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Are fungal strains from salinized streams functionally more efficient than their conspecifics from reference streams?

Tese de Mestrado em Ecologia, orientado por Prof. Dr. Cristina Canhoto e Dr. Ana Gonçalves
e apresentada pelo Departamento de Ciências da Vida da Universidade de Coimbra

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Resumo

Em ribeiros florestados, a decomposição é um processo ecossistémico chave. É largamente promovida por fungos - hifomicetes aquáticos - que fazem a ligação entre as folhas que entram no sistema e os invertebrados consumidores. A salinização dos cursos de água induzida pelo homem é um problema global crescente cujas consequências na função dos ribeiros permanecem, em grande parte, desconhecidas. Neste trabalho foram avaliados os efeitos da salinização ($6gL^{-1}$ NaCl) na perda de massa foliar e parâmetros microbianos associados, promovidos por conjuntos de uma ou múltiplas espécies de estirpes fúngicas de *Heliscus lugdunensis* (HELU), *Tetrachaetum marchalianum* (TEMA) e *Flagellospora curta* (FLCU) isoladas a partir de um ribeiro de referência (R) ou salinizado (S). A morfologia, crescimento fúngico e interações ecológicas estabelecidas em ambos os contextos também foram avaliados. A contaminação por sal tendeu a inibir a decomposição das folhas de choupo e a biomassa fúngica associada, mas não foram observadas diferenças entre espécies, estirpes ou comunidades. As taxas de esporulação não foram afetadas pela presença de sal, mas foram diferentes entre as espécies (FLCU> HELU> TEMA), com as estirpes S a produzirem mais conidia. Apesar dos conjuntos mistos de fungos não mostrarem diferenças significativas na produção total de conidia (entre estirpes ou meios), a dominância das espécies foi afetada pela adição de sal no meio. Na presença de sal, a esporulação foi dominada por HELU, que apresentou consistentemente o maior crescimento e comportamento antagonista. TEMA (R ou S) foi a espécie menos antagonista e teve a menor tolerância ao sal. Os resultados sugerem que as comunidades fúngicas, independentemente da sua origem, são capazes de manter a sua eficiência funcional em ribeiros salinizados, garantindo assim a incorporação foliar na produção secundária. As interações antagonistas parecem ser mais fortes na presença de sal e tendem a ser semelhantes entre espécies e estirpes (exceto HELU-R). Esperam-se efeitos em cascata ao longo das cadeias alimentares, considerando as perdas de diversidade e potenciais mudanças na qualidade do material foliar para os invertebrados consumidores de folhas previsíveis em ribeiros contaminados com sal.

Palavras-Chave: Salinização; Tolerância Hifomicetes Aquáticos; Decomposição; Interações.

Abstract

In forested streams, decomposition is the pivotal ecosystem-level process. It is mainly carried out by fungi - aquatic hyphomycetes - that link leaf litter and invertebrate consumers. Human-induced salinization of watercourses is a globally growing problem of which the consequences on stream function remain largely unknown. Here we evaluated the effects of salinization ($6gL^{-1}$ NaCl) on leaf mass loss and associated microbial parameters promoted by mono- and multispecies assemblages of fungal strains of *Heliscus lugdunensis* (HELU), *Tetrachaetum marchalianum* (TEMA) and *Flagellospora curta* (FLCU) isolated from a reference (R) or salinized (S) stream. Fungal morphology, growth and ecological interactions established in both contexts were also assessed. Salt contamination tends to inhibit poplar leaf decomposition and associated fungal biomass, but no differences were observed between species, strains or assemblages. Sporulation rates were not affected by the presence of salt but were different among species (FLCU > HELU > TEMA), with S strains usually releasing more conidia. Despite mixed fungal assemblages not showing significant differences in total conidia production (either between strains or media), the dominance of species was affected by salt addition in the medium. In the presence of salt, sporulation was dominated by HELU, which consistently presented the highest growth and antagonistic behavior. TEMA (R or S) was the least antagonistic species and had the lowest salt-tolerance. Results suggest that fungal communities, irrespective of their origin, are able to maintain their functional efficiency in salinized streams, thereby guaranteeing leaf incorporation into secondary production. Antagonism seemed to be stronger in the presence of salt and tended to be similar across species and strains (except HELU-R). Cascading effects throughout stream food webs are expected considering the losses of diversity and potential changes in leaf litter quality to leaf-consumers that can be expected in salt-contaminated stream.

Keywords: Salinization; Tolerance; Aquatic Hyphomycetes; Decomposition; Interactions.

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Chapter 1
Introduction

1.Introduction

1.1 Headwaters

Headwaters are watercourses located in the origin of the river network (Gomi et al., 2002). These small streams are quite abundant, accounting for more than 80% of the total length of the fluvial net (Meyer and Wallace, 2001; Wipfli et al., 2007; Clarke et al., 2008). Their quantity, location, unique and diverse biota make them paramount for the downstream reaches and streams (Gomi et al., 2002; Meyer et al., 2007; Clarke et al., 2008) influencing the structure, function, and productivity of larger watercourses (Wipfli et al., 2007). The relevance of these systems *per se* and to the global fluvial system, make them crucial for the health of the all system and preferential targets for the implementation of freshwater protective measures and activities (Lowe and Likens, 2005; Meyer et al., 2007; Wipfli et al., 2007)

Small forest streams (Graça et al., 2015) present high margin:surface area ratios as most of them are covered by treetops, which prevent light from reaching the surface of the water. Therefore, they are heterotrophic systems where the organic matter from terrestrial origin (wood, seeds, invertebrates, but mainly leaves) plays a fundamental role as source of nutrients and energy to the stream biota (Abelho, 2001; Graça, 2001; Ferreira et al., 2011). The riparian zone is though crucial to the ecology and productivity of these headwaters (Naiman and Décamps, 1997; Wallace et al., 1999; Meyer and Wallace, 2001), controlling the seasonality, quality and quantity of the organic matter supplied to the stream as well as most of the physico-chemical characteristics of the water (Casotti et al., 2015; Lidman et al., 2017).

1.2 Leaf Decomposition

Low-order streams are very retentive systems - about 70% of the organic matter that falls annually is retained (Abelho 2001) and then processed. In these systems, leaf litter decomposition is a (the) key ecosystem-level process (Gessner et al., 1999).

Once in the water, leaves are subjected to abiotic and biotic-driven transformations, converting senescent leaf litter in live biomass, CO_2, inorganic compounds, fine particulate organic matter and dissolved organic matter (Gessner et

al., 1999; Hieber et al., 2002). The decomposition of these leaves proceeds in three distinct phases that normally occur in a temporal sequence: leaching, conditioning and fragmentation (Webster et al., 1986; Abelho, 2001; Canhoto et al., 2015).

Leaching. Upon immersion, leaf leaching begins. The efficiency of this abiotic phase depends mainly on the characteristics of the organic matter, but also on other environmental characteristics such as the temperature, turbulence and water chemistry (Abelho, 2001). Leaching is characterized by the rapid loss of soluble constituents of the leaf (e.g. amino acids, simple sugars and phenols) and usually occurs within the first 48h after immersion, leading to a loss of foliar mass that frequently reaches 30% (Webster et al., 1986; Gessner et al., 1999; Abelho, 2001). Despite this, the loss of soluble compounds continues to occur during the remaining decomposition process (Webster et al., 1986; Abelho, 2001).

Conditioning. The conditioning phase corresponds to the leaf colonization by microorganisms and facilitates posterior consumption by the invertebrates. However, fungi and bacteria can colonize the terrestrial organic material before entering the water; upon immersion the activity of these microorganisms is suppressed (Abelho, 2001; Graça & Canhoto, 2006). During this phase leaves are "modified" by microorganisms, mainly aquatic hyphomycetes, but also by bacteria (Abelho, 2001; Bärlocher et al., 2004; Pascoal et al., 2004; Gonçalves, et al., 2014). The colonization usually begins with the settlement of asexual spores (conidia), but it can also be promoted by direct contact (some outgrown hyphae in a colonized leaf touches another leaf), or at a distance (detached hyphae fragments) (Dang et al., 2007). Aquatic hyphomycetes conidia germinate and grow in the foliar substrate (Canhoto and Graça, 1999; Dang et al., 2007), and incorporate nitrogen and phosphorus from the water, promoting the nutritional enrichment of the leaf material. The fungal enzymatic activity is also responsible for the degradation of cell wall compounds, such as cellulose, hemicelluloses, pectin and lignin (Gessner et al., 1999; Graça, 2001; Krauss et al., 2011) and for the softening of the leaf tissue. This process thus increases the palatability and the nutritional value of the leaves for the detritivores, particularly for the shredders - invertebrates that feed on course particulate organic matter (Cummins 1974).

Leaves are a shared substrate for fungi, so it has been pointed out the possibility of different species being able to compete for the substrate or to collaborate in their degradation (Yuen et al., 1999; Gonçalves et al., 2014). Some studies demonstrate the importance that fungal interactions have on the organization and composition of the fungal assemblages, and therefore on leaf litter decomposition (Wong et al., 1998; Yuen et al., 1999; Treton et al., 2004; Gessner et al., 2010). Fungi can collaborate in litter decomposition, for example when different species (with different enzymes production) contribute with complementary enzymes to degrade plant polymers (Gessner et al., 2010). Antagonistic interactions, able to inhibit growth through the production of antibiotic substances, have also been observed (Shearer and Zare-Maiven, 1988; Khan, 1987; Asthana and Shearer, 1990). Shearer and Zare-Maiven (1988) showed that almost all interspecific interactions result in an inhibition of growth of one or two colonies in treatments with two isolates.

Nevertheless, little is known about the relationships that are established between fungi during colonization and much less about the effect that stressors have on these relationships.

Fragmentation. Leaf litter fragmentation corresponds to foliar physical degradation, resulting from the abrasion and stress exerted by the force of the water or sediments (Gessner, 1999), and the removal of pieces through feeding and digestion by the macroinvertebrates, mainly shredders (Graça, 2001; Canhoto and Graça, 2008). Detritivores prefer to feed on leaves colonized by fungi, not only because the increased nutritional value of the detritus and leaf softness, but also because most invertebrates can't digest energy-rich foliar compounds (cellulose). In this case, they may rely on fungal exoenzymes that can remain active in their digestive system (Canhoto and Graça, 2008).

Invertebrates show fungal or fungal/leaf preferences, being able to discriminate between leaf substrates with different fungal species (Arsuffi and Suberkropp, 1985; Gonçalves et al., 2014). These preferences can be related with the distinct nutritional characteristics among species of aquatic hyphomycetes (Canhoto and Graça, 2008) and/or with the production of attractive or rejection compounds that may also shape fungal interactions (Arsuffi and Suberkropp, 1984; Rong et al., 1995) and the establishment of specific communities. Fungal stoichiometry (Cross et al.,

2005; Danger et al., 2016) has been pointed as crucial for the stimulation or inhibition of consumption by detritivores. Gonçalves et al. (2014) suggest that the interactions occurring between fungi could be hiding the preference or rejection by invertebrates, since the leaf consumption by invertebrates tended to be higher in the treatments with a single species of aquatic hyphomycetes than in treatments with several species of aquatic hyphomycetes.

In addition, the lipids and carbohydrates incorporated in the conditioning phase may be necessary for the metamorphism and reproduction of some invertebrates (Canhoto and Graça, 2008). For these reasons, leaves colonized by fungi are the preference of invertebrates, providing greater survival, growth and fecundity (Bärlocher and Kendrick, 1975; Graça, 2001; Canhoto and Graça, 2008).

1.3 Threats to Headwater systems: The special case of Streams Salinization

Water is paramount for man, either for direct use or for activities such as agriculture and industry. However, freshwater systems are considered the most endangered ecosystems due to their overexploitation, pollution, destruction, degradation of habitats and even due to invasion by exotic species (Dudgeon et al., 2006; Geist, 2011). Headwaters in particular are very sensitive to disturbances due to their small dimensions, isolation and close relationship with their drainage basin (Gomi et al., 2002; Meyer et al., 2007).

The salinization of streams and rivers has been expanding and intensifying by global warming and climate change, since an increase in temperature (increased evaporation) and decrease in precipitation, along with extreme events are expected, leading to important losses of biodiversity, ecosystem functions and associated services provided by these systems. Therefore, salinization is considered one of the most important stressors for watercourses and is currently a widespread concern (Williams, 2001; Kaushal et al., 2005; Millennium Ecosystem Assessment, 2005; Piscart et al., 2005; Cañedo-Argüelles et al., 2013; Szöcs et al., 2014; Timpano et al., 2018).

Salinization is defined as the concentration of salts (inorganic ions) dissolved in water or soil (Kaushal et al., 2005; Kefford et al., 2012). Salinity may result from natural events such as the dissolution of the watershed (Williams, 2001; Cañedo-Argüelles et

al., 2013) and even precipitation by evaporation of sea water (Williams, 1999; Herczeg et al. al., 2001). Despite the above-mentioned natural causes, anthropogenic activities (called secondary salinization) have been one of the main causes of increased salinity in rivers.

Secondary salinization includes several activities, such as agriculture, mining, deforestation and even salt (NaCl) use for ice-melting. These activities can lead to a mobilization or extraction of salts found in soils, that can reach directly the surface of the water or the groundwater and then drain into the rivers (Irrigation - Williams, 2001; Hart et al., 2003; Cañedo-Argüelles et al., 2013; Mining activity - Williams, 2001; Cañedo-Argüelles et al., 2012, Cañedo-Argüelles et al., 2013; Deforestation - Williams 2001; Kefford et al 2006; Van Dijk et al., 2007; Ice-Melting -Williams et al., 2000; Kaushal et al., 2005; Cañedo-Argüelles et al., 2013).

By definition, freshwater courses have values of salinity <0.5 g / L (Teixeira et al., 2007; Cañedo-Argüelles et al., 2013) and several publications point to adverse effects on biota when salinity values exceed 1g / L (Hart et al., 1991; Dunlop et al., 2005; Kaushal et al., 2005). However, very little is known on how streams salinization affects some of the key players of ecosystem processes such as decomposition and its protagonists.

1.4 Impacts of Streams' Salinization

It is generally accepted that salinization of freshwaters can significantly affect the biota, ecosystem functions and provided services (Silva et al., 2000; Kefford et al., 2011; Canhoto et al. 2017). Nonetheless, there is still no consensus on the subjacent reasons for its effects. This may be due to distinct approaches and environmental contexts but also due to the fact that salinity may be due to different ions. Furthermore, salt-contamination usually does do occur "isolated", but in parallel with other stressors, that also have specific effects on watercourses and on their biodiversity increasing or decreasing the effects of salinity by itself. For example, very high pH values (pH = 11) increase organism sensitivity to salinity (Cañedo-Argüelles et al., 2013), but low temperatures increase tolerance (Hall et al., 2002; Kennedy et al., 2004). In addition, salinity contamination can be sensed in different ways – it can be acute or chronic, occur discrete and sharply (pulses), rise quickly (in concentration)

before reaching stable level (press) or gradually over time (ramp) – determining oscillations on salt concentration with potentially distinct amplitudes and frequencies (Lake, 2000; Muehlbauer et al., 2011). Consequences of such differences are barely known (Cañedo-Argüelles et al., 2014).

Most studies on the effects of salinization have been biased toward invertebrates. Authors point to a decrease in diversity and abundance in salt-rich environments (Piscart et al., 2005; Pinder et al., 2005; Kefford et al., 2006; Cañedo-Argüelles et al., 2012; Bäthe et al., 2011). Such effects on invertebrates seem to highly depend on their ability to osmoregulate (Cañedo-Argüelles et al., 2013; Zinchenko et al., 2013; Kefford et al., 2016). This adaptation has a great metabolic cost that can affect the viability of organisms (reproduction and growth) and their resilience. Therefore, when the osmoregulatory capacity is exceeded, the death of the organisms may occur (Hart et al., 1991; Cañedo-Argüelles et al., 2013). Streams salinization has been linked to a negative effect on scrapers and shredders, which lead to a decrease of leaf decomposition (Schäfer et al., 2012; Cañedo-Argüelles et al., 2014).

Studies on the effects of salinization on fungi are scarce. However, it is now known that aquatic hyphomycetes are less sensitive to salinity than invertebrates, but even at low levels of salinity, and despite some salinity tolerance, the activity (conidial production, oxygen consumption, mycelial biomass accumulation, fine particulate organic matter production and leaf mass loss) of fungal communities is affected (Hart et al., 1991; Silva et al., 2015; Sauer et al., 2016; Canhoto et al., 2017). This can lead to changes in community composition that can differ in their degradative efficiency and therefore streams functioning (Canhoto et al., 2017). Previous studies suggest that salinization leads to the inhibition of fungal reproduction rather than growth (Byrne and Jones, 1975) while others point to an increase in sporulation at low levels of salinization (1-3g/L) (Sridhar and Bärlocher, 1997). This means that in seawater or brackish water at least some species of fungi can survive, grow and reproduce (Müller-Haeckel and Marvanová, 1979).

Previous studies suggest that aquatic hyphomycetes from watercourses contaminated with different concentrations of heavy metals, have different phenotypes, growth and may establish distinct interspecific interactions, which may correspond to a genetic tolerance or adaptation to the contaminant (Braha et al.,

2007; Ferreira et al., 2010). Since the growth and sporulation of many aquatic hyphomycetes species is affected by salinity and that some species are found in contaminated streams, it seems possible that, like in heavy metal polluted water courses, some species might be tolerant/resistant or adapted to salinized streams. It seems also conceivable that fungal interactions, such as competition and antagonism, may be affected; less severe interactions might be beneficial for the species in such contaminated environments. Nevertheless, there are still few studies on the salinity of watercourses in ecosystem processes, and on the effects on fungal metabolic parameters and ecological interactions. Studying the potential impact of this stressor in streams and on fungi in particular should be a priority, considering their pivotal role on leaf litter breakdown and functioning of the stream ecosystems.

1.5 Objectives

In this study, we aimed to assess the potential effects of salt-addition on leaf mass loss and microbial parameters of single and multispecies assemblages of two strains (isolated from a reference (R) or a historically saline (S) stream) of three aquatic hyphomycetes species (*Heliscus lugdunensis*, *Tetracladium marchalianum* and *Flagellospora curta*). Fungal morphology, growth, and intra- and interspecific interactions were also evaluated.

We hypothesized that the morphology of strains isolated from the salt-rich stream will be distinct of the correspondent strains from the reference stream. Fungal species growth will be higher in the medium with salt (NaCl) concentration similar to the one observed in the stream of origin. Regarding interactions and degradation efficiency, we expect that if the presence of the species in the salt-rich stream is due to their tolerance to the salt, then both strains are likely to react in the same way to a salt-rich and reference media. If the presence is explained due to genetic adaptation, then both strains are expected to react best when kept in the medium with the salt (NaCl) concentration similar to the one observed in the stream of origin.

Chapter 2
Materials and Methods

2. Materials and Methods

2.1 Experimental process

Two strains (R and S) of three AH species - *Heliscus lugdunensis* (HELU), *Tetracladium marchalianum* (TEMA) and *Flagellospora curta* (FLCU) – were isolated from single conidia, released from submerged leaf litter collected from two different sites: S strains were collected in the Pontével stream (Cartaxo; 39°08'40"N; 8°50'04.4"W), which presented a salted water (with 6 g/L of salt) due to the surrounding agricultural activities; R strains were collected from the Candal stream (Lousã; 40°4’44”N, 8°12’10”W), a pristine headwater (0,01 g/L of salt). Pure cultures of both strains of all aquatic hyphomycetes species grown on malt extract agar solid medium (MEA; 2%) at the correspondent salt concentration of origin (0 or 6 g/L NaCl) for 14 days.

2.1.1 Morphology and Growth of R and S fungal species strains

In order to evaluate the morphology and growth of the different strains when subjected to both salt conditions found in the streams of origin of the two AH strains (R and S): an agar plug (ø = 4 mm) was discarded from the edge of the growing pure colonies and placed in the middle of Petri dishes previously filled with reference and salt-rich MEA.

In total, 36 petri dishes were used, where each strain of each species was submitted to both R and S medium, with 3 replicates for each treatment (3 species x 2 strains x 2 treatments x 3 replicates). The colony morphology was compared visually, where it was attempted to find differences between strains in the color, shape and growth of the colony. The diameter of each colony was measured every two days, until one of the colonies had reached the distance of 1cm from the wall of the petri dish, where from that moment all growth stopped. For growth, graphs were obtained with the absolute growth of the colonies per day and radial growth rate was calculated through the slope of the linear regression obtained between the diameter of the colony and the time, being expressed as mm/day.

2.1.2 Fungal Interactions

To evaluate the intra and interspecific interactions among AH, one agar plug (ø = 4 mm) from each pure culture of both strains (R and S) of the different three species grown in R and S MEA medium was placed in petri dishes at the same distance between plugs and between them and the petri dish edge.

All possible interactions, including paired and triplet groups, were performed in both media in a fully factorial design as shown in table I, with 5 replicates per each treatment. The interactions type was evaluated when the interaction was clear, with one colony met each other (as in Ferreira et al, 2010). At that time, two distances from the center of each colony of each petri dish were measured, one in relation to the edge of the petri dish (R1) and the other until the area of contact of the interaction (here called R2). The values of R1 and R2 allowed to calculate the percentage inhibition of each colony in each interaction, through the formula (((R1 – R2) / R1) x 100 (Shearer and Zare-Maivan 1988; Bärlocher 1991)), that is, for each treatment, each colony has its percentage of inhibition (whenever it has occurred), representing how much their growth was inhibited by the other colony. This inhibition could often be observed with the naked eye, but it was always confirmed by observation of the contact area of the colonies under a microscope.

Table I - Complete experimental design of interactions test using both strains (R – reference; S – saline) of three fungal species: HELU - *Heliscus lugdunensis*, TEMA - *Tetracladium marchalianum* and FLCU - *Flagellospora curta* (n = 5).

Intraspecific Interactions

HELU-R x HELU-R	Reference medium and Salted medium
HELU-S x HELU-S	
HELU-R x HELU-S	
TEMA-R x TEMA-R	
TEMA-S x TEMA-S	
TEMA-R x TEMA-S	
FLCU-R x FLCU-R	
FLCU-S x FLCU-S	
FLCU-R x FLCU-S	

Interspecific Interactions

HELU-R x TEMA-R	Reference medium and Salted medium
HELU-S x TEMA-S	
HELU-R x FLCU-R	
HELU-S x FLCU-S	
TEMA-R x FLCU-R	
TEMA-S x FLCU-S	
HELU-R x TEMA-R x FLCU-R	
HELU-S x TEMA-S x FLCU-S	

In addition, the type of interaction was classified according to Yuen et al. (1999), and the antagonism index (IA) was also calculated for each strain of each species in each treatment. The total number of interactions of each type that a particular strain had in each medium was counted, and the following formula was used $IA = B1(n\times1) + B2(n\times1) + C(n\times2) + D(n\times3) + E1(n\times4) + E2(n\times4)$, where the capital letters are the nomenclature of the interaction type and n is the number of times that the strain presented this interactions type.

2.1.3 Degradative Efficiency

To evaluate the degradation efficiency of reference and saline AH assemblages, sets of 20 dried and weighted discs (ø = 12mm) of senescent poplar leaves (*Populus nigra L.*; as the most representative tree species in the present study streams) were immersed in Erlenmeyer flasks (microcosms) with 40mL of nutrient solution (75.5 mg CaCl2, 10 mg MgSO4.7H2O, 0.5 g 3-morpholinopropanesulfonic acid (MOPS), 5.5 mg K2HPO4 and 100 mg KNO3 per liter of sterile distilled water; pH = 7; Dang et al., 2005) at both conditions, reference and salted (where 6g/L of NaCl was added to the nutrient solution to simulate the same concentration of salt than the stream of origin of saline AH strains). A total of 48 microcosms were inoculated with different AH species and/or strains, in a complete factorial design. While 6 replicates were inoculated with each strain of each AH species individually, placing one plug (ø = 12mm) in each microcosm, 6 other replicates were incubated with each type of mixed assemblages (reference - including the R strains of the three species together; saline - with the saline strains of the same three species together). For this purpose, plugs of each species/strain (ø = 7mm) were used, thus ensuring that all treatments (individual or mixed) had the same total inoculum size. Then, half of the replicates of each treatment was exposed to each liquid medium concentration, reference and salted. All microcosms were incubated on orbital shakers at 120 rpm under photoperiod (12 h light: 12 h dark).

After 7 days (inoculation period) the nutrient solution of each microcosm was renewed, and the plugs were excluded. The medium was then replaced every 48h. At the end of 15 days (common sporulation peak period according to Graça et al. 2005) discs from each microcosm were used to evaluate the leaf mass loss, ergosterol content (fungal biomass) and sporulation.

The evaluation of dry mass loss (% DM) after the conditioning period was estimated by the difference between the initial and final leaf dry mass of the 20 discs (previously weighed) from each microcosm.

In order to determine the fungal biomass, the ergosterol concentration (Gessner and Chauvet, 1993; Young, 1995) was analyzed, 5 leaf discs were frozen, then lyophilized, weighed, the ergosterol extracted and measured by liquid

chromatography. The values obtained to the concentration of ergosterol were converted into fungal biomass using the conversion factor of 5.5 μg ergosterol mg^{-1} fungal dry mass (Gessner and Chauvet, 1993). The results were expressed as mg of fungal biomass per g of dry mass.

In the case of sporulation, when the microcosm experiments were stopped, 5 discs were maintained with 25mL of the same medium to induce sporulation for 48h, at which time the suspensions were collected, and conidia fixed with 2ml of formalin (37%). Then, they were homogenized using 100μl Triton X-100 (0,5%), an aliquot was filtered (Millipore SMWP filters, 5μm pore size) and spores stained with 0.05% cotton blue in lactic acid (60%). Finally, the number of spores produced by species/strain in each microcosm was counted under a microscope (at 250x) to calculate the sporulation rate, which was expressed as the total number of conidia produced per mg of leaf dry mass per day.

2.2 Data and Statistical analysis

To evaluate the existence of significant differences in growth (as the colony diameter) among species, between strains and treatments, it was used the ANOVA of Repeated Measures, since there was a time scale of samples, followed by Tukey's test when necessary to clarify the specific differences. Before using ANOVA, the Levene's variance homogeneity test was performed, and data were previously transformed when necessary to achieve the assumptions of ANOVA. The statistical analysis performed to compare the percentage of inhibition of each strain in each interaction was paired t-Test, in order to know if the inhibition between colonies were significant.

To verify the existence of significant differences between the three factors (species, strains and treatments) in relation to mass loss, fungal biomass and sporulation rate, a factorial ANOVA was carried out. When significant statistical differences were found ($P < 0.05$), Tukey's test were used to identify the significant effects. To confirm the homogeneity of variances, all data were previously submitted to Levene's test.

Chapter 3
Results

3. Results

3.1 Morphology and Growth rate of R and S fungal species strains

3.1.1 Morphology

Different responses of the AH strains to the presence of salt can be observed on the fungal morphology, in terms of colony shape and pigmentation (Fig. 1). While HELU and TEMA grown in a perfect circular form, independently of the strain origin's and the presence of salt in the solid medium. In the case of FLCU, R strain in reference medium presented an irregular boundary, but, when exposed to salt, this strain tends to growth as the FLCU-S strain, in a perfect circular shape.

Regarding the color of fungal colonies, they exhibit a considerable diversity depending on the salt medium conditions as well as on the origin of fungal strains. The HELU strains are different, being the HELU-R brown and translucent, and the HELU-S white and completely transparent. The salt addiction does not affect the color of HELU strains. TEMA strains color does not reflect sensitivity to the salt (all colonies are white). On other hand, FLCU strains have different pigmentation, with opaque brown FLCU-R and transparent white FLCU-S colonies. However, salt exposition does not affect the color of FLCU strains.

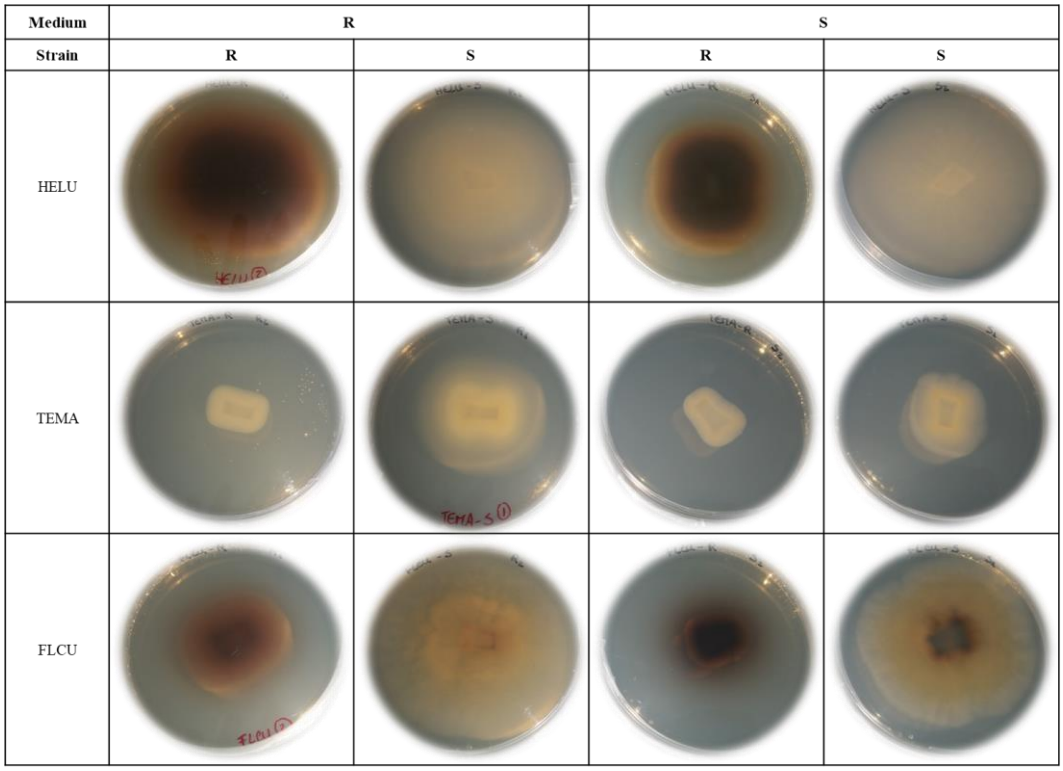

Figure 1 – Morphology of two strains (R – reference; S – saline) of *H. lugdunensis, T. marchalianum* and *F. curta* grown for 21 days in two MEA media, a reference and salted medium (with 6g/L NaCl).

3.1.2 Growth and Growth rate of R and S fungal species strains

The growth of AH colonies shown to be significantly different between species, strains and medium salt concentration (Factorial Repeated Measures ANOVA, F = 4788.60, 733.8, 2725.8, P < 0.001; respectively). In general, fungal hyphae seems to disperse significantly better in the reference medium (Tukey's test P < 0.001; Fig. 2, 3 and 4), with HELU growing faster than the other two species that growth similarly each other (Tukey's test P < 0.001; Fig. 2; Table II). In most cases, saline strains grown faster, independently of the species or medium salt concentration (Tukey's test P < 0.001; Fig. 2, 3 and 4; Table II). The behavior of HELU and FLCU was similar, with both strains growing at the same rate when exposed in the respective salt of origin concentration (see Tukey's significances in Table II). However, if S strains were stimulated in the absence of salt, the R strains were negatively affected by salt, growing slower than all the other treatments (Table II). In opposition, both strains of TEMA prefer to growth without salt in the medium, with R dispersing faster than S strain. Growth rate of TEMA-R colonies was also the slowest (Table II).

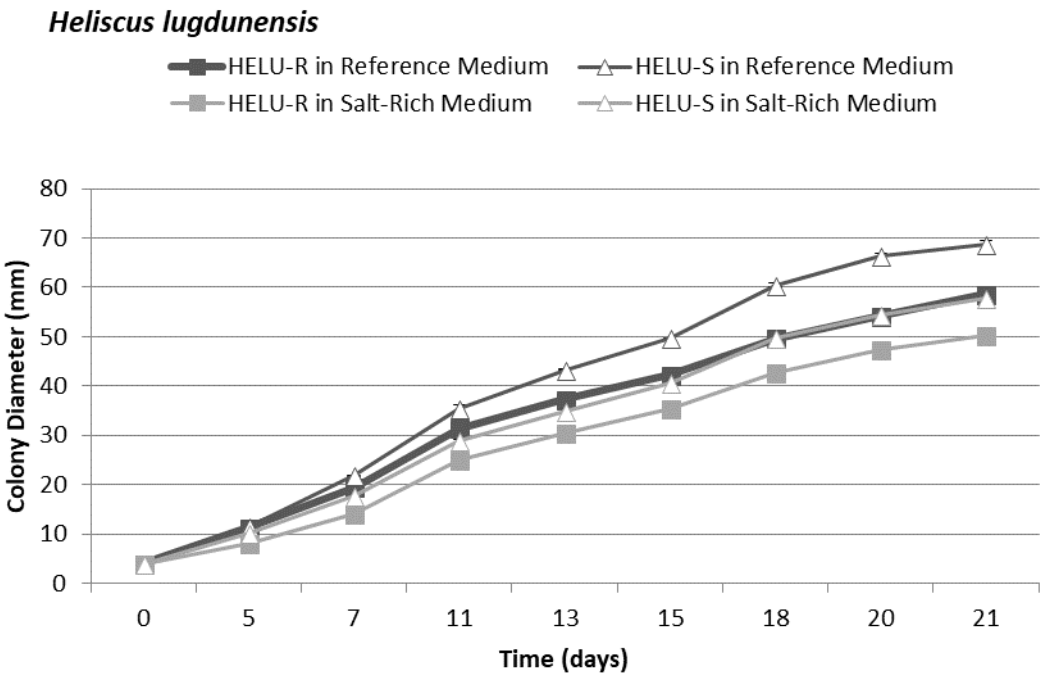

Figure 2 - Absolute growth of two strains (R - reference; S - saline) of *H. lugdunensis* (HELU) for 21 days in two MEA media, a reference and a salted medium (with 6g/L NaCl). The SE bars are smaller than the symbols.

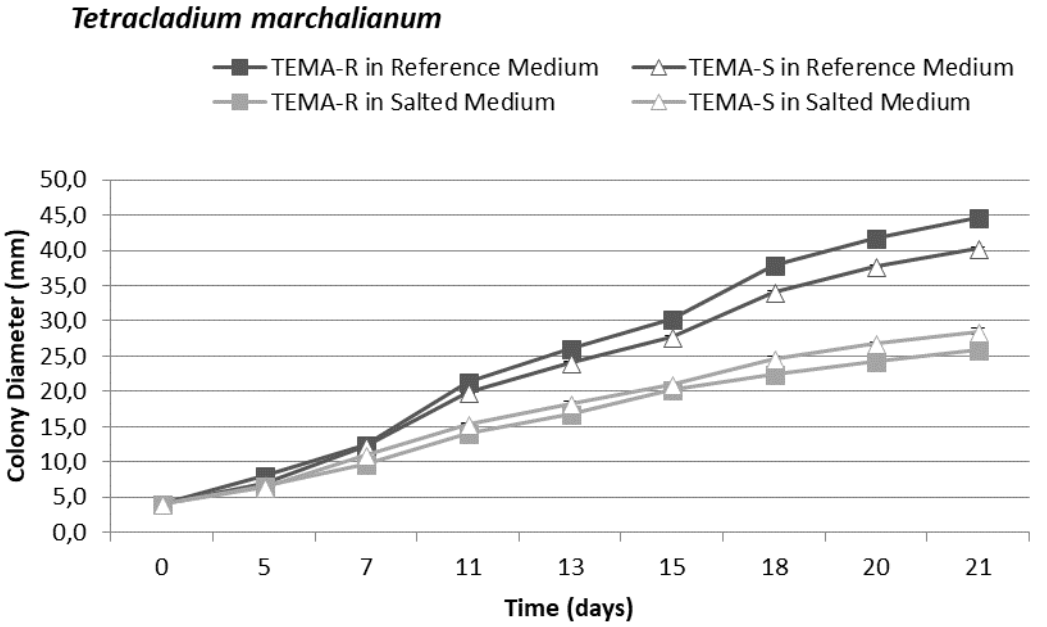

Figure 3 - Absolute growth of two strains (R - reference; S - saline) of *T. marchalianum* (TEMA) for 21 days in two MEA media, a reference and a salted medium (with 6g/L NaCl). The SE bars are smaller than the symbols.

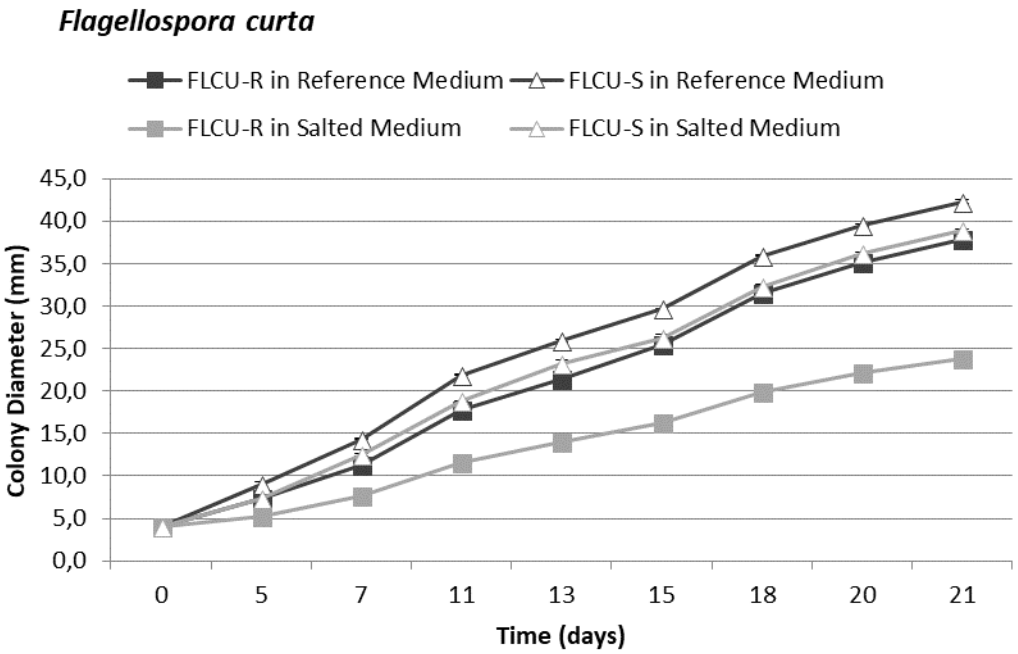

Figure 4 – Absolute growth of two strains (R - reference; S - saline) of *F. curta* (FLCU) for 21 days in two MEA media, a reference and a salted medium (with 6g/L NaCl). The SE bars are smaller than the symbols.

Table II - Growth rate (mm/day) of two strains (R - reference; S - saline) of the three AH species here tested in two MEA media, a reference and a salted medium (with 6g/L NaCl). Comparisons within the same species were made through ANOVA of repeated measures followed by Tukey's test (different letters indicate significant differences for $P < 0.05$).

Species	Strain	Treatment	Growth rate	SE	R^2	P	Tukey's Test
Heliscus lugdunensis	Reference	Reference	2.684	0.090	0.992	< 0.001	b
	Salted	Reference	3.300	0.127	0.990	< 0.001	a
	Reference	Salted	2.359	0.113	0.984	< 0.001	c
	Salted	Salted	2.707	0.106	0.989	< 0.001	b
Tetracladium marchalianum	Reference	Reference	2.052	0.102	0.983	< 0.001	a
	Salted	Reference	1.835	0.091	0.983	< 0.001	b
	Reference	Salted	1.101	0.047	0.987	< 0.001	d
	Salted	Salted	1.228	0.052	0.988	< 0.001	c
Flagellospora curta	Reference	Reference	1.692	0.091	0.980	< 0.001	b
	Salted	Reference	1.885	0.063	0.992	< 0.001	a
	Reference	Salted	1.007	0.065	0.971	< 0.001	c
	Salted	Salted	1.730	0.082	0.985	< 0.001	b

3.2 Fungal Interactions

In more than a half of the total of here tested interactions, independently if it was between species or strains or near 1 or 2 different colonies in the same Petri dish, the observed interaction was the type “mutual intermingling”, with hyphae of both colonies mixing, inducing or not a growth rate reduction of one of the colonies. The other two types of interactions here detected caused a mutual inhibition, most of the times just when the mycelium of both colonies touched each other, ceasing the growth of both species (mutual inhibition at contact; observed 14x), and at a distance (observed 4x).

3.2.1 Intraspecific Interactions

When we tested how the stains of the same species interact with each other, it was found that they do not react very aggressively, varying its inhibition capability between 20.09 and 36.69% in reference medium. Among all the possible combinations, only significant inhibition of growth occurred when the different HELU and TEMA strains faced each other, with the HELU-R being the most aggressive (Paired t-test, $P < 0.001$), while in TEMA it is the strain S (Paired t-test, $P = 0.006$). In both species, the type of interaction is the mutual intermingling (except when TEMA-S faced up to itself), while within FLCU mutual inhibition occurred when at contact.

In general, when in the presence of salt, this aggressiveness tends to decrease even more (9.09-37.56% in salted medium), also attenuating the differences between strains, where only HELU preserved the same significant differences (Paired t-test, $P < 0.001$). Additionally, salinity seems to inhibit the contact between strains colonies, with growth ceasing as soon as they come into contact (HELU and TEMA) or even at a distance (FLCU). FLCU only showed significant inhibition when it came to interaction between different strains in the presence of salt.

3.2.2 Interspecific Interactions

When in reference medium, in all combinations of species including the HELU, regardless of strain, a significant inhibition occurred (Paired t-test, $P < 0.001$), and HELU colonies imposed an inhibition between 26.40 and 38.80% in all the interactions, except when strain S is exposed to FLCU-S, where the growth of HELU-S was inhibited about 30.60% and just caused a 18.19% inhibition. Both strains of TEMA and FLCU species do not react significantly to each other (Paired t-test, $P > 0.256$). In general, the interactions do not change when the three species interacted simultaneously with each other in the same reference medium (only HELU-S and TEMA-S stopped interacting significantly between them, not inhibiting each other).

Salt addition to the medium increased aggressiveness between fungal species of the same strains, both those that already existed (in all interactions with the presence of HELU, with this species significantly inhibiting all other species; Paired t-test, $P < 0.001$), and FLCU also became more competitive facing TEMA (Paired t-test, $P < 0.015$), inhibiting the developing of its colonies between 19.17 (strain S) and 29.98% (strain R). However, in the presence of salt the response between species underwent some changes when all species were simultaneously exposed to each other: when species were from salinized stream (the same salt concentration in medium), there was no significant interactions (Paired t-test, $P > 0.063$) and mycelium of each colony grew toward the other without inhibition or mutually inhibiting the species at a distance. On the other hand, HELU-R strain continues to dominate the other two species, even experiencing a mutual intermingling among the hyphae. It should be noted that in this triage situation, no interaction occurred between strain R of TEMA and FLCU, and that between the S strains there was a mutual inhibition without contact between hyphae (although not statistically different; Paired t-test, $P = 0.307$).

Table III – Induced inhibition of growth (average ± 1 SE) for each intraspecific interacting species and interaction type (see text for definitions).

Medium	Interaction	Species	Strain	Inhibition (%)	Species	Strain	Inhibition (%)	Paired t-test (*P*)	Interaction Type
Reference	**Intraspecific (pairs)**	HELU	R	24.16 ± 1.26	HELU	R	20.09 ± 1.57	0.078	Mutual intermingling
		HELU	R	25.50 ± 1.19	HELU	S	36.69 ± 0.54	< 0.001	Mutual intermingling
		HELU	S	35.88 ± 1.14	HELU	S	35.34 ± 0.33	0.401	Mutual intermingling
		TEMA	R	36.41 ± 0.66	TEMA	R	33.87 ± 0.88	0.050	Mutual intermingling
		TEMA	R	32.95 ± 1.23	TEMA	S	25.62 ± 1.52	0.006	Mutual intermingling
		TEMA	S	32.18 ± 2.08	TEMA	S	30.20 ± 2.56	0.565	Mutual inhibition at contact
		FLCU	R	31.24 ± 1.40	FLCU	R	28.46 ± 1.97	0.315	Mutual inhibition at contact
		FLCU	R	32.41 ± 0.59	FLCU	S	31.50 ± 0.32	0.213	Mutual inhibition at contact
		FLCU	S	31.49 ± 1.42	FLCU	S	30.51 ± 0.93	0.617	Mutual inhibition at contact
	Interspecific (pairs)	HELU	R	0.00 ± 0.00	TEMA	R	35.09 ± 3.51	< 0.001	Mutual intermingling
		HELU	S	0.00 ± 0.00	TEMA	S	26.40 ± 0.49	< 0.001	Mutual intermingling
		HELU	R	14.79 ± 1.48	FLCU	R	38.80 ± 2.19	< 0.001	Mutual intermingling
		HELU	S	30.60 ± 1.40	FLCU	S	18.19 ± 2.83	< 0.001	Mutual inhibition at contact
		TEMA	R	33.88 ± 1.44	FLCU	R	30.39 ± 2.60	0.274	Mutual intermingling
		TEMA	S	27.11 ± 3.52	FLCU	S	32.22 ± 2.05	0.256	Mutual intermingling
	Interspecific (triplet)	HELU	R	0.00 ± 0.00	TEMA	R	33.09 ± 3.94	< 0.001	Mutual intermingling
		HELU	R	0.00 ± 0.00	FLCU	R	10.90 ± 3.62	0.024	Mutual intermingling
		TEMA	R	7.59 ± 4.80	FLCU	R	8.04 ± 5.07	0.950	Mutual inhibition at contact
		HELU	S	21.62 ± 4.20	TEMA	S	23.34 ± 2.32	0.732	Mutual intermingling
		HELU	S	19.15 ± 3.24	FLCU	S	33.15 ± 2.15	0.007	Mutual inhibition at contact
		TEMA	S	13.68 ± 3.62	FLCU	S	9.71 ± 1.74	0.378	Mutual intermingling

Salted	**Intraspecific (pairs)**	HELU	R	27.44 ± 1.28	HELU	R	28.51 ± 1.08	0.543	Mutual inhibition at contact
		HELU	R	17.11 ± 0.79	HELU	S	37.56 ± 1.99	< 0.001	Mutual inhibition at contact
		HELU	S	28.09 ± 1.10	HELU	S	30.61 ± 0.78	0.816	Mutual intermingling
		TEMA	R	21.95 ± 3.19	TEMA	R	23.96 ± 3.12	0.666	Mutual inhibition at contact
		TEMA	R	21.18 ± 1.44	TEMA	S	23.96 ± 1.60	0.231	Mutual inhibition at contact
		TEMA	S	27.47 ± 1.76	TEMA	S	26.23 ± 2.07	0.660	Mutual inhibition at a distance
		FLCU	R	24.08 ± 1.91	FLCU	R	18.14 ± 3.43	0.168	Mutual inhibition at a distance
		FLCU	R	9.09 ± 0.00	FLCU	S	14.02 ± 1.73	0.047	Mutual inhibition at a distance
		FLCU	S	27.00 ± 1.84	FLCU	S	27.081.37	0.975	Mutual inhibition at contact
	Interspecific (pairs)	HELU	R	0.00 ± 0.00	TEMA	R	32.44 ± 2.45	< 0.001	Mutual intermingling
		HELU	S	0.00 ± 0.00	TEMA	S	31.43 ± 2.62	< 0.001	Mutual intermingling
		HELU	R	0.00 ± 0.00	FLCU	R	38.43 ± 0.75	< 0.001	Mutual intermingling
		HELU	S	23.37 ± 0.94	FLCU	S	38.88 ± 2.04	< 0.001	Mutual inhibition at contact
		TEMA	R	29.98 ± 1.73	FLCU	R	15.96 ± 3.79	0.015	Mutual inhibition at contact
		TEMA	S	19.17 ± 2.70	FLCU	S	0.00 ± 0.00	< 0.001	Mutual intermingling
	Interspecific (triplet)	HELU	R	0.00 ± 0.00	TEMA	R	28.24 ± 3.81	< 0.001	Mutual intermingling
		HELU	R	0.00 ± 0.00	FLCU	R	23.88 ± 8.14	0.026	Mutual intermingling
		TEMA	R	-	FLCU	R	-	-	-
		HELU	S	20.72 ± 2.12	TEMA	S	18.25 ± 3.81	0.592	Mutual intermingling
		HELU	S	26.81 ± 4.68	FLCU	S	37.75 ± 1.95	0.063	Mutual intermingling
		TEMA	S	20.23 ± 6.34	FLCU	S	11.36 ± 5.08	0.307	Mutual inhibition at a distance

3.3.3 Antagonism Index

The Antagonism Index (IA) shows the ability of each fungal colony to compete and dominate the others when in contact. In figure 5, it is possible to observe evident differences among the IA of the three species in the reference medium (HELU >> FLCU > TEMA), even though without differences between strains.

Medium salt addition tends to standardize the competitiveness of all strains, except to HELU-R, presenting an IA thereabout 2x higher than the other strains. Independently of the strain origin, almost all of them became more antagonists when exposed to salt, just contrasting with HELU-S. In these conditions, both TEMA strains were stimulated to similar levels of antagonism of the other strains.

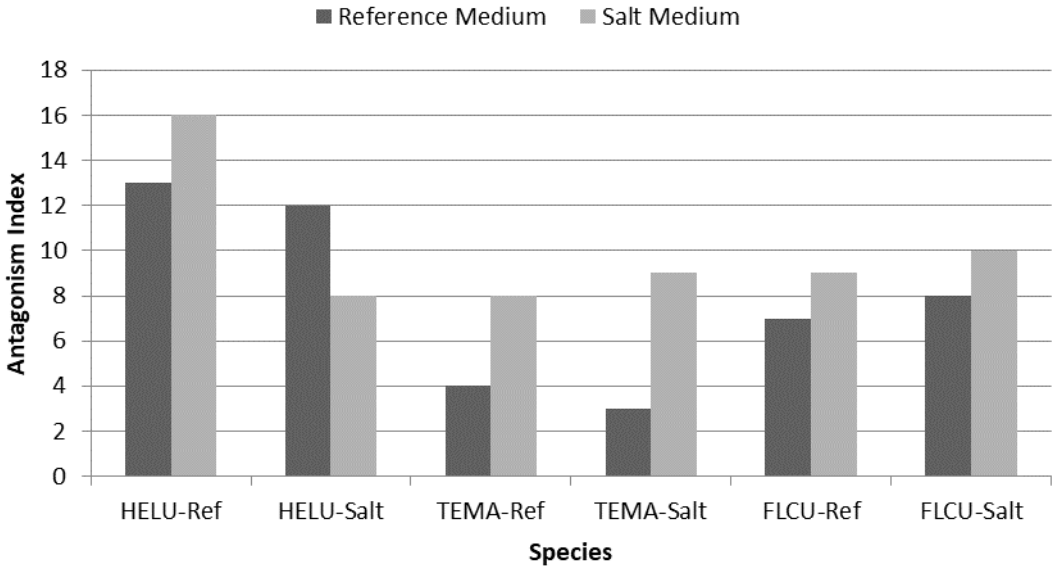

Figure 5 - Antagonism index of two strains (R - reference; S - saline) of *H. lugdunensis* (HELU), *T. marchalianum* (TEMA) and *F. curta* (FLCU) in two MEA media, a reference and a salted medium (with 6g/L NaCl).

3.2 Degradative Efficiency

3.3.1 Mass Loss

In terms of decomposition, mass loss showed to be only significant different between media (Factorial ANOVA F = 9.11, P = 0.005), with strains appear to

decompose more in the absence of salt (Tukey's test, P = 0.005). However, both by looking at the graphs and by statistical analysis between strains and species, differences in decomposition were not significant. Additionally, no differences were detected in the presence of mixed or individual fungal assemblages.

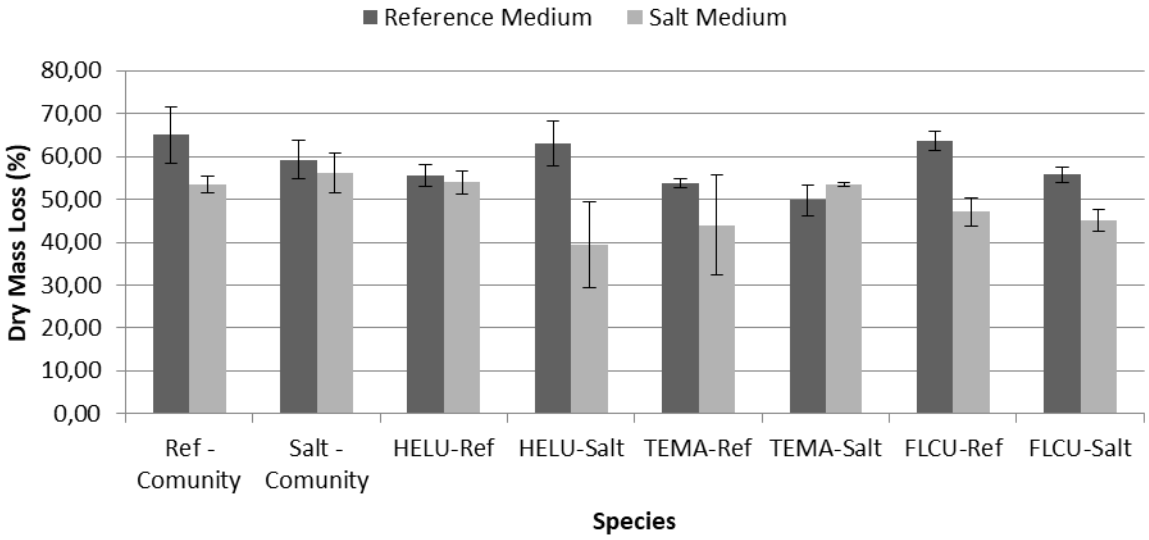

Figure 8 - Mass loss of poplar leaves of two strains (R - reference; S - saline) of *H. lugdunensis* (HELU), *T. marchalianum* (TEMA) and *F. curta* (FLCU), as well as two communities (one formed by the three species coming from the reference stream and another by these same species but coming from the salinized stream) in two MEA media, a reference and a salted medium (with 6g/L NaCl). Comparisons were made through Factorial ANOVA followed by the Tukey's test (different letters indicate significant differences for p <0.05).

3.3.2 Sporulation

The sporulation rate showed to be significantly different between species (Factorial ANOVA F = 42.48; P <0.001) and strains (Factorial ANOVA F = 28.91; P < 0.001), but not between medium salt concentration (Factorial ANOVA F = 0.98; P = 0.33). Globally, S trains usually sporulate more than the R strains (Tukey's test, P < 0.001). Independently of the presence of salt in the medium, FLCU was the species that produced more conidia by day at the individual microcosms, followed by treatments inoculated with mixed assemblages and HELU individually (that sporulate at similar rates; Tukey's test, P = 0.55). TEMA was the species that produced less number of spores (Tukey's test, P < 0.001). Mixed fungal assemblages did not show significant differences in the total conidia production, either between different strains or media (Tukey's test, P > 0.05; Figure 6). All species strains were able to produce

spores, even when exposed to different salt conditions in relation to the origin. However, the relative contribution of each species was differently affected by salt addition in the medium: without salt, the mixture of R-strains is dominated by TEMA conidia (59%), followed by HELU (38%), while in the S strains mix the FLCU was able to produce almost 63% of the total of spores; in the presence of salt, it was favored the reproduction activity of HELU, contributing with 86 and 87% of the total of spores in the mixtures of R and S strains, respectively.

Within individual treatments inoculated with each fungal species, just HELU showed significantly differences between strains, with S sporulating more than 3 times of R strain in the respective concentration of salt of the origin (Tukey's test, P = 0.02). However, when exposed to salt, R strain was not affected, maintaining its sporulation rate much lower than S strain in the same medium (Tukey's test, P = 0.001).

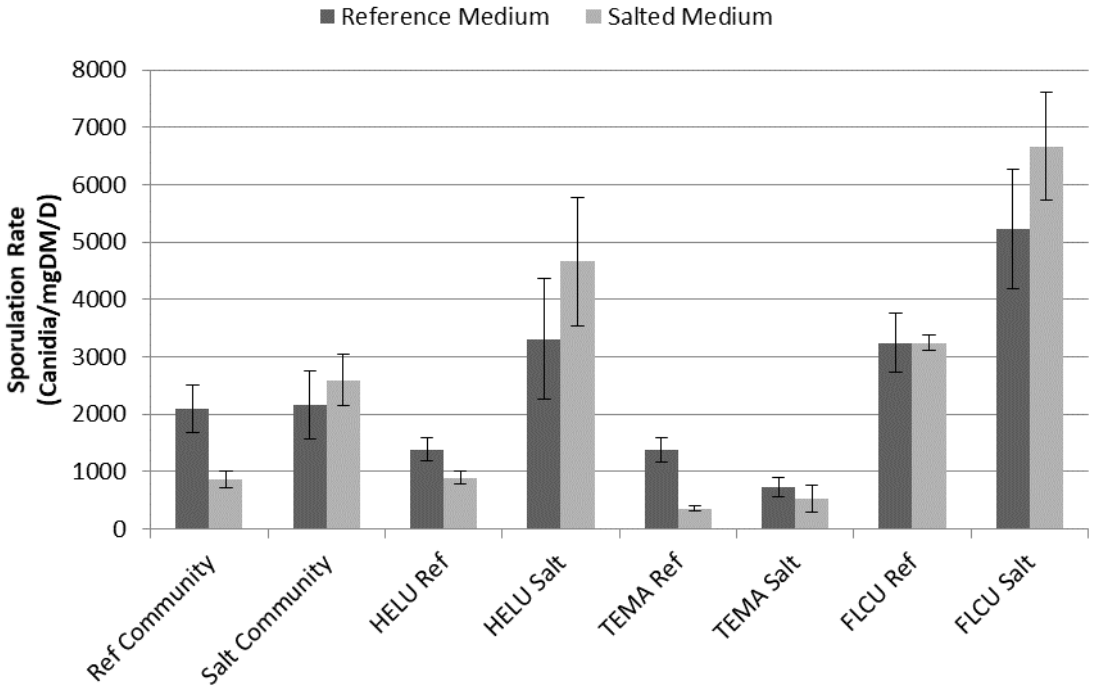

Figure 6 - Sporulation rate of two strains (R - reference; S - saline) of *H. lugdunensis* (HELU), *T. marchalianum* (TEMA) and *F. curta* (FLCU), as well as two communities (one formed by the three species coming from the reference stream and another by these same species but coming from the salinized stream) in two MEA media, a reference and a salted medium (with 6g/L NaCl). Comparisons were made through Factorial ANOVA followed by the Tukey's test (different letters indicate significant differences for $p < 0.05$).

3.3.3 Fungal Biomass

Ergosterol concentration was significantly different in relation to the media (Factorial ANOVA F = 19.28; P < 0.005), with higher fungal biomass in reference media. In community, the reference strains showed a relatively lower percentage of fungal biomass in relation to the saline strains, however, statistically there were no significant differences. HELU-S biomass was negatively affected by the salt addition to the medium (Tukey's test, P = 0.001), while the opposite was verified regarding the TEMA species, being R, the strain negatively affected by salt (Tukey's test, P = 0.002). FLCU did not present significant differences in biomass development in any treatment.

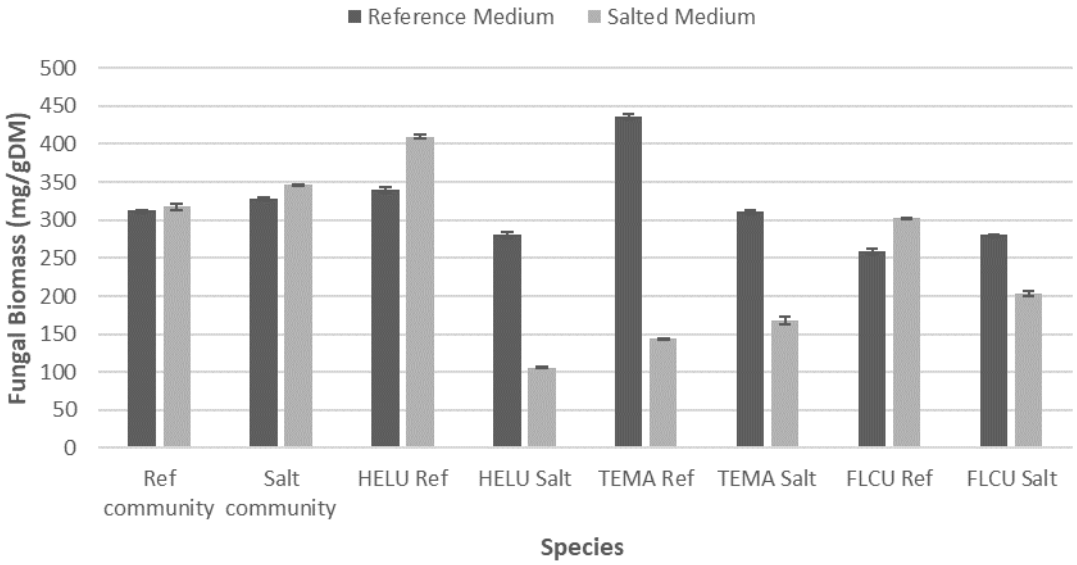

Figure 7 – Fungi Biomass of two strains (R - reference; S - saline) of *H. lugdunensis* (HELU), *T. marchalianum* (TEMA) and *F. curta* (FLCU), as well as two communities (one formed by the three species coming from the reference stream and another by these same species but coming from the salinized stream) in two MEA media, a reference and a salted medium (with 6g/L NaCl). Comparisons were made through Factorial ANOVA followed by the Tukey's test (different letters indicate significant differences for p <0.05).

Chapter 4
Discussion

4. Discussion

Results from this study indicate that the *H. lugdunensis*, *T. marchalianum* and *F. curta* were tolerant to salt-contamination. Salinization didn´t affected the colony morphology of the R or S strains. The morphology in *H. lugdunensis* varied in terms of color between strains, which may suggest intraspecific variability. In the case of *T. marchalianum*, neither R or S strains showed morphologic differences. The morphology of *F. curta* weren't affected by salinity, however the R strain in reference medium had irregular form and the others circular form; strains differed in pigmentation. This suggest that salinity can induce phenotypic changes eventually associated with tolerance. Previous studies have already observed morphological differences in fungal strains subjected to contaminants at different concentrations (e.g. heavy metals - Braha et al., 2007; Ferreira et al., 2010); different phenotypes have been associated with different resistances to the contaminant (Bohannan and Lenski, 2000).

The strains were expected to grow best in their medium of origin. However fungal hyphae seem to grow better in reference medium, with S strains having the higher growth rates in most treatments. This may indicate that S strains have undergone metabolic changes to cope with salt-induced osmotic stress. *H. lugdunensis* and *F. curta* had similar responses: The R strains were negatively affect by salinity while the S strains were stimulated in the absence of salt addition. This may mean that both species have some tolerance to salinity, which guaranties the maintenance of the growth rate even in the presence of salt. Whether this implies the synthesis of osmoprotective compounds allowing them to cope with the increase of salinity is still unknown. Studies on the effects of heavy metals suggest that aquatic hyphomycetes may have developed responses to these contaminants, such as the synthesis of compounds (phytochelatines, sulfur-rich compounds and peptides derived from glutathione) for detoxification (Mirsch et al., 2005; Braha et al., 2007) and creation of mechanisms by bioabsorption and bioaccumulation to avoid heavy metals toxicity (Jaeckel et al., 2005; Krauss et al., 2003). The strains of *T. marchalianum* species had similar growth in both treatments - both are negatively affected by the salinity - suggesting that there was no adaptation under salt contamination.

For the interactions, the growth toward the walls of the Petri dishes was greater than the growth towards the opposite colony. This result was expected since both colonies compete for space and nutrients (Bärlocher, 1991; Yuen et al., 1999). Inhibition of growth was more evident in interspecific than in intraspecific interactions, with *H. lugdunensis* being the most antagonistic. As expected, and according to Bärlocher (1991), self-inhibition was expected to be less aggressive than inhibition in interactions between different strains and species. When in the presence of salt, the percentage of inhibition in intraspecific interactions became lower. In contrast, when considering interspecific interactions, salinity increased the aggressiveness between strains of the same origin. Also, when in assemblages (all three species simultaneously) in salt-rich medium, no significant inhibition occurred between S strains. This may suggest a loss of the competitive capacity of the S strains. Among the R strains *H. lugdunensis* confirmed its dominance while no interaction occurred between *T. marchalianum* and *F. curta*.

The interactions were classified as being of three types, with mutual intermingling the most common; higher differences in the percentage of inhibition (caused and experienced) were observed in this case. Since a solid nutrient-rich medium was used, many faster-growing species extended their mycelium more rapidly against the opposite colony, being the percentage of inhibition higher in interactions where growth rates were most different. This relation between growth and inhibition was also observed by Shearer and Zare-Maivan (1988) and Ferreira et al., (2010), but not by Bärlocher (1991).

In conclusion, differences in morphology and growth between strains and media point to a possible fungal tolerance in watercourses contaminated with salt. Previous studies suggest that strains from contaminated streams may have higher tolerance than their conspecific from reference streams ((Chamier and Tipping 1997, Miersch et al. 1997) – in Ferreira et al., 2010). These differences in tolerance have been related with differences in community structure and function (Ferreira et al., 2010), meaning that those differences can be related to different species composition and abundance in the communities of each stream, leading to different degradative efficiencies. So, according to this point of view, salinized streams can lead to poorer

communities, only composed by species with high salt tolerance, that may have different functional efficiencies in relation to those of the original communities.

In line with previous studies, fungal degradative efficiency was affected by the presence of salt (Silva et al., 2015; Canhoto et al., 2017). The mass loss and fungal biomass was generally lower with the presence of salt with significant differences between media although no significant differences were found between strains and species (including mixed and single assemblages). The lower fungal biomass in the presence of salt corroborates the idea of a negative effect of salt contamination on fungal growth (Canhoto et al., 2017). Also, these results show a positive relationship between the fungal biomass and the decomposition rate, which is in accordance with other authors (Sridhar et al., 2000; Pascoal et al., 2004; Gonçalves et al., 2014). However, for experiments involving salinity, Silva et al. (2015) obtained an opposite result, where salinity did not affect fungal biomass, but negatively affected leaf decomposition. The authors explained these results as a possible energetic tradeoff between enzymatic production and mycelium metabolism in order to maintain the mycelium integrity.

In general, S strains sporulated more than R strains: *F. curta* produced the highest number of conidia, which contrasted with *T. marchalianum*. Even in multispecies assemblages there were no differences in sporulation, despite differences in dominance or identity of the dominant species. In the two assemblages submitted to salinity, *H. lugdunensis* was dominant, while in the assemblages submitted to the reference medium *T. marchalianum* (in the community with R strains) and *F. curta* (in the community with S strains) took his place. Despite the alteration of the dominant species, no changes occurred in the observed decomposition abilities, which suggest functional redundancy among the used species (Suberkropp and Klug 1980; Arsuffi and Suberkropp 1985; Butler and Suberkropp 1986). In addition, several studies indicate that few species of aquatic hyphomycetes are sufficient to maintain decomposition (Gessner et al., 2010)- 1 to 4 species (Silva et al., 2015) and 3 to 5 species (Pascoal et al., 2010; Geraldes et al., 2012; Gonçalves et al., 2015). Our study corroborates this idea.

Sporulation was expected to be the parameter most affected by salinity, but none of the treatments showed changes in sporulation. This was not predicted since

multiple studies point to sporulation being the most sensitive parameter to contaminants (e.g. Salinity -Byrne & Jones, 1975; Müller-Haeckel & Marvanová, 1979; Silva et al., 2015; Canhoto et al., 2017; Heavy Metals- Pascoal et al., 2010). In a study conducted by Canhoto et al. (2017), results showed that sporulation of all tested species was negatively affected by salinity with *H. lugdunensis* and *T. marchalianum* sporulation being ceased at 2g/L. Also, Byrne and Jones (1975) reported that sporulation of two species of aquatic hyphomycetes was inhibited at 10% and 30% seawater while growth was maintained up to 100%. The authors suggested that freshwater fungi, when submitted to salinity, tend to invest more in mycelial growth than in sporulation. The results of the present study indicate that R strains, when submitted to salinity, tend to invest more in growth, which goes in accordance with Byrne and Jones (1975). On other hand, S strains seem invest more in sporulation. This can mean that S strains are more tolerant to salt than R strains; they may not need to allocate so much energy to maintain the structure (i.e. integrity of the cells) of the mycelium being able to invest more in sporulation. In opposition to S strains, while having a certain tolerance to salt, R strains likely need to invest more in maintaining mycelial integrity (e.g. through the production of osmoprotective compounds), which may determine a decrease in the energetic investment in sporulation.

In conclusion salt contamination may inhibit leaf decomposition and fungal biomass with no differences registered between species, strains or species combinations. A functional redundancy is suggested by the present results. Sporulation was not affected by salt addition. Nonetheless, S strains sporulated more than R strains, which suggest that an eventual energetic investment in maintaining mycelial viability was not made at the expenses of conidial production.

Chapter 5
Final Remarks

5. Final Remarks

Freshwaters are one of the most endangered ecosystems and the salinization of streams is now a widespread concern. However, in the case of headwaters, there are still few studies on the effects of this contaminant on ecosystem processes. Since aquatic hyphomycetes play a key role in leaf degradation (key process in forested streams) it becomes essential to understand how salt-contamination affect their function.

This experiment confirmed that aquatic hyphomycetes have high tolerance to salinity maintaining, to a great extent, their functional efficiency in salt-contaminated streams. However, it is probable that fungal communities' composition change with increasing salinity originating poorer communities dominated by tolerant species. This may affect the consumption of leaves by invertebrates, since Invertebrates show fungal or fungal/leaf preferences (Arsuffi and Suberkropp, 1985; Canhoto and Graça, 2008; Gonçalves et al., 2014). Cascading effects on the stream food chains can though be anticipated.

Results gathered by this study are important and open new lines of inquiry. Nonetheless, conclusions need to be faced with caution: as the present results are based on microcosm approaches that provide a "limited" vision of the consequences of salinization at an ecosystem-level. In addition, in this work only 3 species (2 strains each) of aquatic hyphomycetes and a single species of leaf litter were used, once again, this does not reflect a real environment. Furthermore, this study did not consider multiple stressor scenarios. For these reasons, a more extensive characterization and interpretation of the fungal responses to salt-contamination is needed. Field experiments, considering other associated stressors such as temperature would also be advisable, since global warming and climate change seems intensify the effects of salinity (Cañedo-Argüelles et al. 2013).

Despite this, the present study led to a better knowledge and understanding of the effect of salt on fungal ecology. It also strengthens the fundamental role that fungi play in maintaining the ecological functions of saline streams, thus minimizing the effects of this stressor.

Chapter 6
References

6. References

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