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FERNANDA MANCINI NAKAMURA

Microcosms and the role of active microbiota on methane cycle in soils under Forest and Pasture of Eastern Amazon

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To my parents

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"If anything is worth doing, do it with all your heart"
Buddha (563 – 483 BC)

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Fernanda Mancini Nakamura

"The power of intuitive understanding will protect you from harm until the end of your days."
Lao Tzu (- 531 BC)

"*Praticar diariamente uma maneira de*

viver que corresponda à vontade de Deus é, então, resolver os três

problemas do "meio ambiente", "recursos e "paz"."

Masanobu Taniguchi (1951), Ashimoto Kara Heiwa O

ABSTRACT

NAKAMURA, F. M. **Microcosms and the role of active microbiota on methane cycle in soils under forest and pasture of Eastern Amazon**. 2019. 117 p. Tese (Doutorado) – Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba.

Amazonian soils are being converted to agricultural lands. Changes in soil coverage and management drive modifications in the chemical and physical soil attributes, which influences the microbiota structure and biological roles. A great portion of the C and N cycling in soil are driven mostly by microbial activity, and the biological cycle of greenhouse gases (GHGs) on Amazonian soils can be influenced by soil use changes and climate changes. The magnitude of those changes in the Amazon Forest can impact considerably the biogeochemical cycles from this tropical area. Advanced molecular studies allied to stable isotope incorporation by soil microbiota can link the identity to function, besides their ecological relation with soil chemical and physical components. This study aimed to evaluate the dynamics of microbiota associated to the methane cycling of Amazonian soils in transitional land-uses. Also, evaluate the factorial effects of these land-uses subjected to climate changes predictions for this biome in a modified soil moisture and temperature. The specific objetive is evaluate these effects throught methane-enriched atmosphere for detection of methanotrophs and methanogens, besides other groups in a trophic network supplied with derived methyl-compounds. Microcosms were set in biological triplicates with intact core soil samples of Oxisols under Pristine Forest and Pasture of Eastern Amazon, in State of Pará, Brazil. In the first chapter, we had three moisture-modulated groups per point in 17%, 35% and 70% v.v. at field capacity, with factorial incubation in two groups of temperature, 25°C or 30°C, and incubation with natural atmosphere. In the second chapter, we had three moisture-modulated groups per point in 17%, 35% and 70% v.v. at field capacity at 30°C, and two groups of incubation, $^{12}CH_4$ or $^{13}CH_4$. Samples were all stored in closed headspace for 15 days in the dark. Gases CH_4, CO_2 and N_2O were measured in gas chromatography. Marker genes were absolute quantified in Real-time PCR for methanotrophs (*pmoA*), methanogens *(mcrA*), *16S rRNA* genes for *Bacteria* and *Archaea*. In the first chapter it was demonstrated that Forest and Pasture under wetness and 5°C above actual increased the microbial populations and lead to CH4 emission. In addition, Forest under drought release CH4; and Pasture under drought release N2O. We conclude that specific factorial conditions of land-use, soil type, soil moisture and temperature lead to divergent responses by the microbiota associated to the methane cycle in tropical soil.

In the second chapter it was demonstrated that the methane-enrichments, with $^{12}CH_4$ or $^{13}CH_4$, were able to stimulate microbial growth among the three tested moistures from the two land-uses. We addressed taxonomy to methane cycle associated functions and relate them to the system conditions, finding specific land and/or moisture related groups. Overall we conclude that under the predictions for climate changes in Amazonian biome, the drought for Western Amazon and wetness for Eastern Amazon can lead to drastic changes on soil microbiota associated with methane and other GHGs, changing the hitherto known inventory for natural emissions from tropical lands.

Keywords: methanotroph, methanogens, Archaea, Bacteria, pmoA, mcrA, hydrocarbons, organic matter, CH4, CO2, N2O, methyl-reducing methanogenesis, hydrogenotrophs, $^{13}CH_4$, Stable Isotope Probing (SIP), GC fractionation.

RESUMO

NAKAMURA, F. M. **Microcosmos e o papel da microbiota ativa no ciclo do metano em solos sob floresta e pastagem da Amazônia Oriental.** 2019. 117 p. Tese (Doutorado) – Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba.

Solo amazônicos vem sendo convertidos em áreas agrícolas. Mudanças na cobertura do solo e manejo trazem modificações nos atributos físicos e químicos do solo, que por sua vez influencia a estrutura da microbiota e suas funções biológicas. Uma grande porção da ciclagem de carbono e nitrogênio no solo dependem da atividade microbiana, e os ciclos biogeoquímicos dos gases do efeito estufa (GEEs) em solos amaznônicos podem ser influenciados pelas mudanças no uso do solo e pelas mudanças climáticas. A magnitude destas mudanças na Floresta Amazônica podem impactar consideravelmente os ciclos biogeoquímicos desta área tropical. Estudos moleculares avançados, aliados à incorporação de isótopos estáveis pela microbiota do solo, podem ligar a identidade à função, além das relações ecológicas com os componentes físicos e químicos do solo. Este estudo almejou avaliar a dinâmica da microbiota associada ao ciclo do metano em solos amazônicos sob transição de uso do solo. Como também, avaliar os efeitos fatoriais destas mudanças de uso do solo sujeitas às predições de mudanças climáticas para este bioma numa modificação de umidade e temperatura do solo. Os objetivos específicos são avaliar estes efeitos por meio de enriquecimento da atmosfera com metano para detectar metanotróficas e metanogênicas, além de outros grupos numa cadeia trófica suprida por compostos metílicos derivados. Os microcosmos foram estabelecidos em triplicata com amostras intactas de solo de Oxisolos sob Floresta Primária e Pastagem da Amazônia Oriental, Pará, Brasil. No primeiro capítulo, houve três grupos de umidade modulada por ponto em 17%, 35% and 70% v.v. sob capacidade de campo e incubação fatorial em dois grupos de temperatura, 25°C ou 30°C, e incubação com ar atmosférico natural. No segundo capítulo, houve três grupos de umidade modulada por ponto em 17%, 35% ou 70% v.v. em capacidade de campo sob 30°C e dois grupos de incubação, $^{12}CH_4$ ou $^{13}CH_4$. As amostras foram armazenadas em *headspace* fechado por 15 dias no escuro. Gases CH_4, CO_2 e N_2O foram medidos em cromatografia gasosa. Genes marcadores foram quantificados de forma absoluta em Real-time PCR para metanotróficas (*pmoA*), metanogênicas *(mcrA*), genes *16S rRNA* para *Bacteria* e *Archaea*. No primeiro capítulo foi demonstrado que Floresta e Pastagem sob alagamento e 5°C acima do atual levou

ao aumento das populações microbianas, levando à emissão de CH4. Além disso, Floresta sog seca emite CH4, e Pastagem sob seca emite N2O. Conclui-se que condições fatoriais específicas de uso do solo, tipo de solo, umidade e temperatura do solo acarretam respostas diferentes pela microbiota associada ao ciclo do metano em solos tropicais. No segundo capítulo foi demonstradoque os enriquecimentos de metano, com $^{12}CH_4$ or $^{13}CH_4$, foram capazes de estimular o crescimento microbiano ao longo das três umidades em dois usos do solo. A taxonomia do ciclo do metano foi associada às funções e às condições do sistema, encontrando grupos específicos do uso do solo e/ou umidade. No geral, conclui-se que sob as predições das mudanças climáticas no bioma Amazônico, a seca para o Oeste e alagamento para o Leste podem acarretar mudanças drásticas na microbiota do solo associada ao metano e outros GEEs, modificando o inventário conhecido até então para emissões de solos tropicais.

Keywords: metanotróficas, metanogênicas, Archaea, Bacteria, pmoA, mcrA, hidrocarbonetos, matéria orgânica, CH4, CO2, N2O, metanogênesis redutoras de metil, hidrogenotróficas, $^{13}CH_4$, Stable Isotope Probing (SIP), Fracionamento do conteúdo G+C

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1. PRELUDE AND THESIS STRUCTURE

> *"A destruição da natureza é um produto da 'ilusão' que acredita que os seres humanos e a natureza são existências sem ligação, com conflitos de interesse. As pessoas devem estar conscientes, de forma mais intensa, da Imagem Verdadeira de que a natureza e os seres humanos são um na essência"*
>
> *Masaharu Taniguchi(1893 – 1985), Shukyo Wa Naze Tokai O Hanareruka*

This thesis is structured by the purpose of presentation of pre-textual, textual elements - a introductory text, followed by studies presented in extended scientific manuscript format - and post textual supplementary materials available in the Appendices section. All elements attend the conditions of integrated contents which can be examined and consulted in just one document.

1.1 INTRODUCTION

Soil microorganisms are fundamental for the persistence of fertility (KISS et al., 1978; O'NEILL et al., 2009), being essential for the ecosystem functioning through organic matter decomposition, removal of xenobiotics and toxins, besides suppressive activities against pathogens (MOREIRA; SIQUEIRA, 2002). Soils present a notable microorganism metabolic diversity and genetic adaptability (KURTBOKE; SWINGS; STORMS, 2004), an important source of genetic resources for health, fuel, food, and goods industries. The microbial growth is influenced by the soil conditions. Normally, soil is an oligotrophic environment, where nutrients availability is derived from the interaction of soil type, soil coverage, management and climate, and biological properties (MIRANSARI, 2013; CUNHA et al., 2013).

The Amazonian Rainforest is the South America`s largest tropical forest, being the regulator of the rainfall seasons influencing the climate of the whole planet climate. The Brazilian Legal Amazon takes 61% of Brazil`s territory, about 5 mi km^2 across 9 States and 771 cities, comprising 12.3% of Brazilian population (IBGE, 2011). The populaional increase

and territory exploitation has been submitting Amazonia biome to intense deforestarion observed over the past 30 years (PRODES, 2019). These alterations comprise logging, the forest-to-cropland and forest-to-pasture transitions, based on a total deforestation, the "corte raso", and additional slash-and-burn for agricultural land establishment and maintenance (RIVERO et al, 2009). Forest-to-pasture comprises more than 80% of the land-uses changes, which is directly proportional to the increase in cattle number in the Brazilian Legal Amazon (IBGE, 2009). In addition, the selective logging has similar effect of the forest-to-agriculture transition, comprising croplands and pasture (HOUGHTON et al., 2000). The deforestation for agricultural lands places Brazil in 2nd most deforesting country in the world (HANSEN et al., 2013). The Land-use, Land-use Changes and Forestry (LULUCF), or Forestry and other Land-use Changes (FOLU) – UN`s nomenclature includes forest-to-agriculture, deforestation and forest degradation – places Brazil in 5th greenhouse gas emitter, and the 8th total GHGs emitter country (CAIT, 2014). The arisen issues reaches the ecossystem as a whole (TIAN et al., 2015), as the report of decrease in 1/3 of Amazonian rainforest in the capacity to sink C (Exbrayat et al., 2017), as well as the respiratory problems and infectuous diseases aggravation to local citizens (HAHN et al., 2014).

In addition, the climate changes drive alterations in the rain seasons and temperature (IBGE, 2014; UN Environment, 2018), being a further influence to the complex ecological interactions and activity in tropical biogeochemical cycles. Such perturbations promote release of high amounts of C to the atmosphere (EXBRAYAT et al., 2017), deprives the soil in nutrients previously provided by diverse roots exudates, and acidifies the soil, decreasing microorganisms recruitment leading to the loss of rare microbial groups, driving to functional redundancy where different taxa develops the same functions in soil utilizing the same niches (RODRIGUES et al., 2013). The prediction of a worse scenario for future effects of climate changes is challenging for the mitigation policies (EXBRAYAT et al., 2017).

C processing – from soil, up to gas emission and sink into metabolisation and mineralization – is mediated by soil organisms. On a decrescent scale from macrofauna, mesofauna – from worms coprolites to the tannin of plants – to be used by the microorganisms. Around 60 – 80 % of sank C in soil is emitted as CO_2; 15 – 20 % is transformed in non-humic substances and humine, ending up with 3 – 8 % being immobilized by soil microbial biomass. A great portion of the biochemical cycles, the cycling of C and N, are produced in soil by Bacteria, Archaea and Fungi, as a result of abiotic and biotic processes of production and consumption by soil`s microbiota (CONRAD, 2005). Thus, the biological

cycle of greenhouse gases (GHGs) on Amazonian soils can be influenced by soil use changes and climate changes.

Methane is the most impacting GHG for its heat retention potential aside the quantity emitted yearly, the 2nd most emitted correspondent to 17% of the total emitted GHGs (IPCC, 2014). The synergistic relation between methanogens and methanotrophs attract scientific interest for CH_4 modulation studies (Figure 1). Due to the vast dimension of Amazonian territory, and its land-use changes increasing rates, tracking methanogens and methanotrophs in these situations is of great value to the scientific undertanding of Amazonian biome, and to better predict future changes, either by direct anthropic activities or by worldwide climate changes.

Microbial studies are better supported by molecular approaches. Marker genes for key enzymes are screened and quantified with oligonucleotides (primers) to estimate populations size and modulations by system factors. Besides, the target or shotgun sequencing and identification allow te observation of the tendencies of groups and their ecological niches and relations. In addition to molecular studies, the stable isotope incorporation by soil microbiota can link the identity and activity of the consumers of a target substrate. Another trait is the G+C %mol linkage to populations related to the target biogeochemical cycle in soil. In the case of methane, we could observe which groups are present and how they are modulated by land-uses and abiotic factors in the Amazonian biome.

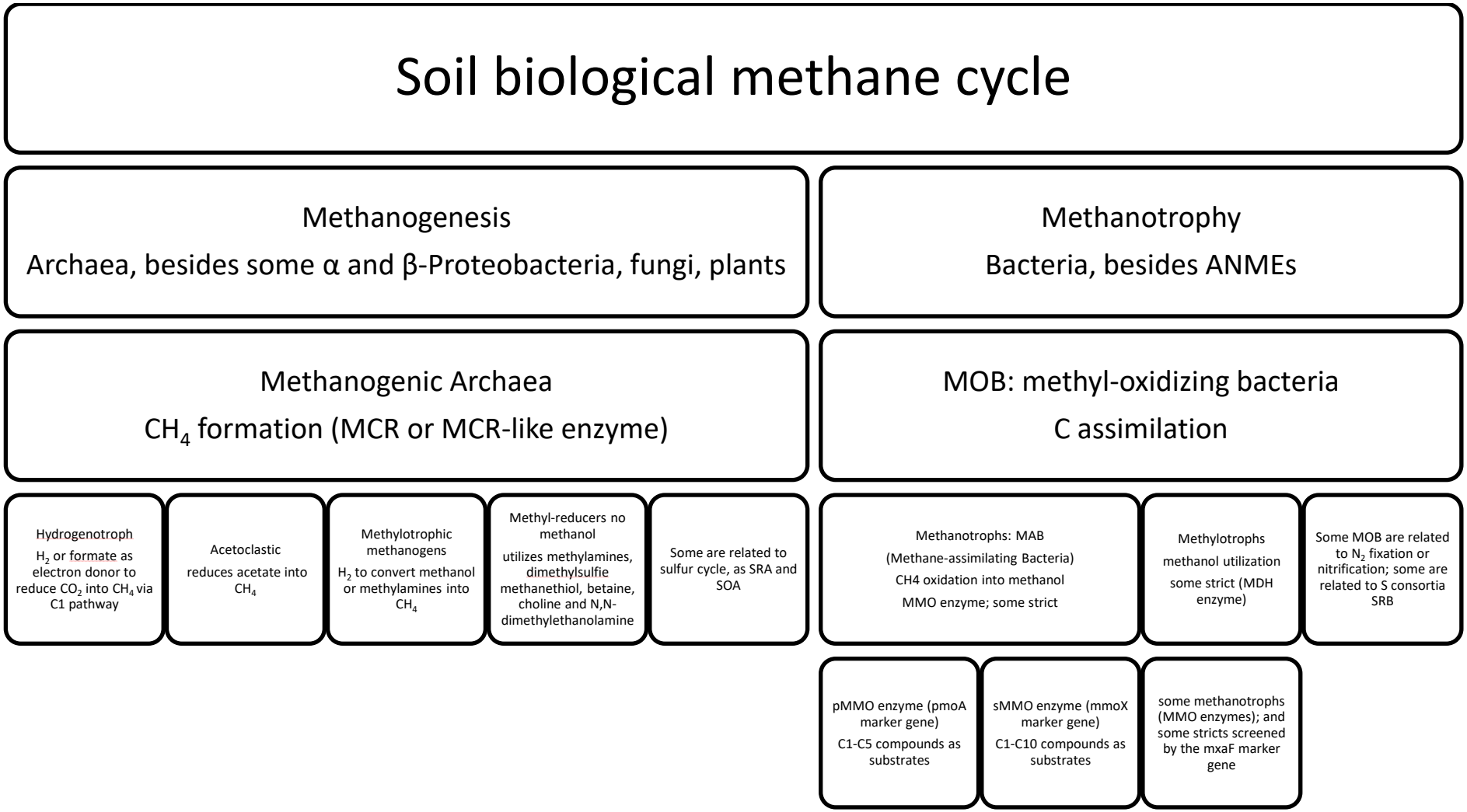

Figure 1 – Nomenclature utilized in this thesis to separate the groups according to the ecological activity in soil methane cycle.

1.2 Hypothesis

Land-use and Land-use Changes in the Eastern Amazonian biome alter methanotrophs and methanogens structures – as richness and abundance – among lands, as well as their relation with soil chemical and physical components are influenced by temperature and moisture changes, once predicted to occur in future as the consequence of the Global Climate Changes.

1.3 Objectives

Evaluate the microbiota related to the methane cycling in Land-use – forest and pasture – , and Land-use Changes – forest-to-pasture – of Eastern Amazonian soils subjected to aspects of the precited Climate Changes to this biome.

1.3.1 Specific Objectives

The aim of the second chapter was evaluate the influence of the isolated and combined factors – drought, drought-heat, wetness and wetness-heat – in the abundance of microbial populations – Bacteria, Archaea, methanotrophs and methanogens – and the abundance of emitted gas – CH_4, CO_2, N_2O – from closed microcosms with soil cores of Oxysols under Pristine Forest and Pasture of Eastern Amazon, in State of Pará, Brazil.

The aim of the third chapter was evaluate the abundance, metataxonomy and metafunction of methanotrophs, methanogens and methyl-consumers among the combined factors drought-heat and wetness-heat through C-isotopic-enrichment of closed microcosms with soil cores of Oxysols under Pristine Forest and Pasture of Eastern Amazon, in State of Pará, Brazil, and DNA isopycnic density separation coupled with G+C fractionation prior to amplicon next generation sequencing.

1.4 Methods

The broader view of the methodological pipeline performed in each of the two main studies (Figure 2), which are presented in chapters in this Thesis.

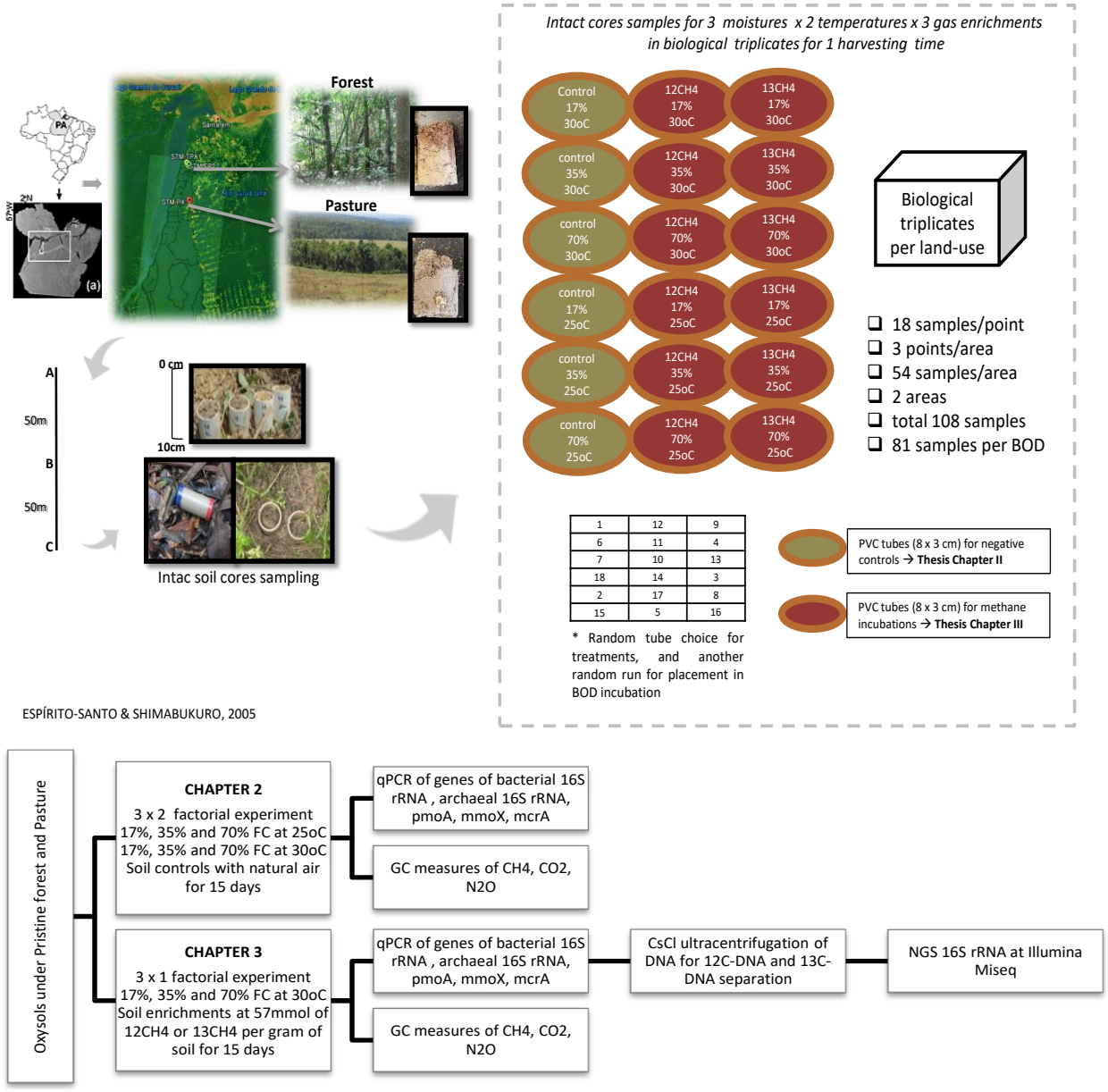

Figure 2 – General panorama of methodology implemented in Thesis.

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CHAPTER 2

CLIMATE CHANGES AND LAND-USE EFFECTS ON METHANE CYCLING OF EASTERN AMAZONIAN SOILS: MOISTURE AND TEMPERATURE DO MATTER

ABSTRACT

Amazonian soils are being converted to agricultural lands over the past 30 years. Changes in soil coverage and management drive modifications in the chemical and physical soil attributes, which influences the microbiota structure and biological roles. A great portion of the C and N cycling in soil are driven mostly by microbial activity, and the biological cycle of greenhouse gases (GHGs) on Amazonian soils can be influenced by soil use changes and climate changes. The magnitude of those changes in the Amazon Forest can impact considerably the biogeochemical cycles from this tropical area. This study aimed to evaluate the dynamics of microbiota associated to the methane cycling of Amazonian soils in transitional land-uses. Also, evaluate the factorial effects of the land-uses subjected to climate changes predictions for this biome in a modified soil moisture and temperature: drought, drought with heat, wetness and wetness with heat. Microcosms were set in biological triplicates with intact core soil samples of Oxisols under Pristine Forest and Pasture of Eastern Amazon, in State of Pará, Brazil. Microcosms of 3 x 2 were established, where three moisture-modulated groups per area point – 17%, 35% and 70% v.v. at field capacity – were further divided in two groups of temperature, 25°C and 30°C. Samples were stored in closed headspace for 15 days in the dark. Gases CH_4, CO_2 and N_2O were measured in gas chromatography. Marker genes were absolute quantified in Real-time PCR for methanotrophs (*pmoA*), methanogens *(mcrA*), *16S rRNA* genes for *Bacteria* and *Archaea*. The findings of this study where based uniquely on a microbial system to isolate the factors soil-microbiota. Besides, the experiment was developed in closed headspace, miming an extreme situation of gases accumulation. We demonstrate that Forest and Pasture under wetness and 5°C above actual increased the microbial populations and lead to CH4 emission. GHGs were all present in forest and pasture among moistures and/or temperatures. In addition, Forest under drought release CH4; and Pasture under drought release N2O. Forest and Pasture under wetness-heat had their methanogens and methanotrophs increased, reflecting on positive CH_4 and N_2O from dry Pasture, and high CH_4 in wet Pasture, which is alarming due to its natural high amount release under actual moisture and temperature conditions. Correlations to soil physico-chemical attributes show important macro and micronutrients as growth factors for methanotrophs and methanogens, mainly affected by moisture. We conclude that specific factorial conditions of land-use, soil moisture and temperature lead to divergent responses by the microbiota associated to the methane cycle in tropical soil. We conclude that under the predictions for climate changes in Amazonian biome, the drought for Western Amazon and wetness for Eastern Amazon can lead to drastic changes on soil microbiota associated with methane and other GHGs, changing the hitherto known inventory for natural emissions from tropical lands.

Keywords: methanotroph, methanogens, Archaea, Bacteria, pmoA, mcrA, hydrocarbons, organic matter, CH4, CO2, N2O.

2.1 INTRODUCTION

"One must still have chaos in oneself
to be able to give birth to a dancing star"
Friedrich Nietzsche (1844-1900)

The Amazonian Rainforest is the South America`s largest tropical forest, being the regulator of the rainfall seasons influencing the whole planet climate. The Brazilian Legal Amazon takes 61% of Brazil`s territory, about 5 mi km^2 across 9 States and 771 cities, comprising 12.3% of Brazilian population (IBGE, 2011). The populaional increase and territory exploitation has been submitting Amazonia biome to intense deforestation observed over the past 30 years (PRODES, 2019). These alterations comprise logging, the forest-to-cropland and forest-to-pasture transitions, based on a total deforestation, the "corte raso", and additional slash-and-burn for agricultural land establishment and maintenance (RIVERO et al, 2009). Forest-to-pasture accounts for more than 80% of the land-uses changes, which is directly proportional to the increase in cattle number in the Brazilian Legal Amazon (IBGE, 2009). The deforestation for agricultural lands inserts Brazil as the 2nd most deforesting country in the world (HANSEN et al., 2013). The Land-use, Land-use Changes and Forestry (LULUCF), or Forestry and other Land-use Changes (FOLU) – including forest-to-agriculture, deforestation and forest degradation – places Brazil in 5th as greenhouse gas emitter country, and the 8th total GHGs emitter country (CAIT, 2014).

Climate change is one of the greatest challenges of the century (BETTS et al., 2008), which alters the rain seasons and temperature (IBGE, 2014; UN Environment, 2018), being a further influence to the complex ecological interactions and activity in tropical biogeochemical cycles. Climate Changes predictions for 2040 – 2100 suggest disastrous consequences to the entire Earth climate regulation. Aside the current average rise of 0.258°C per decade (MALHI; WRIGHT, 2004), temperatures predictions show two worlds: optimistic projections suggest an increase of up to 1.5°C (IPCC, 2018), and pessimist projections suggest an increase of 3 – 8°C up to end of this century (DUFFY et al., 2015). Hence, predictions to Amazon show a threat to the region in a precipitation reduction of 20% in western Amazon, and 40% on eastern Amazon (MALHI et al. 2008; BETTS et al., 2008), causing wetness and drought as a savanna-like system, respectively, in the next 50 – 100 years (DUFFY et al., 2015).

However, there is no data to project the climatic changes influence in the tropical soils such as Amazonian soil microbiota (CLEVELAND & SULLIVAN, 2012; NAYLOR &

COLEMAN-DERR, 2018). Therefore, we aimed to provide additional data to soil changes predictions inserting the soil microbiota as a important trait to fill gaps in tropical ecosystem studies. Throught combined effects, we show the modulation of methanotrophs and methanogens among land-uses and abiotic changes in soil.

2.2 Hypothesis

The following hypothesis were tested: (a) higher moisture coupled with higher temperature will enhance methanogen population and methane emission in both land-uses; (b) methanotrophs will be higher in forest soil, thus there will be lower methane emission reltative to pasture.

2.3 Objectives

To pursue these hypothesis, we performed an experiment for temperature and moisture alterations in soils. The focus of this publication is therefore (a) to evaluate the methane soil microbiota behavior in abundance and GHGs emission in drought, drought-heat, wetness and wetness-heat for each land-use; (b) to evaluate how land-use change affects microbial communities related to GHGs modulation in soil.

2.4 Methods

2.4.1 Sampling and Experimental Design

The expedition occurred in June 2017 in two land-uses at Belterra Plateau, Eastern Amazon, State of Pará, Brazil: a) a preserved Pristine Forest (PF) (S2 51.326 W54 57.501) at km 67, the “Forest 2” of LBA scientific station, in Tapajós National Forest (Floresta Nacional dos Tapajós - Flona Tapajós); b) and a nearby well-maintained and active 15 year old Pasture (PT) (S3 07.830 W54 57.475) located at km 92, near Flona Tapajós border. The soil classification is detailed in Table 1. Soil chemical attributes are shown in Table 2. This region is under a humid climate classified as Am (Köppen) which rainfall annual mean is 936 mm; wet season mean of 1450 mm; and dry season mean of 423 mm; and temperature annual mean of 25.5°C; wet season mean of 24.5°C; and dry season mean of 27.3°C (BDMEP, 2017). A transect per area was sampled in three points distant 50 m from each other, where the first one

was 50 m inside the area to avoid borderline effect. Nine intact core soil samples were collected per point in polyvinyl chloride tubes of 8 x 3 cm. The total population size was 36 samples. Physical analysis samples were taken with one metal ring of 5 x 5 cm per point; and 300 g per point were stored in plastic bags for chemical analysis. All the samples were stored in ventilated bags and sent to the Laboratory of Cellular and Molecular Biology of Center for Nuclear Energy on Agriculture (LBCM – CENA/USP), Piracicaba, State of São Paulo, Brazil. The intact core soil samples were sent to the Laboratory of Prof. Jorge L. M. Rodrigues at the Department of Land, Air and Water Resources, University of California, Davis, CA, USA.

The experiment was a 3 x 2 performed with intact soil cores under three moisture variations – 17%, 35% and 70% FC – and further two temperature variations – 25˚C and 30˚C. First, the actual moisture and dry weight (dw) of each sample was evaluated through subsamples submitted to drying in glass plates in stove for 72 h. Second, the total dry weight of each sample was evaluated, described on item 2.4.4, excluding the weight of the sampling-tubes. Third, three groups of each land-use had their moisture altered by drying in dry stove or adding autoclaved 0.2µm filtered tap water, to reach the three moisture variations, and let set for 3 days to drain, covered with Parafilm M (Sigma Aldrich, USA) to avoid evaporation, and allow gas exchanges. Fourth, 5 g dw equivalent of intact soil cores were collected to 27 ml glass tubes, air tight closed with butyl rubber stoppers and crimped with aluminum caps. Fifth, 30 ml of 0.2µm filtered room air were inserted. Sixth, samples were divided again into two groups to be incubated at 25°C or 30°C. The samples were disposed randomly in tubes racks inside the two equal BODs (Innova2 Incubator Shaker Series, New Brunswick, NJ, USA), covered with dark cloth and let set for 15 days. After incubation, 25 ml of the accumulated headspace were harvested and stored in 15 ml Exetainer vials (Labco, Ceredigion, UK) and cap tops sealed with hot glue. The soils were immediately stored at ultra-freezer at -80°C.

Table 1 – Soil classifications

Site	Study name	GPS coordinates	Soil coverage	Soil Type class (Sousa Jr et al., 2008)	Soil Type class. (WRB/FAO, 1998)	Texture class
FP	Pristine Forest 2, km 67 (Flona Tapajós)	S2 51.326 W54 57.501	low anthropical activity	LAd3: Typic Dystrophic Yellowish "Latossolo", highly clayey, medium clay, flat and medium wavy	Oxisol or Udox/ Ustox; Ferralsol	High Clay
PT	Pasture 3, km 92 (property)	S3 07.830 W54 57.475	15 yo. *Brachiaria* sp. Pasture with cattle activity	LAd22 + PVAd: Typic Dystrophic Yellow "Latossolo", clayey and highly clayey, medium wavy + Typic Dystrophic Red-Yellowish "Argissol"	Oxisol or Udox/ Ustox + Udult/Ustult; Ferralsol	Medium Clay

2.4.4 Soil attributes analysis

Soil samples (300 g) and cylinders with core samples were chemically analyzed (Soil Science Department of ESALQ/USP, Piracicaba, Brazil) and the methodology was as follows: a) chemical analysis - pH in water and KCl by potentiometric method; organic matter (SOM) by Walkley-Black modified for optical density difference; total N by Kjeldahl method and NH4 and NO3 extracted by distillation and vapor; P by Mehlich extractor; K and Na by emission photometry; exchangeable Ca and Mg by atomic absorption spectrometry; potential acidity (H+Al) by potentiometric of SMP (SCHMACKER, MCLEAN, PRATT); cations exchange capacity (CEC) and sum of exchangeable bases (SB) by Embrapa Solos methods (1997); b) physical analysis (Soil Physics Department, CENA/USP, Piracicaba, Brazil) with Buyoucos densimeter (CAMPBELL et al., 2002); c) Field capacity (FC) was determined under the soil gravimetric water content (GWC), or gravimetric water holding capacity "European Method" (WHC): 30 g of soil and 100 ml of water in a funnel with cotton in the bottom, drained and dried for 72 h in stove, and calculated under equation 1 (EMBRAPA, 1997). The actual moisture started with the natural soil moisture and calculated in the same way.

$$U\% = [(Mu - Ms)/Ms] \times 100 \quad (Equation\ 1)$$

U% *= gravimetric moisture;*

Mu *= wet soil mass (g);*

Ms*= dry soil mass (g).*

2.4.5 Gas quantification

Methane and nitrous oxide were quantified in a gas chromatographer (Shimadzu Scientific Instruments with system 1, SC, USA) equipped with System 1 with column of 20m x 0.2 mm. The flame ionization detector (FID) used for CH_4 determination performance is described as follows: heating at 250°C, pressure of 143 kPa, N_2 as carrier gas, H2 pressure of 75 kPa, air pressure of 50 kPa, and retention time of 2.5 minutes. The electron capture detector (ECD) for N_2O determination performance is described as follows: heating at 320°C, pressure of 148.8 kPa, N_2 as carrier gas, argon/methane as make-up gas in 12 kPa.4ml^{-1}, and retention time of 6.1 minutes. Samples were diluted in filtered atmospheric gas insertion from 1:1 to

1:10 to avoid cross-contamination. Besides, one blank comprised by atmospheric air was utilized between samples to avoid super estimation in saturated samples. In addition, gas standards were used every 20 samples for area peak and concentration calculation, which varied from 10 – 100 ppm for CH_4, and 1 – 10 ppm for N_2O (Quadren Cryogenic Processing, California, USA). The measured concentration assignment was perfomed under the equations 2 and 3.

$$\boldsymbol{sample\ CH4\ ppm = 0.0002 * x + 1.5343}\ \textit{(Equation 2)}$$

$$\boldsymbol{sample\ N2O\ ppm = 3\ [\text{^}(-5)] * area - 0.5256}\ \textit{(Equation 3)}$$

The carbon dioxide was analyzed in a single channel non-dispersed infrared (S153 CO2 Analyzer Qubit, MA, USA) supplied by carrier gas N_2 in a insertion of 0.5 -1 ml discounting the baseline. CO_2 standards used were 1,000 - 30,000 ppm (Quadren Cryogenic Processing, California). Between samples, four syringes equivalent to 12 samples were directly inserted to clean the columns to avoid contamination between samples saturated in CO_2. Samples were diluted in filtered atmospheric gas insertion from 1:1 to 1:10 to avoid cross-contamination. Besides, one blank comprised by atmospheric air was utilized between samples to avoid super estimation in saturated samples.

Finally, the accumulated gas data from closed headspace were normalized for µg of C or N per g of soil beyond equation 4.

$$\boldsymbol{GHG\mu g = GHGppm * GHGMM * [(Patm * VL)/(R * TK * soilg)]}\ \textit{(Equation 4)}$$

$\boldsymbol{GHG_{\mu g}}$ *= GHG mass (µg)*
$\boldsymbol{GHG_{ppm}}$ *= Gas chromatograph output (ppm)*
$\boldsymbol{GHG_{MM}}$ *= atomic mass of C or N (g/mol)*
$\boldsymbol{P_{atm}}$ *= environment pressure (atm)*
$\boldsymbol{V_L}$ *= flask volume (L)*
$\boldsymbol{R}$ *= ideal gases constant (0,082)*
$\boldsymbol{T_K}$ *= temperature (K)*
$\boldsymbol{soil_g}$ *= soil dry mass (g)*

2.4.6 DNA extraction

Soil genomic DNA was isolated from treated samples in duplicate with DNeasy PowerLyzer PowerSoil Kit (Qiagen, MD, USA) with modifications for clayey soils adapted from Venturini[1] et al. (2019), where the precipitated was re-extracted and another vortex step of 15 min was applied to enhance the DNA yields. Besides, all incubations were set under -20˚C. Subsamples were then pooled, and evaluated in quality and quantity in electrophoresis 1% gel – stained with GelRed (Biotium, CA, EUA), molecular standard Low Mass (Invitrogen, MA, USA), run at 90V for 45 min –, and finally quantified in a fluorometer Qubit 3.0 (Thermo Fischer Scientific, MA, EUA).

2.4.7 Taxonomical and biochemical marker genes quantification

Standard curves for Real-Time PCR (qPCR) were built from standard microorganisms detailed at Supplementary Material SP A1. Cloning and sequencing was performed to achieve a single amplicon used as standard curve template. Conventional PCR was carried out as follows: 5 µl Phusion HSII High Fidelity Mastermix (Thermo Scientific, MA, USA); 1µl of 10µM of primers (IDT, IA, USA); 10 ng of DNA and H_2O ultrapure to a final volume of 10 µL. Primers sequences and reaction conditions are detailed (SP A1). The amplicons were cloned with TOPO® TA Cloning® Kit for Sequencing (Invitrogen, CA, USA) and plasmids were extracted with Wizard® Plus SV Minipreps DNA Purification Systems (Promega, WI, USA). Amplicon sizes were checked in agarose gel at 1% under 80V for 50 min, and concentration was checked in Qubit (Thermo Scientific, MA, USA). Dillution for absolute quantification were performed – 10 to 10E7 copies for *mcrA* and *pmoA* genes, or 10 to 10E9 for 16S rRNA Bacteria and 16S rRNA Archaea genes. Sequencing reaction with BigDye™ Terminator v3.1 Cycle Sequencing Kit, and primer M13F/M13R were prepared to check the identity of the amplicon. For 16S rRNA Archaea gene standard curve, environmental clone was obtained from soil samples of this study by conventional PCR as described above, followed by 1% agarose gel unique band purification with QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) at the desired size. Amplicons were sequenced at an ABI 3730

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Capillary Electrophoresis Genetic Analyzers with ABI BigDye Terminator v3.1 Cycle and sequenced at the UCDNA Sequencing Facility, UCDavis, Davis, USA. Sequences were confirmed at blatsn (AGARWALA et al., 2018).

The absolute Real-Time quantitative PCR (qPCR) reactions were taken in Bio-Rad Thermocycler (Bio-Rad, CA, USA) as follows: 5µl SSOAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, CA, USA), 10µM of each primer; 10 ng of DNA and H_2O to a final volume of 10µl. Primers and reaction conditions are detailed on SP A1. Data was analyzed for specificity and efficiency at CFX Maestro Software (Bio-Rad, CA, USA). The *16S rRNA Bacteria, 16S rRNA Archaea, pmoA* and *mcrA* genes were calculated by the linear regression of Ct values (threshold cycle) – the first cycle in which amplicon accumulates and its fluorescence intensity (RFU) is possible to detect – against serial dilution of a template in a standard curve at Real-Time PCR Detection Systems software (Bio-Rad, USA).

To ensure equal realiable gene quantification among multiple plates, an inter-run calibration sample (IRS) was used as a parameter of inter-run error comprised by two experiment samples, one of high abundance and other of low abundance of the target genes – carrying the same inhibitors of all samples, if there is one, allowing higher reliability from multiple plates. The quality parameters for qPCR quantification E (reaction efficiency), R2 (coefficient of determination), slope, negative control, and Ct values between technical triplicates were checked. The data was exported to Excel. For the post-amplification analysis, we also accounted for the possibility that different DNA stages of the standard curve used by several freeze-thaw cycles, different plates, reagents and even machines could lead to a vicious bias for each plate. To equal these gene copies for multiple plates of a single experiment under a single gene type, inter-plate calibration (IRC) was also performed with Factor_qPCR software version 2016.0 (RUIJTER et al., 2006), which performs a correction factors by a matrix of geometric means.

2.4.9 Statistical analysis

Factorial experiment 2 x 3 x 2 for forest-to-pasture analysis, detailed on Figure 3, including 2 populations based on land-use type (between-subjects interaction: Forest-PF and Pasture-PT areas) subjected to treatments in within-subjects interactions (moisture 1, moisture 2, moisture 3 : temperature 1, temperature 2) for 7 microbial traits as variables (absolute Real-time qPCR archaea by V4 16S rRNA with 519f/806R primers, Bacteria by V4 16S rRNA 515F/806R with primers, methanotrophs by *pmoA* gene, methanogens by *mcrA* gene; GC of CH4, CO2, N2O). The gas abundance was normalized for µg of C or N per gram of dw. The gene abundance was normalized for copy per gram of dw. All statistical analysis were performed in R software (R-Core-Team, 2018). Normality was evaluated by Shapiro-wilk test with stats package 3.5.1 from R default (R-Core-Team, 2018). The soil chemical and physical attributes, normal data, were evaluated by analysis of variance (ANOVA) with Post hoc Tukey`s test at $p < 0.05$ with agricolae package 1.3-0 (MENDIBURU, 2015). The microbial traits, from the factorial experiment evaluated as non-normal data, were analyzed by mixed-design analysis of variance (split plot ANOVA) with ART package 1.0 (VILLACORTA, 2015) and Post hoc Tukey`s test at $p < 0.05$ with agricolae package 1.3-0 (MENDIBURU, 2015). Spearman's monotonic correlations between soil attributes and microbial traits were generated using the ggplot2 package 3.1.0 (Wickham, 2016). All means, microbial traits summarized as mean ± confidence interval at 95% and significances were organized in tables.

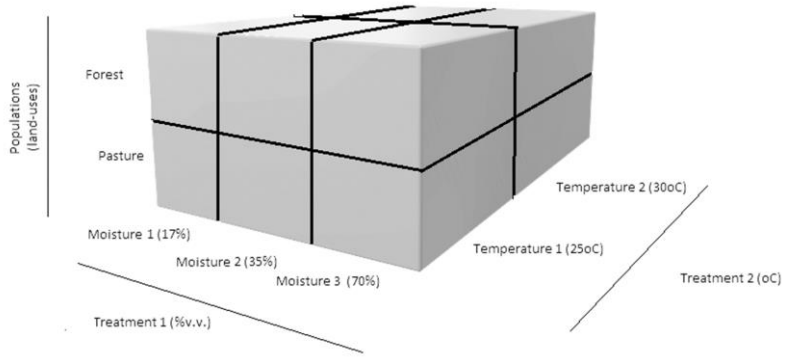

Figure 3 – Variables dependences in the factorial 3 x 2 analysis for each land-use, and 2 x 3 x 2 for land-use change, the forest-to-pasture.

2.5 Results

2.5.1 Land-use soil analysis

All soils present low pH, however PF is the most acid, and also have significantly higher acidity potential (H+Al), Al+3, aluminum saturation (m%), Fe, B, N and CEC; c) Bases saturation (V%) is significantly higher in PT; c) organic matter (MOS), phosphorus (P), sulfur (S), Ca^{+2}, sum of bases (SB), Copper (Cu), Mn^{+2} and Zn, contents are similar for all soils; d) the grade of clay is crescent from PT to PF. The following contents were similarly found in both soils: SOM, P, S, Ca^{+2}, SB, Cu, Mn^{+2} and Zn.

Table 2 - Soil attributes characterization

Soil attributes	Unit	Areas	
		PF2	PT3
pH	$CaCl_2$	3,48a	4.28b
MOS	$g.dm^{-3}$	49a	37.4a
P	$mg.dm^{-3}$	6,6a	6.8a
S	$mg.dm^{-3}$	7a	5.4a
K	$mmolc.dm^{-3}$	0,6a	1.28a
Ca^{+2}	$mmolc.dm^{-3}$	3,6a	11.6a
Mg^{+2}	$mmolc.dm^{-3}$	2,8a	4.6a
Al^{+3}	$mmolc.dm^{-3}$	21,8b	6.2a
H+Al	$mmolc.dm^{-3}$	156,4b	55.6a
SB	$mmolc.dm^{-3}$	6,8a	17.48a
CTC	$mmolc.dm^{-3}$	163,2b	73.08a
V	%	4,2a	21.8b
m	%	76,4b	34a
B	$mg.dm^{-3}$	0,676b	0.408a
Cu	$mg.dm^{-3}$	0,42a	0.48a
Fe	$mg.dm^{-3}$	179,8b	86.2a
Mn	$mg.dm^{-3}$	3,34a	3.9a
Zn	$mg.dm^{-3}$	0,28a	1.4b
N	$mg.dm^{-3}$	2697.8b	1506.4a
Sand	$g.kg^{-1}$	36,2a	741.4b
Clay	disperse $g.kg^{-1}$	818,6a	777.6a
Silt	$g.kg^{-1}$	145,4a	96.2a
Texture class		Highly Clayey	Medium Clayey

Abbreviations: mo, soil organic matter; H+Al, acidity potential; SB, sum of bases (Ca+Mg+K); CEC, cation exchange capacity (Al+Ca+Mg+K); V%, bases saturation [(Ca+Mg+K/CEC) x 100]; m%, aluminum saturation. (2) Means (n=3) from each sampling point, in each site. (3) Letters refer to mean differences (n = 9, P < 0.05, ANOVA Tukey's tes).

2.5.2 Microbial traits quantifications

The quality parameters for absolute gene quantification (qPCR) ranged within the expected for a robust procedure with reaction efficiency (E) between 90-105%; coefficient of determination (R2) around 0.980; and slope inside the range -3.6 and -3.1 indicate an almost entire double amplification of each sequence per cycle (PFAFFL, 2001; LOBO, LOBO, 2014). The copies of the negative control were minimum for all four genes evaluated. Treated treated samples were assigned as positive (above background noise). Ct values between technical triplicates were checked.

Significant alterations among moistures, temperatures and double interaction between samples are summarized in Figure 4. Absolute abundances are detailed in Table 3 and Figure 5, and relative genes abundances detailed in Figure 6. The overall influences of different moistures and temperatures affected all the microbial traits tested for both land-uses (SP A2). Moisture affected all populations except methanogens – Archaea ($p < 0.001$), Bacteria ($p < 0.001$), methanotrophs ($p < 0.05$). Moisture also affected CH4 ($p < 0.001$) and N2O ($p < 0.001$). Temperature affected Archaea ($p < 0.001$), methanogens ($p < 0.05$), methanotrophs ($p < 0.001$), CH4 ($p < 0.001$) and N2O ($p < 0.001$). The factorial combinations of moisture and temperature affected differently the land-uses. In Forest, we had influency of combined factors for Archaea and Bacteria (both, $p < 0.001$), as well as methanogens and CH4 (both, $p < 0.05$). In Pasture, we had combined influence for Archaea ($p < 0.05$), Bacteria ($p < 0.001$), and N2O ($p < 0.01$).

In wet forest soil we found the highest abundances for Bacteria in both temperatures (FN7025 and FN7030, $F= 76.83$, $p < 0.001$) and Archaea under 30oC (F7030, $F = 30.28$, $p < 0.001$). Methanogens were found in similar abundances in all samples, except dry forest soils under both temperatures ($F= 26.42$, $p < 0.01$). CH4 was in highest abundance in dry forest soil under 25oC (FN1725, $F = 6.7$, $p < 0.01$).

For pasture soils, the highest abundances of Archaea was also wet pasture soil under 30oC (PN7030, $F = 5.88$, $p < 0.05$). Bacteria was found in higher abundance in higher temperature ($F= 11.76$, $p < 0.05$). N2O tended to drop with moisture, being higher in dry pasture soils ($F = 7.94$, $p < 0.01$).

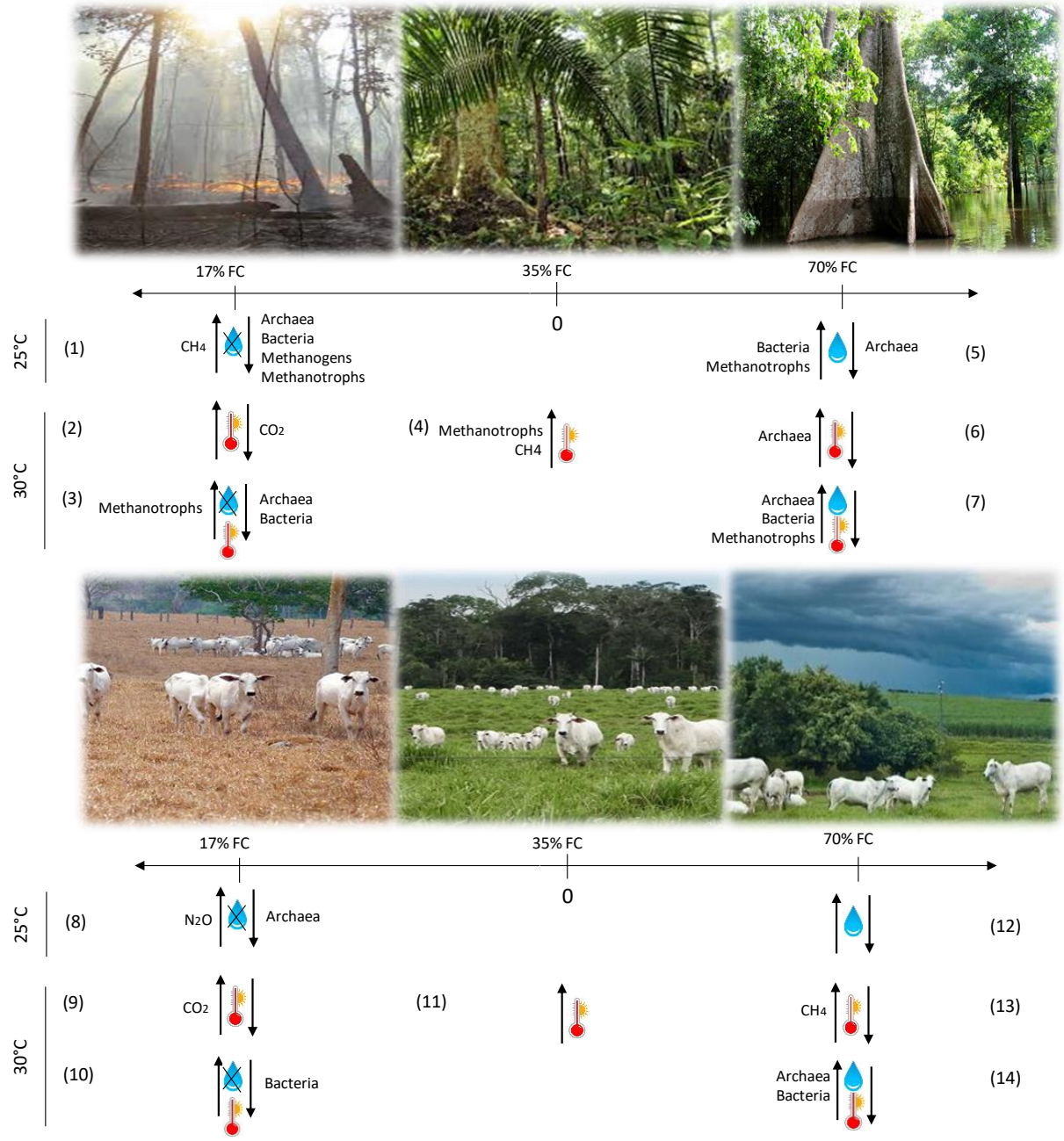

Figure 4 – Summarizing the significant ($p < 0.05$) microbial traits modulation in the transitions of treatments among moistures and temperatures.

Abbreviations: FN, Forest soils; PN, Pasture soils; comparison between samples under same moisture (17%, 35% or 70% v.v. of f.c.) and changing temperature (25°C to 30°C); (1), FN3525 to FN1725; (2), FN1725 to FN1730; (3) FN3530 to FN1730; (4), FN3525 to FN3530; (5), FN3525 to FN7025; (6), FN7025 to FN7030; (7) FN3530 to FN7030; (8), PN3525 to PN1725; (9), PN1725 to PN1730; (10), PN3530 to PN1730; (11), PN3525 to PN3530; (12), PN3525 to PN7025; (13) PN7025 to PN7030; (14) PN3530 to PN7030.

Images sources:Revista FAPESP; Dupont; Copopular, El Territorio; WWF Brasil; Nosnomundo.

Table 3 – Difference of means for the effect of factorial treatments based on quantification of 16S rRNA genes of Archaea and Bacteria, *mcrA* and *pmoA* genes (copies g soil^{-1}), and C from CH_4 and CO_2, and N from N_2O (µg g soil^{-1}) for all sites, followed by the results of F-values, and p-values of the rank-aligned mixed-design ANOVA

Sample	Area	M (%v.v)	T (oC)	16S rRNA of Archaea	Std (±)	F-value	16S rRNA of Bacteria	Std (±)	F-value	*mcrA*	Std (±)	F-value	*pmoA*	Std (±)	F-value	C-CH4	Std (±)	F-value	C-CO2	Std (±)	F-value	N-N2O	Std (±)	F-value
M*T per land-use																								
FN1725	Forest	17	25	9.75E+09a	1.95E+09	30.28*** ↑	9.88E+09a	1.98E+09	76.85*** ↑	3.52E+03ab	1.46E+03	26.42** ↑	1.11E+06a	1.16E+06	3.38	34.33b	34.25	6.70** ↓	4.49E+04a	7.66E+03	0.61	4.78a	0.05	0.92
FN1730			30	6.71E+09a	1.24E+09		5.53E+09a	4.16E+09		4.07E+03a	5.60E+03		4.41E+05a	5.60E+05		0.49ab	0.79		2.18E+04a	1.20E+04		4.81a	6.92	
FN3525		35	25	2.62E+10cd	1.91E+09		3.31E+10b	1.04E+09		2.21E+04b	1.14E+04		4.90E+06a	1.54E+06		0.03a	0.01		2.51E+04a	1.72E+04		0.22a	0.27	
FN3530			30	2.06E+10bc	4.15E+09		5.28E+10b	1.12E+10		6.34E+04b	3.73E+03		7.88E+06a	2.00E+06		0.09ab	0.05		2.65E+04a	1.76E+04		2.13a	3.55	
FN7025		70	25	1.51E+10b	1.86E+09		6.58E+10c	2.42E+09		2.27E+04b	3.16E+03		1.13E+07a	5.04E+06		0.04a	0.00		2.60E+04a	1.29E+04		0.05a	0.06	
FN7030			30	3.69E+10d	4.60E+09		6.65E+10c	1.29E+10		1.74E+04ab	1.19E+04		2.23E+08a	3.69E+08		0.07ab	0.02		2.80E+04a	2.29E+04		0.10a	0.08	
PN1725	Pasture	17	25	3.00E+09a	9.46E+08	5.88* ↑	9.67E+09a	4.87E+09	11.66**	3.43E+07a	2.22E+07	2.44	2.24E+08a	5.09E+07	0.62	0.03ab	0.01	1.69	5.89E+03a	4.47E+03	0.21	0.03a	0.03	7.94** ↓
PN1730			30	3.28E+09a	4.18E+08		8.74E+09a	4.01E+09		4.38E+07a	3.75E+07		3.99E+08a	3.50E+08		0.05b	0.01		1.12E+04a	1.31E+03		0.01a	0.01	
PN3525		35	25	7.10E+09ab	1.80E+09		1.49E+10a	1.30E+10		7.31E+07a	5.16E+07		4.22E+08a	5.24E+08		0.03ab	0.01		7.92E+03a	6.58E+03		0.01a	0.00	
PN3530			30	6.42E+09ab	1.55E+09		2.65E+10a	4.93E+09		7.73E+07a	3.05E+07		3.96E+08a	7.49E+07		0.05ab	0.02		7.43E+03a	5.98E+03		0.01a	0.00	
PN7025		70	25	7.13E+09ab	2.63E+09		3.24E+10a	1.21E+10		1.01E+08a	3.16E+07		6.47E+08a	3.02E+08		0.03a	0.00		9.81E+03a	6.30E+03		0.00a	0.00	
PN7030			30	1.54E+10b	5.86E+09		3.36E+10a	7.21E+09		6.59E+07a	5.29E+07		1.50E+09a	1.69E+09		0.04ab	0.00		5.32E+03a	4.55E+03		0.00a	0.00	
M per land-use																								
FN1725	Forest	17	25	9.75E+09a	1.95E+09	27*** ↑	9.88E+09a	1.98E+09	27*** ↑	3.52E+03a	1.46E+03	7* ↑	1.11E+06a	1.16E+06	12.88** ↑	34.33a	34.25	12.88** ↓	4.49E+04a	7.66E+03	2.09	4.78a	0.05	0.91
FN3525		35		2.62E+10c	1.91E+09		3.31E+10b	1.04E+09		2.21E+04b	1.14E+04		4.90E+06ab	1.54E+06		0.03a	0.01		2.51E+04a	1.72E+04		0.22a	0.27	
FN7025		70		1.51E+10b	1.86E+09		6.58E+10c	2.42E+09		2.27E+04b	3.16E+03		1.13E+07b	5.04E+06		0.04a	0.00		2.60E+04a	1.29E+04		0.05a	0.06	
PN1725	Pasture	17		3.00E+09a	9.46E+08	6.31* ↑	9.67E+09a	4.87E+09	3.59	3.43E+07a	2.22E+07	3.59	2.24E+08a	5.09E+07	1.35	0.03a	0.01	1.58	5.89E+03a	4.47E+03	0.10	0.03a	0.03	27*** ↓
PN3525		35		7.10E+09b	1.80E+09		1.49E+10a	1.30E+10		7.31E+07a	5.16E+07		4.22E+08a	5.24E+08		0.03a	0.01		7.92E+03a	6.58E+03		0.01a	0.00	
PN7025		70		7.13E+09b	2.63E+09		3.24E+10a	1.21E+10		1.01E+08a	3.16E+07		6.47E+08a	3.02E+08		0.03a	0.00		9.81E+03a	6.30E+03		0.00a	0.00	
FN1730	Forest	17	30	6.71E+09a	1.24E+09	27*** ↑	5.53E+09a	4.16E+09	27*** ↑	4.07E+03a	5.60E+03	4.11	4.41E+05a	5.60E+05	12.88** ↑	0.49a	0.79	0.33	2.18E+04a	1.20E+04	0.51	4.81a	6.92	0.51
FN3530		35		2.06E+10b	4.15E+09		5.28E+10b	1.12E+10		6.34E+04a	3.73E+03		7.88E+06a	2.00E+06		0.09a	0.05		2.65E+04a	1.76E+04		2.13a	3.55	
FN7030		70		3.69E+10c	4.60E+09		6.65E+10c	1.29E+10		1.74E+04a	1.19E+04		2.23E+08a	3.69E+08		0.07a	0.02		2.80E+04a	2.29E+04		0.10a	0.08	
PN1730	Pasture	17		3.28E+09a	4.18E+08	27*** ↑	8.74E+09a	4.01E+09	12.88** ↑	4.38E+07a	3.75E+07	0.14	3.99E+08a	3.50E+08	0.91	0.05a	0.01	2.00	1.12E+04a	1.31E+03	2.74	0.01a	0.01	0.51
PN3530		35		6.42E+09a	1.55E+09		2.65E+10b	4.93E+09		7.73E+07a	3.05E+07		3.96E+08a	7.49E+07		0.05a	0.02		7.43E+03a	5.98E+03		0.01a	0.00	
PN7030		70		1.54E+10b	5.86E+09		3.36E+10b	7.21E+09		6.59E+07a	5.29E+07		1.50E+09a	1.69E+09		0.04a	0.00		5.32E+03a	4.55E+03		0.00a	0.00	
T per land-use																								
FN1725	Forest	17	25	9.75E+09a	1.95E+09	3.50	9.88E+09a	1.98E+09	3.50	3.52E+03a	1.46E+03	0.38	1.11E+06a	1.16E+06	1.25	34.33a	34.25	3.50	4.49E+04b	7.66E+03	13.5* ↓	4.78a	0.05	0.38
FN1730			30	6.71E+09a	1.24E+09		5.53E+09a	4.16E+09		4.07E+03a	5.60E+03		4.41E+05a	5.60E+05		0.49a	0.79		2.18E+04a	1.20E+04		4.81a	6.92	
FN3525		35	25	2.62E+10a	1.91E+09	3.50	3.31E+10a	1.04E+09	0.38	2.21E+04a	1.14E+04	0.38	4.90E+06a	1.54E+06	13.5* ↑	0.03a	0.01	13.5* ↑	2.51E+04a	1.72E+04	0.04	0.22a	0.27	0.38
FN3530			30	2.06E+10a	4.15E+09		5.28E+10a	1.12E+10		6.34E+04a	3.73E+03		7.88E+06a	2.00E+06		0.09a	0.05		2.65E+04a	1.76E+04		2.13a	3.55	
FN7025		70	25	1.51E+10a	1.86E+09	13.5* ↑	6.58E+10a	2.42E+09	0.38	2.27E+04a	3.16E+03	0.38	1.13E+07a	5.04E+06	0.04	0.04a	0.00	1.25	2.60E+04a	1.29E+04	0.38	0.05a	0.06	0.38
FN7030			30	3.69E+10b	4.60E+09		6.65E+10a	1.29E+10		1.74E+04a	1.19E+04		2.23E+08a	3.69E+08		0.07a	0.02		2.80E+04a	2.29E+04		0.10a	0.08	
PN1725	Pasture	17	25	3.00E+09a	9.46E+08	0.38	9.67E+09a	4.87E+09	0.04	3.43E+07a	2.22E+07	0.04	2.24E+08a	5.09E+07	0.38	0.03a	0.01	13.50	5.89E+03a	4.47E+03	13.5* ↑	0.03a	0.03	1.25
PN1730			30	3.28E+09a	4.18E+08		8.74E+09a	4.01E+09		4.38E+07a	3.75E+07		3.99E+08a	3.50E+08		0.05a	0.01		1.12E+04a	1.31E+03		0.01a	0.01	
PN3525		35	25	7.10E+09a	1.80E+09	0.38	1.49E+10a	1.30E+10	0.38	7.31E+07a	5.16E+07	0.04	4.22E+08a	5.24E+08	0.38	0.03a	0.01	3.50	7.92E+03a	6.58E+03	0.04	0.01a	0.00	0.04
PN3530			30	6.42E+09a	1.55E+09		2.65E+10a	4.93E+09		7.73E+07a	3.05E+07		3.96E+08a	7.49E+07		0.05a	0.02		7.43E+03a	5.98E+03		0.01a	0.00	
PN7025		70	25	7.13E+09a	2.63E+09	3.50	3.24E+10a	1.21E+10	0.04	1.01E+08a	3.16E+07	0.38	6.47E+08a	3.02E+08	0.04	0.03a	0.00	13.5* ↑	9.81E+03a	6.30E+03	1.25	0.00a	0.00	0.38
PN7030			30	1.54E+10a	5.86E+09		3.36E+10a	7.21E+09		6.59E+07a	5.29E+07		1.50E+09a	1.69E+09		0.04b	0.00		5.32E+03a	4.55E+03		0.00a	0.00	

*Significant values for rank-aligned ANOVA and Bonferroni correction rank coefficients are indicated at *, p <0.05; **, p <0.01; ***, p <0.001.*
Letters indicate mean difference Tukey`s Post hoc test (p < 0.05); ↑ , indicates directly proportional; ↓ , indicates inversely proportional.
*Abbreviations are as follows: FN = Forest samples; PN = Pasture samples; _17_ , _35_, _70_= samples under 17% , 35% or 70% FC moisture, respectively; _25, _30 = samples under 25°C or 30°C, respectively; M = soil moisture variation under field capacity in % v.v. (17%, 35% or 70%); T = atmospheric temperature variation (25°C or 30°C); M*T per land-use = variations of both temperature and moisture among samples per each area; M per land-use = variations of moisture among samples per area; M per land-use = variations of temperature, 25°C or 30°C, per each area; Std ± = Standard deviation.*

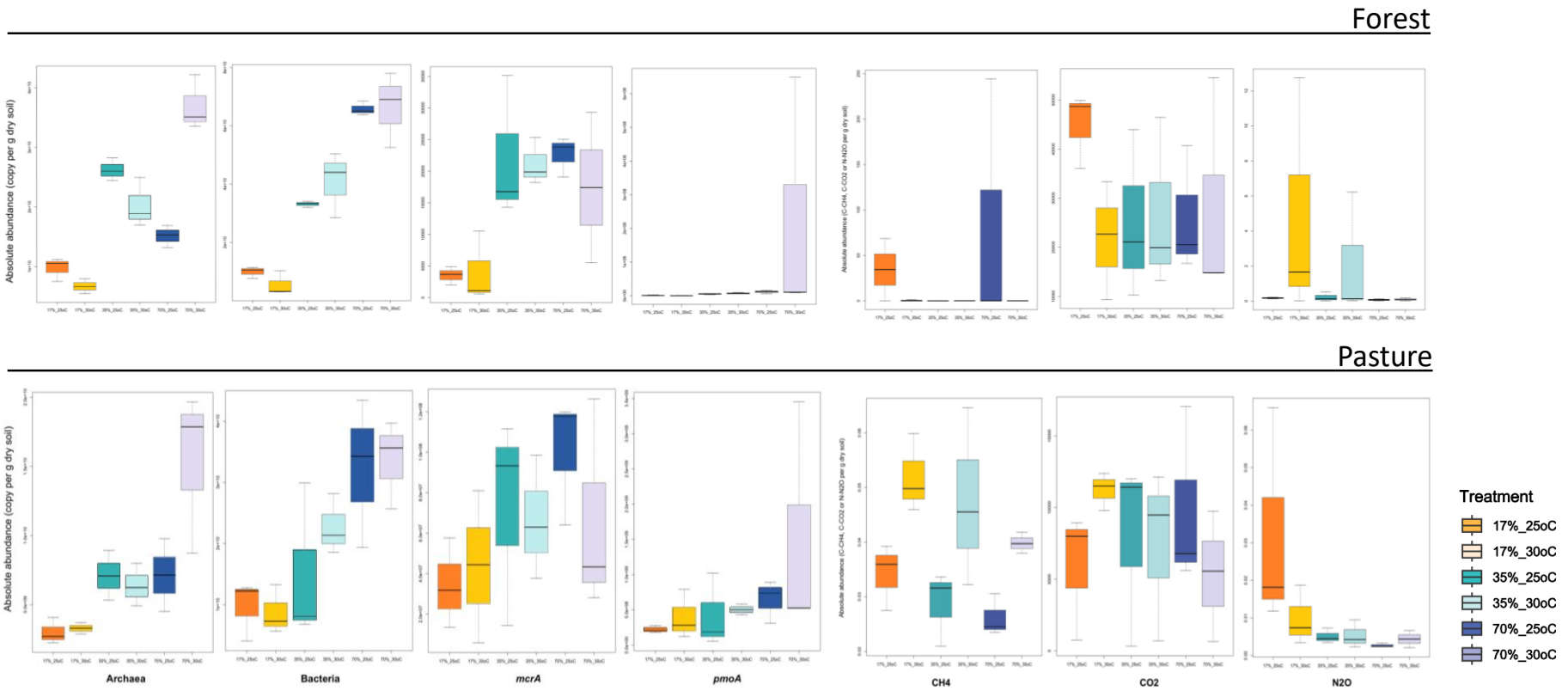

Figure 5 – Box plots of quantification of 16S rRNA genes of Archaea and Bacteria, *mcrA* and *pmoA* genes (copies g soil^{-1}), and C from CH4 and CO2, and N from N2O (µg g soil^{-1}) for all sites. *Abbreviations: 17%_25$^{°C}$= soil moisture at 17% v.v. and 25°C; 17%_30$^{°C}$= soil moisture at 17% v.v. and 30°C; 35%_25$^{°C}$= soil moisture at 35% v.v. and 25°C; 35%_30$^{°C}$= soil moisture at 35% v.v. and 30°C; 70%_25$^{°C}$= soil moisture at 70% v.v. and 25°C; 70%_30$^{°C}$ = soil moisture at 70% v.v. and 30°C. Only significant means difference are shown in letters: (blue) relates to comparison between samples per area; (green) relates to comparison between samples with static temperature (25°C or 30°C) in a changing moisture (17% to 35% to 70% v.v. of f.c.) per area; (black) relates to comparison between samples under same moisture (17%, 35% or 70% v.v. of f.c.) and changing temperature (25°C to 30°C). Plot was made using ggplot2 (Wickham, 2016).*

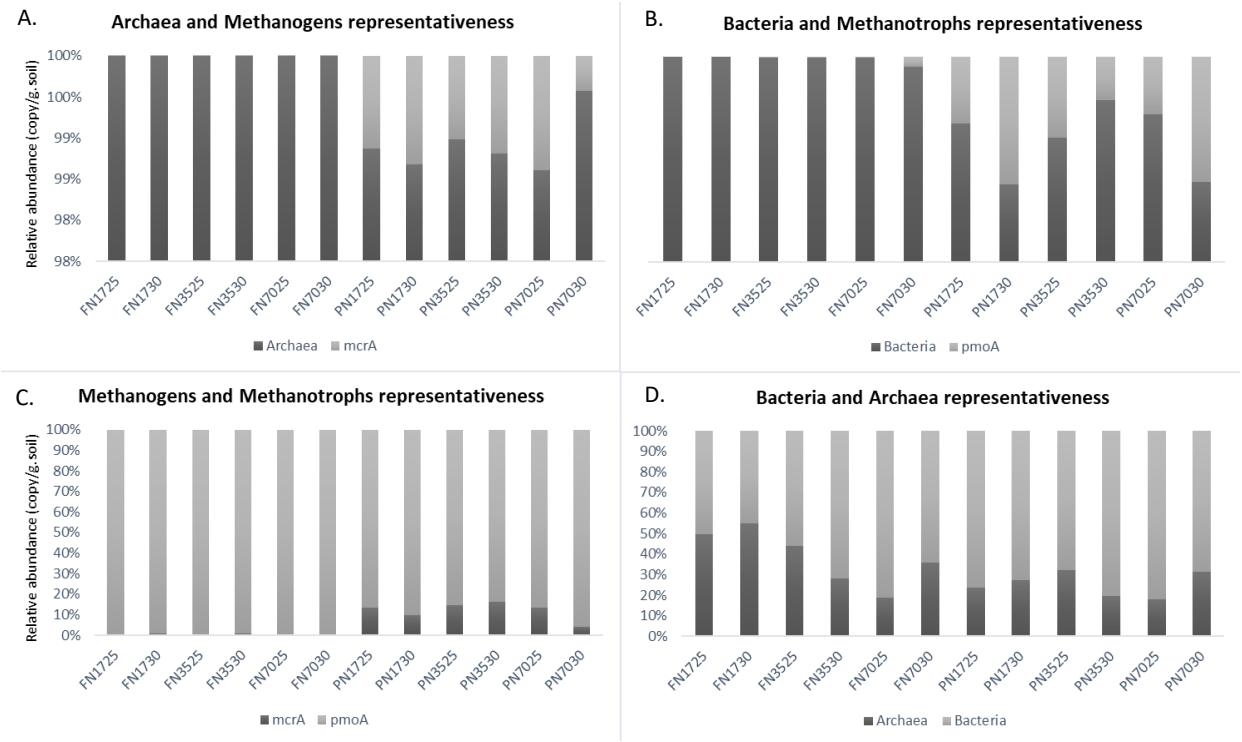

Figure 6 – Representativeness of functional populations and their taxonomy related genes *Abbreviations are as follows: FN = Forest samples; PN = Pasture samples; _17_ , _35_, _70_= samples under 17% , 35% or 70% v.v. f.c. moisture, respectively; _25, _30 = samples under 25°C or 30°C, respectively. Variables: Archaea 16S rRNA gene, Bacteria 16S rRNA gene, mcrA gene, pmoA genes are shown in means of copy per gram of dry soil (n = 9). Relative bars were performed in Excel.*

2.5.4 Microbial traits correlations

The soil physicochemical correlations presented are an indirect evaluation of microbial shifts among treatments and static soil rates since they were measured only in the beginning of experiment, thus we rely our findings on possible modulations occurred with the previous amount of the attribute once tested. Magnifying the view of treatments, we address the significant positive correlations (rho, $p < 0.05$) from previous soil state as well as the gained in the transition of temperature (Figure 7) and moisture (Figure 8), detailed described in SP A3.

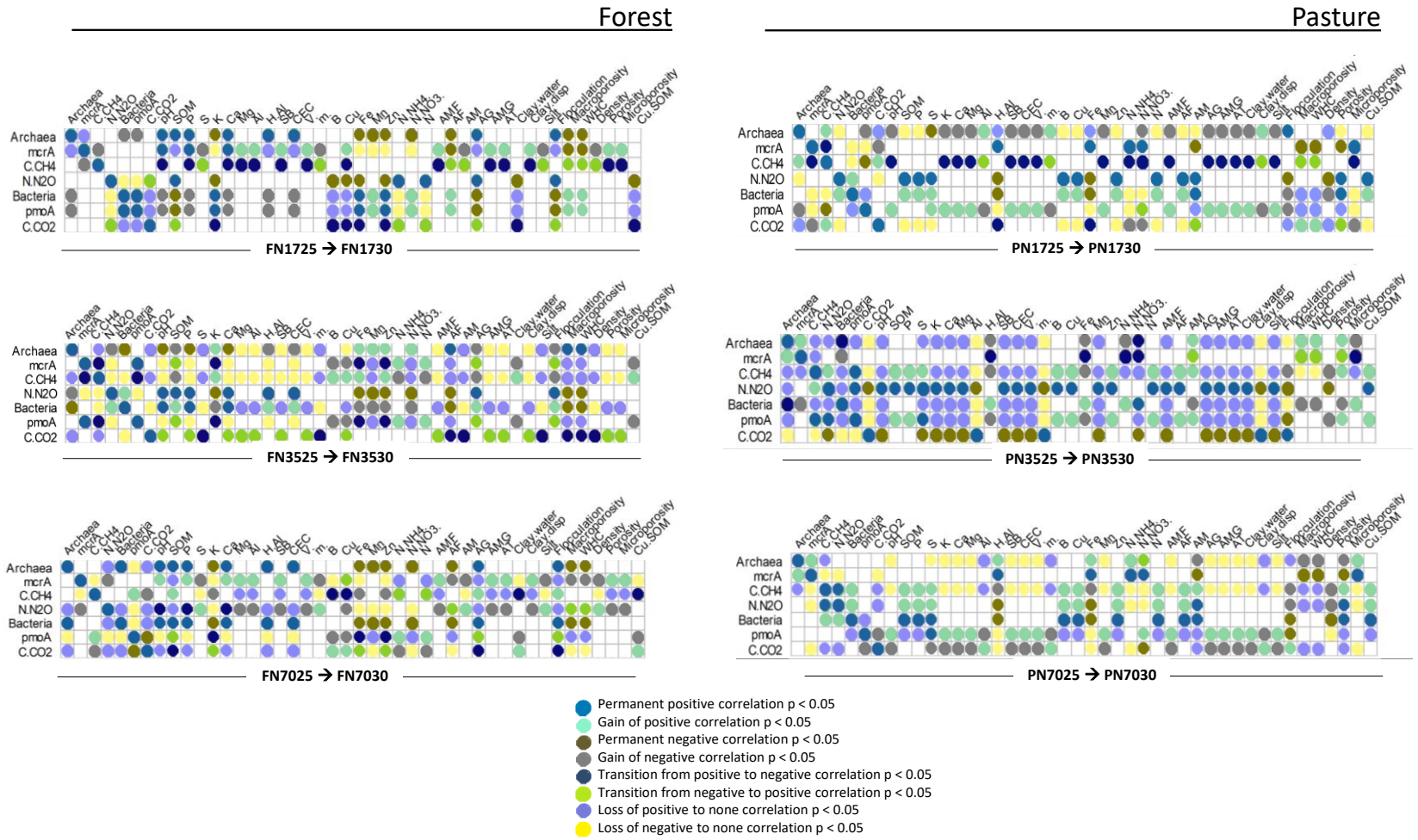

Figure 7 – Superposition of significant ($p < 0.05$) Spearman's rank correlation matrix of the microbial traits among temperature variation under static moisture.
Abbreviations: CH4, N2O and CO2 gases evaluated as C or N per gram of dry soil; and soil attributes analyzed from the six treatments for moistures and temperatures changes. Only significant values ($p<0.05$) are shown. The colors of the dots are formed from superposition of two heatmap matrix transformed for only positive or negative dots, where correlation positive (blue)-negative (yellow) was superposed to other correlation where positive (green)-negative (black), and the final matrix colors denote to different states of correlations between compared samples: remaining positive (blue) or negative (brown) correlations, gain of positive (water green) or negative (grey) correlations, loss of positive (purple) or negative (yellow) correlations, transition positive-negative (dark blue) or negative-positive (green) correlations. Correlations are discussed in the text. Plot was made using R packages Hmisc 3.0-12 and corrplot..

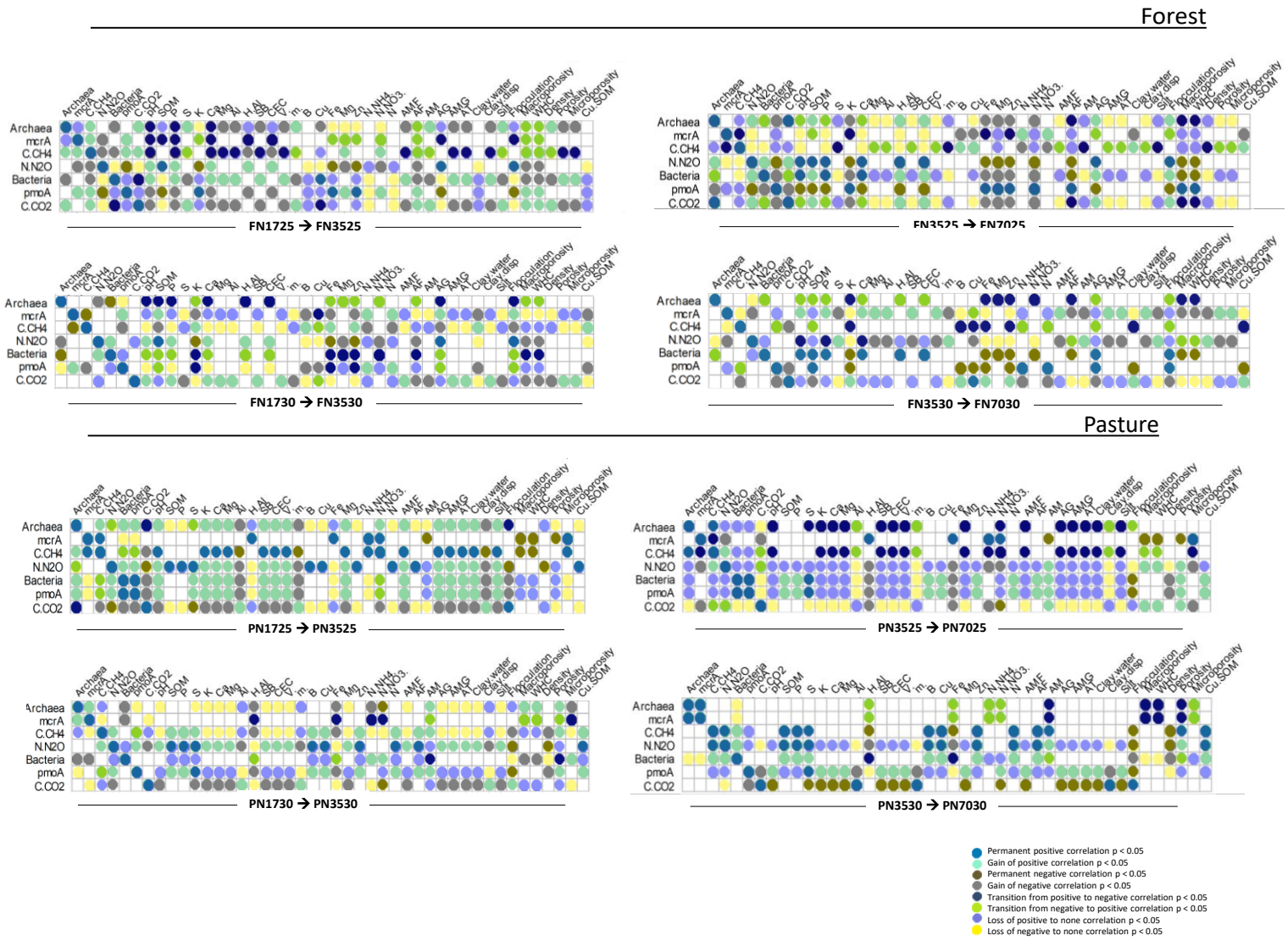

Figure 8 – Superposition of significant ($p < 0.05$) Spearman's rank correlation correlation matrix of the microbial populations among moisture variation under static temperature. *Abbreviations: CH4, N2O and CO2 gases evaluated as C or N per gram of dry soil; and soil attributes analyzed from the six treatments for moistures and temperatures changes. Only significant values ($p<0.05$) are shown. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two microbial populations. Correlations marked with circles are discussed in the text. Plot was made using R packages Hmisc 3.0-12 and corrplot.*

2.6 Discussion

2.6.1 Soil health and methane cycling microbiota

A healthy soil presents all essential components for organisms' presence and activity, ecological relations, hence functioning of biogeochemical cycles. Different concentrations and soil particles structure influence water penetration and nutrients retention. Clay particles tend to form aggregates with SOM, hence a higher water and nutrients retention in soil. However, it is a factor combination. Both soils presented equal elevated rates of SB (Table 2), which indicates fertility for driving to a lower leaching rate in sandy soils (RONQUIM, 2010; GLASER et al., 2002), and even more effect on the clayey ones of this study.

Forest presented higher CEC, hence higher bases saturation (V%) (EMBRAPA, 2011), besides higher rates of B, Fe and N contents, indicatives of fertility. However, Forest presented higher H+Al, Al^{+3} and clay content, reaching an aluminum toxicity state, a negative factor to fertility of this Amazonian pristine forest, which reflects on microbial cell abundances, microbial communities structure and diversity. This high H+Al rates indicates a possibility of high reactivity humic acids (HA) enabled by mineralization and oxidation in this clayey soil, already verified in forests (CUNHA et al., 2007). The state of SOM in Forest is associated to the complexity of its litter composed by leaves and branches of high floristic richness and trees lignin, thus a more recalcitrant older litter full of hydrocarbons which cycling is slow in this oligotrophic environment, being one of the reasons for the low amount of all microorganisms studied (GLASER et al., 2002; CUNHA et al., 2007; MIRANSARI, 2013).

Pasture presented not favorable attributes to fertility as aluminum saturation (m%). On the other hand, Pasture is the less acid soil which means less aluminum availability, presented by the available aluminum (Al^{+3}), acidity potential (H+Al) from solid phase, and aluminum saturation (m%). One of the reasons for a lower acidity of Pasture could be that the organic matter decomposition and humus formation, specially humic acids (HA), contributes to cation exchange capacity (CEC) influencing directly the soil nutrient cycling (CUNHA, 2005). It is evident that pastures have a low complexity litter from a monoculture, hence have a more dynamic cycling, providing an young organic matter (GLASER et al., 2002). Besides, the active pasture hold cattle feces and urine, contributing to SOM with micronutrients and microbial cells, which can increase the amount of nutrients in soil and drive to a more fertile

profile (RONQUIM, 2010; EMBRAPA, 2011). In the case of CH_4 microorganisms, feces could be a source of methanotrophs which could find proper niche in pasture soil clayey aggregates, surviving and augmenting these communities, as previously described (MEYER et al., 2017).

Therefore, the status of higher fertility state for Pasture is acceptable for this land-use changed soil due to its different stress than Forest, thus providing available fertility attributes to microorganisms. Forest provide a more complex ecosystem services controlled by diverse microorganisms with low metabolism redundancy, creating an environment that holds rare groups, and this complexity provides resilience for perturbations. On the other hand, land-use changed areas tend to have a higher metabolism redundancy, where rare groups are lost, presenting higher α-diversity, but lower β-diversity regarding to forests (RODRIGUES et al., 2013; PAULA et al., 2014). However, it is not reflected on microorgamis` abundance for the methane cycling as target system. Forest although being a preserved area, for the targeted microorganism's abundance do not present a high amount of these cells, indicating that the methane cycle is not one of the major metabolisms in this area, as seen in microbial communities abundances (Figures 6). Differently of Pasture, which showed higher amount of cells for all microbial populations evaluated: Bacteria, Archaea, methanotrophs and methanogens (Figure 1, 2) and the higher proportion of groups of the methane cycling, representing an important metabolism of this area (Figure 6).

2.6.2 Climate change effects in biological GHGs in tropical soils

Whereas seasonal methane fluxes are highly variable, having different origins as abiotic and biotic ones, then it should be accounted in methane studies for environmental changes due to climate and land-use changes. Moisture and temperature variations were tested for microbial populations related to biotic methane cycle in soils, leading to changes in populations and gases, regardless to the specific populations proportions (Figure 6) and absolute abundances (Figures 4 and 5, Table 3) of each site, as well as soil attributes correlation (Figures 7 and 8, SP A3). The findings of this study where based uniquely on a microbial system to isolate the factor for only microbiota and soil, not considering plants, fauna and other influences of a complex system as tropical soils systems. Besides, the experiment was developed in closed headspace, an extreme situation of CH_4 and CO_2 accumulation in atmosphere leading to a possible O_2 depletion, hence microaerophilic and anaerobioses conditions. In addition, as we based our studies in genes quantification from total

soil DNA extraction, we are not able to distinguish between alive and necromass, such as the naked DNA adsorbed in mineral fractions of clay and sand (YU et al., 2013). In addition, the association of DNA with other traits as gas, when seen as biological product, leads to some limitations, as the emitted gas is the consequence of a production and consumption in soil, as well as there is no possibility to directly link populations abundance with DNA and production. Thus, gas measurements here were used to indicate the state of a mittigation or emissary of soils under conditions of moisture and temperature in a climate changed based treatment. Besides all these limitations, such attributions are extensively used as a primary panorama for microbial ecology studies, derivating in high scientific value.

Rapid changes in soil moisture strongly affects the entire system. Water percolation and residence are factors for temperature change, aeration, pH and redox potential, dilution and diffusion of nutrients – once adsorbed in aggregates with the association of SOM and mineral soil particles –, and osmosis and diffusion in cells – stimulating growth and activating dormant cells. Moisture was the main driver for direct proportional microbial abundance, where the decrease of microbial cells in drought is known (HUESO et al., 2012; ALSTER et al., 2013; NAYLOR & COLEMANN-DERR, 2018), by the death or dormancy (JENSEN et al., 2003; ALSTER et al., 2013). Drought also promotes aeration, a change in redox, increases the pH and slow down the nutrients availability. Methanogens screened by *mcrA* gene, were only affected by moisture in forest soil under lower temperature, and overall correlated to Archaea, being both overall modulated by pH, SOM, CEC. Methanotrophs were affect by drought in Forest under actual temperature, being overall correlated to Bacteria, SOM, B, Cu in drier Forest (discussed in Redox section, 2.6.2.5).

By contrast, elevated moisture is likely to promote a better solubility of macro and micronutrients as K, P, N, S, Zn, Cu, cations, hence SB and CEC rates, as well as the Mn, stimulating the growth of all tested populations (Tables 3 and 4, Figures 3 and 4). Besides, the elevated temperature stimulates the enzymatic reactions. Methanotrophs screened by *pmoA* gene in dry-heat effect correlated to Mn, Zn and Fe.

Methanotrophs subjected to wetting in Forest seem to be in different niche from total Bacteria, and correlate to SOM, N and NH4+ in wet forest, or SOM, NH4+, in wetting-heat forest. It is evidenced that higher water content increased Bacteria and methanotrophs. On the other hand, the methanotrophs with *pmoA* induction by moisture are likely to be related to nutrients solubility from SOM in the water soil pores.

Temperature increase is associated to the increase in microbial activity (YVON-DUROCHER et al., 2014), as it influences the enzymatic activation energy – soil tropical microbiota optimum 25 to 40◦C (DUNFIELD et al. 1993) –, hence the microbial activity and abundance, as well as the RNA secondary structures and activity (GALTIER; LOBRY et al., 1997). The temperature alone affected the microbial populations, evidenced in the crescent abundance in methanogens and general Archaea, as well as methanotrophs, and variations also in CH_4 and N_2O emissions (Tables 3 and 4, Figures 4 and 5). Overall, Archaea was induced by drought and heat. Archaea and Bacteria populations were stimulated by wet in forest and pasture soils, presenting even higher abundancy under warmer state (Figures 4 and 5). Methanotrophs were also affected by heat in medium moisture forest soil, correlated to NO3-, where N cycle seem to be active in these aerobic soils. However, N_2O is not released in Forest, the opposite to pasture in which is released in wetting treatment (FN3525 to FN7025). Discussed in N2O.

Regarding GHGs, biological CH_4 emission, as well as CO_2 and N_2O are present in forest and pasture among moistures and/or temperatures (Figures 4 and 5, Table 3), and specific aspects of correlation (SP A3). We now investigate the changes in microbial groups and respective GHGs emissions.

2.6.2.1 CO_2 and temperature

CO_2 emission was only affected under heat influence in both dry land-uses, decreasing in Forest, and increasing in Pasture. CO_2 decreasing is related to SOM, NH_4+ and total N, then affecting N cycle in dry forest soil under heat effect (FN1725 to FN1730, $F_{-2.05} = 13.5$, $p < 0.05$); whit the increase of Archaea to Bacteria proportion (Figure 7) (discussed in N_2O section, 2.6.2.4).

On the other hand, CO_2 in dry Pasture under heat effect (PN1725 to PN1730, $F_{19} = 13.5$, $p < 0.05$) is more related to soil physic aspects and CH_4, however not detected in significant change, while increased Archaea to Bacteria proportion (Figure 7), the same effect as Forest. Interestingly, methanogens to Archaea proportion increased. Methanotrophs to Bacteria proportion also increased, as methanotrophs to methanogens increased. This shows perhaps a mixed soil CO_2 emission from other groups as Fungi, as well as some detected prokaryotic modulation, and possible gas leaking from pores in soil.

These results show the high representativeness of heat in the modulation of methanogens and methanotrophs in dry Forest and Pasture soils, allied to biogeochemical cycles congruence with N cycle.

2.6.2.2 CH_4 modulation by temperature

Methane was affected in medium moisture Forest subject to heat (FN3525 to FN3530), and in wetting-heat Pasture (PN3530 to PN7030).

In the case of medium moisture forest subjected to heat, methanotrophs with *pmoA* changed specifically (FN3525 to FN3530, $F_{1.3} = 13.5$, $p < 0.05$) remaining with positive correlation with methanogens, and got positive correlation with NO_3^-, N and SOM, which coincides with increase in Bacteria proportion to Archaea was also affected (increase Bacteria/Archaea), within CH_4 emission increase (FN3525 to FN3530, $F_{2.98} = 13.5$, $p < 0.05$) (Table 4, Figure 5, SP 4 and 5). Thus, CH4 emission in this situation could be related to the classic methanogens. Therefore, the drought-heat in Forest or heat in medium moisture Forest have induced a specific part of prokaryotic population for a favorable niche for methanotrophs. A proper O_2 availability for methanotrophs with *pmoA* in a aerobic, or microaerophilic system, is ragulated by aeration, hence the macroporosity defined by texture and soil structure, such as fractions in clayey soil from wet-dry cycles (LIPSON, 2010).

Furthermore, the heat effect stimulated CH_4 emission in already wet forest and pasture soils. Wet forest under heat had Archaea induced by temperature (FN7025 to FN7030, $F_{2.44} = 13.5$, $p < 0.05$) and positively correlated to Bacteria, besides an increase in Archaea to Bacteria proportion, and a interesting increase in representativeness of methanotrophs to Bacteria, however not ending up emitting CH_4. Similarly, in wet Pasture under heat effect there was an Archaea to Bacteria increase, as occurred in Forest under the same conditions, however with CH4 being emitted emission (PN7025 to PN7030, $F_{1.52} = 13.5$, $p < 0.05$), which coincides with increase of methanotrophs to Bacteria proportion, and increase in methanotrophs to methanogens. Here again there is evidence of CH_4 emission being related to Archaea and a possible MCR-like (described in 2.6.2.1).

In addition, methanotrophs affectd by heat in Forest under medium moisture, as well as heat in wet Forest and Pasture is likely to be related to CH_4 stimulation and consumption. Methanotrophs' products could have lead to the enhance in CH_4 emissions, maybe by methyl-reducing methanogenesis, besides their sinking was not sufficient to uptake methane, and its emission was detected. In addition, maybe methanotrophs with *pmoA* were induced by other

methyl-compounds utilization in Forest not directly related to C-CH_4 utilization as primary carbon and energy source. Methanol is produced directly by methanotrophy, which could have been utilized, as well as other methyl-oxidation by bacteria (MOB) in Forest`s SOM rich in recalcitrant hydrocarbons (discussed at 2.6.1), either utilized for methylotrophic methanotrophs, methylotrophic methanogens, or even broader methyl-reducing methanogens. In addition, deppending on system conditions, conventional methanotrophs with pMMO, the screened in this study, participate in the co-oxidation of short-chain alkanes, alkenes (COLBY, DALTON, 1978; STIRLING, DALTON, 1979). Additional correlation is discussed in Cu section (described at 2.6.2.7). Therefore, in either increase in moisture or temperature, biological methane modulation is affected.

Reinforcing the discussed in 2.6.1, the population of methanogens and methanotrophs represent more in the microbial structure of Pasture, the opposite to Forest (Figure 7). Therefore, the Pasture, also rich in SOM and relatively high rate clay, under water saturation and higher temperature stimulated the aerobic, microaerophilic and anaerobic populations (LIPTZIN et al., 2011), modulating groups of Archaea and Bacteria (Table 3), activating pathways of oxidation and reduction which utilize hydrocarbons, derivating later in methane.

2.6.2.3 CH4 modulation by moisture and moisture-heat effects

Forest soils respond to drought was reported as positive, negative or no effect in GHGs emission (CLEVELAND & SULLIVAN, 2012). Here we evidence that methane emission was affected by moisture in forest soils under actual average temperature, 25°C (BDMEP, 2017), also in medium moisture forest and wet pasture, both submitted to heat.

Specific modulations were found in drought effect in forest in which CH_4 was increased (FN3525 to FN1725, $F_{2.6}$= 12.88, $p < 0.01$) concommitantly to the decrease of all microbial populations, such as the accentuated decrease of Archaea (FN3525 to FN1725, $F_{-2.68} = 27$, $p < 0.001$), also Bacteria (FN3525 to FN1725, $F_{-3.35} = 27$, $p < 0.001$), Methanogens (FN3525 to FN1725, $F_{-6.27} = 7$, $p < 0.05$), and finally Methanotrophs (FN3525 to FN1725, $F_{-4.41} = 12.88$, $p < 0.01$). The overall decrease of prokaryotes in drought evidence a water depletion negative effect on cell functions, depressing cell multiplication independently of land-use. On the other hand, aerobic sites are favored by drought, already reported in dry peatlands where CH4 was negatively correlated to soil depth of ground-water, while CO2 was positively correlated (MOORE; DALVA, 1997), and effectively represented in soil drought study (O'CONNELL et al., 2018). The still CH4 emission and the increase in Archaea to

Bacteria proportion (Figure 7) is related to conventional screened methanogens (SP A3), still active emitting methane in anaerobic sites in dry Forest, or this methane was stored in clayey micropores and release easily being accumulated in headspace and detected.

The factor combination of moisture and heat for microbial cells stimulation had evidences in drought-heat Forest, which FN1730 had the lowest abundances for Bacteria, methanotrophs, and methanogens. An overall prokayote decrease is evidenced in the Archaea (FN3530 to FN1730, $F_{-3} = 27$, $p < 0.001$), and Bacteria (FN3530 to FN1730, $F_{-10} = 27$, $p < 0.001$), while Archaea increased in proportion to Bacteria. Thus, Archaea in dry-hot Forest is favored and related to methane increase, ehich hence have stimulated the methanotrophs, which increased enormously in drought-heat (FN3530 to FN1730, $F_{17.8} = 12.88$, $p < 0.01$).

Regarding the soil under 70% FC, our wet soils on a total different system, an aerobic soil can show spots of O_2 depletion – consumed faster than diffusion in micropores of clayey soils, and in zones of intense decomposition of SOM – reaching a microaerophilic or even anaerobic system, decreasing the redox potential and producing redox-active organic compounds for being reduced in the fermentation, and methanogenesis (LIPSON, 2010; JUGOLD et al., 2012). This was the case of methane emission, which was correlated to SOM in all forest moistures, mainly in wet forest (Figures 7 and 8, SP A3). Besides the correlation to SOM, methane increasing and maintenance was directly correlated to Archaea and Bacteria in wetter Forest (FN35 and FN70 under both temperatures), or even methanogens in dry Forest, which methyl-reducing methanogens and methyl-oxidizing bacteria could be involved. We must cite that SOM could be also degraded by other organisms as saprophytic fungi in aerobic systems producing methane, as well (LENHART et al., 2012), however, not investigated in this study.

The wetting in Forest, from 35% to 70% FC (FN3525 to FN7025), increased Bacteria; decreased Archaea; and methanotrophs remained, which coincides with increase in Bacteria to Archaea proportion (Figure 7). Besides, wetness effect in Pasture (PN3525 to PN7025) increased Archaea; decreased N_2O; increased Bacteria to Archaea; increased Bacteria to methanotrophs; also methanogens to Archaea proportion increased. The methanogens arise, however not statistically confirmed, was the reason for Archaea increase in wet pasture soil, besides the Bacteria increasing seems to be related to N_2O decrease (Discussion in N_2O section, 2.6.2.4).

Methane emission in wet-heat Pasture (PN7025 to PN7030, $F_{1.52} = 13.5$, $p < 0.05$) is likely to be related to anaerobiosis, coinciding with the higher proportion of Archaea to

Bacteria, as occured in forest soil under same conditions. Pasture under wet-heat showed also a higher proportion of Archaea to methanogens within methane emission, which evidences that the methane is related to Archaea other than the methanogens with *mcrA* type screened by the primers utilized. This indicates a possible stimulation of a group of methanogens not detected by the conventional MCR enzyme, such as the MCR-like (LASO-PÉREZ et al., 2016; BOYD et al., 2018).

Besides, the wet-heat in Pasture showed the increase in methanotrophs proportion to Bacteria, and the increase in methanotrophs to methanogens, indicating a achievement of a favorable niche to methanotrophs, either by the methane utilization from a high activity of methanogens, either by a proper O_2 and nutrients availability, as aerobiosis for most of methanotrophs, or anaerobic methanotrophy (KOIZUMI et al., 2003; ETTWIG et al. 2008; KHADEM et al., 2011).

2.6.2.4 N_2O and moisture

Soils account for 56–70% of total global N_2O emissions, and is reported to be mostly from bacterial activity of heterotrophic denitrifier (HINK et al., 2016).

CO_2 was related to N cycle in dry forest soil under heat (FN1725 to FN1730), while increased Archaea to Bacteria proportion. Besides, the positive correlation of CO2 with Bacteria and N_2O in drying-heat Forest (FN3530 to FN1730), and Bacteria and CH_4 correlation with N_2O in wetting Forest under heat or not (FN3525 to FN7025, and FN3530 to FN7030), as well as in drying-heat effect in Pasture (PN3525 to PN1725), and decreasing in Archaea and N_2O emission. CH_4 in wetting-heat Pasture was positively correlated with N_2O, besides Bacteria and CO_2 and nutrients, evidences a nutrient availability in this higher moisture, together with temperature increase. Similarly, N_2O in wet forest is related to Bacteria and CH_4 emission.

Regarding the CH_4 cycle microbiota, N was found to be inhibitory to methanotrophy in forest soils (STEUDLER et al., 1989), however being also reported inhibition, stimulation, or no effect in uplands being subjected to influences as the availability of acetate, CH_4, O_2, N, Cu, pH and temperature (MOHANTY et al., 2006), biomass, and even niches dispute with MOB. Here in this study, methanotrophs increase had positive significant correlation to SOM, N and NH_4+ in wetting Forest (FN3525 to FN7025), and SOM, NH_4+ in wetting-heat Forest (FN3530 to FN7030). In addition, as well as NO_3- in medium moisture Forest subject to heat

(FN3525 to FN3530), indicating that the N cycle is very active in these aerobic Forest soils. However, N_2O is not released, seeming that the denitrication is not completed.

N_2O was emitted only in drying Pasture (PN3525 to PN1725, $F_3 = 27$, $p < 0.001$), and was correlated positively with Bacteria, both in drying-heat and wetting-heat treatments in Pasture (PN3530 to PN1730, and PN3530 to PN7030), and also with Archaea, which correlated to NH_4+ and N in wetting-heat pasture. These populations were probably related to ammonia-oxidizing archaea (AOA) and nitrifying bacteria (AOB), responsible for NH_4+ oxidation in many environments. The pre-existent NH_4+ could have been utilized by nitrifiers converting into NO_2- and NO_3-, which can be either leached under high moisture, or follow the cycle being converted to NO_2- under aerobiosis by deltα-proteobacteria (SMITH et al., 2007), following the denitrification cycle into N_2O emission (LU et al. 2012), in this case of Pasture (IBRAHIM et l., 2016). In N cycling, ammonification is stable regarding to average temperature, however nitrification is driven by thermosensitive microbes, where under low temperature the ammonium is present, or an enhanced nitrification is promoted under higher temperature, such as the case of the increase from 25°C up to 30°C in Forest and Pasture Amazonian soils.

The significant N_2O release in dry pasture and suppression in wet pasture, as well as all correlations in Forest and Pasture evidences that biological influences drive to important changes in N cycle. Thus, there is a relation of C and N cycles as the groups of prokaryotes can act in both and environmental conditions seem to drive shifts in methanotrophs (MAB) and AOB groups in both land-uses. However, we did not screened for N cycle genes.

2.6.2.5 Chemical attributes modulation by moisture and temperature

Important pathways for biochemical cycles of N, C, P, S are controlled by oxygenation rates, hence redox potential (CONRAD, 1989), which determines the nature of the reaction, oxidation or reduction, controlling enzymatic reactions, the speciation, solubility and availability of soil elements (TIEDJE, 1984). Drought raise solute concentrations, limits diffusion (SCHIMEL et al., 2007; LIPTZIN et al., 2011), and decrease ion content such as Na+2, K+, P, and the redox sensitive Al, Fe, Mo (BOUSKILL et al., 2016). In addition, the pH is the main driver for nutrients solubilization – the free H+ associates to extreme parts of soil particles and free ions alter the nutrients solubility, cellular permeation, enzymatic reaction by the redox potential control – being a direct agent on microbial structuration. Aerated soils at pH of 4 to 9 have a elevated redox potential, prevailing the respiration and

facultative anaerobiosis, fitting a high abundance of microorganisms. The initial pH in our soils, 3.48 for PF and 4.2 for PT (Table 2) may have changed due to moistures. Archaea and methanogens correlated to pH in Forest under all moistures and temperatures, and Bacteria only in wet Forest. Drought tends to increase pH, and related to this, especially in dry Forest under 25°C there was an important pH effect correlated to methanogens and CH_4 emission.

Archaea and Bacteria increasing in wet Forest under both temperatures (FN7025 and FN7030) correlated positively with pH, SOM, P and CEC. K, P, and N. Thus, the nutrients availability under acidity such as the pre-existent P, cations, SB and CEC from SOM-mineral fraction might have been altered due to moisture and temperature.

In addition, the acidity potential (H+Al) positive correlations reflects a tolerance to low pH as it relates to the soild phase potential of adsorbed H+ and Al+3, which can turn to exchangeable subjected to the system condition for moisture and temperature. These attributes were important mainly to Forest's Archaea positive correlations since occurred in all moistures and temperatures in single or combined effect (Figures 7 and 8, SP A3), as well as Archaea in drying and drying-heat Pasture (PN3525 to PN1725, and PN3530 to PN7030), and also in the positive correlation with Bacteria increase under wetting and wetting-heat in Forest (FN3525 to FN7025, and FN3530 to FN7030).

As mentioned by Conrad (1989), methanotrophy in the overlaying oxic soil competes for substrates for other metabolisms as nitrate, iron and sulfate reduction. Regarding this, we describe the implications of these cycles with biological methane cycle in Amazonian land-uses.

The positive correlation of Bacteria with S in drying-heat Forest (FN3530 to FN1730); Bacteria and CH_4 in wetting-heat Forest (FN3530 to FN7030), besides Bacteria in Pasture under heat (PN3525 to PN3530) and wetting-heat in Pasture (PN3530 to PN7030), could be related to nutrients availability by pH increased with S in the case of aerobiv systems. Sulfur (S) is modulated by microbial activity being utilized by some bacteria, the sulfur oxidizing bacteria (SOB), which forms hydrogen by SO4-2 oxidation and hence acidifies the soil (MIRANSARI, SMITH 2007), and S reaction which leads to increase in pH and turn some nutrients insoluble, such as Fe (MARSCHNER 1995).

In decreased redox potential systems, as Pasture and Forest under wetting-heat correlated to S, there is also a possibility of direct activity in S-cycle, whereas some Archaea and Bacteria are Sulphur-reducing microorganisms (SRM), and also most sulphate-reducing bacteria (SRB) (POKORNA et al., 2015) are tolerant to O_2 in microaerophilic environments, using S as electron acceptor and turn to H_2S, which is then oxided by Sulphur-oxidizing

prokaryotes (SOP). In addition, there is influence of the Fe(III) dissimilatory bacteria with S utilization as electron acceptor (YONEDA et al. 2012), and some SRB can also reduce NO_3^- (LOVLEY et al. 1995). Thus, drought, wet and heat affects the S cycle in Forest and Pasture, as well.

Phosphorus (P) was highly present in correlations with microbial traits in this study. Moisture changes the soil pH, hence the type of P compound (KHAN et al., 2014). Mostly of P in soil is in insoluble form, and the activity of P can be evidenced in the presence of other soluble attributes (KHAN et al., 2009), such as aluminum (Al+3), Fe and Mn in acid soils (pH < 5) (MCLAUGHLIN et al., 2011; DE SOUZA et al., 2015), strengite ($FePO_4.2H_2O$) and variscite ($AlPO_4.2H_2O$) (KHAN et al., 2014), which is the case of the Amazonian soils studied, evidencing correlations of Al^{+3} and P in Forest under medium moisture with bacterial increase.

High P rates in soil also increase the pH and tend to form insoluble compounds, affecting availability of some micronutrients as the iron (Fe), more available in wet conditions (MARSCHNER, 1995), which can have positively affected the Archaea and Bacteria in wetting and wetting-heat treatments in Forest (FN3525 to FN7025 and FN3530 to FN7030). The activity of some Bacteria can be detected in the inorganic P solubilization by the *Proteobacteria* and *Firmicutes* (GUPTA et al., 1993; MALBOOBI et al., 2009), as well as in the potassium-solubilizing bacteria (KSB) comprised by some *Firmicutes* (ETESAMI, EMAMI and ALIKHANI, 2017; UROZ et al. 2009), and also the iron-chelating molecules for phosphate desorption in deficient environments (ROMANO et al., 2017).

Inorganic forms of P were reported to decrease in drought probably by Fe-P binding after Fe oxidation in a volcanoclastic sediment forest soil around 25°C (O'CONNELL et al., 2018), similar situation of our dry Forest (FN1725 and FN1730) in which Methanogens and CH_4 emissions were positively correlated to P, as well as Archaea in drying-heat Forest (FN3530 to FN1730).

Another correlation found was Methanotrophs in wetting Forest (FN3525 to FN7025), as well as Bacteria in drying-heat (FN3530 to FN1730) with Fe and NO_3-, which can be related to Fe(II) oxidation coupled with NO3- reduction by Delta-proteobacteria (SMITH et al. 2007). Delta-proteobacteria were also related to methanotrophy in Cao et al. (2016), evidencing a diverse methanotrophic groups activity in Amazonian Forest.

Amazonian soils are rich in Fe (Table 2), and once this environment be in doubled moisture, there will be sites of anaerobiosis where decomposition of SOM and mineral fraction and biological Fe solubilization will lead to insoluble Fe oxides to soluble Fe (III),

which, as well as other electropositive electron acceptors SO4-2, NO3- (CONRAD, 2005), in the presence of N_2 and diverse intermediators can compete with CO_2 as extracellular acceptor of electrons in anaerobically respiration, leading to the decrease of methanogenesis potential (TURICK et al., 2002; ZHANG et al., 1999; LOVLEY et al., 1996; LIPSON, 2010), occurring simultaneously with reductions in aerobic sites, likely to happen in clayey soils (LIPSON, 2010). Therefore, CH_4 nule emission in our soils could also be resultant of the abiotic mittigation influence by soil Fe.

Other micronutrients with correlation (Figure 10) where Mn and mainly Zn with methanotrophs and Bacteria in drying-heat effect (FN3530 to FN1730), and only methanotrophs wetting-heat (FN3530 to FN7030). The Mn tend to not be affected by moisture, unlike other nutrients, where oxidation is bacterial, but reduction is both biological and chemical (GHIORSE, 1988). Zn, on the other hand, was correlated to CH_4 emission in wetting and wet-heat treatments, affected by moisture and by microbiota – the Zinc-phosphate solubilizing bacteria (IQBAL et al., 2010), such as *Firmicutes* and *Proteobacteria* (HE et al., 2010).

Finally, Cu availability is also microbial by the synthesis of carboxylates and phenolic compounds (MARSCHNER 1995; BADRI and VIVANCO 2009). Interesting findings were found in dry Forest in the methanotrophs increasing (FN3525 to FN1725, $F_{-4.41} = 12.88$, $p < 0.01$) and correlating to Cu, Cu/SOM, besides SOM, Bacteria and CO_2, which evidences pMMO activity in this dry Forest. Cu is important for regulating bacterial copper enzymes as CuMMOs superfamily, which affects methanotrophs MMOs in cells that produce both enzymes, as pMMO synthesis is directly proportional to the Cu/biomass ratio (STANLEY et al., 1983), and sMMO is negatively proportional to the Cu/biomass ratio during growth, regulating the transcription (Nielsen et al., 1996, 1997).

2.6.2.6 Physical attributes modulation by moisture and temperature

Soil physicochemical properties indicate the importance of activity of ecological balance that works on soils, and interaction of mineral-microbial complexes in soil by and for microbiota. Furthermore, correlations with soil physics can evidence factors that control trace gases diffusion in soil which describe the variation of microbial respiration rates and other processes which are influenced by pore sizes and water percolation, aeration and oxygenation.

Regarding gas dynamics in soil under high moisture, water could also collapse pores, blocking their transit. However, gas measurements did not evidence that, where correlation of

gas were very likely to be related to biotical activity, in which water can also favor the mobility of microbial cells favoring transit, as well as and horizontal gene transfer (HGT), however not measured in this study. Besides, drought lead to a high GHGs release, as CO_2 positive correlation with CH_4 and N_2O emission with macroporosity in dry Pasture subjected to heat (PN1725 to PN1730), as well as large sand particles and highly resilient clay (flocculation) in the same conditions show a physical influence on GHGs release, which could indicate emission of gas stored in soil profile and released by fractures in dry clayey soils (JUGOLD et al. 2012).

Accounting for abiotic agents associated to biotic CH_4 may represent one of the missing soil source needed to understand better the CH_4 cycle in aerobic soils, mainly warm and wet regions subjected to ultraviolet radiation anthropical soils, and it is suggested that this CH_4 source is highly sensitive to global changes (JUGOLD et al., 2012). Regarding the CH_4 emission from dry Forest (FN325 to FN1725), a chemical reaction could be hypothesized, save that there was no light in the experiment, thus the Jugold et al. (2012) evidence for CH_4 emission from SOM degradation with ultraviolet irradiation is discarded in our study.

Specifically, CH_4 cycle was indeed related to the physical aspects clay aggregation, the heavy clay or flocculation, and large sand particles, as well as to the complex associations to chemical attributes and microbial groups – as the discussed SOM, NH4+ and total N, Archaea, Bacteria, methanogens and methanotrophs – which are influenced by the soil physics as well. As already cited, the depletion of O_2 in water saturated soil and clayey aggregates promote microaerophilic and anaerobic sites in soil, evidencing that an entire system condition must be proper to sustain methanogenic and methanotrophic populations.

Additional correlations of microbial populations with soil physical attributes could also indicate some relation about nutrients bounds, cell size and particles sizes modulation of microbial groups (PORTILLO et al., 2013). The correlation of clay aggregates (flocculation) and large sand (GS) to many groups in this study - Archaea and methanotrophs in forest under all moistures and temperatures, as well as Bacteria in wet Forest, besides methanogens in dry Forest - could be somewhat explained. However, these interactions are way too complex for only molecular based studies, since microbial cells plasticity is specific to strains, and that singular interactions hold that cell in that particle, not being directly related to taxonomic based studies only (BAKKEN and OLSEN, 1987; HAHN 2004; PORTILLO et al. 2013).

2.7 Conclusion

This study highlights the influence of changes that Amazonian biome could face on the next decades, which would impact negatively the CH_4 emissions by natural and anthropically Amazonian lands. We can conclude that specific factorial conditions of type of land-use, soil type, soil moisture and temperature lead to divergent responses by methane cycle microbiota. Biological CH_4 emission, as well as CO_2 and N_2O are present in forest and pasture among moistures and/or temperatures. In either increase in moisture or temperature, biological methane modulation is affected.

Forest and Pasture under wetness and heat had their methanotrophs increased, and CH_4 release is likely to origin from methyl-reducing methanogenesis, which is alarming due to its natural high amount release under natural moisture and temperature conditions today.

Overall the population of methanogens and methanotrophs represent more in the microbial structure of Pasture, the opposite to Forest.

The significant N_2O release in pasture under drought and suppresed under wet, as well as all correlations in either Forest and Pasture evidences that biological influence drives to changes in N cycle.

It is evidenced that CH_4 high emission in wet-heat Pasture is related to Archaea increase, however not to methanogens with *mcrA* type screened by the primers utilized.

The drought-heat in Forest or heat in medium moisture Forest have induced a specific part of prokaryotic population for a favorable niche for methanotrophs.

Under the predictions for climate changes in Amazonian biome, the drought for Western Amazon and wetness for Eastern Amazon can lead to drastic changes on soil microbiota associated with methane and other potent greenhouse gas emission, as nitrous oxide specially in drought, changing known the inventory for natural emissions from tropical lands.

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CHAPTER 3

METHANOTROPHS AND METHANOGENS RESPONSE IN DNA STABLE ISOTOPE PROBING COUPLED WITH G+C FRACTIONATION FOR CLIMATE CHANGE BASED VARIATIONS AMONG TWO AMAZONIAN LAND-USES

ABSTRACT

The largest tropical rainforest, the regulator of seasons rainfall regime in South America and entire Earth, has been suffering intensive logging and deforestation over the past 50 years for croplands and pasture, disturbing the entire soil ecology, driving to functions shiftings and even loss. Methanogenesis and methanotroph substrates are diverse and subjected to system conditions as moisture and temperature which influence pH, redox, hence microbial function in soil. Stable isotope incorporation by soil microbiota with molecular studies can answer the identity and activity of the microbial producers and consumers of CH_4 among tropical areas as Amazonian rainforest under land-use changes. Microcosms were set in biological triplicates with intact core soil samples of Oxisols under Pristine Forest and Pasture of Eastern Amazon, in State of Pará, Brazil. Treatments were as follows: 17%, 35% and 70% v.v. at field capacity and 30°C. Samples were incubated in closed headspace enriched in room atmosphere, $^{12}CH_4$ or $^{13}CH_4$ in the dark for 15 days, followed by GC measurements of CH_4, CO_2 and N_2O; populations quantification – methanotrophs (*pmoA*), methanogens *(mcrA*), 16S rRNA genes for Bacteria or Archaea. It was demonstrated that the atmosphere manipulated with $^{12}CH_4$ or $^{13}CH_4$ were able to enrich microbiotas among the three tested moistures from the two land-uses . We were able to address taxonomy to function and relate to soil conditions. The technique utilized is able to filter and highlight punctual variations through a very fine sellection of recently active microorganisms upon treatments, avoiding some necromass in a DNA-based study. We report that forest soils are more subjected to changes in microbial richness related to methyl-compounds degradation, methanotrophy and methanogenesis, and possibly resultant GHGs emissions due to drought and wetness. Evidence of hydrogenotrophic predominancy upon acetoclatic, as well as methyl-reducing, methylotrophic methanogenesis as the responsible for CH_4 emission in dry and wet Pasture soils under 30°C. This study brings a clear vision of the of key microbial functions in methane fluxes, providing data for the understanding the real impacts of climate and soil use changes can cause to the gas fluxes from soils in large tropical areas as the Amazon.

Keywords: methanotrophs, methanogens, Archaea, Bacteria, *pmoA*, *mcrA*, hydrocarbons, hydrocarbons, CH_4, CO_2, N_2O. methyl-reducing methanogenesis, hydrogenotrophs, $^{13}CH_4$, SIP

3.1 INTRODUCTION

"Learn from yesterday, live for today, hope for tomorrow.
The important thing is to not stop questioning."
Albert Einstein (1879-1955)

The Land-use, Land-use Changes and Forestry (LULUCF), or Forestry and other Land-use Changes (FOLU) – including forest-to-agriculture, deforestation and forest degradation – places Brazil in the 2nd for the most deforesting country in the world (HANSEN et al., 2013), in 5th in greenhouse gas emitter, and in 8th total GHGs emitter country (CAIT, 2014). Forest-to-pasture accounts for more than 80% of the land-uses changes in the Brazilian Legal Amazon (IBGE, 2009). A further influence to the complex ecological interactions and activity in tropical biogeochemical cycles, the Climate Changes alters the rain seasons and temperature (BETTS et al., 2008; IBGE, 2014; UN Environment, 2018). Climate Changes predictions for 2040 – 2100 suggest disastrous consequences to the entire Earth climate regulation (MALHI; WRIGHT, 2004, IPCC, 2018), and for the Amazonian biome (MALHI et al. 2008; BETTS et al., 2008; DUFFY et al., 2015). However, there is no data to project the climatic changes influence in the tropical soils such as Amazonian soil microbiota (CLEVELAND & SULLIVAN, 2012; NAYLOR & COLEMAN-DERR, 2018).

Methane is the most impacting GHG for its heat retention potential aside the quantity emitted yearly, the 2nd mostly emitted correspondent to 17% of the total emitted GHGs (IPCC, 2014). The synergistic relation between methanogens and methanotrophs attract scientific interest for CH_4 modulation studies. Due to the vast dimension of Amazonian territory, and its land-use changes increasing rates, tracking methanogens and methanotrophs in these situations is of great value to the scientific undertanding of Amazonian biome, and to better predict future changes, either by direct anthropic activities or by worldwide climate changes.

Microbial studies are better supported by molecular approaches. Marker genes for key enzymes are screened and quantified with oligonucleotides (primers) to estimate populations size and modulations by system factors.

Methane-assimilating Bacteria (MAB) possess the enzymes MMOs particulate methane monooxygenases (pMMOs) are screened by the operon genes *pmoCAB* found in multiple copies restricted to species belonging to the genera *Methylosynus, Methylocapsa, Methylococcus, Methyloacidimicrobium, Methyloacidiphilum, Methylomirabilis.* Soluble

methane monooxygenases (sMMOs) screened by the operon genes *mmoXYZC* found in one copy, where sMMO is restricted to species belonging to the genera *Methylococcus, Methylomonas, Methylomicrobium, Methylosinus and Methylocystis* (NAKAMURA et al., 2007), and *Methyloferula* (VOROBEV et al., 2011). The *Alpahproteobacteria* class in *Rhizobilaes* order owns *Beijerinckiaceae* and *Methylocystaceae* families, mixed groups of methanotrophs and/or methylotrophs. The *Gammaproteobacteria* class owns the *Methylococacceae* and *Methythermaceaea* families, which are entirely methanotroph, and some can be facultative methylotrophs, and able to consume other hydrocarbons since both MMOs have a wider range for C-chain molecules, as pMMO co-oxidize a number of short-chain, from C1-C5, such as alkanes, alkenes, and ammonia (COLBY, DALTON, 1978; STIRLING, DALTON, 1979). The sMMO can react with C1-C10, co-oxidizing alkanes, alkenes, and aromatic compounds (JIANG, WILKINS, & DALTON, 1993). In addition, both MMOs can be found in a same cell, where pMMO is regulated by Cu/biomass rate (Stanley et al., 1983) being directly proportional to concentrations > 4 µM Cu (HAKEMIAN, ROSENZWEIG, 2007); and where sMMO is found to be inversely proportional to Cu/biomass rate (NIELSEN et al., 2003) in concentrations < 0.8 µM Cu (HAKEMIAN, ROSENZWEIG, 2007).

Methanogens are Archaea of four groups, diverse in terms of ecological niches, divided by their C utilization as a part of the anaerobic respiration (KALLISTOVA et al, 2017). Overall, methanogens possess the *mcrBDCGA* operon encoding for methyl-coenzyme M reductase (MCR), which transform diverse substrates utilized as C and/or energy source into methane on the terminal process of this biogeochemical cycle (CONRAD, 2005). There are diverse final electron acceptors utilization, which groups respond differently to the environment (WEBER et al., 2001): a) hydrogenotrophic methanogens utilize H_2/CO_2, or formate, to produce H_2 as electron donor, comprised by archaeal orders *Methanobacteriales, Methanococcales, Methanopyrales, Methanomicrobiales, Methanocellales*; b) aceticlastic methanogens fermentates acetate for C use as energy source for final electron acceptor and produce CH_4 in absence of O_2, sulphates (SO4-2), nitrates (NO3-) and oxidized mineral, or as C source, assimilating and derivate in methyl-compounds, comprised by the archaeal orders *Methanosarcinales*, families *Methanosarcina* (low affinity to acetate, minimum use of 1mM) and *Methanothrix* (*Methanosaeta*) (high affinity to acetate, minimum use of 7-70µM); c) methylotrophic methanogens utilize methanol and methylamines by dismutation, or disproportioning (LANG et al., 2015; KALLISTOVA et al., 2017); d) methyl-reducing

methanogens utilize as C source plenty of C1-methylated other than acetate, e.g. methanol, methylamines, such as dimethylamine, and trimethylamine, and methyl-thiols as methyl-sulfide and methanethiol (MeSH) (CONRAD, 2005) by obligate hydrogen-dependent reduction of methyl (Me, CH3) (Lang et al., 2015; KALLISTOVA et al., 2017). In addition, a related enzyme was identified in methyl-reducing methanogens as the MCR-like owners *Ca. Bathyarchaeota,* and *Ca. Syntrophoarchaeum,* which are able to β-oxides butane, and maybe other types of hydrocarbons, found in anoxic and hydrocarbons rich environments, utilizing this variety of molecules stored in SOM as carbon and energy sources (LASO-PÉREZ et al., 2016; BOYD et al., 2018).

The target or shotgun sequencing for taxonomy and functional studies allow the accessment of which groups are present and how they are modulated by the system, which in our case are the land-uses and abiotic factors in the Amazonian biome. However, DNA based studies cannot exclude the necromass factor (YU et al., 2013). For a better resolution of DNA study, we can utilize the density separation of nucleic acids under stable isotope incorporation by soil microbiota to link the identity and activity of the consumers of a target substrate. Another trait is the G+C %mol linkage to populations related to the target biogeochemical cycle in soil.

The Stable Isotope Probing technique (SIP) was developed for metabolism of specific substrates studies in pure cultures, and has been widely used for the community knowledge metabolism for an specific substrate and is powerful approach for trophic network-based studies (XUAN et al., 2007; CHEN et al., 2011; DUNFORD et al., 2010; GAO et al., 2017). The genomic G+C %mol content employed for taxonomic assignment in DNA based association of close organisms is another important trait for systematic assignment for phyla and lower levels, and ecological role (MANN et al., 2010; WU et al., 2012).

When we consider a buoyant density of a DNA fragment, we need to account for the factors that may affect the separation, such as G+C% mol rate in a DNA fragment adrift, and the irregular isotopic labeling of the complex soil biota. The downstream analyses of gradient fractions and comparison to ^{12}C-fed controls are critical to confirm ^{13}C enrichment of ‘‘heavy’’ gradient fractions. In this way, the protocol for DNA-SIP coupled with G+C fractionation was intended to overcome inherited issues of the technique. Besides, the downstream amplicon-based next generation sequencing and the metataxonomic assignment of prokaryotes through ASVs promoted the accessment of the microbiota related to methane cycle in tropical soil of Eastern Amazon. This study brings a clear vision of the biological

activity of methane fluxes in the forest-to-pasture transition in Amazonia, providing data for the understanding of the real impacts such soil use changes could cause to the gas fluxes from soils in large tropical areas.

3.2 Hypothesis

Based on previous survey of climate changes effects in tropical soil (see Chapter 2), the following hypothesis were tested: (a) Forest and Pasture CH_4 dynamics are different due to different richness and functions linked to organic matter utilization; (b) Forest under drought will show specific group that could address to the high CH_4 emission; (c) $^{12}CH_4$ and $^{13}CH_4$ will enrich the methanotrophs as primary consumers and lead to labeling other populations as methylotrophs, methyl-oxidizing bacteria and methyl-reducing methanogens.

3.3 Objectives

This study aimed to evaluate the abundance, metataxonomy and metafunction of methanotrophs, methanogens and methyl-consumers among the combined factors drought-heat and wetness-heat through C-isotopic-enrichment of closed microcosms with soil cores of Oxysols under Pristine Forest and Pasture of Eastern Amazon, State of Pará, Brazil, and DNA isopycnic density separation coupled with G+C fractionation prior to amplicon-based next generation sequencing.

3.4 Methods

This study is subsequent to the Chapter 2 and the following steps were performed under the same conditions: a) sampling; b) soil attributes analysis; c) gas quantification; d) DNA extraction; e) taxonomical and biochemical marker genes quantification. The additional methodology is detailed described below.

3.4.1 Microcosms experimental design

The experiment was a 3 x 2 x 3 performed with intact soil cores under three moisture variations – 17%, 35% and 70% FC – and further two temperature variations – 25°C and 30°C,

in a total population size of 108 samples. First, the actual moisture and dry weight (dw) of each sample was evaluated through subsamples submitted to drying in glass plates in stove for 72 h. Second, the total dry weight of each sample was evaluated, described on item 2.4.4, excluding the weight of the sampling-tubes. Third, three groups of each land-use had their moisture altered by drying in dry stove or adding autoclaved 0.2µm filtered tap water, to reach the three moisture variations, and let set for 3 days to drain, covered with Parafilm M (Sigma Aldrich, USA) to avoid evaporation, and allow gas exchanges. Fourth, 5 g dw equivalent of intact soil cores were collected to 27 ml glass tubes, air tight closed with butyl rubber stoppers and crimped with aluminum caps. Fifth, three incubation groups were set – control incubations through 30 ml of 0.2µm room air were inserted (Chapter 2), and two methane incubation groups $^{12}CH_4$ (Airgas, USA) and $^{13}CH_4$ (Cambridge Isotope Laboratories Inc., USA) under one concentration of 370,000 ppm, or 37% (v.v.), in a rate of 57 mmol of C- CH_4 per g dry weight soil. Sixth, samples were divided again into two groups to be incubated at 25°C or 30°C. The samples were disposed randomly in tubes racks inside the two equal BODs (Innova2 Incubator Shaker Series, NJ, USA), covered with dark cloth and let set for 15 days. After incubation, 25 ml of the accumulated headspace were harvested and stored in 15 ml Exetainer vials (Labco, Ceredigion, UK) and cap tops sealed with hot glue. The soils were immediately stored at ultra-freezer at -80°C.

3.4.2 DNA extraction and genes quantification

DNA samples were performed the same way as item 2.2.7, however additional 5 extractions were necessary for CH_4-incubated samples. From the gene quantification data, it was possible to select the significant ones to proceed to the next step, the DNA-based isopycnic gradient centrifugation coupled with G+C content separation for accessment of the communities in general and for the differentially isotopic labeled for the oes which metabolize and assimilate C-CH_4. The methodology performed for the genes quantification is described on item 2.2.8. The samples chosen for both land-uses comprised all three moistures, 17%, 35% and 70% under field capacity, at the highest temperature, 30°C. The biological triplicates comprised 36 samples.

3.4.3 DNA-SIP/G+Cf

The treatments filtered by the evalutaion with Real-Time quantification (qPCR), described on item 2.4.7, were designed to the downstream tehcniques. The DNA Stable Isotope Probing coupled wth G+C Fractionation (DNA-SIP/G+Cf) was developed under Neufeld et al. (2007) modified by DNA staining with 8 µl of GelGreen (Bioticum, USA) as described in Gao et al. (2017) with additional 8µl of bis-benzamide per 5 ng DNA sample (CHEN; MURREL, 2010; BUCKLEY et al., 2011). The runs were developed at Beckman Coulter Optima MAX-TL Ultracentrifuge (Beckamn Coulter) with the fixed-angle TLA-110 rotor for 5.1 mL polyallomer Quick-Seal centrifuge tubes hot sealed and centrifuged in batches of 8 samples at 67,000 rpm, or 187,281 x g, for 44 hours at 20˚C with vacuum, as performed in Gao et al. (2017). The batches comprised always the cold-labeled sample enriched with $^{12}CH_4$, and the respective hot-labeled sample enriched with $^{13}CH_4$. The bands were subject to UV transilluminator and the expected bands pattern were: 12C-DNA in first upper band; high GC content DNA as a intermediate thick band for both samples enriched in 12C and 13C; and an additional lower band of 13C-DNA for samples enriched in 13C. A total of 12 fractions were recovered by automatic syringe pump NE-4000 High Pressure Programmable Single Syringe Pump (New Era Pump, USA) at rate of 425 µl.min-1 as described by (Dunford; Neufeld, 2010). Fractions were then evaluated by the following two methods: a) density in refractometer by 5 to 10 µl on top of adhesive tape with a role of 5 mm, as described by Buckley et al (2007); b) correlated to G+C % mol curves pre-generated by the evaluation of melting curve of 2 pure ATCC standards, ATCC51375 *Bacillus methanolicus* (G+C content of 38.5%) and ATCC35069 *Methyosinus sporium* (G+C content of 61.52%) grown in batches of $^{12}CH_4$ and $^{13}CH_4$, and two more samples from the experiment, at termocycler Bio-Rad CFX Connect Real Time System (Bio-Rad) in a 10µl final volume: 5µl SSO Advanced Sybr mix, 10 ng of DNA, water. Based on previous evaluation, fractions were chosen to proceed to DNA precipitation with the use of linear polyacrylamide (Thermo Fischer Scientific, USA) and PEG, as described in Bartram et al. (2009), which were quantified with Qubit (ng/µl) and the ones with > 10 ng.μl^{-1} were then prepared, generating 2 to 4 possible amplifying fractions per sample. Detailed protocol is on Supplementary material SP B8.

3.4.4 Amplicon-based next generation sequencing

DNA-SIP/G+Cf fractions, 2 to 4 per sample, were V4 16S rRNA amplified in conventional PCR carried out as follows to final volume of 20µL: 10 µl Phusion HSII High Fidelity Mastermix (Thermo Scientific, MA, USA); 10 ng of DNA; H_2O; 1µl of 10µM of primers (IDT, IA, USA) with Caporaso primers (2010), 515F primer with 158 barcodes, and unique barcoded 806R primer. Amplicons were organized in pools with 100 ng per sample to final library volume of 100 µl, and purified with UltraClean Purification Kit (Mobio, USA). Pools were then checked in gel electrophoresis 1% and Qubit 3.0 (Thermo Fischer Scientific, MA, EUA). Amplicon-seq was performed at Genome Center UCDavis Facility in unique run at Illumina Miseq PE 250 bp. Reads were quality filtered and grouped by its amplicon sequence variants (ASVs) with DADA2 pipeline (CALLAHAN et al., 2016) in R software with forward reads, length of 200 bp, quality > 30, and SILVA SSU Ref v.132 taxonomy assigned (YILMAZ et al., 2014). Spreadsheets were later joined with dplyr package (WHICKHAM et al., 2014). The metataxonomic assignment of Archaea and Bacteria through ASVs up to Genus level were then assigned with ecological functionality based on cultured and uncultured data from LPSN database (PARTE, 2018) and SILVA database (YILMAZ et al., 2014).

3.4.5 Statistical analysis for gene copies and gases

Factorial experiment 3 x 3 of 2 populations based on land-use type (between-subjects interaction: Forest-PF and Pasture-PT areas) subjected to treatments in within-subjects interactions (moisture 1, moisture 2, moisture 3 under 1 temperature) for 7 microbial traits as variables (absolute Real-time qPCR archaea by V4 16S rRNA with 519f/806R primers, bacteria by V4 16S rRNA 515F/806R with primers, methanotrophs by *pmoA* gene, methanogens by *mcrA* gene; GC of CH_4, CO_2, N_2O). The gas abundance data was normalized for µg of C or N per gram of dry soil. The gene abundance data were normalized for copy per gram of dry weight soil (dw). All statistical analysis were performed in R software (R-Core-Team, 2018.). Normality was evaluated by Shapiro-wilk test with stats package 3.5.1 from R default (R-Core-Team, 2018). Normal and rank-aligned groups (Figure 2) were analyzed by mixed-design analysis of variance (split plot ANOVA) with ART package 1.0 (VILLACORTA, 2015) and Post hoc Tukey`s test at $p < 0.05$ with agricolae package 1.3-0 (MENDIBURU, 2015). All means, microbial traits summarized as mean ± confidence interval at 95%, and significances were organized in tables.

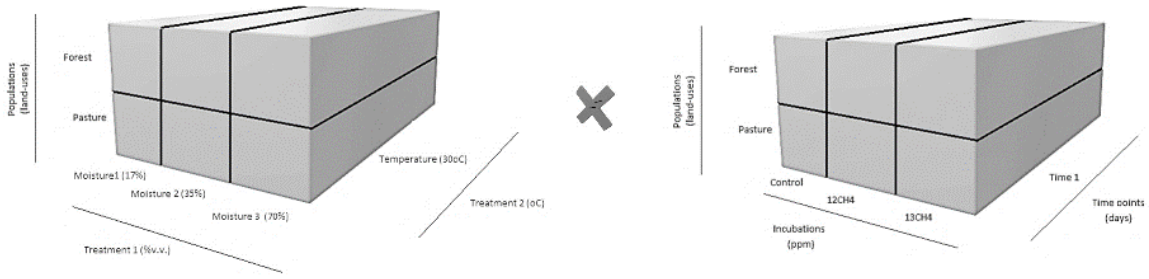


Figure 4 – Variables dependences in the factorial 3 x 3 analysis for each land-use, and 2 x 3 x 3 for land-use change, the forest-to-pasture.

3.5 Results and Discussion

3.5.1 Communities quantification among methane incubations

The absolute gene quantifications from the incubations with $^{12}CH_4$, $^{13}CH_4$, and control incubated samples (see Chapter 2) based on samples before de ultracentrifugation (Tables 4 and SP B3) reveals that we had significant increases in methanotrophs in Forest and CH_4 in Pasture. Overall, the double effect of moisture-gas in Forest and Pasture drove to changes in Bacteria, methanogens, methanotrophs and CH_4 (Figure 13).

For Forest (Table 4), wet-control presented the highest bacterial abundance ($F = 13.96$, $p < 0.001$), besides the ^{13}C-drying showed the highest CH_4 emission ($F = 3.36$, $p < 0.01$). The CH_4 release was higher in either C-isotopes of CH_4, seeming a relative higher abundance in prokaryotic activity in the utilization of both C-isotopes in Forest. In dry and medium Forest there was no significative moisture and gas influence. However in wet Forest, we noticed an increase in Bacteria under $^{12}CH_4$, and even higher under $^{13}CH_4$, besides a natural higher methanotroph abundance in all wet samples.

The wet-control and ^{13}C-wet in Pasture showed the highest bacterial abundance ($F = 10.2$, $p < 0.001$), besides the ^{12}C-medium moisture showed the highest CH_4 emission ($F = 17.4$, $p < 0.01$). Methanotrophs ($F = 4.4$, $p < 0.05$, however low mean difference) presented the highest abundance in all wet Pasture samples.

Therefore, methanotrophs were enriched with CH_4 incubations, in either land-uses under both ^{12}C and ^{13}C isotopes. Overall, there was a ^{13}C-CH_4 preference (Figure 13), seeming a kinetic ^{13}C-CH_4 preference of the microbial communities in both land-uses, when ^{13}C substrates of low C/N ratios are more decomposable by its lower negativity (WERTH & KUZYAKOV, 2010).

Table 4 – Difference of means for the effect of factorial treatments based on quantification of 16S rRNA genes of Archaea and Bacteria, *mcrA* and *pmoA* genes (copies g soil^{-1}), and C from CH_4 and CO_2, and N from N_2O (µg g soil^{-1}) for all sites, followed by the results of F-values, and p-values of the rank-aligned mixed-design ANOVA

Sample	Area	M (%v.v)	Gas	16S rRNA Bacteria	Std (±)	F-value	mcrA	Std (±)	F-value	pmoA	Std (±)	F-value	C-CH4	Std (±)	F-value	C-CO2	Std	F-value	N-N2O	Std	F-value
FN1730			Air	5.53E+09a	4.16E+09		4.07E+03a	5.60E+03		4.41E+05a	5.60E+05		0.49a	0.79		21799.22a	12005.782		4.8089795a	6.9218539	
F121730		17	12CH4	2.76E+09a	1.16E+09		1.22E+04a	3.98E+03		1.09E+05a	6.67E+04		125.27ab	35.36		33276.85a	1745.019		2.1072731a	0.9120284	
F131730			13CH4	9.47E+09a	6.92E+09		4.11E+04a	3.03E+04		8.25E+05a	4.42E+05		184.78b	41.26		30841.55a	6591.708		0.3621574a	0.3367068	
FN3530			Air	4.10E+10abc	1.12E+10		6.34E+04a	3.73E+03		7.46E+06a	2.00E+06		0.09a	0.05		26546.98a	17563.781		2.1309902a	3.5487375	
F123530	Forest	35	12CH4	1.92E+10ab	1.14E+10	13.96***	1.12E+05a	8.53E+04	3*	3.34E+06a	2.23E+06	8.61***	117.89ab	153.57	3.36**	24309.26a	18636.32	0.27	1.829885a	2.0910744	0.93
F133530			13CH4	3.64E+10abc	6.11E+09		2.48E+05a	3.87E+05		4.85E+06a	3.66E+06		124.99ab	33.53		31573.7a	8580.796		1.3142783a	2.1827083	
FN7030			Air	6.65E+10c	1.29E+10		1.74E+04a	1.19E+04		2.23E+08a	3.69E+08		0.07a	0.02		28034.3a	22942.587		0.0971568a	0.0842834	
F127030		70	12CH4	1.87E+10ab	8.82E+09		1.60E+05a	1.89E+05		3.78E+06a	3.86E+06		101.03ab	41.54		25997.38a	13677.758		0.0386986a	0.0423634	
F137030			13CH4	5.28E+10bc	3.23E+10		8.20E+04a	2.68E+04		7.53E+06a	5.00E+06		82.37ab	76.30		32253.37a	5906.882		1.3858323a	2.392427	
PN1730			Air	8.74E+09a	4.01E+09		4.38E+07a	3.75E+07		3.99E+08a	3.50E+08		5.19E-02a	7.19E-03		9.49E+03a	5.42E+03		5.19E-03a	1.22E-03	
P121730		17	12CH4	5.54E+09a	2.28E+09		4.17E+07a	3.46E+07		1.26E+08a	1.73E+08		1.54E+02b	1.67E+01		9.29E+03a	6.70E+03		2.95E-03a	7.85E-04	
P131730			13CH4	6.81E+09a	3.03E+09		2.48E+07a	3.29E+07		3.05E+08a	2.42E+08		6.68E+01ab	3.33E+01		1.64E+04a	2.07E+04		5.13E-02a	8.41E-02	
PN3530			Air	2.27E+10ab	4.93E+09		6.64E+07a	3.05E+07		5.06E+08a	7.49E+07		4.74E-02a	1.63E-02		1.36E+04a	3.48E+03		3.68E-02a	3.46E-02	
P123530	Pasture	35	12CH4	4.83E+09a	1.51E+09	10.2***	4.60E+07a	5.80E+07	0.82	3.02E+08a	1.32E+08	4.4**	8.22E+01bc	4.42E+00	17.4***	7.41E+03a	5.85E+03	1.10	7.34E-03a	1.99E-03	1.70
P133530			13CH4	1.28E+10ab	4.08E+09		3.30E+07a	4.41E+07		8.82E+07a	1.80E+07		1.42E+02abc	1.58E+01		3.86E+03a	4.58E+03		4.26E-03a	1.71E-03	
PN7030			Air	3.36E+10b	7.21E+09		6.59E+07a	5.29E+07		1.50E+09a	1.69E+09		3.98E-02a	1.92E-03		1.12E+04a	1.31E+03		9.88E-03a	7.91E-03	
P127030		70	12CH4	1.65E+10ab	7.00E+09		1.09E+08a	5.48E+07		7.99E+08a	6.65E+08		5.11E+01a	6.43E+01		7.43E+03a	5.98E+03		5.38E-03a	3.73E-03	
P137030			13CH4	3.07E+10b	1.80E+10		3.31E+07a	1.45E+07		1.85E+09a	1.22E+09		6.94E+01ab	4.42E+01		5.32E+03a	4.55E+03		4.42E-03a	2.26E-03	

*Significant values for rank-aligned ANOVA and Bonferroni correction rank coefficients are indicated at *, p <0.05; **, p <0.01; ***, p <0.001.*
Letters indicate mean difference Tukey`s Post hoc test (p < 0.05); ↑ , indicates directly proportional; ↓ , indicates inversely proportional.
Abbreviations are as follows: _17_ , _35_, _70_= samples under 17% , 35% or 70% FC moisture, respectively; Air = incubation with normal atmospheric air (controls); 12CH4 = incubation in methane enriched in light isotope ; 13CH4 = incubation with methane enriched in heavy isotope; Std ± = Standard deviation.

3.5.2 Metataxonomical composition

The protocol for DNA-SIP coupled with G+C fractionation was intended to overcome G+C effect on DNA buoyant density and this study could raise further information for direct assign the G+C %mol content in the 16S rRNA V4 regions of Archaea and Bacteria to density. In this study we utilized band staining to check bands position by naked eye and it was possible to observe one thick band and some smear in the middle of the tubes. We retrieved 12 fractions by automated pump with 4 - 7 gradient fractions per sample, where the ones of < 10% of DNA retrieved were not able to follow downstream techniques. Thus, we ended up with 2 – 4 fractions with sufficient DNA amount (> 10 ng/µl) for the dowstream techniques. Summarizing, all final utilized fractions per sample, together recovered 80 - 90% of the initial DNA, 5µg (data not shown). Specifically, DNA was spread allover 2 to 3 fractions in Forest samples, and 3 to 4 samples in Pasture samples (Figures 14 to 18, Table 4). PCR and quality checkings ended up in the amplicon-based next generation sequencing of the 16S rRNA V4 in unique run, having no G+C content affecting accuracy of sequencing, as reaised by Laursen et al. (2017). The metataxonomic assignment of Archaea and Bacteria through ASVs allowed us to identify multiple strains up to Genus level, resulting on the nomenclature for Archaea (SP B4) and Bacteria (SP B5). Metataxonomy was then mannually assigned to ecological functionality through cultured and uncultured data from literature of the groups associated to CH_4 cycling in soil.

The prokaryotic community differed among the areas (Figures 13 and 14). Within Archaea, the phyla per land-use (Figure 13) show that the archaeal phylum *Thaumarchaeota* (84.52%) and *Diapherotrites* (0.02%) were more representative in Forest relative to Pasture, however *Euryarchaeota* (78.03), *Crenarchaeota* (12.29%), *Nanoarhaeota* (1.26%), and unclassified Archaea (0.09%) were more representative in Pasture soils. Regarding moistures and C-isotope incubations (Figure 13.B), oscillations in relative abundance were observed. For Archaea, *Thaumarchaeota* was dominant to *Euryarchaeota* in Forest under all moistures, seeming a preference for ^{13}C-CH_4. On the other hand, *Euryarchaeota* was dominant in Pasture under all moistures, where *Thaumarchaeota* was supressed in drought and stimulated with moisture, being increased in medium moisture and wet Pasture, as well as modulated by the C-isotope incubation, seeming to prefer ^{13C}-CH4 in dry and wet Pasture, such as in Forest. *Thaumarchaeota* is abundant in tropical oxysols of Amazon (TAKETANI & TSAI, 2010), as well as in Cerrado (BARRETO et al., 2013), both rich in soil organic matter. *Euryarchaeota*

and *Crenarchaeota* were found in clones from Amazonian sediments (PAZINATO et al., 2010), and also the last was found in acidic forest soil of temperate zone (KEMNITZ et al., 2007).

The following bacterial phyla were more representative in Forest (Figure 13): *Proteobacteria* (44.35%), *Actinobacteria* (23.33%), *Acidobacteria* (16.14%), WPS-2 (2.10%), GAL15 (0.3%), *Chlamydiae* (0.13%). The following bacterial phyla were more representative in Pasture relative to Forest (Figure 13.A): *Firmicutes* (34.19%), *Chloroflexi* (13.19%), WS2 (2.10%), *Verrucomicrobria* (1.41%), *Gemmatimonadetes* (0.67%), WSA4 (0.28%), BRC1 (0.19%), *Latescibacteria* (0.07%), *Tenericutes* (0.05%). *Armatimonadetes* (0.05%), *Cyanobacteria* (0.04%), *Rokubacteria* (0.03%), *Bacteroidetes* (0.02%), *Nitrospirae* (0.02%), *Entotheonellaeota* (0.02%), *Patescibacteria* (0.01%), *Omnitrophicaeota* (0.01%), The phyla *Planctomycetes* (7.29% in forest, 6.81% in pasture), *Dependentiae* (0.03% in both areas), and unclassified Bacteria (1.02% in forest, and 0.99% in pasture) were equaly distributed among both land-uses, and also equaly distributed among gradient fractions (Figure 21).

Regarding moistures and C-isotope enrichments (Figure 14.B), oscillations in relative abundance for Bacteria were observed in Forest soils, as *Actinobacteria* was dominant in dry Forest; *Proteobacteria* was dominant in medium moisture ans wet Forest, mostly stimulated by $^{13}CH_4$ in detriment to $^{12}CH_4$, very likely to be related to diverse hydrcarbons degradation, other than methane and methanol. *Acidobacteria* was differentialy observed in wet Forest soils, being highly stimulated in $^{12}CH4$ in detriment to $^{13}CH_4$. *Actinobacteria* and *Deltaproteobacteria* were enriched in drought and possibly related to plants drought tolleration and other abiotical influences (SANTOS-MEDELLÍN et al., 2017), which can be related to a complex polysaccharides degradation ability by these oligotrophic bacteria (BOUSKILL et al., 2016; MARTINY et al., 2016) in utilize inorganic N for extracellular enzymes soils (TRESEDER et al., 2011; NAYLOR & COLEMAN-DERR, 2018), as well as the differenciated capacity to produce osmolytes by Gram- and Gram+ strains (BOUSKILL et al., 2016). In addition, *Actinobacteria* members are highly related to antibiotics production (LANCINI & LORENZETTI, 1993), which are interestingly found in high amount in dry soils (BOUSKILL et al., 2016). Antimicrobians are also produced by members of *Proteobacteria* and *Firmicutes*, which in drought can be related to signaling for biofilm formation (BOUSKILL et al., 2016).

For Bacteria in Pasture, *Firmicutes* was dominant in all moistures, being stimulated by the ^{13}C-CH4. *Chloroflexi* members seem to utilize preferentially the ^{12}C-CH4 in medium moisture and wet Pasture under $^{12}CH_4$. *Acidobacteria* was differently observed in wet Pasture, being highly stimulated also in $^{13}CH_4$ in detriment to $^{12}CH_4$. Interestingly, Pasture soils presented unique phyla, i.e. not present in Forest, however in very low abundance (< 0.00%): *Cloacimonetes, Halanaerobiaeota, Hydrogenedentes, Kiritimatiellaeota, Margulisbacteria, Omnitrophicaeota, Spirochaetes*, WS1, WS4. The phyla *Proteobacteria, Firmicutes, Actinobacteria, Acidobacteria* are the most abundant in tropical soils of Amazon and are related to diverse functions in soil, but mostly to the aromatic hydrocarbons degradation present in the soil organic matter from lignin degradation, as well as plant growth promotion (LEAHY; COLWELL, 1990; PIEPER, 2005; DAANE et al., 2001; NAVARRETE et al., 2010; GERMANO et al., 2011; HAMAOUI et al., 2012; RODRIGUES et al., 2013; DE CARVALHO et al., 2016; LAN et al, 2017; KROEGER et al., 2018), as well as *Proteobacteria* and *Firmicutes* have P solubilizing members (phosphorus-solubilizing bacteria) (GUPTA et al., 1993; MALBOOBI et al., 2009), and *Firmicutes* are also K-solubilizing bacteria (KSB) (ETESAMI et al., 2017). The fact that *Firmicutes* was the most abundant bacterial phylum in Pasture (34.19%), mostly in dry and medium moisture under ^{13}C-CH_4 – despite having very low representativeness in Forest (0.57%) mostly in dry under both C-isotopes incubation – is likely to be related to some pH alteration with drought and modulation of solubilizing bacterial members (see Chapter 2), as well as some tolerance to dehydration such as sporullation ability (TOCHEVA et al., 2016; NAYLOR & COLEMAN-DERR, 2018). *Chloroflexi* and *Proteobacteria* are related to rhizosphere being enriched in root associated-microbiome subjected to drought (SANTOS-MEDELLÍN et al., 2017), thus tolerant to drought.

3.5.2.1 Climate and Land-use specific groups

Besides all gradient fractions distributions, we could address some specific Archaea and Bacteria to moisture and land-use types. Accounting for possible bias since unsufficient sample size to cover variations in soil cores sampling of an highly heterogeneous soil as forest, due to diverse phyllosphere lignocellulosic structure in SOM, underground network of rhizospheres and diverse concentrations and types of exsudates and associations with AMF and their actvity in soil aggregation with soil particles sizes and properties which all this affecting niche of microorganisms and their occurrence and abundance in that specific portion

of soil. Regarding pasture sampling, heterogeinity is also present when in covered and exposed soils, presence of animal activity in waste and soil compaction, besides presence of different grass in the same area which rhizosphere may affect ecological relations. Bias in every step of manipulation can occur, even having optimized techniques applied up to sequences filtering and final analysis standards. Considering all that, this study reports Archaea and Bacteria related to environmental functions that can be lost when absent in negative bioindicators, or gain in the presence of positive biocindicator (DIAZ, 2009).

Soil changes affect soil microbiota, soil fertility and biogeochemical cycles (LAUBER et al., 2008; FOLEY et al., 2005). Some groups were area-exclusive, moisture-exclusive and even fractions specific (item 3.5.3).

Some functions are activated when in either drought and wet Pasture: a) the methanotrophy by *Methylomicrobium (Proteobacteria, γ-proteobacteria, Methylococcales, Methylomonaceae)*, extensively reported as methanotroph alkaliphilic and alkalitolerant found in soda lakes; and some species are halo(alkali)philic and halo(alkali)tolerant (KHMELENINA et al., 2008), where pH of pasture soils in loss or excess of water may change, affecting the cell and promote and entire different system in terms of nutrient solubility in soil; b) Methanotrophy of high affinity to CH_4, therefore low affinity to O_2, *Methylosarcina (Proteobacteria, γ-proteobacteria, Methylococcales, Methylomonaceae)* (KALYUZHNAYA et al. 2005); c) as well as hydrogenotrophy, H_2 or formate utilizing, by unclassified *Methanobacteriaceae (Euryarchaeota, Methanobacteria, Methanobacteriales*).

Some functions are activated when in wet Forest, as the anaerobic methanotrophy by Sh765B-TzT-35 (*Rokubacteria, NC10, Methylomirabilales, Methylomirabilaceae*), as well as methylotrophic methanogenesis (methyl-reducing only methylotrophic methanogens-MRMOM) *Methanomassiliicoccus (Thermoplasmata, Methanomassiliicoccales, Methanomassiliicoccaceae*) in Forest, which could be addressed as positive biological indicators of methanotrophy and methanogenesis in wet forest soils. Interestingly, these groups are present allover moistures in Pasture. Sh765B-TzT-35 has 3242 uncultured reports SILVA database found in freshwater lake sediment, being related to anaerobic methanotrophy (KOIZUMI et al., 2003; ETTWIG et al. 2008; KHADEM et al., 2011). *Methanomassiliicoccus* is reported as obligate methylotroph methanogens H_2-dependent (only methanol, methylamines reduction with H_2) found in intestinal tract of termites, human feces;

bovne rumen, anaerobic sludge reactor (LANG et al., 2015; BORREL et al., 2012; NOEL et al., 2016; Iino et al., 2013).

The exclusive presence of AOA related unclassified Group_1.1c (*Thaumarchaeota*) and the strict methylotroph methanogen (methyl-reducing only methylotrophy methanogen - MRMOM) unclassified *Thermoplasmata (Euryarchaeota*) in wet pasture evidence a positive bioindicator for methanogenesis from methanol and methylamines in SOM, as well as AOA activity in acid wet pasture soils. Group_1.1c (Thaumarchaeota) sequences were found in mixotrophic growth of cultivated AOA (WEBER et al., 2015) and assimilated amino acids by marine ammoniα-oxidizing *Thaumarchaeota* (INGALLS et al., 2006), and also found actetate microcosms, in acidic forest soils in pH range 4.5–6.0 (JURGENS et al., 1997; WEBER et al., 2015), in mixed deciduous forest (KEMNITZ et al., 2007), and also found in tropical savanna soil (CATÃO et al., 2013). Some works with forest-to-pasture or forest-to-deforestation relate changes on microbial communities to increase in pH (NAVARRETE et al., 2015), not on soil coverage itself (CARVALHO et al., 2016).

There was a lost in dry and wet Pasture soils of the strict methylotroph (only methanol and methylamines) *Methyloceanibacter* (*Proteobacteria, α-proteobacteria, Rhizobiales, Methyloligellaceae*), reported as extremophile (TAKEUCHI et al., 2014).

The strict methylotroph *Methyloceanibacter* (*Proteobacteria, α-proteobacteria, Rhizobiales, Methyloligellaceae*), reported as extremophile (INEGAKI et al., 2003), is lost in drought and wet pasture soils.

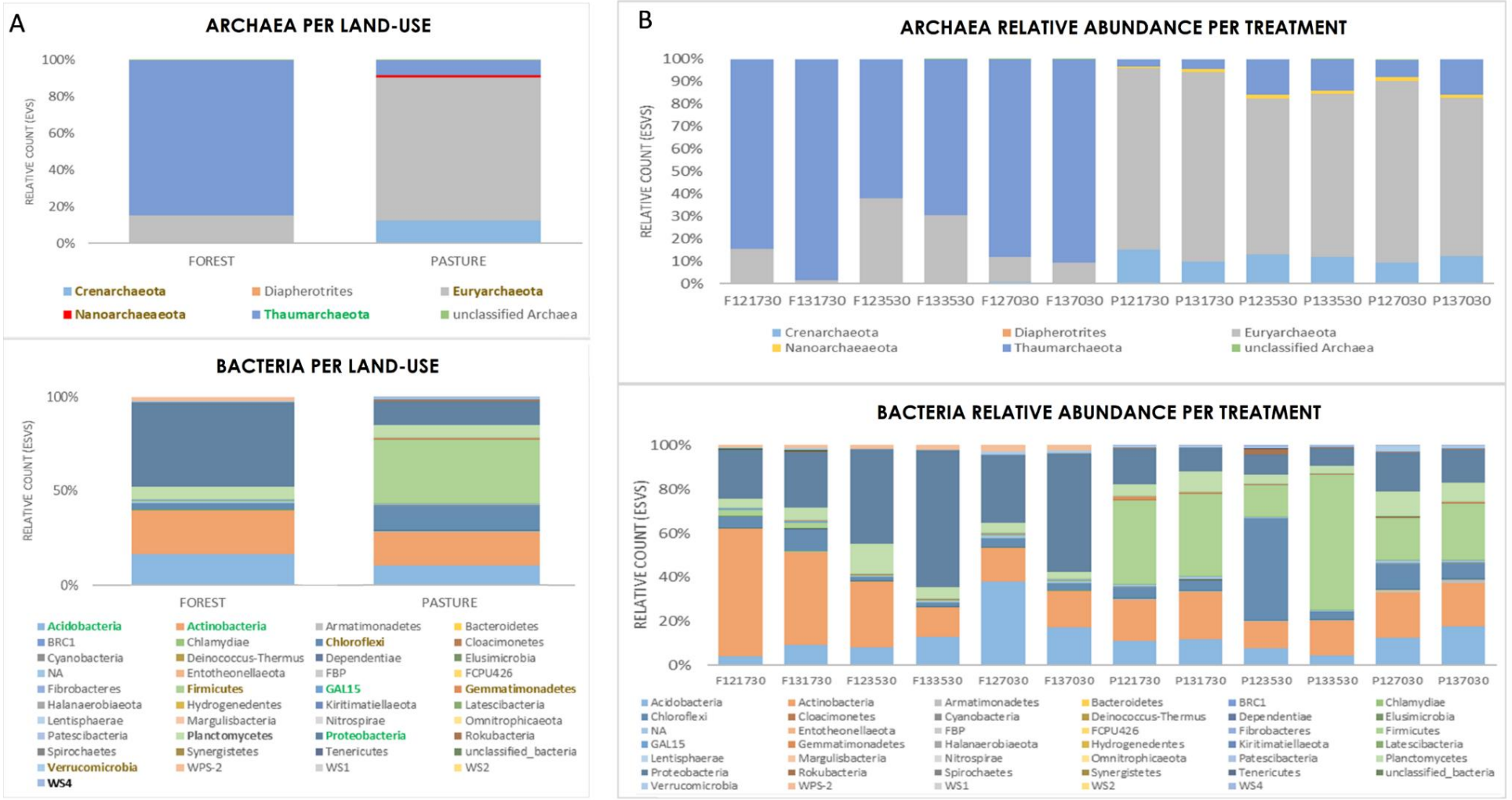


Figure 13.a– The overall metataxonomical assignment for Archaea and Bacteria phyla, showing the diversity per land-use (A), where the representative phyla for both land-uses are in bold, besides the representative phyla for Forest are in bold and green, and the representative phyla in Pasture are in bold and brown; diversity per moisture treatment, being drought, medium moisture and wetness (B). Plots were performed in Excel.

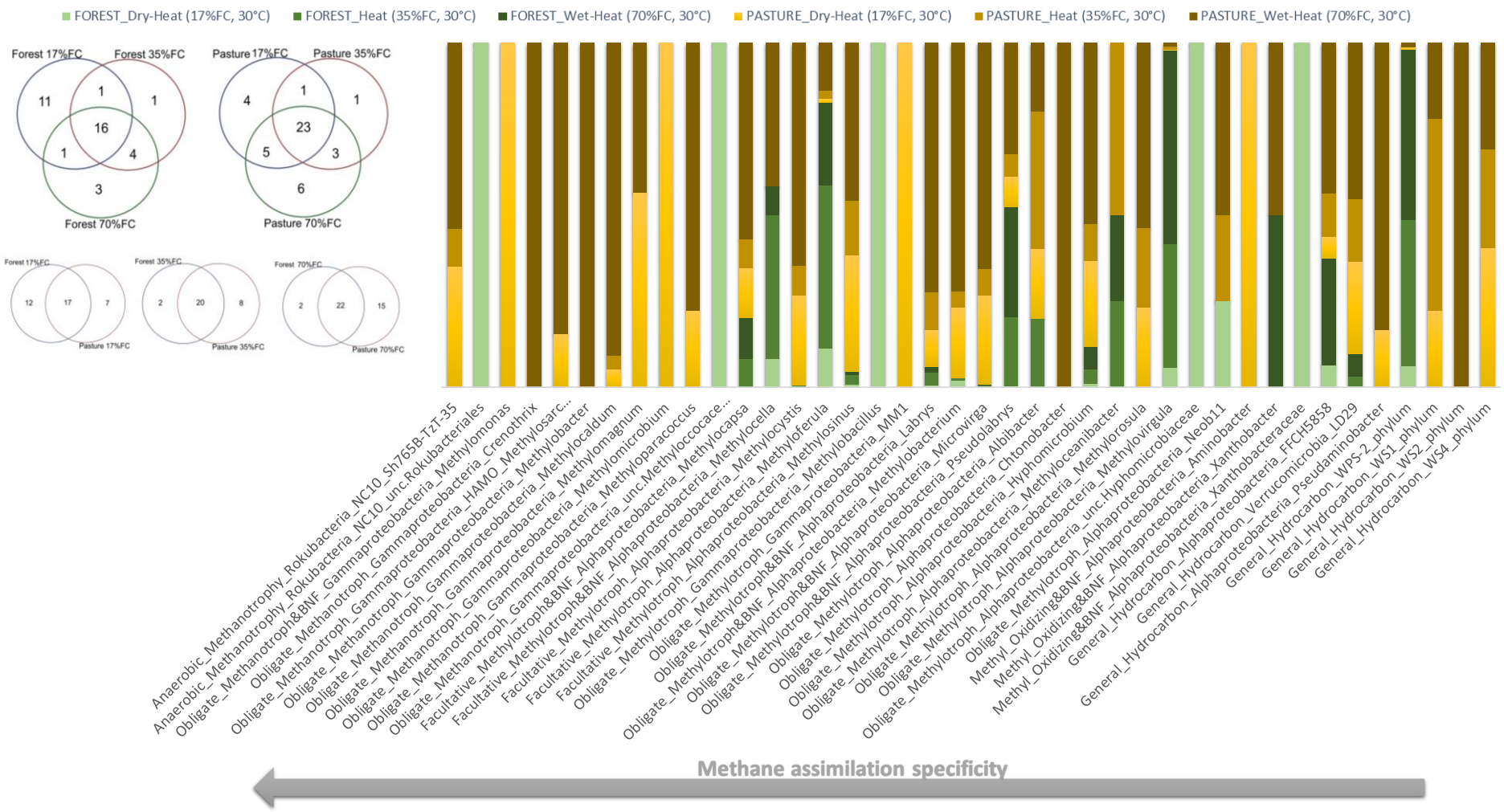


Figure 13.b – Bacterial metataxonomic-based functions related to CH_4 cycle in forest-to-pasture conversion under Climate Changes effects.

Figure 13.c – Archaeal metataxonomic-based functions related to CH_4 cycle in forest-to-pasture conversion under Climate Changes effects.

3.5.3 Gradient fractions metataxonomical composition

Regarding gradient fraction distribution of microbiota associated to CH_4 cycle (SP B2 and B3), Archaea and Bacteria were present among all densities and G+C %mol content (Figures 12 and 13), however Pasture presented higher range for density (g/ml^{-1}) and correlated G+C % mol content. Considering that all samples plotted are positioned due to their G+C %mol content influence on the buoyant density of DNA fragments, therefore we can consider that Pasture prokaryotic community present higher G+C %mol content, and only in Pasture there was sufficient DNA recovery from 4 fractions related to a higher density. Diversity indexes Shannon`s H and E_H (Figures 14 and 15) evidences that median fractions had higher functional diversity, coincident to the higher DNA amount retrievement from the ultracentrifugation.

Based on the selected functions related to CH_4 cycle (Tables 5 and 6, Figures 16 to 20), Pasture, in comparison to Forest, present higher β-diversity (SP B1). The measurement for funtional and taxonomical traits based on Kingdoms Archaea and Bacteria shows that Pasture soils also harbors higher α-diversity. Our results differ from the normally addressed to the diversity of tropical forest conversion, as described in Rodrigues et al. (2013). Shannon`s H of uncertainty (ESLER; REBELO, 2014), hence, show higher presence of rare functions in Pasture than in Forest soils. Hopefuly, the results show a necromass avoidance by the C-CH_4 enrichment, fortunately labeling active populations, and suplanting the non-active cells, which DNA was further processed in gradient fractions, as well as function selecting by taxonomy, thus capturing the recently activated prokaryotic community associated to the use of ^{12}C or ^{13}C inserted in the system.

On the other hand, among the density distribution of Archaea and Bacteria in both land-uses (Figure 13), it was possible to observe a broader range of G+C communities in Forest, which shows a low G+C community not present in Pasture. Thus, the forest-to-pasture conversion drives to a differential community richness in the G+C modulation of species level, as we show further in this work that the same Genus, and the above taxonomic levels, behavior differentially among land-uses.

We found specific functions to corroborate previous discoveries (see also Chapter 2) detailed on Tables 5 and 6, and Figures 15 to 19, in which we describe the findings.

Overall, we recovered general hydrocarbon utilizers from soil organic matter; methanotrophs and methylotrophs (Figure 15.a to 15.f), besides methanogens (Figure 16.a to 16.d). We observed the presence of these groups spread allover areas, moistures and gradient fractions evidencing a broad substrate specificity, being able to utilize both C isotopes and diverse compounds, and/or A+T and G+C richness in 16S rRNA V4 region. Therefore, the abundances in organisms distributed among gradient densities is not merely due to G+C, but to the functionality around CH_4 cycling, captured through genes, gas and metataxonomy.

Specific findings were the alphaI_cluster (alphα-proteobacteria, *Rhizobiales*, *Beijerinckiaceae*) in the light fractions of Forest and Pasture under both isotopes, thus ^{12}C_ATrich-DNA and ^{13}C_ATrich-DNA related to 51 - 58 G+C %mol, meaning that this Genus can be suited as median G+C %mol group. Alphα-Proteobacteria class members have diverse G+C contents. Hitherto there are 1084 reports of partial sequence for uncultured α-I_clsuter (or α-Icluster) and no whole genomic data.

The hydrogenotroph *Methanobrevibacter* was homogeneously found among both land-uses, under all moistures, C-istopes and gradient fractions. This evidences a G+C plasticity likely to be related to species level, besides being a hydrogenotroph commonly found among Amazonian land-uses.

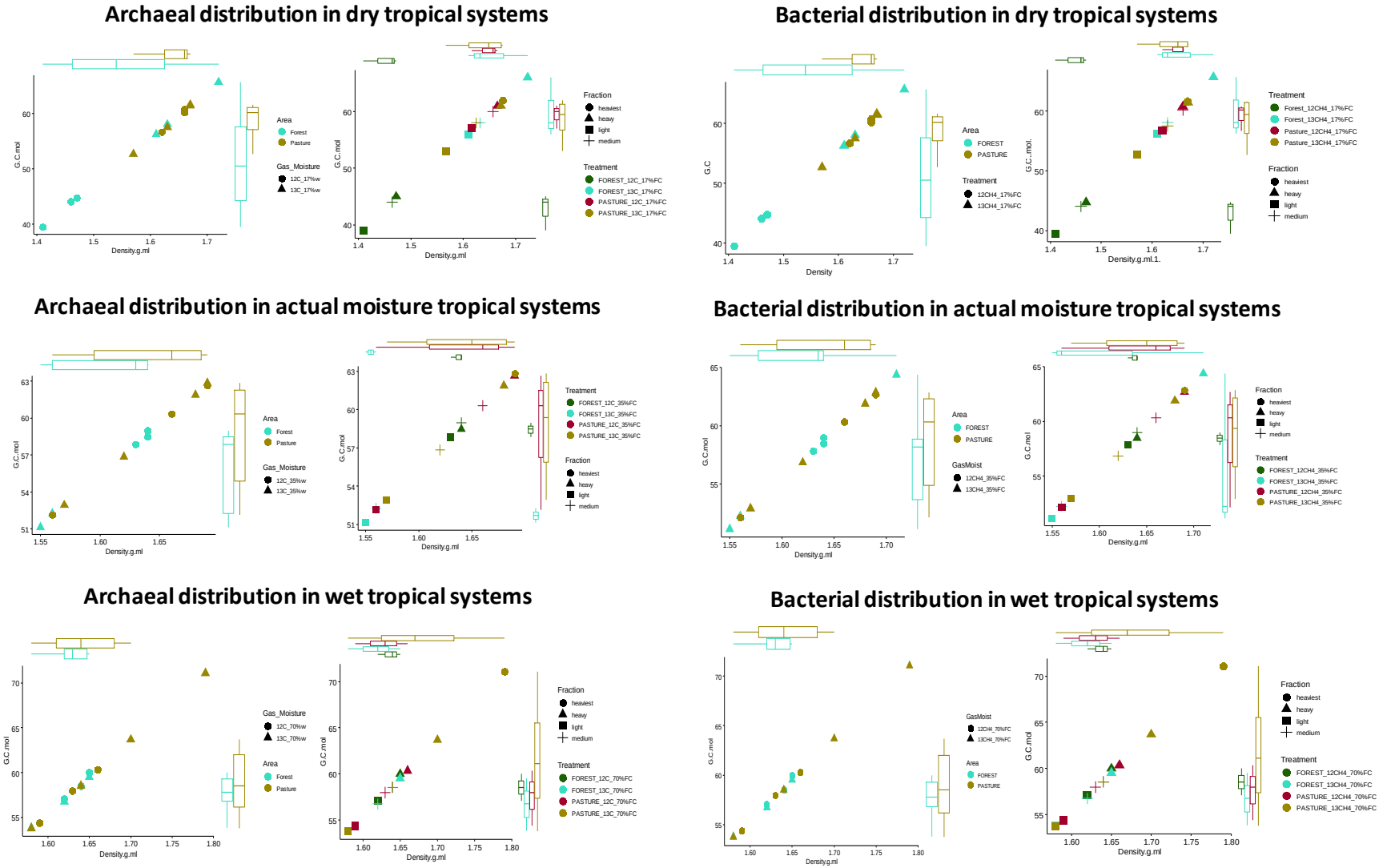


Figure 14.a – Prokaryotes distribution among densities and G+C %mol of the DNA-SIP/G+Cf fractions in Forest and Pasture under moistures. *Factorial treatments are written as follows: 12C= 12C-CH4 enrichment; 13C= 13C-CH4 enrichment; light, medium, heavy and heaviest fractions describe the ranges of each area. Scatter plots performed in R software (R core team, 2018) using ggplot2 (Wickham, 2016).*

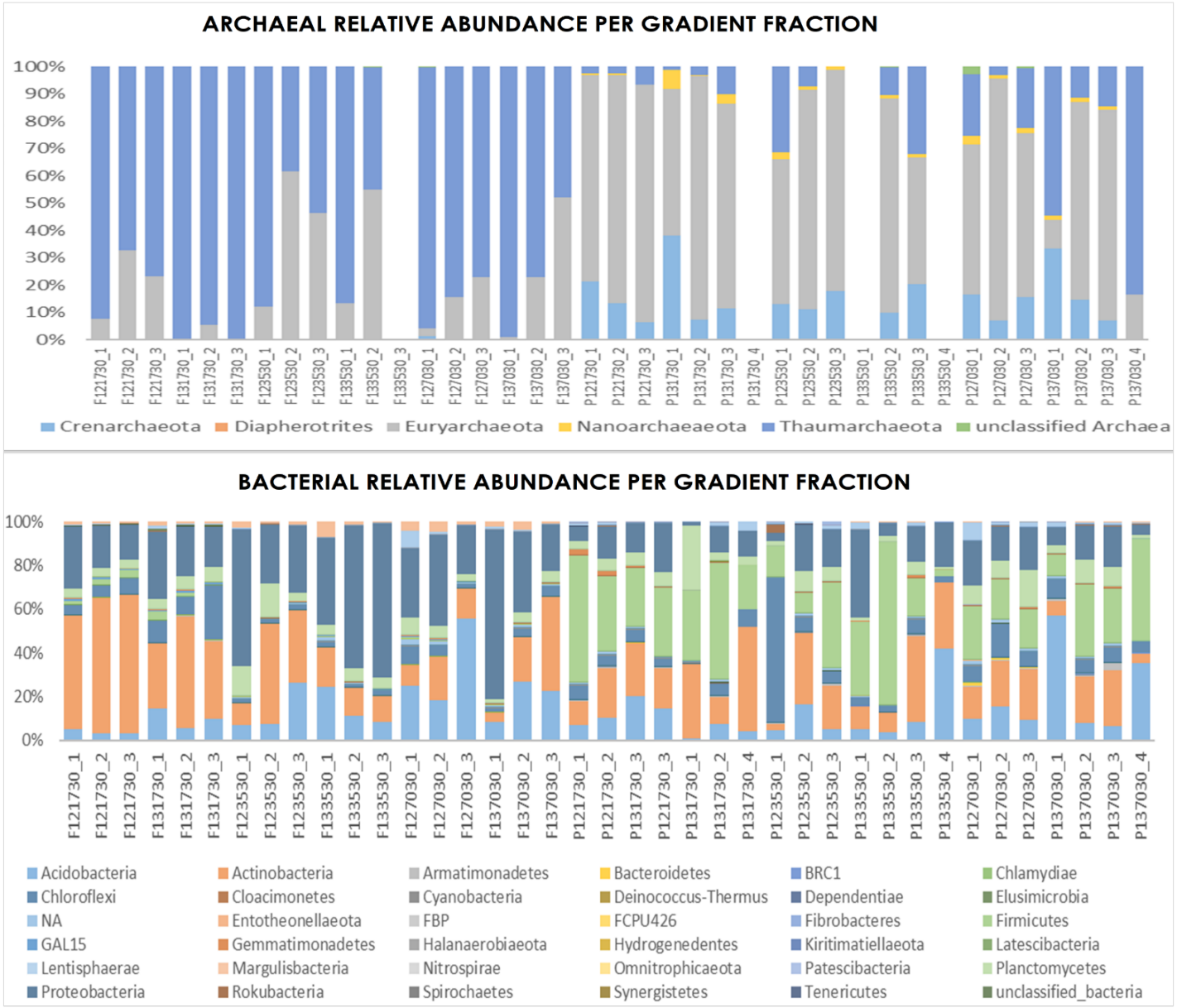


Figure 14 – Prokaryotes distribution among densities and G+C %mol of the DNA-SIP/G+Cf fractions in Forest and Pasture under moistures. *Factorial treatments are written as follows: 12C= 12C-CH4 enrichment; 13C= 13C-CH4 enrichment; light, medium, heavy and heaviest fractions describe the ranges of each area.* Plots were performed in Excel.

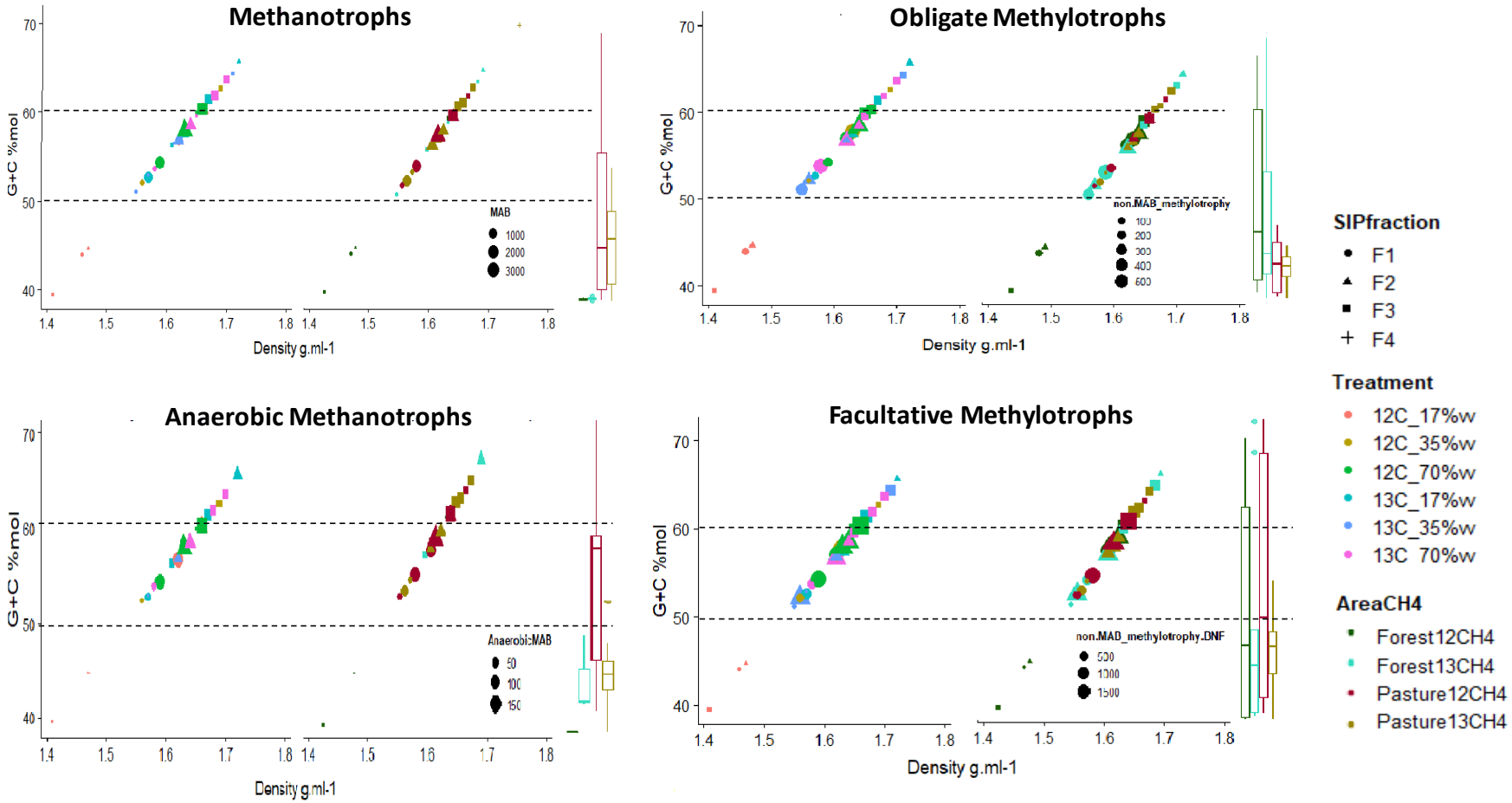


Figure 15.a– Methanotrophs and methylotrophs distributed among densities and G+C %mol of the DNA-SIP/G+Cf fractions in Forest and Pasture under moistures. *Factorial treatments are written as follows: 12C= 12C-CH4 enrichment; 13C= 13C-CH4 enrichment; light, medium, heavy and heaviest fractions describe the ranges of each area. Scatter plots performed in R software (R core team, 2018) using ggplot2 (Wickham, 2016).*

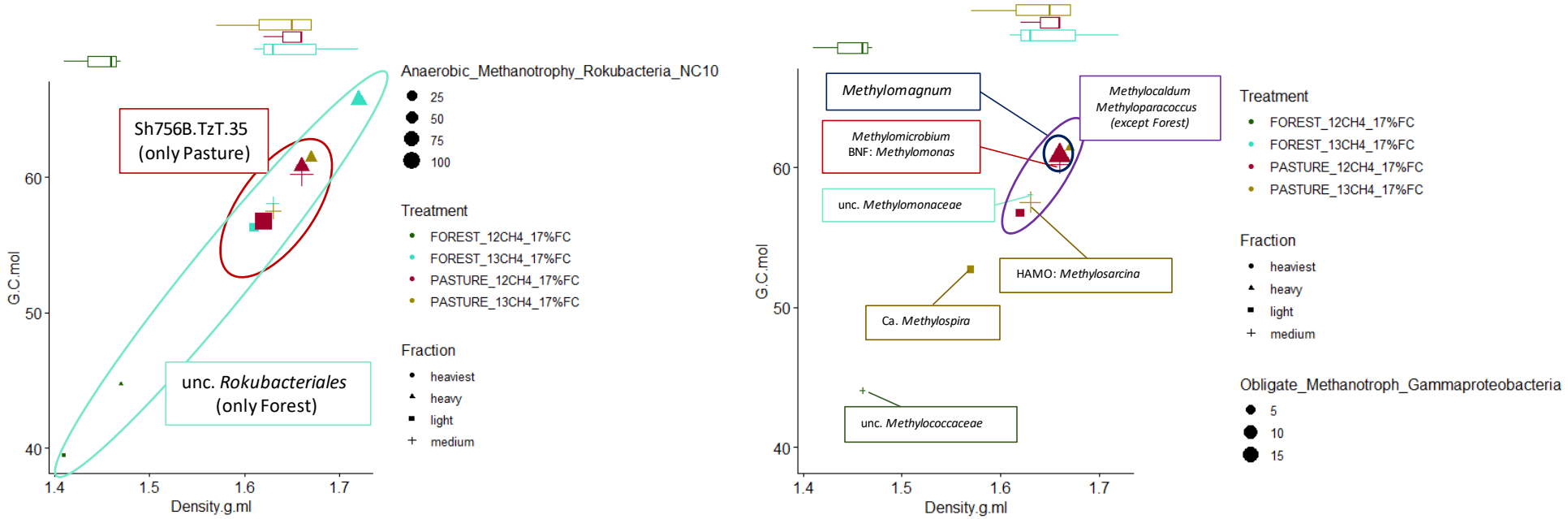


Figure 15.b– Methanotrophs and methylotrophs distributed among densities and G+C %mol of the DNA-SIP/G+Cf fractions in Forest and Pasture under moistures. *Factorial treatments are written as follows: 12C= 12C-CH4 enrichment; 13C= 13C-CH4 enrichment; light, medium, heavy and heaviest fractions describe the ranges of each area. Scatter plots performed in R software (R core team, 2018) using ggplot2 (Wickham, 2016).*

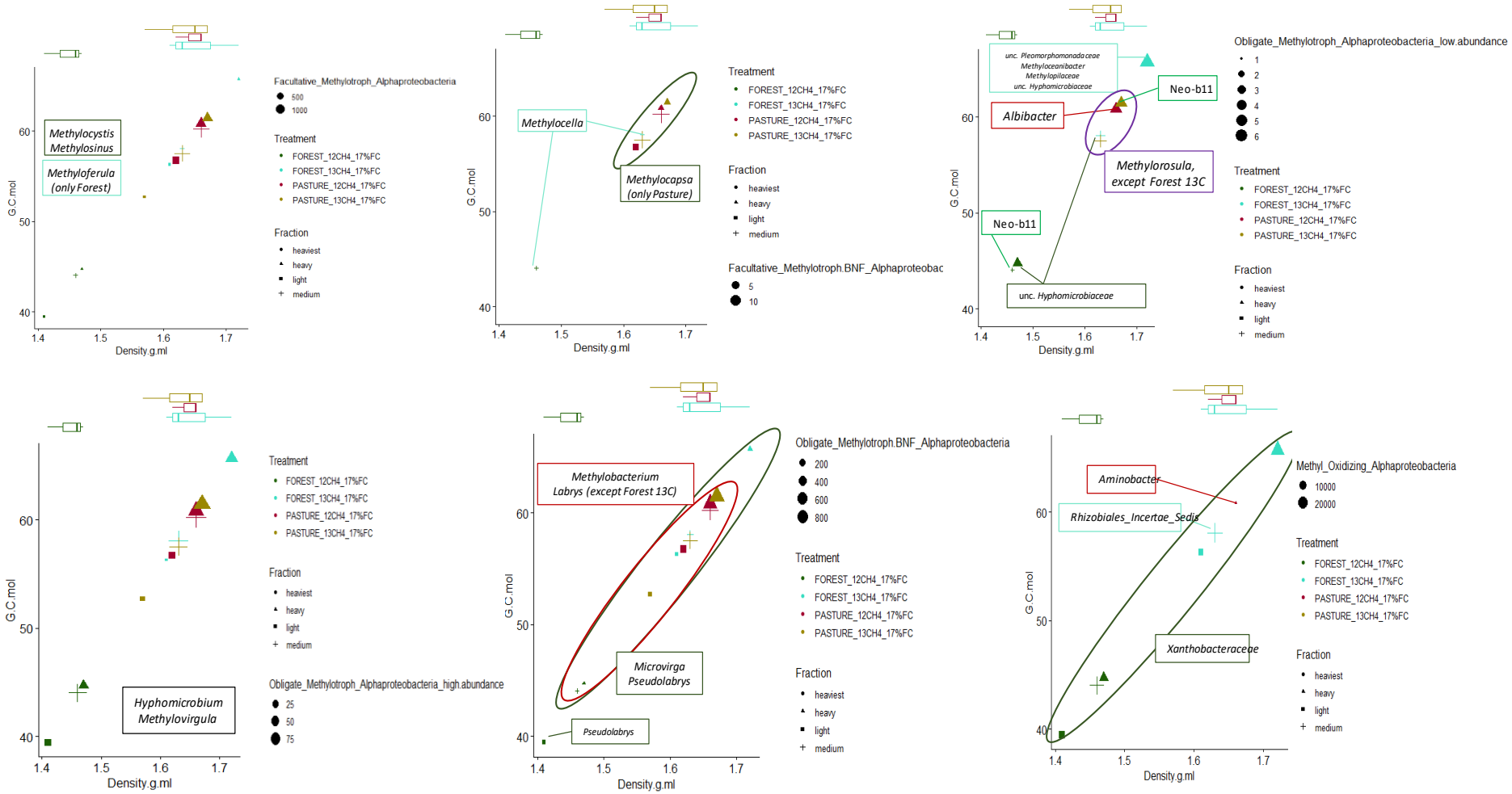


Figure 15.c– Methanotrophs and methylotrophs distributed among densities and G+C %mol of the DNA-SIP/G+Cf fractions in Forest and Pasture under moistures. *Factorial treatments are written as follows: 12C= 12C-CH4 enrichment; 13C= 13C-CH4 enrichment; light, medium, heavy and heaviest fractions describe the ranges of each area. Scatter plots performed in R software (R core team, 2018) using ggplot2 (Wickham, 2016).*

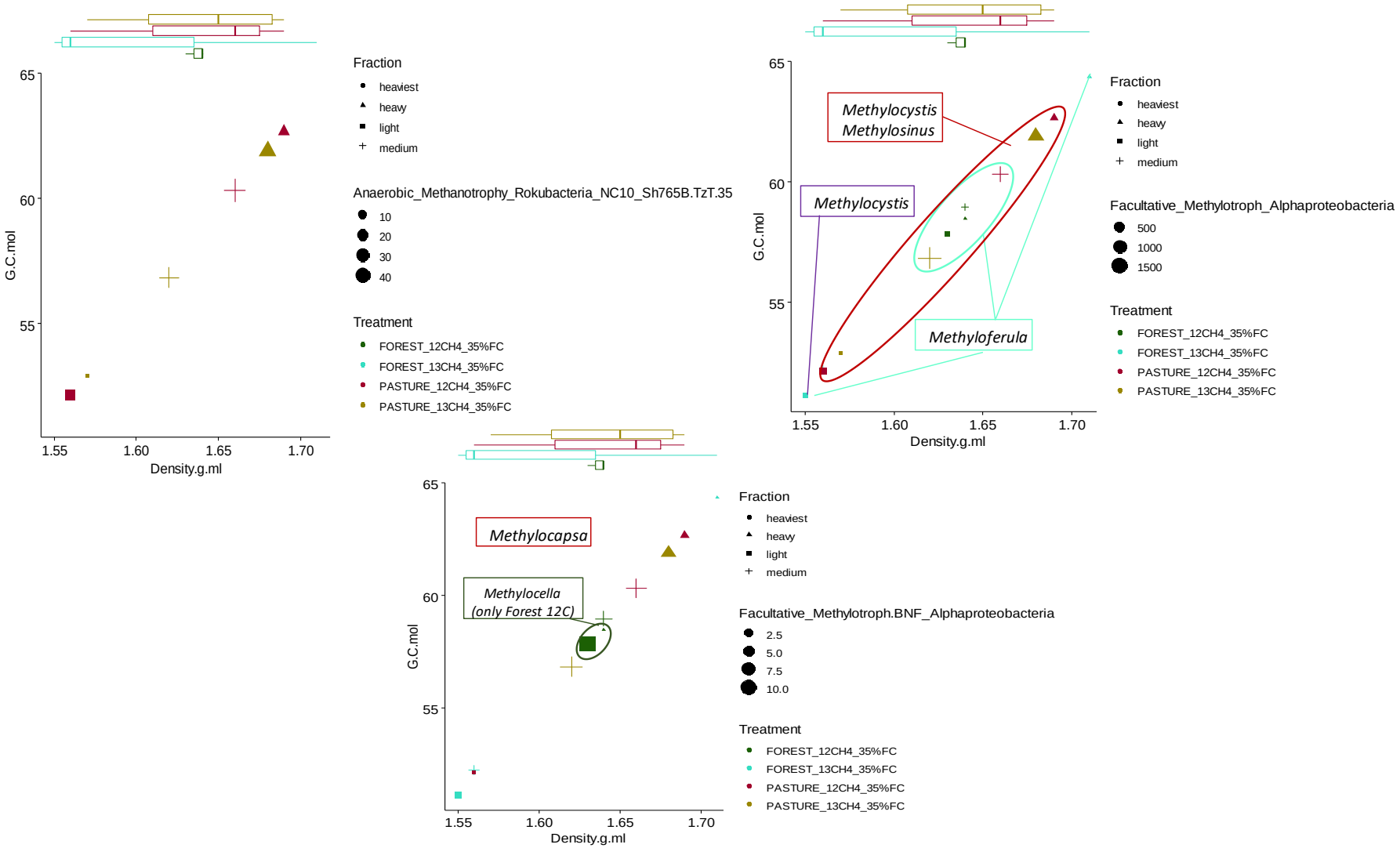


Figure 15.d– Methanotrophs and methylotrophs distributed among densities and G+C %mol of the DNA-SIP/G+Cf fractions in Forest and Pasture under moistures. *Factorial treatments are written as follows: 12C= 12C-CH4 enrichment; 13C= 13C-CH4 enrichment; light, medium, heavy and heaviest fractions describe the ranges of each area. Scatter plots performed in R software (R core team, 2018) using ggplot2 (Wickham, 2016).*

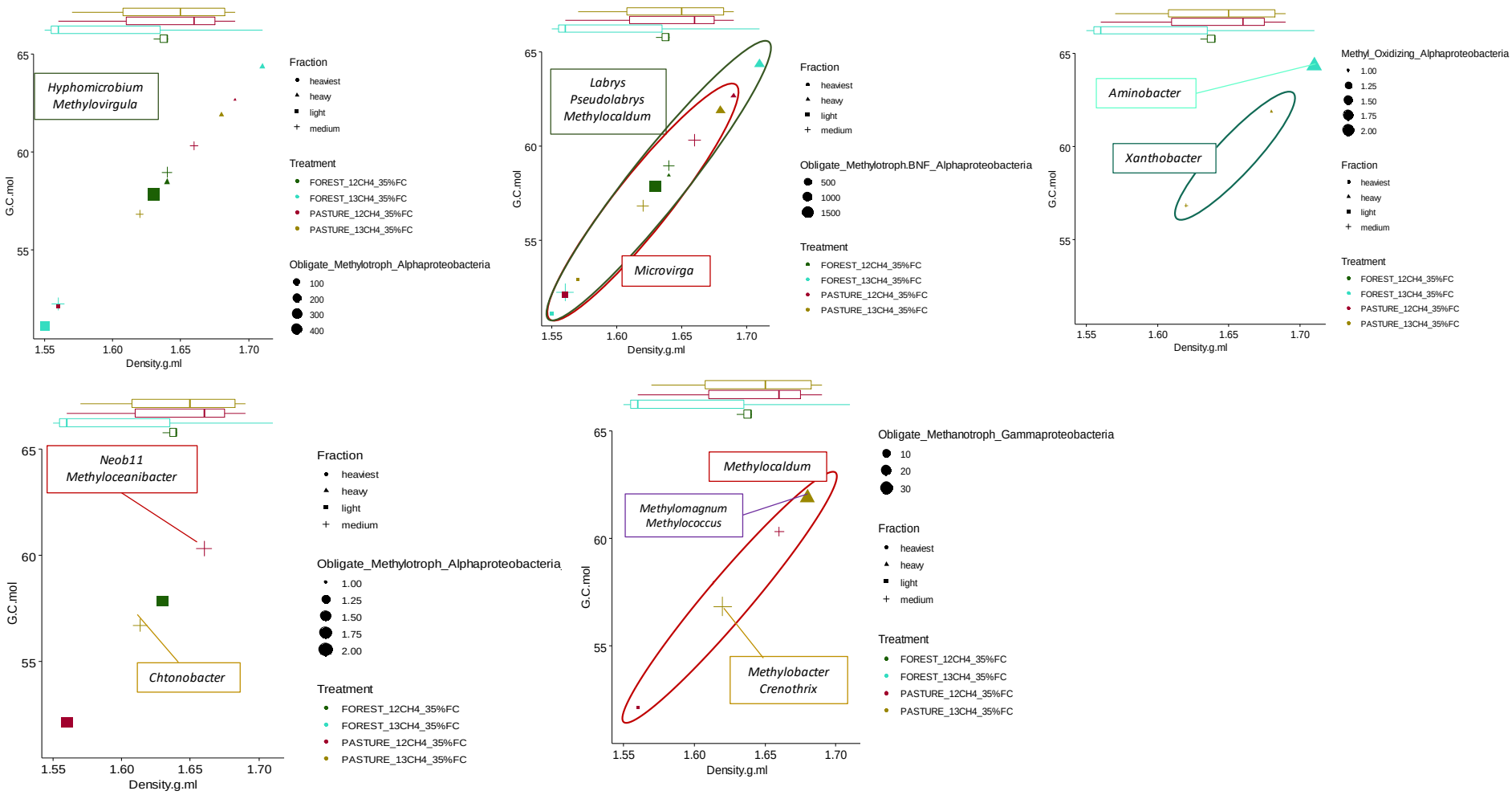


Figure 15.e – Methanotrophs and methylotrophs distributed among densities and G+C %mol of the DNA-SIP/G+Cf fractions in Forest and Pasture under moistures. *Factorial treatments are written as follows: 12C= 12C-CH4 enrichment; 13C= 13C-CH4 enrichment; light, medium, heavy and heaviest fractions describe the ranges of each area. Scatter plots performed in R software (R core team, 2018) using ggplot2 (Wickham, 2016).*

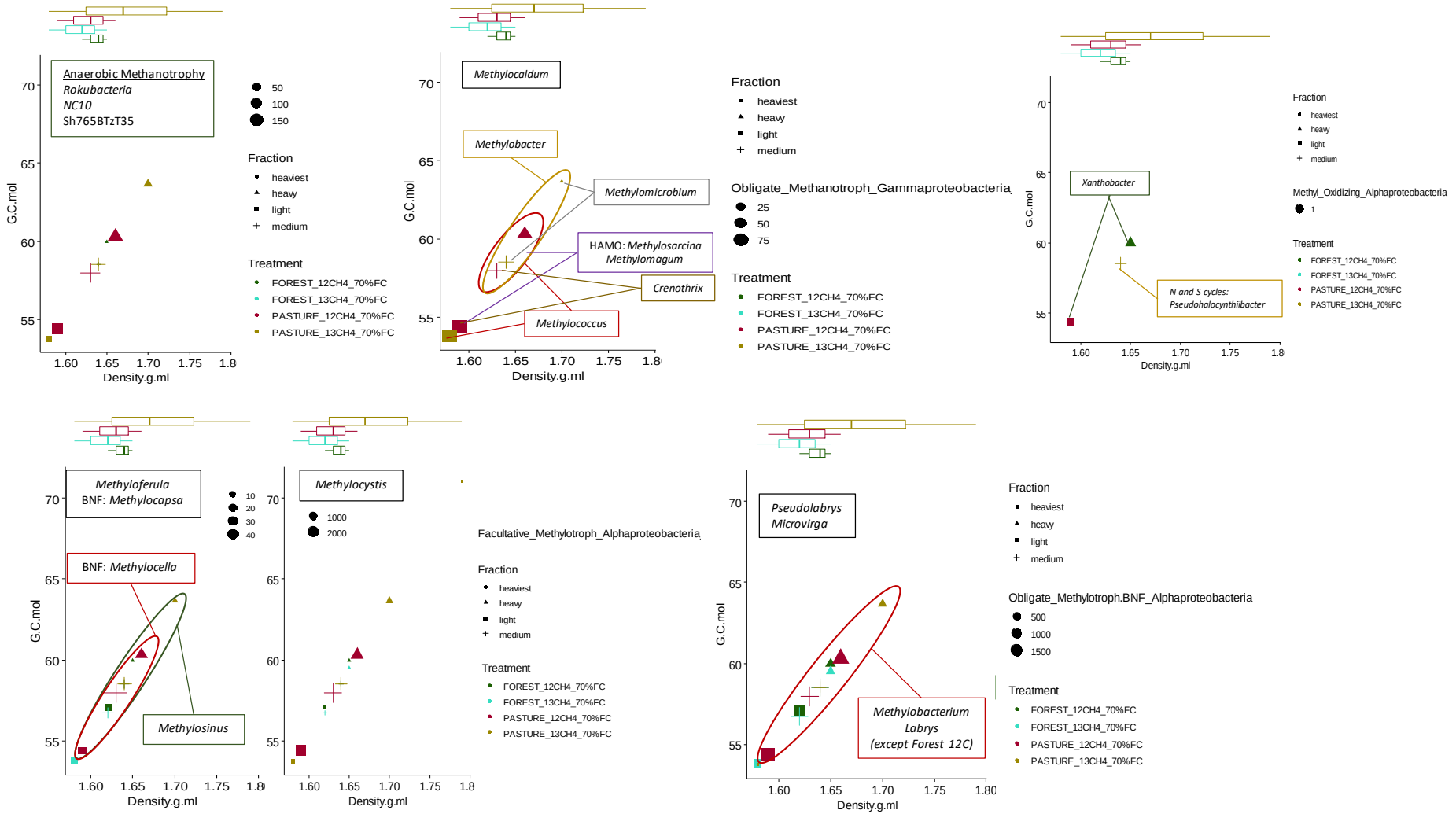


Figure 15.f – Methanotrophs and methylotrophs distributed among densities and G+C %mol of the DNA-SIP/G+Cf fractions in Forest and Pasture under moistures. *Factorial treatments are written as follows: 12C= 12C-CH4 enrichment; 13C= 13C-CH4 enrichment; light, medium, heavy and heaviest fractions describe the ranges of each area. Scatter plots performed in R software (R core team, 2018) using ggplot2 (Wickham, 2016).*

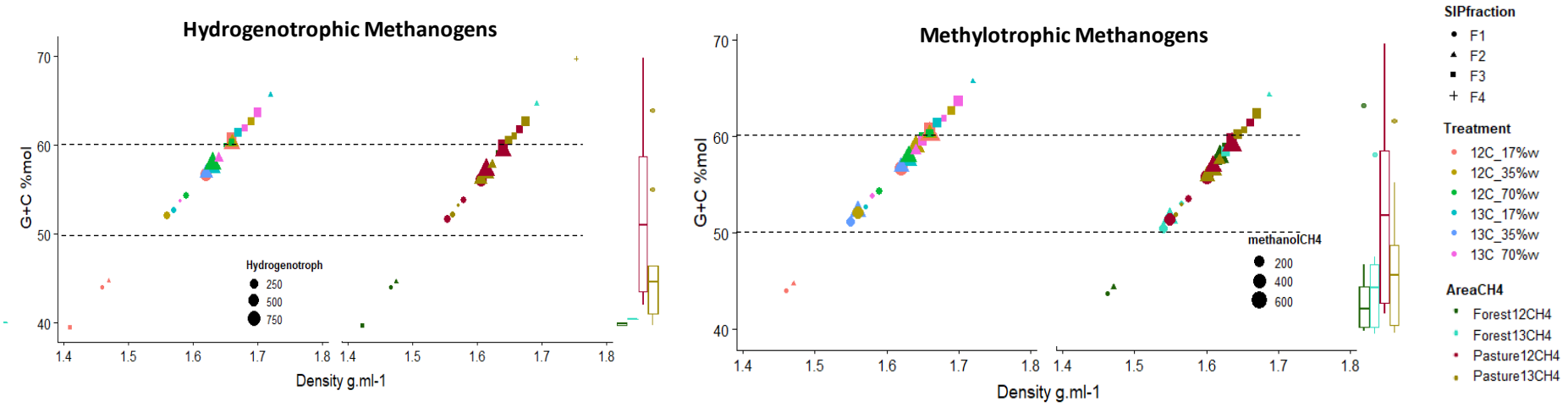
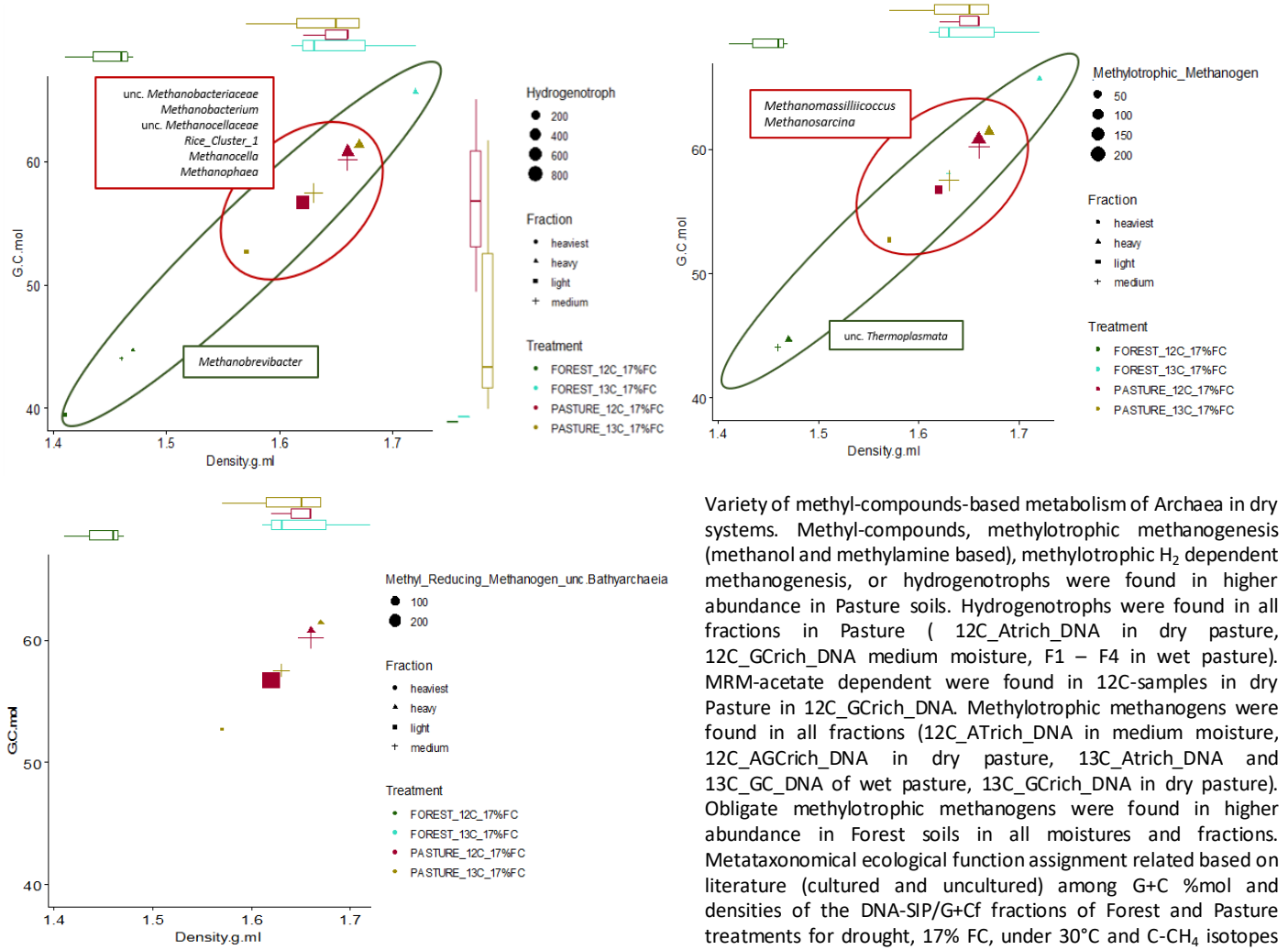


Figure 16a– Methanogens distributed among densities and G+C %mol of the DNA-SIP/G+Cf fractions in Forest and Pasture under variations of moisture. *Factorial treatments are written as follows: 12C= 12C-CH4 enrichment; 13C= 13C-CH4 enrichment; light, medium, heavy and heaviest fractions describe the ranges of each area. Scatter plots performed in R software (R core team, 2018) using ggplot2 (Wickham, 2016).*



Variety of methyl-compounds-based metabolism of Archaea in dry systems. Methyl-compounds, methylotrophic methanogenesis (methanol and methylamine based), methylotrophic H_2 dependent methanogenesis, or hydrogenotrophs were found in higher abundance in Pasture soils. Hydrogenotrophs were found in all fractions in Pasture (12C_Atrich_DNA in dry pasture, 12C_GCrich_DNA medium moisture, F1 – F4 in wet pasture). MRM-acetate dependent were found in 12C-samples in dry Pasture in 12C_GCrich_DNA. Methylotrophic methanogens were found in all fractions (12C_ATrich_DNA in medium moisture, 12C_AGCrich_DNA in dry pasture, 13C_Atrich_DNA and 13C_GC_DNA of wet pasture, 13C_GCrich_DNA in dry pasture). Obligate methylotrophic methanogens were found in higher abundance in Forest soils in all moistures and fractions. Metataxonomical ecological function assignment related based on literature (cultured and uncultured) among G+C %mol and densities of the DNA-SIP/G+Cf fractions of Forest and Pasture treatments for drought, 17% FC, under 30°C and C-CH_4 isotopes incubation.

Figure 16.b – Methanogens distributed among densities and G+C %mol of the DNA-SIP/G+Cf fractions in Forest and Pasture under variations of moisture. *Factorial treatments are written as follows: 12C= 12C-CH4 enrichment; 13C= 13C-CH4 enrichment; light, medium, heavy and heaviest fractions describe the ranges of each area. Scatter plots performed in R software (R core team, 2018) using ggplot2 (Wickham, 2016).*

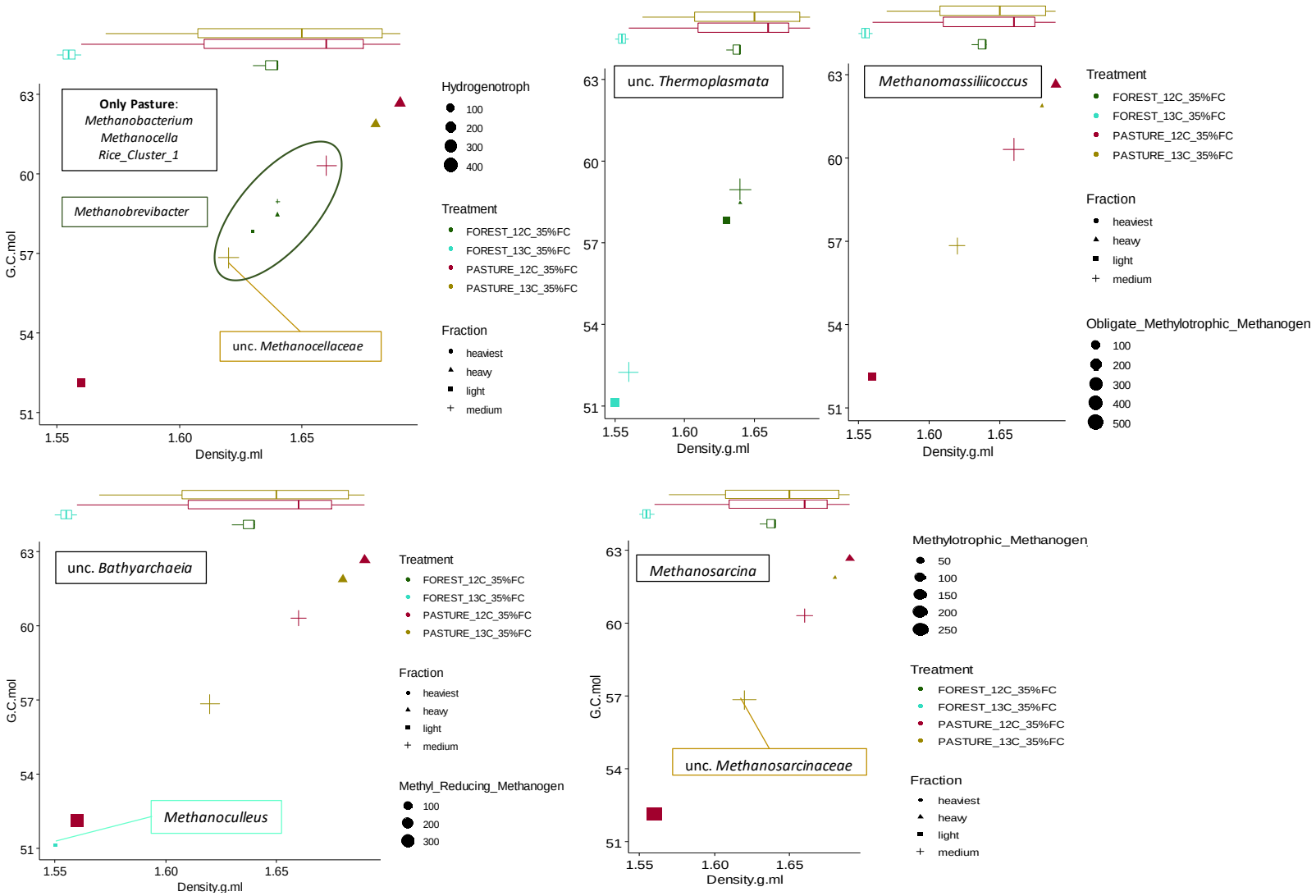


Figure 16.c – Methanogens distributed among densities and G+C %mol of the DNA-SIP/G+Cf fractions in Forest and Pasture under variations of moisture. *Factorial treatments are written as follows: 12C= 12C-CH4 enrichment; 13C= 13C-CH4 enrichment; light, medium, heavy and heaviest fractions describe the ranges of each area. Scatter plots performed in R software (R core team, 2018) using ggplot2 (Wickham, 2016).*

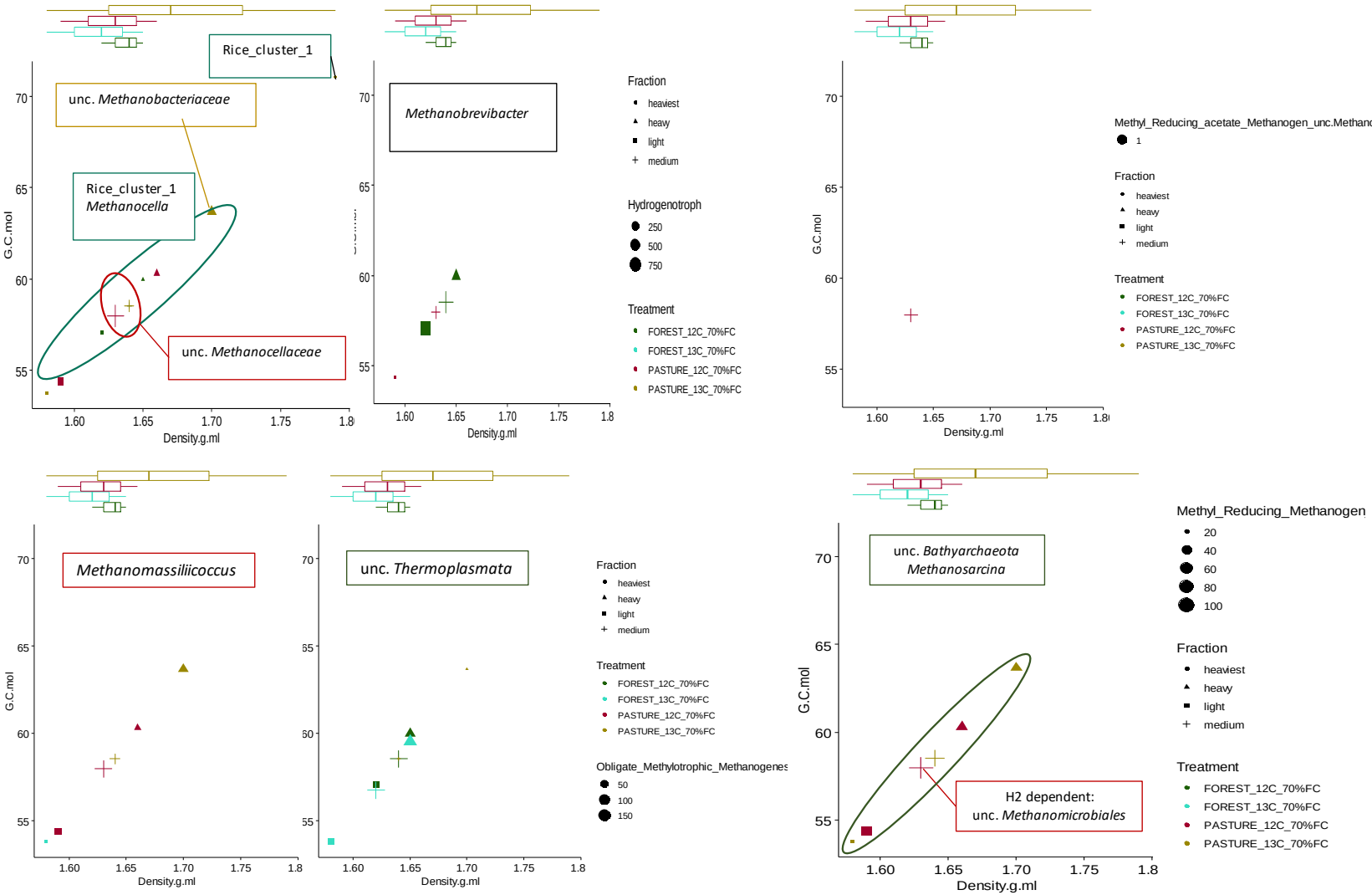


Figure 16.d – Methanogens distributed among densities and G+C %mol of the DNA-SIP/G+Cf fractions in Forest and Pasture under variations of moisture. *Factorial treatments are written as follows: 12C= 12C-CH4 enrichment; 13C= 13C-CH4 enrichment; light, medium, heavy and heaviest fractions describe the ranges of each area. Scatter plots performed in R software (R core team, 2018) using ggplot2 (Wickham, 2016).*

3.5.4. Exclusive groups and functions

3.5.4.1 Exclusive groups and functions in Forest

Higher abundances of Archaea and Bacteria in wet forest were found in the controls and $^{13}CH_4$ samples (Table 4).

Obligate methylotrophic methanogens were found in higher abundance in Forest soils in all moistures and fractions (Figure 16). The methylotrophic methanogen uncultured *Thermoplasmata* was found only in Forest under both C-isotopes among all fractions and in high abundance on the heavy fraction of ^{13}C-DNA samples, indicating a ^{13}C-consumption, hence evidencing that this methylotrophic methanogen was enriched and a is very likely to be active in Forest.

In addition, a very representative presence of methyl-oxidizing diazotrophic bacteria was found as uncultured *Xanthobacteriaceae*, likely to be related to methyl-compounds oxidation, such as methanol and methylamines as products from methanotrophy. Lidstrom et al. (2001) show that the C1 substrate derivated from methanotrophy and from decompostiton of litter has been related to an enormous contribution to methanogenesis in forests.

Only in the heavy fractions of $^{13}CH_4$-DNA of dry forest we found the following *Alphaproteobacteria*: a) the obligate methylotrophs unclassified *Methylopilaceae* (*Rhizobiales* order), and unclassified *Pleomorphomonadaceae* (*Rhizobiales*); b) the methyl-oxidizing bacteria unclassified Rhizobiales_Incertae_Sedis (*Rhizobiales*); c) the methyl-oxidizing bacteria related to N and S cycles unclassified *Rhodobacteriaceae* (*Rhodobacterales*); d) the methanotroph unclasscified *Methylomonaceae* (*Methylococcales*).

Also in the heavy fractions of 13C-DNA we found the phylum WPS-2 spread allover the forest samples under all moistures studied, however in higher amount of medium moisture, 35% FC and wet soil 70% FC. WPS-2 is related to a wide range of polycyclic hydrocarbon degradation, found also in soil with SIP technique (WANG et al., 2015). The fact that in Pasture it was poorly found under the same conditions evidences the abundance of this generalistic hydrocarbon degrader, which could be linked to diversity of substances in forest soils SOM (data not shown).

Interestingly, we found exclusively in dry and medium moisture Forest the unclassified *Micrarchaeia* (*Diapherotrites*), which function is not known, found in the light fractions of both C-isotopes, evidencing a DNA pf low G+C. Besides, the *Methanoculleus*

(*Euryarchaeota, Methanomicrobiales, Methanomicrobiaceae, Methanomicrobia*), which is a known methyl-reducing methanogen, was only found only in 13C_Atrich_DNA "lighter" fraction medium moisture forest, evidencing a DNA of medium G+C content.

We also found uncultured *Gammaproteobacteria* and *Alphaproteobacteria*, as well as unassigned order *Rhizobiales*, and unassigned genus from families *Beijerinckiaceae*, *Rhizobiaceae*, and *Methyloligellaceae*, which future findings will contribute for the understanding of Amazonian forest soils under drought and increased temperature for climate changes effects predictions.

We report that forest soils are more subjected to changes in microbial richness related to methyl-compounds degradation, methanotrophy and methanogenesis, hence the resultant GHGs emissions due to drought and wetness.

3.5.4.2 Exclusive groups and functions in Pasture

DNA from all live beings range from as low 25% up to 75%, which distribution is heterogeneous occuring in islets as repetittive sequences and also flanking ribossomal genes (MANN et al., 2010). The higher G+C influence in Pasture community of Archaea and Bacteria (Figure 14.a) is likely to be related to some cell resiliance face abiotic and biotic stress. Once these soils suffer higher disturbations as low density of soil coverage and surface exposition to ultraviolet radiation, some soil revolving and cattle soil compactation, besides the slash-and-burn method, which could stimulate dehydration resistant cells. G+C abundance in genome was once related to temperature resistance with exceptions (HURST et al., 2001; WU et al., 2012), and overall G+C regions are related to expression regulation, which in prokaryotes have ecological value specific to genus and even strains (MANN et al., 2010).

CH_4 emission was stimulated under $^{12}CH_4$ incubations in dry pasture soil, and no effect was observed in $^{13}CH_4$ incubations, besides no significant changes in genes with slightly higher methanotrophs with pMMO in control (Figure 14). If this CH_4 was from biological origin, thus we could infer that there was a preference for the 12C incorporated to soil by methanotrophs, and the resultant methyl-compounds could have been utilized with H_2 dependency for methanogenesis in dry pasture soil. A preference for the ^{12}C for methanogenesis was assigned to acetatoclastic methanogens (WHITICAR, 1999; CHANTON et al., 2005).

In addition (Figure 16), hydrogenotrophs were found in all fractions in Pasture (12C_Atrich_DNA in dry pasture, 12C_GCrich_DNA medium moisture, F1 – F4 in wet pasture). Methyl-reducing methanogens were found in 12C-samples in dry Pasture in 12C_GCrich_DNA. Methylotrophic methanogens were found in all fractions (12C_ATrich_DNA in medium moisture, 12C_AGCrich_DNA in dry pasture, 13C_Atrich_DNA and 13C_GC_DNA of wet pasture, 13C_GCrich_DNA in dry pasture). Interestingly, acetoclastic are reported as mostly present in DNA in pasture soils (MEYER et al., 2017), and the low acetoclastic methanogens in our samples suggest a very fine sellection of recently active microorganisms upon treatments maybe filtering some necromass. The technique utilized is able to filter and likely to lose information, however is able to highlight punctual variations.

The hydrogenotroph uncultured *Methanobacteriaceae* was found in dry and wet Pasture under both C-isotopes. In addition, the following groups were distributed among all moistures under both C-isotopes: Candidatus *Nitrososphaera*, uncultured *Woesearchaeia;* the hydrogenotrophs *Methanobacterium*, uncultured *Methanocellaceae*, *Methanocella* and Rice_Cluster_I; the methylotrophic methanogens *Methanosarcina*, *Methanomassiliicoccus;* and the methyl-reducing methanogen uncultured *Bathyarchaeia* (Figure 16). The presence of these groups in either $^{12}CH_4$ heavy fractions and $^{13}CH_4$ heavy fractions evidences a high G+C content and either the 13C-methyl-compounds consumption formed in the trophic network under $^{13}CH_4$ enrichment.

Methylosarcina is a generalistic methanogen, utilizing most of the possible substrates being classified as acetoclastic (minimum of 1mM), as well as methyl-reducing methanogens (SOWERS et al., 1984). *Methylosarcina* was found exclusively in Pasture in all moistures, concentrated in wet at 12_GCrich_DNA, and under dry in both higher fractions 13GCrich_DNA (Figure 15, Table 4). Besides, *mcrA* gene was abundantly detected in dry pasture soil, reinforcing the evidence of methyl-reducing, methylotrophic and/or acetoclastic methanogenesis as the responsible for CH_4 emission.

The methanotrophs of high affinity to CH_4 (HAMO) *Crenothrix* (*Proteobacteria, γ-proteobacteria, Methylococcales, Crenothricaceae*) (DUNFIELD, 2007; POL, 2007), and methanotroph *Methylobacter (Proteobacteria, γ-proteobacteria, Methylococcales, Methylomonaceae)* (BOWMAN et al., 1993; WARTIAINEN et al., 2006), as well as the strict methylotroph *Chthonobacter (Proteobacteria, α-proteobacteria, Rhizobiales,*

Pleomorphomonadaceae) (KIM et al., 2017) were present only in medium and wet Pasture soils.

3.6 Conclusions

The downstream amplicon-based next generation sequencing and the metataxonomic assignment of prokaryotes through ASVs from the DNA-based Stable Isotope Probing coupled with G+C Fractionation promoted the accessment of the microbiota related to methane cycle in tropical soil of Eastern Amazon.

The technique utilized is able to filter and likely lose information, however is able to highlight punctual variations through a very fine sellection of recently active microorganisms upon treatments, avoiding some necromass, even in a DNA-based study.

We report that forest soils are more subjected to changes in microbial richness related to methyl-compounds degradation, methanotrophy and methanogenesis, and possibly resultant GHGs emissions due to drought and wetness.

Evidence of hydrogenotrophic predominancy upon acetoclatic, as well as methyl-reducing, methylotrophic methanogenesis as the responsible for CH_4 emission in dry and wet Pasture soils under 30°C.

This study brings a clear vision of the of key microbial functions in methane fluxes, providing data for the understanding the real impacts of climate and soil use changes can cause to the gas fluxes from soils in large tropical areas as the Amazon.

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APPENDICES

APPENDIX A: Supplementary material of Chapter 2

SP A1 – Absolute quantification Real Time PCR (qPCR) reactions conditions

Gene	Standard	Primer	Sequence (5'-3')	Fragment length (pb)	Reference	Conv. PCR condition	qPCR condition
***mcrA*: codifies the α- subunit of metanol coenzyme M reductase (MCR) from methanogens**	*Methanolinea mesophila* (DSM 23604)	mlα-F	GGYGGTGT MGGDTTCA CMCARTA	472	Angel et al. (2012)	95 °C - 4 min; 40 cycles: 95°C - 30 s, 60°C - 45 s, 72°C - 30 s; 72°C- 10 min	95°C 10 min; 45 cycles: 95°C - 30 s, 60°C - 45 s, 72°C - 30 s; Melting curve: 95°C - 15 s, 60°C - 1 min 95 °C -15 s data reading every each 0,5 °C
		mcrA-R	CGTTCATB GCGTAGTT VGGRTAGT T		Steimberg; Regan (2008)		
***pmoA*: codifies the α- subunit of methane monooxygenase (pMMO) from methanotrophs**	*Methylosinus sporum* (ATCC35069)	A189f	GGNGACTG GGACTTCT GG	469	Holmes et al. (1995)	95°C - 4min; 40 cycles: 95°C -30 s, 58°C - 45 s, 72°C - 45 s, 72°C- 10 min	95°C - 10 minutos; 45 cycles: 95 °C - 30 s, 58°C - 30 s, 72 °C - 45 s; Melting curve: 95°C -15 s 58°C - 1 min 95°C - 15 s data reading every each 0,5 °C
		MB661r	CCGGMGC AACGTCYT TAAC		Costello; Lidstrom (1999)		
V4 region 16S rRNA for *Bacteria*	*Methanobrevibacter smithi* (ATCC35061)	515F	GTGCCAGC MGCCGCG GTAA	292	Caporaso et al., (2011)	98°C - 4min; 27 cycles: 98°C -10 s, 54°C - 30 s, 72°C - 15 s; 72°C - 7 min	95°C - 10 minutos; 40 cycles: 98 °C – 3min, 98°C – 10 s, 54 °C - 30 s; Melting curve: 95°C -15 s 54C - 1 min 95°C - 15 s data reading every each 0,5 °C
		806R	GGACTACN SGGGTMTC TAAT				
V4 region 16S rRNA for *Archaea*	*environmental clone*	U519F	CAGYMGC CRCGGKAA HACC	288	Porat et al. (2010)	98°C - 4min; 27 cycles: 98°C -10 s, 55°C - 30 s, 72°C - 15 s; 72°C - 7 min	95°C - 10 minutos; 40 cycles: 98 °C – 3min, 98°C – 10 s, 54 °C - 30 s; Melting curve: 95°C -15 s 54C - 1 min 95°C - 15 s data reading every each 0,5 °C
		806R	GGACTACN SGGGTMTC TAAT		Caporaso et al., (2011)		

SP A2 – Absolute quantification of 16S rRNA genes of Archaea and Bacteria, *mcrA* and *pmoA* genes, and C from CH4 and CO2, and N from N2O for all sites, followed by the results of degrees of freedom (DF), F-values, and p-values of the three-way mixed-design ANOVA of the aligned rank-transformed data.

Sample	Area	M (% v.v)	T (°C)	DF	16S rRNA of Archaea	16S rRNA of Bacteria	mcrA	pmoA	C-CH4	C-CO2	N-N2O
Area	FOREST PASTURE			1	74.92***	39.18***	73.93***	23.93***	37.72***	69.96***	10.62**
Moisture		17 35 70		2	33.41***	14.32***	2.21	4.23*	19.94***	0.81	9.89***
Temperature			25 30	1	17.74***	0.94	4.2*	7.42**	37.87***	1.01	10.54**
Moisture*Temperature				2	3.8*	29.289***	0.29	1.70	6.11**	0.15	2.18
Area*Moisture	Forest	17 35 70		2	34.11***	22.16***	0.611	1.01	3.56	1.23	0.81
	Pasture	17 35 70		2	5.11*	0.95	0.527	0.42	0.961	0.716	1.6
Area*Temperature	Forest		25 30	1	15.35**	0.64	0.25	5.24*	10.78**	1.28	3.96
	Pasture		25 30	1	5.83*	0.32	0.17	2.16	17.75**	0.0014	2.51
Area*Moisture*Temperature	Forest	17 35 70	25 or 30	2	30.28***	76.84***	6.42*	3.37	6.7*	0.61	0.91
	Pasture	17 35 70	25 or 30	2	5.87*	11.66**	2.44	0.62	1.685	0.2114	7.94**

*, $p < 0.05$; 88, $p < 0.01$; ***, $p < 0.001$.

SP A3 – Significant positive genes and gases variations coupled with significant correlations to soil attributes and microbial traits.

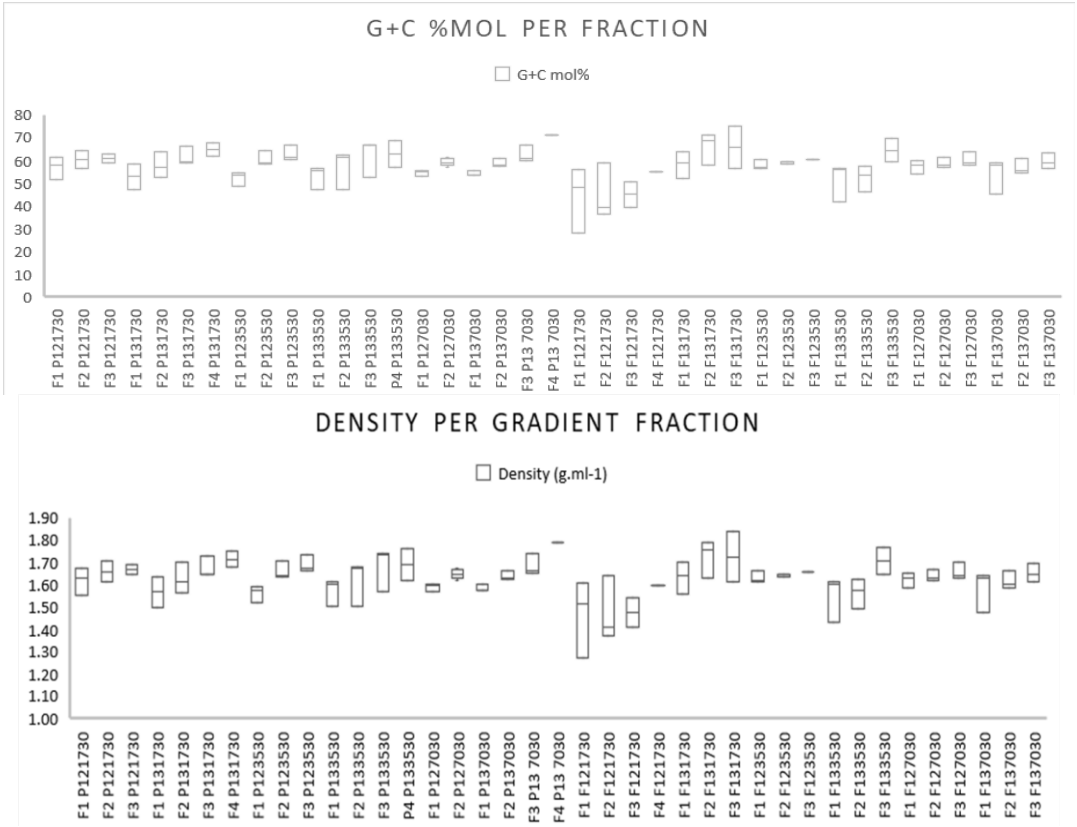
		Archaea					Bacteria				
		35 to 17% v.v.		35 to 70% v.v.		70%	35 to 17% v.v.		35 to 70% v.v.		35%
		25oC	30oC	25oC	30oC	25-30oC	25oC	30oC	25oC	30oC	25-30oC
Significative changes in qPCR		Decreased (FN3525 to FN1725, F-2.68 = 27, p < 0.001)	Decreased (FN3530 to FN1730, F-3 = 27, p < 0.001)	Decreased (FN3525 to FN7025, F-1.73 = 27, p < 0.001)	Increased (FN3530 to FN7030, F1.12 = 27, p < 0.001)	Increased (FN7025 to FN7030, F2.44 = 13.5, p < 0.05)	Decreased (FN3525 to FN1725, F-3.35 = 27, p < 0.001)	Decreased (FN3530 to FN1730, F-10 = 27, p < 0.001)	Increased (FN3525 to FN7025, F1.98 = 27, p < 0.001)	Increased (FN3530 to FN7030, F1.25 = 27, p < 0.001) re	
Forest	Soil chemical attributes	SOM, H+Al, CEC	pH, SOM, P, Ca+2, H+Al, CEC	N2O, SOM, H+Al, CEC, pH, P	pH, SOM, P, Ca+2, H+Al, CEC,	pH, SOM, P, Ca+2, H+Al, CEC,	K, B, Fe, Zn, Cu/SOM	K, Fe, Mn, Zn, NO3-	pH, P, Ca+2, SOM, H+AL, CEC	pH, P, Ca+2, SOM, H+AL, CEC	
	Soil physical attributes	GS, flocculation	GS, flocculation	GS, flocculation	GS, flocculation	GS, flocculation	Labile clay	FS, macroporosity, whc	GS, flocculation	GS, flocculation	
	Microbial traits	mcrA		Bacteria	Bacteria	Bacteria	pmoA	pmoA	Archaea, CO2, N2O	Archaea, CO2, N2O	
Significative changes in qPCR		Decreased (PN3525 to PN1725, F-2.3 = 6.31, p < 0.05)		Remained (PN3525 to PN7025, F1 = 6.31, p < 0.05)	Increased (PN3530 to PN7030, F2.4 = 27, p < 0.001)			Decreased (PN3530 to PN1730, F1.23 = 12.88, p < 0.01)		Remained (PN3530 to PN7030, F1.26 = 12.88, p < 0.01)	
Pasture	Soil chemical attributes	H+Al, Fe,		Al+3, m%,	H+Al, Fe, NH4+, NO3-			SOM, P, S, B, Cu, Zn, N, Cu/SOM		SOM, P, S, B, Cu, Zn, N	
	Soil physical attributes	density, flocculation		labile clay, flocculation.	microporosity			FS, MS, porosity		FS, MS, porosity	
	Microbial traits	CO2		CH4				N2O		CH4, N2O	
		Methanogens					Methanotrophs_pMMO				
		35 to 17% v.v.		35 to 70% v.v.		70%	35 to 17% v.v.		35 to 70% v.v.		35%
		25oC	30oC	25oC	30oC		25oC	30oC	25oC	30oC	25-30oC
Significative changes in qPCR		Decreased (FN3525 to FN1725, F-6.27 = 7, p < 0.05)		Remained (FN3525 to FN7025, F1 = 7, p < 0.05)			Decreased (FN3525 to FN1725, F-4.41 = 12.88, p < 0.01)	Increased (FN3530 to FN1730, F17.8 = 12.88, p < 0.01)	Increased (FN3525 to FN7025, F2.3 = 12.88, p < 0.01)	Increased (FN3530 to FN7030, F28 = 12.88, p < 0.001)	Increased (FN3525 to FN3530, F1.6 = 13.5, p < 0.05)
Forest	Soil chemical attributes	pH, SOM, P, Ca+2		NH4+, N, SOM			B, SOM, Cu, Cu/SOM	K, Fe, Zn, Mn	K, Fe, Mn, Zn, NO3-	SOM, NH4+, N	SOM, N, NO3-
	Soil physical attributes	GS, flocculation		GS, flocculation			GS, flocculation, clay	macroporosity, whc, FS	FS, mcroporosity, whc	GS, flocculation	GS, flocculation
	Microbial traits	Archaea					Bacteria, CO2, N2O	Bacteria		CH4	mcrA

		CH4			CO2
		35 to 17% v.v.	35%	70%	17%
		25oC	25-30oc		25oC-30oC
Significative changes in GC		CH4 Increased (FN3525 to FN1725, F= 12.88, p < 0.01)	CH4 Increased (FN3525 to FN3530, F3 = 13.5, p < 0.05)		CO2 Decreased (FN1725 to FN1730, F-2.05 = 13.5, p < 0.05)
Forest	Soil chemical attributes	pH, P, Ca+2, Mg+2, Al+3, SB, V%, Cu	K, B, Cu, Fe, Zn, Cu/SOM		SOM, NH4+, N,
	Soil physical attributes	MFS, MGS, sand, clay, micro porosity	clay.water		GS, flocculation
	Microbial traits				N2O
Significative changes in GC				CH4 Increased (PN7025 to PN7030, F1.52 = 13.5, p < 0.05)	CO2 Increased (PN1725 to PN1730, F19 = 13.5, p < 0.05)
Pasture	Soil chemical attributes			SOM, P, S, B, Cu, Zn, N, Cu/SOM	
	Soil physical attributes			FS, porosity	MS, microporosity, macroporosity, WHC
	Microbial traits			Bacteria, N2O	CH4
		N2O			
		35 to 17% v.v.	35%	70%	
		25oC	25-30oc		
Significative changes in GC		N2O Increased (PN3525 to PN7025, F3 = 27, p < 0.001)			
Pasture	Soil chemical attributes				
	Soil physical attributes	macroporosity, microporosity, whc			
	Microbial traits	CO2, CH4			

Abbreviations:SOM, soil organic matter; H+Al, acidity potential; SB, sum of bases (Ca+Mg+K); CEC, cation exchange capacity (Al+Ca+Mg+K); V%, bases saturation [(Ca+Mg+K/CEC) x 100]; m%, aluminum saturation.

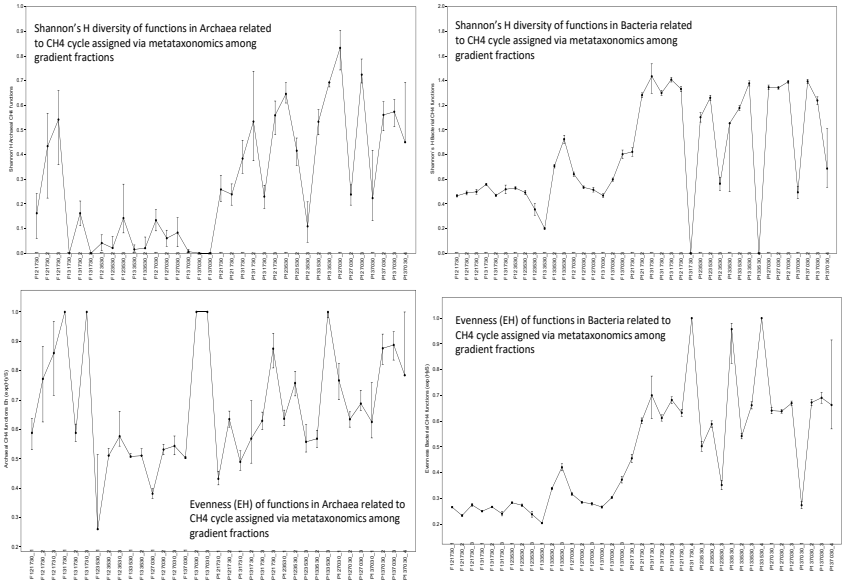
APPENDIX B: Supplementary material of Chapter 3

SP B1 – Distribution of gradient fractions among isopycnic densities and attributed G+C content. The square indicates the terms utilized in this study.



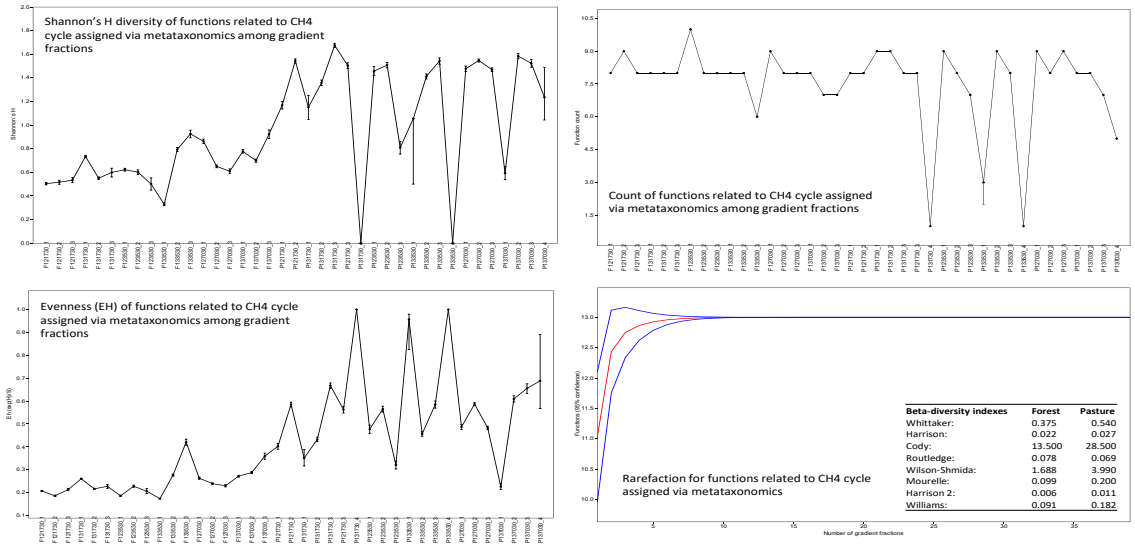
Isotope enriched substrate	Gradient fraction ID	Gradient fraction type	DNA composition	Final nomenclature	Ecological mean	G+C% mol
$^{12}CH_4$	F1	Light (12Clight-DNA)	ATrich-DNA	12C_ATrich-DNA	Non-labeled and/or AT rich DNA fragment	< 40
	F2	Medium (12Cheavy-DNA)	GCrich-DNA	12C_GCrich-DNA	Non-labeled and GC rich DNA fragment	40 – 50
	F3	Heaviest (12Cheavy-DNA)				50 – 60
$^{13}CH_4$	F1	Light (13Clight-DNA)	ATrich-DNA	13C_ATrich-DNA	Non-labeled and/or AT rich DNA fragment	< 50
	F2	Medium (13Cheavy-DNA)	13Crich AND/OR GCrich-DNA	13C_GCrich-DNA	Labeled and/or GC rich DNA fragment	50 – 60
	F3 and F4	Heaviest (13Cheavy-DNA)				>60

SP B2 –



Beta-diversities	Forest Archaea	Pasture Archaea	Forest Bacteria	Pasture Bacteria
Whittaker:	0.77	0.81	0.14	0.18
Harrison:	0.05	0.07	0.01	0.01
Cody:	10.50	5.00	3.00	16.00
Routledge:	0.14	0.10	0.04	0.04
Wilson-Shmida:	3.72	1.51	0.49	3.14
Mourelle:	0.23	0.13	0.03	0.16
Harrison 2:	0.00	0.04	0.00	0.00
Williams:	0.00	0.33	0.00	0.00

α and β--diversity indexes for functions related to CH_4 cycle among gradient fractions. Functions are detailed on Tables 5 and 6. Plots performed in Past 3.x (Hammer et al., 2001).



Beta-diversity indexes	Forest	Pasture
Whittaker:	0.375	0.540
Harrison:	0.022	0.027
Cody:	13.500	28.500
Routledge:	0.078	0.069
Wilson-Shmida:	1.688	3.990
Mourelle:	0.099	0.200
Harrison 2:	0.006	0.011
Williams:	0.091	0.182

α- and β-diversity indexes for functions related to CH_4 cycle among gradient fractions. Functions are detailed on Tables 5 and 6. Plots performed in Past 3.x (Hammer et al., 2001).

SP B3 – Absolute quantification of 16S rRNA genes of Archaea and Bacteria, *mcrA* and *pmoA* genes, and C from CH4 and CO2, and N from N2O for all sites, followed by the results of degrees of freedom (DF), F-values, and p-values of the three-way mixed-design ANOVA of the aligned rank-transformed data.

Treatments	Area land-use	M % v.v	Gas ppm (%)	DF	16S rRNA		mcrA		pmoA		C-CH4		C-CO2		N-N2O	
					MD	F value	MD	F value	MD	F value	MD	F value	MD	F value	MD	F value
Area	Forest			1	b	2.40	a	156.2***	a	102***	a	0.56	b	55.6***	b	41***
	Pasture				a		b		b		a		a		a	
Moisture		17		2	a	40.84***	a	1.55	a	8.19***	a	2.43	a	0.39	a	3.48
		35			b		a		a		a		a		a	
		70			c		a		b		a		a		a	
Gas			Air	2	b	4.96**	a	0.16	a	1.03	a	34.98***	a	0.39	a	0.08
			12CH4		b		a		a		b		a		a	
			13CH4		a		a		a		b		a		a	
Area*Moisture	Forest	17		2	b	20.37***	a	4.52*	a	23***	a	1.24	a	0.006	a	5.53*
		35			a		a		a		a		a		a	
		70			a		a		a		a		a		a	
	Pasture	17		2	a	23.7***	a	2.29	a	15.94***	a	1.91	a	6.51*	a	3.63
		35			a		a		a		a		a		a	
		70			b		a		b		a		a		a	
Area*Gas	Forest		Air	2	a	2.80	a	3.85*	a	1.80	a	11***	a	0.93	a	0.52
			12CH4		a		a		a		b		a		a	
			13CH4		a		a		a		b		a		a	
	Pasture		Air	2	b	4*	a	1.50	a	1.23	a	34.6***	a	0.23	a	2
			12CH4		a		a		a		c		a		a	
			13CH4		ab		a		a		b		a		a	
Area *Moisture *Gas	Forest	17	all	2		13.96***		3*		8.61***		3.36**		0.27		0.93
		35														
		70														
	Pasture	17	all	2		10.92***		0.82		4.4**		17.4***		1.10		1.7
		35														
		70														

SP B4 – Richeness of entire Archaea obtained in metataxonomic assignment from the DNA-SIP/G+Cf samples

Phylum	Class	Order	Family	Genus
Archaea_phylumNA	Archaea_phylumNA	Archaea_phylumNA	Archaea_phylumNA	Archaea_phylumNA
Crenarchaeota	Crenarchaeota_classNA	Crenarchaeota_classNA	Crenarchaeota_classNA	Crenarchaeota_classNA
Crenarchaeota	Bathyarchaeia	Bathyarchaeia_orderNA	Bathyarchaeia_orderNA	Bathyarchaeia_orderNA
Crenarchaeota	Thermoprotei	Thermoprotei_orderNA	Thermoprotei_orderNA	Thermoprotei_orderNA
Diapherotrites	Micrarchaeia	Micrarchaeia_orderNA	Micrarchaeia_orderNA	Micrarchaeia_orderNA
Euryarchaeota	Thermoplasmata	Thermoplasmata_orderNA	Thermoplasmata_orderNA	Thermoplasmata_orderNA
Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter
Euryarchaeota	Methanomicrobia	Methanocellales	Methanocellaceae	Rice_Cluster_I
Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobacterium
Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanomicrobiaceae	Methanoculleus
Euryarchaeota	Thermoplasmata	Methanomassiliicoccales	Methanomassiliicoccaceae	Methanomassiliicoccus
Euryarchaeota	Methanomicrobia	Methanosarcinales	Methanosarcinaceae	Methanosarcina
Euryarchaeota	Methanomicrobia	Methanocellales	Methanocellaceae	Methanocella
Euryarchaeota	Euryarchaeota_classNA	Euryarchaeota_classNA	Euryarchaeota_classNA	Euryarchaeota_classNA
Euryarchaeota	Methanomicrobia	Methanocellales	Methanocellaceae	Methanocellaceae_genusNA
Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanosphaera
Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobacteriaceae_genusNA
Euryarchaeota	Methanomicrobia	Methanosarcinales	Methanosarcinaceae	Methanosarcinaceae_genusNA
Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanomicrobiales_familyNA	Methanomicrobiales_familyNA
Euryarchaeota	WSA2	Ca. Methanofastidiosa	unassigned	Ca. Methanofastidiosum
Nanoarchaeaeota	Woesearchaeia	Woesearchaeia_orderNA	Woesearchaeia_orderNA	Woesearchaeia_orderNA
Nanoarchaeaeota	Nanoarchaeaeota_classNA	Nanoarchaeaeota_classNA	Nanoarchaeaeota_classNA	Nanoarchaeaeota_classNA
Thaumarchaeota	Nitrososphaeria	Nitrososphaerales	Nitrososphaeraceae	Nitrososphaeraceae_genusNA
Thaumarchaeota	Group_1.1c	Group_1.1c_orderNA	Group_1.1c_orderNA	Group_1.1c_orderNA
Thaumarchaeota	Nitrososphaeria	Nitrosotaleales	Nitrosotaleaceae	Nitrosotaleaceae_genusNA
Thaumarchaeota	Nitrososphaeria	Nitrososphaeria_orderNA	Nitrososphaeria_orderNA	Nitrososphaeria_orderNA
Thaumarchaeota	Nitrososphaeria	Nitrososphaerales	Nitrososphaeraceae	Candidatus_Nitrososphaera
Thaumarchaeota	Nitrososphaeria	Nitrososphaerales	Nitrososphaeraceae	Candidatus_Nitrocosmicus
Thaumarchaeota	Nitrososphaeria	Nitrososphaerales	Nitrososphaerales_familyNA	Nitrososphaerales_familyNA
Thaumarchaeota	Thaumarchaeota_classNA	Thaumarchaeota_classNA	Thaumarchaeota_classNA	Thaumarchaeota_classNA

SP B5 – Richeness of methanotrophs and/or methylotrophs obtained in metataxonomic assignment from the DNA-SIP/G+Cf samples

Phylum	Class	Order	Family	Genus
Proteobacteria	A-proteobacteria	Rhizobiales	Beijerinckiaceae	28-YEA-48
Proteobacteria	A-proteobacteria	Rhizobiales	Beijerinckiaceae	α-I_cluster
Proteobacteria	A-proteobacteria	A-proteobacteria_NA	A-proteobacteria_NA	A-proteobacteria_NA
Proteobacteria	A-proteobacteria	Rhizobiales	Beijerinckiaceae	Beijerinckiaceae_NA
Proteobacteria	A-proteobacteria	Rhizobiales	Beijerinckiaceae	FFCH5858
Proteobacteria	A-proteobacteria	Rhizobiales	Beijerinckiaceae	Methylobacterium
Proteobacteria	A-proteobacteria	Rhizobiales	Beijerinckiaceae	Methylocapsa
Proteobacteria	A-proteobacteria	Rhizobiales	Beijerinckiaceae	Methylocella
Proteobacteria	A-proteobacteria	Rhizobiales	Beijerinckiaceae	Methylocystis
Proteobacteria	A-proteobacteria	Rhizobiales	Beijerinckiaceae	Methyloferula
Proteobacteria	A-proteobacteria	Rhizobiales	Beijerinckiaceae	Methylorosula
Proteobacteria	A-proteobacteria	Rhizobiales	Beijerinckiaceae	Methylosinus
Proteobacteria	A-proteobacteria	Rhizobiales	Beijerinckiaceae	Methylovirgula
Proteobacteria	A-proteobacteria	Rhizobiales	Beijerinckiaceae	Microvirga
Proteobacteria	A-proteobacteria	Rhizobiales	Beijerinckiaceae	Neo-b11
Proteobacteria	A-proteobacteria	Rhizobiales	Hyphomicrobiaceae	Hyphomicrobium
Proteobacteria	A-proteobacteria	Rhizobiales	Hyphomicrobiaceae	Hyphomicrobiaceae_NA
Proteobacteria	A-proteobacteria	Rhizobiales	Labraceae	Labrys
Proteobacteria	A-proteobacteria	Rhizobiales	Methyloligellaceae	Methyloligellaceae_NA
Proteobacteria	A-proteobacteria	Rhizobiales	Methyloligellaceae	Methyloceanibacter
Proteobacteria	A-proteobacteria	Rhizobiales	Methylopilaceae	Albibacter
Proteobacteria	A-proteobacteria	Rhizobiales	Methylopilaceae	Methylopila
Proteobacteria	A-proteobacteria	Rhizobiales	Methylopilaceae	Methylopilaceae_NA
Proteobacteria	A-proteobacteria	Rhizobiales	Pleomorphomonadaceae	Pleomorphomonadaceae_NA
Proteobacteria	A-proteobacteria	Rhizobiales	Pleomorphomonadaceae	Chthonobacter
Proteobacteria	A-proteobacteria	Rhizobiales	Rhizobiaceae	Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium
Proteobacteria	A-proteobacteria	Rhizobiales	Rhizobiaceae	Aminobacter
Proteobacteria	A-proteobacteria	Rhizobiales	Rhizobiaceae	Pseudaminobacter
Proteobacteria	A-proteobacteria	Rhizobiales	Rhizobiaceae	Rhizobiaceae_NA
Proteobacteria	A-proteobacteria	Rhizobiales	Rhizobiales_Incertae_Sedis	Rhizobiales_Incertae_Sedis_NA
Proteobacteria	A-proteobacteria	Rhizobiales	Rhizobiales_NA	Rhizobiales_NA
Proteobacteria	A-proteobacteria	Rhizobiales	Xanthobacteraceae	Bradyrhizobium
Proteobacteria	A-proteobacteria	Rhizobiales	Xanthobacteraceae	GAS113
Proteobacteria	A-proteobacteria	Rhizobiales	Xanthobacteraceae	Pseudolabrys
Proteobacteria	A-proteobacteria	Rhizobiales	Xanthobacteraceae	Xanthobacter
Proteobacteria	A-proteobacteria	Rhizobiales	Xanthobacteraceae	Xanthobacteraceae_NA
Proteobacteria	A-proteobacteria	Rhodobacterales	Rhodobacteraceae	Rhodobacteraceae_NA
Proteobacteria	A-proteobacteria	Rhodobacterales	Rhodobacteraceae	Pseudohalocynthiibacter
Proteobacteria	Deltaproteobacteria	Deltaproteobacteria_order	Deltaproteobacteria_family	Deltaproteobacteria_NA
Proteobacteria	Gammaproteobacteria	Gammaproteobacteria_NA	Gammaproteobacteria_NA	Gammaproteobacteria_NA
Proteobacteria	Gammaproteobacteria	Methylococcales	Methylococcaceae	Candidatus_Methylospira
Proteobacteria	Gammaproteobacteria	Methylococcales	Methylomonaceae	Crenothrix
Proteobacteria	Gammaproteobacteria	Methylococcales	Methylomonaceae	Methylobacter
Proteobacteria	Gammaproteobacteria	Methylococcales	Methylococcaceae	Methylocaldum
Proteobacteria	Gammaproteobacteria	Methylococcales	Methylococcaceae	Methylococcaceae_NA
Proteobacteria	Gammaproteobacteria	Methylococcales	Methylococcaceae	Methylomagnum
Proteobacteria	Gammaproteobacteria	Methylococcales	Methylomonaceae	Methylomicrobium
Proteobacteria	Gammaproteobacteria	Methylococcales	Methylomonaceae	Methylomonaceae_NA
Proteobacteria	Gammaproteobacteria	Methylococcales	Methylomonaceae	Methylomonas
Proteobacteria	Gammaproteobacteria	Methylococcales	Methylococcaceae	Methyloparacoccus
Proteobacteria	Gammaproteobacteria	Methylococcales	Methylomonaceae	Methylosarcina
Proteobacteria	Gammaproteobacteria	Methylococcales	Methylomonaceae	pLW-20
Proteobacteria	Gammaproteobacteria	B-proteobacteriales	Methylophilaceae	Methylobacillus
Proteobacteria	Gammaproteobacteria	B-proteobacteriales	Methylophilaceae	MM1
Rokubacteria	NC10	Rokubacteriales	Rokubacteriales_NA	Rokubacteriales_NA
Rokubacteria	NC10	Methylomirabilales	Methylomirabilaceae	Sh765B-TzT-35
Verrucomicrobia	Verrucomicrobiae	Chthoniobacterales	Chthoniobacterales_NA	Chthoniobacterales_NA
Verrucomicrobia	Verrucomicrobiae	Chthoniobacterales	Chthoniobacteraceae	Chthoniobacter
Verrucomicrobia	Verrucomicrobiae	Chthoniobacterales	Chthoniobacteraceae	Chthoniobacteraceae_NA
Verrucomicrobia	Verrucomicrobiae	Chthoniobacterales	Chthoniobacteraceae	LD29
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiae_NA	Verrucomicrobiae_NA	Verrucomicrobiae_NA
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	Verrucomicrobium
WPS-2	unassigned	unassigned	unassigned	unassigned
WS1_	unassigned	unassigned	unassigned	unassigned
WS2	unassigned	unassigned	unassigned	unassigned
WS4	unassigned	unassigned	unassigned	unassigned

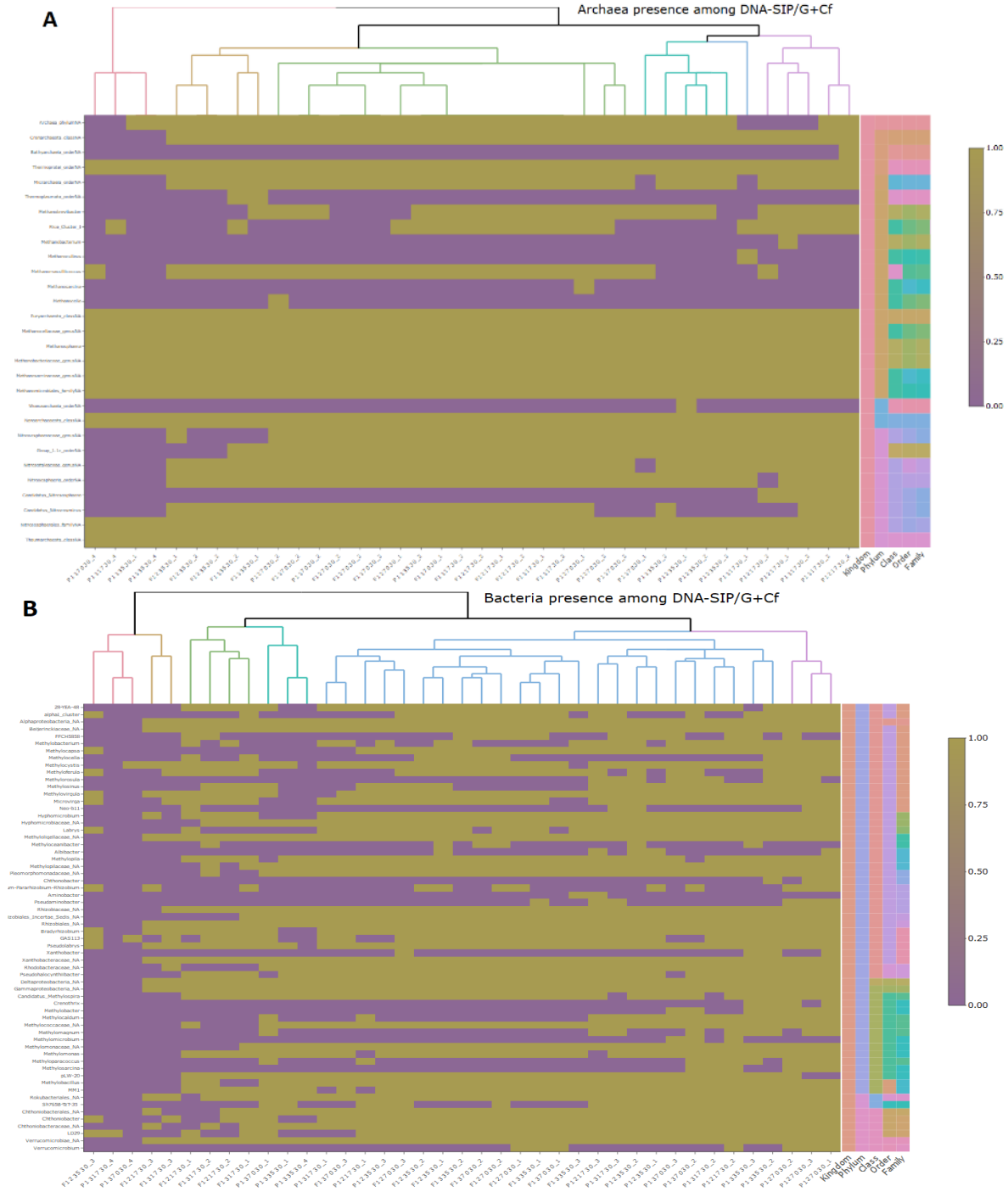
SP B4 – C1 and other hydrocarbon archaeal assimilating groups obtained from metataxonomic assignment of DNA-SIP/G+Cf incubated samples with 12CH4 and 13CH4, 35,000 ppmv

Kingdom	Phylum	Class	Order	Family	Genus
MRM_acetate	Euryarchaeota	Methanomicrobia	Methanosarcinales	Methanosarcinaceae	Methanosarcinaceae_genusNA
AOA	Thaumarchaeota	Group_1.1c	Group_1.1c_orderNA	Group_1.1c_orderNA	Group_1.1c_orderNA
		Nitrososphaeria	Nitrosotaleales	Nitrosotaleaceae	Nitrosotaleaceae_genusNA
			Nitrososphaerales	Nitrososphaeraceae	Ca. Nitrososphaera
					Ca. Nitrocosmicus
					Nitrososphaeraceae_genusNA
			Nitrososphaeria_orderNA	Nitrososphaeria_orderNA	Nitrososphaeria_orderNA
	Nanoarchaeaeota	Woesearchaeia	Woesearchaeia_orderNA	Woesearchaeia_orderNA	Woesearchaeia_orderNA
Hydrogenotroph	Euryarchaeota	Methanomicrobia	Methanocellales	Methanocellaceae	Rice_Cluster_I
					Methanocella
					Methanocellaceae_genusNA
		Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter
					Methanobacterium
					Methanosphaera
					Methanobacteriaceae_genusNA
MRM_H2	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanomicrobiales_familyNA	Methanomicrobiales_familyNA
MRM	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanomicrobiaceae	Methanoculleus
	Crenarchaeota	Bathyarchaeia	Bathyarchaeia_orderNA	Bathyarchaeia_orderNA	Bathyarchaeia_orderNA
MRMAM_acetate	Euryarchaeota	Methanomicrobia	Methanosarcinales	Methanosarcinaceae	Methanosarcina
					Methanosarcinaceae_genusNA
MRMOM	Euryarchaeota	Thermoplasmata	Thermoplasmata_orderNA	Thermoplasmata_orderNA	Thermoplasmata_orderNA
MRMOM	Euryarchaeota	Thermoplasmata	Methanomassiliicoccales	Methanomassiliicoccaceae	Methanomassiliicoccus
Other	Diapherotrites	Micrarchaeia	Micrarchaeia_orderNA	Micrarchaeia_orderNA	Micrarchaeia_orderNA
Parasitism	Nanoarchaeaeota	Nanoarchaeaeota_classNA	Nanoarchaeaeota_classNA	Nanoarchaeaeota_classNA	Nanoarchaeaeota_classNA
Unknown	Crenarchaeota	Crenarchaeota_classNA	Crenarchaeota_classNA	Crenarchaeota_classNA	Crenarchaeota_classNA
		Thermoprotei	Thermoprotei_orderNA	Thermoprotei_orderNA	Thermoprotei_orderNA
	Euryarchaeota	Euryarchaeota_classNA	Euryarchaeota_classNA	Euryarchaeota_classNA	Euryarchaeota_classNA
	Thaumarchaeota	Thaumarchaeota_classNA	Thaumarchaeota_classNA	Thaumarchaeota_classNA	Thaumarchaeota_classNA
	unclassified_Archaea	unclassified_Archaea	unclassified_Archaea	unclassified_Archaea	unclassified_Archaea

SP B5 – C1 and other hydrocarbon bacterial assimilating groups obtained from metataxonomic assignment of DNA-SIP/G+Cf incubated samples with 12CH4 and 13CH4, 35,000 ppmv

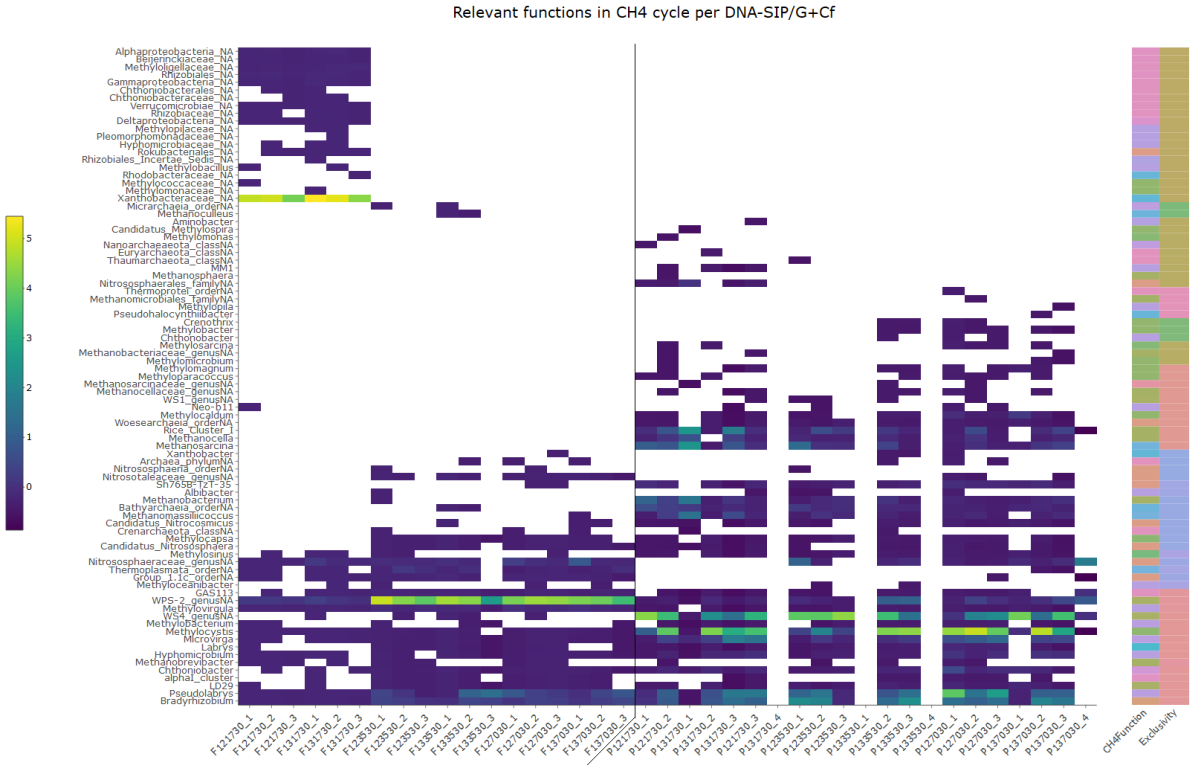
Ecology	Phylum	Class	Order	Family	Genus
Anaerobic Methanotrophs (an_MAB)	Rokubacteria	NC10	Rokubacteriales	Rokubacteriales_NA	Rokubacteriales_NA
			Methylomirabilales	Methylomirabilaceae	Sh765B-TzT-35
Methanotrophs (MAB)	Proteobacteria	Gammaproteobacteria	Methylococcales	Methylococcaceae	Candidatus_Methylospira
					Crenothrix
					Methylobacter
					Methylocaldum
					Methylococcaceae_NA
					Methylomagnum
					Methylomicrobium
					Methylomonaceae_NA
					Methyloparacoccus
MAB_HAMO					Methylosarcina
MAB_BNF					Methylomonas
MAB_methylotrophy		A-proteobacteria	Rhizobiales	Beijerinckiaceae	Methylocystis
					Methyloferula
					Methylosinus
MAB_methylotrophy_BNF					Methylocapsa
					Methylocella
non-MAB_methylotrophy		A-proteobacteria	Rhizobiales	Beijerinckiaceae	Methylorosula
					Methylovirgula
					Neo-b11
				Hyphomicrobiaceae	Hyphomicrobium
					Hyphomicrobiaceae_NA
				Methyloligellaceae	Methyloceanibacter
				Methylopilaceae	Albibacter
					Methylopila
					Methylopilaceae_NA
				Pleomorphomonadaceae	Pleomorphomonadaceae_NA
					Chthonobacter
		Gammaproteobacteria	B-proteobacteriales	Methylophilaceae	Methylobacillus
					MM1
non-MAB_methylotrophy_BNF		A-proteobacteria	Rhizobiales	Beijerinckiaceae	Methylobacterium
					Microvirga
				Xanthobacteraceae	Pseudolabrys
non-MAB_MOB_BNF				Rhizobiaceae	Aminobacter
MOB_BNF				Xanthobacteraceae	Xanthobacter
					Xanthobacteraceae_NA
				Labraceae	Labrys
MOB_NScycle			Rhodobacterales	Rhodobacteraceae	Rhodobacteraceae_NA
					Pseudohalocynthiibacter
		Deltaproteobacteria	Deltaproteobacteria_order	Deltaproteobacteria_family	Deltaproteobacteria_NA
Bacterial Nitrogen Fixers (BNF)		A-proteobacteria	Rhizobiales	Rhizobiaceae	Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium
					Pseudaminobacter
				Xanthobacteraceae	Bradyrhizobium
Hydrocarbon cycling	Proteobacteria	A-proteobacteria	Rhizobiales	Beijerinckiaceae	FFCH5858
				Rhizobiales_Incertae_Sedis	Rhizobiales_Incertae_Sedis_NA
	Verrucomicrobia	Verrucomicrobiae	Chthoniobacterales	Chthoniobacteraceae	LD29
	WS1	unassigned	unassigned	Unassigned	unassigned
	WS2	unassigned	unassigned	Unassigned	unassigned
	WS4	unassigned	unassigned	Unassigned	unassigned
	WPS-2	unassigned	unassigned	Unassigned	unassigned
Rhizosphere	Proteobacteria	Verrucomicrobiae	Chthoniobacterales	Chthoniobacteraceae	Chthoniobacter
			Verrucomicrobiales	Verrucomicrobiaceae	Verrucomicrobium
Unknow Bacteria		A-proteobacteria	Rhizobiales	Beijerinckiaceae	28-YEA-48
					α-I_cluster
					Beijerinckiaceae_NA
				Rhizobiales_NA	Rhizobiales_NA
				Rhizobiaceae	Rhizobiaceae_NA
				Methyloligellaceae	Methyloligellaceae_NA
				Xanthobacteraceae	GAS113
			A-proteobacteria_NA	A-proteobacteria_NA	A-proteobacteria_NA
		Gammaproteobacteria	Gammaproteobacteria_NA	Gammaproteobacteria_NA	Gammaproteobacteria_NA
			Methylococcales	Methylomonaceae	pLW-20
	Verrucomicrobia	Verrucomicrobiae	Chthoniobacterales	Chthoniobacterales_NA	Chthoniobacterales_NA
				Chthoniobacteraceae	Chthoniobacteraceae_NA
			Verrucomicrobiae_NA	Verrucomicrobiae_NA	Verrucomicrobiae_NA

SP B6- Presence and absence of metataxonomic genus assignment of entire population of Archaea (A) and selected methanotrophs and/or methylotrophs populations in Bacteria (B) among fractions of DNA-SIP/G+Cf.



Metataxonomy annotated through ASVs (Dada2) SILVA SSU Ref database version 132 (Callahan et al., 2016). Abundance heatmap with heatmaply package in euclidean distance sample dendrogram. Abbreviations: Barcolor from absence (purple) and presence (brown); genus are written on the left and right sidebar colors represent the richness of phyla, classes, orderes and families.

SP B7 - Unique functions among G+C %mol and densities distribution of the DNA-SIP/G+Cf gradient fractions of Forest and Pasture treatments for moisture variations (17%, 35% or 70% v.v.) under 30°C and CH_4 isotopes incubation.



SP B8 - Protocol for DNA Stable Isotope Probing coupled with G+C fractionation (DNA-SIP/G+Cf)

November, 2018. Developed by Fernanda Mancini Nakamura at Prof. Jorge L. M. Rodrigues` lab of Soil EcoGenomics Laboratory, Department of Land, Air, and Water Resources - University of California, Davis (UCDavis)
Acknowledgments
Lucas Palma P. Braga, Jordan M. Sayre, Rachel E. Danielsen.

Modified from:
Dunford, E. A., & Neufeld, J. D. (2010). DNA stable-isotope probing (DNA-SIP). Journal of Visualized Experiments : JoVE, (42), 1–6. https://doi.org/10.3791/2027/ Video link: https://www.jove.com/video/2027/dnα-stable-isotope-probing-dnα-sip?language=German
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SIP incubation and DNA extraction

1. Expose environmental samples to labeled substrate in the rate of 5–500 µmol ^{13}C (0.5 mmol ^{13}C) per g soil to detect ^{13}C DNA above background. A light labeling (cold labeling) with ^{12}C is required as a control.
2. Extract DNA with a proper technique, and perform quality and quantity evaluations.

NOTE: For rich and complex samples, such as soil, a total of 5µg of DNA is required.

Gradient preparation

1. **GB buffer**: 0.1 M Tris-HCl (pH = 8); 2,1 ml HCl (or 210 µl), 0.37275g for 0.1 M KCl, 10ml of 500mM premade EDTA 1.0 mM EDTA (pH = 8), complete to 500ml with miliQ and autoclave for 15min;
2. **Stock CsCl solution**: 1g CsCl per 1 ml GB in required amount, storing in sealed aliquots;

NOTE: Stock solution of 1g.ml-1 in 500ml. CsCl has a molecular weight of 168.36g/mol. Neufeld et al. (2007) suggest preparing 7.163M of CsCl stock solution to have the final 1.73 g.ml-1 after GB solution, so the count is: 603g in 500ml. The calculation of GB needed to have the volume of DNA and to assure the final density was based on Neufeld et al. (2007).

3. Prepare gradient for 5.1 ml polyallomer Quick-Seal centrifuge tubes by slowly adding with 1ml pipette (tips f 1 ml) the 5 µg DNA, and the GE buffer based on the formule:

$$(\text{stock CsCl} - \text{desired average density of 1.74}) * \text{CsCl stock volume of 4.7ml} * 1.52 \text{ factor}$$

4. Then add 8 µL 10,000X GelGreen™ (Gao et al., 2017), and 8µl of bis-benzimide (Chen; Murrel, 2010; Buckley et al., 2011) and complete to 5 ml with stock CsCl solution up to the base of tube neck.

NOTE: The final density of this solution, once combined with EtBr (for GelGreen is it the same?), is cited as being ~ 1.55 g/ml (see ref. 17), but we find that it tends to be slightly higher than this. The lower density of this solution is required because EtBr binds DNA and decreases its density to approximately 1.55 g/ml.

NOTE: Bis-benzimide is an intercalating agent to A+T regions making this sequences lighter in the buoyant density, inversely proportional to G+C content, allowing to better separate DNA bands rich in G+C. This can be an option for the soil DNAs and for the standard ATCC 35069. This is made in 2 steps, where first a density fraction is made with collection of 13C bands+G+C are acquired and re-fractionated with bisbenzimide (Chen; Murrel, 2010), and also Buckley et al. (2011) applied 8µl of bis-benzimide to the DNA band extracted from first fractionation and re-fractionated this DNA. In my opinion and tests with pure cultures (ATCC35069 12C or 13C-DNAs), there was no difference in inique run or twice run, so due to time and material, I chose the unique run.

NOTE: Try to run the biological tripicates at once to avoid inter-runs bias if you have a heterogeneous sample, e.g. soil cores. On the other hand, if your samples are homogenized, e.g. sewed and well mixed soil samples, then there will not be a inheritated variance and the inter-runs bias can be more tolerable.

5. Balance tube pairs to within 10 mg of difference.
6. Seal the tubes with heat (Beckman Coulter 358312 Tube Topper) according to the manufacturer's instructions. Verify that each tube is leak-free by squeezing forcefully by hand.
7. Double check that tube pairs remain balanced.

NOTE: DNA is stable in CsCl solution and tubes may be stored at room temperature in the dark for several weeks.

8. Place tubes into rotor with balanced pairs opposite each other and seal rotor (TLA-110 fixed-angle Beckamn Coulter) according to the manufacturer's instructions.
9. Run in the ultracentrifuge (Beckman Coulter Optima -Max XP Ultracentrifuge) at 67,000 rpm, or 187,281 x g, for 44 hours at 20°C with vacuum under maximum acceleration and brake in 5. With these conditions, beginning a run mid-after-noon on day 1 permits gradient processing on day 3.

NOTE: The banding efficiency of isotopically distinct nucleic acid species increases with time. Gradient steepness, on the other hand, once established by diffusion, is controlled purely by rotor geometry and does not change with time.

Fractions retrieving

10. After run, retrieve tubes taking care to avoid disturbing the gradients, fix tube to a clamp, check DNA bands illuminating with UV light transluminator placed stand up and mark with a pen the exact place of bands and delimitations of the fractions to recover 12 fractions.

NOTE: Complex and rich samples will not deliver a fine, nor even double bands, but a thick median band instead.

11. Carefully pierce the top with a 23-gauge 1" needle attached to the pump. Next, insert another needle in the bottom of the tube.

NOTE: Practice beforehand until tube piercing can be performed in a smooth and controlled manner to avoid bubbles from neither tube shoulder-down, or bottom-up.

12. Pre annotate 1.5 ml tubes in a rack and perform pumping, changing tubes when exaustion of each fraction is completed. Obtain fractions with automatic syringe pump NE-4000 High Pressure Programmable Single Syringe Pump (New Era Pump, USA) with bromophenol at 0.1% at rate of 430 µl.min-1 similarly to Dunford and Neufeld (2010). Discard the first and last fractions due to CsCl precipitation or bromophenol contamination.
13. Perform density measurement in refractometer (5-10µl with electric adhesive tape with a role of 5mm) or weight the fractions, pre-anotating tubes weight before collection. This confirms that gradients formed in all tubes and allows specific templates detected in downstream analyses to be assigned to the given densities. Correlate the refractometer BRIX to pre-made curves of CsCl/GB density.

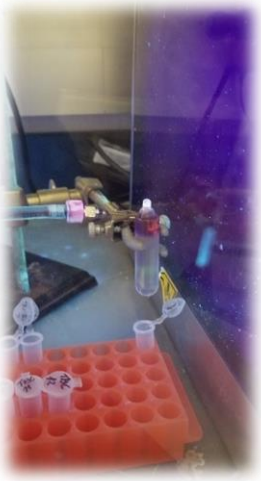
NOTE: Expect the density range to be approximately 1.690–1.760 g/ml, with a median density (for fraction 6 or 7) of approximately 1.725 g/ml. Heavy DNA fraction ought to be heavier than the median density, 1.725g/ml. If weighing fractions, ensure that sample tubes are at a uniform temperature to avoid a drift in the weight measurements.

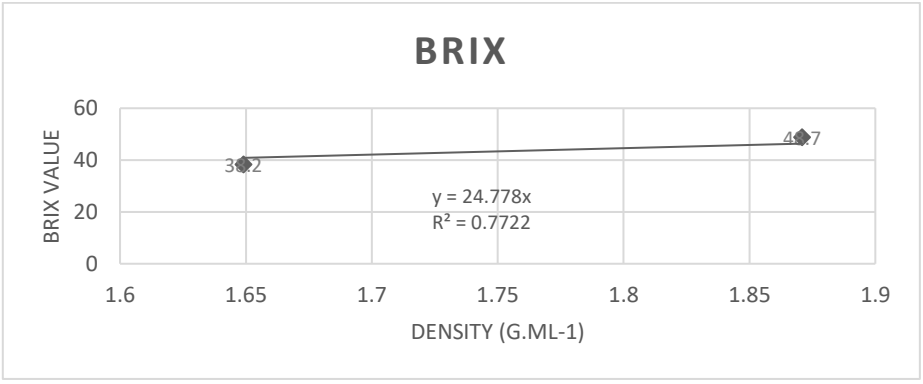
DNA precipitation

14. Precipitate DNA by adding 20µg of linear polyacrylamide, mix by inversion, add 2 volumes of PEG solution (Final volume of 100ml: 30ml of PEG for 30% PEG, 9.344g of NaCl for 1.6M NaCl), mix by inversion
15. Leave at room temperature for 2h. If necessary, precipitated DNA may be stored overnight at room temperature.
16. Ultracentrifuge for 13,000 x g for 30min at 15-20°C. Discard supernatant and see the pellet in the bottom (I don`t remember seeing pellets in all samples).
17. Wash the pellet with 500µl of fresh and cold 70% ethanol, do not disturb. Centrifuge at 13,000 x g for 10 min.
18. Discard ethanol and see the pellet more visible.
19. Dry pellet at room temperature for 15 min (tubes open face up to evaporate).
20. Add 50µl of autoclaved pure Tris-HCl buffer (utilize ultrapure filtered H20, or have a ordered buffer) to store at -20°C for downstream analysis.
21. Allow DNA to dissolve on ice and mix occasionally by gently tapping the tube. DNA is stable for several days at 4ºC. For longer term storage, -20 or -80ºC is recommended.
22. Run fluorometric or PCR-based quantification methods for the specific allocation of density-resolved nucleic acids to gradient fraction and select by the DNA amount required to your downstream analysis;
23. Select the desired fractions to perform the downstream techniques.
24. You can correlate with a G+C curve (pre-made with pure strands 12C-DNA and 13C-DNA melting curve method at termocycler Bio-Rad in a 10µl final volume: 5µl SSO Advanced Sybr mix, 10ng of DNA, water).

SP B8 Table – Standards parameters for SIP optimization

Microorganism	Incubation	Cod. ATCC	DNA (µg)	Volume (µl)	ng/µl	Separation for 5µg	CsCl stock	Desired conc. CsCl	Factor	Final vol. (µl)	GB µl	Vol CsCl input
Methylosinus sporium	12CH4	35069	67.2	600	112	44.6	1.85	1.8	1.52	4700	334.9	4342.8
Methylosinus sporium	13CH4	35069	30.88	400	77.2	64.8	1.85	1.8	1.52	4700	324.8	4342.8
Bacillus methanolicus	13CH4	51375	38.96	400	97.4	51.3	1.85	1.8	1.52	4700	331.5	4342.8





Solution	g.ml-1	BRIX
CsCl stock 1	1.8709	48.7
CsCl stock 2	1.649	38.2

SP B8 Figure – Labeled isotope assimilation and G+C % mol interference in bands separation. DNA GelGreen staining and bands checking in naked eye.