University of São Paulo
"Luiz de Queiroz" College of Agriculture

The microbiome related to carbon and nitrogen cycling in pure and mixed *Eucalyptus grandis* and *Acacia mangium* plantations

Arthur Prudêncio de Araujo Pereira

Thesis presented to obtain the degree of Doctor in Science: Area: Soil and Plant Nutrition

Piracicaba
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versão revisada de acordo com a resolução CoPGr 6018 de 2011

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I learned that courage is not the same as absence of fear, but the triumph over it. The brave man is not the one who never feels afraid, but the one who conquers fear.

~ Nelson Mandela, Long Walk to Freedom.

If you got a dream… You have got to protect it! People cannot do something by themselves, and they want to tell you that you cannot do it either. If you want something, go and get it!

~ Will Smith, The Pursuit of Happyness.

CONTENTS

RESUMO

O microbioma relacionado à ciclagem de carbono e nitrogênio em plantações puras e mistas de *Eucalyptus grandis* e *Acacia mangium*

A inserção de árvores fixadoras de nitrogênio (N_2) em sistemas florestais mistos é uma estratégia recente que pode reduzir o uso de *inputs* externos e aumentar a sustentabilidade das plantações de Eucalipto. Nesses sistemas, existe uma forte interconexão entre as árvores, a qual ocorre por uma complexa rede de interações entre micro-organismos, acima e abaixo do solo. Essas interações resultam em inúmeros processos biológicos e serviços ecossistêmicos, os quais são essenciais para a saúde do solo e das plantas. Além do mais, o resultado da interação Eucalipto-microbioma-Acácia tem sido apontado como essencial no alcance de maiores índices de produtividade do Eucalipto em sistemas mistos. Nosso objetivo foi explorar a dinâmica do microbioma relacionado à ciclagem de nutrientes em plantações puras e mistas de *Eucalyptus grandis* e *Acacia mangium*. Especificamente, nossos esforços focaram nos benefícios do microbioma para a melhoria de funções biológicas, principalmente aquelas promovidas pela introdução da Acácia em plantações comerciais de Eucalipto. Por exemplo, abordamos detalhes como o conhecimento da diversidade, composição e funções desse microbioma pode nos ajudar a compreender sua íntima relação com a ciclagem de carbono (C) e nitrogênio (N) no solo e na serapilheira. Acreditamos que abordagens holísticas, com as quais possamos explorar as interações biológicas em sistemas com plantas de alto valor ecológico (Acácia) e alto valor econômico (Eucalipto) serão inevitáveis no futuro. Se aprendermos a manipular alguns processos mediados pelo microbioma envolvido nessas interações, daremos um passo importante para superar as atuais limitações de recursos, aliando o aumento da produtividade com a intensificação ecológica das plantações florestais e a sustentabilidade do meio ambiente.

Palavras-chave: Florestas mistas; Microbioma; Funções biológicas; Ciclagem de C e N; Sustentabilidade ambiental

ABSTRACT

The microbiome related to carbon and nitrogen cycling in pure and mixed *Eucalyptus grandis* and *Acacia mangium* plantations

The introduction of N_2-fixing trees in mixed forest systems is a recent strategy that can reduce the use of external inputs and increase the *Eucalyptus* plantations sustainability. In these systems, there is a strong interconnection between the trees, which occurs through a complex network of interactions between microorganisms, above and belowground. These interactions result in innumerable biological functions and ecosystem services, which are essential for soil and plant health. Moreover, the result of the *Eucalyptus*-microbiome-*Acacia* interaction has been pointed out as essential in achieving higher *Eucalyptus* productivity indexes in mixed systems. Our aim was to explore the dynamics of microbiome related to nutrient cycling in pure and mixed *Eucalyptus grandis* and *Acacia mangium* plantations. Specifically, our efforts were focused on the microbiome benefits to the biological functions improvement in commercial *Eucalyptus* plantations driving by *Acacia* introduction in the system. We also give details regarding as the knowledge of the microbiome diversity, composition and functions can help us to understand their close relationship with carbon (C) and nitrogen (N) cycling in soil and litter layers. We believe that holistic approaches in which we can explore the biological interactions in systems using plants of high ecological value (*Acacia*) and high economic value (*Eucalyptus*) will be inevitable in the near future. If we learn how to manipulate important processes mediated by the microbiome involved in these interactions, we will take an important step to overcome the current resource constraints, combining increased productivity with the ecological intensification of forest plantations and the environmental sustainability.

Keywords: Mixed forests; Microbiome; Biological functions; C and N cycling; Environmental sustainability

1. BIOLOGICAL PROPERTIES IN FOREST ECOSYSTEMS: WHY ARE THEY IMPORTANT FOR *Eucalyptus* PLANTATION?

The forests habitats: a brief description

Natural forests are considered a specific ecosystem, representing high wood production and comprising huge habitats that support the microbiome's life, which is dynamic and quickly responds to anthropogenic and environmental changes (Baldrian, 2017). Moreover, reactions to the microbiome's metabolism can occur in the most diverse plant organs and locations, such as leaves, flowers, seeds, fruit, wood, inside (endophytic) or on the tree surface (phyllosphere), as well as belowground (soil, roots, rhizosphere and mycorrhizosphere) (Baldrian, 2017). Even more important, habitats differ in properties such as nutrient availability, major environmental conditions, processes and dynamics, which together can alter the microbiome dynamics. The forest microbiome research has been highly focused on soil habitats, emphasising tree roots and their symbionts, while litter and other habitats have been greatly underexplored, mainly in pure and mixed plantations (Pereira et al., 2018).

The forest environment has specific properties that differentiate it from other (e.g., agricultural systems and implanted forest) (Navarrete et al., 2015). One of the most important feature is the huge effect of the dominant trees on the surrounding habitat, which can regulate aboveground and belowground interactions (Wardle et al., 2004). The trees interact with microbial activities and composition, and this is mediated by bulk soil and litter chemistry (Augusto et al., 2015; Šnajdr et al., 2013; Urbanová et al., 2015). Here, we include organic matter contents, the soil pH, nitrogen transformations and other macro and micronutrients (Fierer and Jackson, 2006; Lauber et al., 2008; Prescott and Grayston, 2013; Rousk et al., 2010; Tedersoo et al., 2016; Urbanová et al., 2015). These effects seem to be extremely dependent on forest management (Tedersoo et al., 2016), and all drivers are combined by stochastic effects on microbiome assembly (Bahram et al., 2016), which can contribute to the dynamics of microbiomes in different forest habitats (Štursová et al., 2016).

The native and implanted forest environment

Implanted forests are subject to multiple modes of disturbance, such as insect attacks, fires, nutritional imbalances in soil, among others. In addition, this system is also significantly changed by many anthropogenic factors, as climate change or environmental pollution, water

deficit, and inappropriate management practices, which together may easily shift the balance of carbon and nitrogen cycling processes (Trumbore et al., 2015).

Soils under native forest have characteristics that differentiate them in numerous aspects from the implanted forestry and agricultural soils (Fig. 1A). For example, around 50% of the C fixed by trees in allocated in the soil through their root activity (Högberg et al., 2001), while the litter layers are important organic matter sources for the system, governing important stages of ecosystems services and nutrient cycling (Baldrian, 2017). Besides the C contents, one of the most notorious effects of tree influence is the low pH of the soil solution, potentiated by the release of organic acids through root system exudation (Motavalli et al., 1995). In addition, there is a large root extrusion of enzymes that degrade organic matter which make biogeochemical cycling very active in this environment, with increases in C and N contents above and belowground (Fig. 1A) (Chapter 2).

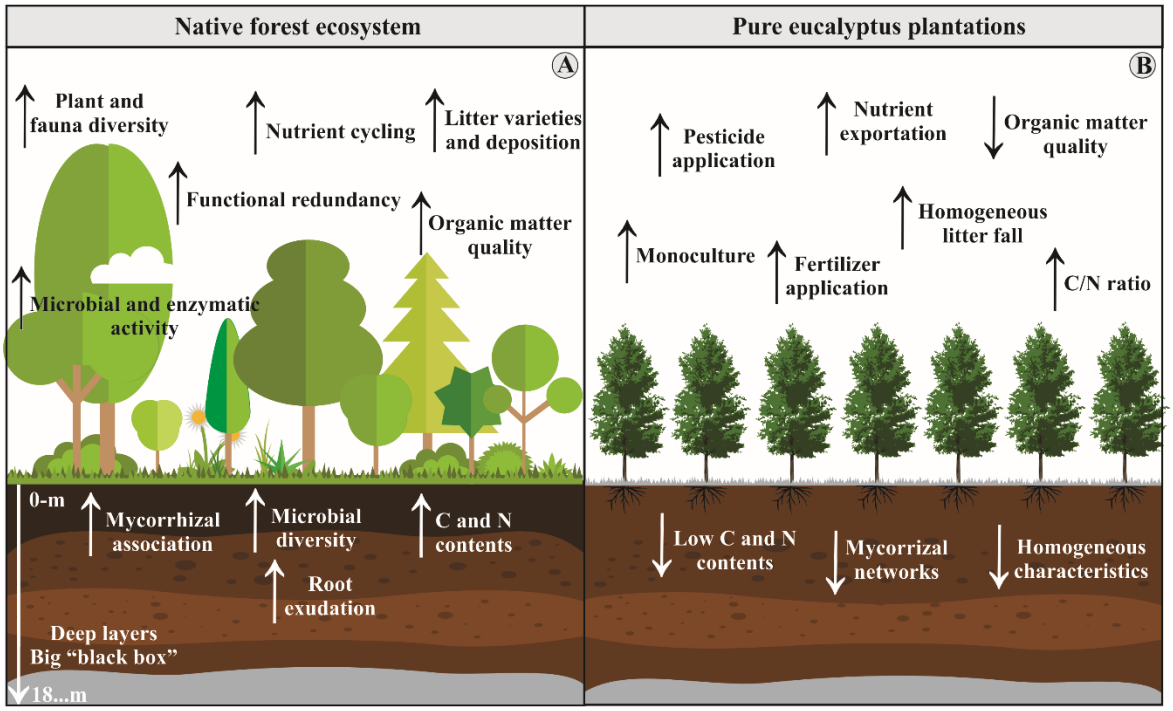

Figure 1. Major differences between a natural forest and an implanted forest ecosystem. Arrows pointing upwards in (A) indicate better soil health than in (B), arrows pointing down, where the opposite is true.

Natural forests can provide several ecosystem services that are fundamental for the maintenance of the surrounding environment, mainly in soil protection (Lal et al., 2014), biogeochemical cycling of nutrients (Laclau et al., 2010), maintenance of microbial

biodiversity, meso and macrofauna (Cardoso et al., 2013), organic matter quality (Pereira et al., 2018) among others (Fig. 1A). On the other hand, the conditions that occur in the implanted forest ecosystems, such as in *Eucalyptus* plantations, differ strongly from their natural state, mainly in terms of diversity and functionality in the soil-plant (micro) biota interface (Fig. 1B).

In planted forests (monocultures) we find deposition of a unique litter type, which may present low nutrient availability (high C/N ratio) (Mercês et al., 2016; Snowdon et al., 2005). Moreover, when compared to natural systems, the depletion of mineralizable nutrients may occur over time (mainly due to wood exportation), and the type and quality of exudates secreted by the roots are extremely selective (Churchland and Grayston, 2014) (Fig. 1B). Thus, forest plantations may differ in some soil properties and determine different kinds of temperature, aeration, porosity and soil water storage capacity (Baldrian, 2017). This behaviour can promote more homogenous conditions for microbial communities, making them less diverse and less efficient in the use of available resources, leading to negative plant-soil feedbacks (PSF) (Mariotte et al., 2017), or "soil fatigue" (Huang et al., 2013). In this sense, little emphasis was placed on studies to minimize PSF using intercropping systems to improve biological functions in different forest niches (Wang et al., 2017).

The mixed-system with N_2-fixing trees: brief importance of biological functions to soil, plant health, and nutrient cycling

There are around 3 trillion trees on the planet Earth (Crowther et al., 2015), which are responsible for covering a large part of the vegetated soil surface. This large volume of biomass is extremely important, especially in the regulation of the world's climate, and the health of soil and bodies of water (Kirilenko and Sedjo, 2007). However, trees are closely dependent on the microbiome to survive, which provides nutrients essential for their development, such as N, P and K (primary macro nutrients), through organic matter cycling in the soil (Baldrian, 2017). For example, it is estimated that N_2-fixing bacteria and mycorrhizal fungi are responsible for providing up to 75% of the nitrogen and 80% of the phosphorus that forests use during their life cycle (Van Der Heijden et al., 2008). In addition, all organic matter transformation steps depend on the activity of microorganisms (Singh et al., 2018).

Acacia trees form symbiotic relationships with N_2-fixing bacteria and provide a key reservoir of N, C and P for the surrounding ecosystem (Bini et al., 2013; Paula et al., 2018; Pereira et al., 2018; Taylor et al., 2017). In this sense, it is possible to integrate trees of high

economic value (*Eucalyptus*) and trees of high ecological value (*Acacia*) in an intercropped system (Laclau et al., 2008; Pereira et al., 2017; Rachid et al., 2015) (Fig. 2).

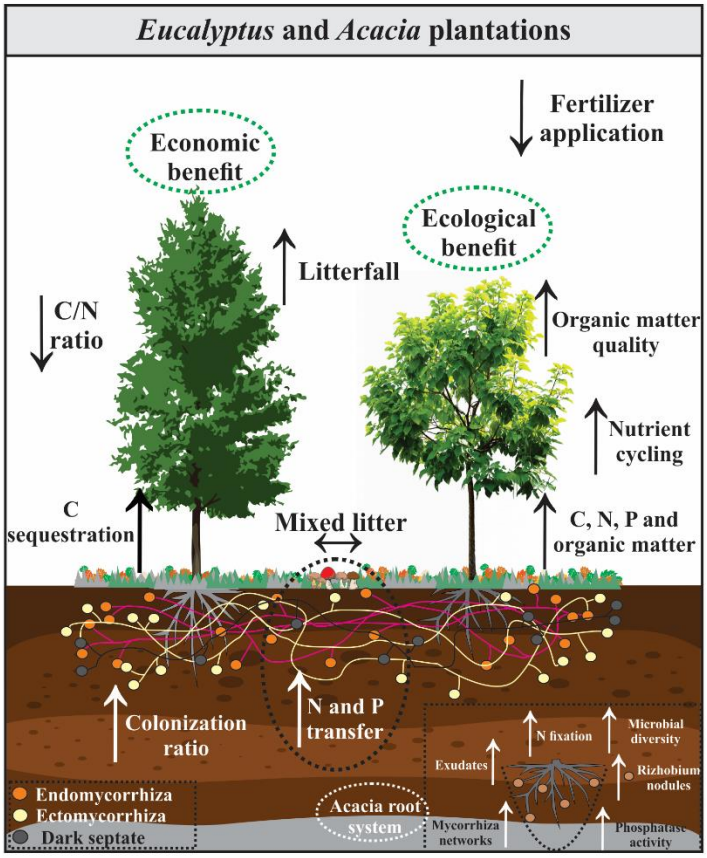

Figure 2. Intercropped *Eucalyptus* and *Acacia* plantations. Upward arrows mean that soil health is better than in pure plantations, and the opposite is true for downward arrows. Belowground networks representing the mycorrhizal associations (endo and ecto mycorrhiza, and dark septate endophytes) and interactions between the two plants.

Recent studies have shown the N_2-fixing potential around 90-120 kg ha^{-1} $year^{-1}$, as well as the direct transfer of N of the roots of *A. mangium* to the *E. grandis* roots (Bouillet et al., 2008; Paula et al., 2015, 2018). In this sense, *Eucalyptus* plants would provide financial benefits and those of *A. mangium*, immeasurable ecological gains (Fig. 2). In this case, the availability of N can occur for *Eucalyptus* also after senescence of *Acacia* plant tissues (litter, fine roots and nodules), root exudation, as well as the cell death of organisms of the soil microbiota, providing N through mineralization process (May and Attiwill, 2003; He et al., 2003; Chalk et al., 2014). However, in spite of the diverse benefits of this association, studies

evaluating the interactions at the soil-plant-microbiome interface in this type of forest management remain poorly understood.

The increase of N in the soil promoted by *A. mangium* is able to promote a significant increase in *E. grandis* productivity, even in the absence of the application of mineral fertilizers (Laclau et al., 2008). In a review published by Forrester et al. (2006), a meta-analysis of 18 studies showed that several trials with mixed cultures were significantly more productive than monocultures, with very few cases showing the opposite. For example, 11 years after the implantation of a mixed *E. globulus* and *A. mearnsii* plantation, mixed stands between species were more productive than monocultures in terms of aerial biomass, volume of wood produced and C allocation in soil, with higher N and P cycling rates in litter (Forrester et al., 2004).

Presently, we are experiencing a big data generation from microbiome studies (Goodrich et al., 2014). In this case, manipulation strategies of this microbiome with the attempt of reducing the application of pesticides and mineral fertilizers should be urgently sought, aiming at obtaining more efficient and sustainable agricultural practices (Mariotte et al., 2017; Pereira and Verma, 2018) (Fig. 3). Few attempts have been made regarding the study of these aspects in areas with the insertion of *Acacia* into *Eucalyptus* systems.

In the near future, the researchers need to focus on manipulations of the Acacia microbiome to increase Eucalyptus plantations sustainability (Fig. 3). In this sense, we have to consider the emerging holobiont theory (Catania et al., 2017; Theis et al., 2016), which states that the symbiont's characteristics need to be considered along with those of the host in order to understand the biological functions of the associated organisms (Fonseca et al., 2017). In this context, the Acacia microbiome represents a very important component of the plant holobiont, and its composition may affect tree productivity.

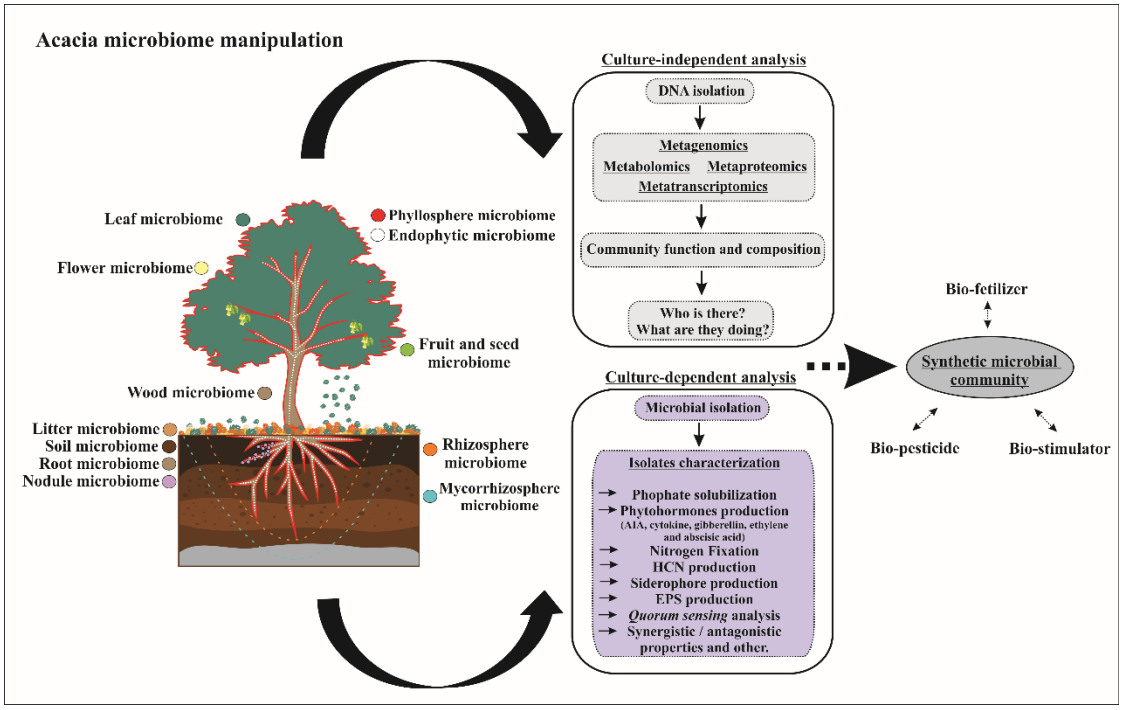

Figure 3. Strategies to explore the Acacia microbiome for development of sustainable technologies and to increase the production of implanted forest plantations.

The above-mentioned microbiome can improve plant growth and health in different ways, including N_2-fixation, phosphorus (P) solubilization, and phytohormone production, among others (Fig. 3) (Vessey, 2003). The manipulation of the diversity of endophytic communities associated with Acacia under different planting and edaphoclimatic conditions will be very important to reduce production inputs and improve the resource efficient use.

General objectives and hypotheses

Our main aim in this thesis is to evaluate changes in soil and litter attributes and to correlate them with microbiological functions 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*). Specifically, we characterize soil organic matter fractions and litter and examine their correlations with the microbial community, in order to further explore the microbial influence on C and N functions and how it can discriminate among plantation treatments. We hypothesized that intercropping of *A. mangium* in *E. grandis* plantations would induce changes in microbial communities (composition, structure, diversity) and drive

biological functions associated with C and N cycles. We also hypothesized that the application of mineral fertiliser would reduce the abundance of key functional genes in soil and litter layers (e.g., *nif*H and *amo*A genes (oxidizing-ammonium bacteria (AOB) and archaea (AOA) communities).

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2. *Acacia* CHANGES MICROBIAL INDICATORS AND INCREASES C AND N IN SOIL ORGANIC FRACTIONS IN INTERCROPPED *Eucalyptus* PLANTATIONS

Abstract

Intercropping forest plantations of Eucalyptus with nitrogen-fixing trees can increase soil N inputs and stimulate soil organic matter (OM) cycling. However, microbial indicators and their correlation with specific fractions of soil OM are poorly understood in tropical sandy soils. Here, we examined the microbial indicators associated with C and N in the soil under pure and intercropped *Eucalyptus grandis* and *Acacia mangium* plantations. We hypothesized that the introduction of *A. mangium* in an Eucalyptus plantation promotes changes in microbial indicators and increases C and N concentrations in labile fractions of the soil OM, when compared to pure Eucalyptus plantations. We determined the microbial and enzymatic activity, and the potential for C degradation by the soil microbial community. Additionally, we evaluated soil OM fractions and litter parameters. Soil (0–20 cm) and litter samples were collected at 27 and 39 months after planting from the following treatments: pure *E. grandis* (E) and *A. mangium* (A) plantations, pure *E. grandis* plantations with N fertilizer (E+N) and an *E. grandis*, and *A. mangium* intercropped plantations (E+A). The results showed that intercropped plantations (E+A) increase 3, 45, and 70% microbial biomass C as compared to A, E+N, and E, at 27 months after planting. The metabolic quotient (qCO_2) showed a tendency toward stressful values in pure *E. grandis* plantations and a strong correlation with dehydrogenase activity. A and E+A treatments also exhibited the highest organic fractions (OF) and C and N contents. A canonical redundancy analysis revealed positive correlations between microbial indicators of soil and litter attributes, and a strong effect of C and N variables in differentiating A and E+A from E and E+N treatments. The results suggest that a significant role of *A. mangium* is to enhance the dynamics of soil microbial indicators, which help in the accumulation of C and N in soil OF in intercropped *E. grandis* plantations. Our results are mostly relevant for plantations in sandy soil areas with low levels of OM, suggesting an efficient method for improving nutrient availability in the soil and optimizing Eucalyptus growth and development.

Keywords: Forest soil; Soil biology; Mixed-systems; C-N cycles; Organic matter

Introduction

Brazil is the world's largest producer of *Eucalyptus* spp., a species of fundamental ecological, social, and financial importance due to its effects in reducing pressures on native forests and generating direct and indirect jobs (ABRAF, 2013). However, the sustainability of Eucalyptus plantations has been intensely debated due to their concentration in south and central Brazil, where low fertility soils prevail, and Eucalyptus is mostly grown in mono-specific plantations (Gonçalves et al., 2013). Additionally, Eucalyptus production areas in Brazil have relatively short-rotation (6-7 years) and high productivity (~37 m^{-3} ha^{-1} $year^{-1}$), which entails a high nutrient exportation due to wood exportation and a negative balance in soil nitrogen over time (Bouillet et al., 2008; Gonçalves et al., 2013; Pulito et al., 2015).

As a result, mineral fertilizers are employed to maintain productivity levels of Eucalyptus plantations, increasing both economic input and risk of environmental pollution. In this context, intercropped *Eucalyptus grandis* and *Acacia mangium* plantations represent a convenient forest management strategy as it minimizes environmental side-effects especially in sandy soils with low organic matter (OM) levels (Laclau et al., 2008). *A. mangium* (family Fabaceae) forms associations with the diazotrophic bacteria Rhizobia, promoting greater N availability in the soil. Several studies have revealed improvements generated by this association, especially in C and N dynamics, and wood productivity (Bouillet et al., 2008; Laclau et al., 2008; Voigtlaender et al., 2012; Paula et al., 2015; Fonseca et al., 2017).

N inputs promoted by *Acacia* can improve biogeochemical cycles and enhance the ecological intensification of ecosystem services (Bouillet et al., 2008; Laclau et al., 2008; Richards et al., 2010; Forrester et al., 2011; Bini et al., 2013; Rachid et al., 2013). Therefore, a better understanding of changes in soil microbial indicators and C and N dynamics in sandy soils is especially important in an intercropped plantation design, as they may reflect OM quality and soil nutrients availability and reduce fertilizer use (Bouillet et al., 2008; Voigtlaender et al., 2012; Cardoso et al., 2013; Pereira et al., 2017). However, little is known about soil microbial indicators in either pure or intercropped *E. grandis* and *A. mangium* plantations in sandy soils.

Bini et al. (2013) published the first evaluation of microbial attributes in an intercropped system at 2, 7, 14, and 20 months after planting, but results were highly variable across times. Additionally, the authors did not highlight the effects of *A. mangium* on enzyme activity associated with C and N cycling in soil, the potential for degradation of C sources, and the reflexes of these indicators on C and N concentrations in soil OM fractions. Thus, important questions remain unanswered such as the potential effect of introduction of *A. mangium* in an intercropped system on soil microbial attributes and soil OM labile fractions. Investigating those questions is crucial to planning strategies of Eucalyptus plantations and their sustainability in tropical soils.

Here, we evaluate changes in soil microbial quality indicators such as microbial activity and enzymatic and metabolic potential associated with C and N cycles in the soil. Additionally, we characterize soil OM fractions and litter attributes and examine their correlations, in order to further explore the microbial influence on C and N cycling and how it can discriminate among plantation treatments. We hypothesized that intercropping of *A. mangium* in *E. grandis* plantations can promote changes in microbial indicators (microbial,

enzymatic, and metabolic activity) and increase C and N concentrations on labile fractions of soil OM.

Materials and Methods

Experimental design and sampling

Location, climate, and soil classification

The study was carried out at the Itatinga Forest Sciences Experimental Station (23°03′00″S−48°37′00″W; 830 m above sea level), Department of Forest Sciences, Luiz de Queiroz College of Agriculture, University of São Paulo, Itatinga, São Paulo state, Brazil (Figure 1). The climate in this region is Cfa (Köppen classification), with an annual rainfall of 1,350 mm mostly concentrated (75%) between March and October (Laclau et al., 2008).

The soil is a red-yellow Latosol (according to the Brazilian soil classification system) or Ferralsol (FAO/WRB) of medium texture, low cation exchange capacity, and typically dystrophic. A complete soil characterization can be found in Table S1.

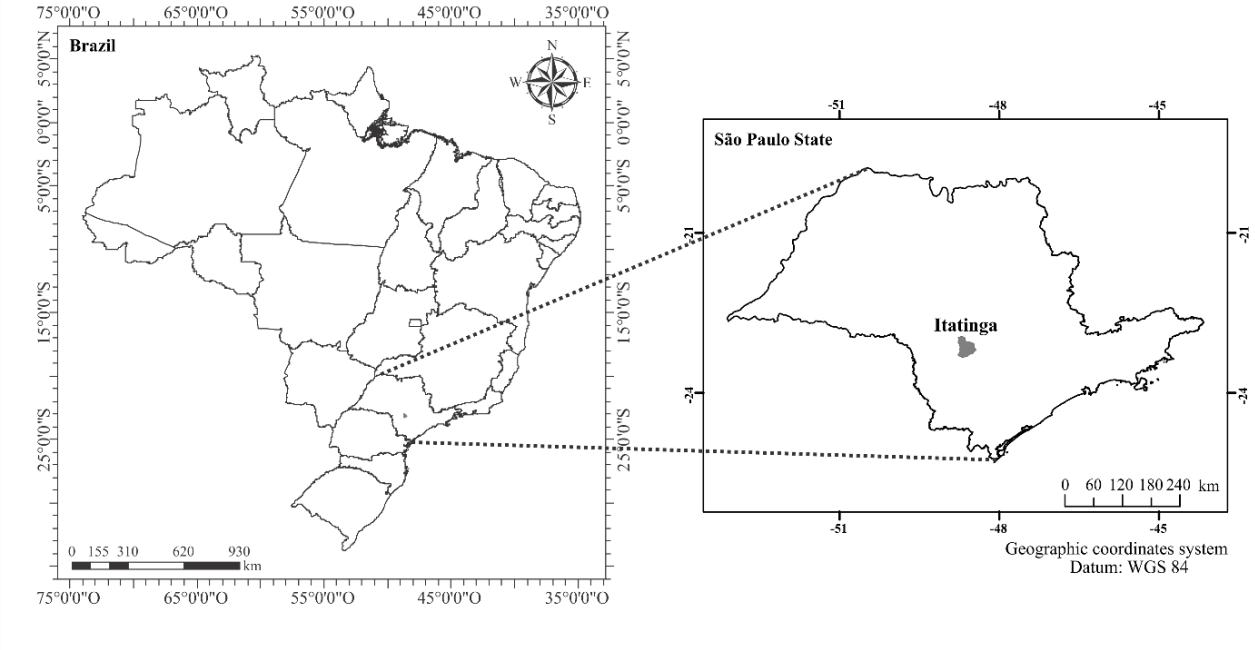

Figure 1. Geographic location of Itatinga municipality, São Paulo state, Brazil.

Soil history, experimental design, planting, and tree fertilization

The experiment was carried out in an area of a first rotation installed in December 2013, which had been previously planted with *E. grandis* (30-50 years) and managed without fertilizer application. After clearing the vegetation, the experiment was implemented as a fully randomized block design with the following four treatments: pure *E. grandis* (E) and *A. mangium* (A) plantations, pure *E. grandis* plantations with N fertilizer (E+N), and *E. grandis* and *A. mangium* intercropped plantations (E+A), with 4 blocks each and totaling 16 plots. Trees were planted in a minimum tillage system with 3 × 3 m spacing (9 m^2 per plant). Total area of plots was 1,296 m2 (36 × 36 m), and useful area eliminating the edge effect was 576 m^2 (24 × 24 m). The intercropped plantation (E+A) was in double rows, with a plant ratio of 1:1. *A. mangium* seedlings were inoculated with *Rhizobium* strains previously selected (Embrapa Agrobiologia) for their high biological N-fixing capacity and high nodulation rates in *Acacia* spp. Nitrogen fertilization in treatment E+N was carried out in December 2013 and 2014, with the application of 10 and 90 kg ha^{-1} of N respectively, using 450 kg ha^{-1} as ammonium sulfate ($(NH_4)_2SO_4$). Other nutrients applied to all treatments are presented in Table S1.

Soil and litter sampling

In March 2016 and 2017, corresponding respectively to the 27th and 39th months of the trees, we collected 120 soil samples (0–20 cm) by drawing a Voronoi polygon, the elementary space defined by the half distances between the sampled tree and its neighbors for each tree (Figure S1; Honda, 1978; Saint-André et al., 2005). We collected samples from six representative trees in each experimental plot, totalizing six subsamples per plot, which were homogenized and mixed into a composite sample (Figure S1). Twelve subsamples were taken from mixed treatments E+A (six trees at the edge of *A. mangium* and six at *E. grandis*), which were homogenized into a composite sample. The sampled litter (including twigs, branches, and leaves) followed the same standardization procedure. However, we used a template (25 × 25 cm) placed on the soil surface and sampled all organic material underneath. As forest rotations in Brazil take about 6 or 7 years, we selected our sampling periods in order to assess differences between the beginning (27 months) and the maximum (39 months) period of litter deposition in the soil, and to contrast possible differences in C and N cycling in the soil.

For microbiological analyses, the soil was sieved to a 2 mm fraction and stored at 4°C. For chemical and physical evaluations, the soil was sieved (2 mm) and air dried for 72 h. Litter was oven-dried at 60°C for 24 h and then ground (1 mm) for chemical analyses.

Analytical Procedures

Microbial Indicators Analyses

Microbial biomass, respiration, and enzymatic activity

Microbial biomass of C (Cmic) and N (Nmic) were estimated by the fumigation-extraction method, using the coefficients *Kc* = 0.40 and *Kn* = 0.54, respectively (Brookes et al., 1985; Vance et al., 1987). Soil respiration (SR) was estimated by quantification of CO_2-C emitted during 28 days of incubation at 28°C (Bonfim et al., 2016). In both tests, soil moisture was maintained at 60% of maximum water retention capacity. The relationship between SR and Cmic was used to calculate the metabolic quotient (qCO_2) (Anderson and Domsch, 1993). In addition, C and N microbial quotients (*q*Mic-C and *q*Mic-N, respectively) were calculated using the relationship between Cmic and total organic carbon (TOC) (Cmic/TOC) and Nmic and total soil N (Nmic/Total-N) (Bini et al., 2014).

The potential of urease (EC 3.5.1.5), L-asparaginase (EC 3.5.1.1), L-glutaminase (EC 3.5.1.2), amidase (EC 3.5.1.4), β-D-glucosidase (EC 3.2.1.21), and dehydrogenase (EC 1.1.1.) activity was determined following Tabatabai (1994). Due to the short time of incubation (1-2 h), toluene was omitted from the analysis (Souza et al., 2016). The six soil enzymes were selected for their participation in C (β-D-glucosidase and dehydrogenase) and N (urease, L-asparaginase, L-glutaminase and amidase) cycling, respectively. All assays were performed in duplicates.

Metabolic profile of the soil microbial community

The potential of soil microbial communities in the degradation of C sources was assessed with Biolog® EcoPlate (Zak et al., 1994). This approach uses colorimetric detection (tetrazolium dye as redox indicator) to measure microbial activity in 31 different C sources, including carbohydrates, polymers, carboxylic acids, amines, amino acids, and miscellaneous compounds. Microbial suspensions were prepared using 5 g of fresh soil from each sample in 45 mL of sterile saline solution (0.85% NaCl) and shaking at 150 rpm during 30 min. Soil suspension was subjected to tenfold dilutions in sterilized saline solution to a final dilution of 10^{-2}. An aliquot (150 µL) was added to each plate well, using one-third of the plate for each composed sample replicate. The plates were incubated at 25°C and inspected every 24 h for 6 days at 590 nm by an ELISA plate reader. The potential of the soil microbial community for degradation of C sources was used to calculate the community niche (CN) index in each treatment. We calculated the CN index by summing over the 31 C sources and the maximal

potential observed for the microbial community in each soil sample and individual C source (Salles et al., 2009).

Soil and litter analyses

To evaluate the influence of microbial indicators on the dynamics of C and N, soil OM was physically fractionated from an air-dried soil mass (20 g) using the granulometric method (Brandani et al., 2017). This process separates organic (OF) (2,000-75 μm), organic-mineral 1 (OMinF1) (2,000-75 μm), organic-mineral 2 (OMinF2) (75-53 μm) and organic-mineral 3 (OMinF3) (<53 μm) fractions. C and N concentrations in each OM fraction were determined by dry combustion with an elemental analyzer (LECO®) (Nelson and Sommers, 1982; Christensen, 2001; Signor et al., 2014). TOC and Total-N contents in soil OM fractions were obtained by multiplying C and N concentrations of each fraction by their corresponding mass (Brandani et al., 2017).

Litter was dried at 60°C for 24 h and ground (1 mm). Total C and N were determined equally by dry combustion in the elemental analyzer (LECO®). In addition, NH^{+}_{4}-N and NO_{3}^{-}-N fractions were extracted by nitric-perchloric digestion and contents were determined using the "Kjeldahl" method (Nelson and Sommers, 1982; Vezzani et al., 2001; Freitas et al., 2013).

Statistical Analyses

The data were tested for homogeneity and normality of variances through Levene and Shapiro–Wilk's tests. The data set was analyzed through one-way ANOVA and means were compared by Tukey's tests at a significance level of 5%. A Canonical Redundancy Analysis (RDA) was performed to identify variables discriminating among treatments (Baretta et al., 2008). In RDA, microbiological indicators (microbial and enzymatic activity) were correlated with soil and litter variables to identify patterns across treatments. In parallel, Monte Carlo test was performed by considering 499 random permutations and displaying "*p*" values and Wilk's lambda (%) of the attributes, resulting in estimates of significance and weight of each correlation (Baretta et al., 2008). Due to collinearity, factors with an inflation factor >20 were removed (Baretta et al., 2008). A heat map and a Principal Coordinates Analysis (PCoA) were performed to characterize the metabolic profile of the soil microbial community, revealing the distribution of consumption across the 31 C sources. An ANOSIM test was applied to identify differences between the treatments showed in PCoA (Ramette, 2007). Univariate analyses and the heat map graphic were prepared using R software (https://www.r-project.org, using

"agricolae," "gplots," and "RColorBrewer" packages), and multivariate analysis was performed on Canoco® software for Windows (v.4.5) and PRIMER-E (Leps and Smilauer, 2003; Baretta et al., 2008).

Results

Microbial biomass (C and N contents), and soil microbial activity

Microbial C content (Cmic) was significantly higher in A and E+A stands and lower in E and E+N stands at 27 months after planting, when mixed stands E+A showed increases of 45 and 70% in soil Cmic compared E+N and E, respectively, and almost similar to A (increase of only 3%) ($p < 0.05$) (Figure 2A). In contrast, at 27 months microbial N content (Nmic) did not differ between E+N, A and E+A stands, but E stands showed the lowest soil Nmic content. E stands also showed the lowest Nmic content at 39 months. In general, soil Nmic contents were highest at 39 months, except in A stands that did not show significant differences at any period. Increases in soil Nmic contents between 27 and 39 months after planting in E, E+N, and E+A stands were, respectively 68, 92, and 51% ($p < 0.05$; Figure 2B).

Soil CO_2-C emission rates did not vary between treatments at 27 months, with a mean value of 22 mg CO_2-C kg^{-1} day^{-1}. At 39 months CO_2-C emissions had significantly increased in all treatments, especially in the E+N treatment where rates more than doubled (50.5 mg CO_2-C kg^{-1} day^{-1}) ($p < 0.05$; Figure 2C). Metabolic quotient (qCO_2) values increased with time in all treatments ($p < 0.05$) (Figure 2D). There was a non-significant trend toward higher values between 27 and 39 months in E and E+N stands.

The C microbial index (*q*Mic-C) was significantly higher at 27 months for treatments A and E+A, but not for E stands. The N microbial index (*q*Mic-N) at 27 months was higher in the E and A treatments ($p < 0.05$; Figure 2F). N fertilization in Eucalyptus (E+N) decreased *q*Mic-N to half the value of E stands at 27 months after planting. At 39 months, *q*Mic-N was significantly higher for all treatments except A ($p < 0.05$; Figure 2F).

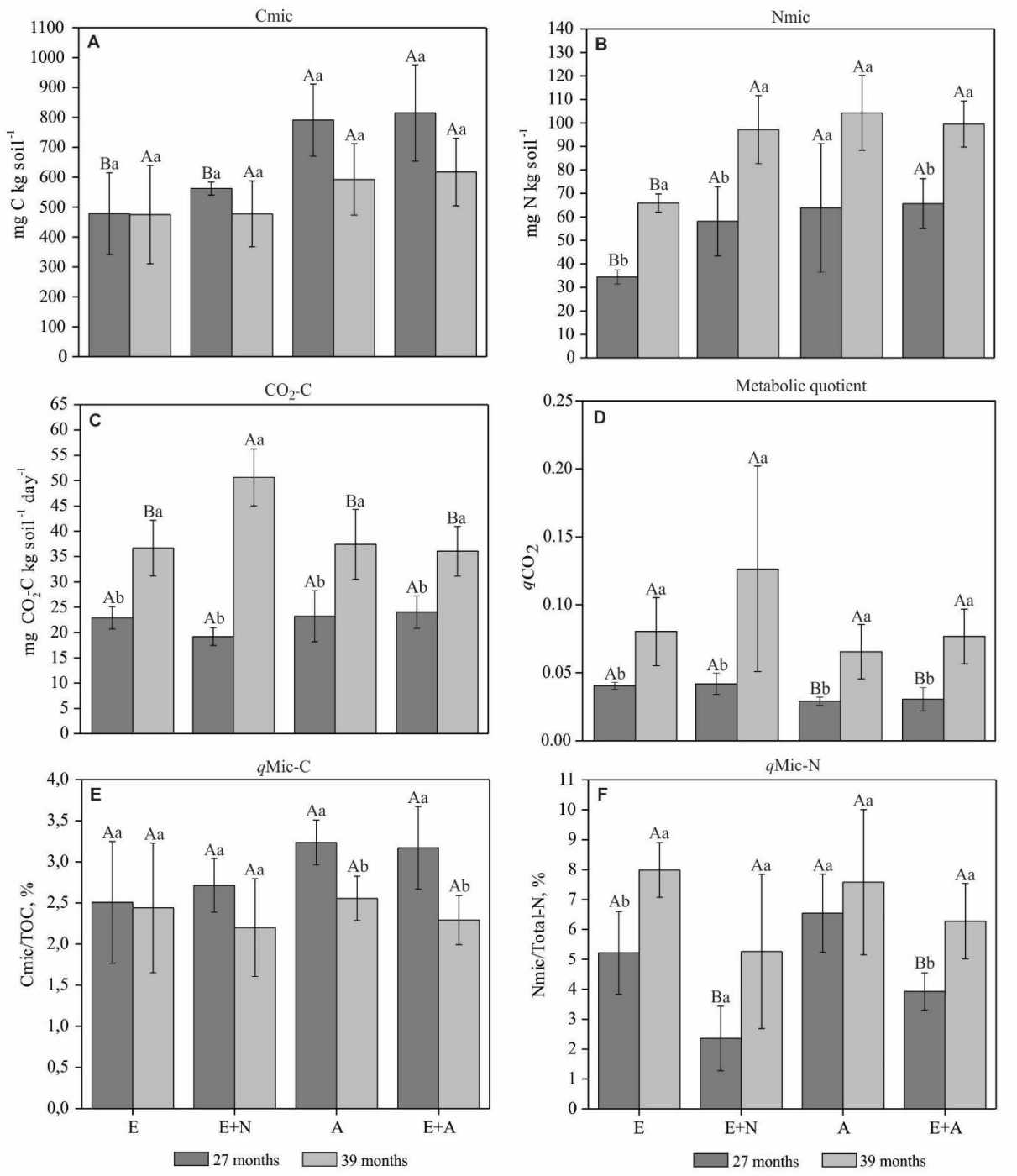

Figure 2. Biological soil indicators in pure and mixed *E. grandis* and *A. mangium* plantations. (E) *E. grandis*, (E+N) *E. grandis* with N fertilization, (A) *A. mangium*, and (E+A) mixed plantation of *E. grandis* and *A. mangium* at 27 and 39 months after planting. (A) Cmic: microbial C; (B) Nmic: microbial N; (C) CO_2-C: Soil Respiration; (D) qCO_2: Metabolic quotient; (E) *q*Mic-C: Microbial C quotient, and (F) *q*Mic-N: Microbial N quotient. Means followed by the same letter do not differ by Tukey's test at a significance level of 5%. Upper case letters compare treatments within each period and lower case letters compare periods within each treatment. Error bars indicate standard deviation; $n = 4$.

Potential activity of soil enzymes

The potential activity of urease was significantly higher in E and E+N treatments in both periods. However, the potential of urease activity was 84, 62, 65, and 84% higher at 39 months in E, E+N, A, and E+A, respectively, compared to 27 months ($p < 0.05$; Figure 3A). There were no significant differences in L-asparaginase activity across treatments at 27 months, with a mean value of 13.8 μg NH_4^+-N g^{-1} $2h^{-1}$. However, the L-asparaginase potential activity was generally higher at 39 months, especially in the E+N and A treatments. Thus, the L-asparaginase potential activity increased by 19, 63, 76, and 33%, respectively in E, E+N, A, and E+A treatments at 39 months ($p < 0.05$; Figure 3B). L-glutaminase activity did not differ among treatments at 27 months after planting. However, there was higher L-glutaminase activity at 39 months, with an increase of ~228% in comparison to 27 months ($p < 0.05$; Figure 3C). The pattern of amidase potential activity was similar to that of urease, with higher activity in E and E+N treatments and lower in A and E+A in both periods. Nevertheless, enzyme activity was 57, 79, 130, and 14% higher in the E, E+N, A, and E+A treatments, respectively at 39 months compared to 27 months ($p < 0.05$; Figure 3D). β-glucosidase activity did not differ among treatments in either period, with mean values of 85.1 and 73.58 mg PNF kg^{-1} h^{-1}, respectively. However, when comparing the same treatment between periods, A showed decreased activity in β-glucosidase activity ($p < 0.05$; Figure 3E). Dehydrogenase activity was higher in E and E+N stands, especially at 27 months. At 39 months, there were also differences in the dehydrogenase activity potential (E > E+N > A = E+A; Figure 3F).

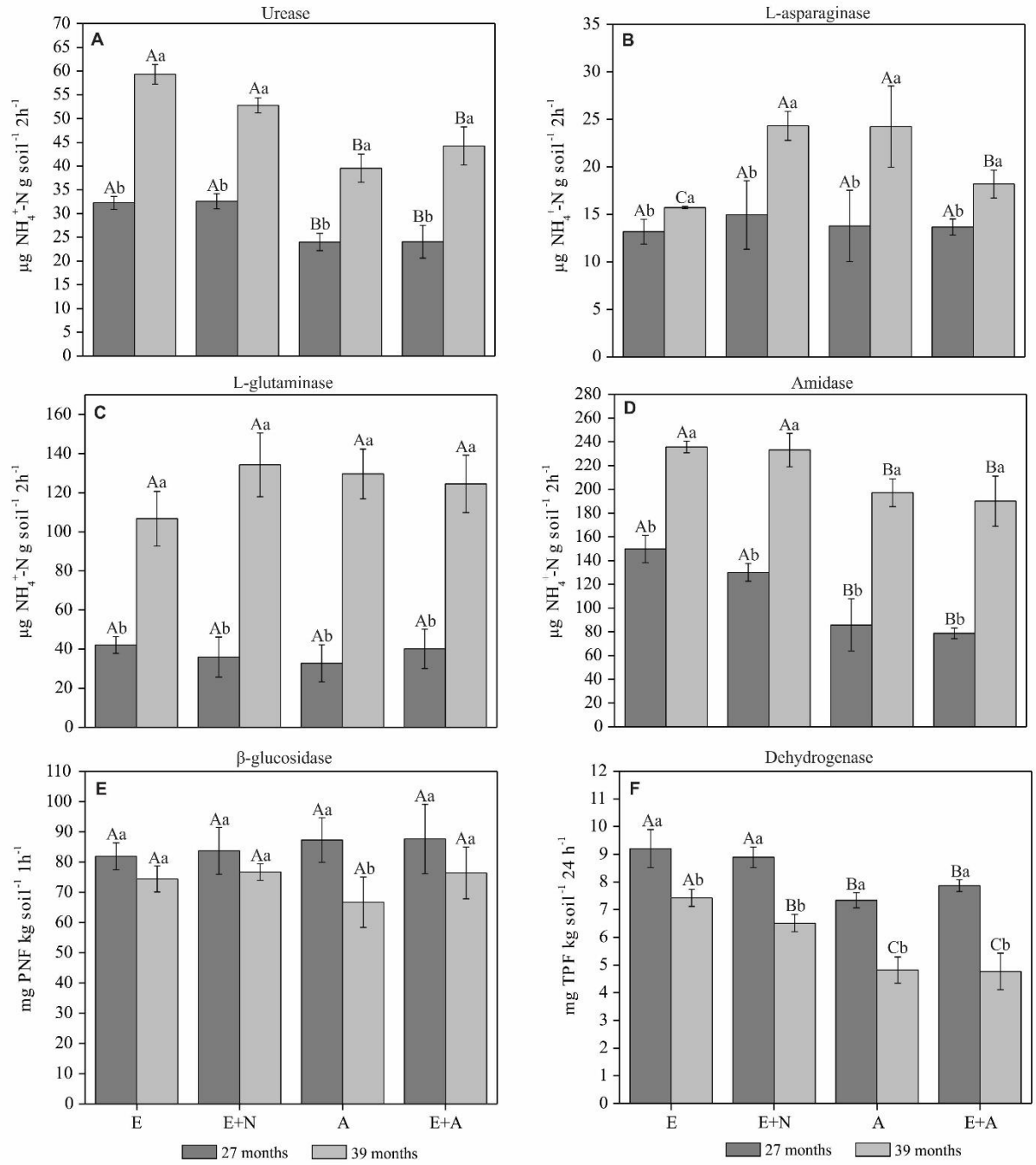

Figure 3. Potential enzymatic soil activity in pure and mixed *E. grandis* and *A. mangium* plantations. (E) *E. grandis*, (E+N) *E. grandis* with N fertilization, (A) *A. mangium*, and (E+A) mixed plantation of *E. grandis* and *A. mangium* at 27 and 39 months after planting. (A) Urease; (B) L-asparaginase; (C) L-glutaminase; (D) Amidase; (E) β-glucosidase, and (F) Dehydrogenase. Means followed by the same letter do not differ by Tukey's test at a significance level of 5%. Upper case letters compared treatments within each period and lower-case letter compared periods within each treatment. Error bars indicate standard deviation; $n = 4$.

Microbial metabolic profile

At 27 and 39 months after tree planting, there were no significant differences between treatments regarding the potential of the 31 C sources degradation (Table S2 and Figures 4A, B). However, we verified significant differences when comparing periods, mainly in A (16 sources) and E+A (5 sources; Table S2 and Figure 4). The PCoA analysis and the ANOSIM test did not show significant differences between the treatments within the same period (Figures S2 A,B). However, was detected significant separation between 27 and 39 months after planting, only for the E+A treatment (R = 0.795, $p < 0.0247$) (Figure S2C). The community niche index (CN) did not show significant differences between treatments, although it showed a slight increase in A and E+A at 27 months. However, there was a significant effect between the sampling times, where treatments A and E+A showed a reduction in the CN index at the 39 months ($p < 0.05$; Figure S4).

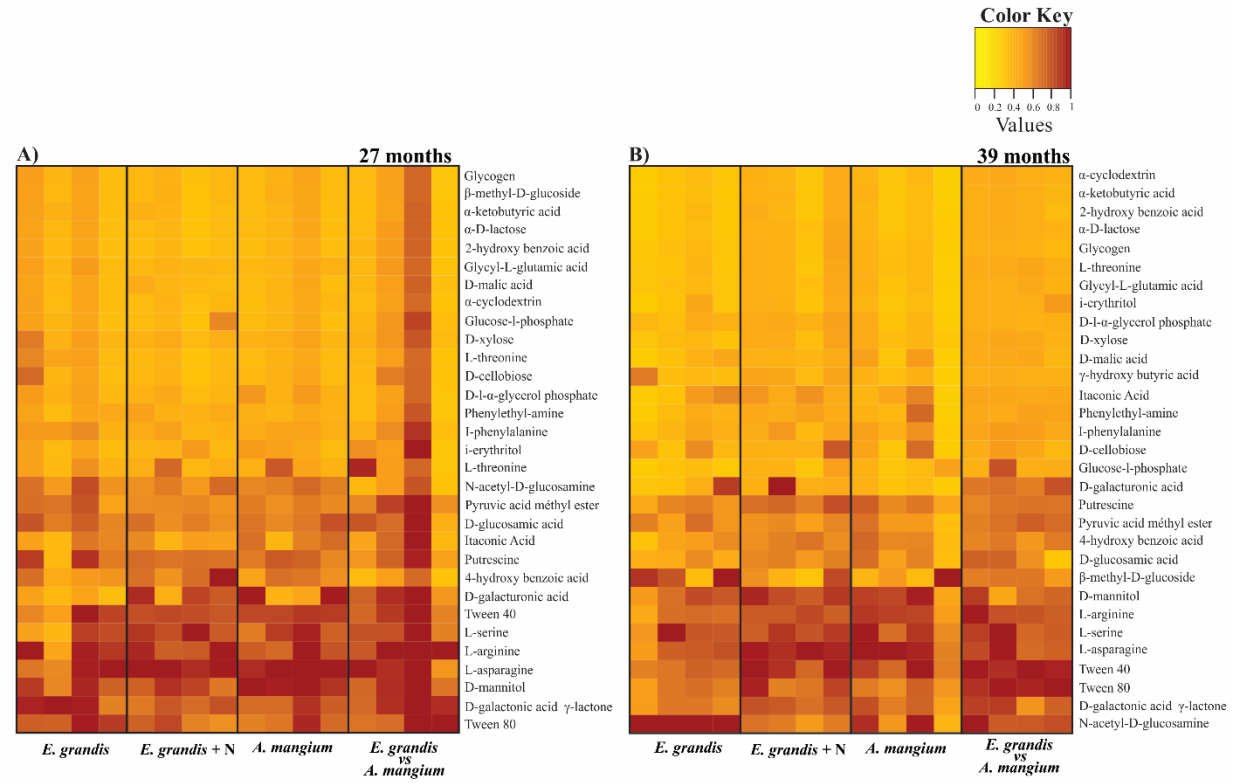

Figure 4. Metabolic profile showed the potential of 31 C sources degradation assessed by the Biolog EcoPlates in pure and mixed *E. grandis* and *A. mangium* plantations at (A) 27 and (B) 39 months after planting the trees. The score in a heat map analysis represents the difference between the consumption of each sample for the same substrate. The highest consumption is identified by a red color and the lowest consumption by a yellow color.

Organic matter fractions and litter attributes

Organic fraction (OF) presented double the mass in *A. mangium* (A) and mixed stands (E+A) when compared to other treatments, especially at 39 months ($p < 0.05$; Table 1). Most of the initial soil mass analyzed (20 g) was found in the OMinF2 fraction (organo-mineral fraction 75-53 μm), which ranged from 63 to 70% and did not present significant statistical differences across treatments ($p < 0.05$; Table 1).

Table 1. Physical characterization and C-N concentrations of soil organic matter (0–20 cm) in (E) *E. grandis*, (E+N) *E. grandis* with N addition, (A) *A. mangium*, and (E+A) mixed plantation between *E. grandis* and *A. mangium* at 27 and 39 months after planting.

	E		E+N		A		E+A	
	27 months	39 months	27 months	39 months	27 months	39 months	27 months	39months
OM FRACTIONS MASS (g kg^{-1})								
OF	12.7±1.8 ^{Ba}	14.93±3.4 ^{Ba}	12.91±1.2 ^{Bb}	18.47±0.6 ^{Ba}	25.44±2.1 ^{Ab}	29.55±0.35 ^{Aa}	25.6±2.1 ^{Ab}	30±0.84 ^{Aa}
OMinF1	12.48±20 ^{ns}	12.42±2.69 ^{ns}	13.89±13.6 ^{ns}	10.0±2.19 ^{ns}	13.31±1.78 ^{ns}	10.09±0.46 ^{ns}	12.5±2.4 ^{ns}	10.4±1.30 ^{ns}
OMinF2	700±55.8 ^{ns}	678.2±59.6 ^{ns}	601.1±53.3 ^{ns}	660.2±66.1 ^{ns}	662.8±20.6 ^{ns}	697.59±52.4 ^{ns}	679.1±92.2 ^{ns}	668.7±66.3 ^{ns}
OMinF3	268.1±46.8 ^{ns}	278.94±55.3 ^{ns}	327.3±61.2 ^{ns}	285±44 ^{ns}	288.2±43.6 ^{ns}	223.6±51.8 ^{ns}	286±56.3 ^{ns}	266.6±68.76 ^{ns}
Mean recovery (%)	97	98	97	97	97	96	97	98
N-CONCENTRATIONS (g kg^{-1})								
N-OF	0.16±0.02 ^{Ba}	0.20±0.05 ^{Ba}	0.17±0.01 ^{Ba}	0.22±0.04 ^{Ba}	0.35±0.04 ^{Aa}	0.35±0.05 ^{Aa}	0.34±0.05 ^{Aa}	0.36±0.06 ^{Aa}
N-OMinF1	0.01±0.004 ^{ns}	0.01±0.008 ^{ns}	0.01±0.003 ^{ns}	0.01±0.01 ^{ns}	0.02±0.01 ^{ns}	0.01±0.003 ^{ns}	0.02±0.01 ^{ns}	0.02±0.006 ^{ns}
N-OMinF2	1.51±0.37 ^{Aa}	0.92±0.69 ^{Aa}	1.25±0.16 ^{Aa}	0.91±0.79 ^{Aa}	1.41±0.21 ^{Aa}	0.80±0.64 ^{Aa}	1.38±0.30 ^{Aa}	1.40±0.23 ^{Aa}
N-OMinF3	0.15±0.02 ^{ns}	0.16±0.05 ^{ns}	0.16±0.02 ^{ns}	0.15±0.03 ^{ns}	0.16±0.02 ^{ns}	0.09±0.01 ^{ns}	0.14±0.07 ^{ns}	0.14±0.04 ^{ns}
Total-N	1.85^{Aa}	1.31^{Bb}	1.60^{Ba}	1.31^{Bb}	1.95^{Aa}	1.27^{Bb}	1.89^{Aa}	1.93^{Aa}
C-CONCENTRATIONS (g kg^{-1})								
C-OF	4.82±0.75 ^{Ba}	6.0±1.54 ^{Ba}	5.27±0.45 ^{Ba}	6.60±0.96 ^{Ba}	10.2±1.29 ^{Aa}	9.80±1.49 ^{Aa}	9.97±1.16 ^{Aa}	10.44±1.98 ^{Aa}
C-OMinF1	0.71±0.11 ^{ns}	0.84±0.32 ^{ns}	0.79±0.19 ^{ns}	0.78±0.35 ^{ns}	0.90±0.34 ^{ns}	0.57±0.07 ^{ns}	0.84±0.30 ^{ns}	0.75±0.16 ^{ns}
C-OMinF2	35.3±10.72 ^{ns}	20.05±17.10 ^{ns}	28.91±3.16 ^{ns}	20.18±17.9 ^{ns}	32.77±2.30 ^{ns}	18.02±15.01 ^{ns}	32.24±10.7 ^{ns}	32.27±5.87 ^{ns}
C-OMinF3	0.87±0.65 ^{ns}	0.64±0.12 ^{ns}	0.76±0.39 ^{ns}	0.85±0.16 ^{ns}	0.83±0.23 ^{ns}	0.66±0.32 ^{ns}	0.70±0.26 ^{ns}	0.67±0.14 ^{ns}
TOC	41.71^{Aa}	27.54^{Bb}	35.75^{Ba}	28.43^{Bb}	44.7^{Aa}	29.07^{Bb}	43.77^{Aa}	44.15^{Aa}

Means followed by the same letter do not differ by Tukey test at a significance level of 5%. Capital letters, in the line, compared treatments within each period and lower-case letter, in the line, compared periods within each treatment. Mean Standard Deviation (±); n=4. ns, not significant; OM, Organic Matter; N, Nitrogen; C, Carbon; OF, OrganicFraction (2,000–75μm); OMinF1, Organic Mineral Fraction 1 (2,000–75μm); OMinF2, Organic Mineral Fraction 2 (75–53μm); OMinF3, Organic Mineral Fraction 3 (<53μm); TOC, Total Organic Carbon.

The A and E+A treatments exhibited higher N contents in the OF (2,000-75 μm), than in E and E+N stands, even though the N fertilizer dose in the E+N treatment was 100 kg ha^{-1} of N. Mixed plantations (E+A), when compared to pure plantations E and E+N, showed increases of 45 and 50% (at 27 months) and 55 and 61% (at 39 months) in N contents in the OF respectively ($p < 0.05$). Total-N concentration (Total-N) did not differ at 27 months except for the E+N treatment, which presented the lowest value. On the other hand, at 39 months the E+A treatment showed the highest content of Total-N. Total-N concentrations were always higher at 27 months except for E+A treatment, pointing to a sampling period effect.

Higher C contents were found in A and E+A stands in OF. The mixed stand E+A showed increases of 48 and 53% (at 27 months), and 57 and 63% (at 39 months) of C in OF,

when compared to the E and E+N treatments respectively ($p < 0.05$). Total soil C (TOC) did not differ at 27 months except for the E+N treatment that showed the lowest value. The highest concentrations of TOC were detected at 27 months, except for the E+A treatment that did not show differences between sampling periods, although it presented higher soil TOC at 39 months.

Total-N contents in litter were significantly higher in the A stand, especially at 39 months after tree planting. E+A stands displayed lower Total-N content than A stands. However, A stands showed higher contents ($p < 0.05$) than E and E+N stand (especially at 39 months). A and E+A stands exhibited the lowest litter C/N ratios, with the smallest values in the A stand at 39 months ($p < 0.05$) (Table 2). Litter NH_4^+-N was higher in A and E+A, followed by E and E+N stands, which in general exhibited highest values at 27 months.

Table 2. Litter chemical characterization in (E) *E. grandis*, (E+N) *E. grandis* with N fertilization, (A) *A. mangium*, and (E+A) mixed plantation between *E. grandis* and *A. mangium* at 27 and 39 months after planting.

	E		E+N		A		E+A	
	27 months	39 months	27 months	39 months	27 months	39 months	27 months	39months
Total-C (g kg^{-1})	521±15 ^{Aa}	518±7 ^{Aa}	526±6.5 ^{Aa}	519±20 ^{Aa}	514±9 ^{Aa}	517±8 ^{Aa}	527±9 ^{Aa}	519±3.5 ^{Aa}
Total-N (g kg^{-1})	8.4±0.5 ^{Ca}	9.1±0.3 ^{Ca}	8.5±0.7 ^{Cb}	10.3±0.4 ^{Ca}	14±0.4 ^{Ab}	16.6±1.2 ^{Aa}	11.5±0.1 ^{Bb}	13.7±1.2 ^{Ba}
C/N ratio	62±4.5 ^{Aa}	58±4.83 ^{Aa}	62±5.5 ^{Aa}	51±1.2 ^{Bb}	37±1.8 ^{Ca}	31±2.0 ^{Cb}	46±1, 1 ^{Ba}	39±3.4 ^{Bb}
NH_4^+-N (mg kg^{-1})	95.2±4.3 ^{Ba}	53.2±14.5 ^{Ab}	60±4.1 ^{Ca}	36.2±2.4 ^{Ab}	145.6±6 ^{Aa}	22.1±5 ^{Bb}	149±3.3 ^{Aa}	22.4±6.7 ^{Bb}
NO_3^--N (mg kg^{-1})	40±7 ^{ns}	95.2±26 ^{ns}	42±3 ^{ns}	86.1±27 ^{ns}	56.3±3.2 ^{ns}	36.8±5.9 ^{ns}	69±3.9 ^{ns}	17.9±6.7 ^{ns}

Means followed by the same letter do not differ by Tukey test at a significance level of 5%. Capital letters, in the line, compared treatments within each period and lower-case letter, in the line, compare periods within each treatment. Mean Standard Deviation (±); n=4. Total-C, Total Carbon; Total-N, Total Nitrogen; C/N, relationship between C and N concentrations; NH_4^+-N, ammonium ion; NO_3^-N, nitrate ion.

Global results by Redundancy Analysis (RDA)

At 27 months, RDA revealed a strong differentiation between *Acacia* (A and E+A) and *Eucalyptus* treatments (E and E+N). The first two canonical axes are responsible for 56.2% of variance (33.9% by axis 1 and 22.3% by axis 2). The vectors that discriminated *A. mangium* (A) and the mixed system (E+A) from the other treatments were the microbial indicators Cmic ($\lambda = 5\%$, $p < 0.0039$), CO_2-C ($\lambda = 2\%$, $p < 0.0039$) and *q*Mic-C ($\lambda = 1\%$, $p < 0.0410$). They had a strong effect (positive correlation) on soil and litter attributes including C-OF ($\lambda = 14\%$, $p < 0.0017$), N-OF ($\lambda = 10\%$, $p < 0.0018$), N-OMinF1 ($\lambda = 9\%$, $p < 0.0010$), TOC ($\lambda = 2\%$, $p < 0.0280$), Total-N (Litter) ($\lambda = 17\%$, $p < 0.0019$), and NH_4^+-N (Litter) ($\lambda = 10\%$, $p < 0.0011$). On the other hand, the attributes that most contributed to the differentiation of *E. grandis* treatments (E and E+N), were the microbial indicators *q*CO_2 ($\lambda = 2\%$, $p <$

0.0441), amidase (λ = 9%, p < 0.0019) and urease (λ = 1%, p < 0.0501), which were positively correlated with litter C/N ratio (λ = 3%, p < 0.001) and dehydrogenase (λ = 7% p < 0.0033) (Table S3 and Figure 5A).

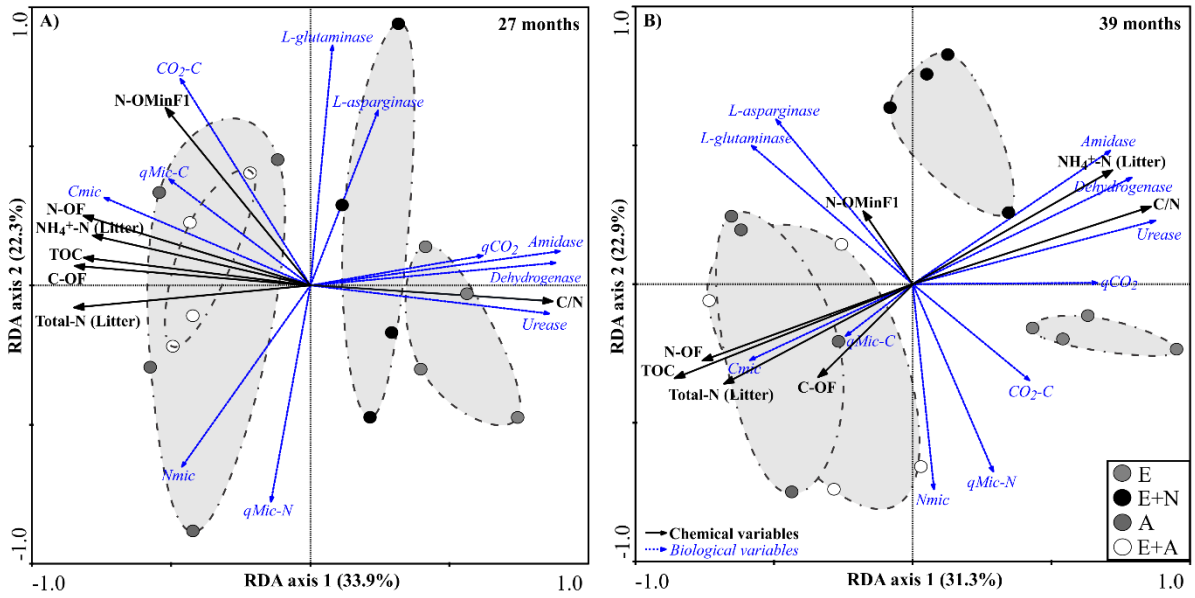

Figure 5. Redundancy analysis (RDA) of soil-litter attributes and soil microbial attributes based on Monte Carlo permutation test (499 permutations). (E) *E. grandis*, (E+N) *E. grandis* with N addition, (A) *A. mangium*, and (E+A) mixed plantation of *E. grandis* and *A. mangium*. Black arrows indicate soil and litter attributes, and blue arrows indicate microbiological attributes. Only significant correlations were fitted in the ordination (p < 0.0067). (A) 27 months, (B) 39 months; n = 4.

At 39 months, the first two canonical axes accounted for 64.2% of the variation in data, with 31.3 and 32.9% deriving from axes 1 and 2, respectively. The overall pattern was similar to that at 27 months, with a strong separation between the treatments with *A. mangium* (A and E+A) and with *Eucalyptus* (E and E+N), but with different factors and weights. The vectors to differentiate the *A. mangium* (A) and the mixed plantation (E+A) from the others were the microbial indicators Cmic (λ = 3%, p < 0.0490), Nmic (λ = 2%, p < 0.0478), and *q*Mic-C (λ = 1%, p < 0.0579), which had a marked influence (positive correlation) on soil and litter attributes. Among such vectors we highlight N-OF (λ = 19%, p < 0.0027), C-OF (λ = 7%, p < 0.0201), TOC (λ = 4%, p < 0.0309), and Total-N (Litter) (λ = 2%, p < 0.0478). On the other hand, the attributes that contributed most to the differentiation between E and E+N

on one side, and A and E+A treatments on the other, were the microbial indicators qCO_2 (λ = 16%, $p < 0.0028$), amidase (λ = 9%, $p < 0.0147$), dehydrogenase (λ = 9%, $p < 0.0147$), and urease (λ = 2%, $p < 0.0489$), which presented a positive correlation with litter attributes such as NH_4^+-N (λ = 9%, $p < 0.0027$) and C/N ratio (λ = 1%, $p < 0.0501$) (Table S3 and Figure 5B).

Discussion

Our results demonstrate that soil microbial indicators associated with C and N cycles showed significant changed in pure and intercropped treatments of *A. mangium* in the first rotation of plantations, even in the juvenile phase (27 and 39 months). Overall, our experiments demonstrate an improvement in soil fertility, confirming our hypothesis that introduction of *A. mangium* in an intercropped Eucalyptus forest plantation promotes changes in microbial indicators (microbial, enzymatic, and metabolic activity) and increases in C and N concentrations in soil OM labile fractions.

Intercropped systems exhibit increased C and N microbial contents

The soil microbial community is highly sensitive to changes in management practices and therefore represents a good indicator of soil quality (Cardoso et al., 2013). The highest concentrations of C and N microbial content in the A and E+A (Figures 2 A,B) suggest a high efficiency of microbial biomass in incorporating C and N, and improved OM cycling in mixed systems with *A. mangium* from 27 months after planting. This phenomenon may be due to *A. mangium* systems depositing N-rich sources (low C/N ratio), which can accelerate the microbial attacks, enhancing labile nutrients amounts in the soil (Bini et al., 2013). Such response is relevant under sandy soil conditions, since microbial biomass (MB) represents the most labile fraction of soil OM, acting directly on the availability of nutrients important during the initial development of plants, thereby reducing the need for mineral fertilizers. In a field experiment with a similar experimental design to ours, with *E. grandis* and *A. mangium* plantations, Bini et al. (2013) found that MB operates as a C drain until 14 months after planting. However, there was a high C cycling rate at 20 months, thus acting as a source of C in the soil. Their results suggest that the presence of *A. mangium* provides better conditions for increases in MB, a conclusion confirmed by our study. However, they did not address other key periods of forest development such as the transitional period from biochemical to

biogeochemical cycling (27 and 39 months, respectively). Moreover, their study also failed to investigate the effect of changes in soil OM fractions caused by the insertion of *A. mangium*.

Dynamics of microbial and enzymes associated with C and N cycling in soil

Enzymes act in the soil by degrading OM, promoting the mineralization of nutrients through hydrolytic and oxidative reactions, and their potential regulated among other factors by the nutritional conditions in the soil (Mendes et al., 2013). The higher potential activity of urease and amidase we identified in E and E+N treatments (Figures 3 A,D) may be related to a low litter chemical quality (high C/N ratio; Table 2) and resulting increase in enzymatic production to promote material cycling. On the other hand, the low litter C/N ratio leads to a higher presence of easily hydrolysable N substrates and reduces the requirement of specific enzymes that make them available in the soil (Tabatabai, 1994), as observed in *A. mangium* treatments. It is possible that the urease and amidase enzymes play a key role in the availability of N in plantations that deposit a nutrient-poor litter, such as *E. grandis* pure forests. However, this mechanism requires further investigations into the status of N mineralization rates, since a low correlation was detected between the potential activity of these enzymes and N fractions in the soil. Additionally, the positive correlation between levels of qCO_2 and dehydrogenase activity suggests stressful conditions (Figure 5), resulting in lower effectiveness of the soil microbial community at storing organic C on microbial biomass, and in higher qCO_2. In this case, high dehydrogenase activity in E and E+N stands may be indicative of a more intense electron flow due to respiration under adverse conditions, in agreement with a higher qCO_2 index (Casida et al., 1964; Cardoso et al., 2013). This would imply that the dynamics of nutrient cycling in pure *Eucalyptus* systems is considerably different from when *Acacia* is present.

A. mangium **increases C and N concentrations in the soil organic fraction**

Treatments A and E+A are associated with changes in soil OM fractions even in the short run (27 and 39 months after planting). Organic matter has a key effect on physical, chemical and biological soil attributes, especially in sandy soils where it can improve soil structure, cation exchange capacity, and nutrient availability (Blanchart et al., 2005; Laclau et al., 2010). Therefore, a better understand of main microbial indicators and their correlation with C and N dynamics in soil may allow a more precise forecast of the effects of forest management on soil functions (Bini et al., 2014).

The highest contents of organic fraction (OF) in *A. mangium* stands (A and A+E) suggested a potential longer maintenance of *Acacia* residues in the labile fraction of soil OM when compared to *Eucalyptus* treatments. Bachega et al. (2016) tested the "Home Field Advantage" hypothesis and examined the effect of litter chemistry on the decomposition of leaves and fine roots of *A. mangium* and *E. grandis* but did not examine the effects of soil microbial community and their correlation with soil OM fractions. However, they showed that decomposition rates were slower in *A. mangium* litter than in Eucalyptus, even though initial N and P concentrations were higher in *A. mangium* residues. This demonstrates that litter decomposition depends partially on the C quality of the litter, primarily in terms of water-soluble compounds and lignin content. In addition, the organic fraction (OF) presents a chemical composition comparable to that of plant material, which may be quickly affected by fluctuations in the quality and quantity of organic inputs due to land-use changes (Koutika et al., 2001), alterations in the management practices (Brandani et al., 2017), or longer periods in forest systems (Epron et al., 2015). Therefore, they are used as quality indicators of soil because they can quickly change the microbial community.

The significant increase in N and C contents in the organic fraction (OF) in the A and E+A treatments is possibly related to the N fixation process performed by diazotrophic bacteria associated with the roots of *A. mangium* (Bouillet et al., 2008). This is corroborated by the higher N content in the litter and higher N mineralization potential (NH_4^+-N) (Voigtlaender et al., 2012). *A. mangium* is a nitrogen-fixing tree recognized for improving soil nutrient status, mostly due to its N-enriched litter (Voigtlaender et al., 2012; Epron et al., 2016; Koutika and Mareschal, 2017). Additionally, the higher N input promoted by N-fixing tree species such as *A. mangium* is directly related to the accumulation of C in the soil, mainly because they exhibit significant increases in the volume of deposited litter and fine roots produced (Binkley, 2005). Thus, the positive correlation between C and N in soil and litter, in both seasons, and in treatments A and E+A (Figures 5A, B), indicates that the higher levels of C (especially in the organic fraction of OM) are associated with a higher N concentration in these treatments.

The treatment with N addition (E+N) promoted an increase in the content of microbial N (Nmic) in both sampling times (Figure 2) but did not necessarily influence the Total-N content of the soil (Table 1). This result demonstrates that the application of soluble nutrient sources can influence soil attributes superficially, without reflecting nutrient accumulation at least in the short term. The same applies to accumulation of C in soil, where the E+N treatment does not reflect the increase of total C. Binkley (2005) points out that the increase

of C in the soil by means of mixed systems is not mediated by a single and strictly chemical process, since the addition of N via fertilization may inhibit the enzymatic functions of the microbial community, especially those associated with lignin degradation (Binkley et al., 2004).

Soil microbial indicators are modulated by climatic conditions and plant age

The study of microbial indicators and associated metabolic process under field conditions is a huge challenge, due to the dynamics of the system in the edaphoclimatic changes along a forest rotation. For example, the reduction in microbial C and N contents, *q*Mic-C index, as well as the potential activity of β-glucosidase (A treatment) and dehydrogenase enzymes at 39 months may have been influenced by soil water stress, since a water deficit of -3 mm was observed in this period (Figure S3). The resulting increases in CO_2-C emissions, as well as a higher qCO_2 index at 39 months, may have stressed the microbial community and reduced its efficiency at this sampling time. The reduction in the community niche (CN) index in treatments A and E+A at 39 months may have occurred for the same reason. The larger the community niche, the more efficiently a community can exploit available resources (Salles et al., 2009). Thus, in regions with prolonged water deficit throughout the year, mixed plantation systems of *E. grandis* and *A. mangium* can present low CN index and inefficiently exploit available soil resources, paralyzing or reducing the dynamics of C and N in the system.

In addition to water deficit, another relevant factor behind the variation among treatments was forest age. At 39 months, plantations generally reach the maximum of litter deposit and increase nutrient cycling in soil (Laclau et al., 2010), which may justify the significant increase in microbial attributes at this stage. In addition, the high values of potential activity of amidohydrolases enzymes at 39 months may also be associated, with the greater contribution of fresh litter to the soil. This may temporarily decrease the efficiency of C use in the soil due to the scarcity of N as energy sources to the microbiota, promoting a stimulus to the activity of these enzymes, degradation of newly deposited substrates (López et al., 2012), and greater immobilization of N in the microbial biomass during this period (Figure 2B). Also, degrading enzymes such as amidohydrolases are strongly regulated by substrate availability (Sinsabaugh, 2010).

It is worth noting that mixed treatment (E+A) showed no differences in total C and N concentrations over time (Table 1). Nonetheless, it is possible that organomineral fractions, such as N-OMinF2 and F3, as well as C-OMin-F2, have increased over time (Table 1). This

means that part of the organic fraction of OM can be converted into more stable fractions of OM in the mixed plantations, slowing down microbial attacks and generating negative loads responsible for increased water retention and nutrient availability in sandy soils (Neumann et al., 2014).

Conclusions

The use of nitrogen-fixing trees such as *A. mangium* in mixed plantation systems with *E. grandis* promoted significant changes in microbial attributes and had a strong effect on C and N dynamics in the soil, even in juvenile stages of growth (27 and 39 months). The higher concentrations of C and N in the microbial biomass demonstrate that mixed plantations promote a more efficient use of those elements by microbial communities. Moreover, microbial changes influenced soil OM fractions, especially the more labile organic fraction (OF, 2,000-75 μm) that significantly increased in the mixed plantations, thereby confirming our hypotheses. In this sense, the higher concentrations of C and N in the organic fraction of mixed plantations indicates this type of planting increases nutrient availability in soils with low levels of OM such as Latosols, commonly found in tropical regions. Eucalyptus monocultures, either N-fertilized or not, present litters with high C/N ratio, resulting in higher values of metabolic quotient and dehydrogenase enzyme in the soil, which provided strong evidence for the presence of a microbial biomass with low resource efficiency. Thus, future research is important to characterize the role of the soil microbiome in areas with pure and mixed plantations under different soil and climate conditions. This type of initiative will be necessary to reduce (partially or completely) the mineral fertilizers use in Eucalyptus plantations, so that mixed plantations may become a more generalized practice, for being more economical and sustainable.

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Supplementary Material

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Acacia Changes Microbial Indicators and Increases C and N in Soil Organic Fractions in Intercropped *Eucalyptus* Plantations

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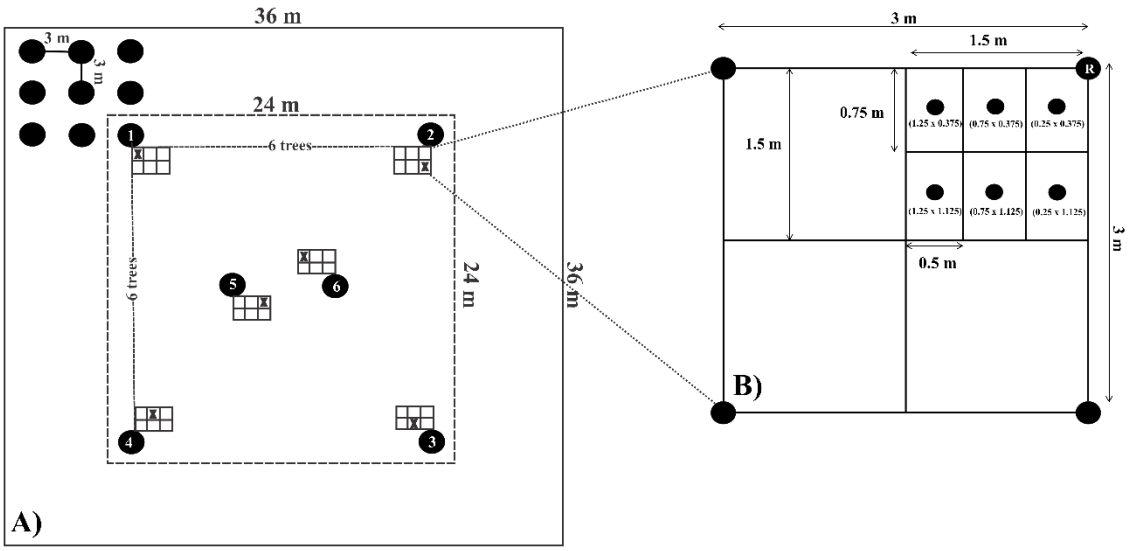
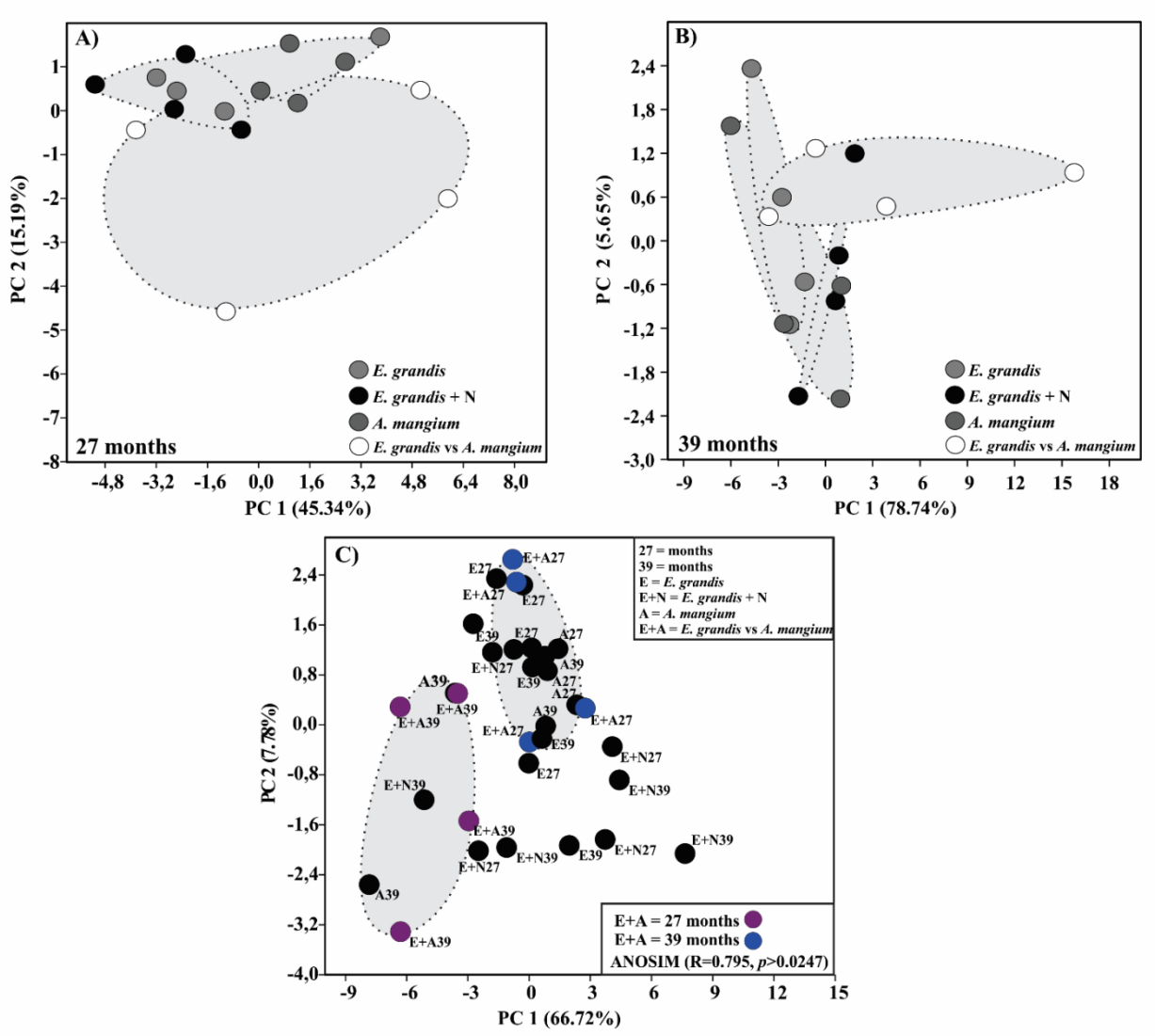
Intercropping forest plantations of *Eucalyptus* with nitrogen-fixing trees can increase soil N inputs and stimulate soil organic matter (OM) cycling. However, microbial indicators and their correlation in specific fractions of soil OM are unclear in the tropical sandy soils. Here, we examined the microbial indicators associated with C and N in the soil resulting from pure and intercropped *Eucalyptus grandis* and *Acacia mangium* plantations. We hypothesized that introduction of *A. mangium* in a *Eucalyptus* plantation promotes changes in microbial indicators and increases C and N concentrations on labile fractions of the soil OM, when compared to pure eucalyptus plantations. We determined the microbial and enzymatic activity, and the potential for C degradation by the soil microbial community. Additionally, we evaluated soil OM fractions and litter parameters. Soil (0–20 cm) and litter samples were collected at 27 and 39 months after planting from the following treatments: pure *E. grandis* (E) and *A. mangium* (A) plantations, pure *E. grandis* plantations with N fertilizer (E+N) and an *E. grandis,* and *A. mangium* intercropped plantations (E+A). The results showed that intercropped plantations (E+A) increase 3, 45, and 70% microbial biomass C as compared to A, E+N, and E, at 27 months after planting. The metabolic quotient (qCO_2) showed a tendency toward stressful values in pure *E. grandis* plantations and a strong correlation with dehydrogenase activity. A and E+A treatments also exhibited the highest organic fractions (OF) and C and N contents. A canonical redundancy analysis revealed positive correlations between microbial indicators of soil and litter attributes, and a strong effect of C and N variables in differentiating A and E+A from E and E+N treatments. The results suggested that a significant role of *A. mangium* enhance the dynamics of soil microbial indicators which help in the accumulation of C and N in soil OF in intercropped *E. grandis* plantations. Our results are mostly relevant to plantations in sandy soil areas with low levels of OM, suggesting and efficient method for improving nutrient availability in the soil and optimizing eucalyptus growth and development.

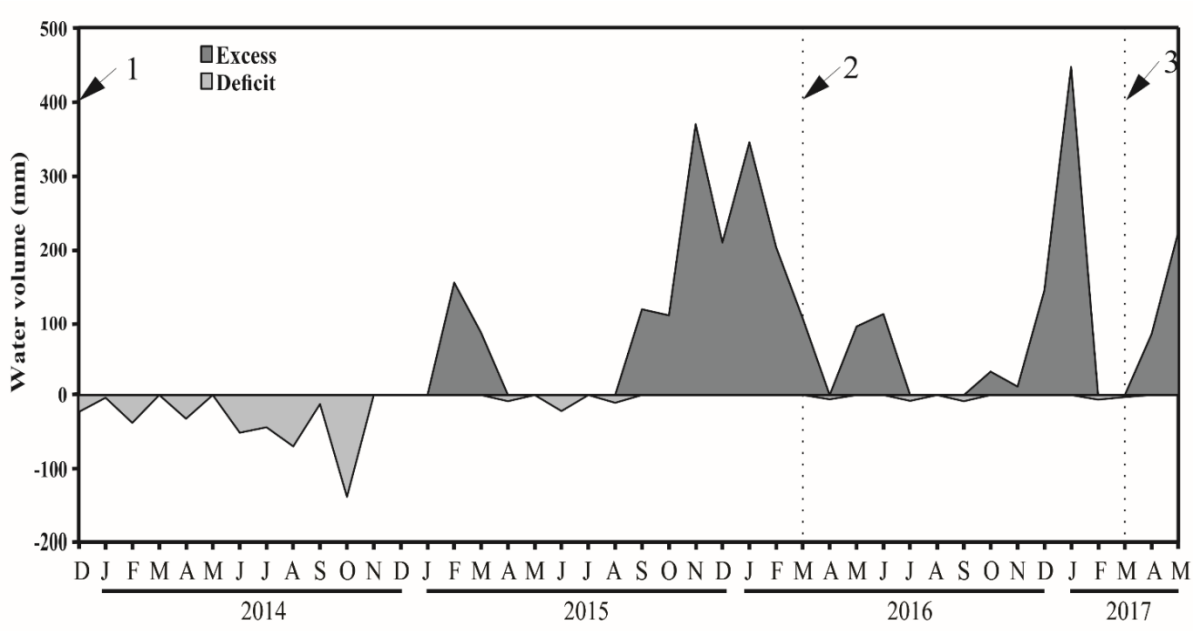
Keywords: forest soil, soil biology, mixed-systems, C-N cycles, organic matter



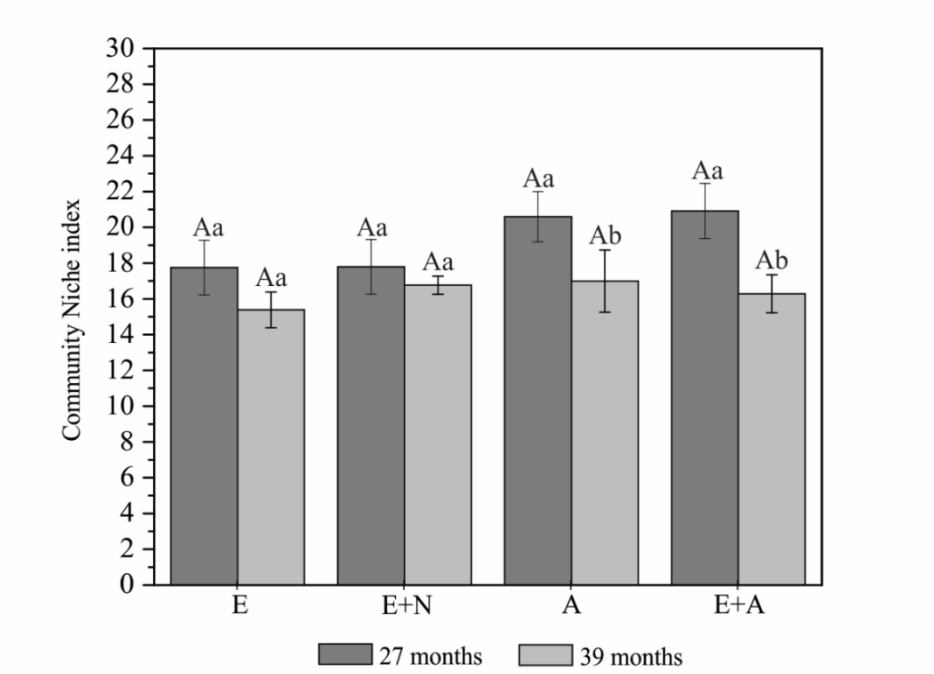
Supplementary Figure S1. Soil and litter sampling based on Voronoi grids. A) total plot area (36 x 36 m), the useful plot (24 x 24 m) and spacing between plants (3 x 3 m). Numbers 1 to 6 indicate the trees selected for sampling and "X" indicts the collection points at each tree; B) "R" represents the reference tree and the six rectangles represent the dimensions of each sampling point.



Supplementary Figure S2. Principal Coordinates Analysis (PCoA) based on the metabolic profile degradation of the soil microbial community accessed by Biolog EcoPlates in pure and mixed *E. grandis* and *A. mangium* plantations. A) 27 and B) 39 months after planting, and C) total profile.



Supplementary Figure S3. Monthly sequential water balance by the method proposed by Thornthwaite and Mather (1955) during the experiment (arrow 1, December 2013) from 2014 to 2017. Arrows 2 and 3 represent the 1st (27 months) and 2nd (39 months) sampling period. Soil water storage capacity = 280 mm.



Supplementary Figure S4. Community Niche index based on the metabolic profile degradation by the soil microbial community 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 between *E. grandis* and *A. mangium* at 27 and 39 months after planting. Means followed by the same letter do not differ by Tukey's test at a significance level of 5%. Upper case letters compare treatments within each period and lower-case letter compare the periods within each treatment. Error bars indicate standard deviation; $n = 4$.

Table S1. A) Soil correctives used in the *E. grandis* pure treatments (E and E+N), (A) *A. mangium* and (E+A) mixed plantation between *E. grandis* and *A. mangium*. B) Initial chemical characterization of the studied soil.

	2013						2014	
	N	K_2O	P_2O_5	Micro.	Limestone	B	N	K_2O
	----------kg ha^{-1}----------							
E	-	30	100	30	2000	4.5	-	120
E+N	10	30	100	30	2000	4.5	90	120
A	-	30	100	30	2000	4.5	-	120
E+A	-	30	100	30	2000	4.5	-	120

Sources:
N: Ammonium sulfate.
K_2O: Potassium Chloride.
P_2O_5: Triple Superphosphate.
Micronutrients: FTE BR12 - granulate.
Limestone: Dolomitic to supply Ca^{2+} and Mg^{2+}.
B: Boron/Borogran®.

Table S1. B) Initial chemical characterization of the studied soil.

Depth	pH	P	S	K	Ca	Mg	Al	H+Al	SB	CTC	V	m
cm		mg kg^{-1}					mmolc kg^{-1}				%	
0-10	3.63	4.90	4.62	0.49	1.05	0.67	16.99	99.98	2.20	102.19	2.87	83.55
10-20	3.82	3.71	4.60	0.38	0.99	0.62	11.45	66.46	1.99	68.45	3.31	82.85
20-40	3.80	3.28	4.96	0.37	1.15	0.63	11.34	63.16	2.16	65.31	3.52	83.81
40-60	3.88	3.05	6.44	0.28	0.88	0.62	8.47	48.65	1.78	50.44	3.67	82.01
60-100	3.93	2.58	8.39	0.23	0.82	0.62	7.02	41.73	1.67	43.40	4.05	80.02
100-200	4.04	2.07	4.54	0.19	0.77	0.62	3.77	30.64	1.58	32.22	5.09	68.28
200-300	4.19	2.15	3.65	0.18	0.80	0.62	1.62	24.64	1.60	26.24	6.54	37.32

Table S2. Metabolic profile assessed by the Biolog 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 and 39 months after planting.

		E		E+N		A		E+A	
	C sources	**27**	**39**	**27**	**39**	**27**	**39**	**27**	**39**
		Average Well Colour Development (AWCD)							
I	α-D-lactose	0.36	0.33	0.37	0.40	0.39 A	0.32 B	0.42 A	0.34 B
	D-cellobiose	0.40	0.41	0.37	0.50	0.40	0.45	0.42	0.44
	D-mannitol	0.79	0.89	0.79	0.85	0.96 A	0.71 B	0.96	0.78
	D-xylose	0.49	0.53	0.41	0.43	0.43 A	0.35 B	0.44 A	0.37 B
	i-erythritol	0.42	0.48	0.43	0.40	0.46	0.35	0.45	0.34
	N-acetyl-D-glucosamine	0.65	1.18	0.62	0.62	0.041 B	0.64 A	0.70	0.71
	β-methyl-D-glucoside	0.43	0.48	0.38	0.53	0.40	0.76	0.42	0.57
II	α-cyclodextrin	0.42	0.48	0.37	0.39	0.40 A	0.32 B	0.45	0.34
	Glycogen	0.42	0.42	0.38	0.41	0.41 A	0.33 B	0.43	0.36
	Tween 40	0.74	0.98	0.82	0.91	0.86 A	0.69 B	0.83	0.80
	Tween 80	0.84	1.01	0.69	0.77	0.73	0.63	0.84	0.63
III	2-hydroxy benzoic	0.42	0.48	0.36	0.43	0.39 A	0.32 B	0.41	0.34
	4-hydroxy benzoic	0.56	0.65	0.73	0.61	0.60	0.47	0.62	0.57
	α-ketobutyric	0.39	0.42	0.38	0.39	0.40 A	0.32 B	0.42	0.34
	D-galactonic γ-lactone	0.88	0.97	0.74	0.76	0.77 A	0.60 B	0.76	0.67
	D-galacturonic	0.43	0.50	0.75	0.57	0.71	0.48	0.69 A	0.35 B
	D-glucosamic	0.66	0.66	0.62	0.60	0.68 A	0.47 B	0.69	0.54
	D-malic	0.39	0.37	0.39	0.42	0.39	0.37	0.43	0.40
	γ-hydroxy butyric	0.46	0.51	0.49	0.45	0.52	0.43	0.52	0.36
	Itaconic Acid	0.58	048	0.50	0.50	0.60	0.43	0.61	0.44
IV	Phenylethyl-amine	0.41	0.48	0.44	0.46	0.42	0.37	0.47	0.45
	Putrescine	0.69	0.67	0.69	0.72	0.69 A	0.56 B	0.42	0.47
V	Glycyl-L-glutamic	0.42	0.46	0.39	0.41	0.41 A	0.33 B	0.44 A	0.35 B
	L-arginine	0.83	0.93	0.86	0.78	0.82	0.64	0.82	0.78
	L-asparagine	0.80	1.03	0.97	0.95	0.98 A	0.72 B	0.96	0.89
	L-phenylalanine	0.51	0.56	0.43	0.45	0.46	0.40	0.49	0.45
	L-serine	0.64	0.95	0.87	0.83	0.80	0.78	0.84	0.79
	L-threonine	0.49	0.49	0.37	0.40	0.40 A	0.33 B	0.43 A	0.35 B
VI	D,L-α-glycerol phosphate	0.51	0.48	0.40	0.45	0.47	0.39	0.46	0.36
	Glucose-l-phosphate	0.39	0.47	0.42	0.41	0.39 A	0.31 B	0.42	0.39
	Pyruvic acid methyl ester	0.66	0.81	0.58	0.56	0.61	0.59	0.65	0.52

I: Carbohydrates; II: Polymers; III: Carboxylic acids; IV: Amines; V: Amino acids; VI: Miscellaneous.

Table S3. Percentage of variation in soil microbiological patterns between treatments correlated with the soil and litter parameters based on Monte Carlo permutation test.

	Conditional effects			
Attributes	Lambda (λ)	Contribution (%)	F-test	*p* value
27 months				
Total-N (Litter)	0.17	17	4.27	0.0019
C-OF	0.14	14	3.27	0.0017
NH_4^+-N (Litter)	0.10	10	5.38	0.0011
N-OF	0.10	10	4.89	0.0018
N-OMF1	0.09	9	4.23	0.0010
Amidase	0.09	9	3.54	0.0019
Dehydrogenase	0.7	7	5.06	0.0014
Cmic	0.05	5	3.30	0.0039
C/N	0.03	3	3.29	0.0033
CO_2-C	0.02	2	3.30	0.0039
TOC	0.02	2	3.75	0.0280
qCO_2	0.02	2	3.17	0.0441
*q*Mic-C	0.01	1	3.14	0.0410
Urease	0.01	1	3.01	0.0501
L-glutaminase	-	-	2.87	0.0578
L-asparaginase	-	-	2.01	0.0935
*q*Mic-N	-	-	1.17	0.1478
Nmic	-	-	1.09	0.2478
39 months				
N-OMF1	0.21	21	3.24	0.0017
N-OF	0.19	19	3.06	0.0027
qCO_2	0.16	16	3.14	0.0028
NH_4^+-N (Litter)	0.09	9	3.93	0.0027
Amidase	0.09	9	3.86	0.0147
Dehydrogenase	0.09	9	3.13	0.0147
C-OF	0.07	7	3.47	0.0201
TOC	0.04	4	3.20	0.0309
Cmic	0.03	3	3.16	0.0490
Total-N (Litter)	0.02	2	3.91	0.0478
L-glutaminase	0.02	2	3.25	0.0421
Nmic	0.02	2	3.08	0.0478
Urease	0.02	2	3.19	0.0489
L-asparaginase	0.01	1	3.73	0.0407
C/N	0.01	1	3.04	0.0501
*q*Mic-C	0.01	1	2.98	0.0579
*q*Mic-N	-	-	2.24	0.0689
CO_2-C	-	-	1.37	0.9789

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3. MIXED PLANTATION INDUCED CHANGES IN MICROBIAL COMMUNITIES INCREASE BIOLOGICAL FUNCTIONS IN THE SOIL AND LITTER LAYERS

Abstract

Mixed plantations of *Eucalyptus* and N_2-fixing trees are beneficial because they are reported to stimulate organic matter cycling and increasing carbon (C) and nitrogen (N) pools. 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 induced 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 E+A and A showed an increase in C and N content 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 E+A and A, compared to pure *E. grandis* (especially E+N) plantation. Our data suggest that the total N content has influenced the bacterial community structure of litter, which undergoes alterations according to treatment and forest age. Equally, *Rhizobium*, *Bradyrhizobium* and *Sphingomonas* showed a positive correlation with *nif*H and soil N. Our study has provided evidence that changes in the microbial community in mixed *A. mangium* and *E. grandis* plantations is linked to increased C and N cycling. These findings have implications for the optimization of mineral fertilizers in forest plantations, and for increased productivity and environmental sustainability.

Keywords: Forest ecosystem; Mixed forest; Nutrient cycling; Microbial diversity and ecology; Eucalyptus sustainability

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 globally promoted as an alternative for industrial raw material, reducing the extractive activity in native forests (Gonçalves et al., 2013). In Brazil alone, around eight 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., 2010; 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 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., 2018; 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). It is closely associated with N dynamics in the soil, starting with the N_2-fixation process and extending to organic matter mineralization and soil greenhouse gas 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 higher 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., 2010; Rocha et al., 2016), this is 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 hypothesised that the mixed plantation would induce changes in microbial community and drive biological functions associated with C and N cycles. We also hypothesised that the application of mineral fertiliser 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 cycles.

Materials and Methods

Experimental site, design and data collection

The study was carried out at the Itatinga Experimental Station of Forest Science (23º03'S - 48º37''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 and a low CEC (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, the area was planted with *E. grandis*, without 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 x 36 m), with three field replicates and spacing between plants of 3 x 3 m. The edge effect was eliminated by sampling in only the central area of each plot, equating to 576 m^2 (24 x 24 m). *A. mangium* seedlings were inoculated with *Rhizobium* strains (BR3609T and BR6009) provided by "Embrapa 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 (December 2013; Fig. 1), following the grid methodology based on the Voronoi polygons, widely accepted for sampling in forest ecosystems (Saint-André et al., 2005).

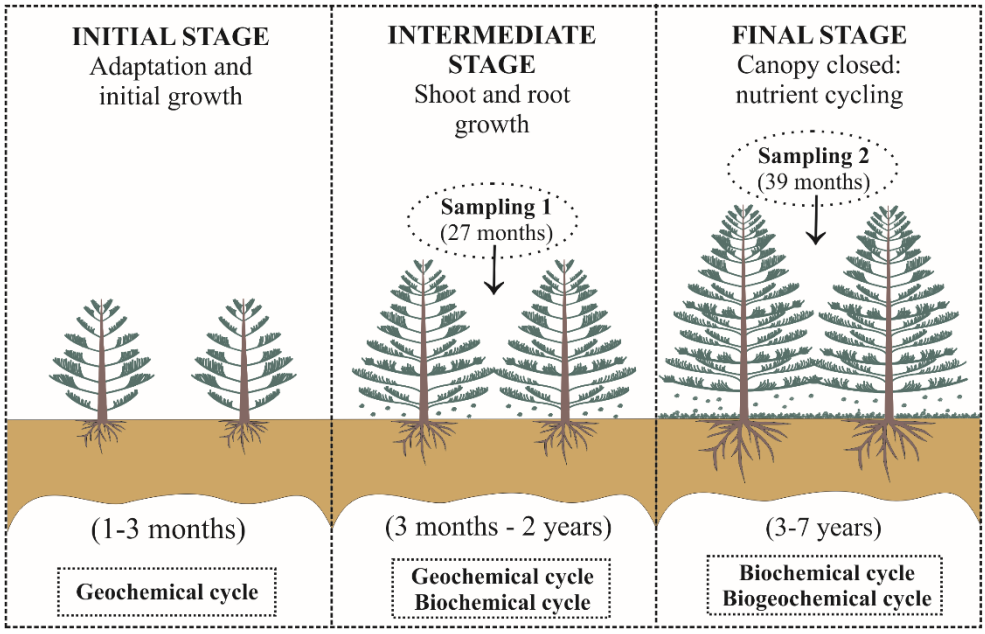

Figure 1. Growth stages and changes in nutrient cycling in a typical *Eucalyptus* plantation in Brazil. Soil and litter sampled at 27 and 39 months after tree planting, when there was a change from the biochemical cycle to the biogeochemical cycle in the soil.

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 Gonçalves 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 (Gonçalves and Benedetti, 2000). For a complete description of the experimental design and data collection, see also Pereira et al. (2018).

Analytical procedures

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., 1991). We determined C (Cmic) and N (Nmic) of the microbial biomass and soil basal

respiration (CO_2-C), as well as qCO_2, *q*Mic-C and *q*Mic-N quotient. We measured the activity of six soil enzymes, two associated with C (β-glucosidase and dehydrogenase) and four with N (urease, L-asparaginase, L-glutaminase, and amidase) cycling (Anderson and Domsch, 1993; Brookes et al., 1985; Vance et al., 1987; Tabatabai, 1994; Pereira et al., 2018). Soil organic matter was physically fractionated, and we evaluated C (OF-C) and N (OF-N) content in the organic fractions (OF - 2,000-75 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; Freitas et al., 2013; Pereira et al., 2018).

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 - 2 mm) was adjusted to 40% of the maximum water holding capacity. Samples were incubated at 25ºC, 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 2M KCl solution and determined following the "Kjeldahl" method. 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).

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 -20ºC and used for later molecular procedures.

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 x 250 bp), following standard Illumina® sequencing protocols.

Bioinformatics processing

Raw forward and reverse reads were joined, and afterward 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; Mercier et al., 2013). 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.

Quantitative PCR analysis (*q*PCR)

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: 95ºC for 10 min, 39 amplification cycles of 94ºC for 60 s, 55ºC for 2 s and 72ºC for 60 s. The standard curve efficiency was 90%. The *amo*A gene of bacteria (AOB) were 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: 95ºC for 10 min, 39 amplification cycles of 94ºC for 60 s, 60ºC for 60 s and 72ºC 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: 95ºC for 10 min, 39 amplification cycles of 94ºC for 45 s, 50ºC for 45 s and 72ºC 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: 95ºC for 10 min, 39 amplification cycles of 95ºC for 27 s, 62ºC for 60 s and 72ºC for 30 s. The standard curve efficiency was 106%.

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 also 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/).

Results

General soil and litter pools

The results of some soil chemical and microbial attributes, enzymatic activity, and litter characterization, were previously presented in Chapter 1 (Pereira et al., 2018). The soil pH ranged from 3.8 to 4.2 and soil nitrate (NO_3^--N) from 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). However, 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).

Bacterial community composition in soil and litter layers

For the soil bacterial compositions, 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%), *Afípia* (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 ($p<0.05$; Figure 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). A total of 142 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 was 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).

Differences in α-diversity metrics (Shannon 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 to 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 E+A (561) and A (554) treatments at 27 months, but there was a significant reduction in E (538) and E+N (486). At 39 months, treatment A showed a higher PD (516) than all others, and E+N (451) 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.

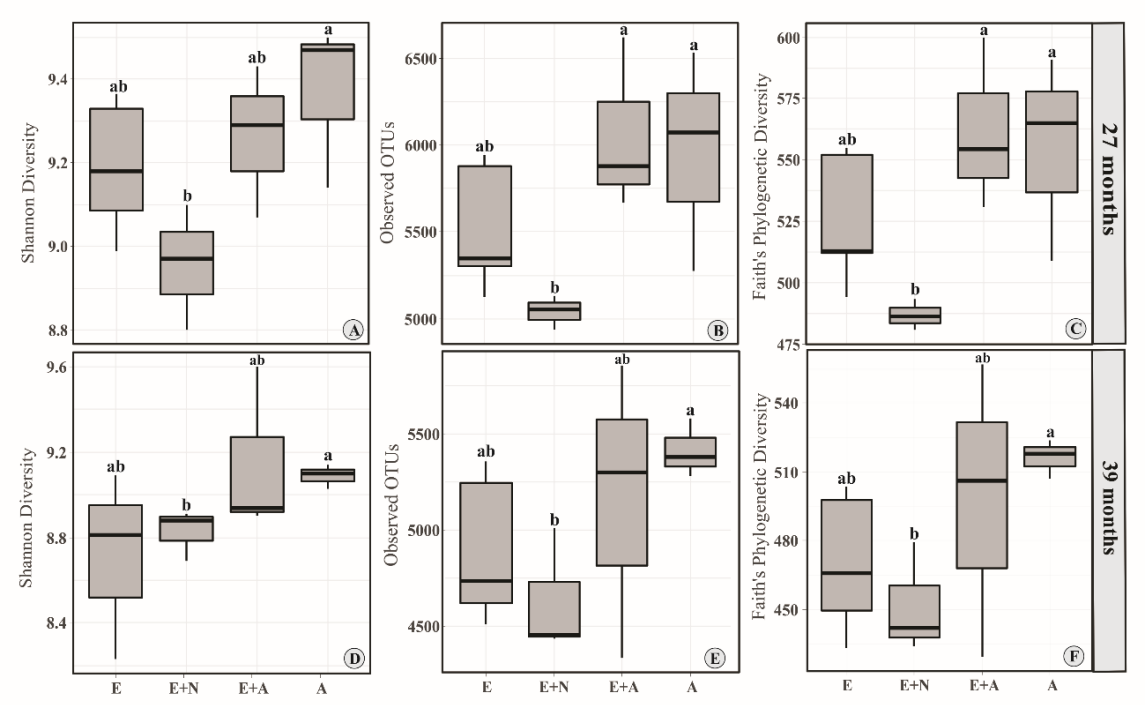

Figure 2. Alpha diversity metrics in soil of pure and mixed *E. grandis* and *A. mangium* plantations. (E) *E. grandis*, (E+N) *E. grandis* with N fertilization, (E+A) intercropped *E. grandis* and *A. mangium* and (A) *A. mangium* plantation at 27 (A, B and C) and 39 (D, E and F) months after planting.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

In the litter layer, Shannon's diversity index was higher in E+A (with a mean of 8.25) and A (8.45) than other treatments at 27 months. At 39 months, Shannon's diversity index was also higher in the intercropped plantation E+A (8.87) than 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 metrics 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).

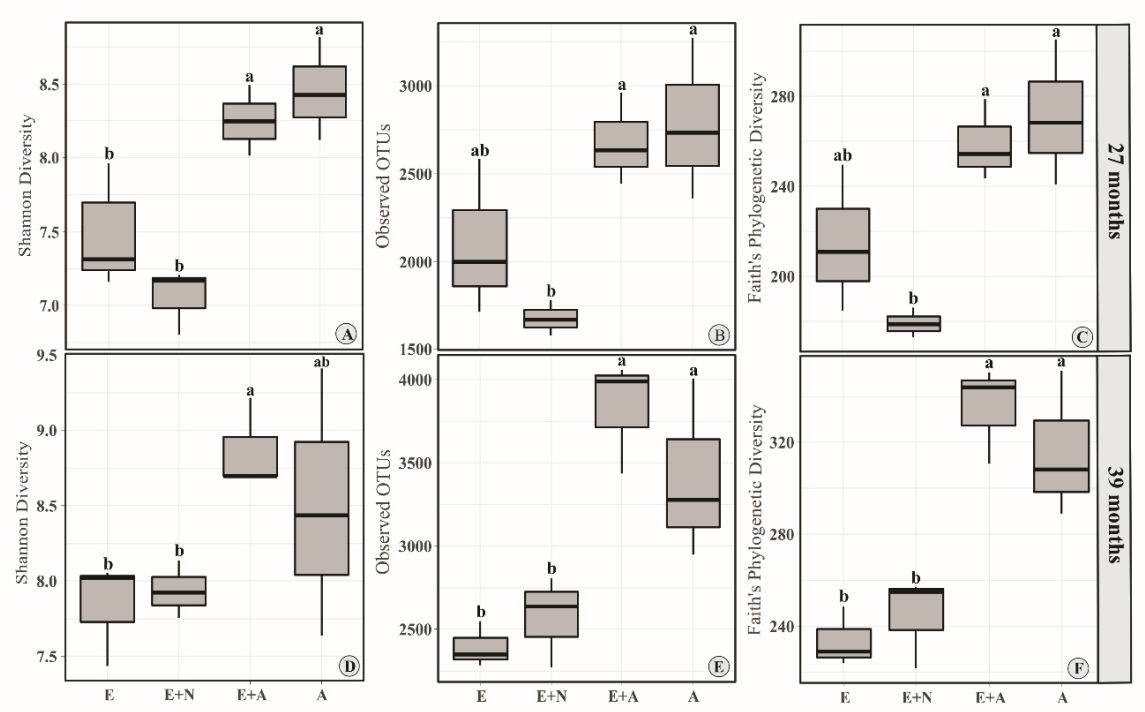

Figure 3. Alpha diversity metrics in litter of pure and mixed *E. grandis* and *A. mangium* plantations. (E) *E. grandis*, (E+N) *E. grandis* with N fertilization, (E+A) intercropped *E. grandis* and *A. mangium* and (A) *A. mangium* plantation at 27 (A, B and C) and 39 (D, E and F) months after planting.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

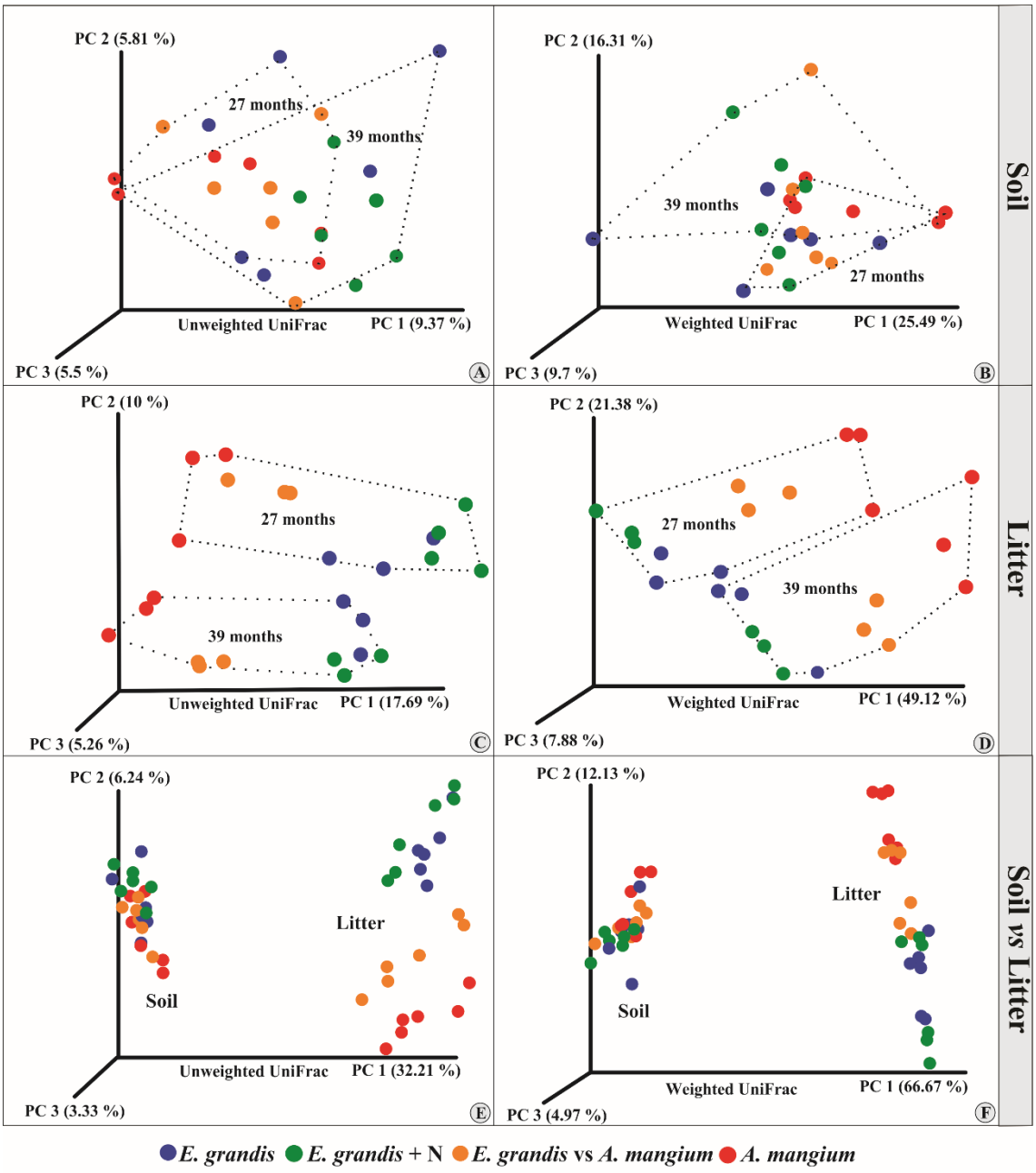

Figure 4. Principal Coordinate Analysis (PCoA) by Unweighted and Weighted UniFrac of soil (A and B) and litter (C and D) of the bacterial community in pure and mixed *E. grandis* and *A. mangium* plantations. (E) *E. grandis*, (E+N) *E. grandis* with N fertilization, (E+A) mixed *E. grandis* and *A. 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).

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).

Abundance of *nif*H, *amo*A (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 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).

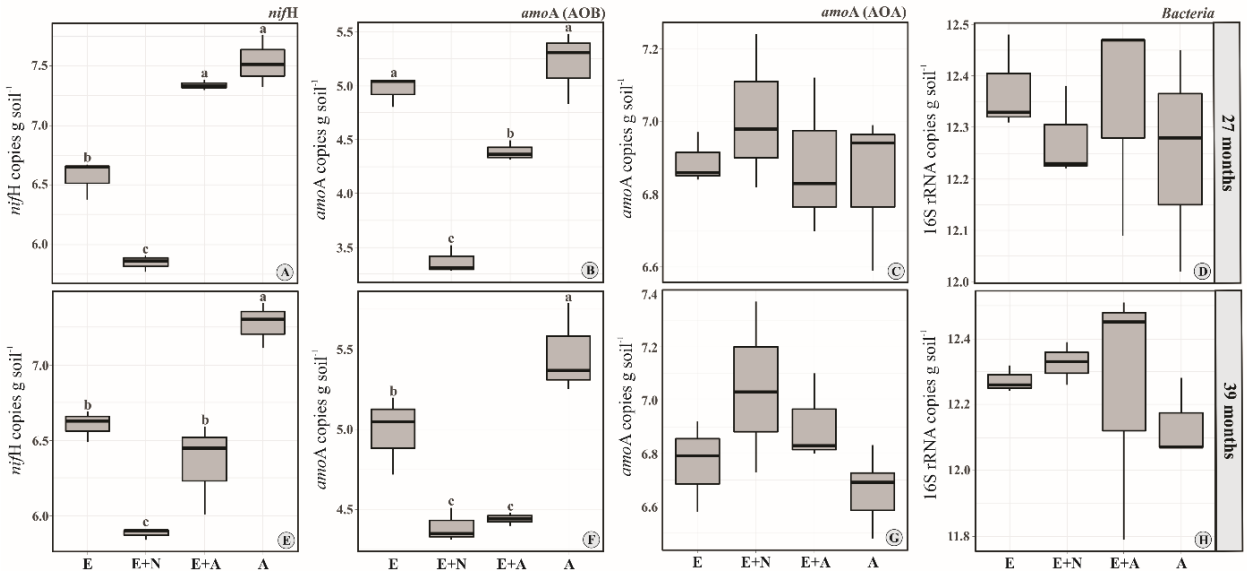

Figure 5. Real-time quantitative PCR (*q*PCR) of *nif*H, *amo*A (AOB and AOA) and total bacteria (16S rRNA) genes abundance in pure and mixed *E. grandis* and *A. mangium* plantations. (E) *E. grandis*, (E+N) *E. grandis* with N fertilization, (A) *A. mangium* and (E+A) mixed plantation of *E. grandis* 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 test at a significance level of 5%. All values are presented in $\log_{10}$ (n = 3).

There was a higher *amo*A (AOB) abundance in E and A than 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 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).

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 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 was positively correlated 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).

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.

	27 months					39 months				
Soil	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).

Spearman's rank correlation coefficients

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. S3 B). 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.

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 to 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 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, 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).

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, is *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.

The α-diversity increased in *Acacia* and mixed plantations, while the N fertilizer application reduced it

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 to the ecological theory for 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). 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 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 community. 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.

Bacterial community structure in soil and litter

Response of soil bacterial structure to treatments and forest age

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. Also, 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).

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).

Pure *Acacia* **and intercropped** *E. grandis* **and** *A. mangium* **plantations increase the functional gene abundance and N contents**

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 can 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, 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 sandy 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 is related to 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 not observed for 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 this process (Prosser and Nicol, 2012; Wen et al., 2017; Yao et al., 2011), which may explain the positive correlation between soil nitrate content and *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 mixed plantations can be used to reduce the amount of N fertilizer and therefore can promote a sustainable increase in the plantation's productivity.

Conclusions

We provide novel evidence that pure *Acacia* and mixed plantations between *Eucalyptus* and *Acacia* promotes 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. 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 driven 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 (only in soil) and microbial activities in litter and soil. In addition, increased bacterial diversity and abundance of functional genes (mainly at 27 months) correlated 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.

Acknowledgments

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Supplementary Material

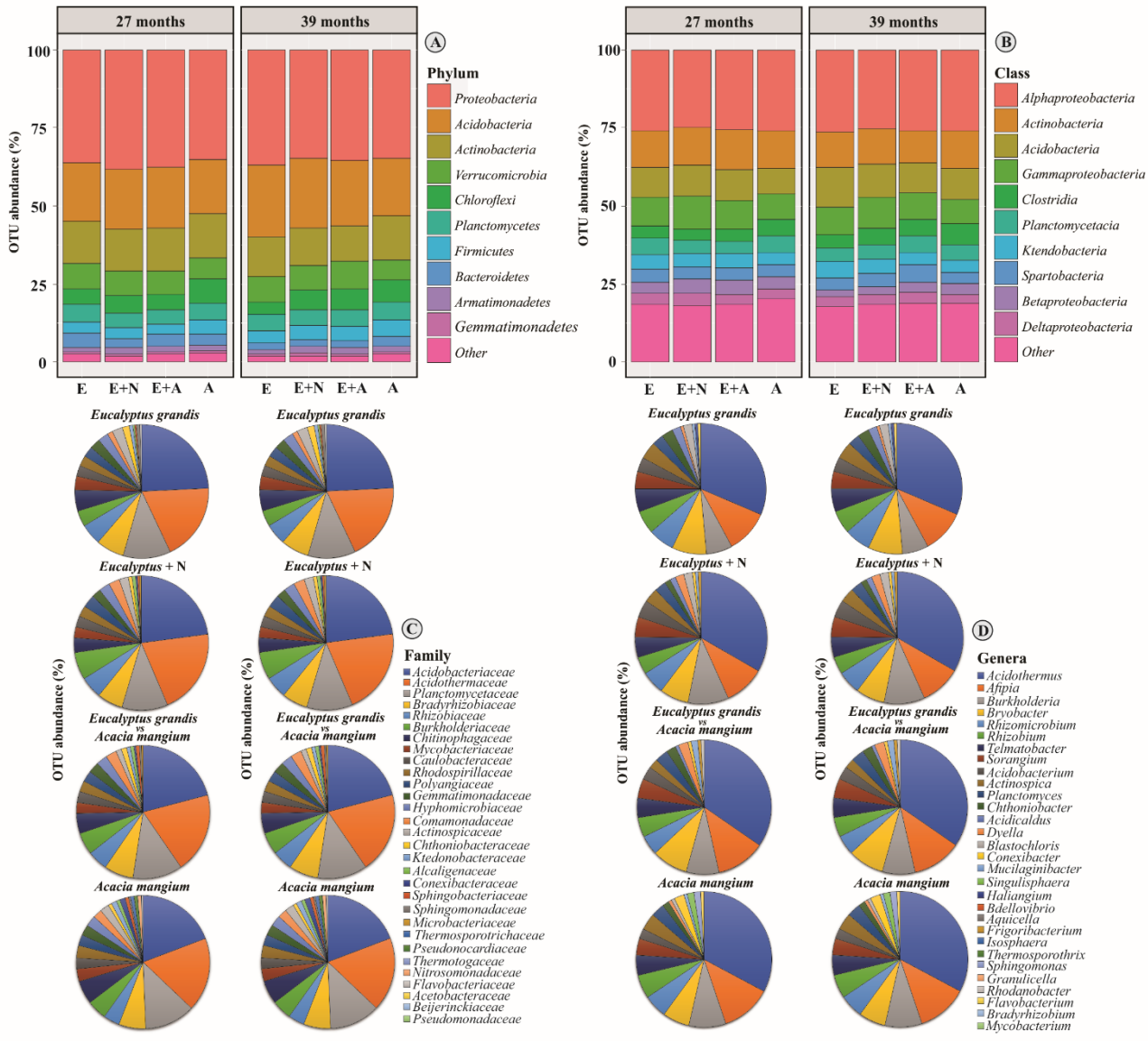

Figure S1. Relative abundance of major bacterial phyla (A), classes (B), families (C) and genera (D) in the soil of pure and mixed *E. grandis* and *A. mangium* plantations. (E) *E. grandis*, (E+N) *E. grandis* with N fertilization, (E+A) mixed *E. grandis* and *A. mangium* and *A. mangium* (A) plantation at 27 and 39 months after planting. Other: members with relative abundance lower than 5%. In (C) and (D), families and genera which represent the 30 highest OTUs number (n = 3).

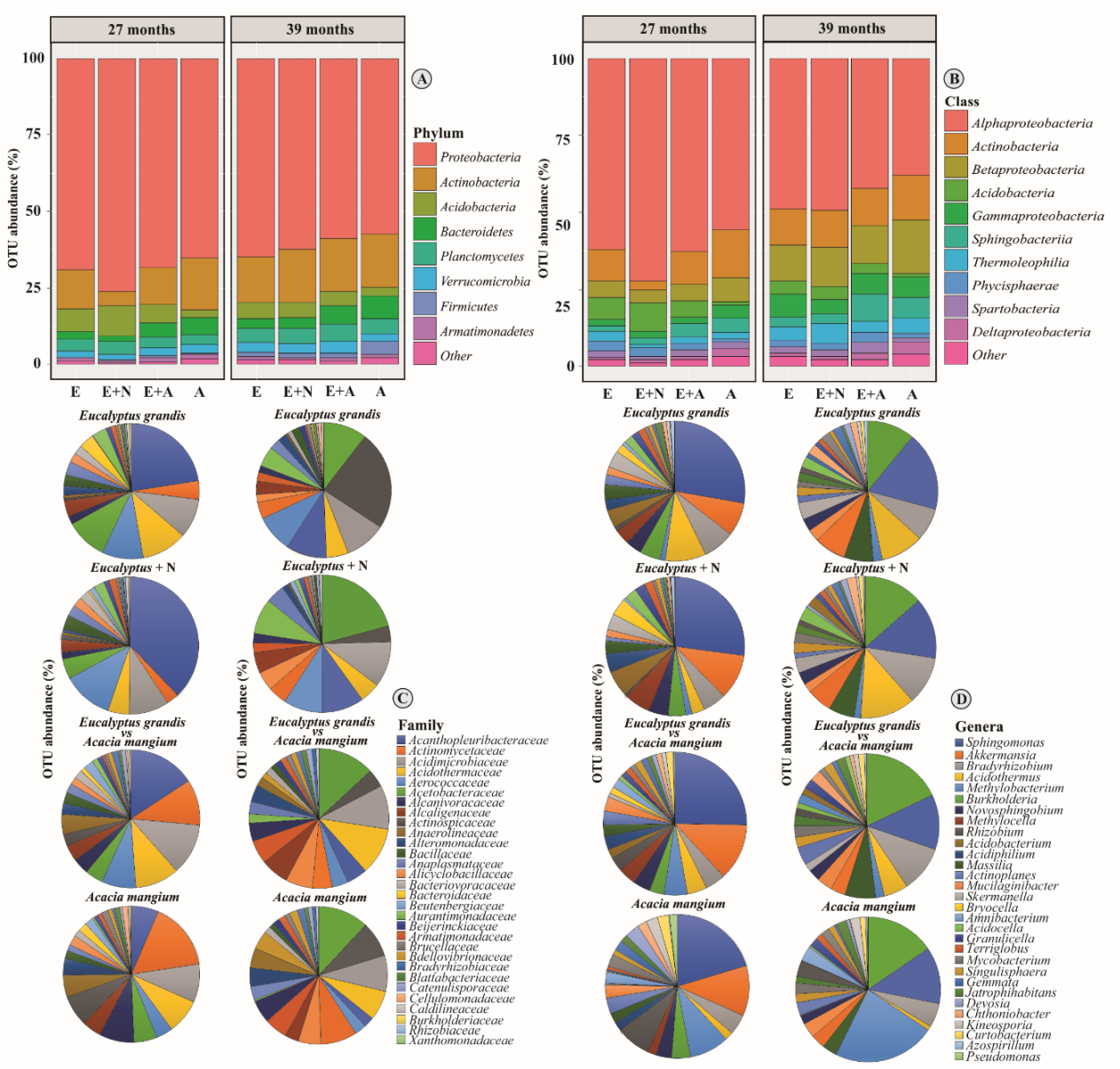

Figure S2. Relative abundance of major bacterial phyla (A), classes (B), families (C) and genera (D) in the litter of pure and mixed *E. grandis* and *A. mangium* plantations. (E) *E. grandis*, (E+N) *E. grandis* with N fertilization, (E+A) mixed *E. grandis* and *A. mangium* and *A. mangium* (A) plantation at 27 and 39 months after planting. Other: members with relative abundance lower than 5%. In (C) and (D), families and genera which represent the 30 highest OTUs number (n = 3).

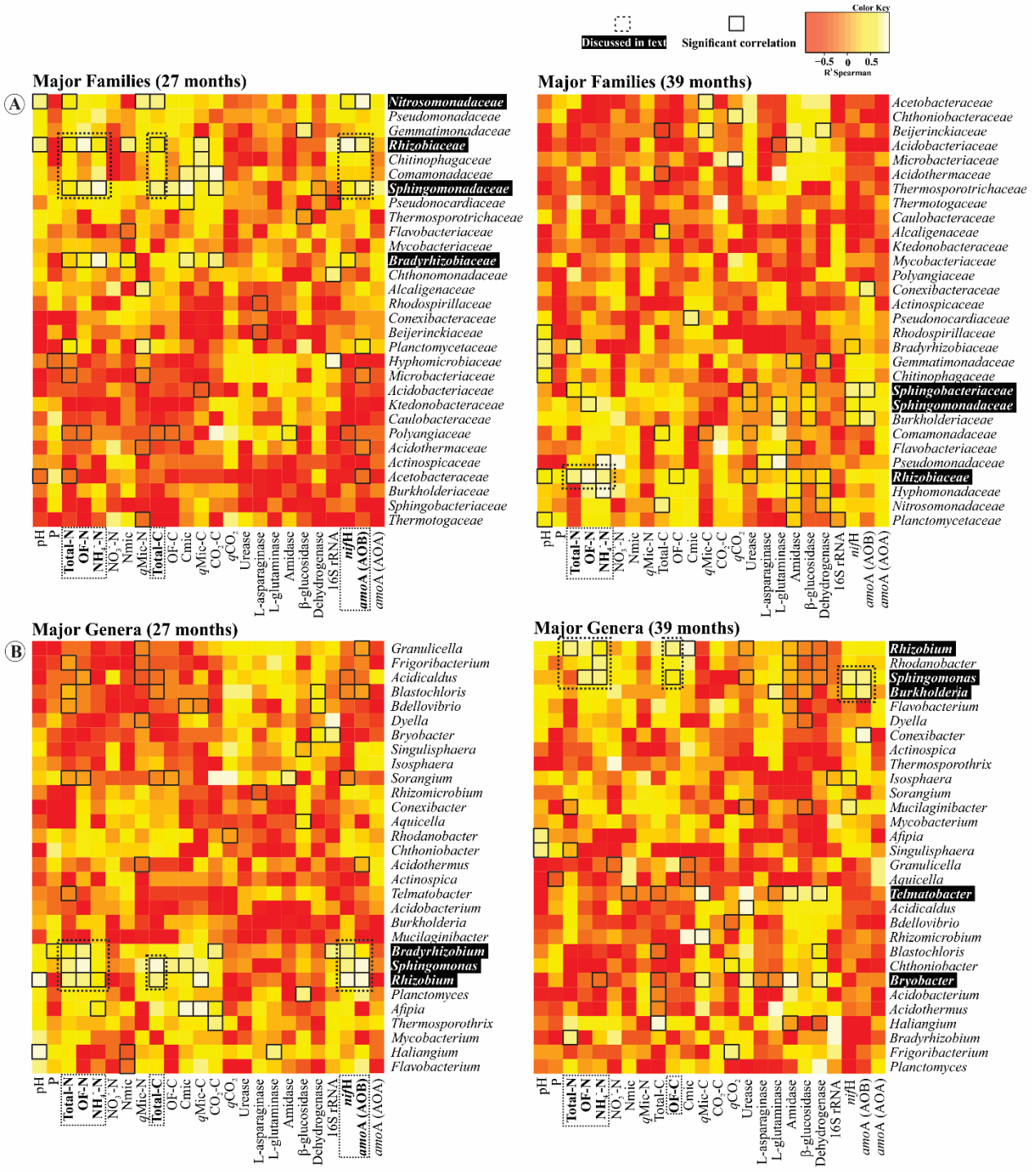

Figure S3. Spearman's rank correlation coefficient between major families (A) and genera (B) of soil bacterial community and soil-microbial attributes associated with C and N cycling in pure and mixed *E. grandis* and *A. mangium* plantations at 27 and 39 months after planting (n = 6).

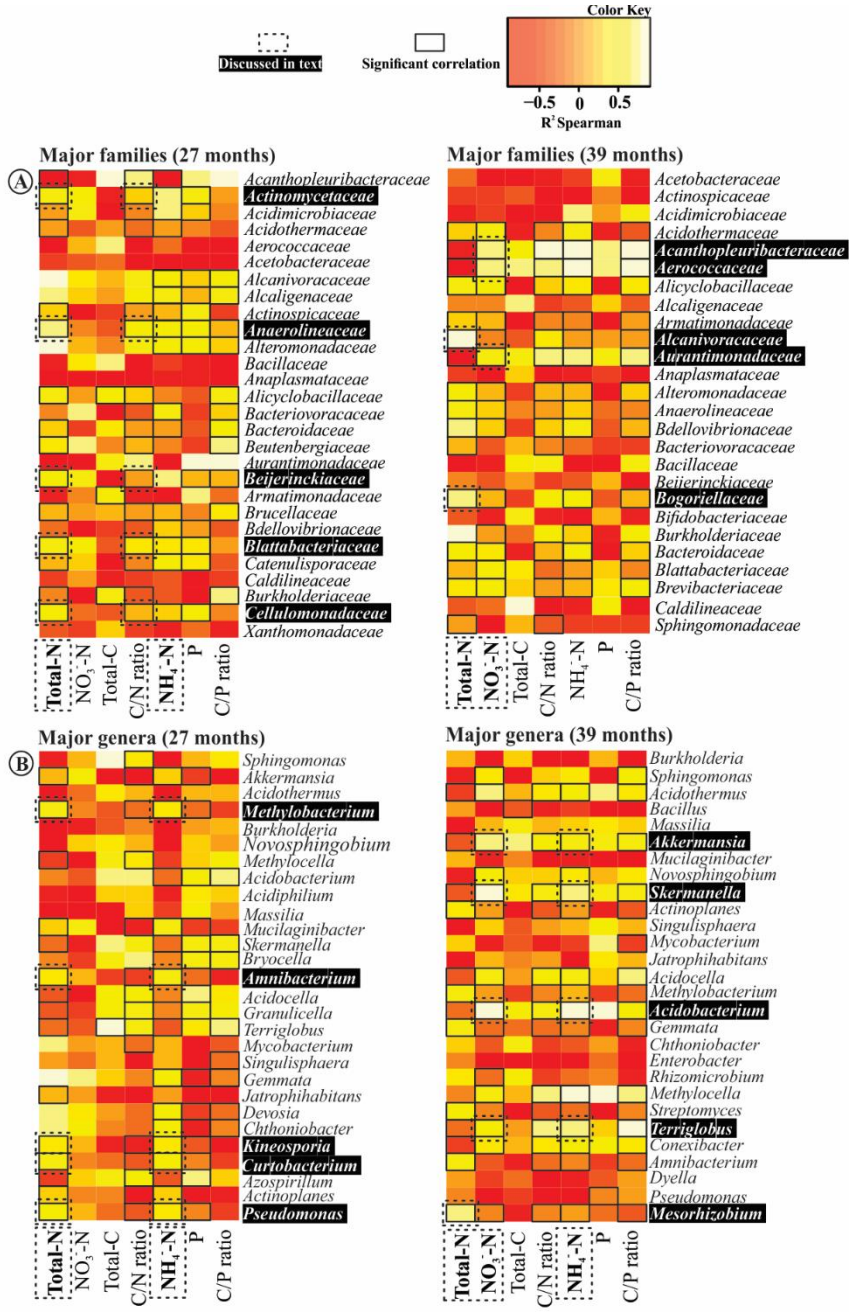

Figure S4. Spearman's rank correlation coefficient between major families (A) and genera (B) of litter bacterial community and litter attributes associated with C and N cycling in pure and mixed *E. grandis* and *A. mangium* plantations at 27 and 39 months after planting (n = 3).

Table S1. Soil (0-20 cm) and litter characterization, and soil microbial and enzymatic activity in (E) *E. grandis*, (E+N) *E. grandis* with N addition, (E+A) mixed plantation between *E. grandis* and *A. mangium* and (A) *A. mangium* at 27 and 39 months.

Soil attributes	27 months				39 months			
	E	E+N	E+A	A	E	E+N	E+A	A
pH	4.2±0.4^{Aa}	3.9±0.1^{Aa}	4.1±0.2^{Aa}	4.1±0.3^{Aa}	3.8±0.1^{Aa}	3.9±0.1^{Aa}	3.9±0.3^{Aa}	4.2±0.4^{Aa}
P	6.25±0.95^{Aa}	5±0.81^{Aa}	5.75±0.95^{Aa}	5.2±0.95^{Aa}	3.9±0.57^{Ab}	3.3±0.47^{Ab}	4±0.81^{Ab}	3.3±0.47^{Ab}
Cmic	478.6±136.5^{Ba}	562.3±21.4^{Ba}	814.8±161^{Aa}	791±120.6^{Aa}	475±164.2^{Aa}	477.5±110^{Aa}	617.5±113^{Aa}	592.5±120^{Aa}
Nmic	33±1.7^{Bb}	57.8±17.7^{Ab}	65.7±10.6^{Ab}	104.2±16^{Aa}	65.6±3.8^{Ba}	96.9±14.5^{Aa}	99.5±9.7^{Aa}	63.9±27.4^{Aa}
CO_2-C	22.7±2.2^{Ab}	19±1.7^{Ab}	24±3.2^{Ab}	23.2±5^{Ab}	36.5±5.5^{Ba}	50.5±5.6^{Aa}	46.1±8.8^{Ba}	37.4±6.8^{Ba}
qCO_2	0.04±0.003^{Ab}	0.041±0.008^{Ab}	0.03±0.009^{Bb}	0.03±0.003^{Bb}	0.08±0.02^{Aa}	0.126±0.07^{Aa}	0.07±0.02^{Aa}	0.06±0.02^{Aa}
*q*Mic-C	2.5±0.7^{Aa}	2.71±0.33^{Aa}	3.17±0.50^{Aa}	3.24±0.27^{Aa}	2.44±0.79^{Aa}	2.20±0.59^{Aa}	2.29±0.30^{Ab}	2.26±0.26^{Ab}
*q*Mic-N	5.22±1.38^{Ab}	2.3±1.0^{Ba}	3.9±0.6^{Bb}	6.5±1.3^{Aa}	7.8±0.9^{Aa}	5.2±2.5^{Aa}	6.2±1.2^{Aa}	7.5±2.4^{Aa}
Urease	32.2±1.3^{Ab}	32.5±1.5^{Ab}	24.0±3.4^{Bb}	24±1.8^{Bb}	59.3±2.0^{Aa}	52.7±1.5^{Aa}	44.2±4.0^{Ba}	39.5±2.9^{Ba}
L-asparaginase	13.1±1.3^{Ab}	14.9±3.6^{Ab}	13.6±0.8^{Ab}	13.7±3.7^{Ab}	15.7±0.1^{Ca}	24.3±1.5^{Aa}	18.1±1.4^{Ba}	24.2±4.2^{Aa}
L-glutaminase	42.1±4.3^{Ab}	35.9±10.2^{Ab}	40.1±10^{Ab}	32.7±9.4^{Ab}	106.7±13.9^{Aa}	134.2±16.3^{Aa}	124.5±16.6^{Aa}	129.5±12.7^{Aa}
Amidase	149.8±11.5^{Ab}	130±7.4^{Ab}	78.6±4.4^{Bb}	85.8±22^{Bb}	235.7±5^{Aa}	233.1±14^{Aa}	190±21^{Ba}	197.2±11.7^{Ba}
β-glucosidase	81.9±4.4^{Aa}	83.7±7.7^{Aa}	87.6±11.4^{Aa}	87.3±7.4^{Aa}	7.4±4.3^{Aa}	76.7±2.7^{Aa}	76.4±8.5^{Aa}	66.7±8.3^{Ab}
Dehydrogenase	9.2±0.6^{Aa}	8.8±0.3^{Aa}	7.8±0.2^{Ba}	7.3±0.2^{Ba}	7.4±0.3^{Ab}	6.5±0.3^{Bb}	4.7±0.6^{Cb}	4.8±0.4^{Cb}
OF	12.7±1.8^{Ba}	12.9±1.2^{Bb}	25.6±2.1^{Ab}	25.4±2.1^{Ab}	14.9±3.4^{Ba}	18.4±0.6^{Ba}	30±0.8^{Aa}	29.5±0.3^{Aa}
N-OF	0.16±0.02^{Ba}	0.17±0.01^{Ba}	0.34±0.05^{Aa}	0.35±0.04^{Aa}	0.20±0.05^{Ba}	0.22±0.04^{Ba}	0.36±0.06^{Aa}	0.35±0.05^{Aa}
Total-N	1.85^{Aa}	1.60^{Ba}	1.89^{Aa}	1.31^{Aa}	1.31^{Bb}	1.31^{Bb}	1.93^{Aa}	1.27^{Bb}
NH_4^+-N	22.7±6.5^{Ba}	26±4.55^{Ba}	56.7±8.5^{Aa}	45.5±11.7^{Aa}	20.5±1.73^{Aa}	17±3.5^{Aa}	37±13.3^{Aa}	31.7±10.5^{Aa}
NO_3^--N	38.7±24^{ns}	18.2±8.2^{ns}	28.2±23.6^{ns}	38.7±24.5^{ns}	26.7±15.8^{ns}	20.5±11.7^{ns}	22±13.9^{ns}	23±11.6^{ns}
C-OF	4.8±0.7^{Ba}	5.2±0.4^{Ba}	9.9±1.1^{Aa}	10.±1.3^{Aa}	6.0±1.5^{Ba}	6.6±0.9^{Ba}	10.4±1.9^{Aa}	9.8±1.5^{Aa}
Total-C	41.7^{Aa}	35.7^{Ba}	43.7^{Aa}	44.7^{Aa}	27.5^{Bb}	28.4^{Bb}	44.1^{Bb}	29.07^{Aa}
Litter attributes								
Total-C	521±15^{Aa}	526±6.5^{Aa}	527±9^{Aa}	514±9^{Aa}	518±7^{Aa}	519±20^{Aa}	519±3.5^{Aa}	517±8^{Aa}
Total-N	8.4±0.5^{Ca}	8.5±0.7^{Cb}	11.5±0.1^{Bb}	14±0.4^{Ab}	9.1±0.3^{Ca}	10.3±0.4^{Ca}	13.7±1.2^{Ba}	16.6±1.2^{Aa}
C/N ratio	62±4.5^{Aa}	62±5.5^{Aa}	46±1.1^{Ba}	37±1.8^{Ca}	58±4.8^{Aa}	51±1.2^{Bb}	39±3.4^{Bb}	31±2.0^{Cb}
NH_4^+-N	95.2±4.3^{Ba}	60±4.1^{Ca}	149±3.3^{Aa}	145.6±6^{Aa}	53.2±14.5^{Ab}	36.2±2.4^{Ab}	22.4±6.7^{Bb}	22.1±5^{Bb}
NO_3^--N	40±7^{ns}	42±3^{ns}	69±3.9^{ns}	56.3±3.2^{ns}	95.2±26^{ns}	86.1±27^{ns}	17.9±6.7^{ns}	36.8±5.9^{ns}
P	2.56±0.14^{Ab}	2.56±0.18^{Ab}	2.58±0.32^{Aa}	2.38±0.41^{Aa}	3.5±0.46^{Aa}	3.5±0.4^{Aa}	3.27±0.63^{Aa}	3.05±0.45^{Aa}
C/P ratio	203.3±15.3^{Aa}	205.5±16^{Aa}	206.3±30^{Aa}	220±36.7^{Aa}	150.3±17^{Ab}	151.6±17.6^{Ab}	165.42±30^{Aa}	171±23.4^{Aa}

Soil: P = mg dm^{-3}, Cmic = mg C kg $soil^{-1}$; Nmic = mg N kg $soil^{-1}$; CO_2-C = mg CO_2 kg^{-1} soil day^{-1}; *q*Mic-C and *q*Mic-N = %; Urease, L-asparaginase, L-glutaminase and amidase = µg NH_4^+-N g $soil^{-1}$ $2h^{-1}$; β-glucosidase = mg PNF kg $soil^{-1}$ $1h^{-1}$; Dehydrogenase = mg TPF kg $soil^{-1}$ $24h^{-1}$; OF, N-OF, Total-N, C-OF and Total-C = g kg^{-1}; NH_4^+-N and NO_3^--N = mg kg^{-1}. Litter: Total-C = g kg^{-1}; Total-N, NH_4^+-N and NO_3^--N = mg kg^{-1}; P = g kg^{-1}. Means followed by the same letter do not differ by Tukey test at a significance level of 5%. Upper case letters compared treatments within each period and lower case letters compared periods within each treatment.

Table S2. Permutational Multivariate Analysis of Variance (PerMANOVA) based on Adonis dissimilarities of phylogenetic distance in soil bacterial community.

Factor	Df	SumsOfSqs	MeanSqs	F.Model	R^2	%	Pr(>F)
Quantitative							
Chemical							
pH	1	0.05019	0.050187	0.70940	0.03143	3.14	0.8649
P	1	0.06845	0.068446	0.96749	0.04286	4.28	0.4464
Total-N	1	0.06888	0.068882	0.97365	0.04313	4.32	0.4471
OF-N	1	0.08084	0.080836	1.14263	0.05062	5.06	0.2583
NH_4^+-N	1	0.04737	0.047369	0.66957	0.02966	2.96	0.9189
NO_3^--N	1	0.05928	0.059278	0.83790	0.03712	3.71	0.6602
Nmic	1	0.06639	0.066392	0.93845	0.04157	4.15	0.4997
*q*Mic-N	1	0.07553	0.075529	1.06761	0.04730	4.73	0.3314
Total-C	1	0.09088	0.090878	1.28458	0.05691	5.69	0.1598
OF-C	1	0.05752	0.057519	0.81303	0.03602	3.60	0.6888
Cmic	1	0.06683	0.066827	0.94461	0.04185	4.18	0.4966
*q*Mic-C	1	0.08658	0.086576	1.22376	0.05421	5.42	0.2053
Residuals	11	0.77820	0.070746	-	0.48731	-	-
Total	23	1.59692	-	-	1.00000	-	-
Microbial activity							
CO_2-C	1	0.06378	0.063784	0.90207	0.03994	3.99	0.5566
*q*CO2	1	0.04826	0.048263	0.68257	0.03022	3.02	0.8987
Residuals	21	1.48488	0.070708	-	0.92984	-	-
Total	23	1.59692	-	-	1.00000	-	-
Enzyme activity							
Urease	1	0.07925	0.079250	1.12320	0.04963	4.96	0.2738
L-asparaginase	1	0.07204	0.072040	1.02102	0.04511	4.51	0.3758
L-glutaminase	1	0.06237	0.062368	0.88393	0.03905	3.90	0.5847
Amidase	1	0.06690	0.066895	0.94810	0.04189	4.18	0.4732
B-glucosidase	1	0.05323	0.053230	0.75443	0.03333	3.33	0.8005
Dehydrogenase	1	0.06367	0.063669	0.90238	0.03987	3.98	0.5509
Residuals	17	1.19947	0.070557	-	0.75111	-	-
Total	23	1.59692	-	-	1.00000	-	-
Quantitative PCR							
*nif*H	1	0.06395	0.063952	0.92533	0.04005	4.00	0.5152
*amo*A (AOB)	1	0.04812	0.048119	0.69624	0.03013	3.01	0.9051
16S rRNA	1	0.10260	0.102596	1.48448	0.06425	6.42	0.0770
Residuals	20	1.38226	0.069113	-	0.86557	-	-
Total	23	1.59692	-	-	1.00000	-	-
Qualitative							
Time	3	0.07445	0.074448	1.06548	0.04662	4.66	0.3258
Treatment	1	0.25822	0.086075	1.23188	0.16170	16.17	0.1158
Time *vs* Treatment	3	0.14629	0.048763	0.69788	0.09161	9.16	0.9891
Residuals	16	1.11796	0.069873	-	0.70007	-	-
Total	23	1.59692	-	-	1.00000	-	-

***p<0.001; **p<0.01 and *p<0.05

Table S3. Permutational Multivariate Analysis of Variance (PerMANOVA) based on Adonis dissimilarities of phylogenetic distance in litter bacterial community.

Factor	Df	SumsOfSqs	MeanSqs	F.Model	R^2	%	Pr(>F)
Quantitative							
Total-N	1	0.9204	0.92035	13.2689	0.27980	27.98	**0.0001*****
NO_3^--N	1	0.1234	0.12342	1.7794	0.03752	3.75	0.1026
Total-C	1	0.3222	0.32225	4.6459	0.09797	9.79	**0.0007*****
C/N ratio	1	0.1029	0.10290	1.4836	0.03128	3.12	0.1713
NH_4^+-N	1	0.5707	0.57069	8.2278	0.17350	17.35	**0.0001*****
P	1	0.0946	0.09461	1.3640	0.02876	2.87	0.2107
C/P ratio	1	0.0453	0.04530	0.6531	0.01377	1.37	0.7007
Residuals	16	1.1098	0.06936	-	0.33739	-	-
Total	23	3.2893	-	-	1.00000	-	-
Qualitative							
Time	1	0.6837	0.68371	10.9331	0.20786	20.78	**0.0001*****
Treatment	3	1.2170	0.40566	6.4868	0.36998	36.99	**0.0001*****
Time *vs* Treatment[1]	3	0.3881	0.12935	2.0685	0.11798	11.79	0.0136*
Residuals	16	1.0006	0.06254	-	0.30419	-	-
Total	23	3.2893	-	-	1.00000	-	-
Time (27 months)[1]							
Total-N	1	0.59590	0.59590	12.5844	0.48162	48.16	**0.0001*****
NO_3^--N	1	0.07484	0.07484	1.5806	0.06049	6.04	0.2050
Total-C	1	0.13219	0.13219	2.7916	0.10684	10.68	0.0760
C/N ratio	1	0.08341	0.08341	1.7614	0.06741	6.74	0.1773
NH_4^+-N	1	0.06294	0.06294	1.3291	0.05087	5.08	0.2640
P	1	0.05599	0.05599	1.1823	0.04525	4.52	0.3126
C/P ratio	1	0.04263	0.04263	0.9002	0.03445	3.44	0.4351
Residuals	4	0.18941	0.04735	-	0.15308	-	-
Total	11	1.23730	-	-	1.00000	-	-
Time (39 months)[1]							
Total-N	1	0.52885	0.52885	5.3573	0.38650	38.65	**0.0006*****
NO_3^--N	1	0.07868	0.07868	0.7971	0.05750	5.75	0.5815
Total-C	1	0.06252	0.06252	0.6334	0.04569	4.56	0.7413
C/N ratio	1	0.10616	0.10616	1.0754	0.07758	7.75	0.3817
NH_4^+-N	1	0.09947	0.09947	1.0076	0.07269	7.26	0.4077
P	1	0.05847	0.05847	0.5923	0.04273	4.27	0.7853
C/P ratio	1	0.03931	0.03931	0.3982	0.02873	2.87	0.9443
Residuals	4	0.39486	0.09871	-	0.28857	-	-
Total	11	1.36831	-	-	1.00000	-	-

$^{***}p<0.001$; $^{**}p<0.01$ and $^{*}p<0.05$

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4. FINAL REMARKS

Research associating the microbiome with pure and mixed forests is only just beginning. The achievement of sustainable Eucalyptus forest systems that meet the wood production demands and maintain an intimate relationship with biodiversity is an important challenge of the 21st century. Although our ability to describe the microbiome in this type of forest remains incomplete, we have already created an integrated view of very important processes mediated by microorganisms and their diversity. We know very little about how to exploit the multifunctionality and multicomplexity of natural ecosystems and apply them in agriculture to increase efficiency in nutrient use, yield and sustainability of forest ecosystems. Understanding the dynamics between tree-soil-microbiome is a complex but very important challenge because this relationship is one of intense mutual cooperation.

This thesis showed new concepts and expressive results about these systems that can be used to develop a better exploration of the soil-plant-microbiome interaction, but some bottlenecks are still eminent. Although the soil deep layers represent an immense black box, it has been demonstrated, for example, that fungi and bacteria can associate and be influenced by these trees up to 8 meters deep (Pereira et al., 2017; de Araujo Pereira et al., 2018). We still do not know what role these communities play in these inhospitable zones for microbial development. Are they active, participate in the nutrients assimilation and cycling, and/or water to the plants? We already know that there is a strong N transference from Acacia trees to Eucalyptus trees (Paula et al., 2015), but we still do not know what the main mechanisms associated with this process are. For example, is mycorrhizal-mediated N transfer between these two tree species? What are the main groups and what is the amount of N that is transferred? Issues such as these represent a huge challenge but need to be thought through in future studies. Moreover, some habitats need to be better explored, as well as microbial groups and their functions, such as plant growth promoting bacteria (PGPR) for the development of bio-fertilizers, bio-pesticides and bio-stimulants (Fig. 3). In addition, there is no studies with the phyllosphere microbiome, as well as of several other habitats within this forest ecosystem (Fig. 3). We have important evaluations of the functioning of bacteria and fungi in this system, but Archaea, an important group of soil microbial life, has not yet been studied.

There is a clear need for intensive studies in the setting up of experimental trials that consider multiple regions and to make relationships between them, even if these studies are initially descriptive. Focusing on the Eucalyptus and Acacia microbiome at the geographic

scale will undoubtedly represent an important and valuable future field of work in the microbial ecology and sustainability of these forest plantations. We must know the functional attributes of plants, soil organisms and relationships, so we can make appropriate predictions of how forest ecosystems will respond to management changes, environmental changes and climatic events in the coming decades.