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Stable isotope probing reveals compositional and functional shifts in active denitrifying communities along the soil profile in an intensive agricultural area

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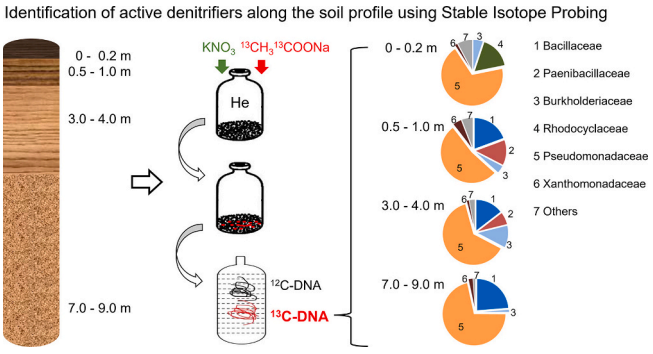
HIGHLIGHTS

- Active denitrifiers in a 9 m agricultural soil profile were identified using SIP.
- Active denitrifiers were compositionally different between the topsoil and subsoil.
- The composition of active denitrifiers could be linked to N transformation function.

GRAPHICAL ABSTRACT



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ABSTRACT

Denitrifying microbial communities in the vadose zone play an essential role in eliminating the nitrate leached from agricultural practices. This nitrate could otherwise contaminate groundwater and threaten public health. Here, we utilized stable isotope probing combined with amplicon sequencing and functional gene quantification to inspect the composition and function of heterotrophic denitrifying microorganisms along a 9-m soil profile in an intensive agricultural area. Dramatic differences in the composition of the active denitrifiers were uncovered between the surface soil and deep layers of the vadose zone. The main denitrifying bacterial taxa identified from ^{13}C-DNA fractions were *Pseudomonadaceae* (*Pseudomonas*), *Rhodocyclaceae* (*Azoarcus*), and *Burkholderiaceae* in the surface soil (0–0.2 m), and were *Pseudomonadaceae* (*Pseudomonas*), *Burkholderiaceae*, *Bacillaceae* (*Bacillus*), and *Paenibacillaceae* (*Ammoniphilus*) in the deep layers (0.5–9.0 m). Analysis of the functional genes (*nirS*, *nirK*, and *nosZ)* in isotope-labeled DNA revealed an upward *nos/nir* ratio with increasing soil depth, which may account for the higher nitrous oxide emission potential in the surface soil, as compared to the deeper sand-rich, low

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organic carbon layers. This study improves our understanding of active denitrifying microbes in the vadose zone and helps in developing techniques to reduce nitrate pollution in groundwater.

1. Introduction

Anthropogenic activities have increased nitrogen (N) inputs in terrestrial ecosystems globally in recent decades (Galloway et al., 2004; Fierer et al., 2011), and elevated reactive N in the environment is perceived as an escalating threat due to its impacts on water quality and the atmosphere (Stark and Richards, 2008). Nitrate leaching in agricultural ecosystems may result in contamination of groundwater and has gained significant attention due to its impacts on public health (Wolfe and Patz, 2002). Denitrification converts nitrate or nitrite to gaseous

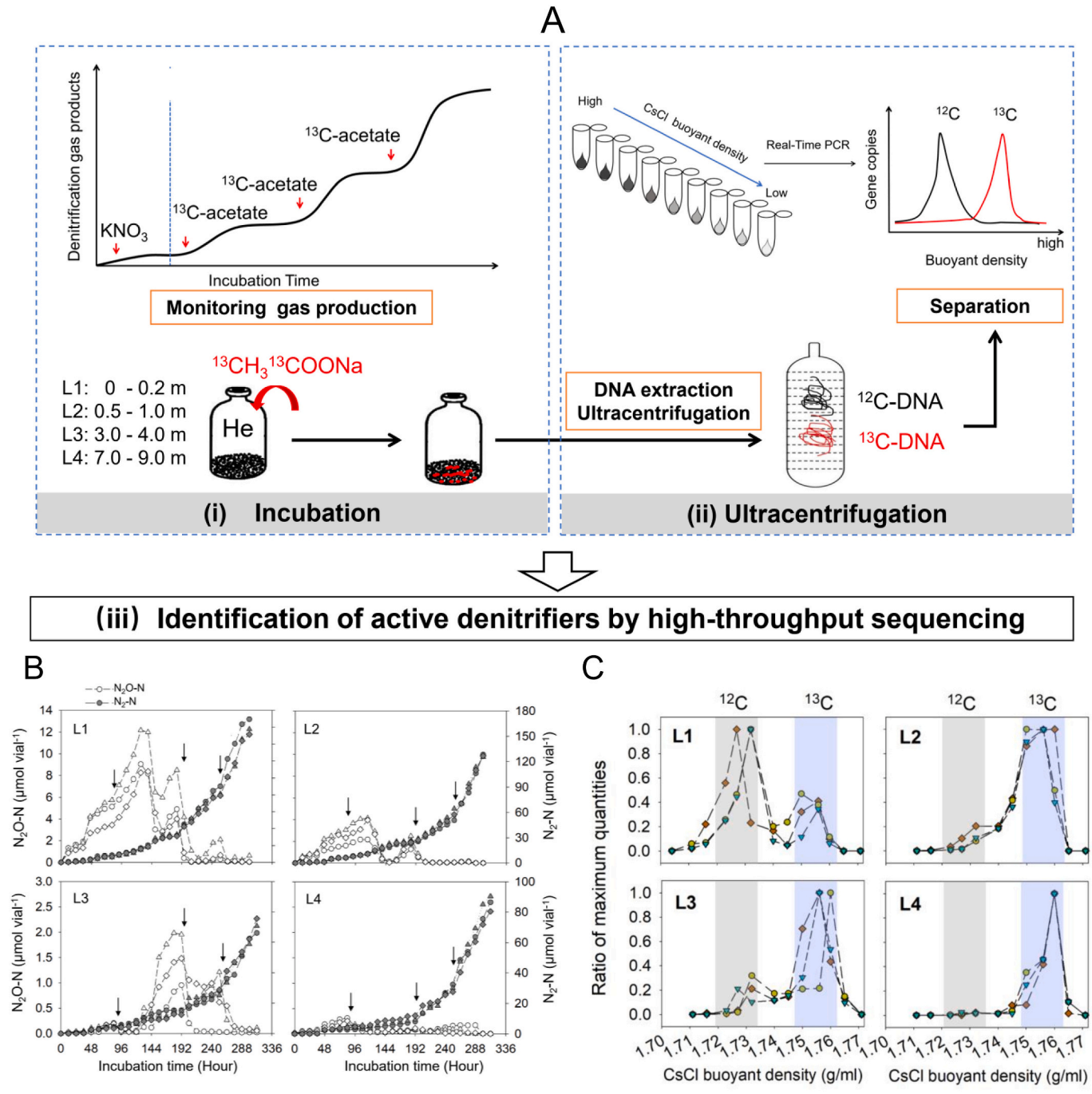

Fig. 1. (A) Schematic diagram of the experimental design in this study. It included three parts: (i) Incubation; (ii) Ultracentrifugation; and (iii) Identification of active denitrifier communities. (B) Kinetics of N_2O and N_2 production of various layer vadose zone samples during the incubation under denitrifying conditions. The arrows indicated the three additions of $^{13}CH_3^{13}COONa$ (at 84 h, 192 h, and 264 h). Three shapes represented three repetitions. (C) Quantitative distribution of the 16S rRNA gene along the CsCl density gradient of various layer vadose zone samples that were incubated for 312 h under denitrifying conditions. Three shapes represented three repetitions.

nitrogen compounds and is therefore an important process for controlling the quantity of nitrate that may leach into groundwater.

A series of studies have focused on the process of denitrification in the vadose zone. Microbial denitrification was observed at depths of several meters and this process is limited in deep layers because of the scarcity of organic carbon and the low abundance of denitrification microorganisms (Chen et al., 2018; Liu et al., 2020; Han et al., 2023). Prior studies have attempted to inspect the composition of denitrifying communities through monitoring shifts in microbial community composition in laboratory incubation experiments where carbon and nitrate was supplemented (Chen et al., 2018; Oh et al., 2023). In these studies, denitrifying communities were identified by comparing the microbial communities before and after incubation under denitrifying conditions. The structure of the microbial community was assessed by analyzing 16S rRNA gene sequences. Although some denitrifying microorganisms have been identified, one limitation of this method is that it may overlook functionally important denitrifiers whose relative abundance did not significantly change during the incubation.

Stable isotope probing (SIP) technique is an effective approach for identifying functionally active communities from background populations (Radajewski et al., 2000; Costa et al., 2020), and has been recently applied to identify active denitrifiers in environmental samples (Bellini et al., 2018; Xing et al., 2018). In the current study, we developed a strategy to inspect the active denitrifiers in the deep vadose zone samples. The soil carbon that can support denitrification was depleted during a pre-incubation stage. Then ^{13}C-carbon was added to the vadose zone samples and taken up by the denitrifiers as the carbon source for denitrification. These active denitrifiers were subsequently identified by separating ^{13}C-DNA from ^{12}C-DNA using ultracentrifugation. The ^{13}C-DNA represents the active denitrifiers in the incubation process, and the ^{12}C-DNA represents the rest of the microbial community present in the system.

2. Materials and methods

The vadose zone samples were taken in a typical intensive agricultural area in the North China Plain. The details of the field experiment and sampling procedure were described in our prior study (Chen et al., 2018). In this study, four layers (0–0.2 m, 0.5–1.0 m, 3.0–4.0 m, and 7.0–9.0 m) vadose zone samples in a long-term fertilization field experiment (600 kg N ha^{-1} yr^{-1}) were selected. The depth of 0–4.5 m consists of a sandy loam layer, while the depth of 4.5–9.0 m is composed of a sand layer. Soil organic carbon decreased significantly with depth, dropping below 2.0 g kg^{-1} in the layers deeper than 1.5 m (Table S1). Moreover, the microbial community composition has been investigated, and significant differences were discovered between depths below and above 2.5 m (Wang et al., 2021).

The experimental procedure is illustrated in Fig. 1A. (i) Incubation. Soil samples weighing 10.0 g were prepared in 120 ml serum vials and made anaerobic using a method described previously (Chen et al., 2018). A pre-incubation was performed with excess potassium nitrate (21 μmol-N g^{-1} soil) to deplete the available soil organic carbon for denitrification (electron donors). During the incubation, the denitrification gas products (N_2O and N_2) were monitored using a robotized incubation system (Molstad et al., 2007). When the dinitrogen (N_2) gas concentration reached a plateau, isotope-labeled carbon ($^{13}CH_3^{13}COONa$) was supplemented as the new electron donor for denitrification. This process was repeated three times, with 5 μmol g^{-1} soil of isotope-labeled carbon being added each time. (ii) Ultracentrifugation. Soil total DNA was extracted after incubation, and mixed with cesium chloride (CsCl) for gradient ultracentrifugation. After fractionation and DNA recovery, real-time quantitative PCR was performed to quantify the abundance of bacterial 16S rRNA gene in each individual fraction to identify the ^{12}C-DNA and ^{13}C-DNA. (iii) Identification of active denitrifier communities. The bacterial community in the ^{13}C-DNA fractions, ^{12}C-DNA fractions, and soil samples without incubation (original soil, O) were analyzed through 16S rRNA gene sequencing, and functional denitrification genes (*nirS*, *nirK*, and *nosZ*) in the ^{13}C-DNA fractions were quantified using real-time PCR. Detailed methods are presented in the Supplementary Materials and Methods.

3. Results and discussion

The vadose zone samples from all four layers exhibited denitrification activity when carbon and nitrate were added (Fig. 1B). A higher rate of denitrification gas production ($N_2O + N_2$) was observed in the surface soil compared to the deeper layers. It should be noted that nitrate ammonifiers, which perform the process of dissimilatory nitrate reduction to ammonium (DNRA), can compete with denitrifiers for nitrate and carbon. However, analyzing the ammonia concentration before and after incubation indicated that the DNRA process was much less effective than denitrification (Table S2). In addition, a noticeable increase in the rate of denitrification gas production was observed every time after isotope-labeled carbon was added (Fig. 1B), suggesting that the denitrification process in the incubation system was limited by the amount of available carbon, and the isotope-labeled carbon was successfully taken up by the active denitrifiers.

The ^{12}C-DNA and ^{13}C-DNA fractions were identified at buoyant densities of 1.721–1.733 g ml^{-1} and 1.749–1.761 g ml^{-1}, respectively, by quantifying the abundance of the bacterial 16S rRNA gene (Fig. 1C). The microbial community compositions of ^{13}C-DNA fractions were dramatically different from those of ^{12}C-DNA fractions and O samples. Phyla *Actinobacteria*, *Acidobacteria*, *Chloroflexi*, *Gemmatimonadetes*, *Rokubacteria*, *Proteobacteria* and *Firmicutes* were found dominant in most of the layers of the vadose zone samples, as indicated in the composition of O samples (Fig. 2A). Among them, phyla *Proteobacteria* and *Firmicutes* were identified the dominant phyla in the ^{13}C-DNA fractions (Fig. 2A), and the relative abundance of these two phyla was greater than 93 % in all the four layer samples, indicating taxa in these phyla were actively performing denitrification and assimilating isotope-labeled carbon during the incubation. Taxonomic classification results at family and genus level (Fig. 2, Table S3) suggested that the main active denitrifying bacterial taxa were *Pseudomonadaceae* (*Pseudomonas*), *Rhodocyclaceae* (*Azoarcus*), and *Burkholderiaceae* in the surface soil and *Pseudomonadaceae* (*Pseudomonas*), *Burkholderiaceae*, *Bacillaceae* (*Bacillus*), and *Paenibacillaceae* (*Ammoniphilus*) in the deep layers. Many strains of these taxa have been previously identified as denitrifiers, and studies have reported that they possess denitrification genes (Ishii et al., 2010; Verbaendert et al., 2011; Wei et al., 2015; Liu et al., 2019; Hetz and Horn, 2021). A prominent finding was that the family *Pseudomonadaceae* was significantly more abundant, accounting for over 53 % of the total microbial community in the ^{13}C-DNA samples for all four layers. However, its abundance was much lower in the ^{12}C-DNA and O samples, especially in the surface soil where it constituted less than 0.5 %. This indicates that the less dominant or even the rare taxa in the system might have important functions.

During the incubation, the surface soil (0–0.2 m) exhibited a higher potential for nitrous oxide emissions compared to the deeper layers (Fig. 1B). This function change could be linked to shifts in the composition of the active denitrifying community. The ratios of *nosZ*/(*nirS* + *nirK*) and *nirK*/*nirS* in the ^{13}C-DNA exhibited upward trends as soil depth increased (Fig. 2C), suggesting that there is an increase in the proportion of N_2O reducers and *nirK*-type denitrifiers in the deeper layers. Although the denitrification process is also regulated at transcriptional and post-transcriptional levels, these changes could be one of the reasons for the variations in N_2O emission potential across different layers (Fig. 1B).

In summary, dramatic compositional and functional differences were discovered between the surface soil and the deep layers of the vadose zone. The active denitrifiers present in the 9-m soil profile were identified using Stable Isotope Probing (SIP). This study has provided a deeper understanding of the composition and function of denitrifiers in the vadose zone. It is also beneficial for the development of bio-

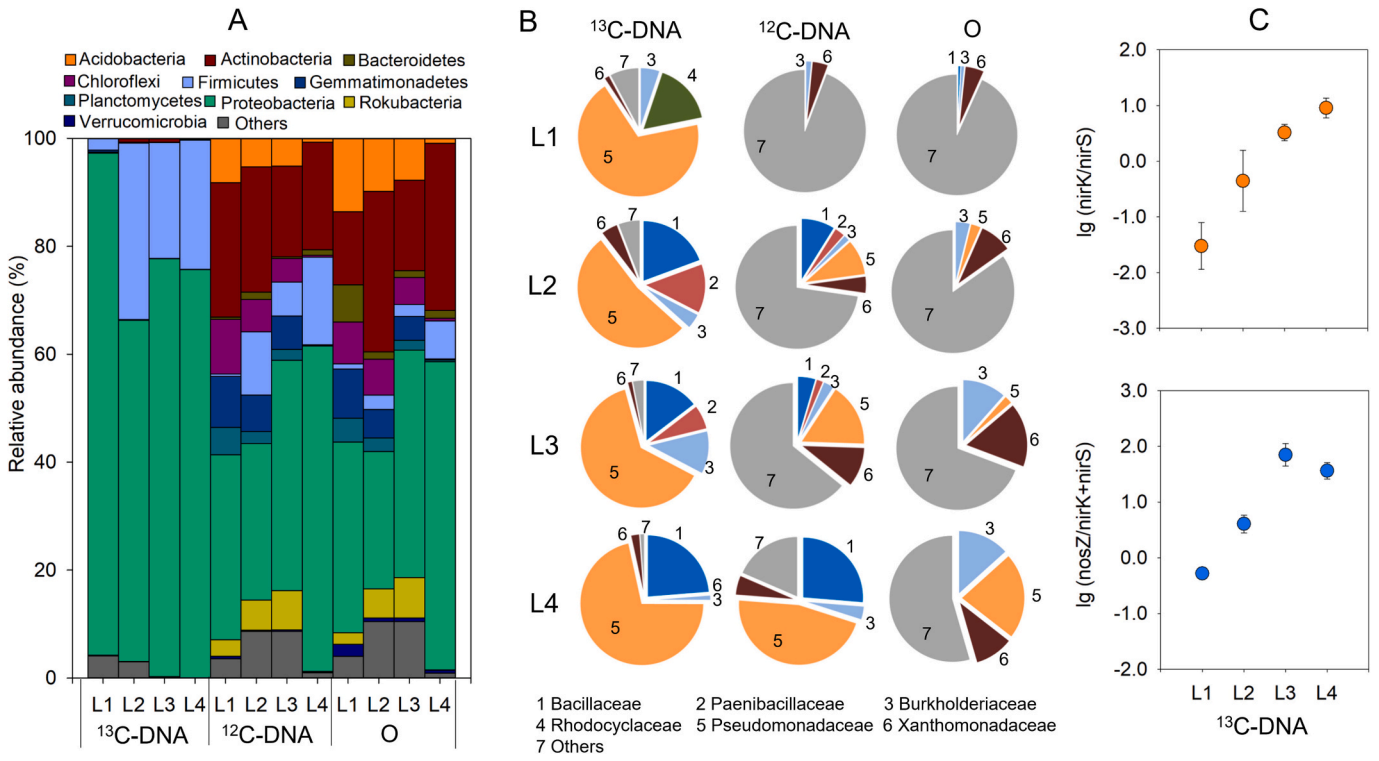

Fig. 2. Bacterial community structure in various layer vadose zone samples at the phylum level (A) and family level (B). Families with relative abundances greater than 1 % were shown, and other families were grouped into Others. Ratios of different functional genes in the ^{13}C-DNA fractions (C). L1: 0–0.2 m; L2: 0.5–1.0 m; L3: 3.0–4.0 m; L4: 7.0–9.0 m; ^{13}C-DNA: ^{13}C-DNA fractions; ^{12}C-DNA: ^{12}C-DNA fractions; O: original sample DNA.

techniques that aim to reduce nitrate leaching to groundwater by enhancing microbial denitrification.

CRediT authorship contribution statement

Shuaimin Chen: Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Eiko E. Kuramae:** Conceptualization, Writing – review & editing. **Zhongjun Jia:** Methodology, Writing – review & editing. **Binbin Liu:** Conceptualization, Writing – original draft, Writing – review & editing, Funding acquisition, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data generated or analyzed during this study are included in this published article and its Supplementary Information files. Sequencing data were deposited in the European Nucleotide Archive under accession number PRJEB55093.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.167968.

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