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SOILS, SEC 1 • SOIL ORGANIC MATTER DYNAMICS AND NUTRIENT CYCLING • RESEARCH ARTICLE

Responses of ureolytic and nitrifying microbes to urease and nitrification inhibitors in selected agricultural soils in Victoria, Australia

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Abstract

Purpose: Urease inhibitors (UIs) such as *N*-(*n*-butyl) thiophosphoric triamide (NBPT) and nitrification inhibitors (NIs) such as 3, 4-dimethylpyrazole phosphate (DMPP) have been reported to improve the efficiency of nitrogen (N) fertilizers. However, their effects on the ureolytic and ammonia-oxidizing microbes in agricultural soils are uncertain. This study aimed to investigate the effects of DMPP and NBPT on the abundance and community composition of ureolytic and nitrifying microbes in selected agricultural soils in Australia.

Materials and methods: Soil was collected from five agricultural farms and used to establish an incubation experiment. Urea, urea + NBPT, urea + DMPP, and urea + NBPT + DMPP were applied on the soils which were incubated under 25 °C and 60% water-filled pore space for 28 days. Sampling was done on different days for DNA extraction and measurement of ammonium (NH_4^+) and nitrate (NO_3^-) concentration.

Results and discussion: NBPT inhibited NH_4^+ accumulation in all the soils but had no significant effect on nitrification in any soil. DMPP alone or DMPP + NBPT significantly inhibited nitrification. The abundances of ammonia-oxidizing bacteria (AOB) and complete ammonia oxidizers (comammox *Nitrospira*), but not ammonia-oxidizing archaea (AOA), were significantly influenced by the application of NBPT, DMPP or DMPP + NBPT. AOA, AOB, and comammox *Nitrospira* clade B might be significant contributors to nitrification in the studied soils.

Conclusions: These findings suggest that NBPT and DMPP can reduce N losses and improve N fertilizer efficiency by directly inhibiting the growth of AOB and comammox organisms in the soils, with implications for our mechanistic understanding of the molecular mechanisms of urease and nitrification inhibitors.

Keywords 3, 4-dimethylpyrazole phosphate (DMPP) • *N*-(*n*-butyl) thiophosphoric triamide (NBPT) • Ammonia-oxidizing archaea • Ammonia-oxidizing bacteria • Comammox

Abbreviations

UIs, Urease inhibitors; NIs, nitrification inhibitors; DMPP, 3,4-Dimethylpyrazole phosphate; NBPT, *N*-(*n*-butyl) thiophosphoric triamide; AOA, Ammonia-oxidizing archaea; AOB, Ammonia-oxidizing bacteria; Comammox, Complete ammonia oxidizers; PCR, Polymerase chain reaction; qPCR, Quantitative polymerase chain reaction; TRFLP, terminal restriction fragment length polymorphism.

1 Introduction

Urea is the most used form of nitrogen (N) fertilizers in agriculture globally, accounting for 50% of the world N consumption (Zaman et al. 2012). Soil-applied urea fertilizer or animal urine is rapidly hydrolyzed to NH_4^+ and ammonia (NH_3) by the urease enzyme, leading a pH increase around the urea granule, which drives NH_3 loss through volatilization. Subsequent transformation of NH_3 or NH_4^+ in the soil involves nitrification which converts NH_4^+ to NO_3^- via nitrite (NO_2^-) (He et al. 2012). Nitrification has previously been thought to be a two-step process involving ammonia oxidation and nitrite 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) (Prosser and Embley 2002; He et al. 2012). 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 of completely oxidizing NH_3 to NO_3^- in a single organism (comammox *Nitrospira*) (Daims et al. 2015; van Kessel et al. 2015). These comammox organisms have been shown to be widely distributed in soils and water and might have an advantage over AOB and AOA under substrate limiting conditions (van Kessel et al. 2015; Hu and He 2017). The NO_3^- produced by nitrification provides a substrate for denitrification, a biological pathway in which N is returned to the atmosphere from soil through the reduction of NO_3^- or NO_2^- to N_2 via nitrous oxide (N_2O)/ nitric oxide (NO) by the nitrite reductase enzyme (Prosser and Embley 2002; Philippot et al. 2007).

Nitrogen use efficiency (NUE) is generally low, rarely exceeding 50% (Chen et al. 2008; Khan et al. 2014) as a consequence of N loss mainly via the nitrification and denitrification pathways (Hu et al. 2015a). These account for economic losses to the

farmer and environmental pollution. Technologies developed to improve urea efficiency and minimize N losses include the use of urea coated with urease and/or nitrification inhibitors (Chen et al. 2008). Urease inhibitors (UIs) are compounds that delay urea hydrolysis by inhibiting the urease enzyme activity, thus preventing localized zones of high pH that drive NH_3 volatilization (Watson et al. 2008; Harty et al. 2016). N-(n-butyl) thiophosphoric triamide (NBPT) is the only urease inhibitor that has been developed for commercial use and is effective in a wide range of soils (Watson et al. 2008; Harty et al. 2016; Cantarella et al. 2018). Once applied to the soil, NBPT is considered to be converted to its oxon analog *N*-(*n*-butyl) phosphoric triamide (NBPTO) (active form), forming a tridentate ligand with the urease enzyme, inactivating the enzyme, and inhibiting urea hydrolysis (Manunza et al. 1999; Watson et al. 2008). NBPT can inhibit hydrolysis for a period of up to two weeks depending on soil and environmental conditions (Cantarella et al. 2008; Suter et al. 2011).

Nitrification inhibitors (NIs) are compounds that delay the oxidation of NH_4^+ to NO_2^-, reducing NO_3^- concentration for some days (Trenkel 1997) by suppressing the activity of AMO in AOA and AOB (Di and Cameron 2011; Florio et al. 2014). This reduces nitrification rates and associated N losses (Chen et al. 2008; Recio et al. 2018). NIs could also reduce denitrification losses indirectly by influencing substrate availability (Florio et al. 2014; Ruser and Schulz 2015). DMPP and Dicyandiamide (DCD) are two NIs that are commercially available. DMPP has some advantages over DCD, e.g., longer efficacy (about 4-10 weeks), lower application rate, no deleterious effects on crops, and less mobile in soil (Wissemeier et al. 2001; Zerulla et al. 2001). Some factors that have been reported to influence the effectiveness of UIs and NIs include soil pH, organic matter content, soil texture and microbial activity (Barth et al. 2001; San Francisco et al. 2011; Thapa et al. 2016).

The NUE of crops can be increased by minimizing N losses but minimizing losses through one pathway could increase losses of another (IFA 2007). For example, when NBPT is used alone, there is inhibition of urea hydrolysis which initially reduces the NH_4^+ concentration, but any NH_4^+ formed is subject to nitrification and denitrification losses (Dougherty 2016). NIs can inhibit NH_3 oxidation but this could lead to prolonged retention of NH_4^+ in soil and increase the risk of volatilization (Kim et al. 2012). Therefore, in systems with multiple N loss pathways, an approach that simultaneously targets all N pathways for an overall economic and environmental benefit is required (IFA 2007). Combining UIs and NIs for use with fertilizers would simultaneously minimize losses from hydrolysis, nitrification, and denitrification. However, limited molecular studies have investigated the effect of combined UIs and NIs on the soil N cycling microbes. Although some studies exist on the effect of NIs on nitrifying microbes (Florio et al. 2014; Kong et al. 2016), there is limited information on the effect of NIs on the urease producing microbes. The effect of NBPT on nitrifying microbes and urease producing microbes has also received little attention (Fan et al. 2018).

The objectives of this study were to investigate: a) the effects of DMPP and NBPT on soil N-cycling microbes and b) how soil properties influence the efficiency of NBPT and DMPP. We hypothesized that: a) use of NBPT and DMPP will reduce the abundance of N cycling microbes in soil and alter the microbial community composition, and b) soil properties will influence the performance of these inhibitors.

2 Materials and methods

2.1 Site description, sampling, and physicochemical characterization

The soil was collected in March 2017 from five agricultural sites in Victoria, Australia: Dookie (DW), 36°22'S, 145°22'E; Horsham (HW) 36°45'S, 142°07'E; Warrnambool (WP) 38°24'S, 142°38'E; Panmure (PP) 38°20'S, 142°44'E; and Clyde (CV) 38°08'S, 145°20'E. The DW soil was classified as sandy loam with a pH of 6.4, HW was classified as clay with a pH of 8.8, WP was classified as loamy sand with a pH of 7.3, PP was classified as sandy loam with a pH of 5.5, and CV was classified as loamy sand with a pH of 7.2. At each site, five soil cores (7.6 cm in diameter and 10 cm in depth) were homogenized to a composite sample and transported to the laboratory on ice. The soil was air-dried, ground and passed through a 2-mm sieve for physical and chemical analyses. Gravimetric water content was determined by oven drying of sub-samples at 105 °C for 24 h. Soil NH_4^+-N and NO_3^--N were extracted using a ratio of 1:5 (fresh soil: 2 M KCl, 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). Details of the selected soil physical and chemical properties are shown in Table 1.

2.2 Microcosm incubation

For each site, sieved (2 mm) soil (55 g air-dried) was placed into a 500-ml vial, wetted to 60% water-filled pore space (WFPS) and incubated at 25 °C for 1 week prior to the commencement of the experiment to stabilize the microbial activity. The microcosm incubation experiment was established with the following treatments with three replicates: Control (CK), Urea (U), Urea + NBPT (UN), Urea + DMPP (UD), and Urea + NBPT+DMPP (UND). Urea was applied at 200 mg N kg^{-1} soil as a solution. NBPT and DMPP were applied in solution form, at a rate of 0.5% and 1% of applied N, respectively. Two ml of each solutions were applied to the soil to make the final WFPS to 60%. The vials were closed immediately after treatment application and incubated at 25 °C in the

dark for 28 days. During the incubation, the microcosms were aerated every 3 days and moisture was replenished as required. The soils were destructively sampled at days 0 (4 h), 1, 2, 4, 7, 14 and 28 after treatment application. On the sampling days, a subsample of 5 g (air-dried equivalent) of soil was collected from each vial and stored separately at -20 °C for DNA extraction while the rest of the soil (50 g) was used for soil NH_4^+-N and NO_3^--N extraction.

2.3 Net nitrification rate

Soil NH_4-N and NO_3-N were extracted from 50 g soil using 2 M KCl as described above. The equation developed by Persson and Wirén (1995) was used to calculate the net nitrification rates in the first week of incubation (0-7 days).

$$n\ (d0–d7) = [\ (NO_3^-\text{-}N)_{d7} – (NO_3^-\text{-}N)_{d0} / 7],$$

Where n is the net nitrification (mg kg^{-1} soil day^{-1}) within a specified period, $(NO_3^-\text{-}N)_{d0}$ and $(NO_3^-\text{-}N)_{d7}$ are the NO_3^--N concentrations (mg kg^{-1} soil) of the soil on days 0 and 7, respectively.

2.4 Soil DNA extraction

DNA was extracted from 0.25 g frozen soil sample using the MoBio PowerSoil™ DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions with slight modifications where a Fastprep beating system (Bio-101 Vista CA, USA) at a speed of 5.5 m s^{-1} for 30 s was used for the initial cell lysis step (Hu et al. 2015b). The DNA concentration was assessed photometrically using the NanoDrop® ND-

2000c Spectrophotometer UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

2.5 Quantitative PCR analyses of N–cycling functional genes

Abundances of the urea-hydrolysing and nitrifying genes in the soil samples 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 cycling conditions shown in Table 2. The 10-µl reaction mixture contained 5 µl of Sensimix (Bioline Sydney, NSW, Australia), 0.25 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) products, and the amplification efficiencies for all qPCR runs ranged between 80 and 110% with R^2 of 0.99.

2.6 Terminal restriction fragment length polymorphism (T-RFLP) analyses

T-RFLP analysis was performed to investigate the changes in community structure of nitrifying microbes focusing on the *amoA* genes of AOA and AOB using fluorescently labeled primers (Hu et al. 2015b). A 25 µl PCR reaction mixture contained 2 µl of diluted template DNA (1– 10 ng), 0.5 µl of each primer (10 µM), 5 µl MyTaq buffer, 1.5 U of MyTaq polymerase (Bioline, Sydney, Australia). The thermal cycling conditions for AOA and AOB *amoA* genes were as follows: 95 °C for 5 min, 35 cycles of 30 s at 95 °C, 30 s at 56 °C, and 60 s at 72 °C, followed by 10 min at 72 °C. The PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, San Luis Obispo, CA,

USA) and quantified using the NanoDrop ND-2000c Spectrophotometer. The restriction digestion was carried out in a 10 μl mixture containing 200 ng of purified PCR products, 0.1 μl of BSA,1 μl of ×10 NE buffer, and 5 U of the restriction enzymes MspI for AOB; RsaI (BioLabs, Sydney, Australia) for AOA. The digests were incubated at 37 °C for 3 h and denatured for 10 min at 95 °C. Terminal restriction fragments (TRFs) were size separated with an ABI PRISM 3500 Genetic Analyzer (Applied Biosystems, CA, USA) and analyzed using a local southern size-calling 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 1 bp were combined into the same TRF (Hu et al. 2015b). The relative fluorescence abundances of all TRFs were exported for further analysis of the community composition.

2.7 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). Data were analyzed using analysis of variance (ANOVA) at $p < 0.05$ followed by the Fisher test to compare treatment means only where 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 *amoA* gene copy numbers and NO_3^--N concentrations. Nonmetric multidimensional scaling (NMDS) plots were used to visualize the Bray-Curtis dissimilarity matrices based on the T-RFLP data of AOA and AOB.

3 Results

3.1 Mineral N and net nitrification rate under different treatments

Urea addition significantly ($p < 0.05$) increased NH_4^+-N concentration compared to CK in all the soils and the NH_4^+-N concentration in the U treatment remained significantly higher than CK for 2 weeks in all soils except in WP and CV (Fig. 1 and Fig. S1). Addition of NBPT (UN) significantly reduced NH_4^+-N accumulation compared to U ($p < 0.05$). The reduction of NH_4^+-N concentration by NBPT varied with 58-69%, 47-55%, 47.4%, 26-29% and 18% in HW, DW, CV, PP and WP soils, respectively. Addition of DMPP (UD) or DMPP + NBPT (UND) resulted in a significant reduction in NH_4^+ -N accumulation compared to U in the initial days for DW and HW and to a small extent in CV and WP (Fig. 1 and Fig. S1).

The NO_3^--N concentration in the CK treatment remained almost unchanged in all the soils throughout the 28-day incubation period. Urea addition significantly increased NO_3^--N concentration compared to CK in all the five soils. Addition of DMPP (UD) alone or DMPP + NBPT (UND) significantly reduced NO_3^--N concentration compared to U in all the soils. However, the addition of NBPT alone did not significantly affect NO_3^--N concentration in any soil compared to U. The significant reduction in NO_3^--N concentration by DMPP alone (UD) and DMPP + NBPT (UND) ranged between 44 to 48.7% and 28.7 to 46.3% for HW soil; 38.2% and 23.9-28.9% for DW soil; 13.2-36.4% and 12.7-34.7% for PP soil; 25.5 to 39% and 7 to 38.6% for CV soil; and 13.5-30.6 % and 11.9 to 23.6% for WP soil, respectively (Fig. 1 and Fig. S1).

In the first week, net nitrification rates were significantly higher in U treatment of all the soils compared to CK. Use of DMPP alone (UD) or DMPP + NBPT (UND) significantly reduced the net nitrification rates by 59.6 and 55.8% in HW soil; 31.6 and 31.9% in PP

soil; 33.4 and 28.4% in WP soil, respectively, compared to U (Table 3) and 43.4% reduction by DMPP (UD) in CV soil compared to U. In DW soil, net nitrification rates were not significantly different between UD or UND compared to U during the first week of incubation. NBPT had no significant influence on net nitrification rates in all the soils compared to U (Table 3).

Overall, our results showed that accumulation of NH_4^+ was significantly suppressed by NBPT but stimulated by DMPP alone or combined with NBPT in all soils. On the contrary, the nitrification process was suppressed by DMPP alone or combined with NBPT but not by NBPT alone in all the soils. The efficacy of NBPT and DMPP varied among the soils.

3.2 Copy numbers of the *ureC* and *amoA* genes

The addition of urea with or without inhibitors had no significant effect on the *ureC* gene copy numbers in WP, HW and PP soils ($p > 0.05$) (Fig. 2 and Fig. S2). UN, UD, and UND treatments had significantly lower gene copy numbers compared to U in DW and CV soils on the last day of incubation ($p < 0.05$) (Fig. 2 and Fig. S2).

There was no significant treatment effect on AOA abundance in all the soils during the sampling period (Fig. 3 and Fig. S3). However, there seemed to be growth of AOA in PP soil during the incubation period compared with much smaller or no growth of AOB or comammox (Fig. S3). Urea addition significantly increased AOB *amoA* gene copy numbers ($p < 0.05$) compared to CK on some incubation days in DW, WP, HW, and CV soils. Addition of DMPP alone or DMPP + NBPT significantly ($p < 0.05$) reduced AOB abundance compared to urea on some incubation days in DW, WP, HW, and CV soils

compared to U (Fig. 3 and Fig. S3). NBPT addition significantly reduced AOB abundance compared to U alone on day 7 in WP soil (Fig. 3). However, there was no significant treatment effect on AOB abundance in PP soil (Fig. S3). There was an increase in AOA: AOB ratio on day 7 with the addition of DMPP alone or DMPP + NBPT except in WP soil.

Urea addition with or without inhibitors had no significant effect on comammox *Nitrospira* clade A *amoA* gene copy numbers in WP and DW soils on any sampling day (Fig. 4). NBPT significantly reduced comammox *Nitrospira* clade A abundance compared to urea on the last day of incubation in PP soil (Fig. S4). The addition of NBPT + DMPP significantly reduced comammox *Nitrospira* clade A abundance compared to urea on some incubation days in the HW, PP and CV soils (Fig. S4). Urea addition significantly increased comammox *Nitrospira* clade B abundance compared to CK on day 7 in DW (Fig. 4). Addition of NBPT, DMPP, or DMPP + NBPT significantly reduced comammox *Nitrospira* clade B abundance on some days in WP, CV, DW, and HW soils compared to urea (Fig. 4 and Fig. S4). However, there was no significant treatment effect on comammox *Nitrospira* clade B gene copy numbers in PP soil (Fig. S4) on any day during the incubation.

Our results showed that except for AOA, all the treatments significantly suppressed the growth of ammonia oxidizers in some soils. A trend of increasing ratio of AOA: AOB was observed when DMPP alone or DMPP + NBPT were added. The *ureC* gene copy numbers were suppressed by all the treatments towards the end of the experiment in most soils.

3.3 Treatment effects on community composition and structure of AOA and AOB

For the T-RFLP analysis, digestion of AOA and AOB *amoA* genes with RsaI and mspI restriction enzymes, respectively, produced different TRFs in the five soils, suggesting that AOA and AOB had different responses to treatment application on day 7. The changes in the relative abundance of major fragments were used to depict treatment effects on AOA and AOB community composition in the five soils.

AOA digestion in DW and WP soils produced a total of five and three distinct TRFs, respectively, with TRFs 41 bp and 56 bp being the most abundant, respectively (Fig. 5). Three distinct TRFs were produced in HW with TRFs 41 bp and 56 bp being the most abundant (Fig. S5). Both PP and CV soils produced five distinct TRFs each with TRF 36 bp in PP soil and TRF 56 bp in CV soil being the most abundant (Fig. S5). In DW soil, the relative abundance of TRF 41 bp was reduced by U compared to CK, and increased with the addition of NBPT, DMPP or NBPT + DMPP compared to U (Fig. 5). In WP soil, the relative abundance of TRF 56 bp was increased by U compared to CK, and reduced by the addition of NBPT or DMPP + NBPT compared to U (Fig. 5). In HW soil, the relative abundance of TRF 41 bp was increased when U was added compared to CK but reduced when DMPP + NBPT was added compared to U. The relative abundance of TRF 56 bp was reduced by U compared to CK and reduced by DMPP compared to U (Fig. S5). In PP soil, the relative abundance of TRF 36 bp was increased by U compared to CK but reduced by NBPT compared to U (Fig. S5). In CV soil, the relative abundance of TRF 56 bp was reduced by DMPP or NBPT + DMPP compared to U (Fig. S5).

Similarly, digestion of AOB produced 8, 6, 9, 9 and 7 distinct TRFs, with 232 bp; 55 bp and 231 bp; 37 bp, 54 bp and 152 bp; 54 bp, 152 bp and 253 bp; and 253 bp being the most abundant in DW, WP, HW, PP, and CV soils, respectively (Fig. 6 and Fig. S6). In DW soil, the relative abundance of TRF 232 bp was reduced by U compared to CK but increased by NBPT, DMPP or DMPP + NBPT compared to U (Fig. 6). In WP soil, there

was no treatment effect compared to urea on the TRF relative abundance (Fig. 6). In HW soil, the relative abundance of TRF 37 bp was reduced by U compared to CK but increased by NBPT, DMPP, or DMPP + NBPT compared to U. TRF 152 bp in HW soil was increased by U compared to CK and reduced by NBPT, DMPP, or DMPP + NBPT compared to U (Fig. S6). In PP soil, the abundance of TRF 54 bp was increased by U compared to CK and reduced by DMPP or DMPP + NBPT compared to urea (Fig. S6). In CV soil, the relative abundance of TRF 253 bp was reduced by U compared to CK but reduced by NBPT and increased by DMPP compared to U (Fig. S6).

The NMDS analysis was used to visualize changes in community structure for both AOA and AOB in the soils following treatment application. In DW and WP AOA NMDS plots, there was no clustering into different groups based on treatment (Fig. 5). In HW- AOA NMDS plot, UD and UND clustered separately from U. However, there was no clear separation of CK or UN from U (Fig. S5). PP-AOA NMDS plot showed that U clustered separately from CK, UN, UD and UND treatments (Fig. S5). CV AOA NMDS plot showed that UND and UD clustered separately from U (Fig. S5). HW, CV, and PP- AOB NMDS plots showed that U clustered separately from UD, UND, and CK but not from the UN (Fig. S6). There was no clustering based on treatment in DW and WP soils (Fig. 6).

The T-RFLP results generally indicated that NBPT alone, DMPP alone or their combination had an influence on the structure and community composition of both AOA and AOB.

4 Discussion

4.1 Treatment effects on mineral N dynamics and net nitrification

The addition of NBPT significantly reduced the accumulation of NH_4^+-N ($p < 0.05$) in the five soils compared to urea alone in the initial days, indicative of urea hydrolysis (Fig. 1 and Fig. S1). This is because NBPT inhibited hydrolysis through suppressing the activity of urease enzymes (San Francisco et al. 2011). Similar results were reported by other researchers who showed that NBPT significantly reduced the accumulation of soil NH_4^+-N (Zaman et al. 2009; Sanz-Cobena et al. 2014). Our results showed varying efficacies of NBPT in inhibiting hydrolysis across the five soils, indicating that NBPT efficacy varied with soil physical and chemical properties. We noted a general trend of a reduction in NBPT efficacy with reducing pH except for WP soil, which can be attributed to increased chemical degradation of NBPT at lower pH (Engel et al. 2013, 2015). In line with our findings, other studies also reported a decrease in NBPT efficacy with reducing pH (Frame 2017; Sunderlage and Cook 2018). Our results also showed that apart from the pH, other factors also determined NBPT efficacy. For example, both PP and WP soils had the highest OC compared to other soils (Table 1), which may account for reduced efficiency of NBPT in these two soils compared to other soils. Moreover, HW soil with the lowest OC (7.3 g kg^{-1}) had the best NBPT performance compared to other soils. Urease activity has been shown to increase with increasing OC, resulting in reduced NBPT efficacy (San Francisco et al. 2011; Suter et al. 2011). This could be attributed to increased microbial degradation of NBPT in high OC soils.

The high NH_4^+-N concentration in DMPP alone (UD) or DMPP + NBPT indicated the suppression of nitrification in these treatments which showed the effectiveness of DMPP in retarding the conversion of NH_4^+ to NO_2^-. This was also supported by the lower NO_3^- concentration in DMPP alone (UD) or DMPP + NBPT compared to U (Fig. 1 and Fig. S1), and the net nitrification rates (Table 3). An increase in soil NH_4^+-N content due to NIs has also been reported by other researches (Zaman et al. 2009; Fisk et al. 2015). Previous

reports have shown that where a NI was combined with NBPT, there was either a reduction in effectiveness of NBPT for reducing NH_4^+-N accumulation/NH_3 emissions compared to NBPT alone or an increase in NH_4^+-N accumulation compared to urea alone (Soares et al. 2012; Pereira et al. 2013). Therefore, our results for increasing NH_4^+-N accumulation in the NBPT + DMPP treatment could be related to the increased NH_4^+-N accumulation by DMPP making NBPT to appear ineffective.

The performance of DMPP in reducing NO_3^--N accumulation in the soils varied among the five soils and reduced with increasing OC. This can be linked to the limited mobility of DMPP due to increased adsorption in high OC soils (Ruser and Schulz 2015; Marsden et al. 2016). Other studies have reported increased performance of NIs when OC was low or removed from soils (Yan et al. 2012; Fisk et al. 2015; Shi et al. 2016). We also noted a reduction in DMPP efficacy with reducing pH except for WP soil. This could be associated with increased degradation of DMPP at lower pH. The other reason for the reduced performance of DMPP at lower pH could be due to heterotrophic nitrification that has been reported to occur in acidic soils (Li et al. 2018; Zhang et al. 2018b). Previous studies have reported an increase in DMPP performance or reduced degradation at high soil pH (Yan et al. 2012; Suter et al. 2016).

4.2 Treatment effect on the *ureC* and *amoA* gene copy numbers

AOA abundance showed no significant response to treatment addition compared to other ammonia oxidizers. However, the growth of AOA in PP soil during the sampling period compared to other ammonia oxidizers suggested their potential contribution to nitrification in PP soil. Previous studies have concentrated on studying the effects of fertilizer addition with nitrification inhibitors on AOA and AOB abundances. Some studies reported that

growth of AOB, but not AOA, was increased by fertilizer addition (Beeckman et al. 2018; Ouyang et al. 2018) or reduced by DMPP addition (Beeckman et al. 2018; Zhang et al. 2018a). Other studies, however, reported a significant reduction in both AOA and AOB abundances by DMPP (Liu et al. 2015). The significant treatment effect on AOB but not AOA abundance reported in our study could be due to the structural difference between AOA and AOB, and the potential heterotrophic growth of AOA (He et al. 2012; Ruser and Schulz 2015; Beeckman et al. 2018; Zhang et al. 2018a). This accounts for less susceptibility of AOA to environmental changes (O'Callaghan et al. 2010). Little is known about the response of comammox organisms to either urease or nitrification inhibitors but inhibition of nitrification in comammox organisms by the NI allylthiourea has been reported (van Kessel et al. 2015; Li et al., 2019). Moreover, DMPP has a similar inhibition mechanism as Allylthiourea, which inhibits nitrification by binding to copper, an important cofactor for the *amoA* gene (Ruser and Schulz 2015; Beeckman et al. 2018; Li et al. 2019). Therefore, it could be possible for DMPP to inhibit nitrification by reducing the growth of comammox organisms as reported in some of our soils.

The reported increase in the ratio of AOA: AOB due to DMPP alone or DMPP + NBPT compared to urea in some soils on day 7 may suggest that the AOB population in these treatments was reduced leading to an increase in AOA population. This is consistent with the findings of other researchers who reported that AOA would thrive when AOB is inhibited due to niche differentiation (Hink et al. 2018; Fan et al. 2019).

The reduction of AOB and comammox *Nitrospira* abundances by NBPT in this study could be explained by the fact that NBPT inhibits urea hydrolysis, therefore, avoiding the accumulation of NH_4^+ that is required as a substrate by these microbes to initiate the nitrification process. In addition, some AOA and AOB may have the ability to utilize urea as they possess some ureolytic genes and activities enabling their dependence on urea as a

substrate for hydrolysis and subsequent nitrification (Burton and Prosser 2001). Ureolytic capacity has also been reported in some comammox organisms (Palomo et al. 2018; Koch et al. 2019). The reduction of comammox *Nitrospira* and AOB by NBPT would therefore be assumed to be linked to the direct effect of NBPT on urease genes in these ammonia oxidizers, influencing the degradation of urea a source of nitrification. Another possible explanation is that NBPT could inhibit the intracellular urease in ammonia oxidizers, blocking hydrolysis and subsequent nitrification due to limited NH_3 availability (Shi et al. 2017). Other studies have shown that NBPT either inhibited both AOA and AOB or stimulated AOA but inhibited AOB or had no effect on ammonia oxidizers (Shi et al. 2017; Fan et al. 2018).

Pearson's correlation results revealed that significant contributors to nitrification in CV soil might be AOA and comammox *Nitrospira* clade B, while for HW, DW and WP, AOB might be a significant contributor to nitrification. For PP soil, AOA might be the dominant contributor to nitrification. Soil pH could be a contributing factor that favored activity and distribution of ammonia oxidizers in these soils. Our results agree with previous findings that AOA are an important player of nitrification in acidic soils compared to AOB which are dominant in alkaline soils (Zhang et al. 2012; Di and Cameron 2016). However, our results also showed positive significant correlations for AOB with nitrification in acidic DW soil, which is not unexpected as some acidophilic AOB strains have also been reported to contribute to nitrification in acidic soils (Li et al. 2018; Zhang et al. 2019). Distribution, abundance and activity of AOB shown in our results could have also been determined by the soil NH_4^+-N concentration which was high for both DW and WP compared to other soils at the beginning of the incubation.

The *ureC* gene copy numbers tended to decrease significantly in some soils towards the end of the incubation period in NBPT, DMPP, or DMPP + NBPT, compared to U ($p <$

0.05). In line with our findings, other researchers reported a reduction in the abundance of *ureC*-containing bacteria in both acid and alkaline soils when they added NBPT to urea in a 25-day incubation experiment (Fan et al. 2018). However, on day 28 of incubation, we expect hydrolysis to have been finished in the soils. Urease enzyme has been shown to exist both inside and outside the cells on release when the cell dies (Fisher et al. 2017; Fan et al. 2018). We would speculate that most of the hydrolysis was conducted by extracellular urease which may absorb and keep NBPT stable in cells for long (Fan et al. 2018).

There is currently no information regarding the effect of DMPP on *ureC* genes. However, the significant reduction of the *ureC* gene copy numbers by UD and UND compared to U as reported in some soils in this study could be explained by the acidic effect of DMPP on soil pH in both treatments. It has been reported that *ureC* gene copy numbers increase with increasing soil pH, while nitrification inhibitors increase NH_4^+-N concentration which enhances acidification (Smoleń et al. 2012; Fisher et al. 2017). Based on these reports and given the fact that the reduction occurred towards the end of incubation, it is possible that the effect of DMPP or DMPP + NBPT on the *ureC* gene abundance was because of the effect of these treatments on soil pH.

5 Conclusions

We reported a significant increase in NH_3 and NO_3 accumulation due to urea addition. NBPT inhibited NH_3 accumulation in all tested soils but had no significant effect on nitrification in any soil. DMPP alone or DMPP + NBPT significantly inhibited nitrification. The abundances of comammox organisms and AOB but not of AOA were significantly influenced by NBPT, DMPP or DMPP + NBPT. Soil pH and organic carbon

might be key factors influencing the activity of urease and nitrification inhibitors in the studied soils. We also found that AOA, AOB and comammox *Nitrospira* clade B might be significant contributors to nitrification in the studied soils. These findings suggest that NBPT and DMPP can reduce N losses and improve N fertilizer efficiency by directly inhibiting the growth of AOB and comammox organisms in the soils, with implications for our mechanistic understanding of the molecular mechanisms of urease and nitrification inhibitors.

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Figure legends

Fig. 1. Changes in the NH_4^+-N (top) and NO_3^--N (bottom) concentrations in the 28-day microcosm incubation in the Dookie wheat (DW) and Warrnambool pasture (WP) soils across the five treatments of *CK* control, *U* urea, *UN* Urea + NBPT, *UD* urea+ DMPP, *UND* Urea + NBPT +DMPP. Error bars represent standard errors from three replicates.

Fig. 2. The *ureC* gene abundance during the 28-day microcosm incubation of Dookie wheat (DW) and Warrnambool pasture (WP) soils across the five treatments of *CK* control, *U* urea, *UN* Urea + NBPT, *UD* urea+ DMPP, *UND* Urea + NBPT +DMPP. Error bars represent the standard errors of three replicates. Means that do not share a letter are significantly different at any sampling time at $p \leq 0.05$ level (Fisher test). Note that y-axes scales differ between charts. There was no significant difference between the treatments on all the sampling days in WP soil.

Fig. 3. The abundance of AOA and AOB during the 28-day microcosm incubation of Dookie wheat (DW), and Warrnambool pasture (WP) soils across the five treatments of *CK* control, *U* urea, *UN* Urea + NBPT, *UD* urea+ DMPP, *UND* Urea + NBPT +DMPP. Error bars represent the standard errors of three replicates. Means that do not share a letter are significantly different at any sampling time at $p \leq 0.05$ level (Fisher test). There was no significant difference between the treatments on all the sampling days in the two soils on AOA gene copy numbers. Note that y-axes scales differ between charts.

Fig. 4. The abundance of comammox *Nitrospira* clade A and clade B during the 28-day microcosm incubation of Dookie wheat (DW), and Warrnambool pasture (WP) soils across the five treatments of *CK* control, *U* urea, *UN* Urea + NBPT, *UD* urea+ DMPP, *UND* Urea + NBPT +DMPP. Error bars represent the standard errors of three replicates. Means that do not share a letter are significantly different at any sampling time at $p \leq 0.05$ level (Fisher test). There was

no significant difference between the treatments on all the sampling days in the two soils on comammox *Nitrospira* clade A gene copy numbers. Note that y-axes scales differ between charts.

Fig. 5. Terminal restriction fragment length polymorphism fingerprint TRFs (top), and Non-metric multidimensional scaling ordinations (bottom) based on the Bray-Curtis dissimilarity matrices of the T-RFLP data of AOA *amoA* genes digested by the RsaI restriction enzyme for Dookie wheat (DW), and Warrnambool pasture (WP), soils across the five treatments of *CK* control, *U* urea, *UN* Urea + NBPT, *UD* urea+ DMPP, *UND* Urea+ NBPT +DMPP on day 7 of incubation.

Fig. 6. Terminal restriction fragment length polymorphism fingerprint TRFs (top), of AOB and Non-metric multidimensional scaling ordinations (bottom) based on the Bray-Curtis dissimilarity matrices of the T-RFLP data of AOB (bottom) genes digested by the msp1 restriction enzyme for Dookie wheat (DW), and Warrnambool pasture (WP), soils across the five treatments of *CK* control, *U* urea, *UN* Urea + NBPT, *UD* urea+ DMPP, *UND* Urea+ NBPT +DMPP on day 7 of incubation. All the stress values for the NMDS plots were lower than 0.20, an indication that these data were well-represented by the two-dimensional ordinations.

Fig. 7

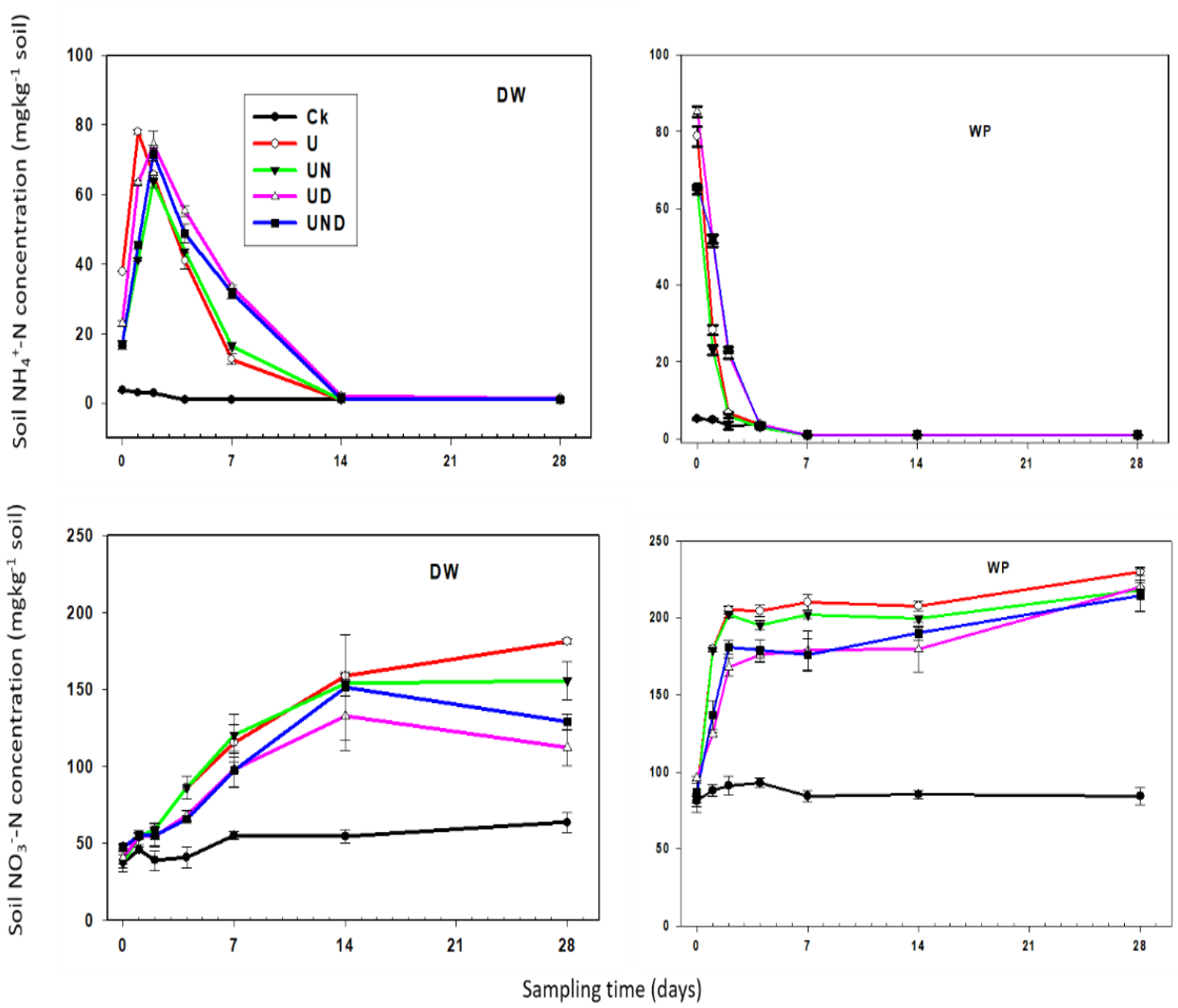

Soil NH_4^+-N concentration ($mgkg^{-1}$ soil)
Soil NO_3^--N concentration ($mgkg^{-1}$ soil)
DW
WP
Ck
U
UN
UD
UND
Sampling time (days)

Fig. 2

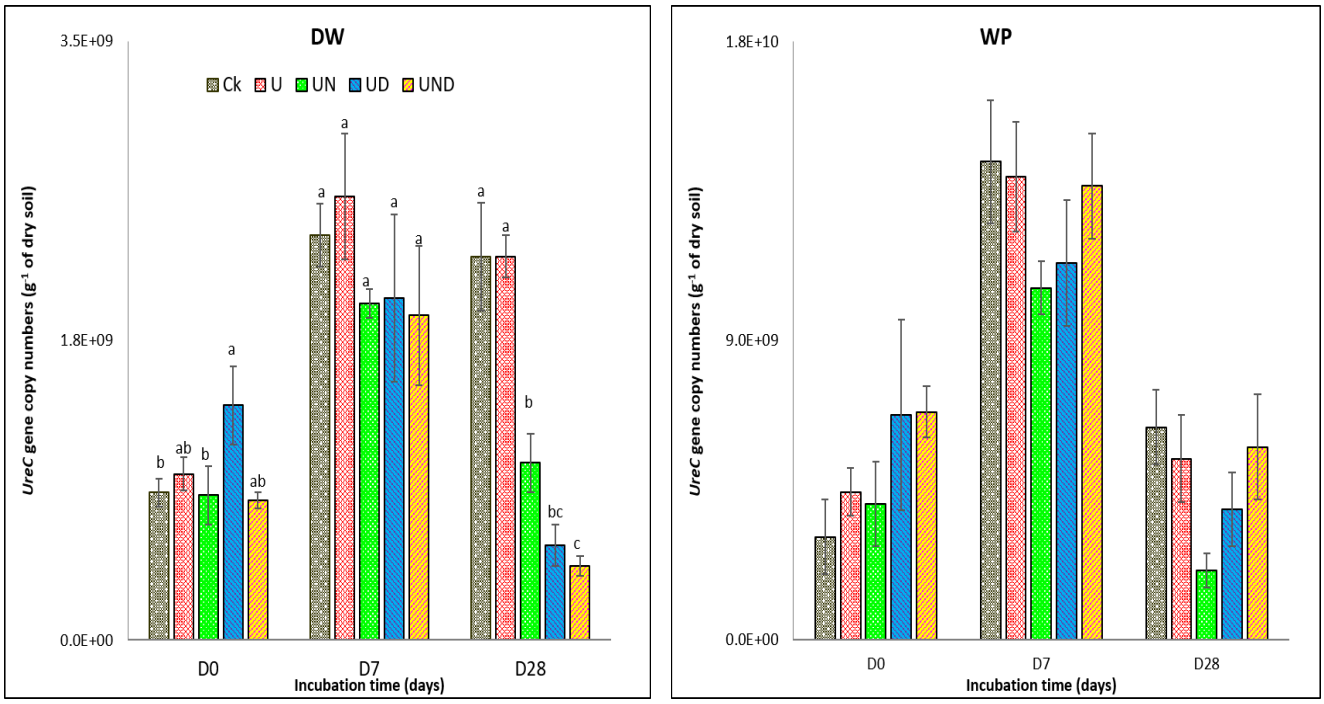

DW
Ck U UN UD UND
3.5E+09
1.8E+09
0.0E+00
UreC gene copy numbers (g⁻¹ of dry soil)
b ab b a ab
a a a a
a a b bc c
D0 D7 D28
Incubation time (days)
WP
1.8E+10
9.0E+09
0.0E+00
UreC gene copy numbers (g⁻¹ of dry soil)
D0 D7 D28
Incubation time (days)

Fig. 3

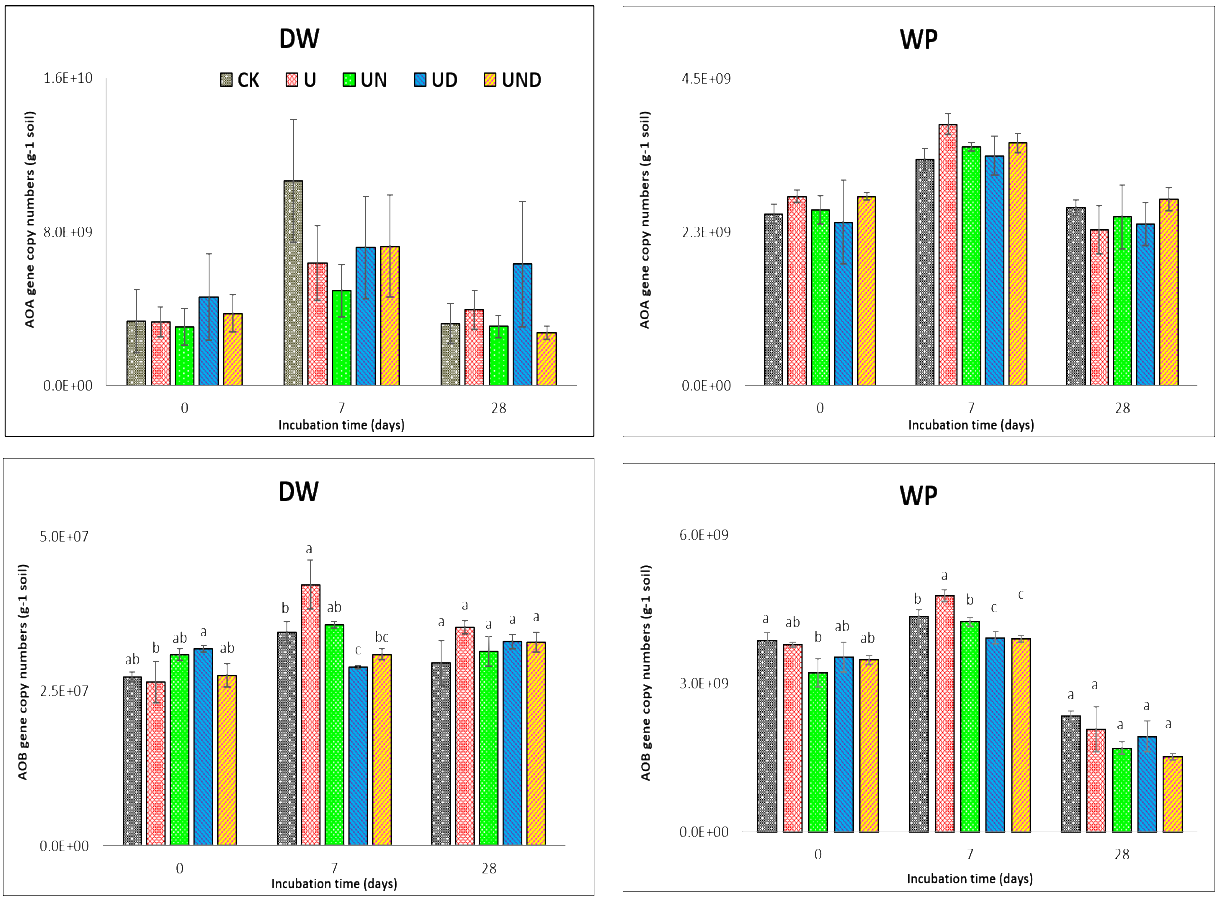
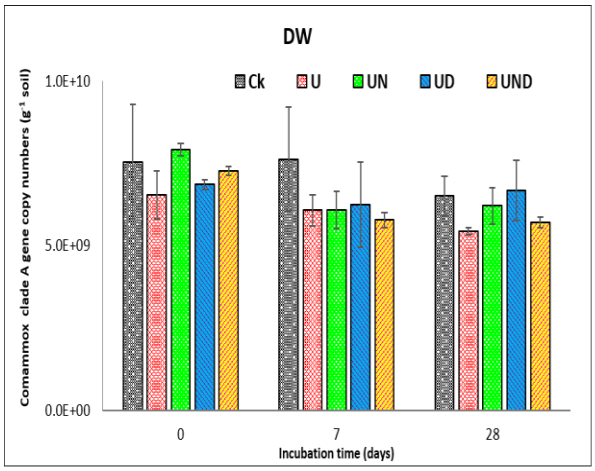
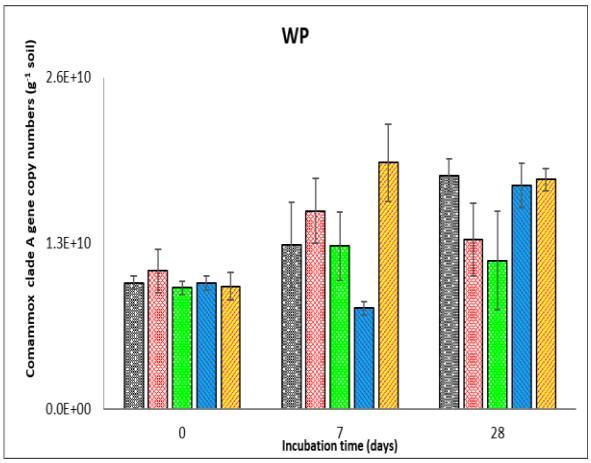
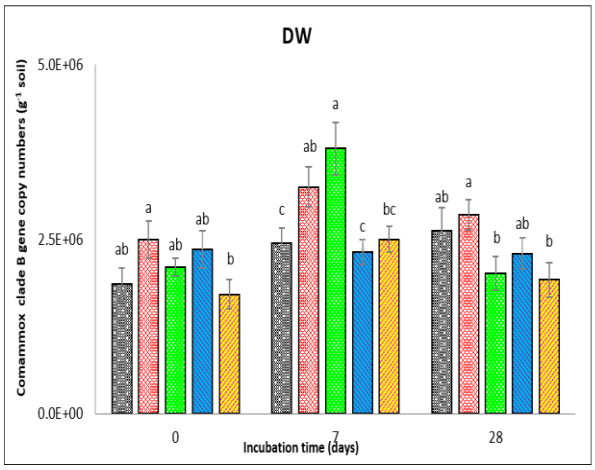

DW
CK
U
UN
UD
UND
1.6E+10
8.0E+09
0.0E+00
AOA gene copy numbers (g-1 soil)
0
7
28
Incubation time (days)
WP
4.5E+09
2.3E+09
0.0E+00
AOA gene copy numbers (g-1 soil)
0
7
28
Incubation time (days)
DW
5.0E+07
2.5E+07
0.0E+00
AOB gene copy numbers (g-1 soil)
ab
b
ab
a
ab
b
a
ab
c
bc
a
a
a
a
a
0
7
28
Incubation time (days)
WP
6.0E+09
3.0E+09
0.0E+00
AOB gene copy numbers (g-1 soil)
a
ab
b
ab
ab
b
a
b
c
c
a
a
a
a
a
0
7
28
Incubation time (days)

Fig. 4


DW
Ck
U
UN
UD
UND
Comammox clade A gene copy numbers (g⁻¹ soil)
1.0E+10
5.0E+09
0.0E+00
0
7
28
Incubation time (days)


WP
Comammox clade A gene copy numbers (g⁻¹ soil)
2.6E+10
1.3E+10
0.0E+00
0
7
28
Incubation time (days)


DW
Comammox clade B gene copy numbers (g⁻¹ soil)
5.0E+06
2.5E+06
0.0E+00
ab
a
ab
ab
b
c
ab
a
c
bc
ab
a
b
ab
b
0
7
28
Incubation time (days)

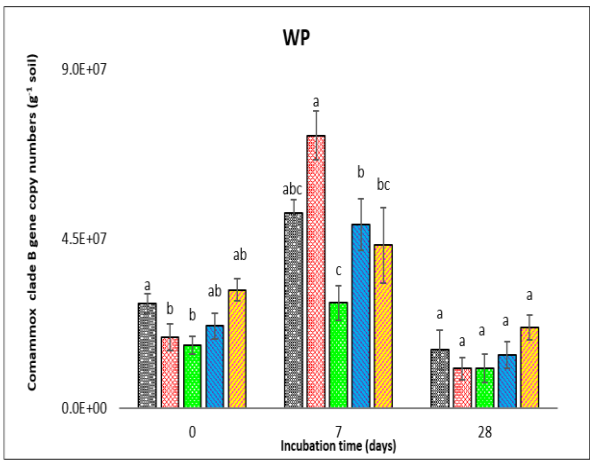
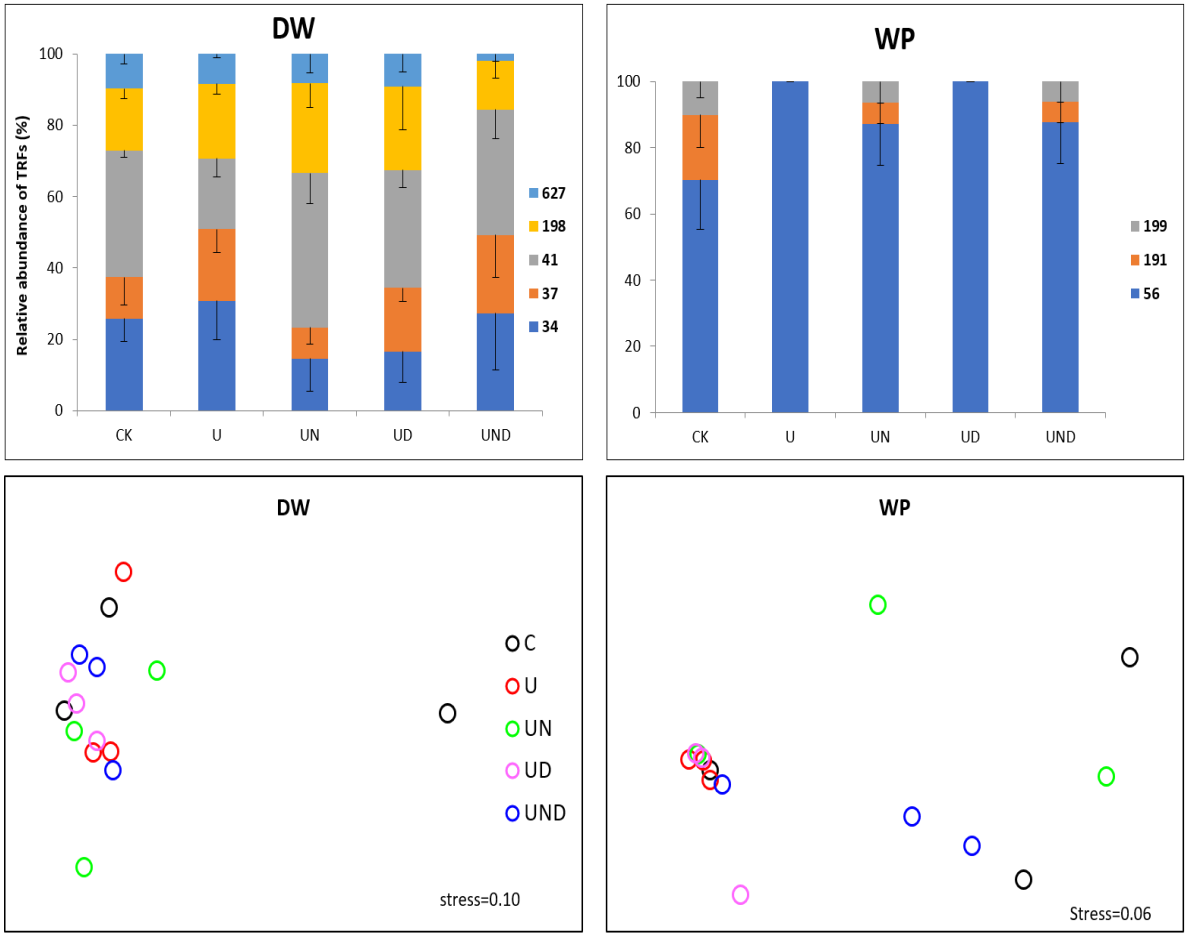

WP
Comammox clade B gene copy numbers (g⁻¹ soil)
9.0E+07
4.5E+07
0.0E+00
a
b
b
ab
ab
abc
a
c
b
bc
a
a
a
a
a
0
7
28
Incubation time (days)

Fig. 5


DW
Relative abundance of TRFs (%)
100
80
60
40
20
0
CK
U
UN
UD
UND
627
198
41
37
34
WP
100
80
60
40
20
0
CK
U
UN
UD
UND
199
191
56
DW
C
U
UN
UD
UND
stress=0.10
WP
Stress=0.06

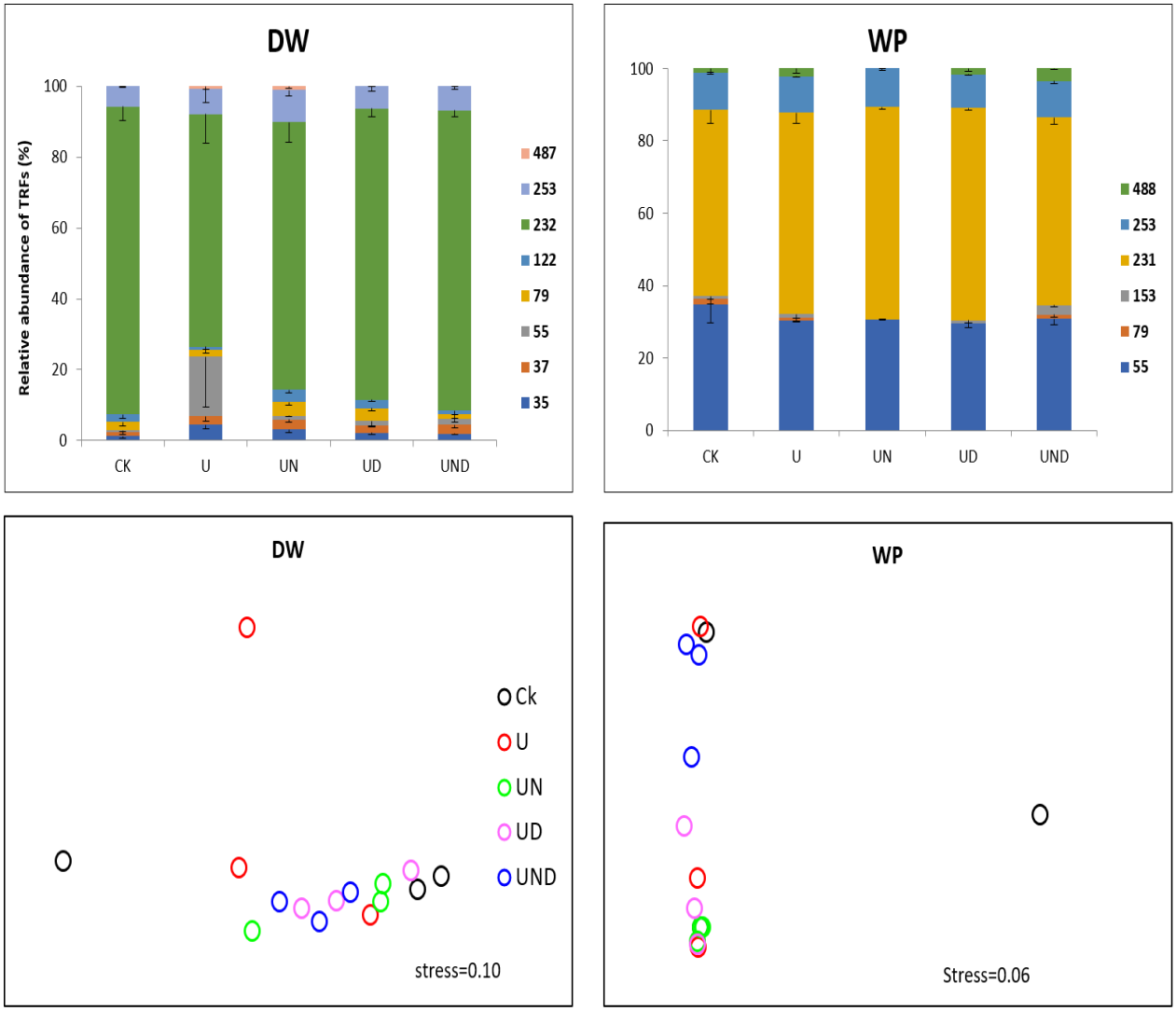

DW
Relative abundance of TRFs (%)
100
80
60
40
20
0
CK
U
UN
UD
UND
487
253
232
122
79
55
37
35
WP
488
253
231
153
79
55
DW
Ck
U
UN
UD
UND
stress=0.10
WP
Stress=0.06

Fig. 6

Table legends

Table 1. Basic properties of the soils used in this study

Table 1. Primers used for quantification of the key N-cycling functional genes

Table 2. Net nitrification rates (mg N kg^{-1} soil day^{-1}) of Horsham wheat (HW), Dookie wheat (DW), Clyde vegetable (CV), Panmure pasture (PP), Warrnambool Pasture (WP) soils during the period from day 0 to day 7. Treatments: Control, Urea, Urea + NBPT, Urea + NBPT + DMPP.

Table 1.

Soil property	Units	Dookie wheat (DW)	Horsham wheat (HW)	Warrnambool pasture (WP)	Panmure pasture (PP)	Clyde vegetable (CV)
Location		36º22'S,145º22'E	36°45′S, 142°07′E	38º24'S, 142º38'E	38º20 'S,142º44E	38°08′S,145°20′E
Cation Exchange Capacity (CEC)	Cmol kg^{-1}	21	45	19.4	10.6	11.0
pH (1:5 $cacl_2$)		5.7	7.9	6.8	4.7	6.5
pH (1:5 water)		6.4	8.8	7.3	5.5	7.2
Organic carbon	g kg^{-1}	2.7	7.3	33	60	18
Nitrate- N	mg kg^{-1}	32	7.2	110	38	51
Ammonium- N	mg kg^{-1}	5.9	0.95	5.8	16	2.2
Particle size						
Sand (0.02- 2mm)	%	59.9	35.7	86.1	68.9	83.7
Silt (0.002- 0.02 mm)	%	21.3	10.1	6.3	17.4	7.5
Clay (< 0.002mm)	%	18.8	54.2	7.6	13.7	8.8

Table 3.

primers	Sequence	Length (bp)	Reference	Thermocycling conditions
ureC gene				
L2F	ATHGGYAARGCNGGNAAYCC	390	(Gresham et al., 2007)	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.
L2R	GTBSHNCCCCARTCYTCRTG			
AOA *amoA*				
CrenamoA-23f/	ATGGTCTGGCTWAGACG	629	(Tourna et al., 2008)	
CrenamoA-616r	GCCATC CATCTGTATGTCCA			
AOB *amoA*				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- 1F	GGGGTTTCTACTGGTGGT	491	(Rotthauwe et al., 1997)	
amoA-2R	CCCCTCKGSAAAGCCTTCTTC			
Comammox A				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-244F	TAYAAYTGGGTSAAYTA	415	(Pjevac et al., 2017)	
comaA-659R	ARATCATSGTGCTRTG			
Comammox B				
comaB-244F	TAYTTCTGGACRTTYTA	415	(Pjevac et al., 2017)	
comaB-659R	ARATCCARACDGTGTG			

Table 4.

Soil type	Net nitrification rate (mg N kg^{-1} soil day^{-1})				
	Control	**Urea**	**Urea + NBPT**	**Urea + DMPP**	**Urea + NBPT + DMPP**
DW	2.55±1.04b	9.64±1.81a	11.92 ±2.39a (NR)	8.26±1.34a (NR)	7.14±1.31ab (NR)
HW	0.89±0.09b	8.44±1.19a	7.98±1.2a (NR)	3.41±0.57b (59.6)	3.74±1.06 b (55.8)
WP	1.2± 0.93c	17.8 ± 2.33a	17.09 ± 0.51a (NR)	11.85± 1.64b (33.4)	12.78± 0.59b (28.2)
PP	0.65 ±0.41 c	6.89 ±0.75 a	6.10 ±0.97ab (NR)	4.72 ± 0.4 b (31.6)	4.7 ± 0.64 b (31.9)
CV	1.45 ±0.11 d	9.90 ±1.36 ab	13.45 ±1.44a (NR)	5.6 ± 1.38 c (43.4%)	6.78 ±1.21 bc (NR)

Values are means ± standard error (n = 3). Values within the same row followed by the different letters indicate a significant difference ($P < 0.05$). The values in the parentheses show the % inhibition by NBPT, DMPP or a combination of both used with urea. Assuming nil inhibition where there is 'NR' in parenthesis.

Supplementary Material
Click here to access/download;Supplementary Material;Electronic Supplementary Material for

ELECTRONIC SUPPLEMENTARY MATERIAL

SOILS, SEC 1 • SOIL ORGANIC MATTER DYNAMICS AND NUTRIENT CYCLING • RESEARCH ARTICLE

Responses of ureolytic and nitrifying microbes to urease and nitrification inhibitors in selected agricultural soils in Victoria, Australia

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Fig. S1 Changes in the NH_4^+-N (top) and NO_3^--N (bottom) concentrations in the 28-day microcosm incubation in the Horsham wheat (HW) and Panmure pasture (PP),and Clyde vegetable (CV) soils across the five treatments of *CK* control, *U* urea, *UN* Urea + NBPT, *UD* urea+ DMPP, *UND* Urea + NBPT +DMPP. Error bars represent standard errors of three replicates

Fig. S2 The *ureC* gene abundance during the 28-day microcosm incubation of Horsham wheat (HW), Panmure pasture (PP), and Clyde vegetable (CV) soils across the five treatments of *CK* control, *U* urea, *UN* Urea + NBPT, *UD* urea+ DMPP, *UND* Urea + NBPT +DMPP. Error bars represent the standard errors of three replicates. Means that do not share a letter are significantly different at any sampling time at $p \leq 0.05$ level (Fisher test). Note that y-axes scales differ between charts. There was no significant difference between the treatments on all the sampling days in HW and PP soils

Fig. S3.The abundance of ammonia oxidizers (AOA, (top), AOB (bottom), during the 28-day microcosm incubation of Horsham wheat (HW), Panmure pasture (PP) and Clyde vegetable (CV) soils across the five treatments of *CK* control, *U* urea, *UN* Urea + NBPT, *UD* urea+ DMPP, *UND* Urea + NBPT +DMPP. Error bars represent the standard errors of three replicates. Means that do not share a letter are significantly different at any sampling time at $p \leq 0.05$ level (Fisher test). There was no significant difference between the treatments on all the sampling days in the HW, PP and CV soils on AOA, and in PP soil on AOB gene copy numbers. Note that y-axes scales differ between charts

Fig. S4 The abundance of ammonia oxidizers (Comammox Clade A, (top), Comammox Clade B (bottom), during the 28-day microcosm incubation of Horsham wheat (HW), Panmure pasture (PP) and Clyde vegetable (CV) soils across the five treatments of *CK* control, *U* urea, *UN* Urea + NBPT, *UD* urea+ DMPP, *UND* Urea + NBPT +DMPP. Error bars represent the

standard errors of three replicates. Means that do not share a letter are significantly different at any sampling time at $p \leq 0.05$ level (Fisher test). There was no significant difference between the treatments on all the sampling days in the PP soil on Comammox Clade B gene copy numbers. Note that y-axes scales differ between charts

Fig. S5 Terminal restriction fragment length polymorphism fingerprint TRFs (top), and Non-metric multidimensional scaling ordinations (NMDS (bottom)) based on the Bray-Curtis dissimilarity matrices of the T-RFLP data of AOA genes digested by the RsaI restriction enzyme for Horsham wheat (HW), Panmure pasture (PP) and Clyde vegetable (CV) soils across the five treatments of *CK* control, *U* urea, *UN* Urea + NBPT, *UD* urea+ DMPP, *UND* Urea+ NBPT +DMPP on day 7 of incubation. All the stress values for the NMDS plots were lower than 0.20, an indication that these data were well-represented by the two-dimensional ordinations

Fig. S6 Terminal restriction fragment length polymorphism fingerprint TRFs (top), and Non-metric multidimensional scaling ordinations (NMDS (bottom)) based on the Bray-Curtis dissimilarity matrices of the T-RFLP data of AOB genes digested by the msp1 restriction enzyme for Horsham wheat (HW), Panmure pasture (PP) and Clyde vegetable (CV) soils across the five treatments of *CK* control, *U* urea, *UN* Urea + NBPT, *UD* urea + DMPP, *UND* Urea+ NBPT + DMPP on day 7 of incubation. All the stress values for the NMDS plots were lower than 0.20, an indication that these data were well-represented by the two-dimensional ordinations

Fig. S1.

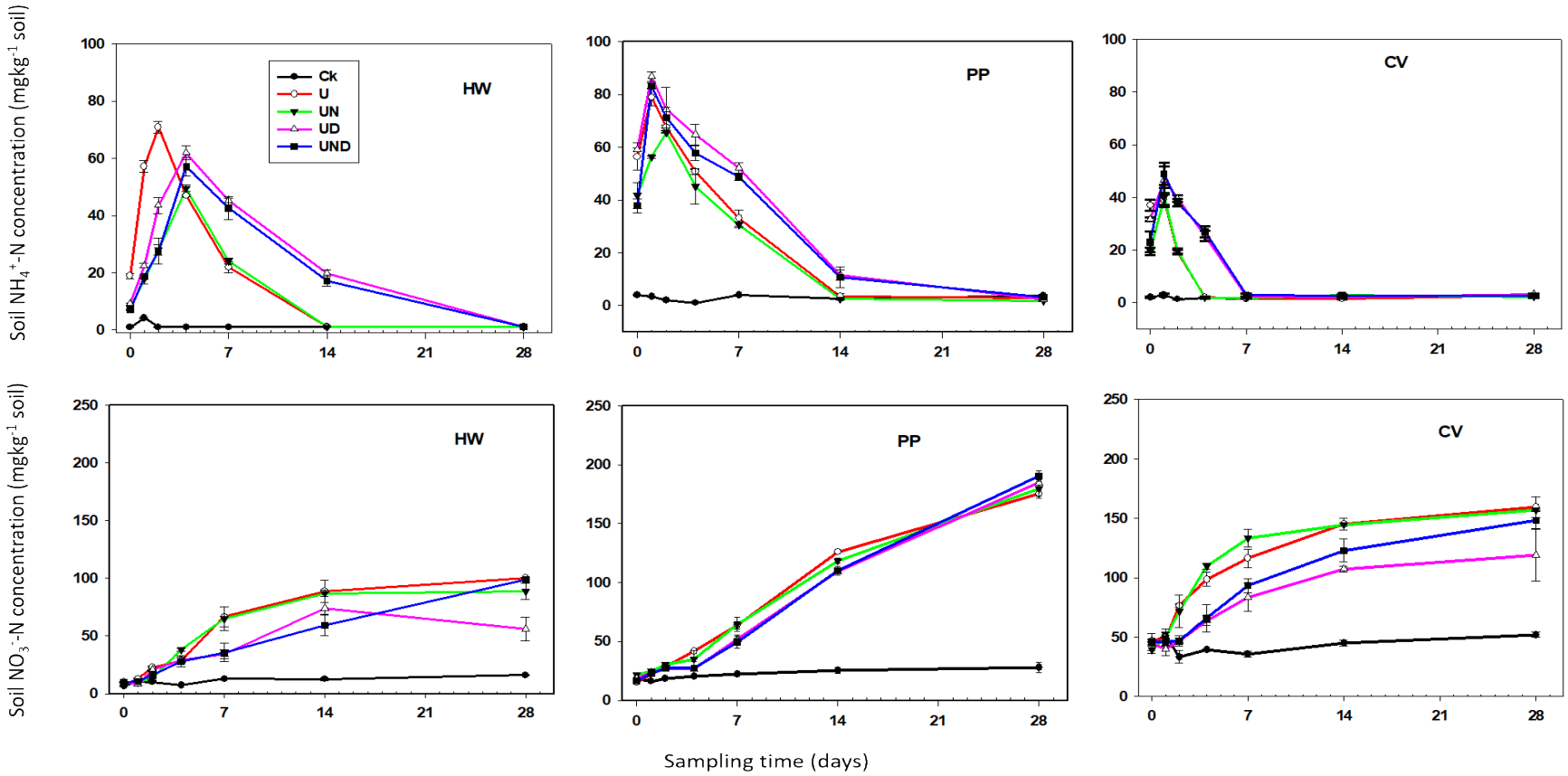
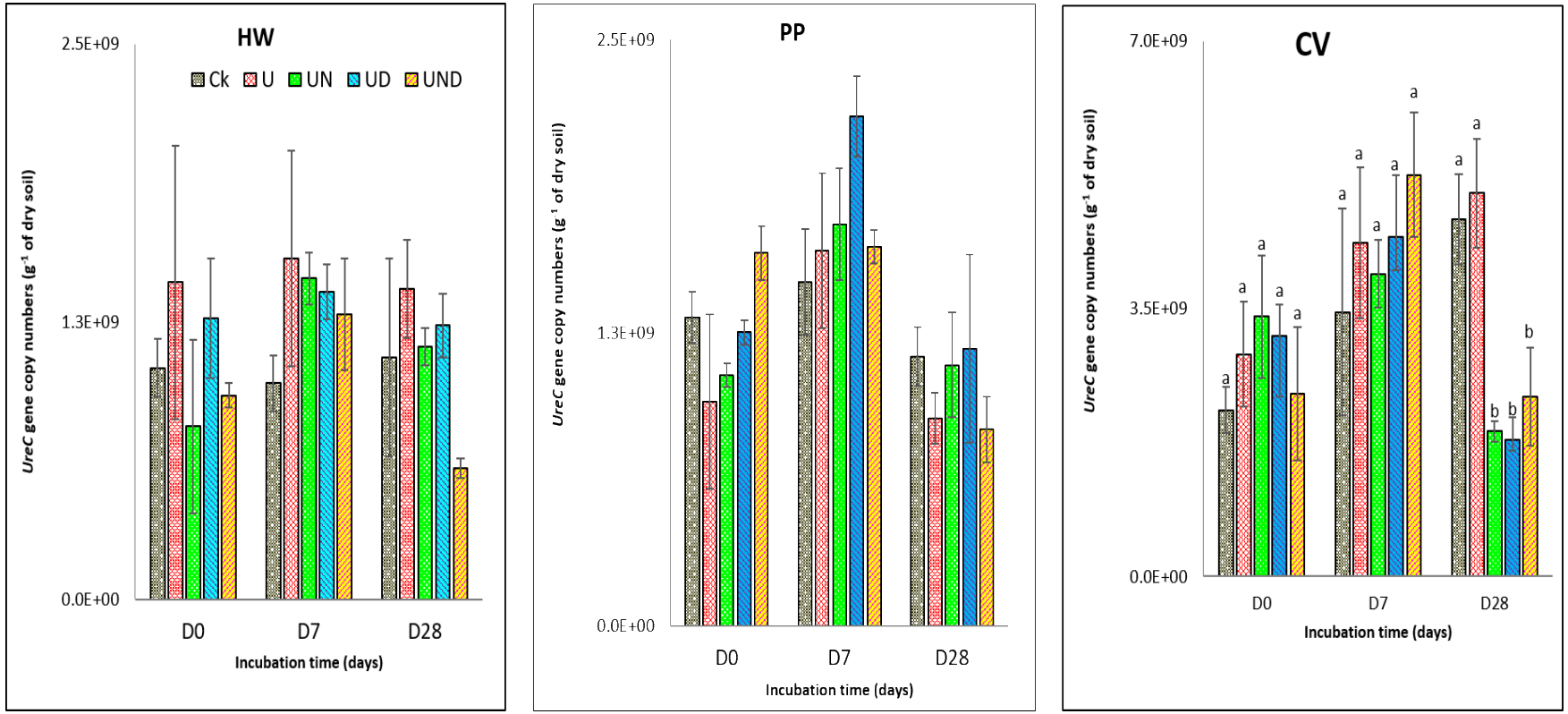

Soil NH_4^+-N concentration (mgkg^{-1} soil)
Soil NO_3^--N concentration (mgkg^{-1} soil)
Ck
U
UN
UD
UND
HW
PP
CV
Sampling time (days)

Fig. S2


HW
Ck
U
UN
UD
UND
2.5E+09
1.3E+09
0.0E+00
UreC gene copy numbers (g-1 of dry soil)
D0
D7
D28
Incubation time (days)
PP
2.5E+09
1.3E+09
0.0E+00
UreC gene copy numbers (g-1 of dry soil)
D0
D7
D28
Incubation time (days)
CV
7.0E+09
3.5E+09
0.0E+00
UreC gene copy numbers (g-1 of dry soil)
a
a
a
a
a
a
a
a
a
a
a
a
b
b
b
D0
D7
D28
Incubation time (days)

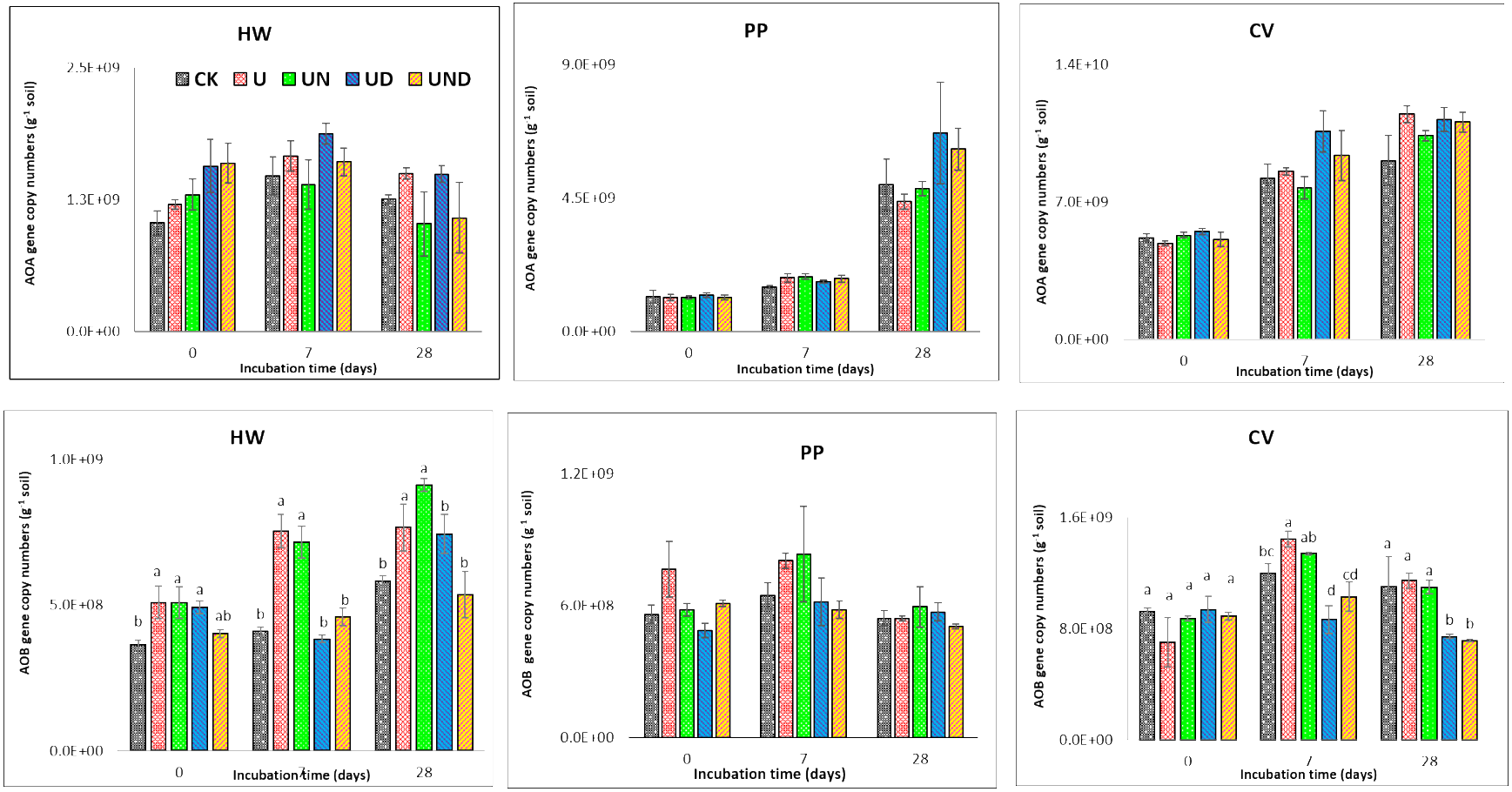

HW
PP
CV
CK
U
UN
UD
UND
AOA gene copy numbers (g-1 soil)
AOB gene copy numbers (g-1 soil)
Incubation time (days)
0
7
28
2.5E+09
1.3E+09
0.0E+00
9.0E+09
4.5E+09
1.4E+10
7.0E+09
1.0E+09
5.0E+08
1.2E+09
6.0E+08
1.6E+09
8.0E+08

Fig. S3.

Fig. S4.

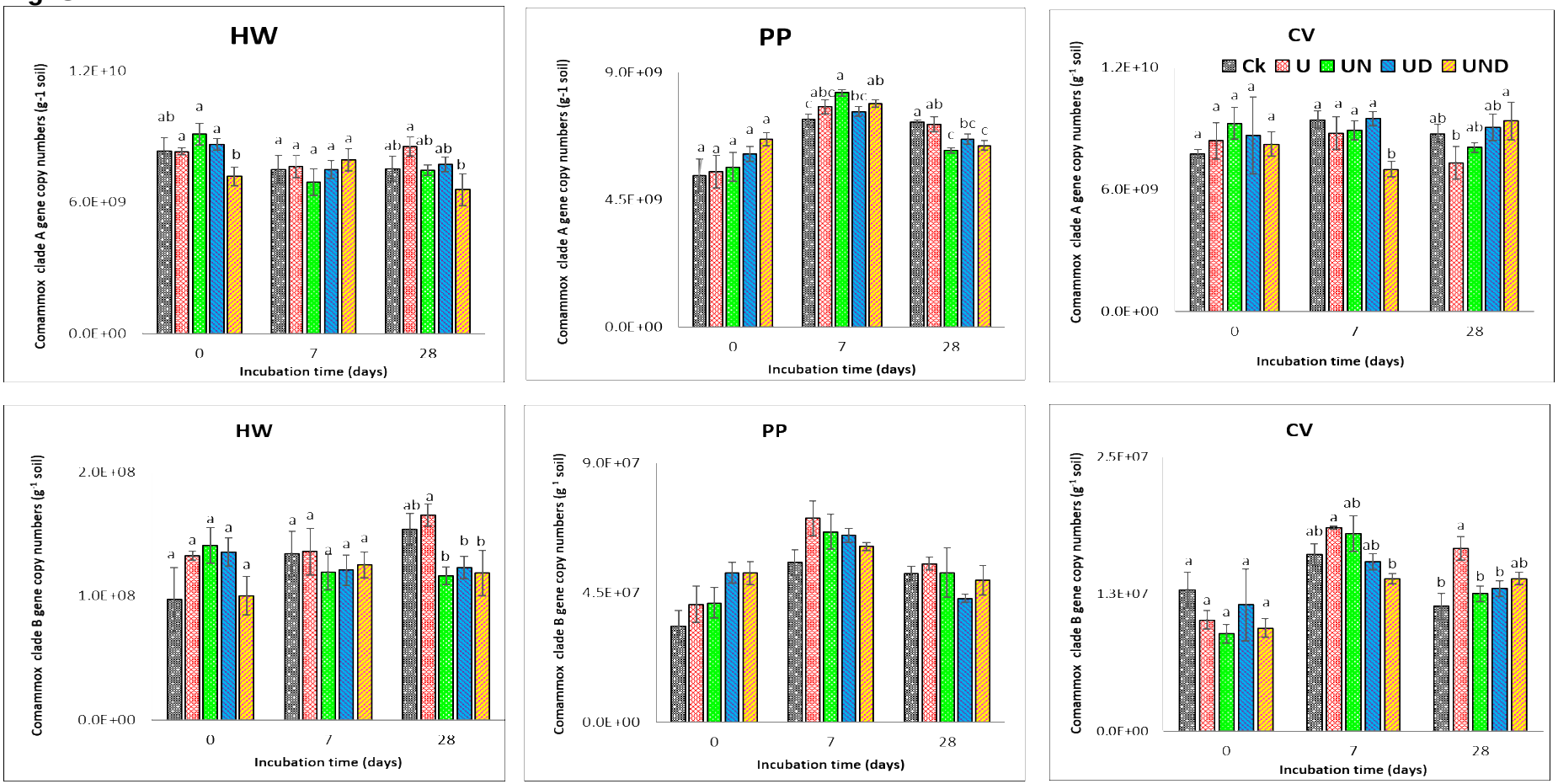

HW
PP
CV
Ck U UN UD UND
Comammox clade A gene copy numbers (g-1 soil)
Comammox clade B gene copy numbers (g-1 soil)
Incubation time (days)
0
7
28
1.2E+10
6.0E+09
0.0E+00
9.0E+09
4.5E+09
2.0E+08
1.0E+08
9.0E+07
4.5E+07
2.5E+07
1.3E+07

Fig. S5.

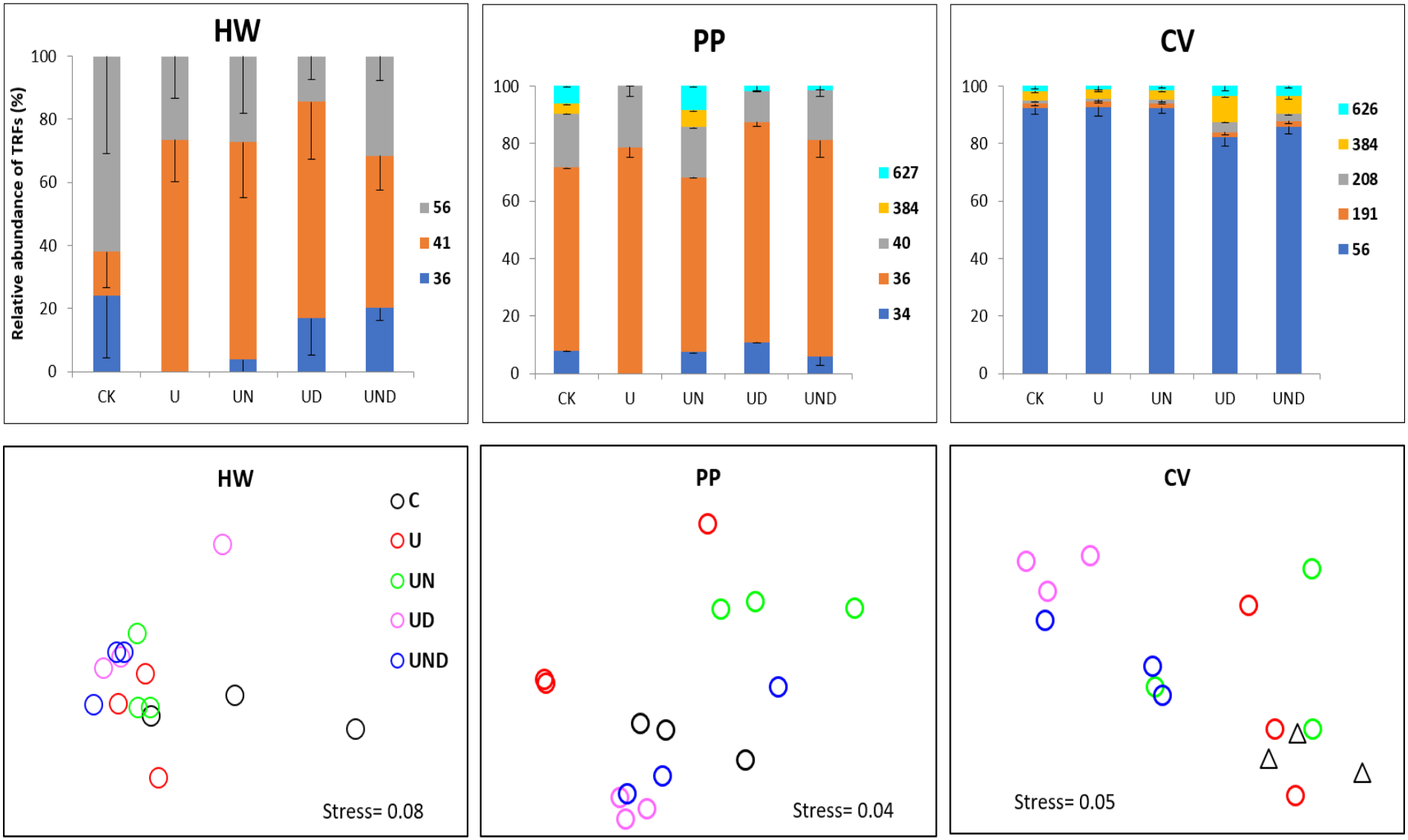
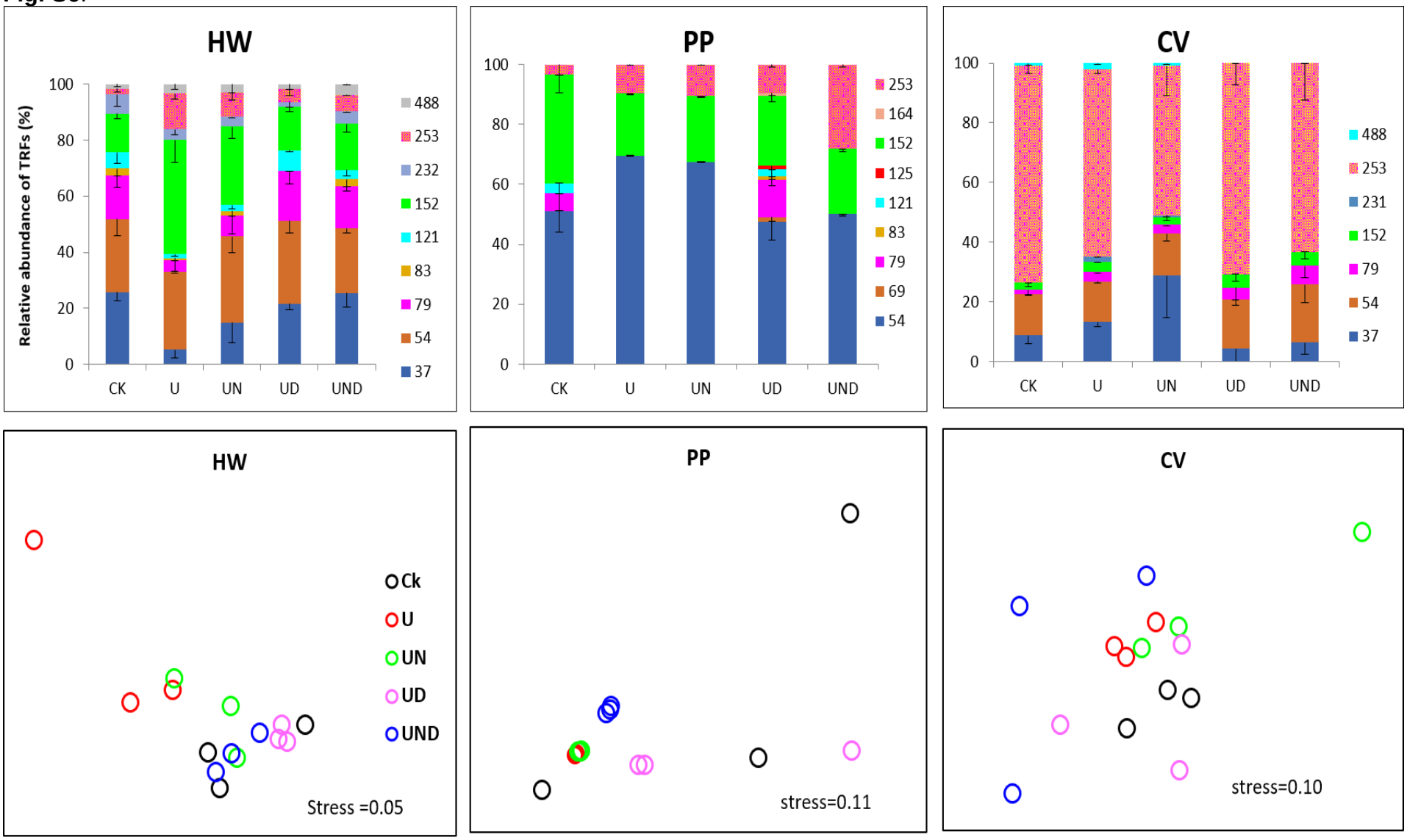

HW
Relative abundance of TRFs (%)
100
80
60
40
20
0
CK
U
UN
UD
UND
56
41
36
PP
627
384
40
36
34
CV
626
384
208
191
56
HW
C
U
UN
UD
UND
Stress= 0.08
PP
Stress= 0.04
CV
Stress= 0.05

Fig. S6.


HW
PP
CV
Relative abundance of TRFs (%)
100
80
60
40
20
0
CK
U
UN
UD
UND
488
253
232
231
164
152
125
121
83
79
69
54
37
Ck
U
UN
UD
UND
Stress =0.05
stress=0.11
stress=0.10