Effects of 1-octyl-3-methylimidazolium nitrate on the microbes in brown soil

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ABSTRACT

The toxicity of ionic liquids (ILs) on soil organisms has aroused wide attention due to their high-solubility. The present investigation focused on the toxicity of 1-octyl-3-methylimidazolium nitrate ($[C_8mim]NO_3$) on the microbial populations (bacteria, fungi, and actinomycetes), soil enzyme (urease, dehydrogenase, acid phosphatase, and β-glucosidase) activities, microbial community diversity using terminal restriction fragment length polymorphism (T-RFLP), and abundance of the ammonia monooxygenase (amoA) genes of ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) using quantitative real-time polymerase chain reaction (q-PCR) in brown soil at each trial with doses of 0, 1.0, 5.0, and 10.0 mg/kg on days 10, 20, 30, and 40. The contents of $[C_8mim]NO_3$ in soil were measured using high performance liquid chromatography with recoveries of 84.3% to 85.2%, and changed less than 10% during the experimental period. A significant decrease was observed from the bacteria, fungi and actinomycetes populations at 10.0 mg/kg, at which the urease activity was inhibited and the β-glucosidase activity was stimulated on days 20, 30, and 40. In addition, $[C_8mim]NO_3$ inhibited the dehydrogenase activity at 10 mg/kg on days 30 and 40 and the acid phosphatase activity on day 20. The diversity of the soil microbial community and the gene abundance of AOA- and AOB- amoA were also inhibited. Furthermore, the present investigation provided more scientific information for the toxicity evaluation of ILs in soil.

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Introduction

For the past few years, ionic liquids (ILs) have been considered "green solvents" due to their low volatility and wide application (Chen et al., 2016; Guo et al., 2016; Liu et al., 2016a; Panda and Gardas, 2015), while the toxicity of ILs on aquatic and soil organisms has aroused wide attention due to high-solubility and good stability. Some previous investigations have been conducted on the toxicity of ILs to aquatic organisms (Du et al., 2012, 2014; Mu et al., 2009; Zhang et al., 2017a, 2017b), earthworms (Guo et al., 2016) and plants (Liu et al., 2016a), while little data is available on the toxicity of ILs to soil microbes. Therefore, the present investigation about the IL on the microbial flora in brown soil is significant.

1-Octyl-3-methylimidazolium nitrate ($[C_8mim]NO_3$) was chosen as the tested IL for the present study. The toxicity of

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the investigated IL on *Chlorella vulgaris* and *Daphnia magna* was studied by Zhang et al. (2017b). The toxicity of the investigated IL on zebrafish (*Danio rerio*) was studied by Zhang et al. (2017a) and on earthworms (*Eisenia foetida*) by Shao et al. (2017). The toxicity of the ILs 1-octyl-3-methylimidazolium chlorine ($[C_8mim]Cl$) and 1-octyl-3-methylimidazolium tetrafluoroborate ($[C_8mim]BF_4$) on soil microbes was studied by Guo et al. (2015b) and Sun et al. (2017), respectively.

As important participants in material circulation in soil and bioprocesses, soil microbes were considered important biological indicators for evaluating soil environmental quality and were studied. Thus, the effects of ILs on soil environmental quality can be reflected by the toxic effects of ILs on soil microbes (Sun et al., 2017).

The present investigation studied the effects of IL $[C_8mim]NO_3$ on the microbial populations (bacteria, fungi, and actinomycetes), soil enzyme (urease, dehydrogenase, acid phosphatase, and β-glucosidase) activities, microbial community diversity using terminal restriction fragment length polymorphism (T-RFLP), and abundance of ammonia-oxidizing archaea (AOA)- and ammonia-oxidizing bacteria (AOB)-amoA genes using q-PCR in brown soil, which was beneficial for further research on the toxicity of ILs to soil ecosystems.

1. Materials and methods

1.1. Soil and chemicals

The tested soil, brown soil, was gathered from 0 to 15 cm of topsoil from the experimental farm of Shandong Agricultural University (Taian, China). A sieve of 2-mm mesh was used to separate the plant roots and bulky grain from the soil samples that were stored in the refrigerator (Siemens BCD-610 W, Bossy China Home Appliance Co., Ltd., China) at 4°C after drying under room temperature. Sixty percent of the maximum water-holding capacity (WHC) was used to incubate the soil samples for 7 days prior to the experiment at 25°C (Guo et al., 2015b). Table 1 lists the physical–chemical properties of the tested soil in the present study. According to the study of Zhang et al. (2014), the tested soil was considered a typical sandy loam soil using the United States Department of Agriculture (USDA) Soil Taxonomy.

The Chengjie Chemical Co. Ltd. (Shanghai, China) provided the ionic liquid 1-octyl-3-methylimidazolium nitrate ($[C_8mim]NO_3$, 99% purity, CAS No. 203389-27-9). Acetonitrile of chromatographic grade, used to determine the contents of the IL $[C_8mim]NO_3$, was obtained from Tianjin Kaitong Chemical Reagent Co., Ltd. (Tianjin, China). The other chemicals and reagents, all of analytical-grade, were purchased from Solarbio Science &Technology Company (Beijing, China) and Shanghai Sangon Biological Engineering Technology and Service Co., Ltd. (Shanghai, China).

1.2. Application of the ionic liquid $[C_8mim]NO_3$ to soils

The toxicant-exposure was carried as Sun et al. (2017) described that listed below. The three IL treatment-groups with the doses of 1.0, 5.0, and 10.0 mg $[C_8mim]NO_3$ per kg soil and the control group using deionized water were applied in the present study. A standard reserve solution of 10.0 g/L was weighed precisely with approximately 1.0 g of IL $[C_8mim]NO_3$using a sensitive balance (BS-210S, Sartorius Industrial Weighing Equipment (Beijing) Co., Ltd., China) in a 5 mL beaker, dissolved and transferred to a 100 mL volumetric flask, which was filled to the mark with deionized water. Next, the standard reserve solution was diluted to the operating fluids of 0.1, 0.5, and 1.0 g/L. In the treatment groups, 0.5 mL of operating fluids was blended uniformly with 50 g of soil samples while 0.5 mL of deionized water was blended uniformly with 50 g of soil samples. Then, the aforementioned soil samples were transferred into the 125 mL brown glass bottles, which were plugged with a tampon. Triplicates were applied to each trial. Each iteration consisted of four brown glass bottles corresponding to four experimental periods. Afterwards, the bottles were weighed and recorded. Then, the bottles were cultured in a constant temperature incubator (HPG-280, Harbin Donglian Electronic Technology Development Co., LTD, China) in the dark at 25°C. The deionized water was added to stop for a water break every two days to maintain the content of water in the soil samples at 60% of the maximum WHC. The samples were determined on days 10, 20, 30, and 40 after initiation of the IL application.

1.3. Content determination of the IL $[C_8mim]NO_3$

Referring to the method of Nichthauser et al. (2009), IL $[C_8mim]NO_3$ was extracted from the tested soil samples. For extraction, the saturated ammonium chloride and methanol were blended at a ratio of 1:9 (*V/V*), in which ethylenediaminetetraacetic acid disodium salt (Na_2EDTA) was added to 1 g/L. Soil samples of 4.0 g from each IL exposure and control group were weighed and extracted using 10.0 mL of the extraction, after which the samples were vortexed for half 1 min, ultrasound extracted for 50 min using an ultrasonic cleaner (KQ-500DB, Kunshan Ultrasonic Instruments Co., Ltd., China), and shaken at 160 revolutions per min (r/min) at 37°C for 1 hr using a thermostat oscillator (THZ-C, Suzhou Peiying Laboratory Equipment Co., Ltd., China). The supernate was transferred into centrifugal tubes that were centrifuged at 4000 r/min using a high-speed freezing centrifuge (Centrifuge

Table 1 – The physical–chemical properties of the tested soil.									
Soil type	pH	TOC (g/kg)	Available N (mg/kg)	Available P (mg/kg)	Available K (mg/kg)	Clay ($<2 \times 10^{-3}$ mm, %)	Sand ($>5 \times 10^{-2}$ mm, %)	Silt (2×10^{-3}–5×10^{-2} mm, %)	WHC (%)
Sandy loam	6.93	13.91	93.41	35.24	73.12	14.62	59.26	26.12	18.51

TOC: total organic carbon; N: nitrogen; P: phosphorus; K: potassium; WHC: water holding capacity.

5804, Eppendorf China Ltd., China) for 10 min. Next, the obtained supernate was filtered using the syringe filters (0.22 μm) and was poured into sample bottles to determine the contents of IL [C_8mim]NO_3.

Referring to the method of Zhang et al. (2017a), the contents of IL [C_8mim]NO_3 were determined using a high performance liquid chromatography (HPLC, Agilent 1260, Agilent Technologies Inc., USA). The determination conditions for the HPLC were listed below. The triethylamine (5.0 mL) was added to 1000 mL volumetric flask, and ultrapure water was added to the mark. Potassium dihydrogen phosphate (KH_2PO_4, 3.4 g) was weighed precisely and dissolved with the 1000 mL of triethylamine solution. Next, phosphoric acid (H_3PO_4) was used to adjust the pH of the solution to 3.0. Afterwards, a phosphate buffer (KH_2PO_4/H_3PO_4, 25 mmol/L) and ultrapure water were considered mobile phases at a ratio of 70:30 (V/V) after filtration and supersonic degassing. The samples were separated using the C_{18} column (Eclipse XDB-C_{18}, 4.6 mm × 250 mm, 5 μm) with the temperature at 30°C. An ultraviolet–visible (UV/VIS) variable wavelength detector (VWD G1314A, Agilent Technologies Inc., USA) was applied to analyze the sample at 212 nm with an injection volume of 10 μL and a flow rate of 0.8 mL/min. Triplicates were applied to each trial.

1.4. Microbial population determination

Referring to the method of Zhang et al. (2014), the populations of soil microbes (bacteria, fungi, and actinomycetes) were assessed using the dilution method of plate counting. Triplicates were applied to each trial. Considering the different needs of the soil microbial growth, there were three different kinds of culture mediums: Gauze's medium No. 1, potato dextrose agar (PDA, Difco) and Luria-Bertani (LB) medium (Guo et al., 2015a; Wang et al., 2015). Samples (10.0 g) from each control group and exposure group were transferred into the 250 mL of Erlenmeyer flasks holding 90 mL of sterile water. Next, the Erlenmeyer flasks were shaken for 20 min using the thermostat oscillator at 200 r/min. After standing for 5 min, the obtained liquid supernatant was considered 10^{-1} of the soil dilution. The 10^{-1} of the soil dilution was transferred into the 10 mL test tube holding 9 mL of sterile water. After 1 min vortex, the obtained solution was considered 10^{-2} of the soil dilution. By analogy, 10^{-4} of the soil dilution was obtained to coat plates. The Gauze's medium No. 1 and 10^{-4} of the soil dilution (0.1 mL) were applied for incubation of bacteria at 30°C for 5 days. The PDA and 10^{-1} of the soil dilution (0.1 mL) were applied for incubation of fungi at 30°C for 48 hr. The LB medium and 10^{-4} of the soil dilution (0.1 mL) were applied for incubation of bacteria at 30°C for 36 hr. The colony-forming unit (CFUs) per gram of dry soil was used as the unit of the populations of bacteria, fungi, and actinomycetes.

1.5. Determination of soil enzyme (urease, dehydrogenase, acid phosphatase, and β-glucosidase) activities

Referring to the methods as previously described (Sun et al., 2017), the determination of the four soil enzyme (urease, dehydrogenase, acid phosphatase, and β-glucosidase) activities was conducted using the spectrophotometry method on

days 10, 20, 30, and 40. Triplicates were applied to each trial. The specific methods were described below.

As for the assay of urease activity, samples (10 g) and methylbenzene (2 mL) were transferred into a 100 mL volumetric flask with a stopper. After standing for 15 min at room temperature, 10% urea (10 mL) and a citrate buffer (20 mL) with a pH of 6.7 were added to the volumetric flasks. After shaking well gently, the samples were cultivated for 6 hr at 37°C followed by adding deionized water (38°C) to the mark. After extracting the methylbenzene, the suspension was filtrated. The obtained filtrates (1 mL) and 19 mL of deionized water were transferred in to each volumetric flask (50 mL). Next, sodium phenate (4 mL) and sodium hypochlorite (3 mL) were added to the volumetric flasks (50 mL). After being shaken well gently and standing for 20 min, the solution was added with deionized water to the mark. Afterwards, the absorbance of the solution was measured using an ultraviolet–visible spectrophotometer (UV spectrophotometer, UV-2600, Shimadzu, Japan) at 578 nm within 1 hr. The microgram of NH_3-N per gram of dry soil per 24 hr was used as the unit for evaluating the activities of the urease.

As for the assay of dehydrogenase activity, samples (5 g) and 2, 3, 5-triphenyl tetrazolium chloride (TTC) solution (0.5% by weight, 5 mL) were transferred into the test tubes with a stopper. After being shaken well gently, the samples were cultivated for 24 hr at 30°C. Next, methanol (40 mL) and the solutions in the test tubes were added into Erlenmeyer flasks that were sealed using sealing film. Afterwards, the suspension was shaken at 220 r/min for 1 hr at 37°C and filtrated. The absorbance of obtained filtrates was measured using the UV spectrophotometer at 485 nm in contrast to methanol and calculated by the curve of triphenylformazan standards. The microgram of TPF per gram of dry soil per 24 hr period was used as the unit for evaluating the activities of the dehydrogenase.

As for the assay of acid phosphatase activity, samples (1 g) from each control group and exposure group were transferred into 50 mL Erlenmeyer flasks. Next, methylbenzene (0.2 mL), modified universal buffer (MUB) with pH of 6.5 (4 mL) and 0.05 mol/L 4-nitrophenyl phosphate disodium salt hexahydrate (1 mL) were added into Erlenmeyer flasks sequentially and were cultivated for 1 hr at 37°C. Afterwards, 0.5 mol/L $CaCl_2$ (1 mL) and 0.5 mol/L NaOH (4 mL) were added to the Erlenmeyer flasks. After being shaken well gently and filtrated, the filtrates of the release of p-nitrophenol phosphate (pNP) were diluted ten times using MUB and were then measured using the UV spectrophotometer at 410 nm and calculated by the curve of p-nitrophenol standards. The microgram of pNP per gram of dry soil per hour was used as the unit for evaluating the activities of acid phosphatase.

The methods to measure the activities of β-glucosidase were similar to those used for acid phosphatase except for the following. First, the chemical was changed from 4-nitrophenyl phosphate disodium salt hexahydrate to p-nitrophenyl-β-D-glucopyranoside (pNG). Second, the obtained filtrates were measured at 400 nm.

1.6. Extraction of total DNA in soil

The total DNA of soil was extracted using the E.Z.N.A.™ Soil DNA Kit (D5625-01, OMEGA, USA) for the analysis of T-RFLP and the quantitative real-time polymerase chain reaction

(q-PCR). According to the manufacturer's instructions, 0.5 g of soil samples were used for extraction. The concentrations obtained were measured using a nucleic acid concentration tester (NanoDrop 2000, Thermo Fisher Scientific Inc., USA), and the DNA selected for the future experiment was stored in the refrigerator (-20°C).

1.7. The analysis of the soil bacteria using T-RFLP

Using the methods described previously (Heuer et al., 1997), the common primers for soil bacteria 1492R (GGTTACCTT GTTACGACT) and 27-F (5′ labeled with 6-FAM, 27F-FAM: AGAGTTTGATCCTGGCTCAG) were selected for the fragment amplification of the 16s rRNA gene. The mixtures of amplification reaction used for the 16s rRNA gene were 5 μL of 10× PCR Buffer including Mg^{2+} (Solarbio Science &Technology Company, China), deoxyribonucleoside triphosphate (dNTP, 1 μL), each of the aforementioned PCR primers (2 μL), template DNA (1 μL), Taq polymerase (0.6 μL) and final distilled water was added to the final volume (50 μL). Triplicates were applied to each trial. The PCR reaction procedure for the 16s rRNA gene amplification was 95°C for 5 min, followed by 30 cycles of 94°C for 60 sec, 57°C for 90 sec and 72°C for 2 min. Next, 72°C for 10 min was applied to the final extension (Maul et al., 2014). Afterwards, a SanPrep® quick PCR purification kit (BBI Life Sciences Corporation, China) was used to purify the obtained PCR products to gain the 16s target fragment that was used for enzyme digestion. The enzyme reaction system for enzyme digestion was 26.5 μL of the targeted 16s fragment, 3.5 μL of 10× PCR Tango and 3 μL of *Msp I* enzymes (TaKaRa Bio Inc., Japan) at 37°C in the dark for 8 hr. The digested products were detected using a DNA analyzer (ABI 3730, BBI Life Sciences Corporation, China) (Yu et al., 2014). Peak Scanner Software (v1.0) was used to analyze the fragment sizes. Next, the terminal restriction fragments (T-RFs) ranging from 50 to 600 bp were selected for calculation as the total peak area (%) and were used for the analyses only if their relative abundances were greater than 1.0% (Chen et al., 2011). Each fragment represented a different bacterial flora. Thus, the analysis of the soil bacteria using T-RFLP can reflect the changes in soil microbial community structure (Schutte et al., 2008).

1.8. Gene expression abundance of AOA and AOB

The quantitative real-time polymerase chain reaction (q-PCR) was applied to assay the effects of IL [C$_8$mim]NO$_3$ on the expression quantity of the soil function genes (AOA- and AOB-amoA) according to the method of SYBR Green I using a Real-Time PCR instrument (StepOnePlus, Thermo Fisher Scientific Inc., USA). According to Chen et al. (2011), the PCR primers used for AOA-amoA genes were Arch-amoAF: STAATGGT CTGGCTTAGACG and Arch-amoAR: GCGGCCATCCATCTGTA TGT, and those used for AOB-amoA genes were amoA-1F: GGGGTTTCTACTGGTGGT and amoA-2R: CCCCTCKGSAAAGCC TTCTTC. The mixtures of amplification reaction used for AOA and AOB in PCR were 25 μL of 2 × PFU PCR MasterMix (Solarbio Science &Technology Company, China), each of the PCR primers (2 μL), template DNA (2.8 μL for AOA-AmoA, 3 μL for AOB-AmoA), and finally distilled water was added to reach the final volume

(50 μL). Triplicates were applied to each trial. The PCR reaction procedure for the amoA gene amplification of AOA was at 94°C for 5 min, followed by 30 cycles of 94°C for 45 sec, 54°C for 60 sec and 72°C for 60 sec. Next, 72°C for 15 min was applied to the final extension. Afterward, the melting curve was analyzed at 55°C to 95°C (0.5°C increments for 5 sec) to verify the specificity of gene amplification. The PCR reaction procedure for the amoA gene amplification of AOB was at 94°C for 1 min, followed by 30 cycles of 94°C for 60 sec, 60°C for 60 sec and 72°C for 60 sec. Next, 72°C for 10 min was applied to the final extension. Afterward, the melting curve was analyzed at 55°C to 95°C (0.5°C increments for 5 sec) to verify the specificity of gene amplification. Using the methods described previously (Sun et al., 2017), the standard curves for AOA and AOB were drawn.

1.9. Statistical analysis

The data obtained in the present study, except T-RFLP and RT-PCR, were processed by Microsoft Excel (Edition 2010, Microsoft Corp., USA) and analyzed by the software program called the Statistical Package for Social Sciences (SPSS, Standard Version 20.0, SPSS Inc., Chicago, Illinois, USA) *via* a one-way analysis of variance (ANOVA). The standard deviation (SD) was illustrated using an error bar. The significant differences among exposed-groups and controls were illustrated using the different letters above the columns at the $p < 0.05$ level via the least significant difference (LSD) test.

As for the analysis of T-RFLP, the software program Bio-Dap was applied to calculate the indexes of Shannon and evenness to assess the diversity of the microbial community structure according to the terminal restriction fragment. Using the method of Burke et al. (2011), the obtained data of T-RFLP was analyzed via a principal component analysis (PCA). For the analysis of T-RFLP, StepOne Software (v2.3, ABI, USA) was applied to analyze the Ct values, melting curve and standard curve, which were used to determine the gene copy numbers of each trial. Triplicates were applied to each result.

2. Results and discussion

2.1. Dynamic variation in the contents of the IL [C$_8$mim]NO$_3$

Fig. 1 shows the dynamic variation in the contents of the IL [C$_8$mim]NO$_3$ in the tested soil samples during the entire experimental periods. No IL [C$_8$mim]NO$_3$ was detected in the control groups. The recoveries of [C$_8$mim]NO$_3$ in the samples ranged from 93.2% to 114.5% and conformed with the standard of 80% to 120% (Zhang et al., 2017a). The concentrations of the tested IL on day 40 were lower than on day 0 with variations of 7.7%, 7.96%, and 4.83% at doses of 1.0, 5.0, and 10.0 mg/kg, respectively. Thus, the tested IL can be considered stable. Sun et al. (2017) observed similar results when they studied the toxicity of IL1-octyl-3-methylimidazolium tetrafluoroborate ([C$_8$mim]BF$_4$) on soil microbes. Liu et al. (2016b) and Guo et al. (2016) have also drawn similar conclusions when they studied the toxic effects of 1-octyl-3-methylimidazolium hexafluorophosphate ([C$_8$mim]PF$_6$) and 1-octyl-3-methylimidazolium chloride ([C$_8$mim]Cl) on earthworms (*Eisenia foetida*).
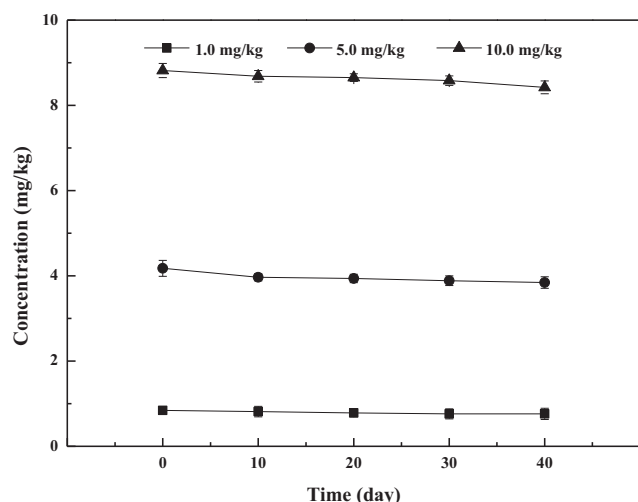
Fig. 1 – Dynamic variation in the contents of the IL [C$_8$mim]NO$_3$ in the tested soil samples during the entire experimental period. The standard deviation (SD) was illustrated using an error bar. However, the SD obtained was too low to be observed in the figure. [C$_8$mim]NO$_3$: 1-octyl-3-methylimidazolium nitrate.

2.2. Effects of IL [C$_8$mim]NO$_3$ on the populations of soil bacteria, fungi, and actinomycetes

The soil-microbial populations are correlated with the soil quality and soil conditions, and maintain the balance of the soil ecosystem and substance circulation in soil. Thus, the populations of soil microbes are considered an ideal index for evaluating the risks of soil pollution (Brookes, 1998; Zhang et al., 2014). The populations of bacteria are the highest and the populations of fungi are the lowest in the three soil microbes in the soil environment (Chen et al., 2012). The effects of IL [C$_8$mim]NO$_3$ on the populations of soil microbes are shown in Fig. 2.

As for the bacteria population shown in Fig. 2a, no notable increase or decrease was observed at the doses of 1.0 or 5.0 mg/kg compared to the control groups during the entire experimental periods, and a notable decrease was observed at the highest dose of 10.0 mg/kg compared to the control groups during the entire experimental periods. For the fungi population shown in Fig. 2b, a notable decrease was observed at the highest dose of 10.0 mg/kg compared to the control groups during the entire experimental period. In addition, a notable decrease was observed at the dose of 5.0 mg/kg on day 10 and at the doses of 1.0 or 5.0 mg/kg on day 30. For the populations of actinomycetes shown in Fig. 2c, a notable decrease was observed at the highest dose of 10.0 mg/kg compared to the control groups on days 10, 30, and 40. In addition, a notable decrease was observed at the dose of 1.0 mg/kg on day 10. Guo (2015) has drawn different conclusions with the present study when they studied the effect of the ILs 1-octyl-3-methylimidazolium bromide ([C$_8$mim]Br) and [C$_8$mim]Cl on soil microorganisms. The difference may be caused by different anions. Tejada et al. (2015) believed

that the cycle of nitrogen (N), phosphorus (P), and sulfur (S) affected the population of soil microorganisms. However, different soil types and soil physicochemical properties can lead to different nutrients and contents of organic matter in soil, which can cause obvious differences in soil microorganisms (Bending and Sonia, 2007). Thus, the effects of [C$_8$mim]NO$_3$ on soil-enzyme activities and the function gene abundance of soil microorganisms were scientifically assessed to evaluate the effects of the tested IL on soil microorganisms.

2.3. Effects of the IL [C$_8$mim]NO$_3$ on the activities of soil enzymes (urease, dehydrogenase, acid phosphatase, and β-glucosidase)

Soil enzymes, considered an important indicator for evaluating the soil quality, play significant roles in biochemical reactions (*e.g.*, the carbon and nitrogen cycles of the soil, the transmission of soil organic matter) in soil (Nayak et al., 2015; Parelho et al., 2016). Soil enzymes are also considered a significant indicator for evaluating soil productivity and soil biological activity. The effects of the IL [C$_8$mim]NO$_3$ on the activities of soil enzymes (urease, dehydrogenase, acid phosphatase, and β-glucosidase) are shown in Fig. 3.

The interferences of the tested IL on the four tested soil enzymes were not obvious. For the urease activity shown in Fig. 3a, no notable increase or decrease was observed at the doses of 1.0 or 5.0 mg/kg compared to the control groups during the entire experimental periods, and a notable decrease was observed at the highest dose of 10.0 mg/kg on day 20, 30, and 40. Sun et al. (2017) observed the IL [C$_8$mim]BF$_4$ induced the urease activity on day 10 and 20, and then inhibited the urease activity on days 30 and 40. The different results may be caused by the different anions. Sarathchandra et al. (1984) believed the activities of urease had a close relationship with the abundance of soil microbes. Guo et al. (2015b) have stated that the IL inhibited the diversity of soil microbes to inhibit the soil-urease activity, which was similar to our finding.

As for the dehydrogenase activity shown in Fig. 3b, no notable increase or decrease was observed in the IL treatment groups compared to the control groups on days 10 and 20, while a notable decrease was observed at the dose of 5.0 mg/kg on day 40 and the dose of 10.0 mg/kg on days 30 and 40. Zhang et al. (2014) believed the activities of dehydrogenase had a close relationship with the populations of soil microbes and the diversity of the microbial community structure. Our study showed that the tested IL inhibited both the soil microbial population and the diversity of the microbial community structure, whose trend is similar to the activities of dehydrogenase. Liwarska-Bizukojc (2011), who studied the effects of ILs on the dehydrogenase activity in activated sludge microorganisms, obtained similar results as ours, that the tested ILs inhibited the activities of dehydrogenase.

As for the acid phosphatase activity shown in Fig. 3c, a notable decrease was observed only at the dose of 5.0 mg/kg on day 30 and at the highest dose of 10.0 mg/kg on day 20 and 30. This finding may be because acid phosphatase is not sensitive to the soil pollutants. However, the acid phosphatase activity was closely related to the populations of soil microbes (Fernández-Calviño et al., 2010).
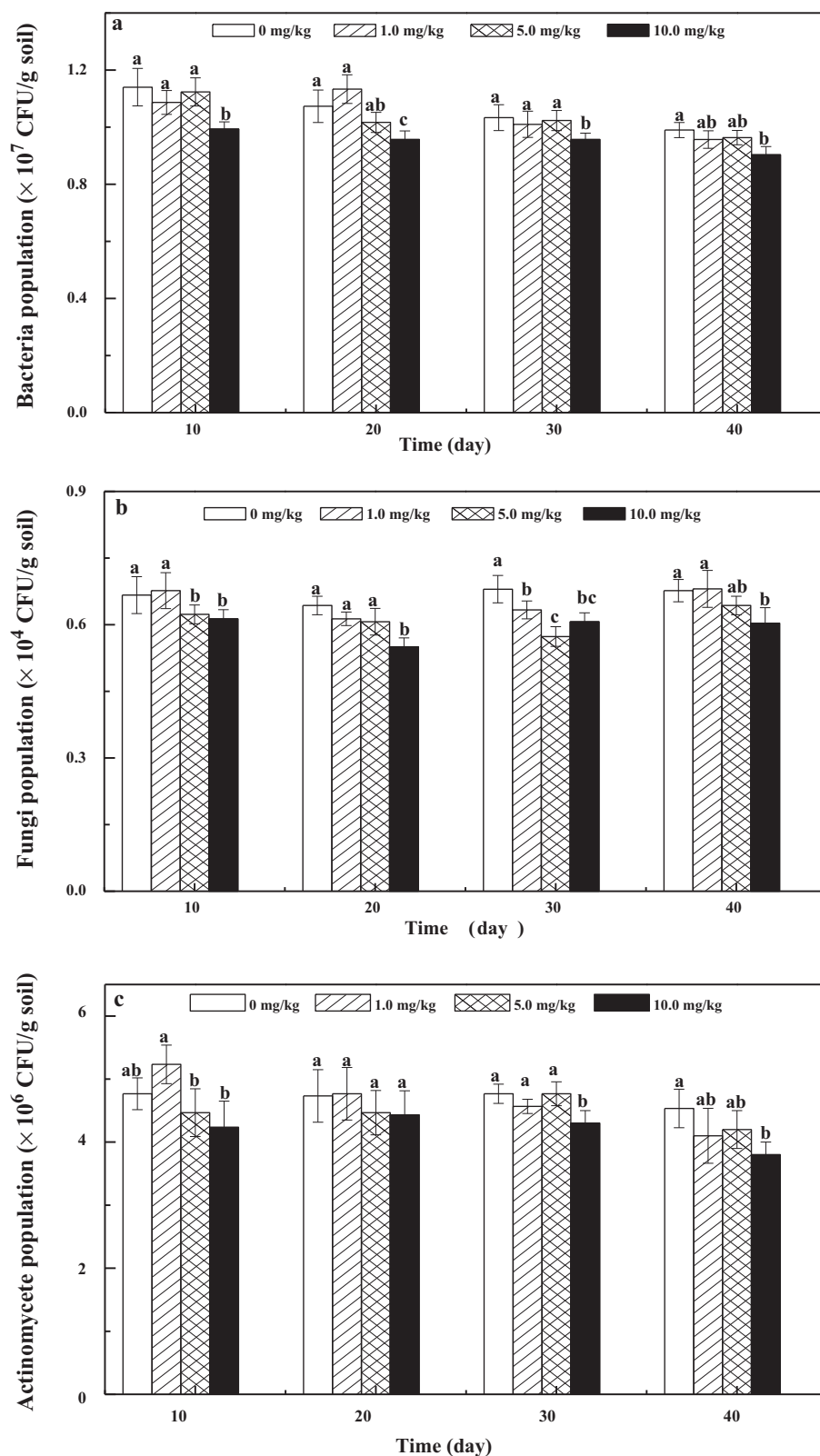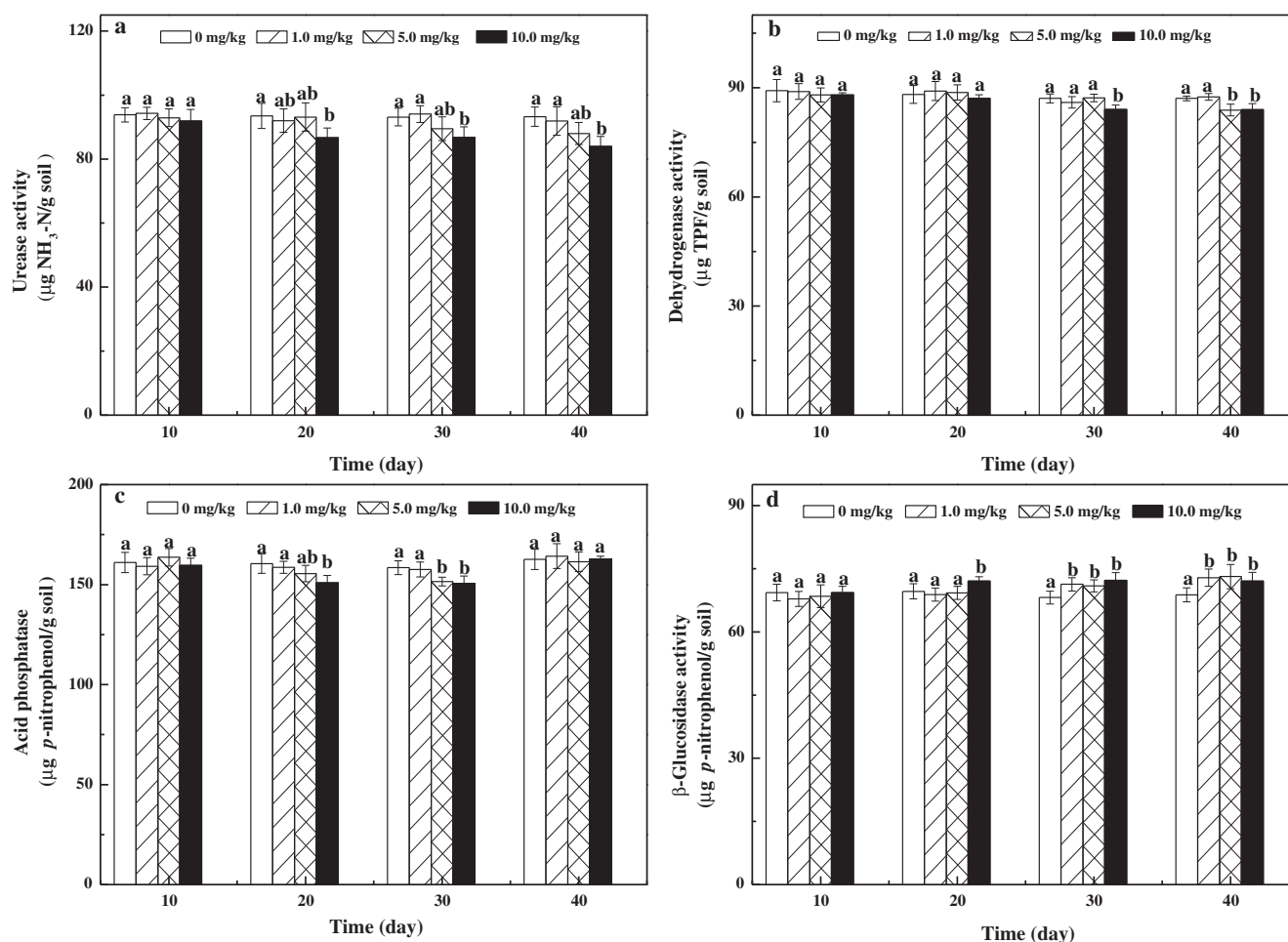
Fig. 2 – Effects of the IL [C$_8$mim]NO$_3$ on the populations of (a) soil bacteria, (b) fungi, and (c) actinomycetes. Each column is the average value of the triplicates. The standard deviation (SD) was illustrated using an error bar. The significant differences among exposed-groups and controls were illustrated using different letters above the columns at the $p < 0.05$ level via the least significant difference (LSD) test.

Fig. 3 – Effects of the IL [C₈mim]NO₃ on soil enzyme activity: (a) urease activity, (b) dehydrogenase activity, (c) acid phosphatase activity, and (d) β-glucosidase activity. Each column is the average value of the triplicates. The standard deviation (SD) was illustrated using an error bar. Significant differences among exposed-groups and controls were illustrated using different letters above the columns at the $p < 0.05$ level *via* the least significant difference (LSD) test.

As for the β-glucosidase activity shown in Fig. 3d, a notable increase was observed at the dose of 10.0 mg/kg on day 20 and at all the IL-treatment groups on days 30 and 40. The higher stimulation of β-glucosidase activity may be due to its association with the production of other extracellular enzymes (Cohen et al., 2005). The study of Li et al. (2013) showed that ILs could induce the activities of soil enzymes, which was similar to the present study.

2.4. Effects of IL [C₈mim]NO₃ on the diversity of soil microbial community structure

Terminal restriction fragment length polymorphism (T-RFLP) was applied to the status of the soil ecology. The effects of the IL [C₈mim]NO₃ on the relative abundance of soil microbial communities are shown in Fig. 4.

Five primary T-RFs including 66, 92, 148, 198, and 432 bp, which existed in each control- and treatment-group during the entire experimental period, were selected to analyze the changes of relative abundance of microbial communities in

soil. Thus, the bacterial flora represented by the five T-RFs may be considered the dominant bacterial community in IL-exposed soil. The relative abundance of bacterial flora represented by 92 bp was the highest (ranging from 20% to 54%) in the five T-RFs. At the 10th day after the IL was exposed to the soil samples, the relative abundance of 66 bp decreased compared to the control groups, especially at the dose of 5.0 mg/kg. The relative abundances of 198- and 432-bp decreased compared to the control groups, especially at the doses of 1.0 and 10.0 mg/kg. In addition, the relative abundances of 92- and 148-bp increased at each IL treatment group compared to the control groups. At the 20th day after the IL was exposed to the soil samples, the relative abundance of 92 bp decreased and that of 66- and 198-bp increased in each IL-treatment group compared to the control groups. In addition, the relative abundance of 148 bp dramatically decreased at the dose of 5.0 mg/kg compared to the control groups. No obvious change was observed from the relative abundance of 432 bp. At the 30th day after the IL was exposed to the soil samples, the relative abundance of 432 bp
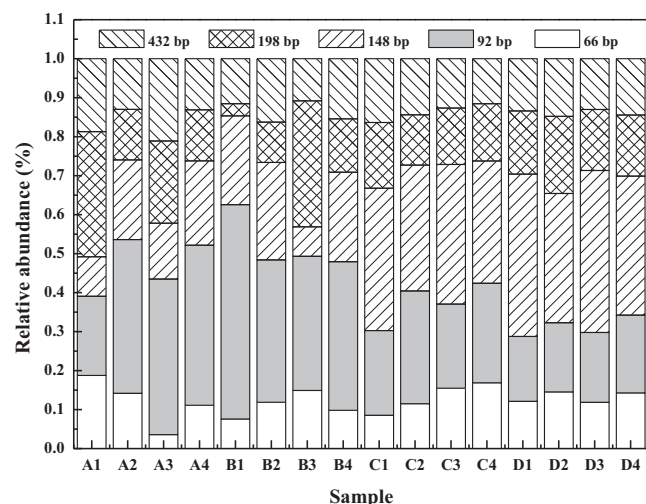
Fig. 4 – Effects of the IL [C₈mim]NO₃ on the relative abundance of soil microbial communities. A, B, C, and D indicate the incubation times of 10, 20, 30, and 40 days, respectively, and the concentrations of [C₈mim]NO₃ were 1, 0 mg/kg; 2, 1.0 mg/kg; 3, 5.0 mg/kg; and 4, 10.0 mg/kg.

decreased and that of 66 bp increased in each IL-treatment group compared to the control groups. The other T-RFs returned to the control levels. The relative abundance of all five T-RFs returned to the control levels on day 40. These results illustrated the tested IL affected the diversity of the soil microbial community structure. However, the microbial community structure returned to the control levels after a period of adaptation. While Sun et al. (2017) stated that the effects of the IL [C₈mim]BF₄ on the diversity of the microbial

community structures couldnot recover in a short period. This discrepancy may be caused by the different anions.

The principal component analysis (PCA) was performed on the T-RFLP profiles of the bacterial community in each control group and IL-treatment group is shown in Fig. 5. The total principal component was 58.7%, with a PC1 of 39.6% and a PC2 of 19.1%. Compared to the control groups at the 10th day after IL was exposed to the soil samples, the PC2 of each trial has a more notable difference than the PC1. Compared to the control-groups on day 20, the highest-dose (10.0 mg/kg) treatment groups scored lowest on both the PC1 and PC2, the dose of the 5.0 mg/kg IL treatments scored lowest on the PC1, and the IL-treatments with the dose of 1.0 mg/kg had no notable difference according to the PC1. Compared to the control groups according to both the PC1 and the PC2 on day 30, no notable difference was observed in the IL treatments with the doses of 1.0 and 5.0 mg/kg, while a notable difference was observed in the highest dose (10.0 mg/kg) of the IL treatments. These results may illustrate that IL-treatments with the doses of 1.0 and 5.0 mg/kg returned to the control levels with the extension of time, which was in accordance with those of T-RFs relative abundance. Compared to the control-groups on day 40, no notable difference was observed in any trial according to the PC1 and the PC2.

The Shannon index and the evenness index were used to illustrate the effects of the IL [C₈mim]NO₃ on the diversity and uniformity of the microbial community structure in soil and are shown in Table 2. Compared to the controls, a notable decrease was observed on days 30 and 40 and in the treatments of 10.0 mg/kg on days 20, 30, and 40. The results of the evenness index were similar to those of the Shannon index except that the evenness index of the treatments (10.0 mg/kg) was notably inhibited on day 10. The result illustrated that a high dose of ILs
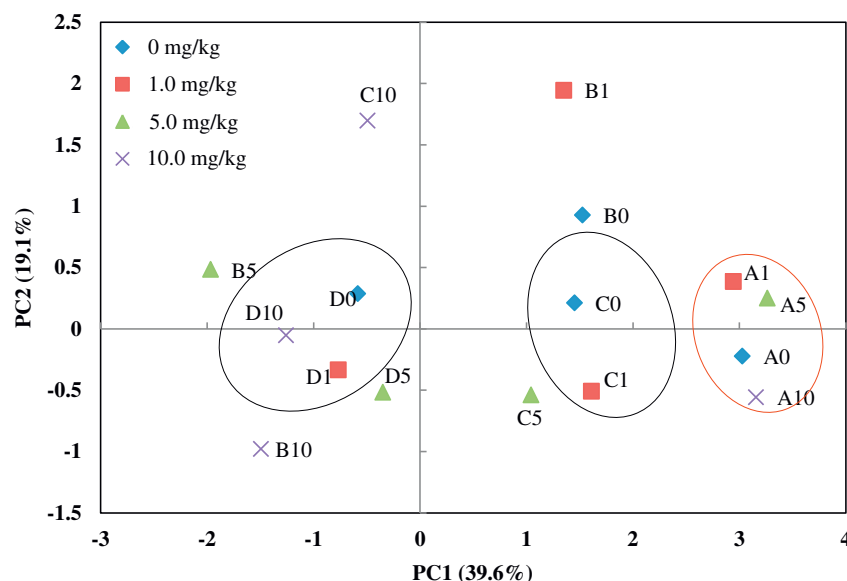
Fig. 5 – Principal component analysis (PCA) conducted on the T-RFLP profiles of the bacterial community in each control- and IL [C₈mim]NO₃ treatment- group. The letters A, B, C, and D represent different incubation times of 10, 20, 30, and 40 days, respectively. The concentrations of [C₈mim]NO₃ were 0, 0 mg/kg; 1, 1 mg/kg; 5, 5 mg/kg; and 10, 10 mg/kg. T-RFLP: terminal restriction fragment length polymorphism.

Table 2 – Effects of [C$_8$mim]NO$_3$ on Shannon index and Evenness index based on the T-RFLP data.

Concentration* (mg/kg)	Shannon index (H′)[a],*				Evenness index (E′)[b],*			
	Day 10	Day 20	Day 30	Day 40	Day 10	Day 20	Day 30	Day 40
0	1.21 ± 0.08A	1.22 ± 0.02A	1.20 ± 0.01A	1.19 ± 0.02A	3.60 ± 0.04A	3.66 ± 0.06A	3.57 ± 0.02A	3.62 ± 0.05A
1.0	1.18 ± 0.11A	1.19 ± 0.05A	1.17 ± 0.05A	1.15 ± 0.04A	3.58 ± 0.08A	3.62 ± 0.08A	3.56 ± 0.08A	3.60 ± 0.04A
5.0	1.19 ± 0.02A	1.17 ± 0.11AB	1.10 ± 0.02B	1.08 ± 0B	3.58 ± 0.05A	3.60 ± 0.03A	3.52 ± 0.09B	3.53 ± 0.08 B
10.0	1.16 ± 0.11A	1.11 ± 0.02B	1.09 ± 0B	1.06 ± 0.01C	3.51 ± 0.02B	3.56 ± 0.01B	3.50 ± 0.11B	3.41 ± 0.09C

[a,b] Different letters denote significant differences among treatments.
* $p < 0.05$.

notably inhibited the diversity of the soil microbial community structure.

These results illustrated that the tested IL notably inhibited the variety of soil bacteria and that the soil microbial community structure was disturbed after IL exposure to the soil samples. After the corresponding strains speculation of the enzyme fragment, 66 bp may represent *Arthrobacter*; 92 bp, *Bacteroide*; 148 bp, *Eubacterium*; 198 bp, *Firmicutes*; and 432 bp, *Ralstonia*.

2.5. Effects of the IL [C$_8$mim]NO$_3$ on gene copy numbers of the function genes (AOA- and AOB-amoA)

Ammonia oxidizing archaea and ammonia oxidizing bacteria are beneficial to the process of ammonia oxidate changing to hydroxylamine and participate in the cycle of nitrogen in soil (Jin et al., 2014). Effects of the IL [C$_8$mim]NO$_3$ on gene copy numbers of the function genes using RT-PCR are shown in Fig. 6.

For the gene copy numbers of AOA-amoA shown in Fig. 6a, no notable change was observed on day 10 compared to the controls. A notable decrease was observed at the dose of 10.0 mg/kg on day 20. On day 30, the values of all IL treatment groups were lower than the control, and notable difference was observed at the dose of 10.0 mg/kg. On day 40, notable decrease was observed in all IL treatment groups, and the values were

dose-dependent. As shown in Fig. 6b, the IL [C$_8$mim]NO$_3$ of 1.0 mg/kg almost had no influence on the gene copy numbers of AOB-amoA during the entire experimental period. A notable increase was observed at the dose of 5.0 mg/kg on day 30. A notable decrease was observed at the dose of 10.0 mg/kg on days 10, 20, and 30. The results of Sun et al. (2017) did not match ours, which may be caused by the discrepancy in anions. Furthermore, the gene copy numbers of AOA-amoA were approximately two orders of magnitude more than AOB-amoA. This result may be due to the unbalanced distribution of AOA and AOB in nature, so the populations of AOA are much higher than AOB. Chen et al. (2011) found the similar results when they studied the populations of AOA and AOB on an acidic paddy soil. Strauss et al. (2014) stated the gene copy numbers of amoA in soil may be related to the abundance of soil microbial species. Thus, we speculated that the tested IL can inhibit the diversity of the soil microbial community to inhibit the gene expression quantity of amoA.

3. Conclusions

A study regarding the toxicity of IL [C$_8$mim]NO$_3$ to the diversity of the soil microbial community and the activity of
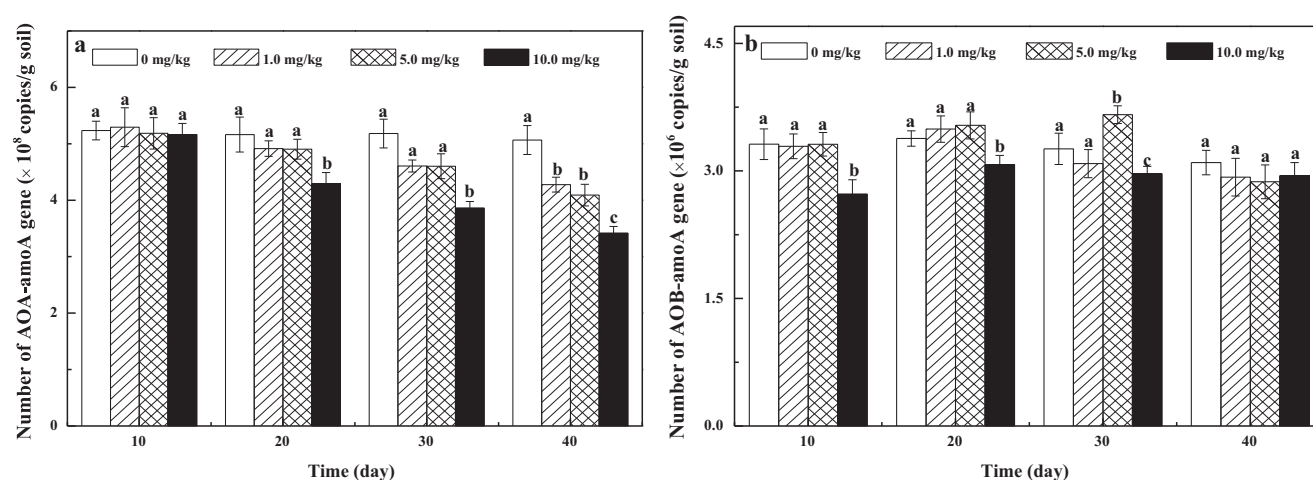
Fig. 6 – Effects of the IL [C$_8$mim]NO$_3$ on gene copy numbers of soil (a) AOA-amoA and (b) AOB-amoA. Each column is the average value of the triplicates. The standard deviation (SD) was illustrated using an error bar. The significant differences among exposed-groups and controls were illustrated using different letters above the columns at the $p < 0.05$ level via the least significant difference (LSD) test.

soil enzymes was performed. The highest dose of 10.0 mg/kg IL treatments had notable effects on the soil microbial community and soil enzyme activity. The specific conclusions of the present study were as follows:

(1) The contents of the tested IL on day 40 were lower than on day 0 with the variation no greater than 10%. The tested IL can be considered stable in the present study.

(2) Significant decreases can be observed for all biomasses of bacteria, fungi and actinomycetes at the highest dose (10.0 mg/kg) of the IL [C$_8$mim]NO$_3$ during the environmental periods. For actinomycetes, a significant decrease was observed at the dose of 5.0 mg/kg on day 10.

(3) The highest dose (10.0 mg/kg) of the tested IL inhibited the activity of urease and stimulated the activity of β-glucosidase on days 20, 30, and 40. In addition, at the dose of 10 mg/kg, [C$_8$mim]NO$_3$ inhibited the activity of dehydrogenase on days 30 and 40 and inhibited the activity of acid phosphatase on day 20.

(4) The tested IL inhibited the diversity of the soil microbial community and the numbers of the genes of AOA-amoA and AOB-amoA, in particular, in the highest dose (10.0 mg/kg) treatment-groups.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgment

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