Influence of fertilisation regimes on a nosZ-containing denitrifying community in a rice paddy soil

Zhe Chen, Haijun Hou, Yan Zheng, Hongling Qin, Yijun Zhu, Jinshui Wu and Wenxue Wei

Abstract

BACKGROUND: Denitrification is a microbial process that has received considerable attention during the past decade since it can result in losses of added nitrogen fertilisers from agricultural soils. Paddy soil has been known to have strong denitrifying activity, but the denitrifying microorganisms responsible for fertilisers in paddy soil are not well known. The objective of this study was to explore the impacts of 17-year application of inorganic and organic fertiliser (rice straw) on the abundance and composition of a nosZ-denitrifier community in paddy soil. Soil samples were collected from CK plots (no fertiliser), N (nitrogen fertiliser), NPK (nitrogen, phosphorus and potassium fertilisers) and NPK + OM (NPK plus organic matter). The nitrous oxide reductase gene (nosZ) community composition was analysed using terminal restriction fragment length polymorphism, and the abundance was determined by quantitative PCR.

RESULTS: Both the largest abundance of nosZ-denitrifier and the highest potential denitrifying activity (PDA) occurred in the NPK + OM treatment with about four times higher than that in the CK and two times higher than that in the N and NPK treatments (no significant difference). Denitrifying community composition differed significantly among fertilisation treatments except for the comparison between CK and N treatments. Of the measured abiotic factors, total organic carbon was significantly correlated with the observed differences in community composition and abundance (P < 0.01 by Monte Carlo permutation).

CONCLUSION: This study shows that the addition of different fertilisers affects the size and composition of the nosZ-denitrifier community in paddy soil.

INTRODUCTION

Nitrous oxide (N₂O) is an important trace gas in the global nitrogen cycle. Increases in the atmospheric concentration of N₂O contribute not only to global warming but also directly to the destruction of the stratospheric ozone layer. Due to its long estimated half-life (approximately 120 years) and a global warming potential of about 296 times that of carbon dioxide, even a small accumulation of N₂O may have destructive effects for centuries. Agricultural soil is a major source of N₂O emission, accounting for 24% of annual global emissions. Although nitrous oxide is produced by microbial processes including nitrification and denitrification, the latter is the major N₂O-producing mechanism.

Denitrification is a dissimilatory process in which oxidised nitrogen, nitrate and nitrite are reduced to gaseous nitric oxide, nitrous oxide and molecular nitrogen when oxygen is limited. The ability to carry out denitrification has been observed for a wide range of taxonomic groups including bacteria, archaea and fungi; for example, more than 50 genera of bacteria have been identified with denitrifying function. To understand the function of denitrifying bacteria in the process of N₂O production in soil, traditional cultural methods are not adequate because only about 1% of bacteria can be identified. Molecular techniques such as terminal restriction fragment length polymorphism (T-RFLP) and denaturing gradient gel electrophoresis (DGGE) have been used to explore the community composition and diversity of soil microorganisms. Because of the high phylogenetic diversity among denitrifiers, 16S rRNA gene-based approaches are also not suitable. Instead, use of functional genes involved in denitrification is a more direct method of detecting or identifying denitrifying bacteria. These genes include nitrate reductase.
(napA and narG), nitrite reductase (nirK and nirS), nitric oxide reductase (gndR and cnorB) and nitrous oxide reductase (nosZ). The reduction of nitrous oxide to molecular nitrogen, catalysed by N2O reductase (nosZ), is the last step in the complete denitrification pathway and represents a respiratory process in its own right, because many denitrifiers can grow at the expense of N2O as the sole electron acceptor.9 nosZ gene was usually used as genetic marker to investigate denitrifying community9–12 due to N2O reductase being a main regulator in controlling N2O emission.13

Research on denitrification in agricultural soil has mainly focused on the effects of fertilisers. Nitrogen fertilisers promote denitrification activity in agricultural soils, and substantial amounts of applied nitrogen fertiliser are lost through denitrification.14–16 Organic fertilisers often promote denitrification more than inorganic fertilisers.12,17–19 Among these organic fertilisers, returning crop residues to the field is highly recommended in China because the annual crop straw production is 7 × 108 t and increases steadily at an annual rate of 1.25 × 107 t.20 Crop straw is often returned to the field rather than burned as a measure to increase the annual crop straw production is 7 × 108 t and increases steadily at an annual rate of 1.25 × 107 t.20 Crop straw is often returned to the field rather than burned as a measure to increase soil fertility and soil organic carbon storage.21 It is also recommended that a certain amount of nitrogen fertiliser is added to maintain the soil carbon–nitrogen balance while returning crop straw to the field. Urea is the main N fertiliser used in rice paddy soil.22 Although researchers have previously compared effects of organic (manure and sewage sludge) and inorganic fertilisers on the composition of the denitrifying community in upland soil,8,12,23 little is known about the effect of straw residues incorporation and urea on the abundance, composition and activity of denitrifying communities.

Paddy fields are important agricultural land and about 70% of them are located in subtropical and tropical areas, which are the major source of nitrous oxide.24 Unlike upland soils, frequently flooded paddy soil supplies an anaerobic condition favouring denitrification, but the microbes responsible for denitrification are not well known. In this study, the main objective was to determine the long-term effects of repeated applications of rice straw residues and mineral fertilisers on the composition and abundance of nosZ-denitrifier community in the paddy soil. The impact of environmental parameters on nosZ-denitrifier community was also determined.

### MATERIALS AND METHODS

#### Field site and soil sampling

Soil samples were collected in March 2007 from a paddy field of a long-term fertilisation experiment initiated in 1990. The experimental site was located in the Taoyuan Ecological Observation Station (110°72′ E, 28°52′ N) in Taoyuan County, China. The soil was developed from Quaternary red soil, the cropping system was double rice and the experimental plot area was 33 m². Four treatments with three randomly distributed replications were sampled: CK (control, without fertilisation), N (urea, 182 kg N ha⁻¹), NPK (urea, 182 kg N ha⁻¹; calcium superphosphate, 39.3 kg P ha⁻¹; potassium chloride, 197 kg K ha⁻¹), and NPK + OM (NPK plus *Astragalus sinicus* L. and 80% straw produced in the plot). At the time of sampling (winter), all the plots were dried without flooding for about 4 months. Soil samples were taken with a clean, sterile sampler from the upper (0–15 cm) soil layer, where the field had remained undisturbed since the previous rice harvest. About 200 g of soil was packed into a sterile bag and frozen in liquid nitrogen immediately and stored at −70 °C. The sample was freeze-dried (Neocool, Yamato) and stored at −70 °C. The rest of the fresh original soil was air-dried for the analysis of soil properties.

#### Physical and chemical properties of the soil

Soil pH was determined at a soil to water ratio of 1:2.5 and soil organic carbon was determined by the K₂Cr₂O₇ oxidation method.25 Nitrate and ammonium were extracted with 2 mol L⁻¹ KCl, and determined using a continuous flow analyser (Flastar 5000 Analyzer). Potential denitrifying activity (PDA) was performed according to the C₂H₂ inhibition method.21,26 The physical and chemical properties of soil samples of the four fertilisation treatments were shown in Table 1.

#### DNA extraction

For each of the 12 samples, three independent DNA extractions were performed according to the previous method.27 To minimise the influence of PCR inhibitors, extracted DNA was further purified by Agarose Gel DNA Purification Kit (Takara Bio Inc., Shiga, Japan).The three extracts from the same replicate plot were pooled before further analysis. DNA concentration and quality was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

#### Primers for nosZ gene fragments

Degenerate primers were designed for amplification of nosZ gene fragments based on the alignment of protein sequences of NosZ distributed in 19 bacterial species (supplementary Table 1). These sequences were collected from the GenBank databases (http://www.ncbi.nlm.nih.gov/), and the corresponding bacterial strains were found in soil. Primer sequences were: 5’-GGCCTBGGGCRCYTGACAYC-3’ for the forward primer (nosZ-1126F) and 5’-CATYTCASAKRTGCAKGCCRTG-3’ for the reverse primer (nosZ-1884R). The numbers included in primer designations indicate nucleotide positions corresponding to the nosZ gene of *Sinorhizobium melloti* 1021 (AE007253). The forward primer

---

### Table 1. The physical and chemical properties of soil samples of the four fertilisation treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pH (H₂O)</th>
<th>Organic carbon (g kg⁻¹)</th>
<th>Total N (g kg⁻¹)</th>
<th>NO₃⁻-N (mg kg⁻¹)</th>
<th>NH₄⁺-N (mg kg⁻¹)</th>
<th>C/N</th>
<th>PDA (mg N₂O-N g⁻¹ ds h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK</td>
<td>5.17 ± 0.08a</td>
<td>18.1 ± 0.7c</td>
<td>1.77 ± 0.09b</td>
<td>0.16 ± 0.01c</td>
<td>3.35 ± 0.52b</td>
<td>10.27 ± 0.56a</td>
<td>68.3 ± 16.8c</td>
</tr>
<tr>
<td>N</td>
<td>5.17 ± 0.11b</td>
<td>20.1 ± 0.8c</td>
<td>1.91 ± 0.17b</td>
<td>3.16 ± 0.12a</td>
<td>6.07 ± 0.68a</td>
<td>10.55 ± 0.49a</td>
<td>220.0 ± 27.3b</td>
</tr>
<tr>
<td>NPK</td>
<td>5.10 ± 0.09a</td>
<td>21.6 ± 0.5b</td>
<td>1.97 ± 0.14a</td>
<td>1.27 ± 0.02b</td>
<td>3.20 ± 0.28b</td>
<td>10.93 ± 0.59a</td>
<td>161.7 ± 31.9b</td>
</tr>
<tr>
<td>NPK + OM</td>
<td>5.05 ± 0.22a</td>
<td>27.4 ± 0.3a</td>
<td>2.69 ± 0.06a</td>
<td>1.26 ± 0.03b</td>
<td>4.74 ± 0.19ab</td>
<td>10.17 ± 0.14a</td>
<td>394.0 ± 23.6a</td>
</tr>
</tbody>
</table>

1 Mean ± standard error (n = 3). Values within the same column followed by the same superscript letter do not differ at P < 0.05, ds, dry soil.
was fluorescently labelled with 6-Fam (6-carboxyfluorescein). In addition, 25 strains were used to evaluate primer specificity (Table 2).

**PCR amplification of nosZ fragments and T-RFLP**

The PCR reaction solution consisted of 5 µL of 10 × reaction buffer, 2 µL of 10 mmol L⁻¹ dNTP, 2 µL of 10 µmol L⁻¹ forward and reverse primers, 50–100 ng of DNA template, 2 U of Taq DNA polymerase (TianGen, Beijing, China) and dH₂O to make a final volume of 50 µL. Touchdown PCR was performed with 5 min of denaturation at 95 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 to 52 °C, and 45 s at 72 °C. The first 2 cycles were at 55 °C, the following three cycles were at 52 °C, and the last 30 cycles were at 50 °C. PCR was completed with 7 min at 72 °C.

Products were digested using the restriction enzyme of Alul (Takara Bio Inc.) by incubating the restriction digest for 4 h at 37 °C and heat inactivation at 65 °C for 10 min. The mixture was submitted to Sangni Corporation (Shanghai, China) for T-RFLP analysis using an ABI Prism 3100 Genetic Analyzer.

**Cloning and sequencing nosZ fragments**

PCR-generated nosZ fragments from soil DNA were purified using Wizard SV gel and PCR clean-up system (Promega, Madison, WI, USA) followed by molecular cloning with pGEM-T vector (Promega) into E. coli strain DH5α. Template DNAs for PCR were pooled from three representative samples of each treatment. Screening for correct inserts was carried out by PCR with M13 forward and reverse primers (Promega). A total of 331 clones (about 83 clones from each treatment) containing inserts of predicted size were randomly selected for sequencing by ABI Prism 3100 Genetic Analyzer according to the manufacturers protocol. NosZ gene sequences were deposited in the GenBank database under accession numbers FJ209365 to FJ209538.

**Quantitative polymerase chain reaction**

The primer pairs nosZ1126qF1/1381R were designed and applied to amplify a 256 bp nosZ fragment. The primer sequences were: 5′-GGGCTBGGGCGRTTGCA-3′ for the forward primer (nosZ-1126qF1) and 5′-GAAGCGRCTTSGARACTGG-3′ for the reverse primer (nosZ-1381R). Numbers included in primer designations indicate nucleotide positions corresponding to the nosZ gene of Sinorhizobium meliloti 1021 (AE007253). Again, 25 strains were also used to evaluate primer specificity (Table 2). The real-time PCR assay was carried out as described previously. Thermal cycling conditions were as follows: an initial cycle of 95 °C for 3 min; 5 cycles of 95 °C for 15 s, 60 °C for 20 s, 72 °C for 20 s, and 30 cycles of 95 °C for 15 s, and 55 °C for 20 s, 72 °C for 20 s. qPCR was performed with ABI Prism 7900 sequence detection system. The standard curves for nosZ were created using 10-fold dilution series (10²–10⁷ copies, supplementary Fig. 1) of the plasmids containing the targeted gene fragments from soil as described by Henry et al.²⁷ The efficiency of the reaction was 97% (based on the slope of the standard curve). One sharp peak was observed in the melt curve for the nosZ standard (supplementary Fig. 2).

---

**Table 2. Bacterial strains used in this study to test the specificity of nosZ primers**

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>nosZ</th>
<th>nosZ</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1126F/1884R</td>
<td>1126QF/1381R</td>
<td></td>
</tr>
<tr>
<td>Denitrifying strains with nosZ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudomonas stutzeri A1501</td>
<td>+</td>
<td>+</td>
<td>CAAS</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>+</td>
<td>+</td>
<td>GIM</td>
</tr>
<tr>
<td>Paracoccus denitrificans</td>
<td>+</td>
<td>+</td>
<td>ACCC</td>
</tr>
<tr>
<td>Achromobacter xylosoxidans</td>
<td>−</td>
<td>+</td>
<td>CGMCC</td>
</tr>
<tr>
<td>Ralstonia eutropha H16</td>
<td>+</td>
<td>+</td>
<td>DSM</td>
</tr>
<tr>
<td>Ralstonia eutropha CH34</td>
<td>+</td>
<td>+</td>
<td>DSM</td>
</tr>
<tr>
<td>Bordetella petrii DSM 12804</td>
<td>+</td>
<td>+</td>
<td>DSM</td>
</tr>
<tr>
<td>Thiobacillus denitrificans DSM12475</td>
<td>−</td>
<td>−</td>
<td>DSM</td>
</tr>
<tr>
<td>Burkholderia thailandensis E264</td>
<td>−</td>
<td>+</td>
<td>DSM</td>
</tr>
<tr>
<td>Chromohalobacter salexigens DSM3043</td>
<td>+</td>
<td>+</td>
<td>DSM</td>
</tr>
<tr>
<td>Herbaspirillum frisingense GSP 30</td>
<td>+</td>
<td>+</td>
<td>DSM</td>
</tr>
<tr>
<td>Herbaspirillum seropedicae Z67</td>
<td>−</td>
<td>−</td>
<td>DSM</td>
</tr>
<tr>
<td>Azospirillum lipofera Sp59b</td>
<td>−</td>
<td>+</td>
<td>DSM</td>
</tr>
<tr>
<td>Azospirillum brasilense Sp7</td>
<td>−</td>
<td>+</td>
<td>DSM</td>
</tr>
<tr>
<td>Azospirillum halopraeferens Aua</td>
<td>−</td>
<td>+</td>
<td>DSM</td>
</tr>
<tr>
<td>Azospirillum doebereinae GSF 71</td>
<td>+</td>
<td>+</td>
<td>DSM</td>
</tr>
<tr>
<td>Sinorhizobium meliloti 1021</td>
<td>+</td>
<td>+</td>
<td>IAE</td>
</tr>
<tr>
<td>Rhizobium meliloti sp.</td>
<td>++</td>
<td>+</td>
<td>IAE</td>
</tr>
</tbody>
</table>

| Denitrifying strains without nosZ (Firmicutes, G⁻) | − | − | IAE |
| Staphylococcus epidermidis sp.                | − | − | IAE |
| Bacillus licheniformis ATCC 14580             | − | − | DSM |
| Bacillus cereus sp.                           | − | − | IAE |
| Bacillus subtilis sp.                         | − | − | ISA  |
| Corynebacterium glutamicum ATCC 13032        | − | − | DSM |
| Pseudonocardia sp.                            | − | − | IAE |
| Actinomadura sp.                              | − | − | IAE |

+, PCR product of the right size obtained; −, no PCR product obtained; ++, PCR product was cloned and sequenced and the sequence was deposited in the data banks. Pseudomonas stutzeri A1501 was provided by Professor Min Lin, The Chinese Academy of Agricultural Sciences.
negative control containing no template DNA gave null values. The presence of PCR inhibitors in DNA extracted from soil was examined by (1) diluting soil DNA extract, and (2) mixing a known amount of plasmid DNA to soil DNA extract before qPCR. In none of the cases inhibition was detected. Standard curves, negative control and all samples were run on a single 384-well plate used for real-time PCR.

**Statistical analysis**

Soil properties and PDA were analysed by analysis of variance (ANOVA) using SPSS 13.0 software. T-RFLP analysis was performed as described by Ulrich and Becker.29 Sizes and relative abundances of terminal restriction fragments (T-RFs) were quantified using PeakScan version 1.0 software (Applied Biosystems, Inc., Foster City, US) Only fragments with a signal above 1% of the sum of all peaks were included in analyses. The peak areas of T-RFs that differed in size by ≤ 1 bp in an individual profile were summed and considered as one fragment. T-RFs in the range of 35–700 bp were used for cluster analysis. Canonical correspondence analysis (CCA) was used to assess the relationship between microbial community profiles and environmental variables. The resulting CCA profiles were significantly correlated with TOC (P = 0.002 by Monte Carlo permutation test within CCA analysis), while no significant correlation was found with the other measured parameters (C/N, NO3−-N and NH4+-N) (data not shown).

**Quantitative polymerase chain reaction assays**

nosZ gene copy numbers were significantly higher in N, NPK, and NPK + OM treatments (8.5 × 10^6, 9.6 × 10^6, and 1.8 × 10^7 gene copies g−1 dry soil, respectively) than CK (4.8 × 10^6 gene copies g−1 dry soil) (Fig. 3). There was no significant difference between the N and the NPK treatments and highest copy number was observed in plots amended with NPK + OM.

**Sequence analysis**

Four clone libraries of nosZ were constructed from the CK, N, NPK and NPK + OM treatments. For each library, about 120 clones were randomly selected and screened for correct inserts and about 83 clones from each library were selected for sequencing. Comparison with the NCBI databases using BLAST search revealed that 289 sequences (87.3%) of the 331 clones isolated were related to known nosZ sequences. Sequence identity ranged from 65 to 98% (nucleotide) and 56 to 99% (amino acid). A matching sequence for the remaining 42 sequences could not be confirmed by BLAST search. Sequences with similarity >98% were treated as individual OTUs, giving 53, 57, 51 and 52 OTUs from CK, N, NPK and NPK + OM treatments, respectively. Among the 171 OTUs, only 28 were found in different treatments and 143 OTUs were unique, and, none of the new sequences was completely identical to known nosZ sequences. The design of nosZ-containing denitrifiers, existing mostly in soils and sediments. nosZ gene fragments were successfully amplified from all 12 samples by the primer sets nosZ1126F/1884R and nosZ1126qF/1381R, respectively. The specificity of the two primer sets was further verified by using phylogenetically diverse denitrifier strains. PCR products of the expected size were obtained with each of the nosZ primer sets for most of the Gram-negative strains (Table 2). In addition, for both sets of the primers, no product was amplified from Gram-positive strains and the deduced amino acid sequence within the amplified region was conserved in all sequenced nosZ clones of the Cuz and the CuA catalytic domains and the active site of the enzyme.31
Potential denitrifying activity and the relation with composition and abundance of \textit{nosZ}-denitrifier community

Long-term fertilisation affected the potential denitrifying activity. All soil samples from the N-fertilised treatments showed statistically higher \((P < 0.05)\) PDA than the control samples. The highest PDA was observed in the NPK + OM treatment (Table 1).

PDA was not coupled to \textit{nosZ}-denitrifier community composition, as determined by CCA analysis. The \textit{nosZ}-denitrifier community did not change significantly after amended with urea only (N treatment) (Fig. 2), while, PDA was significantly increased (Table 1). Contrasting results were found with N and NPK treatments, which demonstrated different \textit{nosZ}-denitrifier community but similar PDA. On the other hand, statistical calculation showed that the differences in PDA were significantly correlated with the differences in \textit{nosZ} gene numbers; \(r = 0.916, P < 0.05\) by the Pearson correlation test.

\textbf{DISCUSSION}

\textbf{Primer validation}

Reliable PCR primers are a prerequisite for molecular analysis of microbial communities, since they ultimately determine what is detected in the environmental sample, and specificity and universality are key criteria in primer design. In this study, the specificity of newly designed primers was verified by amplifying \textit{nosZ} from denitrifiers representing a spectrum of proteobacterial species. The tested primers did not amplify representatives of the Firmicutes, indicating specificity for \textit{nosZ} genes of Gram-negative bacteria. Clones generated using the \textit{nosZ}11126F/1884R primers covered more than 10 genera of alpha-, beta- and gamma-proteobacteria and a large number of uncultured species, demonstrating that amplification was not restricted to a narrow group. Furthermore, the primer set \textit{nosZ}-F/R showed advantage of amplifying \textit{nosZ}-containing alpha- and beta-proteobacteria evenly compared to some \textit{nosZ} primers with bias towards alpha- or beta-proteobacteria.3,4,35

\textbf{Denitrifier community composition}

The composition of \textit{nosZ}-containing denitrifying communities may vary in relation to soil conditions, such as pH and nutrient content. Although reduction of soil pH is considered to be an important factor affecting denitrifier composition, following continuous application of ammonium sulfate and ammonium nitrate,12,23,36 we did not detect significant changes of pH among fertilisation regimes, probably because the urea applied constantly in this study is a physiologically neutral fertiliser and the frequently flooded paddy soil could buffer the soil from pH changes. Hence, soil pH might not be a significant factor in the shift of \textit{nosZ} communities caused by the fertilisation. Instead, CCA analysis demonstrated that organic carbon content (TOC) influenced the composition of the \textit{nosZ}-containing community. Soil amended with rice straw caused significant variations in the community, with associated clustering in phylogenetic analysis, e.g. blocks A and C.
Figure 4. Phylogenetic tree for nosZ based on the partial gene fragments from paddy field with long-term fertilisation. The tree was reconstructed using the neighbour-joining method and bootstrap analysis with 1000 replicates. Genbank accession number followed by treatment abbreviation in parentheses. The scale bar represents 10% estimated sequence divergence. The shading A, B and C represents the aggregation of clones from N or NPK + OM treatments.
Figure 4. (Continued).
et al.\textsuperscript{37} reported that addition of labile organic material altered the microbial communities that are most competitive in terms of growth rate and their ability to absorb nutrients. The amendment with rice straw may, thus, have selected for a more different suite of species compared to what was possible in the other treatments. Modification of the denitrifying community structure has also been observed in response to organic or mineral fertilisation assessed using nosZ, nirK and narG genes as functional markers.\textsuperscript{6,12,23,38}

Furthermore, the NPK + OM treatment generated higher relative abundance of the dominant T-RFs of 144 bp and 288 bp (corresponding to clones in clusters 1 and 2) than the N treatment. Variation in the dominant T-RFs of 144 and 288 bp might be due to their association with the alpha-proteobacterial rhizobiaceae, as found by others.\textsuperscript{39,40} A continuous supply of available nitrogen for many years, as in the N treatment, might reduce the importance of these nitrogen-fixing organisms. These sub-group denitrifiers bearing a so far uncharacterised nosZ gene might play an important role in denitrification and should be given more attention in further research. In contrast, plots amended with rice straw stimulated growth of denitrifiers but application of organic matter also led to reduction of another dominant T-RF, 276 bp, in comparison to other treatments. T-RF 276 bp was closely related to Burkholderiales of beta-proteobacteria. Although nothing is known of its characteristics in relation to soil denitrification, the change suggests that this group of denitrifiers might not favour eutrophic conditions and may not contribute intensively to PDA.

**Abundance of the nosZ gene**

The technology of real-time PCR, which is not limited by the culturability of bacteria, has been successfully applied to the quantification of functional genes as markers for bacteria in the environment.\textsuperscript{28,34,41} Up to now, only one copy number of the nosZ gene per cell has been observed, indicating that the nosZ gene copy numbers are directly correlated with cell numbers.\textsuperscript{42,43} However, the effects of long-term fertilisation on nosZ-denitrifier abundance are poorly understood. In this study, long-term fertilisation regimes changed the abundance of nosZ denitrifiers. Rice straw incorporation (NPK + OM) induced significantly higher copy number of nosZ gene. This may also be associated with high carbon bioavailability, since most denitrifiers are heterotrophs and are often C-limited.\textsuperscript{44} Similarly, Kandel et al.\textsuperscript{45} found the highest correlation existed between the quantity of soil organic carbon and the number of denitrifying genes (narG, nirK and nosZ) in a receding glacier foreland. However, Hallin et al.\textsuperscript{43} found that organic fertiliser alone did not significantly increase the nosZ-denitrifier abundance compared with the ones in the nitrogen treatment in the 50-year fertilisation experiment. The differences with our results are likely due to other nutrient supplements except for the organic carbon, such as nitrogen, sometimes reported as an important or even primary factor affecting denitrification.\textsuperscript{3,24} The remarkably higher carbon and nitrogen concentration existed in the NPK + OM treatment could provide abundant and balanced nutrient for denitrifiers.

It should be noted that the positive effects of the mineral fertilisers and the straw residue on nosZ-denitrifier abundance need long-term accumulation, because Miller et al.\textsuperscript{37} found neither the crop residues and nitrogen fertiliser alone nor the combinations induced a measurable change in the abundance of the nosZ gene in a short term (144 h). Straw residues are the complex carbon source, and need to be decomposed to the soluble compounds (i.e. glucose, glycerol, and mannitol) which could be available for microorganisms. Although the decomposition of rice straw residues was slowly at the first week under field conditions, it can sustain increasing soil organic carbon for more than 1 year,\textsuperscript{48} and provide labile nutrients directly to the denitrifiers, and/or stimulate microbial metabolism, which increases the consumption of O2 and creates conditions favourable for denitrification.\textsuperscript{39}

On the other hand, Hallin et al.\textsuperscript{43} found the number of nosZ genes was significantly decreased in lower pH fertilised soils amended with sewage sludge and ammonium sulfate. It was suggested that pH was a negative factor influenced the denitrifiers abundance and could even counteract the positive effects expected by high organic inputs.\textsuperscript{43} However, no obvious differences of soil pH among the fertilisation regimes in this study indicating that pH differences may play a minor role in altering the abundance and composition of nosZ-community. Among the measured environmental factors, only soil organic carbon was significantly correlated with the number of nosZ gene ($r = 0.873, P < 0.01$). This, however, does not mean the organic carbon was the only dominant factor on denitrifier abundance. Other soil properties might function combinedly and need to be studied in the future research.

Our results showed that the differences of PDA were significantly correlated with the differences in nosZ gene number rather than the variations of nosZ community composition. A similar result was found in fertilised and unfertilised soils.\textsuperscript{43} The correlation between nosZ gene numbers and PDA indicating that density of denitrifiers, are likely to be of importance for denitrification. However, the composition change of nosZ-denitrifiers induced by fertilisation should not be ignored. It might react on N2O reduction process even though we know a little about the importance of the shift of functional denitrifier composition in determining the process rates.

In conclusion, this study demonstrates long-term fertilisation affected both the composition and abundance of the nosZ-containing microbial community. Among the fertilisation regimes, rice straw incorporation remarkably altered the community composition and resulted in the highest abundance of nosZ population, whereas urea alone showed less effect on the nosZ community composition. Soil organic carbon was the dominant factor determining the denitrifying community composition and size. Further study should explore the functional ability of different groups of nosZ-containing denitrifying bacterial community.

**ACKNOWLEDGEMENTS**

We appreciate the helpful comments and English improvements given by Professor J.I. Prosser. This work was financially supported by Chinese Academy of Sciences (KZhX2-YW-T07, KZhX2-YW-BR-01) and the National Science Foundation of China (41071181, 41090282).

**Supporting information**

Supporting information may be found in the online version of this article.

**REFERENCES**

Selected indicators of food and agriculture development in Asia-

10 Guenzi WD, Beard WE, Watanabe FS, Olsen SR and Porter LK, Denitrification.

16 Knowles R, Denitrification.

18 Ellis S, Yamulki S, Dixon E, Harrison R and Jarvis SC, Denitrification

21 Scala DJ and Kerkhof LJ, Nitrous oxide reductase (nitS) gene fragments differ between native and cultivated Michigan soils. 

23 Mounier E, Hallet S, Cheneby D, Benizri E, Gruet Y, Nguyen C, Deklein CAM and Vanlogtestijn RSP, Denitrification in the top-soil of managed grasslands in the Netherlands in relation to soil type and fertilizer level. 


28 Scala DJ and Kerkhof LJ, Nitrous oxide reductase (nosZ) gene-specific PCR primers for detection of denitrifiers and three nosZ genes from marine sediments. 

31 Roesch C, Mergel A and Bothe H, Biodiversity of denitrifying and nitrifying bacterial communities in relation to the plant species in a constructed wetland. 

34 You CB and Zhou FY, Non-nodular endorhizospheric nitrogen-fixation in wetland rice. 

39 Nogales B, Timmis KN, Nedwell DB and Osborn AM, Detection and characterization of denitrifying bacteria from adjacent meadow and forest soils. 

44 Parkin TB, Soil microsites as a source of denitrification variability. 

46 Nogales B, Timmis KN, Nedwell DB and Osborn AM, Detection and characterization of denitrifying bacteria from adjacent meadow and forest soils. 

52 Drenovsky RE, Yo D, Graham KJ and Scow KM, Soil water content and organic carbon availability are major determinants of soil microbial community composition. 

56 You CB and Zhou FY, Non-nodular endorhizospheric nitrogen-fixation in wetland rice. 

58 Dunn RL, Relman DA, Fendorf SE, Martiny JBH, Triplett EE and Stahl DA, Denitrifying and nitrifying bacterial communities in relation to soil pH. 

65 Kandeler E, Deiglmayr K, Tscherko D, Bru D and Philippot L, Denitrifying genes in bacterial and Archaeal genomes.