

Effects of consumers and nitrogen availability on heterotrophic microbial activity during leaf decomposition in headwater streams

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SUMMARY

1. Microbial processes in streams may be influenced by bottom-up factors, such as nutrient availability, and by top-down factors, such as the activity of upper trophic levels. The latter pathway has been well described between autotrophs and grazers, but the effects of consumers on heterotrophic basal trophic levels may also be important. Consumers may influence microbial activity through consumption of microbial biomass and by altering nutrient availability via consumer nutrient recycling (CNR).

2. We investigated the effects of two shredder taxa, *Gammarus* spp. and *Tipula* spp., on nitrogen (N) uptake during organic matter decomposition by leaf-associated heterotrophic microbial assemblages under conditions of ambient and enriched N availability in stream mesocosms. Colonised red maple leaves were placed in mesocosms with low (<20 $\mu\text{g L}^{-1}$) or high (>800 $\mu\text{g L}^{-1}$) nitrate concentration in the presence of amphipods, tipulids or no shredders. To test the importance of consumption and CNR, leaf packs were constructed from both coarse and fine mesh, which respectively allowed or prevented shredders from consuming detritus.

3. Nitrogen enrichment generally increased red maple breakdown rates, while shredder influence on leaf breakdown was taxon specific. Feeding by tipulids increased red maple breakdown rate compared with amphipods, although not significantly. While feeding by amphipods did not directly influence breakdown rates, amphipod nutrient recycling stimulated leaf breakdown rate by the same magnitude as the N-enrichment treatment.

4. Fungal biomass increased with N enrichment. However, this did not lead to changes in N uptake, which was extremely variable and did not respond significantly to either treatment. In general, N uptake (per leaf mass) was greatest under enriched N conditions when tipulids were present.

5. Our results indicate that both top-down and bottom-up factors influence microbial activity in heterotrophic systems and suggest that consumers may play important roles in detrital processing, not only through direct consumption but also through nutrient recycling.

Keywords: consumer nutrient recycling, decomposition, microbes, nitrogen cycling, streams

Introduction

Interactions between bottom-up and top-down factors have been shown to be important in regulating the structure and function of autotrophic aquatic microbial assemblages (Lamberti & Resh, 1983; Power, 1992; Rosemond, Mulholland & Elwood, 1993; Schindler *et al.*,

1997). The relative roles of bottom-up and top-down factors may also be significant in detrital-based food webs. Temperate, forested, headwater streams are classic examples of detrital-based heterotrophic systems. The decomposition and transport of seasonal inputs of allochthonous carbon (C) in these streams occurs in a series of well-described stages (Kaushik & Hynes, 1971):

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chemical leaching, respiration by heterotrophic fungi and bacteria, and the physical fragmentation and respiration by macroinvertebrates. The cycling of nutrients such as nitrogen (N) is coupled with the processing of this detrital C by the metabolic requirements of the organisms feeding on the relatively N-poor leaf material (e.g. Cheever *et al.*, 2013). Heterotrophic cycling of both C and N is probably affected by interactions between bottom-up influences such as nutrient availability and top-down influences such as consumer activity.

Litter decomposition in streams is often limited by nutrient availability. Increasing dissolved nutrient concentration has been shown to stimulate litter breakdown rate as well as microbial community characteristics such as biomass, production and respiration, both in comparisons across natural nutrient gradients (e.g. Meyer & Johnson, 1983; Suberkropp & Chauvet, 1995; Weyers & Suberkropp, 1996) and in experimental enrichments (e.g. Elwood *et al.*, 1981; Gulis & Suberkropp, 2003; Ferreira, Gulis & Graça, 2006). However, other studies have shown little or no influence of nutrient availability on litter breakdown rates or microbial activity (Triska & Sedell, 1976; Newbold *et al.*, 1983; Royer & Minshall, 2001; Pozo *et al.*, 2011). Many factors can modify the relationship between nutrient enrichment and litter decomposition, including temperature (Hynes & Kaushik, 1969; Ferreira & Chauvet, 2011), substrate quality (Gulis *et al.*, 2004; Ferreira *et al.*, 2006), ambient nutrient concentrations (Abelho & Graça, 2006; Ferreira *et al.*, 2006) and interactions with other potentially limiting nutrients (Hynes & Kaushik, 1969; Howarth & Fisher, 1976).

Direct, top-down effects of consumers are explicit in the leaf-processing continuum. The presence of macroinvertebrates, particularly those classified as shredders, has been shown to increase leaf breakdown rate (Cuffney, Wallace & Lughart, 1990) and decrease leaf standing stock at the whole-reach scale (Wallace *et al.*, 1991a). Shredder feeding activity converts litter to fine particulate organic matter (Cummins & Klug, 1979; Wallace *et al.*, 1991b) and may also affect leaf-associated microbial communities. Shredders preferentially consume microbially-conditioned litter (Bärlocher & Kendrick, 1975; Arsuffi & Suberkropp, 1985) and have shown preferences for specific fungal taxa in laboratory feeding experiments (Suberkropp, Arsuffi & Anderson, 1983; Arsuffi & Suberkropp, 1989). Consumers also influence microbial activity indirectly by controlling nutrient availability via consumer nutrient recycling (CNR), the process by which animals convert nutrients from organic to inorganic forms through consumption and mineralisa-

tion (e.g. Vanni, 2002). Inorganic nutrients released via CNR can make significant contributions to dissolved nutrient pools and can account for a large portion of nutrient uptake fluxes (Grimm, 1988; Hudson, Taylor & Schindler, 1999; Vanni *et al.*, 2002; McIntyre *et al.*, 2008). In addition to supplying potentially limiting nutrients, CNR can influence producer assemblage richness, structure and stoichiometry (Evans-White & Lamberti, 2006; Kato, Urabe & Kawata, 2007; Knoll *et al.*, 2009).

Our objectives were to investigate the effects of and interactions between nutrient availability and consumer activity on heterotrophic microbial activity. We manipulated water NO_3^- concentration and the presence of two shredders, tipulid larvae and amphipods, in artificial streams. We predicted that these two shredders would influence microbial activity differently through the feeding and CNR pathways. Insects have been shown to have a greater biomass %N than crustaceans (Evans-White, Stelzer & Lamberti, 2005) and should therefore retain relatively more N (Sterner & Elser, 2002), resulting in a reduced contribution to the dissolved inorganic nitrogen (DIN) pool via recycling. Amphipods and tipulids also have different feeding modes; tipulids often engulf whole chunks of detritus, while amphipods tend to skeletonise leaves by grazing on the surficial microbial assemblage and mesophyll (B. M. Cheever, pers. observ.). Therefore, amphipods may maintain a low but rapidly cycling microbial biomass. To assess the CNR and grazing pathways, we exposed each shredder taxon to detritus under two conditions: detritus available to shredder feeding and detritus protected from shredder feeding but exposed to shredder CNR. Using these two detritus conditions allowed us to isolate the effects of CNR from the effects of direct shredder consumption.

Methods

Mesocosm construction and monitoring

Using recirculating stream mesocosms, we manipulated N availability via two N levels: ambient N (LOW N) and elevated N (HIGH N). We also manipulated shredder presence via three shredder levels: tipulids (TIP), amphipods (AMP) and no shredders (NONE). We used a full factorial design with each treatment replicated three times, giving a total of 18 mesocosms.

Each mesocosm consisted of a 2.1 m length of PVC pipe (0.39 m in diameter) cut longitudinally and placed horizontally. End pieces were cut from hard plastic to fit the inner diameter of the pipe, glued to each end of 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., Pembroke, VA, U.S.A. Little Stony is a soft-water stream with low levels of DIN ($\leq 15.3 \mu\text{g NO}_3\text{-N L}^{-1}$ and $1.2\text{--}15.4 \mu\text{g NH}_4\text{-N L}^{-1}$), dissolved organic carbon ($1.63\text{--}2.60 \text{ mg C L}^{-1}$) and inorganic phosphorus ($\leq 4.7 \mu\text{g PO}_4\text{-P L}^{-1}$ as soluble reactive phosphorus) (Cheever *et al.*, 2013). Small pumps ($1.5 \text{ m}^3 \text{ h}^{-1}$; Beckett Corporation, Irving, TX, U.S.A.) at the foot of each mesocosm 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 over a v-notched piece of plastic into the pump chamber. Flow velocity in the main chamber of all 18 mesocosms averaged (\pm standard deviation) 0.14 m s^{-1} (± 0.05).

Mesocosms were placed in a temperature-controlled chamber at 8°C in the dark. To make the water in the mesocosms more suitable for amphipods, we increased the hardness to a target concentration of $70 \text{ mg CaCO}_3 \text{ L}^{-1}$ by adding the following (ASTM Standard E729-96, 2007): 20 mL L^{-1} of a 0.02 M CaSO_4 and 0.02 M CaCl_2 solution made in Little Stony stream water, $26 \mu\text{L L}^{-1}$ of a $0.5 \text{ M MgSO}_4\cdot 7\text{H}_2\text{O}$ solution made in deionised (DI) water, 1.1 mL L^{-1} of a 1 M NaHCO_3 solution made in DI water and $108 \mu\text{L L}^{-1}$ of a 0.5 M KCl solution made in DI water.

Water levels in the mesocosms were checked daily and additional stream water was added as needed. Filtered (through $0.7\text{-}\mu\text{m}$ nominal pore size glass fibre filters) water samples were taken from each mesocosm twice per week in order to monitor hardness (as $\text{mg CaCO}_3 \text{ L}^{-1}$) and $\text{NO}_3\text{-N}$ throughout the study, while $\text{NH}_4\text{-N}$ was measured once at the end of the study. We measured $\text{NH}_4\text{-N}$ and $\text{NO}_3\text{-N}$ concentrations by the phenate and Cd-reduction method, respectively, using a Lachat Quickchem flow injection analyser (Lachat Instruments, Loveland, CO, U.S.A.; American Public Health Association (APHA), 1999). Hardness of the water in each mesocosm was maintained by adding the solutions described above or stream water based on these biweekly measurements.

Leaf pack construction and deployment

We constructed packs of red maple (*Acer rubrum*) leaves using leaves collected from a single tree shortly after abscission and air-dried to a constant weight. We constructed packs using screen with two different mesh sizes: coarse mesh screen (10 mm mesh size) that allowed shredders access to the leaves and fine mesh

screen (1 mm mesh size) that prevented shredders from feeding on the leaves. Packs of each mesh size contained either 5 g or 10 g dried leaf mass and were placed in Little Stony Creek for 4 days to leach and to become colonised by a natural microbial assemblage. We used 5-g packs to measure leaf breakdown rate and 10-g packs to measure N uptake. After leaching, we placed 18 of the 5-g leaf packs of each mesh size and two of the 10-g packs of each mesh size in each mesocosm. Packs were weighed down with cobbles. An additional 18 of the 5-g packs of each mesh size per mesocosm were placed in bins of stream water collected from Little Stony Creek. We 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 total of 13 days in the mesocosms and bins. The water was completely drained and replaced with fresh, hardness-corrected stream water once during this time. Shredders were added to the mesocosms after the leaching period (see below). Thirteen days after the addition of the animals, 16 L ($\sim 61\%$) of the water was carefully removed from each mesocosm and replaced.

Shredder and nitrogen treatments

We added 44 individuals to each TIP mesocosm and 132 individuals to each AMP mesocosm, for a density of 0.2 tipulids and 0.6 amphipods per g dry leaf mass. These densities were chosen based on previous sampling of a local stream where amphipods were found in densities of 0.6 individuals per g ash-free dry mass (AFDM) leaf standing stock, and average amphipod biomass was $1/3$ that of average tipulid biomass (B.M. Cheever, unpubl. data). Therefore, our tipulid and amphipod treatments contained different numbers of individuals but had realistic shredder biomass per g leaf. Tipulids (*Tipula* spp.) were collected from two local streams, Stonecrop Creek and Tom's Creek. Amphipods (*Gammarus* spp.) were ordered from Carolina Biological Supply Co. (Burlington, NC, U.S.A.). Both taxa were kept in separate aerated tanks of Little Stony stream water with leaves collected from the stream 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 . The water in these holding tanks was changed periodically throughout the experiment.

High N mesocosms were spiked with 21 mL of a 1000 mg L^{-1} NaNO_3 solution (for an addition of 21 mg

NO₃-N per mesocosm) every other day. Our objective was to maintain NO₃-N concentrations of ~800 µg L⁻¹ 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 in the same way as the mesocosms. Low N mesocosms were not spiked.

Leaf breakdown and fungal biomass

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

Within hours of collection, two packs of each mesh size from each mesocosm were dried at 60 °C, weighed and ground in a coffee grinder. A subsample of ground material was weighed, ashed at 550 °C for 2 h and reweighed to obtain AFDM. Ten discs (2 cm diameter) were cut from leaves from the third 5-g pack of each mesh size. The remaining leaf material in these packs was dried and ashed as described above. Five of the leaf discs were placed in 5 mL of methanol and kept frozen and in the dark for ergosterol analysis. The remaining five discs were dried, weighed, ashed at 550 °C and reweighed to obtain AFDM. The AFDM of the removed discs was added to the AFDM of the subsampled packs. We used the slope of the natural logarithm of the per cent AFDM remaining (in relation to the first collection which occurred after the leaching period but before the shredder addition) after each collection over time to calculate the breakdown rate of red maple (Benfield, 2006) for each mesh size in each mesocosm.

Ergosterol was extracted from frozen discs using a liquid-phase extraction, quantified using high-performance liquid chromatography (HPLC) and converted to fungal biomass (Gulis & Suberkropp, 2006). Fungal biomass was expressed as mg fungal biomass per g AFDM of detritus extracted.

Microbial uptake assay

At the end of 8 weeks, one of the 10-g packs was collected from each mesocosm, opened, invertebrates were removed, and the leaf material was placed in individual glass tanks with 4 L of filtered water from the corresponding mesocosm. After a ~12-h acclimation period, we spiked each glass tank with 10 µg of NH₄-N (added as NH₄Cl). Three filtered (Whatman GF/F) 10 mL water samples were collected from each tank before spiking (except for the first assay, see below), immediately after spiking, and 15, 30, 45, 90, 150 and 240 min after spiking. Samples were frozen until analysed for NH₄-N concentration (phenate method) using a Lachat Quickchem flow injection analyser (Lachat Instruments; APHA, 1999). Three post-assay samples were taken at least 4 h after the final sample. Leaves from each tank were dried at 60 °C, weighed and ground. Subsamples of ground material were used to obtain AFDM using the method previously described. We repeated this assay four times over a period of 4 days, twice with fine mesh packs and twice with coarse mesh packs. The packs (fine or coarse mesh) were randomly selected from each mesocosm on each day.

For each trial, we 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 & Dodds, 2008). For each mesh size in each mesocosm, we used the uptake rate from the assay generating the relationship with the largest r^2 for further calculations. r^2 values averaged 0.220 (0.003–0.686) and 0.220 (<0.001–0.854) for relationships used for coarse and fine mesh packs, respectively. If NH₄-N concentrations increased over time (or negative uptake), we used a value of zero for the uptake rate. We 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⁻¹) 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 calculated as the mean of the samples taken from each tank before the addition of the spike, except for the first assay where C_{amb} was calculated as the mean of the samples taken immediately after the spike minus the 2.5 µg L⁻¹ spike addition. We also calculated fungal biomass-specific (FBS) uptake. We divided uptake flux by the total fungal biomass in each tank, which we estimated by scaling the estimates of fungal biomass (mg fungal biomass per g AFDM leaf) from ergosterol extractions to the AFDM of leaves in the tanks.

Animal excretion and body stoichiometry

Macroinvertebrates were collected from each mesocosm at the end of the experiment for excretion assays. Tipulids were placed in whirl packs containing 50 mL of filtered (Whatman GF/F) Little Stoney water. We used five replicate bags for each TIP mesocosm with each bag containing one to three individuals depending on size and five control bags containing filtered water only. Bags were incubated for 1 h at 8 °C. Amphipods were collected from AMP mesocosms and treated similarly, except that we used 30 mL of filtered water and three replicate bags for each mesocosm.

After incubation, the animals were frozen and water from each bag was refiltered and analysed for $\text{NO}_3\text{-N}$ and $\text{NH}_4\text{-N}$ as described above. Animals were dried and weighed with individuals from the same replicate bag pooled into a single sample. Excretion rates were calculated as the change in $\text{NH}_4\text{-N}$ or $\text{NO}_3\text{-N}$ concentration (after correcting for concentration in the controls) over time per dry weight of macroinvertebrate biomass. If N concentrations in the bags were less than the controls, we used a value of zero for the excretion rate. Total DIN excretion was calculated as the sum of NO_3^- and NH_4^+ excretion.

After weighing, animals were analysed for C and N content using a FlashEA 1112 Series Elemental Analyzer where samples were combusted at 975 °C and the products were reduced and measured using a thermal conductivity detector. Tipulid samples were analysed individually, so we used the mean values of the five replicate assays from the same mesocosm to represent C and N content of tipulids from each TIP mesocosm. However, we needed to pool amphipod tissue from the three replicate assays from the same mesocosm in order to obtain enough biomass for analysis. Therefore, the C and N content of amphipods from each AMP mesocosm are from a single pooled sample.

Statistical analysis

Water chemistry over time, including $\text{NO}_3\text{-N}$ concentration, hardness and pH, was compared among shredder treatments in HIGH N and LOW N mesocosms using repeated-measures analysis of variance (rmANOVA) with Tukey's *post hoc* comparisons. $\text{NH}_4\text{-N}$ concentrations at the end of the experiment among shredder treatments in HIGH N and LOW N mesocosms were compared using a one-way analysis of variance (ANOVA).

Means of tipulid and amphipod biomass %C, %N, molar C : N and DIN excretion rates were calculated for

each N treatment ($n = 3$) and compared using two-way ANOVA tests. DIN excretion flux data were natural logarithm-transformed, while biomass C and N data were arcsine square-root-transformed to meet the assumptions of normality.

We used three-way ANOVA tests with Tukey's *post hoc* tests to compare mean leaf breakdown rate, fungal biomass, NH_4^+ uptake rate, uptake flux and FBS uptake flux among N, shredder and mesh treatments. Due to the many zero values, the raw data for the uptake parameters were not normally distributed. However, ANOVA tests are robust to violations of normality (Schmider *et al.*, 2010), the other assumptions (i.e. equal variance) were satisfied, and the residuals were unimodal. When mesh size was a significant factor in the three-way ANOVA, we used a two-way ANOVA test to determine the influence of shredder and N enrichment within each mesh type. Red maple breakdown rate data were natural logarithm-transformed to meet the assumptions of normality. All statistical tests were performed using SIGMAPLOT with SigmaStat Integration (version 10; Systat Software Inc, Chicago, IL, U.S.A.).

Results

The $\text{NO}_3\text{-N}$ concentrations in HIGH N treatments were near our target concentration of $\sim 800 \mu\text{g L}^{-1}$ for the first 4 weeks of the experiment (Table 1). After the second leaf pack collection, N addition exceeded the removal capacity of the system and $\text{NO}_3\text{-N}$ concentrations increased, peaking at levels of approximately $6700 \mu\text{g L}^{-1}$. Within HIGH N treatments, $\text{NO}_3\text{-N}$ concentrations were generally lower in the AMP treatments than in the TIP treatments (rmANOVA, $P = 0.024$), although $\text{NO}_3\text{-N}$ in neither shredder treatment differed significantly from the NONE treatments (rmANOVA, $P > 0.053$). $\text{NO}_3\text{-N}$ concentrations in the ambient N mesocosms were similar among all shredder treatments (rmANOVA, $P = 0.261$) and remained substantially lower than the HIGH N mesocosms, averaging $42.9 \mu\text{g L}^{-1}$ across all sampling times and shredder treatments (Table 1). Neither hardness nor pH differed among shredder treatments in the HIGH N mesocosms (rmANOVA, $P > 0.765$), ranging from 53.3 to 83.3 $\text{mg CaCO}_3 \text{L}^{-1}$ and 8.3 to 9.6, respectively. In LOW N mesocosms, hardness was similar among all shredder treatments (rmANOVA, $P = 0.107$), ranging from 53.3 to 90 $\text{mg CaCO}_3 \text{L}^{-1}$. pH was higher in AMP mesocosms (8.7–9.5) than in the TIP and NONE treatments (7.7–9.1; rmANOVA, $P < 0.017$). $\text{NH}_4\text{-N}$ concentrations at the end of the experiment were similar among all shredder

Table 1 Comparison of N chemistry among shredder treatments in HIGH and LOW N mesocosms over the course of the experiment

	+2 weeks	+4 weeks	+6 weeks	+8 weeks	+10 weeks
HIGH N mesocosms:					
NO ₃ -N (µg L ⁻¹)					
AMP ^a	413.6 (39.1)	261.9 (252.8)	997.0 (903.4)	1744.2 (232.9)	5574.0 (623.5)
TIP ^b	510.7 (33.1)	488.5 (288.4)	2552.0 (186.2)	2751.0 (508.5)	6690.7 (494.1)
NONE ^{ab}	363.7 (320.4)	687.8 (638.8)	2450.5 (854.0)	2133.0 (554.9)	6710.4 (1247.3)
NH ₄ -N (µg L ⁻¹)					
AMP	–	–	–	–	20.4 (3.60)
TIP	–	–	–	–	59.7 (28.2)
NONE	–	–	–	–	21.4 (5.46)
LOW N mesocosms:					
NO ₃ -N (µg L ⁻¹)					
AMP	bd	bd	bd	59.5 (54.3)	6.5 (9.2)
TIP	bd	15.8 (13.7)	2.0 (3.3)	191.3 (229.0)	16.2 (12.6)
NONE	bd	22.0 (21.6)	1.1 (1.7)	145.2 (208.2)	8.2 (14.1)
NH ₄ -N (µg L ⁻¹)					
AMP	–	–	–	–	13.5 (4.59)
TIP	–	–	–	–	30.2 (9.81)
NONE	–	–	–	–	18.0 (7.34)

Values are means (±standard deviation) from three replicate mesocosms. Lowercase letters indicate significant differences ($P < 0.05$) among treatments according to repeated-measures ANOVA. Dashes indicate samples not taken. Note that NH₄ concentrations were obtained from the uptake assays before the addition of the NH₄⁺ spike but after leaves had been incubating overnight.

treatments in both the LOW N and HIGH N mesocosms (ANOVA, $P > 0.064$; Table 1).

Biomass stoichiometry and excretion rates

Tipulids and amphipods had different elemental compositions in our mesocosms (Fig. 1). Tipulids had higher %N, %C and molar C : N than amphipods under both ambient and enriched N conditions (two-way ANOVA; $P < 0.002$). N enrichment did not change biomass elemental composition (two-way ANOVA, $P > 0.104$).

Nitrogen excretion also varied among taxa. Under ambient N conditions, amphipods excreted almost 100× more DIN per min per unit biomass than tipulids (two-way ANOVA; $P = 0.002$; Fig. 2). The same between-taxa patterns existed under enriched N conditions (Fig. 2) with amphipods generating significantly greater DIN excretion fluxes than tipulids (two-way ANOVA; $P < 0.001$). N enrichment did not alter excretion fluxes within either taxa (two-way ANOVA; $P > 0.218$).

Effects of shredder activity and N enrichment on N cycling and leaf breakdown

Neither shredder activity nor N enrichment influenced N uptake rate. NH₄-N uptake rate (min⁻¹) was highly variable and did not differ among shredder, N or mesh treatments (Table 2, Fig. 3a,b). The greatest uptake fluxes occurred in HIGH N treatments with

tipulids, both when tipulids had access to leaves (flux measured from leaves from coarse mesh bags) and were excluded from leaves (flux measured from leaves from fine mesh bags) (Fig. 3c,d), although N uptake flux was statistically similar among all treatments (Table 2).

Nitrogen enrichment stimulated fungal biomass standing stocks across all shredder treatments (Table 2, Fig. 3e,f). Shredders did not influence fungal biomass under either ambient or enriched N conditions, irrespective of mesh size. However, there was a mesh * N interaction. Fungal biomass was greater on leaves from fine mesh packs but only under enriched N conditions (Tukey's test, $P = 0.014$). Differences in fungal biomass did not alter NH₄⁺ uptake among treatments. Fungal biomass-specific uptake (µg NH₄-N mg⁻¹ fungal biomass min⁻¹) was highly variable within and among treatments and did not vary with N enrichment, shredder treatment or mesh size (Table 2, Fig. 3g,h).

Shredder feeding and CNR had different effects on C processing depending on shredder taxa and N availability. Leaves in coarse mesh packs broke down faster than those in fine mesh packs (Table 2). Increased N availability generally stimulated breakdown rates of red maple leaves in coarse packs and leaves exposed to tipulid feeding broke down ~2× faster than leaves exposed to amphipod feeding, although these differences were not statistically significant (two-way ANOVA, Tukey's test, $P > 0.084$; Fig. 4a).

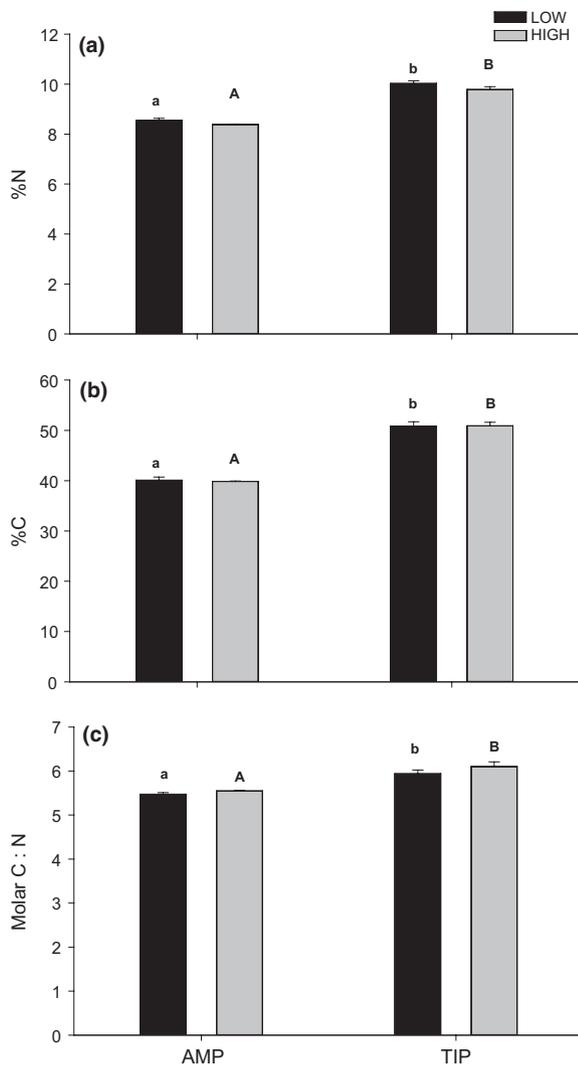


Fig. 1 Elemental composition, including (a) %N, (b) %C, and (c) molar C : N of amphipods (AMP) and tipulids (TIP) in mesocosms under ambient N (LOW) and enriched N (HIGH) conditions. Bars are means (\pm SE) from three replicate mesocosms. Bars within the same N treatment with different letters (lowercase letters within LOW and capital letters within HIGH) are significantly different from each other ($P < 0.05$). Data were arcsine square-root-transformed for analysis.

The effect of shredders was reversed when the CNR pathway was isolated (fine mesh packs; Fig 4b). Leaf breakdown rates were more than twice as fast in AMP mesocosms than in TIP mesocosms under ambient N conditions (two-way ANOVA, Tukey's test, $P < 0.017$; Fig. 4b). N enrichment increased breakdown rates of fine mesh packs in TIP and NONE (two-way ANOVA, Tukey's test, $P < 0.008$) treatments but did not change the breakdown rate in AMP treatments (two-way ANOVA, Tukey's test, $P = 0.549$). The shredder effect also disappeared under high N conditions; breakdown

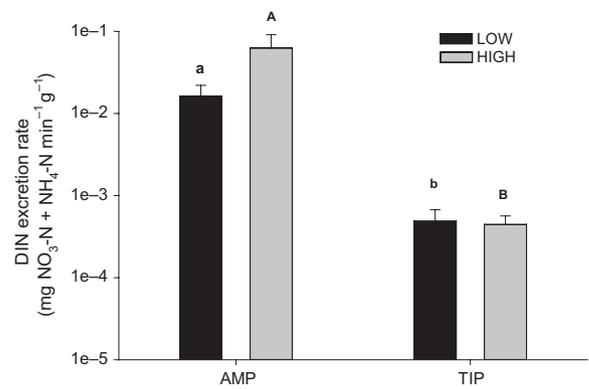


Fig. 2 DIN excretion rates of amphipods (AMP) and tipulids (TIP) in ambient N (LOW) and enriched N (HIGH) mesocosms. Bars are means (\pm SE) from three replicate mesocosms. Bars within the same N treatment with different letters (lowercase letters within LOW and capital letters within HIGH) are significantly different from each other ($P < 0.05$). Data were ln-transformed for analysis. Note the scale of the Y axis.

rates were similar among AMP, TIP and NONE treatments in HIGH N streams (two-way ANOVA, Tukey's test, $P > 0.617$; Fig. 4b). Changes in leaf breakdown rate were partially due to changes in fungal biomass. Fungal biomass at the final collection explained 46% of the variance in breakdown rates across all treatments (linear regression; $r^2 = 0.460$, $P = 0.006$).

Discussion

Without considering shredders, effects of N enrichment on red maple breakdown rate were generally as predicted by established decomposition theory. Many studies in both terrestrial and aquatic ecosystems have shown that litter decomposition responds positively to increased N availability (Hunt *et al.*, 1988; Hobbie, 2000; Cheever *et al.*, 2013; but see Howarth & Fisher, 1976; Triska & Sedell, 1976; Hobbie & Vitousek, 2000). Fungal growth appeared to be N-limited in our mesocosms as N enrichment increased fungal standing stocks in both fine and coarse mesh packs. Red maple breakdown rates were also generally faster under enriched N conditions, presumably due to increased fungal abundance and activity.

However, the positive response of fungal biomass to N enrichment did not alter N uptake in the way predicted by other studies. The second Lotic Intersite Nitrogen eXperiment (LINX II) showed an increase in total areal N uptake but a decrease in the efficiency of N removal from the water column as N availability increased across 72 streams (Mulholland *et al.*, 2008). These results were from isotopic tracer studies

Table 2 Comparison of N uptake parameters and leaf breakdown rates among shredder, N and mesh treatments using three-way ANOVAs

	Uptake rate (min ⁻¹)		Uptake flux (µg NH ₄ -N g ⁻¹ AFDM min ⁻¹)		Fungal biomass (mg g ⁻¹ AFDM)		FBS uptake flux (µg NH ₄ -N mg ⁻¹ fungal biomass min ⁻¹)		Red maple breakdown rate (d ⁻¹)*	
	F	P	F	P	F	P	F	P	F	P
N	0.0399	0.843	1.795	0.193	63.057	<0.001	0.482	0.495	27.852	<0.001
Shredder	0.735	0.490	2.950	0.072	0.123	0.885	0.813	0.457	0.937	0.406
Mesh	0.0392	0.845	0.025	0.875	1.181	0.289	0.048	0.829	10.808	0.003
N * shredder	2.003	0.157	2.947	0.073	1.052	0.366	1.190	0.324	1.479	0.244
N * mesh	0.0056	0.941	0.154	0.699	6.435	0.019	0.187	0.670	2.593	0.120
Shredder * mesh	0.584	0.565	0.169	0.845	0.453	0.642	0.897	0.670	5.705	0.009
N * shredder * mesh	0.495	0.616	0.299	0.744	2.723	0.088	0.651	0.532	2.310	0.121

Significant *P* values (<0.05) are in bold. *n* = three replicate mesocosms.

*ln-transformed for analysis.

conducted at a whole-reach scale. In our study, conducted at the leaf pack scale, N enrichment did not alter N uptake flux by leaf packs. However, our results agree with the LINX II findings in that N uptake efficiency (amount of N taken up per unit fungal biomass) was generally lower in HIGH N mesocosms, although not significantly so.

The lack of significant response of N uptake parameters to N enrichment may be due to several factors. Firstly, we measured uptake 10 weeks after the application of the treatments. Fungal biomass in our mesocosms seemed to plateau after about 7 weeks, particularly in HIGH N treatments (time series data not shown). We might have seen treatment differences if we had measured uptake earlier in the experiment when the fungal growth rates were faster and presumably N demand was greater. Secondly, we may not have seen an uptake response to enrichment because we measured NH₄⁺ uptake but we enriched with NO₃⁻. Recent work has demonstrated that the response of microbial NH₄⁺ and NO₃⁻ uptake depends on the species of N available in ambient and enriched conditions (Bunch & Bernot, 2012; Ribot *et al.*, 2013). We measured NH₄⁺ uptake because NH₄⁺ is often the preferred N species for microbial uptake (Dortch, 1990; Marzluf, 1997) and NH₄⁺ uptake can exceed NO₃⁻ uptake (Bernhardt, Hall & Likens, 2002; Simon *et al.*, 2004). However, there may have been a stronger response of NO₃⁻ uptake if NO₃⁻ enrichment caused microbes to switch to this more abundant N source. Alternatively, the high levels of N enrichment achieved by the time of the uptake assays (~7–8× higher than our target concentrations) may have pushed the system to severe P limitation. Finally, our measurements of NH₄-N uptake rate were extremely variable in all treatments. We are uncertain as to why there was so

much variation, but it made it difficult to detect any treatment effects that might have occurred.

The conversion of leaves to smaller particles by shredder feeding is a key part of both the leaf-processing continuum described by Cummins and Klug (1979) and the river continuum concept (Vannote *et al.*, 1980), and shredders have been shown to play a significant role in organic matter processing in natural streams (Webster, 1983; Mulholland *et al.*, 1985; Cuffney *et al.*, 1990; Wallace & Webster, 1996; Hieber & Gessner, 2002). However, direct feeding by shredders did not significantly increase litter breakdown rates independent of N enrichment in our study. Decomposition rate has failed to respond to invertebrate abundance in other studies (Meyer, 1980; Rosemond, Pringle & Ramirez, 1998; Stockley, Oxford & Ormond, 1998), in some cases due to low relative abundance of shredders (Rosemond *et al.*, 1998). Although there were no significant effects, the general trends seen in our study suggest that tipulid feeding had a stronger effect on leaf breakdown than amphipod feeding. This difference is probably due to differences in feeding mode; tipulids ingest large chunks of leaf mass, while amphipods tend to skeletonise leaves, leaving structural tissue behind (B. M. Cheever, pers. observ.). The lack of significant increases in breakdown rate in response to shredder feeding in our LOW N mesocosms may have been due to insufficient shredder biomass, differences in shredder behaviour compared with natural systems or shredder mortality over the course of the experiment. Surveys of invertebrate density were not conducted during the experiment, but several living individuals of both taxa were found in leaf packs at each collection and leaves collected from packs showed evidence of shredder activity (e.g. skeletonisation).

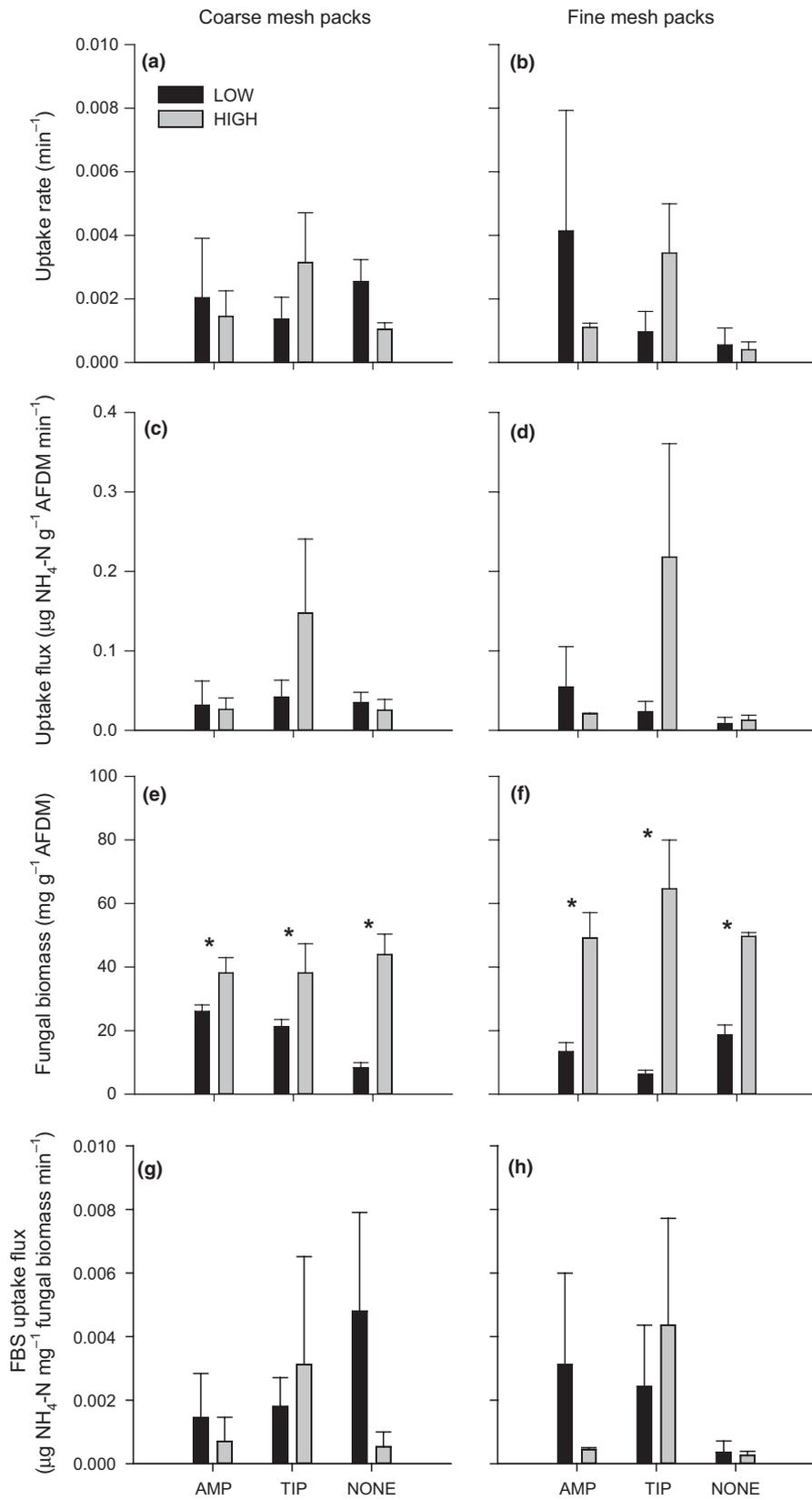


Fig. 3 Nitrogen uptake by microbes and fungal biomass measured at the final collection in coarse (left panels) and fine (right panels) mesh packs in amphipod (AMP), tipulid (TIP) and no shredder (NONE) treatments under ambient (LOW) and enriched (HIGH) N conditions. Bars are means (\pm SE) from three replicate mesocosms. Asterisks denote differences between N treatments within the same shredder treatment.

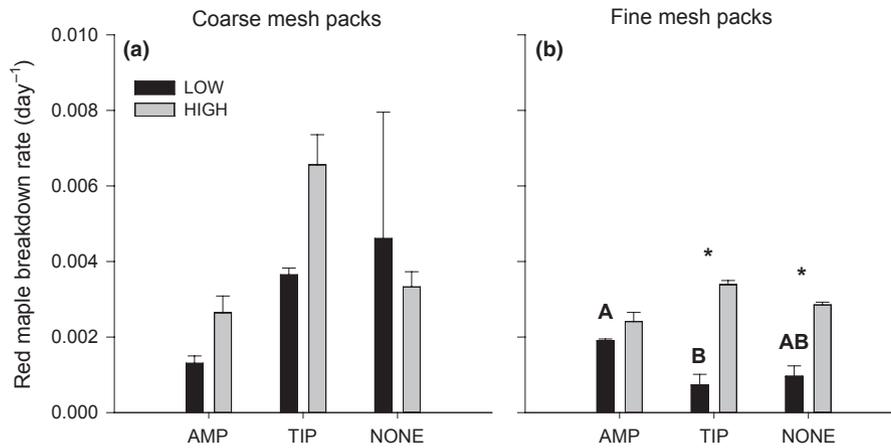


Fig. 4 Red maple breakdown rate in (a) coarse mesh and (b) fine mesh packs in streams with amphipods (AMP), tipulids (TIP) and no shredders (NONE) under ambient (LOW) or enriched (HIGH) N conditions. Bars are means (\pm SE) from three replicate streams. Bars within the same N treatment with different letters are significantly different from each other ($P < 0.05$). Asterisks indicate significantly different means within the same shredder treatment. $n = 3$ replicate mesocosms per treatment.

We predicted that the amphipods would decrease fungal abundance by selectively feeding on mesophyll tissue, while the tendency for tipulids to ingest bulk detritus would lead to little or no effect on the fungal biomass on remaining substrate. However, we saw no difference in fungal biomass between amphipod and tipulid treatments. Shredders may have been selectively feeding on areas of the leaf heavily colonised by microbes (Arsuffi & Suberkropp, 1985), leaving behind substrates with similar microbial biomass. If so, we would expect the fungal biomass in shredder treatments to be lower than in the no-shredder treatment. Although this was generally the case, differences between shredder and no-shredder treatments were not statistically significant.

Grazing has been shown to regulate benthic primary producer abundance and increase primary production in streams (Lamberti & Resh, 1983). However, our results suggest that this paradigm may be less relevant to detritivore–detritus systems. In epilithon–grazer systems, areas of grazed inorganic substrates are available for recolonisation by highly productive taxa. In contrast, shredders often remove pieces of the entire detrital complex, leaving no substrate behind for recolonisation. While it is possible to assess the degree to which an area of inorganic substrate has been grazed, often nothing remains of a heavily ‘grazed’ organic substrate, particularly in the case of shredders such as tipulids. The microbial assemblages on the remaining material may be relatively intact, making it difficult to assess the effects of shredder feeding on the abundance or production of ‘grazed’ assemblages.

The effects of shredder CNR on leaf breakdown were taxon-specific and conformed to ecological stoichiometric theory. Amphipods excreted orders of magnitude more DIN than tipulids, presumably due to their comparatively lower body N content. Correspondingly, leaf breakdown rate was stimulated by amphipod CNR but did not respond to tipulid CNR under low N conditions. A similar stimulation of leaf breakdown rates in HIGH N mesocosms with no shredders suggests that amphipod CNR alleviated N limitation to the same degree as in our N-enrichment treatment. However, unlike in our N-enrichment treatment, this increase in leaf breakdown rate was not due to a corresponding increase in fungal biomass, suggesting that amphipod CNR may have influenced the bacterial component of the heterotrophic assemblage or may have increased fungal production without increasing biomass.

Interactions between top-down and bottom-up factors were particularly evident, although not significant, in the response of N cycling in our study. N uptake flux was greatest in mesocosms with both enriched N and tipulids. High uptake flux indicates that N is required by the microbial assemblage. As fungal biomass was similar across shredder treatments in both fine and coarse mesh packs, differences in fungal production or bacterial abundance or production may be responsible for this increased N demand. Additionally, this pattern was present in both coarse and fine mesh packs, suggesting that both the feeding and CNR mechanisms may be important. A visual comparison of the amount of fine particulate organic matter (FPOM) remaining in the mesocosms after leaf packs were removed suggested that

tipulid feeding generated a large amount of FPOM. This FPOM was likely entrained in leaf packs of both mesh sizes and may have stimulated bacterial colonisation as bacteria are more abundant on small detrital particles (Sinsabaugh & Findlay, 1995; Findlay *et al.*, 2002). The relatively fast turnover times (Findlay & Arsuffi, 1989) and low C : N (Chrzanowski *et al.*, 1996; Cross *et al.*, 2005) typical of bacteria would be expected to increase the N requirements of the microbial assemblage, explaining the observed pattern in N uptake fluxes.

Leaf-associated microbial activity may also be colimited by N and P availability (Howarth & Fisher, 1976) and the failure of amphipods or tipulids in low N conditions to elicit a similar N uptake response could have been due to differences in P excretion between the two shredders. Crustaceans tend to have a higher biomass %P than insects (Evans-White *et al.*, 2005). According to stoichiometric theory, this should result in tipulids retaining less and excreting more P, which may have alleviated P limitation for microbes in mesocosms containing tipulids. Therefore, microbial production in enriched N treatments with tipulid CNR would have both abundant N and P, while microbes experiencing only N enrichment or tipulid CNR would be lacking sufficient P or N, respectively.

Overall, our results suggest that top-down and bottom-up factors, and possibly interactions between the two, can influence N cycling by microbial assemblages associated with decomposing organic matter in streams. Changes in microbial nutrient cycling in response to shredder and nutrient interactions warrant more study. Nutrient enrichment of fresh waters is a continuing environmental concern and is often accompanied by shifts in consumer communities. Understanding the effects these changes will have on microbial nutrient processing is necessary for understanding the transformation and transport of nutrients across landscapes.

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