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Long-term application of lime or pig manure rather than plant residues suppressed diazotroph abundance and diversity and altered community structure in an acidic Ultisol

Yongxin Lin[a,1], Guiping Ye[a,b,1], Deyan Liu[a], Stewart Ledgard[c], Jiafa Luo[c], Jianbo Fan[a], Junji Yuan[a], Zengming Chen[a], Weixin Ding[a,*]

[a] State Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Science, Chinese Academy of Sciences, Nanjing, 210008, China
[b] University of Chinese Academy of Sciences, Beijing, 100049, China
[c] AgResearch Limited, Ruakura Research Centre, Hamilton, 3240, New Zealand

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ABSTRACT

Biological fixation of atmospheric dinitrogen (N_2) is an important process that replenishes biologically available nitrogen (N) in soil and helps minimize the use of inorganic N fertilizer in agricultural ecosystems. Diazotrophs are key fixers of atmospheric N_2 in a range of soil types, however, there is uncertainty about how they respond to long-term fertilization. Here, using the *nifH* gene as a molecular marker, we investigated the long-term effects of inorganic and organic fertilization on diazotroph abundance and community structure in an acidic Ultisol. The field experiment ran for 27 years and comprised seven treatments: no fertilization (control); inorganic NPK fertilizer (N); inorganic NPK fertilizer + lime ($CaCO_3$) (NL); inorganic NPK fertilizer + peanut straw (NPS); inorganic NPK fertilizer + rice straw (NRS); inorganic NPK fertilizer + radish (NR); and inorganic NPK fertilizer + pig manure (NPM). Long-term application of fertilizer reduced the abundance and diversity of *nifH* gene compared with the control ($P < 0.05$), while lime and pig manure increased the inhibitory effects ($P < 0.05$). The abundance and diversity of *nifH* genes were negatively correlated with soil pH, indicating that increasing soil pH potentially affect N fixation ability in acidic Ultisols. Community structure of diazotrophs in the NPS, NRS, and NR treatments were similar and shared most operational taxonomic units (OTUs) with the N treatment, with only a minor difference to that of the control. Thus, there was no effect of plant residue types on diazotroph community structure. In contrast, the application of inorganic fertilizer + liming or pig manure altered the diazotroph community structure with shifts in the dominant genus, from *Bradyrhizobium* in the control to *Azohydromonas* in NL and *Azospirillum* in NPM. Soil pH was the key factor correlated with change in diazotroph community structure. Overall, our results suggested that regular use of lime or pig manure rather than different types of plant residue reduced the abundance and diversity and altered community structure of diazotrophs, that may potentially affect N fixation ability in acidic Ultisols.

1. Introduction

Ultisols are widespread in tropical and subtropical regions (Alvear et al., 2005; Chandran et al., 2005; Hauser et al., 2006) and represent 8.4% of global soil types (Lal, 2004). These soils are generally characterized by low cation exchange capacity (CEC) and pH, high nutrient leaching and Al and Mn toxicities, and, low structural stability with little or no mineral reserves (Clair and Lynch, 2010; Uwah and Iwo, 2011). In order to improve crop production (Huang et al., 2010; Onunka et al., 2012), large amounts of fertilizer, particularly inorganic N fertilizer, have been used to amend Ultisols, which in turn has exacerbated soil acidification (Cai et al., 2015), induced metal toxicity and threatened environmental sustainability. The enhancement of biological N fixation is an alternative approach towards meeting the nutritional requirements of plants under low N fertilizer conditions (Cleveland et al., 1999).

Diazotrophs are agents of biological N fixation, that convert atmospheric N_2 into plant available N via the nitrogenase enzyme and contribute 100–290 Tg N yr^{-1} to the biosphere (Cleveland et al., 1999). The highly diverse diazotrophs include members of the Proteobacteria,

* Corresponding author.
E-mail address: wxding@issas.ac.cn (W. Ding).
[1] Two authors contributed equally to this study.

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Firmicutes, Cyanobacteria and Archaea, however, most of these groups are recalcitrant to laboratory cultivation techniques (Rösch et al., 2002). The *nifH* gene, which encodes a subunit of the nitrogenase enzyme and is highly conserved across the bacterial and archaeal domains, provides a useful marker for studying the diazotroph communities without the need for cultivation (Collavino et al., 2014). Consequently, the characterization of diazotroph communities by *nifH* genes could be a potential indirect approach to the assessment of levels of biological N fixation in soils (Reardon et al., 2014).

Although studies of diazotroph communities in Ultisols are rare, their activity, abundance and community structure in other soil types have been shown to be influenced by various physical and chemical properties, including soil pH (Nelson and Mele, 2006), oxygen partial pressure (Limmer and Drake, 1996), carbon quantity (Collavino et al., 2014), N availability (Pereira e Silva et al., 2013), soil texture (Pereira e Silva et al., 2011), and soil aggregate size (Poly et al., 2001), but there have been inconsistencies among findings. For example, soil pH was positively correlated with *nifH* gene abundance at all sampling times in the Netherlands (Pereira e Silva et al., 2011), but a negative association was reported in subtropical regions of Australia (Bai et al., 2015). Likewise, N availability has been found to have contrasting effects on *nifH* gene abundance, where stimulatory (Perez et al., 2014; Reardon et al., 2014) and inhibitory effects (Wang et al., 2017a; Zhalnina et al., 2015) have been reported. These inconsistent results may derive from specific diazotroph populations that respond variously to environmental factors in different ecosystems. For example, the relative abundance of *Mesorhizobium* was positively correlated with soil pH in alpine meadows with pH range from 5 to 8 (Wang et al., 2017b) while Brígido et al. (2007) found that some isolates of *Mesorhizobium* showed maximum growth at pH 5 from root nodules of chickpea.

Effects of soil fertilization on diazotroph communities is of growing concern (Meng et al., 2012; Nelson and Mele, 2006; Wang et al., 2017a), due to associated changes in soil physiochemical properties (Berthrong et al., 2014). Long-term experiments are suggested to be particularly useful since the succession and stabilization of microbial community structures requires a long period (Reardon et al., 2014). Long-term experiments have shown that diazotroph communities are affected by established inorganic N and phosphorus (P) fertilization in mangrove sediments (Romero et al., 2012) and by long-term inorganic fertilization in an acidic soil (Wang et al., 2017a). The effect of organic fertilization on diazotroph communities, however, remains unclear, as do the effects of combined applications of inorganic and organic fertilizer that are suggested as the best fertilization practice for enhancing crop yields and improving the quality of Ultisols (Huang et al., 2010).

Liming served as an effective way to ameliorate soil acidity, is also an important agricultural management in Ultisols, and has been shown to increase kernel yield of peanuts (Chang and Sung, 2004; Basu et al., 2008). The impact of liming on peanut nodulation was inconsistent from acidic soils, stimulation (Basu et al., 2008) and suppression (Van Rossum et al., 1994) were both reported. However, much less was known about the impact of liming on diazotroph communities in acidic soils, although some efforts have been made by Wang et al. (2017a) in a maize-wheat rotation system.

We established a long-term field experiment in April 1988 to monitor the influences of inorganic and organic fertilizers, and their combination, on crop yield and physicochemical and diazotroph properties of an Ultisol. Using quantitative PCR and high-throughput sequencing, soil samples were analyzed to understand long-term fertilization effects on diazotrophs in this study. Specifically, the objectives of this study were to: (1) evaluate whether long-term fertilization affected diazotroph abundance, diversity or community structure; and (2) determine the response of dominant diazotroph genera to key influencing factors in an acidic Ultisol.

2. Materials and methods

2.1. Experimental site and soil sampling

The fertilization experiment was established in April 1988 at the Yingtan Red Soil Ecology Experimental Station at the Chinese Academy of Sciences, Jiangxi, China (28°15′20″N, 116°55′30″E) in a region that has a subtropical monsoon climate, with a mean annual temperature of 17.6 °C and a mean annual precipitation of 1795 mm. The soil was derived from quaternary red clay and is classified as a Typic Plinthudult (Ultisols) based on USDA Soil Taxonomy (Soil Survey Staff, 1998) and comprised 41.2% clay, 33.2% silt, and 25.6% sand.

The field experiment was arranged as three replicates of seven treatments in a randomized block design, where plots measured 34.6 m^2 and were separated by a concrete wall embedded 100 cm into soil. The treatments comprised Control: no fertilizers; N: inorganic NPK fertilizer; NL: N + lime ($CaCO_3$); NPS: N + peanut straw; NRS: N + rice straw; NR: N + radish residues; and, NPM: N + pig manure aged for 3 months. The field was cultivated with continuous peanut monocropping in summer and fallow in winter. NPK was applied annually as 120 kg N ha^{-1} as urea, 68.7 kg P_2O_5 ha^{-1} as calcium magnesium phosphate, and 108.4 kg K_2O ha^{-1} as potassium chloride. In the NPS, NRS, NR, and NPM treatments, 30% of the inorganic N fertilizer was replaced by organic N. All treatments received the same rates of total N, P, and K, and calcium magnesium phosphate and potassium chloride were added to the organic fertilizer treatments to achieve the same rate of P or K. For the NL treatment, 1500 kg ha^{-1} $CaCO_3$ was applied annually. For all treatments, fertilizer and organic materials were evenly spread onto the soil surface by hand and immediately tilled into the plowed soil prior to sowing. Each year, peanut (cv Ganhua 5) was sown on 10 April by placing two seeds per hole to give a 20 cm plant-to-plant spacing and 30 cm row-to-row spacing per plot.

Soil samples were collected on 13 December 2014, where ten soil cores (10 cm diameter, 0–20 cm depth) were randomly collected from each plot and combined to form one composite sample per plot. Soil samples were transported from the study site to the laboratory in a constant temperature box containing ice. After visible stones and plant residues were removed using forceps, the soil samples were gently broken apart along natural-break points, passed through an 8-mm sieve and thoroughly mixed before being divided into two parts. One part was analyzed for soil physicochemical properties, while the other part was sieved to < 2 mm, to increase homogeneity, and immediately stored at −80 °C for subsequent DNA extraction.

2.2. Soil physicochemical analysis

Air-dried soils were used to determine soil organic carbon (SOC), total nitrogen (TN), available P (AP), available K (AK), and pH, and fresh soil samples were analyzed for content of soil ammonium (NH_4^+-N), nitrate (NO_3^--N), dissolved total N (DTN), dissolved organic carbon (DOC), and dissolved organic nitrogen (DON). Soil pH was measured using a glass electrode in a 1:5 soil to water ratio. Concentrations of SOC and TN were determined by the wet oxidation redox titration and micro-Kjeldahl methods, respectively (Lu, 2000). DOC was extracted by incubating 10 g of fresh soil (on an oven-dried basis) with 50 ml deionized water for 30 min on an end-over-end shaker at 25 °C. The extracted samples were centrifuged at 10000 rpm for 10 min at 4 °C and resulting supernatants were filtered through a 0.45-μm membrane filter (Whatman, Clifton, NJ, USA) and DOC content was quantified using a Shimadzu C analyzer (TOC Vcph, Shimadzu, Kyoto, Japan) (Liu et al., 2014). We extracted NH_4^+-N, NO_3^--N, and DTN in 2.0 M KCl before measurement in a continuous flow analyzer (San^{++}, Skalar, Holland) and DON was calculated as DTN − NH_4^+-N − NO_3^--N. AK in soil was extracted with 1 M ammonium acetate and analyzed by flame photometry (FP640, INASA, China), while AP was extracted with 0.0125 M H_2SO_4 in 0.05 M HCl and determined using the molybdenum blue

method (Lu, 2000).

Six samples of undisturbed soil cores (100-cm^3 cylinder) were collected from each plot from which water retention curves were calculated using a ceramic pressure plate at equilibrium matrix potentials of −0.1, −0.2, −1, −3.5, −6, −10, −33, −50, −100, −200, −500, and −1500 kPa in a pressure chamber. The effective diffusion coefficient of oxygen in the soil (DC_o, $m^2\ s^{-1}$) was calculated following Aachib et al. (2004):

$$DCo = 1/N^2 \times [D_{ao} \times Q_a^p + KH \times D_{wo} \times Q_w^p]$$

where N is the soil porosity; D_{ao} is the free diffusion coefficient of oxygen in air at 20 °C ($1.8 \times 10^{-5}\ m^2\ s^{-1}$); KH is Henry's equilibrium constant at 20 °C (0.03); D_{wo} is the free diffusion coefficient of oxygen in water at 20 °C ($2.2 \times 10^{-9}\ m^2\ s^{-1}$); Q_a and Q_w are the proportions of soil porosity occupied by air and water, respectively ($Q_a + Q_w = 1$); and, p is the power constant (3.4). Soil porosity (N) was calculated as follows:

$$N = 1 - \rho / \rho_0$$

where ρ is the soil bulk density (g cm^{-3}); and ρ_0 is the soil particle density (g cm^{-3}). The proportion of soil porosity occupied by water (Q_w) was calculated as follows:

$$Q_w = \rho \times w / N$$

where w is the soil gravimetric moisture content ($cm^3\ g^{-1}$).

2.3. DNA extraction and quantification

DNA was extracted from 0.5 g soil using the Fast DNA Spin Kit for Soil (MP Biomedicals, CA, USA) and then purified using PowerClean DNA Clean-up Kit (Mobio, CA, USA) according to the manufacturers' protocols. The quality and concentration of the extracted DNA was evaluated by gel electrophoresis (0.8% agarose) and NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, USA), and the extracted DNA was subsequently stored at −20 °C.

Functional marker genes (*nifH*) of diazotrophs were quantified using q-PCR in a CFX96 Optical Real-Time Detection System (Bio-Rad Laboratories Inc., Hercules, CA, USA). Each reaction in 25 μL contained the specific primer set for each group for the diazotrophs: nifHF (AAA GGY GGW ATC GGY AAR TCC ACC AC) - nifHR (TTG TTS GCS GCR TAC ATS GCC ATC AT) (Töwe et al., 2010). Each reaction mixture (25 μL) consisted of 12.5 μL 1 × SYBR Premix Ex Taq (Takara, Tokyo, Japan), 0.25 μL of each primer, and 1 μL of DNA template that contained approximately 1–10 ng of DNA. A negative control was run with sterilized distilled water. Amplification was initiated by denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 10 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, and the plate was read at 80 °C. Standard curves were created using 10-fold dilution series of plasmid DNA containing the *nifH* gene for diazotrophs. A serial dilution of the DNA template was used to assess whether the PCR was inhibited during the amplification; amplification resulted in single peaks with efficiencies of 104.9% and R^2 values of 0.997.

2.4. High-throughput sequencing and bioinformatics analysis

We used a nested PCR approach to amplify *nifH* gene fragments for high-throughput sequencing. The primer set PolF (TGC GAY CCS AAR GCB GAC TC)-PolR (ATS GCC ATC ATY TCR CCG GA) (Poly et al., 2001) was used for the first PCR reaction and RoeschF (ACC CGC CTG ATC CTG CAC GCC AAG G)-RoeschR (ACG ATG TAG ATT TCC TGG GCC TTG TT) (Roesch et al., 2006) was used for the second reaction. The thermal conditions and reaction composition of PCR were conducted according to Pereira e Silva et al. (2013). A unique sample identifying barcode was added to the forward primer in the second PCR amplification and triplicate PCR amplifications for each sample were conducted and pooled as PCR products that were subsequently purified with a Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were generated using the TruSeq DNA PCR-Free Sample Preparation Kit (Illumina, USA) following the manufacturer's recommendations and library quality was assessed on the Qubit 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 systems. Finally, high-throughput sequencing of *nifH* genes was carried out using Illumina MiSeq and 300 bp paired-end reads were generated. All sequences have been deposited in the DNA Data Bank of Japan under the accession number DRA006142.

All paired-end raw reads were initially merged using FLASH (version 1.2.7) (Magoč and Salzberg, 2011), in which forward and reverse reads of same sequence with at least 10 bp overlap and no base mismatches were combined as single sequence. Sequencing data were processed using Quantitative Insights Into Microbial Ecology (QIIME) v1.8.0 (Caporaso et al., 2010) and low quality sequences (quality scores < 20, with ambiguous base, or did not match the primer and barcode) were removed. Barcode sequences were also removed after sample sequences were sorted according to the barcodes. Next, the sequences were compared with the reference database (Edgar et al., 2011) using the UCHIME algorithm to detect chimera sequences that were subsequently removed. The remaining high-quality sequences at 90% similarity were assigned to the same OTUs using the Uparse software (Uparse v7.0.1001) (Edgar, 2013) and the most abundant sequence from each OTU was selected as its representative sequence. All representative OTUs were taxonomically classified from the construction of neighbor-joining phylogenetic trees in MEGA 5.2 using representative sequences of the *nifH* genes, together with taxonomically-determined reference sequences from GenBank (Fig. S1). Heterogeneity of the number of sequences per sample was removed by rarefying sequences prior to calculation of alpha diversity statistics. Alpha diversity was assessed by calculating Chao1 (Chao and Bunge, 2002), Observed species, Shannon (2001) and Simpson (1949) metrics in QIIME.

2.5. Data analysis and statistics

The statistical analyses were conducted using SPSS 18.0 software package for Windows (SPSS Inc., Chicago, IL, USA). All data were checked for normality and homogeneity of variance (Levene's test) prior to testing for treatment differences using one-way ANOVA; data were ln-transformed to meet the assumptions of the ANOVA where necessary. Least significant difference (LSD) calculations at a 5% confidence level were used to test for differences among treatments. Pearson's correlation was used to test the association between soil physicochemical variables and relative abundance of diazotroph genera that occurred > 0.1% across all the treatments. Responses of diazotroph communities to fertilization were estimated using response ratio analysis (Luo et al., 2006), based on the sequence abundance of each genera.

R software (Version 3.4.1) was utilized to conduct the following analyses. First, Spearman's rho correlation analysis and stepwise multiple regressions with Akaike Information Criterion (AIC) were calculated, using the vegan package (Oksanen et al., 2007), to determine the key soil characteristics that affected *nifH* gene copy numbers, using criteria of $P < 0.05$ to accept and $P > 0.1$ to remove a variable from analysis. Aggregated boosted tree analysis (ABT) (De'Ath, 2007) was carried out using the gbm package (with 5000 trees for boosting, 10-folds cross-validation, and three-way interactions) to quantitatively evaluate the relative influence of environmental variables on *nifH* gene abundance and diversity. A multivariate regression tree (MRT) was built to identify the most important abiotic factors for diazotroph diversity and community composition using mvpart package (De'Ath, 2002). Correlations between soil physicochemical variables and diazotroph community composition were calculated in the vegan package using a Mantel test and canonical correspondence analysis (CCA) in which environmental variables with VIF < 20 were selected.

Table 1
Soil physicochemical properties following long-term application of inorganic and organic fertilizers.

Properties	Control	N	NL	NPS	NRS	NR	NPM
pH (1:5 H_2O)	4.96 ± 0.02a	5.20 ± 0.01b	6.59 ± 0.04d	5.13 ± 0.03b	5.16 ± 0.02b	5.17 ± 0.02b	5.58 ± 0.02c
SOC (g C kg^{-1})	5.69 ± 0.10a	6.44 ± 0.26b	6.32 ± 0.08b	6.62 ± 0.06bc	7.13 ± 0.28c	6.76 ± 0.29bc	8.21 ± 0.20d
DOC (mg C kg^{-1})	4.27 ± 0.37a	4.38 ± 0.23a	5.33 ± 0.34a	4.35 ± 0.20a	5.53 ± 0.60a	5.63 ± 0.85a	12.25 ± 1.80b
TN (g N kg^{-1})	0.59 ± 0.01a	0.70 ± 0.01b	0.72 ± 0.02b	0.78 ± 0.02c	0.82 ± 0.02cd	0.82 ± 0.01cd	0.85 ± 0.02d
NH_4^+ (mg N kg^{-1})	0.54 ± 0.17ab	0.66 ± 0.04b	0.56 ± 0.19ab	0.69 ± 0.10b	0.41 ± 0.13ab	0.25 ± 0.07a	0.49 ± 0.03ab
NO_3^- (mg N kg^{-1})	12.50 ± 0.39c	8.60 ± 0.77ab	5.21 ± 0.81a	8.71 ± 0.60ab	8.66 ± 0.08ab	11.56 ± 2.24bc	9.56 ± 0.50bc
DON (mg N kg^{-1})	9.22 ± 0.37c	5.56 ± 0.24ab	4.28 ± 0.75a	5.38 ± 0.57ab	8.90 ± 2.88bc	5.93 ± 0.46abc	5.89 ± 0.45abc
AP (mg P kg^{-1})	5.25 ± 0.54a	15.00 ± 3.12bc	16.40 ± 1.82c	10.30 ± 0.95b	10.78 ± 0.52b	11.63 ± 0.72bc	156.73 ± 8.85d
AK (mg K kg^{-1})	84.65 ± 10.38a	177.59 ± 23.0b	203.10 ± 5.62c	178.02 ± 2.42b	206.69 ± 4.16c	204.84 ± 0.99c	170.77 ± 3.86b
DC_o ($\times 10^{-6}$ m^2 s^{-1})	12.58 ± 0.39a	10.71 ± 0.60b	9.95 ± 0.19b	9.96 ± 0.85b	8.20 ± 0.55c	9.70 ± 0.66bc	2.81 ± 0.35d

Mean ± SEMs (n = 3). Values within the same row followed by different letters indicate significant differences at $P < 0.05$. SOC: soil organic carbon; DOC; dissolved organic carbon; TN: soil total nitrogen; DON: dissolved organic nitrogen; AP: available phosphorus; AK: available potassium; DC_o: effective diffusion coefficient of oxygen.

Permutational multivariate analysis of variance (PERMANOVA), based on Bray-Curtis distance metrics, to quantify the effect size and significance among the treatments was performed using the function 'adonis' in the vegan package. Finally, OTUs as indicators of different treatments were identified using the "indicspecies" package in R (Cáceres and Legendre, 2009) and visualized using the Cytoscape software (Version 3.2.1) (Shannon et al., 2003).

3. Results

3.1. Soil properties

Soil physicochemical properties of the treatments are presented in Table 1. The lowest soil pH (4.96) was recorded from the control, and was lower than those in the other treatments ($P < 0.05$), especially the NL (6.59) and NPM (5.58) treatments. However, compared to the N treatment, pH was higher in the NL and NPM treatments, but not different from those in the NPS, NRS, and NR treatments. The NL treatment increased soil pH from 4.80 in 1988 to 6.56 in 2006 and 6.59 in 2014, indicating that an increase in soil pH might be minor after 20 years. Compared with control, application of fertilizer increased concentrations of SOC, TN, and AP ($P < 0.05$), where they were greatest in the NPM treatment. Concentration of DOC was higher in the NPM treatment (12.25 mg C kg^{-1}) than in the control (4.27 mg C kg^{-1}) ($P = 0.012$), while there were no differences among the other treatments. Concentration of NO_3^- was approximately one order of magnitude greater than NH_4^+ in all treatments and, compared to the control, was lower following long-term (27 years) application of inorganic NPK + lime (NL); there was no difference in NH_4^+ concentration between the control and the treatments. We found that fertilizer application increased AP and AK. The DC_o in the NPM treatment was estimated at 2.81×10^{-6} m^2 s^{-1}, which was lower than in the other treatments ($8.20–12.58 \times 10^{-6}$ m^2 s^{-1}) ($P < 0.05$).

3.2. The nifH gene copy number

The highest number of *nifH* gene copies was recorded in the control (9.92×10^5 copies g^{-1} dry weight soil) and was higher than those in the other treatments ($P < 0.05$), and the NL and NPM treatments had the greatest inhibitory effects on *nifH* gene copy number (Fig. 1). However, the application of plant residues exerted no effect on *nifH* gene abundance, compared to N treatment. Abundance of *nifH* gene was positively correlated with DON, NO_3^--N, and DC_o and negatively correlated with pH, DOC, and AP (Table S1). Stepwise multiple regression analysis showed that the abundance of *nifH* gene was best predicted by a model incorporating soil pH, DC_o and AK ($R^2 = 0.754$, $P < 0.001$), in which pH explained most of the variation in *nifH* gene abundance (42.6%). ABT further confirmed that soil pH was the major factor determining *nifH* gene copy number, accounting for approximately 22% of the relative influence on *nifH* gene copy numbers (Fig. 2).

3.3. Diazotroph diversity

Chao1, observed species, Shannon and Simpson were used to estimate diazotrophic diversity and richness among different treatments (Fig. S2). Compared to the control, diazotroph diversity (Shannon and Simpson indices) and OTU richness (Chao1 and Observed species) were lower in the NL and NPM treatments ($P < 0.05$). However, there was no effect of plant residue types on diazotroph diversity and OTU richness, compared with N treatment. MRT indicated that diazotroph diversity and richness were mainly shaped by soil pH (Fig. 3), while ABT showed that soil pH accounted for more than 32% of the variation (Fig. S3). A further correlation analysis showed that all metrics of diazotroph diversity and richness indices were negatively correlated with soil pH ($P < 0.01$).

3.4. Diazotroph community structure

After quality filtering, 996682 high-quality sequences, ranging from 38920 to 63662 sequences per sample, were obtained, and the numbers of OTUs (at 90% similarity) in the treatment plots ranged from 79 to 189 (Table S2). CCA analysis showed that the diazotrophic communities formed four clusters: Control, NL, NPM, and the other treatments (Fig. 4). PERMANOVA analysis confirmed these results, showing that diazotroph communities differed between the treatments ($P < 0.05$; Table S3). The diazotrophic communities of NL were associated with higher soil pH values, while the communities of the NPM treatment were associated with SOC, DOC, AP, and TN (Fig. 4). The MRT analysis of 446 OTUs from the experimental plots showed that community composition was mainly shaped by soil pH (Fig. 5). Mantel tests also confirmed that soil pH was the primary influencing factor of diazotroph communities ($R = 0.852$, $P = 0.001$), while AP and NO_3^--N were also shown to contribute to community structure ($R = 0.311$, $P = 0.037$ and $R = 0.252$, $P = 0.042$, respectively; Table 2).

OTUs were taxonomically classified into different genera across all the treatments and the eight most abundant genera with relative abundances of > 1% are shown in Fig. S4. *Azospirillum*, *Bradyrhizobium* and *Azohydromonas* were the three most abundant genera across the treatments, together accounting for 77.8–97.4% of the *nifH* gene sequences. The relative abundance of these genera differed among the treatments (Fig. S4), where *Azohydromonas* abundance increased from 11.9% in the control to 77.8% and 33.2% in the NL and NPM treatments, respectively ($P < 0.05$). *Bradyrhizobium* was the most abundant genus in the control, accounting for 44% of the total sequences while long-term fertilization suppressed it. The NL treatment also increased abundance of *Herbaspirillum* and *Azonexus*, but decreased the abundance of *Azospirillum* ($P < 0.05$), whereas the NPM treatment increased the abundance of *Azospirillum*, *Azohydromonas*, *Herbaspirillum*,

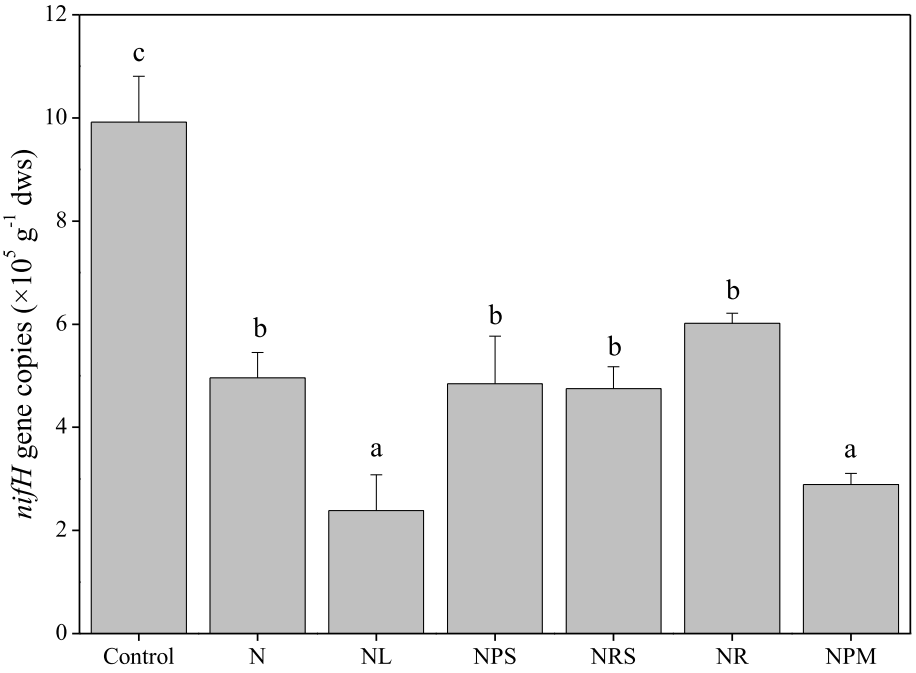

Fig. 1. *nifH* gene copy number in different treatments. Control: control; N: inorganic NPK fertilizer; NL: N + lime ($CaCO_3$); NPS: N + peanut straw; NRS: N + rice straw; NR: N + radish residues; and, NPM: N + pig manure. Vertical bars are SEMs (n = 3). Different letters denote significant differences between treatments ($P < 0.05$).

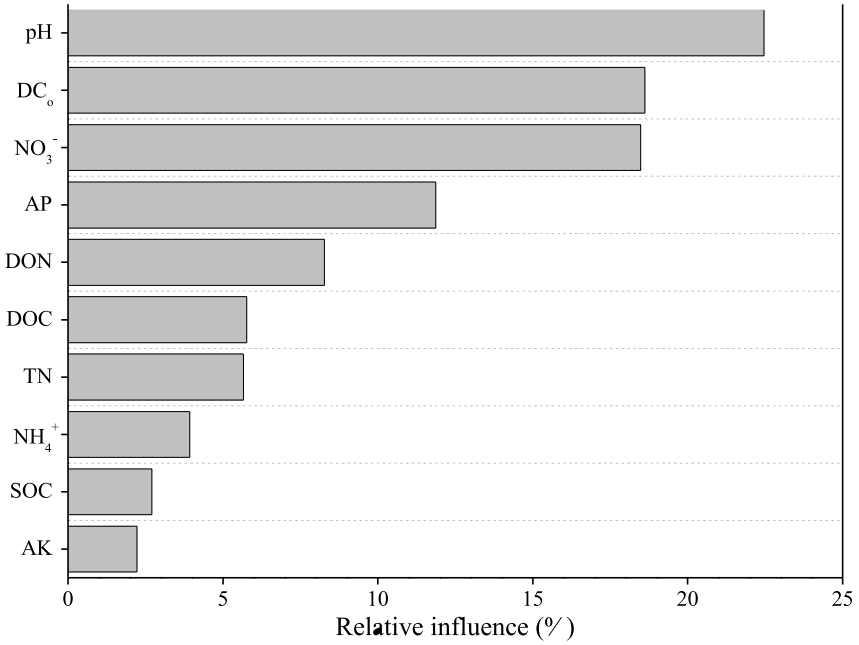

Fig. 2. Relative influence of soil physicochemical properties on abundance of *nifH* genes evaluated using ABT models. DC_o: effective diffusion coefficient of oxygen; AP: available phosphorus; DON: dissolved organic nitrogen; DOC: dissolved organic carbon; TN: total soil nitrogen; SOC: soil organic carbon; and, AK: available potassium.

Azonexus, and *Frankia* ($P < 0.05$, Fig. 6).

We attempted to identify indicator species (OTUs) of the treatments, however, most of the OTUs were shared between the N, NPS, NRS, and NR treatments (Table S4, Fig. 7) and PERMANOVA analysis confirmed there was no difference in diazotrophic communities among these treatments. The indicator species of the remaining treatments are presented in Fig. 8, where OTU355 was the most abundant indicator species in the control treatment, with a relative abundance of 2.89%, and was most closely related to *Bradyrhizobium* that, was negatively correlated with soil pH, SOC, DOC, AP, and AK and positively associated with NO_3^--N and DC_o (Table S5). The most abundant indicator species in the NL treatment was OTU97, with a relative abundance of 2.88% (Table S4), and was most closely related to *Azohydromonas* that, was positively correlated with soil pH and negatively associated with NO_3^--N ($P < 0.01$). Indicator species, OTU88, which was most closely related to *Frankia*, was the most abundant indicator species in the NPM treatment (1.57%, Table S4) and was positively associated with DOC and AP and negatively correlated with DC_o ($P < 0.05$).

4. Discussion

4.1. Effect of long-term fertilization on soil diazotrophic abundance

Long-term N fertilization decreased *nifH* gene abundance in the acidic Ultisol plots and additional inhibitory effects of liming and pig manure were observed (NL and NPM treatments). Previous studies have also showed that N fertilization suppressed the abundance of diazotrophs (Meng et al., 2012; Berthrong et al., 2014; Wang et al., 2017a), and attributed the reductions to high soil NH_4^+ content (Yoch and Gotto, 1982; Lakshminarayana et al., 2000; Wang et al., 2017a). In our

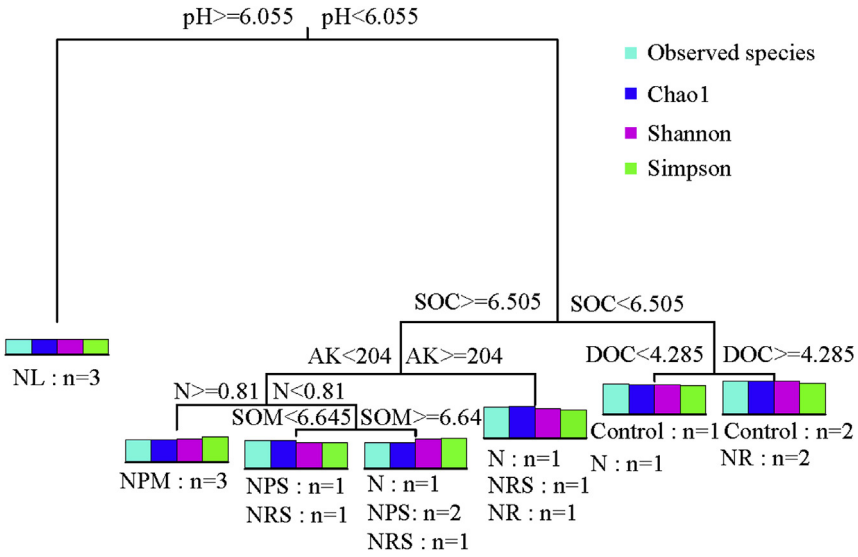

Fig. 3. Multivariate regression tree analysis of alpha diversity of diazotrophs and soil physicochemical variables. Treatments and the number of samples included in the analysis are shown under bar plots.

study, however, NH_4^+ concentration was very low (< 0.7 mg N kg^{-1}) and was not different between the control and N fertilization treatments, primarily due to high plant uptake and N losses triggered by heavy summer rainfall (Cao et al., 2006). In addition, we found that the nitrification activity in the NL treatment was significantly higher than those in the other treatments (data not shown) primarily due to increased soil pH, indicating that NH_4^+ concentration in the NL treatment was more efficiently converted into NO_3^-. Thus, we suggest that NH_4^+ concentration partly, if any, contributed to the reduction of *nifH* gene abundance in the test soil.

Previous studies have shown that growth of most of diazotrophs was generally higher when levels of oxygen in soil were reduced (Limmer and Drake, 1996; Cohen, 2014), since high concentrations of oxygen inactivate nitrogenase necessary for diazotroph growth (Robson and Postgate, 1980). Although diazotrophs could also fix nitrogen under well-aerated environments, they have to develop particular mechanisms to protect nitrogenase from inactivation by oxygen (Oelze, 2000; Ureta and Nordlund, 2002), through costing more energetic resources (Norman and Friesen, 2017). Moreover, previous studies showed that the dominated genus *Bradyrhizobium* in general grows better under low oxygen level environments (Sciotti et al., 2003; Vriezen et al., 2007). In our study, however, there was a positive association between the abundance of *nifH* genes and DC_o, where the reduction in level of oxygen in the NPM treatment did not cause an increase in *nifH* gene abundance. We suggest this may have been due to the over-riding effect of increased soil pH in the NPM treatment.

We found that soil pH, which was negatively correlated with *nifH* gene abundance, was identified as the primary predictor of *nifH* gene abundance. Indeed, some previous studies have suggested that soil pH could greatly influence *nifH* gene abundance. For example, Bai et al.

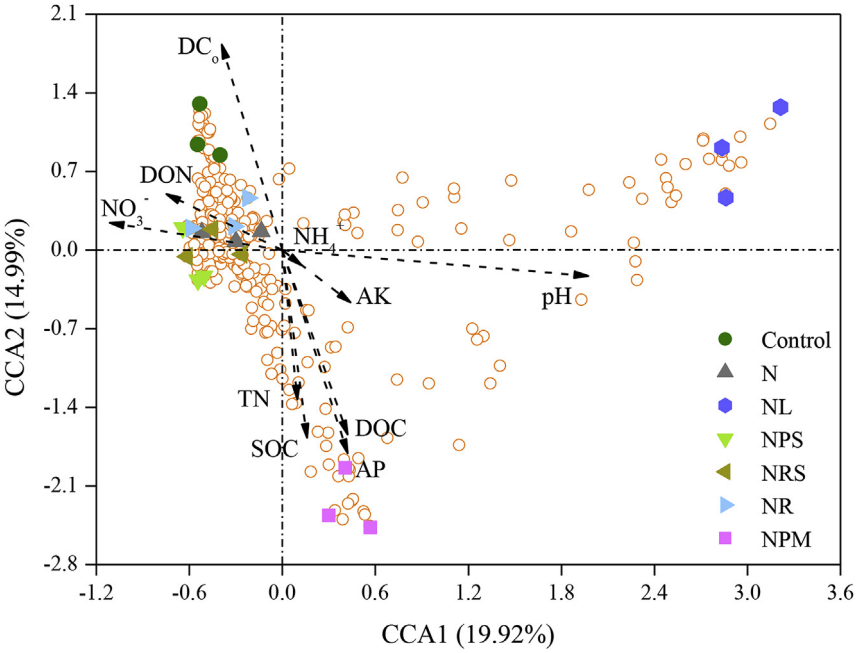

Fig. 4. Canonical correspondence analysis (CCA) of the diazotroph community structures of the treatments. The positions and lengths of the arrows indicate the directions and strengths, respectively, of the effects of soil variables on the diazotroph communities. Open circles are detected OTUs.

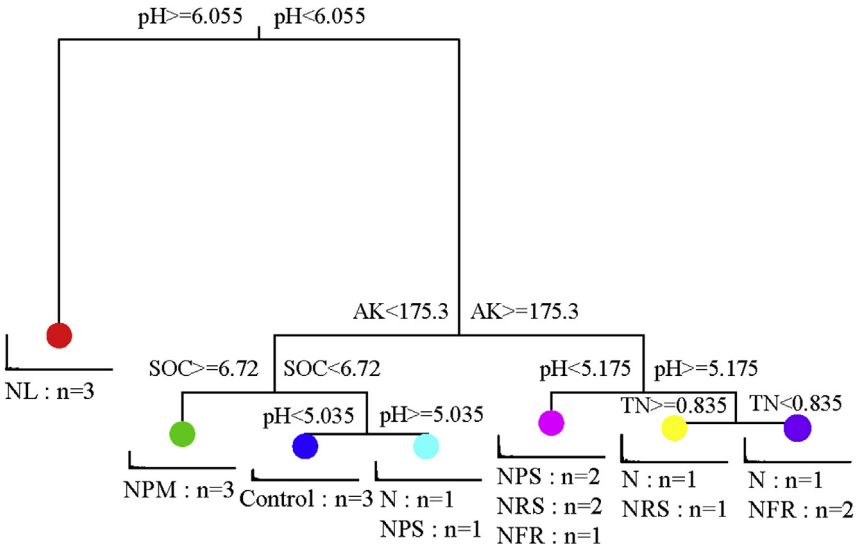

Fig. 5. Multivariate regression tree analysis of diazotroph community structure and soil physicochemical variables. Treatments and the number of soil samples included in the analysis are shown under bar plots.

Table 2
Mantel test correlations between diazotroph community structure and soil physicochemical properties.

Soil variables	*R*	*P*
Soil pH	**0.852**	**0.001**
SOC	0.196	0.098
DOC	0.241	0.080
Total N	0.056	0.296
DON	0.063	0.264
NH_4^+-N	−0.032	0.579
NO_3^--N	**0.252**	**0.042**
AP	**0.311**	**0.037**
AK	−0.035	0.533
DC_o	0.249	0.067

SOC: soil organic carbon; DOC: dissolved organic carbon; Total N: total soil nitrogen; DON: dissolved organic nitrogen; AP: available phosphorus; AK: available potassium; DC_o: effective diffusion coefficient of oxygen. Values in bold indicate significant effects ($P < 0.05$).

(2015) observed that incorporation of wood biochar with a pH of 8.21 into an acid soil decreased the abundance of *nifH*, indicating that increasing soil pH from acid soils had negative impact on abundance of diazotroph. In contrast, Pereira e Silva et al. (2011) found that pH was positively correlated with the overall *nifH* gene abundance in different soils with pH ranging from 4.3 to 7.7.

Decreased *nifH* gene abundance in the NL and NPM treatments was primarily attributed to the reduction in abundance of the genus *Bradyrhizobium* that accounted for 44% of diazotrophs in the control. The *Bradyrhizobium* genus is ubiquitous in soil, and includes symbiotic N-fixing bacteria and free-living soil diazotrophs (Kahindi et al., 1997; Kaneko et al., 2002; Berthrong et al., 2014). Previous studies have demonstrated that the indigenous *Bradyrhizobium* abundance and magnitude of nodulation decreased with an increase in soil pH, especially when soil pH was > 6 (Tang and Robson, 1993; Van Rossum et al., 1994; Zhalnina et al., 2013). The reasons for this include: first, *Bradyrhizobium* could excrete alkaline compounds thus creating a competitive advantage over other bacteria in an acidic environment (Fujihara, 2009); secondly, the expression of *Bradyrhizobium hemA* gene, which encodes the enzyme for δ-aminolevulinic acid formation, is increased by more available iron (Page et al., 1994). The δ-aminolevulinic acid is the universal precursor of heme, an essential molecule for almost all organisms due to its involvement in primary functions, such as cellular differentiation and gene regulation (Franken et al., 2012). However, iron availability decreases with increasing soil pH in acidic soil (Shuman, 1998), so increased soil pH may indirectly inhibit *Bradyrhizobium* growth. Furthermore, indigenous diazotrophs in acidic Ultisols are well adapted and tolerant to these prevailing soil conditions (Bååth, 1996). Thus, increasing pH in acidic soil would suppress diazotroph growth (Bååth et al., 1995; Dumale et al., 2011), resulting in reduction of *nifH* gene abundance.

The abundance of *nifH* genes is correlated with N_2 fixation rates in some soils (Reed et al., 2010), and it is possible that the reduction of *nifH* abundance, induced by increasing soil pH, may compromise the N_2 fixation in these treated soils. However, it should be noted that higher gene abundance did not always resulted in higher metabolic rates in soils (Rocca et al., 2015). Moreover, soil pH < 4.5 was also detrimental for growth of *Bradyrhizobium* (Fujihara and Yoneyama, 1993; Graham et al., 1994), therefore increasing soil pH from extremely acid to 4.5 could promote *nifH* gene abundance (Wang et al., 2017a).

4.2. Effect of long-term fertilization on diazotrophic communities

Our observation that soil pH was the most important determinant in controlling diazotroph diversity in the acidic Ultisols, which is consistent with previous reports from alpine meadows (Wang et al., 2017b). However, in contrast to the alpine meadows, where diazotrophic diversity was greater in high pH soils, we found an opposite trend in the acidic Ultisols, which might be due to differences in the indigenous diazotroph community composition of the two ecosystems. In the alpine meadows, *Mesorhizobium* were the most abundant genus, and the relative abundance of them was positively related to soil pH with pH ranging from 5 to 8 (Wang et al., 2017b), whereas in the test Ultisols, *Mesorhizobium* was undetectable and the relative abundance of predominant genus *Bradyrhizobium* increased with decreasing soil pH.

Optimum pH for growth of different cultivated acidophilic species is known to vary (Edwards et al., 2000; Bräuer et al., 2006) and quantitative proteomic analysis has showed pH-specific niche partitioning of prokaryotes and the importance of pH in regulating acidophilic microbial community structure and function (Belnap et al., 2011). In our study, the change in soil pH, caused by long-term fertilization, selected different types of diazotrophs and subsequently altered community composition, especially in the NL and NPM treatments. We found that *Azohydromonas* was the most abundant indicator species in the liming treatment (NL), accounting for 78% of total sequences. Previous work has showed that this genus was, in general, the dominant N-fixing

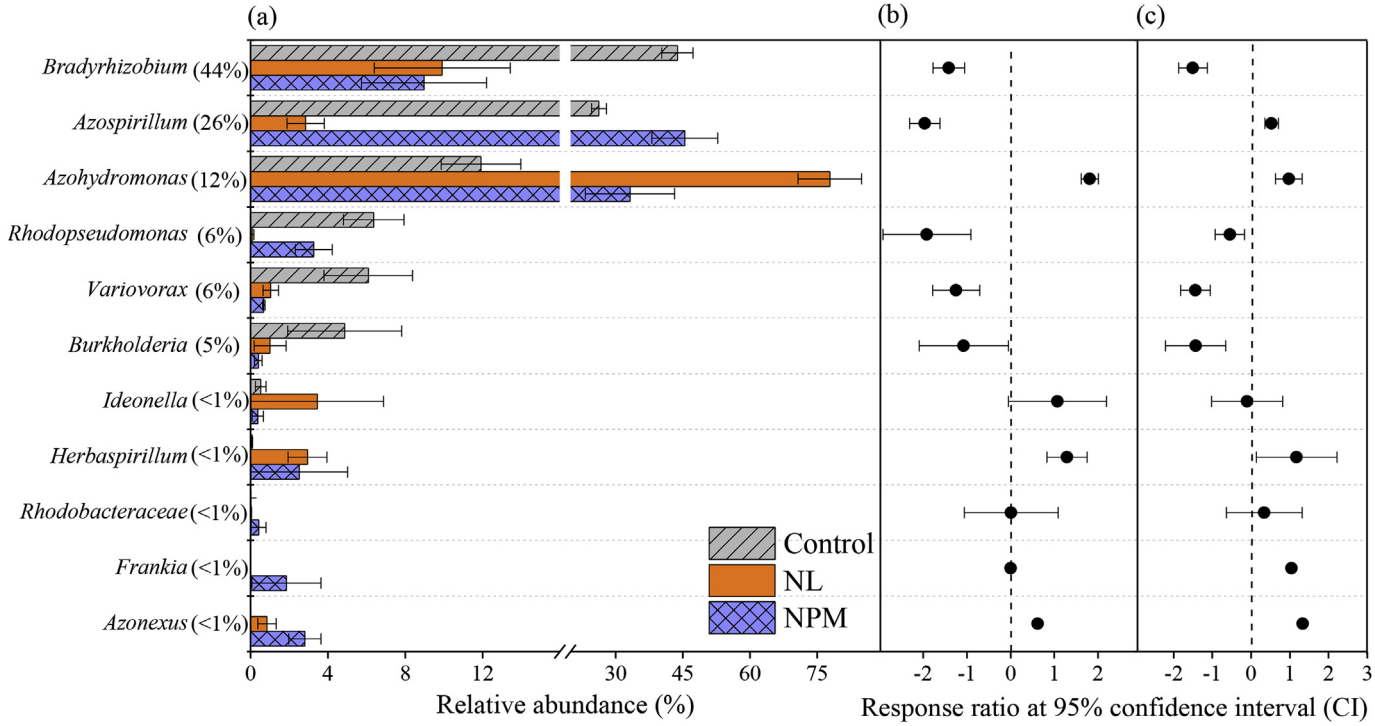

Fig. 6. Dominant diazotroph genera and responses to the treatments. Panel a: distributions of the dominant diazotroph genera in three selected treatments (control, NL, and NPM). Vertical bars are SEMs (n = 3). The percent values in parentheses refer to the relative abundance in the control. Panels b and c indicate degree of diazotrophic genera response to long-term application of NPK + lime (NL) and pig manure (NPM), respectively, based on the sequence size of each genera. Error bars non-overlapped with dashed line indicate significant ($P < 0.05$) responses to fertilization, and error bars at the right and left of dashed line mean significant positive and negative responses, respectively.

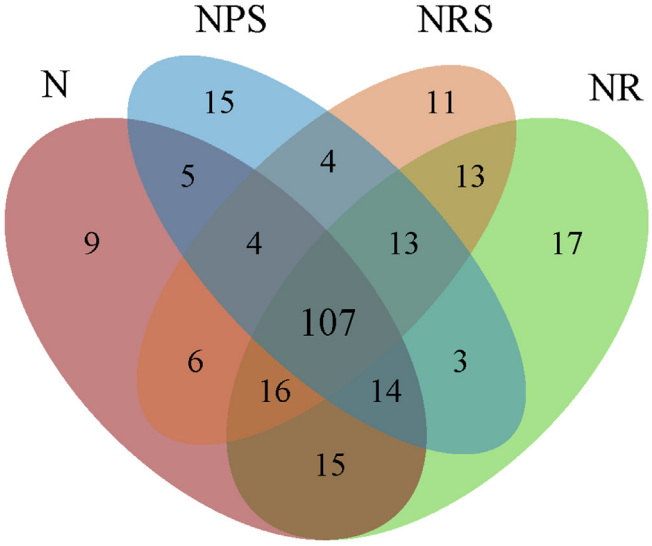

Fig. 7. Venn diagrams showing the distribution of OTUs in NPK (N), and NPK + peanut straw (NPS), rice straw (NRS), and radish (NR) treatments.

bacteria in nearly neutral pH soil (Coelho et al., 2007, 2009). The strong positive correlation between the relative abundance of *Azohydromonas* and soil pH ($R = 0.932$, $P < 0.001$) indicated that increasing soil pH in acidic soils could be beneficial for its growth. However, *Azohydromonas* is renowned for bioremediation of polycyclic aromatic hydrocarbons (PAHs) (Lladó et al., 2009) rather than N fixation, so further research would be required to evaluate the N fixation capacity of *Azohydromonas* in limed soils. In contrast, we found that *Frankia* was only recorded in the NPM treatment, where it was also the most abundant indicator species. As a member of the *Actinobacteria*, *Frankia* is known for its N fixation ability, mainly of trees and shrubs (Tian et al., 2002) and while its maximum N-fixing activities have been recorded from soils with pH of 6.0–6.5 and at temperatures of 25–30 °C (Tjepkema et al., 1981), activity is known to be inhibited by high concentrations of NH_4^+ (Meesters et al., 1985). It is thought that *Frankia* in soil is associated with high levels of organic C (Collavino et al., 2014), which is consistent with our finding that *Frankia* was positively related to DOC concentration ($R = 0.634$, $P < 0.01$). In our study, *Frankia* more readily colonized the NPM treatment, primarily due to the one fold greater DOC concentration than in the other treatments and the highest level of total organic C. In addition, we found that *Azospirillum* was the most abundant genus in the NPM treatment and was significantly higher than in the control and NL treatment. *Azospirillum* is the best characterized genus of plant growth-promoting rhizobacteria (Steenhoudt and Vanderleyden, 2000), and a meta-analysis has suggested that inoculation of wheat with *Azospirillum* could increase seed yield by 8.9% and aboveground dry weight by 17.8% (Veresoglou and Menexes, 2010). However, previous studies have reported that *Bradyrhizobium*, which was frequently found to be symbiotic with peanuts (Munoz et al., 2011; Wang et al., 2013), plays an important role in N fixation in acid soils due to its ability to tolerate high Al and Mn concentration (Indrasumunar et al., 2012; Tu et al., 2016). Thus, the shift of dominant diazotrophic communities in the NPM and NL treatments might affect the symbiotic N fixation ability. Further research on determining N_2 fixation rates in situ is required to compare diazotrophic community structure with N_2 fixation activity.

Plants may influence structuring of diazotrophic communities mainly through root exudates. For example, peanut cultivation was shown to preferentially select *Bradyrhizobium* (Wang et al., 2013), while *Azospirillum* is known to be associated with a wide variety of crops (Steenhoudt and Vanderleyden, 2000). However, the application of NPK fertilizers plus plant residues in our study had only a minor influence on the community structure of diazotrophs, compared to the

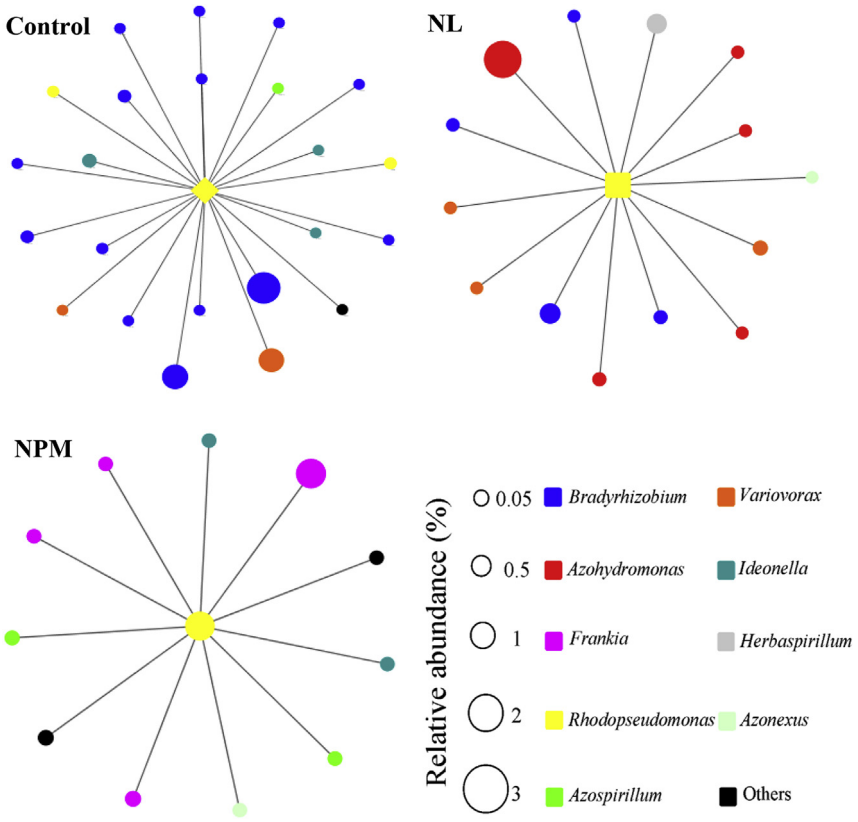

Fig. 8. Indicators species in the control, NL and NPM treatments. Circles represent OTUs, and the size of each circle denotes relative abundance.

NPK fertilizer-only treatment (N). Similarly, Chèneby et al. (2010) did not observe apparent changes in the structure of the *narG* and *napA* nitrate-reducing community under the incorporation of plant residues. Indeed, the alteration of diazotroph community composition under fertilization has previously been attributed to the variation of soil physiochemical properties (Berthrong et al., 2014; Wang et al., 2017a), but in our study, soil pH, NO_3^--N, and AP, which primarily affected the diazotroph communities, were identical between the N and N + plant residues treatments. Thus, the lack of difference in the structure of the diazotroph communities between these treatments may be a result of a lack of effect of plant residues on soil physiochemical properties in the acidic Ultisols.

5. Conclusions

In conclusion, our study demonstrated that pH was the primary driver of abundance, diversity, and community structure of soil diazotrophs in Ultisols. Increasing soil pH by fertilizer + lime or pig manure reduced the abundance and diversity of *nifH* genes. Long-term fertilization shifted the diazotroph composition from one dominated by *Bradyrhizobium* in the control to one dominated by *Azohydromonas* in the NL treatment and *Azospirillum* in NPM treatment. However, application of crop residues in addition to NPK fertilizer had only a minor influence on diazotroph composition. Since *Bradyrhizobium* is frequently considered as key for N fixation in acid soils, we postulate that inoculation of peanut crops with *Bradyrhizobium* in plots treated with lime or pig manure might be worthwhile to increase N fixation. Nonetheless, further research is required to test this hypothesis and better understand N fixation in Ultisols.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2018.05.018.

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