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Diversity and activity of aquatic fungi under low oxygen conditions

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SUMMARY

1. The objective was to test whether a decrease in oxygen concentration in streams affects the diversity and activity of aquatic hyphomycetes and consequently leaf litter decomposition.

2. Senescent leaves of *Alnus glutinosa* were immersed for 7 days in a reference stream, for fungal colonization, and then incubated for 18 days in microcosms at five oxygen concentrations (4%, 26%, 54%, 76% and 94% saturation). Leaf decomposition (as loss of leaf toughness), fungal diversity, reproduction (as spore production) and biomass (ergosterol content) were determined.

3. Leaf toughness decreased by 70% in leaves exposed to the highest O_2 concentration, whereas the decrease was substantially less (from 25% to 45%) in treatments with lower O_2. Fungal biomass decreased from 99 to 12 mg fungi g^{-1} ash-free dry mass on exposure to 94% and 4% O_2 respectively. Sporulation was strongly inhibited by reduction of dissolved O_2 in water (3.1×10^4 versus 1.3×10^3 spores per microcosms) for 94% and 4% saturation respectively.

4. A total of 20 species of aquatic hyphomycetes were identified on leaves exposed to 94% O_2, whereas only 12 species were found in the treatment with 4% O_2 saturation. Multidimensional scaling revealed that fungal assemblages exposed to 4% O_2 were separated from all the others. *Articulospora tetracladia*, *Cylindrocarpon* sp. and *Flagellospora curta* were the dominant species in microcosms with 4% O_2, while *Flagellospora curvula* and *Anguillospora filiformis* were dominant at higher O_2 concentrations.

5. Overall results suggest that the functional role of aquatic hyphomycetes as decomposers of leaf litter is limited when the concentration of dissolved oxygen in streams is low.

Keywords: aquatic hyphomycetes, leaf decomposition, oxygen concentration, streams

Introduction

Aquatic hyphomycetes are a phylogenetically heterogenous group of fungi that have been recorded on decomposing leaves all over the world, including temperate (e.g. Bärlocher & Graça, 2002; Marvanová, Pascoal & Cássio, 2003), alpine (Gessner & Robinson, 2003) and tropical streams (Sridhar & Kaveriappa, 1988; Sridhar, Chandrashekhar & Kaveriappa, 1992; Gusmão, Grandi & Milanez, 2001; Schoenlein-Crusius, 2002; Rajashekhar & Kaveriappa, 2003). These fungi play a key role in the decomposition of leaf litter in aquatic environments (Bärlocher, 1992; Gessner & Chauvet, 1994; Gessner, Chauvet & Dobson, 1999) by

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producing a range of extracellular enzymes capable of degrading the structural components of plant cell walls (Bärlocher, 1992). Although leaves entering streams are already colonized by terrestrial fungi (e.g. Graça & Ferreira, 1995), aquatic hyphomycetes are well adapted to fresh water and rapidly colonize, grow and reproduce on submerged leaves (Bärlocher, 1992; Canhoto & Graça, 1996).

In streams, fungal biomass on decomposing leaves can account for up to 18% of leaf mass (Ferreira, Gulis & Graça, 2006a; Gulis, Ferreira & Graça, 2006) and a large proportion of their production is allocated to asexual reproduction (Gessner & Chauvet, 1994), with up to 7% of leaf mass incorporated into spores (Ferreira *et al.*, 2006a). Fungal activity on leaves is affected by several environmental factors, such as dissolved nutrients in water (Suberkropp & Chauvet, 1995; Gulis & Suberkropp, 2003a), temperature (Chauvet & Suberkropp, 1998), turbulence (Webster, 1975) and pH (Dangles *et al.*, 2004). Moreover, human activities, such as those from agriculture and urbanization, can lead to nutrient enrichment with consequent impacts on aquatic hyphomycetes and plant litter decomposition. Generally, low to moderate nutrient concentrations stimulate fungal activity (Gulis & Suberkropp, 2003b; Gulis *et al.*, 2006); however, high nutrients may lead to eutrophication and oxygen depletion, the combined effects of which on aquatic biota and processes are difficult to predict (Allan, 1995; Pascoal & Cássio, 2004; Elosegi, Basaguren & Pozo, 2006). Chergui & Pattee (1988) reported that decomposition and the number of species of aquatic hyphomycetes was lower in a side arm of the Rhone River than in the main channel, and this difference was attributed to lower water velocity and dissolved O_2 in the side arm. Similarly, Pascoal *et al.* (2003, 2005b), Pascoal, Cássio & Marvanová (2005a), and Pascoal & Cássio (2004) observed accelerated leaf decomposition with increased nutrient loading in a stream, except in a section with low current velocity and dissolved O_2. This raises the question whether concentration of dissolved O_2 is an important factor regulating the activity of aquatic hyphomycetes. However, low concentrations of O_2 are often accompanied by other environmental stressors, making it difficult to assess accurately the effects of dissolved O_2 on fungal activity and leaf decomposition in streams.

In this study, we tested the effect of dissolved O_2, separately from other confounding environmental factors, on diversity and ecological functions of aquatic fungi. We hypothesized that low concentrations of O_2 may reduce aquatic hyphomycete diversity and activity, leading to slower leaf decomposition in streams.

Methods

Colonization of leaves by aquatic hyphomycetes

Senescent leaves of *Alnus glutinosa* were collected from the ground just after abscission in November 2003 and stored dry. Portions of approximately 3.0 g were placed in mesh bags (0.5 mm pore size: $n = 20$) and in May 2004 placed in the São João stream, central Portugal (40°6′N, 8°14′W), a circumneutral fifth order stream running over a schistous substratum. The water has low conductivity (36–50 μS cm^{-1}), 150–180 and 10–12 μg L^{-1} of NO_3-N and SRP respectively (for additional information see Graça, Ferreira & Coimbra, 2001 and Ferreira *et al.*, 2006b). During the incubation of leaves, stream water was saturated with oxygen and mean temperature was 13.2 °C. After 7 days of immersion, leaf bags were collected and transported to the laboratory in an ice chest. Leaves were then individually rinsed with deionized water and cut into discs (7 mm diameter) with a cork borer. Previous studies in the same stream revealed that 7 days were enough for alder leaves to be colonized by aquatic hyphomycetes (Bärlocher & Graça, 2002).

Microcosm assay

We adapted microcosms described by Soares *et al.* (2005) to control oxygen concentration in the water. Microcosms consisted of 350 mL glass jars (11 cm high and 8 cm diameter, Fig. 1) containing 250 mL of a nutrient solution (5.88 g $CaCl_2{\cdot}2HO_2$; 2.46 g $MgSO_4{\cdot}7$-H_2O; 1.30 g $NaHCO_3$; 0.115 g KCl; 8.2 g KH_2PO_4; 16.9 g de $NaNO_3$; 1 L sterile deionized water). Aeration was supplied with two diffusing stones; one connected to an aquarium pump and the other to a nitrogen bottle. Nitrogen gas and air were supplied through a plastic tube inserted through the lid and ending into a diffusing stone. The rate of diffusion of both gases was individually controlled by taps in each jar and adjusted to the following oxygen concentration in the water: 100%, 75%, 50%, 25% and 5%, which corresponds to a range from 0.5 to 10.8 mg L^{-1} of

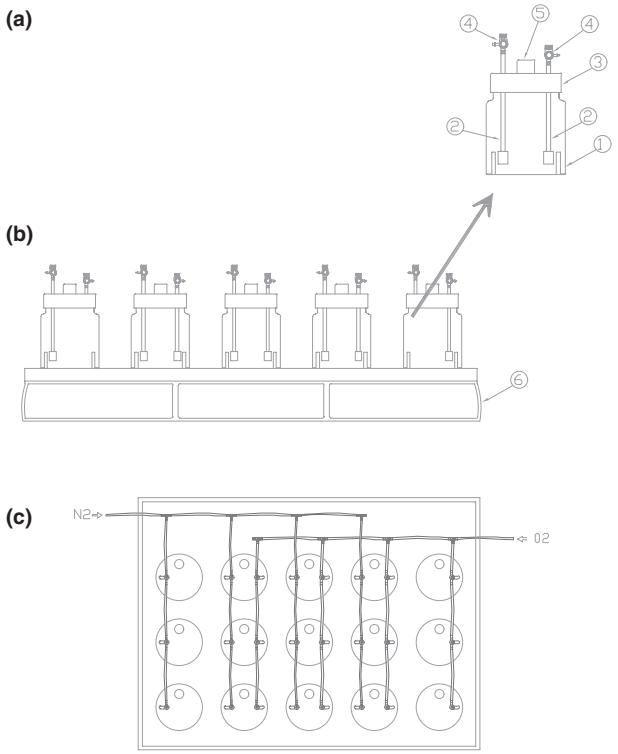

Fig. 1 The system for oxygen manipulation. (a): (1) glass jar microcosms, (2) diffusion stones, (3) lid, (4) tap to control gas flow, (5) hole for the introduction of the oxygen electrode (closed by parafilm). (b): (6) Microcosms over an orbital shaker. (c) Top view of microcosms with gas inflows. The roman numerals I–V represent the concentration of oxygen in the replicate jars (4%, 26%, 54%, 76% and 94% respectively).

dissolved oxygen. However, actual values during the experiment turned out to be 94%, 76%, 54%, 26% and 4% (standard error: 0.1–0.8). For each treatment (oxygen concentration), three replicate jars were deployed. Dissolved oxygen was measured twice per day in each jar using an oxygen meter (WTW 315i; Wissenschaftlich-Technische Werkstätten, Weilheim, Germany). Five leaf discs, from a pool of different leaves, were randomly allocated in each jar and incubated in an orbital shaker (60 rpm, at 15 °C) for 18 days and solutions from each jar were replaced every 3 days.

Leaf decomposition

Decomposition of leaf discs was measured as changes in leaf toughness using the device described by Graça & Zimmer (2005). Arsuffi & Suberkropp (1988) found a linear relationship between decrease in leaf toughness and leaf mass loss. To measure leaf toughness, leaf discs were secured by two pegs: one of the pegs was fixed, whereas the other was connected to a cup to which sand was added until its weight caused the leaf disc to break. Initial toughness was measured in five discs obtained from the leaves collected in the stream after 7 days of immersion. Toughness of leaf discs was measured in all microcosms at the end of the experiment. Results were expressed in terms of the percentage loss of strength relative to the value measured at the beginning of the experiment. To obtain a rough estimation of final ash-free dry mass (AFDM) of leaves, we assumed that decrease in mass loss was proportional to decrease in leaf toughness (Graça & Zimmer, 2005). Initial AFDM of leaves was determined as follows: sets of leaf discs were dried at 60 °C for a minimum of 2 days, weighted (±0.01 mg) ashed (500 °C, 5 h) in a muffle and reweighed.

Fungal reproduction and biomass

When renewing the nutrient solution (every 3 days), suspensions of conidia released from leaf discs were filtered through a 5-μm pore size membrane filter (Millipore Corporation, Bedford, MA, U.S.A.). The conidia retained were stained (0.1% cotton blue in lactophenol) and examined under the microscope (400×) for identification and counting (see Bärlocher, 2005 for details). The results were expressed as the total number of spores in each microcosm.

Fungal biomass was estimated from ergosterol concentration according to Gessner (2005). Sets of five leaf discs were preserved in 10 mL of KOH–methanol and stored at −20 °C until used. Lipids were extracted and saponified at 80 °C. The extracted lipids were partitioned into a non-polar phase and ergosterol was purified by solid-phase extraction. A final purification and quantification of ergosterol was achieved by HPLC (DIONEX Summit P580, Sunnyvale, CA, U.S.A.). Ergosterol concentration was converted to fungal biomass using the conversion factor of 5.5 μg ergosterol mg^{-1} fungal mycelium (Gessner & Chauvet, 1993) and the results were expressed as mg fungi $g^{-1}$of AFDM.

Statistical analyses

Differences in leaf toughness, and fungal biomass and sporulation among O_2 treatments were analysed by one-way ANOVA followed by a Tukey test (Zar, 1996).

Univariate analyses were performed using SIGMASTAT v. 2.03, (SSPS, Chicago, IL, U.S.A.).

To assess how the concentration of dissolved oxygen influenced the community of aquatic hyphomycetes, the fungal data (taxa at each sampling date and oxygen treatment) were subjected to similarity analysis followed by multidimensional scaling ordination (MDS) (Clarke & Warwick, 2001). MDS uses an algorithm, which successively refines the positions of the points until they satisfy, as closely as possible, the dissimilarity between samples. The result is a three-dimensional ordination plot where points that are close together represent samples that are very similar in composition. Points that are far apart correspond to samples with very different composition. Stress values for each MDS plot indicate the goodness of representation of differences among samples. To each grouping criteria, an ANOSIM was applied to check which of the proposed groups were significantly distinct (Clarke & Warwick, 2001). A simper analysis was carried out to determine the contribution of each species to the similarity relationships among treatments (Clarke & Warwick, 2001). Multivariate analyses were performed using PRIMER 5 (Software package; Plymouth Marine Laboratory, Plymouth, U.K.).

Results

Oxygen content was significantly different among treatments (one-way ANOVA, $P < 0.05$), with low variation within treatments (SEM up to 0.8%). Leaf discs from oxygen-saturated microcosms had the highest decrease in leaf toughness (70.4%), indicating faster decomposition than in treatments with lower oxygen concentrations (one-way ANOVA: $P < 0.05$: Fig. 2a). Fungal biomass on leaves was 6 mg g^{-1} AFDM at the beginning of the microcosm experiment and increased during incubation in all treatments (data not shown). Fungal biomass was significantly affected by O_2 concentration (one-way ANOVA: $P < 0.05$; Tukey test $P < 0.5$; Fig. 2b), with a maximum content in the treatment with 94% O_2 (99 mg fungi g^{-1} AFDM) and a minimum with 4% O_2 (12 mg fungi g^{-1} AFDM). Oxygen concentration had a significant effect on fungal sporulation (one-way ANOVA: $P < 0.05$), with the highest spore production (3.1×10^4 per microcosms) found at 94% O_2 and the lowest (1.3×10^3 spores per microcosms) at 4% O_2 (Tukey test, $P < 0.05$; Fig. 2c).

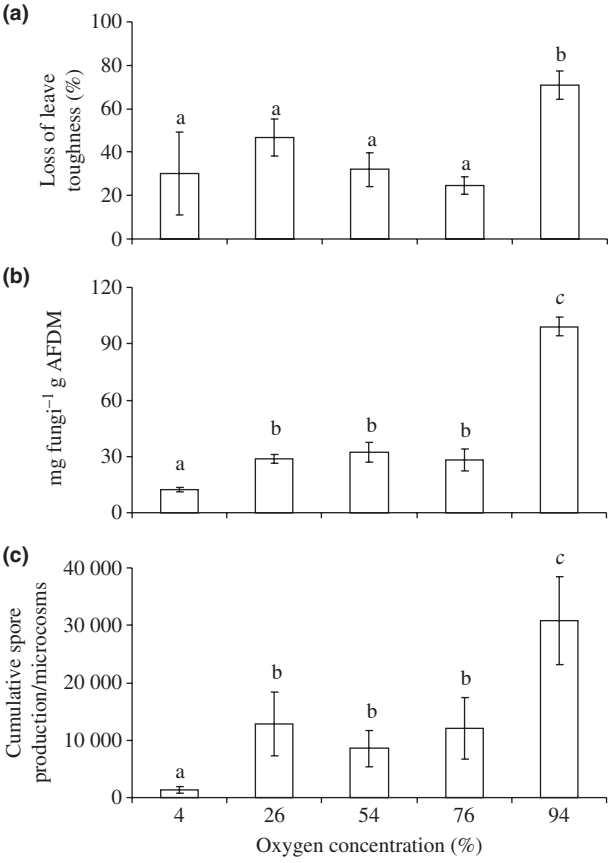

Fig. 2 (a) Loss of leaf toughness, (b) biomass of fungi and (c) cumulative spore production on alder leaves exposed to different oxygen concentrations for 18 days. Mean ± SEM; $n = 3$. Different letters denote significant differences among treatments.

A total of 20 species of aquatic hyphomycetes were identified on leaves (Table 1). The lowest number of fungal species (12) was found in microcosms with the lowest oxygen concentration (4%), whereas ≥16 species were found in treatments with higher oxygen availability. Moreover, *Flagellospora curvula* was a dominant species in microcosms with more than 54% of O_2, whereas *Articulospora tetracladia*, *Cylindrocarpon* sp. and *Flagellospora curta* were dosant in microcosms with the lowest concentration of O_2.

The three-dimensional MDS (Fig. 3) ordination based on aquatic hyphomycetes sporulating on leaves shows that communities in microcosms with 4% O_2 were significantly different from those with 54%, 76% and 94% O_2 (ANOSIM, pairwise test: R: 0.329–0.560, $P = 0.01$, 999 permutations). The taxa *Cylindrocarpon* sp. and *Flagellospora curta* were the main taxa responsible for differences between communities because

Table 1 Contribution of aquatic hyphomycete species to the total production of conidia, during the decomposition of alder leaves exposed to five oxygen concentrations

Species	Oxygen concentration (%)				
	4	26	54	76	94
Alatospora acuminata Ingold					0.5
Anguillospora crassa Ingold					0.2
Anguillospora filiformis Greath	1.5	10.0	18.7	40.7	27.1
Articulospora tetracladia Ingold	18.3	1.1	0.6	0.8	1.0
Clavatospora longibrachiata (Ingold) Marvanová and Sv. Nilsson	0.3		0.3	0.5	
Culicidospora aquatica R. H. Pertesen		0.2		0.3	0.2
Cylindrocarpon sp.	22.1	1.8	2.0	1.9	0.7
Dimorphospora foliicola Tubaki	6.0	4.9	2.2	4.2	1.3
Flagellospora curta J. Webster	23.9	3.4	2.1	2.8	3.4
Flagellospora curvula Ingold	16.3	19.9	64.2	42.3	48.8
Flagellospora sp.		0.7	0.7	0.1	0.8
Heliscella stellata (Ingold & V. J. Cox) Marvanová		1.3	0.1	0.8	0.4
Heliscus lugdunensis Sacc. & Thérry	1.4	0.9	0.4	1.0	0.7
Lunulospora curvula Ingold	1.7	7.0	0.2		0.8
Tetrachaetum elegans Ingold	5.1	22.9	5.6	2.0	8.6
Tetracladium marchalianum De Wild.					1.2
Tricladium chaetocladium Ingold			0.1		0.3
Tripospermum myrti (Lind) S. Hughes		0.2	0.2	0.1	0.1
Triscelosphorus sp.	2.0	12.4		0.5	0.2
Unknown sigmoid	1.3	1.1	1.4	1.0	0.4
Unknown tetraradiate		12.2	1.2	1.1	3.6
Total number of species	12	16	16	16	20

their contribution to total conidial production increased in microcosms with 4% O_2 (simper analysis; $P < 0.05$).

Discussion

In general, low-order streams are saturated with oxygen given the low volume : area ratio and turbulence, which increase the solution of atmospheric oxygen in stream water (Allan, 1995). However, organic and inorganic nitrogen and phosphorus can cause eutrophication, leading to a decrease in dissolved oxygen in the water, particularly in deeper and slowly flowing sections. Some studies have reported that leaves are strongly colonized by fungi in streams under aerobic conditions, while the contribution of bacteria to leaf litter decomposition increases under anoxic or hypoxic conditions (O'Connell *et al*., 2000; Pascoal & Cássio, 2004). Nevertheless, the consequence of a reduction in oxygen availability to aquatic fungi and to plant litter decomposition in streams is not well documented.

The results of our study indicate that leaf litter decomposition is affected by oxygen content in the stream water. As expected, the fastest leaf decomposition was obtained in microcosms with the highest oxygen content; these also had the highest fungal biomass and total spore production. A decrease in oxygen concentration to 76% caused a significant reduction in fungal sporulation and biomass, and in leaf decomposition. Surprisingly, further reductions in oxygen concentration (to 4%) did not lead to an additional inhibition in leaf decomposition, despite the progressive decrease in fungal biomass and spore production. This suggests that other microbial decom-

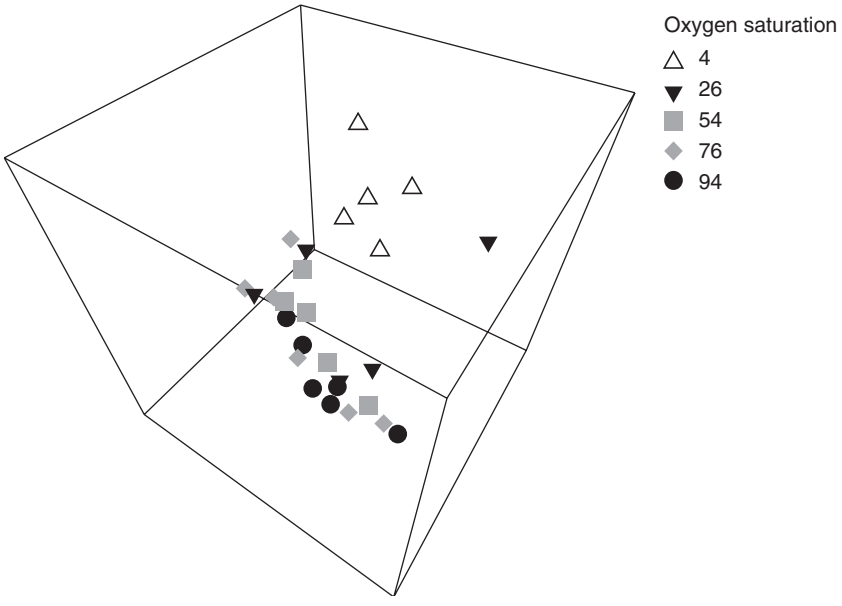

Fig. 3 Multidimensional scaling ordination (Bray Curtis) of fungal taxa sporulating on alder leaves exposed to different oxygen concentrations for 18 days (sampled six times during this period). Stress value 0.09 indicates that values are not randomly distributed in the 3D space.

posers, such as bacteria, might have increased their contribution to leaf decomposition, as has been suggested by others (O'Connell *et al*., 2000; Pascoal & Cássio, 2004). However, leaf decomposition was slow, which agrees with studies pointing to a minor role of bacteria in leaf decomposition (Pascoal & Cássio, 2004; Pascoal *et al*., 2005a,b).

We followed the effect of oxygen on leaf decomposition over 18 days, which might be too short to detect the effects of chronic exposure to low oxygen in the water. Experiments over longer exposure times might be more effective because effects may be amplified under chronic stress, particularly if fungal reproduction is seriously compromised. Results from our microcosm experiment could help to explain field observations of increased decomposition rates with increased nutrient content in the stream water, but only when oxygen concentration remained high (see Pascoal & Cássio, 2004; Pascoal *et al*., 2005b). Fungal biomass in microcosms with the highest oxygen content (maximum of 99 mg g^{-1} AFDM corresponding to 545 μg ergosterol g^{-1} AFDM) was similar to that found for the same leaf species in real streams (e.g. 631 μg ergosterol g^{-1} AFDM, Gessner & Chauvet, 1994; 477 μg ergosterol g^{-1} AFDM, Hieber & Gessner, 2002), suggesting that our microcosms were a reliable system for assessing the effect of oxygen on fungal activity.

Low oxygen concentrations may also selectively eliminate particular species of aquatic hyphomycetes. Twenty species were found on leaves, 14 of which had previously been reported from the stream in which the leaves were initially incubated (Bärlocher, Canhoto & Graça, 1995; Bärlocher & Graça, 2002). All species occurred in treatments with the highest oxygen concentration, but only 12 were found at the lowest. If we extrapolate these findings to the field, it means that decreases in oxygen content may cause changes in the structure of fungal decomposer communities. Indeed, Rajashekhar & Kaveriappa (2003) found a positive correlation between dissolved oxygen and the number of aquatic hyphomycete species in rivers of the Western Ghats of India.

Flagellospora curta and *Cylindrocarpon* sp. seemed to drive the results of MDS in which communities exposed to the least oxygen were separated from all the others, mainly because of high spore production by those species under oxygen-limited conditions. *Flagellospora curta* has been reported as dominant on decomposing alder leaves in polluted rivers from Portugal (Pascoal *et al*., 2003, 2005a; Pascoal & Cássio, 2004), whereas *Cylindrocarpon* sp. has been found in either polluted (Pascoal *et al*., 2003) or non-polluted (Bärlocher & Graça, 2002) streams. This suggests that at least some populations of both taxa may be adapted to harsh conditions and tolerate low oxygen. According to Field & Webster (1983), aquatic fungi potentially tolerate hypoxic conditions in streams because some species of aquatic hyphomycetes are able to grow under anaerobic conditions. In our study, however, the remaining tolerant species did not appear to be efficient in maintaining decomposition. It is important to point out that the initial fungal community came from an undisturbed stream; therefore, fungi able to tolerate low oxygen concentrations are expected to be rare.

In summary, the structure of the fungal community and leaf litter decomposition were strongly affected by a decrease in oxygen content in the water to only 76%. Therefore, dissolved oxygen seems to be an important factor in controlling fungal decomposing activity and its reduction may inhibit ecosystem processes. However, from this study alone we cannot ascertain if aquatic hyphomycete assemblages are able to recover from oxygen depletion; aquatic fungi could reduce their activity when oxygen decreases, resuming their growth and reproduction after conditions ameliorate. If that is the case, the impact of oxygen depletion on ecosystem functioning may be less dramatic than predicted from this study.

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