Response of soil characteristics and bacterial communities to a gradient of N fertilization rates for coastal salt-affected Fluvo-aquic soil under paddy rice-winter wheat rotation

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Abstract

Excessive salts in soil inhibit enzyme activity, decrease microbial growth and constrain biochemical functioning, which could be alleviated by soil management and fertilization. However, the effect of consecutive chemical fertilizer on soil bacterial community structure under saline environment is poorly understood. Here, a field randomized block design under four nitrogen fertilization rates (0, 150, 300, and 450 kg N hm-2 y-1) was conducted on coastal salt-affected Fluvo-aquic soil. Effect of nitrogen fertilization rates on soil properties and bacterial community was characterized using Illumina Miseq sequencing for 16S rRNA gene. Results indicated that consecutive chemical N fertilization accelerated the improvement of soil chemical and microbial properties under the paddy rice - winter wheat rotation. Soil bacterial community well responded to the nitrogen fertilization and community richness and diversity increased with the nitrogen rates. Predominant bacterial phyla belonged to Proteobacteria, Chloroflexi, Acidobacteria, Actinobacteria and Planctomycetes, whereas Deltaproteobacteria, Anaerolineae, Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Actinobacteria and Planctomycetia were dominant bacterial classes. Increasing nitrogen fertilization resulted in an elevation in the relative abundance of classes Alphaproteobacteria, Gammaproteobacteria, Planctomycetia and Nitrospira, and a decline in Anaerolineae, Acidobacteria_Gp6, Cytophagia, Bacilli and Acidobacteria_Gp10. Clear separations in the bacterial communities at class level were observed under different nitrogen fertilization rates. Community structure of classes Alphaproteobacteria, Planctomycetia and Nitrospira was significantly influenced by potential nitrification rate (PNR), and community structure of class Actinobacteria was significantly influenced by carbon mineralization rate (CMR). The results demonstrated that nitrogen fertilization improved nutrients and metabolic activities to more suitable bacterial microhabitats for saline soil.

1. Introduction

With the increasing soil degradation and growing population, soil salinization has been a critical problem to agricultural production and ecosystem sustainability in not only arid and semi-arid areas, but also the coastal ecosystem (Pitman and Läuchli, 2004). This is particularly the case in the marine-terrestrial interlaced zone of the Delta region, where large amount of mudflats were developed from marine sediments and alluvial deposits, and these mudflats are naturally saline and can be used as wet lands or cultivated as reserve land resources (Li et al., 2014; Long et al., 2016). However, soil salts cause high osmotic stress and constrains the water and nutrient uptake by plants (Hagemann, 2011), restrict microbial growth and biochemical functioning which plays a pivotal role in soil organic matter and nutrient cycle (Wichern et al., 2006; Yan and Marschner,

2013). Therefore, improving the microbial biomass and activity in saline soil contributes to increasing soil organic matter input, promoting microbial C mineralization and nutrient cycling, and enhancing nutrient utilization efficiency (Elmajdoub and Marschner, 2015; Meena et al., 2016).

It has been shown that soil salinity is important in shaping bacterial communities in saline soils under halophytic vegetation, irrigation, fertilization regimes and even amendment residue application (Zhao et al., 2018; Rath et al., 2019). Recently, many efforts have been devoted to linking soil bacterial community composition to soil salinity along environmental gradients. Rousk et al. (2011) suggested that soil salinity was not a decisive factor for bacterial growth, and for structuring the decomposer community in an arid agroecosystem. Ren et al. (2018) found that soil salinity shaped microbial communities and contributed to nitrogen cycling and carbon fixation, *Thaumarchaeota* and *Proteobacteria* were crucial for nitrogen cycle and *Proteobacteria* and *Crenarchaeota* played important roles in dicarboxylate-hydroxybutyrate cycle. Study of Zhang et al. (2019) exhibited the importance of environmental filtering in microbial community assembly and suggested that soil salinity was a key determinant for soil microbial community composition and assembly processes in a desert ecosystem. In addition to terrestrial ecosystem, this relationship was also observed for saline lake sediments and wetland. Hollister et al. (2010) found that soil microbial community structure shifted along an ecological gradient of hypersaline sediments, and the greater depth of sequencing resulted in the detection of taxa not described previously. Cong et al. (2014) concluded that soil microbial community structure evolved along halophyte succession in Bohai Bay wetland and the belowground processes were strongly related with aboveground halophyte succession. Under the saline environment, soil microbial community was also found to be well responsive to interactive effect of amendment measurements, including biochar-manure compost (Lu et al., 2015), biogas residue (Shi et al., 2018), flue gas desulfurization gypsum by-products (Li et al., 2012), crude oil contamination (Gao et al., 2015), saline water irrigation (Chen et al., 2017) and even cultivation year (Cui et al., 2018). Furthermore, Baumann and Marschner (2013) stated that the microbial tolerance to soil drying and rewetting stress was salt level dependent, and the adaptation to salt stress could reduce the influence of water stress on microbial community composition only when salt stress was beyond a critical salinity level.

Fertilization is also capable of shaping soil microbial community structure in different scenarios of soil environment and planting patterns. A recent meta-analysis of soil microbial metabolic activity reported that the shift in microbial activity was a crucial mechanism for the change of N transformation rates in N-limited ecosystems with N addition (Zhou et al., 2017). Ikeda et al. (2014) assessed urea-formaldehyde (UF) fertilizer on the diversity of bacterial communities in onion and sugar beet, and revealed that that the community structures in both planting patterns shifted unidirectionally in response to the UF fertilizer. Li et al. (2016) found that nitrogen fertilization rate was one of the main factors influencing rhizosphere microbial community in continuous vegetable cropping within an intensive greenhouse ecosystem. For the alkaline soil, Zhou et al. (2016) revealed that the change in straw chemical properties had impact on the bacterial communities associated with the decomposition of straw in agro-ecosystems. Interactions between soil fertilization and saline water irrigation or precipitation were also observed on soil bacterial community and microbial metabolic activity, and the findings showed similarity under such circumstances, i.e., long-term saline water irrigation altered the bacterial composition of soil in an N-dependent manner (Guo et al., 2018), and alleviated the adverse effects of irrigation salinity on microbial metabolic activity (Chen et al., 2017). However, the non-synergistic effects of N fertilization and precipitation regimes on the microbial functional groups was reported by Sun et al. (2018), and it also showed the negative effect of lower pH induced by N enrichment would be alleviated by precipitation regimes. Dong et al. (2015) discovered that combined additions of N and P fertilizer could promote soil fertility and microbial activity in fir plantations of subtropical area, and suggested β -glucosidase (β G) and N-acetyl-β -D-glucosaminidase (NAG) as useful indicators of the biogeochemical transformation and metabolic activity of soil microbes. More recently, Nguyen et al. (2018) discussed the legacy impacts of extreme weather events and N fertilizer addition on soil bacterial communities and the key processes involved in carbon cycling, and summarized that nitrogen addition did not improve the resilience (rate of recovery) of soil bacterial communities and functions to prolonged-drought event, and a long time was needed for the recovery of the soil microbial community

historically exposed to extreme weather events.

With the above reviews, soil salinity and fertilization have been demonstrated to be the most important influencing factors on microbial composition at a global scale (Lozupone and Knight, 2007; Zhou et al., 2013). The importance of understanding bacterial community evolution in deterministic and stochastic processes is broadly recognized in microbial ecology (Evans et al., 2017), and recent literatures mostly focused on community assembly processes along natural salinity, pH, moisture, nitrogen fertilization, and irrigation water volume gradients (Van Horn et al., 2014; Zhang et al., 2019). However, little is known about soil characteristics and bacterial community assembly processes along a nitrogen addition gradient under saline environment. In this study, effect of N fertilization rates on soil characteristics and bacterial community structure was examined for coastal salt-affected Fluvo-aquic soil. This work was performed in a marine-terrestrial interlaced area, and the soil was reclaimed from mudflats and exposed to seawater immersion before reclamation for cultivation. The main objectives of this study were: (1) to investigate the shifts of soil chemical and microbial properties with cultivation years and N fertilization rates; (2) to determine how N fertilization rates affect the soil bacterial richness and diversity under saline environment; (3) to explore how the composition of bacterial community vary along N fertilization gradients at phylum and class levels; and (4) to determine which environmental factors are responsible for the alteration of the bacterial community structure at the class level.

2. Materials and methods

2.1 Site description

The study location was situated in Tiaozini reclamation area in Dongtai prefecture (32°49.9' ˜ 32deg50.3'N, 120deg56.6' ˜ 120deg57.4'E), north Jiangsu Province, China (Fig. 1). This site was located in marine-terrestrial interlaced area and was enclosed and reclaimed from coastal tidal mudflats in 2013. The distance from this site to the coastline of China Yellow Sea was about 1.6 km (Liu et al., 2019). The experimental site has nearly flat topography with an average elevation of 1.0 m above-sea-level. This site was in subtropical zone and strongly affected by the oceanic monsoon throughout the year. Cold, dry season is from November to March and the hot, wet season is during June and September. The mean annual rainfall is 1048.5 mm and an average of 734.3 mm rainfall occurred from May to September during the period of 2000-2015. Annual air temperature and daily sunshine duration were averagely 15.0 and 5.8 h, respectively. Developed from Yangtze alluvial sediments and marine sediments, the predominant soil is silt loam, classified as a loamy, mixed Typic Halaquepts group of Aquepts in Inceptisols based on soil taxonomy (Soil Survey Staff, 2014). Shallow saline water table (annual average electrical conductivity of 7.6 dS/m and water table of 1.10 m) results in large areas of salt-affected land and poor soil productivity for cropland. The experimental site is a representative of large areas of salt-affected farmlands in coastal alluvial plain of China.

Figure 1

2.2 Land use and management history

The Tiaozini reclamation area had no documented history of cultivation until 2015, and paddy rice and winter wheat rotation was employed in this area (Fig. 1). Paddy rice was initially planted in the cropland to leach soil salinity as the salt levels of the newly-reclaimed farmland exceeded the salt-tolerant thresholds for most agricultural crops. Brackish water with the average electrical conductivity of 3.6 dS/m, as drawn from the river near the site, was used for paddy rice irrigation, and water was supplied using flood irrigation during the rice season. The growing season of paddy rice was from late-June to mid-October and that of rainfed winter wheat was from early November to mid-June. Conventional soil fertility and pest management practices were used and no organic matter inputs were made. Poor soil aggregate structure, soil nutrient pools and microbial activity, as induced by soil salinization, are known as significant limitations to soil productivity in the coastal saline area (Zhang et al., 2014; Shahid et al., 2018), resulting in an annual yield depression of 30˜50% in the experimental site.

2.3 Experiment design

A randomized block design with 16 plots was used in this experiment, including four treatments with four replicate blocks. Sixteen 3 m x 4 m plots were built and a ridge of 50 cm width and 30 cm height was used to separate the adjacent plots. A thick plastic film was vertically buried along the ridge to a depth of 1 m between any two adjacent plots. The four treatments included control (CK, no N fertilization), NF1 (N fertilization with 150 kg N $hm^{-2}year^{-1}$), NF2 (N fertilization with 300 kg hm^{-2} $year^{-1}$) and NF3 (N fertilization with 450 kg N $hm^{-2}year^{-1}$). Prior to the experiment, an amount of 2250 kg hm^{-2} calcium superphosphate was applied to all the plots as soil amendment measurement and phosphorus fertilization supply, according to the local management practice habits in coastal region (Wang et al., 2016; Yang et al., 2018). No potassium fertilization was used and urea (nitrogen content 46.4%) acted as base fertilizer and topdressing fertilizer during the experiment. Crop residues were removed from the plots after the harvest of each season. The amount of nitrogen rates in the paddy rice (*Oryza sativa* L.) season was equivalent to that in the winter wheat (*Triticum aestivum* L.) season. Before the sowing time of each crop season, the base fertilizer was applied and mixed uniformly with 0-15 cm soil layer by manual, and the topdressing fertilizer was added to soil by spraying manually in key stages of crop growth season. This work was repeatedly conducted from late May 2015 to early June 2018. Table 1 presents the scheme of nitrogen management in key stages of crop growth period.

Table 1

2.4 Soil sampling and lab analysis

A field with uniform soil salinity status was selected for the plot experiment, and the vegetation of this field was mostly Aeluropus littoralis and reeds with the coverage of about 30%. This field was leveled using a rototiller to ensure the homogeneous condition of surface soil. Plots were built after the field leveling and soil sampling at 0-15 cm layer was conducted from each plot on late May 2015 after the plots were established (before calcium superphosphate and urea application). Soil samples taken at this time was use as the initial soil conditions. During the experiment, soil sampling were repeatedly performed on early June 2016, early June 2017 and early June 2018, respectively, i.e., after the harvest of winter wheat and before the sowing of paddy rice. In each plot, soil samples were collected using corers (5.0 cm diameter) and three soil cores were taken and then mixed to form one unique representative sample. A total of sixteen composite samples were obtained for each soil sampling.

Soil sample from each plot were subdivided in three subsamples: the first one was air dried, crushed and passed through 1 mm and 0.15 mm sieves for soil physicochemical analysis, the second one was sieved with the mesh size of 2 mm and stored at 4 for soil microbial analysis, and the third one was passed through 2 mm sieves and stored at -80 for soil DNA extraction, amplification and pyrosequencing. The analyzed soil physicochemical and microbial attributes included soil salinity (EC_e), pH, sand (S_A) and clay (C_L) particle content, soil organic carbon (SOC), bulk density (ρ_b), cation exchange capacity (CEC), total nitrogen (TN), available nitrogen (AN), available phosphorous (AP), microbial biomass carbon (MBC), microbial biomass nitrogen (MBN), carbon mineralization rate (CMR), net nitrogen mineralization rate (NMR) and potential nitrification rate (PNR). The soil physical attributes mentioned above were measured only for initial soil samples since these attributes were assumed to be static and not change over a short period of time (Yao et al., 2013). Table 2 gives the analyzed physicochemical and microbial properties as well as the analytical protocols selected.

Table 2

Table 3 presents some measured soil physicochemical properties of initial soil samples across the study location. Apparently, the soils in our experimental site are characterized by high salinity, high soil compaction, low organic matter, and low nutrient supply capacity. This was in line with previous reports for coastal mudflat soil (Yao et al., 2016; Luo et al., 2017).

Table 3

2.5 Soil DNA extraction, amplification and pyrosequencing

According to the manufacturer's instructions of a Power Soil DNA Isolation Kit (MoBio Laboratories Inc., USA), the total genomic DNA was extracted from the fresh soil subsamples collected on early June 2018. The quality and purity of the extracted genomic DNA was quantified using agarose gel electrophoresis and spectrophotometry on a NanoDrop® ND-2000c UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Then the extracted DNA was diluted to about 10 ng μL^{-1} and stored at -80 for the following analytical steps.

Bacterial 16S rRNA gene amplicon sequencing and Miseq library construction was performed by Genesky Biotechnologies Inc. (Shanghai, China). The V4-V5 hypervariable region of the 16S rRNA gene was amplified with dual-indexed Illumina fusion primers: V4for 5'-GTGCCAGCMGCCGCGGTAA-3'and V5rev 5'-CCGTCA ATTCMTTTRAGTTT-3' (Kozich et al., 2013). Polymerase chain reaction (PCR) was performed with GoTaq® Hot Start PCR Master Mix (Promega, Madison, WI, USA) in a 25 µL reaction. Using ABI 2720 Thermal Cycler (Thermo Fisher Scientific, USA), the following thermal cycling scheme was used: initial denaturation step at 94 for 3 min, followed by 35 cycles of denaturation at 94 (45 s), annealing at 50 (30 s), extension at 70 (90 s), and a final extension at 72 for 10 min (Rath et al., 2019). The PCR products were purified with AmpureXPbeads (AGENCOURT) to remove the unspecific products. The average molecule length was determined using the Agilent 2100 bioanalyzer instrument (Agilent Technologies, USA). The library was quantified by real-time quantitative PCR (EvaGreen™). The Qualified libraries are sequenced pair-end on the Illumina MiSeq Benchtop Sequencer (Illumina, San Diego, CA, USA).

All downstream processing of sequences was conducted using the QIIME pipeline v1.7.0 as described in Caporaso et al., (2010). Paired-end reads were assembled using PANDAseq (Masella et al., 2012), and assembled sequences were quality filtered using USEARCH v7 (Edgar, 2010), retaining only sequences ¿ 100 bases in length with expected errors < 2.0. After the filtering step, 4,790,305 sequences were collected with an average length of 376 bases. Sequences were clustered de novo into operational taxonomic units (OTUs) at a 97% similarity level. Taxonomic information was assigned to OTUs and samples were rarefied to 10,000 sequences to correct for differences in sequencing depth. Samples with $<$ 10,000 sequences and OTUs that were observed $<$ 10 times across all samples were excluded in downstream processing, and these criteria resulted in the removal of 5243 out of the total 14601 OTUs.

2.6 Statistical analysis

In this study, each group (nitrogen rates and control treatments) consisted of four physicochemical and biological replicates, and results are shown as the mean value plus/minus standard deviation and the statistical significance is accepted at $p < 0.05$. Using SPSS Statistics 17.0 (IBM Company, Armonk, NY, USA), one-way analyses of variance (ANOVA) and two-way ANOVA comparisons were used to identify the effect of nitrogen rates and cultivation time on soil chemical properties and microbial communities. Using the QIIME software 1.7.0 (https://qiime.org), the diversity indices including observed OTUs, Chao1, ACE, Shannon, Simpson and coverage were obtained and used to evaluate the biodiversity based upon the richness of the species and to estimate the abundance-based richness within the community (Magurran, 2013). Based upon CANOCO version 5.0 (Microcomputer Power, Ithaca, USA), principal coordinates analysis (PCoA) was employed to analyze the abundance of bacterial class to visualize differences in soil bacterial communities, and redundancy analyses (RDA) were conducted to investigate the relationship between environmental factors and bacterial community structure.

3. Results

3.1 Soil chemical and microbial properties

Temporal changes of soil chemical and microbial properties across all the treatments are presented in Table 4 and Figure 2, respectively. Compared with control treatment (CK), consecutive N fertilization significantly

increased SOC, TN, MBC, MBN, CMR, NMR and PRN, whereas significantly decreased soil EC_e. The effect of N fertilization rates on soil pH, CEC and AN was not significant across all the treatments. Soil AP showed temporal decrease trend since calcium superphosphate was applied as soil amendment and phosphorus fertilizer supply for single use prior to the first paddy rice season. Table 5 shows the Pearson correlation coefficient among the soil chemical and microbial properties. Soil EC_e was negatively correlated with SOC, CEC, AP, MBC, MBN, CMR, NMR and PNR, and SOC showed positive correlation with CEC, TN, AN, MBC, MBN, CMR, NMR and PNR. Soil microbial properties were relevant to each other whereas the correlation between pH and other soil attributes was not significant. The two-way ANOVA results across all the treatments are also presented in Table 5. Cultivation time effect of treatments on EC_e, pH, SOC, CEC, AP, MBC, MBN, CMR, NMR and PRN was observed, and the effect of N fertilization rates on SOC, TN, AN, AP, MBC, MBN and NMR was significant. Cultivation time and N fertilization rates had interactive effect on AN, MBC and NMR in this study.

Table 4

Table 5

Figure 2

3.2 Description of Illumina Miseq dataset and bacterial richness and diversity

Ranging from 187,097 to 395,864 sequences with an average of 299,394 sequences per sample, a total of 4,790,305 high-quality sequences were obtained from sixteen soil samples treated with different N fertilization rates. The average length of the sequences analyzed was 376 bases, approximate 80% of the sequences analyzed were assigned at the Phylum level, 45-50% of the sequences at the Order level, and only 30-35% of the sequences were assigned to the Genus level. Figure 3 displays the description of the number of assignments by RDP classifier at each taxonomic level and for each soil sample.

Figure 3

In total, 9358 OTUs occurred with a frequency of at least 10 reads in the dataset. Figure 4 presents the bacterial community richness and diversity indexes calculated from the 16S rRNA gene amplicon sequencing. In comparison with the control soil samples, the OTUs increased by 3.67%, 8.14 and 15.05% at treatments of 150, 300 and 450 kg N hm^{-2} y^{-1} rates, respectively. Alpha diversity based on species richness varied among samples ($p < 0.05$) (Fig. 4). Chao1 and ACE, the community richness indexes, increased with the N fertilization rates, and samples at NF3 (450 kg N hm^{-2} y^{-1}) treatment had significantly higher Chao1 and ACE indexes than those at CK and NF1 (150 kg N hm^{-2} y^{-1}) treatments. The average ratio of OTUs/Chao1 was $83.27\% \pm 1.86\%$, implying that the sequencing efforts were not exhaustive. Shannon index showed similar trend with coverage index, i.e., increasing with the N fertilization rates, whereas Simpson index decreased with N rates. The comparison of the samples based on the richness and diversity indexes showed that bacterial community structures well responded to the N fertilization rates, and greatest bacterial richness occurred in NF3 and NF2 treatments, followed byNF1 and CK treatments.

Figure 4

3.3 Bacterial community structures at phylum and class levels

The bacteria from sixteen soil samples exhibited similar diversity but different abundances. A total of 42 identified phyla were observed, and the 4,790,305 high-quality sequences classified at the phylum level were affiliated to 36 Bacterial phyla and 6 Archaea phyla. Bacteria were numerically dominant relative to Archaea. The dominant phyla across all samples were: *Acidobacteria* , *Actinobacteria* ,*Bacteroidetes* , *Chloroflexi* , *Cyanobacteria* ,*Firmicutes* , *Gemmatimonadetes* , *Ignavibacteriae* ,*Nitrospirae, Planctomycetes* , *Proteobacteria* ,*Thaumarchaeota* and*Verrucomicrobia* (relative abundance more than 0.5%). The dominant taxa in the analyzed soil samples were:*Proteobacteria* (39.17%), *Chloroflexi* (15.81%),*Acidobacteria* (7.19%), *Actinobacteria* (6.34%),*Planctomycetes* (5.82%), followed by a second group with a lower but still important percentage of distribution across all the samples, i.e., *Bacteroidetes* (4.44%), *Firmicutes* (1.98%),*Gemmatimonadetes*

(1.53%), *Ignavibacteriae* (1.51%). The taxa with a lower relative distribution were *Cyanobacteria*(0.99%), *Nitrospirae* (0.95%), *Verrucomicrobia* (0.70%) and *Thaumarchaeota* (0.56%). The relative bacterial community abundance at the phylum level was shown in Fig. 5a. For the CK treatment, *Proteobacteria* accounted for 36.54% of the total soil bacteria, whereas the percentage increased to 39.29%, 39.42% and 40.78% for NF1, NF2 and NF3 treatments, respectively.*Chloroflexi* , another predominant phylum, was 17.12% of the total bacteria in the CK treatment soil, which significantly decreased to17.58%, 15.30%, and 13.57% in NF1, NF2 and NF3 treatment soils, respectively. Moreover, significant shifts were also observed for*Firmicutes* and *Nitrospirae* across all the treatments.

Figure 5

Figure 5b illustrates the relative bacterial community abundance at the class level. In total, 73 classes were classified across all soil samples, of which 23 classes had relative abundance of more than 0.5%. The relative abundance of unassigned classes was 18.68%, and the dominant classes (relative abundance more than 0.5%) across all samples included *Deltaproteobacteria* (11.79%), *Anaerolineae*(10.61%), *Alphaproteobacteria* (10.27%),*Betaproteobacteria* (7.94%), *Gammaproteobacteria* (7.41%),*Actinobacteria* (6.13%) and *Planctomycetia* (5.15%). The bacteria classes with lower but important percentage (relative abundance between 1% and 5%) comprised of *Acidobacteria_Gp* 6 (2.65%),*Ignavibacteria* (1.49%), *Cytophagia* (1.43%),*Sphingobacteriia* (1.33%), *Nitrospira* (1.32%),*Gemmatimonadetes* (1.20%), *Bacilli* (1.19%) and*Acidobacteria_Gp* 10 (1.05%). The relative bacterial community abundance at the class level was presented in Table 6. Increasing trend was observed for classes of *Alphaproteobacteria* ,*Gammaproteobacteria* , *Planctomycetia* and *Nitrospira*with the increment of N fertilization rates. For instance, the relative abundance of *Nitrospira* increased from 1.09% to 1.13%, 1.35% and 1.65% at 150, 300, and 450 kg N $hm^{-2}y^{-1}$ rates, respectively. This trend was also observed for *Nitrospira* at the phylum level. The N fertilization decreased the relative abundance of classes *Anaerolineae* ,*Acidobacteria_Gp* 6, *Cytophagia* , *Bacilli* and*Acidobacteria_Gp* 10. The relative abundance of classes*Deltaproteobacteria* , *Betaproteobacteria* ,*Actinobacteria* , *Ignavibacteria* , *Sphingobacteriia*and *Gemmatimonadetes* was not statistically different among various N fertilization rates, indicating that the effect of N fertilization on the above bacterial classes was not obvious.

Table 6

3.4 Differences in bacterial classes among different N rates

Principal coordinates analysis (PCoA) was performed to determine the extent of treatment differentiation with regard to the N fertilization rates. The PCoA demonstrated clear separations in the bacterial communities at class level under different N fertilization rates (Fig. 6). The first principal component (PC1, 40.7% of contribution rate), which explains the majority of variations in the data, represented presence or absence of N fertilization. The second principal components (PC2), representing N fertilization rates, explained 17.9% of the data variance. In total, 58.6% of variance of species was explained by the two principal components. The two components separated the community composition by differences in the N fertilization rates which was the only difference among the treatments. Obviously, one soil sample with 300 kg N hm^{-2} y^{-1} was clustered into the group of 150 kg N hm^{-2} y^{-1}. Despite this, the PCoA results suggested that soil bacterial classes were well separated from different N application rates, and the change in N rates brought about changes in the bacterial community structure.

Figure 6

The similarity and differences of the sixteen dominant bacterial classes was further presented in the bacterial community heatmap (Figure 7). The cluster structure showed four main groups of class which shared a peculiar composition and abundance among the samples. The control soil samples were discriminated from other samples treated with N fertilization, suggesting clear distinction of bacterial community structure between the treatments with and without N fertilization. Samples with 450 kg N hm^{-2} y^{-1} rate were also discerned easily, such as *Alphaproteobacteria* ,*Anaerolineae* and *Actinobacteria* , whereas the distinction between treatments of 150 kg N $hm^{-2}y^{-1}$ and 300 kg N $hm^{-2}y^{-1}$ rates was not obvious. Actually, most groups of classes, although showing varying abundance, appeared grouped together and uniformly distributed across

all the samples with the increase of N rates. For 150 kg N hm^{-2} y^{-1} and 300 kg N hm^{-2} y^{-1} treatments, all the classes had almost the same abundance degree, resulting in the uncertainty in cluster discrimination. Overall, this indicated that the capacity of N fertilization in shaping bacterial communities was not as important as that of soil salinity. This was also witnessed by the statistics of relative abundance in Table 6.

Figure 7

3.5 Redundancy analysis (RDA) on bacterial community composition

Redundancy analysis method (RDA) was employed to investigate what environmental factors shifted the bacterial community structure and the classes' relative abundance among treatments. Environmental variables included SOC, TN, AN, MBC, MBN, CMR, NMR and PNR, and attributes including EC_e, pH, CEC and AP were not considered since no significant differences were observed across all soil samples (collected on June 10, 2018). Monte Carlo permutation was employed in RDA method to test the significance of soil chemical and microbial parameters in explaining variation in bacterial community structure. Figure 8 gives the environment-species relationship of RDA tests based upon bacterial community data matrix at the class level. Soil bacterial community distribution differed between the four treatments, suggesting that metabolic functions also vary depending on the conditions. The first axis explained 37.8% of the variation ($p < 0.01$), which was correlated with CMR, PNR and TN. It was indicated that the first axis to some extent may characterize the status of soil carbon and nitrogen metabolism (Figure 7A). The second axis explained 12.0% of the variation, which was correlated with SOC, MBC, MBN, AN and NMR. The second axis represented the status of soil carbon and nitrogen content. TN was the strongest factor (P = 0.016) that was correlated with the class distribution of bacterial community. CMR also showed significant correlations with community composition (P = 0.046), whereas the other factors were all not significant (Fig. 7A). The community structure of*Alphaproteobacteria* , *Planctomycetia* and *Nitrospira*was significantly influenced by PNR, and the community structure of*Actinobacteria* was significantly influenced by CMR. Also, SOC, MBC, MBN and AN had significant influence on the community structure of*Deltaproteobacteria* , *Gammaproteobacteria* and*Bacteroidia* . The above results indicated that the N fertilization shifted the environmental factors and the distribution of the bacterial community.

Figure 8

Spearman's rank correlation results, showing the dependence between relative abundance of bacterial classes and the environmental factors, are shown in Table 7. *Nitrospira* showed significant positive correlation with SOC, TN, AN, MBN, CMR, NMR and PRN, whereas*Cytophagia* exhibited significant negative correlation with SOC, TN, AN, MBC and NMR. Relative abundance of *Gammaproteobacteria*was positively correlated with TN, MBC, MBN and NMR. Moreover, significant negative correlation between *Anaerolineae* and TN,*Bacilli* and SOC, *Bacilli* and MBC,*Acidobacteria_Gp* 10 and MBC was observed. Three classes showed a statistically significant dependence on the CMR of soil samples:*Alphaproteobacteria* , *Actinobacteria* and *Nitrospira* . Generally, *Nitrospira* exhibited significant positive correlation with most of the environmental factors, whereas *Cytophagia* showed significant negative correlation with most of the soil properties. No significant correlation between soil properties and*Deltaproteobacteria* , *Betaproteobacteria* ,*Planctomycetia* , *Acidobacteria_Gp* 6,*Sphingobacteriia* , *Ignavibacteria* and*Gemmatimonadetes* was observed, indicating that these bacterial classes were independent from the environmental factors associated with soil carbon and nitrogen metabolism.

Table 7

Effect of N gradient on the relative abundances of the dominant bacterial classes was examined using regression analysis (Figure 9). The N fertilization rates was significantly positively correlated with*Alphaproteobacteria* (R^2=0.270, p<0.05), *Gammaproteobacteria*(R^2=0.375, p<0.01) and *Nitrospira*(R^2=0.650, p<0.01), indicating that the relative abundance of this bacterial classes increased with the N fertilization rates. Significant negative correlation was observed between N gradient and *Cytophagia* (R^2=0.425, p<0.01) and Bacilli (R^2=0.261, p<0.05) and the indication was that the increase of N fertilization rates reduced the relative abundance of the above bacterial classes.

Figure 9

4. Discussion

4.1. Response of soil chemical and microbial properties to cultivation and N fertilization

In this study, time lapse (cultivation years) was found to be influential to most soil properties, including EC_e, pH, SOC, CEC, AP, MBC, MBN, CMR, NMR and PNR (Table 5). Among these, soil EC_e had significantly negative correlation with most of other soil properties, indicating that the changes of soil chemical and microbial properties were mostly ascribed to the decline of soil EC_e induced by consecutive paddy rice- winter wheat rotation. Actually, most of previous literatures (Canfora et al., 2014; Chen et al., 2017) reported that soil salinity was a key determinant for soil microbial communities in desert and coastal ecosystems. Soil organisms, available nutrients, microbial diversity, biomass and metabolic activities linearly decreased along the increase of salinity gradient. This coincided with the findings of this study.

In addition to the change of soil EC_e caused by rotation system, fertilization also played an important role in temporal changes of soil chemical and microbial attributes which was essential to transformation and cycle of soil organic matter and nutrients. Table 5 showed that SOC, TN, AN, AP, MBC, MBN and NMR exhibited clear response to N fertilization. Li et al. (2016) reported that N fertilization affected bacterial communities by strongly driving the shifts of dominant bacteria within an intensive greenhouse ecosystem. Chen et al. (2017) found that N fertilization had significant influence on soil microbial metabolic activity (MMA), and medium (350 kg ha^{-1}) N fertilization coupled with medium amount of saline water irrigation could obtain the optimal MMA. Dong et al. (2015) reported that soil microbial community composition, soil microbial biomass (C and N) and enzyme activities were responsive to N fertilization, but responses often varied depending on N quantity added, and combined additions of N and P fertilization was suggested to promote soil fertility and microbial activity. In a more rigorous study, Liang and MacKenzie (1996) tracked the fate of nitrogen using^{15}N fertilization, and revealed that higher N fertilization rates above normal increased microbial biomass N immobilization with greater N release, and high N fertilization rates significantly increased both the magnitude of soil microbial biomass N and microbial fertilization N recovery in the soil microbial biomass. This was consistent with our findings in this study that high chemical N fertilization rates increased both microbial biomass N (MBN) and N mineralization rate (NMR), and resulted in higher soil total nitrogen and available nitrogen.

4.2. Relative abundances of the bacterial phyla and classes under saline environment and different N fertilization rates

Relative abundance of phyla *Proteobacteria* , *Chloroflexi* ,*Acidobacteria* , *Actinobacteria* , *Planctomycetes* ,*Bacteroidetes* and *Firmicutes* accounted for more than 80% of total bacterial richness. Correlation analysis on relative abundance of dominant bacterial phyla and N fertilization rates showed that N fertilization rates were positively correlated with*Proteobacteria* (r =0.562) and *Planctomycetes*(r =0.492), and negatively correlated with *Chloroflexi*(r =-0.624) and *Firmicutes* (r =-0.688). Most of the bacterial phyla found in this area were also reported by Canfora et al. (2014) in a semiarid Mediterranean environment, Gao et al. (2015) in coastal salt-affected alluvial area, Ahmed et al. (2018) in mine salt stressed soil, Ren et al. (2018) in a salt-affected desert ecosystem, and Rath et al. (2019) along a salt-affected lake ecosystem. Based upon meta-analysis and retrieved sequences from the two databases GenBank and RDP, Ma and Gong (2013) summarized that 90% of the bacterial sequences were categorized into six phyla, including *Proteobacteria* ,*Actinobacteria* , *Firmicutes* , *Acidobacteria* ,*Bacteroidetes* , and *Chloroflexi* . Similarly, Delgado-Baquerizo et al. (2018) concluded that five dominant bacterial phyla in mudflats and paddy soil were *Proteobacteria* ,*Bacteroidetes* , *Chloroflexi* , *Acidobacteria* and*Planctomycetes* . These bacterial phyla were also discovered in our study, although the absolute and relative richness varied among these phyla. This was not unexpected considering that the average EC_e of soil samples decreased from 9.3 dS m^{-1} on late May 2015 to 2.78 dS m^{-1}on early June 2018. This meant that soil salinity decreased from moderately saline level (EC_e, 8-16

dS m^{-1}) to very slightly saline level (EC_e, 2-4 dS m^{-1}) according to soil salinization classification proposed by Soil Survey Division Staff (1993). In addition to the above soil salinity related bacterial phyla, some phyla widely distributed in nonsaline soil were also found in our study. This group consisted of the following phyla:*Gemmatimonadetes* , *Ignavibacteriae* ,*Cyanobacteria/Chloroplast* , *Nitrospirae* ,*Verrucomicrobia* , *Thaumarchaeota* , *Armatimonadetes*and *Euryarchaeota* , although some phyla were classified as "salinity related" by some authors (Naz et al., 2010; Chambers et al., 2016). Correlation analysis also showed that N fertilization rates had significantly positive influence on *Gemmatimonadetes*(r =0.819) and *Nitrospirae* (r =0.895), and negative influence on *Armatimonadetes* (r =-0.529) and*Euryarchaeota* (r =-0.589). On the other hand, some bacterial phyla, showing an equal distribution across all the sites, were not correlated with and influenced by the N fertilization rates. Generally, the capacity of N fertilization in shaping bacterial communities was not as important as that of soil salinity, and this conclusion could be drawn from the comparison among most of previous literatures.

At the class level, more than 60% of total bacterial richness belonged to seven classes: *Deltaproteobacteria* , *Anaerolineae* ,*Alphaproteobacteria* , *Betaproteobacteria* ,*Gammaproteobacteria* , *Actinobacteria* and*Planctomycetia* . As the division of phylum *Proteobacteria* ,*Deltaproteobacteria* , *Alphaproteobacteria* ,*Betaproteobacteria* and *Gammaproteobacteria* were predominant bacterial classes. Decrease trend was found for the relative abundance of *Deltaproteobacteria* when amendments were made to the salt-affected soils (Bello-Lopez et al., 2014). Wu et al. (2006) found that the relative abundance of *Alphaproteobacteria* and the*Gammaproteobacteria* increased with soil salinity, whereas the*Betaproteobacteria* decreased with salinity. The findings of Yousuf et al. (2014) also showed that *Alphaproteobacteria* was the dominant group in the high-salinity soil or water in terms of abundance, whereas *Betaproteobacteria* was more important in nonsaline environment and *Gammaproteobacteria* was a minor component. This was in line with Kirchman et al. (2004) who concluded that marine systems were typically dominated by *Alphaproteobacteria* and*Cytophaga* -like bacteria, whereas *Betaproteobacteria*appeared to be the dominant group in freshwater systems. However, in another study by Valenzuela-Encinas et al. (2008), who found that, for soils from the former lake Texcoco, the dominant class of*Proteobacteria* in both high and low saline soils was*Gammaproteobacteria* , whereas *Alphaproteobacteria* was dominant in medium saline soils. In this study, the relative abundance of bacterial *Alphaproteobacteria* (r =0.520) and*Gammaproteobacteria* (r =0.612) showed significantly positive correlation with N fertilization rates, and positive correlation was also observed between N fertilization rates and*Deltaproteobacteria* , *Actinobacteria* and*Planctomycetia* , whereas *Anaerolineae* exhibited negative correlation with N fertilization rates. When considering the bacteria classes with lower but important percentage, N fertilization rates was found to be negatively correlated with relative abundance of*Cytophagia* (r =-0.652) and *Bacilli*(r =-0.511), and positively correlated with relative abundance of*Nitrospira* (r =0.806). This indicated that, under soil saline environment, N fertilization increased the relative abundance of*Alphaproteobacteria* , *Gammaproteobacteria* and*Nitrospira* , whereas decreased the relative abundance of*Cytophagia* and *Bacilli* (Fig. 8). In the semiarid irrigated area, Guo et al. (2018) found that no significant difference in bacterial composition occurred under slightly saline water irrigation with varying levels of N fertilization, and long-term slightly saline water irrigation (EC= 1.7 dS m^{-1}) increased the abundances of *Actinobacteria* and *Nitrospira* in soils. This was different from the findings of our study. The possible reason was that bacterial community was more sensitive to soil moisture than N fertilization rates for the semi-arid irrigated soil, whereas the level of N fertilization prevailed under the soil saline environment when soil moisture was not a constraining factor (Sun et al., 2018).

4.3. Relationship between bacterial community structure and soil chemical and microbial traits

Changes in soil bacterial community composition are more likely the result of a direct influence of shifts in soil chemical and microbial properties which were associated with different N fertilization rates, whereas compositional changes that did not corresponded with N fertilization were more likely to be the result of indirect factors synergetically changing with N fertilization. According to Lau and Lennon (2012) and Radhakrishnan and Baek (2017), bacterial community composition could vary by replacing less adapted species with better adapted ones, and this was one of the major mechanisms through which the trait distribution

of community changed. Relative abundance of*Anaerolineae* , which belonged to phylum *Chloroflexi* and was a type of carbohydrate and phenols degrading bacteria, showed negative correlation with most soil chemical and microbial properties. This was different from Zhang et al. (2019b) who reported that relative abundance of *Anaerolineae* in coastal salt-affected mudflats tended to increase with increasing years of rice cultivation. The possible reason was that high N fertilization rates and low organic matter resulted in low C/N and the richness of *Anaerolineae* which was important organic matter degraders under anoxic condition. These results agreed with Zhang et al. (2019a) who concluded that and the relative abundance of *Anaerolineae* decreased by 8.1%˜22.7% with the increase of nitrogen application. Relative abundance of*Alphaproteobacteria* and *Gammaproteobacteria* was positively associated with most soil chemical and microbial properties. This showed consistency with Iwaoka et al. (2018) who found that relative abundance of *Alphaproteobacteria* and *Gammaproteobacteria* was positively correlated with total N (TN) and net N mineralization rate (NMR). *Planctomycetia* and *Nitrospira* , which played important roles in soil nitrogen cycle (Fuerst and Sagulenko, 2013), showed positive correlation with most soil attributes in this study, especially class *Nitrospira* . Among them, *Planctomycetia*utilized and transformed ammonium and *Nitrospira* played pivotal roles in nitrification as an aerobic chemolithoautotrophic nitrite-oxidizing bacterium (Daims and Wagner, 2018). This was not unexpected considering that consecutive N fertilization improved the metabolic activities of some soil bacteria associated with N cycle, such as the net N mineralization rate and potential nitrification rate in this study. Interestingly, *Cytophagia* , *Bacilli* ,*Gemmatimonadetes* and *Acidobacteria* (including*Acidobacteria* _*Gp* 6 and *Acidobacteria* _*Gp* 10) were negatively influenced by soil chemical and microbial properties. Actually, relative abundance of *Cytophagia* was noted for an endophytic bacterial population closely associated with soil basic cations, namely soil salinity level (Szymańska et al., 2018), whereas soil salinity was negatively correlated with most of other soil microbial attributes (Table 5). With regard to *Bacilli* , Sharma et al. (2015) used 16S rRNA gene sequencing and fatty acid methyl ester (FAME) for diversity analysis of *Bacilli* , and found that most species possessed the ability to tolerate high salt, form endospores, and withstand harsh environments. Moreover, previous researches reported that flooding favored *Actinobacteria* and *Gemmatimonadetes*in salt-affected soil (de León-Lorenzana et al., 2017), and the relative abundance of *Gemmatimonadetes* was negatively correlated with soil enzyme activity and decreased significantly with cultivation year. This could be ascribed to the halotolerant and oligotrophic characteristics of these bacterial. Gad (2014) isolated halotolerant*Actinobacteria* from hypersaline sediments in Great Salt Plains, Oklahoma and analyzed the phylogenetic diversity and anti-MRSA activity. Siles et al. (2014) also found that amendments to salt-affected soil using crop residues decreased the relative abundance of*Gemmatimonadetes* .

5. Conclusions

This study revealed that consecutive N fertilization accelerated the improvement of soil chemical and microbial properties for coastal salt-affected Fluvo-aquic soil under paddy rice- winter wheat rotation. Results of 16S rRNA gene sequencing showed that soil bacterial community structures well responded to the N fertilization rates and community richness indexes increased with the N fertilization rates. Phyla of*Proteobacteria* , *Chloroflexi* , *Acidobacteria* ,*Actinobacteria* and *Planctomycetes* were the dominant phylum across all soil samples, whereas *Deltaproteobacteria* ,*Anaerolineae* , *Alphaproteobacteria* ,*Betaproteobacteria* , *Gammaproteobacteria* ,*Actinobacteria* and *Planctomycetia* were predominant bacterial classes at the class level. Although the impact of N fertilization on shaping bacterial communities was nowhere near as significant as that of soil salinity, N fertilization resulted in the increase in the relative abundance of classes*Alphaproteobacteria* , *Gammaproteobacteria* ,*Planctomycetia* and *Nitrospira* , and the decrease in*Anaerolineae* , *Acidobacteria_Gp* 6, *Cytophagia* ,*Bacilli* and *Acidobacteria_Gp* 10. Four bacterial classes were well separated from different N fertilization rates. However, community heatmap showed that most groups of classes appeared grouped together and uniformly distributed for different N fertilization rates. Redundancy analysis (RDA) indicated that the community structure of*Alphaproteobacteria* , *Planctomycetia* and *Nitrospira*was significantly influenced by PNR, and the community structure of*Actinobacteria* was significantly influenced by CMR.

Overall, N fertilization improved soil nutrients and metabolic activities to more suitable microhabitats for bacteria, and bacterial community evolved by replacing less adapted species with better adapted ones for coastal salt-affected soil under paddy rice-winter wheat rotation.

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