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1 **Maintaining grass coverage increases methane uptake in Amazonian pasture soils**

2

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20 Running Title: Grass rhizosphere drives soil methane cycle

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21 **Abstract**

22

23 Cattle ranching is the largest driver of deforestation in the Brazilian Amazon. The rainforest-
24 to-pasture conversion affects the methane cycle in upland soils, changing it from sink to
25 source of atmospheric methane. However, it remains unknown if management practices could
26 reduce the impact of land-use on methane cycling. In this work, we evaluated how pasture
27 management can regulate the soil methane cycle either by maintaining continuous grass
28 coverage on pasture soils, or by liming the soil to amend acidity. Methane fluxes from forest
29 and pasture soils were evaluated in moisture-controlled greenhouse experiments with and
30 without grass cover (*Urochloa brizantha* cv. Marandu) or liming. In parallel, we assessed
31 changes in the soil microbial community structure of both bare pasture soil as well as
32 rhizosphere soil through high throughput sequencing of the 16S rRNA gene, and quantified
33 the methane cycling microbiota by their respective marker genes related to methane
34 generation (*mcr*A) or oxidation (*pmo*A). The experiments used soils from eastern and western
35 Amazonia, and concurrent field studies allowed us to confirm greenhouse data. The presence
36 of a grass cover not only increased methane uptake by up to 35% in pasture soils, but also
37 reduced the abundance of the methane-producing community. In the grass rhizosphere this
38 reduction was up to 10-fold. Methane-producing archaea belonged to the genera
39 *Methanosarcina sp., Methanocella sp., Methanobacterium sp.,* and Rice Cluster I. Further, we
40 showed that liming compromised the capacity of forest and pasture soils to be a sink for
41 methane, and instead converted formerly methane-consuming forest soils to become
42 methane sources in only 40-80 days. Our results demonstrate that pasture management that
43 maintains grass coverage can mitigate soil methane emissions, if compared to a bare pasture
44 soil.

45

46 Keyword: climate change, soil methane cycling, environmental microbiology, soil
47 conservation, microbiome management

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52 **Introduction**

53 The establishment of pasture lands is the main cause of deforestation in the Amazon
54 region (Dias et al., 2016; Margulis, 2003). This transformation of rainforest into pastures leads
55 to the net increased emission of the powerful greenhouse gas methane, and turns a methane
56 consuming forest soil into a methane producing pasture soil (Meyer et al., 2020; Fernandes et
57 al., 2002; Steudler et al., 1996; Goreau; De Mello, 1988). The resulting greenhouse gas
58 emissions account for half of Brazil's greenhouse gas production, and have already exceeded
59 national emissions from fossil fuel by more than 20% (Bustamante et al., 2012; Fearnside &
60 Imbrozio Barbosa, 1998). Livestock production is responsible for the emission of several
61 greenhouse gases, and current studies work on minimizing this impact (Herrero et al. 2016,
62 Fiqueiredo et al. 2017). Recently, methane emissions from soil have become a focus of
63 investigations because they might be agriculturally manageable. The impact of land-use
64 conversion on the annual balance of gas fluxes is noticeable considering pastures in western
65 Amazonia can emit up to 270 mg $C\text{-}CH_4/m^2$, while nearby forest soils can consume up to 470
66 mg $C\text{-}CH_4/m^2$ (Steudler et al., 1996).

67 Methane (CH_4) gas has an 86-fold greater potential to retain heat in the atmosphere
68 compared to that of CO_2, calculated over a 20-year period (IPCC, 2013). The global methane
69 emissions are mainly driven by human activities such as livestock, irrigated agriculture, oil
70 and gas production, and landfill decomposition (IPCC, 2013). Soil methane cycling is strongly
71 dependent on the microbiota, since the biogenic source of this gas are methanogenic
72 archaea. The biological consumption of methane is controlled by methanotrophs, mostly
73 bacteria. In soil, the balance between methanotrophic bacteria and methanogenic archaea is
74 related to environmental conditions (i.e., moisture, temperature, soil density, and pH) and is
75 sensitive to changes in agricultural management (Le Mer & Roger, 2001; Liu et al., 2007; Tian
76 et al., 2015).

77 Methanotrophic bacteria in the soil are Gram-negative, belonging to
78 *Gammaproteobacteria* and *Alphaproteobacteria*, *Verrucomicrobia*, and candidates in the
79 phylum NC10 (Hanson & Hanson, 1996; Knief, 2015, Ettwig et al. 2010). The initial step of
80 methane oxidation occurs through its conversion to methanol, which is mediated by the
81 enzyme methane monooxygenase (MMO). Methanogenic archaea traditionally comprise
82 members from eight orders within the phylum *Euryarcheota*: *Methanopyrales,*

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83 *Methanobacteriales, Methanococcales, Methanomicrobiales, Methanocellales,*
84 *Methanosarcinales, Methanomassiliicoccales*, and '*Candidatus* Methanophagales' (Evans et
85 al. 2019), with additional candidates in the phylum *Bathyarchaeota* (Kallistova et al., 2017).
86 Methanogenesis is controlled by archaea and is the final step in an anaerobic pathway that
87 begins with the hydrolysis of organic polymers, fermentation of the resulting monomers and of
88 initial fermentation products, and ends up in the production of CH_4 mostly from acetate,
89 hydrogen, and CO_2. The final step in the methanogenesis pathway is facilitated through the
90 action of the enzyme methyl-coenzyme M reductase, coded by the *mcrA* gene, which can be
91 used as a methanogen-specific marker for molecular studies (Serrano-Silva et al., 2014). The
92 ability to produce methane has recently been demonstrated in cyanobacteria and plants,
93 however, it is believed that this is a by-product from reactions of photosynthesis (Bižić et al.,
94 2020; Keppler et al., 2006).

95 The relationship between changes in land-use and the response of the soil microbial
96 community is not well understood (Nazaries et al., 2013; Tate, 2015), but previous studies
97 have shown significant impacts on microbial diversity in the Amazon region (de Carvalho et
98 al., 2016; Mendes et al., 2015; Navarrete et al., 2015; Rodrigues et al., 2013; Jesus et al.,
99 2009). In well-managed pastures in the Amazon region, the grass root system can redistribute
100 carbon to deeper layers, where it is less susceptible to decomposition (Fearnside & Imbrozio
101 Barbosa, 1998). On the other hand, we can expect that degraded pastures with large bare
102 soil areas can facilitate the release of carbon from the system, with superficial grassroots,
103 higher loss of soil organic matter, and lower carbon stocks (Segnini et al. 2019). Proper
104 management of pasture may involve several practices, such as soil acidity correction and
105 continuous maintenance of grass cover to protect soil from erosion. These practices are
106 particularly important in the Amazon region given the environmental extremes of this area.
107 Altogether, the high soil acidity, high rainfall, and high temperatures combined with exposure
108 of the soil to equatorial solar radiation constitute factors that are associated with increasing
109 erosion and soil degradation (Demattê and Demattê, 1993). Soil degradation is the long-term
110 decline in the soil's productivity and its environment moderating capacity, with soil quality loss
111 and reduction in attributes related to specific functions of value to humans (Lal, 2001).

112 The effect of soil liming on methane fluxes is still poorly understood, and studies of
113 temperate forests show that liming can lead to both an increase and a decrease of methane

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114 consumption (Wang et al. 2021; Borken and Brumme, 1997; Butterbach-Bahl et al., 2002). In
115 wheat-focused agriculture liming has led to an increased consumption of methane in soil
116 (Hütsch et al., 1994). The increased methane consumption after liming was also observed in
117 Mediterranean semiarid soils under lupine, wheat, and triticale, a hybrid of wheat and rye
118 (Barton et al., 2013; García-Marco et al., 2016). However, for tropical soils little information is
119 available regarding what influence liming has on methane production and consumption. In an
120 assessment of greenhouse gas fluxes from soils under soybean cultivation in Brazil, the
121 acidity correction presented no effect on methane fluxes (Lammel et al., 2018). Likewise, in a
122 field experiment in Puerto Rico, soil consumption of atmospheric CH_4 in an intentionally
123 acidified soil was about one-fourth of that at pH 6, and was not restored after liming (Mosier et
124 al., 1998).

125 There is a growing consensus that the key to understand major soil functions lies
126 where plants and soil meet, in the rhizosphere (Lau et al., 2011). The rhizosphere is a micro-
127 environment with differentiated soil conditions and steep overlapping gradients, in which pH
128 can be up to 2 units more acidic or more basic than the soil surrounding the rhizosphere. The
129 rhizosphere can present heterogeneous concentrations of oxygen and moisture and can be
130 enriched in root exudates like sugars and organic acids (Philippot et al., 2013). These factors
131 affect soil methane cycling not only by providing organic substrate for methanogenesis but
132 also by promoting the oxidation of methane in the rhizosphere. Despite its known role in
133 flooded rice soils (Frenzel et al., 1992), little is known about the impact of the rhizosphere on
134 methane cycling in upland soils since these soils are commonly considered to be a methane
135 sink, not a source (Philippot et al., 2009). Thus, improved understanding of how the
136 rhizosphere of land-intensive tropical pastures affects soil methane cycling can yield new
137 strategies to mitigate greenhouse gas emissions related to cattle ranching.

138 The intensive land use in agriculture and cattle ranching in Amazonia leads to soil and
139 pasture degradation ranging from 50% to 70% of total area (Dias-Filho, 2017). Within this
140 context this research aims to evaluate how the management of pastures can affect soil
141 methane cycling. We hypothesized that CH_4 production is reduced by liming soils and by
142 continuous grass coverage due to the influence of the rhizosphere of *Urochloa brizantha* cv.
143 Marandu, a grass widely used in pastures in Brazil. To test this hypothesis, we measured
144 methane fluxes in soils from pasture field sites with and without grass cover, and compared

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145 the microbiota in bare pasture soil to that of the rhizosphere of grass covered soil. These
146 studies were complemented with greenhouse experiments where soil acidity was adjusted
147 and grass was planted, gas flux rates of the soil-air CH_4-fluxes were measured, and shifts in
148 the soil microbial community between bare soil and the rhizosphere of *Urochloa brizantha* cv.
149 Marandu were determined.
150
151 **Materials and Methods**
152
153 **Sampling**
154 These studies were performed with soils from both western and eastern Amazonia. In
155 the western region (hereafter “Ariquemes”) sampling was carried out in April of 2017 at the
156 Fazenda Nova Vida near Ariquemes, RO (10°10'49.5" S, 62°49'23.9" W). While in the eastern
157 region (hereafter “Tapajós”), the samples were taken at the National Forest of Tapajós and
158 the immediately surrounding areas near Belterra, PA (3°07'53.8" S, 54°57'24.2" W), in August
159 of 2019. The sampled soils were used in two rounds of greenhouse experiments at the Center
160 for Nuclear Energy in Agriculture, SP, Brazil (22°42'27.7" S, 47°38'41.0" W). In addition to the
161 soil sampling a field study was performed, but only in the Tapajós region (detailed below).
162 Western Amazonia has been studied for the impacts of conversion from forest to
163 pasture, with extensive scientific literature characterizing ecosystem responses to conversion
164 (de Moraes et al., 1996; Herpin et al., 2002; Reiners et al., 1994) for representing a region
165 with a high degree of exploitation. The Fazenda Nova Vida region has fragments of primary
166 forest and pastures of different ages. The sampled pasture area was established in 1972, and
167 since then managed by cattle rotation, with the use of fire only to control eventual pests,
168 mechanical removal of invasive trees, and at least one record of liming 15 years before the
169 sampling. Soils sampled varied from average clay to sandy texture.
170 Eastern Amazonia represents areas of more recent exploration. The Tapajós National
171 Forest was sampled as a model of a conservation area and the pasture chosen is in a small
172 property in Belterra, PA. The pasture used here was established between 1989-1994 and
173 supported cattle at the time of sampling, had sparse signs of degradation, fire was applied
174 when necessary to control invasive plants, and it has no history of liming. Soils sampled
175 varied from average clay to sandy texture.

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176 During each expedition, we sampled 20-30 kg of soil from the upper 0-10 cm layer of 5
177 equidistant sampling points along a linear gradient of 200 m at each site, from areas under
178 Primary Forest and Pastures with *Urochloa brizantha* cv. Marandu. The sampled soils were
179 transported to the Cell and Molecular Biology Laboratory at the University of Sao Paulo,
180 CENA-USP, with fresh samples for chemical analysis and greenhouse experiments, or were
181 frozen at the end of each sampling day and stored at -20 ºC for future molecular analyses.
182

183 **Field Study**

184 At two pastures in the Tapajós region, 100 m side squares were established and 4
185 points in the square corners, plus a point in the center, were selected to evaluate CH_4 fluxes
186 and to sample soils for molecular and chemical analysis. Those 5 points had grass coverage
187 at the time, and prior to gas flux measurements with static gas collection chambers the grass
188 leaves were cut to their stems (2 cm above soil surface) and removed. Following chamber
189 removal, the roots were collected, and the rhizospheric soil was sampled and stored at -20
190 ºC. Adjacent to each of the five sampling points we had selected, one square meter large
191 areas without grass (bare soil) we used to measure methane fluxes and to collect soil
192 samples for molecular and chemical analysis.
193

194 **Greenhouse experiments**

195 Sampled soil was homogenized, sieved (5 mm), and placed in clay pots with a capacity
196 of 10 liters, resulting in 10 cm high soil columns with 5 kg of soil per pot. The grass was raised
197 from seed in a subsample of the soil, and mature plants were transferred to the experimental
198 clay pots at least 40 days after soil liming. The liming was performed by the addition of $CaCO_3$
199 to reach pH 6.5 (water), calculated for a base saturation of 70-75%. For each treatment, four
200 pots were used to grow *Urochloa brizantha* cv. Marandu with 4 additional pots as no-plant
201 controls (bare soil), both at natural pH and with limed soils (4 pots x 2 soil types x 2 pH
202 situations x 2 plant situations). At the beginning of the experiment soil moisture was
203 standardized to ~70% of the water retention capacity of the soil and adjusted every two to
204 four days, taking as reference the weight variation after drying soil samples for 48 h at 75 ºC.
205 In the experiment with soil from Ariquemes, the plants were removed when they reached
206 approximately 35 cm in height, and by shaking and with the help of a sterilized brush the

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207 rhizosphere soil was collected. Here we defined the rhizosphere as soil that remained
208 attached to the roots even after vigorous plant shaking.
209
210 **Determination of methane fluxes in soil**
211 The measurements of CH_4 fluxes in both the field sampling sites and the greenhouse
212 experiments were carried out using static gas collection chambers (20 cm in diameter, ~6 L
213 inner volume). Over a period of 10 minutes measurements were taken at 10 second intervals
214 using a portable gas analyzer (UGGA, Los Gatos Research, San Jose, CA, USA). Daily flux
215 of gases was estimated from the concentration in the chamber headspace. Daily flux (F, mass
216 of gas $m^{-2}.day^{-1}$) was computed as (Ussiri, Lal and Jarecki, 2009):
217
218 $$F = \Delta gas/\Delta t \times V/A \times k$$
219
220 Where $\Delta gas/\Delta t$ is the rate of change in CH_4 concentration inside the chamber (i.e. mg
221 CH_4-C); V is the chamber volume (m^3); A is the surface area circumscribed by the chamber
222 (m^2) and k is the time conversion factor (1440 min day^{-1}). The cumulative gas emissions were
223 calculated by linear interpolation of average emissions between two successive
224 measurements and the sum of the results obtained over the entire study period. Finally, the
225 data was expressed as differences in the cumulative CH_4 fluxes in relationship to the controls.
226 From this, we subtracted from the accumulated fluxes in the respective treatments (liming,
227 grass coverage, and liming plus grass coverage) the control measurements of the average
228 accumulated flux (bare soils).
229
230 **Characterization of soil chemical properties**
231 About 600 g of soil were analyzed for their physical-chemical properties at the
232 Laboratory of Chemical Analysis in the Soil Science Department at the “Luiz de Queiroz
233 School of Agriculture” (ESALQ / USP) (detailed in van Raij et al., 2001). The soil attributes
234 measured were: pH in $CaCl_2$; concentrations of phosphorus, potassium, calcium, and
235 magnesium by extraction with ion exchange resin; aluminum by extraction of potassium
236 chloride at 1 mol/ L; potential acidity estimated by pH- SMP buffer test; organic matter by the
237 dichromate- titrimetric method; boron by extraction with hot water; copper, iron, manganese

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238 and zinc extracted by the DTPA-TEA extractor (pH 7.3); and by calculating the sum of bases
239 (BS); cation exchange capacity (CEC); base saturation (V%), and aluminum saturation (m%).
240

241 **DNA extraction**

242 DNA was extracted for molecular analyses from greenhouse soils that originated in
243 Ariquemes and Belterra (Tapajós region) and from soils of the field study in the Tapajós
244 regions. Total DNA was extracted from soil samples using the PowerLyzer PowerSoil DNA
245 Isolation Kit (Qiagen, Hilden, Germany) from 250 mg of soil, according to the protocol
246 provided by the manufacturer, except that after adding solution C1 the stirring time was
247 extended to 15 minutes followed by 3 min centrifugation (Venturini et al., 2020). The amount
248 and quality of the DNA extracted was analyzed in a Nanodrop 2000c spectrophotometer
249 (Thermo Fisher Scientific, Waltham, MA, USA) at an optical density of 260 nm. The total DNA
250 extracted was stored at -20 °C.
251

252 **Abundance of methane producers and oxidizers**

253 Real-time quantitative PCR (qPCR) was used to quantify the genes associated with
254 methane cycling *mcrA and pmoA* (Table S1) in total soil DNA samples. For each gene, a
255 standard curve was established spanning each order of magnitude from 10^1 to 10^7 copies of
256 the gene. Target genes were previously obtained by PCR from genomic DNA of *Methanolinea*
257 *mesofila* (DSMZ 23604) for the *mcrA* gene, and *Methylosinus sporium* (DSMZ 17706) for the
258 gene *pmoA*, both obtained from the DSMZ (German Collection of Microorganisms and Cell
259 Cultures). The qPCR was performed in triplicate for each sample on a StepOne Plus cycler
260 (Thermo Fisher Scientific, Walthman, MA, USA), with a final volume of 10 μL, containing 5 μL
261 of SYBR Green ROX qPCR (Thermo Fisher Scientific, MA, USA), 1 μL of each primer (5
262 pmols), 1 μL of soil DNA (adjusted to 10 ng/ μL), 0.8 μL of bovine albumin (20 mg / mL)
263 (Sigma-Aldrich, San Luis, MO, USA), and 1.2 μL of ultrapure water (Milli-Q, autoclaved).

264 In order to minimize bias in the analysis between each qPCR plate run, gene
265 abundance was quantified with the software LinRegPCR (Ramakers et al., 2003). Raw
266 amplification data for each sample were used to calculate individual reaction efficiencies, and
267 detection limits were established for each group of technical replicates. The data generated in
268 arbitrary fluorescence units were converted to the number of copies of the genes using linear

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269 interpolation between the known quantities in the standard curve (5 best points out of 7) and
270 the observed fluorescence measurements, using the curves of each plate as a reference for
271 the respective samples.
272

273 **Sequencing of 16S rRNA gene fragments**

274 The composition of the microbial community was determined with high throughput
275 sequencing (MiSeq Illumina platform with a 600c kit) of the V4 region of the 16S rRNA gene
276 at the Functional Genomics Center of Luiz de Queiroz College of Agriculture (Caporaso et al.,
277 2011). The V4 region was amplified with the primers 515F (Parada et al., 2016) and 806R
278 (Apprill et al., 2015). This sequencing strategy was selected to match the highly diverse soil
279 environment and for the size of the paired-end reads (average 300 bp). Gene library
280 preparation followed the conditions of 95ºC for 3 minutes, followed by 25 cycles at 95ºC for 30
281 seconds, 50ºC for 30 seconds, 72°C for 30 seconds, and a final extension step at 72°C for 5
282 minutes. The DNA concentrations in the samples were adjusted to 10 ng/uL using a
283 Nanodrop 2000c spectrophotometer and the PCR reactions with 2.5 µL of 10x buffer, 1 µL of
284 50 mM MgCl, 1 µL of 10mM dNTPs, 0.5 µL of 10µM Forward and Reverse Primers, 0.5 µL of
285 5 U/ µL Taq Platinum - PCR and water for PCR - 14 µL, in a total volume of 25 µL per
286 reaction. Subsequent DNA purification of the amplicon was performed using AMPure XP
287 beads (Beckman Coulter, Brea, CA, USA) and verified on an agarose gel. In a similarly
288 structured, second PCR the adapters were added, followed by another purification with
289 AMPure XP beads and gel electrophoretic confirmation in agarose. The amplicon pool was
290 normalized using quantification by qPCR with the KAPA Illumina quantification kit (Roche,
291 Basel, Switzerland). The computational processing of these data was performed using
292 QIIME2 2017.11 (Bolyen et al., 2019), with data quality control using the DADA2 tool
293 (Callahan et al., 2017), without clustering into OTUs, and taxonomic identification of the
294 sequences was performed using q2-feature-classifier (Bokulich et al., 2018) and the SILVA
295 v.128 99% database (Quast et al., 2013).
296
297
298
299

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300 **Phylogenetic analyses**

301 Some amplification sequence variants (ASVs) grouped closest with the family
302 Beijerinckiaceae. Since this family represents both methanotrophic and non-methanotrophic
303 genera, we increased our phylogenetic resolution by analyzing phylogenetic trees containing
304 only Beijerinckiaceae sequences that were created with two 16S rRNA primer pairs. One set
305 of primers targeted the region V4, between 515F (Parada et al. 2006) and 806R (Apprill et al.
306 2015), and a second set of primers targeted the V3/V4 region, between 341F and 805R
307 (Herlemann et al., 2011). For this last pair, the amplification protocol was identical to that
308 described above, except the annealing temperature for the second primer pair was 55ºC. The
309 sequences were aligned, and trees were calculated using the software CLC Genomics
310 Workbench 20.0 (QIAGEN, Aarhus, Denmark) at default parameters, and with a maximum
311 likelihood model (PHYML function) with UPGMA (Unweighted Pair Group Method with
312 Arithmetic mean) assuming common replacement frequencies to the bases (Kimura, 1980).
313 The robustness of the final trees was tested with 1000 bootstrap replications. Reference
314 sequences for members of the Beijerinckiaceae family were obtained from the cured
315 database RDP version 11 (Cole et al., 2014) based on the criteria of high-quality reads with a
316 length greater than 1200 bp and representing type strains. The only sequence available for
317 the 16S rRNA gene of the methanotrophic bacterium USCα (Pratscher et al., 2018) was also
318 added.

319

320 **Statistical analysis**

321 All comparative analyses between groups were performed with ANOVA followed by a
322 Tukey Honestly Significant Difference (HSD) test and p-values calculated for a two-tailed
323 distribution of the data using the package *agricolae* version 1.2-8 (R Core Team, 2013).
324 Significant explanatory variables of the methane fluxes were chosen by linear
325 regression and model selection (backward) and by minimizing the Akaike Information
326 Criterion (AIC). The statistical significance was assessed by 1000 permutations of the
327 reduced model. The resulting significant explanatory variables were used to access their
328 contribution to explain the CH_4 fluxes, using the function *varpart* (Peres-Neto et al., 2016) in
329 the vegan package (Oksanen et al., 2015). Statistical analyses were performed in R Studio
330 software (R Core Team, 2013).

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331 The DEICODE tool (Martino et al., 2019) in the QIIME2 2019.10 was used to process
332 the sequencing data. This tool can identify significant changes in the community based on
333 relative abundance data. Next, the software QURRO (Fedarko et al., 2019) was used to
334 assess shifts in the methane cycling community based on transformed abundance data
335 (natural logarithm) and using a minimum of 10 occurrences per taxon.
336

337 **Results**

338 This study tested the effect of acidity correction by liming and the presence of a grass
339 cover by *Urochloa brizantha* cv. Marandu on soil methane fluxes with soils from pasture and
340 forest of different Amazon regions. Two greenhouse experiments were set up. The first
341 experiment was performed with soils from a western Amazon region (Ariquemes, RO) and the
342 second with soils from an eastern region (Tapajós, PA). In both experiments liming resulted in
343 a final pH of ~6.0 ($CaCl_2$, equivalent to pH 6.5 in H_2O), and an increase in calcium availability,
344 as well a decrease in aluminum saturation (Table S2). In the Ariquemes experiment, methane
345 was sink in both bare soils of forest and pasture at their respective natural pH values, with
346 greater uptake in forest soils (Figure 1). In the Tapajós experiment, we observed methane
347 emissions from bare soils from the pasture at natural pH, and methane uptake in bare soils
348 from forest at natural pH (Figure S1). When forest soils from Ariquemes had grass cover they
349 exhibited the highest methane consumption (Figure 1-b; $p = 0.059$), at values close to the
350 naturally acidic forest soil, but significantly lower than both limed soils with or without grass
351 cover (Figure 1-b). The Tapajós soils showed a similar trend compared to Ariquemes soils
352 (Figure S1). Methane uptake in pasture soils increase by 35% on average when they have
353 grass coverage (Figure 1-a; $p = 0.001$). However, liming of pasture soils reduced their
354 methane uptake (Figure 1-a; $p = 0.001$) and turned forest soils from a methane sink into a
355 methane source (Figure S1-b; $p = 0.052$).

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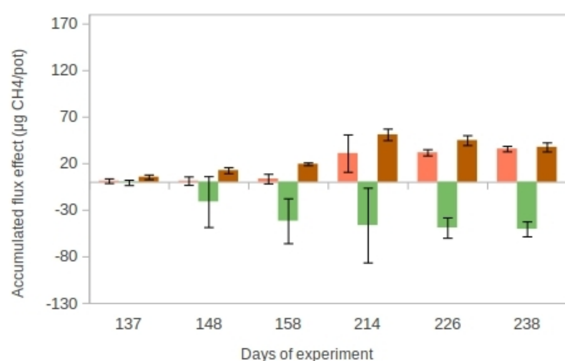

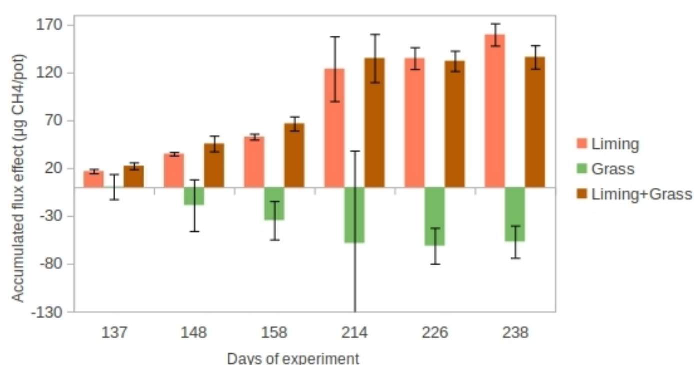

357 Figure 1: Differences in the accumulated methane flux effect compared to untreated control (bare
358 soils at natural pH) in a) pasture and b) forest soils from the Ariquemes experiment in western
359 Amazonia, with and without acidity correction and with and without grass coverage. Bars show
360 standard deviation.
361

362 The validity of our observation that, compared to exposed bare soils, grass coverage
363 improves soil methane uptake was tested in the field. *In situ* measurements of CH_4 fluxes
364 were taken on two pastures in Belterra/PA, Tapajós region, during the end of wet season, at
365 points with and without grass coverage. No significant differences were observed, but the
366 trend is similar to that observed in the greenhouse experiments (Figure S2; p = 0.112).

367 Molecular analyses were performed only with soils from the Ariquemes greenhouse
368 experiment (Figure 2) and from the field study in the Tapajós region (Figure S3). The
369 methane cycling microbiota was evaluated through quantification of their functional marker
370 genes *pmo*A and *mcr*A*,* indicating methane consumers and producers, respectively (Figure
371 2). The rhizospheric community was evaluated only at the end of the experiment (T3=250
372 days). During most of the experimental timeline, we did not observe differences in the
373 abundance of methanotrophs between pasture and forest soils (Figure 2). Regarding
374 methane producers, we observed very low abundance in forest soils compared to pasture
375 soils during the experimental duration. The acidity correction shows a tendency to reduce
376 methanotroph levels in forest soils after 250 days in the grass rhizosphere (p = 0.339) and in
377 the bare soil (p = 0.162) (Figure 2A). Pasture soils had between 100 and 1000-fold more
378 methanogenic archaea than forest soils throughout the experiment, which did not change with
379 acidity correction (Figure 2B). The abundance of methanogenic archaea in the grass
380 rhizosphere in pasture soils was reduced on average by 13 times compared to the bare soil

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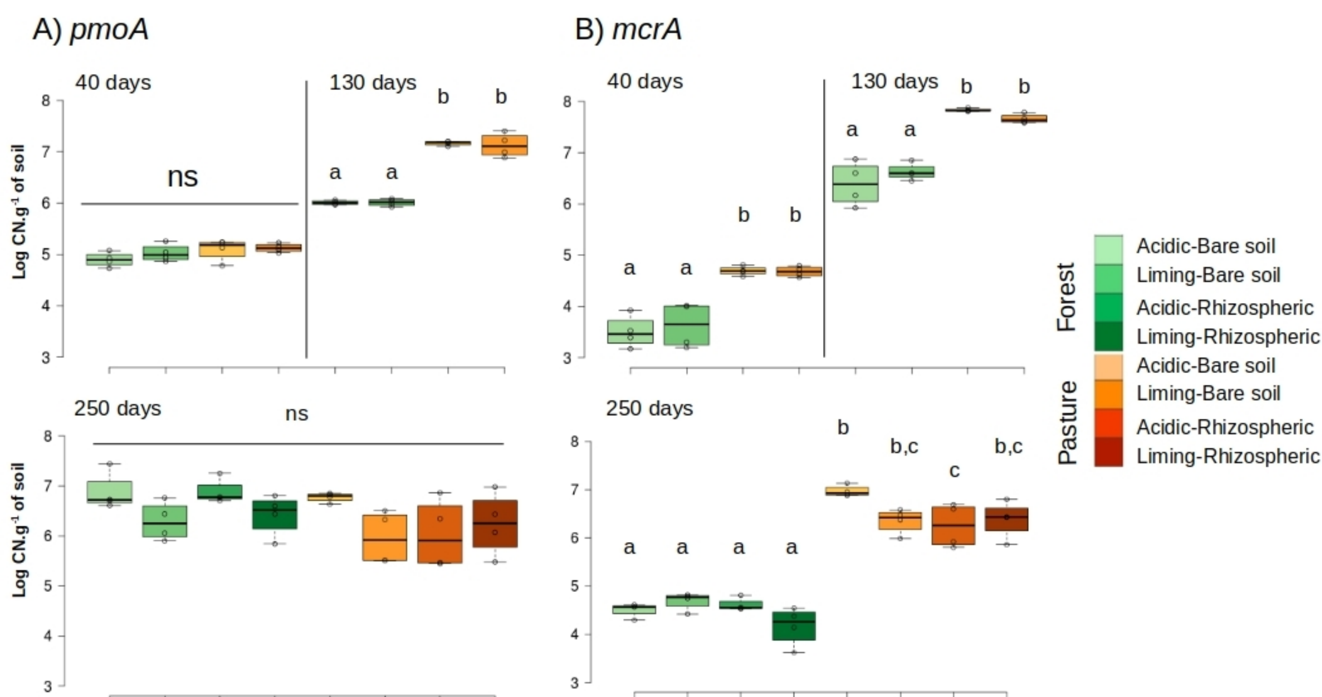
381 (Figure 2B; p = 0.025). No significant changes of methanotrophs were recorded in the
382 rhizosphere (Figure 2A; p = 0.263). This reduction in methanogenic archaea in the grass
383 rhizosphere was not observed in the field study (Figure S3; p = 0.186).
384



385 Figure 2: Gene quantification of the genes (A) *pmoA* and (B) *mcrA* at different times after the
386 beginning of the Ariquemes experiment. T1 = 40 days, T2 = 130 days and T3 = 250 days. Letters
387 above each box plot indicate significant changes ($p < 0.05$). CN = copy number. Ns = Not significant.
388

389 To investigate the effects of acidity correction and grass rhizosphere on specific groups
390 of microorganisms, high throughput DNA sequencing of the 16S rRNA gene was performed.
391 The results show fair sequencing depth, with rarefaction curves leveling off well below the
392 minimal sequencing depth in soils from the Ariquemes experiment (Table S3, Figure S4-a)
393 and in soils from the field studies (Table S4, Figure S4-b).
394 Considering only the community associated with methane cycling in the soil (identified
395 with a minimum of 90% confidence), we filtered the groups recognized as methanotrophs
396 (Knief, 2015) and methanogens (Evans et al., 2019) and observed results similar to those
397 obtained in the quantification of gene copies in total DNA. The number of methanogens in
398 forest soils was smaller, while the abundance of methanotrophs was similar between forest

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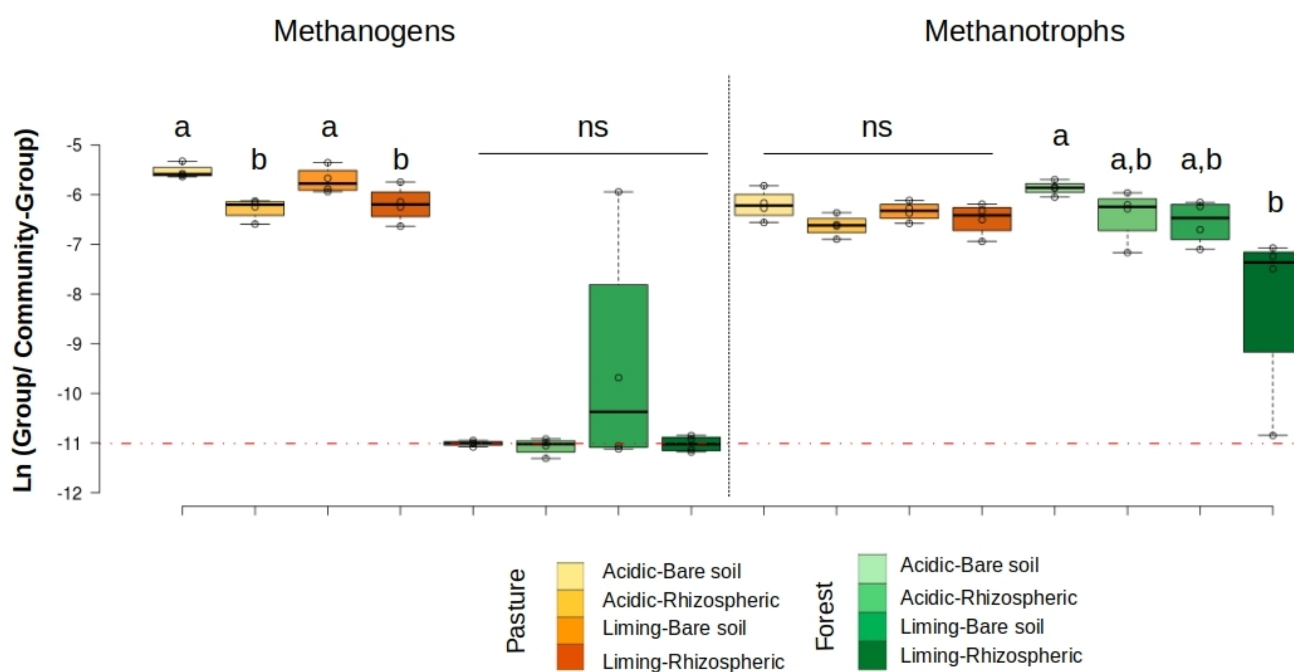
399 and pasture soils (Figure 3). The increase of methanogens in forest soils with acidity
400 correction was not significant. A significant drop in methanotroph abundance was observed
401 only for the combination of acidity correction and grass cover treatments (Figure 3, p=0.024).
402 Methanotrophs in pasture soils did not change with liming or with the presence of grass cover,
403 as previously observed in the quantification of *pmoA* gene copies (Figure 2). However, the
404 number of methanogens was significantly reduced in the grass rhizosphere, with (p=0.017) or
405 without (p=0.007) acidity correction (Figure 3). This last result was similar to that observed in
406 the field, which, although not significant, points to a tendency to reduce methanogenic
407 archaea of different groups in the grass rhizosphere (Figure S5).
408



410 Figure 3: Changes in the logarithmic ratio between methanogenic and methanotrophic groups in
411 relation to the whole community (16S rRNA) in the Ariquemes experiment. The dotted line indicates a
412 calculated ratio of the minimum of 10 counts for each group. The more negative the number of the
413 natural log, the lower the abundance in relation to the total community. Letters indicate significant
414 changes within each treatment for the same land use and group of microorganisms (Tukey HSD; *p*<
415 0.05). Ns = not significant.
416

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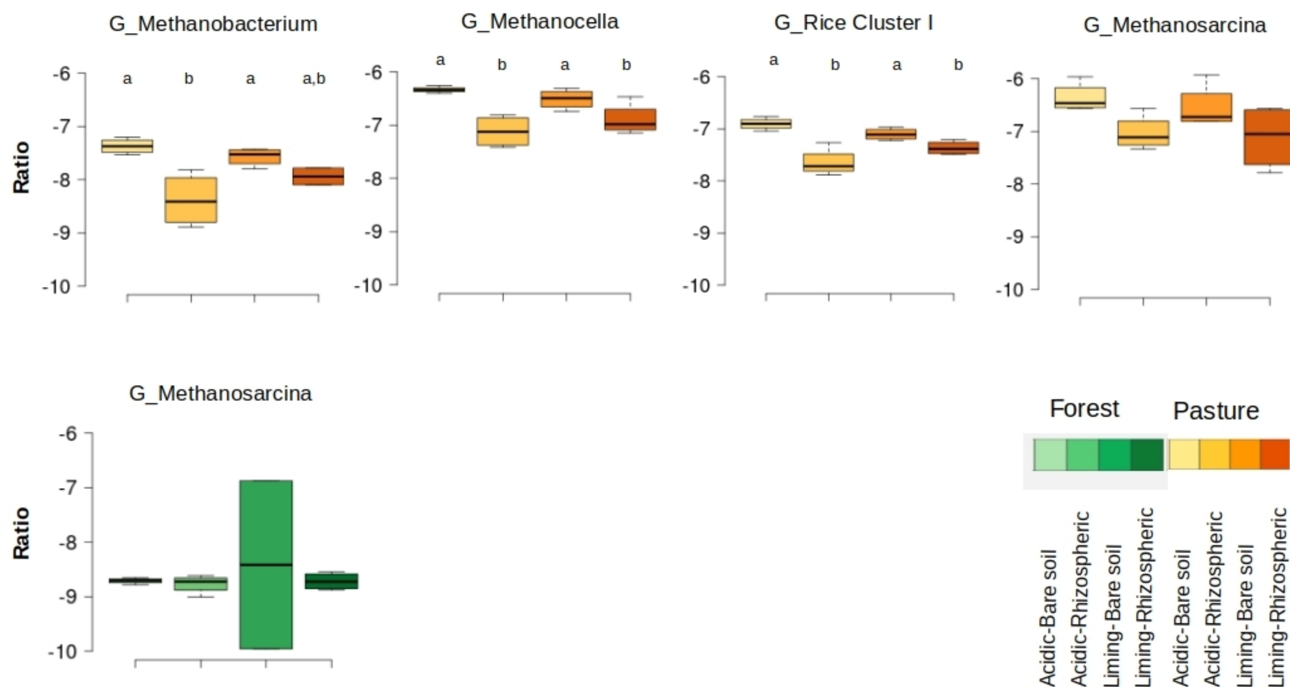
417 Finally, to understand which groups are associated with methane cycling in these soils,
418 a detailed analysis was performed of all groups that presented sequences of the genera
419 known to act in methane cycling (Knief, 2015; Angel et al., 2012) (Figures 4 and 5). In pasture
420 soils, the abundance of members of all methanogenic genera was lower when soil was grass
421 covered. Forest soils showed a low abundance of methanogenic archaea belonging to
422 *Methanosarcina spp*. The Beijerinckiaceae family is abundant in these soils but it was not
423 possible to identify the sequences at the genus level with the database SILVA v.128 (Quast et
424 al., 2012). A new phylogenetic identification was performed comparing all the sequences
425 annotated as Beijerinckiaceae in the RDP database, with the five amplification sequence
426 variants (ASVs) from sequencing with primers 341F/805R and 8 ASVs from primers
427 515F/806R. In this analysis, only sequences of the 16S rRNA gene of this family were used
428 as reference, and the results indicate that they are closely related to the methanotrophic
429 clade, with more than 90% confidence (Figures S6 and S7). Those Beijerinckiaceae are
430 reduced in their relative abundance in forest soils with acidity correction (Figure 5), without
431 changes in pasture. No significant changes were observed in relation to other methanotrophs.
432
433



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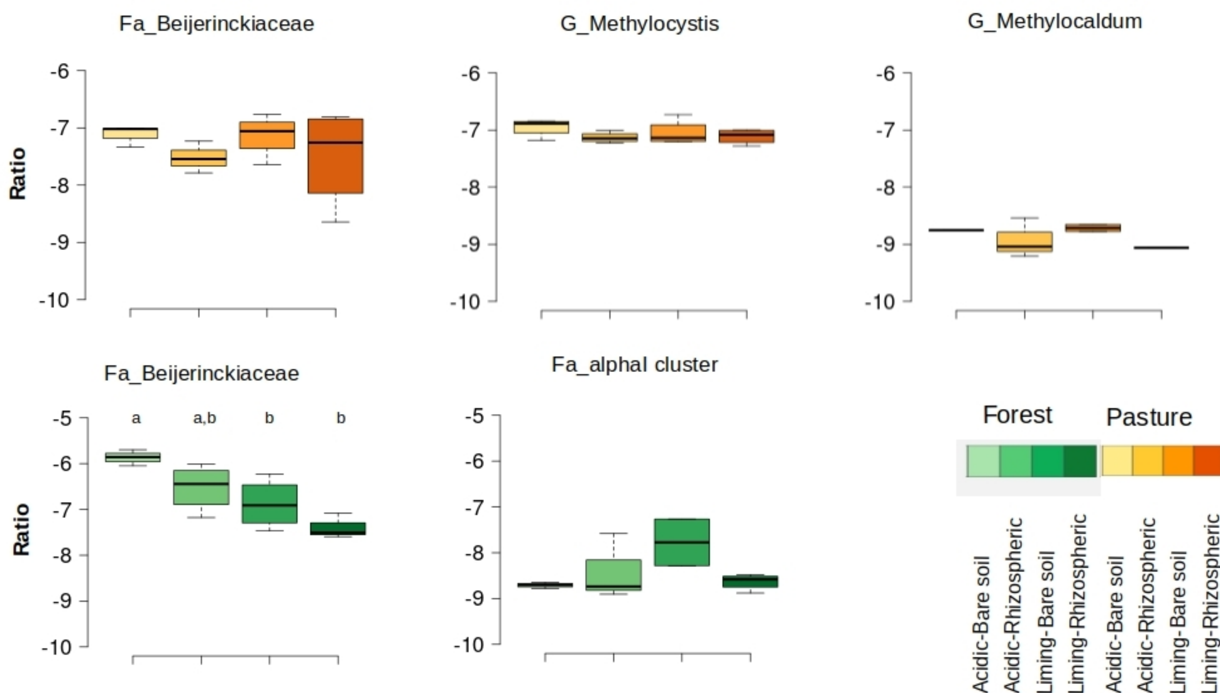
434 Figure 4: Changes in the Log$_e$ ratio between methanogenic microorganisms by genus (G) in relation to
435 the total community in the Ariquemes experiment. All the identified genera are shown. The more
436 negative the numbers, the lower the abundance. Letters indicate significant differences (Tukey HSD;
437 $p<0.05$)



439

440

441 Figure 5: Changes in the Log$_e$ ratio between methanotrophic microorganisms by genus (G) or family
442 (Fa) in relation to the total community in the Ariquemes experiment. The more negative the numbers,
443 the lower the abundance. Letters indicate significant differences (Tukey HSD; $p<0.05$). Analysis
444 showed that a large part of the explained variation (67%) was due to the joint affects.
445

446 To disentangle the effect of soil properties, microbial communities, and the presence of
447 grass on the CH_4 fluxes we performed a variation partitioning analysis. This analysis showed
448 that a large part of the explained variation of the methane fluxes (40%) was due to pH and
449 other soil properties in the first days of the experiment (Figure 6b). However, the grass
450 biomass represented most of the explained variance at the later time-points (Figure 6e-f). The
451 effect of methanogen and methanotroph abundance was minor in the beginning of the
452 incubation (6%) but reached 13% of explained variance at the last time point. These results

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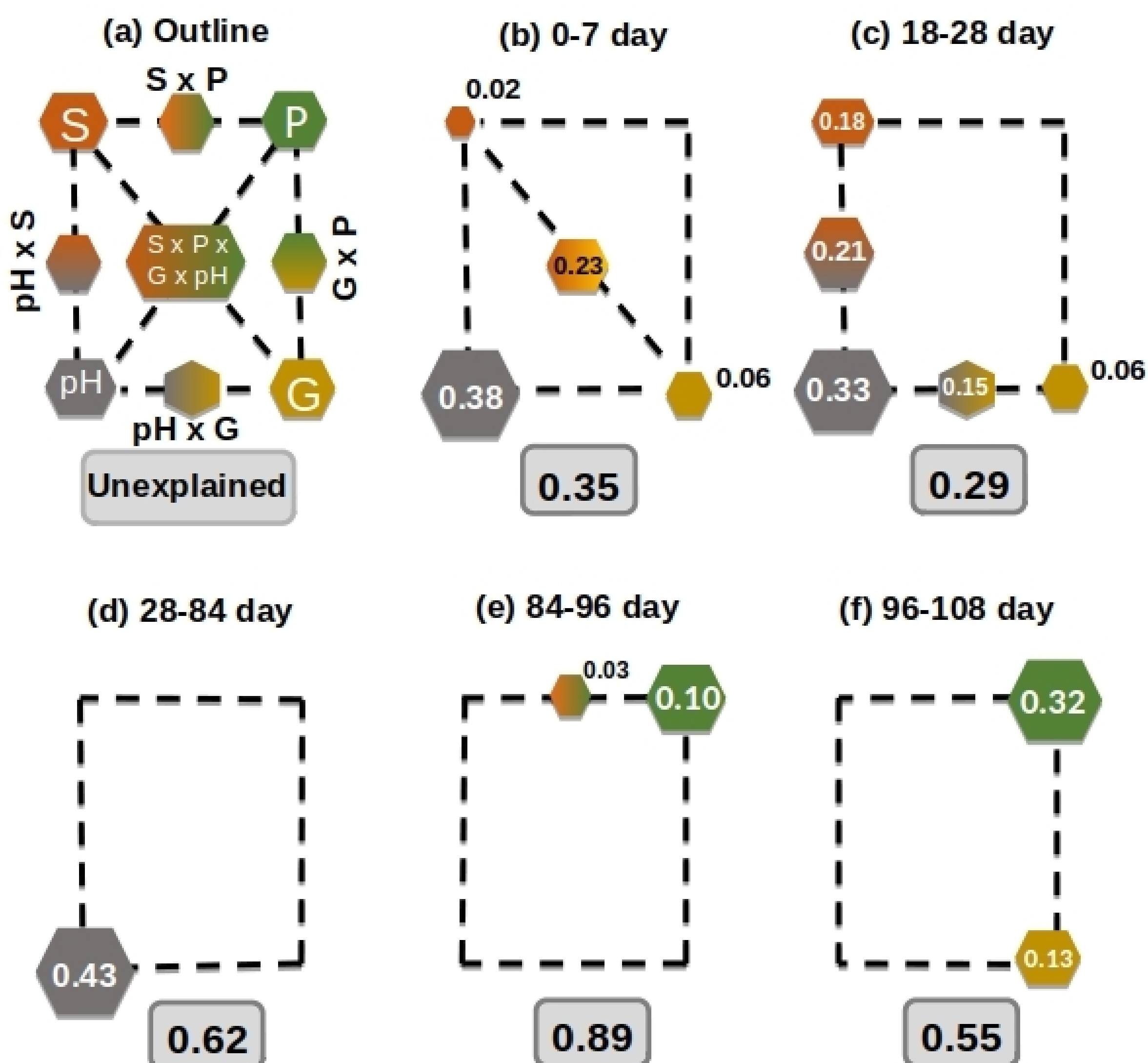
457 indicate that the CH_4 fluxes vary through time and are driven by dynamic factors. Here we
458 decided to separate pH from other soil properties when running this analysis as pH has been
459 previously shown to be a major driver in CH_4 soil fluxes. Our results confirm this as in the
460 beginning of the experiment the pH and other soil physical-chemical properties were the
461 stronger explanatory variables of the CH_4 uptake capacity (Table 1). However, the
462 contribution of the soil properties decreases through time while the presence of the grass and
463 the microbial communities gain in explanatory power.
464



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466 Figure 6: Variation partitioning analysis to determine the drivers of CH_4 fluxes in the Ariquemes soils in
467 time intervals from day 0 to day 7 (b), day 18 to 28 (c), 28-84 (d), 84-96 (e), and 96-1-8 (f). Variance
468 was partitioned into four explanatory variables, soil physical-chemical properties (S), pH, abundance
469 of methanotrophs and methanogens (G), plant biomass (P), and by combinations of these potential
470 predictors as exemplified in the outline (a). Geometric areas are proportional to the respective
471 percentages of explained variation. The corners of the square depict the variation explained by each
472 factor alone, while percentages of variation explained by interactions of two or all factors are indicated
473 on the sides and in the middle of the square, respectively. All numbers represent percentages,
474 graphically represented by the size of the respective hexagons. Only variance fractions ≥ 2% are
475 shown. The variables used for each variation partitioning are indicated in Table 1.

476

477

478 Table 1. Selected explaining variables for the capacity of methane consumption determined
479 with the variation partitioning analyses at five different time-intervals during the
480 Ariquemes experiment.

481

Time interval	Soil properties[a] & pH	Gene abundances	Plant[b]
0-7	pH, OM, Cu, CEC	*mrcA*	NA
18-28	pH, Cu	*pmoA*	NA
28-84	pH, CEC	-	NA
84-96	-	-	Plant biomass
96-108	-	*mcrA*	Plant biomass

482 [a] CEC corresponds to Cation Exchange Capacity, Cu to Copper, and OM to Organic Matter.
483 [b] Plant biomass corresponds to the sum of the above and belowground dry weight.
484

485

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486 **Discussion**

487 Deforestation of the Amazonian forest often followed by the establishment of pastures.
488 This forest-to-pasture conversion affects soil methane cycling, where forest soils that were
489 previously acting as a methane sink now become sources of methane (Fernandes et al.,
490 2002). This study confirms previous field results (Meyer et al., 2020; Fernandes et al., 2002;
491 Goreau; De Mello, 1988; Steudler et al., 1996), although the values obtained in our
492 experiments cannot be directly compared to those reported, since the results are limited to a
493 10 cm surface layer of soil. The trend observed is the same recorded by Steudler et al.
494 (1996), in that forests consume 2.74 more methane than pastures. In our experiments, these
495 values were 0.6-fold in soils from Ariquemes in western Amazonia, and 4.28-fold in soils from
496 Tapajós in eastern Amazonia. This discrepancy may be related to differences in soil
497 microbial communities and chemical properties, but it can also be a consequence of
498 conservation of the forest areas from which these soils originated. While in Ariquemes forests
499 were fragmented and small samples from Tapajós originated from a contiguous forest in a
500 conservation area. Forest fragmentation is known to be associated with increased
501 greenhouse gas emissions (Laurance et al., 1998). Furthermore, the pastures sampled in
502 Ariquemes/RO have a history of long-term management, and the pasture soils sampled in
503 Belterra/PA showed signs of degradation. As management affects the carbon stock in the soil
504 (Fearnside & Imbrozio Barbosa, 1998), it can be expected that it will also affect methane
505 cycling in the soil.

506 The forest-to-pasture conversion alters the physical-chemical properties of the soil,
507 with consequences to microorganisms that produce and consume methane. In field studies in
508 the same region in Ariquemes, a decrease in methanotrophic bacteria and an increase in
509 methanogenic archaea were observed, in addition to changes in the composition of
510 communities, which were attributed at least in part to changes in methane fluxes (Meyer et
511 al., 2017, Meyer et al. 2020). Also, an increase in activity of methanogens in pastures,
512 compared to forest soils, was recorded in soils from the same region (Kroeger et al. 2020).
513 Our results under controlled moisture conditions did not detect significant changes in the
514 methanotrophic community nor changes in the relative abundance of specific methanotrophic
515 groups. However, there was a significant increase in the abundance of methanogenic
516 archaea in pastures compared to forest soils. These results indicate that studies of the

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517 microbiota associated with methane cycling should consider the seasonality of rainfall in the
518 field to better understand the system, such as that developed by Fernandes et al. (2002).
519 Pasture soils in the Amazonian region present a microbial community quite distinct
520 from that observed in forest soils (de Carvalho et al., 2016; Rodrigues et al., 2013; Jesus et
521 al., 2009). This is partly attributed to acidity reduction in the process of establishing pastures.
522 Forest soils in Amazonia have pH (H_2O) values between 3.5 to 4.5 (Demattê and Demattê,
523 1993). The pH is currently understood as one of the main drivers of microbial community
524 structure in soils (Fierer & Jackson, 2006), and increasing it by liming is a strategy to improve
525 fertility and reduce soil toxicity to plants (Oliveira et al., 2013). This process becomes
526 necessary in pasture management to counter the tendency for acidification of pasture soils
527 over time with soil pH reaching values close to those observed in forest areas (de Moraes et
528 al., 1996). In addition, degraded pastures, which can amount to more than 50% of pasture
529 areas in Amazonia (Dias-Filho, 2017), also tend to need acidity correction for their restoration.
530 Little is known about the effects of soil liming on the methane cycling process in
531 tropical soils. It is known that the optimum growth pH of most cultivable methanotrophs and
532 methanogens is neutral (Le Mer & Roger, 2001; Whittenbury et al., 1970), which is why soil
533 pH represents an important explanatory variable for the distribution of methanotrophs.
534 However, methane oxidation is observed in natural environments across a wide pH range
535 (Knief et al., 2003; Kolb, 2009; Nazaries et al., 2013). Our results indicate that the soil acidity
536 correction for pH (H_2O) values close to 6.5 has different effects in pasture or forest soils,
537 possibly because the forest soil has undergone a more intense pH correction, starting at 3.5-
538 5.0 and finishing at 6.5, while in pasture soils the change was from 4.5-5.5 to 6.5. In our forest
539 soils from Ariquemes, we determined a decrease in methane uptake in response to liming,
540 and a shift from uptake to emission in forest soils from Tapajos. Yet, no significant differences
541 were noticed in pasture soils. Thus, liming pasture soils may not impact methane emissions,
542 but still help to maintain the pH of these soils at values suitable for grass biomass
543 productivity. For forest soils, we have shown that the reduction in acidity alone is enough to
544 shift the soil from a methane sink to a source. This change in methane fluxes was not
545 noticeable in the abundance of methanotrophic or methanogenic microorganisms by qPCR,
546 despite a reduction in the relative abundance of methanotrophs that follows the acidity
547 correction.

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548 The identification of microorganisms based on short DNA sequences of the 16S rRNA
549 gene, such as those generated in this study, is limited to the evolutionary information
550 available in that fragment so that it is not always feasible to identify the microorganisms at the
551 genus level. Considering that the ability to oxidize methane is variable at the genus level in
552 the Beijerinckiaceae family, identifying sequences at the family level is not enough to infer if
553 they are methanotrophs. This family also includes generalist bacteria capable of using
554 multiple carbon compounds as an energy source, and here Beijerinckiaceae are more
555 abundant in forest soils than in pastures. Thus, identifying whether they are methanotrophs or
556 not is relevant to understand methane cycling in the forest-to-pasture conversion. The results
557 demonstrate that the Beijerinckiaceae sequences observed in forest soils cluster together in
558 phylogenetic trees. This cluster was observed on the two data sets with high support (> 90%
559 in 1000 bootstraps) and also includes the methanotrophic USCα, which indicates that these
560 sequences are potential methanotrophic Beijerinckiaceae.

561 The differences observed in methane fluxes after liming were not noticed in the
562 abundance of producers and consumers. This discrepancy might be related to a reduction in
563 the activity of forest soil to act as a methane sink after acidity correction, due to the lower
564 availability of Fe and Cu which are necessary as cofactors for the activity of methane-
565 monooxygenase (Semrau et al., 1995). Alternatively, this discrepancy might be due to
566 limitations of the primers, drawn mostly with microbial references from temperate soils, but we
567 applied them to tropical soils. Or, the difference between methane flux and shift in abundance
568 of methane cycling microorganisms can be due to ammonia oxidizers, possibly oxidizing
569 methane at a higher soil pH. We believe that methanotrophs are the group that was affected
570 the most, since acidity correction was followed by a reduction in the consumption of
571 atmospheric methane by the soil (concentrations of ~1.8 ppm). Also, the duration of
572 incubating soil from Ariquemes for 250 days should be long enough to observe compensatory
573 changes due to DNA replication, that should be detected in the DNA quantification analysis.

574 Although there is great natural variability in the methane flux data, the final averages
575 led to the conclusion that pasture soils act as a methane source, which is in fact a commonly
576 reported final result. This observed variability means that pastures could seasonally or by
577 location switch from being a methane source to temporarily becoming a methane sink
578 (Fernandes et al., 2002; Steudler et al., 1996). Our initial hypothesis was that the methane

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579 consuming capacity of pasture soils would be related to intermediate moisture availability in
580 the micro-environments of soil, since soil moisture is a determining factor for methane fluxes
581 in pastures (Verchot et al., 2000). To eliminate moisture variation as a variable in the
582 experiments we set the soil water contents at 70% of the holding capacity in the greenhouse
583 experiments. The variability of pasture gas fluxes could also be explained by grass coverage,
584 a factor associated with pasture management. The management of pastures can influence
585 soil gas fluxes (Figueiredo et al. 2017), since it influences the carbon stocks in the soil
586 (Fearnside & Imbrozio Barbosa, 1998), however the way ongoing pasture management can
587 affect the microbial community remains an open question. Considering that management is
588 performed with the goal of grass productivity, and greater aerial biomass is associated with
589 greater root biomass, we expect that a larger root surface area in pasture would create a
590 more interactive environment with the soil microbiota, and thus enable higher rhizosphere
591 activity. The role of the rhizosphere on methane cycling in upland soils is still poorly
592 understood, and even different plant species can influence the soil by increasing methane
593 oxidation or production, depending on the type of soil or soil conditions (Praeg et al., 2017). In
594 soils of the Ariquemes experiment, we observed that plant cover will lead to a reduced
595 methane flux in both forest and pasture soils compared to those with acidity correction. The
596 methane flux rates with grass cover were similar to those of the original forest soil and tended
597 to be higher than those of pasture without acidity correction. In soils from Tapajós experiment,
598 the same trend was observed in forest soils, but possible due to the shorter duration of this
599 experiment, there were no significant differences in the pasture.

600 When disentangling the contribution of different biotic and abiotic factors to CH_4 soil
601 uptake capacity we found that its drivers change through time, which could explain as
602 previously discussed that soils might change from a source to a sink. While pH and other soil
603 properties explained most of the variance in the beginning of the greenhouse experiment, the
604 abundance of microbial communities related to CH_4 fluxes and plant biomass explained most
605 of the CH_4 uptake at the end of the experiment. These results suggests that our treatments
606 (liming and planting grass) are causing a reorganization of the microbial communities and
607 while the soil properties are initially the main variables explaining the CH_4 fluxes, after a
608 couple of weeks the biotic factors are the main drivers of CH_4 fluxes in these soils. While a
609 previous study showed that peak emissions of the green-house gas N_2O can be driven by the

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610 microorganisms related to the production and reduction of this green-house gas (Domeignoz-
611 Horta, et al., 2017), our results show how microorganisms related to methane cycling and
612 plant cover play a role to understand the temporal dynamics of CH_4 uptake in soils. These
613 results highlight the need for better characterizing microbial communities to increase our
614 understanding of the relationship between abundance and diversity of microorganisms and
615 their corresponding processes.

616 The results presented here demonstrate that soil acidity is an important factor for
617 methane sequestration in tropical soils, as the acidity correction reduces this capacity. In
618 pastures, the effect of the acidity correction is less consequential compared to the presence
619 of grass coverage. This demonstrates that the correction of acidity in pastures, if combined
620 with constant soil coverage with grass, would have little or no impact on methane emissions
621 while improving soil structure and increasing nutrient availability, soil organic matter and grass
622 productivity.

623

624 **Conclusion**

625 Our results suggest that the impact of methane emissions from pastures in the
626 Amazonian region can be mitigated through pasture management, specifically by keeping the
627 soil always covered with grass. The rhizosphere of *Urochloa brizantha* cv. Marandu affects
628 soil microbial communities by lowering the abundance of methanogenic archaea up to 10
629 times compared to the bare soil. The affected methanogens are composed of
630 *Methanobacterium spp., Methanocella spp., Rice Cluster I, and Methanosarcina spp*. In
631 addition, we demonstrate that the correction of acidity in pasture soils can reduce methane
632 sequestration under atmospheric methane concentrations (high-affinity methanotrophs).
633 Therefore, the level of acidity correction should be considered as a factor for additional
634 emissions of greenhouse gases. In the acidic forest soils, an increase in pH reduced methane
635 sequestration by more than 50%, thereby reversing the flux direction to turn forest soil from a
636 methane sink into a source. Field studies with liming and a focus on the grass rhizosphere
637 under seasonal conditions are urgently needed to provide specific recommendations to
638 policymakers and farmers.

639

640

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650

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