



# Variance of microbial composition and structure and relation with soil properties in rhizospheric and non-rhizospheric soil of a flooded paddy

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## Abstract

Soil microbial structure and nutrient properties varied with fertilization and plant growth simultaneously in the rhizosphere. However, the relationships between rhizospheric microbial community structure and soil characteristics were still unclear. This study aimed to explore the impact of nitrogen (N) fertilizer and rice growth on microbial community structure and their relation with soil physiochemical properties. A pot experiment for rice-planting with two levels of N fertilization was conducted in a flooded paddy soil. At tillering and ripening stages, both rhizospheric and non-rhizospheric soils were sampled separately for physiochemical analysis, real-time quantitative PCR assay and terminal restriction fragment length polymorphism (T-RFLP) analysis. The results showed that both N addition and growth stage of rice could affect physiochemical properties of rhizosphere and non-rhizosphere soil simultaneously. At tillering stage, no significant difference of bacterial and archaeal gene abundance was observed, but rhizospheric abundance of microorganisms was significantly different with non-rhizosphere at ripening stage. One-way ANOVA analysis indicated that N addition has greater effect on microbial structure diversity of bacteria rather than archaea, non-rhizosphere than rhizosphere, respectively. Non-metric multidimensional scaling analysis showed two groups of bacterial community both in rhizospheric and in non-rhizospheric soils at tillering stage differing much, but similar at ripening stage. Redundancy analysis showed that the microbial community compositions at tillering stage were most correlated with  $\text{NH}_4^+$ , total N (TN), pH, microbial biomass N ( $N_{\text{mic}}$ ) and microbial biomass carbon ( $C_{\text{mir}}$ ) whereas at ripening stage were more or less lined with Olsen-P, C/N ratio, total C (TC) and available K. Our research suggested that the N addition has more influence on community composition diversity of bacteria and non-rhizosphere. The growth stage might be the main factor affecting bacterial community structure both in rhizospheric and in non-rhizospheric soils affected by different soil parameters.

**Keywords** Rhizosphere microorganisms · Biodiversity · Paddy soil · T-RFLP

## Introduction

Rice is a global food which feeds more than half of the world's population (Zhu et al. 2011). The productivity and health of paddy ecosystem are partly dependent on the functional processes of soil microbial communities (Filip 2002; van der Heijden et al. 2008). Soil microorganisms, both bacteria and archaea, perform an important role in regulating energy and nutrients transformation in the paddy soil (Steenwerth et al. 2002). It is known that land use, management, soil type and rice cultivar may affect microbial community and put these communities at risk (Peixoto et al. 2006; Shen et al. 2010). Hence, the microbial diversity is believed to be more dynamic than those physical and chemical properties, and probably has the potential to serve as early signals

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of soil degradation or improvement (Bell et al. 2005). The understanding of the dynamics of microbial abundance and community structure may improve our insights for sustainability and productivity of paddy ecosystems.

Nitrogen (N) is recognized as an important limiting factor for the production of rice (Kaye and Hart 1997), but it may also result in shifts in the microbial communities (Melero et al. 2008). N input affects rhizospheric microorganisms (Personeni et al. 2005) and then plant growth accordingly. The rhizosphere which is ubiquitous and has a key role in N cycling (Jones and Hinsinger 2008; Kent and Triplett 2002; Richardson et al. 2009); rice rhizosphere is a specific micro-zone of ecosystem where rapid and intensive N turnover occurs through root uptake and microbial activities (Geiseler et al. 2010). However, the mechanisms of rhizospheric microbes by N fertilization remain unclear. Rhizospheric microorganisms are the pivotal components in paddy ecosystem as affected by root uptake and root exudation. The rhizosphere microbial activities are higher compared to those in the non-rhizosphere soil. Moreover, rhizosphere represents a hot spot of nutrient and energy by microbes and plant (Jones and Hinsinger 2008). Recent research showed that rhizosphere microorganisms may be affected by N available, water conditions and growth stage of rice simultaneously (Ma et al. 2007). There is a competition on nutrition between the rice root and the rice rhizosphere microbes at tillering stage and filling stage, leading to a decrease in microbial activity (Wang et al. 2006). The response of N application on rhizosphere microbial community structure at different stages of rice growth is still unclear (Koegel-Knabner et al. 2010). Owing to the difficulty of sampling rhizospheric soil, fewer studies have been conducted to examine the community structure diversity of rhizosphere with different stages of rice growth (Jones and Hinsinger 2008; Roesti et al. 2006; Lu et al. 2006).

Hence, the objectives of the present study were to investigate the effect of N addition and growth stage of plant on the change in microbial community population and structure, the response of the microorganism abundance and its diversity and the relationship between community structure and soil properties both in the rhizosphere and in non-rhizosphere of a typical paddy soil.

## Materials and methods

### Soil sampling and chemical analytical procedures

Paddy soil was collected from subtropical southern China, in Leizhou city, Guangzhou province (N: 20°33'57.9"; E: 110°04'25.2"). Soil samples (0–20 cm) were collected in the field on December 12, 2015 and composited. The soil was a paddy soil from latosol, and the main characteristics of the

soil were as follows: pH (soil/H<sub>2</sub>O, 1: 2.5), 6.42; total organic matter, 23.01 g kg<sup>-1</sup>; total N, 1.07 g kg<sup>-1</sup>; Olsen phosphorus, 6.71 mg kg<sup>-1</sup>; available potassium, 64.81 mg kg<sup>-1</sup>; cation exchange capacity, 185.6 cmol kg<sup>-1</sup>; microbial biomass carbon, 185.6 mg kg<sup>-1</sup>; N<sub>mic</sub>, 41.7 mg kg<sup>-1</sup> and clay content, 28.88%. Prior to the start of the experiment, the soil was air-dried then homogenized and sieved (< 2 mm).

### Experimental design

Rhizo-bags (30- $\mu$ m nylon mesh, 7.5 cm diameter, 12 cm height) filled with 475 g sieved soil were placed in the center of polyvinyl chloride (PVC) pots (15 cm diameter, 23 cm height) which were then filled with 3 kg soil. This allowed the separation of root rhizosphere compartments from the non-rhizospheric soil compartment (Fig. 1). Two treatments (control and N fertilization) were evaluated, and for each treatment, rhizosphere in the rhizo-bag and non-rhizosphere soil in the pot were sampled. In the control treatment, 100 mg N (urea) kg<sup>-1</sup> and 40 mg KH<sub>2</sub>PO<sub>4</sub> kg<sup>-1</sup> dry soil fertilizers were applied as base fertilization. In the treated soil, N was applied as urea (200 mg kg<sup>-1</sup> dry soil) and 40 mg KH<sub>2</sub>PO<sub>4</sub> kg<sup>-1</sup> dry soil fertilizers were applied. The pot experiment was carried out using a randomized design with six replicates for each treatment.

The soil samples were first incubated for one month at 105% water holding capacity (WHC) at 25 °C, then added to pots as described above. Rice seeds (cv. Xiangzaoshan 45) were sterilized in 10% H<sub>2</sub>O<sub>2</sub> for 10 min, and then thoroughly washed with deionized water. One week after germination, uniform seedlings were transplanted into the rhizo-bags. The rice plants were water flooded less than 2 cm during all the growth period in a greenhouse (illumination, 1500 E m<sup>-2</sup> s<sup>-1</sup>; ambient temperature, 35 °C  $\pm$  2 during the day, 30 °C

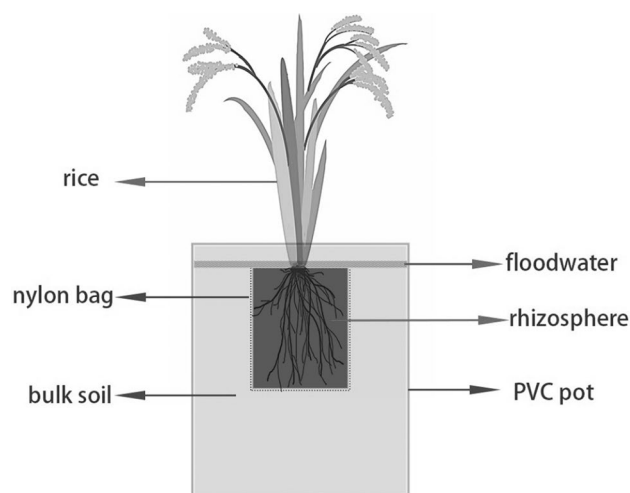


Fig. 1 Rhizo-bag and pot experiment setup for rice growth

at night; humidity, ambient 90%). The rhizosphere and non-rhizosphere soils were sampled taking three replicates at tillering (50 days) and ripening stages (112 days) of rice growth. The soils were divided into two parts. One part was freeze-dried for genomic DNA extraction, and another part was immediately frozen in liquid N<sub>2</sub> and archived at – 80 °C for further analyses.

## DNA extraction and PCR

DNA was extracted from approximately 0.50-g (fresh weight) soil using a FastDNA spin kit (MP Biomedicals, California, America), in accordance with the manufacturer's instructions. Extracted DNA was further purified with the Agarose Gel DNA Purification Kit (Takara Bio Inc., Otsu, Japan) to reduce the influence of PCR inhibitors. DNA concentration and quality were measured by a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Real-time quantitative PCR was performed to assess the abundance of bacterial 16S rRNA gene and archaeal 16S rRNA gene. The primers, PCR reaction mixture and conditions used for amplification of each target gene are listed in Table 1. Three independent quantitative PCR assays were performed for each sample. Thermal cycling and data analysis were performed with a real-time PCR detection system (ABI 7500, Applied Biosystems, Foster City, USA). The reaction mixture consisted of 10 ng DNA as template, 10 µL of SYBR 2 Premix EX *Taq* with 0.4 µL of ROX Reference Dye II (Takara Bio, Otsu, Japan). The standard curves for target genes were made using a tenfold dilution series (ranging from 10<sup>8</sup> to 10<sup>2</sup>) of the plasmids containing the target

gene fragments from the soil. The efficiency of the reaction was from 80 to 110% for the above genes, and a single sharp peak was observed in each of the melting curve. The values of coefficient of determination ( $R^2$ ) for the standard curves were all > 0.99.

A traditional PCR approach was conducted to detect the bacterial and archaeal 16S rRNA genes. The PCR mixture and thermal cycling programs were conducted as shown in Table 1. The reaction mixture consisted of 100 ng DNA as template, and the amplified products were examined by electrophoresis using a 1.0% agarose gel. PCR clone products were ligated into pGEM-T Easy Vector (Promega Corp., WI, USA) and transformed into *Escherichia coli* JM109 competent cells following the manufacture's manual.

## T-RFLP analysis

The terminal restriction fragment length polymorphism (T-RFLP) approach was conducted to detect the diversity of microbial community in rhizosphere and non-rhizosphere. DNA samples were amplified by PCR using the primer pair Ba27f/Ba907r and Ar109f/Ar912rt (Hori et al. 2007) that target bacterial 16S rRNA gene and archaeal 16S rRNA gene, respectively, with the forward primers labeled at the 5' end with 6-carboxy-fluorescein (FAM). For each sample, the labeled PCR products of three reactions were pooled and purified with the agarose gel DNA extraction kit (Tiangen Biotech Co., Beijing, China).

For T-RFLP analysis, approximately 350 ng of purified PCR product was digested by *Msp*I and *Taq*I according to their protocol for further analysis using the GeneMapper v2.1 software (Applied Biosystems, Foster city, CA, USA).

**Table 1** Primers, reaction mixture and thermal profiles used for nested PCR and real-time fluorescence quantitative PCR of bacterial and archaeal 16S rRNA genes

Target gene	Primers	Reaction mixture for traditional PCR	Thermal profile	Reaction mixture for q-PCR	Thermal profile	References
Bacterial 16S rRNA gene	341F and 517R	25 µL Dream <i>Taq</i> , 1.0 µL of forward and reverse primer, BSA (20 mg mL <sup>-1</sup> ) 0.5, 20.5 µL ddH <sub>2</sub> O	95 °C for 2 min; 95 °C for 30 s; 55 °C for 30 s; 35 cycles of 72 °C–30 s; 70 °C for 5 min	10 µL SYBR 2 Premix Ex <i>Taq</i> , 0.8 µL of forward and reverse primers, 0.4 µL ROX Reference Dye II, 6 µL ddH <sub>2</sub> O	95 °C for 30 s; 40 cycles of 95 °C for 5 s and 56 °C for 34 s	Roesti et al. (2006)
Archaeal 16S rRNA gene	Arch21F and Arch958R	25 µL Dream <i>Taq</i> , 1.0 µL of forward and reverse primer, BSA (20 mg mL <sup>-1</sup> ) 0.5, 20.5 µL ddH <sub>2</sub> O	94 °C for 3 min; 94 °C for 45 s; 55 °C for 1 min; 35 cycles of 72 °C for 1 min; 72 °C for 10 min	10 µL SYBR 2 Premix Ex <i>Taq</i> , 0.4 µL of forward and reverse primers, 0.4 µL ROX Reference Dye II, 6 µL ddH <sub>2</sub> O	95 °C for 30 s; 40 cycles of 95 °C for 5 s, 59 °C for 40 s, and 72 °C for 40 s	Lu et al. (2006)

Within each thermal profile, words with gray mark represented fluorescence collecting step, and dissociation stage was added as last step

Relative T-RF abundances were calculated by dividing the individual T-RF areas by the total peak areas (Lukow et al. 2000). Only T-RFs which had a height percentage > 1% in all three replicates were analyzed, and peaks with height percentage above 10% were regarded as dominant T-RFS (Yuan et al. 2012). Shannon-Weiner and evenness indexes were used to compare the biodiversity of bacterial and archaeal community under different N fertilization in rhizosphere and in non-rhizosphere soils (Lukow et al. 2000; Shannon 1948; Yuan et al. 2013).

### Statistical analysis

Results were given on a soil dry weight basis (oven dry, 24 h, 105 °C). Analyses were assessed by SPSS for Windows version 14.0 software (SPSS Inc., Chicago, IL, USA). Multiple comparisons of significant differences were made using Duncan's test ( $P < 0.05$ ). Redundancy analysis (RDA) was done by CANOCO 4.5 (Microcomputer Power, Ithaca, NY, USA) to identify the effects of experimental variables including pH, TC, TN, C/N ratio, Olsen-P, available K,  $C_{\text{mir}}$ ,  $N_{\text{mir}}$ ,  $\text{NH}_4^+$  on T-RFs profile. Non-metric multidimensional scaling (NMDS) was performed by SPSS 13.0 (SPSS Inc., Chicago, IL) to cluster the different sites by the fertilization on phenological stage of rice growth similarities in rhizospheric and non-rhizospheric soils. Multiple comparisons of significant differences were made using Duncan's test ( $P < 0.05$ ). The correlation analysis was explored by using SPSS 13 (SPSS Inc., Chicago, IL) with significance defined at the  $P < 0.05$  and  $P < 0.01$  level.

## Results

### Soil physiochemical properties

The chemical characteristics of the soil are shown in Table 2. Because of addition of N fertilizer as urea, the content of total C, total N,  $C_{\text{mir}}$ ,  $N_{\text{mir}}$  and  $\text{NH}_4^+$  showed increasing trends both in rhizosphere and in non-rhizosphere. Olsen-P was not changed significantly in all treatments. In comparison with tillering stage, soil pH, available K and  $\text{NH}_4^+$  decreased significantly whereas total C, C/N ratio,  $C_{\text{mir}}$  and  $N_{\text{mir}}$  increased at ripening stage. In the rhizosphere, soil pH, total C and  $\text{NH}_4^+$  were lower, but C/N ratio was higher compared with non-rhizosphere at tillering and ripening stages of rice growth, respectively. Moreover, rhizospheric available K,  $C_{\text{mir}}$  and  $N_{\text{mir}}$  were higher at tillering stage while Olsen-P, available K,  $C_{\text{mir}}$  and  $N_{\text{mir}}$  were lower than that in adjacent non-rhizosphere at ripening stage. This indicated that both N addition and growth stage of rice could have effect on soil physiochemical properties of rhizosphere and non-rhizosphere, simultaneously.

### Abundance of bacterial and archaeal 16S rRNA genes

For the quantification of bacterial and archaeal abundance in the flooded paddy soil with N fertilization, quantitative PCR assay was applied for quantification of 16S rRNA gene abundance. Overall, at tillering stage the 16S rRNA gene representing soil bacteria and archaea that ranged from  $2.13 \times 10^9$  to  $2.81 \times 10^9$  and  $2.98 \times 10^7$  to  $5.9 \times 10^7$  copies  $\text{g}^{-1}$  (Table 3), respectively, was almost one order of magnitude lower than those of bacterial and archaeal abundances at ripening stage ( $2.06 \times 10^{10}$  to  $4.84 \times 10^{10}$  and  $2.19 \times 10^8$  to  $6.31 \times 10^8$  copies  $\text{g}^{-1}$ , respectively, Table 3). There was

**Table 2** Soil characteristics with N fertilization at tillering and ripening stages of rice growth both in rhizosphere and non-rhizosphere

Treatments	pH	TC (g $\text{kg}^{-1}$ )	TN (g $\text{kg}^{-1}$ )	C/N	Olsen-P (mg $\text{kg}^{-1}$ )	Available K (mg $\text{kg}^{-1}$ )	$C_{\text{mir}}$ (mg $\text{kg}^{-1}$ )	$N_{\text{mir}}$ (mg $\text{kg}^{-1}$ )	$\text{NH}_4^+$ (mg $\text{kg}^{-1}$ )
RC-50d	6.30 b	12.05 b	1.01 a	11.93 c	6.17 ab	50.77 a	83.52 cd	18.96 d	20.21 c
RN-50d	6.32 b	13.22 ab	1.03 a	12.83 bc	6.61 a	49.76 b	89.10 cd	21.22 d	29.45 bc
NC-50d	6.38 a	11.05 b	1.00 a	11.05 c	6.59 a	56.35 a	105.72 c	24.00 d	37.82 b
NN-50d	6.45 a	11.19 b	1.06 a	10.56 c	6.61 a	54.11 a	154.67 c	35.11 c	82.20 a
RC-112d	5.51 cd	14.51 a	0.85 b	17.07 a	6.63 a	48.63 b	236.19 b	52.62 b	3.66 d
RN-112d	5.36 cd	14.82 a	0.89 b	16.65 a	6.43 a	39.41 c	278.63 a	64.25 a	4.02 d
NC-112d	5.92 bc	13.32 a	0.88 b	15.14 a	5.78 b	32.19 c	214.20 b	47.73 b	6.52 d
NN-112d	5.70 bc	13.86 a	1.03 a	13.46 b	6.07 ab	34.72 c	256.82 a	58.30 a	6.98 d

The soil samples evaluated were (1) rhizosphere in control (RC); (2) rhizosphere in N fertilization (RN); (3) non-rhizosphere in control (NC) and (4) non-rhizosphere in N fertilization (NN). Same as below.  $n = 3$ , Duncan's test at  $P < 0.05$  level, standard error not showed, letters with different labels indicate significant differences of individual parameters between the eight soils

**Table 3** Abundance of bacterial and archaeal 16S rRNA genes in the rhizosphere and non-rhizosphere soil in the greenhouse pot experiment

Soils	Tillering stage		Ripening stage	
	Bacterial 16S rRNA gene ( $\times 10^9$ copies $g^{-1}$ )	Archaeal 16S rRNA gene ( $\times 10^7$ copies $g^{-1}$ )	Bacterial 16S rRNA gene ( $\times 10^{10}$ copies $g^{-1}$ )	Archaeal 16S rRNA gene ( $\times 10^8$ copies $g^{-1}$ )
RC	2.81 $\pm$ 0.31 a	4.46 $\pm$ 0.16 a	2.06 $\pm$ 0.15 d	2.19 $\pm$ 0.10 b
RN	2.13 $\pm$ 0.05 a	2.98 $\pm$ 0.14 a	4.84 $\pm$ 0.04 a	2.67 $\pm$ 0.11 b
NC	2.35 $\pm$ 0.15 a	5.90 $\pm$ 0.30 a	3.61 $\pm$ 0.05 b	6.31 $\pm$ 0.10 a
NN	2.32 $\pm$ 0.03 a	3.52 $\pm$ 0.04 a	2.48 $\pm$ 0.16 c	5.85 $\pm$ 0.19 a

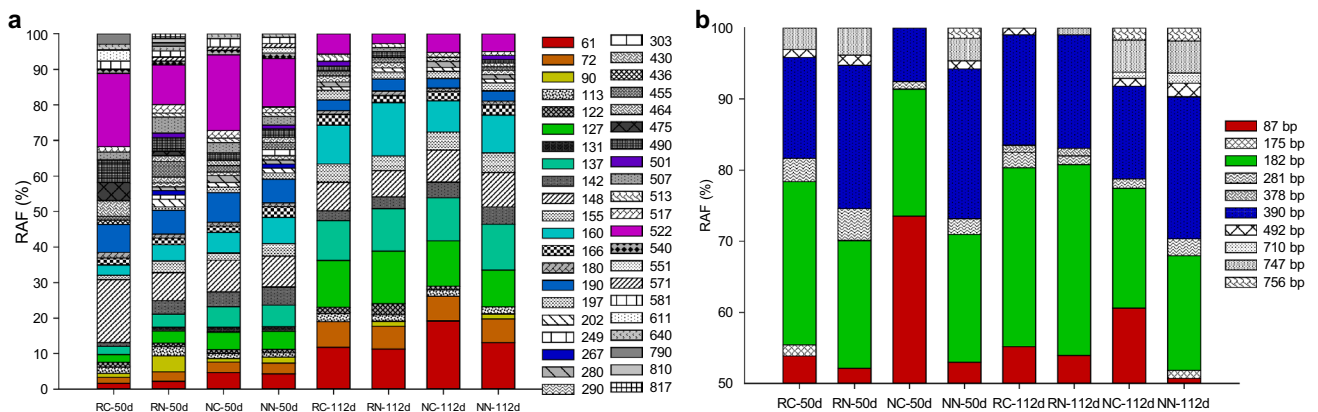
*n* = 3, Duncan’s test at *P* < 0.05 level, letters with different labels indicate significant differences of individual parameters between the four soils

no significant difference of bacterial and archaeal gene abundances at tillering stage of rice growth in two treatments both in rhizosphere and in non-rhizosphere, which may indicate that N application did not change bacterial and archaeal abundances significantly at tillering stage of rice growth. At ripening stage, however, N fertilization increased rhizospheric bacterial abundance and decreased non-rhizospheric bacterial abundance significantly. Furthermore, no significant change was detected for archaeal abundance (Table 3). Moreover, archaeal abundance did not change significantly with N application in the rhizosphere or non-rhizosphere, respectively. In general, N fertilization had no significant effect on the number of microorganism (bacteria and archaea) at tillering stage of rice growth, but changes the bacterial abundance a lot at ripening stage of rice growth.

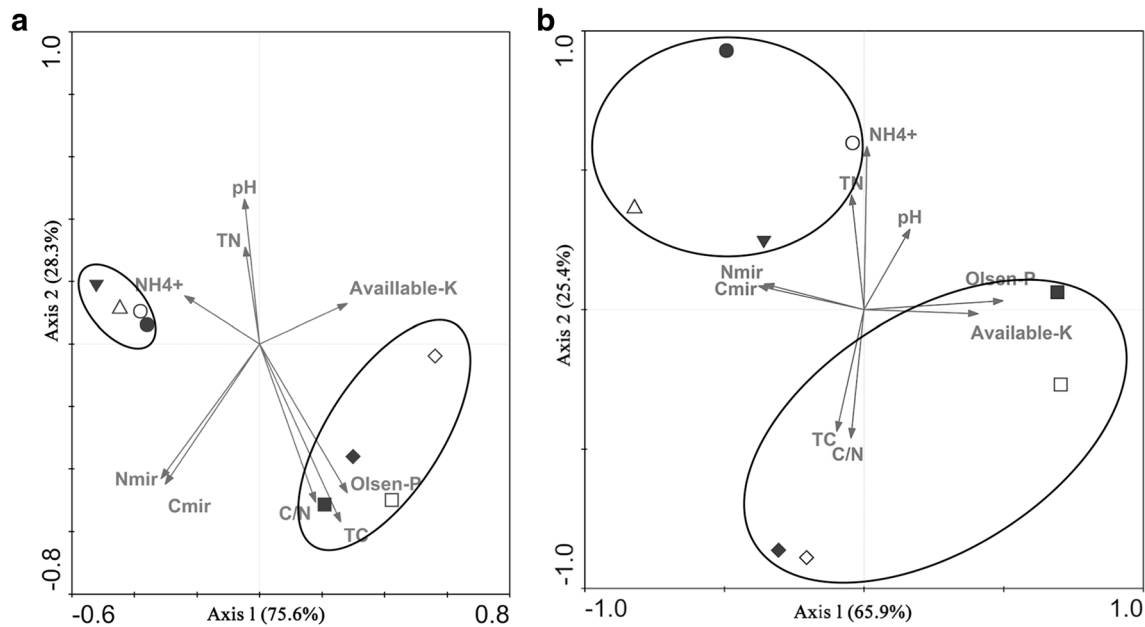
**Bacterial and archaeal community compositions by T-RFLP analysis**

To investigate the community structure of microorganisms, the T-RFLP analysis was conducted for bacterial and archaeal 16S rRNA gene from eight representative soil samples (RC-50d, RN-50d, NC-50d, NN-50d, RC-112d, RN-112d, NC-112d and NN-112d, respectively, Figs. 2,

3). Overall, bacterial 16S rRNA gene T-RFs in both rhizosphere and non-rhizosphere at tillering stage were about twofold higher than those observed at ripening stage of rice growth (Fig. 2). Apparently, the N fertilization had an impact on the presence of bacterial 16S rRNA gene T-RFs in both rhizosphere and non-rhizosphere soils. In the rhizosphere, the T-RFs numbers were 27–30 in control treatment but up to 38 and 34 were observed accordingly in N addition treatments. T-RFs numbers in the non-rhizosphere did not change at 50d but increased with N addition at 112d (from 19 to 23, Fig. 2). Moreover, the dominant T-RFs relative abundance changed with 160, 190, 267 bp in the rhizosphere, and with 90, 127, 160, 501 bp in the non-rhizosphere, respectively. The T-RFs numbers at tillering stage were 27 in the rhizosphere and 30 in the non-rhizosphere, respectively, whereas 38 T-RFs numbers in the rhizosphere and 34 T-RFs numbers in the non-rhizosphere were observed in the N fertilization treatment (Fig. 2). The T-RFs numbers at ripening stage were not changed in the rhizosphere (22 both) and increased from 19 to 23 in the non-rhizosphere in the fertilization treatment. The dominant relative abundance of T-RFs 61, 72, 127, 137 and 160 bp at tillering stage increased compared to ripening stage, but relative abundance of 190 and



**Fig. 2** Relative abundance of T-RFs of bacterial (a) and archaeal (b) 16S rRNA gene in rhizosphere and non-rhizosphere



**Fig. 3** RDA analysis of the T-RFLP profiles for the bacterial (a) and archaeal (b) community compositions in different treatment soils (as referred to RC-50d treatment “diamond”, RN-50d “square”, RC-112d

“filled diamond”, RN-112d “filled square”, NC-50d “triangle”, NN-50d “circle”, NC-112d “inverted triangle”, NN-112d “filled circle”). Arrows represent the environment variables

522 bp decreased. Archaeal 16S rRNA gene T-RFs analysis showed very low community diversity (4–9 T-RFs) compared to bacterial 16S rRNA gene T-RFs (Fig. 2). The dominant T-RFs were 87, 182 and 390 bp which totally accounted for about 90% of the total abundance. The dominant relative abundance of archaeal had a minor difference with N fertilizer in both rhizosphere and non-rhizosphere.

The multiple comparisons of RDA analysis showed the compositions of bacterial and archaeal communities at two stages of rice growth clearly separated from each other (Fig. 3a, b). At tillering stage, the microbial community compositions were mostly correlated with  $\text{NH}_4^+$ , TN, pH,  $N_{\text{mir}}$  and  $C_{\text{mir}}$  (Fig. 3a, b). However, at ripening stage of rice growth, bacteria and archaea were more or less lined with Olsen-P, C/N ratio, TC and available K (Fig. 3a, b).

### Diversity analysis of bacterial and archaeal communities

The diversity variations of the bacterial and archaeal communities based on the T-RFLP results are presented in Table 4. Statistically, significant differences of the diversity index of bacterial and archaeal communities were detected among the N added treatments at two growth stages of rice, both in the rhizosphere and non-rhizosphere. With N application, diversity index of bacterial and archaeal communities increased significantly in the non-rhizosphere. Moreover, bacterial diversity index changed significantly with N addition. These indicated that N addition has more effect on community diversity of bacteria and non-rhizospheric soil.

For more detailed information of the differences in community structure, NMDS analysis was used for the

**Table 4** Community composition diversities of bacterial and archaeal 16S rRNA genes based on T-RFLP profiles

Treatments	Bacterial 16S rRNA gene		Archaeal 16S rRNA gene	
	Diversity	Evenness	Diversity	Evenness
RC-50d	2.80 ± 0.12 b	0.85 ± 0.02 c	1.28 ± 0.08 b	0.66 ± 0.04 ab
RN-50d	3.37 ± 0.25 a	0.93 ± 0.02 a	1.30 ± 0.08 b	0.72 ± 0.10 a
NC-50d	2.97 ± 0.09 b	0.87 ± 0.04 c	0.78 ± 0.05 e	0.56 ± 0.04 c
NN-50d	3.20 ± 0.15 a	0.91 ± 0.03 ab	1.28 ± 0.10 b	0.66 ± 0.03 ab
RC-112d	2.76 ± 0.31 b	0.89 ± 0.01 b	1.14 ± 0.05 d	0.64 ± 0.04 ab
RN-112d	2.69 ± 0.22 c	0.87 ± 0.05 c	1.13 ± 0.04 d	0.63 ± 0.07 ab
NC-112d	2.56 ± 0.13 d	0.87 ± 0.05 c	1.23 ± 0.11 c	0.59 ± 0.30 c
NN-112d	2.74 ± 0.07 b	0.87 ± 0.04 c	1.45 ± 0.09 a	0.66 ± 0.03 ab

similarity analysis of the 16S rRNA gene RFs. The obtained pattern showed two distribution areas at two stages of rice growth in the data of the bacterial 16S rRNA gene RFs samples (Fig. 4). The RFs were dispersed at tillering stage indicating great differences of bacterial community structure. At ripening stage, the RFs were grouped suggesting affinities of the compositions. For the N application, RFs shifted greatly with the tillering stage of rice growth both in the rhizosphere and non-rhizosphere whereas at the ripening stage bacterial community structure had relatively less change. RFs shifted greatly at two sampled stages both in the rhizosphere and non-rhizosphere, indicating that the phenological stage of rice growth may have great impact on bacterial community structure (Fig. 4a). No distinct distributions were found by phenological stage or N amended for the archaeal 16S rRNA gene community structure (Fig. 4b).

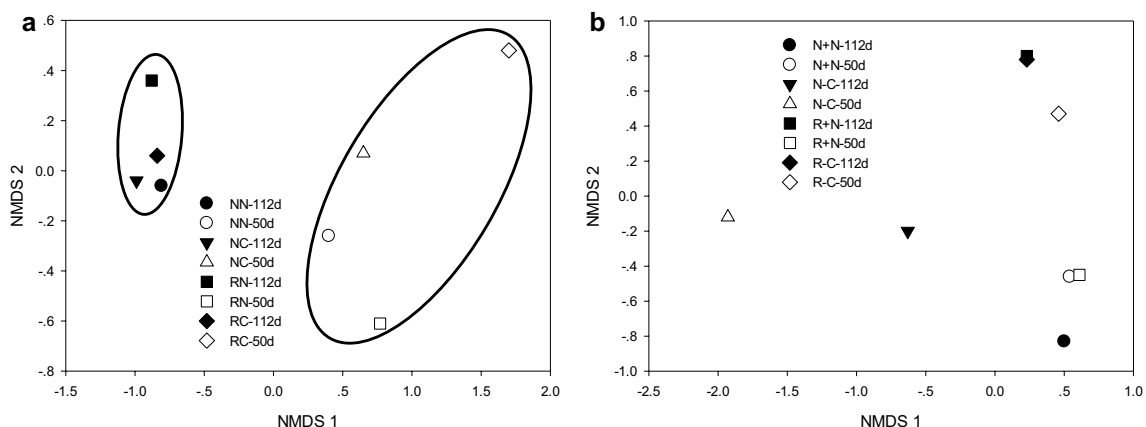
### Correlation parameters

Biological index was significantly correlated with most of the soil physiochemical variables (Table 5). Microbial products ( $C_{\text{mir}}$ ,  $N_{\text{mir}}$ ) and bacterial and archaeal abundances were closely correlated and both positively significantly related to TC, C/N ratio and negatively related to pH, suggesting that they were interrelated (Table 5). Bacterial diversity was mostly affected by pH, C and N nutrients available (TC, TN, C/N,  $C_{\text{mir}}$ ,  $N_{\text{mir}}$ ,  $\text{NH}_4^+$ ), while archaeal diversity was not related to any soil physiochemical characteristics (Table 5). Besides, bacterial abundance was positively correlated with archaeal abundance while negatively related with the diversity (Table 5).

### Discussion

To date, very few researches have been performed to ascertain relationship between rhizospheric soil properties and microbial biodiversity. The reason was probably attributed by the difficulty of rhizosphere sampling. In the present study, we separated non-rhizosphere from rhizosphere using a nylon bag which was commonly adopted in many other studies; (Huang et al. 2015; Jia et al. 2013; Nie et al. 2015; Nie et al. 2014; Steen and Atkinson 1991) thus, rhizosphere can be sampled for soil physical and chemical properties analysis. It should also be noted that in reality the microbial environment around rhizosphere is a continuous redox gradient which extends from the root surface to the non-rhizospheric soil (Brune et al. 2000; Cho 1982; Arth and Frenzel 2000).

In this study, the microbial community diversity in the non-rhizosphere was affected by N application significantly whereas no significant difference was found in the rhizosphere soil, both at tillering and ripening stages of rice growth (Table 4). As urea-N transformed to  $\text{NH}_4^+$ -N in the soil rapidly, the possible reason for these differences may be the  $\text{NH}_4^+$ -N availability in the rhizosphere, which was also found in other researches (Nie et al. 2014; Marschner et al. 1987). In the rhizosphere, no significant difference of ammonium concentration was found with this amount of N application (Table 2). In the non-rhizospheric soil, however, the ammonium concentration with N application was higher significantly than that in the control treatment (Table 2). Hence, we propose that due to root uptake  $\text{NH}_4^+$ -N available in the rhizosphere declined significantly compared to adjacent non-rhizospheric soil, leading to the different impacts of microbial diversity with N application in two zones. Moreover, it



**Fig. 4** NMDS of bacterial (a) and archaeal (b) 16S rRNA gene based on T-RFLP profiles from samples of both rhizospheric and non-rhizospheric soils at different stages of rice growth

**Table 5** Pearson correlations between bio-products index and soil physiochemical properties

	pH	TC	TN	C/N	Olsen-P	Available K	C <sub>mir</sub>	N <sub>mir</sub>	NH <sub>4</sub> <sup>+</sup>	Bacterial abundance	Archaeal abundance	Bacterial diversity	Archaeal diversity
pH	1												
TC	-0.831*	1											
Total N	0.712*	-0.265	1										
C/N	-0.970**	0.820*	-0.764*	1									
Olsen-P	-0.318	0.393	-0.359	0.516	1								
Available K	0.721*	-0.892**	0.065	-0.626	-0.098	1							
C <sub>mir</sub>	-0.995**	0.856**	-0.679	0.966**	0.329	-0.750*	1						
N <sub>mir</sub>	-0.989**	0.899**	-0.615	0.956**	0.318	-0.799*	0.992**	1					
NH <sub>4</sub> <sup>+</sup>	0.944**	-0.718*	0.786*	-0.898**	-0.114	0.578	-0.924**	-0.912**	1				
Bacterial abundance	-0.861**	0.608*	-0.826*	0.885**	0.366	-0.386	0.873**	0.871*	-0.850**	1			
Archaeal abundance	-0.885**	0.845**	-0.413	0.792*	0.106	-0.858**	0.911**	0.916**	-0.777*	0.720*	1		
Bacterial diversity	0.766*	-0.727*	0.713*	-0.767*	-0.208	0.464	-0.769*	-0.758*	0.793*	-0.743*	-0.723*	1	
Archaeal diversity	-0.259	0.327	0.136	0.123	-0.409	-0.579	0.286	0.295	-0.309	0.099	0.352	0.017	1

\*\* , \* Correlation is significant at the 0.01 and 0.05 level, respectively (two tailed)



was indicated that N addition has more effect on bacterial diversity than archaeal diversity (Table 4). The reason for this was probably related to the large difference of their community diversity. The T-RFLP analysis showed that quite more T-RFs (19–38) were identified as bacteria, but only 4–9 dominant T-RFs were considered as archaea (Fig. 2b). Significantly, higher bacterial diversity than archaea was also observed (Table 4). This was in agreement with other studies as reported in wetland soils (Lv et al. 2014; Walsh et al. 2005; Nishizawa et al. 2008).

The phenological stage of rice growth was found to be the main factor affecting the community structure both in the rhizospheric and non-rhizospheric soils (Figs. 2a, 4a). Great difference of community structure was observed by NMDS analysis of their RFs both in the rhizosphere and non-rhizosphere at tillering stage of rice growth in comparison with ripening stage (Fig. 4a). The reason for the great variance might probably be related to root exudation in the rhizosphere. At tillering stage, plant roots and microorganisms interact with, and compete for nutrients with rhizosphere (Jackson et al. 1989). Depending on different carbon and N availability, stimulation or retardation of microbial growth can be expected due to the competition of N in rice (Kaye and Hart 1997). RDA analysis also indicated that  $\text{NH}_4^+$ , TN,  $C_{\text{mir}}$  and  $N_{\text{mir}}$  were the main factors that affect microbial diversity. At ripening stage of rice, however, N availability was very low and microorganisms can use root exudates as N sources. In this process, microbes might partly be influenced by other nutrient parameters like P, C/N ratio, TC and K (Fig. 3a, b). Hence, the microbial structure at ripening stage may be similar with those with N application.

Microbial abundance at tillering stage did not change significantly both in the rhizospheric and non-rhizospheric soils in two treatments. The reason for that was probably related to high available C or N sources at tillering stage in the rhizosphere. At ripening stage, however, the available C or N sources decreased according to the accumulation of rice biomass. Thus, additional N might increase microbial abundance (Table 2). Moreover, large amount of root exudates such as organic acids, sugars and amino acids may stimulate microbial growth. The soil bacterial and archaeal abundances at ripening stage were almost one order higher than those of bacterial and archaeal abundances at tillering stage, respectively.

It should be noted that the microbial community may be affected by many other factors. Further studies on the quantitative research of microbial community as affected by N fertilizers at different stages of rice growth require the application of deep molecular biotechnologies such as clone library analysis or high-speed DNA-sequencing.

## Conclusions

In summary, this study indicates that N fertilizer and phenological stage of rice growth affected bacterial and archaeal community size and composition structure. While N might affect community structure diversity in the bulk soil rather than the rhizosphere, the growth stage of rice probably was the main factor affecting microbial abundance and community structure both in rhizospheric and non-rhizospheric soils.

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