Response of denitrification genes *nirS*, *nirK*, and *nosZ* to irrigation water quality in a Chinese agricultural soil

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Abstract

Purpose Denitrification is an important biochemical process in global nitrogen cycle, with a potent greenhouse gas product N₂O. Wastewater irrigation can result in the changes of soil properties and microbial communities of agricultural soils. The purpose of this study was to examine how the soil denitrification genes responded to different irrigation regimes.

Materials and methods Soil samples were collected from three rural districts of Beijing (China) with three different irrigation regimes: clean groundwater (CW), reclaimed water (RW), and wastewater (WW). The abundance and diversity of three denitrification microbial genes (*nirS*, *nirK*, and *nosZ*) were examined by real-time polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) molecular approaches.

Results and discussion The abundance of *nirS* in the WW treatment was higher than that in the CW treatment, and no significant difference was found between the RW and CW or WW treatments. The abundance of *nirK* gene of the RW

and WW treatments was higher than that of the CW treatment. There was no difference for *nosZ* gene among the three treatments. Correspondence analysis based on the DGGE profiles showed that there was no obvious difference in the *nosZ* gene composition, but *nirS* and *nirK* genes changed with different irrigation regimes.

Conclusions Irrigation with unclean water sources enhanced the soil NO₃⁻ content and changed the abundance and composition of soil denitrifiers, and different functional genes had different responses. Irrigation with unclean water sources increased the abundance of *nirK* gene and changed the community structures of *nirS* and *nirK* genes, while *nosZ* gene was relatively stable in the soil. These results could be helpful to explore the mechanisms of the variation of denitrification processes under long-term wastewater irrigation and partially explain the reason of more N₂O output in the field with wastewater irrigation.

Keywords Agricultural soil · Denitrification gene · DGGE · Irrigation water quality · Real-time polymerase chain reaction

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1 Introduction

Denitrification, an important part of the global nitrogen cycle, is receiving more and more attention because it is one of the primary soil microbial processes leading to the production of nitrous oxide (N₂O), a greenhouse gas and natural catalyst of stratospheric ozone degradation. N₂O concentration in the atmosphere had ascended from 270 to 319 ppb, with an increasing rate of about 0.26% per year from 1750 to 2005 (IPCC 2007), while agricultural fertilization managements were the dominant sources of atmospheric N₂O (Bouwman 1990). Thus, denitrification,

as one of the main pathways for the losses of nitrogen (N) fertilizers from agricultural soils, is of interests for both environmental and economic reasons (Enwall et al. 2005).

Denitrification is a biochemical process involving the stepwise reduction of nitrate (NO_3^-) and nitrite (NO_2^-) to produce the gaseous products of nitric oxide (NO), N_2O , and dinitrogen (N_2) under anaerobic conditions. This process is mediated by physiologically diverse groups of microorganisms. The denitrifying bacteria, namely denitrifiers, are known to belong to more than 50 genera (Zumft 1997), and the nitrate-reducing bacteria comprise an even larger group of phylogenetically unrelated microorganisms (Philippot and Hojberg 1999). Therefore, it is difficult to determine the diversity of nitrate-reducing communities using 16S rRNA gene approaches. Fortunately, genes that encode key enzymes (nitrate-, nitrite-, nitric oxide-, and nitrous oxide reductases) in nitrate reduction processes have been established as molecular markers (Smith et al. 2007). These functional genes include nitrite reductase genes (*nirS* and *nirK*), nitrous oxide reductase gene (*nosZ*), and nitric oxide reductase gene (*norB*).

The abundance and community compositions of the functional genes involved in denitrification can be affected by many factors, such as competition, predation, water content, pH, O_2 , and substrate availability (Ladd et al. 1993; Franklin and Mills 2003). Some studies have investigated the responses of denitrifiers to soil environmental changes. Wallenstein (2004) demonstrated that the abundance of *nirK* and *nirS* genes in the long-term fertilized plots (with $(\text{NH}_4)_2\text{SO}_4$) was lower than that in the control plots. Wolsing and Prieme (2004) found that fertilization with mineral fertilizer and cattle manure caused a shift in the community structure of denitrifiers in an agricultural soil. More recently, it was reported that organic fertilizers (manure, straw) increased the abundance of both *nirK* and *nirS* genes in a tropical agro-ecosystems (Hai et al. 2009). These studies indicated that the abundance and community structure of the soil denitrifiers were influenced by N availability, carbon availability, moisture, pH, and management practices, and these different functional genes could respond differently to environmental changes.

An increase in water shortages has required more frequent use of reclaimed water (RW) or even wastewater (WW) in agricultural irrigation. RW and WW, as complex mixtures, contained many organic, inorganic compounds, and ions. Irrigation with WW and RW could result in the shift of soil basic characteristics that could influence microbial diversities and functions (Friedel et al. 2000; Mapanda et al. 2005; Qian and Mecham 2005). Previous studies have shown that WW irrigation could increase the rates of denitrification and denitrifying enzyme synthesis in a soil ecosystem (Barton et al. 1999, 2000; Friedel et al. 2000; Zou et al. 2009). However, the

responses of soil functional genes involved in denitrification to wastewater irrigation still remain unknown. Thus, in this study, the responses of soil *nirS*, *nirK*, and *nosZ* genes to the different irrigation water sources were investigated based on molecular approaches. The purpose of this study was to examine whether and how these soil functional genes would respond to the long-term and different irrigation regimes. The results would be helpful to improve understanding of the relationships between functional genes of denitrifiers and the denitrification process in the agricultural soil ecosystems.

2 Materials and methods

2.1 Site description and soil sampling

Beijing, the capital of China, is confronted with serious deficiency of water resources. Since 1960s, wastewater (domestic sewage) irrigation has been commenced in agricultural area of Tongzhou District (Yang et al. 2005). In recent years, reclaimed water (treated domestic sewage) has been widely used as an important resource in Beijing. By 2007, about 0.5 billion m^3 reclaimed water was in recycle, including agricultural use that mainly located in southern suburb of the city, namely Daxing District (Cong and Zhao 2009). A reclaimed water (from Xiaohongmen and Huangcun sewage treatment plants) recycling demonstration has been constructed in this area since 2004.

In this study, soil samples were collected from two sites in Daxing (reclaimed water irrigation, RW) and Tongzhou (wastewater irrigation, WW), respectively (Fig. 1). In addition, one site in Changping District was chosen as control (clean water irrigation, CW). The soils in these three sites belonged to the same soil type classified as Eutric Cambisols and had very similar background characteristics before irrigation. All three sites had an annual wheat–maize rotation system and similar agricultural management practices (Zheng et al. 2008).

In each site, 12 surface soils (0–20 cm) were collected randomly in an area of 100 m^2 , and then mixed thoroughly to form one representative sample. Three samples were collected in each site as replicates. Soil samples were kept in an ice box and taken back to the laboratory, and then ground to pass through a 2-mm sieve and stored at -80°C for DNA extraction.

2.2 Determination of soil basic properties and DNA extraction

Soil pH was determined with a soil to water ratio of 2:5. Soil organic matter was measured using the $\text{K}_2\text{Cr}_2\text{O}_7$ oxidation method. Soil nitrate and ammonium were

Fig. 1 Map showing the location of the sampling sites under different irrigation regimes

extracted with 2 mol/L KCl and determined by a Continuous Flow Analyzer (SAN++, Skalar, Holland).

Soil DNA was extracted from 0.5 g fresh soil using MoBio UltraClean™ soil DNA isolation Kits (San Diego, CA, USA) according to the manufacturer's protocol with previously described modification (He et al. 2007).

2.3 Real-time polymerase chain reaction assay

Real-time polymerase chain reaction (PCR) was performed on an iCycler iQ5 thermocycler (Bio-Rad, USA). Amplification was performed in 25-μL reaction mixtures by using SYBR® Premix Ex Taq™ as

Table 2 Basic properties of the soil under different irrigation regimes

Treatment	pH (H ₂ O)	Organic matter (g/kg)	NO ₃ ⁻ (mg/kg)	NH ₄ ⁺ (mg/kg)
CW	7.70±0.05a	19.9±3.2a	22.9±1.6a	8.6±0.5a
RW	7.74±0.12a	20.6±2.0a	34.1±3.0b	8.4±0.2ab
WW	7.74±0.10a	23.4±3.9a	44.7±1.1c	7.5±0.5b

CW clean water irrigated, RW reclaimed water irrigated, WW wastewater irrigated

All values are mean±SD ($n=3$). The different letters (a–c) within the same column indicate significant differences between treatments at $P<0.05$

2.5 Data analysis

One-way analysis of variance (ANOVA) followed by S-N-K-test was performed using SPSS 11.5 to check for quantitative differences between samples. $P<0.05$ was considered to be statistically significant. Correspondence analysis (CA) for community ordination was conducted using CANOCO 4.5 for Windows using relative band intensity data obtained from the Quantity One analysis.

3 Results

3.1 Soil basic properties

Soil basic properties are shown in Table 2. There were no significant differences for the soil pH and organic matter between soil samples from the CW, RW, and WW treatments. Significant differences ($P<0.05$) were detected between NO₃⁻ contents of soil samples from different sites with an order for the treatments of WW > RW > CW. The content of soil NH₄⁺ in the CW site was significantly higher than that of the WW site ($P<0.05$).

3.2 Abundances of *nirS*, *nirK*, and *nosZ* genes

The abundances of *nirS*, *nirK*, and *nosZ* genes of the soil samples are shown in Fig. 2. The copy numbers of *nirS* gene in the soil samples of the CW, RW, and WW treatments were 1.35×10^7 , 1.93×10^7 , and 2.49×10^7 copies g⁻¹ dry soil, respectively. The *nirS* abundance of the WW treatment was significantly higher than that of the CW treatment ($P<0.05$), and no significant variation was observed between the RW and the other two treatments. The copy numbers of *nirK* gene ranged from 5.24×10^7 to 1.78×10^8 copies g⁻¹ dry soil. The *nirK* abundances of the WW and RW treatments were significantly higher than that of the CW treatment ($P<0.05$), and no significant difference was found between the RW and WW treatments. The *nosZ* gene copy numbers in those soil samples ranged from 4.53×10^8 to 7.17×10^8 copies g⁻¹ dry soil, and no significant differences were observed among the CW, RW, and WW treatments.

3.3 DGGE fingerprints of *nirS*, *nirK*, and *nosZ* genes

The community structures of *nirS*, *nirK*, and *nosZ* genes were analyzed by DGGE (Figs. 3a, 4a, and 5a). DGGE profiles with two replicates for each soil sample indicated good reproducibility. High diversities were shown in the DGGE profiles of *nirS*, *nirK*, and *nosZ* genes. For *nirS* and *nirK*, there were obvious variations between soil samples from the three sites (Figs. 3a and 4a). All the samples of the CW treatments showed less bands and lower diversity than those of the RW and WW treatments. The CA ordination diagram based on the DGGE patterns showed that *nirS* and *nirK* communities from the RW and WW treatments had relatively similar structures, and were obviously different from the CW treatment (Figs. 3b and 4b). Furthermore, the *nirK* Shannon values of the RW and WW treatments were significantly higher than that of the CW treatment (Table 3). The DGGE profile of *nosZ* showed minor variations between soil samples (Fig. 5a), and the scattered CA ordination diagram ascertained little difference in the *nosZ* gene communities between treatments (Fig. 5b). The Shannon values of *nirS*, *nirK*, and *nosZ* genes are listed in Table 3. For *nirS* and *nosZ* genes, there was no

Fig. 2 Abundances of *nirS*, *nirK*, and *nosZ* genes in the soils under different irrigation regimes of clean groundwater irrigated (CW), reclaimed water irrigated (RW), and wastewater irrigated (WW) samples. Error bars indicate standard deviations ($n=3$). The different letters indicate significant differences ($P<0.05$)

Fig. 3 **a** DGGE profile of *nirS* gene in the soils under different irrigation regimes of clean groundwater irrigated (CW), reclaimed water irrigated (RW), and wastewater irrigated (WW) samples. **b** Correspondence analysis (CA) ordination diagram of *nirS* gene communities generated by the *nirS* DGGE banding patterns

difference in the Shannon values among the CW, RW, and WW treatments. For *nirK* gene, the Shannon values of the RW and WW treatments were higher than that of the CW treatment ($P < 0.05$).

4 Discussion

4.1 Abundances of *nirS*, *nirK*, and *nosZ* genes under different irrigation regimes

In this study, there was lower *nirS* gene abundance than *nirK* in all samples, which was consistent with some previous studies (Hai et al. 2009; Yoshida et al. 2009). The abundance of *nosZ* gene was the highest one among the three functional genes in this study. This result was higher than those of previously reported: *nosZ* density of 3×10^6 – 2.8×10^8 copies g^{-1} dry soil (Kandeler et al. 2006;

Dandie et al. 2008; Hallin et al. 2009). But Chronakova et al. (2009) found the *nosZ* gene copy number was in a range of 9.1×10^7 – 7.5×10^9 copies g^{-1} soil in an upland pasture soil. Henry et al. (2006) found there was no significant difference between the abundances of *nosZ* and *nirK* in an agricultural soil of France. Thus, the abundances of soil *nirS*, *nirK*, and *nosZ* genes might be closely related to the properties of soil samples, especially when the management practices were different.

Although the copy numbers of *nirK* gene increased as a result of irrigation with wastewater and reclaimed water, no difference was found in the copy numbers of *nirS* gene between the CW and RW treatments. This result indicated that soil *nirK* was more sensitive with the water source quality than *nirS* gene, which brought more in line with the result of Yoshida et al. (2010), who found that the *nirK* gene were more sensitive than *nirS* gene in a paddy soil to management practices. No significant difference was found

Fig. 4 **a** DGGE profile of *nirK* gene in the soils under different irrigation regimes of clean groundwater irrigated (CW), reclaimed water irrigated (RW), and wastewater irrigated (WW) samples. **b**

Correspondence analysis (CA) ordination diagram of *nirK* gene communities generated by the *nirK* DGGE banding patterns

Fig. 5 **a** DGGE profile of *nosZ* gene in the soils under different irrigation regimes of clean groundwater irrigated (CW), reclaimed water irrigated (RW), and wastewater irrigated (WW) samples. **b** Correspondence analysis (CA) ordination diagram of *nosZ* gene communities generated by the *nosZ* DGGE banding patterns

about the *nosZ* gene copy numbers between soil samples. All these results hereby indicated that the functional genes involved in denitrification had different responses to the changes of environment.

Environmental factors affected the abundance of the bacterial denitrification genes greatly (Kandeler et al. 2006; Yoshida et al. 2009). Significantly positive correlations between the soil NO_3^- and the quantity of *nirK* gene ($R=0.682$, $P<0.05$), and between the soil organic matter content and the quantity of *nirS* gene ($R=0.614$, $P<0.05$), were found in this study. This result could imply that NO_3^- and organic matters, as the important substrates for the growth of soil denitrifiers, could stimulate soil denitrifiers. The abundances of soil denitrifiers were closely related to the basic properties of the soil samples, which were influenced by irrigated water greatly in this study.

4.2 Composition and diversity of soil *nirS*, *nirK*, and *nosZ* genes under different irrigation regimes

CA analysis based on the DGGE profiles showed that the composition of *nirS* and *nirK* genes was affected greatly by irrigated water, as well as the copy number of functional genes. However, the community structures of *nosZ* were relatively stable under different irrigation regimes in this

soil. The “unclean” water irrigation might have selective constraints on soil *nirS* and *nirK* communities. Furthermore, the higher *nirK* Shannon values of the RW and WW treatments compared with the CW further confirmed that *nirK* might be more sensitive than *nirS* gene. Some other studies also found the similar phenomena in different soil or under different environmental conditions. For example, Yoshida et al. (2010) found that *nirK* was more sensitive than *nirS* gene to denitrification-inducing conditions (additions of nitrate and organic matters) in a rice paddy soil; Djigal et al. (2010) found that bacterial-feeding nematodes decreased the copy numbers of soil *nirS* and *nirK* genes, but only the *nirK* community was obviously affected by the nematode feeding.

In our study, no obvious difference was found for *nosZ* gene under the different irrigation regimes. Combined with the soil basic properties, this result indicated that *nosZ* gene should be relatively stable under different irrigation regimes and less sensitive to higher nitrate content in this soil.

5 Conclusions and perspectives

This study demonstrated that soil functional genes involved in the denitrification processes responded differently to

Table 3 Shannon and Evenness values of *nirS*, *nirK*, and *nosZ* genes

Treatment	<i>nirS</i>		<i>nirK</i>		<i>nosZ</i>	
	Shannon	Evenness	Shannon	Evenness	Shannon	Evenness
CW	2.55±0.20a	0.988±0.005	2.58±0.22a	0.987±0.013	2.70±0.08a	0.965±0.004
RW	2.87±0.14a	0.983±0.006	3.05±0.05b	0.984±0.012	2.89±0.14a	0.956±0.018
WW	2.77±0.12a	0.959±0.003	3.21±0.06b	0.971±0.006	2.78±0.23a	0.962±0.010

CW clean water irrigated, RW reclaimed water irrigated, WW wastewater irrigated

All values are mean±SD ($n=3$). The different letters (a–b) within the same column indicate significant differences between treatments at $P<0.05$

environmental changes. Wastewater irrigation increased the abundance and changed the community compositions of the soil *nirS* and *nirK* genes. Compared with the direct wastewater irrigation, reclaimed water irrigation only had greater effect on the soil *nirK* gene. If the abundance of denitrifiers had some relationships with the soil denitrification enzyme activity, the increase of *nirK* gene copy numbers with a relative constant abundance of *nosZ* gene under “unclean” water irrigation might be supposed to increase the output of N_2O . This result partially explained the reason of more N_2O output in the field with wastewater irrigation, although the mechanism is still unclear.

There are some arguments about the relationship between the abundance of denitrifiers and the soil denitrification activity. In the future, the relative ratios of those functional genes involved in denitrification should be an important aspect. We need to understand if there is any relationship between the relative ratios and the outputs of products within the soil denitrification processes, and how the ratios change under different environmental conditions. Another important aspect is the study of the community composition of soil denitrifiers. At present, it is difficult to accurately determine the community composition of soil denitrifiers because of their high diversity. Some new and effective methods, e.g., high-throughput sequencing techniques, should be applied in this aspect. The improved understanding of these two questions will shed light on the relationships between the abundances, community compositions of soil denitrifiers, and their soil ecological functions.

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