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Impact of land use, fertilization and seasonal variation on the abundance and diversity of *nirS*-type denitrifying bacterial communities in a Mollisol in Northeast China

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ABSTRACT

Seasonal variations and land use responses of *nirS*-type denitrifying bacterial communities were investigated in a cultivated black soil (0–20 cm) in Northeast China with different fertilization treatments [no fertilizer (NoF), chemical fertilizer (CF) and CF plus manure (CFM)] and in a neighbouring plot of natural restoration (NR) using qPCR and Illumina Miseq sequencing. The NR plot was revegetated naturally with grasses without human disturbance since 1985. In general, the abundance of *nirS*-type denitrifying bacteria did not change significantly between NR and NoF across all sampling times, but fertilization increased the abundance of the *nirS* gene, except for samples collected in October. *Aromatoleum*, *Rhodanobacter*, *Cupriavidus*, *Bradyrhizobium*, unclassified genus of Proteobacteria, *Magnetospirillum* and unclassified genus of Rhodocyclaceae were the main denitrifiers in this study, accounting for 65.6–78.2% of the total *nirS* sequences. *Rhodanobacter* and unclassified genus of Rhodocyclaceae were significantly ($P < 0.05$) affected by the sampling time, and 10 genera (*Aromatoleum*, *Bradyrhizobium,* unclassified genus of Proteobacteria, unclassified genus of Rhodocyclaceae, unclassified genus of Betaproteobacteria, Dechloromonas, Azoarcus, Thiobacillus, Rubrivivax and Halomonas) were significantly ($P < 0.05$) affected by land use and fertilization. The PCoA analyses and pairwise tests revealed that the *nirS*-type denitrifying bacterial communities were affected more by land use than by seasonal change. We also found some OTUs that were detectable in NoF, CF, CFM or NR, and the classification and importance of these OTUs to N cycling merits further investigation.

1. Introduction

Denitrifying bacteria are widely distributed in unrelated phylogenetic groups [1], thus, the commonly used 16S rRNA gene is not suitable for composition analysis of denitrifying bacterial communities. However, several functional genes, such as *nirK*, *nirS*, *cnorB*, *qnorB*, and *nosZ*, involved in the denitrification pathway have been used as marker genes to describe the abundance or diversity of denitrifying bacterial communities in natural environments [2–6]. Among these genes, the *nirK* and *nirS* genes have frequently been used as gene markers to analyse denitrifying communities which can catalyse the reduction of nitrite to nitric oxide by nitrite reductase, i.e., the first step that distinguishes denitrifiers from nitrate-respiring bacteria, which do not reduce nitrite to gas [7]. Although the enzymes encoded by *nirS* and *nirK* are functionally and physiologically equivalent [1], *nirS* is more widely distributed in the environment [8].

Black soils, classified as Mollisols, are typical in Northeast China. Although original black soils are commonly thought to be fertile and productive with a soil organic matter content between 5% and 8%, some of these cultivated black soils have degraded over time due to intensive farming practices [9]. Previous study showed that fertilization practices can change N_2O emissions in arable black soils of Northeast China and the N application treatments resulted in higher cumulative N_2O emissions than no fertilization in the maize and wheat rotation phases, whereas no difference in cumulative N_2O was found between the no fertilization and N treatments in the soybean phase [10]. In addition, the application of both inorganic and inorganic fertilizer plus manure produced greater N_2O emissions was also observed in all three crops. However, to date, no study has focused on an analysis of denitrifying bacterial communities in black soils of this region.

Cultivation alters soil water content, temperature, aeration, organic N and soil physical and chemical properties [11–13], which can influence denitrifying bacterial abundance and communities [14]. Fertilization may increase the availability of N, which also leads to shifts in

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the abundance and community structure of denitrifying bacteria. An investigation about the response of denitrifying bacteria to different N fertilization rates in an alkaline northern Chinese soil found that the numbers of the *nirS* gene were significantly lower in the treatments with more N fertilization ($P < 0.05$) and the *nirS* gene community clearly changed with different N fertilization rates [15]. Our previous study showed that fertilizer inputs have less effect than land use on soil bacterial and fungal communities in black soils [16], but the addition of fertilizer caused changes in the abundance of the ammonia oxidizing bacteria (AOB) detected by *amoA* genes, *e.g.,* cultivated soils with added N fertilizer had a greater frequency of clones in the *Nitrosospira* cluster 10 sequence type but suppressed clones in the *Nitrosospira* cluster 3a.1 sequence type, whereas *Nitrosomonas* sequences were only recovered in the CFM treatment [17]. However, whether the *nirS*-type denitrifying bacterial communities respond in a similar or different manner as other soil microorganisms in the black soils of Northeast China has not been documented. Given that the total C and N contents in the NoF plot were significantly smaller than those in the CF, CFM and NR plots and that soil pH was also significantly different between the NR plots and cultivated plots ($P < 0.05$) [16], we hypothesized that the abundance or diversity of *nirS*-type denitrifying bacterial communities in black soils is influenced by land use and fertilizer regimes because agricultural practices and fertilizers alter the pH, carbon content or soil properties and influence the growth and survival of denitrifying bacteria.

In this study, we used the same soil samples as reported previously [16,17] to analyse changes in the *nirS*-type denitrifying bacterial communities. These samples were seasonally collected from four treatments in an experimental station located in Northeast China. The climate in this region is characterized by extreme seasonal variation in temperature and precipitation (cold and arid in winter, hot and rainy in summer). It is well known that seasonal fluctuations in soil temperature, soil water content and plant cover modulate denitrifying bacterial communities [18–20]. Our previous works also showed that the total bacterial communities and AOB in the same experimental field as this study exhibited seasonal changes associated with plant growth [16,17]. The purpose of this study was: (1) to observe whether land use and fertilization alters the abundance and diversity of *nirS*-type denitrifying bacterial communities in a natural restored grassland and continuously cultivated farmlands in black soils and (2) to examine how seasonality modulated the abundance and diversity of *nirS*-type denitrifying bacterial communities.

2. Materials and methods

2.1. Soil sampling and characteristics

Soils were taken from a long-term experimental field established in 1985 at the Hailun Agro-Ecological Experimental Station (47°26′N, 126°38′E), Heilongjiang Province, at the Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences. The experimental design and soil sampling have been described previously [16,17]. Briefly, an annual crop rotation of maize, soybean, and wheat was repeated every three years on the cultivated field with three fertilizer treatments: no fertilizer (NoF), chemical fertilizer (CF), and CF plus manure (CFM). The fertilizer treatments were randomly arranged within the cultivation field with three replicates. Fertilizers were applied to the treatment plots as NoF, CF (113 kg N ha^{-1}, 45 kg P_2O_5 ha^{-1}, 30 kg K_2O ha^{-1}) and CFM. The pig manure was supplied at 15,000 kg ha^{-1} on straw bedding with average total N, P and K concentrations of 22.1, 2.6 and 2.4 g kg^{-1}, respectively. Adjacent to the cultivated plots was one unreplicated natural restoration (NR) plot with a total area of approximately 1000 m^2. The NR plot was an area without human disturbance (i.e., no fertilizer inputs or tillage), allowing the grasses to grow naturally.

The soil sampling procedure and the sampling time have been described previously [16,17]. Briefly, the samples were collected with a soil auger from the three fertilization plots and the NR plot in October (after maize harvest), March (after snow melt), June (before soybean seeding) and August (soybean reproductive stage). The samples were collected from five locations in each plot and mixed together to form a single composite sample per plot as one replication, then placed in a polyethylene bag and transported to the laboratory and stored at 4 °C. Soon after arrival at the laboratory, the soils were sieved (< 2 mm), and approximately 2 g of each composite soil sample was placed in an autoclaved microcentrifuge tube (2 mL) and stored at −80 °C for molecular microbial analysis. The mean values of soil physiochemical properties from the five sampling dates were reported by previous study [17].

2.2. DNA extraction from soil samples

Soil DNA was extracted from 0.5 g of frozen soils with a Fast DNA SPIN Kit for Soil (Qbiogene Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. The extracted DNA was diluted in a 20 μL TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffer and stored at −20 °C until use.

2.3. Real-time quantitative PCR (qPCR)

Absolute quantification of the *nirS* gene was performed on a LightCycler®480 System (Roche) using primers *nirS*-cd3aF (5′-GTSAACGTSAAGGARACSGG-3′) [21] and *nirS*-R3cd (5′-GASTTCGGRTGSGTCTTGA-3′) [22]. The standard curves were generated using 10-fold serial dilutions of a plasmid containing the *nirS*-insert. Each 20 μL reaction mixture contained 10 μL of SYBR Premix EX Taq TM (Takara, Dalian, China), 0.4 μL each of 10 μM forward and reverse primers, 2 μL of 100-fold diluted template DNA, and 7.2 μL of sterilized Milli-Q water. Thermal cycling conditions were as follows: initial denaturation at 95 °C for 30 s (ramp rate of 4.4C/s), followed by 30 cycles of 95 °C for 5 s for denaturation, 60 °C for 30 s for annealing and elongation, and one final cycle at 50 °C for 30 s for cooling. A melting curve analysis and agarose gel electrophoresis of the PCR products were conducted to confirm that the fluorescence signal was originated from specific PCR products and not from primer-dimers or other artefacts. The copy number of the *nirS* gene was calculated using a regression equation for converting the cycle threshold (C_t) value to the known copy numbers in the standards. All of the real-time PCR reactions were conducted in triplicate using the DNA extracted from each soil sample.

2.4. Illumina MiSeq sequencing

The denitrifying community was also analysed using the primers *nirS*-cd3aF and *nirS*-R3cd, which have been widely used in many studies of denitrifying bacterial communities in environmental samples [2,23,24]. Each sample was tagged by adding an eight-base extension (barcode sequence) to the forward primer used in the PCR. PCR amplification was performed in triplicate using a Gene Amp PCR-System® 9700 (Applied Biosystems, Foster City, CA, USA) in a total volume of 20 μL containing 4 μL of 5 × FastPfu Buffer (TransGen, China), 2 μL of dNTPs (2.5 mM, TransGen, China), 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase (TransGen, China) and 10 ng of template DNA. The mixture was filled to the required volume with Milli-Q water. Thermal cycling conditions were as follows: an initial denaturation at 95 °C for 2 min, 25 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension at 68 °C for 10 min. Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions, and quantified using QuantiFluor™ -ST (Promega, USA). The purified amplicons were pooled in an equimolar ratio and paired-end sequenced (2 × 250) on an Illumina MiSeq platform according to the standard protocols.