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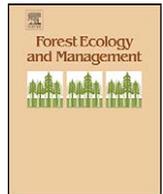
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## Soil microbial community response to nitrogen enrichment in two scrub oak forests

Jennifer Adams Krumins<sup>a,\*</sup>, John Dighton<sup>a,b</sup>, Dennis Gray<sup>b</sup>, Rima B. Franklin<sup>c</sup>, Peter J. Morin<sup>a</sup>, Michael S. Roberts<sup>d</sup>

<sup>a</sup> Department of Ecology, Evolution and Natural Resources, School of Environmental and Biological Sciences at Rutgers University, New Brunswick, NJ 08901, USA

<sup>b</sup> Pinelands Field Station, Rutgers University, New Lisbon, NJ 08064, USA

<sup>c</sup> Department of Biology, Virginia Commonwealth University, Richmond, VA 23284, USA

<sup>d</sup> Dynamac Corporation, Space Life Sciences Laboratory, Mail Code: Dyn-3 Kennedy Space Center, FL 32899, USA

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### ABSTRACT

Microbial communities play a pivotal role in soil nutrient cycling, which is affected by nitrogen loading on soil fungi and particularly mycorrhizal fungi. In this experiment, we evaluated the effects of allochthonous nitrogen addition on soil bacteria and fungi in two geographically distinct but structurally similar scrub oak forests, one in Florida (FL) and one in New Jersey (NJ). We applied allochthonous nitrogen as aqueous  $\text{NH}_4\text{NO}_3$  in three concentrations ( $0 \text{ kg ha}^{-1} \text{ yr}^{-1}$  (deionized water control),  $35 \text{ kg ha}^{-1} \text{ yr}^{-1}$  and  $70 \text{ kg ha}^{-1} \text{ yr}^{-1}$ ) via monthly treatments over the course of 1 yr. We applied treatments to replicated  $1 \text{ m}^2$  plots, each at the base of a reference scrub oak tree (*Quercus myrtifolia* in FL and *Q. ilicifolia* in NJ). We measured microbial community response by monitoring: bacterial and fungal biomass using substrate induced respiration, and several indicators of community composition, including colony and ectomycorrhizal morphotyping and molecular profiling using terminal restriction fragment length polymorphism (TRFLP). Bacterial colony type richness responded differently to nitrogen treatment in the different sites, but ectomycorrhizal morphotype richness was not affected by nitrogen or location. Both experimental sites were dominated by fungi, and FL consistently supported more bacterial and fungal biomass than NJ. Bacterial biomass responded to nitrogen addition, but only in FL. Fungal biomass did not respond significantly to nitrogen addition at either experimental site. The composition of the bacterial community differed between nitrogen treatments and experimental sites, while the composition of the fungal community did not. Our results imply that bacterial communities may be more sensitive than fungi to intense pulses of nitrogen in sandy soils.

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### 1. Introduction

Soil microbial processes play a critical role in shaping plant community structure and function (Bever et al., 1997; Simard et al., 1997; van der Heijden et al., 1998; Packer and Clay, 2000; Baxter and Dighton, 2001; Bever, 2003). For example, mycorrhizal fungi can help defend a plant against pathogens in experimental systems (Smith and Read, 1997), and there is often a direct relationship between mycorrhizal diversity and plant productivity (Baxter and Dighton, 2001) or plant diversity (van der Heijden et al., 1998). Energy transfer and metabolic activity in the soil food web hinges

on the obligate exchange of carbon and inorganic nutrients between producers, their microbial symbionts and consumers. Mycorrhizae helper bacteria (MHB) can promote the relationship between mycorrhizal fungi and the host plant by improving root receptivity to the fungus, facilitating fungal growth and improving rhizosphere soil conditions (Garbaye, 1994). This response is not universal, and differences in environmental conditions or species composition may reduce the benefits of the mutualism (Jumpponen and Egerton-Warburton, 2005).

Nitrogen loading associated with fertilizer use and atmospheric deposition can accelerate the decline of plant diversity and affect the soil organisms in the rhizosphere (Vitousek et al., 1997; Galloway and Cowling, 2002). This may have profound influences on nutrient cycling and influence the biotic and abiotic interactions of soil organisms and the environment. Arnolds (1991) first noted the relationship between nitrogen loading and declining soil diversity of ectomycorrhizal fungi (EMF) in Europe. Since that time, multiple field experiments using both natural nitrogen deposition

\* Corresponding author at: Department of Multitrophic Interactions, Netherlands Institute of Ecology, Boterhoekstraat 48, Heteren 6666GA, The Netherlands. Tel.: +31 026 4791111; fax: +31 026 4723227.

E-mail addresses: [jennkrumins@gmail.com](mailto:jennkrumins@gmail.com), [j.krumins@nioo.knaw.nl](mailto:j.krumins@nioo.knaw.nl) (J.A. Krumins).

gradients and fertilization manipulations have confirmed shifts in diversity and community composition of mycorrhizae with increasing nitrogen concentration. These studies have found a negative relationship between nitrogen concentration in the soil and diversity of EMF colonizing host trees (Taylor et al., 2000; Lilleskov et al., 2002; Dighton et al., 2004). Some even describe a shift in community composition and the identity of dominant EMF species with the decline in diversity (Lilleskov et al., 2002). Further, this idea has been extended (through molecular profiling) to show that decomposer fungi are sensitive to allochthonous nitrogen input as well (Allison et al., 2007).

The spatial distribution of microbial species and diversity is the subject of debate and comparison to macroorganism patterns (Martiny et al., 2006). Indeed, fungi (Green et al., 2004) and bacteria (Franklin et al., 2000; Franklin and Mills, 2003; Horner-Devine et al., 2003) demonstrate local and regional biogeographic patterns. However, very little is known about these factors or the relationship between geographic distribution and function in the environment. This is important because microbes mediate the bulk of biogeochemical processes, particularly nitrogen cycling. Environmental heterogeneity and regional distribution of microbial diversity may cause soil microbial communities to respond differently to nitrogen loading in different locations. For this reason, we carried out the following experiments in two structurally similar but distinct oak forests.

Fungi, particularly mycorrhizal fungi, may be more sensitive than bacteria to allochthonous nitrogen inputs due to their relatively higher C:N and obligate relation with host plants. Our work will simultaneously examine the effects of nitrogen loading on bacterial and fungal communities. Further, this work is novel because we evaluate bacterial and fungal response to nitrogen loading in oak forests characterized by oligotrophic, sandy soils as opposed to coniferous stands.

The objective of this study was to evaluate the simultaneous response of bacterial and fungal communities to allochthonous nitrogen loading in two structurally similar but geographically distinct scrub oak forests. The results of this work show that geographic context and environmental influences interact with the microbial community response to nitrogen loading. We manipulated

nutrients by adding  $\text{NH}_4\text{NO}_3$  in high and low concentration over the course of 1 yr to replicate experimental plots in Florida (FL) and New Jersey (NJ). We then measured the microbial community response using the following methods: substrate induced respiration (SIR) to determine total microbial biomass (bacterial and fungal), bacterial colony morphotyping, EMF morphotyping, and molecular analysis of bacterial and fungal communities using terminal restriction fragment length polymorphism (TRFLP). The molecular analysis and biomass measures captured both saprotrophic and mycorrhizal fungi; when discussing these results we use the word 'fungi' to refer to the entire fungal community. The EMF morphotyping only examined the ectomycorrhizal fungi colonizing root tips. Therefore, when discussing these results, we use the acronym EMF to differentiate a subset of the fungal community.

## 2. Methods

### 2.1. Site characteristics

Both experimental sites have dry, low-nutrient, sandy soils (see bulk densities in Table 1). Both sites are fire prone and contain structurally similar scrub oak communities. Prior to starting experiments, we surveyed plant community composition in all plots at each site. Composition was measured as percent cover of each plant within the each plot; those numbers were summed to create a relative rank of each plant across the entire site. The rank dominance of plants is presented in Table 1. The FL study site is in the NASA Kennedy Space Center/Merritt Island National Wildlife Refuge, an approximately 57,000 ha managed area comprised of brackish estuaries, marshes, scrub oaks, pine forests, and oak/palm hammocks on the Atlantic Coast of central Florida. The research plots are in scrub habitat, adjacent to a brackish marsh, dominated by *Quercus myrtifolia* with *Serenoa repens* (saw palmetto) in the under story. The NJ site is within the Rutgers University Pinelands Field Station that is part of the greater New Jersey Pinelands Preserve in south-central NJ. The Pinelands includes approximately 304,000 ha of land with heavily restricted development as part of the 445,000 ha NJ Pine Barrens ecosystem. The research plots in NJ are dominated by *Q. ilicifolia* with *Vaccinium angustifolia*

**Table 1**  
Comparison of biotic and abiotic characters from the New Jersey and Florida experimental sites.

	New Jersey Pinelands	Cape Canaveral Florida
Ranked dominance of vegetation across all plots at each site <sup>a</sup>	<i>Quercus ilicifolia</i> <i>Q. prinus</i> <i>Q. velutina</i> <i>Vaccinium angustifolium</i> <i>Carex striata</i> <i>Q. alba</i> <i>Pinus echinata</i> <i>Q. coccinea</i> <i>Q. stellata</i> <i>P. rigida</i> <i>Gaylussacia</i> sp.	<i>Quercus myrtifolia</i> <i>Q. incana</i> <i>Serenoa repens</i> <i>Q. chapmanii</i> <i>Rhynchospora megalocarpa</i> <i>Vaccinium myrsinites</i> <i>Ximenea americana</i> <i>Aristida stricta</i> <i>Tellansia</i> sp. <i>Galactia elliptica</i>
Average depth to O horizon (cm ± SE) <sup>b</sup>	3.67 ± 0.3255	2.47 ± 0.5259
Average total C:N of soil (±SE)	61.19 ± 5.28	76.38 ± 11.41
Average bulk density of soil (±SE)	0.888 ± 0.027	0.748 ± 0.035
Fungal/bacterial biomass ratio (±SE)	1.32 ± 0.015	1.37 ± 0.028
Rainfall June 2005–May 2006 <sup>c</sup>	94.43 cm	132.91 cm
NH <sub>4</sub> deposition June 2005–May 2006 <sup>c</sup>	4.1 mg l <sup>-1</sup>	1.71 mg l <sup>-1</sup>
NO <sub>3</sub> deposition June 2005–May 2006 <sup>c</sup>	19.18 mg l <sup>-1</sup>	8.77 mg l <sup>-1</sup>
Latitude and longitude	39.958 and -74.628	28.615 and -80.694
Average annual temperature <sup>d</sup>	12.3 °C	22.4 °C
Soil series <sup>e</sup>	Evesboro (mesic, coated lamellic quartzipsamments)	Pomello (sandy, siliceous, hyperthermic oxyaquinc alorthods)

<sup>a</sup> Percent cover was measured for each plant in each plot; these numbers were summed to create a ranked abundance across the site.

<sup>b</sup> Values significantly different by *t*-test ( $P < 0.05$ ).

<sup>c</sup> National Atmospheric Deposition Program (NADP), Champaign, IL.

<sup>d</sup> National Oceanic and Atmospheric Administration (NOAA).

<sup>e</sup> Web soil survey: <http://websoilsurvey.nrcs.usda.gov/app/>.

(low bush blueberry) in the under story. The two sites are similar in gross vegetation structure and soil types, though differ in species composition and experience very different seasonal and climatic influences (Table 1).

## 2.2. Experimental design and sampling

This experiment used a  $2 \times 3$  factorial design with two geographically distinct treatment sites (factor 1) and three different nitrogen addition treatments (factor 2). Over the course of 1 yr beginning in May 2005, we simulated different levels of nitrogen deposition by dispensing aqueous  $\text{NH}_4\text{NO}_3$  each month in doses of  $70 \text{ kg ha}^{-1} \text{ yr}^{-1}$ ,  $35 \text{ kg ha}^{-1} \text{ yr}^{-1}$  and  $0 \text{ kg ha}^{-1} \text{ yr}^{-1}$  (deionized water control). We chose these levels of nitrogen as they are comparable to or in excess of levels affecting Europe today (Arnolds, 1991). We replicated each treatment combination five times for a total of 15 plots at each treatment site. Each experimental plot measured  $1 \text{ m}^2$  and was at the base of a distinct, tree tag numbered scrub oak tree, *Quercus ilicifolia* in NJ and *Q. myrtifolia* in FL.

We randomly removed three 5 cm diameter soil cores from each plot after 12 months of nitrogen additions. We removed each of the three cores for: (1) EMF morphotyping (stored at  $4^\circ\text{C}$  prior to analysis), (2) bacterial colony morphotyping, biomass measurements using SIR (immediate analysis) and molecular profiling (soil stored at  $-20^\circ\text{C}$  prior to extraction) and (3) nutrient analysis (immediate analysis). Regarding the second and third core retained for bacterial, SIR and nutrient analyses, we retained the top 10 cm of soil from each core and homogenized the mineral and organic layers. We chose to do this rather than separate organic and mineral horizons because many of the FL plots had a negligible organic layer.

## 2.3. Soil nutrient analysis

We measured soil nutrients by collecting the litter, humus and mineral soil fractions from each soil core to a depth of 10 cm. We homogenized this material for further analysis. The moisture content was determined by drying soil at  $70^\circ\text{C}$ . We extracted samples from each core using 2.0 M KCl and analyzed for  $\text{NH}_4^+$  by ion selective electrode (ISE). We also extracted samples with deionized water (DI) and analyzed for  $\text{NO}_3^-$  and  $\text{PO}_4^{3-}$  using the Dionex DX90 ion chromatograph, (Dionex Corp, Sunnyvale CA). We performed all extractions on an approximate 4:1 extractant/dry weight material basis within 24 h of sample collection. We performed ISE analysis of  $\text{NH}_4^+$  and IC analysis of  $\text{PO}_4^{3-}$  and  $\text{NO}_3^-$  according to Standard Methods protocols (Clesceri et al., 1998). We analyzed oven-dried samples for total carbon by infrared  $\text{CO}_2$  detection and total nitrogen by  $\text{N}_2$  thermal conductivity detection following high temperature combustion using a Leco TruSpec carbon/nitrogen analyzer (Leco Corp., St. Joseph MI).

## 2.4. Microbial community characterization

We enumerated cultivable bacteria using standard plating techniques on 10% nutrient agar (Difco Labs, Detroit, MI). We characterized the colony morphotypes that grew after 48 h at room temperature ( $\sim 25^\circ\text{C}$ ) by their color, size, margin and elevation. These counts provided a proxy measure of bacterial diversity and composition, and they have been used successfully to capture relative differences in bacterial community structure (Garland et al., 2001; Muller et al., 2002; Krumins et al., 2006). We recognize that only a small fraction of the community is cultivable on solid media (e.g. in soils, Olsen and Bakken, 1987), but we can still make useful comparisons of the cultivable bacteria among treatments.

We removed a random and representative sample of root fragments from an intact core designated for EMF analysis and suspended it in water in a gridded petri dish. We characterized the EMF community through direct examination of root tips and ectomycorrhizal morphotyping following the methods of Agerer (1987–1999) using a Nikon SMZ dissecting microscope. We counted between 200 and 400 root tips from each core and quantified the relative abundance of each type.

We used a modified SIR method (Beare et al., 1991; Sparling, 1995) to separately quantify bacterial and fungal biomass in the soil. We lightly homogenized approximately 13 g of wet soil and placed it into 250 ml media jars. We then treated soil with either 5 ml of  $0.064 \text{ g ml}^{-1}$  (320 mg) aqueous cyclohexamide (Sigma–Aldrich, St. Louis, MO) to inhibit fungi and isolate the bacterial community, or 5 ml of  $0.013 \text{ g ml}^{-1}$  (65 mg) aqueous streptomycin (Sigma–Aldrich, St. Louis, MO) to inhibit bacteria and isolate the fungal (eukaryotic) community. We treated another set of soil in jars with DI water (positive control for full microbial activity) or cyclohexamide and streptomycin together (negative control assuming a near sterile jar). For simplicity, we present the results of treated jars and not controls. All treated and control jars were incubated with their antimicrobial compound (or deionized water) for 12 h at  $4^\circ\text{C}$ . After incubation, we combined an excess of dry glucose ( $>300 \text{ mg}$ , a preliminary dose response experiment determined the saturating mass of glucose) with the soil and attached the jars to an infra-red gas analyzer (Columbus Instruments, Columbus, OH) to measure  $\text{CO}_2$  evolution. Under the assumption that respiration and  $\text{CO}_2$  evolution correlate with microbial biomass, we calculated bacterial or fungal biomass using the regression equations of Beare et al. (1991) as  $\mu\text{g C fungal gdw}^{-1} \text{ soil}$  or  $\mu\text{g C bacterial gdw}^{-1} \text{ soil}$ . We used the percent moisture of a proximate soil core to calculate dry weight based on the known wet weight of soil added to the jar.

Following collection, samples for molecular analysis were stored at  $-20^\circ\text{C}$ . Later, we extracted whole community DNA from 0.25 g sub-samples using the Ultra Clean Soil DNA Isolation Kit according to their guidelines for maximum yield (MoBio Laboratories, Solana Beach, CA). We analyzed both fungal and bacterial communities for composition differences by amplifying extracted DNA using PCR followed by terminal restriction fragment length polymorphism (TPFLP) (Liu et al., 1997). Targeting the fungal community, we used a 6FAM (fluorescently labeled) forward primer, ITS1-F (CTTGGTCATTTAGAGGAAGTAA), and an unlabeled reverse primer, ITS4 (TCCTCCGCTATTGATATGC). These primers amplify the intergenic transcribed spacer region (ITS) of ribosomal DNA and have been used successfully to amplify ascomycete and basidiomycete fungi (Klamer et al., 2002; Allison et al., 2007). Therefore, we assume our molecular profiling captured mycorrhizal as well as saprotrophic fungi. Targeting the bacterial community, we used a 6FAM (fluorescently labeled) forward primer, SSU 27F (AGAGTTTGATCCTGGCTCAG), and an unlabeled reverse primer SSU 1492R (GGTACCTTGTTACGACTT). These primers amplify the small subunit 16 s of ribosomal DNA, and are used extensively to characterize bacterial community structure (e.g., Blum et al., 2004).

We carried out the bacterial community PCR in 50  $\mu\text{l}$  reactions that included:  $1 \times$  PCR buffer, 2.0 mM  $\text{MgCl}_2$ , 200  $\mu\text{M}$  dNTP (each), 1.0  $\mu\text{M}$  primer (forward and reverse), 0.4  $\mu\text{g } \mu\text{l}^{-1}$  BSA (bovine serum albumin) (Roche Diagnostics, Indianapolis, IN), and 1.25 U DNA polymerase per 50  $\mu\text{l}$  reaction. Unless stated, all PCR reagents were obtained from Applied Biosystems (Foster City, CA). We performed amplification reactions in an MJ Research PTC-200 Thermocycler (Waltham, MA) using the following reaction conditions: initial denaturation at  $94^\circ\text{C}$  for 5 min followed by 34 cycles of 0.5 min at  $94^\circ\text{C}$ , 1 min at  $62^\circ\text{C}$  and 2 min at  $72^\circ\text{C}$  and a final elongation for 3 min at  $72^\circ\text{C}$ . We carried out fungal

community PCR under identical reagent conditions, but within the 34 cycles, the reaction conditions included an annealing temperature of 50 °C for 2 min and elongation at 72 °C for 3 min. The final elongation was held for 5 rather than 3 min at 72 °C. We validated all PCR reactions on a 1.5% agarose gel.

We digested amplified fungal and bacterial DNA using the restriction enzyme *Hha1* (New England Biolabs, Beverly, MA). We desalted and purified the restriction fragments using the QIAquick Nucleotide Removal Kit (Qiagen, Hilden, Germany) then denatured the fragments at 95 °C for 10 min prior to electrophoretic analysis. We separated denatured restriction fragments using capillary electrophoresis with an ABI310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Capillary electrophoresis produces an array of multiple terminal fragments of varying length that are detected by their fluorescent marker. Each fragment theoretically represents a unique fungal or bacterial taxa or operational taxonomic unit (OTU). We used Applied Biosystems' GeneScan software to analyze the fragment patterns of each sample and produced a binary array of presence or absence of each OTU in each of our treatment combinations. We established a minimum response threshold of 50 relative fluorescence units for a fragment to be considered an OTU.

### 2.5. Data analysis

We used a two-way analysis of variance (ANOVA) to test for effects of nitrogen treatment and geographic location on: soil nutrients, bacterial colony and EMF morphotype richness, fungal and bacterial biomass (F/B), and the fungal/bacterial biomass ratio (SIR). When appropriate we separated means between nitrogen treatments with a Bonferroni test.

We were able to separate differences in microbial community structure for the following parameters: colony morphotypes of cultivable bacteria, EMF morphotypes and molecular profiles for bacteria and fungi using principle components analysis (PCA). We used a separate PCA for each parameter. The relative abundance of colony morphotypes and EMF morphotypes for bacteria and fungi respectively served as variables for the PCA that separated the communities based on visual morphotype. The presence or absence of OTU served as variables for the PCA that separated bacterial and fungal communities based on molecular profile. We followed all PCA with a multivariate analysis of variance (MANOVA) of the first three component scores to determine significant effects of nitrogen treatment or geographic location. All statistical analyses were conducted in SAS Version 9.1 (SAS Institute, Inc. Cary, NC).

## 3. Results

### 3.1. Soil nutrient response

Concentrations of extractable soil nutrients were not affected by additions of  $\text{NH}_4\text{NO}_3$  within either the NJ or the FL sites

(Table 2). However, across all plots concentrations of  $\text{NO}_3$  are significantly higher in NJ than FL ( $F_{1,29} = 10.19$ ,  $P < 0.01$ ), and concentrations of  $\text{PO}_4$  are significantly higher in FL than NJ ( $F_{1,29} = 24.37$ ,  $P < 0.0001$ ) (Table 2).  $\text{NH}_4$  concentrations were not affected by nitrogen treatment nor did they differ between sites. Therefore, FL soils have less available nitrogen (as  $\text{NO}_3$ ) than NJ soils, and NJ soils have less available phosphorus relative to FL soils. The higher  $\text{PO}_4$  concentrations in the FL soil may have resulted in part from abiotic effects like sea spray and geology, or alternatively, biotic effects arising from an inability of the microbial community to utilize the  $\text{PO}_4$  due to possible nitrogen limitation (*per* Liebig's Law) (Liebig, 1840) may be the proximal cause. Total soil carbon, total nitrogen and the ratio of the two (C:N, Table 1) were not significantly different between sample sites or across nitrogen treatments. However, these data were high and variable likely due to the low N content in the soil and patchy distribution of vegetation and litter (cores used for analysis also included the litter layer).

### 3.2. Microbial community characterization

We found a significant interaction between site and nitrogen treatment affecting bacterial colony morphotypes richness (Fig. 1A,  $F_{2,24} = 3.82$ ,  $P < 0.05$ ). FL supports significantly higher richness of bacterial morphotypes (Fig. 1A,  $F_{1,29} = 33.93$ ,  $P < 0.0001$ ), and there was no effect of nitrogen treatment on bacterial colony morphotypes. We found no significant interaction between site and nitrogen treatment affecting ectomycorrhizal morphotype richness (Fig. 1B), and there was no significant difference in EMF morphotype richness between FL and NJ or among the nitrogen treatments. These interactions refer to different effects of nitrogen depending on geographic site. Bacterial morphotype richness is lower in nitrogen treated plots than control plots in NJ, but it increases with nitrogen in FL (Fig. 1A). EMF morphotype richness was consistent across sites and nitrogen treatments (Fig. 1B).

We plotted sampling area (assuming each 5 cm diameter soil core removed is equivalent to 19.6 cm<sup>2</sup> of area sampled) versus richness of colony morphotypes (Fig. 2A) and EMF morphotypes (Fig. 2B) described. These results follow logically from Fig. 1. The number of bacterial and EMF morphotypes increased with increasing area sampled, and FL supports a higher richness of bacterial morphotypes than NJ. Our sampling effort may not have been adequate to completely characterize the bacterial and fungal communities in these sites. However, we can still make meaningful comparisons between the sites and treatments.

Both fungal (Fig. 3A,  $F_{1,29} = 8.79$ ,  $P < 0.01$ ) and bacterial (Fig. 3B,  $F_{1,29} = 18.97$ ,  $P < 0.001$ ) biomass determined by SIR were significantly greater in FL than NJ. Fungal biomass did not respond significantly to nitrogen at either site. In FL, there was a significant difference in bacterial biomass between the low and high nitrogen treatments but not the control (Fig. 3A,  $F_{1,29} = 4.63$ ,  $P < 0.05$ ). The fungal to bacterial biomass ratio did not significantly change with

**Table 2**  
Average soil nutrient concentration after 1 yr of nitrogen additions. Values indicate the mean  $\pm$  SE ( $n = 5$ ).

Site	Nitrogen treatment	$\text{NO}_3\text{-N}$ ( $\mu\text{g g}^{-1}$ soil)	$\text{NH}_4\text{-N}$ ( $\mu\text{g g}^{-1}$ soil)	$\text{PO}_4\text{-P}$ ( $\mu\text{g g}^{-1}$ soil)
Florida	0 kg ha <sup>-1</sup> yr <sup>-1</sup>	0.17 $\pm$ 0.02	3.34 $\pm$ 1.96	1.16 $\pm$ 0.47
	35 kg ha <sup>-1</sup> yr <sup>-1</sup>	0.15 $\pm$ 0.01	0.52 $\pm$ 0.07	1.45 $\pm$ 0.49
	70 kg ha <sup>-1</sup> yr <sup>-1</sup>	0.15 $\pm$ 0.03	1.66 $\pm$ 0.68	1.48 $\pm$ 0.13
New Jersey	0 kg ha <sup>-1</sup> yr <sup>-1</sup>	0.13 $\pm$ 0.03	3.00 $\pm$ 0.59	0.31 <sup>a</sup>
	35 kg ha <sup>-1</sup> yr <sup>-1</sup>	0.10 $\pm$ 0.01	1.95 $\pm$ 0.49	bdl
	70 kg ha <sup>-1</sup> yr <sup>-1</sup>	0.14 $\pm$ 0.02	2.17 $\pm$ 0.39	bdl

<sup>a</sup>Only one replicate was above detection limit.

bdl: below detection limit (for  $\text{PO}_4$  detection limit = 0.04 mg  $\text{PO}_4\text{-P l}^{-1}$ ).

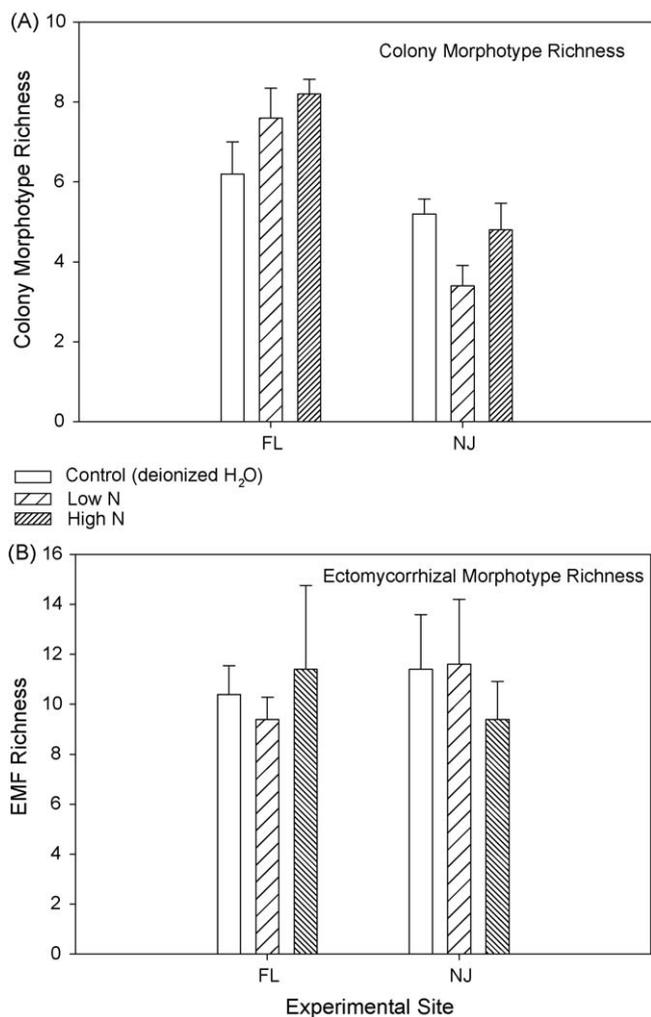


Fig. 1. Bacterial colony morphotype richness (A) and ectomycorrhizal morphotype richness (B). All bars represent mean ± SE and n = 5.

nitrogen addition and only showed a non-significant trend (Table 1,  $F_{1,29} = 2.96$ ,  $P = 0.098$ ) to be higher in FL than NJ.

Bacterial community composition was significantly different between the two experimental sites. This result was seen both in community characterization of colony morphotypes (Fig. 4A, Wilk's Lambda  $F_{1,29} = 8.24$ ,  $P < 0.001$ ) and molecular fingerprints using TRFLP (Fig. 4B, Wilk's Lambda  $F_{1,29} = 3.37$ ,  $P < 0.05$ ). We found significant effects of nitrogen treatments in the bacterial colony morphotypes in NJ only (Fig. 4A, Wilk's Lambda  $F_{2,29} = 2.35$ ,  $P < 0.05$ ), but not in the molecular profiles. Interestingly, fungal community composition was not different at either site or under nitrogen treatments. We found this result through both ectomycorrhizal morphotypes (Fig. 5A) and TRFLP (Fig. 5B).

#### 4. Discussion

##### 4.1. Soil nutrient response

The soil from FL (Schmalzer and Hinkle, 1996, Schortemeyer et al., 2000) and NJ (Tedrow, 1998) is highly porous, sandy, and known to leach soluble nutrients. Bulk densities of soils in this experiment support this conclusion (Table 1). Data from greenhouse experiments using native soil from the NJ site shows that additions of NH<sub>4</sub>NO<sub>3</sub> (in concentrations comparable to this study) did not result in a change in oak seedling (*Quercus rubra*) biomass

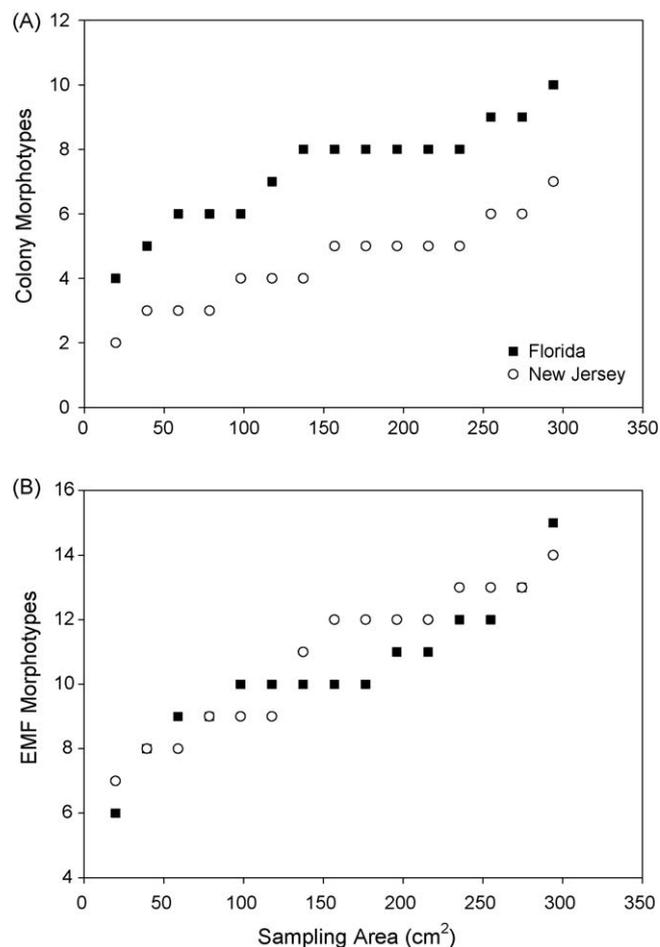
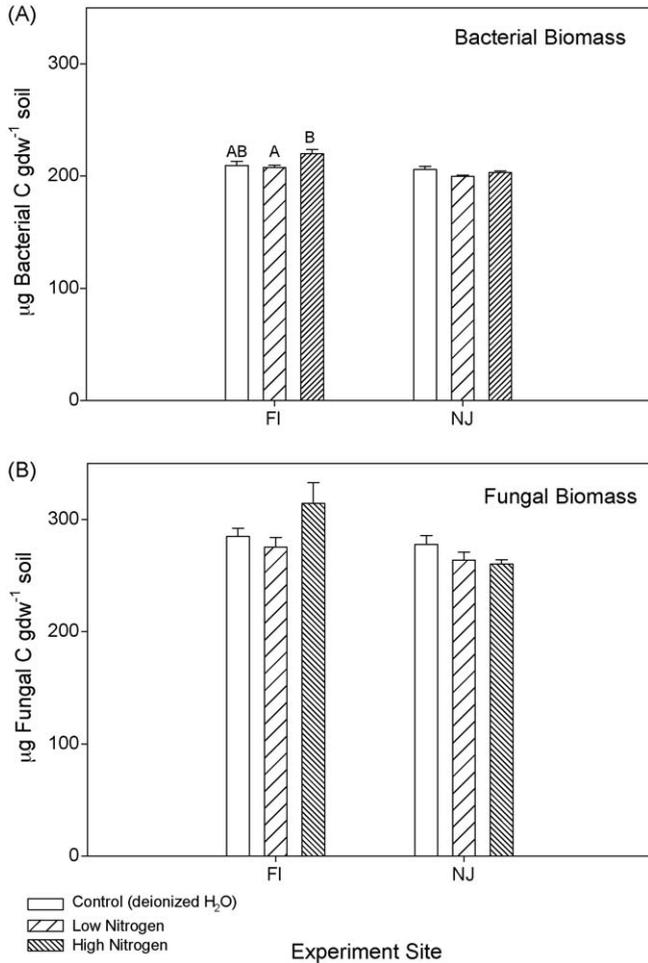


Fig. 2. Sampling effort versus morphotype accumulation curves for (A) colony morphotypes and (B) EMF morphotypes from both FL and NJ. Each point represents one soil core within one sampled plot.

relative to controls (J.A. Krumins, unpublished data). Therefore, we do not believe the nitrogen added in the present study was assimilated by the plants. However, even if it was taken up by plants, indirect effects of the nitrogen on the microbial component of the detrital food web should have been seen as the 'brown world' (detrital based food webs), and 'green world' (producer based food webs) connect in the rhizosphere (Wardle, 1999, Moore et al., 2003, Moore et al., 2004). We suspect that our aqueous nitrogen additions quickly leached from the biologically active portion of the soil, before effects on the biota could take place. This conclusion has very important environmental implications. Soluble nitrogen not assimilated into biotic components of soil will be transported to waterways and groundwater where it can lead to eutrophication (Aber et al., 2003; Galloway et al., 2003).

##### 4.2. Microbial community response

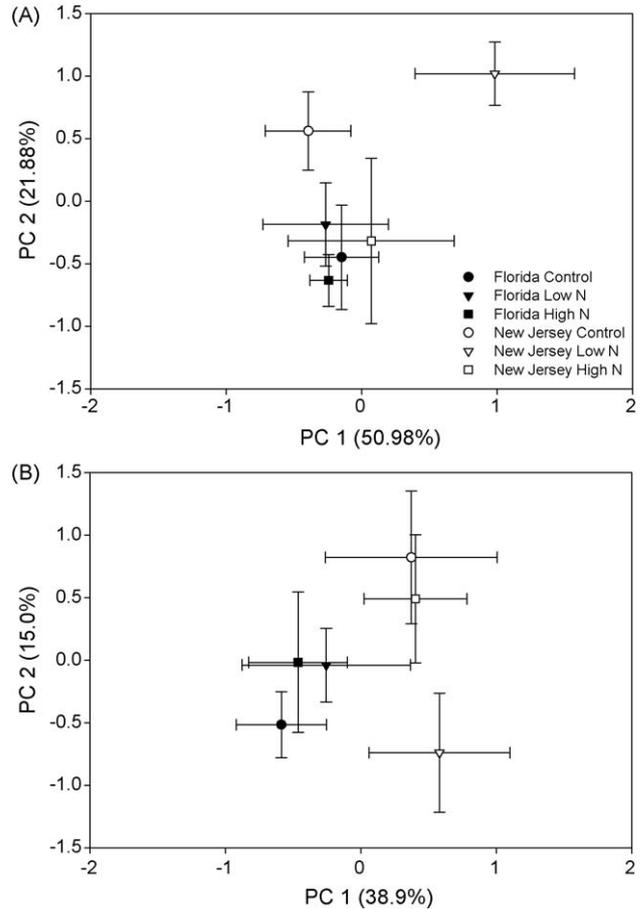
There was no difference in composition of the EMF community (Fig. 5A) or molecular profiles of fungi (Fig. 5B) with nitrogen concentration or between sites. Porous soil at these sites may explain the lack of fungal response to allochthonous nitrogen additions even though other studies have found an effect of nitrogen additions on EMF morphotype diversity (Dighton et al., 2004), spores of vesicular arbuscular mycorrhizae (VAM) (Johnson, 1993) and molecular profiles of fungal communities (Allison et al., 2007). In fact, most of the evidence for an effect of nitrogen deposition on mycorrhizae comes from naturally occurring



**Fig. 3.** Bacterial (A) and fungal (B) biomass as measured by SIR. Different letters over FL bars indicate significant effects of the nitrogen treatments (means separation by the Bonferroni test). All bars represent the mean  $\pm$  SE of all treatments at each site and  $n = 5$ .

deposition gradients that have been affecting the environment for extended periods of time (Arnolds, 1991; Egerton-Warburton and Allen, 2000; Lilleskov et al., 2002; Dighton et al., 2004; Lilleskov, 2005). Relative to the time scale of the industrial age and modern nitrogen deposition, our treatments were an intense pulse onto soils known to leach nutrients. Further, ambient nitrogen deposition is higher in NJ than FL (Table 1). Between site differences may in part be attributable to the press of ambient nitrogen deposition. Significant declines in EMF diversity have been observed across naturally occurring, shallow nitrogen deposition gradients (Dighton et al., 2004). The results of Dighton et al. (2004) contrasted with the findings we present here speak to the importance of time and the long-term effects of even a small amount of nitrogen on a fungal community. Furthermore, our study examines the response of EMF to nitrogen in association with oaks. It is believed that fungi in association with hardwoods may be less sensitive to excess nitrogen (Taylor et al., 2000). The field results of Dighton et al. (2004) were from data collected from mature pitch pine (*Pinus rigida*) within the NJ Pine Barrens ecosystem, and they found significant differences in EMF morphotype richness.

As opposed to EMF, bacterial colony morphotypes were affected by an interaction between nitrogen concentration and geographic location (Fig. 1A). In FL, colony morphotype richness increased with increasing nitrogen concentration. In NJ, the response was mixed; the lowest diversity of colony morphotypes was found in the low

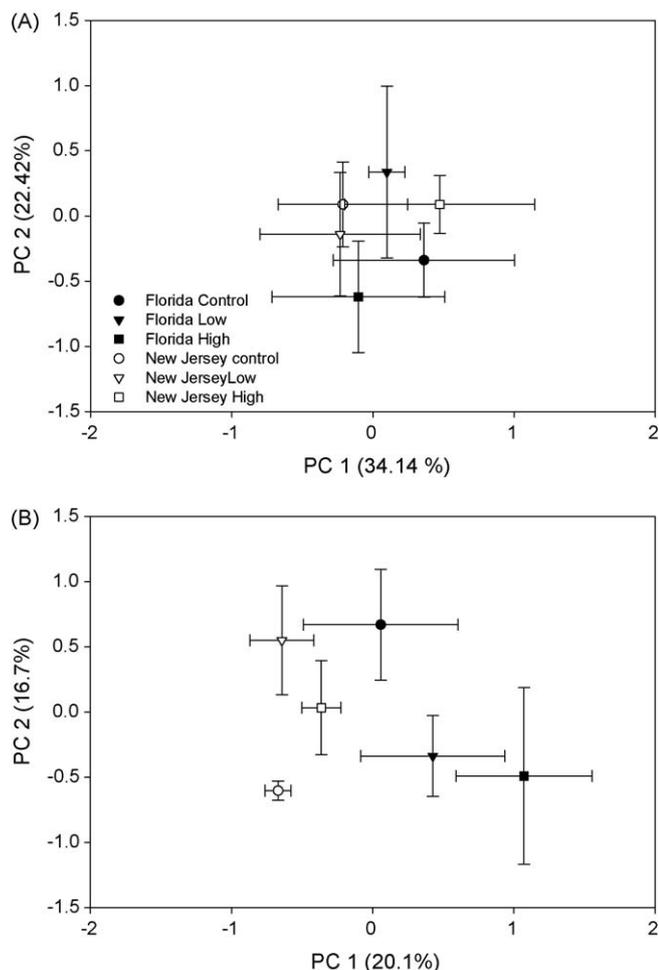


**Fig. 4.** PCA plot of bacterial community characterization of colony morphotypes (A) and molecular fingerprints using TRFLP (B). Each symbol represents the mean  $\pm$  SE of the component scores and  $n = 5$ .

nitrogen treatment and not the control. Interestingly, we found a similar response in FL when bacterial biomass decreased in the low treatment relative to the control, but increased in the high treatment relative to the control (Fig. 3A). This result is difficult to interpret because neither nitrogen treatment was different than the control. Non-linear responses to nitrogen addition may be due to the spatially patchy concentration of soluble nutrients in these plots (see standard error of the mean for NH<sub>4</sub> and PO<sub>4</sub> in Table 2). We think the biomass change was seen in the bacterial community and not the fungal community due to differences in their individual growth patterns. Individual bacteria can access nutrients and divide quickly. Fungi grow more slowly and may not have been able to access soluble nutrients that were quickly leached from the soil.

#### 4.3. Synthesis and implications

The divergent responses of bacterial and fungal communities may have a significant impact on the health of forest communities and ecosystem functioning. Bacterial biomass responded to nitrogen addition in FL (Fig. 3A), and bacterial community composition changed in both FL and NJ (Fig. 4A). Nitrogen addition appears to be differentially affecting bacterial and fungal communities, and for bacteria, this may depend on their environmental or geographic context. The outcome of diverging bacterial and fungal communities will have a significant impact on functional relations in soil. Changes in the bacterial but not fungal community could alter long standing symbioses between bacteria and EMF (Garbaye, 1994), or it could disrupt soil processes like decomposition and nutrient cycling by altering the balance



**Fig. 5.** PCA plot of fungal community composition characterized by EMF morphotype (A) and molecular fingerprint using TRFLP (B). Each symbol represents the mean  $\pm$  SE of the component scores and  $n = 5$ .

between the fungal and bacterial energy channels in soil (Moore and Hunt, 1988).

The number of bacterial and EMF morphotypes increased with each additional plot sampled (Fig. 2) underscoring the highly diverse (Torsvik et al., 2002) and patchy nature (Franklin and Mills, 2003) of microbial communities in soil. The incomplete sampling of these communities may have limited our ability to detect differences between the two sites or in the response to the nitrogen treatments. All microbial sampling methods are selective (Hughes et al., 2001). Hence, it is important to view microbial communities through multiple 'lenses' as we have done here. The molecular methods may not have resolved differences in the communities to the extent the microscopic or culture based methods did due to the challenges of amplifying whole community DNA from environmental samples. The NJ samples in particular were difficult to amplify due to relatively low microbial biomass. In spite of the sampling variability we encountered in this experiment, meaningful trends in microbial community response (or non-response) emerge. This emphasizes the importance of studying microbial community response to environmental change within the context of different geographic locations.

#### 4.4. Conclusions

Bacterial and fungal communities responded differently to allochthonous nitrogen inputs. This perhaps reflects their differing stoichiometry, growth rates and ability to acquire nutrients.

However it is interesting that bacterial community composition changed with nitrogen addition and the fungal community did not. We think this differential response is due to the limited ability of these soils to retain soluble nitrogen. Bacteria utilize resources and grow faster; possibly they were able to incorporate some of the nitrogen whereas the fungi were not. This was particularly the case at the FL sites where phosphorus is in excess allowing fast growing bacteria to possibly immobilize the nitrogen. The results presented here have important implications for understanding microbial communities and forest ecosystem health. Our results show that the microbial response to environmental perturbations like excess nitrogen loading can vary between geographic locations. In a changing world, microbial communities are likely to respond to environmental perturbation in complex and unpredictable ways.

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