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Responses of activities, abundances and community structures of soil denitrifiers to short-term mercury stress

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Abstract

The responses of activities, abundances and community structures of soil denitrifiers to mercury (Hg) stress were investigated through a short-term incubation experiment. Four soil treatments with different concentrations of Hg (CK, Hg25, Hg50, and Hg100, denoted as 0, 25, 50, and 100 mg Hg/kg dry soil, respectively) were incubated for 28 days. Soil denitrification enzyme activity (DEA) was measured at day 3, 7 and 28. The abundances and community structures of two denitrification concerning genes, *nirS* (cd_1-nitrite reductase gene) and *nosZ* (nitrous oxide reductase gene), were analyzed using real-time PCR and denaturing gradient gel electrophoresis (DGGE). Results showed that soil DEA was significantly stimulated in the treatments of Hg25 and Hg50 compared with others at day 7. Meanwhile, no difference in the abundances of soil *nirS* and *nosZ* was found between Hg spiked treatments and CK, except the lower abundance of *nirS* ($P < 0.05$) in the Hg added treatments compared with that in the CK at day 28. The community structures of denitrifiers based on *nirS* gene presented obvious change at day 7 along with the Hg additions, however, no variation was found in all treatments based on the *nosZ* gene. The results indicated that Hg (Hg25 and Hg50) had a strongly short-term stimulation on soil DEA, and *nirS* gene is more sensitive than *nosZ* gene to Hg stress.

Key words: Hg; denitrification enzyme activity; *nirS*; *nosZ*; DGGE; real-time PCR
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Introduction

Denitrification, one of the most important processes of the global nitrogen cycle, is mainly controlled by the primary soil microbial processes, in which nitrous oxide (N_2O), a greenhouse gas and natural catalyst of stratospheric ozone degradation is produced. This biochemical process is driven by physiologically unrelated diverse groups of microorganisms, and through which, nitrate (NO_3^-) and nitrite (NO_2^-) are reduced to gaseous products of nitric oxide (NO), N_2O, and nitrogen (N_2) under suboxic conditions (Zumft, 1997; Philippot and Hojberg, 1999). To date, functional genes encoding key enzymes in denitrification, including *nirS* (cd_1-nitrite reductase gene), *nirK* (Cu-containing nitrite reductase gene), *nosZ* (nitrous oxide reductase gene), and *norB* (nitric oxide reductase gene), have been established as molecular markers in studies of the soil denitrifiers (Smith et al., 2007).

It is well known that functions of soil ecosystem are closely related to soil microorganisms, and then the responses of soil microorganisms to the stresses have been considered as indicators of soil ecosystem health (Yang et al., 2001; Bulluck and Ristaino, 2002; Garbeva et al., 2006). By changing conformation of enzymes, inactivating essential functional groups or exchanging metal ions, heavy metals can influence the functions of soil microorganisms (Tyler, 1981). Some researchers had studied the effects of heavy metals on soil denitrification (Bollag and Barabasz, 1979; Sakadevan et al., 1999; Throbäck et al., 2007), and demonstrated the inhibition of heavy metals to denitrification. For example, Throbäck et al. (2007) described the immediate inhibitory effect of silver on soil denitrification activity by a sigmoid dose-response curve, and found novel *nirK* genotypes in the soil under silver stress. These studies indicated that denitrifying community responsed significantly to the stress of heavy metals and might be used as model to assess the effects of heavy metals on microorganisms in soil microcosms.

Mercury (Hg) is one of the most toxic elements to all living organisms. Due to its persistence, bioaccumulation and toxicity, Hg is listed as a priority pollutant by many international agencies. China has a long history of mining and use of Hg. During the last two decades, the consumption of Hg is more than 900 tons, almost 50% of worldwide production in 2000 (Jiang et al., 2006). Thus Hg emissions in China are widespread and the Hg contents

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in the atmospheres and soils in some areas are much higher than the global background values (Liu et al., 2002; Zhang et al., 2006). Qiu et al. (2004) reported that the total soil Hg contents ranged from 5.1 to 790 mg/kg in the mining area of Guizhou Province, Southwest of China. In the nearby paddy and upland fields, the maximum total soil Hg contents were up to 89 and 264 mg/kg, respectively (Qiu et al., 2006). Therefore, it is worthy to study the mechanisms and responses of soil microorganisms to Hg stress, for the determination of Hg toxicity to soil microorganisms.

The effects of Hg on the community structures of soil bacteria in short and long-term time spans had been reported previously (Müller et al., 2001; Ranjard et al., 2006; Ruggiero et al., 2011). Recently, the responses of soil ammonia oxidizers and denitrifiers to Hg stress were studied by several researchers. Liu et al. (2010) found mercury inhibited the activities and changed the community compositions of soil ammonia oxidizers. Philippot et al. (2008) found Hg (100 mg Hg/kg soil) obviously increased the soil denitrification enzyme activity (DEA) at day 45 and changed the community structure of soil *narG* (periplasmic nitrate reductase gene) at day 120 during the soil incubation. Nevertheless, the relationship between soil DEA and soil denitrifiers and the responses of other functional genes involved in denitrification under Hg stress were less available.

In this study, through a short-term incubation experiment, the effect of gradient concentrations of Hg on the activity of soil denitrifiers was detected. Simultaneously, the abundances and community structures of soil *nirS* and *nosZ* genes under Hg stress were investigated. The objective was to explore the response of the denitrification activity and community compositions of soil *nirS* and *nosZ* based denitrifiers to Hg stress.

1 Materials and methods

1.1 Microcosms and experimental design

Topsoil (0–20 cm) classified as Eutyic Cambisols was collected in July 2010 from a wheat-maize rotation field located in Shunyi District of Beijing (China). The soil basic properties were pH 7.75; organic matter 22.76 g/kg; NO_3^- 21.33 mg/kg; NH_4^+ 11.28 mg/kg; Hg 0.02 mg/kg. The soil sample was air-dried and ground to pass through a 2-mm sieve for the incubation experiment.

The effects of Hg on soil denitrifiers were studied using four different Hg treatments (0, 25, 50, and 100 mg Hg/kg dry soil, represent CK, Hg25, Hg50, and Hg100, respectively) with three replicates. The soil samples were spiked with $HgCl_2$ solutions to give the final Hg concentrations of 0, 25, 50, and 100 mg/kg dry soil. A total of 60 g each soil sample was placed in a tube (8 cm diameter, 15 cm height) in triplicate for each Hg concentration and each harvest time. In each tube, soil water content was adjusted to 35% maximum water holding capacity and was maintained throughout the experiment. Each tube was weighed weekly and sterile distilled water was added where needed. Finally, each tube was loosely covered with plastic cap allowing air-exchange and incubated in a dark incubator at 25°C for 28 days. Soil samples were collected from the tubes at day 3, 7 and 28 for DEA, real-time PCR and DGGE analysis.

1.2 Measurement of soil DEA

Each of 5 g fresh soil with 5 mL sterile distilled water was placed in a glass serum bottle and sealed with rubber septa and aluminum crimp cap. The headspace was replaced with high-purity N_2 gas to achieve anaerobic condition. Approximately 15% (V/V) of the headspace N_2 was replaced with acetylene gas (C_2H_2). Bottles were shaken on a longitudinal shaker for 30 min to evenly distribute the C_2H_2 throughout the soil slurry (White and Reddy, 1999). Bottles were incubated in the dark at 25°C for 12 hr, and then the headspace gas samples were taken and analyzed for N_2O by a gas chromatograph (GC, Model SP3410, Beijing Analytical Instrument Factory, China).

1.3 Soil DNA extraction and real-time PCR assay

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).

Real-time 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 described by the suppliers (Takara Bio, Otsu, Shiga, Japan). The DNA extracts were diluted 5-fold and used as template with a final content of 1–10 ng in each reaction mixture. The primer sets (*nirS*: cd3aF/R3cd; *nosZ*: nosZ-F/nosZ1622R) listed in Table 1 were used to amplify each target gene with real-time PCR. The thermal profile used for *nirS* and *nosZ* amplification was as follows: 94°C/2 min; 6 cycles of 94°C/30 sec, 57°C/30 sec (–1°C/cycle), 72°C/45 sec; 30 cycles of 94°C/30 sec, 52°C/30 sec, 72°C/45 sec. Following the thermal profile, by measuring the fluorescence continuously as the temperature increased from 55 to 95°C, a melting curve analysis was performed

Table 1 Primer sets used for the real-time PCR and DGGE analyses

Target gene	Primer set	Sequence (5′–3′)	Reference
nirS	cd3aF	GTSAACGTSAAGGARACSGG	Michotey et al., 2000
	R3cd	GASTTCGGRTGSGTCTTGA	Braker and Tiedje, 2003
	R3cd-GC[a]		
nosZ	nosZ-F	CGYTGTTCMTCGACAGCCAG	Kloos et al., 2001
	nosZ1622R	CGSACCTTSTTGCCSTYGCG	Throbäck et al., 2004
	nosZ1622R-GC[a]		

[a] (GGCGGCGCGCCGCCCGCCCCGCCCCCGTCGCCC) was attached to the 5′ end of the primers for DGGE.

to confirm the specificity of the PCR product for each real-time PCR amplification. Data analysis was carried out with iCycler software (version 1.0.1384.0 CR). The parameter C_t (threshold cycle) was determined as the cycle number at which a statistically significant increase in the reporter fluorescence was detected.

Standard curves for real-time PCR assays were developed as described previously (He et al., 2007). Briefly, the *nirS* and *nosZ* genes were PCR-amplified from extracted DNA with the primer sets (*nirS*: cd3aF/R3cd; *nosZ*: nosZ-F/nosZ1622R) listed in Table 1, and then the PCR products were cloned into the pGEM-T Easy Vector (Promega Madison, USA). Plasmids used as standards for quantitative analyses were extracted from the correct insert clones of each target gene. The concentration of plasmid DNA was determined on a Nanodrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, USA). Then the copy numbers of the target genes were calculated. To generate an external standard curve, ten-fold serial dilutions of a known copy number of the plasmid DNA were subjected to real-time PCR assay in triplicate.

1.4 PCR amplification and DGGE analysis

For DGGE analysis, the amplification was performed in 50 μL reaction mixtures including 1×PCR buffer, 400 μmol/L each dNTP, 2.5 U hot star Taq DNA polymerase (Takara Bio, Otsu, Shiga, Japan) plus primers (*nirS*: cd3aF/R3cd-GC; *nosZ*: nosZ-F/nosZ1622R-GC) (Table 1). The touchdown thermal profile used for *nirS* amplification was as follows: 94°C/2 min; 10 cycles of 94°C/30 sec, 57°C/30 sec (–0.5°C/cycle), 72°C/45 sec; 30 cycles of 94°C/30 sec, 52°C/30 sec, 72°C/45 sec; 72°C/10 min. The thermal profile for *nosZ* amplification was similar to that for *nirS* amplification, while 58°C was the starting temperature for touchdown (10 cycles of –0.5°C/cycle) and 53°C was the following annealing temperature.

DGGE analysis of each PCR products was performed with the DCode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, USA). PCR products of *nirS* and *nosZ* genes were loaded onto 6% (*W*/*V*) polyacrylamide (37.5:1, acrylamide:bisacrylamide) gels with a denaturing gradient of 50%–75% and 50%–70% (100% denaturant contains 7 mol/L urea and 40% (*V*/*V*) formamide), respectively. Electrophoreses were run at 60°C and 100 V for 15 hr for *nirS* and 13 hr for *nosZ*. The gels were stained for 30 min according to the manufacturer's instructions (Invitrogen 20 Molecular Probes, Eugene, USA), scanned by a GBOX/HR-E-M (Gene Company Limited, Syngene, UK). The obtained results were analyzed using Quantity One Software 4.5.2 (Bio-Rad Laboratories, Hercules, USA) and UPGMA algorithms were used to cluster the DGGE patterns.

1.5 Data analysis

One-way analysis of variance (ANOVA) followed by S-N-K-test was performed using SPSS 11.5 (SPSS Inc., Chicago, USA) to check for quantitative differences between samples. $P < 0.05$ was considered to be statistically significant.

2 Results

2.1 Effect of Hg on the soil denitrification enzyme activity (DEA)

The effects of Hg on the soil DEA are shown in Fig. 1. At the beginning of the incubation (day 3), the lowest DEA were detected in the Hg100, followed by the Hg25 treatment. No significant difference was found between CK and Hg50 treatment. But at day 7, the DEA in the Hg50 and Hg25 were almost two times higher than that in CK, while no difference was found between CK and Hg100 treatment. At the end of the incubation (day 28), Hg100 had a significantly higher DEA than CK, and no difference was found between CK and the other two Hg addition treatments.

2.2 Effects of Hg on the abundances of soil *nirS* and *nosZ* genes

The abundances of soil *nirS* and *nosZ* genes in the treatments with different Hg additions are shown in Fig. 2. At day 3 and 7, the abundances of soil *nirS* gene had no difference between CK and the other Hg addition treatments. While at the end (day 28) of the incubation, the *nirS* abundance in the CK was obviously higher than those in all the Hg treatments (Fig. 2a). As to the abundance of soil *nosZ* gene, no significant difference was found among all the treatments during the incubation (Fig. 2b).

2.3 Effects of Hg on the community structures of soil *nirS* and *nosZ* genes

At day 7 and 28, the community structures of *nirS* and *nosZ* genes were analyzed by DGGE (Figs. 3a and 4a) and the DGGE profiles with three replicates for each treatment indicated good reproducibility.

The *nirS* DGGE profiles showed obvious variations between CK and all Hg addition treatments at day 7, as some bands in the dotted line rectangles only clearly existed in the Hg addition treatments, while the variations disappeared at day 28 (Fig. 3a). The UPGMA cluster analysis based on the DGGE patterns also showed that *nirS*

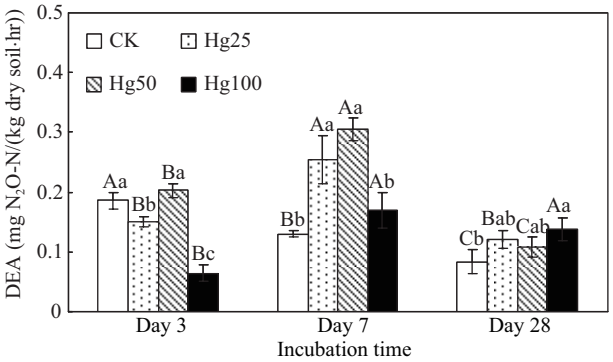

Fig. 1 Soil denitrification enzyme activity (DEA) in the treatments with different concentrations of Hg (CK, Hg25, Hg50, and Hg100, represent 0, 25, 50, and 100 mg Hg/kg dry soil, respectively) during the incubation. Error bars indicate standard deviations ($n = 3$). The different uppercase and lowercase letters above bars indicate significant differences ($P < 0.05$) among the same treatment at day 3, 7 and 28 and all the treatments at the same sampling days, respectively.

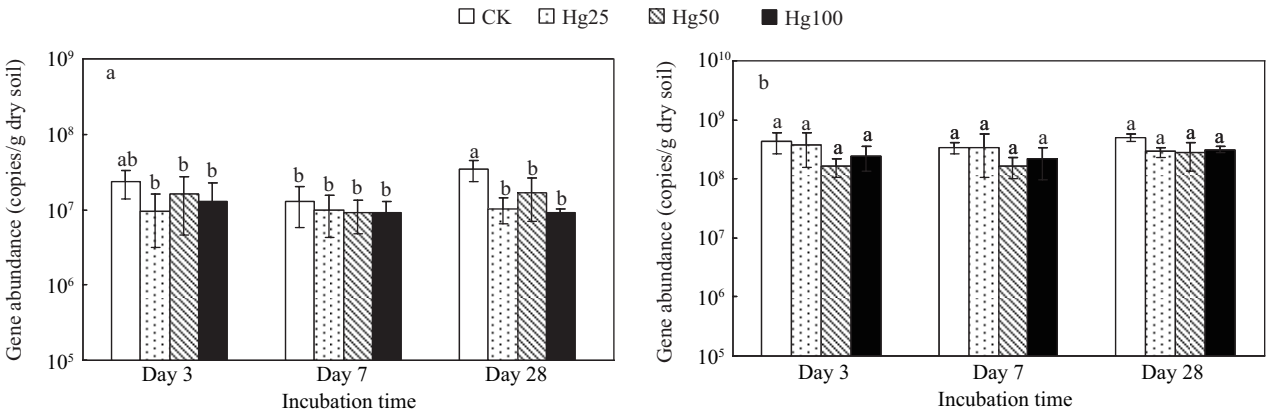

Fig. 2 Quantification of soil *nirS* gene (a) and *nosZ* gene (b) copy numbers in the treatments with different concentrations of Hg during the incubation. Error bars indicate standard deviations ($n = 3$). The different letters above bars indicate significant differences ($P < 0.05$) among treatments during the incubation.

community structures in all Hg addition treatments had relatively similar structures at day 7, which were obviously different from those in CK at day 7 and all the treatments at day 28 (Fig. 3b).

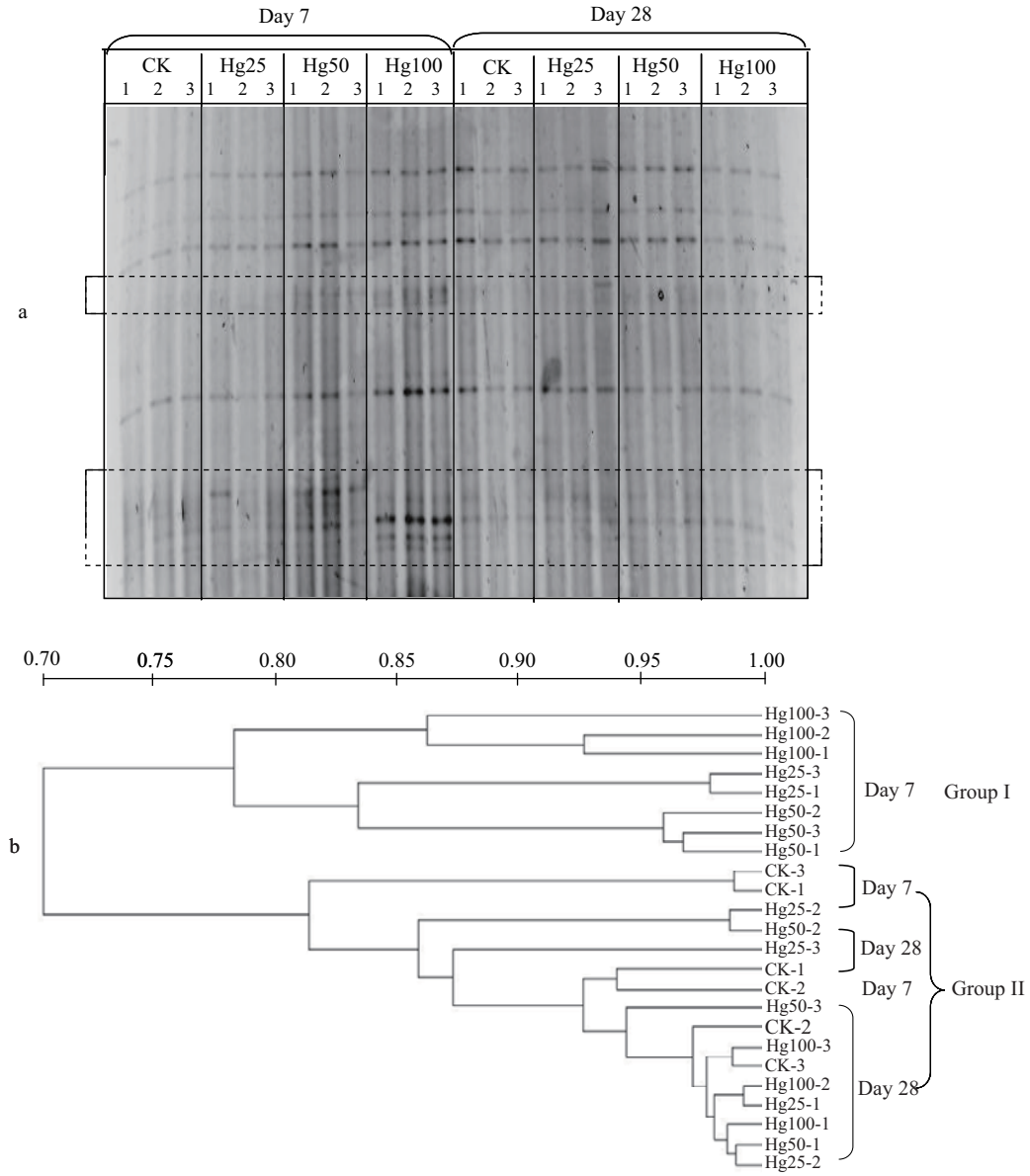

Fig. 3 (a) DGGE profiles of soil *nirS* gene amplified from the treatments with different concentrations of Hg at day 7 and 28. The districts in the rectangles with dotted line showed the special bands in the Hg treatments at day 7; (b) cluster analysis of *nirS* gene communities generated by the *nirS* DGGE banding patterns.

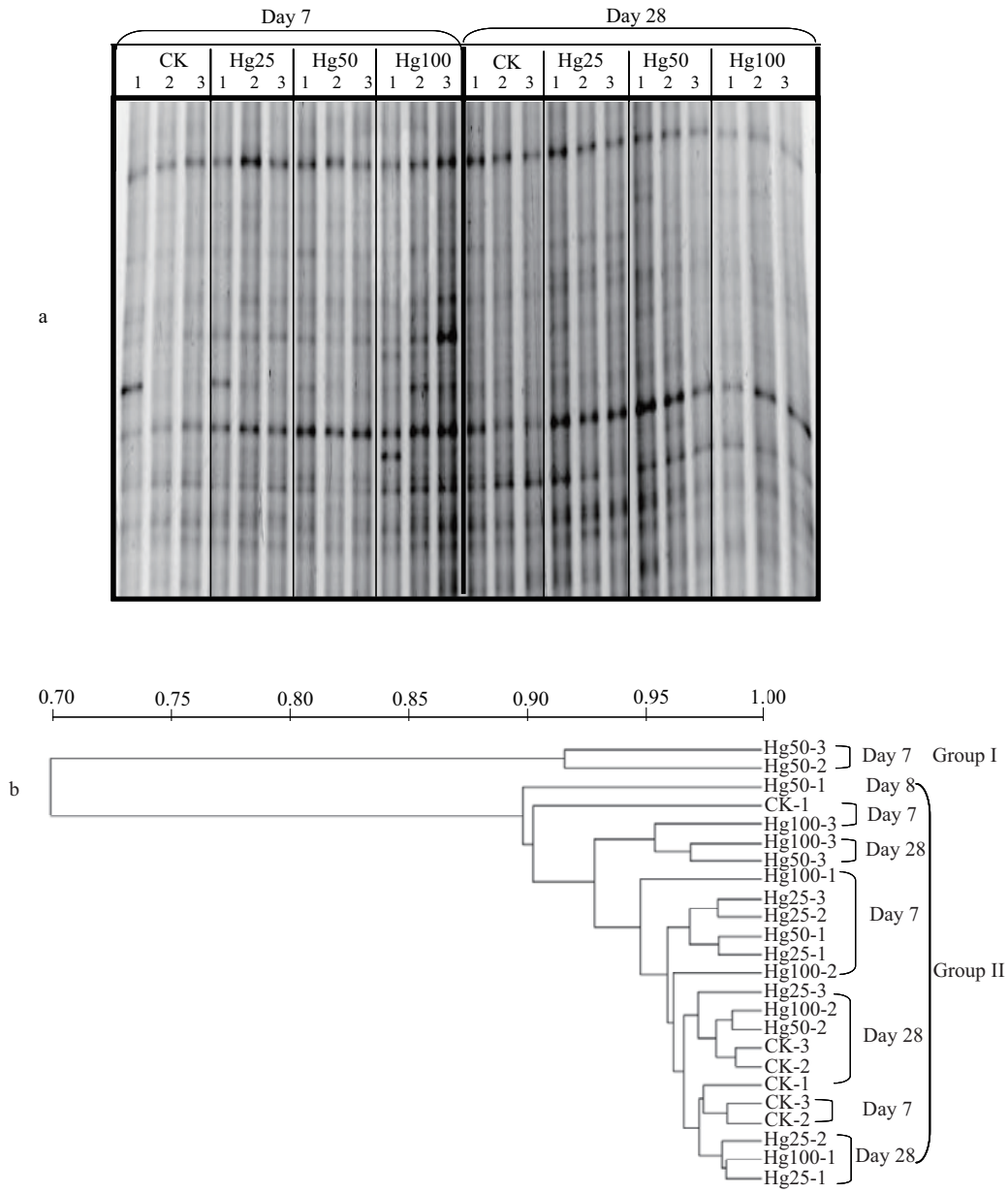

Fig. 4 (a) DGGE profiles of soil *nosZ* gene amplified from the treatments with different concentrations of Hg at day 7 and 28; (b) cluster analysis of *nosZ* gene communities generated by the *nosZ* DGGE banding patterns.

The *nosZ* DGGE profiles showed minor variations among all the treatments at day 7 and 28 (Fig. 4a), and the UPGMA cluster analysis of the *nosZ* DGGE patterns also indicated that *nosZ* gene community structures had no obvious differences among these treatments (Fig. 4b).

3 Discussion

3.1 Effect of Hg on soil DEA

In this study, the soil DEA was obviously inhibited by the highest Hg concentration treatment (Hg100) at day 3, and then recovered at day 7. The other two lower Hg additions (Hg25 and Hg50) sharply stimulated the soil DEA compared with CK at day 7, and had no obvious effect on the soil DEA at the end of the incubation (Fig. 1). The rapid inhibition of the highest Hg concentration on the soil DEA indicated the toxicity of Hg to the activity of soil denitrifying microorganisms, while the following increased DEA confirmed the resilience of soil microorganisms under Hg stress. The inhibiting effect of heavy metals to soil DEA had been reported proviously (Holtan-Hartwig et al., 2002; Magalhães et al., 2007; Ruyters et al., 2010). The significantly higher soil EDA in the Hg25 and Hg50 treatments was in accord with the result of Philippot et al. (2008), who found that Hg (100 mg Hg/kg) resulted in a three to four-fold increase of soil DEA within 2 weeks compared with CK (without Hg addition). The stimulation of Hg on soil DEA could attribute to the increased organic matter due to the lysis of microorganisms under the toxicity of Hg (Philippot et al., 2008). In fact, the wide distribution of soil denitrifiers could not be ignored, because some Hg-resistant bacteria might have the denitrification ability. For example, Kesseru et al. (2002) found *Ochrobactrum anthropi*, a well known denitrifier, was tolerant to Hg and lead (Pb). Chiu et al. (2007) isolated two Hg-resistant bacterial

strains with capacity of denitrification from a Taiwanese estuary. Therefore, the soil Hg-resistant bacterial harboring denitrification ability could be dominant and promote the soil DEA with the increased available substrates under Hg stress. The mechanism of the stimulation of Hg on soil DEA was still unclear, and it might be a combined action of complex soil biological and chemical processes under Hg stress.

3.2 Effects of Hg on the abundances and community structures of soil *nirS* and *nosZ* genes

The results of DGGE profiles indicated that *nirS* based denitrifier responded more sensitively than *nosZ* based denitrifier to the Hg stress, which was in a good agreement with previous findings that different denitrification concerning genes responded differently to environmental stresses (Hai et al., 2009; Yoshida et al., 2010). The stability of soil *nosZ* gene was also confirmed by Ruyters et al. (2010), who found minor changes in soil *nosZ* community structure under zinc stress. Furthermore, the result of obviously increased diversity in *nirS* gene under the Hg stress at day 7 was in accord with the result of Throbäck et al. (2007), who found that the diversity of the other nitrite reductase gene (Cu-nitrite reductase gene, *nirK*) obviously increased under silver stress. Therefore, our results here illustrated that some Hg-resistant bacterial harboring denitrification genes in soil were selected under Hg stress, and changed from minor into major populations enough to be detected by DGGE.

3.3 Relationship between soil DEA and soil denitrifiers under Hg stress

The DEA in Hg25 and Hg50 obviously increased, compared with that in CK at day 7, but no difference in the abundances of soil *nirS* and *nosZ* genes was found between these two Hg addition treatments and CK at the same sampling day. This result indicated that the soil DEA might have no direct relationship with the abundances of soil *nirS* or *nosZ* gene. By contrast, the obvious variation in *nirS* community structure was corresponding to the significantly higher DEA in the Hg25 and Hg50 treatments at day 7. These results indicated that the community structures, but not the abundance controlled the soil DEA. There are always some arguments about the relationship between the abundances and community structures of denitrifiers and the soil denitrification activity. Rich et al. (2003) found a strong relationship between the community composition of denitrifiers and DEA in two soils. However, Boyle et al. (2006) found no relationship between the denitrifier community composition and denitrification activity. Our result illustrated that there was obvious relationship between the structures of denitrifiers and soil denitrification activity under Hg stress.

4 Conclusions

In conclusion, our results demonstrated that Hg could influence the activity and community composition of soil denitrifiers. The highest (Hg100) and lower (Hg25, Hg50) concentrations of Hg had a rapid inhibition and short-term stimulation on the soil DEA, respectively. Furthermore, the soil *nirS* gene responded more sensitively than *nosZ* gene to the Hg stress, and only the community structure of *nirS* gene revealed a direct relationship with the soil DEA. To clarify the effect of heavy metals on soil denitrifiers, some mRNA depended and *in situ* methods should be applied in this field, and the long-term effects of heavy metals on soil denitrifiers should be another important aspect in the future.

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