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

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Application of synthetic nitrogen (N) fertilizer and manure can increase nitrous oxide (N_2O) emissions whereas the underlying microbial mechanisms remain unclear. In this study, 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. 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. Results showed the addition treatments did not affect Operational Taxonomic Unit (OTU) richness or Shannon diversity index of soil microbial community but change soil microbial community structure. Nitrifying enzyme activities (NEA) were unaffected by the addition treatments, whereas denitrifying enzyme activities (DEA) were 39-59 times greater in manure (U+M and Manure) than non-manure amended (Control and Urea) treatments. Real-time quantitative PCR revealed that increased DEA with manure application was highly correlated (r=0.70-0.84, P<0.01) with increased abundance of nitrate reducer (*narG)*, and denitrifies 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. In contrast with manure, urea application had no impact on abundances of nitrifier and denitrifier gene, DEA and NEA, likely due to a limitation of C availability. 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 synthetic nitrogen (N) fertilizer and manure can increase nitrous oxide (N_2O) emissions whereas the underlying microbial mechanisms remain unclear. In this study, 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. 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. Results showed the addition treatments did not affect Operational Taxonomic Unit (OTU) richness or Shannon diversity index of soil microbial community but change soil microbial community structure. Nitrifying enzyme activities (NEA) were unaffected by the addition treatments, whereas denitrifying enzyme activities (DEA) were 39-59 times greater in manure (U+M and Manure) than non-manure amended (Control and Urea) treatments. Real-time quantitative PCR revealed that increased DEA with manure application was highly correlated (r=0.70-0.84, P<0.01) with increased abundance of nitrate reducer (*narG)*, and denitrifies 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. In contrast with manure, urea application had no impact on abundances of nitrifier and denitrifier gene, DEA and NEA, likely due to a limitation of C availability. 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.

Keywords Nitrifier, Denitrifier, Manure, Drip irrigation, Microbial community structure, Denitrifying enzyme activitie

INTRODUCTION

Nitrous oxide (N_2O) accounts for nearly 8% of warming impact of anthropogenic activities and cause 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 greater N_2O emission with manure compared with inorganic (*Watanabe et al., 2014*; *Zhou et al., 2017*), confirming our recent observations on a drip-irrigated cotton field with low soil organic carbon (*Kuang et al., 2018*). However, it remains unclear whether the increased emissions 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 are converted to nitrate via nitrite ($NH_3 \rightarrow NH_2OH/HNO \rightarrow NO_2^- \rightarrow NO_3^-$). The steps of nitrification are conducted 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 is considered the usual rate-limiting step for the entire nitrification reaction (*Kowalchuk & Stephen, 2001*). Applications of manure or inorganic N can exert significant impact on nitrification through their effect 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 mineral 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 were main drivers of nitrification and denitrification activities, rather than the abundance and community structure of functional gene groups (*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 nitrate reducers and a range of denitrifiers under oxygen (O_2) limited conditions. Each step of the reaction is regulated by specific reductases encoded by functional genes, 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*). In addition to bacterial, fungi also play important roles in N_2O emissions from agricultural systems. For example, a recent field study reported N_2O emissions from an intensively managed vegetable field was dominant by fungi (*Ma, Shan & Yan, 2017*). 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-old 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*- 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 synthetic 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 with manure, inorganic N application generally reduces soil microbial community and diversity. For example, *Zhang et al. (2017)* recently reported that application of chemical fertilizers to acidic and near-neutral soils in a maize-vegetable rotation in southwest China significantly reduced bacterial Operational Taxonomic Unit (OTU) richness and Shannon diversity index. *Sun et al. (2015)* also reported that soil bacterial richness and diversity were significantly decreased following 30 years' application of mineral fertilizer to a wheat-soybean rotation in central China. Application of inorganic fertilizer affected the soil microbial community mainly by a decreasing soil pH (*Geisseler & Scow, 2014*).

As a dominant cash crop in NW China, cotton production has received intensive inputs of synthetic fertilizers and water recently as drip-irrigation due to its benefits in improving water and nutrient use efficiency (*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*). Still, it remains unclear how additions of organic manure or synthetic 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 mineral fertilizer and manure on abundance and activities of nitrifying and denitrifying communities, as well as microbial community structure in a drip-irrigated cotton field. We hypothesized that animal manure application would increase the abundance of functional genes due to increased supply of substrate carbon and N inputs, and thus result in greater activities of nitrification and denitrification than the conventional urea.

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)*.

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. Treatments included (1) an unfertilized control, and application of 240 kg of available N ha^{-1} in the form of (2) granular urea (Urea), (3) mixture of sheep and cattle manure (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 used manure was a compost mixture from cattle and sheep and was all applied before planting by broadcast-incorporation at 10 cm depth. The manure had a 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. In each year, cotton (c.v. Xinluzao 48) was planted 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.

146 **Soil sampling**

147 Soil samples (0-20 cm depth) were collected with a hand auger (2.5 cm diameter) in September
148 2016 with cotton at boll opening stage. In each plot, four soil cores were collected next to the drip
149 tape and mixed thoroughly together for one composite sample per plot. Each sample was passed
150 through a 2 mm mesh screen and partitioned into three subsamples. One subsample was air-dried
151 at room temperature for chemical analysis. The second subsample for analysis of denitrifying
152 enzyme activity (DEA) and nitrifying enzyme activity (NEA) was stored at -20 °C. The third
153 subsample for microbial molecular analysis was stored at -80 °C.

154

155 **Soil chemical properties**

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

165

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

Soil DEA was expressed as the rate of N_2O production (μg N h^{-1} g^{-1} soil) and determined using the anaerobic slurry technique (*Beauchamp & Bergstrom, 1993*). Briefly, 25 g defrost soil samples was placed into 125 ml plasma flasks with 25 ml solution including 10 mM KNO_3, 10 mM glucose, 50 mM K_2HPO_4 and 0.1 g L^{-1} chloramphenicol to inhibit new protein production. The flasks were evacuated and flushed with a 90:10 He-C_2H_2 gas mixture to create anaerobic conditions and suppress N_2O-reductase activity. Flasks were then shaken for 60 min and gas samples taken 0, 15, 30, 45 and 60 min after onset of mixing using an 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).

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 defrost 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 using the Power Soil Total DNA Isolation Kit (QIAGEN, Inc., MD, USA) according to the manufacturer's instructions. The quality and concentration of DNA were estimated using a Nanodrop 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.

Real-time 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, 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 real-time qPCR reactions are given in Table 1. Plasmids that containing respective sequences of the targeted genes were generated by cloning the targeted gene fragments into plasmid pMD™ 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 was 92% (R^2=0.990) for archaeal *amoA*, 105% (R^2=0.999) for bacterial *amoA,* 90% (R^2=0.999) for *narG,* 85% (R^2=0.997) for *nirS*, 96% (R^2=0.998) for *nirK*, and 80% (R^2=0.990) for *nosZ*, respectively.

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™ 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, and finally PRINSEQ software was used for the quality control of the merged reads. Chimeras were filtered by comparing the sequences with those in the reference database using the UCHIME algorithm. After the above screening, the remaining high-quality sequences were clustered into Operational Taxonomic Units (OTUs) at a ≥ 97% similarity identity threshold. The Ribosomal Database Project (RDP) classifier 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 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 implemented with the R language, which was based on bacterial weighted UniFrac metric matrix. Canonical correspondence analysis (CCA) was performed with the Vegan package Version 1.17-7 (*Oksanen et al., 2011*) implemented with the R language to determine the relationships between soil physiochemical properties and microbial communities. Untransformed data were used for the PCoA and CCA analyses. The differences phylum in the relative abundances of categories between treatment were calculated using the Welch's t-test with STAMP (Statistical Analysis of Metagenomic Profiles). Corrected p-values were calculated with FDR (False Discovery Rate) Multiple test correction.

RESULTS

Soil chemical characteristics

Manure application 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, soil C:N ratio were 37-100% greater in Manure and U+M than in Urea and Control treatments.

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. 1). 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.

Microbial community, and nitrifier and denitrifier gene response

Richness and diversity of the soil microbial (prokaryote) community of treatments is presented in Table 3. 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. 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 microbial community in response to addition treatments (Fig. 2). The microbial 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 microbial community, with PCoA1 and PCoA2 explaining 61% and 12%, respectively. At bacterial phylum level, *Planctomycetes*, *Bacteroidetes* and *Ignavibacteriae* increased with manure additions, whereas *Latescibacteria*, *Acidobacteria*, *Armatimonadetes*, *Actinobacteria,* and *candidate* division WPS-2 decreased (Table 4). At archaeal phylum level, *Thaumarchaeota and Euryarchaeota* decreased with manure application.

Real-time qPCR was further used to analyze changes in abundances of nitrifying and denitrifying functional genes. 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.

Canonical correspondence analysis (CCA) revealed clear relationships between microbial β-diversity and soil environmental variables (Fig. 4). The variance in microbial community structure was explained by the first and second axes to 38.6% and 13.9%, respectively. The microbial 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 microbial community of treatments without manure addition (Control and Urea) was mainly associated with soil pH.

DISCUSSION

This study assessed the effect of different N addition strategy on nitrification, denitrification and associated functional gene abundance and soil microbial community from cotton field under drip irrigation and clearly showed that manure application exerted significant effect on microbial communication and greatly increased the DEA compared with conventional urea. We further

linked the increase of DEA by manure application with changes in denitrifier abundance. It was found the increased denitrification activity with manure application was in accordance with the increasing abundance of nitrate reducer (*narG*), and *nirK*- or *nosZ*-type denitrifiers. These results demonstrate that the previously reported greater N_2O emissions with manure application at the same experimental site by *Kuang et al. (2018)* were most likely associated with denitrification. 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 hinders the investigations of temporal changes in soil microbes and could also cause uncertainties in correlating with N_2O emissions.

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.

335 In contrast to DEA, NEA was not affected by manure application in the current study. Similarly,
336 *Shen et al. (2008)* also reported that organic manure did not affect potential nitrification rates of
337 an alkaline sandy loam soil in northern China. Several studies suggested that soil pH is the
338 dominant factor for nitrification as it determines the availability of NH_4^+, which is the primary
339 substrate for ammonia oxidation, the initial and rate-limiting step of nitrification (*Fan et al., 2011*;
340 *Nicol et al., 2008*). In our study, both pH and the availability of NH_4^+ were not affected by N
341 addition strategy, confirming the insensitivity of NEA to N sources.
342 In contrast with manure, urea did not significantly affect DEA and NEA. Our results agree with
343 those of *Yin et al. (2015)* who reported that manure but not chemical fertilizer increased
344 denitrification. In contrast, several studies reported that application of chemical N fertilizers
345 increased the activity of nitrification (*Fang et al., 2018*; *Shi et al., 2016*) and denitrification (*Duan*
346 *et al., 2017*; *Wang et al., 2018*). The absence of the inorganic fertilizer effect in the current study
347 was associated with the minor to no effect by urea application on soil properties such as pH, DOC
348 and inorganic N (NO_3^- and NH_4^+) compared with Control. It is likely the buildup of C and N
349 substrates by urea application were not sufficient enough to affect the activities of functional genes.
350

351 **Impact of N addition strategy on abundance of functional genes and microbial community**
352 **structure**

353 In the current study, the positive relationships of the abundances of *narG*, *nirK* and *nosZ* with DEA
354 and further with soil DOC, total C and total N suggest that manure significantly increased gene
355 abundance by providing C and N substrate. This result is in line with the previous findings that the

356 denitrifiers abundance could be used as a predictor of DEA (*Morales, Cosart & Holben, 2010*).
357 Our findings also agree with previous studies which reported that organic manure increased
358 abundance of *nosZ*-type denitrifier compared to synthetic fertilizers (*Hallin et al., 2009*; *Tao et al.,*
359 *2018*). Also being consistent with previous studies (*Zhou et al., 2011*), abundance of *nirK* but not
360 *nirS* was increased by manure application in this study, suggesting that *nirK* was more susceptive
361 to fertilizer regimes than *nirS*-type denitrifier. *Hallin et al. (2009)* also reported that denitrification
362 rates were not correlated with the abundance of *nirS* genes in soils treated with different fertilizer
363 regimes for 50 years. A possible reason for the lack of correlation could be that the denitrifier
364 harbouring the *nirS* gene might play a minor functional role for DEA (*Attard et al., 2011*). In the
365 current study, the nitrate reducer (*narG*), and *nirK* and *nosZ*-type denitrifiers, which encodes the
366 main catalytic enzymes responsible for nitrate reduction, nitrite reduction and N_2O reduction
367 respectively, were more sensitive to manure application. This implies the increased DEA with
368 manure application were mainly associated with steps of nitrate, nitrite and N_2O reduction.

369 It is interesting to note that manure application increased *AOA* and *AOB* whereas had no effect
370 on NEA in this study, suggesting the abundances of ammonia-oxidizers are not necessarily
371 associated with nitrification potential. *Nicol et al. (2008)* reported the activity of ammonia-
372 oxidizers was more associated with the relationships among transcription, translation and enzyme
373 function rather than abundance of functional genes. It is also likely that the complicated subsequent
374 hierarchical regulation of enzyme expression resulted in an uncouple effect between NEA and
375 *amoA* gene abundance (*Röling, 2010*). Consistent with previous studies (*Fan et al., 2011*; *Tian et*
376 *al., 2014*), N addition reduced the *AOA*/*AOB* ratio in this study, suggesting that *AOA* and *AOB*

may occupy different soil niches due to the differences in physiological and metabolic pathways. Previously, *AOA* has been shown to prefer low NH_3 substrate conditions for growth whereas *AOB* prefers higher NH_3 levels (*Di et al., 2010*), thus potentially resulting in a lower *AOA*/*AOB* ratio following N addition.

Agreement with previous studies (*Ji et al., 2018*; *Kumar et al., 2018*; *Wang et al., 2019*), manure application showed significant impact on soil microbial community in the current study. Clearly, the increased N and C substrates with manure application have increased the growth and reproduction of some specific microbial groups and suppress others and thus changed the composition of soil microbial community. It is also likely that manure application had caused high loads of exogenous microbes and thus resulted in the community disturbance (*Durso et al., 2011*). The absence of urea treatment on microbial community was not unexpected as the soil was low in organic matter of only 6.6 g kg^{-1}, suggesting C other than N was limiting microbial activities under the conditions in this study. In addition, manure or urea applications did not influence soil microbial diversity. *Fierer & Jackson (2006)* reported that soil pH is the main driver for determining microbial diversity. In the current study, no treatment effect on soil pH was detected.

In the current study, the changes of soil microbial community structure in response to manure application were attributed to increasing abundance of *Planctomycetes*, *Bacteroidets*, *lgnavibacteriae* and decreasing abundance of *Actinobacteria, Acidobacteria, Latescibacteria*, *Armatimonadetes* and *candidate division* WPS-2. These results highlight the change of eutrophic and oligotrophic bacteria. For example, *Fierer et al. (2007)* found that *Bacteroidetes* were typically copiotrophic bacteria and could thrive in soil with high available organic carbon. *Planctomycetes*

398 are involved in the turnover of soil organic carbon and nutrient availability and the reproduction
399 of this microbial group may increase intensively in response to the application of manure (*Lupatini*
400 *et al., 2016*). The phyla which were negatively influenced by manure application were considered
401 as slow-growing oligotrophs accustomed to nutrient-limited environments. For example, several
402 studies had shown that *Acidobacteria* strains grew slowly with their growth being limited with
403 substrate additions (*Goldfarb et al., 2011*). Further studies are needed to examine how the changes
404 of the soil microbial community affect soil N transformation processes.
405

406 **CONCLUSIONS**

407 This study investigated the effect of N addition strategy on the abundances of denitrfiers, nitrifiers
408 and their relative contribution to soil denitrification and nitrification under drip-irrigated
409 conditions. We found that manure application significantly elevated the abundances of nitrate
410 reducer (*narG*), and *nirK- and nosZ*-type denitrifiers, in accordance with a substantial increase of
411 denitrifying enzyme activity. Additionally, Soil DOC, total C and total N contents were highly
412 correlated with the abundance of *narG*, *nirK and nosZ* genes, suggesting manure stimulated the
413 functional genes via providing C and N substrates. In contrast, urea application did not exert
414 significant impacts on the abundances of nitrifier and denitrifiers. High throughout pyrosequencing
415 clearly showed that two years of manure application significantly altered community composition.
416 Consequently, our study demonstrated a strong link between abundances of nitrate reducer (*narG*),
417 *nirK*- and -*nosZ* type denitrfiers and enhanced denitrifying enzyme activity by manure application
418 under the drip-irrigated conditions, indicating that denitrification is likely the key process

419 determining manure-induced N_2O emissions.

420

421

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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 real-time qPCR 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 Richness and 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 Richness and 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	Chao	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 $P < 0.05$.

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Table 4(on next page)

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

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

	Bacteria								Archaea	
	Planctomycetes	***Acidobacteria***	***Actinobacteria***	***Bacteroidetes***	***Armatimonadetes***	***Ignavibacteriae***	***candidate division WPS-2***	***Latescibacteria***	***Thaumarchaeota***	***Euryarchaeota***
Control	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	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	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	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.

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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.

3

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) and nitrifying enzyme activity (NEA) 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 $P < 0.05$.

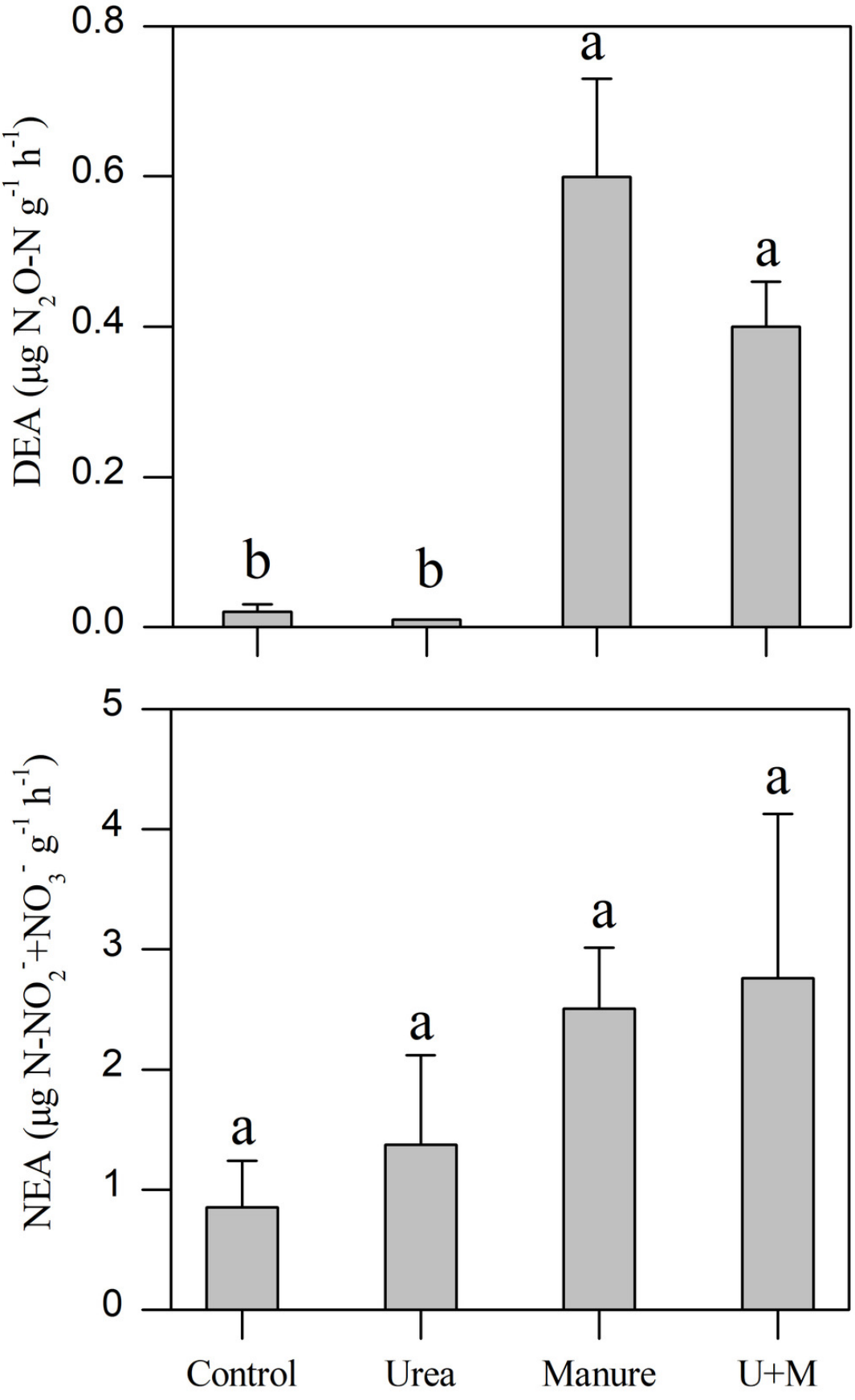

DEA (μg N_2O-N g^{-1} h^{-1})
0.0
0.2
0.4
0.6
0.8
b
b
a
a
NEA (μg N-NO_2^-+NO_3^- g^{-1} h^{-1})
0
1
2
3
4
5
a
a
a
a
Control
Urea
Manure
U+M

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.

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.

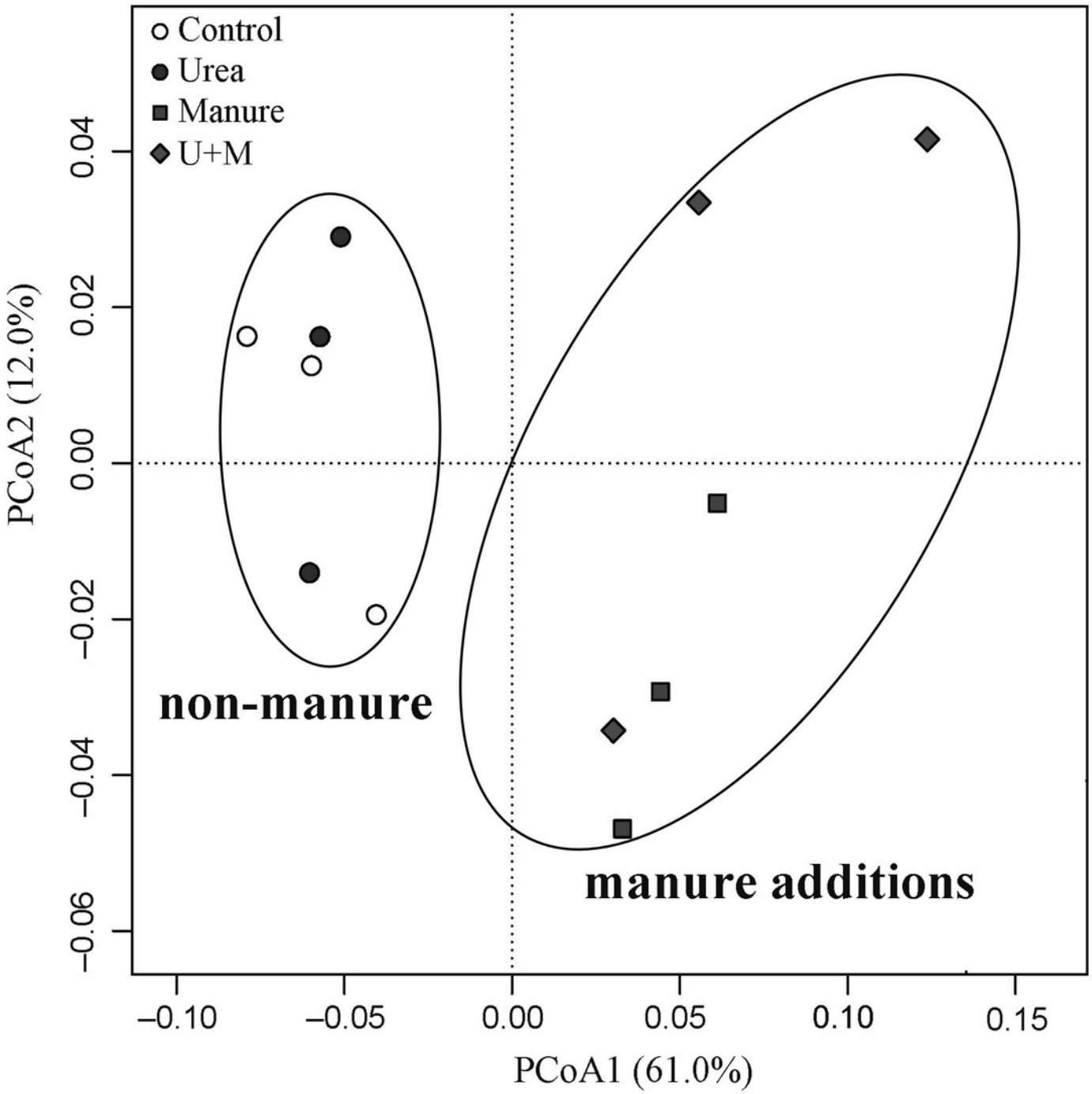

Control
Urea
Manure
U+M
non-manure
manure additions
PCoA1 (61.0%)
PCoA2 (12.0%)
0.04
0.02
0.00
−0.02
−0.04
−0.06
−0.10
−0.05
0.00
0.05
0.10
0.15

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 $P < 0.05$.

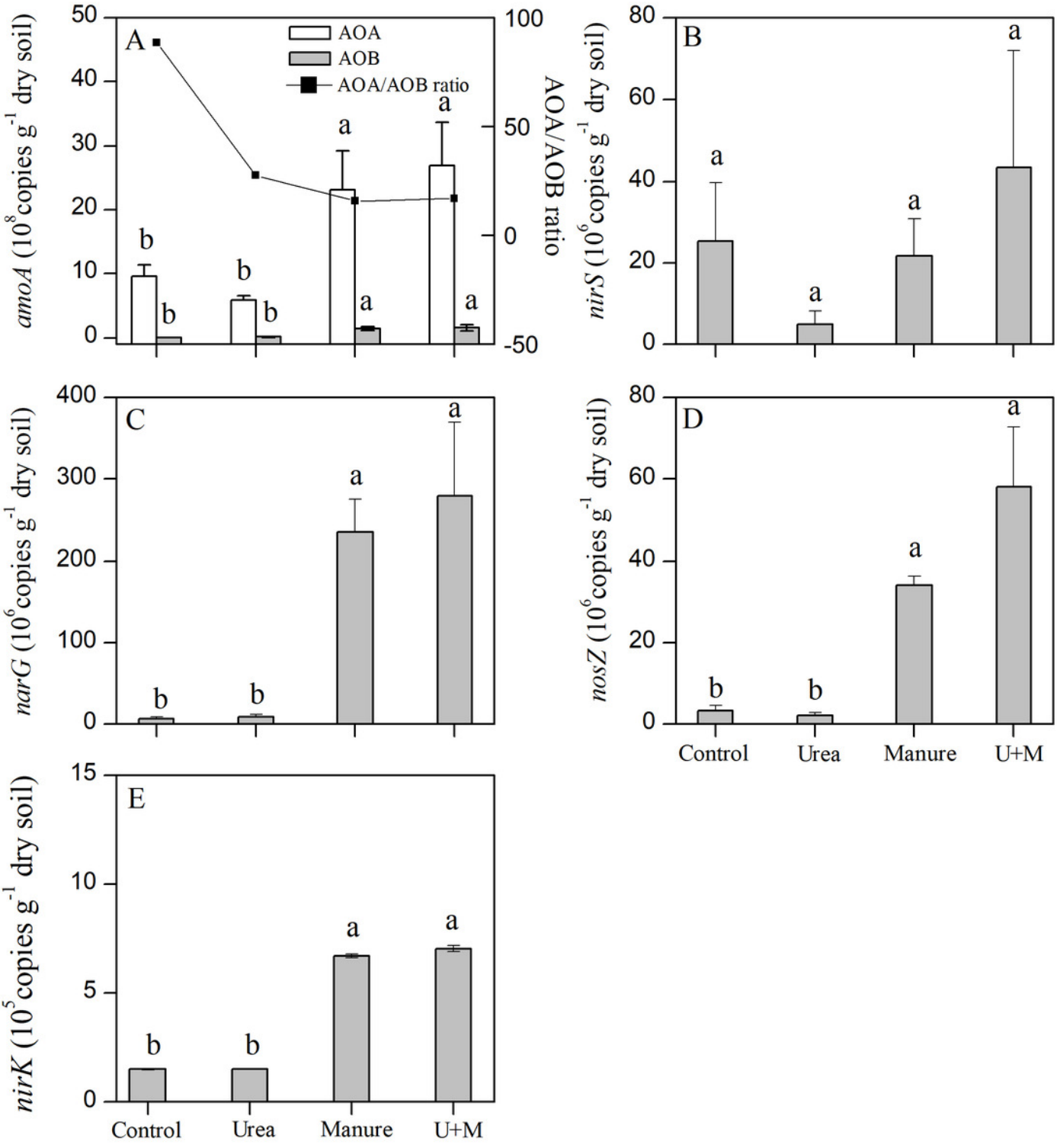
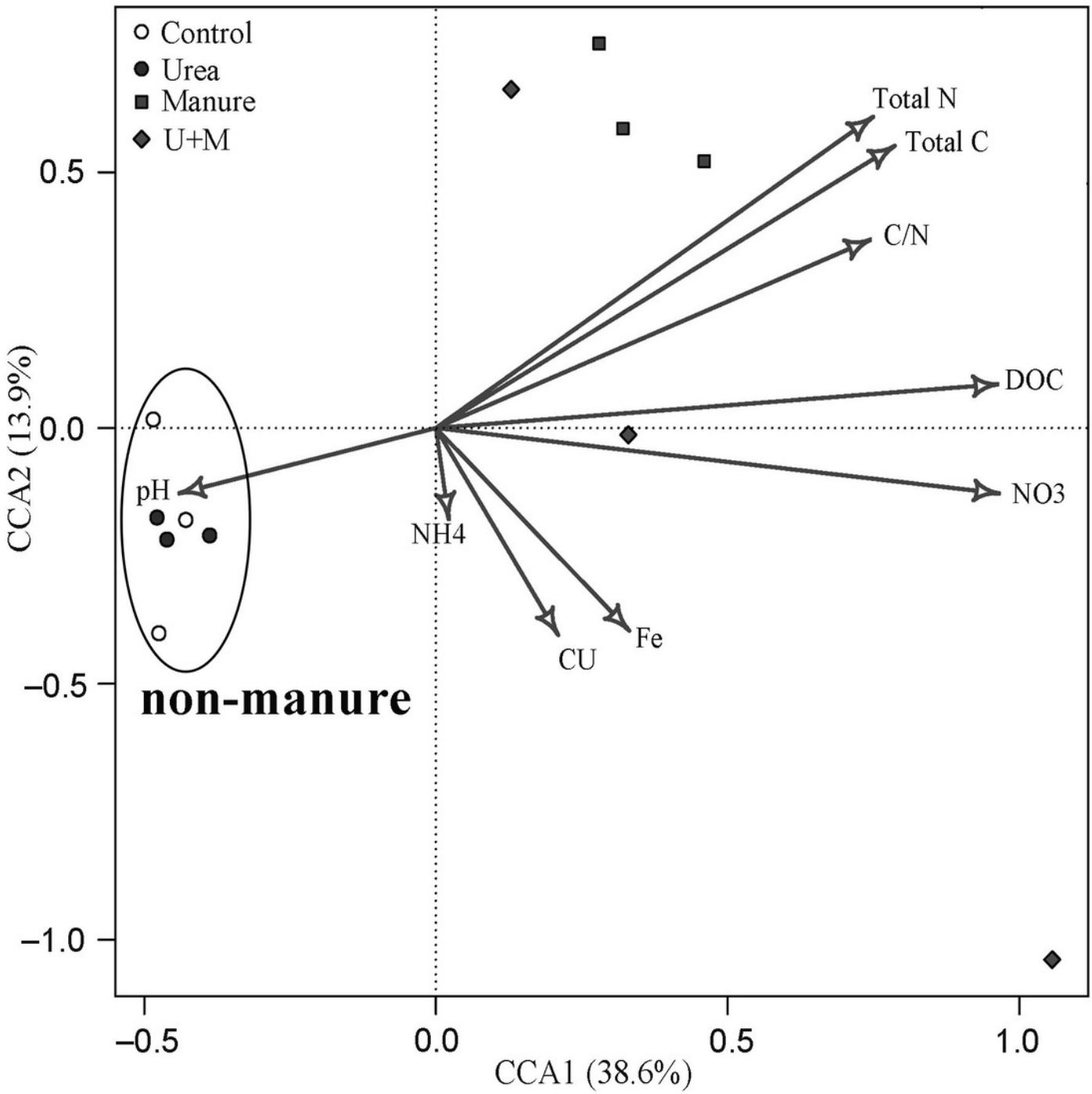

A
AOA
AOB
AOA/AOB ratio
amoA (10^8 copies g^-1 dry soil)
AOA/AOB ratio
B
nirS (10^6 copies g^-1 dry soil)
C
narG (10^6 copies g^-1 dry soil)
D
nosZ (10^6 copies g^-1 dry soil)
E
nirK (10^5 copies g^-1 dry soil)
Control
Urea
Manure
U+M

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.

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.


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