

Microbial and metazoan effects on nutrient dynamics during leaf decomposition in
streams

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ABSTRACT

I investigated the drivers of nutrient cycling by heterotrophic microbes during leaf decomposition in streams. My research addressed two overarching questions: 1) how do exogenous and endogenous factors interact to drive microbial nitrogen (N) cycling during organic matter decomposition in stream ecosystems, and 2) what affect will the global increase in biologically active N have on these factors and resulting fluxes? I conducted studies in natural streams and laboratory mesocosms to address these questions and used general stoichiometric theory to conceptualize diverse microbial assemblages as a single functional unit within stream ecosystems.

First, I described spatial and temporal patterns of N and phosphorus uptake and mineralization by leaf-associated microbial assemblages in five southern Appalachian streams which spanned a gradient of nitrate availability. I found wide variations in nutrient fluxes across time and space, perhaps due to macroinvertebrate-induced changes in microbial assemblage composition. Secondly, I explored the roles of endogenous and exogenous N in meeting microbial requirements. I isolated microbial biomass from leaves that had been labeled with ^{15}N and incubated in the same five Appalachian streams. The importance of exogenous N increased as decomposition progressed and was particularly important in streams with high N availability. Finally, I tested potential interactions between two exogenous drivers of microbial nutrient cycling: N availability and animal activity. I used mesocosms to test the effects of consumer nutrient recycling (CNR) and grazing by two shredders on microbial uptake under different N regimes. Animals only influenced microbial uptake under low N conditions. Shredder CNR generally stimulated uptake while grazing had a negative effect.

My research provides a robust model describing N cycling by detritus-associated microbes over the course of decomposition. According to this model, microbes assimilate endogenous N during the initial stages of decomposition and immobilization of

exogenous N becomes more important as decomposition progresses. The labeled substrate technique that I used to generate this model is an elegant way of testing the applicability of this model in other ecosystems. My results also suggest that anthropogenic activities that increase exogenous N availability have implications for N and C cycling in lotic systems.

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Attributions

Chapter 2: Immobilization and mineralization of N and P by heterotrophic microbes during leaf decomposition

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The published article included two additional authors:

Erika B. Kratzer: contributed to the conceptual design of the study and assisted with the field work.

Jackson R. Webster: contributed to the conceptual design of the study, participated in sample collection and analysis in an advisory capacity, and contributed to the development of the manuscript.

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Chapter 1: General Introduction

Nitrogen (N) is one of the fundamental building blocks of living biomass. It is an important component of all proteins and many major macromolecules, including DNA and RNA. Therefore the processes that dictate the amount of N that is biologically active in an ecosystem can limit the distribution and biomass of organisms and may influence ecosystem function. The majority of N on the planet is found in the atmosphere as N_2 gas, which is not available to most organisms. N is made biologically available via microbial fixation, and organisms that transform N from inorganic to organic forms make N available to higher trophic levels. Organisms take up inorganic forms of N, such as NO_3^- and NH_4^+ , from their environment to support growth and reproduction. N is returned to inorganic pools by the mineralization of these and other organisms within the ecosystem.

Organisms couple N with other biogeochemical cycles by simultaneously obtaining N and other nutrients, such as C, in proportions dictated by their biomass stoichiometry and metabolic requirements. According to general stoichiometric theory, the relative rates of nutrient uptake and mineralization by organisms are dictated by the biochemical stoichiometry of their biomass (Sterner and Elser 2002). Although often applied to single organisms or taxa, this theory can be useful for predicting nutrient transformations in ecosystems if all of the uptake organisms in the system of interest are considered as a single functional unit with a uniform nutrient requirement.

Microorganisms dominate the cycling of nutrients in many ecosystems and are important drivers of N transformations in ecosystems with no vascular plants (such as the open ocean) and ecosystems supported by allochthonous carbon. Temperate, forested, headwater streams experience episodic inputs of allochthonous C in the form of leaf litter during autumn. The microbes that colonize litter drive in-stream N and organic matter decomposition.

Nutrient cycling in streams is conceptualized by the nutrient spiraling concept (Webster and Patten 1979) where nutrients travel a certain distance downstream in dissolved form before being taken up by microbes. Nutrients then travel downstream in organic or particulate form before being mineralized back into the water column. Spiraling metrics and nutrient dynamics are often measured at the reach or whole stream

scale (Newbold et al. 1981, Peterson et al. 2001, Mulholland et al. 2008). The process of organic matter decomposition and the factors that drive decomposition rates have also been well described in streams (Kaushik and Hynes 1971, Petersen and Cummins 1974, Webster and Benfield 1986). Organic matter decomposition is often assessed by measuring mass loss from single leaf packs (Benfield 2006). Although recent simulations have coupled N and C cycling during decomposition in terrestrial ecosystems (Manzoni et al. 2008, Manzoni et al. 2010), robust descriptions of N cycling by microbes during organic matter decomposition based on empirical data are rare in lotic systems.

Understanding the role of biota in stream nutrient dynamics is increasingly important as anthropogenic activity continues to alter N storage and transformations in ecosystems worldwide. Anthropogenic fixation of nitrogen (N) has increased the amount of N available for biological uptake (Galloway et al. 2003) and much of this excess N is transported and processed in stream networks. It is unclear how the global increase in N availability may influence microbial uptake and mineralization in streams or the potential for cascading effects on the rates of organic matter decomposition and CO₂ production.

I investigated drivers of N cycling by heterotrophic microbes over the course of organic matter decomposition in streams by addressing the following questions: 1) how do exogenous and endogenous factors interact to influence nutrient uptake and mineralization by heterotrophic microbes, and 2) how do these fluxes respond to greater availability of biologically reactive N. I addressed these questions using leaf breakdown studies in 5 southern Appalachian streams spanning a gradient of N availability and a manipulative laboratory experiment in recirculating stream mesocosms.

In Chapter 2, I quantified uptake and mineralization fluxes by leaf-associated heterotrophic microbes using an adaptation of a method developed for autotrophic biofilms (O'Brien and Dodds 2008). This research contributes to the understanding of mineralization by stream biota. Although the spiraling concept describes the transport of both dissolved and particulate nutrients, most spiraling studies have focused on quantifying biotic uptake from the water column and mineralization is rarely measured. I described temporal patterns of both fluxes for both N and phosphorus. I also generated a conceptual model that organizes the potential drivers of nutrient cycling by heterotrophic microbial assemblages. In Chapter 3, I investigated the relative importance of substrate

and water-derived N in supporting microbial growth and explore the implications for organic matter decomposition rate using an isotopic tracer. Finally, in Chapter 4, I used laboratory mesocosms to investigate the influence of two exogenous drivers of nutrient cycling: N availability and animal activity. I used the results of this research to add N dynamics to existing models of organic matter decomposition in lotic systems.

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Chapter 2: Immobilization and mineralization of N and P by heterotrophic microbes during leaf decomposition

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Abstract

The rate and stoichiometry of microbial mineralization depend, in part, on nutrient availability. For microbes associated with leaves in streams, nutrients are available from both the water column and the leaf. Therefore, microbial nutrient cycling may change with nutrient availability and during leaf decomposition. I explored spatial and temporal patterns of mineralization by heterotrophic microbes by placing packs of red maple leaves at sites in 5 Appalachian streams spanning a range of N and P availability. Leaf packs were collected 4 times from each site. Leaf disks from these packs were incubated in microcosms, and uptake rates and steady-state concentrations of NH_4^+ and soluble reactive P (SRP) were used to calculate mineralization rates. N uptake peaked between 50 and 60 d, whereas P uptake peaked ~10 d later. I did not observe clear patterns in fungal biomass-specific uptake or mineralization fluxes of either nutrient over time or space, but the microbes grown in the site with the lowest nutrient availability had the highest fungal biomass-specific cycling. These results suggest that the ability of microbes to access nutrients from their substrate may prevent dissolved nutrient availability from being a strong driver of microbial nutrient cycling.

Introduction

Understanding the flow of nutrients, such as N and P, through ecosystems is increasingly important because anthropogenic activities are altering these cycles at a global scale (Vitousek et al. 1997). Organisms have certain nutrient demands or requirements based on the composition of their biomass, and these demands must be satisfied by assimilating nutrients from available resources. Therefore, nutrient flow at the organismal level is driven by the availability of the nutrient relative to the organism's demand for that

nutrient (Sternner and Elser 2002). Organisms will either retain or release nutrients depending on the flexibility of their demand for nutrients and the relative availability of nutrients in their environment. Organisms retain nutrients in situations of high demand or low availability and release nutrients in situations where demand is low or nutrients are readily available (Vanni et al. 2002, Evans-White and Lamberti 2006). Nutrient demand and availability may be important drivers of nutrient cycling at the ecosystem level as well.

In ecosystems, organisms that are capable of incorporating inorganic nutrients into their biomass drive nutrient cycling by transforming nutrients from inorganic to organic forms, and making them available to higher trophic levels. The identity and diversity of these uptake organisms vary widely across ecosystems. However, all of these organisms present in a given ecosystem could be conceptualized as a single functional unit. In this case, immobilization and mineralization fluxes in the ecosystem should depend on the nutritional demand of this unit, which would be determined by the nutritional composition of its biomass (excluding recalcitrant or inactive portions) relative to nutrient availability. In this scenario, the uptake functional unit is analogous to an organism for which nutrient cycling is driven by its nutritional demand.

Redfield (1958) used this approach to explain the similarity between the nutrient composition of plankton biomass and dissolved nutrient concentrations in the open ocean. He hypothesized that dissolved nutrient concentrations in the oceans were biologically regulated through plankton nutrient cycling. The relationship between the nutritional composition of uptake organisms and their resources has been explored most completely in planktonic ecosystems (Hecky et al. 1993, Elser et al. 1995, Elser et al. 2000, Sternner et al. 2008). Much of the C in these ecosystems is fixed by photosynthetic algae that sequester limiting and non-limiting nutrients (Sternner and Elser 2002). However, nutrient cycling in ecosystems fed by detrital C sources is driven by heterotrophic microbes. These organisms are limited in their ability to store non-limiting nutrients (Sternner and Elser 2002) and, therefore, are much more static in nutrient composition and demand (Persson et al. 2010).

Temperate forested headwater streams are classic examples of ecosystems dominated by heterotrophic processes, particularly in autumn when a large input of C

enters the streams with leaf fall. These leaves are rapidly colonized and conditioned by aquatic fungi and bacteria (Cummins 1974). Microbial nutrient demand peaks during this time of high C availability. Microbes may satisfy part of this demand by removing nutrients from the water column (Kaushik and Hynes 1971), and stream nutrient concentration often decreases during leaf fall in response to high microbial demand (Mulholland 2004).

Leaf-associated microbes should shift their nutrient demand over time coinciding with leaf breakdown and changes in relative availability of C and N. Microbial nutrient demand should be greatest during the initial stages of decomposition while microbes are actively growing and need nutrients to sustain that growth. As decomposition progresses, demand for nutrients should peak and then decline as microbes become established, grow, and eventually senesce. Nutrient immobilization and mineralization should respond to these changes in demand, resulting in a shift from net uptake of N and P to net mineralization during decomposition. This shift in function has been demonstrated during autotrophic biofilm development (Teissier et al. 2007) and has been shown in models of leaf decomposition (Webster et al. 2009).

A similar shift from net uptake to net mineralization should occur across a gradient of nutrient availability. Net uptake should occur under when nutrient availability is low because microbes should retain potentially limiting nutrients. Mineralization should be greater when nutrients are more available and exceed microbial demand. My objective was to observe patterns in nutrient uptake and mineralization by leaf-associated microbes during decomposition in sites spanning a gradient of N and P availability. I then used these observations to assess the usefulness of an organism-based conceptual model for explaining nutrient cycling in heterotrophic streams.

Methods

Study sites

The 5 study streams are in the Appalachian region of Virginia and North Carolina (USA). All are small, shaded, 1st- or 2nd-order streams with riparian vegetation dominated by deciduous hardwoods. Hugh White Creek (HW), Stonecrop Creek (SC), and Little Stony Creek (LS) are on public or private forested land, whereas sites at Little Back

Creek (LB) and Smith Creek (SM) are along roadsides and are forested only on one bank. HW drains a reference watershed at Coweeta Hydrologic Laboratory.

The 5 streams span a gradient of N and P concentration (Table 1) that ranges from below detection to 896 $\mu\text{g NO}_3^-/\text{L}$ and 8.4 μg soluble reactive P (SRP)/L. The molar ratio of total inorganic N (DIN = $\text{NH}_4^+ + \text{NO}_3^-$ concentration) to SRP ranges from ~2.5 to ~600.

Deployment and collection of leaf packs

I collected red maple (*Acer rubrum*) leaves from one tree shortly after abscission and dried them at room temperature to constant mass. I placed 10 g of leaves into mesh packs (1.5-cm mesh), which I anchored to the beds of the study sites in mid-December 2008. I collected 8 packs from each site 4, 6, 8, and 10 wk after deployment by removing them from the water column and placing each pack in a separate Zip-Lock[®] bag of stream water. I transported packs to the laboratory on ice and stored them at 4°C until analysis (≤ 48 h later). I filtered (Whatman GF/F) water samples from the sites on each collection date (except the first) and analyzed them for NH_4^+ (phenate method), NO_3^- (Cd-reduction method), and SRP (ascorbic acid method) concentrations with a Lachat Quickchem flow-injection analyzer (Lachat Instruments, Loveland, Colorado; APHA 1999). I also collected 8 L of filtered stream water (Whatman GF/F) from each site on each date for use in laboratory microcosms to measure uptake and mineralization. I stored this water at 4°C.

Laboratory analysis

I modified a method proposed by O'Brien and Dodds (2008) to measure nutrient cycling by heterotrophic microbes associated with leaves. I cut leaf packs open ≤ 48 h after each collection and placed the contents in pans of stream water to keep them moist. I used a cork borer to cut disks (2 cm diameter) from leaves but avoided stems and skeletonized areas. I reserved 30 disks from each site for determination of leaf breakdown rate and fungal biomass.

Breakdown rate.— I compared disks cut from leaves before deployment and disks cut from leaves retrieved on each collection date to calculate breakdown rate (/d) of red

maple leaves at each site. I dried (45°C for ≥ 24 h) 3 replicates of 5 disks from each site on each collection date, weighed them, combusted them (550°C for 2 h), and reweighed them to obtain ash-free dry mass (AFDM). I calculated breakdown rate as the slope of the line describing $\ln(\% \text{ mass remaining})$ over time in each site. I intentionally selected intact disks for this analysis, so these breakdown rates represent only mass loss caused by chemical and microbial processes. I calculated the half life (d) of red maple in each site from these rates. I used the fraction of half life (number of days in stream/half life) for comparisons across sites.

Fungal biomass.—I froze 3 replicates of 5 disks from each site and collection date in 5 mL of methanol. I extracted ergosterol from these samples with a liquid-phase extraction method, quantified it with high-performance liquid chromatography, and converted values to fungal biomass (Gulis and Suberkropp 2006).

Nutrient uptake.—I used the remaining disks from each site to measure nutrient uptake. I used 2 sets of 50-mL tubes for each site on each collection date. Both sets consisted of 27 tubes each with 35-mL of filtered stream water and 6 leaf disks collected from the same site. I set aside 3 tubes in each set as no-spike controls and did not add nutrients to them. I filled 8 additional tubes per set with filtered stream water only to serve as container controls. Approximately 12 h after filling the tubes, I spiked them with nutrients (N or P). I added 1 mL of 1000 $\mu\text{g/L}$ $\text{NH}_4\text{-N}$ stock solution to each tube (for a total addition of 28.5 $\mu\text{g/L}$ of $\text{NH}_4\text{-N}$ /tube) in the 1st set (except the no-spike controls) and 1 mL of 1000 $\mu\text{g/L}$ $\text{PO}_4\text{-P}$ stock solution to each tube (for a total addition of 28.5 $\mu\text{g/L}$ $\text{PO}_4\text{-P}$) in the 2nd set (except the no-spike controls). I incubated tubes in an environmental chamber on shaker tables (1000 rpm) at 15°C. I destructively sampled 3 tubes containing leaf disks and 1 container-control tube 0, 15, 30, 45, 75, 120, 180, and 240 min after spiking for each nutrient set. I sampled the no-spike controls after the 240-min time step. At each time step, I removed leaf disks from the tubes with hemostats and placed them in labeled Al pans. Then I capped the tubes and froze them until analysis. I measured NH_4^+ or SRP concentrations in tubes as described above (APHA 1999), and I dried and combusted leaf disks to estimate AFDM as described above.

Uptake and mineralization assays

I calculated uptake rate (k_t , /min) as the slope of the line describing the $\ln(\text{nutrient concentration}/\text{AFDM of the leaf disks})$ over time. I calculated uptake flux (U ; $\mu\text{g nutrient min}^{-1} \text{g}^{-1} \text{AFDM}$) from uptake rate and the average ambient nutrient concentration in the site from which the leaves were collected (C_{amb} ; $\mu\text{g/L}$) as:

$$U = k_t C_{amb} V / L$$

where V is the volume of water in each tube (L) and L is the average leaf mass in the tubes (g AFDM). After 4 h, nutrient concentration ($\mu\text{g/L}$) approached a steady-state concentration (C' ; $\mu\text{g/L}$), where uptake equaled mineralization. Therefore, I calculated mineralization flux (M ; $\mu\text{g nutrient min}^{-1} \text{g}^{-1} \text{AFDM}$) as:

$$M = k_t C' V / L$$

(O'Brien and Dodds 2008). I used the average nutrient concentration of the 3 no-spike control tubes as the steady-state concentration.

To compare across sites and time, I calculated fungal biomass-specific (FBS) uptake and mineralization. I divided uptake and mineralization fluxes by the total fungal biomass in each tube, which I estimated by scaling the estimates of fungal biomass (mg fungal biomass/g AFDM leaf) from ergosterol extractions to the AFDM of leaf disks in the tubes.

Statistical analysis

I compared fungal biomass and ambient nutrient concentration among sites with repeated measures analysis of variance (rm ANOVA). When ANOVAs were significant I used Tukey post hoc tests to identify those means that were different. I $\ln(x)$ -transformed ratios to meet assumptions of normality. I used regression analysis to assess the relationships between breakdown rate for red maple and fungal biomass and to assess changes in N and P dynamics over time and space. All regressions were linear unless stated otherwise in the text. I selected nonlinear models by comparing the Akaike information criterion (AICc) among several candidate models (SAS version 9.2; SAS Institute, Cary, North Carolina). I used SigmaPlot with SigmaStat Integration version 10; Systat Software Inc, Chicago, Illinois) for all other statistical tests.

Results

Ambient N and P concentrations varied across sites and over time (Table 1). Ambient NH_4^+ concentration was low in all sites, whereas NO_3^- concentrations varied 1000-fold across the gradient. NO_3^- concentrations decreased slightly over time in HW, LS, and LB, but not in SC and SM, which had the highest NO_3^- concentrations (rm ANOVA, $p < 0.001$). SRP was below detection in all sites on the 1st sampling date and remained low in most sites. SRP concentrations were higher in LB and SC than in the other sites in the later stages of the study (rm ANOVA, $p < 0.001$). DIN:SRP also varied considerably among sites and was higher in SM than in the other sites (rm ANOVA, $p < 0.001$).

Fungal biomass on red maple leaves generally increased over time in all 5 sites. Leaves accumulated more fungal biomass over 10 wk in SM than in HW and LS (Fig. 1; rm ANOVA with Tukey post hoc test, $p = 0.010$). Fungal biomass increased linearly with site NO_3^- concentration and site SRP concentration but not site NH_4^+ concentration (Table 2). Red maple decomposed at different rates among sites (Table 3). Red maple had the slowest breakdown rate in HW and the fastest rate in SM. Breakdown rates were $>2\times$ faster in SM than in HW. Leaves in HW, LB, LS, and SC had completed ~40 to 60% of their half lives by the final collection date, whereas leaves in SM had completed $>80\%$ of their half life by that date (Table 3). Differences in breakdown rates were positively related to microbial activity. Total fungal biomass accumulated over 10 weeks explained $>70\%$ of the variation in breakdown rates, but this relationship was only marginally significant (Fig. 2).

Nutrient dynamics

Nitrogen cycling varied spatially and temporally (Fig. 3A-H). N uptake rate in microcosms varied widely among sites (Fig. 3A). Peak N uptake rate of leaves collected from HW was $>2\times$ that of leaves collected from LB and SC. Peak N uptake rate of leaves collected from LS and SM were intermediate. When all sites were analyzed together, a significant peak in N uptake rate occurred between 50 and 60 d in stream (cubic polynomial regression, $r^2 = 0.42$, $p = 0.049$). This relationship disappeared when N uptake rate values were standardized for stage of decomposition (Fig. 3E). Peak N uptake rate occurred after ~30% of the half life in 2 low-nutrient sites (HW and LS) but occurred

later in SC and LB (~40 and 48% of the half life, respectively).

Steady-state NH_4^+ concentrations in the container-control tubes generally were greater than ambient concentrations, although not always. Ambient and steady-state NH_4^+ concentrations differed by up to 13 $\mu\text{g/L}$ (Table 4). SRP steady-state concentrations were also generally greater than ambient SRP concentrations, which were often below detection (Table 4).

Temporal patterns of FBS N uptake differed among sites. FBS N uptake peaked after <30 d in SM and after >50 d in LS (Fig. 3B). FBS N mineralization in LS peaked after 50 d and then declined, whereas FBS N mineralization at the other sites stayed relatively constant and then declined by the last collection date (Fig. 3C). Fungal biomass was lowest in LS, and FBS N uptake and mineralization were ~3 to 4 \times greater in LS than at the other sites. Standardization for stage of decomposition did not appear to influence the overall pattern of FBS N uptake or mineralization (Fig. 3F, G). Leaves were further along in decomposition in SM than in LS and HW, but FBS N uptake and mineralization remained greatest in LS and HW.

I was unable to identify a pattern in net FBS N flux (uptake – mineralization) across time, space, or stage of decomposition. Net N flux varied widely across time and space (Fig. 3D). I observed net N uptake or steady state on most collection dates across most sites, but occasionally, N mineralization flux exceeded uptake. Standardization for stage of decomposition did not influence this pattern (Fig. 3H).

P dynamics were also variable over time and space (Fig. 4A-H). P uptake rate increased linearly after 30 days in stream at HW, LB, and LS ($r^2 = 0.57$, $p = 0.005$; Fig. 4A). This linear relationship was not observed when P uptake rate across sites was standardized for stage of decomposition (Fig. 4E). P uptake rate was consistently faster at HW than the other sites on every collection date and was 2 \times that of LB by the final collection date. P uptake rate by leaves collected from LS was initially slow but increased to a rate similar to that of HW by the final collection date. I was unable to measure P uptake or to estimate steady-state concentration in several microcosm experiments (Table 4). Therefore, I did not include data from SC and SM in my analysis of P cycling. Some data from the other 3 sites also were missing because concentrations were below detection.

Temporal patterns in FBS P uptake also differed among sites. FBS P uptake increased over time in HW and LS but peaked after 50 d in LB (Fig. 4B). FBS P mineralization peaked in LB and LS after ~50 d but continued to increase in HW (Fig. 4C). I saw a spatial trend in P fluxes similar to that of N. FBS P uptake was >2× greater in LS than the other 2 sites by the end of the study. However, FBS P mineralization differed from FBS N mineralization in that it was more similar between LS and the other sites. Net P flux (uptake – mineralization) also differed from net N flux in that I observed net P mineralization at several sites (Fig. 4D). As with N dynamics, accounting for the stage in decomposition did not change the overall patterns in FBS P uptake, mineralization, or net flux (Fig. 4F–H).

Microbial nutrient cycling responded differently across the gradients in NH_4^+ and NO_3^- availability (Fig. 5A–F). FBS N uptake or mineralization and NH_4^+ availability did not appear to be related (Fig. 5A, B). FBS N uptake and mineralization were greatest at low NO_3^- availability (Fig. 5D, E). LS seemed to be an outlier because this site had very low fungal biomass (Fig. 1) and very high FBS N fluxes. I observed net mineralization only at low NH_4^+ or NO_3^- availability (Fig. 5C, F).

Ambient SRP concentration was quite low at all sites. Thus, the SRP gradient was much more constricted than the NO_3^- gradient. However, FBS P uptake and mineralization were greatest at low SRP concentrations and decreased exponentially as SRP availability increased (Fig. 6A, B; with uptake: $r^2 = 0.84$, $p = 0.001$; with mineralization: $r^2 = 0.80$, $p = 0.001$). Net P mineralization was only observed at low SRP availability (Fig. 6C).

Discussion

Leaf breakdown and fungal biomass

Breakdown of leaf material is the net result of several processes including chemical leaching, physical breakage, microbial decomposition, and macroinvertebrate feeding (Webster and Benfield 1986). Direct comparisons between rates measured in my and other studies are difficult to make for most of my sites. However, rates measured in other Coweeta reference sites are similar to my measurements in HW (0.0041/d). Red maple breakdown rates ranged from ~0.006 to 0.018/d over several years in the stream

draining reference WS 53 (Eggert and Wallace 2003) and were 0.0048/d in WS 54 (Gulis and Suberkropp 2003). In these and most other studies of leaf breakdown, rates were calculated by measuring mass lost from leaf packs over time (Benfield 2006). However, I calculated breakdown rate by measuring mass loss from leaf disks cut from intact, unskeletonized leaf surfaces in leaf packs. Thus, my breakdown rates incorporate only mass loss from chemical leaching and microbial decomposition, and the influences of most macroinvertebrate feeding and physical breakage were excluded. The breakdown rates reported here are slower than what actually occurs in these sites, but they are useful for comparing microbial processes.

Given my method for estimating breakdown rates, it is not surprising that microbial growth drove red maple breakdown rates in my study. The pattern of colonization of leaves by fungi has been described by others (Gulis and Suberkropp 2006, Gessner et al. 2007). Direct comparisons between my study and others are difficult to make, but the maximum fungal biomass I observed in HW was slightly <20 mg/g AFDM after 66 d in the site. Gulis and Suberkropp (2003) reported a maximum of ~35 mg/g AFDM on red maple leaves in Coweeta reference WS 54 after 120 d in the stream. Fungal biomass on red maple leaves in HW might have continued to increase had I continued my collections, but it seemed to be declining by the end of my study.

Differences in fungal biomass among sites can be caused by several extrinsic factors including temperature and nutrient availability. Temperature influences microbial activity directly (Suberkropp et al. 1975, Webster and Benfield 1986, Chauvet and Suberkropp 1998, Ferreira and Chauvet 2011). I did not measure water temperature during my study, but intersite variation in water temperature probably contributed to differences in total fungal biomass. Nutrient availability can increase fungal growth (Suberkropp and Chauvet 1995, Suberkropp 1998, Grattan and Suberkropp 2001, Gulis and Suberkropp 2003, Baldy et al. 2007). Ambient NO_3^- concentration controlled fungal biomass in my study and explained ~50% of the variation across sites. Fungal biomass also increased with ambient SRP concentration, but this gradient was much more constrained.

Nutrient dynamics

I observed net mineralization of both N and P on several collection dates and at several sites. Net P mineralization was particularly common and occurred more frequently than net P uptake. In contrast, net N uptake was more common than net N mineralization. Net mineralization suggests that microbial biomass is not increasing or that it is limited by other nutrients. Ambient SRP was relatively low at all sites, and microbes probably were P limited. However, the net mineralization fluxes were quite low ($<0.002 \mu\text{g NH}_4\text{-N min}^{-1} \text{mg}^{-1}$ fungal biomass and $<0.003 \mu\text{g PO}_4\text{-P min}^{-1} \text{mg}^{-1}$ fungal biomass), and these fluxes might be an artifact of the precision of my analytical methods.

Measurements of mineralization are scarce in stream literature. Stream ecologists have well developed and relatively straightforward methods for measuring and comparing nutrient uptake at the reach scale (Payn et al. 2005, Webster and Valett 2006, Mulholland et al. 2008). However, measuring mineralization at similar scales requires use of stable or radioactive isotopes (Newbold et al. 1983a, Peterson et al. 2001, Simon et al. 2004), which is logistically impractical for most field studies. Webster et al. (2009) used a computer model that considered N and P content of the water, microbial biomass, and leaf material to simulate microbial nutrient cycling during leaf decomposition in HW. Their simulation produced a shift for both N and P from net retention to net mineralization during decomposition. I was unable to demonstrate a relationship between net mineralization and stage of decomposition, possibly because my method was not sensitive enough to measure very low levels of net mineralization.

Microbial retention or regeneration of inorganic nutrients in headwater streams has implications for downstream nutrient processing. Nutrients exported by retentive headwaters would be largely refractory organic forms, potentially causing downstream communities to become nutrient limited. Regenerative headwaters potentially would alleviate nutrient limitation of downstream communities by exporting excess inorganic nutrients. However, in many conceptual and practical models, streams are considered as being at steady state and nutrient concentrations often are maintained over longitudinal distance (Brookshire et al. 2009). Steady state might be observed at low resolution temporal and spatial scales because of the temporal and spatial heterogeneity of microbial activity. For instance, at any one time, leaves in streams are in various stages of

decomposition (e.g., Cummins et al. 1989) and, therefore, might be supporting microbial communities with varying functions with respect to nutrient retention. This functional heterogeneity could lead to relatively consistent nutrient concentrations over time and space.

Uptake and mineralization did not follow the pattern I predicted, but some consistent trends were present in my data. Sites with low nutrient availability had low fungal biomass and, consequently, high FBS nutrient fluxes. For example, LS had the lowest fungal biomass but the greatest FBS uptake and mineralization of both N and P. Microbes were cycling nutrients faster per unit fungal biomass in the low- than in high-nutrient sites. I think that differences in shredder assemblages among my sites may have contributed to the differences in fungal nutrient-cycling efficiencies. Grazing can influence both structure and function of primary producers in autotrophic ecosystems (McNaughton 1979, Gregory 1983, Lamberti and Moore 1984). Specifically, intermediate levels of grazing can enhance production by removing dead or inactive biomass. Shredding macroinvertebrates in some of my sites may have been contributing to enhanced activity by heterotrophic microbes in a similar way. Not all macroinvertebrates classified as shredders exhibit the shredding or chewing behavior typical of this functional feeding group. Several Plecoptera taxa, including peltoperlids, have been described as “microshredders”, or shredders that scrape superficial microbes and mesophyll tissue from the leaves (Wallace et al. 1970, King et al. 1988). Microshredders might enhance production by removing dead or senescent cells, which would result in a low but productive fungal biomass that would cycle nutrients at a relatively fast rate. The abundance of microshredders probably varied across sites. All of my sites have similar riparian vegetation and local conditions (with the exception of nutrient availability), but they drain catchments with different land uses. LS and HW are in forested catchments, whereas SM is in an agricultural catchment. As an order, Plecoptera are often most abundant in cool, forested streams. I did not formally assess Plecoptera abundance, but I noticed more peltoperlids and other stonefly taxa in leaf packs collected from LS and HW than in packs collected from the other sites, an observation suggesting that microbes in LS and HW may have experienced the type of grazing that may cause high cycling rates but low biomass.

Evaluation of conceptual model

I did not see the patterns in nutrient cycling that I predicted from the organism-based ecosystem model. This failure to generate accurate predictions may indicate that the model is not appropriate for nutrient cycling at the ecosystem level. Nutrient cycling in the organism-based model is driven by demand and availability. For organisms, particularly animals, demand often is defined as the nutrient content of the body of the individual organism and availability as the nutrient content of its food source. I included all uptake organisms in a single functional unit analogous to a single organism and attempted to predict its nutrient cycling based on its demand and the availability of nutrients. However, I made several simplifying assumptions that might have influenced both demand and availability (Fig. 7).

First, I considered the microbe–substrate complex as a single functional unit with a single nutrient demand. However, this complex is actually a consortium of many different types of organisms including fungi, heterotrophic bacteria, protozoans, and probably some autotrophic cells. These organisms, along with the extracellular enzymes and products they produce, form a matrix within and on the surface of the leaf substrate. Each of the groups of organisms in this matrix has a specific nutrient composition, growth efficiency, growth rate, and enzyme production rate, all of which contribute to a specific nutrient demand. My model assumes that each of these groups of organisms will respond similarly to changes in nutrient availability, either over time or across a gradient. However, interactions among these organisms and their matrix may so alter conditions within the detritus so that broad measures of nutrient availability are not relevant.

Second, I assumed that the nutrient content of the microbes was homeostatic and, therefore, the response in microbial nutrient demand was driven by changes in nutrient availability across space or by changes in microbial biomass over time. However, the nutrient content of microbes may be temporally or spatially variable at the individual and the assemblage level. Autotrophic microbes can store nutrients in specific compounds or in vacuoles within their cells, a process referred to as luxury uptake. This ability makes the nutrient composition of autotrophs, and potentially their nutrient demand, very responsive to nutrient availability in the environment (Sturner and Elser 2002). Algae can colonize detritus, particularly when light levels are favorable (Rier et al. 2007, Artigas et

al. 2009). I did not measure the algal content of the microbial assemblage, but it was probably fairly low, particularly in HW and LS, which were heavily shaded by *Rhododendron*. In comparison, heterotrophic microbes are considered to be stoichiometrically homeostatic. That is, the nutrient composition of heterotrophic microbes does not respond to changes in nutrient availability (Sternner and Elser 2002, Makino et al. 2003). However, results of a recent meta-analysis suggest that homeostasis should be considered as a continuum and some heterotrophic groups may be weakly plastic (Persson et al. 2010). The heterotrophic microbes associated with the leaves in my study might have exhibited some plasticity over decomposition or the nutrient gradient. The nutrient composition of the microbes may have also changed because of shifts in the composition of the microbial assemblage over time and space. Bacterial cells are much richer in N and P than fungal hyphae because of their relative lack of structural material and faster growth rate (Sternner and Elser 2002). Bacteria become more abundant in the later stages of decomposition (Suberkropp and Klug 1976), and this increase potentially could cause a shift in the stoichiometry of the microbial assemblage over time. Differences in the responses of fungi and bacteria to nutrient availability (Suberkropp et al. 2010) might cause differences in microbial stoichiometry among streams. Changes in the nutrient composition of the microbial biomass, whether caused by individual or assemblage-level mechanisms, should be considered as drivers of microbial nutrient demand.

The final simplifying assumption of my model was that immobilization of dissolved inorganic nutrients reflects microbial demand. Several lines of evidence suggest that microbes use nutrients dissolved in the water column. The concentrations of dissolved nutrients in the water column decrease significantly during times of high microbial demand, such as peak leaf fall (Mulholland 2004, Goodale et al. 2009). Dissolved nutrient uptake is positively correlated with detrital standing stocks (e.g., Mulholland et al. 1985). Dissolved nutrients can stimulate microbial abundance (Meyer and Johnson 1983, Suberkropp and Chauvet 1995, Grattan and Suberkropp 2001, Baldy et al. 2007), reproduction (Suberkropp 1998, Grattan and Suberkropp 2001), and function (Meyer and Johnson 1983, Suberkropp and Chauvet 1995, Grattan and Suberkropp 2001). Studies with stable-isotope tracers have supplied direct evidence for use of

dissolved nutrients by heterotrophic microbes (Tank et al. 2000, Sanzone et al. 2001). A substantial portion of microbial demand might also be satisfied by organic pools. Several authors have suggested that some forms of dissolved organic N may be readily available and used quickly by stream microbes (Brookshire et al. 2005, Johnson and Tank 2009, Johnson et al. 2009).

Microbes assimilate nutrients as well as dissolved organic matter from organic substrates. The fact that nutrient content of leaves can drive breakdown rates suggests that substrate nutrients support at least a portion of microbial nutrient demand (Ostrofsky 1997, Richardson et al. 2004, Lecerf and Chauvet 2008). Aquatic microbes produce a suite of exoenzymes that liberate nutrients from the leaves (Sinsabaugh et al. 1991). These enzymes could be used to mine nutrients as well as to acquire C (Craine et al. 2007). Knowledge of the relative importance of water-column and leaf-derived nutrients in satisfying microbial demand over the course of decomposition and at different nutrient levels would enable development of more accurate predictive models describing microbial nutrient processing in streams.

My study showed wide variation in the rates of microbial immobilization and mineralization of N and P in 5 forested sites. I did not detect strong patterns in the fluxes of either nutrient during decomposition or across a nutrient gradient. I suggest that future investigations of microbial nutrient cycling would benefit from both methodological and conceptual improvements. First, obtaining refined measurements of microbial mineralization is difficult as immobilization and mineralization occur simultaneously and the limitations of analytical methods often make detecting subtle concentration changes difficult. Stable isotopes may be useful in parsing out these simultaneous processes, despite the expense and difficulties often associated with their use. Second, I suggest that the simple organism-based model is insufficient when describing nutrient cycling at the ecosystem level. This model needs to be modified to include temporal and spatial variability of nutrient demand based on microbial requirements for production and growth as well as alternative sources of nutrients available to satisfy that demand.

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Table 1. Mean nutrient concentrations ($\mu\text{g/L}$) at 5 study sites across 4 collection dates. NO_3^- was not measured on the first collection date. Dissolved inorganic N (DIN) = NH_4^+ + NO_3^- . Ratios are molar and were not given if soluble reactive P (SRP) was below detection limits (bd). HW = Hugh White Creek, LS = Little Stony Creek, LB= Little Black Creek, SC = Stonecrop Creek, SM = Smith Creek, Co = county, NC = North Carolina, VA = Virginia. $n = 3$ water samples collected at each site on each collection date.

Date/variable	HW	LS	LB	SC	SM
13–14 Jan 09					
NH ₄ -N	2.6	19.4	12.9	bd	10.0
SRP	bd	bd	bd	bd	bd
26–27 Jan 09					
NH ₄ -N	8.5	4.8	25.8	7.5	5.7
NO ₃ -N	5.2	45.0	97.8	260.0	896.0
SRP	2.7	2.9	7.3	7.4	3.3
DIN:SRP	11.4	37.6	37.3	79.8	599.0
09–10 Feb 09					
NH ₄ -N	10.5	4.8	6.4	5.8	6.8
NO ₃ -N	3.7	20.0	47.3	178.7	782.3
SRP	bd	2.2	4.0	3.4	bd
DIN:SRP	–	24.6	29.8	119.6	–
23–24 Feb 09					
NH ₄ -N	2.8	1.5	3.2	1.9	7.5
NO ₃ -N	bd	18.5	41.5	251.7	890.0
SRP	2.5	2.4	6.2	8.4	3.8
DIN:SRP	2.5	18.6	15.8	66.8	527.5

Table 2. Linear regression coefficients for breakdown rates of red maple leaves and fungal biomass as functions of ambient water-column nutrient concentrations ($\mu\text{g/L}$). Bolded p values are significant at the 0.05 level. SRP = soluble reactive P.

Nutrient	Leaf breakdown rate (/d)		Fungal biomass (mg/g AFDM)	
	r^2	p	r^2	p
NH ₄ -N	0.002	0.949	0.001	0.894
NO ₃ -N	0.86	0.024	0.49	0.004
SRP	0.001	0.968	0.30	0.012

Table 3. Breakdown metrics for red maple leaves at each site. Fraction of half life was calculated based on the final collection date. Fraction of half life is the number of days in site (given in parentheses)/half life. See Table 1 for site codes.

Site	Leaf breakdown rate (/d)	Half life (d)	Fraction half-life
HW	0.0041	169	0.39 (66)
LB	0.0062	112	0.59 (66)
LS	0.0056	124	0.54 (67)
SC	0.0052	133	0.50 (67)
SM	0.0101	69	0.86 (59)

Table 4. Ambient (C_{amb}) and steady-state (C') concentrations ($\mu\text{g/L}$) from $\text{NH}_4\text{-N}$ and $\text{PO}_4\text{-P}$ uptake and mineralization assays. C' was obtained from no-spike control tubes. C_{amb} was obtained from filtered water used in assays. See Table 1 for site codes.

Date/site	$\text{NH}_4\text{-N}$		$\text{PO}_4\text{-P}$	
	C_{amb}	C'	C_{amb}	C'
13–14 Jan 2009				
HW	2.6	5.7	bd	4.2
LS	19.4	13.4	bd	4.7
LB	12.9	10.0	bd	11.0
SM	10.0	5.1		
26–27 Jan 2009				
HW	8.5	8.8	2.7	4.8
LS	4.8	6.0	2.9	5.1
LB	25.8	12.0	7.3	10.2
SC	7.5	5.7	–	–
SM	5.7	5.8	–	–
09–10 Feb 2009				
HW	10.5	5.3	bd	4.8
LS	4.8	3.1	2.2	5.7
LB	6.4	9.5	4.0	11.5
SC	5.8	4.8	3.4	8.3
SM	6.8	2.9	bd	3.6
23–24 Feb 2009				
HW	2.8	1.4	2.5	6.6
LS	1.5	4.4	2.4	1.2
LB	3.2	6.0	6.2	4.4
SM	7.5	1.4	3.8	3.6

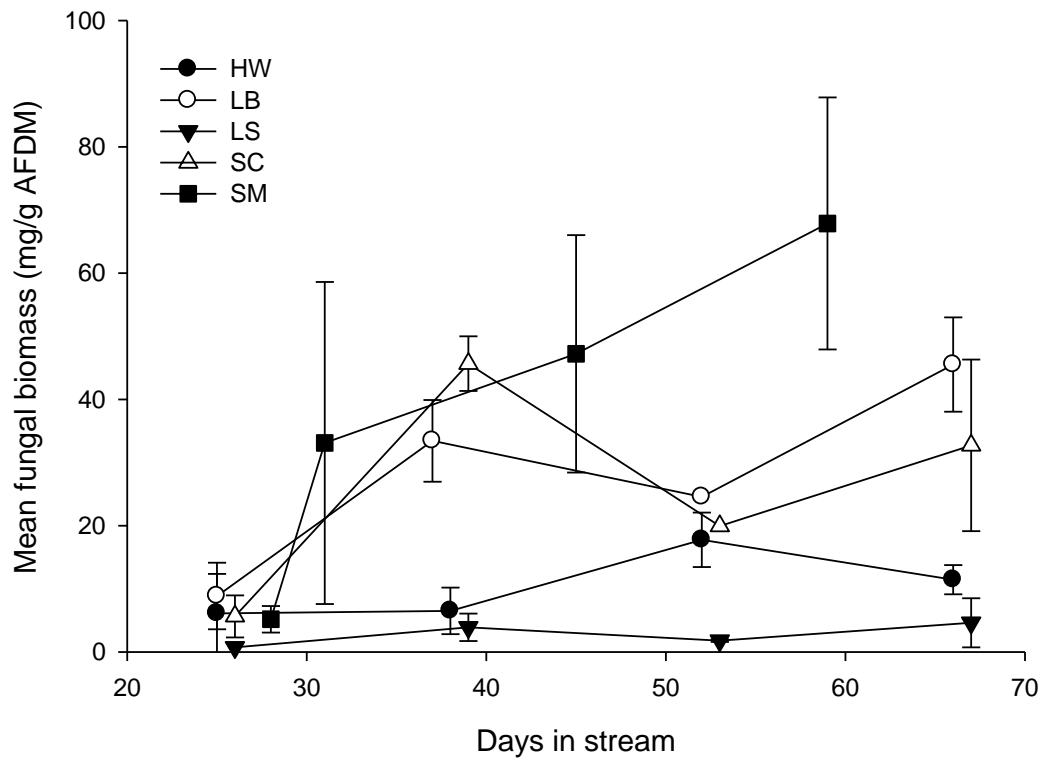


Figure 1. Mean (± 1 SD, $n = 3$) fungal biomass (mg/g ash-free leaf dry mass [AFDM]) extracted from leaves incubated in each of the 5 sites over time. The first collections began on 13–14 January 2009, after leaves had been in sites for 4 wk. See Table 1 for site codes.

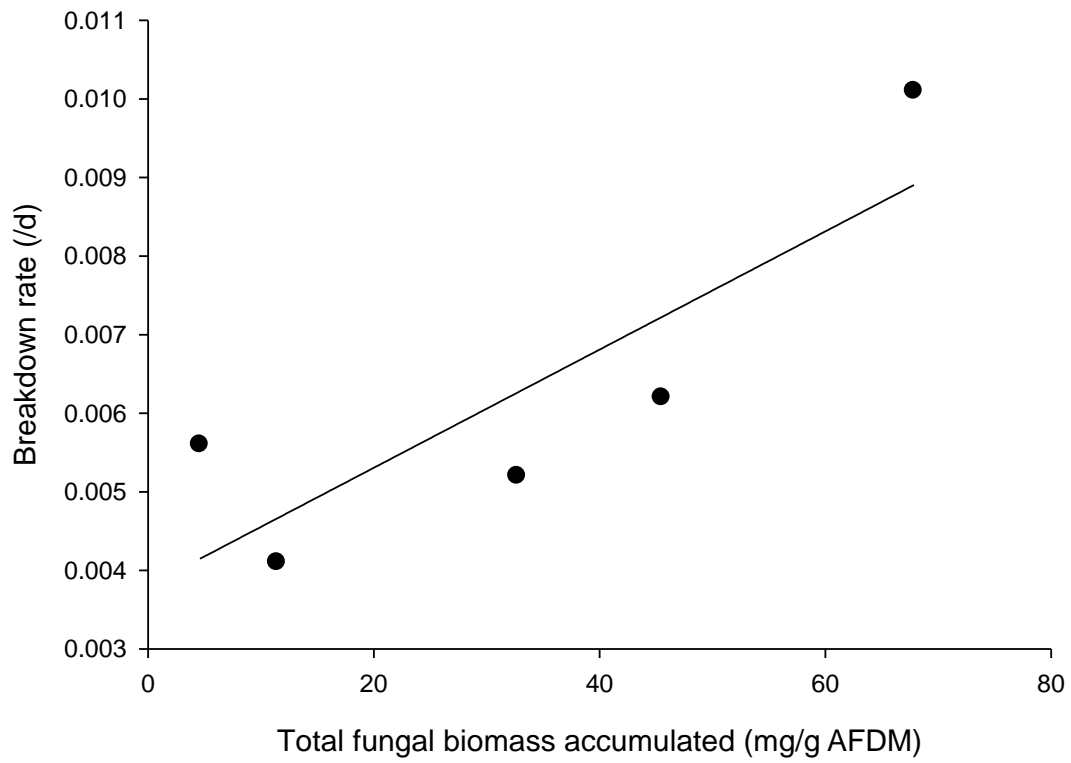


Figure 2. Regression for breakdown rates of red maple leaves as a function of fungal biomass accumulated over 10 wk ($r^2 = 0.71$, $p = 0.072$).

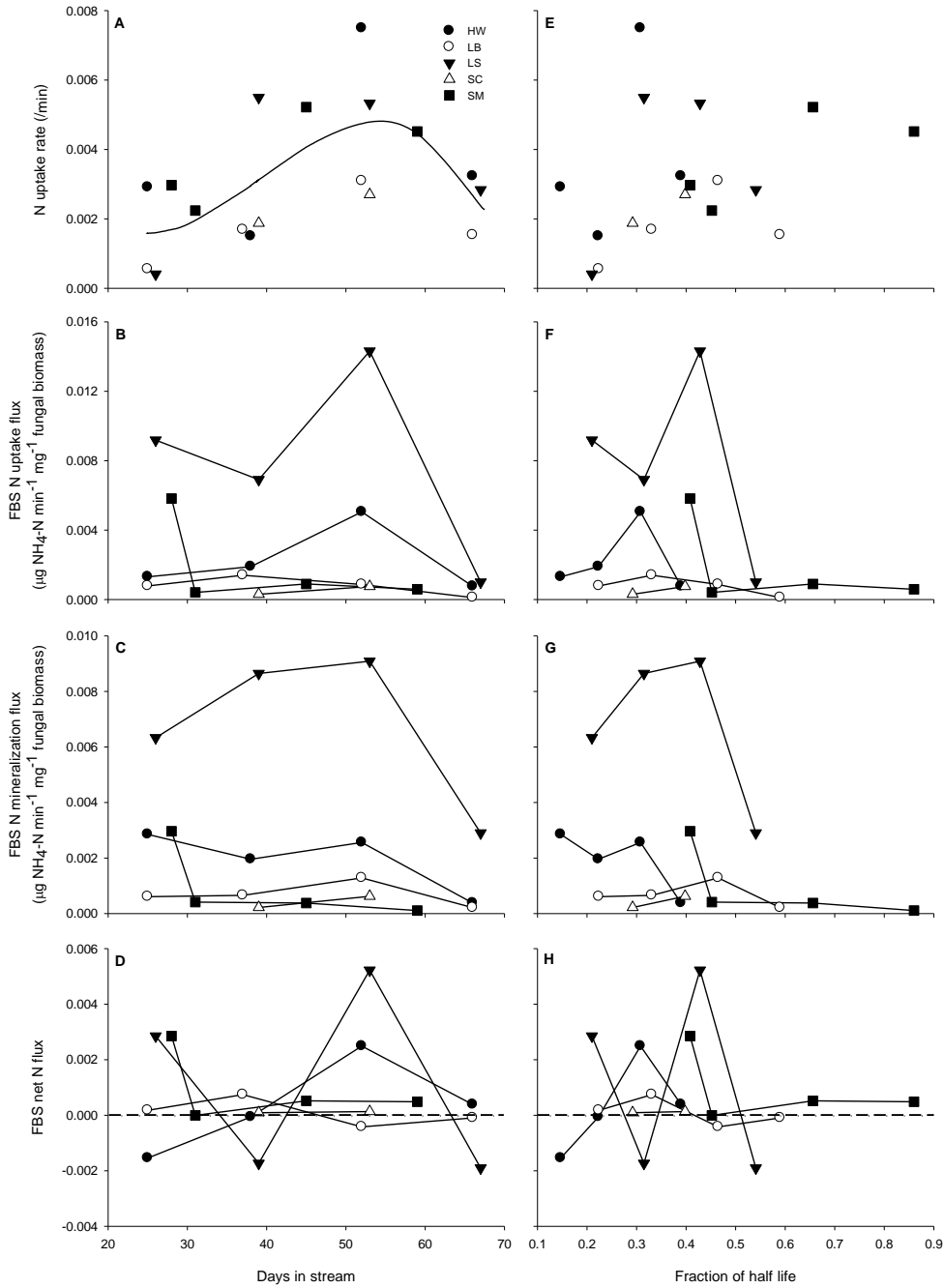


Figure 3. N uptake rate (A, E), fungal biomass-specific (FBS) uptake flux (B, F), FBS mineralization flux (C, G), and FBS net N flux (uptake – mineralization) (D, H) of microbes from each site over time (A–D) and stage of decomposition (fraction of half life) (E–H). The curve in panel A is a cubic polynomial regression ($r^2 = 0.52$, $p = 0.049$, $y = 0.0125 - 0.001x + 0.0000206x^2 + 0.000000259x^3$). Dashed lines in panels D and H separate net uptake (above line) from net mineralization (below line). Missing points were below detection. See Table 1 for site codes.

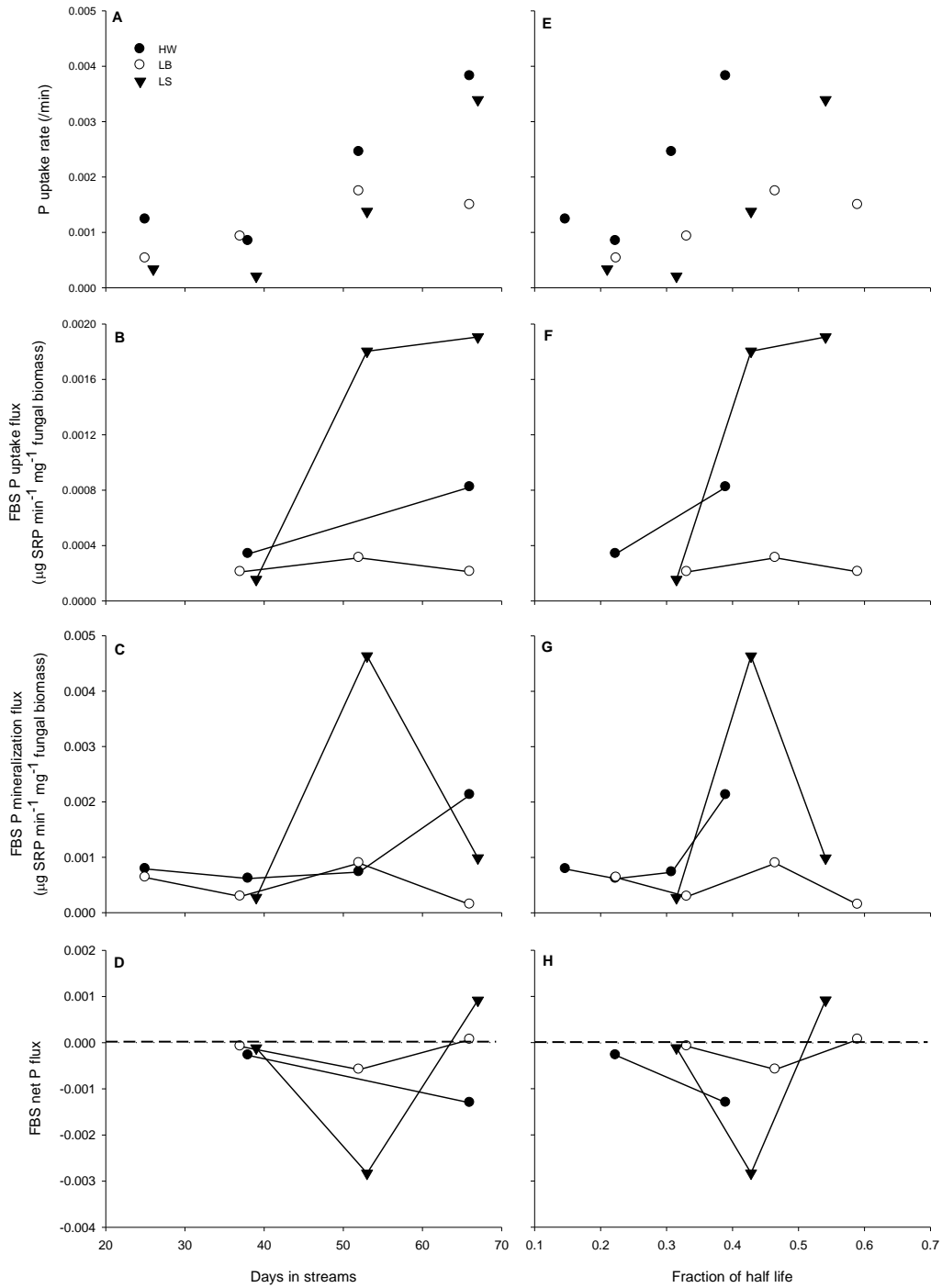


Figure 4. P uptake rate (A, E), fungal biomass-specific (FBS) uptake flux (B, F), FBS mineralization flux (C, G), and FBS net N flux (uptake – mineralization) (D, H) of biofilms from each site over time (A–D) and stage of decomposition (fraction of half life) (E–H). Dashed lines in panels D and H separate net uptake (above line) from net mineralization (below line). Missing points were below detection. See Table 1 for site codes.

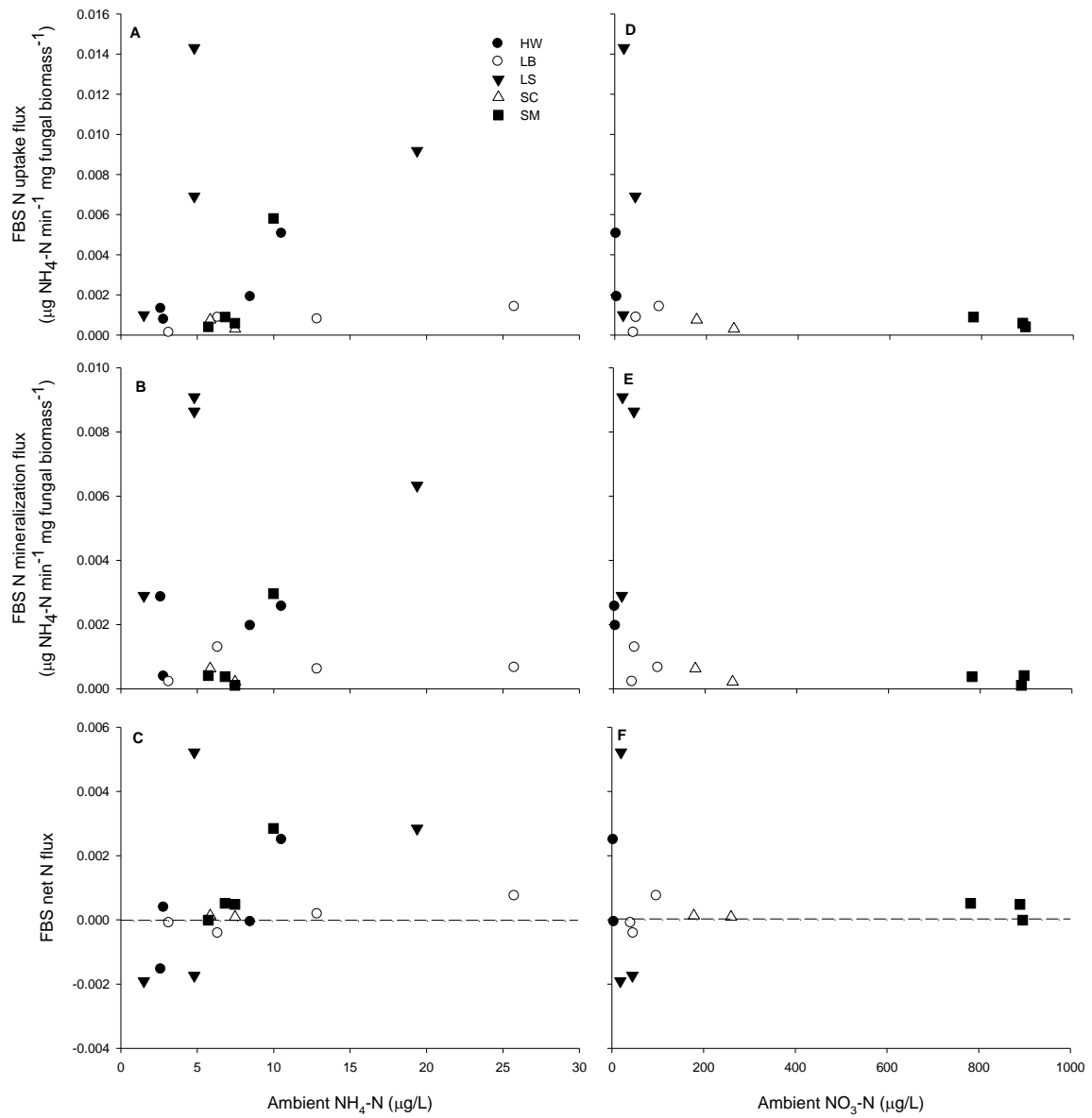


Figure 5. Fungal biomass-specific (FBS) N uptake flux (A, D), mineralization flux (B, E), and net flux (uptake – mineralization) (C, F) along the gradient of NH_4^+ (A–C) and NO_3^- (D–F) availability. Dashed lines in panels C and F separate net uptake (above line) from net mineralization (below line). See Table 1 for site codes.

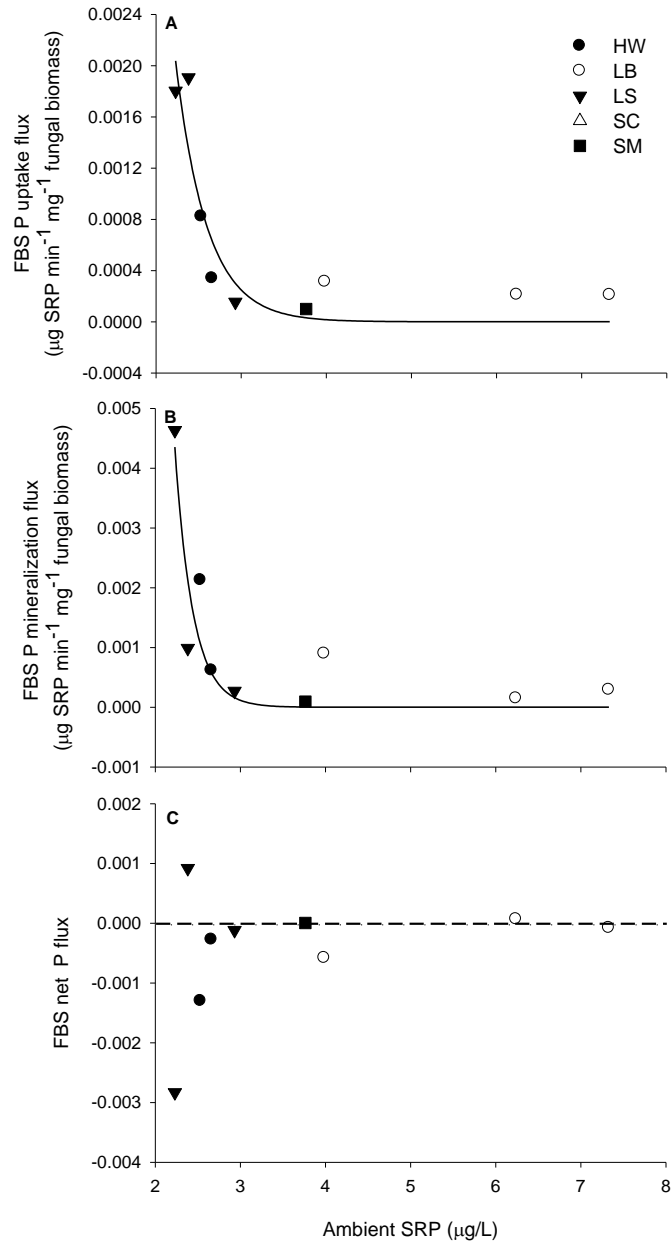


Figure 6. Fungal biomass specific (FBS) P uptake flux (A), mineralization flux (B), and net flux (uptake – mineralization) (C) along the gradient of soluble reactive P (SRP). Solid lines in panels A and B are exponential decay regressions where FBS uptake and mineralization = $ae^{(-b[\text{ambient SRP}])}$ (FBS uptake: $r^2 = 0.84$, $p = 0.001$; FBS mineralization: $r^2 = 0.80$, $p = 0.001$). Dashed line in panel C separates net uptake (above line) from net mineralization (below line). See Table 1 for site codes.

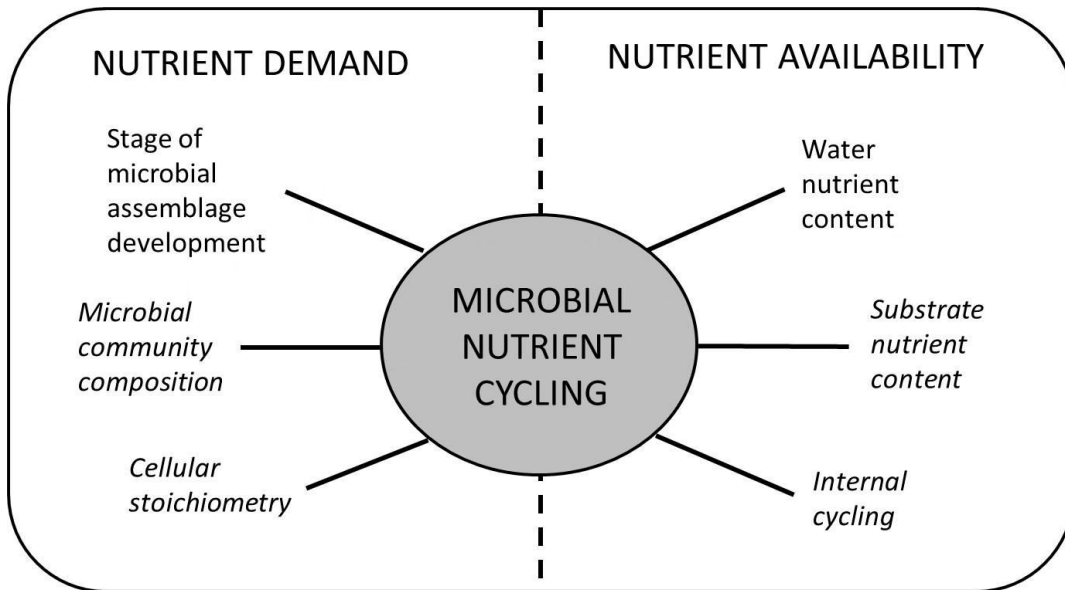


Figure 7. Conceptual model of factors influencing nutrient cycling by leaf-associated heterotrophic microbes in streams. Microbial nutrient cycling is the product of nutrient demand and nutrient availability. At the ecosystem level, demand and availability are influenced by several factors. Italicized type indicates factors that were assumed constant or not included in my study.

Chapter 3: Where do microbes get nitrogen: relative importance of water and leaf-derived nitrogen in satisfying heterotrophic microbial requirements.

Abstract

Heterotrophic microbes colonizing detritus may obtain nitrogen (N) for growth in 2 ways: assimilating N from their substrate or immobilizing exogenous inorganic N. Microbial use of these two pools has different implications for N cycling and organic matter decomposition. I quantified the relative importance of substrate and exogenous N to microbial growth in 5 streams spanning a gradient of inorganic N availability. I used sugar maple leaves labeled with ^{15}N to differentiate between microbial N that had been assimilated from the leaf substrate (enriched with ^{15}N) or immobilized from the water (natural abundance $^{15}\text{N}:^{14}\text{N}$). Packs of labeled leaves were incubated in five Appalachian streams with ambient nitrate concentrations ranging from about 5 to 900 $\mu\text{g NO}_3\text{-N/L}$. Leaves were collected periodically over time and microbial N was extracted from leaves at each collection using chloroform fumigation. Both detritus and microbial biomass became more depleted in ^{15}N during decomposition in all streams. The % microbial N derived from leaves decreased over time in all streams and was the least in the stream with the greatest ambient $\text{NO}_3\text{-N}$ concentration. These results suggest that immobilization of exogenous N becomes more important as decomposition progresses and may be the predominant source of N for meeting microbial requirements when available in sufficient concentrations.

Introduction

The amount of biologically available nitrogen (N) in the biosphere has increased due to anthropogenic activities (Vitousek et al. 1994). This increase may alter the rates of N cycling (Galloway et al. 2003) as well as the interactions of N with other elemental cycles (Finzi et al. 2011). The N cycle is driven and coupled with other elements, such as carbon (C), by organisms that incorporate elements into their biomass in specific ratios determined by their growth requirements (Sterner and Elser 2002). For example, heterotrophic microbes drive both N cycling and organic matter decomposition in many ecosystems. These microbes often need more N than is readily available in detritus to

support growth, requiring microbes to access a supplementary source of N (Parton et al. 2007).

Potential sources of N available to microbes include N bound in more recalcitrant substrate compounds and exogenous dissolved inorganic N (DIN) from the surrounding environment. The relative importance of these two N pools (substrate-derived and exogenous) in meeting microbial requirements may determine how microbes respond to increasing amounts of biologically available N and may have implications for organic matter decomposition rates and CO₂ evolution. There is support for microbial utilization of both pools in terrestrial ecosystems. Measures of initial substrate quality are often correlated with microbial activity (Melillo et al. 1982, Berg et al. 2003, Parton et al. 2007), and microbes produce exoenzymes that break up recalcitrant compounds such as lignin; compounds which can complex with N (Moorhead and Sinsabaugh 2006, Sinsabaugh and Shah 2011). However, exogenous nutrient availability can also influence microbial activity (Hunt et al. 1988, Vivanco and Austin 2011), and N content of detritus often increases during the initial stages of decomposition (Aber and Melillo 1982, McClaugherty et al. 1985, Berg and McClaugherty 1989), presumably due to microbial immobilization of exogenous DIN. Recent simulations of nutrient dynamics during organic matter decomposition assume microbial use of DIN (Webster et al. 2009, Manzoni et al. 2010), although the magnitude of this flux seems to be at least partially dependent on initial substrate quality (Parton et al. 2007).

I investigated the relative roles of substrate and exogenous N pools in meeting heterotrophic microbial N requirements in headwater streams. Temperate, forested headwater streams are detrital-based ecosystems (Fisher and Likens 1973). Whereas the exogenous N supply available to litter-associated microbes in terrestrial ecosystems is highly dependent on precipitation and is therefore temporally variable, the dissolved exogenous nutrient pool in lotic ecosystems is constantly renewed from upstream. Microbial activity in lotic systems has been shown to respond to both the concentration of DIN in the water column and initial litter quality (e. g. Hynes and Kaushik 1969, Suberkropp and Chauvet 1995, Suberkropp et al. 2010). My objectives were to determine 1) the predominant source of N for stream heterotrophic microbes over the course of leaf litter decomposition, and 2) the influence of DIN availability on this relationship.

I used a natural gradient of DIN availability and leaves labeled with a stable isotope of N to address these objectives (Figure 1). Leaf litter collected from the streams at any given time (called detritus from here on) can conceptually be separated into two pools: leaf tissue and microbial biomass. Nitrogen enters microbial biomass from leaf tissue via assimilation or from the water via immobilization and is returned to the water from the microbial biomass via mineralization. I used sugar maple leaves that had been labeled with ^{15}N in order to differentiate N in microbial biomass that had been assimilated (enriched ^{15}N) or immobilized (natural abundance $^{15}\text{N}:^{14}\text{N}$) in five streams spanning a gradient in ambient DIN concentration. Using this design, I considered the following alternative temporal responses of microbial biomass from a single site: 1) if microbes relied predominantly on recalcitrant leaf compounds as their supplemental source of N, microbial biomass would remain heavily labeled over the course of decomposition, or 2) if microbes relied predominantly on DIN as their supplemental N source, the isotopic signature of the microbial biomass would become more reflective of the water over the course of decomposition. The influence of DIN availability on microbial N sources can also be investigated using this design. If high DIN availability caused microbes to rely more heavily on immobilization of exogenous N, then the isotopic signature of the microbial biomass in streams with high DIN concentration would be less labeled compared to microbial biomass in low DIN streams.

Methods

Study sites

Field research was conducted in five forested headwater streams from November 2009-February 2010. Stonecrop Creek (SC), Little Stony Creek (LS), Little Back Creek (LB), and Smith Creek (SM) are located in the Appalachian region of Virginia, and Hugh White Creek (HW) drains a reference watershed at Coweeta Hydrologic Laboratory in the Appalachian region of North Carolina. All five streams are shaded on at least one bank with deciduous riparian vegetation. The five streams span a gradient of DIN and phosphorus concentration, ranging from below detection to approximately 900 $\mu\text{g/L}$ and 7.0 $\mu\text{g/L}$ respectively. The molar ratio of DIN (measured as $\text{NH}_4\text{-N} + \text{NO}_3\text{-N}$ concentration) to soluble reactive phosphorus (SRP) also varied across these sites,

ranging from approximately 2.5 to over 600. More detailed descriptions of stream chemistry were described by Cheever et al. (2012).

Leaf pack deployment and collection

Sugar maple leaves labeled with ^{15}N were obtained from Timothy Fahey, Department of Natural Resources, Cornell University. The labeling process was described by Fahey et al. (2011). Briefly, sugar maple saplings were fertilized with 99 atom % enriched $^{15}\text{NH}_4\text{Cl}$ ($0.25\text{g }^{15}\text{N}/\text{m}^2$) during the summer growing season. Abscised leaves were collected the following fall and one year later. Leaves were dried at room temperature to a constant weight.

I gently crushed the dried leaves into small fragments ($\sim 1\text{-}2\text{ cm}^2$) and sieved (2 mm mesh size) to remove small particles. I crushed and mixed the leaves in order to decrease variability in ^{15}N label distribution within the leaf packs. After sieving, 2 or 10 g of leaf fragments were sealed into screen packs (2 mm mesh size). Twenty packs of each weight were anchored to the beds of the five study streams on 8 Nov 2009. Four packs of each weight were collected from each stream 24 hours after deployment and periodically over the next 12-15 weeks. An additional set of packs was transported to and from the sites but not deployed in order to correct for mass loss due to handling and to establish initial nutrient conditions. Packs were collected by removing them from the water column and placing each pack in a Zip-Lock[®] bag of stream water. Packs were transported to the laboratory on ice and stored at 4°C until analysis (no more than 48 hours later).

Filtered water samples (Whatman GF/F) for nutrient analysis were collected in triplicate from each site at each collection. Water samples were analyzed for $\text{NH}_4\text{-N}$ (using the phenate method), $\text{NO}_3\text{-N}$ (using the cadmium reduction method), and SRP (using the ascorbic acid method) concentration using a Lachat Quickchem flow injection analyzer (Lachat Instruments, Loveland, CO, USA; APHA, 1999). Samples were also analyzed for dissolved organic carbon (DOC, using a heated persulfate oxidation method) with an OI Model 1010 Total Organic Carbon Analyzer (APHA, 1999).

Laboratory analysis

Leaf breakdown rate – Leaves from each 2 g pack were dried at 45°C for at least 24 h, weighed, ashed at 550°C for two hours, and reweighed in order to obtain ash free dry mass (AFDM). The breakdown rate of sugar maple leaves in each stream was calculated as the slope of the natural logarithm of percent AFDM remaining over time (Benfield 2006).

Fungal biomass – On each collection date, leaves from the 10 g packs collected from each stream were pooled and divided into several subsamples. Three replicate paired subsamples of 0.2 g each were used for ergosterol analysis. One subsample from each pair was placed in 5 ml of methanol and kept frozen until analysis. Ergosterol was extracted from these samples using a liquid phase extraction, quantified using high performance liquid chromatography, and converted to fungal biomass (Gulis and Suberkropp 2006). AFDM was obtained from the remaining subsamples from each pair according to the method described above. Fungal biomass is expressed as mg/g AFDM of detritus.

Detrital N and ¹⁵N content -- Four subsamples from the 10-g leaf packs of between 15-30 mg each were dried at 45°C for at least 24 hours, ground to a fine powder, and analyzed for carbon and nitrogen content using a FlashEA 1112 Series Elemental Analyzer where samples were combusted at 975°C, the products were reduced, and measured using a thermal conductivity detector. Four additional subsamples were dried and ground in a similar manner, packaged in tin capsules and sent to UC Davis Stable Isotope Facility for ¹⁵N content analysis by mass spectrometry. All values of ¹⁵N content are expressed in terms of atom fraction or atom %:

$$\text{Atom fraction} = {}^{15}\text{N}_{\text{sample}} / ({}^{15}\text{N}_{\text{sample}} + {}^{14}\text{N}_{\text{sample}}) \quad (1)$$

$$\text{Atom \%} = ({}^{15}\text{N}_{\text{sample}} / ({}^{15}\text{N}_{\text{sample}} + {}^{14}\text{N}_{\text{sample}})) * 100 \quad (2)$$

Microbial biomass N and ¹⁵N content -- Additional material from the 10 g leaf packs was used to determine total N and ¹⁵N content of the microbial biomass. Microbial N was isolated from the detritus using chloroform fumigation. This technique has been used in soils (Brookes et al. 1985a) and has also been used to extract nutrients from biofilms associated with leaves in streams (Sanzone et al. 2001). Four 5-g subsamples were fumigated and four 5-g subsamples were left unfumigated for each stream at each

collection. Fumigated subsamples were placed in a dessicator with liquid chloroform. The dessicator was sealed, evacuated using a vacuum pump to boil the chloroform, and samples were left for three days. Fumigated and unfumigated samples were then extracted with 60 ml of 0.5 M K₂SO₄. Nitrogen in the extracts was converted to nitrate using the alkaline persulfate oxidation method. I analyzed 10-ml extractant samples for NO₃-N concentration by the cadmium reduction method using a Lachat Quickchem flow injection analyzer. The remaining extractants were brought to a volume of 75 ml with additional K₂SO₄. The nitrate in these samples was converted to ammonia gas by adding Devarda's Alloy and increasing the pH to ~13 by adding 2 ml of 10M NaOH and trapped on acidified filters (Stark and Hart 1996). Filters were then encapsulated in tin capsules and sent to the University of Nebraska-Lincoln Water Center for ¹⁵N analysis by mass spectrometry.

Microbial N (N_{microbial}) was calculated according to the following equation:

$$N_{\text{microbial}} = (N_{\text{fumigated}} - N_{\text{unfumigated}}) * 1.61 \quad (3)$$

where N_{fumigated} and N_{unfumigated} are the mass of total N extracted from the fumigated and unfumigated samples respectively, after accounting for the N load of the K₂SO₄ matrix. The factor 1.61 was used to correct for N that may not have been released from microbial cells during the 3 day fumigation process (Brookes et al. 1985a, Brookes et al. 1985b).

The atomic fraction of the microbial biomass (F(B)) was calculated according to the following equation:

$$F(B) = ({}^{15}\text{N}_{\text{fumigated}} - {}^{15}\text{N}_{\text{unfumigated}}) / (N_{\text{fumigated}} - N_{\text{unfumigated}}) \quad (4)$$

where ¹⁵N_{fumigated} and ¹⁵N_{unfumigated} are the masses of ¹⁵N found on the acidified filters from the fumigated and unfumigated samples, respectively, after accounting for the ¹⁵N load of the K₂SO₄ matrix and the Devardas Alloy.

Leaf-derived microbial N -- The mass of microbial N derived from the leaf substrate at each collection from each stream was calculated as:

$$\text{microbial N derived from leaves} = (N_{\text{microbes}} * (F(B) - F(W)) / (F(L) - F(W)) \quad (5)$$

where F(B) is the atomic fraction of the microbial biomass, F(W) is the atomic fraction of the water, and F(L) is the atomic fraction of the leaf material. F(B) was measured at each collection as previously described. F(W) was calculated from the mean background stream water atomic fraction observed in the LINX I Appalachian stream sites (Peterson

et al. 2001). This value was used for all collections from all sites. I used the leaves collected after 24 h from each site as a stream-specific F(L). I assumed that most of the leaching of soluble material occurred during the first 24 h (Petersen and Cummins 1974), and there was negligible microbial growth during this time. I also assumed physical, chemical, and biological processing of the leaf material after 24 h did not further change the isotopic signature of the leaf material.

Statistics

Mean $\text{NH}_4\text{-N}$ concentration, SRP concentration, TOC concentration, peak and final fungal biomass, and peak and final % detrital N were compared among sites using one-way analysis of variance (ANOVA) with Tukey post-hoc pair-wise comparisons. $\text{NO}_3\text{-N}$ concentration, natural log of % AFDM remaining, detrital molar C:N, and microbial TN were compared among sites using analysis of covariance (ANCOVA) tests with site as the categorical variable and days in stream as the continuous variable. Detrital atom % and microbial atom fraction were compared among sites using ANCOVA with site as the categorical variable and % AFDM remaining as the continuous variable. The microbial atom fraction data were arcsin-square root transformed prior to analysis to meet the assumption of normality.

Relationships between sugar maple breakdown rates and fungal biomass and $\text{NO}_3\text{-N}$ concentration were analyzed by Pearson correlations. Patterns in N, ^{15}N , and % leaf-derived N in microbial biomass over decomposition and across the NO_3^- gradient were assessed by regression analysis. The value of $0.1 \mu\text{g NO}_3\text{-N/L}$ was used if $\text{NO}_3\text{-N}$ concentrations were below detection. All regressions were linear unless stated in the text. All statistical tests were conducted using SigmaPlot with SigmaStat Integration (version 10; Systat Software Inc, Chicago, Illinois) except for the ANCOVA tests which were performed in JMP (version 9.0.0; SAS Institute Inc, Cary, North Carolina).

Results

Stream chemistry

There was a significant gradient of $\text{NO}_3\text{-N}$ concentration across the five sites (Fig. 2) with the greatest $\text{NO}_3\text{-N}$ concentrations in SM (average \pm SE over all collections: 1396

± 123.0 $\mu\text{g/L}$), intermediate concentrations in SC and LB (average \pm SE over all collections: 358.6 ± 133.7 and 164.1 ± 37.6 respectively), and low concentrations in HW and LS (average \pm SE over all collections: 9.2 ± 3.8 and below detection respectively). Although $\text{NO}_3\text{-N}$ concentrations were temporally variable, this gradient was maintained over time (ANCOVA, $p < 0.0001$).

Spatial distributions of other nutrients varied depending on the nutrient (Table 1). $\text{NH}_4\text{-N}$ concentration was generally low and did not differ among sites (one-way ANOVA, $P = 0.14$). There were differences in SRP concentration among the sites, but the spatial pattern was different than that of $\text{NO}_3\text{-N}$ and the gradient was more constrained. Mean SRP concentration was greatest in SC, lowest in HW and LS, and intermediate in LB and SM (one way ANOVA, $p < 0.001$). Mean TOC also varied spatially with the lowest concentrations in HW, intermediate levels in LB and SC, and the greatest concentrations in LS and SM (one way ANOVA, $p < 0.001$).

Sugar maple breakdown rates and fungal biomass

Sugar maple breakdown rate (d^{-1}) generally increased over the NO_3^- gradient (linear regression, $r^2 = 0.866$, $p = 0.022$). However, only leaves in HW had a statistically significantly different rate (ANCOVA, $p = 0.0053$), breaking down $\sim 1.5\text{-}3\text{x}$ slower than leaves at the other sites (Table 2). There was no relationship between sugar maple breakdown rate calculated per degree day and $\text{NO}_3\text{-N}$ concentration. However, breakdown rate (degree-day^{-1}) was significantly slower in HW compared to other sites (ANCOVA, $p = 0.0032$; Table 2).

Fungal biomass on leaves varied over time and among sites (Fig. 3). Fungal biomass generally peaked between 15-60 days in all sites, with the exception of LB, where fungal biomass was still increasing at the end of the study. I found no significant differences in peak fungal biomass among sites (one way ANOVA, $p = 0.304$) or in the fungal biomass at the final collections among sites (one way ANOVA, $p = 0.462$). There was no relationship between sugar maple breakdown rate and peak fungal biomass (Pearson correlation, $r = 0.448$, $p = 0.449$) or final fungal biomass (Pearson correlation, $r = 0.307$, $p = 0.616$).

N pools during decomposition

Total detrital nitrogen content – The %N of detrital AFDM increased during the initial stages of decomposition in all sites (Fig. 4A). Detrital %N peaked and began to decline after 37 days in all sites except SC, which peaked after 59 days. Peak %N was significantly different among all sites (one way ANOVA, $p < 0.001$). Detrital %N remained above initial values throughout the study in all streams except HW. There was no relationship between %AFDM remaining and detrital %N (linear regression, $r^2 = 0.112$, $p = 0.119$).

Molar C:N of detrital AFDM also changed over time (Fig. 4C). Detrital C:N decreased after 24 h in all of the sites and generally continued to decline over time in all sites. This decline occurred at a faster rate in LB compared with other 4 sites (ANCOVA, $p = 0.006$). Detrital C:N was influenced by stage of decomposition across all sites, with % AFDM remaining explaining 48% of the variation in C:N (linear regression, $r^2 = 0.481$, $p < 0.001$; Fig. 4D).

The change in absolute N mass of detritus ($\Delta N_{\text{detritus}}$) between collections was highly variable over time and among sites (Table 3). $\Delta N_{\text{detritus}}$ ranged from approximately 0.05 mg to over 27 mg. Detritus from LS and SM showed a net gain in N mass over the entire study (1.745 and 11.576 mg respectively) while detritus from the other sites showed a net loss in N.

Total microbial N content -- Microbial TN increased linearly over time in each site ($r^2 = 0.540$, $p < 0.001$; Fig. 4E). Total microbial N accumulation rate was significantly greater in LB (ANCOVA, $p = 0.0015$) and significantly slower in HW (ANCOVA, $p = 0.014$) compared to the other sites. Microbial TN also increased linearly as decomposition progressed ($r^2 = 0.601$, $p < 0.001$; Fig. 4F).

The change in absolute N mass in microbial biomass ($\Delta N_{\text{microbial}}$) between collections was also variable among sites (Table 3). However, unlike the change in detrital N, the $\Delta N_{\text{microbial}}$ over the entire study was positive in all sites, which shows a net increase in microbial biomass.

Detrital ^{15}N content -- The atom % of the bulk detritus declined during decomposition in all streams (Fig. 5A). Detrital atom % declined over decomposition at a similar rate in each stream with the exception of SC (ANCOVA, $p = 0.0007$). When all

sites were analyzed together, % AFDM remaining explained over 55% of the variance in atom % (linear regression $r^2 = 0.552$, $p < 0.001$). All observed values of detrital atom % were between the atom % of the dry leaves before incubation (0.8653 ± 0.0941 SD) and the background atom % of the water (0.3660 ± 0.004 SD).

The change in absolute ^{15}N mass in detritus ($\Delta^{15}\text{N}_{\text{detritus}}$) varied over time and among sites (Table 3). Detritus from most sites showed a net loss in ^{15}N over the entire study, and detritus lost ^{15}N mass between collections in several sites.

Microbial ^{15}N content -- Microbial biomass also became more depleted in ^{15}N over time (Fig. 5B). Microbial atom fraction decreased in all of the sites (with the exception of SC), although this decrease was only significant when all of the sites were analyzed together ($r^2 = 0.376$, $p = 0.003$; Fig. 5B). The rate of decrease over decomposition was similar in all sites, again with the exception of SC (ANCOVA, $p = 0.0118$). All observed values of microbial atom fraction were between the atom fraction of the dry leaves before incubation and the background signal of the water for all of the collections from all of the sites with the exception of the first collection from HW. The microbial biomass from this collection had a greater atom fraction than the dry leaves before incubation and was therefore excluded from the analyses.

Microbial biomass gained ^{15}N mass between most collections in all sites (Table 3). The change in ^{15}N mass in microbial biomass ($\Delta^{15}\text{N}_{\text{microbial}}$) was positive over the entire study in all sites.

Leaf-derived microbial N -- The reliance of microbes on leaf-derived N generally decreased as decomposition progressed (Fig. 5C). The % microbial N derived from the leaves decreased during decomposition in each individual site, with the exception of SC. When all of the sites were analyzed together, the % microbial N derived from leaves decreased linearly during decomposition ($r^2 = 0.332$, $p = 0.008$; Fig. 5C). I excluded the first collection from HW from all analyses because the unreasonably high microbial biomass atomic % (as previously noted) caused the % leaf-derived N in the microbial biomass to be over 200%.

N pools over NO₃⁻ gradient

Total nitrogen content -- Neither detrital %N or C:N seemed to be related to the nitrate gradient. I found no relationship between the mean water column NO₃⁻ and detrital %N after 2 wk ($r^2 = 0.273$, $p = 0.478$), after the final collection ($r^2 = 0.095$, $p = 0.614$), or at peak detrital %N ($r^2 = 0.568$, $p = 0.141$). There also were no relationships between the mean water column NO₃⁻ and detrital C:N after 2 wk ($r^2 = 0.040$, $p = 0.800$), after the final collection ($r^2 = 0.322$, $p = 0.318$), or at peak C:N ($r^2 = 0.061$, $p = 0.689$). The microbial TN at 2 wk increased as NO₃⁻ availability increased ($r^2 = 0.964$, $p = 0.018$). There was no response in either peak microbial TN ($r^2 = 0.176$, $p = 0.482$) or final microbial TN ($r^2 = 0.178$, $p = 0.479$) to increasing NO₃⁻ availability.

¹⁵N content -- The relative abundance of ¹⁵N in both the detritus and microbial biomass varied over the NO₃⁻ gradient (Fig. 6). The atom % of the detritus after 2 wk in each stream declined linearly as NO₃⁻ availability increased (Fig 6A). The mean water column NO₃-N concentration explained over 80% of the variance in detrital atom % at two weeks (linear regression, $r^2 = 0.848$, $p = 0.026$). The relative abundance of ¹⁵N in the microbial biomass also decreased as ambient NO₃⁻ availability increased but not linearly (Fig 6B). The atom % of the microbial biomass at two weeks decreased drastically as NO₃⁻ availability increased above the detection limit. The rate of decrease in microbial biomass atom % decreased drastically once NO₃-N concentration was above detection, according to a hyperbolic decay function. This function explained over 90% of the variation in microbial biomass atom % over the NO₃⁻ gradient (hyperbolic decay regression, $r^2 = 0.999$, $p = 0.022$).

Leaf-derived microbial N -- The degree to which microbes used leaf-derived N also responded to the ambient NO₃⁻ availability (Fig. 6C). Similar to microbial ¹⁵N, the % microbial N derived from the leaf decreased sharply as NO₃-N concentration became detectable. The % microbial N derived from the leaf continued to decrease as NO₃-N concentration increased above the detection limit but at a much slower rate. Water column NO₃-N concentration explained over 90% of the variation in % microbial N derived from the leaves at 2 wk (according to a hyperbolic decay relationship, $r^2 = 0.996$), but this relationship was not statistically significant ($p = 0.063$).

Discussion

Where do microbes get their N?

I used leaves labeled with ^{15}N to determine the predominant source of N for leaf-associated microbes during decomposition. As decomposition progressed, both the detritus and the microbial biomass became more depleted in ^{15}N and a smaller percentage of the microbial N was derived from the leaf material. These data suggest that microbes in streams supplement the labile N supply in their substrate by immobilizing N from the water column.

Determining the influence of DIN availability on microbial immobilization from my study is less clear. Although reliance on water column N occurred in all sites across the N availability gradient, $\text{NO}_3\text{-N}$ concentration explained over 80% of the variation in ^{15}N content of detritus after 2 weeks in the streams, suggesting that exogenous N availability may influence when microbes begin supplementing leaf-derived N via immobilization. However, the rate of decomposition varied among sites, resulting in large differences in % AFDM remaining among sites after 2 weeks. For example, leaves from the two end members of the $\text{NO}_3\text{-N}$ gradient, HW and SM, had approximately 90% and 70% AFDM remaining after 2 weeks respectively. Therefore the differences among detrital atom% measurements (Fig. 6A) may reflect stage of decomposition instead of N availability. However, if I consider points where leaves from the end member sites were at similar stages of decomposition, I did see a difference in the proportion of leaf-derived N in the microbial biomass (Fig. 5C). For example, leaves collected from HW after 59 days and leaves collected from SM after 16 days had similar % AFDM remaining (75% and 68%, respectively) but the % leaf-derived N in the microbial biomass differed greatly between the sites (58% and 18%, respectively).

While these results suggest that microbes in sites with low N availability may rely on leaf-derived N more heavily than those in high N sites, they are based on a natural gradient of NO_3^- availability. Several studies have shown microbial responses to experimental DIN enrichment. Gulis and Suberkropp (2003) showed significantly greater fungal biomass and higher microbial respiration rates in stream reaches enriched with nitrogen and phosphorus on two different leaf litter types compared to upstream control reaches. Suberkropp et al. (2010) show similar responses of microbial activity on a g^{-1}

substrate basis to a long term N and P enrichment. However, Howarth and Fisher (1976) reported similar respiration rates on leaf disks in microcosm streams enriched with NO_3^- compared to the control. They only saw a response in microbial respiration when $\text{PO}_4\text{-P}$ and $\text{NO}_3\text{-N}$ concentrations were both increased, suggesting that microbes may be co-limited by both nitrogen and phosphorus.

I propose the following conceptual model of microbial N acquisition during decomposition. When microbes initially colonize leaves, they utilize the readily available N contained in labile leaf compounds. This results in an initial microbial biomass with a high percentage of leaf-derived N. Microbial biomass increases rapidly, labile material is depleted, and microbes begin immobilizing exogenous DIN. If exogenous DIN availability is sufficient to meet microbial N requirements, the percentage of microbial N that is derived from the leaf declines and immobilization becomes the predominant source of N for microbial growth. If the exogenous N availability does not meet microbial requirements, microbes may access the more recalcitrant leaf material and leaf-derived N may remain a significant proportion of microbial biomass.

The above model conceptualizes the microbial assemblage as a “black-box” compartment with a uniform N requirement, dominant function (i. e. immobilization or assimilation), and response to exogenous N availability. However, the microbial biomass growing on leaf litter is a diverse assemblage of organisms from at least two domains (Suberkropp and Klug 1976). The increasing reliance on water-derived N seen in my study may be interpreted not only as a microbial functional shift from assimilation to immobilization, but may also reflect a compositional shift in microbial assemblage. Fungi dominate microbial biomass in the early stages of decomposition (Suberkropp and Klug 1976, Weyers and Suberkropp 1996) and produce exoenzymes that metabolize leaf compounds (Suberkropp and Klug 1980, Chamier 1985) and therefore may be able to better utilize N bound in these compounds. Bacterial biomass and production are less than fungi during the early stages of decomposition, but can exceed fungi on smaller more processed fragments (Sinsabaugh and Findlay 1995) and may rely more heavily on immobilization of exogenous DIN. Although it is unlikely that decomposition had progressed far enough for bacteria to dominate the microbial assemblages in any of my sites (Gulis and Suberkropp 2003), it is possible that smaller fragments with high

bacterial abundance were trapped in the leaf packs and were included as part of the detritus. If so, the depletion of the isotopic signature and the decrease in the proportion of leaf-derived N in the microbial biomass may have been due to an increasing influence of bacterial activity.

Consequences for stream nutrient cycling

The results of my study confirm the importance of microbial immobilization as a major pathway for DIN removal from the water column and emphasize the importance of in-stream nutrient processing in determining water column nutrient concentrations. Uptake of nutrients by stream biota is an integral flux in stream nutrient cycling models (Newbold et al. 1981, Webster et al. 2009) and several studies have shown decreases in stream nutrient concentrations corresponding with autumnal leaf fall (Mulholland 2004, Goodale et al. 2009) and correlations between nutrient uptake flux and leaf standing stocks (Mulholland et al. 1985). Microbial immobilization has implications for N cycling and transport along stream networks as the fate of immobilized N is likely different from DIN. Although biotic immobilization does not remove N from the ecosystem permanently (Baulch et al. 2011), much of immobilized N may be transported downstream as particulate organic N instead of the relatively more labile dissolved inorganic form (Webster 2007). These particulates generally become more refractory as they are processed and transported downstream (Peters et al. 1989, Yoshimura et al. 2008). Therefore, immobilization of DIN by heterotrophic microbes in the headwaters may result in the downstream transport of N in less available or more refractory forms, which may influence nutrient dynamics downstream.

Microbial immobilization was likely not the only flux of N from the water column to the detritus occurring in my study. The change in detrital N mass in my study was often greater than the corresponding change in microbial N mass as calculated from the chloroform fumigations (Table 3). The chloroform fumigation method may have underestimated the microbial N. My measurements of microbial TN from the fumigations were not well correlated with fungal N (Pearson correlation, $r = 0.232$, $p = 0.313$) as estimated by assuming a fungal biomass of 50% C and 3% N (Sternner and Elser 2002). However, the changes in fungal biomass N between collections (ΔN_{fungal}) were generally still

insufficient to account for the corresponding changes in detrital N. When both detrital and fungal N changed in the same direction (i.e. both gained or both lost N), the change in fungal N exceeded the change in detrital N in only one instance. In other cases, change in fungal N accounted for less than half of the gain in detrital N (Table 4).

In light of this imbalance, I revised the chloroform fumigation estimates of microbial N by assuming the microbial biomass after 24 h was composed of 100% fungal biomass. I then compared the 24 h microbial N estimates from the fumigations to the fungal N estimates from the ergosterol assays (as noted above) and calculated a revised estimate of microbial N (N_{revised}) for each collection from each stream according to the following equation:

$$N_{\text{revised}} = (1-a) * N_{\text{fungal}} + N_{\text{fumigation}} \quad (6)$$

where N_{fungal} is the fungal N estimated from the ergosterol analysis, $N_{\text{fumigation}}$ is the microbial N estimated from the chloroform fumigations, and a is the average inefficiency of the chloroform fumigation for capturing fungal N. I calculated this inefficiency as

$$a = N_{\text{fumigation at 24 h}} / N_{\text{fungal at 24 h}} \quad (7)$$

The gain in N_{revised} between collections still only accounted for the gain in detrital N for one collection. In other cases where detritus and the revised microbial biomass both gained or lost N, the change in N_{revised} accounted for less than 1% to almost 80% of the change in detrital N (Table 4).

I was unable to account for the change in detrital N with changes in microbial N whether I estimated microbial N using the fumigations, fungal biomass, or attempted to correct for fumigation inefficiencies. However, detritus could have gained N through abiotic uptake processes, such as nitrogenous compounds complexing with the lignin fraction of the leaf biomass (Suberkropp et al. 1976). Although these nitrogenous compounds may be microbially-derived (e.g. exoenzymes), they would not have been included in my microbial biomass estimates. N bound in lignin could represent a substantial portion of total detrital N; Suberkropp et al. (1976) reported an average of over 18% and 32% of the total detrital N was found in the lignin + cellulose fractions of hickory and oak detritus respectively. If I assume that 32% of the $\Delta N_{\text{detritus}}$ was due to abiotic uptake into lignin complexes, this flux combined with microbial immobilization could account for the N gained by detritus in LS over the course of the study, but does

not account for the changes in N mass between collections and still leaves 42% of the total $\Delta N_{\text{detritus}}$ in SM unexplained.

Consequences for organic matter decomposition

The relative importance of exogenous versus substrate-derived N in meeting microbial growth requirements predicts different consequences for organic matter decomposition. If the microbial assemblage only used exogenous N to meet their growth requirements, the resulting decomposition rate of the colonized litter would be slower than if those microbes were degrading complex leaf compounds to obtain N. The implications of the importance of these two pools extend to the response of organic matter decomposition to the increasing exogenous N availability due to anthropogenic N fixation. If microbes meet their N requirements by immobilizing from this exogenous pool, then increasing exogenous N availability may lead to increased microbial biomass and activity, resulting in faster organic matter decomposition compared to the baseline condition. However, if microbes rely on recalcitrant leaf N to meet their N requirements, microbial biomass and activity may not respond to increasing exogenous N availability (because they are not utilizing this pool), or microbes may respond by shifting from assimilating leaf N to using exogenous N, a more labile and now more abundant pool (Moorhead and Sinsabaugh 2006, Craine et al. 2007). Organic matter decomposition may remain the same in the former scenario, or may become slower in the latter scenario as microbes stop degrading the leaf compounds in search of N. The response of litter decomposition rate to increased N availability in other studies is unclear, with organic matter in both terrestrial and freshwater ecosystems showing faster decomposition (Hunt et al. 1988, Hobbie 2000, Vestgarden 2001, Gulis and Suberkropp 2003) and others slower rates or no response (Triska and Sedell 1976, Newbold et al. 1983, Magill and Aber 1998, Hobbie and Vitousek 2000).

I have shown that immobilization of DIN is an important flux supporting microbial growth in streams, even when DIN availability is relatively low. Sugar maple decomposition rates in my study responded positively across the NO_3^- gradient, which supports the prediction that increasing N availability associated with anthropogenic acceleration of the global N cycle could potentially contribute to more rapid

decomposition and increased CO₂ flux from detrital pathways in stream ecosystems. However, the use of a natural gradient limits my ability to infer a direct relationship between decomposition rate and increasing N availability. I think that experiments combining the labeled substrate method I employed here and enrichments of the exogenous N pool would address not only the response of organic matter decomposition to increased N availability, but would also elucidate the microbial processes driving the response.

Conclusions

My results demonstrate the importance of immobilization of exogenous DIN for leaf-associated heterotrophic microbes in headwater streams. Substrate-derived N decreased as a proportion of microbial N over the course of decomposition, and immobilization appeared to be important even in streams when DIN availability was extremely low. The acceleration of leaf decomposition across the gradient of NO₃⁻ availability also supports microbial reliance on exogenous DIN as a potential mechanism coupling the increasing availability of biologically available N to changes in the rates of organic matter decomposition and CO₂ evolution from stream ecosystems.

One of the present challenges of ecosystem ecology is to understand the ways in which biota couple nutrient cycles and to predict how these cycles may be altered by several the facets of global change. I think that the use of labeled substrates is an approach that will lead to further understanding of the microbial processes that drive N dynamics during decomposition and the implications for coupled nutrient cycles under conditions of global change. Future experiments can use this approach to focus on the role of exogenous N availability on organic matter decomposition in systems where exogenous N pools are more temporally variable, such as terrestrial ecosystems where exogenous N availability is intermittent, and lentic systems where exogenous N pools can be exhausted. I also suggest that labeled substrates will be useful in experiments combining manipulations of both exogenous and leaf N pools.

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Table 1. Mean nutrient concentration ($\mu\text{g/L}$) in five study sites over the course of the study. Means are from three water samples collected at each date. bd = below detection. Dashes indicate samples were not taken. Superscript letters indicate significant differences ($p < 0.05$) among sites. See text for site abbreviations.

		HW	LS	LB	SC	SM
07 Nov 09	NH ₄ -N	bd	bd	bd	7.6	7.2
	SRP	bd	4.7	16.6	13.9	7.2
	TOC	1380	1630	1803	2325	2400
22-23 Nov 09	NH ₄ -N	-	11.9	13.4	14.7	16.5
	SRP	-	bd	9.4	9.7	bd
	TOC	-	1760	1790	1611	2055
14-16 Dec 09	NH ₄ -N	14.7	15.4	14.3	13.2	36.9
	SRP	bd	bd	5.1	11.6	16.8
	TOC	619	2602	1380	1161	2394
05 Jan 10	NH ₄ -N	bd	-	bd	bd	bd
	SRP	bd	-	bd	5.0	bd
	TOC	555		1056	1110	1641
26 Jan 10	NH ₄ -N	bd	bd	-	bd	-
	SRP	bd	bd	-	12.5	-
	TOC	461	2397	-	1074	-
02 Feb 10	NH ₄ -N	-	bd*	bd	-	bd
	SRP	-	bd*	bd	-	bd
	TOC	-	-	897	-	1704
Mean across all collections	NH ₄ -N	3.8 ^a	5.5 ^a	5.6 ^a	7.1 ^a	12.2 ^a
	SRP	bd ^a	bd ^a	6.3 ^b	10.5 ^c	6.3 ^{ab}
	TOC	754 ^a	2097 ^b	1385 ^c	1456 ^c	2039 ^b

*collected on 23 Feb 2010

Table 2. Sugar maple leaf breakdown rate per day and per degree day for each site. Starred rates were significantly different than other sites (ANCOVA with $p < 0.05$).

Site	Decomposition rate	
	(d ⁻¹)	(degree-day ⁻¹)
HW	0.0028*	0.00037*
LS	0.0041	0.00065
LB	0.0057	0.00092
SC	0.0057	0.00074
SM	0.0089	0.00110

Table 3. Change in mass (mg) of total detrital N ($\Delta N_{\text{detrital}}$) and microbial N ($\Delta N_{\text{microbial}}$) that occurred between collections (day interval). Change in ^{15}N mass for detritus ($\Delta^{15}\text{N}_{\text{detrital}}$) and microbial biomass ($\Delta^{15}\text{N}_{\text{microbial}}$) also shown. Bolded values are changes in mass over the entire study.

	Day interval	$\Delta N_{\text{detritus}}$	$\Delta N_{\text{microbial}}$	$\Delta^{15}\text{N}_{\text{detritus}}$	$\Delta^{15}\text{N}_{\text{microbial}}$
HW	1-16	6.575	0.363	0.055	0.002
	16-37	-0.604	0.709	-0.016	0.004
	37-59	-6.825	-0.260	-0.051	-0.002
	59-80	-10.374	-0.070	-0.081	0.0002
	1-80	-11.229	0.741	-0.093	0.005
LS	1-16	4.087	-0.104	0.012	-0.001
	16-37	0.058	0.275	0.002	0.002
	37-80	-7.699	0.872	-0.059	0.003
	80-107	5.299	0.201	0.024	0.001
	1-107	1.745	1.244	-0.021	0.005
LB	1-37	-2.015	0.995	-0.025	0.005
	37-86	-6.906	2.174	-0.058	0.009
	1-86	-8.922	3.169	-0.083	0.014
SC	1-16	7.078	0.546	0.041	0.002
	16-37	-2.907	0.098	-0.001	0.002
	37-59	9.275		0.047	
	59-80	-27.545		-0.194	
	1-80	-14.101	0.588	-0.107	0.003
SM	1-16	-3.979	1.237	-0.064	0.005
	16-39	20.812	-0.495	0.148	-0.002
	39-86	-5.258	2.187	-0.042	0.010
	1-86	11.576	2.929	0.042	0.013

Table 4. Change in mass (mg) of detrital N ($\Delta N_{\text{detrital}}$) and microbial N estimated from fumigations ($\Delta N_{\text{fumigations}}$) and fungal biomass (ΔN_{fungal}) that occurred between collections (day interval). $\Delta N_{\text{revised}}$ is the change in microbial N mass between collections estimated from the fumigations and corrected for the inefficiency of the chloroform in lysing fungal cells (described in text). A negative Δ indicates a loss of N mass. Parenthetical values are the percent of the change in detrital N accounted for by the change in microbial N. Percentages were only calculated when detritus and microbial biomass both gained or both lost N. Bolded values are changes in mass over the entire study.

	Day interval	$\Delta N_{\text{detritus}}$	$\Delta N_{\text{fumigation}}^*$	ΔN_{fungal}	$\Delta N_{\text{revised}}$
HW	1-16	6.575	0.363 (5.52)	0.752 (11.4)	0.809 (12.3)
	16-37	-0.604	0.709	-0.288 (47.6)	0.538
	37-59	-6.825	-0.26 (3.8)	-0.022 (0.32)	-0.273 (4.00)
	59-80	-10.374	-0.07 (0.67)	0.075	-0.026 (0.25)
	1-80	-11.229	0.741	0.517	1.048
LS	1-16	4.087	-0.104	0.191 (4.70)	0.009 (0.22)
	16-37	0.058	0.275 (47.4)	0.804 (138)	0.753 (129)
	37-80	-7.699	0.872	-0.838 (10.9)	0.375
	80-107	5.299	0.201 (3.79)	0.08 (1.50)	0.248 (4.68)
	1-107	1.745	1.244 (71.3)	0.237 (13.6)	1.385 (79.4)
LB	1-37	-2.015	0.995	0.493	1.288
	37-86	-6.906	2.174	0.23	2.311
	1-86	-8.922	3.169	0.724	3.599
SC	1-16	7.078	0.546 (7.71)	1.469 (20.7)	1.419 (20.0)
	16-37	-2.907	0.098	-0.987 (33.9)	-0.488 (16.8)
	37-59	9.275		-0.238	-0.94
	59-80	-27.545			
	1-80	-14.101	0.588	-0.463	0.313
SM	1-16	-3.979	1.237	0.409	1.48
	16-39	20.812	-0.495	-0.033	-0.515
	39-86	-5.258	2.187	0.194	2.303
	1-86	11.576	2.929 (25.3)	0.570 (4.92)	3.268 (28.3)

* $\Delta N_{\text{fumigation}}$ is the same as the $\Delta N_{\text{microbial}}$ in Table 3 and described in the Results text.

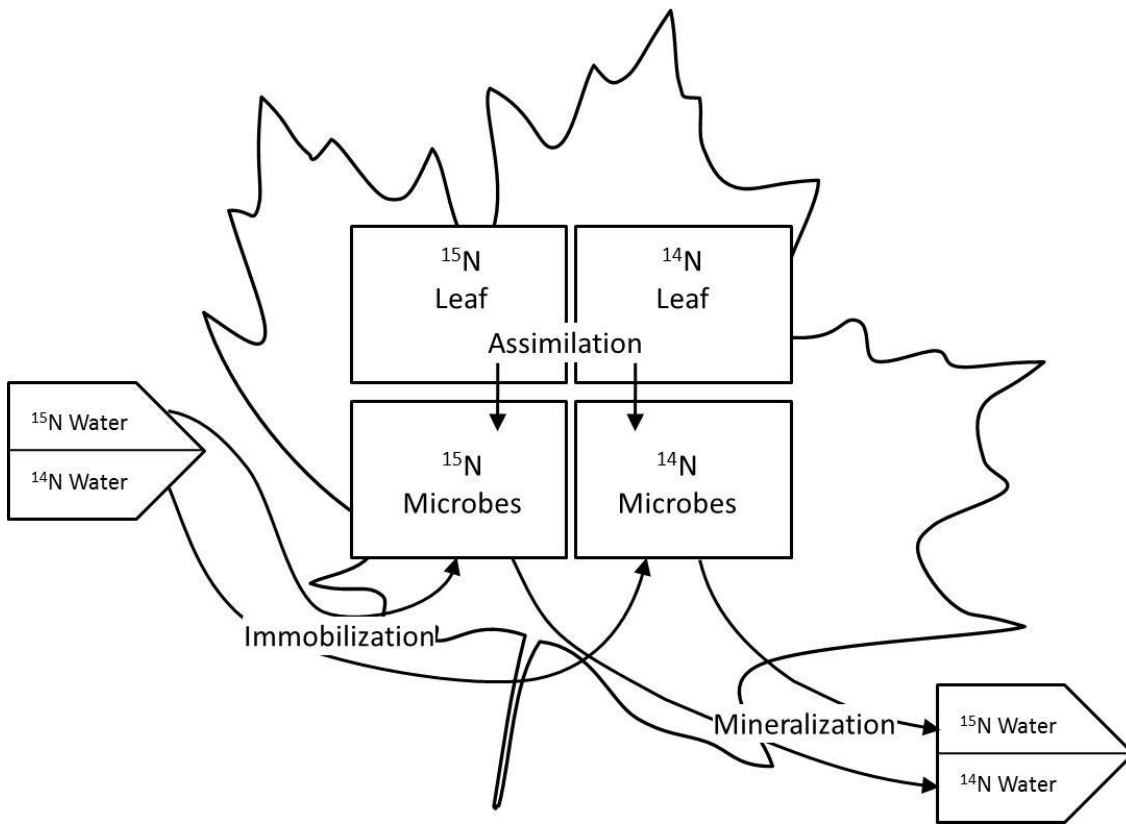


Figure 1. Conceptual diagram tracing the movement of ^{14}N and ^{15}N . Boxes represent N standing stocks (mass) and arrows represent N fluxes (mass/time).

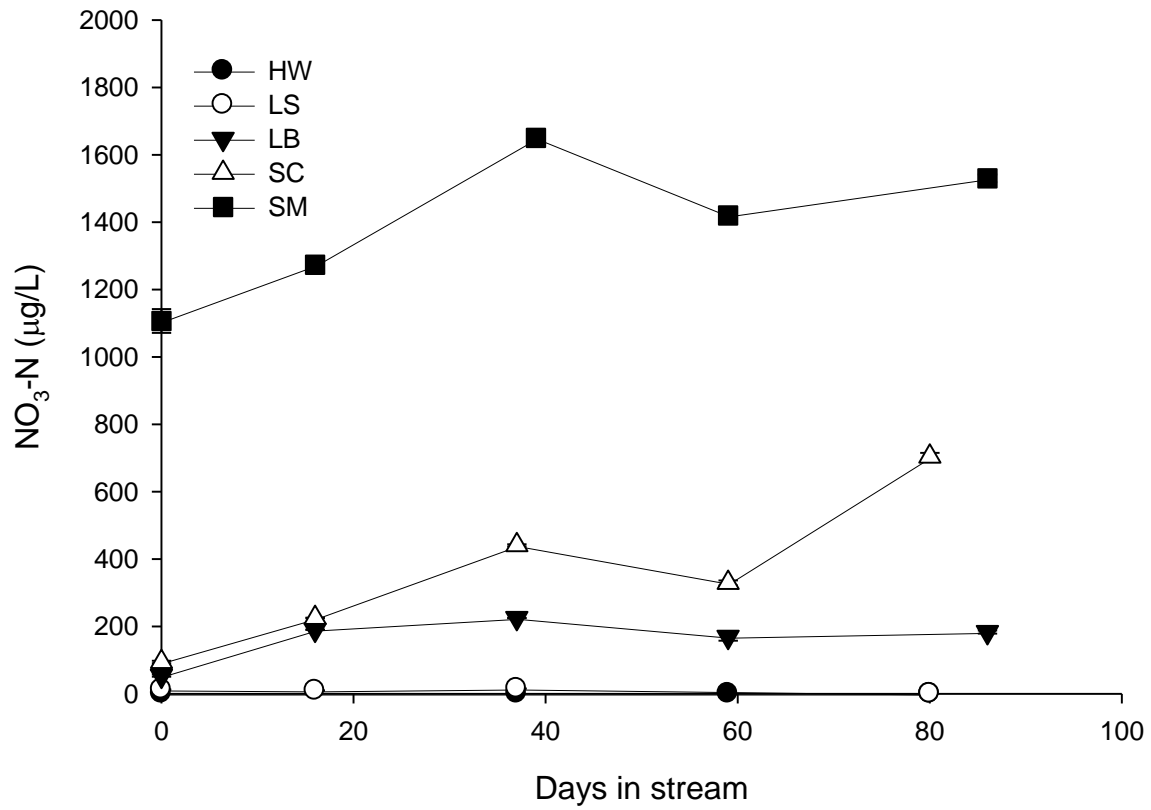


Figure 2. Mean (± 1 SE) water-column $\text{NO}_3\text{-N}$ concentration ($\mu\text{g/L}$) over time in all five study sites. $n = 3$ for each collection for each site. Symbol size exceed SE for nearly all collections.

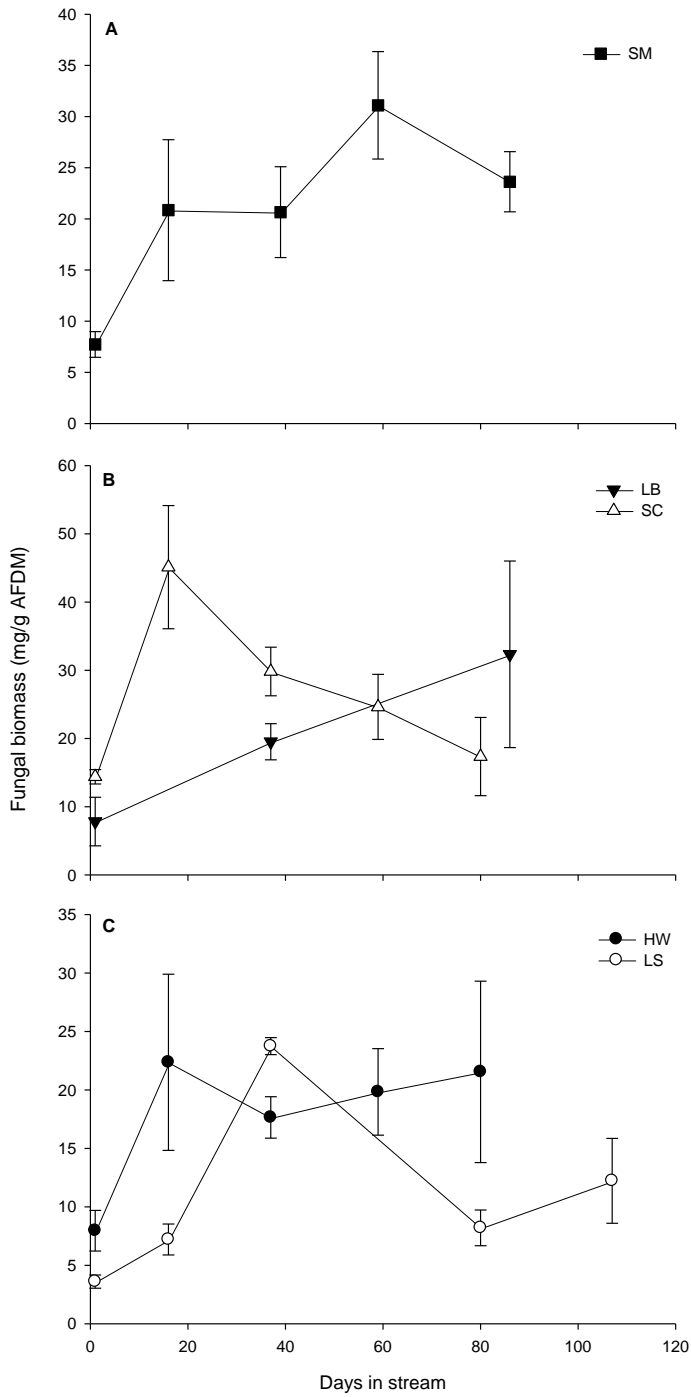


Figure 3. Mean (± 1 SE) fungal biomass (mg/g AFDM) of detritus collected from high (A), medium (B), and low (C) N streams over time. $n = 3$ for each collection from each site.

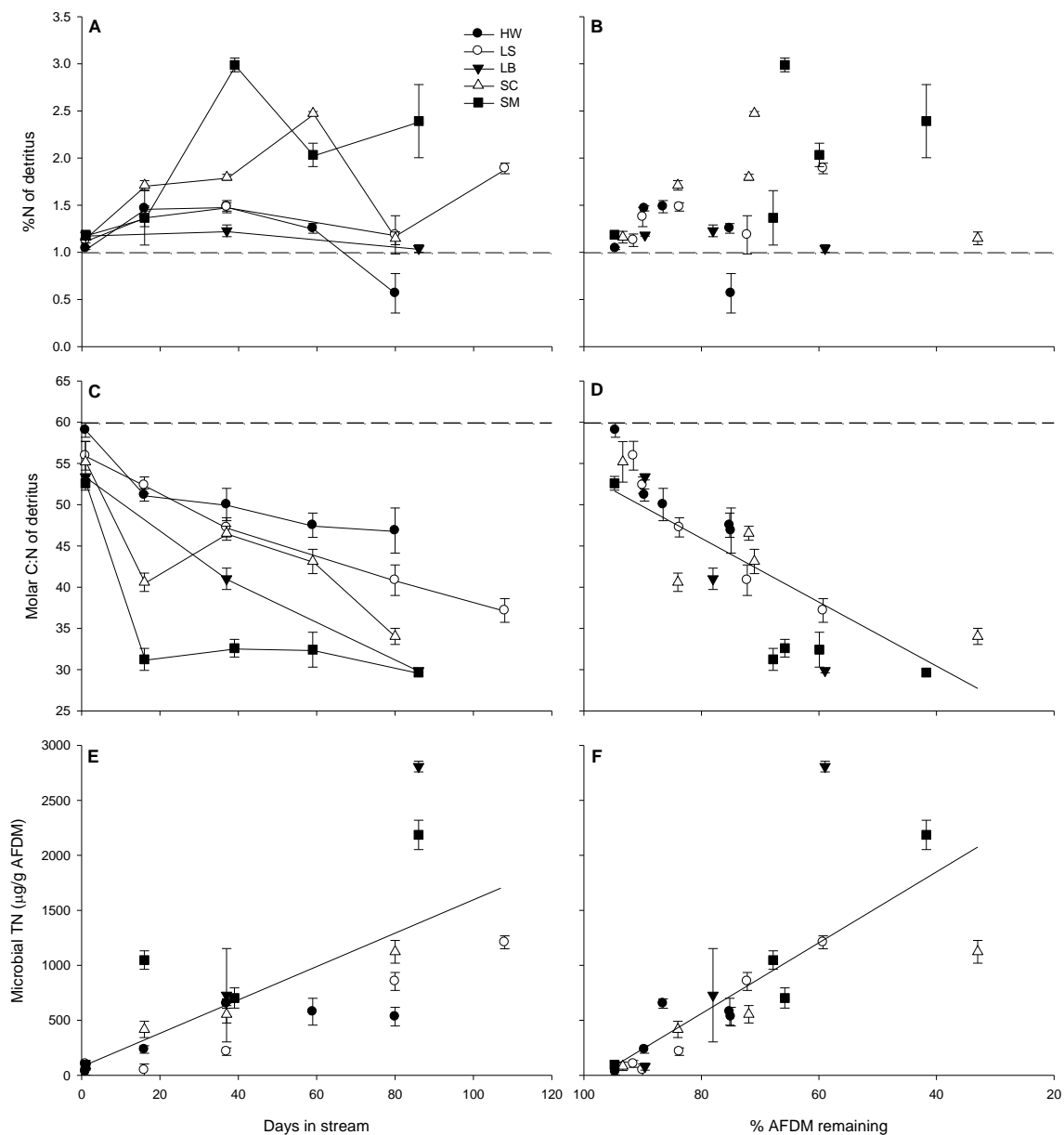


Figure 4. N standing stocks as %N (A-B) and molar C:N (C-D) of detrital AFDM and microbial biomass (E-F) over time (A,C,E) and decomposition (B,D,F). Symbols in A-D are means (± 1 SE) of 4 samples. Symbols in E-F are differences between means of 4 fumigated samples and 4 unfumigated samples ($\pm 95\%$ CI) for each collection for each site. The dashed lines in A-D represent the condition of leaves before deployment and solid lines in D-F are linear regressions including all sites with $\text{C:N} = 0.387 * \% \text{AFDM remaining} + 14.961$ ($r^2 = 0.481$, $P < 0.001$), $\text{microbial TN} = 15.173 * \text{days in stream} + 78.268$ ($r^2 = 0.540$, $P < 0.001$) and $\text{microbial TN} = -32.202 * \% \text{AFDM remaining} + 3137.561$ ($r^2 = 0.601$, $P < 0.001$). Note: % AFDM decreases from left to right in panels B,D,F.

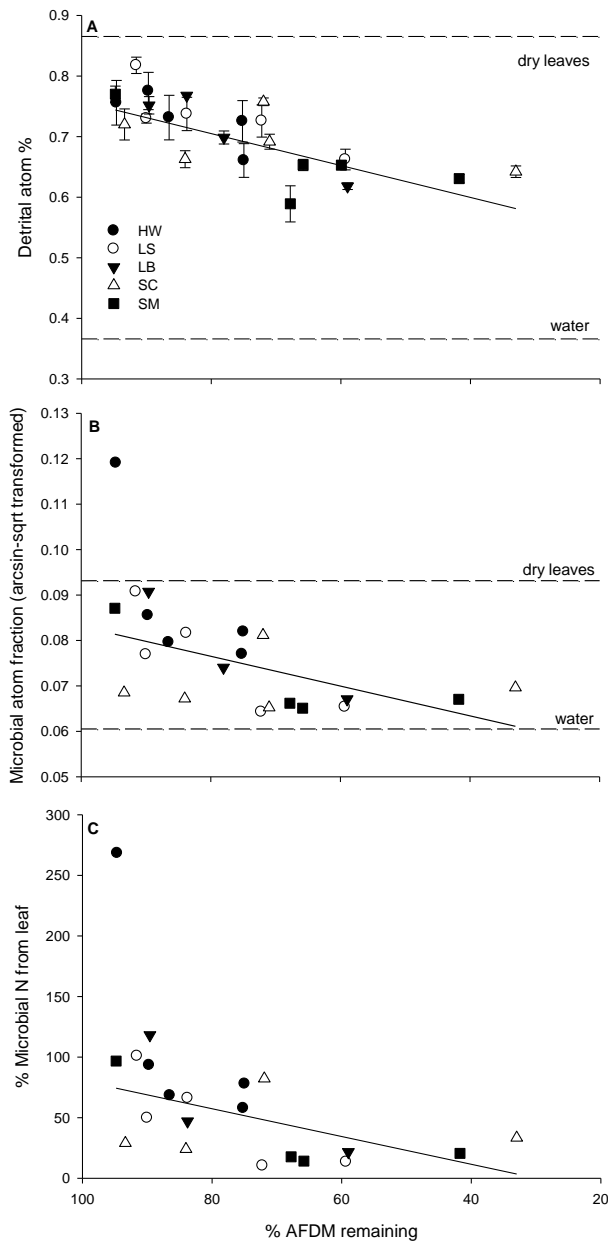


Figure 5. Relative ^{15}N abundance in detritus (A) and microbial biomass (B) and the % microbial N derived from leaves (C) over decomposition in each site. Symbols in (A) are means (± 1 SE) of 4 samples. Solid lines are regressions with detrital atom% = $0.00265 * \% \text{ AFDM remaining} + 0.505$ ($r^2 = 0.552$, $P < 0.001$), microbial atom fraction (arcsin-sqrt transformed) = $0.000328 * \% \text{ AFDM remaining} + 0.0503$ ($r^2 = 0.359$, $P = 0.004$), and % microbial N from leaf = $1.146 * \% \text{ AFDM remaining} - 34.326$ ($r^2 = 0.332$, $P = 0.008$). Regressions in B-C exclude 1st collection from HW. Dashed lines (A-B) are the values of leaves before deployment (upper line) and of stream water (lower line). Note: % AFDM decreases from left to right along X-axis.

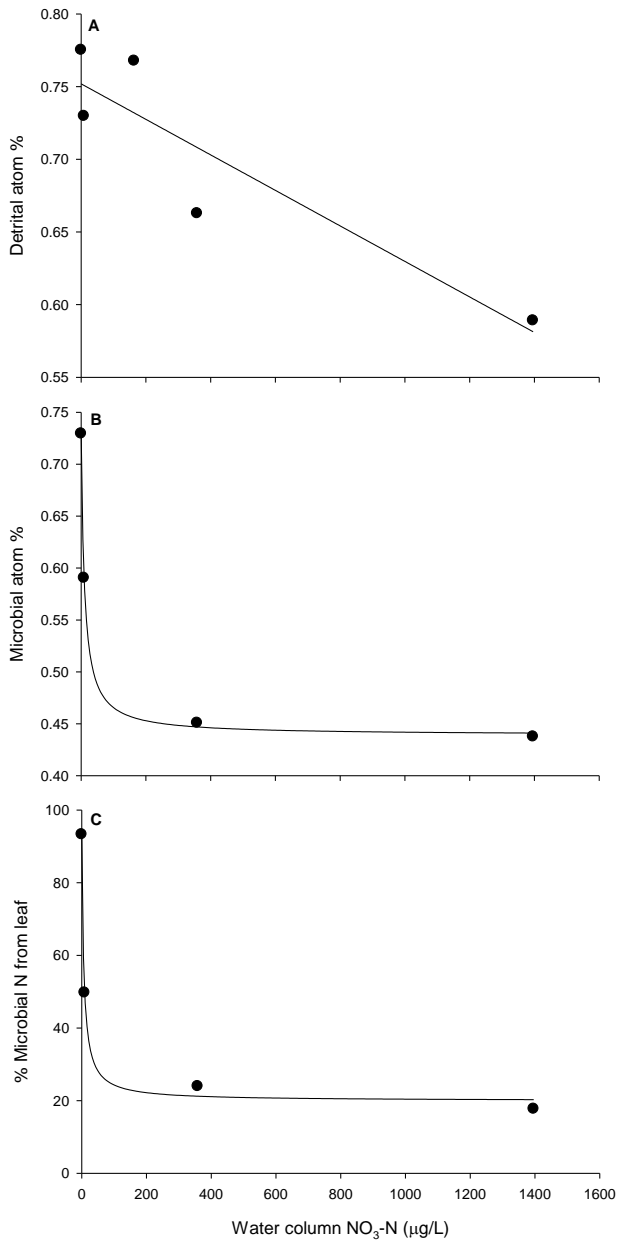


Figure 6. Response of detrital atom % (A), microbial atom % (B), and % microbial N from the leaves (C) after two weeks in stream across the gradient in NO_3^- . Ambient NO_3^- -N is the mean NO_3^- concentration in each stream over the entire study. Line in (A) is a linear regression with detrital atom % = $0.000183 * \text{ambient } \text{NO}_3\text{-N} - 0.285$ ($r^2 = 0.848$, $P = 0.026$). Lines in B-C are hyperbolic decay regressions with microbial atom % = $0.4390 + 2.924/(10.0587 + \text{ambient } \text{NO}_3\text{-N})$ ($r^2 = 0.999$, $P = 0.022$) and % microbial N from leaf = $19.9479 + 464.437/(6.3266 + \text{ambient } \text{NO}_3\text{-N})$ ($r^2 = 0.996$, $P = 0.063$). LB data in B-C were lost.

Chapter 4: Influence of animals on nutrient cycling by stream heterotrophic microbes

Abstract

Understanding the role of animals in ecosystem nutrient cycles is becoming more important as global biodiversity continues to decline. Nutrient recycling by animals can significantly contribute to inorganic nutrient standing stocks, and animal grazing can alter microbial assemblage structure and function. I investigated the effects of animal nutrient recycling and grazing on carbon and nitrogen cycling by heterotrophic microbial assemblages associated with leaf litter in streams. I manipulated the presence of two shredding macroinvertebrate taxa (amphipods and tipulids) in artificial stream mesocosms. I also manipulated nitrate concentrations in the mesocosms to determine the role of dissolved N availability in mediating animal affects. N enrichment significantly increased leaf breakdown rates. Breakdown rates did not respond to the shredder treatments. Nutrient recycling by tipulids stimulated fungal biomass specific N uptake but only under low N conditions. Grazing by amphipods suppressed fungal biomass specific N uptake, but again, this effect was only present under low N conditions. My results suggest that different shredders have may influence nutrient cycling in natural streams in different ways. Shredder influences on nutrient cycling appear to be most important when N availability is low.

Introduction

Microorganisms drive the flow of material and energy through many ecosystems. The activity of these organisms is dependent on exogenous factors, such as nutrient availability. Dissolved inorganic nitrogen (DIN) availability can increase organic matter breakdown rate (Meyer and Johnson 1983, Suberkropp and Chauvet 1995), respiration (Ferreira and Chauvet 2011), and nitrogen (N) uptake (Mulholland et al. 2008, Cheever et al. *in preparation*).

Activity by organisms of upper trophic levels may also be an important exogenous driver of microbial activity. Microbial nutrient cycling is dependent on

nutrient availability in the environment and the microbial nutrient requirements (demand) (Cheever et al. 2012). Animals may influence both of these factors through two different pathways. First, animals may influence nutrient availability through consumer nutrient recycling (CNR), the process by which animals convert nutrients from organic to inorganic forms through consumption and mineralization (e. g. Vanni 2002). The rate of regeneration of specific elements by CNR depends on the amount of this element needed to sustain the growth of the animal as well as the availability of the element in the diet relative to this requirement (Sterner and Elser 2002). CNR can contribute significantly to inorganic nutrient standing stocks. For example, zooplankton assemblages across 20 North American lakes regenerated 20% of the particulate P pool daily (Hudson et al. 1999), and Grimm (1988) showed that the invertebrate assemblage in a desert stream cycled between 15-70% of the total N retained by the system over 24 hours. Inorganic nutrients produced via CNR can also account for a large portion of the nutrient uptake flux in various types of ecosystems. Nutrients regenerated by the fish assemblage in Rio Las Marias in Venezuela accounted for 49% of the algal N demand and 126% of the algal P demand (Vanni et al. 2002) and over 75% of the demand for dissolved inorganic nutrients (McIntyre et al. 2008). In addition to supplying potentially limiting nutrients, CNR can also influence producer assemblage richness, structure, and stoichiometry (Evans-White and Lamberti 2006, Kato et al. 2007, Knoll et al. 2009).

Secondly, animals may influence microbial nutrient cycling by altering microbial nutrient demand. Animal feeding can modify the microbial assemblage composition and function. The influence of grazing on microbial assemblages has been investigated mostly in autotrophic systems where grazing can alter producer assemblage composition (Rosemond et al. 1993) and may stimulate primary production (McNaughton 1979, Gregory 1983, Lamberti and Moore 1984). Intermediate levels of grazing increased standing stocks of plant available N and stimulated microbial mineralization in African savannah ecosystems (Seagle et al. 1992, McNaughton et al. 1997, Augustine et al. 2003, Augustine and McNaughton 2006). The potential for grazing to influence heterotrophic processes such as organic matter breakdown and nutrient cycling by heterotrophic microbes is less clear. Connelly et al. (2011) found that the presence of tadpoles that graze heterotrophic biofilms associated with decomposing leaf litter did not influence

decomposition or microbial respiration rates. However, Cheever et al. (2012) suggested that differential grazing regimes may have explained differences in nutrient cycling by leaf-associated heterotrophic microbes among streams. Fungal biomass-specific N uptake and mineralization observed in this study were greatest in streams with shredders that feed by scraping mesophyll and skeletonizing leaves rather than engulfing pieces of the entire detrital complex. The former feeding mode may have maintained a low but rapidly turning over fungal biomass.

Despite the potential contribution of animals to nutrient standing stocks and fluxes, most ecosystem models have constrained the role of animals to a food-web view and not incorporated them into nutrient dynamics. My objective was to investigate the effects of and interactions between two exogenous drivers of microbial N cycling in streams, DIN availability and animal activity. I manipulated the water NO_3^- concentration and the presence of two shredders, an insect and a crustacean, in artificial streams. These two shredders may influence microbial activity through CNR and grazing pathways differently. Insects have been shown to have a greater biomass %N compared to crustaceans (Evans-White et al. 2005) and should therefore retain relatively more N (Sterner and Elser 2002), resulting in less of a contribution to the DIN pool via recycling. Amphipods and tipulids also have different feeding modes; tipulids often engulf whole chunks of detritus while amphipods tend to skeletonize leaves by grazing on the surficial microbial assemblage and mesophyll (*BMC, personal observation*). In order to assess the CNR and grazing pathways, I exposed each shredder taxa to detritus under two conditions: detritus available to shredder feeding (or shredder grazing of the microbial assemblage) and detritus protected from shredder feeding but exposed to shredder CNR. Using these two detritus types allowed me to isolate the effects of CNR as manifested in DIN availability from the effects of direct shredder grazing.

Methods

Mesocosm construction and monitoring

I used recirculating stream mesocosms to manipulate N availability and shredder presence. I had two N levels: ambient N (LOW) and elevated N (HIGH), and three

shredder levels: tipuids (+TIP), amphipods (+AMP), and no shredders (NONE). Each treatment was replicated three times, for a total of 18 mesocosms.

Each mesocosm consisted of a 2.1 m length of PVC pipe (0.39 m in diameter) cut longitudinally. End pieces were cut from hard plastic to fit the inner diameter of the pipe, glued to each end the pipe with adhesive, and sealed with silicon. Mesocosms were filled with 26 L of stream water collected from Little Stony Creek, a forested headwater stream in Giles Co., VA. A small pump (405 gal/h; Beckett Corporation, Irving, TX) was placed at the foot of each mesocosm and pumped water through black tubing to the head of the mesocosm where water flowed evenly over a square, plastic blade into the main chamber. Water flowed evenly through the main chamber and through a v-notched piece of plastic into the pump chamber. Flow in the main chamber of all 18 mesocosms averaged (\pm standard deviation) 0.14 m/sec (\pm 0.05).

Mesocosms were placed in a temperature-controlled growth chamber at 8°C. I increased the hardness of the water in the mesocosms to approximately 70 mg CaCO₃/L to make it more suitable for amphipods by adding the following solutions per L of stream water in each mesocosm (ASTM 2007): 20 ml/L of a calcium sulfate + calcium chloride solution (0.02 M of CaSO₄ and 0.02 M CaCl₂ made in Little Stony stream water), 26 µl/L of magnesium sulfate solution (0.5 M MgSO₄·7H₂O made in DI water), 1.1 ml/L of a sodium carbonate solution (1 M NaHCO₃ made in DI water), and 108 µl/L of a potassium chloride solution (0.5 M KCl made in DI water).

Water levels in the mesocosms were checked daily and additional stream water was added as needed. Hardness (as mg CaCO₃/L), concentrations of total organic carbon (TOC), NO₃-N, NH₄-N, and temperature were also monitored periodically (at least 2x per week) throughout the study. I measured NH₄-N and NO₃-N concentrations by the phenate method and the Cd-reduction method, respectively using a Lachat Quickchem flow-injection analyzer (Lachat Instruments, Loveland, Colorado; APHA 1999). TOC was measured using a heated persulfate oxidation method with an OI Model 1010 Total Organic Carbon Analyzer (APHA, 1999). Hardness of the water in each mesocosm was maintained by adding the solutions described above or stream water based on these measurements.

Leaf pack construction and deployment

I constructed packs of red maple (*Acer rubrum*) leaves using leaves collected from a single tree shortly after abscission and dried to a constant weight. I constructed packs of two different masses of dried leaves, 5 g and 10 g, and with two different mesh sizes, large meshed screen that allowed shredders access to the leaves and small meshed screen that prevented shredders from feeding on the leaves. Packs were placed in Little Stony Creek for 4 days to leach. After leaching, I placed 18 of the 5 g leaf packs per mesh size and 2 of the 10 g packs per each mesh size in each mesocosm. Packs were weighed down with cobbles. An additional 18 of the 5 g packs per mesh size per mesocosm were placed in bins of stream water collected from Little Stony Creek. I increased the hardness of the water in the bins in the same manner as the water in the mesocosms. Bins were aerated and kept in the same temperature controlled room as the mesocosms.

Leaves were allowed to leach for a week in the mesocosms and bins. After the leaching period, water in both the mesocosms and the bins was replaced with fresh stream water that had been corrected for hardness as previously described. After an additional week of leaching, 10 L of water was replaced in each mesocosm.

Shredder and nitrogen treatments

Shredders were added to the appropriate streams after the second leaching period. I added 44 tipulids to each +TIP mesocosm and 132 amphipods to each +AMP mesocosm. These densities were chosen based on previous sampling of a local stream where amphipods were found in densities of 0.6 individuals / g AFDM leaf standing stock, and average amphipod biomass was 1/3 that of average tipulid biomass (unpublished data). Therefore, my shredder treatments contained different shredder densities but had equal and realistic shredder biomass per g leaf. Tipulids (*Tipula abdominalis*) were collected from two local streams, Stonecrop Creek and Tom's Creek. Amphipods (*Gammarus spp.*) were ordered from Carolina Biological Supply Co. (Burlington, NC USA). Both species were kept in separate tanks of Little Stony stream water with leaves and aerators until being placed in mesocosms. Invertebrates that were

not placed in mesocosms were kept in aerated tanks of stream water with leaves at 8°C. Water in the tanks was changed periodically throughout the experiment.

High N mesocosms were spiked with 21 ml of a 1000 mg/L NaNO₃ spiking solution (for an addition of 21 mg NO₃-N per mesocosm) every other day. My objective was to maintain NO₃-N concentrations of approximately 800 µg/L in the high N mesocosms. The spiking solution was added to the main chamber just in front of the blade in each mesocosm. Half of the bins containing the extra leaf packs were also spiked with the same addition of spiking solution as the mesocosms. Low N mesocosms were not spiked. NO₃-N concentration in Little Stony is typically ~45 µg/L. A more complete nutrient analysis of Little Stony was presented by Cheever et al. (2012).

Leaf breakdown and fungal biomass

I collected three large mesh and three small mesh 5-g packs from each mesocosm every 2 wk following the addition of the shredders. Packs were removed from mesocosms and placed in zip-lock bags to be processed the same day. I replaced each pack with one of the same mesh size from bins with the same N treatment. I replaced the packs in order to avoid depleting the resources in mesocosms and concentrating the macroinvertebrates on fewer packs as the experiment progressed. I also added 3 tipulids and 9 amphipods to the appropriate mesocosms after each collection to replace individuals that may have been removed with the leaf packs. I based these numbers on the number of individuals added to each treatment per g leaf material at the beginning of the experiment.

For each collection day, for each mesocosm, two packs of each mesh size were dried at 60°C, weighed, and ground in a coffee grinder. A subsample of each ground pack was weighed, ashed at 550°C for 2 h, and reweighed to obtain ash free dry mass (AFDM).

Leaves from the third 5-g pack of each mesh size were subsampled for ergosterol. I cut 10 leaf disks (2 cm diameter) from each pack and placed 5 disks in 5 ml of methanol. These samples were frozen and later analyzed for ergosterol. Ergosterol was extracted from these samples using a liquid phase extraction, quantified using high performance liquid chromatography (HPLC), and converted to fungal biomass (Gulis and

Suberkropp 2006). AFDM was obtained from the remaining 5 disks according to the method described above. Fungal biomass was expressed as mg fungal biomass per g AFDM of detritus extracted. The remaining leaf material in the packs was dried and ashed according to the method described above. The AFDM of the removed disks was added to the final AFDM of the subsampled packs.

The breakdown rate of red maple leaves in each mesocosm was calculated as the slope of the natural logarithm of the percent AFDM remaining after each collection over time (Benfield 2006).

Microbial uptake assay

At the end of 8 wk, the 10-g packs were removed over a period of 4 days (1 per day). Upon removal, packs were opened, any invertebrates were removed, and the leaf material collected from each mesocosm was placed in individual glass tanks with 4 L of filtered water from the corresponding mesocosm. After a ~12 h acclimation period, I spiked each glass tank with 10 µg of NH₄-N (added as NH₄Cl). I collected 3 filtered (Whatman GF/F) 10 ml samples from each tank before spiking, immediately after spiking, and 15, 30, 45, 90, 150, and 240 min after spiking. Samples were frozen until analyzed for NH₄-N concentration (using the phenate method) using a Lachat Quickchem flow injection analyzer (Lachat Instruments, Loveland, CO, USA; APHA, 1999). 3 post-assay samples were taken at least 4 h after the final sample. Leaves from each tank were dried at 60°C, weighed, ground, and subsampled. Each subsample was ashed and weighed to obtain AFDM. I repeated this assay twice for each mesh size for each mesocosm over 4 days.

For each trial, I calculated NH₄⁺ uptake rate (k_t , min⁻¹) as the slope of the line describing the natural logarithm of nutrient concentration over time for each mesocosm (O'Brien and Dodds 2008). I calculated uptake flux (U ; µg nutrient min⁻¹ g⁻¹ AFDM) from uptake rate and the average ambient nutrient concentration in mesocosm from which the leaves were collected (C_{amb} ; µg/L) as:

$$U = k_t C_{amb} V / L$$

where V is the volume of water in each tank (L) and L is the leaf mass in the tanks (g AFDM). C_{amb} was estimated from the samples taken from each tank before the addition

of the spike. I also calculated fungal biomass-specific (FBS) uptake. I divided uptake flux by the total fungal biomass in each tank, which I estimated by scaling the estimates of fungal biomass (mg fungal biomass/g AFDM leaf) from ergosterol extractions to the AFDM of leaves in the tanks.

Statistical analysis

I used two-way analysis of variance (ANOVA) tests to compare mean leaf breakdown rate, fungal biomass, NH_4^+ uptake rate, FBS uptake flux, and FBS mineralization flux among N and shredder treatments for leaf packs of each mesh size. All statistical tests were done using SigmaPlot with SigmaStat Integration (version 10; Systat Software Inc, Chicago, Illinois).

Results

Fungal biomass generally increased through time for both mesh sizes in all treatments (Fig. 1A). Increased $\text{NO}_3\text{-N}$ concentration influenced fungal biomass accrual differently depending on the mesh size. Fungal biomass on leaves in large mesh packs was significantly greater in the high N streams compared to the low N streams (repeated measures ANOVA, $p = 0.037$). This relationship was present for the +AMP and NONE treatments ($p < 0.029$), and was marginally significant for the +TIP treatment ($p = 0.056$). Shredders did not influence fungal biomass within N treatments. There were no significant differences in fungal biomass among the shredder treatments in the high N mesocosms ($p > 0.304$) or the low N mesocosms ($p > 0.583$). There was no effect of N enrichment on fungal biomass on leaves in small mesh packs (Fig. 1B). Fungal biomass was similar among N treatments ($p = 0.063$) and among shredder treatments within high N treatments ($p = 0.628$).

The response of leaf breakdown rate to N treatments also differed among mesh sizes. Leaves in large mesh packs broke down over 2x as fast in mesocosms with elevated N compared with leaves in low N mesocosms regardless of shredder treatment (Fig. 2A). Breakdown rates in high N mesocosms were significantly faster for the +AMP (two-way ANOVA with Tukey post-hoc; $p = 0.015$) and +TIP (two-way ANOVA with Tukey post-hoc; $p = 0.006$) treatments. The response of leaf breakdown in large mesh packs to N

treatment was nearly significant in mesocosms with no shredders (two-way ANOVA with Tukey post-hoc; $p = 0.053$). Breakdown rate of leaves in small mesh packs did not respond to elevated N or to the presence of shredders (Fig. 2B). Breakdown rate was similar among N treatments (two-way ANOVA; $p = 0.079$) and shredder treatments (two-way ANOVA; $p = 0.107$) with no significant interaction between treatments ($p = 0.465$).

N cycling by leaves in large mesh packs responded to the $\text{NO}_3\text{-N}$ concentration in the mesocosms. $\text{NH}_4\text{-N}$ uptake rate (min^{-1}) was highly variable but was faster in mesocosms with low compared to high NO_3^- availability (two-way ANOVA, $p = 0.008$; Fig. 3A). This trend was significant in mesocosms with amphipods (two-way ANOVA with Tukey post-hoc; $p = 0.012$) but not in the +TIP or NONE mesocosms (two-way ANOVA with Tukey post-hoc; $p > 0.110$). Leaves from large mesh packs also had a greater uptake flux ($\mu\text{g NH}_4\text{-N min}^{-1} \text{g}^{-1} \text{AFDM}$) under high N conditions when shredders were present. The uptake flux from leaves in +AMP and +TIP was 10x greater in mesocosms with high NO_3^- concentration (two-way ANOVA with Tukey post-hoc; $p < 0.012$; Fig. 4A). There was no difference in uptake flux from leaves in large mesh packs among high and low N treatments in mesocosms with no shredders present (two-way ANOVA with Tukey post-hoc; $p = 0.289$).

N cycling by leaves from small mesh packs responded differently to NO_3^- availability compared to leaves from large mesh packs. Uptake rate (min^{-1}) was faster in mesocosms with high compared to low NO_3^- availability (Fig 3B). Uptake rate in high N streams was over 2x faster compared to low N mesocosms in +AMP and +TIP treatments, although neither N treatment nor shredder treatment was significant (two-way ANOVA, $p > 0.215$). Uptake flux ($\mu\text{g NH}_4\text{-N min}^{-1} \text{g}^{-1} \text{AFDM}$) by leaves in small mesh packs was also similar among N and shredder treatments (two-way ANOVA, $p > 0.656$; Fig. 4B).

However, this response of N cycling by leaves in small mesh packs reversed when I calculated uptake flux per mass fungal biomass instead of per mass detritus (Fig 5). FBS uptake flux ($\mu\text{g NH}_4\text{-N min}^{-1} \text{g}^{-1} \text{fungal biomass}$) by fungi on leaves from small mesh packs was 5-10x greater in the low N compared to high N mesocosms. The difference between FBS uptake in low versus high N treatment was significant in the +TIP mesocosms (two-way ANOVA with Tukey post-hoc; $p = 0.004$; Fig. 5B). Unlike uptake

based on detrital mass, FBS uptake responded to shredder treatment within the low N mesocosms for both mesh sizes, although the pattern of the response differed depending on mesh size. For large mesh packs, fungi in low N streams with no shredders took up significantly more $\text{NH}_4\text{-N}$ per g fungal biomass than fungi exposed to amphipod grazing (two-way ANOVA with Tukey post-hoc; $p = 0.036$; Fig. 5A). For small mesh packs, FBS uptake was over 2x that of both the +AMP and NONE treatments, although the difference was only significant between the +TIP and NONE treatments (two-way ANOVA with Tukey post-hoc; $p = 0.047$; Fig. 5B).

Discussion

This study explored the effects of two exogenous drivers of microbial activity during decomposition: N availability and the activity of animals. My results indicate that microbial activity responds to both of these factors but in different ways. I also observed interactions between these factors.

Influence of animal activity

Shredders did not appear to influence leaf breakdown rate in either the small or large packs. The lack of effect of shredder treatments on leaf breakdown in large mesh packs was unexpected. Macroinvertebrate feeding plays a significant role in mass loss from leaf packs in natural streams (Webster and Benfield 1986, Cuffney et al. 1990). Although there was no significant effect of shredder treatment on leaf breakdown in my study, I did see generally lower breakdown rates in small packs (where shredders were excluded) compared to large mesh packs. The lack of significant shredder influence on leaf breakdown in my study may have been due to insufficient shredder biomass in the mesocosms or shredder mortality. Surveys of invertebrate density were impossible during the course of the experiment, but several living individuals of both taxa were found in leaf packs at each collection and were collected at the end of the experiment. Shredders may not have been actively feeding in the mesocosms. I did not measure the growth of individuals, but leaves collected from packs did show evidence of shredder activity (e. g. skeletonization).

Animal activity did influence N cycling through both CNR and grazing. Shredder

CNR stimulated FBS uptake. This response may have been due to an increased DIN availability resulting from the contribution of shredder CNR. Greater N availability can increase N uptake flux to the benthos in natural streams (Earl et al. 2006, Mulholland et al. 2008), and Evans-White and Lamberti (2005) demonstrated an increase in inorganic N uptake by algae due to macroinvertebrate CNR. However, the CNR-induced higher FBS uptake was only significant in the +TIP treatments in my study. This result was unexpected; I predicted that CNR by amphipods would contribute more to the DIN pool and would thus affect N uptake more strongly compared to tipulids. Amphipods are crustaceans, which have been shown to have a lower biomass %N compared to insect taxa (Evans-White et al. 2005). Ecological stoichiometry theory predicts that body stoichiometry dictates the rate at which organisms recycle nutrients (Sterner and Elser 2002). The greater influence of CNR by tipulids in my study may indicate that amphipods were feeding at different rates in the mesocosms than in natural streams. If amphipod feeding was inhibited in the mesocosms, their rates of nutrient recycling may not have been comparable to what has been seen in natural systems.

Grazing of the microbial assemblage also influenced N cycling. Lamberti and Moore (1984) presented a general application of optimal grazing theory (McNaughton 1979) to epilithic autotrophic biofilms in streams. According to their model, intermediate levels of grazing maintains biofilms with smaller biomass but high productivity. However, the response of heterotrophic assemblages to shredder feeding did not follow this model in my study. Shredder feeding generally decreased FBS uptake. This response was significant in the amphipod treatments. Shredders did not suppress fungal biomass, which was statistically similar among shredder treatments but was generally higher in treatments containing shredders compared to the non-shredder control. Shredder feeding in my study was not intense enough to suppress fungal biomass, but also did not seem to illicit the functional response predicted by intermediate grazing theory. Intermediate grazing theory may not apply to shredders who engulf detritus, such as tipulids. Tipulids remove whole fragments of detritus, leaving the microbial assemblage on the remaining detritus relatively intact. Therefore, the microbial assemblage assessed for N cycling in our study had not been grazed by tipulids. However, although amphipod feeding is more analogous to grazers who feed on autotrophic biofilms (i.e. they remove surficial

microbial biomass), amphipods did not produce the expected response.

Interaction with N availability

N availability was a more important driver of microbial decomposition compared to animal activity, generally stimulating leaf breakdown rate. Several studies have shown a similar response of leaf breakdown rates to N availability (Meyer and Johnson 1983, Suberkropp and Chauvet 1995, Gulis and Suberkropp 2003), suggesting that microbial decomposition may be N limited in some streams. However, my results suggest that grazing by shredders may mediate the response of leaf breakdown to N availability. Differences in leaf breakdown between N treatments were only significant when animals were present and allowed access to the leaves (large mesh packs). The mechanism by which animal feeding may mediate the influence of N availability is unclear. Shredders in mesocosms may have increased the turnover rate of the microbial biomass, resulting in increased microbial respiration in these treatments supported by the elevated N availability. The interaction between N availability and macroinvertebrate feeding may be an artifact of the mesocosms. Breakdown rate of maple species in natural streams can respond to N availability without the influence of macroinvertebrates (Cheever et al. *in preparation*).

While macroinvertebrates seemed to alter the effect of N availability on leaf breakdown, N availability altered the response of N uptake to both shredder CNR and feeding activity. Differences in FBS uptake among shredder treatments disappeared in the high N mesocosms for both the large mesh and small mesh packs. High N availability significantly suppressed FBS uptake and generally increased fungal biomass for both mesh sizes compared to ambient N conditions. These results agree with models that suggest that assemblages with high biomass may be less productive and cycle nutrients less efficiently. Cheever et al. (2012) described a similar trend among a series of natural streams spanning a gradient of ambient N availability. They found the greatest FBS uptake by heterotrophic microbes on leaves collected from the streams with the lowest NO₃-N concentrations.

Conclusions

I found that animals have the potential to influence nutrient cycling by heterotrophic microbes in streams via both CNR and grazing. CNR stimulated biomass-specific nutrient cycling. Shredder feeding also influenced N cycling but not in the ways predicted by optimal grazing theory. My results indicate that animals do not influence heterotrophic and autotrophic assemblages in the same way. This suggests that nutrient cycling in streams that change from heterotrophic to autotrophic states due to land use shifts in the catchment or riparian areas will respond differently to an exogenous driver.

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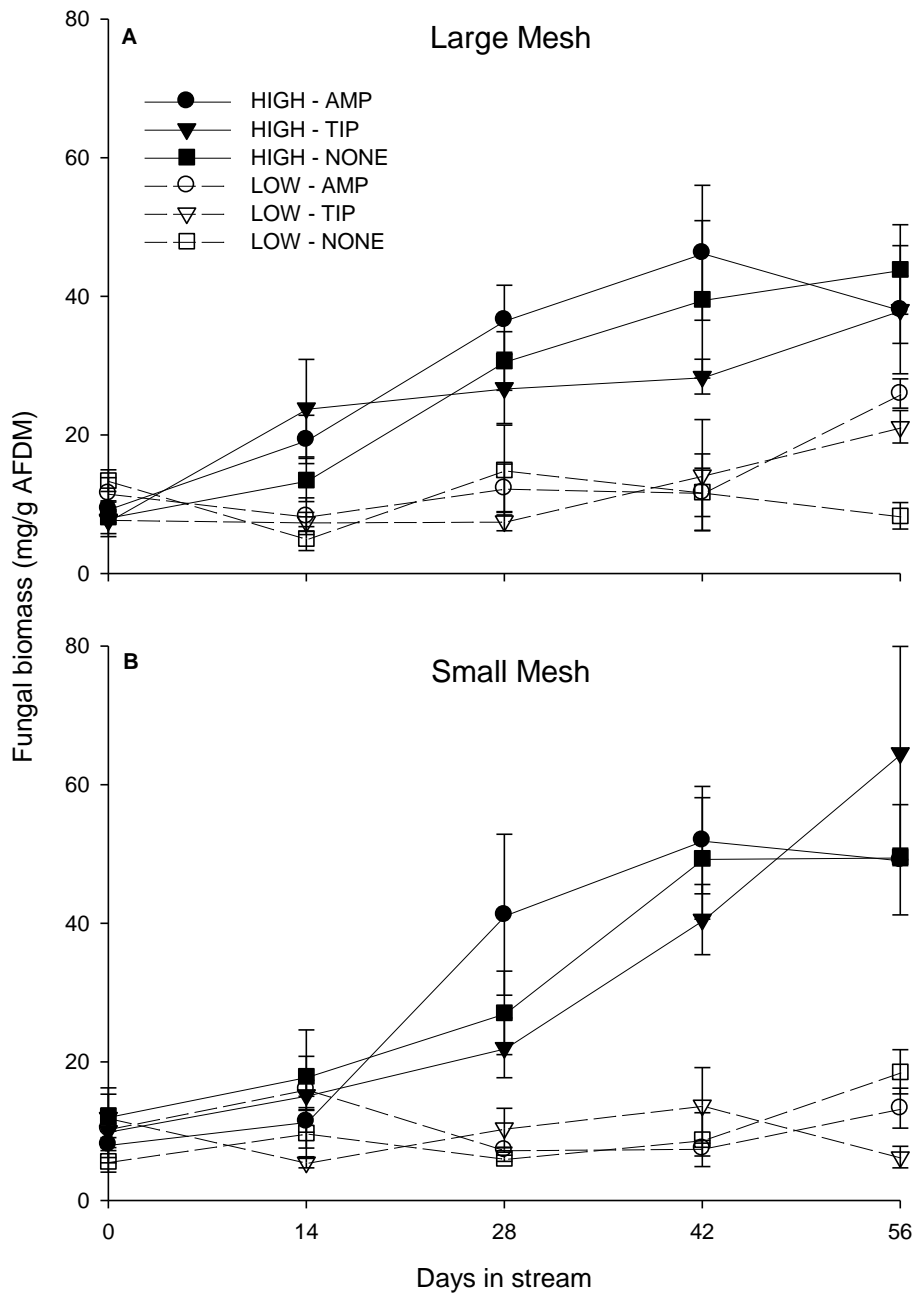


Figure 1. Mean fungal biomass (\pm SE) from leaves in large mesh packs (A) and small mesh packs (B) for N and shredder treatments over the course of the study. $n = 3$ for each collection.

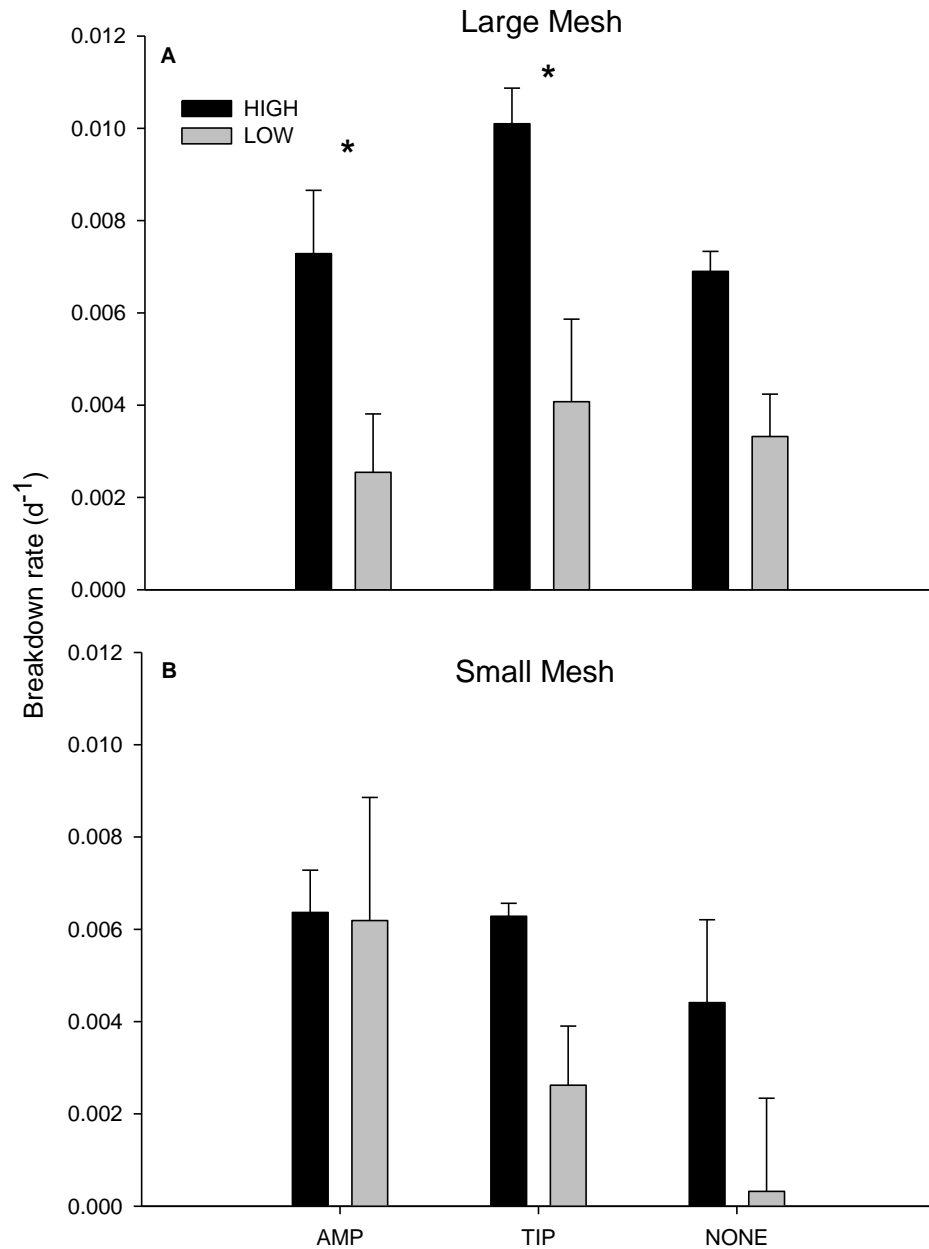


Figure 2. Mean (\pm SE) breakdown rate (d^{-1}) of leaves from large mesh (A) and small mesh (B) packs for each treatment. $n = 3$ mesocosms for each treatment. Asterisks indicate significant differences (two-way ANOVA, $p < 0.05$) between high and low N (HIGH and LOW) treatments within the same shredder treatment.

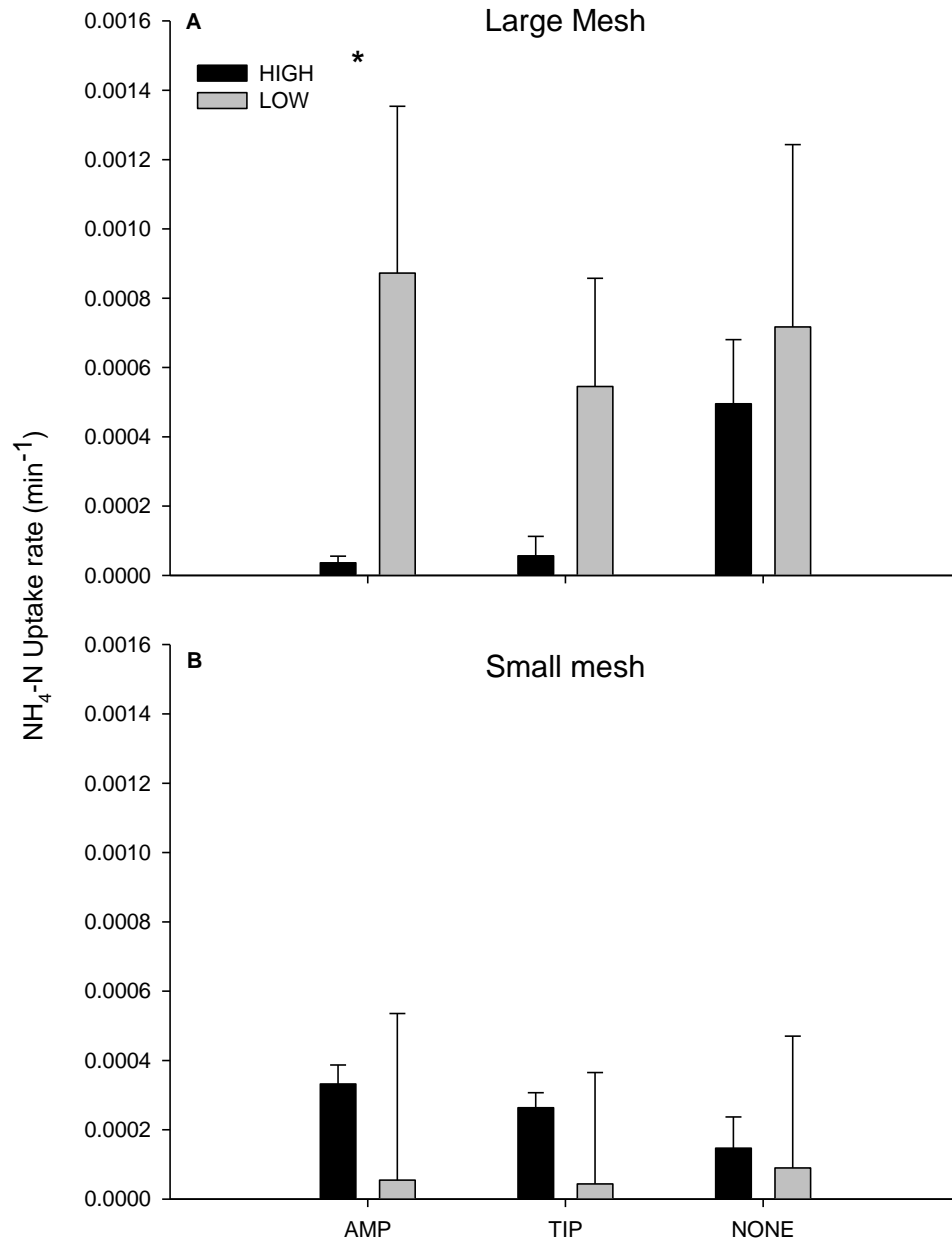


Figure 3. Mean NH₄-N uptake rate (\pm SE) by leaves in large mesh (A) and small mesh (B) packs in each treatment. $n = 3$ mesocosms for each mean. Asterisk indicates significant differences (two-way ANOVA, $p < 0.05$) between HIGH and LOW treatments within the same shredder treatment.

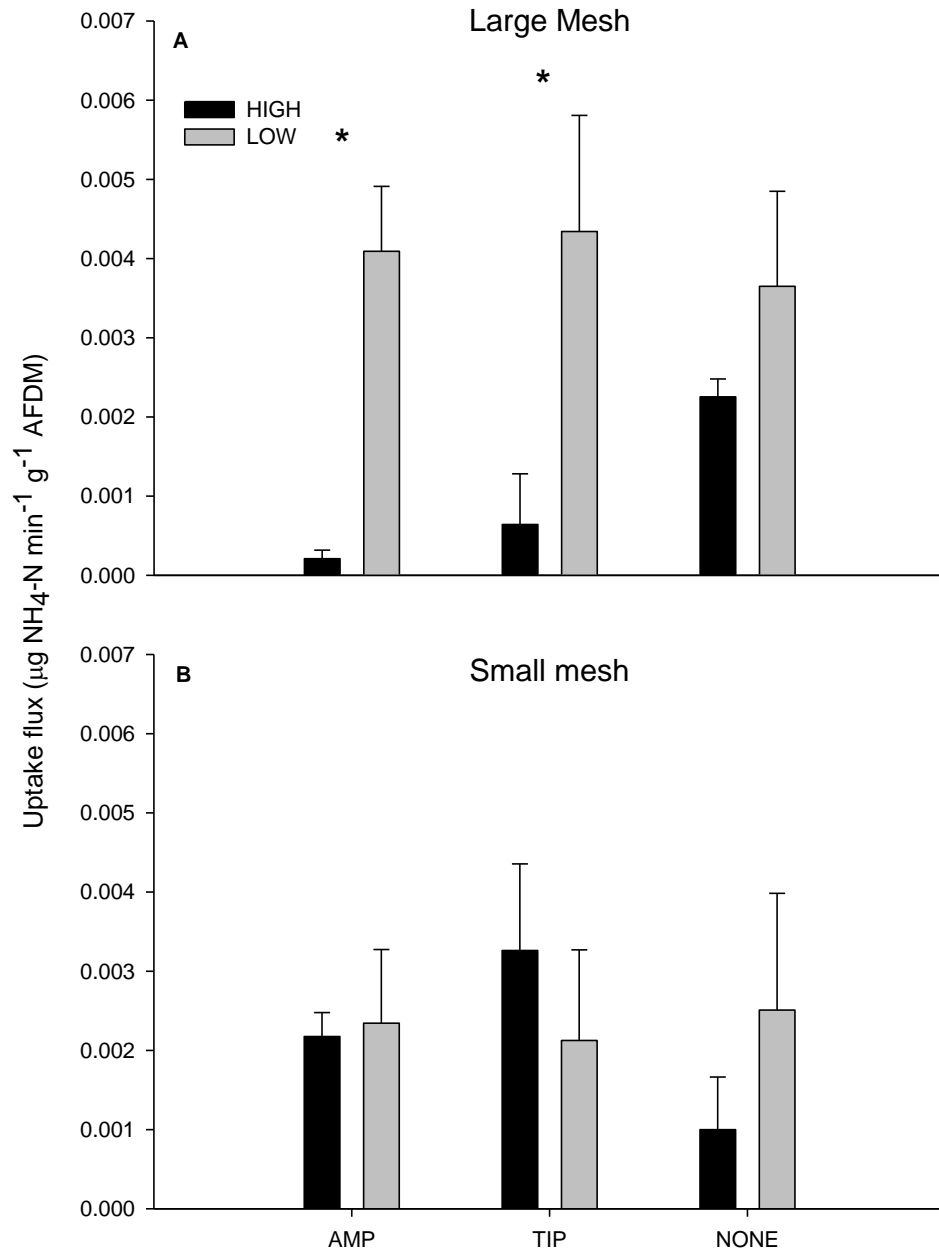


Figure 4. Mean NH₄-N uptake flux (\pm SE) by leaves in large mesh (A) and small mesh (B) packs in each treatment. $n = 3$ mesocosms for each mean. Asterisk indicates significant differences (two-way ANOVA, $p < 0.05$) between HIGH and LOW treatments within the same shredder treatment.

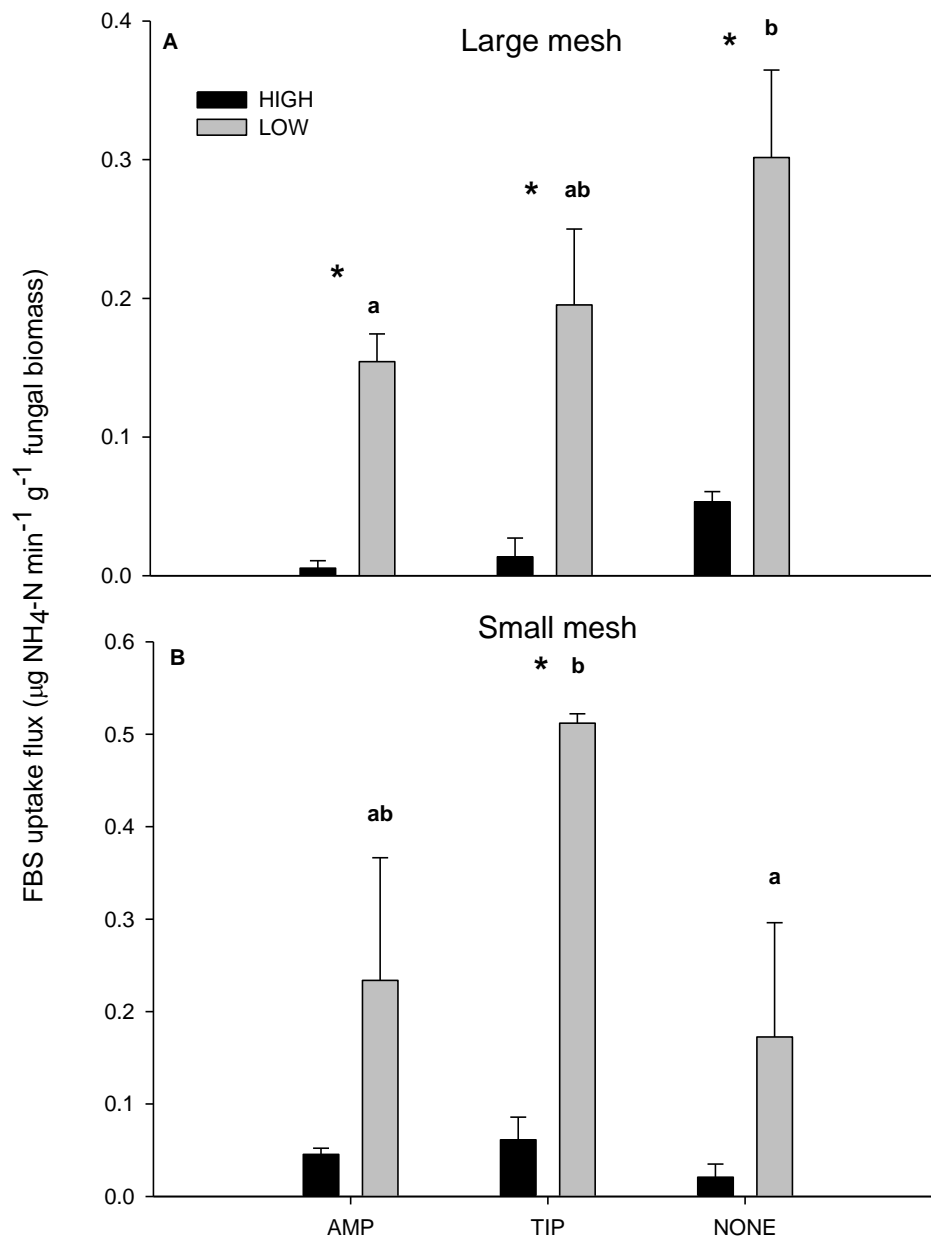


Figure 5. Mean FBS NH₄-N uptake flux (\pm SE) by leaves in large mesh (A) and small mesh (B) packs in each treatment. $n = 3$ mesocosms for each mean. Asterisk indicates significant differences (two-way ANOVA, $p < 0.05$) between HIGH and LOW treatments within the same shredder treatment. Lowercase letters indicate significant differences among shredder treatments within the same N treatment (two-way ANOVA, $p < 0.025$).

Chapter 5: Synthesis

Ecosystem ecology seeks to understand the flow of nutrients and energy through ecosystems and to predict how these flows are likely to respond to anthropogenic perturbations. Biotic transformations of matter are at the heart of these flows; therefore understanding the factors that drive the rates and outcomes of biotic processes is essential to accomplishing these goals.

I used general ecological stoichiometry theory to investigate the factors influencing microbial nutrient processing during organic matter decomposition. My conceptual approach treats the detritus-associated microbial assemblage as a “black boxed” unit whose use of N (and other nutrients) is analogous to that of a single organism where N acquisition and release are determined by assemblage requirements relative to N availability. While this approach reduces a taxonomically and perhaps functionally diverse assemblage to a single functional unit, it is necessary for systems such as detritus-associated microbial assemblages where measuring functions of individual taxa is not feasible, and provides useful insights regarding nutrient cycling at the ecosystem scale.

This research centered around two common themes. First, microbial N dynamics are driven by both the N demand of the microbial assemblage and the availability of N in various environmental pools. The study that quantified N and P immobilization and mineralization over decomposition in the 5 study sites used stream NO_3^- concentration as an indication of N availability and the accumulation of fungal biomass over the course of decomposition as an indication of microbial demand. Results of this survey suggested that the water column nutrient pool may not be the dominant pool for meeting microbial nutrient demand. The second study investigated an additional pool available for microbial use: the substrate. Results demonstrated that the pool used to meet microbial requirements changes over decomposition. This study showed that microbes use substrate-derived N during the initial stages of decomposition and immobilization of N from the water column becomes more important as decomposition progresses. Finally, the laboratory mesocosm study investigated the influence of upper trophic levels on microbial nutrient dynamics. Direct manipulations of stream water DIN and shredders showed both the strong influence of water column N on microbial activity and potential

for animals to alter microbial function through nutrient recycling and grazing of the microbial assemblage.

These results can be placed in the context of heterotrophic microbial assemblage development during organic matter decomposition (Figure 1). Organic matter decomposition occurs in three main phases: the leaching phase where soluble compounds are lost via chemical leaching, the conditioning phase where microbes colonize detritus and mineralize carbon, and the animal processing phase where animal foraging contributes to mass loss (Petersen and Cummins 1974). Heterotrophic microbial assemblages develop in the context of this continuum. Microbial biomass is low during the colonization phase, increases rapidly using labile detrital carbon pools, and levels off as detrital carbon becomes more recalcitrant.

My research makes a significant contribution to this model by describing N fluxes over this continuum. I propose that microbes acquire leaf-derived N primarily from leached materials. Initial colonizers use leachates to become established on detrital surfaces, resulting in assimilation as a dominant flux and a corresponding high proportion of leaf-derived N in microbial biomass during the first 24 h of decomposition. The importance of assimilation quickly declines once the leaching phase is over, although it is important to note that it never completely stops in my model; leaching, and therefore the assimilation of leachates, continues during decomposition as microbial activity exposes new surfaces. As assimilation declines, microbes begin to immobilize exogenous N to support rapid growth. This shift occurs rapidly, and immobilization is the dominant source of microbial N soon after the leaching phase is completed. Immobilization continues to be an important source of N as microbial biomass continues to accumulate, but may plateau or even decline during the later stages as detritus begins to release or mineralize N (Manzoni et al. 2010).

This model is a robust description of N cycling by detritus-associated heterotrophic microbes during organic matter decomposition that can be assessed across ecosystems. The immobilization of exogenous N is likely important in all ecosystems due to the stoichiometric imbalance between microbial biomass and detritus common in many systems (Sturner and Elser 2002). However, the shape and slopes of the assimilation and immobilization curves in the model may vary across terrestrial, lentic, and lotic

ecosystems. The availability and temporal variability of dissolved exogenous N is different among these ecosystem types. Exogenous N in terrestrial ecosystems is dependent on precipitation regime and the hydrologic storage capacity of the litter layer. Endogenous N sources may be more important in these ecosystems. Exogenous N in lentic ecosystems may be abundant, particularly in eutrophic systems, but this pool may become depleted over time and may limit immobilization during the later stages of decomposition. Microbes in this situation may ramp up assimilation, or may be limited by a lack of N. The isotopic labeling method that I employed in this research provides a novel and an elegant way of testing this model in these ecosystems.

The second theme of my research is to place microbial N cycling during decomposition in the broader context of global change, specifically increases in biologically available N. Results of my work revealed two implications of increased biologically available N. First, I observed a general increase in the importance of water-column derived N in supporting microbial growth across the natural gradient of NO_3^- availability. This result suggests that the shape of the curves describing the assimilation of endogenous N and immobilization of exogenous N in Figure 1 may change as N loading increases. However, I also observed the greatest uptake and mineralization fluxes in situations of low N availability in both natural streams and mesocosms. These results suggest that while water-column derived N may become more important with elevated N availability, an overall decrease in microbial turnover may also occur. In other words, increases in N availability may result in greater amounts of microbial biomass that cycle N less efficiently. This synthesis agrees with the conclusion of the LINX II (Lotic Inter-site Nitrogen Experiment) study, which showed that v_f , a metric often used as a measure of uptake efficiency, decreased as stream NO_3^- concentration increased and areal uptake of N by stream reaches increased from low to intermediate NO_3^- concentrations, but plateaued at high concentrations (Mulholland et al. 2008). Mine and the LINX II findings suggest that the ability of streams to process N will be inhibited as anthropogenic N loading to lotic systems continues. My results also suggest that increased N loading to lotic systems may override the influence of other drivers, such as consumer nutrient recycling or feeding on microbial activity.

My results also have implications for feedbacks between increasing N availability and carbon processing. My results show that increasing N availability may lead to faster organic matter decomposition and greater CO₂ fluxes from stream ecosystems. Results from the natural gradient and direct manipulations of NO₃⁻ concentrations support this conclusion.

In conclusion, my use of isotopically labeled organic matter substrates provides an elegant and straightforward method for describing N dynamics in heterotrophic systems. Results from my research provide a robust model of N cycling by heterotrophic microbes during organic matter decomposition and reveal implications of alterations in the global N cycle for N processing, coupled C and N cycles, and the role of animals in heterotrophic ecosystems.

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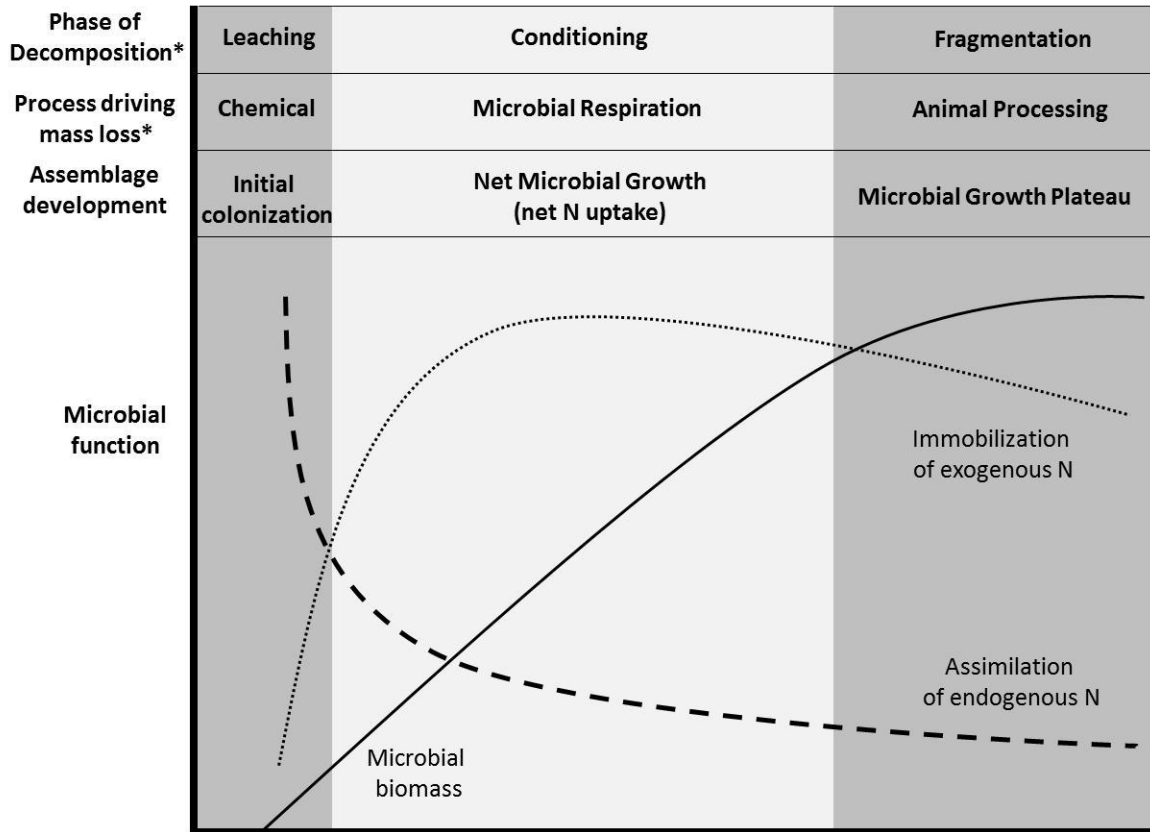


Figure 1. Conceptual model describing changes in microbial functions including immobilization of exogenous N (dotted line) and assimilation of endogenous N (dashed line) as microbial biomass (solid line) develops over the course of decomposition. Top panels provide stage of decomposition, mass loss processing, and heterotrophic microbial development context.

*Stage of decomposition and processes are adapted from Petersen and Cummins (1974).