

# Changes of diazotrophic communities in response to cropping systems in a Mollisol of Northeast China

Jiaxun Zou<sup>Equal first author, 1, 2</sup>, Qin Yao<sup>Equal first author, 1</sup>, Junjie Liu<sup>1</sup>, Yansheng Li<sup>1</sup>, Fuqiang Song<sup>2</sup>, Xiaobing Liu<sup>1</sup>, Guanghua Wang<sup>Corresp. 1</sup>

<sup>1</sup> Key Laboratory of Mollisols Agroecology, Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, Harbin, China

<sup>2</sup> College of Life Science, Heilongjiang University, Harbin, China

Corresponding Author: Guanghua Wang

Email address: wanggh@iga.ac.cn

Nitrogen-fixing microorganisms play important roles in N cycling. However, knowledge related to the changes in the diazotrophic community in response to cropping systems is still rudimentary. In this study, the *nifH* gene was used to reveal the abundance and community compositions of diazotrophs in the cropping systems of continuous cropping of corn (CC) and soybean (SS), and soybean-corn rotation for growing corn (CSC) and soybean (SCS) in a black soil of Northeast China. The results showed that the abundance of the *nifH* gene was significantly higher in cropping soybean than in cropping corn under the same cropping system, while remarkably increased in the rotation system under the same crop. The Shannon index in the CC treatment was significantly higher than that in the other treatments, but the OTU number and Chao1 index had no significant change among the four treatments. *Bradyrhizobium japonicum* was the dominant diazotrophic species, and its relative abundance was at the lowest value in the CC treatment. In contrast, *Skermanella* sp. had the highest relative abundance in the CC treatment. A PCoA showed that the diazotrophic communities were separated between different cropping systems, and the variation caused by continuous corn cropping was the largest. Among the tested soil properties, the soil available phosphorus was a primary factor in determining diazotrophic community compositions. Overall, the findings of this study highlighted that the diazotrophic communities in black soils are very sensitive to cropping systems.

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**Author names:** Jiaxun Zou<sup>1,2,†</sup>, Qin Yao<sup>1,†</sup>, Junjie Liu<sup>1</sup>, Yansheng Li<sup>1</sup>, Fuqiang Song<sup>2</sup>, Xiaobing Liu<sup>1</sup>, Guanghua Wang<sup>1,\*</sup>

**Affiliation:**

1. Key Laboratory of Mollisols Agroecology, Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, Harbin 150081, China

2. College of Life Science, Heilongjiang University, Harbin 150080, China

† These authors contributed equally to this work

**\* Corresponding author:**

Guanghua Wang

No.138 Haping Road, Harbin, Heilongjiang, 150081, China

E-mail address: [wanggh@iga.ac.cn](mailto:wanggh@iga.ac.cn)

**Abstract:** Nitrogen-fixing microorganisms play important roles in N cycling. However, knowledge related to the changes in the diazotrophic community in response to cropping systems is still rudimentary. In this study, the *nifH* gene was used to reveal the abundance and community compositions of diazotrophs in the cropping systems of continuous cropping of corn (CC) and soybean (SS), and soybean-corn rotation for growing corn (CSC) and soybean (SCS) in a black soil of Northeast China. The results showed that the abundance of the *nifH* gene was significantly higher in cropping soybean than in cropping corn under the same cropping system, while remarkably increased in the rotation system under the same crop. The Shannon index in the CC treatment was significantly higher than that in the other treatments, but the OTU number and Chao1 index had no significant change among the four treatments. *Bradyrhizobium japonicum* was the dominant diazotrophic species, and its relative abundance was at the lowest value in the CC treatment. In contrast, *Skermanella* sp. had the highest relative abundance in the CC treatment. A PCoA showed that the diazotrophic communities were separated between different cropping systems, and the variation caused by continuous corn cropping was the largest. Among the tested soil properties, the soil available phosphorus was a primary factor in determining diazotrophic community compositions. Overall, the findings of this study highlighted that the diazotrophic communities in black soils are very sensitive to cropping systems.

## 1. Introduction

The black soil (Mollisol) zone of Northeast China is one of the four largest black soil regions in the world and plays an important role in maintaining food security in China (Liu et al., 2008). In this region, soybean and corn are two major crops that are either continuously cropped or grown annually in rotation with each

other. It is well known that continuous cropping can decline soil quality, which seriously decreases the quantity and quality of crop products (Zhang et al., 2007). Previous studies have shown that continuous cropping has led to several problems in soils, for example, deficiencies in soil nutrition (Ashworth et al., 2018), decreases in soil enzyme activity (Chavarría et al., 2016), increases in the autotoxicity of root exudates (Huang et al., 2013), increases in pests and diseases (Torres et al., 2018) and imbalances in soil microbial communities (Bennett et al., 2012). Although the barriers of continuous corn cropping are not as serious as those of continuous soybean cropping (Xu et al., 2004), several studies have revealed that continuous cropping of corn has caused the lodging and surge of pests, which have damaged corn yield (Jirak-Peterson & Esker, 2011; Liang et al., 2017). The cause of the barriers of continuous cropping are very complex, and some biotic and abiotic factors are commonly related to crop-yielding decline. Among these factors, biotic factors, such as changes in soil microbial communities, are considered to be the major factor for barriers to continuous cropping (Dias et al., 2015). Studies have shown that continuous cropping can lead to the breakdown of the intrinsic balance of soil microorganisms and the increase of crop pathogens such as an accumulation of soil-borne *Fusarium* spp. (Xiong et al., 2015; Zhu et al., 2018).

Biological nitrogen fixation (BNF) is the main route of inputting nitrogen (N) in natural ecosystems. Approximately 110 Tg of N is input into terrestrial ecosystems annually through the BNF method (Gruber et al., 2008). BNF is carried out by a wide range of microorganisms containing nitrogen-fixing enzymes. The enzymes are regulated by multiple genes, and the *nifH* gene is one of the most conserved functional genes and is commonly used as a biomarker gene for studying nitrogen-fixing microorganisms or diazotrophic communities in soils (Coelho et al., 2009). Previous studies have shown that the diazotrophic

community is highly sensitive to variations in soil factors such as pH (Fan et al., 2018), organic matter (Calderoli et al., 2017) and available nutrient content (Collavino et al., 2014). Since soil factors are greatly affected by different cropping systems (Jha et al., 2004; Moisaner et al., 2012), whether different cropping systems have a direct or indirect impact on diazotrophic communities in soils, especially in black soils, is less reported.

Using molecular fingerprinting methods, such as polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and the terminal restriction fragment length polymorphism (T-RFLP) method, several studies have investigated the changes in diazotrophic communities in response to cropping systems. For example, Silva et al. (2013) revealed that  $\text{NH}_4^+\text{-N}$ ,  $\text{NO}_3^-\text{-N}$ , and soil pH were the determining factors in shifting the diazotrophic community structure by using the PCR-DGGE method. Using the T-RFLP method, Xiao et al. (2010) revealed that diazotrophic community diversity in continuous cropping of soybean fields was different from that in rotational cropping. However, the low resolutions of molecular fingerprinting methods restricted in-depth data analysis and did not allow us to fully understand the changes in diazotrophic community structures. Recently, high-throughput sequencing has become the mainstream method for studying diazotrophic communities. Using this method, Wang et al. (2017) revealed that soil pH and nutrient availability had a cooperative effect on diazotroph abundance, while soil nutrient availability was the main factor. In addition, studies analyzing changes in microbial community structures in response to different cropping systems have mainly focused on bacterial and fungal communities (Bai et al., 2015; Liu et al., 2017) but are rarely related to the diazotrophic community.

In this study, we comparatively investigated the abundance, diversity and community structures of

diazotrophs under different cropping systems in the black soil region of Northeast China using real-time PCR and Illumina MiSeq sequencing methods. Specifically, the purposes of this study were 1) to compare the effect of different cropping systems on soil chemical properties; 2) to assess the responses of diazotroph abundance and diversity to different cropping systems; and 3) to examine the contributions of crop types and soil factors to the changes of diazotrophic community structures.

## 2. Materials and methods

### 2.1 Study site descriptions and soil sampling

A fixed experimental field was established in 2013 at Guangrong Village (47°21'N, 126°49'E), Hailun County, Heilongjiang Province of Northeast China, to evaluate the long-term effectiveness of crop rotation and continuous cropping systems. At the experimental site, the annual mean temperature and precipitation were 1.5 °C and 530 mm, respectively. The soil in the experimental site was typical black soil (classified as Mollisol according to the U.S. soil taxonomy).

Four cropping treatments were selected in this study: continuously cropped corn (CC), continuously cropped soybean (SS), soybean-corn rotation for growing corn crops (CSC) and soybean-corn rotation for growing soybean crops (SCS). The cropping sequence for the four treatments from the starting year of 2013 to the sampling year of 2017 is shown in Fig. S1. The commercial fertilizers of urea, diammonium phosphate, and potassium sulfate were used as N, P and K fertilizers. The dosage of chemical fertilizers used for growing soybean and corn was based on the local farming practice. For cropping soybean, the chemical fertilizers supplied as base fertilizers before sowing were 35.2 kg P ha<sup>-1</sup>, 55.2 kg N ha<sup>-1</sup> and 22.4

kg K ha<sup>-1</sup>. For corn cropping, the chemical fertilizers supplied as base fertilizers before sowing were 35.2 kg P ha<sup>-1</sup>, 66.9 kg N ha<sup>-1</sup> and 22.4 kg K ha<sup>-1</sup>, and 105 kg N ha<sup>-1</sup> was applied as top dressing at the V6 stage (6th leaves) of corn.

Each treatment had four randomly arranged replicate plots. The area for each plot was 75.6 m<sup>2</sup>, which contained 12 rows with a row of 70 cm in width and 9 m in length. Soil samples were collected on 14 September 2017 from the field when soybean was nearly at maturity and corn was at wax maturity. From each plot, five points of soil within a depth of 0-20 cm were randomly collected and combined into a single sample. The soil was placed into individual sterile plastic bags, which were placed in ice boxes and transferred back to the laboratory immediately. After soil samples arrived at the laboratory, the soil was sieved through a 2-mm mesh and divided into two groups: one part was stored at -80 °C for DNA extraction, and the remaining soil was kept at 4 °C for soil chemical analysis.

## 2.2 Soil chemical property analysis

Soil pH was determined using a pH meter in the soil-water suspension (1:2.5 w/v). The concentrations of soil total carbon (TC) and total nitrogen (TN) were determined using an elemental analyzer at 950 °C (VarioEL III, Germany). Soil NH<sub>4</sub><sup>+</sup>-N and NO<sub>3</sub><sup>-</sup>-N extracted with 2.0 M KCl solution, total phosphorus (TP) digested by HClO<sub>4</sub>-H<sub>2</sub>SO<sub>4</sub> and available phosphorus (AP) extracted with NaHCO<sub>3</sub> were measured with a continuous flow analytical system (SKALAR SAN++, The Netherlands). Soil total potassium (TK) digested with HNO<sub>3</sub>-HClO<sub>4</sub>-HF and available potassium (AK) extracted with 1.0 M NH<sub>4</sub>Ac were estimated using inductively coupled plasma-atomic emission spectrometry (ICPS-7500, Shimadzu, Japan) (Lu, 2000).

### 2.3 Soil DNA extractions and quantification of *nifH* gene abundance

The total soil DNA of each sample was extracted from 0.5 g of fresh soil using a FastDNA® SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) following the manufacturer's protocols. The extracted DNA was diluted in DES buffer (DNA Elution Solution-Ultra Pure Water). DNA concentration was determined with the NanoDrop method (NanoDrop 2000, Thermo Scientific, USA) and then stored at -20 °C until further use.

Quantitative real-time PCR (qPCR) was performed using the primers *nifH*-F (5'-AAA GGY GGW ATC GGY AAR TCCA CCA C-3') and *nifH*-R (5'-TTG TTS GCS GCR TAC ATS GCC ATC AT-3') to measure the abundance of the *nifH* gene in a LightCycler®480 (Roche Applied Science, Basel, Switzerland). Each qPCR reaction mixture contained 10 µL of SYBR Premix Ex Taq™ (Takara, Dalian, China), 1 µL of extracted soil DNA, 1 µL of 10 µM forward primer, 1 µL of 10 µM reverse primer and 7.0 µL of sterilized MilliQ water (Rösch et al., 2002). For each sample, qPCR amplification was performed in triplicate following a program of denaturation at 95 °C for 30 s (ramp rate of 4.4 °C/s), 30 amplification cycles of 95 °C for 5 s, 60 °C for 30 s and 50 °C for 30 s. The copy number of *nifH* gene was calculated using the standard curve with a known number (Yao et al., 2017).

### 2.4 Illumina MiSeq sequencing

The diazotrophic community was analysed using the *nifH* gene-specific primers *nifH*-F/*nifH*-R with 7-bp unique barcodes at the 5' end, which produced an approximately 432 bp PCR product. The PCRs were performed in triplicate for each sample in ABI GeneAmp® 9700 with a volume of 20 µL, which comprised



4  $\mu$ L of 5  $\times$  FastPfu Buffer, 2  $\mu$ L of 2.5 mM dNTPs, 0.8  $\mu$ L (5  $\mu$ M) of each primer, 0.4  $\mu$ L of FastPfu Polymerase, 0.2  $\mu$ L of BSA (Bovine Serum Albumin) solution, 1.0  $\mu$ L of template DNA (10 ng) and ddH<sub>2</sub>O to reach 20  $\mu$ L. The PCR amplification program started with 95 °C for 3 min for initial denaturation, followed by 35 cycles of 95 °C for 30 s for denaturation, 55 °C for 30 s for annealing, 72 °C for 45 s, and ended with one final cycle at 72 °C for 10 min for extension. Equimolar amounts of the purified PCR products were pooled and paired-end sequenced on an Illumina MiSeq platform at Majorbio BioPharm Technology Co., Ltd., Shanghai, China.

## 2.5 Sequence data analysis

After sequencing, QIIME Pipeline (Version 1.9.0) was used to process the raw data (<http://qiime.org/tutorials/tutorial.html>) (Caporaso et al., 2010). Sequences which were shorter than 200 bp or had an average base quality score below 20 were removed before further analysis. Removal of the trimmed sequences was done with the UCHIME algorithm (Edgar et al., 2011). Clustering of the high-quality sequences into operational taxonomic units (OTUs) was performed through the UPARSE software (<http://drive5.com/uparse/>) at a 97% similarity level. Representative sequences from each OTU were translated into amino acid sequences and taxonomic identification was performed using a BLASTp search tool at the NCBI website (<https://blast.ncbi.nlm.nih.gov/>). Amino acid sequences were aligned, and a relaxed neighbor-joining tree was built with MEGA7 (Kumar et al., 2016). All raw sequences generated in this study have been deposited in NCBI under the accession PRJNA516581.

## 2.6 Statistical analysis

To compare the difference between samples, the lowest sequence number of 6545 was randomly selected from each sample as subset data for diazotrophic diversity analyses. The alpha diversity of diazotrophic community was presented by Shannon, Simpson, OTU number and Chao1 indices. SPSS software (Version 22.0) was used to evaluate the significant differences in soil properties, diazotrophic abundance and alpha diversity between treatments by one-way ANOVA analysis, as well as to calculate the correlations between the community compositions of diazotroph and soil properties. Based on a UniFrac distance matrix, the beta diversity of diazotrophic community was analyzed through principal coordinate analysis (PCoA) (Gower et al., 1966) in the R environment (version 3.2.5) (R Development Core Team, 2016). The difference in the diazotrophic community between treatments was analyzed using the Adonis method based on Bray-Curtis distances (Clarke et al., 1993), which was conducted using the “vegan” library in the R environment. The relationships between the soil properties and composition of the diazotrophic community were analyzed by Redundancy Analysis (RDA) and envfit analysis in the R environment using the “vegan” library.

## 3. Results

### 3.1 Bulk soil properties

The effects of different cropping systems on soil chemical properties are summarized in Table 1. The soil pH, TK, AK and TC contents were not significantly changed among the treatments. The AP content varied significantly among treatments ( $P < 0.05$ ), with the highest and the lowest values in the SCS and CC

treatments, respectively. C/N was significantly higher in the CC and SS treatments than in the CSC and SCS treatments ( $P < 0.05$ ). The contents of soil TN,  $\text{NH}_4^+\text{-N}$  and  $\text{NO}_3^-\text{-N}$  were significantly changed among the treatments ( $P < 0.05$ ), with the highest values in the CSC treatment. Overall, this result indicated that different cropping systems significantly changed the soil nutrient content, especially the chemical properties related to nitrogen nutrition.

### 3.2 Abundance of the *nifH* gene

The abundance of the *nifH* gene varied significantly among treatments ( $P < 0.01$ ), and it ranged from an average of  $2.3 \times 10^6$  to  $11.1 \times 10^6$  gene copies per gram of soil under different cropping systems (Fig. 1a). The abundance of the *nifH* gene in the SCS treatment increased 57.4%, 123.8% and 382.3% compared to that in SS, CSC and CC, respectively. Pearson's correlation analysis showed that the abundance of the *nifH* gene was marginally correlated with the values of C/N ( $r = -0.541$ ,  $P = 0.031$ ),  $\text{NO}_3^-\text{-N}$  ( $r = -0.602$ ,  $P = 0.014$ ), and AP ( $r = 0.597$ ,  $P = 0.015$ ) (Fig. 1b).

### 3.3 Alpha diversity index of the diazotrophic community

The alpha diversity of the diazotrophic community showed that the Shannon index in the CC treatment was higher than that in the other three treatments, while the Simpson index had the opposite result. The OTU numbers and Chao1 values were not significantly different among treatments (Fig. 2). Pearson's correlation analysis showed that the Shannon index had a significant positive correlation with C/N ( $r = 0.517$ ,  $P = 0.040$ ) and a significant negative correlation with AP ( $r = -0.664$ ,  $P = 0.005$ ). In contrast, the Simpson index showed the opposite trend (Table 2).

### 3.4 Diazotrophic community composition

A total of 205,696 high quality sequences were obtained across all samples, with an average of 12,853 sequences per sample. A random subset of 6545 (minimum number of sequences) sequences was selected from each sample for downstream analysis. Based on 97% similarity, 51 different OTUs were obtained across all samples. A neighbor-joining phylogenetic tree showed that 49 OTUs in this study fell into the orders of Burkholderiales, Desulfuromonadales, Myxococcales, Nostocales, Rhizobiales and Rhodospirillales, and two OTUs (OTU3 and OTU9) grouped with uncultured bacteria (Fig. 3).

Proteobacteria was the dominant phylum in all treatments, with relative abundances ranging from 99.08% to 100% across all samples. Within this phylum, the Alphaproteobacteria was the dominant class, with an average relative abundance ranging from 94.35% to 96.13%, while the average relative abundance of Betaproteobacteria and Deltaproteobacteria was only 0.65% to 1.71%, respectively. In addition, a very low abundance of Cyanobacteria was detected in some samples (Table 3).

At the order level, six groups, Rhizobiales, Rhodospirillales, Desulfuromonadales, Burkholderiales, Nostocales and Myxococcales, were detected (Table 3). Among them, Rhizobiales was the dominant order, with an average relative abundance ranging from 81.97% to 92.47% across treatments. Rhodospirillales was the second most abundant order, with average relative abundance ranging from 2.09% to 10.09%. The other orders had very lower abundances. The relative abundance of Rhizobiales was significantly lower, but Rhodospirillales was significantly higher in the CC treatment than in the other three treatments ( $P < 0.05$ ).

At the family level, Bradyrhizobiaceae and Rhodospirillaceae were the two most abundant diazotrophs, and their average relative abundances ranged from 63.20% to 87.25% and from 2.09% to 10.09% across treatments, respectively (Table 3). The relative abundances of Bradyrhizobiaceae and Rhodospirillaceae were significantly lower and higher ( $P < 0.05$ ) in the CC treatment than in the other three treatments, respectively.

At the OTU level, although 51 OTUs were detected in this study, only 15 OTUs had a relative abundance of more than 0.3% in at least one treatment (Table 4). Among them, OTU17 was a dominant member with an average relative abundance ranging from 63.01% to 87.26% across all treatments, and its relative abundance was significantly lower in the CC treatment than in the other three treatments. In the rotation system, OTU17 was lower in the CSC treatment than in the SCS treatment. A BLAST search at the amino acid level showed that OTU17 had 100% identity with *Bradyrhizobium japonicum* (ABO27443). OTU13 was the second most abundant member of the diazotroph and had 99% identity with *Bradyrhizobium* sp. (AKN21127). The abundance of OTU13 was at the highest level in CC than in the other treatments. Similarly, other more abundant OTUs were significantly higher in CC than in the other treatments. In particular, several OTUs classified into the genus *Skermanella* (OTU5, 10, 26, 30, 31), which showed the highest abundance in the CC treatment (Table 4).

### 3.5 Changes of diazotrophic community structures

The PCoA plot of all diazotrophic community structures is shown in Fig. 4a. PCoA1 and PCoA2 explained 80.87% and 8.40% of the variation of the community structures, respectively, indicating that the

diazotrophic community structures mainly changed along the PCoA1 axis. All samples were clearly separated into two main groups: one group contained samples of CC, and the other group consisted of samples from SS, CSC and SCS. Noticeably, although the diazotrophic communities among the treatments of SS, CSC and SCS were grouped closely with each other, the Adonis analysis indicated that the communities were significantly different between SS and CSC ( $P = 0.028$ ), between CSC and SCS ( $P = 0.035$ ), but not significant different between SS and SCS ( $P = 0.055$ ) (Table S1). The envfit analysis showed that three soil factors were significantly correlated with changes in community structures (Table S2). Of these,  $\text{NO}_3^-$ -N was positively correlated with the CC treatment, and AP was positively correlated with the SS, SCS and CSC treatments (Fig. 4b). In addition, the Mantel test showed that AP ( $r = 0.26$ ,  $P = 0.038$ ) was the most important factor in shifting the diazotrophic community structures (Table S3).

## 4. Discussion

### 4.1 Changes in soil chemical properties in different cropping systems

Previous studies have shown that different cropping systems significantly change the soil chemical properties (Liu et al., 2017). For example, Jagadamma et al. (2008) and Miao et al. (2007) found that soil pH, TN, TC and available nutrient contents were significantly increased after long-term rotation of soybean. However, in this study, we found that soil pH and TC had no significant changes among the four treatments. Meriles et al. (2009) stated that the change in soil pH was related to the sampling times, and the soil pH was significantly changed at planting time in different cropping systems, while no change was observed at harvest time. In this study, the soils were collected near crop maturity, which may have resulted in no

significant change in soil pH among the treatments. In addition, the finding of no significant change in soil TC content in this study is consistent with previous studies (Spargo et al., 2008; Liu et al., 2017), which may be related to the short-term experimental period (only 5 years) in this study. However, compared with the continuous cropping treatments of SS and CC, TN content in the rotation was significantly increased under the same crop (Table 1). The reasons for this may be related to two aspects: one is the rotation benefit for the growth of soybeans, which can fix more nitrogen from the air and promote the increase of TN content in soils (Adeboye et al., 2006), and the other is that rotations including corn put more N fertilizer into the soil compared with the SS treatment, which can increase TN content in soil. Similarly, the changes in available nutrients, such as AP,  $\text{NH}_4^+\text{-N}$  and  $\text{NO}_3^-\text{-N}$ , may also be related to different fertilizer regimes between corn cropping and soybean cropping.

#### 4.2 Effects of cropping system on the abundance of the *nifH* gene and diazotroph diversity

Previous studies have shown that different cropping systems significantly change the abundance of the *nifH* gene in agricultural soils (Reardon et al., 2014). In this study, we found that the abundance of the *nifH* gene was higher in cropping rotation than in continuous cropping, and it was also higher when the growth crop was soybean than when the crop was corn (Fig. 1a), suggesting that the abundance of the *nifH* gene is influenced by both cropping system and crop type. In addition, the finding that the *nifH* gene abundance was negatively correlated with  $\text{NO}_3^-\text{-N}$  in this study (Fig. 1b) is consistent with the result reported by Mirza et al. (2014), who stated that the increase in  $\text{NO}_3^-\text{-N}$  content in soil satisfied the demand of microorganisms for nitrogen, and the abundance of diazotrophs containing the *nifH* gene was significantly decreased. The finding that the *nifH* gene abundance is also negatively correlated with C/N (Fig. 1b) is consistent with the

results of Wang et al. (2012), which stated that the levels of C and N influence the activity and distribution of nitrogen-fixing bacteria. Furthermore, the abundance of the *nifH* gene is positively correlated with AP (Fig. 1b), suggesting that AP is an important constraint in adjusting the ability of N<sub>2</sub> fixation, and this result is consistent with a report by Tang et al. (2017).

The soil microbial community structures and the diversity index were affected by different cropping systems (Yin et al., 2010). Based on the meta-analysis of the soil microbial diversity index in different cropping systems, Venter et al. (2016) stated that only 15.1% and 3.4% of the microbial community richness and diversity index in the rotation system showed an increasing trend, and the changes in the soil microbial diversity index were closely related to the different rotation systems and the years of continuous cropping. In this study, the Shannon index of diazotrophs in the CC treatment was significantly higher than that in the other treatments (Fig. 2), indicating that continuous cropping of corn significantly increased the alpha diversity of the diazotrophic community. This phenomenon may be related to the significant increase in the number of OTU17 in cropping systems including soybean (Table 4), since the increase of a single bacterium can lead to decrease community evenness and Shannon diversity index (Ortiz-Burgos, 2016). Consistent with the results of Navarro et al. (2013), there was no significant difference in the diversity index between the SS and SCS treatments (Fig. 2). In addition, there was no significant difference in the Chao1 and OTU number index among the treatments, suggesting that the crop systems significantly changed the abundance rather than species of the diazotrophic community. Furthermore, the finding that the Shannon index of the diazotrophic community was significantly negatively correlated with AP content (Table 2) was consistent with the results of Eisenhauer (2016). The diazotrophic community is highly sensitive to P content, and P



is an important element for microorganism growth and development (Jean et al., 2013).

### 4.3 Effects of cropping systems on diazotrophic community structures

The diazotrophs in the black soil ecosystem are dominated by Proteobacteria (Ding et al., 2016). Meanwhile, Silva et al. (2013), based on PCR-DGGE analysis, has shown that *Bradyrhizobium* is the most abundant genus of diazotrophs in long-term rotation systems. In this study, we found that compared with the CC treatment, the abundance of Rhizobiales was significantly increased in the SS, SCS and CSC treatments (Table 3), which is mainly because most Rhizobiales are symbiotic with the roots of legumes, leading to better breeding. Inderjit (2005) reported that the quantity and quality of crop secretions from different crops could lead to changes in diazotroph colonization. Consistent with the results of Silva et al. (2013), this study found that *Bradyrhizobium* sp. is the dominant species of the diazotrophs in all treatments. We found that the most abundant OTU17, which was classified as *Bradyrhizobium japonicum*, was significantly higher in SS, CSC and SCS than in CC (Table 4), suggesting that cropping systems, including soybean, are beneficial to the multiplication of this bacterium. Noticeably, although continuous cropping of corn was conducted for 5 years in this study, the members belonging to the *Bradyrhizobium* sp. (OTU17 and OTU13) were still in higher abundance than other OTUs in the CC treatment (Table 4). We observed that this result is not surprise since *Bradyrhizobium* sp. can act as a soil saprophyte and survive in soil without legume plants (Silva et al., 2013). Recently, a study also showed that *Bradyrhizobium* sp. is the dominant member of the diazotrophic community in the annual rotation of summer corn with winter wheat in southern China (Wang et al., 2017). One of the interesting findings is that the CC treatment led to a higher abundance of OTU13 than in the other three treatments (Table 4). This finding was not reported

previously, and the reasons need to be revealed with further studies. Another noteworthy finding was that the OTUs classified in the *Skermanella* genus had the highest abundance in the CC treatment (Table 4). This finding was consistent with the results of Hu et al. (2018), who detected that *Skermanella* was the dominant diazotroph under 35 years of monoculture of corn in a black soil. Therefore, we speculate that the relative abundance of *Skermanella* will increase with years of continuous corn cropping in the black soils.

The diazotrophic community structure in CC was significantly different from that in the other treatments (Fig. 4a). Meanwhile, the structure of diazotrophs in SCS was markedly different from that in the CSC treatment, suggesting that different crops significantly change the diazotrophic community structure (Kent and Triplett, 2002; Wardle et al., 2004). Inderjit (2005) reported that the main reason for forming different microbial communities is the allelopathic effects between plants and microorganisms. Soybean and corn secrete different root exudates into the soil, indicating different microbial colonization and a correspondingly change the diazotrophic community structure in soils. Interestingly, there was no significant difference in the diazotrophic community structures between the SS and SCS treatments, indicating that when the growing crop is soybean, the diazotrophic community structures are not influenced by the cropping system. However, given only five years of continuous soybean cropping involved in this study, whether long-term cropping, such as longer than 10 years of continuous soybean cropping, could change the diazotrophic community structures compared with SCS needs to be addressed in future studies.

Both RDA and the Mantel test results showed that AP had the greatest contribution to the changes of the diazotrophic community structures (Fig. 4b; Table S3), indicating that AP is the main driving factor for

the variation of diazotrophic communities after changes in cropping systems. It is well known that P is an essential element of microorganisms and an important component of nucleotides, cell membranes, and energy substances (Tang et al., 2017). Although soil enzyme activity was not determined in this study, a previous report has shown that soil phosphatase activity was significantly increased in crop rotation compared with continuous cropping systems (He et al., 2010) since crop rotation can allow more organophosphate mineralization to be available as phosphorus for plant and microorganism growth, development, and reproduction (Alvey et al., 2001). In addition, previous studies have shown that the form and content of phosphorus in soil have a significant effect on the growth of different types of diazotrophs (Zhang et al., 2013), indicating that the AP content in soil is closely related to the diazotrophic community structure.

## 5. Conclusions

In summary, this study revealed that the abundance of the *nifH* gene in crop rotation was higher than that in continuous cropping, and higher in soybean cropping than in corn cropping. Continuous cropping of corn increased the Shannon diversity of the diazotrophic community but had no significant influence on the OTU number and Chao1 index. Moreover, members belonging to *Bradyrhizobium* sp. were significantly increased under the cropping systems including soybean. However, *Skermanella* sp. were increased under continuous corn cropping. The diazotrophic community structures differed with cropping systems, especially continuous corn cropping, which significantly changed the community structures. The soil parameters of available phosphorus played a critical role in shaping the diazotrophic communities. Overall, our results demonstrated that continuous cropping and rotation systems significantly altered the abundance,

diversity and community structures of the diazotrophic community in black soils.

# References

Adeboye MKA, Iwuafor ENO, Agbenin JO. 2006. The effects of crop rotation and nitrogen fertilization on soil chemical and microbial properties in a Guinea Savanna Alfisol of Nigeria. *Plant and Soil* 281:97-107 DOI: 10.1007/s11104-005-3828-5.

Alvey S, Bagayoko M, Neumann G, Buerkert A. 2001. Cereal/legume rotations affect chemical properties and biological activities in two West African soils. *Plant and Soil* 231: 45-54 DOI: 10.1023/a:1010386800937.

Ashworth AJ, Allen FL, Debruyn JM, Owens PR, Sams C. 2018. Crop rotations and poultry litter affect dynamic soil chemical properties and soil biota long term. *Journal of Environmental Quality* 47:1327-1338 DOI: 10.2134/jeq2017.12.0465.

Bai L, Cui J Q, Jie WG, Cai BY. 2015. Analysis of the community compositions of rhizosphere fungi in soybeans continuous cropping fields. *Microbiological Research* 180: 49-56 DOI: 10.1016/j.micres.2015.07.007.

Bennett AJ, Bending GD, Chandler D, Hilton S, Mills P. 2012. Meeting the demand for crop production: the challenge of yield decline in crops grown in short rotations. *Biological Reviews* 87: 52-71 DOI: 10.1111/j.1469-185X.2011.00184.x.

Calderoli PA, Collavino MM, Kraemer FB, Morras HJM, Aguilar OM. 2017. Analysis of *nifH*-RNA reveals phylotypes related to Geobacter and Cyanobacteria as important functional components of the N<sub>2</sub>-

fixing community depending on depth and agricultural use of soil. *MicrobiologyOpen* 6: e00502 DOI: 10.1002/mbo3.502.

Caporaso JG, Kuczynski J, Stombaugh J. 2010. QIIME allows analysis of high-throughput community sequencing data. *Nature Methods* 7: 335-336 DOI: 10.1038/nmeth.f.303.

Chavarria DN, Verdenelli RA, Serri DL, Restovich SB, Andriulo AE, Meriles JM, Vargas-Gil S. 2016. Effect of cover crops on microbial community structure and related enzyme activities and macronutrient availability. *European Journal of Soil Biology* 76: 74-82 DOI: 10.1016/j.ejsobi.2016.07.002.

Clarke KR. 1993. Nonparametric multivariate analyses of changes in community structure. *Austral Ecology* 18: 117-143 DOI: 10.1111/j.1442-9993.1993.tb00438.x.

Coelho MRR, Marriel IE, Jenkins SN, Lanyon CV, Seldin L, O'Donnell AG. 2009. Molecular detection and quantification of *nifH* gene sequences in the rhizosphere of sorghum (*Sorghum bicolor*) sown with two levels of nitrogen fertilizer. *Applied Soil Ecology* 42: 48-53 DOI: 10.1016/j.apsoil.2009.01.010.

Collavino MM, Tripp HJ, Frank IE, Vidoz ML, Calderoll PA, Donato M, Zehr JP, Aguilar OM. 2014. *nifH* pyrosequencing reveals the potential for location-specific soil chemistry to influence N<sub>2</sub>-fixing community dynamics. *Environmental Microbiology* 16: 3211-3223 DOI: 10.1111/1462-2920.12423.

Dias T, Dukes A, Antunes PM. 2015. Accounting for soil biotic effects on soil health and crop productivity in the design of crop rotations. *Journal of the Science of Food and Agriculture* 95: 447-454 DOI: 10.1002/jsfa.6565.

Ding JL, Jiang X, Ma MC, Zhou BK, Guan DW, Zhao BS, Zhou J, Cao FM, Li L, Li J. 2016. Effect of 35 years inorganic fertilizer and manure amendment on structure of bacterial and archaeal communities

in black soil of northeast China. *Applied Soil Ecology* 105: 187-195 DOI: 10.1016/j.apsoil.2016.04.010.

Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. 2011. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27: 2194 DOI: 10.1093/bioinformatics/btr381.

Eisenhauer N. 2016. Plant diversity effects on soil microorganisms: Spatial and temporal heterogeneity of plant inputs increase soil biodiversity. *Pedobiologia* 59: 175-177 DOI: 10.1016/j.pedobi.2016.04.004.

Fan KK, Weisenhorn P, Gilbert JA, Shi Y, Bai Y, Chu HY. 2018. Soil pH correlates with the co-occurrence and assemblage process of diazotrophic communities in rhizosphere and bulk soils of wheat fields. *Soil Biology and Biochemistry* 121: 185-192 DOI: 10.1016/j.soilbio.2018.03.017.

Gower J. 1966. Some distance properties of latent root and vector methods used in multivariate analysis. *Biometrika* 53: 325 DOI: 10.1093/biomet/53.3-4.325.

Gruber N, Galloway JN. 2008. An Earth-system perspective of the global nitrogen cycle. *Nature* 451: 293-296 DOI: 10.1038/nature06592.

He ZQ, Honeycutt CW, Griffin TS, Larkin RP, Olanya M, Halloran JM. 2010. Increases of soil phosphatase and urease activities in potato fields by cropping rotation practices. *Journal of Food Agriculture and Environment* 8: 1112-1117 DOI: 10.3168/jds.2010-93-4-1785.

Hu XJ, Liu JJ, Zhu P, Wei D, Jin J, Liu XB, Wang GH. 2018. Long-term manure addition reduces diversity and changes community structure of diazotrophs in a neutral black soil of northeast China. *Journal of Soils and Sediments* 18: 2053-2062 DOI: 10.1007/s11368-018-1975-6.

- 405 Huang LF, Song LX, Xia XJ, Mao WH, Shi K, Zhou YH, Yu JQ. 2013. Plant-Soil feedbacks and soil  
406 sickness: from mechanisms to application in agriculture. *Journal of Chemical Ecology* 39: 232-242  
407 DOI: 10.1007/s10886-013-0244-9.
- 408 Inderjit. 2005. Soil microorganisms: An important determinant of allelopathic activity. *Plant and Soil* 274:  
409 227-236 DOI: 10.1007/s11104-004-0159-x.
- 410 Jagadamma S, Lal R, Hoeft RG, Naffiger ED, Adey EA. 2008. Nitrogen fertilization and cropping system  
411 impacts on soil properties and their relationship to crop yield in the central Corn Belt, USA. *Soil and*  
412 *Tillage Research* 98: 120-129 DOI: 10.1016/j.still.2007.10.008.
- 413 Jean ME, Phalyvong K, Forestdrolet J, Bellenger JP. 2013. Molybdenum and phosphorus limitation of  
414 asymbiotic nitrogen fixation in forests of Eastern Canada: Influence of vegetative cover and seasonal  
415 variability. *Soil Biology and Biochemistry* 67: 140-146 DOI: 10.1016/j.soilbio.2013.08.018.
- 416 Jha MN, Prasad AN, Misra SK. 2004. Influence of source of organics and soil organic matter content on  
417 cyanobacterial nitrogen fixation and distributional pattern under different water regimes. *World*  
418 *Journal of Microbiology and Biotechnology* 20: 673-677 DOI: 10.1007/s11274-004-2157-9.
- 419 Jirak-Peterson JC, Esker PD. 2011. Tillage, crop rotation, and hybrid effects on residue and corn  
420 anthracnose occurrence in Wisconsin. *Plant Disease* 95: 601-610 DOI: 10.1094/PDIS-11-10-0837.
- 421 Kent AD, Triplett EW. 2002. Microbial communities and their interactions in soil and rhizosphere  
422 ecosystems. *Annual Review of Microbiology* 56: 211-236 DOI:  
423 10.1146/annurev.micro.56.012302.161120.
- 424 Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular evolutionary genetics analysis version 7.0 for  
425 bigger datasets. *Molecular Biology and Evolution* 33: 1870-1874 DOI: 10.1093/molbev/msw054%20.

426 Liang AZ, Zhai ZL, Mclaughlin NB, Yang XM, Zhang XP, Chen XW, Sun BJ. 2017. Lodging in corn  
427 varies with tillage and crop rotation: A case study after Typhoon Bolaven pummeling over the black  
428 soil zone in northeast China. *Pakistan Journal of Agricultural Sciences* 54: 539-545 DOI:  
429 10.21162/PAKJAS/17.4156.

430 Liu JJ, Yu ZH, Yao Q, Hu XJ, Zhang W, Mi G, Chen XL, Wang GH. 2017. Distinct soil bacterial  
431 communities in response to the cropping system in a Mollisol of northeast China. *Applied Soil Ecology*  
432 119: 407-416 DOI: 10.1016/j.apsoil.2017.07.013.

433 Liu XB, Jin J, Wang GH, Herbert SJ. 2008. Soybean yield physiology and development of high-yielding  
434 practices in Northeast China. *Field Crops Research* 105: 157-171 DOI: 10.1016/j.fcr.2007.09.003.

435 Lu RK. 2000. Analytical methods for soil and agro-chemistry. *Beijing: China Agricultural Science and*  
436 *Technology Press* (In Chinese).

437 Meriles JM, Gil SV, Conforto C, Figoni G, Lovera E, March GJ, Guzman CA. 2009. Soil microbial  
438 communities under different soybean cropping systems: characterization of microbial population  
439 dynamics, soil microbial activity, microbial biomass, and fatty acid profiles. *Soil and Tillage Research*  
440 103: 271-281 DOI: 10.1016/j.still.2008.10.008.

441 Miao SJ, Qiao YF, Hang XZ. 2007. Review of researches on obstacles of continuous cropping of soybean.  
442 *Chinese Journal of Eco-Agriculture* 15: 203-206 (In Chinese).

443 Mirza BS, Potisap C, Nüsslein K, Bohannan BJM, Rodrigues JLM. 2014. Response of free-living nitrogen-  
444 fixing microorganisms to land use change in the Amazon rainforest. *Applied and Environmental*  
445 *Microbiology* 80: 281-288 DOI: 10.1128/AEM.02362-13.



446 Moisander PH, Cheshire LA, Braddy J, Calandrino ES, Hoffman M, Piehler MF, Paerl HW. 2012.  
447 Facultative diazotrophy increases *Cylindrospermopsis raciborskii* competitiveness under fluctuating  
448 nitrogen availability. *FEMS Microbiology Ecology* 79: 800-811 DOI: 10.1111/j.1574-  
449 6941.2011.01264.x.

450 Navarro NYE, Gómez AS, Montoya CN, Rojas VA, Suarez AMC, Valenzuela EC, Jimenez BN, Verhulst  
451 N, Govaerts B, Dendooven L. 2013. Relative impacts of tillage, residue management and crop-rotation  
452 on soil bacterial communities in a semi-arid agroecosystem. *Soil Biology and Biochemistry* 65: 86-95  
453 DOI: 10.1016/j.soilbio.2013.05.009.

454 R Development Core Team. 2006. R, a language and environment for statistical computing. R 21.  
455 Foundation for Statistical Computing, Vienna, Austria.

456 Reardon CL, Gollany HT, Wuest SB. 2014. Diazotroph community structure and abundance in wheat–  
457 fallow and wheat–pea crop rotations. *Soil Biology and Biochemistry* 69: 406-412 DOI:  
458 10.1016/j.soilbio.2013.10.038.

459 Rösch C, Mergel A, Bothe H. 2002. Biodiversity of denitrifying and dinitrogen-fixing bacteria in an acid  
460 forest soil. *Applied and Environmental Microbiology* 68: 3818-3829 DOI: 10.1128/AEM.68.8.3818-  
461 3829.2002.

462 Ortiz-Burgos S. 2016. Shannon-Weaver Diversity Index. *Springer Netherlands* DOI: 10.1007/978-94-017-  
463 8801-4\_233.

464 Silva MCPE, Schloterhai B, Schloter M, Elsas JD, Salles JF. 2013. Temporal dynamics of abundance and  
465 composition of nitrogen-fixing communities across agricultural soils. *PLoS One* 8: e74500 DOI:  
466 10.1371/journal.pone.0074500.

467 Spargo JT, Alley MM, Follett RF, Wallace JV. 2008. Soil nitrogen conservation with continuous no-till  
468 management. *Nutrient Cycling in Agroecosystems* 82: 283-297 DOI: 10.1007/s10705-008-9190-2.

469 Tang YF, Zhang MM, Chen AL, Zhang WZ, Wei WX, Sheng R. 2017. Impact of fertilization regimes on  
470 diazotrophy community compositions and N<sub>2</sub>-fixation activity in paddy soil. *Agriculture Ecosystems  
471 & Environment* 247: 1-8 DOI: 10.1016/j.agee.2017.06.009.

472 Torres FZV, Souza DA, Lira ED, Faria M, Sujii E, Lopes RB. 2018. Occurrence of the anamorphic stage  
473 of *Ophiocordyceps myrmicarum* on a non-Formicidae insect in integrated crop-livestock farming  
474 systems. *Fungal Ecology* 34: 83-90 DOI: 10.1016/j.funeco.2018.05.009.

475 Venter ZS, Jacobs K, Hawkins HJ. 2016. The impact of crop rotation on soil microbial diversity: A meta-  
476 analysis. *Pedobiologia* 59: 215-223 DOI: 10.1016/j.pedobi.2016.04.001.

477 Wang C, Zheng M, Song WF, Wen SL, Wang BR, Zhu CQ, Shen RF. 2017. Impact of 25 years of inorganic  
478 fertilization on diazotrophic abundance and community structure in an acidic soil in southern China.  
479 *Soil Biology and Biochemistry* 113: 240-249 DOI: 10.1016/j.soilbio.2017.06.019.

480 Wang S, Pablo GP, Ye J, Huang DF. 2012. Abundance and diversity of nitrogen-fixing bacteria in  
481 rhizosphere and bulk paddy soil under different duration of organic management. *World Journal of  
482 Microbiology and Biotechnology* 28: 493-503 DOI: 10.1007/s11274-011-0840-1.

483 Wardle DA, Bardgett RD, Klironomos JN, Setälä H, Putten WH, Wall DH. 2004. Ecological linkages  
484 between aboveground and belowground biota. *Science* 304: 1629-1633 DOI:  
485 10.1126/science.1094875.

- Xiao CH, Tang H, Pu LJ, Sun DM, Ma JZ, Yu M, Duan RS. 2010. Diversity of nitrogenase (*nifH*) genes pool in soybean field soil after continuous and rotational cropping. *Journal of Basic Microbiology* 50: 373-379 DOI: 10.1002/jobm.200900317.
- Xiong W, Zhao QY, Zhao J, Xun WB, Li R, Zhang RF, Wu HS, Shen QR. 2015. Different continuous cropping spans significantly affect microbial community membership and structure in a vanilla-grown soil as revealed by deep pyrosequencing. *Microbial Ecology* 70: 209-218 DOI: 10.1007/s00248-014-0516-0.
- Xu YL, Li CJ, Li ZL. 2004. Effects of corn rotation and continuous cropping system on weed population. *Chinese Journal of Ecology* 23: 37-40 (In Chinese).
- Yao Q, Liu JJ, Yu ZH, Li YS, Jin J, Liu XB, Wang GH. 2017. Three years of biochar amendment alters soil physiochemical properties and fungal community composition in a black soil of northeast China. *Soil Biology and Biochemistry* 110: 56-67 DOI: 10.1016/j.soilbio.2017.03.005.
- Yin CT, Jones KL, Peterson DE, Garrett KA, Hulbert SH, Paulitz TC. 2010. Members of soil bacterial communities sensitive to tillage and crop rotation. *Soil Biology and Biochemistry* 42: 2111-2118 DOI: 10.1016/j.soilbio.2010.08.006.
- Zhang B, Liang C, He HB, Zhang XD. 2013. Variations in soil microbial communities and residues along an altitude gradient on the northern slope of Changbai Mountain, China. *PLoS One* 8: e66184 DOI: 10.1371/journal.pone.0066184.
- Zhang XY, Sui YY, Zhang XD, Meng K, Herbert SJ. 2007. Spatial variability of nutrient properties in black soil of northeast China. *Pedosphere* 17: 19-29 DOI: 10.1016/S1002-0160(07)60003-4.

506 Zhu SY, Wang YZ, Xu XM, Liu TM, Wu DQ, Zheng X, Tang SW, Dai QZ. 2018. Potential use of high-  
 507 throughput sequencing of soil microbial communities for estimating the adverse effects of continuous  
 508 cropping on ramie (*Boehmeria nivea* L. Gaud). *PLoS One* 13: e0197095 DOI:  
 509 10.1371/journal.pone.0197095.

# Figure 1

Effect of different cropping systems on diazotrophic *nifH* gene abundance (a) and the bivariate correlation between the abundance of the *nifH* gene and soil factors in black soil (b)

CC and SS represent the treatments of continuous cropping corn and soybean, respectively; CSC and SCS represent the treatments of soybean-corn rotation for growing corn and soybean, respectively. Error bars show the standard deviation of abundance, and the bars marked with different letters show the significant difference at  $P < 0.05$ .

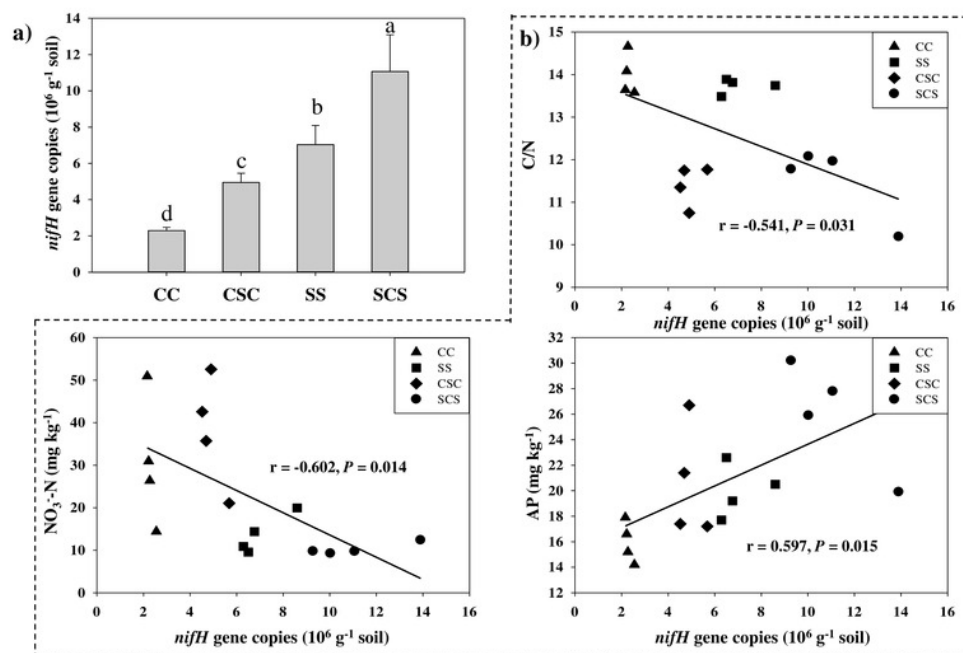


Figure 1

# Figure 2

Effect of different cropping systems on the alpha diversity indices of diazotrophic communities calculated based on a randomly selected subset of 6545 sequences per sample.

The abbreviations of CC, SS, CSC and SCS are described in Figure 1. The bars marked with different letters show the significant difference at  $P < 0.05$ .

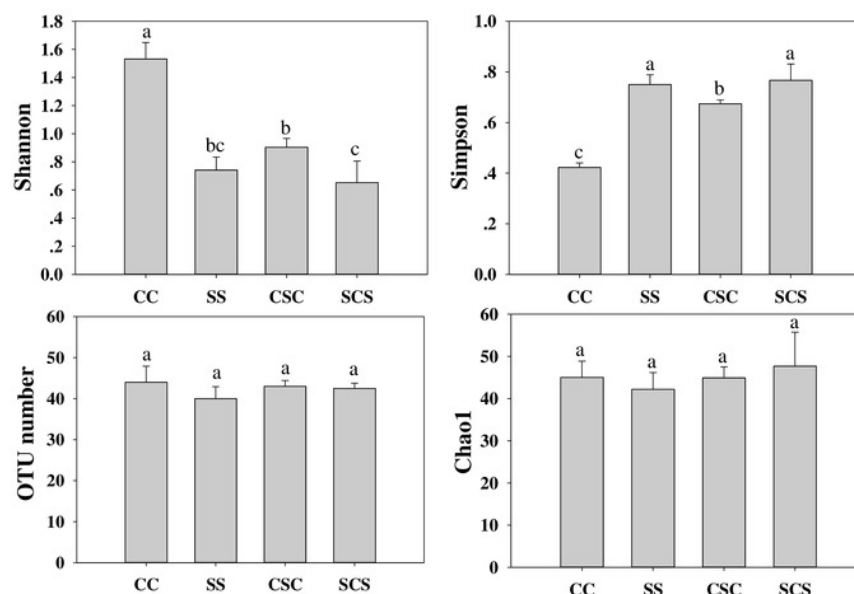


Figure 2

# Figure 3

Neighbor-joining phylogenetic tree showing the relationships of the *nifH* gene obtained in this study with their closest represented microbes at the amino acid level.

Numbers in the parentheses are the accession numbers of the amino acid sequences on the NCBI website. Bootstrap values less than 50 are not shown. The scale bar represents the abundance of amino acid substitutions per residue.

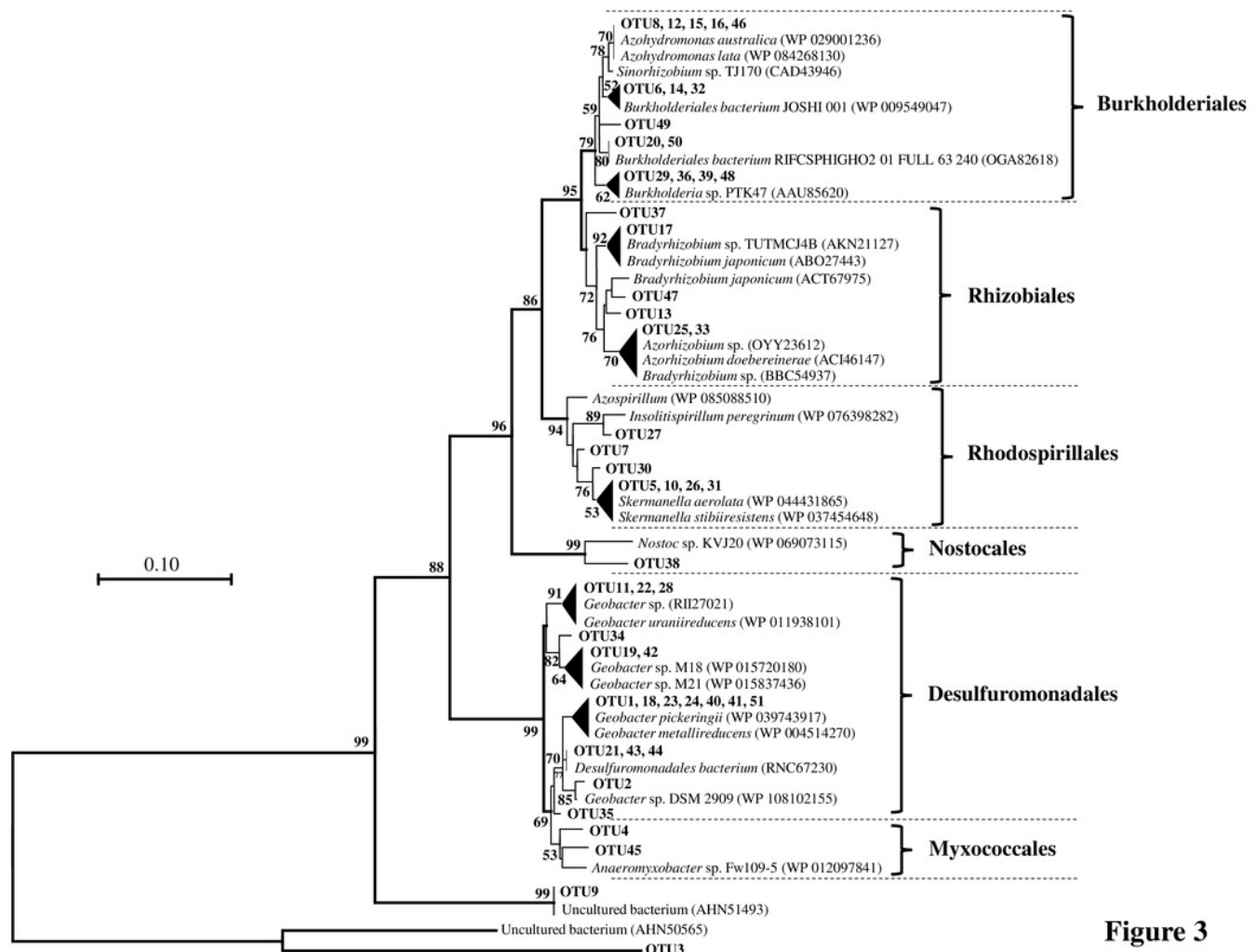
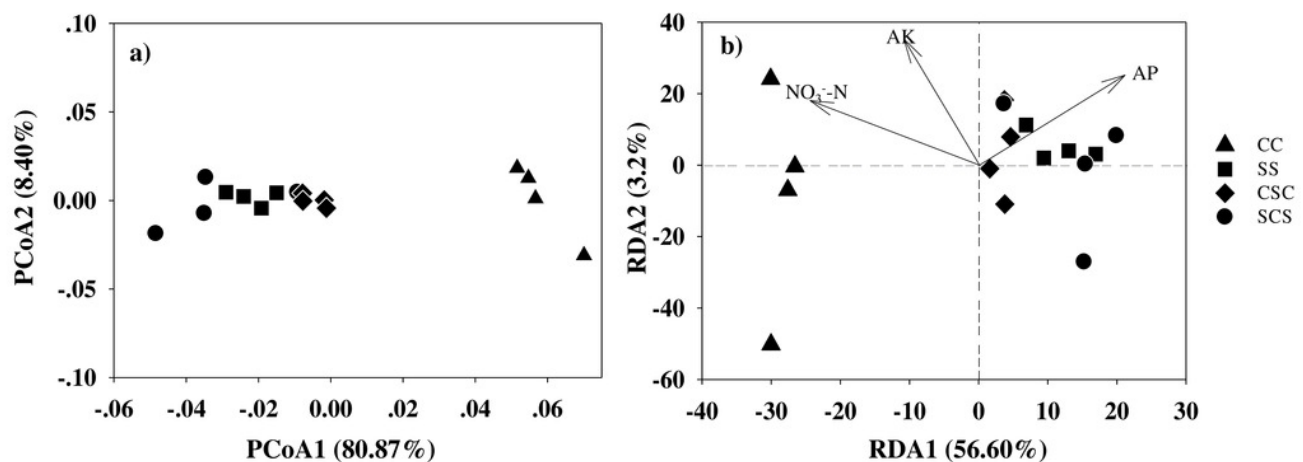


Figure 3

# Figure 4

Principal coordinate analysis (a) and redundancy analysis (b) of soil diazotrophic communities in different cropped systems.

The abbreviations of CC, SS, CSC and SCS are described in Figure 1.



**Figure 4**



**Table 1** (on next page)

Effects of different cropping systems on soil chemical properties.

**Table 1** Effects of different cropping systems on soil chemical properties

Treatment	pH	TN (g kg <sup>-1</sup> ) <sup>b</sup>	TC (g kg <sup>-1</sup> ) <sup>b</sup>	C/N	TP (g kg <sup>-1</sup> ) <sup>b</sup>	TK (g kg <sup>-1</sup> ) <sup>b</sup>	NH <sub>4</sub> <sup>+</sup> -N (mg kg <sup>-1</sup> )	NO <sub>3</sub> <sup>-</sup> -N (mg kg <sup>-1</sup> )	AP (mg kg <sup>-1</sup> ) <sup>b</sup>	AK (mg kg <sup>-1</sup> ) <sup>b</sup>
CC <sup>a</sup>	6.39 ± 0.12 a <sup>c</sup>	1.57 ± 0.16 bc	21.94 ± 2.62 a	13.99 ± 0.50 a	0.56 ± 0.029 a	15.64 ± 0.25 a	18.83 ± 1.37 b	30.66 ± 5.21 b	15.98 ± 1.62 c	231.05 ± 14.11 a
SS <sup>a</sup>	6.36 ± 0.01 a	1.52 ± 0.12 c	20.90 ± 1.88 a	13.73 ± 0.17 a	0.56 ± 0.035 a	15.79 ± 0.20 a	17.74 ± 1.68 b	13.69 ± 3.64 c	20.00 ± 2.08 b	219.55 ± 11.29 a
CSC <sup>a</sup>	6.36 ± 0.16 a	1.84 ± 0.15 a	20.97 ± 2.40 a	11.40 ± 0.48 b	0.47 ± 0.023 b	15.97 ± 0.16 a	24.22 ± 1.35 a	37.98 ± 3.23 a	20.68 ± 2.46 b	223.57 ± 9.84 a
SCS <sup>a</sup>	6.34 ± 0.09 a	1.78 ± 0.13 ab	20.47 ± 2.67 a	11.50 ± 0.86 b	0.56 ± 0.035 a	15.68 ± 0.40 a	18.13 ± 1.12 b	10.28 ± 1.42 c	25.96 ± 2.40 a	234.96 ± 12.21 a

<sup>a</sup> CC, SS, CSC and SCS represent continuous corn cropping, continuous soybean cropping, soybean-corn rotation for growing crop was corn and soybean-corn rotation for growing crop was soybean, respectively.

<sup>b</sup> TN, TC, TP, TK, AP and AK represent soil total nitrogen, total carbon, total phosphorus, total potassium, available phosphorus and available potassium, respectively.

## **Table 2**(on next page)

The bivariate correlation between the alpha diversity of diazotrophic communities and soil factors.

**Table 2** The bivariate correlation between alpha diversity of diazotrophic communities and soil factors.

Diversity index <sup>a</sup>	pH	TN <sup>b</sup>	TC <sup>b</sup>	C/N	NH <sub>4</sub> <sup>+</sup> -N	NO <sub>3</sub> <sup>-</sup> -N	AP <sup>b</sup>	AK <sup>b</sup>	TP <sup>b</sup>	TK <sup>b</sup>
OTU number	-0.163	-0.095	-0.245	-0.121	0.146	-0.020	-0.297	-0.063	-0.009	-0.048
Shannon	0.095	-0.329	0.167	<b>0.517<sup>*c</sup></b>	0.006	0.356	<b>-0.664<sup>**c</sup></b>	0.037	0.107	-0.146
Simpson	-0.099	0.292	-0.171	<b>-0.478<sup>*</sup></b>	-0.032	-0.421	<b>0.627<sup>**</sup></b>	-0.096	-0.111	0.161
Chao1	-0.088	0.253	0.072	-0.174	-0.170	-0.267	-0.013	0.065	0.187	0.117

<sup>a</sup> All indices are calculated based on the minimum number of 6545 sequences per sample.

<sup>b</sup> TN, TC, TP, TK, AP and AK represent soil total nitrogen, total carbon, total phosphorus, total potassium, available phosphorus and available potassium, respectively.

<sup>c</sup> \* and \*\* represent significant correlation at  $P < 0.05$  and  $P < 0.01$  level, respectively.

**Table 3**(on next page)

Relative abundances of the dominant diazotrophic bacteria at different taxonomic levels in different cropping systems.

**Table 3** Relative abundance of the dominant diazotrophic bacteria at different taxonomic levels in different cropping systems.

Taxa	Treatments			
	CC <sup>a</sup>	SS <sup>a</sup>	CSC <sup>a</sup>	SCS <sup>a</sup>
<b>Phylum</b>				
Cyanobacteria	0.08±0.01a <sup>b</sup>	0.09±0.03a	0.00±0.01b	0.03±0.02a
Proteobacteria	99.92±0.13b	99.91±0.13b	100.00±0.01a	99.97±0.05b
<b>Class</b>				
Alphaproteobacteria	94.35±1.87a	95.33±0.55a	94.93±0.83a	96.13±1.45a
Betaproteobacteria	1.37±0.37a	0.97±0.28ab	1.30±0.16a	0.65±0.30b
Deltaproteobacteria	1.56±0.93a	1.20±0.48a	1.71±0.84a	0.85±0.20a
<b>Order</b>				
Burkholderiales	1.47±0.37a	0.98±0.28bc	1.30±0.13ab	0.55±0.32c
Desulfuromonadales	1.48±0.92a	1.15±0.49a	1.65±0.88a	0.80±0.18a
Myxococcales	0.16±0.16a	0.05±0.01a	0.06±0.05a	0.05±0.03a
Nostocales	0.01±0.01a	0.02±0.01a	0.01±0.01a	0.03±0.02a
Rhizobiales	81.97±1.45b	92.14±1.26a	90.78±1.90a	92.47±2.58a
Rhodospirillales	10.09±2.63a	2.75±0.57b	4.09±1.15b	2.09±1.37b
<b>Family</b>				
Alcaligenaceae	1.47±0.37a	0.98±0.28bc	1.31±0.13ab	0.55±0.32c
Bradyrhizobiaceae	63.20±1.19b	86.43±2.27a	81.71±0.89a	87.25±3.92a
Geobacteraceae	1.48±0.92a	1.22±0.57a	1.65±0.88a	0.80±0.18a
Cystobacterineae	0.16±0.16a	0.05±0.01a	0.06±0.05a	0.05±0.03a
Nostocaceae	0.08±0.01a	0.09±0.03a	0.00±0.01b	0.03±0.02a
Rhodospirillaceae	10.09±2.63a	2.75±0.57b	4.09±1.15b	2.09±1.37b

<sup>a</sup> Abbreviation for treatments are described in Table 1.

<sup>b</sup> Different letters within the same row indicate significant difference between treatments tested by one-way ANOVA ( $P < 0.05$ ).

# **Table 4**(on next page)

Identification of the abundant OTUs (relative abundance > 0.3% at least in one treatment) at the amino acid sequence level by BLASTp on the NCBI website and changes in their relative abundances (%) as influenced by different cropping treatments.

**Table 4** Identification of the abundant OTUs (relative abundance > 0.3% at least in one treatment) at amino acid sequence level by BLASTp on the NCBI website and changes of their relative abundance (%) as influenced by different cropping treatments.

OTU ID	Closest relatives	Access number	Identity	CC <sup>a</sup>	SS <sup>a</sup>	CSC <sup>a</sup>	SCS <sup>a</sup>
OTU1	<i>Geobacter pickeringii</i>	WP_039743917	99%	0.56±0.46a <sup>b</sup>	0.19±0.03a	0.28±0.14a	0.15±0.11a
OTU5	<i>Skermanella aerolata</i>	WP_044431865	99%	2.06±0.75a	0.43±0.06b	0.77±0.11b	0.41±0.32b
OTU6	<i>Burkholderiales</i> bacterium JOSHI_001	WP_009549047	100%	4.74±3.06a	0.44±0.06b	1.75±0.97b	0.40±0.16b
OTU7	<i>Azospirillum</i>	WP_085088510	97%	0.40±0.23a	0.15±0.04b	0.23±0.04b	0.10±0.07b
OTU9	Uncultured bacterium	AHN51493	100%	1.42±1.14a	0.34±0.14b	0.85±0.48ab	0.35±0.17b
OTU10	<i>Skermanella stibiirensistens</i>	WP_037454648	99%	2.05±0.64a	0.64±0.17b	1.00±0.32b	0.42±0.24b
OTU13	<i>Bradyrhizobium</i> sp. TUTMCJ4B	AKN21127	99%	12.86±3.04a	4.98±1.30b	7.19±2.08b	4.77±3.02b
OTU14	<i>Burkholderiales</i> bacterium JOSHI_001	WP_009549047	99%	1.20±0.22a	0.71±0.34b	0.70±0.39b	0.42±0.22b
OTU16	<i>Azohydromonas lata</i>	WP_084268130	99%	0.70±0.15a	0.40±0.13bc	0.50±0.26ab	0.16±0.09c
OTU17	<i>Bradyrhizobium japonicum</i>	ABO27443	100%	63.01±1.22c	86.59±2.22a	81.73±0.94b	87.26±4.10a
OTU26	<i>Skermanella stibiirensistens</i>	WP_037454648	99%	0.34±0.06a	0.10±0.05b	0.15±0.09b	0.07±0.07b
OTU30	<i>Skermanella stibiirensistens</i>	WP_037454648	97%	2.30±0.69a	0.68±0.14b	0.97±0.30b	0.48±0.30b
OTU31	<i>Skermanella stibiirensistens</i>	WP_037454648	99%	3.41±0.87a	0.83±0.17b	1.31±0.37b	0.68±0.49b
OTU32	<i>Burkholderiales</i> bacterium JOSHI_001	WP_009549047	99%	0.42±0.10a	0.06±0.02b	0.07±0.04b	0.03±0.02b
OTU41	<i>Geobacter metallireducens</i>	WP_004514270	99%	0.68±0.64a	0.22±0.12a	0.61±0.83a	0.16±0.07a

<sup>a</sup> Sample as described in Table 1.

<sup>b</sup> Different letters within the same row indicate significant difference systems samples tested by one-way ANOVA ( $P < 0.05$ ).