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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 increases in soil methane (CH_4) emission. To better understand the drivers of this change, we measured soil CH_4 flux, environmental conditions, and belowground microbial community structure across primary forests, cattle pastures, and secondary forests in two Amazonian regions. We show that pasture soils emit high levels of CH_4 (mean: 3454.6 ± 9482.3 μg CH_4 m^{-2} d^{-1}), consistent with previous reports, while forest soils on average emit CH_4 at modest rates (mean: 9.8 ± 120.5 μg CH_4 m^{-2} d^{-1}), but often act as CH_4 sinks. We report that secondary forest soils tend to consume CH_4 (mean: −10.2 ± 35.7 μg CH_4 m^{-2} d^{-1}), demonstrating that pasture CH_4 emissions can be reversed. We apply a novel computational approach to identify microbial community attributes associated with flux independent of soil chemistry. While this revealed taxa known to produce or consume CH_4 directly (i.e. methanogens and methanotrophs, respectively), the vast majority of identified taxa are not known to cycle CH_4. Each land use type had a unique subset of taxa associated with CH_4 flux, suggesting that land use change alters CH_4 cycling through shifts in microbial community composition. Taken together, we show that microbial composition is crucial for understanding the observed CH_4 dynamics and that microorganisms provide explanatory power that cannot be captured by environmental variables.

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1. Introduction

After a decade of slowing rates of forest loss, the Amazon rainforest is again undergoing high rates of deforestation, driven primarily by agricultural expansion for cattle pasture (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; Petersen et al., 2019; de Carvalho et al., 2016) as well as microbially-mediated biogeochemical cycles (Neill et al., 2005, 1997; Verchot et al., 1999), including the methane (CH_4) cycle (Neill et al., 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 (Fernandes et al., 2002; Steudler et al., 1996), 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 (Verchot and Davidson, 2000; Keller et al., 1986), 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 (Meyer et al., 2017; de Carvalho, 2016; Fernandes et al., 2002; Steudler et al., 1996; Verchot and Davidson, 2000; Keller et al., 1986), 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 and Whitman, 2013; Evans et al., 2019). Methanogens have been shown to increase in relative abundance following conversion of rainforest to cattle pasture (Kroeger et al., 2020), 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 Gammaproteobacteria 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 decreased population abundance and altered community composition in cattle pasture (Meyer et al., 2017; Lammel et al., 2015a, 2015b).

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 (Evans et al., 2019; Müller and Frerichs, 2013; Thauer et al., 2008). 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 and Lin, 1993; Klüber and 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 and Steenbergh, 2014; Ho et al., 2016), or predation by protozoa or viruses (Tyutikov et al., 1980; Murase and 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 and Treseder, 2010; Bier et al., 2015; Hall et al., 2018; Malik et al., 2020), 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., 2020). 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., 2020). One way to do so uses principle components analysis to derive environmental, spatial, and community structure covariates for incorporation into statistical models (Morris et al., 2020; Price et al., 2006). 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 important insights into a poorly understood phenomenon that possibly already dominates the 53 million hectares of pasture present in the Brazilian Amazon (http://mapbiomas.org, v4.1).

2. Materials and methods

2.1. Site description, sampling design, sampling dates

Our study was performed in two regions of the Brazilian Amazon: the state of Rondônia in the Western Amazon, and the state of Pará in the Eastern Amazon. Both states have experienced high rates of forest loss, largely driven by agricultural expansion for cattle ranching (Carvalho et al., 2019; Ometto et al., 2011). In Rondônia, we surveyed three primary forest sites (5 sampling locations each), three cattle pasture sites (5 sampling locations each), and two secondary forest sites (5 sampling locations in one site, 4 in the other, between 18 and 35 years post-abandonment, Supp. Table 1), totaling 39 sampling locations, all in or directly adjacent to Agropecuaria Nova Vida, about 250 km south of Porto Velho. The climate at Agropecuaria Nova Vida is humid tropical, and receives 2200 mm annual mean precipitation (Steudler et al., 1996; Alvares et al., 2013). Soils are red-yellow podzolic latosol with sandy clay loam texture, and are described in detail elsewhere (Neill et al., 1997). Vegetation type is open moist tropical forest with palms, and is described elsewhere (Pires et al., 1985). In Pará we surveyed two primary forest sites (5 sampling locations each), two cattle pasture sites (5 sampling locations each), and three secondary forest sites (5 sampling locations each for two sites and 3 sampling locations for one site, between 19 and 50 years post-abandonment, Supp. Table 1), totaling 33 sampling locations. The primary forest sites had no indication of recent disturbance from logging or wildfires. Pará sites were in or around Tapajós National Forest, 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 (Keller et al., 2005; Silver et al., 2000; Parrotta et al., 1995; Espírito-Santo et al., 2005; Berenguer et al., 2015). We strove to sample forests, pastures, and secondary forests equally, but faced restrictions due to 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 min 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. Immediately following the removal of the chamber, air temperature, soil temperature and soil moisture were measured and then used in the flux calculations. This sampling procedure took place once for each sampling location. 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 CH_4 rate of change. If trends were non-linear, we used the linear portion of the data near the time of chamber placement to calculate CH_4 rate of change (Salimon et al., 2004; Pirk et al., 2016). The final CH_4 flux for each chamber was calculated from the rate of change converted to moles of CH_4 using the ideal gas law, based on the concentration change, chamber volume, air temperature, and pressure (measured in real time by the Gasmet), divided by the surface area enclosed in the chamber.

Directly following gas flux measurement, soil samples were taken with a sterilized corer (5 cm diameter × 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.

2.2. 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, 2001). Soil chemical parameters included pH, organic matter, P, S, K, Ca, Mg, Al, H + Al, sum of exchangeable bases, cation exchange capacity, base saturation (% V), Al saturation, Cu, Fe, Mn, Zn, and total N. Soil pH was assessed using 0.01 M $CaCl_2$. Organic matter was measured using colorimetry. P was extracted using ion exchange resin and determined with colorimetry. S was extracted with 0.01 M $Ca_3(PO_4)_2$ and assessed by turbidimetry. K was extracted using ion exchange resin and determined using atomic emission spectrophotometry. Ca and Mg were extracted using ion exchange resin and determined using atomic absorption spectrophotometry. Exchangeable Al was extracted with 1 M KCl and determined by colorimetry. H + Al (potential acidity) was determined using the Shoemaker-McLean-Pratt buffer. Cu, Fe, Mn, and Zn were extracted with DTPA and determined by atomic absorption spectrophotometry. Total N was determined using the Kjeldahl method. The sum of exchangeable bases, cation exchange capacity, base saturation, and Al saturation were assessed based on calculations from the abovementioned procedures. 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).

2.3. 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. Soil samples from Pará sites required two subtle modifications (Venturini et al., 2020): (1) vortexing was performed for 15 min, instead of 10 min, 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).

2.4. 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 30 s (initialization), 98 °C for 10 s (denaturation), 61 °C for 20 s (annealing), and 72 °C for 20 s (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 (Fadrosh et al., 2014; Kozich et al., 2013), 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 and 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. The resulting community matrix contained 5,833,719 counts of bacteria or archaea, 5,972 of which matched to known methanogens and 19,169 to known methanotrophs (Supplemental Table 2).

2.5. 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 and Regan, 2008). For methanotrophs, we targeted the *pmoA* gene using the A189 – mb661 primer combination (Bourne et al., 2001; Kolb et al., 2003). 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 and by altering template concentration and addition of bovine serum albumin (BSA). *mcrA* reactions included 2 ng of DNA (10 ng/µl) in a 10 µl reaction with 0.2 µl BSA added, whereas *pmoA* reactions used 1 µl of DNA template (10 ng/µl) in a 20 µl reaction with no BSA addition. Both reactions took place under the following conditions: 98 °C 10 min (initialization), 98 °C 15 s (denaturation), 55.6 °C 15 s (annealing), 72 °C 60 s (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 calculate gene copy number. To account for plate-to-plate variation (among technical replicates) gene count values for each sample were residualized (by subtracting the mean copy number per plate), then averaged.

2.6. Statistical methods

All statistics were performed in the R statistical environment (Core and Team, 2018). CH_4 flux and community differences among regions and land types were assessed using a Kruskal-Wallis test, which does not rely on assumptions of distribution and can handle imbalanced sampling designs. Pairwise differences among groups were assessed using Dunn's test for multiple comparisons. Differences in community structure were assessed with a PERMANOVA test using the 'adonis' function in the vegan package in R (Oksanen et al., 2015).

Sequence depth per sample ranged from 62,865 to 148,053 sequences per sample, median: 77,653. To account for these differences in sampling depth, the community matrix was rarefied to 62,800 counts per sample ten times and averaged, which did not exclude any samples. The rarefied community matrix was also subsetted for known methanogens and methanotrophs (Supplementary Table 2). We compiled a table of microbial community attributes that represent putative controls on CH_4 emissions, including abundance, diversity, and composition (Table 1).

We tested for a relationship between the relative abundance of each taxon and CH_4 flux. To account for systematic differences in taxon relative abundances due to species interactions, local environmental selection, dispersal history between sites, and other factors unrelated to CH_4 dynamics, we performed a principal components (PC) correction using the community, environmental, and spatial variables with the 'prcomp' function in R (Morris et al., 2020; Price et al., 2006). For the environmental covariates, we included all soil chemical variables that were shared across samples and that had no missing values and scaled them to unit variance (to account for differences in units of measurement). To account for community structure, we performed principal components analysis (PCA) on the rarefied 16S rRNA gene community matrix following Hellinger transformation and after scaling for unit variance. Spatial coordinates (latitude and longitude) of each sample were assigned a PC score following the same procedure as the soil chemical variables. CH_4 fluxes, the relative abundance of each taxon in the community matrix, and each community attribute were then adjusted by the principal components for each covariate (community, environment, and geography). This principal components correction removed the correlation between CH_4 flux and community similarity, environmental similarity, and spatial proximity as well as the correlation between taxon relative abundance and each of the covariates, allowing us to test the unique contribution of each taxon to CH_4 flux independent of these underlying factors (Morris et al., 2020; Price et al., 2006). We regressed each corrected taxon or community attribute against $\log_{10}$-transformed CH_4 fluxes, and applied a Bonferroni correction (alpha = 0.05) to conservatively address the issue of false positives associated with large numbers of comparisons. Taxa significantly correlated with CH_4 flux were subsetted from the rarefied community matrix and reduced to a single variable using PCA, and then regressed against $\log_{10}$-transformed CH_4 fluxes. Model fit (R^2) was assessed after confirming normal distribution of residuals. In several instances one or two high leverage outliers, i.e. "influential outliers" (as defined by (Aguinis et al., 2013)), were removed due to their strong and disproportionate influence on model fit (R^2).

Table 1
Microbial community attribute measurements used to identify relationships between communities and CH_4 flux. ASV = Amplicon sequence variant.

Attribute	Variables
Abundance	qPCR (of *mcrA* & *pmoA* genes) Relative abundance (in prokaryotic 16S rRNA gene - based community) of methanogens/methanotrophs
Composition	Matrix of 16S rRNA gene-based prokaryotic community Principle Components (PCs) 1, 2, 3, 4, 5, & 6 of 16S rRNA gene community matrix
Diversity	ASV richness of prokaryotic community Shannon (ASV) diversity of prokaryotic community ASV richness of methanogen/methanotroph community subsets Shannon diversity of methanogen/methanotroph community subsets

3. Results

3.1. CH_4 flux and microbial community attributes differ across land use types

CH_4 fluxes were significantly different across land use types in both regions (Kruskal Wallis Chi-squared = 33.98, df = 5, $p < 0.001$, Fig. 1A). In both regions, pasture soils emitted CH_4 at higher rates than primary forest or secondary forest soils (Dunn test for multiple comparisons $p < 0.001$). Combining land use types from the two regions, the same pattern emerged, i.e. CH_4 emissions varied by land use type (Chi-squared = 25.11, df = 2, $p < 0.001$, Fig. 1B) and pastures emitted CH_4 at significantly higher rates than primary or secondary forests (Pasture average: 3454.6 ± 9482.3 μg CH_4 m^{-2} d^{-1}, Primary forest average: 9.8 ± 120.5 μg CH_4 m^{-2} d^{-1}, Secondary forest average: −10.2 ± 35.7 μg CH_4 m^{-2} d^{-1}, Dunn test $p < 0.001$). Of the 25 pasture measurements, only one exhibited CH_4 uptake (−11 μg CH_4 m^{-2} d^{-1}). In Rondônia, all pasture fluxes were positive, with rates 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 Pará, pasture emissions were lower, ranging from − 11 to 400 μg CH_4 m^{-2} d^{-1} (mean 93.6 ± 157.9 μg CH_4 m^{-2} d^{-1}). CH_4 fluxes in the Rondônia primary forests ranged from − 160 to 550 μg CH_4 m^{-2} d^{-1} (mean = 22 ± 156.2 μg CH_4 m^{-2} d^{-1}), with four of the fifteen measurements exhibiting uptake, three exhibiting near zero fluxes, and eight emitting CH_4. Six out of the ten measurements in Pará primary forests exhibited CH_4 uptake, one had a near zero flux, and three had low levels of emission, ranging from −30 to 8 μg CH_4 m^{-2} d^{-1} (mean − 8.6 ± 13.2 μg CH_4 m^{-2} d^{-1}). Secondary forests in both regions exhibited CH_4 uptake on average (Rondônia: mean −17.8 ± 37.7 μg CH_4 m^{-2} d^{-1}, Pará: mean − 4.9 ± 34.8 μg CH_4 m^{-2} d^{-1}). Flux values for secondary forest soils in Rondônia ranged from − 80 to 30 μg CH_4 m^{-2} d^{-1}, while fluxes from secondary forest soils in Pará ranged from − 54 to 61 μg CH_4 m^{-2} d^{-1}.

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

Land use change drove numerous alterations to the diversity, abundance, and composition of CH_4-cycling communities. Methanogen ASV-level richness significantly increased in pastures relative to primary and secondary forest in both regions (Chi-squared = 28.86, df = 2, $p < 0.001$, Fig. 2A). The abundance of methanogens (copies of *mcrA* per g soil) varied by land use type and region (Chi-squared = 45.62, df = 5, $p < 0.001$), and was higher in pastures relative to primary forests (Rondônia: $z = -4.24$, $p < 0.001$, Pará: $z = -3.91$, $p < 0.001$) and secondary forests

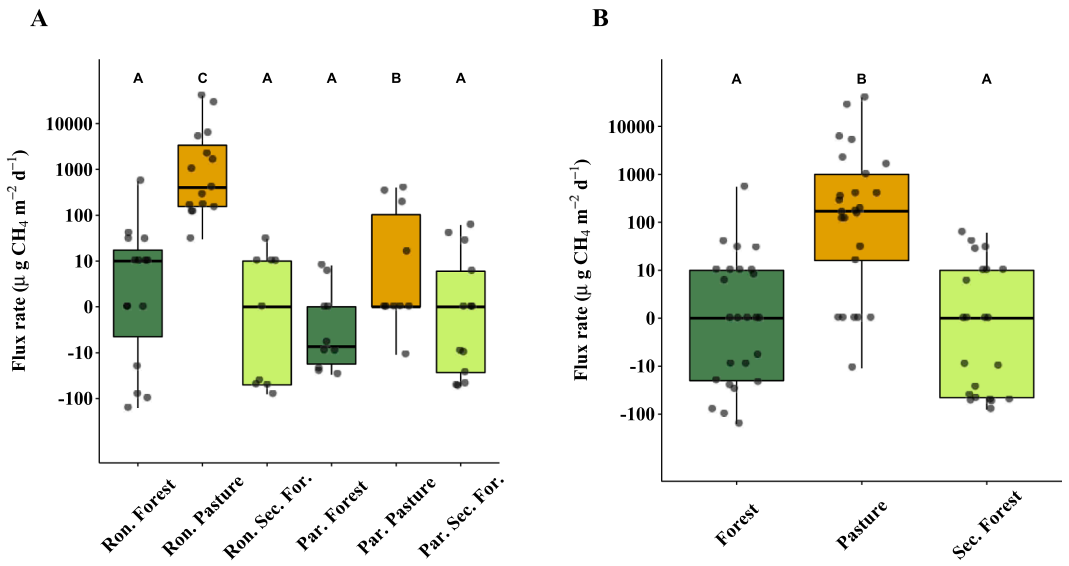

Fig. 1. Increased rates of CH_4 emission in cattle pasture relative to primary forest and secondary forest. Note the $\log_{10}$ scale of y-axis values. A) CH_4 emission rates in primary forest, cattle pasture, and secondary forest (Sec. For.) across two regions of the Amazon Basin: Rondônia (Ron.) and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

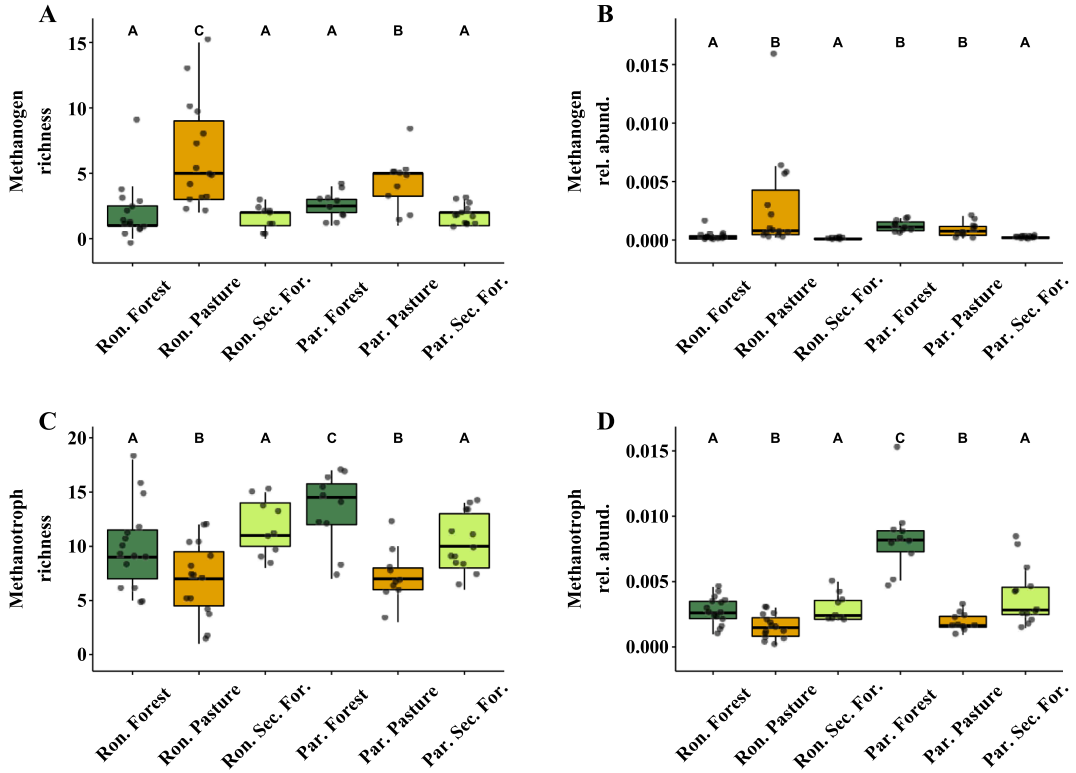

Fig. 2. CH_4-cycling taxa respond to land use change in two regions of the Amazon: Rondônia (Ron.) and Pará (Par.). (A) The ASV-level taxonomic richness of methanogens by region and land use type (inferred from 16S rRNA gene sequences). (B) The relative abundance of methanogens in the 16S rRNA gene-inferred prokaryotic community across land use type 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.

of Pará relative to pasture ($z = -4.37$, $p < 0.001$, Supp. Fig. 3A). A similar trend was observed for methanogen relative abundance (in the 16S rRNA gene-derived community) in Rondônia, but in Pará, primary forest and pasture, levels were indistinguishable ($p > 0.05$), while 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 use 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 much less pronounced trend across regions and land types (Chi-squared = 10.87, df = 5, $p = 0.05$, Supp. Fig. 3B). 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 in pastures relative to forest in both regions (*Methylocella*: Rondônia: z = 3.6, $p < 0.001$; Para: z = 2.13, $p < 0.05$; *Methylogaea*: Rondônia: z = 3.51, $p < 0.001$; Pará: z = 3.86, $p < 0.001$), and increased in secondary forests (*Methylocella*: Rondônia: z = −3.85, $p < 0.001$; Pará: z = −1.65, $p < 0.05$; *Methylogaea*: Rondônia: z = −2.67, $p < 0.01$; Pará: z = −1.91, $p < 0.05$). Lastly, the proportion of methanotrophs in the CH_4-cycling community (i.e. methanotroph relative abundance divided by the combined relative abundances of methanotrophs and methanogens) was lower in pastures in both regions, but this was only significant in Rondônia (z = 4.71, $p < 0.001$), and secondary forest levels were higher than pasture levels in both regions (Rondônia: z = −5.51, $p < 0.001$; Pará z = −3.22, $p < 0.001$).

3.2. Microbial abundance and diversity are associated with CH_4 flux

We first asked whether measurements of abundance or diversity (of CH_4-cycling taxa or the community as whole) could explain variance in CH_4 flux after accounting for sample covariance structure. Among the best predicting attributes were the ASV-level richness ($R^2 = 0.42$, $p < 0.001$, Fig. 3A) and relative abundance ($R^2 = 0.42$, $p < 0.001$, Fig. 3B) of methanogens. These were both positive relationships, whereby sites with more abundant and/or diverse populations of methanogens tended to emit CH_4 at higher rates. The proportion of methanotrophs in the CH_4-cycling community was negatively associated with CH_4 flux ($R^2 = 0.36$, $p < 0.001$, Supp. Fig. 4); however, this relationship was no longer significant after accounting for covariance structure ($p = 0.07$). No other methanotroph community attributes were related to CH_4 flux, despite exhibiting strong changes across sites. The only environmental variables significantly associated with CH_4 flux were pH ($R^2 = 0.08$, $p < 0.05$), Zn ($R^2 = 0.21$, $p < 0.001$), and Mn ($R^2 = 0.20$, $p < 0.001$), all exhibiting positive relationships.

3.3. Taxa associated with CH_4 flux in each land type

We next sought to identify taxa associated with CH_4 fluxes independent of environmental, spatial, and community covariance structure. We performed our analysis on two datasets: 1) subsets by land use type (i.e. primary forest, pasture, and secondary forest) to ask if emissions are controlled by different community members across land use types, and 2) across all samples combined. In primary forest sites we identified 41 (Supp. Table 3) ASVs that together explained 55% of the forest CH_4 flux variance ($p < 0.001$, Table 2). None of the taxa are canonically associated with CH_4 cycling. These taxa included one member of the Thaumarchaeota (Nitrosphaera), and members of eight bacterial phyla, including Acidobacteria, Actinobacteria, Chloroflexi, Firmicutes, Gemmatimonadetes, Planctomycetes, Proteobacteria (divisions Alpha, Beta, Delta, and Gamma), and Verrucomicrobia.

526 taxa across 25 phyla (Supp. Table 4) were associated with pasture CH_4 fluxes. Only 9 of these taxa are known to directly cycle CH_4, including 6 methanogens belonging to the genera *Methanocella*, *Methanobacterium*, and *Methanomassiliicoccus*, and 3 Gammaproteobacteria methanotrophs belonging to the genera *Methylobacter*, *Methylocaldum*, and *Methylococcus*. Two members of the Crenarchaeota (genus *Thermofilum*) were also among the taxa selected. Collectively the 526 taxa explained 87% of pasture emission variance (regression of subsetted PC1, $p < 0.001$, Table 2), following removal of one high leverage outlier. There was no overlap at the ASV level between the taxa identified for the primary forest and the pasture sites.

For the secondary forest sites, no taxa passed the p value cutoff from our Bonferroni correction ($p < 3.95 \times 10^{-6}$). We relaxed this threshold to $p < 0.001$ and identified six taxa (Supp. Table 5), including a member of *Acidobacteria* group 13, and members of the genera *Gaiella* (Actinobacteria), *Actinallomurus* (Actinobacteria), *Rhodoplanes* (Alphaproteobacteria), *Nitrospirillum* (Alphaproteobacteria), and *Desulfacinum*

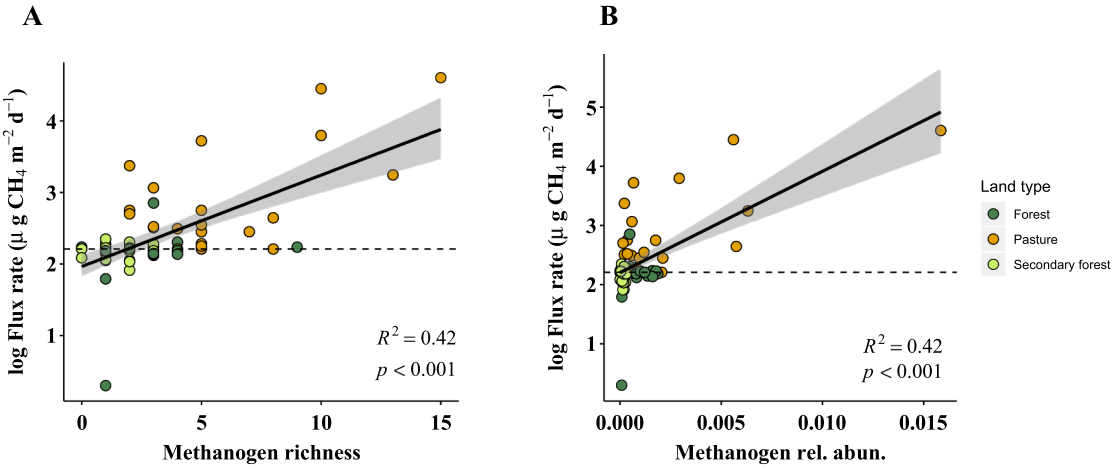

Fig. 3. Changes to the (A) diversity and (B) relative abundance of methanogen taxa are significantly associated with CH_4 flux across land use types and regions, even after accounting for sample covariance structure. R^2 values represent the proportion of CH_4 flux variance explained by methanogen attribute, 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.

Table 2
Community members highly associated with CH_4 for each land use type and all sites combined, numbers in parentheses indicate number of sites per land type. * indicates where *p*-value cutoff was adjusted to < 0.001 to identify any associated taxa.

	Primary Forest (5)	Cattle Pasture (6)	Secondary Forest (5)	All Sites (16)
Number of identified taxa	41	526	6*	654
Associated phyla	Thaumarchaeota, Acidobacteria, Actinobacteria, Chloroflexi, Firmicutes, Gemmatimonadetes, Planctomycetes, Proteobacteria, and Verrucomicrobia	Crenarchaeota, Euryarchaeota, Acidobacteria, Actinobacteria, Armatimonadetes, Bacteroidetes, BRC1, Candidate Division WPS-1, Candidatus Saccharibacteri, Chlamydiae, Chloroflexi, Elusimicrobia, Firmicutes, Gemmatimonadetes, Latescibacteria, Lentispharae, Nitrospirae, Parcubacteria, Planctomycetes, Proteobacteria (Alpha, Beta, Delta, Gamma divisions), Spirochaetes, Synergistetes, Tenericutes, Verrucomicrobia	Acidobacteria, Actinobacteria, Proteobacteria (Alpha and Delta divisions)	Crenarchaeota, Euryarchaeota, Acidobacteria, Actinobacteria, Armatimonadetes, Bacteroidetes, BRC1, Candidate Division WPS-1, Candidatus Saccharibacteri, Chlamydiae, Chloroflexi, Elusimicrobia, Firmicutes, Gemmatimonadetes, Latescibacteria, Lentispharae, Nitrospirae, Parcubacteria, Planctomycetes, Proteobacteria (Alpha, Beta, Delta, Gamma divisions), Spirochaetes, Synergistetes, Tenericutes, Verrucomicrobia
Associated methanogen genera	None	*Methanocella*, *Methanobacterium*, *Methanomassiliicoccus*	None	*Methanocella*, *Methanobacterium*, *Methanosarcina*, *Methanomassiliicoccus*
Associated methanotroph genera	None	*Methylobacter*, *Methylocaldum*, *Methylococcus*	None	*Methylocystis*, *Methylobacter*, *Methylocaldum*, *Methylococcus*
Proportion of variance explained by subset PC1	55%	87%	38%	50%

(Deltaproteobacteria). None of these taxa were associated with primary forest fluxes and only one (*Gaiella*) was associated with pasture fluxes. Collectively, these taxa when reduced to a single variable explain 38% of the CH_4 flux variance in secondary forests ($p = 0.001$, Table 2).

3.4. Taxa associated with CH_4 flux across land types

Lastly, we performed the above-detailed procedure across all three land use types in both regions and identified 654 taxa associated with CH_4 flux (Supp. Table 6). We subsetted all significant taxa from the community matrix, ordinated them, and regressed their PC1 against CH_4 flux, and the resulting model explained 50.0% ($p < 0.001$, Table 2) of the CH_4 flux variance after removal of one high leverage sample (Fig. 4). Many taxa identified were found in the pasture subset, indicating that pasture samples have a large influence over which taxa are chosen.

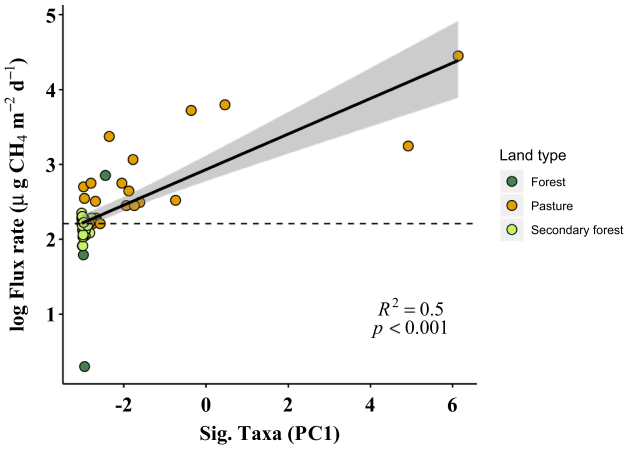

Fig. 4. CH_4 flux is related to a subset of highly associated taxa. Position of points on the X-axis represents the Principle Component 1 (PC1) score representing the 654 taxa that were identified to be highly associated with CH_4 fluxes, after accounting for sample covariance. R^2 values represent the 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.

Eleven methanogen taxa were identified, including members of the genera *Methanocella*, *Methanobacterium*, *Methanosarcina,* and *Methanomassiliicoccus,* comprising 1.7% of identified taxa. Four methanotroph taxa 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).

4. Discussion

Microbial communities drive biogeochemical cycles, 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 and subsequent land management are driving changes to soil CH_4-cycling dynamics in Amazon soils, and thus play a role in the switch between CH_4 sink and source.

The identity of community members can be an important determinant of ecosystem function (Díaz et al., 2016; Wardle et al., 2011; Bannar-Martin et al., 2018). 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 use 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 (Knief, 2015; Ho et al., 2013). Our results thus suggest that a better understanding of the characteristics of these taxa including their responses to environmental changes could improve predictions of CH_4 cycling. Moreover, recent reports of novel clades and physiologies of methanogens (Bižić et al., 2020; Borrel et al., 2019) and methanotrophs (Chiri et al., 2020; Leu et al., 2020) excitingly suggest further possible discoveries in the CH_4 cycle. Future work could examine phylogenetic relationships of Amazonian methanogens and methanotrophs using *mcrA*- or *pmoA*-specific primers.

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. Six of the taxa associated with CH_4 flux belong to known acetogenic genera (Müller and Frerichs, 2013), which is consistent with suggestions that syntrophic interactions with methanogens could regulate the CH_4 cycle (Conrad, 1996). We also identified several taxa that could impact the thermodynamic favorability of methanogenesis, or the nutritional demands of methanotrophs. For instance, our identified taxa included members of the ammonia oxidizing genera *Nitrosospira* (Betaproteobacteria) and *Nitrosococcus* (Gammaproteobacteria), members of the nitrite-oxidizing genera *Nitrospira* (Nitrospirae) and *Nitrolancea* (Chloroflexi), and the iron-reducing and manganese-reducing genus *Geothrix*. N-cycling activity could impact the activity of methanotrophs by providing nutrients required for growth (Bodelier et al., 2000; Bodelier and Steenbergh, 2014). We also identified denitrifier and diazotroph taxa as important predictors of CH_4 flux, underscoring the interdependence of the C and N cycles. Taken together, these findings suggest that the observed CH_4 dynamics could depend on the activity of taxa involved in redox processes and/or changes to nutrient availability from other community members.

Beyond compositional controls, our results suggest that changes in methanogen abundance and diversity could also be driving increased CH_4 fluxes in cattle pasture. Methanogen abundance and diversity levels were higher in cattle pasture, which is consistent with another study using metagenomics (Meyer et al., 2017) and a recent study using stable isotope probing (Kroeger et al., 2020). This suggests that the soil environment of pastures could be favorable for methanogenesis, perhaps due to an additional supply of labile carbon from grass root exudates and/or decreased O_2 concentrations throughout the soil column due to compaction (Fernandes et al., 2002). Methanogenesis has been positively associated with methanogen abundance and diversity in Congo Basin wetland soils (Meyer et al., 2020) as well as anaerobic digesters (Sierocinski et al., 2018), suggesting that abundance- and diversity-controls may be common in the CH_4 cycle.

Our study supports past findings that land use change impacts methanotrophs (Meyer et al., 2017; Lammel et al., 2015a, 2015b; Knief et al., 2005; Singh et al., 2007), but how these community changes influence CH_4 flux is less clear. The observed negative correlation between the proportion of methanotrophs and CH_4 flux was not significant after controlling for environmental variation, suggesting that the influence of methanotrophs on CH_4 flux depends on environmental conditions. Importantly, we cannot ascertain whether the methanotrophy process is altered by land use change, as our measurements of CH_4 flux are the net result of both methanogenesis and methanotrophy. One possibility is that the changes to methanotroph communities that we observed do predict CH_4 oxidation rates, but that methanotrophy largely does not control CH_4 fluxes relative to methanogenesis or other processes. Methanotrophy rates have been shown to only predict CH_4 fluxes when soils are dry and CH_4 fluxes are negative (Von Fischer, 2007).

Our study uncovered several relationships between CH_4 fluxes and soil chemical variables, but the majority of soil chemical variables were not predictive. We saw positive relationships between CH_4 flux and total soil Zn and Mn levels. Zn plays an important role in the activation of methyl-coenzyme M, a key intermediate for CH_4 production by all methanogens (Sauer and Thauer, 2000). Increased Zn levels have also been shown to stimulate CH_4 production in tropical alluvial soils under rice production (Mishra et al., 1999). Mn has recently been demonstrated to be involved in anaerobic methane oxidation (Leu et al., 2020), and has been shown to stimulate methanogenesis in an anaerobic digester system by acting as an electron donor (Qiao et al., 2015). Zn has also been shown to stimulate methanogenesis (Belay and Daniels, 1990). The general lack of correspondence between the soil chemistry and CH_4 flux could result from assessing soil chemistry at too coarse of a scale. Microsite conditions are important for anaerobic processes such as methanogenesis, and it has been suggested that better quantifying soil chemistry at microscales could improve our ability to predict CH_4 emissions (Von Fischer, 2007). Moreover, our community analysis results suggest that measuring certain additional ecosystem processes such as soil ammonia oxidation, nitrite oxidation, iron reduction or manganese reduction may provide further insights into the regulation of the CH_4 cycle.

In both Rondônia and Pará, we see a recovery of CH_4 uptake rates in secondary forest, and on average secondary forest soils in Rondônia consume CH_4 at rates higher than the primary forests we surveyed. A recent meta-analysis has similarly concluded that CH_4 sinks can be restored by allowing native ecosystem regeneration on abandoned pasture (Wu et al., 2020). Our microbial analyses indicate that secondary forest microbial communities begin to resemble primary forest in the composition and diversity of both CH_4-cycling organisms as well as the broader community, even in secondary forests that are aged 18–50 years, and in which other ecosystem components such as vertebrate, plant, and invertebrate biodiversity and above ground biomass have not recovered (Lennox et al., 2018; Berenguer et al., 2014). Therefore, pasture abandonment and forest recovery could have advantages that go beyond aboveground carbon sequestration for climate change mitigation, and microorganisms seem to be mediating this response. A final consideration across land use types is the role that trees may play in the exchange of soil gases produced at depth. Tree-mediated CH_4 emissions have been reported to comprise a substantial portion of the Amazon CH_4 budget, particularly in seasonally inundated zones (Pangala et al., 2017). Thus, an untested possibility is that the removal of trees could redirect CH_4 fluxes through the soil and that secondary forest generation may redirect these fluxes through tree tissue.

Ongoing deforestation and forest-to-pasture conversion in the Amazon Basin is resulting in increased soil CH_4 emissions. Understanding the mechanism for this change is important not only for our fundamental understanding of global biogeochemical cycles but also for how we manage ecosystems and model future climate impacts of land use change. 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 commonly measured environmental variables.

CRediT authorship contribution statement

Kyle M. Meyer: Conceptualization, Data curation, Formal analysis, Writing - original draft, Writing - review & editing. **Andrew H. Morris:** Formal analysis, Writing - original draft, Writing - review & editing. **Kevin Webster:** Conceptualization, Methodology, Formal analysis, Writing - original draft, Writing - review & editing. **Ann M. Klein:** Formal analysis, Writing - original draft, Writing - review & editing. **Marie E. Kroeger:** Methodology, Writing - original draft, Writing - review & editing. **Laura K. Meredith:** Conceptualization, Methodology, Formal analysis, Writing - original draft, Writing - review & editing. **Andreas Brændholt:** Methodology, Writing - original draft, Writing -

review & editing. **Fernanda Nakamura:** Methodology, Writing - original draft, Writing - review & editing. **Andressa Venturini:** Methodology, Writing - original draft, Writing - review & editing. **Leandro Fonseca de Souza:** Methodology, Writing - original draft, Writing - review & editing. **Katherine L. Shek:** Methodology, Writing - original draft, Writing - review & editing. **Rachel Danielson:** Methodology, Writing - original draft, Writing - review & editing. **Joost van Haren:** Methodology, Formal analysis, Writing - original draft, Funding acquisition, Writing - review & editing. **Plinio Barbosa de Camargo:** Resources, Methodology, Funding acquisition, Writing - review & editing. **Siu Mui Tsai:** Resources, Funding acquisition, Writing - review & editing. **Fernando Dini-Andreote:** Funding acquisition, Writing - review & editing. **José M.S. de Mauro:** Methodology, Writing - original draft, Writing - review & editing. **Jos Barlow:** Resources, Writing - original draft, Writing - review & editing. **Erika Berenguer:** Resources, Writing - original draft, Writing - review & editing. **Klaus Nüsslein:** Conceptualization, Methodology, Writing - original draft, Funding acquisition, Writing - review & editing. **Scott Saleska:** Conceptualization, Methodology, Writing - original draft, Funding acquisition, Writing - review & editing. **Jorge L.M. Rodrigues:** Conceptualization, Methodology, Writing - original draft, Funding acquisition, Writing - review & editing. **Brendan J.M. Bohannan:** Conceptualization, Methodology, Writing - original draft, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

The sequence data, ASV community matrix, community attribute matrix, and metadata pertaining to this study can be found at: https://doi.org/10.6084/m9.figshare.12980912. Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2020.106131.

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