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Title page

Title: **Temporal response of ureolytic and ammonia-oxidizing microbes and pasture yield to urea and NBPT at Leigh Creek of Victoria in Australia**

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Abstract

The urease inhibitor *N*-(*n*-butyl) thiophosphoric triamide (NBPT) has been reported to effectively reduce nitrogen (N) losses by inhibiting urea hydrolysis. However, the effect of NBPT on soil ureolytic and ammonia-oxidizing microbes is not well understood, with inconsistent effects on crop yield and nitrogen use efficiency (NUE). Further, repeated use or overuse of chemical fertilizers can have environmental implications like soil acidification. A field experiment was conducted at Leigh Creek, Victoria at a site with a history of repeated application of urea alone (40U) or with NBPT (as Green Urea NV® (40GU)) at 40 kg N ha^{-1}, and urea applied at 80 kg N ha^{-1} (80U), to perennial ryegrass *(Lolium perenne* L.). We aimed to investigate the temporal effects of NBPT applied with urea on ureolytic and ammonia-oxidizing microbes, pasture dry matter (DM) yield, and NUE within a season following treatment applications. The abundance of ureolytic microbes was higher in the control (CK) compared to all N treatments on all sampling days. The *ureC* gene copy numbers in 40 GU were significantly lower than in 40U on sampling days seven and 45. There was no significant effect of NBPT on the abundance of ammonia oxidizers, but increasing urea application rate to 80 kg N ha^{-1} (80U) significantly increased the abundance of ammonia-oxidizing bacteria (AOB) on days 7 and 45, and complete ammonia oxidizers (comammox *Nitrospira*) clade B on day 7 compared to 40U. There was no significant effect of NBPT on pasture DM yield, N-uptake, or NUE. Increasing N application rate significantly increased pasture DM yield and N- uptake but this did not influence the pasture NUE.

Keywords: Green Urea NV®, NBPT, Nitrogen use efficiency, comammox *Nitrospira,* Ammonia- oxidizing archaea, Ammonia- oxidizing bacteria.

1.0. Introduction

By 2050, the world population is expected to increase by 2-3 billion (DESA, 2017; Foley, 2011). This implies an expected increase in demand for agricultural land and N fertilizers (Tilman et al., 2001). Nitrogen is an essential plant nutrient that contributes to increased crop yields (Chen et al., 2008; Giannoulis et al., 2020). However, much of the applied fertilizer nitrogen (N) is lost from the plant-soil system through ammonia (NH_3) volatilization and nitrous oxide (N_2O) emissions, nitrate (NO_3^-) leaching, and surface run-off (Castaldelli et al., 2019; Chen et al., 2008; Fuertes-Mendizábal et al., 2019), causing adverse environmental and economic consequences (Galloway et al., 2008; Stark and Richards, 2008).

The N cycling process is largely microbially mediated. For instance, once urea fertilizer is applied to the soil, it quickly undergoes hydrolysis, which is catalysed by the urease enzyme, producing ammonium (NH_4^+) or ammonia (NH_3) depending on pH (Singh et al., 2008). The *ureC* gene that encodes the alpha subunit of the urease enzyme has been used to identify and study the functional ureolytic microbial communities (Wang et al., 2018). The NH_3 then undergoes oxidation to nitrite (NO_2^-) by ammonia-oxidizing archaea (AOA) or ammonia-oxidizing bacteria (AOB) using the ammonia monooxygenase (AMO) enzyme encoded by the *amoA* gene. The NO_2^- is then oxidized to NO_3^- by nitrite-oxidizing bacteria (NOB) (Caceres et al., 2018; Hayden et al., 2014; Kits et al., 2017). Recently, comammox organisms in the *Nitrospira* were discovered with the ability to undertake complete nitrification, oxidizing ammonia to NO_3^- (Daims et al., 2015; van Kessel et al., 2015). However, the contribution of comammox organisms to nitrification in many soils is not yet investigated.

Urea is the most widely used form of N globally due to its high N content (46%) and ease of handling (Chen et al., 2008; Glibert et al., 2006). However, high N losses have been reported from urea compared to other forms of N due to urea hydrolysis and the associated elevation of soil pH (Schwenke, 2014). Urease inhibitors, such as N-(n-butyl) thiophosphoric triamide (NBPT), have been developed to minimize N loss by inhibiting the urease enzyme activity, delaying urea hydrolysis, and preventing the elevated pH that drives NH_3 loss (Akiyama et al., 2010; Harty et al., 2016). By inhibiting urea hydrolysis, NBPT slows the formation of NH_4^+, a precursor for nitrification, and therefore could indirectly also influence nitrification and subsequent denitrification (Menendez et al., 2009).

Several studies at field and laboratory levels have reported the effect of NBPT on reducing hydrolysis rates and associated NH_3 loss, as well as its contribution to reducing the emissions of N_2O (Salazar et al., 2014; Silva et al., 2017). However, few studies have investigated the effect of NBPT on ureolytic, ammonia-oxidizing, or denitrifying microbes (Shi et al., 2017; Xi et al., 2017). Conflicting results exist regarding the impact of NBPT on yield and NUE, with some showing no effect (Abalos et al., 2012; Dougherty, 2016) and others reporting positive effects (Dawar, 2011; Vistoso, 2012). The effects of repeated application of urea and NBPT on soil N cycling microbial communities and yield is important as this has implications for soil health.

We investigated the effects of urea application rate and the use of NBPT on a) the abundance of soil N-cycling microbial communities involved in urea hydrolysis and nitrification, and b) pasture dry matter (DM) yield, N uptake, and nitrogen use efficiency (NUE). We hypothesized that: i) increasing the urea application rate would increase the abundance of ureolytic and nitrification organisms, ii) application of NBPT would reduce the abundance of ureolytic and nitrifying microbes by inhibiting urea hydrolysis, and iii) use of NBPT would improve pasture DM yield, N uptake, and NUE by slowing the supply of plant-available forms of N, thereby maximizing plant uptake while reducing the risk of N loss.

Materials and methods

The experimental site, soil sampling, and analysis

Our field campaign commenced in mid-February 2018 on an established long-term experimental site with perennial ryegrass (*Lolium perenne* L.) pasture at Leigh Creek, Victoria (37°55'S, 143°95'E). The area has a mean annual rainfall of 686.9 mm with mean maximum and minimum annual temperatures of 17.4 ^{o}C and 7.1 ^{o}C respectively (BOM, 2019). The soil contains 27.1% clay, 26.7% silt, and 46.2 % sand in the topsoil (0-10 cm) and is classified as a Chromosol (Isbell, 2016). Prior to the commencement of our measurements, the soil properties in the 0-10 cm layer were measured and are as follows: pH_{water} of 6.0, organic carbon of 3.0 %, NH_4^+-N and NO_3^- -N of 5.1 and 20 mg kg^{-1} soil respectively, cation exchange capacity of 12 cmol(+)/kg. The field site had a history of repeated surface application of urea and NBPT coated urea (Green Urea NV®) under the treatments of control (CK), granular urea (46% N) applied at 40 kg N ha^{-1} (40U), and 80 kg N ha^{-1} (80U), and urea coated with the urease inhibitor NBPT (as Green Urea NV® at 40 kg N ha^{-1} (Agrotain® @ 5 L t^{-1} urea)) (40GU). The proportion of NBPT in the GU was 0.14% N on a weight basis. The long-term experiment was established using a completely randomized block design with each treatment replicated three times. The first application of treatments occurred on 5th October 2014, and fertilizer was then applied after every pasture cut with about eight to 10 fertilization events per year.

On 19th February 2018, our measurement campaign commenced with the plots (4 x 1 m) receiving the treatments outlined above after pasture had been harvested to a height of 5 cm. Irrigation was applied on day 0 (19th February 2018), day two, and day 17 after fertilizer application. Soil samples were collected on days 0 (1-3 hours), 1, 2, 3, 4, 5, 6, 7, 10, 13, 17, 24, 31, 38, and 45 at a depth of 0–10 cm with an auger of 7.6 cm internal diameter by taking three cores per plot and homogenizing them to form one composite sample for each plot. After sampling, the soil was transported on ice to the lab. The collected soil samples were analysed for physicochemical properties and microbial composition (stored at -20^{o}C for DNA extraction). Gravimetric water content was determined by oven drying sub-samples at 105 °C for 24 h. Five grams of soil were used for soil pH determination on a ratio of 1:5 (weight/volume, soil: water) with a pH meter (Mettler Toledo Switzerland). Soil urea, NH_4^+-N, and NO_3^--N were extracted from 5 g of soil using a ratio of 1:5 (fresh soil: 2 M KCl- Phenyl Mercuric acetate (PMA), w/v) by shaking at 175 rpm for 1 h. The solution was filtered through a Whatman 42 filter paper and measured by a Segmented Flow Analyzer (SAN++, Skalar).

Plant sampling and analysis

The pasture was harvested on day 45 (4th April 2018) by cutting a strip of 4 x 0.7m (2.8 m^2) within each plot, leaving 5 cm of stubble on the ground. Pasture dry matter (DM) was determined by oven-drying the subsamples at 60 °C until constant weight

(Rayment and Higginson, 1992). The dried samples were then ground and passed through 1 mm sieves for analysis of total N to calculate pasture N uptake and NUE. Shoot total N was determined by the Dumas combustion method (LECO-Trumac). The shoot N uptake and NUE were calculated using the equations below:

$$Shoot\ N\ uptake\ (kg\ N\ ha^{-1}) = \frac{\text{Shoot DM}\ (kg\ \ ha^{-1})\ x\ N\ concentration(\%)\ in\ plant\ shoots}{100} \qquad [1]$$

$$\text{NUE}\ (\%) = \frac{\text{NUf} - \text{NU0}}{N\ rate}\ X\ 100 \qquad [\ 2]$$

where NUf and NU_0 are the shoot N uptake in the treatments with and without N fertilizer, respectively (Wang et al., 2019).

Soil DNA extraction

Soil DNA was extracted from a 0.25 g soil sample collected on days 0, seven, and 45, using the MoBio PowerSoil™ DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA USA) following the manufacturer’s instructions with slight modifications where a fast prep beating system (Bio-101 Vista CA, USA) at a speed of 5.5 ms^{-1} for 30s was used for the initial cell lysis step (Hu et al., 2015). The DNA concentration was assessed photometrically using the NanoDrop® ND-2000c Spectrophotometer UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Quantitative PCR analysis of N–cycling functional genes

The abundance of the ureolytic and ammonia-oxidizing microbes was quantified on a Bio-Rad CFX384 optical real-time PCR detection system (Bio-Rad, Laboratories Inc., Hercules, CA, USA) using the primer sets and thermal conditions shown in Table 1.

The 20-µl reaction mixture contained 10 µl of Sensimix (Bioline Sydney, NSW Australia), 0.5 µl of each primer (10 µM), and 1 µl of template DNA. Standard curves were generated using 10-fold serial dilutions of plasmids containing correct inserts of the target genes. Melting curve analysis was performed between 72 and 94.5°C at the end of each amplification assay to evaluate the specificity of quantitative PCR (qPCR), and the amplification efficiencies for all qPCR runs ranged between 80 and 110% with R^2 of 0.99.

Statistical analysis

Data are represented as the means of three replicates. The gene copy numbers were calculated using the equation described in (Behrens et al., 2008) (see equation 3).

$$\text{No. of gene copies /volume of sample} = \frac{no.of\ gene\ copies\ per\ reaction\ volume\ X\ volume\ of\ extracted\ DNA\ (\mu l)}{volume\ of\ DNA\ per\ reaction\ mix\ (\mu l)\ X\ weight\ of\ sample\ used\ for\ extraction\ (g)} \quad [3]$$

Data were analysed in Minitab18 using a one-way analysis of variance (ANOVA) at $p < 0.05$ followed by the Fisher test to compare treatment means only where there was a significant effect within each sampling day. Pearson's correlation was performed to assess the correlation between the soil microbial communities and soil physical and chemical properties.

2.0. Results

Temporal changes in soil pH and soil N content during the pasture growth period

Overall, soil pH ranged from 5.1 to 6.5 for the whole campaign period across all the treatments. The control plots had the highest pH (6.3 to 6.4), which remained relatively unchanged over the 45 sampling days, followed by 40 U, 40 GU, and 80 U (Fig. 1).

The pH increased in response to fertilizer application over the first seven days for 40U (from 5.9 to 6.2) and 40GU (from 5.8 to 5.9) and over the first two days for U80 (from 5.4 to 6.1). Despite the rise in pH, the pH for all the fertilizer treatments remained lower than the control ($p < 0.05$). Following the elevation in pH associated with fertiliser application, the pH started declining in all the fertilizer treatments. The greatest pH reduction occurred in the 80U treatment (by 0.9 pH units) followed by 40GU (by 0.3 pH units) and 40U (by 0.2 pH units) (Fig. 1). However, in all the fertilised treatments, the pH dropped to the starting pH by the end of the sampling time.

The urea concentration in soil responded to fertiliser application and was higher than the control for all fertilised treatments, dropping to the background levels by day five regardless of the amount of N applied or use of the inhibitor (Fig. 2 A). Compared to 40U, 40GU had higher urea concentration, but it was only significantly higher on day one with 34.45 and 47.03 (mg Kg^{-1} of soil) respectively. There was a significantly higher urea concentration in 80U compared to 40U and 40GU for the first three days (Fig. 2 A).

The addition of urea significantly increased soil NH_4^+-N concentration up to sampling day 31 (40U) and 38 (80U, 40GU) compared to the control (Fig. 2 B). The use of NBPT (40GU) significantly reduced the NH_4^+-N concentration compared to N alone (40U) from day two to, day four. The NH_4^+-N concentration generally remained lower for 40GU compared to 40U until day 38 when it became higher. Increasing the N application rate to 80 kg N ha^{-1} significantly increased soil NH_4^+-N concentration compared to that at 40 kg N ha^{-1} between day one and day seven (Fig. 2 B).

The addition of N significantly increased soil NO_3^--N concentration compared to the control from day four to 31 (Fig. 2 C). Soil NO_3^--N concentration increased after sampling day three to a peak on day 17 before declining steadily to day 38 in all fertilised treatments. The NO_3^--N concentration was lower than NH_4^+-N concentration for all fertilised treatments. The addition of NBPT did not significantly alter the NO_3^--N concentration compared to urea alone. Increasing N application rates significantly increased NO_3^--N concentrations from sampling day three onwards (Fig. 2 C).

Temporal changes in N-cycling microbial gene copy numbers

The addition of urea significantly reduced *ureC* gene copy numbers compared to the control on all sampling days (Fig. 3). Increasing the urea application rate did not significantly change *ureC* gene copy numbers. The use of NBPT significantly reduced *ureC* gene copy numbers compared to urea alone by day 7 of the sampling period (Fig.3). Pearson's correlation revealed that soil pH was positively correlated with the *ureC* gene copy numbers on day seven (p <0.05) and day 45 (p <0.01) of sampling (Table 2).

AOA and comammox *Nitrospira* clade A gene copy numbers were not significantly affected by the addition of urea, N application rate, or use of NBPT (Fig.4 A and C). AOB gene copy numbers were not significantly affected by using urea alone or with NBPT at an application rate of 40 kg N ha^{-1} but significantly increased with an increasing N application rate (Fig. 4 B).

The addition of N did not significantly increase comammox *Nitrospira* clade B gene copy numbers except for the application rate of 80kg N ha^{-1} on day seven. The use of NBPT did not influence comammox *Nitrospira* clade B gene copy numbers (Fig. 4 D).

Pearson's correlation analysis revealed that NO_3^--N concentration was positively correlated to AOB gene copy numbers on day 7 ($p < 0.01$). Soil pH was negatively correlated to the AOB gene copy numbers on sampling day seven ($p < 0.05$) and day 45 ($p < 0.01$) (Table 2).

Pasture DM yield, N uptake, and NUE

The addition of N at 40 kg ha^{-1} increased pasture DM yield significantly compared to the control. Increasing the N application rate to 80 kg ha^{-1} significantly increased pasture DM yield compared to the control and U40 treatments. The use of NBPT did not significantly change pasture DM yield relative to N alone applied at 40 kg ha^{-1} (Table 3). The addition of N at 40 kg ha^{-1} increased N uptake significantly relative to control. Increasing the N application rate to 80 kg ha^{-1} increased N uptake in the pasture compared to the control and U40 treatments. The use of NBPT did not change pasture N uptake relative to N alone applied at 40 kg ha^{-1}. Increasing the N application rate to 80 kg ha^{-1} or the use of NBPT did not significantly influence pasture NUE (Table 3).

3.0. Discussions

Temporal changes in soil pH and soil N content

The initial lower soil pH in the fertilizer treatments compared to the control indicated that the repeated applications of urea since 2014 has led to acidification, and this was more noticeable with higher inputs of urea. The pH reduction due to N-fertilizer addition results from the release of protons during nitrification, and when NH_4^+-N is taken up by crops (Geisseler and Scow, 2014; Tian and Niu, 2015). A reduction in pH with repeated fertilizer application or increasing N application rates has been reported previously (Luchibia et al., 2020a; Dai et al., 2018; Chen et al., 2019). The rapid increase in pH after the urea was applied is due to the hydrolysis of the urea granule (Sherlock et al., 1987).

The concentration of urea declined quickly in the fertilizer treatments and by day five, it had reached background levels. Although the urease enzyme activity was not measured in this experiment, it is likely in this pasture system that the high urease activity contributed to the quick decline in urea concentration (Suter et al., 2011). This rapid hydrolysis is reflected in the pH increase observed over the first 7 days. Further, this could be linked to NH_3 loss via volatilization due to this localized pH increase. Under acidic conditions, urea can also be taken up preferentially by AOB (described below) (Allison et al., 1991; Marsh et al., 2005). Other factors that might have contributed to such a quick decline in urea concentration include direct urea uptake by the crop, although if this did occur it would be a minor component (Dawar, 2011). While the concentration of urea appears to be essentially the same for the U40 and GU40 treatments, the impact of the NBPT is shown in the lower pH increase compared to the straight urea treatment, and the lower concentration of NH_4^+. The impact of the NBPT in slowing urea hydrolysis may be limited somewhat by i) the high urease activity likely to exist in this pasture system (Suter et al., 2011), and ii) the acidic conditions of the soil which could accelerate the degradation of NBPT (Engel et al., 2015; Engel et al., 2013).

The significantly lower NH_4^+-N concentration in the NBPT (40GU) treatment compared to urea alone in the initial days indicates that the NBPT is inhibiting urea hydrolysis (Silva et al., 2017; Cantarella et al., 2018).

The increase in soil NH_4^+-N concentration towards the end of the experiment in the NBPT treatment compared to urea alone could be due to the release of NH_4^+-N initially preserved due to the inhibition of the hydrolysis by NBPT as NBPT activity had been finished. It could also be linked to the lower pH in the NBPT treated soil compared to that of N alone which could influence the equilibrium between NH_3 and NH_4^+ in the soil preventing NH_3 loss as gas and increasing the NH_4^+-N concentration (Sigurdarson et al., 2018). This was confirmed by the negative correlation between soil pH and NH_4^+-N concentration on sampling days seven and 45 (p <0.05, n=12 (Table 2)), which showed that treatments with lower pH had higher NH_4^+-N concentration.

Treatment effect on N cycling microbes

The reduction of *ureC* gene copy numbers with N addition is contrary to our expectations and could be linked to the decreased soil pH observed as a result of repeated applications of urea and NBPT. This is supported by the lower *ureC* gene copy numbers in the fertilizer treatments on day 0, compared to the control (Fig. 3) which corresponded to the lower soil pH (Fig. 1). This pH effect was further confirmed by the positive correlation of *ureC* gene copy numbers with soil pH on day seven (p <0.05, n=12) and day 45 (p <0.01, n=12 (Table 2)). In line with our field results, *ureC* gene copy numbers have been shown to be higher in alkaline conditions (Fisher et al., 2017). Compared to urea alone, NBPT significantly reduced *ureC* gene copy numbers on both day seven and day 45. The delay in urea hydrolysis by NBPT has been reported to last up to 15 days (Cantarella et al., 2018; Khan et al., 2013). Therefore, the reduction in *ureC* gene copy numbers reported in this experiment may not be solely due to the NBPT inhibition of urea hydrolysis as NBPT is not expected to still be active in soil by day 45. The reduction in *ureC* gene copy numbers may be better explained by the decline in soil pH which was supported by the trend in *ureC* copy numbers of CK> 40U> 40GU (Fig.3, Fig.1). Our finding that increasing N rate did not affect *ureC* gene copy numbers contradicts previous work where an increase in *ureC* gene copy numbers was observed with increasing N (Zhang et al., 2019). However, the study of Zhang et al. (2019) was undertaken in alkaline soil (pH 7.2 to 8.4) whereas our soil was acidic (pH 5.2 to 6.3), indicating that pH had a much greater effect on *ureC* than N inputs. The *ureC* gene copy numbers have been found to be positively correlated with ureolytic activity in soils (Fisher et al., 2017; Sun et al., 2019) so the reduction in *ureC* gene copy numbers with the addition of urea compared to the control, and with urea + NBPT compared to urea alone implies that repeated application of these treatments may reduce ureolytic activity in this soil.

The increase in AOB and comammox *Nitrospira* clade B gene copy numbers with increasing N application rate (Fig. 4 B and D) indicated that these microbes were more responsive to increased nutrient availability in this soil environment compared to AOA and comammox *Nitrospira* clade A (Fig. 4 A and C). In line with our findings, other researchers have reported an increase in AOB abundance with increased N input (Jia and Conrad, 2009).

The significant positive correlation between soil NO_3^--N concentration and the AOB gene copy numbers and not AOA/comammox *Nitrospira* implied that AOB were the key players in nitrification in this soil. Soil pH has been shown to be a key player in shaping ammonia-oxidizing communities (Gubry-Rangin et al., 2010; Li et al., 2018). In this study, the abundance of AOB, but not other ammonia oxidizers, was significantly negatively correlated with soil pH, (Table 2). This could be linked to the preferential uptake of urea by ureolytic AOB under acidic conditions leading to NO_3^--N accumulation (Marsh et al., 2005). This phenomenon could explain the increased AOB gene copy numbers in the treatment with an increased N application rate due to increased substrate availability. This indicated that AOB could be favoured by high N inputs in soils that have become acidic due to repeated application of urea N.

There was no effect of NBPT on the abundance of ammonia oxidizers which is in line with the reports by other authors (Xi et al., 2017). However, our results contradict some of our previous findings where NBPT significantly reduced AOB and comammox *Nitrospira* (Luchibia et al. 2020b). We previously concluded that this was due to inhibition of urea hydrolysis by NBPT which influenced substrate availability for these ammonia oxidizers or the direct effect of NBPT on the intracellular urease within these ammonia oxidizers, influencing their growth. The reason for the lack of response of ammonia oxidizers to NBPT application on a repeated basis in this study is not clear, but it could be due to the lack of susceptibility of ammonia oxidizers to NBPT under these conditions which could be influenced by other soil or environmental conditions.

Effects on pasture DM yield, N uptake, and NUE

Pasture DM yield and N uptake increased with increased addition of N as expected. The use of NBPT did not increase pasture DM yield, N uptake, or NUE compared to N alone, which has previously been reported in pasture systems (Dougherty, 2016; Suter et al., 2013). A previous study reported an increase in pasture DM yield, NUE, and N-uptake with the use of NBPT compared to urea applied alone at two application rates in a pastoral system (Zaman et al., 2013). These authors attributed their findings to reduced urea hydrolysis and subsequent nitrification by NBPT, but we did not observe any significant contribution of NBPT to nitrification despite it having reduced NH_4^+-N concentration. The use of NBPT was also reported to increase ryegrass NUE, N uptake, and yield compared to urea alone and this was attributed to the direct absorption of urea and delayed hydrolysis (Dawar, 2010; Dawar et al., 2012). Some possible explanations for the lack of pasture yield response to NBPT in our study include: first, the initial soil mineral N level could be high such that the conserved N due to NBPT was not sufficient to improve crop yield (Cantarella et al., 2018; Li et al., 2015; Suter et al., 2013). However, the soil N level was unlikely excessive for pasture growth as there was an increase in DM yield, N uptake, and NUE with increasing N application rate. This N response has been reported by others in ryegrass systems with N rates up to 300 kg Nha^{-1} per year (Mora et al., 2007). Secondly, the N loss in the absence of NBPT could have been small, such that NBPT induced reduction in this loss was not beneficial to pasture yield. Thirdly, edaphic factors, such as soil pH, texture, moisture, and other nutrients, or environmental factors, such as temperature, may have limited the response of the pasture growth and yield to NBPT (Lam et al., 2018).

Conclusion

Our results demonstrated that the application of urea with NBPT reduced the rate of urea hydrolysis and mineral N production but had minimal effect on yield, N-uptake, and NUE. Increasing the urea application rate increased DM yield and N uptake but not NUE. The study also reported that AOB were important players in nitrification in acidic soils with a history of repeated fertilizer and NBPT application. The recommendation to use urea with NBPT needs further experimentation under different cropping, environmental, and soil conditions, to understand the possible contribution of NBPT to crop production in the long-term. It is important for future studies to quantify the effects of repeated /long term applications of urea applied with NBPT on enzyme activities. For a better understanding of the effects of N fertilization with NBPT application on pasture yield, N-uptake, NUE, and the underlying microbial mechanisms, it may need to sample in each growth season of the long-term experiments to carry out the measurements. This will contribute to instructive and informative fertilizer management recommendations in long-term experiments.

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Figure Legends

Fig. 1. soil pH during the 45-day sampling period across the four treatments: CK, control; 40U, N applied at 40 kg N ha^{-1} as urea; 40GU, NBPT treated urea applied at 40 kg N ha^{-1} as Green Urea NV®; 80U, N applied at 80 kg N ha^{-1} as urea. Error bars represent standard errors from three replicates.

Fig. 2. Soil urea (A), NH_4^+-N (B), and NO_3^--N (C), concentration during the 45-day sampling period across the four treatments: CK, control; 40U, N applied at 40 kg N ha^{-1} as urea; 40GU, NBPT treated urea applied at 40 kg N ha^{-1} as Green Urea NV®; 80U, N applied at 80 kg N ha^{-1} as urea. Error bars represent standard errors from three replicates. Note that y-axes scales differ between charts.

Fig. 3. The abundance of *ureC* genes across the four treatments: CK, control; 40U, N applied at 40 kg N ha^{-1} as urea; 40GU, NBPT treated urea applied at 40 kg N ha^{-1} as Green Urea NV®; 80U, N applied at 80 kg N ha^{-1} as urea, at three sampling days. Error bars represent the standard error of three replicates. Means that do not share a letter within a sampling day are significantly different at $p < 0.05$ level.

Fig. 4. The abundance of AOA (A), AOB (B), comammox *Nitrospira* clade A (C), and comammox *Nitrospira* clade B (D) genes across the four treatments: CK, control; U, Urea; and Green Urea NV®, applied at rate 40 kg N ha^{-1}, and urea applied at 80 kg N ha^{-1}, at three sampling days. Error bars represent the standard error of three replicates. Means that do not share a letter within a sampling day are significantly different at $p < 0.05$ level. Note that y-axes scales differ between charts. There were no significant differences

across treatments on AOA (A), and Comammox *Nitrospira* clade A (C) gene copy numbers.

Fig. 1.

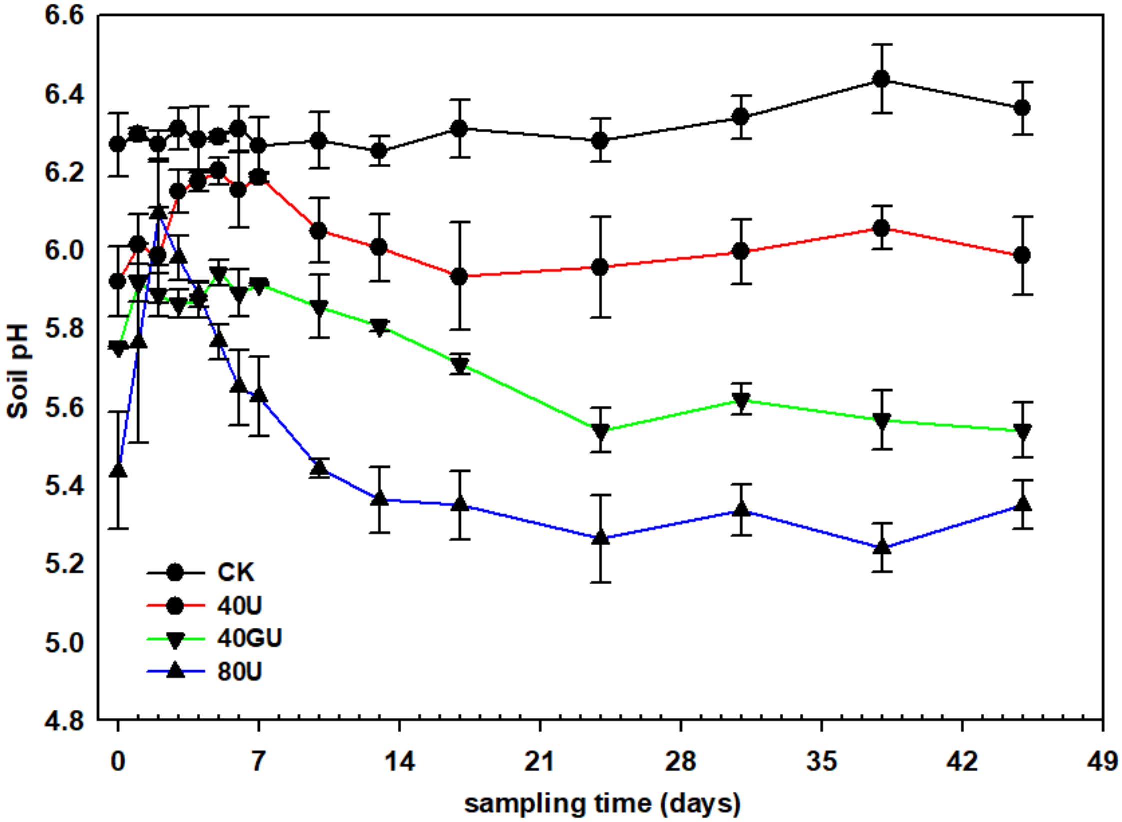

6.6
6.4
6.2
6.0
5.8
5.6
5.4
5.2
5.0
4.8
Soil pH
0
7
14
21
28
35
42
49
sampling time (days)
CK
40U
40GU
80U

Fig. 2.

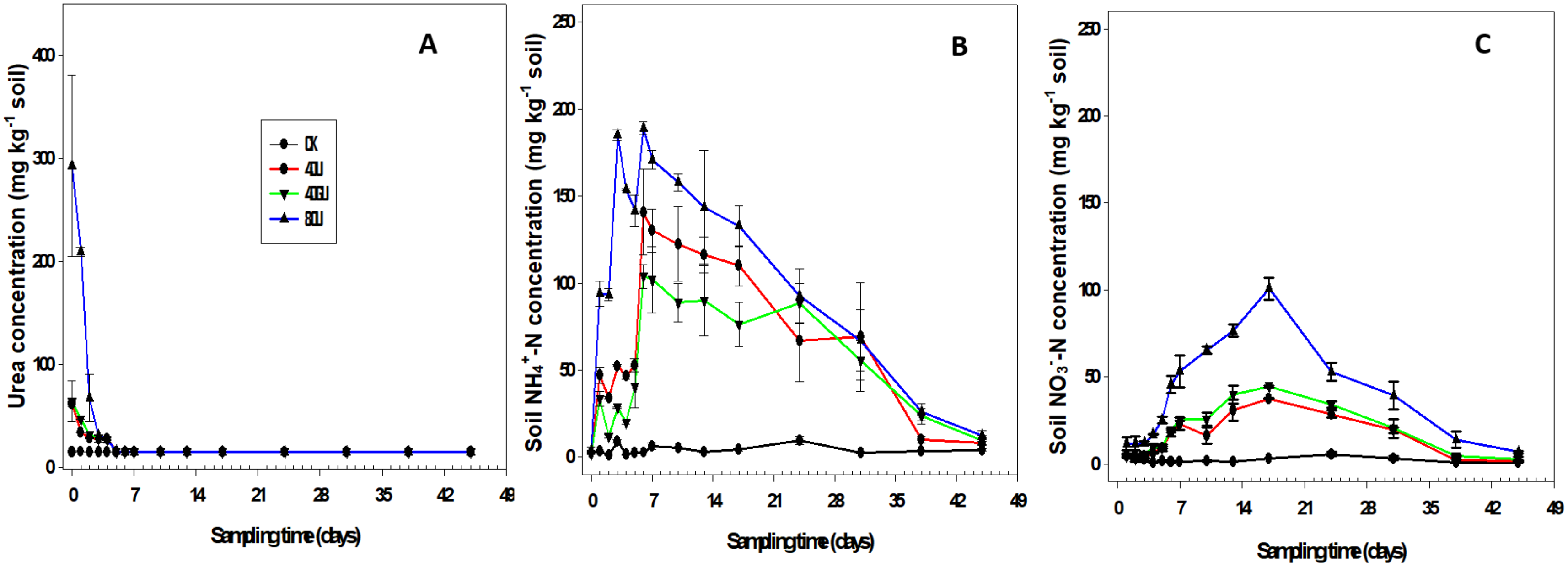

A
Urea concentration (mg kg^{-1} soil)
0
100
200
300
400
CK
40U
40GU
80U
0
7
14
21
28
35
42
49
Sampling time (days)
B
Soil NH_4^+-N concentration (mg kg^{-1} soil)
0
50
100
150
200
250
0
7
14
21
28
35
42
49
Sampling time (days)
C
Soil NO_3^--N concentration (mg kg^{-1} soil)
0
50
100
150
200
250
0
7
14
21
28
35
42
49
Sampling time (days)

Fig. 3.

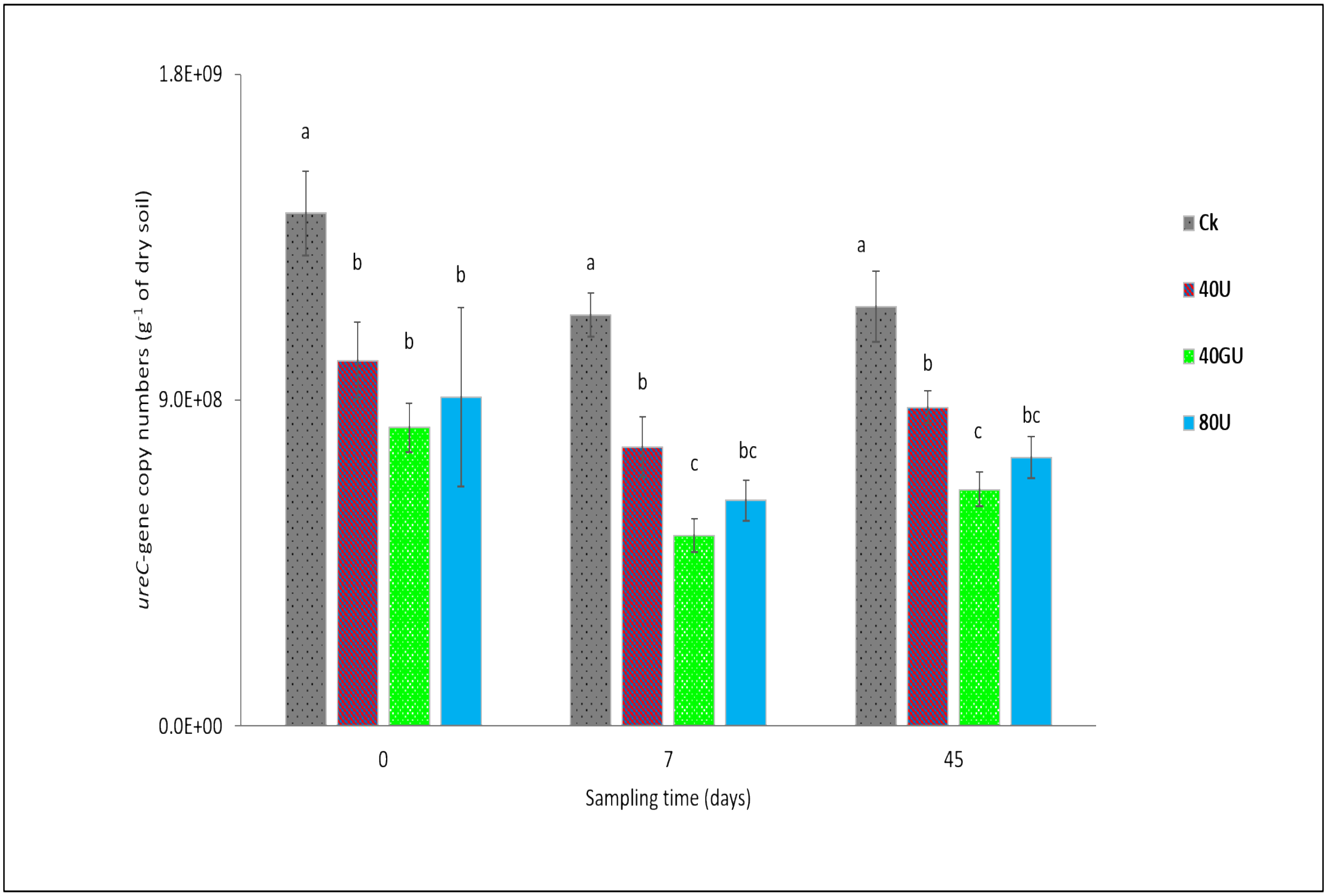
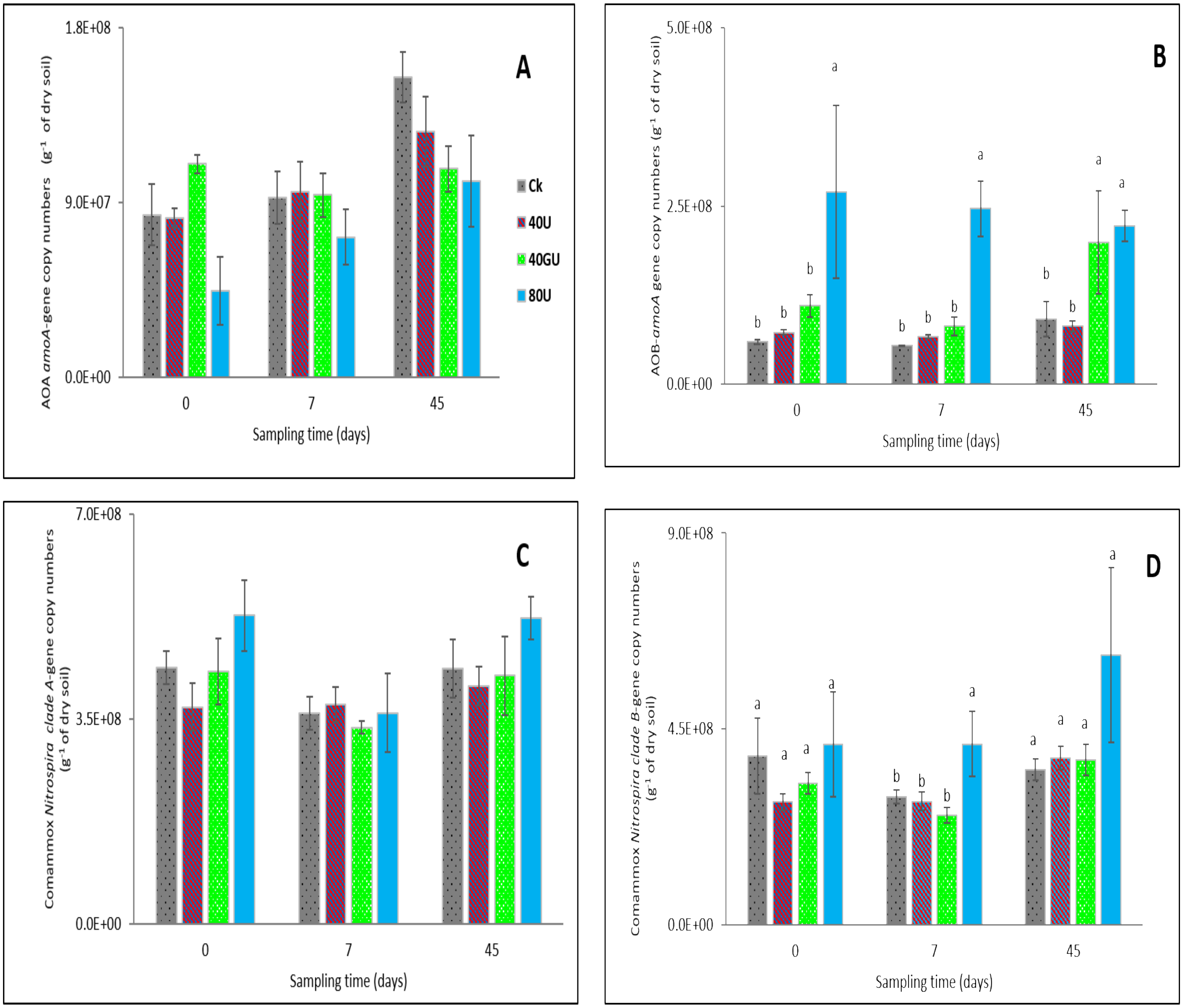

1.8E+09
9.0E+08
0.0E+00
ureC-gene copy numbers (g⁻¹ of dry soil)
a
b
b
b
a
b
c
bc
a
b
c
bc
0
7
45
Sampling time (days)
Ck
40U
40GU
80U

Fig. 4.



A
AOA *amoA*-gene copy numbers (g⁻¹ of dry soil)
1.8E+08
9.0E+07
0.0E+00
0
7
45
Sampling time (days)
Ck
40U
40GU
80U
B
AOB-*amoA* gene copy numbers (g⁻¹ of dry soil)
5.0E+08
2.5E+08
0.0E+00
b
b
b
a
b
b
b
a
b
b
a
a
0
7
45
Sampling time (days)
C
Comammox *Nitrospira clade A*-gene copy numbers (g⁻¹ of dry soil)
7.0E+08
3.5E+08
0.0E+00
0
7
45
Sampling time (days)
D
Comammox *Nitrospira clade B*-gene copy numbers (g⁻¹ of dry soil)
9.0E+08
4.5E+08
0.0E+00
a
a
a
a
b
b
b
a
a
a
a
a
0
7
45
Sampling time (days)

Table Legends

Table 1. The primers and thermocycling used for different gene qPCR amplification

Table 2. Pearson's correlation between soil pH, soil mineral N and soil microbial gene abundance

Table 3. Pasture dry matter yield, N uptake, and nitrogen use efficiency (NUE)

Table 1.

Gene	Primer	Sequence	Length (bp)	Thermocycling conditions	References
ureC	L2F L2R	ATHGGYAARGCNGGNAAYCC GTBSHNCCCCARTCYTCRTG	390	10 min at 95 °C, 40 cycles of (30 s at 95 °C, 45 s at 55 °C, and 45 s at 72 °C), 10 min at 72°C.	(Gresham et al., 2007)
AOA *amoA*	CrenamoA-23f CrenamoA-616r	ATGGTCTGGCTWAGACG GCCATC CATCTGTATGTCCA	629	10 min at 95 °C, 40 cycles of (30 s at 95 °C, 45 s at 55 °C, and 45 s at 72 °C), 10 min at 72°C.	(Tourna et al., 2008)
AOB *amoA*	amoA- 1F amoA-2R	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	491	10 min at 95 °C, 40 cycles of (30 s at 95 °C, 30 s at 56 °C, and 30 s at 72 °C), 10 min at 72°C.	(Rotthauwe et al., 1997)
Comammox *Nitrospira* clade A	comaA-244F comaA-659R	TAYAAYTGGGTSAAYTA ARATCATSGTGCTRTG	415	10 min at 95°C, 25 cycles of (30 s at 94 °C, 45s at 42-52 °C, and 60s at 72 °C), 10 min at 72°C.	(Pjevac et al., 2017)
Comammox *Nitrospira* clade B	comaA-244F comaA-659R	TAYAAYTGGGTSAAYTA ARATCATSGTGCTRTG	415	10 min at 95°C, 25 cycles of (30 s at 94 °C, 45s at 42-52 °C, and 60s at 72 °C), 10 min at 72°C.	(Pjevac et al., 2017)

Table 2.

	Soil pH			NO_3^--N		
	day 0	day7	day 45	day 0	day7	day 45
NH_4^+-N	0.44	-0.67*	-0.62*			
NO_3^--N	-0.64**	-0.91**	-0.67**			
ureC	0.4	0.66**	0.75**			
AOA	0.47	0.27	0.4	-0.30	-0.17	-0.53
AOB	-0.45	-0.77**	-0.73**	-0.19	0.76**	0.51
comammox *Nitrospira* clade A	-0.29	-0.13	-0.22	-0.27	0.24	0.22
comammox *Nitrospira* clade B	-0.22	-0.32	-0.47	0.05	0.30	0.56

**significant at the 0.01 probability level.

*Significant at the 0.05 probability level.

Table 3.

Treatment	Pasture dry matter yield (kg ha^{-1})	Shoot N uptake (kg ha^{-1})	NUE (%)
CK	1696.6±136.5 c	32.6±2.7 c	-
40U	2333.1±112.7 b	53.1±5.8 b	51.8±16.4a
40GU	2661.5±196.5 b	60.8±6.7 b	71.2±10.9a
80U	3564.5±51.8 a	88.4±1.4 a	70.0±5.1a

Treatments: CK, control; 40U, N applied at 40 kg N ha^{-1} as urea; 40GU, NBPT treated urea applied at 40 kg N ha^{-1} as Green Urea; 80U, N applied at 80 kg N ha^{-1} as urea. Values are mean (± standard error) (N = 3). Values within the same column followed by different letters are significantly different at $p < 0.05$ level.

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