Soil Biology & Biochemistry 107 (2017) 156–163

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Biological nitrification inhibition by *Brachiaria* grasses mitigates soil nitrous oxide emissions from bovine urine patches

Ryan C. Byrnes [a, b], Jonathan Nùñez [a], Laura Arenas [a], Idupulapati Rao [a], Catalina Trujillo [a], Carolina Alvarez [c], Jacobo Arango [a], Frank Rasche [d], Ngonidzashe Chirinda [a, *]

[a] International Center for Tropical Agriculture (CIAT), A.A. 6713, Cali, Colombia
[b] Department of Land, Air and Water Resources, One Shields Avenue, University of California, Davis, CA 95616, USA
[c] National Institute of Agricultural Technology (INTA), Córdoba, Argentina
[d] Institute of Agricultural Sciences in the Tropics (Hans-Ruthenberg-Institute), University of Hohenheim, 70593 Stuttgart, Germany

ARTICLE INFO

Article history:
Received 22 September 2016
Accepted 31 December 2016
Available online 14 January 2017

Keywords:
Biological nitrification inhibition
Bovine urine patches
Brachiaria humidicola
Ammonia oxidizing bacteria and archaea
N_2O emission factor

ABSTRACT

High nitrogen (N) concentration in bovine urine, which generally exceeds plant N uptake rates, results in the formation of hotspots of N loss when bovine urine is deposited on grazed pasture soils. High spatial variability in the distribution of urine patches in grazed pastures poses a major challenge to mitigate N losses. Some exudates from the roots of several tropical forage grasses were shown to inhibit the activity of soil nitrifiers; a process known as biological nitrification inhibition (BNI). We hypothesized that nitrate (NO_3^-) production and nitrous oxide (N_2O) emissions from urine patches deposited on soils under forage grasses with high BNI capacity are lower than those with forage grasses with low BNI capacity. This hypothesis was tested using field plots of two tropical forage grass cultivars, *Brachiaria humidicola* cv. Tully (BT) and interspecific *Brachiaria hybrid* cv. Mulato (BM) which, correspondingly, have high and low BNI capacity. Nitrification rates and *amo*A gene copy numbers of ammonia oxidizing archaea (AOA) and bacteria (AOB) in soils under the two forage grasses were quantified before and after urine and water (control) application, as well, an additional experiment was conducted to quantify denitrification potential. Moreover, soil N_2O emissions from simulated urine (0.123 kg N m^{-2}) and water patches were monitored over a 29-day period. Results showed a greater suppression of nitrification, denitrification and AOA abundance in soils under BT than those under BM. Positive relationships ($p < 0.05$) existed between AOA and AOB abundance and NO_3^- contents in soils under BM. Bovine urine resulted in higher cumulative N_2O fluxes from soils under BM (80 mg N_2O-N m^{-2}) compared to those under BT (32 mg N_2O-N m^{-2}). Consequently, N_2O emission factors were higher for soils under BM (0.07%) than under BT (0.00002%). We conclude that tropical forage grasses with high BNI capacity play a key role in mitigating N_2O emissions from bovine urine patches in archaea-dominated soils. This suggests that wide-spread adoption of tropical forage grasses with high BNI capacity may have a great potential to tighten N cycling in grazed pastures.

1. Introduction

The atmospheric concentration of nitrous oxide (N_2O), a persistent greenhouse gas with a global warming potential 298 times higher than carbon dioxide (CO_2) is continuously increasing (Butterbach-Bahl et al., 2013). Recent estimates show that, in 2014, average atmospheric N_2O levels (327.1 ± 0.1 ppb) were 121% higher than pre-industrial levels (World Meteorological Organization, 2015). In soils, N_2O is produced as an intermediary in several microbially driven processes consisting of nitrification and denitrification (Braker and Conrad, 2011). It has been acknowledged that nitrification and denitrification represent critical pathways for nitrogen (N) loss (van Groenigen et al., 2015; Zhang et al., 2015).

Globally, animal waste contributes about 10% of the annual N_2O emissions from agricultural soils (Mosier et al., 1998). According to the IPCC guidelines, the default N_2O emission factor for manure and urine deposited on pastures is 2% of N excreted (IPCC, 2006). The main N component in excreted urine is urea which, when deposited on soil, is rapidly hydrolyzed to ammonium (NH_4^+), and transformed into nitrate (NO_3^-) enabling N_2O production (Whitehead,

* Corresponding author.
E-mail address: n.chirinda@cgiar.org (N. Chirinda).

http://dx.doi.org/10.1016/j.soilbio.2016.12.029

1995). Bovine urine patches are characterized by high concentrations (500–1000 kg ha^{-1}) of readily available N (Barneze et al., 2014; Haynes and Williams, 1993). The high N concentrations in voided urine are generally higher than the rates at which tropical grasses can accumulate N (Di et al., 2014; Rao et al., 1995). Therefore, a significant proportion of the N in deposited urine is vulnerable to loss in different forms such as N_2O (Di and Cameron, 2002).

In the mid-1980s, researchers at the International Centre for Tropical Agriculture (CIAT) observed that soils of grass alone pastures of *Brachiaria humidicola* cv. Tully (CIAT 679) have markedly lower nitrification rates than those under legume-alone pastures or bare soil (Sylvester-Bradley et al., 1988). The mechanism underlying the suppression of soil nitrification is based on the particular characteristic of *Brachiaria* roots to release a substantial amount of exudates composed of inhibitory compounds such a brachialactone which blocks the ammonia monooxygenase (AMO) and hydroxylamino oxidoreductase (HAO) ammonia oxidizing enzymatic pathways (Subbarao et al., 2009; 2007). Whereas evidence on the use of *Brachiaria* grass as a biological nitrification inhibition (BNI) tool has mounted, there remains a critical research gap on its efficacy to suppress nitrification in urine patches, an area where synthetic nitrification inhibitors (NI) have been relatively successful albeit at a cost and with site-specific efficacy challenges (Mazzetto et al., 2014).

Interest on the relative roles of ammonia oxidizing archaea (AOA) and bacteria (AOB) in the nitrification process has grown. Research has shown that AOA, while dominating soil ammonia oxidizers (Leininger et al., 2006), are also particularly adapted to stressful environments of extreme pH, temperature, salinity and fertility, while AOB may favor more moderate conditions (Valentine, 2007). More recent, studies have demonstrated that AOA and AOB may differ markedly in the oxidation of ammonia and may dissimilarly contribute to the nitrification process (Prosser and Nicol, 2008; Di et al., 2009). However, Di et al. (2009) found that nitrification was driven primarily by AOB in N rich, temperate grasslands while Zhang et al. (2012) found the opposite to be true in highly acidic soils, suggesting that AOA and AOB may have dissimilar roles in ammonia oxidation. Subbarao et al. (2009), demonstrated contrasting abundances of AOA and AOB between soils under different *Brachiaria* species, suggesting dissimilar suppression of nitrifiers.

Our objectives were to: (i) evaluate N_2O emissions from urine patches of two forage grass cultivars: *Brachiaria humidicola* cv. Tully (BT) and *Brachiaria hybrid* cv. Mulato (BM), (ii) establish urine patch N_2O emission factors for both forage grass cultivars, and (iii) determine potential relationships between the abundance of prokaryotic ammonia oxidizers nitrate (NO_3^-) production and N_2O emission. Our main hypotheses were that (i) N_2O emissions from bovine urine patches are lower in soils under BT than BM which is due to the higher BNI capacity of BT, and that (ii) this difference is related to a dissimilar suppression of archaeal and bacterial nitrifiers and subsequently reduced nitrification rates.

2. Materials and methods

2.1. Experimental site

The field plots used in this study were part of a long-term field experiment (10 years-old) at CIAT, located at Palmira-Valle of Cauca (3°30′7″N 76°21′22″W and approximately 1000 m above sea level), in Colombia. The experiment was established in 2006 to evaluate the BNI potential of several tropical forage grass cultivars. Soil at the experimental site is classified as a Mollisol (fine-silty, mixed, isohyperthermic Aquic Hapludoll - Soil Survey Staff, 1999) with a silt clay loam texture with clay content of 40–60% in the plough layer (0–25 cm); total N content of 1.49 g kg^{-1}; pH of 6.2 (1:1 water) and a bulk density of about 1.44 g cm^{-3}. The site has a mean annual rainfall of 894 mm and a mean annual air temperature of 24 °C. During the course of the current study, mean daily temperature was 26 °C, the highest temperature was 28 °C (24 August, day 21) and the lowest was 24 °C (9 August, day 6) (Fig. 1). During the 29 days of monitoring period only one rain event (4.6 mm) was recorded. Baseline mean soil pH, total C, total N and mineral N concentrations in the treatment plots were similar (Table 1). Soil bulk density was lower in the BT than in the BM plots.

2.2. Site set-up and treatments

The experiment was organized as a completely randomized block design with three replicates per cultivar and plot size of 10 m × 10 m. Within each plot of BT or BM, we established 1 m × 1 m subplots. Bovine urine was collected from cows at the reproductive stage and weighing between 480 and 500 kg. The urine was pooled and a total of 900 ml was applied within areas demarcated by cylindrical PVC static chamber bases with a 26-cm internal diameter and 10 cm height, achieving a urine-N application rate of 0.123 kg N m^{-2}. A control that received similar amounts (900 ml) of deionized water was included. Therefore treatments included: *Brachiaria humidicola* cv. Tully (BT) and *Brachiaria hybrid* cv. Mulato (BM) with urine (BT + U and BM + U) and water (BT + W and BM + W) patches. Therefore, a total of 24 static chamber bases (12 per forage cultivar) were inserted at the centre of each 1 m × 1 m area to a depth of 5 cm, seven days prior to the start of gas and soil sampling. For each forage cultivar, chamber bases were equidistributed in the three replicate plots and soils within the six chamber bases received either urine or water.

The two forage grass cultivars BT and BM were chosen based on prior research indicating contrasting BNI capacities (Subbarao et al., 2009) and the extent of use as forages in tropical and subtropical cattle production systems. To simulate grazing, grass in each plot was cut to approximately 5 cm sward height, seven days prior to the beginning of the gas and soil sampling. The experimental plots had not been fertilized for 5 months prior to the current study.

2.3. Soil properties and microbial community dynamics

Prior to starting the experiment, soils (0–10 cm) from each plot were characterized for total nitrogen, total carbon, pH, and electrical conductivity. Within each plot an area equal to the size of an individual gas chamber base was established for soil sampling, which received the same + U or + W treatments in order to characterize soil chemical parameters and changes in soil microbial activity.

Soil inorganic N in the form of NH_4^+ and NO_3^- concentrations were determined five times during the experimental period; 1 day before urine application, and subsequently, 1 h and 7, 21 and 29 days after urine application. We collected and composited two subsamples (0–10 cm) from each plot. A total of 200 ml of 1M KCl were added to 20 g of the composite sample and shaken on a rotary shaker for 1 h, to extract mineral N. The extract was then filtered and stored in a frozen state (−20 °C) until spectrophotometric determination of NH_4^+ and NO_3^-. The soil moisture content in each sample was measured gravimetrically after oven drying at 105 °C for 48 h.

The ammonia oxidizing archaea (AOA) and bacteria (AOB) were estimated through qPCR using the *amo*A gene marker (Subbarao et al., 2009). DNA was isolated from 500 mg fresh soil using the Fast DNA™ SPIN Kit for Soil (MP Biomedicals, Solon, Ohio, USA) according to the manufacturer's instructions. The mix for qPCR

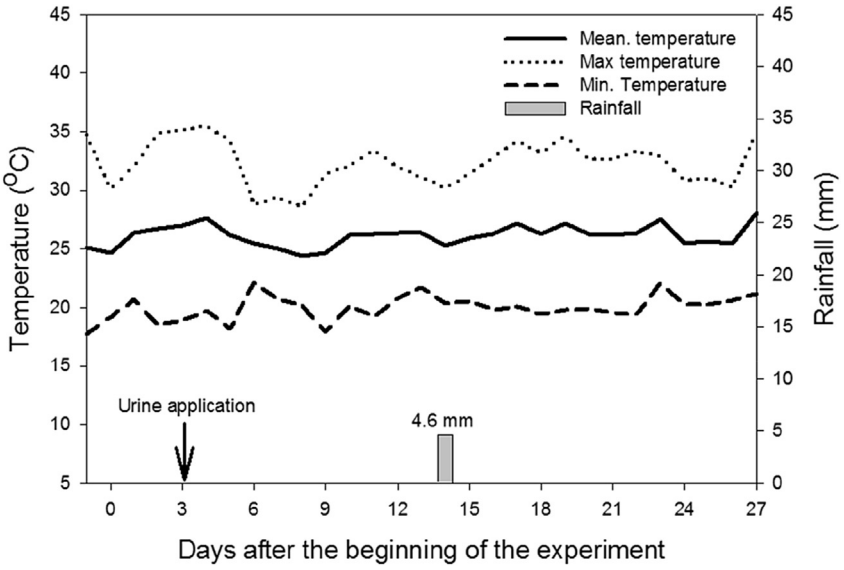

Fig. 1. Mean daily temperatures and rainfall during the course of sampling campaign of 29 days during the dry season at CIAT-Palmira, Colombia.

Table 1
Soil properties (0–10 cm) in field plots of *Brachiaria* hybrid cv. Mulato (BM) and *Brachiaria humidicola* cv. Tully (BT) grown in a Mollisol at CIAT-Palmira, Colombia.

Forage type	pH	Bulk density	$N\text{-}NO_3^-$	$N\text{-}NH_4^+$	Total N	Total C
		g cm^{-3}	(mg N kg^{-1})	(mg N kg^{-1})	(g N kg^{-1})	(g C kg $soil^{-1}$)
BM	6.5	1.52	0.61	n.d[a]	1.5	20.0
BT	6.1	1.39	0.64	n.d[a]	1.5	27.0

[a] Below the instrument detection limit.

contained 10 ng of DNA, 10 μl of brilliant sybr mix (promega), primers (0.5 μM) *amo*A-1F/*amo*A-2R for AOB (Rotthauwe et al.,, 1997) and amoA19F/amoA643R for AOA (Leininger et al., 2006) according to the methods of Rasche et al. (2011). Seven point standard curves ranging from 10^3 to 10^9 molecules were used to obtain an absolute quantification of amoA gene in the samples. Gene copy numbers and reaction efficiencies were obtained using the stepOne software version 2.2.2 (applied biosystem).

A separate experiment was conducted to determine the denitrification potential. In this assay, the denitrifying enzyme activity (DEA) under non-limiting conditions is assumed to be proportional to the denitrification potential (Tiedje, 1994). The method used is described in detail by Chirinda et al. (2011), briefly, 10 g of fresh soil were placed in 150 ml bottles.

A 25-mL solution with excess potassium nitrate (1 mM), glucose (1 mM), and 1 g L^{-1} of chloramphenicol (a bacteriostatic agent to inhibit de novo protein synthesis) was added to all soil samples. The bottles were made anaerobic by evacuating and flushing them with helium three times, ending up with a headspace of helium at atmospheric pressure. A total of 10 ml helium was drawn from each bottle using a syringe, and 10 ml acetylene generated from calcium carbide was then added to block N_2O reduction to N_2. The soil slurries were incubated on a rotary shaker at 25 °C and rotating at 125 rpm. A total of 10 ml gas samples were taken from each bottle at 15 min intervals over a 1 h period. N_2O concentration in each sample was analyzed using a gas chromatographs (GC-2014 Shimadzu) equipped with a Porapak K column and electron capture detector (ECD ^{63}Ni). Denitrifying enzyme activity was calculated from N_2O accumulation using linear regression.

Soil nitrification rates were determined through a soil incubation assay described by Subbarao et al. (2006a). Briefly, the rhizospheric soil was collected and dried at room temperature for 2 days before being passed through a sieve (2 mm mesh size). The soil incubation assay was composed of a 10 ml-amber flask, hermetic cap with one hole, 3 g of homogenized soil supplemented with 0.8 ml of ammonium sulfate (27 mM), to maintain the field capacity at 60% and three replicates were used per incubation time. A baseline nitrate concentration was obtained with KCl (1M) extraction of the samples after 4 and 12 days of incubation. The nitrate concentration was determined using an auto-analyzer as described by Subbarao et al. (2006b). The nitrification rate was expressed as a rate of nitrate production per kilogram of soil per day according to:

$$NR = \frac{\left(T4\ N-NO_3^-\ -\ N-NO_3^-\ basal\right)\ -\ \left(T12\ N-NO_3^-\ -\ N-NO_3^-\ basal\right)}{Incubation\ days} \quad (1)$$

where NR is nitrification rate, T4 $N-NO_3^-$ and T12 $N-NO_3^-$ is the N derived from nitrate obtained from the incubation time 4 and 12 days, and N-NO_3^- basal is the baseline N concentration before NH_4^+ application.

2.4. BNI potential estimation through bioassay

Three plants from each plot of the two forage grasses (BT, BM) were used to obtain root samples for the bioassay. The root tissue was dried by lyophilization and then ground to a fine powder. A sub-sample (100 mg) of the ground roots had 2 ml of 100% methanol solution added and placed on a strong vortex with a paint mixer machine for 3 min (Harbil paint mixer). The methanolic extraction was filtered through a syringe-driven 0.22 mm membrane filter (Millex, Millipore USA), evaporated by vacuum, re-suspended in 50 μl of dimethyl sulfoxide (DMSO) and 2 μl used in the BNI bioassay. Control for methanolic extraction was subjected to the same procedure as for sample (methanol + beads + shaking without plant tissue). The bioassay was performed with a recombinant Nitrosomonas strain that was transformed with a plasmid carrying the luciferase gene (Iizumi et al., 1998) and standardized for estimation of BNI potential by Subbarao et al. (2006b).

The bacteria was grown on 200 ml of P-media (final concentration: KH_2PO_4 5.14 mM, Na_2HPO_4 95.1 mM, $(NH_4)_2SO_4$ 18.91 mM, $NaHCO_3$ 5.95 mM, $CaCl_2$-$2H_2O$ 0.034 mM, $MgSO_4$-$7H_2O$ 0.041 mM, Fe (III) EDTA 0.0027 mM, pH 7.8), during 7 days at 50RPM and 28 °C supplemented with 100 μl of Kanamycin 50 mg ml^{-1}. The pellet was collected by centrifugation at 4000 RPM for 20 min, and suspended in 50 ml of fresh P-media. For the bioassay a mix with 2 μl of tissue extract was evaluated with 198 μl of distilled water and 250 μl of bacteria, incubated for 15 min at 15 °C with continuous shaking at 900 RPM (Fisher vortex genie 2). A sample of 100 μl was used to measure the luminescence using a luminometer glomax 20/20 (promega) with injection of 25 μL of decil-aldehyde (1%). The luminescence was registered as an integration time between 2 and 10 s.

The inhibition capacity was calculated as the percentage of light emitted by Nitrosomonas. The ATU units was calculated considering an inhibition of 80% of luminescence of 0.22 μM of allylthiourea according to the methods described by Subbarao et al. (2006b).

2.5. Soil N_2O fluxes

From 31 July to 27 August 2015, closed non-vented static chambers (10 cm height) were used for measuring N_2O emissions from each plot (de Klein et al., 2003). On each sampling campaign, PVC chambers were fitted to the chamber bases and sealed with an airtight rubber belt. Gas sampling was conducted on 10 occasions: once before the application of urine or water, 2 h after urine or water application, daily for the first three days following urine or water application, three times during the second week and twice per week during the last weeks of the experiment. Syringes (15 ml) fitted with hypodermic needles were used to collect fours gas samples (0, 10, 20 and 30 min) during each chamber deployment time (09:00–12:00 a.m.). Collected samples were transferred to pre-evacuated 8-ml headspace glass vials fitted with rubber butyl septa crimp caps.

The N_2O concentrations in each sample were analyzed by gas chromatography (GC-2014 Shimadzu). The N_2O detection limit of the GC, which was calculated as proposed by Christiansen et al. (2015), was 0.128 ppm. The daily gas fluxes were calculated by regressing mean N_2O emissions for each sampling date against time. All flux data were checked for linearity by visual inspection during data analysis. In addition, as suggested by Clemens et al. (2016), when the coefficient of determination (R^2) of the linear regression was greater than 0.7, the slope was taken to be the N_2O flux over the 0–30 min sampling interval. Cumulative fluxes were calculated from mean N_2O emissions by interpolation between measurement days (Dobbie et al., 1999).

The N_2O-N emission factor for urine patches in the grasses were calculated according to Sordi et al. (2014):

$$EF(\%) = \frac{(N_2O - N_{emitted}) - (N_2O - N_{control})}{N_{applied}} \times 100 \quad (2)$$

where EF is the emission factor, N_2O-$N_{emitted}$ and N_2O-$N_{control}$ are the cumulative N_2O emissions from urine and water patches over the 29-day monitoring period. $N_{applied}$ represents the amount of N in applied urine.

2.6. Statistical analyses

Statistical analyses were done using RStudio (RStudio Team, 2015) and SAS version 9.4. Data were checked for linear model assumptions, data that did not meet initial assumptions were log transformed (base 10) to achieve variance homogeneity and normal distribution. Analysis of variance tests were used to determine if significant treatment effects existed and relationships between NO_3^- production and AOA and AOB communities were determined through stepwise variable elimination in RStudio. Post-hoc Tukey means separation tests were used to determine if differences in N_2O emissions existed between treatments. Studentized t-tests were used in the determination of differences of BNI concentration and denitrifier activity. The multiple linear regression procedure PROC MIXED of SAS (SAS Institute, 1996) was used. The statistical model with the lowest Akaike's Information Criteria value used was to determine effects of forage grasses and urine or water application on soil NH_4^+ and NO_3^- content, nitrification rates and gene copy numbers and mean daily N_2O fluxes. The fixed effects were forage grasses (BT and BM), treatment (urine and water) and forage grasses × treatment interactions. Block was included as a random effect and sampling data was included as a repeat function.

3. Results

3.1. Soil inorganic N

High urea-N application rates (0.123 kg m^{-2}) resulted in an increase in soil NH_4^+ following urine application in both the BT and BM plots. The NH_4^+ level initially increased following urine application then decreased within 7 days but remained higher than the control for the rest of the monitoring period (Fig. 2a). Where no urine was applied (+W), NH_4^+ concentrations remained similar throughout the monitoring period. Soil NH_4^+ concentrations in BM + U remained higher than those in BT + U treatment plots throughout the monitoring period. Towards the end of the sampling campaign, soil NO_3^- concentrations where generally higher in BM + U than BT + U, BT + W and BM + W for which similar soil NO_3^- concentrations were observed (Fig. 2b). Both the forage grass type ($P = 0.0108$) and applied treatment ($P < 0.0001$) affected soil NH_4^+ levels. Forage grass and treatment interactions were observed for soil NH_4^+ ($P = 0.0093$) but not for NO_3^-.

3.2. Root tissue BNI, soil nitrifier abundance, nitrate production rates and nitrate content

Nitrate productions rates were influenced by forage type and treatment ($P < 0.0001$). The highest nitrate production rates were observed 5 days after urine application, for soils under BM (Fig. 3). Nitrifier activity remained low in the BT plots. The BNI concentration was found to be significantly ($p < 0.05$) greater in the root tissue of BT compared to BM (Fig. 4a). Potential denitrification was significantly higher ($p < 0.05$) in BM compared to BT plots (Fig. 4b). Prior to urine application, the gene copy numbers of AOA and AOB

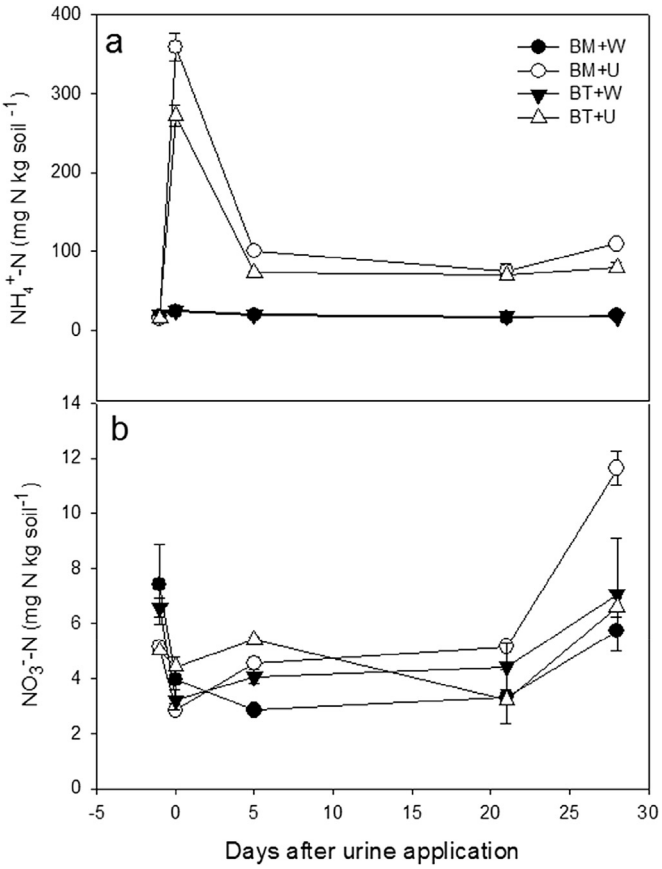

Fig. 2. Soil inorganic N dynamics in the *Brachiaria humidicola* cv. Tully with urine (BT + U) and without urine (BT + W) treatments and *Brachiaria* hybrid cv. Mulato with urine (BM + U) and without urine (BM + W) treatments a) Ammonium, and b) Nitrate. Error bars represent standard error of the mean.

for BT and BM treatments were significantly different ($p < 0.05$). The very low gene copy number of AOA in BT + U before urine application suggests long-term suppression of AOA in these plots. Following the addition of urine, AOA copy numbers in BT + U plots were always lower than in BM + U plots, while that of AOB was much higher in BT + U plots compared to BM + U on two out of the three sampling days (Fig. 5). Results on the gene copy number for the water control treatments showed similar trends with the urine treatments. Specifically, the BT + U treatment always had the highest AOB gene copy number post water application and AOA gene copy number for BT + W was always the lowest over the sampling period (data not shown).

The relationship between soil nitrifier gene copy numbers and soil nitrate contents was best explained through linear regression

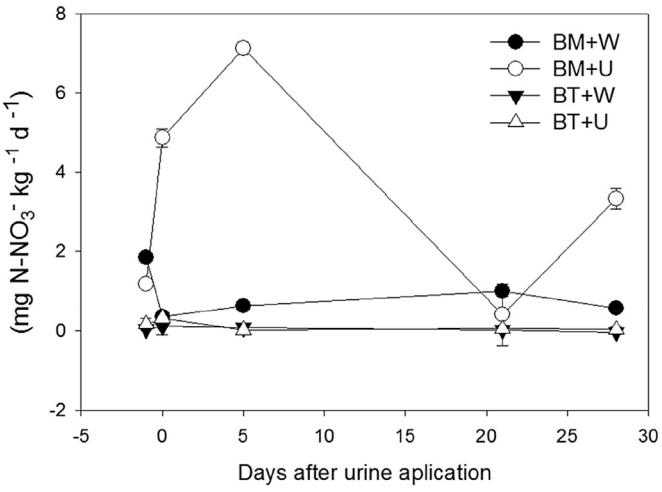

Fig. 3. Soil nitrification rates from *Brachiaria humidicola* cv. Tully (BT) plots with urine (BT + U) and without urine (BT + W) treatments, and from *Brachiaria* hybrid cv. Mulato (BM) plots with urine (BM + U) and without urine (BM + W) treatments. Error bars represent standard error of the mean.

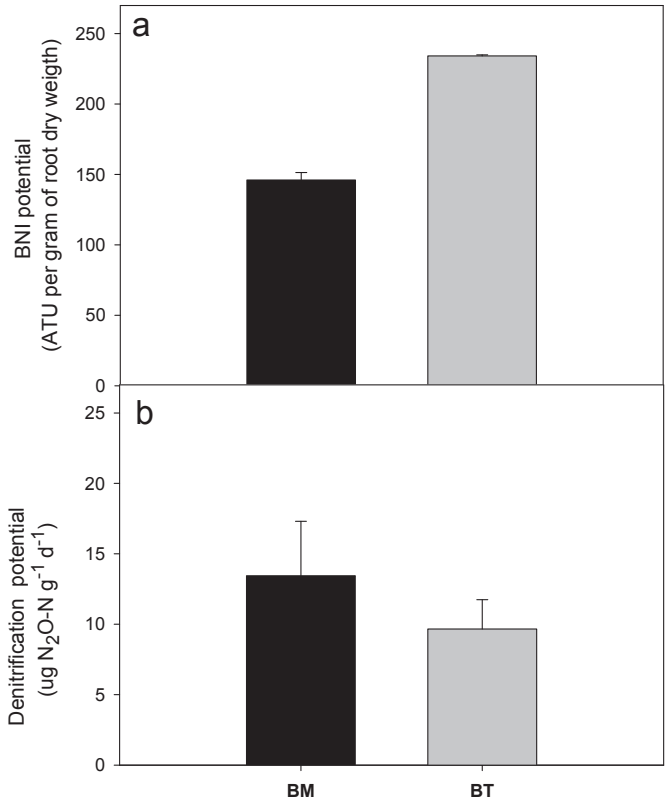

Fig. 4. a) Biological nitrification inhibition potential in the root tissue of *Brachiaria* hybrid cv. Mulato (BM) and *Brachiaria humidicola* cv. Tully (BT) and b) Denitrification potential in soils under BM and BT. Error bars represent standard error of the mean.

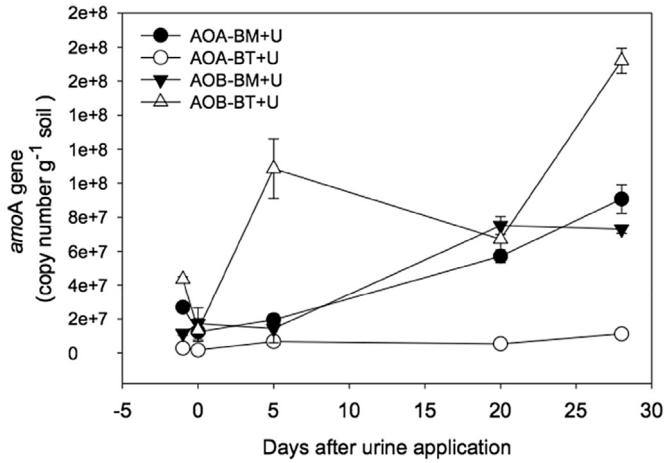

Fig. 5. AOA and AOB gene copy number in *Brachiaria* hybrid cv. Mulato (BM) and *Brachiaria humidicola* cv. Tully (BT) plots. Error bars represent standard error of the mean.

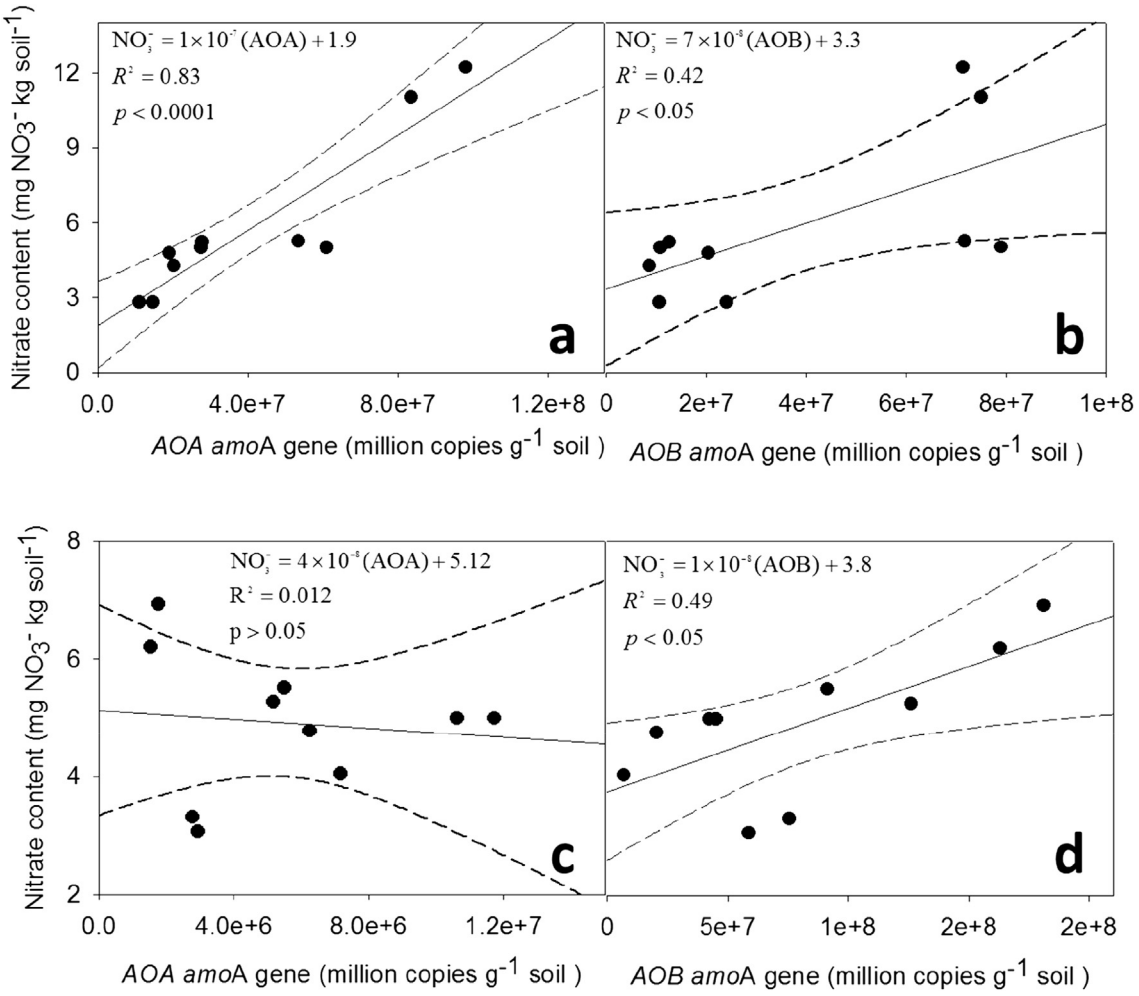

Fig. 6. Relationship between NO_3^- concentration and AOA and AOB *amoA* gene copy number in *Brachiaria* hybrid cv. Mulato (BM) plots (a, b) and *Brachiaria humidicola* cv. Tully (BT) plots (c, d).

(Fig. 6 a–d). The R^2 values suggested that for soils under BM and BT, the AOB could only account for less than 50% of the variation in nitrification rates (Fig. 6b and d). For the BM and BT cultivars, AOA correspondingly accounted for 83% and 1% of the variation in nitrification rates, respectively (Fig. 6a and c). Soil nitrifier abundance was not related to N_2O emissions (data not shown).

3.3. Gas emissions and emissions factors

For soils under BM, peak N_2O emissions were observed two days after urine application. Nitrous oxide emissions from soil where water was applied were significantly ($P < 0.001$) lower than those where bovine urine was applied (Fig. 7). Forage grass type had a significant effect on daily N_2O emissions ($P < 0.05$). The cumulative N_2O fluxes, which followed the order BM + U > BT + U > BT + W > BM + W, were significantly higher ($P < 0.05$) in the BM + U (80 mg N_2O-N m^{-2}) compared to the BT + U (32 mg N_2O-N m^{-2}). Significant interactions ($P < 0.01$) where observed between forage type and treatment (urine or water application). The N_2O-N emissions factors for BM and BT were 0.07% and 0.00002%, respectively.

4. Discussion

The main objectives of this study were to evaluate N_2O emissions from urine patches on soil under two forage grass cultivars, BT and BM, establish urine patch N_2O emission factors for the two forage grass cultivars, and determine relationships between the abundance of prokaryotic ammonia oxidizers and NO_3^- production.

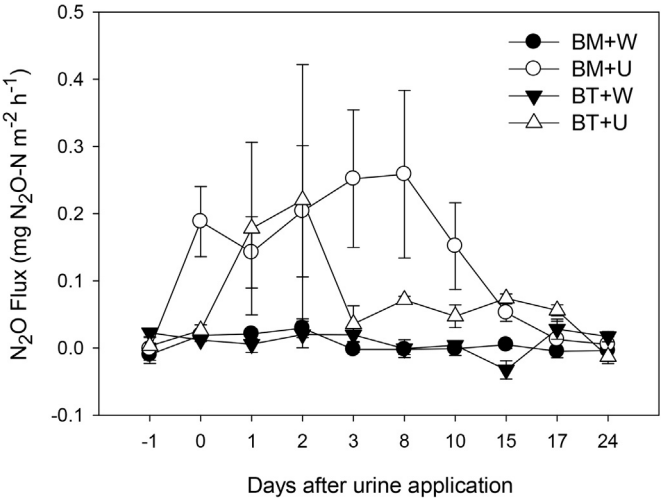

Fig. 7. Soil N_2O emissions from two forage grass plots of *Brachiaria humidicola* cv. Tully, BT + U/+W with urine (BT + U) and without urine (BT + W) treatments; and *Brachiaria* hybrid cv. Mulato, with urine (BM + U) and without urine (BM + W) treatments. Error bars represent standard error of the mean.

It was a key highlight of our study that cumulative N_2O emissions in soils under BT were significantly lower than under BM. This finding was explained by the high BNI potential of BT suppressing the activity of prokaryotic nitrifiers as revealed by determination of *amoA* gene abundance. Likewise, BM revealed higher direct N_2O EFs for urine-N than BT.

4.1. Soil N_2O emissions and emission factors for urine patches

The initial surge in NH_4^+ levels in soils where urine was applied resulted from the high N content in the urine. The inability of soil NH_4^+ content did not drop below 50 mg N kg^{-1} soil after the initial surge suggests that nitrification was generally inhibited by both forages. While we expected that the higher BNI capacity of BT would correspond to high soil NH_4^+content, the higher soil NH_4^+ in BM plots was possibly due to dissimilar soil N uptake rates between the two forages. Unpublished data collected at the same study site showed that root biomass was higher in soil under BT than BM (Jacobo Arango, personal communication). High root biomass suggested that BT roots explore a larger soil volume and thus possibly have higher N uptake rates. This might partially explain observed differences in soil NH_4^+ levels implying that besides high BNI capacity, differences in soil N uptake most likely contributed to observed differences in soil N_2O emission.

The EFs observed in the current study were lower than those reported in studies conducted under comparable tropical and subtropical conditions, which ranged between 0.1 and 1.2% (Barneze et al., 2014; Mazzetto et al., 2015; Pelster et al., 2016; Sordi et al., 2014). This may be attributed to the dry soil conditions experienced during the monitoring period of our experiment that was characterized by only one light precipitation event (4.6 mm). Previous studies suggested that short-term evaluations (4 weeks) were generally sufficient to capture the main urine-dependent N_2O emissions from grazed pasture soils (Van der Weerden et al., 2014; Velthof and Oenema, 1994; Monaghan and Barraclough, 1993). Therefore, whereas longer N_2O monitoring campaigns may enable the capturing of the remainder of urine-dependent N_2O emissions (Mosier et al., 1998), short-term studies, as reported here, provide important insights on the major urine-dependent N_2O emissions after urine placement. Low N_2O emissions in soils under *Brachiaria* cultivars suggests that, besides climatic factors; animal breed, diet (Pelster et al., 2016) and forage type may partially explain the variability of soil N_2O emissions from urine patches. Lessa et al. (2014) made a similar suggestion for a study conducted in Brazil.

4.2. Relationships between microbiological properties and soil nitrate content

Copy numbers of *amoA* genes collected prior to urine application showed that AOA and AOB were the dominant population of soil nitrifiers in BM + U and BT + U, respectively. The former observation supports earlier reports stating that AOA are better adapted than AOB to low N environments, such as pasture soils (Leininger et al., 2006; Prosser and Nicol, 2008; Valentine, 2007). The lower AOA gene copy numbers for BT than BM, pre-urine application, suggests long-term AOA suppression in BT plots due to higher BNI inhibition capacity. While an increase in AOB was observed in both BM and BT plots, AOA still demonstrated a stronger positive relationship ($R^2 = 0.83$, $p < 0.0001$) to soil nitrate production compared to AOB ($R^2 = 0.42$, $p < 0.05$) in BM plots. This is in contrast to BT plots where AOB were the primary drivers of soil N concentrations ($R^2 = 0.49$, $p < 0.05$) whereas AOA had no relationship to nitrate production ($R^2 = 0.012$, $p > 0.05$). This finding supports previous studies that have shown that exudates such as brachialactone, reduced the activity and abundance of soil AOA populations (Subbarao et al., 2009) but also reveals that in tropical pastures cultivated with non-BNI forage grasses and even with high N deposition, AOA may be the dominant nitrifying population as shown in the BM plots. The observed lower denitrification potential in soils under BT compared to BM further indicated that high BNI activity restricted the production of NO_3^-, a key substrate for inducing denitrifier enzyme activity (Saggar et al., 2013).

5. Conclusions

We conclude that tropical forage grasses with high BNI capacity play a key role in mitigating N_2O emissions from bovine urine patches in pasture soils. Accordingly, the wide-spread adoption of tropical forage grasses with high BNI capacity across humid and sub-humid tropics may have a great potential to tighten N cycling in grazed pastures mitigating climate change. Since our study was based on a short-term monitoring period only to determine the initial responses of soil nitrifiers and their respective feedbacks on soil N_2O emission under two different *Brachiaria* cultivars, we strongly suggest the need for longer monitoring periods to rule out the long-term effects on targeted soil properties, particularly active AOA.

Acknowledgements

This work was undertaken as part of the LivestockPlus Project funded by CGIAR Research Program on Climate Change, Agriculture and Food Security (CCAFS), which is a strategic partnership of CGIAR and Future Earth. We would like to thank the United States Agency for International Development (USAID) and the University of California, Davis Research for Innovation Fellowship in Agriculture (RIFA) that supported this research and all donors who supported this work through their contributions to the CGIAR Fund. We thank Dr. G. V. Subbarao, JIRCAS, Japan for collaborative research on the theme of biological nitrification inhibition in *Brachiaria* grasses and Dr. Meryl Richards, CCAFS, for reading through the manuscript and providing valuable insights for improvement.

References

Barneze, A.S., Mazzetto, A.M., Zani, C.F., Misselbrook, T., Cerri, C.C., 2014. Nitrous oxide emissions from soil due to urine deposition by grazing cattle in Brazil. Atmospheric Environment 92, 394–397.

Braker, G., Conrad, R., 2011. Diversity, structure, and size of N_2O-producing microbial communities in soils – what matters for their functioning? Advances in Applied Microbiology 75, 33–70.

Butterbach-Bahl, K., Baggs, E.M., Dannenmann, M., Kiese, R., Zechmeister-Boltenstern, S., 2013. Nitrous oxide emissions from soils: how well do we understand the processes and their controls? Philosophical Transactions of the Royal Society B: Biological Sciences 368, 20130122.

Chirinda, N., Olesen, J.E., Porter, J.R., 2011. Post cold storage conditioning time affects soil denitrifying enzyme activity. Communications in Soil Science and Plant Analysis 42, 2160–2167.

Christiansen, J.R., Outhwaite, J., Smukler, S.M., 2015. Comparison of CO_2, CH_4 and N_2O soil-atmosphere exchange measured in static chambers with cavity ring-down spectroscopy and gas chromatography. Agricultural and Forest Meteorology 211, 48–57.

Clemens, S., Rowlings, D.W., Grace, P.R., 2016. Non-linear response of soil N_2O emissions to nitrogen fertilizer in a cotton–fallow rotation in sub-tropical Australia. Soil Research 54, 494–499.

de Klein, C.A.M., Barton, L., Sherlock, R.R., Li, Z., Littlejohn, R.P., 2003. Estimating a nitrous oxide emission factor for animal urine from some New Zealand pastoral soils. Australian Journal of Soil Research 41, 381–399.

Di, H.J., Cameron, K.C., 2002. The use of a nitrification inhibitor, dicyandiamide (DCD), to decrease nitrate leaching and nitrous oxide emissions in a simulated grazed and irrigated grassland. Soil Use and Management 18, 395–403.

Di, H.J., Cameron, K.C., Shen, J.P., Winefield, C.S., O'Callaghan, M., Bowatte, S., He, J.Z., 2009. Nitrification driven by bacteria and not archaea in nitrogen-rich grassland soils. Nature Geoscience 2, 621–624.

Di, H.J., Cameron, K.C., Podolyan, A., Robinson, A., 2014. Effect of soil moisture status and a nitrification inhibitor, dicyandiamide, on ammonia oxidizer and denitrifier growth and nitrous oxide emissions in a grassland soil. Soil Biology and

Biochemistry 73, 59–68.
Dobbie, K.E., McTaggart, I.P., Smith, K.A., 1999. Nitrous oxide emissions from intensive agricultural systems: variations between crops and seasons, key driving variables, and mean emission factors. Journal of Geophysical Research 104, 26891–26899.
Haynes, R.J., Williams, P.H., 1993. Nutrient cycling and soil fertility in the grazed pasture ecosystem. Advances in Agronomy 49, 119–199.
Iizumi, T., Mizumoto, M., Nakamura, K., 1998. A bioluminescence assay using Nitrosomonas europaea for rapid and sensitive detection of nitrification inhibitors. Applied and Environmental Microbiology 64, 3656–3662.
IPCC, 2006. IPCC Guidelines for National Greenhouse Gas Inventories. IPCC, Geneva, Switzerland, p. 2006.
Leininger, S., Urich, T., Schloter, M., Schwark, L., Qi, J., Nicol, G.W., Schleper, C., 2006. Archaea predominate among ammonia-oxidizing prokaryotes in soils. Nature 442, 806–809.
Lessa, A.C.R., Madari, B., Paredes, D., Boddey, R., Urquiaga, S., Jantalia, C.P., Alves, B.J.R., 2014. Bovine urine and dung deposited on Brazilian savannah pastures contribute differently to direct and indirect soil nitrous oxide emissions. Agriculture, Ecosystems and Environment 190, 104–111.
Mazzetto, A.M., Barneze, A.S., Feigl, B.J., Groenigen, J.W., Van Oenema, O., Cerri, C.C., 2014. Soil Biology & Biochemistry Temperature and moisture affect methane and nitrous oxide emission from bovine manure patches in tropical conditions. Soil Biology and Biochemistry 76, 242–248.
Mazzetto, A.M., Barneze, A.S., Feigle, B.J., van Groenigen, J.W., Oenema, O., de Klein, C.A.M., Cerri, C.C., 2015. Use of the nitrification inhibitor dicyandiamide (DCD) does not mitigate N_2O emission from bovine urine patches under Oxisol in Northwest Brazil. Nutrient Cycling in Agroecosystems 101, 83–92.
Monaghan, R.M., Barraclough, B., 1993. Nitrous oxide and dinitrogen emissions from urine-affected soil under controlled conditions. Plant and Soil 151, 127–138.
Mosier, A.R., Kroeze, C., Nevison, C., Oenema, O., Seitzinger, S., Van Cleemput, O., 1998. Closing the global N_2O budget: nitrous oxide emissions through the agricultural nitrogen cycle. OECD/IPCC/IEA phase II development of IPCC guidelines for national greenhouse gas inventory methodology. Nutrient Cycling in Agroecosystems 52, 225–248.
Pelster, D.E., Gisore, B., Goopy, J., Korir, D., Koske, J.K., Rufino, M.C., Butterbach-Bahl, K., 2016. Methane and nitrous oxide emissions from cattle excreta on an East African Grassland. Journal of Environmental Quality. http://dx.doi.org/10.2134/jeq2016.02.0050.
Prosser, J.I., Nicol, G.W., 2008. Relative contributions of archaea and bacteria to aerobic ammonia oxidation in the environment. Environmental Microbiology 10, 2931–2941.
Rao, I.M., Ayarza, M.A., Garcia, R., 1995. Adaptive attributes of tropical forage species to acid soils I. Differences in plant growth, nutrient acquisition and nutrient utilization among C4 grasses and C3 legumes. Journal of Plant Nutrition 18, 2135–2155.
Rasche, F., Knapp, D., Kaiser, C., Koranda, M., Kitzler, B., Zechmeister-boltenstern, S., Sessitsch, A., 2011. Seasonality and resource availability control bacterial and archaeal communities in soils of a temperate beech forest. ISME Journal 5, 389–402.
Rotthauwe, J.H., Witzel, K.P., Liesack, W., 1997. The ammonia monooxygenase structural gene amoA as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Applied and Environmental Microbiology 63, 4704–4712.
RStudio Team, 2015. RStudio: Integrated Development for R. RStudio, Inc, Boston, MA.
Saggar, S., Jha, N., Deslippe, J., Bolan, N.S., Luo, J., Giltrap, D.L., Kim, D.-G., Zaman, M., Tillman, R.W., 2013. Denitrification and N_2O: N_2 production in temperate grasslands: processes, measurements, modelling and mitigating negative impacts. Science of the Total Environment 465, 173–195.
SAS Institute, 1996. SAS/STATTM Software: Changes and Enhancements through Release 6.11. SAS Institute, Cary, NC.
Soil Survey Staff, 1999. Soil Taxonomy: a Basic System of Soil Classification for Making and Interpreting Soil Surveys, second ed. Natural Resources Conservation Service. U.S. Department of Agriculture Handbook.
Sordi, A., Dieckow, J., Bayer, C., Amaral, M., Thiago, J., Acordi, J., Moraes, A. De, 2014. Nitrous oxide emission factors for urine and dung patches in a subtropical Brazilian pastureland. Agriculture, Ecosystems and Environment 190, 94–103.
Subbarao, G.V., Nakahara, K., Hurtado, M., del, P., Ono, H., Moreta, D.E., Salcedo, A.F., Yoshihashi, A.T., Ishikawa, T., Ishitani, M., Ohnishi-Kameyama, M., Yoshida, M., Rondon, M., Rao, I.M., Lascando, C.M., Berry, W.L., Ito, O., 2009. Evidence for biological nitrification inhibition in Brachiaria pastures. Proceedings of the National Academy of Sciences 106, 17302–17307.
Subbarao, G.V., Ito, O., Sahrawat, K.L., Berry, W.L., Nakahara, K., Ishikawa, T., Suenaga, K., Rondon, M., Rao, I.M., 2006a. Scope and strategies for regulation of nitrification in agricultural systems—challenges and opportunities. Critical Reviews in Plant Sciences 25, 303–335.
Subbarao, G.V., Ishikawa, T., Ito, O., Nakahara, K., Wang, H.Y., Berry, W.L., 2006b. A bioluminescence assay to detect nitrification inhibitors released from plant roots: a case study with *Brachiaria humidicola*. Plant and Soil 288, 101–112.
Subbarao, G.V., Rondon, M., Ito, O., Ishikawa, T., Rao, I.M., Nakahara, K., Lascano, C., Berry, W.L., 2007. Biological nitrification inhibition (BNI) - is it a widespread phenomenon? Plant and Soil 294, 5–18.
Sylvester-Bradley, R., Mosquera, D., Méndez, J.E., 1988. Inhibition of nitrate accumulation in tropical grassland soils: effect of nitrogen fertilization and soil disturbance. Journal of Soil Science 39, 407–416.
Tiedje, J.M., 1994. Methods of Soil Analysis, Part 2, Microbiological & Biochemical 260 Properties. SSSA, pp. 256–257.
Valentine, D.L., 2007. Adaptation to energy stress dictate the ecology and evolution of the Archaea. Nature Reviews Microbiology 5, 316–323.
Van der Weerden, T.J., Manderson, A., Kelliher, F.M., de Klein, C.A.M., 2014. Spatial and temporal nitrous oxide emissions from dairy cattle urine deposited onto grazed pastures across New Zealand based on soil water balance modelling. Agriculture, Ecosystems and Environment 189, 92–100.
van Groenigen, J.W., Huygens, D., Boecks, P., Kuyper, T.W., Lubbers, I.M., Rutting, T., Groffman, P.M., 2015. The soil N cycle: new insights and key challenges. Soil 1, 235–256.
Velthof, G.L., Oenema, O., 1994. Effect of nitrogen fertilizer type and urine on nitrous oxide flux from grassland in early spring. In: Mannetje, L., Frame, J. (Eds.), Proceedings of the 15th General Meeting of the European Grassland Federation. Grassland and Society, pp. 458–462.
Whitehead, D.C., 1995. Grassland Nitrogen. CAB International, Wallingford, UK.
World Meteorological Organization, 2015. WMO Greenhouse Gas Bulletin: the State of Greenhouse Gases in the Atmosphere Based on Observations through 2014. http://www.wmo.int/gaw/.
Zhang, L.-M., Hu, H.-W., Shen, J.-P., He, J.-Z., 2012. Ammonia-oxidizing archaea have more important role than ammonia-oxidizing bacteria in ammonia oxidation of strongly acidic soils. The ISME Journal 6, 1032–1045.
Zhang, J., Müller, C., Cai, Z., 2015. Heterotrophic nitrification of organic N and its contribution to nitrous oxide emissions in soils. Soil Biology and Biochemistry 84, 199–209.