Title: Rainforest-to-pasture conversion stimulates soil methanogenesis across the Brazilian Amazon

Marie E. Kroeger[1]*, Laura K. Meredith[2,3], Kyle M. Meyer[4,10], Kevin D. Webster[5], Plinio Barbosa de Camargo[6], Leandro Fonseca de Souza[6], Siu Mui Tsai[6], Joost van Haren[3,7], Scott Saleska[8], Brendan J.M. Bohannan[4], Jorge L.M. Rodrigues[9], Klaus Nüsslein[1#]

1. Department of Microbiology, University of Massachusetts Amherst, Amherst, MA, USA
2. School of Natural Resources and the Environment, University of Arizona, Tucson, AZ, USA
3. Biosphere 2, University of Arizona, Tucson, AZ, USA
4. Institute of Ecology and Evolution, University of Oregon, Eugene, OR, USA
5. Planetary Science Institute, Tucson, AZ, USA
6. Center for Nuclear Energy in Agriculture, University of São Paulo, SP, Brazil
7. Honors College, University of Arizona, Tucson, AZ, USA
8. Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ, USA
9. Department of Land, Air and Water Resources, University of California Davis, CA, USA
10. Department of Integrative Biology, University of California – Berkeley, Berkeley, CA, USA

*Current address: Bioenergy and Biome Sciences, Los Alamos National Laboratory, Los Alamos, NM, USA

Author for correspondence: nusslein@microbio.umass.edu

Running Title: Tropical deforestation stimulates methanogenesis

ABSTRACT

The Amazon rainforest is a biodiversity hotspot and large terrestrial carbon sink that is threatened by agricultural conversion. Rainforest-to-pasture conversion leads to the release of a potent greenhouse gas by converting soil from a methane sink into a source. The biotic methane cycle is driven by microorganisms; therefore, this study focused on active methane-cycling microorganisms and their functions across land-use types. We collected intact soil cores from three land use types (primary rainforest, pasture, and secondary rainforest) of two geographically distinct areas of the Brazilian Amazon (Santarém, Pará and Ariquemes, Rondônia) and performed DNA stable-isotope probing coupled with metagenomics to identify the active methanotrophs and methanogens. At both locations, we observed a significant change in the composition of the isotope-labeled methane-cycling microbial community across land use types, specifically an increase in the abundance and diversity of active methanogens in pastures. We conclude that a significant increase in the abundance and activity of methanogens in pasture soils could explain the greater methane flux. Furthermore, we found that secondary rainforests recovered as methane sinks, indicating the potential for reforestation to offset greenhouse gas emissions in the tropics. These findings are critical for informing land management practices and global tropical rainforest conservation.

INTRODUCTION

Climate change, caused by the anthropogenic release of greenhouse gases[1], is affecting every ecosystem on Earth. Although the majority of greenhouse gases released to the atmosphere are associated with the industrial revolution and fossil fuel combustion, land use change is a significant contributor. Specifically, tropical deforestation in the last decade has released ~1 Pg C yr^{-1}, an equivalent to 10% of anthropogenic carbon dioxide emissions[1], and 78% of total

47 greenhouse gas emissions in Brazil are caused by land use change[2-3]. In addition to being
48 biodiversity hotspots of plants and animals, tropical rainforests are large terrestrial carbon sinks.
49 However, rainforest deforestation to create cattle pastures or agricultural fields releases large
50 amounts of stored carbon, converting former terrestrial carbon sinks into major carbon sources[3-4].
51 In the Amazon rainforest particularly, over 1 Mha of forest has been lost in 2017 alone[5]. The
52 conversion of primary rainforest (i.e. mature rainforest older than 150 years) to cattle pasture is a
53 main cause of deforestation in Brazil and not only changes plant diversity but also the
54 microorganisms that drive soil biogeochemical cycling[6].

55 The methane (CH_4) biogeochemical cycle is of interest because of its potency as a
56 greenhouse gas with 86-times the global warming potential of carbon dioxide over a 20-year
57 timescale[1]. Biotic CH_4 cycling is controlled by microorganisms, specifically methanogenic
58 archaea that produce CH_4, and methanotrophic bacteria that consume CH_4[7-8]. The balance
59 between these two functional groups determines whether the soil acts as a CH_4 source or sink.
60 Under anoxic conditions, soil methanogenic archaea generally metabolize fermentation products
61 such as acetate (acetoclastic methanogenesis) or reduce carbon dioxide with hydrogen
62 (hydrogenotrophic methanogenesis) to produce CH_4[9-11]. Methanotrophs are commonly aerobic
63 bacteria located at anoxic/oxic boundaries from either *Gammaproteobacteria*,
64 *Alphaproteobacteria*, or *Verrucomicrobia*, corresponding to Type I, II, and III methanotrophs,
65 respectively[12-13]. Previous research into the different growth conditions of Type I versus Type II
66 methanotrophs found that Type II methanotrophs generally dominate high CH_4, low oxygen
67 environments along with nitrogen- and copper-limiting conditions[14-16]. However, Type II
68 methanotrophs have also been found in soils with low CH_4 concentrations[17-19] likely due to two

69 isoenzymes of the particulate methane monooxygenase that have different affinities for CH_4[20]
70 making them more versatile metabolically.

71 Researchers have focused on the impact of rainforest-to-pasture conversion on CH_4
72 cycling for decades[21-23]. Measurements of in-field gas flux generally show soil CH_4 consumption
73 across seasons in mature rainforest, while pasture soils emit CH_4[24-25]. Over the last decade,
74 further research into how tropical land use change influences CH_4 cycling microorganisms found
75 varied results. One study observed that the functional biomarkers for methanotrophy (*pmoA* and
76 *mmoX*) decreased in cattle pastures with no change to the methanogenesis biomarker (*mcrA*),
77 while another study observed a decrease in *pmoA* abundance from Type II methanotrophs and an
78 increase in *mcrA* in cattle pastures[6,19]. These previous studies investigated how land use change
79 in the Brazilian Amazon impacts the genomic potential of the soil methane-cycling microbial
80 community, but no study has directly targeted the active community.

81 Metatranscriptomics, metaproteomics, and stable isotope probing are increasingly
82 common techniques to target the active microorganisms in an environmental sample[26-29].
83 Previous research by our group using metatranscriptomics and metaproteomics was unable to
84 determine if soil CH_4 cycling genes and proteins were differentially expressed between land use
85 types due to low counts (unpublished data). Therefore, for this study we used stable isotope
86 probing to determine the active fraction of the soil microbial community cycling CH_4, referred to
87 henceforth as members of the active community. Stable isotope probing is commonly applied to
88 study CH_4 cycling in soil given the specific nature of the substrate and its relevance to climate
89 change[30-32]. This technique uses the less abundant isotope of an atom, such as ^{13}C-carbon, to
90 label the microorganisms capable of consuming the ^{13}C and, via their anabolic metabolism,
91 incorporating it into their DNA, which then can be separated by ultracentrifugation from the

community DNA. Subsequently, next generation sequencing enables the identification of active community members and provides insight into their functional potential.

The central goal of this study was to determine how the members of the active CH_4-cycling microbial community, their functions, and CH_4-related metabolic pathways changed across land use types (primary rainforest, cattle pasture, and secondary rainforest) and geographically distinct regions of the Brazilian Amazon. We hypothesized that the cause of increased soil methane production in cattle pastures was caused by a decrease in active methanotrophy. To test this hypothesis, we sampled sites at the most active deforestation frontiers in northeastern and southwestern Amazonia in the states of Pará (Tapajós National Forest) and Rondônia (Fazenda Nova Vida), respectively. To determine the community composition and functions of the active methane-cycling microorganisms, we coupled stable isotope probing (DNA-SIP) with metagenomics, using either ^{13}C-labeled methane (CH_4), carbon dioxide (CO_2), or sodium acetate (NaAOc) as a substrate. Overall, we observed significant shifts in the active microbial community compositions and their methane-cycling functional genes between land-use types, geographic location, and substrates. Specifically, the abundance and diversity of active methanogens increased with conversion to pasture. Ultimately, this significant increase in active methanogens in pasture soils significantly correlated with the in-field methane gas flux. Therefore, we conclude that an increased abundance and diversity of active methanogens is causing the overall net positive methane flux in cattle pastures.

METHODS

Site description and sampling: At each sampling site, we first took soil gas flux measurements as detailed below, followed by collecting 5 to 6 soil cores. Seventy-two soil cores (5 cm diameter ×

10 cm depth) were collected from the Tapajós National Forest and its adjacent areas in the State of Pará in June 2016 for DNA-SIP. Another 72 soil cores were collected from Fazenda Nova Vida and its adjacent areas in the State of Rondônia in April 2017 for DNA-SIP (GPS coordinates in Supplemental Methods). For each location, 18 soil cores were collected from each land use type, two primary rainforests (PF1 or PF2), one cattle pasture (P), and one secondary rainforest (SF). Soil cores were collected along a transect ranging from 100 to 200 meters with five equidistant sampling points. Three adjacent soil cores were taken from each sampling point with a fourth soil core taken from sampling points 2, 3, and 4 along the transect (Supplemental Figure 1). The cores were stored at 4°C until incubation with stable isotopes in the laboratory (up to 2 weeks later due to travel). The two additional soil cores collected at each sampling point were immediately homogenized, and then divided into two volumes for total prokaryotic community analyses from extracted DNA and for soil physical-chemical characterization.

Stable isotope probing: During incubation with stable isotopes, the intact soil cores (~200 g depending on soil density) were stored in gas-tight glass jars in the dark. For each combination of location (Pará or Rondônia) and transects across land use type (two primary rainforests, one pasture, one secondary rainforest), five soil cores were incubated with ^{13}C-substrate and one additional core with ^{12}C-substrate as the control. For each sampling site this resulted in a total of six cores for each of the three substrates, or 72 cores total for each location tested. Soils were incubated at 25°C for ~7 months due to the low gas exchange at the surface top of the undisturbed soil column (20 cm^2) compared to homogenized soil (20-32x lower rates; unpublished data). Either 25 mL of ^{13}C-carbon dioxide (3% headspace concentration), 1 mL of ^{13}C-sodium acetate (1 mM final concentration, added to the top of each soil core), or 25 mL of

^{13}C-methane (3% headspace concentration) were added every two weeks. Equal volumes (1 mL) of sterile water were added to carbon dioxide and methane incubations. Air was added once a week to the ^{13}C-methane incubations to ensure an oxic headspace. Pressure was released periodically prior to substrate injection from all jars. The duration of incubation was determined by monitoring the methane gas flux and attempting to ensure 20 mM of substrate was incorporated, following published recommendations to apply 5-500 µM ^{13}C per g of soil[33]. Our target was to incorporate ~100 µM ^{13}C per g of soil, rendering shorter incubation times insufficient. Methane production or consumption was monitored throughout the incubation experiment by gas chromatography (Shimadzu GC-17A, Kyoto, Japan). After incubation, each soil core was sectioned longitudinally into five 2-cm tall segments (numbered 1-5 from top to bottom) and stored frozen at -20°C until DNA extraction.

DNA extraction, quantification, and sample processing: DNA was extracted from 0.25 g of soil from all segments from two of the five ^{13}C soil cores using the DNeasy PowerSoil DNA Extraction kit (Qiagen, Hilden, Germany) to determine the segment with the highest abundance of methanogens or methanotrophs based on the respective functional marker genes using qPCR as described below. Upon identifying the segment with the highest genomic abundance of methanogens or methanotrophs, DNA was extracted from 4 g of soil from the identified segment of three ^{13}C soil cores and from the ^{12}C-control for each substrate/sample site combination using the DNeasy PowerMax Soil Kit (Qiagen). DNA was quantified fluorometrically using the Qubit dsDNA Broad-Range assay (Invitrogen, Carlsbad, CA). A total of 5 µg of DNA was subjected to ultracentrifugation according to a previously described protocol[33], followed by fractionation of the density gradient into 12 fractions of equal volume. The continuity of the density gradient was

161 confirmed with a refractometer. DNA was precipitated following the published protocol[33] except
162 for the addition of 20 µg linear acrylamide (Invitrogen) instead of glycogen and each fraction
163 was quantified using fluorometry via the Qubit dsDNA High-Sensitivity assay (Invitrogen). To
164 identify the fractions with ^{13}C-labeled DNA, we quantified the abundance of methanogens or
165 methanotrophs in each fraction using qPCR of the respective functional gene marker for a subset
166 of samples compared to their respective ^{12}C-controls (details in Supplementary Methods). We
167 pooled the ^{12}C (~1-5) and ^{13}C (~6-12) fractions, respectively. Since the GC content of microbial
168 DNA can influence DNA density, we sequenced both the light and heavy DNA fractions from
169 our ^{12}C-controls for a total of 12 ^{12}C-light DNA, 12 ^{12}C-heavy DNA, and 36 ^{13}C-heavy DNA
170 samples per location.
171
172 Quantitative PCR: The particulate methane monooxygenase alpha subunit gene (*pmoA*) was
173 amplified using the primer pair A189f/mb661r[34-35], and the gene for the methyl coenzyme M
174 reductase alpha subunit (*mcrA*) was amplified using the primer pair mlas/mcra-rev[36]. Standard
175 reaction mixtures and thermocycler conditions are specified in Supplemental Methods.
176
177 Sequencing: All DNA library preparation and sequencing were performed at the University of
178 Oregon Genomics and Cell Characterization Core Facility (Eugene, OR) (Supplementary
179 Methods). Briefly, the three genes of interest (16S rRNA, *pmoA*, and *mcrA*) were amplified using
180 custom dual-indexed PCR primers designed by the core facility. For each location, paired-end
181 300 bp amplicon sequencing of the pooled heavy fractions for three ^{13}C-samples per sample
182 site/substrate combination and the pooled heavy and light fractions for all ^{12}C-controls was
183 completed on an Illumina MiSeq sequencer (Illumina, San Diego, CA). For metagenomes,

sequencing of the heavy fraction of two ^{13}C-samples per sampling site/substrate combination and all ^{12}C-controls was performed on an Illumina HiSeq4000 across two flow lanes for each location. All sequences were demultiplexed at the core facility.

Soil physical-chemical analysis: Homogenized soil samples stored at 4ºC were processed as described in detail previously[37].

Methane Gas Flux Measurements: In-field soil CH_4 fluxes were measured using a field-deployable Fourier transform infrared spectrometer (Gasmet, DX 4015, Vantaa, Finland) sampling a recirculating flow-through soil flux chamber placed on soil collars (aluminum, inner area of 284 cm^2) that were installed in soil (5 cm deep) at least 20 minutes beforehand. Fluxes were calculated from the rate of headspace CH_4 accumulation or depletion in a 30-minute period. We fit linear models to concentration vs. time trends, limiting the extend of the time series used to the initial linear decline in cases when nonlinear behavior was observed at late stages in the measurement[38-39].

Data and statistical analysis: Amplicon sequences were processed and analyzed using the DADA2 pipeline in QIIME2[40-41]. Metagenome sequences were processed and annotated using MG-RAST[42]. GenBank and SEED Subsystem were used for the organismal and functional annotations, respectively. The SEED Subsystem annotation “Methanogenesis strays” is described as “several additional genes and clusters from methanogens.” The influence of homogeneous dispersion within each sample and heterogeneous dispersion between samples was assessed using the Permdisp and Adonis functions, respectively, from the ‘vegan’ package in R

on dissimilarity matrices made from the annotation tables[43-44]. To specifically target the active microbial community, the metagenomic annotations were rarefied (vegan:rrarefy) and counts were normalized to the ^{12}C-control for each substrate (see Supplementary Methods). After rarefication, the dissimilarity between land use types within each substrate were analyzed (vegan:Adonis). STAMP v2.1.3 was then used to identify active microorganisms and functions by comparing each individual ^{13}C-sample to their respective ^{12}C-control using Fisher's exact test[45]. All figures were made in R v3.5.1 using ggplot2[43,46]. Soil physical-chemical data were analyzed using ANOVA with a Tukey-Kramer post-hoc test. Correlation analyses between the in-field methane flux and the abundance of active methane-cycling taxa or functional annotations were completed using a Pearson correlation (cor.test)[43].

Active Fraction Analysis: In this study, "active" means that the cells were actively growing (anabolically incorporating ^{13}C) and not just metabolically active (catabolic turnover of ^{13}C substrate independent of growing). The incubations with ^{13}C-labeled substrates determine both actively growing and metabolically active community members, and we used our ^{12}C incubation controls to correct for the metabolically active part. Therefore, an annotation was deemed active, if it was significantly higher ($p < 0.05$) in the ^{13}C-sample compared to the ^{12}C-control. Samples were normalized to their respective ^{12}C-control with features that had less abundance in the ^{13}C- than ^{12}C-samples being marked as 0 counts. Samples from the same substrate were compared between land use types in STAMP using the multigroup stats function (ANOVA with Tukey-Kramer Post-hoc test). All significantly different annotations were checked again to see if they were active in the samples.

Data Accessibility: Metagenomes are available publicly on MG-RAST under the following project accession numbers: mgp88468 and mgp86794. All raw amplicon sequence files have been deposited on figshare under the following DOI: 10.6084/m9.figshare.10565552, 10.6084/m9.figshare.10565690, 10.6084/m9.figshare.10565870, 10.6084/m9.figshare.10565897, 10.6084/m9.figshare.10565957, 10.6084/m9.figshare.10565801.

RESULTS AND DISCUSSION

Active Methane-Cycling Community Changes with Land-Use

To understand the active methane-cycling microbial community composition and abundance, we analyzed sequences of both PCR-amplified marker genes (16S rRNA, *mcrA*, and *pmoA*) and metagenomes. The amplification-based approach makes our data comparable to many microbial studies that use these biomarkers, but this method comes with the potential issues of primer bias allowing for missed taxonomic groups, lower phylogenetic resolution, and no additional information on ecosystem processes[47-48]. Therefore, after confirming that enough label was present in the target sample using amplicon-based sequencing, we used metagenomics to gain a deeper understanding of the ^{13}C-labeled methane-cycling community and its supporting members[49-51]. The composition of the total soil microbial community, based on 16S rRNA, significantly differed between geographic locations (Rondônia vs Pará; p=1e-03 r^2=0.118), individual land-use types (primary rainforest(PF), pasture(P), secondary rainforest(SF), p=1e-03 r^2=0.08), and added substrates (CH_4, CO_2, NaAOc, p=1e-03 r^2=0.08) (Figure 1). When we specifically targeted the active community, we found that only samples incubated with CO_2

253 significantly differed between locations (Pará CO_2 p=3e-03; Rondônia CO_2 p=1.8e-02;
254 Supplemental Figures 2-3). It was unsurprising that location is the main driver to differentiate the
255 total microbial community since Rondônia and Pará are separated by ~1500 km. Also, abiotic
256 factors such as seasonal differences and/or soil physical-chemical properties could be driving
257 these locational differences[52-54]. The significant differences in CO_2 incubated samples may be
258 due to the similarity of the overall active community across samples, while the methane-
259 consuming or -producing community makes up only a small fraction of that community and the
260 signal is lost when we look at the community composition broadly.

261 When we investigated the richness of active methane-cycling communities, we found that
262 pasture samples had the highest active methanogen richness in metagenomes from both locations
263 and regardless of substrate (CO_2 or NaAOc); however, it was only significant in Rondônia
264 NaAOc samples (P vs PF p=9.6e-03, P vs SF p=7.9e-03; Figure 2, Supplemental Table 1). All
265 active methanogens that significantly changed abundance between land-use types were
266 associated with pasture soils in both locations (Table 1). Specifically, *Methanosarcina* spp.
267 dominated the active methanogens for most samples in both locations regardless of substrate
268 (Figure 3, Supplemental Tables 2-3; 5-6). These archaeal species are known to have multiple
269 methanogenesis pathways making them capable of utilizing both $^{13}CO_2$ and ^{13}NaAOc, likely
270 explaining their dominance in both locations and substrates[55-58].

271 We observed a significantly higher abundance of total active methanogens in Rondônia
272 pasture soils compared to both primary and secondary rainforest samples in ^{13}NaAOc samples
273 (p=1e-03, p=3.8e-02, respectively) and compared to secondary rainforest in $^{13}CO_2$ samples
274 (p=9e-03). There was no significant difference in the abundance of total active methanogens
275 between land-use types for either methanogenic substrate in Pará, but many taxa did significantly

change abundance (Table 1). Previous research studies showed mixed findings on methanogen communities response to tropical land-use change ranging from no change to increased *mcrA* gene abundance in pastures[59,6,19]. By targeting the active community, we directly show that pasture soils have a higher richness of active methanogens and specific methanogenic taxa significantly increase abundance. This increase in methanogen abundance and richness is likely due to the increased soil carbon cycling occurring in pasture soils[60-61].

Previous research into methanotrophy across Amazonian land uses found methanotroph abundance to be lower in pasture relative to primary forest soils[6,59,19]. Based on these studies, we hypothesized that pasture soils would have the lowest abundance and richness of active methanotrophs. Unlike the active methanogen community, we did not find a consistent association between active methanotroph richness and land-use types across locations. The highest richness was either found in pasture or secondary rainforest for Pará and Rondônia, respectively, but it was not significant (Figure 2). The total active methanotroph abundance did not significantly change between land-use types. In both locations and all land-use types, Type II methanotrophs (Alphaproteobacteria) dominated the active methanotroph community (Figure 4, Supplemental Tables 4, 7). Only one primary rainforest sample from Pará was dominated by Type I methanotrophs and only one Type III methanotroph was found to be active, *Methylacidiphilum*, but remained rare (Supplemental Tables 4, 7).

Although, the total abundance of active methanotrophs did not significantly change between land-use types, the abundance of specific methanotrophs changed in Pará and Rondônia associating with pasture and secondary rainforest, respectively (Table 1). This was surprising and not what we hypothesized based on previous studies[6,19,59]. Several factors should be considered to address this discrepancy. First, our study targeted the microorganisms actively consuming CH_4

299 rather than looking at the total microbial community. Studies of the total microbial community
300 can be influenced by the potential presence of extracellular DNA, which may affect estimates of
301 abundance and diversity[62-64]. Additionally, we incubated our samples at CH_4 concentrations
302 above those in the atmosphere due to the inability to label the community at low concentrations.
303 Although necessary for the technique, this could influence the composition and activity of the
304 CH_4-consuming community. Furthermore, there is a possibility that we incorrectly assumed
305 primary rainforests would have the highest methanotroph richness and abundance since these
306 forests are known to be methane sinks[21-22]. Here, our findings enable the next question to test
307 what environmental variables govern the active CH_4-consuming community between land-use
308 types in the Brazilian Amazon.
309
310 Dominant Active Methanogenesis Pathways Differed Between Locations
311 We next asked which CH_4-related metabolic pathways were active across land-use types
312 and how they changed in response to deforestation. We observed active methanotrophy based on
313 the abundance of the genes for particulate methane monooxygenase (*pmmo*) and soluble methane
314 monooxygenase (*smmo*) in all ^{13}C-labeled samples and in both locations (Figure 5; Supplemental
315 Table 8). The *pmmo* genes were abundant and active in 94% of samples while *smmo* was active
316 in most secondary rainforest samples and Rondônia-PF1 (Figure 5; Supplemental Table 8). The
317 secondary rainforest and pasture samples at Rondônia significantly increased in *pmmo*
318 abundance (p=6e-03; p=3e-02, respectively) compared to primary rainforest (Supplemental
319 Table 8). We found no significant difference in the abundance of any active methanotrophy-
320 related genes across land-use types in Pará (Figure 5). Overall, Rondônia had a significantly
321 higher relative abundance of *pmmo* to total methanotrophy annotations compared to Pará

(Supplemental Table 8) (p=1e-04). Soil physical-chemical properties are known to influence the activity of these different methane monooxygenases[65]. Copper is a key component regulating the activity and abundance of these methane monooxygenases having a positive relationship with *pmmo* abundance [66-67,12]. We observed a significantly higher concentration of copper (9x) in Rondônia compared to Pará (p=7.42E-06) which may explain the increased abundance of active *pmmo* genes.

Regardless of location, the abundance of active methanogenesis genes dominated in pasture compared to other land-use types. Interestingly, in Pará we observed these significant increases in the $^{13}CO_2$ incubation, while in Rondônia the ^{13}NaAOc incubation accounted for the increased abundance (Figure 6; Supplemental Table 9). The Pará ^{13}NaAOc incubation presented some significant changes in methanogenesis-related genes including Coenzyme F420 synthesis (p=8e-05), methanopterin biosynthesis2 (p=2e-03), and methanogenesis strays (p=1e-03). The two pasture samples in Pará ^{13}NaAOc incubations performed very differently. Although pastures are considered to be more biotically homogeneous[68], these two samples differed strongly with one sample having about 8.5x more active methanogens (Supplemental Table 4). When the relative abundance of active methanogenesis genes to total annotations was investigated, we identified a significant difference between land-use types in the Pará $^{13}CO_2$ incubations (PF v P p=1e-03, SF v P p=1.8e-02) and in the Rondônia ^{13}NaAOc incubations (PF v P p=7e-02, SF v P p=1.8e-02) (Supplemental Table 10). In addition to methanogenesis genes changing between land-use types, we observed an increase in carbon cycling activity in Pará pasture soils incubated with $^{13}CO_2$ (Glycolysis and gluconeogenesis p=1e-03, Pentose phosphate pathway p=2e-03, Entner-Doudoroff pathway p=3e-02). Overall, we found that active methanogenesis was driven by methanogens using the hydrogenotrophic pathway in Pará and the acetoclastic pathway in

Rondônia (Supplemental Table 9). This shift in the dominant methanogenesis pathway between locations may be due to differences in the physical-chemical soil parameters or a result of the types of fermentation leading to either more acetate or hydrogen production. Interestingly, the active methanogen community was dominated by *Methanosarcina* spp. in both locations. Members of the genus *Methanosarcina* are known to require three different types of hydrogenases for the reduction of CO_2 to CH_4 with electrons derived from H_2[58]. The significantly increased activity of multiple types of hydrogenases (Energy conserving hydrogenase ferrodixin Ech p=1.6E-08; membrane bound hydrogenases p=4.6e-02; Archaeal membrane bound hydrogenases p=0.048; Coenzyme F420 hydrogenase p=5e-02) in soils from Pará compared to soils from Rondônia indicates a selection for the hydrogenotrophic pathway. This selection is supported by the increased availability of trace metals (iron) in soils from Pará which are needed by methanogenic hydrogenases[58].

Land-Use Change Alters Key Redox-Cycling Active Taxa

In the context of highly complex soil microbial communities, methanogens need other microorganisms to produce the substrates necessary for this redox reaction to occur. Methanogenesis is one of the least thermodynamically favorable anaerobic reactions; therefore, other redox reactions must transpire prior to methanogenesis[69]. Although we were targeting active methane-cycling microorganisms in this study, the methanogenic substrates used, $^{13}CO_2$ and ^{13}NaAOc, are not exclusively used by methanogens. Therefore, we investigated which coexisting microorganisms were actively consuming these substrates and thereby interacting with methanogens. Many non-methanogenic but active microbial taxa changed significantly in abundance between land use types in both geographic locations. In the Pará $^{13}CO_2$ SIP

368 incubations, we observed a significant increase of active *Syntrophus aciditrophicus* (p=1E-06) in
369 pasture along with many known sulfate-reducing bacteria (Supplemental Table 10. *Syntrophus*
370 *aciditrophicus* is known to promote the growth of *Methanospirillum* spp., which accounted for
371 2.76% of the active methanogen community in Pará $^{13}CO_2$ pasture samples[70]. In the secondary
372 rainforest, we found a significantly higher abundance of various active nitrifying and sulfur-
373 oxidizing bacteria, such as *Nitrobacter* and *Thioalkalivibrio*. Many of these microbial groups are
374 known to utilize CO_2 and have thermodynamically preferred redox potentials[71-76]. Rondônia
375 pastures increased in active ammonia-oxidizing microorganisms including *Nitrosococcus* and
376 *Geobacillus* species ($^{13}CO_2$ samples; Supplemental Table 10). One potential cause of increased
377 *Geobacillus* species is the slash and burn process used to create pastures that deposits
378 hydrocarbons in the soil, which these microorganisms are known to use[77-79].
379 The abundance of active *Geobacillus, Clostridium,* and *Sulfolobus* spp. increased in Pará
380 ^{13}NaAOc incubated pasture soils (Supplemental Table 11). Some *Geobacillus* and *Clostridium*
381 spp. are known to utilize acetate, which may explain their increased abundance in the ^{13}C-labeled
382 community[80-81]. The denitrifying bacterium *Hyphomicrobium denitrificans* was active and
383 significantly increased abundance in Pará primary rainforest samples along with the genes
384 associated with denitrification (p=0.02). In the ^{13}NaAOc Rondônia soils, we observed a
385 significant increase in both sulfate-reducing and sulfur-oxidizing microorganisms along with
386 nitrate-reducers in secondary rainforest with many competitors for acetate as a carbon source[82,74]
387 (Supplemental Table 11). It is well documented that before methanogenesis is able to occur
388 nitrate and sulfate must be depleted as electron acceptors[69]. The increased abundance of active
389 sulfate and nitrate reducers in the Rondônia secondary rainforest and overall lack of active
390 methanogenesis indicates that these more favorable electron acceptors were still available in the

soil during incubation with ^{13}NaAOc inhibiting methanogenesis through substrate competition[83-84].

Soil Physical-Chemical Parameters Increase Potential Methane Production

Land-use change is one of the strongest drivers to alter soil ecosystems. Parallel changes to the soil physical-chemical parameters, physical structure, and aboveground vegetation may provide additional support for increased methanogenesis in pasture soils. Specifically, the compaction caused by cattle grazing creates more anoxic microsites providing more opportunity for methanogenesis to occur[85]. The comparison of soil physical-chemical parameters between the geographic locations presented several significant differences (Supplemental Tables 12-13). Of note were increased concentrations of sulfur (p=2.95E-15) and copper (p=7.42E-06) along with higher pH (p=1.35E-07) in Rondônia compared to Pará, and total soil acidity (p=9.29E-11) and total nitrogen (p=2.31E-06) were significantly higher in Pará soils. For both locations, the soil pH was significantly higher in pasture compared to primary rainforests. Soil bulk density was found to be highest in pasture from both locations (Supplemental Figure 5). The increased pH in pasture soils likely helps support methanogenesis since optimum process activity is at near neutral pH and quickly decreases as the pH becomes more acidic[86]. Another contributing factor to the increased methanogenesis in pasture soils is due to *Urochloa brizantha* excreting large amounts of carbon as root exudates into the soil[87]. With increased carbon availability in pasture soils, there is overall increased soil microbial activity[88]. All of these changes to the soil in pastures could contribute to the increased methanogenic activity observed in our SIP study.

413 Relating Functional Activity from In-Field Methane Gas Flux to Functional Potential from
414 Metagenomics

415 Research into modeling biological activity to help predict future climate scenarios has
416 continued to grow over the past few decades[89-91]. Since this study focused on the active methane-
417 cycling microbial community across land use types, we performed a correlation between the
418 abundance of the active community or of functional biomarkers with the gas fluxes measured in
419 our sites. Tentatively, we found the strongest correlation between total active methanogen
420 abundance and field methane flux (p=6e-04, cor=0.573) (Figure 7). However, these findings will
421 need to be further investigated across more sampling points to confirm their validity broadly.
422 Overall, only Rondônia showed significant differences in field methane flux across land-use
423 types, with pasture having significantly higher methane emissions (PF vs P p=1e-04, SF vs P
424 p=2e-03, SF vs PF p=9.9e-01; Supplemental Figure 6). The correlation observed between active
425 methanogen abundance and methane flux in the field provides further support that an increase in
426 methanogenesis activity is driving the change in methane flux between land-use types.
427
428 Minimal Enrichment of Methane-Cyclers After Incubation

429 In SIP studies specifically, we target the active microbial groups involved in a
430 biogeochemical process; however, the majority of soil SIP studies use homogenized soil where
431 soil columns get sieved[92-97]. It is clear from the literature that soil structure is an important aspect
432 of microbial activity and carbon cycling; therefore, if possible, microbial activity should be
433 studied under environmentally-relevant conditions. This study shows the feasibility of keeping
434 soil and its assembled microbial communities more similar to the natural environment by
435 incubating soil cores in-tact. We observed that even after seven months of incubation, the

436 abundance of functional marker genes (*pmoA* and *mcrA*) did not become greatly enriched.
437 Compared to field soils at the time of sampling, there was a small but significant increase of
438 *mcrA* gene copies in Pará $^{13}CO_2$ SIP soil (p=4.6e-03), but no significant difference in soils from
439 Para amended with ^{13}NaAOc (p=7.2e-01) (Supplemental Figure 7). Overall, there was no
440 significant difference between the Rondônia SIP and field soils' *mcrA* gene abundance. The only
441 significant difference found was between $^{13}CO_2$-incubated primary forest and pasture samples
442 (p=4.6e-02). Interestingly, *pmoA* gene abundance decreased significantly in SIP incubated soils
443 from Pará (p=1E-05) and Rondônia (p=4E-07, Supplemental Figure 8). One possible explanation
444 for the decreased *pmoA* gene abundance between SIP incubated and field soil is that during the
445 incubation the methanotrophic community was potentially altered. Our comparative analysis of
446 the metagenome data supports this possibility as a 7.7-fold and 4.0-fold increase from Rondônia
447 and Pará, respectively, were observed in obligate methanotroph abundance between ^{13}C vs ^{12}C
448 heavy fraction samples. Since primer bias is a common problem, as previously discussed, the
449 change in community could alter the compatibility of the primer to the *pmoA* sequences of the
450 changed community; thus, potentially presenting a lower *pmoA* abundance in the SIP than field
451 soils.
452
453 CONCLUSIONS
454 Land use change from rainforest to pasture stimulates the soil methanogenic community
455 in the Brazilian Amazon. Using undisturbed soil columns for SIP incubations, we were able to
456 ascertain that methanogen abundance and activity is significantly higher compared to both
457 primary and secondary rainforests which could drive methane emissions from the soil of
458 Brazilian cattle pastures. Future studies should focus on identifying what specific environmental

459 factors are responsible for increased methanogenesis in pasture soils (i.e. pH, vegetation,
460 compaction, carbon or trace element availability, etc.), so that land management can better
461 mitigate CH_4 emissions. Another important finding was that secondary rainforests in both
462 locations have recovered as CH_4 sinks with an active methanotrophic community. Through large
463 forest restoration efforts occurring in the tropics, there is potential to see these forests recover
464 with enough time to overcome excess CH_4 production. It is currently unknown how long
465 secondary rainforests take to recover as a CH_4 sink, and how widespread this recovery is
466 geographically. Adoption of best management practices in pastures can compensate for a small
467 fraction of the impact of deforestation on net emission of greenhouse gases and the loss of
468 carbon from Amazonia. With the currently accelerating expansion of land-use change in
469 Amazonia understanding which players might assist mitigation of concomitant greenhouse gas
470 production is increasingly important for all agricultural management.
471
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ETHICS DECLARATIONS. Conflict of interest. The authors declare that they have no conflict of interest.

FIGURE AND TABLE LEGENDS

Figure 1. Composition of the total soil microbial community based on 16S rRNA. Non-metric dimensional scaling plot of rarefied 16S rRNA SILVA annotations at genus-level from all samples. The dotted lines outline samples from each geographic location (Pará and Rondônia). The colored lines connect samples from the same land-use type to the centroid (primary rainforest = green, pasture = orange, and secondary rainforest = blue). The circles represent the standard error dispersion of samples for each substrate (NaAOc=sodium acetate, CO_2=carbon dioxide, CH_4=methane). The p-values and r^2 values for each variable (Location, Substrate, Land Use) are derived from the Adonis function in the vegan package.

Figure 2. Richness of active methane cycling taxa (methanotroph or methanogen) from two geographic locations (Pará or Rondônia), three land-use types (primary rainforest = green,

pasture = orange, and secondary rainforest = blue) incubated with one of three substrates (methane, carbon dioxide, and sodium acetate).

Figure 3. Abundance of active methanogen genera in the ^{13}C metagenome samples. (a) The abundance of active methanogens in soils from Pará incubated with $^{13}CO_2$, (b) the abundance of active methanogens in soils from Rondônia incubated with $^{13}CO_2$, (c) the abundance of active methanogens in soils from Pará incubated with ^{13}NaAOc, (d) the abundance of active methanogens in soils from Rondônia incubated with ^{13}NaAOc. Samples on the x-axis are colored by land use type (primary rainforest (PF) = green, pasture (P) = orange, and secondary rainforest (SF) = blue).

Figure 4. Relative Abundance of methanotroph types I, II, and III across both geographic locations (Pará and Rondônia) and land use types (primary rainforest, pasture, and secondary rainforest). Type I = grey, Type II = blue, Type III = pink.

Figure 5. Heatmap visualizing the average relative abundance of active genes involved in methanotrophy pathways. The scale is from lowest relative abundance (blue) to highest relative abundance (red) of the genes and is normalized to each gene (i.e. column). The metagenome samples are on the y-axis are colored by land use type (primary rainforest (PF) = green, pasture (P) = orange, and secondary rainforest (SF) = blue) and have the location (Rondônia or Pará) in the label. The genes involved in methanotrophy are on the x-axis. The dendrogram shows the Euclidean distance between samples.

Figure 6. Dot chart illustrating the relative abundance of active methanogenesis genes. The samples are grouped by location in descending order and include both methanogenic substrates (CO_2 and NaAOc). The colors correspond to the land use type (green = primary rainforest,

526 orange = pasture, blue = secondary rainforest). The shapes of the dots correspond to substrate
527 (circle=CO_2, triangle = NaAOc). Active methanogenesis genes includes SEED subsystem
528 annotations as "Methanogenesis," "Methanogenesis from methylated compounds," and
529 "Methanogenesis strays," Methanogenesis strays are "additional genes and clusters from
530 methanogens". The specific genes associated with "Methanogenesis strays" can be found by
531 searching for the subsystem on the SEED viewer
532 *(http://rast.theseed.org/FIG/seedviewer.cgi?page=SubsystemSelect).*

533 Figure 7. The field gas flux for methane (CH_4 mg-C/m^2/hr) correlated to active methanogen
534 abundance in $^{13}CO_2$ and ^{13}NaAOc SIP incubations. Primary rainforest = green, pasture = red, and
535 secondary rainforest = blue. Samples from Rondônia are circles and samples from Pará are
536 triangles.

537 Table 1. Methanogens and methanotrophs that are both active and significantly different between
538 land use types (primary rainforest, pasture, secondary rainforest). The term 'Land use
539 association' signifies which land use is associated with a significantly higher abundance of the
540 taxon. Mean relative abundance (%) depicts the average relative percent of each taxon in each
541 land use.
542

543 **Supplemental Table and Figure Legends**

544 Suppl. Figure 1. Visualization of the sampling scheme for each geographic location and land-use
545 type (two primary rainforests, one cattle pasture, and one secondary rainforest). Soil cores (18 x)
546 were collected along a transect ranging from 100 to 200 m with five equidistant sampling points.
547 Three adjacent soil cores were taken from each sampling point with a fourth soil core taken from
548 sampling points 2, 3, and 4 along the transect.

549

550 Suppl. Figure 2. Non-metric multidimensional scaling indicating how similar the active soil
551 microbial communities are for the substrates (a) $^{13}CH_4$, (b) $^{13}CO_2$, (c) ^{13}NaAOc from Pará. The
552 plot was created using a Bray–Curtis dissimilarity matrix among all samples. Primary rainforest
553 = green, pasture = red, and secondary rainforest = blue.

554

555 Suppl. Figure 3. Non-metric multidimensional scaling indicating how similar the active soil
556 microbial communities are for the substrates (a) $^{13}CH_4$, (b) $^{13}CO_2$, (c) ^{13}NaAOc from Rondônia.
557 The plot was created using a Bray–Curtis dissimilarity matrix among all samples. Primary
558 rainforest = green, pasture = red, and secondary rainforest = blue.

559

560 Suppl. Figure 4. Abundance of active methanotroph genera in samples from (a) Pará or (b)
561 Rondônia incubated with $^{13}CH_4$. The active metagenomes are colored based on land-use type
562 with primary rainforest = green, pasture = orange, and secondary rainforest = blue.

563

564 Suppl. Figure 5. The soil bulk density (g/cm^3) for each land use (primary rainforest, pasture,
565 secondary rainforest) and geographic location (Rondônia and Pará).

566

567 Suppl. Figure 6. The field gas flux for methane (CH_4 mg-C/m^2/hr) correlated significantly with
568 active methanogen abundance in SIP incubations for either substrate $^{13}CO_2$ and ^{13}NaAOc.
569 Primary rainforest = green, pasture = red, and secondary rainforest = blue. Samples from
570 Rondônia are circles and samples from Pará are triangles.

571

572 Suppl. Figure 7. The log *mcrA* gene copies per ng of DNA for soils from three land-use types
573 (primary rainforest, pasture, and secondary rainforest) in Rondônia and Pará incubated with
574 $^{13}CO_2$, ^{13}NaAOc, or no incubation (field soil). $^{13}CO_2$ samples = red, ^{13}NaAOc samples = blue,
575 unaltered samples = green.
576
577 Suppl. Figure 8. The log *pmoA* gene copies per ng of DNA for soil from three land-use types
578 (primary rainforest, pasture, and secondary rainforest) in Rondônia and Pará incubated with
579 $^{13}CH_4$ or no incubation (field soil).
580
581 Suppl. Table 1. Significance values (p-values) from ANOVA with Tukey Honestly Significant
582 Difference Test comparing the richness of active methane-cycling taxa.
583
584 Suppl. Table 2. The abundance of active methanogen species found in each $^{13}CO_2$ SIP incubation
585 from Rondônia.
586
587 Suppl. Table 3. The abundance of active methanogen species found in each ^{13}NaAOc SIP
588 incubation from Rondônia.
589
590 Suppl. Table 4. The abundance of active methanotroph species found in each $^{13}CH_4$ SIP
591 incubation from Rondônia. The Type column specifies whether that methanotroph species is
592 Type I, II, or III.
593

Suppl. Table 5. The abundance of active methanogen species found in each $^{13}CO_2$ SIP incubation from Pará.

Suppl. Table 6. The abundance of active methanogen species found in each ^{13}NaAOc SIP incubation from Pará.

Suppl. Table 7. The abundance of active methanotroph species found in each $^{13}CH_4$ SIP incubation from Pará. The Type column specifies whether that methanotroph species is Type I, II, or III.

Suppl. Table 8. The relative abundance of active methanotrophy or methanotrophy-related genes for samples incubated with $^{13}CH_4$. Location indicates whether the sample is from Rondônia or Pará. Land use states whether the sample is from a primary rainforest, pasture, or secondary rainforest. The within and between location values show the p-value from a two-tailed t-test comparing land-use types.

Suppl. Table 9. The relative abundance (%) of active methanogenesis genes (methanogenesis + methanogenesis strays + methanogenesis from methylated compounds) to the total methanogenesis gene annotations for each sample incubated with either 13CO2 or 13NaAOc. Location indicates whether the sample is from Rondônia or Pará. Land use states whether the sample is from a primary rainforest, pasture, or secondary rainforest. SIP Incubation indicates whether the sample was incubated with 13CO2 or 13NaAOc. PF = primary rainforest, P = pasture, SF = secondary rainforest. Methanogenesis strays are described by SEED Subsystem as

617 "additional genes and clusters from methanogens". The specific genes associated with these
618 SEED Subsystems can be found by searching for the subsystem on the SEED Viewer
619 (http://rast.theseed.org/FIG/seedviewer.cgi?page=SubsystemSelect). The two-tailed t-test values
620 are p-values with significant ($p<0.05$) highlighted in red.
621
622 Suppl. Table 10. Microbial species implicated in the sulfur, nitrogen, or carbon cycle that were
623 active and significantly different between land use types in Rondônia or Pará $^{13}CO_2$ -supported
624 SIP samples. The term "Land use association" indicates the land use type that had (1) a
625 significantly higher abundance than the other land use types, and (2) the microbial species was
626 active in that land use type.
627
628 Suppl. Table 11. Microbial species implicated in the sulfur, nitrogen, or carbon cycle that were
629 active and significantly different between land use types in Rondônia or Pará ^{13}NaAOc-
630 supported SIP samples. The term "Land use association" indicates the land use type that had (1)
631 a significantly higher abundance than the other land use types, and (2) the microbial species was
632 active in that land use type.
633
634 Suppl. Table 12. Soil physico-chemical properties of samples collected in pasture, primary
635 rainforest, and secondary rainforest in the State of Pará, Brazil . The mean values and results
636 from an ANOVA with a post-hoc Tukey-Kramer test are tabulated. M.O. = organic matter. H+Al
637 = total soil acidity, SB = sum of exchangeable bases (Ca + Mg + K), CTC = cation exchange
638 capacity, m = aluminum saturation. mmolc = millimoles of charge,V = base saturation as a
639 percentage of CTC, m = aluminum saturation as a percentage of CTC.

Suppl. Table 13. Soil physico-chemical properties of samples collected in pasture, primary rainforest, and secondary rainforest in the State of Rondônia, Brazil. The mean values and results from an ANOVA with a post-hoc Tukey-Kramer test are tabulated. M.O. = organic matter, H+Al = total soil acidity, SB = sum of exchangeable bases (Ca + Mg + K), CTC = cation exchange capacity. m = aluminum saturation, mmolc = millimoles of charge, V = base saturation as a percentage of CTC, m = aluminum saturation as a percentage of CTC.

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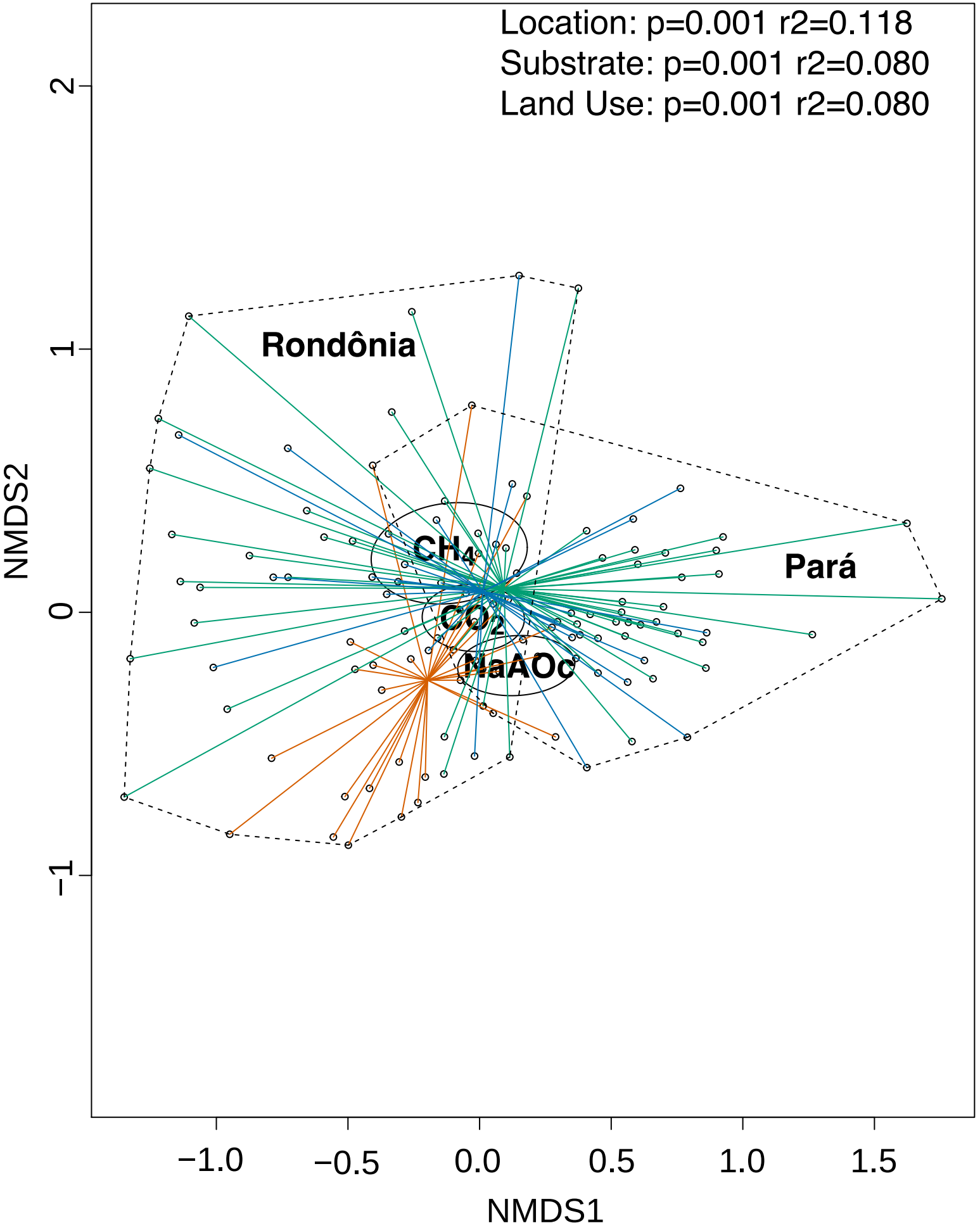
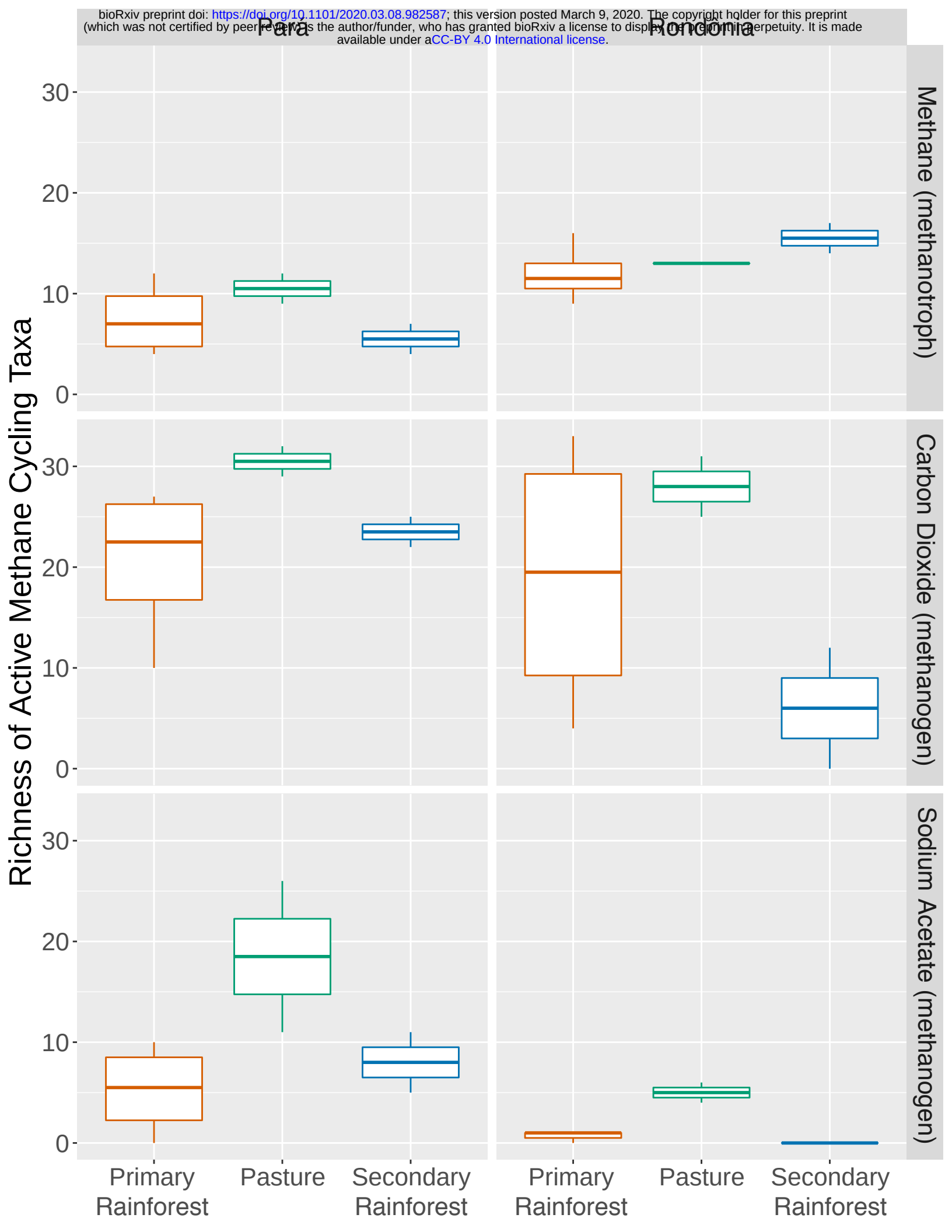




Genus
Methanobacterium
Methanobrevibacter
Methanocaldococcus
Methanococcoides
Methanococcus
Methanocorpusculum
Methanoculleus
Methanohalobium
Methanohalophilus
Methanoplanus
Methanoregula
Methanosaeta
Methanosarcina
Methanosphaera
Methanosphaerula
Methanospirillum
Methanothermobacter
Methanothermococcus
Methanothermus

a

Abundance of Active Methanogens

12500
10000
7500
5000
2500
0

PF1−2
PF1−3
PF2−3
PF2−5
P−1
P−3
SF−4
SF−5

b

Abundance of Active Methanogens

60000
40000
20000
0

PF1−4
PF1−5
PF2−2
PF2−3
P−4
P−5
SF−1
SF−2

c

Abundance of Active Methanogens

15000
10000
5000
0

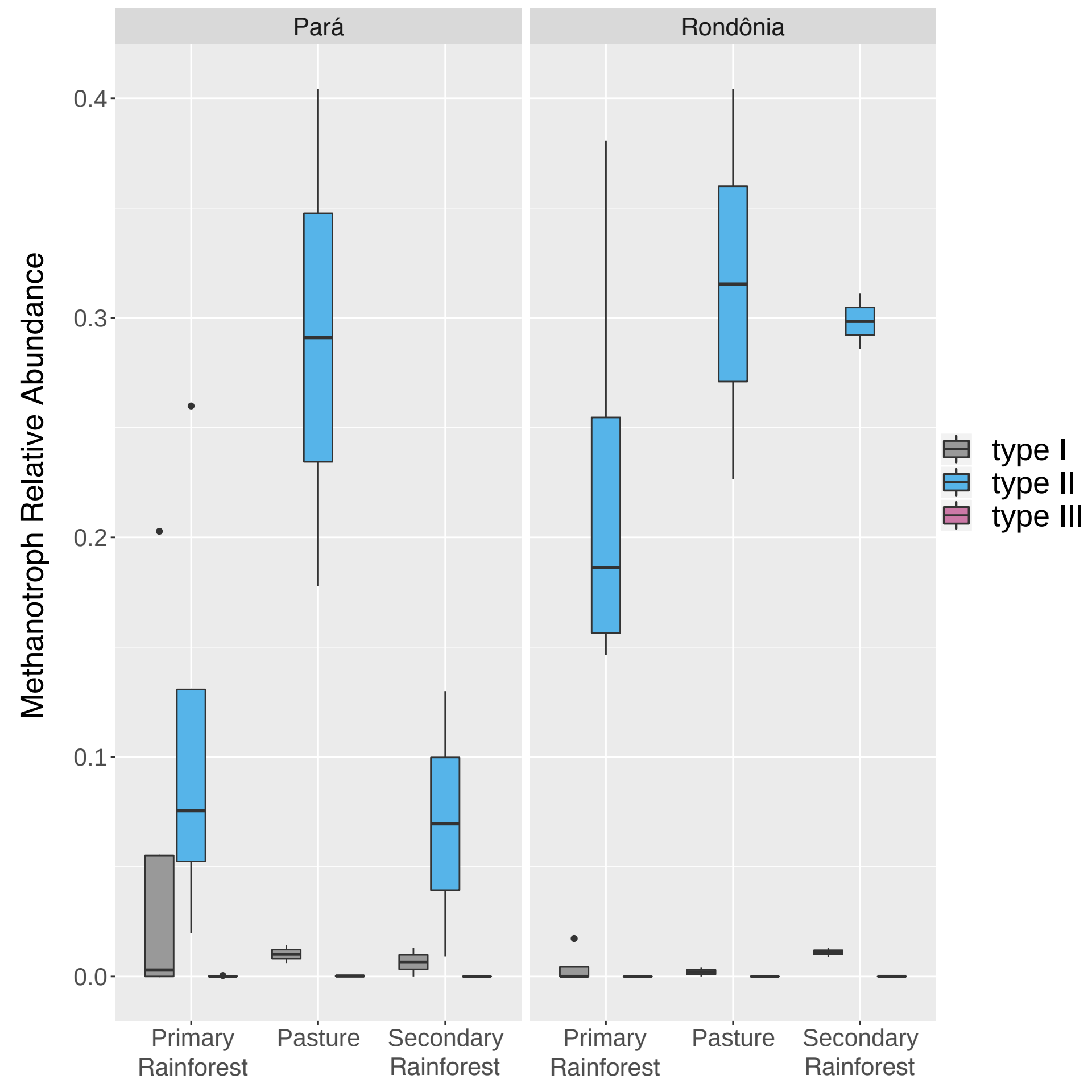
PF1−1
PF1−3
PF2−3
PF2−4
P−1
P−3
SF−2
SF−4

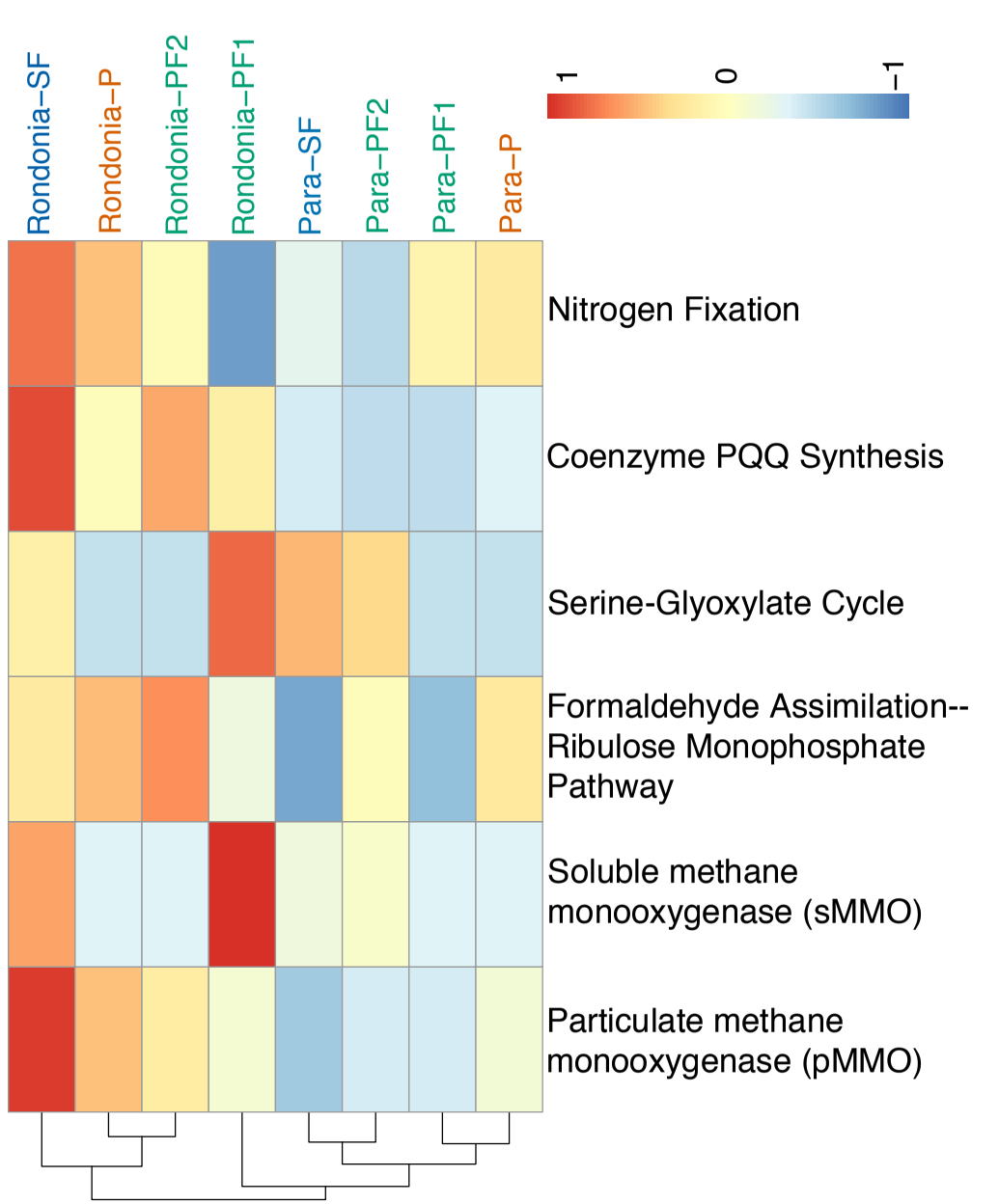
d

Abundance of Active Methanogens

15000
10000
5000
0

PF1−3
PF1−5
PF2−2
PF2−4
P−1
P−3
SF−1
SF−2


Pará
Rondônia
Methanotroph Relative Abundance
0.4
0.3
0.2
0.1
0.0
Primary Rainforest
Pasture
Secondary Rainforest
Primary Rainforest
Pasture
Secondary Rainforest
type I
type II
type III


Rondonia–SF
Rondonia–P
Rondonia–PF2
Rondonia–PF1
Para–SF
Para–PF2
Para–PF1
Para–P
1
0
−1
Nitrogen Fixation
Coenzyme PQQ Synthesis
Serine-Glyoxylate Cycle
Formaldehyde Assimilation--
Ribulose Monophosphate
Pathway
Soluble methane
monooxygenase (sMMO)
Particulate methane
monooxygenase (pMMO)