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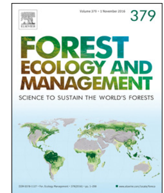

Mixed *Eucalyptus* plantations induce changes in microbial communities and increase biological functions in the soil and litter layers

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ABSTRACT

Mixed plantations of *Eucalyptus* and N_2-fixing trees are ecologically beneficial because they stimulate organic matter cycling and increase carbon (C) and nitrogen (N) pools in the soil. However, the microbial mechanisms that contribute to the improvement of C and N dynamics remain poorly understood in managed forest ecosystems. Here, we evaluated interactions between the bacterial community and biological functions involved in C and N cycles in the soil and litter layers resulting from pure or mixed *Eucalyptus grandis* and *Acacia mangium* plantations. We hypothesized that the mixed plantations induce changes in the bacterial community that would drive increases in C and N pools in soil and litter layers. We established a field experiment with treatments including pure *E. grandis* without (E) and with nitrogen fertilization (E + N), pure *A. mangium* (A), and mixed *E. grandis* and *A. mangium* (E + A). Soil and litter from all treatments were sampled 27 and 39 months after planting. We evaluated the soil and litter bacterial community and biological functions involved in C and N cycles (i.e., microbial and enzyme activities, functional gene abundance, and soil-litter nutrient cycling). The treatments A and E + A showed an increase in C and N contents in the organic soil fractions. We found higher bacterial diversity and OTU richness in soil and litter, and higher *nif*H gene abundance in the soil under A and E + A, when compared to pure *E. grandis* (especially E + N) plantation. Our data suggest that the total N content influences the bacterial community structure of the litter, which undergoes alterations according to the treatment and forest age. Equally, *Rhizobium*, *Bradyrhizobium* and *Sphingomonas* showed a positive correlation with *nif*H and soil N. Our study provided evidence that changes in the microbial community in mixed *A. mangium* and *E. grandis* plantations is correlated with increased C and N cycling. These findings have implications for increased productivity and environmental sustainability, besides allowing for the optimization of mineral fertilization in forest plantations.

1. Introduction

Land management practices can significantly improve plant productivity and can shift soil biophysical properties with significant consequences for ecosystem functions in forests and reforestation (Colombo et al., 2016). *Eucalyptus* forest plantations are important as an alternative for industrial raw material, reducing the extractive activity in native forests (Gonçalves et al., 2013). In Brazil alone, around seven million hectares are under *Eucalyptus* plantations, and the total area is increasing every year (Ibá, 2015). Such forest plantations require considerable amounts of mineral nutrients, due to a harvest system with quite short intervals (about seven years), which export (via biomass removal) more N than the amount of N fertilizer generally added at each new rotation (Laclau et al., 2010a,b; Pulito et al., 2015; Voigtlaender et al., 2012). Thus, there is an increasing demand to develop alternatives for *Eucalyptus* fertilization, focusing on plantation sustainability (Forrester et al., 2006; Laclau et al., 2008).

Previous studies have shown that the introduction of *Acacia*

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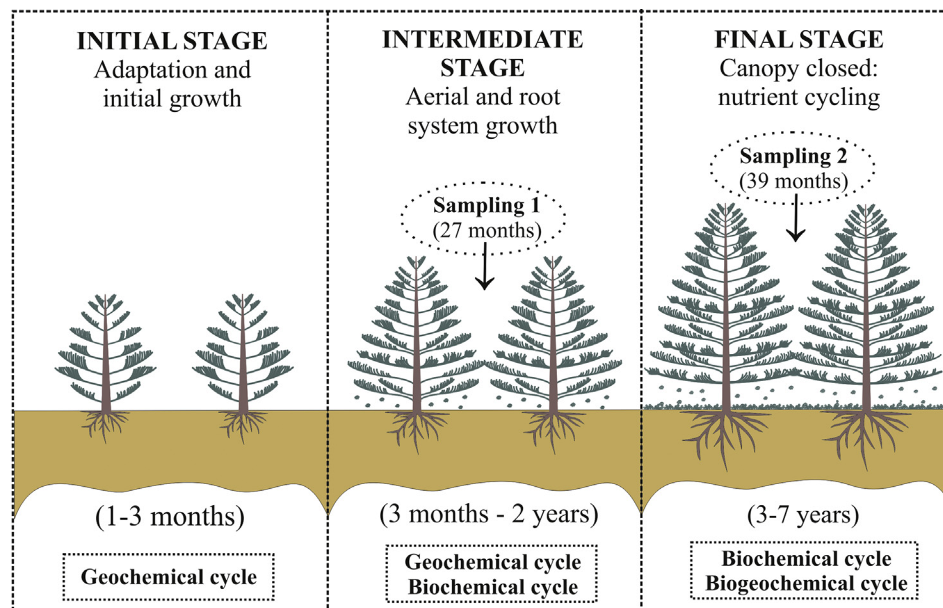

Fig. 1. Growth stages and changes in nutrient cycling in a typical *Eucalyptus* plantation in Brazil. Soil and litter were sampled at 27 and 39 months after tree planting, when there was a change from the biochemical to the biogeochemical cycle in the soil.

mangium trees could reduce fertilizer use in *E. grandis* plantations (Forrester et al., 2011; Laclau et al., 2008; Rachid et al., 2013; Richards et al., 2010; Voigtlaender et al., 2012; Epron et al., 2016). *A. mangium* roots form an association with diazotrophic bacteria and thus enhance the N content in the soil organic fraction by means of N_2-fixation (Laclau et al., 2008; Bouillet et al., 2008; Paula et al., 2018; Pereira et al., 2018). More recently, Pereira et al. (2017) reported that mixed plantations could influence the soil bacterial structure down to 300 cm depth. However, the principal soil and litter variables that regulate bacterial structure, diversity and composition and their linkage to essential biological functions in the forest remains poorly understood.

The bacterial community plays a critical role in regulating core ecosystem processes, such as C and N cycling (Baldrian, 2017; Delgado-Baquerizo et al., 2016). Bacteria are closely associated with N dynamics in the soil, starting with the N_2-fixation process and extending to organic matter mineralization and soil greenhouse gases emissions (Martins et al., 2015; Nelson et al., 2016; Ward and Jensen, 2014). The bacterial community also plays an equally important role in C soil cycling, improving organic matter decomposition and nutrient release (Liang et al., 2017). Previous research highlighted the availability of plant nutrients as a key benefit in mixed *E. grandis* and *A. mangium* plantations (Laclau et al., 2008; Pereira et al., 2018). However, limited studies have examined the response of the litter microbial community (Emilson et al., 2017), and none of these included mixed *E. grandis* and *A. mangium* plantations. Given that litter is a primary source of soil organic matter and is linked to C and N dynamics in forest ecosystems and tree nutrition (Bothwell et al., 2014; Laclau et al., 2010a,b; Rocha et al., 2016), this constitutes a critical knowledge gap.

Here, we evaluated the soil and litter bacterial community in a first rotation of pure *E. grandis* (with and without N addition) and *A. mangium* plantations, and in an intercropped system (*E. grandis vs A. mangium*). We hypothesized that the mixed plantation would induce changes in microbial community and drive biological functions associated with C and N cycles. We also hypothesized that the application of mineral fertilizer would reduce the abundance of key functional genes in soil and litter layers of pure *E. grandis* plantations. In soil and litter layers, we identified the main drivers of the bacterial community composition, structure, and diversity, the abundance of functional *nif*H and *amo*A genes (oxidizing-ammonium bacteria (AOB) and archaea (AOA) communities), and some biological functions related to C and N cycling.

2. Materials and methods

2.1. Experimental site and design, sampling and analyses

The study was carried out at the Itatinga Experimental Station of Forest Science (23°03′S–48°37″W; 830 m above sea level), University of São Paulo, Brazil. According to the Köppen-Geiger classification, the climate in the region is Cfa (i.e., humid subtropical, with a hot summer, but lacking a dry season), annual precipitation of 1350 mm (75% concentrated between March and October) and the mean relative air humidity of 83% (Alvares et al., 2013). The soil is a Yellow Latosol (Brazilian soil classification system) or Ferralsol (FAO/World Reference Base for Soil Resources), typically dystrophic, with a medium texture (~83% sand) (Laclau et al., 2008; Pereira et al., 2018). This soil type represents the majority of the soils where large commercial *Eucalyptus* plantations have been set up in Brazil (Gonçalves et al., 2013).

Historically, *E. grandis* occupied the area and there was no special management or fertilizer application, for ~30–50 years. After clear-cutting, an experiment with four treatments was planted in this area, consisting of pure *E. grandis,* without (E) and with N addition (E + N), pure *A. mangium* (A), and a mixed system (1:1 ratio) of *E. grandis* and *A. mangium* (E + A). The experiment was set up in a complete block design (36 × 36 m), with three field replicates and a spacing between plants of 3 × 3 m. We eliminated the edge effect by sampling only in the central area of each plot, equivalent to 576 m^2 (24 × 24 m). *A. mangium* seedlings were inoculated with *Rhizobium* strains (BR3609T and BR6009) from "Embrapa Agrobiologia" (https://www.embrapa.br/agrobiologia), selected for their high N_2-fixation and nodulation rates in *Acacia* spp.

Soil (0–20 cm) and litter (including twigs, branches, and leaves) were sampled at 27 and 39 months after planting (Fig. 1), corresponding to March 2016 and 2017 (rainy season), following the grid methodology based on the Voronoi polygons, widely accepted for sampling in forest ecosystems (Saint-André et al., 2005). We collected 24 soil and 24 litter samples, totalling 48 samples, complying with four treatments, three blocks and two sampling periods. N fertilization (E + N) was based on Gonçalvez and Benedetti (2000), with application rates of 50 and 400 kg ha^{-1} of ammonium sulfate in December 2013

and 2014, respectively. The low N application in 2013 was to account for the initial growth of the young plants, avoiding N losses. For the second fertilization, in 2014, there was a higher dose (400 kg ha^{-1}), to meet the increased requirements for tree growth (Gonçalvez and Benedetti, 2000). For a complete description of the experimental design and data collection, see also Pereira et al. (2018).

2.2. Analytical procedures

2.2.1. Soil, litter, and microbial attributes

We measured more than 20 soil microbial and seven litter attributes associated with C and N cycling. Physical and chemical attributes of the soil and litter layers can be found in Table S1 and in Pereira et al. (2018). Briefly, the soil pH was determined in a suspension with 0.01 mol L^{-1} $CaCl_2$ (1:2.5 soil: water ratio), and available P through ion-exchange resin (Raij et al., 2001). We determined C (Cmic) and N (Nmic) of the microbial biomass and soil basal respiration (CO_2-C), as well as $q$$CO_2$, qMic-C and qMic-N quotient (Anderson and Domsch, 1993; Brookes et al., 1985; Vance et al., 1987). We measured the activity of six soil enzymes, two associated with C (β-glucosidase and dehydrogenase) and four with N cycling (urease, L-asparaginase, L-glutaminase, and amidase) (Tabatabai, 1994). Soil organic matter was physically fractionated, and we evaluated C and N contents in the organic fractions (OF-C) and (OF-N). Organic fractions varied in size from 2000 to 75 µm (OF - 2000–75 µm). We also determined other C, N and P attributes (Total-C, Total-N, NH_4^+-N, NO_3^--N, P, C/N and C/P ratio) in litter samples (Brandani et al., 2017; Christensen, 2001; de Freitas et al., 2013).

To explore the potential of soil N transformations, we included a soil organic matter mineralization assay. N mineral fractions (NH_4^+-N and NO_3^--N) were determined following the aerobic incubation method proposed by Hart et al. (1994). For this purpose, soil (10 g to 2 mm) was adjusted to 40% of the maximum water holding capacity. Samples were incubated at 25 °C, and N extractions were performed at 0 and 28 days after incubation. The NH_4^+-N and NO_3^--N contents were extracted using 50 mL of 2 M KCl solution and determined following the "Kjeldahl" method. The liquid mineralization ratio was obtained by subtracting the initial (zero-day) and final (28 days) ammonium (NH_4^+-N) and nitrate (NO_3-$^-$N) concentrations (Hart et al., 1994).

2.2.2. Total DNA extraction in soil and litter

The soil samples were sieved (2 mm), and litter samples rinsed in sterile running water and ground in liquid N before DNA extraction. Total DNA was extracted from 400 mg soil and from 100 mg litter, using the MoBio Power Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA), according to the manufacturer's instructions. The extracted DNA was quantified using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific®). The DNA samples were stored at -20 °C and used for later molecular procedures.

2.2.3. Illumina amplicon sequencing

The 16S rRNA gene library was constructed according to the 16S metagenomics sequencing library preparation protocol (Illumina®, San Diego, CA, USA) targeting the V4 hypervariable region. The KAPA 2x HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington, MA, USA) and AMPure XP beads Kit were used for PCR reactions and purification, respectively. The first PCR was performed with soil and litter DNA templates using region-specific primers 515F/806R (5 µL) shown to have compatibility with Illumina® index and sequencing adapters (Caporaso et al., 2011). Amplified products were sequenced on the MiSeq sequencing platform (Illumina®) using the V3 kit (600 cycles) and paired-end approach (2 × 250 bp), following standard Illumina® sequencing protocols.

2.2.4. Bioinformatics processing

Raw forward and reverse reads were joined, and afterwards paired-end sequences were quality filtered. Chimeric sequences were identified and chloroplast sequences in litter samples were removed. Good quality sequences were binned into operational taxonomic units (OTU) at 97% of sequence similarity using Sumaclust (Kopylova et al., 2014). Representative sequences for each OTU were taxonomically classified with SILVA's ribosomal gene database (version 123) (Quast et al., 2013). We also filtered out singleton sequences, and α and β-diversity metrics were measured using the core_diversity_analyses.py script. Upstream analyses were done with the Quantitative Insights into Microbial Ecology software (QIIME, version 1.9) (Caporaso et al., 2010; Lozupone et al., 2011). A total number of 2,315,968 16S rRNA gene sequences was obtained in soil samples (~96,498 sequences per sample), and the raw OTU table was rarefied to a depth of 54,580 good quality sequences. In the litter layer, 2,175,076 16S rRNA sequences (~90,628 sequences per sample) were obtained, and the raw OTU table was rarefied to a depth of 36,230 good quality sequences.

2.2.5. Quantitative PCR analysis (qPCR)

Functional genes associated with the N_2-fixation (*nif*H) and the nitrification process (*amo*A, AOB and AOA) as well the phylogenetic 16S rRNA bacterial genes were quantified on the StepOne™ Real-Time PCR System platform. The reactions had a final volume of 20 µL, and we used SYBR Green PCR Master Mix 2x as a fluorescent marker (10 µL) (Applied BiosSystems®), BSA (Bovine Serum Albumin) (0.5 µL, 20 mg mL^{-1}), DNA template (1 µL) and the specific primers for each target region. Duplicate reactions were performed for each DNA sample, and four control samples (DNA free) were added at each run. Standard curves were constructed using ten-fold serial dilutions of plasmids containing the target gene product (insert) cloned following *p*GEM-T Easy Kit (Promega®) instructions. Amplification specificity was confirmed using the melting curve analysis and the fragment size, which was checked by electrophoresis in agarose gel (1.5%). Amplification efficiency (E) was calculated according to the equation: $E = [10(-1/slope)^{-1}]$, and gene copies (g $soil^{-1}$) were expressed in Log_{10}.

The *nif*H gene was quantified using FGHP19/POLR primers (1.6 µM) (Simonet et al., 1991; Poly et al., 2001). The thermal cycle conditions were as follows: 95 °C for 10 min, 39 amplification cycles of 94 °C for 60 s, 55 °C for 2 s and 72 °C for 60 s. The standard curve efficiency was 90%. The *amo*A gene of bacteria (AOB) was quantified using amoA-1F/amoA-2R primers (1:10–0.25 µM) (Stephen et al., 1999; Rotthauwe and Witzel, 1997). The thermal cycle conditions were as follows: 95 °C for 10 min, 39 amplification cycles of 94 °C for 60 s, 60 °C for 60 s and 72 °C for 60 s. The standard curve efficiency was 99%. The *amo*A gene of archaea (AOA) was quantified using amo23F/crenamoA616r (0.2 µM) (Nicol et al., 2008; Tourna et al., 2008). The thermal cycle conditions were as follows: 95 °C for 10 min, 39 amplification cycles of 94 °C for 45 s, 50 °C for 45 s and 72 °C for 45 s. The standard curve efficiency was 83%. The *q*PCR reaction of 16S rRNA gene was carried out using FP16S/RP16S primers (0.8 µM) (Bach et al., 2002), and the thermal cycle conditions were as follows: 95 °C for 10 min, 39 amplification cycles of 95 °C for 27 s, 62 °C for 60 s and 72 °C for 30 s. The standard curve efficiency was 106%.

2.2.6. Statistical analyses

We examined the homogeneity and normality of the variances by Levene and Shapiro-Wilks tests. The dataset was analyzed using ANOVA, and significant attributes were compared through Tukey test ($p < 0.05$). A Principal Coordinates Analysis (PCoA) based on the Unweighted and Weighted UniFrac metric distance was performed to visualize changes in the bacterial community structure using make_emperor.py (EMPeror) in QIIME (Edgar, 2010; Ramette, 2007; Vázquez-Baeza et al., 2011). Permutational Multivariate Analyses of Variance (PerMANOVA) were performed to examine treatment effects and correlations between soil, microbial and litter attributes with the bacterial community structure at 27 and 39 months after planting

(Adonis function - 9999 permutations) (Anderson, 2001). We correlated all soil-litter functions with bacteria diversity (Shannon index), *nif*H, *amo*A (AOB and AOA) and 16S rRNA genes abundance through Pearson's test. We used the Spearman ranking to assess correlations between families and bacterial genera (for 30 which presented the greatest OTU abundance) and soil and litter C-N functions. Upstream analyses were performed using R software (version 3.4.2) employing "*agricolae*", "*ggplot2*", "*vegan*", "*multtest*" and "*biobase*" packages (https://www.r-project.org/).

3. Results

3.1. Bacterial community composition in soil and litter layers

For the soil bacterial composition, there were no significant differences between treatments and sampling periods (27 and 39 months), including relative abundances at phylum, class, family and genus levels (Fig. S1 A-D). Around 50 phyla and 175 different bacterial classes were obtained. The three most dominating phyla were *Proteobacteria* (36%), *Acidobacteria* (20%) and *Actinobacteria* (13%), and the three most numerous classes were *Alphaproteobacteria* (22%), *Actinobacteria* (10%) and *Acidobacteria* (9%) (Fig. S1 A and B). These OTUs were classified into 750 families and 1429 different bacterial genera. The three most abundant families were *Acidobacteriaceae* (8%), *Acidothermaceae* (7%) and *Planctomyceae* (5%), while the three most abundant genera were *Acidothermus* (7%), *Afipia* (2%) and *Burkholderia* (2%) in the soil bacterial composition (Fig. S1 C and D).

In the litter layer, we found a significant effect of treatments and sampling periods on the bacterial composition ($p < 0.05$; Fig. S1). In this case, the bacterial OTUs were classified into 45 phyla and 141 different bacterial classes. The three phyla *Proteobacteria* (64%), *Actinobacteria* (14%) and *Acidobacteria* (5%), and the three classes *Alphaproteobacteria* (50%), *Actinobacteria* (10%) and *Betaproteobacteria* (9%) were most abundant in litter samples (Fig. S2 A and B). One hundred and forty two families were classified from the litter. At 27 months, the families *Acanthopleuribacteraceae* (2%), *Actinomycetaceae* (1%) and *Acidimicrobiaceae* (1%) were most abundant, while at 39 months, *Acetobacteriaceae* (2%), *Actinospicaceae* (2%) and *Acidimicrobiaceae* (2%) were the most abundant families (Fig. S2 C). A total number of 252 genera were classified in the litter compartment. At 27 months, *Sphingomonas* (12%), *Akkermansia* (5%) and *Bradyrhizobium* (3%), while at 39 months, *Burkholderia* (7%), *Sphingomonas* (7%) and *Bradyrhizobium* (4%) were the three most abundant genera in the litter bacterial composition, respectively (Fig. S2 D).

3.2. Differences in Shannon's index, OTU numbers and Phylogenetic Diversity in soil and litter layers

In soil, Shannon's diversity index demonstrated highest values in A treatment (with a mean of 9.23) and showed a decline in treatment E + N (8.89) ($p < 0.05$) (Fig. 2 A and D). The treatments E + A and A showed the highest OTU numbers (5895) at 27 months, but there was a significant reduction in E + N (5040). At 39 months, treatment A showed the highest number of OTUs (5412), and E + N presented the lowest number of OTUs (4635) (Fig. 2 B and E). Faith's Phylogenetic diversity index (PD) showed the highest index in the E + A (5 6 1) and A (5 5 4) treatments at 27 months, but there was a significant reduction in E (5 3 8) and E + N (4 8 6). At 39 months, treatment A showed a higher PD (5 1 6) than all others, and E + N (4 5 1) had the lowest value ($p < 0.05$) (Fig. 2 C and F). Within treatments, we found no time effect on Shannon's index, OTU numbers and PD.

In the litter layer, Shannon's diversity index was higher in E + A (with a mean of 8.25) and A (8.45) than in other treatments at 27 months. At 39 months, Shannon's diversity index was also higher in the intercropped plantation E + A (8.87) than in other treatments ($p < 0.05$) (Fig. 3 A and D). The observed OTU number was higher in E + A and A in both sampling periods (E + A = 2678 and 3731, and A = 2786 and 3411 at 27 and 39 months, respectively) (Fig. 3 B and E). The PD was higher in E + A (with a mean of 306) and A (with a mean of 293) (Fig. 3 C and F). Within treatments, the E + N and E + A showed an increase in alpha diversity and OTU number between 27 and 39 months. For example, Shannon's diversity ranged from 7.05 to 7.93, and from 8.24 to 8.86 in E + N and E + A at 27 and 39 months, respectively, following the OTU numbers (from 1676 to 2572, and from 2678 to 3831) and PD (from 179 to 244, and from 258 to 335), respectively ($p < 0.05$).

3.3. Shifts in bacterial community structure in soil and litter layers

A Principal Coordinate Analysis, based on the Unweighted and Weighted UniFrac distance matrices, explained 20.68% (axes x: 9.37%, y: 5.81% and z: 5.5%) and 51.5% (axes x: 25.49%, y: 16.31% and z: 9.7%) of the variation in the soil bacterial community structure, respectively (Fig. 4 A and B). No significant correlation was found between C and N attributes and the soil bacterial structure (Table S1). Moreover, there was no significant group separation, either between treatments (PerMANOVA, $R^2 = 16\%$, $p = 0.1158$) or sampling periods (PerMANOVA, $R^2 = 4.66\%$, $p = 0.3258$) (Table S2).

In litter layers, the PCoA explained 32.95% (axes x: 17.69%, y: 10.0% and z: 5.26%) and 78.38% (axes x: 49.12%, y: 21.38% and z: 7.88%) of the variation in the litter bacterial structure, respectively (Fig. 4 C and D). The PerMANOVA test showed a positive correlation between the litter bacterial structure at both periods with Total-N ($R^2 = 28\%$, $p < 0.0001$), NH_4^+-N ($R^2 = 17\%$, $p < 0.0001$) and Total-C ($R^2 = 9\%$, $p < 0.0001$) contents (Table S3). There was a significant group separation between treatments (PerMANOVA, $R^2 = 37\%$, $p < 0.0001$) and sampling time (PerMANOVA, $R^2 = 20.7\%$, $p < 0.0001$). In this case, litter Total-N content explained 48.1% and 38.6% of the data variation ($p < 0.0001$) at 27 and 39 months, respectively (Table S3).

Comparing soil-litter bacterial structure, including all treatments and sampling periods, PCoA explained 41.78% (Unweighted; axes x: 32.21%, y: 3.33% and z: 6.24%) and 83.77% (Weighted, axes x: 66.67%, 12.13% and 4.97%) of the community structure (Fig. 4 E and F). In this case, the PerMANOVA test showed significant separation between the soil and litter bacterial structure ($R^2 = 83\%$, $p < 0.0001$).

3.4. Abundance of nifH, amoA (AOB and AOA) and 16S rRNA genes

The *nif*H abundance was highest in A, followed by E + A at 27 months (7.48 and 7.34 copies g soil^{-1}, respectively) ($p < 0.05$) (Fig. 5 A). At 39 months, the A treatment also showed the highest *nif*H abundance (7.24 copies g soil^{-1}) ($p < 0.05$). However, there was a reduction in E + A (6.38 copies g soil^{-1}), and E (6.63 copies g soil^{-1}) ($p < 0.05$) (Fig. 5 E). It is important to highlight that in the pure *E. grandis* plantations and even more in (E + N), with N fertilizer, there was a significant decline in *nif*H abundance, independently of the sampling time (with a mean of 5.9 copies g soil^{-1}) ($p < 0.05$) (Fig. 5 A and E).

There was a higher *amo*A (AOB) abundance in E and A than in other treatments at 27 months, with no significant differences between them (5.99 and 5.11 copies g soil^{-1}, respectively) ($p < 0.05$) (Fig. 5 B). In the same period, E + N presented the lowest *amo*A abundance, differing from all other treatments (3.36 copies g soil^{-1}), $p < 0.05$ (Fig. 5 B). At 39 months, A and E showed a higher *amo*A (AOB) abundance than E + A and E + N, ($p < 0.05$; Fig. 5 F). In this period, E + N and E + A showed the lowest *amo*A (AOB) abundance, with no significant differences between these two treatments (4.39 and 4.45 copies g soil^{-1}, respectively) ($p < 0.05$; Fig. 5 F).

Regarding the sampling period, there was a significant effect only in E + A, which also reduced *nif*H the gene abundance from 7.34 (at 27 months) to 6.35 (at 39 months) *nif*H copies g soil^{-1}. Conversely, the

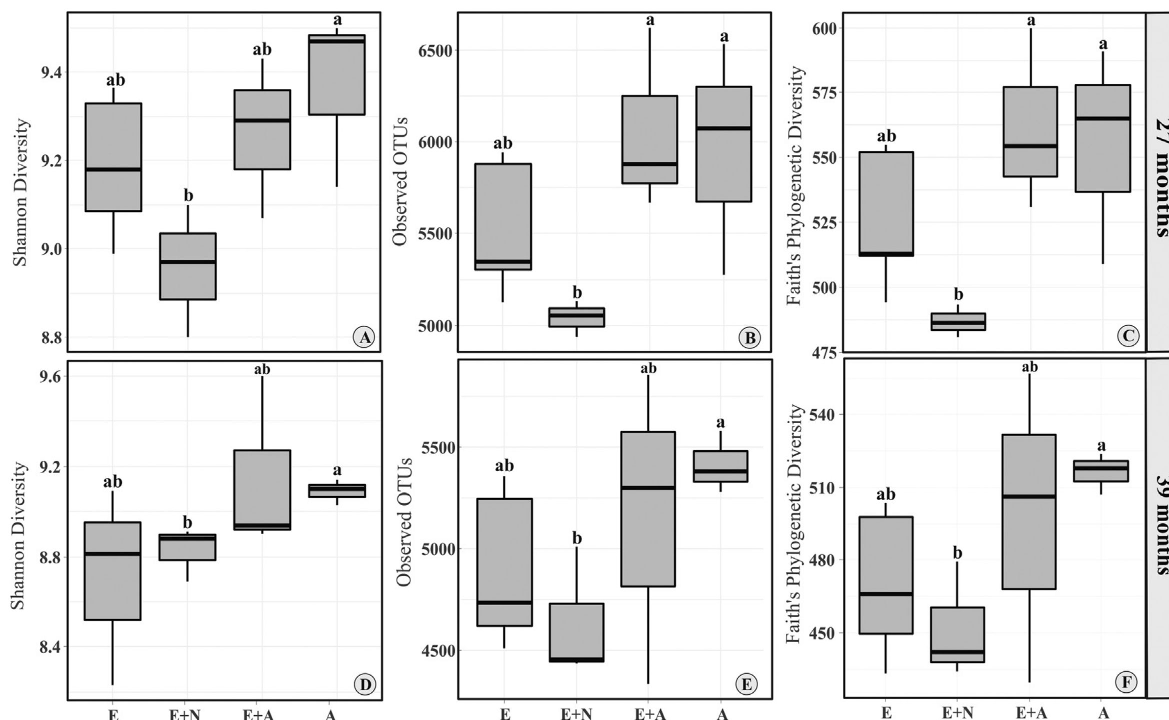

Fig. 2. Shannon diversity, observed OTUs and Phylogenetic Diversity metrics in soil of pure and mixed *E. grandis* and *A. mangium* plantations. (E) *E. grandis*, (E + N) *E. grandis* with N fertilization, (E + A) intercropped *E. grandis* and *A. mangium* and (A) *A. mangium* plantation at 27 (A, B and C) and 39 (D, E and F) months after planting. Means followed by the same letter do not differ by Tukey's test at a significance level of 5% (n = 3).

E + N treatment showed an increase in *amo*A (AOB) at 27 months, from 5.37 to 6.39 at 39 months ($p < 0.05$). The *amo*A (AOA) and total bacterial 16S rRNA genes did not differ between treatments, independently of the sampling time (with a mean of 5.87 and 12.26 copies of *amo*A (AOA) and 16S rRNA genes g soil^{-1}, respectively) (Fig. 5 C-D and G-H).

3.5. General soil and litter pools

The soil pH ranged from 3.8 to 4.2 and soil nitrate (NO_3^--N) from

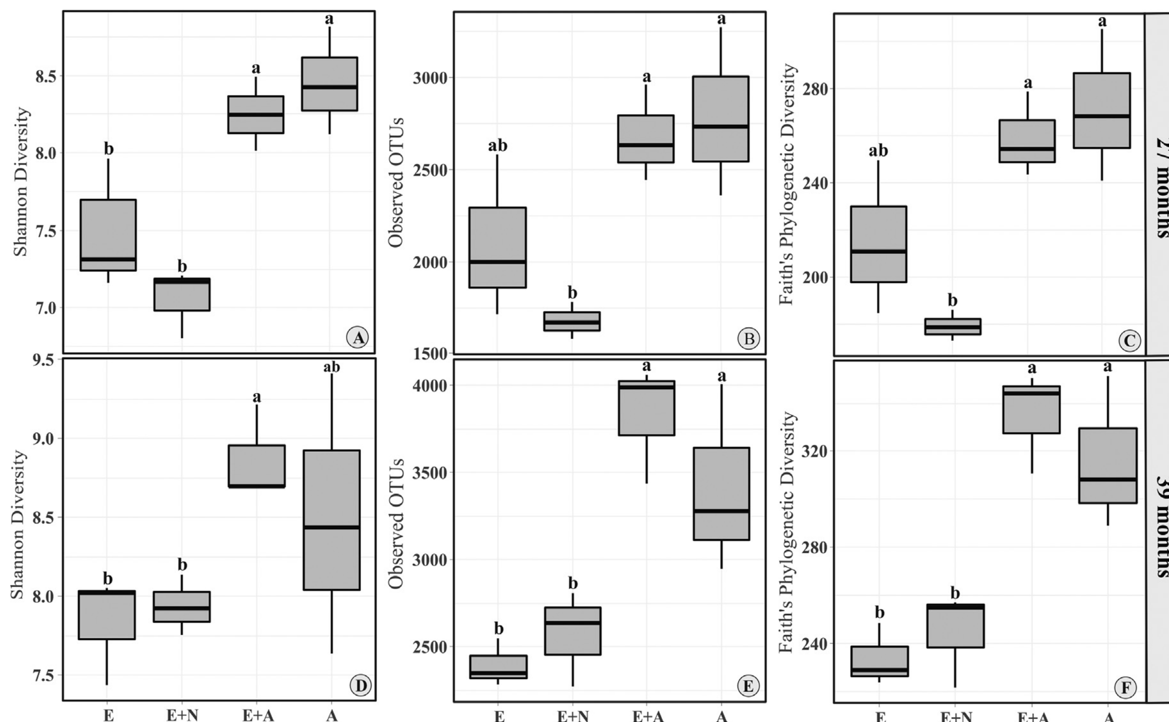

Fig. 3. Shannon's diversity index, observed OTUs and Phylogenetic Diversity metrics in litter of pure and mixed *E. grandis* and *A. mangium* plantations. (E) *E. grandis*, (E + N) *E. grandis* with N fertilization, (E + A) intercropped *E. grandis* and *A. mangium* and (A) *A. mangium* plantation at 27 (A, B and C) and 39 (D, E and F) months after planting. Means followed by the same letter do not differ by Tukey's test at a significance level of 5% (n = 3).

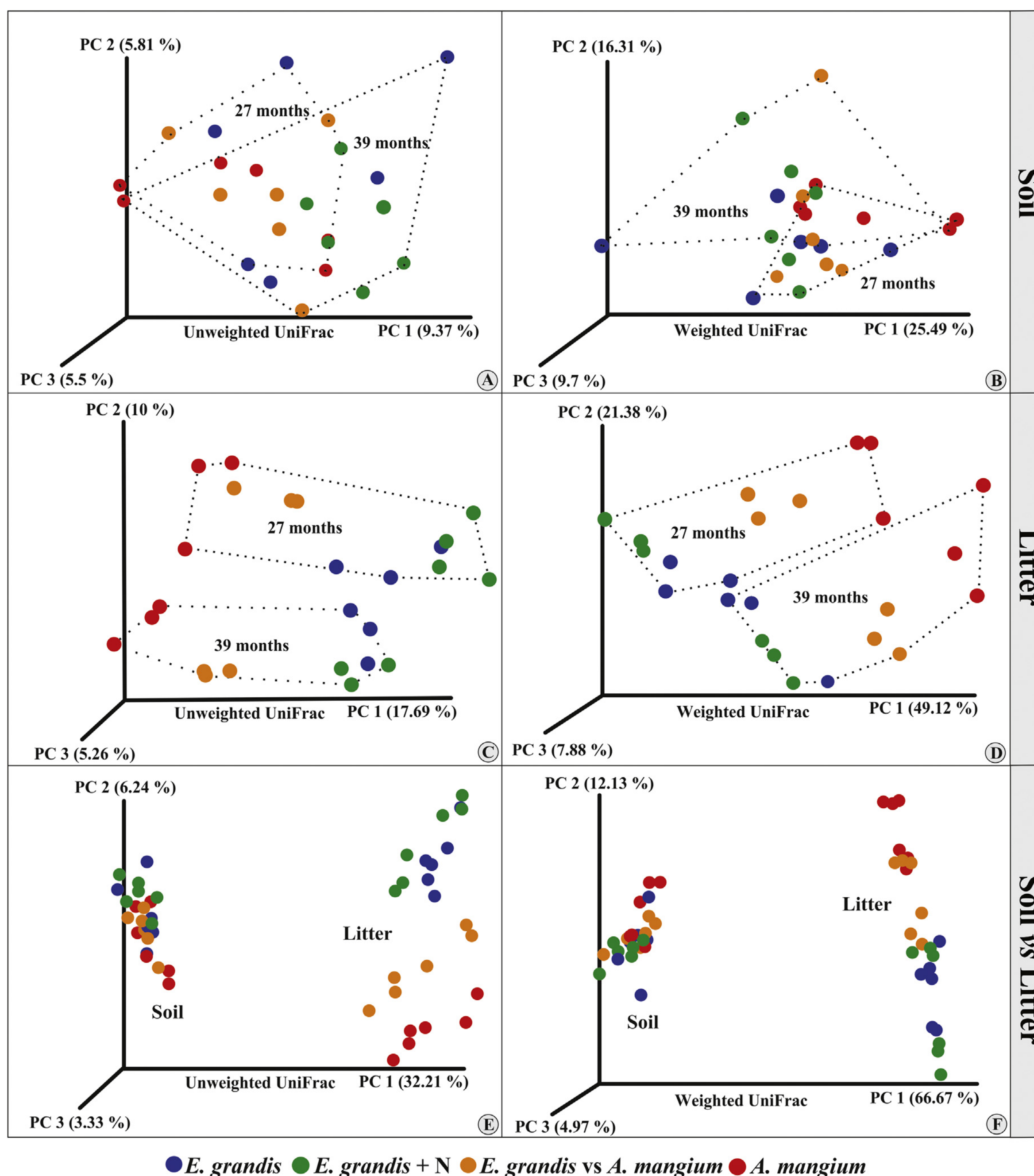

Fig. 4. Principal Coordinate Analysis (PCoA) by Unweighted and Weighted UniFrac of soil (A and B) and litter (C and D) of the bacterial community in pure and mixed *E. grandis* and *A. mangium* plantations. (E) *E. grandis*, (E + N) *E. grandis* with N fertilization, (E + A) mixed *E. grandis* and *A. mangium* and *A. mangium* (A) plantations at 27 and 39 months after planting (n = 3). The figures E and F compare soil and litter using all treatments and sampling times (n = 6).

18.2 to 38.7 mg kg^{-1}, and no variation was detected between treatments and sampling periods in either case ($p < 0.05$). However, the soil NH_4^+-N content was higher in A and E + A, mainly at 27 months (56.7 and 45.5 mg kg^{-1}, respectively) ($p < 0.05$). The P content did not change between treatments. However, the P content was higher at the first sampling, with a mean of 5.5 mg dm^{-3}, than at the second, with a mean of 3.6 mg dm^{-3} ($p < 0.05$). Litter P content showed no significant differences between treatments at both sampling periods (mean of 2.52 and 3.33 at 27 and 39 months, respectively). Nevertheless, litter P content in E and E + N treatments were lower at 27 months ($p < 0.05$). The litter C/P ratio did not change between treatments at both sampling periods, but treatments E and E + N presented the lowest values at 39 months ($p < 0.05$) (Table S1).

The results for some soil chemical and microbial attributes, enzymatic activity, and litter characterization, were previously presented by Pereira et al. (2018). Briefly, A and E + A showed higher microbial C content (Cmic) and C microbial quotient (qMic-C) than E and E + N, especially at 27 months ($p < 0.05$). The amidohydrolases (especially urease and amidase) and dehydrogenase enzyme activity were highest in E and E + N. In contrast to dehydrogenase, all amidohydrolases showed higher activity at 39 than at 27 months ($p < 0.05$). The mass of the soil organic fraction (OF) (2000–75 μm) in A and E + A treatments were higher (twice as much) than in E and E + N, especially at 39 months. Also, the OF in A and E + A showed higher C and N concentrations than in E and E + N ($p < 0.05$). In this case, the mixed treatment (E + A), when compared with E and E + N, showed increases in N concentrations of 45% and 50% (at 27 months), and of 55% and 61% (at 39 months) (OF-N), respectively. The same was

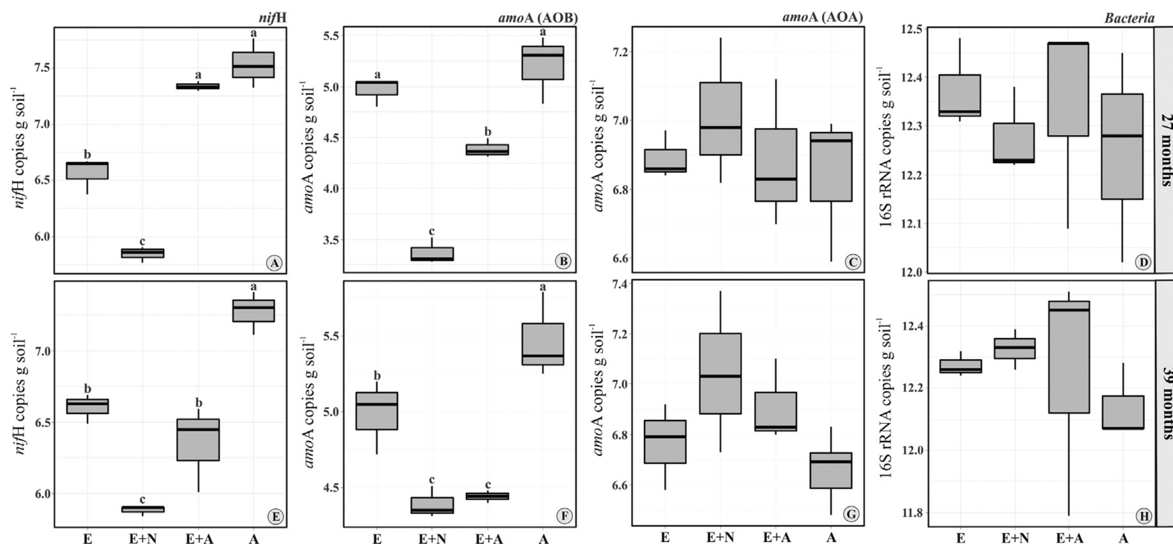

Fig. 5. Real-time quantitative PCR (*q*PCR) of *nif*H, *amo*A (AOB and AOA) and total bacteria (16S rRNA) genes abundance in pure and mixed *E. grandis* and *A. mangium* plantations. (E) *E. grandis*, (E + N) *E. grandis* with N fertilization, (A) *A. mangium* and (E + A) mixed plantation of *E. grandis* and *A. mangium* at 27 (A, B, C and D) and 39 (E, F, G and H) months after planting. Means followed by the same letter do not differ by Tukey's test at a significance level of 5%. All values are presented in log_{10} (n = 3).

true for C content in the OF. E + A showed increases of 48% and 58% (at 27 months) and 57% and 63% (at 39 months) in C concentrations (OF-C). In the litter, Total-N contents were higher in A and E + A, than in E and E + N, especially at 39 months. Also, A and E + A had the lowest C/N ratio in both sampling periods, and the litter NH_4^+-N contents were higher in the same treatments when compared to E and E + N ($p < 0.05$) (Table S1).

3.6. Relationship between the gene abundance and soil-litter nutrient pool with soil functions

Pearson's test showed a strong positive correlation between the *nif*H gene abundance and soil, microbial and litter C and N attributes, such as Total-N, OF-N, NH_4^+-N, Nmic, *q*Mic-N, Total-C, OF-C, Cmic, *q*Mic-C, CO_2-C, litter Total-N and NH_4^+-N, mainly at 27 months. At 39 months, the *nif*H gene had a positive correlation with six attributes, three in the soil (Total-N, NH_4^+-N, and Nmic) and three in the litter (Total-N, NH_4^+-N and C/N ratio), respectively. Shannon's diversity index showed a positive correlation with Total-N, OF-N, OF-C, Litter Total-N and NH_4^+-N (Table 1).

The *amo*A (AOB) abundance correlated positively with soil Total-C and litter NH_4^+-N at 27 months. The *amo*A (AOB) gene abundance also correlated with soil and litter Total-N in both sampling periods. The gene *amo*A (AOA) abundance correlated with urease, L-asparaginase and L-glutaminase activity (only at 27 months), and with NO_3^--N at both sampling periods. Only three attributes showed correlation with abundance of the bacterial 16S rRNA genes, including pH and L-glutaminase at 27 months, and NH_4^+-N at 39 months (Table 1).

3.7. Spearman's rank correlation coefficients

3.7.1. Soil microbial community vs. nutrient pools

Twenty-two families (belonging to the 30 most abundant families) showed significant correlations with soil-microbial C and N attributes. Five of these families had the highest correlation values, such as *Sphingomonadaceae*, *Rhizobiaceae*, *Bradyrhizobiaceae*, *Nitrosomonadaceae* and *Sphingobacteriaceae* were among those with the highest correlation number (Fig. S3 A). In this case, there was a strong correlation between *Rhizobiaceae* and *Sphingomonadaceae* with Total-N (R = 0.85 and 0.82, respectively), OF-N (R = 0.74 and 0.67), NH_4^+-N (R = 0.52 and 0.61), Total-C (R = 0.56 and 0.83), *nif*H (R = 0.88 and 0.86) and *amo*A (AOB) (R = 0.61 and 0.65) at 27 months. No families correlated positively with available P and nitrate in the soil.

Six of the most abundant genera showed strong correlations with five different soil-microbial C and N attributes. For example, *Rhizobium*, *Sphingomonas*, *Bryobacter*, *Bradyrhizobium*, *Telmatobacter* and *Burkholderia* showed the highest number of correlations (Fig. S3B). In this case, at 27 months, *Rhizobium* and *Sphingomonas* showed stronger correlations with Total-N (R = 0.75 and 0.86), OF-N (R = 0.64 and 0.66), Total-C (R = 0.56 and 0.81), *nif*H (R = 0.78 and 0.76) and *amo*A (AOB) (R = 0.61 and 0.67) (Fig. 3 B). Likewise, there was no correlation between any bacterial genus and available P or NO_3^--N in the soil.

3.7.2. Litter microbial community vs. nutrient pools

At 27 months, nine families (belonging to the 30 most abundant) showed four significant correlations with litter chemical attributes, and *Anaerolineaceae* alone showed five correlations. No litter families correlated with NO_3^--N content. However, 14 families showed significant correlation with Total-N and NH_4^+-N, such as *Actinomycetaceae* (R^2 = 0.80 and 0.93, respectively), *Anaerolineaceae* (R^2 = 0.86 and 0.84), *Beijerinckiaceae* (R^2 = 0.73 and 0.93), *Blattabacteriaceae* (R^2 = 0.85 and 0.76) and *Cellulomonadaceae* (R^2 = 0.78 and 0.75), respectively (Fig. S4 A). At 39 months, 14 families (belonging to the 30 most abundant), demonstrated up to five significant correlations with litter nutrients. No family correlated with litter Total-C and P content. However, *Acanthopleuribacteraceae*, *Aerococcaceae*, and *Aurantimonadaceae* showed a strong correlation with NO_3^--N (R^2 = 0.60, 0.71 and 0.58, respectively) and NH_4^+-N (R^2 = 0.67, 0.82 and 0.61) respectively. Also, seventeen families had significant correlations with Total-N, and *Alcanivoracaceae* (R^2 = 0.88) and *Bogoriellaceae* (R^2 = 0.87) correlated the most strongly (Fig. S4 A).

Twelve genera (belonging to the 30 most abundant) showed between four and five significant correlations with the litter chemical attributes (Fig. S4 B). At 27 months, no genus correlated with NO_3^--N and only *Terriglobus* showed significant correlation with Total-C (R^2 = 0.60). However, the Total-N and NH_4^+-N content had a positive correlation with 15 and 18 litter bacterial genera, highlighting *Methylobacterium* (R^2 = 0.88 and 0.74, respectively) and *Amnibacterium* (R^2 = 0.79 and 0.89), respectively (Fig. S4 B). At 39 months, thirteen genera (belonging to the 30 most abundant) showed between four and five significant correlations with the litter chemical attributes (Fig. S4 B). Fourteen genera showed significant correlations with Total-N,

Table 1
Pearson's correlation test between soil, microbial and litter attributes with Shannon's diversity and gene abundance (16S rRNA, *nif*H and *amo*A (AOB and AOA)) at 27 and 39 months after planting.

Soil	27 months					39 months				
	Shannon	16S rRNA	*nif*H	*amo*A (AOB)	*amo*A (AOA)	Shannon	16S rRNA	*nif*H	*amo*A (AOB)	*amo*A (AOA)
pH	ns	0.58^{*}	ns	ns	ns	ns	ns	ns	ns	ns
P	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Total-N	0.60^{**}	ns	0.88^{***}	0.85^{*}	-0.24^{**}	0.50^{**}	ns	0.12^{*}	-0.50^{*}	ns
OF-N	0.53^{*}	ns	0.85^{***}	ns	-0.04^{*}	0.47^{***}	ns	0.31^{*}	ns	ns
NH_4^+-N	ns	ns	0.71^{***}	ns	ns	ns	-0.57^{**}	ns	ns	ns
NO_3^--N	ns	ns	ns	ns	0.23^{***}	ns	ns	ns	ns	0.15^{**}
Nmic	ns	ns	0.60^{**}	ns	ns	ns	ns	-0.57^{*}	-0.67^{**}	ns
*q*Mic-N	ns	ns	0.61^{**}	0.83^{***}	ns	ns	ns	0.54^{*}	ns	ns
Total-C	ns	ns	0.90^{*}	0.81^{***}	ns	ns	ns	ns	ns	ns
OF-C	0.65^{*}	ns	0.84^{***}	ns	ns	0.50^{*}	ns	ns	ns	ns
Cmic	ns	ns	0.64^{***}	ns	ns	ns	ns	ns	ns	ns
*q*Mic-C	ns	ns	0.49^{**}	ns	ns	ns	ns	ns	ns	ns
CO_2-C	ns	ns	0.45^{*}	ns	-0.54^{*}	ns	ns	0.40^{*}	-0.52^{*}	ns
qCO_2	ns	ns	-0.68^{**}	ns	ns	ns	ns	ns	ns	ns
Urease	-0.56^{**}	ns	-0.83^{***}	ns	0.32^{**}	-0.63^{**}	ns	ns	ns	ns
L-asparaginase	-0.54^{*}	ns	ns	ns	0.09^{*}	ns	ns	ns	ns	ns
L-glutaminase	ns	0.60^{*}	ns	ns	0.07^{*}	ns	ns	ns	ns	ns
Amidase	ns	ns	-0.77^{***}	ns	ns	-0.60^{**}	ns	ns	ns	ns
β-glucosidase	ns	ns	ns	ns	ns	-0.52^{*}	ns	ns	-0.64^{**}	ns
Dehydrogenase	ns	ns	-0.79^{***}	ns	ns	-0.58^{**}	ns	ns	ns	ns
Litter	–	–	–	–	–	–	–	–	–	
Total-N	0.84^{*}	ns	0.83^{***}	0.48^{*}	ns	0.87^{*}	ns	0.93^{***}	0.48^{*}	ns
NH_4^+-N	0.60^{**}	ns	0.82^{***}	0.50^{*}	-0.32^{**}	0.35^{*}	ns	0.89^{***}	ns	ns
NO_3^--N	ns	ns	ns	ns	ns	-0.61^{**}	ns	ns	ns	ns
Total-C	0.27^{*}	ns	ns	ns	ns	-0.59^{*}	ns	ns	ns	ns
C/N ratio	ns	ns	-0.82^{***}	-0.45^{*}	ns	-0.68^{***}	ns	-0.54^{*}	ns	ns
P	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
C/P ratio	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Significance codes: $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$. ns: not significant. Parameters: P (available phosphorus), OF-N (nitrogen – soil organic fraction), NH_4^+-N (ammonium), NO_3^--N (nitrate), Nmic (microbial N), *q*Mic-N (microbial N quotient), OF-C (carbon - soil organic fraction), Cmic (microbial C), *q*Mic-C (microbial C quotient) and qCO_2 (metabolic quotient).

including *Mesorhizobium* ($R^2 = 0.86$). Moreover, the NO_3^--N and NH_4^+-N content correlated with 16 and 14 genera, respectively, especially *Akkermansia* ($R^2 = 0.68$ and 0.64, respectively) and *Skermanella* ($R^2 = 0.72$ and 0.72) (Fig. S4 B).

4. Discussion

The mixed plantation system with *Eucalyptus* and *Acacia* is considered an important strategy for minimizing the use of mineral fertilizer in commercial forest plantations (Bouillet et al., 2008; Laclau et al., 2008; Pereira et al., 2018; Voigtlaender et al., 2012; Forrester et al., 2011). One of the key reasons for this fact is that *Acacia* forms associations with N_2-fixing bacteria and can provide N for the growth of both tree species (Paula et al., 2018). We provide evidence that mixed plantations induce changes in the microbial community, improving C and N cycles. More importantly, we provide novel evidence that soil and litter microbiomes differ, and they respond differently to plantation management practices from phylum to genus levels.

Acacia stands (A) and mixed plantations (E + A) increase α-diversity (Shannon diversity, OTU numbers, and phylogenetic diversity), however the application of N fertilizer (E + N) reduced it in both sampling periods. The association between *E. grandis* and *A. mangium* can promote a more heterogeneous environment above (mixed litter) and below ground (root exudation, mycorrhizal associations) (Baldrian, 2017; de Araujo Pereira et al., 2018; Laclau et al., 2013). Thus, in addition to the increase in C and N content in soil and litter, the number of ecological niches (microhabitats) can increase to support a more diverse microbial community in mixed *E. grandis* and *A. mangium* plantations. This finding provides support for the ecological theory of possible links between microhabitats and biodiversity (Beckers et al., 2017; Griffiths and Philippot, 2018; Jansson and Hofmockel, 2018).

On the other hand, pure *E. grandis* plantations showed a low nutrient quality (high C/N ratio) both in litter and soils (Pereira et al., 2018; Bini et al., 2013). Furthermore, the application of high N fertilizer rates can affect soil and litter community functions. For example, the ammonium sulfate fertilizer ($(NH_4)_2SO_4$) produces instantaneous acidification in the soil. In this case, after hydrolysis, the ammonium ion (NH_4^+) can be converted to nitrate [$2\ NH_4^+ + 3O_2 \rightarrow 2NO_3^- + 2H_2O + 4H^+$] by soil nitrifying microbes (IPNI, 2014). This process releases large amounts of H^+ and can promote acidification in the surrounding soil (Yu et al., 2016). The low soil pH is known to reduce the α-diversity of bacterial communities (Fierer and Jackson, 2006; Lauber et al., 2009). The antagonistic effect of N-fertilizers on soil bacterial communities in forest plantations corroborates with previous work (Colombo et al., 2016; He et al., 2013; Zheng et al., 2017). Here, we provide evidence that the negative impact of N on microbial α-diversity is also relevant in litter layers.

Overall, the soil α-diversity index showed no changes between 27 and 39 months after planting. However, there was a time effect in the litter layers, where the E + N and E + A treatments presented increases in α-diversity. For example, both treatments, E + N and E + A, showed a constant increase in Shannon's index, OTU numbers and phylogenetic diversity between 27 and 39 months. We suggest that the litter bacterial community responds directly to the management system at shorter durations than the overall soil communities. In mixed plantations, the α-diversity increases further with time. Therefore, litter communities seem to adapt their response to the available resources. A stronger response in E + A may be due to increases in litter total-N, as supported by the strong correlation between the bacterial litter community and total-N.

The microbial community structure and composition are governed by several factors including soil pH and nutrient concentration in soil

and litter (Delgado-Baquerizo et al., 2018; Delgado-Baquerizo et al., 2016; Lauber et al., 2008), that can be influenced by the climate and age of the trees within the plantation (Classen et al., 2015). The PerMANOVA test showed no differences in bacterial community structure between treatments in the soil, as well as no significant correlation between community structure and soil attributes in both sampling periods. This result contrasts with previous findings from a similar experimental design (Pereira et al., 2017). In contrast, we found a substantial effect of time and treatments on the litter bacterial community structure and composition. The litter bacterial community response to Total-N and NH_4^+-N content may be associated with the highest concentrations of these attributes in A and E + A treatments (Pereira et al., 2018).

Moreover, the bacterial community from the mixed treatment (E + A) was intermediate between the monocultures in the PCoA, suggesting that the litter bacterial community in mixed plantations has characteristics belonging to both monoculture microbiomes. Equally, the litter diversity may represent an important factor in the soil bacterial community variations, mainly because of the strong influence on the soil biology and nutrient attributes (Baldrian, 2017). This suggests that the litter diversity has an immediate impact on the litter microbiome even when the soil microbiomes remain unchanged. Our results also suggest that the plant-mediated change in the soil microbiome can take longer than the experimental duration of our study (Pereira et al., 2017). Interestingly, Bini et al. (2013) studying pure and mixed *E. grandis* and *A. mangium* plantations showed bigger microbial activity in litter than soil, independently to treatment. These findings corroborate our results, since we found different bacterial structure, composition and diversity in litter when compared to soil. This may be the result of the mixed plantations, which deposit more easily degraded high quality litter, generating litter with higher N contents and lower C:N ratio, which can, consequently, stimulate the microbial activity and diversity (Bini et al., 2013).

Although the litter and soil interface are intimately connected through the exchange of energy and nutrients (Fanin et al., 2012), the distinction between the microbial structure in the litter layer and the underlying soil was rarely made clear, particularly in mixed plantations. Litter deposited on the soil can modulate climatic factors, nutrient quality and availability, and microhabitats influencing the soil bacterial community structure (Prevost-boure et al., 2011) and the rhizospheric effect (Pereira et al., 2017). Further, the litter is primarily a source of C and N as well as the habitat of many microorganisms, but the impact on the soil bacterial community is slow and mediated by processes of redistribution of the resources by fragmentation, leaching or incorporation into the mineral soil (Carrillo et al., 2015). In this case, litter influence can increase with time, suggesting that the long-term effects may not be predictable from short-term experiments, as in a young forest (Cleveland et al., 2014). In addition, the material composing the litter layer displays a much wider C and N range, as well as distinct C quality and quantity compared to that of soil organic matter, which may be another reason for considerable differences between litter and soil bacterial communities (Fanin et al., 2012). However, the litter-derived materials, once processed and incorporated into the soil, can change the soil bacterial communities over time (Carrillo et al., 2011).

The *nif*H gene regulates nitrogenase synthesis, an enzymatic complex that catalyzes the N_2-fixation to plant available NH_3 (Gaby and Buckley, 2014). We found that the *nif*H gene abundance was highest in A and E + A treatments, what actually would be expected. Recently, Paula et al. (2018) reported that interspecific interactions in intercropped plantations with *E. grandis* increased N_2-fixation rates by diazotrophic bacteria associated with *A. mangium* roots. Moreover, we found a positive correlation between *nif*H genes and Total-N, OF-N, NH_4^+-N, Total-C and Cmic contents in the soil, especially at 27 months. On the other hand, the *nif*H reduction in E + N treatments may be attributed to the greater NH_4^+ availability in the soil that can reduce the abundance of free-living N_2-fixing bacteria (Fonseca et al., 2017; Florence et al., 2016). However, the soil microbiome variations promoted by the acidic fertilizer reaction seems to be more coherent, since the N application also reduced the OTU numbers and α-diversity in soil and litter in both sampling periods. These results show the importance of *A. mangium* in the mixed system with *E. grandis*, particularly in weathered soil, containing low organic matter content, because it can increase N pools and minimize the requirement of mineral fertilizers, improving soil and plant health (Richards et al., 2010).

The *amo*A gene correlates with the synthesis of the ammonium monooxygenase enzyme (AMO), which oxidizes ammonium to nitrate, a reaction promoted by bacteria and archaea (Verhamme et al., 2011). The abundance of the *amo*A (AOB) gene differed between treatments, but soil nitrate levels did not show significant changes between treatments and sampling periods, which was different from the *amo*A (AOA) gene. These results differ from those reported previously by Rachid et al. (2013) in a similar forest plantation. The authors reported an increase of soil nitrate in mixed plantations. However, the nitrate levels reported by Rachid et al. (2013) were quite low and ranged from ~0 to $4\,g\,kg^{-1}$ at soil pH = 5.5. In our experiment, however, nitrate concentrations ranged from 18.2 to $38.7\,g\,kg^{-1}$ at soil pH = 4.0. Thus, the possibility of nitrate increasing in soil is higher in the first case than in the second, mainly because the low nitrate concentrations and the higher soil pH may have favored nitrifiers (Kyveryga et al., 2004). Moreover, under more acidic soil pH levels, below 5.5, *Archaea* (AOA) were reported to play a dominant role in ammonia oxidation (Prosser and Nicol, 2012; Wen et al., 2017; Yao et al., 2011), which may explain the positive correlation between soil nitrate content and the *amo*A (AOA) gene. In addition, the autotrophic nitrification rates are slower in extremely acid soils, and investigations suggest that heterotrophic nitrification can be very important in tropical soil, mainly governed by the fungal community (Pajares and Bohannan, 2016; Zhu et al., 2014).

Moreover, Pereira et al. (2018) showed that mixed *A. mangium* and *E. grandis* plantations increase C and N concentrations in the soil organic fractions. This fact is very relevant because it stimulates nutrient cycling, providing energy for soil microbial activities. In the soil, *Rhizobium*, *Bradyrhizobium*, and *Sphingomonas* had a strong correlation with the *nif*H gene abundance and other important C and N attributes. This provides strong evidence that mixed plantations promote the abundance of bacteria directly linked to N_2-fixation and other C-N functions, which results in increased availability of N for tree species, suggesting that in mixed plantations one can reduce the amount of N fertilizer and therefore there is a promotion of a sustainable increase in the plantation's productivity.

5. Conclusions

We provide novel evidence that pure *Acacia* and mixed plantations between *Eucalyptus* and *Acacia* promote soil microbial diversity and activities. Specifically, we showed that mixed *E. grandis* and *A. mangium* plantations significantly increase bacterial community diversity, functional gene abundance and key biological functions. This, in turn, improves C and N cycling in the soil and litter interface. Furthermore, our results suggest that the litter microbiome is distinct from the soil microbiome and responds more rapidly to management practices than the soil microbiome. Shifts in the litter bacterial community are modulated by total-N content, which alter according to treatment and forest age. We showed improvement in bacterial diversity (Shannon's index) and functional gene abundance in *Acacia mangium* and mixed plantations, mainly at 27 months. The improvement of soil microbial properties by *Acacia* seems to remove some metabolic or biochemical constraints imposed by *Eucalyptus* monocultures. Further, the use of mineral N fertilizers proves to be an inferior alternative for long-term soil health, because mineral N can reduce the abundance of functional genes, bacterial diversity and microbial activities. In addition, increased bacterial diversity and abundance of functional genes correlates strongly

with increased rates of measured biological functions and C-N pools, suggesting that management practices that favor these characteristics have to be promoted for profitable and sustainable foresting.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foreco.2018.11.018.

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