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Differentiated responses of nirS- and nirK-type denitrifiers to 30 years of combined inorganic and organic fertilization in a paddy soil

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ABSTRACT
The responses of soil denitrifiers to inorganic and organic mixed fertilization in paddies have not been well evaluated. The abundance and diversity of nirS- and nirK-type denitrifiers in paddies after 30-year fertilization were estimated using quantitative PCR and Illumina MiSeq sequencing. The nirS gene abundance was two orders of magnitude higher than the nirK gene and both were reduced by fertilization. The high manure addition (NPK + 60% OM) decreased nirK gene abundance by 32.7% compared to inorganic fertilization alone (NPK) and decreased its community Chao1 index by 30.2% compared to unfertilized control (CK). Fertilization significantly changed the proportion of dominant (mean proportion > 1%) operational taxonomic units (OTUs) of both denitrifiers. Fertilization increased the proportion of OTUs belonged to cluster III (48.7–97.3%) of the nirS-type denitrifiers compared to CK. NPK + 60% OM increased that belonged to cluster II (48.0%) compared to the low manure addition (NPK + 30% OM). Total nitrogen and organic carbon correlated significantly with the abundance, diversity and community structure of the nirK-type denitrifiers, while they solely correlated with the nirS-type denitrifier community structure. Our results demonstrate that abundance and diversity of the nirK-type denitrifiers are more, while the community structure is less, sensitive to inorganic and organic mixed fertilization than the nirS-type in paddy soils.

Introduction
Nitrous oxide (N2O) is a great potent greenhouse gas (GHG) with 298 times of global warming potential that of carbon dioxide (CO2) in a 100-year horizon (Butterbach-Bahl et al. 2013; IPCC 2014) and is the single most important stratospheric ozone-depleting substance (Ravishankara et al. 2009). Atmospheric N2O concentrations have been increased by 19% since the pre-industrial times and significantly associated with the overuse of N fertilizer and unmanaged manure in terrestrial ecosystems (Syakila and Kroeze 2011). N2O emissions from agricultural soils account for approximately 60% of the global anthropogenic N2O emitted to the atmosphere (IPCC 2007; Syakila and Kroeze 2011) and soil microbial mediated denitification is supposed to be a crucial contributor to the anthropogenic N2O emissions.

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Denitrification is a four-step reduction process in which nitrate (NO$_3^-$) is reduced to dinitrogen (N$_2$), via nitrite (NO$_2^-$), nitric oxide (NO) and nitrous oxide (N$_2$O) (Zumft 1997; Jones et al. 2008). Reduction of NO$_2^-$ to NO is the key process to distinguish denitrifying bacteria from other nitrate-respiring bacteria and in which dissolved N turns into gaseous N for the first time in denitrification (Zumft 1997; Braker et al. 2000). This step is catalyzed by the enzyme nitrite reductase (NIR) which has two structural differentially but functionally equivalent forms: cytochrome cd1-containing reductase (NirS) and copper-containing reductase (NirK) encoded by the nirS and nirK genes (Cutruzzola et al. 2001; Philippot et al. 2007), respectively. The denitrifiers containing the nirS or nirK genes responding differently to environmental gradients or fertilization practices in rice paddies (Yoshida et al. 2010; Azziz et al. 2017; Herold et al. 2018; Hou et al. 2018), suggesting they are suitable markers for ecological behavior investigation of the denitrifier communities.

Applications of inorganic fertilizers and organic manure are crucial practices to improve soil fertility and increase crop yields (Shang et al. 2014), while combinedly use of inorganic and organic fertilizers has been proved to be a better approach than inorganic fertilization alone to sustain soil productivity and crop yields with less environmental footprints (Zhang et al. 2016). Recently, impacts of different fertilization on the abundance, diversity and community structure of the denitrifiers have been extensively emphasized (Sun et al. 2015; Yin et al. 2015; Cui et al. 2016). The nirK-type denitrifiers have been reported to be more sensitive to fertilization or environmental variables than the nirS-type (Chen et al. 2010; Yoshida et al. 2010; Herold et al. 2018). However, responses of the two denitrifier communities to inorganic fertilizers and organic manure were inconsistent (Sun et al. 2015; Hou et al. 2018; Tao et al. 2018). Furthermore, most investigations were obtained from one combination of inorganic and organic fertilization compared to inorganic or organic fertilization alone (Yin et al. 2014; Cui et al. 2016), the effect of different combinations of inorganic and organic fertilization was not well known. To understand the responses of the nirK- and nirS-type denitrifier communities to combined inorganic and organic fertilization in paddy fields might be critical to reveal their adaptive mechanisms to fertilization.

In this study, we investigated the abundance, diversity and community structure of the nirS- and nirK-type denitrifiers in a 30-year fertilized paddy field, to compare the impacts of inorganic fertilizers alone and two combinations of inorganic fertilizers and organic manure. We hypothesized that (i) the abundance of the nirK gene was higher than that of the nirS gene in paddy soils and (ii) the changes in abundance, diversity and community structure of the nirK-type denitrifiers were more sensitive to fertilization strategies than the nirS-type.

**Materials and methods**

**Site characteristics and soil properties**

The field experiment was conducted from 1986 at the Agricultural Technology Extension Center of Ningxiang city (28°07ʹN, 112°18ʹE; 36 m asl), Hunan Province, China (Yang et al. 2019). The study area has a sub-tropical continental and monsoon climate with distinct seasons. The long-term annual average temperature, precipitation and frost-free period are 16.8 °C, 1553 mm and 274 d, respectively. The soil in the experimental site belongs to the waterloggogenic paddy soil and is derived from quaternary red clay (FAO 1974). Basic properties of the 0–20 cm soil layer were: soil pH 6.85 (H$_2$O), soil organic matter (SOM) 29.39 g kg$^{-1}$, total nitrogen (TN) 2.01 g kg$^{-1}$, available phosphorus (Avail-P) 12.87 mg kg$^{-1}$ and available potassium (Avail-K) 33.0 mg kg$^{-1}$ before the experiment started.

**Experiment setup**

In this study, four treatments: unfertilized control (CK), NPK fertilizers (NPK), NPK fertilizers plus 30% organic manure (NPK + 30% OM) and NPK fertilizers plus 60% organic manure (NPK + 60% OM) were compared. The plot size was 66.7 m$^2$ (10 m × 6.67 m) and each plot was separated by a cement ridge
with 1.0 m in depth (35 cm above and 65 cm below the surface). The N, P and K fertilizers were applied at a rate of 300 kg N ha\(^{-1}\), 81 kg P\(_2\)O\(_5\) ha\(^{-1}\) and 144 kg K\(_2\)O ha\(^{-1}\) during the double rice growing seasons (early rice: 142.5 kg N ha\(^{-1}\), 54.0 kg P\(_2\)O\(_5\) ha\(^{-1}\) and 63.0 kg K\(_2\)O ha\(^{-1}\); and late rice: 157.5 kg N ha\(^{-1}\), 27.0 kg P\(_2\)O\(_5\) ha\(^{-1}\) and 81.0 kg K\(_2\)O ha\(^{-1}\)) for both the inorganic fertilization alone and combined inorganic and organic fertilization treatments. The nitrogen (N), phosphorus (P) and potassium (K) fertilizers were applied in form of urea, calcium superphosphate and potassium chloride, while organic manure was applied with chicken manure (N 1.77%, P\(_2\)O\(_5\) 0.80% and K\(_2\)O 1.12%; dry weight), respectively. Fertilizers N and K were applied with 70% as base fertilizer and 30% as top-dressing on the seventh day after transplanting, respectively, while fertilizer P and chicken manure were all applied as base fertilizer, for both rice growing seasons. Other field practices were done as local recommend.

**Soil sampling and analyses**

The 0–20 cm layer soils were collected on 27 October 2016 after the late rice was harvested. Five soil cores were randomly taken and mixed to obtain representative samples for each treatment. Collected soils were sieved (< 2.0 mm) and divided into two parts: one part for soil property measurements and the other part was stored at −80°C for the denitrifier community analyses.

Soil pH was measured using a pH meter (FE28, Mettler Toledo, Shanghai, China) at soil to water ratio of 1:2.5 (m/v). Soil organic carbon (SOC) and total nitrogen (TN) were determined using the K\(_2\)Cr\(_2\)O\(_7\) oxidation-reduction titration method and the Kjeldahl digestion method (Bao 2000), respectively. Soil NH\(_4\)+-N and NO\(_3\)-N were extracted from fresh soil using 2 M KCl solution at soil to solution ratio of 1:5 (m/v) and detected using a Continuous Flow Analyzer (Skalar + Analytical, Breda, The Netherlands) (Yang et al. 2019).

**DNA extraction and quantitative PCR**

Soil microbial DNA was extracted from 0.3 g fresh soil using the EZNA® Soil DNA Kit (Omega, Norwalk, GA, USA) according to the manufacturer’s instructions. The purity of DNA solutions was checked on 1% agarose gel and the concentrations were determined using a NANO Quant (Tecan, Männedorf, Switzerland).

Quantitative PCR (qPCR) of the denitrifier communities was performed on an ABI 7500 thermocycler (Applied Biosystems, Foster City, CA, USA) based on the nitrite reductase genes (nir\(_S\) and nir\(_K\)) using the SYBR® Premix Ex Taq\(^{TM}\) (Takara, Dalian, China) according to the manufacturer’s instructions. The 25 \(\mu\)L PCR mixture included 12.5 \(\mu\)L of 2× SYBR Premix Ex Taq\(^{TM}\), 0.5 \(\mu\)L of each primer (10 \(\mu\)M) and 1–10 ng of template DNA. The primers used for nir\(_S\) and nir\(_K\) gene quantification were Cd3aF/R3CdR (Michotey et al. 2000; Braker and Tiedje 2003) and F1aCu/R3aCu (Hallin and Lindgren 1999), respectively. The qPCR amplification conditions were: 95 °C (10 min), followed by 40 cycles of 95 °C (30 s), 50 °C (40 s), 68 °C (40 s) for the nir\(_S\) gene and 95 °C (10 min), followed by 40 cycles of 95 °C (30 s), 56 °C (40 s), 68 °C (40 s) for the nir\(_K\) gene (Yang et al. 2017).

Standard curves of the nir\(_S\) and nir\(_K\) gene quantification were conducted using ten-fold dilution series of known copy number plasmids (ranged from 10\(^8\) to 10\(^3\)) containing the nir\(_S\) and nir\(_K\) genes that were PCR amplified as described previously by Yang et al. (2018). The qPCR amplification efficiencies were 92% and 97%, and \(R^2\) values were 0.994 and 0.996 for the nir\(_S\) and nir\(_K\) genes, respectively.

**Illumina MiSeq sequencing and analyses**

PCR amplification of the nir\(_S\) and nir\(_K\) genes was performed on an ABI GeneAmp\(^{®}\) 9700 PCR thermocycler (ABI, Foster, CA, USA), using the same primers (nir\(_S\) gene: Cd3aF/R3CdR and nir\(_K\) gene: F1aCu/R3aCu) as used in the qPCR amplification. The 5’ end of forward primers Cd3aF and
F1aCu contained an eight-base sequence barcode unique to each sample for the nirS and nirK genes, respectively. The 25 μL PCR mixtures contain 5 μL of 5x TransStart FastPfu Buffer, 0.5 μL of each primer (10 μM), 2.5 μL of 2.5 mM dNTPs, 0.5 μL of TransStart FastPfu DNA Polymerase, 1 μL of template DNA (< 0.5 μg) and finally ddH₂O up to 25 μL (TransGen, Beijing, China). The PCR condition was 5 min at 95 °C for initial denaturing, followed by 35 cycles of 95 °C (30 s), 55 °C (30 s) and 72 °C (45 s) and a final elongation step of 72 °C (5 min) for both the nirS and nirK genes.

PCR products of the nirS and nirK genes were extracted from 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer’s instructions. Purified PCR products were quantified using Quantifluor™-ST (Promega, Madison, USA) and pooled in equimolar and paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Majorbio, Shanghai, China) with standard protocols.

The raw fastq files were de-multiplexed and quality-filtered using QIIME 1.8.0 (Fadrosh et al. 2014). Operational taxonomic units (OTUs) with 97% similarity cutoff were clustered using the UPARSE pipeline 7.1 (Edgar 2013), and chimeric sequences were identified and removed using UCHIME (Edgar et al. 2011). The phylogenetic affiliation of nirS and nirK genes was analyzed by RDP Classifier against the FGR functional gene database (Fish et al. 2013), using a confidence threshold of 70%. The Shannon diversity index and Chao1 richness estimator were chosen to evaluate the alpha diversity of the nirS- and nirK-type denitrifier communities using MOTHUR 1.30.1 (Schloss et al. 2011). The representative nirS and nirK gene sequences with an average proportion of > 1% (abundant OTUs) among all the treatments were selected for the phylogenetic-tree construction in this study. Reference sequences for the nirS and nirK genes were obtained from the NCBI (http://www.ncbi.nlm.nih.gov/) after blasting using the dominant OTU sequences with an average proportion of > 1%. Phylogenetic analyses of the nirS and nirK genes were conducted by MEGA 5.1 using the neighbor-joining method with 1000-fold bootstrap support (Tamura et al. 2011). Principal coordinate analysis (PCoA) was used to compare the differences in community structure of the nirS- and nirK-type denitrifiers and redundancy analysis (RDA) was performed to reveal the relationships between soil properties and community structure of the nirS- and nirK-type denitrifiers using R statistical 3.2.1 (R Core Team 2014).

**Statistical analysis**

Statistical analyses were performed using SPSS Statistics 20.0 (IBM Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was performed to compare the differences in soil properties, gene abundance, alpha diversity index and abundant OTU proportion of the nirS- and nirK-type denitrifier communities between different fertilization treatments using the least significant difference test (LSD) at a significance level of p < 0.05. Pearson correlation analysis (significance level α = 0.05) was used to identify the relationships between soil properties and gene abundance, alpha diversity index and abundant OTU proportion of the nirS- and nirK-type denitrifier communities. Analysis of similarities (ANOSIM) was performed to evaluate the community differences of denitrifiers using R statistical 3.2.1 (R Core Team 2014). Figures of gene abundance, alpha diversity index and abundant OTU composition of the nirS- and nirK-type denitrifier communities were created using SigmaPlot 12.5 (Systat Software Inc., San Jose, CA, USA).

**Results**

**Soil properties**

Soil pH values in NPK + 30% OM and NPK + 60% OM were 0.08 and 0.09 units lower than that in CK (p < 0.01) (Table 1). The NH₄⁺--N and NO₃⁻–N concentrations in the fertilized treatments (NPK, NPK + 30% OM and NPK + 60% OM) were 0.77–0.82 and 1.08–1.96 times as much as that in CK (p < 0.05 and p < 0.001), respectively. The TN ranked in a significantly decreasing order of NPK + 60% OM > NPK +
Table 1. Properties of the soils in different fertilization treatments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pH (H2O)</th>
<th>NO3−-N (mg kg−1)</th>
<th>NH4+-N (mg kg−1)</th>
<th>TN (g kg−1)</th>
<th>SOC (g kg−1)</th>
<th>C/N ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK</td>
<td>6.78 ± 0.02a</td>
<td>1.99 ± 0.18a</td>
<td>2.14 ± 0.07c</td>
<td>2.15 ± 0.04d</td>
<td>13.23 ± 0.49c</td>
<td>6.15 ± 0.14a</td>
</tr>
<tr>
<td>NPK</td>
<td>6.73 ± 0.1a</td>
<td>1.74 ± 0.04b</td>
<td>2.45 ± 0.23b</td>
<td>3.21 ± 0.06c</td>
<td>14.33 ± 0.22c</td>
<td>6.15 ± 0.20a</td>
</tr>
<tr>
<td>NPK+30% OM</td>
<td>6.70 ± 0.01b</td>
<td>1.64 ± 0.07b</td>
<td>2.04 ± 0.25b</td>
<td>2.33 ± 0.06c</td>
<td>6.26 ± 0.16a</td>
<td></td>
</tr>
<tr>
<td>NPK+60% OM</td>
<td>6.69 ± 0.01b</td>
<td>1.54 ± 0.08b</td>
<td>2.04 ± 0.25b</td>
<td>2.33 ± 0.06c</td>
<td>6.26 ± 0.16a</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± standard error (n = 3). Different lowercase letters within the same column indicate significant differences at the 0.05 level of significance. Abbreviations: TN, total nitrogen; SOC, soil organic carbon; CK, unfertilized control; NPK, NPK fertilizers; NPK + 30% OM, NPK fertilizers plus 30% organic manure; and NPK + 60% OM, NPK fertilizers plus 60% organic manure.

30% OM > NPK > CK (p < 0.001, Table 1). The SOC contents in NPK + 30% OM and NPK + 60% OM were 1.51–1.69 and 1.40–1.56 times as much as those in CK and NPK (p < 0.001), respectively.

Abundance of the nitrite reductase genes

The nirS gene abundances in NPK and NPK + 60% OM were 27.3–32.3% lower than that in CK (p < 0.05), whereas no significant difference was observed between NPK, NPK + 30% OM and NPK + 60% OM (Figure 1). The nirK gene abundances in NPK, NPK+30% OM and NPK + 60% OM were 33.6–49.9% as much as that in CK and that in NPK + 60% OM was 0.32–0.33 times less than those in NPK and NPK + 30% OM (p < 0.05), respectively. Additionally, the nirS gene abundances were two orders of magnitude (249–546 folds) higher than the nirK gene in all the treatments (p < 0.001).

The nirK gene abundance and ratio of nirS to nirK gene abundance correlated significantly to soil pH (r = 0.833, p < 0.01; r = −0.793, p < 0.01), NH4+-N concentration (r = 0.749, p < 0.01; r = −0.629, p < 0.05), NO3−-N concentration (r = −0.867, p < 0.001; r = 0.892, p < 0.01), TN content (r = −0.733, p < 0.01; r = 0.913, p < 0.01) and SOC content (r = −0.740, p < 0.01; r = 0.833, p < 0.01); however, no significant correlation was observed between the nirS gene abundance and any soil properties. Furthermore, ratio of nirS to nirK gene abundance correlated significantly to the abundance of the nirK gene (r = −0.814, p < 0.01) (Table S1).

Alpha diversity of the nirS- and nirK-type denitrifier communities

Each sample generated 27,473 nirS gene reads and 29,025 nirK gene reads after normalization, which produced 27–31 and 99–300 OTUs at the 97% similarity, respectively, for the nirS- and nirK-type denitrifier communities. The OTU numbers in NPK, NPK + 30% OM and NPK + 60% OM were 39.3–42.6% lower than that in CK for the nirK-type denitrifiers (p < 0.05), while no significant difference was obtained in OTU number for the nirS-type denitrifiers. Additionally, the OTU number of the nirK-type denitrifiers correlated significantly to soil pH (r = 0.823, p < 0.01) and NH4+-N concentration (r = −0.639, p < 0.05) (Table S1).

The Chao1 richness estimator in NPK + 60% OM was 42.5% lower than that in CK for the nirK-type denitrifiers (p < 0.05, Figure 2D), while no significant difference was observed for the nirS-type denitrifier communities (Figure 2C). There was no significant difference in the Shannon diversity index for both the nirS- and nirK-type denitrifiers (Figure 2A, B). Furthermore, the Chao1 richness estimator of the nirK-type denitrifiers correlated significantly to soil pH (r = 0.876, p < 0.01), NO3−-N concentration (r = −0.589, p < 0.05), TN content (r = −0.590, p < 0.05) and SOC content (r = −0.582, p < 0.05).

Composition of the nirS- and nirK-type denitrifier communities

Eleven and twelve abundant OTUs (average relative abundance of > 1%) were observed for the nirS- and nirK-type denitrifier communities, respectively (Figure 3). For the nirS-type
denitrifiers, OTU21 had the highest proportion of 20.8%, followed by OTU23, OTU12, OTU36 and OTU29, with an average proportion of 16.6%, 15.3%, 14.6% and 12.7% among all the treatments. The OTU21 abundance in NPK + 60% OM was 0.4–1.3 folds more than those in the other treatments ($p < 0.05$). The OTU36 and OTU3 abundances in NPK, NPK + 30% OM and NPK + 60% OM were 2.0–3.0 and 0.2–0.4 times as much as that in CK ($p < 0.01$ and $p < 0.01$), respectively. The OTU31 abundance in NPK was 3.2–10.0 times greater than those in the other treatments ($p < 0.01$) (Figure 3A). As for the nirK-type denitrifiers, OTU301 was the predominant OTU that represented 37.8% of the total reads, followed by OTU10, OTU322, OTU399 and OTU282, with a mean proportion of 22.2%, 9.8%, 6.6% and 4.8%. The proportions of OTU282, OTU427 and OTU436 in NPK + 60% OM were 1.2–30.0, 1.9–7.0 and 1.4–10.3 folds higher than those in the rest treatments ($p < 0.05$, $p < 0.01$ and $p < 0.01$), respectively. The OTU410 abundance in NPK was 4.0–695.7 folds as much as those in the rest treatments ($p < 0.01$) and the OTU281 abundance in CK was 0.8–3.1 folds greater than those in the rest treatments ($p < 0.01$) (Figure 3B).

Both nirS and nirK gene phylogenetic trees were divided into three clusters (Figure 4). Six nirS gene OTUs (37.9% of the total reads, hereafter only percentage) were grouped into cluster I and they belonged to bacteria at the domain level and that cannot be classified at lower taxonomic levels.

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Figure 1. Abundance of the nirS (A) and nirK (B) genes in different fertilization treatments. Values are mean ± standard error ($n = 3$). Different lowercase letters indicate significant differences at the 0.05 level of significance.

Abbreviations: CK, unfertilized control; NPK, NPK fertilizers; NPK+30% OM, NPK fertilizers plus 30% organic manure; and NPK+60% OM, NPK fertilizers plus 60% organic manure.
except for OTU31 (phylum Proteobacteria). OTU21 and OTU23 were grouped into cluster II (37.5%) and OTU3, OTU14 and OTU36 were grouped into cluster III (21.7%), respectively. Proportion of the OTUs grouped into cluster II in NPK + 60% OM was 40.0% higher than that in NPK ($p < 0.05$) and those belonged to cluster III in the fertilized treatments (i.e. NPK, NPK + 30% OM and NPK + 60% OM) were 48.7–97.3% greater than that in CK ($p < 0.001$) (Figure 4A). When it came to the nirK genes, seven OTUs were grouped into cluster I (71.6%) which can be classified only at the domain level with the exception for OTU301 (genus Bradyrhizobium). OTU322 was grouped into cluster II (10.5%) and the rest four OTUs were grouped into cluster III (10.5%) which processed the multiple sequences affiliated to environmental samples, bacteria, Proteobacteria, and Nitrosospira at different taxonomic levels (Figure 4B). Differ from the nirS genes, no significant difference was observed in the proportion of the nirK gene OTUs that grouped into any clusters among different fertilization treatments.

**Community structure of the nirS- and nirK-type denitrifiers and their relationships with soil properties**

There were apparent impacts of fertilization on the community structure of the nirS- and nirK-type denitrifiers (ANOSIM: nirS, $R = 0.8272$, $p < 0.01$ and nirK, $R = 0.8704$, $p < 0.01$) that were evident upon the principal coordinate analysis (PCoA) based on the OTU level (Figure 5). The principal components (PCs) calculated from nine and ten variables for the nirS- and nirK-type denitrifier communities, respectively. The first two principal components (PC1 and PC2) explained 68.5% and 63.6% of the

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**Figure 2.** Shannon diversity index (A, B) and Chao1 richness estimator (C, D) of the nirS- and nirK-type denitrifier communities in different fertilization treatments.

Values are mean ± standard error ($n = 3$). Different lowercase letters indicate significant differences at the 0.05 level of significance. Abbreviations: NS, non-significant difference; CK, unfertilized control; NPK, NPK fertilizers; NPK+30% OM, NPK fertilizers plus 30% organic manure; and NPK+60% OM, NPK fertilizers plus 60% organic manure.
The PC1 (38.8% and 38.5%) and PC2 (27.9% and 25.1%) contributed similarly to variations of the nirS- and nirK-type denitrifiers, respectively. However, distributions of the nirS- and nirK-type denitrifiers were inconsistent according to PC1 and PC2, respectively. Furthermore, the top two components explained 62.8% and 36.8% of the nirS- and nirK-type denitrifier community structure in redundancy analysis (RDA) based on the OTU level, respectively (Figure 6). Among all the properties, soil pH \((R = 0.8571, p < 0.001)\) correlated best, followed by TN content \((R = 0.7205, p < 0.01)\), NO\(_3^-\)-N concentration \((R = 0.7172, p < 0.01)\) and SOC content \((R = 0.6821, p < 0.05)\), to the nirS-type denitrifier community structure. Whereas only TN content \((R = 0.6367, p < 0.05)\) and SOC content \((R = 0.5681, p < 0.05)\) correlated significantly to the nirK-type denitrifiers (Table S2).

**Discussion**

Although some studies showed that the nirK-type denitrifiers were more abundant than the nirS-type in agroecosystem (Yoshida et al. 2010; Cui et al. 2016; Azziz et al. 2017), opposite results were also found in other studies (Santoro et al. 2006; Yin et al. 2014; Pan et al. 2016; Yang et al. 2018). This indicated that nirS- and nirK-type denitrifier communities are not ecologically equivalent under different experimental conditions. Correspondingly, niche differentiation was proposed to be the major reason for the distinct behaviors of the two communities (Jones et al. 2008; Jones and Hallin 2010) and different management
practices associated with changes in soil conditions drove the selection of these two communities. In this study, we found that the abundances of nirS gene were much higher than the nirK gene, which may be attributed to the preference of more constant anaerobic conditions for the nirS-type denitrifiers (Ligi et al. 2014; Azziz et al. 2017; Yang et al. 2018). It has been proved that there was a significantly higher...
frequency of co-occurrence of the nirS gene with both the nitric oxide reductase (nor) and the nitrous oxide reductase (nosZ) gene than the nirK gene (Graf et al. 2014). This indicated that the higher nirS-type denitrifiers might result in lower N\textsubscript{2}O emissions due to the complete denitrification pathway than the nirK-type. Moreover, the ratio of nirS to nirK genes was proposed to be a significant indicator of the N\textsubscript{2}O sink capacity of a given soil (Jones et al. 2014). Although denitrification rate and abundance of denitrifiers were not always correlated (Yoshida et al. 2009; Yin et al. 2014), the abundance of denitrifiers can represent the potential of denitrification to some extent. These results indicated that the nirS-type

*Figure 5.* Principal coordinate analysis (PCoA) of the nirS- (A) and nirK-type (B) denitrifier community structure in different fertilization treatments.

 Abbreviations: CK, unfertilized control; NPK, NPK fertilizers; NPK+30% OM, NPK fertilizers plus 30% organic manure; and NPK+60% OM, NPK fertilizers plus 60% organic manure.

*Figure 6.* Redundancy analysis (RDA) reveals the relationships between the nirS- (A) and nirK-type (B) denitrifier community structure and soil properties.

 Abbreviations: TN, total nitrogen; SOC, soil organic carbon; CK, unfertilized control; NPK, NPK fertilizers; NPK+30% OM, NPK fertilizers plus 30% organic manure; and NPK+60% OM, NPK fertilizers plus 60% organic manure.
denitrifiers might play a more important role than the nirK-type in soil denitrification and there might be a low N\textsubscript{2}O emission potential in the paddy field.

The nirS- and nirK-type denitrifier communities were found to be differentially influenced by fertilization practices (Yoshida et al. 2009; Chen et al. 2010; Yang et al. 2017; Tao et al. 2018). This is because the nirS and nirK genes mostly belong to different bacterial strains that are thought to represent two typically ecologically distinct groups (Jones and Hallin 2010); nonetheless, some denitrifying bacteria strains are reported to harbor both genes (Jang et al. 2018; Sánchez and Minamisawa 2018). Both inorganic and organic fertilization greatly changed the community composition of the nirS- and nirK-type denitrifiers, which has been proved by most previous studies (Chen et al. 2010; Zhang et al. 2016; Tao et al. 2018). Significant differences were observed in some abundant OTUs between treatments and the proportion of OTUs grouped into clusters II and III in NPK + 60% OM were significantly higher than that in CK for the nirS-type denitrifiers (Figure 4), indicating that fertilization induced changes in the denitrifiers community composition and high rate of organic manure amendment (NPK + 60% OM) can enrichment some ecological groups due to the niche differentiation of denitrifiers (Jones et al. 2008; Hou et al. 2018). However, no difference was observed in the proportion of OTUs that belonged to any clusters for the nirK-type denitrifiers. These results revealed that these two communities processed differentially responding mechanisms to fertilization practices and the nirS-type denitrifiers were more sensitive to organic manure application as revealed by some previous studies (Yoshida et al. 2010; Duan et al. 2018).

Fertilization significantly affected the soil properties, which in turn greatly influenced the abundance, diversity and community structure of denitrifiers. Organic manure amendment was reported to increase the abundance of the denitrifier communities in previous studies (Tatti et al. 2013; Duan et al. 2018), probably via greater C resource availability in the organically amended soils. However, inorganic fertilization alone and combined inorganic and organic fertilization significantly decreased both the nirS and nirK gene abundances. Decreasing in soil pH was an important reason as it was supposed to be correlated to metabolic substrate concentrations of NO\textsubscript{3}–N and dissolved organic carbon (Bárta et al. 2010; Herold et al. 2018), which can both directly and indirectly, affect the denitrifier communities. In our study, the nirK gene abundance correlated positively to soil pH and correlated negatively to SOC content, indicating that soil pH and SOC were two dominant drivers for the lower gene abundances in the fertilized soils (Bru et al. 2011; Yin et al. 2014; Sun et al. 2015). Besides, the significantly lower NO\textsubscript{3}–N concentration in the fertilized treatments was another reason for the lower nirK gene abundances, even though the range was narrow, as it was the substrate of denitrification which can strongly affect the denitrifier communities (Zhou et al. 2011; Francis et al. 2013). Fertilization led to a significant decrease in nirS gene abundance, but no correlation was observed between the nirS gene abundance and any detected soil properties, indicating that there might be other factors affecting the nirS gene abundance.

Based on the PCoA profiles (Figure 5), distinguishable differences were observed in the community structure of the nirS-type denitrifiers between fertilized treatments and unfertilized control, while minor differences were found for the nirK-type. This indicated that the community structure of the nirS-type denitrifiers was more sensitive to fertilization practices than the nirK-type, which has been proved previously (Yin et al. 2015; Cui et al. 2016; Yang et al. 2017). The differences in community structure of the two communities might be explained by changes in soil pH, NO\textsubscript{3}–N concentration, TN content and SOC content, as they had significant correlations with the nirS-type denitrifiers, while only TN content and SOC content correlated significantly to the community structure of the nirK-type (Figure 6). This might be associated with the different sensitives of the two communities to soil properties (Yoshida et al. 2009; Yang et al. 2018). Furthermore, significant variances were detected in the combined inorganic and organic fertilized treatments than inorganic fertilization alone compared to unfertilized control for the nirS-type denitrifiers, indicating that organic manure application had a more significant effect than inorganic fertilizers on the community structure of the nirS-type denitrifiers.
Conclusions

Long-term application of inorganic fertilizer alone and combined with organic manure significantly decreased the *nirS* and *nirK* gene abundances and profoundly influenced the composition and structure of the two communities in paddy soil. The abundances of *nirS* gene were two orders of magnitude higher than that of the *nirK* gene, regardless of fertilization treatments. NPK + 60% OM decreased the *nirK* gene abundance compared to the rest fertilized treatments and decreased the Chao1 index of the *nirK*-type denitifiers compared to unfertilized control, respectively. In contrast, no difference was detected in abundance and Chao1 index of the *nirS*-type denitifier communities within various fertilization treatments. Fertilization has stronger effects on the community structure of the *nirS*-type than the *nirK*-type denitifiers. Combined organic and inorganic fertilization had a greater effect than inorganic fertilization alone on the community structure of the *nirS*-type. Abundance and Chao1 index of the *nirK*-type denitifiers and community structure of the *nirS*-type were primarily influenced by soil pH, NO<sub>3</sub>−–N, TN and SOC. However, the community structure of the *nirK*-type was mainly associated with TN and SOC. Our findings suggest that abundance and diversity of the *nirK*-type denitifiers are more sensitive, but community structure is less sensitive than the *nirS*-type to combined inorganic and organic fertilization in the paddy soil.

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Disclosure statement

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Y. YANG ET AL.


