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Diazotrophs Show Signs of Restoration in Amazon Rain Forest Soils with Ecosystem Rehabilitation

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ABSTRACT Biological nitrogen fixation can be an important source of nitrogen in tropical forests that serve as a major CO_2 sink. Extensive deforestation of the Amazon is known to influence microbial communities and the biogeochemical cycles they mediate. However, it is unknown how diazotrophs (nitrogen-fixing microorganisms) respond to deforestation and subsequent ecosystem conversion to agriculture, as well as whether they can recover in secondary forests that are established after agriculture is abandoned. To address these knowledge gaps, we combined a spatially explicit sampling approach with high-throughput sequencing of *nifH* genes. The main objectives were to assess the functional distance decay relationship of the diazotrophic bacterial community in a tropical forest ecosystem and to quantify the roles of various factors that drive the observed changes in the diazotrophic community structure. We observed an increase in local diazotrophic diversity (α-diversity) with a decrease in community turnover (β-diversity), associated with a shift in diazotrophic community structure as a result of the forest-to-pasture conversion. Both diazotrophic community turnover and structure showed signs of recovery in secondary forests. Changes in the diazotrophic community were primarily driven by the change in land use rather than differences in geochemical characteristics or geographic distances. The diazotroph communities in secondary forests resembled those in primary forests, suggesting that at least partial recovery of diazotrophs is possible following agricultural abandonment.

IMPORTANCE The Amazon region is a major tropical forest region that is being deforested at an alarming rate to create space for cattle ranching and agriculture. Diazotrophs (nitrogen-fixing microorganisms) play an important role in supplying soil N for plant growth in tropical forests. It is unknown how diazotrophs respond to deforestation and whether they can recover in secondary forests that establish after agriculture is abandoned. Using high-throughput sequencing of *nifH* genes, we characterized the response of diazotrophs' β-diversity and identified major drivers of changes in diazotrophs from forest-to-pasture and pasture-to-secondary-forest conversions. Studying the impact of land use change on diazotrophs is important for a better understanding of the impact of deforestation on tropical forest ecosystem functioning, and our results on the potential recovery of diazotrophs in secondary forests imply the possible restoration of ecosystem functions in secondary forests.

KEYWORDS diazotrophs, functional distance decay, recovery of diazotrophs in secondary forest

The Amazon rain forest acts as a major sink of CO_2 by absorbing 0.4 to 0.6 Pg C annually, which represents a quarter of the absorption by all forests globally (1–4). Global emission of the greenhouse gas carbon dioxide (CO_2) is a serious environmental concern. The uptake of CO_2 requires significant input of nitrogen, which comes from the mineralization of organic material, atmospheric deposition, and biological nitrogen fixation (BNF). In terrestrial nonmanaged ecosystems, 97% of natural nitrogen input comes from biological nitrogen fixation (5, 6) carried out by diazotrophs (N_2-fixing *Bacteria* and *Archaea*) (7, 8). To assess variations in the diazotrophic species, the marker gene *nifH* has been widely used as a target gene (9–17), and alterations in its composition and abundance have been directly correlated with nitrogen fixation rates (18–22).

The Amazon is the largest equatorial forest in the world, and it controls vital biogeochemical cycles. Changes in its land use can significantly influence a wide range of ecosystem processes (23, 24), such as the N cycle, in which decreases in mineralization, nitrification, and denitrification processes have been observed in response to deforestation (25–27). Currently, Amazon forests are being cleared for cattle ranching and soybean fields at an alarming rate, and about 20% of the area of the Brazilian Amazon rain forest was cleared between 1970 and 2015 (28). Ramankutty et al. (29) estimated that most of the deforested area is being used for pasture (62%) and crop production (6%), with the remaining area being secondary forests (32%) that were established after pasture abandonment. Owing to the extent of deforested area (7,418,000 ha) (30) that is currently pasture and secondary forest, it is important to assess how changes in land use have influenced microbial functional groups and whether restoration of microbial communities in secondary forests occurs. Previous studies, primarily focused on plants and animals, have demonstrated that secondary forests invariably have much lower biodiversity than undisturbed primary forests (31, 32) and that the effects of losing primary forests are irreparable (32). So far, detailed information on the recovery of microbial communities in general and those responsible for specific ecological functions (e.g., diazotrophs) is limited.

Previous molecular microbial studies in the Amazon region have mainly focused on the effect of deforestation on bacterial communities as a whole (33, 34), with a limited number of studies focused on specific functional groups (35–37). These studies have generally been limited in terms of both the number of soil samples collected across different land use systems and depth of sequencing (i.e., the number of DNA sequences per sample using Sanger DNA sequencing) (14, 38–40). Previously, Mirza et al. (14) reported alterations of diazotrophic community structure in the Amazon following deforestation and land use change, but they did not include large-scale spatial analyses. Therefore, in the present study, we used a spatially explicit, nested-sampling approach to understand the spatial variation in diazotrophic community structure and the factors that influence it over a wide geographical area (up to 10 km²). Second, we investigated the extent of their recovery in secondary forests that were established after the abandonment of pasture. High-throughput sequencing was used to generate 443,600 *nifH* gene sequences across 89 soil samples for the detailed comparison of diazotrophic community structure across three different land use types.

The specific objectives of the current study were (i) to evaluate a functional distance decay relationship of the diazotrophic bacterial community in a tropical forest ecosystem and (ii) to quantify the roles of various factors that drive the changes in the diazotrophic community structure and play a major role in the recovery of diazotrophs in secondary-forest ecosystems.

RESULTS AND DISCUSSION

Diazotrophic α-diversity and spatial turnover or β-diversity. There was a significant increase in the diazotrophic α-diversity (for both taxonomic and phylogenetic metrics), based on the *nifH* gene sequences, in response to the forest-to-pasture conversion (Fig. 1; also, see Fig. S1 in the supplemental material) ($P < 0.01$). The increase in diazotrophic α-diversity at the pasture sites established after the defores-
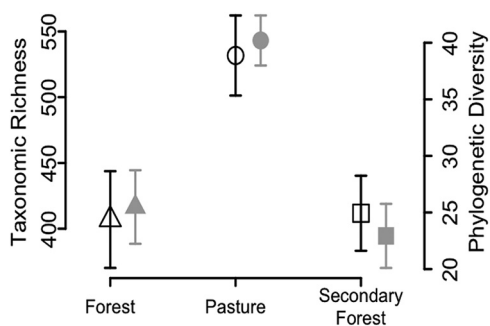
FIG 1 Differences in the taxonomic (black) and phylogenetic (gray) α-diversity of the *nifH* genes at 97% DNA identity in response to land use change in the Amazon. Taxonomic diversity was calculated as the number of unique genes, and phylogenetic diversity was calculated as Faith's phylogenetic diversity (66). Error bars represent 95% CI.

tation of the primary forests can be due to the enhanced availability of slowly released labile carbon (41) or an enhanced supply of easily degradable C sources in the form of root exudates of fast-growing grass species (*Urochloa brizantha* and *Panicum maximum*) in the pastures (42). On average, we observed higher soil C content in the pasture sites (mean $\pm$ 95% confidence interval [CI], 2.93 ± 0.65 g kg of soil^{-1}) than in the forest sites (1.52 ± 0.42 g kg of soil^{-1}), which suggests that higher soil C content might have selectively favored a diverse diazotrophic community. Consistent with our observation, Cenciani et al. (43) also reported an increase in soil C content in pasture soil compared to primary-forest sites in the Amazon region.

Previous studies (44–46) have suggested that the presence of high plant diversity supports the growth of diverse microbial populations in soil mainly because of the differences in the nature organic material produced by different plants. In contrast, several microbial studies have also observed that despite the presence of high plant diversity, bacterial α-diversity was low in soil (47–50) and it increased after deforestation (33, 34, 48). Here, we observed a similar trend for the diazotrophic community, which we assessed using a genetic marker for nitrogen fixation. Furthermore, we also observed a decrease in the diazotrophic α-diversity at the secondary-forest sites, which had higher plant diversity than the pasture sites (Fig. 1; also, see Fig. S1) ($P < 0.05$) (51).

We also examined patterns of β-diversity by comparing the degree of community turnover between samples using both taxonomic and phylogenetic data. We found that the diazotrophic rate of turnover based on taxonomic diversity was the lowest in the pastures (slope = 0.22 ± 0.01 [95% bootstrap CI]; $R^2 = 0.04$) (Fig. 2), intermediate in the secondary-forest sites (slope = 0.34 ± 0.02; $R^2 = 0.22$), and highest in the primary-forest sites (slope = 0.45 ± 0.03; $R^2 = 0.55$). This indicates that the pasture diazotrophic community was more homogenous, whereas the communities in the forested sites displayed greater patchiness. This trend was consistent at the different DNA and protein similarity levels (see Fig. S2A to C) as well as for the phylogenetic analysis (Fig. 2). A conceivable justification of the observed spatial patterns of diazotrophic species can be the presence of unique ecological niches or patchiness in the primary forests. Similar outcomes for general bacterial community turnover rates have been noticed in response to the deforestation of primary forest for pasture (34, 52). The present study not only extends this observation to a specific functional group but also shows that the spatial turnover rates partially recovered in secondary forest (Fig. 2).

The driver of changes in the diazotrophic community structure. Overall, there was a significant shift in the diazotrophic microbial community composition at 97% DNA similarity across three different land use systems for both complete data sets (Fig. 3) (PERMANOVA, $F_{(2,87)} = 11.81$; $P = 0.001$) and subsampled data sets (see Fig. S3 and S4) (PERMANOVA, $F_{(2,87)} = 6.92$; $P = 0.001$). Similarly, an increase in abundance of the diazotrophic community, quantified through quantitative PCR (qPCR), was also significantly altered as a result of forest-to-pasture conversion (see Fig. S5) ($P < 0.001$).
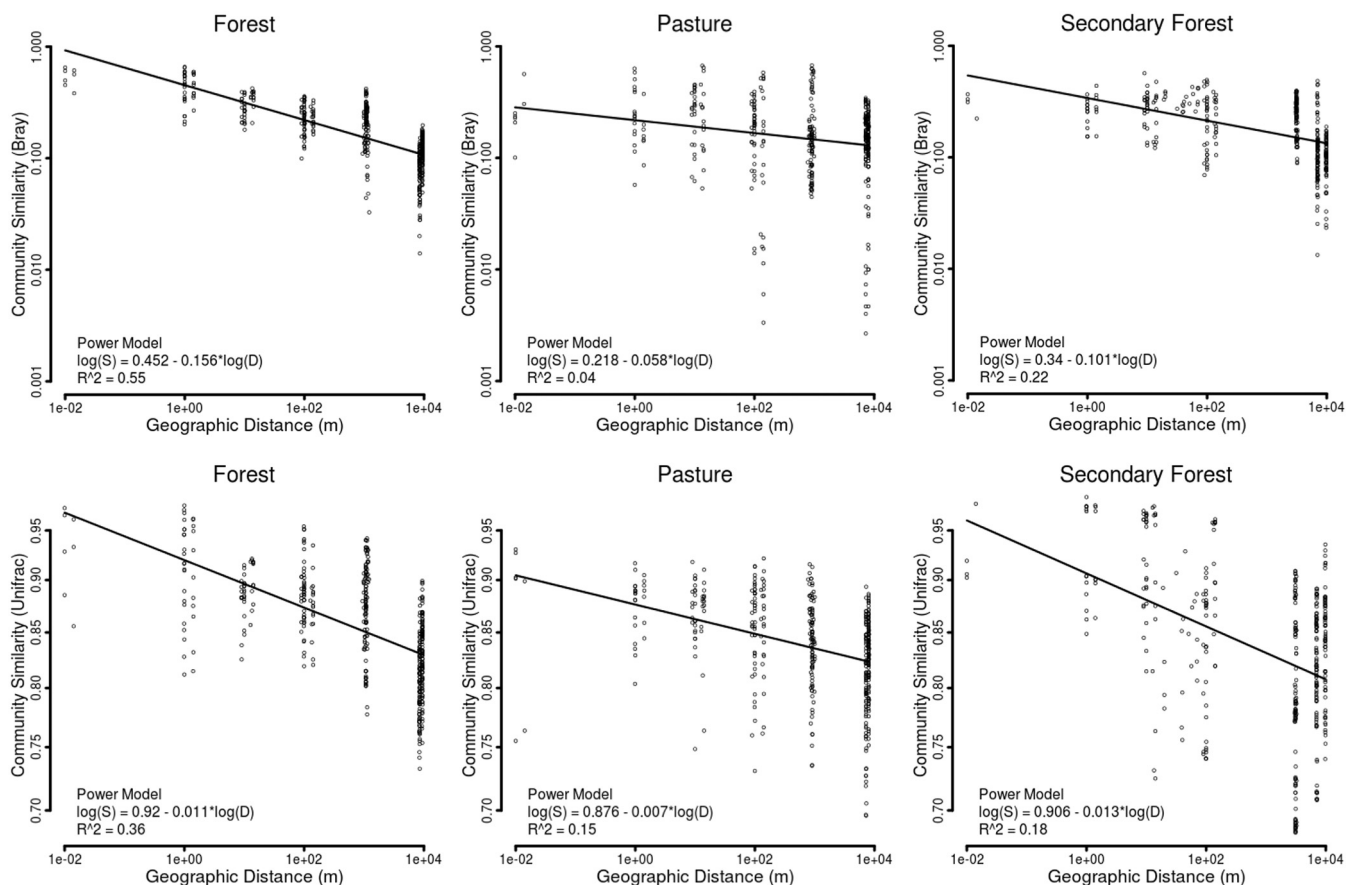
FIG 2 Diazotrophic community β-diversity turnover at 97% DNA identity, representing a distance decay relationship in response to land use change in the Amazon. Differences in the slopes across different land use systems were compared by a power model. The top row shows the variations in the taxonomic β-diversity and the bottom row shows the phylogenetic β-diversity across different land use systems.

However, this abundance was not different between primary and secondary forests (see Fig. S5) ($P > 0.05$).

To further explore the question of what factors would have caused this variation in the diazotrophic community structure, we performed variance-partitioning analysis, in which three matrices of explanatory variables (the factors land use, physicochemical characteristics, and geographic distance) were evaluated. Using this approach, we were
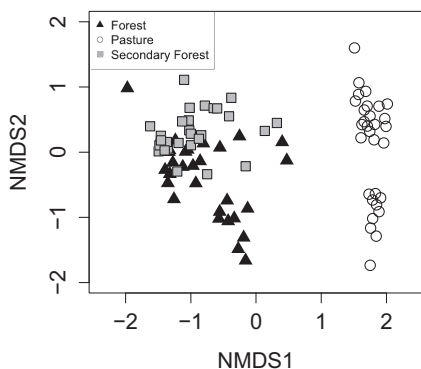

FIG 3 Ordination based on a dissimilarity matrix of the *nifH* gene sequences comparing three different land use systems in the Amazon. The nonmetric multidimensional scaling representation was based on 97% DNA identity. The complete data set contains a total of 443,600 *nifH* gene sequences, with the following abundance distribution of sequences retrieved from different treatments: primary-forest samples, 158,261; pasture samples, 143,700; secondary-forest samples, 131,640.
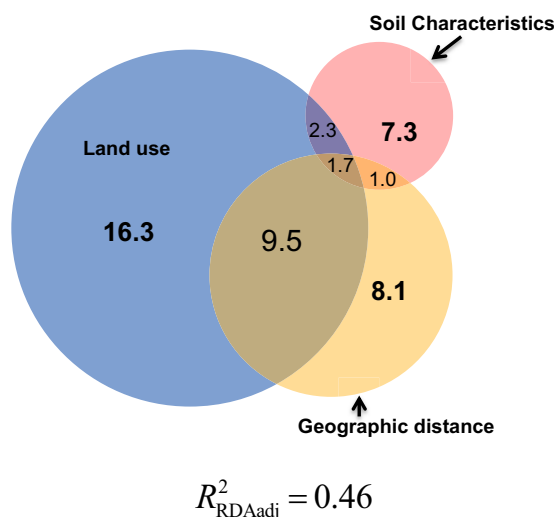
$$R^2_{\text{RDAadj}} = 0.46$$

FIG 4 Variance partitioning analysis to determine how land use, geochemical characteristics, and geographic distance explain the variation in the diazotrophic community. Each circle represents the portion of variation accounted for by each of the three factors.

able to assess the influence of each factor while controlling the confounding influence of the other two factors. Results of variation partitioning analysis showed that about 46% of the total variation in the diazotrophic community structure was explained by these three matrices (Fig. 4). Land use or plant species was the primary driver of the observed changes in the diazotrophic community and explained about 29% of the variation through its individual influence (16.3%) and shared influences with the geographic distance (9.5%) and soil characteristics (2.3%). Geographic distance and soil properties had similar individual fractions of explained variation (approximately 10%), and their effects were much smaller than the effect of land use.

This change in the diazotrophic community in response to land use change could be due to the direct influence of plant species across different systems, i.e., plant root exudates and/or their detritus controlling diazotrophic community rather than their indirect effect by altering geochemical characteristics. Soil characteristics explained only about 7% of the variation in the diazotrophic community (Fig. 4). In contrast, Jesus et al. (33) found in the western part of the Amazon that soil attributes explained about 31% of the variation observed in general microbial communities from forest-to-pasture conversion, and the influence of land use was not significant. In our study, geographic distance explained only about 8% of variations in the diazotrophic community (Fig. 4). The geochemical characteristics, including organic matter content, pH, potential acidity (H+Al), potential cation exchange capacity (T), N, P, K, S, Ca, Mg, Al, Zn, Fe, Mn, Cu, and B (see Table S2), were measured in both studies. Most of the soil characteristics measured in this study were same as those measured by Jesus et al. (33), with a few exceptions, such as soil texture, base saturation, Al saturation, and equilibrium phosphorus. Other differences between these two studies were as follows: (i) the sampling depth in our study was 0 to 10 cm, whereas in the study by Jesus et al. (33), it was 0 to 20 cm, and (ii) we analyzed 108 individual soil samples, whereas Jesus et al. (33) analyzed 26 soil samples, and each was a composite of 12 individual soil samples. It is not possible to point out a factor or factors that contributed to the differences between the study results. One possible explanation for our results could be that several other factors as mentioned above (33), along with the available iron (53), light, and other variables (54) that were not measured in this study, might have contributed to the observed variations in the diazotrophic community. In contrast, several previous studies suggested that soil characteristics are the main driver of the changes in general microbial community compositions (33, 48, 55, 56).

The changes in the plant composition predominantly caused significant changes in the diazotrophic community structure. The most diverse forest sites may contain up to 300 different plant species per hectare (57), secondary forests exhibited an intermediate level of plant diversity, up to 15 to 18% of woody plant species (51), and pasture sites were least diverse, with only two fast-growing grass species. Previously, higher plant diversity has often been associated with higher N mineralization rates, which lead to a higher net N supply (58, 59), and significantly higher N mineralization rates have been observed upon forest-to-pasture conversion in this region (25). This was also consistent with the lower diazotrophic community abundance quantified in the primary forest in comparison to pasture (see Fig. S5). About 80% of the N requirements of the primary forests is fulfilled by the mineralization of diverse plant materials (60), and a small amount of N in forest soil is also contributed by atmospheric N depositions (61). Hence, tropical forests seem less dependent on the diazotrophic community to fulfill their N requirements. In contrast, pastures containing two fast-growing grass species (*Urochloa brizantha* and *Panicum maximum*) obtain about 40% of their N requirements through biological N_2 fixation (42). N deficiency caused by continued depletion due to grazing of aboveground biomass and the presence of high-C resources supplied by the grass species in the form of root exudates may provide selectively favorable conditions for the diazotrophic community and enhance BNF. Future *in situ* estimates of BNF in the Amazon rain forest could help to improve our understanding of N flux in this terrestrial ecosystem and also indicate that high *nifH* gene abundance truly represents high N fixation rates, as were reported previously (21).

Recovery of diazotrophs in the secondary forest. Diazotrophic communities in approximately 12- to 17-year-old secondary forests showed signs of partial recovery in terms of α-diversity, turnover rates, and community structure (Fig. 1 to 3). First, as we described above, there was a decrease in the diazotrophic α-diversity at the secondary-forest sites (Fig. 1; also, see Fig. S1) ($P < 0.05$) (51). Second, we observed an intermediate level of turnover in the secondary forest (i.e., a lower level than in primary forests and higher than in pasture), which indicates the onset of potential restoration of confined ecological niches in the secondary forests that might have been lost in the pasture lands due to biotic homogenization (34). Third, the distributions of diazotrophic community composition of primary and secondary forests were significantly different ($P < 0.05$) at 97% DNA similarity. Nevertheless, the secondary-forest diazotrophic community composition was more closely related to that of the primary forest than that of pastures (Fig. 3). At other DNA (95% and 90%) and amino acid (99%, 97%, and 95%) base similarity levels, the differences in diazotrophic community composition between the primary and secondary forests were nonsignificant (see Fig. S4) ($P > 0.05$), while both sites were significantly different from pastures. Similarly, *nifH* gene abundance was also similar between primary forests and secondary forests (see Fig. S5) ($P > 0.05$). Lastly, phylogenetic analysis (Fig. S6) also indicated a high similarity in the diazotrophic groups at the primary- and secondary-forest sites, which were different from those detected in the pasture sites. Likewise, the overall diversity estimated by the accumulation of taxa with the number of soil samples also suggested a similarity between primary- and secondary-forest ecosystems compared to pasture sites (see Fig. S7).

It was evident from these results that the diazotrophic community was influenced by changes in land use. However, we observed potential recovery of the diazotrophic community in 12- to 17-year-old secondary forests, indicating their high resilience. This suggests that changes in land use from forest to pasture did not cause permanent loss of most of the diazotrophic species (extinction); rather, it caused a shift in their relative abundance in the pasture lands. These results highlight the need for more comprehensive spatial-scale studies to understand the true impact of land use on functional microbial communities and have improved our understanding of the effect of the transition from forest to pasture lands and from pasture to secondary forest on the diazotrophic community. Overall, this outcome is very encouraging; it indicates that, in parallel to the recovery of a few plant species, unique ecological niches for diazotrophic

community can be restored and may eventually lead to the recovery of functions. To verify the complete recovery of function in the secondary forests, studies focused on the *in situ* estimates of BNF in the Amazon rain forest, pasture lands, and secondary forest would improve our understanding of this process and of the extent to which the recovery of diazotrophs translates into a function. This is a detailed study in this region that indicates the recovery of diazotrophic species in 12- to 17-year-old secondary forests in tropical regions.

Overall, we observed a decrease in β-diversity in response to the forest-to-pasture conversion. Land use was the single most important driver of changes in community structure (composition and abundance) relative to differences in geography and soil. We also observed the recovery in the diazotrophic β-diversity and community structure in the 12- to 17-year-old secondary forests, which has important ecological implications about the possible restoration of ecosystem function in secondary forests. Future *in situ* estimates of BNF in the Amazon rain forest could help to improve our understanding of N flux in this terrestrial ecosystem and of the extent to which the recovery of diazotrophs translates into a function.

MATERIALS AND METHODS

Sampling design and total soil DNA extraction. Soil samples were collected from the state of Rondônia (10°10′5″S, 62°49′27″W), Brazil, in 2010. This site was selected because it was one of the three states (Rondônia, Mato Grosso, and Pará) which accounted for more than 85% of all Amazon deforestation from 1996 to 2005 (62). Primary forest had been cleared by removing large trees for timber and burning the remaining vegetation to create pasture lands that were vegetated with two fast-growing grasses (*Urochloa brizantha* and *Panicum maximum*). Once pastures became unproductive, they were abandoned, allowing secondary vegetation to grow. These secondary forests have much lower plant diversity than the primary forests (51, 63).

A spatially explicit sampling design was established for three plots within each of the primary-forest, adjacent pasture (38 years old), and secondary-forest (12 to 17 years old) areas (see Fig. S8). Totals of nine sites, three per land use system, and 12 samples per site were used. The nested-quadrat sampling design established 0.01-, 0.1-, 1.0-, and 10-m² quadrats (shown as a series of squares in Fig. S8) nested within a 100-m² quadrat, and 12 samples were collected within each 100-m² plot. Overall, three 100-m² quadrats were established 0, 1, and 10 km apart within both primary-forest and pasture sites (36 samples per land use system). No secondary-forest site was available at exactly a 1-km distance, so the second 100-m² sampling plot for secondary forest was at about 3 km (0, 3, and 10 km). Soil cores (5-cm diameter) collected at a 10-cm depth were used for DNA extraction and physicochemical analysis as described previously (34).

Amplification and sequencing of a functional gene. The *nifH* gene was amplified using the PCR primers PolF and PolR, developed by Poly et al. (64), and products were sequenced using the 454 GS FLX Sequencer (Michigan State University Genomics Facility, East Lansing, MI). We used the primers PolF and PolR because these are the most widely used primers to assess the diversity of diazotrophs in terrestrial ecosystems. Previously (14), we tested three other *nifH* gene-specific PCR primer sets (Ueda19F-Ueda470R, PicenoF-PicenoIR, and Z-primer) along with PolF and PolR on 15 soil samples from the Amazon. Only PolF and PolR resulted in successful amplification of the *nifH* gene from all soil samples along with a single band of the appropriate size. Therefore, in this study, we used PolF and PolR. It is important to mention that like any other PCR primers, PolF and PolR may have some biases toward certain specific diazotrophic groups, such as *Alpha-*, *Beta-*, and *Gammaproteobacteria*, *Firmicutes*, and *Actinobacteria* (64).

Raw DNA sequences (456,211) were initially processed for quality control using the functional gene pipeline of the Ribosomal Database Project II (RDP) (65) (http://rdp.cme.msu.edu). Chimeric sequences were identified and removed using the USEARCH 6 chimera check. Frameshift errors were adjusted by running FrameBot, and protein sequences were aligned through the hidden Markov model HMMER3 aligner. After initial filtering, high-quality sequences (443,600), both DNA and translated proteins, were aligned and clustered into operation taxonomic units (OTUs) at 99, 97, 95, and 90% for DNA and operational protein units at 99, 97, and 95% for translated proteins by complete linkage clustering using RDP's mcClust tool. Identification of the *nifH* gene sequences was carried out by running NCBI local BLAST and comparing against a *nifH* gene reference database of about 1,100 sequences obtained from Wang et al. (16). A total of 19 soil samples resulted in <1,500 *nifH* gene sequences that were not included in this study. The details of these 19 samples are provided in the supplemental material (Table S2).

Diversity metrics. Because analyses of α- and β-diversities can be influenced by differences in the number of DNA sequences retrieved per sample, we subsampled the data set with a total of 1,500 *nifH* gene sequences per sample (45,000 per land use type) to evaluate differences in the diazotrophic diversity in response to land use change. Subsamples were generated by randomly selecting 1,500 DNA sequences per sample, based on the sample with the fewest DNA sequences, using a Python script developed in-house. Differences in the α-diversity across the three different land use systems were compared by calculating both taxonomic and phylogenetic diversity. Taxonomic diversity was calculated as total species richness, Shannon diversity, and Margalef species richness using OTU abundance data

(97%). Phylogenetic diversity was calculated as Faith's phylogenetic diversity (66) by constructing a phylogenetic tree of representative *nifH* gene sequences using FastTree 2.15 (67). The significance of the differences was assessed through one-way analysis of variance (ANOVA).

Species turnover and variance partitioning. We examined spatial variation in diazotrophic community turnover using three distinct approaches: (i) distance decay analysis (68) to compare the patterns and rates of decay in diazotrophic community similarity across the three land uses, (ii) nonmetric multidimensional scaling (NMDS) (69) to explore variability in taxonomic composition across all three land uses simultaneously, and (iii) variation partitioning (70) to estimate the individual and shared components of variation in species turnover due to land use, geography, and soil physicochemistry. For NMDS and variation partitioning analyses, we used both the complete data set (containing 443,600 *nifH* gene sequences) and a subsampled data set (135,000 *nifH* gene sequences) to assess the changes in the diazotrophic community composition. We used the abundance-based Bray-Curtis metric as a measure of taxonomic similarity, which was calculated using the function vegdist in the R package vegan (71). For phylogenetic similarity, the Fast Uni-Frac distance metric was used (72).

We computed the distance decay relationship (DDR) separately for each type of land use by estimating the rate of change in taxonomic and phylogenetic community similarity as a function of geographic distance. To estimate the rate of distance decay, we fitted the two most common models of the DDR: the exponential and power models (73). To test the differences between distance decay slopes, we compared the 95% bootstrapped confidence intervals for the slope parameters using 10,000 bootstrap iterations, which avoids distributional assumptions about the test statistic (i.e., the slope). If the confidence intervals did not overlap, the slopes were considered significantly different. We computed the DDR for 97, 95, and 90% cutoffs for defining OTUs (see the supplemental material). We tested whether the sample centroids were located in significantly different locations of multidimensional space via PERMANOVA (74) using the function adonis in R (71). We carried out the variation partitioning analysis using the varpart function in R (71).

Data availability. The *nifH* sequences were deposited in the NCBI Sequence Read Archive (SRA) under BioProject no. PRJNA329012.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 4.5 MB.

ACKNOWLEDGMENTS

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