

1 **Title page**

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

4 Authors: Aineah Obed Luchibia <sup>a\*</sup>, Helen Suter<sup>a</sup>, Shu Kee Lam<sup>a</sup>, Lee Menhenett<sup>b</sup>, Ji-  
5 Zheng He<sup>a\*</sup>

6 <sup>a</sup> *School of Agriculture and Food, Faculty of Veterinary and Agricultural Sciences,*  
7 *The University of Melbourne, Victoria 3010, Australia.*

8 <sup>b</sup> *Incitec Pivot fertilizers, Leigh Creek, Victoria, Australia*

9 *\*Corresponding authors: Aineah Obed Luchibia or Ji-Zheng He*

10 *E-mail: ineahobed@gmail.com or jizheng.he@unimelb.edu.au*

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12 **Abstract**

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

33 Keywords: Green Urea NV<sup>®</sup>, NBPT, Nitrogen use efficiency, comammox *Nitrospira*,  
34 Ammonia- oxidizing archaea, Ammonia- oxidizing bacteria.

35 **1.0. Introduction**

36 By 2050, the world population is expected to increase by 2-3 billion (DESA, 2017;  
37 Foley, 2011). This implies an expected increase in demand for agricultural land and N  
38 fertilizers (Tilman et al., 2001). Nitrogen is an essential plant nutrient that contributes  
39 to increased crop yields (Chen et al., 2008; Giannoulis et al., 2020). However, much of  
40 the applied fertilizer nitrogen (N) is lost from the plant-soil system through ammonia  
41 ( $\text{NH}_3$ ) volatilization and nitrous oxide ( $\text{N}_2\text{O}$ ) emissions, nitrate ( $\text{NO}_3^-$ ) leaching, and  
42 surface run-off (Castaldelli et al., 2019; Chen et al., 2008; Fuertes-Mendizábal et al.,  
43 2019), causing adverse environmental and economic consequences (Galloway et al.,  
44 2008; Stark and Richards, 2008).

45 The N cycling process is largely microbially mediated. For instance, once urea fertilizer  
46 is applied to the soil, it quickly undergoes hydrolysis, which is catalysed by the urease  
47 enzyme, producing ammonium ( $\text{NH}_4^+$ ) or ammonia ( $\text{NH}_3$ ) depending on pH (Singh et  
48 al., 2008). The *ureC* gene that encodes the alpha subunit of the urease enzyme has  
49 been used to identify and study the functional ureolytic microbial communities (Wang  
50 et al., 2018). The  $\text{NH}_3$  then undergoes oxidation to nitrite ( $\text{NO}_2^-$ ) by ammonia-oxidizing  
51 archaea (AOA) or ammonia-oxidizing bacteria (AOB) using the ammonia  
52 monooxygenase (AMO) enzyme encoded by the *amoA* gene. The  $\text{NO}_2^-$  is then  
53 oxidized to  $\text{NO}_3^-$  by nitrite-oxidizing bacteria (NOB) (Caceres et al., 2018; Hayden et  
54 al., 2014; Kits et al., 2017). Recently, comammox organisms in the *Nitrospira* were  
55 discovered with the ability to undertake complete nitrification, oxidizing ammonia to  
56  $\text{NO}_3^-$  (Daims et al., 2015; van Kessel et al., 2015). However, the contribution of  
57 comammox organisms to nitrification in many soils is not yet investigated.

58 Urea is the most widely used form of N globally due to its high N content (46%) and  
59 ease of handling (Chen et al., 2008; Glibert et al., 2006). However, high N losses have  
60 been reported from urea compared to other forms of N due to urea hydrolysis and the  
61 associated elevation of soil pH (Schwenke, 2014). Urease inhibitors, such as N-(n-  
62 butyl) thiophosphoric triamide (NBPT), have been developed to minimize N loss by  
63 inhibiting the urease enzyme activity, delaying urea hydrolysis, and preventing the  
64 elevated pH that drives NH<sub>3</sub> loss (Akiyama et al., 2010; Harty et al., 2016). By inhibiting  
65 urea hydrolysis, NBPT slows the formation of NH<sub>4</sub><sup>+</sup>, a precursor for nitrification, and  
66 therefore could indirectly also influence nitrification and subsequent denitrification  
67 (Menendez et al., 2009).

68 Several studies at field and laboratory levels have reported the effect of NBPT on  
69 reducing hydrolysis rates and associated NH<sub>3</sub> loss, as well as its contribution to  
70 reducing the emissions of N<sub>2</sub>O (Salazar et al., 2014; Silva et al., 2017). However, few  
71 studies have investigated the effect of NBPT on ureolytic, ammonia-oxidizing, or  
72 denitrifying microbes (Shi et al., 2017; Xi et al., 2017). Conflicting results exist  
73 regarding the impact of NBPT on yield and NUE, with some showing no effect (Abalos  
74 et al., 2012; Dougherty, 2016) and others reporting positive effects (Dawar, 2011;  
75 Vistoso, 2012). The effects of repeated application of urea and NBPT on soil N cycling  
76 microbial communities and yield is important as this has implications for soil health.

77 We investigated the effects of urea application rate and the use of NBPT on a) the  
78 abundance of soil N-cycling microbial communities involved in urea hydrolysis and  
79 nitrification, and b) pasture dry matter (DM) yield, N uptake, and nitrogen use efficiency  
80 (NUE). We hypothesized that: i) increasing the urea application rate would increase

81 the abundance of ureolytic and nitrification organisms, ii) application of NBPT would  
82 reduce the abundance of ureolytic and nitrifying microbes by inhibiting urea hydrolysis,  
83 and iii) use of NBPT would improve pasture DM yield, N uptake, and NUE by slowing  
84 the supply of plant-available forms of N, thereby maximizing plant uptake while  
85 reducing the risk of N loss.

## 86 **Materials and methods**

### 87 **The experimental site, soil sampling, and analysis**

88 Our field campaign commenced in mid-February 2018 on an established long-term  
89 experimental site with perennial ryegrass (*Lolium perenne* L.) pasture at Leigh Creek,  
90 Victoria (37°55'S, 143°95'E). The area has a mean annual rainfall of 686.9 mm with  
91 mean maximum and minimum annual temperatures of 17.4 °C and 7.1 °C respectively  
92 (BOM, 2019). The soil contains 27.1% clay, 26.7% silt, and 46.2 % sand in the topsoil  
93 (0-10 cm) and is classified as a Chromosol (Isbell, 2016). Prior to the commencement  
94 of our measurements, the soil properties in the 0-10 cm layer were measured and are  
95 as follows: pH<sub>water</sub> of 6.0, organic carbon of 3.0 %, NH<sub>4</sub><sup>+</sup>-N and NO<sub>3</sub><sup>-</sup>-N of 5.1 and 20  
96 mg kg<sup>-1</sup> soil respectively, cation exchange capacity of 12 cmol(+)/kg. The field site had  
97 a history of repeated surface application of urea and NBPT coated urea (Green Urea  
98 NV<sup>®</sup>) under the treatments of control (CK), granular urea (46% N) applied at 40 kg N  
99 ha<sup>-1</sup> (40U), and 80 kg N ha<sup>-1</sup> (80U), and urea coated with the urease inhibitor NBPT  
100 (as Green Urea NV<sup>®</sup> at 40 kg N ha<sup>-1</sup> (Agrotain<sup>®</sup> @ 5 L t<sup>-1</sup> urea)) (40GU). The proportion  
101 of NBPT in the GU was 0.14% N on a weight basis. The long-term experiment was  
102 established using a completely randomized block design with each treatment

103 replicated three times. The first application of treatments occurred on 5<sup>th</sup> October 2014,  
104 and fertilizer was then applied after every pasture cut with about eight to 10 fertilization  
105 events per year.

106 On 19<sup>th</sup> February 2018, our measurement campaign commenced with the plots (4 x 1  
107 m) receiving the treatments outlined above after pasture had been harvested to a  
108 height of 5 cm. Irrigation was applied on day 0 (19<sup>th</sup> February 2018), day two, and day  
109 17 after fertilizer application. Soil samples were collected on days 0 (1-3 hours), 1, 2,  
110 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  
111 cm internal diameter by taking three cores per plot and homogenizing them to form  
112 one composite sample for each plot. After sampling, the soil was transported on ice to  
113 the lab. The collected soil samples were analysed for physicochemical properties and  
114 microbial composition (stored at -20°C for DNA extraction). Gravimetric water content  
115 was determined by oven drying sub-samples at 105 °C for 24 h. Five grams of soil  
116 were used for soil pH determination on a ratio of 1:5 (weight/volume, soil: water) with  
117 a pH meter (Mettler Toledo Switzerland). Soil urea, NH<sub>4</sub><sup>+</sup>-N, and NO<sub>3</sub><sup>-</sup>-N were extracted  
118 from 5 g of soil using a ratio of 1:5 (fresh soil: 2 M KCl- Phenyl Mercuric acetate (PMA),  
119 w/v) by shaking at 175 rpm for 1 h. The solution was filtered through a Whatman 42  
120 filter paper and measured by a Segmented Flow Analyzer (SAN++, Skalar).

### 121 **Plant sampling and analysis**

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

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

$$129 \text{ Shoot N uptake (kg N ha}^{-1}\text{)} = \frac{\text{Shoot DM (kg ha}^{-1}\text{)} \times \text{N concentration(\%)} \text{ in plant shoots}}{100} \quad [1]$$

$$130 \text{ NUE (\%)} = \frac{\text{NUf} - \text{NU}_0}{\text{N rate}} \times 100 \quad [2]$$

131 where NUf and NU<sub>0</sub> are the shoot N uptake in the treatments with and without N  
132 fertilizer, respectively (Wang et al., 2019).

### 133 **Soil DNA extraction**

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

### 141 **Quantitative PCR analysis of N-cycling functional genes**

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

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

## 152 **Statistical analysis**

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

$$155 \text{ No. of gene copies /volume of sample =} \\ 156 \frac{\text{no. of gene copies per reaction volume} \times \text{volume of extracted DNA } (\mu\text{l})}{\text{volume of DNA per reaction mix } (\mu\text{l}) \times \text{weight of sample used for extraction } (g)} \quad [3]$$

157 Data were analysed in Minitab18 using a one-way analysis of variance (ANOVA) at  $p$   
158  $<0.05$  followed by the Fisher test to compare treatment means only where there was a  
159 significant effect within each sampling day. Pearson's correlation was performed to  
160 assess the correlation between the soil microbial communities and soil physical and  
161 chemical properties.

## 162 **2.0. Results**

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

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

167 The pH increased in response to fertilizer application over the first seven days for 40U  
168 (from 5.9 to 6.2) and 40GU (from 5.8 to 5.9) and over the first two days for U80 (from  
169 5.4 to 6.1). Despite the rise in pH, the pH for all the fertilizer treatments remained lower  
170 than the control ( $p < 0.05$ ). Following the elevation in pH associated with fertiliser  
171 application, the pH started declining in all the fertilizer treatments. The greatest pH  
172 reduction occurred in the 80U treatment (by 0.9 pH units) followed by 40GU (by 0.3 pH  
173 units) and 40U (by 0.2 pH units) (Fig. 1). However, in all the fertilised treatments, the  
174 pH dropped to the starting pH by the end of the sampling time.

175 The urea concentration in soil responded to fertiliser application and was higher than  
176 the control for all fertilised treatments, dropping to the background levels by day five  
177 regardless of the amount of N applied or use of the inhibitor (Fig. 2 A). Compared to  
178 40U, 40GU had higher urea concentration, but it was only significantly higher on day  
179 one with 34.45 and 47.03 (mg Kg<sup>-1</sup> of soil) respectively. There was a significantly higher  
180 urea concentration in 80U compared to 40U and 40GU for the first three days (Fig. 2  
181 A).

182 The addition of urea significantly increased soil NH<sub>4</sub><sup>+</sup>-N concentration up to sampling  
183 day 31 (40U) and 38 (80U, 40GU) compared to the control (Fig. 2 B). The use of NBPT  
184 (40GU) significantly reduced the NH<sub>4</sub><sup>+</sup>-N concentration compared to N alone (40U)  
185 from day two to, day four. The NH<sub>4</sub><sup>+</sup>-N concentration generally remained lower for  
186 40GU compared to 40U until day 38 when it became higher. Increasing the N  
187 application rate to 80 kg N ha<sup>-1</sup> significantly increased soil NH<sub>4</sub><sup>+</sup>-N concentration  
188 compared to that at 40 kg N ha<sup>-1</sup> between day one and day seven (Fig. 2 B).

189 The addition of N significantly increased soil NO<sub>3</sub><sup>-</sup>-N concentration compared to the  
190 control from day four to 31 (Fig. 2 C). Soil NO<sub>3</sub><sup>-</sup>-N concentration increased after  
191 sampling day three to a peak on day 17 before declining steadily to day 38 in all  
192 fertilised treatments. The NO<sub>3</sub><sup>-</sup>-N concentration was lower than NH<sub>4</sub><sup>+</sup>-N concentration  
193 for all fertilised treatments. The addition of NBPT did not significantly alter the NO<sub>3</sub><sup>-</sup>-N  
194 concentration compared to urea alone. Increasing N application rates significantly  
195 increased NO<sub>3</sub><sup>-</sup>-N concentrations from sampling day three onwards (Fig. 2 C).

### 196 **Temporal changes in N-cycling microbial gene copy numbers**

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

204 AOA and comammox *Nitrospira* clade A gene copy numbers were not significantly  
205 affected by the addition of urea, N application rate, or use of NBPT (Fig.4 A and C).  
206 AOB gene copy numbers were not significantly affected by using urea alone or with  
207 NBPT at an application rate of 40 kg N ha<sup>-1</sup> but significantly increased with an  
208 increasing N application rate (Fig. 4 B).

209 The addition of N did not significantly increase comammox *Nitrospira* clade B gene  
210 copy numbers except for the application rate of 80kg N ha<sup>-1</sup> on day seven. The use of  
211 NBPT did not influence comammox *Nitrospira* clade B gene copy numbers (Fig. 4 D).  
212 Pearson's correlation analysis revealed that NO<sub>3</sub><sup>-</sup>-N concentration was positively  
213 correlated to AOB gene copy numbers on day 7 ( $p < 0.01$ ). Soil pH was negatively  
214 correlated to the AOB gene copy numbers on sampling day seven ( $p < 0.05$ ) and day  
215 45 ( $p < 0.01$ ) (Table 2).

### 216 **Pasture DM yield, N uptake, and NUE**

217 The addition of N at 40 kg ha<sup>-1</sup> increased pasture DM yield significantly compared to  
218 the control. Increasing the N application rate to 80 kg ha<sup>-1</sup> significantly increased  
219 pasture DM yield compared to the control and U40 treatments. The use of NBPT did  
220 not significantly change pasture DM yield relative to N alone applied at 40 kg ha<sup>-1</sup>  
221 (Table 3). The addition of N at 40 kg ha<sup>-1</sup> increased N uptake significantly relative to  
222 control. Increasing the N application rate to 80 kg ha<sup>-1</sup> increased N uptake in the  
223 pasture compared to the control and U40 treatments. The use of NBPT did not change  
224 pasture N uptake relative to N alone applied at 40 kg ha<sup>-1</sup>. Increasing the N application  
225 rate to 80 kg ha<sup>-1</sup> or the use of NBPT did not significantly influence pasture NUE (Table  
226 3).

## 227 **3.0. Discussions**

### 228 **Temporal changes in soil pH and soil N content**

229 The initial lower soil pH in the fertilizer treatments compared to the control indicated  
230 that the repeated applications of urea since 2014 has led to acidification, and this was  
231 more noticeable with higher inputs of urea. The pH reduction due to N-fertilizer addition  
232 results from the release of protons during nitrification, and when  $\text{NH}_4^+\text{-N}$  is taken up by  
233 crops (Geisseler and Scow, 2014; Tian and Niu, 2015). A reduction in pH with repeated  
234 fertilizer application or increasing N application rates has been reported previously  
235 (Luchibia et al., 2020a; Dai et al., 2018; Chen et al., 2019). The rapid increase in pH  
236 after the urea was applied is due to the hydrolysis of the urea granule (Sherlock et al.,  
237 1987).

238 The concentration of urea declined quickly in the fertilizer treatments and by day five,  
239 it had reached background levels. Although the urease enzyme activity was not  
240 measured in this experiment, it is likely in this pasture system that the high urease  
241 activity contributed to the quick decline in urea concentration (Suter et al., 2011). This  
242 rapid hydrolysis is reflected in the pH increase observed over the first 7 days. Further,  
243 this could be linked to  $\text{NH}_3$  loss via volatilization due to this localized pH increase.  
244 Under acidic conditions, urea can also be taken up preferentially by AOB (described  
245 below) (Allison et al., 1991; Marsh et al., 2005). Other factors that might have  
246 contributed to such a quick decline in urea concentration include direct urea uptake by  
247 the crop, although if this did occur it would be a minor component (Dawar, 2011). While  
248 the concentration of urea appears to be essentially the same for the U40 and GU40

249 treatments, the impact of the NBPT is shown in the lower pH increase compared to the  
250 straight urea treatment, and the lower concentration of  $\text{NH}_4^+$ . The impact of the NBPT  
251 in slowing urea hydrolysis may be limited somewhat by i) the high urease activity likely  
252 to exist in this pasture system (Suter et al., 2011), and ii) the acidic conditions of the  
253 soil which could accelerate the degradation of NBPT (Engel et al., 2015; Engel et al.,  
254 2013).

255 The significantly lower  $\text{NH}_4^+$ -N concentration in the NBPT (40GU) treatment compared  
256 to urea alone in the initial days indicates that the NBPT is inhibiting urea hydrolysis  
257 (Silva et al., 2017; Cantarella et al., 2018).

258 The increase in soil  $\text{NH}_4^+$ -N concentration towards the end of the experiment in the  
259 NBPT treatment compared to urea alone could be due to the release of  $\text{NH}_4^+$ -N initially  
260 preserved due to the inhibition of the hydrolysis by NBPT as NBPT activity had been  
261 finished. It could also be linked to the lower pH in the NBPT treated soil compared to  
262 that of N alone which could influence the equilibrium between  $\text{NH}_3$  and  $\text{NH}_4^+$  in the soil  
263 preventing  $\text{NH}_3$  loss as gas and increasing the  $\text{NH}_4^+$ -N concentration (Sigurdarson et  
264 al., 2018). This was confirmed by the negative correlation between soil pH and  $\text{NH}_4^+$ -  
265 N concentration on sampling days seven and 45 ( $p < 0.05$ ,  $n=12$  (Table 2)), which  
266 showed that treatments with lower pH had higher  $\text{NH}_4^+$ -N concentration.

### 267 **Treatment effect on N cycling microbes**

268 The reduction of *ureC* gene copy numbers with N addition is contrary to our  
269 expectations and could be linked to the decreased soil pH observed as a result of  
270 repeated applications of urea and NBPT. This is supported by the lower *ureC* gene

271 copy numbers in the fertilizer treatments on day 0, compared to the control (Fig. 3)  
272 which corresponded to the lower soil pH (Fig. 1). This pH effect was further confirmed  
273 by the positive correlation of *ureC* gene copy numbers with soil pH on day seven ( $p$   
274  $<0.05$ ,  $n=12$ ) and day 45 ( $p <0.01$ ,  $n=12$  (Table 2)). In line with our field results, *ureC*  
275 gene copy numbers have been shown to be higher in alkaline conditions (Fisher et al.,  
276 2017). Compared to urea alone, NBPT significantly reduced *ureC* gene copy numbers  
277 on both day seven and day 45. The delay in urea hydrolysis by NBPT has been  
278 reported to last up to 15 days (Cantarella et al., 2018; Khan et al., 2013). Therefore,  
279 the reduction in *ureC* gene copy numbers reported in this experiment may not be solely  
280 due to the NBPT inhibition of urea hydrolysis as NBPT is not expected to still be active  
281 in soil by day 45. The reduction in *ureC* gene copy numbers may be better explained  
282 by the decline in soil pH which was supported by the trend in *ureC* copy numbers of  
283 CK > 40U > 40GU (Fig.3, Fig.1). Our finding that increasing N rate did not affect *ureC*  
284 gene copy numbers contradicts previous work where an increase in *ureC* gene copy  
285 numbers was observed with increasing N (Zhang et al., 2019). However, the study of  
286 Zhang et al. (2019) was undertaken in alkaline soil (pH 7.2 to 8.4) whereas our soil  
287 was acidic (pH 5.2 to 6.3), indicating that pH had a much greater effect on *ureC* than  
288 N inputs. The *ureC* gene copy numbers have been found to be positively correlated  
289 with ureolytic activity in soils (Fisher et al., 2017; Sun et al., 2019) so the reduction in  
290 *ureC* gene copy numbers with the addition of urea compared to the control, and with  
291 urea + NBPT compared to urea alone implies that repeated application of these  
292 treatments may reduce ureolytic activity in this soil.

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

299 The significant positive correlation between soil NO<sub>3</sub><sup>-</sup>-N concentration and the AOB  
300 gene copy numbers and not AOA/comammox *Nitrospira* implied that AOB were the  
301 key players in nitrification in this soil. Soil pH has been shown to be a key player in  
302 shaping ammonia-oxidizing communities (Gubry-Rangin et al., 2010; Li et al., 2018).  
303 In this study, the abundance of AOB, but not other ammonia oxidizers, was significantly  
304 negatively correlated with soil pH, (Table 2). This could be linked to the preferential  
305 uptake of urea by ureolytic AOB under acidic conditions leading to NO<sub>3</sub><sup>-</sup>-N  
306 accumulation (Marsh et al., 2005). This phenomenon could explain the increased AOB  
307 gene copy numbers in the treatment with an increased N application rate due to  
308 increased substrate availability. This indicated that AOB could be favoured by high N  
309 inputs in soils that have become acidic due to repeated application of urea N.

310 There was no effect of NBPT on the abundance of ammonia oxidizers which is in line  
311 with the reports by other authors (Xi et al., 2017). However, our results contradict some  
312 of our previous findings where NBPT significantly reduced AOB and comammox  
313 *Nitrospira* (Luchibia et al. 2020b). We previously concluded that this was due to  
314 inhibition of urea hydrolysis by NBPT which influenced substrate availability for these  
315 ammonia oxidizers or the direct effect of NBPT on the intracellular urease within these

316 ammonia oxidizers, influencing their growth. The reason for the lack of response of  
317 ammonia oxidizers to NBPT application on a repeated basis in this study is not clear,  
318 but it could be due to the lack of susceptibility of ammonia oxidizers to NBPT under  
319 these conditions which could be influenced by other soil or environmental conditions.

### 320 **Effects on pasture DM yield, N uptake, and NUE**

321 Pasture DM yield and N uptake increased with increased addition of N as expected.  
322 The use of NBPT did not increase pasture DM yield, N uptake, or NUE compared to N  
323 alone, which has previously been reported in pasture systems (Dougherty, 2016; Suter  
324 et al., 2013). A previous study reported an increase in pasture DM yield, NUE, and N-  
325 uptake with the use of NBPT compared to urea applied alone at two application rates  
326 in a pastoral system (Zaman et al., 2013). These authors attributed their findings to  
327 reduced urea hydrolysis and subsequent nitrification by NBPT, but we did not observe  
328 any significant contribution of NBPT to nitrification despite it having reduced  $\text{NH}_4^+\text{-N}$   
329 concentration. The use of NBPT was also reported to increase ryegrass NUE, N  
330 uptake, and yield compared to urea alone and this was attributed to the direct  
331 absorption of urea and delayed hydrolysis (Dawar, 2010; Dawar et al., 2012). Some  
332 possible explanations for the lack of pasture yield response to NBPT in our study  
333 include: first, the initial soil mineral N level could be high such that the conserved N  
334 due to NBPT was not sufficient to improve crop yield (Cantarella et al., 2018; Li et al.,  
335 2015; Suter et al., 2013). However, the soil N level was unlikely excessive for pasture  
336 growth as there was an increase in DM yield, N uptake, and NUE with increasing N  
337 application rate. This N response has been reported by others in ryegrass systems  
338 with N rates up to  $300 \text{ kg N ha}^{-1}$  per year (Mora et al., 2007). Secondly, the N loss in

339 the absence of NBPT could have been small, such that NBPT induced reduction in this  
340 loss was not beneficial to pasture yield. Thirdly, edaphic factors, such as soil pH,  
341 texture, moisture, and other nutrients, or environmental factors, such as temperature,  
342 may have limited the response of the pasture growth and yield to NBPT (Lam et al.,  
343 2018).

#### 344 **Conclusion**

345 Our results demonstrated that the application of urea with NBPT reduced the rate of  
346 urea hydrolysis and mineral N production but had minimal effect on yield, N-uptake,  
347 and NUE. Increasing the urea application rate increased DM yield and N uptake but  
348 not NUE. The study also reported that AOB were important players in nitrification in  
349 acidic soils with a history of repeated fertilizer and NBPT application. The  
350 recommendation to use urea with NBPT needs further experimentation under different  
351 cropping, environmental, and soil conditions, to understand the possible contribution  
352 of NBPT to crop production in the long-term. It is important for future studies to quantify  
353 the effects of repeated /long term applications of urea applied with NBPT on enzyme  
354 activities. For a better understanding of the effects of N fertilization with NBPT  
355 application on pasture yield, N-uptake, NUE, and the underlying microbial  
356 mechanisms, it may need to sample in each growth season of the long-term  
357 experiments to carry out the measurements. This will contribute to instructive and  
358 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<sup>-1</sup> as urea; 40GU, NBPT treated urea applied at 40 kg N ha<sup>-1</sup> as Green Urea NV<sup>®</sup>; 80U, N applied at 80 kg N ha<sup>-1</sup> as urea. Error bars represent standard errors from three replicates.

**Fig. 2.** Soil urea (A), NH<sub>4</sub><sup>+</sup>-N (B), and NO<sub>3</sub><sup>-</sup>-N (C), concentration during the 45-day sampling period across the four treatments: CK, control; 40U, N applied at 40 kg N ha<sup>-1</sup> as urea; 40GU, NBPT treated urea applied at 40 kg N ha<sup>-1</sup> as Green Urea NV<sup>®</sup>; 80U, N applied at 80 kg N ha<sup>-1</sup> 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<sup>-1</sup> as urea; 40GU, NBPT treated urea applied at 40 kg N ha<sup>-1</sup> as Green Urea NV<sup>®</sup>; 80U, N applied at 80 kg N ha<sup>-1</sup> 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<sup>®</sup>, applied at rate 40 kg N ha<sup>-1</sup>, and urea applied at 80 kg N ha<sup>-1</sup>, 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.

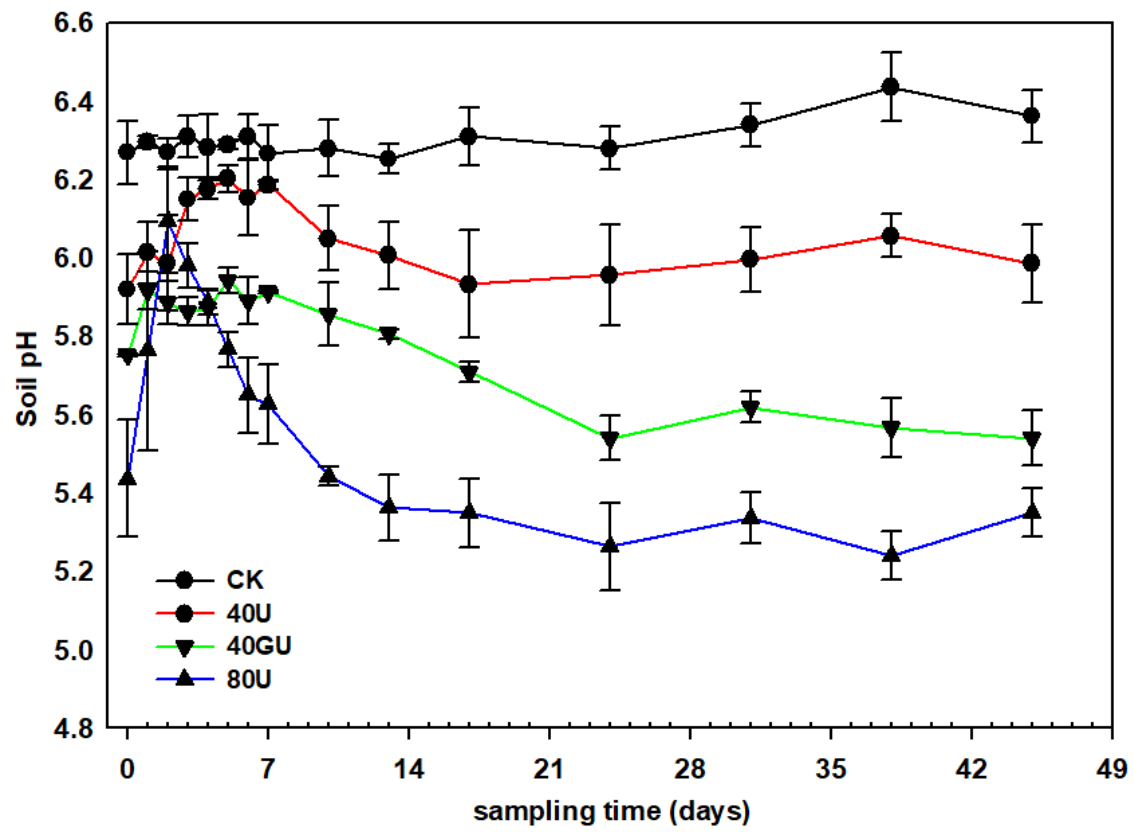
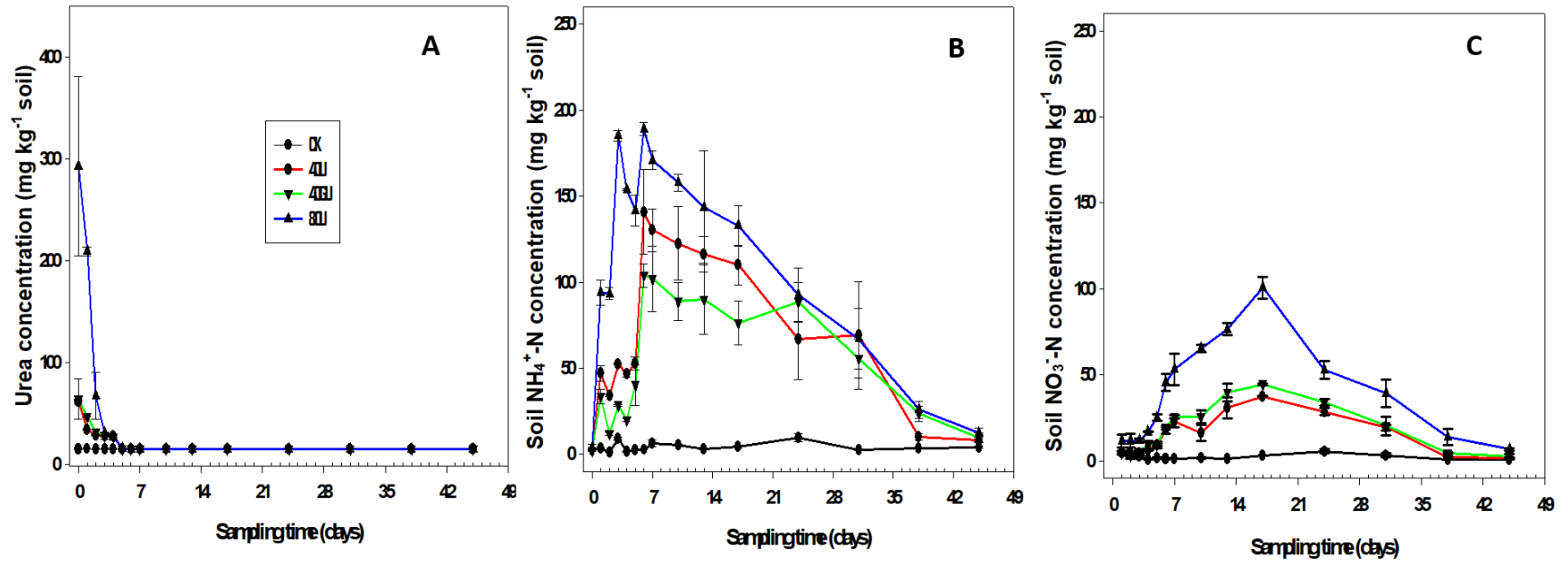
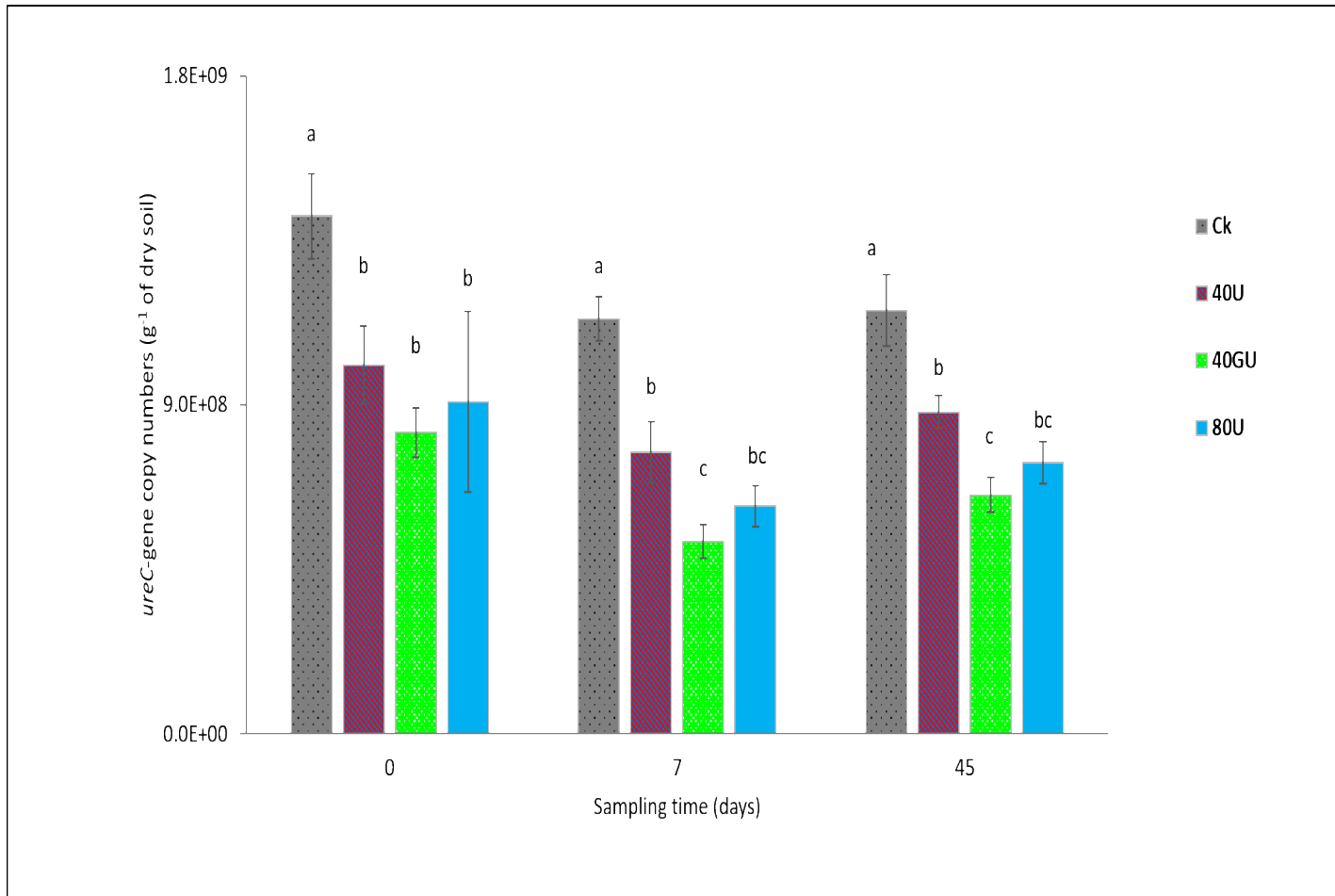


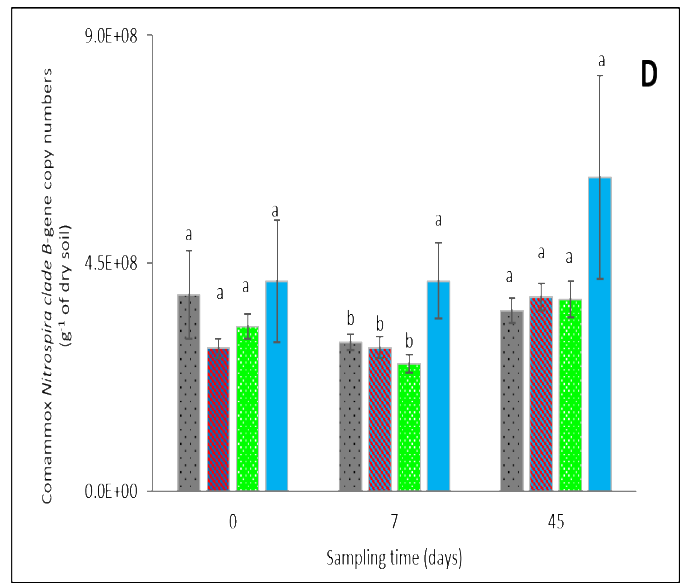
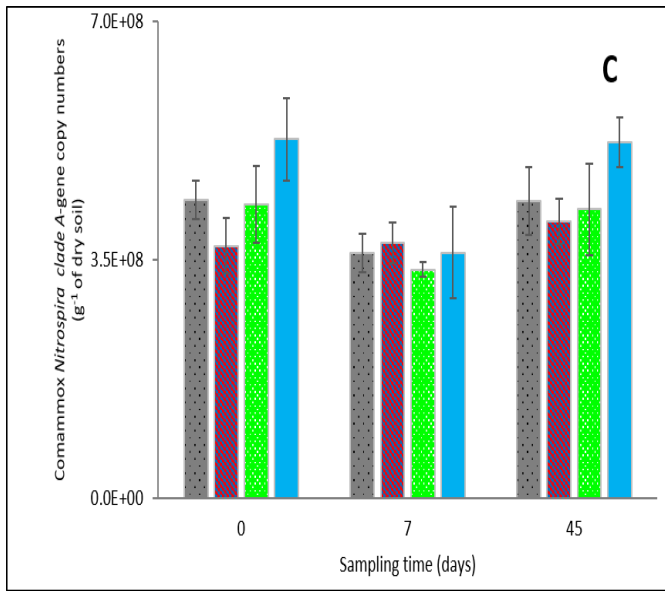
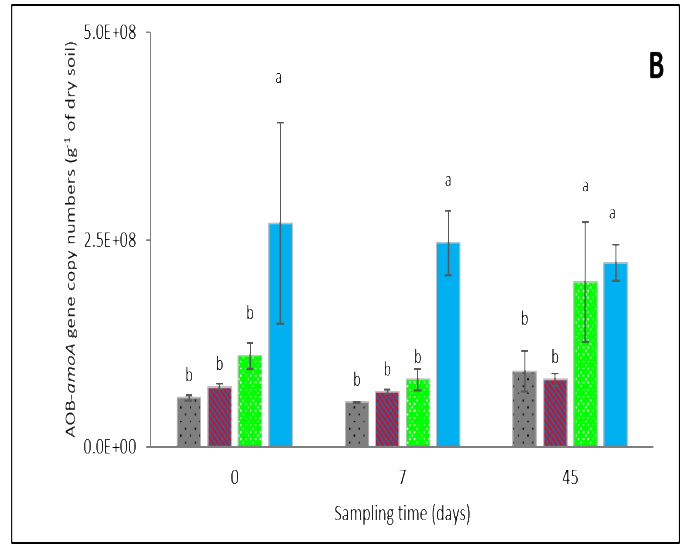
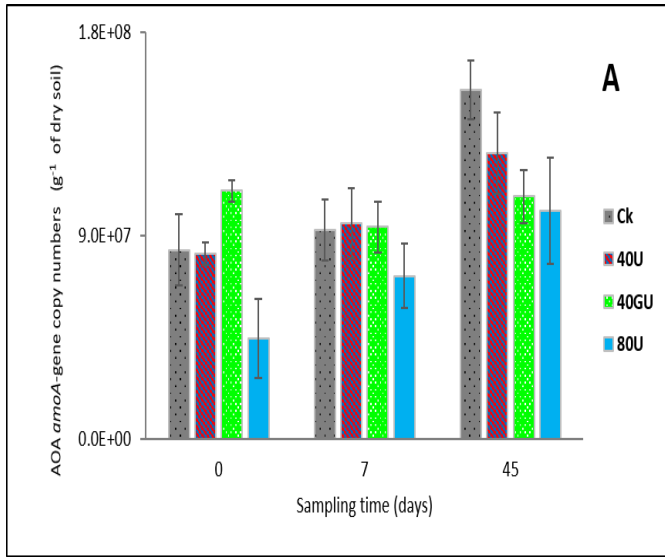
Fig. 2.



**Fig. 3.**



**Fig. 4.**



## **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
<i>ureC</i>	L2F	ATHGGYAARGCNGGNAAYCC	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)
	L2R	GTBSHNCCCCARTCYTCRTG			
AOA <i>amoA</i>	CrenamoA-23f	ATGGTCTGGCTWAGACG	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)
	CrenamoA-616r	GCCATC CATCTGTATGTCCA			
AOB <i>amoA</i>	amoA- 1F	GGGGTTTCTACTGGTGGT	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)
	amoA-2R	CCCCTCKGSAAAGCCTTCTTC			
Comammox <i>Nitrospira</i> clade A	comaA-244F	TAYAAYTGGGTSAAAYTA	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)
	comaA-659R	ARATCATSGTGCTRTG			
Comammox <i>Nitrospira</i> clade B	comaA-244F	TAYAAYTGGGTSAAAYTA	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)
	comaA-659R	ARATCATSGTGCTRTG			

**Table 2.**

	Soil pH			NO <sub>3</sub> <sup>-</sup> -N		
	day 0	day7	day 45	day 0	day7	day 45
NH <sub>4</sub> <sup>+</sup> -N	0.44	-0.67*	-0.62*			
NO <sub>3</sub> <sup>-</sup> -N	-0.64**	-0.91**	-0.67**			
<i>ureC</i>	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 <i>Nitrospira</i> clade A	-0.29	-0.13	-0.22	-0.27	0.24	0.22
comammox <i>Nitrospira</i> 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 <sup>-1</sup> )	Shoot N uptake (kg ha <sup>-1</sup> )	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<sup>-1</sup> as urea; 40GU, NBPT treated urea applied at 40 kg N ha<sup>-1</sup> as Green Urea; 80U, N applied at 80 kg N ha<sup>-1</sup> 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.

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