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 the hypothesis that soil N_2O flux with manure was linked with the nitrifying and denitrifying enzyme activities, and further with the abundance of N_2O-producing functional genes and the bacterial community structure. A field experiment was conducted in a drip-irrigated cotton field in an arid region of northwestern China. 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 was broadcast-incorporated into soil before seeding while urea was split-applied with drip irrigation (fertigation) over the growing season. The addition treatments did not, as assessed by nextgen sequencing of PCR-amplicons generated from rRNA genes, effect alpha diversity of bacterial communities but changed beta diversity. 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, suggesting that manure application increased N_2O emission by increasing denitrification

and the population of bacteria that mediate that process.

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ABSTRACT

Application of inorganic nitrogen (N) fertilizer and manure can increase nitrous oxide (N_2O) emissions. We tested the hypothesis that soil N_2O flux with manure was linked with the nitrifying and denitrifying enzyme activities, and further with the abundance of N_2O-producing functional genes and the bacterial community structure. A field experiment was conducted in a drip-irrigated cotton field in an arid region of northwestern China. 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 was broadcast-incorporated into soil before seeding while urea was split-applied with drip irrigation (fertigation) over the growing season. The addition treatments did not, as assessed by nextgen sequencing of PCR-amplicons generated from rRNA genes, effect alpha diversity of bacterial communities but changed beta diversity. 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, suggesting that manure application increased N_2O emission by increasing denitrification and the population of bacteria that mediate that process.

Keywords Nitrifier, Denitrifier, Manure, Drip irrigation, Bacterial community structure, 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 (*Ravishankara, Daniel & Portmann, 2009*). 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*). 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. 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 of fertilized forest soils

in China found 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*). Changes in the abundance and community of denitrifiers can largely explain the increase in denitrification associated with fertilizer application (*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*).

Nitrogen additions can affect soil microbial community directly by supplying substrates for microorganisms or indirectly by changing soil properties. Animal manure 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 receives intensive inputs of inorganic fertilizers and water recently as drip-irrigation (*Dai & Dong, 2014*). Cattle and sheep manure are also often used as nutrient sources due to the nearby livestock production. Manure application greatly increased N_2O emissions compared with conventional urea from this area, 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 at -80 °C.

Soil chemical properties

Soil NH_4^+ and NO_3^- was extracted using 0.01 M $CaCl_2$ and measured with a continuous flow

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

Determination of denitrifying and nitrifying enzyme activity

The frozen soil samples were pre-incubated to thaw at 25 °C for 2 days before analysis of DEA and NEA. 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 thawed soil samples was placed into 125 ml plasma flasks. 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 was added to each plasma flask. 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, 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™ 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™ 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 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

Manure and U+M treatment significantly ($P < 0.001$) increased DEA levels, compared to Urea and Control treatments (Fig. 1A). In contrast, NEA levels did not respond significantly to fertilizer treatment (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 addition treatments resulted in a clear clustering in β-diversity of the soil bacterial community (Fig. 2). Two groups with (Manure and U+M) and without (Control and Urea) manure application occurred 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.

Manure and U+M treatments doubled the gene copy number of *AOA* (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*.

The bacterial β-diversity was highly associated with changes in 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

Manure application exerted significant effect on microbial abundance and beta 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

316 activity with manure application was in accordance with the increasing abundance of nitrate
317 reducer (*narG*), and *nirK*- or *nosZ*-type denitrifiers. It should be noted, however, soil samplings
318 were conducted for only one time over the growing season for determination of soil microbial
319 activities in the current study, which hindered the investigations of temporal changes in soil
320 microbes and could also cause uncertainties in correlating with N_2O emissions. Still, sampling was
321 done in a representative field for the local cotton production where we compared farmer's
322 management practices of applying manure relative to inorganic fertilizers. The sampling depth (0-
323 20 cm) for microbial analysis was also in accordance with previous findings that soil N_2O
324 emissions following N addition were mostly attributed to the top soils (*Wagner-Riddle et al., 2008*;
325 *Kuang et al., 2019*).
326
327 **Impact of N addition strategy on denitrification and nitrification**
328 The increased activity of soil denitrifying enzymes with manure in the current study is in consistent
329 with our findings at the same field where we reported more N_2O emissions from manure compared
330 with urea application under drip irrigation conditions (*Kuang et al., 2018*). It is likely that the
331 increased NO_3^- and carbon supply with manure application could have provided primary substrate
332 for denitrification and increased the $N_2O/(N_2O+N_2)$ ratio (*Francis et al., 2013*). *Chantigny et al.*
333 *(2010)* also suggested that manure can elevate soil respiration and deplete O_2 concentration to
334 create temporary anaerobic conditions, thereby further increasing the proportion of N_2O
335 production through denitrification. These studies highlight the importance of N addition source on
336 soil N transformation processes and suggest that manure induced N_2O emissions are likely
337 attributed to denitrification.
338 In contrast to DEA, NEA was not affected by manure application in the current study. Similarly,

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

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

354

355 **Impact of N addition strategy on abundance of functional genes and bacterial community**
356 **structure**

357 In the current study, the positive relationships of the abundances of *narG*, *nirK* and *nosZ* with DEA
358 and further with soil DOC, total C and total N suggest that manure significantly increased gene
359 abundance by providing C and N substrate. This result is in line with the previous findings that the
360 denitrifiers abundance could be used as a predictor of DEA (*Morales, Cosart & Holben, 2010*).
361 Our findings also agree with previous studies which reported that organic manure increased

abundance of *nosZ*-type denitrifier compared to inorganic fertilizers (*Hallin et al., 2009*; *Tao et al., 2018*). Also being consistent with previous studies (*Zhou et al., 2011*), abundance of *nirK* but not *nirS* was increased by manure application in this study, suggesting that *nirK* was more susceptive to fertilizer regimes than *nirS*-type denitrifier. *Hallin et al. (2009)* also reported that denitrification rates were not correlated with the abundance of *nirS* genes in soils treated with different fertilizer regimes for 50 years. A possible reason for the lack of correlation could be that the denitrifier harbouring the *nirS* gene might play a minor functional role for DEA (*Attard et al., 2011*). In the current study, the nitrate reducer (*narG*), and *nirK*- and *nosZ*-type denitrifiers, which encodes the main catalytic enzymes responsible for nitrate reduction, nitrite reduction and N_2O reduction respectively, were more sensitive to manure application. The increase of the denitrifiers abundance with manure application thus resulted in an increase for the pool of denitrifying enzymes. Even though the limited soil sampling for microbial analysis hindered the possibility of directly linking results from the current study to the *in-situ* measurements of N_2O flux, the positive relationship between DEA and the abundance of denitrifiers suggest the manure-induced N_2O emissions in *Kuang et al. (2018)* was more likely determined by denitrification.

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

Similar to previous studies (*Ji et al., 2018*; *Kumar et al., 2018*; *Wang et al., 2019*), manure application significantly changed β-diversity of soil bacterial community in the current study. The PCoA analysis revealed a dominant contribution of PCoA1 (61%) to total variation and a clear separation of manure vs. non-manure groups along the axis PCoA1. This suggests that addition of manure was a key factor determining the variation in bacterial community among treatment. Clearly, the increased N and C substrates with manure application have increased the growth of some specific microbial groups and suppress others and thus changed the composition of soil microbial community. The absence of urea effect on the β-diversity of bacterial community was attributed to the low organic matter content (6.6 g kg^{-1}), suggesting that the substrate deficiency of C limited microbial activities under the conditions in this study. In this study, manure or urea applications did not influence α-diversity of soil bacterial community, likely due to an absence effect on soil pH. *Fierer & Jackson (2006)* reported that soil pH is the main driver determining the α-diversity and richness of soil bacterial community.

In the current study, the changes of soil bacterial community structure in response to manure application were attributed to the increasing relative 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* are involved in the turnover of soil organic carbon and nutrient availability and the reproduction

of this microbial group may increase intensively in response to the application of manure (*Lupatini et al., 2016*). The phyla which were negatively influenced by manure application were considered as slow-growing oligotrophs accustomed to nutrient-limited environments. For example, several studies had shown that *Acidobacteria* strains grew slowly with their growth being limited with substrate additions (*Goldfarb et al., 2011*).

CONCLUSIONS

Manure application significantly elevated the abundances of nitrate reducer (*narG*), and *nirK- and nosZ*-type denitrifiers, in accordance with a substantial increase of denitrifying enzyme activity. Additionally, soil DOC, total C and total N contents were highly correlated with the abundance of *narG*, *nirK and nosZ* genes, suggesting manure stimulated the functional genes via providing C and N substrates. In contrast, urea application did not exert significant impacts on the abundances of nitrifiers and denitrifiers. High throughout sequencing clearly showed that two years of manure application significantly altered bacterial community composition. Consequently, our study demonstrated a strong link between abundances of nitrate reducer (*narG*), *nirK*- and *nosZ*-type denitrifiers and enhanced denitrifying enzyme activity by manure application under the drip-irrigated conditions, indicating that denitrification is likely the key process determining manure-induced N_2O emissions.

REFERENCES

Attard E, Recous S, Chabbi A, De Berranger C, Guillaumaud N, Labreuche J, Philippot L, Schmid B, Le Roux X. 2011. Soil environmental conditions rather than denitrifier abundance and diversity drive potential denitrification after changes in land uses. *Global Change Biology* **17:**1975-1989 DOI 10.1111/j.1365-2486.2010.02340.x.

Beauchamp EG, Bergstrom DW. 1993. Denitrification. In: Carter MR. (Ed.), *Soil Sampling and Methods of Analysis*. Lewis Publishers, Boca Raton, FL, pp. 351-357.

Bru D, Sarr A, Philippot L. 2007. Relative abundances of proteobacterial membrane-bound and periplasmic nitrate reductases in selected environments. *Applied and Environmental Microbiology* **73:**5971-5974 DOI 10.1128/AEM.00643-07.

Carter MR. 1993. *Soil Sampling and Methods of Analysis*. Lewis Publishers, Boca Raton, FL.

Chantigny MH, Rochette P, Angers DA, Bittman S, Buckley K, Massé D, Bélanger G, Eriksenhamel N, Gasser MO. 2010. Soil nitrous oxide emissions following band-incorporation of fertilizer nitrogen and swine manure. *Journal of Environmental Quality* **39:**1545-1553 DOI 10.2134/jeq2009.0482.

Clark IM, Buchkina N, Jhurreea D, Goulding KW, Hirsch PR. 2012. Impacts of nitrogen application rates on the activity and diversity of denitrifying bacteria in the Broadbalk Wheat Experiment. *Philosophical Transactions of the Royal Society B-Biological Sciences* **367:**1235-1244 DOI 10.1098/rstb.2011.0314.

Dai J, Dong H. 2014. Intensive cotton farming technologies in China: Achievements, challenges and countermeasures. *Field Crops Research* **155:**99-110 DOI 10.1016/j.fcr.2013.09.017.

Di HJ, Cameron KC, Shen JP, Winefield CS, O'Callaghan M, Bowatte S, He JZ. 2010. Ammonia-oxidizing bacteria and archaea grow under contrasting soil nitrogen conditions. *FEMS Microbiology Ecology* **72:**386-394 DOI 10.1111/j.1574-6941.2010.00861.x.

Ding K, Zhong L, Xin, X. Xu Z, Kang X, Liu W, Rui Y, Jiang L, Tang L, Wang Y. 2015. Effect of grazing on the abundance of functional genes associated with N cycling in three types of grassland in Inner Mongolia. *Journal of Soils and Sediments* **15:**683-693 DOI 10.1007/s11368-014-1016-z.

Duan R, Long X-E, Tang Y-f, Wen J, Su S, Bai L, Liu R, Zeng X. 2017. Effects of different fertilizer application methods on the community of nitrifiers and denitrifiers in a paddy soil. *Journal of Soils and Sediments* **18:**24-38 DOI 10.1007/s11368-017-1738-9.

Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. 2011. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **27:**2194-2200 DOI 10.1093/bioinformatics/btr381.

Fan F, Yang Q, Li Z, Wei D, Cui XA, Liang Y. 2011. Impacts of organic and inorganic fertilizers on nitrification in a cold climate soil are linked to the bacterial ammonia oxidizer community. *Microbial Ecology* **62:**982-990 DOI 10.1007/s00248-011-9897-5.

Fang Y, Wang F, Jia X, Chen J. 2018. Distinct responses of ammonia-oxidizing bacteria and archaea to green manure combined with reduced chemical fertilizer in a paddy soil. *Journal of Soils and Sediments* **19:**1613-1623 DOI 10.1007/s11368-018-2154-5.

Fierer N, Bradford MA, Jackson RB. 2007. Toward an ecological classification of soil bacteria. *Ecology* **88:**1354-1364 DOI 10.1890/05-1839.

Francis CA, O'Mullan GD, Cornwell JC, Ward BB. 2013. Transitions in *nirS*-type denitrifier diversity,

community composition, and biogeochemical activity along the Chesapeake Bay estuary. *Frontiers in Microbiology* **4:**237-237 DOI 10.3389/fmicb.2013.00237.

Francis CA, Roberts KJ, Beman JM, Santoro AE, Oakley BB. 2005. Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. *Proceedings of the National Academy of Sciences* **102:**14683-14688 DOI 10.1073/pnas.0506625102.

Geisseler D, Scow KM. 2014. Long-term effects of mineral fertilizers on soil microorganisms – A review. *Soil Biology and Biochemistry* **75:**54-63 DOI 10.1016/j.soilbio.2014.03.023.

Goldfarb KC, Karaoz U, Hanson CA, Santee CA, Bradford MA, Treseder KK, Wallenstein MD, Brodie EL. 2011. Differential growth responses of soil bacterial taxa to carbon substrates of varying chemical recalcitrance. *Frontiers in Microbiology* **2:**94 DOI 10.3389/fmicb.2011.00094.

Hallin S, Jones CM, Schloter M, Philippot L. 2009. Relationship between N-cycling communities and ecosystem functioning in a 50-year-old fertilization experiment. *Isme Journal* **3:**597 DOI10.1038/ismej.2008.128.

Hart SC, Stark JM, Davidson EA, Firestone MK. 1994. *Nitrogen mineralization, immobilization, and nitrification*. In: Weaver RW. (Ed.), Methods of Soil Analysis: II. Microbiological and Biochemical Properties. SSSA, Madison, WI, pp. 985-1018.

Harter J, Krause HM, Schuettler S. Ruser R, Fromme M, Scholten T, Kappler A, Behrens S. 2014. Linking N_2O emissions from biochar-amended soil to the structure and function of the N-cycling microbial community. *Isme Journal* **8:**660-674 DOI 10.1038/ismej.2013.160.

IPCC. 2013. Climate Change 2013: The Physical Science Basis. Cambridge University Press, New York, USA.

Ji L, Wu Z, You Z, Yi X, Ni K, Guo S, Ruan J. 2018. Effects of organic substitution for synthetic N fertilizer on soil bacterial diversity and community composition: A 10-year field trial in a tea plantation. *Agriculture, Ecosystems & Environment* **268:**124-132 DOI 10.1016/j.agee.2018.09.008.

Kowalchuk GA, Stephen JR. 2001. Ammonia-oxidizing bacteria: a model for molecular microbial ecology. *Annual Review of Microbiology* **55:**485 DOI 10.1146/annurev.micro.55.1.485.

Kuang W, Gao X, Gui D, Tenuta M, Flaten DN, Yin M, Zeng F. 2018. Effects of fertilizer and irrigation management on nitrous oxide emission from cotton fields in an extremely arid region of northwestern China. *Field Crops Research* **229:**17-26 DOI 10.1016/j.fcr.2018.09.010.

Kuang W, Gao X, Tenuta M, Gui D, Zeng F. 2019. Relationship between soil profile accumulation and surface emission of N_2O: effects of soil moisture and fertilizer nitrogen. *Biology and Fertility of Soils* **55**:97-107 DOI 10.1007/s00374-018-01337-4.

Kumar U, Nayak AK, Shahid M, Gupta VV, Panneerselvam P, Mohanty S, Kaviraj M, Kumar A, Chatterjee D, Lal B. 2018. Continuous application of inorganic and organic fertilizers over 47 years in paddy soil alters the bacterial community structure and its influence on rice production. *Agriculture, Ecosystems & Environment* **262:**65-75 DOI 10.1016/j.agee.2018.04.016.

Li C, Yan K, Tang L, Jia Z, Li Y. 2014. Change in deep soil microbial communities due to long-term fertilization. *Soil Biology and Biochemistry* **75:**264-272 DOI 10.1016/j.soilbio.2014.04.023.

Lupatini M, Korthals GW, de Hollander M, Janssens TK, Kuramae EE. 2016. Soil Microbiome Is More Heterogeneous in Organic Than in Conventional Farming System. *Front Microbiol* **7:**2064 DOI 10.3389/fmicb.2016.02064.

Ma Z, Gao X, Tenuta M, Kuang W, Gui D, Zeng F. 2018. Urea fertigation sources affect nitrous oxide emission from a drip-fertigated cotton field in northwestern China. *Agriculture Ecosystems & Environment* **265:**22-30

506 DOI 10.1016/j.agee.2018.05.021.

507 **Marusenko Y, Garcia-Pichel F, Hall SJ. 2015.** Ammonia-oxidizing archaea respond positively to inorganic
508 nitrogen addition in desert soils. *FEMS Microbiology Ecology* **91:**1-11 DOI 10.1093/femsec/fiu023.

509 **Morales SE, Cosart T, Holben WE. 2010.** Bacterial gene abundances as indicators of greenhouse gas emission in
510 soils. *Isme Journal* **4:**799-808 DOI 10.1038/ismej.2010.8.

511 **Nicol GW, Leininger S, Schleper C, Prosser JI. 2008.** The influence of soil pH on the diversity, abundance and
512 transcriptional activity of ammonia oxidizing archaea and bacteria. *Environmental Microbiology* **10:**2966-
513 2978 DOI 10.1111/j.1462-2920.2008.01701.x.

514 **Oksanen J. 2011.** *Multivariate analysis of ecological communities in R*: vegan tutorial. R package version 1.17-7.
515 URL:http://cc.oulu.fi/~jarioksa/opetus/metod i/vegantutor.pdf.

516 **Ravishankara AR, Daniel JS, Portmann RW. 2009.** Nitrous oxide (N_2O): the dominant ozone-depleting
517 substance emitted in the 21st century. *Science* **326:**123-125 DOI 10.1126/science.1176985

518 **Röling WF. 2010.** Do microbial numbers count? Quantifying the regulation of biogeochemical fluxes by
519 population size and cellular activity. *FEMS Microbiology Ecology* **62:**202-210 DOI 10.1111/j.1574-
520 6941.2007.00350.x.

521 **Rotthauwe J-H, Witzel K-P, Liesack W. 1997.** The ammonia monooxygenase structural gene *amoA* as a
522 functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. *Applied and*
523 *Environmental Microbiology* **63:**4704-4712 DOI 10.1126/science.284.5411.63.

524 **Scala DJ, Kerkhof LJ. 1998.** Nitrous oxide reductase (*nosZ*) gene-specific PCR primers for detection of
525 denitrifiers and three *nosZ* genes from marine sediments. *FEMS Microbiology Letters* **162:**61-68 DOI
526 10.1111/j.1574-6968.1998.tb12979.x.

527 **Schloss P, Westcott S, Ryabin T, Hall J, Hartmann M, Hollister E, Lesniewski R, Oakley B, Parks D,**
528 **Robinson C, Sahl J, Stres B, Thallinger G, Van Horn D, Weber C. 2009.** Introducing mothur: open-source,
529 platform-independent, community-supported software for describing and comparing microbial communities.
530 *Applied and Environmental Microbiology* **75:**7537-7541 DOI 10.1128/AEM.01541-09.

531 **Schmieder R, Edwards R. 2011.** Quality control and preprocessing of metagenomic datasets. *Bioinformatics*
532 **27:**863-864 DOI 10.1093/bioinformatics/btr026.

533 **Shen JP, Zhang LM, Zhu YG, Zhang JB, He JZ. 2008.** Abundance and composition of ammonia-oxidizing
534 bacteria and ammonia-oxidizing archaea communities of an alkaline sandy loam. *Environmental*
535 *Microbiology* **10:**1601-1611 DOI 10.1111/j.1462-2920.2008.01578.x.

536 **Shi X, Hu H-W, Kelly K, Chen D, He J-Z, Suter H. 2016.** Response of ammonia oxidizers and denitrifiers to
537 repeated applications of a nitrification inhibitor and a urease inhibitor in two pasture soils. *Journal of Soils*
538 *and Sediments* **17:**974-984 DOI 10.1007/s11368-016-1588-x.

539 **Shrewsbury LH, Smith JL, Huggins DR, Carpenter-Boggs L, Reardon CL. 2016.** Denitrifier abundance has a
540 greater influence on denitrification rates at larger landscape scales but is a lesser driver than environmental
541 variables. *Soil Biology and Biochemistry* **103:**221-231 DOI 10.1016/j.soilbio.2016.08.016.

542 **Simon J, Klotz MG. 2013.** Diversity and evolution of bioenergetic systems involved in microbial nitrogen
543 compound transformations. Biochimica et Biophysica Acta (BBA) - Bioenergetics **1827:**114-135 DOI
544 10.1016/j.bbabio.2012.07.005.

545 **Sun R, Guo X, Wang D, Chu H. 2015.** Effects of long-term application of chemical and organic fertilizers on the
546 abundance of microbial communities involved in the nitrogen cycle. *Applied Soil Ecology* **95:**171-178 DOI

10.1016/j.apsoil.2015.06.010.

Tang Y, Yu G, Zhang X, Wang Q, Tian D, Tian J, Niu S, Ge J. 2019. Environmental variables better explain changes in potential nitrification and denitrification activities than microbial properties in fertilized forest soils. *Science of the Total Environment* **647:**653-662 DOI 10.1016/j.scitotenv.2018.07.437.

Tao R, Wakelin SA, Liang Y, Chu G. 2017. Response of ammonia-oxidizing archaea and bacteria in calcareous soil to mineral and organic fertilizer application and their relative contribution to nitrification. *Soil Biology and Biochemistry* **114:**20-30 DOI 10.1016/j.soilbio.2017.06.027.

Tao R, Wakelin SA, Liang Y, Hu B, Chu G. 2018. Nitrous oxide emission and denitrifier communities in drip-irrigated calcareous soil as affected by chemical and organic fertilizers. *Science of the Total Environment* **612:**739-749 DOI 10.1016/j.scitotenv.2017.08.258.

Throbäck IN, Enwall K, Jarvis Å, Hallin S. 2004. Reassessing PCR primers targeting *nirS*, *nirK* and *nosZ* genes for community surveys of denitrifying bacteria with DGGE. *FEMS Microbiology Ecology* **49:**401-417 DOI 10.1016/j.femsec.2004.04.011.

Tian XF, Hu HW, Ding Q, Song MH, Xu XL, Zheng Y, Guo LD. 2014. Influence of nitrogen fertilization on soil ammonia oxidizer and denitrifier abundance, microbial biomass, and enzyme activities in an alpine meadow. *Biology and Fertility of Soils* **50:**703-713 DOI 10.1007/s00374-013-0889-0.

Wagner-Riddle C, Hu QC, Van Bochove E, Jayasundara S. 2008. Linking Nitrous Oxide Flux During Spring Thaw to Nitrate Denitrification in the Soil Profile. *Soil Science Society of America Journal* **72:** 908-916 DOI 10.2136/sssaj2007.0353.

Wang Q, Garrity GM, Tiedje JM, Cole JR. 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology* **73:**5261-5267 DOI 10.1128/AEM.00062-07.

Wang F, Chen S, Wang Y, Zhang Y, Hu C, Liu B. 2018. Long-term nitrogen fertilization elevates the activity and abundance of nitrifying and denitrifying microbial communities in an upland soil: implications for nitrogen loss from intensive agricultural systems. *Frontiers in Microbiology* **9:**2424 DOI 10.3389/fmicb.2018.02424.

Wang Z, Liu Y, Zhao L, Zhang W, Liu L. 2019. Change of soil microbial community under long-term fertilization in a reclaimed sandy agricultural ecosystem. *PeerJ* **7:**e6497 DOI 10.7717/peerj.6497.

Watanabe A, Ikeya K, Kanazaki N, Makabe S, Sugiura Y, Shibata A. 2014. Five crop seasons' records of greenhouse gas fluxes from upland fields with repetitive applications of biochar and cattle manure. *Journal of Environmental Management* **144:**168-175 DOI 10.1016/j.jenvman.2014.05.032.

Yin C, Fan F, Song A, Cui P, Li T, Liang Y. 2015. Denitrification potential under different fertilization regimes is closely coupled with changes in the denitrifying community in a black soil. *Applied Microbiology & Biotechnology* **99:**5719-5729 DOI 10.1007/s00253-015-6461-0.

Zhang J, Kobert K, Flouri T, Stamatakis A. 2014. PEAR: a fast and accurate Illumina Paired-End reAd mergeR. *Bioinformatics* **30(5):**614-620 DOI 10.1093/bioinformatics/btt593.

Zhang Y, Shen H, He X, Thomas BW, Lupwayi NZ, Hao X, Thomas MC, Shi X. 2017. Fertilization Shapes Bacterial Community Structure by Alteration of Soil pH. *Frontiers in Microbiology* **8:**1325 DOI 10.3389/fmicb.2017.01325.

Zhou M, Zhu B, Wang S, Zhu X, Vereecken H, Brüggemann N. 2017. Stimulation of N_2O emission by manure application to agricultural soils may largely offset carbon benefits: a global meta-analysis. *Global Change Biology* **23:**4068-4083 DOI 10.1111/gcb.13648.

589 **Zhou ZF, Zheng YM, Shen JP, Zhang LM, He JZ. 2011.** Response of denitrification genes and to irrigation
590 water quality in a Chinese agricultural soil. *Environmental Science & Pollution Research* **18:**1644-1652 DOI
591 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.

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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.

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 $P < 0.05$.

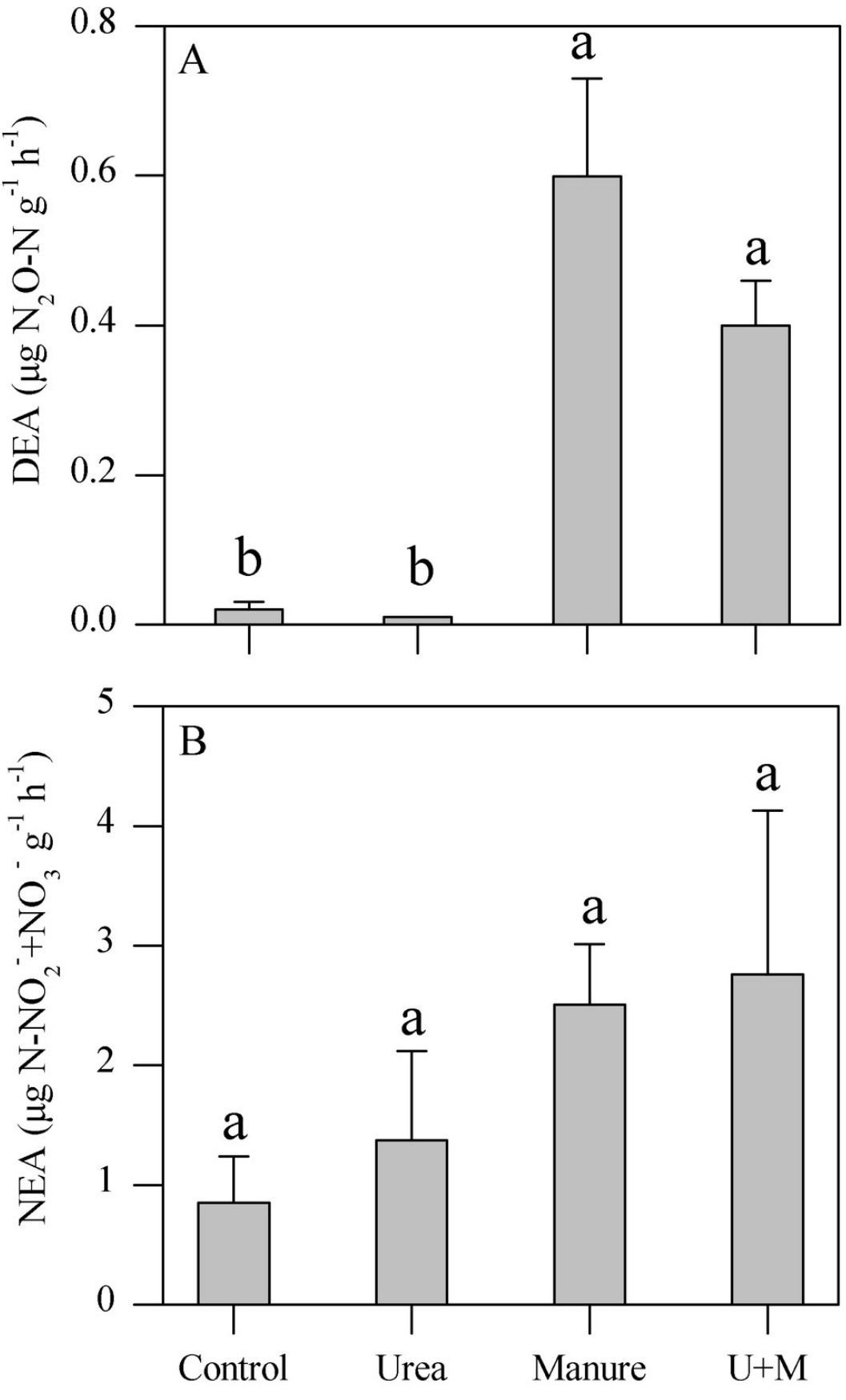
A
B
DEA (μg N_2O-N g^{-1} h^{-1})
NEA (μg N-NO_2^-+NO_3^- g^{-1} h^{-1})
0.0
0.2
0.4
0.6
0.8
0
1
2
3
4
5
a
b
Control
Urea
Manure
U+M

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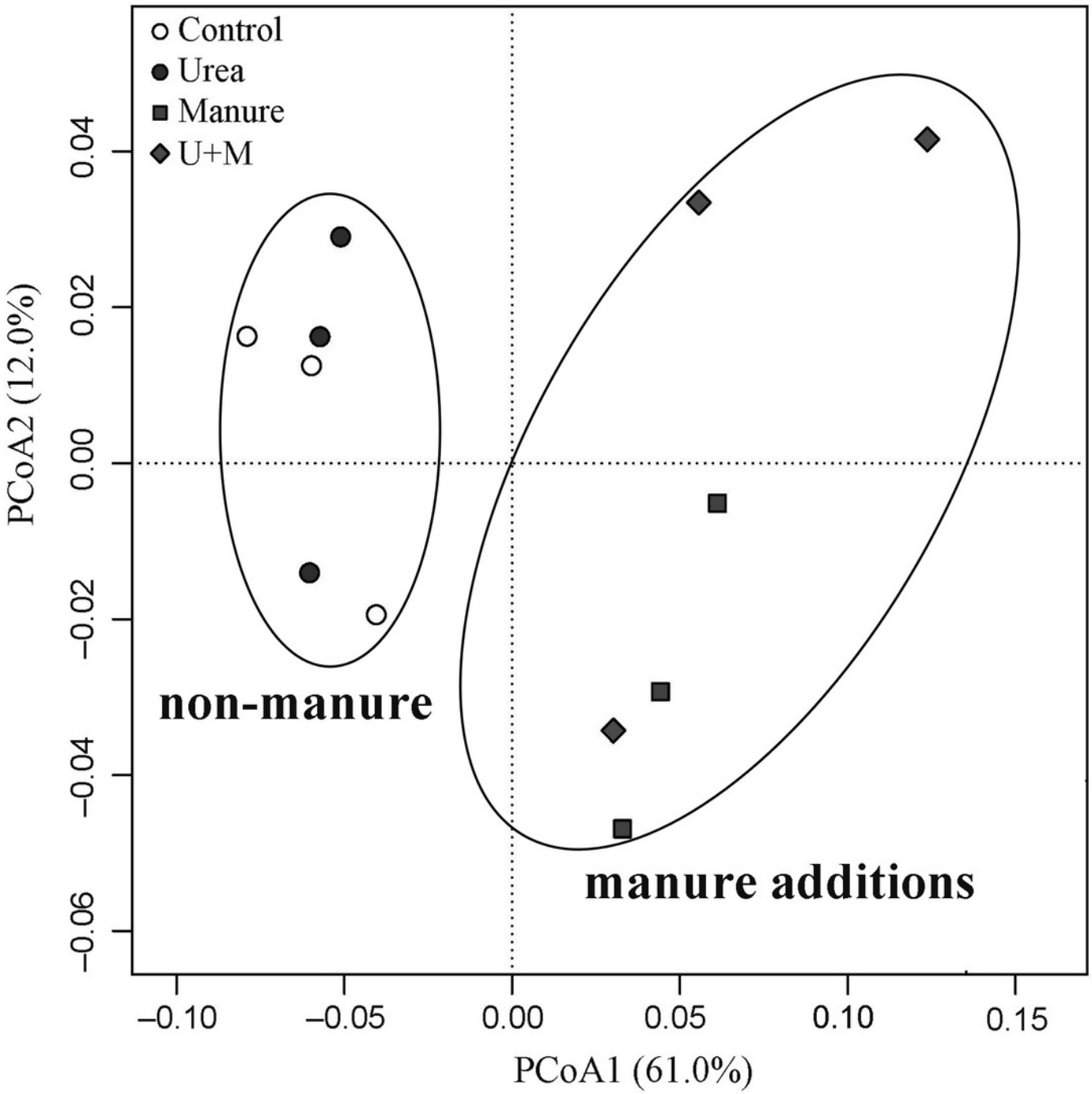
Control
Urea
Manure
U+M
non-manure
manure additions
PCoA2 (12.0%)
PCoA1 (61.0%)
0.04
0.02
0.00
−0.02
−0.04
−0.06
−0.10
−0.05
0.00
0.05
0.10
0.15

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 $P < 0.05$.

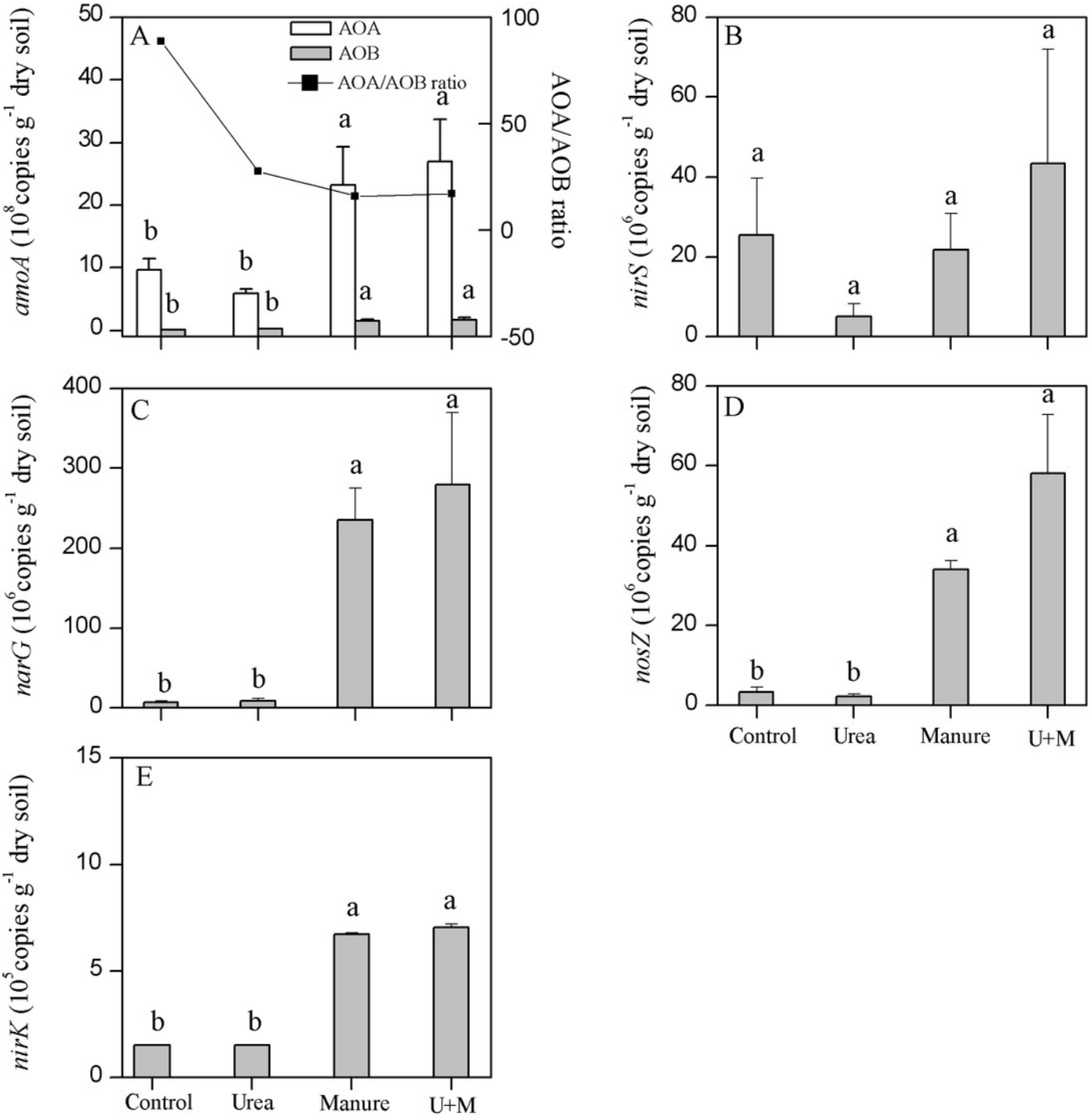
A
AOA
AOB
AOA/AOB ratio
amoA (10^8 copies g^-1 dry soil)
AOA/AOB ratio
B
nirS (10^6 copies g^-1 dry soil)
C
narG (10^6 copies g^-1 dry soil)
D
nosZ (10^6 copies g^-1 dry soil)
E
nirK (10^5 copies g^-1 dry soil)
Control
Urea
Manure
U+M

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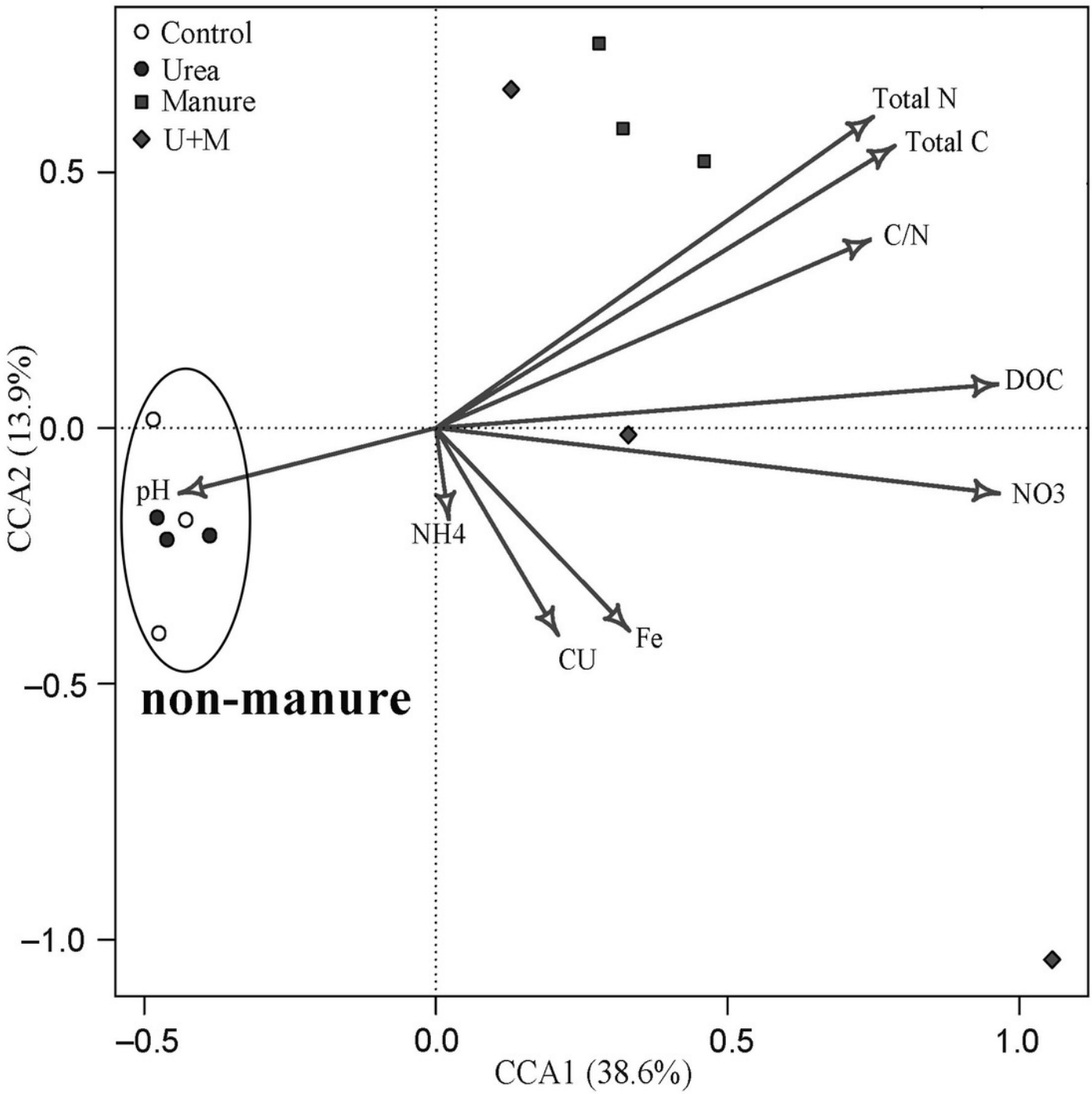
Control
Urea
Manure
U+M
Total N
Total C
C/N
DOC
NO3
pH
NH4
CU
Fe
non-manure
0.5
0.0
−0.5
−1.0
−0.5
0.0
0.5
1.0
CCA2 (13.9%)
CCA1 (38.6%)