Title: Temperature alters interspecific relationships among aquatic fungi

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Abstract

Temperature is a key driver of the structure and activity of fungal assemblages on decomposing plant-litter in streams. However, little is known on how temperature affects interspecific relationships among fungi. We compared the radial growth of 4 widespread aquatic hyphomycete species co-occurring in temperate streams, namely *Articulospora tetracladia*, *Heliscus submersus*, *Lunulospora curvula* and *Variscosporium elodeae*, in all possible combinations of 1 to 4 species when exposed to 5 levels of temperature from 11 to 27 ºC. At 27 ºC, monocultures of *H. submersus*, *L. curvula* and *V. elodeae* had the highest growth rates, while the opposite was observed for *A. tetracladia*. At 27 ºC, the increase in species diversity had no effect on *V. elodeae* growth, increased the growth of *H. submersus* and *L. curvula* and decreased the growth of *A. tetracladia.* Results suggest that within its optimal temperature range, species performance may increase with the increase in fungal diversity.

Keywords: Aquatic hyphomycetes, temperature, interspecific interactions

Introduction

One of the most claimed threats to freshwater biodiversity and ecological processes is the expected temperature increase as a result of global warming (Carpenter *et al.* 1992). During the 21st century, the climate model projections anticipate an increase in the global surface temperature of Earth up to 6.4 ºC in the worst-case scenario (IPCC 2007). Because aquatic ecosystems are particularly vulnerable to climate change (Carpenter *et al.* 1992; Dudgeon *et al.* 2006), addressing how and to what extent the increase in temperature will affect freshwater biodiversity, species interactions and related ecosystem processes is a priority to aquatic ecologists.

Aquatic hyphomycetes are an ecological group of fungi that play a key role in plant-litter decomposition in streams due to its ability to produce extracellular enzymes that soften leaf tissues increasing its palatability to invertebrate detritivores (Suberkropp 1998; Bärlocher 2005). In streams, plant litter is usually colonized by a considerable number of fungal species (up to 20 sporulating species, Pascoal *et al.* 2005a; Duarte *et al.* 2009, 2010), but typically 1 to 4 species appear to ensure community productivity by contributing with more than 90 % to the total spores produced (e.g. Duarte *et al.* 2010).

Aquatic hyphomycetes are found within temperatures ranging from 0 to 34 ºC (Rajashekhar & Kaveriappa 2003; Nikolcheva & Bärlocher 2005) and temperature has been reported as a major factor in determining aquatic hyphomycete distribution in streams (Suberkropp 1984; Iqbal 1997; Bärlocher 2000). In temperate regions, the seasonal change in stream water temperature is the main factor controlling the structure and activity of fungal communities on decomposing plant litter (Suberkropp & Arsuffi 1984; Bärlocher 2000; Nikolcheva & Bärlocher 2005). Some studies addressed the effects of temperature on the growth and sporulation of several aquatic hyphomycete species (Chauvet & Suberkropp 1998; Rajashekhar & Kaveriappa 2000), but few have

considered the concomitant effects of temperature and species interactions. When *Lunulospora curvula* and *Tricladium chaetocladium* were grown in mixed cultures, their optimal temperatures for sporulation were lower than those found in monocultures (Webster *et al.* 1976). Thus, analysing the effects of temperature in species interactions may help to better understand the responses of fungal assemblages to temperature.

Species coexistence implies that they have encountered suitable conditions for survival and reproduction. However, the simple occurrence of a species does not mean that it is under its optimal temperature. We hypothesized that when a species is far from its optimal temperature would be less competitive than under its optimal condition. To test this hypothesis, we constructed fungal assemblages with up to 4 species that coexist in temperate streams of Northwest Portugal where water temperature typically ranges from 6 to 12 ºC in winter (Pascoal *et al.* 2005a, 2005b; Duarte *et al.* 2010) and from 16 to 21 ºC in spring (Pascoal & Cássio 2004). We compared growth rates of fungal species alone and in all possible combinations under 5 different temperatures ranging from 11 to 27 ºC to mimic ambient temperature and to simulate warming conditions.

Materials and Methods

Fungal species

The selected aquatic hyphomycete species were *Articulospora tetracladia* Ingold (UMB-72.01), *Heliscus submersus* H.J. Huds. (UMB-135.01), *Lunulospora curvula* Ingold (UMB-108.01) and *Variscosporium elodeae* W. Kegel (UMB-310.06). Fungi were isolated from foam or decomposing leaves from streams of Northwest Portugal and belong to the culture collection of the Centre of Molecular and Environmental Biology (CBMA), Department of Biology of the University of Minho.

Aquatic hyphomycetes were grown on 1 % (w/v) malt extract with 1.5 % (w/v) agar, before beginning the experiment.

Experimental setup

Malt extract 1 % agar plates were inoculated with culture plugs, collected from the edge of 15 old day colonies of the 4 fungal species, in monoculture or in all possible combinations of 2 to 4 species (4 replicates). Inoculation of single species plates was done with a 5 mm diameter plug. For multiple-species treatments, the total inoculum size was maintained and divided equally among species. Plugs were placed centrally in agar plates and sets of inoculated agar plates were incubated at 11, 13, 16, 21 and 27 ºC. Fungal radial growth was measured every 3 days under a binocular stereoscope during 12 days.

Statistical analyses

Radial growth rates (kr) of each fungal species at each temperature were estimated by linear regression between fungal radial growth and time. Fungal radial growth rates were compared by analysis of covariance (ANCOVA, Zar 2009). The growth rate of each fungal species in multicultures was then expressed as percentage of its growth in monoculture. Two-way ANOVAs were performed to test the effect of temperature and species diversity on the growth of each individual species (Zar 2009). Differences between treatments were analysed by Tukey´s post-tests (Zar 2009). All analyses were performed with $P < 0.05$ as criterion of significance. Linear regressions and ANCOVAs were done with Prism 4.0 for Windows (GraphPad software Inc., San Diego, CA, USA) and ANOVAs were performed with Statistica 8.0 for Windows (Statsoft, Tulsa, OK, USA).

Results

Effect of temperature on the growth of aquatic fungi in monocultures

Radial growth rates of aquatic hyphomycete species were significantly affected by temperature and species identity (ANCOVA, $P < 0.05$, Fig. 1). Generally, growth rates of *H. submersus*, *L. curvula* and *V. elodeae* increased with increasing temperature from 11 to 27 ºC (Table 1, Fig. 1). The growth rate of *V. elodeae* (kr = 1.68 mm d^{-1}) was significantly higher at 27 ºC than at the remaining temperatures (Tukey´s tests, $P < 0.05$). Maximum growth rates of *L. curvula* and *H. submersus* were 1.20 and 0.93 mm d^{-1}, respectively (27 ºC), and minimum growth rates were 0.36 and 0.20 mm d^{-1}, respectively (11 ºC) (Tukey´s tests, $P < 0.05$, Table 1, Fig. 1). Conversely, growth rates of *A. tetracladia* were maxima at lower temperatures (kr = 1.00 mm d^{-1} at 13 ºC; and 1.02 mm d^{-1} at 16 ºC) and minima at 27 ºC (kr = 0.51 mm d^{-1}) (Tukey´s tests, $P < 0.05$, Table 1, Fig. 1).

At the lowest temperature (11 ºC), *A. tetracladia* and *V. elodeae* had higher growth rates than *H. submersus* and *L. curvula* (Tukey´s tests, $P < 0.05$). At 21 ºC, growth rates of *V. elodeae* were higher than those found for all the other species (Tukey´s tests, $P < 0.0001$), while at 27 ºC growth rates increased in the following order: *A. tetracladia*<*H. submersus*=*L. curvula*<*V. elodeae* (Tukey´s tests, $P < 0.01$; Table 1, Fig. 1).

Effects of temperature and assemblage diversity on fungal growth

Growth rates of each aquatic fungus in multicultures were affected by temperature, species diversity and the interaction between both factors (2-way ANOVA, $P < 0.05$, Table 2, Fig. 2). At 11, 16 and 21 ºC, growth of *A. tetracladia* in monoculture did not differ from that in multicultures (Fig. 2, Tukey´s tests, $P > 0.05$), while at 13 ºC growth rates were higher in multicultures of 2 and 3 species than in monocultures (Tukey´s

tests, $P < 0.05$). At the highest temperature (27 ºC) growth rates of *A. tetracladia* strongly decreased in the presence of more than 2 species in multicultures (Tukey´s tests, $P < 0.0001$).

In a general way, growth of *H. submersus* decreased with the increase in species diversity at temperatures from 11 to 16 ºC (Fig. 2). However, significant differences in fungal growth were found only between monoculture and multicultures of 3 and 4 species at 11 ºC, and between dicultures and the remaining multicultures at 13 ºC (Tukey´s tests, $P < 0.01$). At the highest temperatures, the increase in species diversity led to an increase of *H. submersus* growth, with significant differences found between dicultures and multicultures of 4 species (Tukey´s tests, $P < 0.05$).

Similarly to *H. submersus*, growth rates of *L. curvula* decreased with the increase of species diversity at the lowest temperatures (Fig. 2). Significant differences in fungal growth were found between multicultures of 4 species and dicultures and monocultures at 11 ºC, and between multicultures of 4 species and all the remaining treatments at 13 ºC (Tukey´s tests, $P < 0.01$). On the other hand, a slight increase in the growth rate was found in *L. curvula* in multicultures of 3 and 4 species at 27 ºC, but these differences were not significant (Tukey´s tests, $P > 0.05$).

Growth rates of *V. elodeae* were not affected by species diversity at all tested temperatures, with the exception of 13 ºC where growth was significantly higher in all multicultures compared to monoculture (Tukey´s tests, $P < 0.0001$).

Discussion

In the current study, we analyzed the effect of temperature on the growth of 4 widespread aquatic hyphomycetes that coexist in streams of Northwest Portugal (e.g., Pascoal *et al.* 2005b) and, thus, representative of a realistic fungal assemblage on

decomposing plant-litter in temperate streams. Due to technical limitations to visualize and distinguish the mycelium of each species on decomposing leaves, we analysed the interactions between fungi on solid culture medium by following the growth of each species alone and in combination. The growth of aquatic hyphomycetes was affected by temperature and effects differed between fungal species. *Lunulospora curvula* and *H. submersus* had maximum growth rates between 21 and 27 ºC and were strongly inhibited at 11 ºC. This is not surprising since these species are reported in tropical and subtropical streams (Hudson 1961; Abdel-Raheem 1997; Iqbal 1997), and in warmer seasons in temperate streams (Chauvet 1991; Duarte *et al.* 2004; Pascoal & Cássio 2004). This also agrees with the maximum growth and sporulation rates found at 25 ºC (Chauvet & Suberkropp 1998; Rajashekhar & Kaveriappa 2000) and maximum cellulase activity at 28 ºC for *L. curvula* (Chandrashekar & Kaveriappa 1991). In addition, spore production of *L. curvula* in streams rapidly declines in late autumn, when lower temperatures are attained (Suberkropp 1984; Bärlocher 1991). On the other hand, in our study, *A. tetracladia* decreased its growth by approximately 50 % of its maximum at the highest temperature (27 ºC). This species showed maximum growth and sporulation at lower temperatures (15-20 ºC, Chauvet & Suberkropp 1998) and decreased its contribution to the total conidial production on decomposing leaves as temperature increased from 16 to 25 ºC (Fernandes *et al.* 2009; Geraldes *et al.* 2012), which is consistent with the strong inhibition of growth at 27 ºC found in our study. *Varicosporium elodeae* had high growth rates at the majority of temperatures tested in our study (11 to 27 ºC), which is consistent with its occurrence either in temperate or tropical streams in temperatures ranging from 0 to 25.5 ºC (Pascoal & Cássio 2004; Nikolcheva & Bärlocher 2005; Schoenlein-Crusius *et al.* 2009; Duarte *et al.* 2010).

Interspecific interactions have been documented in freshwater fungi (Yuen *et al.* 1999; Treton *et al.* 2004; Ferreira *et al.* 2010). Complementarity has been suggested to occur between aquatic hyphomycetes especially for biomass build-up (Duarte *et al.* 2006; Pascoal *et al.* 2010; Fernandes *et al.* 2011). The extensive intermingling of hyphae of several fungal species on leaves decaying in streams suggests the predominance of cooperative (or at least neutral) interactions, instead of antagonistic interactions, between aquatic hyphomycetes (Shearer & Lane 1983). In our study, *A. tetracladia*, the species with optimal growth at lower temperatures, exhibited higher growth rates in the presence of other species at 13 ºC. However, at the highest temperature (27 ºC) the growth of this species was highly inhibited in multicultures, suggesting that *A. tetracladia* was not able to take benefit of the presence of other species at temperatures that were not ensuring its optimal growth. Consistently, *L. curvula* and *H. submersus*, which showed higher growth rates at higher temperatures, had their growth inhibited by the presence of other species at lower temperatures (11 and 13 ºC), but stimulated by the increase in species diversity at 27 ºC. *Varicosporium elodeae* was the less affected species by the presence of others at most temperatures. Since growth rates of *V. elodeae* in monoculture were similar or higher than those of other species at all tested temperatures, it is conceivable that *V. elodeae* would be less negatively affected in multicultures.

Overall, we found that temperature differentially affected growth rates of four widespread aquatic hyphomycete species. *Articulospora tetracladia* exhibited maximum growth rates at lower temperatures and at the highest temperature its growth was strongly inhibited, mainly in multicultures. Conversely, the growth of warm-temperature tolerant species was stimulated or not affected by the presence of other species at the highest temperature. These findings suggest that within the optimal

temperature range of a species, the increase in fungal diversity increases the growth of that species, while out of this range growth decreases with increasing diversity. If this happens in nature, temperature might be a key factor determining interspecific relationships among aquatic hyphomycetes leading to changes in species dominance. Also, because invertebrate detritivores have preference to feed on certain fungal species (Suberkropp *et al.* 1983; Arsuffi & Suberkropp 1984), alterations in fungal species interactions by increased temperature might have a profound impact in energy flow and nutrient cycling in detritus food-webs in freshwaters.

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Figure legends

Figure 1. Effects of temperature on the radial growth (mm) of aquatic hyphomycetes in monocultures along time. Mean ± SEM, n = 4.

Figure 2. Effects of temperature on the radial growth rates (%) of fungal species in multicultures up to 4 species. Growth rates of each species in multicultures were expressed as % of the growth rate of each species in monoculture. Mean ± SEM, n = 4.

Tables

Table 1. Radial growth rates of four aquatic hyphomycete species at different temperatures. Mean ± SE, n = 4.

	Radial growth rates (mm d^{-1})				
	Temperature (ºC)				
Fungal species	11	13	16	21	27
A. tetracladia	0.79 ± 0.02	1.00 ± 0.04	1.02 ± 0.05	0.94 ± 0.03	0.51 ± 0.07
H. submersus	0.20 ± 0.02	0.61 ± 0.04	0.52 ± 0.02	0.90 ± 0.02	0.93 ± 0.16
L. curvula	0.36 ± 0.02	0.86 ± 0.01	0.89 ± 0.04	1.03 ± 0.02	1.20 ± 0.11
V. elodeae	0.81 ± 0.04	0.77 ± 0.03	1.23 ± 0.07	1.33 ± 0.03	1.68 ± 0.04

Table 2. Two-way ANOVAs of the effects of temperature and species diversity on growth rates of each aquatic fungus in multicultures.

	SS	Df	MS	F	P
A. tetracladia					
Temperature	75258	4	18814	82	<0.000001
Species diversity	9242	3	3081	13	<0.000001
Temperature x Species diversity	41987	12	3499	15	<0.000001
Error	30608	134	228		
H. submersus					
Temperature	83585	4	20896	23	<0.000001
Species diversity	14899	3	4966	5	0.001
Temperature x Species diversity	65519	12	5460	6	<0.000001
Error	126184	137	921		
L. curvula					
Temperature	24088	4	6022	8	0.00001
Species diversity	17648	3	5883	8	0.00009
Temperature x Species diversity	44666	12	3722	5	0.000001
Error	104961	137	766		
V. elodeae					
Temperature	81121	4	20280	118	<0.000001
Species diversity	4415	3	1472	8	0.00003
Temperature x Species	23376	12	1948	11	<0.000001

diversity			
Error	22391	130	172