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Nitrogen transformation and biodiversity characteristics of fluvo-aquic soil from Hebei plain, China

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To reveal the nitrogentransformations and the microbial community in the upper (0-20 cm) and low (20-40cm) layer of fluvo-aquic soil, the effects of microorganisms were investigated through drip irrigations by Chinese cabbage culture experiments. Three treatments of drip irrigation: groundwater (CK), groundwater with five types of bacteria (I#) and groundwater with one type of bacteria (A#) were conducted. 16S rDNA was used to detect the microbial community. Results suggested that drip irrigation with different bacteria significantly affected the nitrogen transformations in the upper soil layer. The total nitrogen (TN) concentration was the lowest (483.94 $mg \cdot kg^{-1}$) in soils using I# water compared with the other two treatments, suggesting that bacteria in I# water enhanced nitrogen transformation. The highest levels of nitrate in the 6^{th} week of 17.45 $mg \cdot kg^{-1}$, 23.98 $mg \cdot kg^{-1}$ and 18.14 $mg \cdot kg^{-1}$ were found for I#, CK and A# water, respectively. The changes in ammonium concentrations were stable (0.19-0.34 $mg \cdot kg^{-1}$) in both soil layers treated with different drip irrigations in week 2-9.The TN, nitrate and ammonium concentrations in the lower layer soil showed the same trend but small variation changes compared with that in the upper soil layer. The *phylum* proteobacterium was most prominent among the bacterial phyla in soil, followed by actinobacteria, whereas thaumarchaeota and phyla proteobacteria were dominant among archaea and ammonia oxidizing bacteria, respectively. *Nitrososphaera, cenarchaeum* and *candidatus* were the most abundance genera across all soil samples for *archaea* community and *nitrosomonas* species were dominant asNH_4^+-N oxidizers in the soil.

Key words: Fluvo-aquic soil, Nitrogen transformation, Biodiversity, PCR-DGGE

INTRODUCTION

Microorganisms play important roles in the biogeochemical cycling of major nutrients, especially nitrogen (N) in fluvo-aquic soil. Due to microbial activity, soil is the most active area of the N cycle. A large amount of organic nitrogenous compounds are easily transformed by microorganisms into ammonium (NH_4^+-N) and then can be oxidized to nitrate(NO_3^--N) through nitrification. NO_3^--N is converted to N_2 which is released into the atmosphere through de-nitrification process [1]. NH_4^+-N oxidation is a key step in the N cycle because NO_3^--N as a final product of nitrification benefits plant growth[2].

Traditionally, nitrification is mainly carried out by ammonia-oxidizing bacteria (AOB)[3], and AOB occurring in soils are known to belong to β-proteobacteria represented by the genera *Nitrosospira* and *Nitrosomonas* [4]. It was also found that ammonia-oxidizing archaea (AOA) may be another group of ammonia oxidizers[5]. The AOA are wide-spread in diverse environments, including soils[6], wastewater treatment bioreactors[7], marine sediments[8] and open ocean water[9]. Several studies indicated that AOA even outnumbered AOB in soils[10, 11], however, it is still not clear which group of ammonia-oxidizing microorganisms plays a more important role in the fluvo-aquic soils.

Previous studies indicated that many factors such as nitrogen fertilization, pH, ammonia/ammonium, soil moisture, organic carbon, irrigation water quality, temperature, and salinity can affect the abundance and community structure of AOA and AOB[5]. Among them, irrigation water qualities are considered as an important factor affecting soil microbial community composition and activity. The impacts of irrigation with different water qualities on the soil microbial community have been partially evaluated and show contrasting results [12]. Therefore, it is important to determine the microbial structure of the AOB and AOA community in response to different irrigation water quality to understand the microorganism responsible for ammonia oxidation pathway occurring influvo-aquic soils. On the other hand, changes in microbial structure and activity in turn have important implications for the N transformation in soil. The analysis of gene abundance of these functional groups of microbes could provide a good estimation of soil N transformations[13].To date, only a few studies have examined the effects of water quality on soil microbes [14-16]. To the best of our knowledge, the effects of drip irrigation with different bacteria on soil microorganisms and soil nitrogen transformations have not been evaluated.

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Thus, the aim of this research was:1) to understand the effect of water quality on the abundance and community compositions of soil microbial communities; 2)to determine the effect of water on soil N transformations and contributions of AOA and AOB in soils. Considering the high complexity and biodiversity of soil microbial communities, a 70-day experiment using typical fluvo-aquicsoil in China was designed and the change inNH_4^+-N and NO_3^--N concentrations and gene abundance of total bacteria was analyzed. Special attention was paid to functional microbes(N-fixation bacteria, AOA, and AOB) using real-time quantitative PCR.

MATERIALS AND METHODS

Soil samples

Fluvo-aquic soils(0-40 cm) were collected from Wuqiang County. The field site was located in the eastern part of China (38°18′32″ N, 115°58′41″ E), comprising a total area of approximately 67 987 km^2 of the Hebei plain, with a population of about 50 million people. The site lays between the west of the Bohai Bay and the east of the Taihang Mountain.

Soils were collected from soil cores (20 cm diam × 40 cm deep) with a split soil corer (AMS, Inc., American Falls, ID, USA) and pooled by split-plot. Cores were arranged in a randomized complete block design with 3 replications. The samples were sieved through a <2mm sieve to remove plant tissue and divided into upper layer (0-20cm) and lower layer (20-40cm) according to the depth of site. All soil samples were kept cool with ice in the field and maintained at 4 ℃ in the lab until processed. Chemical and physical properties of the soil are shown in Table 1.

Experimental design and treatment

Experiments were carried out on soil culture-potted Chinese cabbage from Aug. 7 to Oct. 5, 2015.

They consisted of three treatments of drip irrigation: groundwater(CK), groundwater with five types of bacteria(I#, 100:1, v/v), groundwater with one type of bacteria (A#, 100:1, v/v), each with three replicates. The amount of bacteria in the liquid was 10^6CFU·mL^{-1}. The five types of bacteria in I# were all gram-positive (G^+) bacteria, including *arthrobacter* species, *ensifer* species, *empedobacter* species, *chryseobacterium* species, and *zobellella* species. These five species show high N transformation quality [17-20]. The bacterium in A# was a strain of *bacillus subtilis*. It is a Gram-positive bacterium commonly found in soil and is an excellent model organism for the study of basic cell processes. The application of *Bacillus subtilis* to the soil or *via* seed may provide disease control[21].

All bacteria used in this study were purchased from the Chinese Academy of Sciences, Institute of Life Science, Beijing. The Chinese cabbage was dripped once a day using an irrigation system after budding, and the amount was 50 ml at a time. The Chinese cabbages were protected from heavy rainfall and strong light and removed after incubation of 5 weeks. Soil was regularly loosened. During the study period, soil samples were collected once every 7 days from the upper and lower layers. The samples were dried naturally, sieved, and then analyzed.

Genomic DNA extraction and PCR

Bacterial genomic DNA was isolated from 0.3g soil samples according to the CTAB/SDS method. DNA was diluted to 1 ng·μl^{-1} using sterile water. Its concentration and purity were quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA) by running it on 1% agarose gel with 0.5 M tris-borate-EDTA buffer. The samples were then transported to Novogene Bioinformatics Technology Co. Ltd. (Beijing, China) for PCR optimization and pyrosequencing analysis.

PCR was performed with 16S/18S rRNA gene primers. All PCR reactions were carried out on 30 μl PCR mixtures with 15 μl Phusion® High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, USA), 0.2 μM forward and reverse primers, and about 10 ng template DNA. Thermal cycling procedure consisted of initial denaturation at 98 °C for 1 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. The PCR products were examined by 2% agarose gel electrophoresis and were purified with the GeneJETGel Extraction Kit (Thermo Fisher Scientific, MA, USA). Samples with a banbetween 400–450bp in gel electrophoresis were chosen for further experiments.

Table 1. Physicochemical characteristics and nitrogen concentration of the fluvo-aquic soil samples.

Sampling location (depth)	pH	Organic matter (g/kg)	TN (g/kg)	NO_3^--N (mg/kg)	NH_4^+-N (mg/kg)	NO_2^--N (mg/kg)	Bacterial concentration (CFU/g)
Wuqiang(0–20 cm)	7.8	28.6	1.6	139.2	2.86	0.2	2.6×10^6
Wuqiang(20–40cm)	7.8	17.8	1.0	35.9	0.68	0.3	3.4×10^6

Illumina sequencing and data analysis

Sequencing libraries were generated using NEBNext® Ultra™ DNA Library Prep Kit for Illumina® (New England Biolabs), following the manufacturer's protocol, and index codes were added to them. The library quality was assessed on the Qubit® 2.0 fluorometer (Thermo Fisher Scientific) and Agilent 2100 bioanalyzer. Finally, the library was sequenced in an IlluminaHiSeq platform, and 250 bp paired-end readings were generated.

The relative abundance of major phyla and genera were used to describe the microbial community composition. To compute the alpha diversity, Shannon diversity index was calculated at 97% operational taxonomic unit level.

Chemical analysis

Soil samples were thoroughly mixed in the lab and analysis of chemical and physical properties of soils was done by standard procedures. TN, NO_3^--N, NH_4^+-NandNO_2^--N were analyzed according to Chinese SEPAStandard Methods[22].Soil pH was measured by a single function pH electrode (R-27012-06, Cole-Parmer Inc.) using a soil-to-water ratio of 1:2.5.

RESULTS AND DISCUSSION

Effect of bacteria on soil N concentration

Effect of bacteria on TN concentration

The effect of bacteria on soil TN concentration is shown in Figure 1. TN concentration shows similar trends in the upper soil layers after drip irrigation with different bacteria, showing an initial decrease from weeks 1–5, when the Chinese cabbage absorbed soil nutrients during its growth stage and then the variations became smooth. The minimum TN concentration in the upper soil layer was shown in week 5 when Chinese cabbage was grown up. It was 483.94 mg·kg^{-1} using I# water drip-irrigated treatment, which is lower than CK water drip-irrigated (528.45 mg·kg^{-1}) and A# water drip-irrigated (699.99 mg·kg^{-1}) treatment in upper the soil layer. Reasonable interpretation is that the bacteria in I# water enhanced N transformation, result in higher NO_3^--N and NH_4^+-N release, which were easily absorbed by Chinese cabbage. Therefore the TN in the upper soil layer using I# water was the lowest as N was transferred from soil to the Chinese cabbage. The N in the Chinese cabbage should be further detected.

After the Chinese cabbage was harvested, the TN in the upper layer tended to significantly increase to 932.66 mg·kg^{-1}, 1042.92 mg·kg^{-1}, 1073.59 mg·kg^{-1} for I#, CK and A# water in week 10, respectively. Same trend was reported by other studies when conifer species were harvested in forest soil[23].

The TN concentration in the lower layer soil showed the same trend with that in the upper layer soil. However, the changes cope was small. It was observed during the experiments that the soil was drier than the upper layer soil, indicating that the water with different bacteria did not or little reached the lower layer soil because of the depth.

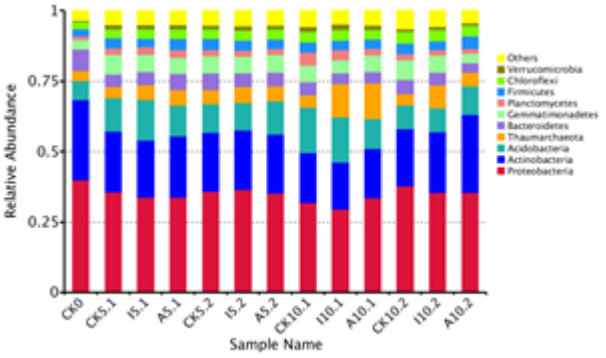

Fig. 1. Effect of bacteria on soil TN change.

Effect of bacteriaon NO_3^--N concentration

Changes in NO_3^--N concentration were similar to those in TN concentration in the upper soil layer after different water drip irrigations (Figure 2). It decreases with the time in week 1-5from 17.4 mg·kg^{-1} (before water drip irrigation) to 6.31 mg·kg^{-1}, 7.22 mg·kg^{-1} and 8.77 mg·kg^{-1} for I#, CK and A# water in week 5, respectively.TheNO_3^--N concentration from week 5 to week 6in the upper soil layer shows a rapid lifting for three different bacteria treatment. This causes the highest levels of NO_3^--N in the soil in the 6th week of 17.45 mg·kg^{-1}, 23.98 mg·kg^{-1} and 18.14 mg·kg^{-1} for I#, CK and A# water, respectively. This relates to the harvest of Chinese cabbage and the effect of the bacteria. However, there was a slightly decrease inNO_3^--N concentration in the upper soil layer since week 7 for these three different treatments. NO_3^--N level at I# water treatment did not differ from the treatment of others despite its level of NO_3^--N is the lowest one (from 17.45 mg·kg^{-1}to 14.43 mg·kg^{-1}).

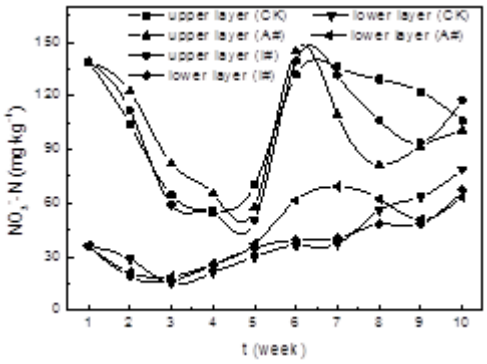

Fig. 2. Effect of bacteria on soil NO_3^--N change.

It is likely that bacteria in I# water stimulate the growth of soil nitrifying bacteria so NO_3^--N is the highest in soil by I# water treatment. Decrease of NO_3^--N by I# water treatment is also the slowest when compared to the other two treatments. As the

experiment continued, the diversity and quantity of microbes, especially bacteria in I# water, increased because of reproduction and organic N was continuously converted to NO_3^--N through nitrification, leading to an increase in NO_3^--N concentration[24].

Effect of bacteria on NH_4^+-N concentration

The effect of different bacteria on NH_4^+-N concentration in the soil is shown in Figure 3.

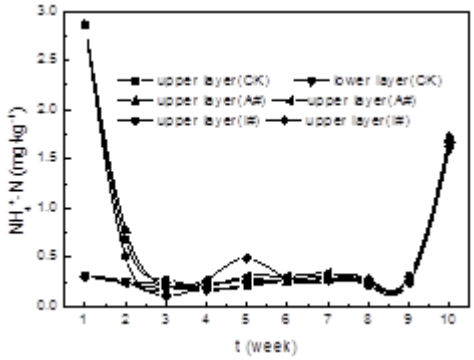

Fig. 3. Effects of bacteria on soil NH_4^+-N change.

The changes in NH_4^+-N concentration were stable (0.19-0.34 mg·kg^{-1}) in the lower soil layers related with different drip irrigations during weeks 2–9. The values did not change overall, probably due to the decrease in nutrient requirements of Chinese cabbage, low diversity and quantity of aerobic microbes, and weakening of the biochemical action of the N cycle[25]. Interestingly, during weeks 9–10, NH_4^+-N concentration greatly fluctuated and the values sharply increased higher than the initial values. Further research should be done to find a reasonable explanation. The changes in NH_4^+-N concentration in the upper soil layer were also similar after treatment with different drip irrigations. There was no significant difference between the upper and lower soil layer.

The changes inNH_4^+-N lever during the experiments maybe due to the nitrification process in the aerobic surface soil [7]. The reaction is:

$$2NH_4^+ + 3O_2 \xrightarrow{\text{Nitrosomonas}} 2NO_2^- + 2H_2O + 4H^+$$
$$2NO_2^- + O_2 \xrightarrow{\text{Nitrobacter}} 2NO_3^-$$

The reprocesses probably also affect the content of TN in the soil as shown in Figure 1.

Analysis of the whole microbial community

The relative abundance of the 10 most abundant phyla is shown in Figure 4. No significant differences in bacterial abundance were observed among the different drip irrigations and incubation times. The bacterial distribution showed that *Proteobacteria* was the most dominant phylum with mean relative abundance of 35.0%, followed by *Actinobacteria* (21.3%) and *Acidobacteria* (11.1%). The same predominant groups have been previously observed in other studies on other soil samples[26].The remaining phyla including *Thaumarchaeota, Bacteroidetes, lanctomycetes, Gemmatimonadetes, Firmicutes, Chloroflexi, and Verrucomicrobia,* accounted for <34% of the relative abundance observed, and were designated as minor. In addition, 5.1% of the clones could not be assigned to any particular phylum, after comparison with RDP and/or GenBank databases, or else had a sequence length too short to be conclusive.

Shannon diversity index (H) was calculated by the formula $H = -\Sigma p_i \ln p_i$, where p_i is the ratio of

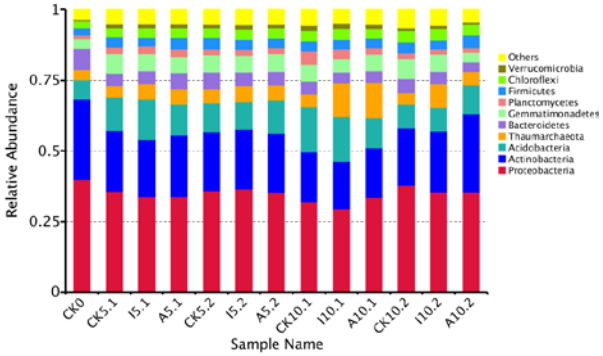

Fig. 4. Relative abundance of 10 most abundant phyla in the bacteria under drip irrigation with CK, I# and A# water.('5' represents the incubation after 5 weeks, '10' represents the incubation after 10 weeks, '1' represents the upper layer, '2' represents the lower layer).

The relative intensity of band i and the relative intensity of the lane, used to estimate the species diversity in the soil are listed in Table 2. Shannon diversity indices of bacteria indicated that different drip irrigations and incubation times affected the soil bacterial community diversity. After 10 weeks of incubation, the values of H for soils with different drip irrigations slightly decreased (e.g., from 9.6 to 9.4 for I5.1 and I10.1, respectively), except the soil irrigated with only CK water, which slightly increased (e.g., from 9.6 to 9.7 for CK5.1 and CK10.1, respectively). However, all H values were higher than that of soil sample CK0, suggesting a high level of species diversity in the soil after irrigation with different bacterial water.

Table 2. Soil microbial functional diversity indices based on the Shannon diversity index

Samples	Bacteria	AOA	AOB
CK0	8.485	4.322	0.36
CK5.1	9.641	4.698	0.196
I5.1	9.635	5.199	0.161
A5.1	9.64	3.5	0.407
CK5.2	9.672	4.312	0.699
I5.2	9.595	4.174	0.183
A5.2	9.635	4.62	0.278
CK10.1	9.743	3.996	0.181
I10.1	9.439	4.063	0.225
A10.1	9.348	5.255	0.242
CK10.2	9.748	4.86	0.666
I10.2	9.543	5.195	0.444
A10.2	9.37	4.038	0.231

The species diversity of AOA had a vastly different trend compared to that of the bacterial community (Table 2).Mean value of H (4.5) was consistently higher in soil samples irrigated with water than in the sample without any treatment (CK0, 4.3), whereas it was lower or higher for each sample in the control treatment as compared to the sample CK0, with no consistent trend.

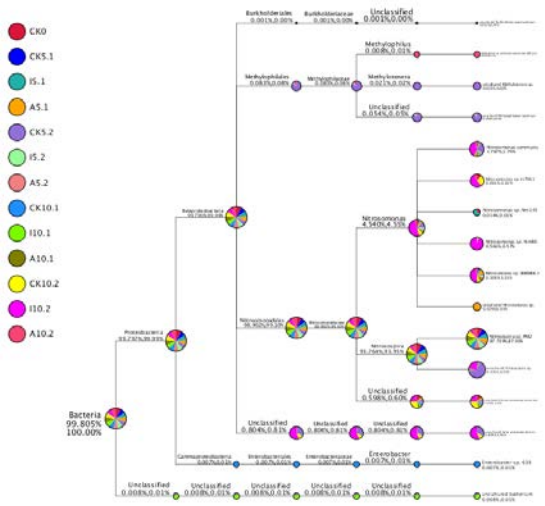

Fig. 5. Species classification tree of 20 predominant AOB in soil samples. the former percentage was of all speciesand the latter accounts for the percentage of the selected species in the sample.

Previous findings have shown that *Nitrosospira*-like species are the dominant species of AOB in paddy soil[27]. Our phylogenetic analysis results provided further evidence that *Nitrosomonas* species were dominant and might be the main ammonia oxidizers in the soil (Figure 5).

CONCLUSIONS

In the upper layer soil, the lowest TN level was 528.45 mg·kg^{-1}, 483.94 mg·kg^{-1}, and 699.99 mg·kg^{-1}for CK, I# and A# water soils in the 5th week, respectively, and the lowest NO_3^--N level was 23.98 mg·kg^{-1},17.45 mg·kg^{-1} and 18.14 mg·kg^{-1}.NH_4^+-N concentrations were stable during the incubation time except a tremendous decrease from the 1st to the 2ed week and increase form 9th to 10th week. The changes of TN, NH_4^+-N, andNO_3^--N in the lower layer soil showed the same trends as the upper layer soil with low level and smooth changes.

Phylum *proteobacteria* was the most dominant phylum with mean relative abundance of 35.0%, followed by *Actinobacteria* (21.3%) and *Acidobacteria* (11.1%). The AOA community was composed mainly of *Thaumarchaeota* in all samples. *Nitrososphaera*, *Cenarchaeum* and *Candidatus* were the most abundant genera, as for AOB community. *Nitrosomonas* species were dominant and might be the main ammonia oxidizers in the soil.

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