Using Inga punctata in forest restoration influences the accumulation of tree soil carbon and nitrogen, and the nitrogen-fixer and lignin degrader bacterial community compositions, complexity and stability.

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Research Article

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Abstract

Aims

This study was designed to determine if planting the leguminous tree *Inga punctata* in cleared former tropical premontane wet forest lands changed the Nitrogen (N)-fixer and Lignin Degrader bacterial community compositions, complexity and stability, to enhance the soil carbon (C) and nitrogen (N) components.

Methods

Soils were collected from old growth, 4, 8 and 11-year-old *I. punctata* trees, and assessed for differences in various soil C and N metrics, and the mean of percent sequences (MPS, as “relative abundance” via Next Generation eDNA Sequencing), composition, stability and complexity of the community of N-fixer and Lignin Degrader genera.

Results

The MPS N-fixer genera decreased and that of the Lignin Degraders increased over time, as did the composition and stability of both groups. There were negative correlations between the MPS levels of the N-fixer and Lignin Degrader genera, positive correlations between the MPS levels of Lignin Degraders, TN, NO_3^-, TOC, and Biomass C, negative correlations between the MPS levels of Lignin Degraders and NH_4^+, and opposite correlations between these C and N metrics and MPS levels of N-fixers.

Conclusions

This is the first evidence that *I. punctata* facilitates changes over time in the amount and composition of tree soil N-fixer and Lignin Degrader genera, enhances the stability and complexity of both groups, increases the accumulation of tree soil C and N, thus, increasing the potential for these soils to serve as C and N sinks, and supporting the future use of this reforestation management strategy.

Introduction

Tropical deforestation has resulted in more than 30% of global terrestrial land area now being represented by cleared tropical forests used for agriculture (Tilman et al. 2001; Gibbs et al. 2010), and more than 50% of all tropical forest land now having experienced some human impact (Jacobson et al. 2019). This practice is thought to negatively impact the soil microbial community structure, the soil nitrogen (N) and carbon (C) cycle activities, and facilitate colonization of scrub and invasive species plant growth, thus, reducing the capacity of these tropical soils to serve as carbon sinks (Leopold et al. 2001; Banning et al. 2011; Bradford and Crowther 2013; Bardgett and van de Putten 2014; Brienen et al. 2015; Mendes et al. 2015; Nottingham et al. 2015; Eaton et al. 2019, 2021 a, b). To remediate these negative impacts, reforestation strategies are being implemented to enhance conservation, climate change mitigation, restoration, and the recovery of C and N cycle processes in damaged lands (Chazdon 2008; Aide et al.

2013; Martin et al. 2013; Locatelli et al. 2015; Powlen and Jones 2019; Philipson et al. 2020; Poorter et al. 2021).

An important part of many reforestation strategies in Central and South America has been the use of leguminous trees (Carpenter et al. 2004; Macedo et al. 2008; Piotto 2008; Orwa et al. 2009; Lojka et al. 2010), in which the N-fixer bacteria within the tree root nodules is presumed to enhance recovery of the soil microbiomes and the associated soil N, C, and biomass components critical for soil and ecosystem health (Carpenter et al., 2004; Lojka et al., 2010; Macedo et al., 2008; Piotto et al., 2003; Pan et al., 2011; Poorter et al. 2016; Chazdon et al. 2016). It has also been suggested that these root nodule N-fixer bacterial activities represent the principal pathway by which deforested tropical lands recuperate the soil N and C (Carpenter et al., 2004; Chazdon et al., 2016; Gehring et al., 2005; Pan et al., 2011) that was depleted during deforestation, and subsequent agricultural uses (Davidson et al. 2007; Amazonas et al. 2011; Groppo et al. 2015; Powers and Marín-Spiotta 2017), and is also in high demand for the rapidly growing vegetation during reforestation (Batterman et al. 2013). However, it's also been suggested that the free-living N-fixer bacterial community may be as important as root nodular N-fixers in early successional stages of tropical forest development, and may even play a more important role in N fixation in older tropical forests than the root nodular N-fixation (Schmidt et al. 2008; Hedin et al. 2009; Reed et al. 2011). Although it appears that both forms of N-fixer bacteria are critical to tropical forest health, it is certain that more work is needed to better understand the roles of N-fixer bacterial soil microbiomes associated with leguminous trees. This information will help fill the extensive knowledge gaps that exist in understanding the long-term beneficial effects that reforestation practices have on tropical forest soil microbial communities and the associated C and N cycle activities, following forest extraction-based management strategies, as this is simply not well-known (Loreau 2010).

Species within the genus *Inga* are leguminous trees that are common throughout the tropical Americas, and are thought to be ecologically important as forage resources, and have also been proposed to enhance the quantity and quality of the soil organic C (Oglesby and Fownes 1992; Leblanc et al. 2005, Lojka et al. 2008 and 2010), N and P (Szott and Palm 1996; Lojka et al. 2008), stimulate greater above ground biomass development than other non-*Inga* tree species in the same habitats (Lawrence et al. 1995, Lojka et al. 2005), and stimulate C sequestration and long-term N accumulation (Oglesby and Fownes 1992; Leblanc et al. 2005). These proposed characteristics, along with *Inga*'s ability to thrive in the high acid soils common in the tropical Americas have resulted in reforestation plans using different species of *Inga* trees to restore degraded Central and South America tropical soils in reforestation practices (Lojka et al. 2008 and 2010). However, these activities are speculative, and it is unknown how *Inga* trees affect the soil microbiota, how this may be correlated with changes in the soil C and N characteristics, or the mechanisms that may be involved. Leblanc et al. (2005) did find 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 the Northern Zone of Costa Rica, *I. edulis* had greater levels of soil NH_4^+, and a less rich and possibly more dominant bacterial community in its microbiome in comparison to another leguminous tree,

Pentaclethra macroloba. These studies suggest that *I. edulis* soils may have reached bacterial community structural equilibrium status quicker than the richer, less dominant *P. macroloba* soil microbiomes. This coincides with other studies that have suggested that some *Inga* species trees could be using more NH_4^+ as a source of N, as it has been shown that *I. edulis* prefers NH_4^+ for growth and root nodule stimulation (Fisher 1995; Omena-Garcia et al. 2015; Justino et al. 2017). However, other than these studies, it appears that not much is known about how *Inga* trees influences the soil ecosystem.

Critical questions still remain concerning the ecological activities and mechanisms associated with *Inga* trees. For example, how does using different species of *Inga* for reforestation help in the recovery of the overall soil ecosystem conditions, including the soil C and N-cycle activities, and the composition of the soil N and C-cycle-associated microbial communities (Eaton et al. 2020a)? How does using *Inga* in reforestation strategies influence rates of regrowth of other adjacent tropical trees (Davidson et al. 2007; Nasto et al. 2014; Menge and Chazdon 2016; Taylor et al. 2017)? How do different species of *Inga* influence the amount and rate of nitrification, the production of nitrite (NO_2^-) and nitrate (NO_3^-), and the structure of the nitrifying bacterial communities that use the NH_4^+ from N-fixation. Given that all three forms of inorganic N (i.e., NH_4^+, NO_2^-, and NO_3^-) are critical for the development of the microbial enzymes needed for the decomposition processes associated with healthy forest and soil recovery (Hedin et al. 2009; Reed et al. 2011; Wallenstein et al. 2011; Burns et al. 2013; Henry 2013; Janusz et al. 2017; Xu et al. 2021), how might using *Inga* for reforestation practices influence rates and types of decomposition? The availability of the inorganic N in the soils is thought to be an important regulator of the decomposition process rates of both labile and recalcitrant forms of organic C (Saiya-Cork et al. 2002; Mack et al. 2004; Wallenstein and Burns 2011; Henry 2013; Burns et al. 2013; Paul 2015; Xu et al. 2021). It is known that increasing the decomposition of the more recalcitrant forms of organic C, such as lignin, produces more long-lasting forms of TOC in the soils, and can provide more material for biomass development than decomposition of more labile forms of organic C (Wallenstein and Burns 2011; Paul 2015; Datta et al. 2017; Janusz et al. 2017; Xu et al. 2021). However, it is unknown how different species of *Inga* influence the soil microbial community involved in decomposition of the more complex organic C, in particular the Lignin Degrader bacterial community, and the dynamics of this component of decomposition.

Bacterial N-fixer activities are known to be facultative and feedback-regulated by the levels of inorganic N in the soils from N-fixation, but also from nitrification, or decomposition (i.e., ammonification). Changes in the rates or levels of these separate activities can result in variations in N-fixation rates that may not necessarily be proportional to the abundance or taxa of either N-fixer tree or N-fixer bacteria species within a reforested area (Barron et al., 2011; Gei et al., 2018). However, it is unclear under what conditions (species of tree, age of tree, seasonality, land management history, etc.) that *Inga* differentially influence the growth, abundance and community composition of N-fixer bacteria, and how they influence the various N and C-cycle activities and associated bacterial communities critical to these activities. This type of information could certainly provide insight into how *Inga* influences both the soil recovery and forest regrowth patterns after reforestation (Potthast et al., 2012; Powers and Marín-Spiotta, 2017; Tischer

et al., 2014). The lack of information on the mechanisms of the likely importance of *Inga* ecologically for use in reforestation practices, and, specifically, how *Inga* species influence the biotic and abiotic components of soil ecosystems, and how this influences the overall forest ecosystem conditions, all support the need to understand more about the relationships between *Inga*, its tree-soil microbiome, and the associated C and N component characteristics. Determining such information concerning *Inga* could help to maximize the efficacy of tropical reforestation and management plans (Berg and Smalla 2009; Kardol and Wardle 2010; Wagg et al. 2011; Mendes et al. 2018; McGee et al. 2019; Eaton et al. 2019 and 2020b).

The goal of this current study was to determine if planting *I. punctata* in a former premontane wet forest site in Costa Rica previously cleared and converted for pasture, then agricultural use (coffee and sugar cane), and then abandoned, successfully increased the potential for the soils to serve as C and N sinks. Specifically, we assessed if the planting strategy enhanced the 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 components of the *I. punctata* tree soil microbiomes along a tree age gradient compared to the same metrics determined from adjacent old growth *I. punctata* soils. All sampled tree soils were in the same forest life zone, at the same elevation, within 100–500 m of each other, and located on the same farm, now a wildlife refuge. Through this work it was envisioned that predictors of the drivers of soil ecosystem recovery would be identified that could suggest the efficacy of this type of *I. punctata* reforestation strategy. To address this goal, soils from old growth forest *I. punctata* trees, and from I. *punctata* trees planted in the adjacent abandoned pasture/agricultural sites 4, 8 and 11 years prior to this study were analyzed for the levels of the various metrics mentioned above. This is part of a restoration effort on the Pacific slope of Monteverde, Costa Rica by the Fundación Conservacionista Costarricense (FCC) and Monteverde Institute (MVI). The *I. punctata* planting regime is part of their program objectives to restore abandoned agricultural land with native and diverse tree species that would align with natural forest compositions

This study was designed to address the following questions: (1) Do the soil C and N-cycle metrics, and C biomass levels change, and in what direction, following 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 complexity and stability of the tree soil microbiomes 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; Figure x), 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 C and 26C (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, the planted *I. punctata* trees used for this study were considered as 3 age groups: 4, 8, and 11-year-old post-planting (Inga 4, 8 and 11-year-old). For comparative purposes, old growth *I. punctata* trees (Old Inga Forest) that were > 50-years-old were also identified in an adjacent forest and used for this study.

Six trees from each of the different tree planting age groups and old growth trees were identified, and the approximate distance from the tree base to the edge of each tree's canopy "drip-line" determined, and 10% of that distance calculated. Soil samples were collected at this 10% distance point from the base of each tree and in the four cardinal directions (N, S, E, W), resulting in four sample locations per tree within the 10% drip-line region. At each of these four locations around each tree, two soil cores (7.5 cm x 1.25 cm x 15 cm) were collected and pooled, providing a pooled sample made of 8 cores for each of the 6 trees per each tree age. Thus, each tree age had 6 replicate pooled soil sample for analysis. 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.

Nutrients

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 (Buchmann 2000; 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. (2018). 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 soil sample per habitat 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.

For this project, the focus was to study 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 in soil microbial studies in the region 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 S1) 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. 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 microbiome communities within the differently treated soils may be associated with changes in genera communities often associated with these certain functions. The %RA of these groups was also determined.

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 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.

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). The compositions of the two functional group communities were compared between each tree soil age using ANOSIM (Analysis of Similarities) routines. 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). ANOSIM is a non-parametric test that determines if the between group differences are greater than the within group differences, based on ranking of the distance measures, and is commonly used for the comparison of differences in taxonomic composition between groups (Clarke 1993; Anderson et al. 2008). An R statistic (from 0 to 1, with 0 being most similar) and *p* value probability are the ANOSIM outputs from this test. The ANOSIM procedure Global R and *p* values measure the degree of separation of the entire bacterial community across tree soil groups (i.e., “main” test), while the pairwise R and *p* values measure the level of separation of the community compositions between different tree 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 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, and to maximize 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) The Similarity Percentage Comparisons: The levels of the percent similarity of the genera composition for each tree soil age microbiome were determined by applying the Similarity Percentage (SIMPER) routine (PRIMER-E) to the different 4th root transformed MPS data (Clarke 1993). The SIMPER identifies the overall similarity in the composition of the genera within the different tree soils, and also identifies the genera that are the greatest contributors to this similarity, and are, thus, considered to be the most typical genera of that tree soil type (Clarke and Gorley 2006; Clarke and Warwick 2001). The lower the percent similarity is across a sample group, the presumed greater the variability of the community structures, which would be expected for an older more complex, stable and perhaps resilient soil community (Derhé et al. 2016; Louca et al. 2016; Eldridge et al. 2016; Eaton et al. 2020, 2021).

e) Evenness Comparisons: Pielou's Evenness indices were calculated using the 4th root transformed MPS data and the PRIMER-E package, and used to estimate the relative proportion or abundance of each genus relative to the other genera in each different tree soil community. The levels of evenness were assessed for critical differences using the Kruskal – Wallis procedure in SPSS (v26). Greater levels of evenness were used to suggest greater diversity, genera that were more dominant and filling more niches, resulting in a more complex community with more niches, less competition, and more resilience and stability (see *f)* below for further discussion), while low levels of evenness were used to represent a community that was less diverse, with fewer niches filled by dominant genera, and generally is communities that were less complex, less resilient, and more susceptible to perturbations (Wilsey and Polley 2002; Tracy and Sanderson 2004; Hooper et al. 2005; Wittebolle et al. 2009; De Roy et al. 2013).

f) Microbiome Community Complexity and Stability: The greater the variability in genera (MPS, ANOSIM, CAP and SIMPER results), and the greater the Pielou's evenness, were presumed to suggest a more heterogenous bacterial community, with greater diversity, which suggests the presence of a greater number of niches and a more stability and complexity (Tilman et al. 2006; Isbell et al. 2009; Pyron 2010; Sasaki and Laurenroth 2011). To further support the determination of the levels of the stability and complexity of each tree age soil microbiome, the methods of Tilman et al. (2006) were used to identify

the temporal stability (*S)* of several key variables. In this case, *S* is defined as the mean/std. dev. of variables of interest, where m is the mean variable value for the different tree age soils, and the std. dev. is the temporal standard deviation of that variable. In each of the tree soils for this project, we determined the *S* values for the MPS levels as “abundance”, or S_{ab}, of the N-fixer and Lignin Degrader bacteria to indicate the stability (and complexity) of the different bacterial communities over time. The greater the *S* value, the greater the stability and complexity of the system.

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.

Relationships Between the C and N Metrics and the Composition of the Bacterial Genera

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

Nutrients

There were several clear patterns observed in the levels of the soil N metrics that were associated with the age of the tree soils (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, $p = 0.009$).

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			

TN ANOSIM Results			NH_4^+ ANOSIM Results			$NO3^-$ ANOSIM
Inga 8, Inga Forest	0.959	0.002	Inga 8, Inga Forest	0.831	0.002	
Inga 11, Inga Forest	0.698	0.002	Inga 11, Inga Forest	0.535	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: There were 52 N-fixer bacterial genera (40 strictly free-living and 12 with both free-living and nodular N-fixer capabilities) and 168 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 2). 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 2). 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.

Table 2

Kruskal – Wallis analysis results of the differences in mean percent 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 Kruskal – Wallis H and p values are presented for both the Main and Pairwise Tests.

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
IF to I4	2.619	0.009	IF to I4	6.55	0.0001
IF to I8	2.386	0.017	IF to I8	7.58	0.0001
IF to I11	2.223	0.035	IF to I11	4.83	0.001
I11 to I4	2.101	0.059	I11 to I4	3.73	0.004
I4 to I8	0.603	0.561	I4 to I8	1.09	0.302
I8 to I11	1.904	0.06	I8 to I11	4.72	0.001

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 3; 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.

Table 3

ANOSIM analysis of the results of the differences in the community composition of the Nitrogen-fixing (N-fixers) genera and Lignin Degrading genera 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). The ANOSIM Global R and *p* values and pairwise R and *p* values are presented for each set of analyses.

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, Inga Forest	0.256	0.037	Inga 4, Inga Forest	0.464	0.002
Inga 8, Inga 11	0.011	0.422	Inga 8, Inga 11	0.057	0.245
Inga 8, Inga Forest	0.181	0.052	Inga 8, Inga Forest	0.207	0.024
Inga 11, Inga Forest	0.194	0.043	Inga 11, Inga Forest	0.191	0.035

There were also differences in the community composition of the Lignin Degrader genera across the different tree soil microbiomes (Table 3; 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.

c) Strength of the Compositional Differences: The 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 4) 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 4). Strong differences were observed in this community between the Old Inga Forest and Inga 4-year-old tree soil microbiomes (R^2 = 0.809), and moderate differences between the Old Inga Forest tree soil community and those of the Inga 8 and Inga 11-year-old tree soils (R^2 = 0.583), as well as between the Inga 4 and

Inga 11-year-old tree soils (R^2 = 0.583), but the differences between the Inga-4-year-old and 8-year-old tree soils and the Inga 8 and 11-year-old tree soils were very weak (R^2 = 0.086).

Table 4
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 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). 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.

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, Inga Forest	0.858	Strong	0.809	Strong
Inga 8, Inga 11	0.446	Weak	0.086	Weak
Inga 8, Inga Forest	0.858	Strong	0.583	Medium
Inga 11, Inga Forest	0.858	Strong	0.583	Medium

d) Differences in the Similarity Percentages: The cumulative percentage cut-off criterion was set at 70% for the SIMPER analyses of the N-fixer bacterial genera in the different tree soil types, which indicated there were 10 genera, in total, across all tree soils that were the top contributors to the similarity in community compositions among the different tree soil, whose patterns of MPS levels typified the different tree soil microbiome community of N-fixer bacteria. The SIMPER results showed (Table 5) the average within group similarity of the N-fixer bacterial community composition in the Inga 4-year-old tree soils was 71.17%, which was reached with 8 genera that could also be considered as those most typifying that community's composition. For the Inga 8-year-old tree soils, the average within group similarity of the N-fixer community was 70.66%, which was approximately reached with 6 genera that also could be considered as typical N-fixer genera within this tree soil microbiome. The average within group similarity of this community in the Inga 11-year-old tree soil microbiomes was 72.26%, and this level was reached with 9 genera that are considered as typical. The Old Inga Forest tree soil community did not reach the 70% cut-off criterion that was set, as it had an average within group similarity of only 52.69% between all replicate pairs, with only 5 genera considered typical for this tree soil type.

Table 5
Results of the Similarity Percentage (SIMPER) routine conducted on the Mean Percent Sequences (MPS) levels of the N-fixing genera of bacteria 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). The average percent similarity of the composition of all N-fixing genera in the different tree soils is presented, as is the percent contribution that each genus made to total composition of the N-fixing bacteria in each tree soil type.

	Inga 4 Soils	**Inga 8 Soils**	**Inga 11 Soils**	**Old Forest Inga Soils**
Avg Similarity of All Samples	71.17%	70.66%	72.26	52.69%
	% Contribution to	% Contribution to	% Contribution to	% Contribution to
Genus	Total Similarity	Total Similarity	Total Similarity	Total Similarity
Bradyrhizobium	14.46	14.91	14.24	17.16
Bacillus	11.56	14.27	12.33	11.63
Anaeromyxobacter	10.74	9.73	6.06	none
Rhodoplanes	8.96	9.14	10.48	12.26
Streptomyces	8.92	none	none	10.25
Rhizobium	7.11	7.60	7.90	9.47
Devosia	6.85	5.94	6.11	none
Cupriavidus	6.53	5.97	5.21	none
Paludibaculum	none	none	6.65	none
Paenibacillus	none	none	6.50	none

There were also patterns in the distribution of the 6 most common N-fixer genera associated with at least 3 of the different ages of tree soils that provided some level of differentiation in the community compositions among the different tree soils (Fig. 4). *Bradyrhizobium, Bacillus, Rhodoplanes*, and *Rhizobium* were top contributors to the community compositions of all 4 tree soil habitats. However, the percent contribution of *Bradyrhizobium, Rhizobium*, and *Rhodoplanes* all increased and that of Bacillus decreased in these tree soils with age. The percent contribution of *Anaeromyxobacter* and *Cupriavidus* decreased with age in the planted tree soils, but these were not important contributors in the Old Inga Forest tree soils. *Devosia* was only a top contributor to the communities in the planted tree soils, but did not change with age. The other 3 of the 10 top contributors to the community compositions were only found in one or two of the different tree soils (Table 4). *Streptomyces* was important in the community

composition of the Inga 4-year-old and Old Inga Forest tree soils, while *Paludibaculum* and *Paenibacillus* were only important contributors to the composition of the Inga 11-year-old tree soil N-fixer community.

For the Lignin Degrader bacterial community, the cumulative percentage cut-off criterion was also set at 70% for the SIMPER analyses in the different tree soil types (Table 6). This showed there were 20 genera, in total, across all tree soils that were the top contributors to the similarity in community compositions among the different tree soil, whose patterns of MPS levels typified the different tree soil microbiome community of Lignin Degrader bacteria. The average within group similarity of the composition of the Lignin Degrader genera between all pairs of replicates of the Inga 4-year-old tree soils was 71.95%, which was reached with 14 genera that typified the community. The average within group similarity of this functional group was 69.30% in the Inga 8-year-old tree soils, which was reached with 13 genera that typified these tree soil microbiomes. The average similarity of the Lignin Degrader community composition in the Inga 11-year-old tree soils was 66.32%, and was reached with 14 genera considered as typical. The Old Inga Forest tree soil community did not reach the 70% cut-off criterion, as it had only a 56.74% within group similarity, which was reached with 9 genera that could be considered as typical for this tree soil type.

Table 6

Results of the Similarity Percentage (SIMPER) routine conducted on the Mean Percent Sequences (MPS) levels of the Lignin Degrading genera of bacteria 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). The average percent similarity of the composition of all Lignin Degrading genera in the different tree soils is presented, as is the percent contribution that each genus made to total composition of the N-fixing bacteria in each tree soil type.

	Inga 4 Soils	**Inga 8 Soils**	**Inga 11 Soils**	**Old Forest Inga Soils**
Avg Similarity of All Samples	71.95%	69.31%	66.32%	56.74%
	% Contribution to	% Contribution to	% Contribution to	% Contribution to
Genus	Total Similarity	Total Similarity	Total Similarity	Total Similarity
Bradyrhizobium	7.93	8.5	8.33	7.2
Solibacter	7.06	7.15	7.48	5.11
Bacillus	7.33	7.4	6.96	5.24
Flavobacterium	3.78	7.35	7.01	11.27
Pedomicrobium	5.15	5.32	5.7	7.14
Mycobacterium	5.64	5.54	4.68	5.37
Sphingomonas	5.54	6.08	5.47	5.24
Nocardioides	4.36	3.41	6.12	6
Gemmatimonas	4.13	5.17	5.75	0
Novosphingobium	0	0	0	6.46
Adhaeribacter	0	3.65	3.79	0
Kribbella	3.77	3.85	3.65	0
Cupriavidus	0	3.51	2.62	0
Streptomyces	4.89	0	0	0
Pseudonocardia	4.3	0	0	0
Labrys	3.71	0	0	0
Koribacter	3.61	0	0	0
Paenibacillus	0	3.92	0	0
Rubrobacter	0	0	2.6	0

	Inga 4 Soils	Inga 8 Soils	Inga 11 Soils	Old Forest Inga Soils
Burkholderia	0	0	2.52	0

There appeared to be greater variation in the bacterial genera considered as important contributors to the Lignin Degrader community composition in the tree soils as compared to the N-fixer genera. There were 12 genera found in 2 or more tree soils representing top contributors that typified the different tree soil communities (Fig. 5). *Bradyrhizobium*, *Solibacter*, *Bacillus, Flavobacterium, Pedomicrobium, Mycobacterium, Sphingomonas*, and *Nocardioides* were top contributors to the Lignin Degrader community composition in all 4 tree soil ages, although the levels of contribution were variable across the soils. In particular, *Bradyrhizobium, Solibacter*, and *Bacillus* showed a trend of decreasing percent contribution to the Lignin Degrader community with tree soil age, while *Flavobacterium*, and *Pedomicrobium* trended towards an increasing percent contribution to this community with tree soil age. The percent contribution of *Nocardioides* to this community increased from that in the Inga 4 and 8-year-old tree soils to the levels found in the Inga 11-year-old and Old Inga Forest tree soils. *Novosphingobium* was only a critical contributor to the composition of this community of the Old Inga Forest tree soils, *Rubrobacter* and *Burkholderia* only in the Inga 11-year-old tree soils, *Paenibacillus* only in Inga 8-year-old tree soils, and *Streptomyces*, *Pseudonocardia*, *Labrys*, and *Koribacter* were all top contributors to the Lignin Degrader community in the Inga 4-year-old tree soils (Table 6).

e) Differences in Evenness, Stability and Complexity: The Kruskal – Wallis analyses of the evenness data showed a clear trend of increasing evenness of both the N-fixer and Lignin Degrader bacterial communities (Table 7) 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.

Table 7
Evenness levels of the Nitrogen-fixing (N-fixers) genera and Lignin Degrading (Lignin Degraders) genera bacterial communities. Mean and Standard Deviation (Std. Dev.) Pielou's Evenness j values are presented. Also, Kruskal-Wallis Main and Pairwise tests were conducted to determine the levels of differences in evenness (j values) between the mean percent sequences of the different functional group 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 Growth *I. punctata* trees (Old Forest Inga). The Kruskal-Wallis H and *p* values are presented for each set of analyses.

	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 Forest	0.989	0.042	Old Forest	0.980	0.039
	Kruskal-Wallis Test			Kruskal-Wallis Test	
Main Test	H value	*p* value	Main Test	H value	*p* value
All Data	4.308	0.001	All Data	4.025	0.005
Pairwise Tests			Pairwise Tests		
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

e calculated Stability (*S*) values showed that the tree soil systems appeared to be undergoing an increase in stability and complexity over time (Table 8). The S_{ab} for the N-fixer bacterial community was about the same in the Old Inga Forest (10.166) and Inga-11-year-old (10.034) tree soils, both of which were greater than that in the Inga 8-year-old (4.141) and 4-year-old (2.310) soils. The S_{ab} levels for the Lignin Degraders increased in the older tree soils, with the levels being about the same in the Inga 8 (8.113) and 4-year-old (8.856) tree soils, followed by the greater values in the Inga 11-year-old (13.004) and Old Inga Forest (16.364) tree soils. The differences in these *S* values along with the differences in the total MPS levels of the two different functional groups of bacteria, the compositional differences determined via ANOSIM, CAP, and the SIMPER analyses, and the differences in the Pielou's Evenness point towards a trend of increasing stability and complexity in the tree soils with age.

Table 8
Temporal Stability (S_{ab}) values for the Nitrogen-Fixing (N-Fixer) and Lignin Degrading (Lignin Degrader) bacterial community compositions were calculated from the mean percent sequences of bacteria 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). The Temporal S_{ab} values were calculated as per Tilman et al. (2006) by dividing the mean MPS values by the standard deviation of the MPS values for each group.

	N-Fixer	**Lignin Degrader**
	S_{ab} values	S_{ab} values
Inga 4	2.310	8.856
Inga 8	4.141	8.113
Inga 11	10.034	13.004
Inga Forest	10.166	16.364

f) Results of the Spearman's and DistLM Analyses: The Spearman's Correlation analyses (Table 9) 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. 6) showed that the variations in the TN levels explained 8.8% of the variation in the N-fixer bacterial community composition (Fig. 6a, Pseudo-F = 2.02, $p = 0.053$), and the TOC levels explained 14.19% of the variation in the Lignin Degrader bacterial community composition (Fig. 6b, Pseudo-F = 3.47, $p = 0.004$). The TN explained 12.17% of the variation in the Lignin Degraer community across the tree soils (Fig. 6c, 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.

Table 9
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	

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 as a result 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 for the production of 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; Ghazoul and Sheil 2010). 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 (Berg and Smalla 2009; Kardol and Wardle 2010; Wagg et al. 2011; Mendes et al. 2018; McGee et al. 2019; Eaton et al. 2019 and 2020a). 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 (Paul 2015). 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 (Paul 2015), 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 (Saiya-Cork et al. 2002; Mack et al. 2004; Wallenstein and Burns 2011; Henry 2013; Burns et al. 2013; Paul 2015; 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 (Paul 2015). 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* sp. 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* sp. 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).

In the current study, the TN, NO_3^-, TOC and Biomass C levels increased in the *I. punctata* tree soils with tree age (4, 8 and 11-year-old trees and Old Growth Forest trees), were positively correlated with the MPS levels of the Lignin Degrader bacteria, and were negatively correlated with the N-fixer bacterial MPS values. Conversely, the NH_4^+ levels, which were positively correlated with the N-fixer bacterial MPS levels and negatively correlated with those of the Lignin Degrader bacteria, decreased along this same tree soil age temporal gradient. In addition, the N-fixer MPS levels (decreased along the gradient) and Lignin Degrader bacterial MPS levels (increased along the gradient) were also negatively correlated to one another. These results provide evidence that *I. punctata* is influencing the soil microbiomes over time such that it is enhances long-term accumulation of N in soils, and also the soil TOC (Oglesby and Fownes 1992; Leblanc et al. 2005; Lojka et al. 2008).

Nitrogen and the Microbiomes

The combined increases in TN and NO_3^- and decreases in NH_4^+ coincide with the decrease in MPS levels of the N-fixer bacterial community genera along the tree soil age gradient from the Inga 4 and Inga 8-year-old soils, to the Inga 11-year-old soils, and then to the Old Inga Forest soils. This suggests that the impact of *I. punctata* on the N-fixer community occurs rapidly, within the first 4–8 years, and begins to decrease sometime after that, with the lowest MPS levels occurring in the old growth *I. punctata* tree soils. Although the role of the N-fixer bacterial community appears to diminish over time in the tree soils, the community is developing into a more long-term, more stable and complex community (i.e., greater *S* index), consistent with successional theory, that will likely undergo baseline levels of N-fixation, until another significant gap or other disturbance requires it to upregulate. This could be reflected in the increasing percent contribution of *Bradyrhizobium*, *Rhizobium, Rhodoplanes* and *Streptomyces* with age, the decreasing percent contribution of *Anaeromyxobacter, Bacillus* and *Cupriavidus* with age to the point of not being important in the Old Inga Forest tree soils, and *Devosia* being a top contributor at a constant level in the planted tree soils, but not in the Old Inga Forest tree soils. These changes suggest the relative

importance of *Bradyrhizobium*, *Rhizobium, Rhodoplanes* and *Streptomyces* in N-fixer in later stages of tree soil development, biotic succession, while *Anaeromyxobacter, Bacillus, Cupriavidus*, and *Devosia* perhaps being more critical in the earlier soil stages. It is likely that younger trees require high levels of NH_4^+ in their early growth stages, which appears to be provided by the N-fixer bacteria becoming active in association with the young *Inga* tree soil microbiomes. It appears that N-fixer root nodules form on very young *I. punctata* seedlings, as revealed in a query of nodule formation that found large white root nodules on all 6 sampled *I. punctata* seedlings that were less than 1 year old, still in plastic planting bags at the MVI nursery (Eaton, pers comm). This is under further investigation.

The decrease in N-fixer bacterial MPS levels in the 11-year-old and Old Inga Forest tree soils, was associated with the positive correlation between the N-fixer community MPS and the NH_4^+ levels, the negative correlation between the MPS of this community and the TN and NO_3^- levels and positive correlation with NH_4^+ levels, and the DistLM evidence for how the TN levels explain the variation in the N-fixer community composition. All of these patterns occurred in association with an increase in the stability and complexity of the N-fixer community, which collectively provide some solid indicators that N-fixer slows down and possibly nitrification increases over time in these tree soils. It has been shown that N-fixation is a facultative process, and over time it is down-regulated in later stages of soil development (Barron et al. 2011; Gei et al. 2018). It is also known that in the presence of NH_4^+, N-fixer bacteria will use that inorganic N that is either already fixed as NH_4^+ or is present as a product of ammonification-produced fixed N, rather than expend the energy needed to fix the N they need (Barron et al. 2008; Cusack et al. 2009; Barron et al. 2011; Reed et al. 2011). As well, the older *I. punctata* trees could be using more NH_4^+ as a source of N, as it has been shown that some species of *Inga* prefer NH_4^+ for growth and root nodule stimulation (Fisher 1995; Omena-Garcia et al. 2015; Justino et al. 2017). This could represent 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. Lastly, the observed decrease in NH_4^+ and the increase in NO_3^- suggests that the bacterial N-fixation occurring could be facilitating nitrification and enhancement of the nitrifier community, which would result in the observed increase in NO_3^- and the reduction in NH_4^+ (Paul 2015). This is currently being studied.

Given that soil cores for this current project were collected from around the tree bases using a 10–15 cm core depth, rather than carefully selecting rhizosphere samples where nodules would be found, it is logical to assume that many of the N-fixer genera would be free-living, or both nodular and free-living in their capacity. This is consistent with the current project findings in which 40 of the 52 N-fixer bacterial genera identified in this study have been listed as being free-living N-fixer bacteria according Weir (2016) and ICSP (2020), with the other 12 listed as both nodular and free-living N-fixers. The relationship between free-living and nodular N-fixer bacteria is interesting, but still not clear. Although it has been suggested that the nodular N-fixation associated with leguminous trees is the principal for N regeneration in damage tropical soils (Carpenter et al., 2004; Chazdon et al., 2016; Gehring et al., 2005; Pan et al., 2011); Chazdon et al. 2016; Powers and Marín-Spiotta 2017), it has also been suggested by others 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). 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, or in earlier stages of recovery of damaged soils (Schmidt et al. 2008; Hedin et al. 2009; Reed et al. 2011). Clearly more work is needed on this topic. That said, it is likely, then, that the enhancement of N-fixation by both free-living and nodular bacteria associated with leguminous trees are 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 (Gehring et al. 2005; Six et al. 2006; Cenciani et al. 2009; Hedin et al. 2009; Reed et al. 2011; Pan et al. 2011; Wallenstein et al. 2011; Burns et al. 2013; Henry 2013; Rodrigues et al. 2013; Chazdon et al. 2016; Janusz et al. 2017; Xu et al. 2021). Thus, in addition to the possible role of the nitrifiers in generating the relatively large amounts of NO_3^- in the older planted and old growth tree soils, the increase in TN over time in the tree soils could also partly be a result of the increased decomposition of N-containing organic compounds (i.e., ammonification) which would be expected to increase along the tree soil age gradient. Thus, it appears that *I. punctata* may be playing an important role in the regulation of the N-fixer, nitrifying, and complex organic C decomposition activities in these tree soils along the tree soil age gradient.

Carbon and the Microbiomes

The increases in TN and NO_3^-, the decreases in NH_4^+ and levels of the MPS of the N-fixer community occurred over time in the tree soils along with increases in the levels of the MPS of the Lignin Degrader community, the TOC and the Biomass C. These patterns and the positive correlations between the MPS of the Lignin Degrader communities and the TN, NO_3^-, TOC and Biomass C levels, and the DistLM evidence for how the levels of TOC and Biomass C explain the variations in the Lignin Degrader community compositions occurred as the Lignin degrader community was increasing in stability and complexity. These patterns collectively suggest that the Lignin Degrader community is upregulated over time in these tree soils resulting in enhanced C accumulation. Thus, it appears that in addition to the possible early recovery stage roles *I. punctata* is playing in enhancing the N-fixer bacterial community, providing inorganic N for the trees and the developing microbial community, these leguminous trees may also be facilitating the development of the complex organic C decomposition processes and the bacteria involved. This is probably occurring through provision of inorganic N for microbial enzyme development and enhancement of the MPS and complexity of the Lignin Degrader bacterial community and the levels of TOC in the soils. In addition, this project showed that this is occurring as the Lignin Degrader community is increasing in stability and complexity. This provides strong evidence that *I. punctata* is critical for recuperation of soil C levels and processes after deforestation and other types of damage. Studies are underway to determine the rates of nitrification, Biomass C development, TOC and TN changes, and decomposition in different ages of tree soils, correlated with the microbial community composition.

The conversion of tropical forests to pasture used for grazing often results in overgrazed areas with altered TOC cycle components and dynamics, and reduced biomass productivity, both below and above ground (Chazdon 2003; Gibbs et al. 2010; Rodrigues et al. 2013; Soares-Filho et al. 2006). This practice damages soils such that it can diminish soil ecosystem successional processes, resulting in colonization of scrub growth, invasive grasses and ferns (Guariguata and Ostertag 2001; Leopold et al. 2001). These changes have been associated with changes in soil microbial communities that have reduced complex C and Lignin Degrader activities and reduced production of organic C and decreased C-use efficiency (Eaton et al. 2019; 2021 a, b). Previous studies have shown that different species of *Inga* trees are important sources of inorganic N (Lojka et al. 2010) that enhance the quantity and quality of the soil organic C (Leblanc et al. 2005; Lojka et al. 2005, 2010), stimulating greater biomass development than other non-*Inga* 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). These Lignin Degrader bacterial genera are representative of the microbial groups that degrade complex organic C, in addition to lignin. As such, enhancing this community likely represents one of the important contributions that *I. punctata* is facilitating the tree soil microbiome that increases 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; Six et al. 2006). 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.

Microbiome Ecosystem Complexity and Stability

The greater levels of complexity and stability of tree soil microbiomes in this project were defined as those with greater variabilities in composition of the genera of the two functional groups, as demonstrated by the MPS, ANOSIM, CAP, and SIMPER results, with greater Pielou's evenness results, and temporal stability *S* values. Collectively, these suggested a more heterogenous tree soil bacterial community, with greater diversity, thus, indicating the presence of a greater number of niches and a more stable and complex microbiome community (Tilman et al. 2006; Isbell et al. 2009; Pyron 2010; Sasaki and Laurenroth 2011). The Old Inga Forest tree soils had the greatest variability in the composition of the genera of both functional groups, the greatest evenness in the communities, and the largest *S* values, thus suggesting the oldest soil environments were more stable with a greater number of niches occupied, less competition, and also suggesting greater complexity in these soils—none of which is surprising. The interesting pattern observed for the planted trees was that the indicators of complexity and stability increased along the gradient from the Inga 4 to 8 to 11-year-old tree soils, providing strong evidence of the influence that *I. punctata* has on tree soil ecosystem regeneration over time.

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). In particular it shows how these interactions enhance 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 (Guariguata and Ostertag 2001; Nichols et al. 2001; 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, more stability and more complexity, with increased deposition 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.

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

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

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Figures

Figure 1a

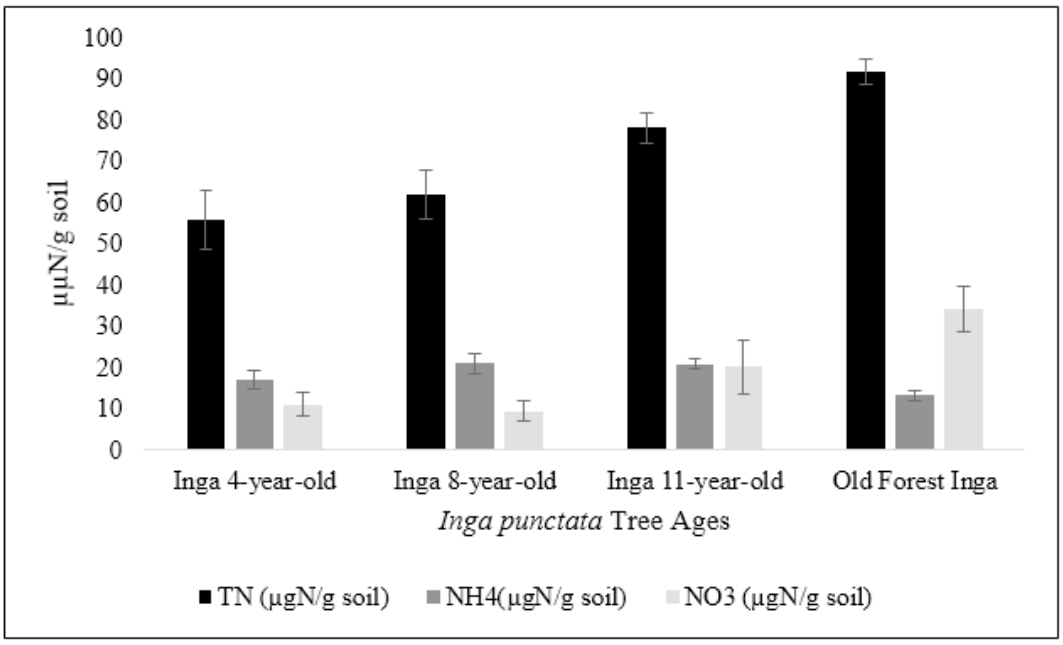


Figure 1b

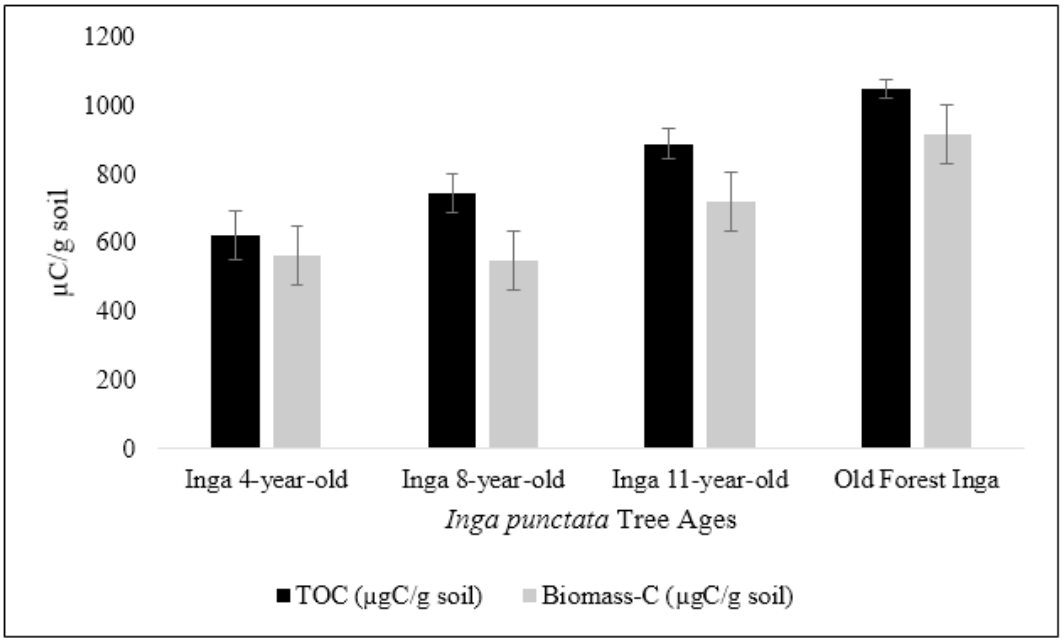


Figure 1

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.

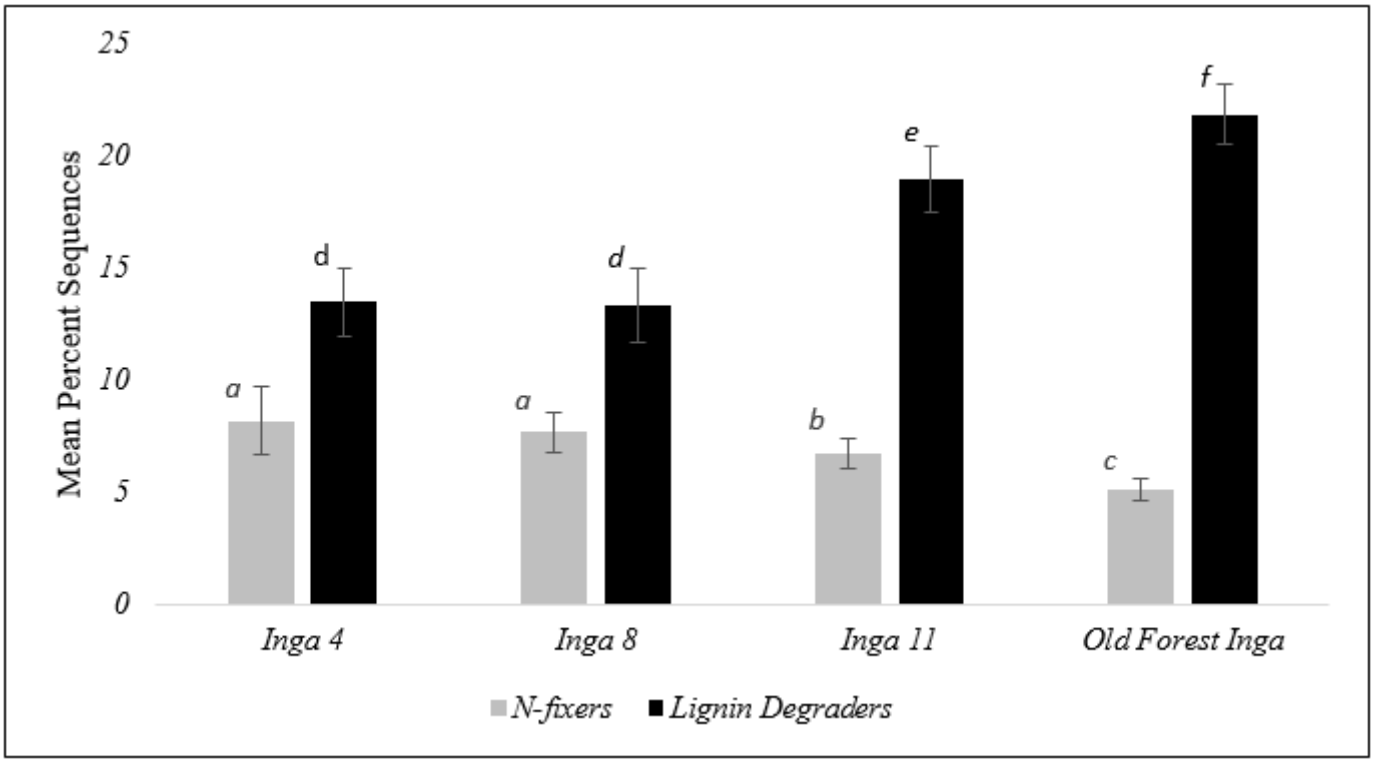


Figure 2

Differences in the mean percent 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.

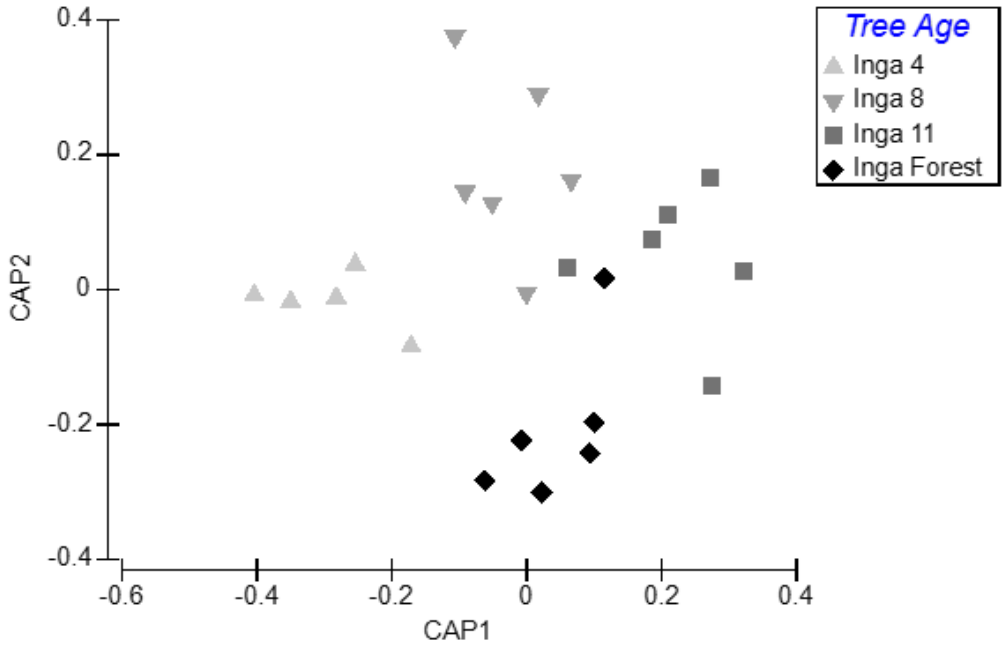


Figure 3b.

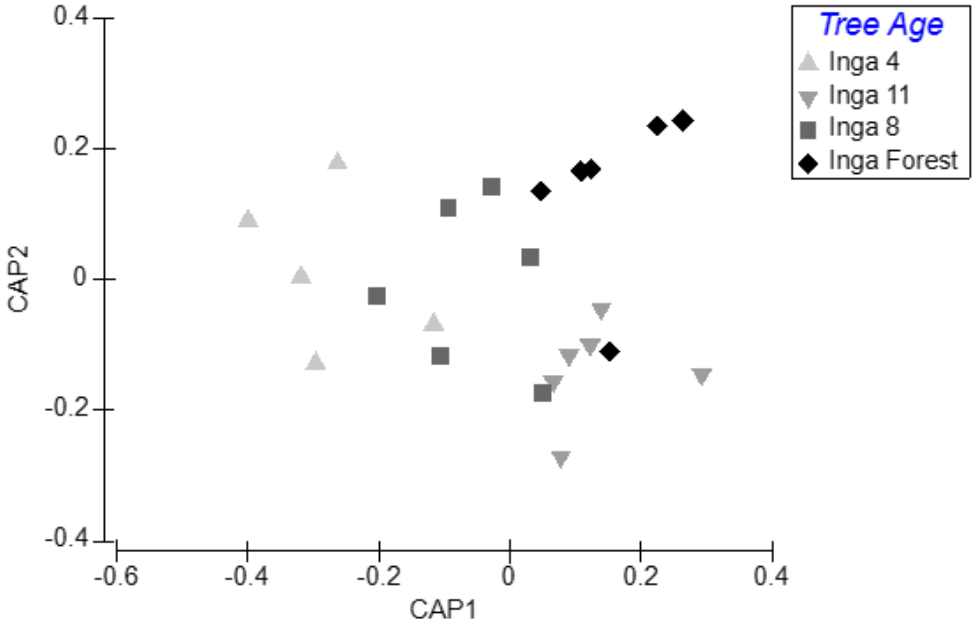


Figure 3

Visualization of the Canonical Analysis of Principal Coordinates (CAP) analyses demonstrating the differences in the community composition of the N-fixing bacterial genera (Figure 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).

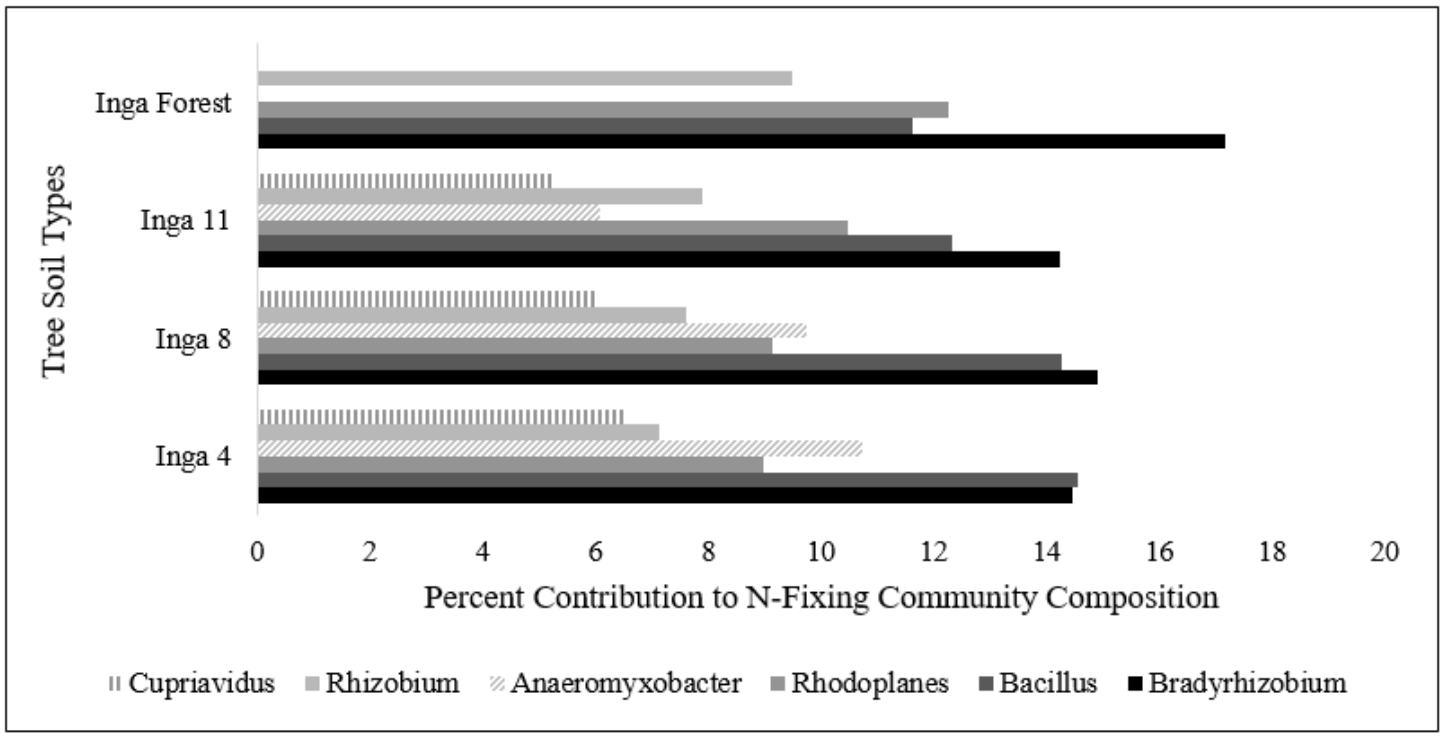


Figure 4

Results of the Similarity Percentage (SIMPER) routine conducted on the Mean Percent Sequences (MPS) levels of the N-fixing genera of bacteria 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) showing the percent contribution of the 6 genera of N-fixing bacteria that were considered as top contributors to the compositions of this community in 3 or more of the different tree soil communities.

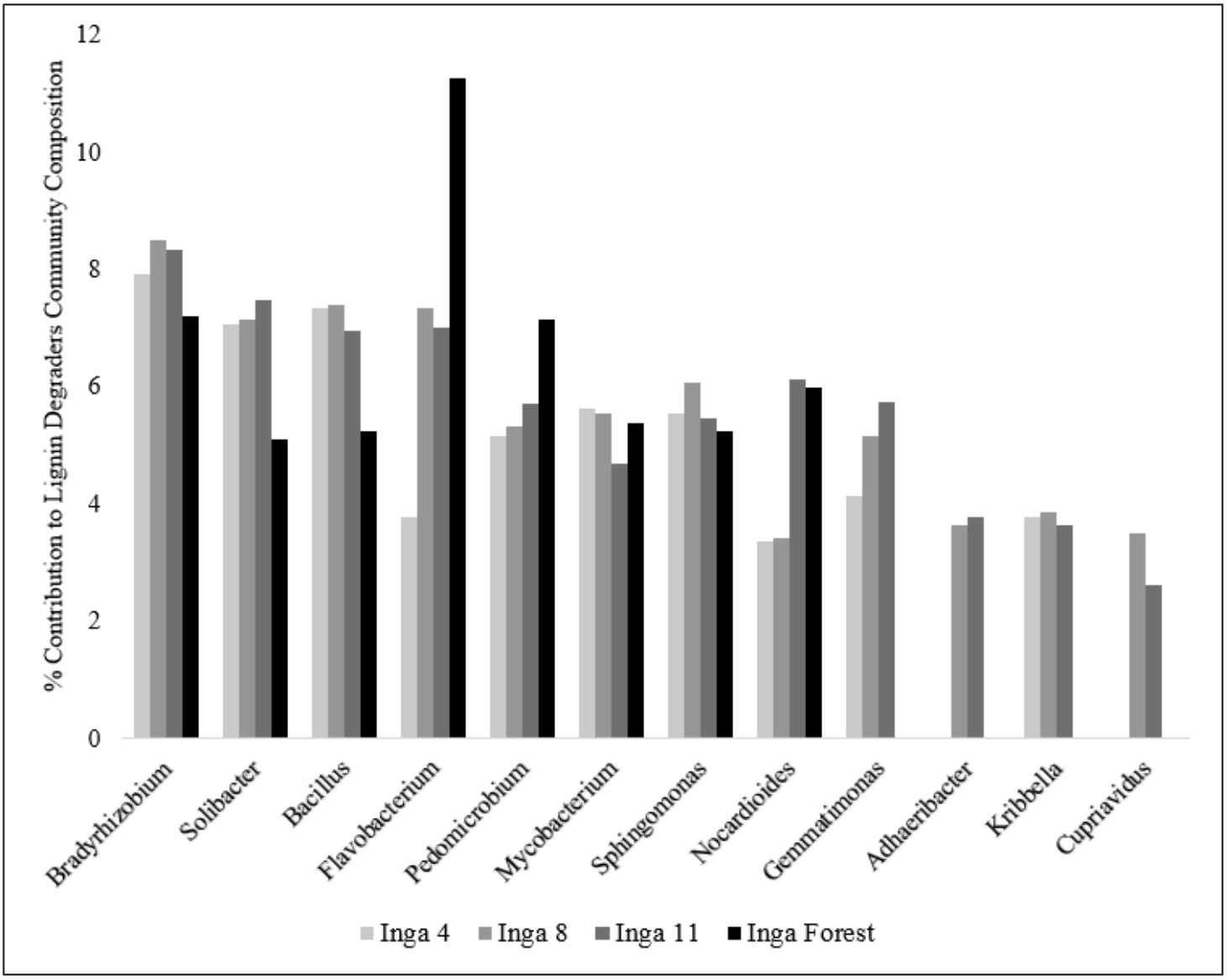
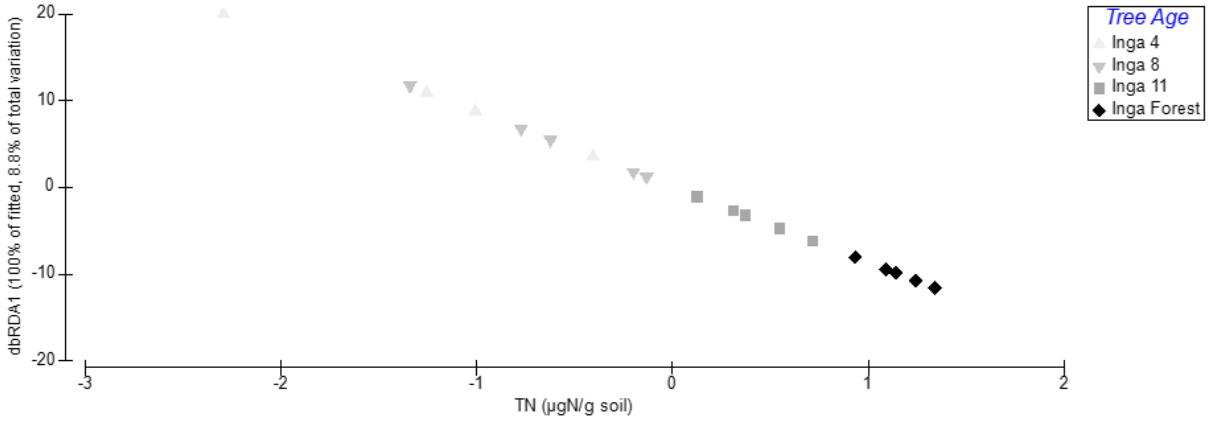


Figure 5

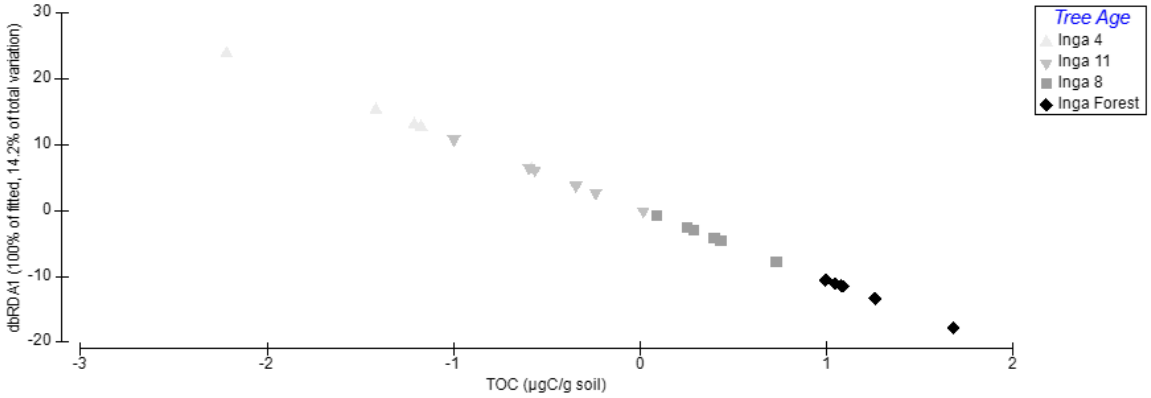
Results of the Similarity Percentage (SIMPER) routine conducted on the Mean Percent Sequences (MPS) levels of the Lignin Degrading genera of bacteria identified in the tree soils of *Inga punctata* trees planted 4, 8, and 11 years prior to the study (Inga 4, 8 and 11) and Old Growth *I. punctata* trees (Old Forest Inga) showing the percent contribution of the 12 genera of Lignin Degrading bacteria that were considered as top contributors to the compositions of this community in 2 or more of the different tree soil communities.

Figure 6a.



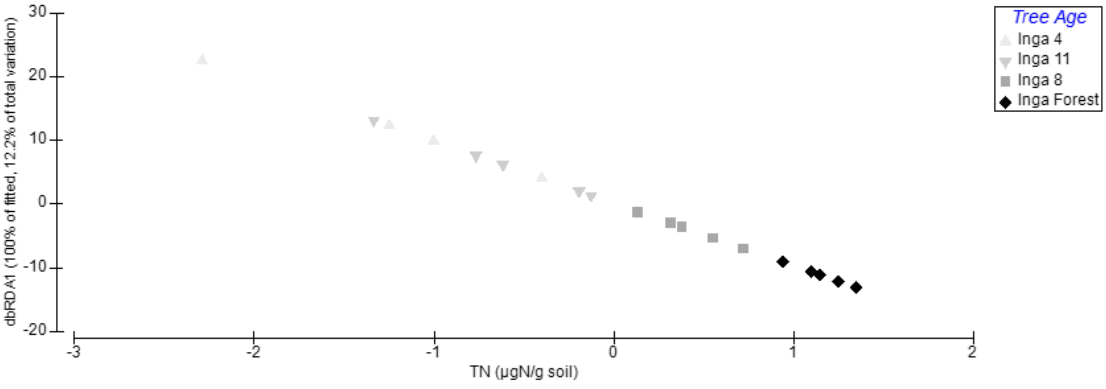
Pseudo-F = 2.024, p value = 0.053, explains 8.79% of N-fixer community variation

Figure 6b.



Pseudo-F = 3.471, p value = 0.004, explains 14.19% of Lignin Degrader community variation

Figure 6c.



Pseudo-F = 2.909, p value = 0.015, explains 12.17% of Lignin Degrader community variation

Figure 6

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 (Figures 6a and 6c) bacterial community compositions, and the influence that the total organic carbon (TOC) had on the lignin degrading bacterial community (Figure 6b) 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

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