Experimental Warming Alters Nitrogen Cycle in a Humid Tropical Forest

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Summary

- Humid tropical forests typically contain high soil nitrogen (N), supporting rapid rates of primary productivity and recovery from disturbances. Nitrogen fixation is regulated by microbial communities, which can be free-living in the soil and leaf litter (asymbiotic) or in symbioses with plants.
- To investigate how warming affects asymbiotic and symbiotic components of the N cycle, we analyzed soil and leaf litter samples as well as annual seedling census data from an experimental warming field site in Puerto Rico.
- 16S and *nifH* sequencing revealed that warming significantly altered bacterial composition. Asymbiotic N fixation rates were 55% greater in soil (0.004 nmol N_2/g/hr) and 525% greater (0.217 nmol N_2/g/hr) in leaf litter from warmed compared to ambient plots. This increase in fixation was associated with changes in the N-fixing bacterial community. Under warming, N fixers experienced a 4.4-fold increase in growth rate compared to non-fixers, yet competition with neighboring N fixers eventually reduced N fixer growth. Seedling growth, especially of N fixers, initially increased following hurricane disturbances before declining.
- Together, our findings suggest that warming increases the flux of N from the atmosphere into this tropical forest, driving changes in both microbial and tree dynamics.

Introduction

Tropical forests have an exceptional capacity to sequester carbon, support biodiversity, and cycle nutrients. Despite covering just 15% of Earth's terrestrial surface, tropical forests account for two-thirds of global carbon (C) storage, one-third of global net primary productivity, and large portions of global nutrient cycling (Pan *et al.,* 2013). These high rates of primary production and large biomass stocks, as well as rapid regeneration rates, are largely attributed to favorable climate, ample soil nitrogen (N), and quick recovery of the N cycle following disturbance (Brookshire *et al.,* 2012). The interactions between N and C cycles within terrestrial ecosystems are likely to affect trajectories of atmospheric C concentrations and associated global climate changes (Thornton *et al.,* 2009; Gerber *et al.,* 2010; Zaehle and Dalmonech, 2011; Wårlind *et al.,* 2014; Xu and Yuan, 2017). Thus, it is essential to study how N cycling in humid tropical forests will be impacted by climate change (Wood *et al.,* 2012) and the altered regime of major disturbances such as hurricanes (Reed *et al.,* 2020; Tunison *et al.*, 2024).

Unlike other major plant nutrients, N is naturally brought into most ecosystems through the biological fixation of atmospheric N gas by microbes. N-fixing microbes are divided into two groups: asymbiotic (free-living) and symbiotic. Asymbiotic N-fixing microbes are hetero- or autotrophic bacteria, as well as some archaea, inhabiting water, soil, and leaf litter (Dynarski and Houlton 2018). Estimates from multiple tropical forests 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.,* 2014): an estimated 0.459 – 0.714 kg N/ha/year in the humid tropical forest of the Amazon, for example (Moreira *et al.,* 2021). Besides asymbiotic N-fixing microbes, others form a symbiotic relationship with specific plants and are housed in nodules in the roots of these plants. These N-fixing plants (N fixers) are relatively abundant in neotropical forests and provide a large potential source of N fixation (Taylor *et al.,* 2017; Tamme *et al.,* 2021). By bringing large inputs of N into these ecosystems, these N fixers may fuel rapid forest growth and carbon sequestration (Brookshire *et al.,* 2019; Menge *et al.,* 2019; Taylor *et al.,* 2019).

At large scales, climate is considered a major driver of the microbial community function and composition (Castro *et al.,* 2010; de Vries and Griffiths, 2018; Jansson and Hofmockel, 2020). Warming is especially important, as it can increase microbial processing, enzyme activity, turnover, and growth efficiency (Castro *et al.,* 2010; Pajares and Bohannan, 2016; Wood *et al.,* 2019). Warming conditions have long been thought to favor N fixation because it is a microbial

process catalyzed by the enzyme nitrogenase (Houlton *et al.,* 2007; Reed *et al.,* 2011). Asymbiotic microbial N fixation rates have been found to increase in response to warming across many biomes, including tropical forests, although some studies reported only minor effects (Bai *et al.,* 2013; Liu *et al.,* 2016; Zheng *et al.,* 2020). Extreme or chronic warming can inhibit asymbiotic N 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). The long-term effects of warming on asymbiotic N fixation are still a critical point of study in lowland, humid tropical forests (Contosta *et al.,* 2015; Pajares and Bohannan, 2016; Zheng *et al.,* 2020).

In addition to influencing the functioning of microbes, warming may influence the composition of microbial communities. Warming can shift the asymbiotic 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 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 the temperate grassland (Penton *et al.,* 2015), and enhanced N-fixing microbial abundance and diversity in tundra ecosystem soils (Feng *et al.,* 2019). While the relationship between asymbiotic N fixation rates and N-fixing microbial community composition has been investigated in terrestrial ecosystems, this relationship remains poorly investigated in tropical forests (Reed *et al.,* 2010, Mirza *et al.,* 2014, Pajares and Bohannan, 2016).

Warming may also affect the growth of N-fixing trees (Cusack *et al.,* 2016; Nottingham *et al.,* 2023). Much previous research on the effects of warming on plant growth in the tropics has centered on changes in photosynthesis and related physiology (Saxe *et al.,* 2001; Cusack *et al.,* 2016, Carter *et al.,* 2021). Studies and meta-analyses have indicated that temperatures in tropical forests are likely already near optimal thresholds for plant metabolism and further warming may reduce photosynthetic rates by creating supraoptimal temperatures (Way and Oren, 2010; Yamori *et al.,* 2014; Doughty *et al.,* 2023). However, optimal temperatures for N fixation in plant symbioses can be higher than optimums for photosynthesis, suggesting plant C and N gain may be decoupled with respect to temperature (Bytnerowicz *et al.,* 2022). Additionally, N fixation in trees may acclimate to growing temperatures in tropical forests (Bytnerowicz *et al.,* 2022). Therefore, climate warming may increase the growth of N fixers in tropical systems.

N fixers affect the growth of neighboring plants and overall forest growth, but it there is limited data on how this relationship between N fixers and their neighbors will change with future warming. Decades of research has shown that N-fixing trees facilitate the growth of neighboring non-fixing trees (Uselman *et al.,* 1999). It has been demonstrated that N can be transferred from tropical N-fixing trees to neighboring plants (Rao and Giller, 1993) via several pathways, including contributions to soil fertility by additions of organic N in plant litter, common mycorrhizal networks, and direct root-to-root transfer via exudation (Munroe and Isaac, 2014; Xu *et al.,* 2019). However, N fixers can also inhibit growth of neighbors in secondary tropical forests (Taylor *et al.,* 2017). These contrasting results might arise if N fixers facilitate growth of neighbors early in succession but inhibit growth later in succession, perhaps by taking light and other resources away from neighboring plants (Chapin *et al.,* 2016; Taylor *et al.,* 2017; Menge *et al.,* 2019). These complex neighboring effects could be altered by climate change, especially if fixers and non-fixers respond differently to high temperature as previously discussed.

Finally, climate warming may affect how non-fixer and N fixer growth respond to additional disturbances, such as hurricanes. Hurricanes can directly increase tree damage and mortality (Walker 1995; Yap *et al.,* 2016) and increase light availability to the less-damaged understory (Battaglia *et al.,* 2001; Umaña *et al.,* 2023). The understory also experiences a pulse of available soil nutrients, including N, associated with canopy-level defoliation (Lodge *et al.,* 1991; Harmon *et al.,* 1995; Xu *et al.,* 2004; May and Oberbauer, 2021). As a result, N might become an excessive nutrient immediately following hurricane before rapidly dissipating (Hasselquist *et al.,* 2010), changing how N fixers grow and interact with neighbors following hurricane disturbances. Studying how hurricane and warming influence plant demographics in N fixers and non-fixers is therefore important for determining future ecosystem resilience (Johnstone *et al.,* 2016; Reed *et al.,* 2020).

Given the contribution of tropical forests to the global N cycle, it is important to determine how N-fixing microbial communities and N-fixing trees in tropical forests will be affected by projected warming. In this work, we present the first comprehensive study of the effects of warming on N cycling dynamics in a tropical forest by utilizing a tropical field warming experiment to examine the two most important components bringing N into this system: asymbiotic N-fixing microbes and symbiotic N-fixing trees. To examine the effect of warming on asymbiotic microbes, we combined N fixation assay with DNA sequencing of soil and litter

samples from the Tropical Responses to Altered Climate Experiment (TRACE), an *in-situ* field warming experiment in Puerto Rico. We hypothesized that warming would increase 1) N-fixing microbial community diversity and N fixation rates, indicating that warmed temperatures favor the growth and activity of N-fixing microbes, 2) growth of N-fixing seedlings compared to non-fixing seedlings, 3) the facilitative and competitive effect N fixers have on neighboring non-fixers and N fixers, respectively, and 4) the growth spike in N fixers following hurricane disturbances.

Materials and methods

Site description

This work took place at TRACE, in Puerto Rico. This study site is in a lowland subtropical wet forest located near the U.S. Forest Service Sabana Field Research Station, which is 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 mean annual rainfall is 3,500 mm (Alonso-Rodriquez *et al.,* 2022). The site contains clay-rich, acidic soils classified as Ultisols, and >60% of root biomass is concentrated in the top 10 cm soil layer. In the study area, the dominant canopy tree species are *Syzygium jambos*, *Ocotea leucoxylon*, and *Casearia arborea*, and the site is also dominated by the palm *Prestoea montana*. The most prevalent N-fixing tree species are *Inga laurina* and *Inga vera*. 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 to 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 site. Warming was halted for a year before restarting in September 2018. Prior to the hurricanes, surface soils (0-10 cm) in warmed 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 ~ +1°C (Tunison *et al.,* 2024). These experimental conditions enable study the effects of warming and hurricane disturbance on various ecosystem processes in this tropical forest (Reed *et al.,* 2020; Tunison *et al.,* 2024).

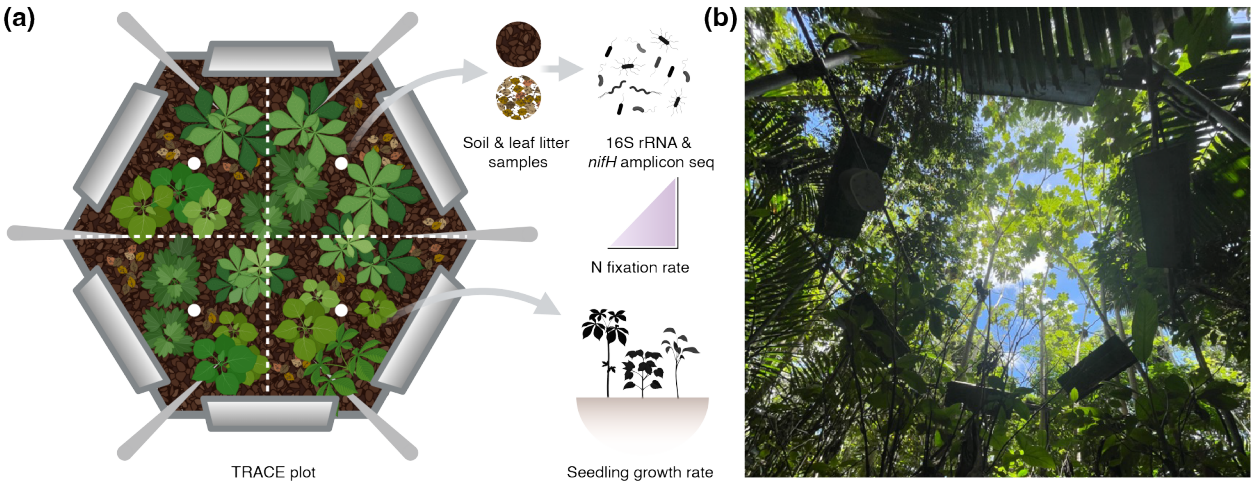


Fig. 1: Experimental setup of the TRACE plots in the Luquillo Forest of Puerto Rico. The TRACE experiment utilizes infrared heaters to warm three 12 m^2 plots within the forests. These warming plots are compared to three control plots experiencing ambient temperatures. (a) Diagram depicting top view 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 N-fixation measurements and microbial sampling. (b) View from underneath one of the TRACE plots in the Luquillo Experimental Forest.

Field collections

In July 2023, one leaf litter sample and one soil sample were collected from each of the 4 quadrants in each of the 6 plots (Fig. 1A). Using sterilized nitrile gloves, approximately 4 grams of leaf litter and 13 grams of soil were collected from the center of each quadrant. Four additional leaf litter and four additional soil samples were collected approximately 2 meters in a random direction away from two of the warmed plots and away from two of the control plots (for baseline ethylene concentration measurements). 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 N fixation rate section). Following this, samples were subsampled, and a portion was dried to calculate moisture content; these subsamples were then ground to calculate nitrogen, carbon, and phosphorous content (see C, N, and P content section). The remaining samples were stored in individual bags in a -80°C freezer

184 before being shipped to Oklahoma State University for characterization of the microbial
185 community composition (see microbial community composition section).
186
187 *N fixation rate*
188 The fixation rates of 24 leaf litter and 24 soil samples were analyzed using an acetylene
189 reduction assay (Hardy *et al.,* 1968; Reed *et al.,* 2010). Each 55-ml tube was sealed with a lid fitted
190 with a septum, injected with 3.0 mL acetylene (creating approximately 10% headspace
191 concentration), and vented to the atmosphere. The additional samples collected away from four of
192 the plots, 8 in total, were not injected with acetylene so measurements of ethylene produced in the
193 absence of acetylene could be determined. Eight blank samples were created by injecting acetylene
194 into tubes without sample inside so measurements of acetylene reduced to ethylene in the absence
195 of sample could be determined. After an approximately 18-hour incubation in the lab at ~26°C,
196 headspaces of all 64 samples (48 plot samples, 8 control samples, and 8 blank samples) were
197 subsampled and injected into Exetainer tubes. These Exetainer headspace gas samples were
198 transported to the USGS Southwest Biological Science Center (SBSC) in Moab, Utah, and
199 analyzed for ethylene concentration on a gas chromatograph (Shimadzu GC-14A equipped with a
200 Flame Ionization Detector, Shimadzu Corporation, Kyoto, Japan).
201 The baseline ethylene concentrations of the additional soil and leaf litter samples, and blank
202 samples were averaged respectively. To account for ethylene production by soil (or leaf litter)
203 alone, as well as for ethylene production in the absence of sample, the average baseline ethylene
204 concentration of the additional soil (or leaf litter) samples and blank samples was subtracted from
205 the baseline ethylene concentration of plot soil (or leaf litter) samples to obtain the adjusted
206 ethylene concentration for each sample.
207 For plot soil samples, which were compact at the bottom of each tube, headspace volume
208 was calculated via the measured height of empty space in each tube: from the soil surface to the
209 bottom of the lid. For plot leaf litter samples, which were packed loosely, a headspace volume of
210 25 mL was assumed; this is 70% of the total volume for an average empty tube. Dry weights for
211 each sample were calculated by dividing each sample's total wet weight by their moisture content
212 (calculated from weighing wet and dried subsamples) + 1. Incubation times for each sample were
213 calculated by measuring time at acetylene injection and time at headspace sampling. The adjusted
214 baseline ethylene concentrations were converted to parts per million (ppm) of ethylene using the

215 calibration curve calculated from standard samples. Then, the nanomoles of ethylene produced per
216 gram of sample per hour were calculated using the adjusted ethylene concentration (ppm),
217 headspace volume (mL), dry weight (g), and incubation time (hr) of each sample.
218 Each mole of ethylene produced is equivalent to one mole of acetylene reduced. acetylene
219 reduction rates were then converted to N fixation rates by dividing moles of ethylene produced by
220 three (Reed *et al.,* 2010).
221
222 *C, N, and P content*
223 After the acetylene reduction treatment, a ~2 g subsample was taken from each soil sample,
224 and a ~1 g subsample was taken from each leaf litter sample, and the remaining portion of each
225 sample was frozen in a -80°C freezer. The subsamples were dried at 105°C for 48 hours. These
226 subsamples were pooled together by type and plot, and then ground using a SPEX SamplePrep
227 8000D dual mixer/mill (SPEX SamplePrep LLC, Metuchen, NJ). All ground samples were sent to
228 the USGS SBSC, and total C and N content was determined using an Elementar Vario Micro Cube
229 (Elementar Americas Inc., Ronkonkoma, NY). The ground samples were then sent to the BYU
230 Environmental Analytical lab, where they were subjected to nitric acid digestion in a Mars 6
231 Microwave (EPA method 3051A; CEM Corporation, Matthews, NC) and total phosphorus content
232 was determined using a Thermo Scientific iCAP 7400 ICP-OES Analyzer (Thermo Fisher
233 Scientific, Waltham, MA).
234
235 *Microbial community composition*
236 Soil and leaf litter samples stored in the -80°C freezer were thawed in 4°C and
237 homogenized. Genomic DNA was extracted from each of the samples in triplicate using a Qiagen
238 DNeasy Power Soil Pro Kit (Qiagen, Hilden, Germany). Additionally, along with both the soil and
239 leaf litter extractions, the DNA extraction kit was run on blank controls containing no sample.
240 Extractions were pooled for each sample and stored at -80°C until downstream processing.
241 DNA extractions were used to characterize the full bacterial community using the 16S
242 rRNA gene and the N-fixing bacterial community using the *nifH* gene. Amplification and
243 sequencing were carried out at the University of Minnesota Genomics Center (UMGC).
244 Specifically, 16S rRNA gene sequences were amplified by PCR using the 16S primer set 515F-
245 806R, which amplifies the variable V4 region of the 16S rRNA gene. The *nifH* gene, which

246 encodes the reductase subunit of nitrogenase, the enzyme that catalyzes the reaction which fixes
247 N, has been widely used as a genetic marker to study the diversity and abundance of N-fixing
248 microbes in gene sequencing studies (Pajares and Bohannan, 2016). *NifH* gene sequences were
249 amplified in two separate PCR steps to minimize the formation of undesirable primer dimers. First,
250 the *nifH* gene was amplified using the *nifH* primer set Ueda19F-R6 (Sigma-Aldrich, St. Louis,
251 MO), a broadly inclusive pair (Angel *et al.,* 2018). This was followed by PCR amplification using
252 Nextera-adapted primers suited for Illumina sequencing.

253 16S and *nifH* gene amplicons underwent v3 Illumina MiSeq 2x300bp paired end
254 sequencing. Soil and leaf litter sequence data were processed separately using the Mothur MiSeq
255 standard operating procedure (Schloss *et al.,* 2009; Kozich *et al.,* 2013). Briefly, paired-end reads
256 were assembled for each amplicon and filtered to the expected amplicon length (291 base pairs
257 (bp) for 16S and 394 bp for *nifH*). The trimmed 16S sequences were aligned to the reference
258 sequence file Silva 132 database. The trimmed *nifH* sequences were aligned to the *nifH* sequence
259 database described by Gaby and Buckley (2014). For both 16S rRNA and *nifH* genes, chimeras
260 were removed using chimera.vsearch (Mothur) and the sequences were grouped into operational
261 taxonomic units (OTUs) at 97% sequence similarity. OTUs with only one observation in a dataset
262 were removed using remove.rare (Mothur). For 16S, taxonomy was classified in Mothur using
263 version 19 of the Ribosomal Database Project (RDP) training set, publicly released in July 2023.
264 For *nifH*, representative sequences from each OTU obtained using get.oturep (Mothur) were
265 taxonomically classified by Basic Local Alignment Search Tool analysis against the NCBI
266 GenBank database.

267

268 *Seedling survey*

269 In June 2015, all seedlings of at least 10 cm height present in the 6 plots were tagged,
270 identified to species, and measured for height from ground level and stem diameter at ground level.
271 After a year of drought (2015), the census was repeated annually starting June 2016 until 2023
272 (with the exception of 2020). Using the census data from 2016-2023, we calculated seedling
273 growth rates (growth rate = $\log(x_{final}/x_{initial})$/days between measurements, where x = height or stem
274 diameter). By investigating both height and diameter growth rates, we can detect shifts in growth
275 strategies such as conservative versus acquisitive (Reich, 2014; Zhao *et al.,* 2016; Fagundes *et al.,*
276 2022). We also measured crowding within quadrants due to fixers and non-fixers, by separating

277 the effects of overall crowding (number of seedlings in the quadrant) from the effects of crowding
278 from fixers calculated as the proportion of neighbors that are fixers (number of fixer seedlings in
279 quadrant/total number of seedlings in quadrant). Using the date of the censuses, we calculated the
280 number of days since last hurricane to investigate how hurricane disturbance affects seedling
281 growth rates.
282
283 <u>*Statistical analyses*</u>
284 *Microbial composition and N fixation*
285 Community composition analyses were conducted in the R programming environment
286 using the *gjam* and *vegan* packages (Clark *et al.,* 2017; Oksanen *et al.,* 2022). Each of the four
287 bacterial communities was analyzed separately: soil 16S, soil *nifH*, leaf litter 16S, and leaf litter
288 *nifH*. Before analyses, all OTUs appearing in the soil or leaf litter blank controls were removed
289 from the soil and leaf litter OTU composition, respectively. Homogeneity in dispersion of
290 sequence relative abundances in OTUs from the warming and ambient groups was confirmed using
291 *betadisper* with the Bray-Curtis distance metric. Distance-based redundancy analyses (dbRDA)
292 were applied to the OTU composition data using *capscale*, again with sequence relative
293 abundances and the Bray-Curtis distance metric. The abiotic (treatment, moisture, C:N ratio, and
294 P content) and biotic (proportion of N-fixing seedlings, Shannon Index, and species richness in the
295 given quadrant) independent variables in the dbRDA model were z-transformed. Permutation tests
296 with 999 permutations determined which of these independent variables had significant effects on
297 overall (16S) and N-fixing (*nifH*) bacterial composition.
298 Generalized joint attribute modeling (GJAM), with plot as a random effect, was used to
299 analyze how the treatment, C:N ratio, and P content affected the bacterial communities. The GJAM
300 analyses were conducted using Gibbs sampler (a Markov chain Monte Carlo technique, Geman
301 and Geman 1984) with 2000 iterations and a burn-in period of 200. Using this approach, we
302 identified OTUs associated with a significantly decreasing or increasing relative abundance in the
303 warming treatment. We jointly analyzed N fixation rate of the samples and N fixing bacterial
304 community to investigate how N fixation was directly affected by the predictor variables and
305 indirectly via changes in bacterial composition.
306
307 *Seedling demography*

To investigate how seedling growth dynamics changed due to their N fixing status and the major disturbances experienced by the plots (warming and hurricane disturbance), the growth rate ($g_{i,s,t}$) in both height and diameter of an individual seedling i from species s under the treatment t (ambient and warming) was modeled with the following equation: $g_{i,s,t} \sim N(\mu = \beta_t x_i + \sigma_{species} + (\sigma_{plot} / \sigma_{seedling}), \sigma)$, where x represents a matrix of independent variables: treatment, N-fixation status (whether the seedling is capable of fixing N), number of seedlings in the given quadrant, the proportion of N-fixing seedlings in the given quadrant, days since hurricane disturbance, seedling's initial height, as well as two-way interactions between N-fixation status and the other covariates (except seedling initial height), and three-way interactions among treatment, N-fixation status, and the other covariates (except seedling initial height). Count variables in the matrix (i.e. number of seedlings in a quadrant and days since hurricane) were log- and then z-transformed, and the independent proportion variable (proportion of N fixers in a quadrant) was z-transformed after taking the arcsine of the square root. Both of these linear mixed-effects models (height and diameter growth rate) included species ($\sigma_{species}$), plot (σ_{plot}), and individual seedling ($\sigma_{seedling}$) random effects to account for repeated measurements.

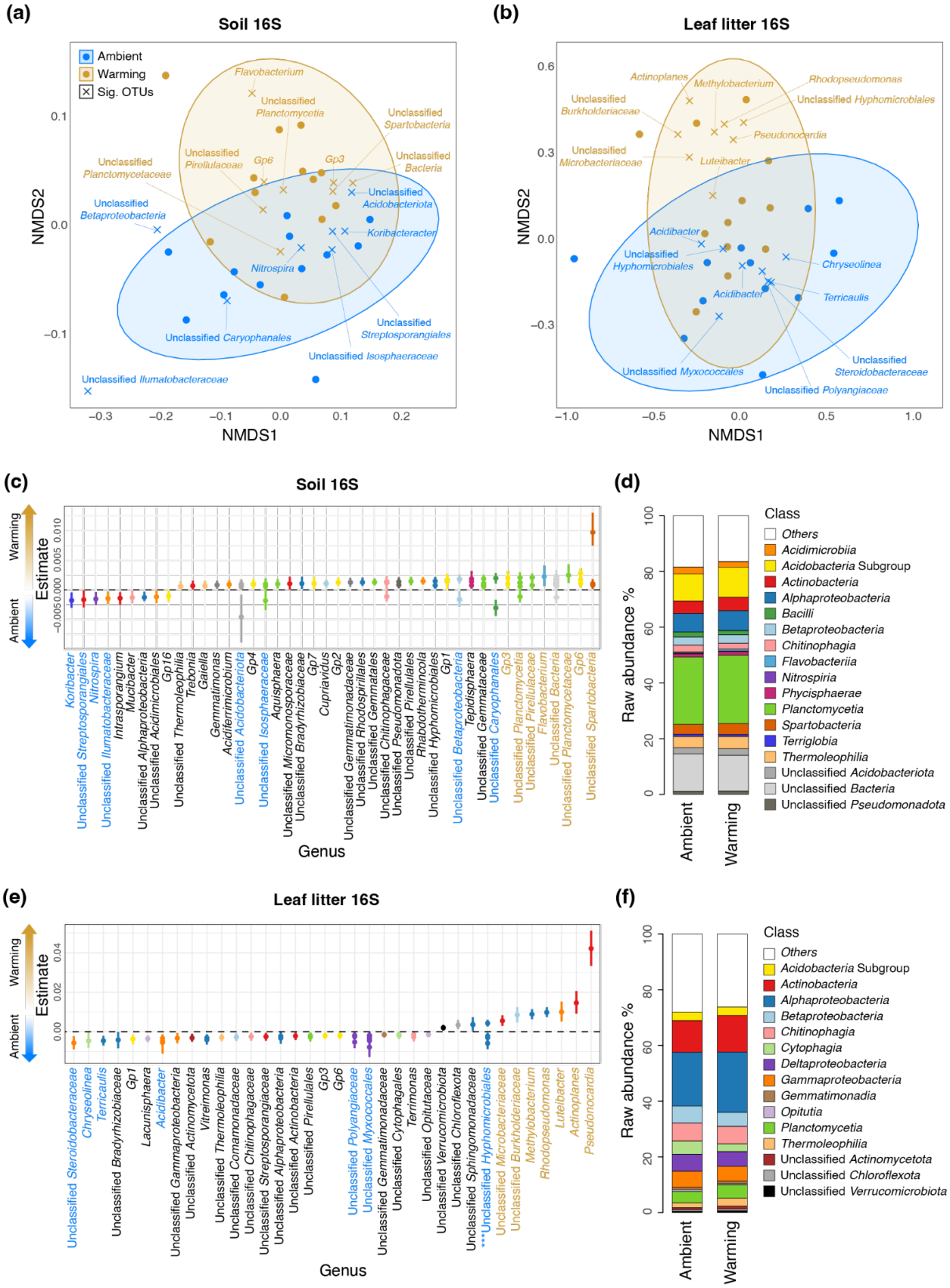
Results

Microbial composition and N fixation

The 16S amplicon library for soil samples recovered 25,094 Operational Taxonomic Units (OTUs) from 1,883,812 rRNA sequences after quality trimming, removal of rare OTUs, and removal of OTUs that appeared in blank controls. 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% respectively. The 16S amplicon library for leaf litter samples recovered 34,857 OTUs from 3,416,750 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% respectively.

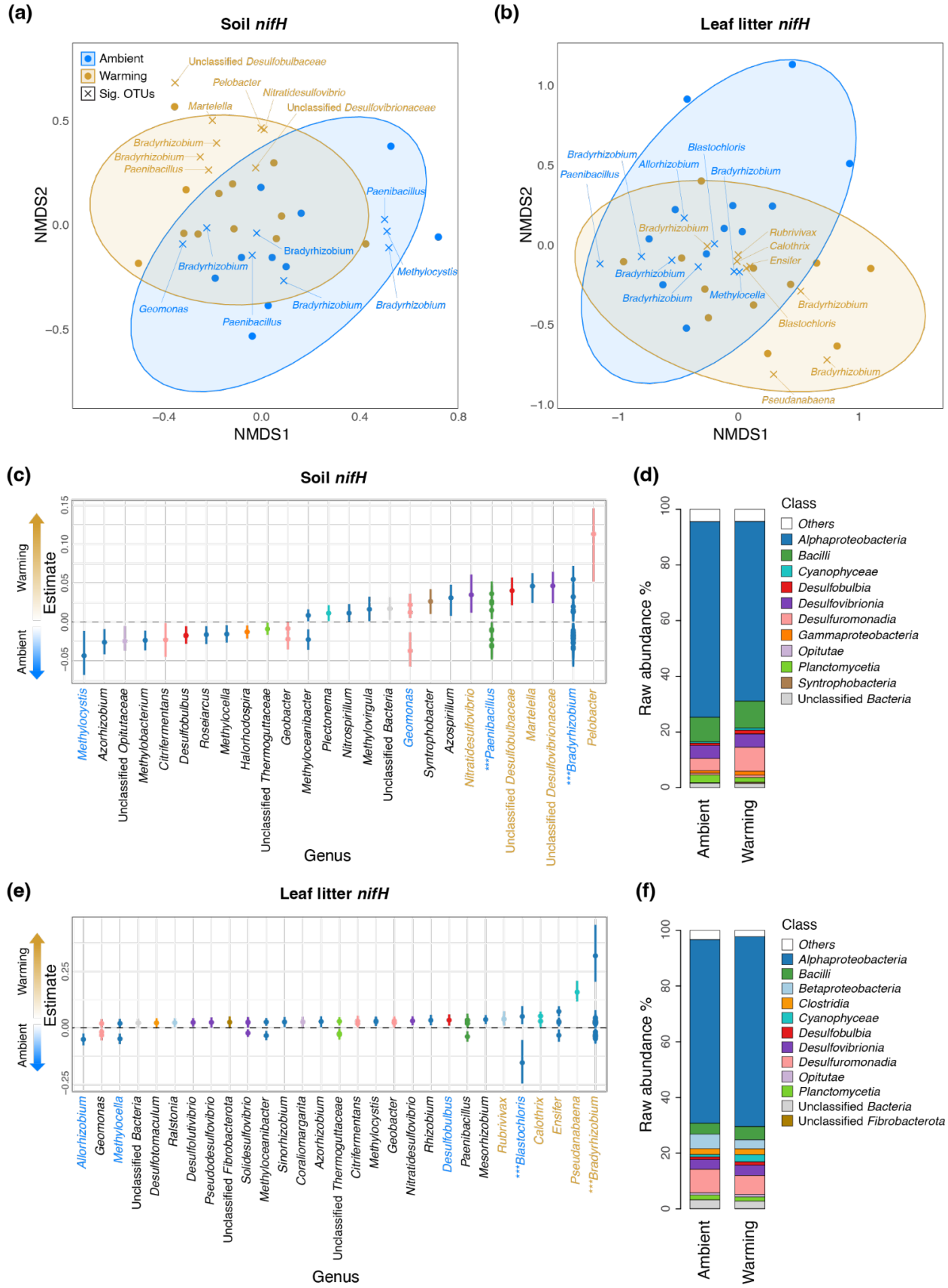
The *nifH* amplicon library for soil samples recovered 4,495 OTUs from 47,158 quality sequences (this total excludes two ambient plot samples with too few quality reads that were

339 excluded from all analyses), and each soil sample yielded an average of 2,143 quality sequences.
340 Among OTUs that were classified (99.16%), here were 208 unique genera identified, which were
341 assigned to 16 bacterial phyla. The phyla Pseudomonadota and Thermodesulfobacteriota
342 dominated both sample types, with an average relative abundance of 71.87% and 10.55%,
343 respectively, in ambient plot samples, and 66.86% and 15.62%, respectively, in warming plot
344 samples. The *nifH* amplicon library for leaf litter samples recovered 8,811 OTUs from 167,914
345 quality sequences and each leaf litter sample yielded an average of 6,996 quality sequences.
346 98.68% of OTUs were assigned to classified, cultured bacteria using BLAST. 223 unique genera
347 were identified, assigned to 17 bacterial phyla. Again, the phyla Pseudomonadota and
348 Thermodesulfobacteriota dominated both sample types, with an average relative abundance of
349 71.89% and 12.81%, respectively.


(a)
Soil 16S
Ambient
Warming
Sig. OTUs
NMDS1
NMDS2
(b)
Leaf litter 16S
(c)
Soil 16S
Estimate
Warming
Ambient
Genus
(d)
Raw abundance %
Class
Others
Acidimicrobiia
Acidobacteria Subgroup
Actinobacteria
Alphaproteobacteria
Bacilli
Betaproteobacteria
Chitinophagia
Flavobacteriia
Nitrospiria
Phycisphaerae
Planctomycetia
Spartobacteria
Terriglobia
Thermoleophilia
Unclassified Acidobacteriota
Unclassified Bacteria
Unclassified Pseudomonadota
Ambient
Warming
(e)
Leaf litter 16S
Estimate
Genus
(f)
Raw abundance %
Class
Others
Acidobacteria Subgroup
Actinobacteria
Alphaproteobacteria
Betaproteobacteria
Chitinophagia
Cytophagia
Deltaproteobacteria
Gammaproteobacteria
Gemmatimonadia
Opitutia
Planctomycetia
Thermoleophilia
Unclassified Actinomycetota
Unclassified Chloroflexota
Unclassified Verrucomicrobiota
Ambient
Warming

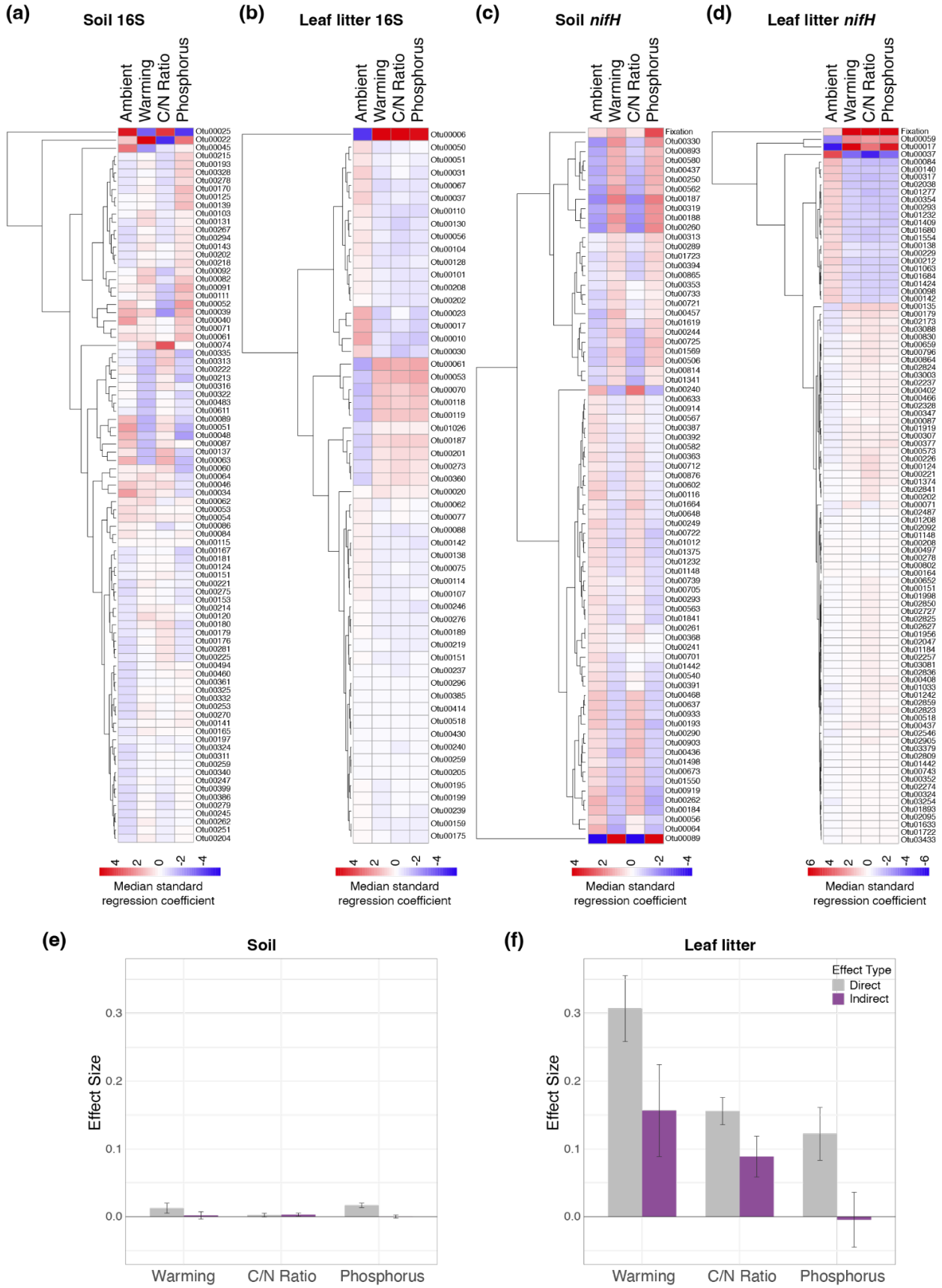
350

351 **Fig. 2**: Differences in overall bacterial community composition elucidated by 16S rRNA gene
352 sequencing of soil and leaf litter samples. NMDS plots depicting samples and significantly altered
353 OTUs show that bacterial communities are distinct between ambient and warming treatments (a-
354 b). Forest and raw abundance plots of significantly altered OTUs, listed by genus and colored by
355 class, show how different genera respond to ambient versus warming conditions in soil (c-d) and
356 leaf litter (e-f). The points on the x-axis represent the median of the standardized regression
357 coefficients associated with warming treatment, derived from the posterior distribution of GJAM
358 model parameters. Segments expand to the 95% credible intervals (CI). OTUs with 95% CI
359 beneath 0 (dashed vertical line) are significantly more abundant under ambient conditions, and
360 OTUs with 95% CI above 0 are more abundant under warming conditions. The 8 most highly
361 altered OTUs in ambient and warming are highlighted. The asterisks indicate class where
362 individual OTUs responded positively and negatively to the warming condition.


(a)
Soil nifH
Ambient
Warming
Sig. OTUs
Unclassified Desulfobulbaceae
Pelobacter
Nitratidesulfovibrio
Martelella
Unclassified Desulfovibrionaceae
Bradyrhizobium
Paenibacillus
Methylocystis
Geomonas
NMDS1
NMDS2
(b)
Leaf litter nifH
Blastochloris
Allorhizobium
Rubrivivax
Calothrix
Ensifer
Methylocella
Pseudanabaena
(c)
Soil nifH
Estimate
Warming
Ambient
Genus
(d)
Raw abundance %
Class
Others
Alphaproteobacteria
Bacilli
Cyanophyceae
Desulfobulbia
Desulfovibrionia
Desulfuromonadia
Gammaproteobacteria
Opitutae
Planctomycetia
Syntrophobacteria
Unclassified Bacteria
(e)
Leaf litter nifH
(f)
Betaproteobacteria
Clostridia
Unclassified Fibrobacterota

363

364 **Fig. 3**: Differences in the N-fixing bacterial community composition elucidated by *nifH* gene
365 sequencing of soil and leaf litter samples. NMDS plots show that bacterial communities are
366 distinct between ambient and warming treatments (a-b). Forest and raw abundance plots of
367 significantly altered OTUs, listed by genus and colored by class, show how different genera
368 respond to ambient versus warming conditions in soil (c-d) and leaf litter (e-f). The points on the
369 x-axis represents the median of the standardized regression coefficients associated with warming
370 treatment, derived from the posterior distribution of GJAM model parameters. Segments expand
371 to the 95% credible intervals (CI). OTUs with 95% CI beneath 0 (dashed vertical line) are
372 significantly more abundant under ambient conditions, and OTUs with 95% CI above 0 are more
373 abundant under warming conditions. The 8 most highly altered OTUs in ambient and warming
374 are highlighted. The asterisks indicate class where individual OTUs responded positively and
375 negatively to the warming condition.


(a)
Soil 16S
(b)
Leaf litter 16S
(c)
Soil nifH
(d)
Leaf litter nifH
Ambient
Warming
C/N Ratio
Phosphorus
Median standard regression coefficient
(e)
Soil
(f)
Leaf litter
Effect Size
Effect Type
Direct
Indirect
Warming
C/N Ratio
Phosphorus

376

377 **Fig. 4**: Individual and global responses to warming conditions as well as contribution of the
378 bacterial community to N fixation. The heatmaps (a-d) represent the median of the standardized
379 regression coefficients of the full GJAM models (warming treatment, C:N ratio, and phosphorus)
380 for the OTUs that were the most affected by these variables for the overall soil and litter bacterial
381 community (a-b) and the soil and leaf litter N-fixing bacterial community (c-d). The N-fixing
382 bacterial community was modeled jointly with N fixation rates and therefore fixation is included
383 in the response variables influence by the model in the heatmaps (c-d). Finally, the direct (gray)
384 and indirect (purple) effects of warming, C:N ratio, and phosphorus content on N fixation rates in
385 e) soil and f) leaf litter samples from the GJAM models. Bars reached the median effect and
386 segments expand to the 95% CI.
387
388 As hypothesized, warming significantly altered both the overall and N-fixing bacterial
389 community composition in soil and leaf litter (Table S1, Fig. 2a-b, Fig. 3a-b). Generalized joint
390 attribute modeling of the overall bacterial community identified 16 OTUs in the soil and 44 OTUs
391 in the leaf litter that were significantly associated with the ambient plots (Fig. 2c-f). These OTUs
392 also show contrasting responses to edaphic factors (C:N ratio and phosphorus, Fig 4a-b). In the
393 soil, OTUs associated with the warming plots tended to be negatively associated with C:N ratio
394 and positively associated with phosphorus content while the opposite was true for OTUs associated
395 with the ambient plots (Fig 4a). Additionally, 71 OTUs in the soil and 12 OTUs in the leaf litter
396 were significantly associated with the warming plots (Fig. 2c-f). In the leaf litter, OTUs associated
397 with ambient or warming plots responded similarly with C:N ratio and phosphorus (Fig 4b); OTUs
398 associated with warming plots were positively associated with soil C:N and phosphorus, and OTUs
399 associated with ambient plots were negatively associated with soil C:N and phosphorus. These
400 OTUs were distributed across several genera and classes. Notably OTUs in the class Spartobacteria
401 and in the genus *Pseudonocardia* were substantially more abundant in warming plots in soil and
402 leaf samples, respectively.
403 Similarly, generalized joint attribute modeling identified many OTUs of N-fixing bacteria
404 that were significantly associated with warming or ambient plots in the soil and leaf litter (Fig 3c-
405 f, Fig. 4c-d). 47 OTUs in the soil and 20 OTUs in the leaf litter were significantly associated with
406 the ambient plots, and 27 OTUs in the soil and 73 OTUs in the leaf litter were significantly
407 associated with the warming plots. Notably, OTUs in the genera *Pelobacter* and *Pseudanabaena*

408 were substantially more abundant in warming plots in soil and leaf samples, respectively. Several
409 OTUs in the genus *Bradyrhizobium* were considerably more abundant in warming, but several
410 were also more abundant in ambient. As with the overall bacterial community, OTUs associated
411 with ambient or warming plots showed contrasting responses to soil C:N ratio and phosphorus (Fig
412 4c-d). In the soil, OTUs associated with the warming plots were positively associated with soil
413 phosphorus and negatively associated with soil C:N ratio, and the opposite was found for ambient
414 plot OTUs (Fig 4c). Finally, as with the overall bacterial community, OTUs in the leaf litter
415 associated with the warming plots also responded positively to soil C:N ratio and phosphorus (Fig
416 4d).

417 In soil, N fixation rates increased an average of 0.004 nmol N_2/g/hr, or 55%, under warming
418 compared to ambient. The direct effect of warming on N fixation was significant, but the indirect
419 effect, which is the effect of warming on N fixation mediated by change in N-fixing bacterial
420 composition, was not (Fig. 4e). Thus, the overall effect of warming was not considered significant
421 in the soil. In leaf litter, N fixation rates increased an average of 0.217 nmol N2/g/hr, or 525%,
422 under warming compared to ambient. Both direct and indirect effects, and thus the overall effect,
423 were significant (Fig. 4f). Specifically, N fixation rate was significantly increased by warming,
424 and this effect was both due to the direct effect of warming on N fixation rate and the indirect
425 effect of warming mediated by changes in the N-fixing bacterial community.

Seedling demography

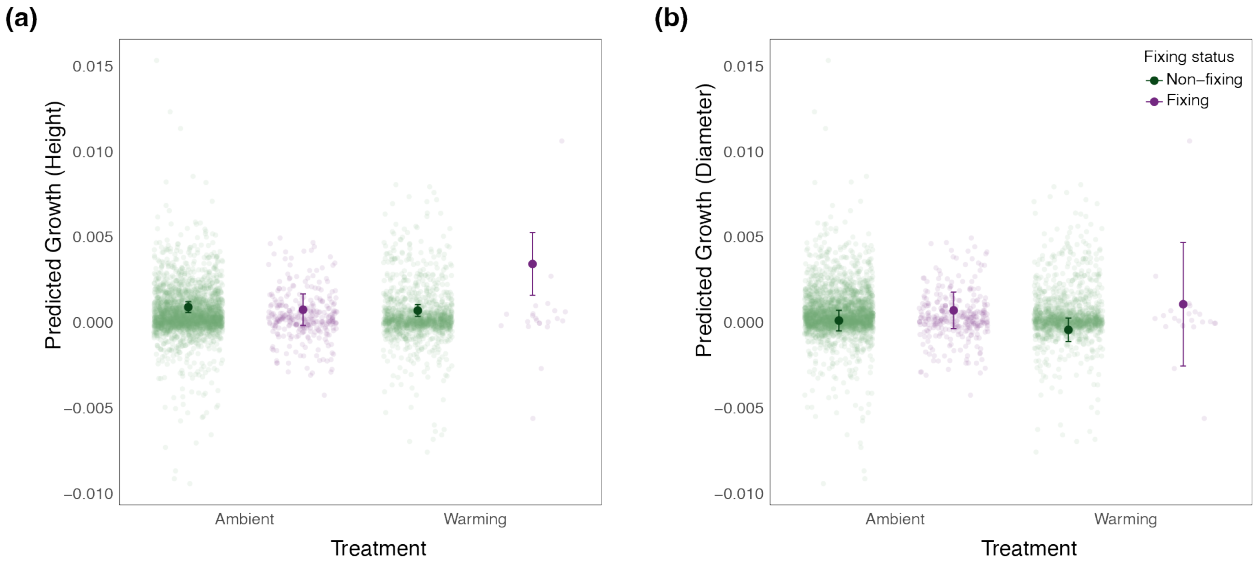


Fig. 5: Growth model predictions in ambient and warming plots for non-fixing and N-fixing species. Both values of height (a) and diameter (b) growth predicted by the growth rate model are shown. Background dots represent individual, scaled seedling growth rates, and foreground dots represent predicted growth rates from the linear mixed-effects model. Segments expanding from the foreground dots represent the 95% CI.

As hypothesized, fixers exhibited a significantly higher rate of height growth than the non-fixers in the warming plots (Fig. 5). Specifically, under warming there was a 4.4-fold increase in the rate of height growth in N fixers compared to non-fixers. However, this fixer growth advantage was only for height growth and in the warming plots. If warming was beneficial to fixer seedlings, it was generally harmful to non-fixers individuals (Fig. 5): the warming treatment marginally and significantly decreased non-fixer height and diameter growth, respectively.

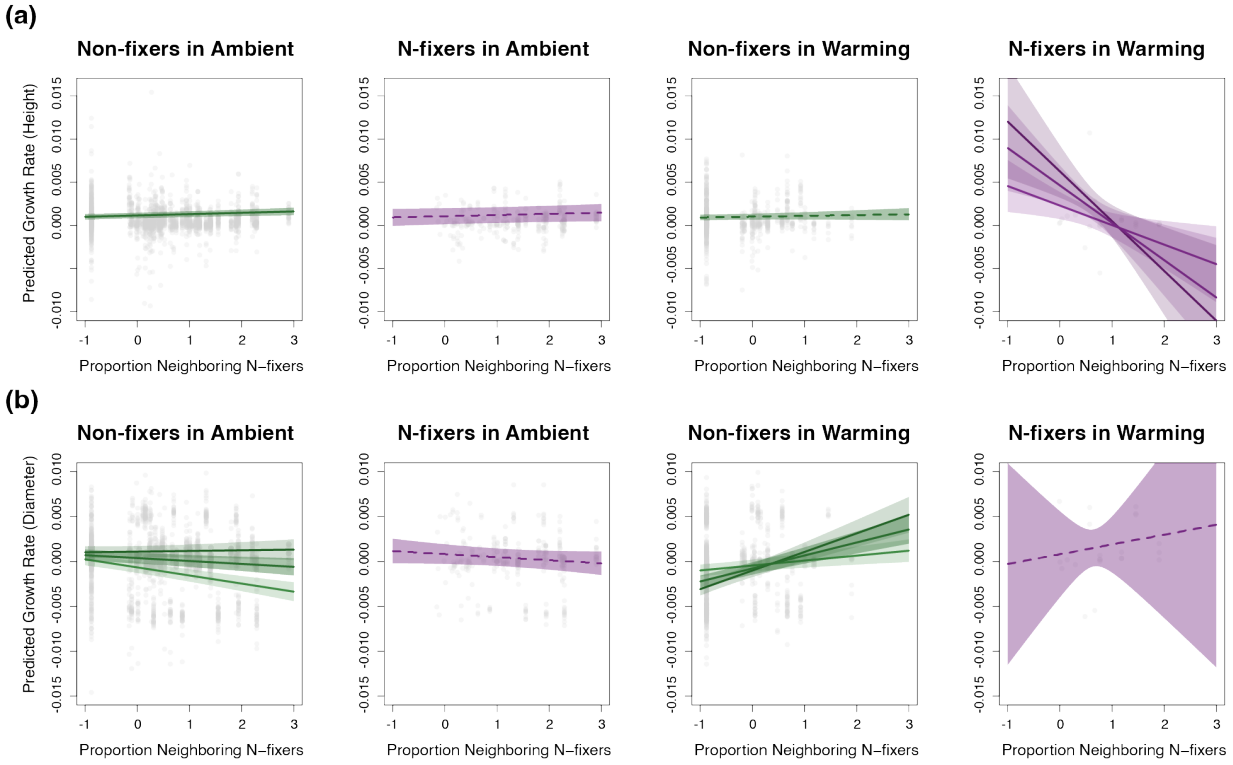


Fig. 6: Values of height and diameter growth in ambient and warming plots predicted for non-fixing and N-fixing seedlings, as influenced by proportion of N-fixing seedlings in the quadrant and total number of seedlings in the quadrant. Background dots (gray) represent the scaled proportion of neighboring N fixers in the same quadrant as each seedling with a measured growth rate. Lines represent growth rates predicted for the proportion of neighboring N fixers by the linear mixed-effects model. Solid lines represent significant effects of neighboring N fixers on growth, while dashed lines represent non-significant effects. Panels with multiple lines indicate that the total number of seedlings in a quadrant also had a significant effect on growth. These lines represent growth rate predictions with low, medium, and high total number of neighboring seedlings, represented by light, medium, and dark coloring, respectively. Shading for all lines represents the 95% CI.

As predicted, growth in height of non-fixer seedlings in ambient plots benefitted from a higher proportion of N fixers in the same quadrant (Fig. 6a). However, this effect was not present in warming. Also consistent with predictions, height growth of N fixers in warming plots decreased with a higher proportion of N fixer neighbors. Additionally, for N fixers in the warming plots, the decrease in height growth with increasing proportion of N fixer neighbors was significantly more

drastic in seedlings with a greater total of neighboring seedlings. In contrast to height growth, N fixer diameter growth was not influenced by the proportion of nearby N fixers, in either the ambient or warming plots (Fig. 6b). Additionally, diameter growth of non-fixers in ambient plots decreased with increasing proportion of neighboring N fixers. Even so, a higher total number of neighboring seedlings was associated with less-decreased growth. Finally, non-fixers in warming experienced increased diameter growth with increasing proportion of N fixer neighbors, and this effect was significantly exaggerated by increasing total number of neighboring seedlings.

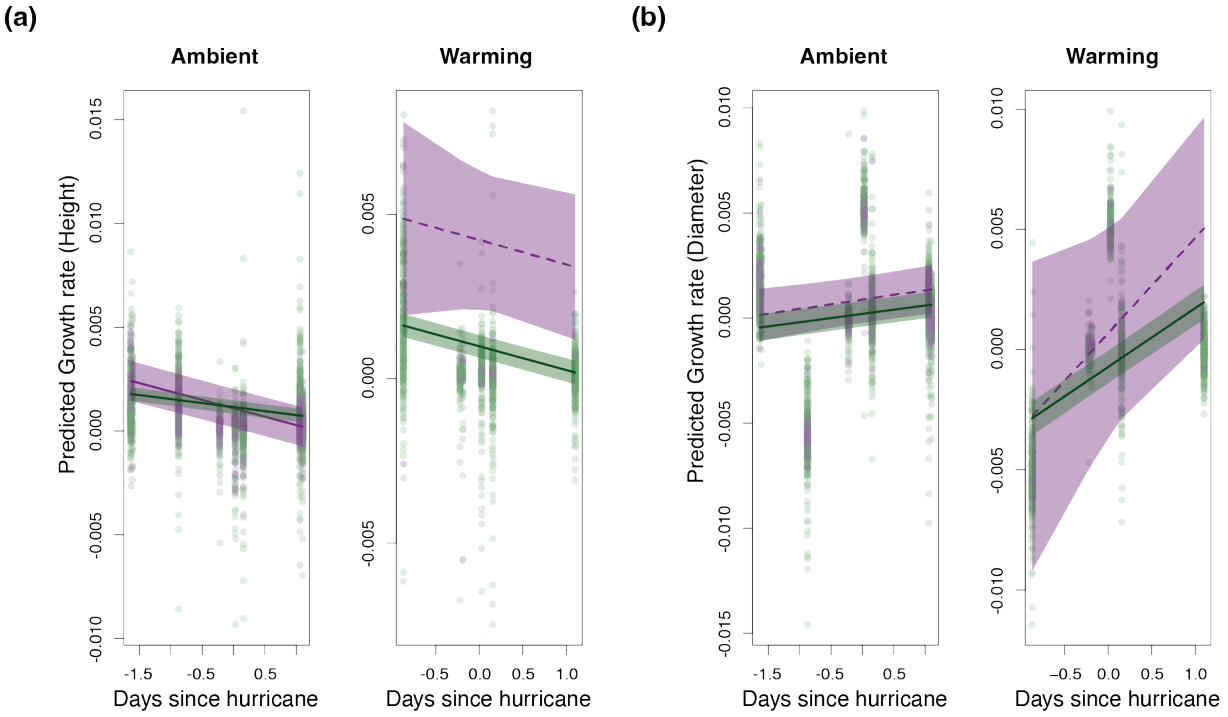


Fig. 7: Values of height and diameter growth in ambient and warming plots predicted for non-fixing (green) and N-fixing (purple) seedlings, as related to number of days since hurricane disturbance, which is scaled. Background dots represent individual, scaled seedling growth rates. Lines represent growth rates predicted for the number of days since hurricane by the linear mixed-effects model. Solid lines represent significant effects of days since hurricane on growth, while dashed lines represent non-significant effects. Shading for all lines represents the 95% CI.

As hypothesized, as the number of days since hurricane increases, height growth of all seedlings significantly decreases, and this effect is significantly exaggerated in N fixers in ambient

475 plots (Fig. 7a). Conversely, diameter growth increases with number of days since hurricane,
476 although this effect is only significant in non-fixers (Fig. 7b).
477
478 **Discussion**
479 We provide the first comprehensive study on the effects of warming on asymbiotic and
480 symbiotic N fixation aspects in a tropical forest by utilizing a tropical forest warming experiment,
481 combining N-fixation assays, genetics, and long-term survey data. We found that warming
482 increased asymbiotic N fixation activity and that this increase was partially mediated by change in
483 the N-fixing bacterial community under warming. Both the overall and N-fixing bacterial
484 communities were highly altered by warming, even more so than by other factors known to
485 influence the asymbiotic bacterial community such as moisture, C:N ratio, and phosphorus.
486 Enhanced asymbiotic N fixation activity under warming was echoed by our results in seedling
487 growth, suggesting that symbiotic N fixation might also be enhanced. We found that N fixers had
488 a growth advantage under warming, and crowding from neighboring plants nullified this advantage
489 or even placed N fixers at a disadvantage. In both N fixers and non-fixers there was generally a
490 shift from height investment to diameter investment following hurricane disturbance and from
491 ongoing warming disturbance. Together, our results suggest that warming may increase N flux
492 from the atmosphere to the ecosystem which if this N flux is coupled with a decrease in fertilizer
493 use might help mitigating predicted increase in nitrous oxide emissions (Aryal *et al.,* 2022).
494
495 *Warming significantly alters bacterial community composition and function*
496 As hypothesized, warming increased N fixation activity: this increase is likely due to the
497 effect of warming on both enzyme activity and bacterial composition. The direct effect of warming
498 on N fixation was significantly positive in both the soil and leaf litter, and the effect of warming
499 mediated by its effect on the N-fixing bacterial community was also significantly positive in the
500 leaf litter. The differential responses in soil and leaf litter may be explained by previous findings
501 that the two environments display different rates of N fixation, with leaf litter showing higher
502 mass-based rates of N fixation (Reed *et al.,* 2013; Van Langenhove *et al.,* 2019) compared to soil.
503 Therefore, leaf litter may have a high capacity for N fixation, allowing warming to increase
504 fixation in leaf litter to a greater extent than in soil. The direct positive effect of warming on
505 fixation observed in both environments may be due to increased enzyme activity and metabolism

506 of N-fixing bacteria (Houlton *et al.,* 2007; Reed *et al.,* 2011; Wood *et al.,* 2019), as the indirect
507 effect accounts for the changing N-fixing bacterial community.

508 This indirect effect of warming is consistent with findings that temperature is a
509 predominant parameter governing the N-fixing community (Zhao *et al.,* 2020) and the N-fixing
510 community is a strong predictor of N fixation activity (Berthrong *et al.,* 2014). *NifH* gene
511 composition doesn't always correlate to fixation rates since bacteria containing *nifH* genes are not
512 necessarily always fixing N (Feng *et al.,* 2019), but in our study warming-induced shifts in N-
513 fixing community composition did lead to increases in fixation. This is consistent with a previous
514 study which found that variation in the N-fixing community accounted for differences in N fixation
515 rates across a climate gradient which included tropical regions (Wu *et al.,* 2020). Finally, while
516 the activity and composition of N-fixing microbes in tropical soils have been found to be regulated
517 by moisture, C:N ratio, and the availability of phosphorus (Pajares and Bohannan, 2016), our study
518 shows that, even accounting for these parameters, warming generally had the strongest effects on
519 activity and composition of N-fixing microbes in leaf litter.

520 The effects of warming on N fixation rates were in part explained by changes in bacterial
521 composition. We found that artificial warming led to significant changes in both the overall and
522 N-fixing bacterial community, as demonstrated by specific genera significantly associated with
523 either the ambient or warming treatment. The taxa most highly associated with ambient plots in
524 the soil include the nitrifying genus *Nitrospira* (Lücker *et al.,* 2010) and the methanotrophic, N-
525 fixing genus *Methylocystis* (Stein *et al.,* 2011), which is also capable of nitrification. Additionally,
526 Spartobacteria and *Pelobacter* were highly associated with warmed soils. Spartobacteria is an
527 abundant, yet lesser-studied class of bacteria that, based on genome analysis, can likely degrade
528 various complex carbohydrates but likely has no role in the N cycle (Herlemann *et al.,* 2013;
529 Cernava *et al.,* 2017). *Pelobacter* is a lesser-studied N-fixing genus that is capable of producing
530 sulfide (Li *et al.,* 2021). The taxa most associated with ambient plots in the leaf litter include the
531 order *Myxococcales,* which has predicted roles in carbon and potassium cycling (Dai *et al.,* 2024),
532 and the symbiotic N-fixing genus *Allorhizobium* (Egamberdieva *et al.,* 2015). Taxa highly
533 associated with warming in the leaf litter include *Pseudonocardia* and *Bradyrhizobium*. The genus
534 *Pseudonocardia* is associated with certain plant and ant species and has been found to be capable
535 of asymbiotic N fixation (Chen *et al.,* 2019). *Bradyrhizobium* is a symbiotic N-fixing genus that
536 is also capable of asymbiotic fixation and denitrification (Zhang *et al.,* 2023). *Bradyrhizobium*

537 may be warm adapted (Zhao *et al.,* 2020), but some *Bradyrhizobium* OTUs were also associated
538 with ambient plots, possibly because OTUs represent different bacterial strains.
539 The possibility of different strains or species of the same genus being affected differently
540 by warming can be demonstrated by the example of Paenibacillus, which, in soil, has several OTUs
541 associated with ambient plots and several associated with warming plots. The genus Paenibacillus
542 is well-studied due to its ability to enhance plant growth by N fixation and P solubilization (Ryu
543 *et al.,* 2003). Further examination of specific OTUs in our study revealed that Paenibacillus species
544 associated with ambient plots were different from species associated with warming plots. Warming
545 may affect bacteria differentially even at the species level, emphasizing the need to further
546 investigate in situ effects of warming on the bacterial community.
547
548 *Warming significantly alters N fixer demographics*
549 Warming had strong effects on the growth dynamics of symbiotic N fixers. While warming
550 4°C above ambient temperatures was generally harmful to non-fixer seedling height and diameter
551 growth, N fixer seedlings experienced increased growth. In contrast, Nottingham *et al.,* (2023)
552 found that seedling growth decreased in tropical soils heated 4°C, particularly in N fixer seedlings.
553 This inconsistency may stem from different heating techniques used. Nottingham *et al.,* (2023)
554 utilized direct heating of soils in experimental plots, whereas in our study, plots were heated above
555 soil level, with infrared heaters starting 1-2 m above soil level and rising to 4 m above soil level
556 as understory vegetation height increased, resulting in the soils being heated to only 1°C above
557 ambient temperatures (Tunison *et al.,* 2024). Different warming temperatures can yield different
558 growth responses. In a plant growth chamber study on the short-term effects of warming on two
559 dominant tree species in a subtropical forest, Ghafoor *et al.,* (2022) found that warming 2.3°C
560 above ambient temperatures was beneficial for the height and diameter growth of the non-fixing
561 species, while it was only beneficial for the diameter growth of the N-fixing species. Warming of
562 4.5°C above ambient temperatures reduced growth in both species, although it increased root
563 nodule development in the N-fixing species.
564 Increased N fixer growth rates under warming suggest that warming affects symbiotic N
565 fixation (Ghafoor *et al.,* 2022; Nottingham *et al.,* 2023), but evidence of symbiotic N-fixation rate
566 changes is needed to show this conclusively. Although temperature effects root N exudation
567 depending on the thermal optimums of species, climate warming has been considered to generally

increase N exudation and play a role in sustaining increases in forest growth (Uselman *et al.,* 1999). However, more recent results suggest that warming can also decrease root N exudation (Nottingham *et al.,* 2023), and the relative importance of symbiotic N fixation in general has been questioned because it is facultative (Menge *et al.,* 2009) and may decline to near zero in mature tropical rainforests (Barron *et al.,* 2011; Van Langenhove *et al.,* 2019). In future studies it will be important to measure the effects of warming on both N fixer growth and symbiotic fixation, as our knowledge of how temperature affects symbiotic N fixation, especially in the tropics, is primitive and based on imprecise methods that can be improved (Bytnerowicz *et al.,* 2022). This will also be important when considering neighborhood effects in future warming studies on N fixer growth.

Our study also highlights differences in how N fixers and non-fixers respond to hurricane disturbances. Biogeochemical changes in tropical forest soils caused by hurricane disturbance will be shaped in concert with future warming (Reed *et al.,* 2020). Our current study shows that, while N fixers likely receive a greater boost in growth after hurricane disturbance compared to non-fixers, the effect of warming, as well as the underlying mechanisms, of this growth response are still unclear. Over the longer term, studying the relationship between hurricane-induced changes in plant growth, symbiotic fixation, and warming will be important in determining future ecosystem resilience (Johnstone *et al.,* 2016; Reed *et al.,* 2020).

In both N fixers and non-fixers, there was a shift from height investment to diameter investment in the presence of other N fixers under warming, as plants potentially shift from acquisitive to conservative growth strategies. Acquisitive strategies take precedence when plants can maximize resource acquisition rates and focus on outcompeting others, leading to fast but vulnerable growth, especially height growth in the case of seedlings vying for sunlight (Zhao *et al.,* 2016; Fagundes *et al.,* 2022). 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). This suggests that resource competition and stress tolerance may be more critical factors under warming. Together, our analysis shows that, besides our hypotheses being supported to at least some extent, there is likely an additional trend of plants shifting from acquisitive to conservative growth following disturbance, including hurricane, and the plants in the warming plots are currently displaying more of a conservative growth pattern, having experienced consistent warming disturbance for over five years.

Conclusion

Our study provides insight into the effects of warming on the asymbiotic N-fixing bacterial community, asymbiotic N fixation rates, and the growth of N-fixing symbioses in tropical forests, and therefore provides a uniquely integrated view of warming effects on N cycling dynamics in this biome. Our combined results suggest that warming will increase N flux from the atmosphere into the ecosystem while also increasing stress responses and competition in plants, possibly because warming elevates plant N demand even beyond microbial N production, resulting in conservative N utilization and growth in plants (Lie *et al.,* 2021). Climate models predict that extremely high temperatures will occur more frequently in tropical regions than in other climatic regions (Anderson *et al.,* 2012; Mora *et al.,* 2013), so it is crucial to predict how warming-induced alterations in the N cycle in these regions will change ecosystem functions and contributions to global nutrient cycles in the future. Future studies should integrate the asymbiotic and symbiotic N fixation components explored in this study, in addition to symbiotic N fixation rates and other elements of the N cycle such as mineralization, nitrification, and denitrification, as well as N assimilation by plants and even N leaching.

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Conflict of interest

The authors declare no conflicts of interest.

Author contributions

PB and BB designed this current study. TEW, SCR, and MAC designed the warming experiment and run it. TEW, IFGP, BB oversee the annual seedling census, and the entire field season associated with this study. PB and SCR conducted the data collection for N fixation assays. SCR measured N fixation rates. PB analyzed all the data and wrote the first draft of the study. SS supported sequencing and data analysis. PB, BB, and SS generated figures for data visualization and edited manuscript. 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 on request.

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996 **Supplementary Information**

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998 **Table S1** The number of OTUs removed from each dataset because they appeared in the blank
999 samples, the number of OTUs remaining for analysis, and the p-values of independent variables,
1000 determined by the distance-based redundancy analysis.

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1002 **Table S2** The fixed effects from the linear mixed-effect model analyzing the effects of various
1003 predictors on the rate of height growth.

1004

1005 **Table S3** The fixed effects from the linear mixed-effect model analyzing the effects of various
1006 predictors on the rate of diameter growth.