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# Placing the Effects of Leaf Litter Diversity on Saprotrophic Microorganisms in the Context of Leaf Type and Habitat

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**Abstract** Because of conflicting results in previous studies, it is unclear whether litter diversity has a predictable impact on microbial communities or ecosystem processes. We examined whether effects of litter diversity depend on factors that could confound comparisons among previous studies, including leaf type, habitat type, identity of other leaves in the mixture, and spatial covariance at two scales within habitats. We also examined how litter diversity affects the saprotrophic microbial community using terminal restriction fragment length polymorphism to profile bacterial and fungal community composition, direct microscopy to quantify bacterial biomass, and ergosterol extraction to quantify fungal biomass. We found that leaf mixture diversity was rarely significant as a main effect (only for fungal biomass), but was often significant as an interaction with leaf type (for ash-free dry mass recovered, carbon-to-nitrogen ratio, fungal biomass, and bacterial community composition). Leaf type and habitat were significant as main effects for all response variables. The majority of variance in leaf ash-free dry mass and C/N ratio was explained after accounting for treatment effects and spatial covariation at the meter (block) and centimeter (litterbag) scales. However, a substantial amount of variability in microbial communities was left unexplained and must be driven by factors at other spatial scales or more complex spatiotemporal dynamics. We conclude that litter diversity effects are primarily dependent on leaf type, rather than habitat type or identity of surrounding leaves, which can guide the search for mechanisms underlying effects of litter diversity on ecosystem processes.

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## Introduction

Understanding the consequences of shifts in biodiversity is a central topic of ecological study [18, 26]. After senescence and abscission, leaves originating from different tree species naturally intermingle to form leaf litter mixtures of varying diversity on the forest floor. These leaf mixtures are the primary carbon and energy resources for decomposer microorganisms, but the impact of varying leaf diversity on the decomposer microbial community has not been explicitly investigated. Previous experimental work on the impacts of litter diversity on decomposition rates has often revealed significant patterns, but the direction and magnitude of litter diversity effects in different studies can be inconsistent (reviewed in [13, 17]). This suggests that diversity effects may be altered by interaction with habitat or leaf characteristics, which often vary among studies. Identification of factors that moderate the effects of litter diversity will ultimately lead to mechanistic predictions of its importance in different situations.

Leaf litter diversity may influence microbial communities and decomposition rates through several mechanisms related to mixing of leaves with differing characteristics [17]. Soluble organic compounds and nutrients may be transferred between leaves via water flow and diffusion. Release of tannins and phenolics can inhibit decomposition of adjacent leaves [12, 30, 38], whereas nutrient mineralization from labile litter can stimulate decay rates of nutrient-poor litter [6, 12, 32]. Translocation of nutrients

from nutrient-rich litter through the action of hyphal organisms can also stimulate decay rates of nutrient-poor litter [41, 44]. Finally, different types of leaves may alter physical characteristics of the leaf litter microenvironment, resulting in, for example, differing levels of moisture retention [49]. Litter diversity *per se* is not a component of these mechanisms, implying that leaves of each species may be affected in unique ways by the diversity or identity of surrounding leaves. Hence, it has been noted that the effects of litter diversity may be masked if analyses are performed on multi-species mixtures or pooled samples, rather than individual leaf types separated from litter mixtures [2, 19].

Habitat characteristics could also potentially alter interactions among leaves of different species. For example, water saturation can affect the relative importance of diffusion versus hyphal transport of nutrients between leaves, as well as other factors, including saprotrophic community composition [36, 46]. To look for habitat interactions with litter diversity, we chose to examine three broadly different habitats (stream channel, riparian forest, upland forest) that are well known to differ in microbial community composition and decomposition rates, thus maximizing the likelihood that the importance of litter diversity will vary between habitats. In addition, smaller-scale environmental variation within habitats (e.g., in nutrient availability) that could alter litter diversity effects was also examined by quantifying spatial covariance within habitats at two spatial scales.

We focus on the beginning stages of decomposition and on early-colonizing microbial saprotroph communities for several reasons. Decomposition rates are much faster in streams than terrestrial environments, so we targeted a short duration in order to include the stream habitat in this study [40]. During early stages, leaves retain many of their original, distinct characteristics before becoming increasingly homogenized chemical resources for microorganisms. Finally, microbial community characteristics in early stages can dramatically affect subsequent decomposition [8, 20].

Direct examination of microbial saprotrophs, and the elements often limiting their activity (C and N), may provide important insight into causes of variation in effects of litter diversity. The mechanisms by which litter diversity may affect saprotrophs differ from hypothesized relationships between plant diversity and primary production [18, 26] because senesced leaves do not constitute a living ecological guild performing an ecosystem process, but instead serve as a resource for microbial saprotrophs. The diversity of leaf litter represents resource and habitat heterogeneity for saprotrophs at a local spatial scale. In theory, increased environmental heterogeneity increases the diversity of organisms that can coexist in a location [42, 47]. However, the diversity of coexisting microorganisms has been found to be so large [10, 50] that it is difficult to make predictions about the effects of a relatively modest increase in heterogeneity without an understanding of both the degree of specialization of microbes for different leaf types, and the spatial scale at which microbial communities tend to vary. Although it is possible to hypothesize that diverse leaf mixtures will favor particular saprotrophs ("diversity specialists"), we suggest that placing leaves of different types in close proximity is more likely to alter colonization and competition dynamics such that the mixed leaf types have more homogeneous saprotrophic communities than when the leaf types are separated. We examine these properties of microbial communities using terminal restriction fragment length polymorphism (T-RFLP), which facilitates quantitative, cultivation-independent comparison of dominant community members in a large number of environmental samples [45].

In this study, we compare decomposition and development of microbial communities over a short period in leaves from four woody species when incubated singly or in mixtures of differing diversity. Our experiment examined if the effects of leaf litter diversity were specific to a particular leaf type or habitat. Specifically, we compared litterbags containing one, three, or four leaf types to test the hypotheses: [1] the importance of litter diversity depends on the habitat in which the leaves decomposed, [2] the importance of litter diversity depends on the leaf type (or species) in question, and [3] the importance of litter diversity depends on the identity of the surrounding leaves. We also tested the hypothesis [4] that mixing leaves reduces the overall variability among leaf types (i.e., leaves in four-species and three-species litterbags would be more similar to each other than they would be when separated into four single-species litterbags).

## Materials and Methods

### Experimental Design and Study Site

During October 2007, senescent American beech (*Fagus grandifolia*), witch hazel (*Hamamelis virginiana*; hereafter referred to as "hazel"), sugar maple (*Acer saccharum*), and pin oak (*Quercus palustris*) leaves were collected from trees in Jennings Woods in northeastern Ohio, USA [31]. Leaves were air dried at room temperature. Representative samples were used to determine percent organic matter and C/N ratio using an Elemental Combustion System (Costech Instruments, Valencia, CA, USA).

Litterbags were created by placing 5 g of leaves in polyethylene mesh bags (16×28 cm, 3-mm mesh openings; Sacramento Bag Manufacturing Co., Woodland, CA, USA), which were then closed with plastic cable ties. For mixed-

species bags, leaf weight was evenly distributed among species. Litter combinations included: each single species alone (beech, hazel, maple, or oak), each combination of three species (beech + maple + oak, beech + hazel + oak, hazel + maple + oak, beech + hazel + maple), and all four species together (beech + hazel + maple + oak), for a total of nine litterbag treatments.

Litterbags were deployed in three habitats at Jennings Woods in November 2007: stream, riparian forest, and upland forest. Litterbags were deployed in four replicate blocks in each habitat ($n$=4 replicates per treatment). In terrestrial habitats, nine litterbags representing the nine different litter combinations were placed in four 0.5×1-m blocks separated by approximately 10 m. Bags were placed on the soil surface after removal of existing leaf litter to avoid confounding the diversity treatments. In the stream, litterbags were attached to four ropes, considered "blocks", separated by approximately 10 m and spanning the width of the stream channel. After 7 weeks, litterbags were collected and brought to the lab on ice.

## Leaf Processing, Mass Loss, and C/N Ratio

For each litterbag, leaves were separated by species and rinsed with sterilized distilled water to remove adherent material. Leaves were then weighed, divided into ~0.5× 0.5-cm pieces, and subsampled for various analyses. One subsample was dried at 68°C for 24 h to determine moisture content. A portion of the oven dried sample was used to determine C and N content as above, and the remainder was heated to 500°C for 5 h to determine leaf ash-free dry mass (AFDM). Another subsample was frozen at −80°C for subsequent DNA extraction. Finally, two subsamples were preserved to determine microbial biomass as described below.

## Bacterial and Fungal Biomass

For bacterial biomass, leaf fragments were preserved in 1× phosphate-buffered saline (Ph 7.2) and 10% formaldehyde, and sonicated for 5 min [31]. Samples were concentrated on 0.2-μm black polycarbonate filters and stained with 15 μg/ml 4′,6-diamidino-2-phenylindole for 3 min [37]. Total bacterial number was determined by counting under epifluorescence microscopy. Biovolumes of approximately 200 cells per sample were calculated from digital images based on cell measurements using CMEIAS/ImageTool 1.27 [24]. Biomass was calculated from allometric equations according to Loferer-Krößbacher et al. [25].

Extraction of ergosterol from leaves was used to determine fungal biomass, following Tank and Webster [43]. Ergosterol content in extracts was determined by HPLC (Alltech, IL) and comparison with peak areas of standards ranging from 0.25 to 20 mg/l (Sigma-Aldrich, Seelze, Germany). Fungal biomass of leaves was calculated assuming an ergosterol content of 5.5 mg/g biomass [14].

## Microbial Community Analyses

Leaves from each single-species and four-species bag were subjected to DNA extraction and community analysis. Briefly, approximately 100 mg of leaf material was ground in a Genogrinder (2000 model; SPEX CertiPrep, Metuchen, NJ, USA), followed by addition of 500 μl CTAB buffer [2% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 2% polyvinyl pyrrolidine 40, 20 mM EDTA, 1.42 M NaCl, 5 mM ascorbic acid, 100 mM Tris, pH 8.0] and 1 μl β-mercaptoethanol buffer. Samples were incubated for 20 min in a 60°C water bath and centrifuged for 10 min at 4,000 rpm. The aqueous layer was then isolated, extracted with an equal volume of chloroform, and then mixed with an equal volume of isopropanol. DNA was precipitated overnight at −20°C followed by centrifugation 10 min at 4,000 rpm. After isopropanol was removed, the pellet was washed with 200 μl of 70% ethanol, centrifuged 5 min at 4,000 rpm, and then DNA was dissolved in 50 μl of sterile distilled water.

Bacterial and fungal T-RFLP was performed as previously described [9]. Bacterial 16S rRNA genes were amplified using primers Eub338F-0-III (labeled with 6-carboxyfluorescein) and 1392R, with annealing at 57°C and 35 PCR cycles [4]. The fungal ribosomal internal transcribed spacer (ITS) region was amplified with NSI1F (labeled with 6-carboxyfluorescein) and NLB4R, with annealing at 60°C and 30 PCR cycles [28]. PCR product was digested with HaeIII prior to separation and detection of terminal restriction fragments as described previously [9]. T-RFs 50–600 bp in size were used for further analysis if their fluorescence was greater than 0.1% and 0.5% cumulative fluorescence for the bacterial and fungal samples, respectively.

## Statistical Analyses

AFDM recovery, C/N ratio of leaves, bacterial biomass, and fungal biomass were analyzed using mixed linear models by Proc Mixed in SAS 9 [39]. Habitat (stream, riparian, or upland), leaf type (beech, hazel, maple, or oak), and litter diversity treatment (one, three, or four species) were fixed effects in a full-factorial design, with block (nested within habitat) and bag (nested within block) included as random effects. A significant diversity treatment × habitat interaction would support hypothesis 1 (diversity effects depend on habitat), and a significant diversity treatment × leaf type interaction would support hypothesis 2 (diversity effects depend on leaf type). Both hypotheses 1 and 2 would also

be supported by a significant three-way interaction between diversity treatment, habitat, and leaf type. If a significant interaction with diversity was found, effects of diversity within individual leaf types or habitats were investigated. Type III tests were used to account for the unbalanced design of the experiment with respect to diversity treatment (different three-species combinations were pooled here to focus on diversity, but separated in an analysis below to test hypothesis 3). Pairwise comparisons were performed using the Tukey multiple comparisons test based on least squares means and standard errors calculated by Proc Mixed.

Hypothesis 3 (diversity effects for one leaf type depend on the particular combination of other leaf types present) was tested separately for each leaf type by a mixed model analysis of three-species litterbags. For each leaf type, there were three unique three-species combinations (which were pooled in the analysis of diversity described in the previous paragraph). Hypothesis 3 is supported if the three-species combinations are significantly different from each other.

Hypothesis 4 (samples of different leaf types mixed in the same litterbag are less variable than when separated into different species-specific litterbags) is supported if, after accounting for all other factors in the model, there is additional variance explained by the fact that certain leaves were together in the same litterbag. We therefore tested whether the covariance accounted for by random factors was significantly different from zero by a Wald-$Z$ test requested with the restricted maximum likelihood estimate of covariance parameters in Proc Mixed (SAS Institute 2004). Wald-$Z$ tests are sensitive to small sample sizes, so results were confirmed by (1) $\chi^2$ tests calculated by comparing −2 log-likelihood values of nested models run with and without the random effects, and (2) exact $F$ tests where the random factors were specified as fixed effects.

T-RFLP peak height relative abundances were square-root transformed to base community comparisons on Hellinger distance [23]. The hypotheses that habitat, leaf type, or litter diversity affected T-RFLP profiles were tested by redundancy analysis using the software Canoco 4.5 (MicroComputer Power, Ithaca, NY, USA) [22]. Interaction effects were interpreted as tests of hypotheses 1 and 2, as described above. Hypothesis 3 could not be tested for T-RFLP profiles because the analysis was not performed on three-species bags.

Hypothesis 4 was tested on T-RFLP data using a permutation test implemented in SAS Proc IML, similar to the multivariate dispersion test of Anderson [1]. This test focuses on a difference between single-species and four-species litterbags in terms of variability in community composition (or multivariate dispersion) independent of any change in average composition (or multivariate location) across habitats or blocks. Hellinger variance, a measurement of multivariate dispersion for a group of communities, was calculated by finding the average Hellinger distance between all sample pairs within a group. Hellinger variance was calculated separately for the following groups of leaves in each block: (1) leaves that were together in a four-species litterbag and (2) leaves that were separated into four single-species litterbags. The difference between one- and four-species Hellinger variances was calculated for each block, and the average difference was then calculated across all blocks. The probability of this difference arising by chance was assessed by comparing the empirical value to a distribution obtained by 999 random permutations. For each permutation, samples within each block were randomly divided into single-species and four-species treatments (maintaining $n$=4), and Hellinger variances were recalculated.

## Results

### Mass Loss and C/N Ratio

As expected, mass loss was strongly influenced by the main effects of leaf type and habitat, and there was also a significant leaf type × habitat interaction (Table 1, Fig. 1). However, the habitat × diversity interaction was not significant (Table 1), resulting in no support for hypothesis 1. In support of hypothesis 2, the diversity × leaf type interaction was marginally significant (Table 1). However, this accounted for a small amount of the variance in leaf AFDM recovered in comparison to main effects of leaf type and habitat (Fig. 2). Mass loss was minimal in beech leaves in upland and riparian habitats, and in oak leaves in all habitats, over the 7-week incubation period (Fig. 1a; we attribute AFDM values over 100% to extrapolation of average % moisture and ash for each unincubated leaf type to all litterbags, when in fact there was likely variation among leaf subsamples). Because lack of mass loss in one leaf type may obscure significant results, the full ANOVA model was repeated without oak, and then again without oak or beech. Diversity was still not significant in these analyses, but the diversity × leaf type interaction was strengthened ($P$<0.05 in both analyses). In analyses of individual leaf types, there were significant differences among diversity treatments only for maple leaves (analysis of maple only, $P$<0.01; Fig. 1b).

The effect of the different three-species combinations (hypothesis 3) on AFDM was not significant ($P$>0.05). Positive covariance of leaf samples from the same litterbag (after accounting for fixed effects; Hypothesis (4) was marginally significant in Wald-$Z$ and $F$ tests, but not the $\chi^2$ test. The effect of block was highly significant in $\chi^2$ and $F$ tests, and marginally significant in the Wald-$Z$ test (Table 2; Fig. 2).

**Table 1** *P* values determined by linear mixed model ANOVA (AFDM recovery, C/N ratio, bacterial biomass, fungal biomass) or redundancy analysis (bacterial and fungal community composition)

| | AFDM | Leaf C/N | Bacterial biomass | Fungal biomass | Bacterial community | Fungal community |
|---|---|---|---|---|---|---|
| Habitat | 0.0038 | 0.0001 | 0.0029 | 0.0009 | 0.001 | 0.001 |
| Diversity | NS | NS | NS | 0.0074 | NS | NS |
| Leaf type | <0.0001 | NS | <0.0001 | <0.0001 | 0.051 | 0.001 |
| Habitat × diversity[a] | NS | NS | NS | 0.0347 | NS | NS |
| Habitat × leaf type | NS | <0.0001 | <0.0001 | <0.0001 | 0.006 | 0.011 |
| Diversity × leaf type[b] | 0.0516 | <0.0001 | NS | 0.0470 | 0.033 | NS |
| Habitat × leaf type × diversity | NS | NS | NS | NS | NS | NS |

*NS* not significant

[a] Interaction term corresponds with hypothesis 1

[b] Interaction term corresponds with hypothesis 2

The initial C/N ratios of beech, hazel, maple, and oak were 66, 66, 56, and 42, respectively. C/N ratios of decomposed leaves were significantly influenced by habitat (Table 1; Fig. 3a) but not main effects of leaf type or diversity treatment. However, hypothesis 2 was supported because there was a significant diversity × leaf type interaction (Table 1), and different leaf types exhibited divergent responses to diversity treatment. The C/N ratio of beech was not different between single- and mixed-species litterbags ($P>0.05$; Fig. 3b), while the C/N ratio of hazel was significantly higher in single-species bags than in three-species and four-species mixed bags ($P<0.01$). In contrast, the C/N ratio of oak was significantly higher in three-species and four-species mixed bags than in single-species bags ($P<0.01$). Finally, the C/N ratio of maple was highest in three-species bags, intermediate in single-species bags, and lowest in four-species bags ($P<0.01$; Fig. 3b).

For some leaf types, the identity of surrounding leaf types affected C/N ratio, supporting hypothesis 3. In beech leaves, there was a significant effect of the different three-species combinations ($P<0.05$). Beech C/N ratio in the beech + hazel + oak combination was significantly lower than in the beech + maple + oak combination, with beech + hazel + maple intermediate. A similar trend was seen in maple C/N ratio, increasing in order of maple + hazel + oak, beech + hazel + maple, beech + maple + oak. The main effect of three-species combination was not significant for maple ($P>0.05$), but there was a significant habitat × three-species combination interaction ($P<0.05$); the difference in C/N ratio between maple + hazel + oak and beech + maple + oak was significant only within the riparian habitat. There was also a significant habitat × three-species combination interaction ($P<0.05$) for hazel leaves, but no significant differences between three-species combinations within a habitat emerged. There were no significant leaf type combination effects for oak.

Hypothesis 4 was supported for C/N ratio because covariance due to litterbag was significant in all three random-effects tests ($P<0.05$), and the effect of block was highly significant in $\chi^2$ and $F$ tests, and marginally significant in the Wald-$Z$ test (Table 2; Fig. 2).

## Microbial Biomass

Bacterial biomass on leaves was significantly different among habitats and leaf types, but there were no statisti-

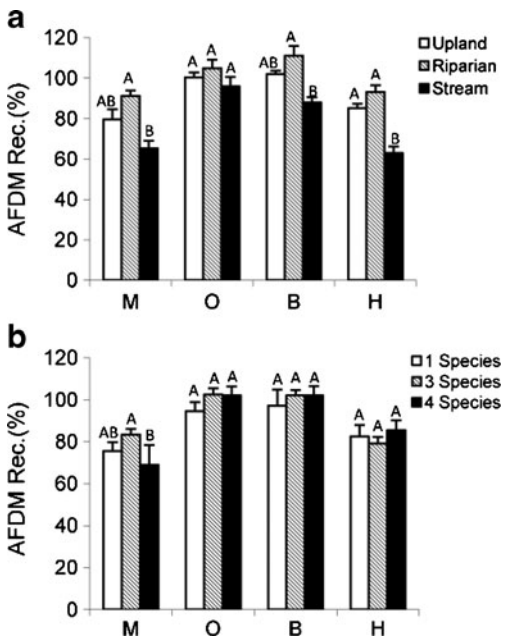


**Figure 1** The percent recovery of ash free dry mass (% AFDM) for leaf types in **a** different habitats (averaged across diversity treatments) and **b** different diversity treatments (averaged across habitats). *B* beech, *H* hazel, *M* sugar maple, *O* pin oak. *Bars* show mean of four replicates + standard error. *Bars* within one leaf type with differing *letters* are significantly different ($P<0.05$)

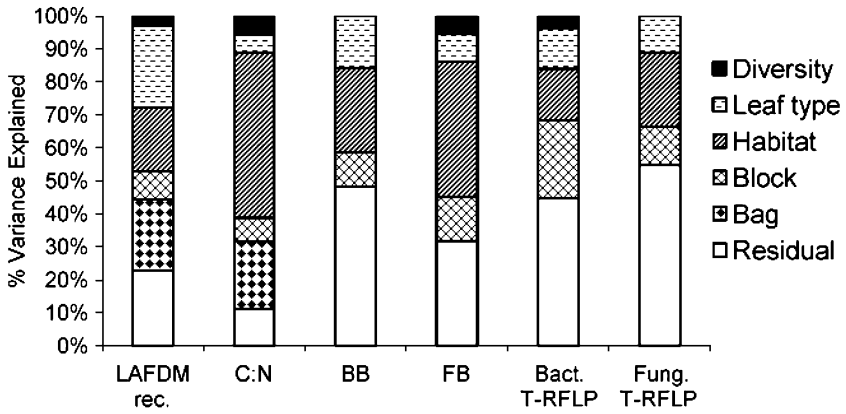


**Figure 2** Percent variance explained by fixed and random factors found to be significant or marginally significant ($P<0.1$; Table 1) by linear mixed model ANOVA [AFDM, C/N ratio, bacterial biomass (*BB*), fungal biomass (*FB*)] or redundancy analysis (bacterial and fungal T-RFLP profiles). Significant diversity × leaf type and diversity × habitat interactions were included in the variance explained by diversity; significant leaf type × habitat interactions were included in the variance explained by leaf type

cally significant differences among diversity treatments. There was also a significant habitat × leaf type interaction; other interactions were not significant (Table 1; Fig. 4). The effect of the different three-species combinations (hypothesis 3) on bacterial biomass was not significant ($P>0.05$). The effect of litterbag on variance in bacterial biomass (hypothesis 4) was not significant ($P>0.05$), whereas the effect of block was highly significant in $\chi^2$ and *F* tests, and marginally significant in the Wald-*Z* test (Table 2).

In contrast to bacterial biomass, fungal biomass was significantly influenced by the main effect of diversity, in addition to habitat and leaf type (Table 1). There were also several significant interactions (Table 1), including habitat × diversity (supporting hypothesis 1) and leaf type × diversity (supporting hypothesis 2). Averaging across leaf types, four-species litterbags had significantly greater fungal biomass (13.1±2.2 mg/g AFDM) than three-species (11.1±1.0 mg/g AFDM) or single-species (9.8±1.4 mg/g AFDM) litterbags. In separate analyses of leaf types, the same pattern was significant for maple leaves ($P<0.01$), and beech leaves behaved similarly but differences were not significant ($P>0.05$; Fig. 5b). For hazel, fungal biomass was highest in three-species mixed bags, intermediate in four-species bags, and lowest in single-species bags ($P<0.05$; Fig. 5b). Fungal biomass in oak leaves was relatively constant ($P>0.05$), although slightly lower in four-species bags (Fig. 5b). The effect of the different three-species combinations (hypothesis 3) on fungal biomass was not significant ($P>0.05$). The effect of litterbag on variance in fungal biomass (hypothesis 4) was not significant, whereas the effect of block was significant in all three random-effects tests (Table 2).

## Microbial Community Composition

Bacterial and fungal community profiles of leaves from single- and four-species litterbags were determined using T-RFLP. Among fixed factors, habitat and leaf type explained significant variability in both bacterial and fungal community composition (Table 1). However, for bacterial community profiles, there was a significant diversity × leaf type interaction supporting hypothesis 2, although this accounted for a small amount of total variance (Fig. 2). This interaction is apparent in the canonical ordination plot (Fig. 6a–d), where one- and four-species treatments are consistently separated for beech and hazel leaves, but not maple or oak. Furthermore, the bacterial communities of beech and hazel responded in opposite ways; in Fig. 6c, single-species hazel communities are shifted to the upper right of four-species hazel communities from the same

**Table 2** *P* values for tests of random factors evaluated by linear mixed model ANOVA

| Factor | Test | AFDM | Leaf C/N | Bacterial biomass | Fungal biomass |
|---|---|---|---|---|---|
| Block | Wald-*Z* | 0.0567 | 0.0557 | 0.057 | 0.0297 |
| | $\chi^2$ | <0.0001 | <0.0001 | <0.0001 | <0.0001 |
| | *F* test | <0.0001 | <0.0001 | 0.0002 | <0.0001 |
| Litterbag[a] | Wald-*Z* | 0.0968 | 0.039 | NS | NS |
| | $\chi^2$ | NS | 0.0404 | NS | NS |
| | *F* test | 0.051 | <0.0001 | NS | NS |

*NS* not significant

[a] Variance explained by litterbag is a test of hypothesis 4

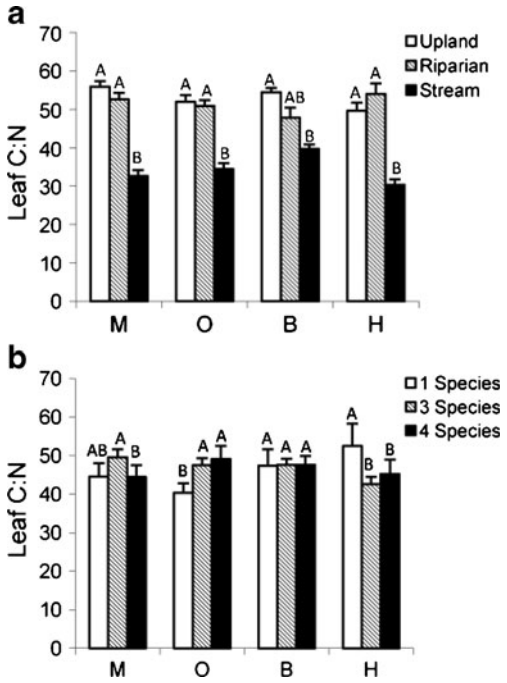


**Figure 3** C/N ratio for leaf types in **a** different habitats (averaged across diversity treatments) and **b** different diversity treatments (averaged across habitats). *B* beech, *H* hazel, *M* sugar maple, *O* pin oak. *Bars* show mean of four replicates + standard error. *Bars* within one leaf type with differing *letters* are significantly different ($P<0.05$)

habitat, whereas in beech communities in Fig. 6d this pattern is reversed.

The effect of block within habitat was also significant and accounted for a large portion of total variability for both bacteria and fungi ($P<0.05$, Fig. 2). Hypothesis 4 was not supported by permutation testing. Hellinger variance was 0.79 for bacteria in single-species litterbags and 0.73 for four-species litterbags; however, this difference was not found to be significant by permutation test ($P>0.05$). For fungi, Hellinger variance was 0.85 and 0.83 for single-species and four-species litterbags, respectively, but the increased variance in the single-species was again not significant ($P>0.05$).

## Discussion

### Effects of Litter Diversity and Interactions with Other Factors

In the absence of strong generalizable patterns, it is important to understand the circumstances in which diversity could have an important impact on biological processes [7]. Previous studies have documented variable effects of plant litter diversity on the processes and organisms involved in decomposition [13, 17]. Here, we showed that the effects of litter diversity are highly dependent on interactions with other factors. Among our six measured responses, we rarely found a main effect of litter diversity (only for fungal biomass), but the interaction between diversity and other factors was significant in four cases (including fungal biomass). The interaction of litter diversity with leaf type seemed to be particularly important: for four out of six response variables, the leaf type × diversity interaction (hypothesis 2) was significant. Hence, litter diversity was important for some leaf types and not others, and in some cases the effects of diversity were opposite for different leaf types. Other factors hypothesized to confound the main effects of litter diversity (see hypotheses 1, 3, and 4) were each found to be significant for only one or two response variables. This suggests that differences between studies observed previously may be due largely to the particular array of leaf types used, rather than differences between habitats. This is surprising given the widely differing habitats investigated here, but suggests that the effects of leaf litter mixture composition depends primarily on the properties of the particular leaf in question, rather than the exact transport mechanism of substances between leaves.

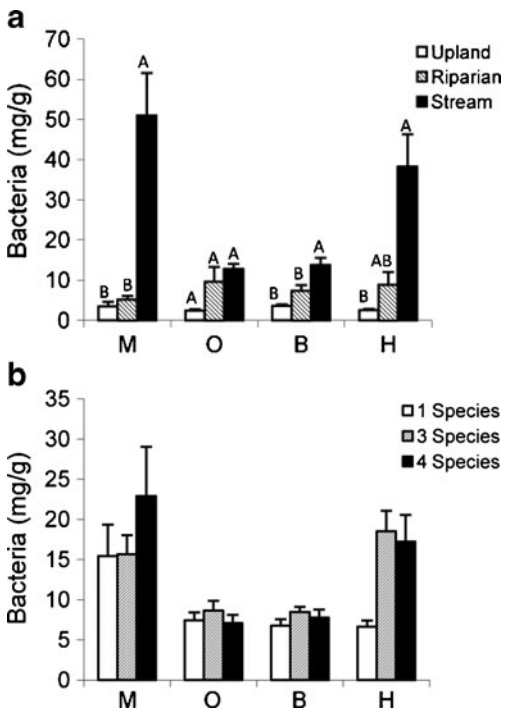


**Figure 4** Bacterial biomass for leaf types in **a** different habitats (averaged across diversity treatments) and **b** different diversity treatments (averaged across habitats). *B* beech, *H* hazel, *M* sugar maple, *O* pin oak. *Bars* show mean of four replicates + standard error. *Bars* within one leaf type with differing *letters* are significantly different ($P<0.05$)

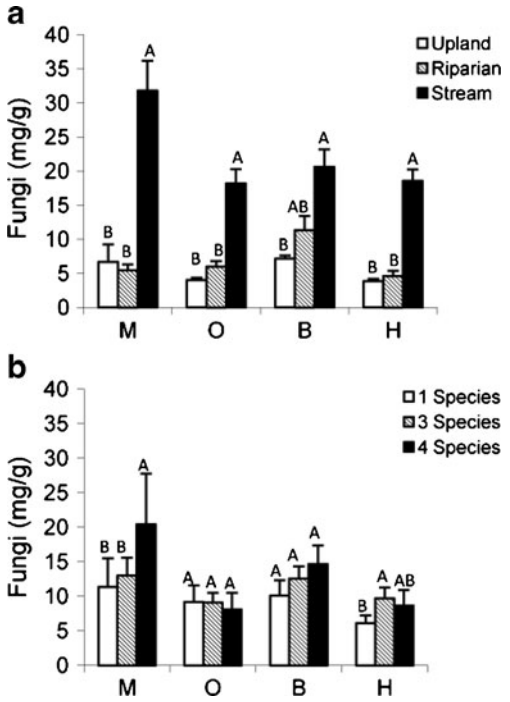


**Figure 5** Fungal biomass for leaf types in **a** different habitats (averaged across diversity treatments) and **b** different diversity treatments (averaged across habitats). *B* beech, *H* hazel, *M* sugar maple, *O* pin oak. *Bars* show mean of four replicates + standard error. *Bars* within one leaf type with differing *letters* are significantly different ($P<0.05$)

Lecerf et al. [21] reached a similar conclusion examining decomposition of litter mixtures in streams, but Madritch and Cardinale [27] found a significant site × litter diversity effect on decomposition across three terrestrial sites. Different conclusions may have been reached because Madritch and Cardinale [27] did not separately quantify mass loss of leaf types in mixtures, so direct impacts of leaf type and leaf type × diversity could not be tested. Our results highlight the importance of physically separating leaf types during sample processing in order to quantify the effects of litter diversity. The effects of litter diversity for some leaf types will be masked by inclusion of other leaf types that do not respond to litter diversity, or that have opposite responses.

Litter diversity did have a relatively consistent positive effect on fungal biomass. Fungi are able to integrate microhabitats, such as leaves and soil, through hyphal transport of resources [5, 11]. Hence, fungal biomass may increase with litter diversity due to more optimal use of resources in any given leaf type. For example, C/N ratio increased with diversity in oak leaves, whereas it decreased in hazel leaves. This could be due to hyphal transport of nitrogen from oak to hazel leaves, resulting in greater biomass on hazel with increased diversity (oak was the only leaf type where diversity had little effect on fungal biomass). Litter diversity also increased fungal biomass on beech and maple leaves, although C/N ratio was not consistently affected. We hypothesize that transfer of other resources may have been involved for these leaf types (e.g., phosphorus, micronutrients). Another possibility is that a wider variety of biochemical compounds available for direct uptake could stimulate fungal growth by alleviating the need for additional biosynthesis through fungal metabolism.

## Main Effects of Leaf Type and Habitat

While our results suggest that litter diversity, and interactions of diversity with other factors, are important in some cases, the amount of variance explained by these factors was relatively small compared to the main effects of leaf type and habitat (Fig. 2). It is not surprising to find differences between stream and terrestrial habitats. Saprotrophs in terrestrial and stream habitats experience vastly different environments in terms of moisture, redox, and nutrient limitation, as well as physical mixing and abrasion in the environment. Leaf type is also well known to affect decomposition rate through differences in contents of nutrients and plant biochemicals (e.g., cellulose, lignin). Leaf types with lower lignin and higher nitrogen contents are expected to decompose more rapidly [3, 33], and this pattern was observed for beech, oak, and maple leaves. However, hazel leaves had unexpectedly high initial lignin and low initial nitrogen contents (unpublished data), and yet decomposed at a rate similar to maple. The cause of this anomaly is not clear, but we hypothesize that structural carbohydrates in hazel are more chemically labile, or physically less well protected by lignin or crystalline structure. In addition, unique leachates in hazel litter may alter decomposition through effects on the microbial community [51].

Leaf type also significantly affected microbial biomass and community composition, supporting the idea that microbial community assembly depends on matching of microbial traits, such as the capacity to produce extracellular enzymes, with the resources present in different leaf types [15, 52]. In this case, litter diversity could have a simple additive effect on microbial diversity, if certain microbial species are only present on given leaf types. However, the strong leaf type × habitat interaction effects also indicate that this filtering by biochemical resources may depend on other environmental conditions, or the pool of potential colonists available in a particular location [29].

## Spatial Analysis of Covariation

Mass loss, C/N ratio, microbial biomass, and community composition are all expected to respond to the biotic and

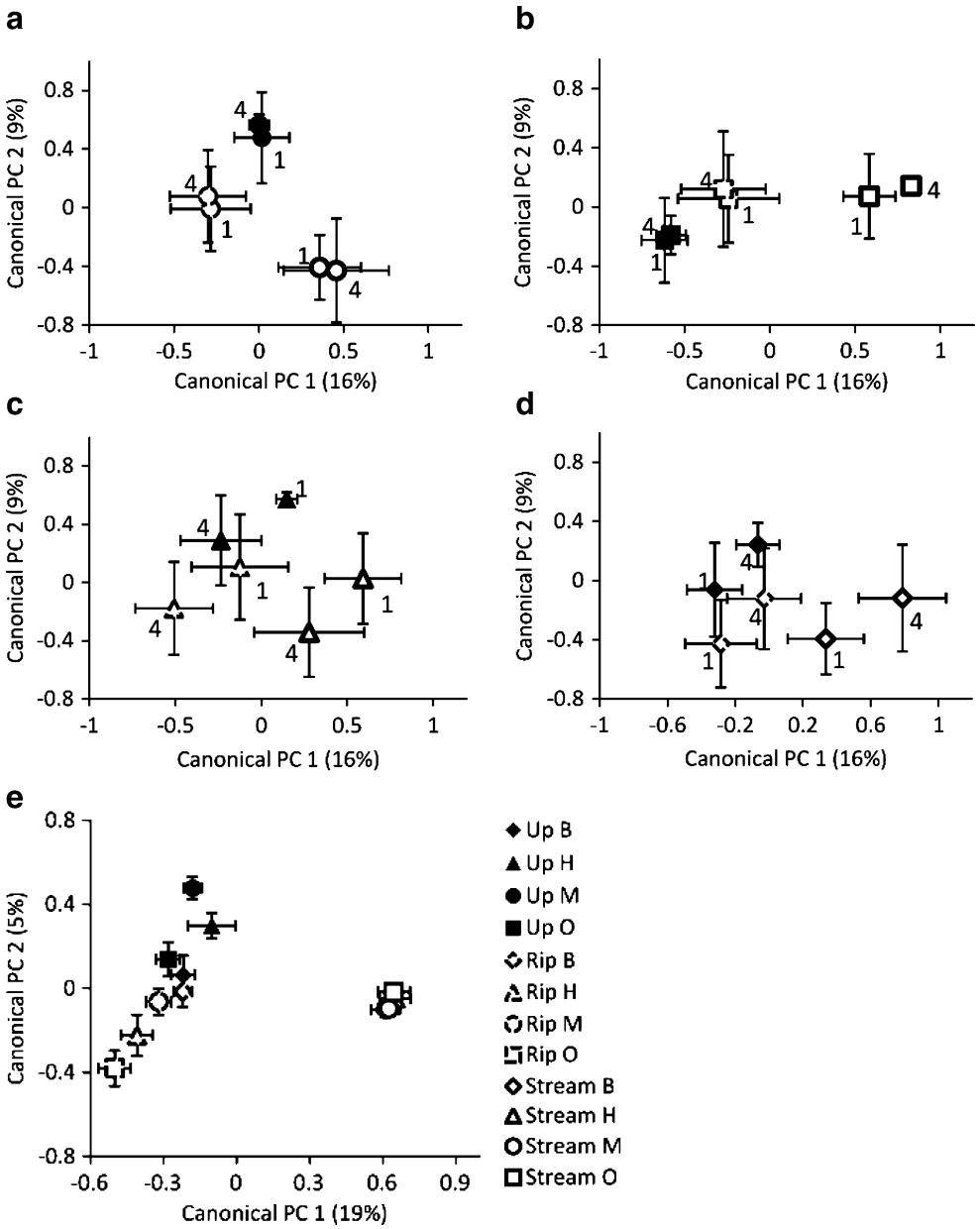


**Figure 6** Effects of habitat, plant species, and diversity treatment on microbial community composition. Bacterial T-RFLP profiles on **a** sugar maple, **b** pin oak, **c** hazel, and **d** beech. **e** Fungal T-RFLP profiles. Bacterial plots were separated by leaf type for clarity, but ordination axes are identical for all bacterial leaf type plots. Only significant fixed effects are shown, averaging across blocks and non-significant effects (Table 1). Values are group centroids (i.e., treatment means) with $N$=4 per point for bacteria, and $N$=8 for fungi. *Error bars* represent standard error around each treatment mean. *Numbers* next to *symbols* in the bacterial plot indicate diversity treatment (number of leaf types) to show the significant leaf type × diversity effects. *Symbols* are defined in panel (**e**), where *Up* upland, *Rip* riparian, *B* beech, *H* hazel, *M* maple, *O* oak. Axes are canonical principal components derived from redundancy analysis of T-RFLP profiles, with percent variation shown on each axis indicating the variation in the full dataset that is explained by the separation of treatment means by that axis

abiotic conditions in the immediate environment of a leaf mixture. However, microbial community composition may additionally depend on the availability of colonists of different species [29]. The presence of spatial structure within microbial communities has recently been detected in some cases, indicating that dispersal limitation or priority effects may play a role in determining the membership of microbial communities (e.g., [16, 35]). Spatial factors in our experiment included habitat, block, and litterbag. The spatial component of habitat is confounded by the clear environmental gradients this factor was meant to capture; however, there were no obvious environmental differences between blocks within habitats or litterbag locations within blocks. The block effect was significant for all variables and explained the most variability in bacterial community composition. Surprisingly, litterbag location (after accounting for treatment effects) was only significant for mass loss and C/N ratio. Because these spatial patterns in biogeochemistry cannot be explained by similar patterns in microbial community composition, we hypothesize that they are driven by patchiness in soil/sediment properties or invertebrate densities. The fact that microbial biomass and community composition on leaves in the same bag were no more similar than expected due to treatment effects suggests that the communities are well mixed at the small plot scale (0.5 $m^2$), but not at larger scales. However, the unexplained variance in community composition was higher than it was for mass loss or C/N ratio, indicating that smaller-scale

structure and subsampling of leaves within bags may mask variability at the plot scale. Experimental artifacts may also be stronger for community composition because, for example, particular species may colonize during litter sorting and litterbag construction, causing variability in community composition that may have little impact on biogeochemical processes due to functional redundancy within the microbial community [34, 48]. Further studies on microbial community assembly with unmanipulated leaves at multiple scales are necessary to clarify this picture.

## Conclusions

While habitat and leaf type had the largest effects on saprotrophic microbes in litter mixtures, the diversity and identity of leaves in the mixtures played a secondary role, important for some leaf types and not others. Hence, differences in the types of leaves used may explain previous disagreement among studies examining the effects of litter diversity on decomposition. In addition, testing of spatial covariance was used to suggest spatial scales at which other important factors, affecting leaf litter in the field but not directly manipulated in our experiment, vary in the environment. Additional variability in mass loss and C/N ratio, but not microbial biomass or community composition, was explained by spatial proximity at the centimeter scale (litterbags). In contrast, spatial proximity at the meter scale (blocks) was important for all variables. Unexplained variability in microbial community composition points to the potential importance of environmental variability on a scale smaller than we manipulated here, as well as more complex spatiotemporal dynamics such as dispersal limitation and priority effects. The importance of litter diversity in these processes and at larger spatial scales remains to be investigated.

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