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IMPACTS OF WARMING ON FRESHWATER DECOMPOSERS ALONG A GRADIENT OF CADMIUM STRESS

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Abstract

Ongoing climate change is considered a driving force for ecosystems in the 21st century. There is a consensus in the scientific community that freshwaters are particularly vulnerable to climate changes. Climate models indicate that the temperature changes will entail increased drought periods followed by intense rainfalls and therefore, runoff from the surrounding soils is expected to increase leading to changes in the levels and bioavailability of contaminants in freshwaters. Contamination by heavy metals is of major concern in aquatic systems. Metal impacts in freshwaters has been the focus of much research, but their effects on the biota under a warming scenario are difficult to predict.

In streams, plant-litter decomposition is an important integrative ecosystem process, and is governed by microbial decomposers and invertebrate detritivores. Microbial decomposers, namely fungi, play a critical role in this process degrading leaf material and increasing leaf palatability for invertebrate shredders. Several studies report that metal pollution depresses plant litter decomposition, and the activity and diversity of aquatic decomposers. Conversely, many biological processes, such as microbial growth and consumption by selected invertebrate shredders, are positively related to temperature.

The aim of this study was to evaluate the interactive effects of cadmium and temperature on leaf decomposition, activity and diversity of leaf-associated fungi, and leaf consumption by the *Limnephilus* sp, a typical invertebrate shredder in streams of the NW Portugal. Freshly fallen leaves were immersed in a stream for 7 days, and then were exposed in microcosms to a gradient of cadmium (11 levels, ≤ 35 mg/L). One set of microcosms was kept at 15ºC, a temperature typically found in Portuguese streams in autumn, and at 21ºC, to simulate a warming scenario.

The increase in temperature stimulated leaf decomposition and sporulation of fungi, and increase the consumption and growth rates of invertebrates. The exposure of naturally colonized leaves to increased concentrations of cadmium showed that metal altered the structure of fungal communities and inhibited reproduction of fungi, particularly at 21ºC. Leaf consumption rates by invertebrates, but not their growth, were affected simultaneously by cadmium and increased temperature. The largest percentage of cadmium was found in leaves or in the cocoon of the shredders.

Overall, the predicted warming scenario may exacerbate cadmium impacts on detritus food-webs further compromising the functioning of freshwater ecosystems.

Resumo

O aquecimento global é considerado um assunto bastante importante no que diz respeito aos ecossistemas. Toda a comunidade científica está de acordo que os ecossistemas de água doce são particularmente vulneráveis às mudanças climáticas, uma vez que os modelos climáticos indicam que as mudanças de temperatura irão implicar um aumento dos períodos de seca seguidos de chuvas intensas. Por conseguinte, o escoamento superficial, a partir do solo circundante, deverá aumentar, levando a mudanças nos níveis e na biodisponibilidade de contaminantes nas águas doces. A contaminação por metais pesados é uma grande preocupação nos sistemas aquáticos. Os impactos dos metais nos sistemas de água doce tem sido foco de muita pesquisa, mas os seus efeitos sobre os ecossistemas sob um cenário de aquecimento global são difíceis de prever.

Nos rios, a decomposição foliar é um processo integrativo do ecossistema que é conduzido por microrganismos decompositores e invertebrados detritívoros. Os microrganismos, mais particularmente os fungos, desempenham um papel fundamental no processo de decomposição, uma vez que degradam as folhas e aumentam a sua palatabilidade para os invertebrados trituradores. Vários estudos demonstram que a poluição por metais inibe a decomposição da folhada e a actividade e diversidade dos decompositores aquáticos. Por outro lado, muitos processos biológicos, tais como o crescimento microbiano e a taxa de consumo de alimentos por invertebrados trituradores, estão positivamente relacionados com a temperatura.

Este estudo tem como objectivos avaliar os efeitos interactivos do cádmio e da temperatura na perda de massa foliar, na actividade e diversidade de fungos associados à folhada e no consumo da folhada pelo *Limnephilus* sp, um invertebrado triturador típico dos rios do noroeste de Portugal. Folhas de amieiro (*Alnus glutinosa L.*) foram imersas num rio durante sete dias e, em seguida, expostas em microcosmos a um gradiente de cádmio (11 níveis, ≤ 35 mg L^{-1}). Um conjunto de microcosmos foi mantido a uma temperatura tipicamente encontrada nos rios portugueses no Outono (15 º C) e outro a 21 º C, para simular um cenário de aquecimento global.

O aumento da temperatura provocou um estímulo na decomposição foliar e na esporulação dos fungos, e aumentou as taxas de consumo e crescimento dos invertebrados. A exposição das folhas colonizadas naturalmente a elevadas concentrações de cádmio mostrou que o metal alterou a estrutura das comunidades de fungos e inibiu a reprodução dos mesmos, especialmente a 21 º C. A taxa de consumo, mas não a de crescimento dos invertebrados, foi afectada simultaneamente pelo aumento

da concentração de cádmio e pela temperatura. Uma grande percentagem de cádmio foi acumulada nas folhas e no casulo dos invertebrados.

Num cenário de aquecimento global, os impactos dos metais nas cadeias alimentares detritívoras podem ser agravados, comprometendo ainda mais o funcionamento dos ecossistemas de água doce.

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Chapter 1

General Introduction

1.1. Leaf litter decomposition in freshwater ecosystems

The main source of organic matter in low-order forested streams is allochtonous and it is mainly constituted by plant detritus, including leaves and twigs that enter the streams and can be used by the stream biota or stored or transported downstream, depending on the retentiveness of the stream reach (Larrañaga *et al.* 2003, Elosegi 2005).

In streams, plant litter breakdown occurs through three distinct stages: i) the leaching, that is characterized by a substantial abiotic loss (up to 30%) of soluble substances, such as phenolics, carbohydrates and amino acids, within 24 hours to up to 7 days after leaf immersion (Graça & Pereira 1995, Canhoto & Graça 1996, Casas & Gessner 1999); ii) the conditioning, corresponding to the colonization and growth of microorganisms on leaf litter, that enhance leaf palatability to invertebrate shredders (Suberkropp 1998, Graça 2001); and iii) physical and biotic fragmentation that usually follows some period of tissue softening by the microbial enzymes (Cummins 1974). Although these stages tend to occur sequentially, leaf decomposition is a complex process and some of these stages can occur simultaneously (Gessner *et al.* 1999). Therefore, the original plant material is transformed into several products, including microbial and invertebrate biomass, fine particulate organic matter (FPOM), dissolved organic matter (DOM), inorganic nutrients and carbon dioxide (Gessner *et al.* 1999).

1.2. Aquatic biota involved in leaf litter decomposition

Leaf litter decomposition is a key process in freshwater ecosystems governed by microbial decomposers, namely bacteria and fungi, and invertebrate detritivores (Gessner *et al.* 2007). Among microbial decomposers, fungi, particularly aquatic hyphomycetes, are known to play an important role in microbial decomposition of plant litter in streams, whereas bacteria are thought to increase their importance only after plant material has been partially broken down (Weyers & Suberkropp 1996, Baldy *et al.* 2002, Pascoal & Cássio 2004). A predominance of fungi in microbial decomposer assemblages has been found in streams (Weyers & Suberkropp 1996, Pascoal *et al*. 2005b, Duarte *el al.* 2009) and in rivers (Baldy *et al.* 1995, 2002, Pascoal & Cássio 2004, Pascoal *et al.* 2005a) under different environmental conditions.

The success of the aquatic hyphomycetes in the leaf litter decomposition is mainly because of their morphological and physiological adaptations. The high conidial production and germination rates, the conidial shapes (tetraradiate or sigmoid) and the mucilage produced by conidial arms allow an efficient dispersion and attachment to new

substrata (Read *et al.* 1992). Besides, these fungi can be active at low temperatures so they are well adapted to streams of temperate climates in winter (Suberkropp 1998). Most species of aquatic hyphomycetes is capable of producing a variety of extracellular enzymes that can degrade complex polysaccharides of plant cell walls, including cellulose, xylan and pectin, making them appropriate sources of carbon and energy for invertebrate shredders (Suberkropp 1998). Invertebrates may benefit from the microbial biomass on leaves by two distinct ways: (i) by directly feeding on microorganisms (Alan & Castillo 2007); and (ii) by eating the modified plant substrates due to the microbial enzymatic action over the structural carbohydrates of plant cell walls (cellulose, hemicellulose, and pectin) (Bärlocher 1985). Furthermore, consumption of leaves by shredders appears to be affected by the type of fungal species colonising leaves (Bärlocher & Kendrick 1973, Arsuffi & Suberkropp 1985, Graça *et al.* 1993a,b, Lecerf *et al.* 2005). Several studies demonstrated that higher shredder abundance and biomass have been associated with faster leaf breakdown (Robinson & Gessner 2000, Graça 2001) and their exclusion in streams by application of an insecticide led to a decrease in leaf breakdown rates (Wallace *et al*. 1996). However, a minor role of shredders in litter breakdown has been found in large rivers (Chauvet *et al*. 1993), tropical streams (Mathuriau & Chauvet 2002) and polluted streams (Pascoal *et al.* 2005a). In these conditions, microorganisms may have a major contribution to leaf decomposition.

The activity of decomposers on leaf litter can be influenced by several stream water variables, such as pH (Dangles *et al.* 2004), nutrient concentrations (Gulis & Suberkropp 2003, Duarte *et al*. 2009, Fernandes *et al*. 2009a) and temperature (Suberkropp & Weyers 1996, Fernandes *et al.* 2009b). Moreover, microbial decomposing activity on leaves can be also affected by leaf litter quality (Sampaio *et al.* 2001) or its state of senescence (Bärlocher 1997).

1.3. Effects of global warming on freshwater ecosystems

Climate change is considered a driving force for ecosystems in the 21st century and simulations considering a doubling in atmospheric CO_2 predict a 1.1–6.4 ºC increase in air temperature by the year 2100 (IPCC, 2007). This increase of temperature will have consequences in several organisms causing changes in their physiology, time of life cycle events, and distribution of individual species with shifts toward higher altitudes or latitudes (Parmesan & Yohe 2003, Root *et al.* 2003). It seems probable that, at least, some species will become extinct, either as a direct result of physiological stress or *via* alterations in competitive interactions with other species (Hughes 2000). Nevertheless, there is a limited

number of species and ecological processes on which we have information on their responses to temperature and other related-climate change events. There is a consensus in the scientific community that several ecosystems would be particularly vulnerable to climate change, including freshwaters (Carpenter *et al.* 1992, Dudgeon *et al.* 2006).

In streams, leaf litter decomposition is expected to be affected by increased water temperature. Laboratory experiments demonstrated that higher water temperatures affect litter decomposition, directly by promoting leaching of soluble compounds (Chergui & Pattee 1990), and indirectly by enhancing microbial activity (Carpenter & Adams 1979) or stimulating leaf consumption by selected invertebrate shredders (Gonzalez & Graça 2003, Azevedo-Pereira *et al.* 2006).

Indeed, increased temperatures have been reported to stimulate the production of fungal assemblages on leaves (Suberkropp & Weyers 1996), and growth and sporulation of some species of aquatic hyphomycetes (Koske & Duncan 1974, Graça & Ferreira 1995, Rajashekar & Kaveriappa 2000, Dang *et al*. 2009, Ferreira & Chauvet 2010), that can result in accelerated litter decomposition rates (Fernandes *et al.* 2009, Ferreira & Chauvet 2010). Dang *et al.* (2009) suggest that effects caused by diel temperature oscillations can be significant even when changes in thecommunity structure are relatively minor (i.e., only relative proportions of species in communities changed) and the dominance of particular species in a diverse community may alter the response of ecosystem process rates to warming under naturally oscillating temperature regimes.

The increase of temperature may also have several effects in invertebrates, it is expected a faster initial growth rate, shorter developmental time and smaller size at maturity (reviewed by Atkinson 1995, Atkinson & Sibly 1997). However, effects of increased water temperature are predicted to be stronger for invertebrates inhabiting cold waters when compared with those inhabiting warmer waters (e.g. highlands vs. lowlands, northward vs. southward, winter vs. summer) (Braune *et al.* 2008), since in cold water environments biological activities are more temperature limited (Brown *et al*. 2004). A recent study showed that increases in temperature lead to alterations in the individual body elemental composition and in the performance of detritivores (Ferreira *et al.* 2009). Changes in consumption rates, lower growth rates, higher mortality and changes in body composition under the future climate scenario may result in a decrease of populations with impacts to the processes in which these organisms are involved (e.g., litter decomposition, nutrient cycling).

Nevertheless, more studies on how aquatic hyphomycetes and invertebrates, and their ecological functions respond to changes in temperature are needed if we want to predict the impacts of global warming to freshwaters.

1.4. Effects of metals on aquatic biota and plant litter decomposition in streams

Among anthropogenic stressors, metals are of major concern in aquatic systems because of i) their toxicity to organisms and ii) their relatively long-term persistence in the environment after a single contamination event. Although metals occur naturally in the composition of earth and can be released into the environment through weathering of rocks, the main source that makes them toxicants is of anthropogenic origin (Ayres 1992). Industrial activities, such as mining, smelting, finishing and plating of metals and dye manufacture (Rand *et al.* 1995), are the major sources of metal contamination in aquatic environments. Some metals such as cadmium (Cd) never occurs in isolation in natural environments, but mostly as a "guest" metal in lead (Pb) and zinc (Zn) mineralization (Baker *et al.* 1990).

Essential metals, such as Zn and Cu, are involved in growth, metabolism, and differentiation of organisms, and those that readily form two different oxidation states (e.g. Cu) are often involved in redox reactions. Other metals like Cd, have no apparent biological function. However, above certain threshold concentrations, both essential and non-essential metals can be toxic, because different metals have different targets in cells and thus different toxicities (Gadd 1993). Consequently, metal identity, bioavailability and timing of exposure will probably determine the community response, which potentially affects ecosystem processes.

High concentrations of metals in the stream water are reported to strongly inhibit leaf decomposition (Bermingham *et al.* 1996, Sridhar *et al.* 2001, 2005, Niyogi *et al.* 2001, 2002, Baudoin *et al.* 2007), microbial activity (Bermingham *et al.* 1996, Niyogi *et al.* 2002, measured as respiration), fungal diversity (Bermingham *et al.* 1996, Sridhar *et al.* 2001, Niyogi *et al.* 2002, Baudoin *et al.* 2007), and to promote shifts in the structure of microbial communities on leaf litter (Maltby & Booth 1991). Metals above certain levels can cause anomalies in the feeding behaviour and growth of macroinvertebrates (Stuhlbacher & Maltby 1992, Tessier *et al.* 2000, Felten *et al.* 2008, Wang *et al.* 2009). Within metals, Cd is considered one of the most toxic, and it is reported be toxic to many aquatic organisms, including zooplankton, even at micro grams per liter level, and it can accumulated in aquatic organisms (Chapman *et al*. 2003).

1.4.1. Aquatic hyphomycetes

Several studies have demonstrated that both essential and non-essential metals can negatively affect the performance of aquatic hyphomycetes. Laboratory experiments showed that metals inhibit the growth (Miersch *et al.* 1997, Guimarães-Soares *et al.* 2005, Azevedo *et al.* 2007, Miersch & Grancharov 2008) and sporulation of several aquatic hyphomycetes species (Abel & Bärlocher 1984, Azevedo *et al.* 2007, Duarte *et al.* 2004) and sporulation was more sensitive than growth and fungal biomass to metal exposure (Abel & Bärlocher 1984, Bermingham *et al.* 1996b, Azevedo *et al.* 2007, Niyogi *et al.* 2002, Baudoin *et al.* 2007).

Aquatic hyphomycetes are mainly documented in clean streams (Bärlocher 1992), but they have also been found in streams affected by metal pollution (Sridhar *et al.* 2001; Pascoal *et al.* 2005a). The ubiquitous and sometimes dominant presence of aquatic fungi in metal-polluted streams (Sridhar *et al.* 2001) has increased the interest in examining the effects of metals on fungi during decomposition of organic matter. If the fungal community colonizing leaves is perturbed, the rate of decomposition and consumption by detritivores may be affected, leading to alterations in nutrient cycling and energy flow, and hence to changes in food-web structure (Maltby 1992).

In metal–polluted streams, a decrease in the richness of aquatic hyphomycete species (Bermingham *et al.* 1996, Niyogi *et al.* 2002) and a reduction in conidium production and fungal biomass (Sridhar *et al.* 2001) were found. However, fungal biomass associated with decomposing leaves is less affected by metals than fungal diversity or reproduction (Niyogi *et al.* 2002, *Duarte et al.* 2004, 2008, 2009). Leaf decomposition rates are usually low in streams affected by mine drainage (Bermingham *et al.* 1996, Niyogi *et al.* 2001). The greatest impacts of metal contamination to aquatic fungal communities on leaves were found during the initial stages of leaf colonization (Sridhar *et al.* 2005).

1.4.2. Macroinvertebrate detritivores

Benthic macroinvertebrates have been extensively used to assess the ecological integrity of freshwater ecosystems (Barbour *et al.* 1999, EU 2000). In streams polluted by metals, a reduced biomass of shredding invertebrates has been found (Niyogi *et al.* 2001, Carlisle & Clements 2003). Metal accumulation in invertebrates has been correlated more with metal content in food than in water or sediment (Kiffney & Clements 1994, Beltman *et al.* 1999).

It is also reported that metals can decrease the longevity and reproduction (Vogt *et al.* 2007, Wang 2009) and affects consumption rates of many invertebrates (Heinis *et al.* 1990, Riddell *et al.* 2005, Felten *et al.* 2008).

Metal accumulation, including Cd, by organisms is currently reported in literature (McGeer *et al.* 2000, Nunez-Nogueira *et al.* 2005, Wang *et al.* 2008), particularly in gammarids species (Wright 1980, Stuhlbacher & Maltby 1992, Zauke *et al.* 2003). In crustaceans, cadmium accumulates primarily in gills and hepatopancreas (Soegianto *et al.* 1999), causing cellular damages (Xu 1995, Soegianto *et al.* 1999, Silvestre *et al.* 2005).

1.5. Aim and outline of the thesis

To better understand the impacts of metals and global warming on plant litter decomposition, the effects of Cd and temperature on the diversity and activity of aquatic fungi and on consumption and growth of invertebrate shredders were investigated. Chapter 1 provides an overview on plant litter decomposition in streams and the associated biota. The impacts of metal pollution and global warming in the aquatic biota involved in plant litter decomposition are also addressed.

In Chapter 2, a microcosm experiment was carried out to assess the effects of Cd and increased temperature on stream-dwelling aquatic hyphomycete assemblages, obtained by immersion of alder leaves in two streams of Northwest Portugal. The following functional parameters were examined: leaf mass loss, fungal biomass, from ergosterol content in leaves, and fungal reproduction, by counting spores released from decomposing leaves. The effects of Cd and temperature on the structure of aquatic hyphomycete assemblages were assessed based on diversity of sporulating fungi and on DNA fingerprints from denaturing gradient gel electrophoresis.

In Chapter 3, we tested how leaf consumption by an invertebrate shredder and its growth was affected by Cd and whether the increase in temperature modulates this relationship by evaluating leaf consumption and animal growth rate.

The main conclusions are presented in Chapter 4, to provide a global perspective of the work and possible lines for future research.

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Chapter 2

Impacts of temperature on aquatic fungal decomposers along a gradient of cadmium stress

2.1. Introduction

Climate change is a driving force for ecosystems in the 21st century (IPCC, 2007), and an increase in air temperature of 2.0–6.3 °C is expected to occur in Europe by 2100 (EEA, 2004). Several climate models show that an increase in extreme weather events will occur, such as increases in extreme high temperatures, decreases in extreme low temperatures, and increases in drought periods followed by intense rainfalls (Jentsch *et al*. 2007). These changes are expected to alter biodiversity (Petchey *et al.* 1999 Castella *et al.* 2001), the structure of biotic communities (Mouthon & Daufresne 2006), species distribution (Eaton & Scheller 1996, Castella *et al.* 2001), interspecific relationships (Webster *et al.* 1976, Beisner *et al.* 1997, Mouritsen *et al.* 2005, Jiang & Morin 2007), and ecological processes (Petchey *et al.* 1999, Baulch *et al.* 2005) in streams and rivers.

In freshwaters, plant litter decomposition is an important ecosystem-level process (Bärlocher 2005), which depends on the activity of invertebrates and microorganisms (Pascoal *et al.* 2005). Among microbial decomposers, fungi, particularly aquatic hyphomycetes are known to play an important role in leaf litter decomposition in streams (Baldy *et al.* 2002, Pascoal & Cássio 2004, Gessner *et al.* 2007) and enhance leaf nutritional value for detritivores (Graça 2001). If the temperature in stream water increases, fungal activity on plant litter in temperate streams may be stimulate. This may shorten the residence time of available substrates for aquatic fungi and, therefore, decrease density and diversity of aquatic hyphomycetes in streams (Bärlocher *et al.* 2008). Occasional freezing may constrain fungal diversity and their ecological functions (Fernandes *et al.* 2009), while warming appears not only to accelerate plant litter decomposition in streams (Fernandes *et al.* 2009, Ferreira & Chauvet 2010), but also to affect the structure of aquatic hyphomycetes assemblages (Fernandes *et al.* 2009, Ferreira & Chauvet 2010). However, studies on how aquatic hyphomycete species and their ecological functions respond to changes in temperature are scarce.

Pollution by metals is another major concern in freshwaters, because of their toxicity to living organisms and their persistence in the environment. However, metals in low doses are reported to have a stimulatory effect on reproduction and growth of several aquatic organisms - hormesis phenomenon (Calabrese and Blain 2005, Lefcort *et al.* 2008, Shen *et al.* 2009).

Several studies report that metal pollution depresses plant litter decomposition, and the activity and diversity of aquatic decomposers (Duarte *et al* 2004, 2008, Sridhar *et al*. 2001). Conversely, many biological processes, such as microbial growth, are positively related to temperature (Rajashekhar & Kaveriappa 2000). Therefore, the knowledge of the

interactive effects of metals and temperature on leaf litter decomposition and the associated biota may contribute to a better understanding of the factors regulating the functioning of freshwater ecosystems.

Aquatic hyphomycetes are mainly documented in clean streams (Bärlocher 1992), but they have also been found in streams affected by metal pollution (Sridhar *et al*. 2001), suggesting that some species can be more tolerant to metals than others. Metals can have a strong effect on the structure of aquatic fungal communities (Duarte *et al.* 2004, 2008) and a decrease in the richness of aquatic hyphomycete species has been found in heavy metal–polluted streams (Bermingham *et al.* 1996, Niyogi *et al.* 2002). Several metals, including cadmium, nickel, copper and zinc, are reported to inhibit the growth (Miersch *et al.* 1997, 2001, Azevedo & Cássio 2010) or reproduction of aquatic hyphomycetes (Abel & Bärlocher 1984, Azevedo & Cássio 2010). It has been proved that conidium production by aquatic hyphomycetes seems to be more sensitive to heavy metals than growth (Abel & Bärlocher 1984, Duarte *et al.* 2004, 2008, Fernandes *et al.* 2009).

The effects of environmental stressors are usually tested individually; however, ecosystems are exposed to several stressors simultaneously, whose impacts on biodiversity and ecosystem processes are difficult to predict (Folt *et al.* 1999, Vinebrooke *et al.* 2004). In this work, the interactive effects of cadmium and temperature on leaf litter decomposition and the associated fungal communities were analyzed. Leaves were immersed in two streams with different communities for 7 days to allow microbial colonization and then were exposed to increasing concentrations of cadmium (up to 35 mg/L, 11 levels) and two temperatures: 15 ºC, a temperature commonly found in streams of Northwest Portugal between spring and autumn; and 21 ºC to simulate a warming scenario. The measured endpoints were leaf mass loss, leaf-associated fungal biomass, fungal reproduction and fungal diversity from sporulating species and DNA fingerprints.

2.2. Material and methods

2.2.1. Sampling site

The study was carried out in two streams in the NW Portugal, the Estorãos stream and the Algeriz stream located in the Ponte de Lima and Braga, respectively. Temperature, pH, conductivity and oxygen dissolved in the stream water were measured in situ with field probes (Multiline F/set 3 no. 400327, WTW). Stream water was collected

into sterile glass bottles, transported in a cold box (4 ºC), and used within 24 h for chemical analyses. Concentration of nitrate (HACH kit, program 355), nitrite (HACH kit, program 371), ammonia (HACH kit, program 385) and phosphate (HACH kit, program 480) were determined (Table 2.1).

Additional stream water samples were collected in both streams, filtered to retain suspended solids, and autoclaved (120 °C, 20 min) for microcosm experiments.

Table 2.1. Physical and chemical parameters of stream water in the Estorãos and Algeriz streams, used in microcosm experiments.

Parameter	Estorãos stream	Algeriz stream
Temperature (°C)	16.8	12
pH	5.83	6.8
Conductivity (µS/cm)	50	43
O^2 dissolved (mg/l)	8.75	10.5
$N–NO_3^-$ (mg/l)	0.43	0.06
$N–NO_2^-$ (mg/l)	<0.1	<0.1
$N–NH_3$ (mg/l)	<0.01	<0.01
$P–PO_4^{3-}$ (mg/l)	<0.01	0.03

2.2.2. Microcosms

In September, leaves of *Alnus glutinosa* (L.) Gaertn (alder), were collected immediately before abscission and dried at room temperature. The leaves were leached in deionised water for 2 days and cut into 12 mm diameter disks. Sets of 50 disks were placed into 0.5 mm mesh bags (16 × 20 cm). Leaf bags were immersed in the selected streams (70 leaf bags per stream) to allow microbial colonization.

After 7 days, leaf bags were retrieved and transported to the laboratory in a cool box. Leaf disks from each bag were rinsed with deionised water and placed in 150 mL Erlenmeyer flasks with 70 mL of sterile stream water. The microcosms were supplemented with eleven concentrations of Cd (added as chlorides, Sigma) ranging from 0 to 35 mg L^{-1} (3 replicates). Microcosms without added metal were used as controls. One set of microcosms was kept at 15ºC and the other set at 21ºC. All microcosms were placed on a shaker at 120 rpm for 20 days. Stream water was changed every 4 days and the conidium suspensions were filtered for species identification and counting.

2.2.3. Leaf dry mass

Leaf disks from each replicate microcosm were freeze dried to constant mass (72 ± 24h) and weighed (± 0.001 g). Sets of leaf disks before fungal colonization were used to estimate initial dry mass of the leaves.

2.2.4. Fungal biomass

Sets of six freeze-dried leaf disks from each microcosm were used to determine ergosterol concentration as a measure of fungal biomass on leaves. Lipids were extracted from leaf disks by heating (80 ºC, 30 min) in 0.8% of KOH/methanol, and purified by solid-phase extraction. Ergosterol was quantified by high-performance liquid chromatography (HPLC) using a LiChrospher RP18 column (250 mm x 4 mm, Merck), connected to a Beckmann Gold liquid chromatographic system. The system was run isocratically with HPLC-grade methanol (Riedel de-Haen) at 1.4 mL min^{-1} and 33 ºC (Gessner 2005).

Ergosterol was detected at 282 nm and its concentration was estimated using standard series of ergosterol (Fluka) in isopropanol. Ergosterol was converted to fungal biomass using a factor of 5.5 mg ergosterol g^{-1} fungal dry mass (Gessner & Chauvet 1993).

2.2.5. Conidial production

Conidial suspensions were mixed with 200 µL of 0.5% Tween 80 and appropriate volumes were filtered (0.45-µm pore size, Millipore). Conidia on the filters were stained with 0.05% cotton blue in lactic acid. Approximately 300 conidia per filter were identified and counted under a light microscope (400x magnification), to determine the contribution of each aquatic hyphomycetes species to the total conidial production.

2.2.6. Diversity of fungi from DNA fingerprints

DNA was extracted from three leaf discs with a soil DNA extraction kit (MoBio Laboratories, Solana Beach, CA) according to the manufacturer's instructions. The ITS2 region of fungal rDNA was amplified with the primer pair ITS3GC and ITS4 (White *et al.* 1990).

For polymerase chain reaction (PCR) of fungal DNA, 12.5 µM of Go Taq, 0.5 µM of each primer, 1 µL of DNA and 10.5 µM of ultra pure water were used in a final volume of 25 µL. Fungal PCRs were carried out in a MyCycler Thermal Cycler (BioRad Laboratories, Hercules, CA, USA) using the following program: initial denaturation at 95 ºC of 2 min; followed by 36 cycles of denaturation at 95 ºC for 30 s, primer annealing at 55 ºC for 30 s and extension at 72 ºC for 1 min. Final extension was at 72 ºC for 5min (Nikolcheva & Bärlocher 2005, Duarte *et al.* 2008).

Denaturing gradient gel electrophoresis (DGGE) analysis was performed using a DCodeTM Universal Mutation Detection System (BioRad Laboratories). For fungal DNA, 20 µL samples of the amplified products with 380–400 bp were loaded on 8% (w/v) polyacrilamide gel in 1x Tris-acetate-EDTA (TAE) with a denaturing gradient from 30 to 70%. The gels were run at 55 V, 56 ºC for 16 h and stained with 10 µL of gel star solution for 10 min. The gel images were captured under UV light in a transiluminator Eagle eye II.

2.2.7. Data analyses

Two-way analyses of variance (Two-way ANOVA) were used to test the effects of Cd and temperature on leaf mass loss, fungal biomass and reproduction (Zar 1996). Data had a normal distribution, and therefore no data transformation was needed.

Correspondence analysis (CA) was used to determine how fungal communities, based on sporulating species or DNA fingerprints, were structured by the environmental variables (temperature and cadmium concentration).

ANOVAs were done with Graph Pad Prism 5 (GraphPad software Inc., San Diego, CA) and the CA analysis with CANOCO 4.5 (Microcomputer Power, NY, USA). DGGE gels were aligned and the relative intensity of the bands in the gel was analyzed with Bionumerics program. Each DGGE band was considered an operational taxonomic unit (OTU).

2.3. Results

2.3.1. Fungal diversity on leaves

From conidial identification during the whole study, 10 aquatic fungal taxa were found on decomposing alder leaves colonized in the Estorãos stream (Table 2.2; Fig. 2.1), while 19 fungal taxa were found in the Algeriz stream (Table 2.2; Fig. 2.1). The dominant fungal species were *Dimorphospora foliicola* in the Estorãos stream and *Articulospora tetracladia* in the Algeriz stream (Annex). The analysis of fungal communities on decomposing

leaves, assessed from DNA fingerprints, showed higher diversity in the Algeriz stream than in the Estorãos stream for both temperatures (Table 2.2).

Generally, higher fungal diversity was found from DGGE analysis than from conidial counts, for both temperatures (Table 2.2). The exposure to increased Cd concentrations led to a decrease in fungal diversity, as numbers of DGGE OTUs or sporulating species (Table 2.2). Temperature and Cd concentrations significantly affected the number of fungal species on leaves from the Estorãos stream (two-way ANOVA, $p < 0.05$). For leaves colonized in the Algeriz stream, only Cd concentrations significantly affected the number of fungal taxa (two-way ANOVA, $p < 0.05$). A significant decrease in the number of fungal species was observed after exposure to concentrations ≥ 3.5 mg L^{-1} Cd for the Estorãos and Algeriz streams, especially in treatments at the highest temperature (Bonferroni pos tests, $p < 0.05$).

Table 2.2. Fungal diversity as number of sporulating species, based on conidial identification, or DGGE OTUs on leaves colonized in the Estorãos and Algeriz streams, and exposed for 20 days in microcosms to increasing Cd concentrations (0, 0.1, 0.5, 1.5, 4.5, and 35 mg L^{-1} Cd) at 15 ºC and 21 ºC.

	Estorãos stream				**Algeriz stream**			
Cd Concentration (mg L^{-1})	**15 ºC**		**21 ºC**		**15 ºC**		**21 ºC**	
	Conidia	OTUs	Conidia	OTUs	Conidia	OTUs	Conidia	OTUs
0	9	14	6	13	15	19	15	17
0.1	2	15	9	14	13	17	13	14
0.5	7	11	9	9	16	18	14	0
1.5	6	8	5	8	12	16	13	14
4.5	6	7	5	7	12	15	9	15
35	5	6	3	7	0	9	2	10

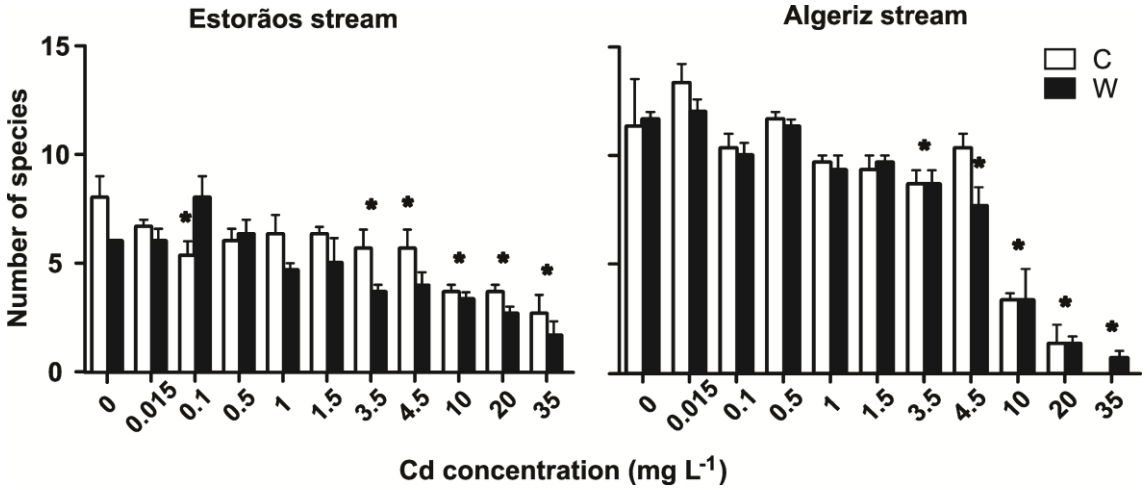

Figure 2.1- Number of fungal species on alder leaves colonized in the Estorãos and Algeriz streams and exposed for 20 days in microcosms to increasing Cd concentrations (0, 0.015, 0.1, 0.5, 1, 1.5, 3.5, 4.5, 10, 20 and 35 mg L^{-1} Cd) at 15 ºC (C) and 21 ºC (W). Asterisk shows treatments that significantly differed from control (Two-way ANOVA $p<0.05$).

The CA ordination of sporulating fungal species (Fig. 2.2A) and DGGE OTUs (Fig. 2.2C) from the Estorãos stream showed that communities exposed to high Cd concentrations were separated from those exposed to low Cd concentrations. Also, the CA ordination of fungal sporulating species from the Algeriz stream separated communities exposed to high and low Cd concentrations (Fig. 2.2B). The CA ordination of DNA fingerprints of fungi from the Algeriz stream did not show any clear trend (Fig. 2.2D). Moreover, temperature affected the number of fungal sporulating taxa (Monte Carlo permutations tests, $p < 0.05$), but not the number of OTUs (Monte Carlo permutations tests, $p > 0.05$). Cadmium only affected the number of fungal sporulating taxa from the Algeriz stream (Monte Carlo permutations tests, $p < 0.05$).

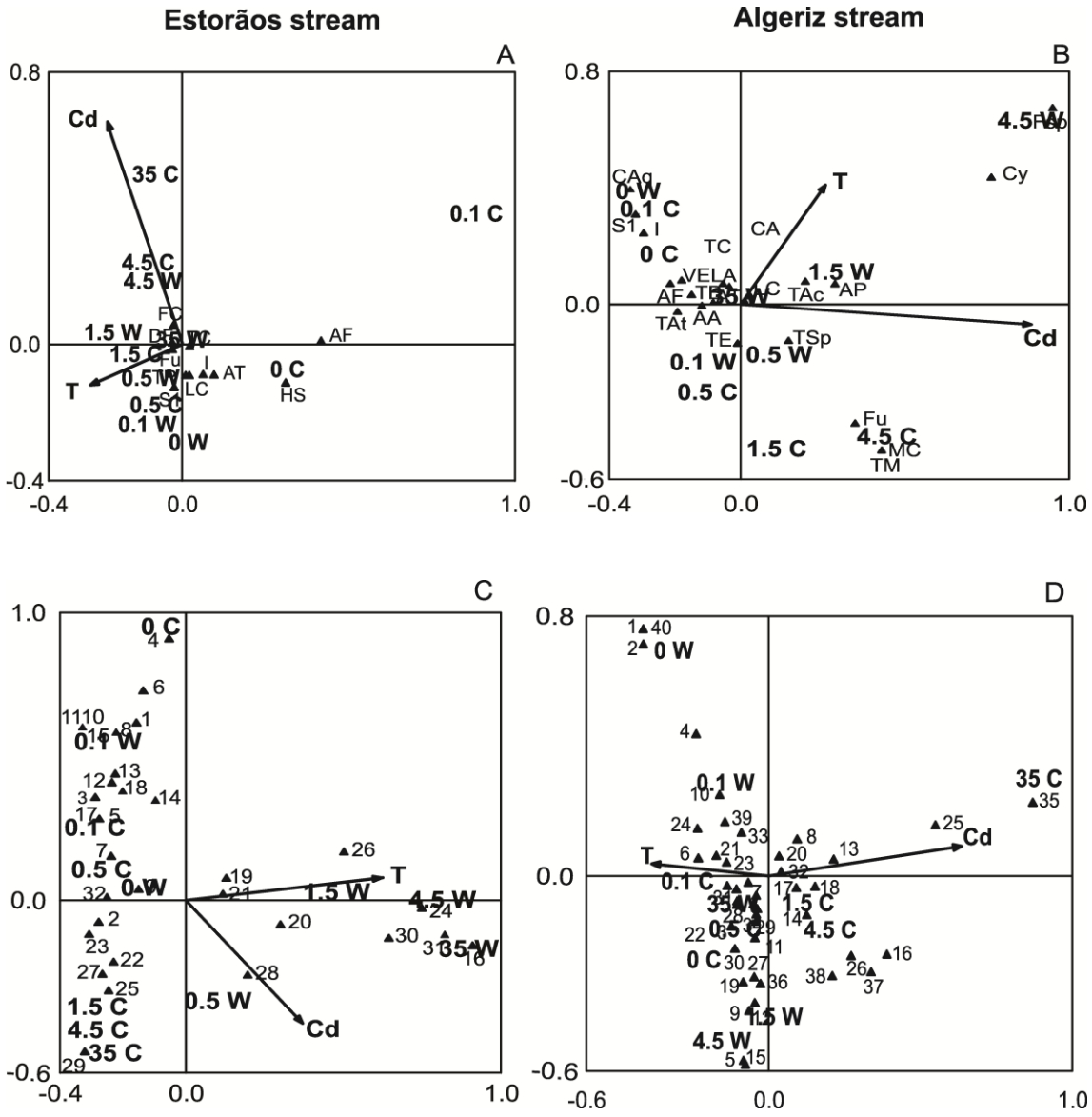

Figure 2.2- CA ordination of fungal sporulating taxa (A, B) and of DGGE OTUs (C, D) on decomposing leaves from Estorãos and Algeriz stream, according to temperature (T) 15ºC (C) and 21 ºC (W) and concentrations of cadmium (Cd). Fungal taxa: AA, *Alatospora acuminata* (Ingold 1942); AF, *Anguillospora filiformis* (Greath. 1961); AP, *Alatospora pulchella* (Marvanová 1977); AT, *Articulospora tetracladia* (Ingold 1942); CA, *Clavariopsis aquatic* (De Wild. 1895); Caq, *Culicidospora aquatica* (R.H. Petersen 1960); Cy, *Cylindrocarpon sp.* (Wollenw. 1926); DF, *Dimorphospora foliicola* (Tubaki 1958); Fcu, *Flagellospora curta* (Ingold 1942); Fu, *Fusarium sp.* (Cooke & Harkn. 1881); H, *Helicosporium sp.*; HS, *Heliscella stellata*; I, *Infundibura* sp. (Nag Raj & W.B. Kendr. 1981); LA, *Lemonniera aquatica* (De Wild. 1894); LC, *Lunulospora curvula* (Ingold 1942); MC, *Mycofalcella calcarata*; TB, *Tetracladium breve* (A. Roldán 1989); TE, *Tetrachaetum elegans* (Ingold 1942); TM, *Tetracladium marchalianum* (De Wild. 1893); TAt, *Tricladium*

attenuatum (S.H. Iqbal 1971); TC, *Tricladium chaetocladium* (Ingold 1974); TSp, *Tricladium splendens* (Ingold 1942); TAc, *Triscelosphorus cf. acuminatus* (Ingold 1942); VE, *Varicosporium elodeae* (W. Kegel 1906).

2.3.2. Fungal sporulation on leaves

After 20 days in microcosms at 15 ºC, fungal sporulation rates on alder leaves non-exposed to Cd were 6 x 10^5 and 8 x 10^5 spores mg^{-1} leaf dry mass d^{-1} for leaves colonized in the Estorãos and Algeriz streams, respectively (Fig. 2.3). At 21 ºC, fungal sporulation rates were 1.9 x 10^5 and 11 x 10^5 spores mg^{-1} leaf dry mass d^{-1} for the Estorãos and Algeriz streams, respectively. Fungal sporulation rates on leaves colonized in the Algeriz stream tended to be higher when compared with those in the Estorãos stream. Cadmium concentration, but not temperature, significantly affected fungal sporulation on leaves colonized in the Estorãos stream (two-way ANOVA, $p < 0.05$, $p > 0.05$, respectively). For leaves colonized in the Algeriz stream, temperature and cadmium significantly affected sporulation rates (two-way ANOVA, $p < 0.05$). Temperature stimulated fungal sporulation on leaves that were immersed in the Algeriz stream (Fig. 2.3). Fungal sporulation rates on leaves colonized in the two streams and for the two temperatures were significantly reduced by Cd, especially for concentrations greater than 1.5 mg L^- (Bonferroni tests, $p < 0.05$).

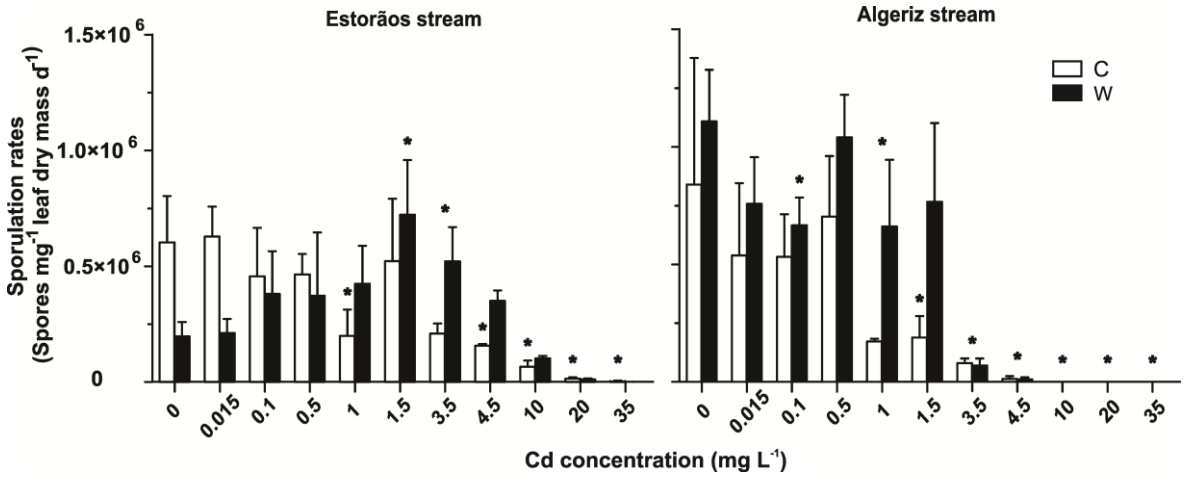

Figure 2.3- Fungal sporulation rates on decomposing alder leaves colonized in the Estorãos and Algeriz streams and exposed for 20 days in microcosms to increasing Cd concentrations (0, 0.015, 0.1, 0.5, 1, 1.5, 3.5, 4.5, 10, 20 and 35 mg L^{-1} Cd) at 15 ºC (C) and 21 ºC (W). Asterisk shows treatments that significantly differed from control (Two-way ANOVA $p<0.05$).

A non linear regression analysis (Gaussian adjustment) applied to sporulation rates from alder leaves colonized in the Estorãos and Algeriz streams showed a significant stimulus, for the lowest concentrations of Cd, followed by a significant decrease at highest Cd concentrations. For the Estorãos stream this was observed up to a concentration of 2.4 mg L^{-1} of Cd at 21 ºC and in the Algeriz stream up to 0.2 mg L^{-1} Cd concentration at 15 ºC ($r= 0.81$, $p < 0.05$) (Fig. 2.4).

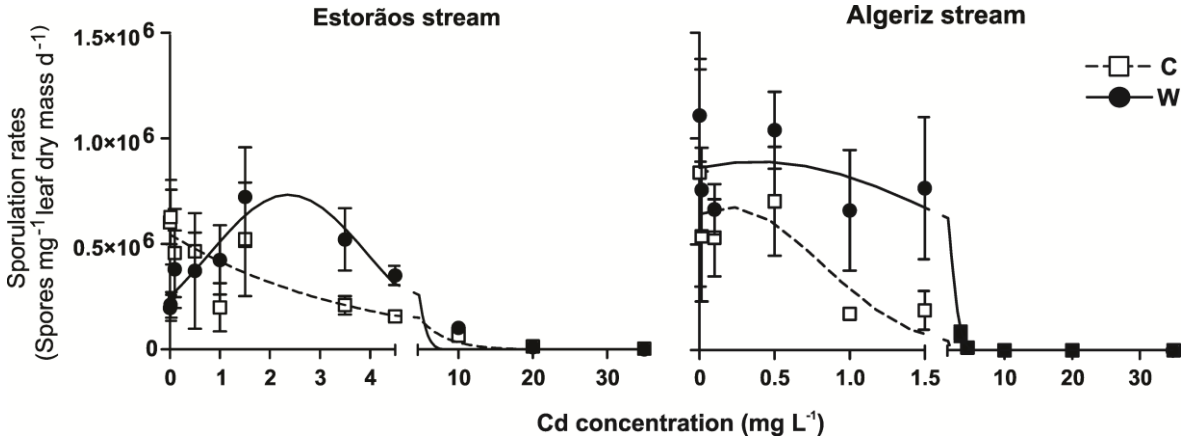

Figure 2.4 – Non-linear regression (Gaussian adjustment) of sporulation rates from alder leaves colonized in the Estorãos and Algeriz streams and exposed for 20 days in microcosms to increasing Cd concentrations at 15 ºC and 21 ºC .

2.3.3. Fungal biomass on leaves

Fungal biomass on alder leaves non exposed to Cd ranged from 217 to 261 µg ergosterol g^{-1} leaf dry mass for leaves colonized in the Estorãos stream and from 185 to 226 µg ergosterol g^{-1} leaf dry mass for leaves colonized in the Algeriz stream (Fig. 2.5). In general, leaves retrieved from the Estorãos stream had higher fungal biomass than those from the Algeriz stream at both temperatures (Fig. 2.5).

Fungal biomass was affected by Cd concentration, but not by temperature, in both streams (two-way ANOVA, $p < 0.05$). The highest fungal biomass was found at 1.5 mg L^{-1} Cd in leaves colonized in the Estorãos stream (353 µg ergosterol g^{-1} leaf dry mass) and in the Algeriz stream (295 µg ergosterol g^{-1} leaf dry mass). Cadmium at concentrations ≥10 mg L^{-1} inhibited fungal biomass on leaves colonized in the Algeriz stream exposed to the highest temperature (Bonferroni tests, $p < 0.05$). In the Estorãos stream, fungal biomass was not significantly decreased by the exposure to Cd.

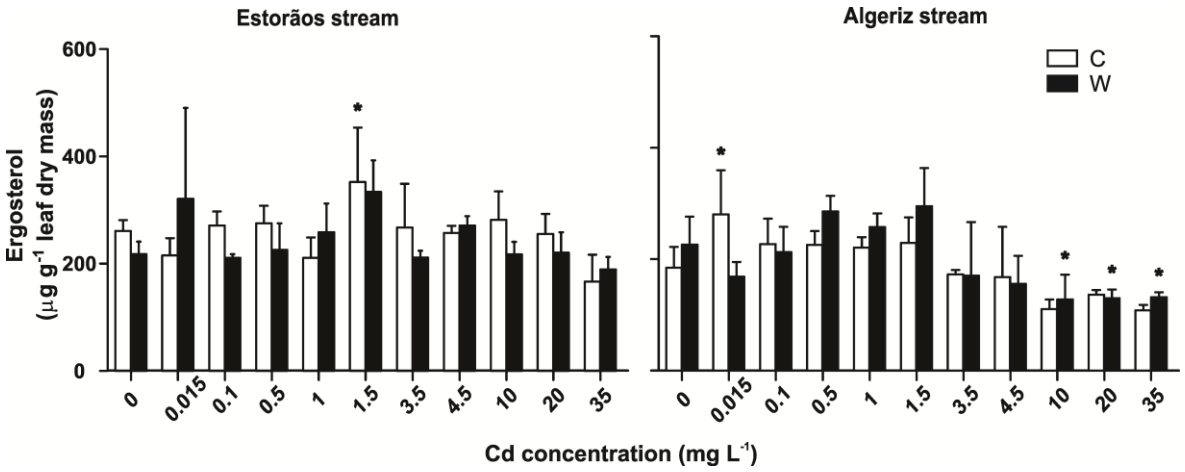

Figure 2.5- Ergosterol concentration on decomposing alder leaves colonized in the Estorãos and Algeriz streams and exposed for 20 days in microcosms to increasing Cd concentrations (0, 0.015, 0.1, 0.5, 1, 1.5, 3.5, 4.5, 10, 20 and 35 mg L^{-1} Cd) at 15 ºC (C) and 21 ºC (W). Asterisks show treatments that significantly differed from control (Two-way ANOVA $p<0.05$).

2.3.4. Leaf decomposition

After 20 days in microcosms at 15 ºC, leaves non-exposed to Cd lost 26 % and 37 % of their initial mass when colonized in the Algeriz stream and in the Estorãos stream, respectively (Fig. 2.6). Mass loss of alder leaves was generally higher at 21 ºC than at 15 ºC for both streams. Temperature and Cd concentration significantly affected mass loss of alder leaves colonized in both streams (two-way ANOVA, $p < 0.05$). For leaves colonized in the Estorãos stream, an increase in leaf mass loss was observed after exposure to Cd at concentrations between 0.1 and 4.5 mg L^{-1} at the highest temperature (Bonferroni tests, $p > 0.05$). For the Algeriz stream, leaf mass loss was stimulated at low Cd concentrations, but reduced at Cd concentrations ≥ 4.5 mg L^{-1} when leaves were exposed to the highest temperature (Bonferroni tests, $p < 0.05$). For leaves exposed to 15 ºC no significant decrease in leaf mass loss was found.

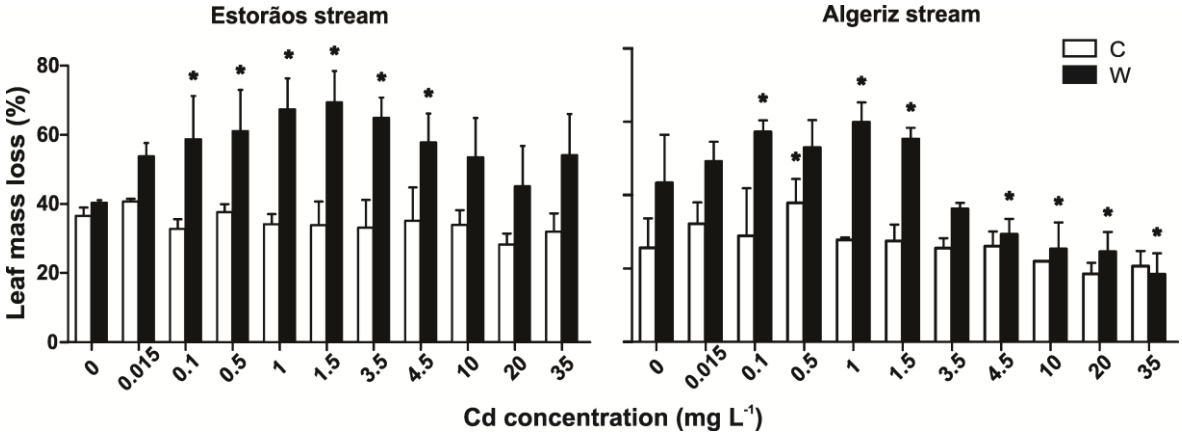

Figure 2.6- Mass loss of alder leaves colonized in the Estorãos and Algeriz streams and exposed for 20 days in microcosms to increasing Cd concentrations (0, 0.015, 0.1, 0.5, 1, 1.5, 3.5, 4.5, 10, 20 and 35 mg L^{-1} Cd) at 15 ºC (C) and 21 ºC (W). Asterisks show treatments that significantly differed from control (Two-way ANOVA $p<0.05$).

Mass loss of alder leaves colonized in both streams was positively correlated with fungal biomass or sporulation (Fig 2.7) at 15 ºC ($r= 0.54$ for biomass, and $r= 0.53$ for sporulation; $p < 0.0001$) and at 21 ºC ($r= 0.57$ for biomass and $r= 0.47$ for sporulation, $p < 0.0001$).

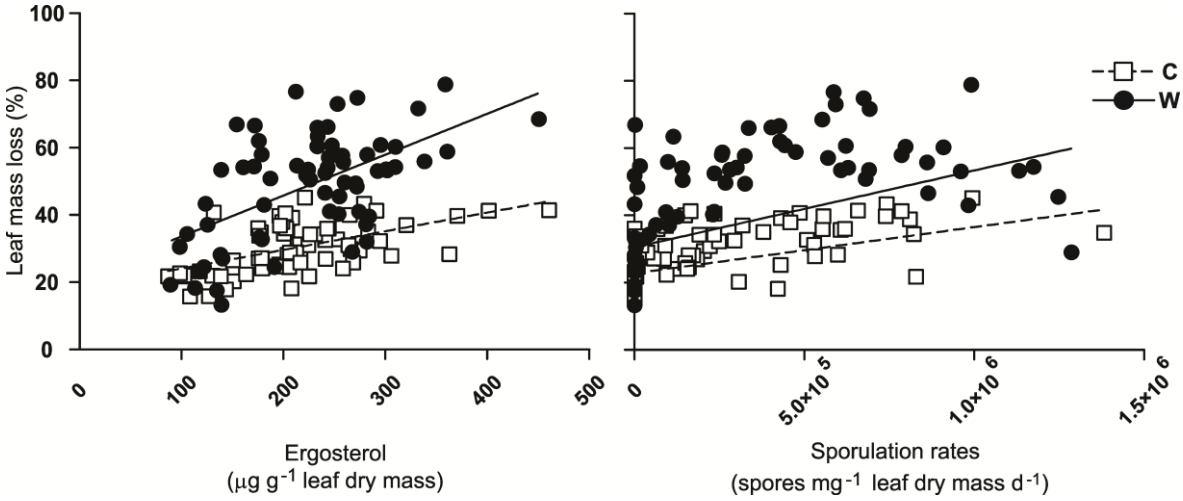

Figure 2.7 – Linear regressions between leaf mass loss and fungal biomass or fungal sporulation rates on alder leaves colonized in the Estorãos and Algeriz streams and exposed for 20 days in microcosms to increasing Cd concentrations (0, 0.015, 0.1, 0.5, 1, 1.5, 3.5, 4.5, 10, 20 and 35 mg L^{-1} Cd) at 15 ºC (C) and 21 ºC (W).

2.4. Discussion

In streams, leaf decomposition and aquatic hyphomycete reproduction and diversity tends to be reduced by the exposure to metals (Bermingham *et al.* 1996, Sridhar *et al.* 2001, Niyogi *et al.* 2002, Duarte *et al.* 2004, 2008). In our study, Cd and temperature significantly affected fungal sporulation rates; the exposure of fungal communities to the highest Cd concentration led to a significant decrease in the conidium production, but not in fungal biomass. This agrees with data reporting that fungal reproduction is more sensitive than fungal growth to metal exposure (Abel & Bärlocher 1984, Rodrigues *et al.* 2002, Duarte *et al.* 2004, 2008, Azevedo *et al.* 2007).

Changes in the community structure and identity of dominant fungal species may have indirect effects on litter decomposition because aquatic detritivores derive a large portion of carbon from fungal mycelium (Chung & Suberkropp 2009), and seem to have preferences to feed on certain fungal species (Suberkropp *et al.* 1983). If dominant species are affected by metal exposure, leaf decomposition and sporulation rates are expected to decrease. In previous studies, metals were able to reduce leaf decomposition but didn't affect fungal biomass (Duarte *et al.* 2004, 2008). Indeed, we cannot infer much about fungal activity from ergosterol measurements alone, since ergosterol is a static measure of standing stock biomass. In our work, only Cd concentrations up to 3.5 mg L^{-1} significantly decreased leaf decomposition, sporulation rates and fungal biomass in both streams.

DNA fingerprintings based on DGGE have been successfully used to analyze diversity of aquatic fungi associated with leaf litter (Nikolcheva & Bärlocher 2005, Duarte *et al.* 2008, 2009), since the contribution of each fungal species based on their reproductive ability can miss fungal taxa that are not sporulating (Nikolcheva *et al.* 2003, Nikolcheva *et al.* 2005) and fungal sporulation is often more sensitive than biomass to environmental stressors; therefore, the diversity of fungi on leaves may be underestimated when taxon identification only rely on the analysis of their reproductive structures (Niyogi *et al.* 2002). Indeed, in our study, the analysis of fungal community showed a higher number of fungal species based on OTUs than on conidium morphology. A relatively high species richness of aquatic hyphomycetes (between 17-24 species) has been reported in rivers polluted with metals (Sridhar *et al.* 2000). In this work, a total of 10 and 19 sporulating aquatic hyphomycete species were found on leaves colonized in the Estorãos and Algeriz streams. However cadmium appeared to lower fungal taxon richness assessed from spore identification and from DNA fingerprinting, particularly at the highest concentration.

Some aquatic hyphomycetes species are relatively tolerant to metals (Abel & Bärlocher 1984, Krauss *et al.* 2001, Miersch *et al.* 1997). Azevedo *et al.* (2009) reported the occurrence of a tightly regulated cell death pathway (programmed cell death), in aquatic hyphomycetes under metal stress as part of the mechanism underlying fungal acclimation in metal-polluted streams, since it would allow the rapid removal of unwanted or damaged cells sparing nutrients and space for the fittest ones. Another study demonstrated a higher production of tiol-containing proteins/peptides in aquatic hyphomycetes when exposed to high metal stress (Guimarães-Soares *et al.* 2006, *Braha et al.* 2007, Guimarães-Soares *et al.* 2007). We observed a stimulation of leaf decomposition and sporulation rates of aquatic hyphomycetes associated with alder leaves immersed in both streams at low Cd concentrations and then a decline above a certain Cd concentration. According to Chapman (2002), under low stress, organisms not only repair any damage, but also overcompensate and reduce background damage more effectively. This may explain why low Cd concentrations appeared to stimulate the development and activity of aquatic hyphomycetes in our study.

Many studies have reported a stimulation of the biological response (e.g. growth) at low doses of an inhibitor (Calabrese *et al.* 2005). This is known as hormesis (Luckey *et al.* 1975), a phenomenon documented in bacteria, plants, algae, fungi and animals (Wainwright 1994, Leading Edge Research Group 1996). There are several theories to explain hormesis. Stebbing (1997) theorizes that homeostatic and homeorhetic control mechanisms are common in living organisms and that during environmental sensing (by receptors common to all organisms) the control mechanisms of organisms cause the hormetic response at low doses of a toxicant, because errors in the control mechanisms increase during sensing loops. This causes the organism to over-respond to the stimulus. At higher concentrations, the toxicant causes the overload of control mechanisms so growth decrease and the organism dies. Thus oscillations in growth rates are a normal response to low-levels of a toxicant. Hart & Frame (1996) point out that because organisms have so many defense mechanisms (e.g. DNA-repair, stress-protein responses, and cell differentiation), each of which is expressed in response to the presence of different toxicants, each case of hormesis may be due to a specific mechanism for a certain organism. Hormesis is often ignored by statistical procedures, e.g. regression analyses (Wainwright 1994) and the ecological role of this phenomenon requires more investigation.

Our study showed that the increase in temperature tended to attenuate the effects of Cd. In the Estorãos stream, leaf decomposition rates increased almost twice when

temperature increased 6 ºC, even for highest Cd concentration and in the Algeriz stream higher sporulation rates were observed at the highest temperature. This was not unexpected because increased temperatures are expected to enhance biological activities. The stimulation of leaf decomposition could be the result of higher enzymatic activities (Brown *et al.* 2004) of some dominant fungal species present at the highest temperature. Even though a stimulation of leaf decomposition was found at the warming treatment, we failed to detect higher overall fungal biomass on leaves. According to Bärlocher *et al.* (2008), if faster leaf decomposition at increased temperature occurs in streams as found in this study, substrate availability for aquatic fungi would decrease further compromising the functioning of detritus-based food webs in freshwaters. Others studies demonstrated that changes in fungal growth and activity may lead to altered energy and nutrient flow through the food chain (Greenwood *et al.* 2006), and might affect aquatic organisms that have their life cycles synchronized with the autumnal litter supply in temperate regions (Bärlocher 2000, Gulis & Suberkropp 2004).

Additionally, in our work fungal species richness was affected by the increase of temperature. If changes in the stream temperature lead to decreases in fungal richness, either because species reach their upper thermal tolerance limit or due to exclusion by competition, this might negatively affect invertebrate performance (Lecerf *et al.* 2005) with further consequences for ecosystem functioning.

In general, fungal communities retrieved from the Algeriz and Estorãos streams had similar responses to the exposure to Cd and to the increase of temperature. An exception can be observed for the reproduction of aquatic hyphomycetes, which reinforces the idea that stressors may primarily affect the reproductive capabilities of these fungi (Bermingham *et al.* 1996, Duarte *et al.* 2008, Krauss *et al.* 2001). Since conclusions were based on responses of only two different fungal communities, further research using communities with different backgrounds may help to better understand the impacts of metal stress and global warming in streams.

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Chapter 3

Effects of temperature and cadmium on leaf consumption by the aquatic invertebrate shredder *Limnephilus sp.*

3.1. Introduction

Nowadays, metal contamination is still an environmental problem in both developing and developed countries throughout the world (USEPA 1999). Metals can reach ground and surface waters by the leaching of ores, and contaminated soils or through industrial activities. The most obvious effects of such contaminants are the reduction of abundance and diversity of aquatic biota, such as several species of aquatic invertebrate shredders (Burton *et al.* 1982, Soucek *et al.* 2000). Aquatic invertebrates are often used to assess biological impacts of trace metal pollution and have been useful for determining the transport and toxicity of contaminants, because they have diverse feeding behaviour, occupying several trophic levels, have rapid metabolism, and bioaccumulate many toxicants (Vuori & Kukkonen 1996, Canivet *et al*. 2001). For example, Cd, a non-essential metal is toxic to many aquatic organisms even at µg L^{-1} level, and can be accumulated by aquatic organisms (Maltby 1992, Chapman *et al*. 2003, Valenti *et al.* 2005, Wang 2008, Wang 2009, Schaller *et al.* 2010). Metals can compromise survival, growth and reproduction of several species of invertebrates at environmentally realistic concentrations (Barata & Baird 2000, Vogt *et al.* 2007). Anomalies or deformities at the morphological level can also be observed for several invertebrate species (Hare & Carter 1976, Donald 1980, Petersen 1986, Vuori & Parkko 1996, Decamps *et al.* 1973, Besch *et al.* 1977, 1979, Petersen & Petersen 1983, 1984, Petersen 1987). A recent study demonstrated that Cd exposure exerts a significant decrease in osmolality and haemolymph Ca^{2+} concentration, and behavioral changes, such as reduction in the feeding rate of the shredder *Gammarus pulex* (Felten *et al.* 2008).

Invertebrate shredders play an important role in plant litter breakdown, since they actively participate in the fragmentation of plant material and decomposition of coarse particulate organic matter (Webster & Benfield 1986), providing a trophic link between headwaters and lower sectors of rivers. The basal food resources for shredders in streams are dead plant tissues (leaves, twigs, and woody debris) from the riparian vegetation (Anderson & Sedell 1979, Webster & Benfield 1986), and leaf fall provides the majority of the annual input of this terrestrial litter (Fisher & Likens 1973, Webster & Meyer 1997). Research in temperate climates has established that shredders prefer certain leaf species over others (Iversen 1974, Irons, Friberg & Jacobsen 1994) and conditioned over unconditioned leaves (Rounick & Winterbourn 1983, Bärlocher 1985, Graça 1993). This pattern can be an advantage in environments where food sources vary seasonally or in an unpredictable way, enabling consumers to use a range of food

sources of variable quality without greatly compromising growth performance. Food preferences are poorly documented for shredders in tropic streams, however tropical shredders may adopt the same feeding tactics as the temperate shredders (Graça *et al.* 2001).

The Intergovernmental Panel on Climate Change (IPCC 2007) predicts an increase to double of the atmospheric CO_2 concentration, potentially affecting autotrophic and heterotrophic pathways in both terrestrial and aquatic systems. It is expected that an increase in water temperature can cause several changes in invertebrates, such as faster initial growth rates, shorter developmental time and smaller size at maturity (Atkinson 1995, Atkinson & Sibly 1997), and reduce the ability of invertebrates to survive on poor nutrient diets (Hanson *et al.* 1983). Other authors have reported a higher body mass for aquatic invertebrates at lower then at higher temperatures (Rempel & Carter 1987, Atkinson 1995, Hogg *et al*. 1995, Hogg & Williams 1996, Blanckenhorn 1997, Turner & Williams 2005). A recent study demonstrated that an increase in temperature alters the individual body elemental composition and affects consumption rate by aquatic detritivores (Ferreira *et al.* 2009). However, all these effects are predicted to be stronger for invertebrates inhabiting cold waters when compared to those inhabiting warmer waters (Braune *et al.* 2008), since biological activities are more temperature limited in cold water environments (Brown *et al*. 2004).

It is probable that the combined effect of metals and increased water temperature may have strong negative impacts on the processes in which invertebrate shredders are involved (e.g. litter decomposition, nutrient cycling), further compromising the functioning of freshwater ecosystems (Ferreira *et al.* 2009).

In this chapter, we tested how leaf consumption by invertebrate shredders and their growth are affected by cadmium and whether increasing temperature modulates this relationship. A common species of invertebrate shredder was collected from an unpolluted stream and acclimated to the laboratory. In one experiment, the animals were allowed to feed on alder leaves, while exposed to increased Cd concentrations (3 levels) and two temperatures: 15 ºC, a temperature commonly found in streams of Northwest Portugal in spring and autumn; and 21 ºC to simulate a warming scenario. In another experiment, the animals were kept under starvation for 4 days while exposed to increasing cadmium concentrations (10 levels) and then were released from the stressor and allowed to feed on alder leaves.

3.2. Material and methods

3.2.1. Tested animals

Experiments were performed with *Limnephilus* sp. (Tricoptera: Limnephilidae) because this genus is known to be widespread in European streams and easy to maintain in the laboratory. The animals were collected from an unpolluted site of the Cávado River (NW Portugal) and maintained in aerated stream water at 15°C, with a supply of *Alnus glutinosa L*. (alder) leaves.

3.2.2. Feeding experiment

The animals were exposed to stream water supplemented or not with Cd concentrations of 0, 0.5 and 10 mg L^{-1}. For each replicate concentration, 1 animal and 12 leaf discs (12 mm diameter) were placed in 250 mL Erlenmeyer flasks containing 150 mL of Cd solutions, which were aerated for 6 days at 15 ºC and 21 ºC (11 replicates). Leaf disks used in the experiment were previously colonized by microbial decomposers by immersing the leaves in a stream for 7 days. Colonized leaves were subsequently exposed for 20 days to Cd concentrations similar to those used in the experiment describe in Chapter 2. Solutions were renewed every 3 days to remove excreted compounds.

At the end of the experiment, leaf disks and animals were separated, counted, freeze dried for 48h and weighed. Just before being used, the length of the cocoon of each individual was measured under a stereoscopic microscope at x16, and individual dry mass of the animals was estimated by the application of the regression model DM = 0.0029 x CO – 0.0293 (R^2 = 0.73, $P < 0.05$, n = 36), where DM is dry mass (g) and CO is the length of the cocoon (mm).

Relative growth rates (RGR) of the animals (g animal dry mass g^{-1} dry mass animal day^{-1}) were calculated as $RGR=DM_g/(DM_f*t)$, where DM_g is the dry mass gained during the elapsed time (t=6 days) given by the difference between final and initial dry mass (mg) and DM_f is the final dry mass (g) (Ferreira *et al.* 2009). Survivorship was registered bi-daily during the experiment.

Relative consumption rates (RCR, g leaf dry mass g^{-1} animal dry mass day^{-1}) were calculated as $RCR=Le/(DM_f*t)$, where Le is the litter dry mass eaten during the elapsed time (t= 6 days) and DMf is the final dry mass of individuals (Ferreira *et al.* 2009). For this, leaf disks were weighed before and after being offered to the invertebrates.

3.2.3. Post-exposure feeding experiment

The animals were kept under starvation for 4 days while exposed to 10 levels of Cd up to 35 mg L^{-1}. For each replicate concentration, 5 animals were placed in each 500 ml Erlenmeyer flask containing 250 mL of solutions of Cd. Water oxygenation and turbulence were induced with air pumps. Cadmium solutions were prepared in filtered stream water.
After 4 days the solutions were replaced by stream water, the animals were allowed to feed on 20 alder leaf disks (12 mm diameter) during additional 5 days.
At the end of the experiment, leaf disks were separated, freeze dried for 48h and weighed to estimate leaf consumption rates.

3.2.4. Accumulation of cadmium on leaves and animals

The samples were oven at 500 ºC (15 h for leaves and cocoon) and (8 h for larvae) and then digested with nitric acid (1 ml, 10%) and hydrochloric acid (1 ml, 10%). After digestion the resulting solutions were washed with ultrapure water and 20 mL were transferred to Falcon tubes for metal analysis. Cadmium content in leaf disks, cocoon and larvae of the animals were analyzed by inductively coupled plasma-atomic emission spectrometry (ICP-AES) (Ehrman *et al.* 2008, Monteiro *et al.* 2009,Takenaka *et al.* 2008, Shi et al. 2008) at the Scientific and Technological Research Assistance Centre (CACTI, University of Vigo).

3.3. Results

3.3.1. Effects of Cd and temperature on invertebrate feeding

After 6 days in microcosms, the relative consumption rate of leaves by the shredder *Limnephilus* sp. corresponded to 0.23 and 0.30 g leaf dry mass g^{-1} animal dry mass day^{-1} at 15 ºC and 21 ºC, respectively (Fig. 3.1). The relative consumption rate was significantly affected by Cd concentration and temperature (two-way ANOVA, $p < 0.05$). Leaf consumption was higher for animals at 21 ºC than at 15 ºC, except when animals were exposed to 10 mg L^{-1} of Cd, in which leaf consumption was strongly inhibited. The exposure to Cd decreased the relative consumption rate by shredders, especially at 15 ºC (Bonferroni tests, $p < 0.05$).

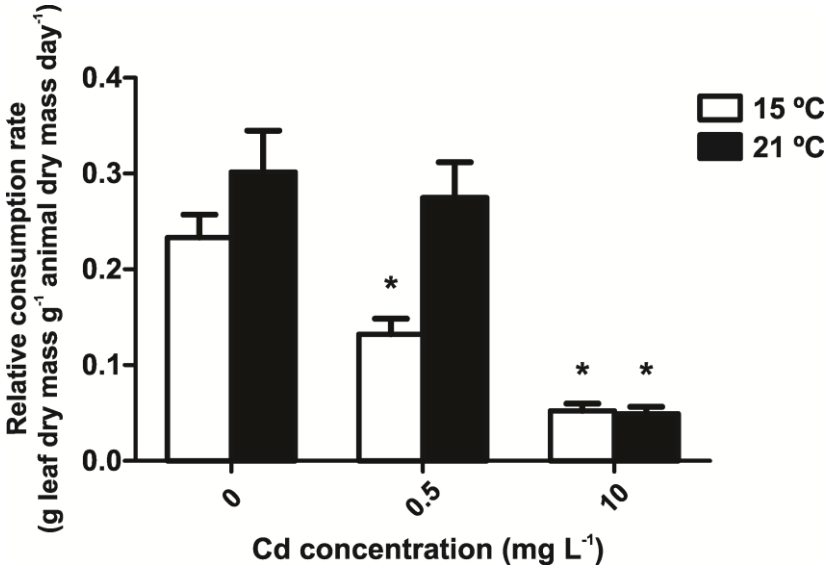

Figure 3.1– Relative consumption rates by the shredder *Limnephilus* sp. exposed or not to Cd at 15 ºC and 21 ºC. Asterisks show treatments that significantly differed from from control (Two-way ANOVA, Bonferroni tests, p<0.05).

The relative growth rate of animals in control microcosms was 0.05 and 0.08 g animal dry mass g^{-1} dry mass animal day $^{-1}$ at 15 ºC and 21 ºC, respectively (Fig. 3.2). Temperature, but not Cd concentration, significantly affected the relative growth rate of the shredder (two-way ANOVA, $p < 0.05$ and $p > 0.05$). Relative growth rate was greater for animals exposed at 21 ºC than at 15 ºC, for both control and Cd treatments (Bonferroni tests, $p < 0.05$).

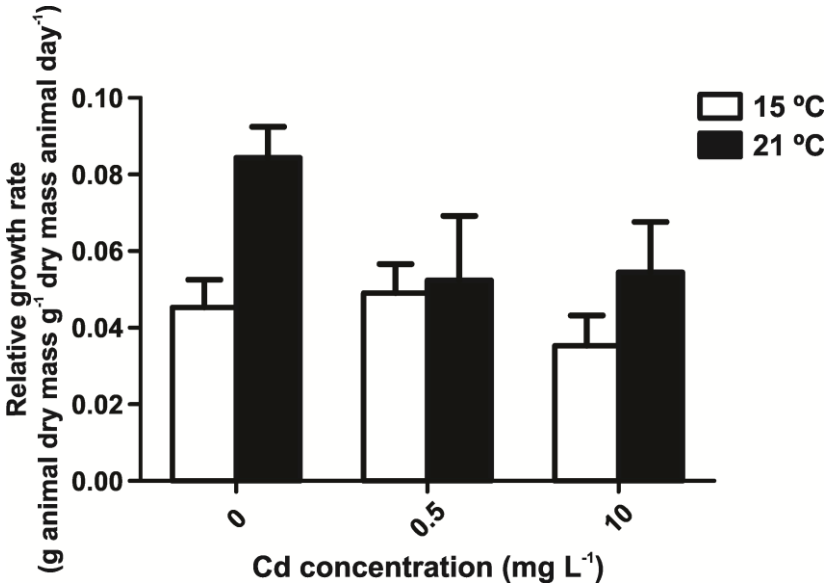

Figure 3.2– Relative growth rates by the shredder *Limnephilus* sp. exposed or not to Cd at 15 ºC and 21 ºC.

3.3.2. Accumulation of cadmium on leaves and invertebrates

After 6 days of Cd exposure, metal was mainly associated with the leaves, attaining a value of 4.30 mg g^{-1} for the highest Cd concentration (Table 3.1). The shredder cocoons have more Cd than the larvae. Moreover, the accumulation of Cd at 21 ºC was consistently higher than at 15 ºC.

Table 3.1- Cadmium concentration (mg g^{-1}) in leaves, and in the cocoon and larvae of the invertebrate shredder *Limnephilus* sp. in microcosms supplemented or not with Cd at 15 ºC and 21 ºC. Data represent the pool of all replicates.

	Temperature	Cd added (mg L^{-1})	Cd measured (mg g^{-1})
Leaves	15 ºC	0	0.02
		0.5	0.25
		10	3.57
	21 ºC	0	0.02
		0.5	0.33
		10	4.30
Cocoon	15 ºC	0	0.01
		0.5	0.05
		10	0.65
	21 ºC	0	0.01
		0.5	0.06
		10	0.89
Larvae	15 ºC	0	0.00
		0,5	0.02
		10	0.05
	21 ºC	0	0.00
		0.5	0.02
		10	0.12

3.3.3. Post-exposure feeding of invertebrate shredders

The invertebrate shredder *Limnephilus* sp. consumed in average 0.04 g leaf dry mass per animal (54 % of the initial leaf mass) in 5 days, after starvation during 4 days without Cd addition. Leaf consumption by the shredders pre-exposed to Cd for 4 days and then

released to the stressor did not significantly differ from control till a concentration of 0.05 mg L^{-1} of Cd (one-way ANOVA, Bonferroni tests, $p > 0.05$). Above this concentration, leaf consumption suffered a drastic inhibition and the animals almost stopped feeding during 5 days when pre-exposed to Cd concentrations ≥ 1 mg L^{-1} (one-way ANOVA, Bonferroni tests, $p < 0.05$).

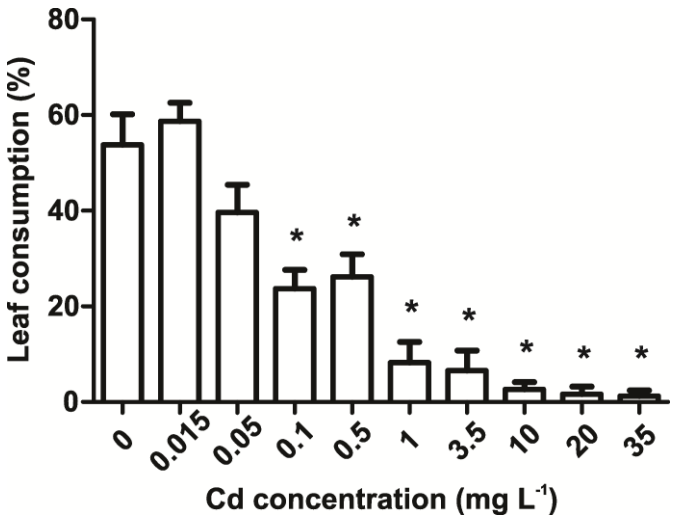

Figure 3.3– Consumption of alder leaves for 5 days by the shredder *Limnephilus* sp. at 15 ºC. The animals were previously exposed to increasing Cd concentrations for 4 days under starvation, before supplied with alder leaves and released from the stressor. Asterisks show treatments that differ significantly from control (One-way ANOVA, Bonferroni tests, $p<0.05$).

3.4. Discussion

In the past few years, a number of studies have used behavioral responses as tools for ecotoxicity testing and water quality monitoring (Mills *et al.* 2006, Felten & Guerold 2001, Maltby *et al.* 2002). These tests are of great importance for ecotoxicology purposes because, in addition to being sensitive, fast, simple to perform and cheap, they allow us to link toxic effects at biochemical/cellular levels to impacts on populations and communities (Wallace & Estephan 2004). In this chapter, we performed ecotoxicological assays to test how leaf consumption by invertebrate shredders and their growth are affected by Cd exposure and whether increasing temperature modulates this relationship. Shredders decompose plant litter from the riparian vegetation (Vannote *et al.* 1980, Cummins *et al.*1979), so they are key components of stream food webs. However, the evaluation of how temperature and Cd will affect shredder performance in streams is still lacking.

Felten *et al.* (2008) found a $LC50_{96h}$ = 82.1 µg L^{-1} for Cd for the aquatic shredder *Gammarus pulex*, and observed a significant Na^+ loss at a range of concentrations between 15 to 1000 µg L^{-1} of Cd. In this study, we used a range of Cd concentrations 10 fold higher than that of Felten *et al* (2008) and we did not observe mortality after 96h of exposure, even at the highest Cd concentration (35 mg L^{-1}). Similar results were found for the saltwater cladoceran *Moina monogolica* (Wang *et al.* 2009). One probable reason for this is the possible influence of the modes of metal exposure. Cadmium accumulation in invertebrates has been more strongly correlated with metal content in the food than in the water or sediment (Hare *et al.* 1991, Kiffney & Clements 1993, Beltman *et al.* 1999, Tessier *et al.* 2000). Metal toxicity is generally assumed to occur through waterborne exposure and environmental regulations do not take into account the potential impact of food as a source of metals to aquatic organisms (Brinkman & Johnston 2008). When metal exposure occurs via food in addition to via water, water quality criteria and standards may underprotect organisms in aquatic environments. Future studies on the effects of dietary versus aqueous exposure on aquatic invertebrates would be helpful to explain metal toxicity and protect ecosystems in more realistic scenarios.

Accumulation of Cd by aquatic organisms is currently reported in literature (McGeer *et al.* 2000, Nunez-Nogueira *et al.* 2005). It has been found that Cd can be stored in the hepatopancreas of the freshwater crab *Sinopotamon yangtsekiense* causing cellular damage (Xu 1995, Soegianto *et al.* 1999, Silvestre *et al.* 2005, Yan *et al.* 2007, Wang *et al.* 2008) or stored in granules and/or lysosomes of invertebrates belonging to Hydropsychidae (Hare 1992). However, if a certain threshold is met, organisms may excrete this toxic metal or trigger an efficient physiological defense (Valenti *et al.* 2005). In our study, Cd accumulated more in the cocoons than in the shredder bodies. Consequently, shredders with coccons might have a survival advantage during short-term exposure to metals. Moreover, our results indicate that alder leaves have a great potential to retain large amounts of Cd and thus they may have a potential for in situ treatments. This is of special interest in view of the low efficiency and very high cost of common metal removal techniques from polluted streams (Gatzweiler *et al.* 2004, Schaller *et al.* 2010).

In this work, the pre-exposure to Cd lowered in 60 % leaf consumption rates by the shredder *Limnephilus* sp. This agrees with other studies reporting a depression in invertebrate feeding rates (17–90%) after a pre-exposure to metals (Soares *et al.* 2005, Moreira *et al.* 2005). Our results suggest that the feeding rates of invertebrates could be considered as an evaluation criterion for toxicity tests. The use of feeding rate as a sublethal endpoint has already been proposed in other studies (Juchelka & Snell 1995,

1994, McWilliam & Baird 2002, Soares *et al.* 2005, Moreira *et al.* 2005, 2006). Alterations in the feeding behaviour are known to influence the organism's physiological performance, interfering with specific life-history events such as development, growth and reproduction, and eventually causing changes at the population and community level (Maltby 1994, Begon *et al.* 1986, Sibley *et al.* 1997, Maltby *et al.* 2002). Although in this study the exposure for 96h to Cd did not lead to animal death, feeding was severely inhibited after metal release suggesting that some animals lost the ability to recover.

In our feeding experiment, Cd caused a significant decrease in the relative consumption rates by the shredder. Moreover, higher leaf consumption rates were found at the highest temperature. Recently, it was reported a higher consumption rate at 15 ºC by medium size winter individuals while spring individuals tended to feed at similar rates at both temperatures, which partially agree with the prediction that increased temperature will strongly affect individuals from colder waters (Braune *et al.* 2008). It is well known the preference of shredders by conditioned leaves (Bärlocher & Kendrick 1973, Arsuffi & Suberkropp 1989, Graça *et al.* 1993, Canhoto & Graça 1996, Graça *et al.* 2001, Ferreira *et al.* 2010), and it is expected that an increase in temperature stimulates fungal biomass on decomposing leaves. This may explain the increased invertebrate feeding activity at higher temperature in our study.

Contrary to other studies (Naylor *et al.* 1989, Felten *et al.* 2008), our results demonstrated that Cd exposure didn't affect the relative growth rate of the animals. Reduced growth after exposure to metals may be due to food avoidance (Hatakeyama 1989, Irving *et al.* 2003, Wilding & Maltby 2006) or reduced food quality (Courtney & Clements 2002, Carlisle & Clements 2003). Studies have shown that fungal colonization on leaves can affect the food choice and growth of shredders (Suberkropp *et al.* 1983) by changing the palatability and food quality of leaves (Bärlocher & Kendrick 1973, Suberkropp 1992). Indeed, invertebrates are capable of detecting differences in the microbial community by selecting the most palatable food (Canhoto & Graça 2008, Chung *et al.* 2009). Since the leaves in our study were previously exposed to Cd it was expected a low associated fungal biomass contributing to lower animal growth rates.

Within non stressful conditions, aquatic invertebrates have been reported to have higher body mass when kept at lower than at higher temperatures (Rempel & Carter 1987, Atkinson 1995, Hogg *et al.* 1995, Hogg & Williams 1996, Blanckenhorn 1997, Turner & Williams 2005, Ferreira *et al.* 2010). Here we found higher relative growth rates for animals exposed to the highest temperature.

In conclusion, our results indicate that the increase in Cd concentration and an increase in temperature (6ºC) affected the feeding behavior and growth performance of invertebrate shredders. This may compromise at longer times the survival of sensitive shredder populations with direct impacts to plant litter decomposition and nutrient cycling in freshwater ecosystems.

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Chapter 4

General discussion and future perspectives

General discussion and future perspectives

Freshwaters are among the most endangered ecosystems in the world (Dudgeon et al. 2006) being metal pollution of great concern due to metal non-degradability, accumulation in the biota and biomagnification along aquatic food webs (Croteau *et al.* 2005, Marie *et al*. 2006). Freshwaters are also particularly vulnerable to climate change (Fernandes *et al.* 2009, Ferreira & Chauvet 2010), because climate models predict that an increase in extreme weather events will occur, such as increases in extreme high temperatures, decreases in extreme low temperatures, and increases in drought periods followed by intense rainfalls (Easterling *et al.* 2000, Jentsch *et al.* 2007). Therefore, runoff from the surrounding soils is expected to increase leading to changes in the levels and bioavailability of contaminants in freshwaters.

In low-order forested streams, plant litter decomposition is a key ecosystem process that is mainly driven by microbial decomposers and invertebrate shredders (Gessner *et al.* 2007). Among microbial decomposers, fungi, particularly aquatic hyphomycetes, seem to have a major role in leaf litter decomposition (Baldy *et al.* 2002, Pascoal & Cássio 2004) and enhance leaf nutritional value to shredder consumption (Graça 2001).

To better understand the impacts of metals and the global warming on plant-litter decomposition, the interactive effects of cadmium (Cd) and increased temperature on the diversity and activity of aquatic fungi and on the performance of invertebrate shredders were investigated.

In this work, leaf mass loss, leaf-associated fungal biomass, fungal reproduction and fungal diversity, assessed from sporulating species and DNA fingerprints, decreased by Cd exposure (Chapter 2). Despite many biological processes, such as microbial growth, are positively related to temperature (Fernandes *et al.* 2009), in this work temperature affected the diversity of fungi and mass loss of leaves colonized in the Algeriz stream and in the Estorãos stream, but not the biomass and sporulation rates of the aquatic hyphomycetes. These effects might have repercussions for energy flow across trophic levels (Chapman 2003), negatively affecting the invertebrate performances and litter decomposition (Lecerf *et al.* 2005), compromising all ecosystem functioning.

In this work, we found a stimulation of leaf decomposition and sporulation rates of aquatic hyphomycetes for low Cd concentrations and then a decline at certain Cd concentrations (Chapter 2). Many studies have reported the stimulation of a biological response (e.g. growth) at low doses of an inhibitor (Calabrese *et al.* 2005); this phenomenon, known as hormesis (Luckey *et al.* 1975), is well documented in bacteria, plants, algae, fungi and animals (Wainwright 1994, Leading Edge Research Group 1996). However, this effect

was not observed upon exposure to Cd in the feeding experiences with the invertebrate shredder *Limnephilus* sp. (Chapter 3).

The feeding rates and growth of the invertebrate shredder were more affected by Cd exposure than by the increase of temperature (Chapter 3). In the literature, nutrition (Brinkman & Johnston 2008) and growth (Hatakeyama 1989, Irving *et al.* 2003, Wilding & Maltby 2006) of invertebrates are commonly reported to be impacted by Cd exposure and by the increase of temperature (Turner & Williams 2005, Ferreira *et al.* 2009).

The results of our work that examined the combined effects to two stressors show negative effects on consumption rates and growth of the invertebrate shredder *Limnephilus* sp. (Chapter 3). This might have relevance on exposure scenarios in natural aquatic systems, as well as consequences on water quality criteria for Cd. However, caution should be taken when extrapolating laboratory results to natural water systems, because the last ones are much more complex and variable (e.g., the presence of organic and inorganic substances can reduce metal bioavailability and toxicity to aquatic biota; Bossuyt *et al.* 2005, Paquin *et al.* 2000).

The evaluation of feeding pos-exposure to the toxicant (Chapter 3) proved to be a very valid and necessary criterion. Alterations in feeding behavior are known to influence the organism's physiological performance, interfering with specific life-history events such as development, growth, and reproduction, and eventually causing changes at the population and community levels (Maltby 1994, Begon *et al.* 1986, Sibley *et al.* 1997, Maltby *et al.* 2002). The use of feeding rate as a sublethal endpoint has becoming more common in ecotoxicological applications, and it might represent a useful tool for the establishment of possible links between the toxic effect of contaminants and their bioavailability in the environment. The changes observed in consumption rates and growth rates, induced by these two stressors (Chapter 3) can result in a decrease in populations (Tuchman *et al.* 2003, Adams *et al.* 2005) that may impact litter decomposition, nutrient cycling and others processes in which these organisms are involved.

Overall, our results showed that the increase in temperature stimulated microbial decomposition of leaf litter, fungal reproduction and leaf consumption by the shredder. Increased cadmium concentrations inhibited reproduction and diversity of fungi, and leaf consumption by the invertebrate. The effects of Cd on fungal activity and diversity were more pronounced at the highest temperature, but an opposite trend appeared to occur for invertebrate feeding.

Since the consequences of metal stress under global warming are still unknown, further research will be needed to better understand the impacts not only on the biota represented here but on other important biota and other ecosystem processes.

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Percentage contribution of each aquatic hyphomycete species to the total conidial production on leaves colonized in the Estorãos stream and exposed for 20 days in microcosms to increasing Cd concentrations (0, 0.015, 0.1, 0.5, 1, 1.5, 3.5, 4.5, 10, 20 and 35 mg L^{-1} Cd) at 15 ºC and 21 ºC.

Species	Cd concentrations (mg L^{-1})																				
	0	**0.015**	**0.1**	**0.5**	**1**	**1.5**	**3.5**	**4.5**	**10**	**20**	**35**	**0**	**0.015**	**0.1**	**0.5**	**1**	**1.5**	**3.5**	**4.5**	**10**	**20**	**35**
	15 ºC											**21 ºC**										
Anguillospora filiformis, Greath. 1961	35.8	27.7	98.6	4.0	1.7	0.6	-	0.1	-	-	-	5.9	4.6	4.3	2.2	0.1	-	-	-	-	-	-
Articulospora tetracladia, Ingold 1942	0.2	0.4	-	0.1	0.1	-	-	-	-	-	-	-	0.1	0.1	0.1	-	-	-	-	-	-	-
Dimorphospora foliicola, Tubaki 1958	53.5	63.5	-	87.5	89.2	92.8	95.7	93.2	78.2	68.8	79.8	81.0	82.9	86.8	88.2	91.9	93.3	97.8	97.1	92.8	86.1	80
Flagellospora curta, Ingold 1942	2.1	1.0	-	1.2	1.5	1.5	1.8	4.9	21.4	29.9	16.7	2.5	1.7	1.3	3.6	4.1	2.1	1.4	2.1	6.6	13.1	15.0
Fusarium sp., Cooke & Harkn. 1881	0.1	-	-	0.1	0.6	0.2	0.2	0.1	-	0.3	-	0.1	0.2	0.1	0.1	-	0.1	-	0.1	-	-	-
Infundibura sp., Nag Raj & W.B. Kendr. 1981	0.7	0.3	1.4	0.3	0.2	-	0.3	0.1	-	0.3	1.2	2.1	1.3	0.5	0.3	0.1	0.6	0.1	0.1	0.3	0.8	5.0
Lunulospora curvula, Ingold 1942	7.3	7.0	-	6.6	6.6	4.6	2.0	1.6	0.4	0.7	1.8	8.4	9.0	6.3	5.2	3.7	3.9	0.6	0.6	0.4	-	-
Sigmoid 1	-	0.2	-	-	-	-	-	-	-	-	-	-	-	0.2	-	-	-	-	-	-	-	-
Tricladium chaetocladium, Ingold 1974	0.1	-	-	-	-	0.2	-	-	-	-	0.6	-	-	-	0.1	-	-	-	-	-	-	-
Triscelosphorus cf. acuminatus, Ingold 1942	0.1	-	-	-	0.1	-	0.1	-	-	-	-	0.2	0.2	0.2	0.2	0.1	-	-	-	-	-	-
No. species	9	7	2	7	8	6	6	6	3	5	5	6	8	9	9	5	5	4	5	4	3	3

Percentage contribution of each aquatic hyphomycete species to the total conidial production on leaves colonized in the Algeriz stream and exposed for 20 days in microcosms to increasing Cd concentrations (0, 0.015, 0.1, 0.5, 1, 1.5, 3.5, 4.5, 10, 20 and 35 mg L^{-1} Cd) at 15 ºC and 21 ºC.

Species	Cd concentrations (mg L^{-1})																				
	0	**0.015**	**0.1**	**0.5**	**1**	**1.5**	**3.5**	**4.5**	**10**	**20**	**35**	**0**	**0.015**	**0.1**	**0.5**	**1**	**1.5**	**3.5**	**4.5**	**10**	**20**	**35**
	15 ºC											**21 ºC**										
Alatospora acuminata, Ingold 1942	0.2	0.4	0.1	0.1	-	-	-	-	-	-	-	0.1	0.1	0.1	0.3	0.1	-	-	-	-	-	-
Alatospora pulchella, Marvanová 1977	4.4	6.0	5.5	8.2	15.5	14.5	36.1	34.5	7.1	-	-	5.9	6.6	8.4	16.3	16.5	26.7	67.9	71.4	-	-	-
Anguillospora filiformis, Greath. 1961	4.0	1.2	3.0	0.7	0.4	1.3	-	-	-	-	-	1.9	1.1	2.3	0.4	0.7	0.1	-	-	-	-	-
Articulospora tetracladia, Ingold 1942	38.7	48.2	42.3	53.8	33.4	28.8	15.9	2.6	14.3	-	-	47.4	45.3	46.7	39.3	44.6	36.6	17.7	8.6	30.8	62.5	-
Clavariopsis aquatica, De Wild. 1895	0.2	-	-	-	-	-	-	-	-	-	-	0.3	0.3	0.1	0.3	0.1	0.2	-	-	-	-	-
Cylindrocarpon sp., Wollenw. 1926	-	-	-	-	0.3	0.3	0.1	0.7	42.9	-	-	-	-	-	-	-	0.1	1.2	4.9	46.2	-	-
Flabellospora acuminata, Descals 1982	-	0.1	-	0.1	-	0.1	0.1	0.4	-	-	-	-	-	-	-	-	-	-	-	15.4	-	50.0
Fusarium sp., Cooke & Harkn. 1881	-	0.1	-	0.1	-	-	0.1	1.3	14.3	16.7	-	-	-	-	-	-	0.1	0.3	-	7.7	25.0	-
Infundibura sp.,Nag Raj & W.B. Kendr. 1981	15.2	12.2	16.5	0.1	-	-	-	-	-	33.3	-	19.7	20.9	3.7	0.2	0.1	-	-	-	-	-	-

Lemonniera aquatica, De Wild. 1894	5.2	4.3	4.1	3.7	8.0	3.4	4.1	3.5	-	-	-	4.3	4.5	4.8	7.3	6.4	5.7	3.0	4.9	-	-	-
Lunulospora curvula, Ingold 1942	4.4	5.8	6.3	4.0	3.2	2.0	2.9	2.2	-	-	-	4.1	3.5	5.0	11.5	12.6	14.3	1.7	3.3	-	-	-
Sigmoid 1	0.1	0.3	-	-	-	-	-	-	-	-	-	0.2	-	-	-	-	-	-	-	-	-	-
Tetracladium elegans, Ingold 1942	25.2	18.6	20.8	28.0	36.4	47.9	37.1	50.9	-	-	-	14.4	14.7	27.3	23.2	17.5	12.4	5.5	4.3	-	-	-
Tetracladium breve, A. Roldán 1989	0.1	0.5	0.2	0.3	0.4	0.1	0.3	0.2	-	-	-	0.4	0.3	-	0.1	-	-	0.1	-	-	-	-
Tricladium attenuatum, S.H. Iqbal 1971	-	0.8	0.1	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tricladium chaetocladium, Ingold 1974	0.8	0.2	0.4	0.3	-	0.4	1.0	0.4	7.1	-	-	0.4	0.1	0.1	0.1	-	0.4	0.3	0.4	-	-	-
Tricladium splendens, Ingold 1942	0.4	0.8	0.6	0.3	1.8	1.0	1.8	2.4	-	-	-	0.1	0.5	0.6	0.3	0.5	0.3	1.2	1.0	-	-	-
Triscelosphorus cf. acuminatus, Ingold 1942	0.4	-	0.1	0.1	0.3	0.1	0.3	1.1	14.3	50.0	-	0.3	1.5	0.6	0.5	0.8	3.1	1.3	1.2	-	12.5	50.0
Varcosporium elodeae, W. Kegel 1906	0.6	0.5	-	0.3	0.4	-	-	-	-	-	-	0.5	0.4	0.2	0.2	0.1	0.1	-	-	-	-	-
No. species	15	16	13	16	11	12	12	12	6	3	0	15	14	13	14	12	13	11	9	4	3	2