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AVALIAÇÃO MOLECULAR DO CICLO DE NITROGÊNIO DO SOLO SOB EMENDAS ORGÂNICAS NA PRODUÇÃO DE ALGODÃO EM LARGA ESCALA

MOLECULAR ASSESSMENT OF SOIL NITROGEN CYCLE UNDER ORGANIC AMENDMENTS IN COTTON BROAD-ACREAGE PRODUCTION

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RESUMO

Introdução: O cinturão de produção de algodão na Austrália abrange vastas áreas, desde regiões subtropicais até temperadas e de pastagens. Os tipos de solo são principalmente diferentes variações de argila, com predominância de solos argilosos pretos, cinzas e vermelhos, contendo proporções variáveis de areia. Os produtores frequentemente cultivam algodão em rotação com outras culturas, como trigo, feijão e milho. A fertilização do solo varia, com alguns produtores usando emendas orgânicas, como esterco ou composto, antes do plantio, como fonte suplementar de nutrientes. **Objetivo:** Este estudo visa entender a influência de várias estratégias de manejo da produção agrícola nas comunidades microbianas do solo e na função microbiana do solo. Assim, auxilia na melhoria da qualidade do solo e na sustentação da produção agrícola com altos rendimentos a um custo menor. **Métodos:** Para atingir o objetivo deste estudo, foi utilizado qPCR para estimar a abundância de genes funcionais envolvidos no ciclo do nitrogênio (*nifH, ureC, nirS, nirK, nosZ*, bem como *amoA* bacteriano e arqueano) no solo. **Resultados:** O estudo mostrou aumento do carbono orgânico do solo em comparação com o solo fertilizado apenas comercialmente. No entanto, houve diferenças nos efeitos do composto e do esterco no carbono orgânico do solo e nas comunidades microbianas do ciclo do nitrogênio. O aumento do carbono orgânico pré-plantio pelo composto correlacionou-se com um aumento no gene *nifH*, enquanto os solos com esterco sustentaram a abundância de *nifH* no solo pós-plantio durante o crescimento das mudas, quando a abundância de *nifH* diminuiu em outros lugares. Em contraste, a maior abundância de micróbios ureolíticos portadores do gene *ureC* foi encontrada em solo fertilizado com ureia inorgânica. Os genes de desnitrificação *nirS, nirK* e *nosZ* também foram mais abundantes em solos de algodão que receberam emendas orgânicas. Os nitrificantes bacterianos e arqueanos responderam de forma diferente ao composto e ao esterco, com maior abundância de *amoA* bacteriano com suplementação de esterco e maior *amoA* arqueano com suplementação de composto. **Discussão:** A emenda orgânica usando cobertura morta ou esterco como suplemento à fertilização comercial melhorou as perspectivas do solo do algodão para funções microbianas necessárias para a entrada e saída de N na biosfera, particularmente a fixação de N e a desnitrificação. **Conclusões:** Concluímos que um maior carbono orgânico do solo sustentou um aumento potencial das funções microbianas essenciais para a entrada e saída de nitrogênio na biosfera, nomeadamente a fixação de nitrogênio e a desnitrificação.

Palavras-chave: *Genes de nitrificação (amoA e amoA arqueano), gene de fixação de nitrogênio (nifH), genes de desnitrificação (nirS, nosZ e nirK), gene urease (ureC).*

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ABSTRACT

Background: The cotton production belt in Australia covers vast areas from subtropical to temperate and grassland. Soil types are mostly different variations of clay, with mainly black, grey, and red clay soil containing variable proportions of sand. Growers often grow cotton in rotation with other crops, such as wheat, beans, and corn, and soil fertilization varies with a number of growers using organic amendments such as manure or compost before planting as a supplementary source of nutrients. **Aim**: The objective of this study is to understand the influence of various crop production management strategies on soil microbial communities and soil microbial function. Thus, it assists in improving the soil quality and sustaining crop production with high yields at a lower cost. **Methods:** To achieve the aim of this study, had been used qPCR to estimate the abundance of functional genes involving nitrogen-cycle (*nifH*, *ureC, nirS*, *nirK*, *nosZ* as well as bacterial and archaeal *amoA*) genes in the soil. **Results:** It showed increased soil organic carbon compared to solely commercially fertilized soil. However, there were differences in the effects of compost and manure on soil organic carbon and nitrogen-cycling microbial communities. Compost-increased organic carbon pre-planting correlated with an increase in the *nifH* gene, whereas manure-amended soils sustained *nifH* abundance in the soil post-planting during seedling growth when *nifH* abundance decreased elsewhere. In contrast, the greatest abundance of ureolytic microbes carrying the *ureC* gene was found in inorganic urea-fertilized soil. Denitrification genes *nirS*, *nirK,* and *nosZ* were also more abundant in cotton soils receiving organic amendments. Bacterial and archaeal nitrifiers responded differently to compost and manure, with bacterial *amoA* abundance greater with manure supplementation and archaeal *amoA* greater with compost supplementation. **Discussion:** Organic amendment using mulch or manure as a supplement to commercial fertilization enhanced cotton soil prospects for microbial functions necessary for N input and output into the biosphere, particularly N fixation and denitrification. **Conclusions:** We concluded that greater soil organic carbon supported an increased potential for microbial functions essential for the input and output of nitrogen into the biosphere, namely nitrogen fixation and denitrification.

Keywords: *Nitrification (amoA and archeal amoA) genes, Nitrogen fixation (nifH) gene, denitrification (nirS, nosZ and nirK) genes, Urease (ureC) gene.*

1. INTRODUCTION:

Conventional agricultural systems meet the high nitrogen (N) needs for intensive crop production by using mineral fertilizers, such as urea and ammonium nitrate. However, the effect of agrochemicals on soil function requires further investigation. Soil sustainability worldwide is threatened by urbanization and intensified agriculture with extensive use of amendments (agrochemicals), irrigation, and heavy machinery (Berry, 1978; Newman *et al*., 2015; Li *et al*., 2015). The structure and function of an important component of the soil, its microbial community, depends on soil profile, water content, temperature, pH, plant cover and rotation, amendments and tillage management (Bossio *et al*., 1998; Saleh-Lakha *et al*., 2005; Kibblewhite *et al*., 2008; Garbeva *et al*., 2008 Jangid *et al*., 2008; Geisseler *et al*., 2010; Quadros *et al*., 2012; de Vries *et al*., 2013; Geisseler and Scow, 2014). While intensive arable farming might affect soil physiochemical and biological parameters (Caravaca *et al*., 2002; Bellamy *et al*., 2005), soil enrichment with organic matter, such as plant compost, straw or manure, can enhance soil organic carbon (SOC) and improve soil quality, increase aggregate stability, biological activity and function alongside with the microbial diversity (Johnston *et al*., 2009; Pérez-Piqueres *et al*., 2006; García-Orenes *et al*., 2016; Pereg *et al*., 2018a), as well as reduce chemical inputs, while improving plant productivity and the sustainability of organically managed agroecosystems (Macci *et al*., 2013).

N cycling in the soil is mainly the function of soil microbes. Teixeira and Yergeau (2012) reviewed functional gene sequences that were found to be suitable targets in the molecular analysis of soil N cycling potential. The abundance of the *nifH* gene in soil samples indicates the potential effect of bacteria responsible for N fixation (Coelho *et al*., 2009), which is considered a major natural input of available nitrogen into the biosphere. N is often applied as urea, or organically bound N, making microbial nitrogen transformation in soils play a vital role in producing N forms available for plants (Fitter *et al*., 2005; Wallenstein and Vigalys, 2005; He *et al*., 2010; Witte 2011;). Urea hydrolysis, responsible for releasing ammonia and carbon dioxide, is catalyzed by bacterial urease, and its alpha subunit is encoded by the *ureC* gene (Koper *et al*., 2004). Microbial assimilation of inorganic nitrogen is critical for improving and enhancing nitrogen retention in soil and reducing the loss of fertilizer nitrogen (Vinten *et al*., 2002; Tahovská *et al*.,

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2013). Soil potential for nitrification, an aerobic prokaryotic process resulting in nitrite, the precursor for nitrate, can be estimated by the abundance of bacterial and archaeal *amoA*, encoding the alpha (A) subunit of the enzyme ammonia monooxygenase (reviewed by Teixeira and Yergeau, 2012). Denitrifier community size has been correlated with denitrification rates (Throbäck *et al*., 2007; Hallin *et al*., 2009; Morales *et al*., 2010; Szukics *et al*., 2010; Petersen *et al*., 2012; Wu *et al*., 2012; Butterbach-Bahl *et al*., 2013), the process removing available nitrate from the biosphere. Denitrifiers contain either a Cu-containing (encoded by *nirK*) or a cytochrome cd1 (encoded by *nirS*) nitrite reductase (Glockner *et al*., 1993; Philippot, 2006; Zumft, 1997). The presence of *nosZ*, encoding nitrous oxide reductase, in the soil may indicate that the denitrifier community can diminish N_2O to N_2 and influence their environmental balance (Philippot, 2006). Being a dominant part of soil microbial communities, the response of denitrifying microbes to agricultural management strategies is important in assessing trends in soil function.

The composition of microbial community has been shown to vary with the use of nitrogen and carbon amendments (Hallin *et al*., 2009; Yin *et al*., 2014; Bastian *et al*., 2009), crop production practices and cropland use (Reeve *et al*., 2010; Bissett *et al*., 2011), and hence being influenced by cover plant species (Bremer *et al*., 2007; Hai *et al*., 2009; Petersen *et al*., 2012). Quantitative polymerase chain reaction (qPCR) was used to quantify genes associated with the N cycle for the analysis of soil microbial functions related to geomorphology and land-use (Colloff *et al*., 2008), pasture management (Wakelin *et al*., 2009), fertilizers (Okano *et al*., 2004; Cavagnaro *et al*., 2008; Pereg *et al*. 2018a) and tillage (Cavagnaro *et al*., 2008). Understanding the impact of N application practices on N cyclers is important for designing agricultural practices that would lead to the increased potential of the soil for transformation of fertilizer N and retention of available N.

Australian cotton production started on a small scale in the 1800's, with increased production in the 1900's and peak production of 3.4 million bales in 2001 (511,077 cultivated ha) and 5.3 M bales in 2012 (566,000 cultivated ha), when yield per hectare of land almost doubled. In 2016, 2.7 million bales were produced on 263,339 cultivated ha, making it a major broad-acreage crop produced on the continent (http://cottonaustralia.com.au/cotton-library/statistics).

Cotton producers in Australia have been using various fertilization strategies, with the majority using agrochemicals but some supplementing with organic matter, including manure and compost. This study aims to determine the impact of over a decade of fertilizers using organic amendment on the N cycling potential in Australian cotton soils during seedling establishment, from sowing until seedlings are at a 6-8 leaf stage, a period when plants are highly vulnerable to diseases (Pereg, 2013). The influence of organic amendments on the community of soil N cycle was investigated by measuring the abundance of genes involved in N-cycling, including *nifH* (N fixers), *ureC* (urea hydrolyzers), *nirS*, *nirK*, *nosZ* (denitrifiers) as well as bacterial and archaeal *amoA* (nitrifiers), to determine the best sustainable management strategy to enhance soil potential for N cycling and promote soil health for sustainable crop production.

2. MATERIALS AND METHODS:

2.1. Study site

Cotton growing areas of New South Wales (NSW) and Queensland (Qld) extend between the southern latitudes of 23° 30' and 32° 30'.

The research was conducted in crop production fields across the cotton-growing belt of Eastern Australia (Figure 1-A), from Qld to NSW. The soil is mostly characterized as vertisol, with soil pH ranging between 7 and 8. The crop is furrow irrigated, and sowing is in elevated beds (called "rows" here) (Figure 1-B). Four locations were sampled: Theodore (Fields B and C) in Qld; Boggabri (Field Q) and Breeza (Field P3) in Northern-Central NSW; and Carrathool (Field T) in the Riverina, Central-Southern NSW (Figure 1A). Information about crops and production practices, including fertilization, crop rotation, soil type, disease, and yield in each field sampled in this work, is given in Table 1, and climatic information for each region in Table 2.

2.2. Soil sampling and experimental design

Soil samples were collected in 2014 in two rounds: immediately before planting (early to mid-spring, pre-planted soil, "pre") and 5-6 weeks later when seedlings reached the 6-8 leaf stage (late spring, post-planted soil, "post"). Samples were extracted from the highest points along elevated rows from the soil using metal corers of 22 mm in diameter and 15 cm deep (Figure 1B). Corers

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were washed and sterilized with 70% ethanol between samples. To overcome difficulties in extracting soil, which is challenging when dealing with vertisols, the soil samples were collected using soil sampling Auger tool (Figure 1B). In each sampling round, a total of 100 soil samples were collected from each field from a 10,000-m2 plot that was 100 m wide and 100 m long. Every 10 samples were collected along a 100 m section of an elevated row, with a distance of 10 m between samples. The rows were separated from each other by 10 m. The insert in Figure 1A shows an example of the sampling design (recorded during sampling using a Garmin GPSMAP 62s GPS).

Samples taken along each row (10 samples) were pooled and mixed thoroughly immediately in the field using a pre-washed pastel and mortar with 70% ethanol. Sub-samples for DNA extractions were frozen at -20 ºC in a field freezer until they reached the laboratory (within 12-48 h), where they were frozen at -80 ºC. Sub-samples for soil property analyses were kept ice cold and stored at 4-16 ºC.

2.3. Soil physical-chemical, microbial, and biochemical properties

Field-moist soil samples were sieved at 2 mm and stored at room temperature for physicochemical analysis. Three independent samples were used to analyze soil properties in each field, with two technical replicates of each sample. SOC was determined as described by Walkley and Black (1934). Available K was extracted with 1N ammonium acetate (Knudsen *et al*. 1982), and Fe, Cu, Zn, and Mn were extracted with DTPA (Lindsay and Norvell 1978) and measured by atomic absorption and emission spectrophotometry. The Kjeldahl method determined total nitrogen (N) (Bremmer and Mulvaney 1982). The availability of phosphorus (AP) was determined by the Burriel-Hernando method (Díez 1982). Inorganic N (NO_3 and NH_4^+) was extracted with 2M KCl (Keeney and Nelson, 1982) and determined by the spectrophotometric method.

2.4. DNA extraction from soil samples

A subsample of 0.25 g soil from each soil sample (10 samples per field, each sample represents 10 grouped samples taken along a 100 m row) was extracted for total DNA using a DNA PowerSoil kit (Mo Bio, Carlsbad, USA) according to the manufacturer's instructions with some modification: Soil was vortexed at 9/10 maximal power on Vortex-Genie2 (Scientific Industries, Inc.) for 30 min, and DNA eluted in a final volume of 65 µL elution buffer. The protocol was modified following rigorously testing various parameters before determining the final DNA extraction protocol.

2.5. Measurement of DNA concentration and quality

DNA yield and purity were measured using a nanodrop (ND-1000 spectrophotometer, NanoDrop Technologies), and the quality of the extracted DNA was further assessed by agarose gel electrophoresis aiming for high molecular weight, intact total soil DNA.

2.6. quantitative-PCR (QPCR)

Quantitative PCR (qPCR) was used to quantify the total bacteria in each soil DNA sample by amplifying the *16S rRNA* gene. It also assessed the abundance of microbes involved in N cycling. The abundance of nitrogen fixers was assessed by amplification of the nitrogenase (*nifH*) gene, abundance of ureolytic microbes by amplification of *ureC*, abundance of denitrifiers by amplification of nitrite reductase (*nirS* and *nirK*) and nitrous oxide reductase (*nosZ*) genes, and abundance of archaeal and bacterial nitrifiers (ammonium-oxidising) by amplification of the archaeal *amoA* or bacterial *amoA* gene (referred to as *amoA*-arch or *amoA*-B respectively). Table 3 provides details of the primer sets and qPCR cycling conditions used in this study.

For qPCR analysis, each sample was assayed in triplicate (three technical replicates of each of the ten independent replicates = 30 qPCR reactions per gene per soil) on a Rotorgene (Qiagen, USA). Each qPCR reaction contained 12.5 µL of 2x iQ SYBR Green Supermix (Bio-Rad Laboratories, CA, USA), 1 µL each of forward and reverse primer (final concentration of 400 nM), 1.25 µL 50 mg/mL ultrapure BSA (Invitrogen, CA, USA), 0.5 µL PCR grade DMSO (Sigma-Aldrich, MO, USA), 1 µL of DNA template and RNase/DNase-free water to a final volume of 25 µL. Duplicate no-template controls were included in each assay. Melt curve analyses and gel electrophoresis supplementary were done to confirm the specificity of the amplicon.
For quantification of target genes in soil samples, all samples were amplified in parallel with a serial dilution (10^1-10^7 gene copies per reaction) of plasmid standards containing the gene of interest that was produced by cloning PCR products into a pGEM-T vector (pGEM-T Easy Vector System II, Promega, WI, USA). Successful cloning of products was confirmed by amplification of the expected product from the plasmids and by

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plasmid sequencing.

The efficiency of each qPCR assay was determined by amplification of a serial dilution of soil DNA (five-fold dilution series, from 5 to 0.008 µL DNA per reaction). Efficiencies ranged from 68.5 to 90.2%, with R^2 values ranging from 0.986 to 0.998. The presence of any PCR inhibitors in the soil DNA, such as humic or fulvic contaminants, was assessed, and the efficiencies and standard curves from amplification of the serial dilution of soil DNA were compared to amplification curves and efficiencies from standard plasmids. Both soil DNA and plasmid standards showed similar efficiencies and standard curve slopes, indicating no inhibitors were present.

Normalization of gene copy number to gram of dry soil was used to give results on a biologically significant scale; this assumes similar DNA isolation efficiency across samples, which is only appropriate when measuring relative (vs. absolute) quantification, as in this study.

2.7. Statistical analysis

The qPCR results and the soil property data were subjected to statistical analysis. The data adjustment to a normal distribution for all properties analyzed was verified with the Kolmogorov-Smirnov test at $P < 0.05$. Measured variables were submitted to a one-way ANOVA, grouped by blocks or treatments as nesting factors, assuming equal variance. The means were separated according to the average post-hoc Tukey test with significant differences at $P < 0.05$. Pearson's correlation coefficients (R) were calculated to quantify the linear relationship between parameters.

The relationships between the abundance of various genes as well as between gene abundance and soil characteristics were analyzed by PCA (principal component analysis) with Varimax normalized rotation to determine the influence of the types of agricultural management on soil microbial N cycling, keeping the number of cases higher than the number of variables (variables ≤N-1) to avoid overparameterization. SPSS software (Statistical Program for the Social Sciences 23.0) was used for all statistical analysis.

3. RESULTS AND DISCUSSION:

3.1. Results

3.1.1 Soil properties

Most soil properties did not differ significantly between Fields B, C, P3, Q, and T, which were prepared for seed sowing (Figure 2). There were no significant differences in %N, NH_4^+ concentrations, or %P among soils, and K concentrations were similar in all soils with slightly higher concentrations in Soil P3. In Qld, SOC was significantly higher in Soil C than in Soil B, and in NSW, it was slightly higher in Soil T and P3 than in Soil Q (Figure 2). Overall, the SOC values were within the range reported by other workers for vertisols in NSW, Australia (Yan *et al*., 2000). While the total N did not differ among fields, the concentrations of NO_3^- were significantly higher in Soils B, P3, and T than in Soils C and Q. The organically amended Soils C and T contained significantly higher concentrations of Fe and Mn than in Soils B, Q and P3 (Figure 2). In Qld, Soil B had slightly higher concentrations of Cu than Soil C, and Soil B and C had similar concentrations of Zn. The concentrations of Cu and Zn across NSW Soils P3, Q, and T were similar.

There was no correlation among the different soil properties, except for Mn, which shared a weak correlation (Pearson correlation significant at the 0.05 level) with Fe (0.948*) (Table 4).

3.1.2 Soil DNA and 16S rRNA gene abundance

In all soils, the abundance of the 16S rRNA genes before planting was similar to that after planting in the presence of seedlings of 6-8 leaves (Figure 3). In Qld, 16S rRNA gene abundance in the inorganically fertilized Soil B was 10-fold lower than in the organically amended Soil C before and after planting (Figure 3). In NSW, Soil P3 and Soil Q had lower 16S rRNA gene abundance than the organically-amended Soil T before and after planting.

DNA concentrations in the Qld soils were similar before and after planting and were four times higher in Soil C than in Soil B (Figure 3). In NSW, DNA concentrations were two-fold higher in Soil T than in Soils P3 and Q before planting. DNA concentrations decreased in Soil T and increased in Soil Q after planting, with little fluctuation in Soil P3 (Figure 3).

3.1.3 Abundance of genes involved in the nitrogen cycle

3.1.3.1 Abundance of nifH gene

Since the *nifH* primers were designed in this work (Table 3), we cloned the amplification products and sequenced 12 of the inserts. We found that the inserts shared high similarity with

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various *nifH* sequences in the databank (PubMed blast). While similar, the amplification products were not identical, suggesting that the primers amplified *nifH* sequences from different bacterial species.

Similar to the 16S rRNA gene, *nifH* abundance in Qld soils was higher in the mulch-treated Soil C than in the inorganically treated Soil B (Figure 4), six-fold higher before planting and 3.5-fold post-planting. However, while the abundance of *nifH* decreased during the six to eight-week period after planting in both Soils B and C, the *nifH* abundances were no longer statistically different (Figure 4).

In NSW, *nifH* abundance was similar in Soils P3, Q, and T before planting. However, *nifH* abundance dropped in Soil Q and P3 but not in Soil T six to eight weeks after planting (Figure 4). In general, the pattern of *nifH* abundance did not necessarily match that of the 16S rRNA gene or the soil DNA concentrations (Figures 3 and 4).

3.1.3.2 Abundance of ureC gene

The abundances of *ureC* in soil samples collected pre-planting were not significantly different from those collected post-planting in all Qld and NSW soils (Figure 5). In Qld, the abundance in Soil C was a magnitude lower than in Soil B, and in NSW, the abundance in Soil T was slightly lower than in Soil P3 and Soil Q (Figure 5).

3.1.3.3 Abundance of nirS, nirK, and nosZ genes

In Qld, all denitrification genes, *nosZ*, *nirK,* and *nirS*, were significantly more abundant in organically amended Soil C than in Soil B, both pre and post-planting (Figure 6). However, the differences in gene abundances between Soils C and B were less prominent in the post-planting soils (Figure 6).

In NSW, *nirS* and *nosZ* were more abundant in Soil T than in Soils P3 and Q at pre and post-planting, but *nirK* was only significantly higher in Soil T post-planting (Figure 6).

In general, numbers before and after planting were at the same level of magnitude for all genes. The abundances of *nirS* were similar to those of *nosZ* in all soils, and *nirK* was slightly higher than both (Figure 6).

3.1.3.4 Abundance of bacterial and archeal amoA genes

In Qld, there was no difference in the abundance of amoA-B between soils B and C, with similar gene copy numbers pre- and post-planting. On the other hand, the abundance of *amoA*-Arch was higher in Soil C than in Soil B at pre and post-planting (Figure 7).

In NSW, *amoA*-B abundance in Soil T, although highly variable, was significantly higher than in Soils P3 or Q at pre and post-planting (Figure 7), while there was no significant difference in *amoA*-Arch abundance among Soils P3, Q and T at pre or post planting (Figure 7).

The abundance of *amoA*-B and *amoA*-Arch has not changed significantly from pre to post-planting in all soils. While there was a high variation in the abundances of *amoA*-Arch, they were two to three levels of magnitudes higher than those of *amoA*-B in all soils (Figure 7).

3.1.3.5 the relationship between soil properties, 16S rRNA gene, and N cycle genes

PCA analyses were performed to evaluate the influence of the organic amendments on physiochemical soil properties and gene abundance (Figure 8). The top two components explained 77.1% of the cumulative variance with eigenvalues > 1.0. The first component, PC1, was loaded with the parameters with the highest scores under PC1, which accounted for a 53.6% variance (Table 5). PC1 separated organically amended Soils T and C from the inorganically fertilized ones (Soils B, P, and Q). Soils Q and P (NSW) were grouped at a distance from Soil B (Qld) (Figure 8A). The second component accounted for 23.5% variance and separated the manure-treated Soil T (NSW) from the mulch-treated Soil C (Qld) (Figure 8A). The loading plot separated *ureC* from all other genes on PC1 (Figure 8B). Several genes, *nifH*, *nirS,* and *nirK*, from pre-planting were clustered around SOC on the loading plot (Figure 8B) and were highly correlated to SOC (0.935*, 0.988** and 0.974**, respectively). On the other hand, the post-planting *nifH* and *nirK* were separated from SOC on PC2, indicating that different parameters may influence the abundance of the genes at pre- and post-planting. PC2 also separated bacterial *amoA*-B from archaeal *amoA*-arch pre-planting, while they were clustered together post-planting (Figure 8B).

Additional PCA analyses, examining the relationship between genes in soils pre-planting, independently from post-planting, showed a similar trend to Figure 8, separating the soils into four groups: B, C, Q/P, and T, thus supporting the

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results observed with the PCA of all parameters (Figure 8). In the PCA of genes pre-planting, the top two components explained 91% of the cumulative variance with eigenvalues > 1.0. The first component, PC1, was loaded with the parameters with the highest scores under PC1, accounting for a 69.5% variance.

3.2. Discussions

Organic amendments in the form of compost or manure have significant effects on the potential of the soil to acquire or release N in otherwise conventionally fertilized cotton-growing soils in Australia. This was shown in this work by the increase of N fixer (nifH) and denitrifier (nirS/K and nosZ) abundances in chemically fertilized soils amended with compost (Farm C, Qld) or manure (Farm T, NSW). This is particularly important at the time of planting and during seedling establishment, which is a critical period in the growth of the cotton plant as it is extremely susceptible to disease and other stressors (Pereg, 2013).

Intensive agricultural practices may lead to land degradation. Therefore, it is necessary to identify cropping systems that would result in high yields and maintain or improve soil ecosystem services, such as soil N cycling, thus ensuring agroecosystem sustainability (Orr et al., 2015). Strictly organic agricultural systems have been shown to improve biodiversity, soil structure, water and nutrient retention, and other properties of soils (Mäder et al., 2002; Hirsch and Mauchline, 2015). However, broad-acreage crop production, such as Australian cotton production, poses many logistical challenges to applying strictly certified organic agriculture practices, mainly due to the sheer size of the fields and their distance from sources, such as feedlots and poultry farms, making it difficult for growers to consider it as an option.

Nevertheless, we showed here that using organic amendments and conventional inorganic fertilizers contributed to the conservation of microbes involved in N cycling. Similarly, Pereg et al. (2018a) also reported a higher abundance of N fixers and denitrifiers in organically fertilized grapevine soils in Spain than inorganically fertilized soil. This leads us to propose that organic amendments could be a solution for sustaining soil N cycling function in various crop production systems.

Application of only 70% of the urea in cotton Field C (350 kg urea ha-1) compared to Field B (500 kg urea ha-1) did not lead to decreased yield, suggesting that either: 1) the lower urea application in Field C was sufficient for supporting the yield, 2) the organic matter applied to Soil C supplied some additional available N, also, 3) reduced level of N fertilization resulted in larger N fixing community in Soil C, which reduced N2 to available NH3. Indeed, nifH abundance was higher in Soil C than in Soil B.

In NSW, Farm T was supplemented with manure and a very high amount of urea (535-600 kg ha-1). Similar to Field B, much of this urea was converted to nitrate since the nitrate concentrations in Soils B and T were the highest of all soils tested. The high concentrations of available N provided by the inorganic N fertilizers in Soil T may be the reason why, before planting, it shows similar levels of nifH to the inorganically fertilized Soils Q and P3. Chicken manure, which was applied to Soil T, has a high content of N, phosphorus, and potassium when compared to other animal manure (e.g., pig manure, kraal manure) and high concentrations of macro-nutrients (Dikinya and Mufwanzala, 2010). During the cotton seedling growth, the slow release of liable carbon and other nutrients from the manure applied in Soil T may have continued to support the N fixing community, resulting in higher N fixer abundance in Soil T compared to Q and P3 following seedling establishment.

Our findings that the concentrations of most soil chemical properties studied in this work fell within narrow ranges and did not differ much between cotton farms, whether organic matter was added or not, are unsurprising. Cotton growers in Australia, similar to crop producers in most developed countries, are following industry recommendations for soil management (for example, the Australian Cotton Production Manual, published by the Cotton Research and Development Corporation: https://www.crdc.com.au/publications/australian-cotton-production-manual) and prepare the soil accordingly for planting. Nevertheless, some differences existed among the various soils within the narrow ranges of results. Higher SOC was observed in Soil C following compost application and, to some extent, in Soil T, where manure was applied before planting. Higher nitrate was observed where the highest concentrations of urea were applied (Soils T and B) and to some extent where ammonium-nitrate was applied (Soil P3). Mn and Fe were highest where organic matter, compost, or manure was applied.

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Like Pereg et al. (2018a), organic amendments in compost and manure increased the overall bacterial loads in cotton-growing soils in Qld and NSW at the time of planting and during seedling establishment. The origin of N cyclers in organically fertilized soil has not been discussed. The manure and mulch compost used in Fields T and C, respectively, contained all of the genes tested in this work (data not shown) and, therefore, may contribute to the diversity of N cyclers in the soil. However, the mass of organic fertilizers per hectare (5 t per ha) was negligible when calculated per 22 mm diameter of the corers. The organic matter was applied at least a month before planting and thus was not considered when estimating the N cycler abundance in the soil.

Thompson et al. (2016) and Wolsing and Primé (2004) discussed the seasonal variation in soil bacterial abundance reported by various groups as well as the dynamics of N-cycling microbial communities and their tendency to follow plant-growing cycles, suggesting they are tightly coupled with seasonal changes in labile C and N pools, temperature, and soil moisture. Moisture was also a major factor in determining N cycler abundance in drip-irrigated citrus orchards (Morugán-Coronado et al., 2019). In the cotton fields sampled, the crop is furrow irrigated, and the time of planting and seedling establishment are in the same season, separated only by approx. 4-6 weeks (mid and late spring). Thus, seasonal/temporal changes in gene abundance were not measured.

In agreement with the discussion by Thompson et al. (2016), our results show that there is a correlation between the overall bacterial abundance and the SOC and nitrate, which are higher in organic fertilized Soils C and T. Our results are also in agreement with Wolsing and Primé (2004), who observed differences in the bacterial populations between soil treated with mineral fertilizers (cattle manure) and soil treated with inorganic fertilizers. Interestingly, Wolsing and Primé (2004) further proposed that the fertilizer type was a more important determinant of the denitrifier community than the fertilizer amount. This suggestion should be examined in future studies.

The high variation in the 16S rRNA gene abundance measurements within fields can be explained by the huge variability in the number of the rRNA genes in various bacterial species and the general heterogenicity of the soil system (Farrelly *et al*., 1995; Klappenbach *et al*., 2001; Sun *et al*., 2013; Vetrovsky and Baldrian, 2013; Pereg *et al*., 2018a). Overall, relatively high variations among field-independent replicates are expected, as the soil is very heterogenic. In this work, we have tested the effect of gradually increasing the number of independent replicates from each soil in the cotton growing system (data not shown) and concluded that, in such wide-acreage systems, it is important to ensure that a large enough number of independent replicate samples (100 independent samples per field, grouped into ten separate samples) is analyzed to obtain statistically meaningful results.

The abundance of N cycling genes in cotton soils changed independently of the total bacterial abundance (16S rRNA gene). Similar phenomena were observed for bacterial communities in grapevine soil in Spain (Pereg *et al*., 2018a) and for denitrifier abundance in corn-soybean soil (Thompson *et al*., 2016), suggesting that bacteria performing different parts of the N cycling were influenced by different proximal regulators to each other and the total bacterial community. In the following sections, we examined the effects of agricultural management on the abundance of various genes involved in N-cycling in Australian cotton soil.

3.2.1 Bacterial involved in acquiring ammonia-nitrogen fixers and urea hydrolyses

Organic compost in Field C increased the abundance of the *nifH* gene in cotton soils in Qld at planting time. Manure in Field T helped maintain the same level of *nifH* before and after planting, whereas in the other soils, the *nifH* abundance reduced after planting. In contrast to Morales *et al*. (2010) and Pereg *et al*. (2018a), high copy numbers of *nifH* under organic fertilization did not correlate with available N, but, in agreement, it did correlate with SOC in cotton soil. This difference could be explained by using mineral N fertilizers in all cotton farms, including in the soils that were also supplemented with organic matter, since high levels of available N suppress nitrogenase, removing the competitive advantage N fixers have in N-poor soils. Higher average temperatures and rainfall in Qld, where Farm C is located, may have supported faster breakdown of the added organic matter, as SOC was higher in Soil C, possibly supporting the presence of larger N fixer populations. However, 6-8 weeks later, the influence of the degraded compost matter can no longer be seen by the seedling establishment stage. The difference in the *nifH* abundance pattern in the manure-amended Soil T, when compared to the other soils tested here, suggests

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that the nature of the organic matter may also influence the N fixer community. The organic matter could have been used as an energy source, increasing the diazotroph abundance pre-planting, as indicated by a higher copy number of *nifH* in the organically supplemented soils. A study conducted by a Spanish on grapevine soil demonstrated that the abundance of the *nifH* gene was also significantly increased when organic fertilizers were used (Pereg *et al*., 2018a). Increased levels of inorganic N fertilizers are associated with a decrease in free-living diazotrophs in the soil (Coelho *et al*., 2009), and the activity of nitrogenase is suppressed by high levels of ammonia and nitrate (available forms of N) (Bisseling *et al*., 1978). Highly available N in soil amended with fertilizers might slow down N fixation considerably, abolishing the competitive advantage diazotrophs may have in N-free environments. N fixation needs large amounts of adenosine triphosphate (ATP) and reducing equivalents (Chan *et al*., 1994). Thus, the availability of a carbon source is essential for diazotrophs. Phosphorus fertilization also stimulates N fixation (Reed *et al*., 2007), possibly due to the high requirements of ATP to fuel the process.

In a constant/permanent system, such as viticulture, organically supplemented soil contains greater levels of available phosphorus (AP), mainly in organic forms, which are gradually released into the soil than soil under commercial fertilization (García-Orenes *et al*., 2016). However, in the cotton system, where the soil in being cultivated and prepared annually for sowing, the use of P fertilizers, as set out in industry guidelines (NUTRIpak, Cotton CRC Australia), maintains similar levels of P (and total N) in all farms, and thus P is not expected to be a factor associated with *nifH* abundance. The organically amended Soils C and T contained significantly higher concentrations of Fe and Mn than Soils B, Q, and P3 (Figure 2). The presence of *nifH* may also be affected by Fe content since the [4Fe-4S] cluster is required as a cofactor of nitrogenase (Gavini and Burgess, 1992). It is important to note that additional parameters, including moisture, compaction, and oxygen level, may also influence the abundance and activity of N-cyclers in the soil (Menneer *et al*., 2004; Meng *et al*., 2017; Morugán-Coronado *et al*., 2019). Our results suggest that increasing SOC using organic amendments helps to sustain the diazotrophic communities in cotton soils.

In contrast to *nifH*, the higher abundance of urease-encoding *ureC* in Soil B, when compared to Soil C, was expected, given the higher concentration of urea applied to Soil B. The *ureC* gene encodes the alpha subunit of the bacterial enzyme urease, which catalyzes the hydrolysis of urea (Koper *et al*., 2004). Koper *et al*. (2004) highlighted the ecological advantage of the urease enzyme, allowing ureolytic bacteria (Urease producing bacteria) to hydrolyze urea as a source of ammonia and CO_2 and modify the pH in their proximity. In fertilized systems, the hydrolysis of urea plays a crucial and significant role in soil nitrogen balance. The high concentrations of nitrate in Soils B and T, where the highest concentrations of urea were applied, lead us to suggest that the ammonia initially produced from the urea was relatively quickly converted to ammonia and then nitrate.

3.2.2 Abundance of denitrifiers

Denitrification gene abundances (*nirK, nirS,* and *nosZ*) were more significant in organically amended Soils C and T than inorganically fertilized soils in Qld and NSW, respectively. Pereg *et al*. (2018a) also showed an increase in denitrifier abundance in grapevine soil treated with organic matter when compared to inorganic fertilization. The great variations in abundance and diversity of denitrifiers in different soils lead to a complex range of influences of fertilizers on denitrifying communities (Hirsch and Mauchline, 2015). Nevertheless, it has been elucidated that overall organic fertilization enhances denitrification in soils (Philippot *et al*., 2007). In contrast to Zhang *et al*. (2013), who demonstrated mixed responses of denitrification genes (*nirS*, *nirK,* and *nosZ*) to various soil amendments, and in agreement with Clark *et al*. (2012) for wheat growing soil at Rothamsted, our results in cotton soils showed overall similar abundance of *nosZ*, *nirK* and *nirS* under all fertilization strategies.

Pereg *et al*. (2018a) discussed the need to measure both *nirS* and *nirK* in terrestrial soil ecosystems. Also, they showed similar patterns of *nirS* and *nirK* abundance under organic and inorganic fertilized grapevine soil in Spain. Clark *et al*. (2012) and Hallin *et al*. (2009) found variations of the *nirS*/*nirK* ratios depending on the treatment and land use, suggesting that the habitats created by the various treatments may select for denitrifiers possessing *nirS* or *nirK*. Hallin *et al*. (2009) suggested that NirS and NirK are not ecologically redundant, although they are functionally equivalent. Nevertheless, we found no greater abundance of any particular denitrification

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gene in cotton soils.

The higher *nosZ* abundance in Soil C, which was supplemented with compost, suggests the presence of larger communities of denitrifiers with the capacity to diminish nitrous oxide (N_2O) to N_2 (Miller *et al*., 2008; Hallin *et al*., 2009). Soil C had a relatively high C:N ratio, which Huang (2004) found to correlate with decreased N_2O emissions. Pereg *et al*. (2018a) also found a larger *nosZ*-containing denitrifier community in organically amended Spanish grapevine soil compared to inorganic fertilization. A larger community of nosZ-containing denitrifiers that can remove greenhouse gas nitrous oxide, supported by higher SOC, suggests that compost amendments can help reduce N_2O emissions from agricultural soils. Interestingly, some microbial species contain a functional *nosZ* gene (Sanford *et al*., 2012) but not other genes related to denitrification. It is important to identify agricultural management practices that support *nosZ*-harboring microbes with the potential to reduce greenhouse gas emissions and provide conditions that enhance denitrification (Hirsch and Mauchline, 2015).

3.2.3 Abundance of nitrifiers

Bacterial and archaeal nitrifiers showed different responses to the addition of compost and manure to cotton soils: higher archaeal *amoA*-arch abundance was observed where compost was added (Soil C) in Qld, but there was no difference between Soils Q, P3, and T in NSW, despite the addition of manure to Soil T. Furthermore, bacterial *amoA*-B abundance was higher where manure was added (Farm T) in NSW, but there was no difference between Soils B and C in NSW, despite the addition of compost to Soil C. Wessen *et al*. (2010), Zhang *et al*. (2010) and Pereg *et al*. (2018a) also found differential responses of bacterial and archaeal groups to soil management, although with contrasting patterns. In agreement with our results, Zhang *et al*. (2017) found significant effects when pig manure was used, particularly on bacterial ammonium oxidizers. Similarly, the size of bacterial but not archaeal nitrifier communities has been shown to increase in response to the application of animal manure (Wakelin *et al*., 2013). Hai *et al*. (2009) also demonstrated an uneven distribution of bacterial nitrifiers in tropical sorghum soils amended with manure or straw with or without urea. However, the archaeal nitrifiers were evenly distributed across these treatments. Similar to Hai *et al*. (2009) and Santoro *et al*. (2008), Pereg *et al*. (2018a) estimated that archaeal nitrifier communities are more stable than bacterial ones and are not as sensitive to variations in environmental conditions. Our observations suggest that in wide-acreage cotton production in vertisols, archaeal communities are also affected by fertilization, but differently from their bacterial counterparts. Some ammonia-oxidizing archaea are capable of autotrophic CO_2 fixation (Pratscher *et al*., 2011), possibly explaining why archaeal *amoA* abundance was independent of SOC as observed in grapevine soils by Pereg *et al*. (2018a) and in Australian cotton field soils in this work.
Our results showed a greater (approx100-fold) archaeal abundance than bacterial nitrifiers in the soil. This is in agreement with Hai *et al*. (2009), Di *et al*. (2009), Zhang *et al*. (2017), and Pereg *et al*. (2018a), and as concluded by Leininger *et al*. (2006). The archaeal nitrifiers require less energy and ammonia than bacterial nitrifiers and are thus expected to be more abundant in unfertilized soils and natural soils such as forest soils (Martens-Habbena *et al*., 2009; Tourna *et al*., 2011). Nevertheless, as shown here for cotton soils, archaeal nitrifiers are more abundant in various arable and agricultural soils (Zhalnina *et al*., 2013; Pereg *et al*., 2018a). It is unclear what the relative contribution of each group to soil nitrification is.

Extensive N fertilization might stimulate nitrifying microbes to produce excess nitrate and lead to the eutrophication of groundwater, aquifers, lakes, and estuaries through leaching, becoming an environmental hazard (Vitousek *et al*., 1997; Galloway *et al*., 2008). As discussed in Section 4.2, the addition of compost to cotton Soil C enhanced the abundance of denitrifiers that can remove nitrate from the soil, in particular *nosZ*-containing bacteria, which also remove the intermediate greenhouse gas nitrous oxide, serving to sustain soil and environmental sustainability.

Some ammonia-oxidizing bacteria are also ureolytic; for example, those isolated from acidic soils can grow at lower pH with urea as an ammonia source. They may also have an advantage in soils receiving animal wastes or urea fertilizers (Koper *et al*., 2004). Nevertheless, we could not detect any relationship between nitrifier and urealytic microbial abundances in our work. If anything, there was a negative correlation between the abundance of *ureC* and *amoA*-arch. The structure of Soil microbial community worldwide is affected and enhanced by pH (Fierer and Jackson, 2006; Noll and Wellinger, 2008; Wakelin *et al*., 2008; Griffiths *et al*., 2011; Zhalnina *et al*., 2015). Nevertheless, there was no

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significant difference in the pH between the organic and the commercial treatments in cotton soils, so this factor cannot explain the differences in N cycler abundance found in cotton soils under various fertilizations.

3.2.4 The relationship between cotton fertilization, soil properties, and N cycle abundances

PCA was performed to evaluate the influence of cotton production management strategies on soil properties and gene abundance. PC1 separated the organic fertilization samples from the inorganic fertilization ones, indicating that SOC, soil bacterial presence, and particularly N fixers and denitrifiers may explain the differences between the soil treated only with inorganic fertilizers and the soil treated also with organic matter. While inorganic fertilization is expected to boost the soil with short-lived nitrate and ammonia at relatively high concentrations, we suggest that organic matter breakdown gradually supplies available N and carbon into the soil, sustaining the communities of diazotrophs and denitrifiers. Indeed, microbial communities in soils are greatly influenced by the choice of soil treatment. For example, organically fertilized systems were characterized by specific microbial guilds that can degrade complex organic compounds, such as compost and manure, whereas systems not treated with manure harbored a dispersed and functionally diverse community characterized by presumably oligotrophic organisms adapted to nutrient-limited environments (Hartmann *et al*., 2015).

In contrast to our findings and the findings by Pereg *et al*. (2018a) that *nirS*/*K*, *nifH,* and *nosZ* showed similar patterns and clustered together with SOC, Hai *et al*. (2009) found a reduced abundance of *nifH*, but not *nirK*/*S*, in straw-treated and control soils when compared to manure-treated tropical agricultural soil. Azziz *et al*. (2017) showed that *nirS*- and *nirK*-containing denitrifiers were differentially influenced by rice cultivar, water management, and soil type. In a study by Morales *et al*. (2010), the abundance of *nifH* had a weak negative correlation with the abundance of *nirS*. In addition, *nirS* abundance exhibited a different trend to *nosZ*. It showed no correlation with organic carbon (Morales *et al*., 2010), highlighting the great variation in soil microbial communities between geographical locations, different soils, land management practices, and environmental conditions. Pereg *et al*. (2018a) noted that comparisons with studies published by other groups might be compromised by the possibility that microbial diversity may also reflect temporal factors and technical differences.

The positive correlation between *nifH* and the denitrification genes cannot be explained by a general increase in soil bacteria since there was no correlation between these genes and the 16S rRNA gene abundance pre-planting. Various soil bacteria, such as rhizobia (Bedmar *et al.,* 2005) and azospirilla (Danneberg *et al*., 1986), contain both the *nifH* and the *nir* genes. They can N fixation and denitrification, although which pathway is functional at any particular time depends on the environmental and cellular conditions. Pereg *et al*. (2018a) concluded that N fertilization strategies should consider ways to maximize N cycling, with higher N fixation than denitrification and nitrification rates, to avoid uptake of the newly fixed N by microbes in competition with plants. Various techniques, such as transcriptomics, proteomics, and situ enzymatic activity, should be used to determine which soil conditions would activate each particular process at any given time. The Australian cotton industry has cofounded Cotton LEADS™, a partnership between the Australian and US cotton industries, demonstrating a commitment to the supply and use of responsibly produced cotton through national and international efforts. (http://cottonaustralia.com.au/cotton-library/fact-sheets/cotton-fact-file-australian-cotton-history1). The results of this study show the potential of organic supplements in contributing to such goals as sustaining healthy soils by adopting responsible agricultural practices.

While we showed here the potential of manure and compost in improving the potential of the cotton soil for N cycling, it is important to note that mineralized organic N originated from animal manures, and crop residues may be lost by the same routes as mineral N fertilizers. There is still the risk of nitrate leaching due to higher denitrification potential than in comparable conventionally fertilized soils (Hirsch and Mauchline, 2015 and references within), especially in soils with low NosZ abundance. Animal and bird manure has also been shown to be a source of human pathogens (Loynachan, 2013) and of antibiotic resistance if sourced from antibiotic-treated animals (Wepking *et al*., 2017). Plant residues can also harbor microbes pathogenic to the grown crop and be a source of inoculum for soilborne diseases (Pereg, 2013). It is, therefore, essential to carefully consider the concentration of the organic matter applied to avoid over-fertilization and N leaching, incorporating the organic matter into the soil to

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avoid N loss, and the source of the organic matter to be free of human, animal, or plant disease.

4. CONCLUSIONS:

Organic amendment using mulch or manure as a supplement to commercial fertilization enhanced cotton soil prospects for microbial functions necessary for N input and output into the biosphere, particularly N fixation and denitrification. This work conducted in broad-acre cotton fields estimated the importance of analyzing adequate numbers of experimental replicates when working in large heterogenic systems. Increased copy numbers of the *nifH*, *nirS*, *nirK,* and *nosZ* genes under organic management correlate with a significant increase in organic carbon, which was the essential factor leading to an increased abundance of N fixers and denitrifiers. Increased *nosZ* abundance is particularly important for diminishing the potent greenhouse gas nitrous oxide. Thus, the environmental benefits of organic soil supplementations go beyond sustaining healthy and fertile agricultural soils, as long as they are applied responsibly, using reliable sources of manure and compost and in appropriate amounts to avoid problems such as the introduction of pathogens or nitrate leaching. Further research is required to determine the best practices when using organic supplements in crop production.

5. DECLARATIONS

5.1. Study Limitations

This study has few limitations in measure used to storage the samples; while freezing at -80°C is often considered the only way to preserve soil samples for microbiome analysis, alternative methods like refrigeration for microbial inoculation studies or air-drying for long-term archives can be valuable depending on the experimental design.
Furthermore, improvement in data normalization and statistics was made, so we may need to address overparameterization between variables. This could involve techniques like dimensionality reduction or principal component analysis.

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5.4. Competing Interests

None.

5.5. Open Access

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(A)

(B)

Figure 1. *Cotton soil sampling locations and method. The provided map of eastern Australia shows the cotton growing belt and sampling location: Soils B and C in Theodore, Qld; Soil P3 in Breeza. Soil Q in Narromine; and Soil T in Riverina, NSW (1A). The insert in Figure A shows an example of the sampling design (recorded during sampling using a Garmin GPSMAP 62s GPS). Soil samples were extracted from the highest points along elevated rows, using metal corers of 22 mm in diameter and 15 cm deep (B).*

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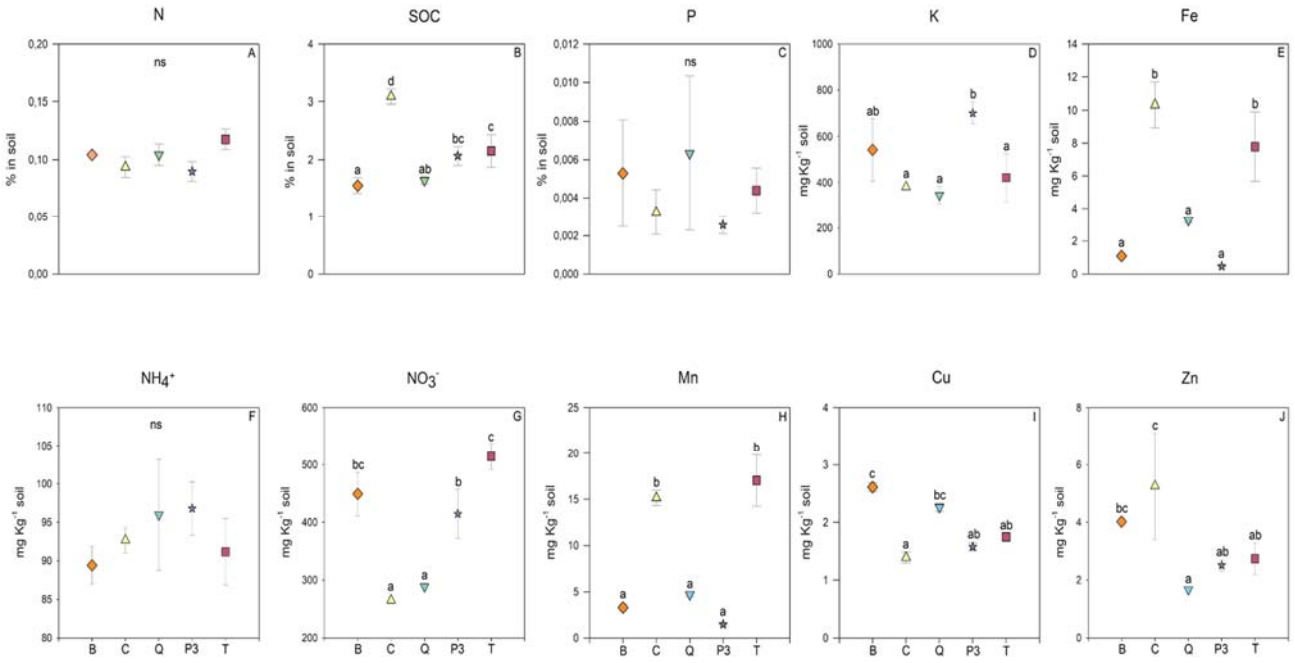

Figure 2. Chemical properties of cotton soils. Mean values (± standard deviation) are presented. Different letters indicate significant differences (one-way ANOVA, $P<0.05$) between treatments. Non-significant differences are shown as "ns". N, available nitrogen; SOC, soil organic carbon; P, available phosphorous; K, potassium; Fe, iron; NH_4^-, ammonium; NO_3^-, nitrate; Mn, manganese; Cu, copper; Zn, zinc. Letters B, C, Q, P3, and T indicate Soils B, C, Q, P3 and T, respectively.

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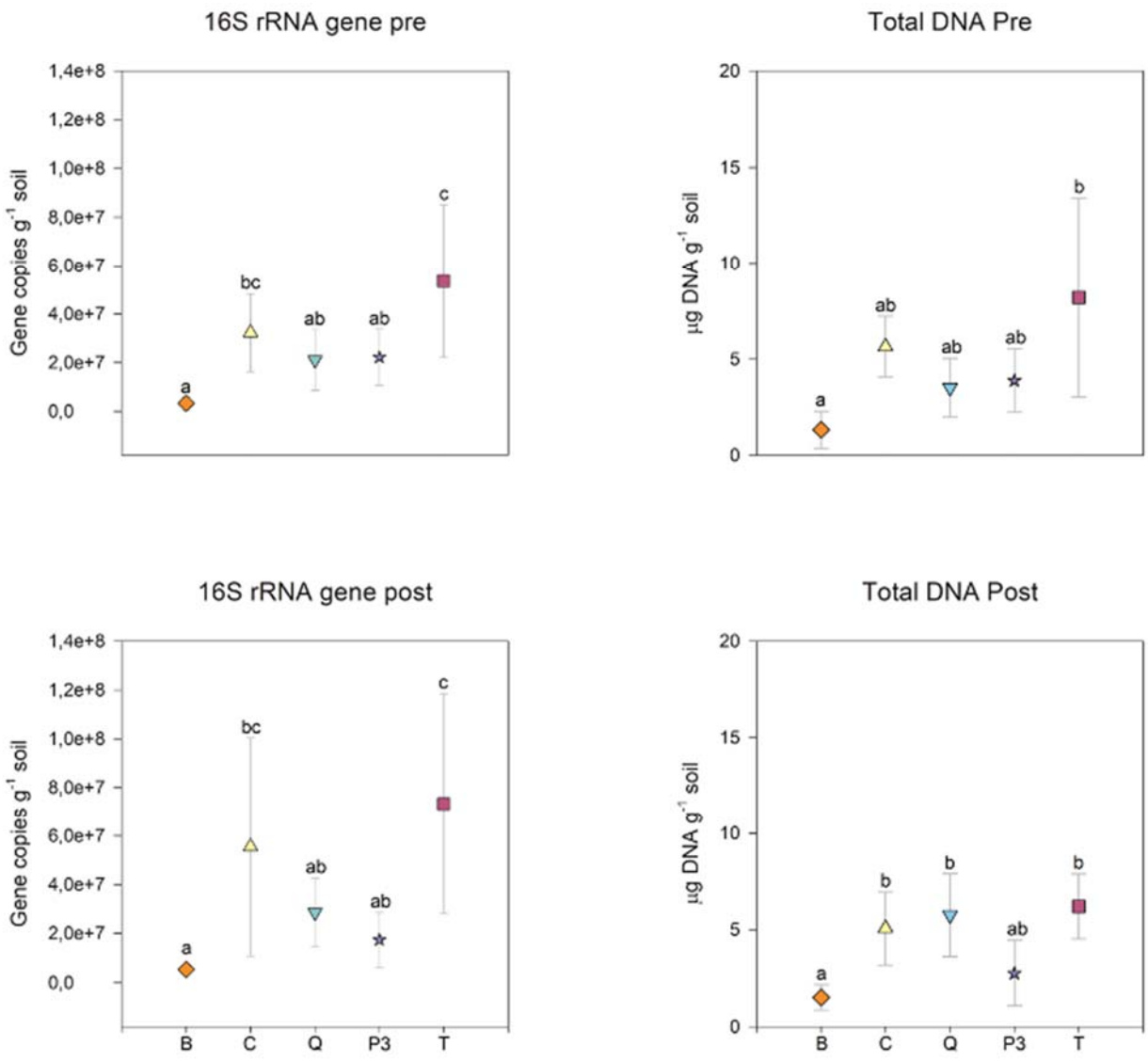

Figure 3. *Mean values (± standard deviation) of total DNA and 16S rRNA gene in cotton soil. Different letters indicate significant differences (one-way ANOVA, P<0.05) between treatments. Letters B, C, Q, P3, and T indicate Soils B, C, Q, P3, and T, respectively. Pre-planting (Pre); Post-planting (Post) at the 6-8 leaf seedling stage.*

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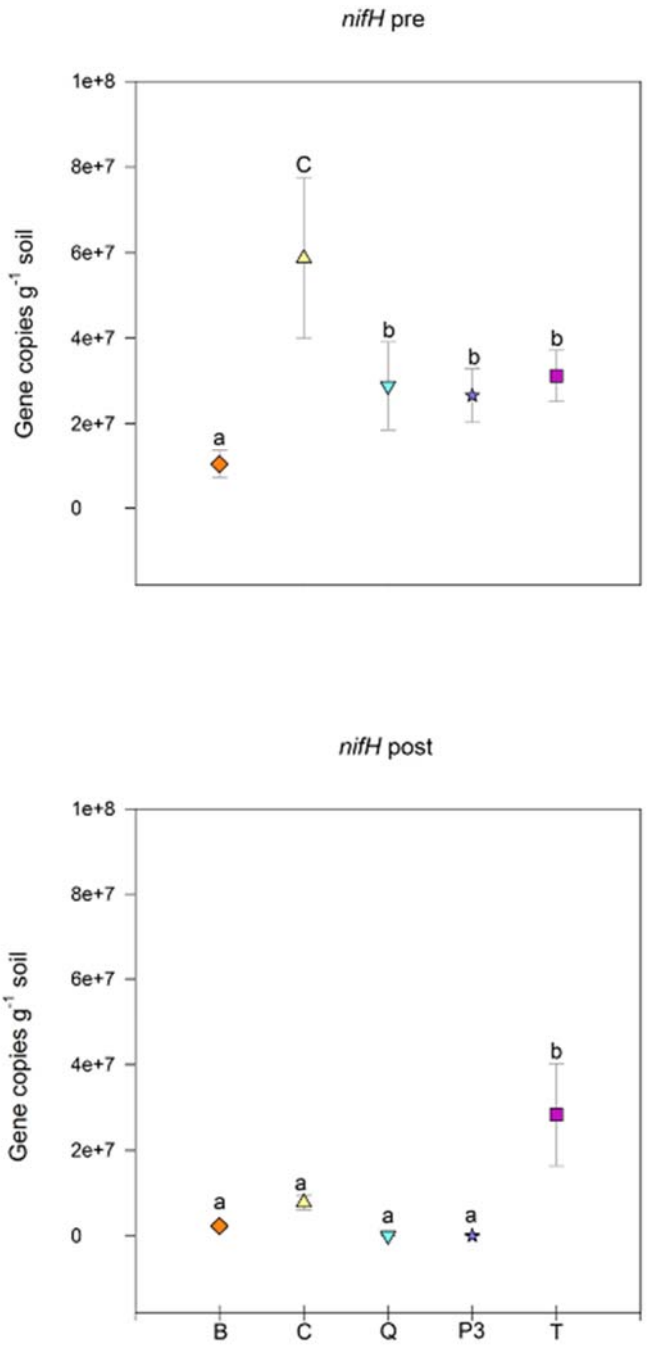

Figure 4. *Mean values (± standard deviation) of nitrogen fixation (nifH) genes in cotton soil. Different letters indicate significant differences (one-way ANOVA, P<0.05) between treatments. Letters B, C, Q, P3, and T indicate Soils B, C, Q, P3, and T, respectively. Pre-planting (Pre); Post-planting (Post) at the 6-8 leaf seedling stage.*

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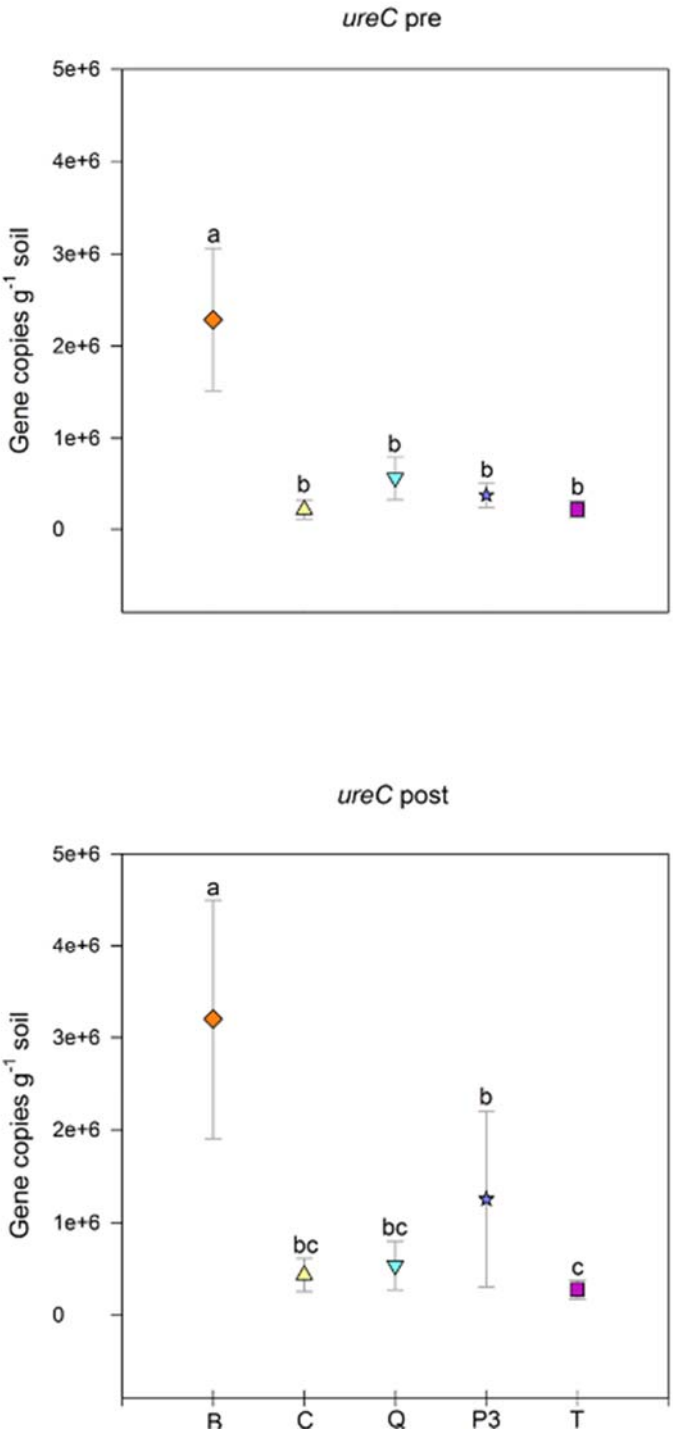

Figure 5. *Mean values (± standard deviation) of urease (ureC) genes in cotton soil. Different letters indicate significant differences (one-way ANOVA, P<0.05) between treatments. Letters B, C, Q, P3, and T indicate Soils B, C, Q, P3, and T, respectively. Pre-planting (Pre); Post-planting (Post) at the 6-8 leaf seedling stage.*

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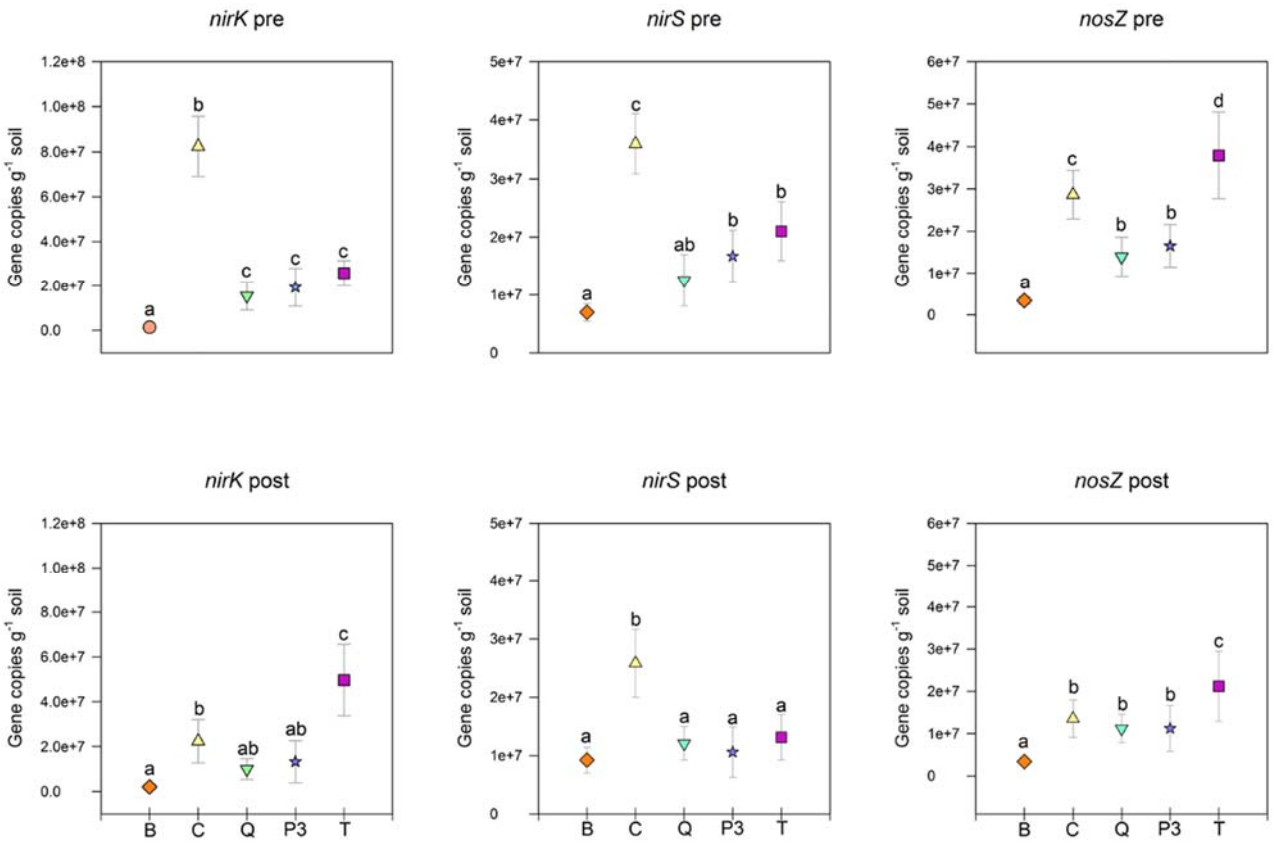

Figure 6. *Mean values (± standard deviation) of denitrification genes in cotton soil. Different letters indicate significant differences (one-way ANOVA, P<0.05) between treatments. Letters B, C, Q, P3, and T indicate Soils B, C, Q, P3, and T, respectively. Pre-planting (Pre); Post-planting (Post) at the 6-8 leaf seedling stage.*

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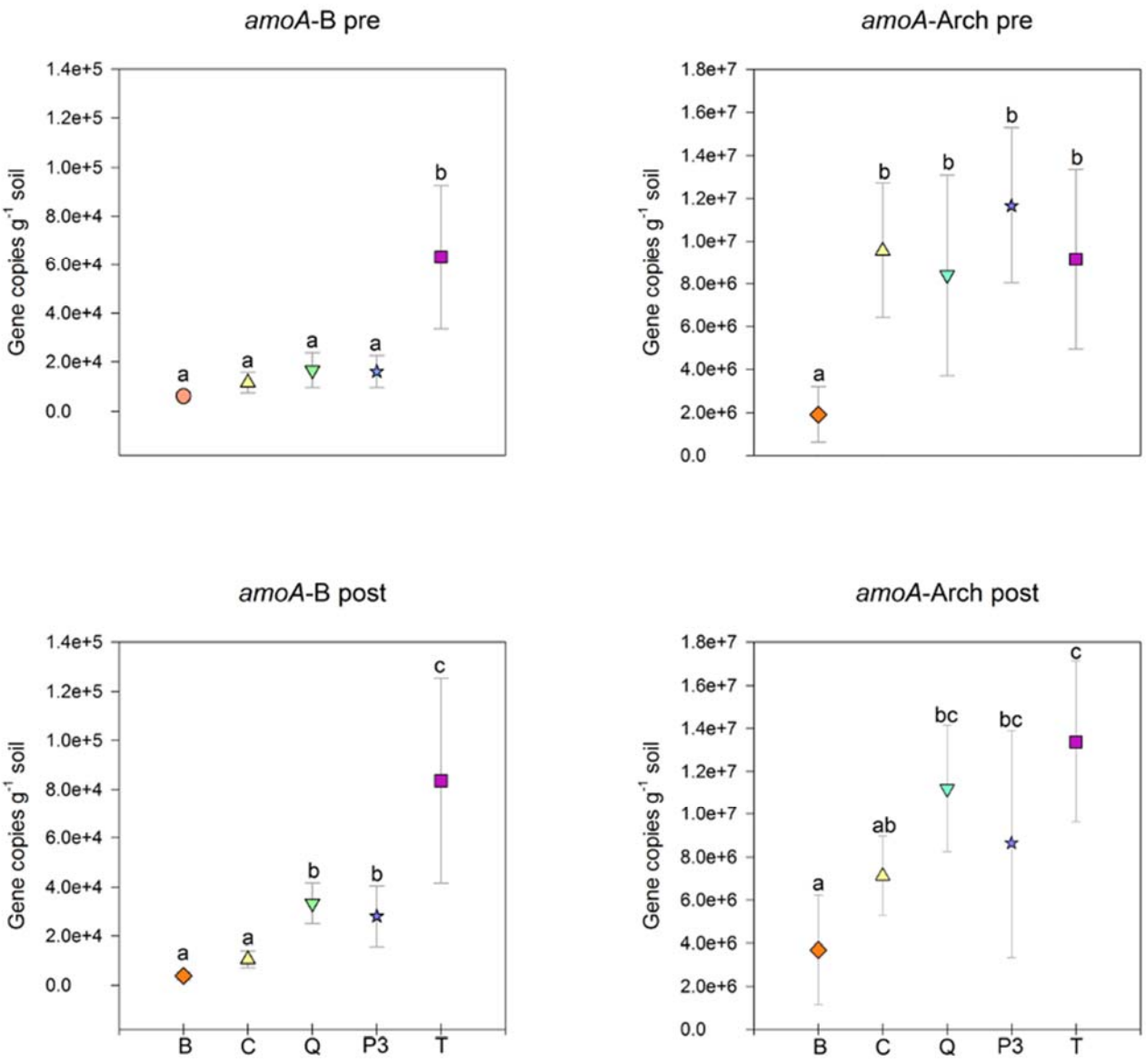

Figure 7. *Mean values (± standard deviation) of bacterial (amoA-B) and archaeal (amoA-arch) nitrification genes in cotton soil. Different letters indicate significant differences (one-way ANOVA, P<0.05) between treatments. Letters B, C, Q, P3, and T indicate Soils B, C, Q, P3, and T, respectively. Pre-planting (Pre); Post-planting (Post) at the 6-8 leaf seedling stage.*

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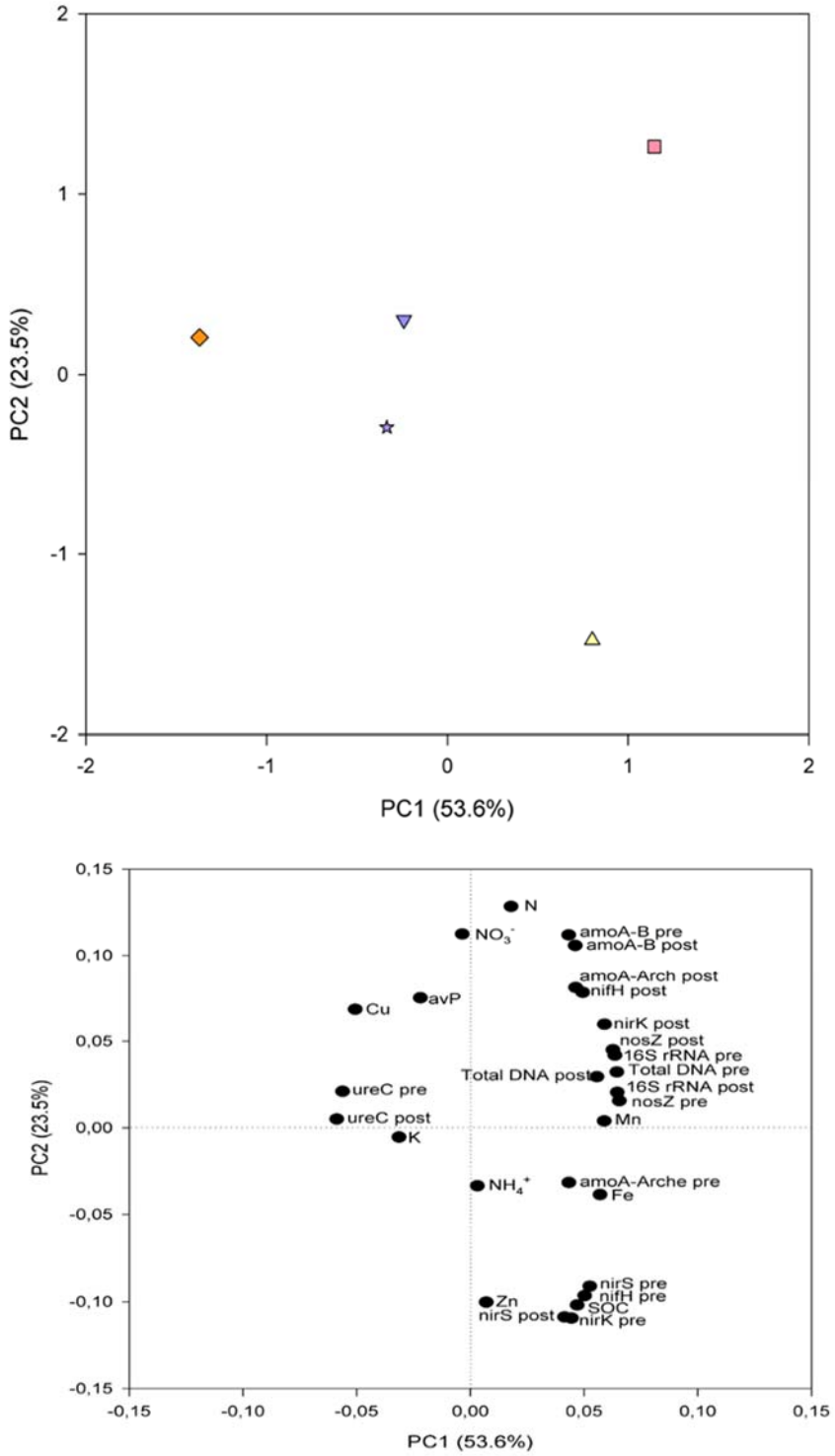

***Figure 8**. Scores (A) and loadings (B) plots from principal component analysis performed on all soil parameters. (▼) Q, (♦) B, (□) T, (☆) P3 and (△) C. SOC: soil organic carbon; N: available nitrogen; NO_3^-: Nitrate; NH_4^+: Ammonium; Av P: available phosphorus; K: potassium; Fe: iron; Mn: manganese; Cu: copper; Zn: zinc. 16S rRNA: 16S rRNA gene; Pre: before planting, from rows prepared for sowing; Post: after planting, at the seedling stage.*

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Table 1. *General information on the 2014/15 cotton season from fields participated in this study*

Soil	**B**	**C**	**Q**	**P3**	**T**
Region	Theodore Qld	Theodore Qld	Boggabri NSW	Breeza NSW	Carrathool NSW
Soil type	Heavy black cracking clay	Light clay with hard-setting properties	Brown/Red	Black Self Mulching cracking clay of basalt origin	Clay-to-clay loam
Rotations	Cotton	Cotton (some years followed by wheat)	Cotton (2013/14 season fallow, 2011/12 season mung bean)	Cotton (2010/11 season wheat)	Cotton (2010/11 season wheat)
Cotton cultivar	74BRF Dynasty/ Genero	71BRF Dynasty/ Genero	CSD-treated seed for cutworm and wireworm	71BRF	74 BRF Dynasty Amparo
Fertilizer	Urea at 500 kg/ha; Rock Phosphate at 300 kg/ha; NTS Stabilized Boron at 25kg/ha	**Seasonal compost at 10 t/ha) pre-planting;** Urea at 350 kg/ha; Previous year: Muriate of Potash (41% K; 18% S) at 150kg/ha	Cotton Sustain (6.1% N; 12% P; 22.5% K; 2.2% S; 0.55% Zink) @ 80kg per ha; 160 kg Big N (82% ammonia N) per ha	Urea-Ammonium Nitrate solution (170KgN/ha); Phosphate (22kg P/ha)	**Seasonal manure at 5 t/ha pre-planting;** 250kgs/ha cotton starter; 535- 600 kg urea Foliar; 5kg/ha Nutrivant cotton (urea 4.1%, nitrate 4.9%; P 9%; K 37%; Mg 2%; Fe 0.2%; Mn 0.1%; B 0.05%; Cu 0.05%; Mo 0.02%; Zn 0.1%)
Max yield	12.5 tone/ha	11.8 bales/ha	10 bales/ha	9.9 bale/ha	11.7 bale/ha
Crop establishment	No problem	No problem	No problem	Not reported	Good
Disease	None	None	None	Black root rot	None

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Table 2. *Climate information sourced from the Australian Government Bureau of Meteorology (BoM).*

Location	Temperature (ºC)	Rainfall (mm)	BoM Temp/humidity	Climate[a]
Theodore, QLD	Mean: 21.15. Mean max: 28.4 Mean min: 13.9 Hottest month: Dec (19.3 min to 33.5 max). Coldest month: July (6.2 min to 21.6 max).	Annual av.: 734 Max: Feb (av. 105.6), Min: Sep (av. 26.3)	Hot, humid summer	Hot Semiarid (BSh) Low latitude
Breeza, NSW	Mean: 18.4 Mean max: 24.6 Mean min: 12.2 Hottest month: Jan (18.9 min to 32 max). Coldest month: July (4.8 min to 16.1 max).	Annual av.: 638 Max: Jan (av. 83.7), Min: August (av. 35.6)	Warm summer, cold winter	Warm oceanic/humid subtropical (Cfa)
Boggabri, NSW	Mean: 18.15. Mean max: 26.1 Mean min: 10.2 Hottest month: Jan (18.3 min to 34.3 max). Coldest month: July (2.1 min to 17.1 max).	Annual av.: 557 Max: Dec (av. 81.5), Min: April (av. 21.6)	Hot dry summer, cold winter	Warm oceanic /humid subtropical (Cfa)
Carrathool, NSW	Mean: 17. Mean max: 23.9 Hottest month: Jan (17.1 min to 33 max). Coldest month: July (3.5 min to 14.5 max).	Annual av.: 402.2 Max: Oct (av. 38), Min: April (av. 27.6)	Hot dry summer, cold winter	Cold Semiarid (BSk), Middle latitude

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***Table 3**. Primer sets and qPCR conditions used in this study*

Target	Primers [a]	Optimized cycle conditions [b]	Primer reference
nirS	Cd3aF/R3cd		Throbäck *et al*. (2004)
nirK	nirk876/nirk1040		Henry *et al*. (2004)
nosZ	nosz2F/nosz2R		Henry *et al*. (2006)
nifH	MMF2: TNATCACCKCNATCACTTCC MMR1: CGCCGGACKWGACGATGTAG	10 min 95ºC; 40 cycles of 15 sec 95ºC, 30 sec 60ºC, 30 sec 72ºC	This work[c]
ureC	ureC-F: TGGGCCTTAAAATHCAYGARGAYT GGG ureC-R: GGTGGTGGCACACCATNANCATRTC	5 min 95°C; 40 cycles of 10 sec 95°C, 30 sec 55°C, 30 sec 72°C	Reed (2001)
amoA-B	amoa1F/amoa2R		Rotthauwe *et al*. (1997)
amoA-Arch	Arch-AmoAF/Arch-AmoAR		Francis *et al*. (2005)
16S rRNA gene	16s Forward/16s reverse		Mohammadi *et al*. (2003)

a: unless specified here, primer sequences were as described by Pereg *et al*. (2018a,b).
b: unless specified here, cycle conditions which were tested and found to be optimal for this work, were as described by Pereg *et al*. (2018a,b)
c: Based on multiple sequence alignment of available *nifH* sequences from GenBank database to identify conserved regions and degenerate primers within these regions designed using Primer3 (http://frodo.wi.mit.edu/). Since the *nifH* primers were designed in this work, 12 random plasmids were selected for sequencing the cloned *nifH*-amplified fragments and were confirmed to contain *nifH* sequences.

***Table 4.** Correlation coefficients (r values) for relationships between soil properties and functional gene abundances.*

Properties	Mn	*nirK*	Total DNA	*ureC*	*nosZ*	*nirS*	*nifH*
SOC		.974**				.988**	.935*
Fe	.948				890		
Mn					.	.977**	
nirK							.973**
amoA-Arch				-.943*			
16S rRNA			.997**		.981**		
Total DNA					.993**		
nirS							.969**

All 11 soil properties measured (N, SOC, NH_4^+, NO_3^-, P, Cu, Mn, Fe, Zn, K, Total DNA) and all genes analyzed (*nifH*, *nirS/K*, *nosZ*, *amoA*-B, *amoA*-arch, *ureC*,16S rRNA gene) pre-planting in this work were included in the correlation analysis. Only those that showed positive correlations are included in the table. Significance: *$P < 0.05$, **$P < 0.01$ (n=15 for soil properties and n=50 for genes analyzed).

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Table 5. *Principal components analysis of soil properties for the five cotton soils: B, C, Q, P3, and T (n=15 for soil properties and n=50 for genes analyzed).* ***Bold*** *means values that significantly explain the variance.*

Variance explained	PC1	PC2
	(53.6%)	**(23.5%)**
***nosZ* pre**	.984	.103
16S rRNA gene post	.971	.136
Total DNA pre	.968	.213
16S rRNA gene pre	.954	.277
***nosZ* post**	.942	.297
***nirK* post**	.886	.394
Mn	.884	.027
***ureC* post**	-.881	.034
Fe	.857	-.253
***ureC* pre**	-.843	.139
Total DNA post	.836	.195
***nirS* pre**	.789	-.600
Cu	-.760	.452
***nifH* pre**	.755	-.636
***nifH* post**	.743	.516
SOC	**.706**	**-.671**
amoA-Arch post	**.696**	.534
amoA-Arch pre	**.651**	-.208
avK	-.471	-.035
N	.269	**.847**
$NO3^-$	-.053	**.741**
amoA-B post	.650	**.738**
nirK pre	.668	**-.721**
nirS post	.622	**-.717**
amoA-B pre	.693	**.698**
avZn	.106	**-.660**
avP	-.330	.495
$NH4^+$	.047	-.220