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Litter chemistry and chemical diversity drive ecosystem processes in forest ponds

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Abstract. Research suggests that a positive relationship exists between diversity and ecological function, yet the multi-trophic effects of biodiversity remain poorly understood. The resource complementarity hypothesis suggests that increasing the trait diversity of resources provides a more complete diet for consumers, elevating consumer feeding rates. Whereas previous tests of this mechanism have measured trait diversity as the variation of single traits or the richness of functional groups, we employed a multivariate trait index to manipulate the chemical diversity of temperate tree litter species in outdoor pond mesocosms. We inoculated outdoor mesocosms with diverse and multi-trophic communities of microbial and macro-consumer species that rely on leaf litter for energy and nutrients. Litter was provided at three levels of chemical trait diversity, a constant level of species richness, and an equal representation of all litter species. Over three months, we measured more than 65 responses, and assessed the effects of litter chemical diversity and chemical trait means (i.e., community-weighted means). We found that litter chemical diversity positively correlated with decomposition rate of leaf litter, but had no effect on biomass or density of producers and consumers. However, the pond communities often responded to chemical trait means, particularly those related to nutrients, structure, and defense. Our results suggest that resource complementarity does have some effect on the release of energy and nutrients from decomposing substrates in forest ponds, but does not have multi-trophic effects. Our results further suggest that loss of tree biodiversity could affect forest ecosystem functionality, and particularly the processes occurring in and around ponds and wetlands.

Key words: amphibians; aquatic-terrestrial linkage; biodiversity; ecosystem functioning; detritivores; ephemeral pond; selection effects; snails; resource subsidies; temperate forests.

Introduction

Declines in global biodiversity have generated an immediate need to find general patterns relating changes in species composition to the functioning of ecological systems (Hector 2011). Early empirical studies suggested a positive relationship exists between diversity and ecosystem processes (Hooper et al. 2005). Despite a wealth of theoretical and empirical research since those studies, diversity-function relationships are not yet generalizable among all ecosystems, trophic levels, or processes (Cardinale et al. 2012). Proximately, this inconsistency is due to the pervasive experimental bias towards simplified, single-trophic ecosystems (e.g., grasslands), whereas the effects of diversity on more complex, multi-trophic ecosystems remains understudied (Duffy 2002, Striebel et al. 2012, Jabiol et al. 2013, Lefcheck et al. 2015). Fundamentally, there is still much debate over which measures of diversity most strongly correlate with function (McGill et al. 2006, Epps et al. 2007, Villéger et al. 2008, Lecerf et al. 2011). In addition, mechanisms underlying diversity–function relationships are often inferred, yet rarely tested (Cardinale et al. 2011), and debate over measurement of diversity has only widened this gap in our knowledge.

Ecosystem function is ultimately driven by the phenotypic traits of organisms that influence ecological interactions (i.e., functional traits; McGill et al. 2006, Díaz et al. 2007). Examples include morphological features (e.g., mouth size), behavior (e.g., mode of resource acquisition), and physiological attributes (e.g., basal metabolic rate). Since many commonly described traits are common to all species within, and even among, trophic levels, understanding the relationship between functional traits and ecosystem function provides a promising avenue for generalizing the diversity–function relationship (McGill et al. 2006, Truchy et al. 2015). Moreover, theories have suggested how variation in expressed functional traits among species may correlate with function. Increased diversity of phenotypic traits in an assemblage is predicted to reduce niche overlap, subsequently reducing competition and improving overall mixture performance (i.e., niche complementarity; Cardinale et al. 2011). Increased diversity might also provide a more complete

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diet for consumers when resources vary in their nutritional quality (i.e., resource complementarity; Gessner et al. 2010).

Testing these mechanisms by manipulating trait diversity has proven challenging. Often, more than one phenotypic trait is responsible for functional responses, yet simultaneously controlling and manipulating the diversity of multiple traits is mathematically and logistically difficult (Epps et al. 2007). Consequently, many studies have only explicitly manipulated single traits (e.g., Schindler and Gessner 2009). Although this method does indirectly manipulate trait diversity since many physical and chemical traits covary, the lack of explicit control over trait diversity creates difficulty in disentangling the effects of single traits and trait diversity. Alternatively, species with similar trait values are grouped (i.e., into functional groups), and trait diversity is manipulated as the number of groups represented by a mixture of species (e.g., Tilman et al. 1997). However, this method results in a substantial loss of information by imposing a discreet structure on continuous traits (Villéger et al. 2008). To eliminate this imposition and allow researchers to include all relevant traits in a single measure of trait diversity, multivariate indices have been proposed (e.g., RaoQ; Schleuter et al. 2010) that incorporate multiple traits into a single, continuous measure. An increasing number of studies have used these measures (e.g., Roscher et al. 2012, Cohen et al. 2014, Frainer et al. 2014) to explain correlations between species richness and ecosystem function, but few have attempted to a priori manipulate diversity along these functional indices.

Past studies of trait diversity–function relationships (i.e., using single traits, functional groups, or multivariate trait indices) have also confounded trait diversity with other variables. Individual species with extreme trait values are more likely to be represented among mixtures with high trait diversity (Dias et al. 2013). This increases the chance that the ecological function of such mixtures will be determined by the presence of a single species possessing a highly influential trait (i.e., trait-driven selection effects; Cardinale et al. 2011, Truchy et al. 2015). Trait diversity may also be confounded with mean trait values, since species mixtures with low trait diversity are more likely to have extreme values of trait means (Dias et al. 2013). Biodiversity–ecosystem function studies based on the traditional approaches of species combinations rather than traits (e.g., Meier and Bowman 2008) fail to explicitly account for these confounding relationships, and thus risk providing an incomplete test of the niche or resource complementarity mechanisms (see Dias et al. [2013] for a review of this subject). One method for removing these relationships is to maintain a low ratio of species mixture richness to species pool richness. This would allow sufficient flexibility to force an equal representation of species across all levels of trait diversity and avoid over- or under-representation of single species across levels of trait diversity. In addition, by avoiding the use of mixtures with low values of trait diversity, extreme trait means can be eliminated, and any confounding relationship of diversity with trait means can be eliminated.

We employed this strategy to explicitly test the mechanism of resource complementarity by manipulating trait diversity of leaf litter in multi-trophic, temperate forest pond mesocosms. These systems receive substantial inputs of leaf litter (Rubbo et al. 2008, Earl et al. 2014) that serve as an energy and nutrient base for myriad consumers (Williams 2005). In these systems, microbial colonization of leaf litter mineralizes nutrients for use by benthic and pelagic primary production. As microbes degrade the leaf litter, a wide array of generalist grazers and filter feeders (e.g., tadpoles, zooplankton, mollusks, arthropods) opportunistically capitalize on both microbial and algal growth, and several consumers can also ingest fragments of litter (Skelly and Golon 2003). Greater rates of microbial activity on leaf litter substrates allow more nutrients to enter producer and consumer trophic levels. Various chemical traits of leaf litter strongly affect microbes and consumers, including nutrient elements that can promote growth as well as toxic and structural compounds that can inhibit growth (Rubbo and Kiesecker 2004, Maerz et al. 2005, Williams et al. 2008, Brady and Turner 2010, Stoler and Relyea 2011, Cohen et al. 2012, 2014, Earl et al. 2014). Reductions in microbial growth will reduce available mineral nutrients for primary production, and reduce overall resource levels for higher consumers.

Mixtures of nutritionally variable litter species are thought to optimize the diet of consumers through diet-switching or through microbial-mediate transfer of nutrients from nutrient-poor to nutrient-rich litter substrates (e.g., through fungal hyphae; Gessner et al. 2010). Consequently, we predicted that chemical trait diversity of leaf litter will positively correlate with rate of litter decomposition, and with amount of primary production and consumer biomass. By testing this hypothesis in forest pond communities, we examined the influence of trait diversity of litter across multiple trophic levels in a system that is relatively novel in the ecosystem function literature, and we explicitly tested an important mechanism underlying diversity-function relationships. We explicitly designed our experiment to control and partition chemical trait means and species presence / absence, both of which may influence ecosystem function; this allowed us to independently explore the effects of these variables.

Methods

The experiment was conducted at the University of Pittsburgh's Pymatuning Laboratory of Ecology in northwestern Pennsylvania. There were three biodiversity treatments: low, medium, and high diversity of leaf litter chemistry. Each treatment was replicated 20 times for a total of 60 experimental units.

Experimental units consisted of 750-L polyethylene mesocosms filled with 500 L of well water. We covered each mesocosm with a 60% mesh cloth to simulate moderate levels of canopy cover and to prevent unwanted escape or entry of organisms. Prior to filling mesocosms with water and litter, we spread 20 L of loamy soil on the bottom of each mesocosm. We filled mesocosms with well water between 3 and 7 May and allowed soil to settle for 1 week before introducing litter.

Collection of leaf litter and analysis of litter chemistry

In autumn 2009, we collected 20 species of broadleaf and coniferous tree litter from western Pennsylvania within 1 week of abscission (Table 1) and analyzed several chemical components of each species. Each species was collected from a single location to reduce intraspecific chemical variation among tree species. Litter was air-dried in an unheated and ventilated garage through the winter. Although some decomposition of leaves might have occurred during this time, we conducted all chemical analyses after drying to ensure that chemical values were representative of leaves used in the experiment. After drying, we used a Wiley mill (Thomas Scientific, Swedesboro, New Jersey, USA) to grind samples of leaf tissue to <0.5 mm. We used these samples to assess litter carbon (C), nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg), calcium (Ca), total phenolics, tannin, lignin, and soluble carbon. C and N were assessed with a CHN analyzer, and P, K, Mg, and Ca were assessed via atomic absorption spectroscopy (Duke Environmental Stable Isotope Laboratory). We assessed total phenolics and tannin via the Folin Ciocalteu method and a radial diffusion assay, respectively (Graça et al. 2005). We assessed lignin and soluble carbon via a modified carbon fractionation method (Moorhead and Reynolds 1993, see details on this method in Appendix S1). All measured chemical components are widely used in forestry studies and many of them have known correlations with litter decomposition rate (Epps et al. 2007)

Calculation and manipulation of litter chemical diversity

We used chemical trait values to calculate and manipulate litter chemical diversity (herein, LCD; Appendix S6: Fig. S1). To manipulate LCD while maintaining constant litter species richness, we first calculated LCD of all possible 4485 four-species litter combinations from the pool of 20 litter species. The choice of four species as the mixture richness value provided sufficient spread among potential values of LCD to delineate distinct ranges of LCD while keeping richness logistically feasible and realistic. We calculated LCD as Rao's quadratic entropy (i.e., RaoQ; Laliberte and Legendre 2010) after reducing trait dimensionality via principal components analysis. Details on the calculation of RaoQ are found in Appendix S2.

After calculating RaoQ values for all possible four-species mixtures, we used the resulting distribution of RaoQ values to delineate ranges of low, medium, and high LCD, corresponding to the three diversity treatments. We selected 20 mixtures from each of these ranges

TABLE 1. The litter species used in the study, including family and species names, and their decomposition rates, measured as the coefficient of decay (*k*; Petersen & Cummins 1974) calculated this value as the slope of non-transformed mass loss over time.

Common name	Family	Species	Abbreviation	*k*
Red maple	Aceraceae	*Acer rubrum*	RM	0.060
Sugar maple	Aceraceae	*Acer saccharum*	SM	0.051
American sweetgum	Altingiaceae	*Liquidambar styraciflua*	SGUM	0.033
Yellow birch	Betulaceae	*Betula alleghaniensis*	BIR	0.056
American beech	Fagaceae	*Fagus grandifolia*	BCH	0.018
American sycamore	Fagaceae	*Plantanus occidentalis*	SYC	0.012
Chinese chestnut	Fagaceae	*Castanea mollissima*	CHCH	0.033
Hybridized chestnut	Fagaceae	*Castanea mollissima x Castanea dentata*	HYCH	0.067
Black oak	Fagaceae	*Quercus velutina*	BOAK	0.023
White oak	Fagaceae	*Quercus alba*	WOAK	0.048
Sassafras	Lauraceae	*Sassafras albidum*	SASS	0.026
Tulip poplar	Magnoliaceae	*Liriodendrum tulipfera*	TP	0.062
Green ash	Oleaceae	*Fraxinus pennsylvanica*	GASH	0.053
Northern tamarack	Pinaceae	*Larix laricina*	TAM	†
Red pine	Pinaceae	*Pinus resinosa*	RP	†
Eastern white pine	Pinaceae	*Pinus strobus*	WP	†
Bigtooth aspen	Salicaceae	*Populus grandidentata*	BASP	0.041
Quaking aspen	Salicaceae	*Populus tremuloides*	QASP	0.040
Black cherry	Rosaceae	*Prunus serotine*	CHER	0.085
Black willow	Salicaceae	*Salix nigra*	BW	0.037

†Decomposition rates were not measured for conifers, because they could not be contained in the litter bags.

which corresponded to the 20 replicates within each diversity treatment. No mixtures were identical in species composition. We avoided confounding LCD with species composition through iterative selection and replacement of species within mixtures that limited the appearance of individual litter species within diversity treatments to between three and five instances. In addition, we avoided using litter species mixtures corresponding to very low or high values of LCD to avoid confounding LCD with chemical trait means. After selection of mixtures, we calculated chemical trait means of each mixture (commonly referred to as community weighted means; Laliberte and Legendre 2010). Preliminary analysis via analysis of variance (ANOVA) verified the lack of difference in trait means of all leaf chemicals across LCD treatments (Appendix S5: Table S1). Details on the method of delineating RaoQ ranges, selecting mixtures, and calculating chemical trait means can be found in Appendix S2.

On 14 May 2010, we placed litter into mesocosms. We placed a total of 200 g into each mesocosm, consisting of 50 g of each of the four component species. This amount is within the range of litter inputs naturally found in these systems (Rubbo et al. 2008) and was generally sufficient to cover the entire benthos. To measure decomposition rate of litter and provide a means of sampling benthic grazers, we also added three mesh bags to each mesocosm containing pre-weighed amounts of litter. The mesh size of bags was 5 mm, which was large enough for consumers to enter the bags, although it likely prevented entry by larger tadpoles during their later developmental stages. Each bag contained a mixture of the four litter species within each mesocosm, including 1.5 g of each litter species (6 g total). Since coniferous needles could not be contained within this mesh size, we excluded them from the bags but still placed them in the water so that all mesocosms had an equal total biomass of litter.

Constructing the aquatic community

In each mesocosm, we constructed aquatic communities that included periphyton, phytoplankton, and consumers of both resource types (Fig. 1). On 16 May, we collected 15 L of water from each of 10 forest ponds to serve as sources of microbes, small plankton, and algae in the mesocosms. From five of these ponds, we also collected larger-bodied zooplankton using a 250-μm plankton tow net. Following removal of all zooplankton predators, we mixed the pond water and zooplankton samples, and introduced equal amounts (2.5 L) of the slurry into each mesocosm. On 23 May, we added 10 μg/L of phosphorus (as Na_2HPO_4) and 72 μg/L of nitrogen (as $NaNO_3$) to each mesocosm. This pulse of nutrients is similar to stoichiometric ratios of freshwater phytoplankton and many consumers (i.e., the Redfield ratio) and adjusted mesocosm nutrient levels to those commonly found in mesotrophic systems (Downing and McCauley 1992), and accelerated growth of phytoplankton, periphyton, and zooplankton. At this time, we also placed three 15 cm^3 clay tiles in each mesocosm to serve as periphyton samplers; we oriented the tiles so that they were standing vertically on their ends, on top of the litter, and on the north side of each mesocosm.

We added 15 individuals of each of three species of spring-breeding larval anurans to the mesocosms: wood frogs (*Lithobates sylvaticus*), American toads (*Anaxyrus americanus*), and spring peepers (*Psuedacris crucifer*). We collected amphibians as newly oviposited eggs from nearby wetlands (9–18 egg masses per species), allowed

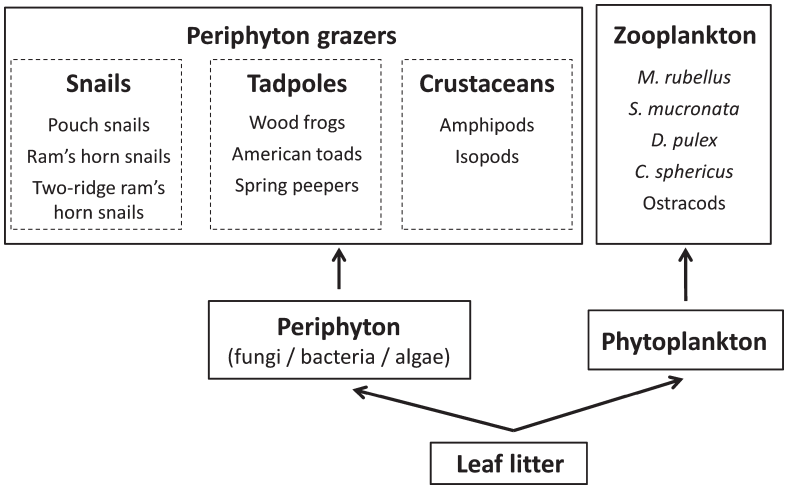

FIG. 1. Diagram of mesocosm communities. Leaf litter served as the basal source of nutrients for periphyton and phytoplankton. Periphyton was consumed by several groups of grazing organisms, including two species of crustaceans, three species of anuran tadpoles, and three species of snails. Phytoplankton was consumed by five species of zooplankton. Zooplankton are *Microcyclops rubellus*, *Schapholeberis mucronata*, *Daphnia pulex*, and *Chydorus sphericus*. See *Methods: Constructing the aquatic community* for further details regarding the construction of aquatic communities.

them to hatch in aged well water, and fed them rabbit chow ad libitum (Purina Mills, St. Joseph, Missouri, USA). Wood frogs and toads were Gosner stage 25 (Gosner 1960) when introduced to the mesocosms and spring peepers were stage 27. Tadpole masses (mean ± SE) were as follows: wood frogs = 65 ± 4 mg, American toads = 29 ± 1 mg, and spring peepers = 50 ± 3 mg. Wood frogs were introduced on 27 May whereas toads and spring peepers were introduced on 28 May. To test for effects of handling on tadpole survival, we assessed 24-h survival in the lab for all three species, which was 100%.

We introduced several species of macroinvertebrates into each mesocosm, including some of the most common consumers in our region. All species are generalist grazers that consume both algae and microbes from substrates. On 23 May, we introduced three species of snails, the pouch snail (*Physa acuta*), the ram's horn snail (*Helisoma trivolvis*), and the two-ridge ram's horn snail (*H. anceps*). We introduced the pouch and ram's horn snail as eggs to avoid potential introduction of parasites common to adult snails in the area. We obtained egg mass from 100 wild-caught adult snails that were kept in the laboratory, and introduced 10 egg masses of each species into each mesocosm. We accidentally introduced two-ridge ram's horn snails as eggs and larvae with zooplankton inoculations. However, since they were introduced in a manner that equally dispersed individuals among mesocosms, we also measured them as a biological response variable. On 2 June, we added ~40 wild-caught individuals of one amphipod species, *Crangonyx pseudogracilis*, and ~40 wild-caught individuals of one isopod species, *Asselus communis*. We collected amphipods and isopods from two ponds where they occurred at high densities. The date of amphipod and isopod introductions marked day 0 of the experiment.

Abiotic measurements

To assess how LCD affected the abiotic conditions of the mesocosms, we measured light attenuation, dissolved oxygen, pH, and temperature every 4 weeks with calibrated meters. We measured light attenuation on days 15, 54, and 85; dissolved oxygen and temperature on days 17, 45, and 77; pH on days 15, 54, and 77. Details of these measurements are found in Appendix S3.

Biotic measurements

We measured several biotic response variables at multiple times during the experiment. Further details regarding the sampling methods are provided in Appendix S3.

We quantified the rate of leaf litter decay as the slope of mass loss over time (Petersen & Cummins 1974). We recorded mass loss of litter in mesh bags in each mesocosm at monthly intervals (i.e., three sample dates; days 27, 54, and 88). After removing a single mesh bag from a mesocosm, we separate litter by species and dried all litter at 60°C for 24 h. We did not replace the litter in the mesocosm. We could not measure the decay rate of conifer litter species, as these species were not included in mesh bags. Although mass loss data is usually log-transformed prior to calculation of decay rate, we found non-transformed data had a better linear fit. Moreover, we found that log-transformation had little effect on estimates of decay rate.

We quantified algal and microbial biomass monthly (i.e., three sample dates; phytoplankton on days 22, 55, and 87; periphyton on days 13, 52, and 83). We estimated phytoplankton as the biomass of chlorophyll *a* (chl *a*) using pipe samples (see Appendix S3 for description of this method) and fluorometric analysis. We estimated periphyton biomass as the oven-dried mass of material scraped from half of a clay tile.

On day 76, we measured bacterial and fungal community structure on leaf and needle fragments using terminal restriction fragment length polymorphism (TRFLP) profiles. Details regarding all methods to quantify the phytoplankton density and periphyton biomass, and microbial community structure are found in Appendix S3.

We quantified the abundance of zooplankton species during the second and third month of the experiment (days 55 and 87). We did not enumerate samples taken during the first month, as zooplankton were not very abundant and it was clear that populations were still growing to carrying capacity. Among collected samples, zooplankton communities were comprised of no more than five species. The most dominant species were the copepod *Microcyclops rubellus*, and the two cladocerans *Schapholeberis mucronata* and *Daphnia pulex* (between 21% and 23%, 15% and 36%, and 60% and 72% of total zooplankton species composition, respectively). A single ostracod species (order Podocopida) and the cladoceran *Chydorus sphericus* were less common (between 1% and 2% of species composition), but were also assessed in our samples.

We quantified the biomass of amphipods, isopods, and all three snail species on a monthly basis. We quantified the biomass of amphipods and isopods on days 27, 54, and 88. To collect amphipods and isopods, we preserved all individuals grazing the leaves contained in the mesh bags used for sampling litter decomposition rate. We corrected all biomass measurements for the total amount of leaf litter in each bag. We quantified the biomass of pouch snails and ram's horn snails on days 36, 68, and 89. We collected and preserved snails in a standardized net sweep along the bottom and up the wall of a mesocosm (see Appendix S3 for details). All subsamples represented a small fraction of total substrate, and the removal of organisms was unlikely to have a strong effect on later community composition.

For the three species of amphibians, we recorded their survival to metamorphosis and mass at metamorphosis. We did not include time to metamorphosis as a response, as many toads and peeper individuals did not metamorphose by the end of the study. We did not measure the mass of individuals that did not metamorphose.

Metamorphosis of larval anurans began on 14 June (day 13). After this date, we checked mesocosms daily for metamorphosing individuals. We ended the experiment on 31 August (day 91), which established an experimental duration well-within the hydroperiod range of ponds common to the area.

Statistical analyses

Our primary goal was to test for the effects of LCD on abiotic and biotic responses in our mesocosm communities. Since our experimental design removed relationships between LCD and chemical trait means (i.e., there was no correlation between trait means and LCD; Appendix S5: Table S1), we also assessed the effects of litter chemical trait means on all responses. Due to the large number of traits used in this study, we first reduced the number of trait means by conducting a principal components analysis. This resulted in two principal components that explained 65% of total variation in trait means. It is worth noting that the use of stoichiometric ratios (i.e., carbon:chemical ratios) yielded similar results, although the use of chemical percentages yielded stronger results. The first principal component (herein PC1) had a positive loading of mean percent lignin in mixture and a negative loadings of mean percent nitrogen, tannin, soluble carbon, and phenolic in mixtures. The second principal component (herein PC2) had positive loadings of nutrient trait means (percent N, P, Mg, Ca, and K in mixtures) and a negative loading of the mean percent carbon in mixtures. Hence, PC1 primarily described the structural and defensive components of leaf litter, whereas PC2 primarily described the nutrient components of leaf litter. Loadings can be found in Appendix S5: Table S2. To explore how litter species arranged along these principal components, we conducted a canonical correspondence analysis (Ter Braak 1986), which demonstrates the relationship between species abundance and environmental variables. Unless otherwise noted, we conducted analyses in R (R Development Core Team 2015) using packages agricolae, nlme, car, and vegan.

Litter decay rate.—We assessed the effects of LCD and chemical trait means on the net litter decay rate of species within mesocosms (i.e., total mass loss of all species within a mesocosm). Because we could not assess the decay rate of conifer species, we first conducted an analysis that assumed no mass loss of conifer needles. This assumption is reasonable given that litter from the Pinaceae is classified as the slowest decomposing litter among all woody plant species (Webster and Benfield 1986). For this analysis, we employed an analysis of covariance (ANCOVA) using a linear mixed model that included LCD (categorical), PC1 (continuous), and PC2 (continuous) as fixed factors, as well as all possible interactions. Since it is possible that our assumption regarding the decomposition of conifer litter species might bias the results, we further examined the data after removing any mesocosm that contained one or more conifer species. Although our results were not quite significant after this omission ($P = 0.098$), the omission did not alter the pattern of our results (see Appendix S6: Fig. S2).

Abiotic, biomass, density, and survival responses.—To assess the effect of treatments on all other responses, we employed MANCOVA with a model that included LCD, PC1, PC2, and all interactions. Due to differences in response types and the number of times we measure each response, we conducted four separate analyses. Analyses included a repeated-measures MANCOVA (rm-MANCOVA) on abiotic measures (measured three times), a rm-MANCOVA on zooplankton species densities (measured two times), a rm-MANCOVA on phytoplankton, periphyton, snail, and benthic detritivore responses (measured three times), and a MANCOVA on amphibian responses. After finding a multivariate effect, we assessed which responses contributed to this effect by conducting univariate analyses for each response within sample dates (when applicable).

Microbial community composition.—We used nonmetric multidimensional scaling (NMS) procedures available through PC-ORD 4 (McCune and Grace 2002) to determine bacterial and fungal community structure. We used terminal restriction fragments (TRFs) as operational taxonomic units for these procedures and subsequently used the proportional abundance of detected TRFs as an indicator of taxa abundance within each sample (Burke et al. 2008). We arcsine square root transformed all proportional abundance data before analysis. After removing outliers and singleton TRFs, NMS analysis of bacterial and fungal communities generated three-dimensional solutions with final stresses of 17.6 and 14.1, respectively. To determine the effects of LCD on community structures, we employed analysis of similarity (ANOSIM) using Euclidean distances. To determine effects of PC1 and PC2 on communities, we used PC-ORD to calculate correlations between the ordinations and the two trait dimensions. See Appendix S4 for further details.

Results

Traits and litter species

Canonical correspondence analysis revealed a homogenous scatter of species in trait space surrounding PC1 and PC2 (Fig. 2). This confirms that there was sufficient similarity and dissimilarity among the 20 litter species to reliably manipulate litter chemical diversity without confounding litter species composition.

Decomposition rate

Decomposition rates varied tremendously among litter species. American beech, American sycamore, and black

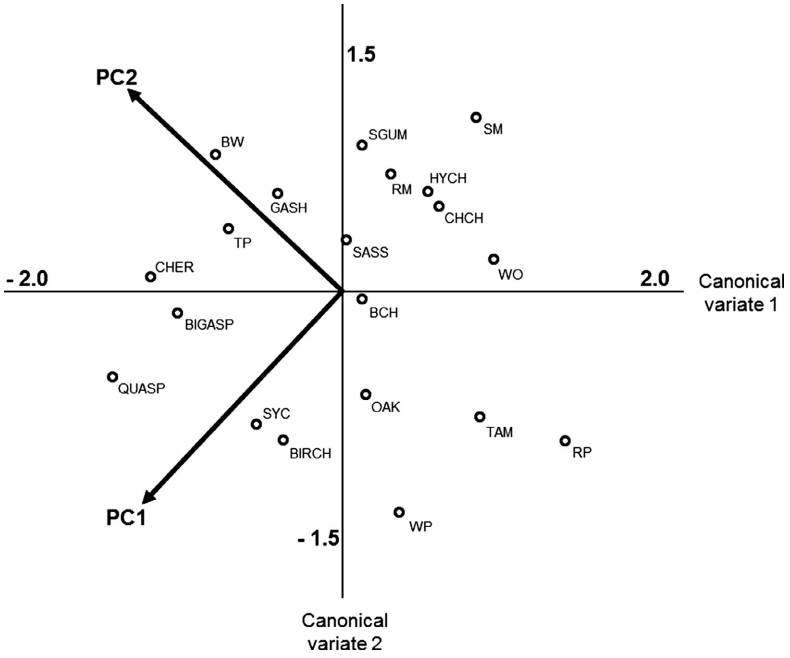

FIG. 2. Canonical correspondence analysis of litter species relations to community-weighted means. Litter chemistry is represented by two principal components, derived from a principal components analysis of community-weighted means. Increasing PC1 refers to an increasing content of structural compounds and a decreasing content of defensive compounds. Increasing PC2 refers to increasing concentrations of N, P, Ca, K, and Mg, and decreasing concentrations of C. Litter species abbreviations are as in Table 1.

oak were among the slowest decomposing species (Table 1). They decomposed 59% to 71% slower than the fastest decomposing species, which were green ash, black cherry, and tulip poplar. Our analysis of litter decomposition rates detected a significant effect of LCD and a positive effect of PC2, but no effect of PC 1 or any interaction (Table 2). Among the three levels of LCD, pairwise comparisons using Tukey's HSD revealed that decomposition rates in the high-diversity treatment were 21% faster than in the low-diversity treatment ($P = 0.013$; Fig. 3).

Abiotic responses

We detected an overall multivariate effect of time on abiotic responses, as well as a time-by-PC1 interaction (Table 2). We did not find an effect of LCD or any other interaction. Univariate analyses revealed significant effects of time for all abiotic responses, and a time-by-PC1 interaction for light attenuation (Appendix S5: Table S3; Appendix S6: Fig. S3). Across all treatments, average pH increased 0.4 units during the experiment. Dissolved oxygen also steadily increased across all treatments, rising from 5.4 mg/L at the start of the experiment to 7.2 mg/L by the last sample date. Average temperature was highest during the second sample date, but it only changed by 1.8°C throughout the study. Across all treatments, light attenuation decreased by 24% during the experiment. Light attenuation exhibited a positive relationship with PC1 on the first sample date, but no relationship on the second or third sample date.

Phytoplankton, periphyton, and macroinvertebrates.— For phytoplankton, periphyton, and macroinvertebrate responses, we detected a multivariate effect of time and PC1, and nearly significant interactions of time-by-LCD and time-by-PC2 (Table 2). Univariate analyses revealed several response relationships with PC1 and PC2, but no effects of LCD (Appendix S5: Table S4; Appendix S6: Figs S4 and S5). Phytoplankton concentrations reached a peak during the middle of the study, increasing by 39% between the first and second sample date, and then decreasing by 37% from the initial value in third sample date. Phytoplankton concentration exhibited a negative relationship with PC1 that was consistent across sample dates, and a negative relationship with PC2 that was present only on the first sample date. In contrast to phytoplankton, periphyton biomass decreased by 42% from the first to last sample date and exhibited a positive relationship with PC1 that was consistent across sample dates. Amphipod biomass doubled between the first and second sample dates, and then dropped slightly on the third sample date. Amphipod biomass also exhibited a negative relationship with PC2 on the first and second sample date, but exhibited a strong positive relationship with PC2 on the third sample date. Isopod biomass exhibited a saturating increase over the study, but no relationship with either PC1 or PC2. The biomass of pouch

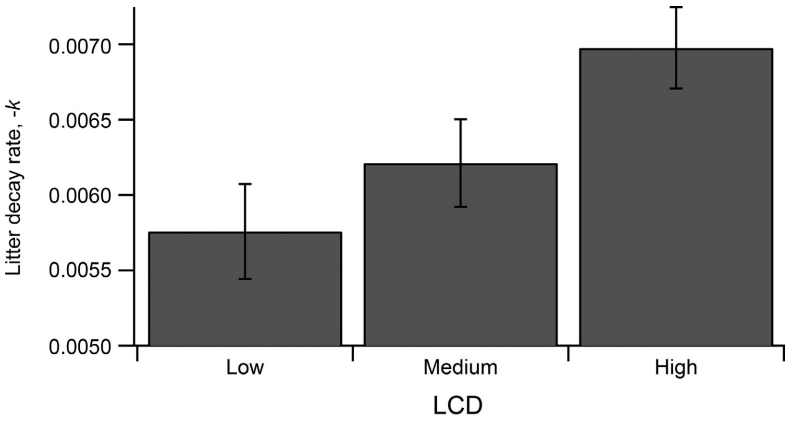

FIG. 3. Effect of litter chemical diversity (LCD) on net decomposition rate (measured as the coefficient of decay [*k*] sensu Petersen and Cummins [1974]) of all species in mixture. Bars represent the mean of net decomposition rate of all individual litter species found within mesocosms of a single diversity treatment, over three months of decomposition. Error bars are ± SE.

snail steadily increased by 75% between the first and last sample date, whereas the biomass of the two-ridge ram's horn snail decreased by 21% between the first and second date and remained constant on the third date. There were no effects of PC1 or PC2 on either snail species. Biomass of the ram's horn snail remained constant throughout the study, but did exhibit a negative relationship with PC1 that was consistent through time.

Zooplankton.—For zooplankton, we detected a multivariate effect of time and a time-by-PC1 interaction (Table 2). Although we did find a nearly significant four-way interaction for *M. rubellus*, we were unable to discern any significant pattern underlying this effect. Univariate analyses revealed significant or nearly significant effects of time for all zooplankton species except *C. sphericus* (Appendix S5: Table S5; Appendix S6: Fig. S6). Of the two dominant cladocerans, the density of *S. mucronata* decreased by 71% whereas the density of *D. pulex* increased by 49% between the two sample dates. We detected a nearly significant negative relationship *D. pulex* density with PC1, but only on the second sample date. The density of ostracods doubled between the two sample dates, but they were consistently the rarest of the zooplankton species.

Amphibians.—Regarding amphibian responses, we found multivariate effects of PC1 and PC2, but no effect of LCD or any interaction (Table 2). PC1 exhibited negative relationships with wood frog survival and biomass, a positive relationship with American toad survival, and a negative relationship with spring peeper biomass. PC2 exhibited a positive relationship with wood frog biomass, spring peeper biomass, and spring peeper survival (Appendix S5: Table S6; Appendix S6: Fig. S7).

Microbial community composition.—After employing ANOSIM, we did not detect any differences in bacteria or fungi community composition between LCD treatments (bacteria $R = 0.029$, $P = 0.115$; fungi: $R = -0.026$, $P = 0.845$). Community ordination did not reveal any clear separation of bacterial or fungal communities based on LCD (Fig. 4). However, we found a significant correlation between PC2 on bacterial community composition, and a significant correlation between of both PC2 and PC1 on fungal community composition (Fig. 4).

DISCUSSION

To our knowledge, this study provides the first explicit test of mechanisms underlying the diversity function-relationship where confounding relationships between trait diversity, trait means, and the presence/absence of individual species were explicitly controlled. In doing so, we were able to examine the independent effects of trait diversity and trait means. Moreover, by manipulating diversity within a multi-trophic system, we were able to assess more realistic mechanisms linking diversity with function relative to studies focused on single trophic levels. In agreement with the hypothesis of resource complementarity as the underlying mechanism, LCD was positively related to the decomposition rate of individual litter species. Surprisingly, neither microbes, producers, nor consumers responded to LCD, although they did respond to litter chemical trait means. This result suggests that the same mechanisms linking diversity with function do not act across multiple trophic levels in decomposer food webs.

Effect of litter diversity on litter decomposition rate

We observed a 21% increase in net decomposition rate of mixtures between low- and high-LCD treatments. It is worth comparing this value to the findings of previous studies, which have found antagonistic, neutral, and synergistic effects of litter mixing, with mass loss of mixtures between −22% and 65% of expected mass (Gartner and Cardon 2004). However, this range is derived entirely from studies that solely manipulated litter species

TABLE 2. Multivariate effects for leaf litter decomposition rate (LCD), abiotic, and biotic responses measured during the study.

Effects	Leaf litter decomposition	Abiotic	Phytoplankton, periphyton, and macroinvertebrates	Zooplankton	Amphibians
LCD	$4.9_{2,47}$*	$0.4_{2,47}$	$0.3_{2,47}$	$1.9_{2,47}$	$0.6_{12,86}$
PC1	$1.6_{1,47}$	$<0.1_{1,47}$	$8.7_{1,47}$**	$0.8_{1,47}$	$11.8_{6,42}$***
PC2	$8.2_{1,47}$**	$<0.1_{1,47}$	$0.1_{1,47}$	$1.0_{1,47}$	$4.4_{6,42}$**
LCD × PC1	$1.1_{2,47}$	$0.6_{2,47}$	$0.7_{2,47}$	$0.2_{2,47}$	$0.5_{12,86}$
LCD × PC2	$1.5_{2,47}$	$0.3_{2,47}$	$1.1_{2,47}$	$1.1_{2,47}$	$0.8_{12,86}$
PC1 × PC2	$0.1_{1,47}$	$0.7_{1,47}$	$1.3_{1,47}$	$<0.1_{1,47}$	$1.3_{6,42}$
LCD × PC1 × PC2	$2.9_{2,47}$	$0.9_{2,47}$	$0.4_{2,47}$	$0.3_{2,47}$	$1.3_{12,86}$
Time		$679.2_{2,47}$***	$18.0_{2,47}$***	$50.9_{1,47}$***	
LCD × Time		$1.0_{4,94}$	$0.8_{4,94}$	$1.6_{2,47}$	
PC1 × Time		$6.9_{2,94}$**	$0.3_{2,94}$	$5.7_{1,47}$*	
PC2 × Time		$1.0_{2,94}$	$0.8_{2,94}$	$2.6_{1,47}$	
LCD × PC1 × Time		$1.6_{4,94}$	$0.8_{4,94}$	$0.1_{2,47}$	
LCD × PC2 × Time		$1.0_{4,94}$	$0.7_{4,94}$	$1.1_{2,47}$	
PC1 × PC2 × Time		$0.3_{2,94}$	$0.1_{2,94}$	$0.3_{1,47}$	
LCD × PC1 × PC2 × Time		$1.0_{4,94}$	$0.2_{4,94}$	$3.0_{2,47}$†	

Notes: The table lists results for five separate analyses, including an ANCOVA on leaf litter decomposition rate, a repeated-measures MANCOVA on abiotic measures (measured three times), a repeated-measures MANCOVAon zooplankton species densities (measured two times), a repeated-measures MANCOVA on phytoplankton, periphyton, snail, and benthic detritivore responses (measured three times), and a MANCOVA on amphibian responses. Univariate analyses for the latter four analyses can be found in Tables S2–S5 in Appendix S5 PC1 primarily describes the structural and defensive components of leaf litter; PC2 primarily describes the nutrient components of the leaf litter. Subscripts indicate degrees of freedom. †$P < 0.10$; *$P < 0.05$; **$P < 0.01$; *** $P < 0.001$.

richness, without maintaining equal representation of individual species across diversity treatments, and without controlling for LCD or chemical trait means. Under these circumstances, the mechanism of resource complementarity is likely to act in concert with selection effects (i.e., effects due to a single species; Gessner et al. 2010). Since such species-specific effects have an equal likelihood of being positive and negative, they should have a neutral effect on function across species mixtures if there is equal representation of individual species among mixtures. This is important, as a balanced representation of species was explicitly built into our experimental design. Although selection effects could still be driven by functional traits, we also designed our experiment such that trait means did not vary across LCD treatments. Given this design, we still observed non-neutral effects of increasing trait diversity; moreover, our observed response of a 21% increase in net mixture decomposition rate is very close to the average of the range reported by Gartner and Cardon (2004). This bolsters our assertion that the observed increase in decomposition rate across diversity levels in our study was due to the effects of resource complementarity.

More recent studies have attempted to test the direct effects of resource complementarity. However, in aquatic systems, responses to diversity have been either non-existent (Schindler and Gessner 2009) or relatively minor in comparison to the influence of other environmental factors, such as temperature (Lecerf et al. 2011). In contrast, in a terrestrial soil system, Meier and Bowman (2008) found positive relationships of LCD with net N mineralization. However, these studies did not explicitly control for known relationships between LCD, chemical trait means, and species composition, thereby making it impossible to determine the direct effects of resource complementarity. Additionally, Schindler and Gessner (2009) and Lecerf et al. (2011) manipulated LCD as the variation in single traits, which may further explain why they observed negligible effects of LCD. The decomposition of litter is controlled by multiple, and often uncorrelated traits (Epps et al. 2007), and the manipulation of single trait variation may lead to undesired variation in another trait. Our use of a multivariate index removed this problem, and subsequently isolated the effect of resource complementarity on the decomposition process.

Effects of diversity on microbes, producers and consumers

Although LCD positively affected litter decomposition rate, we found no effect on the community responsible for that decomposition. Indeed, we found no effect of LCD on microbial community composition, periphyton biomass, phytoplankton concentration, zooplankton densities, macroinvertebrate and amphibian biomass, or amphibian survival. These results are surprising, since evidence suggests that leaf decomposition positively relates to the quality of resources for consumers (Smock and MacGregor 1988). It is possible that microbes or macro-consumers exhibited higher mineralization and ingestion rates in replicates with low-quality litter in an

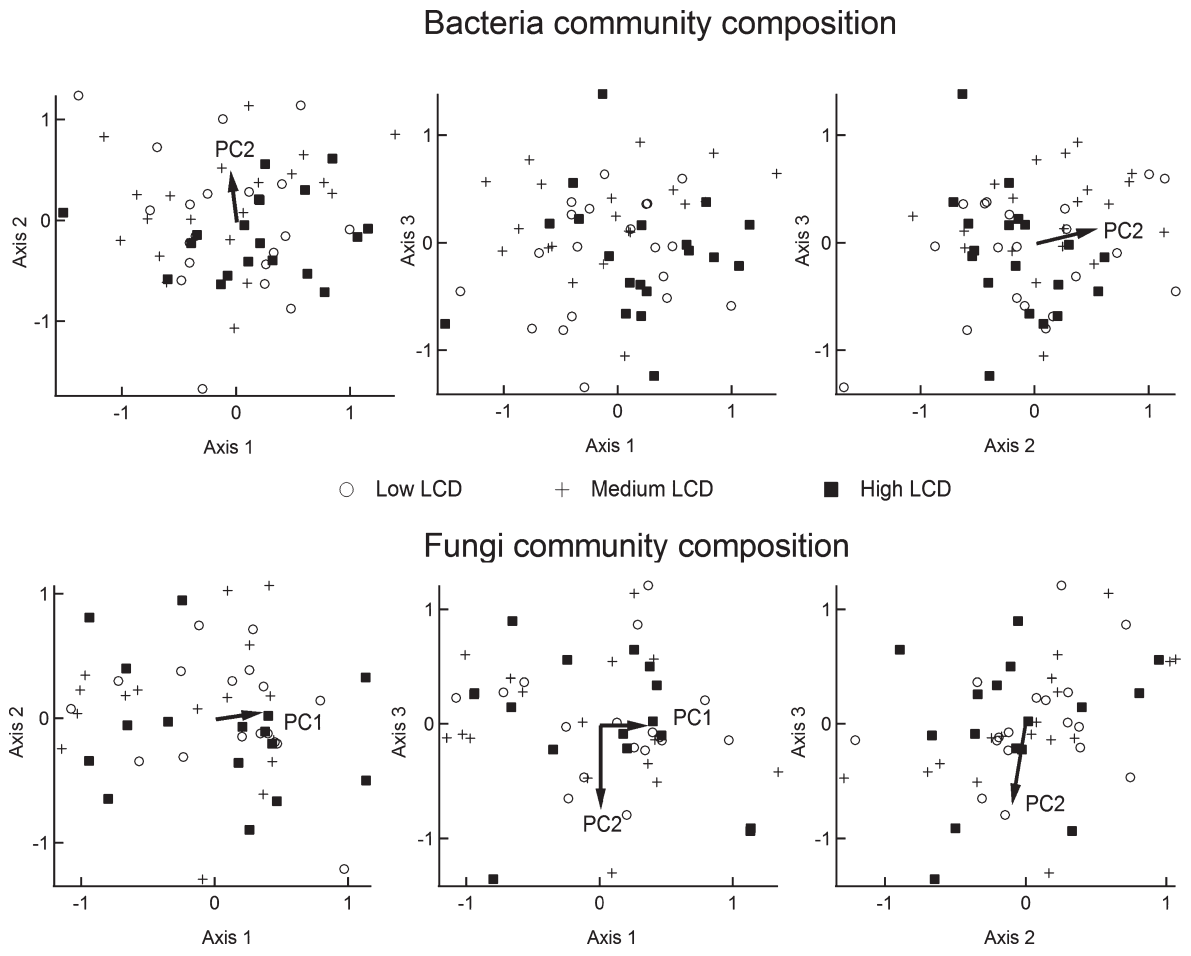

FIG. 4. Nonmetric multidimensional scaling plots for bacteria and fungi community composition. For bacteria and fungi, three axes reduced stress to 17.6 and 14.1, respectively. Vectors represent correlations of axes with PC1 and PC2 (i.e., litter structure and litter nutrients, respectively). Only significant correlations are shown. Note that the vectors are shown at 200% of their length to aid in visual interpretation. Symbols represent samples from low, medium, or high leaf chemical diversity.

effort to meet nutrient demands, which is known to occur among several macroinverbrate and microbial species (Lindroth et al. 1993). Similarly, Carrino-Kyker et al. (2012) found that nutrient additions to vernal ponds caused microbial communities to increase denitrification rates without an associated changed in community structure. Alternatively, plasticity in microbial and macro-consumer stoichiometry may have reduced the apparent influence of LCD on the community. Although consumers are generally considered stoichiometrically homeostatic (Sterner and Elser 2002), there is increasing evidence that many aquatic consumers exhibit stoichiometric plasticity in response to changing resource quality without substantial changes in survival, growth, or fitness (Cross et al. 2005). Such plasticity would lead to changes in litter decomposition as observed in our study, but not increased consumer biomass. These alternative explanations may be explored further by measuring consumer stoichiometry, as well as macro-consumer respiration rates and assimilation efficiencies.

Although consumers showed little response to LCD, we did find that many food web components were highly sensitive to litter chemical traits, and particularly to increasing levels of soluble carbon and phenolics (i.e., decreases in PC1). This is in agreement with previous studies demonstrating sensitivity of several consumers to these compounds, particularly tadpoles (Horne and Dunson 1995, Rubbo and Kiesecker 2004, Maerz et al. 2005). Likewise, the significant correlation between chemical traits and bacterial and fungal community structure is not surprising given the well-accepted observation that microbial community structure and decomposition activity are affected by the nutrient content and structural components of plant litter (Güsewell and Gessner 2009). Mechanistically, the negative effects of phenolics are likely direct, due to the ability of these compounds to bind with active proteins (Maerz et al. 2005). In contrast, the negative effect of soluble carbon on consumers is likely indirect: elevated levels of soluble carbon increases light attenuation, and

decreases primary production, pH, and dissolved oxygen (Stoler and Relyea 2016). Simultaneously, increased soluble carbon is associated with higher levels of microbial aerobic respiration, which can further decrease levels of dissolved oxygen (Pollard 2013). These patterns coincide with our observations of a negative relationship between structural trait means, chl *a*, and dissolved oxygen, and a positive relationship between structural trait means and light attenuation, pH, and periphyton biomass. However, it is worth noting the soluble carbon might actually benefit some organisms that can use the dissolved carbon as an energy source (Williamson et al. 1999, Wetzel 2001).

There were also several relationships between litter nutrient content and consumer responses, particularly with regard to tadpole responses. Biomass of wood frogs, survival of spring peepers, and mass at metamorphosis of spring peepers all increased with nutrient content. Several studies note that the performance of tadpoles and other consumers is positively correlated with litter nutrient content (Moran and Hodson 1989, Cohen et al. 2012). For example, Kupferberg (1997) demonstrated that tadpole growth rate increases with algal protein content. However, the effects of litter nutrients in their study were relatively weak compared to factors such as dissolved oxygen and phenolics, which parallels the findings of Stoler and Relyea (2016). Thus, our study finds partial support for the notion that litter nutrient concentration is an important determinant of consumer biomass (Moran and Hodson 1989), yet we find support for the overriding effect of leached litter components in forest wetlands (Stephens et al. 2013, Stoler and Relyea 2016).

Implications for forest management

The results of our study suggest how nutrient cycling in forests might be altered with changes in tree species composition. Over the past hundred years, temperate forests have undergone massive shifts in composition, such as the complete loss of American chestnut due to invasive fungal disease (Smock and MacGregor 1988). Current changes include the loss of oaks due to overbrowsing by mammals (Abrams 2003), the decimation of eastern hemlock (*Tsuga canandensis*) and ash due to invasive diseases and insects (Orwig and Foster 1998, Kovacs et al. 2010), and massive changes in composition and succession from practices such as fire suppression and selective logging (Abrams 2003). In turn, a few opportunistic species such as black cherry and red maple are encroaching into novel habitats (Abrams 2003). Often, such encroaching species have unique chemical traits that allow them to succeed in novel environments (Cappuccino and Arnason 2006), and are subsequently likely to drive mixture chemistries to extreme values. Similarly, the success of the proposed reintroduction of American chestnut relies on a disease-resistant hybrid species (Thompson 2012) that had the lowest lignin content of all species in our study, the highest phosphorus content, and nearly the highest tannin, phenolic, and soluble carbon content. Our study indicates changing tree species diversity will introduce more extreme traits that will substantially alter consumer assemblages. It is less clear whether changes in diversity will serve to change overall trait diversity, and subsequently alter the complementarity mechanisms. Species composition does not change randomly, and the introduction of a species with one extreme trait might also promote the introduction of a species with the opposite extreme. Additionally, there is increasing evidence that rare species can have a disproportionate effect on ecosystem function due to ecological interactions (e.g., selective grazing; Walker et al. 1999). Hence, to understand how litter chemistry will alter ecosystem functioning through selection and complementarity mechanisms, we must fully understand natural processes involved in forest turnover and how this relates to foliar chemistry.

Conclusions

Our study isolates the effect of litter resource complementarity on wetland ecosystem processes, and reveals how various components of litter diversity, including LCD, trait means, and the presence of individual litter species can alter a forested aquatic environment. We detected directionality with regard to the influence of these diversity components across the food web. In particular, LCD positively correlated with litter decomposition rates, indicating that microbes and macro-consumers increasingly mineralized and ingested litter resources as litter resource diversity increased. However, this was not reflected in producer or consumer responses, which were largely determined by litter trait means. In addition, we found that the presence of chemically unique litter species strongly influences abiotic responses and consumer processes, which serves to bolster conclusions regarding the overriding effects influence of litter chemistry on wetland community components. Given the connectance of wetlands to surrounding riparian zones and to the rest of the forest (Wetzel 2001, Dreyer et al. 2012), our study provides a unique perspective on how changing compositions of forest vegetation are likely to alter the ecosystem ecology of temperate forests.

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