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Research

Experimental warming alters free-living nitrogen fixation in a humid tropical forest

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Summary

- Microbial nitrogen (N) fixation accounts for *c.* 97% of natural N inputs to terrestrial ecosystems. These microbes can be free-living in the soil and leaf litter (asymbiotic) or in symbiosis with plants. Warming is expected to increase N-fixation rates because warmer temperatures favor the growth and activity of N-fixing microbes.
- We investigated the effects of warming on asymbiotic components of N fixation at a field warming experiment in Puerto Rico. We analyzed the function and composition of bacterial communities from surface soil and leaf litter samples.
- Warming significantly increased asymbiotic N-fixation rates in soil by 55% (to $0.002\,\mathrm{kg\,ha^{-1}\,yr^{-1}}$) and by 525% in leaf litter (to $14.518\,\mathrm{kg\,ha^{-1}\,yr^{-1}}$). This increase in N fixation was associated with changes in the N-fixing bacterial community composition and soil nutrients.
- Our findings suggest that warming increases the natural N inputs from the atmosphere into this tropical forest due to changes in microbial function and composition, especially in the leaf litter. Given the importance of leaf litter in nutrient cycling, future research should investigate other aspects of N cycles in the leaf litter under warming conditions.

Introduction

Unlike other major plant nutrients, N is naturally brought into most ecosystems through lightning and the biological fixation of atmospheric N gas by microbes, a process known as N fixation. Given that N limits plant growth in many ecosystems (LeBauer & Treseder, 2008), biological N fixation is a critical process to study (Reed *et al.*, 2011). N-fixing microbes are divided into two groups: asymbiotic (free-living) and symbiotic. Symbiotic microbes form relationships with specific plants and are housed in nodules in the roots and/or stems of these plants. These N-fixing plants (N fixers) are relatively abundant in neotropical forests and can provide a large potential source of N fixation (Batterman *et al.*, 2013; Taylor *et al.*, 2017; Tamme *et al.*, 2021). However, these symbioses are facultative and this, combined with the difficulty of adequately sampling for symbiotic nitrogen fixation (SNF), means it is possible that very little SNF occurs in mature tropical forests and that reported rates are highly uncertain (Menge *et al.*, 2015; Winbourne *et al.*, 2018).

By contrast, asymbiotic nitrogen (N) fixation is easier to sample across space and time. This process is carried out by microbes that are hetero- or autotrophic bacteria, as well as some archaea, inhabiting water, soil, and leaf litter (Dynarski & Houlton, 2018). Estimates from multiple tropical forest studies suggest that a substantial amount of N is fixed by asymbiotic microbes (e.g. Reed *et al.*, 2007; Wurzburger *et al.*, 2012; Matson *et al.*, 2015): an estimated 7-10 $\mathrm{kg\,N\,ha^{-1}\,yr^{-1}}$ in the unmanaged tropical and subtropial moist forests of Latin America (e.g. Reis *et al.*, 2020; Moreira *et al.*, 2021). It is, therefore, important to understand how global changes might alter asymbiotic N fixation in tropical forests.

Biological N fixation is in part regulated by climate. At large scales, climate is considered a major driver of microbial community function and composition (Castro *et al.*, 2010; De Vries & Griffiths, 2018; Jansson & Hofmockel, 2020). Warming is especially important, as it can increase microbial processing, enzyme activity, turnover, and growth efficiency (Castro *et al.*, 2010; Pajares & Bohannan, 2016; Wood *et al.*, 2019; Bytnerowicz *et al.*, 2022). Warming conditions have long been thought to favor N fixation in cold environments because it is a microbial process catalyzed by the enzyme nitrogenase that, like many other enzymes, has reduced activity at very low temperatures (Houlton *et al.*, 2008). Asymbiotic microbial N-fixation rates have been found to increase in response to warming across many biomes, but the majority of the work has been conducted in cold ecosystems (Bai *et al.*, 2013; Liu *et al.*, 2016; Zheng *et al.*, 2020). However, extreme or chronic warming can inhibit asymbiotic N

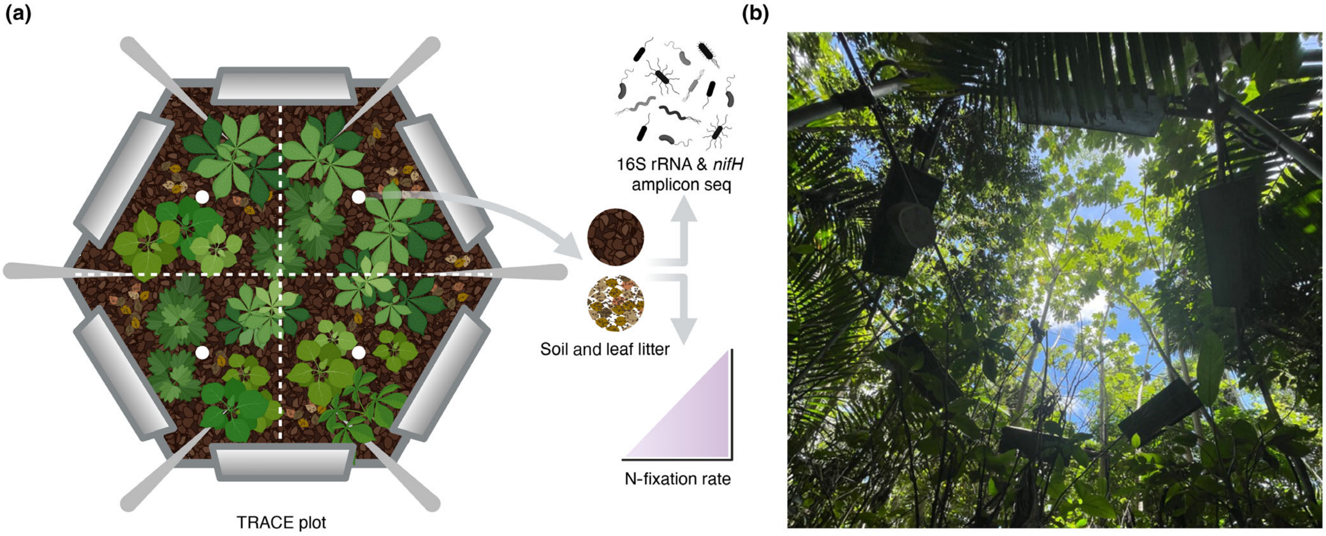

Fig. 1 Experimental setup of the Tropical Responses to Altered Climate Experiment (TRACE) plots in the Luquillo Experimental Forest of Puerto Rico. The TRACE experiment utilizes infrared heaters to warm three 12 m^2 plots within the forests. These warming plots are compared with three control plots experiencing ambient temperatures. (a) Diagram depicting top view of a TRACE plot used for experimental warming. Each TRACE plot is divided into four equal quadrants (represented by dashed lines). The circles at the center of each quadrant represent the approximate location of sites where soil and leaf litter samples were collected for nitrogen (N)-fixation measurements and microbial sampling. Created in BioRender. Shahid, S. (2025) https://BioRender.com/t12j536. (b) View from underneath one of the TRACE plots in the Luquillo Experimental Forest.

fixation, at least in high-latitude ecosystems (Rousk *et al.*, 2018; Zheng *et al.*, 2019), possibly by inducing water limitation of N-fixing microbes (Zheng *et al.*, 2020). In a warmed environment, the nitrogenase optimum temperature might be near 25°C, suggesting that warming might negatively impact N fixation in tropical forests (Deutsch *et al.*, 2024). Thus, the long-term effects of warming on asymbiotic N fixation in lowland, humid tropical forests remain uncertain, highlighting the need for more experimental warming studies in these ecosystems (Contosta *et al.*, 2015; Pajares & Bohannan, 2016; Zheng *et al.*, 2020; Deutsch *et al.*, 2024).

In addition to influencing the functioning of microbes and their enzymatically driven processes, warming may influence the composition of microbial communities. Warming can shift the soil microbial community to microbes adapted to higher temperatures and faster growth rates (Castro *et al.*, 2010; Zhao *et al.*, 2020) and can affect community composition via metabolic carbon (C) or the soil nutrient pool (Ma *et al.*, 2018). Experimental warming studies, which have largely been confined to temperate and higher latitude ecosystems, have found that warming increased the richness of asymbiotic N-fixing microbial communities in temperate grassland (Penton *et al.*, 2015) and enhanced N-fixing microbial abundance and diversity in tundra ecosystem soils (Feng *et al.*, 2019). Whether this is the case in tropical forests remains an open question (Reed *et al.*, 2010; Mirza *et al.*, 2014; Pajares & Bohannan, 2016) and is a focus of the work described here.

In this work, we present a comprehensive study of the effects of warming on N fixation in a tropical forest by utilizing tropical field warming. We examined the effect of warming on asymbiotic microbes by combining N-fixation assays with DNA sequencing of soil and litter samples from the Tropical Responses to Altered Climate Experiment (TRACE), an *in situ* whole-ecosystem warming experiment in Puerto Rico. We hypothesized that warming would increase N-fixing microbial community diversity and N-fixation rates.

Materials and Methods

Site description

This work took place within the Luquillo Experimental Forest in northeastern Puerto Rico (18°18′N, 65°50′W). Located at 100 m elevation, the site has a mean annual temperature of 24°C with relatively low seasonal variation, and a mean annual rainfall of 3500 mm (Alonso-Rodríguez *et al.*, 2022). The site contains clay-rich, acidic soils (described as Ultisol) with > 80% of root biomass concentrated in the top 20-cm soil layer (Yaffar & Norby, 2020). In the study area, the dominant canopy tree species are *Syzygium jambos* (L.), *Ocotea leucoxylon* (Sw.), and *Casearia arborea* (Rich.) Urb., and the dominant understory tree species is the palm *Prestoea montana* (R. Graham) Nichols.

The TRACE experiment, which began in 2015, utilizes infrared (IR) heat to warm three 12 m^2 plots within the forest to 4°C above ambient temperatures, which can be compared with three control ambient plots (Fig. 1; Kimball *et al.*, 2018). Warming treatment started in September 2016 and continued until hurricanes Irma and Maria hit the island in September 2017, causing heavy damage to the experimental infrastructure. Warming was halted for a year before restarting in September 2018. Before the hurricanes, surface soils (0–10 cm) in warming plots were warmed 3.6°C above ambient soils (Kimball *et al.*, 2018). After the hurricanes, the IR heaters were initially 2.5 m above the ground and moved to their max height of 4 m above the ground in July 2020. As understory vegetation height and foliage density increased, the warming effect in surface soils was reduced to *c.* +1°C (Tunison *et al.*, 2024). At the time of the study (summer 2023), surface soils of the warming plots were 2°C warmer than the surface soils of the ambient plots (Wood *et al.*, 2024). Therefore, at the time of the study, soil has experienced a legacy of 7 yr of warming treatment starting at 4°C and slowly decreasing to 1°C and a legacy of hurricane disturbances. These experimental conditions enable the study of the effects of warming and hurricane disturbances on various ecosystem processes in this tropical forest (Reed *et al.*, 2020; Tunison *et al.*, 2024).

Field collections

In July 2023, one leaf litter sample and one soil sample were collected from each of the four quadrants in each of the six plots (Fig. 1a). Using sterilized nitrile gloves, *c.* 4 g of leaf litter and 13 g of soil were collected from the surface at the center of each quadrant with a sterilized spoon. Samples were not collected to a specific depth; rather, we collected the same mass of surface soil and the same mass of leaf litter, ensuring the collection of the entire leaf litter layer (there was no difference in leaf litter thickness visible between ambient and warming plots). Four additional leaf litter and four additional soil samples were collected *c.* 2 m in a random direction away from two of the warming plots and away from two of the control plots (for baseline ethylene concentration measurements used to correct N-fixation rates as described below). Each sample was immediately inserted into a 55-ml clear acrylic tube and returned to the on-site laboratory at Sabana Field Station, where an acetylene reduction assay was used to estimate asymbiotic N-fixation rates (see the N-fixation rate section; Hardy *et al.*, 1968; Reed *et al.*, 2010). Following this, samples were subsampled, and a portion was dried to calculate moisture content; these subsamples were then ground to calculate N, C, and phosphorus (P) content (see the C, N, and P content section). The remaining samples were stored in individual bags in a −80°C freezer before being shipped on dry ice overnight to Oklahoma State University for characterization of the microbial community composition (see the Microbial community composition section).

N-fixation rate

The fixation rates of 24 leaf litter and 24 soil samples were analyzed using an acetylene reduction assay (Hardy *et al.*, 1968; Reed *et al.*, 2010). Each 55-ml tube was sealed with a lid fitted with a septum, injected with 3.0 ml acetylene (creating *c.* 10% headspace concentration), and vented to the atmosphere. The additional samples collected away from four of the plots, eight in total, were not injected with acetylene so measurements of ethylene produced in the absence of acetylene could be determined. Eight blank samples were created by injecting acetylene into tubes without samples inside so measurements of acetylene reduced to ethylene in the absence of samples could be determined. After about an 18-h incubation in the laboratory at *c.* 26°C (exact incubation time measurements described later), headspaces of all 64 samples (48 plot samples, 8 control samples, and 8 blank samples) were subsampled and injected into Exetainer tubes. These Exetainer headspace gas samples were transported to the USGS Southwest Biological Science Center (SBSC) in Moab, Utah, and analyzed for ethylene concentration on a gas chromatograph (Shimadzu GC-14A equipped with a Flame Ionization Detector; Shimadzu Corp., Kyoto, Japan).

The baseline ethylene concentrations of the additional soil and leaf litter samples and blank samples were averaged, respectively. To account for ethylene production by soil (or leaf litter) alone, as well as for ethylene production in the absence of a sample, the average baseline ethylene concentration of the additional soil (or leaf litter) samples and blank samples was subtracted from the baseline ethylene concentration of plot soil (or leaf litter) samples to obtain the adjusted ethylene concentration for each sample.

Soil samples were compacted at the bottom of each tube, and headspace volume was calculated via the measured height of empty space in each tube: from the soil surface to the bottom of the lid. Leaf litter samples were packed loosely and a headspace volume of 25 ml was assumed. Dry weights for each sample were calculated by dividing the total wet weight of each sample by its water content expressed in % plus 1 (see C, N, and P content for method). Exact incubation times for each sample were calculated by measuring time at acetylene injection and time at headspace sampling. The adjusted baseline ethylene concentrations were converted to parts per million (ppm) of ethylene using the calibration curve calculated from standard samples. Nanomoles of ethylene produced per gram of sample per hour were then calculated using the adjusted ethylene concentration (ppm), headspace volume (ml), dry weight (g), and incubation time (h) of each sample.

Each mole of ethylene produced is equivalent to 1 mole of acetylene reduced. Acetylene reduction rates were then converted to N-fixation rates by dividing moles of ethylene produced by three following stoichiometric balance (Hardy *et al.*, 1968). By using three across the samples as commonly found in soil and litter samples, we are assuming that despite changes in the microbial community, the stoichiometric balance remains unchanged (Soper *et al.*, 2021a). Finally, we converted these rates to more commonly reported units to allow comparison across studies. Biological N fixation is often given in kg of N ha^{-1} yr^{-1} using the molar mass of N_2 (28 g mol^{-1}) and making assumptions of homogeneity across spatial and temporal scales (Soper *et al.*, 2021b). In particular, we used the area of the sample (for both soil and leaf litter samples) in the incubation tube to calculate rates over 1 ha.

C, N, and P content

After the acetylene reduction treatment, a 2 ± 0.5 g subsample was taken from each soil sample, and a 1 ± 0.25 g subsample was taken from each leaf litter sample. The remaining sample material was frozen in a −80°C freezer. Subsamples were dried at 60°C for 48 h and were used to calculate % water content. These subsamples were then pooled by type and plot and ground using a SPEX SamplePrep 8000D dual mixer/mill (SPEX SamplePrep LLC, Metuchen, NJ, USA). All ground samples were sent to the USGS SBSC, and total C and N content was determined using an Elementar Vario Micro Cube (Elementar Americas Inc., Ronkonkoma, NY, USA). The ground samples were then sent to the

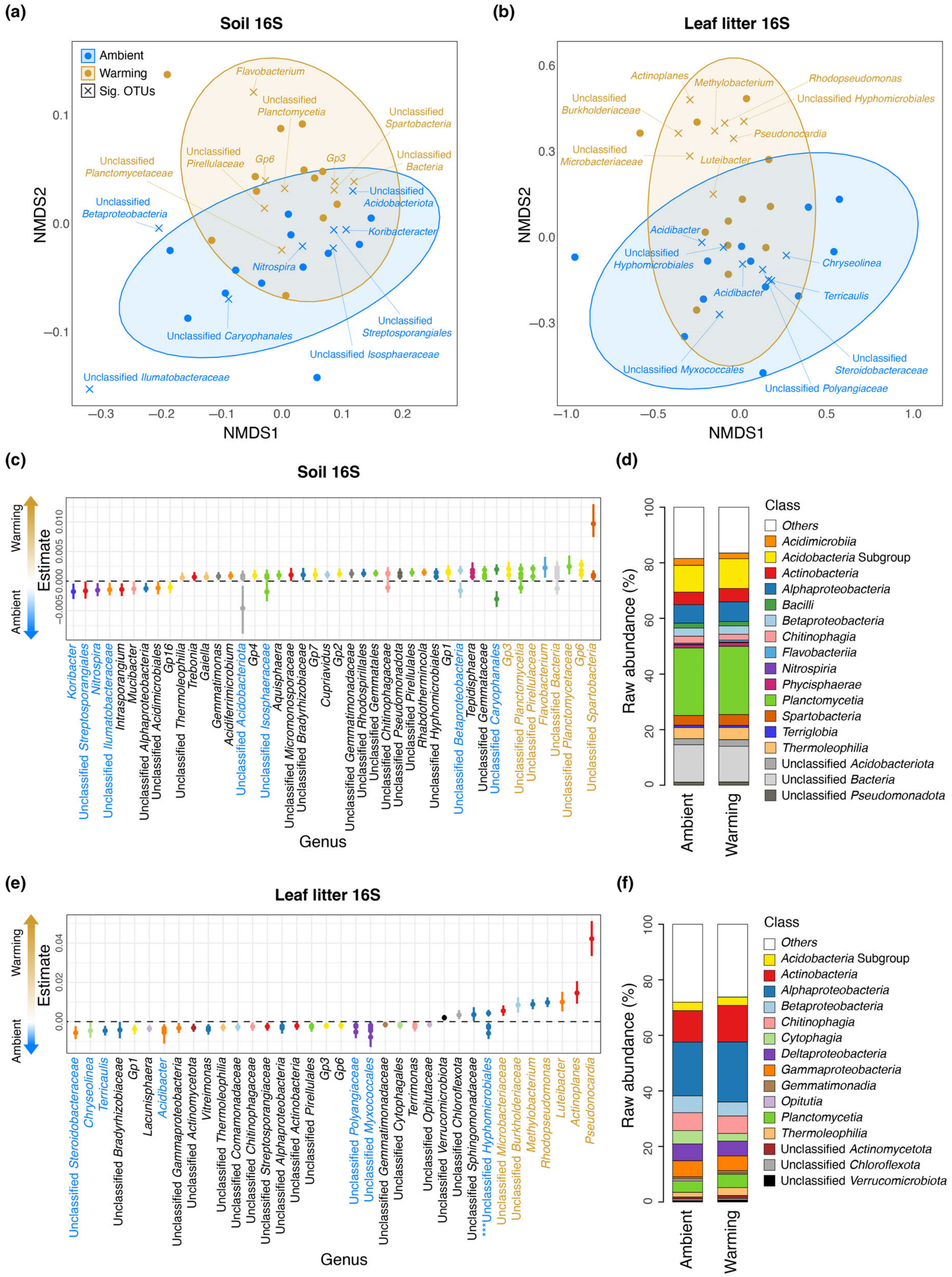

Fig. 2 Differences in overall bacterial community composition elucidated by 16S rRNA gene sequencing of soil and leaf litter samples. Nonmetric multidimensional scaling plots depicting samples and significantly altered operational taxonomic units (OTUs) (Sig. OTUs) show that bacterial communities are distinct between ambient and warming treatments (a, b). Forest plots of significantly altered OTUs, listed by genus and colored by class, show how different genera respond to ambient vs warming conditions in soil (c) and leaf litter (e). When in a genus, several OTUs were significantly altered by warming; we plotted each posterior distribution in the same genus location on the *x*-axis. Raw abundance plots show the composition of the bacterial community at the class level between ambient and warming plots in soil (d) and leaf litter (f). The points on the *x*-axis represent the median of the standardized regression coefficients associated with warming treatment, derived from the posterior distribution of generalized joint attribute modeling (GJAM) model parameters. Segments expand to the 95% credible intervals (CI). OTUs with 95% CI beneath 0 (dashed vertical line) are significantly more abundant under ambient conditions, and OTUs with 95% CI above 0 are more abundant under warming conditions. The eight most highly altered OTUs in ambient and warming are highlighted. The asterisks indicate class where individual OTUs responded positively and negatively to the warming condition.

BYU Environmental Analytical Lab, where they were subjected to nitric acid digestion in a Mars 6 Microwave (EPA method 3051A; CEM Corp., Matthews, NC, USA), and total P content was determined using a Thermo Scientific iCAP 7400 ICP-OES Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). This strong acid digestion method was found to perform well on a range of soil types (Church *et al*., 2017), although it might underestimate total P due to recalcitrant minerals.

Microbial community composition

Soil and leaf litter samples stored in the −80°C freezer were thawed in 4°C and homogenized. Genomic DNA was extracted from each of the samples in triplicate using a Qiagen DNeasy Power Soil Pro Kit (Qiagen). Additionally, along with both the soil and leaf litter extractions, the DNA extraction kit was run on blank controls containing no sample. Extractions were pooled for each sample and stored at −80°C until downstream processing.

DNA extractions were used to characterize the full bacterial community using the 16S rRNA gene and the N-fixing bacterial community using the *nifH* gene. Amplification and sequencing were carried out at the University of Minnesota Genomics Center (UMGC). Specifically, 16S rRNA gene sequences were amplified by PCR using the 16S primer set 515F-806R (Caporaso *et al*., 2011), which amplifies the variable V4 region of the 16S rRNA gene. The *nifH* gene, which encodes the reductase subunit of nitrogenase, the enzyme that catalyzes the reaction that fixes N, has been widely used as a genetic marker to study the diversity and abundance of N-fixing microbes in gene sequencing studies (Pajares & Bohannan, 2016). *NifH* gene sequences were amplified in two separate PCR steps to minimize the formation of undesirable primer dimers. First, the *nifH* gene was amplified using the *nifH* primer set Ueda19F-R6 (Sigma-Aldrich), a broadly inclusive pair (Angel *et al*., 2018). This was followed by PCR amplification using Nextera-adapted primers suited for Illumina sequencing using the UMGC standard PCR conditions.

The 16S and *nifH* gene amplicons underwent v.3 Illumina MiSeq 2 × 300 bp paired-end sequencing. Soil and leaf litter sequence data were processed separately using the MOTHUR MiSeq standard operating procedure (Schloss *et al*., 2009; Kozich *et al*., 2013). Briefly, paired-end reads were assembled for each amplicon and filtered to the expected amplicon length (291 base pairs (bp) for 16S and 394 bp for *nifH*). The trimmed 16S sequences were aligned to the reference sequence file Silva 132 database. The trimmed *nifH* sequences were aligned to the *nifH* sequence database described by Gaby & Buckley (2014). For both 16S rRNA and *nifH* genes, chimeras were removed using chimera.vsearch (MOTHUR), and the sequences were grouped into operational taxonomic units (OTUs) at 97% sequence similarity. OTUs with only one observation in a dataset were removed using remove.rare (MOTHUR). For 16S, taxonomy was classified in MOTHUR using v.19 of the Ribosomal Database Project (RDP) training set, publicly released in July 2023. For *nifH*, representative sequences from each OTU obtained using get.oturep (MOTHUR) were taxonomically classified by Basic Local Alignment Search Tool analysis against the NCBI GenBank database.

Seedling survey

In June 2015, all seedlings of at least 10 cm height present in the six plots were tagged, identified to species, and measured for height from ground level and stem diameter at ground level. After a year of drought (2015), the census was repeated annually starting June 2016 until 2023 (with the exception of 2020). Using the census data from 2023, we calculated species richness and Shannon diversity index within quadrants to see how plant communities in general influenced the shift in microbial community composition. We also measured crowding within quadrants due to N-fixing seedlings as the proportion of neighbors that are N fixers (number of N-fixing seedlings in quadrant/total number of seedlings in quadrant) and used these variables to also explain the shift in microbial community composition as these plants might have distinct effects on microbial communities (e.g. Zhou *et al*., 2017).

Statistical analyses

Community composition analyses were conducted in the R programming environment using the *gjam* and VEGAN packages (Clark *et al*., 2017; Oksanen *et al*., 2022). Each of the four bacterial communities was analyzed separately: soil overall bacterial community (soil 16S), soil N-fixing bacterial community (soil *nifH*), leaf litter overall bacterial community (litter 16S), and leaf litter N-fixing bacterial community (litter *nifH*). Before analyses, all OTUs appearing in the soil or leaf litter blank controls were removed from the soil and leaf litter OTU composition, respectively. Homogeneity in dispersion of sequence relative abundances in OTUs from the warming and ambient groups was confirmed using *betadisper* with the Bray–Curtis distance metric (Anderson & Walsh, 2013). Distance-based redundancy analyses (dbRDA) were applied to the OTU composition data using *capscale*, again with sequence relative abundances and the Bray–Curtis distance metric. The abiotic (treatment, moisture, C : N, and P content) and biotic (proportion of N-fixing seedlings, Shannon Index, and species richness

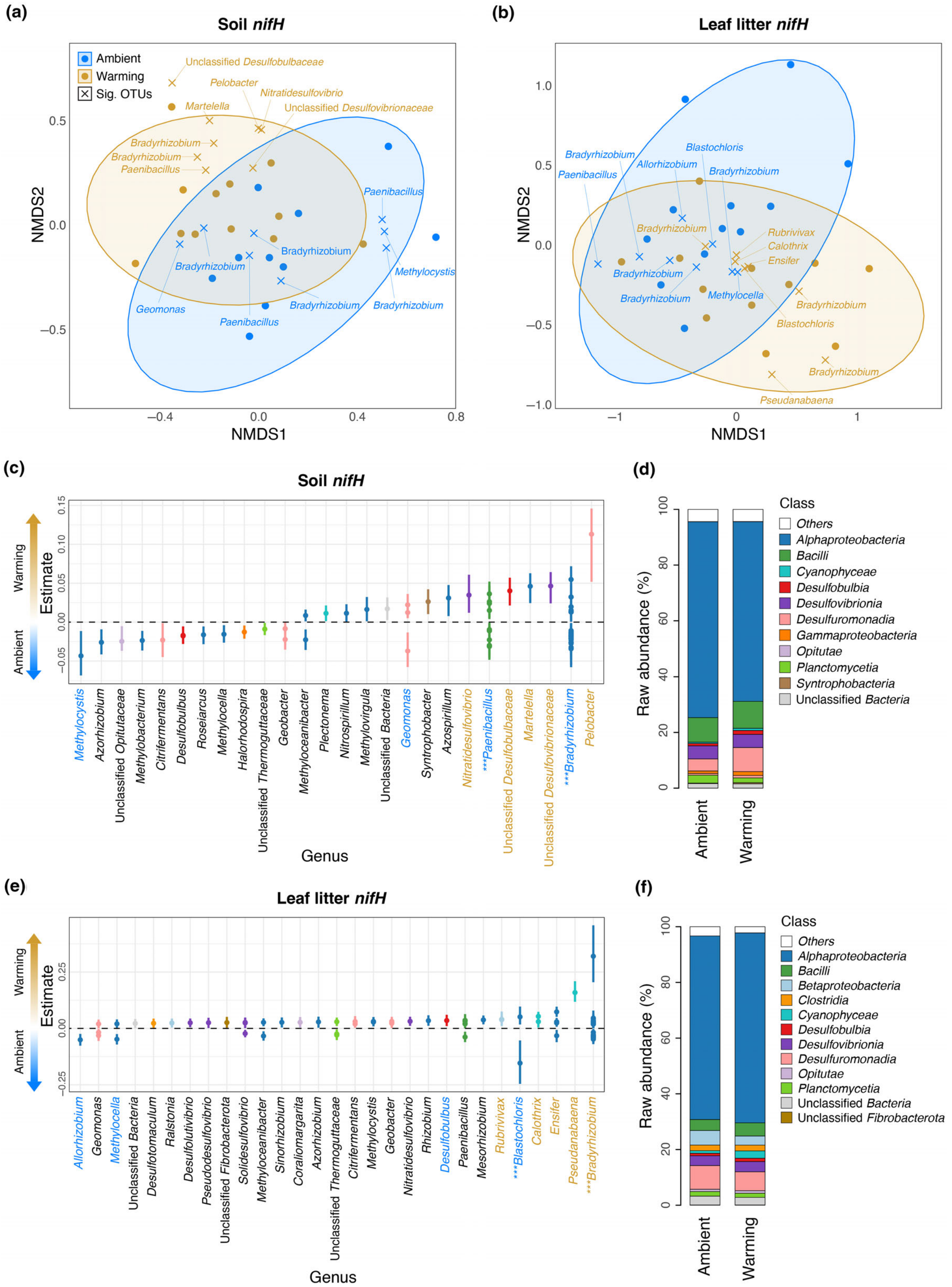

Fig. 3 Differences in the nitrogen (N)-fixing bacterial community composition determined by *nifH* gene sequencing of soil and leaf litter samples. Nonmetric multidimensional scaling plots show that bacterial communities are distinct between ambient and warming treatments (a, b). Forest plots of significantly altered operational taxonomic units (OTUs), listed by genus and colored by class, show how different genera respond to ambient vs warming conditions in soil (c) and leaf litter (e). When in a genus, several OTUs were significantly altered by warming; we plotted each posterior distribution in the same genus location on the *x*-axis. Raw abundance plots of the N-fixing bacterial community composition at the class level in ambient and warming plots in the soil (d) and in the leaf litter (f). The points on the *x*-axis represent the median of the standardized regression coefficients associated with warming treatment, derived from the posterior distribution of generalized joint attribute modeling (GJAM) model parameters. Segments expand to the 95% credible intervals (95% CI). OTUs with 95% CI beneath 0 (dashed vertical line) are significantly more abundant under ambient conditions, and OTUs with 95% CI above 0 are more abundant under warming conditions. The eight most highly altered OTUs in ambient and warming are highlighted. The asterisks indicate class where individual OTUs responded positively and negatively to the warming condition.

in the given quadrant) independent variables in the dbRDA model were z-transformed. Plot was used as a condition to account for the structure of the dataset. Permutation tests with 999 permutations determined which of these independent variables had significant effects on overall (16S) and N-fixing (*nifH*) bacterial composition.

Generalized joint attribute modeling (GJAM), with plot as a random effect to account for the structure in the dataset, was used to analyze how the treatment, C : N, and P content affected the bacterial communities. Briefly, GJAM uses a multivariate normal distribution that accounts for the co-dependence among organisms (Clark *et al.*, 2017). The response variable, Y_i, which is the bacterial composition matrix, is modeled using a latent variable w_i on the continuous scale: $w_i \sim MVN(\beta' X_i, \Sigma)$, where β is the species-by-predictor matrix of coefficients and Σ the organism covariance matrix. A set of partition points and label vectors are then used to assign each w_i to Y_i (Clark *et al.*, 2017). The GJAM analyses were conducted using Gibbs sampler (a Markov chain Monte Carlo technique; Geman & Geman, 1984) with 2000 iterations and a burn-in period of 200. Using this approach, we identified OTUs associated with a significantly decreasing or increasing relative abundance in the warming treatment (OTUs with 95% credible intervals (CI) not overlapping with 0). We jointly analyzed N-fixation rate of the samples and N-fixing bacterial community to investigate how N fixation was directly affected by the predictor variables (β) and indirectly via changes in bacterial composition that influences N fixation through the covariance matrix (Σ).

Results

Mixed linear regressions showed that edaphic conditions differed between warming and ambient plots and across sample type (Supporting Information Table S1; Fig. S1). Soil moisture was marginally lower in the warming than in the ambient plots (degree of freedom = 22, $t = -1.84$, P value = 0.08). Soil C : N ratio did not significantly differ across experimental conditions. Finally, P content was marginally and significantly lower in the warming than in ambient plots both in soil and leaf litter samples (degree of freedom = 1.68, $t = -4.31$, P value = 0.06 in soil, and degree of freedom = 1.88, $t = -6.84$, P value = 0.02 in leaf litter).

The 16S amplicon library for soil samples recovered 25 094 OTUs from 1883 812 rRNA sequences after quality trimming, removal of rare OTUs, and removal of OTUs that appeared in blank controls (Table S1). Each soil 16S sample yielded an average of 78 492 quality sequences. There were 673 unique bacterial genera identified, which were assigned to 27 bacterial phyla. The phyla Planctomycetota and Pseudomonadota dominated both sample types, with an average relative abundance of 25.73% and 18.13% of the full datasets, respectively. The 16S amplicon library for leaf litter samples recovered 34 857 OTUs from 3416 750 quality rRNA reads, and each leaf litter sample yielded an average of 142 364 quality sequences. There were 773 unique bacterial genera identified, which were assigned to 28 bacterial phyla. The phyla Pseudomonadota and Actinomycetota dominated both sample types, with an average relative abundance of 38.74% and 16.47% of the full datasets, respectively.

The *nifH* amplicon library for soil samples recovered 4495 OTUs from 47 158 quality sequences (this total excludes two ambient plot samples with too few quality reads that were excluded from all analyses), and each soil sample yielded an average of 2143 quality sequences. Among OTUs that were classified (99.16%), there were 208 unique genera identified, which were assigned to 16 bacterial phyla. The phyla Pseudomonadota and Thermodesulfobacteriota dominated both sample types, with an average relative abundance of 71.87% and 10.55%, respectively, in ambient plot samples, and 66.86% and 15.62%, respectively, in warming plot samples. The *nifH* amplicon library for leaf litter samples recovered 8811 OTUs from 167 914 quality sequences, and each leaf litter sample yielded an average of 6996 quality sequences. The 98.68% of OTUs were assigned to classified, cultured bacteria using BLAST. Two hundred and twenty-three unique genera were identified, assigned to 17 bacterial phyla. Again, the phyla Pseudomonadota and Thermodesulfobacteriota dominated both sample types, with an average relative abundance of 71.89% and 12.81%, respectively.

Warming significantly alters bacterial community composition and function

Warming significantly altered both the overall and N-fixing bacterial community composition in soil and leaf litter (Figs 2a,b, 3a,b; Table S2). GJAM of the full overall bacterial community identified 16 OTUs in the soil and 44 OTUs in the leaf litter that were significantly associated with the ambient plots (Fig. 2c–f). By contrast, there were 71 OTUs in the soil and 12 OTUs in the leaf litter that were significantly associated with the warming plots (Fig. 2c–f). These OTUs were distributed across several genera and classes. Notably, OTUs in the class Spartobacteria and in the genus *Pseudonocardia* from the class Actinobacteria were significantly more abundant in warming plots in soil and leaf litter samples, respectively. These OTUs also showed contrasting responses to edaphic factors (C : N and P; Fig. 4a,b). In the soil, OTUs associated with the warming plots tended to be negatively associated with soil C : N and positively associated with P content, while the opposite was true for OTUs associated with the ambient plots (Fig. 4a). In the leaf litter, OTUs associated with ambient or warming plots responded similarly with litter C : N and P (Fig. 4b); OTUs associated with warming plots were positively associated with litter C : N and P, and OTUs associated with ambient plots were negatively associated with litter C : N and P.

Similarly, GJAM modeling identified many OTUs of N-fixing bacteria that were significantly associated with warming or ambient plots in the soil and leaf litter (Figs 3c–f, 4c,d; significance occurs when the 95% CIs of the posterior distributions do not overlap with 0). Forty-seven OTUs in the soil and twenty OTUs in the leaf litter were significantly associated with the ambient plots, and 27 OTUs in the soil and 73 OTUs in the leaf litter were significantly associated with the warming plots. Notably, OTUs in the genera *Pelobacter* and *Pseudanabaena* were significantly more abundant in warming plots in soil and leaf litter samples, respectively. Several OTUs in the genus *Bradyrhizobium* were significantly more abundant in warming, but several were also more abundant in ambient. As with the overall bacterial community, OTUs associated with ambient or warming plots showed contrasting responses to soil C : N and P (Fig. 4c,d). In the soil, OTUs of N-fixing bacteria associated with the warming plots were positively associated with soil P and negatively associated with soil C : N, and the opposite was found for ambient plot OTUs of N-fixing bacteria (Fig. 4c). Finally, as with the overall bacterial community in the leaf litter, OTUs of N-fixing bacteria in the leaf litter associated with the warming plots also responded positively to litter C : N and P (Fig. 4d).

Effect of warming on asymbiotic nitrogen fixation rates

In soil, N-fixation rates increased from 0.007 to 0.01 nmol $N_2\,g^{-1}\,h^{-1}$ (or from 0.001 to 0.002 $kg\,ha^{-1}\,yr^{-1}$), or 55%, in the warming plots. GJAM showed that the direct effect of warming on N fixation was significant as assessed by the nonoverlapping of the CIs with 0, but the indirect effect, which is the effect of warming on N fixation mediated by changes in N-fixing bacterial composition, was not (Fig. 4e). Thus, the overall effect of warming was not considered significant in the soil. In leaf litter, N-fixation rates increased from 0.04 to 87.6 $nmol\,N_2\,g^{-1}\,h^{-1}$ (or from 0.007 to 14.518 $kg\,ha^{-1}\,yr^{-1}$), or 525%, in the warming plots. Both direct and indirect effects, and thus the overall effect, were significant (Fig. 4f). Specifically, the N-fixation rate was significantly increased by warming, and this effect was both due to the direct effect of warming on the N-fixation rate and the indirect effect of warming mediated by changes in the N-fixing bacterial community composition.

Discussion

Warming increased asymbiotic N-fixation rates by 55% in the soil and 525% in the leaf litter, and these increases were partially mediated by changes in the N-fixing bacterial community under warming, as shown by our generalized joint attribute model. Our estimates of N-fixation fall within the range of free-living N fixation found in tropical forests (0.1–60 $kg\,N\,ha^{-1}\,h^{-1}$ in Reed *et al.*, 2011), and our lowest value in the soil of the control plots is similar to the ones recently found in French Guiana (0.007 $nmol\,N_2\,g^{-1}\,h^{-1} = 0.001\,kg\,ha^{-1}\,yr^{-1}$; Van Langenhove *et al.*, 2021). Our highest estimate of N-fixation rates in the leaf litter in the warming plots (87.6 $nmol\,N_2\,g^{-1}\,h^{-1} = 2452.8\,ng\,N\,g^{-1}\,h^{-1} = 14.518\,kg\,ha^{-1}\,yr^{-1}$) is twice the rates found in P hot spots in Costa Rica ($< 1200\,ng\,N\,g^{-1}\,h^{-1}$ in Reed *et al.*, 2010). Both the overall and N-fixing bacterial communities were significantly altered by warming, even after accounting for other factors known to influence the asymbiotic bacterial community, such as moisture, soil C : N, and P. Warming explains up to 9% of the variance in bacterial community composition. Together, our results suggest that warming may increase N flux from the atmosphere to the ecosystem.

As hypothesized, warming increased N-fixation activity: This increase is likely due to the effect of warmer temperatures on both the microbial community metabolites and bacterial community composition. The direct effect of warming on N fixation was significantly positive in both the soil and leaf litter, and the effect of warming mediated by its effect on the N-fixing bacterial community was also significantly positive in the leaf litter. It is important to note that we accounted for inherent differences in water content across our samples when calculating N fixation and when investigating shifts in microbial community composition. Since little effect of moisture was detected, differences in soil and leaf litter results might arise from the impacts of warming on the plant communities (changes in plant communities directly lead to changes in leaf litter). For example, changes in plot communities as previously found at this site (Bachelot *et al.*, 2020; Alonso-Rodríguez *et al.*, 2022) could lead to leaf litter with different chemistry or different amounts of leaf litter which could explain the observed differences not only in N-fixation rates but also in total phosphorus (P). Additionally, soil and leaf litter often display different rates of N fixation, with leaf litter showing higher mass-based rates of N fixation (Reed *et al.*, 2013; Van Langenhove *et al.*, 2020) than soil. Therefore, leaf litter may have a high capacity for N fixation, allowing warming to increase fixation in leaf litter to a greater extent than in soil. This is consistent with the important role played by the litter layer in tropical humid forests in nutrient cycling (Wood *et al.*, 2009; Sayer *et al.*, 2024). The direct positive effect of warming on fixation observed in both environments may be due to increased enzyme activity (e.g., via increased enzyme abundance) and metabolism of N-fixing bacteria (Houlton *et al.*, 2008; Reed *et al.*, 2011; Wood *et al.*, 2019), as the indirect effect accounts for the changing N-fixing bacterial community structure.

This indirect effect of warming is consistent with findings that temperature is a predominant parameter governing the N-fixing

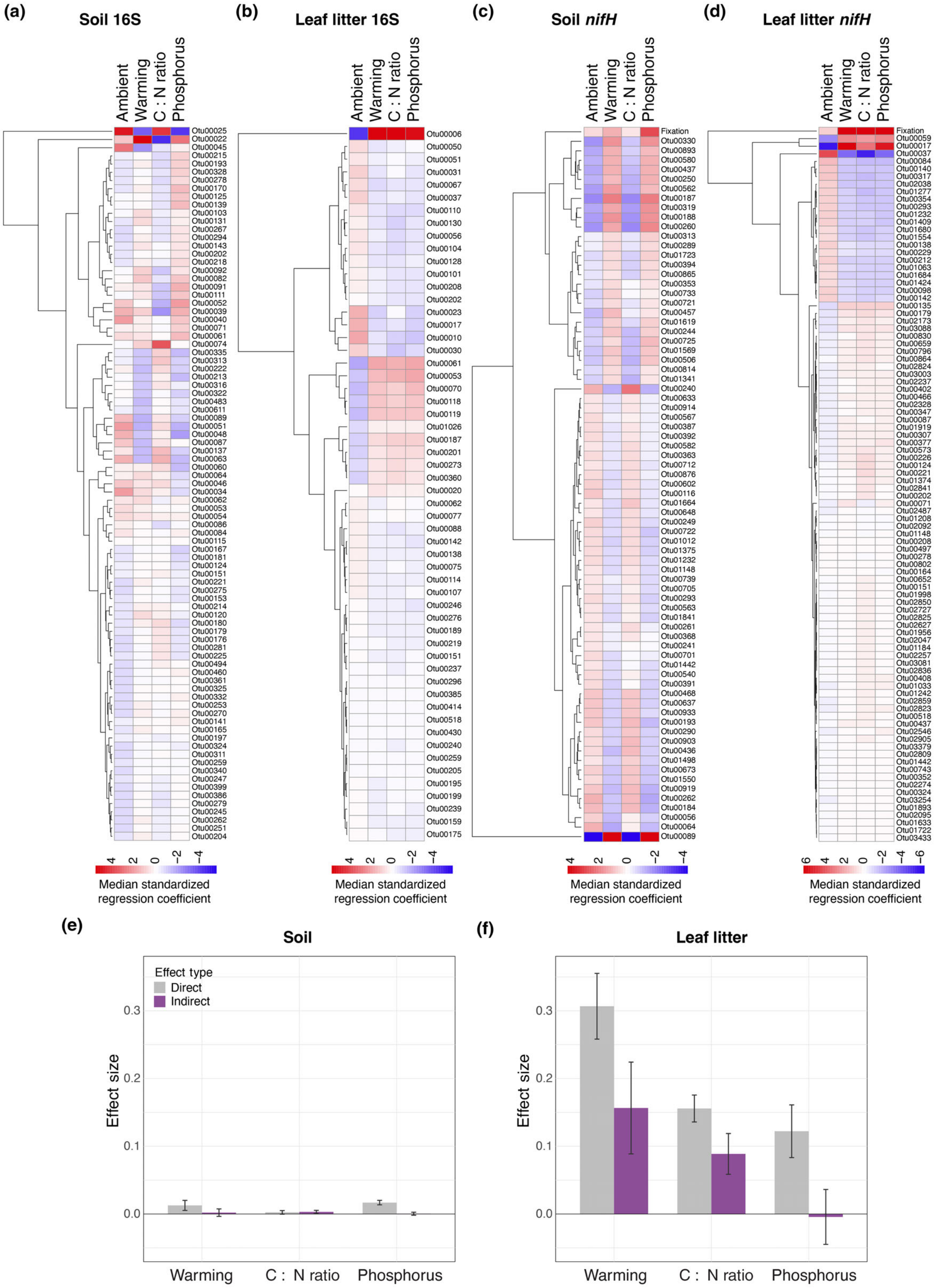

Fig. 4 Individual and global responses to warming conditions as well as contribution of the bacterial community to nitrogen (N) fixation. The heatmaps (a–d) represent the median of the standardized regression coefficients of the full generalized joint attribute modeling (GJAM) models (warming treatment, C : N, and phosphorus (P)) for the operational taxonomic units (OTUs) that were the most affected by these variables for the overall soil and litter bacterial community (a, b) and the soil and leaf litter N-fixing bacterial community (c, d). The N-fixing bacterial community was modeled jointly with N-fixation rates, and therefore, fixation is included in the response variables influenced by the model in the heatmaps (c, d). Finally, the direct (gray) and indirect (purple) effects of warming, C : N, and P content on N-fixation rates in (e) soil and (f) leaf litter samples from the GJAM models. Bars reach the median effect and segments expand to the 95% CI. C, carbon.

community (Zhao *et al*., 2020), and the N-fixing community structure is a strong predictor of N-fixation activity (Berthrong *et al*., 2014). *NifH* gene composition does not always correlate with fixation rates since bacteria containing *nifH* genes are not necessarily always fixing N (Feng *et al*., 2019), but in our study, warming-induced shifts in N-fixing community composition (e.g. warming explained 9% of N-fixing bacterial community composition in the leaf litter) did correlate with an increase in fixation. This is consistent with a previous study that found that variation in the N-fixing community structure accounted for differences in N-fixation rates across a climate gradient that included tropical regions (Wu *et al*., 2021). Finally, while the activity and composition of N-fixing microbes in tropical soils have been found to be regulated by moisture, C : N, and the availability of P (Pajares & Bohannan, 2016), our study shows that, even accounting for these parameters, warming generally had a strong effect on the activity and composition of N-fixing microbes in leaf litter.

The effects of warming on N-fixation rates were in part explained by changes in bacterial composition. We found that *in situ* experimental warming led to significant changes in both the overall and N-fixing bacterial community composition, as demonstrated by specific genera significantly associated with either the ambient or warming treatment. The taxa most highly associated with ambient plots in the soil include the nitrifying genus *Nitrospira* (Lücker *et al*., 2010) and the methanotrophic, N-fixing genus *Methylocystis* (Stein *et al*., 2011), which is also capable of nitrification. Additionally, Spartobacteria and *Pelobacter* were highly associated with warmed soils. Spartobacteria is an abundant, yet lesser studied class of bacteria that, based on genome analysis, can likely degrade various complex carbohydrates but likely has no role in any aspects of the N cycle (Herlemann *et al*., 2013; Cernava *et al*., 2017). *Pelobacter* is a lesser studied N-fixing genus that is capable of producing sulfide (Li *et al*., 2021). The taxa most associated with ambient plots in the leaf litter include the order Myxococcales, which has predicted roles in C and potassium cycling (Dai *et al*., 2024), and the symbiotic N-fixing genus *Allorhizobium* (Egamberdieva *et al*., 2015). Taxa highly associated with warming in the leaf litter include *Pseudonocardia* and *Bradyrhizobium*. The genus *Pseudonocardia* is associated with certain plant and ant species and has been found to be capable of asymbiotic N fixation (Chen *et al*., 2019). *Bradyrhizobium* is a symbiotic N-fixing genus that is also capable of asymbiotic fixation and denitrification (Zhang *et al*., 2023). Some species of *Bradyrhizobium* are known to be able to nodulate with tropical legumes, such as Inga species (Allen & Allen, 1939). *Bradyrhizobium* may be warm-adapted (Zhao *et al*., 2020), but some *Bradyrhizobium* OTUs were also associated with ambient plots, possibly because OTUs represent different bacterial strains. Therefore, an increase in *Bradyrhizobium* relative abundance could be due to increased growth of N-fixing seedlings and/or a preference of these bacteria for warm temperatures.

The possibility of different strains of the same genus being affected differently by warming can be demonstrated by the example of Paenibacillus, which, in soil, has several OTUs associated with ambient plots and several associated with warming plots (Fig. 3c). The genus *Paenibacillus* is well studied due to its

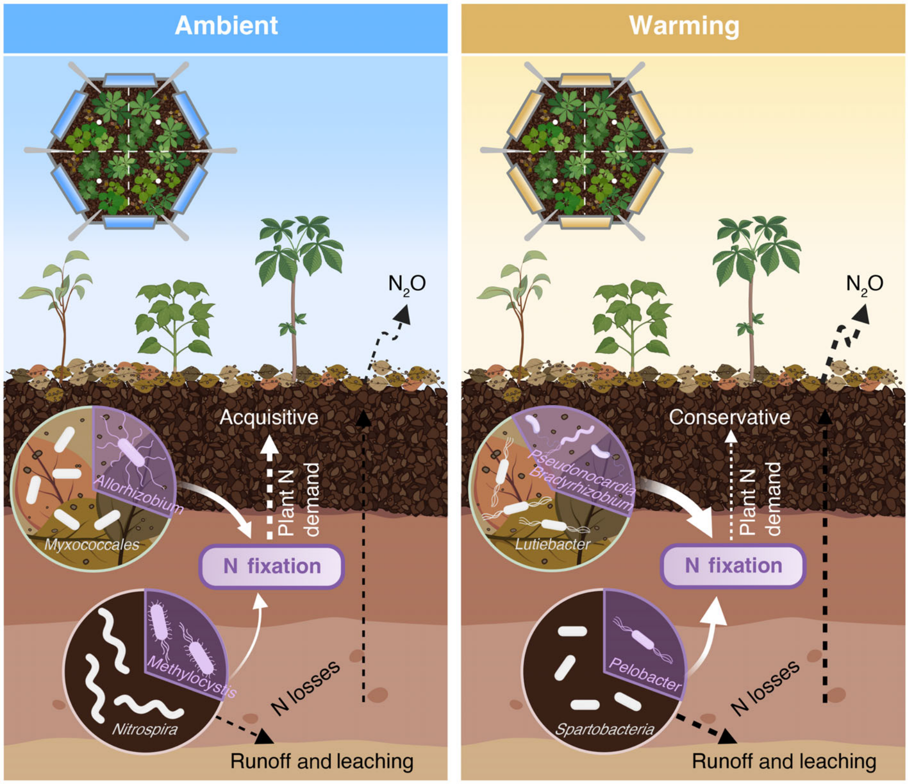

Fig. 5 Conceptual summary of the impacts of warming on the soil and leaf litter microbiome at the Tropical Responses to Altered Climate Experiment (TRACE) site. Warming-induced increase in nitrogen (N) fixation, especially in the leaf litter, in part correlated with change in the N-fixing bacteria community (bacteria in purple, gray bacteria do not fix N). Based on prior studies at the site, warming may also drive a shift from an acquisitive growth strategy to a conservative one (Bachelot et *al*., 2020; Alonso-Rodríguez et *al*., 2022), which suggests a decrease in plant N demand and could lead to an increase in N losses. Additionally, warming is hypothesized to be associated with an increase in nitrous oxide emissions (Aryal et *al*., 2022). Here, dashed arrows indicate hypothesized effects while solid arrows represent the results of this study. Arrow thickness represents the strength of the correlation. Created in BioRender. Bachelot, B. (2025) https://BioRender.com/smi82fq.

ability to enhance plant growth by N fixation and P solubilization (Ryu *et al.*, 2003). Further examination of specific OTUs in our study revealed that *Paenibacillus* strains associated with ambient plots were different from the *Paenibacillus* strains associated with warming plots. Warming may affect bacteria differentially even at the species level, emphasizing the need to further investigate *in situ* effects of warming on the bacterial community.

Understanding the consequences of increased N fixation under warming conditions requires us to investigate the whole-ecosystem level, including changes in plant demographics and microbial dynamics (Fig. 5). Our experimental design enables us to study the whole understory level response to warming as we are not only warming the soil (as in Nottingham *et al.*, 2023) but rather the whole understory, leading to changes in plant demographics and physiology (Bachelot *et al.*, 2020; Alonso-Rodríguez *et al.*, 2022; Hernandes Villani *et al.*, 2025). For example, prior studies at the site highlighted changes in plant demographics (Bachelot *et al.*, 2020) and composition (Alonso-Rodríguez *et al.*, 2022) consistent with a shift toward a conservative growth strategy in the warming plots. Conservative strategies take precedence when limited resources or stressful conditions prioritize minimized resource loss, leading to slow but sturdy and safe growth, as in diameter growth (Reich, 2014; Fagundes *et al.*, 2022). It is possible that N plant demands exceed microbial N production due to increased plant growth rates and/or due to increased N losses, leading to a conservative N utilization in plants (Lie *et al.*, 2021). Warming and anthropogenic N deposition have been associated with increased denitrification consistently across studies (Bai *et al.*, 2013; Elrys *et al.*, 2023; Cui *et al.*, 2024) and increased nitrous oxide emissions (Aryal *et al.*, 2022). It is therefore possible that the increase in N fixation is counterbalanced by increasing N losses through the system due to an overall accelerated N cycle (Cui *et al.*, 2024). Yet, many of these syntheses and meta-analyses emphasized the lack of data from tropical forest warming experiments. Therefore, the net effect of warming on the N cycle remains unclear in tropical forests without further studies integrating the asymbiotic and symbiotic N fixation in addition to mineralization, nitrification, and denitrification, as well as N assimilation by plants and even N leaching.

To conclude, our study provides insight into the effects of *in situ* warming on the asymbiotic N-fixing bacterial community and asymbiotic N-fixation rates, thus providing insights into how warming impacts N fixation in a tropical forest. Our combined results suggest that warming will increase N flux from the atmosphere into the ecosystem. Climate models predict that extremely high temperatures will occur more frequently in tropical regions than in other climatic regions (Anderson, 2012; Mora *et al.*, 2013), so scientists should predict how warming-induced alterations in N fixation in these regions will change ecosystem functions and contributions to global nutrient cycles in the future. Given that N fixation is only one aspect of the N cycle, future studies should investigate how warming influences the entire N cycle in tropical forests (Lie *et al.*, 2021).

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Competing interests

None declared.

Author contributions

PMB and BB designed this current study with inputs from TEW and SCR. TEW, SCR and MAC designed and conducted the warming experiment. TEW, IFG-P, SCR and BB oversaw the entire field season associated with this study. PB and SCR collected the data for N-fixation assays. PB analyzed all the data and wrote the first draft of the study. SS supported sequencing and data analysis. PB, SS and BB generated figures for data visualization and edited the manuscript. BB and SS revised the manuscript, incorporating reviewers' comments. All authors provided regular feedback on the manuscript and data analyses.

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Data availability

All data and scripts associated with this study are available upon request. Sequencing data have been deposited on NCBI:

PRJNA1193036. Additional datasets and scripts for the analyses have been deposited to Zenodo, doi: 10.5281/zenodo.14366667.

References

Allen ON, Allen EK. 1939. Root nodule bacteria of some tropical leguminous plants: II. Cross-inoculation tests within the cowpea group. *Soil Science* 47: 63–76.

Alonso-Rodríguez AM, Wood TE, Torres-Díaz J, Cavaleri MA, Reed SC, Bachelot B. 2022. Understory plant communities show resistance to drought, hurricanes, and experimental warming in a wet tropical forest. *Frontiers in Forests and Global Change* 5: 733967.

Anderson BT. 2012. Intensification of seasonal extremes given a 2°C global warming target. *Climatic Change* 112: 325–337.

Anderson MJ, Walsh DC. 2013. PERMANOVA, ANOSIM, and the Mantel test in the face of heterogeneous dispersions: what null hypothesis are you testing? *Ecological Monographs* 83: 557–574.

Angel R, Nepel M, Panhölzl C, Schmidt H, Herbold CW, Eichorst SA, Woebken D. 2018. Evaluation of primers targeting the diazotroph functional gene and development of NifMAP – a bioinformatics pipeline for analyzing nifH amplicon data. *Frontiers in Microbiology* 9: 703.

Aryal B, Gurung R, Camargo AF, Fongaro G, Treichel H, Mainali B, Angove MJ, Ngo HH, Guo W, Puadel SR. 2022. Nitrous oxide emission in altered nitrogen cycle and implications for climate change. *Environmental Pollution* 314: 120272.

Bachelot B, Alonso-Rodríguez AM, Aldrich-Wolfe L, Cavaleri MA, Reed SC, Wood TE. 2020. Altered climate leads to positive density-dependent feedbacks in a tropical wet forest. *Global Change Biology* 26: 3417–3428.

Bai E, Li S, Xu W, Li W, Dai W, Jiang P. 2013. A meta-analysis of experimental warming effects on terrestrial nitrogen pools and dynamics. *New Phytologist* 199: 441–451.

Batterman SA, Hedin LO, Van Breugel M, Ransijn J, Craven DJ, Hall JS. 2013. Key role of symbiotic dinitrogen fixation in tropical forest secondary succession. *Nature* 502: 224–227.

Berthrong ST, Yeager CM, Gallegos-Graves L, Steven B, Eichorst SA, Jackson RB, Kuske CR. 2014. Nitrogen fertilization has a stronger effect on soil nitrogen-fixing bacterial communities than elevated atmospheric CO_2. *Applied and Environmental Microbiology* 80: 3103–3112.

Bytnerowicz TA, Akana PR, Griffin KL, Menge DNL. 2022. Temperature sensitivity of woody nitrogen fixation across species and growing temperatures. *Nature Plants* 8: 209–216.

Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, Fierer N, Knight R. 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences, USA* 108: 4516–4522.

Castro HF, Classen AT, Austin EE, Norby RJ, Schadt CW. 2010. Soil microbial community responses to multiple experimental climate change drivers. *Applied and Environmental Microbiology* 76: 999–1007.

Cernava T, Erlacher A, Aschenbrenner IA, Krug L, Lassek C, Riedel K, Grube M, Berg G. 2017. Deciphering functional diversification within the lichen microbiota by meta-omics. *Microbiome* 5: 82.

Chen H, Yang ZK, Yip D, Morris RH, Lebreux SJ, Cregger MA, Klingeman DM, Hui D, Hettich RL, Wilhelm SW *et al.* 2019. One-time nitrogen fertilization shifts switchgrass soil microbiomes within a context of larger spatial and temporal variation. *PLoS ONE* 14: e0211310.

Church C, Spargo J, Fishel S. 2017. Strong acid extraction methods for "total phosphorus" in soils: EPA method 3050B and EPA method 3051. *Agricultural & Environmental Letters* 2: 160037.

Clark JS, Nemergut D, Seyednasrollah B, Turner PJ, Zhang S. 2017. Generalized joint attribute modeling for biodiversity analysis: median-zero, multivariate, multifarious data. *Ecological Monographs* 87: 34–56.

Contosta AR, Frey SD, Cooper AB. 2015. Soil microbial communities vary as much over time as with chronic warming and nitrogen additions. *Soil Biology and Biochemistry* 88: 19–24.

Cui J, Deng O, Zheng M, Zhang X, Bian Z, Pan N, Tian H, Xu J, Gu B. 2024. Warming exacerbates global inequality in forest carbon and nitrogen cycles. *Nature Communications* 15: 9185.

Dai W, Liu Y, Yao D, Wang N, Shao J, Ye X, Cui Z, Zong H, Tian L, Chen X *et al.* 2024. Biogeographic distribution, assembly processes and potential nutrient cycling functions of myxobacteria communities in typical agricultural soils in China. *Science of the Total Environment* 906: 167255.

De Vries FT, Griffiths RI. 2018. Impacts of climate change on soil microbial communities and their functioning. In: Horwath WR, Kuzyakov Y, eds. *Developments in soil science, vol. 35.* Amsterdam, the Netherlands: Elsevier, 111–129.

Deutsch C, Inomura K, Luo YW, Wang YP. 2024. Projecting global biological N_2 fixation under climate warming across land and ocean. *Trends in Microbiology* 32: 546–553.

Dynarski KA, Houlton BZ. 2018. Nutrient limitation of terrestrial free-living nitrogen fixation. *New Phytologist* 217: 1050–1061.

Egamberdieva D, Shrivastava S, Varma A, eds. 2015. *Plant-growth-promoting rhizobacteria (PGPR) and medicinal plants, vol. 42.* Cham, Switzerland: Springer.

Elrys AS, Zhu Q, Jiang C, Liu J, Sobhy HH, Shen Q, Sobhy HHH, Uwiragiye Y, Wu Y, El-Tarabily KA *et al.* 2023. Global soil nitrogen cycle pattern and nitrogen enrichment effects: tropical versus subtropical forests. *Global Change Biology* 29: 1905–1921.

Fagundes MV, Souza AF, Oliveira RS, Ganade G. 2022. Functional traits above and below ground allow species with distinct ecological strategies to coexist in the largest seasonally dry tropical forest in the Americas. *Frontiers in Forests and Global Change* 5: 930099.

Feng J, Penton CR, He Z, Van Nostrand JD, Yuan MM, Wu L, Wang C, Qin Y, Shi ZJ, Guo X *et al.* 2019. Long-term warming in Alaska enlarges the diazotrophic community in deep soils. *MBio* 10: e02521-18.

Gaby JC, Buckley DH. 2014. A comprehensive aligned nifH gene database: a multipurpose tool for studies of nitrogen-fixing bacteria. *Database* 2014: bau001.

Geman S, Geman D. 1984. Stochastic relaxation, Gibbs distributions, and the Bayesian restoration of images. *IEEE Transactions on Pattern Analysis and Machine Intelligence* 6: 721–741.

Hardy RWF, Holsten RD, Jackson EK, Burns RC. 1968. The acetylene-ethylene assay for N_2 fixation: laboratory and field evaluation. *Plant Physiology* 43: 1185–1207.

Herlemann DPR, Lundin D, Labrenz M, Jürgens K, Zheng Z, Aspeborg H, Andersson AF. 2013. Metagenomic *de novo* assembly of an aquatic representative of the verrucomicrobial class *Spartobacteria*. *MBio* 4: e00569-12.

Hernandes Villani G, Grullón-Penkova IF, Bartz P, Masanga J, Lasky JR, Cavaleri MA, Wood TE, Bachelot B. 2025. Tropical forest soil microbiome modulates leaf heat tolerance more strongly under warming than ambient conditions. *Ecology and Evolution* 15: e71425.

Houlton BZ, Wang Y-P, Vitousek PM, Field CB. 2008. A unifying framework for dinitrogen fixation in the terrestrial biosphere. *Nature* 454: 327–330.

Jansson JK, Hofmockel KS. 2020. Soil microbiomes and climate change. *Nature Reviews Microbiology* 18: 35–46.

Kimball BA, Alonso-Rodríguez AM, Cavaleri MA, Reed SC, González G, Wood TE. 2018. Infrared heater system for warming tropical forest understory plants and soils. *Ecology and Evolution* 8: 1932–1944.

Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Applied and Environmental Microbiology* 79: 5112–5120.

LeBauer DS, Treseder KK. 2008. Nitrogen limitation of net primary productivity in terrestrial ecosystems is globally distributed. *Ecology* 89: 371–379.

Li X, Bai D, Deng Q, Cao X, Zhou Y, Song C. 2021. DNRA was limited by sulfide and nrfA abundance in sediments of Xiamen Bay where heterotrophic sulfide-producing genus (Pelobacter) prevailed among DNRA bacteria. *Journal of Soils and Sediments* 21: 3493–3504.

Lie Z, Huang W, Liu X, Zhou G, Yan J, Li Y, Huang C, Wu T, Fang X, Zhao M *et al.* 2021. Warming leads to more closed nitrogen cycling in nitrogen-rich tropical forests. *Global Change Biology* 27: 664–674.

Liu Y, Wang C, He N, Wen X, Gao Y, Li S, Niu S, Butterbach-Bahl K, Luo Y, Yu G. 2016. A global synthesis of the rate and temperature sensitivity of soil nitrogen mineralization, latitudinal patterns and mechanisms. *Global Change Biology* **23**: 455–464.

Lücker S, Wagner M, Maixner F, Pelletier E, Koch H, Vacherie B, Rattei T, Damsté JSS, Spieck E, Le Paslier D *et al.* 2010. A *Nitrospira* metagenome illuminates the physiology and evolution of globally important nitrite-oxidizing bacteria. *Proceedings of the National Academy of Sciences, USA* **107**: 13479–13484.

Ma S, Verheyen K, Props R, Wasof S, Vanhellemont M, Boeckx P, Boon N, De Frenne P. 2018. Plant and soil microbe responses to light, warming and nitrogen addition in a temperate forest. *Functional Ecology* **32**: 1293–1303.

Matson AL, Corre MD, Burneo JI, Veldkamp E. 2015. Free-living nitrogen fixation responds to elevated nutrient inputs in tropical montane forest floor and canopy soils of southern Ecuador. *Biogeochemistry* **122**: 281–294.

Menge DN, Wolf AA, Funk JL. 2015. Diversity of nitrogen fixation strategies in Mediterranean legumes. *Nature Plants* **1**: 1–5.

Mirza BS, Potisap C, Nüsslein K, Bohannan BJM, Rodrigues JLM. 2014. Response of free-living nitrogen-fixing microorganisms to land use change in the Amazon rainforest. *Applied and Environmental Microbiology* **80**: 281–288.

Mora C, Frazier AG, Longman RJ, Dacks RS, Walton MM, Tong EJ, Sanchez JJ, Kaiser LR, Stender YO, Anderson JM *et al.* 2013. The projected timing of climate departure from recent variability. *Nature* **502**: 183–187.

Moreira JCF, Brum M, De Almeida LC, Barrera-Berdugo S, De Souza AA, De Camargo PB, Oliveira RS, Alves LF, Rosado BHP, Lambais MR. 2021. Asymbiotic nitrogen fixation in the phyllosphere of the Amazon forest: changing nitrogen cycle paradigms. *Science of the Total Environment* **773**: 145066.

Nottingham AT, Montero-Sanchez M, Slot M, Szczygieł HA, Velasquez E, Meir P. 2023. Seedling growth declines in warmed tropical forest soils. *Biotropica* **55**: 897–910.

Oksanen J, Simpson GL, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, Solymos P, Stevens MHH, Szoecs E *et al.* 2022. *VEGAN: community ecology package (R package v.2.6-4)*. [WWW document] URL https://CRAN.R-project.org/package=vegan

Pajares S, Bohannan BJM. 2016. Ecology of nitrogen fixing, nitrifying, and denitrifying microorganisms in tropical forest soils. *Frontiers in Microbiology* **7**: 1045.

Penton CR, St Louis D, Pham A, Cole JR, Wu L, Luo Y, Schuur EAG, Zhou J, Tiedje JM. 2015. Denitrifying and diazotrophic community responses to artificial warming in permafrost and tallgrass prairie soils. *Frontiers in Microbiology* **6**: 746.

Reed SC, Cleveland CC, Townsend AR. 2007. Controls over leaf litter and soil nitrogen fixation in two lowland tropical rain forests. *Biotropica* **39**: 585–592.

Reed SC, Cleveland CC, Townsend AR. 2011. Functional ecology of free-living nitrogen fixation: a contemporary perspective. *Annual Review of Ecology, Evolution, and Systematics* **42**: 489–512.

Reed SC, Cleveland CC, Townsend AR. 2013. Relationships among phosphorus, molybdenum and free-living nitrogen fixation in tropical rain forests: results from observational and experimental analyses. *Biogeochemistry* **114**: 135–147.

Reed SC, Reibold R, Cavaleri MA, Alonso-Rodríguez AM, Berberich ME, Wood TE. 2020. Soil biogeochemical responses of a tropical forest to warming and hurricane disturbance. In: Dumbrell AJ, Turner EC, Fayle TM, eds. *Advances in ecological research, vol. 62*. Amsterdam, the Netherlands: Elsevier, 225–252.

Reed SC, Townsend AR, Cleveland CC, Nemergut DR. 2010. Microbial community shifts influence patterns in tropical forest nitrogen fixation. *Oecologia* **164**: 521–531.

Reich PB. 2014. The world-wide 'fast–slow' plant economics spectrum: a traits manifesto. *Journal of Ecology* **102**: 275–301.

Reis CRG, Pacheco FS, Reed SC, Tejada G, Nardoto GB, Forti MC, Ometto JP. 2020. Biological nitrogen fixation across major biomes in Latin America: patterns and global change effects. *Science of the Total Environment* **746**: 140998.

Rousk K, Sorensen PL, Michelsen A. 2018. What drives biological nitrogen fixation in high arctic tundra: moisture or temperature? *Ecosphere* **9**: e02117.

Ryu CM, Farag MA, Hu CH, Reddy MS, Wei HX, Paré PW, Kloepper JW. 2003. Bacterial volatiles promote growth in *Arabidopsis*. *Proceedings of the National Academy of Sciences, USA* **100**: 4927–4932.

Sayer EJ, Leitman SF, Wright SJ, Rodtassana C, Vincent AG, Brechet LM, Castro B, Lopez O, Wallwork A, Tanner EV. 2024. Tropical forest above-ground productivity is maintained by nutrients cycled in litter. *Journal of Ecology* **112**: 690–700.

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ *et al.* 2009. Introducing MOTHUR: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology* **75**: 7537–7541.

Soper FM, Simon C, Jauss V. 2021a. Measuring nitrogen fixation by the acetylene reduction assay (ARA): is 3 the magic ratio? *Biogeochemistry* **152**: 345–351.

Soper FM, Taylor BN, Winbourne JB, Wong MY, Dynarski KA, Reis CR, Reis CRG, Peoples MB, Cleveland CC, Reed SC *et al.* 2021b. A roadmap for sampling and scaling biological nitrogen fixation in terrestrial ecosystems. *Methods in Ecology and Evolution* **12**: 1122–1137.

Stein LY, Bringel F, DiSpirito AA, Han S, Jetten MSM, Kalyuzhnaya MG, Kits KD, Klotz MG, Op Den Camp HJM, Semrau JD *et al.* 2011. Genome sequence of the methanotrophic alphaproteobacterium *Methylocystis* sp. strain Rockwell (ATCC 49242). *Journal of Bacteriology* **193**: 2668–2669.

Tamme R, Pärtel M, Kõljalg U, Laanisto L, Liira J, Mander Ü, Moora M, Niinemets Ü, Öpik M, Ostonen I *et al.* 2021. Global macroecology of nitrogen-fixing plants. *Global Ecology and Biogeography* **30**: 514–526.

Taylor BN, Chazdon RL, Bachelot B, Menge DNL. 2017. Nitrogen-fixing trees inhibit growth of regenerating Costa Rican rainforests. *Proceedings of the National Academy of Sciences, USA* **114**: 8817–8822.

Tunison R, Wood TE, Reed SC, Cavaleri MA. 2024. Respiratory acclimation of tropical forest roots in response to *in situ* experimental warming and hurricane disturbance. *Ecosystems* **27**: 168–184.

Van Langenhove L, Depaepe T, Verryckt LT, Fuchslueger L, Donald J, Leroy C, Krishna Moorthy SM, Gargallo-Garriga A, Ellwood MDF, Verbeeck H *et al.* 2021. Comparable canopy and soil free-living nitrogen fixation rates in a lowland tropical forest. *Science of the Total Environment* **754**: 142202.

Van Langenhove L, Depaepe T, Vicca S, Van Den Berge J, Stahl C, Courtois E, Weedon J, Urbina I, Grau O, Asensio D *et al.* 2020. Regulation of nitrogen fixation from free-living organisms in soil and leaf litter of two tropical forests of the Guiana shield. *Plant and Soil* **450**: 93–110.

Winbourne JB, Harrison MT, Sullivan BW, Alvarez-Clare S, Lins SR, Martinelli L, Nasto M, Piotto D, Rolim S, Wong M *et al.* 2018. A new framework for evaluating estimates of symbiotic nitrogen fixation in forests. *The American Naturalist* **192**: 618–629.

Wood TE, Cavaleri MA, Giardina CP, Khan S, Mohan JE, Nottingham AT, Reed SC, Slot M. 2019. Soil warming effects on tropical forests with highly weathered soils. In: Mohan JE, ed. *Ecosystem consequences of soil warming*. Amsterdam, the Netherlands: Elsevier, 385–439. doi: 10.1016/B978-0-12-813493-1.00015-6.

Wood TE, Cavaleri MA, Reed SC. 2012. Tropical forest carbon balance in a warmer world: a critical review spanning microbial- to ecosystem-scale processes. *Biological Reviews* **87**: 912–927.

Wood TE, Cavaleri MA, Reed SC. 2024. Plot-level soil temperature and moisture data for a tropical forest understory warming experiment (TRACE) in Luquillo, Puerto Rico, 2015-2023. Effects of hurricane disturbance and increased temperature on carbon cycling and storage of a Puerto Rican forest: a mechanistic investigation of above- and belowground processes. *ESS-DIVE repository. Dataset*. doi: 10.15485/2478050.

Wood TE, Lawrence D, Clark DA, Chazdon RL. 2009. Rain forest nutrient cycling and productivity in response to large-scale litter manipulation. *Ecology* **90**: 109–121.

Wu C, Wei X, Hu Z, Liu Y, Hu Y, Qin H, Chen X, Wu J, Ge T, Zhran M *et al.* 2021. Diazotrophic community variation underlies differences in nitrogen fixation potential in paddy soils across a climatic gradient in China. *Microbial Ecology* **81**: 425–436.

Wurzburger N, Bellenger JP, Kraepiel AML, Hedin LO. 2012. Molybdenum and phosphorus interact to constrain asymbiotic nitrogen fixation in tropical forests. *PLoS ONE* 7: e33710.

Yaffar D, Norby RJ. 2020. A historical and comparative review of 50 years of root data collection in Puerto Rico. *Biotropica* 52: 563–576.

Zhang N, Jin C-Z, Zhuo Y, Li T, Jin F-J, Lee H-G, Jin L. 2023. Genetic diversity into a novel free-living species of *Bradyrhizobium* from contaminated freshwater sediment. *Frontiers in Microbiology* 14: 1295854.

Zhao W, Kou Y, Wang X, Wu Y, Bing H, Liu Q. 2020. Broad-scale distribution of diazotrophic communities is driven more by aridity index and temperature than by soil properties across various forests. *Global Ecology and Biogeography* 29: 2119–2130.

Zheng M, Zhou Z, Luo Y, Zhao P, Mo J. 2019. Global pattern and controls of biological nitrogen fixation under nutrient enrichment: a meta-analysis. *Global Change Biology* 25: 3018–3030.

Zheng M, Zhou Z, Zhao P, Luo Y, Ye Q, Zhang K, Song L, Mo J. 2020. Effects of human disturbance activities and environmental change factors on terrestrial nitrogen fixation. *Global Change Biology* 26: 6203–6217.

Zhou Y, Zhu H, Fu S, Yao Q. 2017. Variation in soil microbial community structure associated with different legume species is greater than that associated with different grass species. *Frontiers in Microbiology* 8: 1007.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Distribution of the edaphic variables measured in the soil (top row) and in the leaf litter across warming treatment.

Table S1 Description of the edaphic variables measured in each plot.

Table S2 The number of OTUs removed from each dataset because they appeared in the blank samples, the number of OTUs remaining for analysis, and the *P*-values of independent variables, determined by the distance-based redundancy analysis.