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Title page

Title: **Effects of repeated applications of urea with DMPP on ammonia oxidizers, denitrifiers, and non-targeted microbial communities of an agricultural soil in Queensland, Australia**

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Abstract

Nitrification inhibitors have been reported to reduce nitrous oxide emission and nitrate leaching in agricultural systems. The effects of repeated applications of urea alone or in combination with nitrification inhibitors on nitrogen (N) cycling microbes involved in nitrification and denitrification together with non-targeted microbes are not well understood. Therefore, the objective of this study was to investigate the effects of repeated application of urea and DMPP on soil physio-chemistry, ammonia oxidizers and total bacteria in the soil. We collected soil samples from a 4.5-year field experiment under crop rotation with repeated application of seven treatments, namely control (CK), Urea (U), Urea + DMPP (UE) applied at 40, 80 and 120 kg N ha^{-1}, each treatment with three replicates. Ammonia-oxidizing bacteria (AOB) gene copy numbers increased as the N application rate increased (from 0 to 120 kg N ha^{-1}). The use of DMPP significantly reduced AOB and *nirK* gene copy numbers compared to urea alone at an application rate of 120 kg N ha^{-1}. There was no treatment effect on the abundance of ammonia-oxidizing archaea (AOA), Comammox clade A and B, *nosZ* and bacterial *16S rRNA* genes. The community composition of AOB and AOA changed with N addition and use of DMPP but increasing N addition rate changed the composition of AOB only. Addition of N increased potential nitrification rates at 80 and 120 kg N ha^{-1}. There was no significant treatment effect on the relative abundance of bacteria at the phylum level. This experiment demonstrated that the application of N (with or without DMPP) at rates lower than 120kg N ha^{-1} would not result in significant impacts on soil archaeal and bacterial ecology.

Keywords: 3, 4-dimethylpyrazole phosphate (DMPP). Ammonia-oxidizing archaea. Ammonia-oxidizing bacteria. Comammox. Bacterial community.

1.0. Introduction

Soil microorganisms are important players in nutrient transformations and maintenance of soil functions (Aislabie et al., 2013; Brevik et al., 2015; Bei et al., 2018). However, they are highly sensitive to agricultural management practices like applications of fertilizers (Eo et al., 2016; Shen et al., 2016; Chen et al., 2019) and can be used as indicators for soil quality (Sharma et al., 2010).

High input of nitrogen (N) fertilizers has been used to increase crop yields (Duan et al., 2014). However, there is low crop nitrogen use efficiency because applied N fertilizer can be lost through ammonia (NH_3) volatilization, nitrous oxide (N_2O) emissions, nitrate (NO_3^-) leaching, erosion, and runoff processes (Chen et al., 2008; Castaldelli et al., 2019; Fuertes-Mendizábal et al., 2019). Nitrification has previously been thought to be a two-step process involving ammonia oxidation and nitrite (NO_2^-) oxidation. Ammonia oxidation to NO_2^- is the rate-limiting step of nitrification, regulated by the *amoA* gene encoding the alpha subunit of the NH_3 monooxygenase (AMO) within ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) (Gao et al., 2016; Fuertes-Mendizábal et al., 2019; Miao et al., 2019). Nitrite oxidation to NO_3^- is the second step of nitrification and is regulated by nitrite-oxidizing bacteria (NOB). Recently, a group of bacteria within the *Nitrospira* genus was discovered with the capacity to completely oxidize NH_3 to NO_3^- in a single organism (comammox *Nitrospira*) (Daims et al., 2015; van Kessel et al., 2015). Denitrification involves the conversion of NO_3^- back to N_2O and dinitrogen gas (N_2) via NO_2^-, nitric oxide (NO) (Kuypers et al., 2018; Castaldelli et al., 2019). Nitrous oxide can also be formed through nitrification by the chemical decomposition of hydroxylamine (NH_2OH) (Fuertes-Mendizábal et al., 2019). Denitrification is catalyzed by nitrate reductase encoded by the *narG* gene, nitrite reductase

encoded by *nirS / nirK* gene, nitric oxide reductase encoded by *norB* and nitrous oxide reductase encoded by the *nosZ* gene (Braker and Tiedje 2003; Shrewsbury et al., 2016).

Nitrification inhibitors are compounds that delay the oxidation of ammonium (NH_4^+) to NO_3^- thereby preventing N losses through nitrification and denitrification (Suter et al., 2016; Fuertes-Mendizábal et al., 2019). 3, 4-Dimethylpyrazole phosphate (DMPP) is one of the nitrification inhibitors that has gained commercial use (Zerulla et al., 2001).

Fertilizer application has been shown to cause short and long-term effects on soil physicochemical properties which in turn influence soil microbial communities (Shen et al., 2016; Dai et al., 2018). Although several studies have investigated the effects of repeated N fertilizer application on soil microbial communities (Geisseler and Scow 2014; Zhou et al., 2015; Shen et al., 2016), little information is available on the response of soil nitrifying, denitrifying and non-targeted microbes (i.e., who are not supposed to involve in nitrogen cycling processes) following repeated application of N fertilizers with nitrification inhibitors (Shi et al., 2017).

We measured changes in soil chemical properties, and the structure and composition of soil bacterial and N-cycling microbial functional communities, following the repeated applications of urea (U), and Urea + DMPP (UE) at different N rates for 4.5 years. The objective of this study was to investigate the effects of repeated application of urea and DMPP on soil physio-chemistry, ammonia oxidizers and total bacteria in the soil. We tested the following hypotheses to achieve our objectives: a) Repeated applications of urea and DMPP will significantly reduce soil pH and increase soil total carbon (TC) and total nitrogen (TN); and b) The levels of

soil TC, TN, and pH will decrease bacterial composition and increase AOB and *nirK* gene copy numbers in the soil. This study will contribute to our understanding of how agricultural N management practices influence soil microbial ecosystems and their biological functions.

2.0. Materials and methods

2.1. Site description and experimental design

Colonsay experimental site (27°28'S 151°23'E) is situated in the Formartin district of the Darling Downs, southern Queensland. Southern Queensland, Australia. The soil in the area was a grey vertisol (Soil Survey Staff, 2014). The soil was classified as clay with 30.1% sand, 11.4 % silt and 58.5 % clay content. The area receives an average annual rainfall of 530.8 mm (2013-2018, Bureau of meteorology, 2019).

The experiment was established in 1985. Since 2013 when DMPP treated urea (ENTEC®) was introduced into this experiment, the site has been under rainfed production with a crop rotation of wheat (*Triticum aestivum* L.), cotton (*Gossypium hirsutum* L.), grain sorghum (*Sorghum bicolor* (L.) *Moench*), mungbean (*Vigna radiata*) and barley (*Hordeum vulgare* L.) in that order. The experiment had a completely randomized block design with three replicates that had 7 treatments including 3 nitrogen rates (40, 80, and 120 kg N ha^{-1}) with (as ENTEC®) or without DMPP, and the control (no nitrogen, no DMPP), established on 13*2 m^2 plots. Nitrogen was applied as urea. Urea + DMPP treatments were applied as fertilizer brand ENTEC® (Incitec Pivot), granulated urea containing <0.15% DMPP (based on DMPP: urea N). Before each cropping season, treatments were applied in the pre-plant season, i.e., on 10th May 2013 prior to wheat sowing; on 22nd July 2014 prior to cotton sowing; 10th August 2015 prior to grain sorghum; 30th June 2016 prior to

mungbean sowing; and on 7th June 2017 prior to the barley sowing. The plots also received 10 kg P ha^{-1} as triple superphosphate applied at the planting date of each season (in the seed trench in contact with seed). An estimated starting soil water of approximately 65mm was recorded from the on-site rain gauge on 6th June 2017 before barley sowing. Barley was sown on 32cm rows at a rate of 65 kg ha^{-1} to a depth of approximately 6.5 cm on 7th June 2017, using minimum tillage Janke parallelogram coulter disc, spear point tines, and press wheel assemblies on small plot equipment. Five rows of barley spaced 32cm apart were sown between 2m wheel centers across each plot. Post-emergence herbicides were applied on 14th July 2017. The barley crop was harvested on 23rd November 2017. The total growing season rainfall from planting to harvest was 153mm

2.2. Soil sampling

Following harvesting, soil samples were collected on 30th November 2017 from the treatment plots at a depth of 0-10 cm with an auger of 7.6 cm internal diameter by taking 5 cores from the plant line or stubble row per plot and homogenized to form one composite sample for each plot. After sampling, the soil was transported on ice to the lab. A five (5) g subsample from each soil sample was taken for potential nitrification rate (PNR) measurement (extraction was done within 1 week of sampling), and 500 g of soil for physiochemical analysis. Subsamples stored at -20°C immediately and used for soil DNA extraction and molecular analyses.

2.3. Analysis of soil chemical properties

Soil pH was determined at a ratio of 1:5 (weight/volume, soil: water) with a pH meter (Mettler Toledo Switzerland). Soil total carbon (TC) and total nitrogen (TN) were analyzed on the elemental analyzer (Leco Trumac CN) using the Dumas digestion

method. Ammonium and NO_3^--N concentration were extracted at a ratio of 1:5 (w:v, 5 g fresh soil: 25 ml 2M KCl). The extracts were filtered through Whatman paper (42), after shaking for one (1) hour at 175 rpm followed by calorimetric analysis using a segmented flow analyser (Skalar SAN ++).

2.4. Potential nitrification rates measurement

Soil potential nitrification rates (PNR) were determined according to the chlorate inhibition method (Hu et al., 2015). Briefly, fresh soil samples (5 g) were placed in 50-mL falcon tubes with 20 ml ammonium sulfate (1 mM). Potassium chlorate with a final concentration of 10mM was added to the tubes to inhibit nitrite oxidation. The falcon tubes were covered with parafilm with small holes for aeration and incubated in the dark at 25°C for 24 h, and then nitrite was extracted with 10 ml of 2M KCl. The supernatant was measured by spectrophotometry at a wavelength of 540nm with N-(1-naphthyl) ethylenediamine and sulfonic acid).

2.5. Soil DNA extraction

DNA was extracted from 0.25 g of each individual soil sample 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 30 s 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).

2.6. Quantitative PCR analysis of N–cycling functional genes

Abundances of the nitrifying, denitrifying and *16S rRNA* genes were 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.

159 The 10-µl reaction mixture contained 5 µl of Sensimix (Bioline Sydney, NSW
160 Australia), 0.25 µl of each primer (10 µM), and 1 µl of template DNA. Standard
161 curves were generated using 10-fold serial dilutions of plasmids containing correct
162 inserts of the target genes. Melting curve analysis was performed between 72 and
163 94.5°C at the end of each amplification assay to evaluate the specificity of
164 quantitative PCR (qPCR), and the amplification efficiencies for all qPCR runs ranged
165 between 80 and 110% with R^2 of 0.99.

166 **2.7. Terminal restriction fragment length polymorphism (T-RFLP) analysis**

167 T-RFLP analysis of the ammonia-oxidizing microbes was performed on extracted
168 DNA using targeted marker gene PCR amplification separately focusing on the
169 *amoA* genes AOA, and AOB using the fluorescently labeled primers (Hu et al.,
170 2015).

171 A 25 µl PCR reaction mixture contained 2 µl of diluted template DNA (1– 10 ng), 0.5
172 µl of each primer (10 µM), 5 µl MyTaq buffer, 1.5 U of MyTaq polymerase (Bioline,
173 Sydney, Australia). The PCR reaction was conducted using the primer sets and
174 thermal cycling conditions shown in Table 1. The PCR products were purified using
175 the Wizard SV Gel and PCR Clean-Up System (Promega, San Luis Obispo, CA,
176 USA) and quantified using the NanoDrop ND-2000c Spectrophotometer. The
177 restriction digestion was carried out in a 10 µl mixture containing 200 ng of purified
178 PCR products, 0.1 µl of BSA,1 µl of ×10 NE buffer, and 5U of the restriction enzymes
179 MspI for AOB; RsaI (BioLabs, Sydney, Australia) for AOA. The digests were
180 incubated at 37°C for 3 h and denatured for 10 min at 95°C. Terminal restriction
181 fragments (TRFs) were size separated with an ABI PRISM 3500 Genetic Analyzer
182 (Applied Biosystems, CA, USA) and analyzed using a local southern size calling
183 method (peaks >50 bp) and a peak amplitude threshold setting of 50, using

GeneMapper version 4.0 (Applied Biosystems). TRFs with peak height comprising less than 1 % of the total peak height were removed, and peaks that differed by less than 1bp were combined into the same TRF (Hu et al., 2015). The relative fluorescent abundances of all TRFs were exported for further analysis of the community composition.

2.8. Illumina sequencing and analysis of *16S rRNA* gene

The V_4 region of bacterial *16S rRNA* was selected for amplification with primers 515F (5□-GTGCCAGCMGCCGCGGTAA-3□) and 806R (5□-GGACTACVSGGGTATCTAAT-3□) (Caporaso et al., 2011) with Illumina adapter overhang sequences attached. The reaction was carried out in 25 µl mixtures containing 12.5 µl red mix, 0.5 µl each primer and 2 µl template. After the initial enzyme activation for 10 min at 95 °C, 30 cycles of the following program were used for amplification (20 s at 95 °C, 20 s at 55 °C, and 20 s at 72 °C), 5 min at 72°C, on the Bio-Rad C1000 Touch thermal cycler (Bio-Rad, Laboratories Inc., Hercules, CA, USA). The success of the PCR was confirmed by 2% gel electrophoresis and cleaned using the Wizard SV Gel and PCR Clean-Up System (Promega, San Luis Obispo, CA, USA). A second PCR was conducted using a 50 µl reaction volume containing 10.5 µl red mix, 5 µl each of index 1 and 2, and 5 µl of the cleaned PCR product as template DNA. The second PCR was conducted under the conditions of 10 min at 95 °C, 8 cycles of (20 s at 95 °C, 20 s at 55 °C, and 20 s at 72 °C), 5 min at 72°C. The PCR success was again confirmed on a 2 % agarose gel and PCR products purified again using the Wizard SV Gel and PCR Clean-Up System (Promega, San Luis Obispo, CA, USA). The final library was made by mixing all PCR products

in equimolar ratios and quantified using the JetSeq library quantification Lo-ROX Kit (Bioline) then sequenced on an Illumina MiSeq sequencer. Samples were rarefied to a sequence depth of 4806 to ensure 3 replicates per sample and correction of the differences due to sequencing efforts before downstream analysis.

2.9. Statistical analysis

Data are represented as the means of three replicates. Sequence analysis was done using Quantitative Insights Into Microbial Ecology (QIIME) software (Caporaso et al., 2010). The gene copy numbers were calculated using the equation described in (Behrens et al., 2008). Data were analysed using analysis of variance (ANOVA) at $p<0.05$ followed by the Fisher test to compare treatment means only if there was a significant effect as shown by ANOVA in Minitab 18 statistical software. Pearson's correlation was performed to assess the correlation between the soil microbial communities and soil physicochemical properties.

3.0. Results

3.1. Effects of repeated urea with DMPP applications on soil chemical properties and potential nitrification rates

Repeated applications of urea with or without DMPP changed soil pH, TN, TC, NH_4^+-N, NO_3^--N and PNR ($p<0.05$) (Table 2). Soil pH ranged from 9.08 to 7.83. Addition of N significantly reduced soil pH relative to control. The use of DMPP did not affect pH change with N alone addition except when N was applied at higher rates (120 kg Nha^{-1}). Nitrogen addition significantly increased TN compared to Control. Increasing N application rate and use of DMPP had no effect ($p<0.05$) on TN except when N was applied at 120 kg N ha^{-1}. The addition of N significantly increased TC and NH_4^+-

N levels compared to control but there was no significant effect of N application rate and use of DMPP on the levels of TC and NH_4^+-N. Nitrate concentration did not change between urea and urea + DMPP treatments except at the application rate of 120 kg N ha^{-1}. Addition of N did not change PNR except at the application rate of 80 and 120 kg N ha^{-1}. Use of DMPP did not change PNR at any application rate relative to urea applied alone.

Overall, repeated applications of N with DMPP significantly reduced soil pH and increased soil nutrient status. Higher application rates increased PNR.

3.2. Effects of repeated urea with DMPP applications on ammonia oxidizers, denitrifiers, and *16S rRNA* gene copy numbers

AOB gene copy numbers did not change with N addition relative to control. Increasing N application rate to 120 kg N ha^{-1} significantly increased AOB gene copy numbers. The use of DMPP did not change AOB gene copy numbers compared to urea alone except at the application rate of 120 kg N ha^{-1} where DMPP significantly reduced AOB gene copy numbers compared to N alone (Fig. 1a). The addition of N did not change AOA, Comammox clade A and Comammox clade B genes copy numbers relative to control. Increasing the N application rate or use of DMPP did not change AOA (Fig. 1b), Comammox clade A (Fig. 1c), and Comammox clade B genes copy numbers (Fig. 1d).

AOB gene copy numbers were significantly negatively correlated with pH and positively correlated with TN, NH_4^+-N, NO_3^--N concentration, and PNR. No significant relationship was found between AOA, comammox clade A and B with soil chemical properties and PNR at the sampling time (data not shown).

255 *nirK* gene copy numbers did not change with N addition except at the application rate
256 of 120 kg N ha^{-1}. Use of DMPP did not change *nirK* gene copy numbers compared to
257 urea alone except at the application rate of 120 kg N ha^{-1} (Fig. 2a). The addition of N
258 did not change *nosZ* and *16S rRNA* gene copy numbers relative to control (Fig. 2 b
259 and 2c). Increasing the N application rate or use of DMPP did not change *nosZ* and
260 *16S rRNA* gene copy numbers (Fig. 2 b and 2c).

261 These results indicated that only AOB and *nirK* genes were responsive to repeated
262 applications or N with DMPP.

263 **3.3. Effects of repeated Urea with DMPP application on community**
264 **composition of AOA and AOB ammonia oxidizers**

265 Digestion of the AOA *amoA* gene by the RsaI enzyme for TRFLP analysis produced
266 5 specific fragments of which TRFs 34bp and 36bp were the most abundant across
267 all the treatments. The addition of N reduced and increased the relative abundance
268 TRF 34bp and TRF 36bp respectively compared to control. Increasing N application
269 rate did not change the relative abundance of TRF 34bp and 36bp. The use of
270 DMPP increased the relative abundance of TRF 34bp and 36bp at all application
271 rates compared to N alone, and TRF 56bp at an application rate of 40 and 80kg N ha
272 $^{-1}$ respectively compared to N alone. (Fig. 3a).
273 Digestion of the AOB *amoA* by the MspI restriction enzyme for TRFLP analysis
274 produced 6 fragments with TRFs 37 bp and 55 bp being the most abundant. The
275 addition of N reduced the relative abundance of TRF 37bp compared to Control.
276 Increasing N application reduced the relative abundance of TRF 37bp. The use of
277 DMPP increased the relative abundance of TRF 37 bp compared to when N was
278 applied alone at all application rates. The addition of N increased the relative

abundance of TRF 55bp compared to control. The use of DMPP reduced the relative abundance of TRF 55bp compared to N alone at all application rates. High rates of N increased the number of fragments (Fig. 3b).

3.4. Effects of repeated urea with DMPP applications on soil bacterial community composition

The bacterial community within all treatments at Colonsay produced 189,692 sequences (5101-13627 sequences per sample). A total of 33 phyla, 379 families and 612 genera were shared among the 7 treatments. At the phylum level, the microbial community was composed of 10 main phyla that were most abundant across the treatments (with >1% mean in relative abundance in at least one treatment) (Fig. 4). The most abundant phyla included Actinobacteria which occupied about 25.2-27.6%, and *Proteobacteria* with 23.0-26.4% of the total bacterial sequences, followed by *Acidobacteria* (15.0-19.6%), *Chloroflexi* (9.3-11.2%) *Planctomycetes* (6.0-6.7%), *Crenarcheota* (3.2-4.1%), *Verrucomicrobia* (3.1-3.6%), *Bacteroidetes* (4.1-4.6%), *Gemmatimonadetes* (2.2-2.7%), *Nitrospirae* (1.1-1.7%) (Fig.4). There were no significant treatment effects on the relative abundance of soil microbial communities at the phylum level (Fig. 4). However, analysis at the class level in the abundant phyla revealed some significant treatment effects. At the class level, the relative abundance of *Actinobacteria and Thermoleophilia* classes of phylum *Actinobacteria* were significantly influenced by the N application compared to CK (Table 3). The addition of N did not change the relative abundance of *Actinobacteria* class except at the application rate of 80 and 120 kg N ha^{-1} relative to control (Table 3). Increasing the N application rate did not change the relative abundance of *Actinobacteria* class except at the application rate of 120 kg N ha^{-1}. Use of DMPP did not change the relative abundance of *Actinobacteria* class

304 compared to N alone except at the application rate of 120 kg N ha^{-1} (Table 3). The
305 relative abundance of *Thermoleophilia* class (of *Actinobacteria* Phylum) was
306 significantly reduced by N addition compared to control (Table 3). Increasing N
307 addition did not significantly change the relative abundance of *Thermoleophilia* class
308 except at an application rate of 120 kg N ha^{-1}. Use of DMPP did not significantly
309 change the relative abundance of *Thermoleophilia* class except at an application rate
310 of 120 kg N ha^{-1} (Table 3). Class *TK10* (*Chloroflexi* phylum) was significantly
311 reduced by the application of N compared to control (Table 3). There was no
312 significant effect of increasing N application and use of DMPP on the relative
313 abundance of *TK10* class members relative to urea (Table 3).

314 The relative abundance of *Proteobacteria* phylum was negatively correlated with pH
315 ($p<0.05$) and positively correlated with NO_3^--N concentration ($p<0.05$) (Table 4).
316 *Acidobacteria* phylum was negatively correlated to NO_3^--N concentration ($p<0.05$). At
317 the class level, the relative abundance *Actinobacteria* was negatively correlated to
318 pH ($p<0.01$), and positively correlated to TN ($p<0.01$) and NO_3^--N concentration
319 ($p<0.01$). The relative abundance of *Thermoleophilia* class was positively correlated
320 to pH ($p<0.01$) and NH_4^+-N concentration ($p<0.05$), but negatively correlated to TN
321 ($p<0.01$), NO_3^--N concentration and TC ($p<0.05$). *TK10* class (of *Chloroflexi* phylum)
322 was positively correlated with pH and NH_4^+-N concentration but negatively correlated
323 with TN ($p<0.01$) (Table 4).

324 Overall, repeated applications of N and DMPP did not influence bacterial
325 composition at the phylum level but resulted in such changes at higher resolution
326 taxonomic levels within some members of *Actinobacteria* and *Chloflexi* phyla. These

changes in bacterial composition were controlled by soil pH, TN, NH_4^+-N, and NO_3^--N concentration.

4.0. Discussion.

4.1. Effects of repeated urea with DMPP on soil physicochemical properties

The reduction in soil pH with N addition increased N application rate, and the use of DMPP indicated that repeated application of N and DMPP could lead to soil acidification. A reduction in soil pH due to repeated fertilizer application (Guo et al., 2010; Schroder et al., 2011; Dai et al., 2018) or increasing N application rate in repeated fertilizer application experiments (Zhou et al., 2015; Shen et al., 2016; Chen et al., 2019) has been reported. The significant increase in soil pH level by DMPP compared to N alone at a rate of 120 kg N ha^{-1}, indicated the ability of DMPP to counter the extent of pH reduction by N as reported by other authors (Shi et al., 2017).

Application of N alone or with DMPP increased TN, TC, NH_4^+-N, and NO_3^--N and concentration compared to CK. An increase in NO_3^--N, and NH_4^+-N concentration due to increasing N application rate in repeated experiments have been reported (Zhou et al., 2015; Shen et al., 2016; Chen et al., 2019). The significant increase in NO_3^--N at only 120 kg N ha^{-1} could imply that the N applied at the rate of 120 kg N ha^{-1} was in excess of the plant requirement as compared to other N rates therefore, there was N build up in the system in form of NO_3^--N.

The significant low NO_3^--N in DMPP treatment at 120 kg N ha^{-1} compared to N alone at the same rate indicated that although nitrification continued after the DMPP efficacy period, the NO_3^--N remained lower in DMPP treatment compared to urea

alone. However, this may not be because of DMPP since sampling was done 5 months after treatment application, which was longer compared to the reported 100 days of conservation of mineral N by DMPP (Duncan et al., 2017). Future experiments should include quantifying of the inhibitor compounds to ascertain their longevity at field level as this was not done in the current experiment.

4.2. Effects of repeated urea with DMPP on the N-cycling functional groups

Ammonia oxidizers are key players to N cycling, involved in the first and rate-limiting step of nitrification (Carey et al., 2016). Our results generally indicated that AOB are more responsive to increased N addition, or changes in soil pH, TN, and NO_3^- accumulation compared to AOA, (O'Callaghan et al., 2010; Carey et al., 2016; Ouyang et al., 2018) and Comammox. Further, a significant correlation between NO_3^--N concentration and PNR with AOB gene copy numbers (data not shown) implied that AOB could have a greater influence on nitrification in repeated fertilizer applied soils (nutrient-rich soils) than AOA and Comammox. Therefore, the significant difference between AOB gene copy numbers in N alone and N + DMPP at a rate of 120 kg N ha^{-1} would indicate the ability of DMPP to inhibit nitrification by suppressing the growth of AOB genes under repeated application of N and N + DMPP. Other researchers reported similar findings and showed an increase in AOB gene copy numbers due to repeated application of urea alone or a decrease when urea was applied with DMPP (Shi et al., 2017). However, in our case, the results were seen only at a higher application rate of 120 kg N ha^{-1}, which could be linked to the fact that, at this application rate, the N was more than plant needs as explained above. This would imply that at this rate, there was more N than the plant needed resulting in more N build up in the system in the form of NO_3^--N. DMPP prevented NH_3 oxidation by inhibiting the activity of AOB.

375 *nirK* genes have been reported to be contained in AOB and are responsible for the
376 nitrifier denitrification process, therefore, the *nirK* genes amplified in this study could
377 be contained in AOB (Shaw et al., 2006; Cantera and Stein, 2007; Di et al., 2014).
378 This finding was evidenced by the similar trends of AOB and *nirK* gene copy
379 numbers at an application rate of 120 kg N ha^{-1} and the fact that only *nirK*
380 abundance was influenced by treatment application but not *nosZ* genes.

381 **4.3. The effect of repeated urea with DMPP application on soil bacterial**
382 **communities**

383 The lack of significant treatment effects of N addition, application rate and DMPP on
384 the relative abundance of soil total bacteria at the phylum level would suggest that
385 repeated application of N with or without DMPP was not detrimental to soil bacterial
386 composition.

387 Although no significant treatment effect on the relative abundance of soil bacteria
388 was reflected at the phylum level, correlation analysis revealed that soil NO_3^--N
389 concentration was significantly negatively correlated with the relative abundance of
390 *Acidobacteria* phylum (Table 4). Our results are in line with the report of inhibition of
391 *Acidobacteria* by N application from a recent review (Dai et al., 2018), which
392 supports the theory that considers *Acidobacteria* to be oligotrophic (Fierer et al.,
393 2007; Eilers et al., 2010; Fierer et al., 2012).

394 The significant negative and positive correlation of soil pH and NO_3-N concentration
395 respectively with the relative abundance of *Proteobacteria* phylum was in line with
396 reports by other researchers that *Proteobacteria* prefers nutrient-rich soils (Fierer et
397 al., 2012; Zhou et al., 2015; Dai et al., 2018). This is because the increase in N
398 addition in our experiment significantly reduced soil pH.

399 At lower taxonomic levels, we found significant treatment effects on the composition
400 of some members of the major phyla (Table 3). The increase in the relative
401 abundance of the members of *Actinobacteria* phyla with an increase in N application
402 rates is in line with the previous reports that classified *Actinobacteria* as fast-growing
403 Copiotrophs stimulated in high nutrient environments (Fierer et al., 2007, 2012).
404 These results were also confirmed with the significant positive correlation of the
405 relative abundance of *Actinobacteria* class with TN, and NO_3^--N (Table 4).

406 The response of *Thermoleophilia* class and *Actinobacteria* class was different
407 although they are both members of *Actinobacteria* phylum. This indicated that the
408 response to environmental disturbances by the same members of a given taxon can
409 be different (Zeng, 2016). This could also be attributed to the specific responses of
410 different subgroups to different soil chemical properties (Eo et al., 2016). In this
411 study, for example, correlation analysis showed that the relative abundance of
412 *Thermoleophilia* class correlated to soil pH, TN, TC, NH_4^+-N, and NO_3^-N in the
413 opposite way to that of *Actinobacteria* class. Other researchers have reported such
414 trends for different lower taxa of other phyla (Eo et al., 2016; Zeng, 2016). Class
415 *TK10* of *Chloroflexi* phylum decreased when N was added with or without DMPP
416 application regardless of the application rate. The negative correlation of the relative
417 abundance of this subgroup with TN concentration and NO_3^--N further indicated the
418 effect of N addition on *Chloroflexi*. Our findings are in line with the reports of a
419 decrease in the relative abundance of the members of the *Chloroflexi* phylum with
420 the addition of N fertilizer by other researchers (Fierer et al., 2012; Zhou et al., 2015;
421 Eo et al., 2016). The reduction of *Chloroflexi* taxa has been speculated to be due to
422 competition from the bacteria that have been stimulated under high N, or due to the
423 changed soil chemical properties (Eo et al., 2016).

It is expected that changes realized at lower taxonomic levels would be reflected at higher taxonomic levels (Zeng, 2016). However, the lack of reflection of these changes at higher levels, despite their presence at lower levels of some taxa, could be due to the lower relative abundance of the changed groups. For instance, at the phylum level, no significant changes were reflected in the *Chloroflexi* and *Actinobacteria* although some of their members at lower levels were significantly influenced by the treatments.

5.0. Conclusion

It can be concluded that the relatively long term between the treatment application and the sampling date was a key player affecting the soil microbial community composition and size, as NIs and N fertilizer effects on soil physio-chemistry and microbial communities are strongest shortly after application. Therefore care should be taken while interpreting our results as the focus and our findings differ from those of short-term experiments.

This study revealed that when applied at low N rates of 40 kg N ha-1, urea, and DMPP application did not pose negative effects to soil ecosystems. However, at higher application rates of 80 and 120 kg N ha-1, urea and DMPP altered soil bacterial composition at higher resolution taxonomic levels. The application rate of N was shown to be an important factor that contributed to shifting composition, size, and function of N cycling microbial communities (particularly AOB) in repeated applications. For sustainable long-term maintenance of soil microbial composition and function, lower fertilizer rates of 40 kg N ha-1 should be encouraged in repeated N application experiments. This study has improved our understanding that the application of urea and DMPP at lower rates can be used without negatively affecting microbial ecology.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends

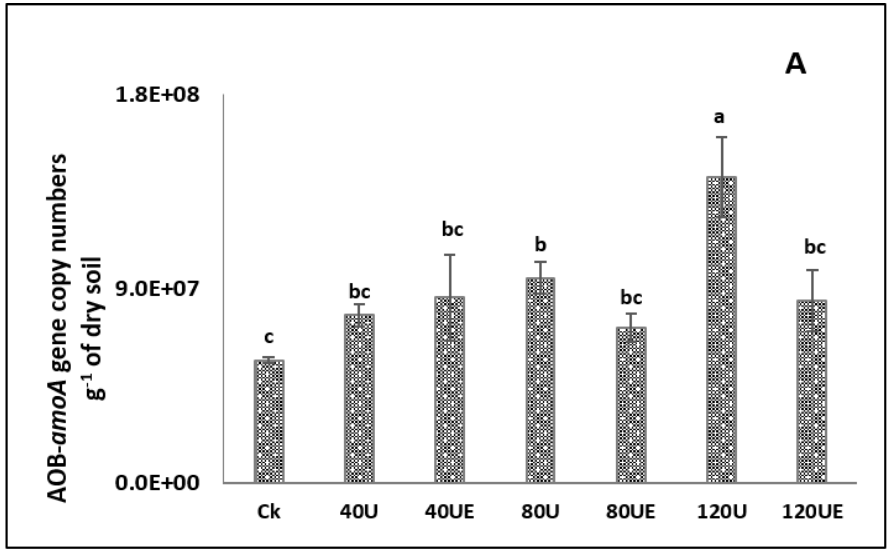
Figure 1. The abundance of AOB (A), AOA (B), Comammox clade A (C) and Comammox clade B (D) genes across the seven treatments: CK, control; U, Urea; and UE, Urea + DMPP applied at rates 40, 80 and 120 kg N ha^{-1}, at Colonsay. Error bars represent standard error of three replicates. Means that do not share a letter are significantly different at $p< 0.05$ level (Fisher Test). Note that y-axes scales differ between charts**.** There were no significant differences across treatments on AOA (A), Comammox clade A (C) and clade B (D) gene copy numbers.

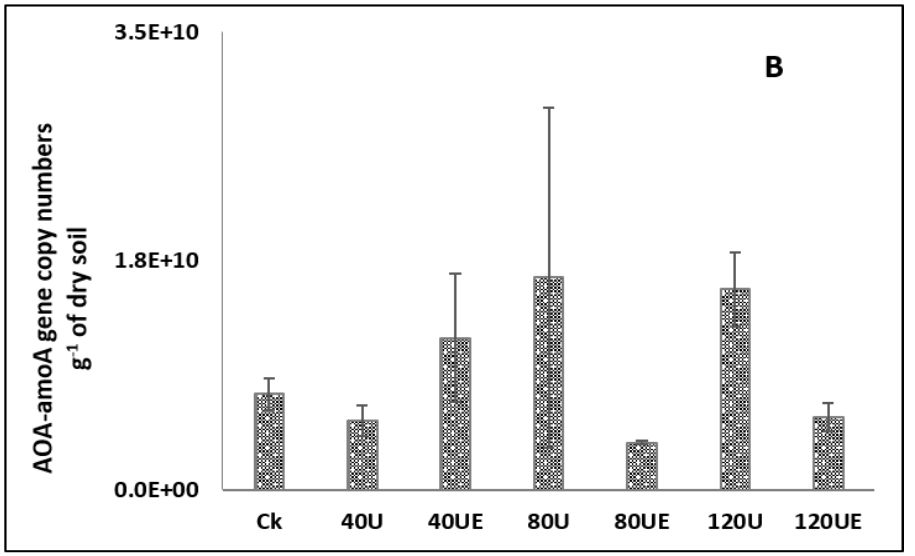
Figure 2. The abundance of *nirK* (A), *nosZ* (B), and bacterial *16S rRNA* (C) genes across the seven treatments: CK, control; U, Urea; and UE, Urea + DMPP applied at rates 40, 80 and 120 kg N ha^{-1}, at Colonsay. Error bars represent standard error of three replicates. Means that do not share a letter are significantly different at $p< 0.05$ level (Fisher Test). Note that y-axes scales differ between charts**.** There were no significant differences across treatments on *nosZ*, *16S rRNA* gene copy numbers.

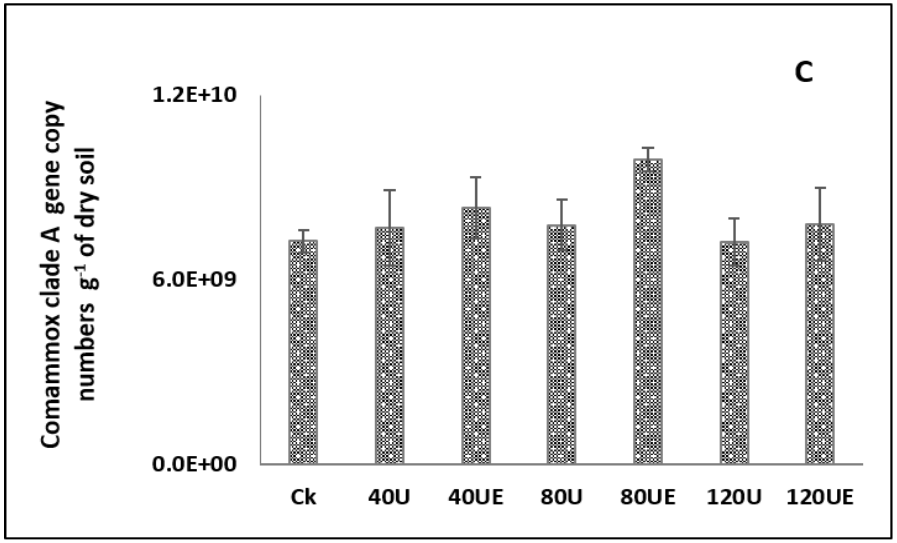
Figure 3. Terminal restriction fragment length polymorphism (T-RFLP) fingerprints of the AOA *amoA* gene (A) digested using the RsaI enzyme and the AOB *amoA* gene (B) digested using the MspI enzyme across the seven treatments**:** CK, control; U, Urea; and UE, Urea + DMPP applied at rates 40, 80 and 120 kg N ha^{-1}**,** at Colonsay.

Figure 4. Relative abundance of the dominant phyla (with abundance > 1% in at least one treatment, only identified sequences classified under a specific taxon were considered) across the seven treatments**;** CK, control; U, Urea and UE, Urea + DMPP applied at rates 40, 80 and 120 kg N ha^{-1}) at Colonsay**.**

Figure 1.


A
AOB-amoA gene copy numbers g-1 of dry soil
1.8E+08
9.0E+07
0.0E+00
c
bc
bc
b
bc
a
bc
Ck
40U
40UE
80U
80UE
120U
120UE


B
AOA-amoA gene copy numbers g-1 of dry soil
3.5E+10
1.8E+10
0.0E+00
Ck
40U
40UE
80U
80UE
120U
120UE


C
Comammox clade A gene copy numbers g-1 of dry soil
1.2E+10
6.0E+09
0.0E+00
Ck
40U
40UE
80U
80UE
120U
120UE

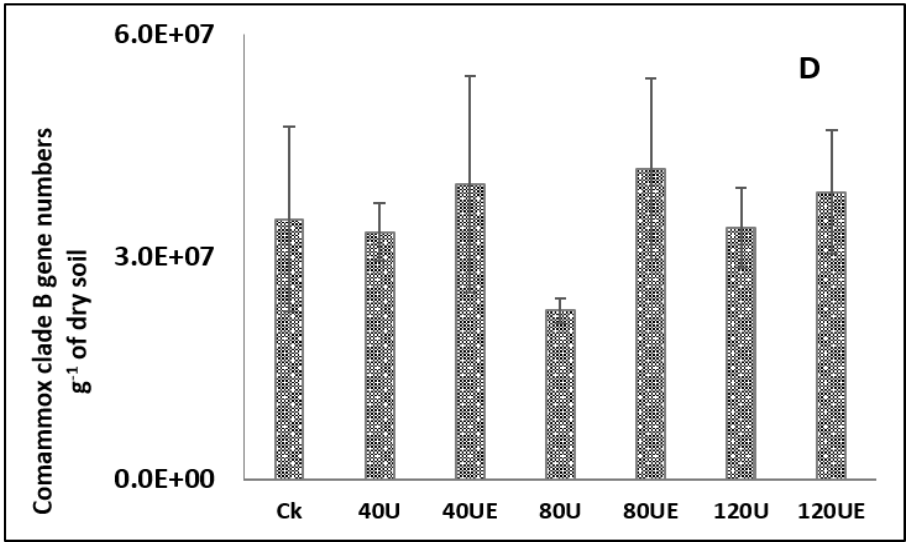

D
Comammox clade B gene numbers g-1 of dry soil
6.0E+07
3.0E+07
0.0E+00
Ck
40U
40UE
80U
80UE
120U
120UE

Figure 2.

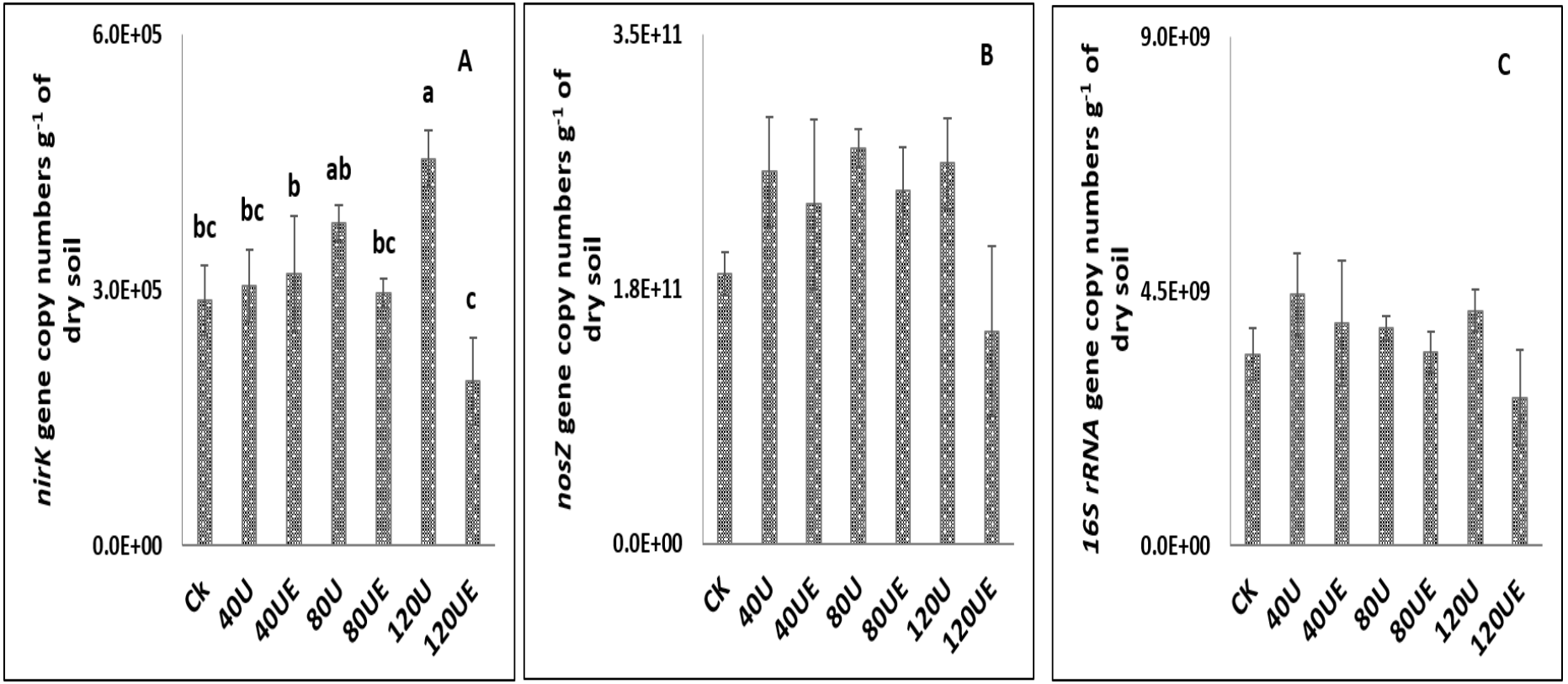

6.0E+05
3.0E+05
0.0E+00
nirK gene copy numbers g-1 of dry soil
A
bc
bc
b
ab
bc
a
c
Ck
40U
40UE
80U
80UE
120U
120UE
3.5E+11
1.8E+11
0.0E+00
nosZ gene copy numbers g-1 of dry soil
B
9.0E+09
4.5E+09
0.0E+00
16S rRNA gene copy numbers g-1 of dry soil
C

Figure 3.

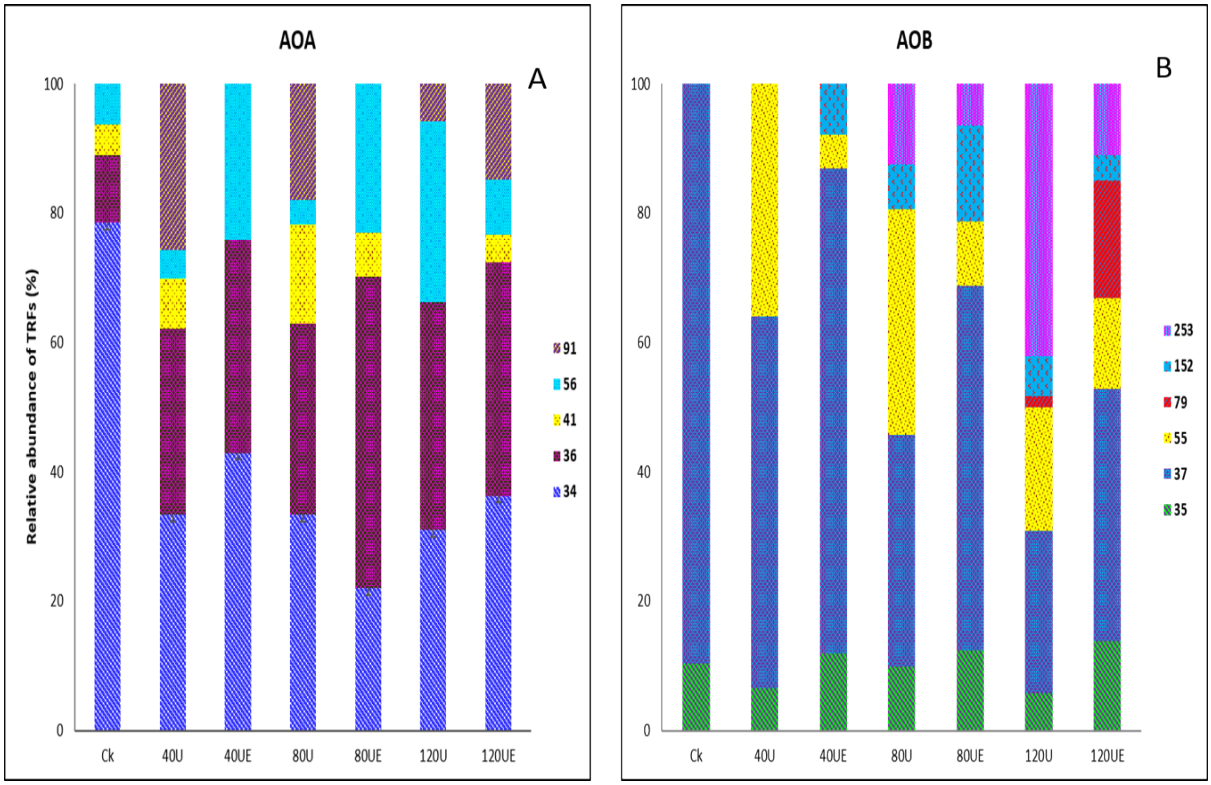

AOA
A
Relative abundance of TRFs (%)
100
80
60
40
20
0
Ck
40U
40UE
80U
80UE
120U
120UE
91
56
41
36
34
AOB
B
253
152
79
55
37
35

Figure 4.

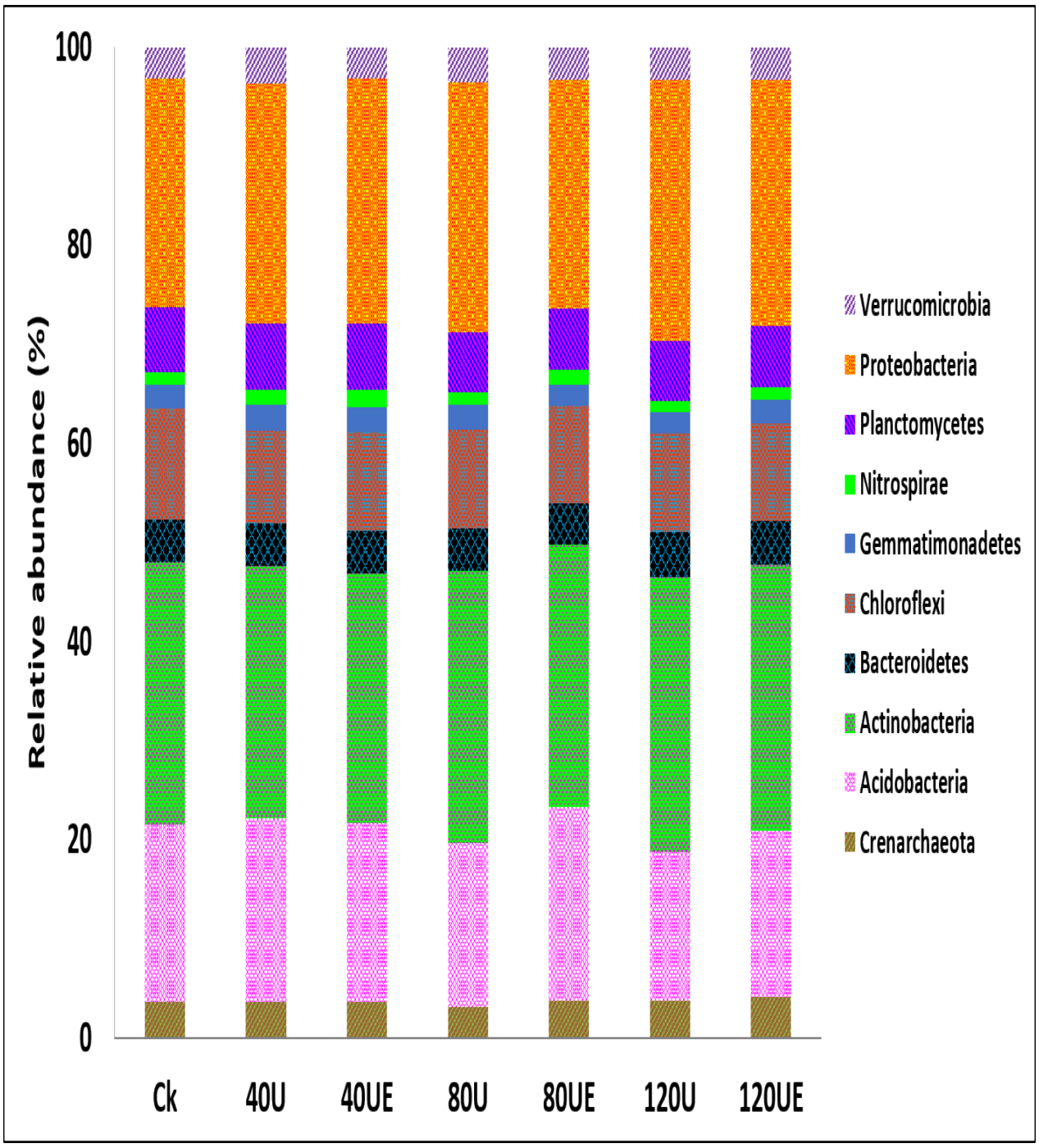

100
80
60
40
20
0
Relative abundance (%)
Ck
40U
40UE
80U
80UE
120U
120UE
Verrucomicrobia
Proteobacteria
Planctomycetes
Nitrospirae
Gemmatimonadetes
Chloroflexi
Bacteroidetes
Actinobacteria
Acidobacteria
Crenarchaeota

Table legends

Table 1. The primers and thermocycling programs used for quantification of the N-cycling functional genes and *16S rRNA* gene

Table 2. Soil chemical properties and potential nitrification rates (PNR) across the seven treatments

Table 3. The relative abundance of soil microbial communities across the seven treatments

Table 4. Pearson's correlation between soil properties and the relative abundance of soil bacterial communities at different taxonomic levels

10 **Table 1.**

Primers	Sequence	Length	References	Thermocycling conditions
		bp		
Bacterial 16 S *rRNA*				
1369F	CGGTGAATACGTTCYCGG	100	(Suzuki et al. 2000)	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.
1492R	CGGTGAATACGTTCYCGG			
AOA *amoA*			(Tourna et al. 2008)	
CrenamoA-23f	ATGGTCTGGCTWAGACG	629		
CrenamoA-616r	GCCATC CATCTGTATGTCCA			
AOB *amoA*				
amoA- 1F	GGGGTTTCTACTGGTGGT	491	(Rotthauwe et al. 1997)	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.
amoA-2R	CCCCTCKGSAAAGCCTTCTTC			
nirK				
nirK876	ATY GGC GGV CAY GGC GA	470	(Bárta et al. 2010)	
nirK1040	GCC TCG ATC AGR TTR TGG TT			
Comammox clade A				
comaA-244F	TAYAAYTGGGTSAAYTA	415	(Pjevac et al. 2017)	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.
comaA-659R	ARATCATSGTGCTRTG			
Comammox clade B				
comaB-244F	TAYTTCTGGACRTTYTA	415	(Pjevac et al. 2017)	
comaB-659R	ARATCCARACDGTGTG			
NosZ 1				10 min at 95°C, 40 cycles of (15s at 95°C, 15s at 60°C, 30s at 72°C, 15s at 82°C), 10 min at 72°C.
nosZ1F,	WCSYTGTTCMTCGACAGCCAG	259	(Henry et al. 2006)	
nosZ1R	ATGTCGATCARCTGVKCRTTYTC			

Table 2.

Treatment	CK	40U	40UE	80U	80UE	120U	120UE
pH	9.08 a†	8.59 b	8.44 b	8.21c	8.24 c	7.83 d	8.21 c
Total N (g kg^{-1})	0.80 c	0.90 b	0.90 b	0.90 b	0.90 b	1.00 a	0.90 b
Total C (g kg^{-1})	12.10 c	14.10 a	14.20 a	13.50 ab	13.10 b	13.40 ab	12.80 bc
NH_4^+–N (mg kg^{-1})	1.19 b	2.31 a	2.89 a	2.89 a	3.00 a	2.98 a	3.01 a
NO_3^-–N (mg kg^{-1})	11.42 c	12.99 c	18.79 bc	35.38 bc	32.55 bc	89.14 a	46.65 b
PNR (mg NO_2^-–N $kg^{-1}hr^{-1}$)	0.61 c	0.96 c	1.46 bc	3.50 a	2.60 ab	3.69 a	3.18 a

Treatments: CK– control; U–Urea, and UE, Urea + DMPP applied at rates 40, 80 and 120 kg N ha^{-1}. Values are means (N = 3).

†Values within the same row followed by the same letter are not significantly different at $p < 0.05$ (Fisher Test).

Table 3.

Phylum	Class	CK	40U	40UE	80U	80UE	120U	120UE
Actinobacteria		25.71 a†	24.73 a	24.48 a	26.67 a	25.74 a	27.02 a	26.05 a
	Actinobacteria	12.12 c	13.70 bc	12.99 c	16.29 ab	14.05 bc	17.62 a	14.45 bc
	Thermoleophilia	10.54 a	8.59 b	8.90 b	8.46 bc	9.37 b	7.44 c	9.15 b
Proteobacteria		22.32 a	23.62 a	24.13 a	24.45 a	22.37 a	25.66 a	24.15 a
Acidobacteria		17.96 a	18.56 a	17.98 a	16.46 a	19.57 a	15.04 a	16.76 a
Chloroflexi		10.88 a	9.04 a	9.72 a	9.71 a	9.60 a	9.61 a	9.59 a
	TK10	2.60 a	1.81 b	1.97 b	1.81 b	1.79 b	1.78 b	1.69 b
Planctomycetes		6.38 a	6.43 a	6.52 a	5.80 a	6.00 a	5.89 a	5.91 a
Crenarchaeota		3.45 a	3.46 a	3.56 a	3.05 a	3.61 a	3.57 a	4.01 a
Verrucomicrobia		3.11 a	3.52 a	3.03 a	3.44 a	3.24 a	3.16 a	3.24 a
Bacteroidetes		4.12 a	4.24 a	4.18 a	4.07 a	3.96 a	4.42 a	4.32 a
Gemmatimonadetes		2.40 a	2.60 a	2.44 a	2.43 a	2.11 a	2.15 a	2.35 a
Nitrospirae		1.24 a	1.42 a	1.69 a	1.28 a	1.40 a	1.09 a	1.21 a

Treatments: CK– control; U–Urea, and UE, Urea + DMPP applied at rates 40, 80 and 120 kg N ha^{-1}. Only for phyla with relative abundance >1% shown. Only classes affected by treatment application within the abundant phyla were included. Values are means (N = 3).

†Values within the same row followed by the same letter are not significantly different at $p< 0.05$ (Fisher Test).

20 **Table 4.**

Phylum	Class	Soil properties				
		pH (1:5$_{water}$)	Total N	Total C	NH_4^+–N	NO_3^-–N
			g kg^{-1}	g kg^{-1}	mg kg^{-1}	mg kg^{-1}
Actinobacteria		–0.30	0.22	–0.12	–0.29	0.22
	Actinobacteria	–0.63**	0.60**	0.12	–0.32	0.67**
	Thermoleophilia	0.70**	–0.81**	–0.48*	0.52*	–0.51*
Proteobacteria		–0.44*	0.40	0.16	–0.11	0.48*
Acidobacteria		0.41	–0.34	0.02	0.15	–0.50*
Chloroflexi		0.35	–0.28	–0.30	0.37	–0.15
	TK10	0.61**	–0.68**	–0.43	1**	–0.16
Planctomycetes		0.39	–0.19	0.14	0.24	–0.36
Crenarchaeota		–0.02	–0.14	–0.08	–0.15	0.02
Verrucomicrobia		–0.08	0.23	0.32	–0.23	–0.10
Bacteroidetes		–0.12	–0.04	–0.20	0.10	0.297
Gemmatimonadetes		0.19	–0.11	0.11	0.13	–0.13
Nitrospirae		0.18	–0.17	0.22	0.08	–0.24

21 **significant at the 0.01 probability level, * Significant at the 0.05 probability level. Only classes affected by treatment
22 application within the abundant phyla were included.

Conflict of interest

The authors have no conflict of interest to declare.