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Belowground changes to community structure alter methane-cycling dynamics in Amazonia

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ABSTRACT

Amazonian rainforest is undergoing increasing rates of deforestation, driven primarily by cattle pasture expansion. Forest-to-pasture conversion has been associated with changes to ecosystem processes, including substantial increases in soil methane (CH_4) emission. The drivers of this change in CH_4 flux are not well understood. To address this knowledge gap, we measured soil CH_4 flux, environmental conditions, and belowground microbial community attributes across a land use change gradient (old growth primary forest, cattle pasture, and secondary forest regrowth) in two Amazon Basin regions. Primary forest soils exhibited CH_4 uptake at modest rates, while pasture soils exhibited CH_4 emission at high but variable rates. Secondary forest soils exhibited low rates of CH_4 uptake, suggesting that forest regrowth following pasture abandonment could reverse the CH_4 sink-to-source trend. While few environmental variables were significantly associated with CH_4 flux, we identified numerous microbial community attributes in the surface soil that explained substantial variation in CH_4 flux with land use change. Among the strongest predictors were the relative abundance and diversity of methanogens, which both increased in pasture relative to forests. We further identified individual taxa that were associated with CH_4 fluxes and which collectively explained ~50% of flux variance. These taxa included methanogens and methanotrophs, as well as taxa that may indirectly influence CH_4 flux through acetate production, iron reduction, and nitrogen transformations. Each land type had a unique subset of taxa associated with CH_4 fluxes, suggesting that land use change alters CH_4 cycling through shifts in microbial community composition. Taken together, our results suggest that changes in CH_4 flux from agricultural conversion could be driven by microbial responses to land use change

in the surface soil, with both direct and indirect effects on CH_4 cycling. This demonstrates the central role of microorganisms in mediating ecosystem responses to land use change in the Amazon Basin.

INTRODUCTION

After a decade of slowing rates of deforestation, the Amazon rainforest is again undergoing high rates of deforestation, driven primarily by agricultural expansion for cattle pasture (Dirzo & Raven 2003; Laurance *et al.* 2014; Barlow *et al.* 2019). Such forms of environmental change are known to alter belowground microbial biodiversity (Rodrigues *et al.* 2013; Mueller *et al.* 2016; Meyer *et al.* 2017) as well as microbially-mediated biogeochemical cycles (Neill *et al.* 1997b, 2005; Verchot *et al.* 1999), including the methane (CH_4) cycle(Neill *et al.* 1997, 2005; Verchot *et al.* 1999). Rainforest soils in the western Amazon Basin switch from acting as a sink for atmospheric CH_4 to a persistent source of CH_4 following conversion (Steudler *et al.* 1996; Fernandes *et al.* 2002), and little is known about whether the CH_4 sink capacity returns following pasture abandonment and secondary forest regeneration. This sink-to-source phenomenon has also been documented in the Eastern Amazon (Keller *et al.* 1986; Verchot *et al.* 2000), suggesting a general functional response to cattle pasture establishment. This is of concern considering recent increases in agricultural conversion throughout the Amazon Basin (Carvalho *et al.* 2019), and the fact that CH_4 is a potent greenhouse gas, with roughly 34 times the global warming potential of CO_2 over a 100-year timeframe (Myhre *et al.* 2013). Although responses of belowground microbial communities and CH_4 flux to land use change have both been documented in the Amazon Basin (Keller *et al.* 1986; Steudler *et al.* 1996; Verchot *et al.* 2000; Fernandes *et al.* 2002; Meyer *et al.* 2017), the relationship between these two responses is not well understood, in part because no study has measured microbial community attributes and CH_4 flux simultaneously.

Soil CH_4 flux results from two counter-acting microbial processes: CH_4 production (methanogenesis) and CH_4 consumption (methanotrophy) (Conrad 2009).

Methanogens are Archaea that anaerobically produce CH_4 using either acetate, methylated compounds, formate, or H_2 and CO_2 (Hedderich & Whitman 2013). Methanogens have been shown to increase in relative abundance following conversion to cattle pasture, as well as undergo compositional changes that may indicate a shift in the predominance of methanogenic pathways (Meyer *et al.* 2017). Aerobic methanotrophs are Bacteria in the Alpha- and Gamma-Proteobacteria and Verrucomicrobia that consume CH_4 via the serine, ribulose monophosphate (RuMP), or Calvin-Benson-Bassham pathways, respectively (Knief 2015). Methanotrophs have also been reported to strongly respond to land use change in the Amazon, including decreases in population abundance and alterations to community composition (Meyer *et al.* 2017).

Methanogens and methanotrophs are the only groups to directly cycle CH_4, but these organisms form complex ecological interactions with other community members and this may influence the rate or directionality of CH_4 flux. For example, methanogens depend on metabolic byproducts (e.g. H_2 and CO_2, or acetate) derived from the activity of other community members such as acetogens or fermentative bacteria (Müller & Frerichs 2013). Methanogens are often outcompeted by other community members for these substrates when more thermodynamically favorable terminal electron acceptors are available, including NO_3^-, NO_2, and Fe (II), (Cord-Ruwisch *et al.* 1988; Chen & Lin 1993; Klüber & Conrad 1998). The activity of aerobic methanotrophs can also depend on community interactions, such as competition for O_2 or soil nitrogen (N) (Bodelier *et al.* 2000; Bodelier & Steenbergh 2014; Ho *et al.* 2016), or predation by protozoa or viruses (Tyutikov *et al.* 1980; Murase & Frenzel 2008). To date, few have sought to relate broader community interactions to soil CH_4 emissions.

There is growing interest in better understanding ecosystem functions using microbial community measurements (McGuire & Treseder 2010; Bier *et al.* 2015; Hall *et al.* 2018), but attempts have generated mixed results (Rocca *et al.* 2015; Graham *et al.* 2016; Louca *et al.* 2018). Microbial taxa can be artefactually related to CH_4 flux due to covariation with environmental conditions that alter function, or through spatial auto-correlation (Legendre 1993), and this covariance structure could blur the connection between communities and function (Morris *et al.* 2019). Accounting for covariance structure has been shown to aid in detecting microbial taxa or community attributes that are causally associated with CH_4 processes (Meyer *et al.* 2019). One way to do so uses principle components analysis to derive environmental, spatial, and community structure covariates for incorporation into statistical models (Price *et al.* 2006; Morris *et al.* 2019). Applying this approach could help clarify the relationship between environmental change and community functional responses, especially in ecosystems such as those of the Amazon Basin, where many variables exhibit change following ecosystem conversion.

This study focuses on a gradient of land use change in two regions of the Amazon Basin. We combine measurements of *in situ* CH_4 flux, soil chemistry, and microbial community structure across primary rainforest, cattle pasture, and secondary forest (derived from abandoned cattle pasture). We first ask how land use change alters soil CH_4 flux and the community structure of bacteria and archaea (including CH_4-cycling organisms). We then investigate the relationships between environmental variables, microbial community attributes, and CH_4 flux, in order to identify mechanisms that link land use change to changes in CH_4 flux. Our study provides an important window into a

poorly understood phenomenon that is likely to become increasingly common throughout the Amazon Basin if rates of land use change continue to increase.

METHODS

Site description, sampling design, sampling dates

Our study was performed in two regions of the Amazon Basin: the state of Rondônia in the Western Amazon, and the state of Pará in the Eastern Amazon. Both states have experienced the highest rates of forest loss in Brazil, largely driven by agricultural expansion for cattle ranching (Soares-Filho *et al.* 2006; Ometto *et al.* 2011; Carvalho *et al.* 2019). In Rondônia, we surveyed three primary forest sites, three cattle pasture sites, and two secondary forest sites, totaling 39 sampling locations, all in or directly adjacent to Fazenda Nova Vida, about 250 km south of Porto Velho. The climate at Fazenda Nova Vida is humid tropical, and receives 2200 mm annual mean precipitation (Steudler *et al.* 1996). Soils are red-yellow padzolic latosol with sandy clay loam texture, and are described in detail elsewhere (Neill *et al.* 1997a). Vegetation type is open moist tropical forest with palms, and is described elsewhere (Pires & Prance 1985). In Pará we surveyed two primary forest sites, three cattle pasture sites, and three secondary forest sites, totaling 33 sampling locations. Pará sites were in or around Tapajós National Park, which receives roughly 2000 mm annual mean precipitation. Soils there have been characterized as ultisols and oxisols in flat areas, and inceptisols in areas with topographic relief, and have been further described alongside floristic descriptions elsewhere (Parrotta *et al.* 1995; Silver *et al.* 2000; Keller *et al.* 2005). We strove to sample forests, pastures, and secondary forests equally, but faced restrictions due to

varying land ownership and logistical issues. At each site, we established a 200 m transect and performed paired sampling of gases and soil at 50 m intervals, with 5 locations for measurements and sampling per transect. Sampling in Pará and Rondônia took place during wet season periods, in June 2016 and March/April of 2017, respectively. GPS coordinates of each sampling point can be found in Supplementary Table 1.

At each sampling location soil CH_4 flux was measured in real time using a field-deployable Fourier-transform infrared spectrometer (Gasmet, DX 4015, Vantaa, Finland) connected to a flow-through soil flux chamber in a closed recirculating loop. Soil collars (aluminum, inner area of 284 cm^2) were installed roughly 5 cm into the soil surface at least 20 minutes before CH_4 concentration measurements began. Soil flux chambers were connected via inlet and outlet ports to the CH_4 analyzer and were placed on the soil collars. CH_4 fluxes were determined by the rate of accumulation or removal of CH_4 in the flux chamber headspace over a 30-minute period. Trends in CH_4 concentration over time varied from linear to non-linear. If trends in CH_4 concentration over time were linear, a linear model was used to calculate flux. If trends were non-linear, we used the linear portion of the data near the time of chamber placement to calculate flux (Salimon *et al.* 2004; Pirk *et al.* 2016).

Directly following gas flux measurement, soil samples were taken with a sterilized corer (5 cm diameter x 10 cm length) positioned under the chamber and another four cores forming a square around the chamber at ~25 cm distance, to capture community heterogeneity surrounding the chamber area. The five soil cores were emptied into a 4 l plastic bag, then mixed by hand from the outside of the bag following root

removal. Two 200 g samples of this soil mixture were placed into new sample bags and either frozen for DNA extraction or stored at 4° C for soil chemical analysis.

Soil chemical analysis

We assessed 19 soil chemical attributes for use as environmental covariates. Soil chemical analyses were performed at the Laboratory of Soil Analysis at "Luiz de Queiroz" College of Agriculture (ESALQ/USP; Piracicaba, Brazil), following the methodology described by (van Raij *et al.* 2001). Soil chemical parameters included pH, organic matter, P, S, K, Ca, Mg, Al, H^+, Al, sum of bases, cation exchange capacity, base saturation (% V), Al saturation, Cu, Fe, Mn, Zn, and total N. All soil chemical data can be accessed in Supplementary Table 1. For one forest site in Pará, soil chemical data are missing due to a sample transport error. These samples were excluded from microbial analyses requiring environmental covariates, but were included for analyses independent of environmental data (i.e. community structure).

Soil DNA extraction

Total DNA from each sample was extracted from 0.25 g soil using the DNeasy PowerSoil kit (Qiagen Inc., Valencia, CA, USA) following manufacturer's instructions. Soils from Pará sites required two subtle modifications, based on Venturini *et al*. (2019): 1) vortexing was performed for 15 minutes, instead of 10 minutes, and 2) all incubations steps were at -20° C degrees, instead of 4° C. It is possible that these subtle modifications could influence our results, but they were necessary to obtain quantifiable amounts of

DNA, likely due to soil inhibitors such as humic acids. DNA yield from each extraction was fluorometrically quantified (Qubit, Life Technologies, USA).

Soil prokaryotic community structure assessment

In order to assess the community structure and diversity of soil prokaryotes in each sample, we performed Illumina Miseq 300 basepair paired-end sequencing of the V4 region of the 16S rRNA gene using the 515F - 806R primer combination (Caporaso *et al.* 2011) at the University of Oregon Genomics Core Facility. PCR mixtures were: 12.5 µl NEBNext Q5 Hot Start HiFi PCR master mix, 11.5 µl primer mixture (1.09 µM concentration), and 1 µl of DNA template (total of 17.5 ng DNA per reaction). Reaction conditions were: 98° C for 30s (initialization), 98° C for 10s (denaturation), 61° C for 20s (annealing), and 72° C for 20s (final extension). Reactions were run for 20 cycles and amplicons were purified using 20 µl Mag-Bind RxnPure Plus isolation beads (Omega Bio-Tek, USA). Sequencing libraries were prepared using a dual-indexing approach (Kozich *et al.* 2013; Fadrosh *et al.* 2014), and samples were pooled at equimolar concentration. The final library was sequenced at a concentration of 3.312 ng/µl.

Paired sequence reads were merged using PEAR (version 0.9.10) with default parameters (Zhang *et al.* 2014). Merged reads were filtered by length (retaining read lengths of 230-350 basepairs) and quality (retaining only reads with quality score >30) using Prinseq (Schmieder & Edwards 2011). Filtered sequences were checked for chimeras, denoised, and collected into amplicon sequence variants (ASVs) using DADA2 (Version 1.6) (Callahan *et al.* 2016) implemented in QIIME2 (Bolyen *et al.* 2019).

Taxonomy was assigned to ASVs using the RDP naïve Bayesian rRNA classifier Version 2.11 (Wang *et al.* 2007; Cole *et al.* 2014) with training set 16.

Quantitative PCR of methanogens and methanotrophs

We estimated the abundance of methanogens and methanotrophs using quantitative PCR (qPCR) of marker genes. For methanogens, we targeted the *mcrA* gene using the mlas-mcraRev primer combination (Steinberg & Regan 2008). For methanotrophs, we targeted the *pmoA* gene using the A189 – mb661 primer combination (Bourne *et al.* 2001). DNA from each soil sample was amplified in triplicate using a blocked design whereby all 72 samples (as well as positive and negative controls) were run in a single 96-well plate, repeated three times. Reactions were run on a Bio-Rad CFX96 real-time qPCR instrument (Bio-Rad, USA), using Sso Advanced Universal SYBR Green Supermix reagents (Bio-Rad, USA). Reaction conditions were optimized using an annealing temperature gradient. For each reaction, 2 ng of DNA were used and reactions took place under the following conditions: 98° C 10 minutes (initialization), 98° C 15 seconds (denaturation), 55.6° C 15 seconds (annealing), 72° C 60 seconds (final extension). For both genes, sample amplification was compared to a standard positive control to calculate gene copy number. For *pmoA* the positive control was genomic DNA from *Methylococcus capsulatus* Foster and Davis (ATCC 33009D-5). For *mcrA* the positive control was a *mcrA* copy ligated into a vector. We used LinRegPCR (Ramakers *et al.* 2003; Ruijter *et al.* 2009) to process amplification data, which calculates individual PCR efficiencies. Individual PCR efficiencies were significantly different among regions (Rondônia versus Pará), so the average PCR efficiency for each region was used to

274 calculate gene copy number. To account for plate-to-plate variation (among technical
275 replicates) gene count values for each sample were residualized (by subtracting the mean
276 copy number per plate), then averaged.
277
278 *Statistical methods*
279 All statistics were performed in the R statistical environment (R Core Team
280 2018). CH_4 flux and community differences among regions and land types were assessed
281 using a Kruskal-Wallis test, which does not rely on assumptions of distribution and can
282 handle imbalanced sampling designs. Pairwise differences among groups were assessed
283 using Dunn's test for multiple comparisons. Differences in community structure were
284 assessed with a PERMANOVA test using the 'adonis' function in the vegan package in R
285 (Oksanen *et al.* 2015).
286 Sequence depth per sample ranged from 62,865 to 148,053 sequences per sample,
287 median: 77,653. To account for these differences in sampling depth, the community
288 matrix was rarefied to 62,800 counts per sample ten times and averaged, which did not
289 exclude any samples. The rarefied community matrix was also subsetted for known
290 methanogens and methanotrophs (Supplementary Table 2). We compiled a table of
291 microbial community attributes that represent putative controls on CH_4 emissions,
292 including abundance, diversity, and composition (Table 1).
293 We tested for a relationship between the relative abundance of each taxon and
294 CH_4 flux. To account for systematic differences in taxon relative abundances due to
295 species interactions, local environmental selection, dispersal history between sites, and
296 other factors unrelated to CH_4 dynamics, we performed a principal components (PC)

297 correction using the community, environmental, and spatial variables with the 'prcomp'
298 function in R (Price *et al.* 2006; Morris *et al.* 2019). For the environmental covariates, we
299 included all soil chemical variables that were shared across samples and that had no
300 missing values and scaled them to unit variance (to account for differences in units of
301 measurement). To account for community structure, we performed principal components
302 analysis (PCA) on the rarefied 16S rRNA gene community matrix following Hellinger
303 transformation and after scaling for unit variance. Spatial coordinates (latitude and
304 longitude) of each sample were assigned a PC score following the same procedure. CH_4
305 fluxes, the relative abundance of each taxon in the community matrix, and each
306 community attribute were then adjusted by the principal components for each covariate
307 (community, environment, and geography). This principal components correction
308 removed the correlation between CH_4 flux and community similarity, environmental
309 similarity, and spatial proximity as well as the correlation between taxon relative
310 abundance and each of the covariates, allowing us to test the unique contribution of each
311 taxon to CH_4 flux independent of these underlying factors (Price et al. 2006; Morris et al.
312 2019). We regressed each corrected taxon or community attribute against $\log_{10}$-
313 transformed CH_4 fluxes, and applied a Bonferroni correction (alpha = 0.05) to
314 conservatively address the issue of false positives associated with large numbers of
315 comparisons. Taxa significantly correlated with CH_4 flux were subsetted from the rarefied
316 community matrix and reduced to a single variable using PCA, and then regressed against
317 $\log_{10}$-transformed CH_4 fluxes. Model fit (R^2) was assessed after confirming normal
318 distribution of residuals. In several instances one or two high leverage outliers, i.e.

319 "influential outliers" (as defined by Aguinis *et al.* 2013), were removed due to their
320 strong and disproportionate influence on model fit (R^2).
321
322 **RESULTS**
323 ***CH_4 flux and microbial community attributes differ across land types***
324 CH_4 fluxes were significantly different across land types in both regions (Kruskal
325 Wallis Chi-squared = 33.98, df = 5, $p < 0.001$, Fig. 1A). In both regions, pasture soils
326 emitted CH_4 at higher rates than primary forest or secondary forest soils (Dunn test for
327 multiple comparisons $p < 0.001$). Combining land types from the two regions, the same
328 pattern emerges, i.e. CH_4 emissions vary by land type (Chi-squared = 25.11, df = 2, $p <$
329 0.001, Fig. 1B) and pastures emit CH_4 at significantly higher rates (Dunn test $p < 0.001$)
330 than primary or secondary forests. Of the 25 pasture measurements, only one exhibited
331 CH_4 uptake (−11 μg CH_4 m^{-2} d^{-1}). In Rondônia, all pasture fluxes were positive, with rates
332 ranging from 30 to 40,000 μg CH_4 m^{-2} d^{-1} (mean = 5,695.3 ± 11,860.5 μg CH_4 m^{-2} d^{-1}). In
333 Pará, pasture emissions were lower, ranging from −11 to 400 μg CH_4 m^{-2} d^{-1} (mean 93.6 ±
334 157.9 μg CH_4 m^{-2} d^{-1}). CH_4 fluxes in the Rondônia primary forests ranged from −160 to
335 550 μg CH_4 m^{-2} d^{-1} (mean = 22 ± 156.2 μg CH_4 m^{-2} d^{-1}), with four of the fifteen
336 measurements exhibiting uptake, three exhibiting near zero fluxes, and eight emitting
337 CH_4. Six out of the ten measurements in Pará primary forests exhibited CH_4 uptake, one
338 had a near zero flux, and three had low levels of emission, ranging from -30 to 8 μg CH_4
339 m^{-2} d^{-1} (mean −8.6 +/− 13.2 μg CH_4 m^{-2} d^{-1}). Secondary forests in both regions exhibited
340 CH_4 uptake on average (Rondônia: mean -17.8 ± 37.7 μg CH_4 m^{-2} d^{-1}, Pará: mean −4.9 ±
341 34.8 μg CH_4 m^{-2} d^{-1}). Flux values for secondary forest soils in Rondônia ranged from −80

342 to 30 μg CH_4 m^{-2} d^{-1}, while fluxes from secondary forest soils in Pará ranged from −54 to
343 61 μg CH_4 m^{-2} d^{-1}.

344 Our taxonomic survey identified 30,809 prokaryotic (bacterial and archaeal)
345 amplicon sequence variants (ASVs) across the 72 soil samples. Prokaryotic community
346 structure differed by land type (i.e. primary forest, cattle pasture, or secondary forest,
347 PERMANOVA on Bray-Curtis dissimilarities: $F_{2,68} = 9.4$, $R^2 = 0.18$, $p < 0.001$), as well
348 as by region (i.e. Rondônia vs Pará, $F_{1,68} = 15.6$, $R^2 = 0.15$, $p < 0.001$, Supp. Fig. 1).
349 Taxonomic richness (ASV level) also differed by region and land type (Chi-squared =
350 54.07, $p < 0.001$, Supp. Fig. 2). Across regions, richness values were higher in Rondônia
351 (across all three land types) than Pará (all comparisons Dunn test $p < 0.001$). In
352 Rondônia, cattle pastures and secondary forests had significantly higher richness than
353 primary forests, while in Pará, pastures were the richest, and primary and secondary
354 forests were statistically indistinguishable, but lower than pasture.

355 Land use change drove numerous alterations to the diversity, abundance, and
356 composition of CH_4-cycling communities. Methanogen ASV-level richness significantly
357 increased in pastures relative to forest and secondary forest in both regions (Chi-squared
358 = 28.86, df = 2, $p < 0.001$, Fig. 2A). The abundance of methanogens (copies of *mcrA* per
359 g soil) varied by land type and region (Chi-squared = 45.62, df = 5, $p < 0.001$), and was
360 higher in pastures relative to primary forests (Rondônia: $z = -4.24$, $p < 0.001$, Pará: $z =$
361 -3.91, $p < 0.001$) and secondary forests of Pará relative to pasture ($z = -4.37$, $p < 0.001$).
362 A similar trend was observed for methanogen relative abundance (in the 16S rRNA gene-
363 derived community) in Rondônia, but in Pará, primary forest and pasture, levels were
364 indistinguishable ($p > 0.05$) and secondary forest abundances were significantly lower

than in primary forests or pastures ($z = 3.73$, $p < 0.001$; $z = 2.55$ $p < 0.01$, Fig. 2B). Methanogen community composition varied by land type (PERMANOVA on Bray-Curtis dissimilarities: $R^2 = 0.18$, $p < 0.001$) and region ($R^2 = 0.06$, $p < 0.001$). Most notably, the genera *Methanocella*, *Methanobacterium*, and *Methanosarcina* were almost exclusively detected in cattle pastures of both regions. The genus *Methanomassiliicoccus* varied by land type and region (Chi-squared = 29.18, df = 5, $p < 0.001$), driven primarily by high abundances in the primary forest sites of Pará ($p < 0.001$ for all comparisons).

Methanotroph ASV-level richness also varied by land use (Chi-squared = 18.03, df = 2, $p < 0.001$), decreasing from primary forest to pasture in both Rondônia and Pará ($z = 2.01$, $p = 0.02$; $z = 3.49$, $p < 0.001$, respectively). Secondary forest values of methanotroph richness in Rondônia recovered to a level that was statistically indistinguishable from forest ($p > 0.05$), while in Pará levels were higher than in pastures ($z = 2.78$, $p < 0.01$), but still lower than primary forests ($z = -1.76$, $p = 0.04$, Fig. 2C). Methanotroph relative abundance was significantly lower in pasture than forest in both Rondônia and Pará ($z = 2.45$, $p < 0.01$; $z = 4.64$, $p < 0.001$, respectively). In Rondônia, secondary forest methanotroph relative abundance levels were indistinguishable from primary forest, whereas in Pará levels were significantly lower than primary forest ($z = -2.56$, $p < 0.01$), but higher than pasture ($z = 2.37$, $p < 0.01$, Fig. 2D). Methanotroph abundance estimates derived from qPCR of the *pmoA* gene showed a similar, but less pronounced trend across regions and land types (Chi-squared = 10.87, df = 5, $p = 0.05$). Methanotroph composition varied by land use ($R^2 = 0.16$, $p < 0.001$), and by region ($R^2 = 0.06$, $p < 0.001$). Most notably, the relative abundance of the genera *Methylocella* (Alphaproteobacteria) and *Methylogaea* (Gammaproteobacteria) were significantly lower

388 in pastures relative to forest in both regions (*Methylocella*: Rondônia: z = 3.6, $p < 0.001$;
389 Para: z = 2.13, $p < 0.05$; *Methylogaea*: Rondônia: z = 3.51, $p < 0.001$; Pará: z = 3.86, $p <$
390 0.001), and increased in secondary forests (*Methylocella*: Rondônia: z = -3.85, $p < 0.001$;
391 Pará: z = −1.65, $p < 0.05$; *Methylogaea*: Rondônia: z = -2.67, $p < 0.01$; Pará: z = -1.91, p
392 < 0.05). Lastly, the proportion of methanotrophs in the CH_4-cycling community (i.e.
393 methanotroph relative abundance divided by the combined relative abundances of
394 methanotrophs and methanogens) was lower in pastures in both regions, but this was only
395 significant in Rondônia (z = 4.71, $p < 0.001$), and secondary forest levels were higher
396 than pasture levels in both regions (Rondônia: z = -5.51, $p < 0.001$; Pará z = -3.22, $p <$
397 0.001).
398
399 ***Microbial abundance and diversity are associated with CH_4 flux***
400 We first asked whether measurements of abundance or diversity (of CH_4-cycling
401 taxa or the community as whole) could explain variance in CH_4 flux after accounting for
402 sample covariance structure. Among the best predicting attributes were the ASV-level
403 richness (R^2= 0.42, $p < 0.001$, Fig. 3A) and relative abundance (R^2= 0.42, $p < 0.001$, Fig.
404 3B) of methanogens. These were both positive relationships, whereby sites with more
405 abundant and/or diverse populations of methanogens tended to emit CH_4 at higher rates.
406 The proportion of methanotrophs in the CH_4-cycling community was negatively
407 associated with CH_4 flux (R^2 = 0.36, $p < 0.001$, Supp. Fig. 3); however, this relationship
408 was no longer significant after accounting for covariance structure (p = 0.07). No other
409 methanotroph community attributes were related to CH_4 flux, despite exhibiting strong
410 changes across sites. The only environmental variables significantly associated with CH_4

411 flux were pH (R^2= 0.08, $p < 0.05$), Zn (R^2= 0.21, $p < 0.001$), and Mn (R^2= 0.20, $p <$
412 0.001), all exhibiting positive relationships.
413
414 ***Taxa associated with CH_4 flux in each land type***
415 We next sought to identify taxa associated with CH_4 fluxes independent of
416 environmental, spatial, and community covariance structure. We performed our analysis
417 on two datasets: 1) subsets by land type (i.e. primary forest, pasture, and secondary
418 forest) to ask if emissions are controlled by different community members across land
419 types, and 2) across all samples combined. In forest sites we identified 41 (Supp. Table 3)
420 ASVs that together explained 55% of the forest CH_4 flux variance ($p < 0.001$). None of
421 the taxa are canonically associated with CH_4 cycling. These taxa included one member of
422 the Thaumarchaeota (Nitrosphaera), and members of eight bacterial phyla, including
423 Acidobacteria, Actinobacteria, Chloroflexi, Firmicutes, Gemmatimonadetes,
424 Planctomycetes, Proteobacteria (divisions Alpha, Beta, Delta, and Gamma), and
425 Verrucomicrobia.
426 526 taxa across 25 phyla (Supp. Table 4) were associated with pasture CH_4 fluxes.
427 Only 9 of these taxa are known to directly cycle CH_4, including 6 methanogens belonging
428 to the genera *Methanocella*, *Methanobacterium*, and *Methanomassiliicoccus*, and 3
429 Gammproteobacteria methanotrophs belonging to the genera *Methylobacter*,
430 *Methylocaldum*, and *Methylococcus*. Two members of the Crenarchaeota (genus
431 *Thermofilum*) were also among the taxa selected. Collectively the 526 taxa explained
432 87% of pasture emission variance (regression of subsetted PC1, $p < 0.001$), following

433 removal of one high leverage outlier. There was no overlap at the ASV level between the
434 taxa identified for the primary forest and the pasture sites.
435 For the secondary forest sites, no taxa passed the p value cutoff from our
436 Bonferroni correction ($p < 3.95 \times 10^{-6}$). We relaxed this threshold to $p < 0.001$ and
437 identified six taxa (Supp. Table 5), including a member of *Acidobacteria* group 13, and
438 members of the genera *Gaiella* (Actinobacteria), *Actinallomurus* (Actinobacteria),
439 *Rhodoplanes* (Alphaproteobacteria), *Nitrospirillum* (Alphaproteobacteria), and
440 *Desulfacinum* (Deltaproteobacteria). None of these taxa were associated with primary
441 forest fluxes and only one (*Gaiella*) was associated with pasture fluxes. Collectively,
442 these taxa when reduced to a single variable explain 38% of the CH_4 flux variance in
443 secondary forests ($p = 0.001$).
444
445 ***Taxa associated with CH_4 flux across land types***
446 Lastly, we performed the above-detailed procedure across all three land types in
447 both regions and identified 654 taxa associated with CH_4 flux (Supp. Table 5). We
448 subsetted all significant taxa from the community matrix, ordinated them, and regressed
449 their PC1 against CH_4 flux, and the resulting model explained 50.0% ($p < 0.001$) of the
450 CH_4 flux variance after removal of one high leverage sample (Fig. 4). Many taxa
451 identified were found in the pasture subset, indicating that pasture samples have a large
452 influence over which taxa are chosen. Eleven methanogen taxa were identified, including
453 members of the genera *Methanocella*, *Methanobacterium*, *Methanosarcina,* and
454 *Methanomassiliicoccus,* comprising 1.7% of identified taxa. Four methanotroph taxa
455 were identified, including members of the genera *Methylocystis* (Alphaproteobacteria),

Methylobacter, *Methylocaldum*, and *Methylococcus* (all in the Gammaproteobacteria), together comprising 0.6% of taxa identified. However, the majority of taxa identified are not known to directly cycle CH_4. Six (0.9%) of the taxa identified are members of the acetogenic genera *Acetonema* (Firmicutes), *Thermacetogenium* (Firmicutes), *Clostridium* (Firmicutes), *Sporomusa* (Firmicutes), and five members of the acetic acid bacteria family Acetobacteraceae. We also identified a member of the anaerobic iron-reducing genus *Geothrix* (Acidobacteria, family Holophagaceae). Lastly, six (0.9%) of the identified taxa play roles nitrogen cycling, including members of the diazotroph genus *Nitrospirillum* (Alphaproteobacteria), a member of the genus of denitrifying bacteria *Denitratisoma* (Betaproteobacteria), ammonia oxidizers from the genera *Nitrosospira* (Betaproteobacteria) and *Nitrosococcus* (Gammaproteobacteria), and members of the nitrite-oxidizing genera *Nitrospira* (Nitrospirae) and *Nitrolancea* (Chloroflexi).

DISCUSSION

Microbial communities drive biogeochemical cycles, including the CH_4 cycle, but understanding how environmental change influences this relationship remains a crucial challenge. Our results suggest that alterations to microbial community structure resulting from land use change are driving changes to soil CH_4-cycling dynamics in Amazon rainforest soils, and thus play a role in the switch from CH_4 sink to source, as well as the recovery following land abandonment and secondary forest regeneration.

The identity of community members can be an important determinant of ecosystem function (Wardle *et al.* 2011; Díaz *et al.* 2016; Bannar-Martin *et al.* 2018), particularly when species differ in physiological traits such as resource use, allocation,

and acquisition (Malik *et al.* 2020). Functional differences among communities can arise when the arrival or persistence of optimal taxa or traits is restricted spatially or temporally (e.g. through dispersal limitation, environmental filtering, or differences in community assembly history). Our results provide compelling evidence for compositional control on the CH_4 cycle. For example, we identified several methanogens and methanotrophs that were highly associated with CH_4 flux, suggesting that these taxa disproportionately influence CH_4 cycling. This included methanogens in the *Methanobacteria*, *Methanocella*, and *Methanosarcina*; all of which increased in relative abundance in pastures relative to forested sites. The methanotrophs identified by our approach also exhibited considerable variation across land types and could influence the flux of CH_4. For instance, pastures showed increased relative abundance of the genus *Methylocaldum*, and decreased relative abundance of the genus *Methylococcus*. These taxa are known to differ from other methanotrophs in traits related to competitive ability and disturbance tolerance (Ho *et al.* 2013; Knief 2015). Although it is not possible to assess the traits of these organisms from our taxonomic survey, our results suggest that a better understanding of the characteristics of these taxa could improve predictions of CH_4 cycling.

The majority of the taxa we identified as associated with CH_4 flux are not known to directly cycle CH_4, highlighting the importance of considering CH_4-cycling organisms in a broader community context. Methanogens utilize and compete for metabolic byproducts, including H_2 and acetate (Westermann *et al.* 1989; Hedderich & Whitman 2013). Six of the taxa associated with CH_4 flux belong to known acetogenic genera (Müller & Frerichs 2013), which is consistent with suggestions that syntrophic

502 interactions could regulate the CH_4 cycle (Conrad 1996). We also identified several taxa
503 that could impact the thermodynamic favorability of methanogenesis, or the nutritional
504 demands of methanotrophs. For instance, the production of NO_3^-, NO_2^-, or the reduction
505 of Fe (III) to Fe (II) are known to limit methanogenesis (Cord-Ruwisch *et al.* 1988; Chen
506 & Lin 1993; Klüber & Conrad 1998; Reiche *et al.* 2008). We identified members of the
507 ammonia oxidizing genera *Nitrosospira* (Betaproteobacteria) and *Nitrosococcus*
508 (Gammaproteobacteria), members of the nitrite-oxidizing genera *Nitrospira* (Nitrospirae)
509 and *Nitrolancea* (Chloroflexi), and the iron-reducing and manganese-reducing genus
510 *Geothrix* as important markers for CH_4 flux. N-cycling activity could impact the activity
511 of methanotrophs by providing nutrients required for growth (Bodelier *et al.* 2000;
512 Bodelier & Steenbergh 2014). We also identified denitrifier and diazotroph taxa as
513 important predictors of CH_4 flux, underscoring the interdependence of the C and N
514 cycles. Taken together, these findings suggest that CH_4 flux in this system could depend
515 on changes to the thermodynamic favorability of methanogenesis as influenced by the
516 activity of taxa involved in redox processes and/or changes to nutrient availability from
517 other community members.

518 Beyond compositional controls, our results suggest that changes in methanogen
519 abundance and diversity could also be driving increased CH_4 fluxes in cattle pasture.
520 Methanogen abundance and diversity levels were higher in cattle pasture, which is
521 consistent with another study using metagenomics (Meyer *et al.* 2017). This suggests that
522 the soil environment of pastures could be favorable for methanogenesis, perhaps due to
523 an additional supply of labile carbon from grass root exudates and/or decreased O_2
524 concentrations throughout the soil column due to compaction (Fernandes *et al.* 2002).

525 Methanogenesis has been positively associated with methanogen abundance and diversity
526 in Congo Basin wetland soils (Meyer *et al.* 2019) as well as anaerobic digesters
527 (Sierocinski *et al.* 2018), suggesting that abundance- and diversity- controls may be
528 common in the CH_4 cycle.

529 Our study supports past findings that land use change impacts methanotrophs
530 (Knief *et al.* 2005; Singh *et al.* 2007; Meyer *et al.* 2017), but how these community
531 changes influence CH_4 flux is less clear. We observed a negative correlation between the
532 proportion of methanotrophs and CH_4 flux. However, after controlling for environmental
533 variation, this relationship was no longer significant, suggesting that the influence of
534 methanotrophs on CH_4 flux depends on environmental conditions. Importantly we cannot
535 ascertain whether methanotrophy is altered by land use change, as our measurements of
536 CH_4 flux are the net result of both methanogenesis and methanotrophy. One possibility is
537 that the changes to methanotroph communities that we observed do predict CH_4 oxidation
538 rates, but that methanotrophy largely does not control CH_4 fluxes relative to
539 methanogenesis or other processes. Methanotrophy rates have been shown to only predict
540 CH_4 fluxes when soils are dry and CH_4 fluxes are negative (Von Fischer & Hedin 2007).

541 Our study uncovered several relationships between CH_4 fluxes and soil chemical
542 variables, but the majority of soil chemical variables were not predictive. We saw
543 positive relationships between CH_4 flux and total soil Zn and Mn levels. Zn plays an
544 important role in the activation of methyl-coenzyme M, a key intermediate for CH_4
545 production by all methanogens (Sauer & Thauer 2000). Increased Zn levels have also
546 been shown to stimulate CH_4 production in tropical alluvial soils under rice production
547 (Mishra *et al.* 1999). Mn has been shown to stimulate methanogenesis in an anaerobic

548 digester system by acting as an electron donor (Qiao *et al.* 2015), and this has also been
549 shown for Zn (Belay & Daniels 1990). We found a weak positive relationship between
550 pH and CH_4 flux, which is consistent with several other studies (Ye *et al.* 2012; Wagner
551 *et al.* 2017). The general lack of correspondence between the soil chemistry and CH_4 flux
552 could result from assessing soil chemistry at too coarse of a scale. Microsite conditions
553 are important for anaerobic processes such as methanogenesis, and it has been suggested
554 that better quantifying soil chemistry at microscales could improve our ability to predict
555 CH_4 emissions (Von Fischer & Hedin 2007). Future work could take a more refined
556 approach by concurrently measuring chemistry and CH_4 production at smaller scales.
557 Our CH_4 flux results provide a sobering look into a potential feedback between
558 climate and land use change. In both regions cattle pastures were sources of CH_4 to the
559 atmosphere. Steudler et al. (1996) and Fernandes et al. (2002) were the first to document
560 the CH_4 sink-to-source transition of Rondônia soils following forest-to-pasture
561 conversion. These studies reported pasture emissions as high as 0.52 mg CH_4-C m^{-2} h^{-1}
562 (12,480 µg CH_4 m^{-2} d^{-1}, converted to the units of this study) and 614 mg CH_4-C m^{-2} yr^{-1}
563 (1682.2 µg CH_4 m^{-2} d^{-1}), respectively. The maximum rate we observed was 40,000 µg
564 CH_4 m^{-2} d^{-1}, and our average CH_4 flux rate across Rondônia pastures was 5,695.3 µg CH_4
565 m^{-2} d^{-1}. Our pasture emission estimates are therefore substantially higher than past
566 estimates in the same region. The highest rates of CH_4 consumption in forest soils from
567 Steudler et al. (1996) were during the dry season, where the maximum uptake rate was
568 0.061 mg CH_4-C m^{-2} h^{-1} (1464 µg CH_4 m^{-2} d^{-1}), with rates two-fold lower during the wet
569 season. Our highest rate of consumption was roughly an order of magnitude lower (160
570 µg CH_4 m^{-2} d^{-1}) than Steudler et al. (1996). Importantly, we sampled during the wet

571 season, when uptake rates would be expected to be lower. Nevertheless, the differences
572 between our uptake rates and Steudler et al. (1996) could represent spatial or temporal
573 variability or the indirect effects of habitat fragmentation due to on-going deforestation
574 activities in the region. Taken together, the immense variability of our CH_4 flux data and
575 the differences between our study and other work highlight the importance of continuing
576 efforts to study the spatio-temporal dynamics of CH_4-cycling in the Amazon Basin.
577 In both Rondônia and Pará, we see a recovery of CH_4 uptake rates in secondary
578 forest, and on average secondary forest soils consume CH_4 at rates higher than the
579 primary forests we surveyed. This suggests that forest regeneration could return
580 ecosystems to CH_4 sinks. Our microbial analyses indicate that secondary forest microbial
581 communities begin to resemble primary forest in the composition and diversity of both
582 CH_4-cycling organisms as well as the broader community. Therefore, pasture
583 abandonment could be a viable strategy for climate mitigation and microorganisms seem
584 to be mediating this response. A final consideration across land types is the role that trees
585 may play in the exchange of soil gases produced at depth. Tree-mediated CH_4 emissions
586 have been reported to comprise a substantial portion of the Amazon CH_4 budget,
587 particularly in seasonally inundated zones (Pangala *et al.* 2017). Thus, an untested
588 possibility is that the removal of trees could redirect CH_4 fluxes through the soil and that
589 secondary forest generation may redirect these fluxes through tree tissue.
590 Ongoing deforestation and forest-to-pasture conversion in the Amazon Basin is
591 resulting in a switch from ecosystems that are net CH_4 sinks to those that are net CH_4
592 sources. Understanding the mechanism for this change is important not only for our
593 fundamental understanding of global biogeochemical cycles but also for how we manage

these ecosystems and model future climate impacts of land use change. With the threat of land use change increasing across the Amazon Basin (Barlow *et al.* 2019; Carvalho *et al.* 2019) it is necessary to improve our understanding of the relationship between community change and ecosystem function. We have shown not only that microbial composition is crucial for understanding CH_4 dynamics, but also that microorganisms provide explanatory power that cannot be captured by easily measured environmental variables.

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FIGURE AND TABLE LEGENDS

Figure 1: Increased rates of CH_4 emission in cattle pasture relative to primary forest and secondary forest. Note the $\log_{10}$ scale of y-axis values. A) CH_4 emission rates in forest, cattle pasture, and secondary forest (Sec. For.) across two regions of the Amazon Basin: Rondônia (Ron.) and Pará (Par.). B) CH_4 fluxes by land type (both regions combined).

Pairwise differences between groups (letters A, B, C) were determined using Dunn's test of multiple comparisons with $p < 0.05$ as significance cutoff.

Figure 2: CH_4-cycling taxa response to land use change in two regions of the Amazon: Rondônia (Ron.) and Pará (Par.). A) The ASV-level taxonomic richness of methanogens by region and land type (inferred from 16S rRNA gene sequences). B) The relative abundance of methanogens in the 16S rRNA gene-inferred prokaryotic community across land use and region. C) The ASV-level taxonomic richness of methanotrophs (inferred from 16S rRNA gene sequences). D) The relative abundance of methanotrophs in the 16S rRNA gene-inferred prokaryotic community. Pairwise differences between groups (letters A, B, C) were determined using Dunn's test of multiple comparisons with $p < 0.05$ as significance cutoff. Sec. For. = Secondary forest.

Figure 3: Changes to the A) diversity and B) relative abundance of methanogen taxa are significantly associated with CH_4 flux across land types and regions, even after accounting for sample covariate structure. R^2 values represent the proportion of CH_4 flux variance explained by methanogen attribute, using a linear model on $\log_{10}$ transformed CH_4 flux data. Y-axis is $\log_{10}$ transformed with the minimum value added (+162). Dashed line indicates 0 μg CH_4 m^{-2} d^{-1} flux rate.

Figure 4: CH_4 flux is related to a subset of highly associated taxa. Position of points on the X-axis represents the Principle Component 1 (PC1) score representing the 654 taxa

638 that were identified to be highly associated with CH_4 fluxes, after accounting for sample
639 covariance. R^2 values represent the proportion of CH_4 flux variance explained by
640 methanogen attribute, using a linear model on log_{10} transformed CH_4 flux data. Y-axis is
641 log_{10} transformed with the minimum value added (+162). Dashed line indicates 0 μg CH_4
642 m^{-2} d^{-1} flux rate.
643
644 **Table 1:** Microbial community attribute measurements used to identify relationships
645 between communities and CH_4 flux. ASV = Amplicon sequence variant.
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