

# Nitrogen (N) Dynamics in the Mineral Soil of a Central Appalachian Hardwood Forest During a Quarter Century of Whole- Watershed N Additions

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## ABSTRACT

The structure and function of terrestrial ecosystems are maintained by processes that vary with temporal and spatial scale. This study examined temporal and spatial patterns of net nitrogen (N) mineralization and nitrification in mineral soil of three watersheds at the Fernow Experimental Forest, WV: 2 untreated watersheds and 1 watershed receiving aerial applications of N over a 25-year period. Soil was sampled to 5 cm from each of seven plots per watershed and placed in two polyethylene bags—one bag brought to the laboratory for extraction/analysis, and the other bag incubated in situ at a 5 cm depth monthly during growing seasons of 1993–1995, 2002, 2005, 2007–2014. Spatial patterns of net N mineralization and nitrification changed in all watersheds, but were especially evident in the treated watershed, with spatial variability changing non-monotonically, increas-

ing then decreasing markedly. These results support a prediction of the N homogeneity hypothesis that increasing N loads will increase spatial homogeneity in N processing. Temporal patterns for net N mineralization and nitrification were similar for all watersheds, with rates increasing about 25–30% from 1993 to 1995, decreasing by more than 50% by 2005, and then increasing significantly to 2014. The best predictor of these synchronous temporal patterns across all watersheds was number of degree days below 19°C, a value similar to published temperature maxima for net rates of N mineralization and nitrification for these soils. The lack of persistent, detectable differences in net nitrification between watersheds is surprising because fertilization has maintained higher stream-water nitrate concentrations than in the reference watersheds. Lack of differences in net nitrification among watersheds suggests that N-enhanced stream-water nitrate following N fertilization may be the result of a reduced biotic demand for nitrate following fertilization with ammonium sulfate.

**Key words:** forest ecosystems; net nitrification; net N mineralization; nitrogen saturation; nitrogen homogeneity hypothesis; N fertilization; forest soils; long-term spatial and temporal trends.

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**Authors' Contributions** FSG conceived of and designed study, performed research, analyzed data, and wrote the paper; CAW performed research, analyzed data, and wrote the paper; MBA conceived of and designed study, and wrote the paper; WTP performed research and wrote the paper.

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## INTRODUCTION

Essential processes that maintain the structure and function of ecosystems vary across scales of time and space. For terrestrial ecosystems, this can be especially pronounced for phenomena, such as nitrogen (N) mineralization, that are mediated by soil microbes which comprise a diverse and spatially and temporally dynamic assemblage that can influence plant community structure and composition (van der Heijden and others 2008). Spatial and temporal scales themselves often covary, such that spatial patterns within an ecosystem can change over time (Aubert and others 2005). With respect to the cycling of N, most undisturbed terrestrial ecosystems exhibit high spatial heterogeneity of N availability, with spatially discrete areas of high N availability referred to as “hot spots” (sensu McClain and others 2003). Similarly, temporal periods of high N mineralization and/or loss of N have been called “hot moments” (McClain and others 2003).

Although few studies have addressed spatial patterns of N dynamics at the whole-watershed scale, studies at much finer spatial scales (for example, 1–10 m<sup>2</sup>) have demonstrated that high spatial heterogeneity in available N can be maintained by “islands of fertility” (sensu Schlesinger and others 1996; see also Stoyan and others 2000; Gilliam and Dick 2010), with patches of high soil N availability driven by the typically clumped and patchy distribution of plant populations. Consequently, these patches provide inputs of organic matter through litterfall, turnover of fine roots, and species-specific variation in the N content of throughfall and stemflow (Crockford and others 1996). Van der Krift and Berendse (2001) demonstrated the strong influence that individual plant species can have on soil N dynamics, finding that nitrophilic species increased rates of net N mineralization and nitrification far more than plant species adapted to low-N habitats. Such a response has been reported for an N-treated watershed in a central Appalachian hardwood forest (see Study Site below), with the nitrophilic *Rubus allegheniensis* increasing in cover by greater than tenfold (Gilliam and others 2016).

Recognizing the importance of spatial patterns of N availability and their effect on forest herbaceous communities, a hypothesis—the *N homogeneity hypothesis*—has been proposed predicting that excess N deposition to forest ecosystems increases the spatial homogeneity of N by decreasing natural patchiness, doing so by essentially filling in the low-N matrix within which discrete high-N patches occur (Gilliam 2006, 2014). Accordingly, a tempo-

ral increase in atmospheric inputs of N should increase N availability within this matrix to approach that within the patches of high fertility. Subsequently, nitrophilic plant species of the forest herbaceous layer should increase in dominance, outcompeting the more numerous N-efficient species and decreasing biodiversity of the forest, up to 90% of which is represented by the herb layer (Gilliam 2007). This hypothesis has been the subject of recent reviews (Lu and others 2008; Fujimaki and others 2009; García-Palacios and others 2012), and its predictions have been generally supported in the literature (for example, Hülber and others 2008; Hedwall and others 2011; Gilliam and others 2016).

Superimposed on this plant-mediated scenario of temporal change in spatial patterns of N in forest ecosystems from enhanced N inputs is the soil microbial community (Ettema and Wardle 2002; Štursová and others 2016). Soil microbial communities often exhibit great spatial variability in forest ecosystems (Štursová and others 2016), and considerable recent work has demonstrated the temporal sensitivity of these communities to prominent facets of global change, especially climate warming (Frey and others 2008, 2013) and excess N (Frey and others 2004, 2014; Treseder 2008; Morrison and others 2016; van Diepen and others 2017). Most microbial biomass comprises fungal groups (Morrison and others 2016), and saprotrophic fungi are the primary decomposers of plant litter in temperate forests. Yet, excess N can inhibit the ability of fungi to decompose litter in forests experiencing high N via atmospheric deposition, including interference with the activity of lignolytic enzymes (Carreiro and others 2000; Frey and others 2014; van Diepen and others 2017).

In spite of the efficacy of the 1977 and 1990 amendments of the Clean Air Act of 1970 in mitigating emissions of N compounds into the atmosphere in the USA, deposition of N remains high in many regions of the conterminous USA (Vet and others 2014). Even in remote areas of the Northern Hemisphere, Holtgrieve and others (2011) demonstrated that the biogeochemical signature of elevated N is evident. For areas where chronic atmospheric deposition of N has led to N saturation (including our Study Site—see below)—when supply of available N exceeds plant and microbial demand for N (Peterjohn and others 1996; Aber and others 2003)—excess N still represents a chronic threat to ecosystem structure and function. This has been especially the case for forest ecosystems, which tend to display a high spatial coinci-

dence with high densities of human populations and associated N pollution (Gilliam 2016).

Despite decreases in N deposition in recent decades (Lajtha and Jones 2013), critical loads for N in the USA are widely exceeded for many ecosystems types, particularly forests (Pardo and others 2011), leading to a variety of negative effects, including loss of biodiversity (Clark and others 2013; Simkin and others 2016). It is likely that recovery of N-impacted sites might require extended periods of time (Strengbom and others 2001; Stevens 2016). Furthermore, recent evidence suggests that global climate change may contribute to further nutrient stress in forest ecosystems, particularly through exacerbating varying combinations of low/limiting availability of phosphorus and calcium, coupled with toxicity from N-mediated increases in aluminum and manganese (Lynch and St. Clair 2004). Therefore, understanding the biogeochemical responses of forest ecosystems to chronically elevated N remains essential.

The purpose of this paper is to document and examine long-term spatial and temporal patterns of net N mineralization and nitrification in mineral soil of a temperate hardwood forest subjected to whole-watershed experimental additions of N for a quarter century. More specifically regarding spatial patterns, we were interested in testing a prediction of the N homogeneity hypothesis, namely that spatial homogeneity of net N mineralization and nitrification would increase on the N-treated watershed relative to two reference watersheds. Predictions of the hypothesis for forest herb communities have been supported in a separate study (Gilliam and others 2016). Regarding temporal patterns, we were interested in determining which climate variables, particularly ambient temperatures and precipitation, best explain the similar long-term trends in net N mineralization and nitrification in mineral soil of all three experimental watersheds.

## METHODS

### Study Site

This study comprises several facets of long-term, ongoing research on the response of a temperate hardwood forest ecosystem to experimental additions of N being carried out at Fernow Experimental Forest (FEF), a 1902-ha area of the Allegheny Mountain section of the unglaciated Allegheny Plateau, and located in Tucker County, West Virginia (39°03′15″N, 79°49′15″W). Mean precipitation at FEF is about 1458 mm/y, occurring

evenly between the growing season and dormant seasons, and increasing with higher elevations; long-term mean annual temperature is approximately 9°C, with temperatures above 30°C uncommon (Gilliam and Adams 1996; Kochenderfer 2006). Length of the frost-free season is about 145 d. Wet deposition of N for this site has historically exceeded 10 kg/ha/y (and increasing with elevation; Gilliam and Adams 1996), which has led to several stands becoming N saturated (Peterjohn and others 1996). Streamflow data suggest that watersheds are still retaining both ambient and added N, though currently at a declining rate (Adams and others 2006).

Three watersheds were used in this study: WS3, WS4, and WS7. As the treatment watershed, WS3 has received aerial additions of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> since 1989. WS4 and WS7 serve as unfertilized reference watersheds of contrasting stand ages (Table 1). Watershed soils are primarily inceptisols of the Berks and Calvin series, both of which are loamy-skeletal, mixed, mesic Typic Dystrachrepts. Mineral soils on these watersheds are derived from sandstone and shale, are mostly coarse-textured sandy loams that are well drained, are about 1 m in depth (Adams and others 2006), and are generally acidic and with moderate organic matter content (Table 1).

Experimental additions of N initiated in 1989 (when WS3, WS4, and WS7 were 20, 20, and ~75 years old, respectively) are administered three times per year, by either helicopter or fixed-wing aircraft. March/November applications are approximately 7 kg/ha of N, whereas July applications are about 21 kg/ha N, for an annual rate of 35 kg N/ha/y, originally chosen as twice the ambient rates of N deposited as throughfall at this site (Adams and others 2006). Currently, WS3 and WS7 support even-aged, 45-year-old stands, whereas WS4 supports an even-aged stand more than 100 years old.

Stands on all watersheds are dominated by mixed hardwood species, including 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 and others 2006). In spite of stand age differences, the composition of the herb layer community was similar between watersheds at the initiation of the study (Gilliam and Turrill 1993; Gilliam and others 1994). Prominent species were *Viola* spp., *Rubus allegheniensis*, mixed ferns, and seedlings of *Acer pensylvanicum* L. and *A. rubrum* L. Currently, *R.*

**Table 1.** Characteristics of Study Watersheds of the Fernow Experimental Forest, WV

Variable	WS3	WS4	WS7
Area (ha)	34.3	38.7	24.0
Stand age in 2015 (years)	~ 45	> 100	~ 45
Stand history	Clearcut N additions	Select cut	Clearcut Herbicide
Aspect	S	S-SE	E
Elevation (m)	735-860	750-870	731-850
Mean annual precipitation (mm)	1473	1473	1473
Total wet N deposition, 1989-2014 (kg/ha)			
Ambient	260	260	260
Added	875	0	0
Total	1135	260	260
Tree basal area (m <sup>2</sup> /ha)	36.0	38.6	28.0
Soil variables			
pH	4.02 <sup>a</sup>	4.41 <sup>b</sup>	4.39 <sup>b</sup>
<i>pH (100 samples/WS in 2011)</i>	<i>4.12<sup>a</sup></i>		<i>4.52<sup>b</sup></i>
Organic matter (%)	11.6 <sup>a</sup>	12.3 <sup>a</sup>	12.1 <sup>a</sup>
Total N (%)	0.34 <sup>a</sup>	0.37 <sup>a</sup>	0.39 <sup>a</sup>
<i>Total N (%) (100 samples/WS in 2011)</i>	<i>0.34<sup>a</sup></i>		<i>0.37<sup>a</sup></i>
Total C (%)	5.8 <sup>a</sup>	5.7 <sup>a</sup>	5.8 <sup>a</sup>
<i>Total C (%) (100 samples/WS in 2011)</i>	<i>5.8<sup>a</sup></i>		<i>5.3<sup>b</sup></i>
C:N	16.9 <sup>a</sup>	15.6 <sup>a</sup>	14.8 <sup>a</sup>
<i>C:N (100 samples/WS in 2011)</i>	<i>17.6<sup>a</sup></i>		<i>14.6<sup>b</sup></i>
Cation exchangeable capacity (meq 100 g <sup>-1</sup> )	8.7 <sup>a</sup>	7.5 <sup>ab</sup>	7.1 <sup>b</sup>

Soil values are watershed means for a mineral soil depth of 0-5 cm as of 2015; means with the same superscript are not different at  $P < 0.05$ . Also shown (in italics) are means for WS3 and WS7 based on a 2011 sampling of 100 plots/watershed (see "Methods" section).

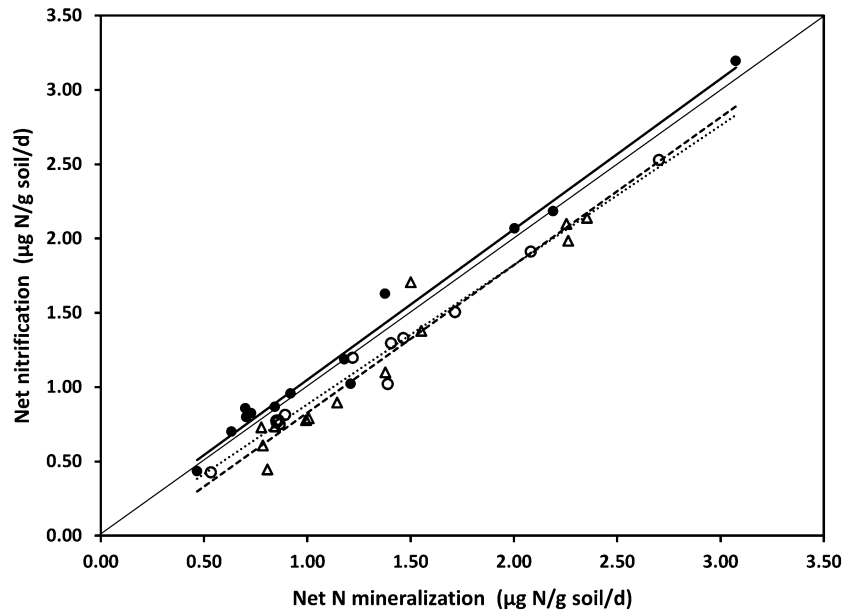
*allegheniensis* has increased significantly on all watersheds, but especially on N-treated WS3, representing nearly 50% of total herb layer cover on WS3 (Gilliam and others 2016). Using densiometer measurements, Walter and others (2017) found canopy closure was equally high on all watersheds. Thus, the response of *R. allegheniensis* is largely a fertilizer effect on a species that typically requires high light and high N availability (Jobidon 1993).

### Field Sampling and Analyses

Within each watershed, seven circular, 0.04-ha permanent sample plots were established to represent the full range of elevation and slope aspect. Mineral soil was collected by hand trowel at five random points within each plot to a depth of 5 cm, composited to a single sample, thoroughly mixed, and then placed in two polyethylene bags. One bag from each pair was returned to the laboratory for immediate extraction and analysis (see below), whereas the other bag was incubated in situ by burying it 5 cm beneath the mineral soil surface for about 30 d. For a given year, specific months of sampling were the same across watersheds, al-

though sample months varied somewhat among years of the study. In 1993, incubations were conducted from June to September. In 1994, sampling was carried out from May to September. Sampling for 1995 was done from April until October. Sampling was repeated in the years 2002, 2005, and then from 2007 to the present during the months May-October.

From 1993 to 1995, soil was extracted and analyzed as described in Gilliam and others (2001). Briefly, subsamples of soil from paired sample bags were extracted separately with both 1 N KCl and with deionized H<sub>2</sub>O (10:1 volume/weight). KCl extracts were analyzed for NH<sub>4</sub><sup>+</sup>, and water extracts were analyzed for NO<sub>3</sub><sup>-</sup> with an Orion 720A pH/ISE meter and NH<sub>4</sub><sup>+</sup>/NO<sub>3</sub><sup>-</sup> electrodes, respectively. Concentrations of NH<sub>4</sub><sup>+</sup> in all samples were well above the detection limit of 0.01 ppm NH<sub>4</sub><sup>+</sup>-N and well within the upper detection limit of 1400 ppm NH<sub>4</sub><sup>+</sup>-N. Concentrations of NO<sub>3</sub><sup>-</sup> in all samples were above the detection limit (0.1 ppm NO<sub>3</sub><sup>-</sup>-N) and well within the upper detection limit of 140 ppm NO<sub>3</sub><sup>-</sup>-N. Separate extractions for NO<sub>3</sub><sup>-</sup> were required to avoid interference of the NO<sub>3</sub><sup>-</sup> signal caused by high concentrations of Cl<sup>-</sup> in 1 N



**Figure 1.** Relative nitrification across study watersheds at Fernow Experimental Forest, West Virginia as annual net nitrification versus net N mineralization, 1993–2014. WS3: solid circles/solid regression line— $y = 1.01x + 0.04$ ,  $r^2 = 0.98$ ; WS4: open circles/dotted line— $y = 0.94x - 0.06$ ,  $r^2 = 0.98$ ; WS7: open triangles/dashed line— $y = 0.99x - 0.17$ ,  $r^2 = 0.95$ .

KCl extracts. Quality assurance procedures for both analyses included random repeat samples (consistently indicating minimal signal drift) and re-calibration for every hour of operation, for an accuracy of  $\pm 2\%$ .

Beginning in 2002, single extracts with 1 N KCl were prepared for simultaneous colorimetric determination of  $\text{NH}_4^+$  and  $\text{NO}_3^-$  with a Bran + Luebbe TrAACs 2000 automatic analysis system and with a Bran + Luebbe AutoAnalyzer 3 in 2007 and thereafter. Quality assurance/control samples (at 1 mg N/L) were routinely run for all methods after every 10–15 samples (accuracy of  $\pm <0.1\%$ ). Net mineralization was calculated as incubated (buried) soil  $\text{NH}_4^+$  plus  $\text{NO}_3^-$  minus initial soil  $\text{NH}_4^+$  plus  $\text{NO}_3^-$ . Net nitrification was calculated as incubated soil  $\text{NO}_3^-$  minus initial soil  $\text{NO}_3^-$ . Relative nitrification was calculated as net nitrification divided by net N mineralization (multiplied by 100).

Because analytical techniques between the 1990s and the 2000s and beyond changed from ion selection to colorimetric methodologies, we tested the possibility that a shift in methods may have significantly affected the observed spatial/temporal changes. Specifically, we tested for temporally consistent spatial patterns in the net nitrification rates among the study plots when measured using the two methods. We performed a Pearson pro-

duct-moment linear correlation (Zar 2009) between the monthly net nitrification rates on an individual sample plot basis in both reference watersheds, based on the mean across the period of 1993–1995 (ion selection period) versus the mean across the period 2002–2007. This correlation was highly significant ( $P < 0.0001$ ,  $r = 0.95$ ,  $N = 42$ ), indicating that the spatial patterns found via ion selection were not different from those found using colorimetry. Furthermore, ion selection revealed spatial/temporal patterns for net nitrification that were correlated with soil water and stream  $\text{NO}_3^-$  that were measured by other methods at that time of the study (1993–1995) (Peterjohn and others 1999; Adams and others 2006; Gilliam and Adams 2011; Gilliam 2014). Thus, these results, along with strict adherence to quality assurance/quality control protocols for all analyses, make it unlikely that a shift in methods significantly biased the initial temporal changes reported in this study.

To test a central prediction of the N homogeneity hypothesis, that is, that excess N increases spatial homogeneity of soil N dynamics, we have devised a homogeneity index (HI) which we calculated as follows for net N mineralization and nitrification for each sample year in each watershed:

$$\text{HI} = 1/(\text{CV}) \times 100$$

where CV is the coefficient of variation of annual means of net N mineralization and nitrification.

An extensive, one-time sampling was conducted to determine whether the lack of a detectable difference in the rates of soil nitrate production between the fertilized (WS3) and an unfertilized (WS7) watershed resulted from the relatively small number of sample locations per watershed ( $n = 7$ ). In late May and early June of 2011, samples from the upper 5 cm of mineral soil were collected from 100 locations per watershed, with locations being selected from a  $50 \times 50$  m grid in WS3 and a  $40 \times 40$  m grid in WS7. Once returned to the laboratory, all samples were sieved to pass a 5.6-mm mesh and then used to measure potential net nitrification rates (28-day laboratory incubations) along with several soil properties that are often associated with differences in net nitrification rates, including the soil C:N ratio determined using a Carlo Erba 1500 NCS elemental analyzer (Peterjohn and others 2015).

Each of the experimental watersheds used in this study is gauged with a V-notch weir. Water samples collected at a fixed location upstream from the weir have been analyzed weekly since 1970 for a variety of analytes, including dissolved nitrate concentrations (Edwards and Wood 2011).

## Data Analysis

This study design is an example of simple pseudoreplication, a common characteristic of watershed ecosystem studies, wherein each watershed represents an experimental condition with a sample size of one (Hurlbert 1984); thus, our data should be interpreted with that in mind. However, we feel that the effects we report, if any, are treatment effects, rather than preexisting differences among watersheds. There are close similarities among watersheds in several soil variables such as texture, organic matter, and total C and N (Table 1).

Spatial patterns of net N mineralization and nitrification in each watershed and for each sample year were characterized with kriging maps. Annual mean rates of net nitrification and net N mineralization were kriged separately in each watershed and each year using an ordinary kriging method with a spherical variogram model and global search radius in R package *gstat* (Pebesma 2004). Each model was fit using a common initial range and sill value and interpolated onto a grid with a cell resolution of  $5 \times 5$  m. Grids were mapped in ArcGIS using 20 equal intervals that spanned the range of kriged values for N mineralization and nitrification,

separately, from 1993 to 2014. Kriging was also used to characterize the spatial patterns present for the potential nitrification rates and soil C:N ratios in the 100 samples collected from WS3 and WS7 in 2011. The 2011 data were analyzed in ArcGIS Spatial Analyst by kriging at a fixed search radius of 150 m to the spatial extent of each watershed using an interpolation grid with a  $2 \times 2$  m cell size. Net nitrification classes were displayed by defining 10 equal intervals.

Climate variables potentially influencing temporal patterns of soil N transformations were assessed first with backwards stepwise linear regression, followed by graphical analysis with polynomial functions. The dependent variables were monthly mean rate of daily net N mineralization and nitrification for each month of sampling. For independent variables, we used two measures of monthly precipitation (current month's total precipitation and previous month's total precipitation) and three measures for monthly temperature: (mean monthly temperature, monthly degree days below  $19^\circ\text{C}$ , and monthly degree days above  $19^\circ\text{C}$ ). All precipitation and temperature data were obtained from the National Oceanic and Atmospheric Administration site for Tucker County, West Virginia (KEKN).

Study period means for relative nitrification and homogeneity indexes for net N mineralization and nitrification were compared across watersheds with analysis of variance and least significance tests (Zar 2009). One-way ANOVA and Mann–Whitney U tests were performed to compare the potential nitrification rates and C:N ratios between WS3 and WS7 using the 100 samples per watershed that were collected in 2011. Significant differences between watersheds were accepted at  $P < 0.05$ .

## RESULTS AND DISCUSSION

### Relative Nitrification

A useful metric to characterize the nature of soil N dynamics is that of expressing rates of net nitrification as a function of net N mineralization, often referred to as *relative nitrification* and expressed as a percent. A distinguishing feature of mineral soil of several watersheds throughout FEF has been markedly high relative nitrification, something that was reported for the initial period (1993–1995) of this study (Peterjohn and others 1996; Gilliam and others 2001) and for forest management plots located throughout other areas of FEF (Gilliam and others 2004). Data for the entire study period (1993–2014) demonstrate that this is a chronic

pattern across all study watersheds, independent of treatment, wherein slopes of linear regression of annual means of net nitrification versus net N mineralization closely approximate the 1:1 reference line (Figure 1). More specifically, calculated means ( $\pm 1$  SE) for the entire study period indicate that these values were  $105 \pm 2.7$ ,  $89 \pm 1.7$ , and  $85 \pm 3.7\%$  for WS3, WS4, and WS7, respectively, significantly higher ( $P < 0.05$ ) on WS3 than on WS4 and WS7, which were not different from each other. It is interesting to note that initial values reported for 1993–1995 by Gilliam and others (2001) were 105, 92, and 91, respectively, indicating that relative nitrification has changed little on these watersheds over time.

A positive linear relationship between net N mineralization and net nitrification is indicative of the predominance of populations of ammonia-oxidizing archaea and bacteria (for example, *Nitrososphaera* and *Nitrosomonas*, respectively), in addition to nitrifying bacteria (for example, *Nitrobacter*) (Banning and others 2015), and arises when  $\text{NH}_4^+$  generated by ammonifying microbes is rapidly converted to  $\text{NO}_3^-$  by these populations. On WS3, however, it is apparent that  $\text{NH}_4^+$  oxidation has, in part, also arisen from nitrification of applied substrates, i.e., aeri ally added  $(\text{NH}_4)_2\text{SO}_4$ , resulting in values of relative nitrification in excess of 100% (Figure 1).

The high values for relative nitrification that are common at the FEF, regardless of treatment, are actually quite rare among published data. A review of 56 studies by Lavoie and Bradley (2003) revealed that, of the 117 sites represented, only five sites had relative nitrification of 90% or more. Forty-three of these sites had relative nitrification rates of 10% or less; among these, 10 sites had rates of 0%. It should be noted further that relative nitrification on WS3 exceeded values from all of studies summarized in Lavoie and Bradley (2003). A controlled incubation study using mineral soil from WS4 at FEF, using temperatures from 4 to 35°C, found that increasing temperature beyond 20°C decreased relative nitrification more than threefold (from  $\sim 100\%$  at 20°C to  $\sim 40\%$  at 35°C), suggesting that about 20°C may be a threshold temperature above which nitrifier activity is inhibited in soils of FEF (Gilliam and others 2015).

## Spatial Patterns

Spatial patterns of net N mineralization and nitrification exhibited a notable degree of variation over time, with absolute rates and associated spatial variability changing through the study period from

1993 to 2014. Because of the high nitrification potential of watershed soils, independent of treatment (see discussion above), it is not surprising that spatial patterns—and their change over time—were almost identical between net N mineralization and net nitrification (Figure 2).

Trajectories of change in spatial pattern contrasted among watersheds in ways that suggests an N-treatment effect. Using coefficient of variation (CV) of annual mean rates as a metric for the degree of spatial variability, both reference watersheds displayed CV maxima at the extremes of the sample period, whereas N-treated WS3 had maximum CV in the middle of this period (data not shown). During the most recent decade (2005–2014), however, spatial variability in net nitrification has changed linearly on all watersheds (note: the patterns were similar for net N mineralization, but the linear relationships were not significant,  $P > 0.17$ ). For fertilized WS3, there has been a significant decrease in CV for net nitrification rate, whereas there have been significant increases in CV for reference WS4 and WS7 (Figure 3).

A central prediction of the N homogeneity hypothesis is that excess N increases spatial homogeneity of soil N dynamics. To test this prediction directly, we calculated a homogeneity index of net N mineralization and nitrification for each sample year in each watershed by taking the inverse of CV expressed as a decimal fraction (see “Methods”). Mean homogeneity indices for each of the watersheds, calculated across the entire study period, were significantly greater for WS3 relative to the reference watersheds for both net N mineralization and net nitrification, supporting this prediction of the hypothesis (Figure 4).

Among the possible mechanisms to explain these results is that added N can stimulate N processing when N availability is low (low-N conditions), but inhibit N processing when N availability is already high (high-N conditions). Evidence for the former is quite common and is essentially a fertilizer effect on soil microbial communities (for example, McNulty and Aber 1993; Rustad and others 1993; Aber and others 1993). Recent evidence for the latter is accumulating (Carreiro and others 2000; Frey and others 2004, 2014; Treseder 2008; Morrison and others 2016; van Diepen and others 2017). These observations suggest that, over time, chronically elevated inputs of N can cause a convergence of rates and patterns of soil N processing at the landscape scale, a central tenet of the N homogeneity hypothesis.

Because fertilization has maintained substantially higher stream-water nitrate concentrations

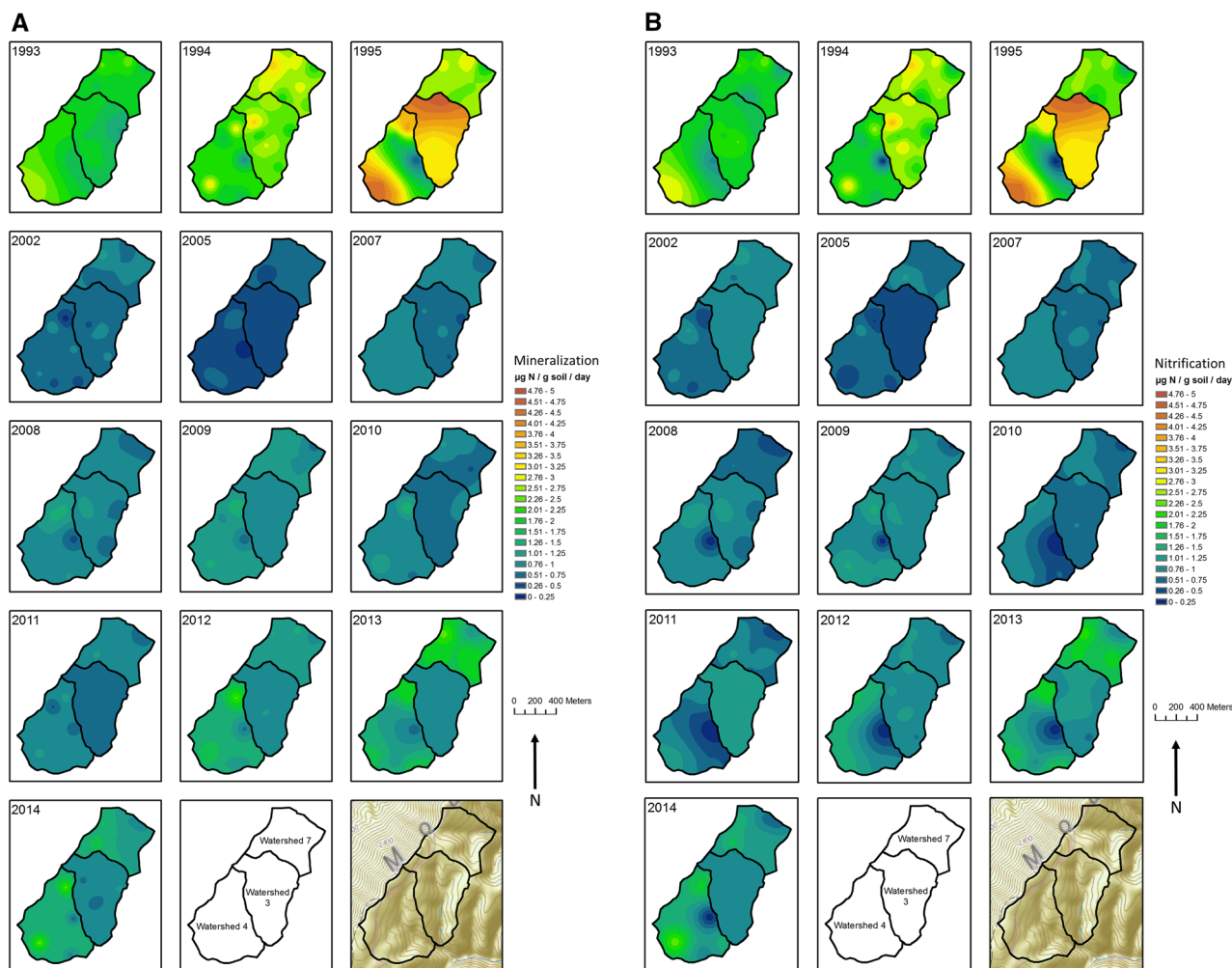


Figure 2. Spatial patterns of the growing season mean net N mineralization (A) and net nitrification (B) in mineral soil for study watersheds at Fernow Experimental Forest, West Virginia, 1993–2014. Shown also for each map set are watershed identification and contour maps.

than those in the unfertilized reference watersheds (Figure 5), a notable result was the lack of a detectable difference (except for 1995) in net nitrification rates between the fertilized (WS3) and unfertilized watersheds. Mean pool size for  $\text{NO}_3^-$  was higher on WS3 than on WS4 and WS7,  $7.5 \pm 0.9$  versus  $5.7 \pm 1.1$  and  $5.1 \pm 0.7 \mu\text{g N/g soil}$ , respectively, suggesting accumulation in soils of WS3 in excess of uptake, allowing for leaching into streams. Consistent with our long-term measurements, the extensive sampling (100, rather than 7, locations per watershed) in 2011 was unable to detect a significant difference in the net nitrification rates between the fertilized and an unfertilized watershed (WS7) (Figure 6;  $P = 0.366$ ), suggesting that greater stream-water nitrate concentrations following N fertilization may be caused by something other than enhanced rates of nitrate pro-

duction. Our extensive sampling did, however, detect lower soil C:N ratios in the fertilized watershed (Figure 7;  $P < 0.001$ ), and a noticeable inverse relationship between the spatial patterns of net nitrification rates and soil C:N ratios (Figure 7). Thus, the balance between C and N in soil organic matter appears to be linked with the differences in nitrate production rates found both within and between our study watersheds.

### Temporal Patterns

Temporal patterns of net N mineralization and nitrification have been published previously for 1993–1995 (Gilliam and others 2001) and 1993–2005 (Gilliam 2014). Herein, we add annual sampling from 2007 to 2014, representing eight additional sample years and a total treatment period of



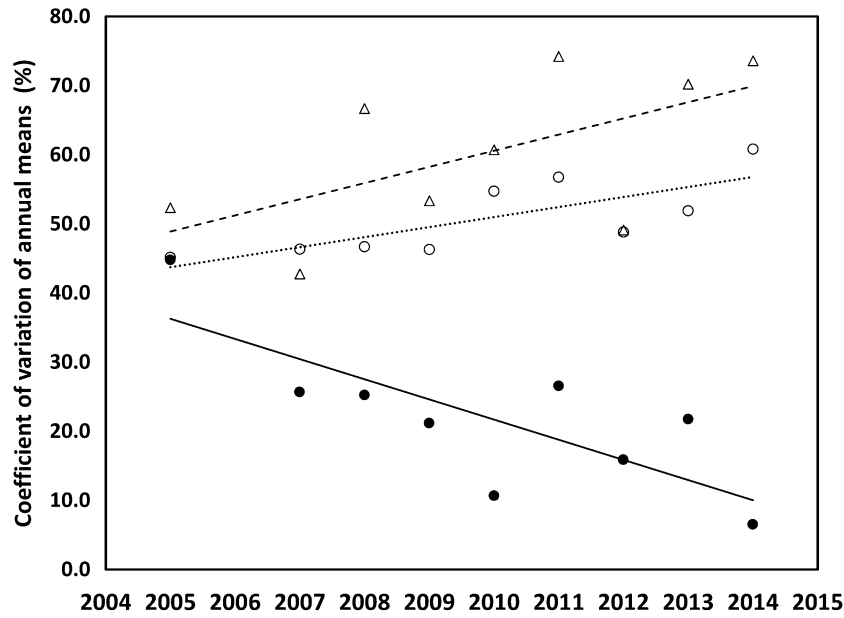


Figure 3. Change in coefficient of variation since 2004 of annual means of net nitrification for study watershed at Fernow Experimental Forest. WS3: closed circles/solid line,  $y = -2.92 + 5885x$ ,  $r^2 = 0.60$ ; WS4: open circles/dotted line,  $y = 1.45 - 2863x$ ,  $r^2 = 0.59$ ; WS7: open triangles/dashed line,  $y = 2.34 - 4640x$ ,  $r^2 = 0.36$ .

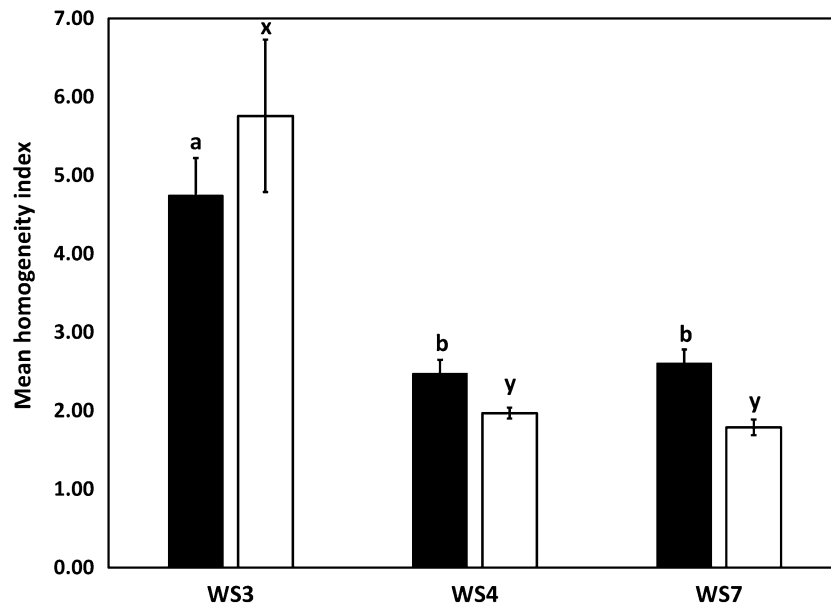


Figure 4. Mean spatial homogeneity index for net N mineralization (solid bars) and net nitrification (open bars) across study watersheds at Fernow Experimental Forest, West Virginia. Shown are means by watershed  $\pm$  1 SE of the mean. Means with the same superscript are not significantly different at  $P < 0.05$ .

25 years. Annual net N mineralization and nitrification increased about 25–30% on all three watersheds during the period 1993–1995, with a significant ( $P < 0.05$ ) N-treatment effect for net nitrification occurring in 1995 (Figure 8A, B). This effect, however, was transient in nature, as the rates of both net N mineralization and nitrification

decreased by more than 50% by 2005 across all watersheds, and no significant treatment effect has been found since 1995. The dramatic decline in net N mineralization and nitrification rates at our sites is consistent with temporal declines in tree-ring  $^{15}\text{N}$  that suggest a widespread reduction in N availability has occurred for many forested sites in the

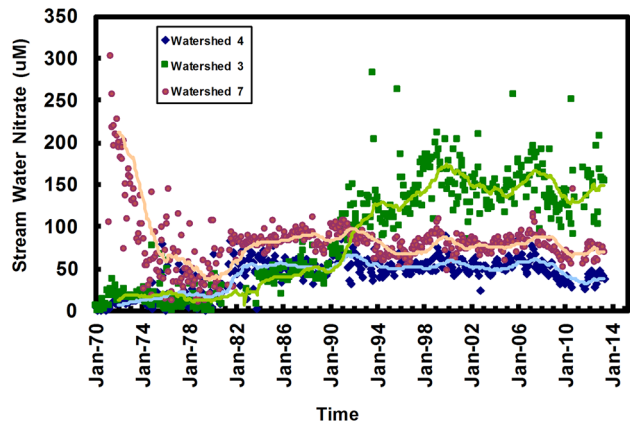


Figure 5. Long-term (40 years) volume-weighted monthly stream-water nitrate concentrations for each of the study watersheds. Ammonium sulfate additions to WS3 (35 kg N/ha/yr) began in 1989. Fitted lines are 24-month running means. Initially high values for WS7 were the result of a clearcutting and herbicide treatment that ended in October of 1969. Based on data from Edwards and Wood (2011) with updates: <https://doi.org/10.2737/RDS-2011-0017>.

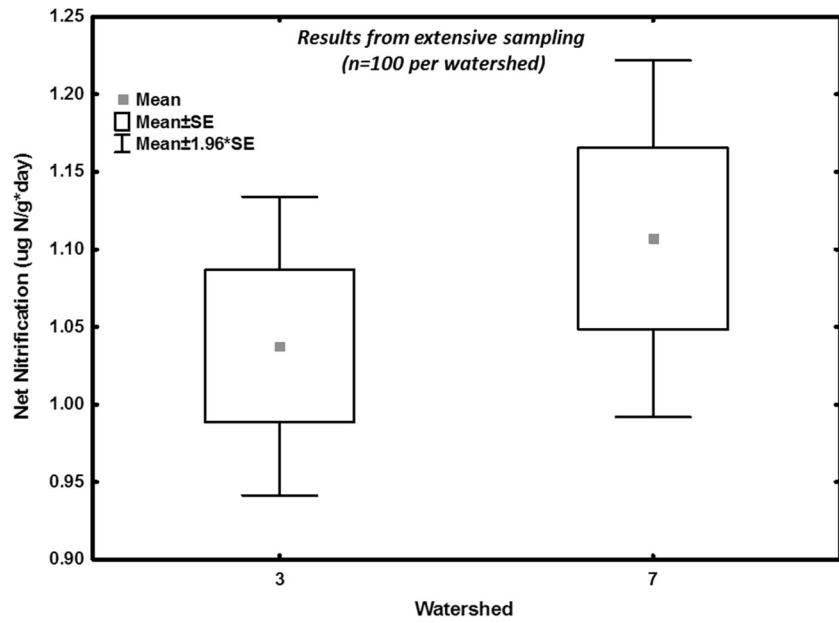
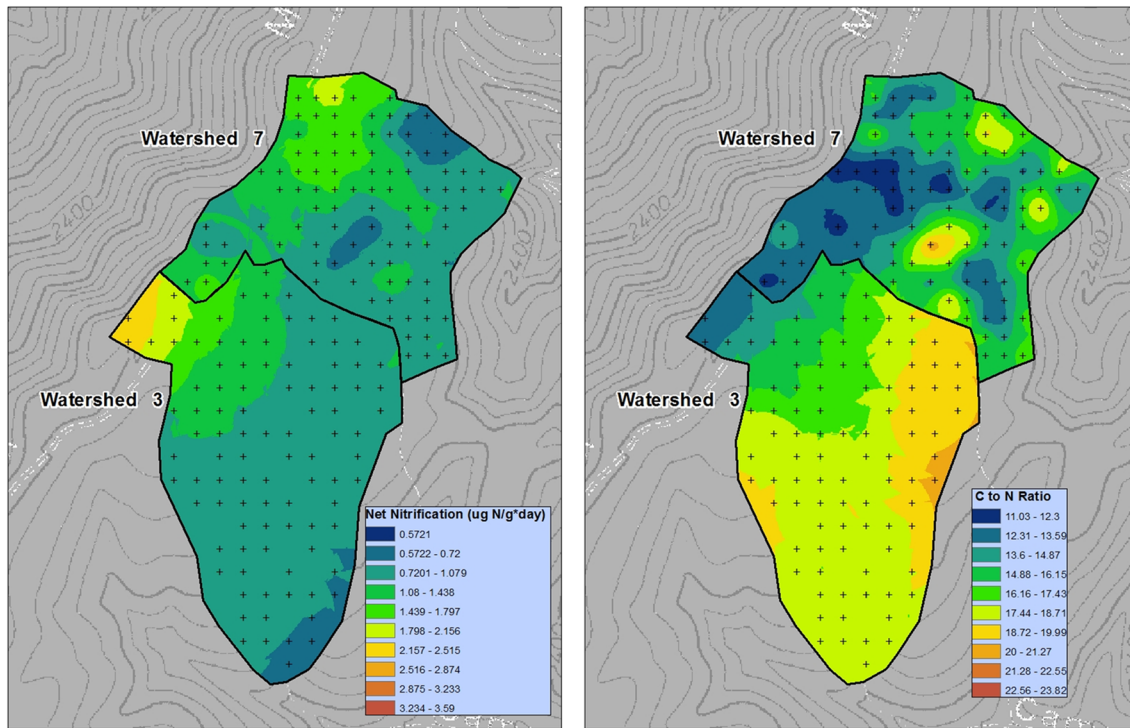


Figure 6. Mean net nitrification rates from an extensive (100 locations per watershed), one-time sampling of the fertilized watershed (WS3) and an unfertilized reference watershed (WS7) during the summer of 2011.

USA (Elmore and others 2016; McLauchlan and others 2017). However, the increased rates of N availability at all our study sites from 2005 to the present ( $P < 0.05$ ; Figure 8A, B) suggest that the temporal patterns observed at other locations—if they are responding for the same reasons as those at our sites—may include significant, shorter-term, multi-year oscillations in N availability.

We used backwards stepwise regression with precipitation and temperature metrics (see “Methods”) as independent variables to determine potential climatic factors that may influence tem-

poral patterns of net N mineralization and nitrification. To identify further which of the independent variables used in the multiple linear regression were more significantly correlated with net rates, this technique eliminates variables from the proposed model sequentially until all the variables remaining in the model produce  $F$  statistics significant at a given probability level ( $P < 0.05$ ) (Zar 2009). For net N mineralization, the regression was significant for all three watersheds; for net nitrification, it was significant for WS3 and WS7, but not WS4 (Table 2). Furthermore, backwards



**Figure 7.** Spatial patterns of net nitrification rates and mineral soil C:N ratios from an extensive (100 locations per watershed), one-time sampling of the fertilized watershed (WS3) and an unfertilized reference watershed (WS7) during the summer of 2011. Plus signs demark sampling locations. For scale, note that distances between adjacent points are 50 m in WS3 and 40 m in WS7.

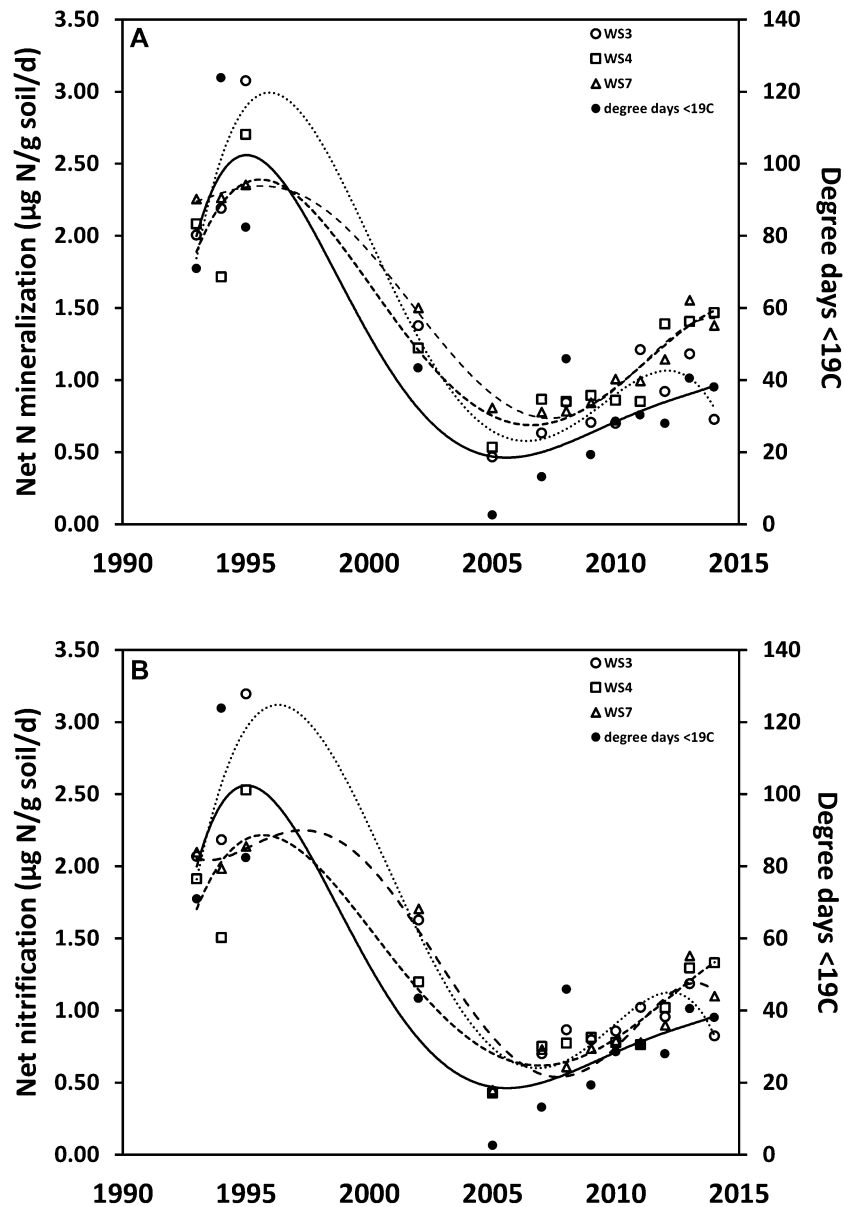
stepwise analysis revealed that, for all significant regressions, only degree days below 19°C contributed significantly to the model. As with net N mineralization and nitrification, degree days below 19°C decreased dramatically from 1995 to 2005 and then increased significantly ( $P < 0.05$ , data not shown) from 2005 to the present (Figure 8A, B).

Although it may appear counterintuitive that degree days below 19°C (that is, rather than degree days  $> 19^\circ\text{C}$ ) would be positively related to net rates N processing, it is important to consider that microbially mediated processes typically exhibit non-monotonic responses to temperature, such that there is an optimum temperature ( $T_{\text{opt}}$ ) that yields maximum rates (Stark 1996; Schipper and others 2014). Previous work with FEF soils from WS4 has shown values for  $T_{\text{opt}}$  of approximately 20°C (Gilliam and others 2015), consistent with results shown in Table 2. Nitrification is carried out solely by specialized archaea and bacteria, two microbial groups that often exhibit narrow tolerance ranges to temperature (Banning and others 2015), typically more narrow than soil fungi (Pietikäinen and others 2005). Thus, it is also notable that relative nitrification, typically very

high in FEF soils (Figure 1), was also shown to be significantly diminished by temperatures above 20°C. That is, in contrast to values of 85–105% reported here under ambient temperatures, relative nitrification declined in laboratory incubations from 120% at 15°C to 40% at 35°C (Gilliam and others 2015).

Because degree days below 19°C was the only variable that was significantly related to long-term annual means of net N mineralization and nitrification, we superimposed degree days below 19°C for each year of soil sampling and, to characterize temporal trends for all variables, used fifth-order polynomials to fit curves to annual data for the entire study period (Figure 8A, B). We are aware that there is no ecological relevance to relating two variables with polynomials greater than second order (that is, a quadratic fit) (Müller and others 2010); a fifth-order model was used simply to produce a representative visual trend over time with a good fit to the data (Figure 8A, B).

The significant relationship between degree days below 19°C and net N mineralization and nitrification underscores the importance of changes in ambient temperature in influencing soil microbial



**Figure 8.** Mean annual growing season net N mineralization (**A**) and net nitrification (**B**) in mineral soil for study watersheds at Fernow Experimental Forest, West Virginia, 1993–2014. Shown also are annual degree days < 19°C. Fitted curves are fifth-order polynomials for all variables to visually characterize temporal trends. Degree days: closed circles/solid curve,  $r^2 = 0.82$ ; WS3: open circles/fine dashes,  $r^2 = 0.94$  and  $0.98$  for net N mineralization and net nitrification, respectively; WS4: open squares/intermediate dashes,  $r^2 = 0.89$  and  $0.89$ ; WS7: open triangles/wide dashes,  $r^2 = 0.96$  and  $0.95$ .

processes, something reported earlier in this study (Gilliam and others 2001), as well as widely in the literature (Stark 1996; Rustad and others 2001; Frey and others 2008, 2013). Although assumptions regarding microbial C and N dynamics are difficult to test (Schimel 1988), the biochemical conditions that lead to decreases in decomposition rates are often correlated with increases in N immobilization (Micks and others 2004). What is

novel about our findings is evidence that microbial activity, especially during the growing season, may be more inhibited by temperatures above  $T_{opt}$ , such that a longer duration of temperature conditions below this (i.e., degree days < 19°C) allows for greater rates of N processing.

Also notable in the 25-year patterns of net N mineralization and nitrification at FEF is their degree of similarity among the three study water-

**Table 2.** Summary of backwards stepwise regression analyses of monthly means of net N mineralization and net nitrification for study watersheds at FEF

Multiple regression models:			
Net N mineralization = Ppt + Pre Ppt + Temp + <19°C + >19°C			
Net nitrification = Ppt + Pre Ppt + Temp + <19°C + >19°C			
Watershed	<i>t</i>	<i>P</i>	Final model
<i>A. Net N mineralization</i>			
WS3	2.49	0.016	Net N mineralization = 0.0044 * < 19°C
WS4	2.09	0.041	Net N mineralization = 0.0032 * < 19°C
WS7	2.49	0.016	Net N mineralization = 0.0047 * < 19°C
<i>B. Net nitrification</i>			
WS3	2.56	0.013	Net N nitrification = 0.0043 * < 19°C
WS4	–	–	Not significant
WS7	2.06	0.045	Net N nitrification = 0.0031 * < 19°C

*Independent variables are as follows: Ppt, total monthly precipitation; Pre Ppt, total precipitation for previous month; Temp, mean monthly temperature; < 19°C, degree days below 19°C; > 19°C, degree days above 19°C. See “Methods” section for further details.*

sheds, regardless of treatment and stand age (i.e., 45-year-old for WS3/WS7, > 100-year-old for WS4). As described in “Methods” section, these watersheds were similar with respect to several state factors, such as elevation, parent material, and soil type/texture, in addition to being similar in some biotic factors, such as soil organic matter, and total soil N and C (Table 1). However, they do differ for other biotic factors, such as dominant tree species (Kochenderfer 2006). Also, some factors (for example, soil C:N ratios and pH) have changed following fertilization. Thus, given the general lack of a N-treatment effect on WS3 (significant only for net nitrification in 1995—Figure 8B), it appears that these state factors exert a more overriding, or mitigating, influence on soil N processing than N amendments over long time periods, with temporal variation in temperature—at least when measured as degree days below 19°C—driving temporal change in rates of N mineralization/nitrification.

Previous microbial work with Fernow soils, especially on WS4, have shown that microbial biomass and community composition can vary sensitively with soil N status, particularly with respect to nitrifying bacteria (Gilliam and others 2011). In addition, soil microbes are influenced by, and indeed acclimate to, site conditions, especially those that affect temperature, such as latitude and elevation (Dalias and others 2002; Waldrop and others 2017). Gilliam and others (2015) compared N dynamics in soils from FEF (mean annual temperature ~ 9.0°C) to soils from a similar forest type

—but at lower latitude and elevation—in southwestern West Virginia (mean annual temperature > 13°C). Using laboratory incubations from 4 to 35°C, they found  $T_{opt}$  for net nitrification at FEF was about 20°C, whereas there was no  $T_{opt}$  for the southwestern WV site, i.e., net nitrification continued to increase exponentially up to 35°C. We suggest that this may explain, in large part, the apparent influence of degree days below 19°C on net N mineralization and nitrification found in our study (Table 2; Figure 8A, B). This may also provide support for the idea (though not the proposed mechanism) that an earlier spring (assuming it means fewer degree days < 19°C) is associated with lower N availability in this region (Elmore and others 2016).

## CONCLUSIONS

Findings of this study demonstrate that soil N processing at this temperate hardwood forest site is both spatially and temporally dynamic, especially in the context of a quarter century of whole-watershed applications of N. Furthermore, although the effects of these N additions on the mean rates of net N mineralization and nitrification have been largely undetectable, their influence on spatial patterns of N dynamics is more long term in nature, and consistent with predictions of the N homogeneity hypothesis.

The surprising observation that greater stream-water nitrate concentrations in the fertilized

watershed are not attributable to elevated rates of nitrate production in the mineral soil suggests that other explanations should be considered. We hypothesize that the observed differences in stream-water nitrate concentrations may result of a reduced biotic demand for nitrate following fertilization with ammonium sulfate. This could occur if trees switch to a greater uptake of ammonium in the fertilized watershed (e.g., Burnham and others 2017), or if chronic additions of ammonium sulfate reduce tree growth (as reported in May and others 2005; DeWalle and others 2006), and thus the overall uptake of all forms of nitrogen. A better understanding of this response to chronic N additions should help us interpret past and future changes in stream-water chemistry in regions that have experienced high levels of N deposition.

Finally, results of this work underscore the importance of long-term field studies. The original design of this study was not to test the N homogeneity hypothesis; rather, the hypothesis arose from observations based on repeated monitoring within the experimental design. Originally articulated as a set of predictions based on the initial phase of the study (1993–1995), nearly 20 years of further observations on spatially explicit plots, comprising a quarter century of experimental N additions, has allowed for an adequate test of these predictions.

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