Effects of Cultivation Years in Protected Vegetable Crop Fields On The Structure and Abundance of Soil *nosZ* Denitrifying Microbial Community

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Abstract

The study of the changes in the structure and quantity of the denitrifying microbial community in the protected vegetable crop field is crucial for evaluating the soil quality of long-term protected cultivation and improving the understanding of nitrogen conversion. In this study, quantitative fluorescent PCR was used, with the help of the Illumina Miseq high-throughput sequencing platform, aiming at the *nosZ* gene, to study the effects of protected cultivation plots 3 a, 5 a, and 7 a, and the outdoor cultivation plot (ck) on the structure and quantity of soil denitrifying microbial community. The results demonstrated that the abundance of *nosZ* gene in ck was significantly higher than the other treatments, and was 1.32, 1.45 and 1.69 times those of Group 3 a, 5 a and 7 a, respectively. As the cultivation year increased, the abundance of *nosZ* gene was decreased, and the α -diversity index was decreased. The Chao1 index and ACE index of ck were the highest. At the phylum level, the dominant group was *Proteobacteria*. While at the genus level, the dominant groups were *Bradyrhizobium* and *Achromobacter*. The relative abundances of *Proteobacteria* and *Bradyrhizobium* were decreased with the increase of protected cultivation time. The principal component analysis (PCA) results showed that the community structure of *nosZ* varied greatly with the increase of planting time. The community structures of Group 3 a and 5 a were similar, but the community structure of Group 7 a was very different from Group 3 a and 5 a. The soil available potassium (AK), ammonium nitrogen and nitrate nitrogen were the primary factors affecting the copy number, α -diversity and community structure of *nosZ* denitrifying microorganisms. As a conclusion, it can be seen that long-term cultivation of vegetable crops in a protected environment significantly reduced the copy number of *nosZ* denitrifying microorganisms and had a significant impact on the community structure.

Introduction

Protected cultivation of vegetable crops is an important branch of the modern intensive agriculture, and is greatly significant for improving natural resource utilization efficiency and boosting farmers' economic benefits. With the growing population and increasing demand for vegetable consumption in China, as well as limited arable land resources, protected cultivation has been dramatically developed in the past 40 years¹. As of 2016, the protected cultivation area in China had exceeded 58 million mu (1 mu = 0.165 acre), ranking first in the world². Currently, protected cultivation production has become a pillar industry in various regions of China. However, protected cultivation has the characteristics of high cropping index, great agricultural investment (such as fungicides, fertilizers, and agricultural films), closed or semi-closed environments with high temperature and high humidity, and no rain leaching. Therefore, the long-term protected cultivation has caused concerns of soil degradation, potential soil and vegetable contamination, and adverse health effects³.

Soil microorganisms are one of the essential components of the soil ecosystem. They contribute to energy circulating, nutrient cycling, organic matter conversion, pollutant degradation, and prevention and control of soilborne diseases in the soil ecosystem⁴. Related studies believed that long-term protected

cultivation had a negative impact on the structure and function of soil microbial community, primarily referring to the community succession from bacteria-dominant to fungi-dominant, and the reduction of the interaction between function-related microbial community members and species, resulting in the replacement of ecologically beneficial microbial community by soilborne pathogens^{5–8}. Currently, scholars are more concerned about the overall changes of soil microbial community or targeted microorganisms related to soil-borne diseases. Due to the high degree of structural and functional diversity of soil microorganisms, various types of soil biological processes are driven by the functional groups of microorganisms involved. So far, the response of soil functional microorganisms to long-term vegetable crop production remains poorly understood, especially that of the microorganisms related to nutrient cycling.

Nitrogen (N) is a primary factor limiting plant growth, and soil nitrogen availability plays a crucial role in determining the nitrogen absorption and yield of plants. Nitrogen conversion mainly includes the processes of N fixation, mineralization, nitrification and denitrification⁹. Among them, denitrification driven by soil microorganisms is an important part of the natural nitrogen cycle and the primary driving force for maintaining the global nitrogen balance. Nitrous oxide (N₂O) produced by denitrification is a powerful greenhouse gas and the primary ozone-depleting substance in the atmosphere¹⁰. It has a global warming potential (GWP) 298 times that of carbon dioxide (CO₂)¹¹. Due to excessive organic fertilizer, nitrogen fertilizer input and flood irrigation, the denitrification in soil was strong, and resulted in a N₂O emission flux 1.41 times higher than that of the field¹². Therefore, how to regulate N₂O conversion through microorganisms has become the focus of many scholars^{13,14}. Currently, the only known biological process in denitrification is the reduction of N₂O by the N₂O reductase encoded by the *nosZ* gene¹⁵. The *nosZ* gene has been used to study the community structure and diversity of denitrifying microorganisms in forests, grasslands and farmlands^{16,17}. However, no reports of the impact of long-term protected cultivation on the structure and quantity of *nosZ*-type denitrifier community in soil are available yet.

Therefore, this study used Illumina MiSeq high-throughput sequencing and real-time PCR to study the structure and abundance of *nosZ*-type denitrifier communities in soil for various years of protected cultivation. The primary aims of this study were: (1) How will the structure and diversity of the denitrifier community change as the planting time increases in protected cultivation plots? Which dominant community members will change? (2) For the long-term protected cultivation, which soil environmental factors have big impacts on the community structure of denitrifying microorganisms? Our study tried to reveal the denitrification of the protected cultivation in the long-term and provided a scientific basis for in-depth understanding of the soil nitrogen cycle of protected cultivation.

Results

Soil chemical properties

Long-term protected cultivation of vegetable crops had a significant impact on the chemical properties of the soil (Table 1). The soil pH, soil organic carbon (SOC) and available potassium (AK) contents of all tested cultivation years were all significantly lower than those of the control, decreasing as the cultivation time increasing. The total nitrogen (TN), available phosphorus (AP), ammonium nitrogen ($\text{NH}_4^+\text{-N}$) and nitrate nitrogen ($\text{NO}_3^-\text{-N}$) of Group 3 a, 5 a and 7 a were all higher than those of ck. The total nitrogen contents of Group 5a and 7a were significantly increased by 38.26% and 51.30% compared with ck, whereas the difference between Group 3 a and ck was not significant. The AP contents of Group 3 a, 5 a and 7 a were respective 2.09, 1.62 and 2.27 times that of AP in ck. The soil ammonium and nitrate nitrogen contents increased by cultivation years, in an order of 7 a > 5 a > 3 a > ck.

Table 1

Soil chemical properties with cultivation age of greenhouse vegetables. Diferent letters afer the mean indicate a signifcant difference between treatments.

Treatments	pH	SOC (g kg ⁻¹)	TN(g kg ⁻¹)	AP(mg kg ⁻¹)	AK(mg kg ⁻¹)	NH ₄ ⁺ -N (mg kg ⁻¹)	NO ₃ ⁻ -N (mg kg ⁻¹)
ck	6.22 ± 0.03a	23.35 ± 0.33a	1.15 ± 0.05c	13.83 ± 1.28c	215.23 ± 3.08a	7.66 ± 0.36d	8.43 ± 0.77d
3 a	5.52 ± 0.27b	21.49 ± 0.64b	1.2 ± 0.02c	28.91 ± 1.81ac	176.51 ± 8.24b	14.48 ± 1.27c	18.76 ± 1.38c
5 a	4.82 ± 0.04c	20.85 ± 0.29bd	1.59 ± 0.04b	22.38 ± 0.68b	130.91 ± 3.13c	17.74 ± 1.37b	24.39 ± 1.34b
7 a	4.53 ± 0.06c	17.75 ± 0.33c	1.74 ± 0.05a	31.42 ± 0.42a	87.25 ± 2.08d	21.56 ± 1.07a	30.67 ± 2.33a

nosZ gene abundance

The range of *nosZ* gene copy number with different years of cultivation was $5.19 \times 10^6 \sim 8.78 \times 10^6$ copies/g soil (Fig. 1). The copy numbers of *nosZ* gene in groups with various years of protected cultivation were significantly less than ck, whose number was respective 1.32, 1.45 and 1.69 times that of Group 3 a, 5 a and 7 a. There was no significant difference in the copy number of *nosZ* gene between Group 3 a, 5 a and 7 a, and the copy number was decreasing as cultivation time increased. The correlation analysis of *nosZ* gene abundance and soil chemical properties revealed that the *nosZ* gene abundance had significant positive correlations with soil pH and SOC, highly significant negative correlations with soil TN, ammonium nitrogen and nitrate nitrogen, a significant negative correlation with AP, and a highly significant positive correlation with AK (Fig. 2).

α-diversity index of nosZ type denitrifiers

The cultivation year had a significant impact on the Chao1 index and ACE index of the *nosZ* community, and it had a highly significant impact on the Shannon's index and the Simpson's index (Table 2). The Chao1 index and ACE index displayed the same trend, ranging from 1,057.51 ~ 1,423.84 and 1,070.06 ~ 1,429.96, respectively. Both indices for various protected cultivation years were smaller than those of ck, decreasing as cultivation time increased. Group 7 a had significantly smaller values compared to all other treatments. The ranges of the Shannon's index and Simpson's index were 6.2 ~ 7.02 and 0.82 ~ 0.86, respectively, with Group 7 a significantly smaller than the other treatments. The Shannon's index was the highest in Group 3 a, in an order of 3 a > ck > 5 a > 7 a. The Simpson's index was the highest in Group 5a, in an order of 5 a > 3 a > ck > 7 a.

Table 2

□ diversity index of *nosZ*-type denitrifying bacterial community with cultivation age of greenhouse vegetables. Different letters after the mean indicate a significant difference between treatments.

Treatments	Chao1 index	ACE index	Shannon index	Simpson index
ck	1423.84 ± 65.53a	1429.96 ± 60.49a	7.02 ± 0.08a	0.84 ± 0.01b
3a	1358.68 ± 73.93a	1366.93 ± 77.77a	7.18 ± 0.24a	0.85 ± 0.02ab
5a	1305.4 ± 97.92ac	1312.08 ± 98.23ac	6.96 ± 0.10ac	0.86 ± 0.01a
7a	1057.51 ± 154.83b	1070.06 ± 157.58b	6.2 ± 0.32b	0.82 ± 0.02c

The Chao1 index and the ACE index were both highly significantly positively correlated with soil pH, SOC and AK, and significantly negatively correlated with TN, AP, ammonium nitrogen and nitrate nitrogen. The Shannon's index was significantly positively correlated with SOC, highly significantly negatively correlated with TN, and significantly negatively correlated with ammonium nitrogen and nitrate nitrogen (Fig. 2).

***nosZ* community composition**

By classifying the *nosZ* community OTUs obtained from the samples, a total of six classes were obtained at the phylum level, including *Proteobacteria*, *Bacteria_unidentified*, *Gemmatimonadetes*, *Bacteroidetes*, *Acidobacteria* and *Chloroflexi* (Fig. 3). The relative abundances of soil *Proteobacteria*, *Bacteria_unidentified*, *Acidobacteria* and *Chloroflexi* were significantly different for treatments with different years of protected cultivation. The *Proteobacteria*, *Bacteria_unidentified* and *Gemmatimonadetes* were the dominant phyla, with relative abundance ranges of 62.27 ~ 70.42%, 16.66 ~ 25.19% and 2.88 ~ 3.82%, respectively. The relative abundances of *Proteobacteria* in Group 5a and 7a were less than that of ck, lowered by 2.69% and 10.34%, respectively. The relative abundance of *Bacteria_unidentified* was the highest in Group 7 a, significantly higher than that of Group 3 a and 5 a. The relative abundance of *Gemmatimonadetes* was the highest in Group 3a and the lowest in Group 5 a, in an order of 3 a > 7 a > ck > 5 a. The protected cultivation exerted an inhibitory effect on the *Proteobacteria* phylum, and the inhibition was increased with the increase of the cultivation time.

A total of ten classes were obtained at the genus level with those with an average relative abundance of < 1% classified into one group (Fig. 4), including *Bradyrhizobium*, *Bacteria_unidentified*, *Achromobacter*, *Aromatoleum*, *Gemmatimonas*, *Ochrobactrum*, *Azoarcus*, *Azospirillum*, *Mesorhizobium* and *Rhodopseudomonas*. Among them, significant differences in the relative abundance of *Bradyrhizobium*, *Bacteria_unidentified*, *Achromobacter* and *Rhodopseudomonas* were seen in the protected cultivations for different years. The *Bradyrhizobium*, *Bacteria_unidentified* and *Achromobacter* were the dominant genera, with relative abundance ranges of 39.01 ~ 47.68%, 18.48 ~ 27.68% and 5.28 ~ 8.57%, respectively. The relative abundance of *Bradyrhizobium* in various protected cultivation groups was higher than that of ck, respective 1.23, 1.12 and 1.07 times that of ck, in an order of 3 a > 5 a > 7 a > ck. The relative abundance of *Achromobacter* was significantly lower in Group 7a than the other treatments, in an order of 5 a > ck > 3 a > 7 a. The relative abundance of *Aromatoleum* was increased with the protected cultivation time increase, and the values of both Group 3 a and 5 a were lower than ck.

nosZ community structure and its relationship with soil chemical properties

Clustering analysis By the PCA conducted for the *nosZ* community, it can be seen that the structure of the *nosZ* community in the soils with various protected cultivation years was significantly different (Fig. 5). The first and second principal components interpreted 48.41% and 21.53% of the bacterial community structure variation, respectively, giving a cumulative contribution rate of 69.94%. Group 7 a was far from Group 3 a and 5 a on PC1 and PC2, indicating a large change of *nosZ* community structure as the cultivation time extended. Group 3 a and 5 a were close, indicating a high community similarity.

The redundancy analysis of *nosZ* community structure and soil chemical indicators were performed (Fig. 6). The physical and chemical properties of soil could explain 46.61% of all the information on the first axis and 22.44% on the second axis for the *nosZ* community, giving a number of 69.05% total information explained by the two axes. The SOC and nitrate nitrogen had significant impacts on the *nosZ* community structure. The soil AP, AK and ammonium nitrogen had highly significant effects on *nosZ* community structure.

Discussion

Long-term protected cultivation of vegetable crops had a significant impact on soil chemical indicators. The TN, ammonium nitrogen and nitrate nitrogen were all increased significantly with the increase of protected cultivation time, and all of them were higher than ck. This may be due to that the intensive planting, continuous high fertilizer input and long-term continuous cropping of protected cultivation restricted the growth of vegetable crops, hindered their nutrient absorption, and thus most of the nutrients accumulated in the soil over time^{18,19}. This study demonstrated that the SOC contents of various protected cultivation years were all lower than ck, decreasing year by year. This was contrary to the results of previous studies^{20,21}, but similar to the results of Song et al²². It may be due to the small amount of organic fertilizer applied and a small return of plant residues to soil in this area. In this study, the soil pH value was decreased as the cultivation year increased, consistent with the results of various

studies^{23,24}. Presumably, it is because of the soil acidification caused by the accumulation of SO_4^{2-} and NO_3^- from long-term excessive application of (nitrogen) fertilizers.

Denitrification is an important part responsible for soil nitrogen loss, greenhouse gas N_2O emission and N_2 conversion. Denitrification is primarily driven by microorganisms and is closely related to soil fertilization²⁵. In this study, the copy numbers of *nosZ* in the soil for various protected cultivation years were all significantly less than that of ck, and the copy number of *nosZ* was decreasing with the extension of planting time. It may be due to the high water and fertilizer input of protected cultivation that the reduction of N_2O by soil microorganisms was declined in greenhouses²⁶. Bergaust et al. found that the N_2O reductase of *Paracoccus* was difficult to assemble at a lower pH, ultimately leading to a decrease in the enzyme activity.²⁷ Hallin et al. believed that the copy number of *nosZ* gene dropped rapidly with increased nitrogen fertilizer application rate²⁵. In this study, the *nosZ* gene abundance had significant positive correlations with soil pH and SOC. Because denitrifying microorganisms are generally chemical heterotrophs, they need substances like organic matter as energy sources²⁸. The study conducted by Matlou and Haynes showed that SOC was closely related to soil microbial community structure, and organic carbon was the necessary substrate and energy source for soil microbial activities²⁹. In our study, the SOC content was decreasing with the increase of protected cultivation time, and thus it could not provide a sufficient energy source for denitrifiers. Bowden et al. believed that the accumulation of SOC was due to the reduction of microbial biomass³⁰. Soil pH is the primary environmental factor responsible for variations in the number of denitrifying microorganisms³¹. As the protected cultivation time increased, the soil pH kept decreasing. Soil acidification causes the accumulation of iron and aluminum. Iron and aluminum hydroxides can reduce the utilization of carbon sources by microorganisms via adsorbing soil soluble carbons³². The stronger the soil acidity, the greater the accumulation of Al^{3+} , and the severer the damage to the soil bacterial cell membrane³³. The research by Bauhus and Khanna showed that the addition of phosphorus promoted soil denitrification in acid forest soils³⁴. In this study, the copy number of *nosZ* gene was significantly correlated with AP, further supporting the regulation of soil phosphorus on denitrifiers. Nevertheless, the mechanism of how phosphorus regulates the growth of denitrifying microorganisms remains elusive. Nitrate nitrogen can serve as the substrate of denitrifiers and the electron acceptor in denitrification, thus affects the growth of denitrifying microorganisms³⁵. The results of this study showed that the abundance of *nosZ* gene was highly significantly negatively correlated with soil TN, ammonium nitrogen and nitrate nitrogen. Comprehensive analysis suggested that changes in soil pH and nitrogen content were the primary factors driving the changes in *nosZ* gene copy number.

In this study, the α -diversity index of *nosZ* denitrifying microbial community was significantly different among different treatments. Both Chao1 and ACE indexes were the highest in ck, and gradually decreased with the extension of planting time. It may be due to the long-term protected cultivation that the soil acidification and salinization became severer, resulting in decreased abundance of soil denitrifier species. The Shannon's and Simpson's indices were higher in Group 3a and lower in Group 7a, indicating decreased soil denitrifying microbial species diversity with the planting time extension. It may be that the

long-term protected cultivation led to a decrease in SOC content, which then could not provide the necessary carbon source for the growth of denitrifiers.

The dominant group of *nosZ* denitrifiers was exclusively *Proteobacteria*, consistent with the results of many studies³⁶. The abundance of soil *Proteobacteria* can represent the nutrient level including soil organic matter to a certain extent, and *Proteobacteria* favor a higher pH soil environment³⁷. In this study, the abundance of *Proteobacteria* showed a decreasing trend with the extension of planting time, and was the highest in ck and Group 3a. It may be related to the high soil nutrient content and high pH that are beneficial to the growth of *Proteobacteria*. At the genus level, the common dominant group was *Brachyrhizobium*, whose relative abundance accounted for 43% of the *nosZ* gene sequences on average. The relative abundances of *Brachyrhizobium* in Group 3a, 5a and 7a were all higher than ck, and it was decreasing with the extension of planting time. According to the PCA results, the *nosZ* community was clustered clearly, and Group 7a was well separated from Group 3a and 5a, while the latter two were clustered relatively closely. It indicated that the *nosZ* community structure varied greatly with the planting time increase for the protected cultivation field.

Related researchers believe that the difference in the response of denitrifiers to fertilization is shown in soil pH, TN, ammonium nitrogen and nitrate nitrogen²⁵. Soil pH is considered to be the primary factor affecting the community structure of denitrifying microorganisms. pH can exert a selective effect on denitrifiers, and its variation can affect the community structure of denitrifying microorganisms and further affect their response to environmental changes³¹. In this study, soil pH was significantly different in the protected cultivations for different years. In addition, soil pH was significantly correlated with the relative abundance of *nosZ*, indicating soil pH as the primary cause of changes in denitrifying microbial community structure. Usually, the SOC, ammonium nitrogen and nitrate nitrogen serve as direct or indirect substrates and energy sources for denitrifying microorganisms, and they may affect the community structure of soil denitrifiers. Nitrate nitrogen is the substrate of denitrification, and its content directly affects the utilization of nitrogen by denitrifying microorganisms³⁸.

In conclusion, the *nosZ* gene abundance, Chao1 index and ACE index demonstrated the same trends that decreased as the protected cultivation time increased. The abundance of *nosZ* gene was not significantly different in different protected cultivation years. At the phylum level, *Proteobacteria* was the dominant group, and the most abundant in Group 3 a. At the genus level, *Bradyrhizobium* and *Achromobacter* were dominant groups. The relative abundance of *Bradyrhizobium* was decreased as the protected cultivation time increased, but was all higher than that of ck. The relative abundance of *Achromobacter* was the highest in Group 5 a, showing a “V” trend as the protected cultivation time increased. The total nitrogen, available potassium, ammonium nitrogen and nitrate nitrogen had great impacts on *nosZ* gene abundance, α -diversity index and community structure. It can be seen that nitrogen fertilizer was an important factor limiting the fast-growing and high-yield of vegetables in protected cultivation fields. Soil nitrogen is conducive to vegetable crop absorption and utilization, and can directly or indirectly regulate the denitrifying microbial community.

Materials And Methods

Site Description and Soil Sampling

The study area was located in Heping Town, Tongren City, Guizhou Province, China. It has a subtropical monsoon climate with an average annual temperature of 18 °C, an annual precipitation of 1,313 mm, and a yellow soil type. Three greenhouses with different planting years were selected as the experimental groups, and the nearby outdoor plot was used as a control (ck). For greenhouse plots, the fertilizers applied included a base fertilizer of 80–100 kg/667 m² of compound NPK (Nitrogen, Phosphorus and Potassium) fertilizer, and a topdressing of nitrogen fertilizer of 40–50 kg/667 m². For the outdoor plot, the fertilizers applied were 40–50 kg/667 m² of base fertilizer and 20–30 kg/667 m² of topdressing. The main planted vegetables were eggplants, cucumbers, zucchini, and cowpeas. The samples were collected in July 2017. The 0–10 cm top-soils were sampled following an “S” shape using the five-point sampling method in each plot, mixed as one soil sample, stored in an ice box and transported to the laboratory as soon as possible. After removing stones and roots, the soil sample was passed through a 2-mm sieve. The prepared soil sample was then divided into three parts, one part of fresh soil was used for the determination of ammonium nitrogen and nitrate nitrogen, one part was stored in a -80 °C freezer for the analysis of *nosZ* community structure and abundance, and one part was used for the determination of soil chemical indicators after air-dried indoors and sieved.

Soil Physicochemical Analysis

Soil pH was measured by the potentiometry method. Soil organic carbon content (SOC) was measured via the potassium dichromate oxidation method. Soil total nitrogen (TN) was determined via Kjeldahl method; Soil available phosphorus (AP) was measured via sodium bicarbonate extraction-molybdenum antimony colorimetric. Soil available potassium (AK) was measured via ammonium acetate extraction-flame photometry. Soil ammonium nitrogen (NH₄⁺-N) was measured via indophenol blue colorimetry. Soil nitrate nitrogen (NO₃⁻-N) was measured via disulfonic acid colorimetry ³⁹.

Soil DNA extraction, *nosZ* gene amplification and quantitative fluorescence PCR (qfPCR)

Soil DNA extraction was conducted using the E.Z.N.A.® soil DNA kit (OMEGA, GA, USA) according to the manufacturer's instruction, using approximately 0.5 g of fresh soil. The purity and integrity of the extracted DNA were assessed via 1% agarose gel electrophoresis, and the concentration and purity of the extracted DNA were evaluated with the help of a nucleic acid quantometer (NanoDrop ND-1000). The *nosZ* gene were amplified using a primer set: *nosZ-F*(5'-GGGCTBGGGCCRTTGCA-3') and *nosZ-R*(5'-GAAGCGRTCCTTSGAARACTTG-3')⁴⁰. The volume of the PCR reaction mixture was 25 µL, containing 5.0 µL 10 × ExTaq, 2.0 µL dNPT (2.5 µmol·L⁻¹), 1.0 µL forward and reverse primer (10 µmol·L⁻¹), 2.0 µL DNA template (1–10 ng), 0.25 µL ExTaq (5U·µL⁻¹) and balanced ultrapure water (ddH₂O). The PCR amplification conditions were as follows: predegeneration for 5 min at 94°C, 32 cycles of amplification at

94°C for 30 s, 57°C for 45 s, and 72°C for 55 s, and extension at 72°C for 10 min⁴¹. After the PCR product was purified and recovered, it was ligated to the pMD8-T vector, and transformed into *E. coli* DH5α competent cells. After cultivation, the positive clones were picked and the *nosZ* gene recombinant plasmid was extracted. The plasmid concentration was determined by a nucleic acid quantifier, and the copy number of the gene was calculated. The ten-fold serial dilutions were prepared to a gene copy number of 10³~10⁸, followed by standard curve preparation.

High-throughput Sequencing

After the amplification of the PCR products, the samples were sent to Shanghai Personalbio Technology Co., Ltd. for sequencing with an Illumina MiSeq System. After the raw reads were available, according to the barcodes sequences and primers sequences, the effective reads were screened. Then the barcodes and primers sequences were trimmed off. FLASH was used to merge the paired-end reads that passed the quality preliminary screening based upon their overlapping regions. At the same time USEARCH software was used to check and remove chimeras to obtain high-quality effective sequences. The uclust tool of QIIME was applied to align sequences, the operational taxonomic units (OTUs) were clustered at a similarity threshold of 97%, and the most abundant sequence would serve as the representative sequence of the corresponding OTU. QIIME was further used to compare the representative sequences of OUT with the Functional Gene Pipeline (FunGene) to obtain taxonomic information of each OTU.

Data analysis

SPSS21.0 was used to conduct the statistical significance analysis and correlation analysis of soil chemical properties, α-diversity index and abundance of denitrifiers, and relative abundance of community composition. R was used to perform principal component analysis (PCA) and redundancy analysis.

Declarations

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Author contributions

H.Z and Z.Y.T. conceived and designed the experiments; H.Z and Z.Y.T. performed the experiments; H.Z analyzed the data and wrote the manuscript.

Competing interests

The authors declare no competing interests.

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Figures

Figure 1

Abundance of *nosZ* genes with cultivation age of greenhouse vegetables.

Figure 2

Correlation analysis between *nosZ* genes abundance, diversity index and soil chemical properties

Figure 3

Phylum composition of nosZ-type denitrifying bacterial community with cultivation age of greenhouse vegetables treatments. * and ** indicate a significance difference at 0.05 and 0.01 levels, respectively.

Figure 4

Genus composition of nosZ-type denitrifying bacterial community with cultivation age of greenhouse vegetables treatments. * and ** indicate a significance difference at 0.05 and 0.01 levels, respectively.

Figure 5

Principal components analysis of nosZ-type denitrifying bacterial community with cultivation age of greenhouse vegetables.

Figure 6

Redundancy analysis (RDA) of relationships among the *nosZ* communities and soil chemical properties