

# Spatial Variability in Soil Microbial Communities in a Nitrogen-Saturated Hardwood Forest Watershed

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The long-term reference watershed (WS4), at Fernow Experimental Forest, West Virginia, displays symptoms of N saturation despite considerable spatial heterogeneity in soil properties, such as texture, N pools, and cycling rates. We identified a weathering gradient of three sites within WS4 by assessing differences in clay content across a common geologic substrate. Across these sites (LN, MN, and HN), NO<sub>3</sub> production rates vary significantly (low, medium, and high nitrification, respectively) and are negatively related to clay content. It is unknown whether microbial communities vary across these sites and contribute to variability in NO<sub>3</sub> production. This study characterized soil microbial communities along this gradient and assessed factors potentially important in explaining microbial composition. We sampled mineral soil from each of the sites, analyzing for moisture, pH, organic matter, extractable N, and microbial biomass and community composition via phospholipid fatty acid (PLFA) analysis. Analysis of variance and canonical correspondence analysis indicated that microbial community composition varied among sites, with a predominance of fungal markers (18:2n6 and 18:1n9c) at the most weathered LN site and Gram negative bacteria (18:1n7c) at the less weathered MN and HN sites. Accordingly, the fungi/bacteria ratio increased in the direction of LN plots in ordination space. Correlations between measured environmental parameters and PLFA data suggest that acidic conditions and low NO<sub>3</sub> abundance at the LN site have selected for fungal dominance, although other important factors known to exert an influence on soil microbial communities, such as differences in plant community and clay and organic matter content, may also be playing a role in determining the observed patterns.

**Abbreviations:** CCA, canonical correspondence analysis; FAME, fatty acid methyl ester; FEF, Fernow Experimental Forest; HN, high nitrification; LN, low nitrification; MN, medium nitrification; PLFA, phospholipid fatty acid.

It is becoming increasingly apparent that excess N in the environment can cause notable declines in the health of forest ecosystems. In areas of high atmospheric inputs of N, N availability can exceed biotic N demand, leading to N saturation, which has been shown to create soil and foliar nutrient imbalances and decrease plant species diversity in a variety of forest ecosystem types (Bobbink et al., 2010; Gilliam, 2006; Lu et al., 2010).

Several hardwood-dominated watersheds at Fernow Experimental Forest (FEF), West Virginia, have become N saturated from chronically elevated N deposition (Peterjohn et al., 1996; Gilliam et al., 2001b). This is particularly the case for the long-term reference watershed at FEF: WS4 (Gress et al., 2007). During the past 40 yr, streamflow from WS4 has exhibited nearly 10-fold increases in annual NO<sub>3</sub> concentrations—from ~0.1 mg NO<sub>3</sub> N L<sup>-1</sup> before 1970 to concentrations consistently ranging from 0.7 to 0.9 mg NO<sub>3</sub> N L<sup>-1</sup> in the past decade (Adams et al., 2006).

The WS4 watershed, however, also displays a considerable degree of spatial heterogeneity in soil N dynamics (Peterjohn et al., 1999; Gilliam et al., 2001b, 2005; Christ et al., 2002). Based on long-term in situ (buried-bag) net nitrification data in the mineral soil, we have identified a gradient of three sites within WS4 that vary in rates of NO<sub>3</sub> production: LN, MN, and HN. In a previous study at this site, field-based gradients of net nitrification were essentially identical to those found under controlled conditions in the laboratory (Gilliam et al., 2010) (Fig. 1a), suggesting that the variability observed

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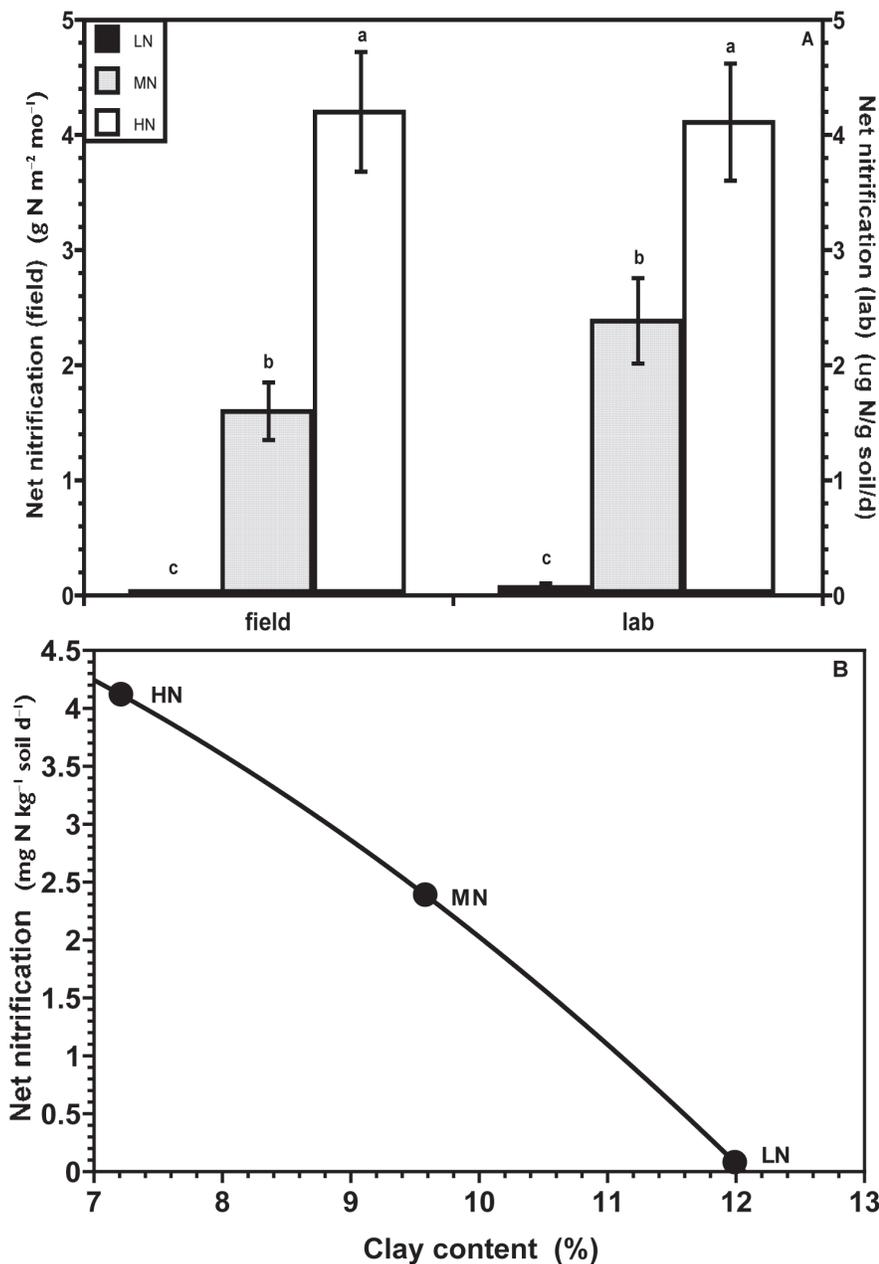


Fig. 1. (A) Net nitrification at three study sites in the long-term reference watershed (WS4), Fernow Experimental Forest, West Virginia, based on field and laboratory incubations for sites identified as low nitrification (LN), medium nitrification (MN), and high nitrification (HN) (field and laboratory data are presented as means  $\pm$  1 SE; means with the same superscript are not statistically different at  $\alpha = 0.05$ ); and (B) relationship between weathering (measured as clay content of soil) and net nitrification for each of the three sites (based on data from Gilliam et al., 2010).

in the field has arisen from variation in soil microbial communities and other soil properties rather than from transient ambient factors, e.g., moisture and temperature, that have otherwise been shown to be important in influencing net nitrification at this site (Gilliam et al., 2001b). Among numerous variables analyzed (e.g., soil and litter C/N ratios and soil fertility), the best predictor of spatial patterning of net nitrification in WS4 is clay content (Fig. 1b). Given that the geologic parent material and clay mineralogy of these three sites is similar (Adams et al., 2006), this pattern suggests that weathering rates may be the potentially ultimate, overriding control on soil N dynamics in this watershed (Gilliam et al., 2005, 2010). Microbial communities are also important controllers of NO<sub>3</sub> production and

consumption in soils (Booth et al., 2005), however, and little is known about the soil microbial communities of this site.

A considerable body of work has been undertaken at FEF focusing on N biogeochemistry, including stream and soil solution chemistry, soil N dynamics, and plant–soil interactions (Adams et al., 2006). By contrast, other than a study by Gilliam et al. (2001a) that used a qualitative method to assess the presence or absence of the *amoA* gene that encodes the active site of ammonia monooxygenase (which catalyzes the initial step in nitrification) and found that *amoA* genes were present in all sites, we are aware of no other studies that have focused on soil microbial communities and their potential contributions to N cycling at FEF.

One analytical microbial technique used to quantify viable microbial biomass and characterize the microbial community composition at the taxonomic level is PLFA analysis (Zelles, 1999; McCulley and Burke, 2004; Kaye et al., 2005; Rousk et al., 2010a,b). Phospholipid fatty acid analysis has been used to examine the soil microbial response to a wide variety of environmental influences, including land use (Kaye et al., 2005; Fraterrigo et al., 2006; Rousk et al., 2010a), plant community composition (Royer-Tardif et al., 2010), soil pH (Rousk et al., 2010b), and landscape heterogeneity (McCulley and Burke, 2004).

Here we present the results of a pilot study aimed at evaluating the relationships between soil parameters (e.g., pH, organic matter, and extractable N) and the microbial community across the weathering–clay–NO<sub>3</sub> availability gradient identified in WS4 at FEF. Because this was the first time microbial community analyses had been performed on soils of FEF, the main purpose of this study was to inform future work; however, this preliminary effort allowed us to evaluate the following basic questions: Do microbial composition and biomass vary along the weathering–clay–NO<sub>3</sub> availability gradient of WS4? What measured environmental factors best predict the observed variation in microbial community composition along the gradient?

## MATERIALS AND METHODS

### Study Site

This study was performed on the long-term reference watershed (WS4) at the FEF, which occupies ~1900 ha of the Allegheny Mountain

section of the unglaciated Allegheny Plateau in Tucker County, West Virginia, adjacent to the Monongahela National Forest (39°03' N, 79°49' W). Mean precipitation at FEF is approximately 1430 mm yr<sup>-1</sup>, varying seasonally and with elevation; in general, precipitation is greater during the growing season and at higher elevations (Adams et al., 2006).

Three sample sites were established in WS4, which supports >100-yr-old mixed-aged hardwood stands. The woody overstory is characterized by a high diversity of tree species, with dominant species including sugar maple (*Acer saccharum* Marshall), black cherry (*Prunus serotina* Ehrh.), and northern red oak (*Quercus rubra* L.). The herbaceous layer of these sites comprises species typical of montane eastern deciduous forests, including blackberry (*Rubus* spp.), violets (*Viola* spp.), stinging nettle [*Laportea canadensis* (L.) Wedd.], and several species of ferns.

As described in greater detail by Adams et al. (2006), a rock layer comprising fractured hard sandstone and shale underlies most watersheds of FEF, including all of WS4. Slopes commonly range from 20 to 50%. Soils are predominantly from the Calvin and Berks soil series (loamy-skeletal, mixed, active, mesic Typic Dystrudepts), originating from uniform parent substrates. Both series are acidic, being moderately deep and well drained, and formed in material weathered from interbedded shale, siltstone, and sandstone. The soil pH of WS4 generally varies between 3.50 and 4.00 (Adams et al., 2006).

## Field Sampling

We sampled the mineral soil from each of the three sites, which represent a gradient of weathering, clay content, and NO<sub>3</sub> status: HN (least weathered–lowest clay content–highest NO<sub>3</sub>), MN (intermediate weathering–clay–NO<sub>3</sub>), and LN (most weathered–highest clay–lowest NO<sub>3</sub>). This gradient appears to have resulted from variations in slope aspect (Table 1), which influences net radiation, a primary factor driving weathering (Tajchman and Lacey, 1986; Tajchman et al., 1988; Rech et al., 2001). Three soil samples were randomly selected (5-cm depth) from a 400-m<sup>2</sup> area at the center of each site, adjacent to the permanent plots on which long-term data are based (Gilliam et al., 2001b, 2005). Soil was removed via a hand trowel and placed in 500-mL, sterile, polyethylene Whirl-Pak bags (Nasco, Fort Atkinson, WI), which were stored on ice for transport back to the laboratory.

## Laboratory Analyses

Soil moisture was measured gravimetrically as the mass difference between moist and oven-dried samples. Soil pH was determined on 1:1 (w/v) slurries with deionized H<sub>2</sub>O (pH<sub>w</sub>) and 1 mol L<sup>-1</sup> KCl (pH<sub>s</sub>) using an Orion 3 Star pH meter with a Ross Ultra combination pH electrode (Thermo Fisher Scientific, Waltham, MA). Soil organic matter was measured from oven-dried soil using the loss-on-ignition method (mass lost during 5 h at 500°C) with a Fisher Isotemp muffle furnace (Thermo Fisher Scientific). Subsamples of field-moist soil were extracted with 1 mol L<sup>-1</sup> KCl at an extract/soil ratio of 10:1 (v/w). The extracts were analyzed colorimetrically for NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> with a SEAL AutoAnalyzer 3 continuous flow analyzer (SEAL Analytical, Mequon, WI).

We assessed the composition and biomass of the microbial community using PLFA analysis, a technique that allows quantification of the viable microbial biomass and taxonomic group composition at the time of sampling based on the total amount of phospholipids extracted and the chemical makeup of the constituent fatty acids (Zelles, 1999; Kaur et al., 2005). Our methodology followed that of Findlay and Dobbs (1993). Each field-moist soil sample was homogenized following thawing for 15 to 30 min. Approximately 5 g of moist soil was extracted in a single-phase, phosphate-buffered dichloromethane solution to remove PLFAs (Bligh and Dyer, 1959). These lipids were further separated by silicic acid chromatography, and phospholipids were derivitized in an alkaline solution to form fatty acid methyl esters (FAMES) (White et al., 1979). The FAMES were further purified with C18 reverse plasma chromatography and then separated and quantified by capillary gas chromatography with a flame ionization detector (Shimadzu 2014 GC, Shimadzu Corp., Japan) equipped with a Restek Rtx-1 column (Restek Corp., Bellefonte, PA). The FAME peaks were identified and concentrations calculated using a Supelco 37-component FAME mix (Sigma-Aldrich Co., St. Louis, MO) as a standard every three samples. The FAMES present in the sample but not part of the Supelco mix were identified based on retention times, the known standards, and comparison with soil FAME chromatographs generated from other independent labs using the Microbial Identification Inc. (MIDI) identification system (MIDI Inc., Newark, DE). The FAMES were described by standard nomenclature (IUPAC-IUB Commission on Biochemical Nomenclature, 1977) (*A:BnC*), where *A* is the total number of C atoms, *B* is the number of unsaturated bonds, and *C* is the number of C atoms between the aliphatic end of the molecule and the first unsaturated bond. The prefixes *i* and *a* indicate differential branching, *cy* indicates that a cyclopropyl group is present, and *10me* indicates a methyl group at the 10th C from the carboxyl end of the molecule. The microbial biomass was calculated as the total extractable PLFA (μmol kg<sup>-1</sup> soil), and PLFAs were classified into microbial taxonomic groups based on previously published PLFA biomarker data (Frostegård et al., 1993; Green and Scow, 2000; Vestal and White, 1989; Zelles, 1997). Fungal/bacterial ratios were calculated (on a μmol kg<sup>-1</sup> soil basis) as: (18:2n6 + 18:1n9)/(i14:0 + i15:0 + a15:0 + i16:0 + i17:0 + a17:0 + 16:1n9 +

**Table 1. Site characteristics of study areas within WS4, Fernow Experimental Forest, West Virginia.**

Characteristic	Nitrification level		
	Low	Medium	High
Elevation, m	808	838	833
Slope aspect, °	240	120	70
Clay, %	12.0 (1.1) a†	9.6 (0.1) b	7.2 (0.5) c
Organic matter, %	44.5 (13.8) a	22.8 (1.3) a	20.1 (2.4) a
Moisture, %	57.4 (7.1) a	42.3 (0.8) b	41.8 (2.0) b
pH (in water)	4.18 (0.20) b	5.01 (0.13) a	4.61 (0.10) ab
pH (in 1 mol L <sup>-1</sup> KCl)	3.10 (0.20) b	3.96 (0.12) a	3.89 (0.04) a
Extractable NH <sub>4</sub> , mg N kg <sup>-1</sup> soil	118.7 (29.4) a	89.2 (2.7) a	80.2 (14.2) a
Extractable NO <sub>3</sub> , mg N kg <sup>-1</sup> soil	0.0 (0.0) b	2.4 (0.4) b	21.1 (6.0) a
Fungal/bacterial ratio	24.2 (6.3) a	10.9 (0.4) b	12.1 (0.5) ab
Microbial biomass, μmol PLFA‡ kg <sup>-1</sup> soil	530 (128) a	386 (27) a	399 (47) a

† Means with 1 SE of the mean in parentheses; different letters denote significant differences (*P* < 0.05) between sites.

‡ Phospholipid fatty acids.

16:1n7 + cy17:0 + 18:1n7 + 18:1n5 + cy19:0 + 14:0 + 15:0 + 17:0 + 18:0 + 10Me16 + 10Me17 + 10Me18 + i17:1n7). We did not include 16:1n5 in this calculation because it is also sometimes reported as a marker for bacteria (Tunlid and White, 1992).

## Data Analysis

The means of all measured soil variables, microbial biomass, fungal/bacterial ratios, and the relative abundance of individual microbial markers (FAMEs) were compared between sites using ANOVA (Zar, 2009). Specific differences among means were determined via LSD tests. Site effects and differences among sites were accepted as significant at  $P < 0.05$ . Soil microbial community similarity among sites was assessed with the Bray–Curtis method (Barbour et al., 1999).

The soil microbial community composition and its potential response to environmental factors were assessed using canonical correspondence analysis (CCA). Canonical correspondence analysis is indirectly derived from principal component analysis, a commonly used analytical method in PLFA studies (McCulley and Burke, 2004; Djukic et al., 2010). Canonical correspondence analysis is a multivariate technique that assesses the similarity of samples with respect to species composition and also combines environmental gradients (e.g., soil moisture and organic matter) directly in the computer algorithms. It is thus a form of direct gradient analysis and identifies environmental factors as vectors that best explain the compositional patterns (Barbour et al., 1999). We also performed a nonmetric multidimensional scaling method on this data set and overlaid the environmental variables on the resulting ordination. The results were essentially identical to that of the CCA approach and are therefore not presented here.

## RESULTS AND DISCUSSION

As anticipated, there were notable differences among sites in the measured soil characteristics. In general, the LN site had more acidic soils with higher moisture and organic matter than either the MN or HN sites, which were not significantly different from each other in these characteristics. Also as expected, the HN soil had the highest level of extractable  $\text{NO}_3$ , whereas the LN soil had no detectable  $\text{NO}_3$  (Table 1).

There was considerable variation in soil microbial community composition among sites, particularly between the LN site and the MN and HN sites, the latter two of which were quite similar to each other. The microbial community for the LN soil had a Bray–Curtis similarity index of 86% with soil from both the MN and HN sites, whereas the microbial communities from the MN and HN soils had a similarity index with each other of 97%.

Some microbial taxonomic groups were relatively ubiquitous among the sites, having both a relatively high mole fraction percentage (high relative abundance) and exhibiting no significant difference among sites; these included the Gram positive i15:0 and the Gram negative cy19:0 and 16:1n7 (Fig. 2). Compared with the MN and

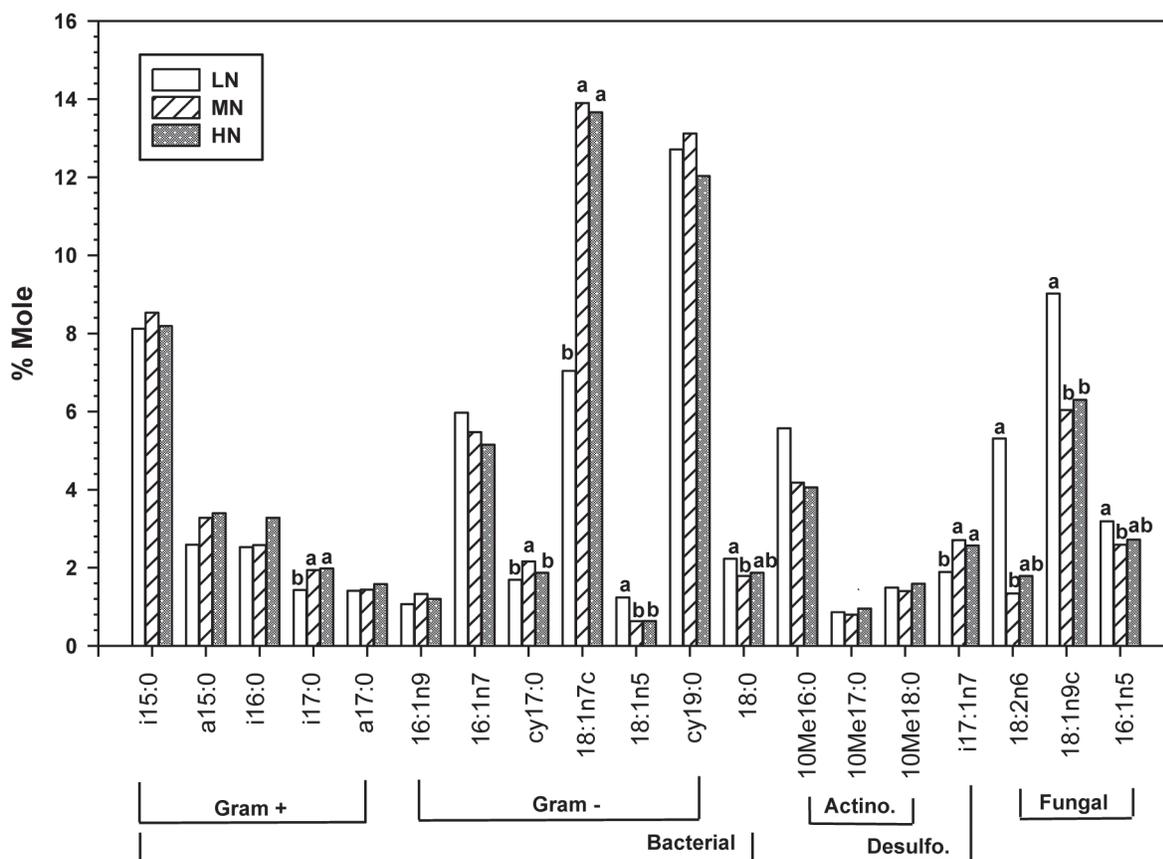


Fig. 2. Average mole fraction percentage (relative abundance) of 19 of the fatty acid methyl esters (FAMEs) that have been associated with microbial taxonomic groups in the literature (bacteria, actinomycetes, desulfovibrio, and fungal). Some FAMEs have been reported as markers for more than one taxonomic group (e.g., 10Me16:0 and 10Me18:0, while listed here as actinomycetes, have also been associated with Gram positive bacteria, and 16:1n5 is used as a marker for arbuscular mycorrhizal fungi and some bacteria). Different letters represent significant differences between sites (LN, low nitrification; MN, medium nitrification; and HN, high nitrification,  $P < 0.05$ ); no letter indicates that the site was not significant for that FAME.

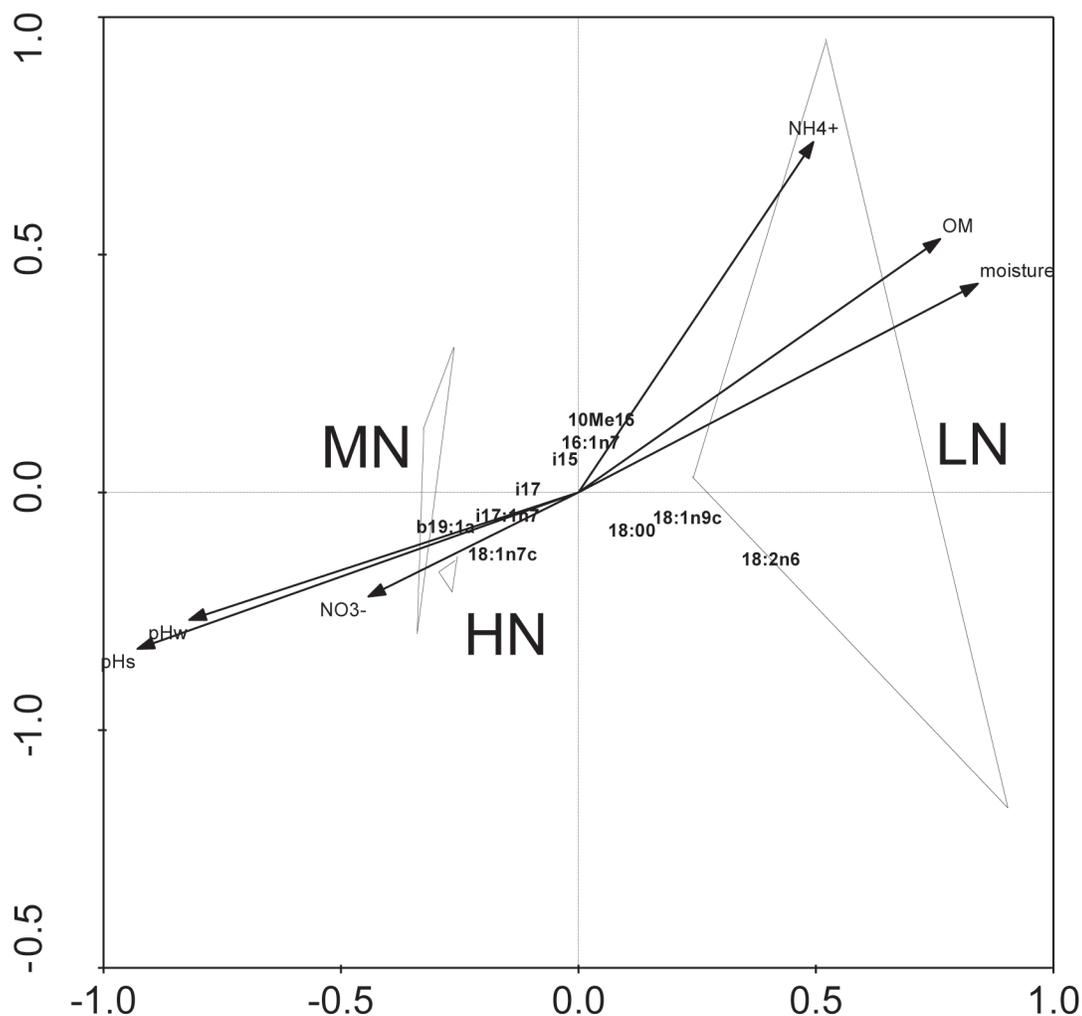


Fig. 3. Canonical correspondence analysis triplot with the 11 most abundant microbial fatty acid methyl esters (FAMES) (see Fig. 2 for identification of FAMES) and the following environmental factors: soil moisture, soil pH extracted by H<sub>2</sub>O and 1 mol L<sup>-1</sup> KCl (pH<sub>w</sub> and pH<sub>s</sub>, respectively), soil organic matter (OM), and extractable NO<sub>3</sub> and NH<sub>4</sub>. Triangles connect ordination space locations of plots for each site type: low (LN), medium (MN), and high nitrification (HN).

HN sites, soils from the highly weathered LN site had a greater predominance of fungal groups (18:2n6 and 18:1n9c; Fig. 2) and higher total microbial biomass, although because of high spatial variability at the LN site (as indicated by the standard error), site differences were not significant for total microbial biomass (Table 1). In contrast, Gram negative bacteria (e.g., 18:1n7c), which can include nitrifying species, tended to be more dominant in the MN and HN than LN soils (Fig. 2). These differences in microbial community were also reflected in the fungal/bacterial ratios (LN > MN = HN; Table 1).

Canonical correspondence analysis revealed patterns of contrast among sites similar to those found using ANOVA and depicted in Fig. 2 (i.e., a greater predominance of fungal groups in the LN soil and bacterial groups in the MN and HN soils). This statistical approach illustrates that the important measured environmental factors explaining these patterns were measures of soil pH, particularly pH<sub>s</sub>, soil moisture, and organic matter content (Fig. 3; Table 2). This result is consistent with work from other systems where pH and various environmental parameters have been shown to be important controlling variables of microbial community composition (De Boer and Kowalchuk, 2001; Bååth and Anderson, 2003; Fierer

and Jackson, 2006; Djukic et al., 2010; Rousk et al., 2010a,b). Soil pH has also been shown to influence watershed-level patterns of NO<sub>3</sub> production (Venterea et al., 2003), although this result is not universally supported (e.g., Ross et al., 2009).

Table 2. Summary statistics for canonical correspondence analysis.

Parameter or environmental variable	Axis 1	Axis 2	All axes
Eigenvalues	0.042	0.010	0.061
Species–environment correlations	0.994	0.990	
Cumulative variance, %			
Species data	68.6	85.3	
Species–environment relation	74.1	92.2	
<i>F</i> ratio	4.367		4.154
<i>P</i> value	0.066		0.036
Correlations of environmental variables			
pH (in 1 mol L <sup>-1</sup> KCl)	-0.92	-0.33	
pH (in water)	-0.81	-0.26	
NO <sub>3</sub> <sup>-</sup>	-0.44	-0.22	
Moisture	0.84	0.43	
Organic matter	0.76	0.53	
NH <sub>4</sub> <sup>+</sup>	0.49	0.73	

By comparing the areas of the site triangles depicted in ordination space in the CCA (i.e., the ordination space enclosed by connecting the three replicate points for each site; Fig. 3), the degree of spatial variability in the soil microbial composition can be assessed for a given site. This comparison illustrates that spatial heterogeneity in the composition of the soil microbial community within the LN site is notably greater than that of the MN site, with the HN site displaying the lowest spatial heterogeneity (Fig. 3). As mentioned above, the standard errors for microbial biomass (Table 1) also suggest that the spatial variability for this parameter is greater in the LN than in the HN or MN sites.

These trends in microbial spatial heterogeneity across the watershed are probably the result of various measured and unmeasured environmental parameters interacting with each other (e.g., variations in plant community, slope, irradiance, and soil temperature). Previous work in this forest has shown that sites with elevated soil N availability tend to have greater homogeneity within the herbaceous layer of the plant community (Gilliam, 2006). In addition, it has been previously reported that the weathered soils at the LN site support a predominance of low-growing ericaceous species in the herbaceous layer, which support ericoid fungal mycorrhizae (Gilliam et al., 2005). The FAME profiles quantified in the study may reflect such plant–microbial community linkages, with fungal markers being most abundant at the LN site and the least spatial heterogeneity of the microbial community at the HN site. Certainly, there is considerable evidence, from many ecosystem types, demonstrating linkages between plant communities and their soil microbial counterparts (Myers et al., 2001; Carney and Matson, 2005; Eisenhauer et al., 2010; Royer-Tardif et al., 2010; Hovatter et al., 2010). It is possible that one of the effects of N saturation or greater NO<sub>3</sub> abundance and cycling in these forests is a homogenization of both microbial and plant communities. Future work will examine this possibility more closely and further evaluate the role of additional environmental variables in producing these spatial patterns.

In general, our results agree with prior work examining how N availability influences forest soil microbial communities and vice versa. For example, DeForest et al. (2004) reported that NO<sub>3</sub> additions reduced the total PLFA biomass in hardwood forest stands in Michigan. We found lower microbial biomass in the sites with more N available (MN = HN < LN, although not statistically significant). Similar to what we observed across our N availability gradient, Waldrop et al. (2004) reported significant alterations in the soil microbial communities of northern hardwood forests in response to N additions, although the responses were forest type dependent. Our observed PLFA response of lower fungal abundance at the high N availability sites is similar to the response measured in the sugar maple–red oak forest of Waldrop et al. (2004) and is also supported by Fraterrigo et al. (2006), who found a negative relationship between fungal dominance and rates of N turnover in Appalachian forests with contrasting land-use history. It is clear that interactions between plant and microbial communities and soil and other environmental parameters ultimately determine the rates and fates of N

cycling in forested watersheds and are sensitive to N inputs via deposition (Bohlen et al., 2001). Additional work investigating the spatial and temporal patterns of these interacting factors is required to understand how these forested ecosystems will respond to future N deposition.

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