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Increasing livestock wastewater application in alternate-furrow irrigation reduces nitrification gene abundance but not nitrification rate in rhizosphere

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Corresponding Author:	Zhongyang Li, PhD Farmland Irrigation Research Institute Xinxiang, Henan CHINA
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Farmland Irrigation Research Institute
Corresponding Author's Secondary Institution:	
First Author:	Yuan Liu, PhD
First Author Secondary Information:	
Order of Authors:	Yuan Liu, PhD
	Andrew L. Neal, PhD
	Xiaoxian Zhang, PhD
	Erping Cui
	Feng Gao
	Xiangyang Fan
	Chao Hu
	Zhongyang Li
Order of Authors Secondary Information:	

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Abstract:	In water-scarce regions, alternate-furrow irrigation (AFI) - alternately wetting half of the plant roots - has proven to be an effective water-saving approach without compromising yield. However, the extent to which AFI with wastewater affects N cycling genes remains poorly studied. We aimed to investigate changes in main N transformation processes, bacterial and fungal community composition, as well as relative abundance of N cycle-associated genes in soil receiving AFI with swine wastewater. The experimental plan included three irrigation rates, irrigating pepper plants with 50%, 65% and 80% of the amount of water required under conventional furrow irrigation to prevent the crop suffering water stress. Each treatment had a groundwater-irrigation control. We measured edaphic factors, microbial community composition and relative abundance of genes in rhizosphere and bulk soils. Altering water use in AFI did not exert a significant effect on bacterial and fungal communities. By increasing the irrigation rate of wastewater, relative abundances of nifH, bacterial and archaeal amoA and nosZ genes decreased whereas those of nirK and nirS genes increased in the rhizosphere soil; nitrification rate did not decrease and the denitrification rate remained unchanged in both rhizosphere and bulk soil, implying that appropriate increase of wastewater use by AFI can improve N use efficiency.	
Suggested Reviewers:	Gupta Vadakattu, PhD Principal Research Scientist, Commonwealth Scientific and Industrial Research Organisation gupta.vadakattu@csiro.au He is an expert in nitrogen cycling genes in soils.	
	Tim George, PhD The James Hutton Institute tim.george@hutton.ac.uk He is a rhizosphere scientist.	

Response to Reviewers

Dear Reviewers/Editor

We are grateful for the time and effort the editor and reviewers have taken to help us improve the manuscript. Our response to the comments of Editor, Reviewer #1 and Reviewer #2 are marked in red, blue and green respectively in the revised manuscript.

Reviewers/Editor comments:

Response to Editor comments

Your manuscript titled "Increasing livestock wastewater application in alternate-furrow irrigation reduces nitrification gene abundance but enhances nitrification rate in rhizosphere" has been reviewed by two referees and it is accepted for publication after revision according to the enclosed referees' comments below.

These are my specific comments:

L. 34, please do not indent;

Reply: We revised as you suggested.

L. 52, "organic C";

Reply: We revised as you suggested.

Please delete commas in the citations at L. 60 and 418;

Reply: We revised as you suggested.

L. 64, the fertilization do not add microbial species unless it is an organic fertilizer. Please reword;

Reply: We added the types of fertilizers. (L. 63-65)

L. 230 and 232, please do not mention not significant differences;

Reply: We deleted the description with no significant differences.

Please replace "nitrogen" with "N" at L. 455, 457 and 844;

Reply: We revised as you suggested.

Please move the reference at L. 781 after that at L. 485

Reply: The reference at L. 781 (L. 795 in the revised manuscript) is talking about N fixation and the related genes, but L. 485 (L. 491 in the revised manuscript) is about nitrification and the associated genes. Therefore, we moved the reference at L. 795 to L. 539 where N fixation is discussed.

Reviewers' comments:

This manuscript has checked by the same reviewers as before, but still some minor revisions required. Authors should check again carefully according to the comments below.

Response to Reviewer comment No. 1

Reviewer #1: The manuscript was revised according to the previous comments and the focus of the study became clear. I think revised version of the manuscript is almost acceptable.

There are some points I would like to make sure before its publication.

1. Did the authors measure soil microbial activities relating to N transformation with samples which had been frozen (L. 143)? To my opinion, this is not appropriate for activity measurement, therefore, if there are any concerns relating to the frozen effects on the microbial activities, it is better to note in the text.

Reply: Yes, the samples had been frozen. We agree with the reviewer that thawing a frozen-stored sample might render the measured soil microbial activities differing from that in the field when the sample was taken, but the reported research in the literature about this is not conclusive, with some showing a little change while others finding no observable alternation at all (Stenberg et al. 1998). Nonetheless, all samples in our work were stored and measured following the same protocol and the methodological effects were thus consistent, as pointed out by Rubin et al. (2013) that methodological consistency is key to ensure accurate characterization and comparison of soil microbial community. As such, the results comparison between different treatments presented in the manuscript is rational. We made this clear in the revised manuscript (L. 147-154).

Rubin BE, Gibbons SM, Kennedy S, Hampton-Marcell J, Owens S, Gilbert JA (2013) Investigating the impact of storage conditions on microbial community composition in soil samples. PloS One 8: e70460

Stenberg B, Johansson M, Pell M, Sjödahl-Svensson K, Stenström J, Torstensson L (1998) Microbial biomass and activities in soil as affected by frozen and cold storage. Soil Biol Biochem 30: 393–402

2. Title, L.27, L. 462-463, L.566; It seems that nitrification rates in rhizosphere soil with wastewater application are not differ among treatments (Fig. 1). Is the relevant explanation in the manuscript accurate?

Reply: Yes, the nitrification rates in rhizosphere soil did not increase significantly with the increase of wastewater application (Fig. 1), we modified the title and the relevant descriptions. (L. 2, 27-28, 466, 468-470, 574)

Response to Reviewer comment No. 2

Reviewer #2: This manuscript has now substantially improved according my and others comments. Especially it is very important that activity data was now provided.

Still the manuscript is very descriptive and some discussion are not well done, for example, analysis in Fig.6 should be done together with activity data.

Reply: We redid the Redundancy Analysis together with the activity data and modified the associated discussion. (L. 363-364, 366, 368, 371, 374, 380, 382, 505, 513-514, 524-525, 550)

Increasing livestock wastewater application in alternate-furrow irrigation reduces nitrification gene abundance but not nitrification rate in rhizosphere

Yuan Liu[a], Andrew L. Neal[b], Xiaoxian Zhang[b], Erping Cui[a], Feng Gao[a], Xiangyang Fan[a] , Chao Hu[a], Zhongyang Li[a,*]

[a] Farmland Irrigation Research Institute, Chinese Academy of Agricultural Sciences, Xinxiang 453002, China

[b] Department of Sustainable Agriculture Sciences, Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, UK

[*]Corresponding author. E-mail address: lizhongyang1980@163.com.

Abstract

In water-scarce regions, alternate-furrow irrigation (AFI) - alternately wetting half of the plant roots - has proven to be an effective water-saving approach without compromising yield. However, the extent to which AFI with wastewater affects N cycling genes remains poorly studied. We aimed to investigate changes in main N transformation processes, bacterial and fungal community composition, as well as relative abundance of N cycle-associated genes in soil receiving AFI with swine wastewater. The experimental plan included three irrigation rates, irrigating pepper plants with 50%, 65% and 80% of the amount of water required under conventional furrow irrigation to prevent the crop suffering water stress. Each treatment had a groundwater-irrigation control. We measured edaphic factors, microbial community composition and relative abundance of genes in rhizosphere and bulk soils. Altering water use in AFI did not exert a significant effect on bacterial and fungal communities. By increasing the irrigation rate of wastewater, relative abundances of *nifH*, bacterial and archaeal *amoA* and *nosZ* genes decreased whereas those of *nirK* and *nirS* genes increased in the rhizosphere soil; nitrification rate did not decrease and the denitrification rate remained unchanged in both rhizosphere and bulk soil, implying that appropriate increase of wastewater use by AFI can improve N use efficiency.

Keywords: Livestock wastewater; Alternate-furrow irrigation; Irrigation amount; Nitrogen transformation genes; Water quality

Introduction

Recycling nutrient-rich livestock wastewaters and reusing them for irrigation (Cai et al. 2013) is an attractive approach to relieve water-shortage pressure, capture N and other nutrients in plant biomass and soil and dispose of wastes in a managed manner. Irrigation with nutrient-rich wastewater is likely to alter N transformations in soils including nitrification, denitrification, N_2-fixation, anaerobic ammonium oxidation (anammox), and complete ammonia oxidation (commamox) to NO_3^--N. During nitrification, NH_4^+ is oxidized progressively to NO_2^- and then to NO_3^-. Ammonia oxidation is a rate-limiting process in nitrification under aerobic condition, mediated by both ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) (Könneke et al. 2005). The sequential reduction of NO_3^- and NO_2^- to nitric oxide (NO), or nitrous oxide (N_2O) or dinitrogen gas (N_2) in denitrification are anaerobic microbial processes, driven by denitrifying microorganisms that involve nitrate reductase (encoded by *narG* and *napA*), nitrite reductase (*nirK* and *nirS*), nitric oxide reductase (*norB* and *norC*), and nitrous oxide reductase (*nosZ*) (Li et al. 2018).

N_2O is a greenhouse gas 300 times more potent than CO_2 and which is also responsible for ozone depletion (Mosier et al. 1998). Reducing its emission from arable soil is thus imperative (Ravishankara et al. 2009), especially since agriculture in China remains a net source of greenhouse gases (Gao et al. 2018). N_2O emission is modulated by functional genes involved in nitrite reduction, such as *nirS* and *nirK*, and *nosZ*, which encodes nitrous oxide reductase (Zehr and Kudela 2011; Hu et al. 2015).

The abundance and activity of nitrifying and denitrifying microorganisms in soil is influenced by organic matter (OM), pH, total N (TN), organic C, temperature, NH_4^+ and NO_3^-, among other factors (Henry et al. 2006; Dong et al. 2009; Li et al. 2018; Shan et al. 2018). Growth of AOB depends on the availability of NH_4^+ (Martens-Habbena et al. 2009). The study on N dynamics in plant-soil system in a

riparian zone showed that there were significant negative correlations between abundance of *nirK*, *nirS*, *nosZ* genes and soil electrical conductivity (EC), while abundance of *nifH* was negatively associated with soil bulk density as opposed to abundance of archaeal *amoA*; it was also found that increasing soil water content led to an increase in *nifH* abundance but decrease in archaeal *amoA* abundance (de Sosa et al. 2018). Increasing the availability of NO_3^- and labile C enhances denitrification (Weier et al. 1993), high NO_3^- concentrations inhibits N_2O reductase activity (Qin et al. 2017), and low C/N ratio or high total N favours bacteria harbouring *amoA* genes (Nugroho et al. 2006; Dong and Reddy 2012). Tillage system induced physicochemical stratification impacts abundance of N cycling microbial communities and net N_2O emissions within the soil profile according to N or C species added during fertilization with an inorganic fertilizer (calcium ammonium nitrate) or two organic fertilizers (liquid dairy slurry and dairy manure compost) (Krauss et al. 2017).

Apart from these edaphic factors, irrigation methods and frequency may also influence the abundance and activity of microorganisms and their associated N transformation genes directly through changes in water and oxygen distributions, or indirectly through changes in diffusion and transport of substrates, pH and temperature *etc.* (Zhou et al. 2011; Wertz et al. 2013; Yin et al. 2015; Hou et al. 2016; Owens et al. 2016; Han et al. 2017; Yang et al. 2018). The effects of irrigation amount on N-related microbial activity and gene abundance are not established (Berger et al. 2013; Zhang et al. 2016; Azziz et al. 2017), suggesting that the effects of irrigation on microbial community and N-transformation genes are poorly understood.

In arid and semi-arid regions, alternate-furrow irrigation (AFI) has been developed as an efficient water-saving irrigation method (Graterol et al. 1993; Kang et al. 2000a, 2000b). AFI irrigates each of two adjacent furrows alternately, and by keeping roots in the dry furrow for a prolonged period,

stimulates synthesis of abscisic acid (ABA) in attempts to reduce leaf stomatal conductance and ultimately plant transpiration. Compared to conventional furrow irrigation (CFI), AFI has the potential to reduce N_2O emissions (Han et al. 2014), but its effect upon the abundance of N cycle genes in soil is poorly understood.

Given the increase in use of AFI and wastewater in irrigation, we investigated the response of abundance of N-cycle genes and main N transformation processes in a pepper field irrigated with different amounts of swine wastewater under AFI. For each treatment, there was a groundwater irrigation control. We hypothesized that (1) wetting-drying cycles associated with AFI exert different effects on N transformation processes and the abundance of associated N-cycle genes in soil from CFI, and (2) the irrigation amounts with AFI change N transformation processes and the abundance of associated N-cycle genes in soil synchronously. In all treatments and controls, we measured the main N transformation activities, distribution of N cycling genes and the composition of bacterial and fungal communities, and analyzed their association with edaphic factors. This will fill the knowledge gap of how AFI with wastewater influences N transformation activities and the related genes, and provide a reference of N management for sustainable use of livestock wastewater in agricultural production in arid and semiarid regions.

Materials and Methods

Soil and water

The field experiment was conducted at the Agriculture Water and Soil Environmental Field Science Research Station of Chinese Academy of Agricultural Science at Xinxiang (Henan Province, 35°15′44″N, 113°55′6″E). All experimental plots were covered by a vinyl shield 5 m above the ground surface to intercept rainwater, and the soil is sandy loam classified as fluvic Cambisol. The main

properties of the top 20 cm of soil were: pH 8.5, electrical conductivity 87.7 mS m^{-1}, OM 9.0 g kg^{-1}, total N 0.7 g kg^{-1}, NO_3^--N 136 mg kg^{-1}, exchangeable NH_4^+-N 7.9 mg kg^{-1}, available K 252 mg kg^{-1}, available P 33.2 mg kg^{-1}, total Cu 25.7 mg kg^{-1}, total Zn 72.4 mg kg^{-1}, total Pb 22.0 mg kg^{-1}, total Cd 0.60 mg kg^{-1}, available Cu 1.5 mg kg^{-1}, available Zn 1.8 mg kg^{-1}, available Pb 1.9 mg kg^{-1}, and available Cd 0.20 mg kg^{-1}.

The groundwater used in the experiment was pumped from a shallow aquifer at the experimental site, and wastewater was taken from a fermentation tank at a near-by hoggery producing approximately 40,000 tons of wastewater annually. The properties of the waters are shown in Table 1.

The plant and the field experiment

The plant used in the experiment was pepper (*Capsicum annuum* L., Fulong F1). The seedling medium was the mixture of perlite and vermiculite at 1:1 ratio (weight), which was packed into seedling-nursing disk consisting of 4 × 8 cavities, each being 5.8 cm high with its internal diameter changing from 5.3 cm on the top to 2.7 cm on the bottom. All pepper seeds were sown on April 14, 2017 and were subsequently supplied with Hoagland and Amon nutrient solutions based on the protocol provided in Li et al. (2010). The field soil was initially amended with a base fertilizer consisting of 180 kg urea ha^{-1} (84 kg N ha^{-1}), 450 kg $Ca(H_2PO_4)_2 \cdot H_2O$ ha^{-1}, and 240 kg KCl ha^{-1}. One month after seed germination, healthy seedlings were transplanted to the field. Seedlings were transplanted into rows in the field spaced 50 cm apart and planted at 50 cm intervals along each row. Each plot was 2 × 8 m, formed of three rows separated by four 30 cm-deep furrows. There was a 50 cm gap between adjacent plots to avoid water flowing from one plot into another. Prior to wastewater irrigation, each plot was irrigated with groundwater at 250 m^3 ha^{-1} via CFI every 7 days until 19 June to establish and maintain healthy plant growth.

The soil was top-dressed with 90 kg of urea ha^{-1} (42 kg N ha^{-1}) on July 21, August 12 and September 3. The total urea applied during the experiment was 450 kg ha^{-1} (210 kg N ha^{-1}). In wastewater treatments, we diluted the wastewater with groundwater at 1:1 volumetric ratio prior to irrigation. There were eight treatments: irrigating with groundwater at 250 m^3 ha^{-1} approximately every 10 days via CFI (GC100), AFI with groundwater using 50% of the water used in GC100 (GA50), AFI with groundwater using 65% of the water used in GC100 (GA65), AFI with groundwater using 80% of the water used in GC100 (GA80), CFI with wastewater using the same amount of the water in GC100 (WC100), AFI with wastewater using 50% of the water used in WC100 (WA50), AFI with wastewater using 65% of the water used inWC100 (WA65), AFI with wastewater using 80% of the water used in WC100 (WA80).

Each treatment has three replicate plots arranged in a completely randomized design. Plots were irrigated on June 19, June 28, July 9, July 21, August 1 and August 12, at approximately the same time. Following Kang et al. (2000b), both adjacent furrows were watered under CFI, but only one furrow was watered under AFI with the same water amount of each CFI furrow: the total water amount of AFI was 50% of CFI. Here, we refer to the water amount in CFI as 100%, thus 50% in AFI. These two treatments with the only difference of irrigated furrows but not the difference in irrigation amount in each furrow were used to examine the effects of AFI. AFI under 50%, 65% and 80% rates were used to study the impact of irrigation amount on N transformation activities as well as distribution and abundance of N cycling genes.

Prior to harvest on 9 October, all plots were irrigated with groundwater at 250 m^3 ha^{-1} *via* CFI every 7 days starting from 23 August. Details of the irrigation schedule are listed in Table S1. At harvest, roots were sampled to a depth of 0 - 20 cm. Soil shaken off the roots was termed bulk soil (BS)

and soil adhering to roots was termed rhizosphere (RS). In each plot, soil collected from 5 randomly selected plant roots was mixed. Sub-samples were stored at -80 ºC for extraction of nucleic acids and determination of nitrification and nitrogen fixation rates (see description in the Supplementary Information), and the rest for measurement of soil chemical properties and denitrification rate (see description in the Supplementary Information). Thawing a frozen-stored sample might render the measured soil microbial activities differing from that in the field when the sample was taken, but the reported research in the literature about this is not conclusive, with some showing a little change while others finding no observable alternation at all (Stenberg et al. 1998). Nonetheless, all samples in our work were stored and measured following the same protocol and the methodological effects were thus consistent, as pointed out by Rubin et al. (2013) that methodological consistency is key to ensure accurate characterization and comparison of soil microbial community. As such, the results comparison between different treatments is rational. The total N of plant roots, stems, leaves and fruits were analyzed on a flow analyzer (AutoAnalyzer 3, Bran Luebbe, Germany) after digestion with concentrated sulfuric acid. N use efficiency of plants was calculated by Eq. (1) (Yang et al. 2017).

$$\text{N use efficiency (\%)} = \text{plant N/added N} \times 100 \quad \text{Eq. (1)}$$

where “plant N” was the sum of N form all tissues in each plot, and “added N” was the sum of N from fertilizer and irrigated water in each plot.

DNA extraction

A FastDNA SPIN Kit for Soil (MP Biomedicals, CA) was used to extract total DNA from about 0.5 g of each soil sample according to the instruction manual, and three replications were conducted for each sample (Vestergaard et al. 2017). We used spectrophotometric analysis (NanoDrop ND-2000c, Thermo Fisher Scientific, Waltham, MA) and 1.5% agarose gel electrophoresis to determine the

concentration and quality of the extracted DNA.

MiSeq pyrosequencing

PCR amplification of the bacterial 16S rRNA gene V3–V4 variable region was performed using the forward primer 5'-ACTCCTACGGGAGGCAGCAG-3' (338F) and the reverse primer 5'-GGACTACHVGGGTWTCTAAT-3' (806R) (Xu et al. 2016). The primers ITS3 (5'-GCATCGATGAAGAACGCAGC-3') (Leaw et al. 2006) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Siddique and Unterseher 2016) were used to amplify the fungal ITS regions. The reaction mixture and the thermal profile of the PCR amplifications were based on Huang et al. (2016). After the PCR products were purified, they were adjusted to equal quantities, and paired-end 2×300 base pair (bp) sequencing was performed on an Illumina MiSeq sequencing platform by Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China).

Sequences were examined for quality using the default arguments in the split_libraries python script apart from increasing primer mismatch from 0 to 2, and were then assigned to each sample based on unique 10-bp barcodes. After removing barcode and primer sequences, the remaining sequences were clustered into operational taxonomic units (OTUs) at a level of 97% sequence similarity (Schöler et al. 2017) and annotated using BLAST searches against the Greengenes (Release 13.8, http://greengenes.secondgenome.com/, bacteria) and Unite (Release 5.0, http://unite.ut.ee/index.php, fungi) databases using the Quantitative Insights into Microbial Ecology (QIIME) software package version 1.8.0 (Caporaso et al. 2010).

Relative quantification of genes

The N-cycle related genes we investigated were involved in N_2 fixation (*nifH*), ammonia oxidation (Archaeal *amoA* and Bacterial *amoA*), nitrite reduction (*nirK* and *nirS*) and nitrous oxide reduction

(*nosZ*). Genes were amplified and quantified using the quantitative polymerase chain reaction (qPCR) and the SYBR Green approach at Shanghai Personal Biotechnology Co., Ltd (Shanghai, China). All qPCR reactions were repeated three times. The primer description can be found in Table S2. All qPCR reactions were performed using the CFX-96 touch real-time PCR detection system (Bio-Rad, USA). Cycle conditions were 95 °C for 5 minutes, followed by 45 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. A threshold cycle (C_t) of 36 was used as the detection limit (Malvick and Impullitti 2007). Generally, the technical triplicates were tested during separate testing occasions (plate and day of testing) as a method of quality control. The $2^{-\Delta\Delta Ct}$ method of comparison (Livak and Schmittgen 2001; Zhu et al. 2013) was used to compare relative gene abundance between samples:

$$\Delta C_t = C_{t,\,(\text{N cycling gene})} - C_{t,\,(16S)}$$

$$\Delta\Delta C_t = \Delta C_{t,(\text{Target})} - \Delta C_{t,(\text{Ref})}$$

where C_t is the threshold cycle, N cycling gene is one of the N cycling gene assays, 16S is the 16S rRNA gene assay, the subscripts Target and Ref refer to experimental sample and reference sample respectively. To identify changes in N-cycle associated gene abundance between soil samples taken from all treatments, the soil before cultivation and fertilization was used as the reference sample in all calculations.

Statistical analysis

We compared the gene abundance and environmental parameters statistically using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA). One-factor analysis of variance (ANOVA) was used to test differences between treatments, and Duncan's multiple range test was used to conduct *post-hoc* pairwise comparisons of treatment-means. A probability of $p < 0.05$ was deemed to be significant. Two-factor ANOVA of gene abundance, nitrification rate, denitrification rate and N use efficiency was

conducted to test the effect of water source and irrigation amount.

OTU data was analyzed using MicrobiomeAnalyst (Dhariwal et al. 2017), using a minimum mean abundance cut-off of 20 across all treatments. A low variance filter was also used to remove OTUs associated with the lowest 10% of coefficients of variance, determined using the inter-quantile range. Cumulative Sum Scaling (CSS) was used to for gene abundance data (Weiss et al. 2017). Principal coordinate analysis (PCoA) of soil bacterial and fungal assemblages, assessed at the OTU-level, was performed using weighted UniFrac distance (Lozupone et al. 2011). The significant OTU divergence between different soils was tested using permutation multivariate analysis of variance (PERMANOVA) based again on weighted UniFrac phylogenetic distance. Whenever detecting a significant divergence between communities, we tested it for homogeneity of multivariate dispersion between the groups using PERMDISP (Anderson and Walsh 2013). Where no significant difference in multivariate dispersion was detected, the significant difference in OTU assemblages was attributed to the imposed treatment. We used the DESeq2 algorithm (Love et al. 2014) to test for OTUs associated with significantly different abundance in response to the significant factors.

The PCoA was used to assess differences between N cycling gene abundance based on the Euclidean distance in PAST 3.20, which, along with the two-factor PERMANOVA with 9,999 permutations conducted in PAST, was used to evaluate the divergence of genes between different treatments. We employed redundancy analysis (RDA) to assess the association between gene abundance and environmental factors using CANOCO 5 (ter Braak 1988), where a significant treatment effect was identified. For the RDA model, statistical predictors of gene abundance were identified from the summarized effects of environmental variables. All environmental variables were transformed to *z*-scores prior to analysis, and statistical significance of the resulting RDA model was

assessed based upon 999 permutations.

Results

Change in soil chemical properties

The changes in soil chemical properties influenced by irrigation are given in Table 2. Soil NO_3^--N contents under AFI using wastewater were lower than CFI with the significant difference at 80% rate in bulk soils. For exchangeable NH_4^+-N in soil, AFI at 50% and 65% rates using groundwater resulted in higher content than CFI in rhizosphere and bulk soils, and AFI at 65% and 80% rates using wastewater in both soil compartments and at 50% rate using wastewater in bulk soils, though not significantly. Soil pH under groundwater irrigation was higher than under wastewater irrigation, as opposed to the content of OM, TN and NO_3^--N as well as C/N ratio. In all treatments, EC and NO_3^--N in bulk soil were higher than in rhizosphere, in contrast to OM and exchangeable NH_4^+-N. There were no significant differences in soil chemical properties between the three AFI treatments.

N transformation activities

The input and uptake of N and plant N use efficiency in different treatments are listed in Table S3. Total N input was higher in wastewater treatments than groundwater treatments, but N use efficiency was significantly lower in wastewater treatments. Water source, but not irrigation amount, had a significant influence upon N use efficiency (Table S4). There were no significant differences in N use efficiency between the wastewater irrigation rates. AFI significantly increased N use efficiency in groundwater treatments compared to CFI, and the efficiency was increased at higher rates of AFI, though not significantly. There were no significant differences in N-uptake by plants under three rates of AFI regardless of water sources, but N-uptake by plants in CFI was

significantly lower than 80% rate of AFI under groundwater irrigation, simultaneously N-uptake by plants in CFI was significantly higher than 50% rate of AFI under wastewater irrigation.

The NO_3^--N content in soil increased from 0h to 16h after adding the ammonium solution and then decreased (data not shown), hence we employed the difference of NO_3^--N content between 0h and 16h to calculate nitrification rate. Nitrification rates were significantly influenced by water source, but not by irrigation rate in rhizosphere (Table S4). In bulk soil, the effects of water source and irrigation amount on nitrification rate were both not significant (Table S4). There were no significant differences in nitrification activity in groundwater-irrigated bulk soils, the nitrification activity in 80% rate of AFI was significantly higher than 50% rate in wastewater-irrigated bulk soils (Fig. 1).

The NO_3^--N content in soil decreased over 4 days of anaerobic culture, and then stayed stable (data not shown), hence we employed the difference of NO_3^--N content between 0d and 4d to calculate denitrification rates. There were no significant differences of denitrification rates between soils irrigated using different water sources or rates (Table S4). In wastewater-irrigated rhizospheres, the denitrification activity in CFI was higher than that under AFI treatments and significantly higher than that at 65% AFI rate. Nitrogen fixation was not detected in any of the soils.

Fig. 1 The nitrification rate and denitrification rate of soil. RS refers to rhizosphere, BS refers to bulk soil, G refers to groundwater, W refers to livestock wastewater, C refers to conventional furrow irrigation, A refers to alternate-furrow irrigation. 100, 50, 65 and 80 refer to 100%, 50%, 65% and 80% of full irrigation amount per plot, respectively. The data are expressed as the mean ± standard deviation. Different lower-case letters above the columns represent significant difference between

treatments at $p < 0.05$

Composition of bacterial and fungal community

Rarefaction curves indicated that the sequencing depth was sufficient to cover the microbial diversity (Fig. S1). We did not detect significant heterogeneity in multivariate dispersion of the OTU assemblages and so used PERMANOVA to test for differences in assemblages between rhizosphere and bulk soil under the different irrigation regimes. Both soil compartment (rhizosphere *vs.* bulk soil, $R^2 = 0.107$, $p = 0.002$) and irrigation water source (groundwater *vs.* wastewater, $R^2 = 0.222$, $p < 0.001$) had significant effects on bacterial OTU assemblage (Fig. 2a). Variability of bacterial OTUs in wastewater-irrigated soil were reduced compared to groundwater-irrigated soil, yet different irrigation amounts did not give rise to significant difference in bacterial OTU assemblages ($R^2 = 0.069$, $p = 0.318$).

For fungi, significant differences in OTU assemblage were observed only between rhizosphere and bulk soils ($R^2 = 0.181$, $p < 0.001$). Neither water source ($R^2 = 0.384$, $p = 0.092$) nor irrigation rate ($R^2 = 0.083$, $p = 0.166$) induced significant changes in OTU assemblages.

Fig. 2 OTU-based unconstrained Principal Coordinate Analysis of soil bacterial (a) and fungal (b) communities using weighted UniFrac distance metrics. RS (circle) refers to rhizosphere, BS (square) refers to bulk soil, G (blue color) refers to groundwater, W (red color) refers to livestock wastewater, C refers to conventional furrow irrigation, A refers to alternate-furrow irrigation. 50, 65, 80 and 100 refer to 50%, 65%, 80% and 100% of full irrigation amount per plot respectively and the colors are changed from light to dark

Significant difference in bacterial and fungal OTUs

We used abundance analysis to identify OTUs whose abundance was significantly increased in response to irrigation water quality or soil compartment between irrigation treatments. For bacteria, we

identified a greater number of OTUs with significantly increased abundance in groundwater-irrigated soil compared to wastewater-irrigated soil (Fig. 3). The number of OTUs whose abundance was significantly higher in the rhizosphere was similar to that in the bulk soil. However, the Phyla and Class of bacteria with significantly different abundance were distinctly different between samples (Tables S5, S6, S7 and S8). For example, OTUs which were significantly more abundant in groundwater irrigated soil were dominated by OTUs classified as either Acidobacteria (23.5% of total OTUs) or Gemmatimonadetes (22.6%). In contrast, those significantly more abundant in wastewater-irrigated soil were predominantly Bacteroidetes (22.2%), α-Proteobacteria (22.2%), γ-Proteobacteria (20.2%) and Actinobacteria (14.1%). Acidobacteria, which were the most numerous OTUs significantly more abundant in groundwater, constituted only 2% of OTUs with significantly greater abundance in wastewater-irrigated soils and Gemmatimonadetes constituted only 1%. Conversely, Bacteroidetes, and α- and γ-Proteobacteria which were numerous in OTUs significantly more abundant in wastewater-irrigated soils constituted only 7.0, 7.0, and 8.7% respectively in groundwater-irrigated soils.

Fig. 3 Number of bacterial (a) and fungal (b) OTUs in the soil with significantly different abundances arising from different water sources or soil compartments. G refers to groundwater-irrigated soil, W refers to wastewater-irrigated soil. RS refers to rhizosphere, BS refers to bulk soil

Differences were also observed in OTUs with significantly different abundance in bulk and rhizosphere soil compartments. In this case, rhizosphere soil was dominated by α-Proteobacteria (30.9% of OTUs with significantly greater abundance in rhizosphere soil) but this class represented only 10.9% of OTUs with significantly greater abundance in bulk soil. In bulk soil, 15.6% of OTUs having significantly greater abundance were classified as Acidobacteria compared to only 1.5% in

rhizosphere soils and 9.4% were classified as Gemmatimonadetes in bulk soil compared to 1.5% in rhizosphere soil.

For fungi, the number of OTUs that showed significant increases in their abundance in groundwater-irrigated soil was higher than that in wastewater-irrigated soil. The number of OTUs associated with a significant increase in abundance in the rhizosphere and bulk soil was the same, similar to that found for the bacterial OTUs, but the numbers for fungi was less. Phyla responding to the different irrigation waters or soil compartments were also the same: Ascomycota, Basidiomycota, Chytridiomycota and Zygomycota (Tables S9, S10, S11 and S12) indicating that the fungi probably originated from soil rather than from the irrigation water, and they were thus less sensitive to development of rhizospheres than bacteria. For OTUs showing significantly increased abundance in the rhizosphere, the number of OTUs showing significant increase under wastewater irrigation (3 OTUs) was higher than that under groundwater irrigation (0 OTU), while the opposite was true in the bulk soil.

Relative abundance of N-cycle related genes

Water quality effects in rhizosphere and bulk soils

The abundance of genes in the rhizosphere and bulk soil was significantly modulated by both water source and irrigation rate (Fig. 4, Tables 3 and 4). In considering N cycle-related gene assemblages in the rhizosphere and bulk soils under different irrigation waters, unconstrained ordination based on the relative abundance of genes indicated separation between groundwater-irrigated and wastewater-irrigated soils (Fig. 5). In rhizosphere, groundwater-irrigation and wastewater-irrigation were separated on the first PCoA axis (associated with 84% of the variation in gene abundance). Assemblages in soils irrigated with wastewater appeared to differ considerably from the groundwater irrigation and vary more.

This difference was less significant in bulk soil, where wastewater- and groundwater-irrigated soils were separated in the second PCoA axis (accounting for only 5% of the variability). Two-factor PERMANOVA (Table 3) indicated a significant divergence in abundance of N cycle-related genes, dependent upon irrigation water source.

Fig. 4 The abundance of N-cycle related genes in soil relative to the soil before fertilization and cultivation, and the gene abundance ratio of *nosZ*/*nirK* and *nosZ*/*nirS*. RS refers to rhizosphere, BS refers to bulk soil, G refers to groundwater, W refers to livestock wastewater, C refers to conventional furrow irrigation, A refers to alternate-furrow irrigation. 100, 50, 65 and 80 refer to 100%, 50%, 65% and 80% of full irrigation amount per plot, respectively. The data are expressed as the mean ± standard deviation. Different lower-case letters above the columns represent significant difference between treatments at $p < 0.05$

Fig. 5 Unconstrained Principal Coordinate Analysis of N-cycle related genes based on relative abundance using Euclidean distance metrics in rhizosphere (a) and bulk soil (b). G refers to groundwater, W refers to livestock wastewater, C refers to conventional furrow irrigation, A refers to alternate-furrow irrigation. 50, 65, 80 and 100 refer to 50%, 65%, 80% and 100% of full irrigation amount per plot, respectively

Fig. 6 Redundancy Analysis presenting the association of N-cycle related gene abundance with environmental factors in rhizosphere (a) and bulk soil (b). G refers to groundwater, W refers to livestock wastewater. C refers to conventional furrow irrigation, A refers to alternate-furrow irrigation. 50, 65, 80 and 100 refer to 50%, 65%, 80% and 100% of full irrigation amount per plot, respectively

Similar patterns were repeated in the constrained ordination using RDA (Fig. 6), which identified strong and significant associations between OM and *nifH*, nitrate and bacterial *amoA*, nitrification rate and *nirS*, as well as between pH and archaeal *amoA* in both rhizosphere and bulk soils. In the rhizosphere, the genes formed three groups: the first comprised of bacterial *amoA*, *nirK* and *nosZ*, showing an increase in abundance with increased nitrate, denitrification rate and at lower pH in

wastewater-irrigated soils; the second, comprised of *nirS* and *nifH* showed a strong association with increased OM and nitrification rate resulting from wastewater irrigation; and the third group, comprised of archaeal *amoA*, showed a strong association with increased pH resulting from groundwater irrigation. These associations were associated with the principal RDA axis, which effectively separated wastewater- and groundwater-irrigated soils, and accounted for 60.5% of the variability described by the model. Soil pH (accounting for 22.5% of variability, *pseudo-F* = 6.4; *p* = 0.006), OM (21.9% of variability, *pseudo-F* = 6.2; *p* = 0.014), nitrate (20.0% of variability, *pseudo-F* = 5.5; *p* = 0.011) and denitrification rate (18.4% of variability, *pseudo-F* = 5.0; *p* = 0.04) were all associated with this separation by water source. No other environmental parameters were identified to account for the significant amount of variability, and only the Archeael *amoA* gene was associated with increases in pH - a salient characteristic of groundwater-irrigated soil.

Such strong associations between environmental factors and gene abundance were not evident in bulk soils, where groundwater and wastewater treatments were separated on the second axis, and accounted for only 3.9% of the variability. Responses of the genes to irrigation were divergent in the bulk soil and there was no evidence of the gene groupings. On the second axis, the abundance of the *nirS* gene was associated with C/N ratio and nitrification rate, while the abundance of *nifH* gene was associated with OM, but neither environmental parameter accounted for a significant amount of variability. An increase in nitrate concentration and reduction in pH did account for significant amounts of variability (nitrate - 39.4% of variability, *pseudo-F* = 14.3; *p* = 0.002; pH - 23.7% of variability, *pseudo-F* = 6.8; *p* = 0.012) and were associated with an increase in bacterial *amoA* gene abundance and a decrease in Archaeal *amoA* gene abundance; the abundance of *nosZ* and *nirK* gene was associated with increases in EC (20.3% of variability, *pseudo-F* = 5.6; *p* = 0.015) and TN (16.3% of variability,

pseudo-F = 4.3; *p* = 0.046), respectively. Nitrate and total N both increased under wastewater irrigation but appeared to be influenced differently by irrigation rate.

The effects of alternate-furrow irrigation and the irrigation amounts

Gene abundance in soil under wastewater irrigation was higher than that under groundwater irrigation except for archaeal *amoA* (Fig. 4). PERMANOVA (Table 3) indicated there were significant differences in gene abundance at different irrigation rates regardless of water resource which was more notable in the bulk soil than in the rhizosphereand evident on the second PCoA axis (Fig. 5). When irrigated with wastewater, relative to CFI, AFI at 50% rate significantly reduced the abundance of bacterial *amoA*, *nifH* and *nirS* in the rhizosphere, and *nosZ*, *nosZ*/*nirK* and *nosZ*/*nirS* in the bulk soil, but increased the abundance of bacterial *amoA* and *nifH* in the bulk soil and archaeal *amoA* in rhizosphere (Fig. 4).

Irrigation rate had significant effects on the abundance of N cycle-related genes in the soils except for *nifH* in the rhizosphere and *nirK* and *nirS* in the bulk soil (Table 4). With reduced AFI rates, the abundance of *nifH* increased in both soil compartments. The abundance of *nirK* increased with the increase of water content in rhizosphere, but *nirS* did not respond in the same manner. In bulk soil, the *nirK* abundance also increased as irrigation rate increased from 50% to 65% and 80%, but the variance of abundance in soil with higher rate were much higher. The bacterial *amoA* abundance increased in both soil compartments when the irrigation amount was reduced from high (80% and 65%) to low (50%), and the Archaeal *amoA* abundance showed same trend with bacterial *amoA* in rhizosphere while opposite in bulk soil. For the *nosZ* gene - coding the nitrous-oxide reductase - the abundance at 50% rate was significantly higher than that at other two rates in rhizosphere. The *nosZ*/*nirK* and *nosZ*/*nirS* ratios has been widely used to estimate the likelihood of N_2O emission (Pereira et al. 2015), and both ratios at 50% rate of AFI using wastewater were higher than those at 65% and 80% rates in both soil

compartments in which the differences of *nosZ*/*nirS* ratio in bulk soil were significant (Fig. 4), suggesting a reduced likelihood of N_2O emission.

Discussion

Nitrification rate in rhizosphere soil and plant N use efficiency were significantly influenced by water source but not by irrigation amount (Table S4), while water source and irrigation rate did not have significant impact on denitrification rate in both soil compartments. Community assemblages in irrigated soils were affected by the imposed treatments, with bacteria being far more sensitive to treatments than fungi, which only differed between soil compartments. Bacterial community assemblages in comparison were significantly impacted by irrigation water quality and soil compartment, but not by irrigation rate. Regarding water quality, irrigation with wastewater has been found to increase *nirK* and *nirS* gene abundance compared to groundwater irrigation (Zhou et al. 2011). In several studies, microbial communities associated with rhizosphere were found to be distinct from those of the bulk soil. The separation of bacterial Phyla and Classes between groundwater- and wastewater-irrigated soil and the different soil compartments is consistent with general traits associated with the different Classes or Phyla. For example, Acidobacteria - which were more associated with groundwater irrigation and bulk soil - have been shown to be sensitive to nutrient concentrations and are more abundant under low nutrient conditions (Fierer et al. 2007). α-Proteobacteria have the opposite response, increasing in abundance under increased nutrient conditions (Gravuer and Eskelinen 2017). This is consistent with their apparent association with wastewater-irrigation and the rhizosphere, where root exudates (Bais et al. 2006) and preference of plants for different nitrogen forms (Stempfhuber et al. 2017) influence microbial abundance. Irrigation rate did not have significant effects on bacterial community assemblages, but its impacts on gene abundance was striking. Soil

compartment, water quality and irrigation rate all had significant effects upon the relative abundance of the N-cycle related genes.

Irrigation effects on N transformation activities in soil

Pepper yield was comparable in all treatments (Table S3). Compared to CFI, AFI with groundwater significantly increased N use efficiency of plants (Table S3). Since AFI wetted only half of the root zone during each irrigation, the difference in water matric potential between soils in the dry and wetted furrows could drive water to flow from the wetted half to the dry half across the root zone, increasing water and N use efficiency (Kang et al. 2000b). However, under wastewater irrigation, the irrigation method and amount did not have significant effects on N use efficiency (Table S3), suggesting that the N input exceeded the uptake of plants, even at the lowest AFI rate. Though N use efficiency was not affected by irrigation amount significantly (Table S4), the highest efficiency was achieved at 80% AFI rate using groundwater (Table S3).

The higher AFI rate (80%) also boosted nitrification activity in both soil compartments regardless of water source (Fig. 1), indicating a reduction in ammonia volatilization losses and an increase in nitrate for denitrification. Denitrification activity did not increase N losses because the denitrification rates under 80% AFI treatments were not increased compared to that under other two AFI rates (Fig. 1). As a result of the high plant N uptake under 80% AFI rate as discussed above, NO_3^--N was not significantly accumulated in the soils (Table 2). Total N content in 80% rate of AFI with wastewater was increased compared to those in other treatments in both soil compartments, and significantly higher than that in CFI and 65% AFI rate in rhizosphere (Table 2), suggesting that soil fertility may have been improved.

The presence of N cycle genes in the soils does not mean the genes expression. Neither the

abundance of bacterial *amoA*, archaeal *amoA* and nitrification rate nor the abundance of *nirK*, *nirS*, *nosZ* and denitrification rate responded similarly to the irrigation methods. Because we targeted soil DNA, the measured gene abundance were not equivalent to actual N transformation activity or mRNA concentration (Bowen et al. 2018). While *nifH* genes were detected in our soils, N fixation was not, consistent with previous studies (Kumar et al. 2017; Wang et al. 2018). The ability of bacteria to fix N is controlled not only by the abundance of organisms carrying *nifH* gene but also by other conditions. Diazotrophs carrying *nifH* gene favor low N habitat, but synthesizing the nitrogenase enzyme is an energetically expensive process and limited by iron, phosphorus or other nutrients (Larson et al. 2018).

When the wastewater used in AFI increased from 50% to 80%, the abundance of bacterial *amoA* and archaeal *amoA* in rhizosphere decreased significantly (Fig. 4), but the nitrification rate did not decrease (Fig. 1), suggesting that the AFI might have alerted the bioavailability of water and other substrates, rendering microbial activity at low rate (50%) differing from that at high rate (80%). Though the differences in nitrification rate in wastewater-irrigated soils between the three AFI treatments were not significant, the nitrification rate at 80% treatment was higher than that at 50% and 65% treatments which made sense for agricultural production. At the 80% rate, the water supply rate exceeded soil infiltration rate, giving rise to surface runoff. As a result, there was no significant difference in soil water content between the irrigated and non-irrigated furrows, and the soil ammonium availability was high for nitrifiers. In contrast, at the 50% rate, the water supply rate was low and only wetted part of the root zone, hence the available ammonium was rather low. Consequently, the 80% AFI rate enhanced nitrification activity even when the abundance of associated genes was low.

The enhanced nitrification activity under 80% AFI rate increased the production of nitrate, but because of leaching, there was no associated increase in nitrate for denitrification. Thus, it was not surprising that we did not find increased NO_3^--N content in both rhizosphere and bulk soil nuder 80% rate compared to 50% and 65% rates (Table 2). Therefore, the denitrification rate under 80% AFI was comparable to that under other two AFI treatments (Fig. 1) even the abundance of *nirK* and *nirS* in the former was slightly higher than that in the latter (Fig. 4). Above results implicate that 80% AFI rate could increase N use efficiency due to a balance between water, oxygen and available N in soil.

Association of enumerated genes with soil chemical parameters

Quantification of relative gene abundance indicated that *nirK* and *nosZ* genes were most abundant in bulk soil, and *nifH* and *nirS* most abundant in the rhizosphere (Fig. 4). Bacterial and archaeal *amoA* were equally abundant in both soil compartments (Fig. 4). This suggests spatial separation of N cycle-related processes, possibly driven by plant root activity. Gene *amoA*, a bioindicator of nitrification potential, was ubiquitous in the soils and the abundance of bacterial and archaeal *amoA* were associated with the increased NO_3^--N and pH respectively (from RDA, Fig. 6). The abundance of bacterial and archaeal *amoA* always respond to the environmental change differently. For example, Han et al. (2017) reported the irrigation amount significantly influenced the copy number of Archaeal *amoA*, but not of bacterial *amoA* in soil. The influence was clearly correlated with the change of soil temperature, water-filled pore space (Liu et al. 2017), pH, NO_3^--N, exchangeable NH_4^+-N and potential nitrification rates. Bacterial *amoA* populations are typically found in greater abundance in agricultural soils, particularly soils with higher fertilizer inputs and soil disturbance (Bruns et al. 1999; Di et al. 2010), while Archaeal *amoA* dominates ammonia oxidation at lower N concentrations (Martens-Habbena et al.

2009) and in undisturbed soils (Nicol et al. 2008). For our experiment, Archaeal *amoA* was more abundant in groundwater-irrigated soils with higher pH and lower concentration of NO_3^--N (Fig. 6). However, it is clear from Fig. 6 that bacterial *amoA* specifically, responded to the greater nutrient addition arising from wastewater-irrigation in both soil compartments. This response to added nutrients is consistent with observations of increased bacterial *amoA* numbers in response to urea addition in soil (Reed et al. 2010; Shen et al. 2011).

The abundance of *nirK* and *nosZ* genes associated with denitrification rate were also associated with increased NO_3^--N concentration in rhizosphere (Fig. 6a), but were most abundant in bulk soil (Fig. 4). The *nirK* and *nirS* genes both regulate transformation of nitrite to nitric oxide, but they are carried by phylogenetically distinct organisms with different niche preferences, and responded to C/N ratio differently (Fig. 6) (Bowen et al. 2018). Quantitative-PCR (Fig. 4) indicated that both abundance of *nirK* and *nirS* got the maximum value in the same treatment (WC100) in rhizosphere, but in bulk soil, the abundance difference of *nirK* between WA65, WA80 and other treatments were much greater than that of *nirS*, suggesting that the abundance of *nirK* was the more responsive functional gene associated with nitrite reduction, as confirmed by Fig. 6a that *nirK* abundance but not *nirS* abundance was closely associated with denitrification rate. Some studies also confirmed that the *nirK*-denitrifying bacterial community was more sensitive to environmental changes (Wertz et al. 2013; Yin et al. 2015). However, one wheat-growing field experiment in a sandy clay loam revealed that *nirS*- and *nosZ*-denitrifying bacterial communities were more sensitive to irrigation managements than *nirK*-denitrifying bacteria (Yang et al. 2018). Denitrification is an anaerobic process and *nirK* and *nirS* abundance in the rhizosphere increased with the increase in irrigation amount generally (Fig. 4). In the bulk soil, the 50% irrigation treatment was associated with the lowest *nirK* and *nirS* relative abundance, but *nirK*

abundance in 100% CFI treatment was also low compared with 65% and 80% AFI treatments (Fig. 4), possibly because the high spatial heterogeneity of bulk soil due to heterogeneous water distribution.

The identification of the N_2O reductase gene (*nosZ*) by qPCR in soil indicated the potential for complete denitrification, thus it is not surprising that the abundance of *nosZ* gene was associated with denitrification rate in bulk soil. N_2O is a product of both nitrification and denitrification, depending on soil moisture, oxygen and other environmental factors (Arp and Stein 2003; Ma et al. 2008), thereby an association between bacterial *amoA* and *nosZ* in rhizosphere in our study (Fig. 6) is reasonable. Nitrification was the main driver of nitrous oxide production in the 0- to 5-cm and 5- to 10-cm soil layers while denitrification was in the 10- to 15-cm and 15- to 20-cm soil horizons (Castellano-Hinojosa et al. 2018). It has been reported that alternating wetting and drying during the rice season in a field rice-wheat rotation system in a paddy soil increased soil aeration status thus increased N_2O emissions from simultaneous nitrification and denitrification during the rice season compared to traditional flooding irrigation (Hou et al. 2016). But flooding lowered soil organic N mineralization during the rice season, thus more mineral N in soil was available to N_2O production and significantly reduced N_2O emissions in the following wheat season compared to traditional irrigation. The N_2O reduction to N_2 is favoured at low redox potential (E_h), and the lower E_h was associated with increased soil moisture (Liu et al. 2012), which could contribute to the high abundance of *nosZ* in 100% CFI treatment in our study (Fig. 4).

The nitrogenase iron protein gene *nifH* is an indicator of N_2 fixation potential (Wang et al. 2018). The gene was correlated with increased organic matter content and reduced concentrations of exchangeable NH_4^+-N in wastewater-irrigated rhizospheres (Fig. 6). This suggests a high biological demand for N in the rhizosphere, rich in organic matter, which could not be satisfied by the available

NH_4^+-N and so N_2 fixation may be used to supplement this high demand. The exchangeable NH_4^+-N content in soil increased with AFI rate (Table 2), which may explain the change in *nifH* abundance with the AFI rate (Fig. 4). A second gene, *nirS*, was also associated with increased organic matter in wastewater receiving rhizosphere soil, and showed correlation with *nifH* (Fig. 6a). Since the NirS protein, encoded by *nirS*, has a secondary function as a hydroxylamine (NH_2OH) reductase (https://www.kegg.jp/dbget-bin/www_bget?K05601+K15864+1.7.99.1+R00143) (Rees et al. 1997; Zumft 1997), which also produces ammonium, it is possible that *nirS* and *nifH* were coupled in response to the decrease in ammonium and that *nirS* was associated with nitrification rate (Fig. 6).

Fungal contribution to N cycling

Nitrogen metabolism in soil is typically associated overwhelmingly with bacterial or archaeal activity. However, fungi may also play a significant role in these processes which should receive more attention. The primers employed in our study are unlikely to amplify fungal genes, but the effects of fungi cannot be ignored. Many fungal species are known to produce N_2O (Shoun et al. 1992; Wei et al. 2014). For example, studies have revealed that *Fusarium oxysporum* and *Cylindrocarpon tonkinese* use NirK to reduce nitrite to nitric oxide (Nakanishi et al. 2010), and fungal *nirK* has close homology to its bacterial ortholog (Kobayashi and Shoun 1995; Kim et al. 2010). A fungal *nirK* primer set nirKfF/nirKfR (Wei et al. 2015) detected *nirK* of Ascomycota, the dominant denitrifying fungal group in soil, and when amplified using these fungal primers, *nirK* clones showed homology to *nirK* of Hypocreales, Sordariales, and Eurotiales of Ascomycota. In this study, the Ascomycota phylum were also identified by amplicon sequencing as having significantly higher abundance in soil regardless of water source (Table S9-S12). Fungi are also indispensable in N mineralization and nitrification (Lang and Jagnow 1986; Boer and Kowalchuk 2001; DeCrappeo et al. 2017), and contribute more to N_2O production

under sub-anoxic and acidic conditions than bacteria (Chen et al. 2015).

Conclusions

Our research studied differences in N transformation activities and the associated N cycling genes distribution in soil following AFI and CFI, comparing groundwater and wastewater sources at different irrigation rates. Compared with CFI, AFI using groundwater increased plant N use efficiency. Water quality had a manifest effect on the propagation of genes abundances: the genes were more responsive to irrigation with wastewater than groundwater. AFI using wastewater reduced the gene abundances in rhizosphere except archaeal *amoA* relative to CFI. Under AFI with wastewater, increasing the irrigation amount could increase the abundance of *nirK* and *nirS* and decrease the abundance of bacterial and archaeal *amoA*, *nifH* and *nosZ* in rhizosphere, but did not decrease the nitrification rate and kept the denitrification rate unchanged in both rhizosphere and bulk soil, revealing that the irrigation amounts with AFI did not change N transformation processes and the abundance of associated N-cycle genes in soil synchronously. We conjectured from our findings that some biophysichemical processes unique to roots induced by water stress under different AFI rates might contribute to the desynchrony between the N transformation processes and the associated N-cycle genes in soil.

We measured the properties of soil only at the harvest and could not get the dynamics of N transformation and gene abundance during the irrigation management. However, the effects of AFI with different irrigation rates on N transformation and gene abundance in soil were clear. Our results have important applications that appropriate irrigation rate in AFI have the potential to increase the N use efficiency.

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Conflicts of interest

The authors declare no conflict of interest.

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Table 1 Properties of groundwater and wastewater used in this study

	pH	EC	COD[a]	TDS[b]	N	P	Ca	Mg	Fe	Zn	Mn
	-	mS cm^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}	μg L^{-1}
Groundwater	8.07	1.985	104	2251	0.550	-	55.5	122	1.07	0.021	178
Wastewater	8.40	2.588	330	1681	325.6	16.6	47.6	38.6	0.88	0.366	120

	Pb	Cd	Cu	Cr	As	Hg	NO_3^-	PO_4^{3-}	SO_4^{2-}	K^+	Na^+
	μg L^{-1}	μg L^{-1}	μg L^{-1}	μg L^{-1}	μg L^{-1}	μg L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}
Groundwater	0.654	0.050	2.45	13.3	9.85	0.065	-	-	844	2.95	514
Wastewater	1.729	0.107	73.16	30.0	2.10	0.178	2.70	4.94	319	212.3	257

Note: a, chemical oxygen demand. b, total dissolved solids; the content of N, P, Ca, Mg, Fe, Zn, Mn, Cu, Pb, Cd, Cr, As, Hg refers to the total content

Table 2 The chemical properties in soil of different treatments

Treatment	Soil compartment	pH	EC (mS m^{-1})	Organic matter (g kg^{-1})	Total N (g kg^{-1})	C/N	NO_3^--N (mg kg^{-1})	Exchangeable NH_4^+-N (mg kg^{-1})
GC100	RS	8.83a	110.55cd	10.90ab	0.69b	6.32a	75.00de	7.00ab
GA50	RS	8.77ab	103.45d	16.36ab	0.84ab	9.49a	68.50e	11.56a
GA65	RS	8.65abcd	108.80cd	11.37ab	0.67b	6.59a	96.50cde	8.72ab
GA80	RS	8.61abcde	119.75bcd	12.22ab	0.77ab	7.09a	124.50bcde	3.28b
WC100	RS	8.50cde	123.65abcd	16.48ab	0.72b	9.56a	146.90abc	6.83ab
WA50	RS	8.48de	125.00abcd	17.48a	0.83ab	10.14a	144.00abc	6.50ab
WA65	RS	8.53bcde	122.05abcd	13.80ab	0.76b	8.01a	135.06abcd	8.61ab
WA80	RS	8.48de	125.25abcd	16.15ab	1.08a	9.37a	142.00abc	10.72a
GC100	BS	8.74abc	142.75abc	8.50b	0.66b	4.93a	114.00cde	5.72ab
GA50	BS	8.65abcd	144.85abc	9.23b	0.73b	5.35a	134.00abcd	7.39ab
GA65	BS	8.50cde	149.65ab	9.33ab	0.64b	5.41a	145.00abc	8.44ab
GA80	BS	8.56bcde	130.45abcd	10.72ab	0.63b	6.22a	139.50abc	5.44ab
WC100	BS	8.39e	159.00a	9.76ab	0.58b	5.66a	195.50a	5.39ab
WA50	BS	8.45de	138.85abcd	13.59ab	0.78ab	7.88a	183.00ab	7.72ab
WA65	BS	8.48de	134.05abcd	11.48ab	0.79ab	6.66a	155.00abc	7.83ab
WA80	BS	8.49de	120.65bcd	12.42ab	0.87ab	7.20a	129.00bcde	8.39ab

Note: G refers to groundwater, W refers to livestock wastewater, C refers to conventional furrow irrigation, A refers to alternate-furrow irrigation. 100, 50, 65 and 80 refer to 100%, 50%, 65% and

80% of full irrigation amount per plot, respectively. RS refers to rhizosphere, BS refers to bulk soil. Different lower case letters above the columns represent significant difference between treatments at $p < 0.05$

Table 3 Permutation multivariate analysis of variance of the relative abundance of all N cycling genes under different irrigation water sources, and different irrigation rates in the rhizosphere and bulk soil

Soil compartment	Source of variation	Genes	
		F	*P*
Rhizosphere	Water source	14.73	<0.001
	Irrigation amount	3.76	0.002
	Interaction	2.73	0.018
Bulk soil	Water source	101.21	<0.001
	Irrigation amount	60.20	<0.001
	Interaction	57.77	<0.001

Table 4 Two-factor analysis of variance of gene abundance in rhizosphere (RS) and bulk soil (BS). Significant treatments effects are indicated in bold type

	Source of	Archaeal *amoA*		Bacterial *amoA*		*nifH*		*nirK*		*nirS*		*nosZ*		*nosZ/nirK*		*nosZ/nirS*	
	variation	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
RS	Water source	**478.93**	**<0.001**	**134.78**	**<0.001**	**9.25**	**0.008**	**17.04**	**0.001**	3.49	0.080	**215.54**	**<0.001**	**35.25**	**<0.001**	**61.20**	**<0.001**
	Irrigation amount	**28.83**	**<0.001**	**44.47**	**<0.001**	2.72	0.079	**3.73**	**0.033**	**3.47**	**0.041**	**36.38**	**<0.001**	**32.21**	**<0.001**	**40.10**	**<0.001**
	Interaction	**95.30**	**<0.001**	**36.09**	**<0.001**	1.62	0.225	**6.30**	**0.005**	**4.51**	**0.018**	**32.85**	**<0.001**	**36.90**	**<0.001**	**41.73**	**<0.001**
BS	Water source	**192.45**	**<0.001**	**48.55**	**<0.001**	**66.03**	**<0.001**	**5.40**	**0.034**	4.19	0.058	**226.84**	**<0.001**	**6.30**	**0.023**	**22.53**	**<0.001**
	Irrigation amount	**95.33**	**<0.001**	**7.82**	**0.002**	**21.09**	**<0.001**	2.70	0.081	0.66	0.588	**141.02**	**<0.001**	**6.56**	**0.004**	**6.69**	**0.004**
	Interaction	**202.20**	**<0.001**	**12.65**	**<0.001**	**22.12**	**<0.001**	2.43	0.103	2.73	0.078	**134.78**	**<0.001**	**5.51**	**0.009**	**6.00**	**0.006**

Figure 1

Figures

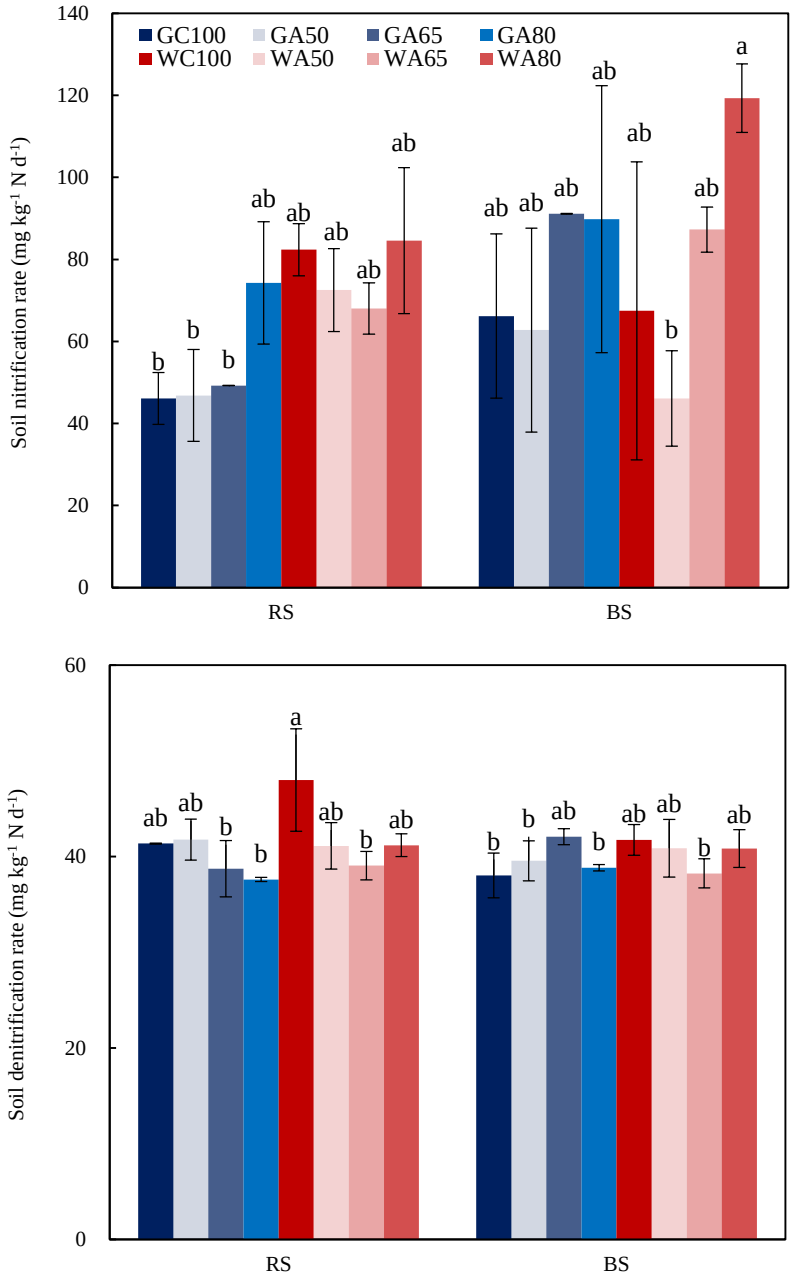


Fig. 1

Figure 2

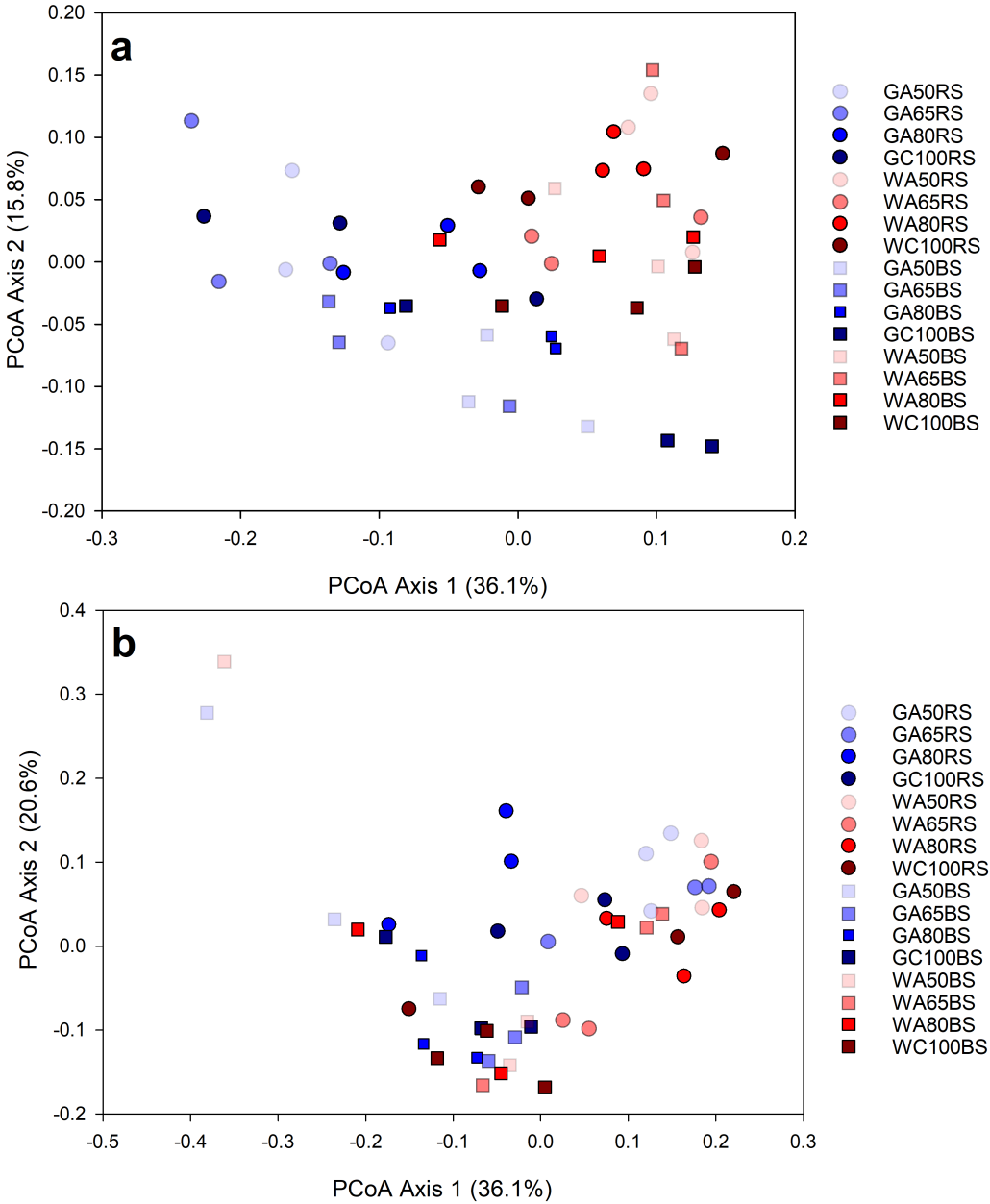
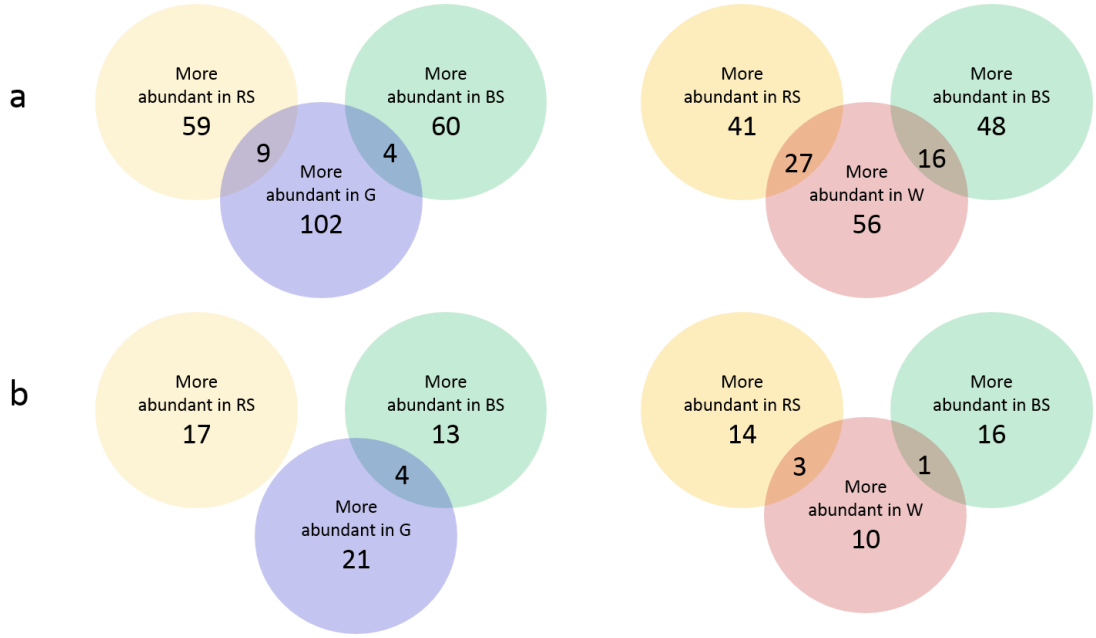


Fig.2


a
More abundant in RS
59
More abundant in BS
60
9
4
More abundant in G
102
More abundant in RS
41
More abundant in BS
48
27
16
More abundant in W
56
b
More abundant in RS
17
More abundant in BS
13
4
More abundant in G
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14
More abundant in BS
16
3
1
More abundant in W
10

Fig. 3

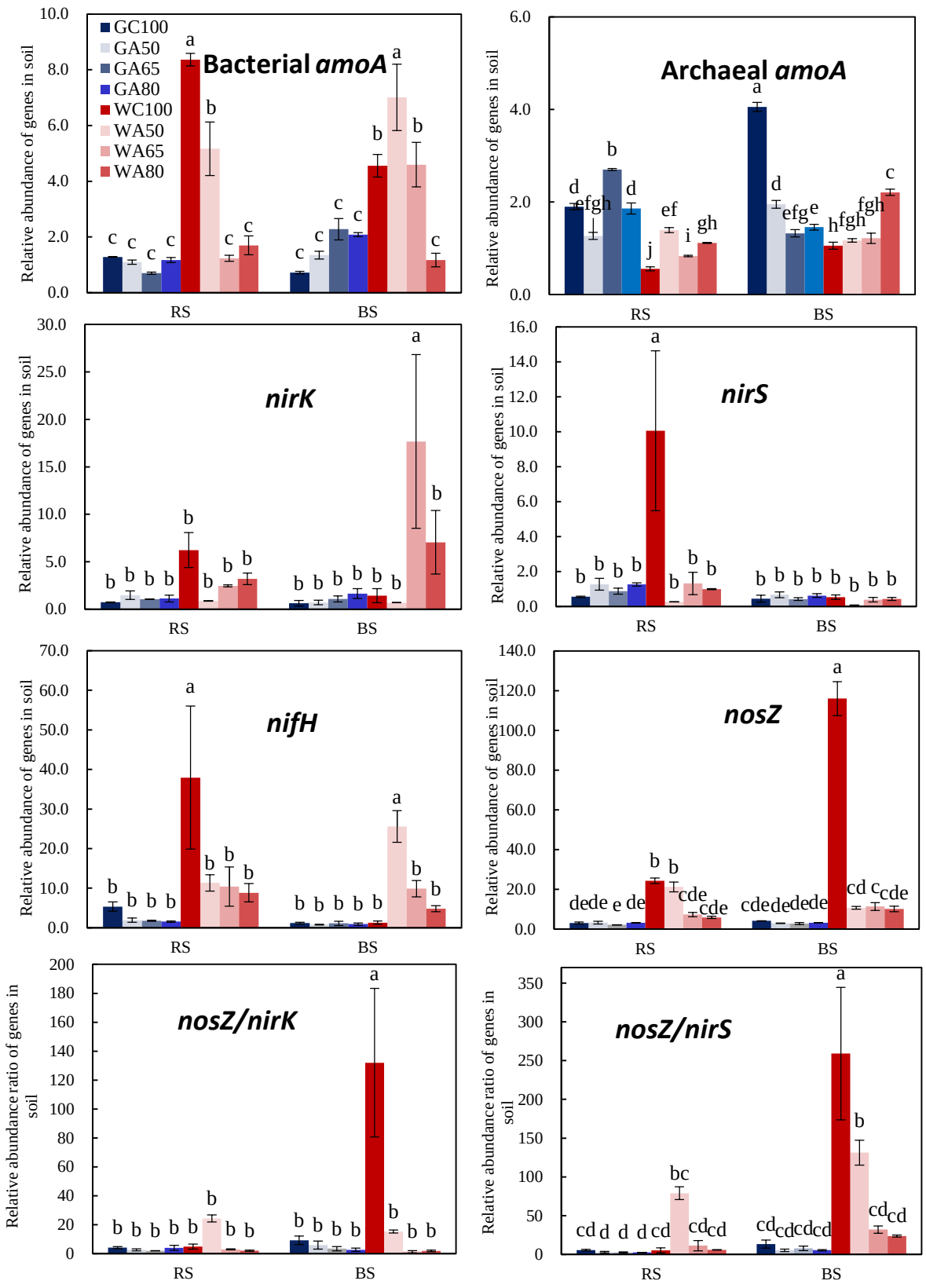
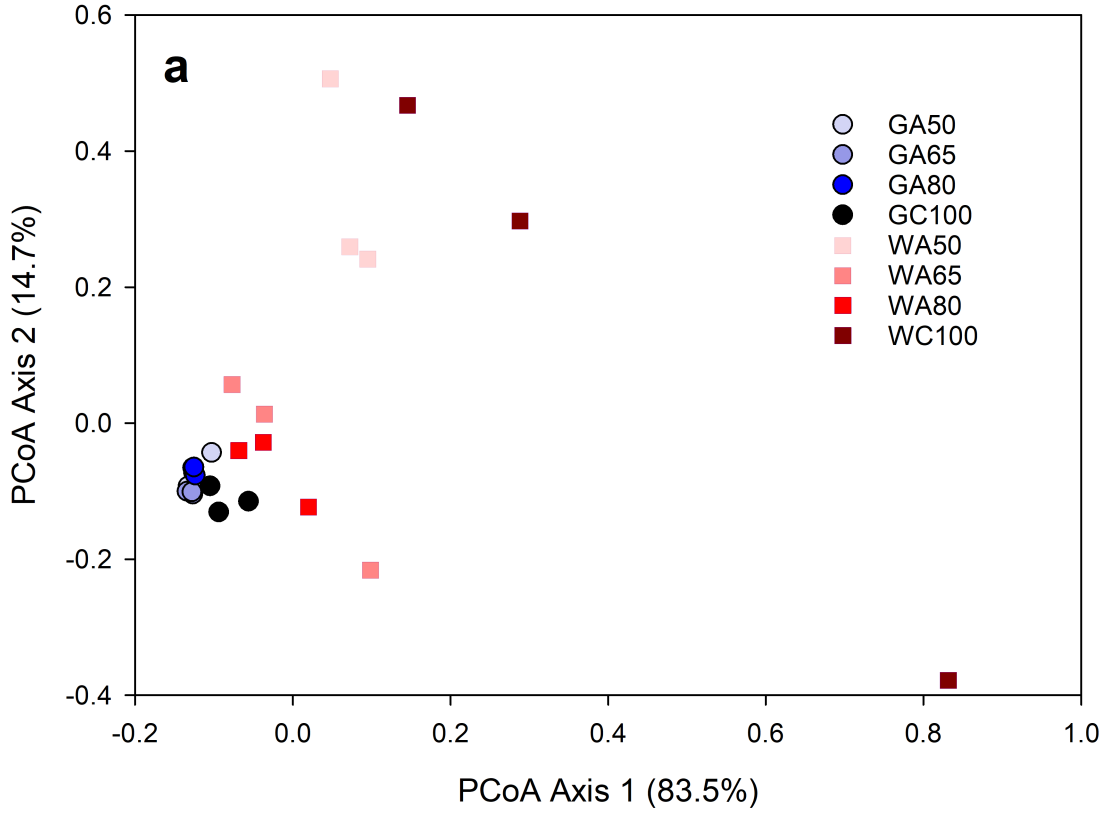

GC100
GA50
GA65
GA80
WC100
WA50
WA65
WA80
Bacterial amoA
Archaeal amoA
nirK
nirS
nifH
nosZ
nosZ/nirK
nosZ/nirS
Relative abundance of genes in soil
Relative abundance ratio of genes in soil
RS
BS

Fig. 4

Figure 5



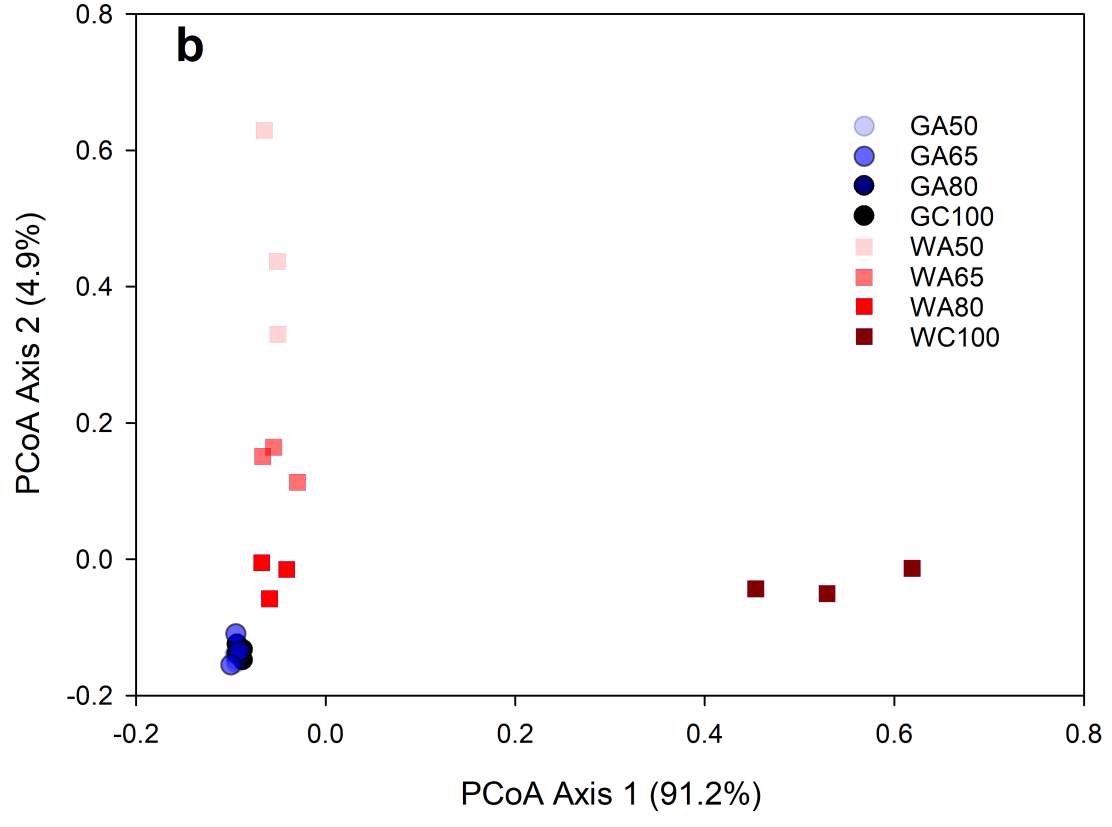


Fig. 5

Figure 6

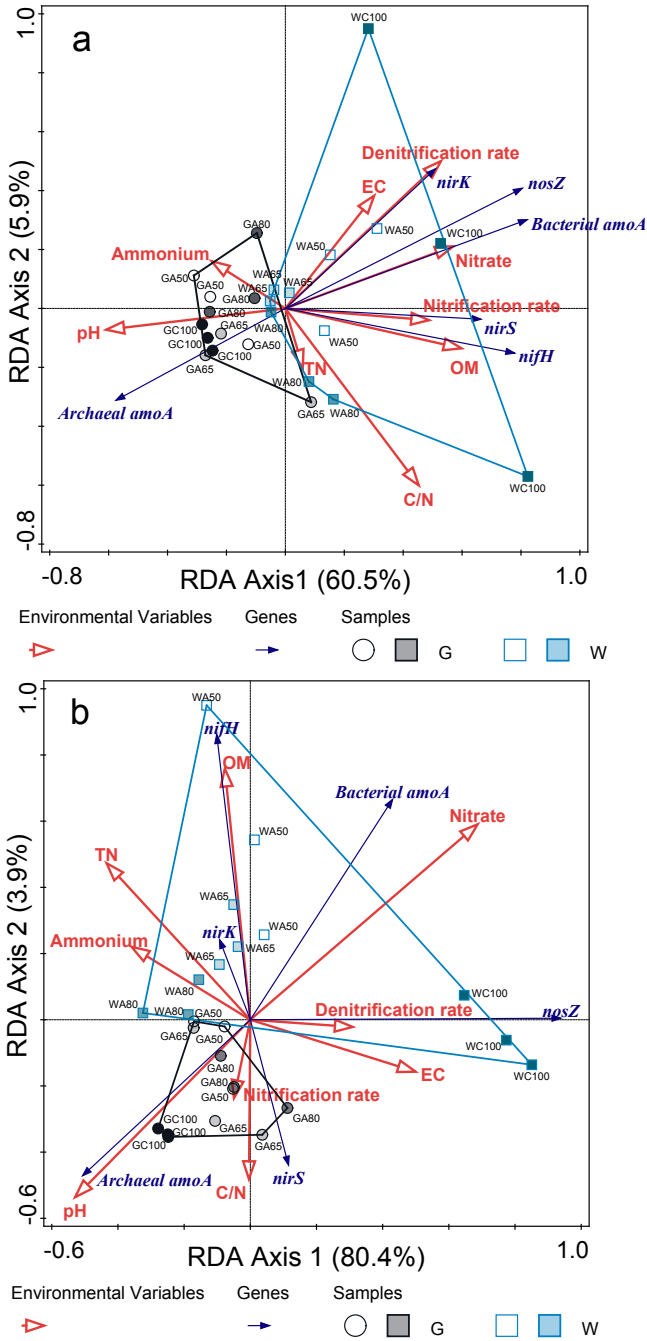


Fig. 6