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## Legacy Effects of Intercropping and Nitrogen Fertilization on Soil N Cycling, Nitrous Oxide Emissions, and the Soil Microbial Community in Tropical Maize Production

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# Legacy Effects of Intercropping and Nitrogen Fertilization on Soil N Cycling, Nitrous Oxide Emissions, and the Soil Microbial Community in Tropical Maize Production

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Maize-forage grasses intercropping systems have been increasingly adopted by farmers because of their capacity to recycle nutrients, provide mulch, and add C to soil. However, grasses have been shown to increase nitrous oxide (N<sub>2</sub>O) emissions. Some tropical grasses cause biological nitrification inhibition (BNI) which could mitigate N<sub>2</sub>O emissions in the maize cycle but the reactions of the N cycle and the microbial changes that explain the N<sub>2</sub>O emissions are little known in such intercropping systems. With this in mind, we explored intercropping of forage grasses (*Brachiaria brizantha* and *Brachiaria humidicola*) with distinct BNI and yield potential to increase N cycling in no-till maize production systems compared to monocrop with two N rates (0 and 150 kg ha<sup>-1</sup>) applied during the maize season. These grasses did not strongly compete with maize during the period of maize cycle and did not have a negative effect on grain yield. We observed a legacy of these grasses on N mineralization and nitrification through the soil microbiome during maize growth. We observed that *B. humidicola*, genotype with higher BNI potential, increased net N mineralization by 0.4 mg N kg<sup>-1</sup> day<sup>-1</sup> and potential nitrification rates by 1.86 mg NO<sub>3</sub>-N kg<sup>-1</sup> day<sup>-1</sup>, while *B. brizantha* increased the soil moisture, fungi diversity, mycorrhizal fungi, and bacterial nitrifiers, and reduced saprotrophs prior to maize growth. Their legacy on soil moisture and cumulative organic inputs (i.e., grass biomass) was strongly associated with enhanced mineralization and nitrification rates at early maize season. These effects contributed to increase cumulative N<sub>2</sub>O emission by 12.8 and 4.8 mg N<sub>2</sub>O-N m<sup>-2</sup> for maize growing after *B. brizantha* and *B. humidicola*, respectively, regardless of the N fertilization rate. Thus, the nitrification inhibition potential of tropical grasses can be outweighed by their impacts on soil moisture, N recycling, and the soil microbiome that together dictate soil N<sub>2</sub>O fluxes.

**Keywords:** intercropping, nitrous oxide-N<sub>2</sub>O, mineralization, nitrification, microbial community

## INTRODUCTION

Agricultural managements that relieve the pressure on deforestation are critical for tropical agriculture and might have global impact on the climate crisis since tropical forests are typically reported as a sink of atmospheric carbon (C). Intercropping maize with tropical grasses have been a common agricultural practice in Brazil (1–3) that increases land use efficiency and spare land for other activities because in this system plants grow almost all year-round (4). In this cropping system, although the forage grass is planted at the same time as maize, their phenology are distinct: maize germinates earlier than the grass and grow faster during the summer, while the forage grass develops slowly (5). After maize is harvested, the forage grass grows unshaded during the fall-winter-spring for 3–7 months, depending on the number of cash crops (crop of economic interest) cultivated in the summer. Typically, forage grasses are included in these systems if maize is the single or second summer cash crop planted in the same growing season. The forage grass is commonly used to feed animals or as a soil cover (mulch) for the next no-till cash crop. The intercropping system does not reduce the grain yield of the crop with economic interest (5), and growing forage grasses in between growing seasons increases land use efficiency (4), improves soil fertility parameters beneficial to the following crop (6) and may also reduce erosion.

Recently, Canisares et al. (1) found that N<sub>2</sub>O emissions were higher during the maize growing season when brachiarias were included in the system than in the sole maize treatments. Despite the widespread adoption by farmers of the maize-brachiaria cropping systems, there is little information on the N cycle and microbial processes that drive N<sub>2</sub>O emission in such systems. These intercropping systems may exhibit the benefits of biological nitrification inhibition (BNI) via this tropical forage grass (7). The conversion of *B. humidicola* grassland to maize has enhances soil N cycling and increased crop nitrogen use efficiency (NUE) when compared with monocropping (8). However, it is unclear whether intercropping systems used in Brazilian maize production can reduce soil nitrification (3, 9). Growing tropical forage grasses in a maize cropping system could impact soil N cycling directly during growth of the tropical grass due to soil N scavenging, root exudation of BNI compounds, and/or stimulation of N mineralization (10, 11) without negative effect on the crop of economic interest (5). Teutscherova et al. (12) showed the same plant genotypes that reduced potential net nitrification rates also had higher root colonization with arbuscular mycorrhizal fungi (AMF) and higher plant root: shoot ratios. Moreover, lower N<sub>2</sub>O emission and AOB abundance, along with higher abundance of AMF, suggests possible competition for N mineralization products between mycorrhizal fungi and ammonium oxidizing organisms (AOO) This tropical forage grass could also impact soil N cycling during its decomposition due to the release of BNI compounds from plant litter or the relative ratio of N immobilization to N mineralization depending on the residue C:N ratio (13). The C:N ratios of the shoot residue may vary with the brachiaria species and growing conditions (1, 14). Thus,

inclusion of this tropical forage grass in a cropping system with maize may lead to substantial relative differences in N immobilization vs. mineralization potential due to the residue C:N stoichiometry while chemically slowing soil nitrification through release of BNIs during decomposition (10, 15, 16), which highlights the importance of the whole microbial community to better understand the effect of plants with BNI potential on soil N dynamics (17).

The impact of agricultural management on microbial communities has been explored in many recent studies (18–20), although using management practices to promote beneficial microbial interactions with crops is an ongoing challenge for the sustainable intensification of agriculture (21). Similarly, linking microbial community changes to important soil functions remains a challenge. Garland et al. (22), found a negative correlation between bacterial alpha diversity and soil multifunctionality based on soil parameters related to structure, fertility, and N cycling potential in agricultural soils. Fungal diversity is typically lower in more stable soil environments (i.e., those with less moisture and temperature fluctuation) but higher with high biomass production and is potentially affected by intercropping or cover cropping (23, 24). However, soil biological functional diversity, as opposed to taxonomic diversity, has been associated with ecosystem multifunctionality (25, 26). Thus, beyond the BNI potential of tropical grasses, increasing plant diversity and land use efficiency through intercropping may lead to higher microbial diversity (23, 27) and potentially enhancing soil functioning related to N mineralization (28).

To understand the potential use of an intercropping system to mitigate the N losses from fertilization, we hypothesized that: (i) tropical grasses (*B. humidicola* and *B. brizantha*) result in lower potential soil net nitrification due to BNI and lower N<sub>2</sub>O emission during maize growing season; (ii) *B. brizantha* increases net mineralization rates, offsetting the benefits of BNI, while *B. humidicola* reduces mineralization, enhancing the BNI effect; (iii) these species cause different changes on soil microbiome diversity and functional groups related to N cycling.

## MATERIALS AND METHODS

A field experiment was set up in December 2016 and conducted at the same location during three growing seasons: 2016/2017, 2017/2018, and 2018/2019. The present study focused on evaluations made during the 2018/2019 growing season. The field experiment was located at an experimental farm in Botucatu, State of São Paulo (22°49'S 48°25'W) at 740 m of altitude in Brazil. The experimental site is classified as “tropical of altitude” (Cwa), which is characterized by dry winters and hot and wet summers, according to Köppen classification (29). The soil is classified as a kaolinitic, thermic Typic Haplorthox (30). Prior to the experiment set up, the soil pH(CaCl<sub>2</sub>) was 4.8, SOM was 31.7 g dm<sup>-3</sup>, P<sub>resin</sub> and S were 23 and 13.4 mg dm<sup>-3</sup>, Al<sup>+3</sup>, potential acidity (H<sup>+</sup> Al<sup>+3</sup>), K, Ca, Mg, cation exchange capacity were 3.5, 45, 4.7, 25, 12, and 86 mmol<sub>c</sub> dm<sup>-3</sup>, base saturation status was 48%, bulk density was 1.4 g cm<sup>-3</sup> and sand, silt and clay content were 173, 235, and 592 g kg<sup>-1</sup> (31).

The experiment was designed as a split plot randomized complete block design with five replicates. The main plots (4.5 × 30 m) consisted of three cropping systems: [1] maize hybrid 2B587 PW monocropped, [2] maize intercropped with *Brachiaria brizantha*, or [3] maize intercropped with *Brachiaria humidicola* cv. Tully. The cropping systems were subjected to two different topdress N fertilization rates, 0 and 150 kg ha<sup>-1</sup>, in subplots of 4.5 × 15 m (Figure 1).

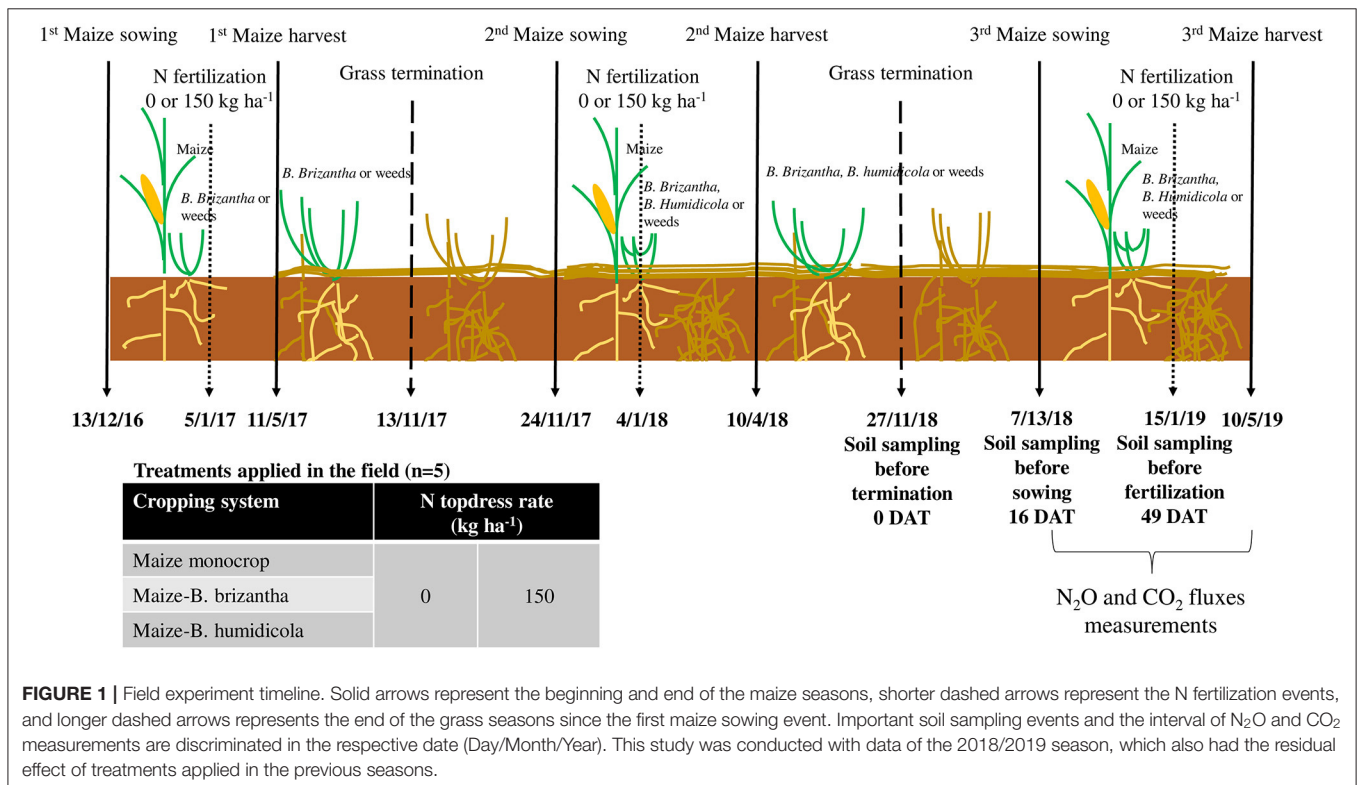
A fertilization supplying 30, 70, and 50 kg of N, P<sub>2</sub>O<sub>5</sub>, and K<sub>2</sub>O ha<sup>-1</sup>, respectively, was performed at maize sowing for all plots on three dates (December 13th, 2016; November 24th, 2017; December 13th, 2018). Maize was sown at 3 cm depth to reach a population of 66,000 plants ha<sup>-1</sup>. During maize sowing, plots receiving the intercropping treatments had seeds of the respective species mixed with fertilizer and band-incorporated 5 cm deep and 5 cm from maize rows aiming to reach a population between 80,000 and 100,000 plants ha<sup>-1</sup>. The N fertilizer treatments (0 or 150 kg N ha<sup>-1</sup>) were top-dressed in narrow bands (~0.05 m) ~0.10 m to the side of the maize rows at the V4–V6 stage. *B. humidicola* did not germinate in the first season (2016/2017) and was replanted in 2017/2018 when maize was seeded. After the maize grain was harvested, *B. brizantha* (both seasons) and *B. humidicola* (2017/2018) grew unshaded during the fall-spring season and were terminated with herbicides ~1 month before a new maize crop started. Data of grain yield, N cycling and N<sub>2</sub>O emission in the first two seasons (2016/17 and 2017/18) were reported in Canisares et al. (1). In the two growing seasons (2016/2017 and 2018/2019) previous to the evaluations performed in this study, the only N removed with plant materials from the plots was that of the maize grains that ranged from

45 to 55.9 and from 116.6 to 168.6 kg N ha<sup>-1</sup> for plots with 0 and 150 kg ha<sup>-1</sup> of N as topdress maize fertilization (1). The *B. brizantha* accumulated 106 kg N ha<sup>-1</sup> more than the plots under fallow during the fall-spring (maize monocrop) (1). The amounts of biomass produced in the first two cycles varied from 8 to 10 t stove ha<sup>-1</sup> yr<sup>-1</sup> for the fertilized maize, to 4–5 t stove ha<sup>-1</sup> yr<sup>-1</sup> for non-fertilized maize; the mulch of *B. brizantha* varied from 6 to 12 t DM ha<sup>-1</sup> yr<sup>-1</sup> and for *B. humidicola* was 2.5 t DM ha<sup>-1</sup> in the second season as there was no production of this grass in the first year (1). In this study, we present the data from the final year of cultivation (2018/2019), in addition to data about the microbial processes resulting from the cumulative effect of the treatments applied in previous years.

Maize grain was harvested in May 2019, after the grain moisture was below 20%. *B. brizantha* and *B. humidicola* grew unshaded after the maize harvest during the fall-winter between maize crops. Twenty-two days before maize sowing on December 13th 2018, all plots were chemically terminated using the combination of 6 and 0.7 L ha<sup>-1</sup> glyphosate, N-(phosphonomethyl) glycine, and clethodim, (RS)-2-[(E)-1-[(E)-3-chloroallyloxyimino]propyl]-5-[2-(ethylthio)propyl]-3-hydroxycyclohex-2-enone, respectively. The desiccated brachiarias or the weeds—in the monocrop treatments were maintained on the soil surface and served as mulch for the no-till maize crop.

### Weather Conditions

During the first 2 months of maize growth, December 2018 and January 2019, the cumulative precipitations were 91 and 181 mm, 48 and 79% of the historic rains for these months. The



**FIGURE 1 |** Field experiment timeline. Solid arrows represent the beginning and end of the maize seasons, shorter dashed arrows represent the N fertilization events, and longer dashed arrows represents the end of the grass seasons since the first maize sowing event. Important soil sampling events and the interval of N<sub>2</sub>O and CO<sub>2</sub> measurements are discriminated in the respective date (Day/Month/Year). This study was conducted with data of the 2018/2019 season, which also had the residual effect of treatments applied in the previous seasons.

precipitation in February, March, and April were 237, 194, and 125 mm, 12, 30 and 99% higher than the historic average for these months (Figure S1A). The total precipitation from December 2018 to April 2019 was 827 mm, 98% of the historic average for these periods, but the lower precipitation observed in December 2018 and January 2019 probably affected the maize yield. The monthly mean air temperature during the same period ranged from 21.8 to 24.8°C while the historic values ranged from 19.3 to 22°C, so the monthly mean air temperature was 0.9–2.8°C higher than the historic average for March and January, respectively (Figure S1B).

### Soil Moisture, Inorganic N Content, and pH

Soil samples were collected in each field plot at three time points: just before termination of forage grasses and weeds (0 DAT, November 20, 2018), just before maize sowing (16 DAT, December 7, 2018), and just before maize N fertilization (49 DAT, January 19, 2019) (Figure 1). Composite soil samples comprised soil from 8 sampling locations of the maize interrow of each plot to a depth of 10 cm using an Edelman auger. We measured soil water filled pore space (WFPS), pH,  $\text{NH}_4^+$  and  $\text{NO}_3^-$  content. The WFPS was calculated with the gravimetric water content determined after drying at 105°C and the soil bulk and particle densities. Soil inorganic N ( $\text{NH}_4$  and  $\text{NO}_3$ ) was extracted from fresh soil with KCl (1M) and quantified by steam distillation (32). Soil pH was measured after shaking 10 g of soil with 50 mL of 0.01M  $\text{CaCl}_2$  for 30 min and waiting for the soil to settle.

### Soil Incubations

#### Potential Net Nitrification Rate ( $\text{NH}_4$ Surplus)

Potential soil net nitrification rates were assessed on the same soil samples above using an aerated microcosm incubation (10, 15). The soil was air dried (25°C) and sieved (2 mm) prior to the incubation assay. Soil samples (5g) of all treatments were incubated with  $(\text{NH}_4)_2\text{SO}_4$  (300 mg  $\text{NH}_4\text{-N kg}^{-1}$  of dry soil) in 50 mL amber flasks to ensure that  $\text{NH}_4$  supply was not limiting for nitrification over the incubation period. This amount of N was expected to be more than enough for more than 7 days even in case of N immobilization since nitrification rates were not expected to exceed 10 mg  $\text{NO}_3\text{-N kg}^{-1} \text{ day}^{-1}$  (10, 33). The incubation was performed at 25°C and soil moisture at 60% of water holding capacity. The soil  $\text{NO}_3\text{-N}$  was extracted using KCl (1 M) at the beginning and 7 days after starting the incubation and was determined by steam distillation (32). Daily rates of nitrification were calculated by the difference between final and initial  $\text{NO}_3\text{-N}$  content divided by the number of incubation days and expressed as mg  $\text{NO}_3\text{-N g-soil}^{-1} \text{ d}^{-1}$ .

We used a randomized design with four out of five replicates of the field treatments since the variability is lower under temperature and moisture-controlled condition in the laboratory than the observed in the field. As the efficacy of nitrification inhibitors depends on edaphoclimatic conditions, a commercially available nitrification inhibitor, dicyandiamide (DCD), was applied at a rate of 5% of the N to soil as an extra treatment to verify the responsiveness of the soil to nitrification inhibitors using the soil samples from the maize monocrop treatment. In this preliminary test we observed that soil of

maize monocrop with DCD had the potential net nitrification rates of  $2.29 \pm 1.41 \text{ mg NO}_3\text{-N g-soil}^{-1} \text{ d}^{-1}$  while the same soil without DCD resulted in  $4.08 \pm 2.15 \text{ mg NO}_3\text{-N g-soil}^{-1} \text{ d}^{-1}$ , confirming the efficacy of nitrification inhibitors in those edaphoclimatic conditions.

### Net N Mineralization

The same soil samples of previous sections were used for the determination of net N mineralization. Five grams of soil from each sample were incubated during 21 days at 20°C and 60% of the water holding capacity. The flasks were randomly arranged with four replicates in an incubation chamber. To best capture the rate of organic N conversion to mineral N, no inorganic N was added to the flasks in this incubation. The net N mineralization rate was the sum of  $\text{NH}_4\text{-N}$  and  $\text{NO}_3\text{-N}$ , accumulated during 21 days of incubation. The daily rates were calculated by the difference of final and initial inorganic N content divided by incubation days. The inorganic N was extracted with KCl (1M) and quantified by steam distillation (32).

### Nitrous Oxide Emissions and Soil Respiration

One static chamber (30 cm diameter) was installed in each field plot to measure nitrous oxide ( $\text{N}_2\text{O}$ ) and carbon dioxide ( $\text{CO}_2$ ) efflux during the 2018/2019 maize growing season (34). The chamber was placed at 5 cm distance from the planted row. Frequent gas sampling (>2 sampling days per week) was undertaken after sowing and N fertilization followed by at least weekly measurements until the end of the maize season (Table S1). The sampling took place between 9:00 and 10:00 am to represent the mean daily gas flux (35). Gases were sampled 0, 15 and 30 minutes after chamber closure and stored in evacuated glass vials protected by a gas-impermeable stopper. The gases were analyzed by gas chromatography (Shimadzu GC-2014), detecting  $\text{N}_2\text{O}$  and  $\text{CO}_2$  concentration by electron capture detector. The gas fluxes were calculated by linear interpolation of concentrations during the sampling period, according with the following equation:

$$F = \left( \frac{\Delta C}{\Delta t} \right) \left( \frac{V}{A} \right) \left( \frac{m}{V_m} \right) \quad (1)$$

where, F is the efflux (mg C- $\text{CO}_2 \text{ m}^{-2} \text{ day}^{-1}$  or  $\mu\text{g N}_2\text{O-N m}^{-2} \text{ day}^{-1}$ ),  $\frac{\Delta C}{\Delta t}$  is the variation of gases concentration while the chamber was closed ( $\text{mol}^{-1} \text{ h}^{-1}$ ), V is the volume of the chamber headspace ( $\text{m}^3$ ), A is the soil area covered by the chamber ( $\text{m}^2$ ), m is the molar mass ( $\text{g mol}^{-1}$ ), and  $V_m$  is the molar volume of the chamber ( $\text{m}^3 \text{ mol}^{-1}$ ).

The flux calculated by equation 1 represents the daily gas flux. To calculate the cumulative gas emission, the linear interpolation of measured fluxes on sampling events was used to estimate the flux on non-sampled events (36).

## Soil DNA Extraction and Quantification, and Abundance of 16S rDNA and AOA Genes

Soil DNA was extracted from 0.3 g of soil sampled from the 0-10 cm layer of four replicates in the field plots at two contrasting time points, 0 and 49 DAT. The DNA extraction was performed using Qiagen Power soil DNA extraction kit and the DNA was quantified using Qubit fluorometer (Life Technologies, Carlsbad, CA, USA), the total extracted DNA was normalized and expressed in ng g<sup>-1</sup> of soil as a proxy for total microbial abundance. The quality of the DNA was measured using NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Montchanin, USA). The bacterial/archaeal 16S rDNA abundance was quantified using primers 515F (5' -GTGCCAGCMGCCGCGGTAA-3') and 806R (5' -GGACTACVSGGGTATCTAAT-3'). Eight nanograms of the DNA samples were diluted in 10 µL of SYBRGreen (2X) 1 µL of the forward and reverse primer and 7 µL of MQ water (Sigma Aldrich) and the abundance of the genes were quantified using real-time PCR with PCR Detection System (Bio-Rad, USA). The thermal conditions were: 1 cycle of 3 min at 95°C for denaturation; 30 cycles of 30 s at 95°C, 45 s at 52°C and 90 s at 72°C for annealing; and 1 cycle of 12 min at 72°C for extension. The qPCR run of DNA samples had a standard curve with serial dilution of known concentration of plasmids measured previous using the Qubit fluorometer (Life Technologies, Carlsbad, CA, USA).

The abundance of ammonia oxidizing archaea (AOA), the group of AOA more sensitive to BNI (37), was quantified by real-time qPCR using the *amoA* gene as reporter. The total reaction volume was 20 µL, using 10 µL of SYBERGreen (2X), 1 µL for each primer, and 2 µL (10 ng total) of DNA template. The primers combination was *amoA19F* (5'-ATGGTCTGGCTWAGACG-3'), *amoA643R* (5'-TCCCACCTWAGACCARGCGCCATCCA-3') for AOA (38). The amplification conditions of PCR were as follows: 1 cycle of 10 min at 95°C; 40 cycles of 60 s at 95°C for denaturation, 30 s at 55°C for annealing, 45 s at 72°C for extension, and 45 s at 72°C for final extension. The melting curve was generated from 65 to 95°C measuring each 0.2°C and maintained for 1 second.

## Soil 16S rRNA Gene and ITS Sequencing

16S rRNA and ITS genes were amplified using PCR. The reaction was prepared using 2.5 µL of PCR buffer (10x), 2 µL of dNTPs mix (2.5 mM), 0.7 of Roche bovine serum albumin (20 mg mL<sup>-1</sup>), 1 µL of the forward and reverse primers (515F/806R and ITS1F/ITS2R), Takara Hot Start Ex Taq polymerase (5 U µL<sup>-1</sup>) and 10 ng of DNA samples. The primers were modified to include the forward and reverse adapters for Illumina Nextera and the reverse barcode contained 12-bp Golay barcode (39). The thermo cycler conditions of 16S amplification were: 1 cycle of 3 min at 95°C for denaturation; 30 cycles of 30 s at 95°C, 45 s at 52°C and 90 s at 72°C for annealing; and 1 cycle of 12 min at 72°C for extension. The set up for ITS amplification were: 1 cycle of 3 min at 95°C for denaturation; 30 cycles of 30 s at 95°C, 30 s at 51°C and 30 s at 72°C for annealing; and

1 cycle of 5 min at 72°C for extension. The products of PCR reactions were purified using SPRI bead purification (AMPure XP, Beckman Coulter Genomics, Danvers, MA) and the quality of the amplicons were checked and the average size distribution on Bioanalyzer 2100 with a DNA 7500 chip (Agilent Technologies, Santa Clara, CA). Purified amplicons of 16S and ITS were quantified using Qubit and pooled in two libraries of 16S and ITS at the same concentration (10 ng of each sample). The libraries were sequenced using v3 Illumina MiSeq at the Vincent J. Coates Genomics Sequencing Laboratory at the University of California, Berkeley.

Forward and reverse reads were aligned and paired using `usearch [v10.0.240] fastq_mergepairs` command (maximum diff = 3) (40). The aligned reads were quality filtered (command `fastq_filter` with `-fastq_trunclen = 250`, `-fastq_maxee = 0.1`), concatenated into a single fasta file, and singletons were removed (command `sortbysize` with `minsize = 2`). The resulting aligned and filtered sequences were processed using the DADA2 pipeline (41) to define amplicon sequence variants (ASVs), by first dereplicating the sequences and then denoising unique sequence pairs using the “`dada`” function with “`err=NULL`” and “`selfConsist=TRUE`.” Taxonomy was assigned to each ASV by a Naïve Bayes classifier using the `assign_taxonomy.py` command in QIIME (42) with reference to the SILVA database accessed from `mothur` (42). For phylogenetic inference of bacterial and archaeal ASVs, representative sequences for each bacterial OTU were aligned to a SILVA SEED sliced alignment using the PyNASt algorithm (43) and archaeal and bacterial phylogeny was inferred using `FastTree` (44). All sequence data were submitted to NCBI under BioProject accession number PRJNA746808.

## Shoot Forage Grass Biomass and Maize Grain Yield

The shoot biomass of *B. brizantha*, *B. humidicola* and weeds (fallow) grown during the fall-winter were sampled from the plots with the respective treatments on November 21st, 2018, at the end of the dry season, using 0.25 m<sup>2</sup> quadrants. The shoot biomass was dried at 65°C for 48 h and the dry masses were extrapolated to Mg ha<sup>-1</sup>. The weeds from plots with both forage grasses were not sampled because visually they covered <5% of the plot area.

Maize grain was harvested from 10 m in each of two central rows in each plot on May 10th, 2019 and weighed at the field moisture content. Grain subsamples of each plot were dried at 105°C for 24 h and the maize grain yield was extrapolated to 1 ha at 13% moisture, which is the standard moisture for maize commercialization in Brazil.

The biomass produced by the forage grasses and maize not harvested as grain was left on the soil surface as mulch for the no-till system. This plant material is expected to gradually decompose and contribute to the stock of soil organic matter (SOM) over the years.

## Statistical Analysis

Descriptive statistics were used to obtain the frequency and distribution of data. We evaluated the effect of cropping system

(maize monocrop, maize intercropped with *B. brizantha* and with *B. humidicola*) and topdress N fertilization (0 and 150 kg N ha<sup>-1</sup>) on cumulative potential net nitrification rate, net N mineralization, N<sub>2</sub>O and WFPS by analysis of variance (ANOVA) using the *sp.plot* function of the *agricolae* package (45) on the R statistical software (46). For significant factors, individual mean comparisons were performed to compare the cropping systems and the topdress N rate within each cropping system using Tukey's test ( $p < 0.1$  or 0.05) in the same R package.

We performed non-metric dimensional scaling analysis (NMDS) to reduce data dimensionality and to observe the variance of community structure aiming to identify differences between the different treatments. Bray-Curtis distances was used for fungi and weighted-unifrac distances for bacteria. The effect of treatments on bacterial and fungi community was tested by perMANOVA test using the *adonis* function of R package *vegan* (47). We used *DESeq* function on *DESeq2* package to identify the taxa with significant difference between pairwise comparisons (mono vs. intercrop and *B. brizantha* vs. *B. humidicola*) by calculating the *log2foldchanges* in relative abundances for each time point, we used the p-value adjusted for multiple comparisons by Storey's Q Value using the *qvalue* function of the *qvalue* package (48). We considered organisms that were identified at least at the order level and showed all the significant taxa in a bar plot.

The function *estimate\_richness* from *phyloseq* package (49) to obtain the Shannon index used as alpha diversity. We used FUNGuild (50) to annotate and classify fungal ASVs into trophic modes and functional guilds such as saprotrophic fungi, taxa with multiple trophic modes (i.e., mixotrophic), diverse types of opportunistic fungal pathogens (e.g., *Fusarium*), endophytes, symbiotic organisms (i.e., *Trichoderma*), arbuscular mycorrhizae (AMF, i.e., *Glomerales*) or ectomycorrhiza (EMF). We performed ANOVA *post-hoc* Tukey test for alpha diversity and relative abundance of each group of fungi as described for other soil parameters.

A principal component analysis (PCA) was performed using *fviz\_pca\_biplot* function from the *factoextra* package (51), with the ellipses drawn using the Euclidean distance from the centroid of each treatment (interaction of cropping system and maize N topdress fertilization) with 95% confidence level. The PCAs were performed for each time point aiming to address the correlations of N cycling parameters (potential net nitrification rates, net mineralization rates and cumulative N<sub>2</sub>O emission), soil edaphic factors and microbial groups (soil inorganic N, NH<sub>4</sub>:NO<sub>3</sub> ratio, WFPS, pH, total DNA extracted from soil, the abundances of 16S rDNA and AOA, fungi and bacterial diversity, cumulative respiration, fungi trophic modes and key fungi and bacteria taxa), and plant parameters (forage grass biomass produced during the fall-winter-spring period and the maize grain yield of the subsequent growing season).

Additionally, multiple linear regression was performed to relate proxy measurements of N cycling (potential net nitrification rate, net N mineralization and N<sub>2</sub>O cumulative emission) using the *lm* function from R version 3.6.2. To predict the cumulative N<sub>2</sub>O emission the considered explanatory variables were potential net nitrification rate, net

N mineralization, WFPS, pH, and inorganic N pools (NH<sub>4</sub> and NO<sub>3</sub>) sampled 0, 16 and 61 DAT as well as total DNA extracted from the soil and the abundance of 16S rDNA from soil sampled 0 and 61 DAT. The explanatory variables for potential net nitrification rate were net ammonification and nitrification, WFPS, pH, inorganic N pools (NH<sub>4</sub> and NO<sub>3</sub>), total DNA extracted from the soil, and the abundance of 16S rDNA and AOA genes. Prediction of net N mineralization was performed using the following explanatory variables: WFPS, pH, inorganic N pools (NH<sub>4</sub> and NO<sub>3</sub>), total DNA extracted from the soil and the abundance of 16S rDNA. The relative importance of each explanatory variable was calculated by Lindeman-Merenda-Gold using *booteval.relimp* function in *relaimpo* package, the 95% confidence intervals were obtained for 1000 iterations.

## RESULTS

### Maize Grain Yield and Forage Grass Biomass

The present study (2018/19) maize grain yield averaged 3.5 Mg ha<sup>-1</sup> across treatments without topdress N fertilization. N application increased maize yield by 125%, but no effect of intercropping system was observed on grain yield (Figure 2).

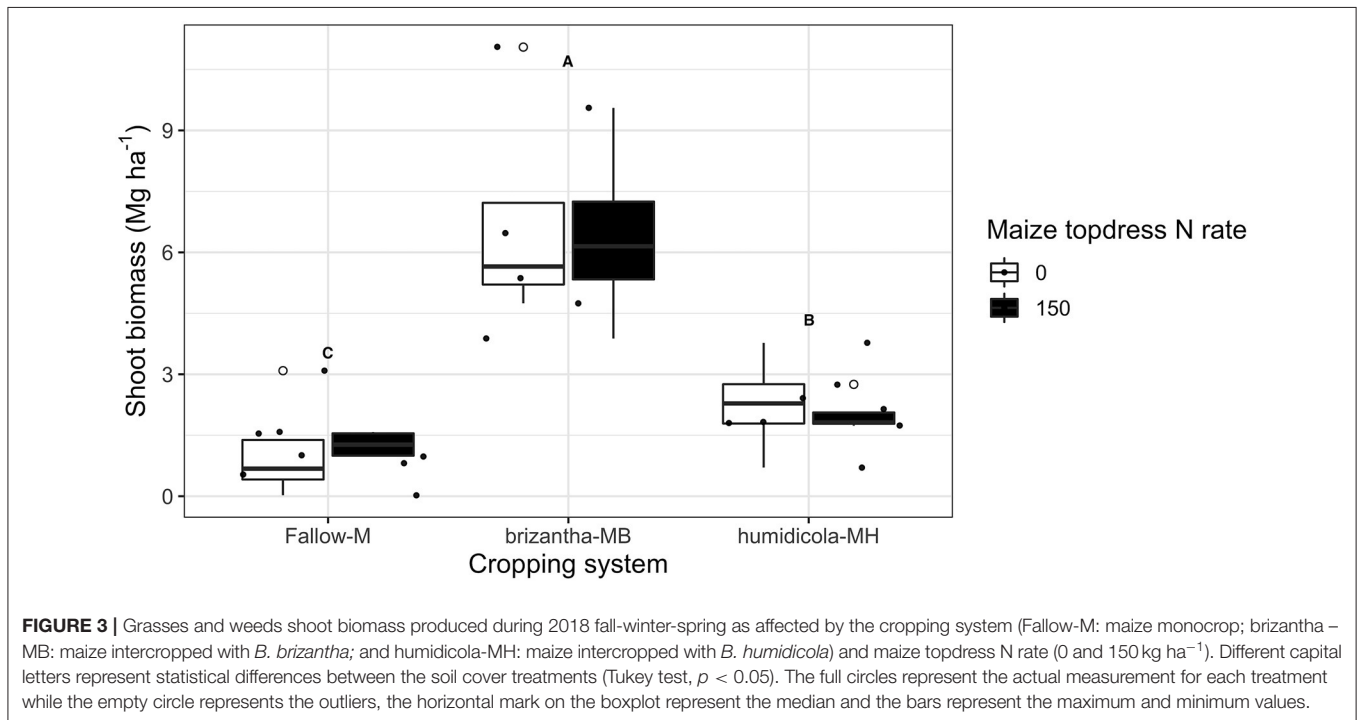
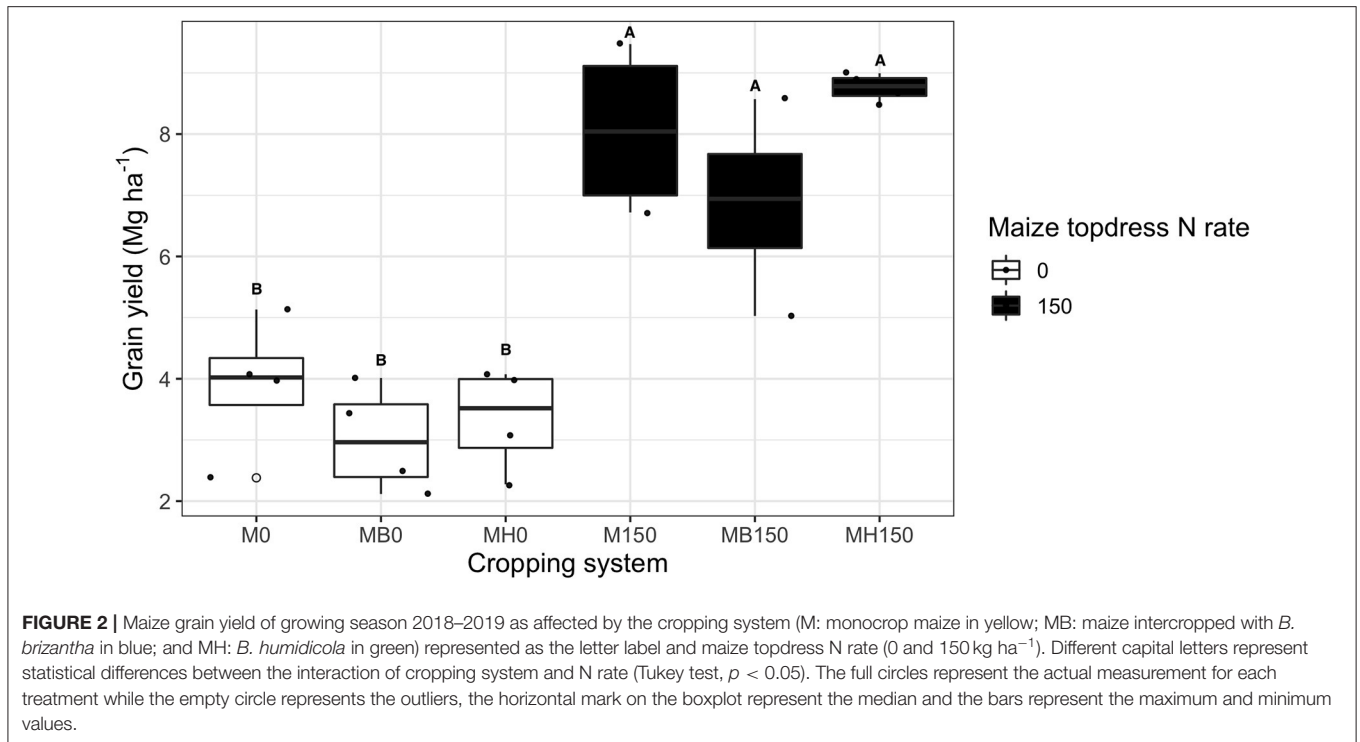
Despite the positive effect of topdress N fertilization on maize yield, there was no effect of N addition on biomass of forage grasses and weeds growing after the maize harvest. The highest biomass dry matter yields was observed for *B. brizantha* (6.6 ± 2.4 Mg ha<sup>-1</sup>); *B. humidicola* accumulated on average 1 Mg ha<sup>-1</sup> more biomass than weeds growing under fallow plots after maize monocrop (Figure 3).

### Soil Parameters Related to N Availability and Losses

Averaged across N rates, *B. humidicola*, which had relatively low shoot biomass yield (Figure 3), increased the potential net nitrification rates before termination (0 DAT) by 1.86 mg NO<sub>3</sub>-N kg<sup>-1</sup> day<sup>-1</sup> compared to fallow and *B. brizantha* treatments. The plots with *B. humidicola* and annual N fertilization resulted in higher nitrification potential (e.g., 2.22 mg NO<sub>3</sub>-N kg<sup>-1</sup> day<sup>-1</sup>) compared to plots without N fertilization (Figure 4). When the soil was sampled 16 and 49 DAT, plots intercropped with grasses had higher potential net nitrification rates regardless of the forage grass species. Intercropping with forage grasses increased potential net nitrification by 2.63 and 1.09 mg NO<sub>3</sub>-N kg<sup>-1</sup> day<sup>-1</sup> at 16 and 49 DAT, respectively compared to maize monocrop (Figure 4).

In addition to increasing potential net nitrification, plots with *B. humidicola* mineralized 0.41 mg kg<sup>-1</sup> day<sup>-1</sup> more inorganic N (NH<sub>4</sub> and NO<sub>3</sub>) than plots under fallow at 0 DAT. At this time point, maize topdress N fertilization increased net mineralization rates of plots by 0.71 mg kg<sup>-1</sup> day<sup>-1</sup> for plots under fallow. At 16 DAT, maize N fertilization had a positive effect only for plots under the intercropping system, increasing net mineralization by 0.36 mg kg<sup>-1</sup> day<sup>-1</sup>. At the later time point (49 DAT), plots under the intercropping system had net mineralization rates 0.24 mg kg<sup>-1</sup> day<sup>-1</sup> higher than monocropping; maize N

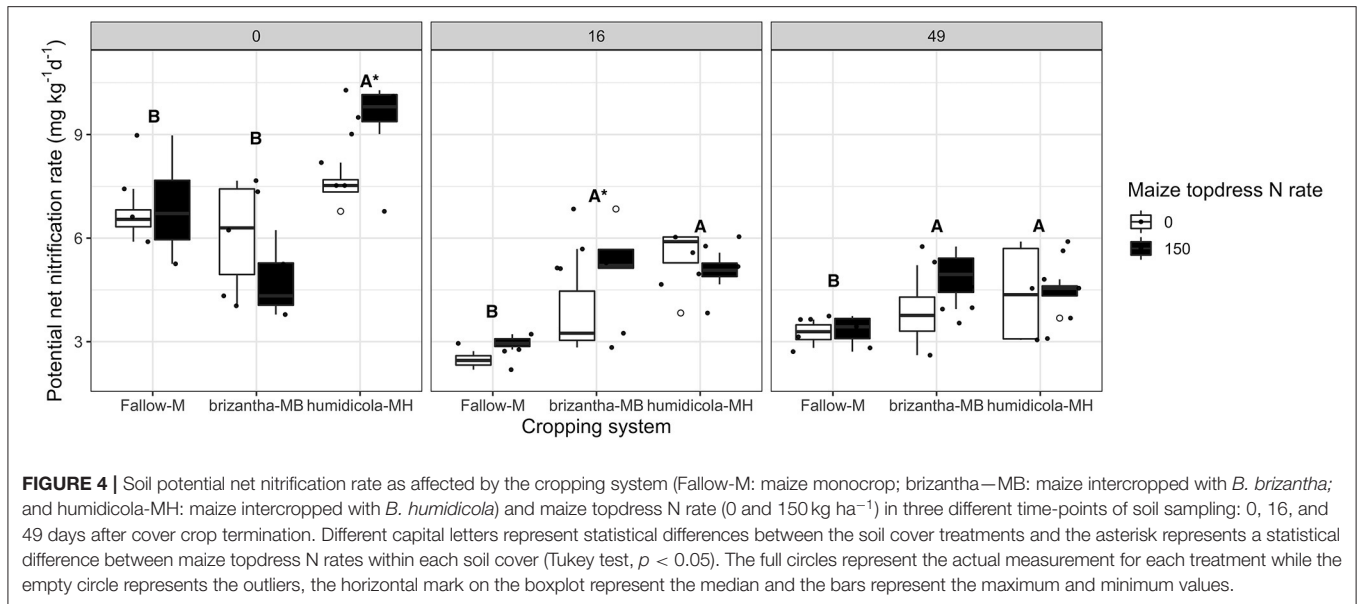




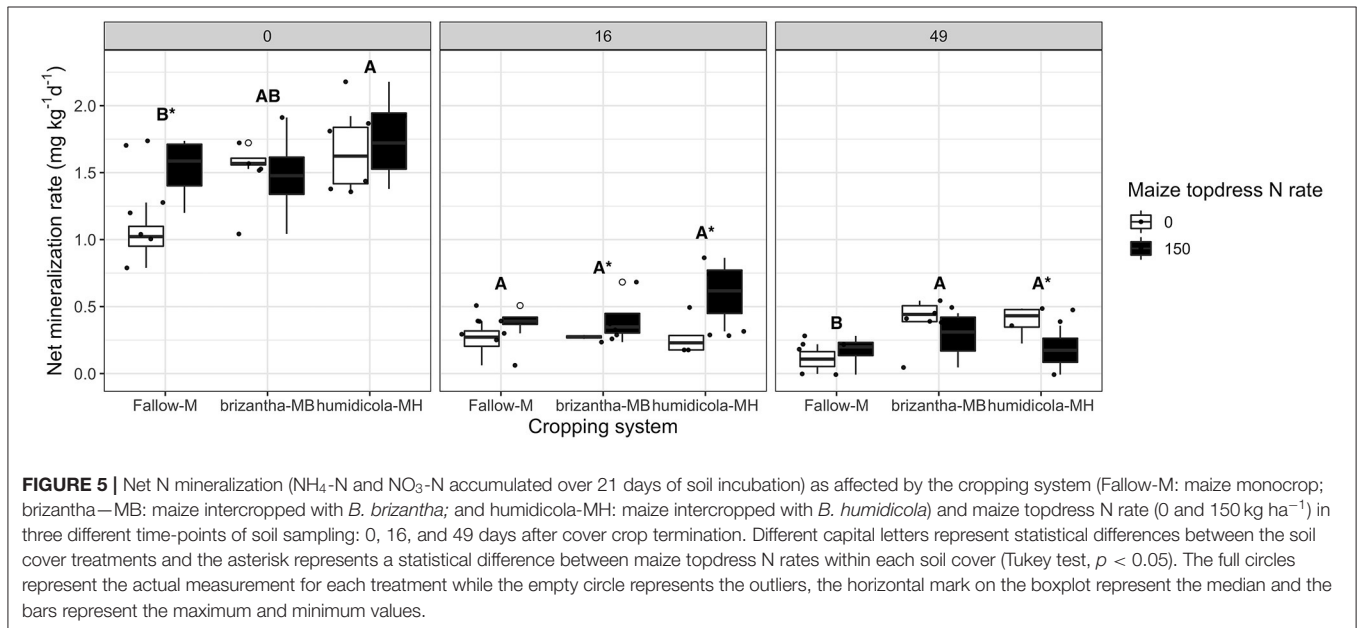
fertilization had a negative impact on mineralization of plots with *B. humidicola*, decreasing the mineralization rates by 0.22 mg kg<sup>-1</sup> day<sup>-1</sup> (Figure 5).

Nitrous oxide emissions were only affected by the cropping system but not by N fertilization. Plots of maize intercropped

with *B. brizantha* and *B. humidicola* emitted 12.8 and 4.8 mg N<sub>2</sub>O-N m<sup>-2</sup> more than that of monocropping system regardless of N fertilization; intercropped plots with *B. brizantha* had higher N<sub>2</sub>O emission than *B. humidicola* (Figure 6). Cumulative soil respiration was higher only for plots of maize intercropped with



**FIGURE 4** | Soil potential net nitrification rate as affected by the cropping system (Fallow-M: maize monocrop; brizantha—MB: maize intercropped with *B. brizantha*; and humidicola-MH: maize intercropped with *B. humidicola*) and maize topdress N rate (0 and 150 kg ha<sup>-1</sup>) in three different time-points of soil sampling: 0, 16, and 49 days after cover termination. Different capital letters represent statistical differences between the soil cover treatments and the asterisk represents a statistical difference between maize topdress N rates within each soil cover (Tukey test,  $p < 0.05$ ). The full circles represent the actual measurement for each treatment while the empty circle represents the outliers, the horizontal mark on the boxplot represent the median and the bars represent the maximum and minimum values.



**FIGURE 5** | Net N mineralization (NH<sub>4</sub>-N and NO<sub>3</sub>-N accumulated over 21 days of soil incubation) as affected by the cropping system (Fallow-M: maize monocrop; brizantha—MB: maize intercropped with *B. brizantha*; and humidicola-MH: maize intercropped with *B. humidicola*) and maize topdress N rate (0 and 150 kg ha<sup>-1</sup>) in three different time-points of soil sampling: 0, 16, and 49 days after cover termination. Different capital letters represent statistical differences between the soil cover treatments and the asterisk represents a statistical difference between maize topdress N rates within each soil cover (Tukey test,  $p < 0.05$ ). The full circles represent the actual measurement for each treatment while the empty circle represents the outliers, the horizontal mark on the boxplot represent the median and the bars represent the maximum and minimum values.

*B. brizantha*, which emitted 39.3 g CO<sub>2</sub>-C m<sup>-2</sup> more than the average of the other treatments (Figure 7) and had higher WFPS values than maize monocrop plots at all three sampling times ( $p < 0.05$ ) (Figure 8).

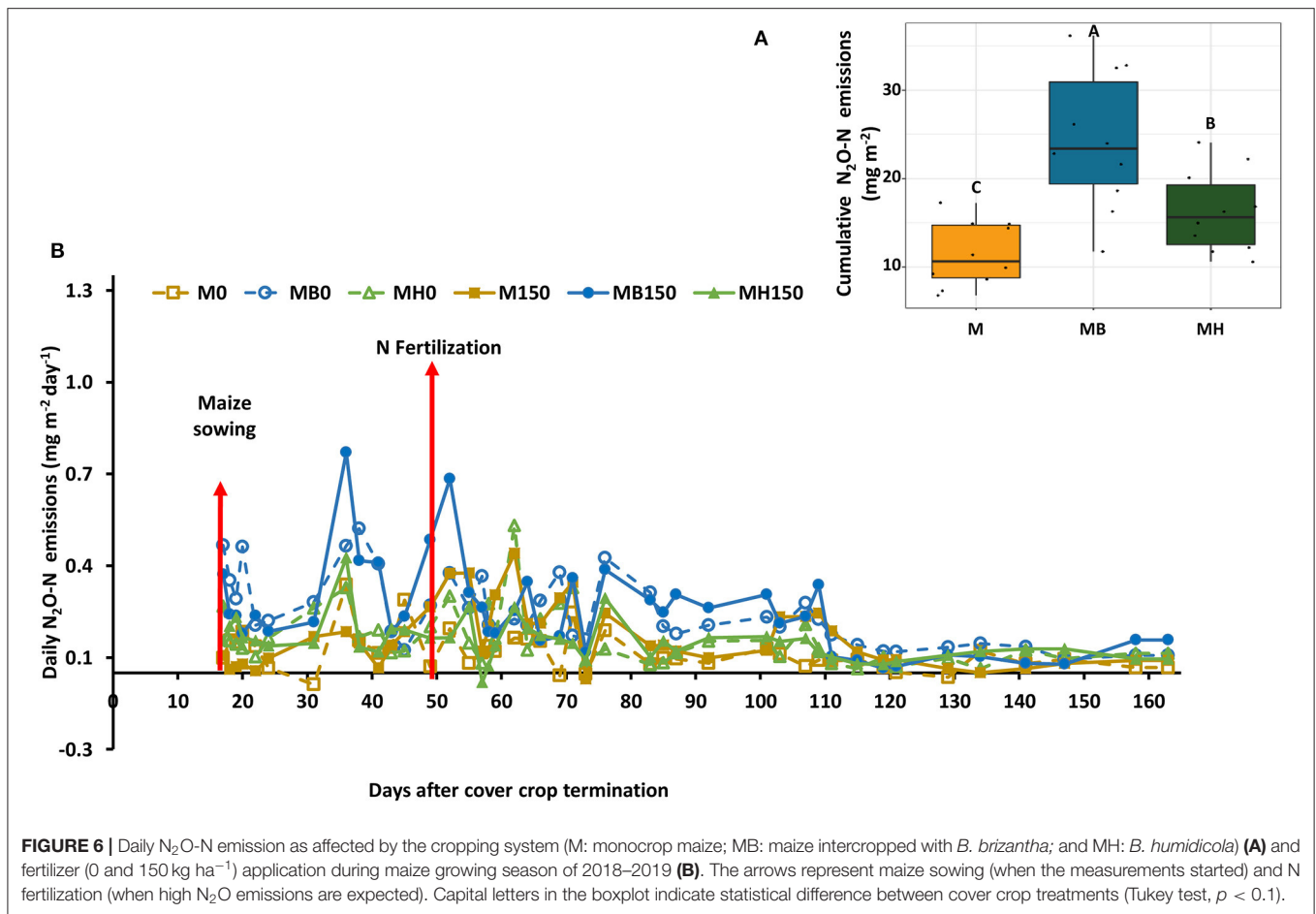
The effect of cropping system and N fertilization on inorganic N was only significant at 49 DAT. At this sampling time, soil with maize and *B. brizantha* intercropping had higher inorganic N, averaging 3.7 mg kg<sup>-1</sup> more than plots of maize monocropping (Figure S2).

No effect of treatment was observed on total extracted DNA or the abundance of AOA genes (Figures S4–S6). The only marginally significant experimental effect was observed before forage grass termination, when *B. humidicola* without maize N

fertilization resulted in a higher number of copies of 16S than with N fertilization ( $p < 0.1$ ).

## Bacterial/Archaeal and Fungi Community

The alpha diversity (Shannon index) of the bacterial community was not influenced by cropping system or N fertilization of the previous maize crops. However, fungal alpha diversity was positively affected by *B. brizantha* regardless of the sampling time (Figure 9). At 0 and 49 DAT, we identified 7 and 24 bacterial, respectively, and 27 and 26 fungal taxa positively associated with the monocrop system (Figure S7) while only 4 and 1 bacterial and 3 and 6 fungi taxa positively associated with intercropping system, respectively (Figure S8).



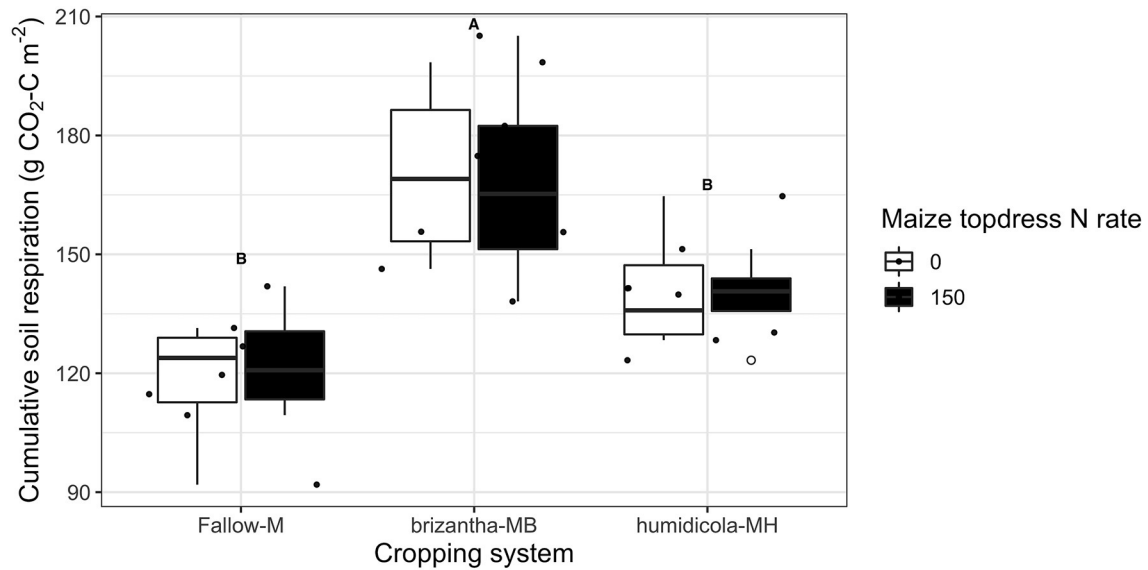
Before termination (0 DAT) *B. humidicola* was positively associated with *Nitrospira*, a nitrite oxidizing organism, while *B. brizantha* was positively associated with *Nitrosomonadaceae mle1-7*, an AOB. However, this effect did not last until the maize season (Figures S9, S10). We also observed that *Myxococcaceae*, positively associated with *B. humidicola* at 0 and 49 DAT (Figure S9), could have a significant functional role for N mineralization and it was used as a possible soil biological indicator of N mineralization for the principal component analysis.

Before forage grass termination, soil samples of the *B. brizantha* plots had greater fungi as well as diversity higher relative abundance of AMF and lower saprotrophs compared to other cropping systems (Figure 9 and Figure S11), however there was no effect of cropping system or maize N rate on any trophic mode for soil sampled at 49 DAT (Figure S11). In our PCA, we observed that AMF vs. EMF and endophyte vs. saprotroph opposed their relative abundances being endophytes the only trophic mode clustered with fungi diversity before the forage grasses termination (0 DAT) (Figure 10). However, at 49 DAT the AMF and endophyte clustered in a direction opposed of that saprotroph while EMF was the only group that clustered in opposition of fungal diversity (Figure 10). Although AMF clustered in opposite direction of C:N ratio at

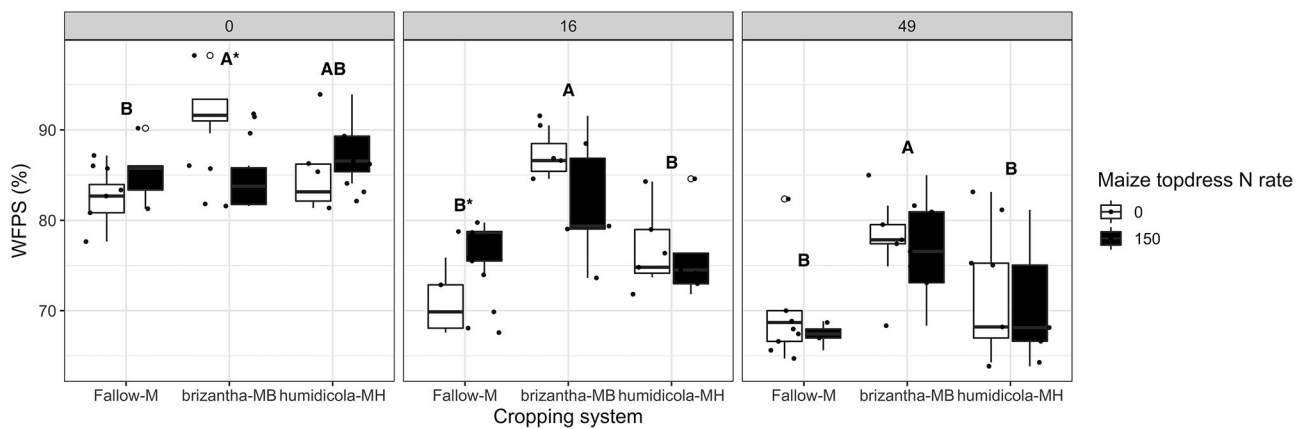
49 DAT, the *Glomerales* was still positively associated with C:N ratio of the grasses even after the termination. The positive effect of *B. brizantha* and *B. humidicola* on different AMF organisms belonging to the *Glomerales* family persisted during the maize growing season (Figures S9, S10). One taxa of the genera *Fusarium*, typically classified as saprotrophic organisms that could be opportunistic pathogenic or endophytic, was positively associated with intercropping system (Figure S8). Only fungal diversity clustered with grass biomass for soil sampled after the termination. The presence of soil cover was related to *Trichoderma spp.* regardless of the time point (Figure S8). *Glomerales* (AMF), *Trichoderma* (endophyte) and *Fusarium* (saprotroph) clustered with the respective trophic mode before the termination (0 DAT) but did not at 49 DAT (Figure 10). As *Glomerales*, *Trichoderma* clustered with C:N ratio in opposite direction of endophytes and AMF while *Fusarium* clustered in opposition to grass biomass and fungi diversity at 49 DAT (Figure 10).

### Correlations of N Cycling Parameters

PCA showed that the intercropping system impacted soil N dynamics differently during the grasses and maize growing season. Before herbicide termination, the potential net nitrification rates clustered with plant C:N ratio was associated



**FIGURE 7** | Cumulative soil respiration as affected by the cropping system (M: monocrop maize; MB: maize intercropped with *B. brizantha*; and MH: *B. humidicola*) and N fertilizer (0 and 150 kg ha<sup>-1</sup>) application during maize growing season of 2018–2019. Capital letters indicate statistical difference between cover crop treatments (Tukey test,  $p < 0.1$ ). The full circles represent the actual measurement for each treatment while the empty circle represents the outliers, the horizontal mark on the boxplot represent the median and the bars represent the maximum and minimum values.

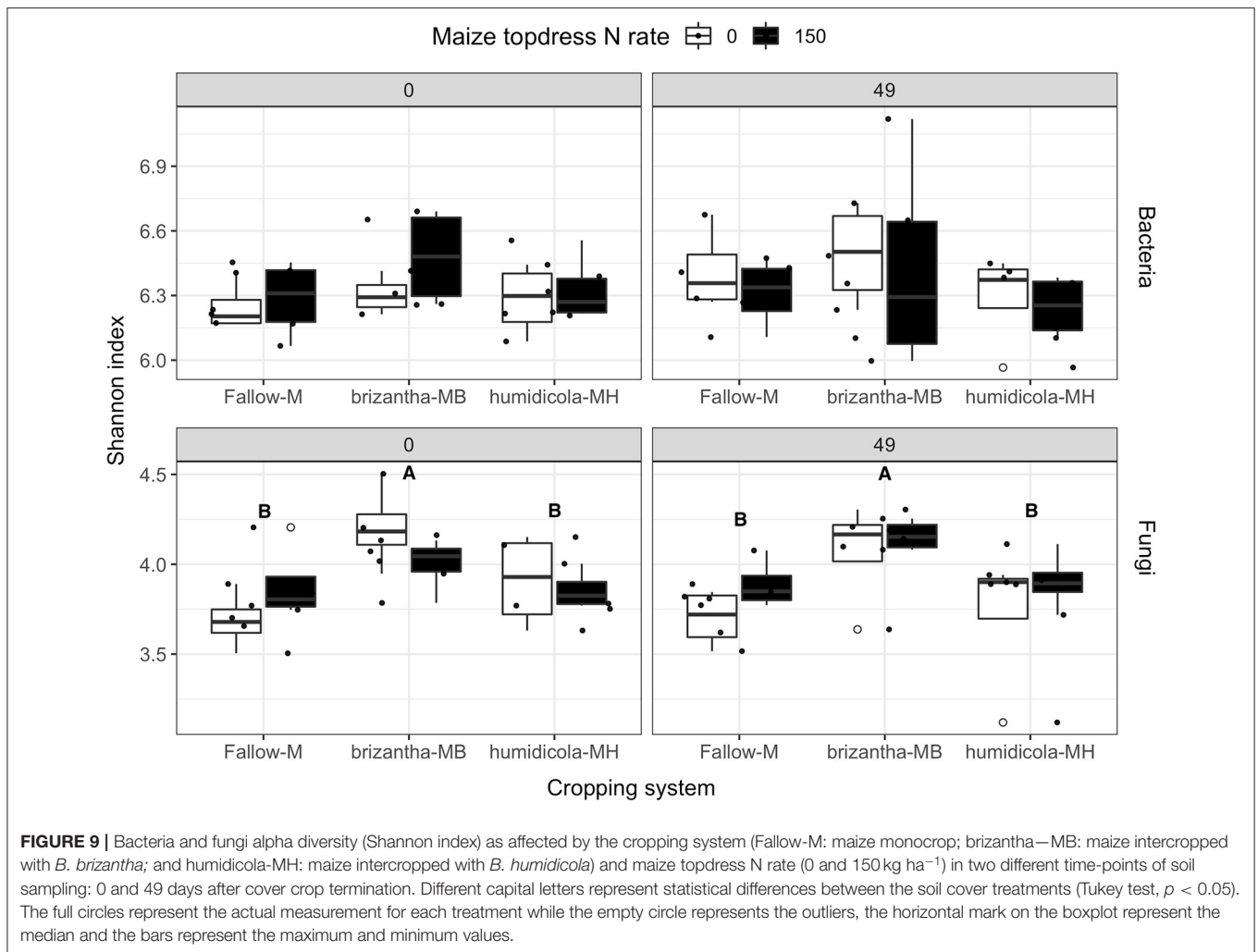


**FIGURE 8** | Soil water filled pore space (WFPS) as affected by the cropping system (Fallow-M: maize monocrop; brizantha—MB: maize intercropped with *B. brizantha*; and humidicola-MH: maize intercropped with *B. humidicola*) and maize topdress N rate (0 and 150 kg ha<sup>-1</sup>) in three different time-points of soil sampling: 0, 16, and 49 days after cover crop termination. Different capital letters represent statistical differences between the soil cover treatments and the asterisk represents a statistical difference between maize topdress N rates within each soil cover (Tukey test,  $p < 0.05$ ). The full circles represent the actual measurement for each treatment while the empty circle represents the outliers, the horizontal mark on the boxplot represent the median and the bars represent the maximum and minimum values.

with *B. humidicola* treatments and negatively correlated with grass biomass associated with *B. brizantha* (Figure 10). The biological indicators positively associated with potential net nitrification rates were relative abundance of saprotroph fungi and *Fusarium*, abundance of AOA, and relative abundance of *Myxococcaceae*. Net mineralization rate was associated with grass biomass and C:N ratio and clustered with relative abundance of AMF, *Nitrosomonadaceae* and *Glomerales*, bacterial diversity, and total DNA. At this time point, the cumulative N<sub>2</sub>O emission during the maize growing season clustered

with fungi diversity, abundance of 16S rDNA and relative abundance of fungal endophytes while negatively associated with soil potential net nitrification rate and abundance of AOA.

However, in soil samples taken during maize growing season, the net mineralization rate was associated with potential net nitrification rate. The N<sub>2</sub>O emission clustered with both potential net nitrification rates and mineralization at 49 DAT, the biological indicators associated with that was fungal and bacteria diversity, and relative abundance of



**FIGURE 9** | Bacteria and fungi alpha diversity (Shannon index) as affected by the cropping system (Fallow-M: maize monocrop; brizantha—MB: maize intercropped with *B. brizantha*; and humidicola-MH: maize intercropped with *B. humidicola*) and maize topdress N rate (0 and 150 kg ha<sup>-1</sup>) in two different time-points of soil sampling: 0 and 49 days after cover crop termination. Different capital letters represent statistical differences between the soil cover treatments (Tukey test,  $p < 0.05$ ). The full circles represent the actual measurement for each treatment while the empty circle represents the outliers, the horizontal mark on the boxplot represent the median and the bars represent the maximum and minimum values.

*Myxococcaceae*. Other soil parameters, such as *Glomerales*, *Trichoderma*, *Nitrosomonadaceae*, *Nitrospira*, saprotroph, AOA, total DNA, abundance of 16S rDNA and AOA were better associated with C:N ratio than biomass and negatively associated with the AMF and endophytes relative abundance.

In the multiple linear regression model, net mineralization rate was the main explanatory variable of potential net nitrification rate, accounting for 27% of the  $R^2$  (Table 1). In general, abundance of AOA was negatively correlated with potential net nitrification rate (12% of the  $R^2$ ), while the total extracted DNA (a proxy for microbial biomass), fungal diversity and pH significantly explained potential net nitrification rates ( $p < 0.05$ ) but explained <10% of the model variance. The full model explained 77% of the variance of potential net nitrification rates. PCA showed that the effect of intercropping was different at 0 and 49 DAT, being associated with saprotrophs and AOA at 0 DAT and with fungal and bacterial diversity at 49 DAT.

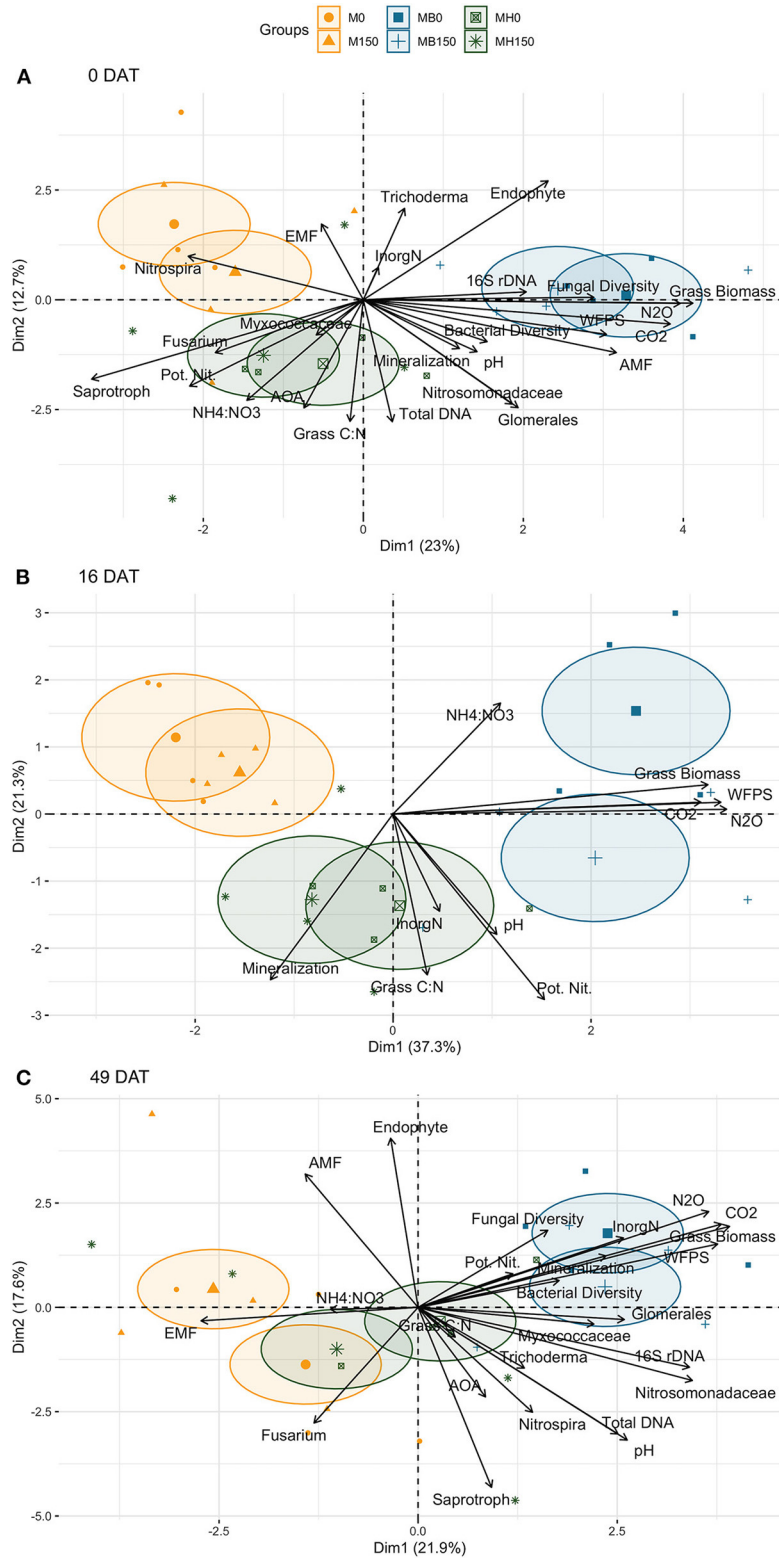
Only soil WFPS was significantly and positively correlated with net N mineralization, accounting for 40% of the model  $R^2$ , and the multiple linear regression model explained 68% of

the data variance (Table 1). The PCAs confirmed the strong correlation between soil water content and mineralization rates, except for soil sampled 16 DAT. The soil WFPS was also associated with treatments where biomass production during the fall-spring by forage grasses were higher (Figure 10).

Potential net nitrification and mineralization rates of soil sampled 49 DAT (1 day before topdress N fertilization) were positively correlated with N<sub>2</sub>O emission and accounted for 8 and 20% of the model  $R^2$ , respectively (Table 2). PCA showed that cumulative N<sub>2</sub>O was mainly correlated with the biomass produced by grasses and weeds before maize growing season and WFPS regardless of sampling time (Figure 10).

## DISCUSSION

In this study, we sought to assess how tropical forage grasses growing in the fall-spring period in a maize intercropping system and chemically terminated before maize season affected potential soil net nitrification, N mineralization and consequently



**FIGURE 10 |** Principal component analysis of N cycling parameters (potential net nitrification rates, net mineralization rates and cumulative N<sub>2</sub>O emission), and other soil (soil inorganic N, NH<sub>4</sub>:NO<sub>3</sub> ratio, WFPS, pH, total DNA extracted from soil, abundance of 16S rDNA and AOA genes, cumulative respiration and bacteria and fungi alpha diversity) and plant parameters (forage grass biomass produced during the spring-fall period and maize grain yield of the subsequent growing season) across cropping systems (M: monocrop maize; MB: maize intercropped with *B. brizantha*; and MH: *B. humidicola*) and maize N fertilization rates (0 and 150 kg ha<sup>-1</sup>) at 3 time points: 0 (A), 16 (B) and 49 (C) days after termination (DAT).

**TABLE 1** | Results of a multiple linear regression model to predict potential net nitrification rate and potential mineralizable N using explanatory variables and the relative importance of each parameter (confidence interval of 95%).

Predicted variables	Explanatory variables	Estimate	Std. error	p	% of R <sup>2</sup>	
Potential net nitrification rate	Net mineralization rate	1.763	0.556	0.000	27 (19–38)	
	Abundance of AOA	−2.982	0.960	0.050	12 (3–27)	
	pH	−1.598	0.577	0.064	8 (2–21)	
	Total DNA	0.034	0.013	0.070	6 (2–16)	
	Fungal alpha diversity	−1.687	0.824	0.099	3 (0–9)	
	Rel. Abund. of Saprotrophs	2.23E+01	1.50E+01	0.112	4 (1–12)	
	Rel. Abund. of EMF	−1.498	1.012	0.149	1 (0–5)	
	Rel. Abund. of <i>Nitrospira</i>	1.68E+02	1.23E+02	0.252	1 (0–2)	
	WFPS	−0.037	0.037	0.282	7 (3–13)	
	Rel. Abund. of AMF	0.800	1.084	0.352	<1	
	Bacterial alpha diversity	0.079	1.007	0.509	1 (0–3)	
	NH <sub>4</sub>	−0.010	0.139	0.542	5 (1–11)	
	Rel. Abund. of <i>Nitrosomonadaceae</i>	1.861	53.472	1 (0–4)		
		NO <sub>3</sub>	0.005	0.143	0.867	1 (0–2)
Full model R <sup>2</sup> : 77%						
Net mineralization rate	WFPS	0.052	0.010	<0.001	40 (28–61)	
	pH	−0.268	0.187	0.162	4 (0.5–13)	
	NH <sub>4</sub>	0.061	0.049	0.218	13 (6–22)	
	EMF	0.421	0.344	0.231	2 (0.2–5)	
	Fungal alpha diversity	0.374	0.318	0.249	3 (0.4–9)	
	Bacterial alpha diversity	−0.428	0.368	0.253	2 (0.2–8)	
	Rel. Abund. of Myxococcaceae	−4.08E+01	3.61E+01	0.268	<1	
	Endophytes	−0.752	1.205	0.537	<1	
	AMF	−0.193	0.365	0.602	<1	
	Rel. Abund. of Fusarium	0.535	1.251	0.672	<1	
	NO <sub>3</sub>	−0.016	0.051	0.749	1 (0.3–5)	
	Rel. Abund. of Trichoderma	−1.730	8.726	0.844	<1	
	Saprotrophs	−0.272	11.070	0.981	<1	
	Total DNA	−5.25E−05	3.11E−03	0.987	3 (0.4–10)	
	Full model R <sup>2</sup> : 68%					

AOA, ammonia oxidizing archaea; WFPS, water filled pore space; NH<sub>4</sub>, soil ammonium content; NO<sub>3</sub>, soil nitrate content.

leave a legacy to either reduce or increase N<sub>2</sub>O emission by maize production in tropical soil. We observed that the forage grasses were associated with organisms related to nitrification and mineralization. However, forage grasses did not reduce the potential net nitrification rates before the termination but instead, *B. humidicola*, with the higher BNI potential, increased the potential net nitrification rates (Figure 4). Net mineralization rates in general followed the same trends (Figure 5), which was the main explanatory variable for the potential net nitrification rate (Table 1). Furthermore, the hypothesis that introducing forage grasses in the maize cropping system could leave a legacy to mitigate the N<sub>2</sub>O emission was denied, showing that the nitrification inhibition potential of tropical grasses may be outweighed by their impacts on soil moisture, N recycling, and the soil microbiome that together dictate soil N<sub>2</sub>O fluxes (Figure 10).

### ***Brachiaria brizantha* and *Brachiaria humidicola* Impact Nitrification and N Mineralization Differently Prior to Maize Cropping**

At 0 DAT we observed for that forage grass intercropping increased net N mineralization and potential nitrification rates. These changes on soil functions related to N cycling are associated with changes in saprotrophic and mycorrhizal fungi, and bacterial nitrifiers prior to maize growth.

Our results of 16S rDNA amplicon sequencing showed that *B. humidicola* at 0 DAT, which resulted in higher potential net nitrification rates (Figure 4), was also associated with the nitrite oxidizer, *Nitrospira* regardless of the maize topdress N rate. The ammonia oxidizing bacteria, *Nitrosomonadaceae mle1-7*, was associated to *B. brizantha*. Although *B. humidicola* was shown to exudate brachialactone that inhibits the activity of *Nitrosomonas*

**TABLE 2 |** Results of a multiple linear regression model to predict cumulative nitrous oxide emissions using explanatory variables and the relative importance of each parameter (confidence interval of 95%) measured in three different time point of soil sampling: 0, 16, and 49 days after cover crop termination (DAT).

Predicted variable	Explanatory variables	Estimate	Std. error	p	% of R <sup>2</sup>
<b>Cumulative N<sub>2</sub>O emission</b>					
<b>0 DAT</b>					
	WFPS	0.042	0.015	0.017	26(2-47)
	Net mineralization rate	0.430	0.344	0.237	12 (4-33)
	NO <sub>3</sub>	0.089	0.077	0.275	6 (2-16)
	Total DNA	0.003	0.003	0.320	3 (1-19)
	Potential net nitrification rate	-0.042	0.052	0.434	7 (2-22)
	pH	-0.176	0.220	0.441	2 (1-11)
	Fungal alpha diversity	0.329	0.434	0.464	14 (3-37)
	NH <sub>4</sub>	0.031	0.065	0.645	1 (1-14)
	Bacterial alpha diversity	-0.114	0.501	0.825	1 (0.5-11)
<b>Full model R<sup>2</sup>: 74%</b>					
<b>16 DAT</b>					
	WFPS	1.104	0.160	< 0.001	65 (54-82)
	pH	-3.055	2.274	0.197	1 (0-3)
	Potential net nitrification rate	0.708	0.621	0.270	8 (0.4-23)
	NO <sub>3</sub>	-0.172	0.319	0.596	<1
	NH <sub>4</sub>	-0.140	0.300	0.645	2 (0-7)
	Net mineralization rate	-0.226	4.965	0.964	4 (0.4-13)
<b>Full model R<sup>2</sup>: 81%</b>					
<b>49 DAT</b>					
	WFPS	0.049	0.010	< 0.001	33 (10-60)
	Net mineralization rate	0.831	0.303	0.016	20 (3-45)
	Potential net nitrification rate	0.140	0.060	0.035	8 (2-27)
	NO <sub>3</sub>	-0.076	0.043	0.095	3 (1-6)
	Fungal alpha diversity	0.357	0.214	0.117	12 (3-31)
	NH <sub>4</sub>	0.058	0.037	0.141	4 (1-14)
	Bacterial alpha diversity	-0.321	0.245	0.210	2 (0-3)
	pH	-0.180	0.160	0.281	1 (0-1)
	Total DNA	0.002	0.004	0.673	<1
<b>Full model R<sup>2</sup>: 83%</b>					

Before maize planting

After maize planting

WFPS, water filled pore space; NH<sub>4</sub>, soil ammonium content; NO<sub>3</sub>, soil nitrate content; AOA, ammonia oxidizing archaea.

*europaeae* (52) and Byrnes et al. (15) observed that *B. humificola* could reduce the abundance of AOO and net nitrification rates, our results showed the opposite effect of forage grasses on nitrifiers organism. However, when Vázquez et al. (11) compared the same cultivar of *B. humificola* used in our experiment with that of the hybrid “Mulato” of the *Brachiaria* genus, they found no negative effect on gross nitrification rates. Along with the effect of the forage grasses on organisms related to nitrification at the end of fall-spring season (0 DAT), *B. humificola* did not reduce but rather increased the potential net nitrification rates, suggesting that forage grass might impact net nitrification rates by increasing inorganic N availability (mineralization).

Although *Nitrosomonadaceae mle1-7* was associated to *B. brizantha*, the net nitrification rates were lower when compared with *B. humificola*. At this time point (0 DAT), *B. brizantha* showed higher relative abundance of AMF and fungi diversity, but lower saprotrophs in comparison with other treatments. One possible mechanism of nitrification inhibition is the competition of soil fungal communities with ammonium oxidizing organisms

(AOO) for substrate (17). Teutscherova et al. (12) found that grasses with high BNI potential also had higher root colonization by AMF. Thus, lower net nitrification and mineralization rates in soils with growing *B. brizantha* when compared to *B. humificola*, may be explained by the higher inorganic N consumption by AMF since its relative abundance was higher in this treatment (Figure S11).

### The Legacy of Forage Grasses on Nitrification and Mineralization During Maize Cropping

The association of *Nitrospira* and *Nitrosomonadaceae mle1-7* with *B. humificola* and *B. brizantha*, respectively, before herbicide termination did not last after the termination (0 DAT), and both forage grasses had higher potential net nitrification and mineralization rates than plots under monocrop. Fanin et al. (13) showed that after an initial N immobilization, different residues tend to have positive net N mineralization in longer



term. Plots under the intercropping system showed a positive effect of intercropping on net N mineralization at 49 DAT. As net mineralization rates were increased in plots with residues of both grasses, the hypothesis of the legacy of forage grasses causing a reduction in net nitrification rates due to N immobilization or residual of BNI activity was rejected.

Using  $^{15}\text{N}$  pool dilution, Vázquez et al. (11) observed that genotypes associated with high BNI potential mineralized and nitrified 2.3 and 0.5  $\mu\text{g g}^{-1} \text{day}^{-1}$  more N, respectively, than genotypes with low BNI potential. Their results suggest that the effect of *B. humidicola* to reduce net nitrification, AOA abundance and  $\text{N}_2\text{O}$  emissions (10, 15, 53, 54) might be due to their effect on gross mineralization/immobilization but not directly on gross nitrification rates. Higher N mineralization provides more substrate for nitrification and consequently increases the nitrification process (55, 56). The potential net nitrification rates are commonly better associated soil organic C than the abundance of AOA and AOB in soil (57), which might be a result of the impact of soil C on mineralization and consequently nitrification rate (55). In the same direction, our study showed that net mineralization rate was a good predictor of potential net nitrification rate (Table 1), supporting the idea that mineralization is an important bottleneck for soil nitrification (55, 58).

The 3-year legacy of the forage grasses to soil under maize cropping was positive on soil mineralization and nitrification rates, regardless of the species, and only *B. brizantha* left a positive legacy on fungi diversity either by rhizodeposition during the fall-spring or plant residues (23). Fungal and bacterial diversity was found to be also associated with residue mineralization rates (28). Our results reinforce that tropical forage grasses can select specific organism related to nitrification that affect potential net nitrification rates during the fall-spring season. However, the legacy of these grasses on N mineralization is the most important parameter influencing the potential net nitrification rate, or the potential of the soil to produce and accumulate  $\text{NO}_3$  (Table 1). Other parameters positively related to potential net nitrification rates were total soil DNA (Table 1). Relative abundance of *Myxococcaceae* also clustered with potential net nitrification rate (Figure 10), this group correspond to a predatory organism that lyses and takes up nutrients from other organisms, and is associated with high microbial biomass (59) and responsive to C and N addition (60), suggesting that changes in microbial biomass known to be positively correlated with N mineralization rates (55). Thus, our results obtained in shorter incubations (7 days) support the evidence that grasses impact the bacterial community related to N cycling before the maize growing season, leaving a positive legacy to N mineralization and nitrification rates, as also observed by Vázquez et al. (11).

## The Legacy of Forage Grasses to $\text{N}_2\text{O}$ Emission During Maize Cropping

We found that that *B. brizantha* produced 5  $\text{Mg ha}^{-1}$  of dry mass more than the fallow plots while *B. humidicola* only increased the shoot biomass production by 1  $\text{Mg ha}^{-1}$ . The

biomass production of forage grasses was not affected by the N fertilization on maize. The high demand for N and fast initial growth of the cereal, shading the brachiaria plants and reducing competition, result in low N uptake by the grasses by the end of the maize growing season. Coser et al. (5) showed that only 2.08% of the  $^{15}\text{N}$  fertilizer applied during maize growing season was taken up by *B. humidicola*. In addition, N mineralization in the clay soil used in our study probably is supplying the nutrient needed for the relatively high grass biomass yields, specially of *B. brizantha*.

The amount of residue left in the field has been shown to be an important factor controlling soil moisture and consequently  $\text{N}_2\text{O}$  emission in tropical soils (61, 62). Thus, the biomass production by forage grasses may be the main factor driving higher soil moisture, recycling N, and adding labile C consequently increasing  $\text{N}_2\text{O}$  emission during the maize growing season. Plots with Brachiaria intercropping had the highest  $\text{N}_2\text{O}$  emissions. On the other hand, the high yielding *B. brizantha* resulted in an annual C input of 3.8  $\text{Mg ha}^{-1}$  more than the monocrop system, considering the shoot and root biomass at the top 40 cm soil layer, *B. brizantha* was shown to accumulate more than 100  $\text{kg ha}^{-1}$  of N in the biomass, but emitted only 0.4  $\text{kg ha}^{-1}$  of  $\text{N}_2\text{O}$ -N over the second maize harvest season of the same experiment in the field (1). Carvalho et al. (63) found that, despite of the higher greenhouse gas emission from the soil as  $\text{N}_2\text{O}$  and  $\text{CH}_4$ , the intercropping system stored 600  $\text{kg ha}^{-1} \text{year}^{-1}$  of C as SOC, resulting in a negative balance of greenhouse gas emission from this cropping system: those authors estimated a net reduction of 360  $\text{kg C-equivalent ha}^{-1} \text{year}^{-1}$  for the intercropping system in a tropical soil.

The stimulatory effect of plant residues on  $\text{N}_2\text{O}$  emission may be attributed to: (i) increased N supply by mineralization (64), (ii) C addition through plant residue (65), and (iii) the presence of soil cover itself (61). Canisares et al. (1) suggested also that N released shortly after grass desiccation with herbicide could increase  $\text{N}_2\text{O}$  emissions. Using a synthetic and sugarcane straw compared to bare soil, Fracetto et al. (61) observed that the soil cover was responsible for retaining soil moisture and consequently increasing  $\text{N}_2\text{O}$  emission regardless of labile C inputs and N mineralization during straw decomposition. The residues left on the field by the forage reduced evaporation and explain the higher soil moisture for soil sampled at 49 DAT (61, 62).

In our study, cumulative  $\text{N}_2\text{O}$  emissions were correlated with WFPS regardless of the time point of soil sampling, and WFPS explained 26, 65, and 33% of  $\text{N}_2\text{O}$  data variance at 0, 16 and 49 DAT, respectively (Table 2). Higher WFPS results in lower  $\text{O}_2$  availability and consequently lower redox potential, creating a more favorable condition for denitrification than the other treatments (66). However, we did not identify strong evidence of anaerobic taxa, which suggest that soil conditions do not match with ideal conditions where denitrification is the main biogeochemical process driving  $\text{N}_2\text{O}$  emission (66).

Soil moisture is also associated with N mineralization and nitrification (55) and our results showed that WFPS was the only significant explanatory variable for net mineralization

rate (Table 1). Besides WFPS, the N<sub>2</sub>O emission was strongly associated with N mineralization rates and soil respiration as well, mainly at 49 DAT (Figure 10). Soil moisture measurements typically follow soil C content (55). However, we did not measure soil C because several years are needed for management treatments to cause significant changes on soil C content (6). The WFPS correlated well with mineralization because of the changes on soil moisture had temporal effects, while using forage grass biomass and C:N ratio as a reference for labile C input did not follow a temporal change. Thus, the higher N<sub>2</sub>O emissions in plots with *B. brizantha* mulch may be attributed to a higher N and labile C pulse after forage grass termination due to its higher biomass yield than *B. humidicola*. The combination of higher biomass with high soil moisture at field conditions, which could not be decoupled in our experimental design, increased N mineralization, consequently supplying substrate for nitrification and denitrification (64).

## CONCLUSION

Our study provided important insights for the understanding of previously unknown aspects of the complex maize-brachiaria intercropping system. We evaluated whether introducing tropical grasses with contrasting BNI potential could reduce nitrification and consequently reduce N<sub>2</sub>O emission during maize cultivation in a tropical soil. This hypothesis was denied. *B. humidicola*, with higher BNI potential, showed to positively impact the potential net nitrification rates at herbicide termination, and both grasses left a positive legacy on potential net nitrification rate 49 days after herbicide termination. *B. humidicola* and *B. brizantha* were associated with nitrifiers prior to termination but no effect on these organisms was observed during the maize season, so this effect was temporary. *B. brizantha* showed a positive effect on AMF and fungi diversity while reducing saprotrophs prior to maize cropping, but the legacy of this grass to maize cropping was a higher fungal diversity. Soil moisture and the forage grass biomass were associated with net mineralization rates, which was related to potential net nitrification rates during early maize growing season. The cumulative emission of N<sub>2</sub>O during the maize growing season was strongly associated with soil moisture, respiration, bacterial and fungal diversity, net mineralization and nitrification rates, and the biomass produced by grasses during the fall-winter-spring, showing that the nitrification inhibition potential of tropical grasses can be outweighed by their impacts on N recycling, soil moisture and the soil microbiome that together dictate soil N<sub>2</sub>O fluxes. This study focused on the initial 3 years after starting this cropping system. The effect of the C input might result in higher soil organic C at the longer term that might compensate the greater N<sub>2</sub>O emission of the intercropping system. Thus, evaluations for longer periods shall be done in the future to measure the net greenhouse gases emission considering the soil C sequestration. Despite the higher N<sub>2</sub>O emissions caused by grasses, brachiarias provided C to the system and did not have a negative effect on maize grain yields. Our findings

may help to devise ways for improving an already sustainable cropping system.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI [accession: PRJNA746808].

## AUTHOR CONTRIBUTIONS

LC, HC, LM, and CC contributed with the study design, run the field and lab experiment, collect the data, data analysis, and writing the manuscript. EB, PS, and UK contributed to microbial amplicon sequencing, data processing and analysis, and writing the manuscript. DV and JA contributed with measuring the abundance of amoA genes and paper writing. HP contributed with data analysis and writing the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fsoil.2021.746433/full#supplementary-material>

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