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Microbial community shifts influence patterns in tropical forest nitrogen fixation

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Abstract The role of biodiversity in ecosystem function receives substantial attention, yet despite the diversity and functional relevance of microorganisms, relationships between microbial community structure and ecosystem processes remain largely unknown. We used tropical rain forest fertilization plots to directly compare the relative abundance, composition and diversity of free-living nitrogen (N)-fixer communities to in situ leaf litter N fixation rates. N fixation rates varied greatly within the landscape, and 'hotspots' of high N fixation activity were observed in both control and phosphorus (P)-fertilized plots. Compared with zones of average activity, the N fixation 'hotspots' in unfertilized plots were characterized by marked differences in N-fixer community composition and had substantially higher overall diversity. P additions increased the efficiency of N-fixer communities, resulting in elevated rates of fixation per *nifH* gene. Furthermore, P fertilization increased N fixation rates and N-fixer abundance, eliminated a highly novel group of N-fixers, and increased N-fixer diversity. Yet the relationships between diversity and function were not simple, and coupling rate measurements to indicators of community structure revealed a biological dynamism not apparent from process measurements alone. Taken together, these data suggest that the rain forest litter layer maintains high N fixation rates and unique N-fixing organisms and that, as observed in plant community ecology, structural shifts in N-fixing communities may partially explain significant differences in system-scale N fixation rates.

Keywords Diversity · Free-living nitrogen fixation · Hotspot · Microbial community structure · *nifH* gene · Phosphorus

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Introduction

All living organisms require nitrogen (N), and one process—biological N fixation—accounts for more than 97% of natural N inputs to terrestrial ecosystems (Galloway et al. 2008). In light of the critical role N fixation plays in the global N cycle, the enormous human alteration of that cycle (Galloway et al. 2008; Townsend et al. 2009), and the fact that N is widely limiting to terrestrial net primary production (Vitousek and Howarth 1991), understanding the controls over biological N fixation is central to future predictions of ecosystem function (Horner-Devine and Martiny 2008). Previous research suggests that the distribution and activity of both symbiotic and free-living N-fixers are regulated by temperature (Hicks et al. 2003) and moisture

(Roskoski 1980), and by the availability of both micro- and macro-nutrients (Vitousek and Howarth 1991), particularly phosphorus (P) (Eisele et al. 1989; Benner and Vitousek 2007; Reed et al. 2007b). Physiological differences among N-fixing organisms can also regulate N fixation rates in natural settings (Sprent and Sprent 1990); however, investigations of organismal controls over terrestrial N fixation rates typically include studies of legumes and the microbial N-fixing organisms residing in their roots (but see Yeager et al. 2004; Hsu and Buckley 2009; Moseman et al. 2009). While symbiotic N fixation may provide the dominant input of biologically fixed N to some ecosystems (Cleveland et al. 1999), research suggests that free-living organisms can account for a significant proportion of total N fixation (Cleveland et al. 1999), particularly in temperate and tropical forests (Parkin 1987; Gehring et al. 2005; Perez et al. 2008; Reed et al. 2007a). Nevertheless, most free-living N-fixing communities remain uncharacterized, and the role of their structure in regulating N fixation rates is unknown.

Free-living N fixation rates vary widely between ecosystems (Cleveland et al. 1999). However, 'hotspots' of N fixation—zones where N fixation rates are much higher than average—are also commonly observed within most ecosystems (e.g., Alexander and Schell 1973; Perez et al. 2008), and these hotspots strongly influence scaled-up estimates of N fixation rates. Such spatial heterogeneity is not uncommon; rates of many microbially mediated processes including denitrification, nitrification, dissimilatory nitrate reduction to ammonium (DNRA), and methanogenesis show marked variation across small spatial scales (Parkin 1987; Silver et al. 2001; Davidson et al. 2004; Hawkes et al. 2005). These hotspots often account for a high percentage of total ecosystem rates (e.g., McClain et al. 2003), and an insufficient understanding of hotspot controls significantly constrains our ability to estimate rates across space and time, to construct elemental budgets, and to model these important processes (Groffman et al. 2009).

Most mechanistic explanations for spatial variation in N fixation rates have focused on differences in resource availability or the high-energy requirements of N fixation (Roskoski 1980; Vitousek and Howarth 1991; Hicks et al. 2003; Reed et al. 2008). However, a growing body of evidence suggests that microbial community composition also regulates fundamental ecosystem processes (Balser and Firestone 2005; Waldrop and Firestone 2006; Reed and Martiny 2007; Strickland et al. 2009), and links between microbial community structure and function have been demonstrated for soil processes such as denitrification (Cavigelli and Robertson 2000), nitrification (Carney et al. 2004), and methanotrophy (Gulledge et al. 1997). Relationships between N-fixer communities and N fixation rates have been investigated for soils in temperate wetlands (Moseman et al. 2009), desert systems (Yeager et al. 2004), and in agricultural lands (Hsu and Buckley 2009), yet links between community and free-living N fixation rates in tropical ecosystems remain poorly studied. Furthermore, we are aware of no study that has explicitly explored the N-fixing communities occupying N fixation hotspots.

In a previous set of experiments in a tropical rain forest in Costa Rica, we found that free-living N fixation contributed ~10 kg N/ha/year and that free-living N fixation rates in the forest's litter layer increased significantly in response to P additions (Reed et al. 2007a), largely owing to a P-induced increase in the magnitude of leaf litter N fixation hotspots. Here, we analyzed leaf litter N fixation rates and simultaneously assessed free-living N-fixer abundance, community composition, and diversity in an attempt to identify possible links between N fixation rates and the structure of the free-living N-fixing community. We examined N-fixer community composition in hotspots and in samples with average N fixation rates in both P-fertilized and unamended plots to assess how small-scale variations in N fixation rates correspond to differences in N-fixer community structure. We hypothesized that small-scale spatial variation in the abundance and community composition of N-fixing organisms could help explain the hotspot and P-induced variation in N fixation rates commonly observed in this site and in others (e.g., Alexander and Schell 1973; Eisele et al. 1989; Perez et al. 2008).

Materials and methods

Site description

The study site is a primary, lowland tropical rain forest on the Osa Peninsula in southwestern Costa Rica, located within the Golfo Dulce Forest Reserve (8°43′N, 83°37′W). Temperature at the site is relatively constant (26 ± 1.5°C) and rainfall is high (>5,000 mm) but seasonally variable, with a 3- to 4-month dry season (months that receive <100 mm rain) between December and April (Cleveland and Townsend 2006). The Osa Peninsula was formed in three large seafloor volcanic events that occurred between 25 and 75 million years ago, and the soils at the site are characterized as relatively P-poor Ultisols (Berrange and Thorpe 1988; Bern et al. 2005). The forest is a stratified, closed canopy, highly diverse (100–200 tree species/ha; Kappelle et al. 2002) rain forest that includes many common neo-tropical tree species [e.g., *Brosimum utile* Kunth Oken. (Moraceae); *Caryocar costaricense* Donn. Sm. (Caryocaraceae); *Hieronyma alchorneoides* Fr. Allem (Phyllanthaceae); *Schizolobium parahybum* Vell. S.F. Blake (Fabaceae); and *Vantanea barbourii* Standl. (Humiriaceae)].

Fertilization experiment and sample collection

In 2001, a nutrient fertilization experiment (N × P in a full-factorial design, 10 plots per treatment) was established at the site to investigate relationships between nutrient availability and tropical rain forest function (Cleveland et al. 2006; Cleveland and Townsend 2006). Forty adjacent plots (5 × 5 m each) were created and were randomly selected to receive fertilization treatments; plots were fertilized twice per year by hand-broadcasting fertilizer onto the leaf litter layer. At the time of this study, the P-fertilized (+P) plots had received 150 kg P/ha/year (as KH_2PO_4) during each of the previous 5 years and the fertilizer had been most recently applied ~6 months prior to this study. Results showed that litter P concentrations and P immobilization in decomposing leaves were significantly greater in the +P plots relative to controls (Cleveland et al. 2006). To examine links between N-fixer community structure and changing P availability, we sampled bulk leaf litter from unamended and +P plots. Samples were collected at the end of the rainy season (November), when N fixation rates are relatively high (Reed et al. 2007a). Wearing nitrile gloves (Fisher Scientific, Pittsburgh, PA, USA), we collected two bulk leaf litter samples by hand (~2 g each; selecting only leaves above the soil surface and avoiding other plant parts) from each of the 10 unamended and P-fertilized plots (for a total of 20 independent unamended and 20 independent +P samples). The 40 individual leaf litter samples were immediately inserted into 55-ml clear acrylic tubes, and the acetylene reduction assay (ARA; Hardy et al. 1968; Belnap 1996) was used to estimate free-living N fixation rates. Briefly, tubes were sealed with a lid fitted with a septum, injected with enough acetylene to create a 10% headspace concentration (by volume), and vented to the atmosphere. After a 16-h incubation on the forest floor (previous tests showed no anoxia with this incubation time), headspace samples were mixed, subsampled, and injected into evacuated Vacutainer tubes (Becton, Dickinson, Franklin Lakes, NJ, USA), and each Vacutainer tube was sealed with non-hydrocarbon silicone sealant (Dow Corning, Midland, MI, USA). Data consistently showed that ethylene production in controls (i.e., litter, no acetylene) was small compared with acetylene reduction rates (<1% of average acetylene reduction rates; data not shown). Following incubation, each of the 40 leaf litter samples was transferred into individual sterile 50-ml centrifuge tubes containing 99% ethanol to preserve DNA (Harry et al. 2000). Following transport to the laboratory at the University of Colorado, litter samples were immediately frozen and Vacutainer headspace gas samples were analyzed for ethylene concentration on a gas chromatograph (Shimadzu, Kyoto, Japan) fitted with a flame ionization detector (330°C) and a Poropak N column (110°C; Supelco, Bellefonte, PA, USA).

The relationship between acetylene reduction and N fixation rates has been assessed using ^{15}N techniques, and while data suggest the relationship is variable (Ley and D'Antonio 1998), it often falls near the theoretical range of 3 moles of acetylene reduced for each mole of N fixed (Hardy et al. 1968; Ley and D'Antonio 1998; Vitousek and Hobbie 2000). Thus, acetylene reduction rates were converted to N fixation rates by dividing moles of acetylene reduced by three (Hardy et al. 1968). Area-based N fixation estimates (kg N/ha/year) were calculated by creating a leaf litter weight-to-area conversion factor determined in the field at the time of sample collection. Briefly, in haphazardly selected areas adjacent to the fertilization plots, a 0.25-m^2 template was placed on the forest floor and all leaf litter beneath the template was collected, dried, and weighed. The process was repeated eight times and produced a consistent mass-to-area relationship. We note that these rates were generated from a single sampling event, and are not necessarily representative of annual rates. However, the N fixation rates reported here are similar to leaf litter estimates measured throughout the year at the same site (Reed et al. 2007a).

DNA extraction, amplification, and sequencing

One goal of this research was to explicitly compare the N-fixer communities associated with leaf litter N fixation rate 'hotspots' to communities that fixed average amounts of N. While hotspots have been qualitatively defined in the literature as areas that show high activity relative to the surrounding area (e.g., Parkin 1987; Christensen et al. 1990; Groffman et al. 2009), there is no general statistical definition of a hotspot; thus, we developed a mathematical approach for grouping our samples. Using the N fixation rate data (treating unamended and +P datasets separately), we quantified the extent to which N fixation rates deviated from the median N fixation rate (we used the median because sample means can be susceptible to skewness with this type of data). We defined 'average' samples as those that deviated from the median by <1 standard error (SE) (regardless of whether they were lower or higher than the median) and 'hotspot' samples as those that exceeded the median by >3 SE. Using these criteria, from the 40 original samples we identified 4 hotspot leaf litter samples from the unamended and 4 from the +P plots. Control and +P plots harbored more than 4 'average' samples (5 and 11, respectively) and from these samples we randomly selected 4 for both 'average' groups (control and +P). These 16 samples were used for genetic analysis, generating a total of 4 N fixation rate-based groups (average unamended, hotspot unamended, average +P, and hotspot +P) that included 4 samples each. From each of the resulting 16 samples, genomic DNA was extracted using a modified

phenol-chloroform-isoamyl alcohol method (Moré et al. 1994), substituting ~0.2 g of leaf litter (dry weight equivalent) for soil. DNA pellets were washed with 70% ethanol, resuspended in water, and purified over a packed Sepharose 4B column (Jackson et al. 1997).

For sequencing analyses, samples were pooled according to group: average unamended, hotspot unamended, average +P, and hotspot +P. Nested PCR methods were adapted from other *nifH* gene surveys (Widmer et al. 1999; Compton et al. 2004), and all methods were tested and modified to optimize for DNA concentration, annealing temperature, and for the minimum number of cycles required for successful amplification. The first PCR reactions contained: (1) 23.25 µl of nuclease free water (NFW; Burdick and Jackson, Morristown, NJ, USA); (2) 10 µl of green GoTaq reaction buffer (Promega, Fitchburg, WI, USA); (3) 2 µl of 25 mM $MgCl_2$ (Promega); (4) 5 µl of 2 mM deoxyribonucleotide triphosphates (dNTPs; Promega); (5) 4 µl 10 µM each of primers nifA (5′-GCHWTHTAYG GNAARGNGG) and nifRev (5′-GCRTAHABNGCCATC ATYTC) (Operon Biotechnologies, Huntsville, AL, USA); (6) 0.25 µl of 5 U GoTaq DNA Polymerase (Promega); and (7) 0.5 µl of 10 mg/ml bovine serum albumin (BSA; Promega). To each reaction, 1 µl of the appropriate leaf litter genomic DNA was added for a total sample volume of 50 µl. PCR parameters were as follows: 95°C for 5 min, followed by 39 cycles of 92°C for 15 s, 48°C for 8 s, 50°C for 30 s, 10 s at 74°C, and 10 s at 72°C with a 72°C final extension step for 10 min. For the 2nd PCRs, primers nifB (5′-GGHTGTGAYCCNAAVGCNGA) and nifRev were used and the reaction composition was the same as for the 1st PCR, except 2 µl of product from the 1st PCR and 22.25 µl of NFW were used. The parameters for the 2nd unamended PCRs were identical to the 1st PCR; however, for the 2nd +P PCRs, only 34 cycles were necessary for amplification. PCR consistently provided *nifH* gene fragments of the expected size, ~370 base pairs, and negative controls for the 1st and 2nd PCR reactions showed no amplification.

All PCR products were purified in a 1% agarose gel and extracted using Qiagen's QIAquick gel purification kit (Qiagen, Valencia, CA, USA). The amplified *nifH* genes were ligated into a pCR 2.1 vector (Invitrogen, Carlsbad, CA, USA) and transformed into *Escherichia coli* using the manufacturer's protocol. Inserted genes from the plasmids were PCR amplified using the primers M13F and M13R (Invitrogen) according to the methods described in Nemergut et al. (2007), and excess nucleotides and primers were removed using Exonuclease I and Shrimp Alkaline Phosphatase (USB, Cleveland, OH, USA; Nemergut et al. 2007). Samples were shipped to Functional Biosciences (Madison, WI, USA) for sequence analysis; 384 clones of the *nifH* gene were sequenced in both directions. The nucleotides described in this study were deposited in GenBank under accession numbers GU945761-GU946066.

Quantitative PCR

We used quantitative PCR (QPCR) methods to quantify the relative abundance of *nifH* genes in 14 of the 16 DNA samples (2 'average' samples—1 from control and 1 from +P plots—did not yield sufficient genomic DNA), as well as for the DNA composite samples for each of the 4 groups. QPCR allows for the quantitative assessment of the relative abundances of specific genes within a genomic DNA sample (Kabir et al. 2003; Fierer et al. 2005), and has been shown to work well for *nifH* genes (Wallenstein and Vilgalys 2005). We note that, while QPCR methodologies assume uniform amplification efficiencies for template DNA, different representatives of a gene (e.g., *nifH*) could produce different efficiencies due to varied stabilities of primer-template pairs. This may be especially possible when using degenerate primers, and thus changes in community composition could in theory affect QPCR gene estimates. We tested amplification efficiencies of three of our most common sequenced *nifH* genes that varied at the QPCR primer site (*nifH* genes matching most closely to *Heliobacterium chlorum*, *Gluconacetobacter diazotrophicus*, and *Desulfovibrio vulgaris*). We observed no notable amplification variation among these genes and, using the three standard curves created by the three different sequences, estimates of *nifH* gene copy number varied by <5%. Nevertheless, whether or not shifts in *nifH* gene composition affected *nifH* gene estimates using QPCR here is unknown and should be considered when evaluating QPCR gene abundance estimates across samples.

We used a Stratagene Mx3000P QPCR system (La Jolla, CA, USA) and methods modified from Wallenstein and Vilgalys (2005). Each QPCR contained 12.5 µl of Brilliant II SYBR Green QPCR Master Mix (Stratagene), 1.25 µl each of the forward (5′-AAAGGYGGWATCGGYAARTC CACCAC) and reverse (5′-TTGTTSGCSGCRTACATSG CCATCAT) *nifH* primers (Operon), 9 µl of NFW (Burdick and Jackson), and 1 µl of genomic DNA. When optimizing the methods for our samples, we used standardized amounts of template mixed with serial dilutions of genomic DNA to test for inhibitory effects on PCR amplification. We found that a DNA concentration of ~10 ng/ml (DNA concentration was quantified on a Nano-Drop UV–Vis Spectrophotometer; Nano-Drop Technologies, Wilmington, DE, USA) resulted in maximum amplification and this concentration was used for all QPCR analyses. The parameters for each reaction were as follows: 95°C for 8 min, followed by 45 cycles of 94°C for 30 s, 53°C for 45 s, 74°C for 10 s, 72°C for 30 s, with a final dissociation step. Each plate included triplicate reactions per DNA sample and per standard. *nifH*

plasmid standards were created using the 4 most common sequences found in our clone libraries. The standard curve was made from a 10× serial dilution ranging from 10^5 to 10^0 gene copies per µl. Also, for each reaction well, we performed a melt curve analysis and assessed the product using agarose gel electrophoresis, and all data were visually inspected to ensure that amplification data were not the result of non-target amplification or primer-dimers (data not shown).

In addition to quantifying *nifH* genes, we performed QPCR analyses on each sample to quantify total bacterial abundance using the same number of replicates and matrix as the *nifH* gene quantification, but substituting forward primer 338F (5′-ACTCCTACGGGAGGCAGCAG), reverse primer 518R (5′-ATTACCGCGGCTGCTGG) (Park and Crowley 2005) and reaction parameters of 95°C for 10 min, followed by 40 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s. A 10× serial dilution (10^5–10^0 gene copies per µl) standard curve was created using bacterial plasmid standards. The *nifH* gene concentration in each sample was divided by its 16S rRNA gene concentration to account for overall shifts in the bacterial population as a whole and for possible differences in amplification efficiency between samples. The *nifH* primers used should amplify both bacterial and archaeal *nifH* DNA (Widmer et al. 1999; Raymond et al. 2004; Wallenstein and Vilgalys 2005), but since none of our sequences matched archaeal sequences, we did not quantify or account for total archaeal abundance. To estimate N-fixer efficiency differences between treatments, we divided N fixation rates by *nifH* gene relative abundance.

Data analysis

Each of the *nifH* gene sequences from our four clone libraries was edited in Sequencher 4.1 (Gene Codes, Michigan, USA), translated into protein (amino acid) sequences, and searched against the GenBank database using blastp (Altschul et al. 1997). Sample sequences, reference sequences (chosen from blastp searches), and the *nifH* gene from outgroup *Nostoc* sp. PCC 7120 (accession# NP_485497; Raymond et al. 2004) were aligned using ClustalX (Thompson et al. 1997). A maximum likelihood phylogenetic tree was created with *nifH* protein sequences using RAxML with the JTT model of substitution (Stamatakis 2006). Rates of N fixation were analyzed in two ways. First, data from all 40 samples were grouped by treatment (unamended or +P) and the effect of fertilization was assessed using a one-way ANOVA. Second, N fixation rates among the four groups (average unamended, hotspot unamended, average +P, hotspot +P) were compared using a one-way ANOVA and LSD post-hoc analyses ($n = 4$ per group). Relative abundances of *nifH* genes among the same four groups were also compared using a one-way ANOVA and LSD post-hoc analyses. We compared N-fixer community composition using UniFrac (Lozupone and Knight 2005), which assesses statistical differences in microbial communities and creates a dendrogram relating communities based on their phylogenetic similarity. All UniFrac *P* values were Bonferroni-adjusted. The diversity of the microbial communities was assessed using both inclusive (PD) and exclusive (*G*) molecular phylogenetic diversity calculations (Lewis and Lewis 2005), which were assessed for each community using PAUP* (Swofford 2001). Both of these diversity indices are a measure of the amount of a phylogenetic tree's branch length that is derived from a particular group (e.g., hotspot +P). The inclusive phylodiversity (PD) is the measure of total branch length that a group maintains, and the exclusive diversity or gain (*G*) is the measure of the branch length that is unique to that group.

Results

Nitrogen fixation rates and N-fixer abundance

When assessing the N fixation rates of all 40 samples grouped by treatment (control and +P), the ANOVA showed that P fertilization significantly stimulated N fixation ($P = 0.017$), eliciting a more than threefold increase relative to unamended (control) plots (13.5 and 3.9 kg N/ha/year, respectively). Defining a N fixation 'hotspot' as a sample that exceeded the median N fixation rate by >3 SE, we found that both the unamended and +P plots had four hotspots of N fixation activity. When assessing N fixation rates separately for each of the four groups ($n = 4$ each), we found: (1) N fixation rates in average samples did not vary significantly between unamended and +P plots; (2) hotspot N fixation rates in unamended plots were significantly ($P < 0.05$) higher than 'average' sample rates; and (3) the hotspots in the +P plots fixed larger quantities of N than any other group (Fig. 1a; $P < 0.05$). We note that while such comparisons allowed us to assess the relationship between N fixation rates and N-fixer community structure, they are not meant to convey any statistical significance or importance of these rates at the ecosystem-scale.

The relative abundance of the *nifH* gene also varied significantly ($P < 0.001$) between the four groups (Fig. 1b), and showed patterns similar to those observed with N fixation rates (Fig. 1a). Namely, samples with average rates of N fixation showed the lowest *nifH* gene relative abundance, unamended hotspots had significantly higher *nifH* gene relative abundances than average samples, and hotspots in the +P plots showed the highest relative abundance of the *nifH* gene. When analyzing samples individually using linear regression analyses, we found a significant ($P = 0.025$)

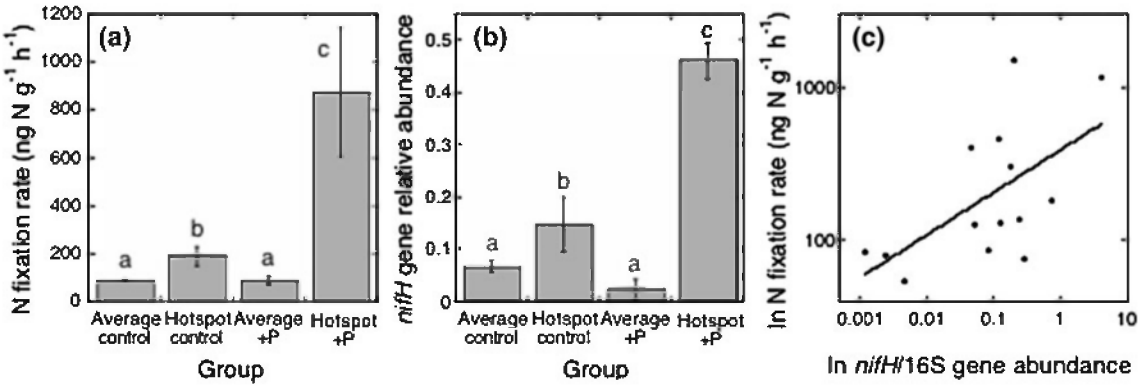

Fig. 1 Patterns in N fixation rates and *nifH* gene relative abundance from unamended and P-fertilized plots. Mean **a** N fixation rates and **b** *nifH* gene relative abundance (relative to 16S rRNA gene abundance) from samples with 'average' and 'hotspot' N fixation activity in control and P-fertilized plots. *Different lowercase letters* depict significant ($P < 0.05$) differences among groups and *error bars* represent ± 1 SE. The N fixation rate data are from samples selected according to rate and are not meant to describe ecosystem-scale differences between groups, but are to show rate variation among the samples used for genetic analyses. **c** Relationship between *nifH* gene relative abundance and N fixation rates ($n = 14$, $P = 0.025$, $r^2 = 0.35$). Note *log scale* used for both axes

linear relationship between N fixation rates and *nifH* gene relative abundance, and *nifH* gene relative abundance explained 35% of the variation in N fixation rates (Fig. 1c). In addition, we assessed the efficiency of the N-fixing community by dividing N fixation rates by *nifH* gene relative abundance. We found that the control plot communities had a lower efficiency ($P = 0.037$) than +P plot communities (0.7 and 1.8 μg N fixed/g/hr/*nifH* gene relative abundance, respectively).

N-fixer community composition

The significant spatial variability in N fixation rates both within and between treatments made it possible to group samples by rate and by treatment, allowing us to compare natural community variability and to directly examine the effects of P fertilization on N-fixer communities. For both the unamended and +P plots, the N-fixer communities associated with average rates of N fixation were significantly different from those associated with hotspots (Fig. 2). The N-fixer community in average samples from unamended plots was dominated by sequences that matched with low sequence similarity to the GenBank database (<86% identity; Altschul et al. 1997), but were most similar to members of the Firmicutes division, specifically organisms in the genus *Heliobacteria* (Electronic Supplementary Material, ESM 1). None of the other samples contained sequences related to this genus, and the overall community composition in the average samples from the unamended plots was significantly different from the communities in all other samples (Fig. 2). Average N fixation rate samples from the +P plots were dominated by sequences related to the γ-Proteobacteria, and had sequences related to the genera *Gluconacetobacter*, *Zymomonas*, *Azospirillum*, and *Rhodopseudomonas*, as well as uncultured organisms. In contrast, the hotspot community in the +P plots was dominated by sequences related to Gluconacetobacter and δ-proteobacteria, and had sequences related to the genera *Methylobacterium*, *Rhizobium* and *Sinorhizobium*. However, the hotspot unamended plot community was not significantly different from either of the P-fertilized N-fixer communities and maintained sequences related to *Gluconacetobacter*, γ-Proteobacteria and the genera *Methylobacterium*, *Azospirillum*, and *Zymomonas*.

The *nifH* gene tree further illustrates how all of the N-fixer community sequences were related phylogenetically (ESM 1). First, sequences from unamended plots and with average N fixation rates grouped separately from all other sequences. Next, sequences from the other three groups (hotspot unamended, average +P, and hotspot +P) were more broadly distributed across the phylogenetic tree. Third, a subset of the sequences from the unamended hotspot, +P hotspot, and +P average samples grouped together, and were most similar to sequences from organisms such as *Gluconacetobacter diazotrophicus*. However, some sequences from the unamended hotspot and +P average samples were not found in the +P hotspot samples and were most similar to sequences related to γ-proteobacterium PAL 386. Finally, a subset of +P hotspot sequences grouped separately from all others, and those sequences were most closely related to *Desulfvibrio vulgaris* (ESM 1).

N-fixer diversity

We used two indices—phylodiversity (PD) and gain (G)—to assess patterns of diversity among the different N-fixing communities (Lewis and Lewis 2005). Both indices provide an estimate of the amount of branch length in a phylogenetic tree that is derived from a particular community (e.g., hotspot unamended or hotspot +P). PD represents the total

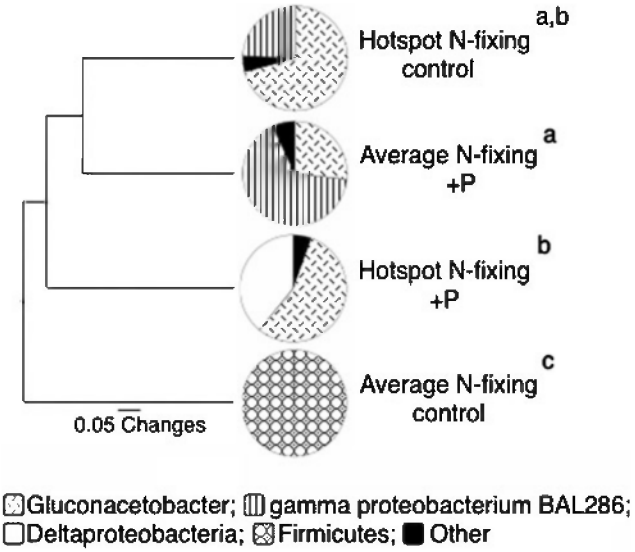

Fig. 2 Comparisons of community composition in N fixation hotspots and from samples maintaining average N fixation rates in unamended and P-fertilized plots. Pie charts depict the relative proportion of sequences grouping within a particular genus according to blastp searches. When the closest blastp matches were <90% identical, phyla or subphyla were indicated instead of genera, and different groups are represented by different patterns. Genera comprising minor components of the community were grouped as '*Other*' and are listed in the "Results". The UniFrac metric was used to cluster the N-fixing communities based on phylogenetic overlap, and communities that are nearer to one another on the UniFrac dendrogram are more similar. Significantly different ($P < 0.05$) communities are depicted by *different lowercase letters*

branch length of the community, while G represents the branch length that is unique to that community. For PD, we found that hotspots in unamended and P-fertilized plots had the highest diversity (PD = 1.37 and 1.16, respectively) compared to PD values of 0.31 and 1.03 in the unamended and the +P average samples, respectively (Table 1). In addition, phylodiversity of the average +P community was more than 3 times the average unamended plot N-fixer diversity. G followed the same patterns: first, diversity was highest in the hotspots of both unamended and +P plots (G = 0.58 and 0.56, respectively) compared to G values of 0.27 and 0.49 in the average unamended and average +P communities, respectively. Second, as with PD, the average community from the +P plots had a higher G than the average unamended plot community.

Discussion

Rates of many important microbially mediated processes such as nitrification, denitrification, DNRA, and methanogenesis frequently vary across relatively small spatial scales (e.g., Silver et al. 2001; Davidson et al. 2004; Hawkes et al. 2005). In particular, 'hotspots'—spatially explicit

Table 1 Inclusive (*PD*) and exclusive (*G*) diversity indices representing the total phylogenetic diversity attributed to a particular group (e.g., hotspot N-fixing control) and the amount of diversity unique to that group, respectively

Group	Diversity indices	
	PD	*G*
Average N-fixing control	0.31	0.27
Hotspot N-fixing control	1.37	0.58
Average N-fixing +P	1.03	0.49
Hotspot N-fixing +P	1.16	0.56

zones where well above average rates occur—can be especially important in regulating overall ecosystem process rates (McClain et al. 2003; Groffman et al. 2009). Not surprisingly, N fixation is also heterogeneous, and hotspots of activity are commonly observed for this process (e.g., Alexander and Schell 1973; Perez et al. 2008). Microbial community composition also varies enormously on small spatial scales (Martiny et al. 2006); therefore, we hypothesized that variations in microbial community composition could help explain observed rate differences in N fixation hotspots, and that the effects of P fertilization may be mediated through shifts in the N-fixer community. We predicted that hotspots of leaf litter N fixation would contain a higher relative abundance of *nifH* functional genes and would be more diverse than samples fixing average amounts of N. In addition, we predicted that P-fertilized leaf litter would have increased *nifH* gene relative abundance, different N-fixer communities, and an increased diversity of N-fixing organisms.

Consistent with our predictions, the phylogenetic data revealed a possible explanation for some of the observed variation in N fixation rates: hotspots harbored very different microbial communities than areas with lower rates (Figs. 1b and 2; Table 1; ESM 1). In the unamended plots, zones with average N fixation rates had a lower relative abundance of the *nifH* gene, suggesting a smaller N-fixing community (Fig. 1b). In addition, the community in the unamended plots with lower leaf litter N fixation rates had lower diversity (Fig. 2; Table 1), and consisted of a phylogenetically restricted clade of *nifH* sequences that appeared to be highly novel: blastp (Altschul et al. 1997) searches resulted in a highest match of only 86% identity to the proteins of *Heliobacterium chlorum* and *H. gestii,* members of the Firmicutes (for reference, most other sequences had >97% identity matches; Table 1; ESM 1). By contrast, N fixation hotspots in unamended plots featured a much more diverse community (Table 1), completely lacked this novel group of organisms (Fig. 2), and had a greater relative abundance of N-fixing organisms (Fig. 1b). Thus, while abiotic factors (e.g., temperature, moisture, and resource availability) are known to strongly regulate N fixation rates (Roskoski 1980; Vitousek and Howarth 1991; Hicks et al.

2003), our data suggest that N-fixer community composition may also play an important role, and likely interacts with abiotic controls to regulate overall rates of N fixation.

When assessing all 40 samples, P fertilization stimulated N fixation ($P = 0.017$), driving rates >3 times higher than in unamended plots (13.5 vs 3.9 kg N/ha/year, respectively) and, as in the unamended plots, P-fertilized plots contained multiple N fixation hotspots. This spatial variability within and across plots allowed us to effectively control for differences in N fixation activity between unamended and fertilized plots, and to directly examine the effects of P fertilization on N-fixer communities. In samples with low N fixation rates, P additions increased N-fixer diversity, and displaced the novel group that dominated the average N-fixer community in unamended plots (Fig. 2). Similarly, a study of Douglas Fir forests in Oregon showed that forest clear-cutting consistently removed unique *nifH* gene phylotypes (Shaffer et al. 2000), suggesting that, in a variety of forests, unique components of N-fixing communities may be wholly displaced in response to perturbation. The N-fixing community in +P samples with average rates of N fixation was far more similar to the community in hotspots from unfertilized plots; both communities were dominated by similar suites of α- and δ-Proteobacteria, including organisms related to *G. diazotrophicus and Raoultella ornithinolytica.* However, the relative abundance of *nifH* genes was similar in average rate samples from both unfertilized and +P plots, while the relative abundance of N-fixers (based on *nifH* gene abundance) in the unamended plot hotspots was far higher (Fig. 1b). Additionally, there was significant overlap in the community composition of unamended and +P hotspots; both included relatives of *G. diazotrophicus and Methylobacterium* sp. 4–46 (Fig. 2), although the +P hotspots also contained sequences distantly related to the δ-proteobacterium *Desulfovibrio vulgaris* that were not present in any of the other samples (Fig. 2; ESM 1).

This study revealed how microbial community dynamics may influence N fixation rates, an important result that was not evident from process-level measurements alone. As predicted, N fixation rates positively correlated with the relative abundance of *nifH* genes (Fig. 1c), but abundance alone accounted for 35% of the N fixation rate variability (Fig. 1c). This observation suggests that other aspects of community structure, as well as the physiological traits of individual organisms, may play important roles in regulating leaf litter N fixation. For example, the average unamended sample was entirely comprised of organisms distantly related to *Heliobacterium* (ESM 1), a genus of anaerobic, photoheterotrophic bacteria commonly found in rice paddy soils. These novel organisms were not detected in any other samples, suggesting they were replaced by other bacteria in hotspots and after P fertilization. Relatives of *G. diazotrophicus,* an obligately endophytic aerobic N-fixer, were found in the unamended hotspots and in the +P samples, accounting for the significant overlap in these communities (Fig. 2). Interestingly, relatives of *Methylobacterium* sp. 4–46, a methylotroph that can nodulate roots of tropical herbaceous plants, were specific to hotspots only, and relatives of the strictly anaerobic sulfate-reducing *Desulfovibrio vulgaris* were unique to +P hotspot samples (ESM 1).

P fertilization appeared to influence leaf litter N fixation on two levels. First, it caused a shift in microbial community composition that made +P plots resemble unamended hotspots, regardless of their N fixation activity (Fig. 2). These data support the notion that both the average and hotspot P-fertilized communities developed from the hotspot community in the unfertilized plots, while the novel community from the unfertilized plots was eliminated by fertilization. In addition, N-fixing communities from +P plots were more efficient than those from unamended samples, displaying higher N fixation rates per *nifH* gene. Second, P additions increased activity in the hotspots, which have a greater relative abundance of *nifH* genes than average samples (Fig. 1b). Differences between the +P and unfertilized hotspots, including the presence of the anaerobic δ-Proteobacteria (Fig. 2) and a higher relative abundance of N-fixers within the overall bacterial community (Fig. 1b), may provide a partial explanation for the dramatic increase in N fixation rates of the P-amended samples. Other work has shown that P fertilization increases soil respiration in these sites (Cleveland and Townsend 2006), perhaps helping to provide anaerobic microsites necessary for the bloom of *D. vulgaris*. Whether the high relative abundance of δ-Proteobacteria in the hotspot samples was responsible for the elevated N fixation remains to be seen, but the spatial restriction of this organism suggests that, as with macroecology, the distribution of particular species may inform our understanding of ecosystem function.

The phylogenetic data also revealed complex relationships between microbial diversity and ecosystem function (Table 1). On unfertilized plots, increases in diversity correlated with increases in N fixation; hotspot communities were notably more diverse than those in low activity zones (Table 1). By contrast, P additions resulted in relatively higher diversity at all levels of activity (Table 1). These data suggest that, while high fertility conditions appear to facilitate greater overall N-fixer diversity, richness alone cannot explain rate variations. Instead, in the +P plots, differences in N fixation rates appear to be at least partially driven by the abundance of N-fixers, and perhaps by the physiological traits of organisms unique to hotspots (such as *D. vulgaris).* For example, *nifH* gene relative abundance explained 59% of the variation in N fixation rates in the +P samples (data not shown), but only 35% of the variation across all samples combined (Fig. 1c). This observation

suggests that, when resources are more available, the abundance of N-fixers may more strongly regulate N fixation rates than when resources are scarce.

Resource availability may also have important consequences for microbial diversity. In macrobial studies, fertilization with limiting nutrients (e.g., N in temperate ecosystems) often elicits a decline in species diversity (Vitousek et al. 1997), but our data suggest the opposite response of N-fixing organisms to P enrichment. As previously suggested by macro-ecological research, this illustrates the importance of accounting for an organism's functional type when considering potential relationships between diversity and function (Ackerly and Cornwell 2007). For example, our data show a pattern analogous to those from a study in a montane forest in Hawai'i. There, P fertilization caused a marked increase in the abundance and richness of one group of organisms (tropical N-fixing epiphytes; Benner and Vitousek 2007), a response that is inconsistent with those often seen for the community as a whole. The increase in diversity we observed in response to P fertilization suggests similar N-fixer responses may exist in the microbial world. Furthermore, the P-induced increase in *nifH* gene relative abundance in the hotspot bacterial community was dramatic (Fig. 1b), suggesting that the response of the N-fixing community to P additions in terrestrial ecosystems may mirror the P-induced N-fixer 'blooms' common in aquatic ecosystems (Howarth et al. 1988).

The fact that the relative abundance (Fig. 1), community composition (Fig. 2), and diversity (Table 1) of free-living N-fixers all varied in concert with N fixation rates has important implications for understanding controls over N fixation. Our data suggest that N fixation hotspots may reflect heterogeneity not only in the abiotic variables that are the traditional focus of many ecosystem studies but also in the community dynamics of N-fixing organisms. Macrobial research demonstrates the importance of community structure in regulating ecosystem function, and the diversity-function debate (Huston 1997; Hooper et al. 2005) often focuses on the presence and abundance of specific traits—not just species numbers (Ackerly and Cornwell 2007). This trait-based approach also seems well suited to microbial ecology, given the ability to focus on communities via relevant genes, and the challenges inherent in linking the dynamics of a vastly diverse microbial world with ecosystem processes.

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