1. Introduction

Aquatic hyphomycetes are an important microbial assemblage in litter decomposition in freshwaters (GESSNER and CHAUVET, 1994; BALDY *et al*., 1995; HIEBER and GESSNER, 2002; PASCOAL *et al*., 2005). The nutrient concentration in water has been shown to affect fungal activity, particularly sporulation (SUBERKROPP, 1998; FERREIRA *et al*., 2006a) and oxygen consumption (STELZER *et al*., 2003; GULIS *et al*., 2004). High fungal activity often facilitates faster rates of litter decomposition as a large amount of initial mass is converted into fungal mycelium and conidia (NIYOGI *et al*., 2003; GULIS and SUBERKROPP, 2003; PASCOAL *et al*., 2005; FERREIRA *et al*., 2006a). Stimulation of fungal activity is thought to be higher in low quality (low nutrients, high lignin) substrates (STELZER *et al*., 2003; GULIS *et al*. 2004; but see FERREIRA *et al*., 2006a).

Wood, being a highly recalcitrant substrate, is usually colonized by fewer species of aquatic hyphomycetes and has lower microbial activity (sporulation, biomass built up, O_2 consumption) when compared with leaves (SIMON and BENFIELD, 2001; STELZER *et al*., 2003; FERREIRA *et al*., 2006a). This lower conditioning of wood can be overcome in the presence of an increase in the concentration of dissolved nutrients. In this case, wood can be an important resource for aquatic food webs when leaves are not available due to their faster decomposition rates or because of seasonal constrains.

The objective of this study was to assess the effect of two contrasting nitrogen levels (ambient and enriched) on the decomposition rates of balsa wood and on the activity (sporulation and oxygen consumption rates) of the associated aquatic hyphomycete communities, using laboratory microcosms. In a broad context, the experiment is expected to elucidate

relationships between nutrient levels in the environment and the functional process of litter decomposition. We expect fungal activity to be stimulated in the microcosms with the highest nutrient concentration, which could be reflected in higher mass loss.

2. Methods

2.1. Conditioning of Balsa Wood in Stream and Incubation in Microcosms

Balsa (*Ochroma pyramidale* (CAV. ex LAM.) URB.) veneers ($100 \times 10 \times 0.1$ cm) were bought from a local supplier and cut into 1.1 cm^2 squares. Each sample was composed of 10 squares (0.1688–0.3547 g) that were placed in fine mesh bags (3×3 cm; 0.5 mm mesh) and incubated in Margaraça stream from April 14 to June 6, 2006. Margaraça stream is a 1st order, circumneutral, SRP rich, N limited (NO_3-N = 104 ± 40 μg L^{-1} and soluble reactive phosphorus (SRP) = 104 ± 34 μg L^{-1}, average ± SD) stream running through a native deciduous forest (Margaraça Forest, Central Portugal, 40°13′ N, 7°56′ W). For more information about the stream see ABELHO and GRAÇA (1998) and FERREIRA *et al.* (2006a).

After 52 days of incubation, the bags containing the veneers (116 in total) were retrieved from the stream, placed in zip lock bags and transported in an ice chest to the laboratory, where they were gently rinsed with distilled water. Half the samples (n = 58) were autoclaved at 120 °C for 15 min. Ten of these samples were oven dried at 105 °C for 24 h, weighed, ashed at 550 °C for 6 h and reweighed to calculate initial air-dry mass to conditioned ash free dry mass (AFDM) conversion factor. The remaining 48 autoclaved samples were split into 2 groups and placed in individual 100 mL Erlenmeyer flasks with 25 mL of stream water (ambient N treatment; 0.16 mg N L^{-1}) or 25 mL of N amended stream water (enriched N treatment; 0.82 mg N L^{-1}; see below). These samples served as controls for fungal activity in the ambient and enriched N microcosms. The remaining non-autoclaved samples (n = 58) were distributed as above and were used to determine the effect of two contrasting N concentrations in fungal activity. Microcosms were incubated in shakers (100 rpm) at 15 °C for 4–24 days. The stream water used in microcosms was collected at the same time as the balsa samples, transported to the laboratory, and filtered using glass fiber filters (Millipore APFF). Half of the water untreated (ambient N treatment), and half had its N concentration amended with $NaNO_3$ so that it would be approx 5× higher than ambient (enriched N treatment). Chemical composition of water was determined from filtered samples by ion chromatography (Dionex DX–120, Sunnyvale, CA); SRP was determined by the ascorbic acid method (APHA, 1995; Table 1).

The solutions in the microcosms were replaced every 4 days, and 4 replicate microcosms of each of the 4 treatments (control + ambient N, control + enriched N, conditioned + ambient N and conditioned + enriched N) were sacrificed for measurements (see below). Fungal activity was determined only from conditioned + ambient N and conditioned + enriched N treatments while mass loss was determined from all 4 treatments.

Table 1. Water chemistry in microcosms (mean ± 1SD; $n = 3$).

	Ambient	Enriched
NO_3-N (mg L^{-1})	0.16 ± 0.03	0.82 ± 0.08
NH_4-N (mg L^{-1})	0.05 ± 0.00	0.03 ± 0.03
SRP (mg L^{-1})	0.07 ± 0.01	0.07 ± 0.03
Na (mg L^{-1})	7.74 ± 0.19	9.21 ± 0.56
Mg (mg L^{-1})	3.64 ± 0.03	3.78 ± 0.23
Ca (mg L^{-1})	3.77 ± 1.13	5.69 ± 4.75
K (mg L^{-1})	0.51 ± 0.25	0.45 ± 0.15
Cl (mg L^{-1})	9.12 ± 7.42	5.27 ± 0.82
SO_4 (mg L^{-1})	6.25 ± 2.38	4.85 ± 0.57

2.2. Aquatic Hyphomycete Sporulation and Microbial Oxygen Consumption

The conidia suspensions of conditioned + ambient N and conditioned + enriched N treatments were decanted into 50 mL centrifuge tubes, and conidia were fixed with 2 mL of 37% formalin to be later counted and identified. When preparing slides for conidia identification, 100 μL of Triton X-100 solution (0.5%) were added to the suspension to ensure a uniform distribution of conidia, stirred and an aliquot (5–10 mL) of the suspension was filtered (Millipore SMWP, 5 μm pore size). Filters were stained with cotton blue in lactic acid (0.05%), and spores were identified and counted with a compound microscope at 200×. Values were expressed as number of conidia microcosm^{-1}.

The 10 balsa squares of conditioned + ambient N and conditioned + enriched N treatments sacrificed every 4 days were used to determine microbial oxygen consumption rates (flow through system; ABELHO and GRAÇA, 2000). The flow-through system was set at 15 °C and consisted of a peristaltic pump with adjustable flow provided with Watson-Marlow orange/green tubes. One end of each tube was connected to a respiration chamber (8 ml glass syringes, covered with aluminum foil) with the 10 balsa squares, and the other end entered a reservoir containing ambient or enriched N stream water 100% oxygenated. Oxygen saturation of water was achieved by pumping air through air-stones. Measurements of oxygen concentrations in water were made only after the chambers' volume was totally replaced. The water flowing through the chambers was collected with a 1 mL syringe and injected into a 0.1 mL micro-chamber adapted to an oxygen electrode (Strathkelvin Inst. 781, Glasgow, Scotland) and readings were made after 30 seconds. After 3 measurements the flow was determined with 5 mL calibrated glass vials for 20 minutes. Oxygen consumptions were expressed as mg O_2 g^{-1} AFDM h^{-1}.

2.3. Decomposition

After the oxygen consumption trial, balsa squares of conditioned + ambient N and conditioned + enriched N treatments were oven dried at 105 °C for 24 h, weighed, ashed at 550 °C for 6 h and reweighed to calculate AFDM remaining. The same procedure was used for balsa squares from control + ambient N and control l + enriched N treatments.

2.4. Data Analysis

Differences in remaining AFDM by day 24 between treatments were assessed by 2-way ANOVA with N concentration (ambient and enriched) and balsa type (control and conditioned) as categorical variables.

Microbial oxygen consumption, aquatic hyphomycete sporulation rates, species richness, and % contribution by selected fungal species to the total conidial production were compared between ambient and enriched N microcosms by 2-way ANOVA (N concentration and time as categorical variables). Cumulative conidial production at each sampling date was calculated by summing values of daily production at each sampling. Comparison of cumulative conidial production between treatments was done by ANCOVA (N concentration as categorical variable and time as continuous variable). Simpson's index (D), which indicates the probability that two individuals chosen at random and independently will be found to belong to the same species (WASHINGTON, 1984), was calculated from conidial abundances (PRIMER 6, Primer-E Ltd, Plymouth, UK; CLARKE and GORLEY, 2001), and comparison between N treatments was done by 2-way ANOVA.

Data was transformed when necessary to achieve normality (ZAR, 1999) and analyses were performed with STATISTICA 6 software (StatSoft, Inc., Tulsa, OK, USA) unless otherwise indicated.

3. Results

3.1. Decomposition

After 52 days in the stream, balsa squares lost *ca*. 40% of their initial mass. The remaining mass was considered the initial mass for the experiment in microcosms. After 24 days

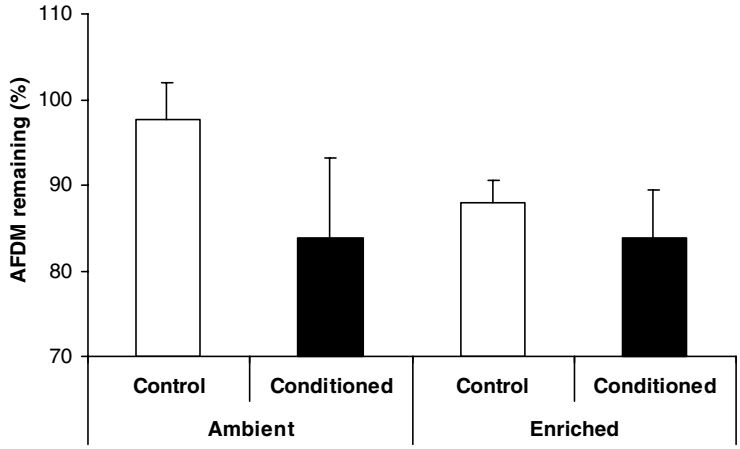

Figure 1. Remaining mass (average ± 1SE) of control and conditioned balsa squares after 24d in ambient and enriched N microcosms.

in microcosms, conditioned balsa squares lost 16% of their initial mass in both N treatments, while control balsa squares lost from 2% (ambient N) to 12% (enriched N) of their initial mass (Fig. 1). By day 24 no significant difference in remaining mass of balsa squares was found between N concentrations (2-way ANOVA, $P = 0.807$) or balsa types (2-way ANOVA, $P = 0.218$).

3.2. Microbial Oxygen Consumption

Oxygen consumption by microbes associated with balsa squares peaked by day 12 in the laboratory in enriched N microcosms (0.26 mg O_2 g^{-1} AFDM h^{-1}), while it decreased over time in ambient N microcosms (0.27 to 0.18 mg O_2 g^{-1} AFDM h^{-1} from d4 to d24) (Fig. 2). However, no significant differences in oxygen consumption rates between N concentrations were found (2-way ANOVA, $P = 0.549$).

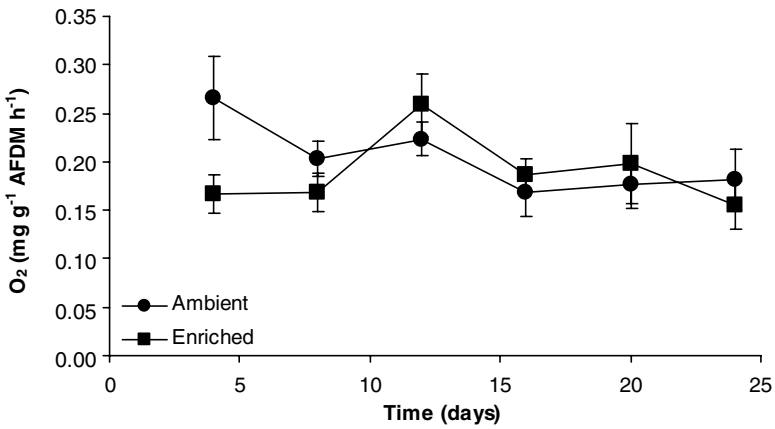

Figure 2. Microbial oxygen consumption (average ± 1SE) in conditioned balsa squares incubated in ambient and enriched N microcosms.

3.3. Aquatic Hyphomycete Sporulation

Conidial production increased through time and was always higher in enriched N microcosms (32902 *vs*. 20380 conidia microcosm^{-1} by d24; 2-way ANOVA, $P = 0.001$; Fig. 3a). Cumulative conidial production was up to 2 times higher in enriched than in ambient N microcosms (108591 *vs*. 59260 conidia by d24; ANCOVA, $P = 0.008$; Fig. 3b).

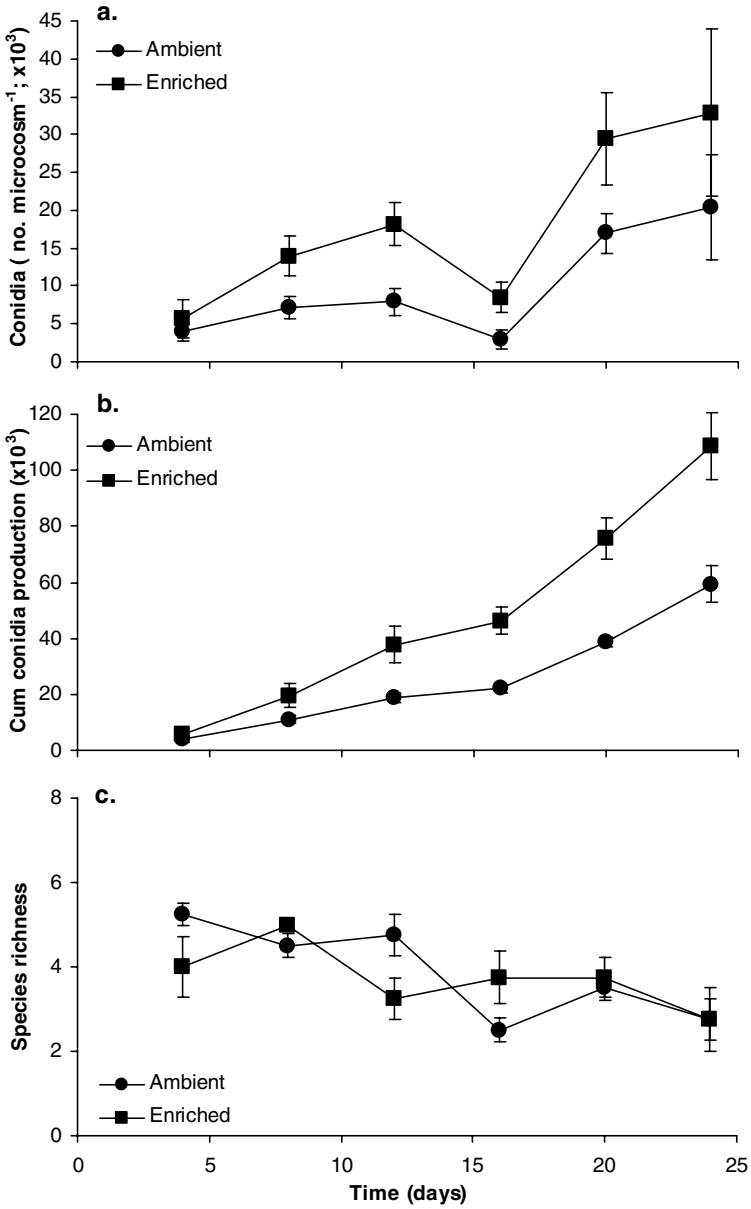

Figure 3. (a) Number of conidia (average ± 1SE), (b) cumulative conidial production (average ± 1SE) and (c) species richness (average ± 1SE) of aquatic hyphomycetes associated with conditioned balsa squares incubated in ambient and enriched N microcosms.

Table 2. Mean relative abundances (%, over all sampling dates) of aquatic hyphomycete conidia in balsa squares incubated in ambient and enriched N microcosms.

	Ambient						Enriched					
Days in water	4	8	12	16	20	24	4	8	12	16	20	24
Alatospora acuminata	8.2	10.6	13.5		0.9	0.7	1.8	7.1	1.0	2.0	5.1	0.6
Anguillospora crassa	75.6	74.8	74.2	96.1	96.3	95.0	85.4	74.8	94.1	94.8	92.0	94.2
Clavariopsis aquatica	3.1	8.7	8.3	3.4	2.4	2.7	5.7	14.1	3.5	0.8	2.1	5.1
Tetrachaetum elegans	0.2		0.5			0.7						
Tricladium chaetocladium	1.9	1.3	0.6		0.1	1.0	1.2	1.1	0.2	0.9	0.6	
Tricladium splendens	11.0	4.6	2.9	0.6	0.3		5.9	2.9	1.1	1.5	0.3	0.2
Simpson's index, D	0.8	0.8	0.8	0.4	0.6	0.5	0.7	0.8	0.6	0.6	0.7	0.6

There were 6 species of aquatic hyphomycetes sporulating in balsa squares in microcosms, but *Anguillospora crassa* dominated the fungal communities across all sampling dates in both N concentration microcosms (75–96% contribution to the total conidial production; Table 2). This was reflected in the relatively high Simpson's index values ($D = 0.5–0.8$; Table 2). The number of species sporulating in balsa squares was similar between ambient and enriched N microcosms (2-way ANOVA, $P = 0.659$) and it decreased through time (Fig. 3c). The percentage contribution of each species to the total conidial production was similar at both N concentrations (2-way ANOVA, $P = 0.103–0.696$). This resulted in similar Simpson's index values between N treatments (2-way ANOVA, $P = 0.701$).

4. Discussion

Given the low nutrient quality of balsa wood (Ferreira *et al*., 2006a), it was expected that an increase in dissolved nitrogen would lead to an increase in mass loss and associated microbial activity (Díez *et al*., 2002; Gulis *et al*., 2004). This seemed particularly likely since dissolved phosphorus was present in high quantity, and previous field experiments had shown that nitrogen was the limiting nutrient for fungal activity associated with this woody substrate (Ferreira *et al*., 2006a). However, decomposition of balsa did not respond to increased nitrogen concentration in microcosms. During the 52 days conditioning period in the stream, balsa squares lost *ca*. 40% of their initial mass, which was higher than reported in a previous experiment (*ca*. 25%; Ferreira *et al*., 2006a). This difference could be attributed to the season during which decomposition trials were carried out; the present study was carried out in late spring, while Ferreira *et al*. (2006a) performed their studies in autumn/winter when the water temperature was lower. Stimulation of litter decomposition and microbial activity due to increased water temperature has been reported before (Chergui and Pattee, 1990; Chauvet and Suberkropp, 1998; Ferreira *et al*. 2006b). The lack of significant differences in mass loss between control and conditioned balsa squares, although unexpected, could be explained by higher desegregation of fibers from wood after being autoclaved than from non-autoclaved squares.

Similarly, no significant differences were found in microbial oxygen consumption between microcosms with contrasting nitrogen concentrations. This was also unexpected, as several studies have reported stimulation of overall microbial activity in the presence of increased dissolved nutrients (GULIS and SUBERKROPP, 2003; NIYOGI *et al*., 2003; STELZER *et al*., 2003; GULIS *et al*., 2004).

Balsa squares were colonized by only 6 species of aquatic hyphomycetes compared to 11 species recorded colonizing this substrate in a previous study at the same stream site (site R; FERREIRA *et al*., 2006a). The difference was probably a result of balsa wood being incubated in spring in the present study, while FERREIRA *et al*. (2006a) incubated it in two consecutive autumns with several sampling dates. The number of species sporulating in balsa squares was similar between microcosms with contrasting nitrogen concentration, which is not surprising since in the stream these species were present in a wide range of nitrogen concentrations (approx 100–1000 $\mu g\ L^{-1}$; FERREIRA *et al*., 2006a). The percentage contribution of each species to the total conidial production was similar in both treatments for all species, which for *Anguillosora crassa* and *Clavariopsis aquatica* was surprising given previous field results. *C. aquatica* dominated fungal communities under high nitrogen conditions ($983 \pm 139\ \mu g\ L^{-1}$), while *A. crassa* dominated in the presence of low nitrogen concentrations ($82 \pm 7\ \mu g\ L^{-1}$) (FERREIRA *et al*., 2006a). The failure of *C. aquatica* to dominate in the high nitrogen microcosms could be explained by its preference for cooler water (BÄRLOCHER, 1992). Between days 8 and 12 of the microcosms experiment, the air conditioning system failed to keep the room at 15 °C and the air temperature raised up to 21 °C for 2 days. In this period, the percentage contribution of *C. aquatica* decreased from 14 to 3.5% in enriched nitrogen microcosms and remained low thereafter.

The relevant finding in this experiment was that, among all the measured parameters, only conidial production differed among treatments: conidial production by aquatic hyphomycetes was higher in enriched nitrogen microcosms, as reported in other studies using wood (FERREIRA *et al*., 2006a) and leaves (SUBERKROPP, 1998; GULIS and SUBERKROPP, 2003) as substrates. The data suggest that changes in the environment are likely to cause biological–physiological modifications at an individual level, before ecosystem functional parameters are altered.

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