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Importance of fungal species on shredders feeding behaviour

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RESUMO

Os ribeiros de baixa ordem dependem da matéria orgânica de origem terrestre como fonte principal de nutrientes e energia. Uma vez no rio, as folhas são decompostas e convertidas em produção secundária. Neste processo, os fungos, denominados hifomicetes aquáticos desempenham um papel chave como ligação entre a matéria orgânica e os invertebrados, nomeadamente os detritívoros. Este grupo alimentar funcional, apesar de generalista, tendo sido referido como reativo ao estado d condicionamento das folhas e capaz de discriminar entre detritos colonizados com diferentes espécies fúngicas. Contudo, muito pouco se sabe sobre os padrões e razões que suportam este comportamento alimentar.

Neste estudo, tentou-se esclarecer a importância da identidade fúngica na alimentação dos invertebrados detritívoros. As experiências foram feitas com o detritívoro endémico *Sericostoma vittatum* Rambur (Trichoptera; Sericostomatidae). Um total de 5 espécies fúngicas (*Anguillospora filiformis, Articulospora tetracladia, Flagellospora curta, Heliscus lugdunensis, Lemonniera aquatica*) foram oferecidas em separado ao triturador como micélio ou colonizando (sozinhas) folhas de carvalho (*Quercus robur*) ou de amieiro (*Alnus glutinosa*).

Os hifomicetes aquáticos apresentaram composição elementar específica de cada espécie. C:N e C:P rácios mais elevados foram observados na *A. tetracladia*, o que determinou um consumo mais elevado não significante de micélio pelo *S. vittatum.*

Como esperado, as folhas de carvalho e amieiro apresentaram composições elementares distintas, sendo o amieiro uma folha mais nutritiva e macia. Contudo, quando colonizadas por uma única espécie de fungo, a perda de massa no carvalho foi significativamente mais elevada, o que pode estar relacionado com uma estimulação geral

das taxas de respiração (e eventualmente degradação enzimática) pelas espécies fúngicas quando na presença d compostos foliares mais recalcitrantes.

O consumo dos invertebrados trituradores foi mais elevado nas folhas de amieiro (vs. carvalho) colonizadas com 4 das 5 espécies de fungos. Diferenças não significativas foram encontradas nas taxas de consumo de amieiro condicionado com as diferentes espécies fúngicas. Apenas a *A. tetracladia* conseguiu estimular o consumo de folhas de carvalho ao mesmo nível que o consumo de amieiro colonizado pelos mesmos fungos. O efeito da colonização e da qualidade dos fungos parece ser mais relevante na presença de material foliar mais recalcitrante.

O número e a identidade das espécies de fungos e de invertebrados utilizadas limita as conclusões dos testes. No entanto, os resultados sugerem que as relações tróficas entre detritívoros e recursos são primariamente determinadas pelas características das folhas. A colonização por espécies de grande qualidade parece promover o consumo de material foliar de pouca qualidade, eventualmente reduzindo as desigualdades estequiométricas entre invertebrados detritívoros e recursos.

ABSTRACT

Low order streams depend on organic matter of terrestrial origin as the main source of nutrients and energy. Once in the stream, leaves are decomposed and converted into secondary production. In this process, fungi, namely aquatic hyphomycetes play a key role as links between the organic matter and invertebrates, namely shredders. This functional feeding group, although generalist, has been referred to respond to leaves conditioning status and to be able to discriminate among detritus colonized by distinct fungal species. However, little is known on the patterns and reasons underlying this feeding behaviour.

In this study we tried to clarify the importance of fungal identity on the consumption by invertebrate detritivores. Tests were performed with the common endemic caddisfly *Sericostoma vittatum* Rambur (Trichoptera; Sericostomatidae). A total of five fungal species (*Anguillospora filiformis, Articulospora tetracladia, Flagellospora curta, Heliscus lugdunensis, Lemonniera aquatica*) were singly offered to the shredder as pure mycelium or as single colonizers of oak (*Quercus robur*) or alder (*Alnus glutinosa*) leaf litter.

Aquatic hyphomycetes presented species-specific elemental composition. Higher C:N and C:P ratios were observed in *A. tetracladia* which determined a non-significant higher consumption of the mycelium by *S. vittatum*.

As expected, oak and alder leaves presented distinct elemental composition, being alder a more nutritious and soft leaf litter. However, when colonized by a single fungal species, mass loss of oak was significantly higher, which maybe related with a general stimulation of the respiration rates (and eventually enzymatic degradation) of the fungal species when in the presence of more recalcitrant leaf compounds.

Shredders' consumption was higher in alder (vs. oak) leaves colonized by 4 out of the 5 colonizing species. Non-significant differences were found in consumption rates of alder conditioned by distinct fungal species. Only *A. tetracladia* was able to stimulate the consumption of oak leaf litter up to the same level as alder colonized by the same fungi. The colonization effect and fungal species "quality" seems to be more relevant in the presence of more recalcitrant leaf litter.

The number and identity of the used fungal and shredder species limit the conclusions of these tests. However, results suggest that trophic relationships between detritivores and resources are primarily determined by leaf traits.

Colonization by high quality fungal species seems to stimulate consumption of low quality leaf litter eventually by reducing stoichiometric imbalances between detritivores and resources.

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-CHAPTER ONE-

INTRODUCTION

1.1. General Introduction

Freshwaters represent up to 0.01% of world's water and only 0.00014% occurs in river systems (Dudgeon *et al.* 2005; Oki & Kanae 2006).

Despite the scarcity of this "blue gold", most running waters are presently impaired systems registering unprecedented losses of diversity (Malmqvist & Rundle 2002; Strayer & Dudgeon 2010; Vörosmarty *et al.* 2010). Along with human growth, that requires more potable water, these resources are also becoming increasingly undermined by anthropogenic activities such as over-exploitation, water resource management, waste disposal, fisheries, introduction of non-native species (Malmqvist & Rundle 2002; Dudgeon *et al.* 2005).

Strategies for the conservation and restoration of natural and impacted watercourses are urgently needed, not only from an ethical point of view, but also taking into consideration the value of the services that freshwater systems provide to humans. To achieve these goals, a primordial understanding of the biota, of its relationships, and of the ecosystem functional properties seems to be needed.

1.2. Low order streams

Small temperate forested streams account for most of the total length of the fluvial network (over 85% of the total length of a lotic system; Anderson & Sedell 1979) and play a crucial role as biodiversity spots for the entire river system (Meyer *et al.* 2007; Wipfli *et al.* 2007). Their abundance and location at the origin of the *continuum* give them

a crucial role in the functioning of the fluvial net (*River Continuum Concept;* Vannote *et al.* 1980).

Low order streams are usually small and generally shaded by the riparian vegetation, which limits stream primary production. In these watercourses, allochthonous material of terrestrial origin constitutes the primary source of carbon and energy for aquatic communities (Abelho 2001).

In temperate systems, this material is mainly supplied in autumn – about 73 % of annual inputs (Abelho & Graça 1998). It is mainly composed of leaves but also (Fisher & Likens 1972) branches, bark, nuts, fruits, flowers and other part plants (Benfield 1997; Abelho 2001). This organic material, usually associated with microorganisms, is designated as detritus (Cummins & Klug 1979; Anderson & Cargill 1987) and is usually divided into three fractions (Cummins 1974): coarse particulate organic matter (CPOM; > 1 mm) that include the leaves, fine particulate matter (FPOM; 0.45 µm - 1 mm) and dissolved organic matter (DOM; < 0.45 µm).

Riparian areas are heterogeneous systems subjected to impacts from aquatic and land factors (Naiman *et al.* 2005; Richardson & Danehy 2007) and its composition determines not channel insolation but also the quantity, quality and seasonality (Gessner & Chauvet 1994; Hättenschwiller *et al.* 2011; Canhoto *et al.* 2013; Bruder *et al.* 2014) with which the litter inputs are supplied to the streams. Riparian subsidies are donor-controlled and depend, among other factors, on riparian plant richness and composition, which may alter the stream biota diversity and organic matter decomposition (e.g. Ferreira *et al.* 2015), through time and along the stream (Naiman *et al.* 2005). Small streams are a source of biodiversity to the fluvial net (Richardson & Danehy 2007).

1.3. Leaf Litter Breakdown

Once in the stream, and immediately upon immersion, leaves are retained and undergo a series of physical and biochemical transformations before being transported downstream, mainly as FPOM and DOM (Gessner *et al.* 2007; Wipfli *et al.* 2007). Litter decomposition is a pivotal ecosystem-level process in streams, determinant for the recycling of organic matter and the transfer of energy. Leaf litter processing (i.e. its incorporation into living biomass) occurs in three phases (Gessner *et al.* 1999), whose intensity and duration depends primarily on leaf intrinsic physic-chemical characteristics (e.g. Lecerf & Chauvet 2008) being modulated by environmental factors (e.g. flow, temperature) (e.g. Gessner *et al.* 1999; Lecerf & Chauvet 2008).

The first phase, **leaching**, occurs immediately after immersion and can last from 48 hours to 7 days (Canhoto & Graça 1996; Gessner *et al.* 1999; Abelho 2001). It consists on the loss of the soluble compounds - phenols, carbohydrates, amino acids, phosphorus and potassium – to the water, and usually leads to a mass loss of 4 to 42%. The quality, intensity and duration of this abiotic process depends on leaf characteristics (Schindler & Gessner 2009): it is usually higher in deciduous (20 to 42%) than in coniferous leaves (7%) (Maloney & Lamberti 1995; Abelho 2001).

Leaf traits as toughness and presence of cuticles are known as important factors in this phase; these characteristics may contribute to reduce the wettability and fragmentation susceptibility of the leaves limiting the solubilisation of the leaf compounds. On the other hand, leaf leaching may reduce the original chemical differences between the leaf species supplied by the riparian areas to the stream (e.g. Rier *et al.* 2002).

Environmental factors such as temperature (Ferreira & Canhoto 2015), nutrients concentration (Shridar & Bärlocher 2000) and current velocity (Ferreira & Graça 2006; Fonseca *et al.* 2013) may affect the intensity of this process with consequences on stream invertebrate communities.

The **conditioning** process corresponds to the colonization of the senescent leaves by microorganisms, namely aquatic hyphomycetes and bacteria.

Leaves are "fully conditioned" when the fungal biomass and activity peak (Boling *et al.* 1975; Canhoto & Graça 2008). The fungal biomass found in detritus can account for up to 7 to 23% of total leaf mass (Bärlocher & Brendelberger 2004; Graça & Canhoto 2006; Krauss *et al.* 2011), and up to 99% of total microbial biomass present on the leaf (Findlay & Arsuffi 1989; Abelho *et al.* 2005).

Fungi dominate over bacteria in terms of biomass, production and degradative capacity in the first stages of decomposition. Bacteria contribution to decomposition trends to increase in latter stages of degradation when the recalcitrant material is more abundant (Hieber & Gessner 2002; Gulis & Suberkropp 2003; Krauss *et al.* 2011).

Aquatic hyphomycetes are a group of polyphyletic fungi dominant in turbulent well-aerated waters. They produce spores generally with sigmoid or tetra radiate shapes (Ingold 1975; Bärlocher 1992; Hieber & Gessner 2002; Bärlocher 2005).

Aquatic hyphomycetes invasion of the leaf material progresses through mechanical (Canhoto & Graça 1999) and enzymatically mediated growth. Fungal growth is known to affect the leaves nutritional quality as it increases leaf total nitrogen (N) and phosphorus (P) (Bärlocher 2005; Gessner *et al.* 2007; Aβman *et al.* 2010). A high area/volume ratio of the mycelium allows an enlarged area of contact with the

environment favouring the accumulation of inorganic nutrients and the nutritional enrichment of detritus. Besides nutrient immobilization capacity, fungi enrich the leaves with their own biomass and degrade the leaf structural components – cellulose, hemicelluloses pectin and in some cases, lignin – making the leaf substrates softer and more prone to lose its integrity. The aquatic hyphomycetes biomass enhances leaf quality by itself since the fungal mycelium possess 2 to 4 times mores digestive nutrients than unconditioned material (Bärlocher 1985); they are also an important source of lipids needed to the metamorphosis and reproduction of most invertebrates (Cargill *et al*. 1995; Mas-Martí *et al.* 2015). Microbial conditioning is frequently recognised as an important transitional phase between the litter and its detritus-feeding invertebrates (Gessner *et al.* 2007; Canhoto & Graça 2008; Krauss *et al.* 2011).

Leaves **fragmentation** is a physical (e.g. abrasion) and biological (e.g. invertebrates feeding, case building), process mainly promoted by shredders, a functional feeding group of aquatic invertebrates that feed on leaves (Cummins 1974; Gessner *et al.* 1999). These detritivores may represent 20% of the total biomass present in a stream (Cummins *et al.* 1989). Its diversity is influenced by the type, amounts and distribution of detritus in the streambed (Abelho 2001).

Leaf litter physical fragmentation depends on leaf resistance and abiotic factors such as flow, type of substratum and turbulence (Molinero *et al.* 1996; Abelho 2000; Gonçalves *et al.* 2007).

The biological fragmentation seems to be mainly influenced both by the leaf characteristics (Aβman *et al.* 2011; Fidalgo *et al.* 2013), conditioning status (Nelson 2011) and by the consuming biota (Graça *et al.* 1993).

Shredders are known to present high ingestion rates and low assimilation efficiencies (Wallace *et al.* 1982). Their importance in leaves decomposition is usually high in temperate streams (but see Gonçalves *et al.*2007). Fine particulate organic matter and DOM (<0.45 µm diameter) are released during their feeding process becoming further available to other functional feeding groups (Cummins 1974) mainly collectors (Cummins 1974; Jonsson & Malmqvist 2005).

1.4. The leaf-fungi-shredders relationships

The use of detritus by shredders seem to be determined by a) species leaf traits, such as C:N ratio (Canhoto & Graça 2008; Fidalgo *et al.* 2013), toughness (e.g. Rincon & Martinez 2006; Gonçalves *et al.* 2015) or the presence of secondary compounds (Canhoto & Graça 1996, 1999); b) identity and diversity of the fungal communities colonizing the leaves (Bärlocher & Kendrick 1973; Jabiol & Chauvet 2012); and c) invertebrate species (Arsuffi & Suberkropp 1989).

It is largely recognized that invertebrates prefer conditioned over unconditioned leaf material (Graça1993, 2001; Graça *et al.* 2001); they are able to discriminate, under laboratory conditions, fungal specific conditioned areas. Moreover they show preference towards mycelium over conditioned leaves (Bärlocher & Kendrick 1973; Arsuffi & Suberkropp 1988; Canhoto & Graça 2008). Higher survival and assimilation rates have been reported on mycelium diets than on unconditioned material (Bärlocher & Kendrick 1973; Kostalos & Seymour 1976; Canhoto & Graça 2008).

Although not fully clarified, shredders seem to be able to respond to detritus (leaves + microbes) stoichiometry. Imbalances between detritus and consumers may determine

these responses: detritus usually have lower N or P content when compared to invertebrates (Cross *et al.* 2006; McManamay *et al.* 2011; Scott *et al.* 2013; Fuller *et al.* 2015). Furthermore, invertebrates may differ in their elemental composition and consequently, required resources (Evans-White *et al.* 2005; Persson *et al.* 2010; Danger & Chauvet 2013).

The fungal/detritus-invertebrates consortium may also depend on characteristics such as body size and/or development stage of the consumer (Evans-White *et al.* 2005; Persson *et al.* 2010). For instance, Chung & Suberkropp (2009) in a study with the Trichoptera *Pycnopsyche gentilis* found out that final larval stages showed a discriminative behaviour towards fungi that provide more lipids and specific enzymes (Chung & Suberkropp 2009). Furthermore, since the invertebrates have a limited capacity to digest leaf polysaccharides, they can use the fungal enzymes that may remain active in the invertebrate's guts (Bärlocher 1982; Bärlocher and Porter 1986; Walters and Smock 1991; Zimmer & Bartolmé 2003).

On a completely conditioned leaf, we can easily find twelve fungal species but not all fungal species appear to have the same value to invertebrates (Canhoto & Graça 2008).

Some species show preferences for certain fungi and reject others (Bärlocher & Kendrick 1973; Suberkropp *et al.* 1983; Arsuffi & Suberkropp 1986; Graça *et al.* 1993; Rong *et al.* 1995; Chung & Suberkropp 2009). For instance, some studies show that the invertebrate *Hesperophylax magnus* present a higher rate of consumption on *Flagellospora curvula* than *Lemonniera aquatica* (Suberkropp *et al.* 1983; Arsuffi & Suberkropp 1986). *Heliscus lugdunensis* also seems to be preferred by a variety of invertebrate species (Arsuffi & Suberkropp 1986; Graça *et al.* 1993).

Preferences may vary according to the invertebrate species. In a study made with two different invertebrates, *Gammarus pulex* and *Asellus aquaticus*, *Tetracladium*

marchalianum was the most consumed species by *A. aquaticus* but it was the least consumed by *G. pulex* (Graça *et al.* 1993).

Under natural conditions these patterns seem difficult to establish as the mycelium of the distinct species forms a close net on the leaf surface and leaf mesophyll.

The reasons subjacent to the still poorly known invertebrates' preferences and rejections towards certain fungal species or fungal-leaf associations are far from clarified (Gonçalves *et al.* 2014). The existence of repellent "flavours" (Arsuffi & Suberkropp 1989), inherent nutritional value (Bärlocher 1985; Grimmet *et al.*2003), ability to detoxify plants allelochemicals (Graça *et al.* 1993), distinct enzymatic potential (Suberkropp *et al.* 1983), may be related with such behaviour. An open field of investigation exists on the chemical cues that may trigger the shredders choices (Adams *et al.* 2009; Webster & Weissburg 2009).

Despite the reasons that determine the choice or preferential consumption of a specific detritus, the relationships established between leaf-fungi-shredders are influenced by abiotic factors such as temperature (Chauvet & Suberkropp 1998; Spänhoff & Meyer 2004), nutrients (Sridhar & Bärlocher 2000; Gulis *et al.* 2006) and pH (Dangles & Chauvet 2003; Schlief & Mutz 2006), among others.

1.5. Main objectives

It is generally accepted that shredders, although generalists, may present a discriminative behaviour towards specific fungal species and detritus. Preferences/rejections for specific fungal species or fungal/leaf associations may though have a cascading effect on stream food-web structure.

In this study, we try to contribute to clarify the importance of fungal identity to leaf consumer invertebrates. We assessed the patterns of preferences of the endemic Trichoptera *Sericostoma vittatum* Rambur (Sericostomatidae) towards selected aquatic hyphomycetes species. The fungal species were offered as pure mycelium and as single colonizers of a tough recalcitrant (*Quercus robur*) and a soft rich (*Alnus glutinosa*) leaf. We hypothesize that fungal preferences will be related with the elemental composition of the mycelium: fungal species with higher N will be more consumed and will stimulate the consumption, particularly of more recalcitrant leaves.

-CHAPTER TWO-

MATERIAL AND METHODS

2.1. Leaves, fungi and shredders

Oak (*Quercus robur*) and alder (*Alnus glutinosa*) senescent leaves were collected in Cioga do Campo, Coimbra (N: 40°14'20.616"; E: -8°31'29.676") and Parque Verde, Coimbra (N: 40º12'5; W: 8º25'30), respectively and stored dry at room temperature until needed. Aquatic hyphomycetes were previously isolated from single conidia in foam or released from submerged leaf litter collected from Candal Stream. Mycelium was grown on Malt Extract Agar (MEA). The used species were ranked preferred (P), rejected (R) or intermediate (I) based on Gonçalves *et al.* (2014) and references therein.

A total of five common species of aquatic hyphomycetes were used in our experiments - *Anguillospora filiformis* (ANFI - preferred), *Articulospora tetracladia* (ARTE - I), *Flagellospora curta* (FLCU - P), *Heliscus lugdunensis* (HELU - P) and *Lemoniera aquatica* (LEAQ - R); Fig. 1. Pure cultures of aquatic hyphomycetes were grown on malt extract agar (MEA; 2%).

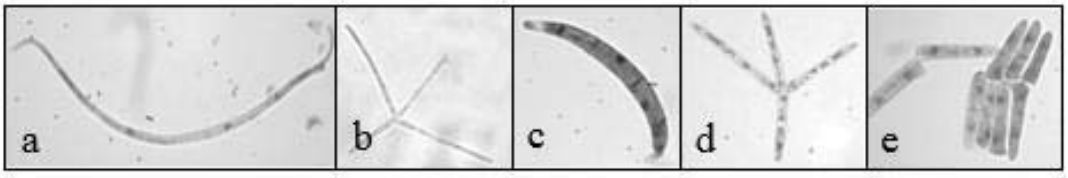

Figure 1 – Aquatic hyphomyecetes used in the experiments: a) *Anguilospora filiformis;* b) *Articulospora tetracladia*; c) *Flagellospora curta*; d) *Heliscus lugdunensis*; e) *Lemoniera aquatica.* (Photos by A. L. Gonçalves)

In order to evaluate the initial litter and fungal mycelium quality, air-dried leaves samples were oven dried (105ºC, 24h), milled (0.5mm powder size) and the mycelium was lyophilized for 24 h. Subsamples of both leaf species and mycelium were weighed and analysed for carbon (C) nitrogen (N) [IRMS Thermo Delta V advantage with a Flash

EA (112 series)], and phosphorus (P, Graça *et al.* 2005) concentrations. Results were expressed as percentage of dry mass (%DM).

In consumption tests, we used *Sericostoma vittatum* (Fig. 2), a common endemic shredder in low order streams of Central Portugal. Larvae were collected from Ribeira de S. João (40º06'N, 8º14'W), Lousã and acclimatized to laboratory conditions (15 ºC; 12:12 h light:dark photoperiod) for 1 week in water from the stream of origin. Invertebrates were fed with a mixture of leaves that were also collected in the same stream.

Figure 2 – *Sericostoma vittatum*, shredder used in the consumption tests. (Photo by A. L. Gonçalves)

2.2. Leaf Conditioning

Oak and alder leaves were cut with 12 mm diameter cork borer and oven-dried (55ºC, 48h). A total of 200 previously leached leaf discs/species were equally distributed by 5 Erlenmeyers filled with 100 mL nutrient solution ((75.5 mg $CaCl_2$, 10 mg $MgSO_4.7H_2O$, 0.5 g 3-morpholino propane sulfonic acid (MOPS), 5.5 mg K_2HPO_4 and 100 mg KNO_3 of sterile distilled water; Dang *et al.* 2005) and autoclaved. Twenty leaf discs of each Erlenmeyer were cut symmetrically in relation to the main vein (so they would have approximately the same weight); pairs were sewed together for further use on the consumption tests. The other group of 20 discs were oven dried (105ºC; 48h) and weighted to determine microbial respiration and leaf mass loss.

Each Erlenmeyer was inoculated with 3 circles of mycelium of each fungal species. The microcosms were incubated on shakers (120 rpm) under a 12h light: 12h dark photoperiod; conditioning occurred for 15 days.

After the conditioning period, pairs of oak and alder discs were saved for the consumption tests, while the other discs were used to assess mass loss and fungal respiration.

2.3. Microbial respiration

To determine leaf (colonized oak and alder) microbial respiration, we used 4 groups of 5 leaf discs from each microcosm. These subsets of 5 leaf discs were put into falcon tubes filled with 50 mL of 100% saturated nutrient solution; the tubes were covered with aluminium foil, and kept in the dark for 12 h.

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.

2.4. Leaf mass loss

After the conditioning period, and after respiration evaluation, the 20 discs from each microcosm were oven dried (105ºC; 48h) and weighed.

Dry mass loss after 15 days was assessed as the difference between initial and final dry mass, and expressed as percentage (%DM).

2.5. Consumption tests

For each test (mycelium, colonized alder/oak consumption), we used a total of 10 invertebrates of similar size (3.010 g ± 0.189 SE). The invertebrates were put in cups (70 mm diameter x 85 mm high cup), that were filled with (200 mL) aerated water of the

stream of origin; the cups had a fine layer of ashed (550 ºC, 6h) stream sediment on their bottom. Each cup had two leaf discs (leaf discs conditioned with each fungal species) – one available to the invertebrate and another enclosed in a small fine mesh (0.5 mm) bag, tied to the edge of the container, to be used as a control. One invertebrate was allocated to each cup (Fig. 3). All consumption tests (mycelium, colonized oak/alder) stopped when half of the mycelium or leaf disc's area was consumed in half the cups. The discs where then withdrawn and oven dried (105ºC; 48h).

Relative consumption rates (RCR) were estimated as the difference between the dry mass of the control discs (enclosed in small fine mesh bags) and the ones given to the invertebrates divided by the dry mass of larvae (Graça *et al.* 2005); RCR were expressed as mg leaf disc consumed per day and per mg of larval dry mass.

$$Consumption\ rate = \frac{Control\ disc\ mass - Consumed\ disc\ mass}{Invertebrate\ dry\ mass * duration\ of\ feeding\ trial}$$

Figure 3 – Consumption test; representative scheme of the cup containing the invertebrate and the leaf discs (from Canhoto *et al.* 2005 adapt by Moreira 2010).

2.6. Statistical analysis

Litter chemical composition was compared between litter types (alder vs. oak) by analysing C, N, P and phenols contents with t-tests.

Aquatic hyphomycetes concentration of C, N and P were compared by 1-way ANOVAs. Comparisons among aquatic hyphomycetes ratios (C:N, C:P and N:P), we made using 1-way ANOVA.

Mycelium consumption was compared among fungal species by 1-way ANOVA.

We evaluated differences among treatments for mass loss, microbial respiration and leaf consumption by 2-way analysis of variance (ANOVA) with leaf type (alder and oak) and fungal species categorical factors followed by planned comparisons to test for the effects of one factor within the other.

Assumptions of normality and homoscedasticity were always respected. Data were transformed (log (x+1) or square root transformation) to achieve normality whenever necessary (Zar 1999).

All statistical analysis was made with the software STATISTICA 7.

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-CHAPTER THREE-

RESULTS

Aquatic hyphomycetes elemental composition

The carbon content of ARTE was significantly lower than the other fungal species (1-way ANOVA, $F=213.350$, $p<0.001$; Tukey's test, $p<0.001$; Table I). The opposed was observed in relation to N and P (1-way ANOVA, $F=37.370$, $p<0.001$; Tukey's test, <0.001 and $F=226.394$, $p<0.001$; Tukey's test, $p<0.001$, respectively).

Accordingly, C:N and C:P ratios in ARTE were lower than the ratios found in the other four species (1-way ANOVA, $F=82.204$, $p<0.001$, Tukey's test, $p<0.001$; 1-way ANOVA, $F=41.712$, $p<0.001$, Tukey's test, $p<0.001$). N:P ratio was significantly lower in ARTE than in ANFI (1-way ANOVA, $F=4.712$, $p<0.022$, Tukey's test, $p=0.012$).

Table I- Chemical composition and elemental ratios (mean±SE; n=3) of aquatic hyphomycetes species: ANFI -*Anguilospora filiformis;* ARTE - *Articulospora tetracladia*; FLCU - *Flagellospora curta*; HELU - *Heliscus lugdunensis*; LEAQ - *Lemoniera aquatica.* Different letters indicate significant differences among species ($p<0.05$).

	Chemical Composition			**Elemental ratios**		
Fungal Species	**C (%)**	**N (%)**	**P (%)**	**C:N**	**C:P**	**N:P**
ANFI	39.881^{b} ±0.128	0.125^{b} ±0.004	0.019^{b} ±0.004	372.857^{b} ±11.082	6010.585^{b} ±1578.989	15.923^{b} ±3.696
ARTE	28.559^{a} ±0.600	0.377^{a} ±0.054	0.327^{a} ±0.014	92.806^{a} ±15.783	219.757^{a} ±14.204	2.453^{a} ±0.254
FLCU	38.691^{b} ±0.108	0.129^{b} ±0.002	0.032^{b} ±0.004	350.177^{b} ±6.056	3115.611^{b} ±449.858	8.862^{ab} ±1.125
HELU	39.930^{b} ±0.216	0.12^{b} 8±0.006	0.024^{b} ±0.001	359.537^{b} ±17.218	4095.378^{b} ±172.719	11.491^{ab} ±1.060
LEAQ	39.007^{b} ±0.323	0.122^{b} ±0.004	0.030^{b} ±0.008	374.894^{b} ±14.081	3452.732^{b} ±1021.553	9.351^{ab} ±3.027
p	<0.001	<0.001	<0.001	<0.001	<0.001	<0.022
F	213.350	37.370	226.394	82.204	41.716	4.712

Initial leaf litter quality

Oak shows a significantly higher percentage of phenolic compounds than alder (Table II). On the other hand, phosphorus and nitrogen content were higher in alder than in oak, even though the percentage of carbon was similar between leaf species (Table II).

For C:N ratio, alder present a significantly higher percentage than oak (Table II). Oak is higher than alder for C:P and N:P ratio (Table II).

Table II- Initial chemical composition (mean ± SE; n=4) of oak and alder leaf litter. Different letters indicate significant differences among species ($p<0.05$).

	Chemical Composition				**Elemental ratios**		
	Phenols (%DM)	**C (%AFDM)**	**N (%AFDM)**	**P (%AFDM)**	**C:N**	**C:P**	**N:P**
Alder	8.893[a] ±0.057	51.500 ±0.300	3.400[a] ±0.150	0.097[a] ±0.019	53.517[a] ±1.824	521.590[a] ±95.123	9.648[a] ±1.457
Oak	23.108[b] ±0.323	56.100 ±1.800	0.960[b] ±0.020	0.050[b] ±0.016	15.897[b] ±0.025	891.550[b] ±18.476	56.086[b] ±1.222
t-Test	43.309	2.316	21.535	-17.751	20.621	-3.818	-24.425
p	<0.001	0.081	<0.001	<0.001	<0.001	<0.001	<0.001

Mycelium Consumption

Larval consumption rate varied between 0.012 ± 0.009 (mean±SE) (HELU) and 0.033±0.013 (ARTE) mg leaf DM/g individual DM/d across fungal species mycelium. No differences were observed among fungal species consumption (1-way ANOVA, F=0.561, p=0.693; Fig. 5).

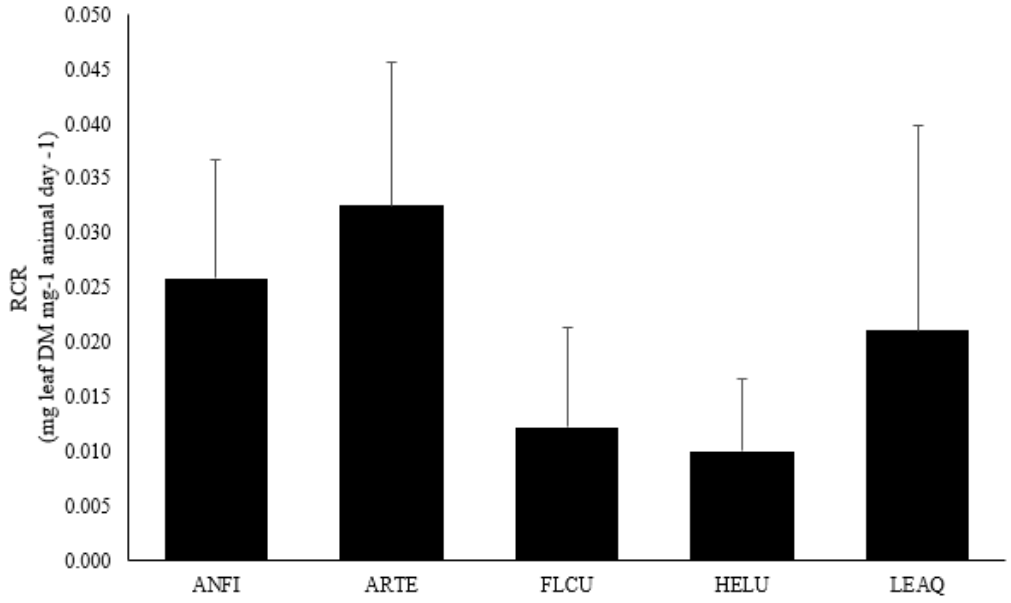

Figure 4 – Relative consumption rate (RCR) of *Sericostoma vittatum* caddisfly larvae fed aquatic hyphomycete mycelium. ANFI -*Anguilospora filiformis;* ARTE - *Articulospora tetracladia*; FLCU - *Flagellospora curta*; HELU - *Heliscus lugdunensis*; LEAQ - *Lemoniera aquatica.* The absence of letters indicate no significant differences among species (p>0.05).

Leaf litter mass loss and microbial respiration

Mass loss was significantly higher on oak than in alder (2-way ANOVA, F=5.187, p=0.030; Tukey's test, p=0.030). Values varied between 20.023 % ± 3.509 (FLCU) and 27.423 ± 2.980 (ANFI) in the case of oak, and 14.840 ± 5.125 (ARTE) and 21.556 ± 1.781 (HELU), in the case of alder. Leaf mass loss was not affected by fungal species (2-way ANOVA, F=0.5789, p=0.680).

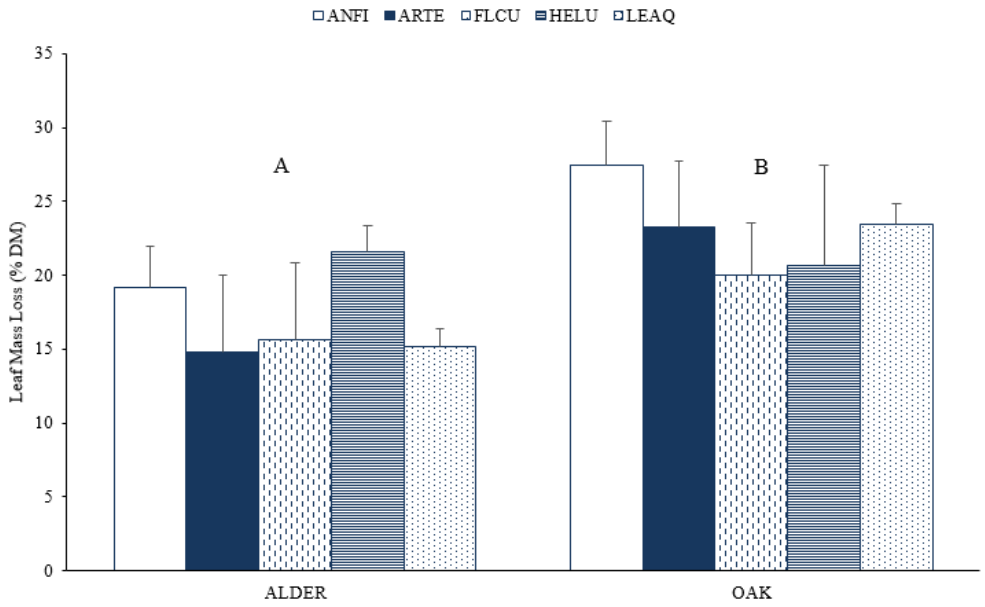

Figure 5 – Leaf mass loss of alder and oak discs in microcosms (mean ±1SE), incubated with single cultures of five aquatic hyphomycetes species. ANFI -*Anguilospora filiformis;* ARTE - *Articulospora tetracladia*; FLCU - *Flagellospora curta*; HELU - *Heliscus lugdunensis*; LEAQ - *Lemoniera aquatica.* Different letters indicate significant differences, referring to polled alder and oak replicates within each fungal species; the interaction effect between the two main factors was not significant.

Respiration rate was significantly affected by both factors, fungal and leaf species (2-way ANOVA, $F=27.288$, $p<0.001$, and $F=8.690$, $p<0.001$, respectively). Alder conditioned with LEAQ presented a significantly lower respiration rate than when colonized by ANFI and HELU (Planned comparisons, $F=6.882$, $p<0.001$; Tukey's test, $p<0.023$), while ANFI, ARTE and HELU tend to stimulate O_2 consumption; no significant differences among them were observed (Tukey's test, $p<0.720$). On the other hand, on oak, ARTE colonization determined a higher respiration than the one presented by FLCU (Planned comparisons, $F=4.747$, $p=0.004$; Tukey's test, $p=0.019$). Respiration rates were significantly higher on oak colonized with ARTE and LEAQ than on alder colonized with the same fungal species (Planned comparisons, $F>5.283$, $p<0.029$; Tukey's test, $p<0.031$). (Fig. 7).

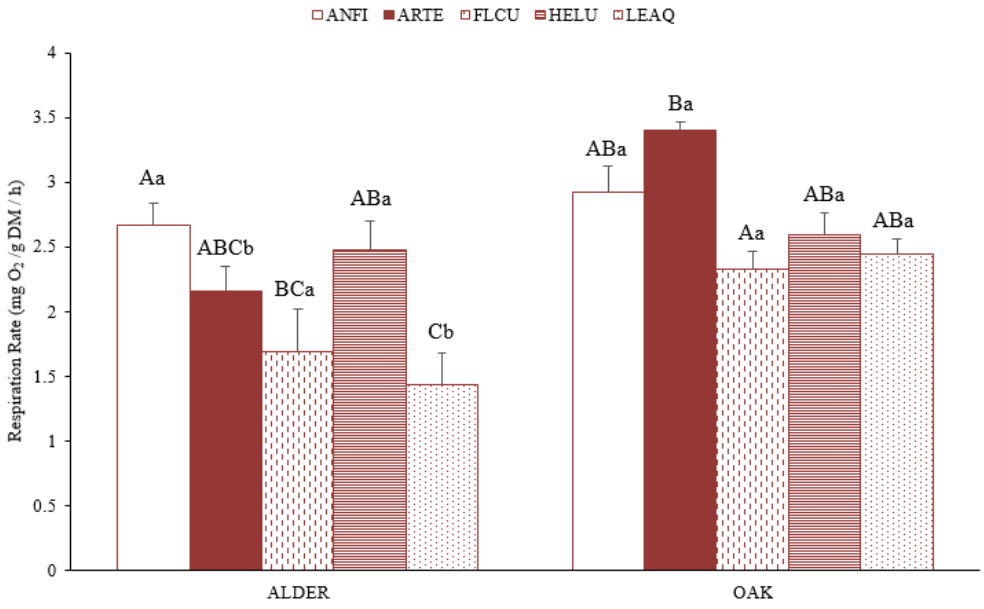

Figure 6 – Microbial respiration of alder and oak discs in microcosms (mean ±1SE), incubated with single cultures of five aquatic hyphomycetes species. ANFI -*Anguilospora filiformis;* ARTE - *Articulospora tetracladia*; FLCU - *Flagellospora curta*; HELU - *Heliscus lugdunensis*; LEAQ - *Lemoniera aquatica.* Bars with the same letters are not significantly different when tested within each leaf species (capital letters) and fungal species (lowercase letters).

Consumption tests

Consumption rate was significantly affected by both factors, leaf species (alder and oak) and colonization by different fungal species (2-way ANOVA, $F=43.877$, $p<0.001$, and $F=2.664$, $p=0.039$, respectively). In oak, ARTE stimulated consumption by the invertebrates when compared to the other four fungal species (Planned comparisons, $F=4.763$, $p=0.002$; Tukey's test, $p<0.044$). Nonetheless, on alder, there were no significant differences determined by the fungal colonization (Planned comparisons, $F=2.622$, $p=0.041$; Tukey's test, $p>0.282$). Consumption rates on leaves colonized with each fungal species was significantly higher on alder colonized with 4 of the 5 fungal species relatively to oak colonized with the same species: ANFI, FLCU, HELU and LEAQ (Planned comparisons, $F>7.397$, $p<0.008$; Tukey's test, $p<0.015$).

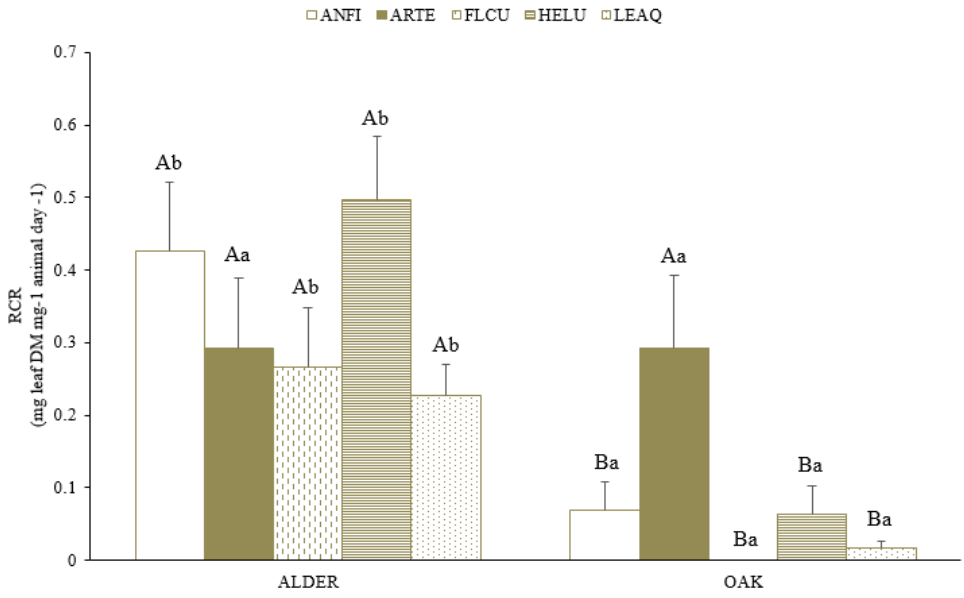

Figure 7 – Relative consumption rate (RCR) of *Sericostoma vittatum* caddisfly larvae fed alder and oak discs conditioned with five aquatic hyphomycete mycelium: ANFI -*Anguilospora filiformis;* ARTE - *Articulospora tetracladia*; FLCU - *Flagellospora curta*; HELU - *Heliscus lugdunensis*; LEAQ - *Lemoniera aquatica.* Bars with the same letters are not significantly different when tested within each leaf species (capital letters) and fungal species (lowercase letters).

-CHAPTER FOUR-

Discussion

Discussion

Little information exists on the elemental composition of aquatic hyphomycetes although the idea of an elemental heterogeneity among fungal species as been pointed by several authors (Leach & Gulis 2010; Danger & Chauvet 2013; Grimmett *et al.* 2013).Our study corroborates these findings.

The present results also strengthens the general belief that such differences, along with fungal distinct metabolism, may help explain invertebrate's preferences for specific mycelium or leaf fungal combinations. In fact invertebrates consumed fungal species like ARTE (especially when given as mycelium) that presented higher values of N and P.

Our results show that fungal ratios were much higher than previously reported which may be related with growth media, age of the mycelium and strains (Danger & Chauvet 2013; Grimmett *et al.* 2013).

Differences in the nutritional composition of the medium are known to affect mycelium composition (Danger & Chauvet 2013). Some authors found fungal species to be highly plastic (i.e. they are not homeostatic) in their elemental composition (Danger & Chauvet 2013) but others defend that aquatic hyphomycetes can maintain their C, N and P composition nearly constant (Leach & Gulis 2010).

Differences in mycelium composition did not determine distinct consumption by *S. vittatum*. However, such lack of significance maybe due to high data variability. Agar plugs saturated with mycelium were offered to the invertebrates. Although able to eat them (pers. obs.), it seems likely that such unusual food item determine a certain "consumption resistance". Furthermore, the agar might have partially masked the identification of the mycelium. In any case a tendency for a higher consumption of ARTE and lower consumption of HELU can be detected. Although higher N and P concentrations may justify the first case, the elemental composition of HELU is not

different from other more consumed species. In this case, it seems that other reasons (e.g. distasteful compounds like secondary metabolites) may underline the results. Further studies seem to be needed to understand if detritivores-resource stoichiometric imbalances (e.g. Hladyz *et al.* 2009; Ferreira *et al.* 2010; Dray *et al.* 2014; Fuller *et al.* 2015; Mas-Marti *et al.* 2015) are main drivers of food choices by invertebrates. In fact, C:N:P ratio may vary among detritivores species in streams (Evans-White *et al.* 2005; Persson *et al.* 2010).

Alder and oak present contrasting chemical composition. Alder is a soft rich leaf while oak is a hard phenolic rich leaf with significant lower values of C:N:P.

Considering field studies, made with leaves colonized by multiple species, these differences should have determined higher mass loss values of alder vs oak (Canhoto & Graça 1996). It seems possible that leaf recalcitrance might have stimulated the enzymatic potential enhancing leaf microbial-mediated degradation. In fact, respiration values in oak are globally higher than in alder which may support this point of view. Contrary to what was expected from laboratory studies (Duarte *et al.* 2006), alder presented lower mass loss values than expected.

Considering fungal species respiration, we can highlight the lower oxygen consumption values of LEAQ and FLCU in both leaves, and high values when leaves were colonized by ARTE (particularly on oak). Oak presented higher values for oxygen consumption (microbial respiration) than alder. This may suggest that aquatic hyphomycetes needed to consume more oxygen in order to grow and produce enzymes that degrade the more recalcitrant leaf material.

Consumption rates by shredders were higher on alder leaves; exception was observed when both leaves were colonized by ARTE. This may be due to the inherent high nutritional quality of alder; ARTE can enhance the quality of oak leaves (i.e. *per se* or through higher enzymatic degradative capacity), stimulating the consumption by invertebrates to levels observed in alder leaves. *A. tetracladia* is ranked as an intermediate species (in terms of palatability) when colonizing leaves (Gonçalves *et al.* 2014) and our experiment attend to support this evidence when colonizing nutritious leaves. Nonetheless, ARTE was preferred when offered as mycelium or in mycelium/oak association, at least to *S. vittatum*.

The value of leaf-fungal combinations to shredders has been proved to vary with the consumer species. However, some general feeding behaviour emerges from our results. For instance, comparatively high consumption rates were observed on ANFI in offered as mycelium and leaf-mycelium combination, which tends to support patterns observed on *Betula papyrifera* (Marshall) colonized by this species and offered to *Gammarus tigrinis, Tipula caloptera* and *Pycnopsyche gutifer* (Rong *et al.* 1995).

L. aquatica is usually rejected by invertebrates and was poorly consumed in various studies made with colonized leaves of *Populus tremuloides* (Michx) (Arsuffi & Suberkropp 1986) and *Ulnus procera* (Salisb) (Graça *et al.* 1993), which is also in line with our observations.

Comparing our consumption rates of colonized alder leaves with previous results of consumption of unconditioned alder leaves (0.23 ± 0.014 (mean±SE) mg leaf DM/g individual DM/day (pers. obs.)), we can notice that alder by itself has quality to be consumed but in some cases, fungal colonization enhances consumption. Looking at oak unconditioned leaves consumption (0.04 ± 0.001 (mean±SE) mg leaf DM/g individual DM/day (pers. obs.)), it becames clear that any fungal colonization promotes oak

consumption. All this supports previous studies that affirm that shredders prefer conditioned over unconditioned leaf material (Graça1993, 2001; Graça *et al.* 2001).

Our microcosm studies tried to clarify the importance of fungal species per se and as modulators of detritus quality to a common shredders of low order stream of Central Portugal. Although this may constitute an important step in the clarification of the feeding behaviour of shredders, results are limited as in natural conditions several fungal species can be found in a fully conditioned leaf (up to 23 species) and the relationships between leaves-fungi and invertebrates are largely determined by the fungal and invertebrate species involved.

The results suggest that trophic relationships between detritivores and resources largely determined by leaf traits. Differences in fungal stoichiometry, metabolic activity, eventually enzymatic potential and presence of other (distasteful or attractive) compounds seem to play a modulator role of the nutritional value of the detritus for the invertebrates, particularly in the case of more recalcitrant leaves as oak.

A long way is still to be covered to clarify the drivers of shredders feeding preferences both in laboratory and field conditions and to understand in what extent fungi may modulate leaf litter incorporation into secondary production.

-CHAPTER FIVE-

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