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ENVIRONMENTAL BIOTECHNOLOGY

Effects of the natural restoration time of abandoned farmland in a semiarid region on the soil denitrification rates and abundance and community structure of denitrifying bacteria

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Abstract

Denitrification accounts for the production of mobile forms of nitrogen (N) for plant uptake, N leaching, and gaseous losses. However, few studies have investigated the potential effects of the natural restoration age on denitrification rates and denitrifying microorganisms, especially in fragile ecosystems in semiarid regions. The potential N gas (N_2O and N_2) emissions and denitrification rates significantly decreased after abandonment (< 9 years) compared to those of active farmland and then steadily increased as the restoration proceeded, leading to an enhanced soil N loss. The total bacterial and *napA* gene abundances significantly decreased after abandonment (< 9 years) compared to that of farmland and then significantly increased as the restoration proceeded. The abundances of the *narG*, *nirK*, *nirS*, *qnorB*, and *nosZ* genes steadily increased with the restoration age of abandoned farmland. The community compositions of denitrifying bacteria exhibited different fluctuating patterns, suggesting different response patterns of community traits of N gas emission–related functional guilds to the restoration age of abandoned farmland. Changes in N gas emissions and in the abundance and diversity of denitrifying microorganisms exhibited similar patterns, suggesting an increased population and diversity of denitrifying bacteria are responsible for the enhanced N gas emissions. We observed clear patterns of plant coverage and denitrifying microorganisms that were associated with increases in the organic C, NH_4^+-N, and NO_3^--N contents and decreases in the soil bulk density as well as increases in the abundance and diversity of denitrifiers with the restoration age of abandoned farmland that were linked to an increase in N gas emissions. It is therefore recommended that effective measures (i.e., modest levels of grazing) may be able to be undertaken to assist with decreasing greenhouse gas nitrous oxide (N_2O) and N loss after 32 years of farmland abandonment.

Keywords Denitrification · Nitrogen lose · Denitrifying microorganism · Abandoned farmland · Natural restoration · Fragile ecosystems

Introduction

Restoring the vegetation of abandoned farmland is an important and effective engineering approach for preventing soil erosion and the restoration of fragile ecosystems, especially in semiarid regions (Barber et al. 2017; Lozano et al. 2014). Vegetation restoration in terrestrial ecosystems is most commonly limited by a shortage of nitrogen (N) (LeBauer and Treseder 2008; Stiles et al. 2017). Denitrification is an important process in soil N loss and nitrous oxide (N_2O) emissions driven by microorganisms associated with soil systems (Petersen et al. 2012). The availability of N can directly constrain net primary production and reshape the composition and diversity of plant species (Stiles et al. 2017; LeBauer and Treseder 2008). However, little is known regarding the potential effects of the natural restoration of abandoned farmland on denitrification and denitrifying microorganisms, especially in the degraded ecosystems of arid to semiarid regions, where N availability is often the limiting factor for ecosystem restoration.

Denitrification is the sequential reduction of soluble nitrate (NO_3^--N → NO_2^--N → NO→N_2O → N_2).

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These various N-related processes involve several functional genes (Megumi et al. 2009; Levy-Booth et al. 2014), which can be used in a functional gene approach to quantify these groups of functionally similar organisms (Canfield et al. 2010). The denitrification process involves several functional genes, including *napA*, *narG*, *nirK*, *nirS*, *qnorB*, and *nosZ* (Canfield et al. 2010). These six functional genes have been commonly adopted as functional markers to investigate the denitrification process, allowing the abundance and community composition of denitrifying bacteria to be ascertained using quantitative polymerase chain reaction (qPCR) and high-throughput sequencing technologies (Saarenheimo et al. 2014; Herrmann et al. 2017). Denitrifiers are influenced by changes in plant communities and by edaphic factors (Tang et al. 2016; Petersen et al. 2012). For instance, recent studies have reported that the most common *nirK* and *nosZ* sequences matched best with proteobacteria, a group of bacteria that may be responsible for denitrification in boreal lake sediments (Saarenheimo et al. 2014). Petersen et al. (2012) reported that significant differences in the abundance of denitrifying bacteria were observed between emergent fen sites and black spruce across a vegetation gradient in Alaska. Management practices (i.e., continuous grazing, mowing, and periodic fencing) can increase the abundance of denitrifying bacteria and stimulate the denitrification process in semiarid grassland ecosystems (Pan et al. 2016). Vegetation and soil characteristics have also been shown to account for different shifts in denitrifying bacterial abundance (Tang et al. 2016; Saarenheimo et al. 2014). However, the degree to which different denitrifying functional genes are controlled by various environmental factors in the fragile ecosystems of semiarid regions is not well characterized, and an understanding of the quantitative relationship between denitrifying genes and denitrification rates ($N_2O + N_2$) is still lacking. The lack of a quantitative link or an empirical relationship between denitrification rates and abundances of functional genes of denitrifying microorganisms limits our ability to take appropriate measures to decrease N loss and N_2O emissions and to maintain the biodiversity, ecosystem service functions, and stability of the vegetation community.

The Chinese Loess Plateau (approximately 640,000 km^2), a temperate arid to the semiarid region, is a traditional agricultural region that suffers from water and wind erosion caused by severe soil degradation (Chen et al. 2015a). Since the 1970s, a large amount of farmland (over 48,300 km^2) has been abandoned to prevent soil erosion and rehabilitate fragile ecosystems (Chen et al. 2015a). These abandoned farmlands can be left to undergo secondary succession, leading to a mosaic of regenerated grassland vegetation in various stages of succession (Chang 1999). Previous studies have addressed the dynamics of soil N and bacterial communities during vegetation restoration of abandoned farmland (Zhang et al. 2016a; Ren et al. 2016), but the dynamics of denitrification rates and their driving mechanisms remain elusive.

In this study, changes in denitrification rates and in the absolute abundance, community composition, and diversity of denitrifying bacteria were investigated across a 45-year (y) chronosequence of vegetation restoration of abandoned farmland in fragile ecosystems of a semiarid region. Specifically, the following three objectives were pursued: (1) to investigate the long-term shifts in denitrification rates, (2) to quantify the absolute abundances of denitrifying functional genes and their compositions and diversities, (3) to determine the quantitative link between denitrification rates and denitrifying functional genes, and (4) to explore linkages among soil properties, plant coverage, N gas emissions, and denitrifying microorganisms along the chronosequence.

Materials and methods

Description of sites and sample collection

The field site was situated within the Zhifanggou Ecological Restoration Watershed (109° 15′ E, 36° 44′ N, 1220-m altitude), a hilly loess landscape on the Loess Plateau. The climate is semiarid, and the mean annual precipitation is 437 mm, with 77% falling from June to September. The soils in this area are primarily Huangmian soils, characterized by a low water-holding capacity, a low organic matter content, and a moderately alkaline pH value.

We assembled a chronosequence (0, 9, 21, 32, and 45 years) of fifteen sites planted between 1971 and 2016. Each site was > 1 ha in size, and the selection process controlled for characteristics such as soil texture (Huangmian soils). We also selected three agricultural fields in which corn and millet (*Setaria italica*) were planted in two and one of the fields, respectively, to represent the prerestoration conditions. The agricultural fields have been in a corn-millet rotation for at least the past decade. We established a 100-m^2 (10 × 10 m) plot within the abandoned farmland and surveyed the plant communities on 10 August 2016 using transects. Because the primary crops were harvested, the vegetation coverage was not investigated at 0-year (y) sites. Vegetation coverage markedly increased with increasing time since the abandonment, ranging from 13.54% at 9-y sites, just after agriculture cessation, to 40.02% at 45-y sites (Supplemental Table S1). At five randomly selected points within each site, we collected approximately 100 g of soil from the top 20 cm with a 5-cm diameter stainless steel corer after the litter horizons were removed. The soil samples were homogenized into a single 500-g sample in a Whirl-Pak bag. We immediately placed the samples on ice for transportation and stored them at − 80 °C until processing. Edaphic variables, such as soil organic carbon (SOC), total nitrogen (TN), NH_4^+-N, NO_3^--N, soil bulk density (BD), and pH, were measured using previously

described methods (Zhang et al. 2016a). The major properties of the experimental sites are shown in Supplemental Table S2.

The in situ temperature and water content in the uppermost 20 cm of soil were continually measured from 23 June 2016 to 26 October 2016 using temperature water monitors (L-99; Hangzhou Luge Science and Technology limited company, Hangzhou, China). The average in situ soil temperature was 23.34 ± 1.80 °C from 8:00 a.m. to 20:00 p.m. and 19.80 ± 0.31 °C from 20:00 p.m. to 8:00 a.m. (Supplemental Fig. S1). The experimental temperature ranged from 20.1 to 25.4 °C from 8:00 a.m. to 20:00 p.m. and from 18.8 to 21.7 °C from 20:00 p.m. to 8:00 a.m.

Soil incubation experiment

The acetylene (C_2H_2) inhibition method was used to measure denitrification rates (Daum and Schenk 1997). In this study, approximately 50 g of fresh soils from each plot were bulked, homogenized, and incubated for 72 h in a digital incubator for accurate temperature settings to create similar in situ environmental conditions (20.1 to 25.4 °C from 8:00 a.m. to 20:00 p.m. and 18.8 to 21.7 °C from 20:00 p.m. to 8:00 a.m.). In half the microcosms for each treatment, 10% (*v*/*v*) of the headspace air was replaced with acetylene (C_2H_2) to measure total denitrification rates ($N_2O + N_2$) by inhibiting N_2O reductase activity. In the remaining microcosms, headspace air was not modified to allow the actual N_2O emission rates to be measured. The change-to-anaerobicity procedure was conducted to quantify denitrification and the N_2O and N_2 gas product potentials. Samples of N_2O and N_2 were collected from the conical flasks after 1, 6, 12, 24, 36, 48, 60, 72, and 96 h. Gas chromatography (Agilent 7890 gas chromatograph equipped with an electron capture detector (ECD) detector, Agilent, Santa Clara, CA, USA) was performed to analyze the concentration of N_2O in the samples. The operating conditions for the N_2O analysis via GC-ECD were as follows: PEG-20M capillary tube chromatographic column (0 m × 0.53 mm × 1.00 mm); column temperature, 40 °C; detector temperature, 250 °C; injection port temperature,150 °C; makeup gas flow, 60 mL/min; column flow, 5 mL/min; and injection volume, 100 mL. The N_2O concentration was calculated by fitting the kinetics of the gas concentration to a linear or exponential model (Kimochi et al. 1998).

DNA extraction and the quantitative polymerase chain reaction

An E.Z.N.A.™ Soil DNA Kit D5625-01 (Omega Bio-tek, Norcross, America) was used to extract the total genomic DNA from the soil samples (0.5–1.0 g). The abundance of the denitrifying bacteria involved in NO_3^--N transformation and N gas production was assessed via qPCR to understand their population dynamics. The absolute abundances of the bacterial 16S rRNA (bacteria), periplasmic nitrate reductase (*napA*), membrane-bound nitrate reductase (*narG*), copper-containing nitrite reductase (*nirK*), cd1-containing nitrite reductase (*nirS*), nitric oxide reductase (*qnorB*), and nitrous oxide reductase (*nosZ*) genes were quantified three times for each sample on a CFX Real-Time polymerase chain reaction (PCR) Detection System (Bio-Rad Laboratories, Singapore City, Singapore) using previously described experimental procedures (Wang et al. 2017; Petersen et al. 2012). The primers, protocol, and parameters used for the seven target genes are shown in the Supplemental Table S3. Each 20 μL quantitative PCR mixture contained 10 μL of SybrGreen qPCR Master Mix (2×), 1 μL of primers (10 μM), and 2 μL of DNA template. Standard curves were constructed with tenfold serial dilutions of a known amount of plasmid DNA containing fragments of the 16S rRNA (total bacteria), *narG*, *napA*, *nirK*, *nirS*, *qnorB*, and *nosZ* genes. The standard curves and amplification efficiencies are shown in Supplemental Table S4.

Illumina MiSeq high-throughput sequencing

Illumina MiSeq high-throughput sequencing was performed to reveal the community composition and diversity of the total bacteria and of the *narG*, *napA*, *nirK*, nirS, *qnorB*, and *nosZ* genes. We used primers sets that encompass the known diversity of the *narG*, *napA*, *nirK*, *nirS*, *qnorB*, and *nosZ* genes. Polymerase chain reaction (PCR) was performed with the primer pairs Eub338f/Eub518r for total bacteria (targeting the V3–V4 hypervariable regions), V17F/4R for *napA* (Bru et al. 2007), 1960 m2f/2050 m2r for *narG* (López-Gutiérrez et al. 2004), 583F/909R for *nirK* (Yan et al. 2003), *nirS*cd3aF/*nirS*R3cd for *nirS*, *qnorB2F*/*qnorB5R* for *qnorB* (Braker and Tiedje 2003), and 1527F/1773R for *nosZ* (Scala and Kerkhof 1998). A representative sequence from each operational taxonomic unit (OTU) was chosen to calculate the relative abundance and to identify the taxonomic group (Blaxter et al. 2005). The detailed protocols and parameters used are described elsewhere (Tang et al. 2016; Barber et al. 2017). The final datasets contained a total of 253,923, 1,079,535, 532,390, 331,131, 227,666, 390,978, and 240,372 sequences of the total bacteria and the *narG*, *napA*, *nirK*, *nirS*, *qnorB*, and *nosZ* genes, respectively (Supplemental Table S5). Quantitative Insights into Microbial Ecology (QIIME v1.8.0, http://qiime.org/) was used to quantify the number of OTUs. The sequences were split into groups according to their taxonomy and assigned to operational taxonomic units (OTUs) at a similarity level of 97% using the UPARSE pipeline (Edgar et al. 2011). Altogether, there were 21,783, 247, 10,963, 7091, 3017, 2697, and 2629 OTUs in the total bacteria *narG*, *napA*, *nirK*, *nirS*, *qnorB*, and *nosZ* libraries, respectively (Supplemental Table S6). All sequence data were submitted to NCBI under BioProject accession number PRJNA394668.

Statistical analysis

One-way analysis of variance (ANOVA) and least significant difference (LSD) multiple comparisons ($P < 0.05$) were used to determine the significant effects of site age on vegetation characteristics, edaphic variables, and the absolute abundance of bacteria and denitrifying functional genes. The standard deviations (SDs) of the absolute abundance data were calculated using three replicates measured via qPCR and plotted as error bars to assess the variation in the data and measurement errors. Linear mixed models were used to determine the quantitative relationships between the N gas emission rates and functional gene groups. Stepwise regression models were constructed to obtain the multiple linear regression equation for the relationship between the abundance of denitrifying bacteria and N gas emission rates using SPSS Statistics 20 (IBM, USA). A rarefaction analysis was used to calculate diversity indices, including the Chao 1 and ACE indices (Schloss et al. 2009). Principal component analysis (PCA) and multidimensional scaling (MDS) were used to evaluate the variation in the total bacterial and denitrifying bacterial community compositions using the R software package v.3.2.3 (Ramette 2007).

Results

Potential N_2O and N_2 emissions

The highest emissions of N_2O (17.46 ± 0.58 μg kg^{-1} day^{-1}) and N_2 (44.04 ± 1.32 μg kg^{-1} day^{-1}) were observed in the 0-y sites, after which the potential N_2O and N_2 emissions significantly increased in the 9- to 45-year sites ($P < 0.05$), with typical emissions ranging from 5.52 ± 0.19 to 13.23 ± 2.09 μg kg^{-1} day^{-1} and from 7.26 ± 0.41 to 24.29 ± 1.24 μg kg^{-1} day^{-1}, respectively (Fig. 1a, b). The denitrification rate (DR) ($N_2O + N_2$) sharply decreased from 61.50 ± 1.80 (0-y sites) to 12.78 ± 0.29 μg kg^{-1} day^{-1} (9-y sites), after which the rate significantly increased from 16.24 ± 1.89 μg kg^{-1} day^{-1} in the 32-y sites to 37.53 ± 1.30 μg kg^{-1} day^{-1} in the 45-y sites (Fig. 1c).

Abundance of denitrifying bacteria

The absolute abundance of the bacterial 16S rRNA gene showed a significant 5.03-fold decrease after the first 9 years of soil restoration, followed by a significant increase as the natural restoration progressed (Fig. 2a). The abundance of the *napA* gene significantly decreased after abandonment (< 9 years) compared to the farmland, significantly increasing as the restoration proceeded. The abundances of the *narG*, *nirK*, *nirS*, *qnorB*, and *nosZ* genes steadily increased with the restoration age of abandoned farmland. The abundance of denitrifying microorganisms in the 32- to 45-y sites

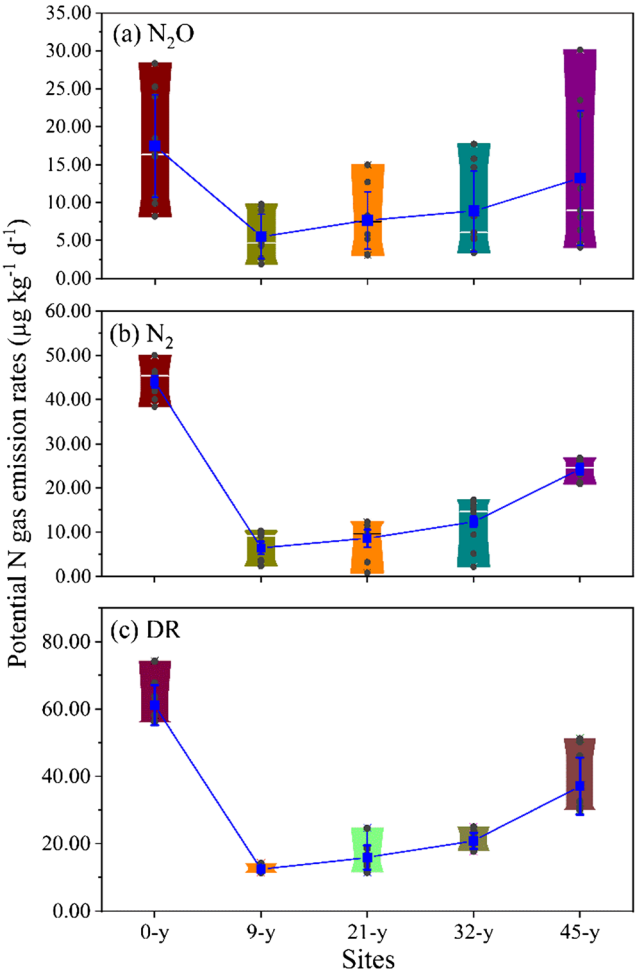

Fig. 1 N gas emission rates during the 45 years of restoration of abandoned farmland. DR denitrification rate (**a**–**c**)

significantly exceeded that observed for the 0-y sites except for *nirK* in the 45-y sites. The changes in the abundances of the *napA* and *narG* genes showed similar tendencies, initially decreasing compared to the 0-y sites (except the *narG* gene), followed by a similar increasing pattern as the restoration progressed (Fig. 2b). The *nirK* and *nirS* genes showed different fluctuating trends with increasing time since abandonment (Fig. 2c). The *qnorB* gene showed similar increasing and decreasing patterns to those of the *nosZ* gene.

Community structure and diversity of denitrifying bacteria

Comparing the total variation, the relative abundances of bacteria suggested there were differences in their community structures, advancing our quantitative understanding of soil microbial communities during 45 years of vegetation restoration of abandoned farmland. We focused on two phyla that were previously identified as predominant players (*Actinobacteria* and *Proteobacteria*) in grassland restorations and cultivated soils. In this study, bacterial communities

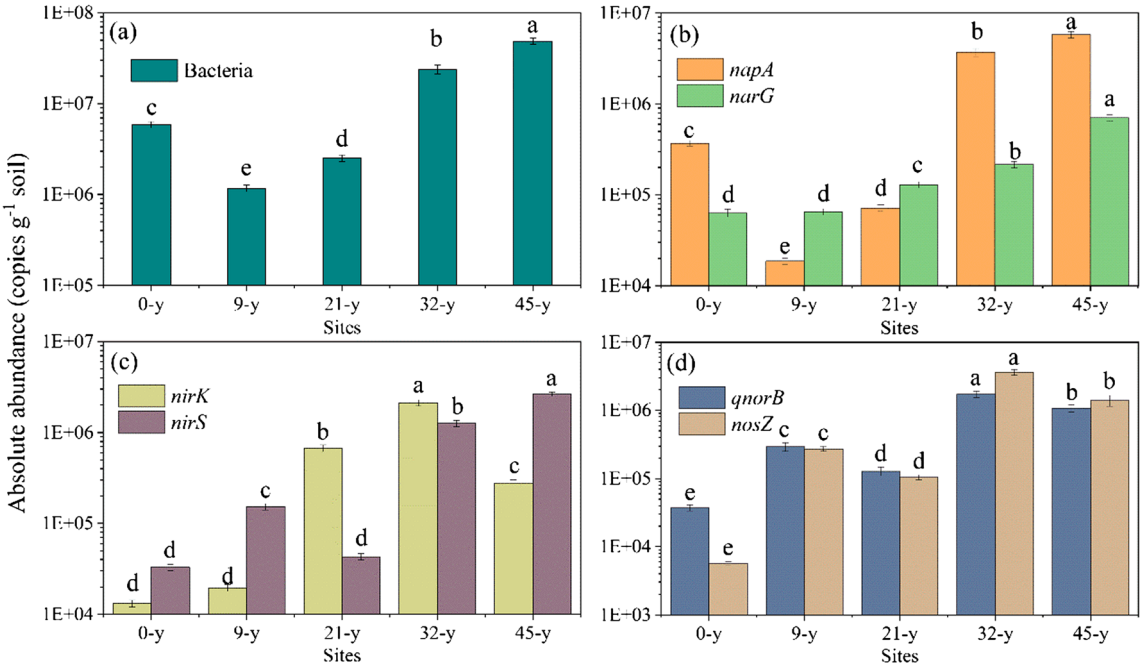

Fig. 2 The absolute abundances of bacteria and denitrifying bacteria during the 45 years restoration of abandoned farmland. (**a**) bacteria; (**b**) *narG* and *napA*; (**c**) *nirK* and *nirS*; (**d**) *qnorB* and *nosZ*. The absolute abundances (copies per gram of soil) are shown on a log10 scale (*y*-axis). The standard deviations of the three replicates are indicated by error bars. Invisible error bars indicate that the standard deviations are smaller than the marker size. Different letters indicate significant differences ($P < 0.05$) among the sites for the individual variables based on a one-way ANOVA followed by an LSD test

transitioned from *Actinobacteria*-dominant to *Proteobacteria*-dominant communities as the vegetation restoration progressed (Supplemental Figs. S2 and S3). The bacterial communities were predominantly composed of *Actinobacteria* (28.8%, on average) and *Proteobacteria* (20.7%) across the vegetation restoration stages.

The denitrifying communities included diverse bacteria from the taxa *Alpha-*, *Beta-*, *Gamma-* and *Deltaproteobacteria*, and *Actinobacteria* and were observed to be different at the genus level (Fig. 3). For the *napA* gene, the dominant bacterial classes were *Alpha-* (56.52%, on average), *Beta-* (27.84%), and *Gammaproteobacteria* (14.96%). For the *narG* gene, the dominant bacterial genus showed similarity to *Pseudomonas* (82.40%) in the class *Gammaproteobacteria*. For the *nirK* gene, the dominant bacterial classes were *Actinobacteria* (33.24%, on average) and *Betaproteobacteria* (19.94%). For the *nirS* gene, the dominant bacterial classes were *Solibacter* (17.17%, on average) and *Acidobacteria* (34.07%). For the *qnorB* gene, the dominant bacterial classes were *Acidimicrobiia* (40.96%, on average) and *Alphaproteobacteria* (17.58%). For the *nosZ* gene, the dominant bacterial classes were *Alpha-* (50.83%), *Beta-* (38.34%), and *Gammaproteobacteria* (7.80%). The characteristics of denitrifying bacteria were clearly separated from each other as the natural vegetation restoration of abandoned farmland progressed (Figs. 4 and 5). The community structure variations in total bacteria and of the *napA* and *narG* genes were strongly enhanced in the first 10 years of soil recovery (exhibiting the highest ratios). The community structure variations with respect to the *nirK*, *qnorB*, and *nosZ* genes at the 9- to 32-year sites also exhibited a strong relationship with soil age. The community structure variations in the *nirS* gene steadily increased with increasing time of abandonment. The variation coefficient calculated using genus-level taxonomy exhibited significant changes (Supplemental Fig. S4), indicating that the functional groups of denitrifying bacteria responded differently to natural vegetation restoration of abandoned farmland in these semiarid regions.

Throughout this study, the diversity of total bacteria increased slightly with increasing site age. The diversity of denitrifying bacteria was marginally significantly related to site age (Fig. 4). The ACE and Chao 1 estimators indicated that the diversity of the denitrifying bacteria at the 9-y site decreased dramatically compared to the farmland (0-y sites) and increased at the 21- to 45-y sites, with the same trend observed for N gas emissions.

Linkages among soil properties, plant coverage, rates, and denitrifying bacteria

Ordination of samples by PCA based on plant coverage, soil properties, and N gas emissions and on the abundance, community composition, and diversity of denitrifying bacteria showed clear differences on the first axis (Fig. 5a, b), with the first two axes explaining 81.19 and 77.53% of total variance, respectively. The ordination supported the trends observed in Supplemental Figs. S5, S6, and S7, showing a strong and positive correlation of some soil properties and plant

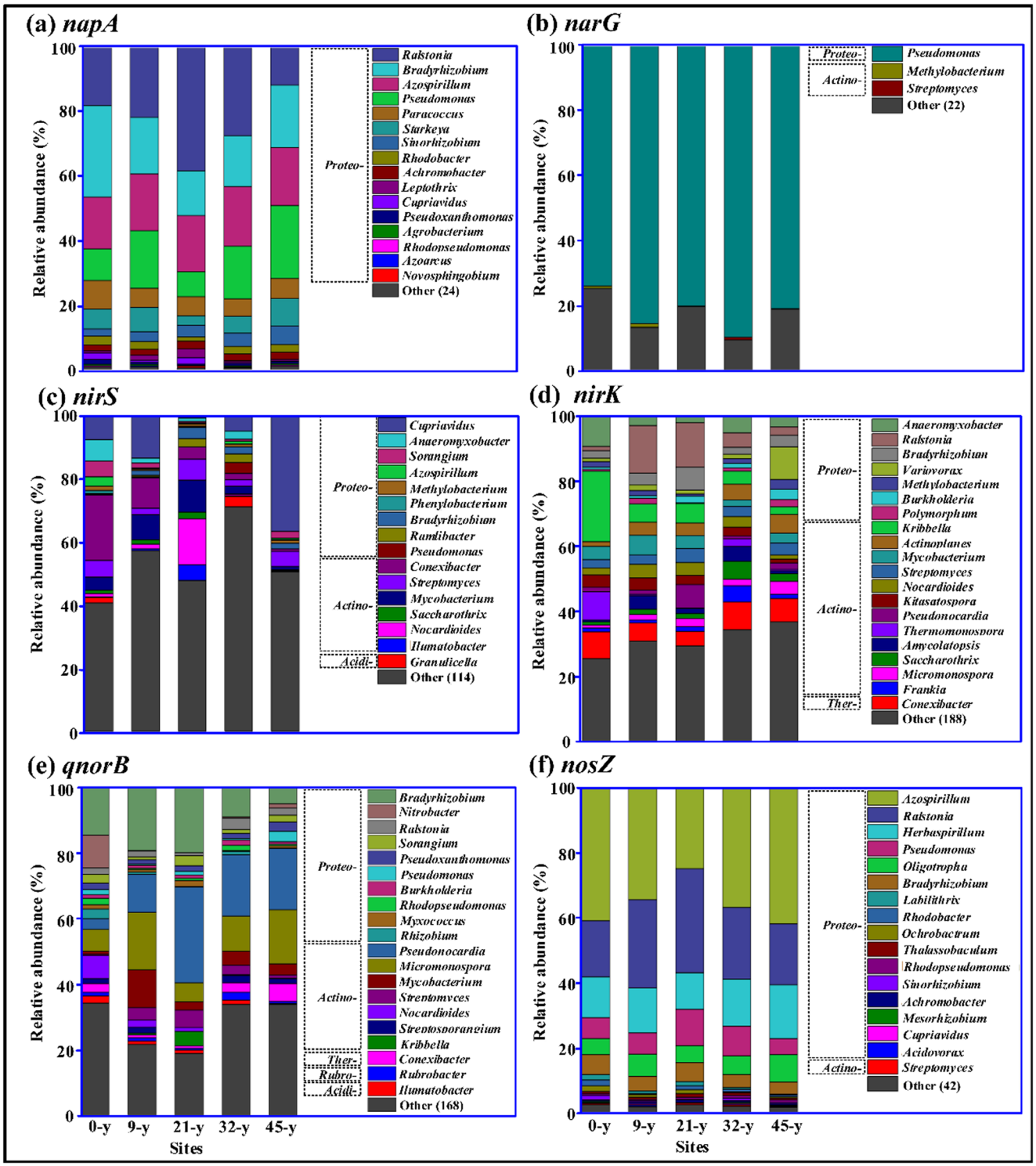

Fig. 3 **a**–**f** Relative abundance of denitrifying functional genes at the genus (or species) level. Proteo- *Proteobacteria*, Actino- *Actinobacteria*, Ther- *Thermotogae*, Rubro- *Rubrobacteria*, Acidi- *Acidimicrobiia*

coverage and for the absolute abundance, community composition, and diversity of denitrifying bacteria. Of particular interest, we observed positive correlations among the increase in plant coverage with time, SOC, NO_3^--N, pH, and C/N and the abundance and diversity of denitrifying bacteria.

The absolute abundance of denitrifying bacteria was observed to be somewhat correlated with the potential N_2O and N_2 emissions (Supplemental Fig. S5). However, we observed that the potential N_2O emissions were significantly correlated with the ratios of (*napA* + *narG*)/bacteria ($R^2 = 0.895$, $P < 0.001$) and

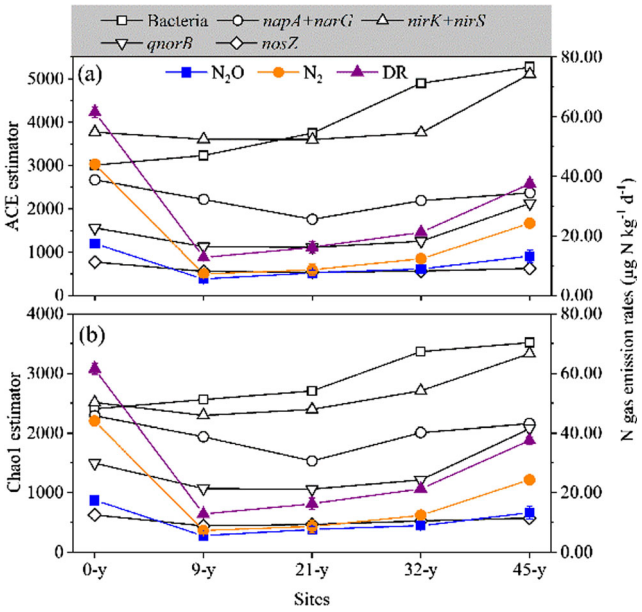

Fig. 4 Comparison of community diversities between N gas emission rates and denitrifying genes during the 45 years of restoration of abandoned farmland. **a** ACE index. **b** the Chao 1 index. The *napA* and *narG* are two functionally equivalent genes, in which the diversity of *napA* and *narG* genes was added together. The *nirK* and *nirS* are two functionally equivalent genes, in which the diversity of *nirK* and *nirS* genes was added together. DR denitrification rate

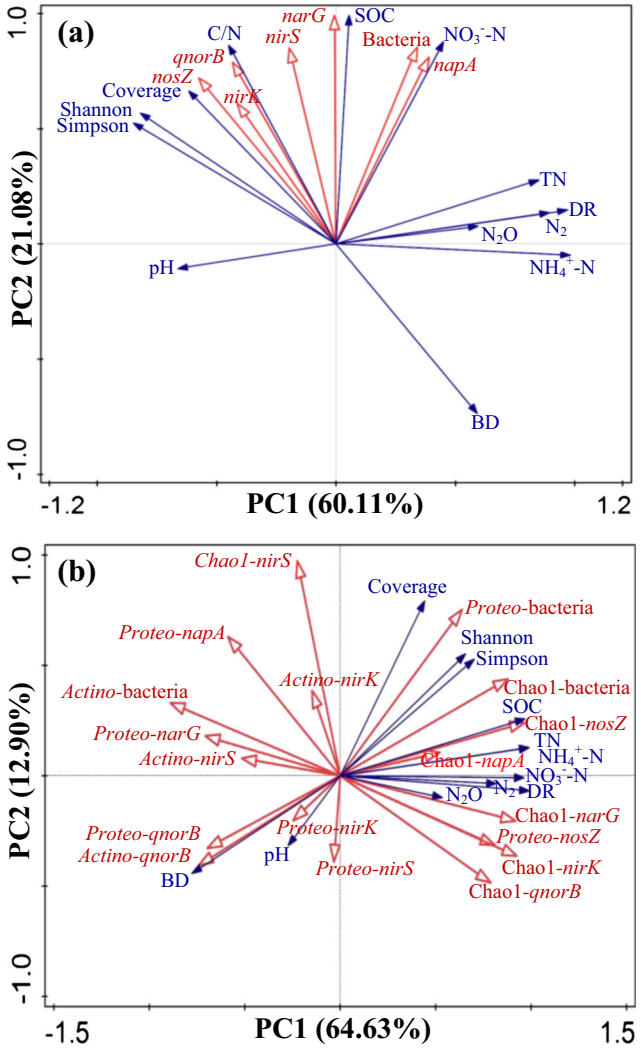

Fig. 5 Principal component analysis of plant, soil properties, N gas emissions, abundances, community composition, and diversity of denitrifying bacteria during the 45 years of restoration of abandoned farmland. SOC soil organic carbon, TN total nitrogen, BD bulk density, Proteo- *Proteobacteria*, Actino- *Actinobacteria*. The first two PCA axes explain 81.19% (**a**) and 77.53% (**b**) of the total variance, respectively

qnorB/bacteria ($R^2 = 0.754$, $P < 0.001$) (Fig. 6a, b). The potential N_2 emissions were significantly correlated with the ratios of (*napA* + *narG*)/bacteria ($R^2 = 0.944$, $P < 0.001$) and (*nirS* + *nirK* + *qnorB*)/*nosZ* ($R^2 = 0.626$, $P < 0.001$) (Fig. 6c, d). The DR was significantly correlated with the ratios of (*napA* + *narG*)/bacteria ($R^2 = 0.855$, $P < 0.001$) and *nosZ*/(*nirS* + *nirK*) ($R^2 = 0.438$, $P < 0.001$) (Fig. 6e, f). To explore the relationships between denitrifying microorganisms and the potential N_2O and N_2 emissions, a stepwise multiple regression was utilized by incorporating all relevant ratios of genes to denitrifying bacterial abundance. The results showed that 91.4% of the variation in the potential N_2O emissions was accounted for by a model that included (*napA* + *narG*)/bacteria and *qnorB*/bacteria (Table 1), and 93.9% of the variation in the potential N_2 emissions was accounted for by a model that included (*napA* + *narG*)/bacteria and (*nirS* + *nirK* + *qnorB*)/*nosZ*. Furthermore, 96.1% of the variation in the DR was accounted for by a model that included (*napA* + *narG*)/bacteria and *nosZ*/(*nirS* + *nirK*).

Discussion

Effects of natural restoration on N gas emissions

The natural restoration of abandoned farmland in a semiarid environment had a strong positive influence on decreasing N gas (N_2O and N_2) emissions during the early stages of restoration. The higher N gas emissions in the 0-y sites (Fig. 1) can be largely attributed to the intense fertilization of these sites (Hu et al. 2015). N gas emissions in farmland soils are primarily governed by the denitrification process, which is mostly limited by soil C and N availability (Kim et al. 2017). In the present study, the termination of input from artificial fertilizers was the primary reason accounting for the decreased content of soil SOC, TN, and NO_3^--N in the 9-y sites (Supplemental Table S2), which is consistent with results of the previous studies (Wang et al. 2017). Long-term fertilization (0-y sites), such as the input of organic manures and N fertilizer, can enhance soil C and N availability, leading to the enhanced denitrifying activity responsible for N gas production (Yin et al. 2017). Thus, the cessation of C and N inputs (9-y sites) suggested that attenuated denitrifying activity accounted for the marked reduction in N gas production.

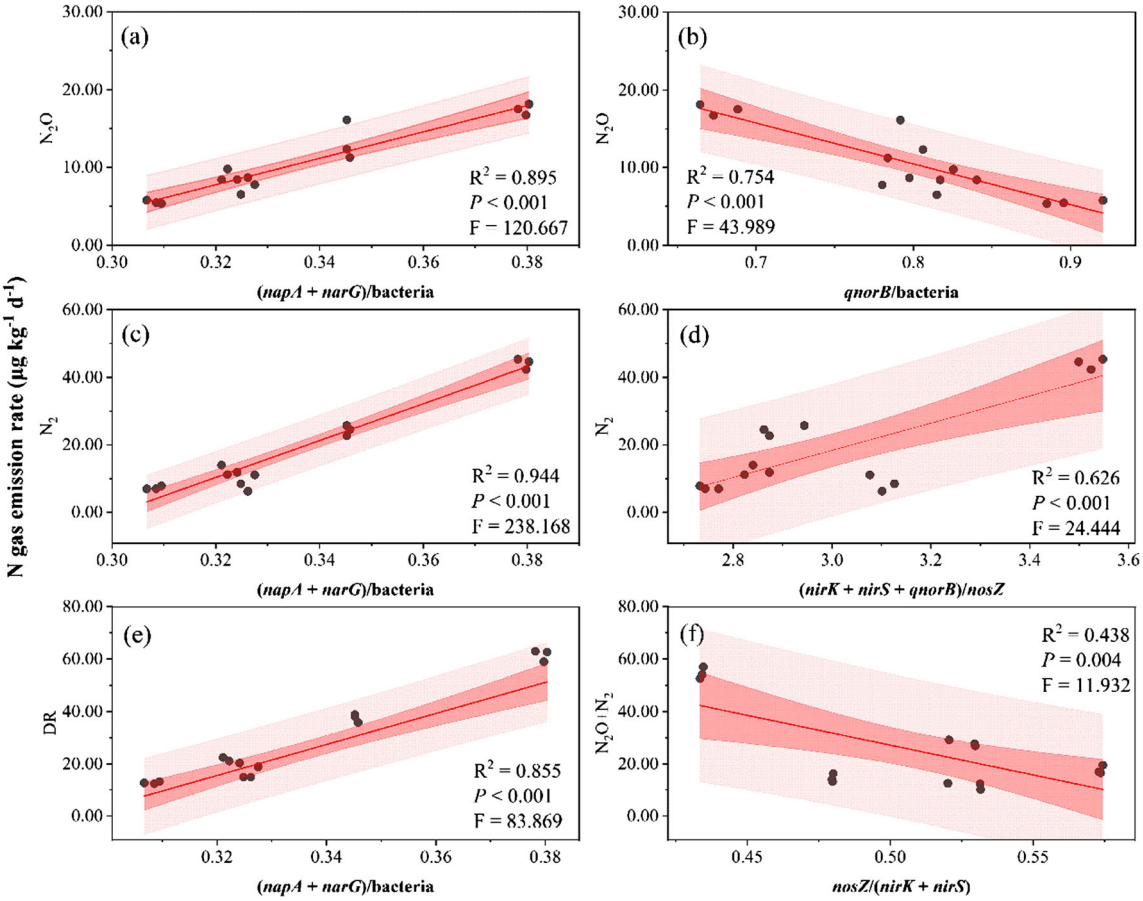

Fig. 6 **a**–**f** Relationships between the N gas emission rates and gene ratios. The bacteria represents the abundance of the total bacterial 16S rRNA gene. The red fitted lines are from ordinary least squares regression. Shaded areas show the 95% confidence interval of the fit. DR denitrification rate

As the natural restoration progressed, the N gas emissions steadily increased in the 9- to 45-y sites (Fig. 1), which led to enhanced soil N loss, especially in the 45-y sites. The denitrification process is predominantly driven by heterotrophic bacteria, which require SOC as an energy source to diversify and grow in soil systems (Levy-Booth et al. 2014). The plant communities in this semiarid environment underwent secondary succession as the restoration progressed, a process that leads to increases in plant cover and litter decomposition, accounting for the accumulation of SOC, TN, and NO_3^--N (Supplemental Table S1 and S2). Soil nutrients, such as SOC, TN, and NO_3^--N, exhibited significant increases after 32 years of natural restoration. This marked increase in SOC and the C/N ratio in 45-y sites led to an enhancement of the enrichment of denitrifying bacteria, suggesting high N gas emissions and N loss (Levy-Booth et al. 2014). Several reports have indicated that ecological associations and symbiotic relationships among denitrifying bacteria occur because of their similar soil environmental adaptations and interactions but distinct ecological niches (Wang et al. 2017; Zhang et al. 2016b). Thus, the increase in N gas emissions in the 45-y sites can largely be attributed to the coupling of soil nutrient availability and denitrification-related pathways (Levy-Booth et al. 2014; Hu et al. 2015; Afreh et al. 2018).

Table 1 Quantitative response relationships between N gas emission rates (μg N kg^{-1} day^{-1}) and functional gene abundances (copies per gram of soil) during the natural restoration of abandoned farmland

Stepwise regression models (equations)	R^2	P value	F
$N_2O = 188.057 \times \frac{(napA+narG)}{\text{Bacteria}} + 7.417 \times \frac{qnorB}{\text{Bacteria}} - 58.818$	0.914	< 0.001	63.609
$N_2 = 546.442 \times \frac{(napA+narG)}{\text{Bacteria}} - 1.583 \times \frac{(nirK+nirS+qnorB)}{nosZ} - 160.237$	0.939	< 0.001	92.387
$DR = 774.000 \times \frac{(napA+narG)}{\text{Bacteria}} + 49.114 \times \frac{nosZ}{(nirK+nirS)} - 256.123$	0.961	< 0.001	148.315

Effects of natural restoration on denitrifying microorganisms

The bacterial abundance clearly decreased during the early stages of restoration and then continuously increased as the restoration proceeded (Fig. 2a). Significant differences in the absolute abundance of bacteria were observed among sites across the 45-y chronosequence of natural restoration of abandoned farmland, which is similar to the results of other studies reporting that the dynamics in vegetation cover and soil nutrients during natural restoration increase the total bacterial abundance (Blaalid et al. 2012; Pereg et al. 2018). In this study, the bacterial communities transitioned from *Actinobacteria*-dominant to *Proteobacteria*-dominant communities as the natural restoration progressed (Supplemental Fig. S2), a transition that has been reported in the previous studies (Barber et al. 2017; Cline and Zak 2015), which suggest that the bacterial community composition was significantly affected by the natural restoration process and that areas that had been restored longer ago harbor communities that are significantly distinct from those that have been restored more recently.

Our results show that the abundances of the six assayed genes involved in denitrification showed dynamic fluctuations as the restoration progressed (Fig. 2). Although these genes do not cover the maximal genetic diversity of each group (Jones et al. 2014; Wei et al. 2015), they still allow for a comparative analysis of the relative abundance of denitrifiers across the 0- to 45-y soils samples. All six genes exhibited overall increases in the 9- to 45-y sites, leading to the enhanced denitrifying activity responsible for N loss and N_2O emissions (Figs. 1 and 2). The similar increasing trend among the six denitrifying functional genes occurred due to their similar environmental adaptations and to their related, yet distinct, and ecological niches (Levy-Booth et al. 2014). The coenrichment of the *narG* and *napA* genes can be attributed to the enhanced availability of SOC and NO_3^--N (Fig. 5 and Supplemental Fig. S5), which is consistent with the results of the previous studies (Kandeler et al. 2009). The *nirK* and *nirS* genes showed dynamic fluctuations across the 45-y chronosequence of natural restoration of abandoned farmland (Fig. 2c), similar to the results of other studies reporting that these two types of *nir*-harboring bacteria were strongly influenced by soil C levels and were sensitive to increases in SOC (Tang et al. 2016; Levy-Booth et al. 2014). The *qnorB* and *nosZ* genes showed similar trends in temporal variation owing to their similar environmental adaptations and ecological interactions (Hallin et al. 2017). Agricultural soils greatly contribute to N_2O emissions, and soils with high ratios of *qnorB*/*nosZ* gene copies may be associated with a high capacity for N_2O production and exhibit high levels of N_2O emissions (Hu et al. 2015). We observed that the ratios of *qnorB*/*nosZ* in the 0-y sites were higher than those in the 9- to 45-y sites (Supplemental Fig. S8), which may explain the correspondingly high N_2O emissions in the 0-y sites.

The most abundant sequences of denitrifying microorganisms best matched those of the phyla *Proteobacteria* and *Actinobacteria* (Fig. 3), consistent with the previous general observations of denitrifying communities (Saarenheimo et al. 2014; Levy-Booth et al. 2014). Previous studies of grassland systems have suggested that the abundance of *Proteobacteria* increases with the progression of natural restoration (Zhang et al. 2016a; Li et al. 2014; Jangid et al. 2013). The results indicated that nutrient limitation, among other factors, favors *Proteobacteria*, which likely have crucial roles in the restoration of soil. Many soil *Proteobacteria* are copiotrophic and become abundant when labile substrates are available (Barber et al. 2017). *Actinobacteria* was the most abundant phylum in the 0- to 45-y sites, and the results also indicate a higher abundance of this phylum in the 0-y sites than in the 45-y sites. This result was consistent with the finding of a previous study where a decrease in the abundance of *Actinobacteria* was observed to be correlated with restoration age (63 years) (Lozano et al. 2014). However, other studies have observed an increase in the abundance of *Actinobacteria* was correlated with restoration age (27 years) (Barber et al. 2017). Thus, the results of this study highlight that the recovery of microbial clades within the *Proteobacteria* and *Actinobacteria* phylum is an important feature of this convergence and that these groups could be targeted for future soil N-focused studies in fragile ecosystems.

Denitrifying bacteria include representatives of more than 60 bacterial genera in soils (Demanèche et al. 2009; Canfield et al. 2010). In our study, the dominant *napA* groups showed the highest similarities to the genera *Ralstonia* (of the class *Betaproteobacteria*), *Bradyrhizobium* and *Azospirillum* (of the class *Alphaproteobacteria*), and *Pseudomonas* (of the class *Gammaproteobacteria*) (Supplemental Fig. S9), similar to other studies reporting that there are different phylogenetic groups of nitrate-reducing bacteria that utilize a *napA*-encoded nitrate reductase (Pohlmann et al. 2000; Hettmann et al. 2004; Smith et al. 2007). These genera accounted for 71.68–78.16% of the all classified sequences as the vegetation recovery progressed. For *napA*, the predominant genus transitioned from *Bradyrhizobium* (0-y sites) to *Pseudomonas* (45-y sites), suggesting that long-term vegetation restoration may be mediated by different phylogenetic groups. Earlier studies of *narG* sequences in soil environments had previously identified sequences affiliated with *Proteobacterial* subclasses (i.e., *Alpha-*, *Beta-*, or *Gammaproteobacteria*) or the phylum *Actinobacteria* (Smith et al. 2007; Carlson and Ingraham 1983; Deiglmayr et al. 2006). The predominant *narG* group in 0- to 45-year sites showed the highest similarity to the *Pseudomonas* genus of the class *Gammaproteobacteria*, suggesting that natural vegetation restoration may not reshape the phylogenetic groups of *narG*. This result was consistent with

those reported by Deiglmayr et al. (2006), who observed that the community structure of *narG* showed a small shift over 100 years of soil formation. Earlier studies of *narG* and *napA* in soil environments had previously identified sequences associated with those from the genus *Pseudomonas* of the class *Gammaproteobacteria* (Smith et al. 2007; Kathiravan and Krishnani 2014), which were consistent with the results of our study (Fig. 3a, b). The dominant *nirK* groups observed showed the highest similarities to the genera *Ralstonia* of (of the class *Beta proteobacteria*), *Conexibacter* and *Kribbella* (of the phylum *Actinobacteria*), and *Bradyrhizobium* (of the class *Alphaproteobacteria*), similar to results of the previous studies (Yoshida et al. 2010; Braker et al. 2000). The dominant *nirS* groups showed highest similarities to the genera *Cupriavidus* (of the class *Betaproteobacteria*), *Conexibacter*, *Mycobacterium*, *Streptomyces*, and *Nocardioides* (of the phylum *Actinobacteria*), and *Gemmatimonas* (of the phylum *Gemmatimonadetes*) (Bourbonnais et al. 2014; Yoshida et al. 2009). The observed abundances of the *nirK* and *nirS* genes confirmed that nitrite reduction in soil environments may be mediated by different phylogenetic groups (Yoshida et al. 2009), leading to significantly fluctuating communities in the 0- to 45-year recovery soils. The dominant *qnorB* groups showed the highest similarities to the genera *Pseudonocardia*, *Mycobacterium*, and *Micromonospora* (of the phylum *Actinobacteria*) and *Bradyrhizobium* (of the class *Alphaproteobacteria*), similar to the results of the previous studies (Casciotti and Ward 2005; Heylen et al. 2007; Jones et al. 2008). The dominant *nosZ* groups showed highest similarities to the genera *Azospirillum* and *Bradyrhizobium* (of the class *Alphaproteobacteria*), *Ralstonia* (of the class *Betaproteobacteria*), and *Pseudomonas* (of the phylum *Actinobacteria*), similar to the results of the previous studies (Delorme et al. 2003; Ishii et al. 2016).

The Chao 1 and ACE indices showed that the diversity of the total bacteria and denitrifying bacteria showed different fluctuating trends as the natural restoration progressed (Fig. 4). Throughout this study, the diversity of total bacteria steadily increased with site age, consistent with the findings of the previous studies, suggesting that increased total bacterial diversity is correlated with increasing site age (Barber et al. 2017; Lozano et al. 2014). The increase in the diversity of total bacteria was attributed to the enhanced soil nutrient supply as the natural restoration progressed (Bernhard and Kelly 2016).

Response relationships between N gas emissions and denitrifiers

Although N gas emissions and the underlying functional genes associated with denitrification are well characterized (Chen et al. 2015b), the roles of these functional genes in N gas emissions are still unclear. Therefore, quantitative response models were constructed to link macroscale N gas emissions and denitrifying functional genes and to promote our quantitative understanding of the key functional genes that govern N gas emissions (Fig. 6). The first variable in the three equations, (*napA* + *narG*)/bacteria, denotes NO_3^--N reduction in the denitrification process and the positive correlation of the variable with N_2O and N_2. The high ratio of this variable represents the extent of NO_3^--N reduction, i.e., greater NO_3^--N reduction indicates a greater availability of the substrate responsible for producing N_2O and N_2. Our results indicate that the *narG* and *napA* genes were the rate-limiting genes that solely that determined the N_2O, N_2 and DR. The variable *qnorB*/bacteria showed a positive relationship with N_2O emission rates. The variable *qnorB*/bacteria was directly involved in N_2O production in the denitrification pathway and was therefore positively correlated with the N_2O emission rates. The N_2 emission rates were negatively correlated with (*nirK* + *nirS* + *qnorB*)/*nosZ*. This relationship occurs because the *nirK*, *nirS*, and *qnorB* genes are involved in NO and N_2O production, whereas the *nosZ* gene is involved in the consumption of N_2O. Therefore, the ratio of production to consumption symbolized the extent or level of NO and N_2O accumulation, i.e., a greater NO and N_2O accumulation translates to lower N_2 production. The DR was collectively determined by the *nirS*, *nirK*, and *nosZ* genes. The variable *nosZ*/(*nirK* + *nirS*) represents the level of NO_3^--N removed via the denitrification pathway, leading to its positive correlation with DR. The results of our study show that the use of functional gene groups is the best approach for explaining the observed N gas emissions with molecular methods. Although gene abundance data are of primary importance in microbial studies and are routinely used to identify genes of interest and to quantify the exact number of copies in the environment (Chen et al. 2015b; Graham et al. 2014), the results presented here strongly indicate that functional gene groups can serve as integrative variables to characterize N gas emission rates.

The changes in microbial diversity had a large influence on the denitrification process (Philippot et al. 2013). Our result indicates that the diversity of denitrifying bacteria in the 9-y sites decreased steadily compared with the 0-y sites and then increased with increasing age after abandonment, with the same trend observed for N gas emissions (Fig. 4a, b). This result indicated that the increased diversity of denitrifying bacteria may enhance N_2O and N_2 emissions. A previous study observed that a loss of diversity in denitrifying communities may have significant impacts on denitrification rates, ultimately governing the fate of N gas production in soils (Manuel et al. 2016). The rates of denitrification are strongly regulated by soil pH, and N_2O:N_2 emission ratios have a significant negative relationship with soil pH within the normal pH range of 5 to 8 in farmland soils (Levy-Booth et al. 2014; Hu et al. 2015). In the present study, the pH (8.73) in the 21-y sites significantly increased, causing a change in the community composition (Fig. 3) and in the diversity of the (*napA* +

narG) and (*nirK* + *nirS*) genes (Fig. 4), leading to the promotion of N_2O emissions. Due to the complexity of interactive relationships, our results therefore highlight the need to further elucidate basic information regarding how changes in the community composition and diversity of denitrifying bacteria affect N gas emissions as vegetation restoration progresses. This is especially important in fragile ecosystems of semiarid regions, where soil N availability is a key limiting factor for plant growth and restoration.

The maintenance of high soil microbial diversity and functional redundancy has been suggested to be important for maintaining soil ecosystem health (Miki et al. 2014; Sun et al. 2016). In this study, better soil conditions, characterized by increased soil C and N, promoted by the increase in plant cover, positively enhancing the abundances of total and denitrifying bacteria (9- to 45-year sites) (Cline and Zak 2015; Tang et al. 2016). An overall increase in the diversity and abundance of total bacteria and denitrifying bacteria was observed in the soils after 21–45 years of restoration, suggesting an enhancement of the overall soil "health" after the natural restoration of abandoned farmland in a semiarid environment over the long term (Li et al. 2013; Levy-Booth et al. 2014). However, the potential risk of the enhanced denitrifying activity, which was responsible for the continuous increase in the greenhouse gas nitrous oxide (N_2O) and N loss after 32 years of natural restoration, should be noted. Scientific and effective measures (i.e., modest levels of grazing) may be undertaken to decrease the excessive organic carbon that accumulates in the soil, partially as a result of the decomposition of litter.

In summary, the results of this study confirmed the natural restoration of abandoned farmland in a semiarid environment has a strong positive influence on decreasing N gas (N_2O and N_2) emissions during the early stages of restoration. N gas emissions were shown to have steadily increased as the natural restoration progressed, which led to enhanced soil N loss. The abundance of denitrifying microorganisms showed dynamic fluctuations as the restoration progressed. The increased abundance and diversity of denitrifying bacteria may enhance N_2O and N_2 emissions. Quantitative response relationships between N gas emissions and denitrifying functional genes were established, and these relationships further confirmed that different denitrification processes were coupled at the molecular level (functional genes) to contribute to N gas emissions.

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Compliance with ethical standards

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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