Organic carbon availability limiting microbial denitrification in the deep vadose zone

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Summary

Microbes in the deep vadose zone play an essential role in the mitigation of nitrate leaching; however, limited information is available on the mechanisms of microbial denitrification due to sampling difficulties. We experimentally studied the factors that affect denitrification in soils collected down to 10.5 meters deep along the soil profile. After an anoxic pre-incubation, denitrification rates moderately increased and the N2O/(N2O+N2) ratios declined while the microbial abundance and diversity did not change significantly in most of the layers. Denitrification rate was significantly enhanced and the abundance of the denitrification genes was simultaneously elevated by the increased availability of organic carbon in all studied layers, to a greater extent in the subsurface layers than in the surface layers, suggesting the severe scarcity of carbon in the deep vadose zone. The genera Pseudomonas and Bacillus, which are made up of a number of species that have been previously identified as denitrifiers in soil, were the major taxa that respond to carbon addition. Overall, our results suggested that the limited denitrification in the deep vadose zone is not because of the lack of denitrifiers, but due to the low abundance of denitrifiers which is caused by low carbon availability.

Introduction

Overuse of chemical nitrogen (N) fertilizer in intensive agricultural areas across the globe has led to serious environmental problems such as nitrate leaching, ammonia volatilization and greenhouse gas emission (Jaynes et al., 2001; Ju et al., 2009). The situation is severe in the China North Plain (CNP), a major farming region that produces over 20% of the national grain output (Li et al., 2007), due to the excessive N fertilization rate (550–600 kg N ha⁻¹ yr⁻¹) and the high rate of N loss through leaching (2.7% and 12.1% of applied N) during the wheat and maize growing seasons (Ju et al., 2009). Leached nitrate from agricultural practices has been demonstrated to accumulate in the subsurface soil, which is more broadly referred to as the vadose zone (Li et al., 2007; Zhou et al., 2016). In the deep vadose zone, denitrification is generally considered a beneficial process that can eliminate accumulated nitrate, and hence is of great ecological importance and has gained considerable attention recently. Although several studies have shown that the vadose soil contains denitrifiers and denitrification activity may occur to several meters deep (less than 2 m) (Paramasivam et al., 1999; Cannavo et al., 2002), there is sparse information about the activity of denitrification enzymes and the corresponding abundance and diversity of the denitrifiers in the deeper vadose zone.



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While the effects of oxygen on denitrification have been well-documented in surface soils, there is a dearth of information on how the oxygen concentration can influence the denitrification and its gaseous products composition in the deep vadose zone. The theories developed to describe the influence of oxygen on denitrification in surface soils may not be applicable to the deep vadose zone due to the dramatic differences in the abundance and diversity of the microorganisms. The water content can strongly affect the oxygen concentration in the soil (Silver et al., 1999), usually the oxygen concentration is unevenly distributed along with the soil profile in the vadose zone. Under natural conditions, due to the changes in soil moisture over time, the oxygen concentration could change dynamically at a time scale of hours to days (Rubol et al., 2012). When oxygen is depleted, facultative bacteria can survive using nitrate as the electron acceptor, which may result in an increased proportion of denitrifiers in the microbial community or an unchanged microbial community with activated or newly synthesized denitrification enzymes. How microorganisms in the vadose zone respond to the change of oxygen concentration is still poorly understood.

Oxygen concentration also strongly affects the composition of the denitrification gases. In an anaerobic microcosm, addition of oxygen resulted in an increased ratio of nitrous oxide to molecular dinitrogen, and this phenomenon was discovered in surface soils (Firestone et al., 1979) and in pure cultures of denitrifiers (Betlach and Tiedje, 1981). In the past several decades, this phenomenon has been rediscovered in a number of studies and has been reviewed (Conrad 1996). Although the mechanism is still not clear, it is not likely due to delayed transcription of N\textsubscript{2}O reductase gene as transcription of the nosZ gene was observed either simultaneously or preceded that of nir genes in batch incubation experiments with either model strains or soils (Bergaust et al., 2010; Liu et al., 2010). The acknowledged explanation is that there are differences in oxygen sensitivities and patterns of regulation of denitrification enzymes synthesis in different denitrifiers. Therefore, when oxygen availability changes in the soil, different effects of the oxygen on the expression of

**Fig. 1.** Schematic diagram of the experimental design of this study.

Two incubation experiments were conducted, the first experiment (Incubation I) was carried out to compare the denitrification activity in N600 and N0 along the soil profile over 12 depths to investigate the effect of carbon addition (C) and anoxic conditions (A) on the original soil (N). The second experiment (Incubation II) was performed using N600 samples from six layers with distinct physico-chemical characters and with four pre-incubation treatments: (i) O: aerobic incubation; (ii) A: anoxic incubation; (iii) OC: aerobic incubation with organic carbon; (iv) AC: anoxic incubation with organic carbon. Molecular analyses were performed only for the Incubation II samples.
denitrification enzymes in various bacteria will determine the composition of the denitrification gases. As nitrification is also an important process which contributes to N$_2$O emission, the alternative explanation is that the increased oxygen in soil can activate nitrifiers, which produce N$_2$O and increase the ratio of N$_2$O to N$_2$. Inspecting the contrasting compositions of microbial communities at different oxygen availability levels in combination with functional analyses is important for understanding the role of the microbial community in response to oxygen concentration fluctuations in the vadose zone.

In the present study, we took soil samples from the surface to the vadose zone at 10.5 meters and performed a series of incubations under strictly controlled conditions to assess the microbial response to organic carbon addition and spells of anoxia. Functional genes abundances and microbial community structure were monitored when the denitrification activities were altered (Fig. 1). The results obtained in this study provide refined information for the understanding of microbial mechanisms of denitrification in the vadose zone, a process that is deemed important for the mitigation of nitrate leaching to groundwater.

**Results**

**Soil characteristics**

The characteristics of the soils were presented in Supporting Information Table S1 (N0) and Supporting Information Table S2 (N600). The pH of the vadose zone soils was 7.7–8.1. Most of the layers were sandy loams comprising 51.8–69.1% sand (> 20 μm), 30.1–49.8% silt (2–20 μm) and 0.3–3.3% clay (<2 μm), except for a sand layer at approximately 4.5–9.0 m, which contained 85.7–92.2% sand. The water contents along the soil profile were roughly correlated to the soil texture and tended to decrease in the coarser textured layers. The lowest water content value was 8.5% and the highest value was 19.8%, which was observed in the sand layer and silt loam layer respectively. The water content did not show a correlation with the soil depth. Soil organic carbon (SOC) decreased significantly with the depth and became less than 2.0 g kg$^{-1}$ with the soil depth. Soil organic carbon (SOC) decreased respectively. The water content did not show a correlation which was observed in the sand layer and silt loam layer content value was 8.5% and the highest value was 19.8%, decrease in the coarser textured layers. The lowest water content was 4.5–9.0 m, which contained 85.7–92.2% sand. The water contents along the soil profile were approximately 4.5–9.0 m, which contained 85.7–92.2% sand. The water contents along the soil profile were roughly correlated to the soil texture and tended to decrease in the coarser textured layers. The lowest water content value was 8.5% and the highest value was 19.8%, which was observed in the sand layer and silt loam layer respectively. The water content did not show a correlation with the soil depth. Soil organic carbon (SOC) decreased dramatically with depth, and became less than 2.0 g kg$^{-1}$ in the layers deeper than 1.5 m, indicating a clear scarcity of the carbon in the deep vadose zone. The concentrations of NH$_4^+$-N in most of the layers were below the detection limit except for surface soils, where the concentration was 0.73 ± 0.11 μg g$^{-1}$ and 1.21 ± 0.13 μg g$^{-1}$ in N0 and N600 respectively. The NO$_3^-$-N concentrations in all layers were dramatically higher in the field where 600 kg N ha$^{-1}$ yr$^{-1}$ was applied (N600) than in the control where no fertilizer was applied (N0) (Fig. 2), indicating that long-term fertilization had caused serious nitrate accumulation not only in the surface soil but also in the deep vadose zone. For N0, the highest NO$_3^-$-N concentration was 7.50 μg g$^{-1}$ in the surface soil (0–0.2 m) and remained at 3.14–4.06 μg g$^{-1}$ in 0.2–10.5 m. For N600, the NO$_3^-$-N concentration was 62.4 μg g$^{-1}$ in the surface soil, declined sharply to 27.26 μg g$^{-1}$ in layers at 0.5–1.0 m and remained at moderately high concentration (34.62–50.39 μg g$^{-1}$) in the layers above 4.5 m before dropping down to 17.4 μg g$^{-1}$ in the sand layer.

**Effect of carbon availability and oxygen concentration on denitrification**

For the first experiment (Incubation I), only the anoxic treatment (A) was pre-incubated for 5 days while for Incubation II all four treatments (A, O, OC and AC) were pre-incubated for 5 days prior to denitrification measurements. In Incubation I, the denitrification rates in the samples with either carbon addition (C) or anoxic incubation (A) were significantly increased as compared with the soil samples without any pre-treatment (N) except in the layers below 1 m of N0 (Fig. 2). The surface soil (0–0.2 m) had the highest denitrification rates (4.28 μg N g$^{-1}$ day$^{-1}$ for N0 and 5.73 μg N g$^{-1}$ day$^{-1}$ for N600), which increased to 7.23 μg N g$^{-1}$ day$^{-1}$ for N0 and 16.96 μg N g$^{-1}$ day$^{-1}$ for N600 by carbon addition and 8.27 μg N g$^{-1}$ day$^{-1}$ for N0 and 15.18 μg N g$^{-1}$ day$^{-1}$ for N600 after anoxic incubation. The influences of both carbon addition and anoxic incubation were much weaker in the deeper layers. The denitrification rates declined quickly with the depth and stabilized at > 1 m depth for both N600 and N0 (Fig. 2).
The denitrification rates and the product ratios of \( \frac{\text{N}_2\text{O}}{(\text{N}_2\text{O} + \text{N}_2)} \) in the 6 layers of N600 from Incubation II are presented in Fig. 3. Although the denitrification rate was highly variable with depth, it was significantly lower (\(< 20 \mu \text{g N g}^{-1} \text{ day}^{-1}\)) in all the treatments without carbon addition (N, O and A), compared with the organic carbon treatments (OC and AC). Anoxic incubation (A) moderately elevated the denitrification rates and decreased the ratios of \( \frac{\text{N}_2\text{O}}{(\text{N}_2\text{O} + \text{N}_2)} \), especially in the surface layers (Fig. 3), compared with N and O. The dramatically increased denitrification rates in OC and AC were closer or even higher in the deep layers than in the surface layer. Much lower denitrification product ratios (\( \frac{\text{N}_2\text{O}}{(\text{N}_2\text{O} + \text{N}_2)} \)) were observed in the layers of 3.0–4.0 m, 7.0–9.0 m and 9.5–10.5 m in AC than in OC, indicating the functional \( \text{N}_2\text{O} \) reductase in the deep layers was successfully upregulated under anoxic conditions.

### Functional genes and 16S rRNA gene quantification

The abundance of the functional genes and 16S rRNA gene were estimated with qPCR for all the treatments in Incubation II to investigate how carbon addition and anoxic incubation influenced the total bacteria and denitrifiers abundance. The amplification efficiencies of qPCR ranged from 85% to 95%. The \( R^2 \) values of the standard curves were more than 0.996 and the melting curves had single peaks in all reactions. The copy numbers of the 16S rRNA gene and the functional genes \( \text{nosZ} \), \( \text{nirS} \) and \( \text{nirK} \) are showed in Supporting Information Fig. S1. The 16S rRNA gene copy numbers, which represent the total number of bacteria, increased approximately 2.0 times in the samples with carbon addition (OC and AC) compared with the original soil N from the surface layer (0–0.2 m). For the deep layers (1.5–10.5 m), the 16S rRNA gene copy numbers were increased 4.2–8.2 times, except in sandy layer (7.0–9.0 m) where a relatively high copy number was found before carbon addition. The abundance of denitrification genes significantly increased in most of the layers with organic carbon addition (AC and OC) (Supporting Information Fig. S1) as compared with control (N). The relative abundances (normalized to total 16S rRNA gene copies) of the functional genes were calculated and shown in Fig. 4A–C. Carbon addition increased the relative abundance of \( \text{nosZ} \) gene in several layers, suggesting the proportion of the microorganisms harboring this gene was elevated. Higher \( \text{nos:nir} \) ratios [\( \text{nosZ}/(\text{nirS} + \text{nirK}) \)] were observed in AC than OC in all the layers (Fig. 4D), indicating that microbes harboring the \( \text{nosZ} \) gene might be enriched faster over the other denitrifiers under anoxic conditions, which could be one reason, among the other reasons at transcriptional and posttranscriptional levels, for the low \( \frac{\text{N}_2\text{O}}{(\text{N}_2\text{O} + \text{N}_2)} \) ratios in the anoxic treatments.

### Microbial community adaptation to carbon addition and anoxic incubation

The soil samples from Incubation II were analysed for the microbial community shifts from the four treatments (O, A, OC and AC) compared with N. Sequencing of the V3-V4 hypervariable region of the 16S rRNA gene generated approximately 4.1 million paired reads (approximately 46 k
Of those, 22.2% was discarded during the assembly of the paired reads, quality control and chimera detection steps (Supporting Information Table S3). The remaining fragments were subjected to the QIIME pipeline, and an average of 1962 operational taxonomic units (OTU) per sample was discovered.

Regarding the phylum level composition, Actinobacteria and Proteobacteria were dominant phyla in the original soils (N) for all 6 layers (Fig. 5). Actinobacteria, which favours aerobic conditions (Hamamura et al., 2006), had a relative abundance of 9.9–28.2%, except in the sand layer (7.0–9.0 m), which was 50.3%. The relative abundance of Proteobacteria was 26.8–45.1%, which was also found to be the dominant phylum in other Chinese subsoils (Li et al., 2014). Bacteroidetes was also present in all layers, but with lower abundance (<9.0%). Acidobacteria, Chloroflexi, Gemmatimonadetes, Nitrospirae and Planctomycetes mainly inhabited the layers of 0–0.2 m and 0.5–1.0 m (<8.2%). Firmicutes mainly inhabited in layers of 1.5–2.0 m, 3.0–4.0 m, 7.0–9.0 m and 9.5–10.5 m (5.0–35.0%). Carbon addition (OC and AC) increased the relative abundance of Proteobacteria. The relative abundance of Proteobacteria was 26.2–52.4% in N, O and A, and increased to 45.4–68.5% in OC and 50.9–74.8% in AC.

At genus level, for samples in layers 0–0.2 m and 0.5–1.0 m, approximately 83.0% of the sequences from N, O and A, and 50.5% of the sequences from OC and AC were not identified, and an average of 25.0% of the sequences was not identified in the rest of soil samples. Seventeen abundant (>0.5%) bacterial genera were listed in Fig. 6. *Ammoniphilus, Nitrospira, Cupriavidus, Rhodoplanes* and *Kaistobacter* mainly existed in the upper two layers. *Rhodococcus, Enterococcus, Lactococcus, Acinetobacter, Methylotenera* and *Stenotrophomonas* mainly presented in the deeper four layers. Carbon addition obviously increased the relative abundance of *Pseudomonas* and *Bacillus* in all layers. In the original soil (N), the relative abundance of *Pseudomonas* was 1.0–21.3%, which increased to 21.3–54.0% in OC and 27.2–60.5% in AC. The relative abundance of *Bacillus* was 0.5–6.2% in N and increased to 0.7–18.7% in OC and 2.4–18.3% in AC.

Shannon-Wiener indices were calculated for the six layer samples from all the treatments in Incubation II, the microbial diversity declined with soil depth and also declined (significantly in most layers) in the organic carbon treatments (OC and AC) compared with treatments without carbon addition (A, O and N) (Fig. 7A). Principal Coordinate Analysis (PCoA) was performed within the QIIME
pipeline with the Bray-Curtis distance of the OTUs from the six layer samples of all the treatments in Incubation II (Fig. 7B). The samples from treatments without carbon addition (N, O and A) clustered together in most of the layers, suggesting that the community structure was not dramatically altered by either anoxic or aerobic incubation without carbon addition. Pairwise PERMANOVA analysis also indicated that, in most of the layers, treatments with carbon addition (AC and OC) changed the community structure significantly \( (p < 0.05) \) compared with the treatments without carbon addition (A and O) and the control soils (N). The treatments without carbon addition (A and O) in most of the layers did not significantly alter the microbial community structure when compared with the control sample (N) (Supporting Information Table S4).

**Discussion**

In this study, shifts in denitrification rates, microbial community structure and functional genes abundances in response to carbon addition or reduced oxygen concentration were investigated along the soil profile in the deep vadose zone. A number of recent studies have investigated denitrification in the vadose zone due to its importance in attenuating nitrate, which may leach into the groundwater. These studies found that carbon amendment significantly promoted denitrification in the subsoil either in lab incubation (Jahangir et al., 2012; Peterson et al., 2013) or in field experiment (Qin et al., 2017). The microbial community composition has been investigated down to 2 meters using phospholipid fatty acid (PLFA) analysis (Fierer et al., 2003; Allison et al., 2007) and the relationship of denitrification gases flux and denitrification genes abundances was reported in three pedological depths (Barrett et al., 2016). However, due to sampling difficulties, few studies have focused on how the denitrifiers abundance and the microbial community composition respond to the factors affecting denitrification rates in even deeper layers. To the best of our knowledge, this is the first study to investigate the factors affecting denitrification rates and the corresponding microbial community responses down to 10.5 meters in depth in the vadose zone in Chinese farmland soils.

Although the denitrification activity increased in all the layers by approximately 2.58 to 6.12 times after anoxic incubation (A) compared with the control N (Fig. 3), the denitrification genes abundances (Supporting Information Fig. S1) and microbial community composition did not change dramatically in most of the layers, as shown by the phylogenetic composition at the phylum (Fig. 5), order (Supporting Information Fig. S2) and family (Supporting Information Fig. S3) levels, and the microbial diversity at OTU level (Fig. 7). The carbon availability in the investigated samples, especially in the deep layers was limited, and the growth rate of microorganisms under substrate-
Fig. 6. The relative abundances of the bacterial genus in the original soils and the samples after pre-incubations in Incubation II. Seventeen abundant (>0.5%) bacterial genera from the six investigated layers are shown. The area indicates the percentage of the relative abundance of each bacterial genus. N, original soil; O, aerobic incubation; A, anoxic incubation; OC, aerobic incubation with organic carbon; AC, anoxic incubation with organic carbon.
limited condition is determined by the low concentration of the single nutrient (Boer et al., 2003). The soil organic carbon in the studied samples was 11.6 g kg\(^{-1}\) in 0–0.2 m, 3.6 g kg\(^{-1}\) in 0.5–1.0 m and less than 1.8 g kg\(^{-1}\) in the other four deep layers (Supporting Information Table S2). Therefore, for treatment A and O, the growth rates of the microbes were mainly controlled by the limited electron donors (carbon source) instead of the electron acceptors (oxygen or nitrate). Additionally, the similar community structures between A and O suggested that most of the microorganisms that can use oxygen as an electron acceptor can switch to heterotrophic denitrification and use nitrate as the electron acceptor. The higher denitrification rates in A than in O were most likely achieved by enhanced transcription and protein assembly processes instead of by augmentation of the denitrifier populations. This notion is also supported by the much lower variations of the functional gene abundance between A and O compared with the AC or OC (Supporting Information Fig. S1).

Anoxic treatments (A and AC) in Incubation II resulted in lower \(N_2O/(N_2O + N_2)\) ratios in most of the layers compared with the oxic treatments (O and OC), confirming that oxygen concentration could be a major regulator for the synthesis of denitrification enzymes (Tiedje, 1988). Dramatically higher denitrification rates in AC and OC compared with the treatment without carbon (Fig. 3) indicated that carbon addition had resulted in de novo synthesis of the denitrification enzymes during the pre-incubation period. The denitrification enzymes induction in OC could be due to the development of anoxic microsites (B. Parkin, 1987). Distinct \(N_2O/(N_2O + N_2)\) ratios were found between upper and lower layers in OC, suggesting differential induction in relative \(N_2O\) reductase activity during oxic pre-incubation. The gene quantification could provide clues to the molecular mechanisms behind this phenomenon. The increment of abundance of \(nir\) and \(nos\) genes was much higher in the lower three layers than in the upper three layers (Supporting Information Fig. S1) during the pre-incubation, suggesting the microbes grown in the lower three layers had successfully developed enzymes for \(N_2O\) production (i.e., NAR, NIR and NOR), but almost completely failed to develop functional \(N_2OR\) enzyme. The remarkably lower \(N_2O/(N_2O + N_2)\) ratio in AC from same layers could suggest the malfunctioning of \(N_2OR\) in the lower three layers in OC was due to oxygen inhibition, probably at transcriptional or posttranslational levels (Bergaust et al., 2010, Bakken et al., 2012).

The abundances of the denitrification genes and the 16S rRNA gene increased after incubation with organic carbon (Supporting Information Fig. S1). The addition of glutamate increased the 16S rRNA gene copy number about 4–8 times in the deeper layers, while it increased only two times in the surface layer (0–0.2 m). The reason could be that microorganisms under low substrate conditions could incorporation substrate into the biomass constituents in a more efficient way (Boer et al., 2003; Allison et al., 2007; Sagova-Mareckova et al., 2016). These microbes could grow faster and end up with similar or even high microbial abundance when exposed to the adequate carbon sources. Higher \(nos:nir\) ratios were detected in AC than in OC (Fig. 4). As the nitrous oxide reductase is more sensitive to oxygen than the other denitrification enzymes (Knowles, 1982), we hypothesize that the microbes harboring \(nzs\) gene (express \(N_2O\) reductase) could obtain energy by reducing \(N_2O\) and grow faster in AC than in OC treatment. But gene transcription and translation were not investigated in this study, so further work is required to test this hypothesis in the future.

Sequencing analysis revealed that relative abundance of \textit{Pseudomonas} and \textit{Bacillus} responded quickly to the carbon addition, suggesting their growth were constrained by substrate starvation in soil, especially in deeper layers. A number of species in these genera have been reported as denitrifiers (Verbaendert et al., 2011; Salles et al., 2012; Pajares and Bohannan, 2016). The augmentation of the same taxa in AC and OC (Fig. 6) suggests that the facultative aerobic heterotrophic bacteria, like the species from
genera *Pseudomonas* and *Bacillus*, were the potentially active denitrifiers in the deep vadose zone.

Through the addition of carbon and incubation either aerobically or anaerobically (Incubation II, OC and AC), dramatically higher denitrification rates were achieved compared with the treatments without organic carbon addition (N, O and A). Anoxic incubation without carbon addition (A) also increased denitrification rates, but they were much lower than that from AC/OC, especially in the deep layers (Fig. 3). These results suggest that, although oxygen concentration can influence the denitrification in the vadose zone to a certain extent, the main reason for the low denitrification rates was a lack of organic carbon. Previous studies also reported the scarcity of carbon as the main reason for limited denitrification in subsoils (McCarty and Bremner, 1992; Devito et al., 2000; Murray et al., 2004) and had been previously reviewed (Holden and Fierer, 2005). If measured immediately after carbon addition (Incubation I), the denitrification rates also increased, but the increase was much lower than in AC and OC in Incubation II, suggesting a low microbial abundance in the original soils. An increase in denitrification concurrent with an increase in denitrification genes abundance in AC and OC suggested that the limited denitrification is not due to a lack of denitrifying microorganisms, but to the low denitrifier abundance which is caused by the low available organic carbon. This conclusion is in agreement with previous subsoil studies in Iowa, USA (McCarty and Bremner, 1992; 1993).

**Conclusion**

Mitigation of leached nitrate through microbial denitrification in the vadose zone has gained increasing attention recently, especially in agricultural lands where nitrogen fertilizer was used intensively. In this study, we found that nitrate was accumulated along the soil profile in plots with high N-fertilizer input (600 kg N ha\(^{-1}\) yr\(^{-1}\), N600) compared with the control plots (0 kg N ha\(^{-1}\) yr\(^{-1}\), N00). Anoxic incubations could promote the denitrification rates in both the surface and the deep vadose zone soils. But the abundance of 16S rRNA gene and denitrification genes and the microbial community structure were not significantly changed in most of the layers. A possible explanation for this phenomenon is that the microbial community responds to the spell of anoxia by developing new denitrification enzymes instead of by altering the community structure. Carbon addition significantly promoted the denitrification rates in all layers, concomitant with the increase in the abundance of denitrification genes. Genera *Bacillus* and *Pseudomonas* were enriched during these processes and were likely the main facultative denitrifiers contributing to denitrification under anoxic conditions. Taken together, our results showed that although oxygen concentration can affect the denitrification, especially the composition of the denitrification gases, the influence is trivial compared with the effects of carbon availability. The major reason for the limited denitrification in the deep vadose zone is the low abundance of denitrifiers which is caused by carbon scarcity.

**Experimental procedures**

**Soil sampling**

Soils were sampled from a long-term fertilization field experiment in the Luancheng Argo-ecosystem Experimental Station in the North China Plain (37°53’ N, 114°41’ E, elevation 50 m). This area is a major grain production area in China. The field experiment was established in 1998 using a range of 0–600 kg N ha\(^{-1}\) yr\(^{-1}\) nitrogen fertilization intensities in triplicate plots (Qin et al., 2012). A high amount fertilizer treatment with 600 kg N ha\(^{-1}\) yr\(^{-1}\) (N600) and a control treatment with 0 kg N ha\(^{-1}\) yr\(^{-1}\) (N0) were chosen for this study. Nitrate in N600 was accumulated along the soil profile in the vadose zone (Li et al., 2007; Zhou et al., 2016). Samples from 0 to 10.5 m in depth were taken sequentially with a drill-rig (Geoprobe 54DT, USA) that collected intact cores using polyvinylchloride columns measuring 43 mm in diameter and 1.2 m in length. The samples under 1.2 m were taken by adding multiple extension steel bars to the drill-rig. The 12 layers were separated based on the soil texture, as the soil organic carbon decreases dramatically with the depth at 0–1 m (Dong et al., 2016), more layers were separated in this depth as shown in Fig. 1. Triplicate sampling sites were randomly chosen in each plot, and the soil samples were transported in icebox to the laboratory and stored at 4°C.

**Soil physical and chemical analyses**

Soil water content was determined by oven drying the samples at 105°C for 12 h. Soil pH was measured at a soil: water ratio of 1:5 using a pH meter (PHS-3C, Shanghai INESA). Soil nitrate was extracted with 1M KCl solution (extracting ratio, 1:5 w/v) and the NO\(_3\)-N concentration was measured by a dual-wavelength ultraviolet spectrophotometer (UV-2450, SHIMADZU). The concentration of NH\(_4\)\(^+\)-N was measured by indophenol blue spectrophotometer (625 nm) method. Soil organic carbon was measured using K\(_2\)Cr\(_2\)O\(_7\)-H\(_2\)SO\(_4\) oxidation method (Nelson and Sommers, 1982). Soil particle size was determined using a laser particle analyser (Mastersizer 3000, Malvern).

**Denitrification rates measurement**

Two incubation experiments were conducted in this study, as illustrated in Fig. 1. Denitrification rates were calculated based on the sum of N\(_2\)O and N\(_2\) accumulated during the measurement period. The first experiment (Incubation I) was performed to compare the denitrification activity in N600 and N0 and to determine to what extent carbon addition (glutamate sodium) or anoxic incubation improve the soil denitrification rate along the 12-layer of soil. Glutamate was chosen as a representative of leached organic carbon.
because it is one of the most abundant amino acids in soil (Forde and Lea, 2007), generated from plant litter degradation and root exudation (Paynel et al., 2001), and is a central molecule in amino acid metabolism in plants (Forde and Lea, 2007). Three sets of soil samples (N, A and C) were prepared. Triplicate soil samples (10 g wet weight) from each layer without any pre-treatment (N) were transferred to 120 ml serum vials and sealed with butyl-rubber septa and aluminium caps. For testing the carbon effect, one more set of soil samples was prepared and glutamate sodium was added to a concentration of 300 μg C g⁻¹ soil (C). Using a concentration of 300 μg C g⁻¹ was based on a preliminary experiment where a range of glutamate sodium concentrations were tested for promoting denitrification, and the denitrification rate positively correlated with the concentration when it was less than 300 μg C g⁻¹, but not changed when the concentration was greater than 300 μg C g⁻¹. In order to inspect the effect of anoxic spills in the vadose zone on denitrification, another set of vials was prepared and incubated anaerobically for 5 days before denitrification rates measurement (A). To measure the denitrification rate, the vials were evacuated (0.1 kPa) and flushed with helium (99.999%, 101.3 kPa) four times to make an anoxic atmosphere and displace N₂ in the vials. Overpressure in the vials was released by a 5 ml syringe filled with 2 ml distilled water to monitor gas release. A preliminary experiment showed that the N₂O and N₂ produced from many samples (mostly deep layers from N0) are under or close to the detection limit of the GC. Therefore, 50 μg N KNO₃ g⁻¹ soil was added to each vial containing 10 g (wt) of soil to reveal the variance of denitrification enzyme activity along the soil profile and between the treatments. Nitrous oxide and nitrogen gases were monitored with a robotized incubation system. All incubation experiments were done at 18°C, which was controlled by a thermostatic water bath. Headspace gases in the vials were sampled every 6 h through an autosampler (Gilion Model 222, LeBel, France) and guided with a peristaltic pump (Gilion Minipuls 3, LeBel, France) to a gas chromatograph (GC, Agilent 7890A). The GC measured the concentration of N₂O and an ECD detector and N₂, CO₂ and O₂ with a TCD detector. The details of this system and routines for calculating net gas production/consumption were described above (Molstad et al., 2007). Denitrification rates were calculated with the data collected at the first 36 h of incubation where the production rates of N₂O and N₂ were fairly constant. In this study, the denitrification rate was defined as the sum of N₂O and N₂ emission rates, if not stated otherwise, this refers to the potential denitrification rate as the nitrate was added in order for the production of N₂ to be above the detection limit of the instrument.

As nitrate accumulated was only observed in the samples from N600 (Fig. 2) which is necessary for enhanced denitrification, six layers were selected, according to the physico-chemical characters including SOC, nitrate and soil texture as showed in Supporting Information Table S2, for a refined test of the effect of carbon availability and anoxic incubation on the denitrification and the response of the microbial community (Incubation II). Four different pre-incubation treatments were designed for all six layers of samples: (i) O: aerobic incubation; (ii) A: anoxic incubation; (iii) OC: aerobic incubation with 300 μg C glutamate sodium g⁻¹ soil; and (iv) AC: anoxic incubation with 300 μg C glutamate sodium g⁻¹ soil. All samples were incubated at 18°C in a thermostatic chamber for 5 days. Then, the gases in the headspace were replaced with helium and the denitrification rates were measured using the method described above. The soils before (N) and after incubations for each treatment (O, A, OC and AC) from the six depth layers (Incubation II) were subjected to molecular analyses.

**Soil DNA extraction and quantitative PCR**

Soil samples described above (O, A, OC and AC) and the initial soil (N) from Incubation II were selected for functional genes quantification and microbial community structure analyses through 16S rRNA gene sequencing. Soil total DNA was extracted using E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Norcross, GA) according to the manufacturer’s instructions. The quality and quantity of the extracted DNA was examined with a Nanodrop spectrophotometer (Nanodrop ND-2000c Technologies, Wilmington, DE) and visually checked on agarose gel (1%). Extracted DNA was stored at −20°C until further analysis.

Denitrification genes (nirK, nirS and nosZ) and 16S rRNA gene were quantified using primers as followed: F1aCu: R3Cu for nirK (Hallin and Lindgren, 1999), cd3aF:R3cd for nirS (Michotey et al., 2000; Throbäck et al., 2004); nosZ-F:nosZ-1622R for nosZ (Kloost, et al., 2001; Throbäck et al., 2004). Standard curves were constructed using a 10-fold series dilution of the plasmids for seven gradients carrying the respective target genes. Quantitative PCR (Q-PCR) was carried out in a volume of 25 μl, containing 2 × SYBR Premix Ex Taq (Takara Biotech, Dalian, China), 1 μM of each primer and template DNA. For samples from layers deeper than 1 meter, DNA concentrations were below the detection limit of Nanodrop and invisible on the agarose gel, 16S rRNA gene was amplified to confirm the presence of DNA while having a negative control where no DNA was added. For samples from the top two layers, the DNA was diluted to a final concentration of 20 ng μl⁻¹ and 1 μl was used as the template. For the other four layers, 2.5 μl of DNA was used due to the low concentration. After running Q-PCR, the copy numbers were normalized to copies per gram of soil based on the dilution rates and the volumes of the template DNA. The Q-PCR program consisted of an initial cycle of 95°C for 2 min; 40 cycles of 30 s at 95°C for denaturation, 40 s for annealing (57°C for nirK, 58.6°C for nirS and 59°C for nosZ), 30 s at 72°C for extension and 10 s at 85°C for collection of the fluorescent signals. Melting curves were generated with continuous fluorescence acquisition from 57 to 95°C at a rate of 0.5°C per 10 s. For the bacterial 16S rRNA gene, the primer pair 1369F and 1492R and the probe TM1389F were used. In a final 25 μl volume, reaction mixtures contained 2 × premix Ex Taq™ (Takara Biotech, Dalian, China), 2 μM of each primer, 3 μM of probe TM1389F and DNA template as described above. The reaction conditions were an initial cycle of 95°C for 2 min and 40 cycles of 15 s at 95°C and 60 s at 56°C (Suzuki et al., 2000).

**Amplicon sequencing**

Microbial community structures were analysed through sequencing of the 16S rRNA gene from the initial soil (N) and samples after the 4 different pre-incubations (O, A, OC and
AC) from Incubation II to investigate how the microbial communities respond to carbon addition or anoxic incubation. PCR was performed using primers targeting V3 and V4 regions with overhang adapters attached (FwOvAd-341F: TC GTCCGCGAGCTCAGATGTATAAGAGACAGCCCTACGGG NGGCWGCA; ReOvAd-785R: GTCTCGTGGGCTCGAGA GTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) (Yasir et al., 2015). In a 25 µl volume, reaction mixtures contained 2 × premix Ex Taq™ (Takara Biotechnology, Dalian, China), 5 µl of each primer and DNA template was the same as denitrification genes described above. The reaction conditions were an initial cycle of 95°C for 3 min; 23 cycles of 30 s at 95°C, 30 s at 55°C and 30 s at 72°C and a final extension at 72°C for 10 min. The PCR products were visualized on agarose gel to confirm a successful amplification and then purified with AMPure XP beads (Beckman Coulter Inc, Brea, CA) to remove residual primers and primer dimers following the manufacturer’s protocol. Then, using a subsequent eight-cycle PCR, Illumina sequencing adapters and dual-index barcodes were added to each amplicon. After purification on AMPure beads, the libraries were then normalized according to the Nextera XT (Illumina) protocol. The pooled samples were sent to Shanghai Jiao Tong University, Shanghai, China, and sequenced on a MiSeq platform (Illumina, San Diego, CA, USA).

Bioinformatics analysis

Raw sequence data were processed with our own custom shell script which combined several other software packages. The reads from both ends of the PCR product were merged using FLASH (version 1.2.11) (Magocˇ and Salzberg, 2011) with the default settings except the maximum overlap length was set to 170. Merged sequences were filtered by the fastx-toolkit (http://hannonlab.cshl.edu/fastx_toolkit/), and only the sequences with more than 80% of the bases that had quality scores higher than 20 were kept. The quality of the sequences was examined with fastQC program (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Sequences with ambiguous bases (N) were discarded for further analysis. The sequences with length outside 414–506 bp (460% ambiguous bases (N) were discarded for further analysis. The sequences with length outside 414–506 bp (460% ambiguous bases (N) were discarded for further analysis. The sequences were renamed and pooled in one file, which was then input into the Quantitative Insights Into Microbial Ecosystems (QIME) software suite (Caporaso et al., 2010). OTU classification and taxonomy assignment were performed using the subsampled open-reference workflow with default settings and uclust (Edgar, 2010) as the OTU-picking method. The Greengenes database (DeSantis et al., 2006) was used as the reference database and the default cutoff (97%) was used. Sequencing data were deposited in the European Nucleotide Archive under the accession number PRJEB19668.

Statistical analyses

The denitrification rate and gene copies were calculated on a dry soil weight basis. Statistical analyses of the gene copies were conducted with the IBM SPSS Statistics 20.0 for Windows (SPSS, USA). Analysis of variance (ANOVA) and least significant difference (LSD) analysis were performed to assess the significant effects of depth on the denitrification rate, and the different treatments on the abundance of denitrification genes and 16S rRNA gene. Significant differences in microbial community structure between treatments in each layer were determined with PERMANOVA (Anderson, 2001; Hell et al., 2013) using PRIMER6 and PERMANOVA+ (version 6.1.18 and version 1.0.8).

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Supporting information
Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. The abundance of the functional genes and the 16S rRNA genes in soil samples from Incubation II. N, original soil; O, aerobic incubation; A, anoxic incubation; OC, aerobic incubation with organic carbon; AC, anoxic incubation with organic carbon. Error bars indicate the standard deviation of the three replicates. Letters indicate the significant difference between treatments in each layer.

Fig. S2. The relative abundances of bacterial order of the 6 layers from treatments in Incubation II. N, original soil; O, aerobic incubation; A, anoxic incubation; OC, aerobic incubation with organic carbon; AC, anoxic incubation with organic carbon.

Fig. S3. The relative abundances of bacterial family of the 6 layers from treatments in Incubation II. N, original soil; O, aerobic incubation; A, anoxic incubation; OC, aerobic incubation with organic carbon; AC, anoxic incubation with organic carbon.

Table S1. Characteristics of the 12 layers of soil in N0
Table S2. Characteristics of the 12 layers of soil in N600
Table S3. Number of read sequences from the 6 layers of soil of each treatment (N, O, A, OC and AC) in Incubation II obtained by high throughput sequencing of V3-V4 hypervariable regions of prokaryotic 16S rRNA gene.
Table S4. Pairwise PERMANOVA comparisons of microbial community structures across the treatments in each layer.