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# Effects of excess nitrogen on biogeochemistry of a temperate hardwood forest: Evidence of nutrient redistribution by a forest understory species

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## HIGHLIGHTS

- Excess nitrogen (N) deposition negatively impacts eastern U.S. hardwood forests.
- Our study studied biogeochemical effects via foliar analysis of herb layer species.
- Aerial N additions were made to an entire watershed for a 25-yr period.
- Early-dominant Viola and late-dominant Rubus were higher in N, lower in Ca.
- N-mediated increases in Rubus appeared to redistribute Mn to surface soils.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Excess nitrogen (N) in terrestrial ecosystems can arise from anthropogenically-increased atmospheric N deposition, a phenomenon common in eastern US forests. In spite of decreased N emissions over recent years, atmospheric concentrations of reactive N remain high in areas within this region. Excess N in forests has been shown to alter biogeochemical cycling of essential plant nutrients primarily via enhanced production and leaching of nitrate, which leads to loss of base cations from the soil. The purpose of our study was to investigate this phenomenon using a multifaceted approach to examine foliar nutrients of two herbaceous layer species in one N-treated watershed (WS3—receiving aerial applications of 35 kg N/ha/yr as ammonium sulfate, from 1989 to the present) and two untreated reference watersheds at the Fernow Experimental Forest, WV, USA. In 1993, we analyzed foliar tissue of *Viola rotundifolia*, a dominant herb layer species and prominent on all seven sample plots in each watershed. In 2013 and 2014, we used foliar tissue from *Rubus allegheniensis*, which had become the predominant species on WS3 and had increased, though to a lesser extent, in cover on both reference watersheds. Foliar N and potassium (K) were higher and foliar calcium (Ca) was lower on WS3 than on the reference watersheds for both species. Magnesium (Mg) was lower on WS3 for *Viola*, but was not different among watersheds for *Rubus*. Results support the stream chemistry-based observation that excess N lowers plant-available Ca and, to a lesser degree, Mg, but not of K. Foliar manganese (Mn) of

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*Rubus* averaged >4 times that of *Viola*, and was >50% higher on WS3 than on the reference watersheds. A Mn-based mechanism is proposed for the N-meditated increase in *Rubus* on WS3. Data suggest that excess N deposition not only alters herb community composition and biogeochemical cycling of forest ecosystems, but can do so simultaneously and interactively.



## 1. Introduction

Foliar nutrient concentrations of wild plants often, though not always, reflect the availability of nutrients in mineral soil, since foliar nutrients are generally indicative of the balance between nutrient supply in the soil and immediate demand by the plants (Chapin, 1980; Schreeg et al., 2014). Sources of variation in this generalization include uptake of nutrients beyond plant demand—luxury uptake—and a high degree of species-specific variability in nutrient use and allocation, including resorption (Chapin and Kedrowski, 1983; Killingbeck, 1996; May et al., 2005; Baeten et al., 2010). To the extent that this generalization is relevant, foliar nutrient analyses can yield insight into factors (e.g., anthropogenic disturbance) that influence nutrient availability, and others (e.g., plant-soil feedbacks) that control nutrient dynamics (Reiners, 1992; Schreeg et al., 2014).

In the United States, the 1977 and 1990 amendments of the Clean Air Act of 1970 have been effective in decreasing emissions of nitrogen (N) compounds into the atmosphere. Despite this, however, high concentrations of reactive nitrogen (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) persist, as do high levels of atmospheric deposition of N, in several regions throughout the world (Galloway et al., 2008; Sutton et al., 2014; Vet et al., 2014; Keene et al., 2015), although N remains the nutrient that most commonly limits or co-limits plant growth globally (Vitousek et al., 1997; Elser et al., 2007). Conversely, chronic atmospheric deposition of N in many areas supplies available N in excess of plant and microbial demand, leading to a phenomenon known as N saturation (Aber et al., 2003).

As discussed by Gilliam (2006), N saturation is a biogeochemical phenomenon that has direct, sometimes immediate, consequences for plant communities, thus integrating the ecological disciplines of both biogeochemistry and vegetation science. Biogeochemically, excess N alters mobility of a variety of essential nutrients, beginning with increased predominance of $NO_3^-$, the highly mobile form of available N. As $NO_3^-$ accumulates in the available N pool in excess of plant uptake, it becomes susceptible to leaching below the active rooting zone, accompanied by cations, particularly $Ca^{++}$ and $Mg^{++}$. The result is an imbalance of increasing availability of N leading to decreasing availability of $Ca^{++}$ and $Mg^{++}$ (Peterjohn et al., 1996; Gilliam et al., 1996; Moore and Houle, 2013). Studies have also found that N saturation can initiate phosphorus (P) limitation forest ecosystems, although the specific mechanism is different than for cations (Güsewell, 2004; Gress et al., 2007; Vitousek et al., 2010).

Regarding the plant response to excess N, there are several possible direct and indirect effects on the species composition of forest herb strata via alteration of interspecific competition, herbivory, mycorrhizal infection, pathogenic fungal infection, and invasive species (Gilliam, 2006). This can be especially relevant for the herbaceous layer of forests considering that (1) many, perhaps most, N-saturated ecosystems are forests (Holland and Lamarque, 1997; Aber et al., 2003; Gilliam, 2014), and (2) the herb layer is potentially the most sensitive of forest strata to changes in nutrient availability (Muller, 2014). In addition, the herb layer merits special attention as the forest stratum with highest plant diversity (Gilliam, 2007).

The site for the current study—Fernow Experimental Forest (FEF), West Virginia—has been used for several past and on-going investigations into the ecological sustainability of Appalachian hardwood forests in the context of natural and anthropogenic disturbances, one of which is chronically-elevated N deposition (Adams et al., 2006). Peterjohn et al. (1996) provided clear evidence that several symptoms of N saturation (cf., Aber, 1992) had developed on the long-term reference watershed for on-going studies at FEF (WS4). One such symptom is high absolute and relative (to net N mineralization) rates of net nitrification, which were shown by Gilliam et al. (2001) to exist on an N-treated watershed (WS3) and two untreated reference watersheds (WS4 and WS7). Another symptom relevant to the present study is increased mobility and leaching of $Ca^{2+}$ and $Mg^{2+}$ associated with enhanced nitrification and leaching of $NO_3^-$ (Peterjohn et al., 1996; Gilliam et al., 1996), along with evidence of decreased growth rates of dominant tree species (May et al., 2005; DeWalle et al., 2006). More recent work using root in-growth bags filled with nutrient-amended soil suggests that N saturation has led to P limitation in several FEF watersheds (Gress et al., 2007).

The purpose of this study was to enhance insight into the effects of excess N on the biogeochemistry of a temperate hardwood forest by examining foliar nutrient concentrations of two dominant herb-layer species on one N-treated watershed and two untreated watersheds at two time periods following initiation of N treatments—4 years and 24–25 years post-treatment. Because there has been an unprecedented N-mediated shift in herb layer dominance on these watersheds (Gilliam et al., 2016), this involves an unavoidable confounding of species and time (i.e., from *Viola rotundifolia* Michx. to *Rubus allegheniensis* Porter dominance—see Methods). Nevertheless, this study is unique in assessing biogeochemical responses to experimental N additions over such a time period and doing so using foliar nutrients on the same sample plots.

## 2. Methods

### 2.1. Study site

This study is part of long-term, on-going research on the effects of experimental additions of N on a temperate hardwood forest ecosystem carried out at FEF, located in Tucker County, West Virginia (39° 03′ 15″N,7 9° 49′ 15″W). FEF is a ~1900 ha area of the Allegheny Mountain section the unglaciated Allegheny Plateau. Precipitation for FEF averages ~1430 mm $yr^{-1}$, with precipitation generally increasing through the growing season and with higher elevations. Ambient wetfall deposition of N is ~10 kg N/ha/yr, and has changed little over the study period (Gilliam and Adams, 1996), other than declines in $NO_3^-$ concentrations (Adams et al., 2006).

Soils of the study watershed are predominantly Inceptisols of the Berks (loamy-skeletal, mixed, mesic Typic Dystrochrept) and Calvin series (loamy-skeletal, mixed, mesic Typic Dystrochrept), derived from sandstone, and are generally coarse-textured sandy loams, well-drained, and ~1 m in depth (Adams et al., 2006). Three watersheds were used for the location of sample plots: WS3, WS4, and WS7, with WS3 serving as the treatment watershed, receiving aerial additions of $(NH_4)_2SO_4$, and WS4 and WS7 serving as unfertilized reference watersheds.

Applications of $(NH_4)_2SO_4$ to WS3 began in 1989, are currently on-going, and are made three times per year; historically, these have been administered by either helicopter or fixed-wing aircraft. March and November applications are 33.6 kg/ha of fertilizer, or 7.1 kg/ha of N. July applications are 100.8 kg/ha fertilizer (21.2 kg/ha N). This rate was originally chosen as approximately twice the ambient rates of N deposited on the watersheds via throughfall. It is also within the range predicted for future increases in N deposition for this region (Bobbink et al., 2010). Stands on WS3 and WS7 were ~45 yr-old at the time of most recent sampling in this study; these are even-aged and developed following clearcutting. WS4 supports even-aged stands >100 yr old.

All study watersheds generally support mixed hardwood stands. Overstory dominant species include sugar maple *(Acer saccharum* Marsh.), sweet birch *(Betula lenta* L.), American beech *(Fagus grandifolia* Ehrh.), yellow poplar *(Liriodendron tulipifera* L.), black cherry *(Prunus serotina* Ehrh.), and northern red oak *(Quercus rubra* L.) (Adams et al., 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 et al., 2006), including species of *Viola*, *Rubus*, mixed ferns, and seedlings of *Acer pensylvanicum* L. and *A. rubrum* L. Currently, *R. allegheniensis* (hereafter, *Rubus*) has increased significantly on all watersheds, but especially on N-treated WS3, where it represents nearly 50% of total herb-layer cover, in contrast to <15% on reference watersheds (Gilliam et al., 2016).

### 2.2. Field sampling and laboratory analyses

Sampling took place within seven circular 0.04-ha plots in each watershed, for a total of 21 plots. Plots were located to span the extremes of aspect and elevation of each watershed (Fig. 1). Thus, the range of elevation was closely similar for sample plots among watersheds: 735–860 m, 750–870 m, and 731–850 m for WS3, WS4, and WS7, respectively.

Foliar material of *V. rotundifolia* (hereafter, *Viola*), the dominant herb-layer species and present on all 21 plots, was sampled in July 1993; these results were reported in part in an earlier paper (Gilliam et al., 1996). At that time, *Rubus* was minor component of the forest herb community of low (~1–2%) cover and frequency. Similar sampling was repeated in July of 2013 and 2014. However, by this time, *Rubus* had replaced *Viola* as the dominant species on all plots except in one plot in each of reference watersheds WS4 and WS7 (Gilliam et al., 2016). By this time, *Viola* was of low (~5%) cover and frequency. Accordingly, foliar material of *Rubus* was taken in 2013 and 2014. Foliar material was sampled in the field by hand-harvesting using surgical gloves, placed in sterile polyethylene bags, and stored in chilled, insulated coolers.

Upon return to Marshall University, all foliar material was oven-dried at 50 °C overnight and ground in a Wiley mill to pass a 40-mesh screen. Samples were analyzed at the University of Maine Soil Testing Service and Analytical Laboratory for macronutrient (N, P, Ca, Mg, K), micronutrient (B, Cu, Fe, Mn, Zn), and Al concentrations. Total Kjeldahl N was determined with autoanalysis following block digestion with $H_2SO_4$ and $K_2SO_4/CuSO_4$; NBS1 572 Citrus Leaf was used as standard. All other elements were analyzed with plasma emission spectrophotometry following dry ashing and extraction with HCl and $HNO_3$.

We assessed changes in concentrations and spatial patterns of extractable Mn on WS3 and WS7 (but not WS4) at two points in time. For the earlier period, we accessed archived data from 1991 wherein extractable Mn was determined at 15 locations spanning all elevations and slope aspects of each watershed (see Gilliam et al., 1994 for methodology). For the later period, as part of a separate investigation in 2011 into within-watershed variation in soil nutrients and using the same methodology (i.e., surface mineral soil sampled with O horizons excluded, extracted by $NH_4CH_3CO_2$), extractable Mn was determined at 100 locations arrayed in a grid in treated WS3 and untreated WS7.

### 2.3. Data analysis

Our study design is an example of simple pseudoreplication (Hurlbert, 1984), so interpretation of data should take that into account. Our contention, however, is that any effects reported are best interpreted as treatment effects, rather than pre-existing differences among watersheds. Indeed, the three experimental watersheds are similar with respect to several site characteristics, e.g., overstory basal area, soil pH, and cation exchange capacity (Adams et al., 2006; Gilliam et al., 2016).

For the *Viola* data, means of all measured elements were compared for significant differences among watersheds using analysis of variance (ANOVA) and least significant difference (LSD) tests. For the *Rubus* data, means were compared for significant differences among watersheds and sample year using ANOVA and LSD tests. *A priori* significant differences were accepted for all statistical tests at $P < 0.10$ to accommodate natural spatial variability at the watershed landscape scale (Zar, 2009).

Data from both 1991 and 2011 soil sampling events were spatially interpreted with kriging to create maps displaying spatial variation in concentrations of extractable soil Mn for both time periods (Stein, 1999). The spatial data were kriged to the spatial extent of WS3 and WS7 at each sample time using ArcGIS Spatial Analyst, then clipped using the watershed boundaries as a mask. Data from 2011 were kriged to a $2 \times 2$ m cell size with a fixed search radius of 150 m. Data from 1991 were kriged to a $50 \times 50$ m cell size with a 300-m fixed search radius, then resampled to $2 \times 2$ m cell size to match the 2011 kriged data. The Mn classes for both maps were defined by 10 equal intervals, ranging from the lowest to highest Mn value in each watershed. The display of both maps was smoothed using a surface bilinear interpolation.

## 3. Results

For *Viola* in 1993, mean foliar concentrations on treatment WS3 varied significantly from either or both reference watersheds for all macronutrients, except P, with N and K higher and Ca and Mg lower on WS3 relative to WS4 and/or WS7 (Fig. 2). Foliar micronutrient and Al concentrations did not vary significantly among watersheds,

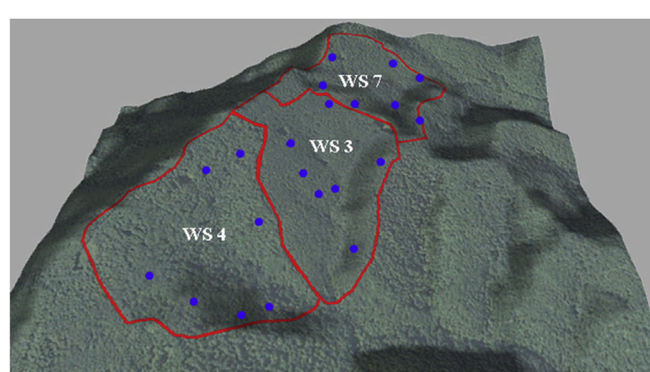


**Fig. 1.** Locations of sample plots on study watersheds at Fernow Experimental Forest, West Virginia: N-treated WS3 and reference WS4 and WS7.

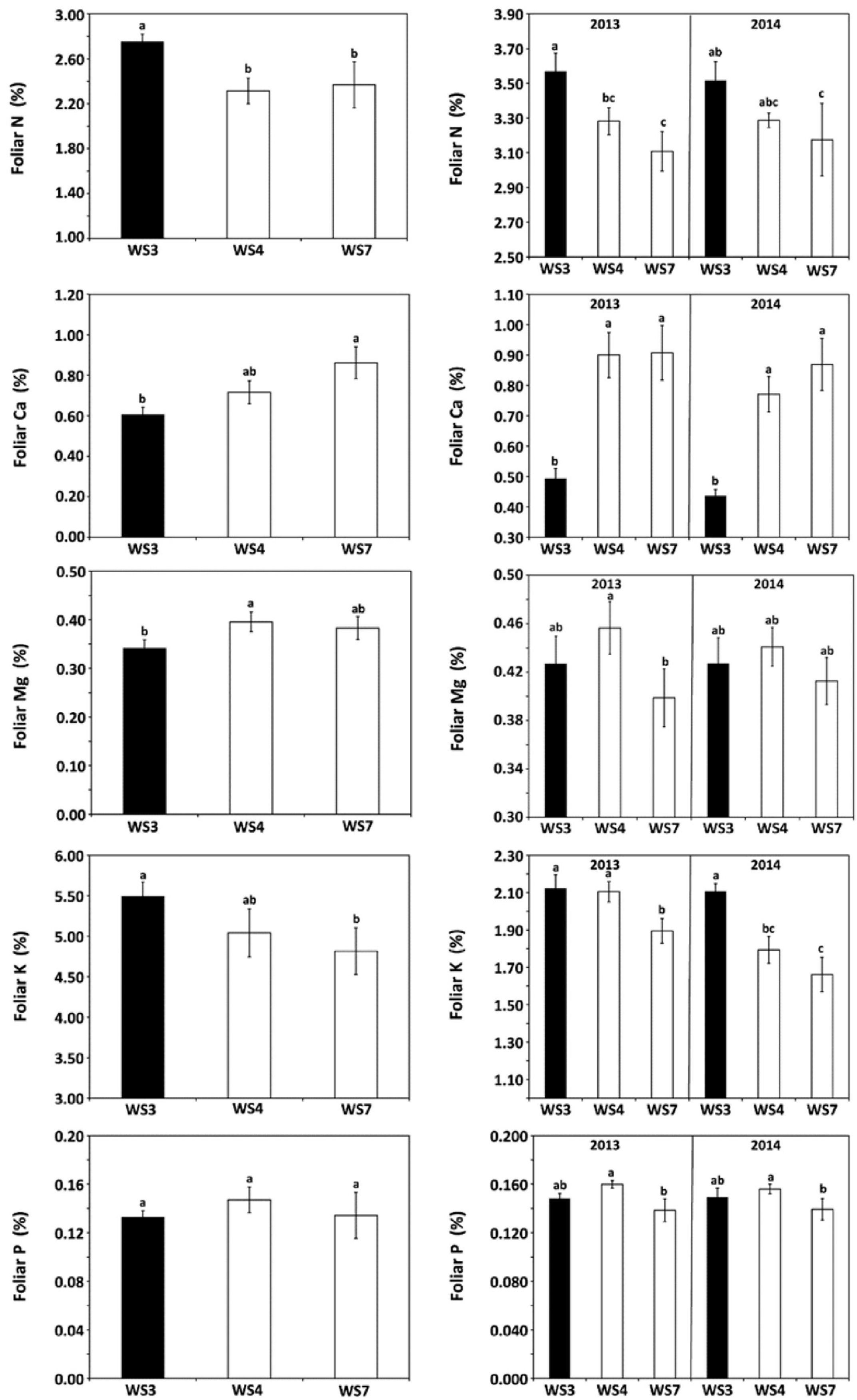


**Fig. 2.** Mean foliar concentrations of macronutrients for *Viola rotundifolia* on N-treated WS3 and reference WS4 and WS7 (left panels) and for *Rubus allegheniensis* on these watersheds in each of 2013 and 2014. For *V. rotundifolia*, means with the same superscript are not significantly different among watersheds at $P < 0.10$. For *R. allegheniensis*, means with the same superscript are not significantly different among watersheds and years at $P < 0.10$.

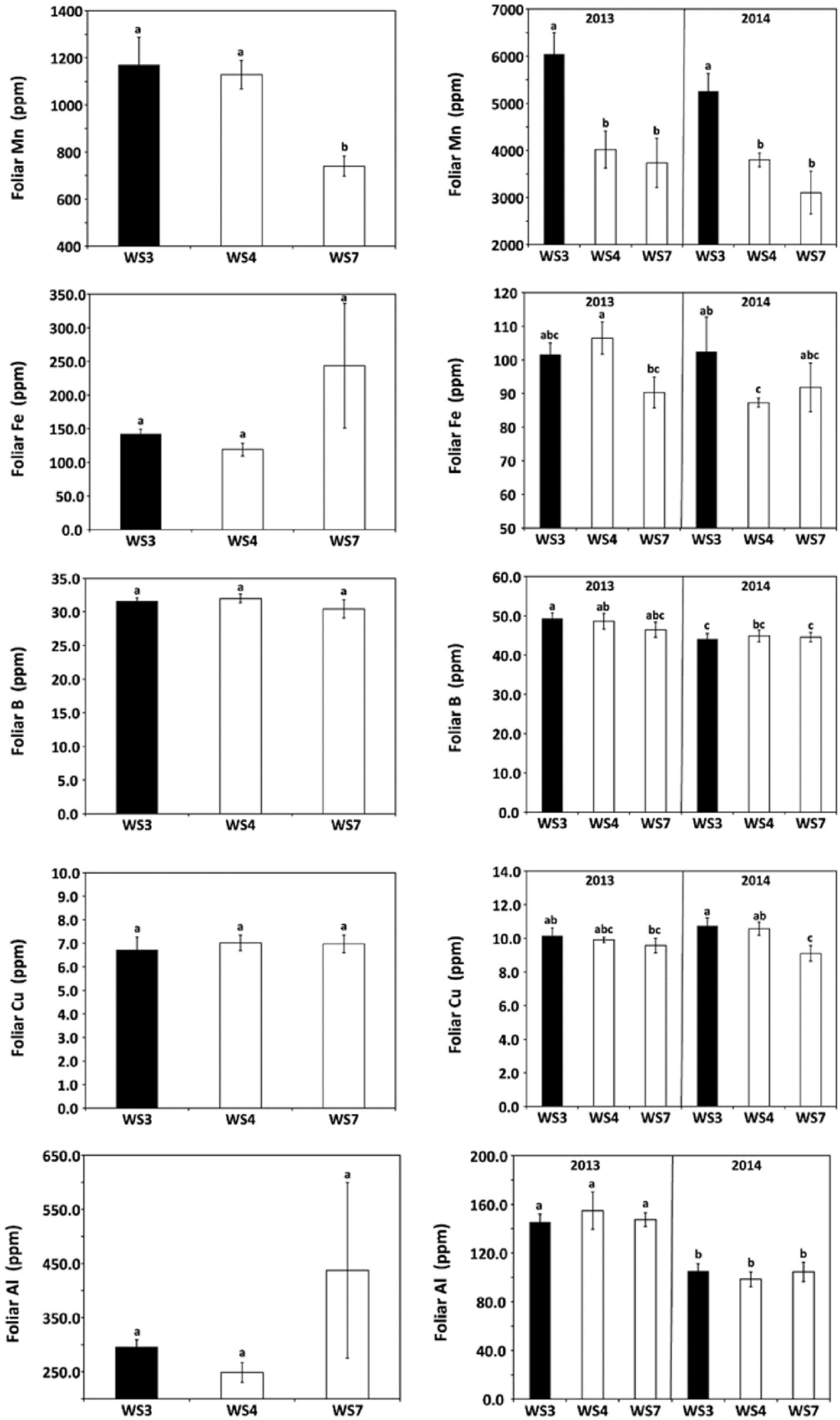


**Fig. 3.** Mean foliar concentrations of micronutrients and Al for *Viola rotundifolia* on N-treated WS3 and reference WS4 and WS7 (left panels) and for *Rubus allegheniensis* on these watersheds in each of 2013 and 2014. For *V. rotundifolia*, means with the same superscript are not significantly different among watersheds at $P < 0.10$. For *R. allegheniensis*, means with the same superscript are not significantly different among watersheds and years at $P < 0.10$.

with the exception of Mn, which was significantly lower on WS7 than on WS3 and WS4 (Fig. 3).

Mean foliar nutrient concentrations for *Rubus* did not vary significantly between 2013 and 2014 for any of the macronutrients. There were significant N-treatment effects for N, Ca, and K, with N and K being higher and Ca lower on WS3 versus WS4, WS7, or both (Fig. 2). Among foliar micronutrient and Al concentrations, only B and Al exhibited significant inter-annual variation, both generally lower in 2014 than in 2013 (Fig. 3). There were no significant N-treatment effects, except for Mn, which was higher on WS3 than on both reference watersheds in each of 2013 and 2014 (Fig. 3).

Additions of N significantly influenced Ca/Al ratios in *Viola*, with WS3 being lower than WS7, whereas the mean for WS4 was intermediate between WS3 and WS7 and significantly different from neither (Table 1). For *Rubus*, Ca/Al ratios were significantly lower on WS3 than on WS4 and WS7 for both years and did not vary between years. Ca/Al did not vary between WS7 and WS4 for either year, but did vary significantly between years for both watersheds (Table 1).

There was a significant effect of N addition on N/P ratio of *Viola*, with WS3 being higher than both WS7 and WS4 (Table 2). For *Rubus*, N/P ratios were significantly higher on WS3 than on WS4 for both years, with WS7 being intermediate between the two watersheds (Table 2).

Kriging of extractable soil Mn data revealed contrasts in both concentrations and spatial patterns between sample periods on WS7 and WS3. For WS7, soil Mn averaged ~0.15 meq/100 g in 1991 with little spatial variation; a similar pattern was found in 2011, with a watershed mean of ~0.13 meq/100 g and even less spatial variation (Fig. 4a). In 1991, Mn was <~0.20 meq/100 g soil throughout most of WS3, whereas values ranged from nearly 0 meq/100 g up to as high as ~0.50 meq/100 g in 2011, with higher concentrations occurring in relatively discrete patches (Fig. 4b).

## 4. Discussion

For two herb-layer species with otherwise sharply contrasting growth forms, life histories, and habitat requirements (Goodwillie and Jolls, 2014; Strik, 2008), *Viola* and *Rubus* exhibited notably similar patterns of N treatment effects on foliar nutrients. Similarities in response of foliar macronutrients to added N include significantly higher N and K concentrations and higher N/P ratios, significantly lower Ca concentrations, and a lack of effect on P (Fig. 2).

**Table 1**
Mean molar ratios (±1 SE of mean) of foliar Ca to foliar Al (mol:mol) for (A) *Viola rotundifolia*; means with the same superscript are not significantly different among watersheds at P < 0.10; and (B) *Rubus allegheniensis*; means with the same superscript are not significantly different among watersheds and years at P < 0.10.

| Watershed | Ca/Al ratio |
|---|---|
| | mol/mol |
| A. *Viola rotundifolia* | |
| WS3 | $13.8 \pm 0.7^{b}$ |
| WS7 | $25.6 \pm 7.9^{a}$ |
| WS4 | $19.9 \pm 1.8^{ab}$ |
| B. *Rubus allegheniensis* | |
| **WS3** | |
| 2013 | $15.5 \pm 1.2^{c}$ |
| 2014 | $19.3 \pm 1.4^{c}$ |
| **WS7** | |
| 2013 | $28.4 \pm 3.8^{b}$ |
| 2014 | $38.8 \pm 4.5^{a}$ |
| **WS4** | |
| 2013 | $27.6 \pm 3.5^{b}$ |
| 2014 | $37.1 \pm 5.3^{a}$ |

**Table 2**
Mean ratios (±1 SE of mean) of foliar N to foliar P (%:%) for (A) *Viola rotundifolia*; means with the same superscript are not significantly different among watersheds at P < 0.10; and (B) *Rubus allegheniensis*; means with the same superscript are not significantly different among watersheds and years at P < 0.10.

| Watershed | N/P ratio |
|---|---|
| | %:% |
| A. *Viola rotundifolia* | |
| WS3 | $20.9 \pm 0.8^{a}$ |
| WS7 | $18.3 \pm 1.0^{b}$ |
| WS4 | $15.9 \pm 0.6^{c}$ |
| B. *Rubus allegheniensis* | |
| **WS3** | |
| 2013 | $24.1 \pm 0.7^{a}$ |
| 2014 | $23.7 \pm 0.7^{a}$ |
| **WS7** | |
| 2013 | $22.7 \pm 1.1^{abc}$ |
| 2014 | $23.0 \pm 1.6^{ab}$ |
| **WS4** | |
| 2013 | $20.6 \pm 0.6^{bc}$ |
| 2014 | $21.1 \pm 0.5^{c}$ |

### 4.1. Nitrogen

Patterns of contrast between watersheds for foliar N are strongly suggestive of N-enhanced luxury uptake in both *Viola* and *Rubus* (Chapin, 1980). Interestingly, results for *Viola* were unrelated to growth response, as mean cover did not vary significantly between watersheds (Gilliam et al., 1994, 2006). In contrast, N-meditated increases in foliar N for *Rubus* were associated with significantly higher cover—by as much as 10-fold—on treatment WS3 (Gilliam et al., 2016). Luxury nutrient uptake is the uptake of a nutrient beyond the minimum requirement for immediate growth (Lipson et al., 1996), and is usually associated with increased availability. Although it is most commonly assessed via foliar analysis, other studies have examined other plants structures, such as stems and rhizomes (Lipson et al., 1996; Muller, 2014). Muller (2014) discussed nutrient uptake for forest herbs, pointing to the potential importance of enhanced N uptake, especially in early spring, as a mechanism for ecosystem N retention.

### 4.2. Calcium and magnesium

Calcium has a multifaceted role in biochemical function and cell structure in plants, from its requirements in several cellular metabolic processes (Kauss, 1987) to its use in Ca-pectate salts to bind plant cell walls (Jarvis, 1984). Furthermore, trees take up and bind a considerable amount of Ca from forest soils (Thomas, 1969; McLaughlin and Wimmer, 1999; Juice et al., 2006). Accordingly, factors that limit or decrease access of plants to soil Ca can negatively impact forest ecosystems. Nitrogen-mediated decreases in foliar of Ca in both *Viola* and *Rubus* are consistent with earlier observations suggesting that N-enhanced leaching of $NO_3^-$ has facilitated leaching of Ca (Peterjohn et al., 1996; Adams et al., 2006), and has decreased tree foliar and bolewood Ca (Gilliam et al., 1996; Jensen et al., 2014).

Although response of foliar Ca is in itself an important metric to assess effects of excess N on the biogeochemistry of forests, an additional index of relevance is that of molar ratios of Ca/Al in foliar tissue. In perhaps the most complete review on the topic, Cronan and Grigal (1995) discussed the use of ratios of Ca/Al as indicators of environmental stress in forest ecosystems, using samples that included soil solution, fine roots of woody species, and foliage.

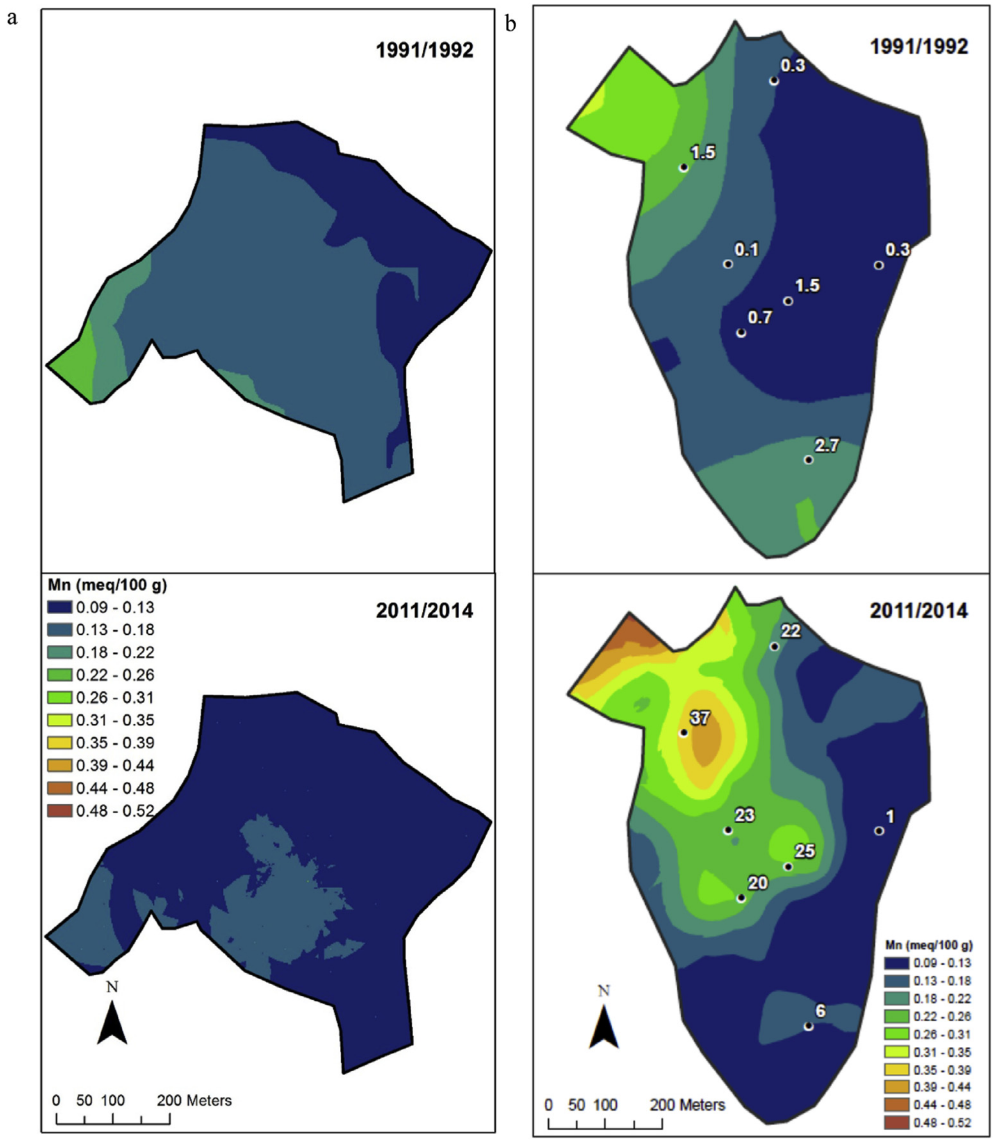


**Fig. 4.** Kriged extractable soil Mn from two sample periods (1991/1992 and 2011/2014) for (a) reference WS7 and (b) N-treated WS3. Soil sampling for the first period was in 1991, whereas sampling for the second period was in 2011 (see Methods). Shown also for WS3 (data not available for WS7) are spatially-explicit mean cover (%) values for *Rubus* in the seven permanent sample plots. Cover for the first period is the mean of 1991 and 1992 sampling, whereas cover for the second period is the mean of 2013 and 2014.

Cronan and Grigal (1995) concluded that molar ratios of foliar Ca/Al $\leq$12.5 represent of 50% risk of Al stress to affect adversely such processes as tree growth. It is, thus, notable that, although Ca/Al ratios for neither *Viola* nor *Rubus* were below this threshold, both exhibited significant decreases in the ratio in response to added N (Table 1). So, our forest stands are not likely experiencing Al stress, but data suggest that further increases in N deposition may lead to it.

Magnesium is biogeochemically similar to Ca (e.g., often being found in identical parent materials such as dolomite), and previous studies at FEF found similar results for Mg as they did for Ca regarding N-mediated leaching and tree foliar deficiencies (Peterjohn et al., 1996; Gilliam et al., 1996; Adams et al., 2006). Although this pattern was generally supported in *Viola* data, it is unclear neither why this was not seen in data for *Rubus* in general, nor, in particular, why there was a significant difference between reference watersheds in 2013 (Fig. 2).

### 4.3. Potassium

Although long-term stream chemistry at FEF suggest N-enhanced leaching of K (Adams et al., 2006), our data indicate increased plant uptake of K from experimental additions of N, as foliar K was significantly higher on WS3 versus WS4 and/or WS7 for both *Viola* and *Rubus* (Fig. 2). Uptake of K has been shown to alleviate membrane damage and chlorophyll degradation, thus mitigating abiotic stress in plants, such as drought, chilling, and high light intensity (Cakmak, 2005). In addition, we interpret our results to indicate the form of N being taken up by forest herbs at FEF, considering that uptake of K by plant roots can be inhibited by $NH_4^+$ (Haynes and Goh, 1978; Waring and Schlesinger, 1985; Mäser et al., 2002). Thus, we might expect that the N addition of $(NH_4)_2SO_4$ to WS3 would result in less K uptake. However, FEF soils have extremely high nitrification potential, with relative nitrification (i.e., percent net nitrification relative to net N mineralization) high on all watersheds, but far greater on WS3 (Gilliam et al., 2001; 2015). Indeed, soil $NO_3^-$ pools—another index of N availability—average >40% higher on WS3 than on the reference watersheds, suggesting that higher availability of $NO_3^-$ relative to $NH_4^+$ may allow greater $K^+$ uptake via less inhibition by $NH_4^+$ (Mäser et al., 2002) and that the luxury uptake of N observed on WS3 (Fig. 2) was in the form of $NO_3^-$ (Truax et al., 1994). As with Mg, it is not clear why there was a significant difference for *Rubus* foliar K between reference watersheds (Fig. 2).

### 4.4. Phosphorus

Several recent studies have shown that alleviation of N limitation often results in P limitation (Elser et al., 2007; Vitousek et al., 2010; Zhu et al., 2013). Gress et al. (2007) demonstrated the onset of excess N-driven P limitation on WS3 using several approaches, including analysis of root-associated activity of phosphomonoesterase (PME—an enzyme produced by plants under extreme P limitation—Duff et al., 1994) in *V. rotundifolia* (finding higher PME in plants on WS3), as well as root in-growth bags, wherein higher fine root biomass was found in bags treated with P on WS3. Thus, we expected N-related differences in foliar P, yet found no differences for either species. Because PME increases the supply of P by releasing organically-bound P, it may be that the increased "mining" of P was sufficient to meet plant demand, though at a greater cost associated with enzyme production (Gress et al., 2007).

A more appropriate index of P limitation is the foliar N/P ratio (Schreeg et al., 2014), with P limitation being positively correlated with N/P ratio (Garten, 1978; Koerselman and Meuleman, 1996; Güsewell, 2004). Higher N/P ratios on WS3 (Table 2) support observations of Gress et al. (2007) regarding N-mediated increases in P limitation. Indeed, Tessier and Raynal (2003) reported an N/P ratio threshold (i.e., ~15) for another species of *Viola* (*Viola macloskeyi* F. Lloyd) that is similar to those found for our reference watersheds. *Rubus* not only had generally higher N/P ratios than *Viola*, but also exhibited less distinct contrasts between treatment and reference watersheds (Table 2). Our values for *Rubus* exceed the range reported by Güsewell (2004) for sites worldwide (~9–18), and for many manipulation studies as indicative of P limitation (Tessier and Raynal, 2003). We conclude that, by the time of recent sampling of *Rubus* foliage, all watersheds had become limited, or at least co-limited (Koerselman and Meuleman, 1996), by P, consistent with chronically-elevated N deposition at FEF (Adams et al., 2006). Although *Rubus* foliar P varied significantly between reference watershed (Fig. 2), the relative amount of this difference was small (~10%).

### 4.5. Micronutrients/Al

In general, micronutrients and Al displayed far fewer responses to experimental additions of N than did macronutrients in both *Viola* and *Rubus*, with significant variation among treatment and reference watersheds found only for Mn and, for *Rubus*, only B and Al varying between years (Fig. 3). Furthermore, for *Viola*, N-related variation in foliar Mn was significant only for WS7. The N-mediated variation in foliar Mn in *Rubus* is notable, and merits further consideration, particularly because of (1) the range of foliar concentrations found in *Rubus* (~3000 to 6000 ppm versus ~700 to 1200 for *Viola*—Fig. 2), and (2) the profound growth response of *Rubus* to N treatments on WS3 (i.e., from 1 to 2% cover in 1991–1994 to ~20% by 2014) in contrast to WS4 (~1% to 4% over the same period) (Gilliam et al., 2016).

Plant micronutrients vary considerably among each other regarding biogeochemistry and physiological function in a given species, but all share the trait of being used by plants in extremely low concentrations (Kabata-Pendias, 2010). Many are classified as heavy metals (e.g. Pb, Ag, Cu, Zn, Cd, Mn) and, because of their need at such low levels, can shift in function from essential element to phytotoxin, even at moderate concentrations (Kowalenko, 2005; Nagajyoti et al., 2010). This seems especially apparent for Mn, an essential element for plants used in several metabolic processes, including photosynthesis and enzyme function (e.g., antioxidant-cofactor). However, at high enough concentrations, Mn toxicity leads to oxidative stress and reduction of photosynthesis and biomass (Lynch and St. Clair, 2004; Millaleo et al., 2010).

Although typical ranges of foliar concentrations of micronutrients are published (e.g., Nagajyoti et al., 2010), there is often considerable interspecific variability. Kula et al. (2012) reviewed Mn concentrations in various tissues of >20 plant species of a temperate European hardwood forest, including a species of *Rubus*. They found foliar Mn concentrations varying from a low of <500 ppm in sorrel (*Rumex acetosa* L.) to a high of >8000 ppm in blackberry (*Rubus fruticosus* L.). As the latter corroborates our observations for *Rubus* at FEF, we suggest that *Rubus* may act to accumulate Mn from soil when Mn mobility is enhanced, e.g., by N deposition.

### 4.6. Nutrient (Mn) redistribution hypothesis

Kriging maps reveal sharp contrasts between reference WS7 and N-treated WS3 (note: similar analysis was not performed on WS4) with respect to both the spatial heterogeneity and change over time in soil Mn. Soil Mn was relatively low in concentration and heterogeneity on WS7, varying minimally over the 20-yr period from 1991 to 2011 (Fig. 4a). By sharp contrast, these maps demonstrate increases in both concentration and spatial heterogeneity in extractable soil Mn during this same period on WS3 (Fig. 4b). The pattern for Mn on WS3 is also in sharp contrast to increased spatial homogeneity in both N and herb community dynamics in response to experimental N additions to the watershed (i.e., the N homogeneity hypothesis—Gilliam et al., 2016). Superimposing mean cover of *Rubus* in permanent sample plots of WS3 suggests that *Rubus* cover and the patchiness in soil Mn are spatially highly correlated. Based on this observation, we propose the following—*the nutrient redistribution hypothesis*—as a mechanism to explain this pattern.

Mobility of Mn is enhanced by increased acidity (Barber, 1995; Blake and Goulding, 2002), and nitrification in the absence of uptake of $NO_3^-$ by plants is an acidifying process (Barber, 1995; Marschner, 1995). Thus, it is likely that the N treatment to WS3, wherein net nitrification is ~100% of N mineralization (Gilliam et al., 2015), has enhanced mobility and availability of Mn. At the same time, *Rubus* at FEF responds interactively with both N and light (Walter et al., 2016), similar to results for other forest herbs (Elemans, 2004). Although the forest canopy on WS3 is decidedly closed, there is notable heterogeneity in light availability via canopy gaps. An unpublished study by G.G. Parker showed that mean gap fraction for the two plots with lower *Rubus* cover on the 2011/2014 map (i.e., 1 and 6%, Fig. 4b) was 7-times lower than the mean for the remaining plots wherein *Rubus* cover ranged from 20 to 37% (0.2% versus 1.5% gap fraction, respectively). The high Mn tolerance of *Rubus* allows foliar accumulation and subsequent release of Mn during decomposition (Keiluweit et al., 2015), redistributing extractable Mn from the depths of the rooting zone to the O horizon. Root systems of many species of *Rubus* have been shown to be particularly expansive, both laterally and with depth (Böhm, 1979).

Nutrient redistribution has long been observed for dominant tree species and macronutrients in both native forests and plantations (Thomas, 1969; Jobbágy and Jackson, 2004); more recently, it has been observed for Mn. Jobbágy and Jackson (2003) used afforestation of native temperate humid grassland in the Pampas of

Argentina with *Eucalyptus* plantations as an experimental system, wherein grasslands and adjacent plantations of up to 100 yr old had identical soil types, yet contrasting distributions of macro- and micronutrients. Their results showed that Mn availability in surface soils was enhanced three-fold via redistribution by *Eucalyptus* roots. Our findings suggest that this may be occurring via *Rubus* in response to experimental additions of N to an entire watershed.

## 5. Conclusions

Several studies, including past and on-going work at FEF, have demonstrated the multifaceted responses of forest ecosystems to excess N. Although this often has been shown biogeochemically via stream and soil water chemical responses (Peterjohn et al., 1996; Driscoll et al., 2003; Adams et al., 2006), increasingly numerous studies also show profound changes in forest herb layer communities (Gilliam, 2006; Bobbink et al., 2010; Clark et al., 2013; Verheyen et al., 2012; Dirnböck et al., 2014; Gilliam et al., 2016). Results from the present study indicate that foliar nutrient data from dominant herb layer species provide an additional—indeed, unique—perspective, providing insights that cannot be elucidated from solution chemistry alone, including the replacement of N-efficient *Viola* with nitrophilic *Rubus* and the redistribution of Mn by *Rubus*.

To our knowledge, this is the first study to suggest nutrient redistribution for a forest herbaceous layer species. Thus, the nutrient redistribution hypothesis predicts that herb layer species, such as *Rubus*, that respond positively and heterogeneously to increased N can alter the spatial distribution of other nutrients in surface soils. Because our particular case involves a micronutrient with its potential for phytotoxicity, our results have important implications for forest herb community structure and composition, given the highly species-specific nature of Mn tolerance (Kula et al., 2012). Not only is it clear that additions of N to WS3 have created a competitive advantage for *Rubus* over more N-efficient species (e.g., *Viola*), but the potential redistribution of Mn by *Rubus* may further act to create a positive feedback for dominance, considering that most other herb layer species exhibit a lower tolerance for Mn.

*Rubus* and other *R*-selected species (sensu Grime, 2006) typically respond sensitively to chronic additions of N and spatial variation in light (Elemans, 2004; Hedwall et al., 2011; Strengbom and Nordin, 2012; McDonnell et al., 2014; Neufeld and Young, 2014). Accordingly, predictions of the nutrient redistribution hypothesis can be tested for a variety of macro- and micronutrients using these species, especially given the wide species-specific variation in nutrient demand among forest herbs (Muller, 2014).

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