Applied Soil Ecology 72 (2013) 69–78

Contents lists available at SciVerse ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Impacts of chloride de-icing salt on bulk soils, fungi, and bacterial populations surrounding the plant rhizosphere

Changrong Ke[a], Zhouyuan Li[b], Yingmei Liang[c,*], Wanqiang Tao[d], Mengchan Du[a]

[a] College of Environmental Science and Engineering, Beijing Forestry University, Beijing 100083, China
[b] School of Environment, Tsinghua University, Beijing 100084, China
[c] Museum of Beijing Forestry University, Beijing Forestry University, Beijing 100083, China
[d] Forestry Protection Station of Beijing, Beijing 100029, China

ARTICLE INFO

Article history:
Received 23 January 2013
Received in revised form 9 June 2013
Accepted 11 June 2013

Keywords:
De-icing salt
Salinity
Bacteria
Fungi
Rhizosphere

ABSTRACT

This study was conducted to investigate the dynamic changes of bulk soil and microbial populations in the surrounding plant rhizosphere under the influence of chloride de-icing salt. Each experimental plant was given 0.25 L de-icing salt solution with an irrigation concentration of 200 g/L, once every 3 days for 27 days. Samples were taken weekly from each soil layers (0–15 cm, 15–30 cm and 30–45 cm). The results revealed that de-icing salt led to different effects in different soil layers in one month. A trend of increasing electric conductivity (EC) values, Na^+ concentrations, and Cl^- concentrations in the upper soil layer, compared with the other two soil layers, was apparent, which proved a stronger effect of de-icing salt on the upper soil layer. Soil cation exchange capacity (CEC) decreased with the accumulation of the de-icing salt. Denaturing gradient gel electrophoresis (DGGE) analysis indicated that de-icing salt decreased the Shannon diversity (*H*) and Evenness (*E*) of fungal populations in three soil layers and led to a decrease of bacterial *H* in the upper soil layers. However, a contrary trend for *H* and *E* of bacterial populations was seen in the middle and lower soil layers. Both the unweighted pair group mean average (UPGMA) cluster and canonical correspondence analysis (CCA) analysis indicated that the bacterial populations were more stable than the fungal populations in response to salinity stress. CCA analysis showed the dynamic changes of microbial populations and the soil properties (EC, CEC, Na^+ and Cl^-) were strongly correlative. The microbial populations were obtaining a new dynamic equilibrium at the end of the study. The dominant microbial populations were originally *Crepidotus*, *Metarhizium*, *Penicillium* and *Acidobacterium*, but converted to *Pythium*, Boletaceae, Cystofilobasidiales, *Phacidium*, *Acidobacterium*, *Pseudomonas* and Chloroflexi with the addition of de-icing salt. The obtained results suggest that the uses of chemical agents leading to soil salinization, such as de-icing salt, can destroy the micro-ecosystem of bulk soil surrounding plant rhizosphere and influence soil health.

© 2013 Elsevier B.V. All rights reserved.

1. Introduction

De-icing salt is an indispensable auxiliary or main approach for snow clearing and skid prevention in winter due to its high efficiency and low cost in many countries (Ramakrishna and Viraraghavan, 2005). Annual rock salt use for road de-icing increased from 163,000 tons in 1940 to over 23,000,000 tons in 2005 in the USA (Jackson and Jibbagy, 2005). In China, at least 600,000 tons of de-icing salt have been used on average each year in recent years. However, the application of de-icing salt over time has produced adverse impacts on roadside vegetation and the urban environment (Godwin and Gallagher, 2003; Kayama et al., 2003; Ramakrishna and Viraraghavan, 2005; Viskari and Kärenlampi, 2000). De-icing salt damages plants through both direct contact and indirect absorption from soil (Fostad and Pedersen, 2000; Hans, 2011). Munns and Tester (2008) pointed out that the mechanism of damage to vegetation may include osmotic effects leading to the accumulation of toxic concentrations of specific elements that are absorbed through the roots and subsequently translocated. Other effects such as soil fertility and soil structure also contribute (Green et al., 2008; Galuszka et al., 2011), since de-icing salt permeates the soil and changes the soil environment (Ramakrishna and Viraraghavan, 2005). The impacts of de-icing salt on vegetation have been extensively reported, including leaf chlorosis and necrosis, bud mortality, stunted growth, cell plasmolysis, disorders in photosynthesis, damage to cell membrane function, and osmotic

* Corresponding author. Tel.: +86 010 62336316; fax: +86 010 62336316.
E-mail addresses: kechangrong.2007@163.com (C. Ke), zhouyuanli2009@gmail.com (Z. Li), liangym812@126.com (Y. Liang), twq@bjfb.gov.cn (W. Tao), dream0904@126.com (M. Du).

0929-1393/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.apsoil.2013.06.003

stress (Galuszka et al., 2011; Graves and Gallagher, 2003; Jonsson, 2006; Kayama et al., 2003; Paludan Müller et al., 2002). However, the impacts of de-icing salt on soil microorganisms in the terrestrial ecosystem have not been extensively assessed and discussed.

Soil microorganisms are very important for maintaining the structure, quality, and fertility of the soil (Černohlávková1 et al., 2008) and can respond to environmental stress by adjusting their activity, biomass, and community structure (Schloter et al., 2003). Furthermore, plant–microorganism interactions in the rhizosphere environment are essential to plant health and soil fertility (Moulin et al., 2001). Thus, studies on the response of soil microbial populations to pollutants in the plant rhizosphere are important both for the understanding of the impact of de-icing salt on the sustainability of the plant and soil environment and for the identification of possible solutions or ways to mitigate this impact. While there have been many studies on the influences of de-icing salt on soil, they have almost all focused on physicochemical properties and soil fertility (Bäckström et al., 2004; Bowers and Hesterberg, 1976; Cunningham et al., 2008; Fritzsche, 1992; Green et al., 2008; Norrström and Jacks, 1998; Norrstrom, 2005; Rasa et al., 2006). Few studies have considered the influences of de-icing salt on soil microorganisms. Green et al. (2008) concluded that accumulation of de-icing salt may alter the ecological structure of soil organism populations. Černohlávková1 et al. (2008) found that microbial biomass and respiration activity could be decreased by de-icing salt, which could stimulate *Pseudomonas putida* growth. However, the effects on microbial community diversity in response to de-icing salt and the alteration of interaction between microbial populations have been not been extensively documented and analyzed. Such investigations would have the additional benefit of improved characterization of the influence of salinity on microorganisms.

Denaturing gradient gel electrophoresis (DGGE) is free of interference by incision enzymes in the outputs, and universal primers for bacterial 16S rDNA and fungal 18S rDNA suitable for the assessment of microbial diversity are readily available (Agnelli et al., 2004), we chose this method for the present study.

Since the acute plant toxicity of de-icing salt upon Japanese spindle (*Euonymus japonicas*) had been undertaken and a dose–effect relationship had been revealed (Li et al., 2012), we continued to investigate the effects from the agent to the rhizospheric ecosystem with the assessment end-point and its corresponding concentration of NaCl solution. In our study, the responses of soil properties and of fungal and bacterial populations were assessed. We hypothesized that de-icing salt should lead to a shift in microbial populations and a decrease in the diversity of microbial populations.

2. Materials and methods

2.1. Materials and location of investigation site

Standard chloride de-icing salt (China National Salt Industry Corporation, China), containing over 95% sodium chloride (NaCl), was used. The study population consisted of 35 2-year-old healthy saplings, *E. japonicas*, with an average height of 70.0 ± 5.5 cm for their aboveground part, and an average depth of 10.0 ± 3.0 cm for the underground roots.

The study was conducted in the experimental nursery garden of Beijing Forestry University (116°20′27″ E and 40°32″ N).

2.2. Processing of sample plot and collection of soil sample

The 35 study saplings were planted in the experimental nursery garden 1 month in advance to ensure their natural qualities before salt exposure. Each plant was given 0.25 L de-icing salt solution in an area of 0.023 m^2 (0.15 m × 0.15 m) with an irrigation concentration of 200 g/L. Each plant received the same treatment once every 3 days for 27 days.

We transferred the surveyed and estimated data of the real case of using de-icing salt in China (Dao et al., 2008; Wen et al., 2010; Wang et al., 2011) (see Supplement 1) and referred the results in the pre-experiment toxicity test (Li et al., 2012) to chose the exposure dose and duration of NaCl solution. The lethal effect on the tested plants was taken as the assessment end-point in the processing.

Sampling was done once every 7 days. Three soil samples were collected from each layer (0–15 cm, 15–30 cm and 30–45 cm) with a soil sampler. The samples were collected from five randomly selected points and then were mixed together in the manner of traditional mixed sampling. Sand, stone, and visible animal and plant residues (>2 mm) were sieved out of the collected soil. The sieved soil samples were divided into two groups. One was air-dried for physicochemical analyses, and the other was placed in a sterile bag and stored at −20 °C for microbial analysis.

2.3. Soil characteristics

The soil is cinnamon soil according to Chinese soil classification system, and approximately equivalent to Ustalf in the USDA Soil Taxonomy. The soil textures of three soil layers are sandy loam.

Soil pH and electric conductivity (EC) were measured in a 1:1 soil to water solution (Rousk et al., 2011). Concentration of sodium ion (Na^+) was extracted with NH_4OAc-NH_4OH (pH 9.0) and determined by flame photometry. Soil chloride ion (Cl^-) in the extractant with a solution of 3 mmol $NaHCO_3$ and 1.8 mmol Na_2CO_3 at a ratio of 5 g soil/25 mL, shaking for 1 h (Li et al., 1994) and was determined following the protocols of Tabatabai and Dick (1983), using an Ion Chromatograph. Organic matter (OM) was determined by the $K_2Cr_2O_7$ oxidation method and cation exchange capacity (CEC) by the NH_4Cl–NH_4OAc method (Lu, 2000).

2.4. PCR-DGGE

Total soil DNA was extracted from sieved soil (~0.5 g wet weight) using a soil DNA kit (Omega, D5625-01, USA) following the manufacturer's protocol. The extracted DNA was used as a template for amplification of the bacterial 16S rDNA gene using the primers GC-338f (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG-3′) and 534r (5′-ATT ACC GCG GCT GCT GG-3′), as described by Muyzer et al. (1993) and the fungal 18S rDNA gene using the primers GC-fungf (5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CAT TCC CCG TTA CCC GTT G-3′) and NS1r (5′-GTA GTC ATA TGC TTG TCT C-3′), as described by May et al. (2001). The final 50 μL PCR mixture contained 1 × PCR buffer (Mg^{2+}) 5 μL, 10 μmol each of the forward and reverse primers, 2.5 units of ex taq DNA polymerase, and ~50–100 ng of template DNA. The PCR amplification of bacterial 16s DNA was performed in a thermocycler (BIO-RAD, S1000™, USA) under the following reaction conditions: 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 45 s, 72 °C for 1 min, and a final extension step at 72 °C for 8 min. The PCR amplification of fungal 18s DNA was performed under the following reaction conditions: 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 45 s, 65 °C for 1 min, and a final extension step at 65 °C for 8 min. All products were checked by agarose gel electrophoresis on a 1 × TAE (Tris Acetate EDTA buffer) agarose gel (1%, w/v). The 16S rDNA-DGGE was performed on a Dcode System (Universal Mutation Detection System, Bio-rad, USA). Then, 25 μL of the products (~150 ng DNA, with 5 μL loading buffer) were loaded on a 8% polyacrylamide gel (Acrylamide/Bis-acrylamide 37.5:1) containing a denaturing gradient of 35–65% (100% denaturant solution

containing 7 mol urea and 40% (V/V) formamide) in 1 × TAE buffer. Electrophoresis was carried out at constant temperature (60 °C) and voltage (80 V) for 18 h. The DGGE gel was dyed with Syber Green I (USA) for 30 min. Then, the gel was scanned using an Alpha Imager (Bio-rad, USA) with UV light and photographed. The photo was saved for further analysis. 18S rDNA-DGGE was performed as described above for 16S rDNA-DGGE with slight modifications. The loading volume was also 25 μL (~150 ng DNA, with 5 μL loading buffer), but the products were loaded on a 6% polyacrylamide gel containing a denaturant gradient of 20–45%. Electrophoresis was carried out at constant temperature (60 °C) and voltage (50 V) for 20 h. The other steps were the same as those undertaken for 16S rDNA, described above.

2.5. Sequencing

The typical DGGE bands were excised from the gels and immersed in 30 μL TE buffer overnight at 4 °C to extract the DNA from the gel. These DNA solutions were then used as amplification templates with the primers 338f (5′-ACT CCT ACG GGA GGC AGC AG-3′) and 534r for bacteria and fungf (5′-CAT TCC CCG TTA CCC GTT G-3′) and NS1r for fungi. The amplicons were visualized by 1.2% (w/v) agarose gel electrophoresis and then were extracted using a gel Midi Purification Kit (Tangen, China) according to the manufacturer's instructions. The purified amplicons were cloned into the PMD19-T vector (Takara, Dalian, China) and then transformed into *Escherichia coli* DH5α cells (Takara, Dalian, China) in accordance with the manufacturer's instructions. The transformed *E. coli* were cultured on LB medium containing ampicilline (100 μg/mL), IPTG (24 μg/mL), X-gal (40 μg/mL) for 16 h at 37 °C, which allowed blue-white screening. To confirm the presence of inserts, 20 white colonies were then picked and inoculated into 1 mL liquid LB medium respectively per sample for 8 h at 37 °C. 1 μL was subsequently used as template DNA for PCR in a volume of 25 μL with the primers 338f and 534r for bacteria, as well as the primers fungf and NS1r for fungi. All PCR products were checked by electrophoresis in 1.2% (w/v) agarose gels to select positive colonies. Three positive colonies were selected randomly and sequenced with primer M13f (5′-CGC CAG GGT TTT CCC AGT CAC GAC-3′) by Yingjun Biological Engineering Technology Company (Shanghai, China).

The sequences were characterized using BLAST search of the GenBank database of the National Center of Biotechnology Information (NCBI). Our sequences were deposited in the NCBI nucleotide sequence database under accession numbers JX215266–JX215327 and JX195654–JX195657.

2.6. Statistical analyses

The DGGE profiles were analyzed with Quantity One Software (version 4.5, Bio-Rad). The Shannon diversity index (H) and evenness index (E) were used to estimate the microbial community diversity in each sample based on the following equations:

$$H = \sum_{i}^{s} p_i \ln pi = \sum_{i=1}^{s} \left(\frac{N_i}{N}\right) \ln \left(\frac{N_i}{N}\right) \quad (1)$$

$$E = \frac{H}{H_{\max}} = \frac{H}{\ln S} \quad (2)$$

where N_i is the abundance (relative quality) of the ith ribotype; N is the total abundance of all ribotypes in each lane of the DGGE gels; and S is the number of bands in each lane of the DGGE gels (Zhou et al., 2011). The similarity between each lane was calculated by the Dice coefficient method and the dendrograms were generated by the unweighted pair group mean average (UPGMA) cluster analysis using Quantity One Software (version 4.5, Bio-Rad) (Yao et al., 2012).

Canonical correspondence analysis (CCA) was used to evaluate the correlations between investigated environmental factors and microbial populations, by the software Canoco for Windows 4.5. Six soil properties including CEC, pH, Na^+, Cl^-, OM and EC which would reflect the effect of deicing salt to soil were investigated as environmental factors. They were visualized in a CCA diagram as vectors, and longer vectors represented factors that were more important to the diversity of microbial populations. In addition, the angle between two vectors indicated the degree of correlation of the soil properties. Also, the changes of the samples as well as the degree to which the microorganisms were influenced as the deicing salt been added could be identified through the CCA diagram.

3. Results

3.1. Soil properties

Na^+ concentration, Cl^- concentration, and EC increased in all three soil layers, with a faster rate of increase in the upper soil layer than in the other two soil layers (Fig. 1a–c). In the upper soil layer, Na^+ concentration elevated from 0.05 mg/g dry soil to 5.04 mg/g dry soil and EC was 30 times higher on Day 28 than on Day 0, with a very high salinization (Fig. 1c). However, CEC showed a downward trend in the three soil layers (Fig. 1d). OM content was relatively stable in the upper soil layer, but decreased on Day 14 in the middle soil layer and on Day 7 in the lower soil layer, followed by an increase for both layers (Fig. 1e). The pH values declined slightly in the upper and middle soil layers, but increased slightly in the lower soil layers (Fig. 1f).

3.2. Responses to de-icing salt by fungal populations

Genetic fingerprints of fungal 18S rDNA by DGGE revealed a relatively abundant diversity of fungi in the three soil layers (Fig. 2). DGGE band intensities and the dominant populations varied with differing sampling times. In the first two samples, bands appeared relatively uniform in density. By contrast, fewer, more intense bands were predominant (about four to five) in the last two samples.

A UPGMA similarity dendrogram of DGGE banding patterns indicated a range of similarity, from 32% to 75%, in all samples (Fig. 3). In this tree, the first and second samples belonged to one clade, and the last three samples clustered together, with the exception of sample 3 M. The similarity among samples increased with sampling time in the same soil layer. The first samples had a relatively lower similarity, 47%, with the second samples in all soil layers, but the fourth samples had a similarity of 72%, 55%, and 51% with the last samples in the upper, middle, and lower layers, respectively.

The Shannon diversity index (H) and evenness index (E) of fungi are shown in Fig. 4. The fungal H decreased with increasing amounts of de-icing salt in the three soil layers (Fig. 4a). Before the de-icing salt treatment, the fungal H in the middle soil layer was higher than that in the other two soil layers. After the de-icing salt treatment, it decreased in all soil layers to a similar level, about 1.8. The E of the fungal community in the three soil layers also decreased overall, but this decrease was especially evident in the middle soil layer (Fig. 4b).

A total of 31 bands were excised and sequenced. Each band was labeled with an Arabic numeral, as shown in Fig. 2. Comparison of the sequences revealed 94–100% similarity with sequences available in the GenBank database. Of the 31 bands, 20 could be classified to the level of genus. They fell into 14 genera: *Geomyces*, *Trichosporon*, *Crepidotus*, *Metarhizium*, *Penicillium*,

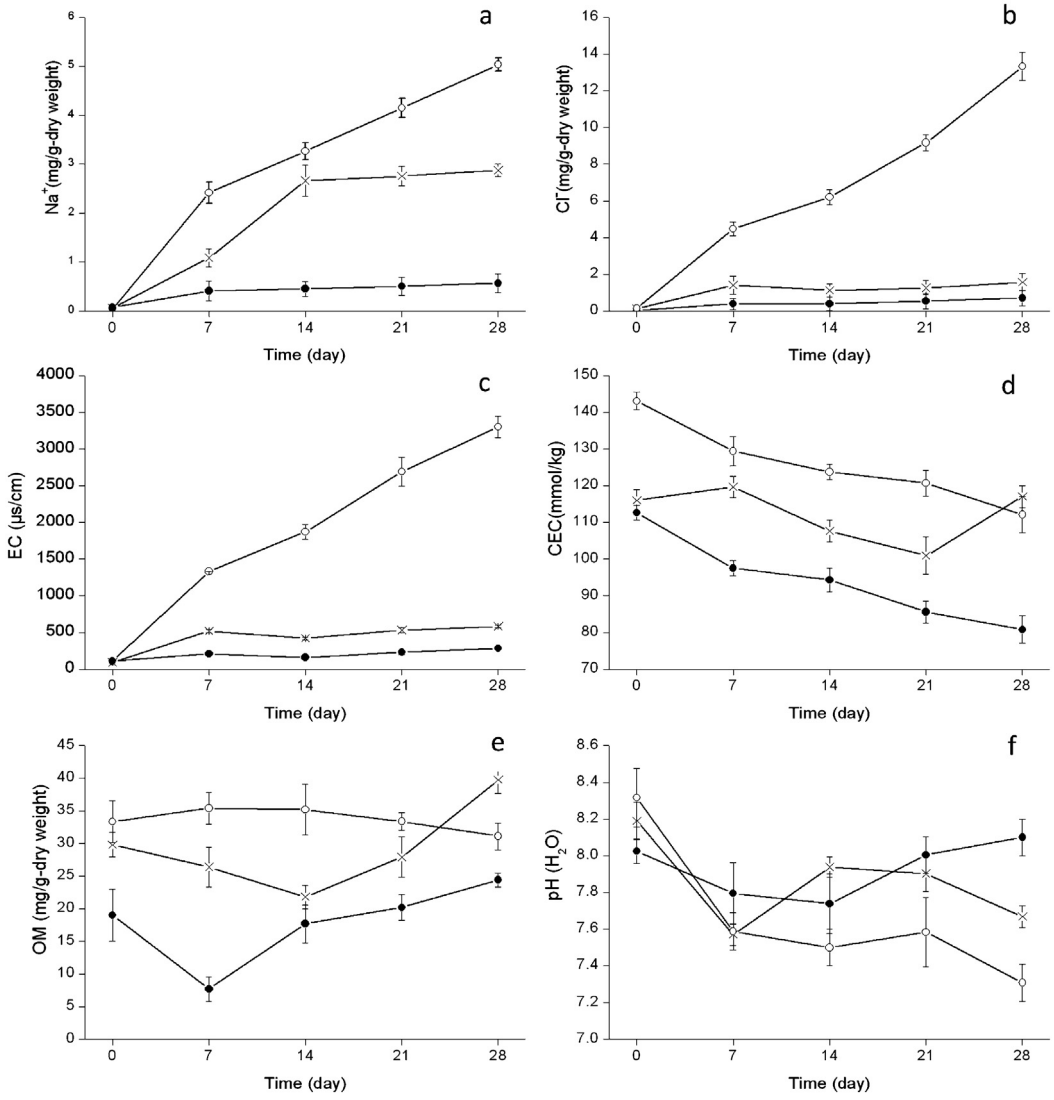

Fig. 1. Physical and chemical properties of upper soil layer (—○—), middle soil layer (—×—), and lower soil layer (—●—) at different sampling times.

Verticillium, *Phaeosphaeria*, *Pythium*, *Ramicandelaber*, *Phacidium*, *Rhizoctonia*, *Acremonium*, *Phyllactinia*, and *Dictyostelium*. Of the remaining 11 bands, seven could be classified either to the level of family (Ascobolaceae, Boletaceae, and Filobasidiaceae), order (Cystofilobasidiales), or phylum (Ascomycota). The four bands for which identity could not be determined to at least the level of phylum were identified as uncultured fungi (Table 1). The dominant populations of fungi were originally *Crepidotus* (band 2), *Metarhizium* (band 3) and *Penicillium* (band 4), but by Day 28, the dominant populations had changed to *Pythium* (band 15), Boletaceae (band

Table 1
Sequence identifications of excised bands from fungal DGGE gel.

Band No.	NCBI Accession No.	Classification	Similarity (%)	Band No.	NCBI Accession No.	Classification	Similarity (%)
1	JX215297	*Trichosporon*	100%	17	JX215313	*Ramicandelaber* sp.	99%
2	JX215298	*Crepidotus*	99%	18	JX215314	Uncultured fungus	99%
3	JX215299	*Metarhizium*	98%	19	JX215315	Boletaceae	97%
4	JX215300	*Penicillium*	100%	20	JX215316	Uncultured fungus	99%
5	JX215301	Ascomycota	100%	21	JX215317	Cystofilobasidiales	99%
6	JX215302	Ascomycota	100%	22	JX215318	*Acremonium*	99%
7	JX215303	*Verticillium*	99%	23	JX215319	*Phacidium*	99%
8	JX215304	Ascobolaceae	99%	24	JX215320	Uncultured fungus	94%
9	JX215305	Boletaceae	98%	25	JX215321	*Phyllactinia*	99%
10	JX215306	*Phaeosphaeria*	99%	26	JX215322	*Geomyces*	100%
11	JX215307	*Acremonium*	99%	27	JX215323	*Acremonium*	98%
12	JX215308	*Metarhizium*	99%	28	JX215324	*Metarhizium*	100%
13	JX215309	*Pythium*	97%	29	JX215325	*Rhizoctonia*	99%
14	JX215310	Filobasidiaceae	100%	30	JX215326	Uncultured fungus	99%
15	JX215311	*Pythium*	99%	31	JX215327	*Dictyostelium*	99%
16	JX215312	*Phyllactinia*	99%				

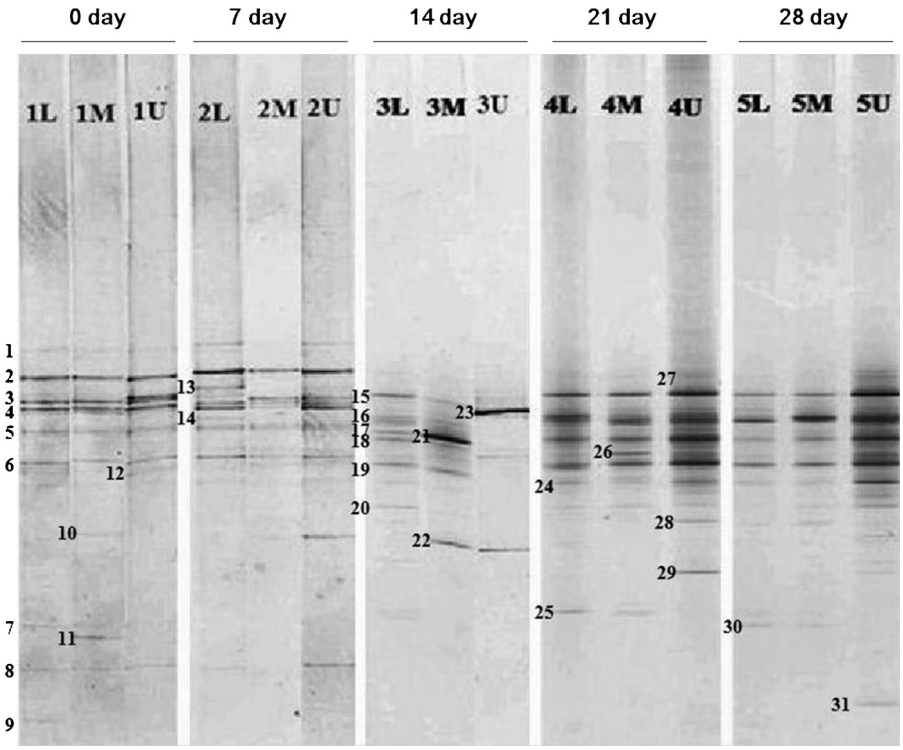

Fig. 2. DGGE fingerprints of fungal communities in three soil layers at different sampling times. The capital letters represent soil depth: "U", "M", and "L" refer to the upper (1–15 cm), middle (15–30 cm), and lower (30–45 cm) soil layers, respectively. The Arabic numerals before the letter refer to sampling timepoint.

19), Cystofilobasidiales (band 21), and *Phacidium* (band 23) (Fig. 2, Table 1).

3.3. Reponses to de-icing salt by bacterial populations

Genetic fingerprints of bacterial 16S rDNA by DGGE indicated a very rich diversity in all three soil layers (Fig. 5). The DGGE patterns of the fourth (Day 21) and fifth (Day 28) samples, however, revealed an obvious narrowing of this range of diversity compared with the first three samples; the DGGE bands of the last two samples were primarily distributed in the middle of the profiles. Furthermore, the fourth lanes (Day 21) showed more abundant dominant populations than the other lanes.

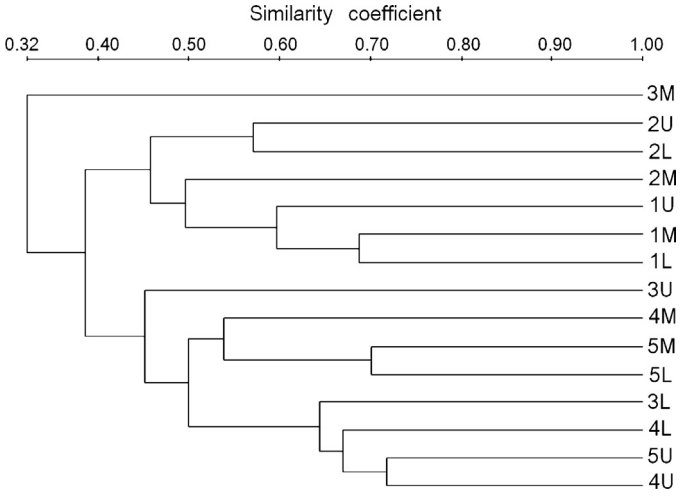

Fig. 3. Cluster analysis (UPGMA, Dice coefficient of similarity) of DGGE profiles of fungi.

DGGE profiles were separated into two distinct clusters using UPGMA cluster analysis. The first three samples belonged to a single large clade, except for 2 M, and the last two samples all clustered together (Fig. 6). The similarity dendrogram revealed a range of similarity, from 35% to 83%, in all samples (Fig. 6). The same tendency was detected in the fungal dendrogram; the similarity of samples increased with sampling time in each soil layer, with the exception of the lower soil layer. In the upper soil layer, the similarity of the first and second samples was 58%, compared with 70% for the last two samples. In the middle layer, the similarity increased from 49% to 69%. However, an opposite trend was observed in the lower soil layer, where similarity decreased from 74% to 65%.

The Shannon diversity index (H) and evenness index (E) of bacteria are shown in Fig. 7. The upper soil layer had a much higher initial bacterial diversity (Fig. 7a). In the first 2 weeks, the bacterial H decreased in the upper soil layer, but increased in the middle and lower soil layers. In later stages, the H of bacteria began to decrease in the middle soil layer, but showed a pattern of steady fluctuation in the other two soil layers. The E of bacteria exhibited a trend of increasing in all soil layers, especially in the middle and lower ones (Fig. 7b). The E of bacteria in all soil layers eventually reached a steady level of around 0.95.

A total of 35 bands were excised from the bacterial DGGE gel and sequenced. The bands were labeled as shown in Fig. 5 (Arabic numerals). The sequences were compared to entries in the GenBank database using BLAST search, which revealed 92–100% similarity between the sequenced amplicons and entries in the database. The BLAST results are shown in Table 2. The sequences could be classified into seven genera, two orders, three phyla, and two classes. The bands classifiable to the level of genus included 14 bands belonging to seven genera, including *Acinetobacter*, *Acidobacterium*, *Actinobacterium*, *Tessaracoccus*, *Clostridiaceae*, *Pseudomonas*, and *Bacillus*. Before the de-icing salt treatment, the dominant populations of bacteria were *Acidobacterium* (bands 4 and 5). By Day 28, however, dominance had shifted to *Acidobacterium* (band 21), *Pseudomonas* (band 28) and Chloroflexi (band 29) (Fig. 5, Table 2).

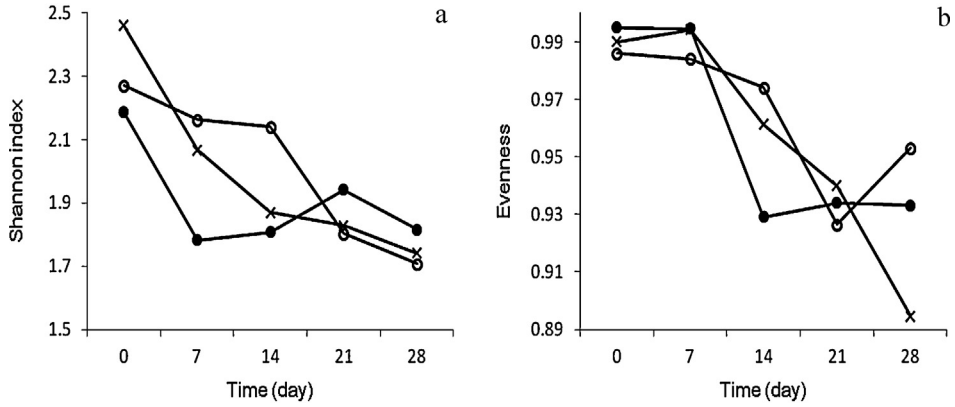

Fig. 4. Shannon diversity index (a) and evenness index (b) of fungi in three soil layers at different sampling times. "—○—", upper soil layer; "—×—", middle soil layer; "—●—", lower soil layer.

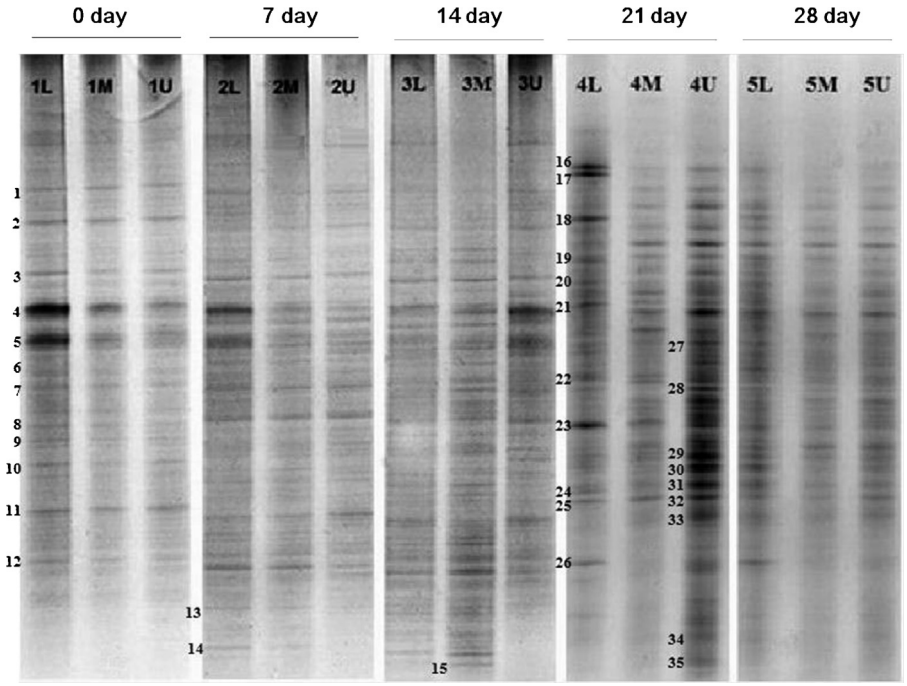

Fig. 5. DGGE fingerprints of bacterial communities in three soil layers at different sampling times. The capital letter represents soil depth: "L", "U", and "M" refer to the upper (1–15 cm), middle (15–30 cm), and lower (30–45 cm) soil layers, respectively. The Arabic numerals before the letter refer to sampling timepoint.

Table 2
Sequence identifications of excised bands from bacterial DGGE gel.

Band No.	NCBI Accession No.	Classification	Similarity (%)	Band No.	NCBI Accession No.	Classification	Similarity (%)
1	JX195656	Planctomycetales	97%	19	JX215280	Bacteroidetes	100%
2	JX195657	*Acinetobacter*	100%	20	JX215281	*Acidobacterium*	99%
3	JX195654	*Acidobacterium*	98%	21	JX215282	*Acidobacterium*	98%
4	JX195655	*Acidobacterium*	99%	22	JX215283	Chloroflexales	98%
5	JX215267	*Acidobacterium*	100%	23	JX215284	Proteobacteria	99%
6	JX215268	Chloroflexales	93%	24	JX215285	Actinobacteria	100%
7	JX215266	Proteobacteria	98%	25	JX215286	Chloroflexales	94%
8	JX215269	Proteobacteria	98%	26	JX215287	Proteobacteria	100%
9	JX215270	Gemmatimonadetes	99%	27	JX215288	Firmicutes	93%
10	JX215271	*Actinobacterium*	100%	28	JX215289	*Pseudomonas*	100%
11	JX215272	Chloroflexales	100%	29	JX215290	Chloroflexales	100%
12	JX215273	*Tessaracoccus*	97%	30	JX215291	Chloroflexales	98%
13	JX215274	*Clostridiaceae*	100%	31	JX215292	*Pseudomonas*	100%
14	JX215275	Firmicutes	100%	32	JX215293	*Bacillus*	100%
15	JX215276	*Actinobacterium*	90%	33	JX215296	Proteobacteria	99%
16	JX215277	Proteobacteria	96%	34	JX215294	*Bacillus*	100%
17	JX215278	Proteobacteria	92%	35	JX215295	Planctomycetales	98%
18	JX215279	Firmicutes	100%				

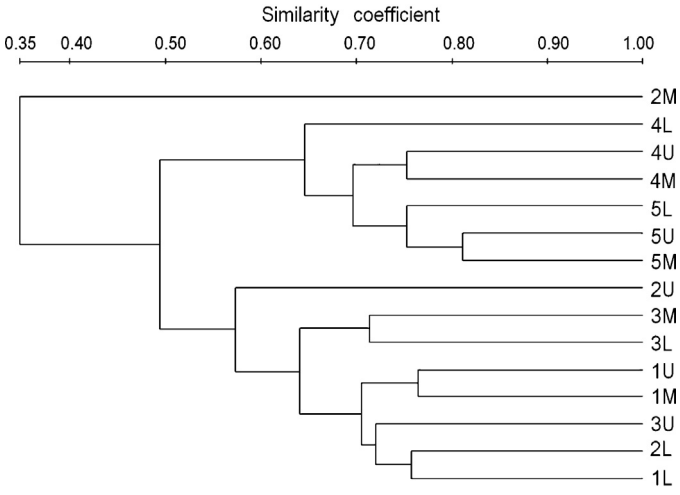

Fig. 6. Cluster analysis (UPGMA, Dice coefficient of similarity) of DGGE profiles of bacteria.

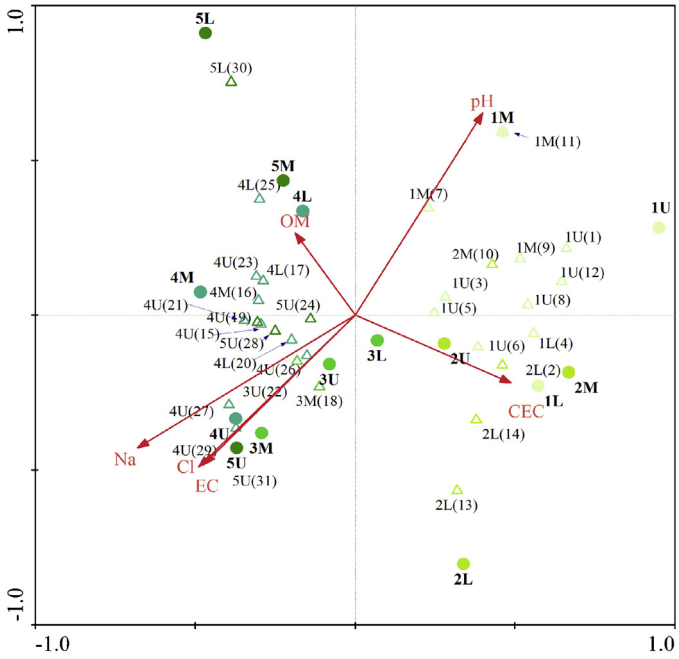

Fig. 8. Canonical correspondence analysis (CCA) biplot of fungal community composition. Arrows represent environmental variables, triangles represent populations, and circles represent samples.

3.4. Correlations between abiotic and biotic factors

The distance among samples in different soil layers shortened with elevated de-icing salt, but became longer again at last (Fig. 8). At the same time, the populations represented by bands 1–14 were distributed in samples 1U, 1M, and 1L and samples 2U, 2M, and 2L, which indicated that those populations are relatively enriched in the samples mentioned above. The populations represented by bands 15–31 were distributed closely within samples 3U, 3M, 3L, 4U, 4M, 4L, 5U, 5M, and 5L. Also, Na^+, Cl^-, EC, and pH had greater effects on the fungal populations than OM and CEC (Fig. 8). In addition, there was a strong correlation among these three factors through small angles between arrows representing Na^+, Cl^-, and EC. Among the top four contributing factors, Na^+, Cl^-, and EC were negatively correlated with the populations of samples 1U, 1M, 1L, 2U, and 2M, though not 2L. However, they were positively correlated with the abundance of populations of samples 3U, 3M, 3L, 4U, 4M, 4L, and 5U, though not 5L and 5M. The pH was positively correlated with the first, second and fifth samples with the exceptions of 2L and 5U, but was negatively correlated with third and fourth samples as well as 5U.

The distributions of samples in different soil layers became more concentrated over time (Fig. 9). The distributions of bacterial populations were similar to those of fungal populations. The populations numbered from 1 to 14 plus 35 were dominant in the first and second samples, and the populations numbered from 15 to 34 were dominant in the samples numbered 3U, 3M, 3L, 4U, 4M, 4L, 5U, 5M, and 5L. However, the third samples still included many of the common populations seen in the second samples; for example, populations 6, 21, 14, 35, and 13. In addition, all of the environmental factors investigated in this study had effects on the microbial populations, and each of the factors made similar contributions since the arrows representing these environmental factors had similar lengths. A strong correlation among Na^+, Cl^-, and EC also existed, as it did in the case of fungi, but no obvious correlations were seen between the other three factors. Moreover, Na^+, Cl^-, and EC were negatively correlated with the populations in samples 1U, 1M, 1L, 2U, 2M, 2L, but were positively correlated with the populations in samples 4U, 4M, 4L, 5U, 5M, and 5L. However, Na^+, Cl^-, and EC had almost no influence on samples 3U, 3M, and 3L. CEC was negatively correlated with the populations of samples 4U, 4M, 4L, 5U, 5M, and 5L, but was positively correlated with the populations of samples 1L, 1M, and 1U. pH was positively correlated with the populations of samples 1U, 1M, 1L, 5M, and 5L, but negatively correlated with the populations of samples 4U, 4M, 4L, and 5U. The

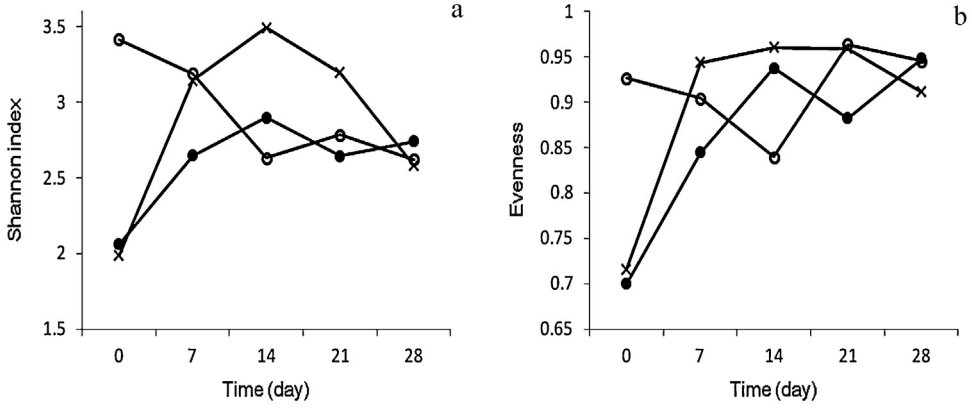

Fig. 7. Shannon diversity index (a) and evenness index (b) of bacteria in three soil layers at different sampling times. "—○—", upper soil layer; "—×—", middle soil layer; "—●—", lower soil layer.

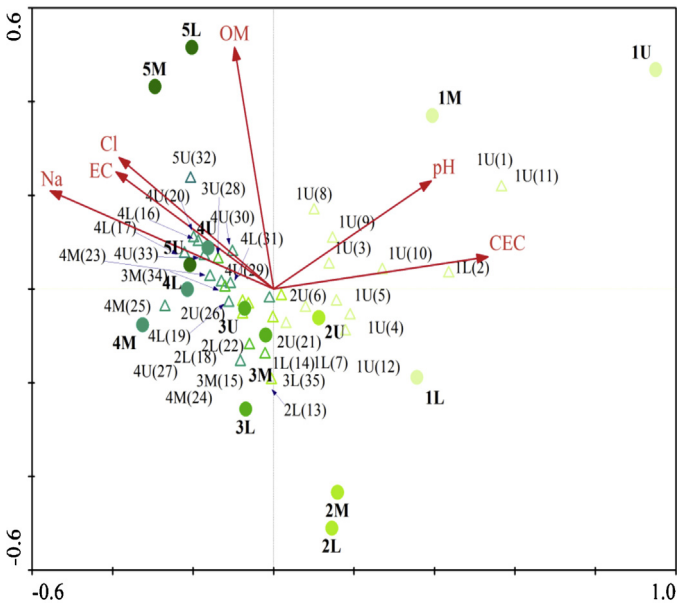

Fig. 9. Canonical correspondence analysis (CCA) biplot of bacterial community composition. Arrows represent environmental variables, triangles represent populations, and circles represent samples.

effects of pH on samples 2U, 2M, 2L, 3U, 3M, and 3L were not obvious, but very small positive effects on samples 2U, 2M, and 2L and very small negative effects on samples 3U, 3M, and 3L could be seen.

4. Discussion

4.1. Influences on abiotic soil factors

De-icing salt leads to soil salinization, as observed by Cunningham et al. (2008), increasing Na^+ and Cl^- concentration and elevating EC. However, the degree of salinity in different soil layers has not been investigated. From our results (Fig. 1), we concluded that de-icing salt produced stronger impacts on the upper soil layers in a month. This partly was because of no rain in Beijing during the trial period which led to slower osmosis. On the other hand, there was stronger ion exchange adsorption in the upper soil due to abundant negatively charged OM (Lavahun et al., 1996), which promoted the adsorption of Na^+. Moreover the accumulation of Na^+ can decrease the soil permeability (Ramakrishna and Viraraghavan, 2005). We therefore summarized that these discriminating influences are related to soil properties and rainfall. The CEC values decreased because Na^+ decreased the soil permeability (Ramakrishna and Viraraghavan, 2005) and occupied the ion exchange sites. Salinity stress alters the composition and activity of microbial populations, which leads to a decrease in soil organic carbon mineralization (Gros et al., 2003; Ghollarata and Raiesi, 2007; McCormick and Wolf, 1980; Sarig et al., 1993; Setia et al., 2010; Setia et al., 2011; Tripathi et al., 2007). On the other hand, salinity alters the amount of plant material entering the soil. In this study, OM content in the upper soil layer had only a small fluctuation, presumably due mainly to lower decomposition rates counteracting the effect of poor plant growth. Rietz and Haynes (2003) drew a similar conclusion. As for the middle and lower soil layers, plant production had little influence on OM in the short term, since the root depth of the plants was only about 10 cm. The OM content decreased in early stages mainly due to little influence on microbial decomposition at this phase, but increased in later stages because the microorganisms were disturbed by the de-icing salt. This disturbance was confirmed by the decreases in fungal and bacterial diversity (Fig. 4). Wichern et al. (2006) found that the amount of recovered OM increased with increasing salinity, exhibiting reduced decomposition of substrate, which more completely confirmed our conclusion. The alteration of pH in upper and middle soil layers was in contrast to the findings of Černohlávková1 et al. (2008), who found that salinity led to increased pH. This may reflect the complexity of factors influencing pH; the fact that pH is not determined simply by individual environmental factors such as degree of salinity and relative ion concentration. Rousk et al. (2011) also observed that pH value is not significantly different in soils with different degrees of salinization.

4.2. Influences on soil microorganisms

The narrowing distribution of the DGGE bands (Figs. 2 and 5) indicates that fewer microorganisms can survive in the contaminated soil. In previous research results, high salinity and sodicity resulted in a smaller and more stressed microbial community (Rietz and Haynes, 2003; Yuan et al., 2007), which is in accord with our result. In addition, compounds added to the de-icing salt have biotoxicity to microorganisms. The more dominant populations of microorganisms in the later stages (Figs. 2 and 5) illustrated that salinity weakened the competitiveness of surviving populations and promoted their common development. As the levels of de-icing salt increased, the similarity of fungal populations was observed to be up among three soil layers. It was the same for the bacterial populations. Fungal similarity increased from 32% to 75% (Fig. 3) and bacterial similarity increased from 35% to 83% (Fig. 6), indicating an obvious change in the microbial populations' structure. A change in similarity >18% indicates a significant change in community structure (Duineveld et al., 1998). Cluster analysis of the fungal and bacterial DGGE profiles illustrated that the samples from later periods had a higher level of similarity, suggesting that the microbial community had fewer changes in later periods than in early days. This indirectly illustrated that the microbial populations were moving into a new dynamic equilibrium. Van Bruggen and Semenov (2000) also found that a microbial community will achieve a new dynamic equilibrium in response to long-term stress.

De-icing salt resulted in a decrease in fungal diversity in the three soil layers and a decrease in bacterial diversity in the upper soil layer. Van Bruggen and Semenov (2000) came to similar conclusions; long-term stress resulted in decreasing fungal diversity. Crecchio et al. (2004) found lower ATP production and microbial metabolic activities in saline soils and postulated that the reason for this was a reduction of microbial populations, which supports our conclusion from another perspective. However, it is worth noting that, in our study, the variation in fungal and bacterial diversity was different in middle and lower soil layers. This may reflect the greater sensitivity of fungi to salinity compared with bacteria, which was also reported by Sardinha et al. (2003), so fungal diversity decreased in response to a lower level of salinization. On the other hand, this suggested that Na^+ can be used as a nutrient substance for bacteria when the concentration does not exceed a certain limit, but that the high Na^+ concentration would produce stress and high osmotic pressure, which is harmful to microorganisms. Furthermore, the decrease in fungal populations' evenness indicated that the fungal community structure was influenced greatly by de-icing salt. As for the bacterial community evenness, because the declines in sensitive populations decreased, competitiveness in the surviving populations and abundance of surviving populations increased. Along with this, the uniformity of the distribution increased.

4.3. Ecological effects on soil of the chloride de-icing salt

Since de-icing salt produces stress on the soil microbial populations, the populations that cannot adapt to this environmental change would be expected to decrease or die. In response, the structure of the surviving microbial community will become relatively simple and the diversity of the microbial community relatively low, which will lead to an increase in the similarity of microbial populations in different soil layers. Furthermore, the distributions of microbial populations have all became concentrated (Figs. 8 and 9), and we inferred that the surviving microorganisms should be more tolerant to the stress and have similar biological properties. From Fig. 8, we inferred that the de-icing salt did not significantly affect the fungal populations before 14 days, since the first two samples contained highly similar populations. In contrast to the fungal populations, the strong influences on bacterial populations were took place after 21 days, for the third samples (Day 21) still shared many common populations with the second samples (Fig. 9). UPGMA cluster analysis provided similar results; the first and second samples belonged to a large clade in the fungal cluster map (Fig. 3), while in the bacterial cluster map, the first three samples belonged to a large clade (Fig. 6). Therefore, we concluded that the stability of bacterial populations was stronger than that of fungal populations. Many studies (Sardinha et al., 2003; Pankhurst et al., 2001; Badran, 1994) have reported that fungi are less tolerant of salinity than bacteria, which explains these results to a certain extent. On the other hand, the bacteria were more diverse than the fungi, so the complexity of the bacterial populations was relatively higher. Complexity is positively correlated with stability, so bacterial populations are more stable than fungal populations.

From CCA analysis, we found that EC, Na^+, and Cl^- were three main factors influencing microbial populations. The toxicities of Na^+ and Cl^- inhibited microbial growth (Zahran, 1997). Gryndler et al. (2008) found Cl^- concentration would affect indigenous microorganism. In addition, Rietz and Haynes (2003) also concluded that EC was significantly negatively correlated with microbial biomass. It was worth noting that the three factors mentioned above were positively correlated with the first two samples except for fungal sample 2L, but were negatively correlated with the last two samples, with the exception of fungal samples 5L and 5M (Figs. 8 and 9). This indicated that microbial populations changed to a salt-tolerant one in response to the presence of the de-icing salt. Most native-born soil microbial populations are adapted to non-saline habitats initially, so the amount of sensitive microorganisms decreased along with increasing de-icing salt. Then, increasing EC, Na^+, and Cl^- abated the pressure of competition of the surviving salt-tolerant microorganisms, which stimulated their growth. For the few exceptions above, this may be explained as follows: the fungal populations in the lower soil layer were sensitive to the stress, and populations less tolerant to stress decreased in the earlier stages of the study (the previous 14 days during the study), so the surviving fungal populations were more tolerant to the stress. As for the fungal populations in middle and lower soil layers, at 28 days, the degree of salinity may exceed the levels that fungi can bear.

5. Conclusions

The results of this study indicated that de-icing salt caused different influences on soil properties in different soil layers, with a stronger effect of de-icing salt on the upper soil layer in one month. These different influences were related to soil properties and rainfall. De-icing salt changed original microbial populations and led to a decrease in diversity of microorganisms. At the same time, de-icing salt also influenced the evenness of the microbial community. These dynamic changes of microbial populations were related to the soil properties (EC, CEC, Na^+ and Cl^-). In response to environmental stress, microbial populations were obtaining a new dynamic equilibrium to adapt to the new environment. Furthermore, bacterial populations seem to be more stable than fungal populations under salinity stress. The obtained results suggest that the use of chemical agents leading to soil salinization, such as de-icing salt, can destroy the micro-ecosystem of bulk soil surrounding plant rhizosphere and influence soil health.

Acknowledgements

The authors thank Dr. Panyue Zhang and Dr. Chengming Tian for comments and criticisms on this manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apsoil.2013.06.003.

References

Agnelli, A., Ascher, J., Corti, G., Ceccherini, M.T., Nannipieri, P., Pietramellara, G., 2004. Distribution of microbial communities in a forest soil profile investigated by microbial biomass, soil respiration and DGGE of total and extracellular DNA. Soil Biol. Biochem. 36, 859–868.
Bäckström, M., Karlsson, S., Bäckman, L., Folkeson, L., Lind, B., 2004. Mobilisation of heavy metals by deicing salts in a roadside environment. Water Res. 38, 720–732.
Badran, R.A.M., 1994. Cellulolytic activity of some cellulose decomposing fungi in salinized soils. Acta Mycol. 29, 245–321.
Bowers, M.C., Hesterberg, J.H., 1976. Environmental implications of highway agents on white pine in Marquette County. Michigan Bot. 15, 75–89.
Černohlávková1, J., Hofman, J., Bartoš, T., Sáňka, M., Anděl, P., 2008. Effects of deicing salt on soil microorganisms. Plant Soil Environ. 54 (11), 479–485.
Crecchio, C., Gelsomino, A., Ambrosoil, R., Minati, J.M., Ruggiero, P., 2004. Functional and molecular responses of soil microbial communities under differing soil management practices. Soil Biol. Biochem. 36, 1873–1883.
Cunningham, M.A., Snyder, E., Yonkin, D., Ross, M., Elsen, T., 2008. Accumulation of deicing salt in soils in an urban environment. Urban Ecosyst. 11, 17–31.
Dao, X., Zhou, F., Guo, H.C., 2008. Driving forces analysis of transportation land use/land cover change in Beijing. Res. Environ. Sci. 21 (5), 207–213 (in Chinese).
Duineveld, B.M., Rosado, A., Van Elsas, s.J.A., 1998. Analysis of the dynamics of bacterial communities in the rhizosphere of the chrysanthemum via denaturing gel electrophoresis and substrate utilization patterns. Appl. Environ. Microbiol. 64, 4950–4957.
Fostad, O., Pedersen, P.A., 2000. Container-grown tree seedling responses to sodium chloride applications in different substrates. Environ. Pollut. 109, 203–210.
Fritzsche, C.J., 1992. Calcium magnesium acetate deicer. Water Environ. Technol. 4, 44–51.
Galuszka, A., Migaszewski, Z.M., Dolegowska, S., Michalik, A., 2011. The influence of chloride deicers on mineral nutrition and the health status of roadside trees in the city of Kielce, Poland. Environ. Monit. Assess. 176, 451–464.
Ghollarata, M., Raiesi, F., 2007. The adverse effects of soil salinization on the growth of *Trifolium alexandrinum* L. and associated microbial and biochemical properties in a soil from Iran. Soil Biol. Biochem. 39, 1699–1702.
Godwin, K.S., Gallagher, J.L., 2003. Long-term trends in sodium and chloride in the Mohawk River, New York: the effect of fifty years of road-salt application. Environ. Pollut. 124, 273–281.
Graves, W.R., Gallagher, J.L., 2003. Resistance to salinity of *Alnus maritima* from disjunct wetlands: symptoms of salt injury, comparison to other shrubs, and effect of inundation. Wetlands 23, 394–405.
Green, S.M., Machin, R., Cresser, M.S., 2008. Effect of long-term changes in soil chemistry induced by road salt applications on N-transformations in roadside soils. Environ. Pollut. 152, 20–31.
Gros, R., Poly, F., Jocteur Monrozier, L., Faivre, P., 2003. Plant and soil microbial community responses to solid waste leachates diffusion on grassland. Plant Soil 255, 445–455.
Gryndler, M., Rohlenová, J., Kopecký, J., Matucha, M., 2008. Chloride concentration affects soil microbial community. Chemosphere 71, 1401–1408.
Hans, M.H., 2011. Short-term effects of alternative deicing chemicals on tree sapling performance. Urban For. Urban Green. 10, 53–59.
Jackson, R.B., Jibbagy, E.G., 2005. From icy roads to salty streams. Proc. Natl. Acad. Sci. U.S.A. 102, 14487–71448.
Jonsson, T.H., 2006. Terminal bud failure of black cottonwood (*Populus trichocarpa*) exposed to salt-laden winter storms. Tree Physiol. 26, 905–914.
Kayama, M., Quoreshi, A.M., Kitaoka, S., Kitahashi, Y., Sakamoto, Y., Maruyama, Y., Kitao, M., Koike, T., 2003. Effects of deicing salt on the vitality and health of

two spruce species, *Picea abies* Karst and *Picea glehnii* Masters planted along roadsides in northern Japan. Environ. Pollut. 124, 127–137.
Lavahun, M.F.E., Joergensen, R.G., Meyer, B., 1996. Activity and biomass of soil microorganisms at different depths. Biol. Fert. Soils 23, 38–42.
Li, Y.M., Chaney, R.L., Schneiter, A.A., 1994. Effect of soil chloride level on cadmium concentration in sunflower kernels. Plant Soil 167, 275–280.
Li, Z.Y., Zhou, J.H., Li, Y.M., 2012. Lethal dose–effect correlations of chloride snow melting agent on *Euonymus japonicus*. J. Beijing For. Univ. 34, 64–69 (in Chinese).
Lu, R.K., 107–108 2000. Analytical Methods for Soil and Agro-Chemistry. China Agricultural Science and Technology Press, Beijing, pp. 27–28 (in Chinese).
May, L.A., Smiley, B., Schmidt, M.G., 2001. Comparative denaturing gradient gel electrophoresis analysis of fungal communities associated with whole plant corn silage. Can. J. Microbiol. 47, 829–841.
McCormick, R., Wolf, D., 1980. Effect of sodium chloride on CO_2 evolution, ammonification, and nitrification in a sassafras sandy loam. Soil Biol. Biochem. 12, 153–157.
Moulin, L., Munive, A., Dreyfus, B., Boivin Masson, C., 2001. Nodulation of legumes by members of the β-subclass of proteobacteria. Nature 411, 948–950.
Munns, R., Tester, M., 2008. Mechanisms of salinity tolerance. Annu. Rev. Plant Biol. 59, 651–681.
Muyzer, G., de Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59, 670–695.
Norrström, A.C., Jacks, G., 1998. Concentration and fractionation of heavy metals in roadside soils receiving de-icing salts. Sci. Total Environ. 218, 161–174.
Norrstrom, A.C., 2005. Metal mobility by deicing salt from an infiltration trench for highway runoff. Appl. Geochem. 20, 1907–1919.
Paludan Müller, G., Saxe, H., Pedersen, L.B., Randrup, T.B., 2002. Differences in salt sensitivity of four deciduous tree species to soil or airborne salt. Physiol. Plant. 114, 223–230.
Pankhurst, C.E., Yu, S., Hawke, B.G., Harch, B.D., 2001. Capacity of fatty acid profiles and substrate utilization patterns to describe differences in soil microbial communities associated with increased salinity or alkalinity at three locations of South Australia. Biol. Fert. Soils 33, 204–217.
Ramakrishna, D.M., Viraraghavan, T., 2005. Environmental impact of chemical deicers – a review. Water Air Soil Pollut. 166, 49–63.
Rasa, K., Peltovuori, T., Hartikainen, H., 2006. Effects of de-icing chemicals sodium chloride and potassium formate on cadmium solubility in a coarse mineral soil. Sci. Total Environ. 366, 819–825.
Rietz, D.N., Haynes, R.J., 2003. Effects of irrigation-induced salinity and sodicity on soil microbial activity. Soil Biol. Biochem. 35, 845–854.
Rousk, J., Elyaagubi, F.K., Jones, D.L., Godbold, D.L., 2011. Bacterial salt tolerance is unrelated to soil salinity across an arid agroecosystem salinity gradient. Soil Biol. Biochem. 43, 1881–1887.
Sardinha, M., Müller, T., Schmeisky, H., Joergensen, R.G., 2003. Microbial performance in soils along a salinity gradient under acidic conditions. Appl. Soil Ecol. 23, 237–244.
Sarig, S., Roberson, E., Firestone, M., 1993. Microbial activity–soil structure: response to saline water irrigation. Soil Biol. Biochem. 25, 693–697.
Setia, R., Marschner, P., Baldock, J., Chittleborough, D., 2010. Is CO_2 evolution in saline soils affected by an osmotic effect and calcium carbonate? Biol. Fert. Soils 46, 781–792.
Setia, R., Marschner, P., Baldock, J., Chittleborough, D., Verma, V., 2011. Relationships between carbon dioxide emission and soil properties in salt-affected landscapes. Soil Biol. Biochem. 43, 667–674.
Schloter, M., Dilly, O., Munch, J.C., 2003. Indicators for evaluating soil quality. Agric. Ecosyst. Environ. 98, 255–262.
Tabatabai, M.A., Dick, W.A., 1983. Simultaneous determination of nitrate, chloride, sulfate, and phosphate in natural waters by ion chromatography. J. Environ. Qual. 12, 209–213.
Tripathi, S., Chakraborty, A., Chakrabarti, K., Bandyopadhyay, B., 2007. Enzyme activities and microbial biomass in coastal soils of India. Soil Biol. Biochem. 39, 2840–2848.
Van Bruggen, A.H.C., Semenov, A.M., 2000. In search of biological indicators for soil health and disease suppression. Appl. Soil Ecol. 15, 13–24.
Viskari, E.L., Kärenlampi, L., 2000. Roadside Scots pine as an indicator of deicing salt use–a comparative study from two consecutive winters. Water Air Soil Pollut. 122, 405–419.
Wang, Y.C., Bai, X.W., Li, F., 2011. Effect of snow melt agent containing chloride-salt on soil chemical characters of urban road greenbelt. Environ. Sci. Technol. 34 (11), 59–63 (In Chinese).
Wen, J., Guo, K., Chen, Q., Zhang, L., Qian, W.H., Ren, F., 2010. Spreading 30,000 tons of deicing salt and over the sum of the past two years. Beijing Times, January 4 2010(4) (in Chinese).
Wichern, J., Wichern, F., Joergensen, R.G., 2006. Impact of salinity on soil microbial communities and the decomposition of maize in acidic soils. Geoderma 137, 100–108.
Yao, Y.L., Lu, Z.M., Min, H., Gao, H.C., Zhu, F.X., 2012. The effect of tetrahydrofuran on the enzymatic activity and microbial community in activated sludge from a sequencing batch reactor. Ecotoxicology 21, 56–65.
Yuan, B.C., Li, Z.Z., Liu, H., Gao, m., Zhang, Y.Y., 2007. Microbial biomass and activity in salt affected soils under arid conditions. Appl. Soil Ecol. 35, 319–328.
Zahran, H.H., 1997. Diversity, adaptation and activity of the bacterial flora in saline environments. Biol. Fert. Soils 25, 211–223.
Zhou, Z.F., Zheng, Y.M., Shen, J.P., Zhang, L.M., He, J.Z., 2011. Response of denitrification genes *nir*S, nirk, and *nos*Z to irrigation water quality in a Chinese agricultural soil. Environ. Sci. Pollut. Res. 18, 1644–1652.