Brachiaria humidicola Cultivation Enhances Soil Nitrous Oxide Emissions from Tropical Grassland by Promoting the Denitrification Potential: A \(^{15}\)N Tracing Study

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Abstract: Biological nitrification inhibition (BNI) in the tropical grass Brachiaria humidicola could reduce net nitrification rates and nitrous oxide (\(\text{N}_2\text{O}\)) emissions in soil. To determine the effect on gross nitrogen (N) transformation processes and \(\text{N}_2\text{O}\) emissions, an incubation experiment was carried out using \(^{15}\)N tracing of soil samples collected following 2 years of cultivation with high-BNI Brachiaria and native non-BNI grass Eremochloa ophiuroides. Brachiaria enhanced the soil ammonium (\(\text{NH}_4^+\)) supply by increasing gross mineralization of recalcitrant organic N and the net release of soil-adsorbed \(\text{NH}_4^+\), while reducing the \(\text{NH}_4^+\) immobilization rate. Compared with Eremochloa, Brachiaria decreased soil gross nitrification by 37.5% and \(\text{N}_2\text{O}\) production via autotrophic nitrification by 14.7%. In contrast, Brachiaria cultivation significantly increased soil \(\text{N}_2\text{O}\) emissions from 90.42 \(\mu\)g \(\text{N}_2\text{O}-\text{N}\) kg\(^{-1}\) under Eremochloa cultivation to 144.31 \(\mu\)g \(\text{N}_2\text{O}-\text{N}\) kg\(^{-1}\) during the 16-day incubation \((p < 0.05)\). This was primarily due to a 59.6% increase in \(\text{N}_2\text{O}\) production during denitrification via enhanced soil organic C, notably labile organic C, which exceeded the mitigated \(\text{N}_2\text{O}\) production rate during nitrification. The contribution of denitrification to emitted \(\text{N}_2\text{O}\) also increased from 9.7% under Eremochloa cultivation to 47.1% in the Brachiaria soil. These findings confirmed that Brachiaria reduces soil gross nitrification and \(\text{N}_2\text{O}\) production via autotrophic nitrification while efficiently stimulating denitrification, thereby increasing soil \(\text{N}_2\text{O}\) emissions.

Keywords: biological nitrification inhibition; Brachiaria humidicola; \(\text{N}_2\text{O}\) emissions; gross N transformation processes; denitrification

1. Introduction

Nitrous oxide (\(\text{N}_2\text{O}\)) concentrations in the atmosphere have increased by more than 20% since pre-industrial times and are responsible for 6% of current global warming [1]. \(\text{N}_2\text{O}\) emissions are also an important factor in stratospheric ozone depletion [2], with agricultural soil accounting for approximately 66% of global anthropogenic \(\text{N}_2\text{O}\) emissions, mainly due to the excessive input of synthetic N fertilizers [3,4]. The increasing use of synthetic fertilizers is also causing increased nitrifier activity, transforming modern agricultural systems into high-nitrifying environments [5,6].

Ammonia oxidation is the rate-limiting first step of nitrification, producing \(\text{N}_2\text{O}\) as a by-product [7]. Biological nitrification inhibition (BNI) is a rhizospheric process whereby specific inhibitors exuded or released from the plant’s roots suppress the activity of...
nitrifying bacteria [8]. This process is widely found in major crops, such as sorghum [9], rice [10], wheat [11,12] and maize [13], as well as in certain forage species [14] and trees [15]. *Brachiaria humidicola*, a tropical grass native to East and Southeast Africa, has a strong BNI capacity due to the release of the specific compound brachialactone in its root exudates [14,16]. Previous studies have shown that soil collected from established *Brachiaria* plots shows a remarkable decrease in the net nitrification rate during incubation compared with soil cultivated with non-BNI plants [16–19]. Meanwhile, Subbarao et al. [14] found that both the soil ammonia oxidation rates and cumulative N$_2$O emissions were reduced by almost 90% after *Brachiaria* pasture planting compared with soybean or plant-free plots during a three-year field experiment in Colombia. However, in contrast, Vazquez et al. [20] found no apparent differences in the gross nitrification rates in the soil in which different *Brachiaria* genotypes with differing BNI capacities were grown.

N$_2$O is produced by a number of simultaneous N transformation processes [21]. Denitrification produces N$_2$O as an intermediate product during the reduction in nitrate (NO$_3^-$) to N$_2$ and is considered a much more potent source of N$_2$O than nitrification in grassland soil [22]. However, it remains unclear whether cultivation of exotic *Brachiaria* in tropical pastures results in a reduction in soil denitrification potential and N$_2$O emissions due to the decrease in supply of NO$_3^-$ substrates for denitrifiers. In this study, we therefore established an incubation experiment using a $^{15}$N tracing technique with soil samples collected from an experimental field cultivated with *Brachiaria* and the native grass *Eremochloa ophiuroide*, which has no BNI capacity. The objectives were to: (1) determine the effect of *Brachiaria* on soil N transformation rates in terms of gross nitrification and denitrification rates; and (2) understand how cultivation of *Brachiaria* affects soil N$_2$O emissions. We hypothesized that *Brachiaria* cultivation would reduce nitrification by releasing biological nitrification inhibitors, thereby reducing the availability of NO$_3^-$ for denitrification and together with nitrification, decreasing soil N$_2$O emissions.

2. Materials and Methods
2.1. Field Experiment and Soil Sampling

The field experiment was established in Danzhou, Hainan Province, China (109°29′ E, 19°30′ N), in August 2015. The area has a tropical monsoon climate, with an annual mean air temperature of 23.1 °C and annual precipitation of 1823 mm. The soil was developed from granite and classified as Latosol according to the US soil taxonomy. The field experiment involved eight treatments consisting of two forage grasses and four N application rates, with three replicates each. The two forage species were the introduced exotic grass *Brachiaria humidicola* CIAT679, which has a high-BNI capacity [14], and the native tropical grass *Eremochloa ophiuroide*, which has no BNI capacity. The four N application rates were 0, 150, 300 and 450 kg N ha$^{-1}$ year$^{-1}$. Plot size was 4 × 3 m and all plots were arranged according to a randomized block design. N fertilizer urea was surface applied prior to irrigation. In the first growing season, 60% urea was applied in August 2015 as a basal fertilizer, with the remaining 40% applied in April 2016 as a top-dressing. In the second growing season, 40% urea was applied in August 2016 as a basal fertilizer, while 30% was applied in March and 30% in June 2017 as a top-dressing. The grasses were harvested using a lawnmower one day before each top-dressing. Phosphorus and potassium application rates were 120 kg P$_2$O$_5$ ha$^{-1}$ year$^{-1}$ (calcium superphosphate) and 120 kg K$_2$O ha$^{-1}$ year$^{-1}$ (potassium sulfate), respectively, with both applied annually as a basal fertilizer in August.

In March 2017, approximately 2 years after establishment of the field experiment and 6 months after the last fertilization, surface soil (0–20 cm) was collected from 10 different positions in each *Brachiaria* and *Eremochloa* plot treated with 150 kg N ha$^{-1}$ year$^{-1}$. The samples were then pooled to form a composite sample for each treatment. After removal of visible roots and litter, the fresh soil was sieved through a 2 mm mesh then divided into two subsamples, one of which was stored at 4 °C for incubation and the other which was air-dried for further analysis. Soil pH was measured in a 1:2.5 soil:water sample ratio using a DMP-2 mV/pH detector (Quark Ltd., Nanjing, China). Soil organic C (SOC) and total
N (TN) were determined by wet-digestion with H$_2$SO$_4$-K$_2$Cr$_2$O$_7$ and on a CN analyzer (Vario Max CN, Elementar, Hanau, Germany), respectively, while NH$_4^+$ and NO$_3^-$ were extracted using 2 M potassium chloride (KCl) at a 1:5 soil:solution ratio then analyzed using a continuous-flow autoanalyzer (Skalar, Breda, The Netherlands). Dissolved organic carbon (DOC) was extracted using deionized water at a soil:water ratio of 1:5 with shaking for 0.5 h then analyzed using a TOC analyzer (Vario TOC cube, Elementar, Hanau, Germany). Soil available K$^+$ was extracted with ammonium acetate and analyzed using a flame photometer (FP640, INASA, China). Soil properties are presented in Table 1.

### Table 1. Properties of the test soils after approximately 2 years of cultivation with *Brachiaria* and *Eremochloa*.

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>TN (g N kg$^{-1}$)</th>
<th>SOC (g C kg$^{-1}$)</th>
<th>NH$_4^+$-N (mg N kg$^{-1}$)</th>
<th>NO$_3^-$-N (mg N kg$^{-1}$)</th>
<th>DOC (mg C kg$^{-1}$)</th>
<th>Available K$^+$ (mg K kg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Brachiaria</em> soil</td>
<td>6.40 ± 0.30 a</td>
<td>0.55 ± 0.02 a</td>
<td>7.32 ± 0.10 a</td>
<td>4.24 ± 0.11 a</td>
<td>6.57 ± 0.10 a</td>
<td>29.79 ± 0.90 a</td>
<td>153.13 ± 10.67 a</td>
</tr>
<tr>
<td><em>Eremochloa</em> soil</td>
<td>6.10 ± 0.1 a</td>
<td>0.54 ± 0.01 a</td>
<td>7.01 ± 0.09 b</td>
<td>4.38 ± 0.13 a</td>
<td>3.70 ± 0.11 b</td>
<td>22.64 ± 1.01 b</td>
<td>44.28 ± 1.18 b</td>
</tr>
</tbody>
</table>

Values represent means ± standard errors (n = 3). Values within the same column with different lowercase letters represent a significant difference at $p < 0.05$. TN: total N; SOC: soil organic C; DOC: dissolved organic C.

### 2.2. $^{15}$N Tracing Experiment

The soil incubation experiments consisted of two NH$_4$NO$_3$ treatments with three replications each, with labelling of either ammonium ($^{15}$NH$_4$NO$_3$, 10.23 atom % excess) or nitrate (NH$_4$$^{15}$NO$_3$, 10.28 atom % excess) with $^{15}$N. Six sets of 250 mL incubation bottles (six bottles per set) were prepared with 30 g fresh soil (on oven-dried basis). After 24 h pre-incubation, 2 mL of $^{15}$NH$_4$NO$_3$ solution or NH$_4$$^{15}$NO$_3$ solution was then added at a rate of 50 mg NH$_4^+$-N kg$^{-1}$ soil and 50 mg NO$_3^-$-N kg$^{-1}$ soil, respectively. The bottles were sealed with cling film punctured with seven pin holes to allow gas exchange then incubated for 16 d at a water holding capacity (WHC) of 60% and a temperature of 25 °C in the dark. Water lost during incubation was compensated for by adding deionized water using a mini pipette to maintain a constant weight. Prior to incubation, a pre-experiment was conducted to confirm the optimal incubation time and gas sampling time interval for identifying the N$_2$O flux peaks and meeting the requirement of data-input for the $^{15}$N tracing model.

Gas sampling and destructive soil sampling were carried out 2, 98, 194, 290, and 386 h after NH$_4$NO$_3$ application, respectively. At each sampling point, gas samples were collected using a 50 mL syringe from a specific set of bottles at 0 and 6 h after sealing with an air-tight lid. The samples were then immediately injected into two pre-evacuated gas vials with a butyl-rubber stopper for analysis of N$_2$O concentrations and the isotopic composition of $^{15}$N$_2$O. In advance of the first gas collection, the bottles were injected with 50 mL of fresh gas to maintain air pressure then after the second collection, the lids were replaced with the punctured cling film. At the same time as gas sampling, NH$_4^+$ and NO$_3^-$ were extracted from another set of soil samples using 100 mL 2 M KCl. After extraction, the soil was rinsed repeatedly with deionized water to remove any residual inorganic N then oven-dried at 50 °C for soil organic N testing. The soil and solution samples were both stored at −20 °C until use.

N$_2$O concentrations in the sampled gas samples were measured using a gas chromatograph (Agilent 7890, Agilent Technologies, Santa Clara, CA, USA) equipped with a $^{63}$Ni electron capture detector. For isotopic analysis, extracted NH$_4^+$ was separated by distillation with MgO, thereafter NO$_3^-$ was converted to NH$_4^+$ with Devarda’s alloy in another distillation [23]. Released ammonia was absorbed in boric acid solution, and NH$_4^+$ concentration was measured using 0.02 M sulfuric acid. After acidification, the solution was dried in an oven at 50 °C and $^{15}$N enrichment of NH$_4^+$ was determined using an isotope ratio mass spectrometry (IRMS 20-22, SerCon, Crewe, UK). While $^{15}$N enrichment of N$_2$O and organic N were measured using a MAT 253 mass spectrometer (Thermo Finnigan, Bremen, Germany).
2.3. \(^{15}\)N Tracing Model

A full process-based \(^{15}\)N tracing model (Figure 1) was used to simultaneously quantify the gross N transformation rates in each soil sample [24]. Average \(\text{NH}_4^+\) and \(\text{NO}_3^-\) concentrations and \(^{15}\)N excess values (average \(\pm\) standard deviations) from the two \(^{15}\)N-labeled treatments were included in the model. The model calculated the gross N transformation rates by simultaneously optimizing the kinetic parameters for the various N transformation processes to minimize misfit between the modeled and observed \(\text{NH}_4^+\) and \(\text{NO}_3^-\) concentrations and respective \(^{15}\)N enrichments. A Markov chain Monte Carlo metropolis algorithm (MCMC-MA) was used for parameter optimization, since it is known to be efficient to simultaneously estimate a large number of parameters [25,26]. This algorithm performed a random walk in model parameter space in order to find the global minimum and was shown to be robust against local minima [24]. The optimization procedure produced a probability density function for each model parameter, from which the mean and standard deviation of three parallel sequences were then calculated [25]. To obtain the best parameter set for \(^{15}\)N tracing analysis that was able to simulate the observed data, various combinations of kinetic settings of individual processes were evaluated (Table 2 shows the final version of the parameter set). The most appropriate model to describe the measured N dynamics was then selected according to the Akaike information criterion for each model version [25]. The \(^{15}\)N tracing model was performed using MATLAB (Version 7.2, The MathWorks Inc., Natick, MA, USA), which used models individually constructed in Simulink (Version 6.4, The MathWorks Inc., Natick, MA, USA).

Figure 1. The \(^{15}\)N tracing model used to determine gross N transformation rates (a) [24] and \(\text{N}_2\text{O}\) production pathways from specific N pools (b). \(\text{N}_{\text{org}}\): soil organic N (including soil labile organic N and recalcitrant organic N), \(\text{NH}_4^+\): ammonium, \(\text{NO}_3^-\): nitrate, \(\text{NH}_4^+\text{ads}\): ammonium adsorbed to soil, \(\text{NO}_3^-\text{sto}\): stored nitrate, SOM: soil organic matter. Abbreviations for the transformations are as in Table 2.
### Table 2. Descriptions and average gross N transformation rates (mean ± standard deviation, µg N g⁻¹ soil d⁻¹) in the Brachiaria and Eremochloa soils.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Kinetics</th>
<th>Gross N Transformation Rates</th>
</tr>
</thead>
<tbody>
<tr>
<td>M_{Nrec}</td>
<td>Mineralization of N_{rec} to NH₄⁺</td>
<td>0</td>
<td>Brachiaria Soil: 2.02 ± 0.05 a, Eremochloa Soil: 1.57 ± 0.03 b</td>
</tr>
<tr>
<td>I_{NH₄⁺}N_{rec}</td>
<td>Immobilization of NH₄⁺ to N_{rec}</td>
<td>1</td>
<td>Brachiaria Soil: 2.94 ± 0.06 b, Eremochloa Soil: 3.52 ± 0.07 a</td>
</tr>
<tr>
<td>M_{Nlab}</td>
<td>Mineralization of N_{lab} to NH₄⁺</td>
<td>0</td>
<td>Brachiaria Soil: 0 ± 0, Eremochloa Soil: 0 ± 0</td>
</tr>
<tr>
<td>I_{NH₄⁺}N_{lab}</td>
<td>Immobilization of NH₄⁺ to N_{lab}</td>
<td>0</td>
<td>Brachiaria Soil: 0.02 ± 0.001 b, Eremochloa Soil: 0.006 ± 0.007 a</td>
</tr>
<tr>
<td>O_{Nrec}</td>
<td>Oxidation of N_{rec} to NO₃⁻</td>
<td>1</td>
<td>Brachiaria Soil: 0.20 ± 0.03 b, Eremochloa Soil: 0.88 ± 0.01 a</td>
</tr>
<tr>
<td>I_{NO₃}</td>
<td>Immobilization of NO₃⁻ to N_{rec}</td>
<td>1</td>
<td>Brachiaria Soil: 1.44 ± 0.02 b, Eremochloa Soil: 1.98 ± 0.04 a</td>
</tr>
<tr>
<td>O_{NH₄⁺}</td>
<td>Oxidation of NH₄⁺ to NO₃⁻</td>
<td>1</td>
<td>Brachiaria Soil: 0.0005 ± 0.0002 b, Eremochloa Soil: 0.0011 ± 0.0007 a</td>
</tr>
<tr>
<td>D_{NO₃}</td>
<td>Dissimilatory NO₃⁻ reduction to NH₄⁺</td>
<td>1</td>
<td>Brachiaria Soil: 0.07 ± 0.06 b, Eremochloa Soil: 35.43 ± 4.65 a</td>
</tr>
<tr>
<td>A_{NH₄⁺}</td>
<td>Adsorption of NH₄⁺</td>
<td>1</td>
<td>Brachiaria Soil: 0.71 ± 0.10 b, Eremochloa Soil: 35.76 ± 3.36 a</td>
</tr>
<tr>
<td>R_{NH₄⁺}</td>
<td>Release of adsorbed NH₄⁺</td>
<td>1</td>
<td>Brachiaria Soil: 0 ± 0, Eremochloa Soil: 0 ± 0</td>
</tr>
<tr>
<td>A_{NO₃}</td>
<td>Adsorption of NO₃⁻</td>
<td>1</td>
<td>Brachiaria Soil: 0 ± 0, Eremochloa Soil: 0 ± 0</td>
</tr>
<tr>
<td>R_{NO₃}</td>
<td>Release of adsorbed NO₃⁻</td>
<td>1</td>
<td>Brachiaria Soil: 0 ± 0, Eremochloa Soil: 0 ± 0</td>
</tr>
</tbody>
</table>

Values followed by different lowercase letters within the same row indicate a significant difference between treatments (no overlap of 85% confidence intervals). N_{lab}: soil labile organic N, N_{rec}: soil recalcitrant organic N. Kinetic types: 0 = zero order, 1 = first order.

The initial pool sizes for soil NH₄⁺-N and NO₃⁻-N were estimated by extrapolating the first two sets of data back to the time point zero [27]. Based on the kinetic settings and the final parameters set, average gross transformation rates were then calculated over the whole incubation period and presented in units of µg N g⁻¹ soil d⁻¹ (Table 2).

#### 2.4. Calculations

The N₂O flux (F, µg N₂O-N kg⁻¹ h⁻¹) was calculated as follows:

\[
F = \frac{\rho \times \Delta C \times V \times 273}{W \times \Delta t \times T}
\]  

(1)

where \(\rho\) is the density of gas under standard conditions (1.25 kg N₂O-N m⁻³); \(\Delta C\) is the variation in gas concentrations during the 6 h gas sampling period (ppbv); \(V\) is the volume of the flask (m³); \(T\) is the incubation temperature; \(\Delta t\) is the incubation time (h); and \(W\) is the dry weight of the soil (kg).

Cumulative N₂O emissions (E, µg N₂O-N kg⁻¹) were calculated as follows:

\[
E = \sum \left( \frac{F_i + F_{i+1}}{2} \right) \times (t_{i+1} - t_i) \times 24
\]  

(2)

where \(F\) is the N₂O flux (µg N₂O-N kg⁻¹ h⁻¹); \(i\) is the \(i\)th measurement; and \(t_{i+1} - t_i\) represents the time interval between the two adjacent measurements.

N₂O is thought to be derived from three N transformation process: autotrophic nitrification, heterotrophic nitrification, and denitrification. The relative contributions of each process to the N₂O emissions were therefore calculated as follows [28]:

\[
a_{N₂O} = f_{AN} \times a_d + f_{HN} \times a_h + f_{DN} \times a_d
\]  

(3)

\[
f_{AN} + f_{HN} + f_{DN} = 1
\]  

(4)

where \(AN, HN\) and \(DN\) represent autotrophic nitrification, heterotrophic nitrification and denitrification, respectively; \(a_{N₂O}, a_d, a_h\) and \(a_d\) represent the ¹⁵N atom % excess of N₂O-N, NH₄⁺-N, organic N and NO₃⁻-N from the paired ¹⁵NH₄NO₃ and NH₄¹⁵NO₃ treatments, respectively; and \(f_{AN}, f_{HN}\) and \(f_{DN}\) represent the respective fractions of N₂O derived from \(AN, HN\), and \(DN\).
The average rate of N₂O production from heterotrophic nitrification (N₂Oₗ), autotrophic nitrification (N₂Oₐ), and denitrification (N₂O₅) were then calculated as follows:

\[ N₂Oₐ = f_{AN} \times N₂OT \]  
\[ N₂Oₕ = f_{HN} \times N₂OT \]  
\[ N₂O₅ = f_{DN} \times N₂OT \]  

where \( N₂OT \) is the total N₂O production rate during the entire incubation time.

2.5. Statistical Analyses

Statistical analysis was not applied to the parameter results since the \(^{15}\)N tracing model contained plenty of iterations [24]. Accordingly, differences between treatments were considered significant at an alpha level of 0.05 if the 85% confidence intervals did not overlap. Differences in soil properties and N₂O emissions between treatments were determined using an independent t-test. All statistical analyses were carried out using SPSS Statistics (version 26.0, IBM corp., Armonk, NY, USA) for Windows.

3. Results

3.1. Soil N Pool Sizes and \(^{15}\)N Enrichment

\( \text{NH}_4^+ \) concentrations decreased while \( \text{NO}_3^- \) concentrations increased during incubation of both the Brachiaria and Eremochloa soils (Figure 2). \( \text{NH}_4^+ \) concentrations decreased more rapidly in the Eremochloa soil, with a decrease of 95.0% during the first 8 d of incubation, with a reduction of only 49.8% in the Brachiaria soil at the end of the 16-day incubation period (Figure 2a). \( \text{NO}_3^- \) concentrations in the Eremochloa soil reached a maximum on day 8 after the application of NH₄NO₃, while a continuous increase was observed up until the end of the incubation in the Brachiaria soil (Figure 2b).

\[ h = \frac{T}{60} \]  
\[ f = \frac{1}{3} \]  
\[ n = 3 \]  

\[^{15}\text{N} \] enrichment of the \( \text{NH}_4^+ \) pool decreased, while that of the \( \text{NO}_3^- \) pool increased following the addition of \( {^{15}}\text{NH}_4^+ \), suggesting that mineralization of soil organic N and \( \text{NH}_4^+ \) oxidation occurred simultaneously (Figure 2c,d). Meanwhile, \(^{15}\)N enrichment of \( \text{NO}_3^- \) decreased after the application of \( \text{NH}_4^{15}\text{NO}_3 \), suggesting that natural or a low
abundance of NO$_3^-$ entered this pool. In contrast, $^{15}$N enrichment of NH$_4^+$ increased slightly after the application of NH$_4^{15}$NO$_3$, suggesting that the direct conversion from $^{15}$NO$_3^-$ to $^{15}$NH$_4^+$ was negligible.

3.2. Gross N Transformation Rates

The $^{15}$N tracing model described the measured data in the test soil with a correlation coefficient ($R^2$) of 0.99. The estimated gross rates of the 12 N transformation processes are shown in Table 2. The dynamic rates of labile organic N (labile organic N mineralization into NH$_4^+$ and immobilization of NH$_4^+$ into labile organic N) and adsorbed NO$_3^-$ (adsorption of NO$_3^-$ and release of adsorbed NO$_3^-$) were negligible in both test soils. Meanwhile, the gross mineralization rate of recalcitrant organic N in the Brachiaria soil was 2.02 µg N g$^{-1}$ soil d$^{-1}$, which was significantly higher than that in the Eremochloa soil. In contrast, the gross rate of mineral NH$_4^+$ immobilization in the Brachiaria soil decreased to 2.94 µg N g$^{-1}$ soil d$^{-1}$ from 3.57 µg N g$^{-1}$ soil d$^{-1}$ in the Eremochloa soil (Figure 3), and as a result, Brachiaria planting increased the mineralization–immobilization turnover of NH$_4^+$ (M/INH$_4^+$) from 44.6% in the Eremochloa soil to 68.7%. Both the adsorption rate of NH$_4^+$ and the release rate of adsorbed NH$_4^+$ were significantly lower in the Brachiaria compared to Eremochloa soil. Meanwhile, the net exchange (release minus adsorption) of mineral NH$_4^+$ between these two pools was 0.64 µg N g$^{-1}$ soil d$^{-1}$ in the Brachiaria soil, almost double that in the Eremochloa soil (0.33 µg N g$^{-1}$ soil d$^{-1}$), suggesting an increase in NH$_4^+$ supply.

![Diagram](https://via.placeholder.com/150)

**Figure 3.** Nitrogen cycles in the *Brachiaria* and *Eremochloa* soil. Values shown represent gross N transformation rates (mean ± SD, µg N g$^{-1}$ d$^{-1}$). The thickness of the arrows indicates the strength of each process. Different lowercase indicates denote a significant difference between treatments at $p < 0.05$. NH$_4^{+\text{ads}}$: ammonium adsorbed to soil.
Autotrophic nitrification was a dominant NO$_3^-$ production process in both sets of soils, while heterotrophic nitrification was negligible. Brachiaria planting reduced the autotrophic nitrification rate to 1.44 μg N g$^{-1}$ soil d$^{-1}$ from 1.98 μg N g$^{-1}$ soil d$^{-1}$ in the Eremochloa soil. The Brachiaria soil also showed a lower nitrification capacity (ONH$_4^+$/M) than the Eremochloa soil (71.3 vs. 126.0%, respectively). Immobilization of NO$_3^-$ overwhelmingly surpassed the rate of dissimilatory nitrate reduction to ammonium (DNRA) in both sets of soil, representing the primary NO$_3^-$ consumption process under our experimental conditions. The NO$_3^-$ immobilization rate in the Eremochloa soil was 0.88 μg N g$^{-1}$ soil d$^{-1}$, nearly four times greater than that in the Brachiaria soil. Meanwhile, the NO$_3^-$ retention capacity and availability in the Eremochloa soil, expressed as the ratio of NO$_3^-$ consumption to production, was significantly higher than in the Brachiaria soil (44.4 vs. 13.9%, respectively).

3.3. N$_2$O Production Pathways and Emissions

The N$_2$O flux peak occurred on day 4 of the incubation in the Eremochloa soil and on day 12 in the Brachiaria soil (Figure 4), although there was no apparent difference in the N$_2$O production rates from heterotrophic nitrification between the Eremochloa and Brachiaria soil (Table 3). The average N$_2$O production rate of autotrophic nitrification was 3.19 μg N$_2$O-N kg$^{-1}$ d$^{-1}$ in the Eremochloa soil, while it was significantly lower at 2.78 μg N$_2$O-N kg$^{-1}$ d$^{-1}$ in the Brachiaria soil. In contrast, the average N$_2$O production rate during denitrification increased sharply from 0.55 μg N$_2$O-N kg$^{-1}$ d$^{-1}$ in the Eremochloa soil to 4.25 μg N$_2$O-N kg$^{-1}$ d$^{-1}$ in the Brachiaria soil, representing a 7.7-fold increase.

![Figure 4.](image)

(a) Fluxes (a) and cumulative emissions (b) of N$_2$O in the Brachiaria and Eremochloa soil during the 16-day incubation. Different lowercase indicates denote a significant difference between treatments at $p < 0.05$. Vertical bars denote the standard deviation of the mean ($n = 3$).

<table>
<thead>
<tr>
<th>N$_2$O Production Rate (μg N$_2$O-N kg$^{-1}$ d$^{-1}$)</th>
<th>Relative Contribution to N$_2$O (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>Autotrophic Nitrification</td>
</tr>
<tr>
<td>Brachiaria soil</td>
<td>9.02 ± 0.05 a</td>
</tr>
<tr>
<td>Eremochloa soil</td>
<td>5.65 ± 0.22 b</td>
</tr>
</tbody>
</table>

Values represent means ± standard deviation ($n = 3$). Different lower case letters within the same column denote a significant difference between treatments at $p < 0.05$. $f_{AN}$, $f_{HN}$ and $f_{DN}$ denote the contribution of autotrophic nitrification, heterotrophic nitrification and denitrification to N$_2$O production, respectively.

Cumulative N$_2$O emissions during incubation were significantly higher in the Brachiaria soil (144.31 μg N$_2$O-N kg$^{-1}$) than that estimated as 90.42 μg N$_2$O-N kg$^{-1}$ in the Eremochloa soil. Meanwhile, denitrification contributed to 47.1% of the emitted N$_2$O exceeding the contributions...
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4. Discussion
4.1. Brachiaria Humidicola Cultivation Enhanced the Soil NH$_4^+$ Supply

This study revealed that the gross mineralization rate of soil recalcitrant organic N was significantly enhanced by 28.7% under cultivation of *Brachiaria* compared with *Eremochloa*, accelerating the renewal of soil organic N due to its slight increase. This is consistent with the findings of Teutscherová et al. [29,30] who revealed a positive priming effect of high-BNI *Brachiaria* on native soil organic N decomposition in Colombian pastures compared with a low-BNI genotype. It is suggested that grasses with a dense root system stimulate organic N mineralization by enhancing microbial biomass and activity through the release of large amounts of dead roots and exudates into the soil [31,32]. It is thought that the increase in organic C accelerates the formation of aggregates and reduces the effective diffusion coefficient of oxygen in the soil, in turn inducing a shift in dominant microbes from aerobes (Gram-negative bacteria) to facultative and/or anaerobic microbes (Gram-positive bacteria and fungi) [33–36]. In general, Gram-positive bacteria and fungi preferentially utilize soil recalcitrant organic matter [37,38]. It is therefore likely that the increase in soil organic C observed here was mainly due to the increase in organic C input from the high biomass of dead roots and exudates under *Brachiaria* cultivation, resulting in more efficient growth of Gram-positive bacteria and fungi and an increase in the turnover of recalcitrant organic C compared with *Eremochloa* cultivation.

In contrast, the immobilization rate of NH$_4^+$ in the *Brachiaria* soil decreased compared with the *Eremochloa* soil. This differs from the results of Vazquez et al. [20] who showed that gross NH$_4^+$ immobilization was enhanced in the soil cultivated with high-BNI *Brachiaria* genotypes, while the NO$_3^-$ concentration and N losses remained low. It has been reported that a high C/N ratio in high-BNI plant soil reduces N availability for microbial N immobilization when no N fertilizers are added or when only limited (animal) urine deposition occurs [14,29,39,40]. In contrast, cultivation of high-BNI *Brachiaria* genotypes in the same field experiment results in a significant reduction in microbial NH$_4^+$ immobilization rates at 7 and 21 days after application of N fertilizers at a rate 50 kg N ha$^{-1}$ [41]. These results suggest that microbial NH$_4^+$ immobilization is dependent on soil NH$_4^+$ availability.

Compared with *Eremochloa*, *Brachiaria* more efficiently reduced the gross rate of soil NH$_4^+$ adsorption than the release rate of adsorbed NH$_4^+$, causing an increase in the net release rate of adsorbed NH$_4^+$. This may have been due to two possible reasons. Firstly, roots of *Brachiaria* can distribute within the 20–40 cm soil layer, allowing effective absorption of non-exchangeable K$^+$ from deeper soil layers [42], dramatically increasing available K$^+$ in the surface soil. The higher availability of K$^+$ also outcompetes NH$_4^+$ for soil adsorption sites, thereby reducing soil adsorption of NH$_4^+$ since both have a similar ionic radius and physical properties [43,44]. To date, however, the influence of *Brachiaria* planting on the soil available K$^+$ is less studied. Further study is required to evaluate how *Brachiaria* cultivation affects the soil available K at the different K application rates. Secondly, the adsorption capacity of NH$_4^+$ in soil is also affected by the content of clay and organic matter [45,46]. Organic matter with plenty of polar atom groups, such as carboxyl and phenolic hydroxyl, contribute to the negative charge and is the main source of variable soil charge. It is therefore likely that NH$_4^+$ as a cation is not as efficiently adsorbed, while NH$_4^+$ from decomposed organic N is released during the mineralization of native recalcitrant organic C under cultivation of *Brachiaria*.

Overall, cultivation of *Brachiaria* therefore reduced the rates of NH$_4^+$ immobilization and adsorption and enhanced the rates of organic N mineralization and adsorbed NH$_4^+$ release, in turn increasing soil NH$_4^+$ availability and supply compared with *Eremochloa* cultivation. These results suggest that in the *Brachiaria* soil, reduced application rate of K fertilizer may increase the adsorption of NH$_4^+$ from the test soil.
4.2. Effect of Brachiaria humidicola Cultivation on Soil N₂O Emissions

As expected, cultivation of Brachiaria significantly reduced autotrophic nitrification and related N₂O production, compared with Eremochloa. This is consistent with the findings of Subbarao et al. [14] who reported that Brachiaria soil reduced the ammonia oxidation rate by 90% during a 3-year field experiment compared with soybean and plant-free soil. Subbarao et al. [47] revealed that the release of brachialactone exudate by Brachiaria blocked the activities of both ammonia monooxygenase and hydroxylaminooxidoreductase, thereby reducing ammonia oxidation in pure culture with the ammonia oxidizer Nitrosomonas europaea. In line with this, a reduction in the abundance of ammonia oxidizers was observed in a previous field study of Brachiaria cultivation [18,40]. It was also revealed that BNI compounds were able to persist for a long time and tended to accumulate over time, remaining effective even after Brachiaria pasture was subsequently replaced with maize [48–50]. However, Vazquez et al. [20] and Teutscherová et al. [41] found no comparable differences in gross nitrification rates between Brachiaria genotypes with differing BNI capacities in soil with a high organic C content in the tropical savanna in Colombia. Moreover, they attributed the reduced inhibition of potential net nitrification rates to strong microbial immobilization of NH₄⁺, and a subsequent reduction in soil NH₄⁺ availability for ammonia oxidizers.

In the present study, the reduction in N₂O production in the Brachiaria soil via autotrophic nitrification was at the lower end of the range of 16.8–90.0% reported by previous studies [51]. This suggests that less NH₄⁺ was converted into N₂O during autotrophic nitrification in test acidic soil. In general, competition for available NH₄⁺ exists between autotrophic nitrification and microbial immobilization or adsorption of NH₄⁺ [25,52]. As discussed above, reduced rates of NH₄⁺ immobilization and NH₄⁺ adsorption together with higher mineralization rates of organic N provided more NH₄⁺ substrates for ammonia oxidizers in the Brachiaria compared to Eremochloa soil. This suggests that the suppression effect of high-BNI Brachiaria on N₂O emissions may also depend on soil NH₄⁺ availability, with greater suppression in soil with relatively low available NH₄⁺ [20,29,53,54].

In contrast to autotrophic nitrification, Brachiaria did not alter the N₂O production rate via heterotrophic nitrification. In general, heterotrophic nitrification is carried out by a large variety of bacteria and fungi [55], with heterotrophic nitrifiers using both organic and inorganic N as a substrate, and possibly producing more N₂O than autotrophic nitrifiers [56,57]. In the present study, we supposed that heterotrophic nitrifiers would use organic N as a unique substrate since the model only can select one substrate pool for running. Thus, it is very likely that the gross rate of heterotrophic nitrification in the Brachiaria and Eremochloa soil was underestimated. In general, fungal nitrification in acidic soil is not inhibited by popular synthetic nitrification inhibitors such as acetylene (C₂H₂), dicyandiamide (DCD) and nitrapyrin [58]. Therefore, our results suggest that the 2-year cultivation with Brachiaria had no effect on N₂O production via heterotrophic nitrification.

As unexpected, cumulative N₂O emissions were significantly higher in the Brachiaria soil compared to the Eremochloa soil as measured in the field (4.30 and 1.54 kg N₂O-N ha⁻¹ under Brachiaria and Eremochloa cultivation, respectively), although N₂O emissions via autotrophic nitrification decreased. This is in contrast with previous results in which Brachiaria establishment was found to reduce soil N₂O emissions by 20–90% compared with plants without BNI capacity [14,47]. In the present study, the N₂O production rate in the Brachiaria soil during denitrification increased 6.7-fold, while the contribution ratio of denitrification to emitted N₂O dramatically increased compared with Eremochloa. These results clearly suggest that the enhanced N₂O emissions in the Brachiaria soil were primarily due to an increased denitrification potential. There were two suggested possibilities. Firstly, compared with Eremochloa, Brachiaria cultivation sharply reduced the NO₃⁻ immobilization rate, which outnumbered the rate of DNRA as the primary consumption process of NO₃⁻. This suggests that cultivation of Brachiaria increased soil NO₃⁻ availability by reducing the ratio of total NO₃⁻ consumption through microbial immobilization of NO₃⁻ and dissimilatory NO₃⁻ reduction to NH₄⁺ (I_NO₃ + D_NO₃) to total NO₃⁻ production (O_NH₄ + O_Nrecc), thereby providing more
NO$_3^-$ for denitrification. It was previously suggested that higher NO$_3^-$ concentrations in the soil tend to result in a higher N$_2$O/N$_2$ ratio during denitrification, thereby favoring N$_2$O emissions [59]. Secondly, in this study, Brachiaria cultivation significantly enhanced soil organic C, notably dissolved organic C, due to the increase in plant biomass and especially biomass of roots and exudates. Increases in soil organic C were also found to promote the formation of anaerobic microsites for denitrification by stimulating aggregation and soil respiration [60,61]. Meanwhile, an increase in organic C was also found to reduce the minimum soil moisture threshold for the occurrence of denitrification [62,63]. For example, Wan et al. [64] found that the addition of starch to sandy loam soil treated with nitrate-based fertilizers stimulated N$_2$O production through denitrification. The results of this study therefore suggest that although cultivation of Brachiaria suppressed autotrophic nitrification, it significantly increased the soil denitrification potential and subsequent N$_2$O production by increasing soil organic C, notably labile organic C, through an increase in plant biomass, thereby stimulating soil N$_2$O emissions.

5. Conclusions

This study examined the effect of the exotic tropical grass Brachiaria, which has a high-BNI capacity, on soil N transformation processes and N$_2$O emissions. Cultivation of Brachiaria significantly decreased the gross rate of autotrophic nitrification and N$_2$O production during nitrification. In contrast, Brachiaria also increased the gross mineralization rate of soil recalcitrant organic N and reduced microbial NH$_4^+$ immobilization and NH$_4^+$ adsorption, thereby increasing the NH$_4^+$ supply for nitrification compared with native Eremochloa. Brachiaria planting caused a significant increase in soil N$_2$O emissions, primarily due to an increased denitrification potential as a result of reductions in NO$_3^-$ immobilization and an increase in soil labile organic C. Further studies are now required to determine the effects of K fertilizers on the adsorption and availability of NH$_4^+$. The effect of synthetic nitrification inhibitors together with the biological nitrification inhibitors released from Brachiaria on the mitigation of N$_2$O emissions in tropical pastures also requires further clarification.

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