wallace, 2000; Lowe & Likens, 2005); they also serve as a spawning habitat, or a rearing habitat for the young (Meyer et al., 2007). Headwaters represent, therefore, important source areas for biodiversity (Gomi et al., 2002; Meyer et al., 2007; Wipfli et al., 2007).

One of the main features of temperate headwater streams is their high edge to area ratio. This results in a high degree of shading, due to the deep closed forest canopy; with low light, primary production is reduced, meaning that these systems are heterotrophic, highly dependent on allochthonous inputs of energy (Abelho, 2001; Graça, 2001). These inputs occur in form of wood, seeds, flowers or terrestrial invertebrates, but mainly of leaf litter, which can account for up to 98% of total allochthonous energy (Abelho, 2001); this organic matter will be incorporated into secondary production through the leaf decomposition process. Leaf litter decomposition is a key-ecosystem process (Gessner et al., 2007) dependent on the activity of three main players: the leaves, the decomposers and the detritus-feeding invertebrates (Graça, 2001; Gomi et al., 2002). All three are interdependent, and their activity will also result in the release of organic matter that will supply nutrients and energy to other functional feeding groups (Cummins, 1974).

Leaf litter decomposition

Leaves are the primary source of energy of low order streams (Abelho, 2001; Bärlocher, 2005); the decomposition of leaf litter occurs in three phases that may overlap in time (Gessner et al., 1999; Abelho, 2001).

The first step – leaching – corresponds to the release of soluble compounds as amino acids, simple sugars and phenolics to the water. This phase frequently leads to a mass loss higher than 30%

(Gessner et al., 1999). This abiotic phase depends on factors such as water temperature, turbulence and, primarily, species identity and traits (Abelho, 2001, Lecerf & Chauvet, 2008).

The second phase of leaf decomposition, conditioning, corresponds to the colonization of leaf tissue by microorganisms, namely bacteria and aquatic hyphomycetes (Abelho, 2001). These fungi are dominant in well aerated and turbulent freshwater systems, where they play an essential role in leaf litter decomposition; they possess the enzymes needed to degrade the main components of leaves, as cellulose, hemicelluloses, pectin and lignin (Gessner et al., 1999; Graça, 2001; Krauss et al., 2011). That degradation, along with the mechanical action of the hyphae (Gessner et al., 2007) results in a physically softened leaf. The addition of fungal biomass to the leaf also increases its nutritional value and palatability to the invertebrates (Canhoto & Graça, 2008). It may require weeks to months to reach the point where the leaf is fully conditioned, i.e., with the highest fungal biomass and activity peak (Canhoto & Graça, 2008). The conditioning process is also determined by species identity and affected by physical and chemical characteristics of the water, as nitrogen and phosphorus concentration (Sridhar & Bärlocher, 1997), dissolved oxygen (Medeiros et al., 2009) or nutrients availability and increased temperature (Ferreira & Chauvet, 2011)

The third step of the litter decomposition is referred as fragmentation. It occurs due to two processes: physical fragmentation, as a result of abrasion from flowing water or sediments, and biotic fragmentation. In this case, the loss of leaves' integrity is promoted by the feeding activity of invertebrates – scrapers and, most importantly, shredders (Cummins, 1974). It has been shown that invertebrates have the ability to recognize and prefer conditioned leaves over non-conditioned ones, and that ingestion of this conditioned material can lead to advantages in growth, survival and fecundity (Bärlocher & Kendrick, 1975; Graça, 2001; Canhoto & Graça, 2008, Christian et al., 2011). Through their activity, detritivores promote the release of fine particulate organic matter (Gessner et al., 1999; Graça, 2001), which will then be used by other organisms (Richardson & Danehy, 2007), such as collectors (Canhoto & Graça, 2008).

Secondary salinization as a main stressor of freshwaters

Salinity is a chemical component of all aquatic systems, defined as the total concentration of dissolved inorganic ions, namely Na^+, Ca^{2+}, Mg^{2+}, K^+, SO_4^{2-}, CO_3^{2-} and HCO_3^-, in the water (Cañedo-Argüelles et al., 2013). The process of increasing the levels of those ions on land or aquatic systems,

above normal levels – undisturbed freshwaters are characterized by a salinity value of <0.5 g/L (Teixeira et al., 2007) – is called salinization; primary when it occurs by natural processes, secondary when it is due to anthropogenic action.

Primary salinization is the result of long-term natural processes, as the deposition, in the soil or groundwater, of salts via sea spray or rain (Mayer et al., 2005), or the weathering of materials that release soluble salts (Cañedo-Argüelles et al., 2013).

Whenever there is salt deposition from other origins, or the rate at which those salts accumulate is changed due to anthropogenic activities, we are facing secondary salinization. Some anthropogenic sources for salts entering freshwaters are mining activity (Williams, 1999; Kefford et al., 2011; Cañedo-Argüelles et al., 2013) and industry (Kefford et al., 2012; Cañedo-Argüelles et al., 2013), as well as the use of salts as de-icing agents for roads in the winter. Those salts are then washed away by rainwater, and transported to adjacent streams (Silva et al., 2000; Cañedo-Argüelles et al., 2013).

The rate at which salts accumulate can be increased due to land clearing, for agricultural or other purposes (Silva et al., 2000; Kefford et al., 2011; Cañedo-Argüelles et al., 2013). The removal of trees will increase the amount of rainwater that reaches the soil; also, it will decrease the amount of groundwater that is absorbed. This will lead to the rising of groundwater tables, until intersecting the ground surface and discharging salts into streams and rivers (Mayer et al., 2005). Also for agricultural purposes, irrigation could lead to the rising of groundwater tables; linked to crop production, that absorb only a small fraction of the salt, it will lead to more saline water intersecting our rivers (Cañedo-Argüelles et al., 2013). Water with salinity values over 1 g/L is considered useless for agricultural purposes, and values slightly higher are no longer suitable for drinking and industrial supplies (Williams, 1999). Economic impacts of salinization are also important; for instance, in Western Australia, by 2050, up to $400 million are predicted to be lost per year in agricultural production due to salinity (Mayer et al., 2005), which is accounted for as one of the most limiting environmental factors for agricultural production (Pitman & Läuchli, 2002).

Impact of salinization in the aquatic biota

Freshwater salinization is considered one of the most important stressors of freshwaters, being known to have direct and indirect impacts on the biota and to impair ecological processes (Williams, 1999; Silva et al., 2000; Kefford et al., 2011), with lethal and sublethal effects from values as low as 1

g/L for aquatic plants and invertebrates (Kaushal et al., 2005). Salinization is even more threatening considering that its effects are not limited to the aquatic zone as riparian areas are also affected (James et al., 2003).

To the riparian vegetation, an excess of ions or water deficiency, due to higher salinity values, result in toxic effects: reduced growth rates, changes in calcium to sodium ratio and potassium uptake, breaking of enzymes and proteins pathways, among other effects such as inhibition of seed germination and lesions on leaves (Hart et al., 1991); ultimately, the disappearance of riparian trees (Williams, 1999). Such effects could occur beyond salinities of 2 g/L. In headwater streams, the decrease of riparian vegetation would eventually lead to more light entering the stream, changing the system from heterotrophic to autotrophic (Cañedo-Argüelles et al., 2013). In the water, the addition of salt may have important physic-chemical effects direct and indirectly important to the biota, such as increases in sedimentation and decreases in the dissolved oxygen concentration (James et al., 2003), increases in the total phosphorous but decreases NO_x (Kefford, 1998) and effects in nitrification rates (Garcia, 2015).

High salinity values cause osmotic stress to organisms (Hart et al., 1991). If salt concentration becomes too high, cells end up dehydrating and collapsing, leading to death (Cook, 2012). Therefore, the salinity tolerance of a species is determined by their ability to maintain the optimal internal osmotic concentration despite external changes (Hart et al., 1991). Freshwater organisms can sustain osmoregulatory processes; however, this always means metabolic costs, at the expense of other processes such as growth or reproduction (Hassel et al., 2006).

The most sensitive species of invertebrates (e.g. Crustacea, Insecta and Mollusca), show adverse effects at salinities of 1 g/L; but 9 g/L seems like a reasonable threshold value, beyond which toxic effects occur to most of the species (Hart et al., 1991). Concerning invertebrates' communities, Piscart et al. (2005) referred a loss of species specialization, changes in reproductive traits (mainly a shift to ovoviviparity) and functional feeding groups proportion (the frequency of scrapers decreased, being replaced by filter-feeding and deposit-feeding) in sites with higher levels of salinity (i.e. approximately 1.37 g/L).

Concerning bacteria, it has been suggested that with increases in salinity, freshwater species might be replaced by their marine forms, without changes in community balance and functioning of the ecosystem (Hart et al., 1991). Also, some studies indicate that bacteria can easily and quickly adapt to low levels of increasing salinity (Hart et al., 1991 and references therein). However, salinity interacts

with other physical and chemical factors, and we cannot know for sure how bacterial communities would respond to changes in such context. For fungi, very few studies have been developed, but results suggest that salinization leads to some inhibition in growth rates and, more strongly, in the reproductive effort (Byrne & Jones, 1975; Müller-Haeckel & Marvanová, 1979). Related to the effect of stream salinization on the process of leaf breakdown, studies are also scarce, but some authors have reported a reduced breakdown rate at higher salinity levels (Sangiorgio et al., 2007; Cañedo-Argüelles et al., 2014), probably by reducing the diversity of invertebrate assemblages (Fritz et al., 2010; Schäfer et al., 2012) or due to higher levels of sedimentation (Blasius & Merritt, 2002).

The effects of salinization on stream aquatic biota are still largely unknown, particularly in what concerns ecosystem-level processes. Understanding the importance of worldwide threats like salinization on streams biodiversity and its outcomes for the ecosystem functioning (and consequent services provided to man) should constitute a priority for stream ecologists.

Objectives

Although being a major contaminant of freshwaters, very little is known on the effect of increased salinity on freshwater systems structure and, especially, function. This lack of knowledge is especially true in Portugal, where, as far as we know, no work has already been done on that subject.

The main goal of this dissertation is to study the effect of increased water-levels of salt (NaCl), on microbial-mediated decomposition of oak (*Quercus robur* L.). We will assess the effects of salinization on the a) growth and reproductive output of 14 aquatic hyphomycetes species and on b) litter decomposition dynamics promoted by salt-tolerant fungal communities, under laboratory conditions.

CHAPTER 2

EFFECTS OF STREAM SALINIZATION ON FUNGAL-MEDIATED LEAF DECOMPOSITION: A MICROCOSM APPROACH

This chapter corresponds to the following paper in preparation:

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Abstract

The ecological status of woodland streams is continuously threatened by human activities. Salinization is of major global concern due to its effects on stream biota and/or processes it maintains; however, there is still very little information on its effects on the leaf breakdown process. This is a key-ecosystem-level process in small streams, driven by decomposers, mainly fungi (aquatic hyphomycetes), that link litter and invertebrates. Here, we assess the effects of an ecological relevant gradient of salt concentrations (0 – 20 g/L NaCl) on (1) fungal growth and species reproductive output and (2) fungal-mediated decomposition of *Quercus robur* leaves by salt-tolerant assemblages. Growth rate was affected by NaCl, decreasing for the majority of species at >4 g/L. EC50s were species-specific, varying from 7.80 g/L to 40.86 g/L. Sporulation rate was more sensitive: it occurred in five out of nine species at 2 g/L, and only one at 8 g/L. Significant decreases in mass loss and sporulation of the salt-tolerant assemblages occurred only from 4 g/L NaCl; respiration was depressed at 2 g/L, being almost 2-times higher in assemblages with no added salt. Results suggest that stream salinization may induce a decrease of fungal diversity with deleterious consequences on streams ecosystem function.

Introduction

Salinization of streams and rivers is a growing global threat (Williams, 2001) and it is expected to be exacerbated by climate change and other anthropogenic effects (Cañedo-Argüelles et al., 2013). Salts may enter freshwater ecosystems through natural pathways (primary salinization) or due to human activities (secondary salinization), such as clearing of native vegetation, agricultural irrigation, rising groundwater, mining activity, industrial discharge and use of salts (particularly NaCl) as deicing and anti-icing agents (Cañedo-Argüelles et al., 2013). Even though this is considered one of the most important stressors for aquatic systems (Kefford et al., 2011), very little information is available on its effects on the biota and related processes.

In temperate headwaters, the breakdown of allochthonous leaf litter is a key ecosystem process, in which decomposers (i.e., aquatic fungi and bacteria) and invertebrates are the main drivers of energy flow. Aquatic hyphomycetes dominate the first stages of this process (Nikolcheva & Bärlocher, 2005), facilitating the feeding behavior of macroinvertebrates, which will carry on with the breakdown of organic matter (Canhoto & Graça, 2008).

The studies of the effect of secondary salinization on stream biota has mainly focused on

invertebrates. Their ecophysiology (Blasius & Merritt, 2002; Hassel et al., 2006; Piscart et al., 2006; Cañedo-Argüelles et al., 2012; Szöcs et al., 2014), density (Cañedo-Argüelles et al., 2012), richness (Pinder et al., 2005; Piscart et al., 2006; Bäthe & Coring, 2011; Braukmann & Böhme, 2011) and trophic structure (Piscart et al., 2005 and 2006) have been shown to be negatively affected by salinization. Few studies assessed the importance of this contaminant on the microbial breakdown of organic matter (Blasius & Merritt, 2002; Sangiorgio et al., 2007; Fritz et al., 2010; Schäfer et al., 2012; Cañedo-Argüelles et al., 2014); results stand for a high susceptibility of the process to this stress factor. In fact, previous information indicate that sporulation is negatively affected when exposed to salinity at values ≥ 2% (Sridhar & Kavariappa, 1988), ≥ 10% or 30% (*Tetracladium setigerum* and *Heliscus lugdunensis;* Byrne & Jones, 1975) or >20% (Tsui & Hyde, 2004). However, a study performed by Müller-Haeckel & Marvanová (1979) indicate that freshwater hyphomycetes are able to survive, grow and sporulate in brackish or seawater.

Herein we evaluated the sensitivity of growth and sporulation rates of 14 common fungal species to a salt (NaCl) gradient. Based on the single species sporulation results, we evaluated if distinct fungal assemblages, composed of the salt-resistant species at each salinity level, were able to maintain similar functional properties and processes under the different contamination levels – 0, 2, 4, 8 g/L NaCl. We used, as endpoints, oak leaf mass loss and associated metabolic descriptors (respiration, reproduction, nucleic acids concentrations). We hypothesize that the effect of salt will be species-specific, with distinct thresholds for growth and reproduction. Within tolerance limits for aquatic hyphomycetes, increasing salt concentrations will decrease fungal richness; poorer salt-tolerant assemblages will show a weakened performance in leaf degradation. We hypothesize a decline in all fungal parameters evaluated, as well as a loss of assemblage's performance along the salinity gradient and loss of species diversity.

Materials and Methods

Salinity effects on growth and sporulation rates of individual fungal species

Pure cultures of 14 aquatic hyphomycetes (AH) species (Table I), grown on malt extract agar (2% MEA = 20 g ME/L H_2O), were used to obtain 3 mm diameter agar plugs, using a cork borer in aseptic conditions. One agar plug was placed in the middle of each petri dish, previously filled with salt

rich MEA, with NaCl concentrations of 0, 2, 4, 8, 12 and 20 g/L NaCl. A total of 252 petri dishes (14 species x 6 NaCl concentrations x 3 replicates) were inoculated and maintained under laboratorial conditions (12h light:12h dark photoperiod; 16°C).The colony diameters were measured every two days, until reaching 1 cm distance from petri dish walls. Growth rate was determined as the slope of the linear regression between colony diameter and time, and expressed as mm/day.

Table I. Aquatic hyphomycetes species used in this study and its abbreviations.

Species	Abbreviation
Anguillospora filiformis Greath	ANFI
Articulospora tetracladia Ingold	ARTE
Clavariopsis aquatica de Wild.	CLAQ
Flagellospora curta Webster	FLCU
Flagellospora curvula Ingold	FLCURV
Fontanospora fusiramosa Marvanová, Fisher & Descals	FOFU
*Heliscus lugdunens*is Sacc. & Thérry	HELU
Lemonniera pseudofloscula Diko	LEPS
Lemonniera aquatica de Wild.	LEAQ
Tetrachaetum elegans Ingold	TEEL
Tetracladium marchalianum de Wild.	TEMA
Tricladium chaetocladium Ingold	TRCH
Tricladium splendens Ingold	TRSP
Varicosporium elodeae Kegel	VAEL

Additionally, in order to evaluate the effect of salinity in the reproductive output of these fungal species, 3 agar plugs from each replicate were immersed in 100 ml Erlenmeyer flasks containing 40 ml of nutrient solution (75.5 mg $CaCl_2$, 10 mg $MgSO_4.7H_2O$, 0.5 g 3-morpholinopropanesulfonic acid (MOPS), 5.5 mg K_2HPO_4 and 100 mg KNO_3 per liter of sterile distilled water; Dang et al. 2005) enriched with the correspondent NaCl concentrations. The microcosms were incubated on shakers (120 rpm) under a 12 h light: 12 h dark photoperiod. The nutrient solution was replaced every 24 h until peak sporulation was achieved. Subsamples of conidial suspensions were then filtered (Millipore SMWP, 5 µm pore size) and stained with 0.05% cotton blue in lactic acid (60%), and the total conidia produced by each species was counted under a compound microscope at 250x (Graça et al. 2005).

Sporulation rate was expressed as the total number of conidia released per day.

Salinity effects on leaf decomposition

Microcosms and experimental setup

Oak (*Quercus robur* Labill.) leaves were collected after abscission, air-dried at room temperature and evaluated for chemical parameters: phosphorus (0.027 ± 0.002%; Graça et al., 2005), total phenols (23.1 ± 0.32%; Graça et al. 2005), carbon and nitrogen [56.1 ± 1.8% and 0.96 ± 0.02%, respectively; IRMS Thermo Delta V advantage with a Flash EA (1112 series)].

Litter discs, cut out with a cork borer (12mm diameter) avoiding the central vein, were oven-dried (105 °C, 24 h); 42 sets of 20 randomly chosen discs were weighed (± 0.1 mg) and placed in individual 100 mL Erlenmeyer flasks filled with 20ml of distilled water and then autoclaved (121°C, 15 min). Three of these 42 microcosms were sacrificed, to account for mass loss due to the leaching process that occurred during sterilization. Distilled water of remaining microcosms was discarded and replaced by 40 ml of nutrient solution enriched with different salt concentrations. Microcosms, closed with cotton bungs, were maintained on an orbital shaker (100 rpm) for 24 h at 16°C to allow additional leaching. The medium of each microcosm was then replaced and inoculation was performed with different fungal assemblages, established based on the sporulating capacity previously found at each NaCl concentration. Microcosms with single species and no salt addition were used as controls (Table II).

A total of 5000 conidia (Treton et al., 2004) were used to inoculate each of the 39 microcosms. In the case of mixed fungal assemblages conidia were equitably divided among the used fungal species. Conditioning was allowed for 35 days. After this period, the oak discs from each microcosm were used to determine mass loss, fungal respiration, sporulation and nucleic acid concentrations.

Leaf mass loss

A total of 20 leaf discs from each microcosm were oven-dried (48 h at 105 °C) and weighted (± 0.1 mg) to obtain dry mass remaining (DMr). Dry mass loss (%DM) was estimated as the difference between the initial and final dry mass remaining in the microcosms after 35 days.

Table II. Salt concentration and fungal assemblages in microcosms. Assemblages were established based on the sporulation results of individual aquatic hyphomycetes (AH) species tested under a gradient of salinity (see above).

# Microcosm	NaCl concentration (g/L)	Number of AH Species	AH species identity
1-3	0	1	FLCU
4-6	0	1	ANFI
7-9	0	1	ARTE
10-12	0		CLAQ
13-15	0	1	TEEL
16-18	0	1	HELU
19-21	0	1	TEMA
22-24	0	1	LEPS
25-27	0	1	TRCH
28-30	0	9	FLCU, ANFI, ARTE, CLAQ, TEEL, HELU, TEMA, LEPS, TRCH
31-33	2	5	ANFI, ARTE, CLAQ, FLCU, TEEL
34-36	4	1	FLCU
37-39	8	1	FLCU

Fungal respiration

Respiration rate was evaluated using a subset of 5 discs from each microscosm. Discs were immersed in 50 ml falcon tubes filled with the correspondent O_2 saturated nutrient solution. Tubes were kept in the dark for 24 h, after which the final O_2 concentration was measured. Oxygen consumption was obtained by the difference between the initial and the final values. Respiration rates were expressed as mg O_2/ g DM/ h.

Sporulation of aquatic hyphomycetes

Each 2 days, the conidial suspensions from the 39 microcosms were transferred into 1.5 L bottles and preserved with 2 ml of 37% formalin. At the end of the experiment, the conidial suspensions were mixed with a magnetic stirrer with 1 mL of Triton 0.5%; an aliquot was filtered (Millipore SMWP, 5 µm pore size) and stained with 0.05% cotton blue in lactic acid (60%). Total conidia were identified and counted under a compound microscope at 250x (Graça et al., 2005). Results were expressed as the total number of conidia/microcosm.

Nucleic acid concentrations

A subset of two leaf discs were freeze-dried overnight and weighted for DNA and RNA extraction (Norgen's RNA/DNA/Protein purification kits; Norgen Biotek, Thorold, Canada). Nucleic acid (NA) concentrations (i.e. RNA and DNA concentrations) in the decomposed leaf discs were measured with a NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Scientific, Delaware, USA) following manufacturer's instructions and expressed as ng NA/ mg DM.

Statistical analysis

Differences in growth and sporulation rates were analyzed using a two-way ANOVA (AH species and NaCl concentrations as categorical variables). Data from growth rate were previously transformed into ranks due to lack of homocedasticity, and was analyzed by a two-way ANOVA; since both tests (with no transformation and with rank transformation) gave the same result, we proceeded with the first one (Zar, 2009). Further analysis was done by planned comparisons tests, to investigate one factor within the other. Growth rate data from each species was fitted to a Gompertz, Logistic or Hormetic model in order to calculate EC50s (effective concentration that led to a 50% inhibition in growth rates). Comparisons between EC50s for each species were performed through the likelihood ratio test.

For the fungal assemblages' experiments, one-way ANOVAs were used to assess differences in microbial respiration, sporulation and mass loss, with NaCl concentration as the categorical variable. A post-hoc Tukey's test was applied whenever necessary. Differences between nucleic acid concentrations at different NaCl concentrations were analyzed trough a Kruskal-Wallis test (due to lack of homocedasticity) followed by a Tukey's test.

All statistical analyses were conducted with STATISTICA 7 software (StatSoft, OK, USA).

Results

Salinity effects on growth and sporulation rates of individual fungal species

Growth rate was species-specific and negatively affected by salinity (two-way ANOVA, $F_{species(1, 13)} = 421.71$, $P < 0.01$, and $F_{NaCl(1, 5)} = 714.91$, $P < 0.01$; Fig. 1). A significant decrease was observed in concentrations equal and above 4 g/L (planned comparisons, $F_{(5)} = 714.9$, $P < 0.01$). The only exception

was *H. lugdunensis,* which showed a significant increase in its growth rate at 20 g/L (planned comparisons, $F_{(5)} = 3.58$, $P < 0.01$).

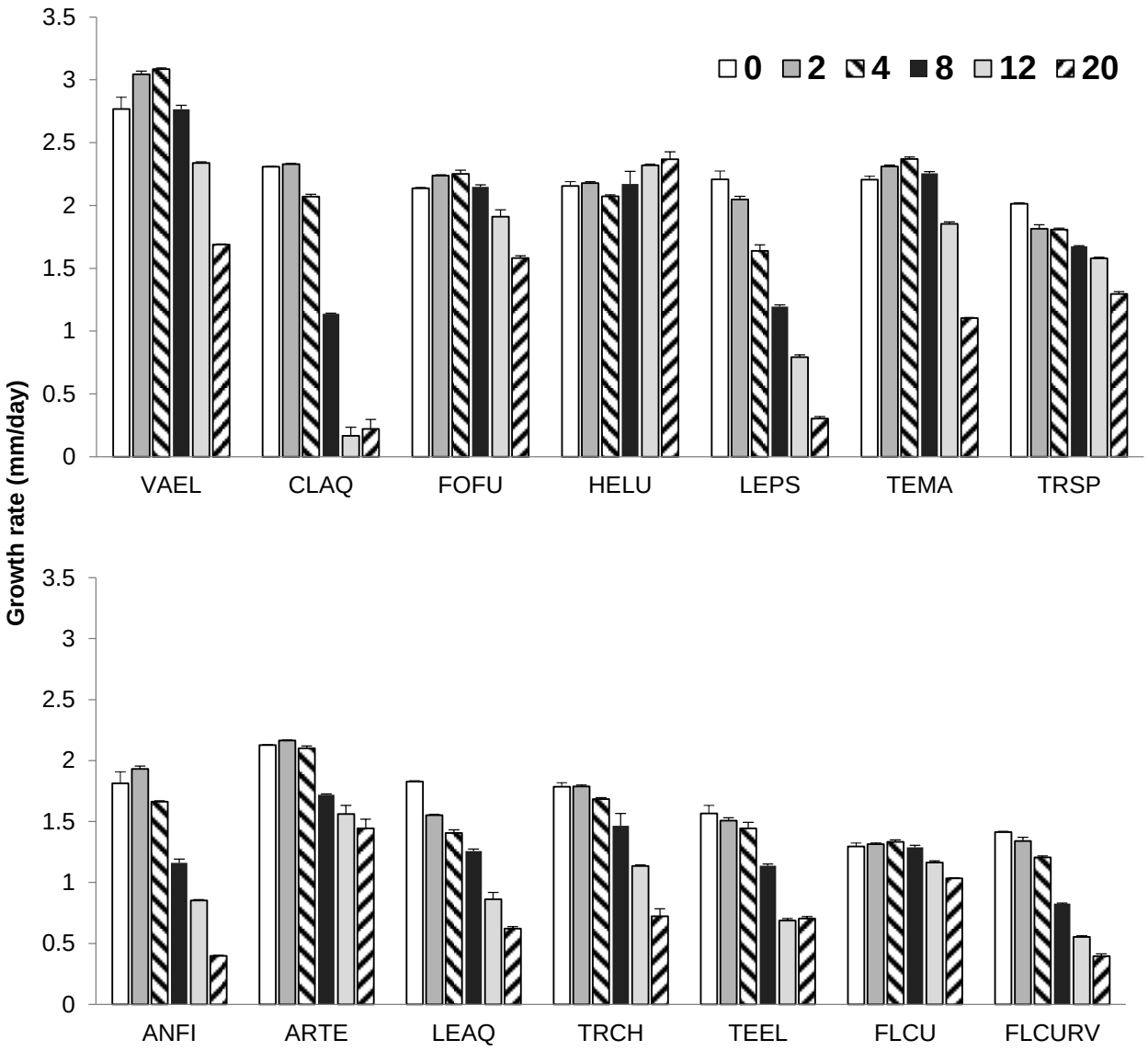

Figure 1 – Growth rate for the 14 aquatic hyphomycetes species tested, grown in MEA with increasing concentrations of NaCl (0, 2, 4, 8, 12 and 20 g/L). Colony diameter was measured every two days until reaching 1 cm distance from the petri dish walls.

EC50s for growth rate were species-specific and varied between 7.80 g/L NaCl for *C. aquatica* and 40.86 g/L NaCl for *F. fusiramosa* (Table III). As *H. lugdunensis* did not show any decrease in its growth rate with increased salinity, EC50 calculation for this species was not possible.

Table III. Effective concentration (EC50) values for the growth rates of the 14 aquatic hyphomycetes species grown in NaCl rich media (0, 2, 4, 8, 12 and 20 g/L) and respective 95% confidence-intervals (CI). Different letters indicate significant differences between species.

Species	EC50	CI
CLAQ	7.80 [a]	7.24 - 8.35
LEPS	8.61 [a]	7.94 – 9.27
FLCURV	10.23 [b]	9.09 – 11.36
ANFI	10.54 [b]	9.84 - 11.25
LEAQ	12.57 [c]	10.44 – 14.69
TEEL	13.99 [c,d]	11.01 – 16.97
TRCH	16.20 [d]	15.59 - 16.81
TEMA	20.0	19.57 - 20.41
FLCU	25.34 [e,f,g]	16.77 - 33.91
VAEL	25.78 [h]	22.97 - 28.58
ARTE	31.45 [e,h,i,j]	23.03 - 39.87
TRSP	36.94 [f,i,k]	29.49 – 44.38
FOFU	40.86 [g,j,k]	29.39 - 52.33
HELU	-	-

Sporulation rates were also species-specific and negatively affected by all tested NaCl concentrations (two-way ANOVA, $F_{(1,8)} = 168.37$, $P < 0.01$; $F_{(1,3)} = 72.82$, $P < 0.01$, respectively). No differences were found in any case between 4 and 8 g/L NaCl (Tukey's test, $P > 0.6$). Only 4 out of the 14 tested species sporulated at 2 g/L, and only one species (i.e. *F. curta*) sporulated at 4 and 8 g/L (Fig. 2).

For all the species that sporulated both at 0 and 2 g/L, only *A. filiformis* presented differences between those two concentrations (planned comparisons, $F_{(1)} = 39.67$, $P < 0.01$). *F. curta* produced the highest number of spores in the control – along with *H. lugdunensis* (planned comparisons, $F_{(1)} = 0.81$, $P > 0.1$) – and in the first NaCl concentration (2 g/L). Sporulation rates for *F. curta* were significantly reduced only at 8 g/L (planned comparisons, $F_{(1)} = 44.16$, $P < 0.01$).

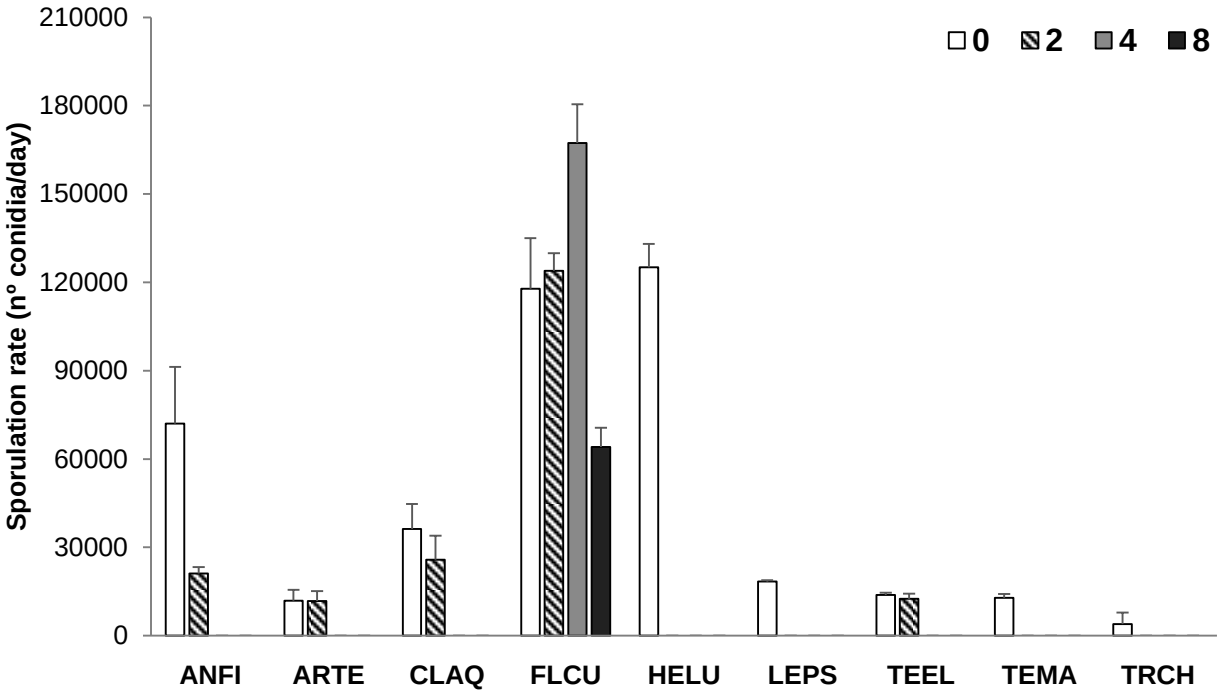

Figure 2 – Sporulation rate of the 14 aquatic hyphomycetes tested, grown in nutrient solution with increasing NaCl concentrations (0, 2, 4 and 8 g/L). Species not shown did not sporulate at any concentration; no sporulation occurred >8 g/L NaCl.

Salinity effects on leaf decomposition

Both % DM loss and sporulation rates were negatively affected by salinity (one-way ANOVA, $F_{mass\ loss(1,\ 3)} = 104.21$, $P < 0.01$ and 1-way ANOVA, $F_{sporulation(1,\ 3)} = 28.99$, $P < 0.01$; Fig. 3 a) and b)). A clear reduction in both parameters was observed at NaCl ≥ 4 g/L (Tukey's test, P < 0.01; Figs. 3 a) and b)). Respiration rates were also negatively affected by salinity (one-way ANOVA, $F_{(1,\ 3)} = 18.4$, $P < 0.01$), being almost two times higher at 0 g/L than in the other treatments (i.e. 2 g/L; Tukey's test, P < 0.01; Fig. 3c)).

A. filiformis, *L. pseudofloscula* and *H. lugdunensis* were the main producers of spores at 0 g/L, making up for 83% of the total assemblages' spore production. (Fig. 4a)). With the increase in salinity (i.e. at 2 g/L) they were replaced by *A. tetracladia*, which alone made up for 63% of total spore production (Fig. 4b)).

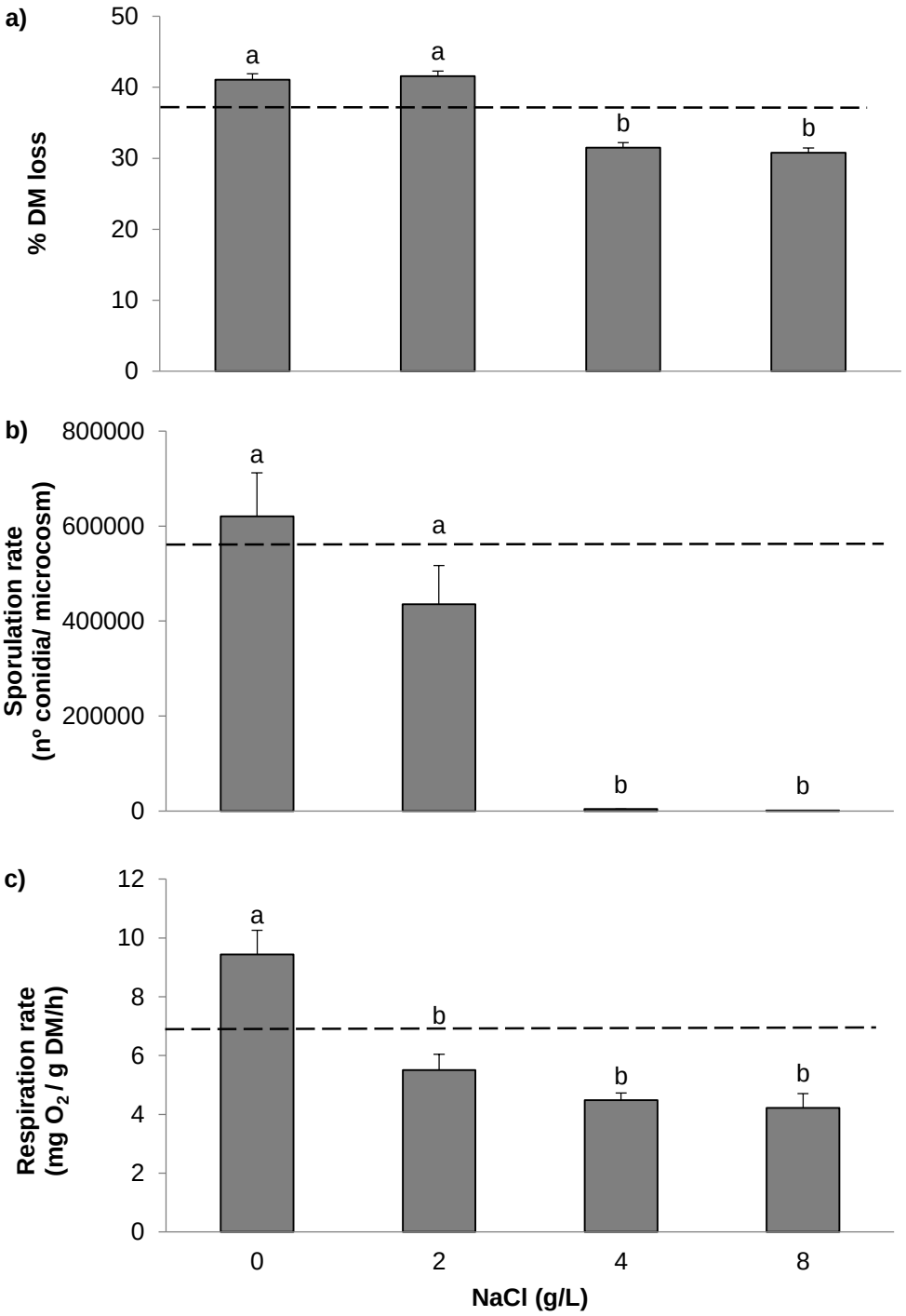

Figure 3 – a) % DM loss, b) sporulation rates, and c) respiration rates observed for the different aquatic hyphomycetes' assemblages: 9 species in the control, 5 at 2 g/L and 1 at 4 and 8 g/L NaCl (*vide* Table II). Different letters indicate significant differences among treatments. (--) corresponds to the mean value of all single-species used as controls.

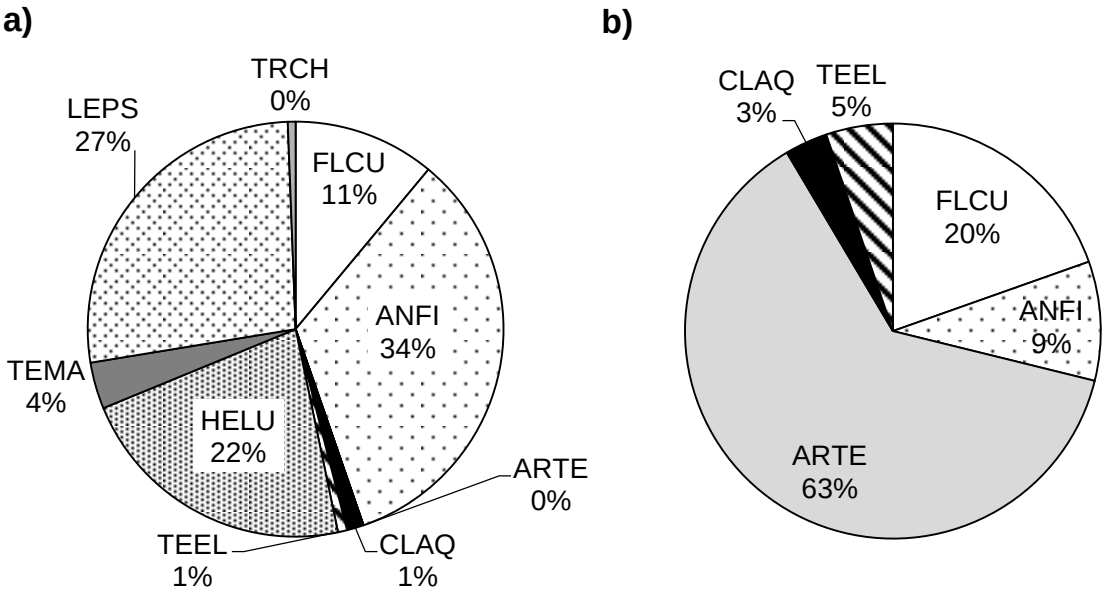

Figure 4 – Mean relative contribution of the different fungal species to the total spore production present on mixed assemblages, at a) 0 g/L and b) 2 g/L after 36 days incubation. Microcosms were inoculated with 5000 conidia distributed equitably from all species.

Both DNA and RNA concentrations associated with the oak leaf disks were significantly affected by NaCl (Kruskal-Wallis H test, $H = 27.79$, $P < 0.01$, and $H = 8.25$, $P < 0.05$, respectively). DNA peaked at 2 g/L NaCl while RNA tended to increase until 4 g/L NaCl (Fig. 5). RNA:DNA ratio significantly increased with NaCl increase (Fig. 6)

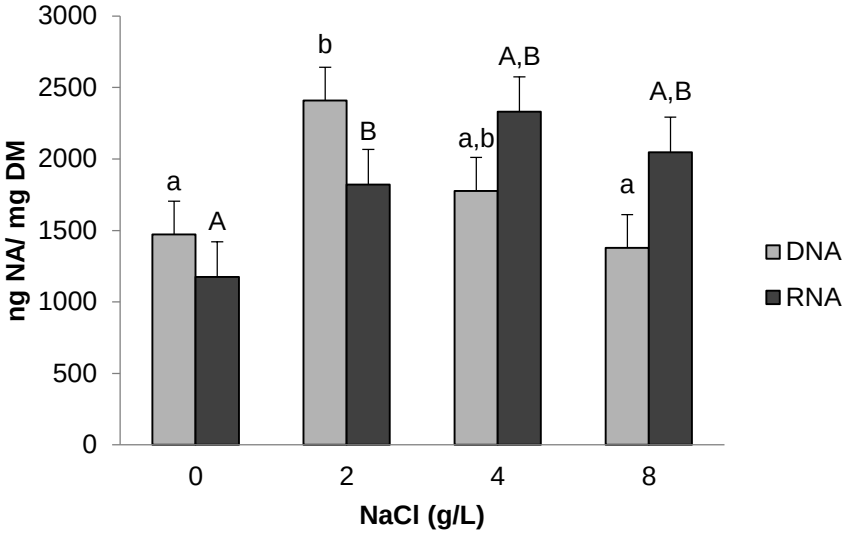

Figure 5 – Mean nucleic acid concentrations obtained from leaves colonized by different aquatic hyphomycetes' assemblages: 9 species in the control, 5 at 2 g/L and 1 at 4 and 8 g/L NaCl. Lower case letters represent significant DNA differences among treatments; upper case letters relate to RNA.

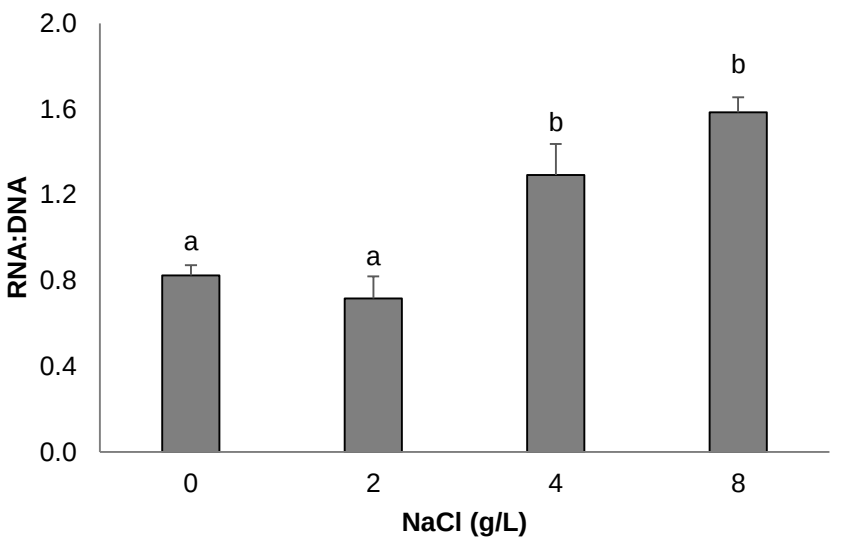

Figure 6 – RNA:DNA ratios at distinct NaCl concentrations (9 species in the control, 5 at 2 g/L and 1 at 4 and 8 g/L NaCl). Lower case letters represent statistically significant differences between NaCl concentrations.

Discussion

Our study indicates that, under laboratory conditions, an increase in NaCl may affect fungal growth and conidial production. However, according to EC50 values, and in agreement with previous studies (Müller-Haeckel & Marvanová, 1979), some species seem to tolerate values found in brackish and estuarine areas, possibly also contributing to leaf litter processing in fluvial-estuarine transitional areas. In spite of this tolerance, sporulation of the mycelium of individual-species was depressed at low salt concentrations (≥ 2 g/L). In fact, this is in agreement with previous studies where sporulation is usually the parameter most sensitive to salinity – in Müller-Haeckel & Marvanová (1979), sporulation ceased at about 40% seawater, but growth was maintained until higher salinities; Byrne & Jones (1975) reported sporulation of two AH species until 10 and 30% seawater, but growth was maintained until 100%) – and even in response to other contaminants (e.g. metals; Bärlocher, 2005; Pascoal et al., 2010). Although leaves' colonization may occur through leaf mycelial outgrowth, the impairment of conidia production (and eventually germination; Byrne & Jones, 1975) by salt addition may clearly affect fungal diversity and, in accordance with ours and previous results (Sangiorgio et al., 2007; Cañedo-Argüelles et al., 2014), the leaf litter decomposition process, at least at values > 2 g/L.

In our study, tested concentrations of NaCl ranged from a freshwater (0 g/L) to a estuarine (≈ 20 g/L) system, and were environmentally realistic: aquatic hyphomycetes seem to be less sensitive to NaCl than invertebrates, in which the most sensitive taxa (i.e., Ephemeroptera, Plecoptera and Pulmonate snails) show maximum 72-h LC50s of 10 g/L (Cañedo-Argüelles et al., 2013). The EC50s

obtained in this work show that such salinity values are still tolerated by most fungal species. This would mean that, in case of a salt discharge, even with some biodiversity loss, aquatic hyphomycetes would be the primary drivers of leaf decomposition, maintaining the organic matter recycling until reaching their salinity threshold.

In fact, in our work, fungal species loss was not followed by lower leaf decomposition (communities composed of 9 species [at 0 g/L] accomplished the same decomposing capacity as those composed of 5 species [at 2 g/L], with similar total spore production), following the redundancy hypothesis (Walker, 1992). Although no comparisons can be made with other studies addressing the effects of reduced biodiversity due to salinity on microbial-mediated decomposition, the effect of salinity as a stressor seems to follow the same pattern as the one observed in field studies, where loss of fungal species due to pollution was not followed by decreased leaf decomposition (Raviraja et al., 1998; Pascoal et al., 2005) or laboratory experiments where assemblages with different levels of species richness (1-8) didn't result in different values of leaf (alder/oak) mass loss (Dang et al., 2005). Significant decreases were, nevertheless, observed on the assemblages' respiration rates at 2 g/L NaCl, in agreement with Connolly et al. (2014) that found a significantly decreased microbial respiration in *Phragmites* when immersed in 0.5% seawater. An obvious (but not significant) decrease in the number of conidia was also observed. This may suggest, based on the growth rate tests and on the maintenance of similar RNA:DNA values at 0 and 2 g/L, an investment of the reduced energetic capacity on growth or degradative capacity, eventually at the expense of reproduction. Ultimately, even if the aquatic hyphomycetes are able to grow in a salinized medium, without reproductive potential their colonizing capacity will be reduced, with important consequences on leaf processing and nutrient cycling in the system.

The present work used a balanced number of spores to colonize the microcosms. After the conditioning period, the communities formed in no salt or 2 g/L NaCl were different and dominated by distinct species. In a laboratorial study, Ferreira & Chauvet (2012) reported that changes in species dominance of fungal assemblages (by manipulation of conidia proportions among 3 species) did not result in changes in decomposition rates. However, it is generally accepted that, besides a common pool of enzymatic potential, fungal species may present differences in their enzymatic capacities (Zemek et al., 1985; Duarte et al., 2006) and that degradative capacities are species-specific (Gonçalves et al., 2015). Whether distinct patterns of dominance in low salt media allowed to

compensate the richness reduction (9 vs 5 species) is not known, but conceivable. In fact, from all the species that showed sporulation at 2 g/L, the two most abundant ones (i.e. *A. tetracladia* and *F. curta*) were also the ones who showed the maximum EC50 values on salted media, along with the highest sporulation rates in the case of *F. curta*.

F. curta was, consequently, the most tolerant species to salinity, when considering growth and reproductive capacity. However, when alone at higher salt concentrations (i.e. 4 and 8 g/L), this species was unable to guaranty a similar leaf degradation as richer communities at lower salinities. It is interesting to notice that a similar "inefficiency" of a single species (in comparison with poor or richer communities) was observed by Gonçalves et al. (2015), in a study on the importance of richness as a buffer against temperature oscillations.

Also, the importance of species identity over species richness on litter decomposition has been frequently suggested (Duarte et al., 2006). Considering the important role that fungi play in the increase of leaf's palatability for the shredders, fungal identity may play a key role in streams' metabolism: invertebrates are selective feeders, preferentially consuming leaves conditioned by some fungal species (Bärlocher & Kendrick, 1973; Arsuffi & Suberkrop, 1986 and 1989). Likewise, species richness has also been referred as being essential for invertebrates' performance: Gessner et al. (2007) referred that higher fungal species richness leads to an improved resource quality for invertebrates, leading to an enhanced decomposition.

Typically, and contrary to what was simulated in the present work, salt pollution occurs by pulses (Cañedo-Argüelles et al., 2014), allowing the communities to recover from that stress until a threshold limit is reached. Also, streams' salinization by NaCl are frequently promoted by the use of salts as deicing agents; in cold temperature conditions, an increase in NaCl could also lead to lower values of dissolved oxygen, which is highly limiting to fungal survival (Bärlocher, 2005), and to what we weren't able to account for. Despite such limitations, our results allow us to conclude that stream salinization may induce a decrease in fungal diversity and affect fungal ecology, with deleterious consequences for the streams' ecosystem function.

CHAPTER 3

FINAL REMARKS

Even though the threat of secondary salinization on freshwater systems has been acknowledged for quite some time, not much is known on its effects on freshwater communities and the processes they operate. This is especially true for the first drivers of the decomposition process, the aquatic hyphomycetes – and, considering this is the key-process for small streams, which will deliver energy to the entire aquatic system, this lack of knowledge should be promptly resolved.

Microcosms' experiments, such as the present study, allow for a better understanding of the true effects of a specific stress on organisms. Even though they do not reflect the actual conditions of ecosystems, where multiple natural or anthropogenic variables interact with each other to an extent we cannot account for, they are the best compromise between significant results and reasonable costs and work involved.

However, in this work we only tested the community's decomposing capacity with a single plant species, and with a limited number of aquatic hyphomycetes species, which do not reflect the conditions of true streams. Also, in natural systems, the addition of salt to freshwaters leads to changes in other chemical parameters, as are suspended solids, total phosphorus or NO_x (Kefford, 1998); all of which could have consequences in aquatic hyphomycetes' ecology. There is also an important role that fungi play in leaf decomposition that is not accounted for in this paper – the increase of leaf's palatability for the shredders.

Therefore, to a better understanding of NaCl increase in streams communities, we suggest the development of field studies, accounting for salt-related changes in physical-chemical components of the water, as well as wide-ranging tests, accounting for cascade-effects.

Considering all the limitations discussed throughout this thesis, the present work should be only viewed as a starting point; further studies should be made to infer how this negative effect works on organisms, and what the long-term consequences are. Despite such limitations, our study has an ecologic relevance due to the climate-change predictions: higher temperatures and lower annual rainfall values. The first will lead to evaporation and drying of stream channels (Bruder et al., 2011), increasing the concentration of dissolved ions (Cañedo-Argüelles et al., 2013); the latter will influence the degree of salinity increase (Bari & Smettem, 2006). Hence, our results suggest that changes in climate conditions will result in lower fungal species richness in streams, followed by a reduced decomposing capacity, leading to energy-deprived aquatic systems.

CHAPTER 4

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