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Effects of dissolved nutrient ratios and concentrations on litter-associated microbial
activity in streamside channels

by
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Abstract

Heterotrophic consumers, such as microorganisms and invertebrates, play a fundamental role in the flow of carbon and energy in streams. The effects of dissolved nutrient concentrations, and especially ratios, on litter-associated microorganisms and decomposition rates of detritus are poorly understood. This study addressed the responses of heterotrophic microbes to a wide range of concentrations and ratios of dissolved inorganic nitrogen (N) and phosphorus (P) in streamside channels simulating headwater streams. Two main questions were: (1) do microbial parameters and litter decomposition rates peak at a dissolved N:P ratio that approaches the nutrient ratio of microbial biomass, and (2) does microbial activity stabilize the nutrient content of decomposing litter possessing different initial nutrient content and carbon quality (i.e., leaf litter vs. wood)? The study was conducted using an array of 30 streamside channels that replicated the width, depth and flow rate of headwater streams at Coweeta Long Term Ecological Research Site situated in the southern Appalachian Mountains (Macon County, NC). Dissolved nutrients were added in low, medium and high concentrations at 2:1, 16:1 and 128:1 molar N:P ratios. Microbial parameters (respiration, fungal biomass, fungal and bacterial production) and litter decomposition rates peaked at a dissolved nutrient ratio of 16:1, a value that approaches the stoichiometry of fungal biomass (*ca.* 10:1). Multiple microbial parameters correlated with dissolved inorganic N and/or P concentrations but rarely with dissolved N:P ratios. Decreases in detrital C:N and C:P ratios strongly correlated with fungal biomass accrual due to fungal immobilization of dissolved nutrients. Microbial colonization stabilized detrital stoichiometry via more drastic changes in C:nutrient ratios of resources with high initial C:nutrient ratios (e.g., wood)

compared to high quality (low initial C:nutrient ratios) leaf litter. Nutrient ratios and concentrations control microbial activity, plant litter decomposition and detrital stoichiometry, altering the flow of carbon, nutrients and energy in headwater streams.

Introduction

Atmospheric deposition coupled with agricultural and urban activities has proven to be a major contributor to nutrient enrichment in many freshwater ecosystems (Carpenter et al. 1998). These sources are described as “non-point” sources and can be difficult to measure and, consequently, complicated to regulate. For instance, since its inception in 1972, the Clean Water Act resulted in vast improvements of water quality across the United States and has limited pollution problems due to untreated water discharge. However, the efforts to reduce and manage the consequences of non-point source nutrient loading are hindered by the lack of information on the responses of aquatic heterotrophic organisms, such as fungi and bacteria, to nitrogen and phosphorus inputs. Elser et al. (2007) performed a comprehensive meta-analysis of nutrient addition experiments and showed that both N and P often co-limit primary producers across many different ecosystems. These findings highlight the importance of anthropogenic activity leading to increased nutrient availability in controlling the biomass, and presumably the productivity, of autotrophs in aquatic and terrestrial ecosystems worldwide (e.g., Vitousek et al. 1997, Carpenter et al. 1998, Galloway et al. 2003). Our understanding of the responses of primary producers to nutrient enrichment has grown from initial experiments on nutrient limitation to studies addressing environmental variables that modify these responses (e.g. light availability and scouring floods; Biggs 2000, Kocum et al. 2002), to applications of basic research findings to the broader goal of determining nutrient criteria for streams and rivers in the U.S. and other countries (Dodds and Welch 2000). While there have been many studies that have dealt with primary producers, few have tested the effects of nutrient enrichment on heterotrophic microorganisms.

The organisms present in heterotrophic aquatic environments, such as streams and small rivers, rely mainly on allochthonous organic material as a source of carbon and energy. Microbes, more specifically aquatic fungi and to a lesser extent bacteria, play an important role in biogeochemical cycling within these ecosystems by mediating the transfer of carbon, other nutrients and energy to higher trophic levels (Kaushik and Hynes 1971, Bärlocher, 1985, Suberkropp 1992a). Therefore, any effects of dissolved inorganic nutrients on the activity of heterotrophic microbes may have direct consequences for food webs relying on stream detritus. Although Elser et al. (2007) illustrated the similarity and interdependence of N and P across ecosystems, there is a fundamental difference between autotrophic and heterotrophic responses to nutrient enrichment. The standing stock of carbon increases in systems dominated by autotrophs while the opposite is true for heterotrophic systems (Dodds 2007, Benstead et al. 2009). While the majority of both basic and applied studies have focused on pathways that originate with increased carbon fixation from primary producers (e.g., Dodds and Cole 2007), recent work has shown that heterotrophic pathways can also be profoundly affected by nutrient enrichment in both aquatic and terrestrial systems (Mack et al. 2004, Cleveland and Townsend 2006, Mallin et al. 2006). For example, in streams, positive effects of nutrient enrichment on plant litter decomposition (e.g., Elwood et al. 1981, Chadwick and Hury 2003, Gulis and Suberkropp 2003b), as well as biomass and production of detritus-associated microbes (Suberkropp 1995, Rosemond et al. 2002, Gulis and Suberkropp 2003a, Gulis et al. 2008) have been demonstrated. However, our conceptual understanding of the effects of nutrient concentrations and, especially, nutrient ratios on heterotrophic microbes and pathways currently lags far behind the body of theory and data related to nutrient effects

on primary producers and the food webs they support. Plant litter (leaves and wood) that enters stream ecosystems has C:P and C:N ratios considerably higher than that of microbial decomposers (e.g. fungi) (Stelzer et al. 2003, Gulis et al., unpublished). In contrast to autotrophs, such as algae, microbial decomposers associated with plant detritus in streams can obtain N and P from both the organic substrate and the water column (Suberkropp 1995). Thus, the stoichiometric imbalance between the organic substrate and microbial biomass can be alleviated by the uptake of external dissolved N and P. The stoichiometric imbalance and nutrient enrichment in streams can affect not only microbes but by extension the ecosystem-level processes such as decomposition and C and nutrient transfer to higher trophic levels (Elser and Urabe 1999, Sterner and Elser 2002).

In lotic ecosystems, plant litter decomposition is driven by microbial decomposers (primarily fungi such as aquatic hyphomycetes) and aquatic invertebrates (e.g., Gessner et al. 2007). As detritus decomposes, microbial biomass accrual leads to changes in overall detrital stoichiometry (decreases in C:N and C:P ratios) and an increase in substrate quality and palatability of decaying material to detritivorous invertebrates (Bärlocher 1985, Gessner et al. 2007). These changes in litter stoichiometry and resource quality can be explained by much lower C:N and C:P ratios of microbial biomass compared to uncolonized plant litter. From a stoichiometric perspective (Elser and Urabe 1999, Sterner and Elser 2002), fungal growth and plant litter decomposition rates should be then maximized on high quality leaf litter with relatively low C:nutrient ratios. Indeed, breakdown rates can be affected by initial litter nutrient content, C:N ratio, lignin content (Taylor et al 1989, Gessner and Chauvet 1994), and lignin:nitrogen ratio (Melillo et al.

1982). High quality leaves (e.g., nutrient-rich low-lignin alder leaves) will decompose faster than low quality leaves (e.g., nutrient-poor high-lignin conifer needles) or wood. Although fungi can obtain some N and P from the plant litter they grow on, they can also immobilize inorganic N and P from the water column (Suberkropp 1995, Gulis et al. 2006). Thus, dissolved nutrient availability (both concentrations and ratios of inorganic N and P) has a potential to modify fungal activity, biomass accrual and detrital stoichiometry. Unfortunately, our understanding of fungal biomass nutrient stoichiometry, and, as such, nutrient requirements for optimal growth, is rudimentary. In addition, until recently, it was not known if microbial decomposers were homeostatic or not with respect to their C:N and C:P ratios, i.e. if microbial biomass stoichiometry changes depending on external nutrient supply. Recent studies have indicated that microbial decomposers in terrestrial ecosystems (Fanin et al. 2013) and aquatic fungi (Gulis et al. unpublished) can be non-homeostatic with regards to their C:P ratio, suggesting possible P storage. If fungi are indeed not homeostatic and are capable of copious uptake of P from the water column, it may have serious implications for plant litter stoichiometry via two mechanisms: (i) stimulation of fungal biomass accrual within the detritus and (ii) increase in P content of fungal mycelium, which would both lead to changes in overall detrital stoichiometry. This could have profound effects on invertebrate consumers that rely on detritus and associated microorganisms as food, with the effects possibly propagating to higher trophic levels (Sturner and Elser 2002, Gessner et al. 2007). Despite these implications, data on detrital C:nutrient ratios at different levels of nutrient availability (from the substrate and the water column) and associated microbial activity are scarce.

In streams, fungi dominate decomposition of plant detritus while the role of bacteria is minor (Gulis et al. 2006, Gessner et al. 2007). For example, fungi contributed 95-99.7% of total microbial biomass and 88-95% of total microbial production on submerged leaf litter in southern Appalachian streams at Coweeta Hydrologic Laboratory, NC (Gulis and Suberkropp 2003b, Suberkropp et al. 2010). A recent experiment completed at Coweeta demonstrated the profound effects of nutrient enrichment on heterotrophic organisms and carbon resources in aquatic systems (Benstead et al. 2009, Suberkropp et al. 2010). This whole-stream nutrient addition experiment resulted in dramatic increases in carbon loss due to downstream export, increased microbial activity and leaf litter N and P content. Further, litter decomposition rates, fungal biomass and sporulation rates in higher-order streams can be similar to those found in headwater streams (Baldy et al 1995) suggesting that the importance of aquatic fungi in regulating litter quantity and quality extends beyond the reaches of headwater streams.

In this study, I tested the effects of dissolved nutrient concentrations and ratios on microorganisms associated with plant litter that differ in initial C:N and C:P ratios. I hypothesized that: (1) microbial activity and litter decomposition rates will peak at dissolved N:P ratio that approaches nutrient stoichiometry of microbial biomass and (2) microbial activity will alter C:nutrient ratios of detritus to a greater extent in substrates that differ more in their initial nutrient stoichiometry from microbial biomass (e.g. low-nutrient wood) while changes for high nutrient substrates (e.g. maple leaves) will be less pronounced. The response of heterotrophic microbes to a range of concentrations and ratios of dissolved inorganic nutrients (N and P) was tested in streamside channels

simulating headwater streams at Coweeta Hydrologic Laboratory Long Term Ecological Research site in Macon County, North Carolina. Streamside channels are man-made constructs that replicate flow patterns of headwater streams and allow for easy set up of multiple treatments (concentrations and ratios of nutrients) including replicates and controls. Since N and P enrichment is the most common source of impairment to the U.S. surface and coastal waters (Carpenter et al. 1998, Howarth et al. 2000), this study advances our understanding of how aquatic systems are affected by nutrient enrichment via heterotrophic pathways, a perspective that is currently lacking in management decisions related to eutrophication (Dodds 2007).

Experimental Design and Methods

Site Description

All fieldwork was conducted at the Coweeta Hydrologic Laboratory (CHL) Long Term Ecological Research site in Macon County, North Carolina. This site, a research facility of the USDA Forest Service, is in the Blue Ridge geological province and spans 2185 ha. Because of the relatively resistant crystalline bedrock, stream nutrient concentrations are very low: $\text{NO}_3\text{-N} < 0.04 \text{ mg L}^{-1}$ and $\text{PO}_4\text{-P} < 0.002 \text{ mg L}^{-1}$ (Swank and Waide 1988). Vegetation is dominated by mixed hardwoods and the stream food webs are dependent on allochthonous energy sources (Wallace et al. 1997, Webster et al. 1997). The terrestrial detritus provides over 90% of the organic matter available for secondary production, even under nutrient-enriched conditions (Cross et al. 2007).

Streamside Channels and Experimental Design

Thirty streamside channels were constructed below the main weir on Shope Fork at CHL (Fig. 1) as a part of collaborative project with researchers from the University of Georgia, the University of Alabama and Coastal Carolina University. The channels are 4 × 0.15 m and were set to a flow rate of 0.1 L/s each (emulating headwater streams at Coweeta). Stream water was pumped to the primary holding tank and then to the secondary tanks and finally into the individual channels. Nine nutrient treatments (N:P at 3 ratios, with each ratio at 3 concentration levels, see Fig. 2, Table 1) plus control (unenriched stream water) were achieved by dosing concentrated nutrient solutions to each channel using peristaltic pumps. These treatments represent realistic nutrient concentrations that were chosen to range from 37 to 975.5 $\mu\text{g L}^{-1}$ of dissolved inorganic nitrogen (DIN) and 2.5 to 135 $\mu\text{g L}^{-1}$ of soluble reactive phosphorus (SRP) that can be found in pristine to polluted streams (Allan 1995, Dodds and Welch 2000). Each treatment was replicated 3 times resulting in the total of 30 channels. Water samples from each channel were taken every two weeks, filtered through glass fiber filters (Whatman GFF, 45-mm diameter) in the field and analyzed for $\text{NH}_4\text{-N}$, $\text{NO}_3\text{+NO}_2\text{-N}$ and SRP at the Analytical Chemistry Laboratory, Odum School of Ecology, University of Georgia. Achieved (actually measured) concentrations in the streamside channels ranged from 44.2 to 878.4 $\mu\text{g L}^{-1}$ DIN and 3.8 to 98.7 $\mu\text{g L}^{-1}$ SRP (Table 1). Each experimental channel received litter bags with several types of plant material (see below). Litter decomposition rates and associated microbial parameters were followed for 5 months of continuous nutrient enrichment.

Litter bags and sampling

The decomposition experiment used fine mesh litter bags constructed from window screening (20 cm x 14 cm, 1-mm mesh size) with 3 ± 0.25 g of plant material per bag. Two leaf species (*Acer rubrum* and *Rhododendron maximum*) were chosen due to differences in initial C:N:P ratio and carbon quality along with a standardized substrate, wood veneers (*Quercus alba*), that has extremely low N and P content and high lignin concentration. Differences in carbon quality, nitrogen, and phosphorus concentrations of plant detritus can account for up to 89% of variances in decomposition rates (Enriquez et al. 1993); therefore, five sampling dates were staggered during the experiment to standardize decomposition stages across the different substrate types. Sampling began with 3/9/2012 serving as day 0 for all substrates; maple leaves were sampled on days 14, 28, 44, 77, 111 while rhododendron leaves and wood veneers were sampled on days 28, 44, 77, 111, 144. On each sampling date, litter bags were retrieved, brought to the laboratory on ice and sets of leaf disks (12-mm diameter) or wood veneer rectangles (1 x 2 cm) cut for microbial analyses. Respiration measurements and incubations to determine bacterial and fungal production were performed on fresh material, while samples for fungal biomass (ergosterol) were preserved in 5 mL of methanol and stored at -20 C until extraction. The remaining bulk material was used to determine litter decomposition rates and C, N and P content.

Fungal biomass and production

Fungal biomass was determined from ergosterol concentrations of plant material (liquid-to-liquid lipid extraction followed by quantification with high performance liquid chromatography (HPLC); Gulis and Suberkropp 2006). A Shimadzu 10-VP HPLC

system equipped with a reverse phase C18 column (Kinetex 2.6 μm core-shell, 4.6 x 150 mm, Phenomenex, Torrance, CA) was run isocratically at 0.75 mL min⁻¹ of methanol at 35 C; ergosterol in 20- μL injections of lipid extracts was detected at 282 nm and eluted at 5.0 min. HPLC areas were converted to ergosterol concentrations using external ergosterol standards (Acros Organics, Morris Planes, NJ) and a conversion factor of 5.5 mg ergosterol per g of fungal dry mass. Fungal production was estimated by the ¹⁴C-acetate incorporation into ergosterol technique (Suberkropp and Weyers 1996, Gulis and Suberkropp 2006). Leaf disks with associated fungal biomass were incubated for four hours in 4 mL of corresponding streamside channel water amended with sodium [1-¹⁴C]acetate (Vitrox, Placentia, CA) (1.0 MBq/sample, 5 mM final concentration). After incubation on a shaker (100 rpm) at stream temperature for 4 h, disks were preserved in 5 mL methanol and kept at -20C until ergosterol extraction. After lipids were extracted as described for fungal biomass, ergosterol was separated and quantified by HPLC using Whatman Partisphere reverse phase C18 column (4.6 x 250 mm) run isocratically at 1.2 mL min⁻¹ of methanol at 35 C; ergosterol in 100- μL or 250- μL injections of lipid extracts was detected at 282 nm and eluted at 8.0 min. The ergosterol fraction was collected (Advantec SF-2120 fraction collector), mixed with Ecolume scintillation cocktail (MP Biomedicals, Solon, OH) and radioactivity quantified using a scintillation counter (Triathler, LabLogic, Brandon, FL). Each sample was then spiked with known amount of ¹⁴C (6000 dpm), radioactivity measured again, and raw counts corrected for quenching. Instantaneous growth rates and fungal production were calculated assuming the exponential growth model as described previously by Gessner et al. (1997), and empirical

conversion factors of 19.3 µg fungal biomass per nmol acetate incorporated (Suberkropp and Weyers 1996).

Bacterial production

Bacterial production was determined from the rate of incorporation of ^3H -leucine into protein (modified from Kirchman 1993, Suberkropp and Weyers 1996). Samples were incubated for 30 min with 4 ml of filtered stream water and 30 µCi of [4,5 ^3H]-leucine (1 µM final concentration). Radiolabel incorporation was stopped by the addition of trichloroacetic acid (TCA, 5% final concentration). In addition, 1 mg of bovine serum albumin (BSA) per sample was added to aid in the subsequent precipitation of protein. Samples were then heated to 95°C for 1 h, cooled to room temperature and stored at 4° C until the extraction of radiolabeled protein. Samples were sonicated for 5 min (FS20D sonication bath, Fisher Scientific) and then centrifuged (4 min at 5000 g), supernatant decanted and 4 ml of 5% TCA added to precipitated protein. This washing step was repeated twice with 5% TCA, twice with 80% ethanol and once with distilled water to remove any excess radiolabel not incorporated into protein. Then protein was solubilized in alkaline extraction solution (0.5 M NaOH, 0.1% SDS and 25mM EDTA) with heating (90°C for one hour). 0.1 mL aliquots were removed and decolorized with hydrogen peroxide. Ecolume (5 mL, MP Biomedicals) was added as the scintillation fluor and radioactivity was quantified using a scintillation counter (Triathler, LabLogic, Brandon, FL). Samples were then spiked with known amount of ^3H (6179 dpm), radioactivity measured again, and raw counts corrected for quenching.

Microbial respiration

All microbial analyses were performed on sets of 5 or 10 leaf disks, or 3 to 6 wood veneer rectangles, to standardize per unit of plant AFDM from corresponding sets that were dried (60° C) and weighed. Microbial respiration rates were determined in the lab from microbial oxygen uptake of plant litter in sealed respiration chambers with dissolved oxygen meters (Orion 5-Star Plus, ThermoScientific, Beverly, MA or YSI 5100, Yellow Springs, OH) at streamside channel water temperature for that date (Gulis and Suberkropp 2003a). The temperature was maintained with a recirculating cooling water bath. Respiration rates were determined from the slope of linear regression of O₂ concentrations over time and then corrected by subtracting the average slope of blanks (stream water) for each respirometer.

Litter mass loss

Detrital mass loss was determined by weighing samples after drying bulk plant material at 60°C for at least 48 h. Dry material was then ground (Ball Mill, Spex Industries 8000) and ash content was determined by ashing pre-weighed ground subsamples at 500°C for 4 h, reweighing and calculating ash-free dry mass (AFDM) of bulk plant material. Decomposition rates (k) were calculated using a negative exponential model (Webster and Benfield 1986).

Nutrient Analyses

Subsamples of ground plant material were used to determine C, N, P content. Nitrogen analyses were performed with a CHN elemental analyzer (Carlo Erba NA1500) at the Analytical Chemistry Laboratory, Odum School of Ecology, University of Georgia. Phosphorus content of litter was determined using a modification of the combustion and

hot HCl extraction procedure of Andersen (1976) followed by spectrophotometric (Beckman DU520) quantification of SRP using ascorbic acid method (APHA 1998). All nutrient analyses were performed using NIST C, N and P standards as a reference.

Statistical Analyses

Since levels of nutrient concentrations (low, medium and high) could not be the same among nutrient ratio treatments (2:1, 16:1 and 128:1) in our experimental design (Fig. 2, Table 1), a regression approach instead of ANOVA was used for the full data sets (all 10 nutrient treatments including control). Specifically, the effects of dissolved nutrient ratios or concentrations (DIN or SRP) on microbial activity (microbial respiration, fungal biomass and production, bacterial production) and decomposition rates (k) were assessed by linear regression using raw or \log_{10} -transformed data. Relationships between microbial parameters and detrital stoichiometry were also explored by linear regression. Data were tested for normality, using Shapiro-Wilks test, and homoscedasticity, using Breusch-Pagan test.

Results

Fungal biomass accrual over time was apparent on all three substrates with higher quality leaf litter supporting earlier biomass increases than more recalcitrant wood veneers (Fig. 3). On most sampling dates, the highest amount of fungal biomass occurred in streamside channels that received dissolved nutrients at a 16:1 ratio. This pattern was even more pronounced for fungal production; 16:1 dissolved N:P ratio typically resulted in the highest amount of fungal production at early stages of the decomposition, d. 28 (Fig. 4). In general, fungal production was maximized at the early stages of litter

breakdown for the most labile substrate, maple leaf litter. The most recalcitrant substrate, wood veneers, demonstrated steady fungal production throughout the experiment, while rhododendron showed a significant increase on the latter (d. 77) sampling date. Fungal biomass on d. 28 and notably in later stages of decomposition (d. 77) showed a significant relationship with DIN for maple leaf litter (Fig. 5, linear regression, $R^2= 0.74$, $p=0.013$). Fungal production showed a significant positive relationship with DIN on d. 28 for both maple and rhododendron leaf litter ($R^2= 0.60$, $p=0.041$; $R^2= 0.58$, $p=0.048$, respectively) but not wood veneers (Fig. 5). No significant relationship with SRP concentrations have been found for either fungal biomass or production.

Bacterial production yielded similar pattern to that of fungal production, with production reaching higher values earlier on maple than on other substrates (Fig. 6). The dissolved nutrient ratio of 16:1 resulted in the highest bacterial activity on all substrates for most of the sampling dates. In contrast to fungal production, bacterial production associated with maple leaf litter showed a significant positive relationship with dissolved P availability (Fig. 7, linear regression, d. 77, $R^2= 0.75$, $p= 0.011$). No relationship of bacterial production with DIN has been found for any substrate or date.

Respiration rates, although varying greatly among nutrient treatments, were generally higher across all substrate types at 16:1 dissolved N:P ratio at early stages of decomposition (d. 28) (Fig. 8). Respiration rates associated with rhododendron leaf litter and wood veneers showed significant positive relationships with DIN, especially at later stages of decomposition, d. 77 (Fig. 9, linear regression, $R^2= 0.61$, $p= 0.008$; $R^2= 0.47$, $p= 0.029$, respectively) when fungal biomass was relatively high (Fig. 3). No relationship between microbial respiration and SRP was found for any dates.

Decomposition rates varied among substrate types as expected, and also among dissolved nutrient concentrations and ratios. Due to the differences in initial C:nutrient ratio (Table 2) and lignin content of the substrates, maple leaf litter showed the highest rate of decomposition followed by rhododendron leaves and wood veneers (Fig. 10). Decomposition rates for each substrate type tended to be higher at a dissolved nutrient N:P ratio of 16:1. Decomposition rates were significantly correlated with DIN availability only for rhododendron leaf litter (Fig. 11, $R^2 = 0.71$, $p = 0.002$). In contrast, decomposition rates of maple leaf litter and wood veneers showed a significant positive relationship with SRP concentration ($R^2 = 0.57$, $p = 0.011$; $R^2 = 0.56$, $p = 0.013$, respectively). Effects of nutrient concentrations on decomposition rates were most pronounced for wood veneers, followed by rhododendron then maple, by comparison of linear regression slopes for each substrate.

Changes in detrital C:N ratios of the three substrates followed an inverse pattern to that of decomposition rates, i.e. C:N ratios of wood veneers (the most recalcitrant, slowly decomposing substrate) decreased the most at higher dissolved N:P ratios (16:1 and 128:1) followed by rhododendron and maple leaf litter (Fig. 12). The lowest C:N ratios, or the greatest changes from initial (Table 2) to final substrate quality, were observed at the dissolved nutrient ratio of 16:1 for maple and rhododendron leaf litter and wood veneers (Fig. 12). Detrital C:P ratios (Fig. 13) followed the pattern described for C:N ratios above; in addition, the magnitude of difference between nutrient treatments and control was more pronounced for detrital C:P. Strong statistically significant negative relationships between log-transformed DIN and log-transformed detrital C:N at later stages of decomposition (d. 77) were found for all litter types (Fig. 14, linear regression,

$R^2 = 0.63$, $p = 0.006$; $R^2 = 0.73$, $p = 0.002$; $R^2 = 0.75$, $p = 0.001$; maple and rhododendron leaves and wood veneers, respectively). Similarly, detrital C:P was controlled by SRP availability (Fig. 14, $R^2 = 0.73$, $p = 0.002$; $R^2 = 0.89$, $p = 5.0 \times 10^{-8}$; $R^2 = 0.88$, $p = 5.0 \times 10^{-5}$; maple and rhododendron leaves and wood veneers, respectively). In general, differences in initial substrate C:nutrient values (Table 2) were greatly reduced over time as substrates were colonized by microorganisms, especially if dissolved nutrient availability was high (Fig. 14). Increases in dissolved N:P ratios did not result in identical increases in N:P ratios of rhododendron leaves and wood veneers (which are driven mostly by fungal nutrient immobilization, see below), suggesting that fungi are not homeostatic with respect to their biomass N:P ratio (Fig. 15). Further, litter-associated fungal biomass accrued by d. 28 or 77 demonstrated very strong inverse relationships with detrital C:N ratios for all types of plant litter (Fig. 16, data for d. 77 are shown, linear regression, all litter types combined, $R^2 = 0.92$, $p = 9.0 \times 10^{-12}$). A similar statistically significant relationship has been found between fungal biomass and detrital C:P ratio for all plant litter types (Fig. 17, data for d. 77 are shown, linear regression, all litter types combined, $R^2 = 0.64$, $p = 1.0 \times 10^{-5}$).

Discussion

Early studies testing the effects of whole-stream nutrient addition on microbially-driven decomposition produced variable results (Elwood et al. 1981, Newbold et al. 1983), with only recent studies successfully demonstrating the effect of nutrients (Rosemond et al. 2002, Gulis and Suberkropp 2003b, Benstead et al. 2009). However, these studies tend to be logistically complicated. Correlative studies (e.g. Woodward et

al. 2012) suffer from the confounding effects of other pollutants found in the nutrient enriched streams. Lab microcosm studies, although showing the effect of nutrients on litter breakdown, cannot accurately portray the complexity of the stream environment. My manipulative study that used man-made streamside channels amended with various combinations of nutrients offered a balanced approach that provided both control and greater ecological realism.

Fungal biomass associated with decomposing plant litter in this study was similar to that from both nutrient addition experiments in laboratory microcosms (Sridhar and Bärlocher 2000) as well as in-stream data (Grattan and Suberkropp 2001, Gulis and Suberkropp 2003b). Both fungal parameters, biomass and production, peaked at 16:1 dissolved N:P ratio during early stages of litter breakdown, which is comparable to previously estimated average nutrient stoichiometry of fungal biomass (mean N:P \approx 10, Gulis et al. unpublished). Fungal parameters showed little variation among levels of nutrient concentrations (low to high) within same dissolved nutrient ratios. This suggests that even relatively low concentrations or small increases in the availability of a limiting nutrient may satisfy fungal demand and have strong consequences for fungal activity and, hence, litter decomposition. Indeed, Ferreira et al. (2006) demonstrated that fungal parameters and litter decomposition were stimulated by nitrate-N but saturated at concentrations as low as about 200 μ g/L in a stream with naturally high non-limiting levels of P availability. In this experiment, fungal biomass associated with maple leaf litter and fungal production associated with maple and rhododendron leaves, showed significant linear relationships with DIN (Fig. 5). An apparent lack of fungal response to DIN on wood veneers in our study could be due to one or a combination of the following

factors: lower carbon quality (higher lignin content), lower surface area to volume ratio that delays fungal colonization (Allen 2007) or severe P limitation in 128:1 N:P ratio treatments. SRP concentration showed no significant relationship with fungal parameters, which can be explained by recent studies that observed non-homeostasis of microbes with increasing SRP (Fanin et al. 2013, Gulis et al. unpublished), suggesting a possible storage of P by microorganisms. This was corroborated in my study where increases in dissolved N:P ratios affected fungal and by extension detrital stoichiometry, yet did not yield similar (1:1) increases in detrital N:P ratios, which would be the pattern in case of extreme non-homeostatic microbial plasticity (Fig. 15). In addition, non-homeostasis of fungal biomass with respect to P can explain why the relationship between fungal biomass accrual and detrital C:N (Fig. 16) is considerably stronger than the similar, but somewhat weaker, relationship between fungal biomass and detrital C:P (Fig. 17). These data (Fig. 16 and Fig. 17) demonstrate that fungi exert strong control over nutrient stoichiometry of decomposing plant litter.

Bacterial production associated with decaying plant litter also peaked at 16:1 dissolved N:P ratio, although maximum values were attained at the later stages of litter breakdown compared to fungal production. The increased bacterial production at more advanced stages of decomposition can be attributed to skeletonization of detritus that increases the surface area available for bacterial colonization (Gessner et al. 2007). On maple leaf litter, bacterial production showed a significant positive correlation with SRP, a relationship that has not been found for fungal production. The effect of SRP on bacterial production can be explained by higher bacterial P demand due to a lower bacterial biomass N:P ratio which stems from higher growth rates and, hence, higher

concentration of P-rich RNA (mostly in ribosomes) in bacterial cells (Elser et al. 2000; Elser et al. 2003).

Microbial respiration followed the pattern of fungal production and biomass, with peak respiration at 16:1 dissolved N:P ratio, which is in agreement with previous studies describing fungal dominance in the decomposition of submerged plant detritus in streams (Gulis et al. 2006, Gessner et al. 2007, Gulis et al. 2008). Respiration rates of plant litter showed significant positive correlation with DIN, but not SRP availability, a general pattern consistent with fungal, but not bacterial, parameters. All of the microbial parameters discussed above support my first hypothesis that microbial activity is maximized at a dissolved nutrient ratio that approaches that of microbial biomass.

Direct stimulation of microbial activity by dissolved nutrients in this study resulted in higher litter decomposition rates as reported earlier in other experiments (e.g. Gulis and Suberkopp 2003b, Ferreira et al. 2006). Variation in decomposition rates among litter types, even in the control treatment, can be attributed to disparities in initial nutrient content (or C:nutrient ratios) of the substrates, i.e. nutrient-rich low-lignin leaves decompose faster than low-quality high-lignin wood (Petersen and Cummins 1974, Gessner and Chauvet 1994). The magnitude of the increase in plant litter decomposition rates under nutrient enrichment vs. unamended condition is similar to that reported in previous studies (Gulis and Suberkropp 2003b, Benstead et al. 2009). The magnitude of the stimulation depended on litter type, i.e. decomposition rates of low quality substrates (wood veneers, rhododendron leaves) were enhanced by nutrients to a greater extent than that of higher quality litter (maple leaves). This can be attributed to a greater microbial

demand for external N and P on nutrient-poor litter types. Similar patterns have been reported by Stelzer et al. (2003), Gulis et al. (2004) and Ferreira et al. (2006).

Acceleration of litter breakdown can be continued once P is provided in addition to N enrichment (Kaushik and Hynes 1968, Tank and Webster 1998) due to possible co-limitation of microbial decomposers and a mismatch between the resource and biomass nutrient stoichiometry (Elser et al. 2007). This study demonstrated that when nutrients are added at a ratio that approaches microbial biomass stoichiometry (e.g. dissolved N:P ratio of 16:1), decomposition rates are maximized due to easing of stoichiometric constraints and alleviation of nutrient limitation of microbially-driven decomposition. Further, our results showed little variation in decomposition rates among levels of nutrient concentrations within the 16:1 N:P ratio, suggesting that high breakdown rates can be achieved even at relatively low dissolved nutrient availability. Thus, microbially-mediated rapid disappearance of plant litter in streams experiencing nutrient loading due to human activity can lead to drastic changes in the availability of plant detritus to higher trophic levels (e.g. shredding invertebrates) in these ecosystems (Benstead et al. 2009, Woodward et al. 2012).

The effect of dissolved inorganic nutrients on detrital stoichiometry was profound, with all litter types showing a significant decrease in C:nutrient ratios with increasing DIN or SRP concentrations (Fig. 14) at later stages of decomposition (d. 77) due to accumulation of microbial (mostly fungal) biomass. Further, the lower the initial substrate quality, or the higher the C:nutrient ratio at day 0, the greater was the decrease in C:nutrient ratio during decomposition. In other words, by d. 77, in treatments with high DIN or high SRP concentrations, C:N or C:P ratios of all types of plant litter converged

to values around 40:1 to 80:1 (C:N) and 1000:1 (C:P) (Fig. 14), despite large differences in initial nutrient ratios among plant litter types (Table 2). Thus, my second hypothesis was supported; microbial activity and fungal biomass accrual led to the convergence of detrital C:nutrient ratios by late stages of decomposition. This phenomenon can be explained once one considers the effect of fungal biomass accrual on detrital stoichiometry. Since fungi can obtain nutrients from both the plant litter they grow on and the water column (Suberkropp 1995, Gulis et al. 2006), it is apparent that these microbes have a dramatic effect on detrital stoichiometry by incorporating dissolved N and P into their biomass, which then contributes to the overall detrital stoichiometry. Principles of microbial metabolism suggest that acquisition of dissolved inorganic N and P may require less energy than mining of organic material to cleave phosphate or amino groups, which would involve production of a suite of extracellular enzymes. Thus, one can speculate that fungi will preferentially rely on dissolved inorganic nutrients which are available in a continuous supply in stream water. Consequently, nutrient supply, fungal nutrient immobilization and fungal biomass accrual are the major factors driving the changes from initial detrital stoichiometry (Cheever et al. 2013). The ability of aquatic fungi to control and stabilize detrital stoichiometry as plant litter decomposes may have important, yet poorly understood, consequences to stream detritivores, which rely on plant-associated microbial biomass rather than plant material alone as a major source of nutrients (Suberkropp 1992b, Chung and Suberkropp 2009).

Conclusions

This study demonstrated that nutrient enrichment, which is a widespread issue in freshwater ecosystems, can directly affect the activity of litter-associated microorganisms and subsequently, decomposition of plant litter. The effect of dissolved nutrients is maximized when they are supplied at a ratio that approaches stoichiometry of microbial (fungal) biomass, even if the concentrations are relatively low. Microbes, mostly fungi, play a key role in the breakdown of submerged plant litter and can accrue high levels of biomass relatively quickly when receiving co-limiting nitrogen and phosphorous at a preferred ratio. These fungi are capable of immobilizing N and P from the water column and, thus, by incorporating them into the biomass, they also control the bulk nutrient content and C:nutrient stoichiometry of detritus as decomposition progresses. These findings provide additional insights into the key importance of aquatic fungi in carbon and nutrient flow in stream ecosystems and some clues about the possible consequences of excessive nutrient loading or eutrophication to lotic ecosystems.

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Table 1. Target and actual concentrations and ratios of dissolved inorganic nitrogen and soluble reactive phosphorous in 10 experimental treatments. Actual concentrations and ratios are means, \pm SD, for the whole experimental period (n=7-10).

Target N:P ratio (molar)	Concentration level	Target DIN ($\mu\text{g L}^{-1}$)	Target SRP ($\mu\text{g L}^{-1}$)	Actual DIN ($\mu\text{g L}^{-1}$)	Actual SRP ($\mu\text{g L}^{-1}$)	Actual N:P ratio (molar)
Control	-	-	-	48.5 \pm 54.3	4.4 \pm 3.8	24.3 \pm 25.3
2:1	L	40.6	45.0	44.2 \pm 38.1	46.3 \pm 13.5	2.1 \pm 2.7
2:1	M	81.3	90.0	56.6 \pm 25.4	48.5 \pm 18.5	2.6 \pm 9.0
2:1	H	121.9	135.0	115.0 \pm 29.8	94.0 \pm 59.3	2.7 \pm 1.0
16:1	L	325.2	45.0	331.3 \pm 122.1	51.5 \pm 17.7	14.2 \pm 5.7
16:1	M	650.3	90.0	602.5 \pm 145.9	58.2 \pm 28.1	22.9 \pm 7.1
16:1	H	975.5	135.0	824.2 \pm 211.2	86.2 \pm 32.9	21.2 \pm 7.5
128:1	L	325.2	5.6	338.0 \pm 105.7	11.7 \pm 11.1	64.0 \pm 34.3
128:1	M	650.3	11.3	518.0 \pm 149.2	13.3 \pm 5.6	86.6 \pm 93.1
128:1	H	975.5	16.9	878.4 \pm 227.9	17.7 \pm 6.5	110.1 \pm 64.9

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

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



Figure 1. Thirty streamside channels fed with water pumped from the Shope Fork at Coweeta LTER site in NC used for decomposition experiments.

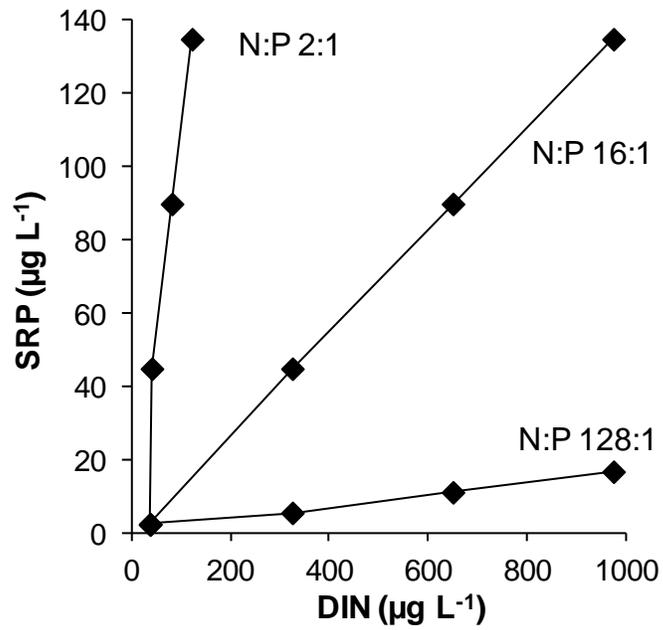


Figure 2. Target dissolved inorganic nitrogen (DIN) and soluble reactive phosphorus (SRP) concentrations and ratios used in this study. Diamonds represent experimental treatments differing in concentrations and ratios. See also Table 1 for details.

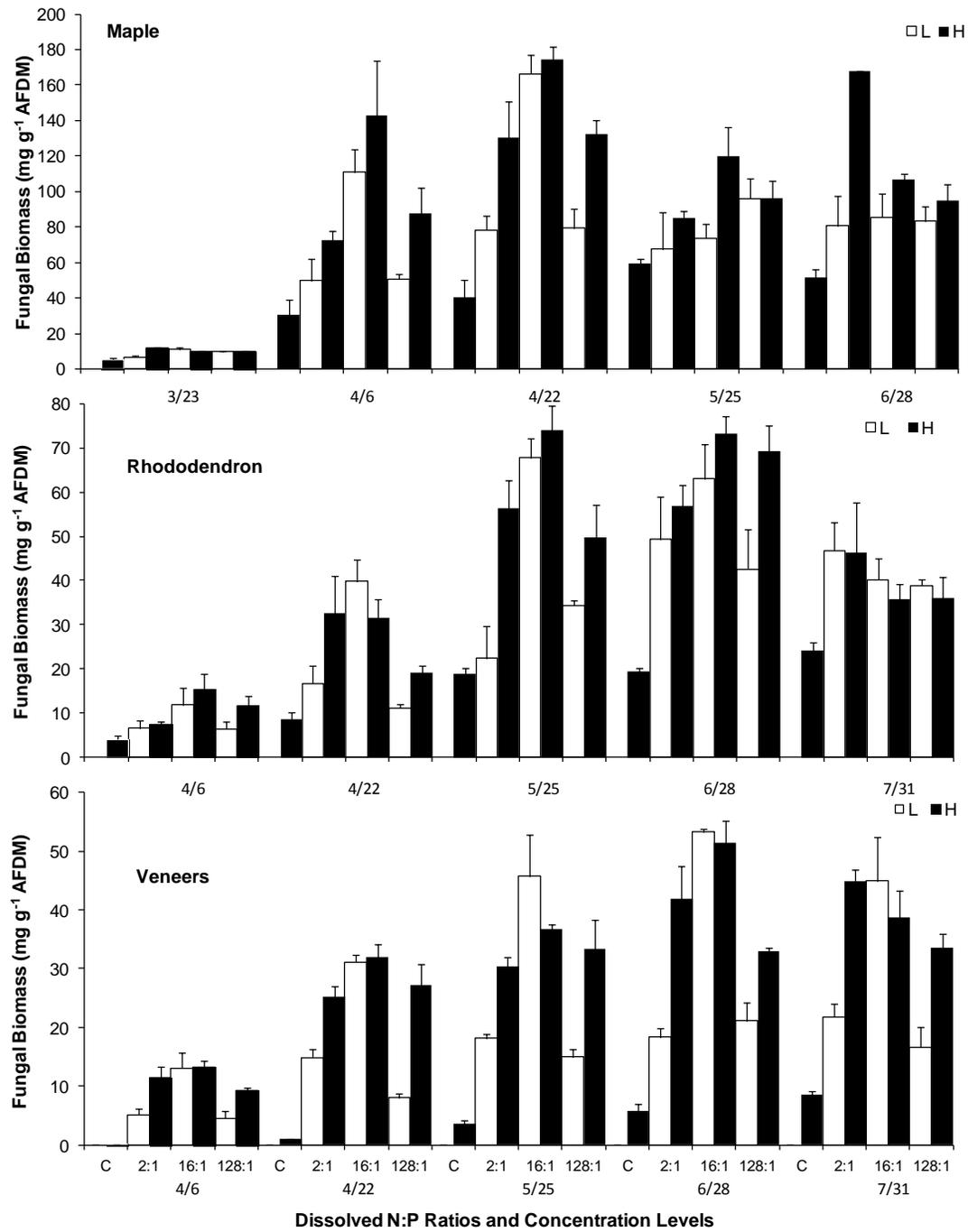


Figure 3. Fungal biomass accrual at low (L) and high (H) nutrient concentration levels for all nutrient ratios throughout the experiment. Error bars show +1 SE. C = unamended stream water (control).

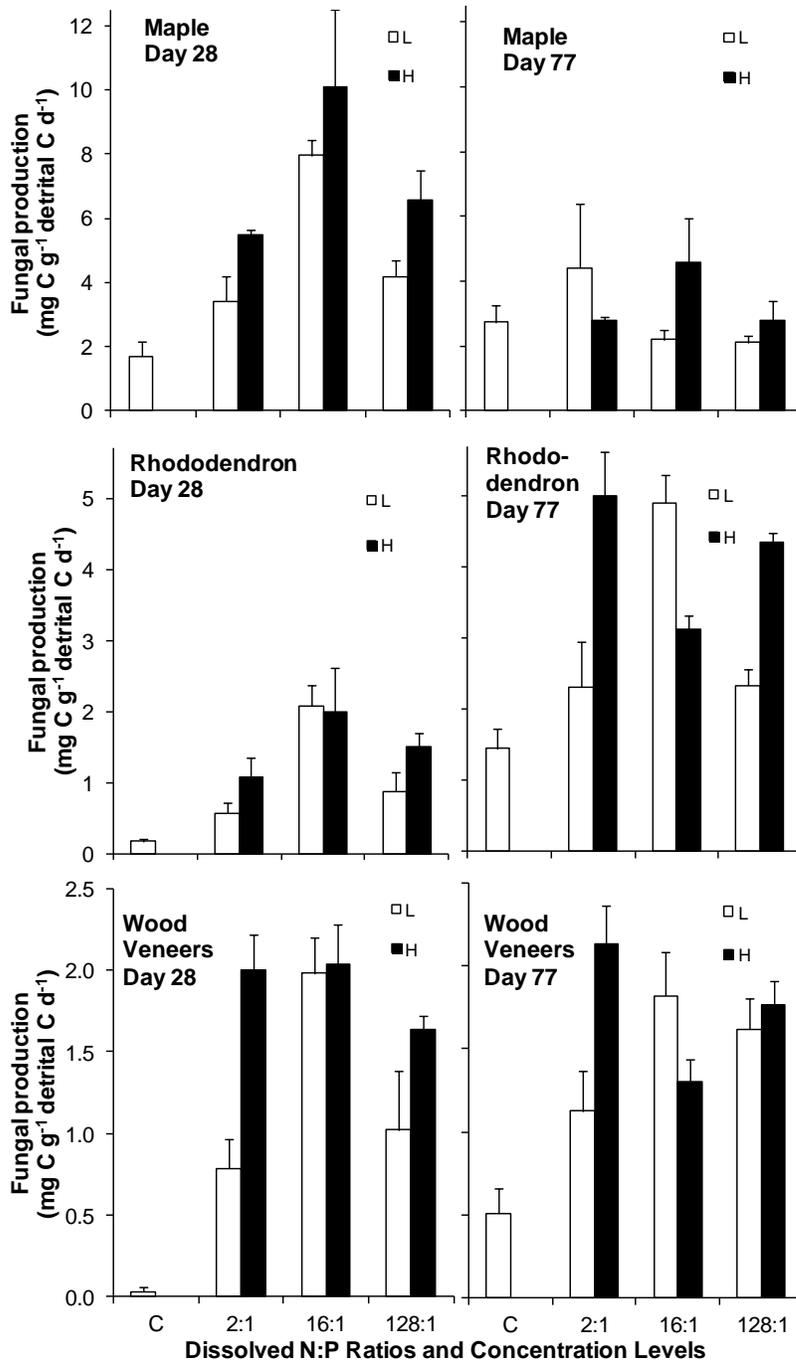


Figure 4. Fungal production for all three substrates on day 28 and 77 at low (L) and high (H) nutrient concentration levels for all nutrient ratios. Error bars show +1 SE. C = unamended stream water (control).

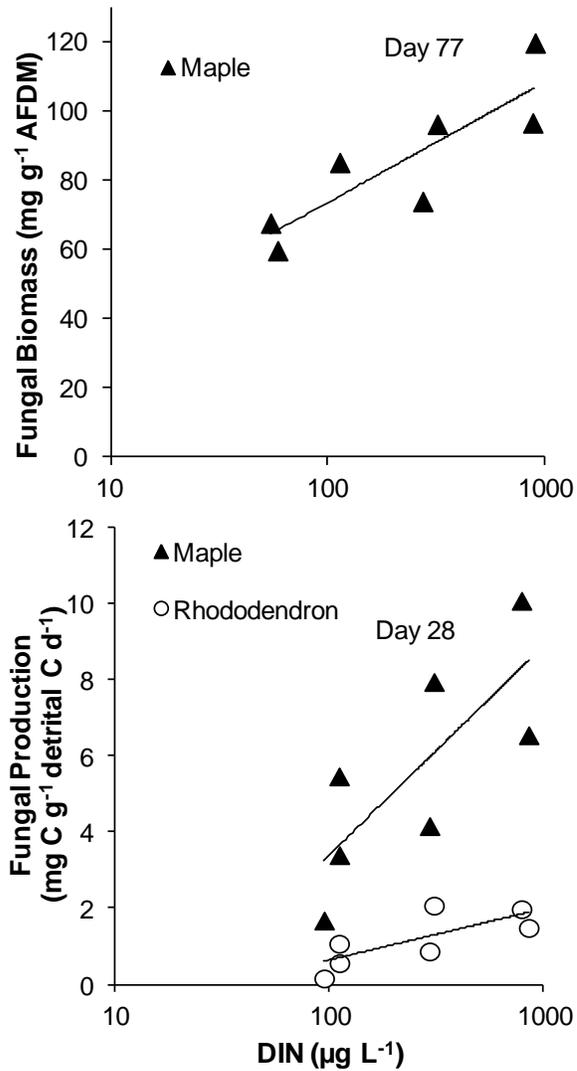


Figure 5. Relationship between fungal biomass associated with maple leaves and dissolved inorganic nitrogen concentration (upper panel) ($R^2 = 0.74$, $p = 0.013$, $n = 7$), and between fungal production and dissolved inorganic nitrogen concentration (lower panel) on maple ($R^2 = 0.60$, $p = 0.041$, $n = 7$) and rhododendron ($R^2 = 0.58$, $p = 0.048$, $n = 7$) leaf litter.

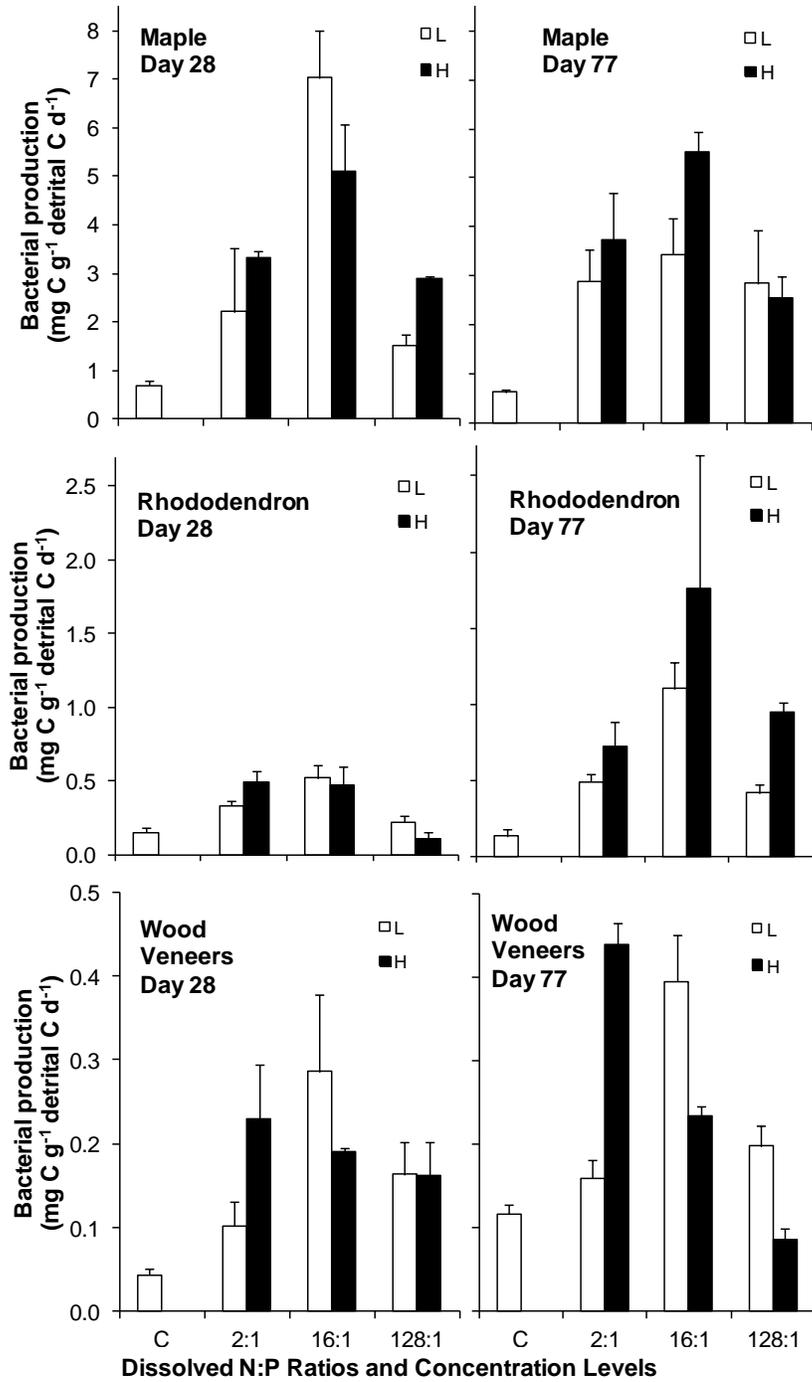


Figure 6. Bacterial production for all three substrates on day 28 and 77 at low (L) and high (H) nutrient concentration levels for all nutrient ratios. Error bars show +1 SE. C = unamended stream water (control).

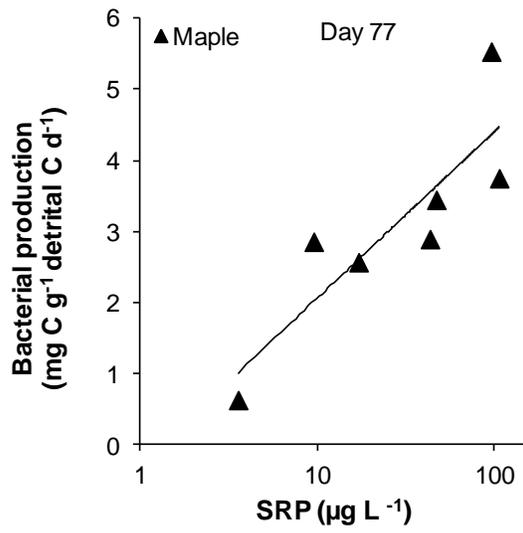


Figure 7. Changes in bacterial production associated with maple leaf litter with increasing soluble reactive phosphorus concentration ($R^2 = 0.75$, $p = 0.011$, $n = 7$).

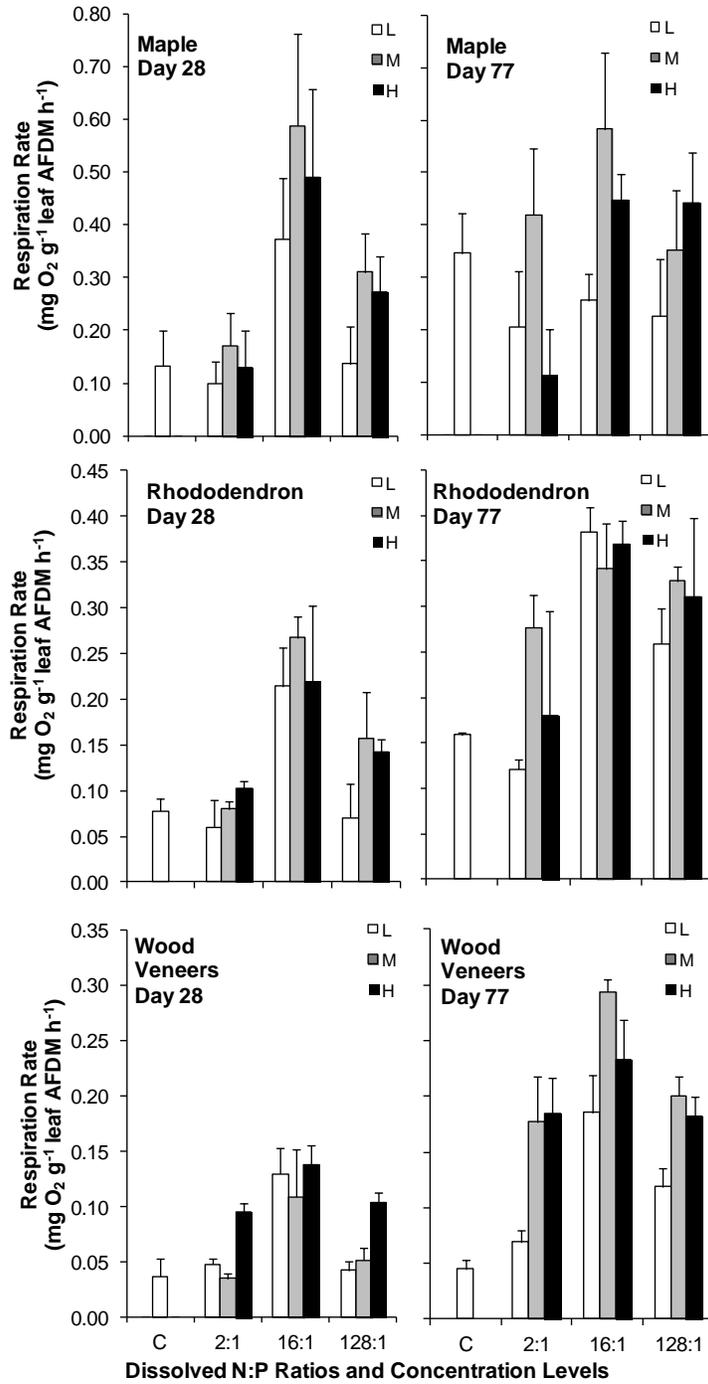


Figure 8. Microbial respiration rates associated with all three substrates on day 28 and 77 at all ten nutrient ratios and concentration levels. Error bars show +1 SE. L = low, M = medium, H = high nutrient levels and C = unamended stream water (control).

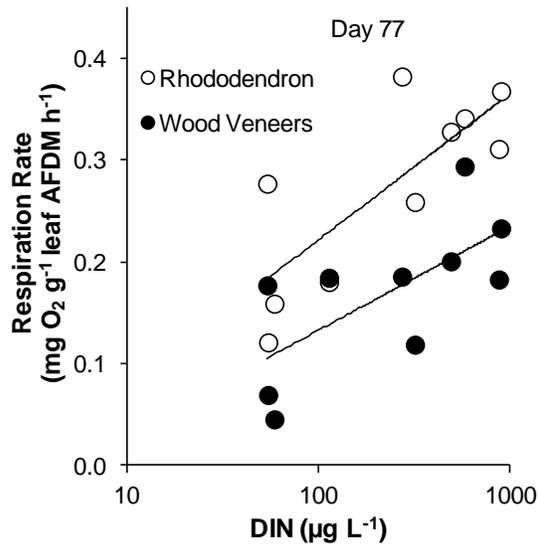


Figure 9. Changes in microbial respiration rates associated with decaying plant litter with increasing dissolved inorganic nitrogen concentrations ($R^2= 0.61$, $p= 0.008$, $n= 10$ and $R^2= 0.47$, $p= 0.029$, $n= 10$ for rhododendron and wood veneers, respectively).

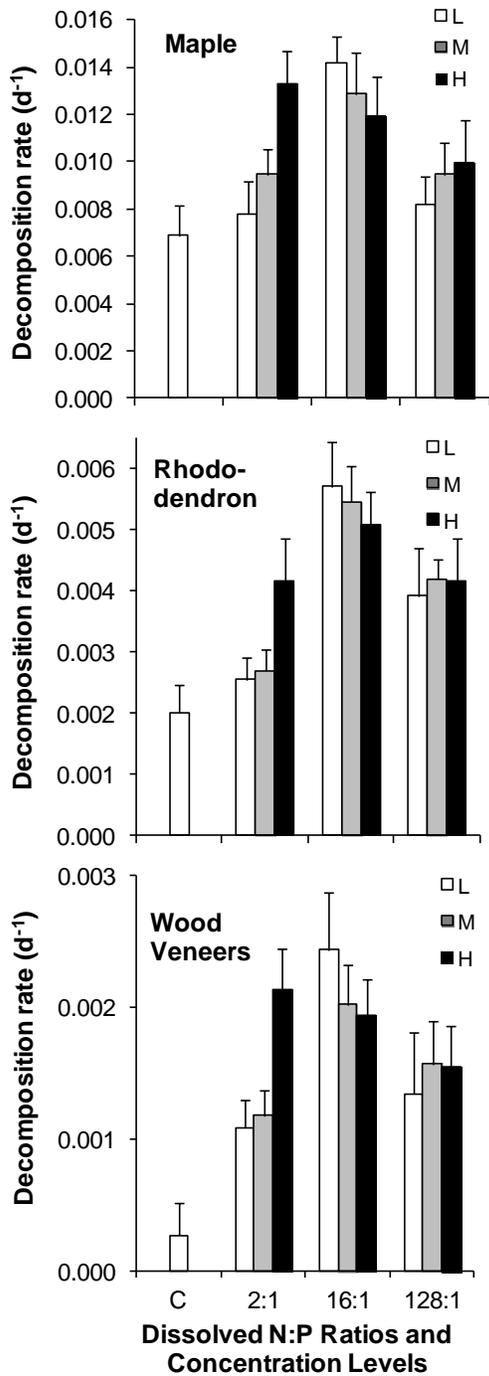


Figure 10. Decomposition rates of maple and rhododendron leaves and wood veneers at ten nutrient ratios and concentration levels. Error bars show +95% confidence intervals. L = low, M = medium, H = high nutrient levels and C = unamended stream water (control).

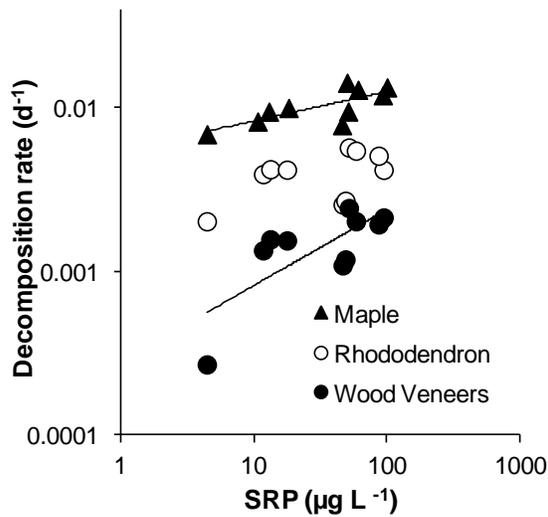
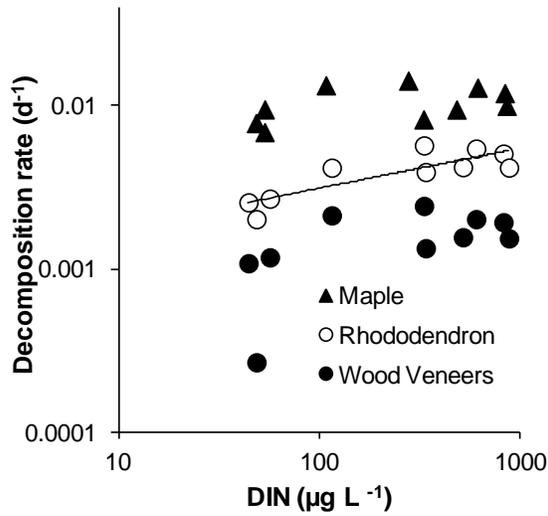


Figure 11. Relationship between litter decomposition rates and dissolved inorganic nitrogen concentrations (upper panel) ($R^2= 0.71$, $p= 0.002$, $n= 10$ for rhododendron leaves) and between litter decomposition rates and soluble reactive phosphorus concentrations (lower panel) ($R^2= 0.57$, $p= 0.011$, $n= 10$ and $R^2= 0.56$, $p= 0.013$, $n= 10$ for maple leaves and wood veneers, respectively). Regression lines are not shown for non-significant relationships.

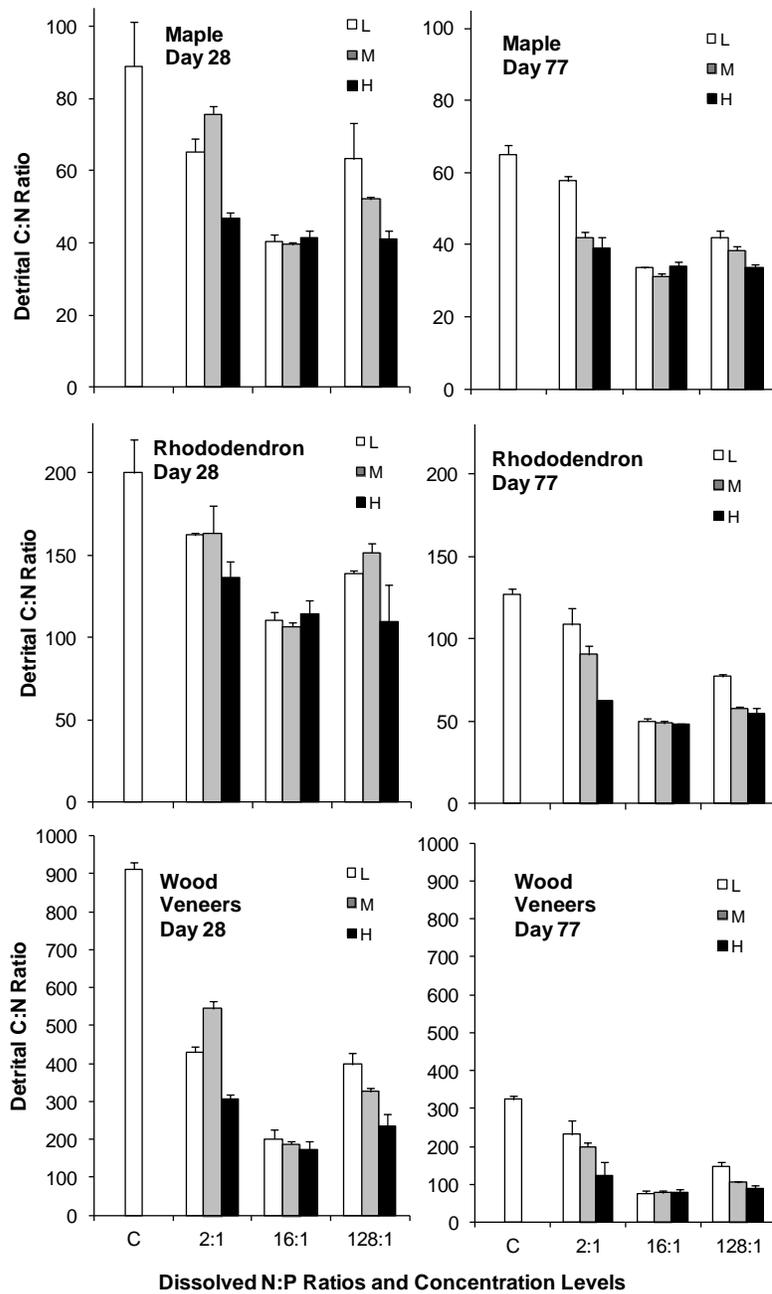


Figure 12. Detrital C:N ratios (molar) for three substrates on day 28 and 77 at all ten nutrient ratios and concentration levels. Error bars show +1 SE. L = low, M = medium, H = high nutrient levels and C = unamended stream water (control).

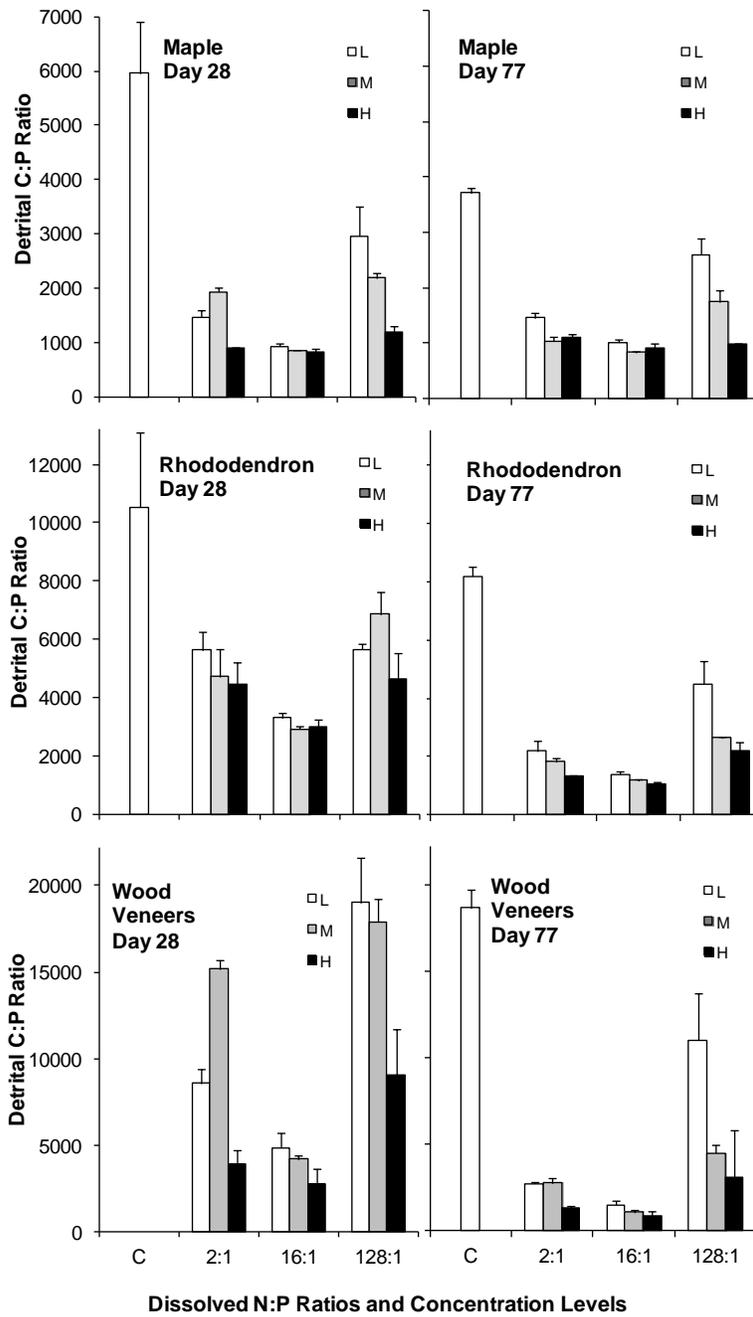


Figure 13. Detrital C:P ratios (molar) for three substrates on day 28 and 77 at all ten nutrient ratios and concentration levels. Error bars show +1 SE. L = low, M = medium, H = high nutrient levels and C = unamended stream water (control).

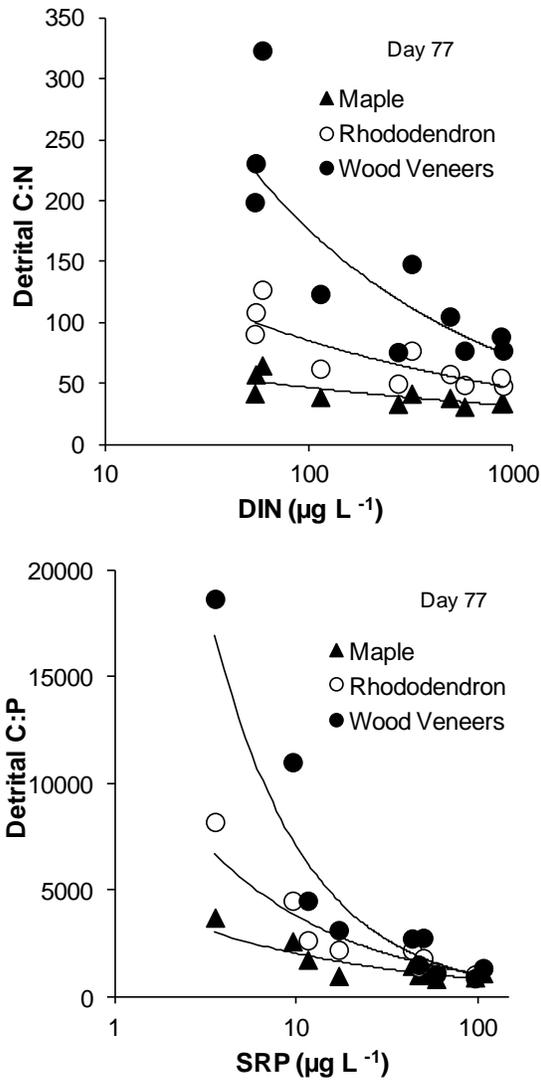


Figure 14. Relationship between detrital C:N ratios and dissolved inorganic nitrogen concentrations (upper panel) (linear regression of \log_{10} -transformed parameters, $R^2=0.63$, $p=0.006$, $n=10$, $R^2=0.73$, $p=0.002$, $n=10$ and $R^2=0.75$, $p=0.001$, $n=10$ for maple and rhododendron leaves and wood veneers, respectively) and between detrital C:P ratios and soluble reactive phosphorus concentrations (lower panel) (linear regression of \log_{10} -transformed parameters, $R^2=0.73$, $p=0.002$, $n=10$, $R^2=0.89$, $p=5.0 \times 10^{-8}$, $n=10$ and $R^2=0.88$, $p=5.0 \times 10^{-5}$, $n=10$ for maple and rhododendron leaves and wood veneers, respectively).

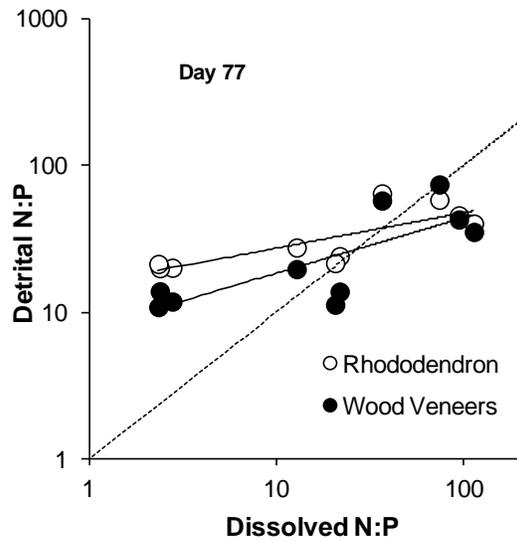


Figure 15. Changes in detrital N:P ratios with increasing dissolved N:P ratios for rhododendron leaves ($R^2 = 0.63$, $p = 0.006$, $n = 10$) and wood veneers ($R^2 = 0.61$, $p = 0.008$, $n = 10$). 1:1 line is also shown (dashed).

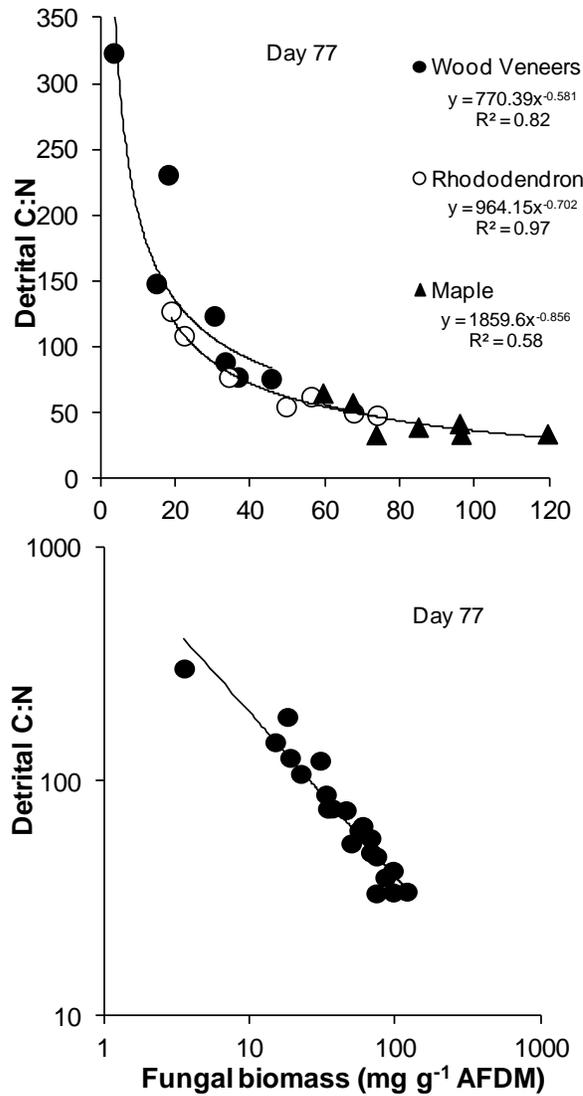


Figure 16. Relationship between litter-associated fungal biomass and detrital C:N ratios on day 77 for three substrate types separately (upper panel) and all substrates combined (lower panel, linear regression $R^2 = 0.91$, $p = 9.0 \times 10^{-12}$, $n = 21$).

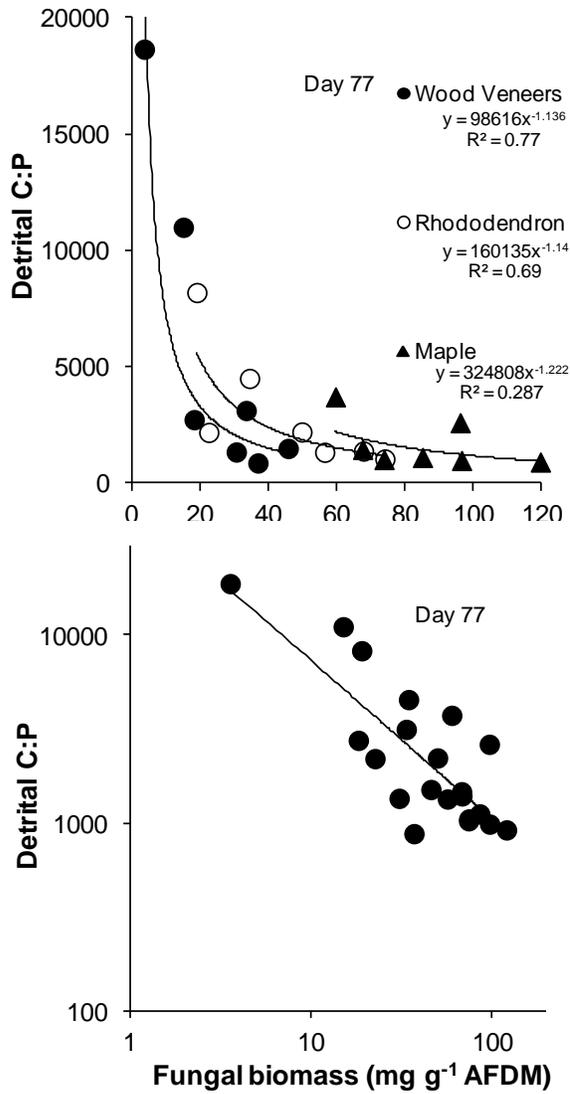


Figure 17. Relationship between litter-associated fungal biomass and detrital C:P ratios on day 77 for three substrate types separately (upper panel) and all substrates combined (lower panel, linear regression $R^2 = 0.64$, $p = 1.0 \times 10^{-5}$, $n = 21$).