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Dissolved Nutrient Ratios and Concentrations Affect Fungal Reproduction and Community Structure Associated with Submerged Leaf Litter and Wood

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DISSOLVED NUTRIENT RATIOS AND CONCENTRATIONS AFFECT FUNGAL REPRODUCTION AND COMMUNITY STRUCTURE ASSOCIATED WITH SUBMERGED LEAF LITTER AND WOOD

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Abstract

Litter-associated fungi are important intermediaries in carbon and energy flow in streams. They can obtain nitrogen (N) and phosphorus (P) from both the substrate and the water column. I tested the effects of dissolved nutrients (dissolved inorganic nitrogen (DIN) 40-975 µg/L, soluble reactive phosphorus (SRP) 2-135 µg/L, N:P ratios 2:1, 16:1, 128:1) on fungi associated with leaf litter (maple and rhododendron) and wood in streamside channels at Coweeta Long Term Ecological Research site, North Carolina. This study addressed two main questions: (1) will fungal reproduction (sporulation rate of aquatic hyphomycetes) peak at a dissolved N:P ratio similar to nutrient stoichiometry of fungal biomass, and (2) what are the effects of dissolved nutrients on fungal community structure and sporulation associated with plant litter that differs in initial N and P content (*e.g.*, leaves *vs.* wood). The highest fungal sporulation rates were found at a dissolved N:P ratio of 16:1, which is comparable to the stoichiometry of fungal biomass (~10:1). Fungal sporulation rates and cumulative spore production per unit of plant litter by the end of the experiment generally showed statistically significant relationships with DIN rather than SRP. The greatest effects of dissolved nutrients were observed on wood compared to leaf litter. Very strong relationships between fungal production and sporulation rates and between cumulative spore production and plant litter decomposition rates were found for all substrates, suggesting that fungal activity is an important driver of plant litter decomposition. Finally, dissolved nutrients affected the relative abundances of dominant species in litter-associated fungal communities. Thus, dissolved inorganic nutrients affect reproductive output and community structure of aquatic fungi, which may have important consequences for plant litter decomposition and the flow of carbon, nutrients and energy in streams.

Introduction

Non-point sources of pollution such as atmospheric deposition, agricultural and urban activities are major contributors to nutrient enrichment in many freshwater ecosystems (Carpenter *et al.* 1998). These non-point sources are difficult to measure and as such complicated to regulate. Since 1972, with the introduction of the Clean Water Act there have been vast improvements in water quality throughout the United States and a decrease in pollution problems, including those from untreated water discharge. However, the lack of research and knowledge on the responses of aquatic heterotrophic organisms to nutrient loading has hindered the management of non-point source input. A meta-analysis of nutrient enrichment experiments (Elser *et al.* 2007) revealed nitrogen and phosphorus are often co-limiting to primary producers in both aquatic and terrestrial ecosystems. These results emphasize the importance of increased nutrient availability and its role in influencing biomass and productivity of autotrophs (Vitousek *et al.* 1997, Carpenter *et al.* 1998). While we have a good understanding of how nutrients affect ecosystems where the main energy source is primary productivity, we know very little about the responses of heterotrophic microorganisms and detrital food webs to dissolved inorganic nutrients such as nitrogen (N) and phosphorus (P) (Rosemond *et al.* 2002, Woodward *et al.* 2012).

Headwater forest streams receive a significant amount of allochthonous organic matter from the riparian zone in the form of leaf litter and woody debris, which is the primary source of carbon (C) and energy for stream communities (Wallace *et al.* 1997). The breakdown of this organic matter is a key process in the metabolism of these streams (Cummins 1988, Gessner and Chauvet 1994). Fungi, rather than bacteria, are the dominant microorganisms in terms of biomass and production that decompose the course particulate organic matter (CPOM), including leaves

and wood, and drive nutrient and energy transfer to higher trophic levels in streams (Gessner *et al.* 2007).

The most active fungal decomposers of plant litter in streams are aquatic hyphomycetes (Bärlocher 1992, Gessner *et al.* 2007). Aquatic hyphomycetes produce extracellular enzymes necessary to break down complex recalcitrant plant polymers. They are well adapted to the stream environment and form tetraradiate, sigmoid, or variously branched conidia, which are adaptations to water dispersal and also facilitate the attachment to substrate in flowing water (Dang *et al.* 2007, Gessner *et al.* 2007). Conidia then germinate quickly, the fungal hyphae extend inside the leaf matrix, and mycelial biomass accumulates. Closely following mycelial growth is the production of conidiophores which release more conidia into the water (Gessner *et al.* 2007). It has been demonstrated in both experimental and natural communities that sporulation rates often peak earlier than fungal biomass (Gulis and Suberkropp 2003, Gessner *et al.* 2007). Sporulation rates often rapidly increase to a maximum and then decline. The maximum rates of sporulation are controlled by leaf litter quality and stream water temperature, alkalinity, pH, and nutrient availability (Gessner *et al.* 2007).

Ecological stoichiometry deals with the relative balance of key elements in trophic interactions. Within this framework, elements are passed through trophic levels and may or may not be in balance with the consumer's elemental requirements (Cross *et al.* 2003). When leaf litter and woody debris enter a stream, they are colonized by microorganisms, primarily aquatic hyphomycetes, who are responsible for initiating the decomposition process (Gessner *et al.* 2007) and energy transfer to higher trophic levels (Bärlocher 1985). This accrual of microbial biomass increases N and P content and lowers C:N and C:P ratios of decomposing detritus by adding N and P to the initial N and P of the plant litter through microbial immobilization of

dissolved inorganic nutrients from the water column (Gessner *et al.* 2007). Thus, microbial colonization and nutrient immobilization brings detrital C:nutrient ratios closer to those values of consumer biomass, such as detritivorous aquatic invertebrates. Aquatic hyphomycetes also break down recalcitrant plant polysaccharides into more easily digestible subunits for invertebrate consumers (Bärlocher 1985). These changes improve the palatability and nutritional value of plant litter as a food source for invertebrate consumers. The process of changing leaf detritus into a suitable food source is commonly referred to as "conditioning" (Bärlocher 1985). Fungi account for 88 – 99.9% of the microbial biomass (fungi + bacteria) associated with plant detritus when it is fully conditioned (Gessner *et al.* 2007). The specific dynamics of conditioning vary depending on chemical and physical characteristics of plant material and environmental parameters of a particular location (Suberkropp 1992b). The energy flow and trophic dynamics of detrital-based food webs have been studied through research on fungi and invertebrate interactions and the role these interactions may play in shaping fungal and invertebrate communities (Kaushik and Hynes 1971, Bärlocher 1982a, Arsuffi and Suberkropp 1989).

Several studies have found that fungal activity (*e.g.*, biomass accrual and sporulation) associated with leaves can be affected by nutrient concentrations in the water (*e.g.*, Gulis and Suberkropp 2003, Ferreira *et al.* 2006, Suberkropp et al. 2010). These effects can then propagate to higher tropic levels (*i.e.*, to invertebrate detritivores). Robinson and Gessner (2000) found that higher-quality resources resulting from nutrient addition increased invertebrate shredder abundance and biomass on leaves in coarse-mesh litter bags. However, fungal biomass and sporulation, although stimulated, were not significantly different among treatments. The authors suggested that the increased feeding by shredders may remove some biomass accumulated due to nutrient stimulation of fungal production with no detectable effect on net biomass standing crop.

Chung and Suberkropp (2009) also found that nutrient enrichment accelerated leaf breakdown rates and increased macroinvertebrate biomass and abundance, but neither fungal biomass standing crop nor sporulation rate was affected by shredder feeding.

Whole-stream nutrient additions may also lead to changes in fungal community structure (*i.e.*, relative abundances of individual species) (Gulis and Suberkropp 2004). These nutrient-related changes in litter-associated fungal community structure have a potential to affect litter decomposition and feeding patterns of detritivorous invertebrates and, by extension, energy and nutrient transfer in stream food webs. This can be attributed to species-specific differences in enzymatic activities (Zemek *et al.* 1985) or elemental composition of fungal biomass (Danger and Chauvet 2013), which suggests that litter colonized by certain fungal species will have distinct nutritive quality and could be preferentially consumed by detritivores. Experiments with detritivorous invertebrates demonstrated that not only can they find and preferentially feed on patches of plant litter colonized by fungi they can also discriminate against or in favor of patches colonized by certain fungal species (Suberkropp *et al.* 1983, Arsuffi and Suberkropp 1985).

Nutrient enrichment experiments carried out in streams suggest that elevated inorganic nutrient availability can result in higher concentrations of aquatic hyphomycete conidia in transport, changes in fungal community structure, and higher species richness (Grattan and Suberkropp 2001, Gulis and Suberkropp 2003a,b, 2004). Gulis and Suberkropp (2004) found a shift in co-dominance pattern of aquatic hyphomycetes following whole-stream nutrient addition. Specifically, they reported an increase in relative abundance of conidia that belong to species producing relatively large conidia. *Alatospora acuminata* and *Articulospora tetracladia*, which produce small spores, were dominant in the reference stream and in the treatment stream before enrichment. Following nutrient addition, *Anguillospora filiformis, Tetrachaetum elegans*, and

Tricladium chaetocladium became co-dominant in the enriched stream (Gulis and Suberkropp 2004). This suggests species producing conidia with higher biovolume may require additional N and/or P supply. In addition, higher availability of dissolved nutrients increased conidia concentration in transport (*i.e.*, stream water) due to stimulation of fungal biomass accrual and sporulation rates (Gulis and Suberkropp 2003b, 2004). This stimulation of fungal activity and decomposition of plant litter together with increased concentrations of conidia in stream water may result in higher transport of fine particulate organic matter (FPOM) from and reduced standing stock of coarse particulate organic matter (CPOM) within the stream reach (Benstead *et al.*, 2009). Fine particulate organic matter and coarse particulate organic matter are critical resources for many aquatic invertebrates. Thus, changes in fungal activity and community structure due to altered nutrient availability may affect plant litter decomposition, palatability and standing stock, with the effects propagating to higher trophic levels in streams.

Allochthonous leaf litter and submerged wood in streams harbor diverse, yet distinct, communities of aquatic fungi (Gulis 2001, Shearer *et al.* 2007). Aquatic fungi show substrate preference and therefore leaves and wood harbor different communities. Since previous ecological studies have focused more on fungi associated with submerged leaves than wood, there are relatively few estimates of fungal activity (*e.g.*, production or sporulation) from woody substrates (Ferreira *et al.* 2006, Gulis *et al.* 2008). Differences in fungal community structure and activity between leaf litter and wood are driven by dissimilarity in their chemical composition (*i.e.*, higher lignin content of wood and its lower N and P content, or higher C:N and C:P ratios (Gulis *et al.* 2008). From a stoichiometric perspective, fungi that colonize wood are more nutrient limited than those that colonize leaves. Consequently, fungal communities associated with wood would be expected to rely on external nutrient supply to a greater extent and would

show a greater response to increases in dissolved inorganic nutrients. Stelzer *et al.* (2003) and Gulis *et al.* (2004) confirmed this experimentally finding that microbial response to nutrient enrichment depended upon the nutrient content of the detritus, with wood exhibiting a greater response to nutrient enrichment than leaves. These substrate specific preferences may affect whole-ecosystem functioning depending upon the relative availability of different types of detritus, due to variation in leaf and wood input and standing crop, and factors affecting microbial activity (Gulis *et al.* 2008).

Identification of many aquatic hyphomycetes is possible based on detached conidia owing to their characteristic shapes (Gulis *et al.* 2005, Gessner *et al.* 2007). Therefore, community structure and diversity of these fungi are often inferred from the relative abundances of conidia filtered from the stream water or from conidia suspensions obtained by inducing sporulation from field collected substrates in the lab (Gessner *et al.* 2003, Gulis *et al.* 2006). Such data are based on the assumption that sporulation rates correlate with the biomass of fungal species inside a leaf. Much of the current knowledge of aquatic hyphomycetes communities has been derived from this approach. However, it may not necessarily be a true representation of the community based on intramatrical mycelia (Gessner *et al.* 2007). Molecular approaches based on the analysis of fungal rDNA extracted from litter samples (e.g. Nikolcheva et al. 2003, Nikolcheva and Bärlocher 2005) offer some advantages, such as detection of non-sporulating species, but, for leaf-associated communities of aquatic hyphomycetes, they generally produce results comparable to those obtained from spore identification and counting.

The aim of this study was to determine the responses of fungal communities associated with decaying submerged plant litter to a range of dissolved inorganic nitrogen (N) and phosphorus (P) concentrations and ratios. The effects of nutrients and changes in fungal

community structure and reproductive success (sporulation) were assessed experimentally in streamside channels that simulated stream conditions. This study answers two main questions: (1) will fungal sporulation rate peak at a dissolved N:P ratio similar to nutrient stoichiometry of fungal biomass and (2) what are the effects of dissolved nutrients on fungal community structure and sporulation associated with plant litter that differs in initial N and P content (*e.g.,* leaves vs. wood).

My study is being conducted as part of a larger National Science Foundation funded project "Collaborative research: Defining ecosystem heterotrophic response to nutrient concentrations and ratios". This project aims to elucidate how microbial communities, detrital food-webs, and stream ecosystem carbon dynamics react to changes in dissolved inorganic nutrient (N and P) concentrations and ratios. Nitrogen and phosphorus are the major nutrients affecting surface and coastal waters in the U.S. (Carpenter *et al.* 1998, Howarth *et al.* 2000). This study will further our understanding of how aquatic systems are affected by nutrient enrichment through heterotrophic pathways, which may help us make more informed management decisions in relation to eutrophication.

Methods

All field work took place at the Coweeta Long Term Ecological Research (LTER) site in Macon County, North Carolina between March and August 2012. The Coweeta LTER is a 2,185 ha research site dominated by hardwoods where *Rhododendron maximum* forms a dense understory, which results in shading of streams throughout the year. Relatively resistant crystalline bedrock in the area makes nutrient concentrations in streams very low: NO_3-N<0.04 mg L^{-1} and PO_4-P< 0.002 mg L^{-1} (Swank and Crossley 1988). Stream food-webs in this area are

dependent on allochthonous energy sources (Wallace *et al.* 1997; Webster *et al.* 1997) and terrestrial detritus provides over 90% of the organic matter available for secondary production (Cross *et al.* 2007).

To assess the responses of litter-associated fungal communities in forest headwater streams to dissolved inorganic nutrient concentrations and ratios, 30 streamside channels simulating stream conditions (Figure 1) were set up below the main weir on Shope Fork. Each channel (made of rain gutters) was 4 m long × 0.15 m wide while the depth was maintained at ~ 0.1 m with a flow rate set to 0.1 L/s to imitate headwater streams at Coweeta. Channels were fed with stream water that was pumped to a primary holding tank, then distributed to three secondary tanks, and finally into the individual channels. Three dissolved nutrient ratio treatments (N:P of 2:1, 16:1, and 128:1) each with two levels of nutrient concentrations (low and high) and an unenriched control (stream water) were replicated three times (Table 1) and used in this study (remaining channels were used for other concurrent experiments within a larger project). Elevated experimental nutrient concentrations were maintained by dosing concentrated nutrient solutions individually into each of 18 channels with peristaltic pumps. Ammonium nitrate and phosphoric acid were used for enrichments. Each channel received five litter bags of each litter type containing 3.0 ± 0.25g of material in the form of rhododendron (*Rhododendron maximum*) leaf litter, maple (*Acer rubrum*) leaf litter, or oak (*Quercus alba)* wood veneers. Both rhododendron and maple plant species are commonly found at the Coweta LTER. Litter bags were 20 cm × 14 cm and constructed with 1mm mesh window screening. Maple and rhododendron leaf litter were chosen for the experiment due to differences in their initial C:N and C:P ratios and lignin content along with a standardized substrate (*i.e.,* wood veneers) that has

exceptionally low N and P content and carbon quality (Table 2, data from our litter samples, provided by Analytical Chemistry Lab, University of Georgia).

Decomposing plant material was collected on five sampling dates, each about a month apart. Collection of maple litter bags began two weeks earlier than for other substrates because maple is a more labile material and has a faster decomposition rate than rhododendron and wood. After the second collection of maple, which occurred on the same date as the first collection of rhododendron and veneer litter bags, all substrate types were sampled on the same dates until day 111, which was the last day of maple collection, while rhododendron and veneer samples were also collected on day 144. On each sampling date, litter bags were retrieved, brought to the laboratory on ice, and subsampled for microbial analyses. All microbial analyses were performed on sets of 5 or 10 leaf disks (12 mm diameter), or 3 to 6 wood veneer rectangles (1 × 2 cm), to standardize per unit of plant ash free dry mass (AFDM). Ash free dry mass was determined by weighing, ashing (500 °C for 4 h), and reweighing corresponding sets of litter samples.

The rates of fungal spore production and fungal community structure were estimated following induction of sporulation from plant litter in the laboratory. Ten leaf disks or six wood rectangles were placed in sterile plastic containers with 25 mL of water from the corresponding nutrient treatment and incubated for 24 hours at stream temperature (recorded at the time of collection) on an orbital shaker (125 rpm) in the environmental chamber. This procedure simulated stream conditions and induced spore production by fungal mycelia associated with plant litter. Spore suspensions were preserved with formalin (2% final concentration) and stored at room temperature until analyses. 100 µL of Triton X-100 solution was added to each sample and stirred to achieve uniform distribution of conidia. Five to 20 mL aliquots were filtered through a 25 mm diameter, 8 µm pore size membrane filter (type SCWP, Millipore). Conidia on

filters were then stained (0.05% trypan blue in 85% lactic acid) and mounted on microscope slides. Using a compound microscope at 10 – 40x magnification, conidia of aquatic hyphomycetes were counted and identified (modified from Gulis and Suberkropp 2006). At least seven microscopic fields and at least 200 conidia were counted per sample. The number of conidia counted, number of fields scanned, total volume of conidia suspension and the aliquot filtered, time of incubation and AFDM of sample were used to calculate the sporulation rate following the methods in Gulis and Suberkropp (2006).

Since levels of nutrient concentrations (low and high) could not be the same among nutrient ratio treatments (2:1, 16:1 and 128:1) in our experimental design (Figure 2, Table 1), linear regression was used for statistical analyses in most cases instead of ANOVA. Cumulative spore production per unit of initial plant litter by the end of experiment was calculated by averaging sporulation rates between sampling dates and then summing up daily spore production values. One-way ANOVA (seven nutrient treatments) was run on $\log_{10}$-transformed cumulative spore production by the end of experiment for each substrate followed by Tukey's test for pairwise comparisons. The effects of dissolved nutrient ratios or concentrations on fungal sporulation rates and cumulative conidia production by the end of experiment was assessed by linear regression.

Differences in fungal community structure among nutrient treatments were assessed by calculating diversity and evenness indices as well as using Multidimensional Scaling (nMDS) ordination. Evenness of conidia distribution among taxa and Shannon-Wiener diversity index were calculated as:

$$E = \frac{H}{H_{\max}} = \frac{-\sum_{i=1}^{S} p_i \ln p_i}{\ln S}$$

where E is evenness, H is Shannon-Wiener index, S is the number of species, p_i is the relative abundance of species i in the community (Magurran 1988). Non-metric multidimensional scaling (nMDS) was run on dissimilarity matrix (1-Sorensen similarity) calculated based on the relative abundances of fungal taxa (21 samples, seven treatments × three substrates) using Primer 5.2.6 software. ANOSIM was used to assess differences in aquatic hyphomycetes communities among treatments and substrates.

Results

Fungal sporulation rates tended to peak 1 – 2 months after litter submergence on all three substrates with an earlier peak on higher quality, faster decomposing maple leaf litter (Figure 3). Higher quality leaf litter (maple and rhododendron) supported sporulation rates more than an order of magnitude higher than that of wood veneers, suggesting that fungal sporulation on wood was limited by N and P availability from the substrate itself (Table 2) and also by lower C quality (*i.e.*, higher lignin content) of wood compared to that of leaves. On most sampling dates, the highest sporulation rates occurred in streamside channels that received nutrients at a 16:1 ratio. Sporulation rates showed a significant positive relationship with dissolved inorganic nitrogen (DIN) for maple (on day 28) and rhododendron (on day 77) leaf litter (Figure 4, linear regression, $R^2 = 0.638$ and 0.575, p=0.031 and 0.048, respectively). While sporulation from wood veneers did not show a significant relationship with DIN the positive trend also existed ($R^2 = 0.491$, p = 0.079). Sporulation rates on all litter types did not show a significant correlation with soluble reactive phosphorus (SRP). However, 2:1 L treatment (severe N limitation of fungal activity) acted as an outlier and when it was removed from the analysis, significant positive

relationships of fungal sporulation and SRP was evident for rhododendron leaf litter and wood veneers (R^2 = 0.760 and 0.663, p = 0.024 and 0.056, respectively).

The cumulative spore production per unit of initial plant litter increased sharply at early stages of decomposition and tended to level off at later stages of decomposition (Figures 5 – 7) as sporulation rates declined (Figure 3). The effect of nutrient availability on cumulative spore production by the end of the experiment (day 111 for maple leaf litter and day 144 for rhododendron leaf litter and wood veneers) was statistically significant (one-way ANOVA, $F_{6,14}$ = 29.4, p<0.0001 for maple leaf litter; $F_{6,14}$ = 55.0, p<0.0001 for rhododendron leaf litter; and $F_{6,14}$=112.0, p<0.0001 for wood veneers). Tukey's tests showed that cumulative spore production was generally higher in treatments with a dissolved N:P ratio of 16:1 and treatments with higher nutrient concentrations within the same nutrient ratio (Figure 5 – 7). However, cumulative spore production from maple leaf litter by the end of the experiment was highest in treatment 2:1 H (Figure 5). This can be explained by the high initial N content of maple litter (or low C:N ratio, Table 2), suggesting that fungi could have obtained sufficient N from this substrate while relying on high levels of dissolved inorganic P supplied in this treatment. This observation was further supported when we ran linear regression analyses to show the relationships between cumulative spore production and SRP or DIN (Figure 8). We found a significant positive relationship between cumulative spore production by the end of experiment and SRP for maple (R^2 = 0.641, p = 0.031) but not other substrates. However, both rhododendron leaf litter and wood veneers showed a significant stimulation of cumulative spore production by DIN (R^2 = 0.588 and 0.577, p = 0.044 and 0.047, respectively). This suggests that fungal reproduction on maple leaf litter is more limited by P while fungal sporulation on rhododendron leaf litter and wood veneers are limited by N or co-limited by N and P since some stimulation of reproduction by SRP, although

not significant (R^2 = 0.544 and 0.548, p = 0.058 and 0.057 for rhododendron leaf litter and wood veneers, respectively), was also observed.

A very strong positive relationship between fungal mycelial production and sporulation rate of aquatic hyphomycetes at early stages of substrate colonization was found for all types of plant litter (Figure 9, linear regression, R^2 = 0.952, 0.816, and 0.871, p = 0.0001, 0.005 and 0.007 for maple and rhododendron leaf litter and wood veneers, respectively). Also, a significant relationship between cumulative fungal spore production by the end of the experiment and litter decomposition rate was found for all litter types (Figure 10, linear regression, R^2 = 0.677, 0.837, and 0.908, p = 0.023, 0.004, and 0.0009 for maple and rhododendron leaf litter and wood veneers, respectively), suggesting that fungal activity controls plant litter decomposition.

At least 18 taxa of aquatic hyphomycetes produced spores on plant litter in the streamside channels during the study (Tables 2 – 4). We did not find apparent differences in total species richness among treatments. Despite the fact that we observed changes in dominant species and relative contributions of individual fungal species to the total conidia pool (see below), species richness, diversity, and evenness of conidia distribution among taxa were similar between the control and treatments for maple and rhododendron leaf litter (see Tables 2 and 3). However, on wood veneers, aquatic hyphomycete diversity and evenness in low nutrient treatments (unamended control, 2:1 L and 128:1 L) were about 2 – 3 fold higher than those in treatments with higher dissolved nutrient availability (Table 4). These changes can be attributed to stimulation of *Tricladium chaetocladium* sporulation by nutrients in 2:1 H, 16:1 L, 16:1 H, and 128:1 H treatments, while relative abundances of other species (especially *Casaresia sphagnorum*, which was co-dominant in low-nutrient treatments) declined (Table 4).

Six species of aquatic hyphomycetes dominated the conidia pool (mean percent contribution of at least 5% on at least one litter type). The relative abundances of the dominant species at early and late stages of plant litter decomposition are illustrated in Figure 11, while relative abundances at intermediate sampling dates are given in Appendices I – III. Overall, there were differences in fungal communities driven by litter type, stage of decomposition and nutrient treatment. Leaf litter and wood supported different microbial communities. *Anguillospora filiformis* was one of the most important species on leaves, but not on wood, whereas *C. sphagnorum* dominated fungal communities in some treatments on wood, while it was a rare species on leaves. Successional patterns in fungal communities were also obvious. *Anguillospora filiformis* was largely replaced by *T. chaetocladium* on maple leaf litter at late stages of decomposition, while *Dimorphospora foliiciola*, an early successional species, was replaced by *C. sphagnorum* on wood. Some species were sensitive to nitrogen availability in water and changed their sporulation rates and, consequently, relative abundances in the conidia pool. The most drastic example is the co-dominance of *D. follicola* or *C. sphagnorum* in treatments with low nutrient availability (unamended control, 2:1 L and 128:1 L) at early and late stages of wood decomposition, respectively, whereas these species were almost completely replaced by *T. chaetocladium* in high nutrient treatments, so that its spores accounted for 59 – 98% of the total conidia pool (Figure 11).

Multidimensional scaling ordination of samples based on relative abundances of fungal species separated them by both substrate type and nutrient availability (Figure 12, 1000 iterations, 2D configuration, stress 0.08). In line with trends described above, leaf litter samples were grouped together and were quite distant from wood veneer samples. nMDS ordination also demonstrated that, for all substrates, samples from different nutrient treatments were generally

arranged across Dimension 1 of the ordination from right to left from low nutrient treatments (*i.e.,* control, 2:1 L, 128:1 L) to higher nutrient treatments. Also, for maple and rhododendron leaf litter, samples from the same nutrient treatment (*e.g.*, M-16:1 H and R-16:1 H, Figure 12) tended to be grouped together due to similar fungal assemblages. ANOSIM analysis demonstrated that not only substrate preferences but also dissolved nutrient availability affected fungal community structure of decaying plant litter (substrate type, $R = 0.762$ and $p = 0.001$, nutrient treatment, $R = 0.792$ and $p = 0.001$).

Discussion

The results of this experiment generally supported our hypothesis that the sporulation rates of aquatic hyphomycetes peak in nutrient treatments that have a dissolved inorganic nutrient ratio (N:P) closest to the stoichiometric ratio of fungal biomass. Indeed, sporulation rates of aquatic hyphomycetes were the highest in 16:1 dissolved N:P treatments for all three substrate types, especially during the peak of sporulation (day 28 for maple leaf litter and day 77 for other substrates) (Figure 3). This is further confirmed by the highest cumulative spore production by the end experiment in 16:1 dissolved N:P ratio treatments for rhododendron leaf litter and wood (Figure 4). This ratio is the closest to stoichiometric ratio of fungal biomass estimated to range from 11:1 to 16:1 (Grimmett *et al.* 2013) in laboratory experiments on rich nutrient medium (*i.e*., no nutrient limitation). In experiments that grew fungi at different levels of external N and P availability (either N or P limitation), fungal biomass nutrient stoichiometry varied more broadly, from 5:1 to 55:1 (Danger and Chauvet 2013) or 3:1 to 27:1 N:P ratio (Gulis *et al.*, unpublished) suggesting that fungi are not homeostatic with respect to their nutrient content. However, the average fungal biomass N:P ratio was estimated at approximately 10:1 by Gulis *et al.*

(unpublished), which is comparable to the dissolved N:P ratio (16:1) that resulted in the highest fungal reproductive output in our experiments. Interestingly, cumulative spore production by the end of the experiment from maple leaf litter in the 2:1 H treatment was higher than that of 16:1 H treatment. This can be explained by high N content of maple leaf litter compared to other substrates (Table 2) and, consequently, the ability of fungi to satisfy their N requirements from the organic substrate, rather than the water column in the 2:1 H (*i.e.*, low inorganic dissolved N) treatment.

After leaf litter is colonized by aquatic hyphomycetes, sporulation rates often rapidly increase to a maximum and then decline (Gessner *et al.* 2007). We observed a similar pattern for fast decomposing maple leaves. However, fungal reproductive output was delayed for rhododendron leaf litter and wood veneers. This could be explained by lower N and P content (or higher C:N and C:P ratios, Table 2) and also lower C quality (*e.g.*, higher lignin content) (Gessner and Chauvet 1994, Gessner *et al*. 2007) of these recalcitrant litter types that may negatively affect fungal activity and ultimately litter decomposition. The extent to which cumulative conidia output was stimulated by dissolved inorganic nutrients was considerably higher for wood than leaf litter types. This agrees with previous reports (Gulis and Suberkropp 2004, Ferreira *et al.* 2006) that stronger stimulation of fungal sporulation by nutrient enrichment occurs on lower quality substrates, such as wood, compared to leaf litter. Overall, our study showed fungal sporulation rates from maple and rhododendron leaves were comparable to those reported by Ferreira *et al.* (2006) for alder and oak leaves. Sporulation rates for oak wood veneers were considerably lower than those from leaf litter in our experiment and also lower than reported by Ferreira *et al.* (2006) for balsa veneers. This pattern can be explained by low N and P content and high lignin content of wood (Table 2, Stelzer *et al.* 2003, Gulis *et al.* 2004)

compared to leaves, which results in fungal nutrient and carbon limitation on wood veneers. In addition, veneers have lower surface area-to-volume ratio than leaves, which impedes fungal colonization and access to substrate nutrients and carbon.

The results of this experiment are comparable with other microbial data gathered during the streamside channel nutrient addition project at Coweeta LTER. Burns *et al*. (unpublished) found that fungal biomass and production associated with maple and rhododendron leaves were stimulated by DIN, while no apparent relationship between SRP and fungal parameters has been recorded. We found similar trend for the effect of DIN, but generally not SRP, on fungal reproductive output (Figures 4 and 8). Recent studies that observed non-homeostasis of microbes with increasing SRP (Fanin *et al.* 2013, Gulis *et al.* unpublished) due to, presumably, P storage may explain why SRP generally did not stimulate fungal sporulation rates in our experiments. However, cumulative spore production from maple leaf litter was significantly affected by SRP while those from rhododendron leaf litter and wood veneers were significantly related to DIN availability. This is not surprising because maple leaves have higher N content than rhododendron leaves or wood, and, therefore, fungi on maple leaf litter can rely on N from the substrate while immobilizing P from the water column. This agrees with results of Ferreira *et al.* (2006) who found that decomposition of balsa wood veneers (low quality and low nutrient substrate) was most N limited while decomposition of alder leaf litter (high N substrate) was least limited by dissolved inorganic N.

A strong significant positive correlation was found between fungal mycelial production, determined based on rates of ^{14}C-acetate incorporation into ergosterol (Burns *et al*, unpublished), and sporulation rate of aquatic hyphomycetes determined from the same samples for all litter types (Figure 9). This relationship suggests that mycelial biomass production and reproductive

output in aquatic hyphomycetes at early stages of litter colonization (when fungal activity is the highest) are tightly coupled. This is the first observation of this kind and is interesting since it refutes the idea that vegetative growth and reproduction are often decoupled. We also found significant relationships between cumulative spore production and decomposition rates for all substrates (Figure 10), which suggests that fungal activity is the major driver in decomposition of plant litter in streams. Earlier studies reported that fungi can directly assimilate up to 35% – 48% of initial litter carbon as plant litter decomposes (Gulis and Suberkropp 2003a, Gulis *et al*. 2006), invest up to 81% of production into spores translating into 12% of litter mass loss (Suberkropp *et al*. 1991), or channel up to 7 % of initial plant litter carbon into their spores (Gulis and Suberkropp 2003a, Ferreira *et al.* 2006).

The fungal assemblages colonizing all three substrates in different nutrient treatments had similar species richness, but generally differed in the relative abundances of dominant species. We found that *A. filiformis* and *T. chaetocladium* increased its spore output in treatments with higher nutrient availability. It is not clear why elevated nutrient concentrations affected the relative abundance of dominant species, but we speculate that production of conidia with higher biovolume requires additional N and P which they can obtain from the water column. Gulis and Suberkropp (2004) reported similar findings for *A. filiformis, T. chaetocladium,* and *Tetrachaetum elegans,* which had significantly higher abundance of conidia in stream water after nutrient addition. However, in a laboratory experiment, Sridhar and Bärlocher (2000) did not find drastic shifts in the structure of fungal assemblages in response to nutrient addition. Further research on the nutrient requirements of individual species would be required to better understand the effects of dissolved nutrients on fungal community structure.

The fungal assemblages colonizing wood veneers were different from those colonizing maple and rhododendron leaves, which led to clear separation in nMDS analysis (Figure 8). Differences between fungal communities that colonize leaves versus wood have been previously reported by Gulis (2001) and Ferreira *et al.* (2006) suggesting the existence of certain substrate preferences among species of aquatic hyphomycetes. However, Nikolcheva and Bärlocher (2005) did not find fungal communities to be strongly influenced by substrate type using both traditional and molecular techniques. The nMDS ordination of our samples was also influenced by nutrient availability, with lower nutrient treatments (including unamended control) corresponding to higher Dimension 1 values and vice versa (Figure 12). This suggests that community structure of aquatic hyphomycetes is controlled by both the substrate type and dissolved nutrient availability. Changes in fungal community structure may affect higher trophic levels and nutrient transfer due to differences in elemental stoichiometry among fungal species and feeding preferences of invertebrate consumers (Suberkropp *et al.* 1983, Arsuffi and Suberkropp 1985, Danger and Chauvet 2013).

In conclusion, the findings showed that sporulation rates of aquatic hyphomycetes peak in nutrient treatments that have a dissolved inorganic nutrient ratio (N:P of ~16:1) closest to the stoichiometric ratio of fungal biomass. In addition, we found that dissolved nutrients, especially DIN, stimulate fungal sporulation rates and cumulative output of spores as litter decomposition progresses. Nutrients had stronger effect on fungal sporulation associated with lower quality plant litter (*i.e.,* wood). Finally, dissolved nutrients affected the relative abundances of dominant species in litter-associated fungal communities. Thus, dissolved inorganic nutrients affect reproductive output and community structure of aquatic fungi, which may have important consequences for plant litter decomposition and the flow of carbon, nutrients and energy in

streams. These findings contribute to our understanding of the possible consequences of excessive nutrient loading or anthropogenic eutrophication in stream ecosystems.

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Table 1. Target and actual concentrations and ratios of dissolved inorganic nitrogen and soluble reactive phosphorus in experimental streamside channels. Actual concentrations and ratios are means ± standard deviation (SD), for the whole experimental period (n=7).

Target N:P ratio (molar)	**Concen-tration level**	**Target DIN ($\mu g\ L^{-1}$)**	**Target SRP ($\mu g\ L^{-1}$)**	**Actual DIN ($\mu g\ L^{-1}$)**	**Actual SRP ($\mu g\ L^{-1}$)**	**Actual N:P ratio (molar)**
Control	-	-	-	48.5 ± 54.3	4.4 ± 3.8	24.3 ± 25.3
2:1	L	40.6	45.0	44.2 ± 38.1	46.3 ± 13.5	2.1 ± 2.7
2:1	H	121.9	135.0	115.0 ± 29.8	94.0 ± 59.3	2.7 ± 1.0
16:1	L	325.2	45.0	331.3 ± 122.1	51.5 ± 17.7	14.2 ± 5.7
16:1	H	975.5	135.0	824.2 ± 211.2	86.2 ± 32.9	21.2 ± 7.5
128:1	L	325.2	5.6	338.0 ± 105.7	11.7 ± 11.1	64.0 ± 34.3
128:1	H	975.5	16.9	878.4 ± 227.9	17.7 ± 6.5	110.1 ± 64.9

Table 2. Initial detrital C:N, C:P and N:P ratios (molar) for all substrate types (mean ± standard error (SE)).

	C:N ratio	**C:P ratio**	**N:P ratio**
Maple leaves	123.4 ± 4.6	6456 ± 482	52.3 ± 3.9
Rhododendron leaves	162.2 ± 6.3	7240 ± 297	44.6 ± 3.3
Wood veneers	528.2 ± 7.3	184376 ± 9487	349.0 ± 19.5

Table 3. Mean relative abundances (%) of aquatic hyphomycetes associated with maple leaf litter in different dissolved nutrient treatments throughout the experiment.

Species	Treatment						
	C	2:1 L	2:1 H	16:1 L	16:1 H	128:1 L	128:1 H
Anguillospora filiformis	35.5	23.9	28.1	24.2	20.0	42.4	29.0
Articulospora tetracladia	4.4	3.2	5.7	7.4	4.8	2.7	3.4
Casaresia sphagnorum	11.4	4.5	0.4	0.5	0.4	0.8	0.2
Clavariopsis aquatica		0.1		0.2	0.1	0.2	0.2
Clavatospora longibrachiata		0.1	0.1	0.2	0.6		0.1
Culicidospora aquatica	1.3	3.2	1.6	1.2	0.5	0.8	1.7
Dimorphospora foliicola	3.0	5.1	15.3	10.7	18.4	8.0	14.1
Heliscella stellata					0.5		0.2
Heliscus lugdunensis	2.5	2.6	0.9	1.3	1.6	0.9	0.5
Lunulospora curvula	11.8	3.7	2.6	3.2	5.2	6.7	2.3
Monotosporellamicro aquatic				0.1	0.2	0.3	0.2
Mycofalcella calcarata							
Tetrachaetum elegans	1.6	11.4	11.9	12.2	12.9	5.6	9.8
Tricladium chaetocladium	27.9	38.6	32.0	37.0	32.4	30.3	36.2
Triscelophoruskonajensis	0.2	3.0	0.5	1.0	0.5	0.7	0.5
small sigmoid (<60 μm)	0.3	0.5	0.9	0.8	1.7	0.6	1.7
inter sigmoid (60-120 μm)	0.1	0.3		0.1			
large sigmoid (>120 μm)		<0.1					
Total	**100**	**100**	**100**	**100**	**100**	**100**	**100**
Total no. of species	12	12	11	13	14	12	14
Evenness (E)	0.693	0.680	0.656	0.637	0.685	0.600	0.621
Diversity (H)	1.721	1.841	1.730	1.765	1.854	1.585	1.680

Table 4. Mean relative abundances (%) of aquatic hyphomycetes associated with rhododendron leaf litter in different dissolved nutrient treatments throughout the experiment.

Species	Treatment						
	C	2:1 L	2:1 H	16:1 L	16:1 H	128:1 L	128:1 H
Anguillospora filiformis	44.6	25.8	30.0	28.9	28.7	45.9	35.0
Articulospora tetracladia	0.8	0.6	1.6	1.2	0.8	1.6	1.6
Casaresia sphagnorum	17.6	14.7	0.2	0.4	0.2	2.4	1.2
Clavariopsis aquatica				0.1		0.1	0.1
Clavatospora longibrachiata			3.2		1.1	0.9	
Culicidospora aquatica	3.4	13.2	12.9	9.0	18.7	3.9	3.7
Dimorphospora foliicola	11.8	0.9	3.1	4.3	2.1	3.6	2.1
Heliscella stellata			2.6		1.2		2.2
Heliscus lugdunensis	1.1	8.6	1.3	1.1	0.5	8.6	1.4
Lunulospora curvula			0.6		0.1	0.5	
Monotosporellamicro aquatic							
Mycofalcella calcarata							
Tetrachaetum elegans	0.5						
Tricladium chaetocladium							0.1
Triscelophorus konajensis							
small sigmoid (<60 μm)	4.1	3.8				0.4	
inter sigmoid (60-120 μm)	10.9	23.2				29.4	
large sigmoid (>120 μm)	1.1						
Total	**100**	**100**	**100**	**100**	**100**	**100**	**100**
Total no. of species	10	8	9	7	9	11	9
Evenness (E)	0.675	0.776	0.663	0.663	0.600	0.574	0.558
Diversity (H)	1.731	1.990	1.750	1.646	1.585	1.554	1.472

Table 5. Mean relative abundances (%) of aquatic hyphomycetes associated with wood veneers in different dissolved nutrient treatments throughout the experiment.

Species	Treatment						
	C	2:1 L	2:1 H	16:1 L	16:1 H	128:1 L	128:1 H
Anguillospora filiformis	7.1	2.7	1.0	0.8	0.1	6.3	1.5
Articulospora tetracladia	0.6	0.8	0.5	0.3	0.1	0.6	0.8
Casaresia sphagnorum	36.1	33.0	1.7	1.1	0.3	19.2	1.7
Clavariopsis aquatica		0.9	3.8	5.2	2.9	0.4	3.5
Clavatospora longibrachiata							
Culicidospora aquatica	2.7		0.2		1.5		
Dimorphospora foliicola	14.6	12.4	2.9	2.2	1.2	10.7	0.5
Heliscella stellata							
Heliscus lugdunensis		8.5	0.2	0.3	0.2		0.2
Lunulospora curvula	0.8	0.5	0.1			0.8	
Monotosporellamicro aquatic							
Mycofalcella calcarata							
Tetrachaetum elegans	5.6	1.3	4.9	1.4	0.3	1.2	1.0
Tricladium chaetocladium	25.5	35.8	83.2	83.4	89.6	51.0	90.4
Triscelophorus konajensis	5.7	2.6	0.3	0.4	0.4	1.9	0.2
small sigmoid (<60 μm)	0.6	1.1	0.1	0.1		3.5	
inter sigmoid (60-120 μm)		0.2				2.2	
large sigmoid (>120 μm)							
Total	**100**	**100**	**100**	**100**	**100**	**100**	**100**
Total no. of species	10	12	12	10	10	11	9
Evenness (E)	0.725	0.636	0.294	0.282	0.198	0.599	0.210
Diversity (H)	1.739	1.631	0.777	0.744	0.522	1.582	0.484

Figure 1. Thirty streamside channels fed with water pumped from the Shope Fork at Coweeta Long Term Ecological Research site in North Carolina used for decomposition experiments under varying dissolved nutrient concentrations.

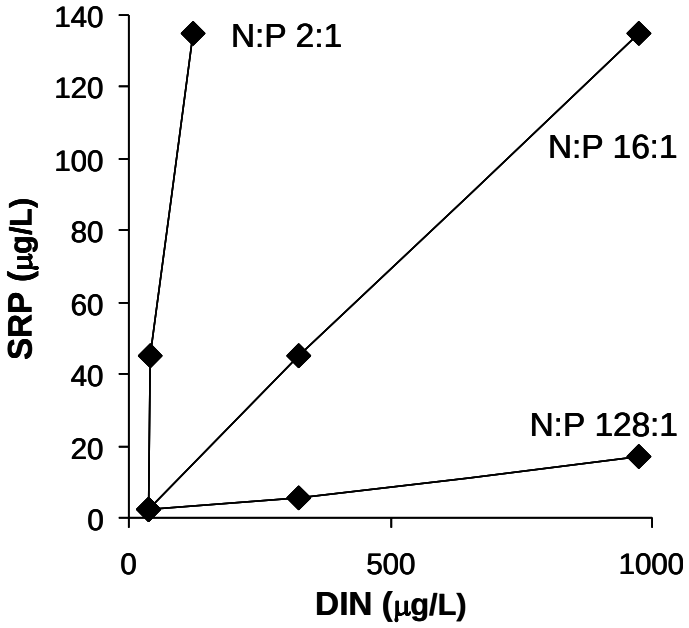


Figure 2. Target dissolved inorganic nitrogen (DIN) and soluble reactive phosphorus (SRP) concentrations and ratios used in experimental streamside channels. Diamonds represent experimental treatments differing in concentrations and ratios. See Table 1 for details.

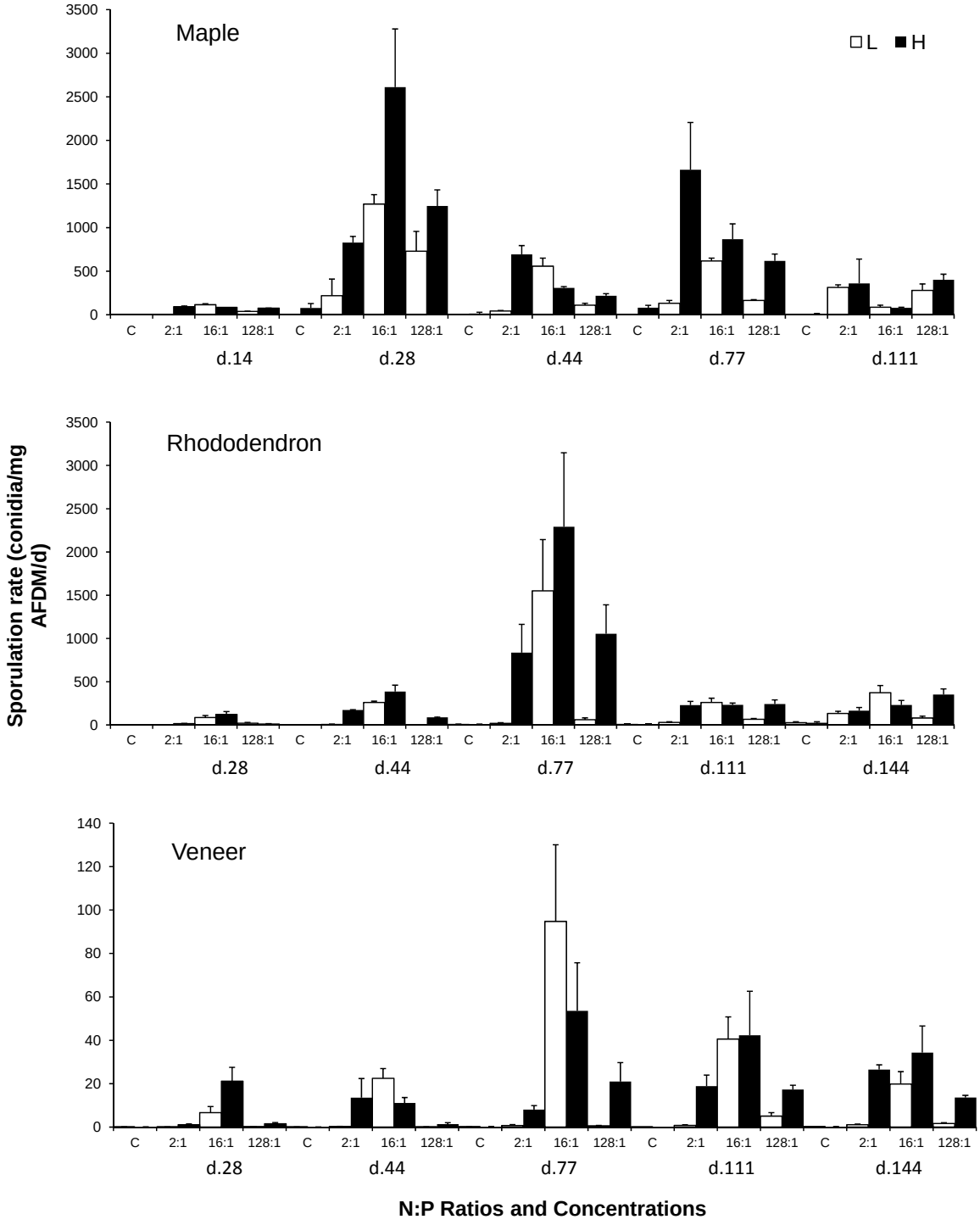


Figure 3. Sporulation rates of aquatic hyphomycetes at low (L) and high (H) nutrient concentration levels for all nutrient ratios throughout the experiment. Error bars show +1 standard error (SE). Control (C) = unamended stream water.

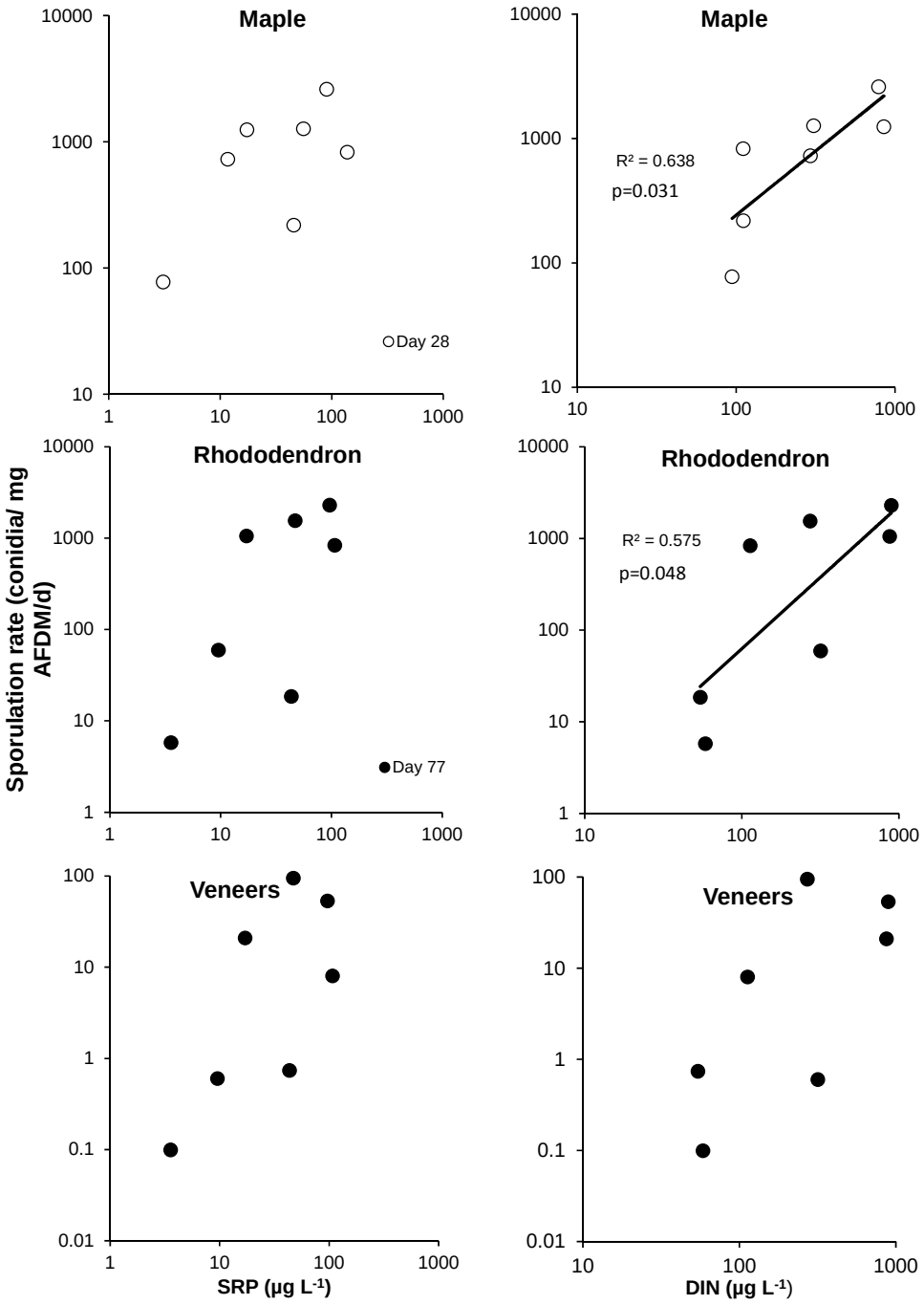


Figure 4. Relationship between sporulation rate and soluble reactive phosphorus (SRP) concentration (left panels) or dissolved inorganic nitrogen (DIN) concentrations (right panels) during peak spore production dates (day 28 for maple, day 77 for rhododendron and wood veneers).

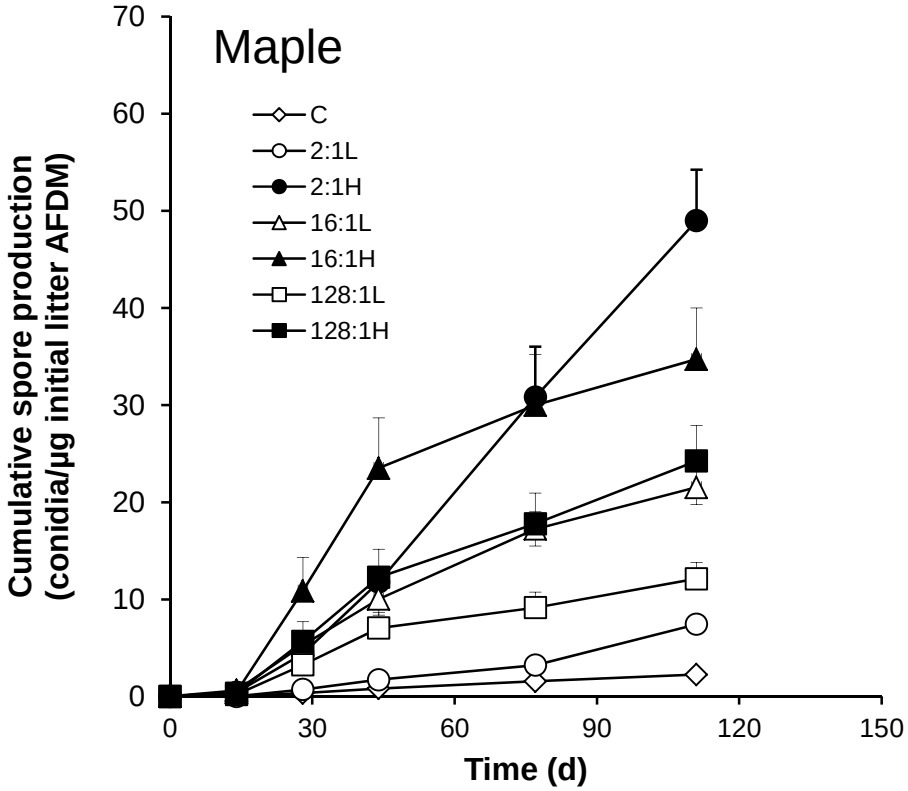


Figure 5. Cumulative spore production of aquatic hyphomycetes on maple in the control and nutrient-enriched streamside channels. See Table 1 for abbreviations and nutrient concentrations. Error bars represent standard error (SE), some are omitted for clarity.

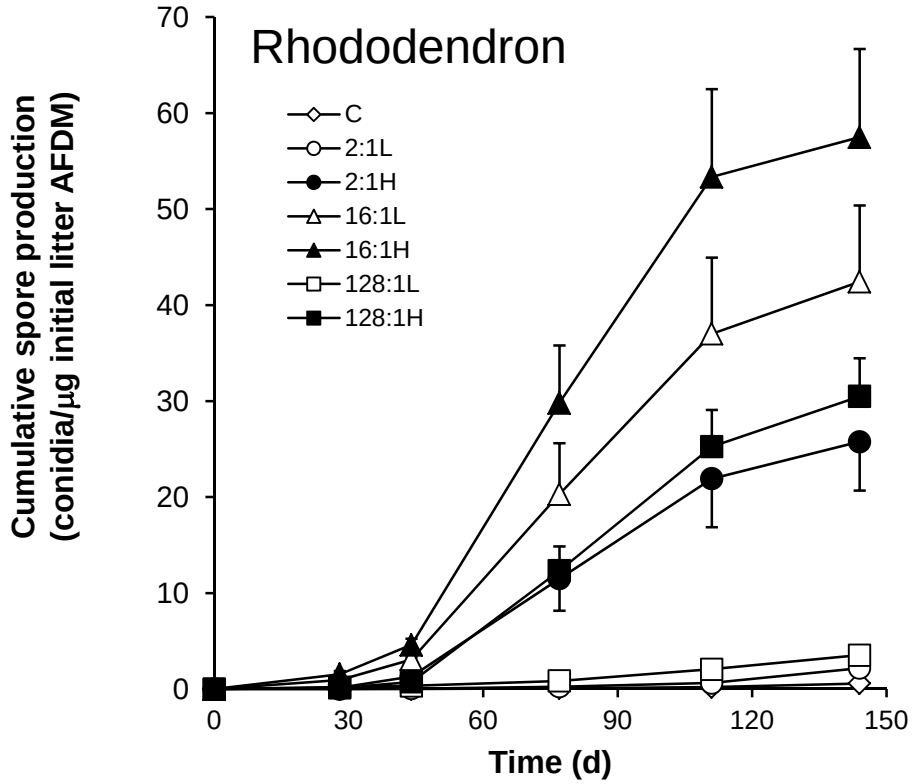


Figure 6. Cumulative spore production of aquatic hyphomycetes on rhododendron in the control and nutrient-enriched streamside channels. See Table 1 for abbreviations and nutrient concentrations. Error bars represent standard error (SE), some are omitted for clarity.

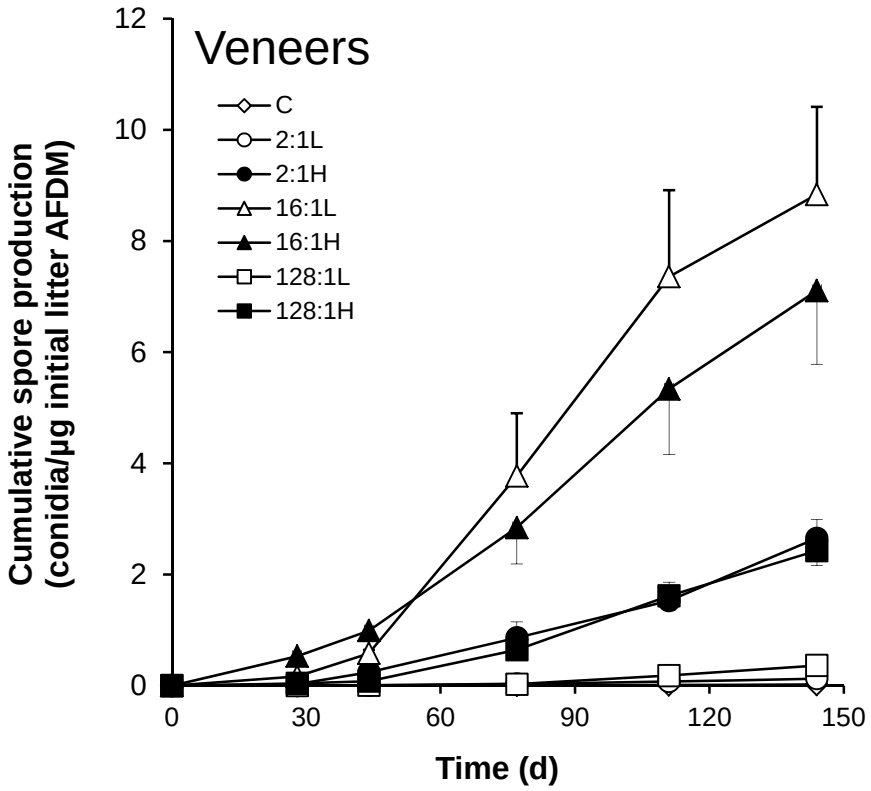


Figure 7. Cumulative spore production of aquatic hyphomycetes on wood veneers in the control and nutrient-enriched streamside channels. See Table 1 for abbreviations and nutrient concentrations. Error bars represent standard error (SE), some are omitted for clarity.

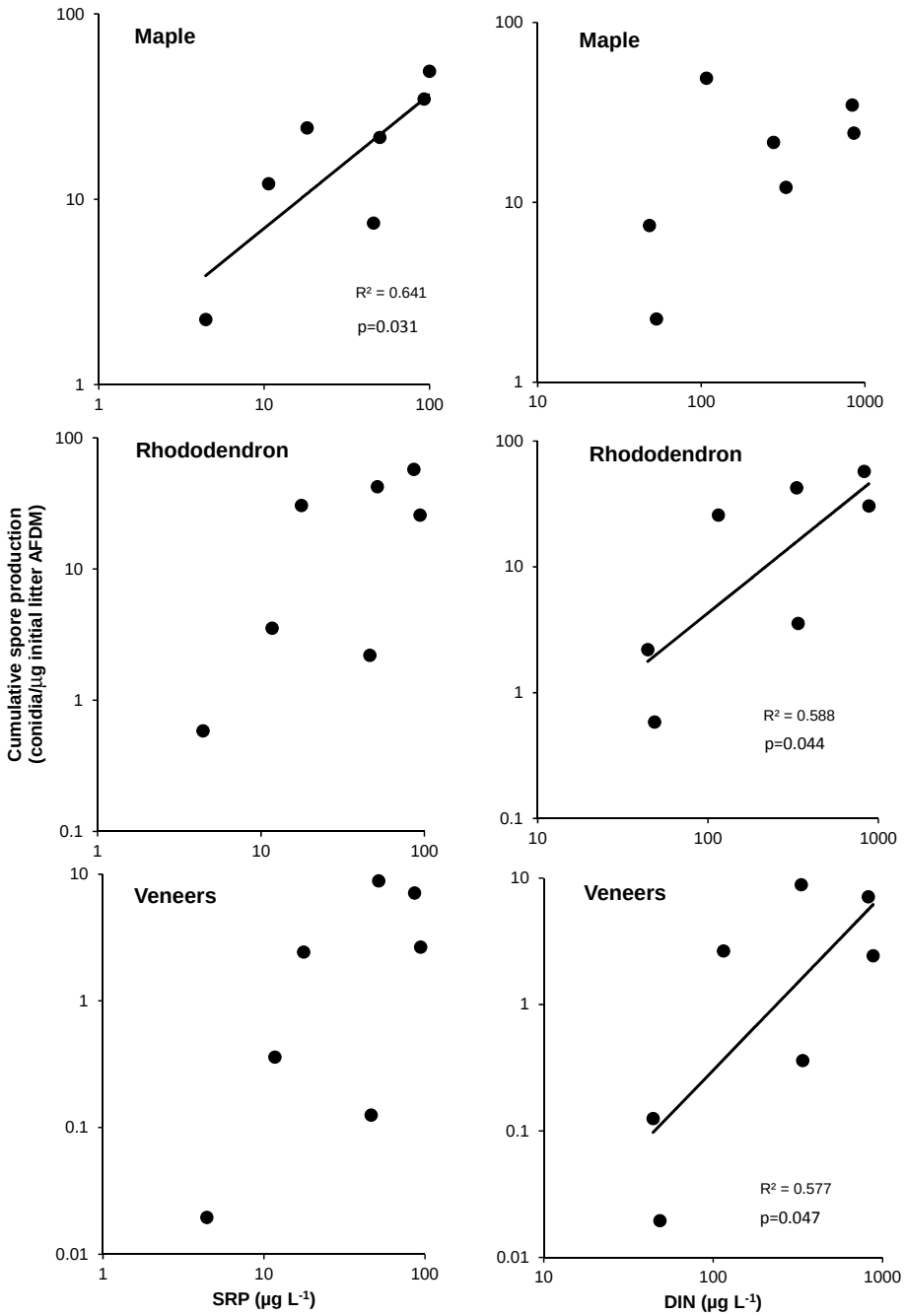


Figure 8. Relationship between cumulative fungal spore production from plant litter by the end of the experiment (d. 111 for maple leaf litter and day 144 for rhododendron leaf litter and wood veneers) and average soluble reactive phosphorus concentration(SRP) (left panels) or dissolved inorganic nitrogen concentrations (DIN) (right panels) during the corresponding time periods.

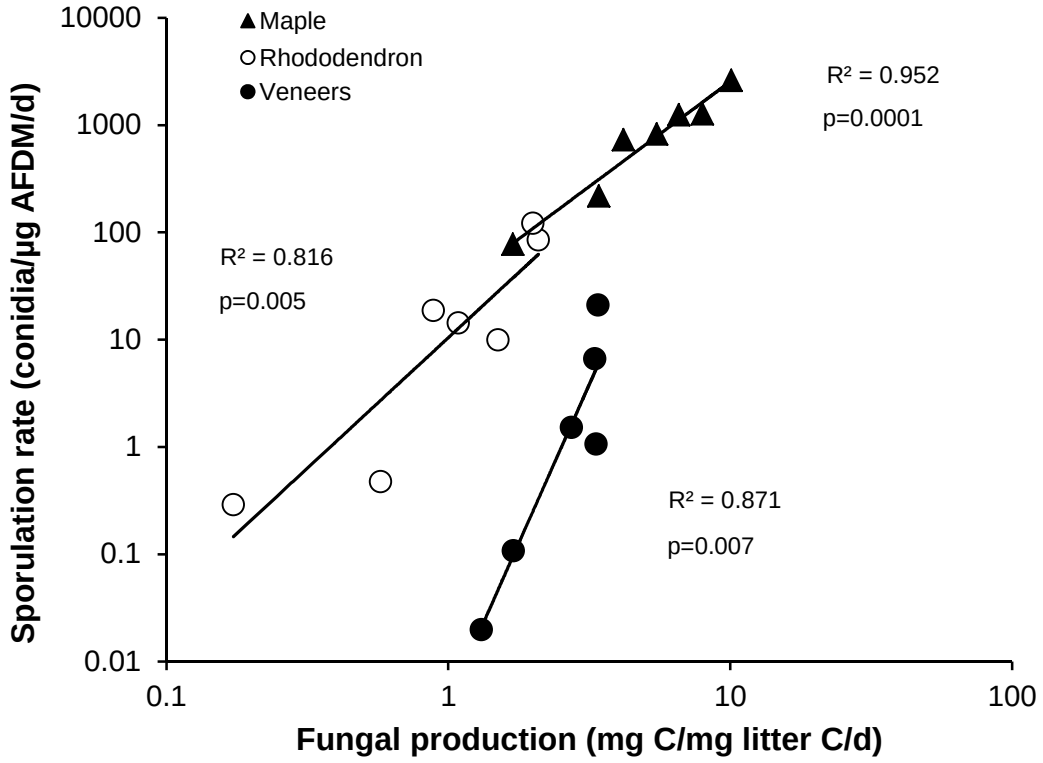


Figure 9. Relationship between fungal production and sporulation rate of aquatic hyphomycetes for all litter types on day 28. R^2 and p values for linear regression between $\log_{10}$-transformed data are shown.

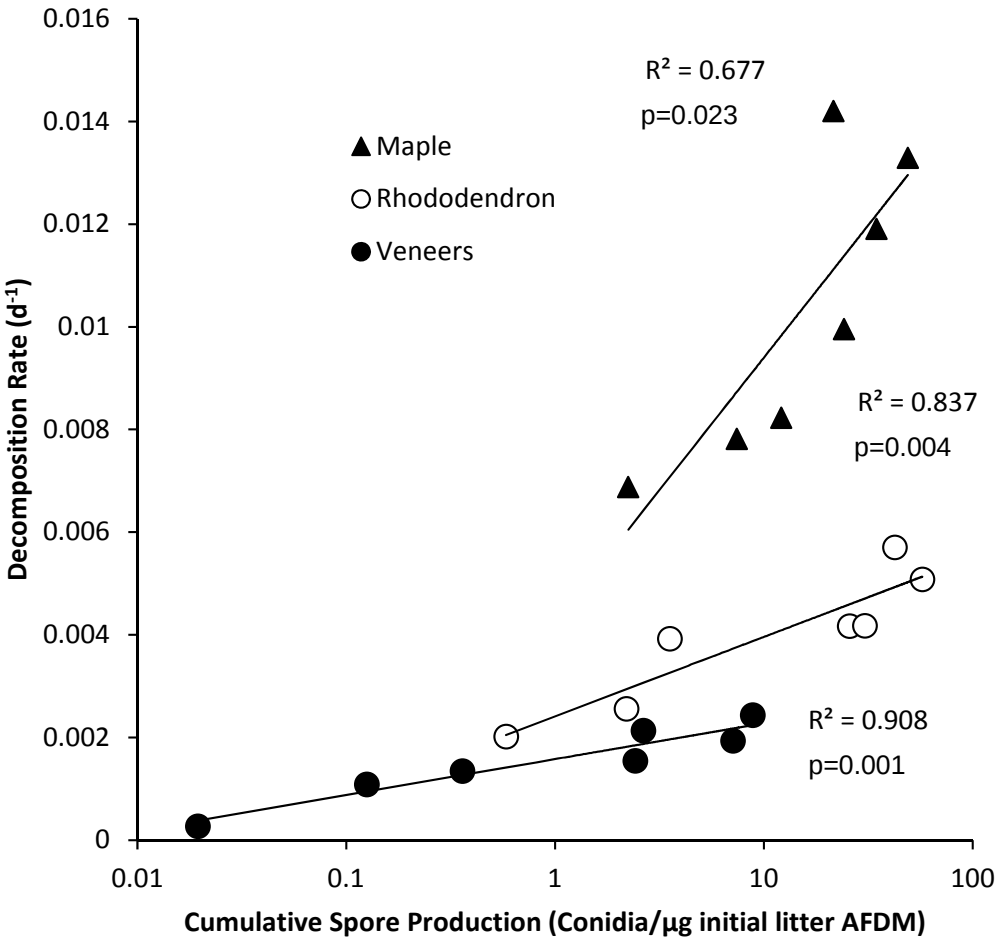


Figure 10. Relationship between decomposition rate and cumulative spore production of aquatic hyphomycetes for all litter types by the last day of the experiment (day 111 for maple, day 144 for rhododendron and wood veneers). R^2 and p values for linear regression are shown.

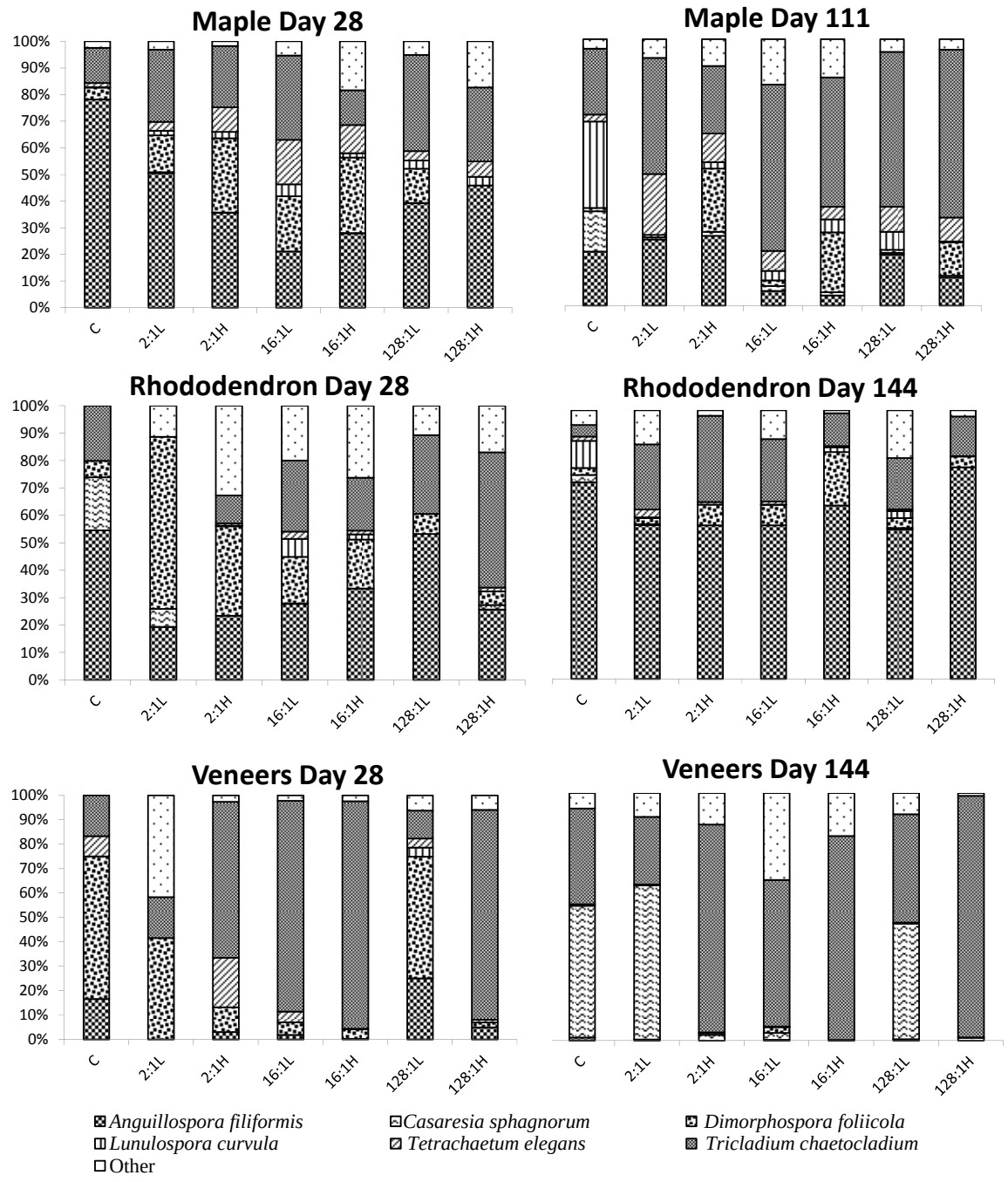


Figure 11. Relative contribution of dominant aquatic hyphomycetes to total conidia pool from each substrate type in the control and nutrient enriched streamside channels at the early (day 28) and late stages of litter decomposition (last sampling date). A dominant species contributed greater than 5% to the total conidia pool on at least one litter type. See Appendix I – III for intermediate dates.

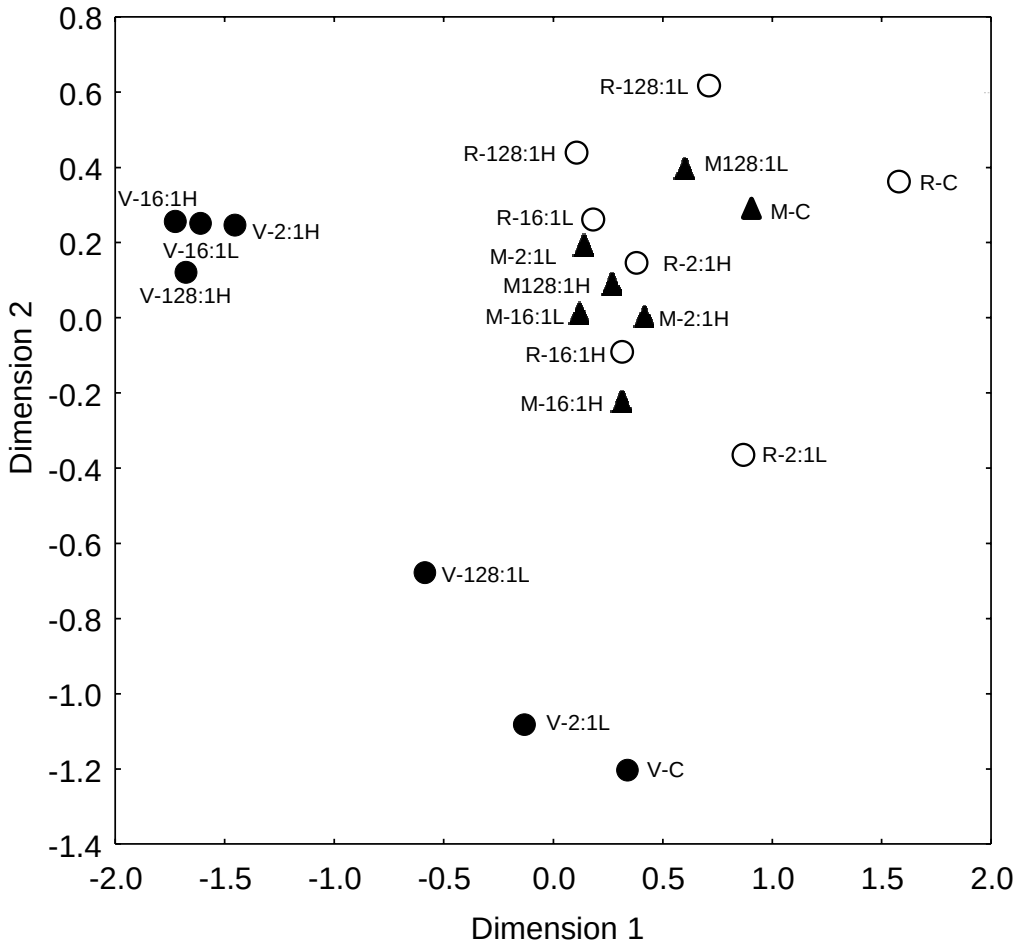
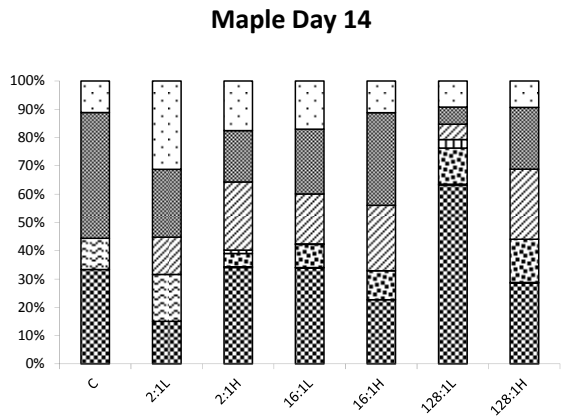
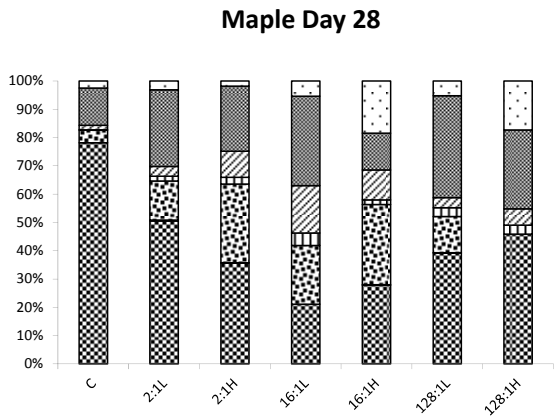
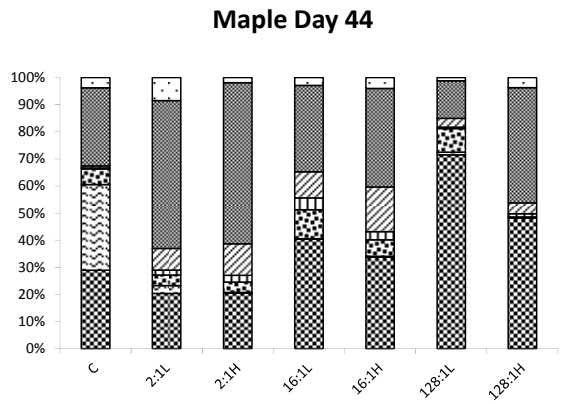


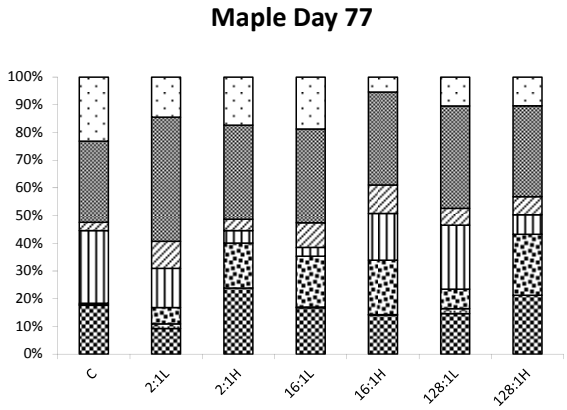
Figure 12. Ordination of litter samples from different nutrient treatments by multidimensional scaling based on fungal assemblage structure. M=maple leaf litter, R=rhododendron leaf litter, V=wood veneer; See Table 1 for nutrient treatment codes.

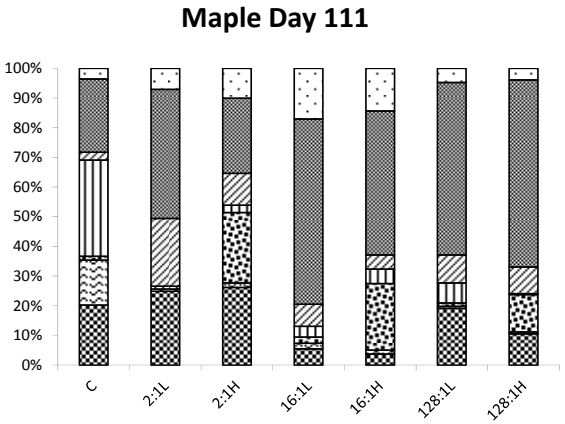
APPENDIX I. Relative contribution of dominant aquatic hyphomycetes to total conidia pool from maple leaf litter.

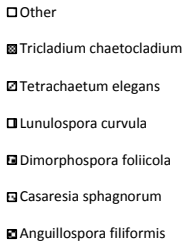




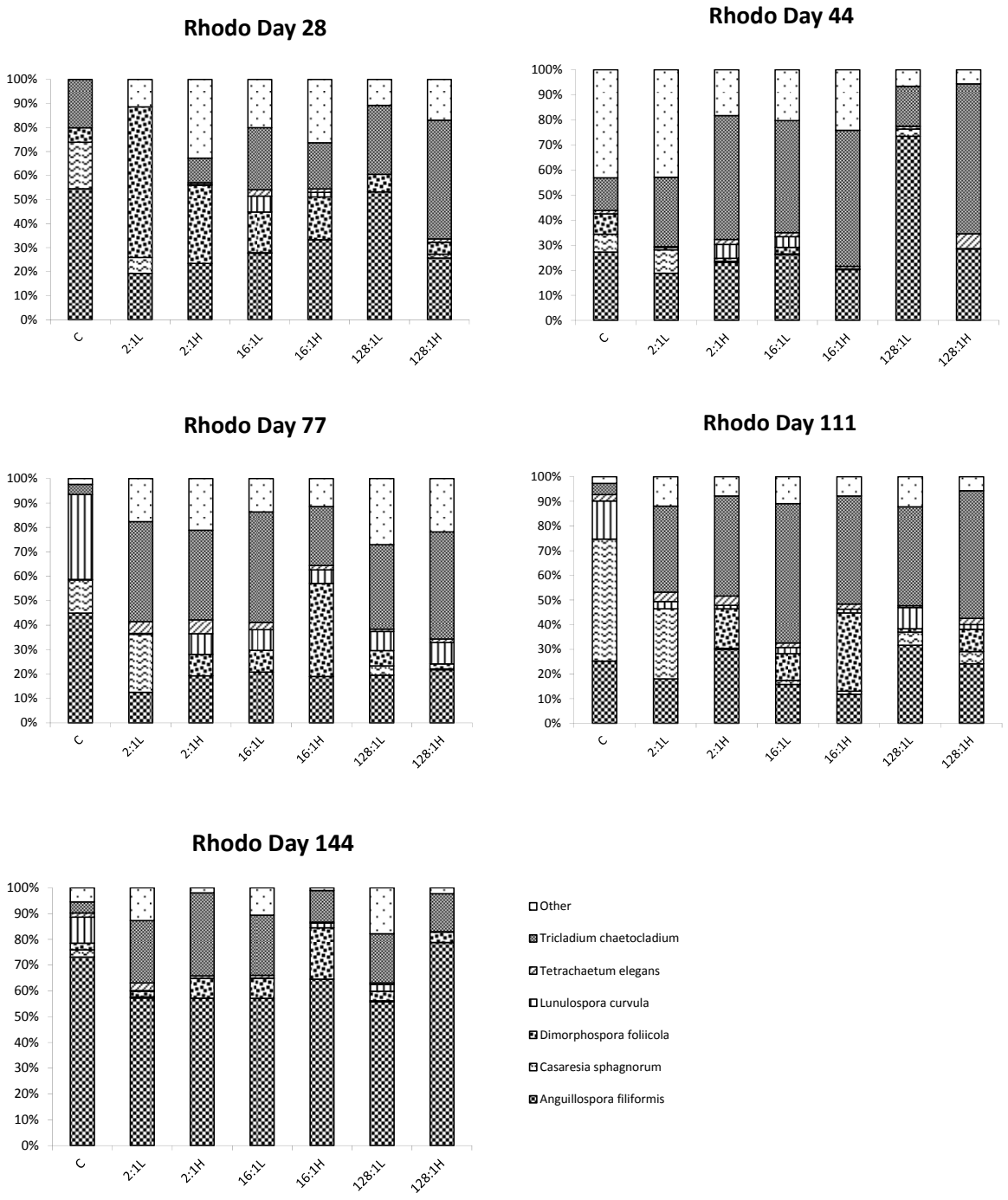








APPENDIX II. Relative contribution of dominant aquatic hyphomycetes to total conidia pool from rhododendron leaf litter.



APPENDIX III. Relative contribution of dominant aquatic hyphomycetes to total conidia pool from wood veneers.