Fungal growth and leaf decomposition are affected by amount and type of inoculum and by external nutrients

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Mass loss and ergosterol level of *Tilia cordata* leaves were studied in microcosms inoculated with 1, 2 or 4 of the following aquatic hyphomycete species: *Anguillospora longissima, Clavariopsis aquatica, Heliscus lugdunensis,* or *Tetracladium setigerum.* The amount of inoculum (4 levels) and nutrient concentrations (N and P, 3 levels) were also varied. Nutrient level, the amount of inoculum, fungal species numbers and identity all significantly affected mass loss over 21 days and final ergosterol levels. The magnitude of the effect was greatest with nutrient levels. There was no evidence of niche complementarity among the four species in this study.

Key words: aquatic hyphomycetes, diversity effects, niche complementarity, sampling effect, mass loss.

The accelerating rate of species extinction is raising concerns about the impact of decreasing biodiversity on ecological functions and services, and has stimulated a tremendous amount of research in the past decade (Huston 1994, Schulze & Mooney 1994, Kinzig *et al.* 2001, Loreau *et al.* 2002). Most studies have investigated the relationship between terrestrial plant diversity and primary production. Typically, there is a positive correlation, at least if the number of species is below 10 – 20 (Hooper *et al.* 2005). Less work has been done on the impact of microbial diversity (Wardle 2002), and only a handful of studies have investigated potential correlations between fungal diversity and decomposition in streams. Deciduous leaves or conifer needles represent one of the major food and energy sources for temperate stream communities (Allan 1995). Their decomposition is dominated by aquatic hyphomycetes, a phylogenetically heterogeneous community of asexually reproducing

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fungi (Bärlocher 1992, 2005), though recent molecular studies have suggested the involvement of other fungal groups (Nikolcheva & Bärlocher 2004). Field studies have failed to reveal correlations between the number of aquatic hyphomycete species in a stream and rates of leaf decomposition, suggesting considerable functional redundancy within these fungi or even among different stream microbial groups (e.g., Raviraja *et al.* 1998, Bärlocher & Graça 2002, Mathuriau & Chauvet 2002, Gulis & Suberkropp 2003, Pascoal & Cássio 2004). Experiments with pure cultures have given contradictory results. Species diversity and identity positively affected decomposition rate (up to 5 species, Bärlocher & Corkum 2003), or fungal biomass accumulation and spore production (4 species, Duarte *et al.* 2006). In the last study, decomposition rate was affected by species identity but not by species diversity. In a study with two species, leaf decomposition also increased in mixed cultures, but spore production was negatively affected (Treton *et al.* 2004). Dang *et al.* (2005) did not find any correlation between species number and decomposition or spore production (up to 8 species). Potentially confounding factors include the supply of inorganic nutrients, the type (mycelium or spores) and amount of inoculum. Increased nitrogen and phosphorus levels stimulate fungal growth, reproduction and degradational activity both in streams (e.g., Suberkropp & Chauvet 1995, Gulis & Suberkropp 2003, Pascoal & Cássio 2004) and in pure culture experiments (Sridhar & Bärlocher 2000, Bärlocher & Corkum 2003). Accelerated growth can increase competition among species and, in plants, this often results in a hump-shaped curve when species are plotted against nutrients or growth (Weldon & Slauson 1986, Huston 1994, Guo & Berry 1998). Increasing the amount of inoculum may further accelerate substrate colonization and the build-up of fungal biomass capable of releasing spores (Treton *et al.* 2004), thereby intensifying interspecific competition. This may lead to an impoverished fungal community without impairment of ecological functions. In the current study, we investigated effects of fungal diversity on the colonization and decomposition of linden leaves and how these functions may be modified by external nutrient supply and amount of inoculum.

Materials and Methods

Cultures and leaves

Four species of aquatic hyphomycetes, isolated from single spores, were provided by L. Marvanová from the Czech Collection of Microorganisms. They were *Anguillospora longissima* (Sacc. et Syd.) Ingold (reference number: CCM F-00980), *Clavariopsis aquatica* De

Wild. (CCM F-10791), *Heliscus lugdunensis* Sacc. et Thérry (CS-950), and *Tetracladium setigerum* (Grove) Ingold (CCM F-20987). They were maintained on 1 % Malt Extract Agar at 18 °C.

Freshly fallen leaves of *Tilia cordata* Mill. were collected from a single tree, soaked in tap water and cut into 19 mm disks with a cork borer. They were leached in distilled water for 24 h, freeze dried and stored until used.

Experimental procedures

A replicate microcosm consisted of a 250 mL Erlenmeyer flask supplied with 100 mL of mineral solution and 15 preweighed leaf disks. Mineral solutions were autoclaved separately, cooled and 100 mL aliquots were poured aseptically into flasks containing sterilized leaf disks. The basic mineral solution contained 0.01 g Na_2SO_4 and 0.0027 g $CaCl_2 . 2H_2O$ L^{-1}. It was supplemented with three levels of KNO_3 (0.14, 1.4 and 14 mg NO_3 L^{-1}) and K_2HPO_4 (0.05, 0.5 and 5 mg PO_4 L^{-1}). Microcosms were inoculated with agar plugs overgrown with a 7 – 14 day old culture. The basic unit of inoculum was one 7-mm disk cut with a corkborer. Four levels of inoculum were chosen, corresponding to 25, 50, 75 and 100 % of one plug. In mixed cultures, the inoculum of each species was reduced to maintain a constant amount per flask (e.g., two half disks when two cultures were used in a 1-plug treatment). For each treatment, three replicates were used. Three replicates without inoculum served to estimate mass loss in the absence of fungi. Flasks were incubated on an orbital shaker (120 rpm, 17 °C). After 21 days, all coarse leaf material was captured on preweighed Whatman filter paper No. 2, freeze-dried and weighed.

Ergosterol analysis

The method for microwave-assisted ergosterol extraction was modified from Young (1995). Freeze-dried leaf disks (50 – 100 mg) were ground in liquid nitrogen, suspended in 2.0 mL methanol with 0.5 mL of 2 M NaOH and tightly sealed in 15 mL glass tubes with Teflon lined screw cap. Extraction tubes were placed in 250 mL plastic bottles and tightly sealed. This combination was then microwaved at 50 % power for 95 s (Kenmore Microwave, Model No. 85055), and tubes were removed from the plastic bottle after attaining room temperature. The solution was neutralized with 1 mL of 1 M HCl and ergosterol was extracted with three consecutive hexane washes. The combined hexane fractions were evaporated to dryness and the residue was redissolved in 1 mL methanol. Aliquots

were injected into a high performance liquid chromatography C_{18} column (Varian, Palo, CA) and eluted with methanol at 1.5 mL min^{-1} (5.3 min retention time). Ergosterol content was estimated by comparison of peak areas with those of standard solutions.

Statistical analyses

Mass loss due to fungal activity was estimated by subtracting control values from values with inoculum and expressed as percentage mass loss. The difference was transformed (arcsine square root) and analysed by ANOVA (Fridley 2002). Analysis of non-transformed values gave the same pattern of significance. Individual values were compared with Tukey's Honestly Significant Difference. Statistical analyses were done with SYSTAT 5.3.1. for Macintosh (Kirby 1993).

In a first analysis, the effects of inoculum size (4 levels), nutrient addition (3 levels), and inoculum identity (four pure cultures and combined inoculation by all four species, 5 levels) on mass loss and ergosterol levels were considered. In a second analysis, the effects of species diversity (1, 2 or 4 species), species identity (nested within diversity) and nutrient levels on mass loss and ergosterol levels were analyzed (Jonnson & Malmquist 2000, Bärlocher & Corkum 2003).

Results

Effect of inoculum size

Fig. 1 shows mass loss of leaves as function of the amount of inoculum, nutrient level, and inoculum identity (one of four species, or equal proportions of all four). All main factors significantly affected mass loss (ANOVA, Table 1), as did interactions between nutrient and inoculum amount and nutrient and inoculum identity. Varying nutrient supply had the greatest impact: all three levels were significantly different from each other ($P = 0.05$), and the mass loss between lowest and highest levels increased by 10.8 % (averages for three nutrient levels across all other treatments). Mass loss was lowest with the smallest amount of inoculum ($P = 0.05$); the three higher levels did not differ significantly from each other. The greatest difference of mass loss (3.4 %) was between leaves inoculated with 0.25 or 1.0 plug inoculum (again, averages for four inoculum levels across all other treatments). Mass losses were also influenced by the identity of the inoculum. Mass loss was highest for *Tetracladium setigerum* and lowest for *Anguillospora longissima* (effect size of 3.2 %). Mass loss when all four species were inoculated was not significantly different from the *T. setigerum* treatment ($P > 0.05$).

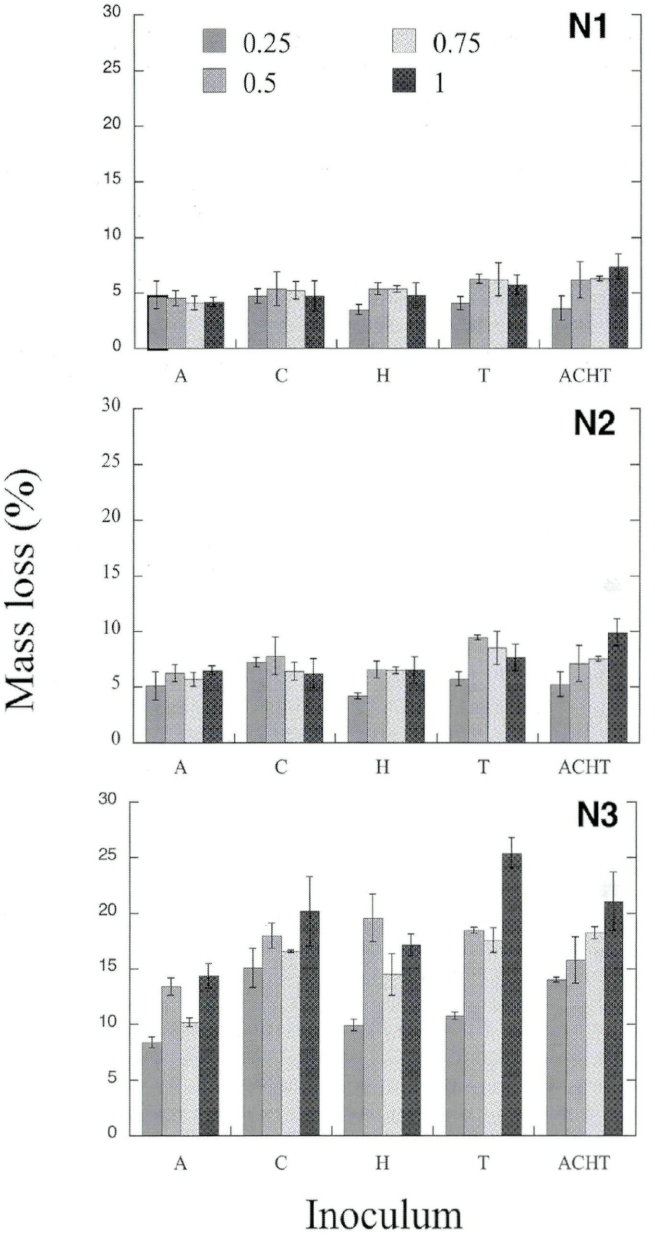

Fig. 1. Percentage mass loss of linden leaf disks inoculated with pure cultures of *Anguillospora longissima* (A), *Clavariopsis aquatica* (C), *Heliscus lugdunensis* (H), or *Tetracladium setigerum* (T), or all four species combined (ACHT), and incubated for 21 d at 17 °C. Total inoculum per flask was 0.25, 0.5, 0.75 or 1.0 of a 7-m plug overgrown with a fungal culture. N1, N2, N3: low, medium and high nutrient level. Each column represents three replicates, ± SE.

Fig. 2 shows final ergosterol levels of leaves as function of the amount of inoculum, nutrient level, and inoculum identity (one of four species, or equal proportions of all four). All main factors had a highly significant effect (ANOVA, Table 1), as did interactions between nutrient and inoculum amount and nutrient and inoculum identity. Varying nutrient supply again had the greatest and most consistent impact: ergosterol increased from low to intermediate to high level, and the largest difference of 88 µg g^{-1} was between low and high nutrient level. By contrast, changing the level of inoculum had a maximum effect of only 14 µg g^{-1}. Ergosterol levels were lowest with an inoculum amount of 0.25, and highest with 0.5. Inoculation with 0.5, 0.75 or 1.0 did not result in significantly different ergosterol levels. The effect of inoculum identity mirrored the results for mass loss. Ergosterol level was highest in treatments with *Tetracladium setigerum* and lowest for *Anguillospora longissima* (difference of 35.1 µg g^{-1}). Ergosterol levels in the treatment with all four species did not differ significantly from the *T. setigerum* treatment.

The combined effects of nutrient level, species diversity (three levels: 1, 2 or 4 species) and identity (nested within species number) on mass loss and ergosterol levels are presented in Fig. 3. Species identity and diversity, and nutrient level, all significantly affected mass loss (Table 1). Again, the greatest effect was achieved by varying nutrient levels (Fig. 3): mass loss at the highest nutrient level was 19.7 %, which was significantly greater ($P < 0.05$) than the 5.4 and 7.4 % found at low and intermediate levels. Across diversity levels, mass losses were 10.3, 6.4 and 12.8 % for 1, 2 and 4 species, respectively. When the identity of the inoculum was considered, the greatest overall loss was achieved by *Tetracladium setigerum* (13.0 %), and the lowest by the pair of *Anguillospora longissima* and *Heliscus lugdunensis* (6.1 %).

Nutrient level, species diversity and identity significantly affected ergosterol levels (Fig. 3). Ergosterol varied between 111 µg g^{-1} at the highest and 19.7 µg g^{-1} at the lowest nutrient level ($P < 0.05$). Like mass loss, ergosterol was lowest when species pairs were inoculated (60, 57 and 71 µg g^{-1} for 1, 2 and 4 species). Looking at the identity of the inoculum, the highest levels were reached in single cultures of *Tetracladium setigerum* (87 µg g^{-1}), and the lowest by the pair of *Clavariopsis aquatica* and *Heliscus lugdunensis* (35 µg g^{-1}).

Discussion

In a study with oak leaves, increasing the nutrient supply had a more pronounced effect on mass loss than modifying the number of fungal species (Bärlocher & Corkum 2003). The current study found the same pattern for linden leaves, and extended it to fungal biomass

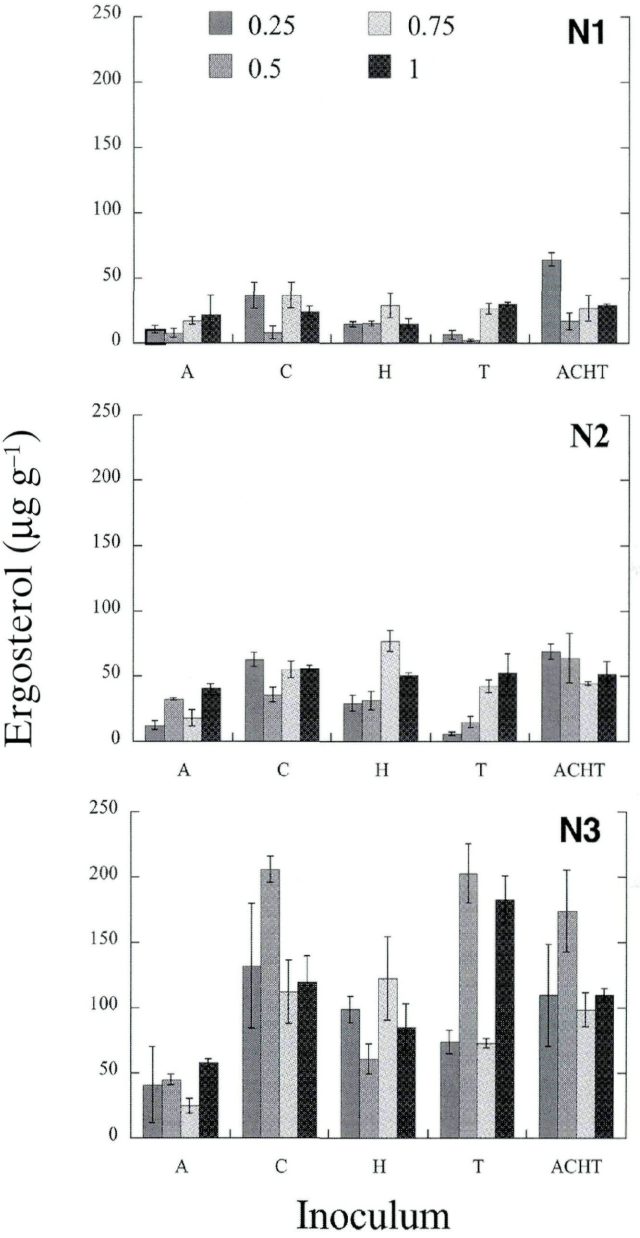

Fig. 2. Ergosterol levels of linden leaf disks inoculated with pure cultures of *Anguillospora longissima* (A), *Clavariopsis aquatica* (C), *Heliscus lugdunensis* (H), or *Tetracladium setigerum* (T), or all four species combined (ACHT), and incubated for 21 d at 17 °C. Total inoculum per flask was 0.25, 0.5, 0.75 or 1.0 of a 7-m plug overgrown with a fungal culture. N1, N2, N3: low, medium and high nutrient level. Each column represents three replicates, ± SE.

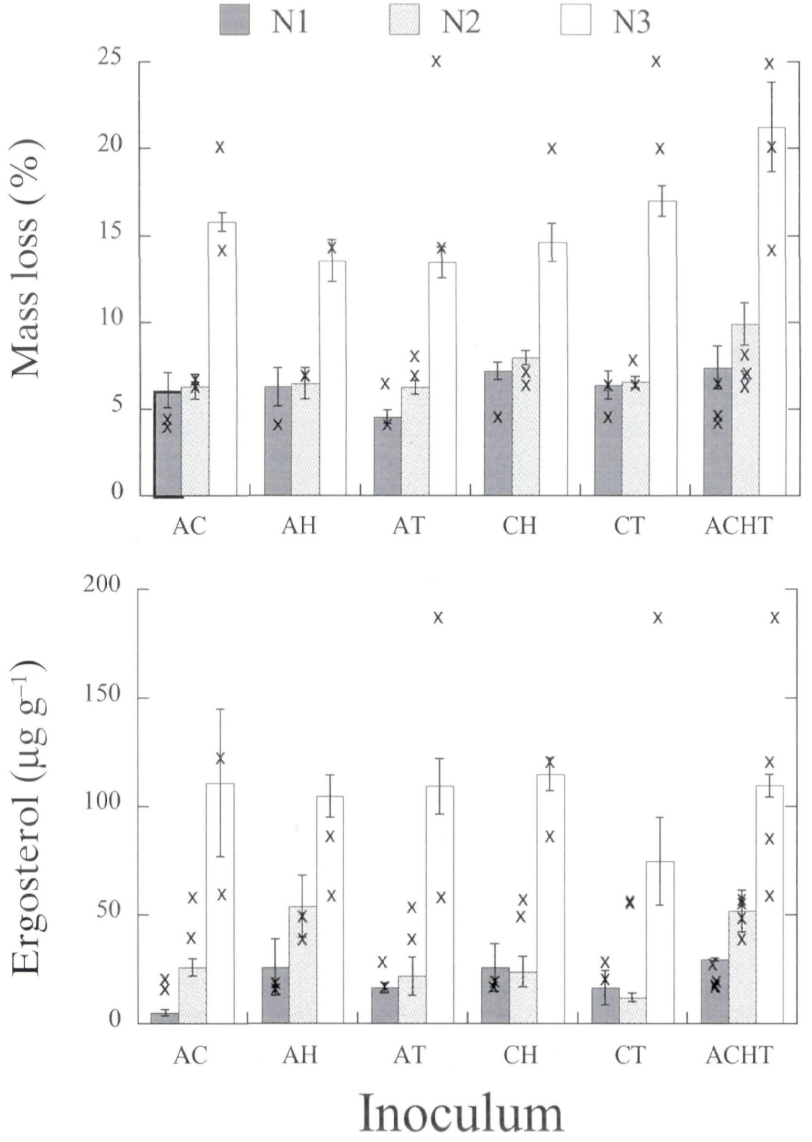

Fig. 3. Percentage mass loss (1) and ergosterol levels (2) of linden leaf disks inoculated with combinations of 2 or 4 of *Anguillospora longissima* (A), *Clavariopsis aquatica* (C), *Heliscus lugdunensis* (H), and *Tetracladium setigerum* (T). Total inoculum per flask was one 7-mm plug overgrown with a fungal culture. N1, N2, N3: low, medium and high nutrient level. Crosses indicate corresponding values for monocultures of the component species at an inoculum size of 1 plug. Each column represents three replicates, ± SE.

accumulating on the leaves. Dang *et al.* (2005) found no effect of nutrient level on alder leaf decomposition or fungal spore production. However, alder leaves contain large initial level of nitrogen and decompose rapidly (e.g., Bärlocher & Graça 2002). In addition, the lower nutrient levels chosen by Dang *et al.* (2005) was already quite high (10 mg L^{-1} KNO_3 and 0.55 mg L^{-1} K_2HPO_4). In many similar studies with plants (Huston 1994, 1997), the variability of resources, such as nitrogen, phosphorus or light, generally has a greater impact than plant diversity on primary production. Fridley (2002) found soil fertility to be a stronger predictor of productivity than plant diversity. In more general terms, diversity effects are context-specific (Cardinale *et al.* 2000, Klironomos *et al.* 2000, Jonsson et al. 2001), and environmental factors often override the importance of species diversity.

Both decomposition rate and fungal biomass increased significantly when more than 0.25 of a plug was used as inoculum (Figs. 1, 2; Table 1), but the three top levels (0.5 – 1.0) resulted in the same outcome when averaged over all levels of the other factors, indicating that a saturation point had been reached. However, interactions between nutrients and inoculum size and nutrients and inoculum identity were significant for both mass loss and ergosterol levels (Table 1), suggesting species-specific responses to combination of these factors. There is some indication that mass loss and ergosterol responded differently. For example, in *C. aquatica* and *T. setigerum* mass loss was greatest when a high nutrient level was combined with high inoculum (1.0 plug). On the other hand, ergosterol levels for these two species peaked at the highest nutrient level combined with an inoculum size of 0.25 (Figs. 1, 2). Possibly, greater levels of inoculum resulted in self-inhibition of fungal growth, while the release and activities of degradative enzymes were not affected.

Treton *et al.* (2004) also demonstrated a pronounced effect of inoculation size on subsequent reproductive investment in two aquatic hyphomycete species. They used conidia as source, and varied inoculum level by two orders of magnitude. In their study, final output depended on the amount of inoculum, though it is unclear if the system had reached an equilibrium at the end of their experiment.

It is difficult to extrapolate these findings to the field, where colonization is dominated by conidia, which are continuously being replaced by the flowing water. Conidial concentrations range from undetectable to 30 000 L^{-1} of stream water (Bärlocher 1992), while Treton *et al.* (2004) used one-time inocula of between 2 500 and 250 000 L^{-1} in their microcosms. Nutrients affect spore production from leaves or mycelia, as well as germination of released spores (Sridhar & Bärlocher 2000, Kempt *et al.* 2002). Nevertheless, these

results strongly suggest that environmental conditions such as nutrient supply and type and quantity of inocula are crucial during the early stages of colonization and influence the subsequent course of decomposition. The importance of these early events is supported by data from Nikolcheva *et al.* (2005), who showed that the arrival and attachment of conidia on leaves slows down dramatically within a few days of immersion in a stream. Similarly, fungi established on leaves are remarkably persistent even when leaves are subsequently removed from their original location and exposed in streams with different chemistries and fungal communities (Suberkropp 1984, Rosset & Bärlocher 1985, Sridhar *et al.* 2005).

In the current study, both diversity and species identity significantly affected leaf mass loss and fungal production at the highest level of inoculum (Fig. 3, Table 1), but their effects were small compared to the impact of the three nutrient levels. In contrast to an earlier study (Bärlocher & Corkum 2003), there were no monotonous increases in function with species diversity. Both ergosterol levels and leaf mass loss with four species were not significantly greater than corresponding values with two species. In fact, the greatest mass loss was found in a monoculture of *Tetracladium setigerum*. When it was combined with other species, mass loss declined.

Mass loss and ergosterol levels of multicultures rarely exceeded the maximum achieved by any of the constituent species in pure culture (Fig. 3). As a result, we cannot exclude the possibility that sampling effects were responsible for the observed functional increases at higher diversity. Sampling effects occur when species that are most active in monoculture dominate and displace less active species in mixed cultures (Aarsen 1997, Huston 1997, Tilman *et al.* 1997). True complementarity, such as facilitation or niche complementarity, should result in greater overall efficiency in resource use and raise the performance of mixed cultures above the level expected from the weighted sum of performances by the constituent species as monocultures (Tilman & Lehman 2001). Unfortunately, there is no simple and accurate method of tracking the biomasses of individual speices through the experiment, which would allow such estimates. Some progress has been made by combining ELISA with monoclonal antibodies against selected fungal species (Bermingham *et al.* 1997). Dang *et al.* (2005) assessed community composition in multicultures by measuring total spore production by the various species, on the assumption that spore output of any species is proportional to leaf mass loss caused by this species. Strong correlations between exponential decay rates and maximum fungal biomasses or sporulation rates have indeed been reported (e.g., Gessner & Chauvet 1994), however, in pure culture experiments, some species may fail to produce spores (Maharning & Bärlocher 1996). In joint cultures of

Flagellospora curvula and *Tetrachaetum elegans,* the spore output of both species dropped (more so in the case of *F. curvula*) while overall mass loss of leaves increased. A third approach is based on quantitative extraction and amplification of specific gene sequences. Some success has been reported in estimating relative biomassses of broad fungal groups (Nikolcheva & Bärlocher 2004), or, of certain species of aquatic hyphomycetes (Nikolcheva *et al.* 2005) by a combination of PCR with specific primers, followed by DGGE or T-RFLP. We attempted this approach in the current study. Preliminary results showed that in mixed cultures, biomass was typically dominated by one species (≥ 80 %, based on relative band intensity on DGGE). However, the age and source of inoculum appeared to have a profound effect on which species dominated the final community of multicultures (unpubl. obs.).

To summarize our knowledge of diversity effects in aquatic hyphomycetes, field studies have not provided convincing evidence that it has a dominant impact on relatively crude measures of leaf decomposition (Chauvet *et al.* 1997, Raviraja *et al.* 1998, Bärlocher & Graça 2002). Studies with pure cultures have shown a positive (Bärlocher & Corkum 2003, Treton *et al.* 2004; this study) or neutral (Duarte *et al.* 2006, Dang *et al.* 2005) effect on leaf mass loss; a positive (Duarte *et al.* 2006), neutral (Dang *et al.* 2005) or negative (Treton *et al.* 2004) effect on spore production; and a positive (Duarte et al. 2006, this study) effect on fungal biomass accrual. These discrepancies may be due to strong identity effects, i.e., the specific composition of communities may be more important than the number of different species they contain. This is revealed, for example, by the fact that in mixed cultures, observed mass loss in different species combinations was shown to be at, above, or below values expected from weighted additions of pure culture values, indicating, that complementary, antagonistic and neutral interactions can occur (Bärlocher & Corkum 2003). As pointed out in Duarte *et al.* (2006), leaves in a stream are typically colonized by between 10 – 20 species. Even ten species allow for 45 distinct species pairs and 120 species triplets. The crucial questions are whether certain combinations are more common than others, and whether in these combinations, complementary or inhibitory effects predominate. Finally, fungal impact extends beyond mass loss. Leaf-eating invertebrates differentiate among leaves (Bärlocher & Kendrick 1973a) and even leaf patches (Arsuffi & Suberkropp 1985) colonized by different fungal species, and their growth and survival vary with the identity of fungi in their food (Bärlocher & Kendrick 1973b, Arsuffi & Suberkropp 1986). It is possible (though as yet untested) that fungal diversity loss in streams may affect higher trophic levels.

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Tab. 1. – Summary of statistical analyses. Data from Figs. 1 – 2: factors were amount of inoculum (Inoculum), nutrient level (Nutrient), and inoculum identity (Identity). Data from Fig. 3: factors were nutrient level (Nutrient), species number (diversity) and species identity nested within diversity (identity{diversity}). Only significant effects are listed.

Dependent variable	Source	F	P
Fig. 1: Mass loss	Inoculum	112.7	0.000
	Nutrient	515.0	0.000
	Identity	15.7	0.000
	Inoculum*Nutrient	2.3	0.000
	Identity*Nutrient	37.4	0.000
Fig. 2: Ergosterol	Inoculum	5.7	0.001
	Nutrient	359.0	0.000
	Identity	34.0	0.000
	Inoculum*Nutrient	15.3	0.000
	Identity*Nutrient	15.8	0.000
Fig. 3: Mass loss	Nutrient	143.4	0.001
	Diversity	11.1	0.001
	Identity{Diversity}	3.6	0.002
	Nutrient*Diversity	5.8	0.000
Fig. 3: Ergosterol	Nutrient	43.0	0.000
	Diversity	6.8	0.000
	Identity{Diversity}	3.2	0.006