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A short running title: Biological nitrification inhibition by *Brachiaria humidicola*

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Title of the paper:

The short-lived inhibitory effect of *Brachiaria humidicola* on nitrous oxide emissions following sheep urine application in a highly nitrifying soil

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Key words:

*Brachiaria humidicola*; *Brachiaria ruziziensis*; nitrogen gas; carbon dioxide; nitrifier; denitrifier.

**Abstract**

***Background***

*Brachiaria humidicola* (Bh) has the ability to produce biological nitrification inhibitors (NIs) and release NIs from the root to the soil.

***Aims***

To compare the effects of growing Bh with *Brachiaria ruziziensis* (Br, which is not able to produce NIs) on soil nitrogen (N) dynamics, N gases and carbon dioxide (CO2) emissions, and nitrifiers and denitrifiers following sheep urine application, a laboratory incubation was conducted in a He/O2 continuous flow Denitrification System (DENIS). This incubation was conducted in the absence of light. Hence the measured effects of Bh and Br on N cycling were the residual effect of biological NIs released into the soil prior to the incubation and released via root death.

***Methods***

The treatments were: 1) Bh with water application (Bh + W); 2) Bh with sheep urine (Bh + U); 3) Br with water application (Br + W); 4) Br with sheep urine (Br + U).

***Results***

Results showed that soil NO3- concentration increased significantly in the soil with sheep urine application after the incubation. Soil nitrous oxide (N2O) and nitric oxide (NO) emissions increased immediately after the sheep urine application and peaked twice during the incubation. Cumulative emissions for the first peak were significantly lower from the Bh + U treatment (0.054 kg N ha-1) compared with the Br + U treatment (0.111 kg N ha-1), but no significant differences were observed in the total cumulative N2O and NO emissions between the Bh + U and Br + U treatment at the end of the incubation. Sheep urine addition did not affect the AOA, *nirS* and *nosZ* gene copies, but significantly increased the AOB gene copies after the incubation.

***Conclusions***

We conclude that the residual effect of Bh to mitigate N2O emissions in a highly nitrifying soil is short-lived.

**1. Introduction**

Nitrification and denitrification are key processes of the soil nitrogen (N) cycle. Nitrification is a two-step microbially mediated process carried out by chemo-autotrophic nitrifying bacteria, first oxidising ammonium (NH4+) to nitrite (NO2-) which is further oxidised to nitrate (NO3-) (*Firestone and Davidson,* 1989). During the nitrification and subsequent denitrification, other gaseous forms of N are produced and lost from agricultural soils, such as nitrous oxide (N2O), nitric oxide (NO) and dinitrogen (N2). N2O has been attributed to nitrification, denitrification and nitrifier denitrification processes depending on the soil environmental conditions, such as water-filled pore space (WFPS), O2 availability, soil pH and temperature (*Bateman and Baggs,* 2005; *Wrage* et al. 2005; *Loick* et al., 2016; *Lai* et al., 2019). Some studies present NO emitted from soils during nitrification process (*Wang* et al., 2016; *Caranto and Lancaster,* 2017; *Kang* et al., 2020). However, denitrification can also be a major source of NO from soils at high water content and/or under the presence of a carbon (C) source (*Loick* et al., 2016; *Wu* et al., 2017; *Ji* et al., 2020), whilst N2 is the final product of denitrification (*Knowles,* 1982).

Synthetic nitrification inhibitors (NIs) have been widely researched and used to inhibit soil nitrification, e.g. dicyandiamide (DCD), 3,4-dimethylpyrazole phosphate (DMPP) (*Weiske* et al., 2001; *Chen* et al., 2014; *Chadwick* et al., 2018). Following concerns of synthetic NIs passing into human food chains (*Study* et al., 2014; *Lin* et al., 2015; *Welten* et al., 2016), there has been increasing interests in the role of biological NIs to reduce N2O emissions and NO3- leaching. Some grass species (*Subbarao* et al., 2008; *Gopalakrishnan* et al., 2009; de Cerqueira Luz et al., 2014) and crop plants (*Subbarao* et al., 2013; *Huérfano* et al., 2016; *Sun* et al., 2016) have the ability to release compounds from their roots to suppress the nitrifier activity which is termed biological nitrification inhibition (BNI) (*Subbarao* et al., 2006). *Brachiaria humidicola* (Bh), a typical tropical pasture grass used for grazing livestock, has been reported to release biological NIs from its roots. Active inhibitory compounds have been isolated from the root tissues (e.g. methyl-p-coumarate and methyl ferulate) (*Gopalakrishnan* et al., 2007), root exudates (e.g. brachialactone) (Subbarao et al., 2009), and shoot tissues (e.g. linoleic acid and linolenic acid) (*Subbarao* et al., 2008) of Bh.

Previous studies have focused on the effects of pure inhibitory compounds identified from the pasture grass or the root exudates of Bh on soil NH4+ transformation and N2O emissions (*Subbarao* et al., 2008; *Gopalakrishnan* et al., 2009; *Meena* et al., 2014). Whilst experiments have been conducted to explore nitrification inhibition and N2O emissions from soil planted with *Brachiaria* grasses, including pasture that receive bovine urine deposition (*Byrnes* et al., 2017; *Simon* et al., 2020). Only a few studies have explored the legacy effects of Bh on N cycling and grain yield of subsequent crops, supplied with N fertiliser, e.g. maize (Karwat et al. 2017), and little is known about the residual effect of biological NIs in the rhizosphere after plants like Bh start to die, on N emissions, soil mineral N, and soil nitrifiers and denitrifiers.

There is strong evidence that other *Brachiaria* species, e.g. *Brachiaria ruziziensis* (Br),are not capable to produce NIs (*Fernandes* et al., 2011). In this study, Br was selected to compare with Bh (which has the ability to release biological NIs from the roots) to: 1) explore the residual effect of Bhand Br on soil NH4+ and NO3- concentrations; 2) quantify the N2O, NO, N2 and CO2 emissions in soil sown with these two *Brachiaria* varieties; and 3) determine the residual effect of Bh and Br on soil nitrifiers and denitrifiers. Based on current research, we hypothesised that i) soil under Bh retains soil NH4+, and results in lower NO3- concentrations than soil under Br, ii) Bh results in lower N2O and NO emissions than soil under Br due to the higher BNI capacity of Bh, and iii) AOA and/or AOB gene copies may be lower in the soil under Bh treatments than those in the soil under Br treatments.

**2. Materials and methods**

*2.1. Soil sampling and physicochemical analysis*

A sandy clay loam textured Eutric Cambisol was collected from a typical sheep-grazed grassland in North Wales (53o24’N, 4o02’W). The soil had not been previously grown with Bh and Br. The soil was selected for its known high nitrification rate (*Jones* et al., 2004) and not necessarily as a typical tropical soil where *Brachiaria* species would be grown. Square intact turves of soil (30×30 cm, depth of 10 cm) were collected from 3 spatially discrete points (at least 10 m apart), which were retained as 3 replicates. Soil was sieved (2 mm) to remove roots and stones before analysis for a range of chemical properties: 19.4% moisture content (105 °C, 24 h), 6.7% organic matter (450 °C, 16 h) (*Ball,* 1964), 2.7% total C and 0.25% total N (CHN2000 Analyzer), pH of 5.9, 1.7 mg N kg-1 dry soil as NH4+-N (*Mulvaney,* 1996) and 30.4 mg N kg-1 dry soil as NO3--N (*Miranda* et al., 2001).

*2.2. Establishment of Brachiaria humidicola and Brachiaria ruziziensis*

To investigate the residual effect of Bh and Br on soil nitrification, greenhouse gas emissions (GHG, N2O and CO2), NO and N2 emissions, and nitrifiers and denitrifiers after sheep urine application, two varieties of *Brachiaria* species (Bh and Br) were sown separately in pots containing the field soil. Seeds of Bh and Br were germinated on wetted tissue paper in an incubator (20 °C). 1.7 kg field fresh soil were added to each pot (diameter: 15 cm; depth: 15 cm) at the same bulk density as the soil at the field site (1.6 g cm-3) (*Marsden* et al., 2016), and 10 geminated seeds were placed onto the soil surface before covering with 100 g soil. There were 12 pots in total, 6 pots were grown withBh and 6 pots with Br. To stimulate grass growth, the plants were cut to 2 cm above the soil level on day 33 and day 75. At the same time, the equivalent of 25 kg N ha-1 as (NH4)2SO4 was added to each pot 3 days after each cut to promote the release of the inhibitory compounds (*Subbarao* et al., 2007b).  50 mL of tap water was added to each pot twice per week to maintain plant growth prior to the incubation experiment. The establishment of Bh and Br was from the beginning of July to the end of November. To stimulate the growth of the tropical grasses, the lights above the plots in the greenhouse were on from October until the end of the cultivation. On day 150 after sowing, the plants and soils were harvested for the incubation experiment (described below).

*2.3. Experimental setup*

The 23 day incubation experiment was conducted in the Denitrification System (DENIS) at Rothamsted Research (North Wyke) (*Cárdenas* et al., 2003), using the top (0-7.5 cm) of the intact (12 cm deep) soils including plants (obtained from section 2.2). The soil cores were placed into 12 stainless vessels (diameter: 14.1 cm) and sealed with stainless steel lids fitted with double ‘O’ rings. The incubation experiment comprised 4 treatments with 3 replicates: 1)Bh with water application (Bh + W); 2)Bh with sheep urine (Bh + U); 3)Br with water application (Br + W); 4)Br with sheep urine (Br + U). The sheep urine used in this experiment had been collected from 6 Welsh Mountain ewes that had been grazing a permanent pasture at the same site the soil was collected from. The urine had been frozen immediately after collection to avoid N losses during storage. The sheep urine was defrosted the day before application to the soil cores, and the individual urine samples (n=6) were pooled and mixed to generate one urine source (total C, 25.3 g L-1; total N, 11.7 g L-1, NH4+-N, 1.09 mg L-1; NO3--N, 3.09 mg L-1) of which 670 mg N kg-1 dry soil (equivalent to 374 kg N ha-1)were added in the treatments.

The incubation experiment followed a similar approach to previous experiments using this DENIS (*Loick* et al., 2016; *Wu* et al., 2017). Briefly, to remove the native N2 from the soil cores and the headspace, the soil cores were flushed from the base at a flow rate of 30 mL min-1 for 48 hours using a mixture of He: O2 (80: 20), with the outlet flow from each chamber directed to a number of gas detectors. Once the N2, N2O and NO concentrations had reached very low levels, the airflow was decreased to 12 mL min-1 to measure the baseline emissions before being switched from the flow through the base to a flow over the soil surface. The sheep urine and water amendments were contained in sealed stainless steel vessels above the lid of each incubation vessel. In previous protocols, these amendment vessels are usually flushed with He/O2 (80:20) to remove N2 (*Cárdenas* et al., 2003). However, in this experiment, the vessels containing the urine and water were not flushed with He/O2, to avoid the N losses (via NH3 volatilisation) from the sheep urine. After the urine and water had attained room temperature, the amendments were applied to the soil by opening the ball-valve connecting the 2 vessels. At the start of the soil incubation, the soil moisture content was increased to 65% WFPS to optimize conditions for nitrification (Mosier et al. 1996), taking the volume of the urine or water amendments into account. The temperature of the vessels was maintained at 15 °C during the flushing phase and the 23 day incubation period after the urine and water applications.

*2.4. Soil sampling and analysis*

During the incubation, the system was totally sealed, with all the soil gases displaced initially via mix of He:O2 (80:20) passed through the soil from below and the outlet flow from each chamber was directed to a number of gas detectors. Thus, fresh soil samples were only collected for analysis before the sheep urine application and at the end of the incubation period. Soil characteristics before sheep urine application and the after the incubation are presented in Table 1. Soil moisture content was measured after oven drying (105 °C, 24 h), and the soil organic matter was determined by loss on ignition of dried soil in a muffle furnace (450 °C, 16 h) (*Ball,* 1964). Total soil C and N concentrations were determined on milled oven dried soil samples using a CHN2000 Analyzer (Leco Corp., St. Joseph, MI). Soil pH and electrical conductivity (EC) were measured on fresh soil using standard electrodes (1:2.5 (w/v) soil-to-distilled water). Extractable NH4+-N and NO3--N were analysed in the filtrates after extracting 5 g of fresh soil with 25 ml K2SO4 (0.5 M) using the colorimetric methods of *Mulvaney* (1996) and *Miranda* et al., (2001), and total dissolved C and N were analysed with the Multi N/C 2100 (AnalytikJena, Jena, Germany). Data were expressed on a per kg dry soil basis.

Table 1 Soil characteristics before sheep urine application (d 0) and after the incubation (d 23).

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Soil property |  | Bh + W | |  |  | Bh + U | |  |  | Br + W | |  |  | Br + U | |  |
| d 0 | | d 23 | | d 0 | | d 23 | | d 0 | | d 23 | | d 0 | | d 23 | |
| Moisture content (%) | 30.3±0.23a | | 27.7±0.78B | | 30.6±0.11a | | 30.1±0.54A | | 29.4±0.60a | | 29.4±0.79AB | | 30.2±0.36a | | 28.0±0.34AB | |
| Organic matter (%) | 6.5±0.15a | | 6.4±0.06AB | | 6.4±0.21a | | 6.6±0.05A | | 6.3±0.05a | | 6.3±0.07B | | 6.3±0.13a | | 6.5±0.03A | |
| pH | 6.6±0.03a | | 6.0±0.02A | | 6.6±0.04a | | 5.3±0.05B | | 6.3±0.08b | | 6.0±0.05A | | 6.5±0.04ab | | 5.2±0.04B | |
| Electrical conductivity  (µS cm-1) | 116.8±16.7a | | 147.8±6.84B | | 109.3±1.84a | | 802.3±21.8A | | 111.0±4.63a | | 158.3±11.0B | | 104.5±6.02a | | 755.3±22.0A | |
| Total carbon  (g kg-1 dry soil) | 21.4±0.43a | | 23.3±0.50A | | 23.2±1.00a | | 24.9±1.79A | | 23.5±0.49a | | 24.1±0.06A | | 23.0±0.49a | | 25.1±0.81A | |
| Total nitrogen  (g kg-1 dry soil) | 2.6±0.04b | | 2.8±0.09B | | 2.7±0.05ab | | 3.1±0.04AB | | 2.8±0.10a | | 2.8±0.08B | | 2.7±0.02ab | | 3.2±0.14A | |
| NH4+-N  (mg N kg-1 dry soil) | 3.3±0.17a | | 1.3±0.36B | | 2.7±0.13a | | 3.2±0.43A | | 3.1±0.39a | | 0.15±0.05B | | 3.3±0.46a | | 3.6±0.97A | |
| NO3--N  (mg N kg-1 dry soil) | 3.7±0.20a | | 16.0±2.61B | | 1.8±0.41a | | 235.7±15.8A | | 2.8±0.65a | | 17.3±3.48B | | 2.6±0.99a | | 213.9±9.63A | |

Values represent means ± SEM. Different letters indicate the significant differences between treatments at d 0 (lower case) and d 23 (upper case) respectively (n=3, *P*<0.05)

At the same time, 5 g fresh soil from each vessel were collected and stored at -80 °C prior to DNA extraction. Soil (0.25 g) was extracted by the the DNeasy PowerSoil kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. After extraction, the purity and concentration of extracted soil DNA were determined by the Nanodrop spectrophotometer ND-1000 (Labtech, UK). Polymerase chain reaction (PCR) was carried out on real-time quantitative PCR (QPCR) using the QuantStudioTM 6 flex real-time PCR system (Thermo Fisher Scientific, UK). Three independent QPCR were performed for each gene and each soil replicate. The 20 µL reaction mixture comprised 10 µL TB Green Premix Ex Taq (TaKaRa, Tokyo, Japan), 0.3 µL of each primer, 0.4 µL ROX Reference dye, 7 µL of sterilized deionised water and 2 µL template DNA. The primers for quantifying nitrification and denitrification function genes were presented in Table 2. The thermal conditions for the AOA, AOB, *nirK*, *nirS* and *nosZ* were the same as those used in previous studies (Henry et al. 2006; Bei et al. 2021). The standard curves for QPCR were generated by 10-fold serial dilutions of linearized plasmids containing cloned AOA, AOB, *nirK*, *nirS*, and *nosZ* genes. The PCR amplification efficiencies of standard curves were 93 to 98% with R2 value of 0.990 to 0.999.

Table 2 Primer sets used for the real-time PCR.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Targeting gene** | | **Primer set** | **Sequence (5'-3')** | **Reference** |
| AOA | Arch-amoAF | | STAATGGTCTGGCTTAGACG | (Robinson et al. 2014) |
| Arch-amoAR | | GCGGCCATCCATCTGTATGT |
| AOB | amoA-1F | | GGGGTTTCTACTGGTGGT | (Robinson et al. 2014) |
| amoA-2R | | CCCCTCKGSAAAGCCTTCTTC |
| *nirK* | FlaCu | | ATCATGGTSCTGCCGCG | (Zulkarnaen et al. 2019) |
| R3Cu | | GCCTCGATCAGRTTGTGGTT |
| *nirS* | cd3aF | | GTSAACGTSAAGGARACSGG | (Zulkarnaen et al. 2019) |
| R3cd | | GASTTCGGRTGSGTCTTGA |
| *nosZ* | 2F | | CGCRACGGCAASAAGGTSMSSGT | (Zulkarnaen et al. 2019) |
| 2R | | CAKRTGCAKSGCRTGGCAGAA |

*2.5. Gas sampling and analysis*

The airflow from each vessel was automatically directed to a valve that directed the sample to different gas detectors, resulting in one sample being analysed every 8 minutes from each of the 12 vessels. Thus, one measurement was made every 1.5 hours from each vessel. The N2O and CO2 concentrations were determined using a gas chromatograph equipped with an electron capture detector (ECD), and a second GC with a helium ionization detector (HID, VICI AG International, Schenkon, Switzerland) was used to analyse N2 concentrations. For NO concentrations, a chemiluminescence analyser was used (Sievers NOA280i, GE Instruments, Colorado, USA). The gas flow rate through each vessel was measured daily to calculate the volume of gas required for the flux calculation. The gaseous fluxes were corrected for the surface area and flow rate through the vessels and are presented in the unit of kg N or C ha-1 d-1. Cumulative gaseous fluxes were calculated by the area under the curve after linear interpolation between sampling points using the Genstat 19th edition (VSN International Ltd) (*Meijide* et al., 2010).

*2.6. Statistical analysis*

One-way analysis of variance (ANOVA) followed by the LSD test at 5% confidence was used to determine the effect of Bh and Br on soil NH4+ and NO3- concentrations, cumulative gas emissions (N2O, NO, N2 and CO2) and gene abundance (AOA, AOB, *nirK*, *nirS*, *nosZ*) at the start (day 0) and end (day 23) of the incubation respectively. All statistical analyses were performed in SPSS Statistics 25.0 (IBM Inc., Armonk, NY).

**3. Results**

*3.1.* *Soil ammonium and nitrate concentrations*

At the start of the incubation, there were no significant differences between all the treatments (Bh + W, Bh + U, Br + W, Br + U) for the soil NH4+ and NO3- concentrations, with average concentrations of 3.1 (ranging from 2.7 to 3.3 mg kg-1 soil) and 2.7 (ranging from 1.8 to 3.7 mg kg-1 soil) mg kg-1 soil, respectively (Table 1). In the Bh + W and Br + W treatments, after the 23 day incubation the NH4+ concentration decreased (Bh + W, 3.3 to 1.3 mg kg-1 soil; Br + W, 3.1 to 0.15 mg kg-1 soil) and NO3- increased (Bh + W, 3.7 to 16.0 mg kg-1 soil; Br + W, 2.8 to 17.3 mg kg-1 soil). 23 days after the sheep urine application, there was a small increase in the NH4+ concentration in the urine treatments (Bh + U, from 2.7 to 3.2 mg kg-1 soil; Br + U, from 3.3 to 3.6 mg kg-1 soil) and a large increase in the NO3- concentration in the same treatments (Bh + U, from 1.8 to 235.7 mg kg-1 soil; Br + U, from 2.6 to 213.9 mg kg-1 soil).

*3.2. Gas emissions*

**Nitrous oxide**: N2O emissions increased immediately after the sheep urine application, with maximum fluxes of 0.12 and 0.22 kg N ha-1 d-1 in the Bh + U and Br + U treatments, respectively (Fig. 1a). These fluxes decreased rapidly within the following 23 h and then reached another peak after day 13, with what seem to be broad peaks lasting up to 9 days (day 10 to 19). Fluxes, however, remained high until the end of the incubation. N2O emissions in the Bh + W and Br + W treatments were much lower than that in the treatments with sheep urine application, with average fluxes of 0.009 and 0.006 kg N ha-1 d-1, respectively. The cumulative N2O emission for the first peak in the Br + U treatment (0.11 kg N ha-1) was significantly higher than that in the Bh + U (0.05 kg ha-1) treatment, although no significant differences were observed in the cumulative N2O emissions for the entire 23 days incubation between the Bh + U and Br + U treatments (Table 3). The cumulative N2O emissions in the Bh + W and Br + W treatments were significantly lower than that from both urine treatments during both the first peak period and the whole incubation period.

**Nitric oxide**: the pattern of NO emissions was similar to the N2O emissions for all treatments during the 23 days incubation, with the exception that the maximum NO fluxes in the sheep urine application treatments occurred during the second peak on day 14-16 (Fig. 1b). The first peak of NO emissions appeared 7.0 h and 10.6 h after the urine application in the Bh + U and Br + U treatments, respectively, which was a little later than the peak time of maximum N2O emissions (3.6 and 5.3 h, respectively) reaching values up to 3 g N ha-1 d-1. Cumulative NO emissions in the treatments with the sheep urine application including the two peaks (Bh + U, 0.114 kg N ha-1; Br + U, 0.103 kg N ha-1) were significantly higher than those in the water only treatments (Bh + W, 0.007 kg N ha-1; Br + W, 0.003 kg N ha-1). Nevertheless, no significant differences in NO emissions were observed between the Bh + U and Br + U treatments, or the Bh + W and Br + W treatments during the first peak period or for the whole incubation period. The second NO peak was broader than the initial one (reaching up to ~8 g N ha-1 d-1) and had not reached background values at the end of the incubation, but clearly showed fluxes were decreasing from d 16 onwards.

**Nitrogen gas**: Fluxes of N2 were low and decreased continuously from the start of the incubation (data not shown), indicating incomplete flushing of the vessels with contribution of the N2 that entered the DENIS when non-flushed (He/O2) urine and water were applied to the soil. Soil-borne N2 emissions were not observed during the incubation, as expected, as soil moisture conditions were managed to favour nitrification (65% WFPS) (Loick et al. 2021).

**Carbon dioxide**: in the Bh + U and Br + U treatments, the CO2 emissions increased rapidly and peaked at 10.8 h after the urine application (similar to the NO peak in the urine treatments), with the maximum fluxes of 207.2 and 198.9 kg C ha-1 d-1, respectively (Fig. 1c). The CO2 emissions decreased afterwards and remained stable (less than ca. 30 kg C ha-1 h-1) from day 3.5 to end of the incubation in the Bh + U and Br + U treatments. After the incubation, the cumulative CO2 emissions in the soil under Br treatments were significantly lower than those in the soil under Bh treatments, following the series: Br + W < Bh + W < Br + U < Bh + U, with the cumulative fluxes of 333.5, 428.5, 654.6, 768.5 kg C ha-1, respectively (Table 3).



Fig. 1 Gaseous emissions (average) of N2O (panel a), NO (panel b) and CO2 (panel c) during the incubation (urine and water were applied on day 0). Error bars represent standard error of the mean (n=3).

Table 3 Cumulative emissions of N2O and NO (in kg N ha-1) and CO2 (in kg C ha-1) after 23 days incubation and during the first peak period.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Gas | Bh + W | Bh + U | Br + W | Br + U |
| N2O (23 d) | 0.216 ± 0.026 b | 1.73 ± 0.316 a | 0.128 ± 0.068 b | 1.72 ± 0.324 a |
| N2O (first peak) | 0.003 ± 0.000 c | 0.054 ± 0.010 b | 0.004 ± 0.001 c | 0.111 ± 0.017 a |
| NO (23 d) | 0.007 ± 0.001 b | 0.114 ± 0.009 a | 0.003 ± 0.001 b | 0.103 ± 0.015 a |
| NO (first peak) | 0.0003 ± 0.0001 b | 0.0015 ± 0.0001 ab | 0.0003 ± 0.0001 b | 0.0025 ± 0.0007 a |
| CO2 (23 d) | 422.0 ± 10.5 c | 761.9 ± 15.7 a | 328.5 ± 13.4 d | 649.0 ± 7.4 b |
| CO2 (first peak) | 97.83 ± 3.34 b | 350.0 ± 10.28 a | 84.56 ± 3.26 b | 328.6 ± 12.59 a |

Values represent means ± SEM. Different letters indicate a significant difference between treatments (n=3, *P*<0.05).

*3.3. Nitrifiers and denitrifiers gene copies*

At the start of the incubation (day 0), there were no significant differences in the AOA, AOB, *nirK*, *nirS* and *nosZ* gene copies between the different treatments (Fig. 2). After the incubation (day 23), no significant differences were observed in the AOA, *nirS* and *nosZ* gene abundance between the treatments with the sheep urine application and without urine application (Fig. 2a, d, e). The sheep urine application increased the soil AOB and *nirK* gene copies at the end of the incubation (Fig. 2b, c). The AOB gene copies in the Bh + U treatment (7.7×106 copies g-1 soil) were significantly higher than that in the Br + U treatment (4.7×106 copies g-1 soil). The *nirK* gene copies in the Br + W (2.1×104 copies g-1 soil) was significantly lower than other treatments, but no significant differences were observed in the *nirK* gene copies between the Bh + W, Bh + U and Br + U treatments (3.3×104, 5.0×104, 3.7×104 copies g-1 soil, respectively).

图片包含 游戏机, 钢琴

描述已自动生成

Fig. 2 Average AOA (panel a), AOB (panel b), *nirK* (panel c), *nirS* (panel d) and *nosZ* (panel e) gene abundance at d 0 and d 23. Error bars represent standard error of the mean (n=3). Different letters indicate significant differences between treatments at d 0 (lower case) and d 23 (upper case), respectively (*P*<0.05).

**4. Discussion**

*4.1. Effect of Bh and Br on soil NH4+-N and NO3--N concentrations*

The decrease of NH4+ and increase of NO3- in the treatments without sheep urine application was caused by the nitrification of residual soil NH4+. In the treatments with sheep urine application, the slight increase of NH4+ and marked increase in NO3- (over 200 mg N kg soil-1) were caused by the hydrolysis of urea and further nitrification of the NH4+ from the urine-N applied (*Byrnes* et al., 2017). It was excepted that soil with Bh retained significantly higher NH4+ and lower NO3-concentrations than soil with Br after the incubation, due to the biological NIs released from its (Bh) root to suppress the transformation of NH4+ to NO3- (*Subbarao* et al., 2007a; *Gopalakrishnan* et al., 2009; *Nuñez* et al., 2018). However, no significant differences were observed in the soil NH4+ and lower NO3-concentrations between the Bh and Br treatments in this study (Table 1).

Previous studies reported that soil applied with different amount of root exudates or compounds (which have been identified as biological NIs) from Bh retained higher soil NH4+ and lower NO3-concentrations compared with the bare soil treatments (Subbarao et al. 2006, 2008; Nuñez et al. 2018). Ma et al. (2021) found that soil applied with different concentrations of biological NIs (linoleic acid and linolenic acid) only decreased soil NO3- concentration but did not affect the soil NH4+ concentration due to the nitrification inhibition and/or N immobilization. As for the effects of different *Brachiaria* species on soil nitrification, Castoldi et al. (2013) suggested that the levels of NH4+ and NO3- determined in the soil were similar among the *Brachiaria* species. This is consistent with the results in this study and also supported by the study by Castoldi et al. (2017), in which no significant differences were observed in the soil NH4+ and NO3- concentrations between *Brachiaria* species. Because of the need to retain air-tight seals throughout the incubation for the measurement of soil derived N2 emissions, it was impossible to collect soil samples during the incubation period. A greater number of time points to explore the dynamics of soil NH4+ and NO3- during the incubation, would have helped to explain the effects of Bh and Br on the transformation of soil NH4+ to NO3-. Previous studies reported that the rates of nitrification inhibition increased with increasing concentrations of the biological NIs (Gopalakrishnan et al. 2009; Sun et al. 2016; Ma et al. 2021). The low stability of biological NIs released from Bh may be also one reason for the unexcepted results in this study. Ma et al. (2021) confirmed that biological NIs (linoleic acid and linolenic acid) identified from the shoot tissue of Bh were much more rapidly mineralized than synthetic NIs (such as DCD, less than 5% of mineralization rate even after 40 days incubation) in a highly nitrifying soil, reaching 40% in about 10 days incubation.

*4.2. Effect of Bh and Br on soil N-gas and CO2 emissions*

N2O and NO are known products of both nitrification and denitrification processes, which dominate under different optimal soil environment conditions such as soil moisture (*Loick* et al., 2016; *Wu* et al., 2017), pH (*Robinson* et al., 2014), temperature (*Lai* et al., 2019), O2 availability (*Zhu* et al., 2013; *Senbayram* et al., 2019) and C availability (*Miller* et al., 2008; *O’Neill* et al., 2020). At the beginning of the incubation experiment, the initial soil water content was set as 65% WFPS which would have favoured nitrification of the NH4+ from the hydrolysed urea in the urine treatments causing the initial observed N2O and NO emission peaks (first smaller peak). It is also supported by the study of Loick et al. (2021), in which nitrification was contributing the most to N2O emissions at 70% WFPS. In addition, the initial CO2 peak coincided with those of N2O and NO, as a result of the amendment application, and provides evidence of aerobic respiration (*Lee* et al., 2011). The duration of this peak is similar to the first N2O and NO peaks.

Soil grown with Bh is assumed to have lower cumulative N2O and NO emissions than that with Br due to the high BNI capacity in Bh (*Gopalakrishnan* et al., 2007; *Subbarao* et al., 2008). In this study, the cumulative N2O in the Bh + U treatment during the first peak was significantly lower than that in the Br + U treatment, which may be due to the nitrification inhibition caused by the biological NIs released from the Bh as previously reported (*Subbarao* et al., 2006, 2007a; *Meena* et al., 2014). In addition, N2O emissions factors (EFs) from sheep urine in the soil grown with Bh and Br were 0.41% and 0.43%, respectively, which is consistent with the literature review conducted by *López-Aizpún* et al. (2020) (with mean value of 0.39%, range from 0.04% to 1.80%). However, there was no significant difference in the cumulative N2O and NO emissions during the whole soil incubation between the Bh + U treatment and Br + U treatment. It is possible that a reason for the short-lived effect of the Bh may have been the death of the grasses in the DENIS (there were no lights present in the incubation vessels). The residual biological NIs produced by the living plants prior to the incubation may have inhibited nitrification temporarily, but may not have remained effective after the death of the grasses.

*4.3. Effect of Bh and Br on Nitrifiers and denitrifiers*

Synthetic NIs, such as DCD and DMPP, have been confirmed to inhibit the AOA and/or AOB genes copies, which play an important role in controlling the nitrification rates and dominate at different conditions (*Chen* et al., 2014; *Shi* et al., 2016; *Li* et al., 2019). NIs have also been shown to inhibit denitrifying microbes, *nirS* and/or *nirK* and/or *nosZ* and/ *narG* (*Shi* et al., 2017; *Zhou* et al., 2018; *Li* et al., 2019). The biological NI, 1,9-decanediol (identified from rice), has also been shown to suppress the nitrification through impeding both AOA and AOB, when applied at high concentrations (≥500 mg kg-1 soil) (*Lu* et al., 2019). A study conducted by Gopalakrishnan et al. (2009) also suggested that biological NIs released by the roots of Bh inhibited nitrifying bacteria, but did not negatively affect other major soil microorganisms. In this study, the controls (Bh + W and Br + W), did not influence the AOA, *nirS* and *nosZ* gene copies, but soil with Bh (with high BNI capacity) with sheep urine application significantly increased the AOB gene copies (responsible for the oxidation of NH4+) compared with Br (Fig. 2). The AOA and AOB gene copies were not lower in the Bh treatments than Br treatments as excepted, which may be because biological NIs inhibit nitrification rates by reducing the cell-specific activity of AOA and/or AOB, rather than affecting ammonia oxidiser populations, as well as non-target soil microorganisms or functions (*Kong* et al., 2016).

In order to retain air-tight seals throughout the incubation for the measurement of soil derived N2 emissions, soil samples were not collected during the incubation period. A greater number of time points to explore the dynamics of soil NH4+ and NO3-, as well as gene copies data during the incubation, or specific stable isotope approaches (such as 15N labelling) would have helped to confirm the sources of gaseous N from soil grown with these two grasses, and nitrification inhibition mechanism of Bh. Gopalakrishnan et al. (2009) suggested that BNI by roots of Bh varies with soil type. In addition, soil moisture content is an important factor related to the release of N-gas emissions (Loick et al. 2016; Wu et al. 2017). The effects of Bh on soil nitrification and GHG emissions under different soil moisture levels and soil types could be explored in the future studies.

**5. Conclusion**

In this highly nitrifying soil, N2O emissions dominated rather than NO emissions, from the soil sown with Bh and Br after the sheep urine application. Bh inhibited N2O emissions during the first peak compared with Br, however, no significant differences were observed in the cumulative N2O and NO emissions between the Bh + U and Br + U treatments over the entire 23 days incubation period. And there were also no significant differences in the soil NH4+ and NO3- concentrations between the Bh and Br treatments. We conclude that the residual biological NIs may inhibit the nitrification temporarily, but not last long enough in a highly nitrifying soil.

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