

Subsoiling and conversion to conservation tillage enriched nitrogen cycling bacterial communities in sandy soils under long-term maize monoculture

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ABSTRACT

Desertification degrades soil health and severely reduces crop productivity. Conventional tillage practices can amplify these problems in arid and semi-arid regions. For semi-arid regions in Inner Mongolia, China, the effects of introducing different tillage practices such as subsoiling (SS), straw mulching (SM), and no-tillage (NT) on otherwise long-term conventional tilled maize monoculture systems, were examined in the context of sandy soil bacterial and archaeal community diversity. Results showed that after three to four years of introduction, subsoiling and conversion to conservation tillage practices had no immediate effect on the alpha-diversity of the soil microbial communities. The beta-diversity of the soil microbial communities was less affected by the introduced tillage practices than by the growing season conditions, soil moisture, total nitrogen, soil macro-aggregate, and organic matter. Importantly, the introduced tillage practices had a notable effect on soil microbial communities associated with nitrogen (N) cycling processes, especially N fixation, nitrate reduction, nitrification, and denitrification. In particular, several years of tillage change from long-term conventional tillage enhanced the abundance of KEGG orthologs (KOs) associated with N fixation function involving species in *Rhodoplanes*, *Nitrospira*, *Skermanella*, and *Rhizobium* according to PICURSt prediction. *Rhodoplanes* spp. are involved in nitrate reduction and denitrification processes, and *Nitrospira* spp. associated with nitrite oxidation. We conclude that, for maize monoculture systems in semi-arid sandy soils, the soil bacterial and archaeal communities associated with many beneficial N cycling processes can be significantly impacted by only three to four years of introduced conservation and subsoiling tillage practices.

1. Introduction

Aeolian desertification often occurs in arid and semi-arid regions as a result of wind erosion and excessive natural vegetation denudation. These regions are often climatically and edaphically marginal for agricultural production (Ai et al., 2015; Arora et al., 1991). Intensive and continuous conventional tillage can amplify soil compaction and reduce water and nutrient availability, leading to long-term soil physicochemical and biodiversity degradation (Aciego Pietri and Brookes, 2009; Bayer et al., 2000; Lapen et al., 2001; Lupwayi et al., 2001; Raper et al., 2005; Sun et al., 2018). By contrast, conservation tillage practices, such as zero tillage (no-till), reduced or minimum tillage, mulch tillage, ridge and contour tillage (Busari et al., 2015), and subsoiling (Comia et al.,

1994) have been shown to stimulate the formation and preservation of water-stable aggregates leading to improved soil water availability (Bottinelli et al., 2017; Dairon et al., 2017). Conservation tillage is advocated broadly also for its potential to promote topsoil organic matter accrual and nutrient availability, to enhance microbial biomass and activity (Chávez-Romero et al., 2016; Kinoshita et al., 2017; Nunes et al., 2018; Sharma et al., 2013; Zhang et al., 2009), and consequently to improve soil fertility and productivity (Busari et al., 2015; Derpsch and Friedrich, 2009; Wang et al., 2017).

In agroecosystems, microbial communities provide critical ecosystem services associated with nitrogen (N), carbon, and other nutrient cycling (Anderson et al., 2017; Dorr de Quadros et al., 2012; Guo et al., 2016). It is critical to understand the microbiological

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responsiveness of soil microbial communities to changes in tillage activities, as such information will promote adoption of tillage practices that optimize productivity and soil biodiversity. In other words, if tillage induced impacts on soil bacterial communities and soil physicochemical properties do not manifest in a positive direction over relatively short time periods (a few seasons), there may be less will to deploy, support, and promote these tillage regimes as beneficial management practices. However, Frey et al. (1999) reported that there were no consistent effects of tillage on bacterial abundance or biomass under a 30-year tillage plot, while several other studies indicated that tilled soil may or may not have greater bacterial diversity than no-tilled fields (Ferreira et al., 2000; Øvreås and Torsvik, 1998). Similarly, consensus could not be drawn from more recent studies that have focused on the effects of no-till, minimum tillage, and crop residue on soil microbial community abundance, diversity, and composition (Anderson et al., 2017; Dong et al., 2017; Hariharan et al., 2017). These findings underscore that such associations are complex, multifactorial, and possibly associated with the time a soil is under a specific tillage practice (Dong et al., 2017; Nivelle et al., 2016). For agricultural sandy soils that have relatively low soil biodiversity to begin with (Dornelas, 2010; Köberl et al., 2011; Øvreås and Torsvik, 1998), conventional tillage practices could denude further critical soil biotic and abiotic properties to a point where many important microbial functions in the soil become inconsequential with respect to crop productivity (Mau et al., 2015; Trivedi et al., 2017; Van Horn et al., 2013). With N being the most limiting element in crop growth, adopting tillage practices that promote the functional potential of bacteria in the N biogeochemical cycle will likely be equally, if not more important than enhanced microbial genetic diversity writ large (Lupwayi et al., 2001; Øvreås and Torsvik, 1998). However, despite decades of research on microbial activity in semi-arid and agro-pastoral aeolian sandy agricultural systems, the influences of long and short-term changes in soil management on the microbiome are still not well understood.

With rapid advances in high-throughput sequencing (HTS) technologies and bioinformatics tool development, it is possible to characterize the complex microbiota and the functional role of soil bacterial communities (Caporaso et al., 2010; Langille et al., 2013; Louca et al., 2016). Coupling information on soil biophysical-chemical properties, environmental stressors, tillage practice conversion, crop productivity, and soil microbiota will help us untangle some of the cause-effect relationships, and better identify indicators on the state of soil health and fertility in response to new agricultural tillage regimes (Hariharan et al., 2017; Hermans et al., 2017).

We hypothesized that conversion of long-term conventional tillage practices to conservation tillage and subsoiling on sandy soils, would enhance and maintain soil microbial diversity and beneficial N cycling bacterial communities, and improve many other soil physicochemical properties necessary for augmenting maize production and improving overall soil health. To test these hypotheses, we established a plot-based study in the Horqin sandy land, a typical semi-arid region in northern China, to investigate how newly introduced conservation and subsoiling tillage practices, on otherwise historically conventionally tilled agricultural sands, would affect soil biotic and abiotic properties and maize production. The Horqin sandy land has been recognised as a highly desertified region due to excessive agricultural reclamation and overgrazing (Wang, 2000). This region is situated in an agro-pastoral transitional terrain zone (Huang et al., 2008), where farmers remove crop residues for livestock feed and predominately employ continuous maize monoculture. Conventional tillage is a common practice in this region, comprising of moldboard plowing soils to a depth of 15 cm prior to planting maize in the spring. The specific objectives of this study were to 1) examine the associations among the newly introduced tillage practices, key soil physicochemical properties, and crop yield; 2) evaluate the impact of four tillage practices and soil properties on the topsoil microbiota diversity, composition, and functional shifts using a metabarcoding approach; and 3) characterize the soil microbial communities

affiliated specifically with N cycling in response to tillage practice change.

2. Materials and methods

2.1. Study site

The experiment was conducted in Ganqika (42°40'~43°42'E, 121°30'~123°42'N), situated in the Golden-Maize-Belt in Horqin sandy land, Tongliao City, Inner Mongolia, China (Supplementary Figure 1-A). This region has a temperate semi-arid monsoon climate with an average annual temperature of 6.1 °C and 451 mm of total annual precipitation. The majority of rainfall occurs in the summer (from June to August), which accounts for approximately 70% of total annual precipitation. Daily precipitation and air temperature in 2016 and 2017 are shown in Supplementary Figure 1-B. The land on which these experiments took place was under conventional tillage (defined below) and maize monoculture for > 10 years. The soil is aeolian sand (arenosols, WRB, 2014) with 10.2 g kg⁻¹ soil organic matter and 2.5% of clay content at 0–20 cm depth.

2.2. Experimental design

The new tillage practices were introduced in the spring (2013) on long-term (> 10 yr) conventionally tilled soils. The trial was laid out in a randomized complete block design with four tillage practices: conventional tillage (CT) (no change in historic tillage practices), subsoiling (SS), no-till (NT), and straw mulching (SM). Each treatment was replicated in three blocks, with individual plots being 15 m long by 5 m wide. At harvest in fall, plots under CT, SS, and NT treatments were left with 10–15 cm of stubble and the remaining crop straw was removed. For plots under SM treatment, all maize straw was chopped to 10–15 cm and spread at ~7500 kg ha⁻¹ on the ground. At sowing in spring, plots under the CT and the SM treatments were tilled to a depth of 15 cm (traditional plowing depth under CT) using a moldboard plow; soils under the SS treatment were loosened to a depth of 35 cm using a subsoiling chisel without mixing crop residues; plots under the NT treatment were direct-injection seeded using an NT seeding-machine (2BMG-2/4, Debont Corp., Beijing, China). The maize (*Zea mays* L.) variety NK718 (purchased from Hefei Fengle Seed Co., Ltd, China) was used in this study and was planted at a density of 72 × 10³ plants ha⁻¹ at the end of April each year. The sowing depth was 5 cm and row spacing was 60 cm. No irrigation was applied to any experimental plots during the study period. Urea as N fertilizer was applied once at 450 kg ha⁻¹ at the jointing stage of maize (mid of July). Maize was harvested at the end of September each year. Grain yield was determined by harvesting plants from the two innermost rows with a length of 10 m of each experimental plot. The average air-dried grain yield per hectare was calculated after drying the grains to a moisture content of 14%.

2.3. Soil sampling and soil physicochemical properties measurement

Soil sampling was carried out two times in the years 2016 and 2017 (the third and the fourth year after tillage changes were made): sampling during each year occurred one week after sowing (AS, 7th May), and at harvest (AH, 29th September). We collected two replicates for each treatment with a total of 16 samples each year. In each plot, five bulk soil cores in close proximity to plant rows, were collected at 0–20 cm depth using a soil auger (5-cm in diameter). The augers were cleaned after each sampling using a 70% ethanol solution. The five soil cores per plot were then combined and homogenized into a composite soil sample for that treatment, and subsequently sieved through a 2 mm mesh to remove plant residues and stones and to extract the soil fraction. The composite soil sample was then divided into two parts: one was immediately stored on ice and transported to a laboratory for DNA extraction within 24 h post sampling, and the other sample was transported to a

laboratory and air-dried for physicochemical characterization.

The soil physical and chemical analyses included soil pH, soil moisture content, bulk density (BD), soil macro-aggregate fraction, soil organic matter (SOM), as well as total N (TN), available N, available phosphorous (P), and available potassium (K) of soil. The soil pH was measured by a pH meter with a 1:2.5 soil to water ratio (Peech, 1965). Soil moisture content was examined gravimetrically (ISSCAS, 1978), and BD was determined using the auger-hole method (ISSCAS, 1978). Soil aggregate fraction was determined by dry-sieving using the method described by Zhang et al. (2003). The SOM was determined with a K_2CrO_7 - H_2SO_4 oxidation procedure (Nelson and Sommers, 1982) and TN was measured by the Kjeldahl method (Sparks et al., 1996). A conversion factor of 1.724 was used to calculate soil organic carbon (SOC) from SOM (Allison, 1965). Soil C:N was calculated as the ratio of SOC to TN. The available N was analyzed using the alkaline diffusion method, and available P was measured by the Bray method (ISSCAS, 1978). The available K was detected by flame spectrophotometry (ISSCAS, 1978).

2.4. Soil DNA extraction and PCR amplification

Microbial DNA was extracted from 0.5 g fresh soil with the E.Z.N.A. DNA Kit (Omega Bio-Tek, Norcross, GA, USA) following the manufacturer's instruction. All DNA extracts were eluted with 50 μ L TE buffer and stored at -20°C until further analysis. The bacterial 16S rRNA gene V4 region was amplified using the primer pair 515F (5'-GTG CCA GCM GCC GCG G-3') and 806R (5'-CCG TCA ATT CMT TTR AGT TT-3') (Berthrong et al., 2013), which were designed to be universal for bacterial and archaeal taxa (Ramirez et al., 2012). Each forward and reverse primer contained a unique barcode to distinguish the samples. All PCR reactions were performed on ABI GeneAmp[®]9700 PCR System (Applied Biosystems, Foster City, CA, USA) consisting in an initial temperature of 95°C for 3 min, followed by 27 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 45 s and a final extension step at 72°C for 10 min. The PCR amplification was performed in 20 μ L PCR reaction system using TransGen AP221-02, consisting of TransStartFastPfu DNA Polymerase (TransGen Biotech, Beijing, China), containing 4 μ L $5 \times$ FastPfu Buffer, 2 μ L 2.5 Mm dNTPs, 0.8 μ L forward primer and 0.8 μ L reverse primer, 0.4 μ L FastPfu polymerase (TransStartFastPfu DNA Polymerase, TransGen) and 10 ng of the template DNA. The PCR products were extracted from 2% agarose gels and all PCR products of each sample were pooled and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, US). The amplicons were sent to Shanghai Major Bio-Pharm Technology Co., Ltd. (Shanghai, China) for sequencing library preparation and paired-end sequencing on Illumina MiSeq platform.

2.5. Processing of sequencing data

QIIME (Quantitative Insights Into Microbial Ecology, version 1.7.0) was used to process the sequencing data (<http://qiime.org/>) (Caporaso et al., 2010). Primers, low-quality 16S rRNA sequences, and meta-barcode were trimmed using the Trimmomatic (Bolger et al., 2014). FLASH (Fast Length Adjustment of Short reads) (Magoč and Salzberg, 2011) was used to join the forward (R1) and reverse (R2) paired-end sequences with the following criteria: (i) 300-bp reads were truncated at any site receiving an average quality score < 20 over a 10-bp sliding window, and any truncated reads shorter than 50 bp were discarded; (ii) reads without exact barcode matching, containing ≥ 2 nucleotide mismatches to the primers, or containing ambiguous bases were removed; and (iii) only paired-end sequences that overlapped by more than 10 bp were assembled; any reads that could not be so assembled were discarded. Chimera sequences were removed using UCHIME algorithm against the Gold database for 16S rRNA gene metabarcodes (Edgar et al., 2011). The total number of merged paired-end 16S rRNA gene sequences was 2,359,971, ranging from 61,542 to 89,891 sequences per sample, and then 2,274,478 high-quality sequences were retained.

Pick open OTU strategy was used to cluster the high-quality sequences into 71,658 operational taxonomic units (OTUs) at 97% similarity cut-off using `pick_open_reference_otus.py` and compared against the GreenGenes database (v13.5) preclustered at 97% similarity cut-off (McDonald et al., 2012) with CD-HIT method (Li and Godzik, 2006). Taxonomic assignment was carried out using the RDP classifier (Wang et al., 2007) against the GreenGenes database (v13.5) with 80% confidence. The singleton OTUs and OTUs classified to chloroplast, mitochondria, and plants were removed. The final OTU table contained 65,700 non-singleton bacterial and archaeal OTUs. The samples were rarefied to an equal sequencing depth ($n = 56,218$) using `rarefy` function in `vegan` (Oksanen et al., 2018) before subsequent analysis. The remaining OTUs were assigned to 48 phyla (99.27% assigned to known phyla), 142 classes (98.25%), 230 orders (90.64%), 289 families (67.50%), and 474 genera (33.93%).

Functional annotation of remaining OTUs was inferred using FAPROTAX (Louca et al., 2016) and PICRUSt v1.0.0 (Langille et al., 2013).

2.6. Statistical and bioinformatics analysis

All statistical analyses were performed in the R environment (version 3.4.3) (R core team, 2017). Figures were created using R or Sigmaplot 12.5 (Systat Software, Inc., San Jose, CA, United States). Soil physicochemical properties were checked for normality with R using `shapiro.test` function and transformed using the Box-Cox power transformation when necessary. One-way analysis of variance (ANOVA) was used to determine if soil physicochemical properties differed significantly under different tillage regimes. In addition, Duncan's new multiple range test was used to compare means for these parameters. The importance of soil physicochemical properties on maize yield was evaluated through stepwise regression analysis using R package `olsrr` (<https://rdocumentation.org/packages/olsrr/versions/0.5.3>).

Sequencing reads were randomly subsampled to the same sample size. The alpha-diversity indices, including species number (richness), Shannon true diversity (Shannon-TD), Simpson true diversity (Simpson-TD), and Chao1, were calculated using `OTU.diversity` function in `RAM` package (Chen et al., 2018). The effects of tillage regimes on alpha-diversity indices and the relative abundance of taxa were determined using `Kruskal-Wallis` rank sum test, a non-parametric method for non-normal numeric data. Functions in R package `ggpubr`, e.g. `ggboxplot` and `stat_compare_means` with "wilcox" method, were used to perform multiple mean comparisons between treatments. The linear-based constrained ordination method, distance-based redundancy analysis (db-RDA), was carried out to evaluate the overall impact of tillage practices, soil physicochemical properties, and sampling time on microbial community heterogeneity using the function `capsale` in `vegan`. The Mantel tests (Mantel and Valand, 1970) were used to evaluate the relationships between the bacterial communities (based on Bray-Curits distance) and soil physicochemical properties (based on Euclidean distance). Function `adonis` in the `vegan` package was used to perform permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2017) for determining the effects of tillage treatments on the community heterogeneity of individual functional groups annotated by FAPROTAX. Spearman's rank-order correlation coefficients between the abundance of microbial taxa and soil properties were performed using the `corr` function in the `emulator` package (Hankin, 2005). The correlation matrices were visualized using `ggcorrplot` function in `ggcorrplot` package (Kassambara, 2019).

2.7. Sequence accession numbers

The raw sequencing data in FASTQ format are accessible from the NCBI Sequence Read Archive under the BioProject accession number PRJNA733810.

3. Results

3.1. Crop yield, soil physicochemical properties

In comparison with the CT practice, NT, SM and SS all significantly increased maize yields by 19.0%, 14.9%, and 13.5%, respectively ($P < 0.05$) (Table 1). Tillage practices had a significant influence on soil BD ($P < 0.001$) which was highest under CT. Compared with the CT treatment, NT, SM, and SS treatments significantly increased soil moisture contents, SOM, TN, as well as available N, P, and K of soil ($P < 0.05$). No difference was detected in soil C:N ratio and soil pH among the treatments. Stepwise regression indicated that maize yield was firstly associated with available N (adj. $R^2 = 0.58$), and secondly with available P (accumulated adj. $R^2 = 0.68$).

3.2. Soil microbiota diversity and compositional structure

The alpha-diversity indices of the soil microbial communities were not significantly affected by tillage regimes ($P > 0.05$) (Supplementary Table S1). Soil C:N ratio was negatively correlated with richness (OTU number) (Spearman's rho correlation coefficient = -0.48), the Shannon index-based true diversity (Shannon-TD) (-0.64), and the Simpson index-based true diversity (Simpson-TD) (-0.59) ($P \leq 0.05$).

The db-RDA analysis showed that the soil microbial community structure differed significantly between sampling year or season ($P = 0.001$), but was less influenced by tillage practices ($P = 0.072$), based on the Bray-Curtis dissimilarity matrix of the hellinger-transformed OTU abundance table. This can be demonstrated by the db-RDA plot (Fig. 1) on which the soil samples under CT were separated from those under SS or conservation tillage practices at each sampling time point, especially at harvest. Soil physicochemical properties also showed significant impact on bacterial and archaeal community structure ($P = 0.001$) (Fig. 1). Mantel tests indicated a significant correlation between the microbial community compositional heterogeneity and the soil

Table 1

Maize yield and soil physicochemical properties^a at 0–20 cm depth under four tillage practices^b.

Edaphic attributes ^c	CT	NT	SM	SS	Significance (P values) ^d
pH	7.97 ± 0.07 ^a	8.02 ± 0.16 ^a	7.96 ± 0.15 ^a	8.04 ± 0.23 ^a	ns
Macro (%)	41.87 ± 1.35 ^a	42.5 ± 1.24 ^a	43.87 ± 1.63 ^a	41.81 ± 1.18 ^a	ns
BD (g cm ⁻³)	1.57 ± 0.01 ^a	1.55 ± 0.02 ^{a,b}	1.53 ± 0.01 ^b	1.53 ± 0.02 ^b	0.01
SMC (%)	6.87 ± 0.52 ^b	7.56 ± 0.84 ^{a,b}	8.03 ± 0.83 ^a	7.47 ± 0.97 ^{a,b}	0.01
SOM (g kg ⁻¹)	9.47 ± 0.41 ^b	11.87 ± 0.80 ^a	12.14 ± 0.92 ^a	11.23 ± 0.60 ^a	< 0.001
TN (g kg ⁻¹)	0.44 ± 0.02 ^b	0.56 ± 0.04 ^a	0.57 ± 0.05 ^a	0.54 ± 0.04 ^a	< 0.001
C:N	12.38 ± 0.42 ^a	12.27 ± 0.84 ^a	12.48 ± 1.29 ^a	12.17 ± 1.00 ^a	ns
AN (mg kg ⁻¹)	50.6 ± 1.92 ^b	60.38 ± 9.00 ^{a,b}	61.4 ± 5.72 ^a	56.9 ± 5.01 ^b	< 0.001
AP (mg kg ⁻¹)	10.29 ± 0.77 ^b	12 ± 0.88 ^a	12.76 ± 1.30 ^a	13.04 ± 1.29 ^a	< 0.001
AK (mg kg ⁻¹)	90.39 ± 2.03 ^b	101.44 ± 4.26 ^a	100.5 ± 6.91 ^a	99.35 ± 3.36 ^a	< 0.001
Maize yield (kg ha ⁻¹)	9,068 ± 498 ^b	10,416 ± 305 ^a	10,789 ± 620 ^a	10,293 ± 389 ^a	< 0.001

^a Values are expressed as MEAN ± SD. Means followed by the same letter in each row are not significantly different at $\alpha = 0.05$ ($n = 8$).

^b CT, conventional tillage; NT, no-till; SM, straw mulching; SS, subsoiling.

^c Macro, macro-aggregate (> 250 μ m); SMC, soil moisture content; BD, bulk density; SOM, soil organic matter; TN, total nitrogen; AN, available nitrogen; AP, available phosphorus; AK, available potassium.

^d ns, not significant at $P \geq 0.05$.

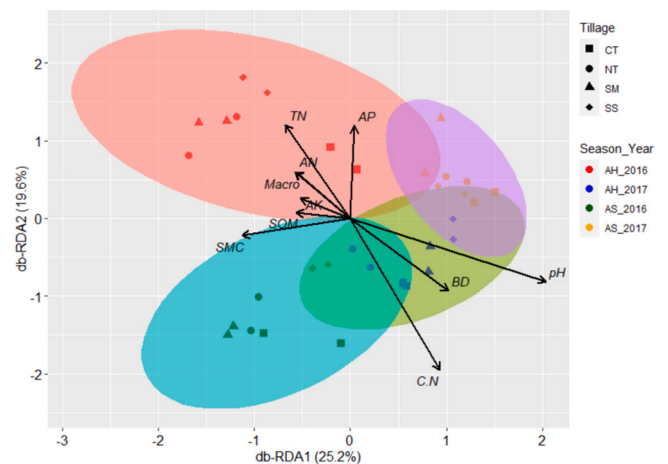


Fig. 1. Distance-based redundancy analysis (db-RDA) of soil bacterial and archaeal community as influenced by tillage, growing season, and the soil physicochemical properties. AH, at harvest; AS, after seeding; CT, conventional tillage; NT, no-till; SM, straw mulch; SS, subsoiling.

physicochemical properties ($r = 0.29$, $P \leq 0.05$), in particular TN ($r = 0.26$), soil moisture ($r = 0.24$), soil macro-aggregate fraction ($r = 0.21$), soil organic matter ($r = 0.19$), and available nutrient content ($r = 0.18$ for available N, $r = 0.17$ for available P, $r = 0.20$ for available K) ($P \leq 0.05$).

Across all samples, the bacterial community was dominated by *Proteobacteria* ($34.07 \pm 1.64\%$ in abundance, 22663 OTUs), *Actinobacteria* ($25.46 \pm 1.61\%$, 10210), followed by *Chloroflexi* ($7.29 \pm 0.61\%$, 5361), *Acidobacteria* ($7.23 \pm 0.84\%$, 3976), *Firmicutes* ($6.50 \pm 0.81\%$, 3834), and *Bacteroidetes* ($5.95 \pm 0.60\%$, 3559). In addition, *Gemmatimonadetes*, *Planctomycetes*, *Verrucomicrobia*, *TM7*, and *Nitrospirae* were recovered in all soil samples in low abundance (< 5%) (Fig. 2-A). The archaeal community was dominated by phylum *Crenarchaeota* ($2.21 \pm 0.26\%$, 264 OTUs), in which, *Candidatus Nitrososphaera*, an ammonia-oxidizing archaeal genus, was most abundant.

Compared with CT, SM significantly enriched *Proteobacteria*, while NT and SM significantly decreased *Planctomycetes* abundance ($P < 0.05$) (Fig. 2-A). Spearman's rank-order correlation analysis showed that *Proteobacteria*, *Bacteroidetes*, and *Gemmatimonadetes* were positively correlated with soil macro-aggregate fraction, moisture content, and nutrient levels, but negatively correlated with soil pH. By contrast, *Chloroflexi*, *Firmicutes*, *Planctomycetes* and *Crenarchaeota* showed an opposite trend. *Acidobacteria* was negatively correlated to soil moisture content (Fig. 2-B).

The predominant genera (> 1% in abundance) included *Arthrobacter* ($3.14 \pm 0.35\%$), *Candidatus Nitrososphaera* ($2.20 \pm 0.26\%$), *Kaistobacter* ($1.90 \pm 0.14\%$), and *Rhodoplanes* ($1.29 \pm 0.05\%$) (Fig. 2-C). *Rhodoplanes* and *Steroidobacter* were significantly more abundant in soils under NT, SM, and SS relative to CT. Compared with CT, NT also significantly enriched *Bradyrhizobium* and depleted *Rubrobacter* in soils ($P < 0.05$).

3.3. Soil microbial communities associated with N cycling

Functional guilds in soil bacterial and archaeal communities were first predicted with FAPROTAX. Overall, 8282 out of 65,700 bacterial OTUs (12.61%) were assigned to at least one functional group, with a total of 72 function groups being identified. Of these, 15 functional groups were affiliated with N cycling (Fig. 3-A), which included 1249 OTUs mainly from *Proteobacteria*, *Crenarchaeota*, and *Nitrospirae* (supplementary Figure 2-A). Tillage practices showed a statistically significant effect on the compositional structure of the bacterial communities (such as *Rhodoplanes* spp.) affiliated with nitrate reduction and denitrification (Fig. 3-A, Table 2). For example, *Rhodoplanes* was

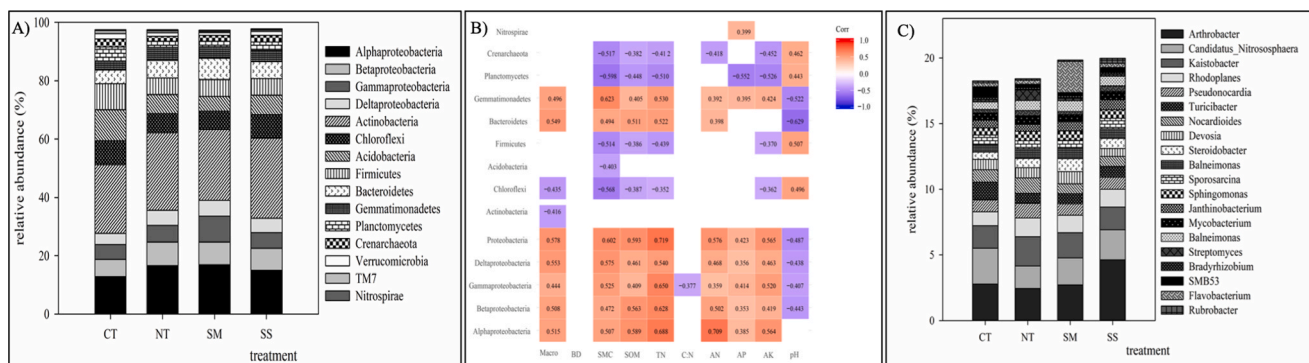


Fig. 2. Soil microbial community compositional structure. A) The relative abundance of the dominant bacterial phyla and the three classes in *Proteobacteria* (*Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*) under different tillage practices. B) Spearman's rank-order correlation coefficients between the soil properties and the abundance of bacterial phyla and three classes in *Proteobacteria*. Correlation coefficients that were statistically insignificant at $\alpha = 0.05$ were left blank. C) The relative abundance of the dominant bacterial genera under different tillage practices. CT, conventional tillage; NT, no-till; SM, straw mulch; SS, subsoiling.

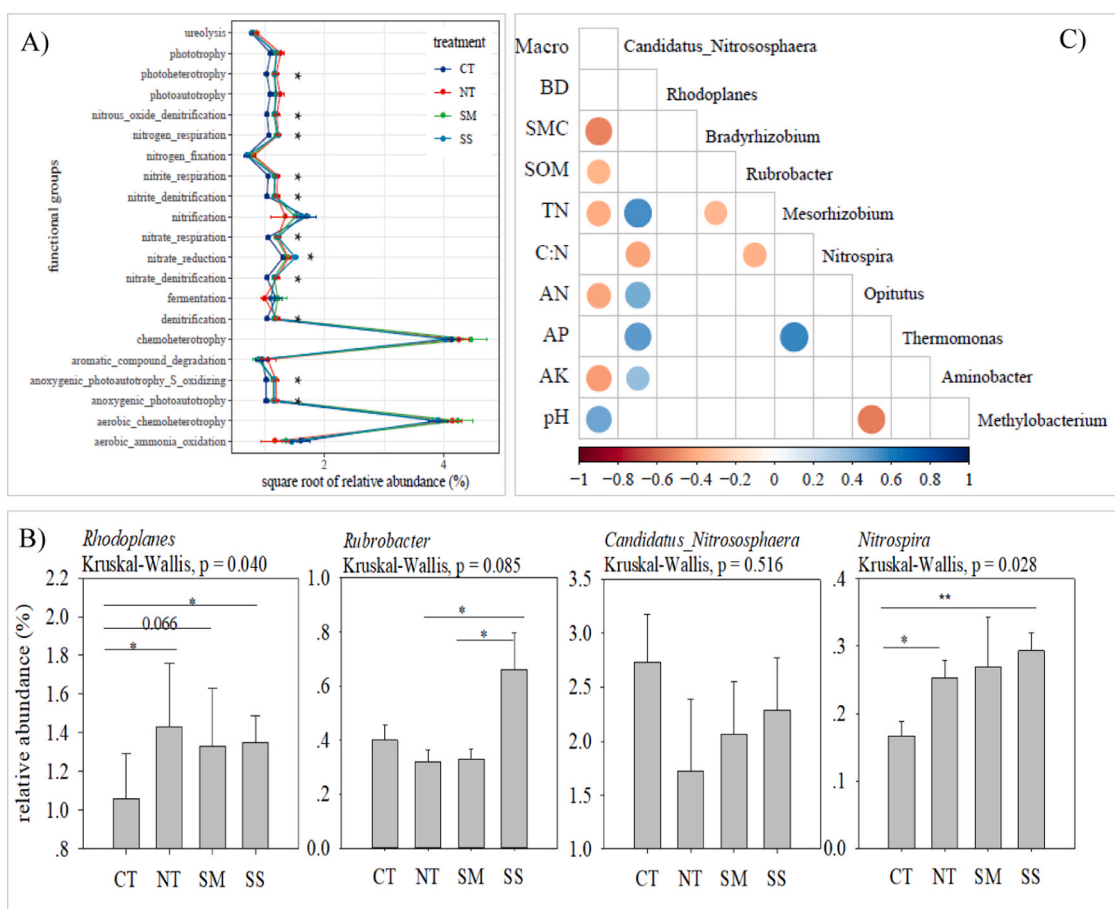


Fig. 3. Bacterial functional groups annotated by FAPROTAX. A) The Hellinger-transformed abundance of the functional guilds under different tillage practices. The error bars represented the standard errors of respective means. A star (*) indicates that the tillage regimes had a significant impact on the abundance of a functional group (Kruskal.test, $P < 0.05$). B) The relative abundance of key genera affiliated to N cycling under different tillage regimes (Kruskal.test, $P < 0.05$). C) The Spearman's rank-order correlation coefficients between the soil properties and the abundance of bacterial genera affiliated to N cycling ($P < 0.05$). Correlation coefficients that were statistically insignificant at $\alpha = 0.05$ were left blank. CT, conventional tillage; NT, no-till; SM, straw mulch; SS, subsoiling.

significantly more abundant under NT and SS ($P < 0.05$), and marginally enriched under SM ($P = 0.066$), compared to CT (Table 2, Fig. 3-B). *Rhodoplanes* was also found to be negatively correlated with soil C:N ratio, but positively correlated to soil TN, available N, available P and available K (Fig. 3-C). *Actinobacteria* and *Rubrobacter* (affiliated with nitrate reduction) were more abundant under SS than under NT and SM

($P < 0.05$) (Table 2, Fig. 3-B). A negative correlation was observed between *Rubrobacter* abundance and TN (Fig. 3-C).

The nitrifying communities (affiliated with aerobic ammonia oxidation, aerobic nitrite oxidation, and nitrification) were dominated by *Candidatus Nitrososphaera* and *Nitrospirae* (Table 2). *Candidatus Nitrososphaera* (209 OTUs, ammonia-oxidizing archaea) was not

Table 2

The abundance and diversity of functional groups affiliated with N metabolism based on FAPROTAX annotation.

Group	No. of OTUs	relative abundance (%) ^a	beta-diversity ^b	Main contributors ^c
nitrification	580	2.65 ± 1.60	0.309	<i>Candidatus_Nitrososphaera</i> , <i>Nitrospira</i> *
aerobic_ammonia_oxidation	284	2.22 ± 1.47	0.459	<i>Candidatus_Nitrososphaera</i>
nitrate_reduction	486	1.97 ± 0.38*	0.03	<i>Rhodoplanes</i> *, <i>Paracoccus</i> , <i>Rubrobacter</i> , <i>Opitutus</i> , <i>Paenibacillus</i>
nitrogen_respiration	215	1.42 ± 0.34*	0.088	<i>Rhodoplanes</i> *
nitrate_respiration	207	1.40 ± 0.34*	0.063	<i>Rhodoplanes</i> *
nitrite_respiration	187	1.35 ± 0.27*	0.066	<i>Rhodoplanes</i> *, <i>Paracoccus</i>
nitrate_denitrification	172	1.32 ± 0.28*	0.031	<i>Rhodoplanes</i> *
nitrite_denitrification	172	1.32 ± 0.28*	0.031	<i>Rhodoplanes</i> *
nitrous_oxide_denitrification	172	1.32 ± 0.28*	0.031	<i>Rhodoplanes</i> *
denitrification	172	1.32 ± 0.28*	0.031	<i>Rhodoplanes</i> *
nitrogen_fixation	64	0.58 ± 0.18	0.883	<i>Bradyrhizobium</i> , <i>Sphingomonas</i> , <i>Azospirillum</i> , <i>Magnetospirillum</i> , <i>Phaeosporillum</i> , <i>Clostridium</i>

^a The relative abundance is expressed as MEAN ± SD. *, tillage practice showed significant impact on the total abundance of bacterial taxa affiliated with respective functional group at $\alpha = 0.05$.

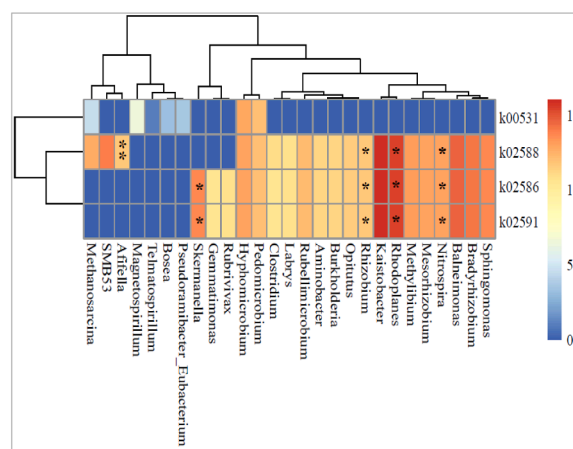
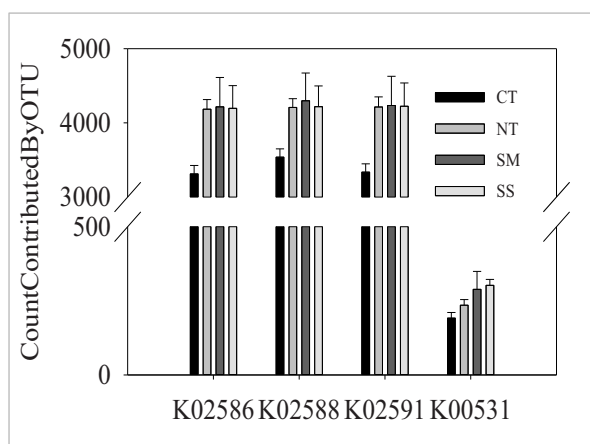
^b P value from PERMANOVA test based on Bray-Curtis distance of hellinger-transformed abundance matrix. The test shows the impact of tillage practices on the beta diversity of the bacterial community affiliated with respective functional group. Values in bold represent significance at $\alpha = 0.05$.

^c The most abundant bacterial genera affiliated with respective function. * tillage practice showed significant impact on the abundance of respective bacterial genus at $\alpha = 0.05$

significantly affected by tillage (Fig. 3-B), but was negatively correlated with soil moisture content, SOM, and soil nutrient content (TN, available N and K) and positively correlated to soil pH (Fig. 3-C). *Nitrospira* (82 OTUs, nitrite-oxidizing bacteria) was found more abundant under NT

and SS than under CT ($P < 0.05$) (Fig. 3-B). There was a positive correlation between *Nitrospira* abundance and available P (Fig. 3-C). FAPROTAX also annotated 64 OTUs to be N fixing bacteria, predominantly assigned to *Bradyrhizobium*, *Sphingomonas* (in *Proteobacteria*), and

A) 4 KOs from Module M00175 relating to N₂ fixation.



B) 7 KOs not from Module M00175 relating to N₂ fixation.

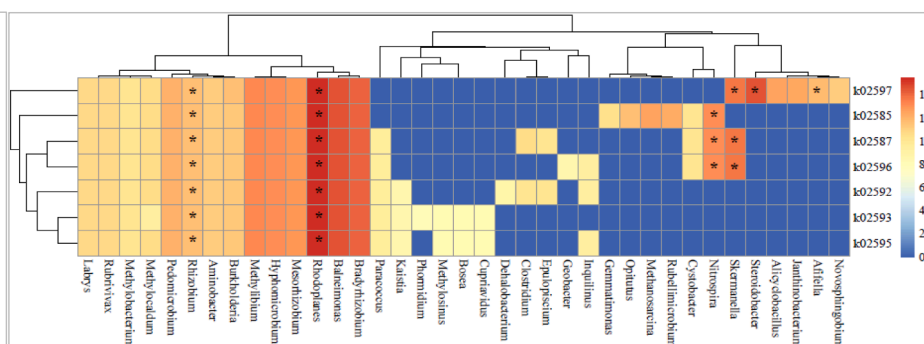
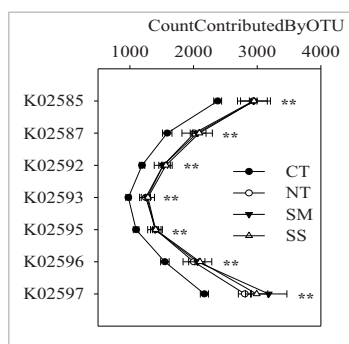


Fig. 4. Functional genes affiliated with N fixation predicted by PICRUSt. A) Left panel: bar plots showing the abundance of four KOs (K02586, K02588, K02591, K00531) from KEGG module M00175 (N fixation) that were significantly impacted by tillage practices ($P < 0.05$). Right panel: a heatmap showing the abundance of the bacterial and/or archaeal genera containing the four KOs. C) Left panel: a line plot showing the abundance of seven KOs affiliated to N fixation but not included in KEGG module M00175 that were affected by tillage regimes ($P < 0.05$). Right panel: a heatmap showing the abundance of the bacterial genera containing these KOs. The stars in the heatmaps indicate significant differences in abundance under different tillage practices (*, $P \leq 0.05$; **, $P \leq 0.01$).

Clostridium (in *Firmicutes*). Their abundance was not significantly affected by tillage practices ($P > 0.05$).

Furthermore, out of the 65 KEGG orthologs (KOs) involved in the N metabolism pathway map00910, 41 were predicted by PICRUST in this study with six KOs (highlighted in red in [Supplementary Figure 2-B](#)) being significantly impacted by tillage regimes. Of the six KOs, four were affiliated with KEGG module M00175 (N fixation, 2633 OTUs) for encoding nitrogenase. They were K02586 (*nifD*), K02588 (*nifH*), K02591 (*nifK*), and K00531 (*anfG*). We further identified seven nitrogenase-encoding KOs (associated with 2529 OTUs) that may modulate N fixation, which, however, were not included in KEGG module M00175. These KOs were K02585 (*nifB*), K02587 (*nifE*), K02592 (*nifN*), K02593 (*nifT*), K02595 (*nifW*), K02596 (*nifX*), and K02597 (*nifZ*). All the 11 nitrogenase-encoding KOs were more abundant under conservation tillage regimes in comparison with CT (Kruskal-Wallis test, $P < 0.05$) ([Fig. 4-A, B](#)). These KOs were predominantly found in *Rhodoplanes*, *Nitrospira*, *Skermanella*, *Steroidobacter*, *Afifella*, and *Rhizobium* ([Fig. 4-A, B](#)). Compared with CT, conservation tillage and SS enriched *Rhodoplanes* and *Nitrospira*, SS enriched *Skermanella*, while NT enriched *Rhizobium* ($P < 0.05$). The N fixing archaea included Methanobacteriaceae (*Methanobacterium* and *Methanobrevibacter*), Methanomicrobiaceae (*Methanococcus*), and anaerobic Methanosarcinaceae (*Methanosarcina*). All the KOs related to N fixation were in general positively correlated with soil TN, available N, P, and K contents, but negatively correlated with soil BD ([Table 3](#)).

4. Discussion

4.1. Crop yield and soil physicochemical properties in response to tillage practices

This study was conducted on a typical aeolian sandy soil with poor water holding capacity (~5–9% of soil moisture content), low soil organic matter content (9–12 g kg⁻¹), and available N and P contents. Conservation tillage practices including NT and SM, and the soil ameliorating SS significantly decreased surface soil bulk density, and increased soil moisture content, soil organic matter, TN, and available nutrients (N, P, K) after 3- and 4-yr of cultivation change from long-term CT. Our results suggested that these conservation tillage practices can potentially improve soil quality over relatively short time frames (few years), and thus, from a physicochemical perspective at least, are viable alternatives to CT in alleviating the problems associated with aeolian sandy soils and crop production in the region (notwithstanding that such improvements may have manifested over shorter post-CT time frames). Higher soil moisture content under all three conservation tillage practices was consistent with [Blevins et al. \(1983\)](#) who observed higher soil moisture under no-till in relation to conventional tillage. Tillage breaks up aggregates, decreases pore sizes/pore connectivity, which can

decrease infiltration and increase bulk density ([Manyiwa and Dikinya, 2014](#)); while minimum tillage and crop residue cover were found to improve aggregate stability and water infiltration ([Verhulst et al., 2011](#)). The soil physical property changes induced by reduced tillage and crop residue management resulted in an increase of topsoil organic matter and nutrient availability.

We acknowledge that the comparison of the SOC and C:N in the present study was at a fixed surface soil depth, which can be error-prone relevant to equivalent soil mass-based methods, as emphasized by [Rovira et al. \(2015\)](#) and [von Haden et al., \(2020\)](#). These authors used experimental and simulated data to demonstrate that quantifying mass-based soil properties or ratios should use equivalent mineral soil mass as reference for repeated measurements or calculations, thus reducing skewness in data interpretation caused by concomitant changes in BD and/or SOM across time, space, or treatments. Despite that many approaches have been developed to correct soil compaction or expansion for accurate estimation of SOC, limitations related to both methods have been discussed extensively elsewhere ([Sanderman and Chappell, 2013](#); [Sollins and Gregg, 2017](#); [Zhang et al., 2019a](#)). Nonetheless, our report on the influences of tillage regimes on soil carbon was in line with [Chávez-Romero et al. \(2016\)](#) and [Zhang et al. \(2009\)](#).

Conservation tillage practices could increase ([Jin et al., 2007](#); [Zhang et al., 2015](#)), decrease ([Campbell et al., 1984](#); [Salem et al., 2015](#)), or maintain ([Messiga et al., 2012](#)) maize production, depending on soil physicochemical properties, weather conditions, and cultivation years. In the present study, conservation tillage practices enhanced maize yield, as consistent with previous studies conducted in sandy soils or dryland ([Arora et al., 1991](#); [Jin et al., 2007](#)). In this study, maize yield was primarily driven by available N according to the stepwise regression analysis; underscoring the importance of understanding the microbially-mediated N cycling processes as affected by tillage practices.

4.2. Soil microbiota diversity and compositional structure in response to tillage practices

The present study suggested that tillage practices had negligible impact on the alpha-diversity of the soil microbial communities. This is in line with a previous study which reported that tillage practices did not affect the species richness and diversity of bacterial communities in semi-arid agro-ecosystems ([Navarro-Noya et al., 2013](#)). [Delgado-Baquerizo et al. \(2017\)](#) found that bacterial diversity was primarily driven by variation in soil resource stoichiometry (total C:N:P ratios). Significant negative associations were detected between alpha-diversity indices of the bacterial community (richness, Shannon-TD, Simpson-TD) and soil C:N ratio, which was in agreement with [Noah and Jackson \(2006\)](#), who reported soil C:N had a negative correlation with bacterial Shannon index in soils from a wide array of ecosystems in

Table 3

Spearman's rank-order correlation coefficients^a between the soil physicochemical properties and the abundance of KEGG Orthologues (KOs) affiliated with N fixation predicted by PICRUST.

soil properties ^b	K02586	K02588	K02591	K00531	K02585	K02587	K02592	K02593	K02595	K02596	K02597
Macro	0.39*	0.38*	0.40*	-0.18	0.32	-0.03	0.06	0.09	0.07	-0.01	0.39
BD	-0.33	-0.32	-0.35	-0.44*	-0.32	-0.40*	-0.41*	-0.44*	-0.36*	-0.42*	-0.57***
SMC	0.29	0.24	0.32	-0.24	0.26	0.03	0.02	-0.07	-0.00	-0.01	0.23
SOM	0.46**	0.44*	0.46**	-0.10	0.44*	0.18	0.26	0.25	0.30	0.23	0.45**
TN	0.67***	0.59***	0.68***	0.08	0.56***	0.40*	0.43*	0.42*	0.41*	0.39*	0.68***
C:N	-0.39*	-0.32	-0.40*	-0.20	-0.24	-0.30	-0.26	-0.28	-0.18	-0.24	-0.43*
AN	0.57***	0.53**	0.56***	0.02	0.48**	0.27	0.42*	0.47**	0.49**	0.38*	0.67***
AP	0.54**	0.51**	0.57***	0.44*	0.55**	0.57***	0.52**	0.48**	0.43*	0.51**	0.60***
AK	0.49**	0.41*	0.51**	0.10	0.42*	0.35	0.40*	0.37*	0.42*	0.39*	0.55**
pH	-0.12	-0.13	-0.15	0.27	-0.07	0.10	0.13	0.19	0.24	0.19	-0.06

^a Statistical significance is indicated as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

^b Macro, macro-aggregate (> 250 μm); BD, bulk density; SMC, soil moisture content; SOM, soil organic matter; TN, total nitrogen; AN, available nitrogen; AP, available phosphorus; AK, available potassium.

North and South America. We found that conservation tillage and subsoiling increased both SOM and TN, and consequently resulted in similar C:N ratios across treatments; this could possibly explain the lack of significant change in alpha diversity of soil bacterial and archaeal communities associated with the introduced tillage practices.

In arid regions, soil water availability may be the most limiting factor for bacterial growth and proliferation, litter decomposition and nutrient mineralization (Deangelis et al., 2015), and their motility and access to nutrients (Long and Or, 2009). Therefore, climatic conditions and agronomic practices (such as tillage regimes) that positively impact soil moisture reserves would be expected to influence soil microbial community composition (Drenovsky et al., 2010; Grayston et al., 2001). Indeed, the significant shift in soil bacterial and archaeal community structure from year 2016–2017 may be attributed to differences in precipitation over these two years (Supplementary Figure 1-B). The differences in community structure between the two sampling seasons (Fig. 1) could be attributed to greater TN, available N and P observed at harvest than after seeding, as also demonstrated by previous studies (Sun et al., 2018; Wang et al., 2014), confirming the important role of soil nutrients in shaping soil microbial community structure.

Our study showed as well, that tillage regimes had smaller impact on soil microbial community structure than sampling year and season; perhaps related to precipitation inputs as previously noted. Nonetheless, soil samples under CT were differentiated from those under SS, SM, and NT based on community compositional heterogeneity. This could be attributed to tillage practices that improved soil TN, soil moisture content, and SOM and overall soil adversity ‘stressors’ (Dong et al., 2017; Dorr de Quadros et al., 2012). The improvement in soil moisture and nutrient content caused by conservation tillage and SS practices also led to the enrichment of *Proteobacteria* spp. and the depletion of *Planctomycetes* spp. This is not surprising, as members of *Proteobacteria* (in particular *Alpha*- and *Beta*-*proteobacteria*) are considered copiotrophs and tend to proliferate and grow well under nutritional conditions (Fierer et al., 2007; Peiffer et al., 2013). We found that the abundance of *Proteobacteria* (positively) and *Planctomycetes* (negatively) correlated with organic matter and TN, as reported in a study under long-term rice cultivation (Zhang et al., 2019b). Recently, however, Xu et al. (2020) showed that higher soil moisture contents and addition of N-fertilizer significantly enriched *Planctomycetes* abundance in sandy wheat fields in northern China, which was incongruent with what we observed in our study.

We conclude that, in arid and semi-arid regions, the beneficial impacts of SS and conservation tillage regimes on plant (maize in this study) productivity and sandy soil microbial diversity could be profoundly compromised or enhanced by other environmental conditions,

in particular precipitation, by modulating mainly soil moisture reserves and organic matter (Wang et al., 2017, 2019; Wezel et al., 2000).

4.3. Potential bacterial functions associated with N cycling in response to tillage practices

Microbes critically drive ammonification, N fixation, nitrification, denitrification, and nitrate reduction (Yoon et al., 2014). Our study found that the conservation tillage practices and subsoiling, significantly increased TN and available N. Crop yield was also primarily driven by available N. Evaluation of tillage effect on the potential bacterial and archaeal functions associated with these N cycling processes using FAPROTAX and PICRUST, elucidated microbial mechanisms in N cycling in response to tillage practice change. A summary of key bacterial and archaeal taxa involved in N cycling processes which were significantly influenced by tillage practices based on the prediction of FAPROTAX and PICRUST are provided in Fig. 5.

Biological N fixation provides a substantial input of fixed N into soils and compensates for the losses that are incurred owing to denitrification (Dixon et al., 2004). The community diversity of soil N-fixing bacteria shifts quickly and is a strong predictor of N fixation activity (Reed et al., 2010). Based on FAPROTAX annotation, nine genera in low abundance were detected to be affiliated with N fixation, including *Bradyrhizobium*, *Sphingomonas*, *Clostridium*, *Azospirillum*, *Magnetospirillum*, *Phaeosporillum*, *Sinorhizobium*, *Beijerinckia*, *Methanosarcina*. Some methanogens such as *Methanobacteriales*, *Methanococcales*, and *Methanosarcinales* were identified as N fixing archaea (Tsou et al., 2016), in agreement with our findings. In addition, PICRUST predicted many bacterial and archaeal taxa (> 5000 OTUs) containing genes encoding proteins involved in the formation of nitrogenase or other enzymes associated with N fixation. Conservation tillage and SS practices increased the abundance of these OTUs, demonstrating a positive effect of conservational/ameliorating tillage practices on the biological N fixation process, as also reported in Singh et al. (2021). All these KOs were principally and positively correlated with TN and available N, P, and K, suggesting nutrient levels regulated the presence and growth of the functional guilds containing these genes, such as *Rhodoplanes*, *Nitrospira*, *Rhizobium*, and *Skermanella*. With 11 predicted KOs affiliated with N fixation, *Rhodoplanes* spp. showed functional importance in improving soil fertility, as also reported by Buckley et al. (2007). *Nitrospira* spp. are usually considered as nitrite-oxidizing bacteria, however, 19 *Nitrospira* OTUs were predicted to have the ability to fix atmospheric N_2 . *Rhizobium* spp. are known as rhizobia that can fix atmospheric N_2 through the action of the nitrogenase enzyme (Mahmud et al., 2020). Both *Rhodoplanes* and *Nitrospira* were enriched by all three introduced tillage practices (SS, SM, and NT),

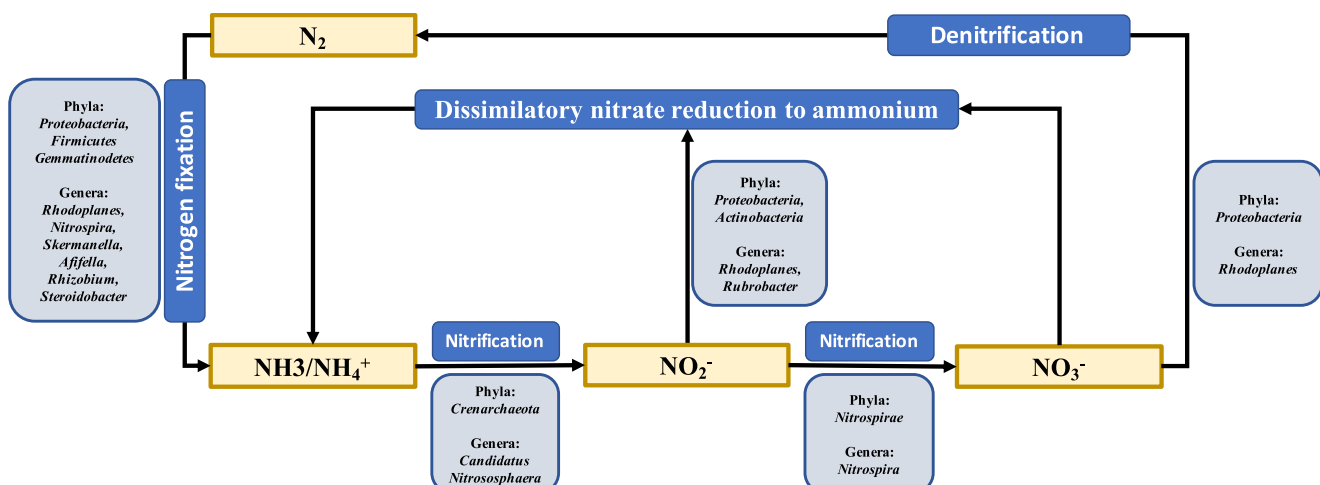


Fig. 5. Bacterial and archaeal phyla and genera affiliated with N metabolism that were affected significantly by tillage practices in this study.

while *Rhizobium* was more abundant under NT and *Skermanella* under SS, in comparison with CT. These results demonstrated that the newly introduced tillage practices can produce a positive effect on the N-fixing bacterial communities, but specific bacterial taxon group could respond rather differently to different conservation/ameliorating tillage regimes.

In addition to N-fixing microorganisms, bacteria and archaea affiliated with other N-cycling processes (such as nitrate reduction, nitrification, and denitrification) were also identified using the FAPROTAX database. Of the two groups of microorganisms that work in close partnership to accomplish nitrification, we identified *Candidatus Nitrososphaera* (in *Crenarchaeota*) as the ammonia-oxidizing archaea (AOA) in the soil, which was in line with [Leininger et al. \(2006\)](#) who reported this genus as the most represented AOA. We observed that *Candidatus Nitrososphaera* spp. tended to decrease in abundance under conservation tillage and SS regimes (especially NT in comparison with CT) potentially because of the higher soil available N under less disturbed soil conditions. The impact of available N may attribute to AOA's high ammonia affinity and their adaptability to low ammonia concentrations ([Herrmann et al., 2009](#)). In addition, we identified *Nitrospira* as the nitrite-oxidizing bacteria (NOB), as also reported by [Hanna et al. \(2015\)](#), [Luo et al. \(2017\)](#), [Toledo-Cervantes et al. \(2016\)](#), and [Wu et al. \(2016\)](#). Compared to CT, NT and SS enriched *Nitrospira*, which exhibited a positive correlation with soil available P, suggesting P availability could be an important factor modulating the proliferation and growth of *Nitrospira* spp.

As a competing process to nitrate reduction pathways, denitrification is the main biological process converting nitrate or other N-containing small molecules (nitrite, ammonia), to N gas ([Tyx et al., 2016](#)). Denitrification genes are often found to be more prevalent in poorly aerated environments with high quantities of organic matter and nitrates ([Strong and Fillery, 2002](#); [Zhang et al., 2016](#)). We found that bacterial communities relating to this function were more abundant in NT, SS and SM treatments than in CT, in agreement with [Wang and Zou, \(2020\)](#) who found that NT enhanced the activity and abundance of the soil denitrifying communities based on a meta-analysis. *Proteobacteria*, in particular *Rhodoplanes* in this phylum, was the most important taxon group contributing to the regulation of denitrification (as affected by tillage practices). *Rhodoplanes* spp. were also found to be capable of respiring nitrite in dark and anaerobic conditions thus competing denitrification ([Cui et al., 2017](#)). Therefore, *Rhodoplanes* spp. can be involved in multiple steps in N cycling, including N fixation, nitrate reduction, and denitrification, and potentially, *Rhodoplanes* spp. could increase (via N fixation) or decrease (via denitrification) N availability in the soil (N fixation) or lead to N loss (denitrification) from soils. There is no evidence from the current study on the actual N turnover in response to tillage practices. However, we observed a positive correlation between *Rhodoplanes* and soil TN and available N, in agreement with previous studies demonstrating that *Rhodoplanes* could have a positive effect in enhancing N availability induced by conservation tillage and subsoiling ([Malique et al., 2019](#); [Sun et al., 2017](#)). Further study is necessary to elucidate the mechanism of *Rhodoplanes* spp. function for different processes of N cycling.

5. Conclusions

In this study, we found that conservation tillage (NT, SM) and subsoiling (SS) enhanced soil moisture content, SOM, TN, and nutrient availability and decreased soil BD after three/four-years of tillage treatment (on otherwise long-term conventional tilled soils). The enhanced maize yield by conservation tillage and subsoiling was principally driven by increased available N. Tillage practices had no significant effect on the alpha-diversity of the soil microbial communities and had smaller influences on community heterogeneity, relative to soil nutrient and moisture content (the later strongly influenced by seasonal precipitation inputs over years of study). However, we observed a notable effect of the introduced tillage practices on soil microbial

communities associated with N cycling processes, including N fixation, nitrate reduction, nitrification, and denitrification. Conservation tillage practices and subsoiling enhanced the abundance of KOs associated with N fixation function involving keystone genera of *Rhodoplanes*, *Nitrospira*, *Skermanella*, *Rhizobium*, according to PICURSt prediction. *Rhodoplanes* spp. were also predicted to be involved in nitrate reduction and denitrification processes, while *Nitrospira* in nitrite oxidization. These microorganisms, therefore, played an important role in enhancing N availability induced by conservation and/or subsoiling tillage practices on aeolian sandy soils. Therefore, conservation tillage and subsoiling would support sustainable agriculture in arid and semi-arid regions, where intensification and expansion of agriculture, important determinants in desertification, are primarily conducted to help meet food consumption demands.

Declaration of Competing Interest

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.still.2021.105197](https://doi.org/10.1016/j.still.2021.105197).

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