Manure application increased denitrifying gene abundance in a drip-irrigated cotton field

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Application of inorganic nitrogen (N) fertilizer and manure can increase nitrous oxide (N_2O) emissions. We tested whether manure addition can affect the abundance of N_2O-producing nitrifier and denitrifier functional genes and the diversity of microbial communities. A field experiment was conducted in a drip-irrigated cotton field in arid region of northwestern China to investigate how two years' heavy applications (240 kg N ha^{-1}) of urea and animal manure affected the abundance of the *amoA* gene involved in nitrification, and *narG, nirS, nirK* and *nosZ* genes involved in denitrification, as well as the structure and diversity of the bacterial community. Treatments included plots not amended (Control), and plots amended with urea (Urea), animal manure (Manure) and a 50/50 mix of urea and manure (U+M). Manure were broadcast-incorporated into soil before seeding while urea was split-applied with drip irrigation (fertigation) over the growing season. The addition treatments did not affect Operational Taxonomic Unit (OTU) richness or Shannon diversity index of soil bacterial community but change soil bacterial community structure. Compared to Control, addition of manure (U+M and Manure) significantly increased the abundance of nitrate reducer (*narG)*, and denitrifiers of *nirK* and *nosZ*. Manure addition (U+M and Manure) did not affect nitrifying enzyme activity (NEA) but resulted in 39-59 times greater denitrifying enzyme activity (DEA) compared to the non-manure amended (Control and Urea) treatments. In contrast, urea application had no impact on abundances of nitrifier and denitrifier gene, DEA and NEA, likely due to a limitation of C availability. DEA with manure application was highly correlated (r = 0.70 - 0.84, $P < 0.01$) with abundance of nitrate reducer (*narG)*, and denitrifiers of *nirK* and *nosZ*. Increase in abundance of these functional genes were further correlated with soil NO_3^-, dissolved organic carbon, total C, total N and

C:N ratio. These results demonstrated a positive relationship between the abundances of denitrifying functional genes (*narG*, *nirK*, and *nosZ*) and denitrification potential in response to manure application, which could contribute to the increased N_2O emissions.

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ABSTRACT

Application of inorganic nitrogen (N) fertilizer and manure can increase nitrous oxide (N_2O) emissions. We tested whether manure addition can affect the abundance of N_2O-producing nitrifier and denitrifier functional genes and the diversity of microbial communities. A field experiment was conducted in a drip-irrigated cotton field in arid region of northwestern China to investigate how two years' heavy applications (240 kg N ha^{-1}) of urea and animal manure affected the abundance of the *amoA* gene involved in nitrification, and *narG, nirS, nirK* and *nosZ* genes involved in denitrification, as well as the structure and diversity of the bacterial community. Treatments included plots not amended (Control), and plots amended with urea (Urea), animal manure (Manure) and a 50/50 mix of urea and manure (U+M). Manure were broadcast-incorporated into soil before seeding while urea was split-applied with drip irrigation (fertigation) over the growing season. The addition treatments did not affect Operational Taxonomic Unit (OTU) richness or Shannon diversity index of soil bacterial community but change soil bacterial community structure. Compared to Control, addition of manure (U+M and Manure) significantly increased the abundance of nitrate reducer (*narG)*, and denitrifiers of *nirK* and *nosZ*. Manure addition (U+M and Manure) did not affect nitrifying enzyme activity (NEA) but resulted in 39-59 times greater denitrifying enzyme activity (DEA) compared to the non-manure amended (Control and Urea) treatments. In contrast, urea application had no impact on abundances of nitrifier and denitrifier gene, DEA and NEA, likely due to a limitation of C availability. DEA with manure application was highly correlated (r = 0.70 - 0.84, $P < 0.01$) with abundance of nitrate reducer (*narG)*, and denitrifiers of *nirK* and *nosZ*. Increase in abundance of these functional genes were

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41 **Keywords** Nitrifier, Denitrifier, Manure, Drip irrigation, Bacterial community structure,
42 Denitrifying enzyme activity

INTRODUCTION

Nitrous oxide (N_2O) accounts for nearly 8% of warming impact of anthropogenic activities and contributes to the depletion of ozone in the stratosphere. N_2O concentration in the atmosphere has increased at a rate of 0.26% per year, with more than 80% of the emissions associated with agricultural activities where organic (e.g. animal manures) or inorganic (e.g. synthetic fertilizers) sources of nitrogen (N) are added to soil (*IPCC, 2013*). Many studies have reported that manure application resulted in more N_2O emissions than inorganic N fertilizers (*Watanabe et al., 2014*; *Zhou et al., 2017*), confirming our recent observations on a drip-irrigated cotton field with low soil organic carbon in arid northwestern China (*Kuang et al., 2018*). However, it remains unclear whether the increased emissions with manure are linked with changes in microbial community, especially those involved in the processes of nitrification and denitrification.

Nitrification is a biological oxidation process in which ammonia is converted to nitrate via nitrite ($NH_3 \rightarrow NH_2OH/HNO \rightarrow NO_2^- \rightarrow NO_3^-$). The steps of nitrification are controlled by nitrifier functional genes, including (1) ammonia-oxidizing bacterial (*AOB*) and (2) archaea (*AOA*) genes, and (3) nitrite-oxidizing bacterial genes. The first step in oxidation of ammonia to NH_2OH limits the entire nitrification reaction (*Kowalchuk & Stephen, 2001*). Applications of manure or inorganic N can exert significant impact on nitrification through their effects on soil properties. For example, *Tao et al. (2017)* reported fertilizer N was the key driver for the abundance, community structure and activity of nitrifying bacteria. Long-term application of manure and inorganic fertilizers reduced the copy number of *AOA* but increased that of *AOB* for agricultural soils in cold climate of China (*Fan et al., 2011*). For a desert topsoil in Arizona of USA, long-term inorganic N addition did not affect the community structure of ammonia-oxidizing microorganisms but increased the *amoA* gene abundance of both *AOA* and *AOB* (*Marusenko, Garcia-Pichel & Hall, 2015*). In

contrast, a recent study for fertilized forest soils in China founded that soil factors such as NH_4^+ concentration and pH controlled nitrification and denitrification activities, rather than the abundance and community structure of N-cycling prokaryotes (*Tang et al., 2019*). Overall, there is very few information about how addition of N might affect the abundance of nitrifiers and nitrification activities for agricultural soils under drip irrigation.

Denitrification is a multi-step reduction process of NO_3^- to N_2 ($NO_3^-\rightarrow NO_2^-\rightarrow NO\rightarrow N_2O\rightarrow N_2$) mediated by a range of denitrifiers under oxygen limited conditions. Specific reductases encoded by functional genes regulate each step of the reaction, including, nitrate reductase (e.g. *narG*, *napA*), nitrite reductase (e.g. *nirS*, *nirK*), nitric oxide reductase (e.g. *cnorB*, *qnorB*) and nitrous oxide reductase (*nosZ*; *Simon & Klotz, 2013*). Previous studies suggested that the fertilizer effect on denitrification activity was primarily attributed to the changes in the abundance and community of denitrifiers (*Yin et al., 2015*). In a 160-year-long field experiment, *Clark et al. (2012)* reported long-term manure application increased denitrification compared to inorganic N fertilizer, which was mainly attributed to an increased abundance of *nirK*- but not *nirS*-type denitrifier. In contrast, several other studies reported that soil properties including soil water content and total N, other than denitrifier, were more important in determining rate of denitrification (*Attard et al., 2011*; *Shrewsbury et al., 2016*). The inconsistent results indicated that further studies are required to clarify the linkage between the environmental factors, denitrifying bacteria and N addition-induced denitrification activity.

Nitrogen additions including inorganic fertilizer and animal manure can affect soil microbial community directly by supplying substrates for microorganisms or indirectly by changing soil properties. Animal manure is a primary source of soil organic matter and its application can increase microbial biomass and diversity by providing carbon sources for microorganisms. In

contrast, inorganic N application generally reduces soil microbial community diversity. For example, *Zhang et al. (2017)* recently reported that application of inorganic fertilizers to acidic and near-neutral soils in a maize-vegetable rotation in southwest China significantly reduced bacterial diversity. *Sun et al. (2015)* also reported that the application of inorganic fertilizer to a wheat-soybean rotation for 30 years in central China reduced soil bacterial richness and diversity. Application of inorganic fertilizer affected the soil microbial community mainly by a decreasing soil pH (*Geisseler & Scow, 2014*).

As a dominant cash crop in northwestern China, cotton production has received intensive inputs of inorganic fertilizers and water recently as drip-irrigation due to its benefit in improving water and nutrient use efficiency by the crop (*Dai & Dong, 2014*). Cattle and sheep manure are also often used as nutrient sources due to the nearby livestock production. Our recent studies from this area clearly showed manure application greatly increased N_2O emissions compared with conventional urea, although emissions under drip irrigation were generally low (*Kuang et al., 2018*; *Ma et al., 2018*). Both nitrification and denitrification could play a role in production and emission of N_2O under field conditions, in response to varying soil conditions such as temperature, moisture, and nutrient availability. It remains unclear how additions of organic manure or inorganic fertilizer affect the gene abundances and activity of nitrifier and denitrifier communities under drip irrigated conditions.

The objective of this study was to determine the influence of inorganic fertilizer and manure application on the abundance and activities of N_2O-producing functional genes, as well as bacterial community structure in a drip-irrigated cotton field. We hypothesized that N_2O emissions from manure application were attributed to the increase of the abundance of denitrifiers and thus greater denitrification activity.

MATERIALS & METHODS

Site description and experimental design

Plot based field experiment was conducted at the Cele Research Station (37°01'N, 80°43'E) of the Chinese Academy of Sciences in the 2015-2016 growing seasons. The region has a typical arid continental climate with an extremely low long-term average annual precipitation of only 42 mm, mainly distributed between May and July. The long-term average mean annual air temperature is 12.7 °C. The soil is classified as Aridisols in the USDA soil taxonomy system. At the start of the study, the surface soil (0-20 cm) was a fine sand texture (sand 90%, silt 4%, clay 6%) with bulk density 1.46 Mg m^{-3}, pH_{H2O} 8.0, electrical conductivity (EC) 144.4 μS cm^{-1}, total Kjeldahl N 0.31 g kg^{-1}, extractable NO_3^--N 25.7 mg kg^{-1}, 0.5 M $NaHCO_3$-extractable P 14.6 mg kg^{-1}, 1.0 M ammonium acetate K 153 mg kg^{-1}, and organic matter 6.9 g kg^{-1}. Analysis of soil characteristics were based on *Carter (1993)*. Prior to this study, the experimental field was cropped to cotton for over 5 years and received both manure and urea applications in each year, in accordance to farmer's practices.

The experimental design was previously described in *Kuang et al. (2018)* and only treatments under drip irrigation was used in the current study. Briefly, the study used a randomized complete block design of four treatments with four replicate plots, giving a total of 16 plots. Each plot was 10 m long × 6 m wide and was separated from the other plots by a 1.1-m buffer zone. Treatments included (1) an unfertilized control, and application of 240 kg of available N ha^{-1} in the form of (2) granular urea (Urea, 46-0-0), (3) mixture of sheep and cattle compost (Manure), and (4) 50% urea with 50% manures (U+M). Such N application rate is commonly used by local producers for high-yielding cotton fields. For urea, 20% N was banded in the plant row before planting and the

rest was applied with irrigation water as a schedule of 5% at 9 weeks, and 15% each at 11, 14, 15, 16 and 17 weeks after planting. The manure was all applied before planting by broadcast-incorporation at 10 cm depth. The manure had a moisture content of 25% and a dry weight-based total N, P, K content of 15.6, 2.0, and 16.8 g kg^{-1}, respectively. Analysis of manure was done on subsamples digested with a mixture of perchloric, sulfuric and hydrofluoric acid. Total P and K in the acid digestion were measured using the Mo-Sb colorimetric method and atomic absorption spectrometry (Thermo Fisher, USA), respectively. Total N was determined colorimetrically after Kjeldahl digestion. The manure had an available N concentration of 28.7 mg N kg^{-1}, determined by the alkaline hydrolyze method. In each year, cotton seed (c.v. Xinluzao 48, Huiyuan Tech, Shihezi, China) was planted at 75 kg ha^{-1} in early to middle April under the plastic-mulch and drip-irrigation system, which is common for cotton production in the region. Details on the system was described by *Kuang et al. (2018)*. Before seeding, all plots received broadcast-incorporated application of 120 kg P_2O_5 ha^{-1} as calcium phosphate and 60 kg K_2O ha^{-1} as K_2SO_4.

Soil sampling

Soil samples (0-20 cm depth) were collected with a hand auger (2.5 cm diameter) in September 2016 with cotton at boll opening stage. In each plot, four soil cores were collected next to the drip tape and mixed thoroughly together for one composite sample per plot. The auger was cleaned using 95% alcohol and wiped with sterile paper before collecting the next soil sample. Each sample was passed through a 2 mm mesh screen and partitioned into three subsamples. One subsample was air-dried at room temperature for chemical analysis. The second subsample for analysis of denitrifying enzyme activity (DEA) and nitrifying enzyme activity (NEA) was stored at -20 °C and analyzed within one week. The third subsample for microbial molecular analysis was stored

158 at -80 °C.

159

160 **Soil chemical properties**

161 Soil NH_4^+ and NO_3^- was extracted using 0.01 M $CaCl_2$ and measured with a continuous flow
162 analyzer (SEAL Analytical, Norderstedt, Germany). Soil pH was measured at 1:2.5 soil:water
163 ratio. Soil total C was measured using by wet oxidation method with potassium dichromate. Total
164 N was analyzed by Kjeldahl acid-digestion method with a Kjeltec 1035 analyzer (Tecator AB,
165 Sweden). Available Fe and Cu were extracted with DPTA (0.005 M diethylenetriamine
166 penetaacetic acid + 0.1 M triethanolamine + 0.01 M $CaC1_2$ set to pH 7.3) and analyzed using ICP-
167 OES (VARIAN, USA). Soil dissolved organic carbon (DOC) was extracted using deionized water
168 (1:5 soil:water ratio) and analyzed using a TOC analyzer (Aurora 1030W, OI, USA). Soil C:N
169 ratio was calculated on the mass basis of total C and total N.

170

171 **Determination of denitrifying and nitrifying enzyme activity**

172 The frozen soil samples were pre-incubated to thaw at 25 °C for 2 days before analysis of DEA
173 and NEA. Soil DEA was expressed as the rate of N_2O production (μg N h^{-1} g^{-1} soil) and determined
174 using the anaerobic slurry technique (*Beauchamp & Bergstrom, 1993*). Briefly, 25 g thawed soil
175 samples was placed into 125 ml plasma flasks. 25 ml solution including 10 mM KNO_3, 10 mM
176 glucose, 50 mM K_2HPO_4 and 0.1 g L^{-1} chloramphenicol to inhibit new protein production was
177 added to each plasma flask. The flasks were evacuated and flushed with a 90:10 He-C_2H_2 gas
178 mixture to create anaerobic conditions and suppress N_2O-reductase activity. Flasks were then
179 shaken for 60 min and gas samples taken 0, 15, 30, 45, and 60 min after onset of mixing using an
180 orbital shake (180 rpm). Concentrations of N_2O in gas samples were immediately analyzed using

gas chromatography equipped with an electron capture detector (Agilent 7890A, Agilent Technologies, Santa Clara, CA, USA).

Soil NEA was expressed as μg NO_3^--N h^{-1} g^{-1} dry soil and determined according to *Hart et al. (1994)*. Briefly, a thawed soil sample (15 g dry soil equivalent) was placed into a 250 ml plasma flask with 100 ml solution of 1.5 mM $(NH_4)_2SO_4$ and 1 mM phosphate buffer (pH = 7.2). The flask was incubated at room temperature under constant agitation (180 rpm). Samples of the slurry were taken at 2, 4, 8, 12, and 24 h during incubation. Concentrations of NO_2^- and NO_3^- in the samples was then determined using the continuous flow analyzer. NEA rate was calculated from the linear slope of the regression of NO_2^- plus NO_3^- concentrations with time.

Soil DNA extraction and real time PCR

Soil DNA was extracted from 0.3 g of the soil sample using the Power Soil Total DNA Isolation Kit (MO-BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. The quality and concentration of DNA were estimated using a Nanodrop 1000 Spectrophotometer (Thermo Fisher, USA) and gel electrophoresis (1.0% agarose). The DNA extracts were diluted at a ratio of 1:10 with double-distilled water (ddH_2O) to reduce potential for PCR inhibition and then stored at -20 °C until use.

Quantitative PCR was used to quantify archaeal *amoA* and bacterial *amoA, narG, nirK*, *nirS* and *nosZ* gene in triplicate. All reactions were carried out in a CFX96™ (BIO-RAD, Laboratories Inc., Hercules, CA, USA). Each PCR reaction mixture contained 1μl of 10-fold diluted soil DNA as template, 10 μl SYBR® Premix Ex Taq™ II (TaKaRa, Japan), 0.8 μl of primer (10 μM) and 7.4 μl ddH_2O in a total volume of 20 μl. Primers and thermocycling conditions used in the qPCR reactions are given in Table 1. Plasmids that containing respective sequences of the targeted genes

were generated by cloning the targeted gene fragments from soil DNA into plasmid pMD^{TM} 19-T Vector (TaKaRa, Japan). Standard curves for each gene were created from 10-fold serial dilutions (10^8-10^1) of the known quantities of linearized plasmid DNA harboring aim gene sequences. All qPCR reactions were conducted in triplicate. The qPCR efficiency and slope were 92% and -3.5 ($R^2 = 0.990$) for archaeal *amoA*, 105% and -3.2 ($R^2 = 0.999$) for bacterial *amoA,* 90% and -3.7 ($R^2 = 0.999$) for *narG,* 85% and -3.7 ($R^2 = 0.997$) for *nirS*, 96% and -3.4 ($R^2 = 0.998$) for *nirK*, and 80% and -3.5 ($R^2 = 0.990$) for *nosZ*, respectively. The generally low qPCR efficiency for *nirS* and *nosZ* genes agreed with previous studies reporting similar ranges (74-90%, *Ding et al., 2014*; *Harter et al., 2014*).

High-throughput sequencing

The 16S rRNA gene of the V3-V4 hypervariable region was analyzed by MiSeq sequencing on the Illumina Miseq 2×300 bp platform at Shanghai Sangon Biotech Co., Ltd. with the universal primers 515F (GTGCCAGCMGCCGCGG) and 907R (CCGTCAATTCMTTTRAGTTT) that amplify both bacteria and archaea (*Li et al., 2014*). Both forward and reverse primers were added with a barcode. The thermocycling program were set as: an initial denaturation at 94 °C for 3 min, 5 cycles at 94 °C for 30 s, 45 °C for 20 s, 65 °C for 30 s of extension, then 20 cycles of 94 °C for 20 s, 55 °C for 20 s, 72 ℃ for 30 s, with a final extension at 72 ℃ for 5 min. The reactions were set as: 15 μl 2×Taq master Mix (Thermo Scientific, USA), 2 μl of DNA template (about 20 ng), 1μl of each appropriate primer (10 μM), 11 μl of ddH_2O. The PCR products were purified and quantified by Agencourt AMPure XP (Beckman Coulter, USA) and Qubit^{TM} ssDNA Assay Kit (Life Technologies, CA, USA), respectively. Finally, the purified PCR products of each sample were equally combined based on their concentrations and produced a DNA pool which included

16S rRNA gene amplified fragments for sequencing.

Sequencing reads were allocated to each sample based on their unique barcodes. Raw sequences were firstly processed using cutadapt software to trim the barcodes of primers. Two short Illumina reads were then merged with PEAR (v 0.9.6) software (*Zhang et al., 2014*), and finally PRINSEQ software (v 0.20.4, *Schmieder & Edwards, 2011*) was used for the quality control of the merged reads. Only sequences > 200 bp in length with an average quality score > 40 were used for further analyses. Chimeras were filtered by comparing the sequences with those in the reference database using the UCHIME algorithm (v 4.2.40, *Edgar et al., 2011*). After the above screening, the remaining high-quality sequences were clustered into Operational Taxonomic Units (OTUs) at a ≥ 97% similarity identity threshold. The singletons and low abundance OTUs were removed before further analyses. The Ribosomal Database Project (RDP) classifier (*Wang et al., 2007*) was used to identify taxonomic information at the bootstrap cutoff of 80%. Based on the OTUs output, α-diversity, and β-diversity, and canonical correspondence analysis were performed. Species richness and diversity indices including coverage, Chao1, ACE, Simpson, and Shannon were calculated using mothur (v 1.30.1, *Schloss et al., 2009*) to estimate α-diversity of each sample.

Statistical analysis

Treatment effects on soil properties, NEA, DEA, α-diversity indices, and bacterial abundance were conducted using a one-way ANOVA. Pearson correlation analysis was conducted to assess the relationships between the functional gene abundances, NEA, DEA and selected soil properties. ANOVA and Pearson correlation analysis were performed with SAS 9.3 (SAS Institute, Cary, NC) and differences were considered significant at $P < 0.05$. Principal coordinates analysis (PCoA) was performed to determine the community β-diversity of bacteria using the Vegan package

Version 1.17-7 (*Oksanen, 2011*) implemented with the R language, which was based on bacterial weighted UniFrac metric matrix. Canonical correspondence analysis (CCA) was performed with the Vegan package implemented with the R language to determine the relationships between soil physiochemical properties and bacterial communities. Untransformed data were used for the PCoA and CCA analyses. The relative abundances of bacterial community at the phylum level between treatments were compared using the Welch's t-test with STAMP (Statistical Analysis of Metagenomic Profiles). Corrected p-values of the Welch's t-test were calculated using the FDR (False Discovery Rate) for multiple testing correction.

RESULTS

Soil chemical characteristics

Manure application (both Manure and U+M treatments) increased soil total N content by half compared to the unfertilized control (Table 2). Soil NO_3^- concentrations with Manure and U+M treatments were 120 and 103 mg kg^{-1}, respectively, being 2.4-4.8 times greater than Urea and Control treatments. In contrast, soil NH_4^+ concentrations were not affected by the treatments. Soil total C and DOC were also greater in Manure and U+M compared to the Control and Urea treatments. As a result, treatments with manure addition (Manure and U+M) had 37-100% higher soil C:N ratios, compared to Urea and Control.

Denitrifying enzyme activity and nitrifying enzyme activity

DEA values of Control, Urea, Manure, and U+M treatments were 0.02, 0.01, 0.60, and 0.40 μg N_2O-N g^{-1} h^{-1} respectively, with values significantly ($P < 0.001$) greater in Manure and U+M than Urea and Control treatments (Fig. 1A). In contrast, NEA values were not affected by N addition

treatments in spite of a numerical increase for Manure and U+M over other treatments (Fig. 1B).

Bacterial community, and nitrifier and denitrifier genes

The sequence coverage index ranged between 0.93 and 0.94, suggesting that the sequencing depth was sufficient to obtain the majority of genetic diversity of samples (Table 3). The average number of effective sequences were similar for treatments, being 27,131, 28,812, 26,413, and 30,405, for Control, Urea, Manure, and U+M, respectively, with a mean read length of 376 bp. The number of OTUs was not affected by N addition and ranged between 4,072 and 4,295. The indexes of richness and diversity, Chao1, ACE, Simpson and Shannon were also not affected by the treatments.

The PCoA plot revealed a clear clustering in β-diversity of the soil bacterial community in response to addition treatments (Fig. 2). The bacterial communities under different fertilizer treatments were separated into two groups with (Manure and U+M) and without (Control and Urea) manure application along axis PCoA1, with a significant dissimilarity ($P < 0.001$). The PCoA explained 73% of the total variation in the composition of bacterial community, with PCoA1 and PCoA2 explaining 61% and 12%, respectively. At bacterial phylum level, the abundance of *Planctomycetes*, *Bacteroidetes* and *Ignavibacteriae* increased with manure additions, whereas that of *Latescibacteria*, *Acidobacteria*, *Armatimonadetes*, *Actinobacteria,* and *candidate* division WPS-2 decreased (Table 4). There was no treatment effect on the abundance of *Proteobacteria*. At archaeal phylum level, the abundance of *Thaumarchaeota and Euryarchaeota* decreased with manure application.

The gene copy number of *AOA* varied from 5.90×10^8 to 2.69×10^9 copies g^{-1} dry soil, with values for manure addition treatments (U+M and Manure) being 1.4-3.6 times greater than non-manure

addition (Urea and Control) treatments (Fig. 3A). Similarly, manure application also significantly ($P < 0.001$) increased the copy number of the *AOB* gene in manure than non-manure amended treatments. The copy number of *AOA* was generally one order of magnitude greater than that of *AOB*. Further, *AOB* copy number responded more to treatment additions than that of *AOA*. As a result, manure addition reduced the ratio of *AOA*/*AOB*, being 88.7, 27.6, 15.8, and 17.0 for Control, Urea, Manure, and U+M, respectively.

Manure addition significantly ($P < 0.001$) increased the copy number of *narG, nirK* or *nosZ* genes, but did not affect that of *nirS* (Fig. 3B-E). Copy number of *narG* was 27.5-39.0 times greater with manure (U+M and Manure) than non-manure (Control and Urea) addition treatments. Copy number of *nirK* was 3.4-3.7 times greater with manure than non-manure addition treatments. Similarly, copy number of *nosZ* gene were 9.6-25.2 times greater in manure than non-manure addition treatments.

Relationships between DEA, NEA, soil properties and microbial abundance

Copy number of nitrifier *(AOB* and *AOA*), nitrate reducer (*narG*), *nirK,* and *nosZ*-type denitrifier genes, but not *nirS*-type denitrifier gene, were positively correlated with NO_3^-, DOC, total N, total C, and C:N ratio (Table 5). In contrast, soil NH_4^+ and pH were not significantly correlated with copy number of any of the functional genes. There were also significantly positive correlations between DEA and abundance of *AOB*, *AOA, narG*, *nirK,* and *nosZ* genes. NEA, however, was not correlated with the abundance of any functional gene, except for a positive correlation with *nirK*.

Canonical correspondence analysis (CCA) revealed clear relationships between bacterial β-diversity and soil environmental variables (Fig. 4). The variance in bacterial community structure was explained by the first and second axes to 38.6% and 13.9%, respectively. The bacterial

community of manure addition treatments (Manure and U+M) were mainly associated with soil concentrations of NO_3^-, DOC, total C, total N, and C:N ratio. In contrast, the bacterial community of treatments without manure addition (Control and Urea) was mainly associated with soil pH.

DISCUSSION

This study clearly showed that manure application exerted significant effect on microbial abundance and diversity and greatly increased the DEA compared with conventional urea. We further linked the increase of DEA by manure application with changes in denitrifier abundance. The increased denitrification activity with manure application was in accordance with the increasing abundance of nitrate reducer (*narG*), and *nirK*- or *nosZ*-type denitrifiers. It should be noted, however, soil samplings were conducted for only one time over the growing season for determination of soil microbial activities in the current study, which hindered the investigations of temporal changes in soil microbes and could also cause uncertainties in correlating with N_2O emissions. Still, sampling was done in a representative field for the local cotton production where we compared farmer's management practices of applying manure relative to inorganic fertilizers. The sampling depth (0-20 cm) for microbial analysis was also in accordance with previous findings that soil N_2O emissions following N addition were mostly attributed to the top soils (*Wagner-Riddle et al., 2008*; *Kuang et al., 2019*).

Impact of N addition strategy on denitrification and nitrification

The increased activity of soil denitrifying enzymes with manure in the current study is in consistent with our findings at the same field where we reported more N_2O emissions from manure compared with urea application under drip irrigation conditions (*Kuang et al., 2018*). It is likely that the

increased NO_3^- and carbon supply with manure application could have provided primary substrate for denitrification and increased the $N_2O/(N_2O+N_2)$ ratio (*Francis et al., 2013*). *Chantigny et al. (2010)* also suggested that manure can elevate soil respiration and deplete O_2 concentration to create temporary anaerobic conditions, thereby further increasing the proportion of N_2O production through denitrification. These studies highlight the importance of N addition source on soil N transformation processes and suggest that manure induced N_2O emissions are likely attributed to denitrification.

In contrast to DEA, NEA was not affected by manure application in the current study. Similarly, *Shen et al. (2008)* also reported that organic manure did not affect potential nitrification rates of an alkaline sandy loam soil in northern China. Several studies suggested that soil pH is the dominant factor for nitrification as it determines the availability of NH_4^+, which is the primary substrate for ammonia oxidation, the initial and rate-limiting step of nitrification (*Fan et al., 2011*; *Nicol et al., 2008*). In our study, both pH and the availability of NH_4^+ were not affected by N addition strategy, confirming the insensitivity of NEA to N sources.

In contrast with manure, urea did not significantly affect DEA and NEA compared to Control. Our results agree with those of *Yin et al. (2015)* who reported that manure but not inorganic fertilizer increased denitrification potential. In contrast, several studies reported that application of inorganic N fertilizers increased the activity of nitrification (*Fang et al., 2018*; *Shi et al., 2016*) and potential denitrification (*Duan et al., 2017*; *Wang et al., 2018*). The absence of the inorganic fertilizer effect in the current study was associated with the minor to no effect by urea application on soil properties such as pH, DOC and inorganic N (NO_3^- and NH_4^+) compared with Control. It is likely the buildup of C and N substrates by urea application were not sufficient enough to affect the activities of functional genes.

365

366 **Impact of N addition strategy on abundance of functional genes and bacterial community**
367 **structure**

368 In the current study, the positive relationships of the abundances of *narG*, *nirK* and *nosZ* with DEA
369 and further with soil DOC, total C and total N suggest that manure significantly increased gene
370 abundance by providing C and N substrate. This result is in line with the previous findings that the
371 denitrifiers abundance could be used as a predictor of DEA (*Morales, Cosart & Holben, 2010*).
372 Our findings also agree with previous studies which reported that organic manure increased
373 abundance of *nosZ*-type denitrifier compared to inorganic fertilizers (*Hallin et al., 2009*; *Tao et*
374 *al., 2018*). Also being consistent with previous studies (*Zhou et al., 2011*), abundance of *nirK* but
375 not *nirS* was increased by manure application in this study, suggesting that *nirK* was more
376 susceptive to fertilizer regimes than *nirS*-type denitrifier. *Hallin et al. (2009)* also reported that
377 denitrification rates were not correlated with the abundance of *nirS* genes in soils treated with
378 different fertilizer regimes for 50 years. A possible reason for the lack of correlation could be that
379 the denitrifier harbouring the *nirS* gene might play a minor functional role for DEA (*Attard et al.,*
380 *2011*). In the current study, the nitrate reducer (*narG*), and *nirK*- and *nosZ*-type denitrifiers, which
381 encodes the main catalytic enzymes responsible for nitrate reduction, nitrite reduction and N_2O
382 reduction respectively, were more sensitive to manure application. The increase of the denitrifiers
383 abundance with manure application thus resulted in an increase for the pool of denitrifying
384 enzymes. Even though the limited soil sampling for microbial analysis hindered the possibility of
385 directly linking results from the current study to the *in-situ* measurements of N_2O flux, the positive
386 relationship between DEA and the abundance of denitrifiers suggest the manure-induced N_2O
387 emissions in *Kuang et al. (2018)* was more likely determined by denitrification.

388 It is interesting to note that manure application increased *AOA* and *AOB* whereas had no effect
389 on NEA in this study, suggesting the abundance of ammonia-oxidizers are not necessarily
390 associated with nitrification potential. *Nicol et al. (2008)* reported the activity of ammonia-
391 oxidizers was more associated with the relationships among transcription, translation and enzyme
392 function rather than abundance of functional genes. It is also likely that the complicated subsequent
393 hierarchical regulation of enzyme expression resulted in an uncouple effect between NEA and
394 *amoA* gene abundance (*Röling, 2010*). Consistent with previous studies (*Fan et al., 2011*; *Tian et*
395 *al., 2014*), N addition reduced the *AOA*/*AOB* ratio in this study, suggesting that *AOA* and *AOB*
396 may occupy different soil niches due to the differences in physiological and metabolic pathways.
397 Previously, *AOA* prefer low NH_3 substrate conditions for growth whereas *AOB* prefers higher NH_3
398 levels (*Di et al., 2010*), thus potentially resulting in a lower *AOA*/*AOB* ratio following N addition.
399 Similar to previous studies (*Ji et al., 2018*; *Kumar et al., 2018*; *Wang et al., 2019*), manure
400 application significantly changed β-diversity of soil bacterial community in the current study. The
401 PCoA analysis revealed a dominant contribution of PCoA1 (61%) to total variation and a clear
402 separation of manure vs. non-manure groups along the axis PCoA1, suggesting that the addition
403 of manure was a key factor determining the variation in bacterial community among treatment.
404 Clearly, the increased N and C substrates with manure application have increased the growth of
405 some specific microbial groups and suppress others and thus changed the composition of soil
406 microbial community. The absence of urea effect on the β-diversity of bacterial community was
407 attributed to the low organic matter content (6.6 g kg^{-1}), suggesting that the substrate deficiency of
408 C limited microbial activities under the conditions in this study. In this study, manure or urea
409 applications did not influence α-diversity of soil bacterial community, likely due to an absence
410 effect on soil pH. *Fierer & Jackson (2006)* reported that soil pH is the main driver determining the

411 α-diversity and richness of soil bacterial community.

412 In the current study, the changes of soil bacterial community structure in response to manure
413 application were attributed to the increasing relative abundance of *Planctomycetes*, *Bacteroidets*,
414 *lgnavibacteriae* and decreasing abundance of *Actinobacteria, Acidobacteria, Latescibacteria*,
415 *Armatimonadetes,* and *candidate division* WPS-2. These results highlight the change of eutrophic
416 and oligotrophic bacteria. For example, *Fierer et al. (2007)* found that *Bacteroidetes* were typically
417 copiotrophic bacteria and could thrive in soil with high available organic carbon. *Planctomycetes*
418 are involved in the turnover of soil organic carbon and nutrient availability and the reproduction
419 of this microbial group may increase intensively in response to the application of manure (*Lupatini*
420 *et al., 2016*). The phyla which were negatively influenced by manure application were considered
421 as slow-growing oligotrophs accustomed to nutrient-limited environments. For example, several
422 studies had shown that *Acidobacteria* strains grew slowly with their growth being limited with
423 substrate additions (*Goldfarb et al., 2011*).

424

425 **CONCLUSIONS**

426 Manure application significantly elevated the abundances of nitrate reducer (*narG*), and *nirK- and*
427 *nosZ*-type denitrifiers, in accordance with a substantial increase of denitrifying enzyme activity.
428 Additionally, soil DOC, total C and total N contents were highly correlated with the abundance of
429 *narG*, *nirK and nosZ* genes, suggesting manure stimulated the functional genes via providing C
430 and N substrates. In contrast, urea application did not exert significant impacts on the abundances
431 of nitrifiers and denitrifiers. High throughout sequencing clearly showed that two years of manure
432 application significantly altered bacterial community composition. Consequently, our study
433 demonstrated a strong link between abundances of nitrate reducer (*narG*), *nirK*- and *nosZ*-type

434 denitrifiers and enhanced denitrifying enzyme activity by manure application under the drip-
435 irrigated conditions, indicating that denitrification is likely the key process determining manure-
436 induced N_2O emissions.

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600 10.1007/s11356-011-0482-8.

Table 1(on next page)

Table 1 The primer sets and thermocycling conditions used for real-time qPCR reactions.

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Table 1 The primer sets and thermocycling conditions used for quantitative PCR reactions.

Target gene	Primer set	Sequence (5'–3')	Product size (bp)	Annealing time and temperature	Elongation time and temperature	Reference
Archaeal amoA	Arch-amoAF Arch-amoAR	STA ATG GTC TGG CTT AGA CG GCG GCC ATC CAT CTG TAT GT	635	30 s, 55℃	30 s, 72℃	*Francis et al. (2005)*
Bacterial amoA	amoA1F amoA2R	GGG GTT TCT ACT GGT GGT CCC CTC KGS AAA GCC TTC TTC	491	30 s, 56℃	30 s, 72℃	*Rotthauwe, Witzel & Liesack (1997)*
narG	narGG-F narGG-R	TCGCCSATYCCGGCSATGTC GAGTTGTACCAGTCRGCSGAYT CSG	173	30 s, 55℃	30 s, 72℃	*Bru, Sarr & Philippot (2007)*
nirS	nirS4QF nirS6QR	GTS AAC GYS AAG GAR ACSGG GAS TTC GGR TGS GTC TTSAYGAA	465	30 s, 60℃	30 s, 72℃	*Throback et al. (2004)*
nirK	FlaCu R3Cu	ATCATGGTSCTGCCGCG GCCTCGATCAGRTTGTGGTT	474	30 s, 63℃	30 s, 72℃	*Throback et al. (2004)*
nosZ	nosZF nosZ-1622R	CGYTGTTCMTCGACAGCCG CGSACCTTSTTGCCSTYGCG	453	30 s, 61℃	35 s, 72℃	*Scala & Kerkhof (1998)*

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Table 2(on next page)

Table 2 Soil (0-20 cm) properties of addition treatments in the drip-irrigated cotton field. U+M: 50% urea + 50% manure. Values are mean ± 1 standard error, n = 4.

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Table 2 Soil (0-20 cm) properties of addition treatments in the drip-irrigated cotton field. U+M: 50% urea + 50% manure. Values are mean ± 1 standard error, n = 4.

Treatment	Total N content (g kg^{-1})	NO_3^- (mg kg^{-1})	NH_4^+ (mg kg^{-1})	Total C content (g kg^{-1})	DOC (mg g^{-1})	C:N
Control	0.9±0.1 c	21±3 b	14.1±0.1 a	7.8±0.5 b	0.21±0.01 b	9.2±0.7 bc
Urea	1.0±0.2 bc	30±2 b	17.9±3.2 a	6.8±0.4 b	0.20±0.02 b	6.8±1.3 c
Manure	1.3±0.1 ab	120±29 a	15.4±1.5 a	15.9±1.4 a	0.37±0.04 a	12.6±0.8 ab
U+M	1.4±0.1 a	103±7 a	17.9±2.6 a	19.1±1.8 a	0.36±0.02 a	13.6±1.3 a

2 Means within a column followed by the same letter are not significantly different at $P < 0.05$.

Table 3(on next page)

Table 3 Diversity of the microbial community at a similarity level of 97% as affected by addition treatments in a drip-irrigated cotton field used in this study. Values are means ± 1 standard error, n = 3.

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Table 3 Diversity of the microbial community at a similarity level of 97% as affected by addition treatments in a drip-irrigated cotton field used in this study. Values are means ± 1 standard error, n = 3.

Treatment	Coverage index	Reads	OTUs	Shannon	ACE	Chao1	Simpson
Control	0.93±0.00 a	27,131±854 a	4,353±100 a	7.0±0.12 a	7,001±662 a	6,354±240 a	0.0070±0.0023 a
Urea	0.94±0.00 a	28,812±63 a	4,395±70 a	6.9±0.07 a	7,001±385 a	6,429±63 a	0.0068±0.0011 a
Manure	0.94±0.01 a	26,413±2,397 a	4,072±164 a	7.1±0.04 a	6,106±147 a	5,972±151 a	0.0031±0.0002 a
U+M	0.94±0.00 a	30,405±1,280 a	4,291±171 a	7.1±0.07 a	6,413±262 a	6,323±278 a	0.0031±0.0004 a

2 Means within a column followed by the same letter are not significantly different at $P < 0.05$.

Table 4(on next page)

Table 4 Relative abundances (%) of selected bacterial and archaeal taxa at the phyla level as affected by addition treatments.

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Table 4 Relative abundances (%) of selected bacterial and archaeal taxa at the phyla level as affected by addition treatments.

	Bacteria									Archaea	
	Proteobacteria	***Planctomycetes***	***Acidobacteria***	***Actinobacteria***	***Bacteroidetes***	***Armatimonadetes***	***Ignavibacteriae***	***candidate division WPS-2***	***Latescibacteria***	***Thaumarchaeota***	***Euryarchaeota***
Control	32.5 a	8.9 b	11.5 a	11.6 a	5.6 b	0.62 a	0.13 b	0.07 a	0.12 a	0.58 a	0.77 a
Urea	32.2 a	8.3 b	10.7 a	12.1 a	6.2 b	0.67 a	0.14 b	0.04 a	0.08 ab	0.50 ab	0.87 a
Manure	34.6 a	11.1 a	8.8 b	9.0 b	11.4 a	0.37 b	0.25 a	0.01 b	0.06 b	0.24 bc	0.24 b
U+M	35.1 a	10.0 a	7.5 b	9.4 b	8.3 ab	0.27 b	0.20 ab	0.01 b	0.04 b	0.30 b	0.24 b

2 Means followed by the same letter are not significantly different at $P < 0.05$.

Table 5(on next page)

Table 5 Pearson correlation coefficients (*r*) between copy number of N_2O-related functional genes and soil characteristics, n = 16.

1

Table 5 Pearson correlation coefficients (*r*) between copy number of N_2O-related functional genes and soil characteristics, n = 16.

	AOA	***AOB***	***narG***	***nosZ***	***nirS***	***nirK***
NO_3^- (mg kg^{-1})	0.65**	0.61*	0.81***	0.65**	0.24	0.77***
NH_4^+ (mg kg^{-1})	-0.29	0.15	-0.58	-0.14	-0.24	0.01
DOC (mg g^{-1})	0.70***	0.73***	0.86***	0.76***	0.47	0.73***
pH	-0.22	-0.30	-0.40	-0.25	-0.28	-0.33
TN (g kg^{-1})	0.63*	0.71**	0.66**	0.58**	0.21	0.63**
TC (g kg^{-1})	0.85***	0.82***	0.82***	0.72**	0.31	0.76***
C/N	0.72**	0.61*	0.67**	0.58*	0.29	0.62**
DEA (ug N_2O-N g^{-1} h^{-1})	0.57*	0.85***	0.84***	0.70**	0.31	0.76***
NEA (ug N-NO_2^-+NO_3^- g^{-1} h^{-1})	0.19	0.26	0.18	0.06	-0.22	0.64**

2 *, **, *** indicate significance at $P < 0.05$, < 0.01 and < 0.001, respectively.

Figure 1

Figure 1 Denitrifying enzyme activity (DEA) and nitrifying enzyme activity (NEA) as affected by addition treatments in the drip-irrigated cotton field.

Figure 1 Denitrifying enzyme activity (DEA, A) and nitrifying enzyme activity (NEA, B) as affected by addition treatments in the drip-irrigated cotton field. U+M: 50% urea +50% manure. Data are means +1 standard error, n = 4. Means followed by the same letter are not significantly different at $P < 0.05$.

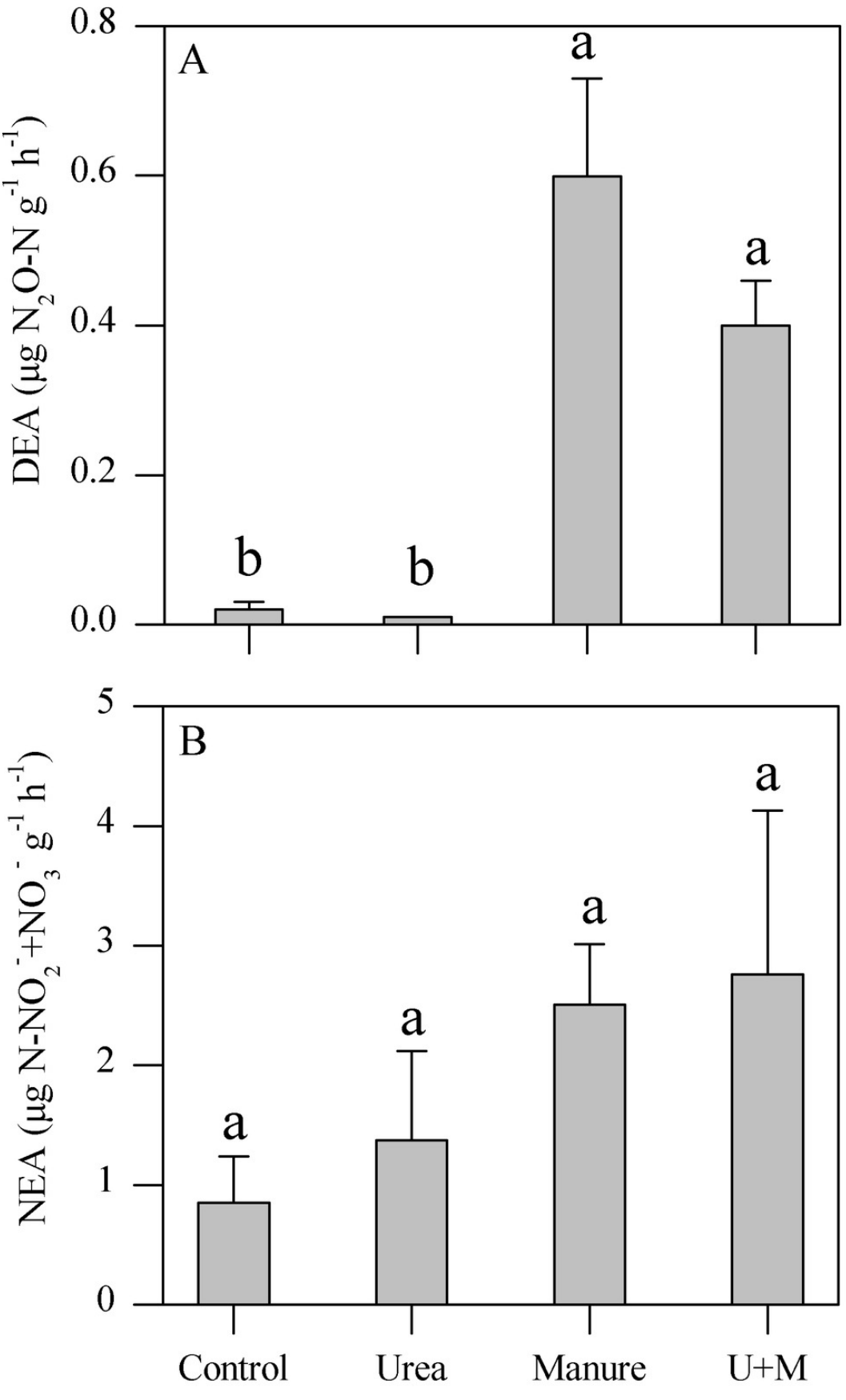
A
B
DEA (μg N_2O-N g^{-1} h^{-1})
NEA (μg N-NO_2^-+NO_3^- g^{-1} h^{-1})
0.0
0.2
0.4
0.6
0.8
0
1
2
3
4
5
a
b
Control
Urea
Manure
U+M

Figure 2

Figure 2 Principal Coordinates Analysis (PCoA) plot of weighted Unifrac distance matrix showing patterns of β-diversity in microbial communities as affected by addition treatments in the drip-irrigated cotton field. U+M: 50% urea + 50% manure.

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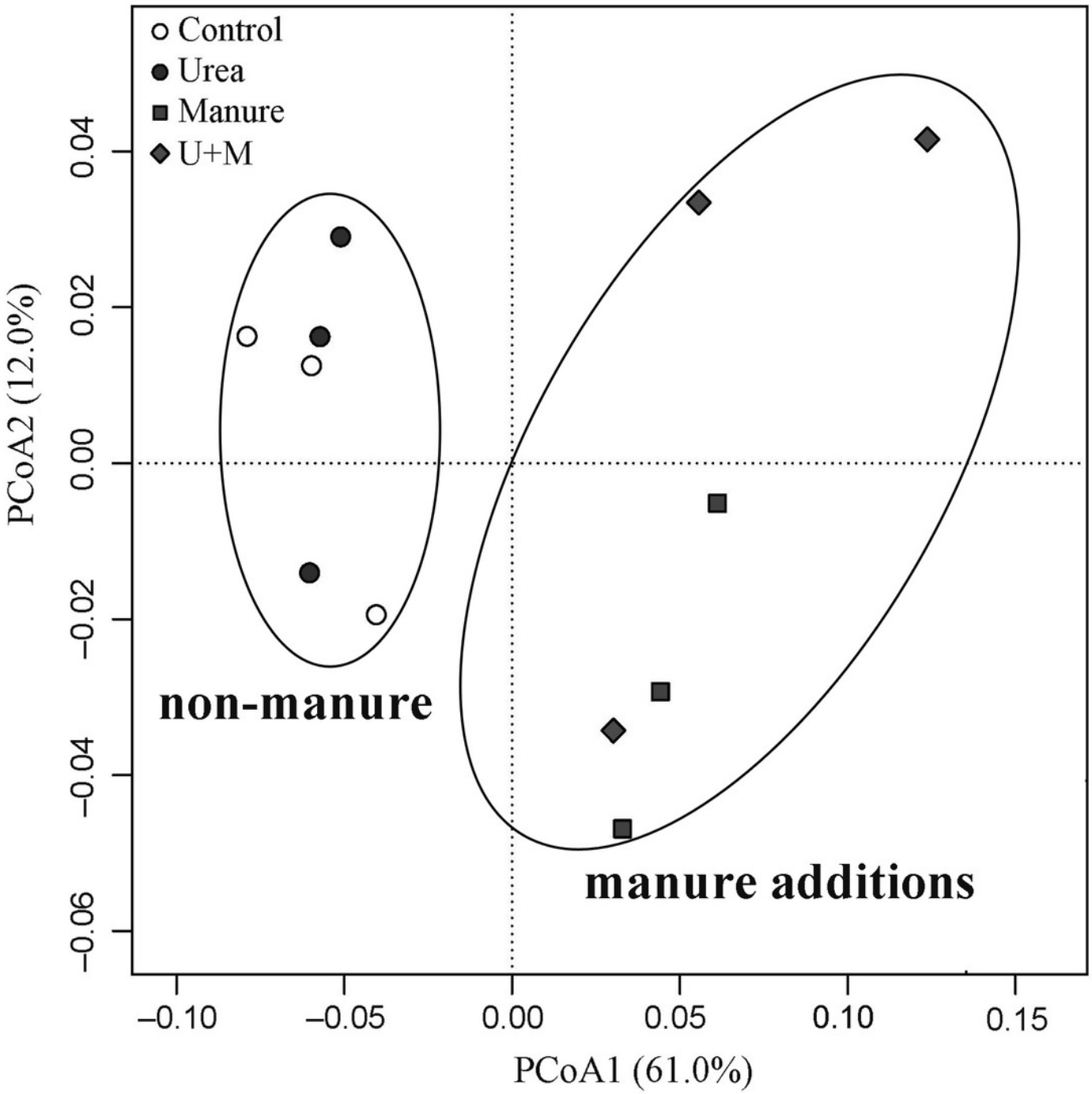
Control
Urea
Manure
U+M
non-manure
manure additions
PCoA2 (12.0%)
PCoA1 (61.0%)
0.04
0.02
0.00
−0.02
−0.04
−0.06
−0.10
−0.05
0.00
0.05
0.10
0.15

Figure 3

Figure 3 Copy numbers of archaeal (*AOA*) and bacterial *(AOB) amoA* (A), *nirS* (B), *narG* (C), *nosZ* (D) and *nirK* (E) genes in the soil as affected by addition treatments in the drip-irrigated cotton field.

Figure 3 Copy numbers of archaeal (*AOA*) and bacterial *(AOB) amoA* (A), *nirS* (B), *narG* (C), *nosZ* (D) and *nirK* (E) genes in the soil as affected by addition treatments in the drip-irrigated cotton field. Data are means +1 standard error, n = 4. Means followed by the same letter are not significantly different at $P < 0.05$.

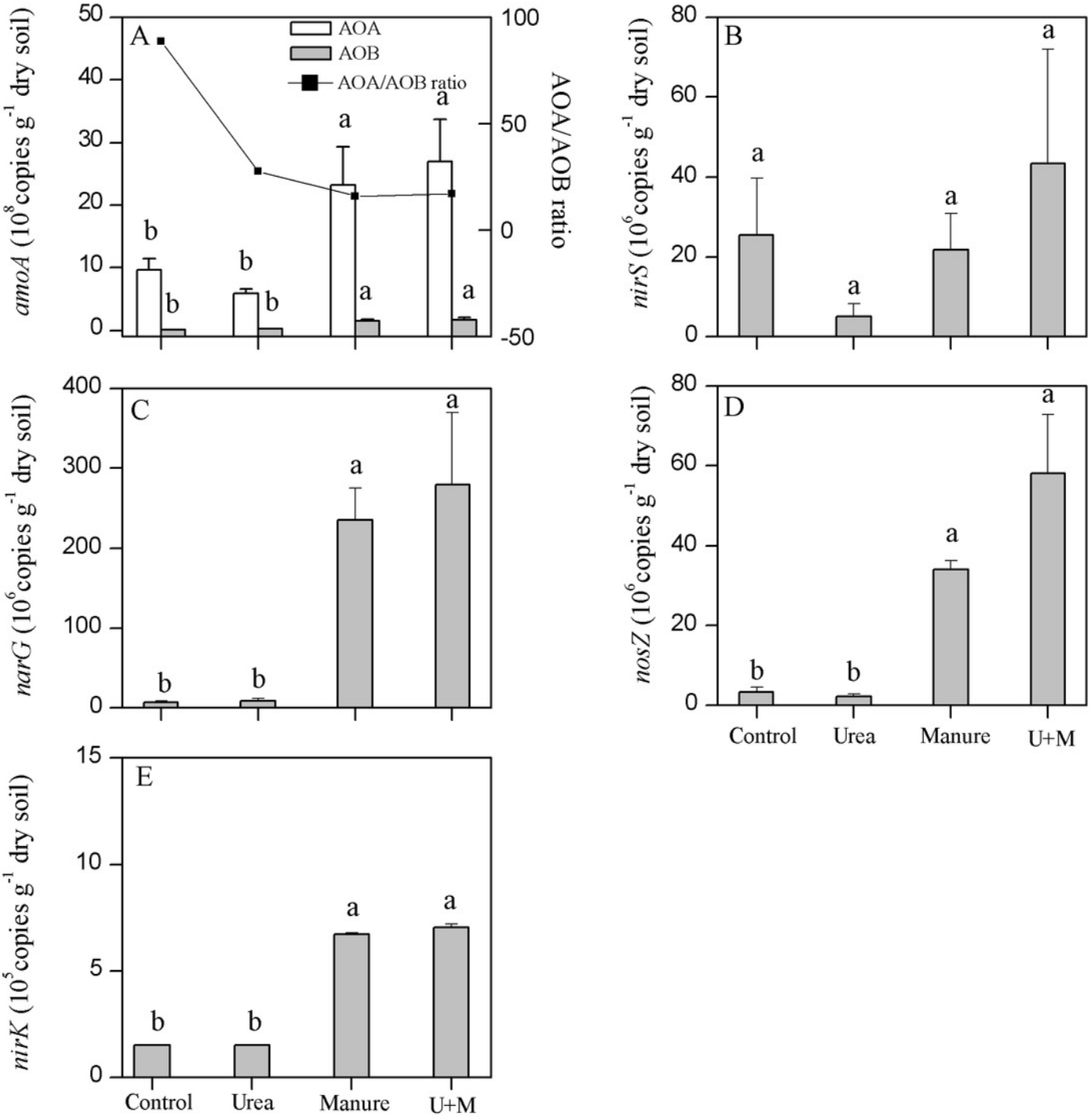
A
AOA
AOB
AOA/AOB ratio
amoA (10^8 copies g^-1 dry soil)
AOA/AOB ratio
B
nirS (10^6 copies g^-1 dry soil)
C
narG (10^6 copies g^-1 dry soil)
D
nosZ (10^6 copies g^-1 dry soil)
E
nirK (10^5 copies g^-1 dry soil)
Control
Urea
Manure
U+M

Figure 4

Figure 4 Canonical correspondence analysis (CCA) of soil properties in relation to microbial OTUs as affected by addition treatments in the drip-irrigated cotton field.

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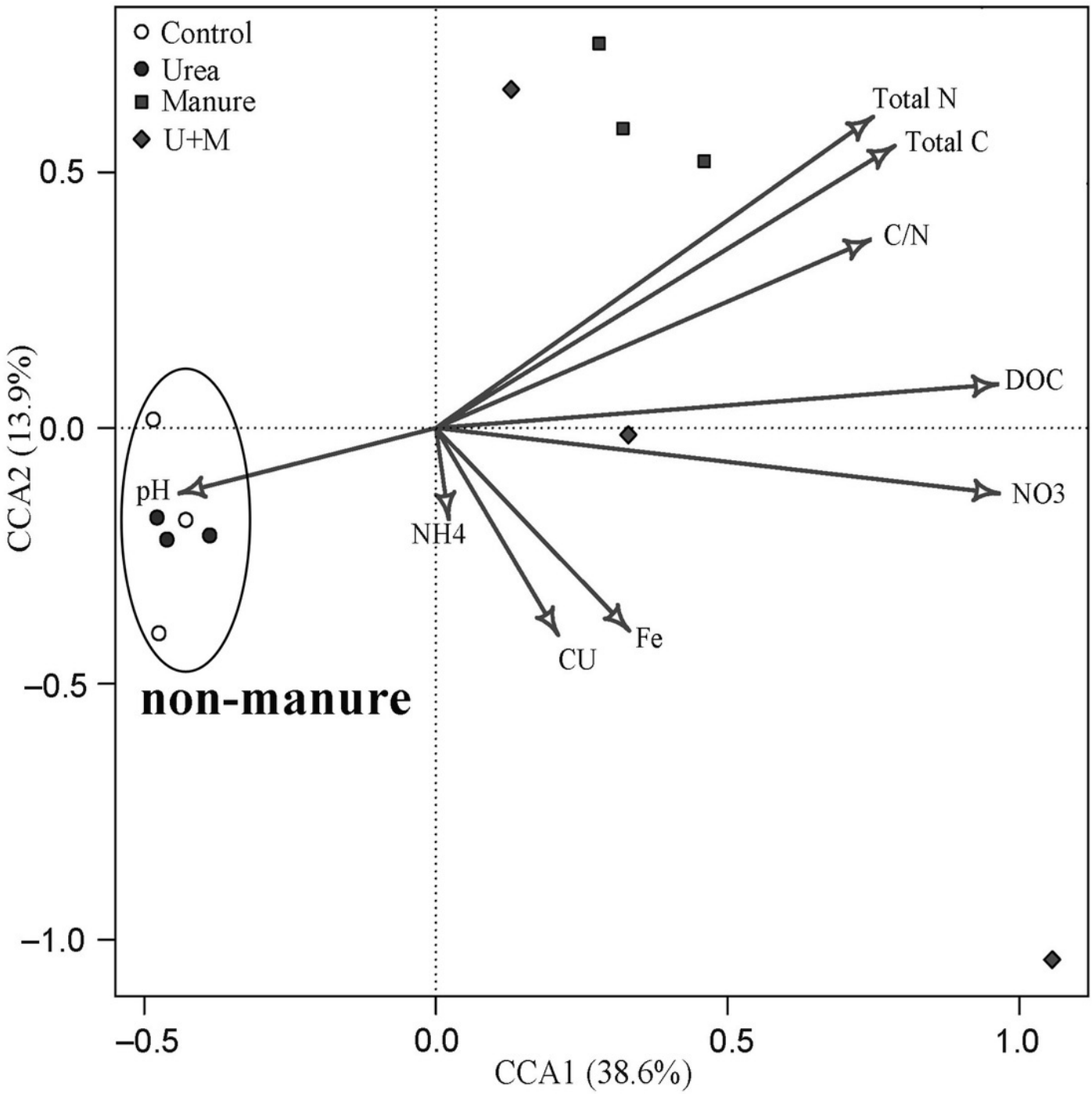
Control
Urea
Manure
U+M
Total N
Total C
C/N
DOC
NO3
pH
NH4
CU
Fe
non-manure
CCA2 (13.9%)
CCA1 (38.6%)
0.5
0.0
−0.5
−1.0
−0.5
0.0
0.5
1.0