Tree soil carbon, nitrogen and associated bacterial community compositions, stability, evenness and diversity are enhanced along an age gradient of planted Inga punctata trees.

William Eaton (weaton@pace.edu)
Pace University https://orcid.org/0000-0002-6596-0331
Debra A. Hamilton

Research Article

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Abstract

Aims

This study was designed to determine if planting the leguminous tree *Inga punctata* in formerly cleared former tropical premontane wet forests changed the Nitrogen (N)-fixer and Lignin Degrader community compositions resulting in increased accumulation of soil carbon (C) and N components.

Methods

Soils were collected from old growth (> 50 years old), 4, 8 and 11-year-old *I. punctata* trees, and assessed for differences in various soil C and N metrics, and the mean proportion of sequences (MPS, as “relative abundance” via 16S rRNA V3, V4 sequencing), composition, stability and evenness of the community of N-fixer and Lignin Degrader genera.

Results

The N-fixer MPS decreased, and Lignin Degraders increased, while evenness and stability of both increased along the tree soil age gradient. The Lignin Degrader MPS were positively correlated with TN, NO_3^-, TOC, and Biomass C, and negatively correlated with N-fixer MPS and soil NH_4^+. The N-fixer MPS were negatively correlated with TN, NO_3^-, TOC, Biomass C and Lignin Degrader MPS, and positively correlated with NH_4^+. Evenness, stability, and diversity increased for both groups along the tree soil age gradient.

Conclusions

This is the first evidence that *I. punctata* facilitates changes the tree soil N-fixer and Lignin Degrader communities over time by increasing the diversity, evenness and stability of both groups, and increases the accumulation of tree soil C and N. This suggests *I. punctata* facilitates soil recovery such that it can serve as C and N sinks, and support its future use in reforestation management strategy.

Introduction

Decades of deforestation have damaged more than 50% of all tropical (Gibbs et al. 2010; Jacobson et al. 2019). This deforestation and subsequent agricultural uses have depleted the soil N and C (Amazonas et al. 2011; Brienen et al. 2015; Groppo et al. 2015; Powers and Marín-Spiotta 2017) and presumably damaged the soil microbial communities associated with these critical cycles impacted, thus reducing the capacity of these tropical soils to serve as carbon sinks (Bardgett and van de Putten 2014; Mendes et al. 2015; Eaton et al. 2019, 2021 a, b). Reforestation strategies are now more commonly being

implemented to remediate the damage done to these forests and their soils (Aide et al. 2013; Locatelli et al. 2015; Philipson et al. 2020; Poorter et al. 2021). Such strategies in Central and South America often include planting leguminous trees, as the N-fixing bacteria within the tree root nodules have been presumed to enhance recuperation of the soil N and C cycle dynamics and associated microbial community compositions and activities required for the recovery of these forests (Batterman et al. 2013; Chazdon et al. 2016; Lojka et al., 2005, 2010; Macedo et al., 2008; Poorter et al. 2016;).

It has been suggested that either these root nodule N-fixing bacterial activities (Chazdon et al., 2016; Gehring et al., 2005; Pan et al., 2011) or those of the free-living N-fixing bacterial activities (Hedin et al. 2009; Reed et al. 2011; Schmidt et al. 2008) represent the principal pathway by the damaged tropical lands recuperate the lost soil N and C. Regardless, both groups likely perform this function to some extent, the levels to which are unclear, as is the. This is only one area of study concerning leguminous tree soils that is needed to help understand the long-term beneficial effects of reforestation (Loreau 2010). Another critical area centers around the knowledge that bacterial N-fixing activity rates are feedback-regulated by the levels of inorganic N in the soils from N-fixation, and also from nitrification, or decomposition (ammonification), but these rates may not necessarily be proportional to the abundance or taxa of either the leguminous trees or N-fixing bacterial species (Barron et al. 2011; Gei et al. 2018). This leads to critical community ecology questions concerning activity in leguminous tree soils, such as what are the actual drivers for this up or down-regulation and what roles do the leguminous trees play? How do conditions such as tree species, age of tree, seasonality, climate change, land management history, etc. differentially influence the community composition, growth, and abundance of N-fixer bacteria and their rates of functional activity? How might all these factors influence the N and C-cycle dynamics in the tree soils?

More recently there have been questions posed as to whether tropical forest leguminous trees have some level of positive or negative influence on the abundance and diversity of their neighbors in reforested tropical areas? Some have said they have a positive influence, mostly associated with N cycle components (Xu et al. 2019 and 2020), while others have suggested perhaps the opposite might be true, at least at some point in time (Lai et al. 2018; Taylor et al. 2017). Still others suggest the answer is probably not straight-forward as it could very well involve many multifactorial, co-varying biotic and abiotic components (which are likely different between study sites) that might have influence forest regeneration pathways for all trees (Arroyo-Rodríguez et al. 2017; Eaton et al. 2020b). Certainly this topic is important to understanding how leguminous trees influence both the soil recovery and forest regrowth patterns after reforestation, which would help to maximize the efficacy of tropical reforestation and management plans (Wagg et al. 2011; Mendes et al. 2018; Eaton et al. 2019 and 2020b).

Members of the genus *Inga* are leguminous trees commonly found throughout the tropical Americas, thought to be ecologically important as forage resources, and proposed to enhance accumulation and sequestration of soil N and C, and stimulate above ground biomass development (Oglesby and Fownes 1992; Lawrence et al. 1995; Leblanc et al. 2005, Lojka et al. 2005, 2008 and 2010). These proposed characteristics, along with *Inga*'s ability to thrive in the high acid soils common in the tropical Americas

have resulted in the use of *Inga* spp. in reforestation strategies in Central and South America tropical lands (Lojka et al. 2005, 2008 and 2010). However, most of these C and N cycle proposed functions are speculative, while it is also unknown how *Inga* trees affect the soil microbial communities and their mechanisms that would be perform most of these functions. Yet, some preliminary work has been conducted. Leblanc et al. (2005) found four strains of symbiotic bacteria closely related to *Bradyrhizobium japonicum* and *B. liaoningense* associated with *I. edulis* root nodules in South America. Eaton et al. (2020a) found that in a recovering secondary forest in Costa Rica, *I. edulis* had greater levels of soil NH_4^+, and a less rich but possibly more dominant bacterial community in its microbiome in comparison to another leguminous tree, *Pentaclethra macroloba*, suggesting *I. edulis* soils may have reached bacterial community equilibrium status quicker than in the *P. macroloba* soils. This coincides with other studies that have shown that some species of *Inga* trees may be using more NH_4^+ as a preferred source of N that has enhanced growth and root nodule stimulation (Fisher 1995; Omena-Garcia et al. 2015; Justino et al. 2017). However, there is little else in the literature concerning *Inga* trees influences the soil ecosystems.

The goal of this current study was to determine if planting *I. punctata* in an abandoned pasture that formerly was premontane wet forest 4, 8 and 11 years prior to this study enhanced the tree soil total organic C (TOC), total Nitrogen (TN), nitrate (NO_3^-), and ammonium (NH_4^+) by facilitating changes in the N-fixer and Lignin Degrader bacterial functional group communities along the planted tree age gradient as compared to adjacent old growth *I. punctata* tree soils, thus suggesting that this tree species recuperated the potential of the soil to serve as C and N sinks. It was envisioned that predictors of soil ecosystem recovery would be identified that could infer the efficacy of this reforestation strategy that is part of a larger restoration effort on the Pacific slope of Monteverde, Costa Rica by the Fundación Conservacionista Costarricense (FCC) and Monteverde Institute (MVI) done with the objective restoring abandoned agricultural land with native and diverse tree species that would align with natural forest compositions

To address the project goal, the following questions were asked: (1) Do the soil C and N-cycle metrics, and C biomass levels change, and in what direction, along a trend of increasing tree soil age? (2) Are there differences in the community compositions of the N-fixer and Lignin Degrader bacterial communities in the different age tree soils that follow any trends in the C, N and Biomass C levels? (3) Does the stability of the tree soil bacterial functional group communities change along the tree soil age gradient? (4) Do the levels of C, N, and/or Biomass C predict the changes in the soil microbial communities, suggesting their use as indicators of a successful soil recovery strategy? (5) Do the results support future use of similar types of reforestation practices in the region?

Materials And Methods

Inga punctata Reforestation Site Description and Soil Sample Collection

Two organizations, the FCC and the MVI have been planting trees to restore abandoned pastures in the Monteverde Cloud Forest Region of Costa Rica since 1998 and 2016, respectively. Three restoration sites were planted on abandoned pasture lands in the Finca Rodríguez Ecological Reserve in 2008, 2011, and 2015, located in Monteverde, Costa Rica (10° 18’ 55.3” N and 84° 50’ 29.8”W; Supplemental Fig. 1), at an elevation of between 1250-1280m asl, and included seedling *I. punctata* trees in their species selection (Hamilton 2022). The land was previously used as dairy farming pasture, then converted to coffee and sugar cane production, which had been discontinued between 2–3 years before planting. The planting locations are within the Premontane Wet Life Zone (Holdridge and Poveda 1975), which is typified as evergreen forest with few deciduous species and moderate epiphyte load (Haber 2000). The annual precipitation of this life zone is stated as 2000–2500 mm, yet a private weather station has published an average annual precipitation of up to 2883.6 mm (LaVal 2020). A pronounced dry season occurs from February through April (< 100mm per month) and average temperatures range between 14 ^{o}C and 26 ^{o}C (Clark et al. 2000). According to Clark et al. (2000), soils are classified as a type of Andisol, Udands, which formed from volcanic parent material under wet, or udic, conditions. In August, 2019, 4 groups of *I. punctata* trees were used for this study: trees planted 4, 8, and 11 years prior to the (hereafter referred to as Inga 4, 8 and 11-year-old) and *I. punctata* older trees from an adjacent forest that had not been managed in recent memory (> 50 years), hereafter referred to as Old Inga Forest trees. All planted trees were located in specific plots within the managed land and were composed of only that age specific group of trees (described above and Fig. 1) and were separated from any other group of planted trees. The 4- and 8-year-old trees chosen for this study were > 50 m apart and > 400m distant form the 8-year-old and the Old Inga Forest trees. Trees from the latter two categories separated by > 50 m.

Six trees from each of the 4 different tree groups were identified for use. The distance from each tree base to the edge of each tree’s canopy “dripline” was determined, and 10% of that distance was calculated. At each of the four cardinal directions (North, South, East, West) and within this 10% drip-line distance, two soil cores (7.5 cm x 1.25 cm x 15 cm) were collected, and pooled, resulting in an 8-core soil sample per tree from within the 10% drip-line region. This method was used to ensure there were no other tree canopy driplines within this 10% drip-line distance, thus, minimizing the potential effect of any adjacent trees on an individual *I. punctata* tree – soil sample. This process resulted in 6 replicate pooled soil samples for, each tree age to be used for analysis. All soils were aseptically collected by disinfecting all gloves and collection tools with 70% ethanol between trees. All soil samples were passed through sterilized 5-mm sieve at field moist conditions prior to any analysis (see Supplementary Material 2 for a list of methods used).

Nutrient Analyses:

Standing pools of soil C and N metrics were used in this study, rather than measurement of fluxes in C and N metrics. Pools are considered better indicators of the long-term influence of “treatments”, such as tree planting and time since planting, whereas fluxes tend to represent rapid turnover rates of nutrients that are more associated with short-term changes in the soil biota (Davidson et al. 2004; Kuzyakov 2010; Plante et al. 2010). About 200 g of field moist soil from each pooled soil sample were analyzed at the

Center for Tropical Agriculture Research and Education (CATIÉ) Laboratories in Turrialba, Costa Rica for determination of all C and N values. Total organic C (TOC) levels were analyzed by the dry combustion method (Anderson and Ingram 1993), total nitrogen (TN) by the Kjeldahl method, NH_4^+ and NO_3^- were measured from 2 M KCl extracts using the spectrophotometric methods of Alef and Nannipieri (1995), and the soil Biomass C was determined by standard chloroform fumigation methods.

DNA Extraction, Sequencing, and Bioinformatics

Environmental microbial DNA was extracted from three 0.33g soil per each replicate soil sample, for a total of 1g for each soil sample, using the MoBio PowerSoil DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA). A NanoDrop 1000 spectrophotometer (ThermoFisher Scientific, Waltham, MA) was used to determine the concentration and purity (A_{260}/A_{280} ratio) of extracted soil eDNA prior to downstream. All the following methods are described in detail by McGee et al. (2019). Briefly, the different eDNA extracts from the soil samples were used for a 2-step PCR amplification of eDNA targeting the v3 and v4 of 16S ribosomal RNA gene region for bacteria and archaea (Caporaso et al. 2011). All generated soil amplicons were dual indexed and sequenced in MiSeq runs using a V3 MiSeq sequencing kit (600 cycles – 300 bp x 2; FC-131-1002 and MS-102-3003). The subsequent Illumina-generated sequences were analyzed using a standard QIIME protocol (Caporaso et al. 2011) with in house Python data management scripts. Input reads were processed through QIIME's open reference clustering with standard parameters and 97% similarity to the GreenGenes database. The open reference clustering performed all operational taxonomic unit (OTU) groupings compared to the database as well as de novo OTU generation and assigned known taxonomy where possible using the UCLUST algorithm. All generated sequencing data were submitted to the NCBI-Gene Expression Omnibus (GEO) repository on August 1, 2019, (Submission: SUB6145149, BioProject: PRJNA559202). This process identified about 790,113 taxonomically identifiable bacterial DNA sequences in the 4 different tree soil microbiomes, which were then categorized into 1476 successfully identified genera. The variable genera library sizes for each tree age soil samples were normalized by converting the read number of each specific genus sequence to the mean proportion of the sequences (MPS) per sample (Weiss et al. 2017), as the number of sequences from within each soil sample, divided by the total number of sequences per that sample.

The focus of this project was to assess the possible influence that *I. punctata*, as a common leguminous tree in the Monteverde region, had on the N-fixer and Lignin Degrader bacterial communities, as it is believed these groups are critical for soil ecosystem health. As there are no complete lists of the microbes within these groups currently available, we used a reference-based approach that makes associations between certain microbial groups and functional genes, which has been used to connect functional genes with specific microbial genera or species (Shim et al., 2017; Shin and Lee, 2017, 2015). Such efforts have resulted in valuable databases that, while not exhaustive nor said to be 100% accurate, provides one of the only means of linking a specific microbial genus identified by 16 S rDNA sequence only to a specific function. Recently, this approach has been used for soil microbial studies in other Costa Rican forests to approximate the community compositions of different bacterial and fungal functional groups, including N-fixer and Lignin Degrader fungi (Eaton et al. 2019, 2020 a, b, 2021 a, b). The same approach was used

for this project in which the databases www.genome.jp/kegg/, http://rdp.cme.msu.edu/hierarchy/, and www.zoology.ubc.ca/louca/FAPROTAX/lib/php/index.php, and 37 literature sources (Supplementary Material 3) were used to identify various genera of soil bacteria in the tree soils that have been previously associated with the different functional groups of N-fixer and/or Lignin Degrading bacteria. In these databases and articles, either the measured functional activity of bacteria in pure cultures or the presence of genes for specific enzymes (such as the *nif*H gene complex for N-fixer, the different laccase or oxidase genes associated with lignin degradation) have been published to identify the potential for the specific functional activity to be associated with the different genera. Only genera that had been clearly connected to the functional group were used, and hereafter for this study, these genera are being identified as N-fixer and/or Lignin Degrader bacteria (see Supplemental Material 4 for the list of bacteria in each functional group used in this study). This is grouping does not guarantee that any one specific member of a genus identified in the study positively had the function of interest. However, it does provide a valid suggestion that possible changes in the microbial communities within the tree age soils may be associated with changes in genera often associated with these certain functional group.

Nutrient Comparisons

The mean and standard deviation were determined for the TOC, Biomass C, TN, NO_3^- and NH_4^+ from all tree soil samples. Differences across the various age tree soils in the mean levels of the TOC and Biomass C together, and the TN, NO_3^- and NH_4^+ together, and also for each of these metrics individually were identified using the ANOSIM routine in the PRIMER-E package. The analyses were conducted on the Euclidean Distance resemblance matrices of log (X + 1) transformed and normalized C and N metric data, as recommended by Clarke and Gorley (2006), using 9,999 permutations.

Analysis of Microbial Community Structure

Several tests were conducted to observe for critical differences in the community structure of both the N-fixer and Lignin Degrader bacterial communities along the tree soil age gradient. We examined the bacterial community structures for differences in the total MPS levels of both functional groups of bacteria, differences in the compositions of both functional groups of bacteria, the strength of any compositional differences, differences in the percent similarity of the genera for each functional group community and in the number and taxonomy of the genera most typifying each tree soil type (SIMPER analyses), the levels of evenness of the communities, and the stability and complexity of the different soil microbiome systems.

a) The MPS and Similarity of Taxonomic Diversity Comparisons: The MPS values and the standard deviation of the total N-fixer and the Lignin Degrader bacteria were calculated and then visualized in bar charts. Kruskal – Wallis non-parametric tests were conducted in SPSS (v. 26) on the average MPS value raw data for these groups to determine if there were critical differences observed between tree soil age types. The Kruskal – Wallis H statistic and *p* value for the comparisons are outcomes of these tests.

Older, more established habitats have vast amounts of coexisting but taxonomically distinct microorganisms living in the same niche space that can perform the same metabolic functions, but the taxonomic identity encoding these functions can be highly variable (likely due to lateral gene transfer and ecological drift) with little to no effect on the function, indicating the extreme levels of functional redundancy that occurs in microbial communities. This often results in samples from older or more established habitats (such as an older forest soil) having greater overall taxonomic diversity, or lower taxonomic similarity, within microbial functional groups as compared to those from younger or more recently disturbed communities (Allison and Martiny 2008; Eaton et al. 2020a, 2021a; Louca and Doebeli 2016; Louca et al 2016, 2018; Morrissey et al 2016; Wohl et al 2004). For the current work, the focus was to use the multivariate SIMPER routine (PRIMER-E v.6) to assess the overall level of similarity of the diversity of the microbial taxa that are the most important community-level contributors to the composition of the N-fixer and Lignin Degrader communities to show either a decline or increase in overall taxonomic similarity (Clarke 1993; Clarke and Gorley 2006; Magierowski and Johnson 2006; Anderson et al. 2008). The multivariate SIMPER routine in PRIMER-E was applied to the different 4th root transformed MPS data converted into a Bray – Curtis similarity matrix (Clarke 1993; Clarke and Gorley 2006; Anderson et al. 2008) to assess the *a priori* assumption that greater community level taxonomic diversity would be represented by lower SIMPER similarity values, which would be expected in an older more stable and soil community.

b) The Compositional Comparisons: All bacterial group MPS values were then transformed using a 4th root transformation to account for dominant and rare taxa, as recommended by Anderson et al. (2008), and these data were converted into Bray-Curtis similarity matrices in PRIMER-E (v6) to quantify the similarity between all pairs of samples (Clarke and Warwick 2001). Differences in the compositions of the two functional group communities were compared between each tree soil age group using the multivariate ANOSIM (Analysis of Similarities) routine (Clarke 1993; Anderson et al. 2008). These were conducted on the Bray-Curtis resemblance matrices of the 4th root transformed MPS data in the PRIMER-E package. The number of permutations for the ANOSIM analyses was set to 9,999 (Clarke 1993; Clarke and Gorley 2006), with the R statistic (from 0 to 1, with 0 being most similar) and *p* value as the outputs from this test to measure degree of separation of bacterial communities. The ANOSIM procedure included Global R and *p* values as the “main” test results, and the pairwise R and *p* values for comparisons between different soil groups (Clarke and Warwick 2001).

c) The Strength of the Compositional Differences: The strength of any compositional differences was determined between tree age soil types using the multivariate Canonical Analysis of Principal Coordinates (CAP) ordination method (Clarke and Gorley 2006; Clarke and Warwick 2001) in the PRIMER-E package with the add-on PERMANOVA+. This analysis was applied to the same Bray-Curtis similarity matrices of the 4th root transformed MPS data mentioned above. The number of permutations for the CAP analyses was set to 9,999, and the program was allowed to select the number of PCO axes (m) that would provide the best separation between groups, while maximizing correct allocations of the groups to the different tree age soil habitats. The distances between the centroids were calculated in PCO space for any resemblance matrix between each tree soil group. The distances between centroids and the CAP axis

squared canonical correlation (R^2) values provided an approximation of the strength of any differences observed between tree soil age groups. Strong differences in the bacterial community compositions between the different soil groups were indicated by CAP axis R^2 values > 0.7, moderate differences are indicated by R^2 > 0.5 to 0.69, and weak differences represented by R^2 values < 0.5 (Anderson and Willis 2003).

d) Microbiome Community Evenness and Stability (Constancy) Comparisons: Increases in community evenness have been associated with increases in community stability (Grimm and Wissel 1997; Hillebrand et al 2008; Isbell et al. 2009; Tilman et al. 2006; van Rujiven and Berendse 2007; Wittebolle et al. 2009; De Roy et al. 2013). This has also been referred to as "constancy" of an ecological metric, which refers to the tendency for that metric to remain essentially unchanged after a disturbance or other system modification (Grimm and Wissel 1997; Shade (et al. 2012). Shade et al. (2012) and Griffths and Philipott (2013) provide reviews of stability related to microbial communities and provide indicators previously used to measure microbial community stability. However, these indicators didn't assess the variations that may occur in mean microbial community taxonomic metrics over time along successional gradients. Our interest was in finding an indicator to use along with evenness to measure changing stability or constancy that may be occurring within tree soil functional group microbial communities over successional time, as it would imply the development more successionally advanced microbial communities. Tilman et al (2006) and van Rujiven and Berendse (2007) used the index "*S*" to measure the stability of a variety of plant components, including species abundance, productivity, root biomass, etc., measured over time, and used these to suggest overall temporal stability of different ecosystem components. The calculation involves the concept that the less deviation there is from a mean value of some metric, the more stable the metric appears to be. From this, as an ecological metric *S* value (defined as mean metric value/standard deviation) increases in community A compared to community B, it implies that the values of this specific ecological metric are more stable in community A than in community B.

Based on this information from the literature, it appears that two reasonable indicators that could be used to demonstrate the development of more stable microbial communities were Pielou's evenness index and the *S* index. Pielou's evenness values were calculated using the 4th root transformed MPS data for the N-fixer and Lignin Degrader functional groups using the PRIMER-E package, while the *S* index was calculated dividing the mean values of the 4th root transformed MPS data for the N-fixer and Lignin Degrader functional groups for each tree age soil set by the standard deviation of the means. The resulting *S* values were labelled as *Sab* (for "abundance). Critical differences in the Pielou's evenness and *Sab* values between tree age soils were assessed using the Kruskal – Wallis procedure in SPSS (v26).

Relationships Between the C and N Metrics and Composition of the Functional Group Communities

To determine the strength and directions of the associations between metrics used in this study, the non-parametric Spearman's correlation method was performed (SPSS, v.26) between the MPS values of the

both bacterial functional group community compositions and the TN, NO_3^-, NH_4^+, TOC, and Biomass C data from the tree soils across the age gradient. The Distance-Based Linear Model (DistLM) permutation tests and distance-based redundancy analyses (dbRDA) were also implemented to explore how much of the patterns of the C and N metrics influenced the composition of the two different bacterial functional groups within the different tree age soil samples. The DistLM and dbRDA analyses were conducted using the 4th root transformed MPS data as the response variables and the Log (X + 1) transformed and normalized C and N metrics as the predictor variables in the DistLM and dbRDA routines in the PRIMER-E and PERMANOVA + packages.

Results

Nutrient Analyses

There were several clear patterns observed in the levels of the soil N metrics that were associated with the soils from the different tree age groups (Fig. 1a, Table 1). The TN levels were about the same in the Inga 4 and 8-year-old soils ($p = 0.335$) but were greater in the Inga 11-year-old soils (R = 0.850, p = 0.002, and R = 0.809, p = 0.002, respectively), and greatest in the Old Inga Forest tree soils (R > 0.933, all p values = 0.002). The NH_4^+ levels increased from that in the Inga 4-year-old soils to the greater levels found in the Inga 8 and Inga 11-year-old tree soils (R = 0.365, $p = 0.024$, and R = 0.535, p 0.006, respectively), the two of which were similar ($p = 0.524$). However, the Old Inga Forest trees had the least amount of NH_4^+ in their tree soils (R = 0.528, $p = 0.006$, R = 0.943, $p = 0.002$, and R = 0.996, $p = 0.002$, respectively).The pattern of NO_3^- levels in the tree soils was similar to that of the TN, with the Inga 4 and 8-year-old soils having about the same amount ($p = 0.543$), which was less than in either the Inga 11-year-old soils or the Old Inga Forest tree soils (R = 0.494 to 0.996, $p = 0.002$ to 0.015). Similar to the TN results, the NO_3^- levels were also greater in the Old Inga Forest tree soils (R = 0.470, $p = 0.009$).

There were also clear patterns in the levels of the soil C metrics that were associated with the age of the tree soils (Fig. 1b, Table 1). The TOC levels increased with each age of tree soil from the Inga 4-year-old to the Old Inga Forest soils (R = 0.272 to 0.998, p = 0.002 to 0.026). The pattern in the Biomass C levels between the tree soils was similar to that of the Biomass C levels were about the same in the Inga 4 and Inga 8-year-old soils (R = 0.596, $p = 0.442$), and then increased sequentially in the Inga 11-year-old tree soils (R = 0.596, $p = 0.004$, and R = 0.407, $p = 0.006$, respectively), and again in the Old Inga Forest tree soils (R = 0.985, $p = 0.002$, and R = 0.831, $p = 0.002$). As well, the Biomass C levels in the Old Inga Forest tree soils were greater than those in the Inga 11-year-old tree soils ($p = 0.002$).

Microbial Community Structure

a) Differences in MPS and Similarity of Taxonomic Diversity: There were 51 N-fixer bacterial genera (40 strictly free-living and 12 with both free-living and nodular N-fixer capabilities) and 155 genera of Lignin

Degrader bacterial genera identified in the tree soil eDNA samples. Differences were observed in the MPS levels of the communities of genera within the different functional groups (Fig. 2, Table 2a). Analysis of the differences in the MPS levels of the different genera using the Kruskal–Wallis procedure showed clear differences in the levels of these functional groups in the microbiomes of the different age tree soils. The MPS levels of the N-fixer bacteria were less in the Old Inga Forest tree soils than in the Inga 4, 8 or 11-year-old tree soils (H = 2.619, 2.386, 2.223, and p = 0.009, 0.017, 0.035, respectively), less in the Inga 11-year-old tree soils than found in either the Inga 4 or Inga 8-year-old tree soils (H = 2.101 and 1.904, p = 0.059 and 0.060, respectively), and were about the same in the Inga 4 and Inga 8-year-old tree soils. The patterns observed for the MPS of the Lignin Degrader bacterial communities across the different tree soils were opposite to that observed for the N-fixer bacteria (Fig. 2, Table 2a). Specifically, the MPS levels of the Lignin Degrader genera were greater in the Old Inga Forest tree soils than in any of the other three tree soils (H = 4.83 to 7.58, p = 0. 001 to 0.0001), greater in the Inga 11-year-old tree soils than either the Inga 4 or Inga 8-year-old tree soils (H = 3.73 to 4.72, p = 0.001 to 0.004), and, as with the N-fixers, not different between the Inga 4 and Inga 8-year-old tree soils.

For the SIMPER analyses, the maximum cumulative percentage cut-off criterion was set at 70% for both the N-fixer and Lignin Degrader bacterial communities in the different tree soil types. The SIMPER results showed (Table 2b) the average taxonomic similarity in the N-fixer bacterial community composition was 71.17% for the Inga 4-year-old tree soil samples, 70.66% for the Inga 8-year-old tree soil samples, 72.26% Inga 11-year-old tree soil samples, and only 52.69% for the Old Inga Forest tree soil samples, indicating that the diversity of this functional group is likely lower in the planted tree soils and increases in the older more established tree soils. Similarly, The SIMPER results for the analysis of the Lignin Degrader bacterial community composition was 71.95% for the Inga 4-year-old tree soil samples, 69.30% for the Inga 8-year-old tree soil samples, 66.32% for the Inga 11-year-old tree soil samples, and 56.74% for the Old Inga Forest tree soil samples. As above, this large decrease in % similarity suggests the diversity of this functional group is likely lower in the planted tree soils and increases with tree soil age along the gradient.

b) Microbial Community Compositional Differences: There were differences in the overall composition of the N-fixer bacterial genera communities between the different tree soil microbiomes (Table 3a; Global R = 0.175; p = 0.008). The differences in genera composition of this group were greatest between the Old Inga Forest tree and Inga 4, 8 and 11-year-old tree soils (R = 0.256, 0.181, 0.194, p = 0.037, 0.052, 0.043, respectively). There were no differences in the community composition of this community between the Inga 8 and 11-year-old tree soils or between the Inga 4 and 8-year-old tree soils, but there was a difference in this community composition between the Inga 4 and 11-year-old tree soils (R = 0.168, p = 0.056), again providing evidence that changes in community composition are occurring along the age gradient of the tree soil microbiomes, at least in the two older tree soils.

There were also differences in the community composition of the Lignin Degrader genera across the different tree soil microbiomes (Table 3a; Global R = 0.235, p = 0.0003). Similar to the N-fixer bacteria, the composition of the Lignin Degrader community was most different between the Old Inga Forest tree soils

and Inga 4, then the 8 and then the 11-year-old tree soils (R = 0.464, 0.207, and 0.191, p = 0.002, 0.024, and 0.035, respectively). As well, the Inga 4 and Inga 11-year-old tree soil Lignin Degrader community compositions were also quite different (R = 0.445, p = 0.002). However, there were no definitive differences found in the Lignin Degrader community structures between the Inga 4 and 8-year-old tree soils or those of between the Inga 8 and 11-year-old tree soils. As with the N-fixer bacterial data, these results provide evidence that the Lignin Degrader community composition changes with tree soil age.

These differences in tree soil community composition were further supported by the CAP assessments. The CAP model (p = 0.004) showed that the differences in the N-fixer community compositions (Fig. 3a, Table 3b) between the Old Inga Forest and the other tree soil types were strong (R^2 = 0.857), those between the Inga-4 and 8-year-old and 4 and 11-year-old tree soils were moderate (R^2 = 0.682), and those between the Inga 8-year-old and 11-year-old tree soils were weak (R^2 = 0.446). The CAP model (p = 0.0025) also showed there were differences in the community compositions of the Lignin Degrader bacterial genera along the tree soil age gradient (Fig. 3b, Table 3b). There were strong differences in Lignin Degrader community composition between the Old Inga Forest and Inga 4-year-old tree soils (R^2 = 0.809), moderate differences in composition between the Old Inga Forest and Inga 8 and Inga 11-year-old tree soils (R^2 = 0.583), and between the Inga 4 and Inga 11-year-old tree soils (R^2 = 0.583). However, the differences in community composition were very weak (R^2 = 0.086) between the Inga-4-year-old and 8-year-old tree soils and between the Inga 8 and 11-year-old tree soils.

c) Differences in Microbiome Community Evenness and Stability: The Kruskal – Wallis analyses of the evenness data (Table 4a) showed a clear trend of increasing evenness of both the N-fixer and Lignin Degrader bacterial communities along the tree soil age gradient. The evenness values of the Old Inga Forest tree soils were greater than in the Inga 11, 8, and 4-year-old tree soils (H = 2.0235, 2.289, 4.139, p = 0.042, 0.022, < 0 0.0001, respectively). The evenness values were also greater in the 8 and 11-year-old Inga Forest soils were greater than in the 4-year-old soils (H = 2.035, 2.289, p = 0.042, 0.022, respectively), but were not different from each other.

Similarly, the stability (S) values showed a trend of increasing community stability along the tree soil age gradient (Table 4b). The S_{ab} for the N-fixer bacterial community was about the same in the Old Inga Forest and Inga11-year-old tree soils, however, both were greater than that in the Inga 8-year-old (H = 5.86 and 5.44, p = 0.0002 and 0.0003, respectively) and 4-year-old (H = 6.70 and 6.32, p = 0.0001 and 0.0001, respectively) soils. The S_{ab} levels for the Lignin Degraders also increased along the same gradient, as the values were greater in the Old Inga Forest and Inga 11-year-old tree soils than in either the Inga 4 or Inga 8-year-old tree soils (H range = 2.45 to 5.47, p range = 0.0003 to 0.034), with both latter groups having about the same S_{ab} levels. These collective results point towards a trend of increasing stability in the soil microbial communities along the tree age gradient. The differences in these S values along with the differences in Pielou's Evenness and increase diversity of the functional group genera point towards a trend of increasing stability (constancy) in the tree soils with age.

Relationships Between the C and N Metrics and Composition of the Functional Group Communities:

The Spearman's Correlation analyses (Table 5) showed that the MPS levels of the Lignin Degrader bacterial genera and N-fixer bacterial genera were negatively correlated (Coefficient = -0.448, $p = 0.032$). In addition, the MPS values of the Lignin Degrader bacteria were positively correlated with the levels of the TN, NO_3^-, TOC, and Biomass C (Coefficient range = 0.682 to 0.815, all p values = < 0.001), and negatively correlated with the levels of NH_4^+ (Coefficient = -0.475, $p = 0.022$). The MPS levels of the N-fixer bacterial group were negatively correlated with the TN, NO3-, TOC and Biomass C data (Coefficient range = -0.539 to -0.623, p values = 0.002 to 0.023), and positively correlated with the NH_4^+ levels (Coefficient = 0.473, $p = 0.023$). The DistLM and dbRDA analyses (Fig. 4) showed that the variations in the TN levels explained 8.8% of the variation in the N-fixer bacterial community composition (a) Pseudo-F = 2.02, $p = 0.053$), and the TOC levels explained 14.19% of the variation in the Lignin Degrader bacterial community composition (b), Pseudo-F = 3.47, $p = 0.004$). The TN explained 12.17% of the variation in the Lignin Degrader community across the tree soils (c), Pseudo-F = 2.91, $p = 0.015$). No other C or N metrics validly explained any of the variation in these communities, as all their p values were > 0.07.

Discussion

Overview

Both the goals and questions of this project stated earlier were successfully addressed, in that the results showed that *Inga punctata* appears to be important in increasing the N cycle activity in soils early in the growth of the tree, specifically through the first decade, by enhancing the activity of the N-fixer bacteria in the tree soil microbiomes. It also appears that because of this enhanced N-cycle activity, the trees are associated with increases in the organic C components in the tree soil microbiomes through enhancing the Lignin Degrader bacterial community, especially after a decade. This, in part, is likely a result of the increased inorganic N that bacteria need to produce the enzymes for this complex organic C decomposition. This project provides some of the first evidence that *I. punctata* is facilitating long-term TN and TOC accumulation in these soils through its influence on the two specific functional groups of bacteria studied.

Members of the genus *Inga* are important leguminous trees common in the forests of Central and South America, including the Monteverde Cloud Forests of Costa Rica (Holdridge and Poveda 1976; Hartshorn et al. 1994; Lojka et al. 2005 and 2010; Orwa et al. 2009). As they are thought to help recuperate C and N components in soils damaged by deforestation and pasture grazing, they are being used as part of various reforestation strategies (Oglesby and Fownes 1992; Leblanc et al. 2005, Lojka et al. 2005, 2008 and 2010). However, little is known of the specifics of how members of this genus influence the below ground biotic and abiotic components of the soil ecosystems. Understanding such information concerning *Inga* sp. could help in development of the most efficacious tropical reforestation and recovery

plans following deforestation of some tropical forests, followed by grazing use (Eaton et al. 2019 and 2020a; McGee et al. 2019; Mendes et al. 2018; Wagg et al. 2011). It has been presumed that *Inga*, and other leguminous trees, influence the soil N cycle dynamics through production of NH_4^+, as it is the outcome of N-fixation. This has also been shown to be the preferred form of inorganic N for *Inga* root nodule development and production (Fisher 1995; Omena-Garcia et al. 2015; Justino et al. 2017). In addition, it would be expected that the NH_4^+ produced from N-fixation associated with *Inga* root nodules would stimulate the production of NO_3^- and NO_2^-, as it serves as the needed substrate for the oxidation of NH_4^+ by groups of nitrifying bacteria. For these reasons, the abundance and composition of the N-fixer community was examined in this project as an indicator of the influence of *I. punctata* on the reforested tree soil microbiomes over time.

There is also an important connection between the inorganic N produced in these ways and the organic C components in soils. Not only are these different forms of inorganic N needed by soil microbiota to produce the nitrogenase enzyme systems for N-fixation, they are also critical for the production of the suite of enzymes needed for all the various kinds of organic C compound decomposition, which regulates the decomposition process rates associated with the oxidation of both the simple, more labile forms (i.e., carbohydrates, cellulose, etc.) and more complex and recalcitrant forms (i.e., lignin, hemicelluloses, etc.) of organic C (Burns et al. 2013; Henry 2013; Wallenstein and Burns 2011; Xu et al. 2021). Enhancing decomposition of the more recalcitrant forms of organic C produces more long-lasting forms of TOC in the soils, and can provide more material for biomass development, while oxidation of the more labile forms of organic C are generally thought of as a source of energy for maintenance. Both, however, are critical to forest soil and overall ecosystem health. As lignin is considered the most recalcitrant of the complex organic C compounds and requires bacteria and fungi that have special enzymes for degradation of this compound (Datta et al. 2017; Janusz et al. 2017), the abundance and composition of the Lignin Degrader bacteria were also considered as an indicator of the influence that *I. punctata* has on ecosystem recovery in the reforested tree soil microbiomes over time in this current project.

The probable roles of *Inga* spp. in the recuperation of both N and C in damaged soils, and in facilitating long-term N accumulation and C sequestration in soils, are the likely reasons it is being used in some restoration strategies. Thus, enhancing our understanding of how different *Inga* spp. might influence both the N-fixer and Lignin-Degrading groups of bacteria, along with levels of various C and N components in soils could provide some insight into the potential roles of this important leguminous tree in both natural forests and reforested areas, which would be valuable for developing intact and damaged tropical forest management plans. This current study focused on how different ages of *I. punctata* planted as part of the FFC/MVI reforestation program ((https://monteverde-institute.org/reforestation-program.html) might be influencing the tree soil microbiomes and ecosystems over time. In particular, the focus was on how *I. punctata* might influence the tree soil C and N cycle components, and the associated soil N-fixer and Lignin Degrader bacterial community compositions. This work is the first, to our knowledge, that provides clear evidence of the significant impact that *I. punctata* has on the tree soil ecosystems, which changes along a gradient of time since tree planting (i.e., tree age). More specifically, it provides evidence to

support the idea that an *Inga* species is influencing the soil microbiomes over time such that it is enhances long-term accumulation of N in soils, and the soil TOC, as proposed by Oglesby and Fownes (1992), Leblanc et al. (2005) and Lojka et al. (2008).

Nitrogen and the Microbiomes

The results in this study strongly linked the N cycle metrics to the N-fixer bacterial functional group composition in the tree soils. Specifically, the TN levels were the best predictor of the N-fixer community composition, and greater levels of TN and NO_3^- and lower levels of NH_4^+ were positively correlated with greater MPS levels of the N-fixer functional group genera. It was also found that MPS levels of the N-fixer bacterial community and NH_4^+ levels were generally greater in the Inga 4 and Inga 8-year-old soils than in the Inga 11-year-old and the Old Inga Forest soils. This suggests that the younger *I. punctata* trees might require greater levels of NH_4^+ in their early growth stages, which appears to be provided by the greater levels of the N-fixer bacterial community, but that both these levels of diminish over time. These patterns are consistent with the theory that low levels of NH_4^+ serve to feedback up-regulate the levels of N-fixation in soils, which would likely result in a greater abundance of N-fixers (Barron et al. 2010; Gei et al. 2018), as would occur in soils early in recovery. Moreover, the N-fixer functional group genera developed into a more stable community with greater evenness of distribution of the functional group taxa (i.e., greater *S* and Pielou's evenness indices) along the tree age gradient, which would be expected following successional theory in soil microbial communities.

Interestingly, it also was found that the taxonomic diversity of the N-fixer community increased with the age of tree soils (as suggested by SIMPER results). Although this may seem counterintuitive considering above ground macroecological theory, it is consistent with soil microbial ecological theory. Specifically, this pattern of greater diversity of microbial functional groups in older, established habitats has been reviewed by Louca et al (2016). These authors point out that in these more established habitats, such as older forest soils, the composition of taxa of microbial functional groups becomes more diverse due to advanced stages of functional redundancy resulting from the evolutionary adaptation of microbes to respond to environmental changes through horizontal transfer of metabolic activity genes, which when combined with very rapid generation times results in taxonomic and ecological drift, thus, providing a high degree of flexibility and stability within these functional groups in older habitats (Allison and Martiny 2008; Eaton et al. 2020a, 2021a; Louca and Doebeli 2016; Louca et al 2016, 2018; Morrissey et al 2016; Wohl et al 2004). Thus, the results of the current project give strong indication that the impact of planting *I. punctata* on the N-fixer community occurs rapidly, within the first 4–8 years, and begins to decrease sometime after that, resulting in development of a more stable and diverse functional group community.

The relationship between N-fixer and nitrifier bacteria is a critical one for soil N recovery in tropical forests (Pajares and Bohannan 2016). The current results suggest the presence of the high levels of NO_3^- and low levels of NH_4^+ occurring along with the low MPS levels of N-fixers could indicate a point along the successional tree age gradient at which the nitrification process becomes more dominant than N-fixation

in these tree soils (Pajares and Bohannan 2016). The specific cause of this functional shift is unclear as there are several factors at play here associated with the changing levels of NH_4^+. The previously mentioned known feedback regulation of N-fixation by NH_4^+ levels (Barron et al. 2011; Gei et al. 2018) will play an important role in up or down-regulating the levels of N-fixation activity, and, thus, indirectly the nitrification process. It is also known that in the presence of excess NH_4^+, N-fixer bacteria will use this inorganic N rather than expend the energy needed to fix new N_2 into NH_4^+ (Barron et al. 2011; Cusack et al. 2009; Reed et al. 2011). Additionally, older *Inga* trees have been found to use more NH_4^+ as a preferred source of N for growth and root nodule stimulation (Fisher 1995; Omena-Garcia et al. 2015; Justino et al. 2017), which represents an interesting example of a plant-microbiome interaction in which the tree may be stimulating development of a specific microbiome that influences the success of nodulation and tree growth. Consequently, it is unclear what specifically is causing the increased levels of NO3- with the decreased levels of NH4 + and N-fixer MPS, as it is likely most of these factors are somehow involved. These relationships deserve much more student in the future.

It is unclear the relative importance of free-living N-fixer bacteria as compared to the root nodule-associated ones. In the current study 40 of the 52 N-fixer bacterial genera identified have been listed as being free-living N-fixer bacteria and 12 listed as both nodular and free-living N-fixers according to Weir (2016) and ICSP (2020). Thus, both groups appear to be important in the N cycle within the *I. punctata* tree soil microbiome. Some have suggested that the nodular N-fixation associated with leguminous trees is the principal pathway for N regeneration in damaged tropical soils (Chazdon et al., 2016; Gehring et al., 2005; Pan et al., 2011; Powers and Marín-Spiotta 2017), yet it has also been suggested by that free-living N-fixer bacteria may be more important in older tropical forests than the nodular N-fixers (Hedin et al. 2009; Reed et al. 2011). Still others suggest that free-living N-fixer soil bacteria are more active than nodular N-fixers in tropical soils under conditions of high N-demands, such as after large amounts of leaf litter deposition in older forests, or in earlier stages of recovery of damaged soils (Hedin et al. 2009; Reed et al. 2011; Schmidt et al. 2008). Clearly more work is needed on this topic. None the less, it is likely that the enhancement of N-fixation by both free-living and nodular bacteria associated with leguminous trees are both part of the primary pathways for recovery of tropical soil inorganic N that is needed to enhance enzyme production for organic C decomposition, biomass development activities, and growth of all cells (Burns et al. 2013; Cenciani et al. 2009; Gehring et al. 2005; Hedin et al. 2009; Henry 2013; Janusz et al. 2017; Pan et al. 2011; Reed et al. 2011; Rodrigues et al. 2013; Wallenstein et al. 2011; Xu et al. 2021). Clearly more work is needed concerning these two groups of N-fixers.

Carbon and the Microbiomes

The above-mentioned increases in TN and NO_3^-, the decreases in NH_4^+ and levels of the MPS of the N-fixer community in the tree soils were positively correlated with increases in the MPS levels of the Lignin Degrader bacterial community, the TOC, and the Biomass C. The TOC and Biomass C levels were observed to increase sequentially in the soils from the different tree age soil groups from the Inga 4-year-old to the Old Inga Forest soils, and the MPS of the Lignin Degrader group increased from the Inga 4 and

8-year-old soils to the Inga 11-year-old soils and then to the Old Inga Forest soils. Further, the levels of TOC and Biomass C were the best predictors of the Lignin Degrader community composition. Consistent with the observations associated with the N-fixer community, the Lignin Degrader community diversity (see the SIMPER results), stability, and evenness increased along the tree age gradient. These are predictable outcomes based on previous soil microbial ecological results and theories showing that microbial functional group diversity increases with successional time (reviewed by Louca et al. 2016), and also the recognition that increases in evenness and the stability suggest development of greater constancy, resilience, and resistance over time, in general (Grimm and Wissel 1997; Tilman et al. 2006; van Rujiven and Berendse 2007; Isbell et al. 2009; Wittebolle et al. 2009; Shade et al 2012; De Roy et al. 2013), and more specifically within microbial communities (Shade et al. 2012; Griffths and Philipott 2013). These observations collectively suggest that *I. punctata* up-regulates the Lignin Degrader community in the tree soils over time, which develops into a more stable and diverse community that is enhances the soil TOC and Biomass C accumulation. These processes are likely a result of *I. punctata* facilitating the microbial-based development of inorganic N for microbial enzyme development, in particular for the use by the Lignin Degrader bacterial community (and likely other critical complex organic C degrader groups), ultimately resulting in the accumulation of greater levels of TOC in the soils.

Previous studies have shown that different species of *Inga* trees are important sources of inorganic N (Lojka et al. 2010; Eaton et al. 2020b) that enhance the quantity and quality of the soil organic C (Leblanc et al. 2005; Lojka et al. 2005, 2010), the development of complex organic C and Lignin Degrading communities (Eaton et al. 2020b), stimulate greater biomass development than other non-*Inga* tree species in their habitats (Lawrence et al. 1995; Lojka et al. 2005), and are thought to stimulate C sequestration and long-term N accumulation (Oglesby and Fownes 1992; Leblanc et al. 2005). The Lignin Degrader bacterial community in the current study are subsets of the larger microbial functional groups that degrade complex organic C, including lignin. As such, enhancing this community likely represents one of the important contributions by *I. punctata* as it is facilitating the tree soil microbiome to increase development of soil organic C and biomass-C, ultimately increasing the soil TOC stabilization and storage, and providing forms of organic C that are more usable for biomass development, all of which are necessary for soil ecosystem recovery after disturbance (Herpin et al. 2002; Hafich et al. 2012; Kivlin et al. 2016). This project has provided some of the first evidence that the planting of *I. punctata* enhances the tree soil TOC and Lignin Degrader microbiome community, resulting in enhancement of soil ecosystem recovery within the first 4 to 8 years after tree planting, and which becomes more beneficial after 11 years post-planting, following a trajectory towards tree soil ecosystems of Old Growth *I. punctata* in the region.

Conclusions

The results of this project provide the first evidence that *I. punctata* facilitates changes in the amount and composition of both the N-fixer and Lignin Degrader bacterial communities over time, both of which also increase in stability and complexity in the tree soils. This occurs in association with the negative correlation between the MPS levels of the N-fixers and Lignin Degrader communities, the positive

correlations between the MPS of the Lignin Degraders and TN, NO_3^-, TOC, and Biomass C, the negative correlations between the MPS of the Lignin Degraders and the NH_4^+ levels, and the opposite directions of correlations between these C and N metrics and the MPS of the N-fixer communities. This suggests the strong relationship and critical interplay between the N-fixer and Lignin Degrader communities, and the successional changes that occur among the two groups, resulting in changes in the various C and N cycle metrics observed along the tree soil gradient (over time), as these interactions enhanced the accumulation of TN and TOC in the *I. punctata* tree soils as the trees age. These interactions represent a classic example of how leguminous trees can facilitate the levels of N and associated soil microbiomes, preparing the soils to support conditions in which specific groups of decomposers can utilize the more complex forms of organic C, ultimately increasing the soil C and N (Paul 2015).

The results of this project provide some of the first evidence that fully supports the ideas that *Inga* sp. may enhance long-term TN and TOC deposition and sequestration (Oglesby and Fownes 1992; Lojka et al. 2005, 2008), which is likely due to the changing tree soil microbiomes. Given this, it seems probable that the *I. punctata* - tree soil microbiome relationships are facilitating the soil recovery of the C and N cycle processes in these former pasture lands and enhancing the potential for these soils to serve in the future as more efficacious C and N sinks (Gehring et al. 2005; Pan et al. 2011; Poorter et al. 2016; Chazdon et al. 2016). Thus, this reforestation strategy used by the MVI is effective at soil ecosystem improvement and should be continued. The results of this work also suggest that increases in TN, NO_3^-, TOC, Biomass C, and Lignin Degrader bacterial MPS, and decreases in $NH_4^+$and N-fixer bacterial MPS, along with increased complexity and stability of both functional groups of bacteria can serve as good indicators of recovery in these soil ecosystems and should be monitored for efficacy evaluations. What is not clear is at what point between 8 and 11-years post-planting do the tree soil microbial communities begin shifting towards greater variation and more stability, with increased accumulation of TN and TOC. This warrants further investigation, as it would be valuable to know for the purposes of planning forestry management strategies and reforestation assessment plans. In addition, studies are underway to determine how *I. punctata* influences the nitrifying bacteria and nitrification dynamics in their tree soils over time.

Declarations

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Conflicts of Interest The authors have no relevant financial or non-financial conflicts of interest to declare.

Availability of Data The generated DNA sequence data is publicly available as described in text, and were submitted to the NCBI-Gene Expression Omnibus (GEO) repository on August 1, 2019, (Submission: SUB6145149, BioProject: PRJNA559202). All other data is available from the corresponding author by request.

Author Contributions Both authors contributed to the study conception, design, and soil sampling. Data collection and analysis was performed by William D. Eaton. Drafts of the manuscript were written by William D. Eaton, with edits made by Debra A. Hamilton. Both authors read and approved the final manuscript.

Ethics Approval Not applicable, as no humans or other animals were used in this work.

Consent to participate Both authors have consented to participate in this work and subsequent publication

Consent for publication All authors agree to have all data published.

References

1. Aide TM, Clark ML, Ricardo Grau H, Lopez-Carr D, Levy MA, Redo D, Bonilla-Moheno M, Riner G, Andrade-Nunez MJ, Muniz M (2013) Deforestation and reforestation of Latin America and the Caribbean (2001–2010). Biotropica 45:262–271
2. Alef K, Nannipieri P (1995) Methods in Applied Soil Microbiology and Biochemistry. Elsevier, Academic Press
3. Allison SD, Martiny JBH (2008) Colloquium paper: resistance, resilience, and redundancy in microbial communities.Proc Natl Acad Sci USA105:11512–11519
4. Amazonas NT, Martinelli LA, Piccolo MDC, Rodrigues RR (2011) Nitrogen dynamics during ecosystem development in tropical forest restoration. For Ecol Manag 262:1551–1557
5. Anderson JM, Ingram JSI(1993) Tropical Soil Biology and Fertility: a handbook of methods 2nd edition Wallingford Oxshire UK: Cambridge University Press CAB International
6. Anderson MJ, Gorley RN, Clarke KR(2008) PERMANOVA + for PRIMER: guide to software and statistical methods PRIMER-E Ltd. Plymouth UK
7. Arroyo-Rodriguez V, Melo F, Martinez-Ramos M, Bongers F, Chazdon R, Meave J, Norden N, Santos B, Leal I, Tabarelli M (2017) Multiple successional pathways in humanmodified tropical landscapes:

New insights from forest succession, forest fragmentation and landscape ecology research. Biol Rev 92:326–340

8. Bardgett RD, van der Putten WH (2014) Belowground biodiversity and ecosystem functioning. Nature 515:505–511
9. Barron AR, Purves DW, Hedin LO (2011) Facultative nitrogen fixation by canopy legumes in a lowland tropical forest. Oecologia 165:511–520
10. 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
11. Brienen RJW, Phillips OL, Feldpausch TR et al (2015) Long-term decline of the Amazon carbon sink. Nature 519:344–348
12. Burns RG, DeForest J, Marxsen J, Sinsabaugh RL, Stromberger ME, Wallenstein MD, Weintraub MN, Zoppini A (2013) Soil enzymes in a changing environment: current knowledge and future directions. Soil Biol Biochem 58:216–234
13. 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. PNAS 108:4516–4522
14. Cenciani K, Lambais MR, Cerri CC, Azevedo LCB, Feigl BJ (2009) Bacteria diversity and microbial biomass in forest, pasture and fallow soils in the southwestern Amazon basin. Revista Brasileira de Ciência do Solo 33:907–916
15. Chazdon RL, Broadbent EN, Rozendaal DMA, Bongers F, Zambrano AMA, Aide TM, Balvanera P, Becknell JM, Boukili V, Brancalion PHS, Craven D, Almeida-Cortez JS, Cabral GAL, de Jong B, Denslow JS, Dent DH, DeWalt SJ, Dupuy JM, Duran SM et al (2016) Carbon sequestration potential of second-growth forest regeneration in the Latin American tropics. Sci Adv 2:e1501639–e1501639
16. Clark K, Lawton RO, Butler PR(2000) In: Nadkarni N, Wheelwright NT (eds) Monteverde: Ecology and Conservation of a Tropical Cloud Forest Bowdoin Scholars' Bookshelf. Book 1.http://digitalcommons.bowdoin.edu/scholars-bookshelf/1
17. Clarke KR (1993) Non-parametric multivariate analyses of changes in community structure. Aust J Ecol 18:117–143
18. Clarke KR, Gorley RN (2006) PRIMER v6: User manual. PRIMER-E Ltd Plymouth UK
19. Clarke KR, Warwick RM (2001) A further biodiversity index applicable to species lists: variation in taxonomic distinctness. Mar Ecol Prog Ser 216:265–278
20. Cusack DF, Silver W, McDowell WH (2009) Biological nitrogen fixation in two tropical forests: ecosystem level patterns and effects of nitrogen fertilization. Ecosyst 12:1299–1315
21. Datta R, Kelkar A, Baraniya D, Molaei A, Moulick A, Meena RS, Formanek P (2017) Enzymatic Degradation of Lignin in Soil: A Review. Sustainability 9:1163
22. Davidson EA, Reis de Carvalho CJ, Vieira ICG, Figueiredo Rde O, Moutinho P, Yoko Ishida F, Primo dos Santos MT, Benito Guerrero J, Kalif K, Tuma Sabá R (2004) Nitrogen and phosphorus limitation of

biomass growth in a tropical secondary forest. Ecol Appl 14:150–163

23. De Roy K, Marzorati M, Negroni A, Thas O, Balloi A, Fava F, Verstraete W, Daffonchio D, Boon N (2013) Environmental conditions and community evenness determine the outcome of biological invasion. Nat Comm 4:1–5
24. Eaton WD, McGee KM, Donnelly R, Lemenze A, Karas O, Hajibabaei M (2019) Differences in the soil microbial community and carbon-use efficiency following development of *Vochysia guatemalensis* tree plantations in unproductive pastures in Costa Rica. Rest Ecol. https://doi.org/10.1111/rec.12978
25. Eaton WD, McGee KM, Alderfer K, Jimenez AR, Hajibabaei M (2020a) Increase in abundance and decrease in richness of soil microbes following Hurricane Otto in three primary forest types in the Northern Zone of Costa Rica. Special Top Issue: "Microbial Ecol Chang Environments" PLoS ONE 15:e0231187. https://doi.org/10.1371/journal.pone.0231187
26. Eaton WD, McGee KM, Hoke E, Lemenze A, Hajibabaei M (2020b) Influence of Two Important Leguminous Trees on Their Soil Microbiomes and Nitrogen Cycle Activities in a Primary and Recovering Secondary Forest in the Northern Zone of Costa Rica. Soil Syst 4:65. https://doi.org/10.3390/soilsystems4040065
27. Eaton WD, McGee KM, Larimer M, Hoke E, Karas O, Hernandez B, Wayland NA(2021a) Changes in soil bacterial communities, and carbon and nitrogen metrics as potential indicators of land use effects in a humid tropical forest.Pedobiologia (Jena)85–86, 150730
28. Eaton WD, McGee, Donnelly R, Lemenze A, Larimer M, Hajibabaei M (2021b) The impacts of a logging road on the soil microbial communities, and carbon and nitrogen components in a Northern Zone Costa Rican Forest. Appl Soil Ecol 164:103937
29. Fisher RF (1995) Amelioration of Degraded Rain Forest Soils by Plantations of Native Trees. Soil Sci Soc Am J 59:544549
30. Gehring C, Vlek PLG, de Souza LAG, Denich M (2005) Biological nitrogen fixation in secondary regrowth and mature rainforest of central Amazonia. Agric Ecosyst Environ 111237–111252. https://doi.org/10.1016/j.agee.2005.06.009
31. Gei M, Rozendaal DMA, Poorter L, Bongers F, Sprent JI, Garner MD, Aide TM, Andrade JL, Balvanera P, Becknell JM, Brancalion PHS, Cabral GAL, César RG, Chazdon RL, Cole RJ, Colletta GD, de Jong B, Denslow JS, Dent DH et al (2018) Legume abundance along successional and rainfall gradients in Neotropical forests. Nat Ecol Evol 2:1104–1111
32. Gibbs HK, Ruesch AS, Achard F, Clayton MK, Holmgren P, Ramanjutty N, Foley JA (2010) Tropical forests were the primary sources of new agricultural land in the 1980s and 1990. PNAS 107:16732–16737
33. Griffiths BS, Philippot L (2013) Insights into the resistance and resilience of the soil microbial community. FEMS Microbiol Rev 37:112–129
34. Grimm V, Wissel C (1997) Babel, or the ecological stability discussions: an inventory and analysis of terminology and a guide for avoiding confusion. Oecologia 109:323–334

35. Groppo JD, Lins SRM, Camargo PB, Assad ED, Pinto HS et al (2015) Changes in soil carbon, nitrogen, and phosphorus due to land-use changes in Brazil. Biogeosci 12:4765–4780
36. Haber W(2000) An introduction to cloud forest trees. In Nadkarni N and Wheelwright NT (eds) Monteverde: Ecology and Conservation of a Tropical Cloud Forest Bowdoin Scholars' Bookshelf. Book 1 http://digitalcommons.bowdoin.edu/scholars-bookshelf/1
37. Hafich K, Perkins EJ, Hauge JB, Barry D, Eaton WD (2012) Implications of Land Management on Soil Microbial Communities and Nutrient Cycle Dynamics in the Lowland Tropical Forest of Northern Costa Rica. Trop Ecol 53:215–224
38. Hamilton D(2022) Offsetting Destruction: The Important Functional Contribution of Carbon Sequestration in the Restoration of a Tropical Forest in Monteverde, Costa Rica. Reference Module in Earth Systems and Environmental Sciences, Elsevier, 2022. ISBN 9780124095489. https://doi.org/10.1016/B978-0-12-821139-7.00198-7
39. Hartshorn GS, Hammel B, McDade LA, Bawa KS, Hespenheide HA, Hartshorn GS (1994) Vegetation types and floristic patterns. La Selva: ecology and natural history of a neotropical rain forest. The University of Chicago Press, Chicago, IL
40. Hedin LO, Brookshire ENJ, Menge DNL, Barron AR (2009) The nitrogen paradox in tropical forest ecosystems. Annu Rev Ecol Syst 40:613–635
41. Henry HA (2013) Soil extracellular enzyme dynamics in a changing climate. Soil Biol Biochem 56:53–59
42. Herpin U, Cerri CC, Conceicão Santana Carvalho M, Markert B, ¸ Enzweiler J, Friese K, Breulmann G (2002) Biogeochemical dynamics following land use change from forest to pasture in a humid tropical area (Rondônia, Brazil): a multi-element approach by means of XRF-spectroscopy. Sci Total Environ 286:97–109
43. Hillebrand H, Bennett DM, Cadotte MW (2008) Consequences of dominance: a review of evenness effects on local and regional ecosystem processes. Ecology 89:1510–1520
44. Holdridge LR, Poveda LJ(1975) Árboles de Costa Rica. Centro Cientı´fico Tropical, San Jose´, Costa Rica, 546 pp
45. The International Committee for Systematics of Prokaryotes, Subcommittee on the Taxonomy of Rhizobia and Agrobacteria (ICSP) (2020) http://edzna.ccg.unam.mx/rhizobial-taxonomy/
46. Isbell FI, Polley HW, Wilsey BJ (2009) Biodiversity, productivity and the temporal stability of productivity: patterns and processes. Ecol Lett 12:443–451
47. Jacobson AP, Riggio JM, Tait A, Baillie JEM (2019) Global areas of low human impact ('Low Impact Areas') and fragmentation of the natural world. Sci Rep 9::14179
48. Janusz G, Pawlik A, Sulej J, Świderska-Burek U, Jarosz-Wilkołazka A, Paszczyński A (2017) Lignin degradation: microorganisms, enzymes involved, genomes analysis and evolution. FEMS Microbiol Rev 41:941–962
49. Justino G, Omena-Garcia R, Santos A, de dos, Camargos L, Sodek L, Gonçalves J (2017) Nitrogen use strategies of nodulated amazonian legume: *Inga edulis*. J Trop For Sci 29:1–9

50. Kivlin SN, Hawkes CV (2016) Tree species spatial heterogeneity and seasonality drive soil fungal abundance richness and composition in Neotropical rainforests. Environ Microbiol 18:4662–4673
51. Kuzyakov Y (2010) Priming effects: Interactions between living and dead organic matter. Soil Biol Biochem 42:1363–1371
52. Lai HR, Hall JS, Batterman SA, Turner BL, Breugel M (2018) Nitrogen fixer abundance has no effect on biomass recovery during tropical secondary forest succession. J Ecol 106:1415–1427
53. Lawrence A, Pennington TD, Hands MR, Zuniga RA (1995) In: Evans DO, Szott LT (eds) *Inga*: high diversity in the neotropics. In Nitrogen Fixing Trees for Acid Soils. pp. Nitrogen Fixing Tree Research Reports, Special Issue, pp 130–141
54. LaVal R(2020) Datos casa LaVal [Online]. Available at: https://monteverde-institute.org/mvi-digital-collections.html
55. Leblanc HA, McGraw RL, Nygren P, Roux CL (2005) Neotropical legume tree *Inga edulis* forms N_2-fixing symbiosis with fast-growing *Bradyrhizobium* strains. Plant Soil 275:123–133
56. Locatelli B, Catterall CP, Imbach P, Kumar C, Lasco R, Marín-Spiotta E, Mercer B, Powers JS, Schwartz N, Uriarte M (2015) Tropical reforestation and climate change: Beyond carbon. Rest Ecol 23:337–343
57. Lojka B, Preininger D, Lojkova J, Banout J, Polesny Z (2005) Biomass growth and farmer knowledge of *Inga edulis* in Peruvian Amazon. Agricultura Trop Et Subtropica 38:44–51
58. Lojka B, Lojkova J, Banout J, Polesny Z, Preininger D (2008) Performance of an improved fallow system in the Peruvian Amazon— modelling approach. Agrofor Syst 72:27–39
59. Lojka B, Dumas L, Preininger D, Polesny Z, Banout J (2010) The use and integration of *Inga edulis* in agroforestry systems in the amazon – review article. Agricultura Trop Et Subtropica 43:352–359
60. Loreau M (2010) From Populations to Ecosystems: theoretical foundations for a new ecological synthesis. Monographs in Population Biology 46. Princeton University Press, Princeton NJ
61. Louca S, Doebeli M (2016) Transient dynamics of competitive exclusion in microbial communities. Environ Microbiol 18:1863–1874
62. Louca S, Jacques SMS, Pires APF, Leal JS, Srivastava DS, Parfre LW, Farjalla VF, Doebeli M (2016) High taxonomic variability despite stable functional structure across microbial communities. Nat Ecol Evol 1:0015
63. Louca S, Polz MF, Mazel F, Albright MBN, Huber JA, O'Connor MI, Ackermann M, Hahn AS, Srivastava DS, Crowe SA, Doebeli M, Parfrey LW (2018) Function and functional redundancy in microbial systems. Nat Ecol Evol 2:936–943
64. Macedo MO, Resende AS, Garcia PC, Boddey RM, Jantalia CP, Urquiaga S, Campello EFC, Franco AA (2008) Changes in soil C and N stocks and nutrient dynamics 13 years after recovery of degraded land using leguminous nitroge-fnixer trees. For Ecol Manag 255:1516–1524
65. McGee KM, Eaton WD, Porter TM, Shokralla S, Hajibabaei M (2019) Soil microbiomes associated with two dominant Costa Rican tree species, and implications for remediation: A case study from a Costa Rican conservation area. Appl Soil Ecol 137:139–153

66. Mendes LW, Tsai SM, Navarrete AA, de Hollander M, van Veen JA, Kuramae EE (2015) Soil-borne microbiome: linking diversity to function. Microb Ecol 70:255–265
67. Mendes MS, Latawiec AE, Sansevero JBB, Crouzeilles R, Moraes LFD, Castro A, et a (2018) Look down-there is a gap-the need to include soil data in Atlantic Forest restoration. Rest Ecol 27:361–370
68. Morrissey EM, Mau RL, Schwartz E, Caporaso JG, Dijkstra P, van Gestel N, Koch BJ, Liu CM, Hayer M, McHugh TA, Marks JC-, Price LB, Hungate BA (2016) Phylogenetic organization of bacterial activity. ISME J 10:2336–2340
69. Oglesby KA, Fownes JH Effects of chemical composition on nitrogen mineralization from green manures of seven tropical leguminous trees. Plant Soil 143:127–132Omena-Garcia R, Justino G(1992) Araujo
70. Omena-Garcia RP, Justino GC, Ferreira de Araujo VB, deSouza LAG, Camargos LS, de Carvalho Goncalves JF (2015) Mineral nitrogen associated changes in growth and xylem-N compounds in Amazonian legume tree. J Plant Nutr 4:584–595
71. Orwa C, Mutua A, Kindt R, Jamnadass R, Anthony S (2009) Agroforestry tree Database: a tree reference and selection guide. version 4.0. World Agroforestry Centre Kenya
72. Pan Y, Birdsey RA, Fang J, Houghton R, Kauppi PE, Kurz WA, Phillips OL et al (2011) A large and persistent carbon sink in the world's forests. Science 333(6045):988–993
73. Pajares S, Bohannan BJM (2016) Ecology of nitrogen fixing, nitrifying, and denitrifying microorganisms in tropical forest soils. Front Microbiol 7:1045
74. Philipson C, Cutler M, Brodrick P, Asner G, Boyd D, Moura Costa P, Fiddes J, Foody G et al (2020) Active restoration accelerates the carbon recovery of human-modified tropical forests. Science 369:838–841
75. Plante AF, Conant RT, Carlson J, Greenwood R, Shulman JM, Haddix ML, Paul EA (2010) Decomposition temperature sensitivity of isolated soil organic matter fractions. Soil Biol Biochem 42:1991–1996
76. Poorter L, Craven D, Jakovac CC, van der Sande MT, Amissah L, Bongers F, Chazdon RL et al (2021) Multidimensional tropical forest recovery. Science 374:1370–1376
77. Powers JS, Marín-Spiotta E (2017) Ecosystem Processes and Biogeochemical Cycles in Secondary Tropical Forest Succession. Ann Rev Ecol Evol Syst 48:497–519
78. Reed SC, Cleveland CC, Townsend AR (2011) Functional ecology of free-living nitrogen fixation: a contemporary perspective. Annu Rev Ecol Evol Syst 42:489–512
79. Rodrigues JLM, Pellizari VH, Mueller R, Baek K, Jesus EC, Paula FS, Mirza B et al (2013) Conversion of the Amazon rainforest to agriculture results in biotic homogenization of soil bacterial communities. PNAS 110:988–993
80. Schmidt SK, Reed SC, Nemergut DR, Grandy AS, Cleveland CC et al (2008) The earliest stages of ecosystem succession in high elevation (5000 metres above sea level), recently deglaciated soils. Proc R Soc B 275:2793–2802

81. Shade A, Peter H, Allison SD, Baho DL, Berga M, Bürgmann H, Huber DH, Langenheder S, Lennon JT, Martiny JB, Matulich KL (2012) Fundamentals of microbial community resistance and resilience. Front Microbiol 3:417
82. Shim JE, Lee T, Lee I (2017) From sequencing data to gene functions: co-functional network approaches. Anim Cells Syst 21:77–83
83. Shin J, Lee I (2015) Co-inheritance analysis within the domains of life substantially improves network inference by phylogenetic profiling. PLoS ONE 10:e0139006. https://doi.org/10.1371/journal.pone. 0139006
84. Taylor BN, Chazdon RL, Bachelot B, Menge DNL (2017) Nitrogen-fixer trees inhibit growth of regenerating Costa Rican rainforests. PNAS 114:8817–8822
85. Tilman D, Reich PB, Knops JM (2006) Biodiversity and ecosystem stability in a decade-long grassland experiment. Nature 441:629–632
86. van Ruijven J, Berendse F (2007) Effects of Diversity on the Temporal Stability of Plant Populations. Oikos 116:1323–1330
87. Wagg C, Jansa J, Schmid B, van der Heijden MG (2011) Belowground biodiversity effects of plant symbionts support aboveground productivity. Ecol Lett 14:1001–1009
88. Wallenstein MD, Burns RG(2011) Ecology of extracellular enzyme activities and organic matter degradation in soil: a complex community-driven process. In: Dick R (ed) Methods of soil enzymology. 9:35–55
89. Weiss S, Xu ZZ, Peddada S, Amir A, Bittinger K, Gonzalez A, Lozupone C, Zaneveld JR et al (2017) Normalization and microbial differential abundance strategies depend upon data characteristics. Microbiome 5. https://doi.org/10.1186/s40168-017-0237-y
90. Weir BS(2016) The current taxonomy of rhizobia. NZ Rhizobia website. https://www.rhizobia.co.nz/taxonomy/rhizobia Last updated: Jan,2016
91. Wittebolle L, Marzorati M, Clement L, Balloi A, Daffonchio D, Heylen K, De Vos P, Verstraete W, Boon N (2009) Initial community evenness favours functionality under selective stress. Nature 458:623–626
92. Wohl D, Arora S, Gladstone J (2004) Functional redundancy supports biodiversity and ecosystem function in a closed and constant environment. Ecology 85:1534–1540
93. Xu CY, Du C, Jian JS, Hou L, Wang ZK, Wang Q, Geng ZC (2021) The interplay of labile organic carbon, enzyme activities and microbial communities of two forest soils across seasons. Sci Rep 11:5002
94. Xu H, Detto M, Li Y, Li Y, He F, Fang S (2019) Do N-fixing legumes promote neighbor diversity in the tropics? J Ecol 107:229–239
95. Xu H, Detto M, Fang S, Chazdon RL, Li Y, Hau BCH et al(2020) Soil nitrogen concentration mediates the relationship between leguminous trees and neighbor diversity in tropical forests. *Commun Biol* **3**, 317 (2020). https://doi.org/10.1038/s42003-020-1041-y

Tables

Table 1. ANOSIM analysis results of the differences in mean values of Total Nitrogen (TN), Ammonium (NH_4^+), Nitrate (NO_3^-), Total Organic Carbon (TOC), and Biomass C measured in the tree soils of *Inga punctata* trees planted 4, 8, and 11 years prior to the study (Inga 4, 8 and 11-year-old trees) and Old Growth *I. punctata* trees (Old Forest Inga). The ANOSIM Global R and *p* values and pairwise R and *p* values are presented for each analysis.

TN ANOSIM Results			NH_4^+ ANOSIM Results			$NO3^-$ ANOSIM		
Global R: 0.754, *p* = 0.001			Global R: 0.553, p = 0.001			Global R: 0.616, *p* = 0.001		
Pairwise Tests	*R value*	*p value*	*Pairwise Tests*	*R value*	*p value*	*Pairwise Tests*	*R value*	*p value*
Inga 4, Inga 8	0.019	0.335	Inga 4, Inga 8	0.365	0.024	Inga 4, Inga 8	-0.03	0.543
Inga 4, Inga 11	0.85	0.002	Inga 4, Inga 11	0.535	0.006	Inga 4, Inga 11	0.494	0.015
Inga 4, Inga Forest	0.985	0.002	Inga 4, Inga Forest	0.528	0.006	Inga 4, Inga Forest	0.983	0.002
Inga 8, Inga 11	0.809	0.002	Inga 8, Inga 11	-0.026	0.524	Inga 8, Inga 11	0.58	0.009
Inga 8, Inga Forest	0.993	0.002	Inga 8, Inga Forest	0.943	0.002	Inga 8, Inga Forest	0.996	0.002
Inga 11, Inga Forest	0.933	0.002	Inga 11, Inga Forest	0.996	0.002	Inga 11, Inga Forest	0.47	0.009
TOC ANOSIM Results			Biomass C ANOSIM Results					
Global R: 0.710, *p* = 0.001			Global R: 0.543, *p* = 0.001					
Pairwise Tests	*R value*	*p value*	*Pairwise Tests*	*R value*	*p value*			
Inga 4, Inga 8	0.469	0.002	Inga 4, Inga 8	-0.019	0.442			
Inga 4, Inga 11	0.722	0.002	Inga 4, Inga 11	0.596	0.004			
Inga 4, Inga Forest	0.998	0.002	Inga 4, Inga Forest	0.985	0.002			
Inga 8, Inga 11	0.272	0.026	Inga 8, Inga 11	0.407	0.006			
Inga 8, Inga Forest	0.959	0.002	Inga 8, Inga Forest	0.831	0.002			
Inga 11, Inga	0.698	0.002	Inga 11, Inga	0.535	0.009			

Forest	Forest

Table 2. Differences in the MPS and taxonomic similarity among the tree soil groups. a) Kruskal – Wallis analysis results of the differences in mean proportion of sequences (MPS) of the Nitrogen-fixing (N-fixer) genera and Lignin Degrading (Lignin Degrader) genera in the soils of *Inga punctata* trees planted 4, 8, and 11 years prior to the study (Inga 4, 8 and 11) and Old Inga Forest *I. punctata* trees (Old Inga). The Kruskal – Wallis H and p values are presented for both the Main and Pairwise Tests. b) Results of the assessment of the percent similarity in taxonomic diversity (as determined by the SIMPER routine) of the mean proportion of sequences (MPS) of the N-fixer and Lignin Degrader functional group genera identified in the tree soils of *Inga punctata* trees planted 4, 8, and 11 years prior to the study (Inga 4, 8 and 11) and Old Growth *I. punctata* trees (Old Forest Inga).

2a.

Kruskal-Wallis Main Test N-Fixer MPS			Kruskal-Wallis Main Test Lignin Degrader MPS		
H value	8.483		H value	15.65	
p value	0.037		*p* value	0.0001	
Kruskal - Wallis Pairwise Tests			Kruskal - Wallis Pairwise Tests		
Comparisons	H value	p value	Comparisons	H value	p value
Old Inga to Inga 4	2.619	0.009	Old Inga to Inga 4	6.55	0.0001
Old Inga to Inga 8	2.386	0.017	Old Inga to Inga 8	7.58	0.0001
Old Inga to Inga 11	2.223	0.035	Old Inga to Inga 11	4.83	0.001
Inga 11 to Inga 4	2.101	0.059	Inga 11 to Inga 4	3.73	0.004
Inga 4 to Inga 8	0.603	0.561	Inga 4 to Inga 8	1.09	0.302
Inga 8 to Inga 11	1.904	0.06	Inga 8 to Inga 11	4.72	0.001

2b.

	Inga 4	Inga 8	Inga 11	Old Inga
	Average Similarity of Taxonomic Diversity Among Each Tree Soil Group (%)			
N-Fixer Genera	71.17	70.66	72.26	52.69
Lignin Degrader Genera	71.95	69.31	66.32	56.74

Table 3. Multivariate-level differences in the composition of the N-fixer and Lignin Degrader communities in the 4 groups of *Inga punctata* trees: those planted 4, 8, and 11 years prior to the study (Inga 4, 8 and 11) and Old Inga Forest I. *punctata* trees (Inga Forest). a) ANOSIM analysis of the results of the differences in the mean proportion of sequences (MPS) of the Nitrogen-fixing (N-fixers) genera and Lignin Degrading (Lignin Degraders) genera. The ANOSIM Global R and *p* values and pairwise R and *p* values are presented for each set of analyses. B) Canonical Analysis of Principal Coordinates (CAP) results demonstrating the strength of the differences in the community composition of the N-fixing bacterial and Lignin Degrading bacterial genera. Model *p* values and Canonical Correlation Squared (R^2) values presented for the comparisons. R^2 values > 0.7 = Strong differences, 0.50 to 0.69 = Medium differences, < 0.49 = Weak differences.

3a)

ANOSIM Results of N-Fixer Bacteria			ANOSIM Results of Lignin Degrading Bacteria		
Global R: 0.175, *p* = 0.008			Global R: 0.235, *p* = 0.0003		
Pairwise Tests	*R value*	*p value*	*Pairwise Tests*	*R value*	*p value*
Inga 4, Inga 8	0.132	0.105	Inga 4, Inga 8	0.445	0.002
Inga 4, Inga 11	0.168	0.056	Inga 4, Inga 11	0.141	0.169
Inga 4, Old Inga	0.256	0.037	Inga 4, Old Inga	0.464	0.002
Inga 8, Inga 11	0.011	0.422	Inga 8, Inga 11	0.057	0.245
Inga 8, Old Inga	0.181	0.052	Inga 8, Old Inga	0.207	0.024
Inga 11, Old Inga	0.194	0.043	Inga 11, Old Inga	0.191	0.035

3b)

CANONICAL ANALYSIS RESULTS				
	N-Fixing Bacteria		Lignin Degrading Bacteria	
Model p value	0.004		0.0025	
	CAP R^2	Strength	CAP R^2	Strength
Inga 4, Inga 8	0.682	Medium	0.086	Weak
Inga 4, Inga 11	0.682	Medium	0.583	Medium
Inga 4, Old Inga	0.858	Strong	0.809	Strong
Inga 8, Inga 11	0.446	Weak	0.086	Weak
Inga 8, Old Inga	0.858	Strong	0.583	Medium
Inga 11, Old Inga	0.858	Strong	0.583	Medium

Table 4. Evenness (a) and stability (b) levels of the Nitrogen-fixing (N-fixers) genera and Lignin Degrading (Lignin Degraders) bacterial communities in the tree soils of *Inga punctata* trees planted 4, 8, and 11 years prior to the study (Inga 4, 8 and 11) and Old Inga Forest trees (Old Inga) *I. punctata* trees. Pielou's Evenness mean j values and stability mean *Sab* values, with their standard deviations are presented. Kruskal-Wallis Pairwise tests were conducted to determine the levels of differences in evenness (j values) of the different functional group communities between the different age tree soils. The Kruskal-Wallis H and *p* values are presented for each set of analyses.

4a)

	Evenness of N-Fixers			Evenness of Lignin Degraders	
	j value	Std. Dev.		*j* value	Std. Dev.
Inga 4	0.704	0.057	Inga 4	0.776	0.052
Inga 8	0.847	0.042	Inga 8	0.885	0.046
Inga 11	0.865	0.055	Inga 11	0.879	0.035
Old Inga	0.989	0.042	Old Inga	0.980	0.039
	Kruskal-Wallis Test			Kruskal-Wallis Test	
Pairwise Test	H value	*p* value	Pairwise Test	H value	*p* value
Inga 4 to Inga 8	2.035	0.042	Inga 4 to Inga 8	3.699	0.005
Inga 4 to Inga 11	2.289	0.022	Inga 4 to Inga 11	3.938	0.003
Inga 4 to Old Inga	4.139	<0.0001	Inga 4 to Old Inga	7.49	< 0.0001
Inga 8 to Inga 11	0.267	0.791	Inga 8 to Inga 11	0.255	0.802
Inga 8 to Old Inga	2.289	0.022	Inga 8 to Old Inga	3.88	0.003
Inga 11 to Old Inga	2.035	0.042	Inga 11 to Old Inga	4.742	0.001

4b)

	Sab value of N-Fixers		*Sab* value of Lignin Degraders		
	Sab value	Std. Dev.		*Sab* value	Std. Dev.
Inga 4	2.31	2.13	Inga 4	8.86	2.88
Inga 8	4.14	1.62	Inga 8	8.11	2.85
Inga 11	10.03	2.1	Inga 11	13	2.98
Old Inga	10.17	1.93	Old Inga	16.36	2.35
	Kruskal-Wallis Test			Kruskal-Wallis Test	
Pairwise Tests	H value	*p* value	Pairwise Tests	H value	p value
Inga 4 to Inga 8	2.77	0.021	Inga 4 to Inga 8	0.453	0.66
Inga 4 to Inga 11	6.32	0.0001	Inga 4 to Inga 11	2.45	0.034
Inga 4 to Old Inga	6.7	0.0001	Inga 4 to Old Inga	4.92	0.001
Inga 8 to Inga 11	5.44	0.0003	Inga 8 to Inga 11	2.95	0.016
Inga 8 to Old Inga	5.86	0.0002	Inga 8 to Old Inga	5.47	0.0003
Inga 11 to Old Inga	0.12	0.907	Inga 11 to Old Inga	2.17	0.055

Table 5. Spearman's non parametric Correlation analysis of the Lignin Degrader (Lig.Deg.) and Nitrogen-Fixing (N-Fix) mean percent sequence (MPS) values, and the levels of Total Nitrogen (TN), Total Organic Carbon (TOC), ammonium (NH_4^+), Nitrate (NO_3^-), and Biomass Carbon (Biomass C) from the tree soils of *Inga punctata* trees planted 4, 8, and 11 years prior to the study (Inga 4, 8 and 11) and Old Growth *I. punctata* trees (Old Forest Inga). Spearman's *rho* value and *p* values are presented (* = significant at $p < 0.05$, ** = $p < 0.01$).

	Lig.Deg.	N-Fix	TN	TOC	NH_4^+	NO_3^-	Biomass C
Lig.Deg.		-.448*	.797**	.815**	-.475*	.682**	.783**
		0.032	< 0.001	< 0.001	0.022	< 0.001	< 0.001
N-Fix	-.448*		-.623**	-.605**	.473*	-.539**	-.620**
	0.032		0.002	0.002	0.023	0.008	0.002
TN	.797**	-.623**		.979**	-.424*	.845**	.880**
	< 0.001	0.002		< 0.001	0.044	< 0.001	< 0.001
TOC	.815**	-.605**	.979**		-0.387	.800**	.844**
	< 0.001	0.002	< 0.001		0.068	< 0.001	< 0.001
NH_4^+	-.475*	.473*	-.424*	-0.387		-.568**	-0.384
	0.022	0.023	0.044	0.068		0.005	0.07
NO_3^-	.682**	-.539**	.845**	.800**	-.568**		.745**
	< 0.001	0.008	< 0.001	< 0.001	0.005		< 0.001
Biomass C	.783**	-.620**	.880**	.844**	-0.384	.745**	
	< 0.001	0.002	< 0.001	< 0.001	0.07	< 0.001	

Figures

Figure 1a

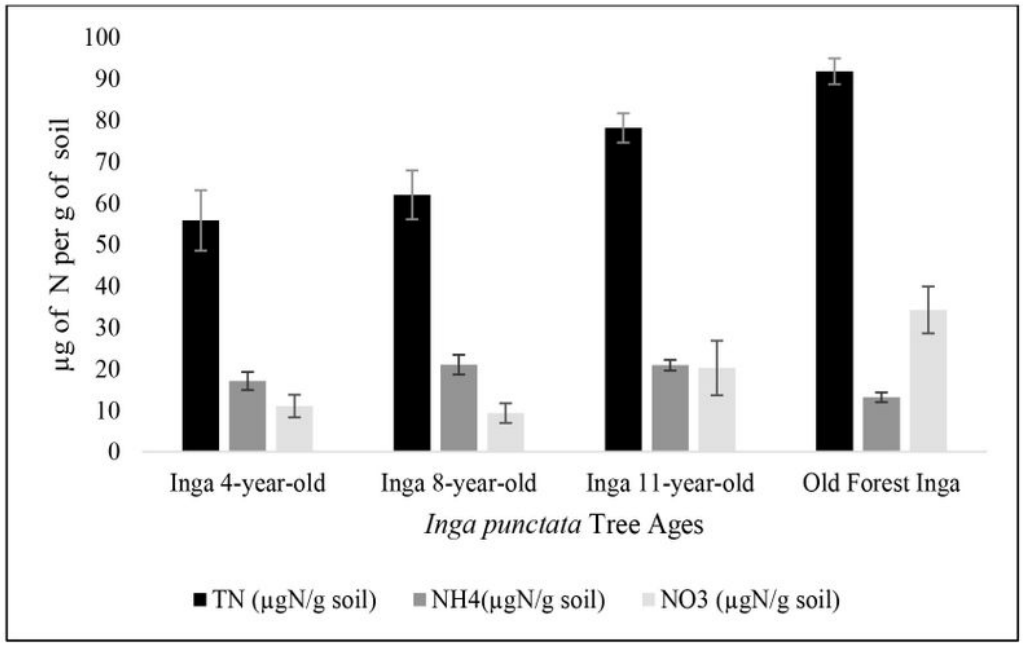

Figure 1b

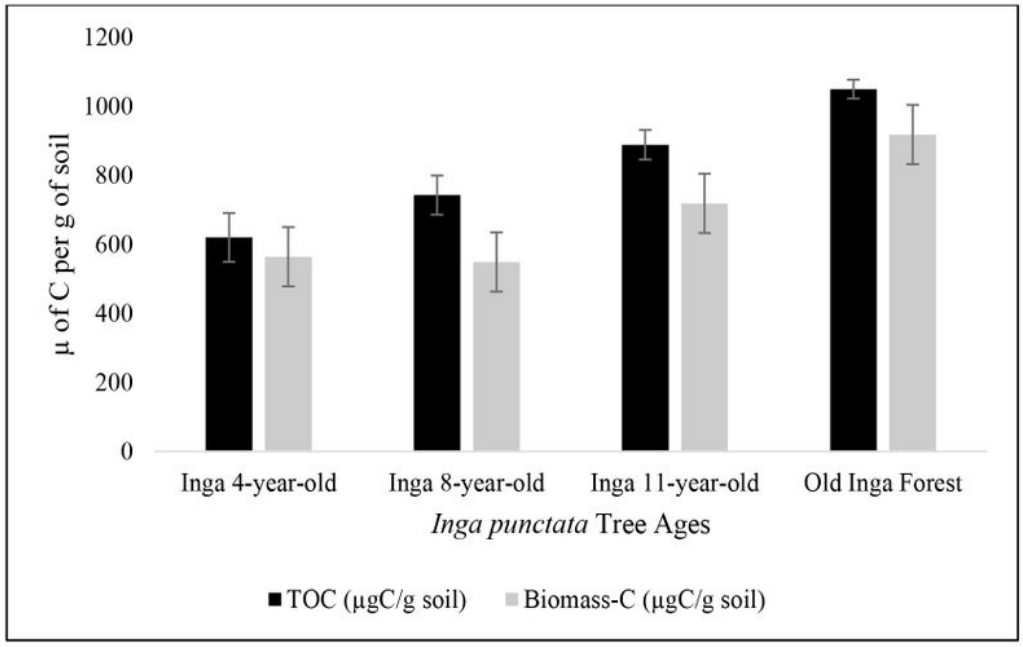

Figure 1

Differences in the mean levels of Nitrogen (1a) and Carbon (1b) metrics measured in the tree soils of Inga punctata trees planted 4, 8, and 11 years prior to the study (Inga 4, 8 and 11-year-old trees) and Old Growth *I. punctata* trees (Old Forest Inga). Mean levels of Total Nitrogen (TN), Ammonium (NH_4^+), Nitrate (NO_3^-), Total Organic Carbon (TOC), and Biomass C, along with standard deviations are presented.

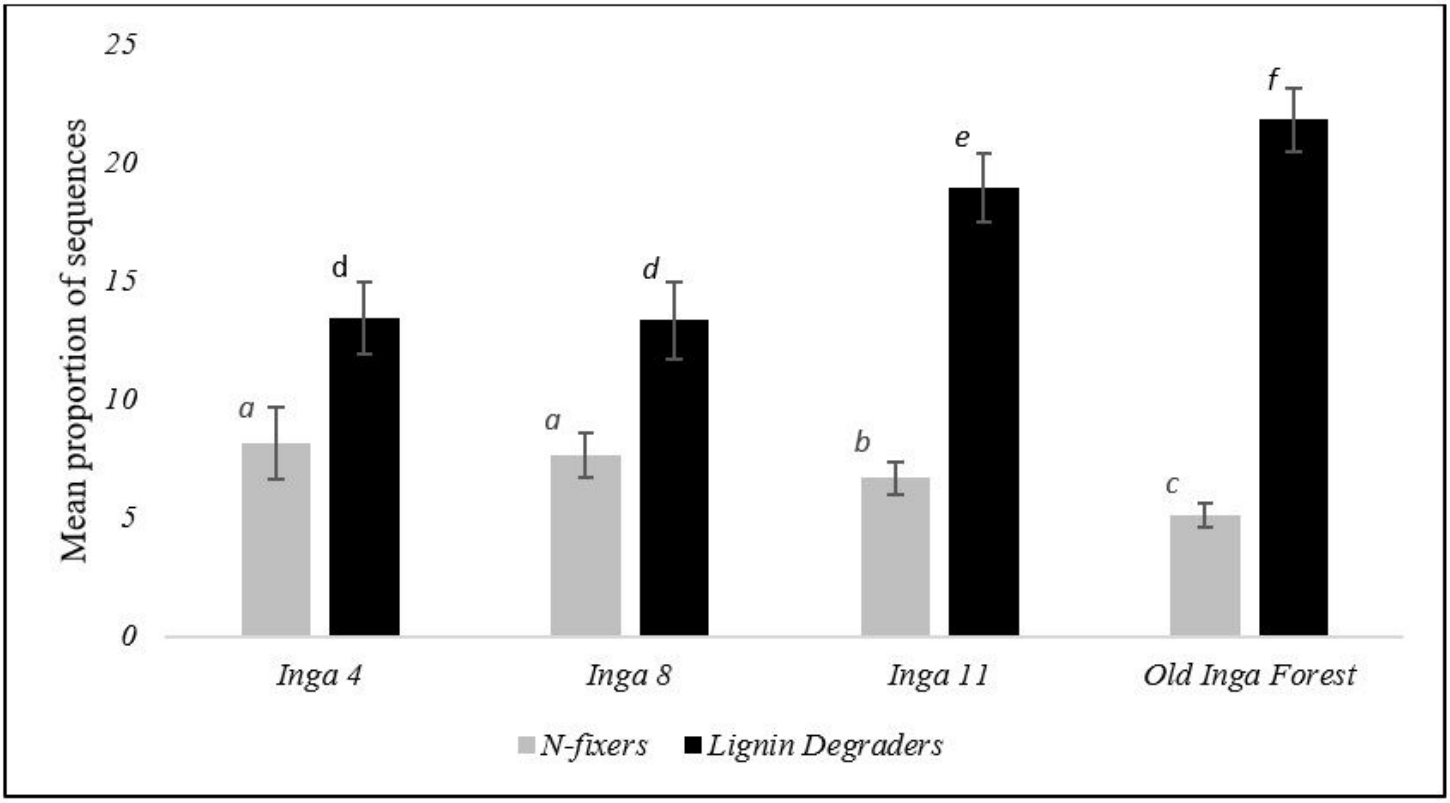

Figure 2

Differences in the mean proportion of sequences (MPS) of the Nitrogen-fixing (N-fixer) genera and Lignin Degrading (Lignin Degrader) genera in the soils of *Inga punctata* trees planted 4, 8, and 11 years prior to the study (Inga 4, 8 and 11) and Old Growth *I. punctata* trees (Old Forest Inga). The MPS values and standard deviations are presented. The MPS values are considered not different if the Kruskal- Wallis p values > 0.06. Those with no difference in MPS values have the same letter a, b, c, d, e, or f.

Figure 3a.

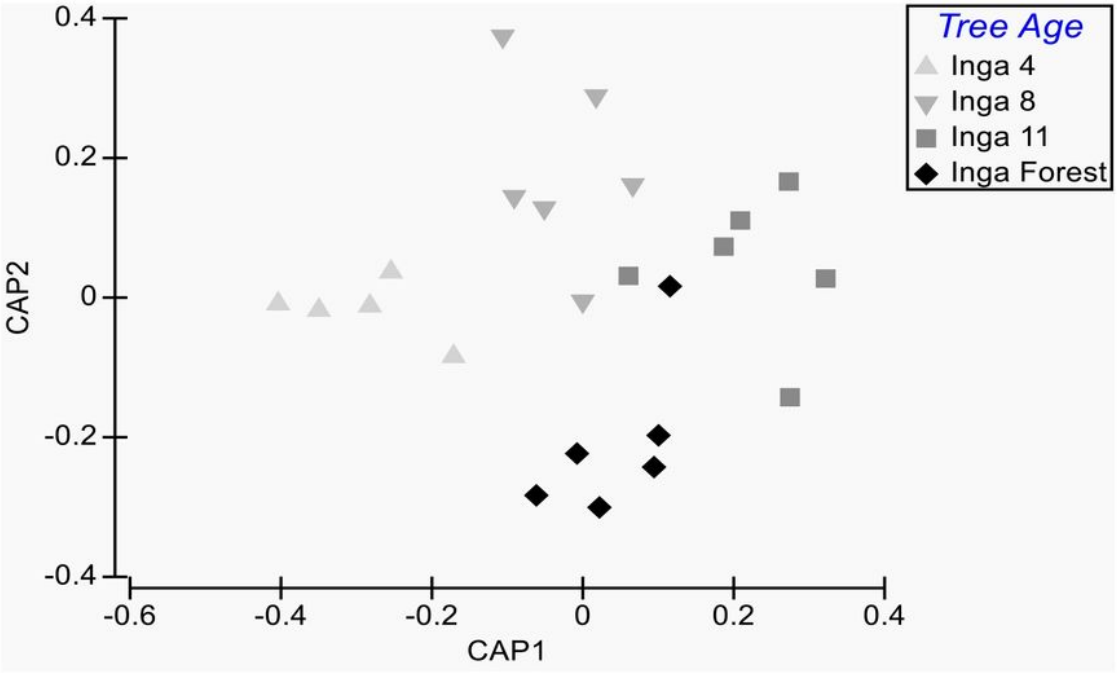

Figure 3b.

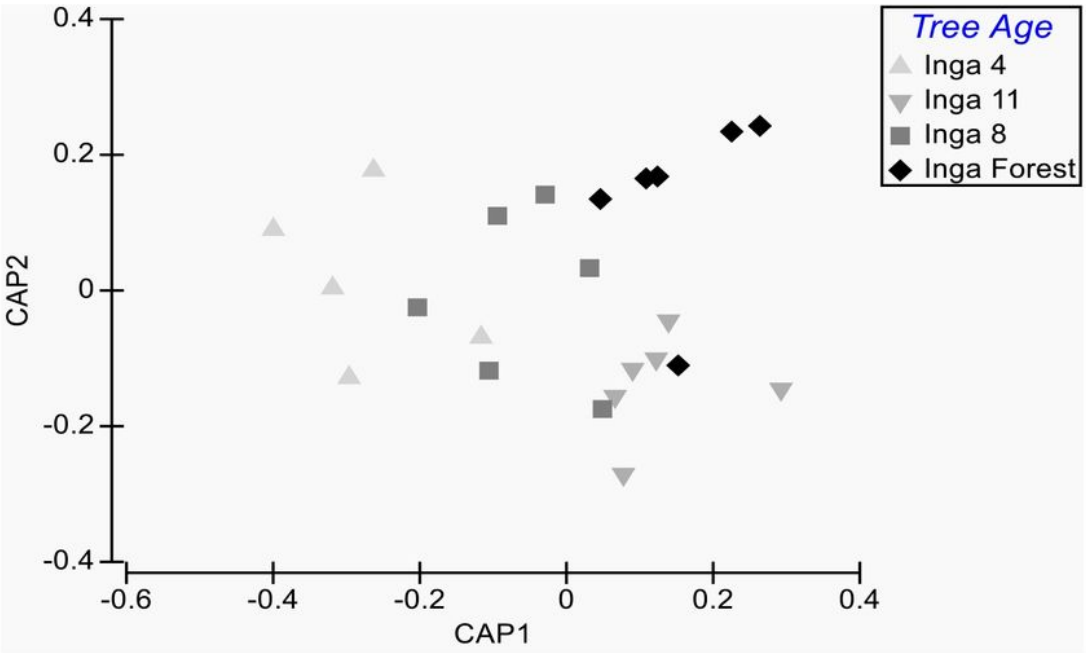

Figure 3

Visualization of the Canonical Analysis of Principal Coordinates (CAP) analyses demonstrating the differences in the community composition of the N-fixing bacterial genera (3a) and Lignin Degrading bacterial genera (Figure 3b) in the tree soils of *Inga punctata* trees planted 4, 8, and 11 years prior to the study (Inga 4, 8 and 11) and Old Growth *I. punctata* trees (Old Forest Inga).

Figure 4

Results of Distance base linear models (DistLM) and dbRDA analyses determining the influence that the total nitrogen levels (TN) had on the Nitrogen-fixer (N-fixer) and Lignin Degrader bacterial community compositions (a and c), and the influence that the total organic carbon (TOC) had on the lignin degrading bacterial community (b) from tree soils of *Inga punctata* trees planted 4, 8, and 11 years prior to the study and Old Growth *I. punctata* trees.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementalFigure1.jpg
- SupplementalMaterials2.MethodsPerformedonSoilSamples.docx
- EatonandHamiltonSupplMaterial3.doc
- SupplementalMaterial4.docx