

Twenty-five-year response of the herbaceous layer of a temperate hardwood forest to elevated nitrogen deposition

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Abstract. Increasing rates of atmospheric deposition of nitrogen (N) present a novel threat to the biodiversity of terrestrial ecosystems. Many forests are particularly susceptible to excess N given their proximity to sources of anthropogenic N emissions. This study summarizes results of a 25-yr treatment of an entire central Appalachian hardwood forest watershed via aerial applications of N with a focus on effects of added N on the cover, species richness, and composition of the herbaceous layer. Research was carried out on two watersheds of the Fernow Experimental Forest (FEF), West Virginia. The long-term reference watershed at FEF (WS4) was used as a reference; WS3 was experimentally treated, receiving three aerial applications of N per year as $(\text{NH}_4)_2\text{SO}_4$ totaling 35 kg N ha⁻¹ yr⁻¹, beginning in 1989. Cover of the herbaceous layer (vascular plants ≤ 1 m in height) was estimated visually in five circular 1-m² subplots within each of seven circular 400-m² sample plots spanning all aspects and elevations of each watershed. Sampling was carried out in early July of each of the following years: 1991, 1992, 1994, 2003, and 2009–2014, yielding 10 yr of data collected over a 23-yr period. It was anticipated that the N treatment on WS3 would decrease species richness and alter herb layer composition by enhancing cover of a few nitrophilic species at the expense of numerous N-efficient species. Following a period of minimal response from 1991 to 1994, cover of the herb layer increased substantially on N-treated WS3, and remained high thereafter. There was also a coincidental decrease in herb layer diversity during this period, along with a sharp divergence in community composition between WS4 and WS3. Most changes appear to have arisen from unprecedented, N-mediated increases of *Rubus* spp., which are normally associated with the high-light environment of openings, rather than beneath intact forest canopies. These findings support the prediction that N-mediated changes in the herbaceous layer of impacted forests are driven primarily by increases in nitrophilic species.

Key words: eastern deciduous forest; forest ecosystems; forest strata; herbaceous layer; nitrogen saturation.

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INTRODUCTION

Despite the success of the Clean Air Act of 1970, and especially its amendments of 1977 and 1990, in decreasing emissions of nitrogen (N)-bearing compounds into the atmosphere, atmospheric concentrations of reactive N (N_r) remain high, as does atmospheric deposition of N_r in many regions of the world (Sutton et al. 2014, Vet et al. 2014). Numerous chemical forms of N_r exist, including NH_3 , NH_4^+ , NO, NO_2 , NO_3^- , $2N_2O_5$, HNO_3 , and several forms of peroxyacetyl nitrates (Horii et al. 2005), a fact that adds to both the complexity of the N cycle and the challenges of addressing problems associated with excess N in ecosystems. Many factors contribute to these problems, including N volatilization from animal feedlots and, more prominently, increases in anthropogenic N-fixation, primarily comprising fossil-fuel combustion and the Haber–Bosch process for production of reduced N in fertilizers. The latter is particularly troubling because, despite recent decreases in emissions of oxidized N, emissions of reduced N from use of fertilizer N are increasing in the United States (Pinder et al. 2011). Paradoxically, the sum of all forms of anthropogenic N fixation currently exceeds the amount of N fixed via natural (nonanthropogenic) processes (Vitousek et al. 1997).

Estimates from modeling (Galloway et al. 2004) have suggested that total atmospheric deposition of NH_4^+ and NO_3^- to terrestrial ecosystems increased from 17 Tg N yr^{-1} in 1860 to 64 Tg N yr^{-1} in the early 1990s, and project future deposition to be 125 Tg N yr^{-1} by 2050. This is an unprecedented increase in rates of atmospheric deposition of N_r and one that presents a myriad of novel threats to terrestrial ecosystems that are often limited, or co-limited, by the lack of available N (Elser et al. 2007, Lebauer and Treseder 2008, Reich et al. 1997). Not surprisingly, understanding the nature of such threats has been the impetus for several recent publications on this topic, including global syntheses (Bobbink et al. 2010, Clark et al. 2013b), analyses of effects of N deposition in North America (Suding et al. 2005, Pardo et al. 2011, Clark et al. 2013a) and Europe (Stevens et al. 2010, De Schrijver et al. 2011, Verheyen et al. 2012, Dirnböck et al. 2014, Ferretti et al. 2014), and a research volume (Sutton et al. 2014).

Although this is an issue that spans both biogeochemistry and vegetation science (Aber et al. 2003, Gilliam 2006, 2014a), much of the recent focus on effects of excess N on terrestrial vegetation—using approaches ranging from experimental plots to synoptic-scale syntheses and meta-analysis of published data—has been on impacts on plant biodiversity. Indeed, recent evidence supports earlier experimental work in grassland communities in Rothamsted, England, and Cedar Creek Natural History Area, Minnesota (Silvertown et al. 2006 and Tilman 1989, respectively) that found that excess N alone can cause profound declines in biodiversity of terrestrial plant communities. Regrettably, there remains a paucity of studies of the herbaceous layer of forest ecosystems (De Schrijver et al. 2011, Lan et al. 2015), which is an unfortunate oversight considering that (1) forests tend to be species rich, (2) most (up to 90%) of the plant diversity of forests is found in the herb layer (Whigham 2004, Gilliam 2007), and (3) the herb layer serves many ecosystem-level functions in forests (Muller 2014, Elliott et al. 2014).

The predicted reduction in forest herb species richness from exposure to excess N has been developed into a mechanistic hypothesis to explain the response of forest herb biodiversity to excess N—the *nitrogen homogeneity hypothesis* (Gilliam 2006, Gilliam 2014a). This hypothesis is based on the competitive interplay between nitrophilic species (competitive only with high N availability) and N-efficient species (competitive under N-limited conditions), the observation that there are far fewer nitrophilic than N-efficient species, the idea that spatial heterogeneity in soil resources can create and maintain high species diversity in plant communities (Hodge et al. 2000, McClain et al. 2003, Hutchings et al. 2003, Bartels and Chen 2010, Costanza et al. 2011), and the observation that experimental additions of N can decrease spatial heterogeneity (i.e., increase homogeneity) of available N, as seen in extractable NO_3^- pools, soil water NO_3^- concentrations, among other metrics of N availability (Gilliam et al. 2001). More specifically, the N homogeneity hypothesis predicts the following sequence of events: (1) An initial increase in herb layer cover—which is essentially a fertilizer-type response as the system shifts from N limitation toward N saturation—and a concurrent decrease in species richness

resulting from loss of N-efficient species, which are numerous relative to nitrophilic species; (2) A subsequent decrease in species evenness because of increased dominance of the relatively few nitrophilic species; and (3) The competitive replacement of N-efficient species by high-cover nitrophilic species that will alter the species composition of herb communities and decrease diversity. It is also expected that the response time of herb layer species to increases in N availability should be inversely related to ambient N deposition. So, a rapid response is expected in areas receiving lower levels of atmospheric deposition of N (e.g., New York and Massachusetts—Hurd et al. 1998 and Rainey et al. 1999, respectively), but a more delayed response should occur in areas receiving higher N deposition, such as many areas found in West Virginia (Gilliam et al. 2006).

The N homogeneity hypothesis has been discussed in review papers (e.g., Lu et al. 2008, Fujimaki et al. 2009, García-Palacios et al. 2012) and either explicitly or implicitly addressed in several published studies. Other than Bernhardt-Römermann et al. (2010), who characterized plant functional types in a deciduous forest of southern Germany and found that the greatest numbers were associated with high-N plots, these studies have supported predictions of the hypothesis (e.g. Hülber et al. 2008, Hedwall et al. 2011).

The purpose of this study was to assess the effects of 25 yr of aerial applications of N on the herbaceous layer of a central Appalachian hardwood forest ecosystem. Although longer studies of effects of N additions have been carried out in herb-dominated systems (e.g., Tilman 1989, Silvertown et al. 2006), we know of no other study that has examined plant responses to added N over this length of time in a forest ecosystem. Furthermore, as such studies are usually carried out on the plot scale (Magill et al. 2000, Lu et al. 2010, 2014) or using natural gradients in N deposition (McNulty et al. 1991, Strengbom et al. 2003), this study is unique in its use of watershed-scale aerial N treatments to assess herb layer response. On the basis of N homogenization hypothesis, we predict the following: (1) added N will increase the spatial homogeneity of N availability, which will lead to increased sample homogeneity of the herb layer, ultimately resulting in N-mediated loss of species diversity and alterations in herb

layer community composition, (2) the herb layer for the N-treated watershed will initially exhibit increases in total cover, (3) N-mediated increases in cover will simultaneously involve increases in nitrophilous species and decreases in N-efficient species, relative to the untreated reference watershed, and (4) both species richness and species evenness will decline (from increases in relatively few nitrophilic species), resulting in decreases in herb layer diversity.

MATERIALS AND METHODS

The FEF is located in north-central West Virginia (39°03' N, 79°49' W), occupying ~1900 ha of montane hardwood forests in the Allegheny Mountain section of the unglaciated Allegheny Plateau. Precipitation averages ~1430 mm yr⁻¹, with most occurring between April and September (Adams et al. 2006). Three adjacent watersheds were used in this study: WS3, WS4, and WS7, with most comparisons being made between WS3 and WS4, based on permanent vegetation sample plots established on both watersheds (see below). Stands on WS3 and WS7 were ~45-yr old at the time of most recent sampling in this study, and are even-aged stands which developed following clear-cutting. WS4 supports even-aged stands >100-yr old (Table 1). The overstory of all three watersheds is composed of mixed hardwood species, and essentially all tree species are found on all watersheds; relative importance, however, varies with stand age. Species typical of earlier successional stands (e.g., *Betula lenta*, *Prunus serotina*, and *Liriodendron tulipifera*) are more dominant on WS3 and WS7, whereas species more typical of later successional stands (*Acer saccharum* and *Quercus rubra*) are more dominant on WS4 (Kochenderfer 2006). In the initial phase of this study, species composition of the herbaceous layer was quite similar between watersheds, despite differences in stand age (Gilliam and Turrill 1993, Gilliam et al. 2006), including species of *Viola*, *Rubus* spp., mixed ferns, and seedlings of *Acer pensylvanicum* and *A. rubrum*. Although we are certain that virtually all *Rubus* encountered in this study is *R. allegheniensis*, specific confirmation requires floral characters, and flowering is rare among these plants at FEF. Therefore, in this study we use the single taxonomic designation, *Rubus*.

Table 1. Characteristics of study watersheds of the Fernow Experimental Forest, WV (data taken from Gilliam et al. 1994 and Adams et al. 2006). Soil values are for a mineral soil depth of 0–10 cm as of 1994.

Variable	WS3	WS4	WS7
Area (ha)	34.3	38.7	24.0
Stand age (yr)	~45	>100	~45
Stand history	Clear-cut N additions	Select cut	Clear-cut Herbicide
Aspect	S	S-SE	E
Elevation (m)	735–860	750–870	731–850
Mean annual precipitation (mm)	1473	1473	1473
Wet N deposition, 1989–2014 (kg ha ⁻¹)			
Ambient	260	260	260
Added	875	0	0
Total	1135	260	260
Tree basal area (m ² ha ⁻¹)	36.0	38.6	28.0
Soil texture (%)			
Sand	65.7	66.0	68.8
Silt	22.2	23.3	21.7
Clay	12.0	10.7	9.5
Soil pH	4.34	4.34	4.50
Organic matter (%)	14.2	13.8	13.4
Cation exchangeable capacity (meq 100 g ⁻¹)	5.1	4.1	4.0

Soils are similar among study watersheds, relatively thin (<1 m in depth), acidic, sandy-loam Inceptisols (loamy-skeletal, mixed, mesic Typic Dystrichrepts) of the Berks and Calvin series (Adams et al. 2006). Sand, silt, and clay fractions on all watersheds are approximately 66, 23, and 11% of mineral soil volume, respectively. These soils are generally acidic (pH ~4.3) and high in cation exchange capacity (~5 meq 100 g⁻¹) (Table 1).

Beginning in January 1989, WS3 has received three aerial applications (by either helicopter or airplane) of (NH₄)₂SO₄ per year, totaling 35 kg N ha⁻¹ yr⁻¹. Applications in March (occasionally April) and November are approximately 7 kg N ha⁻¹; July applications are approximately 21 kg N ha⁻¹. Initial studies confirmed that these treatments increased N availability on WS3 relative to WS4 and WS7, with annual net nitrification averaging 144, 114, and 115 kg N ha⁻¹ yr⁻¹, respectively, from 1993 to 1995, and that relative nitrification (net nitrification relative to net N mineralization) was >90% for all watersheds (Gilliam et al. 2001). Subsequent work indicates that nitrification has decreased over the past 10–15 yr on

all watersheds, no longer varying between them (Gilliam 2014b).

Field sampling

The herbaceous layer was sampled within seven circular 400-m² sample plots in each of WS3 and WS4 (representing the full range of elevation and slope aspect) by identifying and visually estimating cover (%) of all vascular plants ≤1 m in height within five 1-m² circular sub-plots in each sample plot (Walter et al. 2015), for a total of 70 1-m² sub-plots. Sub-plots were located within sample plots using a stratified-random polar coordinates method, which was employed to avoid over-sampling the center region of circular plots (Gaiser 1951). Sampling was carried out in early to mid-July of each of the following years: 1991, 1992, 1994, 2003, and 2009–2014, yielding 10 yr of data taken over a 23-yr period. Nomenclature follows Gleason and Cronquist (1991).

Biomass was estimated for the herb layer at FEF by employing an empirically-derived exponential model: $y = 0.18x^{1.29}$ ($r^2 = 0.71$, $P < 0.001$) where y is aboveground biomass in g m⁻² and x is cover in %. This was based on previous work on these same sample plots (Gilliam and Turrill 1993). We assessed herb community diversity with Hill's N2 index (Hill 1973, Magurran 2003).

To assess the effects of excess N deposition on the spatial homogeneity of N availability, we measured potential rates of net nitrification in the upper 5 cm of mineral soil at 100 locations arrayed in a grid in both treated WS3 and WS7. Soils collected at each location (five, 2.2-cm diameter cores per location), were sieved (5.6 mm mesh) and incubated at field moisture levels in the dark at ~22° C for 28 d. Pre and postincubation soil extracts (1 M KCl) were analyzed for nitrate using a Lachat QuickChem AE autoanalyzer (Lachat Method 10-107-04-1-O). Measurements of potential net N mineralization were also made, and essentially 100% of mineralized N was nitrified which allows us to use net nitrification as an overall index of N availability at these sites.

Data analysis

Because the study design is an example of simple pseudoreplication (Hurlbert 1984), interpretation of data should take that into

account. It is our contention, however, that any effects reported are best understood as treatment effects, rather than pre-existing differences among watersheds. The three watersheds are closely similar with respect to several site variables such as overstory basal area and, earlier in the study, soil pH and cation exchange capacity (Table 1).

We are also aware of the potentially confounding effects of the N treatment with stand age differences. However, published results from the initial sampling of the herb layer shortly after initiation of N additions to WS3 failed to document any significant differences between WS3 and WS4 that are related to differences in stand age (Gilliam and Turrill 1993, Gilliam et al. 1994, 1995). Thus, it is our further contention that effects reported herein are treatment effects, rather than stand-age differences between watersheds.

Effects of the N treatment were assessed in two ways. First, repeated measures analysis of variance (ANOVA) was used to determine treatment effects on total herb layer cover, cover of *Rubus*, and species richness. For the repeated measures ANOVA, N treatment was used as the between-subject factor, plots were used as the subject factor, and year was used as the within-subject factor (Zar 2009). Variation in other variables with time throughout the study period was assessed with linear regression.

Second, changes over time and variation associated with N treatment in species composition of the herbaceous layer were determined with detrended correspondence analysis (DCA) using CANOCO 4.5. Inter-annual patterns were assessed by running DCA on all data (all years and both watersheds) combined, followed by calculation of centroids (two-dimensional means for both DCA axes simultaneously) of seven plots per watershed for each of the 10 years sampled in the study. This approach allows for determination of trajectories of species compositional change over the period 1991 to 2014 for both reference WS4 and N-treated WS3. In addition, output options in CANOCO 4.5 were used to generate sample heterogeneity (root mean square of standard deviation), species diversity, and species evenness per plot. To test predictions of the N homogeneity hypothesis (Gilliam 2006, 2014a), we also calculated a homogeneity

index as the reciprocal of sample heterogeneity (i.e., sample homogeneity = 1/sample heterogeneity).

RESULTS

Effects of N on spatial heterogeneity of soil N availability and herb composition

Based on measurements of net nitrification in mineral soil at 100 locations in both treated WS3 and untreated watershed WS7, the coefficient of spatial variation for potential net nitrification in WS7 was 51% (data not shown), with values ranging from 0.07 to 118 mg N m⁻² d⁻¹. The coefficient of spatial variation in WS3 was 33% (data not shown) and showed a greater degree of homogeneity at moderate to high levels (~40 mg N m⁻² d⁻¹) of net nitrification throughout its 35 ha landscape (Fig. 1).

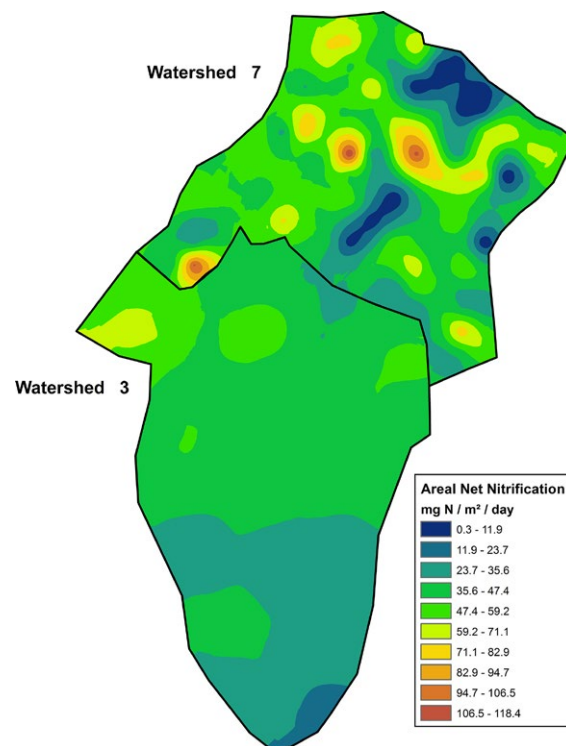


Fig. 1. Spatial patterns of potential net nitrification measured in 2011 using surface soils (top 5 cm of mineral soil) collected from the reference (WS7) and N-treated (WS3) watersheds at the Fernow Experimental Forest, West Virginia.

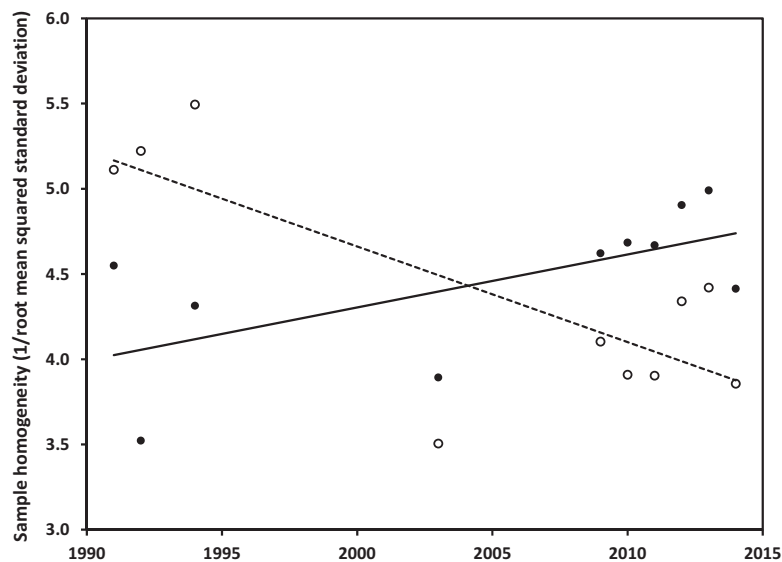


Fig. 2. Change over time in spatial homogeneity for herb layer composition, 1991–2014, on reference WS4 (open symbols/dashed line: $y = 116.84 - 0.06x$, $r^2 = 0.59$, $P < 0.01$) and N-treated WS3 (solid symbols/solid line: $y = -57.82 + 0.03x$, $r^2 = 0.40$, $P < 0.05$).

Sample homogeneity of the herb layer community (calculated as the reciprocal of sample heterogeneity) was initially higher on reference WS4 than treatment WS3 and decreased significantly ($P < 0.01$) through time. In contrast, WS3 exhibited a significant ($P < 0.05$) increase in homogeneity throughout the study period (Fig. 2).

Effects of N on cover of the herbaceous layer

Following an initial period (1991 to 1994), when there were no significant responses of cover of the herbaceous layer to the N treatment, cover increased significantly by 2003 and remained higher on WS3 than on reference WS4 (Fig. 3). Although cover has varied inter-annually throughout the 25-yr period of the study, there was an increase on treated WS3 in 2003, both significantly higher than the 1991–1994 period on that watershed and higher than on reference WS4 in 2009 and 2010 (Fig. 3).

For the sake of comparison both between watersheds and among published data for herb layer cover and/or biomass, the study was divided into early (1991–1994) and late periods (2009–2014), with means for these periods compared with separate t -tests for each watershed (Table 2). Cover and biomass were significantly higher ($P < 0.05$) in the late period for both

watersheds (Table 2). For cover there was a twofold increase on WS4 and a threefold increase on WS3; for biomass there was a twofold and fourfold increase for WS4 (12.9 g m^{-2}) and WS3 (23.9 g m^{-2}), respectively. Cover and biomass for the 2009–2014 period at FEF watersheds are among the highest values for data reported in the literature for individual studies (Table 2).

To more clearly quantify temporal changes in cover of individual herb layer species we divided data into early and late periods, and performed t -tests between periods for the top eight species found in each watershed. Half of these species exhibited no change on untreated WS4, whereas two increased (*Rubus* and *Quercus rubra*) and two decreased (*Viola* spp. and *Acer saccharum*) (Table 3A). On N-treated WS3, three species did not change, whereas two increased (*Rubus* and *Acer rubrum*) and three decreased (*Viola* spp., *Sassafras albidum*, and *Prunus serotina*) (Table 3B).

Temporal change in cover of *Rubus* was especially dramatic and individually assessed via repeated measures analysis of variance, which revealed contrasting patterns between watersheds. The initial period (1991–1994) exhibited little variation in *Rubus* cover between watersheds or over time on either watershed, with *Rubus* cover averaging ~1%. Thereafter, *Rubus* cover increased

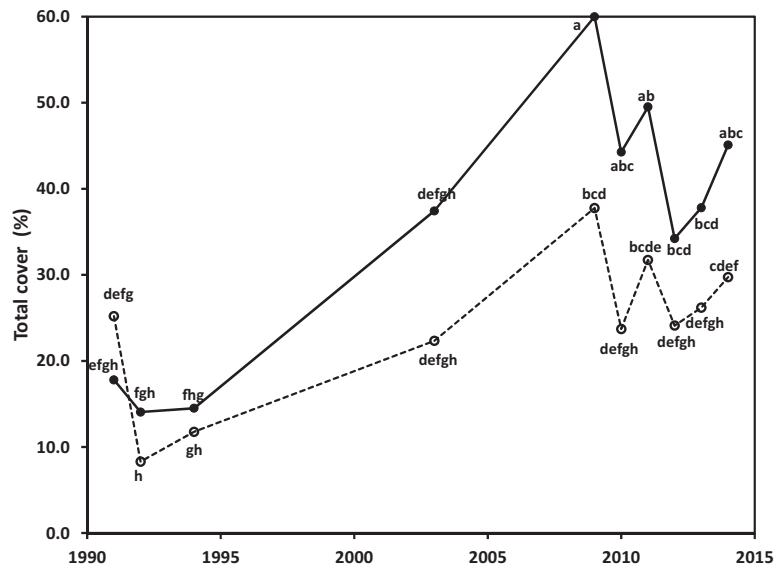


Fig. 3. Changes from 1991 to 2014 in mean total cover of the herbaceous layer on reference WS4 (open symbols/dashed line) and N-treated WS3 (solid symbols/solid line). Means with the same subscript are not significantly different at $P < 0.05$, using repeated measures ANOVA.

Table 2. Cover and biomass of the herbaceous layer from WS4 (reference) and WS3 (N treated) at Fernow Experimental Forest, WV for early (1991–1994) and late (2009–2014) sampling periods, along with data from selected eastern deciduous forest sites.

Site	Forest type	Cover %	Biomass $g\ m^{-2}$
Appalachian hardwood forest	WS4 (1991–1994)	15.1*	6.2*
	WS4 (2009–2014)	27.2	12.9
	WS3 (1991–1994)	15.4*	6.2*
	WS3 (2009–2014)	44.0	23.9
Northern hardwood forest, NH†	Maple-beech	24.0	7.0
Appalachian oak forest, VA‡	Oak-hickory	9.6	11.0
Northern hardwood forest, NJ§	Oak-hickory	31.0	–
Northern hardwood forest, IL¶	Oak-hickory	–	24.5
Northern hardwood forest, WI	Birch	–	51.0

* Indicates significant difference between periods ($P < 0.05$).
 † Siccama et al. (1970).
 ‡ McEvoy et al. (1980).
 § Davison and Forman (1982).
 ¶ Peterson and Rolfe (1982).
 || Zavitkovski (1976).

significantly on WS3, but not on WS4; more recent values were ~4% on WS4 versus ~20% on WS3 (Fig. 4).

Effects of N on species richness and composition of the herbaceous layer

Species richness of the herbaceous layer—measured as mean number of species per sample plot—initially exhibited great inter-annual variability, but no significant differences between watersheds during the initial period of the study (1991–1994) (Fig. 5). Beginning in 2003, however, richness significantly increased on WS4, but did not change on WS3. Since this time, species richness has generally remained significantly lower on WS3 than on WS4 (Fig. 5).

Linear regression was used to quantify temporal change in herb diversity in each watershed separately, and showed a significant increase in species diversity over the sample period on reference WS4 ($y = -119.03 + 0.06x$, $r^2 = 0.37$, $P < 0.05$), whereas diversity decreased on N-treated WS3 ($y = 107.59 - 0.05x$, $r^2 = 0.41$, $P < 0.05$) (Fig. 6). Species evenness did not change significantly over time on WS4, but decreased significantly during this period on WS3 ($y = 11.17 - 0.01x$, $r^2 = 0.63$, $P < 0.01$) (Fig. 7).

Detrended correspondence analysis (DCA)—used to assess temporal changes in species composition of the herb layer of both untreated WS4 and N-treated WS3—revealed close similarities between watersheds in the initial

Table 3. Change in importance (relative cover) of prominent herbaceous layer species on reference WS4 and treatment WS3 from early (1991–1994) to late (2009–2014) periods. Values shown are means for a given period \pm 1 SE of the mean. Means were compared between periods with a t-test: \uparrow indicates a significant increase at $P < 0.05$; \downarrow indicates a significant decrease at $P < 0.05$. \leftrightarrow indicates no significant change at $P < 0.05$.

Species	Early (1991–1994)	Change	Late (2009–2014)
(A) WS4			
<i>Viola</i> spp.	13.5 \pm 1.6	\downarrow	5.4 \pm 1.1
<i>Prunus serotina</i>	9.4 \pm 3.5	\leftrightarrow	4.6 \pm 0.7
<i>Acer pensylvanicum</i>	9.1 \pm 2.7	\leftrightarrow	9.8 \pm 0.5
<i>Rubus</i>	6.5 \pm 0.5	\uparrow	13.2 \pm 0.9
<i>Smilax rotundifolia</i>	5.3 \pm 2.3	\leftrightarrow	8.9 \pm 0.8
<i>Acer saccharum</i>	4.9 \pm 1.9	\downarrow	1.2 \pm 0.2
<i>Polystichum acrostichoides</i>	4.9 \pm 3.0	\leftrightarrow	1.8 \pm 0.5
<i>Quercus rubra</i>	0.7 \pm 0.5	\uparrow	4.9 \pm 0.5
(B) WS3			
<i>Viola</i> spp.	18.9 \pm 4.1	\downarrow	1.8 \pm 0.4
<i>Smilax rotundifolia</i>	15.2 \pm 6.9	\leftrightarrow	15.5 \pm 1.5
<i>Acer pensylvanicum</i>	10.1 \pm 1.8	\leftrightarrow	10.8 \pm 1.0
<i>Rubus</i>	8.9 \pm 1.2	\uparrow	40.9 \pm 2.5
<i>Sassafras albidum</i>	8.1 \pm 1.9	\downarrow	0.1 \pm 0.1
<i>Prunus serotina</i>	4.1 \pm 1.9	\downarrow	0.3 \pm 0.1
<i>Polystichum acrostichoides</i>	0.9 \pm 0.9	\leftrightarrow	1.5 \pm 0.3
<i>Acer rubrum</i>	0.2 \pm 0.2	\uparrow	3.8 \pm 0.3

period of the study (1991–1994) with a sharp departure in trajectories occurring in 2003, and remaining greatly dissimilar thereafter (Fig. 8). The general trend for WS4 was confined primarily to DCA axis 1, with a change toward decreasing scores, significantly and negatively correlated with increases in several herb layer species, including *Rubus*, *Acer pensylvanicum*, *Smilax rotundifolia*, *Acer rubrum*, *Quercus rubra*, and *Dryopteris intermedia* (Table 4).

By contrast, the trajectory of compositional change for WS3 was more pronounced and along both DCA axes 1 and 2 (Fig. 8). Decreases along axis 1 were positively correlated with *Viola* spp., *Sassafras albidum*, and *Prunus serotina*, indicating declines in these species over time, whereas they were negatively correlated with *Rubus*, *Dennstaedtia punctilobula*, and *Polystichum acrostichoides*, indicating increases in these species (Table 4). Increases along axis 2 were positively

correlated with *Rubus*, *Smilax rotundifolia*, *Acer pensylvanicum*, *Dennstaedtia punctilobula*, and *Acer rubra*, indicating increases over time; increases along this axis were negatively correlated with *Viola* spp., *Sassafras albidum*, and *Prunus serotina* (Table 4).

DISCUSSION

The initial predictions of the N homogeneity hypothesis were made before they could be tested adequately at FEF. Now, 25 yr after initiation of N treatments to WS3, we can more fully address the prediction that excess N deposition will increase homogeneity of N availability, and do so in a way that increases homogeneity of the herbaceous layer community.

Earlier work at FEF (Gilliam et al. 2001) gave rise to the prediction that excess N inputs (via atmospheric deposition or fertilizer additions) increase spatial homogeneity of N availability. Compared to both reference watersheds (WS4 and WS7), spatial variability was lowest on WS3 in virtually all measures of soil N status, including soil solution NO_3^- , extractable NO_3^- pools, plant tissue N, litter N, total soil N, and soil C/N ratios (Gilliam et al. 2001). Results of the extensive grid sampling in 2011 (Fig. 1) support this prediction. Spatial heterogeneity in net nitrification—which in our sites was equal to net N mineralization—was notably higher on WS7, with a coefficient of spatial variation of 51% and the values exhibiting a patchy spatial pattern of N hotspots (up to $118 \text{ mg N m}^{-2} \text{ d}^{-1}$) and cold spots (down to $0.07 \text{ mg N m}^{-2} \text{ d}^{-1}$). The coefficient of spatial variation in WS3 was 33% and showed a greater degree of homogeneity of net nitrification at moderate to high levels ($\sim 40 \text{ mg N m}^{-2} \text{ d}^{-1}$) throughout its 35 ha landscape (Fig. 1).

Results from other studies show that spatial heterogeneity of soil resources (e.g., moisture, nutrients) creates and maintains high plant diversity in terrestrial ecosystems (Hutchings and de Kroon 1994, Hutchings et al. 2003, Lundholm 2009, Bartels and Chen 2010, Costanza et al. 2011, Laliberté et al. 2013). Consistent with these results, the N homogeneity hypothesis predicts that increases in the spatial homogeneity of N availability will lead to decreases in biodiversity of the herb layer of impacted forests. In this study,

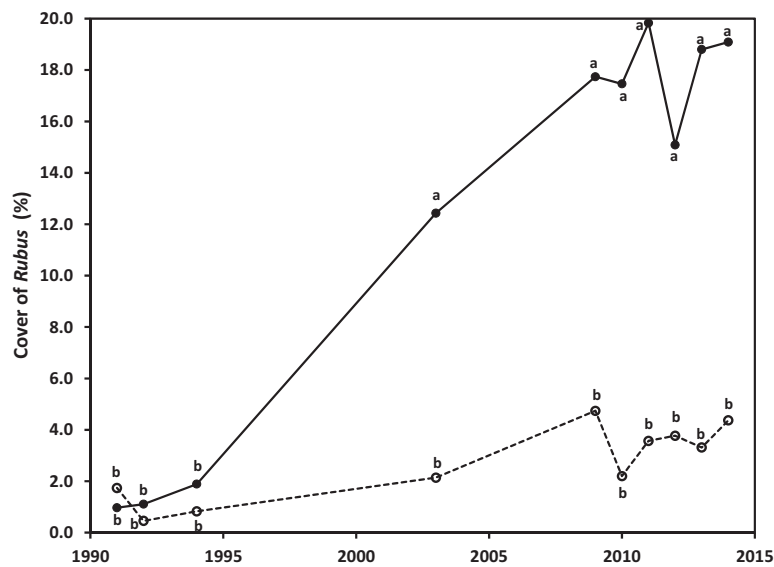


Fig. 4. Changes from 1991 to 2014 in mean cover of *Rubus* on reference WS4 (open symbols/dashed line) and N-treated WS3 (solid symbols/solid line). Means with the same subscript are not significantly different at $P < 0.05$, using repeated measures ANOVA.

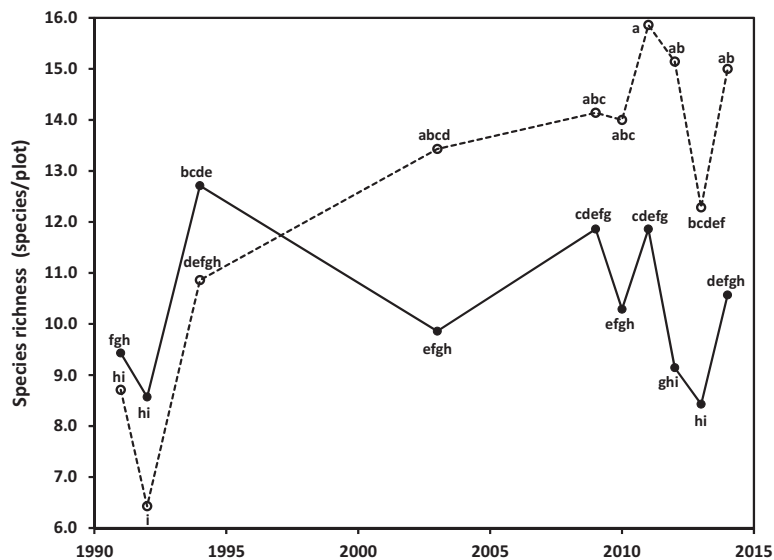


Fig. 5. Changes from 1991 to 2014 in mean species richness on reference WS4 (open symbols/dashed line) and N-treated WS3 (solid symbols/solid line). Means with the same subscript are not significantly different at $P < 0.05$, using repeated measures ANOVA.

sample homogeneity of the herb-layer plant community—calculated as the reciprocal of sample heterogeneity—was initially higher on reference WS4 than treatment WS3, and decreased in WS4

significantly ($P < 0.01$) through time. In contrast, WS3 exhibited a significant ($P < 0.05$) increase in homogeneity throughout the study period (Fig. 2).

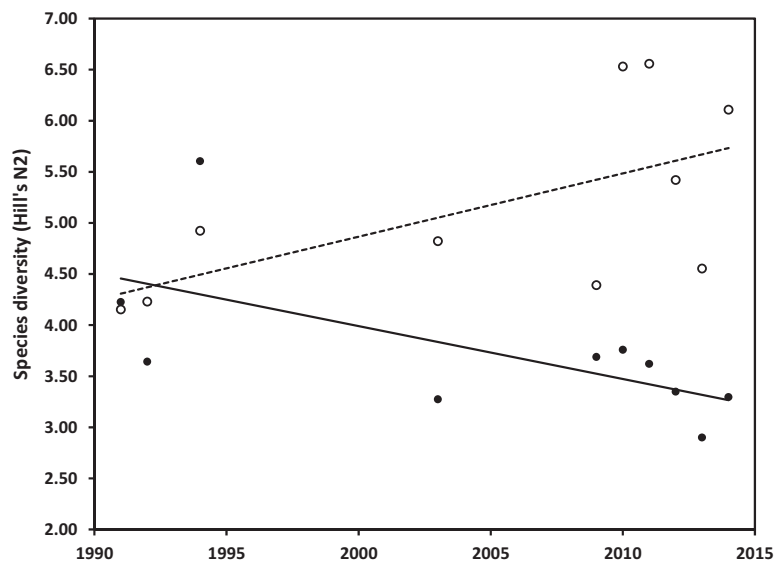


Fig. 6. Change over time in species diversity, 1991–2014, on reference WS4 (open symbols/line: $y = -119.03 + 0.06x$, $r^2 = 0.37$, $P < 0.05$) and N-treated WS3 (solid symbols/solid line: $y = 107.59 - 0.05x$, $r^2 = 0.41$, $P < 0.05$).

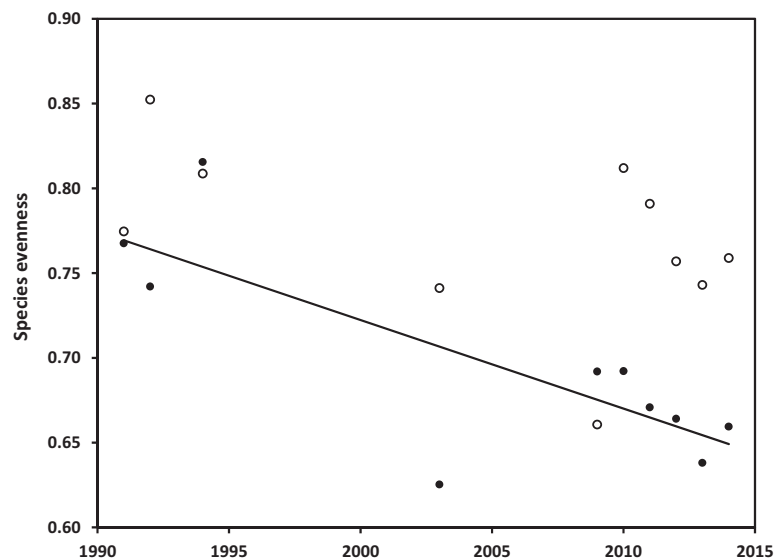


Fig. 7. Change over time in species evenness, 1991–2014, on reference WS4 (open symbols: not significant at $P > 0.05$) and N-treated WS3 (solid symbols/solid line: $y = 11.17 - 0.01x$, $r^2 = 0.63$, $P < 0.01$).

We expected that the response time of herb layer species to increases in N availability should be inversely related to ambient N deposition because other studies suggest a rapid loss of sensitive species as N inputs increase above $\sim 10 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ (Emmett 2007), and since ambient rates

of N deposition at FEF have been relatively high ($15\text{--}20 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ total deposition; Gilliam et al. 2006). Consistent with this expectation, we found little response of herb cover to added N on WS3 during the first 5 yr of fertilizer additions, a lag that is much longer than what occurs in

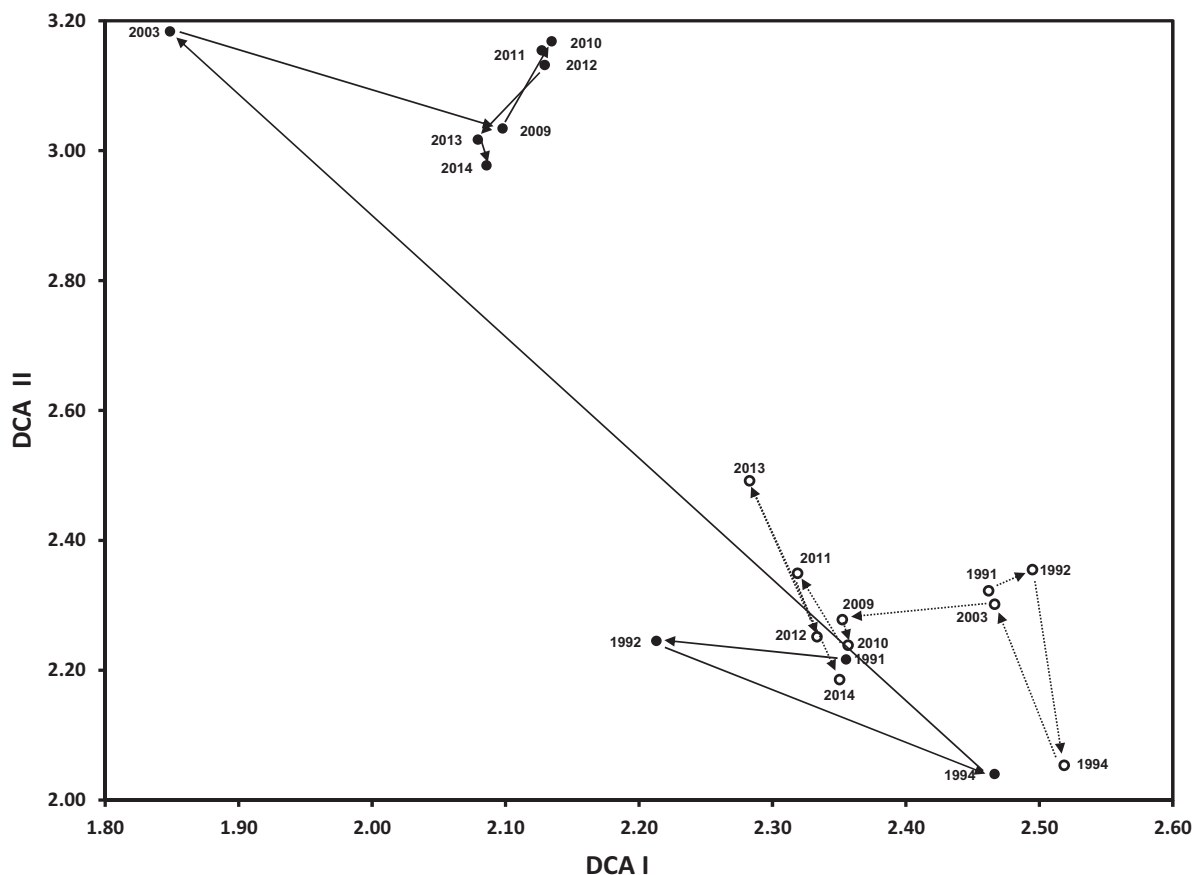


Fig. 8. Detrended correspondence analysis (DCA) for herbaceous layer communities from 1991 to 2014 on reference WS4 (open symbols/dashed line) and N-treated WS3 (solid symbols/solid line). Values shown are centroids (two-dimensional means) for each year per watershed.

Table 4. Correlations of individual herb layer species with DCA axis scores (see Fig. 6) for WS4 and WS3. Values shown are Pearson product-moment correlation coefficients (r) and corresponding P values in parentheses. Significant correlations are indicated in bold.

Species	WS4		Species	WS3	
	DCA I	DCA II		DCA I	DCA II
<i>Laportea canadensis</i>	0.07 (0.85)	-0.21 (0.57)	<i>Rubus</i>	-0.64 (0.05)	0.91 (0.00)
<i>Rubus</i>	-0.85 (0.00)	0.21 (0.56)	<i>Smilax rotundifolia</i>	-0.57 (0.08)	0.67 (0.03)
<i>Acer pensylvanicum</i>	-0.70 (0.03)	0.30 (0.39)	<i>Acer pensylvanicum</i>	-0.24 (0.51)	0.68 (0.03)
<i>Smilax rotundifolia</i>	-0.85 (0.00)	0.37 (0.29)	<i>Dennstaedtia punctilobula</i>	-0.71 (0.02)	0.69 (0.03)
<i>Viola</i> spp.	0.03 (0.94)	-0.10 (0.78)	<i>Viola</i> spp.	0.66 (0.04)	-0.78 (0.01)
<i>Prunus serotina</i>	-0.24 (0.50)	0.00 (1.00)	<i>Acer rubrum</i>	-0.53 (0.12)	0.83 (0.00)
<i>Acer rubrum</i>	-0.87 (0.00)	0.24 (0.51)	<i>Polystichum acrostichoides</i>	-0.80 (0.01)	0.55 (0.10)
<i>Quercus rubra</i>	-0.87 (0.00)	0.18 (0.61)	<i>Sassafras albidum</i>	0.72 (0.02)	-0.85 (0.00)
<i>Dryopteris intermedia</i>	-0.67 (0.03)	0.04 (0.92)	<i>Prunus serotina</i>	0.73 (0.02)	-0.71 (0.02)

regions of lower levels of ambient N deposition. For example, many studies reported responses to experimental N additions occur within one to three years—even when N additions exceeded those added to WS3 (Hurd et al. 1998, Rainey et al. 1999, Price and Morgan 2007, Lu et al. 2010). However, following a lengthy, initial delay, there was an increase in cover on treated WS3 in 2003 that was both significantly higher than the 1991–1994 period on that watershed and significantly higher than on reference WS4. Subsequently, cover not only continued patterns of inter-annual variability, exhibiting essentially parallel patterns between watersheds, but was also significantly higher on WS3 than on WS4 in 2009 and 2010 (Fig. 3), consistent with our prediction of a fertilizer-type response of the herb layer on WS3.

The most notable change in cover on WS3 has been that of *Rubus* (Fig. 4). Since most species of *Rubus* have been well-established as being nitrophilic (Jobidon 1993, Truax et al. 1994, Strik 2008), it is not surprising that its cover increased in response to N additions. However, what is surprising is that it did so beneath the intact canopy of a > 30-yr-old hardwood stand, given its typical requirement of high light conditions (Landhäusser et al. 1997). *Rubus* would be classified as a ruderal, *R*, species (Grime 2006), and thus is typically associated with disturbance-maintained conditions, including high light availability, with cover generally decreasing over time with stand development (Marks 1974, Hédli 2004, Roberts 2004, Reiners et al. 2012). Anecdotal observation indicates that the significant increase in *Rubus* cover on WS3 is not associated with appreciable increases in flowering. Thus, it appears that the N additions to WS3 have alleviated N limitation to *Rubus*, allowing expansive growth, but that the intact canopy limits flower production.

After the initial period from 1991 to 1994, species diversity and its components (species richness and evenness) in the two watersheds diverged, with lower values of diversity, richness, and evenness being found in the fertilized watershed. Furthermore, the temporal trends for richness (increasing in WS4 with no trend in WS3) and evenness (decreasing in WS3 with no trend in WS4) strongly suggest that the decline of herb diversity to added N was largely the result of significant, N-mediated decreases in species evenness (Figs. 5–7).

Species richness is a dynamic component of herb communities in forest ecosystems, changing in response to several environmental factors, and certainly changing through time in secondary forests, as was shown for long-term reference WS4 (Fig. 5). It is possible that added N on WS3 altered the trajectory of increasing richness that would have occurred in the absence of N additions, supporting conclusions of Siddique et al. (2010), who examined the effects of nutrients (N and P) on trajectories of tree species richness and composition in a tropical forest ecosystem. In agreement with our results, most global and regional syntheses point to a general decline in plant diversity with increasing N deposition (Bobbink et al. 2010, Clark et al. 2013b, Suding et al. 2005, Clark et al. 2013a, Stevens et al. 2010, De Schrijver et al. 2011, Verheyen et al. 2012, Dirnböck et al. 2014, Roth et al. 2015), but more specific responses vary with individual studies. For responses to experimental N additions, the outcome may depend on levels of ambient N deposition (Nordin et al. 2005, 2006, Hülber et al. 2008, Diwold et al. 2010, Hedwall et al. 2013, Strengbom et al. 2003).

Similar to species diversity, temporal changes in species composition of the herb layer in both untreated WS4 and N-treated WS3 suggests close similarities between watersheds in the initial period of the study (1991–1994) with a sharp departure in trajectories over time occurring in 2003, and remaining greatly dissimilar thereafter (Fig. 8). The general trend for WS4 was towards increases in several herb layer species, including *Rubus*, *Acer pensylvanicum*, *Smilax rotundifolia*, *Acer rubrum*, *Quercus rubra*, and *Dryopteris intermedia* (Table 4). In contrast, the trajectory of compositional change for WS3 was more pronounced than for WS4 and included both declines in *Viola* spp., *Sassafras albidum*, and *Prunus serotina*, and increases in *Rubus*, *Dennstaedtia punctilobula*, and *Polystichum acrostichoides* (Table 4). Thus, experimental additions of N appear to have resulted in profound changes in species composition, consistent with our prediction.

SYNTHESIS AND CONCLUSIONS

Data from 25 years of aerial additions of N to an entire watershed generally support the chronology of responses of the herbaceous layer

of forests to excess N that is articulated by the N homogenization hypothesis (Gilliam 2006, 2014a): (1) *the herb layer for the N-treated watershed will initially exhibit increases in total cover—following a lag period of no response*; (2) *N-mediated increases in cover will simultaneously involve increases in nitrophilous species and decreases in N-efficient species, relative to the untreated reference watershed—although *Rubus* was not the only species to increase, the notable increase in this nitrophilous species on WS3 coincided with decreases in several species, including *Viola* spp.*; (3) *both species richness and species evenness will decline (from increases in relatively few nitrophilic species), resulting in decreases in herb layer diversity—herb layer diversity decreased on WS3, whereas it increased on WS4, due to both a N-mediated reduction in species richness and evenness over time for WS3*; and (4) *added N will increase the spatial homogeneity of N availability, which will lead to increased sample homogeneity of the herb layer, ultimately resulting in N-mediated loss of species diversity and alterations in herb layer community composition—WS3 displayed a lower level of spatial variability in an index of N availability, with homogeneity of vegetation increasing significantly through the study period.*

Although it is clear from other studies that excess N deposition represents a potential disturbance to the structure and function of forest ecosystems via effects on the herb layer communities they support, N effects are not the only threat they face. Bellemare and Moeller (2014) reviewed a wide range of negative responses of forest herb communities to several facets of climate change (e.g., increased temperatures, lengthened growing seasons, altered phenologies, and limited dispersal), and they concluded that modern climate change seriously threatens forest herb biodiversity. Thus, studies of multiple factor interactions are needed to better assess the applicability of the effects we observed in response to excess N because herb layer communities are currently experiencing conditions far different from those under which they developed, and they continue to adapt to environmental changes occurring at unprecedented rates. Thus, because present-day communities represent “ecological moving targets” (sensu Gilliam 2007), determining the generality of any changes in the past remains a current and ongoing challenge.

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