Research

Non-additive effects of litter mixing are suppressed in a nutrient-enriched stream

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Investigations of how species compositional changes interact with other aspects of global change, such as nutrient mobilization, to affect ecosystem processes are currently lacking. Many studies have shown that mixed species plant litters exhibit non-additive effects on ecosystem functions in terrestrial and aquatic systems. Using a full-factorial design of three leaf litter species with distinct initial chemistries (carbon:nitrogen; C:N) and breakdown rates (*Liriodendron tulipifera*, *Acer rubrum* and *Rhododendron maximum*), we tested for additive and non-additive effects of litter species mixing on breakdown in southeastern US streams with and without added nutrients (N and phosphorus). We found a non-additive (antagonistic) effect of litter mixing on breakdown rates under reference conditions but not when nutrient levels were elevated. Differential responses among single-species litters to nutrient enrichment contributed to this result. Antagonistic litter mixing effects on breakdown were consistent with trends in litter C:N, which were higher for mixtures than for single species, suggesting lower microbial colonization on mixtures. Nutrient enrichment lowered C:N and had the greatest effect on the lowest- (*R. maximum*) and the least effect on the highest-quality litter species (*L. tulipifera*), resulting in lower interspecific variation in C:N. Detritivore abundance was correlated with litter C:N in the reference stream, potentially contributing to variation in breakdown rates. In the nutrient-enriched stream, detritivore abundance was higher for all litter and was unrelated to C:N. Thus, non-additive effects of litter mixing were suppressed by elevated streamwater nutrients, which increased nutrient content of all litter, reduced variation in C:N among litter species and increased detritivore abundance. Nutrients reduced interspecific variation among plant litters, the base of important food web pathways in aquatic ecosystems, affecting predicted mixed-species breakdown rates. More generally, world-wide mobilization of nutrients may similarly modify other effects of biodiversity on ecosystem processes.

Predictions of global change effects must account for multiple anthropogenic stressors on ecosystem processes. Paramount among global issues are increased nutrient mobilization and species loss, which are both expected to alter rates of key functions across ecosystems (e.g. primary production, nutrient cycling and decomposition; Cardinale et al. 2006, Elser et al. 2007). In detritus-based ecosystems, evidence suggests that both nutrient loading (Cleveland and Townsend 2006) and species loss (Wardle et al. 1997) can significantly alter rates of organic matter breakdown, an important ecosystem function that integrates biotic and abiotic stressors and effects across multiple trophic levels (Gessner and Chauvet 2002). However, no work to date has addressed these two factors simultaneously. This is despite dramatic current and predicted changes in species composition of forested ecosystems (e.g. loss of dominant tree species to disease, logging, etc.) affecting litter quality (Ellison et al. 2005, Ibáñez et al. 2006), and elevated levels of nutrient runoff and deposition (Galloway et al. 2004, MEA 2005), both of which are occurring on a global scale.

A first step in predicting species effects on ecosystem processes is determining whether the effects of individual species can be used to predict mixed-species effects (i.e. additivity). Determining when important ecosystem functions, such as litter breakdown rates, can be predicted by additive models of species effects is critical to creating predictive models of non-random species loss. The vast majority of studies that have tested for additivity have found non-additive effects of litter mixing on breakdown in both terrestrial (Wardle et al. 1997, Gartner and Cardon 2004, Ball et al. 2008) and aquatic systems (Swan and Palmer 2004, LeRoy and Marks 2006, Moore and Fairweather 2006, Kominoski et al. 2007). Of aquatic studies finding non-additive effects, many have found antagonistic effects (Swan and Palmer 2004, Kominoski et al. 2007) or evidence of both synergistic and antagonistic effects (Lecerf et al. 2007, Swan et al. 2009). The mechanisms underlying these effects potentially include nutrient transfer between litters, stimulation or suppression of microbial activity, and changes in detritivore colonization and feeding behavior (Hättenschwiler et al. 2005). These mechanisms may

depend on interspecific differences in litter quality (e.g. carbon:nitrogen [C:N], polyphenolic content). Therefore, any extrinsic factor that alters substrate quality, such as nutrient loading, is predicted to influence the interactions generating non-additive breakdown of species-rich litter.

Nutrient mobilization is one of the most important factors affecting terrestrial and aquatic systems world-wide (Smith et al. 1999, Matson et al. 2002). Although effects of N deposition in terrestrial environments are predicted to vary depending on soil and vegetation types (Matson et al. 2002), a relatively robust prediction is that plant litter is likely to have lower C:N as N deposition increases (Aber 1992). Likewise, in aquatic systems, basal food resources that consist of terrestrially-derived organic matter or primary producers generally exhibit lower carbon:nutrient ratios in response to nutrient enrichment (Cross et al. 2003). For dead organic matter, such changes occur via microbial colonization and associated nutrient sequestration (Gulis et al. 2004). Resultant changes in nutrient content may affect diversity of food resources, particularly if the unique qualities of resources converge due to nutrient enrichment.

We tested whether breakdown of mixtures of three dominant litter species in southern Appalachian watersheds was additive (could be predicted from monoculture rates) or non-additive, and whether nutrient enrichment changed interactions among litters. We chose litter species that differ in initial chemistries (C:N) and breakdown rates. These species are current co-dominants but whose relative dominance is likely to change with future species composition shifts in these forested ecosystems (Ellison et al. 2005). Species chosen were *Rhododendron maximum*, *Acer rubrum* and *Liriodendron tulipifera* (slow, medium and fast, respectively, in breakdown rate sensu Peterson and Cummins 1974, Ardón et al. 2009). Predicted changes in forest structure include some losses of foundation forest tree species such as eastern hemlock *Tsuga canadensis* and whitebark pine *Pinus albicaulis* (Ellison et al. 2005). In eastern North American forests, changes in species dominance are predicted to occur in response to declines in eastern hemlock, including increased spread of rhododendron *R. maximum* or replacement by other riparian species such as black gum *Nyssa sylvatica* or tulip poplar *L. tulipifera*, depending on latitude and current distributions (Ford and Vose 2007). Increased dominance of red maple *A. rubrum* is also predicted in response to declines in oaks, increased fire suppression and other factors (Abrams 1998). Thus, understanding how species-specific traits of dominant litter species and litter mixing effects interact with environmental change drivers, such as nutrient loading, is needed to predict future trends in detrital processing.

We predicted that we would observe non-additive effects of litter mixing on breakdown rates and that these effects would be reduced under elevated nutrient concentrations. A previous study in a lower elevation stream in the same region showed non-additive effects of mixing litter species on breakdown rates with some litter species (Kominoski et al. 2007). Nutrients potentially reduce non-additive effects of litter mixing by homogenizing variation among litter types. For example, increased nutrient concentrations accelerate breakdown rates of leaf litter and these effects are dependent on specific litter types: low quality organic matter resources (high C:N) responded more positively to nutrient enrichment than those of higher quality (Stelzer et al. 2003, Greenwood et al. 2007). In addition, microbial colonization increases %N of plant litter, which is elevated under nutrient-enriched conditions (Gulis and Suberkropp 2003). Thus, nutrient enrichment can potentially blur the distinction in substrate quality among litter species through enhanced microbial colonization and nutrient sequestration, which may affect feeding activity of higher order consumers and subsequent detrital processing. We predicted that we would observe: (1) non-additive effects of litter mixing on breakdown rates (due to interspecific variation in litter quality) under reference conditions and (2) homogenization of a key aspect of litter quality, litter C:N, due to nutrient enrichment which could lead to (3) greater colonization by detritivores across single species and litter mixtures, resulting in suppression of non-additive effects of litter mixing. We investigated these interactive effects of litter mixing and nutrient availability as part of a larger experiment assessing the long-term effects of nutrient enrichment on the structure and functioning of heterotrophic streams.

Material and methods

Study site

We deployed single species and mixed species litterbags in two streams that were similar physically, but differed in nitrogen (N) and phosphorus (P) concentrations due to an experimental addition of nutrients at the ecosystem scale (Greenwood et al. 2007). Research was conducted at Coweeta Hydrologic Laboratory Long-Term Ecological Research facility (Coweeta) located in Macon County, North Carolina, USA ($35°00'$N, $83°30'$W). Coweeta is a 2185 ha forested basin in the Blue Ridge physiographic province of the southern Appalachian mountains (Swank and Crossley 1988). Vegetation is mixed hardwood (dominated by *Quercus* spp., *Acer* spp. and *L. tulipifera*), with a dense understory of *R. maximum* that provides year-round shading of streams. Reference (catchment 53) and treatment (catchment 54) streams drain catchments of similar size (ca 5.5 ha), aspect and elevation (ca 850 m), and have similar water chemistry, discharge and physical characteristics (Greenwood and Rosemond 2005). Ambient nutrient concentrations were <30 μg l^{-1} dissolved inorganic N (DIN) and <10 μg l^{-1} soluble reactive P (SRP) in both the reference and treatment streams prior to enrichment with a N:P of 29:1 (Rosemond unpubl., Rosemond et al. 2008).

Nutrient enrichment

Beginning on 11 July 2000, inorganic N and P (NH_4NO_3 and $KH_2PO_4 + K_2HPO_4$; molar N:P ratio of 11:1) were added along a 190 m reach of the treatment stream. We maintained flow-proportional continuous dosing using a solar-powered metering pump that received a signal from an ISCO flow meter (see Greenwood and Rosemond 2005 for additional details). Bimonthly streamwater nutrient concentrations were measured in both streams for a period

of seven years. Nutrient concentrations were consistently elevated in the treatment stream (long-term (five years) means in treatment stream: DIN 506.2 ± 36.3 µg l^{-1}, SRP 80.8 ± 36.3 µg l^{-1}, compared to the reference: DIN 26.2 ± 9.8 µg l^{-1}, SRP 8.1 ± 3.8 µg l^{-1}). During the study period (December 2006–March 2007), concentrations were similarly elevated in the treatment versus reference (treatment: DIN 391 ± 156 µg l^{-1}, SRP 60 ± 19 µg l^{-1} (n = 6) and reference: DIN 8.2 ± 5.5 µg l^{-1}, SRP 3.5 ± 2.9 µg l^{-1} (n = 8)). Our enrichment concentrations are well within the range of values found in the larger geographical area of our study sites that included moderate agricultural and urban land uses (Scott et al. 2002) and within the lowest values of SRP and DIN measured in a synoptic survey of streams in the US (Dodds 2003). Low concentrations of a fungicide had been applied in the treatment stream for ca 10 months as part of another experiment, which was completed four months prior to the start of this study, on 19 August 2006. Due to the short half-life of the fungicide used (Chlorothalonil; half lives <1 day to 3 days depending on light availability (Kwon and Armbrust 2006)), we are confident that there was no continuous presence in the stream. Our experiment employed a blocked design to determine spatial variation in effects. One block in the treatment stream included an area that had no previous exposure to fungicide. No effect that corresponded to prior fungicide treatment was observed (Rosemond et al. unpubl.).

Experimental design

Senescent leaves (hereafter litter) of *L. tulipifera* (L), *A. rubrum* (A), and *R. maximum* (R) were collected from the ground just after senescence and air-dried for ca 2 months in the laboratory (spread in a single layer). After initial air-dried mass was measured, approximately nine grams total of all single- (L, A, R) and mixed-species (A+L, A+R, L+R, A+L+R) combinations of litter in equal mass proportions were placed in plastic mesh litterbags (19.1×38.1 cm, 5×5 mm mesh), resulting in high litter species evenness on a mass basis. On 13 December 2006, 210 litterbags (7 single- and mixed-species treatments $\times 3$ replicates $\times 2$ streams $\times 5$ sampling days) were randomly deployed in three replicate blocks along reaches (ca 100 m) of both reference and treatment streams, and an additional 28 litterbags (7 single- and mixed-species treatments $\times 2$ replicates $\times 2$ streams) were used to estimate handling loss, air-dried mass to ash-free dry mass (AFDM) conversions, and initial litter chemistry. Litterbags were retrieved 21, 28, 42, 56 and 98 days after incubation, brought to the laboratory on ice, and processed within 12 h. Litter was rinsed over nested sieves (1 mm and 250 µm) to remove sediment and debris, and to collect macroinvertebrates. Litter was oven-dried at 60°C for >48 h and sub-samples of litter from sample days 0, 21 and 56 were ball-milled prior to litter chemistry analyses.

Litter C:N and mass remaining

Litter C:N was measured with an elemental analyzer. Oven-dried litter was weighed and combusted at 500°C for four hours to estimate AFDM remaining. Litter AFDM was measured by subtracting ash weight from dry weight, and proportion AFDM remaining on each sampling day was calculated as the ratio of $AFDM_t$ to $AFDM_0$.

Detritivores

Macroinvertebrate detritivores retained on a 1-mm sieve were collected from leaf packs on day 56, preserved in 70% ethanol, identified to genera and classified as taxa that belonged in the functional feeding group classification of shredders versus non-shredders (Merritt and Cummins 1996, Wallace et al. 1999). We then expressed shredder abundance per g AFDM of leaf material.

Statistical analyses

Since one reference and one treatment stream were used, this study was not strictly replicated, violating assumptions of inferential statistics (Hurlbert 1984). However, we felt that the realism gained by testing the effects of nutrient enrichment on the level of the whole system was necessary to address this question in a field setting (Carpenter et al. 1995). Previous studies in these paired watersheds found effects of nutrient enrichment on litter quality (Cross et al. 2003) and breakdown of single-species plant litters (Greenwood et al. 2007). Here, we tested whether species effects in mixtures on litter mass loss were non-additive and if those effects changed under elevated nutrient concentrations. Our statistical approach was to (1) use a general linear model (GLM) to explain variation in mass loss due to time, litter species composition, nutrient treatment, and litter mixing, (Kominoski et al. 2007, Swan et al. 2009) and (2) compare mass remaining of mixtures to that predicted using single-species decay rates for each day $\times$ mixture $\times$ nutrient treatment combination. This analysis was followed by an analysis of treatment effects (species composition, nutrients, mixing) on litter C:N.

Inspection of the data suggested that breakdown rate was an exponential process. We used a GLM, analyzing $\ln(AFDM_t / AFDM_0)$ as a function of day (continuous) interacting with treatment effects (single species presence/absence, species mixtures, with/without added nutrients; Kominoski et al. 2007, Swan et al. 2009). Post-hoc analyses of day $\times$ treatment interaction terms were made to estimate litter breakdown rate, k, and associated standard errors. These are more precise compared to a simple linear regression approach (Kominoski et al. 2007, Swan et al. 2009). The post hoc test was an orthogonal contrast between all single species treatments and mixture treatments separately for reference and nutrient treatments.

We tested for single-species litter presence/absence and non-additive effects (as mixtures) on mass loss using a GLM, with effects evaluated using type I SS. We first tested for block (three levels) and day (continuous) effects, with species (A, L, R) tested at two levels (present, absent), and nutrients tested at two levels, (ambient, elevated). A mixture term (four levels) tested for non-additivity by accounting for additional variance not attributable to species presence. Species $\times$ nutrient (e.g. day $\times$ A $\times$ nutrient) and non-additivity terms (day $\times$ mixture $\times$ nutrient)

were then tested in the model. If a significant interaction term (either mixture, mixture × nutrient, or both) was found, we further investigated non-additive effects.

Non-additive effects of litter mixing on breakdown were estimated using a factorial ANOVA to compare observed versus expected day × nutrient combinations for AFDM remaining. Mass remaining of each of the four litter species mixtures were analyzed separately with three-way factorial ANOVAs, with nutrients and day (five dates) as discrete variables, and a two-level non-additivity treatment (observed vs expected litter AFDM). For a mixture, the expected fraction of mass remaining for each of our sample dates was taken as the average of the fraction of mass remaining estimated using the decay coefficients (k) for each single-species treatment obtained above. We did not use average decay rates (k) from single-species and compare them to observed k-values for mixtures, as has been criticized by others (Ostrosfky 2007). Rather, we used decay rates to estimate mass remaining for each single-species in a mixture on each sample day, averaged the predicted mass remaining for each date, then compared that to the mass remaining of the corresponding mixed-species treatment (Lecerf et al. 2007).

We tested the effects of species presence, additivity and nutrient enrichment on litter quality as C:N using a similar model to mass loss (above) but with day as a discrete variable. This tested for the effects of day (four levels), block (three levels), species (A, L, R) (two levels), and nutrients (two levels) before testing for additivity (mixtures) and all interactions. To assess whether nutrient enrichment reduced variation in C:N among single and mixed species litters, we calculated coefficients of variation (CVs) of mean C:N among single- (A, L, R) and mixed-species (A+L, A+R, L+R, A+L+R) groupings. Finally, we used regression analysis to assess relationships between macroinvertebrate shredder abundance and litter C:N content.

Analyses were carried out using PROC MIXED in SAS (ver. 9.1). Assumptions of normality of residuals were met (Shapiro–Wilkes test), but we did observe unequal variances among some treatment combinations. Therefore, we grouped residual variances when appropriate using the GROUP option (Littell et al. 1996), and adjusted denominator degrees of freedom using the Kenward–Roger approximation. All post GLM and ANOVA comparisons were orthogonal, thus not requiring adjustment of the interpretation of significance.

Results

Litter breakdown rates

We found a significant effect of litter mixing on breakdown rates (Table 1; day × mixture term: $F_{3,167} = 10.50$, $p < 0.0001$) that was dependent on nutrient enrichment (Table 1; day × mixture × nutrient term: $F_{3,167} = 4.07$, $p < 0.008$). Non-additive effects in our model were apparently derived from a suppression of mass loss in species mixtures compared to mass loss rates for single species litters. This non-additive effect did not occur in the nutrient-enriched stream (Fig. 1a). Breakdown rates

Table 1. GLM results of ln(fraction of initial AFDM remaining) as a function of days in the stream, leaf species and mixing. Species designations are *A. rubrum* (A), *L. tulipifera* (L) and *R. maximum* (R). Species effects are based on presence/absence. A significant interaction with day indicates a significant change in litter breakdown rate due to the interacting variable. Values are from type I tests of fixed effects.

Effect	Num DF	Den DF	F-value	Pr > F
Block	2	143	11.16	<0.0001
Day	1	191	509.33	<0.0001
Day × A	1	166	74.07	<0.0001
Day × L	1	167	12.57	0.0005
Day × R	1	168	209.73	<0.0001
Day × nutrient	1	167	288.09	<0.0001
Day × mixture	3	167	10.50	<0.0001
Day × A × nutrient	1	166	0.05	0.8253
Day × L × nutrient	1	166	18.35	<0.0001
Day × R × nutrient	1	167	7.29	0.0077
Day × mixture × nutrient	3	167	4.07	0.0080

calculated from parameter estimates from the model in Table 1 reflected the effects of mixing as well as nutrients: faster breakdown rates occurred in the nutrient-enriched stream than in the reference (Fig. 1b), with some species and combinations having a greater relative response than others.

Further analysis of non-additive effects comparing observed versus predicted AFDM remaining for all two- and three-species mixtures yielded significant differences that were dependent on nutrients (Table 2; nutrient × non-additivity terms). Significant observed versus predicted AFDM differences for A+L were also dependent on time (Table 2). We used parameter estimates of our non-additivity model to determine predicted versus observed mass remaining in species mixture leaf packs. We found that observed mass remaining was typically greater than predicted in the reference stream (the majority of values were at or above the 1:1 line), indicating that mixing leaf species resulted in slower breakdown than predicted by species monocultures (Fig. 2). For all four litter combinations, there was significantly higher observed versus predicted mass remaining in the reference stream, but there were no significant differences between observed versus predicted values in the nutrient-enriched stream (inset graphs, Fig. 2).

Litter C:N

Nutrient enrichment significantly reduced litter C:N, which was higher for mixtures than means of single species (Fig. 3a). The direction of this trend (mixed-species C:N > single-species C:N) was consistent with trends in breakdown rates, which were slower in mixtures than single species under reference conditions. Litter C:N values were consistently lower in the nutrient addition stream versus reference and decreased for all treatments through time (Fig. 3b). In contrast to the interactive effects of nutrients and litter mixing that we observed on litter breakdown rates, litter-mixing effects (mixture term; $F_{3,50.9} = 30.89$, $p < 0.0001$) on litter C:N were not
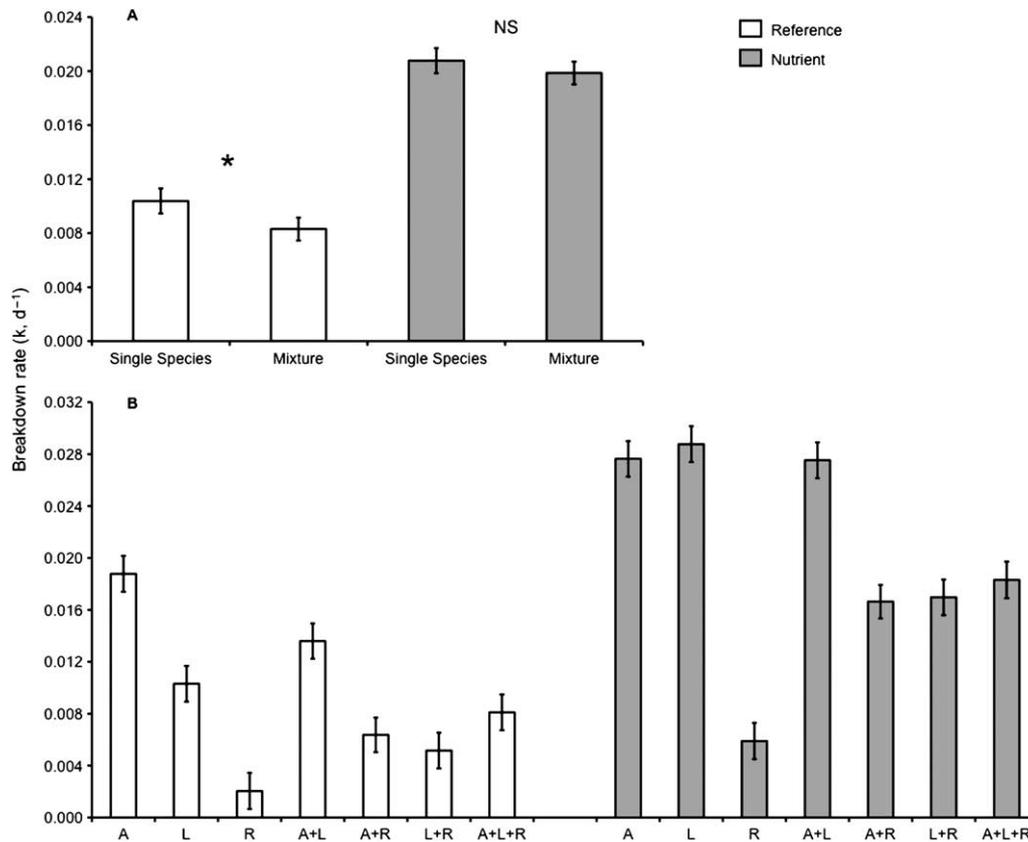
Figure 1. (A) Mean (± 1 SE) breakdown rates of singles species and mixtures separately for the reference and nutrient-enriched streams. Values are based on pooled data from single species and mixtures. Standard errors based on pooled GLM variance estimates. Significant differences (*) between single and mixed litter were determined separately for each nutrient treatment following a significant mixture $\times$ nutrient interaction in the model (Table 1). NS = no significant mixture effect. (B) Mean (± 1 SE) breakdown rates for all litter compositions (single species and mixtures) in the reference and nutrient-enriched streams.

dependent on nutrients (Table 3; mixture $\times$ nutrient term; $F_{3,51.1} = 0.89$, $p = 0.45$).

Changes in litter C:N due to nutrient enrichment were somewhat greater for single- and mixed-species litter containing R than for single- and mixed-species litter containing L (Fig. 3b). Nutrient effects on litter C:N were interactive with litter type for litters R and L but not A (Table 3). To further examine the role of individual species in driving the observed patterns in C:N, we plotted C:N for single species and combinations for A, L, and R with those species present or absent (Fig. 4). We found that the least change due to nutrient enrichment was with L present and the greatest change due to nutrient enrichment was with R present (open us filled circular symbols and solid lines). The converse was also true: when R was absent, there was the least change in C:N with nutrients and when L was absent, there was the greatest change in C:N with nutrients (open us filled square symbols and dashed lines). Changes in litter C:N were similar regardless of whether A was present or absent. We also found that CVs of C:N were consistently lower among both single- and mixed-species litter in the nutrient-enriched versus reference stream after 21 d (for both subsequent sampling dates; unpubl.), suggesting convergence of C:N among litter types in the nutrient-enriched stream.

Detritivore abundance

There was a significant negative correlation between shredder abundance and litter C:N for the reference stream (lower abundance on lower quality litter; Fig. 5). Shredder abundance was not related to litter C:N in the nutrient-enriched stream and was significantly higher than in the reference stream (t-test, $t = 2.3$, $DF = 21.1$, $p = 0.03$).

Discussion

Effects of nutrient enrichment on species interactions

We found that litter mixing had antagonistic effects on breakdown rates under reference conditions, but these effects were suppressed under elevated nutrient concentrations. Many of the potential mechanisms driving the emergent effects of leaf litter species mixing on breakdown rates depend on interspecific variation in litter quality. These mechanisms include variation in compounds that promote (e.g. nutrients, fatty acids) or suppress microbial activity among litter species or in behavioral responses of detritivores to quality (Hättenschwiler et al. 2005, Lecerf et al. 2007). We had hypothesized that nutrient enrichment would reduce variation in litter quality. Specifically, we had

Table 2. ANOVA results of AFDM fraction remaining as a function of nutrient regime, non-additivity treatment (observed versus predicted fraction AFDM remaining on any particular day) and days in the stream for each litter mixture, (a) *A. rubrum+L. tulipifera*, (b) *A. rubrum+R. maximum*, (c) *L. tulipifera+R. maximum*, and (d) *A. rubrum+L. tulipifera+R. maximum*.

(a) A+L Effect	Num DF	Den DF	F-value	Pr >F
Nutrient	1	44	329.85	<0.0001
Non-additivity	1	44	6.04	0.0180
Nutrient ×non-additivity	1	44	12.61	0.0009
Day	5	44	438.92	<0.0001
Nutrient ×day	5	44	15.49	<0.0001
Day ×non-additivity	5	44	2.86	0.0255
Nutrient ×day ×non-additivity	5	44	3.87	0.0054
(b) A+R Effect	Num DF	Den DF	F-value	Pr >F
Nutrient	1	42.7	90.68	<0.0001
Non-additivity	1	42.7	1.21	0.2783
Nutrient ×non-additivity	1	42.7	8.15	0.0066
Day	5	42.7	81.67	<0.0001
Nutrient ×day	5	42.7	4.86	0.0013
Day ×non-additivity	5	42.7	0.32	0.8980
Nutrient ×day ×non-additivity	5	42.7	0.94	0.4637
(c) L+R Effect	Num DF	Den DF	F-value	Pr >F
Nutrient	1	31.5	135.08	<0.0001
Non-additivity	1	31.5	0.03	0.8678
Nutrient ×non-additivity	1	31.5	6.04	0.0196
Day	5	31.5	71.42	<0.0001
Nutrient ×day	5	31.5	5.52	0.0009
Day ×non-additivity	5	31.5	0.24	0.9430
Nutrient ×day ×non-additivity	5	31.5	0.41	0.8410
(d) A+L+ R Effect	Num DF	Den DF	F-value	Pr >F
Nutrient	1	42	86.74	<0.0001
Non-additivity	1	42	1.02	0.3194
Nutrient ×non-additivity	1	42	4.64	0.0370
Day	5	42	74.82	<0.0001
Nutrient ×day	5	42	5.61	0.0005
Day ×non-additivity	5	42	0.53	0.7548
Nutrient ×day ×non-additivity	5	42	1.71	0.1529

hypothesized that nutrient enrichment would lower C:N content of litter, particularly of our poorest quality litter (e.g. *R. maximum* in this study), reducing this source of variation among species, and thus suppressing mixing effects. Our results were consistent with this hypothesis. The potential mechanisms proposed by which non-additive effects of litter mixing occur are: nutrient transfer between litter types (Schimel and Hättenschwiler 2007), variation in stimulation or suppression of microbes (Bardgett and Shine 1999) and variation in detritivore presence or behavior (Hättenschwiler and Gasser 2005, Swan and Palmer 2006). Our data on changes in litter C:N and detritivore density support all three of these mechanisms. Specifically, 1) nutrient content of litters converged and thus transfer of nutrients among species was likely less important, 2) convergence of nutrient content suggested that microbial colonization acted to make litters more similar rather than more different and 3) convergence of litter quality suggested less variation for detritivore feeding to act upon. By considering the mechanisms underlying non-additive effects of mixing under reference conditions, we also gained

insights into how these effects may change under elevated nutrient concentrations.

The non-additive effects on breakdown rates that occurred under reference conditions are consistent with evidence from both terrestrial and aquatic systems. However, in a summary of terrestrial studies, only 20% found antagonistic effects, whereas roughly half found synergistic effects of litter mixing (Gartner and Cardon 2004). No similar synthesis has been conducted for aquatic studies, but both synergistic (Lecerf et al. 2007, Swan et al. 2009) and antagonistic effects of mixing have been shown (Swan and Palmer 2004, Kominoski et al. 2007, Lecerf et al. 2007). Our study did not test the effects of species richness per se as we used a limited number of species in our design. However, studies that have included a greater number of species and have explicitly tested richness effects conclude that species traits are likely more important than species richness in driving non-additive responses (Wardle et al. 1997, Lecerf et al. 2007). Such non-additive responses thus appear to depend on particular groupings of species and can occur via inherent functional qualities of the species tested, as well as
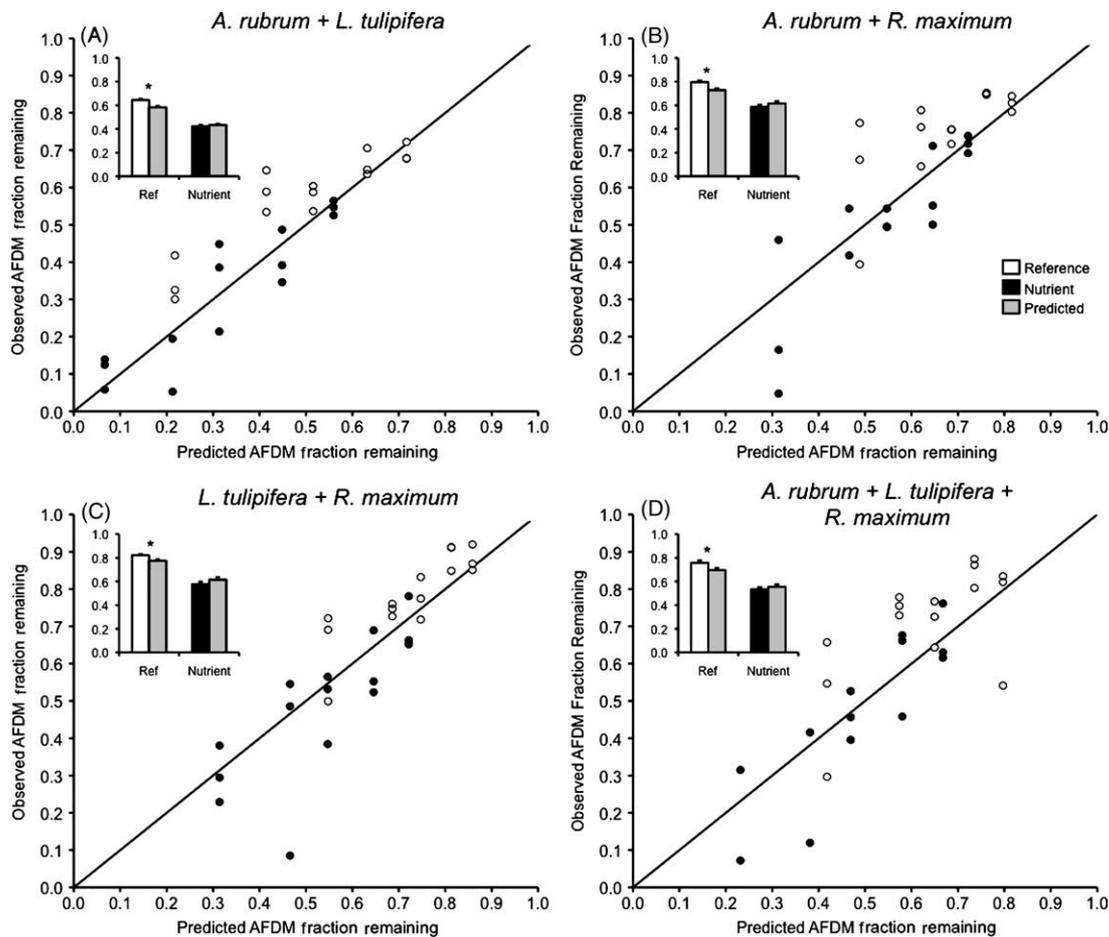
Figure 2. Observed versus predicted (from single-species breakdown estimates) litter mass remaining for each litter mixture: (A) *A. rubrum* + *L. tulipifera*, (B) *A. rubrum* + *R. maximum*, (C) *L. tulipifera* + *R. maximum*, and (D) *A. rubrum* + *L. tulipifera* + *R. maximum*. The line is the 1:1 line. The inset plots are the average (± 1 SE) observed and predicted mass fraction remaining across sample dates, for the reference and nutrient-enriched streams. Significant differences between observed and predicted estimates ($p < 0.05$) are indicated by asterisks. Open symbols are from the reference stream, solid symbols are from the nutrient enriched stream.

associated effects on decomposer and detritivore activity and diversity (Wardle et al. 1997, Lecerf et al. 2007).

The antagonistic, non-additive effects of litter mixing on breakdown rates were consistent with mixing effects on litter C:N, for which mixtures were higher in C:N than predicted based on single species. Many studies in terrestrial systems found that decomposition rates were related to initial litter N content or C:N (Wardle et al. 1997, Hättenschwiler and Gasser 2005). The higher C:N in mixtures suggests conditions inhibitory for microbial colonization when species occurred in mixtures versus singly, or alternatively, could have been the result of preferential feeding by detritivores. Although there was no corresponding nutrient × mixture interaction on litter C:N (C:N of mixtures was greater for single species than mixtures under both reference and elevated nutrient concentrations), changes in litter C:N that occurred with enrichment may provide a mechanism by which mixing effects on breakdown rates differed between high and low nutrient availability. Specifically, under high nutrient conditions, litters with initially low C:N changed the least, and litters with initially high C:N changed the most. This resulted in a lower coefficient of variation of litter C:N

under elevated nutrient conditions than under reference conditions. Thus, variation in litter quality was reduced, potentially through differential microbial colonization among litters. However, we have no direct measures of microbial mass or activity to substantiate this hypothesis. Other work in streams has shown that organic substrates with relatively high C:N (wood vs leaves; leaves ranging in C:N) that have associated slow breakdown rates exhibited a relatively greater increase in breakdown with nutrient addition than faster decomposing, lower C:N substrates (Stelzer et al. 2003, Gulis et al. 2004). Thus, initial nutrient content of detritus is likely to be homogenized as a general result of nutrient loading to aquatic systems, resulting in more homogenous breakdown rates among species.

Variation in C:N may be a potential critical catalyst that can drive detritivore colonization behavior under reference conditions, but this relationship may change under elevated nutrient concentrations. Most evidence suggests that detritivores contribute to mass loss disproportionately across differences in litter quality. Some studies have shown that aquatic detritivores selectively feed on high quality litter, particularly due to differential microbial colonization in aquatic systems (Arsuffi and Suberkropp 1989, Graca 2001,
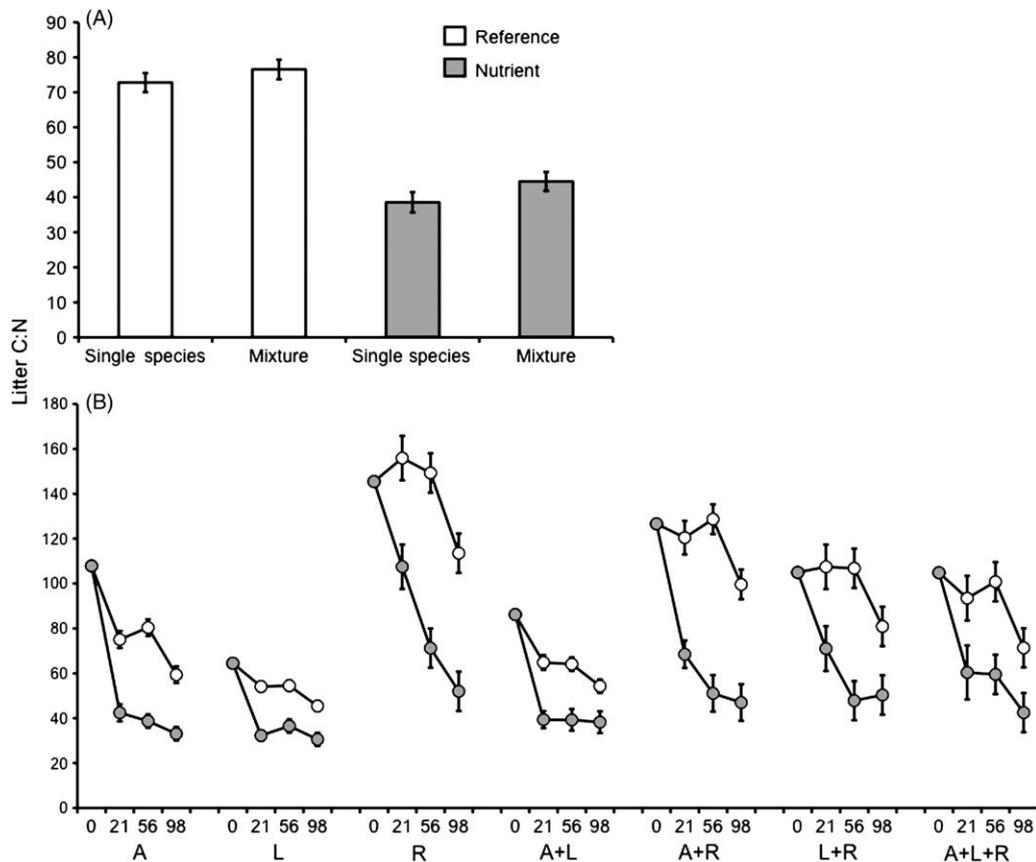
Figure 3. (A) Mean (± 1 SE) C:N of singles species and mixtures for the reference and nutrient-enriched streams on day 98. No comparisons were made between single- and mixed-species litter for each nutrient treatment due to the lack of a significant mixture × nutrient interaction in the model (Table 3). (B) Mean (± 1 SE) of litter C:N of single species and litter mixtures in the reference and nutrient-enriched streams collected on days 21, 56 and 98. Abbreviations as in Fig. 1. Standard errors based on pooled ANOVA variance estimates. Our full model of effects on C:N indicated significant effects of nutrients and mixture but no significant nutrient × mixture interaction. Open symbols are from the reference stream, solid symbols are from the nutrient enriched stream.

Wright and Covich 2005). Feeding preference by soil detritivores has been found to be related to C:N of substrates (Hendriksen 1990, Schönholzer et al. 1998) and to determine the size and direction of litter diversity effects (Hättenschwiler and Gasser 2005). In the latter study, detritivores increased mass loss of low quality species and reduced mass loss of high quality species. Although we did not manipulate detritivores in this study, we would predict that detritivores would have had effects consistent with aquatic studies: that is, contributing relatively more to mass loss of higher quality litter and thus contributing to litter mixing effects under reference conditions (Swan and Palmer 2006). In this study, variation in shredder abundance with litter C:N occurred only under reference conditions and potentially 'magnified' the variation in mass loss due to litter C:N. When nutrients were elevated, shredder abundance was higher and was similar across litter types (since litters had become similar in C:N), suggesting a reduced role of detritivores at elevated nutrient concentrations at driving variation in loss rates among litter types.

The mixing effect that we observed in the reference stream was likely driven by both microorganisms and detritivores, both of which are important in driving break-down processes in most stream ecosystems (Hieber and Gessner 2002). Kominoski et al. (2007) found that higher bacterial and fungal biomass were associated with the presence of *L. tulipifera* in mixed species packs, whereas bacterial and fungal biomass were consistently lower in packs with *R. maximum*. Nutrient enrichment can stimulate both microbial and macroinvertebrate biomass, and changes in breakdown rate due to nutrient variation can be a function of both processes (Rosemond et al. 2002). We suggest that similarly, nutrient enrichment in this study affected species mixtures via effects on both microorganisms and higher order consumers. Although our evidence for microbial change can only be inferred from changes in litter C:N, previous data from our treatment stream indicate that litter-associated fungal biomass and %N concomitantly increased with nutrient addition (Gulis and Suberkropp 2003). Thus, both microbial biomass and detritivore biomass were likely higher in the nutrient-enriched stream, potentially homogenizing variation in initial litter C:N and detritivore colonization. This resulted in higher breakdown rates that were additive based on species composition in the high nutrient stream, rather than non-additive in the reference stream.

Table 3. ANOVA results of litter C:N content as a function of days in the stream, leaf species presence/absence and mixing. Type I tests of fixed effects.

Effect	Num DF	Den DF	F-value	Pr >F
Day	3	52.3	141.54	<0.0001
Block	2	34.3	4.21	0.0231
Block ×day	5	24.6	27.28	<0.0001
A	1	47.9	26.41	<0.0001
L	1	51.6	227.57	<0.0001
R	1	54.9	455.90	<0.0001
Nutrient	1	52.0	489.40	<0.0001
Mixture	3	50.9	30.89	<0.0001
A ×nutrient	1	48.1	0.92	0.3429
L ×nutrient	1	51.4	31.94	<0.0001
R ×nutrient	1	55.0	34.95	<0.0001
Mixture ×nutrient	3	51.1	0.89	0.4529
A ×day	3	47.9	5.76	0.0019
L ×day	3	51.5	6.30	0.0010
R ×day	3	55.2	5.87	0.0015
Nutrient ×day	2	51.9	8.30	0.0007
Mixture ×day	9	53.6	1.91	0.0697
A ×nutrient ×day	3	48.1	0.19	0.9031
L ×nutrient ×day	3	51.0	3.25	0.0290
R ×nutrient ×day	3	55.5	4.24	0.0090
Mixture ×nutrient ×day	9	53.9	0.26	0.9824

Species traits

Our study was also an evaluation of how species traits, using species of interest in deciduous forests, affected litter breakdown rates and associated variables. To relate our results specifically to non-random species loss, it appears that the loss of species on the extremes of 'trait axes' (e.g. high or low C:N) might affect predictions of effects of multi-species on process rates more than losses of species with intermediate traits. Specifically, our mixing effects were likely due to the large variation in litter quality between *R. maximum* and *L. tulipifera*. Changes in litter C:N were dependent on species type, except for *A. rubrum*, for which changes in C:N were similar whether or not it was present. Thus, predicted changes in forest cover in the southern Appalachians (Ellison et al. 2005) in regard to changes in *R. maxium* or *L. tulipifera* may have greater effects on recipient stream ecosystems than changes in distributions of *A. rubrum*.

Species traits and global change

Our study illustrates the potential effects of nutrient mobilization on multi-species interactions. Here, a trait that differentiated the litter species we used (nutrient content) was related to the environmental change driver that we were testing (elevated streamwater nutrient concentrations). Thus, our study showed that when species traits are likely to be affected by specific environmental changes in ways that homogenize traits among species (i.e. in this case where nutrient enrichment led to homogenization of leaf C:N due to differential responses among species), then expressions of species traits in mixtures are predicted to be suppressed. This may be a non-conservative way to determine how aspects of global change affect species trait mixtures; if environmental change drivers were unrelated to the traits, potentially no change in the
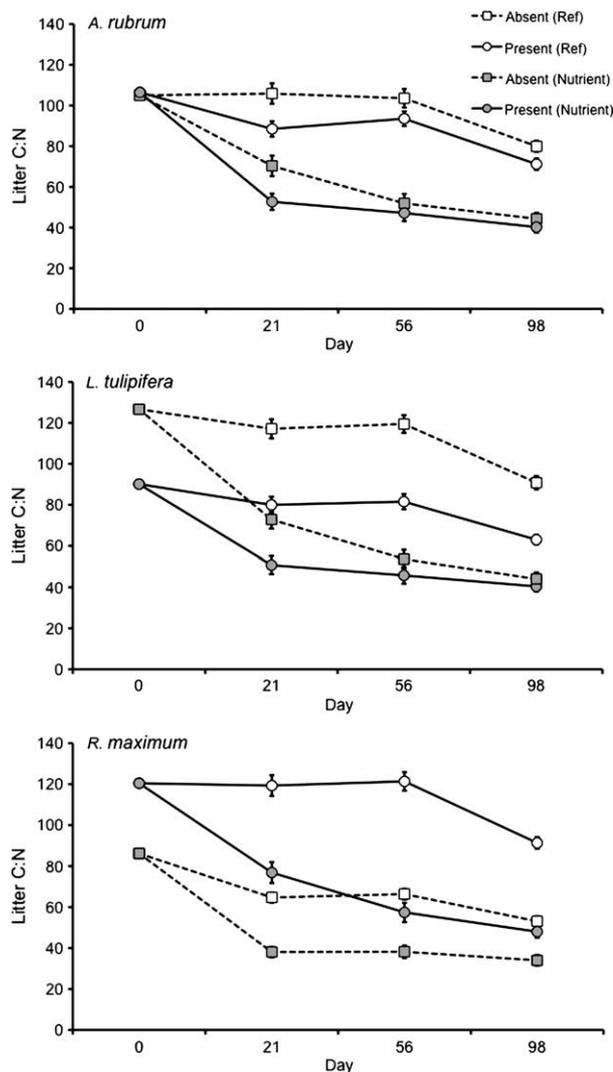

Figure 4. Mean and standard error of litter C:N calculated with each litter type present or absent. Standard errors based on pooled ANOVA variance estimates. 'Present' means based on ANOVA least-squares means for the four litter treatments containing the species, and 'Absent' for the three litter treatments without the species.

interaction of species traits would be predicted. However, this approach is a useful first step in making predictions concerning the relationships between biodiversity and ecosystem functioning under different kinds of environmental change. For example, the response observed in our study can be put in terms suggested previously by Naeem and Wright (2003): litter C:N might serve as both an effect trait (contributing to some specific ecosystem function, e.g. breakdown rate) as well as a response trait (determining the response to environmental change). In this case, the 'match' between response and effect traits might be one scenario where the effects of global change on multi-species trait expressions can be expected and predicted. Additional studies are needed to make generalizations about which (effect) traits drive multi-species interactions and whether those same traits (as response traits) are predicted to change with other kinds of global change.
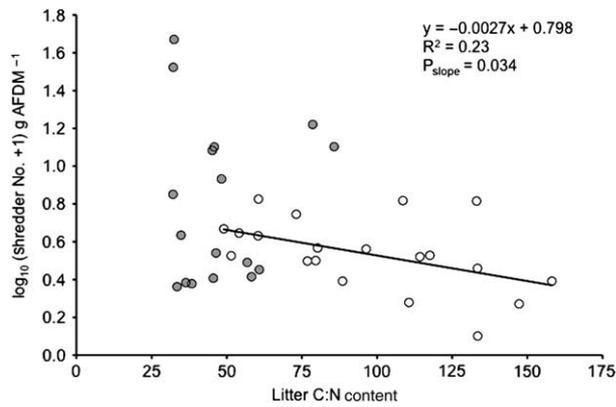
Figure 5. Shredder abundance on litter versus litter C:N under reference (open symbols) and nutrient-enriched (solid symbols) conditions. There was a significant correlation between shredder abundance and litter C:N in the reference stream (line and equation shown), but not in the nutrient-enriched stream.

Our study also illustrates that the effect of nutrient enrichment, even at relatively low concentrations, was greater in magnitude than the effect of species mixing on litter breakdown. Some studies have found relatively large effects of species mixing on breakdown rates, but on average these effects were 17% and 9% respectively, for synergistic and antagonistic effects (summarized by Hättenschwiler et al. 2005). The change in breakdown rates due to non-additivity in this study was also of moderate size (average 20% reduction) compared to the large changes we observed due to nutrient enrichment (average 148% increase, e.g. more than a doubling in rate). Thus, human modification of landscapes can trump effects due to natural variability in species traits in determining rates of biotic processes. That said, other studies have found non-additive effects of litter mixing on breakdown rates in streams, although small in size, at higher nitrogen concentrations than reported here (Lecerf et al. 2007). However, the relative influence of non-additive effects versus environmental change is unknown for most systems and will likely be determined by the effect and response traits of species (sensu Naeem and Wright 2003), as well as the degree of environmental change from some reference state.

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