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ANDRESSA MONTEIRO VENTURINI

Forest-to-pasture conversion in the Eastern Amazon: impacts on the soil methane microbial communities

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Marilia Ribeiro Garcia Henyei
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“Science and everyday life cannot and should not be separated.”

“In my view, all that is necessary for faith is the belief that by doing our best we shall come nearer to success and that success in our aims (the improvement of the lot of mankind, present and future) is worth attaining.”

Rosalind Franklin (1920-1958)

ABSTRACT

VENTURINI, A. M. **Forest-to-pasture conversion in the Eastern Amazon: impacts on the soil methane microbial communities**. 2019. 103 p. Tese (Doutorado em Ciências) - Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2019.

Forest-to-pasture conversion in the Amazon alters the physical, chemical, and biological properties of the soil. However, the impacts of this process on the methane (CH_4) fluxes – the second most important greenhouse gas of anthropogenic origin – and microorganisms responsible for its production (methanogenic *Archaea*) and consumption (methanotrophic *Bacteria*) from soils are still little known. Both groups can also be influenced by the moisture content of these soils, due to the seasonality and occurrence of extreme events in the region. Therefore, the objective of this thesis was to evaluate the impacts of forest-to-pasture conversion on the microbial CH_4 cycle in soils of the Tapajós National Forest and its surroundings, in the Brazilian Eastern Amazon, as well as its response to changes in moisture. In the first study, soil samples were collected in three forests and three pastures during the dry and rainy seasons. In the second, soil samples from both land-uses were used in the development of a microcosm experiment with four moisture levels (original moisture; 60%, 80%, and 100% of moisture at field capacity) for a 30-day period. The soil samples from both studies had their properties determined, whereas their microbial communities were evaluated by quantitative real-time PCR of CH_4 marker genes and metagenomic sequencing. Gas samples from the microcosm experiment were also periodically collected and analyzed by gas chromatography. As main results, the pastures had a higher pH and nutrient levels than the forests, but the field samples revealed a decrease in porosity and an increase in soil density. This has led to changes in the diversity, evenness, and abundance of the CH_4 microbial communities, composed by methanogenic organisms of the phylum *Euryarchaeota* and methanotrophs of the phyla *Proteobacteria* and *Verrucomicrobia*. Pastures consistently showed a greater abundance of methanogens than forests, as well as a higher rate of methanogens by methanotrophs, usually close to or above one. Moisture further intensified this effect, resulting in high CH_4 emissions from the soils of the experiment under 100% of moisture at field capacity. The main methanotrophic groups exhibited different responses to the studied factors, according to their ecological characteristics. In

summary, the data from both studies indicate that forest-to-pasture conversion increases the CH_4 emission potential of the soil, which is enhanced by increasing moisture.

Keywords: Land-use change. Soil moisture. Molecular microbial ecology. Soil methane cycle. Methanogenesis. Methanotrophy. Quantitative real-time PCR. Metagenomics.

RESUMO

VENTURINI, A. M. **Conversão floresta-pastagem na Amazônia Oriental: impactos sobre as comunidades microbianas do metano do solo**. 2019. 103 p. Tese (Doutorado em Ciências) - Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2019.

A conversão floresta-pastagem na Amazônia altera as propriedades físicas, químicas e biológicas do solo. Contudo, os impactos desse processo sobre os fluxos de metano (CH_4) – o segundo mais importante gás do efeito estufa de origem antropogênica – e microrganismos responsáveis pela sua produção (arquéias metanogênicas) e consumo (bactérias metanotróficas) em solos ainda são pouco conhecidos. Ambos os grupos ainda podem ser influenciados pelo teor de umidade desses solos, devido à sazonalidade e ocorrência de eventos extremos na região. Dessa forma, o objetivo dessa tese foi avaliar os impactos da conversão floresta-pastagem sobre o ciclo microbiano do CH_4 em solos da Floresta Nacional do Tapajós e seus arredores, na Amazônia Oriental Brasileira, bem como sua resposta a mudanças na umidade. No primeiro estudo, amostras de solo foram coletadas em três florestas e três pastagens durante as estações seca e chuvosa. No segundo, amostras de solo de ambos os usos foram utilizadas no desenvolvimento de um experimento de microcosmos com quatro níveis de umidade (umidade original; 60%, 80% e 100% de umidade na capacidade de campo) por um período de 30 dias. As amostras de solo de ambos os estudos tiveram suas propriedades determinadas, enquanto as suas comunidades microbianas foram avaliadas por PCR quantitativo em tempo real de genes marcadores do CH_4 e sequenciamento metagenômico. Amostras de gases do experimento de microcosmos também foram coletadas periodicamente e analisadas por cromatografia gasosa. Como resultados principais, as pastagens apresentaram pH e teor de nutrientes mais elevados do que as florestas, mas as amostras do campo revelaram uma diminuição da porosidade e um aumento da densidade do solo. Isso acarretou em mudanças na diversidade, equitatividade e abundância das comunidades microbianas do CH_4, compostas por organismos metanogênicos do filo *Euryarchaeota* e metanotróficos dos filos *Proteobacteria* e *Verrucomicrobia*. As pastagens apresentaram sistematicamente maior abundância de metanogênicos do que as florestas, assim como maior razão de metanogênicos por metanotróficos, comumente próxima ou acima de um. A umidade intensificou ainda mais esse efeito,

resultando na emissão elevada de CH_4 nos solos do experimento sob 100% de umidade na capacidade de campo. Os principais grupos metanotróficos exibiram respostas variadas aos fatores estudados, de acordo com suas características ecológicas. Em resumo, os dados de ambos os estudos indicam que a conversão floresta-pastagem aumenta o potencial de emissão de CH_4 do solo, o que é reforçado pelo aumento da umidade.

Palavras-chave: Mudança de uso do solo. Umidade do solo. Ecologia microbiana molecular. Ciclo do metano no solo. Metanogênese. Metanotrofia. PCR quantitativo em tempo real. Metagenômica.

SUMMARY

1 INTRODUCTION

The Brazilian Amazon has an approximate extension of 4.2 million km^2, which corresponds to 49% of the national territory, and harbors the largest reserve of biological diversity and hydrographic network of the planet (IBGE, 2004). The Amazon is also the largest biome in extension in Brazil (IBGE, 2004) but 20% of its original cover has already been cleared (NOBRE et al., 2016) – a process that was particularly intensified after the 1970s, from which high rates of deforestation were observed due to the expansion of agriculture and livestock, logging, fires, and increase of the human population (FEARNSIDE, 2005; ARRAES; MARIANO; SIMONASSI, 2012). Satellite data collected since 1988 has shown that the extent of deforestation varies among the states that constitute this biome: Pará has the highest clearing rates (34%), followed by Mato Grosso (33%) and Rondônia (14%) (INPE, 2019). Most of these lands (63%) have been converted into pastures – considered the main anthropogenic use of the region – but agricultural (6%) and secondary forest (23%) sites are also present (EMBRAPA, 2016). Although several efforts have been made since 2004 to overcome this issue (NEPSTAD et al., 2014), deforestation rates in the Amazon are still significant and have grown again. Compared to the lowest value ever recorded (2012), this rate increased by 73% in 2018, reaching 7,900 km^2 $year^{-1}$ (INPE, 2019).

Land-use changes are known to alter the physical, chemical, and biological characteristics of the soil (ABDELKADIR; YIMER, 2011), as previously described in other studies in the Amazon (RODRIGUES et al., 2013; MIRZA et al., 2014; PAULA et al., 2014; MENDES et al., 2015; NAVARRETE et al., 2015; RANJAN et al., 2015; HAMAOUI JUNIOR et al., 2016; MEYER et al., 2017). These processes, together with agricultural and livestock activities, are also considered as one of the major sources of greenhouse gases (GHG) in Brazil (BRASIL, 2017). Land-use, land-use change, and forestry themselves were responsible for 78% of the anthropic emissions in 2000 and, not surprisingly, the Amazon accounted for about 65% of this total (BRASIL, 2016). This biome also produces the largest methane (CH_4) emissions from this category, mainly due to the burning of biomass (BRASIL, 2017).

The study of CH_4 emissions from the Amazon is crucial since this gas is the third most important GHG after water vapor and carbon dioxide (CO_2) (HEIMANN, 2010) and has direct and indirect impacts on Earth's climate (CICERONE; OREMLAND, 1988). CH_4 absorbs infrared radiation and influences the atmospheric abundance of

other GHGs, such as water vapor, CO_2, and ozone (O_3), through chemical interactions (CRUTZEN, 1973; CICERONE; OREMLAND, 1988), thus having a global warming potential of 28 and a global temperature change potential of 4 relative to CO_2 over a 100-year period (MYHRE et al., 2013). Recent results based on updated calculations of the CH_4 radiative forcing even suggested that both of these metrics are in fact 14% higher than previously estimated (ETMINAN et al., 2016). In addition, as a precursor of O_3 (CRUTZEN, 1973), this gas also has a significant role in the productivity (COLLINS et al., 2018) and air quality of the terrestrial ecosystems (FIORE et al., 2002, 2008; DENTENER et al., 2005; WEST; FIORE, 2005; WEST et al., 2006).

CH_4 is formed through the metabolism of methanogens (methane-producing microorganisms), geological processes, and incomplete burning of biomass; and regardless the origin, from natural and anthropogenic sources (KIRSCHKE et al., 2013; SAUNOIS et al., 2016). Several studies have shown that wetlands are the main natural source of CH_4, but it can also be derived from freshwaters, geological sources, animals, termites, wildfires, oceans, coastal sediments, methane hydrates, and permafrost; while its anthropogenic sources include agriculture and waste, such as livestock, landfills and wastewater treatment, and rice cultivation; production, distribution, and use of fossil fuels; and burning of biomass and biofuels (CIAIS et al., 2013; KIRSCHKE et al., 2013; SAUNOIS et al., 2016; DEAN et al., 2018). On the other hand, the most important sink of this gas is its oxidation by hydroxyl radicals (OH) in the troposphere, a process that removes about 90% of the atmospheric CH_4 (EHHALT, 1974). CH_4 is also oxidized in soils by methanotrophs (methane-consuming microorganisms), the most important form of CH_4 loss of biological origin (POTTER; DAVIDSON; VERCHOT, 1996); lost in chemical reactions with atomic oxygen ($O(^1D)$), chlorine (Cl), fluorine (F), and OH in the stratosphere (CICERONE; OREMLAND, 1988), and with Cl in the marine boundary layer (ALLAN; STRUTHERS; LOWE, 2007). However, regarding the Amazon basin itself, although several studies have addressed the relationship between anthropogenic activities in the region (e.g., biomass burning, agricultural and livestock activities) and CH_4 emissions, little is known about how land-use changes – in particular, the process of forest-to-pasture conversion – impact soil CH_4 fluxes and the microbial groups responsible for its production and consumption.

CH_4 fluxes from soil and consequently its role as a source or a sink are related to the balance between methanogens and methanotrophs (CHEN et al., 2019). Methanogens are strictly anaerobic *Archaea* from the phyla *Euryarchaeota* (LYU; LIU,

2018), *Bathyarchaeota* (EVANS et al., 2015), and *Verstraetearchaeota* (VANWONTERGHEM et al., 2016) that produce CH_4 as the end-product of their respiration, using as substrate CO_2 and H_2 (or formate and few alcohols), methylated compounds, and acetate (LYU; LIU, 2018). In contrast, aerobic methanotrophs are *Bacteria* from the phyla *Proteobacteria* (HANSON; HANSON, 1996) and *Verrucomicrobia* (DUNFIELD et al., 2007; POL et al., 2007; ISLAM et al., 2008) capable of oxidizing CH_4 as their only carbon and energy source (HANSON; HANSON, 1996). Methanotrophy can also occur under anoxic conditions, but only in methane-oxidizing denitrifying bacteria of the candidate phylum *NC10* (ETTWIG et al., 2010) and archaea in consortia with sulfate-reducing bacteria by a reverse methanogenesis pathway (TIMMERS et al., 2017). A nitrate-dependent anaerobic CH_4 oxidation was also described for the archaeal species '*Candidatus Methanoperedens nitroreducens*' (HAROON et al., 2013; ARSHAD et al., 2015). In any case, both methanogenic and methanotrophic communities have distinct biochemical and ecological characteristics so that several environmental factors can influence them as well as soil CH_4 fluxes, especially moisture, water-filled pore space, and porosity (NAZARIES et al., 2013).

Currently, several molecular methods are available to access the attributes of the soil CH_4 microbial communities, but more realistic results can be obtained through integrative studies using various types of datasets (SEGATA et al., 2013). Quantitative real-time PCR (qPCR) has been widely used in Microbial Ecology to detect and quantify genes of interest in environmental samples (SMITH; OSBORN, 2009); whereas shotgun metagenomics allows the direct analysis of the DNA contained in them, revealing the taxonomic and functional potential of the microbiota (CARVALHAIS et al., 2012). In this thesis, both methods were applied to explore the impacts of forest-to-pasture conversion in the Eastern Amazon and the influence of environmental factors – moisture in particular – on the abundance, taxonomic and functional diversity of soil CH_4 microbial communities. This goal was achieved through two studies carried out in the field (Field Study) and in microcosms (Microcosm Study).

This thesis is part of the thematic project entitled "Dimensions US-BIOTA - São Paulo: Integrating dimensions of microbial biodiversity across land-use change in tropical forests", financed by the São Paulo Research Foundation (FAPESP, Process 2014/50320-4), which aims to understand the microbial CH_4 cycle along land-use gradients in the Amazon through a multidimensional approach, combining several methods of Environmental Biogeochemistry, Microbiology, and Molecular Biology.

1.1 Hypothesis

The general hypothesis of this thesis is that soil CH_4 microbial communities (methanogens and methanotrophs) are influenced by the process of forest-to-pasture conversion in the Eastern Amazon, also varying temporally according to the soil moisture.

1.2 Objectives

1.2.1 General objective

The main objective of this thesis was to evaluate the impacts of land-use change on the abundance, taxonomic and functional diversity of the CH_4 microbial communities from forest and pasture soils of the Tapajós National Forest, in the state of Pará, in the Brazilian Eastern Amazon, as well as their response to changes in moisture, considered a major driver of soil CH_4 fluxes.

1.2.2 Specific objectives

1. Chapter 2 - Field Study: To determine the impacts of land-use change on the abundance, taxonomic and functional diversity of the CH_4 microbial communities from forest and pasture soils of the Tapajós National Forest, in the Brazilian Eastern Amazon, during the dry and rainy seasons, through qPCR of CH_4 marker genes (*mcrA* and *pmoA*) and metagenomic shotgun sequencing.
2. Chapter 3 - Microcosm Study: To determine the effect of moisture on the CH_4 fluxes and the abundance, taxonomic and functional diversity of its related microbial communities from forest and pasture soils of the Tapajós National Forest, in the Brazilian Eastern Amazon, through a 30-day microcosm experiment with four moisture levels (original field moisture content, and 60%, 80%, and 100% of moisture at field capacity), followed by gas chromatography, qPCR of archaeal and bacterial 16S rRNA and CH_4 marker genes (*mcrA*, *pmoA*, and *mmoX*), and metagenomic shotgun sequencing.

1.3 Structure of the thesis

This thesis is composed of an introductory text followed by two studies in the form of scientific articles. The supplementary materials of both studies are available in the appendix section.

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2 SOIL METHANE MICROBIAL COMMUNITIES ARE INFLUENCED BY LAND-USE AND SEASONALITY IN THE EASTERN AMAZON

Abstract

Previous studies in the Amazon have revealed that forest-to-pasture conversion has the potential to turn soils from sinks to sources of CH_4. However, little is known about how this conversion influences CH_4-related soil microbial communities, composed of producers (methanogens) and consumers (methanotrophs) of this gas. This study explored the impacts of land-use change on the CH_4 microbial communities from forest and pasture soils of the Brazilian Eastern Amazon over two seasons. Soil samples were collected in transects established in three forests and three pasture sites in the Tapajós National Forest and its surroundings during the dry and rainy seasons. Following soil DNA extraction, the abundance, taxonomic and functional diversity of the methanogenic and methanotrophic communities were evaluated by quantitative real-time PCR of *mcrA* and *pmoA* genes, respectively, and metagenomic sequencing. For each sampling point, a set of textural, physical, and chemical properties was also determined. The pasture sites were characterized by higher pH and nutrient content, as well as greater soil compaction in relation to the forests. The communities of methanogenic *Archaea* and methanotrophic *Bacteria* found in our samples – the former from the phylum *Euryarchaeota* and the latter from *Proteobacteria* and *Verrucomicrobia* – responded to these changes and increased in diversity and evenness. The abundance of methanogens was highly influenced by land-use, but also by season and the interaction of both factors, presenting the highest results in pasture soils from the rainy season. Despite the variation observed in the methanotrophy data, the abundance ratios of the methanogenic by the methanotrophic community were always higher in the pastures. The main methanotrophic groups were also analyzed separately, showing distinct responses according to their ecological characteristics. Our results indicate that the soil CH_4 microbial communities of the Brazilian Eastern Amazon are mainly influenced by changes in land-use, also varying temporally according to the dry and rainy seasons.

Keywords: Tropical soils. Forest-to-pasture conversion. Moisture. Methanogens. Methanotrophs. Quantitative real-time PCR. Metagenomics.

2.1 Introduction

The transformation of forests and other natural environments into agricultural and pasture lands alters the physicochemical properties of the soil (SMITH et al., 2016) as well as threatens the biodiversity above- and below-ground (SALA et al., 2000; BARNES et al., 2017) and its ecological functions. Most of the converted areas worldwide have been used for livestock and related activities (such as croplands for feed production) so that pastures occupy 26% of the ice-free land surface and even greater proportions in the tropics (FAO, 2009). At one of the most important agricultural frontiers in the world – the Brazilian Amazon – (MAIA et al., 2010; NOLTE et al., 2017; GARRETT et al., 2018), the same pattern is observed. Recent data from the TerraClass project have shown that pastures are the main anthropogenic land-use in the basin (63%), followed by secondary forest (23%) and agriculture sites (6%); and this result may be even greater, since pastures can be included in sites with varying land-use (2%) (occupation mosaics) as well as due to the presence of unobserved areas (4%) (EMBRAPA, 2016). However, regardless of the purpose of the conversion, deforestation in the region, which had been reduced by more than 70% since 2004, has increased again, reaching in 2018 the highest levels in ten years (INPE, 2019).

This scenario is problematic since changes in land-use are also a major source of GHGs to the atmosphere, accounting for about 24% of the net emissions in Brazil in 2015 (BRASIL, 2017). The Amazon biome itself is the third-largest source of GHGs from this sector and the first of CH_4, mainly due to biomass burning (BRASIL, 2017). CH_4 is the third most important GHG after water vapor and CO_2 (HEIMANN, 2010) and has a global warming potential of 28 compared to CO_2 on a 100-year time horizon (MYHRE et al., 2013). Besides, studies in the Amazon have previously revealed that, after forest-to-pasture conversion, upland soils (generally considered as CH_4 sinks) started to exhibit lower consumption rates or even emission of this gas (STEUDLER et al., 1996; VERCHOT et al., 2000; FERNANDES et al., 2002). Even so, little is still known about how this conversion impacts methane-related soil microbial communities.

Two main groups are responsible for the microbial CH_4 cycle in soils: methanogens, strictly anaerobic methane-producing *Archaea* from the phyla *Euryarchaeota* (LYU; LIU, 2018), *Bathyarchaeota* (EVANS et al., 2015), and *Verstraetearchaeota* (VANWONTERGHEM et al., 2016), and methanotrophs, aerobic methane-consuming *Bacteria* from the phyla *Proteobacteria* (HANSON; HANSON,

1996) and *Verrucomicrobia* (DUNFIELD et al., 2007; POL et al., 2007; ISLAM et al., 2008). Methanogens and methanotrophs can be analyzed through several molecular methods using the genes encoding the enzymes that, respectively, catalyze the reduction of a methyl group bound to coenzyme-M and the release of CH_4 – methyl-coenzyme M reductase (MCR) (LUTON et al., 2002) – and the oxidation of CH_4 to methanol – particulate (pMMO) and soluble methane monooxygenases (sMMO) (HANSON; HANSON, 1996). The former MMO is present in all methanotrophic species, except from the genera *Methylocella* and *Methyloferula*; whereas the latter has a variable presence within the group (KNIEF, 2015; DEDYSH; KNIEF, 2018).

The main objective of this work was to determine the impacts of land-use change on the abundance, taxonomic and functional diversity of the CH_4 microbial communities from forest and pasture soils of the Tapajós National Forest, in the Brazilian Eastern Amazon. This goal was achieved through the collection of soil samples in three forest and three pasture sites, followed by DNA extraction, qPCR of CH_4 marker genes (*mcrA* and *pmoA*), and metagenomic shotgun sequencing. Since it is well known that moisture is one of the major factors influencing soil CH_4 fluxes (NAZARIES et al., 2013), the soil from each sampling point was analyzed twice: during the dry and rainy seasons. A diverse set of soil textural, physical, and chemical properties were also determined for each point and correlated with the molecular data.

2.2 Materials and methods

2.2.1 Site description, soil sampling, and determination of physicochemical properties

The studied sites are located in the Tapajós National Forest (FLONA Tapajós) and its surrounding areas, in the state of Pará, Brazil, Eastern Amazon. FLONA Tapajós is a federal conservation unit that comprises an area of 544,927 hectares (IBAMA, 2004). The region has a tropical monsoon climate (Am, according to the Köppen's classification), which is the most representative climate of Brazil and the Pará state itself, and is characterized by the average temperature of the coldest month equal or higher than 18 °C (ALVARES et al., 2013) and two well-defined seasons: rainy (between December and June) and dry (from the end of June) (DE OLIVEIRA JUNIOR et al., 2015). The mean annual air temperature is 25.5 °C (minimum of 21.0 °C and

maximum of 30.6 °C) and the mean annual precipitation is 1,820 mm (IBAMA, 2004). The study was conducted during the dry (November 2015) and rainy (May 2016) seasons in three forests (F1, F2, and F3) and three cattle pastures (P1, P2, and P3) (Figure 2.1). At each site, soil samples from 0 to 10 cm depth were collected in a 200-m transect composed of five sampling points, separated by 50 m each (Supplementary Table S2.1). The samples were transported to the laboratory in a refrigerated compartment and kept at -20 °C until further use.

Figure 2.1 - Google Earth satellite image comprising the city of Santarém, the Tapajós National Forest, and the three forest and three pasture sites sampled in this study, located in the state of Pará, Brazil, Eastern Amazon

About 600 g of each soil sample was sent to the Department of Soil Science of the Luiz de Queiroz College of Agriculture (ESALQ/USP), Piracicaba, in the state of São Paulo, Brazil, for the determination of the following physicochemical properties: pH determined in 0.01 M calcium chloride ($CaCl_2$); soil organic matter (SOM) determined by colorimetry; phosphorus (P) extracted with ion exchange resin and determined by the colorimetric method; sulfur (S) extracted with 0.01 M calcium phosphate ($Ca_3(PO_4)_2$) and determined by turbidimetry; potassium (K) extracted with ion exchange resin and determined by atomic emission spectrophotometry; calcium

(Ca) and magnesium (Mg) extracted with ion exchange resin and determined by atomic absorption spectrophotometry; exchangeable aluminum (Al) extracted with 1 M potassium chloride (KCl) and determined by the colorimetric method; potential acidity (H + Al) determined with the Shoemaker-McLean-Pratt (SMP) buffer; boron (B) extracted with hot water and determined by colorimetry; copper (Cu), iron (Fe), manganese (Mn), and zinc (Zn) extracted with DTPA and determined by atomic absorption spectrophotometry; sand, silt and clay contents determined by the hydrometer method and classified according to the United States Department of Agriculture textural classification system (USDA, 2019). The calculations of sum of exchangeable bases (SB), cation-exchange capacity (CEC), base saturation (V), and aluminum saturation (m) were made based on these previous results. In addition, undisturbed soil cores from each sampling point were previously collected in 2015 using a metal cylinder and sent to the Laboratory of Soil Physics of the Center for Nuclear Energy in Agriculture (CENA/USP), Piracicaba, São Paulo, Brazil, for the determination of soil density, total porosity (microporosity + macroporosity), microporosity, and macroporosity, using the methods described by Donagema et al. (2011). The soil physicochemical properties of the forest and pasture sites were compared for each sampling by the Kruskal-Wallis test on aggregated data in R studio 1.0.153 (RSTUDIO TEAM, 2016), as recommended by Logan (2010) for nested non-normal data. The soil chemical properties of the forest and pasture sites of both samplings were subjected to a non-metric multidimensional scaling (NMDS) based on the Gower's distance using the vegan package 2.5-1 (OKSANEN et al., 2018). The NMDS plot was generated using the ggplot2 package 3.0.0 (WICKHAM, 2016).

2.2.2 DNA extraction and quantification

The total DNA from each soil sample was extracted in duplicate using the PowerLyzer PowerSoil DNA Isolation Kit (MO Bio Laboratories, Inc., Carlsbad, CA, USA). The manufacturer’s protocol was followed, using 250 mg of each soil sample per extraction, except for three adjustments, previously optimized by Venturini et al. (personal communication[1]) for Amazon soils: at the initial stage, after adding the solution C1, the samples were vortexed for 15 min at maximum speed and centrifuged

[1]VENTURINI, A. M. et al. Robust DNA protocols for tropical soils. Submitted for publication.

for 3 min at 10,000 x g, as well as all the incubations after adding the solutions C2 and C3 were made at -20 °C instead of 4 °C. The concentration and purity of the DNA samples were evaluated by 1% agarose gel electrophoresis in sodium boric acid (SB) buffer (BRODY; KERN, 2004) stained with ethidium bromide and on a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The DNA samples were stored at -20 °C for molecular analysis.

2.2.3 Quantitative-real time PCR of CH_4 marker genes

The CH_4 marker genes methyl-coenzyme M reductase (*mcrA*) and particulate methane monooxygenase (*pmoA*) were quantified by quantitative real-time PCR through the standard-curve method using the primer pairs mlas-mod-F (5'-GGYGGTGTMGGDTTCACMCARTA-3') (ANGEL; CLAUS; CONRAD, 2012) and mcrA-rev (5'-CGTTCATBGCGTAGTTVGGRTAGT-3') (STEINBERG; REGAN, 2008) and A189 (5'-GGNGACTGGGACTTCTGG-3') (HOLMES et al., 1995) and MB661 (5'-CCGGMGCAACGTCYTTACC-3') (COSTELLO; LIDSTROM, 1999), respectively. The standard curves were prepared by the PCR amplification of each target gene and its serial dilution from 10^6 to 10^1 copies, using the DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) strains *Methanolinea mesophila* (DSM 23604) for *mcrA* and *Methylosinus sporium* (DSM 17706) for *pmoA*. For both, the qPCR of each DNA sample was performed in triplicate in a 10-µL reaction on a StepOnePlus equipment (Thermo Fisher Scientific, Inc., Waltham, MA, USA), containing 5 µL of SYBR Green ROX qPCR Master Mix (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 0.2 µl of BSA (20 mg ml^{-1}) (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 1 µl of each primer (5 pmol), 1 µl of DNA (10 ng), and ultra-pure H_2O to complete 10 µl. The amplification conditions for *mcrA* consisted of 95 °C for 10 min, 45 cycles of 95 °C for 30 s, 60 °C for 45 s and 72 °C for 30 s; and for *pmoA*, 95 °C for 10 min, 45 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 45 s. These were followed by a melting curve of 95 °C for 15 s, the annealing temperature of each gene for 1 min, and 95 °C for 15 s. The results were analyzed in StepOne Software v2.3 (Thermo Fisher Scientific, Inc., Waltham, MA, USA), exported as a spreadsheet, and converted into the number of gene copies per ng of DNA.

The abundance of each gene as well as the ratio of *mcrA* by *pmoA* was aligned rank-transformed and analyzed by linear mixed-effects models using the ARTool

package 0.10.5 (KAY; WOBBROCK, 2018) in R studio 1.0.153 (RSTUDIO TEAM, 2016), with sampling point (nested within site) and site (nested within land-use) included as random factors. Plots were generated using the ggplot2 package 3.0.0 (WICKHAM, 2016). The Spearman's rank correlation coefficient was calculated to determine the correlations between the abundances of both CH_4 marker genes and soil chemical properties, using the psycho package 0.3.6 (MAKOWSKI, 2018).

2.2.4 Metagenomic shotgun sequencing and bioinformatics

The DNA samples from the sampling points 1, 3, and 5 of all forest (F1, F2, and F3) and pasture (P1, P2, and P3) sites for both samplings were sent to Novogene Co. Ltd. (Beijing, China) for library construction using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) and paired-end shotgun sequencing (2 x 150 bp) on an Illumina HiSeq platform (Illumina, Inc., San Diego, CA, USA), with an expected raw data output of 12 Gb per sample. The forward reads were uploaded and analyzed on the MG-Rast server 4.0.3. (MEYER et al., 2008) using the default parameters. The taxonomic and functional classifications of the forward reads were performed against RefSeq (O'LEARY et al., 2016) and SEED (OVERBEEK et al., 2005) databases, respectively, using the default parameters, except for the alignment length, modified from 15 to 30 bp. On the MG-Rast server, the hits of each sample were filtered according to the RefSeq taxonomy in two groups: methanogenic *Archaea* and methanotrophic *Bacteria*. From the results, the methanogenic and methanotrophic communities were filtered again to obtain the functional hits related to the processes of methanogenesis – genes involved in the methanogenesis pathway and encoding the MCR enzyme – and methanotrophy – genes encoding pMMO and sMMO – according to the SEED subsystems, respectively. The relative abundance of each individual hit was calculated by its division by the total number of hits of each sample.

The parameters of the methanogenic and methanotrophic communities, such as species richness, Shannon's and Simpson's diversity, and Pielou's evenness were calculated using the vegan package 2.5-1 (OKSANEN et al., 2018) in R studio 1.0.153 (RSTUDIO TEAM, 2016). These indices, together with the relative abundances of the taxonomic and functional categories of interest, were analyzed by linear mixed-effects models, as previously described in this work. Plots were generated using ggplot2 3.0.0 (WICKHAM, 2016).

2.3 Results

2.3.1 Soil physicochemical properties

The studied sites varied in texture according to the USDA classification (2019): forest soils were classified as clay and sandy clay loam; and pasture soils, as clay, sandy clay, and sandy clay loam (Supplementary Tables S2.2 and S2.3). The results of micro- and macroporosity were similar between land-uses, considering a p of at least < 0.05, but the total porosity decreased, and the soil density increased after forest-to-pasture conversion (Supplementary Table S2.4). Soil samples from both land-uses, forests in particular, were acidic and of low fertility (RONQUIM, 2010), presenting distinct patterns as to their chemical properties (Figure 2.2). In both seasons, the pH, Ca, and Mg levels were higher in the pastures and, therefore, the calculations SB and V were as well; while the contents of Al, H+Al, and Fe together with CEC and m were higher in the forests. Although similar patterns were found for both seasons, the levels of S, K, and B changed with the land-use only during the dry season (S and B higher in the forests and K higher in the pastures) (Supplementary Tables S2.5 e S2.6).

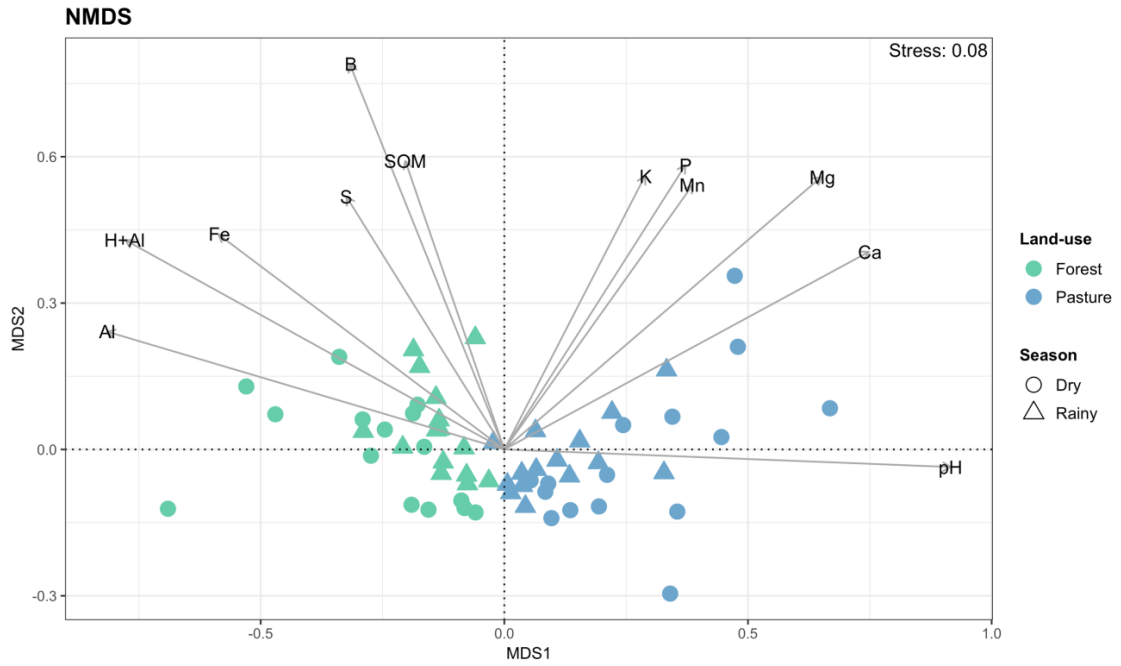

Figure 2.2 - Non-metric multidimensional scaling (NMDS) based on the Gower's distance of the soil chemical properties of the forest and pasture sites in the Tapajós National Forest and its surroundings during the dry and rainy seasons. Significant soil properties ($p < 0.05$) are shown in the arrows. SOM, soil organic matter; H+Al, potential acidity

2.3.2 Quantification of CH_4 marker genes and correlations with soil chemical properties

The qPCR assays presented R^2 values above 0.97 and amplification efficiencies between 90% and 102%, calculated based on the regression lines of each standard curve. The average number of copies per ng of DNA (copies ng DNA^{-1}) ranged from 8.40×10^{-1} to 6.59×10^{2} for *mcrA* and 6.45×10^{0} to 8.23×10^{1} for *pmoA*. The abundances of the CH_4 marker genes, as well as the ratio of *mcrA* by *pmoA*, were influenced by land-use, season, and their interaction (Table 2.1 and Figure 2.3).

Table 2.1 - Results of the non-parametric linear mixed-effects models (degrees of freedom, F-ratios, and p-values) for the quantification of CH_4 marker genes by qPCR, and taxonomic and functional profiling of the CH_4 microbial communities by metagenomics

Data	Land-use			Season			Land-use × Season		
	df	F	p	df	F	p	df	F	p
Gene quantification									
mcrA	1, 4	26.790	0.007	1, 28	35.417	< 0.001	1, 28	35.417	< 0.001
pmoA	1, 4	9.182	0.039	1, 28	20.448	< 0.001	1, 28	16.906	< 0.001
mcrA:*pmoA* ratio	1, 4	17.534	0.014	1, 28	14.177	< 0.001	1, 28	12.593	0.001
Taxonomic profiling									
Methanogenic taxa									
Shannon's diversity	1, 4	18.715	0.012	1, 16	2.893	0.108	1, 16	2.444	0.138
Simpson's diversity	1, 4	24.054	0.008	1, 16	2.445	0.137	1, 16	3.058	0.099
Pielou's evenness	1, 4	24.054	0.008	1, 16	2.445	0.137	1, 16	3.058	0.099
Relative abundance	1, 4	14.550	0.019	1, 16	7.221	0.016	1, 16	5.635	0.030
Methanotrophic taxa									
Shannon's diversity	1, 4	25.598	0.007	1, 16	2.027	0.174	1, 16	3.751	0.071
Simpson's diversity	1, 4	23.984	0.008	1, 16	1.098	0.310	1, 16	4.035	0.062
Pielou's evenness	1, 4	23.984	0.008	1, 16	1.098	0.310	1, 16	4.035	0.062
Relative abundance	1, 4	63.425	0.001	1, 16	2.757	0.116	1, 16	0.149	0.705
Methanogens:methanotrophs ratio									
Relative abundance	1, 4	40.313	0.003	1, 16	1.343	0.264	1, 16	3.066	0.099
Functional profiling									
Methanogenesis	1, 4	39.572	0.003	1, 16	0.026	0.873	1, 16	1.081	0.314
MCR	1, 4	17.876	0.013	1, 16	5.152	0.037	1, 16	7.026	0.017
pMMO	1, 4	1.187	0.337	1, 16	1.229	0.284	1, 16	0.121	0.732
sMMO	1, 4	0.008	0.932	1, 16	0.394	0.539	1, 16	0.831	0.375
MCR:(pMMO+sMMO) ratio	1, 4	48.682	0.002	1, 16	0.536	0.475	1, 16	1.797	0.199

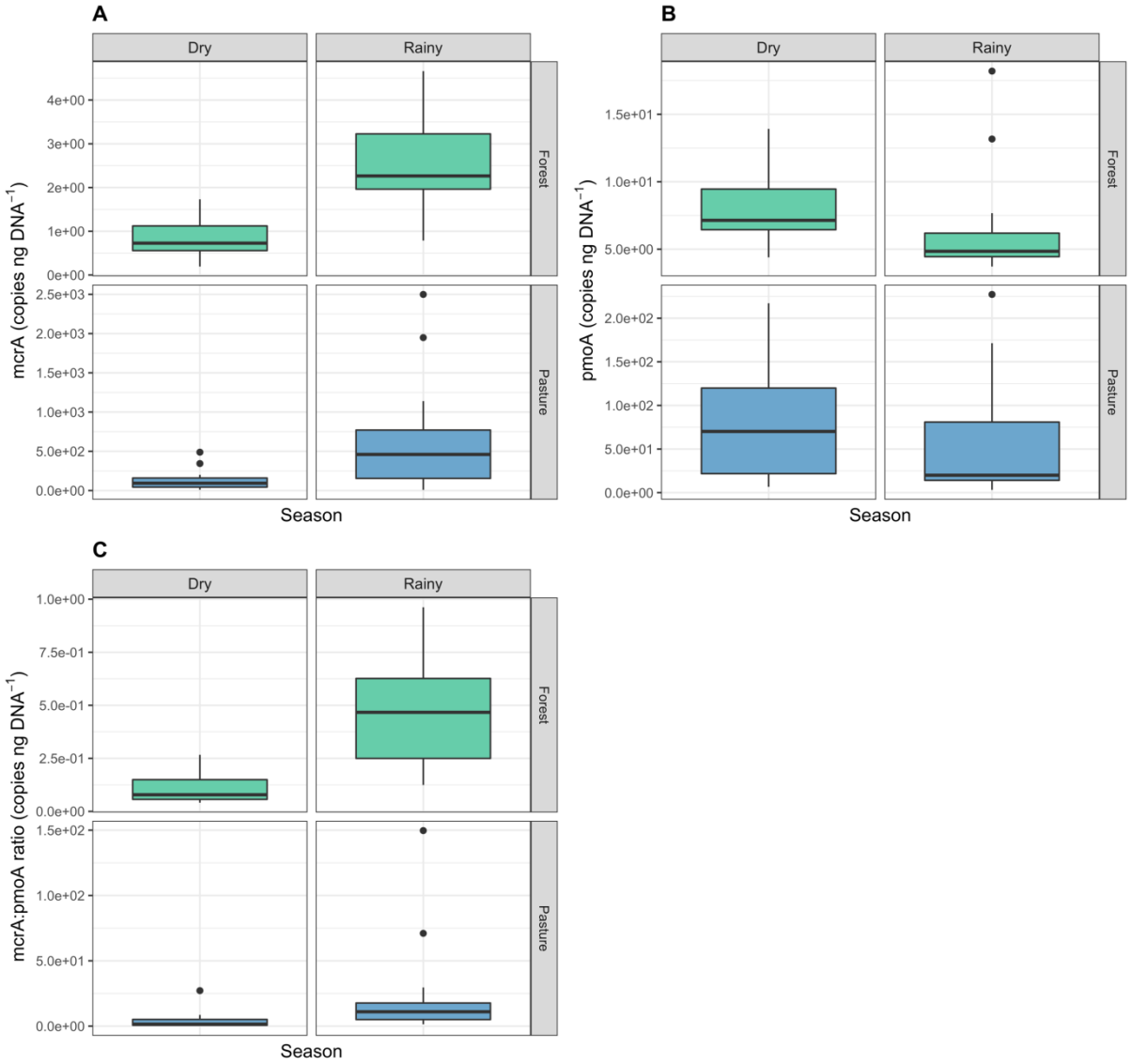

Figure 2.3 - Number of copies per ng of DNA (copies ng DNA^{-1}) obtained by qPCR of (A) *mcrA*, (B) *pmoA*, and (C) *mcrA*:*pmoA* ratio in the forest and pasture soils during the dry and rainy seasons

Pasture soils presented the highest quantification for both genes. Thus, the abundance ratio of most of the samples from this land-use was higher than one, particularly during the rainy season, indicating the presence of more methanogens than methanotrophs. In contrast, forest soils always exhibited results lower than one. The Spearman's test (p at least < 0.05) showed that the abundance of both genes shared a positive correlation (Table 2.2). These were also positively correlated with the most abundant soil properties in the pastures – pH, Ca, Mg, SB, and V – and thus negatively correlated with Al, H+Al, CEC, m, and Fe. The other soil properties were only associated with one or none of the genes.

Table 2.2 - Spearman's correlations between the qPCR abundances of CH_4 marker genes and soil chemical properties of the forest and pasture soils during the dry and rainy seasons

Data	*mcrA*	*pmoA*	*mcrA*:*pmoA* ratio
Gene quantification			
mcrA	-		
pmoA	0.61***	-	
mcrA:*pmoA* ratio	0.92***	0.32	-
Soil chemical properties			
pH	0.76***	0.72***	0.6***
SOM	-0.25	-0.24	-0.17
P	0.38	-0.07	0.47*
S	-0.47*	-0.2	-0.4
K	0.22	0.29	0.17
Ca	0.76***	0.58***	0.65***
Mg	0.65***	0.58***	0.54***
Al	-0.78***	-0.76***	-0.6***
H+Al	-0.82***	-0.67***	-0.66***
SB	0.73***	0.59***	0.62***
CEC	-0.78***	-0.6***	-0.65***
V	0.82***	0.64***	0.69***
m	-0.78***	-0.68***	-0.64***
B	-0.41	-0.63***	-0.21
Cu	0.21	0.17	0.2
Fe	-0.49**	-0.54**	-0.31
Mn	0.41	0.2	0.42
Zn	0.35	0.4	0.25

*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

2.3.3 Taxonomic profiling of the CH_4 microbial communities

The metagenomic shotgun sequencing – carried out based on 18 sampling points in forest and pasture sites of the Eastern Amazon during the dry and rainy seasons – resulted in an average of 47.7 million reads per sample, composed of bases with Phred quality score mostly above 20 (95.4%) and 30 (89.1%) (Supplementary Table S2.7). Of this total, an average of 41.8 million reads with about 150 bp in length remained per sample after quality control on the MG-Rast server 4.0.3 (MEYER et al., 2008) (Supplementary Table S2.8). Detailed information about the results of the annotations can be found in the Supplementary Table S2.9. The taxonomic classification made against RefSeq (O'LEARY et al., 2016) revealed that our libraries

were dominated by *Bacteria* (98.22%), *Archaea* (0.95%), and *Eukaryota* (0.80%). Other groups, including viruses and viroids, were related to only 0.03% of the hits.

The methanogenic microorganisms found belong to the archaeal phylum *Euryarchaeota*, from the classes *Methanomicrobia* (76.6%), *Methanococci* (10.3%), *Methanobacteria* (10.2%), and *Methanopyri* (3.0%) (Supplementary Table S2.10). The most abundant genus was *Methanosarcina*, followed by *Methanoregula*, *Methanoculleus*, *Methanothermobacter*, and *Methanocaldococcus* (Figure 2.4A). This community had the same richness among treatments, but its diversity and evenness indices were higher in the pastures (Tables 2.1 and 2.3). Its relative abundance changed according to the land-use, season, and their interaction (Figure 2.5A), presenting the highest values for pasture soils, particularly from the rainy season.

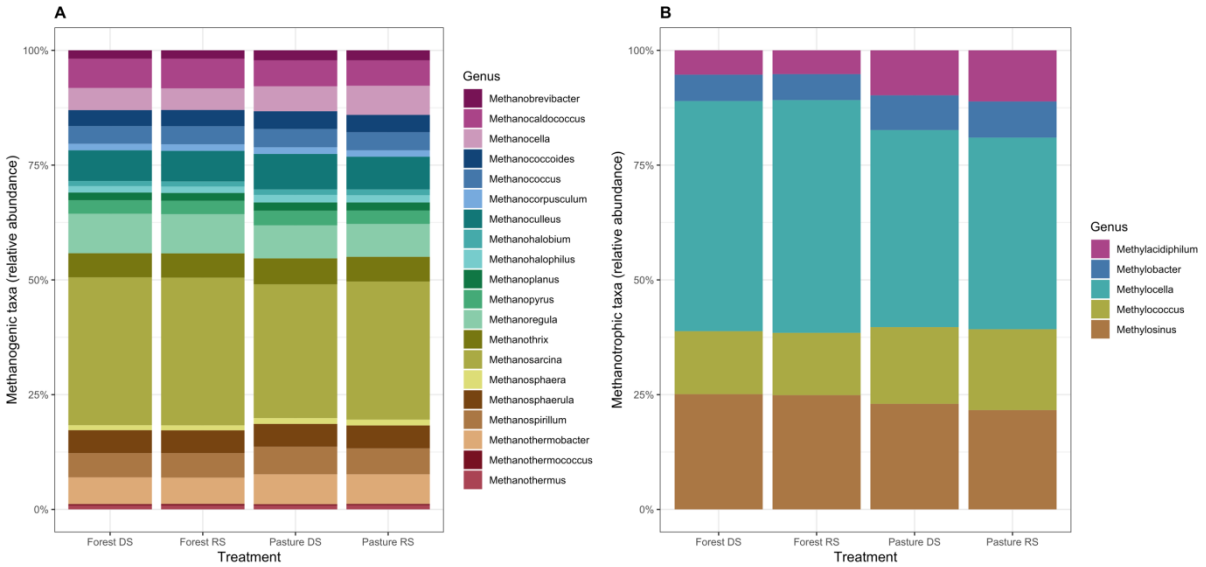

Figure 2.4 - Taxonomic composition obtained by metagenomics of the (A) archaeal methanogenic and (B) bacterial methanotrophic communities at the genus level in the forest and pasture soils during the dry and rainy seasons. DS, dry season; RS, rainy season

Table 2.3 - Richness, Shannon's diversity, Simpson's diversity, and Pielou's evenness (mean and standard deviation) of the archaeal methanogenic and bacterial methanotrophic communities at the genus level in the forest and pasture soils during the dry and rainy seasons

Land-use	Season	Methanogenic taxa				Methanotrophic taxa			
		Nº	Shannon	Simpson	Pielou	Nº	Shannon	Simpson	Pielou
F	DS	20	2.469 ± 0.019	0.862 ± 0.004	0.288 ± 0.001	5	1.284 ± 0.025	0.660 ± 0.010	0.410 ± 0.006
	RS	20	2.471 ± 0.020	0.862 ± 0.005	0.288 ± 0.002	5	1.276 ± 0.028	0.656 ± 0.011	0.408 ± 0.007
P	DS	20	2.537 ± 0.032	0.878 ± 0.007	0.293 ± 0.002	5	1.419 ± 0.039	0.718 ± 0.017	0.446 ± 0.011
	RS	20	2.520 ± 0.016	0.874 ± 0.004	0.292 ± 0.001	5	1.442 ± 0.046	0.727 ± 0.021	0.452 ± 0.013

F, forest; P, pasture; DS, dry season; RS, rainy season

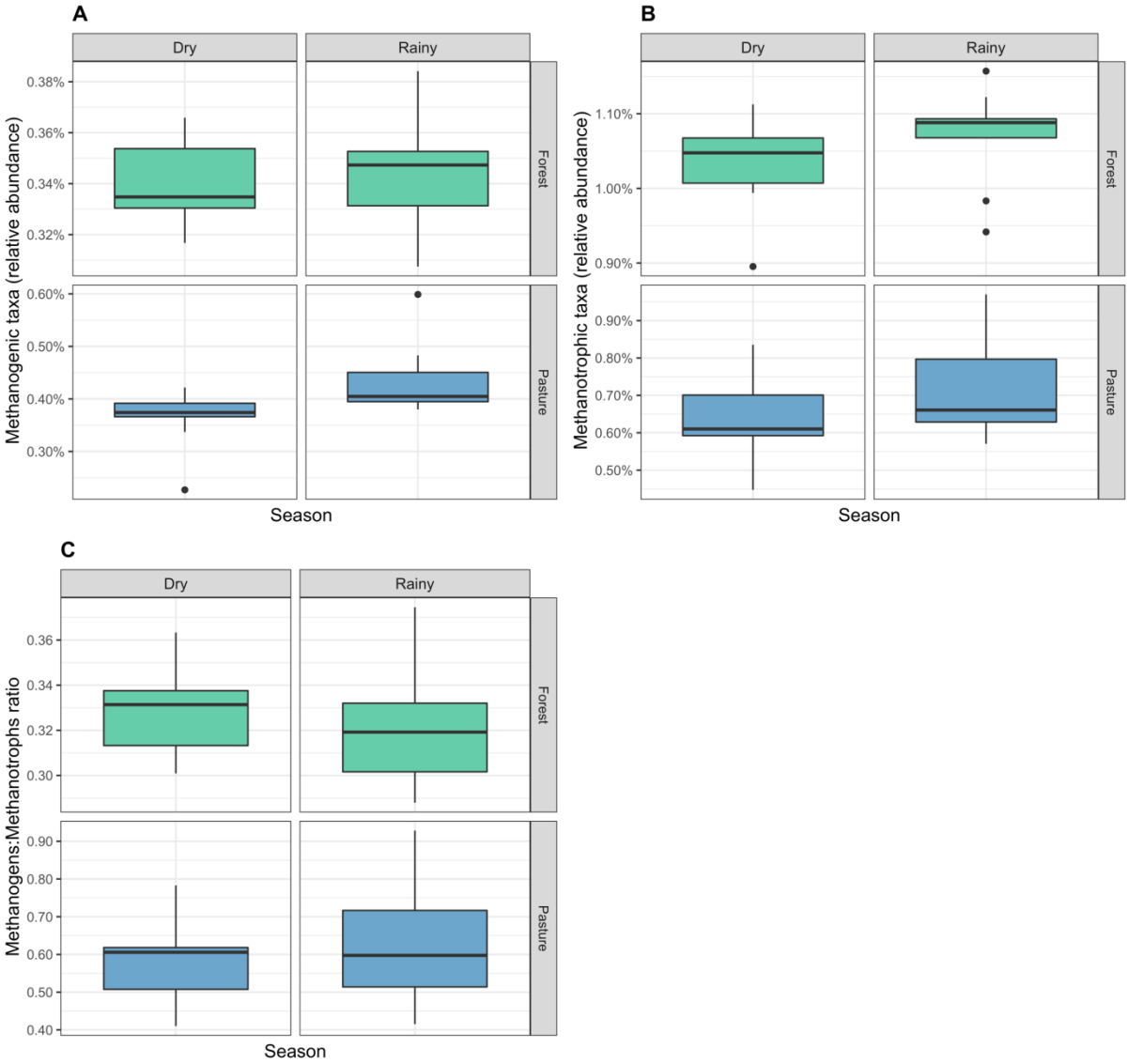

Figure 2.5 - Relative abundance obtained by metagenomics of the (A) methanogenic taxa, (B) methanotrophic taxa, and (C) methanogens:methanotrophs ratio in the forest and pasture soils during the dry and rainy seasons

The methanotrophic microorganisms belong to the bacterial phyla *Proteobacteria*, from the classes *Alphaproteobacteria* (70.0%) and *Gammaproteobacteria* (22.1%), and *Verrucomicrobia*, from *Methylacidiphilae* (7.8%) (Supplementary Table S2.11). *Methylocella* was the most abundant genus, followed by *Methylosinus*, *Methylococcus*, *Methylacidiphilum*, and *Methylobacter* (Figure 2.4B). Similar to the methanogens, this community presented higher diversity and evenness indices in the pastures (Tables 2.1 and 2.3). However, its relative abundance was higher in forest soils (Figure 2.5B). Not surprisingly, the abundance ratio of methanogens by methanotrophs was significantly higher in the pastures, with values closer to one (Figure 2.5C). The main methanotrophic groups were also

analyzed separately and presented distinct trends regarding their attributes, as shown in the Supplementary Tables S2.12 and S2.13 and Supplementary Figure S2.1.

2.3.4 Functional profiling of the CH_4 microbial communities

The functional classification made against the SEED database (OVERBEEK et al., 2005) revealed that the relative abundance of the genes of the methanogenic community involved in methanogenesis was significantly higher in the pastures (Table 2.1 and Figure 2.6A); while the genes encoding the MCR enzyme were significantly influenced by land-use, season, and their interaction, presenting the highest values in the pasture soils, especially during the rainy season (Figure 2.6B). On the other hand, the genes of the methanotrophic community encoding pMMO and sMMO were similar among treatments (Figures 2.6C and 2.6D). The MCR:(pMMO+sMMO) ratio was higher in pastures, with mean values higher than one for both seasons (Figure 2.6E).

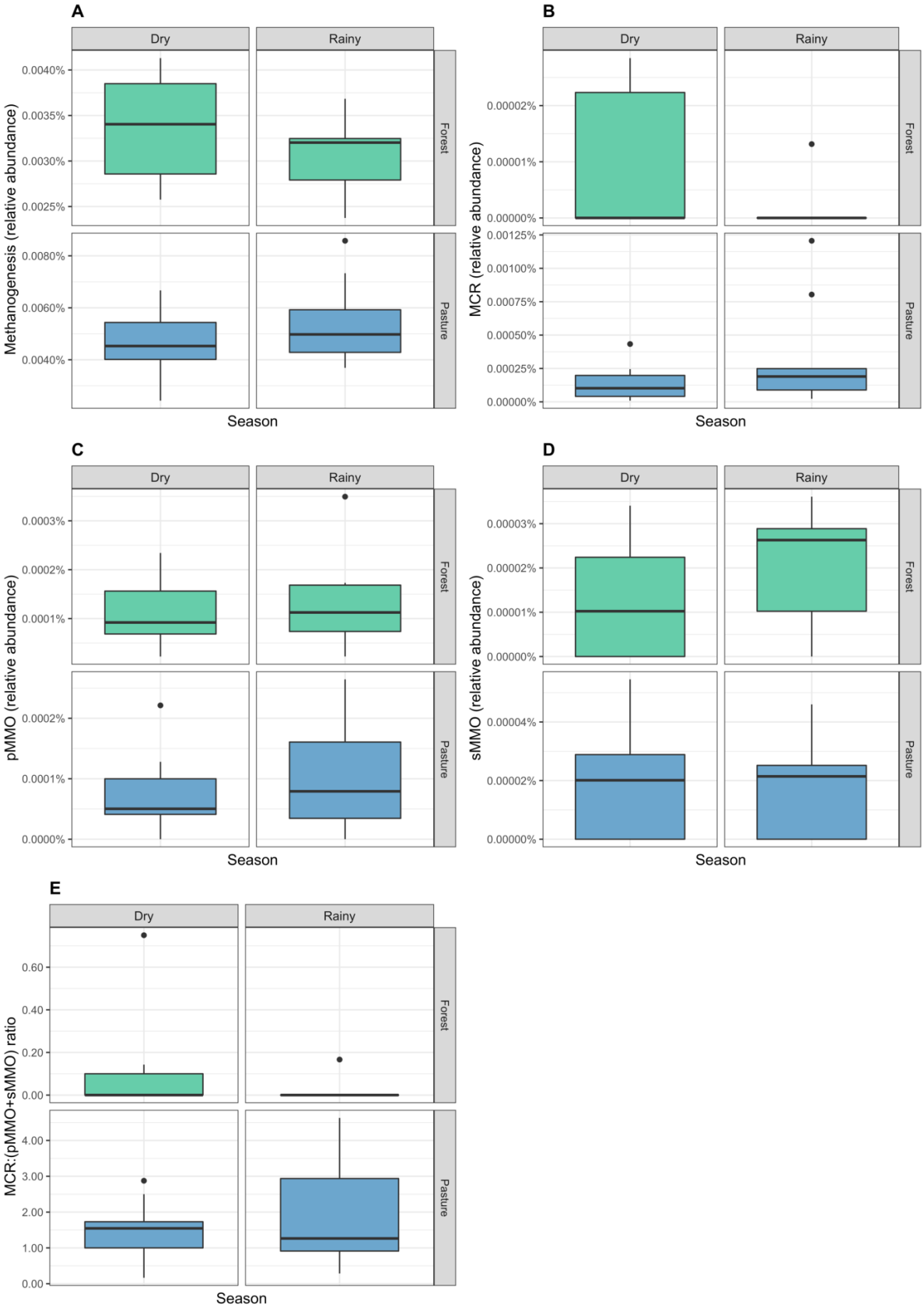

Figure 2.6 - Relative abundance obtained by metagenomics of the genes (A) involved in methanogenesis, (B) encoding the methyl-coenzyme M reductase enzyme (MCR), (C) encoding the particulate methane monooxygenase (pMMO), (D) encoding the soluble methane monooxygenase (sMMO), and (E) MCR:(pMMO+sMMO) ratio in the forest and pasture soils during the dry and rainy seasons

2.4 Discussion

In this work, in order to evaluate the impacts of land-use change on the abundance, taxonomic and functional diversity of the soil CH_4 microbial communities of the Brazilian Eastern Amazon, we collected samples from three forests and three pastures in the Tapajós National Forest and its surrounding areas during the dry (November 2015) and rainy (May 2016) seasons. The soil samples had their textural, physical, and chemical properties determined, whereas their archaeal methanogenic and bacterial methanotrophic communities were evaluated by qPCR of CH_4 marker genes and metagenomic shotgun sequencing.

Similar to most of the Amazon basin, the Tapajós National Forest is mainly composed of naturally weathered, highly acidic, and low-fertile (MANN, 2002; GLASER; BIRK, 2012) oxisols and ultisols (SILVER et al., 2000; IBAMA, 2004). Accordingly, our forest sites had lower pH and higher values of Al, H+Al, and m compared to the pastures, in amounts (m higher than 45%) even considered to be as extremely harmful to plants (OSAKI, 1991). The CEC results were also higher for the forests due to their Al and Fe contents, which are the main colloids responsible for this property in tropical soils (RONQUIM, 2010). However, the process of forest-to-pasture conversion, typically carried out in this region by cutting and burning the natural vegetation (known as slash-and-burn method), provides nutrients and raises soil pH after ash deposition (JUO; MANU, 1996; GIARDINA et al., 2000; ARAÚJO et al., 2011), as observed in our pastures (e.g., pH, K, Ca, Mg, SB, and V were higher in the pastures in at least one season). Juo and Manu (1996) demonstrated that Ca, K, and Mg, in that order, are the most important nutrients released in the soil through these processes.

The forest-to-pasture conversion also influenced the physical properties of our samples, which resulted in a decrease in total porosity and an increase in soil density, thus revealing a tendency for compaction. These changes were previously associated in studies in the Amazon with the use of heavy machinery and cattle trampling in pastures (MÜLLER et al., 2001; ARAÚJO et al., 2004; 2011; VALLADARES; BATISTELLA; PEREIRA, 2011; MELO et al., 2017), which can increase according to the age of the system (ARAÚJO et al., 2011). On the other hand, the forest soils had higher porosity, since this environment is less disturbed and more protected from the weather as well as from drying and wetting cycles, as explained by Araújo et al. (2004).

The impacts of the forest-to-pasture conversion on the physical and chemical properties of our soil samples have led to several changes in their CH_4 microbial communities. The reduction of soil porosity – recognized as one of the most important drivers of soil CH_4 fluxes (NAZARIES et al., 2013) – and in consequence, its aeration, favors the activity of methanogenic microorganisms in pastures (STEUDLER et al., 1996). As a result, the taxonomical attributes of the methanogenic community and the abundance of genes related to the CH_4 production in our sites, evaluated either by qPCR or by shotgun metagenomics, were influenced by land-use. Therefore, this community – mostly dominated by members of the class *Methanomicrobia* of the phylum *Euryarchaeota* – showed higher diversity and evenness in the pastures in comparison to the forests as well as abundance, which is in agreement with previous findings (RADL et al., 2007; BRADLEY et al., 2012; KOUBOVÁ et al., 2012; CHROŇÁKOVÁ et al., 2015; WANG et al., 2016), including one study in the Western Amazon (MEYER et al., 2017). These results, which can also be associated with the input of methanogens into the pasture soils through the cattle manure (GATTINGER et al., 2007; RADL et al., 2007; PREM; REITSCHULER; ILLMER, 2014; CHROŇÁKOVÁ et al., 2015) and even their pH preferences (MICHAŁ et al., 2018), indicate that this community (considering the factors analyzed in this work) were primarily influenced by long-term changes. However, most of its abundance data were also influenced by season (and its interaction with the land-use was also significant), presenting more pronounced values in the samples from the rainy season due to the increase in soil moisture. Depending on the dataset, for example, the abundance found in the pastures was up to five times higher in the rainy season than in the dry season.

The results of the methanotrophic community – composed by members of the classes Alpha and Gamma of *Proteobacteria* and *Methylacidiphilae* of *Verrucomicrobia* – reinforced the impacts of the forest-to-pasture conversion on the microbial CH_4 cycle, revealing a higher diversity and evenness of this group in the pastures in comparison to the forests. Analyzing the main methanotrophic groups separately, *Gammaproteobacteria* presented similar results to the entire community. On the other hand, although *Alphaproteobacteria* showed a comparable tendency, its results were only significantly influenced by season (considered higher during the dry period). Through a microcosm experiment, Ho et al. (2016) demonstrated that this methanotrophic group is resistant to severe drought and heat stress, surviving better

in dry conditions, whereas gammaproteobacterial members are known to present the opposite behavior (SILJANEN et al., 2011).

The abundance of the methanotrophic community was also influenced by land-use, but distinct results were found between our datasets. A recent meta-analysis by McDaniel et al. (2019) exhibited a negative correlation between the number of methanotrophs (using a *pmoA* dataset) in the soil and alterations in land-use, which is similar to our metagenomic data for the total methanotrophic community but contrary to the qPCR results. In addition to both of these datasets being composed of distinct methanotrophs, this variation can be due to the fact that the *pmoA* primers used in our study do not capture members of the *Verrucomicrobia* phylum (DEDYSH; DUNFIELD, 2011; GHASHGHAVI; JETTEN; LÜKE, 2017). Separately, alphaproteobacterial methanotrophs were more abundant in forest soils, as described by Meyer et al. (2017) due to their advantage in low-nutrient environments. The abundance of *Gammaproteobacteria* was influenced by land-use, season, and their interaction, while *Verrucomicrobia* was higher in the pasture sites. Ranjan et al. (2015) reported similar results regarding the total community of this phylum in forest and pasture soils in the Western Amazon. Lastly, the comparison between the abundance of methanogens by methanotrophs produced homogeneous results: pastures always had higher abundance ratios than forests, often presenting more CH_4 producers than consumers.

2.5 Conclusions

Our results demonstrate that the soil CH_4 microbial communities of the Brazilian Eastern Amazon are mainly influenced by the forest-to-pasture conversion, which increased the diversity and evenness of both methanogenic and methanotrophic communities. Physicochemical changes led to an increase in the abundance of methanogens, which were further stimulated during the rainy season. The main methanotrophic groups responded differently to the factors studied, but the pastures always presented a higher ratio of methanogens by methanotrophs than the forests.

Our work is the first to evaluate these communities based on samplings carried out in site replicates of the same land-use and over two seasons. The use of this methodology ensured that the results found were not simply related to specific characteristics of the study sites or the sampling date, thus reinforcing previous

evidence that changes in land-use impact the soil CH_4 microbial communities of this region and showing that these groups are also influenced by seasonal variation.

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3 MOISTURE DIFFERENTLY AFFECTS METHANE FLUXES AND MICROBIAL COMMUNITIES FROM FOREST AND PASTURE SOILS OF THE EASTERN AMAZON

Abstract

Climate changes in the Amazon have the potential to alter the precipitation regime on a regional scale and, consequently, soil moisture. Moisture is a major driver of CH_4 fluxes from soil, which are controlled by the balance between the activity of microbial producers (methanogens) and consumers (methanotrophs). Our study focused on the influence of soil moisture on the CH_4 fluxes and microbial communities from the main land-uses of the Brazilian Eastern Amazon: forest and pasture. Based on the moisture and field capacity (FC) of each soil, a microcosm experiment was established with four treatments: original moisture; 60%, 80%, and 100% of FC. The experiment was carried out for a 30-day period, in which gas samples were periodically collected for gas chromatographic analysis. Following DNA extraction, the abundance of *Archaea*, *Bacteria*, methanogens (*mcrA*), and methanotrophs (*pmoA* and *mmoX*) was evaluated by qPCR; and their taxonomic and functional profile, by metagenomic sequencing. As main results, cumulative CO_2 emissions did not change among treatments. CH_4 daily fluxes presented positive and negative results for forest and pasture, demonstrating that soil can act as a source and a sink for this gas, while its cumulative fluxes were strongly correlated with the moisture content. These results, as well as the abundance of methanogenesis genes and related groups, analyzed by qPCR and metagenomics, were influenced by land-use, moisture, and their interaction, with the highest emission and abundance values found in pasture soils of the 100% FC treatment. Although the methanotrophy results showed greater variation between the datasets, similar trends were found considering the ratio of methanogens by methanotrophs, so that even under field capacity conditions, pasture soils presented higher values compared to the forest. The methanogenic community was composed exclusively of *Archaea* from the *Euryarchaeota* phylum and did not change in diversity and evenness after the experimental period. *Proteobacteria* of the alpha (72.1%) and gamma (20.7%) classes were the most dominant methanotrophs followed by *Verrucomicrobia* (7.3%). The diversity and evenness indices of the methanotrophic community were affected by both studied factors, presenting the highest values for pasture soils under 100% FC. Taken

together, our results showed that soil CH_4 fluxes and related microbial communities responded to changes in land-use and moisture so that pasture exhibited a greater emission potential than forest soils, which was enhanced by increasing moisture.

Keywords: Tropical soils. Land-use change. Forest-to-pasture conversion. Methanogens. Methanotrophs. Gas chromatography. Quantitative real-time PCR. Metagenomics.

3.1 Introduction

The Amazon rainforest is considered one of the Earth's most important reservoirs of biodiversity (HECKENBERGER et al., 2007), in addition to playing crucial roles at local, regional and global scales, such as water and nutrient recycling, watershed protection, erosion and fire control, climate regulation, and carbon storage (FOLEY et al., 2007; ANDERSEN, 2015). Especially since the 1970s, this region has been deforested and transformed into lands for agricultural and livestock activities (FEARNSIDE, 2005; ARRAES; MARIANO; SIMONASSI, 2012), in a process that has already resulted in the removal of 20% of the original forest cover (NOBRE et al., 2016), with about 60% of this total subsequently converted into pastures (INPE, 2016).

However, forest-to-pasture conversion is not the only environmental change of anthropogenic origin that is occurring in the Amazon. Its average temperature has increased by 0.6-0.7 °C in the last 40 years, reaching around 6 °C by 2100 according to climate change predictions, and even higher values in the Eastern region (MARENGO et al., 2018). Some studies have also shown that the precipitation has increased in the rainy season and throughout the year over the last decades in some parts of the basin but has decreased in the dry season, so that the contrast between both seasons is growing (GLOOR et al., 2013; GLOOR et al., 2015; ALMEIDA et al., 2017). The occurrence of extreme events, such as floods and droughts, although part of the natural climate of the region (MARENGO et al., 2013), has also increased (GLOOR et al., 2015). In this sense, these changes in the precipitation regime and, consequently, in the soil moisture content, can impact GHG emissions from the Amazon since moisture is a major driver of soil CH_4 fluxes (NAZARIES et al., 2013).

Soil CH_4 fluxes are controlled by the balance between the activity of microbial producers – methanogens – and consumers – methanotrophs – (CHEN et al., 2019), which differ in their taxonomic, functional and ecological characteristics. Methanogens are strictly anaerobic *Archaea* that produce CH_4 as the final product of their respiration using as substrate CO_2 and H_2 (or formate and few alcohols), methylated compounds, and acetate, and are therefore classified as hydrogenotrophs, methylotrophs, and acetotrophs, respectively (LYU; LIU, 2018). Most known methanogens belong to the phylum *Euryarchaeota* (LYU; LIU, 2018), but new members of the phyla *Bathyarchaeota* (EVANS et al., 2015) and *Verstraetearchaeota* (VANWONTERGHEM et al., 2016) were also described. These microorganisms can be detected, quantified

and characterized through the *mcrA* gene, which encodes the alpha subunit of MCR, the terminal enzyme in the CH_4 formation common to all methanogenic pathways (LUTON et al., 2002; LYU et al., 2018).

On the other hand, aerobic methanotrophs are *Bacteria* from the phyla *Proteobacteria* (HANSON; HANSON, 1996) and *Verrucomicrobia* (DUNFIELD et al., 2007; POL et al., 2007; ISLAM et al., 2008) capable of oxidizing CH_4 as their only source of energy and carbon (HANSON; HANSON, 1996). Its proteobacterial members of the classes *Gammaproteobacteria* and *Alphaproteobacteria* are also commonly known as Type I and II methanotrophs, mostly due to their main carbon fixation pathway (KNIEF, 2015). Similar to methanogens, these microorganisms can be studied through functional genes related to MMO, the enzyme responsible for the oxidation of methane to methanol: *pmoA* encodes the beta subunit of its particulate form (pMMO) and is present in all methanotrophs except *Methylocella* and *Methyloferula*; whereas *mmoX* encodes the alpha subunit of its soluble form (sMMO), but has a variable presence among methanotrophs even within the same genus (KNIEF, 2015; DEDYSH; KNIEF, 2018). Methanotrophy has also been reported under anaerobic conditions in bacteria of the candidate phylum *NC10* (ETTWIG et al., 2010), methane-oxidizing archaea in consortia with sulfate-reducing bacteria (TIMMERS et al., 2017), and in the archaeal species '*Candidatus Methanoperedens nitroreducens*' (HAROON et al., 2013; ARSHAD et al., 2015).

The main objective of this work was to determine the effect of moisture on the abundance, taxonomic and functional diversity of the CH_4 microbial communities from forest and pasture soils of the Tapajós National Forest, in the Brazilian Eastern Amazon, through a microcosm experiment using four moisture levels (original moisture, as found in the field; 60%, 80%, and 100% of moisture at field capacity), followed by soil DNA extraction, qPCR of archaeal and bacterial 16S rRNA and CH_4 marker genes (*mcrA*, *pmoA*, and *mmoX*), and metagenomic shotgun sequencing. Throughout the experimental period, gas samples were also periodically collected for gas chromatographic analysis and later correlated with the molecular data.

3.2 Materials and methods

3.2.1 Site description, soil sampling, and determination of physicochemical properties

The studied sites are located in the Tapajós National Forest (known as FLONA Tapajós) and its surroundings, in the state of Pará, in the Brazilian Eastern Amazon. In July 2015, soil samples from 0 to 10 cm depth were collected in a forest (3°17'44.4"S 54°57'46.7"W) and an adjacent active cattle pasture (3°18'46.7"S 54°54'34.8"W), as previously described in Chapter 2 (Field Study). This pasture site was cleared more than 25 years before the sampling date and, according to the owners, converted into pasture in 2007 after being used for agriculture (Figure 3.1). The soil was transported to the laboratory and sieved through a 5 mm mesh sieve to remove litter material.

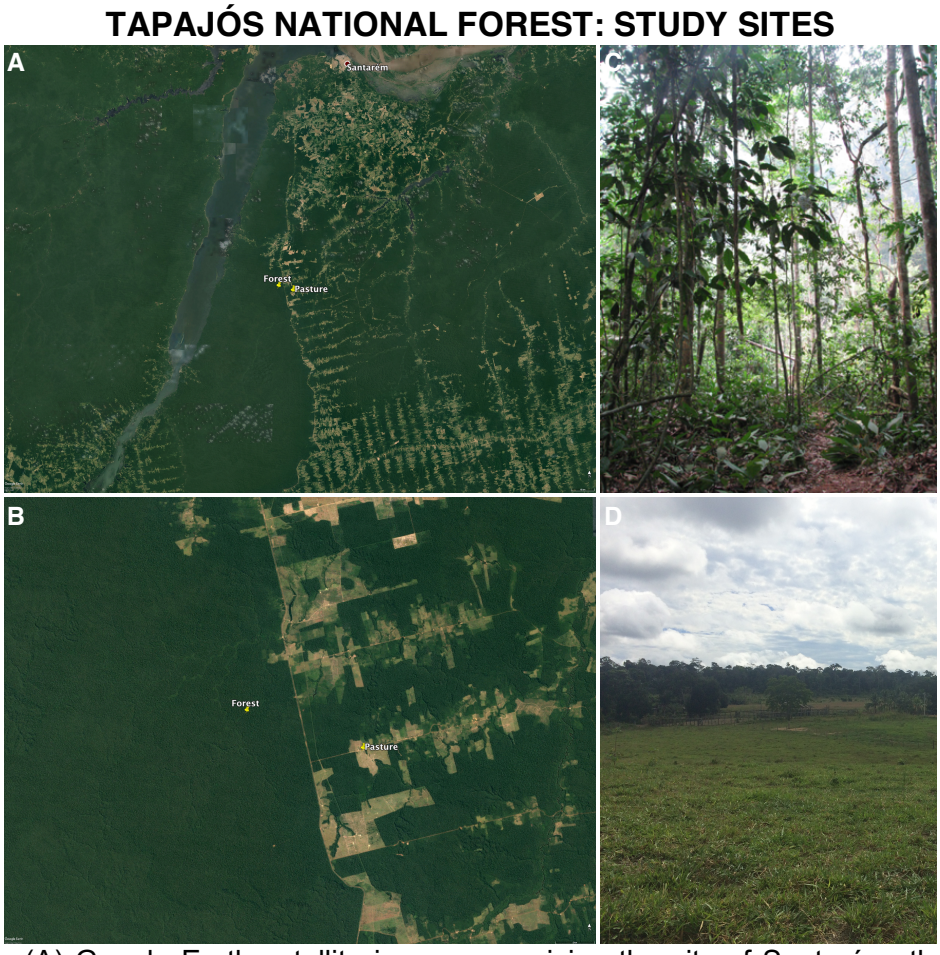

Figure 3.1 - (A) Google Earth satellite image comprising the city of Santarém, the Tapajós National Forest, and the studied forest and pasture sites, located in the state of Pará, Brazil, Eastern Amazon. (B) Google Earth satellite image of the studied sites. (C) Photo of the forest and (D) the pasture site in 2015

Soil aliquots of 600 g were sent to the Department of Soil Science of the Luiz de Queiroz College of Agriculture (ESALQ/USP), Piracicaba, in the state of São Paulo, Brazil, for the determination of the following physicochemical properties: pH determined in 0.01 M $CaCl_2$; SOM determined by colorimetry; total nitrogen (N) determined by the Kjeldahl method; P extracted with ion exchange resin and determined by the colorimetric method; S extracted with 0.01 M $Ca_3(PO_4)_2$ and determined by turbidimetry; K extracted with ion exchange resin and determined by atomic emission spectrophotometry; Ca and Mg extracted with ion exchange resin and determined by atomic absorption spectrophotometry; Al extracted with 1 M KCl and determined by the colorimetric method; H + Al determined with the SMP buffer; B extracted with hot water and determined by colorimetry; Cu, Fe, Mn, and Zn extracted with DTPA and determined by atomic absorption spectrophotometry; sand, silt and clay contents determined by the hydrometer method and classified according to the USDA classification (USDA, 2019). The calculations of SB, CEC, V, and m were made based on these previous results. The gravimetric moisture of each soil was determined in triplicate by its weighing before and after drying at 105 °C for 72h (CLAESSEN, 1997); while the gravimetric FC was determined in triplicate by its saturation, drainage undercover at room temperature, and weighing before and after drying at 105 °C for 72h (GARDNER, 1986; NJUGUNA et al., 2015). The soil physicochemical properties of the forest and pasture sites were compared by a two-sample Student's t-test in R studio 1.0.153 (RSTUDIO TEAM, 2016). The assumptions of normality and homoscedasticity were previously checked by the Anderson-Darling's test in SAS 9.3 (SAS Institute Inc., Cary, NC, USA) and the Levene's test using the car package 3.0-0 (FOX; WEISBERG, 2011) in R studio 1.0.153 (RSTUDIO TEAM, 2016), respectively.

3.2.2 Microcosm experiment, soil and gas sampling

The microcosm experiment had a 2 × 4 factorial design: 2 soils (forest and pasture) and 4 moisture levels based on the FC of each soil (original moisture, as found in the field, determined as 22% for forest and 24% for pasture; and 60%, 80% and 100% of moisture at FC, determined as 50% for forest and 63% for pasture) (Figure 3.2). Each treatment was established in triplicate in 1.5 L jars filled with 350 g of soil. The experiment was maintained at 25 °C, the mean annual temperature of the studied region (IBAMA, 2004), in a BOD incubator for 30 days. The soil moisture of each

microcosm was checked daily by its weighing and corrected by the addition of water to the soil using a spray bottle, followed by its mixing. Gas samples from each microcosm were collected for 30 min (1, 10, 20, and 30 min after closing the jars) before the establishment of the treatments (T0) and at 1, 2, 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30 days after using 20-mL nylon syringes. This procedure was always done in the morning, before moisture checking and correction. Soil samples from each microcosm were frozen in liquid nitrogen and stored at -80 °C at the end of the experiment.

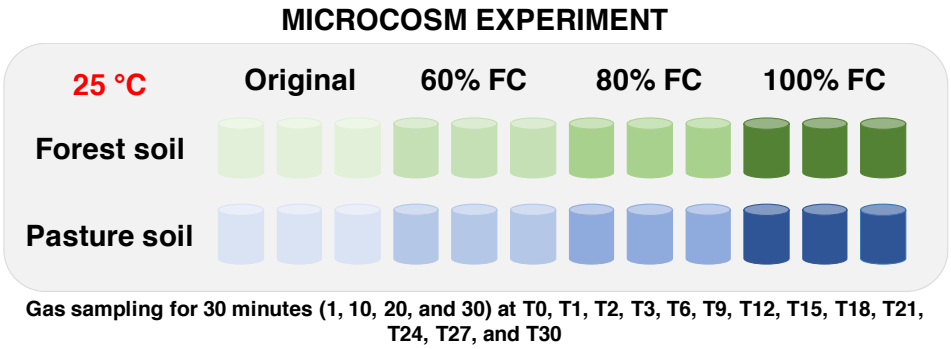

Figure 3.2 - Scheme of the microcosm experiment. The experiment had a 2 × 4 factorial design with two soils and four moisture levels based on their field capacity (FC) and was maintained at 25 °C in a BOD incubator for 30 days. Gas samples from each microcosm were collected for 30 min (1, 10, 20, and 30 min after closing the jars) before the establishment of the treatments (T0) and at 1, 2, 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30 days after

3.2.3 Measurement of CO_2 and CH_4 fluxes by gas chromatography

The gas samples were analyzed on an SRI 8610C gas chromatograph (SRI Instruments, Torrance, CA, USA). Gas fluxes from each jar were calculated according to their concentration as a function of the incubation time (1, 10, 20, and 30 min). Based on these results, daily CO_2 and CH_4 emissions were determined by the mean of their fluxes from the same treatment; and total cumulative emissions were determined through the linear interpolation of the daily emissions between two successive samplings and the sum of the results obtained throughout the experimental period. The cumulative fluxes of both gases were aligned rank-transformed and analyzed by two-way factorial analysis of variance (ANOVA) using the ARTool package 0.10.5 (KAY; WOBBROCK, 2018) in R studio 1.0.153 (RSTUDIO TEAM, 2016). Post-hoc tests (Holm-adjusted) were carried out whenever necessary using the lsmeans package 2.27-62 (LENTH, 2016) and plots were generated using ggplot2

3.0.0 (WICKHAM, 2016). The daily fluxes were plotted using Origin 7 software (OriginLab Corporation, Northampton, MA, USA).

3.2.4 DNA extraction and quantification

The total DNA from each microcosm from the last day of the experiment (T30) was extracted in duplicate using the PowerLyzer PowerSoil DNA Isolation Kit (MO Bio Laboratories, Inc., Carlsbad, CA, USA). The manufacturer's protocol was followed, using 250 mg of each sample per extraction, except for three adjustments, previously optimized (personal communication[2]) for Amazon soils, as described in Chapter 2 (Field Study). The concentration and purity of the DNA samples were evaluated by 1% agarose gel electrophoresis in SB buffer (BRODY; KERN, 2004) stained with ethidium bromide and on a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The DNA samples were stored at -20 °C for molecular analysis.

3.2.5 Quantitative-real time PCR of 16S rRNA and CH_4 marker genes

The 16S rRNA genes of *Archaea* and *Bacteria* and the CH_4 marker genes *mcrA*, *pmoA*, and soluble methane monooxygenase (*mmoX*) were quantified by qPCR through the standard-curve method using the primer pairs listed in Table 3.1. The standard curves were prepared by the PCR amplification of each target gene and its serial dilution from 10^8 to 10^0 copies, using the following strains from DSMZ - German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany): *Methanolinea mesophila* (DSM 23604) for the archaeal 16S rRNA and *mcrA*, *Gordonia sp.* (DSM 11192) for the bacterial 16S rRNA, and *Methylosinus sporium* (DSM 17706) for *pmoA* and *mmoX*. For all genes, the qPCR of each DNA sample was performed in triplicate in a 10-µL reaction on a StepOnePlus equipment (Thermo Fisher Scientific, Inc., Waltham, MA, USA), containing 5 µL of SYBR Green ROX qPCR Master Mix (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 0.2 µl of BSA (20 mg ml^{-1}) (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 1 µl of each primer (5 pmol), 1 µl of DNA (10 ng), and ultra-pure H_2O to complete 10 µl.

[2]VENTURINI, A. M. et al. Robust DNA protocols for tropical soils. Submitted for publication.

Table 3.1 - Sequences of the primer pairs used in the quantitative real-time PCR

Gene	Primer	Sequence (5'-3')	Reference
16S rRNA *Archaea*	ARC787F	ATTAGATACCCSBGTAGTCC	YU; LEE; HWANG, 2005
	ARC1059R	GCCATGCACCWCCTCT	
16S rRNA *Bacteria*	926F	AAACTCAAAKGAATTGACGG	DE GREGORIS et al., 2011
	1062R	CTCACRRCACGAGCTGAC	
mcrA	mlas-mod-F	GGYGGTGTMGGDTTCACMCARTA	ANGEL; CLAUS; CONRAD, 2012
	mcrA-rev	CGTTCATBGCGTAGTTVGGRTAGT	STEINBERG; REGAN, 2008
pmoA	A189	GGNGACTGGGACTTCTGG	HOLMES et al., 1995
	MB661	CCGGMGCAACGTCYTTACC	COSTELLO; LIDSTROM, 1999
mmoX	mmoX1	CGGTCCGCTGTGGAAGGGCATGAAGCGCGT	MIGUEZ et al., 1997
	mmoX2	GGCTCGACCTTGAACTTGGAGCCATACTCG	

The amplification conditions for the archaeal 16S rRNA gene consisted of 95 °C for 10 min, 40 cycles of 95 °C for 45 s, 57 °C for 45 s and 72 °C for 45 s; for the bacterial 16S rRNA gene, 95 °C for 10 min, 40 cycles of 95 °C for 45 s, 60 °C for 15 s and 72 °C for 20 s; for *mcrA*, 95 °C for 10 min, 45 cycles of 95 °C for 30 s, 60 °C for 45 s and 72 °C for 30 s; for *pmoA*, 95 °C for 10 min, 45 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 45 s; and for *mmoX*, 95 °C for 10 min, 45 cycles of 95 °C for 10 s, 66 °C for 20 s and 72 °C for 30 s. These conditions were followed by a melting curve of 95 °C for 15 s, the annealing temperature of each gene for 1 min, and 95 °C for 15 s. The results were analyzed in StepOne Software v2.3 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and converted into the number of gene copies per ng of DNA.

The abundance of each gene, as well as the ratio of *mcrA* by the sum of *pmoA* and *mmoX*, were analyzed by non-parametric two-way factorial ANOVA, as described above. Plots were generated using ggplot2 3.0.0 (WICKHAM, 2016) in R studio 1.0.153 (RSTUDIO TEAM, 2016). The Spearman's rank correlation coefficient was used to determine the correlations among the abundances of CH_4 marker genes, cumulative CH_4 fluxes, and moisture, using the psych package 1.8.4 (REVELLE, 2018). The correlation plot was generated using the corrplot package 0.84 (WEI; SIMKO, 2017).

3.2.6 Metagenomic shotgun sequencing and bioinformatics

The DNA samples from the microcosms with forest and pasture soils under the treatments of original moisture and 100% FC from the T30 sampling were sent to Novogene Co. Ltd. (Beijing, China) for library construction using the NEBNext Ultra II

DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) and paired-end shotgun sequencing (2 x 150 bp) on an Illumina HiSeq platform (Illumina, Inc., San Diego, CA, USA), with an expected raw data output of 12 Gb per sample. The forward reads were uploaded and analyzed on the MG-Rast server 4.0.3. (MEYER et al., 2008) using the default parameters. The taxonomic and functional classifications of the forward reads were performed against RefSeq (O'LEARY et al., 2016) and SEED (OVERBEEK et al., 2005) databases, respectively, using the default parameters, except for the alignment length, modified from 15 to 30 bp.

On the MG-Rast server, the hits of each sample were filtered according to the RefSeq taxonomy in the following groups: *Archaea*, *Bacteria*, methanogenic *Archaea*, and methanotrophic *Bacteria*. From the results, the methanogenic and methanotrophic communities were filtered again to obtain the functional hits related to methanogenesis – genes involved in the methanogenesis pathway and encoding the MCR enzyme – and methanotrophy – genes encoding pMMO and sMMO – according to the SEED subsystems, respectively. The functional hits of the *Archaea* and *Bacteria* communities as a whole were also obtained. The relative abundance of each individual hit was calculated by its division by the total number of hits of each sample.

Differences in the composition of the communities at the genus and functional level were statistically tested through permutational multivariate ANOVA (PERMANOVA) on Bray-Curtis dissimilarities using the vegan package 2.5-1 (OKSANEN et al., 2018) in R studio 1.0.153 (RSTUDIO TEAM, 2016). The parameters of the methanogenic and methanotrophic communities, such as species richness, Shannon's and Simpson's diversity, and Pielou's evenness were also calculated using the same package. These indices, as well as the relative abundance of the taxonomic and functional categories of interest, were also analyzed by non-parametric two-way factorial ANOVA. Plots were generated using ggplot2 3.0.0 (WICKHAM, 2016).

3.3 Results

3.3.1 Soil physicochemical properties

Forest and pasture soils were classified as sandy clay and clay, respectively, according to the USDA textural classification system (USDA, 2019) (Table 3.2). The pH of both soils was found to be acidic and ranged from 3.5 to 4.4, but the forest had

lower values, considering a p of at least < 0.05, compared to the pasture. Forest soils had higher S, Al, H+Al, and Fe levels than pasture soils and pasture soils presented higher contents of soil organic matter, N, Ca, Mn, and Zn than forest soils. Although both soils are low fertile and classified as dystrophic (RONQUIM, 2010), the pasture site had higher SB and V values in comparison to the forest, while the forest site had higher CEC and m values compared to the pasture, mainly due to its elevated Al levels.

Table 3.2 - Soil physicochemical properties (mean and standard deviation) of the forest and pasture sites used in the microcosm experiment

Soil properties	Units	Forest	Pasture
pH	-	3.57 ± 0.06 a	4.33 ± 0.06 b
SOM	g dm^{-3}	32.7 ± 0.6 a	39.3 ± 1.2 b
N	mg kg^{-1}	2,095.3 ± 161.8 a	2,510.7 ± 196.3 b
P	mg dm^{-3}	6.0 ± 0.0 a	7.7 ± 1.2 a
S	mg dm^{-3}	6.7 ± 1.2 a	< 4.0 ± 0.0 b
K	mmolc dm^{-3}	0.60 ± 0.00 a	0.73 ± 0.06 a
Ca	mmolc dm^{-3}	< 3.0 ± 0.0 a	8.0 ± 1.7 b
Mg	mmolc dm^{-3}	2.0 ± 0.0 a	2.0 ± 0.0 a
Al	mmolc dm^{-3}	10.3 ± 0.6 a	6.7 ± 1.2 b
H+Al	mmolc dm^{-3}	85.0 ± 5.2 a	56.0 ± 3.5 b
SB	mmolc dm^{-3}	4.27 ± 1.15 a	10.73 ± 1.79 b
CEC	mmolc dm^{-3}	89.27 ± 5.86 a	66.73 ± 4.63 b
V	%	4.7 ± 1.2 a	16.0 ± 2.0 b
m	%	71.0 ± 6.1 a	38.3 ± 6.5 b
B	mg dm^{-3}	0.490 ± 0.035 a	0.420 ± 0.036 a
Cu	mg dm^{-3}	0.60 ± 0.00 a	0.77 ± 0.21 a
Fe	mg dm^{-3}	206.0 ± 8.7 a	112.7 ± 5.9 b
Mn	mg dm^{-3}	4.20 ± 0.20 a	5.80 ± 0.17 b
Zn	mg dm^{-3}	0.57 ± 0.06 a	1.07 ± 0.12 b
Sand	g kg^{-1}	573.0 ± 12.1 a	143.0 ± 9.0 b
Silt	g kg^{-1}	66.7 ± 11.9 a	124.0 ± 8.5 b
Clay	g kg^{-1}	361.0 ± 1.0 a	733.0 ± 0.0 b
Texture	-	Sandy Clay	Clay

Means preceded by the less than sign (<) presented values lower than the quantification limit. Different letters indicate a significant difference between forest and pasture sites according to the Student's t-test at $p < 0.05$. SOM, soil organic matter; H+Al, potential acidity; SB, sum of exchangeable bases; CEC, cation-exchange capacity; V, base saturation; m, aluminum saturation

3.3.2 Soil gas fluxes

Cumulative CO_2 fluxes throughout the experimental period were considered similar among land-use and moisture treatments, with average fluxes ranging from 130.60 to 515.48 µg C-CO_2 soil g^{-1} (Table 3.3, Figure 3.3A, and Supplementary Figure S3.1A). CH_4 daily fluxes presented positive and negative emission results for both forest and pasture, demonstrating that soil can act as a source and a sink for this gas (Supplementary Figure S3.1B). Cumulative CH_4 fluxes were influenced by land-use, moisture level, and their interaction, with average fluxes ranging from -1,421.04 to 6,815.99 ng C-CH_4 soil g^{-1} (Figure 3.3B). Post-hoc analysis (Holm-adjusted) showed that the difference between forest and pasture CH_4 fluxes in the 100% FC treatment was significantly different ($p < 0.05$) compared to the difference in the original, 60%, and 80% FC treatments. Thus, the microcosms with pasture soils under 100% FC had the highest emission values of all treatments, followed by forest soils under the same conditions but which presented average emission 95.5% lower. Most soils from the other treatments were sinks for CH_4.

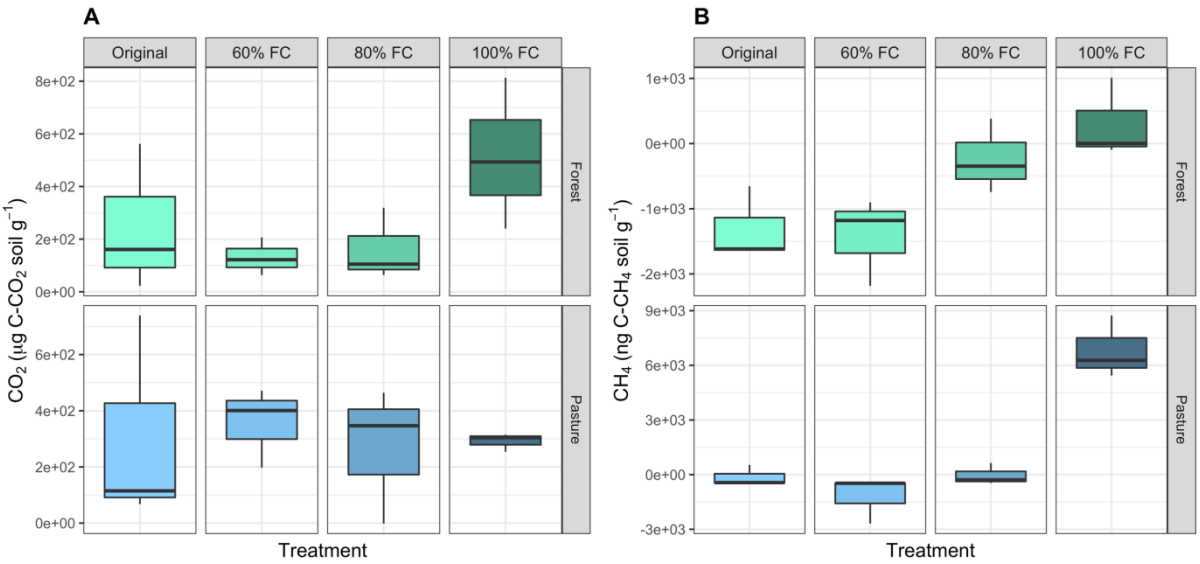

Figure 3.3 - Cumulative fluxes of (A) CO_2 (µg C-CO_2 soil g^{-1}) and (B) CH_4 (ng C-CH_4 soil g^{-1}) over the 30-day period of the microcosm experiment with forest and pasture soils under four moisture levels (original moisture; 60%, 80%, and 100% of moisture at FC). FC, field capacity

Table 3.3 - Results of the non-parametric two-way factorial ANOVAs (degrees of freedom, F-ratios, and p-values) for the cumulative gas fluxes, gene quantification by qPCR, and taxonomic and functional profiling of the metagenomes

Data	Land-use			Moisture			Land-use × Moisture		
	df	F	p	df	F	p	df	F	p
Cumulative gas fluxes									
CO_2	1, 16	0.194	0.665	3, 16	1.147	0.360	3, 16	0.914	0.456
CH_4	1, 16	30.537	< 0.001	3, 16	15.558	< 0.001	3, 16	26.596	< 0.001
Gene quantification									
16S rRNA of *Archaea*	1, 16	0.421	0.525	3, 16	6.437	0.005	3, 16	2.406	0.105
16S rRNA of *Bacteria*	1, 16	44.317	< 0.001	3, 16	1.646	0.218	3, 16	1.365	0.289
mcrA	1, 16	50.330	< 0.001	3, 16	8.400	0.001	3, 16	69.819	< 0.001
pmoA	1, 16	21.022	< 0.001	3, 16	6.812	0.004	3, 16	20.355	< 0.001
mmoX	1, 16	41.350	< 0.001	3, 16	16.233	< 0.001	3, 16	32.121	< 0.001
mcrA:(*pmoA*+*mmoX*) ratio	1, 16	13.444	0.002	3, 16	2.512	0.096	3, 16	2.490	0.097
Taxonomic profiling									
Methanogenic taxa									
Shannon's diversity	1, 8	1.029	0.340	1, 8	3.125	0.115	1, 8	3.125	0.115
Simpson's diversity	1, 8	1.044	0.337	1, 8	1.910	0.204	1, 8	2.512	0.152
Pielou's evenness	1, 8	1.044	0.337	1, 8	1.910	0.204	1, 8	2.512	0.152
Relative abundance	1, 8	27.000	< 0.001	1, 8	25.920	< 0.001	1, 8	7.539	0.025
Methanotrophic taxa									
Shannon's diversity	1, 8	24.923	0.001	1, 8	24.923	0.001	1, 8	1.873	0.208
Simpson's diversity	1, 8	24.923	0.001	1, 8	24.923	0.001	1, 8	12.190	0.008
Pielou's evenness	1, 8	24.923	0.001	1, 8	24.923	0.001	1, 8	12.190	0.008
Relative abundance	1, 8	25.920	< 0.001	1, 8	17.000	0.003	1, 8	9.278	0.016
Methanogens:methanotrophs ratio									
Relative abundance	1, 8	27.000	< 0.001	1, 8	17.785	0.003	1, 8	9.677	0.014
Functional profiling									
Methanogenesis	1, 8	27.000	< 0.001	1, 8	25.920	< 0.001	1, 8	17.785	0.003
MCR	1, 8	17.190	0.003	1, 8	5.669	0.044	1, 8	4.751	0.061
pMMO	1, 8	3.366	0.104	1, 8	25.920	< 0.001	1, 8	0.742	0.414
sMMO	1, 8	26.722	< 0.001	1, 8	1.418	0.268	1, 8	4.100	0.077
MCR:(pMMO+sMMO) ratio	1, 8	26.315	< 0.001	1, 8	6.022	0.040	1, 8	4.830	0.059

3.3.3 Quantification of 16S rRNA and CH_4 marker genes

The qPCR assays presented R^2 values above 0.98 and amplification efficiencies between 75% and 120%, calculated based on the regression lines of each standard curve. At the end of the microcosm experiment, the average number of copies

per ng of DNA (copies ng DNA^{-1}) ranged from 4.38×10^3 to 6.78×10^3 for the 16S rRNA gene of *Archaea*; 1.38×10^6 to 3.77×10^6 for the 16S rRNA gene of *Bacteria*; 2.19×10^0 to 9.91×10^2 for *mcrA*; 6.38×10^0 to 2.69×10^2 for *pmoA*; and 2.16×10^{-1} to 1.70×10^0 for *mmoX* (Figure 3.4).

The abundance of the archaeal 16S rRNA gene differed according to the moisture level, found in post-hoc tests (Holm-adjusted) to be lower ($p < 0.05$) under original moisture than in the other treatments; while the abundance of the bacterial 16S rRNA gene was higher in pasture than in forest soils (Table 3.3). The CH_4 marker genes were influenced by land-use, moisture level, and their interaction. Post-hoc analysis (Holm-adjusted) showed that the differences between their abundances in the 100% FC treatment were significantly different ($p < 0.05$) compared to the differences in the original, 60%, and 80% FC treatments. For the *mmoX* gene, this was also true for the comparison between the original and 80% FC treatment. Therefore, the microcosms with pasture soils, especially under 100% FC, presented the highest quantification of CH_4 genes. The abundance ratio of *mcrA* by the sum of *pmoA* and *mmoX* genes had significantly higher values in pasture than in forest soils. In this sense, pasture soils of all treatments, as well as forest soils of the 100% FC treatment, exhibited a ratio higher than one, indicating a greater abundance of methanogenic microorganisms than methanotrophs.

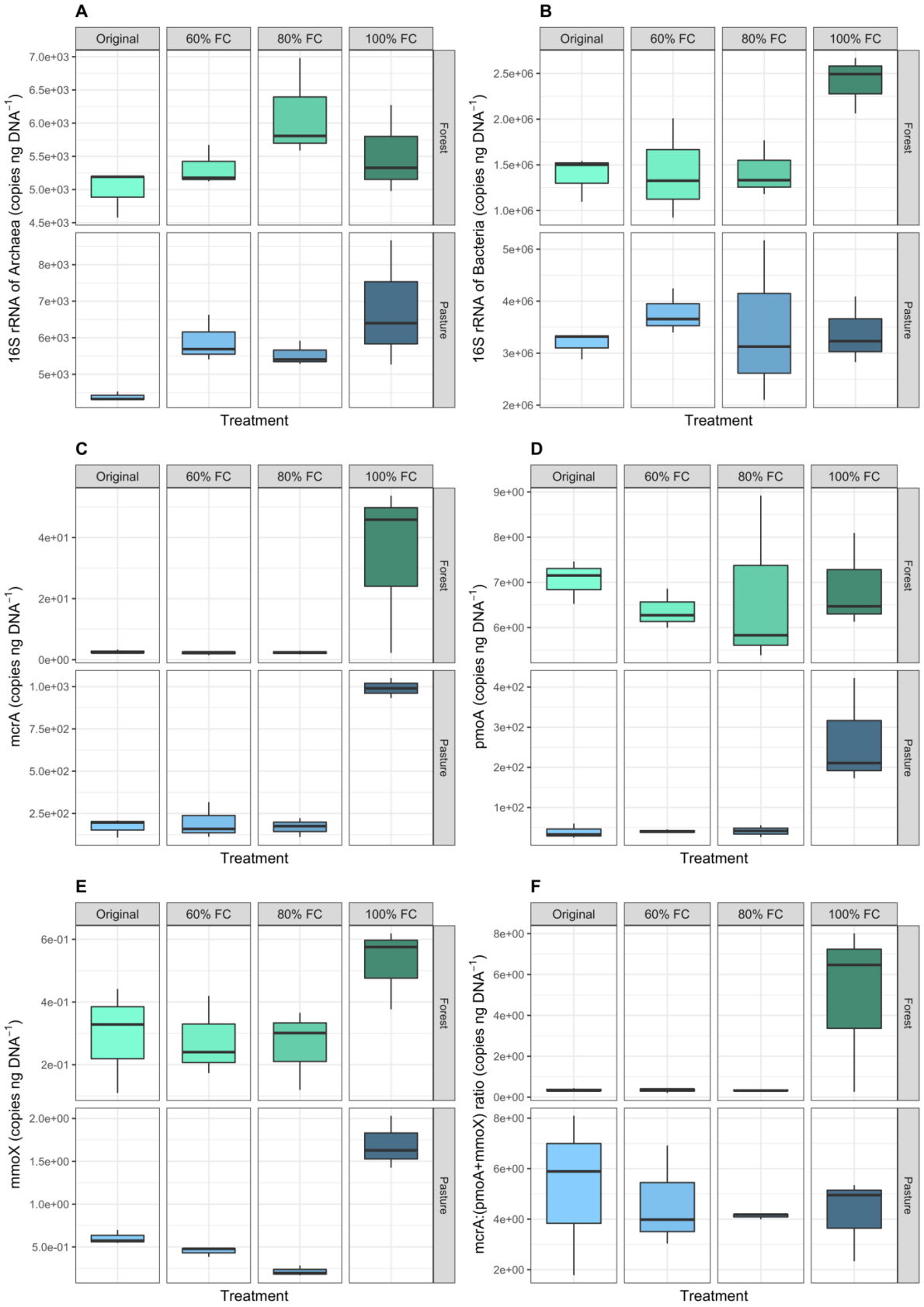

Figure 3.4 - Number of copies per ng of DNA (copies ng DNA^{-1}) obtained by qPCR of the (A) 16S rRNA of *Archaea*, (B) 16S rRNA of *Bacteria*, (C) *mcrA*, (D) *pmoA*, (E) *mmoX*, and (F) *mcrA*:(*pmoA*+*mmoX*) ratio after the 30-day period of the microcosm experiment with forest and pasture soils under four moisture levels (original moisture; 60%, 80%, and 100% of moisture at FC). FC, field capacity

3.3.4 Correlations among the abundance of CH_4 marker genes, CH_4 fluxes, and soil moisture

The Spearman's test (p at least < 0.05) showed that the abundance of the CH_4 marker genes shared a positive correlation (Figure 3.5). The quantification of *mcrA* and *mmoX* was positively correlated with the *mcrA:*(*pmoA*+*mmoX*) ratio and cumulative CH_4 fluxes. The former also presented a positive correlation with the moisture content, which was itself strongly correlated with the CH_4 fluxes.

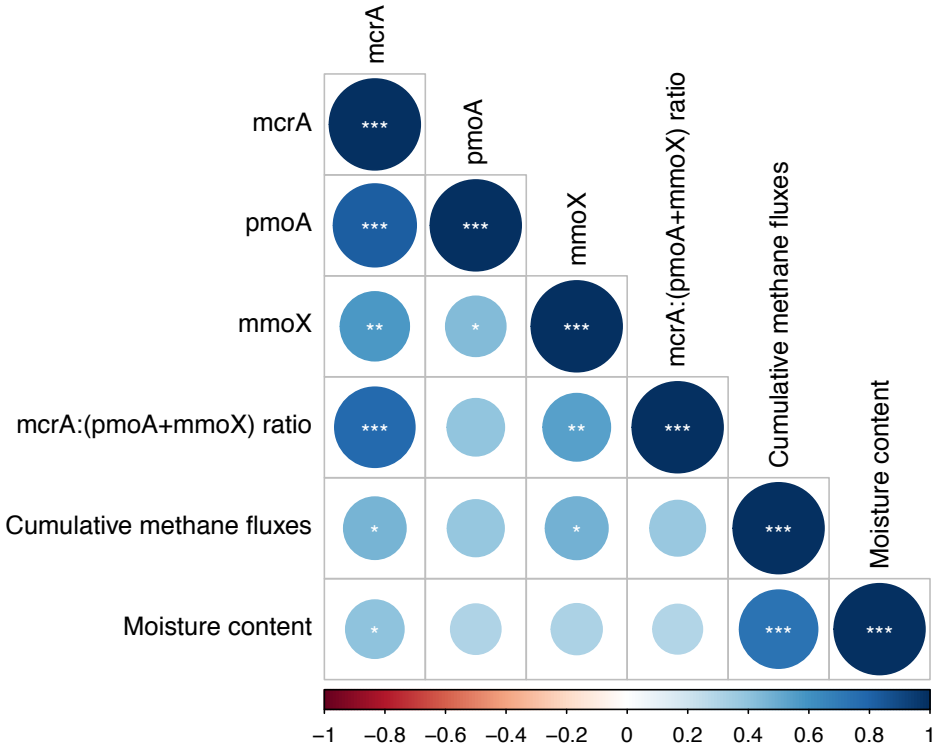

Figure 3.5 - Spearman's correlations among the qPCR abundances of CH_4 marker genes, cumulative CH_4 fluxes, and soil moisture of the microcosm experiment. *, p < 0.05; **, p < 0.01; ***, p < 0.001

3.3.5 Metagenomic sequencing results

The metagenomic sequencing resulted in about 594.9 million reads for 12 samples, with an average of 49.6 million per sample (Supplementary Table S3.1). Approximately 96.8% and 91.8% of the sequenced bases presented a Phred quality score above 20 and 30, respectively. After quality control on the MG-Rast server 4.0.3. (MEYER et al., 2008), about 520.4 million reads remained, averaging 43.4 million per sample and 150 bp in length (Supplementary Table S3.2). Less than

0.1% of the total contained rRNA genes, and 38.6% and 61.3%, predicted proteins with known and unknown functions, respectively (Supplementary Table S3.3).

3.3.5.1 Taxonomic profiling of the metagenomes

The metagenomic libraries, based on the taxonomic classification made against RefSeq (O'LEARY et al., 2016), were dominated by *Bacteria* (98.23%), but also presented sequences matching *Archaea* (0.93%), *Eukaryota* (0.82%), and Virus (0.03%). Less than 0.001% of the hits were classified as belonging to other groups. Based on Bray-Curtis dissimilarities, both *Archaea* and *Bacteria* communities were considered to be compositionally different at the genus level according to the land-use, moisture and their interaction (land-use$_{Archaea}$: $F_{1,8}$ = 59.609, R^2 = 0.690, $p < 0.001$; moisture$_{Archaea}$: $F_{1,8}$ = 12.741, R^2 = 0.148, $p = 0.004$; interaction$_{Archaea}$: $F_{1,8}$ = 5.999, R^2 = 0.069, $p = 0.039$; land-use$_{Bacteria}$: $F_{1,8}$ = 180.775, R^2 = 0.713, $p < 0.001$; moisture$_{Bacteria}$: $F_{1,8}$ = 49.769, R^2 = 0.196, $p < 0.001$; interaction$_{Bacteria}$: $F_{1,8} = 14.917$, $R^2 = 0.059$, $p = 0.002$). The structures of both microbial communities at the phylum level are shown in the Supplementary Figure S3.2.

The archaeal methanogenic community found belong exclusively to the phylum *Euryarchaeota*, from the *Methanomicrobia* (78.7%), *Methanococci* (9.8%), *Methanobacteria* (9.1%), and *Methanopyri* (2.5%) classes, divided into six orders and 12 families (Supplementary Table S3.4). The most abundant genus was *Methanosarcina*, followed by *Methanoregula* and *Methanoculleus* (Figure 3.6A). This community was considered compositionally different at the genus level according to the land-use and moisture level (land-use: $F_{1,8}$ = 37.320, R^2 = 0.586, $p < 0.001$; moisture: $F_{1,8}$ = 14.307, R^2 = 0.224, $p = 0.003$; interaction: $F_{1,8} = 4.105$, $R^2 = 0.064$, $p = 0.072$), but presented the same richness for all soils, as well as did not differ in diversity or evenness (Tables 3.3 and 3.4). However, the relative abundance of the group differed according to the land-use, moisture, and their interaction (Figure 3.7A). Similar to the qPCR quantification, pasture soils, particularly of the 100% FC treatment, had the highest abundance of this gene.

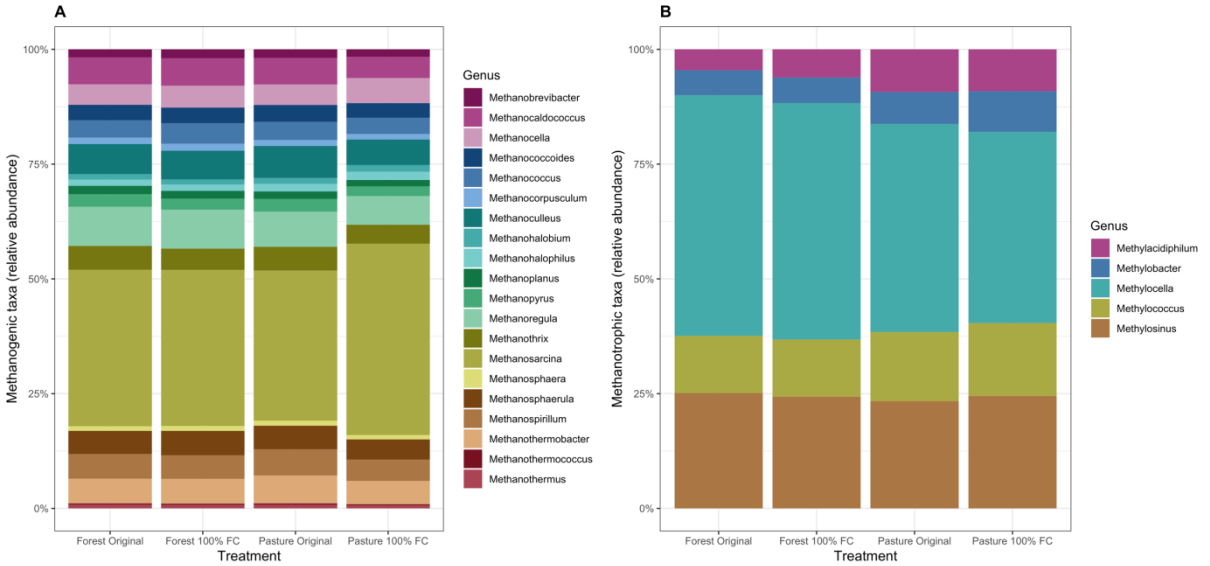

Figure 3.6 - Taxonomic composition obtained by metagenomics of the (A) archaeal methanogenic and (B) bacterial methanotrophic communities at the genus level after the 30-day period of the microcosm experiment with forest and pasture soils under two moisture levels (original moisture and 100% of moisture at FC). FC, field capacity

Table 3.4 - Richness, Shannon's diversity, Simpson's diversity, and Pielou's evenness (mean and standard deviation) of the archaeal methanogenic and bacterial methanotrophic communities at the genus level after the 30-day period of the microcosm experiment with forest and pasture soils under two moisture levels (original moisture and 100% of moisture at FC)

Land-use	Moisture	Methanogenic taxa				Methanotrophic taxa			
		Nº	Shannon	Simpson	Pielou	Nº	Shannon	Simpson	Pielou
F	OM	20	2.432 ± 0.003	0.851 ± 0.001	0.284 ± 0.000	5	1.244 ± 0.003	0.641 ± 0.002	0.399 ± 0.001
	100%	20	2.434 ± 0.003	0.852 ± 0.000	0.284 ± 0.000	5	1.277 ± 0.009	0.653 ± 0.003	0.406 ± 0.002
P	OM	20	2.466 ± 0.035	0.859 ± 0.011	0.287 ± 0.004	5	1.390 ± 0.007	0.704 ± 0.003	0.437 ± 0.002
	100%	20	2.269 ± 0.178	0.798 ± 0.058	0.266 ± 0.019	5	1.434 ± 0.006	0.725 ± 0.002	0.451 ± 0.001

F, forest; P, pasture; OM, original moisture; 100%, 100% of moisture at field capacity

The bacterial methanotrophic community belong to the phyla *Proteobacteria*, from the *Alphaproteobacteria* (72.1%) and *Gammaproteobacteria* (20.7%) classes, and *Verrucomicrobia*, from *Methylacidiphilae* (7.3%), distributed in three orders and four families (Supplementary Table S3.5), with *Methylocella*, followed by *Methylosinus* and *Methylococcus*, as the most abundant genus (Figure 3.6B); and was also compositionally different at the genus level according to the land-use and moisture (land-use: $F_{1,8}$ = 479.730, R^2 = 0.947, p < 0.001; moisture: $F_{1,8}$ = 18.749, R^2 = 0.037, p = 0.002; interaction: $F_{1,8}$ = 0.247, R^2 < 0.001, p = 0.580). Although had the same richness, the microcosms with soils from the pasture site and of the 100% FC treatment presented significantly higher Shannon's diversity (Tables 3.3 and 3.4). For Simpson's diversity, Pielou's evenness, and its relative abundance (Figure 3.7B),

a significant interaction between both factors was also found. Interestingly, while these indices were higher for pasture soils, the methanotrophic abundance was higher in forest soils, but with an increase of all these attributes under 100% FC. The attributes of the methanotrophic community separated among *Alphaproteobacteria*, *Gammaproteobacteria*, and *Verrucomicrobia* taxa were also influenced by both factors and their interaction, as exhibited in the Supplementary Tables S3.6 and S3.7, and Figure S3.3. Exceptionally, the relative abundances of Alpha- and *Gammaproteobacteria* methanotrophs were not affected by moisture and the factors' interaction, respectively. The former presented the highest values in forest soils; and the latter, in soils of the 100% FC treatment.

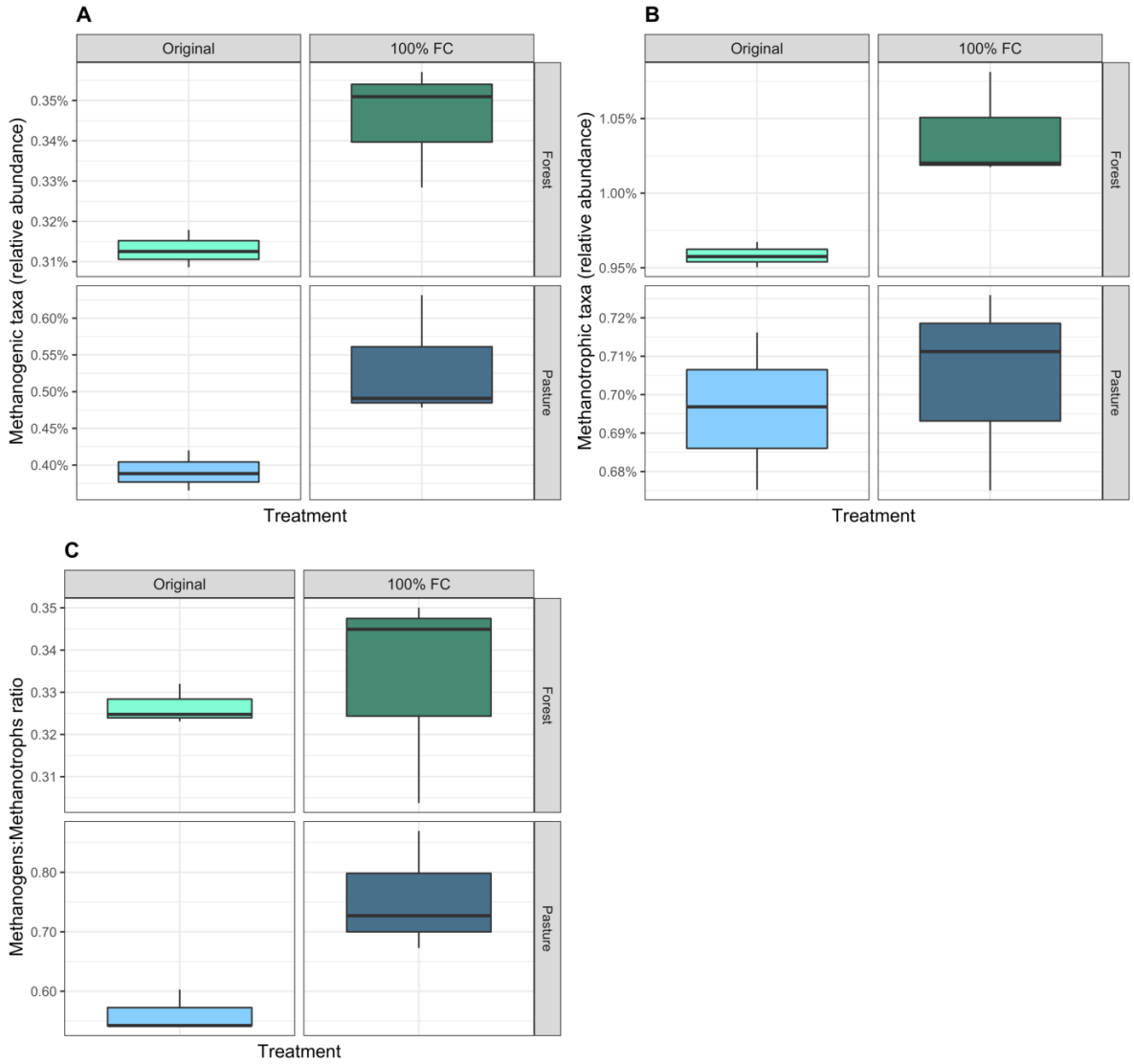

Figure 3.7 - Relative abundance obtained by metagenomics of the (A) methanogenic taxa, (B) methanotrophic taxa, and (C) methanogens:methanotrophs ratio after the 30-day period of the microcosm experiment with forest and pasture soils under two moisture levels (original moisture and 100% of moisture at FC). FC, field capacity

The relative abundance ratio of methanogens by methanotrophs differed according to the land-use, moisture, and their interaction (Figure 3.7C); and even though distinct results were found regarding the methanotrophic abundance through both molecular techniques used in our work (qPCR and metagenomics), similar trends were found, with pasture soils presenting the highest ratio values, closer to one.

3.3.5.2 Functional profiling of the metagenomes

Based on Bray-Curtis dissimilarities, the genes from the *Archaea* and *Bacteria* communities annotated using the SEED database (OVERBEEK et al., 2005) were considered to be compositionally different at the functional level according to the land-use and moisture (land-use$_{Archaea}$: $F_{1,8} = 26.435$, $R^2 = 0.621$, $p < 0.001$; moisture$_{Archaea}$: $F_{1,8} = 5.913$, $R^2 = 0.139$, $p = 0.006$; interaction$_{Archaea}$: $F_{1,8} = 2.223$, $R^2 = 0.052$, $p = 0.099$; land-use$_{Bacteria}$: $F_{1,8} = 91.881$, $R^2 = 0.719$, $p < 0.001$; moisture$_{Bacteria}$: $F_{1,8} = 23.247$, $R^2 = 0.182$, $p = 0.001$; interaction$_{Bacteria}$: $F_{1,8} = 4.601$, $R^2 = 0.036$, $p = 0.054$). The functional categories associated with the archaeal and bacterial communities at the Subsystems Level 1 are exhibited in the Supplementary Figure S3.4.

The relative abundances of the genes of the methanogenic community involved in methanogenesis and encoding the MCR enzyme were significantly influenced by land-use and moisture, and the former, also by their interaction (Table 3.3, Figures 3.8A and 3.8B). Again, the highest results were found for pasture soils, especially under 100% FC. In contrast, the genes of the methanotrophic community encoding pMMO and sMMO presented significantly higher abundances in the 100% FC treatment and in pasture soils, respectively (Figures 3.8C and 3.8D). The MCR:(pMMO+sMMO) ratio also increased in both conditions (Figure 3.8E), but only the pasture microcosms exhibited values above one.

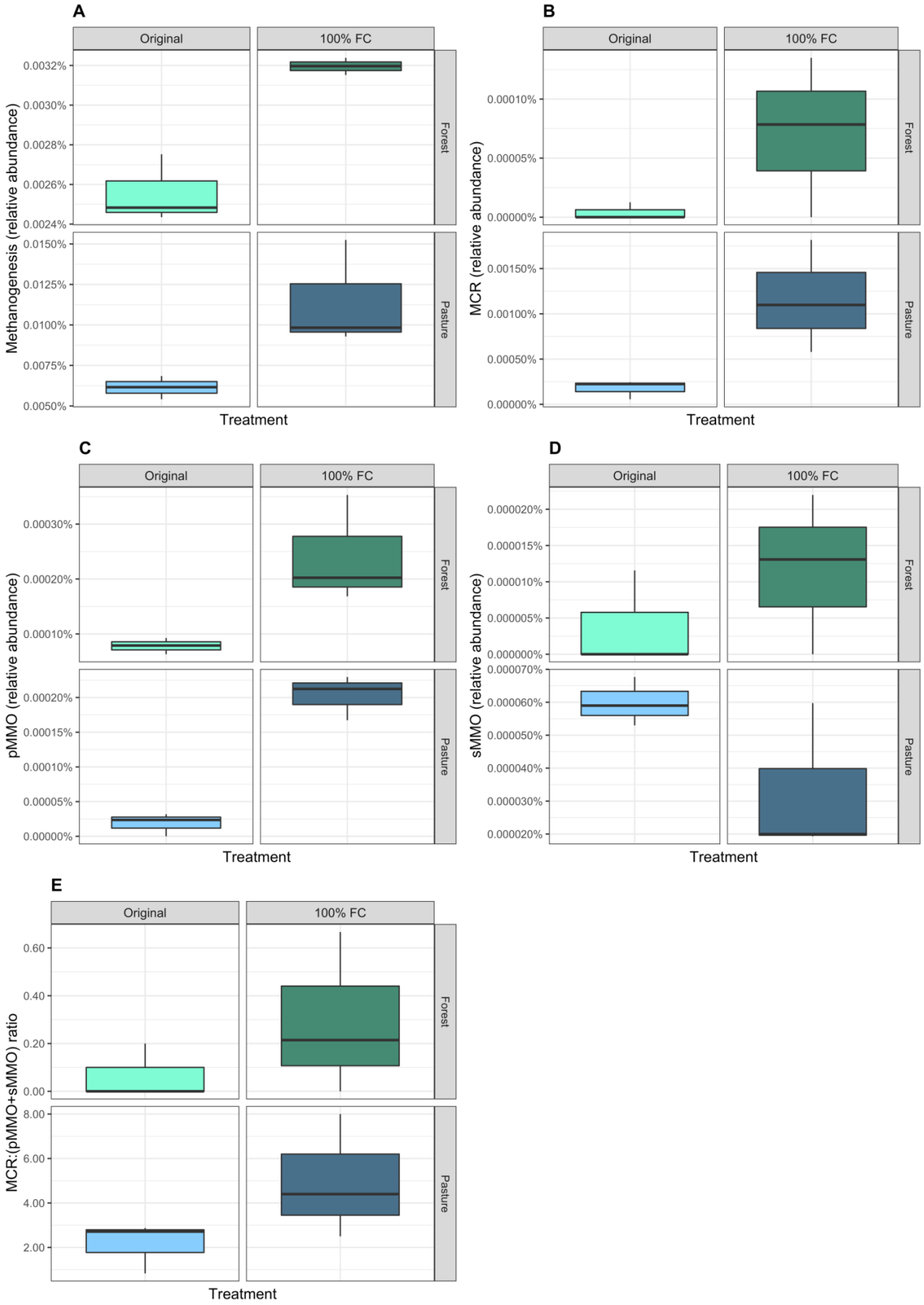

Figure 3.8 - Relative abundance obtained by metagenomics of the genes (A) involved in methanogenesis, (B) encoding the methyl-coenzyme M reductase enzyme (MCR), (C) encoding the particulate methane monooxygenase (pMMO), (D) encoding the soluble methane monooxygenase (sMMO), and (E) MCR:(pMMO+sMMO) ratio after the 30-day period of the microcosm experiment with forest and pasture soils under two moisture levels (original moisture and 100% of moisture at FC). FC, field capacity

3.4 Discussion

In this study, forest and pasture soils of the Tapajós National Forest, in the Brazilian Eastern Amazon, were collected for the development of a microcosm experiment using four moisture levels (original moisture; 60%, 80%, and 100% of moisture at FC). We analyzed gas and soil samples from the experiment through gas chromatography and molecular methods (qPCR of the archaeal and bacterial 16S rRNA and CH_4 marker genes followed by metagenomic sequencing), respectively, to evaluate how moisture influenced CH_4 fluxes and the abundance, taxonomic and functional diversity of their related microbial communities across both land-uses, which are mainly composed of methanogenic *Archaea* and methanotrophic *Bacteria*.

The transformation of forests into agricultural and pasture lands in the Amazon is typically carried out by the slash-and-burn method, which increases soil pH and nutrient content and availability (JUO; MANU, 1996; GIARDINA et al., 2000). Accordingly, several studies have revealed that forest-to-pasture conversion alters the taxonomic (RODRIGUES et al., 2013; MENDES et al., 2015) and functional (PAULA et al., 2014) profile of soil microbial communities from this region, including specific microbial taxa (NAVARRETE et al., 2015; RANJAN et al., 2015) and ecological functions (MIRZA et al., 2014; HAMAOUI JUNIOR et al., 2016; MEYER et al., 2017). Our results showed similar trends, indicating that all soil properties (except P, K, Mg, B, and Cu), as well as the taxonomic and functional composition of the archaeal and bacterial communities, changed according to the land-use. In addition, these also responded to moisture, known to have a strong influence on the activity and diversity of soil microorganisms (LENNON et al., 2012); and considering only the taxonomic composition of our samples, its interaction with land-use was also significant. However, the abundance of both communities changed differently, with higher amounts of *Archaea* under 60%, 80% and 100% FC; and of *Bacteria*, in pasture soils.

Both land-use and moisture also affected methane-related microorganisms, including the taxonomic composition of the archaeal methanogenic community, which was dominated by members of the *Methanomicrobia* class, in particular, *Methanosarcina*. This genus is composed of versatile species found in several environments that, unlike most methane-producing organisms, can use up to nine substrates and have all three methanogenic pathways (hydrogenotrophic, methylotrophic, and acetoclastic) (GALAGAN et al., 2002). Similar to our results,

Meyer et al. (2017) reported compositional differences in the methanogenic community from forest and pasture soils of the Western Amazon, which were also similar in diversity and evenness.

The abundance of genes and microbial groups involved in methanogenesis, as observed in the qPCR and most of the metagenomic data, were also influenced by the studied factors and their interaction, with much higher results in pasture than in forest soils, particularly under 100% FC. Several studies have demonstrated that the use of heavy machinery for forest clearing associated to cattle trampling in pasture sites leads to soil compaction, resulting in a decrease of porosity and an increase of anaerobic sites, suitable for the growth of methanogenic microorganisms (RADL et al., 2007; FREY et al., 2011; BRADLEY et al., 2012; KOUBOVÁ et al., 2012; CHROŇÁKOVÁ et al., 2015; WANG et al., 2016). Radl et al. (2007) and Frey et al. (2011) also showed that this impact is significant in the long-run so that even months after removing the cause of the compaction, the soil can still maintain a high abundance of methanogens and thus its CH_4 emission potential. In addition, the pH increase after slash-and-burn, intensified by the deposition of cattle urine and manure, makes pasture soils a more suitable environment for the development of methanogenic taxa (RADL et al., 2007; CHROŇÁKOVÁ et al., 2015), which grow under minimal pH between 4 and 7 (MICHAŁ et al., 2018). Cattle manure is also a nutrient-rich organic source for these organisms, and more easily degradable than the forest litter (LAMMEL et al., 2015a), besides serving as input of methanogens into the soil (GATTINGER et al., 2007; RADL et al., 2007; PREM; REITSCHULER; ILLMER, 2014; CHROŇÁKOVÁ et al., 2015). Previous results in the Amazon have also shown that pasture sites have a higher abundance of genes encoding the MCR enzyme than forests (MEYER et al., 2017).

Regarding the moisture treatments of the microcosm experiment, no major differences in the qPCR quantification of the *mcrA* gene were detected among the intermediate moisture levels. In the samples under 100% FC, both molecular methods used in our study showed a clear increase in the abundance of methanogenesis genes and its related groups due to the lower availability of oxygen in these soils (NAZARIES et al., 2013). Nevertheless, although this response was observed for both land-uses, the abundance of methanogens remained higher in pasture than in forest soils even under field capacity conditions. Accordingly, the cumulative CH_4 fluxes were strongly correlated with the moisture content and influenced by land-use, moisture, and their interaction. Therefore, our results indicate that Amazon soils are mostly sinks but can

become considerable sources of CH_4 under conditions of high saturation, which is particularly true for pastures. Considering only the 100% FC treatment, this land-use had emission rates about 22 times higher than the forest soils. Other long-term studies in the basin have revealed a source-to-sink shift after forest-to-pasture conversion, so that pasture soils often consume CH_4 in the dry season but emit it in the wet season with increasing soil moisture (STEUDLER et al., 1996; VERCHOT et al., 2000; FERNANDES et al., 2002). It is also worth mentioning that, although soils contribute to a small part of the CH_4 emissions from the Amazon, dominated by emissions from livestock and biomass burning, the transformation of forests into pastures is estimated to result in a net source of about 1 g C-CH_4 m^{-2} $year^{-1}$ from the soil (STEUDLER et al., 1996), which may grow with increasing deforestation and precipitation changes caused by global warming, as previously described.

In contrast, the taxonomical composition of the bacterial methanotrophic community, composed of *Proteobacteria* of the alpha and gamma classes and *Verrucomicrobia* was also influenced by land-use and moisture. A similar change was previously described for Amazon forest and pasture soils (MEYER et al., 2017). *Methylocella* was the dominant methanotrophic genus in all soils of the experiment, particularly those from the forest site. This group of the *Beijerinckiaceae* family is composed of facultative species that can utilize several multicarbon compounds (and even prefer acetate over CH_4) (DEDYSH; DUNFIELD, 2016), which gives an advantage in environments where there is variation in CH_4 emissions (DEDYSH; KNIEF; DUNFIELD, 2005). *Methylocella* species contain only the soluble form of the MMO enzyme (DEDYSH; DUNFIELD, 2016), while all other taxa found in our soils possess its particulate form (KNIEF, 2015; DEDYSH; KNIEF, 2018). Methanotrophic diversity and evenness also varied according to the studied factors, and a significant interaction between them was also found for Simpson's and Pielou's indexes (as well as for the results of *Alpha-* and *Gammaproteobacteria*, as shown in the Supplementary Tables S3.6 and S3.7), presenting the highest values for pasture soils, especially of the 100% FC treatment. Other studies carried out in forest, agricultural and pasture lands have produced comparable results (KRAVCHENKO et al., 2005; KIZILOVA; YURKOV; KRAVCHENKO, 2013; KRAVCHENKO; SUKHACHEVA, 2017; SENGUPTA; DICK, 2017).

Unlike methanogens, the abundance response of the methanotrophic community and its related genes varied between qPCR and metagenomic datasets.

Most of the available studies have reported a decrease in the *pmoA* quantification after cattle grazing (LAMMEL et al., 2015a; WANG et al., 2016; MEYER et al., 2017), and the association of this gene with Amazon forest soils was also found (PAULA et al., 2014). However, other papers detected no quantitative changes, (ZHENG et al., 2012; YANG et al., 2013) and even an opposite tendency in the Southern Amazon (LAMMEL et al., 2015b). In our work, the results can be associated to the large abundance of methanotrophs found in our samples that do not possess the pMMO enzyme (*Methylocella* species) as well as the limitations of the chosen molecular methods, including the bias of the qPCR primers – since the most important primer pair specific for *pmoA* does not capture members of the *Verrucomicrobia* phylum (DEDYSH; DUNFIELD, 2011; GHASHGHAVI; JETTEN; LÜKE, 2017) and amplify less efficiently some *Alphaproteobacteria* (DENG et al., 2013) – and the annotation of short metagenomic reads. Furthermore, the primers used for the detection of the *mmoX* gene were designed and evaluated based on strains of *Methylococcus* and *Methylosinus* (MIGUEZ et al., 1997) and, according to results obtained using the FunGene pipeline (FISH et al., 2013), do not capture all the *Methylocella* diversity (data not shown). Despite the smaller number of data available, several land-use studies could not detect this gene in upland soils (KNIEF; LIPSKI; DUNFIELD, 2003; KNIEF et al., 2005; DÖRR; GLASER; KOLB, 2010; KIZILOVA; YURKOV; KRAVCHENKO, 2013; KRAVCHENKO; SUKHACHEVA, 2017). Nevertheless, independently of the dataset used, the methanotrophic community tended to increase in our soil samples of the 100% FC treatment, probably due to the more considerable amount of CH_4 available.

Considering the most prevailing methanotrophic taxa found in our soils separately, more clear patterns emerge. The relative abundance of *Alphaproteobacteria* was influenced by land-use (62.3% higher in forest than in pasture soils), which is in accordance with other studies that described its dominance in natural environments and decrease after deforestation (KRAVCHENKO et al., 2005; SINGH et al., 2007; SINGH et al., 2009; MALGHANI et al., 2016; MEYER et al., 2017). The life strategy of this group is advantageous in low-nutrient habitats (STEENBERGH et al., 2009; MEYER et al., 2017) such as our forest soils, besides having several members capable of fixing nitrogen (DEDYSH; KNIEF, 2018), but its response to moisture varied since an interaction was found. *Gammaproteobacteria* showed significantly higher values in soils from the forest and under 100% FC. Steenbergh et al. (2009) and Ho et al. (2013) suggested that these organisms rapidly respond to

disturbances and therefore can be favored under increasing moisture conditions (SILJANEN et al., 2011; SILJANEN et al., 2012).

Regardless the variation among our methanotrophy results, all datasets indicated that the abundance ratio of methanogens by methanotrophs changed according to the land-use, with higher values in pasture than in forest soils, which is analogous to the results of Meyer et al. (2017) for the Western Amazon. The taxonomic and functional ratios based on the metagenomic data were also influenced by moisture (and a significant interaction between both studied factors was found for the former), but again, these results continued to be higher in pasture than in forest even under field capacity conditions.

3.5 Conclusions

Taken together, our experimental results showed that soil CH_4 fluxes and related microbial communities – methanogens and methanotrophs – responded to changes in land-use and moisture. Our data exhibited patterns similar to those obtained in the field in the previous chapter (Field Study) and showed that the increase of moisture enhances the alterations caused by the forest-to-pasture conversion, which can lead to high CH_4 emissions. The development of this study in a microcosmic scale allowed the isolation of moisture as a study factor and the homogenization of the other environmental conditions. To our knowledge, this is the first report on the microbial CH_4 cycle in upland soils of the Brazilian Amazon that combined long-term gas measurements with advanced molecular methods.

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APPENDICES

Appendix A - Supplementary materials of Chapter 2 (Field Study)

Supplementary Table S2.1 - Geographical coordinates and elevation (m) of the sampling points in the forest and pasture sites

Land-use	Site	Sampling point	Geographical coordinates	Elevation (m)
Forest	F1	1	2°51'19.6"S 54°57'30.1"W	205
		2	2°51'18.8"S 54°57'28.9"W	207
		3	2°51'18.4"S 54°57'27.2"W	208
		4	2°51'18.0"S 54°57'25.6"W	208
		5	2°51'17.4"S 54°57'23.8"W	207
	F2	1	3°01'08.3"S 55°00'23.3"W	104
		2	3°01'08.1"S 55°00'22.1"W	107
		3	3°01'07.4"S 55°00'20.1"W	111
		4	3°01'07.4"S 55°00'18.4"W	116
		5	3°01'07.4"S 55°00'17.0"W	119
	F3	1	3°17'44.4"S 54°57'46.7"W	132
		2	3°17'45.8"S 54°57'47.6"W	133
		3	3°17'47.2"S 54°57'48.4"W	132
		4	3°17'48.1"S 54°57'49.7"W	130
		5	3°17'49.6"S 54°57'50.6"W	131
Pasture	P1	1	3°18'46.7"S 54°54'34.8"W	123
		2	3°18'48.3"S 54°54'34.9"W	128
		3	3°18'49.9"S 54°54'35.1"W	133
		4	3°18'51.5"S 54°54'35.0"W	134
		5	3°18'53.1"S 54°54'34.7"W	134
	P2	1	3°07'49.8"S 54°57'28.5"W	100
		2	3°07'51.3"S 54°57'28.4"W	100
		3	3°07'52.9"S 54°57'28.1"W	101
		4	3°07'54.4"S 54°57'27.8"W	102
		5	3°07'56.0"S 54°57'27.5"W	103
	P3	1	3°07'44.9"S 54°57'15.5"W	99
		2	3°07'43.9"S 54°57'14.2"W	101
		3	3°07'42.7"S 54°57'13.0"W	100
		4	3°07'41.1"S 54°57'12.4"W	98
		5	3°07'39.5"S 54°57'11.8"W	98

Supplementary Table S2.2 - Soil textural properties (mean and standard deviation) of the forest and pasture sites

Soil properties	Units	Forest			Pasture			Statistics
		F1	F2	F3	P1	P2	P3	Forest × Pasture
Sand	g kg^{-1}	36.2 ± 10.1	65.6 ± 13.3	652.6 ± 59.5	126.4 ± 31.6	741.4 ± 25.1	462.0 ± 34.1	NS
Clay	g kg^{-1}	818.6 ± 37.2	787.8 ± 20.7	298.0 ± 41.8	777.6 ± 46.0	221.8 ± 20.9	471.0 ± 23.0	NS
Silt	g kg^{-1}	145.4 ± 43.1	146.8 ± 29.4	49.6 ± 20.4	96.2 ± 25.1	36.8 ± 11.4	67.2 ± 19.7	NS

NS, non-significant at $p < 0.05$

Supplementary Table S2.3 - Soil texture of the sampling points in the forest and pasture sites according to the United States Department of Agriculture classification system (USDA, 2019)

Land-use	Site	Sampling point	Texture
Forest	F1	1	Clay
		2	Clay
		3	Clay
		4	Clay
		5	Clay
	F2	1	Clay
		2	Clay
		3	Clay
		4	Clay
		5	Clay
	F3	1	Sandy Clay Loam
		2	Sandy Clay Loam
		3	Sandy Clay Loam
		4	Sandy Clay Loam
		5	Sandy Clay Loam
Pasture	P1	1	Clay
		2	Clay
		3	Clay
		4	Clay
		5	Clay
	P2	1	Sandy Clay Loam
		2	Sandy Clay Loam
		3	Sandy Clay Loam
		4	Sandy Clay Loam
		5	Sandy Clay Loam
	P3	1	Sandy Clay
		2	Sandy Clay
		3	Sandy Clay
		4	Clay
		5	Clay

Supplementary Table S2.4 - Soil physical properties (mean and standard deviation) of the forest and pasture sites

Soil properties	Units	Forest			Pasture			Statistics
		F1	F2	F3	P1	P2	P3	Forest × Pasture
Total porosity	%	64.5 ± 5.0	64.8 ± 2.2	52.5 ± 2.7	50.5 ± 3.1	45.5 ± 5.1	49.9 ± 4.3	*
Microporosity	%	17.6 ± 8.0	20.4 ± 3.5	21.7 ± 5.3	9.0 ± 3.7	15.0 ± 8.7	20.7 ± 7.6	NS
Macroporosity	%	46.9 ± 3.0	44.4 ± 2.7	30.8 ± 2.9	41.5 ± 0.9	30.5 ± 4.5	29.2 ± 3.4	NS
Density	g cm^{-3}	0.82 ± 0.11	0.85 ± 0.05	1.17 ± 0.07	1.20 ± 0.08	1.34 ± 0.13	1.33 ± 0.11	*

NS, non-significant at $p < 0.05$

Supplementary Table S2.5 - Soil chemical properties (mean and standard deviation) of the forest and pasture sites during the dry season (November 2015)

Soil properties	Units	Forest			Pasture			Statistics
		F1	F2	F3	P1	P2	P3	Forest × Pasture
pH	-	3.42 ± 0.15	3.46 ± 0.05	3.64 ± 0.15	4.72 ± 0.54	4.98 ± 0.39	4.74 ± 0.57	*
SOM	g dm^{-3}	66.4 ± 16.0	56.0 ± 7.2	27.8 ± 1.9	34.2 ± 3.1	47.6 ± 5.2	40.8 ± 5.1	NS
P	mg dm^{-3}	1.7 ± 1.0	5.2 ± 1.9	1.3 ± 1.2	4.2 ± 1.8	11.0 ± 13.1	10.8 ± 7.2	NS
S	mg dm^{-3}	15.2 ± 2.8	14.2 ± 5.8	9.0 ± 1.9	5.6 ± 1.1	8.8 ± 2.5	7.0 ± 1.0	*
K	mmolc dm^{-3}	0.84 ± 0.09	0.84 ± 0.09	0.40 ± 0.12	1.62 ± 1.05	3.02 ± 3.47	2.56 ± 2.38	*
Ca	mmolc dm^{-3}	3.2 ± 0.4	3.4 ± 0.9	< 3.0 ± 0.0	19.0 ± 11.0	21.0 ± 6.9	23.4 ± 12.4	*
Mg	mmolc dm^{-3}	3.0 ± 0.0	3.2 ± 0.4	2.0 ± 0.0	6.4 ± 3.6	13.6 ± 7.5	8.6 ± 4.5	*
Al	mmolc dm^{-3}	31.0 ± 21.3	20.4 ± 1.7	10.8 ± 3.1	2.6 ± 1.8	1.4 ± 0.5	2.4 ± 1.5	*
H+Al	mmolc dm^{-3}	238.0 ± 85.7	206.2 ± 21.5	105.0 ± 29.4	47.6 ± 16.1	42.0 ± 8.1	51.0 ± 11.4	*
SB	mmolc dm^{-3}	6.24 ± 1.19	6.84 ± 1.60	3.20 ± 0.51	27.02 ± 13.79	37.62 ± 15.02	34.56 ± 18.61	*
CEC	mmolc dm^{-3}	244.24 ± 84.93	213.04 ± 22.07	108.20 ± 29.56	74.62 ± 6.21	79.62 ± 7.76	85.56 ± 13.05	*
V	%	2.6 ± 1.1	3.2 ± 0.8	3.2 ± 0.8	36.6 ± 19.8	46.2 ± 15.2	39.0 ± 18.0	*
m	%	79.6 ± 7.7	74.8 ± 4.7	76.4 ± 4.7	11.2 ± 9.2	4.6 ± 3.6	9.8 ± 9.2	*
B	mg dm^{-3}	0.650 ± 0.173	0.558 ± 0.087	0.348 ± 0.052	0.304 ± 0.034	0.232 ± 0.054	0.346 ± 0.114	*
Cu	mg dm^{-3}	0.36 ± 0.13	0.44 ± 0.32	0.12 ± 0.04	0.26 ± 0.05	1.92 ± 2.35	1.82 ± 2.56	NS
Fe	mg dm^{-3}	234.6 ± 79.9	189.0 ± 62.5	173.6 ± 62.5	54.0 ± 17.1	73.2 ± 32.5	135.8 ± 28.9	*
Mn	mg dm^{-3}	2.90 ± 2.21	4.46 ± 3.62	0.66 ± 0.42	2.54 ± 0.83	13.74 ± 5.41	8.76 ± 3.96	NS
Zn	mg dm^{-3}	5.52 ± 3.89	0.38 ± 0.19	0.26 ± 0.09	0.72 ± 0.08	2.34 ± 1.57	0.56 ± 0.23	NS

Means preceded by the less than sign (<) presented values lower than the quantification limit. NS, non-significant at $p < 0.05$. SOM, soil organic matter; H+Al, potential acidity; SB, sum of exchangeable bases; CEC, cation-exchange capacity; V, base saturation; m, aluminum saturation

Supplementary Table S2.6 - Soil chemical properties (mean and standard deviation) of the forest and pasture sites during the rainy season (May 2016)

Soil properties	Units	Forest			Pasture			Statistics
		F1	F2	F3	P1	P2	P3	Forest × Pasture
pH	-	3.48 ± 0.16	3.56 ± 0.09	3.44 ± 0.15	4.28 ± 0.24	4.66 ± 0.17	4.42 ± 0.34	*
SOM	g dm^{-3}	49.0 ± 10.6	45.6 ± 11.2	35.0 ± 4.4	37.4 ± 14.7	39.0 ± 3.2	33.0 ± 4.9	NS
P	mg dm^{-3}	6.6 ± 0.5	9.6 ± 1.7	6.2 ± 0.8	6.8 ± 3.5	5.8 ± 1.5	10.0 ± 6.2	NS
S	mg dm^{-3}	7.0 ± 1.6	11.2 ± 6.9	7.4 ± 2.6	5.4 ± 2.2	6.4 ± 2.5	8.8 ± 3.8	NS
K	mmolc dm^{-3}	0.60 ± 0.19	0.68 ± 0.15	0.36 ± 0.05	1.28 ± 0.81	0.44 ± 0.13	0.62 ± 0.20	NS
Ca	mmolc dm^{-3}	3.6 ± 0.5	6.6 ± 2.8	3.0 ± 0.0	11.6 ± 11.5	13.6 ± 3.4	14.6 ± 9.8	*
Mg	mmolc dm^{-3}	2.8 ± 0.8	3.8 ± 1.8	1.4 ± 0.5	4.6 ± 3.6	8.4 ± 2.7	6.4 ± 2.6	*
Al	mmolc dm^{-3}	21.8 ± 1.9	19.6 ± 3.4	14.8 ± 3.4	6.2 ± 3.1	2.0 ± 1.7	4.0 ± 3.0	*
H+Al	mmolc dm^{-3}	156.4 ± 41.7	139.4 ± 25.6	97.6 ± 21.7	55.6 ± 3.3	42.8 ± 9.1	46.4 ± 15.7	*
SB	mmolc dm^{-3}	6.80 ± 1.74	11.08 ± 4.30	4.36 ± 1.05	17.48 ± 15.22	22.44 ± 5.76	21.62 ± 12.36	*
CEC	mmolc dm^{-3}	163.20 ± 42.28	150.48 ± 27.11	101.96 ± 21.09	73.08 ± 13.86	65.24 ± 6.61	68.02 ± 9.23	*
V	%	4.2 ± 1.3	7.4 ± 2.7	4.6 ± 1.3	21.8 ± 13.8	35.0 ± 9.3	32.4 ± 19.7	*
m	%	76.4 ± 4.6	64.2 ± 10.5	76.8 ± 7.4	34.0 ± 20.3	9.4 ± 9.7	19.2 ± 16.1	*
B	mg dm^{-3}	0.676 ± 0.043	0.790 ± 0.126	0.424 ± 0.033	0.408 ± 0.027	0.394 ± 0.052	0.488 ± 0.081	NS
Cu	mg dm^{-3}	0.42 ± 0.13	0.32 ± 0.11	0.26 ± 0.05	0.48 ± 0.24	0.44 ± 0.15	0.24 ± 0.05	NS
Fe	mg dm^{-3}	179.8 ± 48.2	103.4 ± 48.0	113.0 ± 38.8	86.2 ± 13.8	79.2 ± 30.5	95.8 ± 27.2	*
Mn	mg dm^{-3}	3.34 ± 1.53	6.30 ± 4.54	2.86 ± 2.26	3.90 ± 5.15	5.50 ± 2.51	3.56 ± 0.81	NS
Zn	mg dm^{-3}	0.28 ± 0.08	0.14 ± 0.09	0.50 ± 0.12	1.40 ± 0.92	1.46 ± 1.47	0.48 ± 0.19	NS

Means preceded by the less than sign (<) presented values lower than the quantification limit. NS, non-significant at $p < 0.05$. SOM, soil organic matter; H+Al, potential acidity; SB, sum of exchangeable bases; CEC, cation-exchange capacity; V, base saturation; m, aluminum saturation

Supplementary Table S2.7 - Detailed information about the metagenomic sequences uploaded to the MG-Rast server 4.0.3. (MEYER et al., 2008): number of raw reads and base pairs; read length and GC content (mean and standard deviation); base error rate; and proportion of bases with Phred quality score above 20 and 30

Sample	Season	Land-use	Site	No. of reads	No. of base pairs	Mean read length (bp)	Mean GC content (%)	Base error rate (%)	Bases of Q > 20 (%)	Bases of Q > 30 (%)
C1	Dry	Forest	F1	50,391,249	7,558,687,350	150 ± 0	62 ± 8	0.02	96.22	90.60
C2	Dry	Forest	F1	55,700,040	8,355,006,000	150 ± 0	63 ± 8	0.02	96.56	91.32
C3	Dry	Forest	F1	48,294,413	7,244,161,950	150 ± 0	63 ± 8	0.03	94.76	87.88
C4	Dry	Forest	F2	54,620,913	8,193,136,950	150 ± 0	62 ± 8	0.02	96.00	90.13
C5	Dry	Forest	F2	57,458,707	8,618,806,050	150 ± 0	64 ± 8	0.02	96.30	90.77
C6	Dry	Forest	F2	50,682,023	7,602,303,450	150 ± 0	62 ± 8	0.02	96.25	90.63
C7	Dry	Forest	F3	51,968,207	7,795,231,050	150 ± 0	62 ± 8	0.02	96.09	90.25
C8	Dry	Forest	F3	48,194,355	7,229,153,250	150 ± 0	62 ± 8	0.02	96.31	90.69
C9	Dry	Forest	F3	33,069,369	4,960,405,350	150 ± 0	62 ± 7	0.03	93.75	86.18
C10	Dry	Pasture	P1	36,877,677	5,531,651,550	150 ± 0	65 ± 8	0.03	94.06	86.70
C11	Dry	Pasture	P1	37,136,069	5,570,410,350	150 ± 0	66 ± 8	0.03	94.00	86.58
C12	Dry	Pasture	P1	48,873,529	7,331,029,350	150 ± 0	66 ± 7	0.03	93.97	86.56
C13	Dry	Pasture	P2	45,616,386	6,842,457,900	150 ± 0	67 ± 8	0.03	93.78	86.25
C14	Dry	Pasture	P2	41,199,241	6,179,886,150	150 ± 0	67 ± 8	0.04	93.21	85.28
C15	Dry	Pasture	P2	48,413,365	7,262,004,750	150 ± 0	66 ± 8	0.01	96.94	92.06
C16	Dry	Pasture	P3	50,104,828	7,515,724,200	150 ± 0	64 ± 8	0.01	96.76	91.68
C17	Dry	Pasture	P3	57,565,627	8,634,844,050	150 ± 0	64 ± 8	0.01	96.85	92.03
C18	Dry	Pasture	P3	46,697,931	7,004,689,650	150 ± 0	67 ± 8	0.01	96.98	92.21
C19	Rainy	Forest	F1	44,907,671	6,736,150,650	150 ± 0	62 ± 8	0.01	97.09	92.50
C20	Rainy	Forest	F1	55,608,768	8,341,315,200	150 ± 0	62 ± 8	0.01	97.04	92.45
C21	Rainy	Forest	F1	50,026,199	7,503,929,850	150 ± 0	63 ± 8	0.01	97.25	92.84
C22	Rainy	Forest	F2	56,713,568	8,507,035,200	150 ± 0	62 ± 8	0.01	96.91	92.16
C23	Rainy	Forest	F2	43,622,933	6,543,439,950	150 ± 0	63 ± 8	0.03	94.69	87.53
C24	Rainy	Forest	F2	42,746,571	6,411,985,650	150 ± 0	62 ± 8	0.03	94.69	87.57
C25	Rainy	Forest	F3	40,431,173	6,064,675,950	150 ± 0	62 ± 8	0.02	95.23	88.63
C26	Rainy	Forest	F3	41,515,485	6,227,322,750	150 ± 0	63 ± 8	0.03	93.96	86.17
C27	Rainy	Forest	F3	48,840,395	7,326,059,250	150 ± 0	62 ± 8	0.03	94.19	86.89
C28	Rainy	Pasture	P1	42,652,101	6,397,815,150	150 ± 0	66 ± 9	0.02	94.82	87.94
C29	Rainy	Pasture	P1	45,720,045	6,858,006,750	150 ± 0	64 ± 9	0.03	94.56	87.48
C30	Rainy	Pasture	P1	54,221,253	8,133,187,950	150 ± 0	65 ± 8	0.03	94.21	86.87
C31	Rainy	Pasture	P2	51,984,014	7,797,602,100	150 ± 0	65 ± 9	0.02	94.81	88.00
C32	Rainy	Pasture	P2	49,373,901	7,406,085,150	150 ± 0	64 ± 8	0.02	94.62	87.69
C33	Rainy	Pasture	P2	46,267,199	6,940,079,850	150 ± 0	64 ± 9	0.02	95.09	88.46
C34	Rainy	Pasture	P3	43,222,163	6,483,324,450	150 ± 0	63 ± 8	0.02	95.09	88.50
C35	Rainy	Pasture	P3	46,610,016	6,991,502,400	150 ± 0	65 ± 8	0.02	95.50	89.27
C36	Rainy	Pasture	P3	48,285,554	7,242,833,100	150 ± 0	64 ± 8	0.02	95.06	88.43

The base error rate and the proportion of bases with Phred quality score above 20 and 30 were supplied by Novogene Co. Ltd. (Beijing, China). Q, Phred quality score.

Supplementary Table S2.8 - Detailed information about the metagenomic sequences after quality control on the MG-Rast 4.0.3. server (MEYER et al., 2008): number of artificial duplicate reads; number of remaining reads and base pairs after quality control; and read length and GC content (mean and standard deviation)

Sample	Season	Land-use	Site	No. of artificial duplicate reads	No. of reads	No. of base pairs	Mean read length (bp)	Mean GC content (%)
C1	Dry	Forest	F1	4,607,870	45,459,317	6,807,288,754	150 ± 2	62 ± 8
C2	Dry	Forest	F1	5,537,586	49,637,917	7,432,861,048	150 ± 2	63 ± 8
C3	Dry	Forest	F1	4,705,210	43,272,018	6,482,477,791	150 ± 1	63 ± 8
C4	Dry	Forest	F2	5,376,940	48,716,360	7,297,833,036	150 ± 1	62 ± 8
C5	Dry	Forest	F2	5,411,776	51,682,759	7,739,712,477	150 ± 2	64 ± 8
C6	Dry	Forest	F2	4,645,394	45,853,608	6,866,175,591	150 ± 2	62 ± 8
C7	Dry	Forest	F3	5,267,609	46,499,800	6,965,415,277	150 ± 1	62 ± 8
C8	Dry	Forest	F3	4,787,052	43,092,682	6,455,546,955	150 ± 1	62 ± 8
C9	Dry	Forest	F3	4,065,638	28,562,187	4,278,631,949	150 ± 2	62 ± 7
C10	Dry	Pasture	P1	4,636,103	31,859,851	4,772,449,479	150 ± 2	65 ± 8
C11	Dry	Pasture	P1	4,912,695	31,958,111	4,787,748,254	150 ± 2	66 ± 8
C12	Dry	Pasture	P1	6,916,325	41,222,502	6,175,112,387	150 ± 2	66 ± 7
C13	Dry	Pasture	P2	6,696,713	38,458,770	5,760,337,402	150 ± 2	67 ± 8
C14	Dry	Pasture	P2	5,718,492	35,192,814	5,271,302,937	150 ± 2	67 ± 8
C15	Dry	Pasture	P2	4,649,602	42,979,499	6,440,194,522	150 ± 1	66 ± 8
C16	Dry	Pasture	P3	5,062,077	44,634,455	6,688,420,609	150 ± 1	64 ± 8
C17	Dry	Pasture	P3	6,580,650	50,126,318	7,511,296,389	150 ± 1	64 ± 8
C18	Dry	Pasture	P3	5,162,614	40,761,913	6,107,029,035	150 ± 1	67 ± 8
C19	Rainy	Forest	F1	5,502,829	39,043,698	5,850,590,345	150 ± 1	62 ± 8
C20	Rainy	Forest	F1	7,045,263	48,084,999	7,204,765,951	150 ± 1	62 ± 8
C21	Rainy	Forest	F1	6,386,993	43,059,419	6,451,721,751	150 ± 1	63 ± 8
C22	Rainy	Forest	F2	7,434,933	48,784,237	7,309,283,294	150 ± 2	62 ± 8
C23	Rainy	Forest	F2	4,351,140	38,887,244	5,825,534,870	150 ± 1	63 ± 8
C24	Rainy	Forest	F2	4,548,061	37,807,475	5,663,891,210	150 ± 2	62 ± 8
C25	Rainy	Forest	F3	4,152,084	35,897,344	5,377,841,827	150 ± 1	62 ± 8
C26	Rainy	Forest	F3	4,072,878	36,962,110	5,535,725,329	150 ± 2	63 ± 8
C27	Rainy	Forest	F3	5,366,425	42,735,362	6,400,363,249	150 ± 1	62 ± 8
C28	Rainy	Pasture	P1	4,587,358	37,379,478	5,598,327,063	150 ± 1	66 ± 8
C29	Rainy	Pasture	P1	4,998,193	39,958,078	5,984,454,788	150 ± 1	65 ± 9
C30	Rainy	Pasture	P1	6,378,174	46,918,680	7,028,312,998	150 ± 1	65 ± 8
C31	Rainy	Pasture	P2	5,580,539	45,283,038	6,782,995,087	150 ± 1	65 ± 9
C32	Rainy	Pasture	P2	5,508,346	43,010,673	6,442,326,577	150 ± 1	65 ± 8
C33	Rainy	Pasture	P2	4,929,159	40,772,645	6,108,070,347	150 ± 1	65 ± 9
C34	Rainy	Pasture	P3	4,670,034	38,149,156	5,714,958,860	150 ± 1	63 ± 8
C35	Rainy	Pasture	P3	5,161,185	41,034,729	6,147,785,606	150 ± 1	65 ± 8
C36	Rainy	Pasture	P3	5,390,468	42,305,214	6,337,851,660	150 ± 1	64 ± 8

Supplementary Table S2.9 - Detailed information about the results of the metagenomic sequence annotation on the MG-Rast 4.0.3. server (MEYER et al., 2008): number of predicted rRNA and protein features; number of identified rRNA and protein features; and number of identified functional categories

Sample	Season	Land-use	Site	No. of predicted rRNA features	No. of predicted protein features	No. of identified rRNA features	No. of identified protein features	No. of identified functional categories
C1	Dry	Forest	F1	37,165	41,023,478	12,642	13,768,518	10,741,905
C2	Dry	Forest	F1	41,496	44,051,546	13,615	15,135,778	11,905,868
C3	Dry	Forest	F1	38,435	38,587,377	12,434	13,257,903	10,378,632
C4	Dry	Forest	F2	41,166	43,506,912	12,925	14,676,650	11,516,941
C5	Dry	Forest	F2	46,088	46,380,169	14,754	15,999,603	12,448,706
C6	Dry	Forest	F2	37,512	41,121,527	12,774	13,558,370	10,537,201
C7	Dry	Forest	F3	38,001	41,961,443	12,136	13,956,349	10,858,168
C8	Dry	Forest	F3	33,763	38,674,610	10,997	12,776,343	9,889,774
C9	Dry	Forest	F3	22,191	25,401,016	6,983	8,439,595	6,570,029
C10	Dry	Pasture	P1	30,926	28,922,074	10,494	10,402,717	8,065,118
C11	Dry	Pasture	P1	33,023	29,157,255	10,658	10,848,832	8,417,631
C12	Dry	Pasture	P1	42,055	38,157,493	13,519	14,059,315	10,966,241
C13	Dry	Pasture	P2	45,905	34,769,391	16,935	13,515,017	10,505,635
C14	Dry	Pasture	P2	42,546	32,887,040	15,837	12,727,130	9,945,530
C15	Dry	Pasture	P2	51,361	39,444,776	19,161	14,532,213	11,248,927
C16	Dry	Pasture	P3	48,460	41,112,328	19,335	15,118,231	11,772,351
C17	Dry	Pasture	P3	46,841	44,817,944	16,378	15,777,579	12,073,822
C18	Dry	Pasture	P3	64,743	36,939,133	29,120	16,443,612	Undefined
C19	Rainy	Forest	F1	31,577	34,860,595	10,422	11,537,475	9,005,790
C20	Rainy	Forest	F1	39,863	42,931,375	12,160	14,469,419	11,367,543
C21	Rainy	Forest	F1	40,400	37,797,985	12,051	12,857,581	10,121,128
C22	Rainy	Forest	F2	41,370	42,948,939	12,401	14,401,723	11,356,286
C23	Rainy	Forest	F2	37,206	34,366,685	12,867	11,474,836	8,935,834
C24	Rainy	Forest	F2	30,070	30,607,978	9,500	9,424,383	7,240,757
C25	Rainy	Forest	F3	32,628	32,436,430	10,437	10,819,955	8,452,688
C26	Rainy	Forest	F3	35,682	33,343,688	11,337	11,565,442	9,082,974
C27	Rainy	Forest	F3	35,932	38,155,278	11,555	12,779,556	Undefined
C28	Rainy	Pasture	P1	50,778	34,593,971	18,983	13,519,109	Undefined
C29	Rainy	Pasture	P1	47,308	36,654,186	16,174	13,156,904	10,252,376
C30	Rainy	Pasture	P1	50,898	43,065,010	17,593	15,543,127	12,058,170
C31	Rainy	Pasture	P2	62,692	41,462,972	23,050	14,876,623	11,652,574
C32	Rainy	Pasture	P2	52,503	39,681,127	18,483	14,124,605	11,055,414
C33	Rainy	Pasture	P2	54,905	37,607,378	17,548	13,053,089	10,096,866
C34	Rainy	Pasture	P3	35,772	34,539,336	12,806	11,916,071	9,305,781
C35	Rainy	Pasture	P3	45,638	36,898,315	16,698	13,403,514	10,259,757
C36	Rainy	Pasture	P3	43,628	38,499,560	15,382	13,624,412	10,543,343

Supplementary Table S2.10 - Methanogens identified through the RefSeq database (O'LEARY et al., 2016) on the MG-Rast 4.0.3. server (MEYER et al., 2008)

Domain	Phylum	Class	Order	Family	Genus
Archaea	*Euryarchaeota*	*Methanobacteria*	*Methanobacteriales*	*Methanobacteriaceae*	*Methanobrevibacter*
					Methanosphaera
					Methanothermobacter
				Methanothermaceae	*Methanothermus*
		Methanococci	*Methanococcales*	*Methanococcaceae*	*Methanococcus*
					Methanothermococcus
				Methanocaldococcaceae	*Methanocaldococcus*
				Methanocorpusculaceae	*Methanocorpusculum*
			Methanomicrobiales	*Methanomicrobiaceae*	*Methanoculleus*
					Methanoplanus
				Methanoregulaceae	*Methanoregula*
					Methanosphaerula
		Methanomicrobia		*Methanospirillaceae*	*Methanospirillum*
				Methanosaetaceae	*Methanothrix*
			Methanosarcinales	*Methanosarcinaceae*	*Methanococcoides*
					Methanohalobium
					Methanohalophilus
					Methanosarcina
			Methanocellales	*Methanocellaceae*	*Methanocella*
		Methanopyri	*Methanopyrales*	*Methanopyraceae*	*Methanopyrus*

Supplementary Table S2.11 - Methanotrophs identified through the RefSeq database (O'LEARY et al., 2016) on the MG-Rast 4.0.3. server (MEYER et al., 2008)

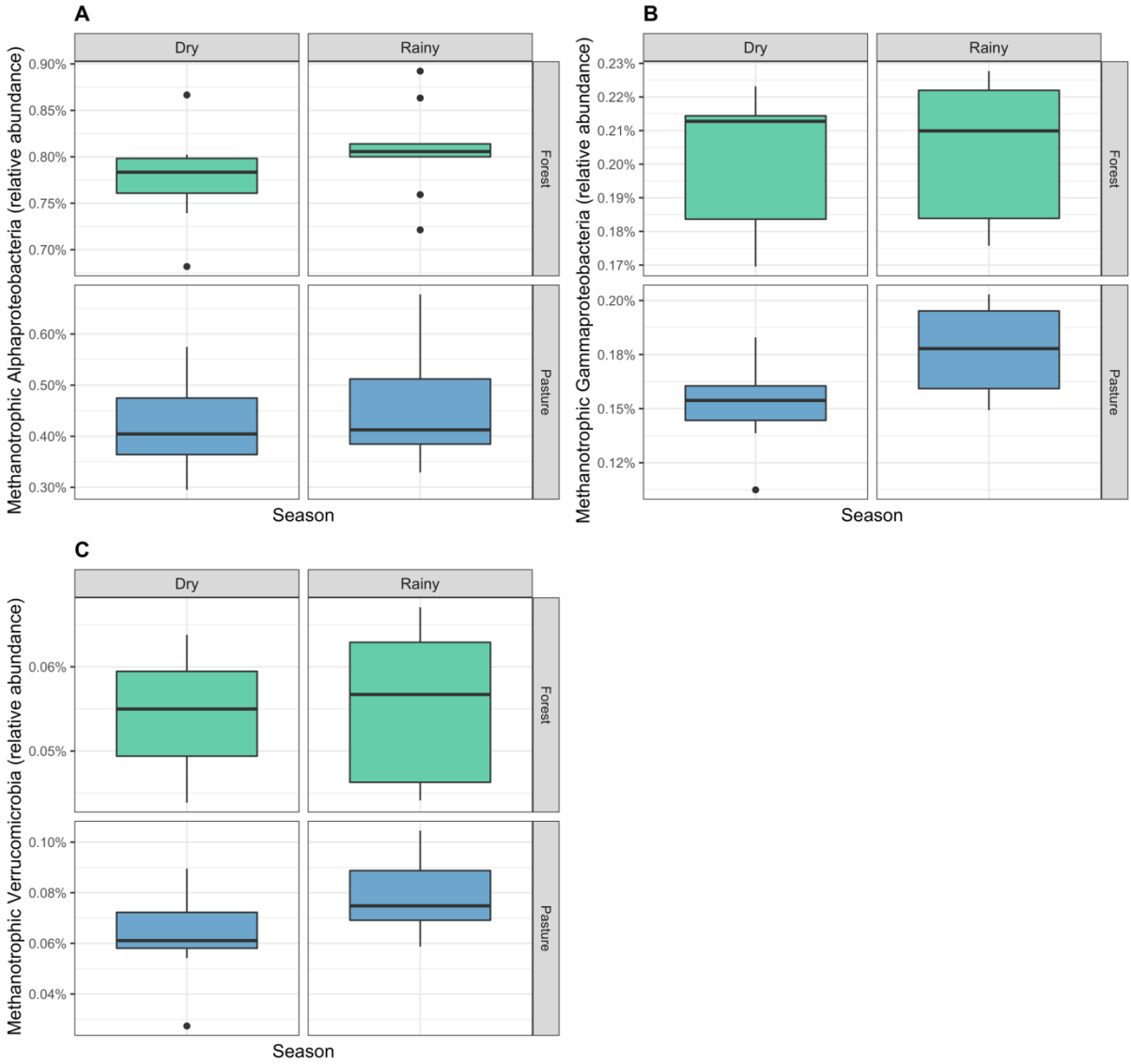
Domain	Phylum	Class	Order	Family	Genus
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Methylocystaceae*	*Methylosinus*
				Beijerinckiaceae	*Methylocella*
		Gammaproteobacteria	*Methylococcales*	*Methylococcaceae*	*Methylobacter*
					Methylococcus
	Verrucomicrobia	*Methylacidiphilae*	*Methylacidiphilales*	*Methylacidiphilaceae*	*Methylacidiphilum*

Supplementary Table S2.12 - Results of the non-parametric linear mixed-effects models (degrees of freedom, F-ratios, and p-values) for the taxonomic profiling of the methanotrophic *Alphaproteobacteria*, *Gammaproteobacteria*, and *Verrucomicrobia* by metagenomics

Data	Land-use			Season			Land-use × Season		
	df	F	p	df	F	p	df	F	p
Taxonomic profiling									
Methanotrophic taxa									
Alphaproteobacteria									
Shannon's diversity	1, 4	4.924	0.091	1, 16	7.274	0.016	1, 16	1.878	0.189
Simpson's diversity	1, 4	4.924	0.091	1, 16	7.036	0.017	1, 16	1.878	0.189
Pielou's evenness	1, 4	4.924	0.091	1, 16	7.036	0.017	1, 16	1.878	0.189
Relative abundance	1, 4	69.325	0.001	1, 16	1.690	0.212	1, 16	0.005	0.945
Gammaproteobacteria									
Shannon's diversity	1, 4	30.559	0.005	1, 16	2.102	0.166	1, 16	1.657	0.216
Simpson's diversity	1, 4	30.559	0.005	1, 16	2.102	0.166	1, 16	1.657	0.216
Pielou's evenness	1, 4	30.559	0.005	1, 16	2.102	0.166	1, 16	1.657	0.216
Relative abundance	1, 4	14.202	0.020	1, 16	10.183	0.006	1, 16	5.831	0.028
Verrucomicrobia									
Relative abundance	1, 4	14.169	0.020	1, 16	4.497	0.050	1, 16	3.672	0.073

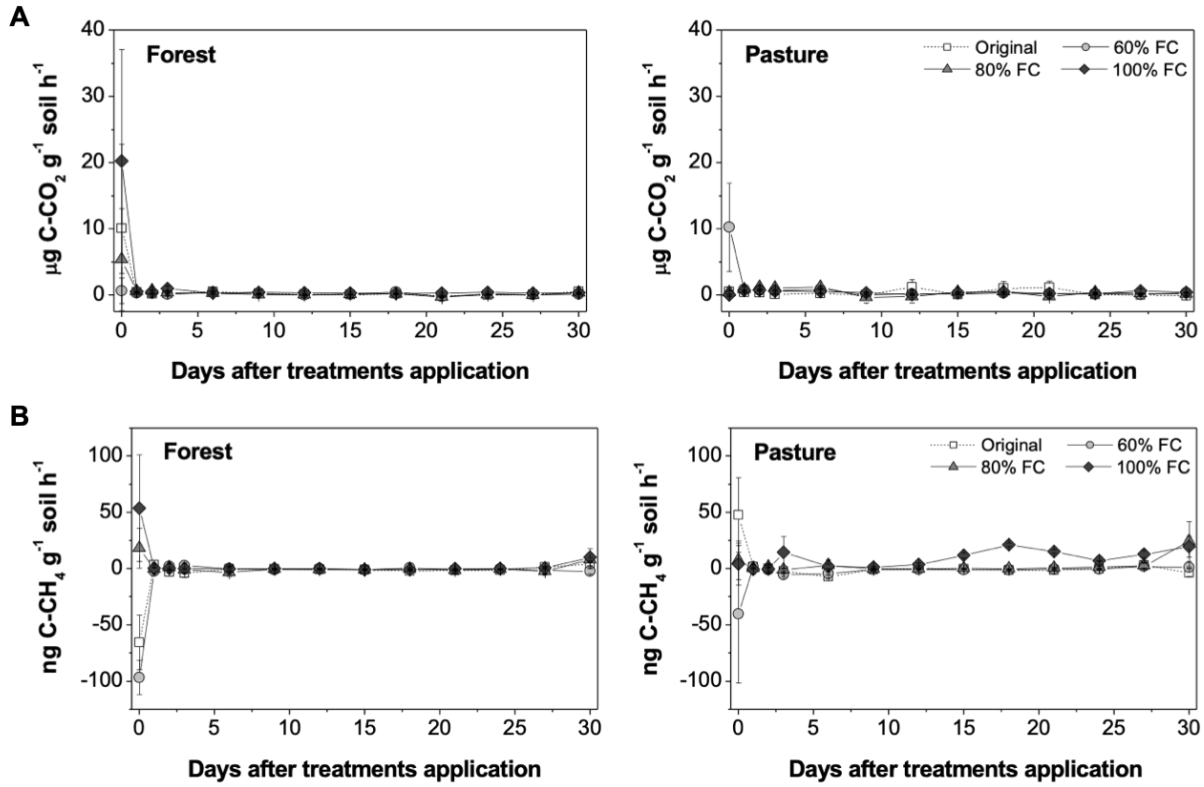
Supplementary Table S2.13 - Richness, Shannon's diversity, Simpson's diversity, and Pielou's evenness (mean and standard deviation) of the methanotrophic *Alphaproteobacteria* and *Gammaproteobacteria* at the genus level in the forest and pasture soils during the dry and rainy seasons

Land-use	Season	Methanotrophic *Alphaproteobacteria*				Methanotrophic *Gammaproteobacteria*			
		Richness	Shannon	Simpson	Pielou	Richness	Shannon	Simpson	Pielou
Forest	Dry	2	0.637 ± 0.005	0.444 ± 0.004	0.641 ± 0.006	2	0.607 ± 0.004	0.416 ± 0.003	0.601 ± 0.005
	Rainy	2	0.633 ± 0.006	0.441 ± 0.006	0.637 ± 0.008	2	0.606 ± 0.005	0.415 ± 0.005	0.599 ± 0.007
Pasture	Dry	2	0.647 ± 0.007	0.454 ± 0.007	0.655 ± 0.010	2	0.621 ± 0.005	0.429 ± 0.005	0.619 ± 0.007
	Rainy	2	0.642 ± 0.012	0.450 ± 0.011	0.649 ± 0.016	2	0.617 ± 0.008	0.426 ± 0.008	0.614 ± 0.011



Supplementary Figure S2.1 - Relative abundance obtained by metagenomics of the methanotrophic (A) *Alphaproteobacteria*, (B) *Gammaproteobacteria*, and (C) *Verrucomicrobia* in the forest and pasture soils during the dry and rainy seasons

Appendix B - Supplementary materials of Chapter 3 (Microcosm Study)



Supplementary Figure S3.1 - Daily fluxes of (A) CO_2 (µg C-CO_2 g^{-1} soil h^{-1}) and (B) CH_4 (ng C-CH_4 g^{-1} soil h^{-1}) over the 30-day period of the microcosm experiment with forest and pasture soils under four moisture levels (original moisture; 60%, 80%, and 100% of moisture at FC). FC, field capacity

Supplementary Table S3.1 - Detailed information about the metagenomic sequences uploaded to the MG-Rast server 4.0.3. (MEYER et al., 2008): number of raw reads and base pairs; mean and standard deviation of read length; mean and standard deviation of GC content; base error rate; and proportion of bases with Phred quality score above 20 and 30

Sample	Land-use	Treatment	No. of reads	No. of base pairs	Mean read length (bp)	Mean GC content (%)	Base error rate (%)	Bases of Q > 20 (%)	Bases of Q > 30 (%)
M1	Forest	Original	51,326,483	7,698,972,450	150 ± 0	63 ± 8	0.01	96.86	91.91
M2	Forest	Original	44,067,727	6,610,159,050	150 ± 0	63 ± 8	0.01	96.44	91.10
M3	Forest	Original	48,555,098	7,283,264,700	150 ± 0	63 ± 8	0.01	96.41	91.00
M10	Forest	100% FC	39,895,293	5,984,293,950	150 ± 0	62 ± 8	0.01	96.60	91.38
M11	Forest	100% FC	42,252,834	6,337,925,100	150 ± 0	62 ± 8	0.01	96.52	91.34
M12	Forest	100% FC	79,929,510	11,989,426,500	150 ± 0	62 ± 8	0.01	97.61	93.78
M13	Pasture	Original	44,290,138	6,643,520,700	150 ± 0	64 ± 8	0.01	96.79	91.83
M14	Pasture	Original	49,885,855	7,482,878,250	150 ± 0	64 ± 8	0.01	96.94	92.14
M15	Pasture	Original	45,344,207	6,801,631,050	150 ± 0	65 ± 8	0.01	96.81	91.84
M22	Pasture	100% FC	51,409,497	7,711,424,550	150 ± 0	64 ± 8	0.01	96.68	91.63
M23	Pasture	100% FC	42,729,731	6,409,459,650	150 ± 0	64 ± 9	0.01	96.68	91.61
M24	Pasture	100% FC	55,247,399	8,287,109,850	150 ± 0	64 ± 9	0.01	96.72	91.72

The base error rate and the proportion of bases with Phred quality score above 20 and 30 were supplied by Novogene Co. Ltd. (Beijing, China).
FC, field capacity; Q, Phred quality score

Supplementary Table S3.2 - Detailed information about the metagenomic sequences after quality control on the MG-Rast 4.0.3. server (MEYER et al., 2008): number of artificial duplicate reads; number of remaining reads and base pairs; mean and standard deviation of read length; and mean and standard deviation of GC content

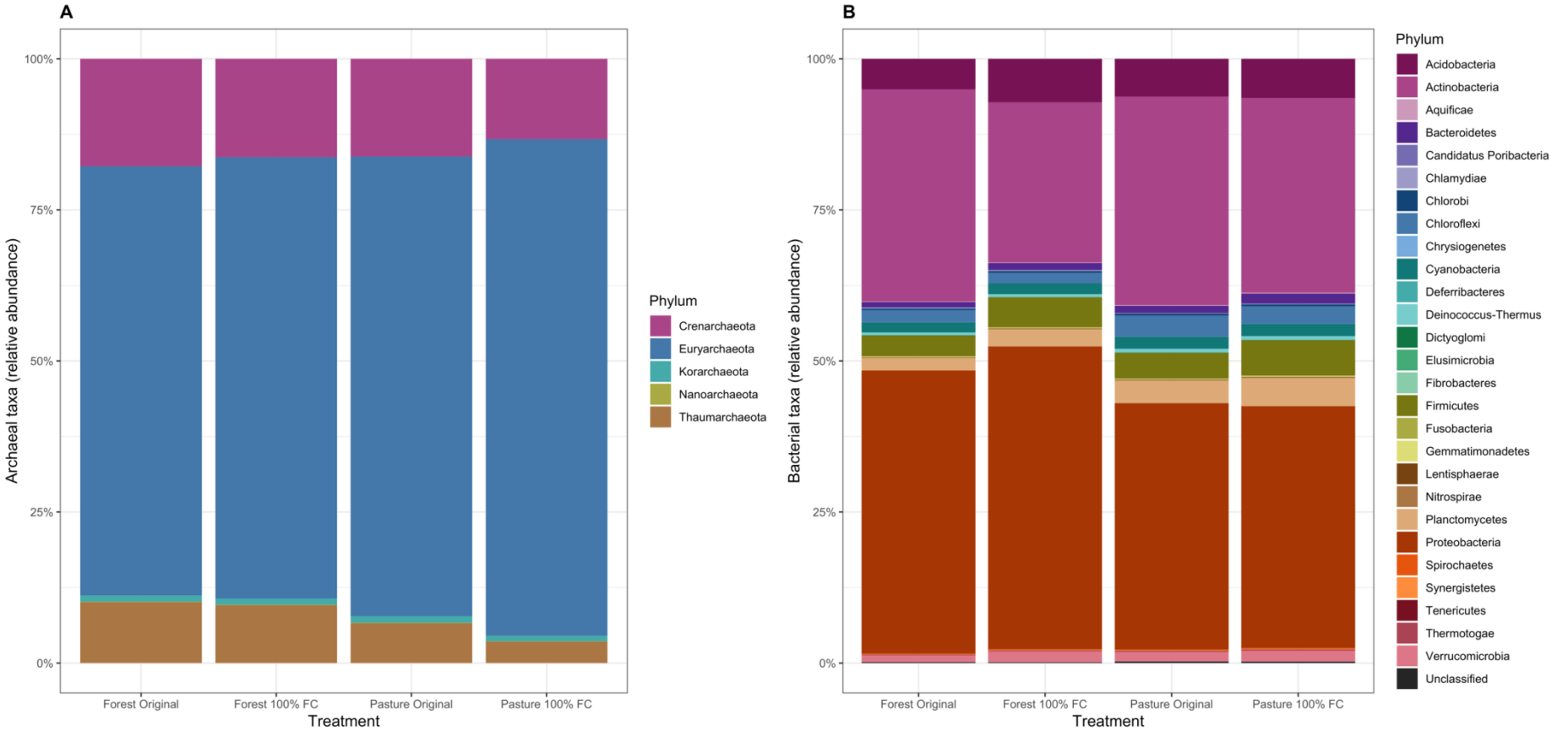
Sample	Land-use	Treatment	No. of artificial duplicate reads	No. of reads	No. of base pairs	Mean read length (bp)	Mean GC content (%)
M1	Forest	Original	6,396,681	44,544,330	6,669,936,333	150 ± 2	63 ± 8
M2	Forest	Original	4,263,012	39,226,710	5,875,429,152	150 ± 2	63 ± 8
M3	Forest	Original	4,763,573	43,106,041	6,456,225,393	150 ± 2	63 ± 8
M10	Forest	100% FC	3,330,606	35,994,725	5,391,733,505	150 ± 2	62 ± 8
M11	Forest	100% FC	4,577,484	37,366,246	5,598,585,981	150 ± 2	62 ± 8
M12	Forest	100% FC	11,729,922	67,062,618	10,050,309,623	150 ± 1	62 ± 8
M13	Pasture	Original	4,743,985	38,891,800	5,826,121,667	150 ± 2	64 ± 8
M14	Pasture	Original	5,487,386	43,877,900	6,574,170,448	150 ± 2	64 ± 8
M15	Pasture	Original	5,110,388	39,896,763	5,977,830,967	150 ± 2	65 ± 8
M22	Pasture	100% FC	5,900,855	44,783,323	6,709,509,667	150 ± 2	64 ± 8
M23	Pasture	100% FC	4,793,074	37,620,116	5,635,340,398	150 ± 2	64 ± 9
M24	Pasture	100% FC	6,691,376	47,985,396	7,189,386,940	150 ± 2	64 ± 9

FC, field capacity

Supplementary Table S3.3 - Detailed information about the results of the metagenomic sequence annotation on the MG-Rast 4.0.3. server (MEYER et al., 2008): number of predicted rRNA and protein features; number of identified rRNA and protein features; and number of identified functional categories

Sample	Land-use	Treatment	No. of predicted rRNA features	No. of predicted protein features	No. of identified rRNA features	No. of identified protein features	No. of identified functional categories
M1	Forest	Original	41,488	40,167,498	13,831	13,619,328	10,645,770
M2	Forest	Original	37,408	35,207,974	12,758	12,145,942	9,490,117
M3	Forest	Original	37,86	38,486,157	13,111	13,193,091	10,283,799
M10	Forest	100% FC	36,556	32,760,633	14,054	11,538,305	9,004,217
M11	Forest	100% FC	36,507	33,854,364	14,249	11,840,650	9,240,516
M12	Forest	100% FC	55,872	60,428,559	19,793	21,068,829	16,466,676
M13	Pasture	Original	39,046	35,555,485	13,922	13,082,304	10,070,050
M14	Pasture	Original	41,659	39,775,043	14,612	14,475,139	11,105,234
M15	Pasture	Original	40,118	36,321,365	14,631	13,538,622	10,413,736
M22	Pasture	100% FC	49,929	41,154,054	18,942	15,375,051	11,816,025
M23	Pasture	100% FC	44,316	34,553,338	16,972	12,843,938	9,886,006
M24	Pasture	100% FC	54,084	44,064,784	19,864	15,989,307	12,222,822

FC, field capacity



Supplementary Figure S3.2 - Taxonomic composition obtained by metagenomics of the (A) archaeal and (B) bacterial communities at the phylum level after the 30-day period of the microcosm experiment with forest and pasture soils under two moisture levels (original moisture and 100% of moisture at FC). FC, field capacity

Supplementary Table S3.4 - Methanogens identified through the RefSeq database (O'LEARY et al., 2016) on the MG-Rast 4.0.3. server (MEYER et al., 2008)

Domain	Phylum	Class	Order	Family	Genus
Archaea	*Euryarchaeota*	*Methanobacteria*	*Methanobacteriales*	*Methanobacteriaceae*	*Methanobrevibacter*
					Methanosphaera
					Methanothermobacter
				Methanothermaceae	*Methanothermus*
		Methanococci	*Methanococcales*	*Methanococcaceae*	*Methanococcus*
					Methanothermococcus
				Methanocaldococcaceae	*Methanocaldococcus*
				Methanocorpusculaceae	*Methanocorpusculum*
		Methanomicrobia	*Methanomicrobiales*	*Methanomicrobiaceae*	*Methanoculleus*
					Methanoplanus
				Methanoregulaceae	*Methanoregula*
					Methanosphaerula
				Methanospirillaceae	*Methanospirillum*
			Methanosarcinales	*Methanosaetaceae*	*Methanothrix*
				Methanosarcinaceae	*Methanococcoides*
					Methanohalobium
					Methanohalophilus
					Methanosarcina
			Methanocellales	*Methanocellaceae*	*Methanocella*
		Methanopyri	*Methanopyrales*	*Methanopyraceae*	*Methanopyrus*

Supplementary Table S3.5 - Methanotrophs identified through the RefSeq database (O'LEARY et al., 2016) on the MG-Rast 4.0.3. server (MEYER et al., 2008)

Domain	Phylum	Class	Order	Family	Genus
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Methylocystaceae*	*Methylosinus*
				Beijerinckiaceae	*Methylocella*
		Gammaproteobacteria	*Methylococcales*	*Methylococcaceae*	*Methylobacter*
					Methylococcus
	Verrucomicrobia	*Methylacidiphilae*	*Methylacidiphilales*	*Methylacidiphilaceae*	*Methylacidiphilum*

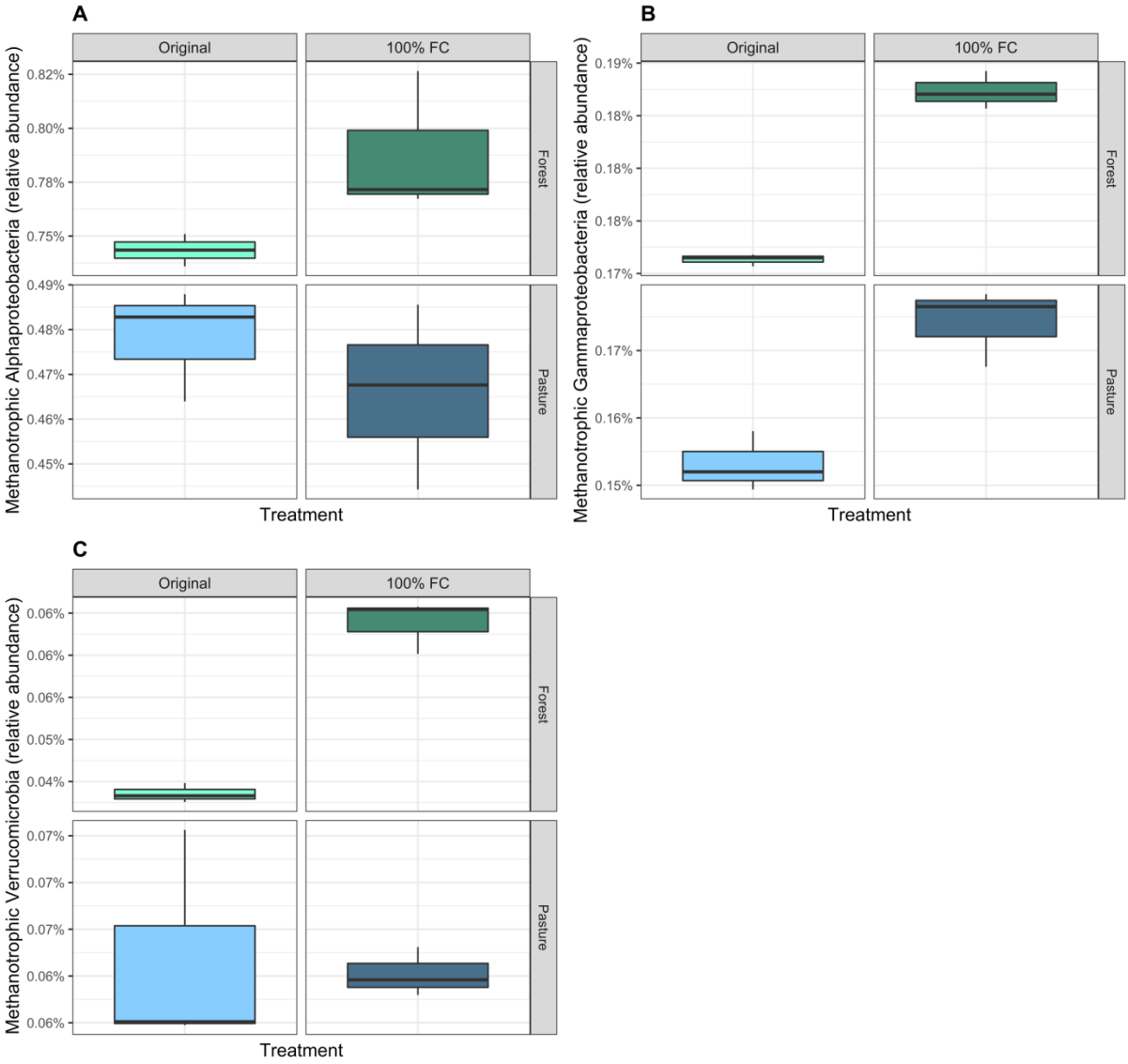
Supplementary Table S3.6 - Results of the non-parametric two-way factorial ANOVAs (degrees of freedom, F-ratios, and p-values) for the taxonomic profiling of the methanotrophic *Alphaproteobacteria*, *Gammaproteobacteria*, and *Verrucomicrobia* by metagenomics

Data	Land-use			Moisture			Land-use × Moisture		
	df	F	p	df	F	p	df	F	p
Taxonomic profiling									
Methanotrophic taxa									
Alphaproteobacteria									
Shannon's diversity	1, 8	25.920	< 0.001	1, 8	25.920	< 0.001	1, 8	24.923	0.001
Simpson's diversity	1, 8	25.920	< 0.001	1, 8	25.920	< 0.001	1, 8	24.923	0.001
Pielou's evenness	1, 8	25.920	< 0.001	1, 8	25.920	< 0.001	1, 8	24.923	0.001
Relative abundance	1, 8	25.920	< 0.001	1, 8	0.742	0.414	1, 8	9.574	0.015
Gammaproteobacteria									
Shannon's diversity	1, 8	25.920	< 0.001	1, 8	25.920	< 0.001	1, 8	25.920	< 0.001
Simpson's diversity	1, 8	25.920	< 0.001	1, 8	25.920	< 0.001	1, 8	25.920	< 0.001
Pielou's evenness	1, 8	25.920	< 0.001	1, 8	25.920	< 0.001	1, 8	25.920	< 0.001
Relative abundance	1, 8	24.923	0.001	1, 8	27.000	< 0.001	1, 8	1.037	0.338
Verrucomicrobia									
Relative abundance	1, 8	27.000	< 0.001	1, 8	27.000	< 0.001	1, 8	24.923	0.001

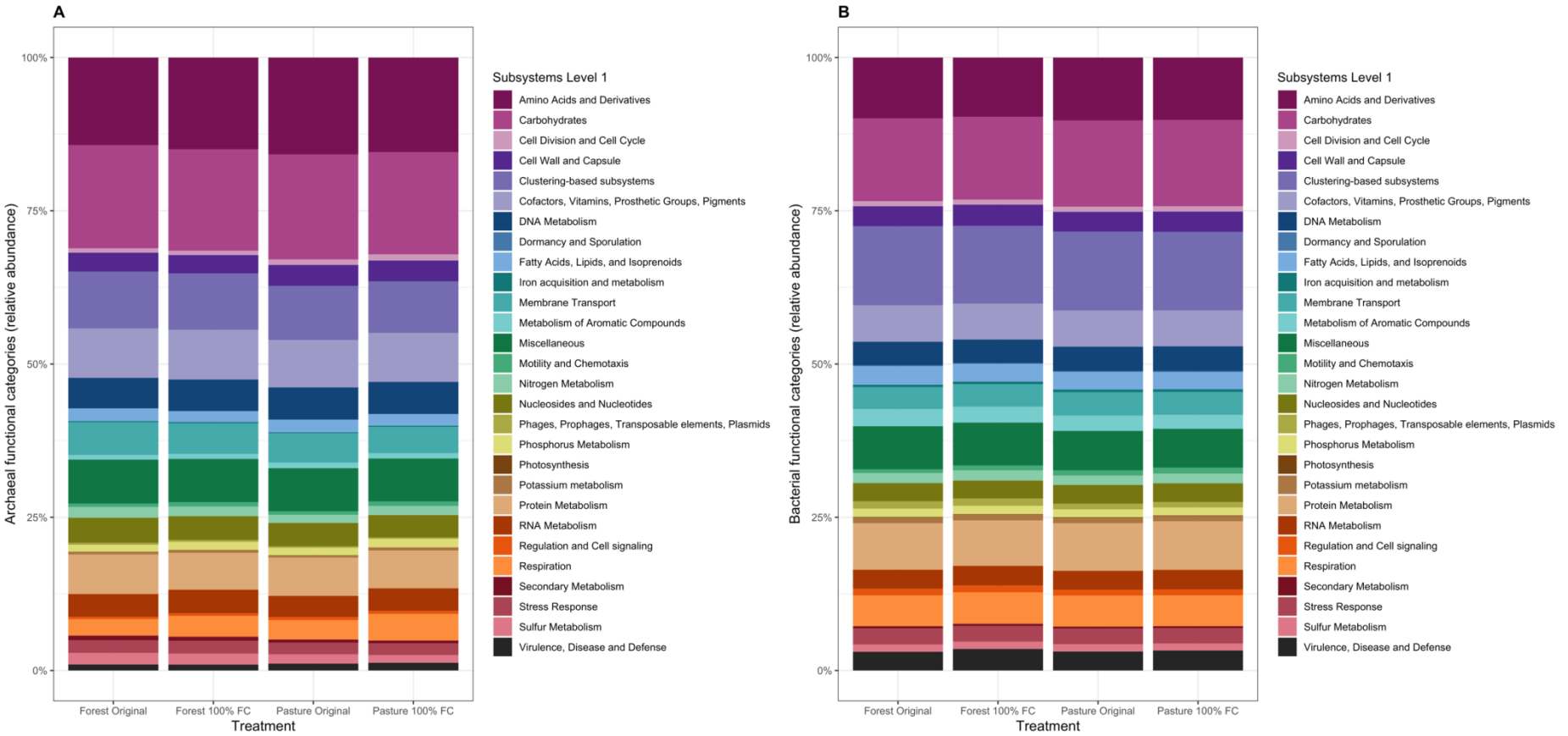
Supplementary Table S3.7 - Richness, Shannon's diversity, Simpson's diversity, and Pielou's evenness (mean and standard deviation) of the methanotrophic *Alphaproteobacteria* and *Gammaproteobacteria* at the genus level after the 30-day period of the microcosm experiment with forest and pasture soils under two moisture levels (original moisture and 100% of moisture at FC)

Land-use	Moisture	Methanotrophic *Alphaproteobacteria*				Methanotrophic *Gammaproteobacteria*			
		Nº	Shannon	Simpson	Pielou	Nº	Shannon	Simpson	Pielou
Forest	Original	2	0.630 ± 0.002	0.438 ± 0.002	0.632 ± 0.003	2	0.615 ± 0.002	0.424 ± 0.002	0.611 ± 0.003
	100% FC	2	0.628 ± 0.002	0.436 ± 0.002	0.629 ± 0.002	2	0.618 ± 0.001	0.427 ± 0.001	0.616 ± 0.002
Pasture	Original	2	0.641 ± 0.001	0.449 ± 0.001	0.648 ± 0.002	2	0.624 ± 0.001	0.432 ± 0.001	0.624 ± 0.001
	100% FC	2	0.659 ± 0.002	0.466 ± 0.002	0.673 ± 0.003	2	0.652 ± 0.007	0.459 ± 0.007	0.663 ± 0.010

FC, field capacity



Supplementary Figure S3.3 - Relative abundance obtained by metagenomics of the methanotrophic (A) *Alphaproteobacteria*, (B) *Gammaproteobacteria*, and (C) *Verrucomicrobia* after the 30-day period of the microcosm experiment with forest and pasture soils under two moisture levels (original moisture and 100% of moisture at FC). FC, field capacity



Supplementary Figure S3.4 - Functional categories obtained by metagenomics of the (A) archaeal and (B) bacterial communities at the Subsystems Level 1 after the 30-day period of the microcosm experiment with forest and pasture soils under two moisture levels (original moisture and 100% of moisture at FC). FC, field capacity