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Impact of Repeated Applications of Metalaxyl on Its Dissipation and Microbial Community in Soil

Fenghua Wang · Lusheng Zhu · Xiuguo Wang · Jun Wang · Jinhua Wang

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Abstract Metalaxyl, an important phenylamide fungicide, is widely used for controlling fungal diseases caused by pathogens of the orders *Peronosporales* and *Pythiales*. Under laboratory conditions, metalaxyl was applied to soil samples at the recommended field rate (1×FR) and double of recommended field rate (2×FR) for two and three times. Soil subsamples were taken at 0, 1, 3, 7, 14, 28, and 45 days after the last application of metalaxyl for determination of metalaxyl residues and 7, 14, 28, and 56 days for enumeration of cultivable microorganisms and DGGE profile of soil microbial community. Soil incubation experiments revealed that metalaxyl was degraded faster in the third application than in the second application of the fungicide, half-lives of metalaxyl decreasing from 16.2 to 9.9 days for recommended field rate and 22.1 to 20.0 days for double of recommended field rate. Soil bacterial and fungal populations decreased in the first 14 days and then recovered to the control levels; population of actinomycetes did not alter in the first 28 days but increased at the end of the experiment after the second application. However, after the third treatment, temporary increase in soil bacteria population, nonsignificant inhibition effect on fungal population, and obvious stimulation effect on actinomycetes number were observed. DGGE results showed that successive inputs of metalaxyl altered the bacterial community structure. There were differences in the persistence and effects of metalaxyl on microbial community between the second and the third metalaxyl treatments.

Keywords Metalaxyl · Repeated application · DGGE · Degradation

F. Wang · L. Zhu (✉) · J. Wang · J. Wang
National Engineering Laboratory for Efficient Utilization of Soil and Fertilizer Resources, Key Laboratory of Agricultural Environment in Universities of Shandong, College of Resources and Environment, Shandong Agricultural University, Taian 271018, China
e-mail: lushzhu@sdau.edu.cn

L. Zhu
e-mail: lushzhu@vip.163.com

X. Wang
Tobacco Research Institute of Chinese Academy of Agricultural Sciences (CAAS), Qingdao 266101, China

1 Introduction

Metalaxyl [methyl N-(2,6-dimethylphenyl)-N-(methoxyacethyl)-DL-alaninate], an important phenylamide fungicide, characterized by low toxicity and long residual period with half life values in the range of 10–73 days (Jones and Ananyeva 2001; Sukul and Spiteller 2001; Fernandes et al. 2006; Sukul 2006; Sukul et al. 2008; Rodríguez-Cruz et al. 2012), is widely used to control plant diseases of downy mildew, late blight, damping off and stem and fruit rots of many plants caused by *Oomycetes*, *Phycomycetes*, and other fungi (Wang et al. 2014; Celis et al. 2015). Metalaxyl is soluble in water (8.4 g L^{-1}) and available in several commercial formulations (Wilson et al. 2001). It has the stability toward hydrolysis and photolysis in water and

soil and is resistant in a wide range of pH and temperature (Massoud et al. 2008). On account of its physiochemical properties and broad-spectrum systemic activity, metalaxyl is used on a broad range of crops such as vegetables, fruits, and horticultural crops (Zhang et al. 2014a). Metalaxyl has been registered for use worldwide including in Asia, Europe, USA, Australia, India, and Egypt (Malhat 2012). However, the application of this agrochemical can influence soil microbial community. Following application of metalaxyl, Sukul et al. (2008) reported a distinct increase of the population of total bacteria, thiosulfate-oxidizing bacteria, aerobic nonsymbiotic nitrogen-fixing bacteria, actinomycetes, and phosphorus-solubilizing microorganisms and an inhibition on fungal number. Metalaxyl also caused a significant reduction in microbial biomass, initially increased and then decreased dehydrogenase, phosphatase, arylsulphatase, and β-glucosidase activities, and a gradual decrease in urease activity during 60 days of experimental period (Sukul 2006). Monkiedje et al. (2002) observed that metalaxyl exerted an adverse effect on enzymatic activities and the population of soil total bacteria and free nitrogen-fixing bacteria. Results of Ferreira et al. (2009) indicated that a single metalaxyl application significantly impacted the PCR-DGGE profiles of cultivable soil bacterial communities (CSBC), and the differences were observed in different soils. Soil microorganisms as well as extracellular enzyme could transfer metalaxyl into extractable and nonextractable metalaxyl residues (Kalathoor et al. 2015). In addition, there were differences in the degradation rates of metalaxyl in different soils (Jones and Ananyeva 2001; Sukul and Spiteller 2001; Sukul 2006; Sukul et al. 2008). The above results were obtained under the condition of single application of metalaxyl. The consequences of continuous applications of fungicide to soil may differ from those of a single application (Yu et al. 2009; Wu et al. 2012; Tortella et al. 2013; Xu et al. 2014). In fact, repeated applications of metalaxyl are necessary to control the fungal diseases in field. However, studies focusing on the variation of degradation rates and effects on soil microbial community following repeated applications of metalaxyl are limited (Papini and de Andrea 2001; Celis et al. 2015). Therefore, it is essential to investigate the degradation rate of metalaxyl and the influence of the fungicide on soil microbial community as influenced by repeated applications to soil.

The aims of the present study were to evaluate the dissipation of metalaxyl and to evaluate the impacts of repeated metalaxyl applications on the microbial populations and community structure in soil.

2 Materials and Methods

2.1 Chemicals and Reagents

Metalaxyl (98 % purity) was obtained from Zhejiang Heben Pesticide and Chemicals Co., Ltd, China. HPLC-grade acetonitrile (ACN) was obtained from Sigma-Aldrich (Steinheim, Germany). Analytical grade sodium chloride (NaCl), anhydrous magnesium sulfate ($MgSO_4$), and ACN were purchased from Tianjin Kaitong Chemical Reagent Co., Ltd. (China). Ultrapure water was prepared by a Milli-Q system (Millipore, Bedford, MA). All other chemicals and solvents used in the experiments were of analytical grade and purchased from Tianjin Kaitong Chemical Reagent Co., Ltd. (China) and Shanghai Sangon Biological Engineering Technology and Service.

2.2 Soil

Soil used in the study that had not been previously applied with metalaxyl and other pesticides was collected from the top layer (0–20 cm) of farms in Taian, China. Soil samples were air-dried at room temperature, mixed thoroughly, sieved (2 mm mesh) and stored at 4 °C before use. Physical and chemical properties of the soil are presented in Table 1.

2.3 Experimental Design

Prior to metalaxyl treatment, the soil samples were maintained at 25 °C for 7 days for activation. Soil samples were treated with metalaxyl solution (in methanol). The soil treatment of adding metalaxyl in methanol to soil referred to methods of Bailey and Coffey (1985, 1986), Monkiedje and Spiteller (2002, 2005), and Sukul and Spiteller (2001). Methanol is toxic to microbes, and to avoid the potential effects of solvents upon the microbiological activity of the soil, the treated soil samples were put into the fume hood until the solvent had completely evaporated before incubation in the dark. Each sample of 50.00 g sieved soil was artificially

Table 1 Physicochemical properties of the soil used in the experiment

Soil type	Organic matter (g kg^{-1})	Available nitrogen (mg kg^{-1})	Available phosphorus (mg kg^{-1})	Available potassium (mg kg^{-1})	pH	Clay (<2 μm) (%)	Sand (>50 μm) (%)	Silt (50–2 μm) (%)	Water-holding capacity (%)
Sandy loam soil	13.91	93.41	35.24	73.12	6.93	14.62	59.26	26.12	18.51

contaminated by spiking 1.00 mL of metalaxyl solution (in methanol) to give a final concentration of the recommended field rate (1×FR) and double of the recommended field rate (2×FR) of metalaxyl, respectively (Sukul 2006; Sukul et al. 2008). The highest single recommended field application rate of metalaxyl is 600 g a.i./ha (China Pesticide Information Network 2010) in 25 % wettable powders, and two or three applications of each treatment were included in the field. For this study, based on the parameters of soil depth (5 cm) and density (1.5 g cm^{-3}), the single application rate under laboratory conditions corresponds to a weighed amount of 0.20 mg metalaxyl kg^{-1} soil (dry weight). Soil samples were repeatedly treated with metalaxyl for 2 and 3 number of application, and the interval between applications was 10 days. An equal volume of pure methanol (1.00 mL) was added to nonmetalaxyl soils for two and three times used for controls (CK). Fungicide-treated soil samples were thoroughly mixed to assure homogeneous metalaxyl distribution and then kept for 2 h in a well-aerated room to allow evaporation of the methanol. Three replicates were tested for all samples, including the treatments and the control. Soil water content was adjusted to 60 % water-holding capacity (WHC), and then, the soil samples were incubated in the dark at 25 °C after the first metalaxyl treatment. During the incubation, water content was adjusted constantly by addition of distilled water every 2 days. Soil subsamples were taken at 0, 1, 3, 7, 14, 28, and 45 days after the second and third application for the determination of metalaxyl residues and 7, 14, 28, and 56 days for soil microbial numbers and bacterial community.

2.4 Determination of Metalaxyl Residues

Metalaxyl in soil was extracted according to the methods of Li et al. (2013) and Liu et al. (2012). The soil sample (2.00 g) was weighed into a 50-mL Teflon centrifuge tube and then 10 mL acetonitrile was added. The tube was vortexed vigorously for 1 min at 2000 r/min, 4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, and 0.5 g citric acid disodium salt were added and immediately vortexed vigorously for 2 min at 2000 r/min and then centrifuged for 10 min at 4000 r/min. The supernatant liquid (1 mL) was decanted into a 1.5-mL centrifuge tube, 150 mg $MgSO_4$, and 25 mg primary secondary amine (PSA) were added and vortexed vigorously for 2 min at 2000 r/min, then centrifuged for 2 min at 6000 r/min. Then, 200 μL of clean supernatant was transferred into the tube containing 800 μL acetonitrile and filtered through 0.22-μm filter membrane for analysis. The quantitation of metalaxyl was performed by HPLC-MS/MS equipped with electrospray ionization (ESI) source. A Thermo Hypersil GOLD C18 column (2.1×100 mm, 3.0 μm) was used. The mobile phase consisted of acetonitrile (solvent A) and 0.1 % (*v*/*v*) formic acid in water (solvent B) with a flow rate of 0.2 mL min^{-1}. The gradient was programmed as follows: 0–0.5 min, 40 % A; 0.5–2.5 min, 40–80 % A; 2.5–10.0 min, 80 % A; 10.0–12.1 min, 80–40 % A; 12.1–15.0 min, 40 % A. The injection volume was 10 μL. Multiple reaction monitoring (MRM) and positive ion mode were used for detection. The conditions were as follows: the capillary voltage and cone voltage at 3000 and 30 V; source temperature at 120 °C; desolvation temperature at 350 °C; quantitative ions (m/z) 280.2/220.2; qualitative ions (m/z) 280.2/192.2; collision energy at 12 and 21 V for m/z of 220.2 and 192.2, respectively; retention time 3.8 min. Calibration was performed with external matrix-matched standards. The calibration curve obtained for metalaxyl (0.01, 0.05, 0.1, 0.5, 1.0 μg ml^{-1}) in soil matrix was $y=9.8731x+0.981$, $R^2=0.9934$. The recoveries of the procedure ranged from 84.3 to 96.9 %, with relative standard deviations (RSDs) from 3.8 to 6.4 %. The limit of detection (LOD) is 0.0001 mg kg^{-1}.

2.5 Enumeration of Cultivable Soil Microorganisms

Soil cultivable microbial populations were measured by the plate counting technique on selective media following the method of Zhang et al. (2014b). Three specific media were prepared to culture each microorganism. Beef-extract–peptone-agar medium was used to culture bacterial colonies, Potato Dextrose Agar (PDA, Difco) for fungi and Gauze's medium no. 1 for actinomycetes. Sample (10.00 g) from each replicate of every treatment was placed into Erlenmeyer flasks (250 mL) containing sterile water (90.00 mL), mechanically shaken for 20 min at 250 rpm, and stationed for 5 min. Serial dilutions were then prepared for plate counts. Soil suspension of 10^{-4} was used for bacteria, 10^{-2} for fungi, and 10^{-4} for actinomycetes. The agar plates were inoculated with 0.10 mL of soil suspension and incubated at 30 °C for 36 h for bacteria, 48 h for fungi, and 5 days for actinomycetes. Analyses were performed in three replicates. Data from triplicates were expressed as colony-forming units (CFUs) per gram of dry soil.

2.6 DNA Extraction

Soil total DNA was extracted with PowerSoil DNA Isolation kit (MO BIO Laboratories, West Carlsbad, CA, USA) as described by the manufacturer. The yield and quality of extracted DNA were verified by 1.0 % agarose gel electrophoresis. Extracted DNA was stored at −20 °C.

2.7 PCR-DGGE

A nested PCR method was used to amplify 16S rDNA fragments of soil bacteria (Zhang et al. 2014b). The universal bacterial primers 27 F and 1492 R were used in the first PCR amplification, and 338 F-GC and 518 R were used in the second PCR reaction for amplification of the variable V3 region of 16S rDNA (Muyzer et al. 1993). The PCR reaction solution (50 μL) contained 1 μL of template DNA (10 ng), 0.2 μM of each primer, 0.3 mM of each deoxyribonucleoside triphosphate (dNTP), 1× PCR buffer (free of Mg^{2+}), 5 μL of $MgCl_2$ (25 mmol/L), and 5 U of Taq polymerase. PCR was performed on a Bio-Rad iCycler Thermal Cycler. The 16S rDNA was amplified with an initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 57 °C for 30 s, extension at 72 °C for 1 min, and a final extension of 10 min at 72 °C. The PCR products were examined by electrophoresis on 1× TAE agarose gel (1 %, *w*/*v*) to confirm the size and approximate quantity of amplicons.

DGGE was performed with a D-Code universal mutation detection system (Bio-Rad Laboratories, Hercules, CA, USA). Samples of 35 μL of PCR products were loaded on an 8 % (*w*/*v*) polyacrylamide gel (acrylamide/bisacrylamide, 37.5:1) with a denaturing gradient varying from 30 to 60 % of the denaturing agents, with 100 % denaturant defined as 7 M urea and 40 % (*v*/*v*) formamide. Electrophoresis was run at a constant voltage of 150 V for 5 h at 60 °C in 1× TAE running buffer. After electrophoresis, the gel was stained with SYBR Green I for 30 min and visualized under UV and photographed with Gel Doc XR (Bio-Rad, USA).

2.8 Sequencing and Phylogenetic Analysis

The special DGGE bands were excised with a sterile razor blade and eluted in 50-μL sterile deionized water overnight at 4 °C. The eluent (1 μL) was used as template for reamplification with primers 338–17 and 518 R under the same PCR conditions as described above. The PCR products were purified by TIANgel Midi Purification Kit (Tiangen Biotech (Beijing) Co., Ltd. The purified PCR products were ligated into a pMD18-T Vector and then cloned into *E. coli* DH5α according to the manufacturer's instructions. Transformants were screened on LB agar plates supplemented with ampicillin (100 mg L^{-1}), X-gal (20 mg L^{-1}), and IPTG (100 μM). The clones possessing the correct insert were sequenced by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd, China. The nearest relatives of the sequenced 16S rRNA gene were identified by comparison to the sequences in GenBank with the Blast program, and a phylogenetic tree was constructed by MEGA 5.0.

2.9 Statistical Analysis

Populations of bacteria, fungi, and actinomycetes were analyzed according to a one-way analysis of variance (ANOVA). Values reported are the mean±standard error

(SE). The degradation of metalaxyl followed a pseudo-first-order kinetic reaction, and the degradation rate constant (k) and half-life ($t_{1/2}$) were estimated by Eqs. 1 and 2:

$$c = c_0 e^{-kt} \quad (1)$$

$$t_{1/2} = 0.693/k \quad (2)$$

where C_0 and C are concentrations (mg kg^{-1}) of metalaxyl at time 0 (day) and t (day).

Cluster analysis of the DGGE patterns and dendrogram were analyzed by unweighted pair group method using arithmetic averages (UPGMA) using Quantity One software (Bio-Rad, USA). Shannon–Wiener H′ were calculated according to the equation $H' = -\sum P_i \ln P_i$, where P_i is the ratio of the density of a band divided by the total density of all the bands in a lane. The principal component analysis (PCA) was performed according to the intensity and position of bands of DGGE profiles.

3 Results

3.1 Dissipation of Metalaxyl in Soil After Repeated Applications

The dissipation of metalaxyl in soil is shown in Table 2. The degradation rates of metalaxyl were dependent on the application frequencies and initial concentrations, and the half-lives were 16.2 and 9.9 days for recommended field rate and 22.1 and 20.0 days for double of recommended field rate after the second and third treatments, respectively. In the soil treated with metalaxyl, the dissipation rates were 0.043 and 0.07 mg kg^{-1} day^{-1} for recommended field rate, and 0.031 and 0.034 mg kg^{-1} day^{-1} for double of recommended field rate for the second and third treatments, respectively. These results showed that dissipation of metalaxyl was accelerated with its increasing application frequency.

3.2 Influence of Repeated Application of Metalaxyl on Soil Microbial Population

The effects of repeated metalaxyl applications on soil cultivable microbial population are shown in Fig. 1. The similar variation in soil bacteria and fungal population were observed in treatments with two successive inputs of metalaxyl to soil compared with the control. Soil bacteria and fungi were inhibited by metalaxyl significantly ($p<0.05$) during the first 14 days of incubation but recovered to control levels on days 28 and 56. The effects of 2×FR of metalaxyl application were the same as that of 1×FR of metalaxyl, except for fungal population on day 7. For actinomycetes, the soil microbial number did not significantly change during the first 28 days of incubation compared with the control (p 0.05). Nevertheless, at the end of the experiment, the actinomycic population was stimulated compared to the control ($p<0.05$). However, after the third treatment, the influences of metalaxyl on the cultivable microbial population were different from that after two successive metalaxyl additions. No obvious change in soil bacteria population was observed on day 7, followed by significant increase on day 14 ($p<0.05$); then, the number of bacteria decreased to control levels. The successive applications of metalaxyl did not inhibit significantly the growth of the fungi. However, for actinomycetes, microbial number increased during initial treatment (i.e., the first 14 days) ($p<0.05$), followed by recovering to control levels, then increased again compared to the control at the end of the experiment.

Table 2 Half-life for metalaxyl degradation in soil after two and three repeated applications

Application frequencies	Application rates	Regression equation	Correlation coefficient	Half-life (days)
Two repeated applications	1×FR	$c=0.1067e^{-0.0428t}$	0.9919	16.2
	2×FR	$c=0.2014e^{-0.0313t}$	0.9545	22.1
Three repeated applications	1×FR	$c=0.1935e^{-0.0702t}$	0.9642	9.9
	2×FR	$c=0.3674e^{-0.0347t}$	0.9987	20.0

1×FR the recommended field rate, *2×FR* double of the recommended field rate

3.3 Influence of Repeated Application of Metalaxyl on Soil Bacterial Community

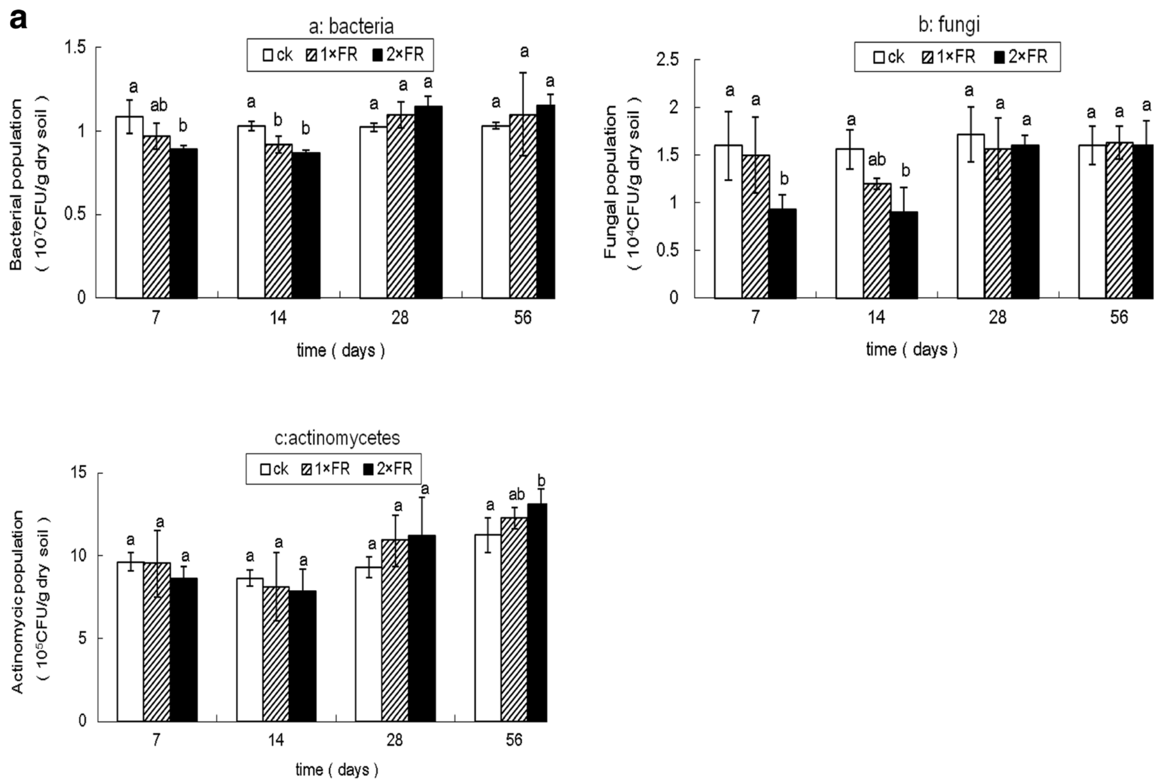
DGGE profiles of 16S rDNA gene fragments amplified from the soils treated with metalaxyl and without metalaxyl are shown in Fig. 2. Between 16 and 48 bands were present in each sample. A strong shift in DGGE banding patterns was observed after the second application of metalaxyl (Fig. 2a). The numbers of dominant bands were lower in metalaxyl-treated samples than in nontreated ones in the first 28 days, and there were least bacterial bands on day 14. Thereafter, increased bands were observed and the dominant bands in soils with metalaxyl treatments were enhanced compared with the controls at the end of the experiment (day 56). Figure 2b showed the obvious changes of the band number and position in the bacterial community occurred in the third metalaxyl treatment and controls. Sixty-eight different band positions were observed for all bacterial patterns (21–48 bands per lane). In the first 7 days of incubation, there was no significant differences in the numbers of bacterial bands in 1×FR of metalaxyl treatments, but there were significant changes in the numbers, positions, and intensities of bands in 2×FR of metalaxyl applications. 2×FR of application resulted in more bands than in 1×FR on 14 days, but the results differed after 28 days of incubation. The different band positions of DGGE profiles showed different responses of bacterial communities to metalaxyl treatments with different application rates and exposure time (Figs. 2a, b). Some bands were preferentially associated with a specific metalaxyl treatment. In contrast, the other bands were common to all treatments, despite of the applied rates and sampling time, suggesting that these bands may represent a well-established group or groups of bacteria not disturbed by the fungicide treatment.

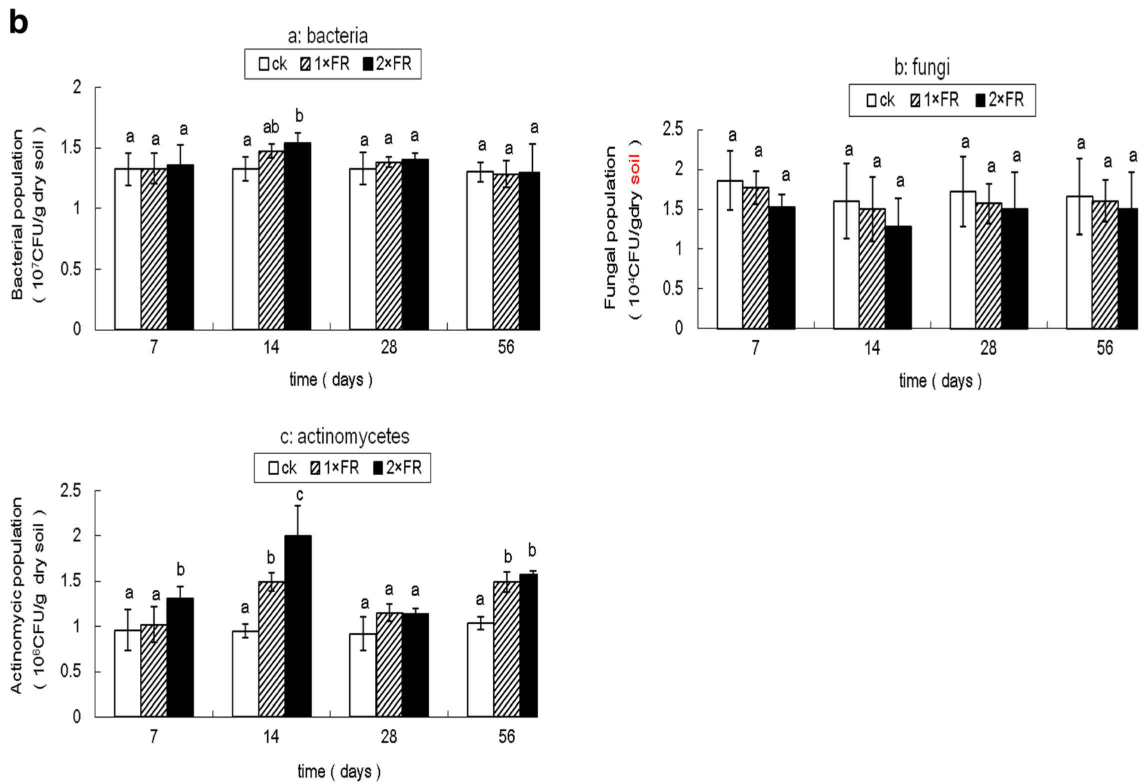
DGGE clustering analysis (Fig. 3a) showed that three major clusters were observed after the second metalaxyl treatment. The second major cluster grouped all control samples except for the sample with 1×FR of metalaxyl on 7 days. The first and third cluster grouped all soils treated with metalaxyl and were separated according to the sampling times. Also, two smaller clusters separated the samples according to the sampling date within the third cluster. The above results indicated that the incubation time influenced the bacterial communities. Although cluster analysis of DGGE patterns with three repeated applications of metalaxyl (Fig. 3b) was slightly different from that of Fig. 3a, the controls separated the fungicide treatments. And, both the sampling time and application rate contributed the clusters in all soils treated with metalaxyl.

Fig. 1 Effects of two and three repeated applications of metalaxyl ▶ on the numbers of bacteria (*a*), fungi (*b*), and actinomycetes (*c*) in soil. **a** Two repeated applications of metalaxyl; **b** three repeated applications of metalaxyl; *1×FR* the recommended field rate, *2×FR* double of the recommended field rate. *Error bars* represent the standard deviation (SD). *Different letters* indicate significant differences compared to the control at $p<0.05$ level

PCA of DGGE patterns (Fig. 4a) showed that dimension 1 explained 38.5 % of the variance after the second metalaxyl treatment, and all samples were divided into three categories along the axis; samples 1, 4, 7, 10, 2, 8, and 9 were separated from samples 3, 5, 6, 11, and 12, showing obvious differences among the samples. Especially, samples 11 and 12 were grouped together, showing very similar soil microbial community structure on 56 days after two successive applications of the fungicide, and irrelevant to the applied dosages. The second principal components axis explained 33.4 % of the variance, samples treated with metalaxyl were separated from the controls suggesting that soil bacterial community were changed by two repeated applications of metalaxyl. Treatments with three repeated metalaxyl applications were separated from controls and migrated to the negative side of PC1 (Fig. 4b), giving the further explanation of the effects of the third metalaxyl additions on soil bacterial community. The second principal components axis explained 22.0 % of the variance showing that the changes of bacterial community depended on the application rates and incubation time.

Several dominant bands were excised, sequenced, and identified. The phylogenetic relationships of sequences of cloned DNA derived from excised bands in combination with 16S rDNA gene sequences obtained from the National Center for Biotechnology Information (NCBI) are shown in Fig. 5. Table 3 shows the nearest reference strains from BLAST searches. After the second metalaxyl treatment, the major sequences derived from excised bands were most similar to organisms in three different groupings of bacteria: *Proteobacteria*, *Firmicutes*, and *Chloroflexi*. The sequences of bands 1, 2, 4, and 7 belonged to *Methylobacillus arboreus*, *Methylotenera mobilis*, and *Sphingomonas canadensis* group of *Proteobacteria*. The sequences of bands 3, 5, and 6 clustered in *Bacillus* group of *Firmicutes*. Band 8 was found to be 100 % similar to 16S rRNA genes of


a
a: bacteria
ck 1×FR 2×FR
Bacterial population (10⁷CFU/g dry soil)
time (days)
b: fungi
Fungal population (10⁴CFU/g dry soil)
c:actinomycetes
Actinomycic population (10⁵CFU/g dry soil)


b
a: bacteria
ck 1×FR 2×FR
Bacterial population (10⁷CFU/g dry soil)
time (days)
b: fungi
Fungal population (10⁴CFU/gdry soil)
c: actinomycetes
Actinomycic population (10⁶CFU/g dry soil)

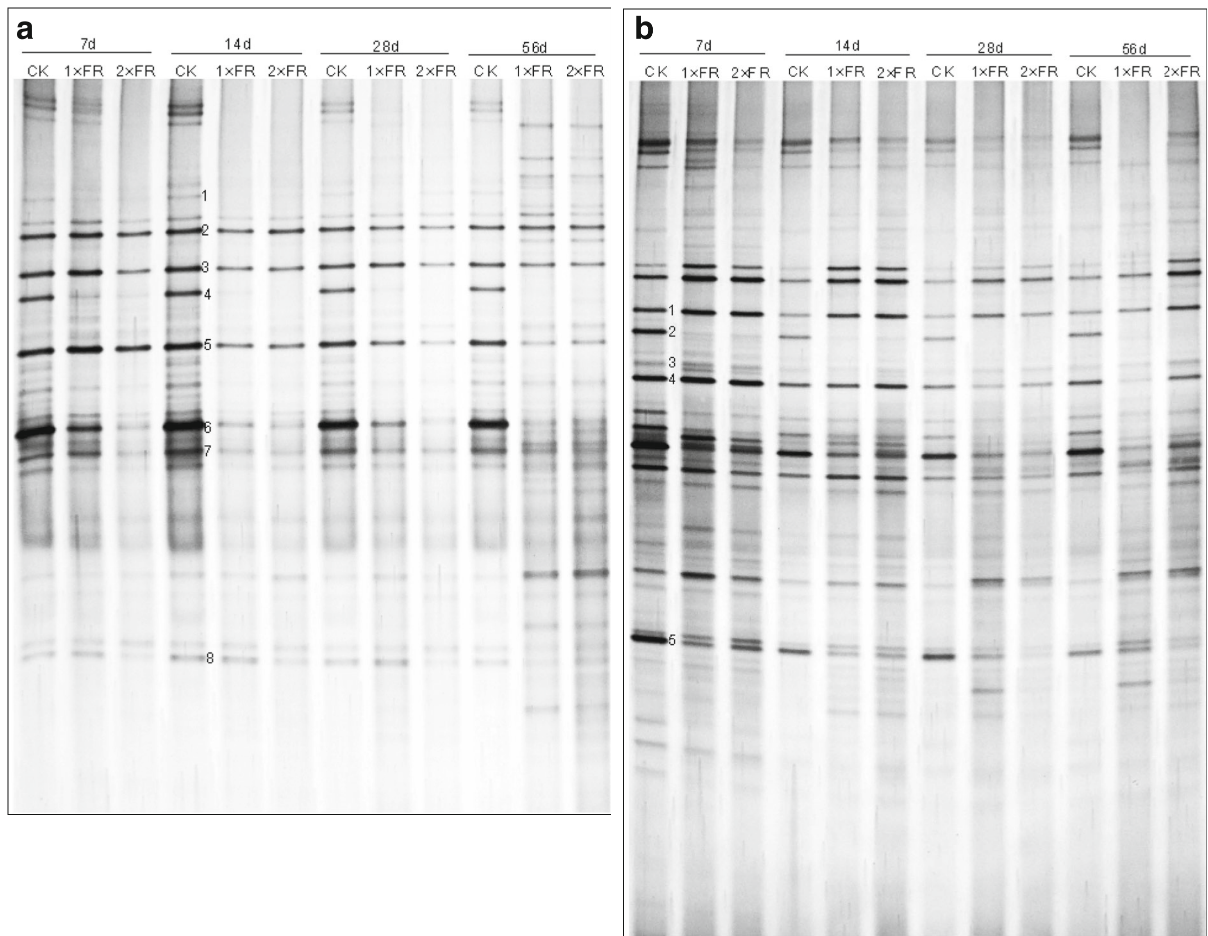


Fig. 2 DGGE patterns of bacterial community in soil treated with metalaxyl and controls during the experimental period. **a** Two repeated applications of metalaxyl; **b** three repeated applications of metalaxyl. *1×FR* the recommended field rate, *2×FR* double of the recommended field rate. The *numbers* indicated the bands excised and sequenced

Roseiflexus sp. *RS-1*. After three repeated metalaxyl applications, five major bands were excised and sequenced; the five bands were identified as *Firmicutes* and *Proteobacteria* related to *Bacillus*, *Fictibacillus*, and *Methylophilus*.

4 Discussion

4.1 Variation in Degradation Rate of Repeated Applications of Metalaxyl

The degradation of single metalaxyl application in soil has been previously studied, but studies focusing on the variation of degradation rates of repeated applications of metalaxyl are limited (Papini and de Andrea 2001; Vischetti et al. 2008). The dissipation of metalaxyl with single application in soils indicated that metalaxyl degradation was inhibited under higher concentrations (Sukul et al. 2008), but in this study, no data have been shown regarding the dissipation of metalaxyl with single application. This is because that metalaxyl should be applied for two or three times to control pathogen for the resistance of *Phytophthora parasitica* var. *nicotianae*, *Pseudoperonospora cubensis*, and *Phytophthora infestans* to metalaxyl (Mukalazi et al. 2001; Liu et al. 2011; Pavelková et al. 2014), and no single application was conducted in order to simulate the real situation. The result of an enhancement in the degradation of metalaxyl with the application frequency in this study was in agreement with other studies (Papini and de Andrea 2001; Vischetti et al. 2008), which showed the occurrence of enhanced degradation of metalaxyl in biobed mixture and soil with repeated metalaxyl application. Also,

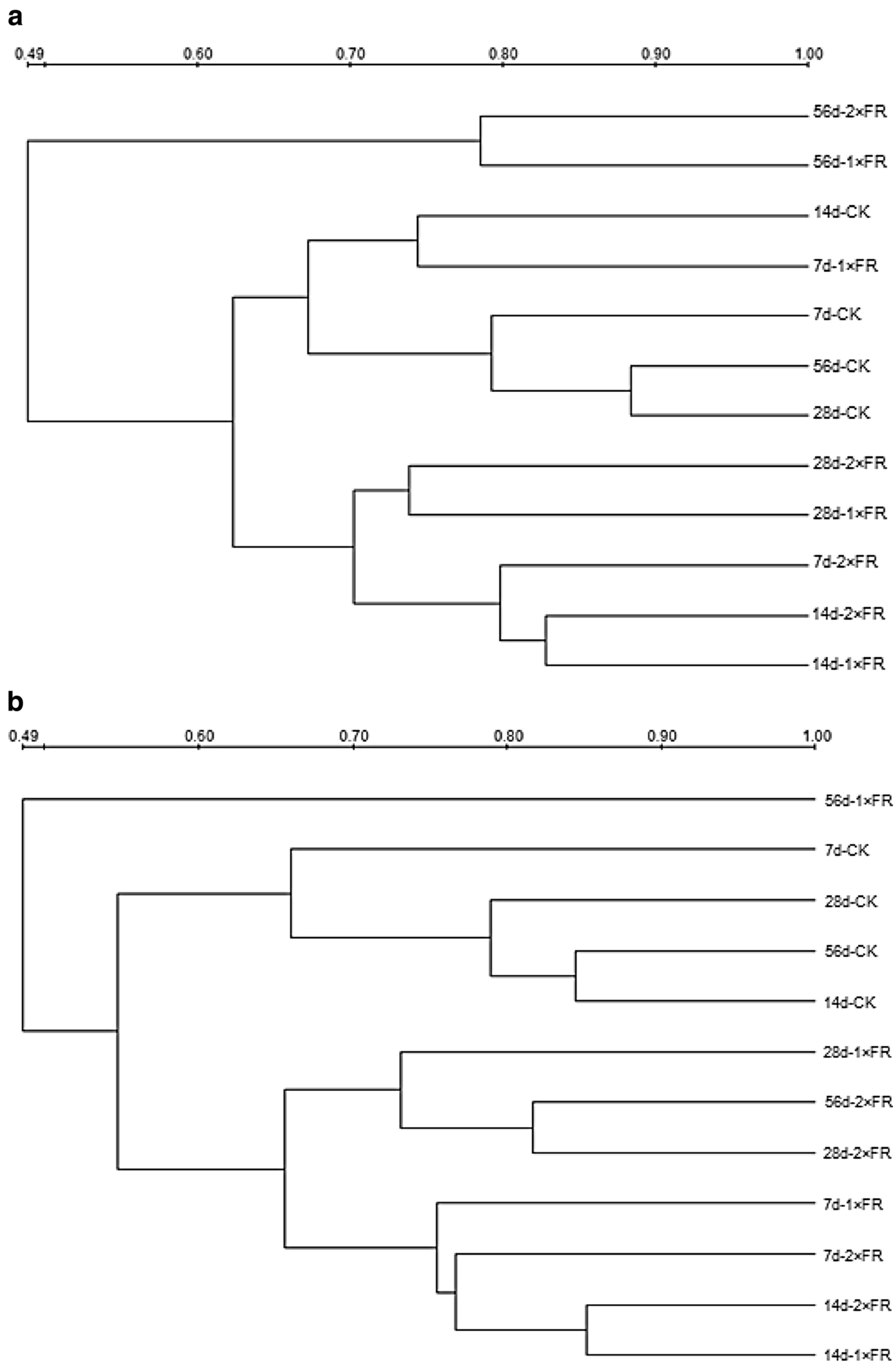


Fig. 3 Cluster analysis of bacterial community structure in metalaxyl treated and untreated soil. **a** Two repeated applications of metalaxyl; **b** three repeated applications of metalaxyl. *1×FR* the recommended field rate, *2×FR* double of the recommended field rate

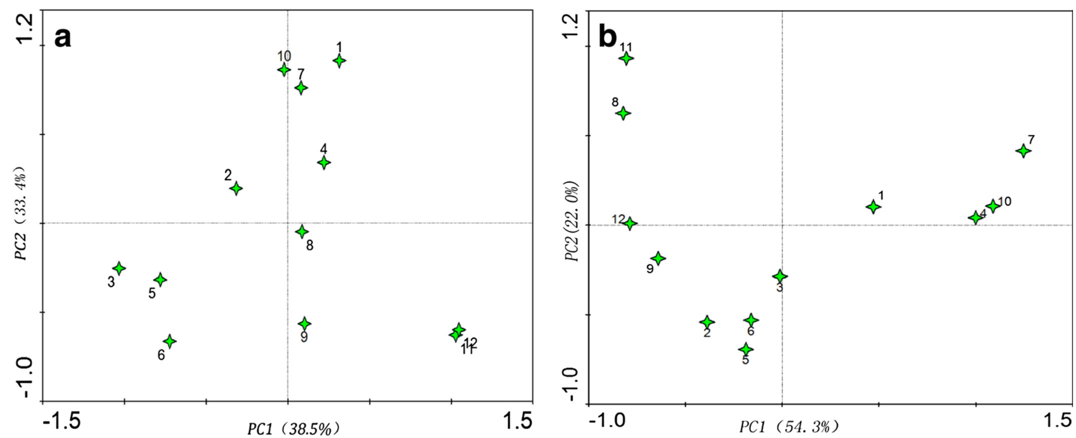


Fig. 4 Principal component analysis (PCA) performed on DGGE profiles of bacterial community in soil with/without repeated applications of metalaxyl. **a** Two repeated applications of metalaxyl; **b** three repeated applications of metalaxyl. *Numbers* refer to the number of samples: 1=7d-CK; 2=7d-1×FR; 3=7d-2×FR; 4=14d-CK; 5=14d-1×FR; 6=14d-2×FR; 7=28d-CK; 8=28d-1×FR; 9=28d-2×FR; 10=56d-CK; 11=56d-1×FR; 12=56d-2×FR. *1×FR* the recommended field rate, *2×FR* double of the recommended field rate

biodegradation of metalaxyl was accelerated in a soil with a history of the fungicide treatment (Bailey and Coffey 1986; Droby and Coffey 1991). The significantly increased degradation rate of metalaxyl following reapplication was also observed in other pesticides (Baxter and Cummings 2008; Morimoto et al. 2008; Yu et al. 2009; Triky-Dotan et al. 2010; Fang et al. 2012). Metalaxyl is a stable compound, resistant to a wide range of pH, temperature, and light (Massoud et al. 2008), so, soil microorganisms played a significant role in the dissipation of metalaxyl (Bailey and Coffey 1986; Sukul and Spiteller 2001). The increase in dissipation rate of metalaxyl in this study indicated that a specific microbial strain or community capable of degrading metalaxyl was enriched by repeatedly applying the fungicide in the soil (Morimoto et al. 2008; Vischetti et al. 2008; Yu et al. 2009; Lancaster et al. 2010; Triky-Dotan et al. 2010; Xu et al. 2014). Metalaxyl could be mineralized in soil with repeated applications and transformed into nonextractable residues (Papini and de Andrea 2001; Kalathoor et al. 2015); further research will be required to determine the quantities of CO_2 produced by metalaxyl mineralization and nonextractable residues of metalaxyl.

4.2 Effects of Repeated Application of Metalaxyl on Soil Microbial Population

The effect of single metalaxyl application on soil bacterial communities has been previously studied, but studies focusing on the influences of repeated application of metalaxyl on soil microbial communities are still relatively scarce (Vischetti et al. 2008). In this study, both cultivation-dependent and cultivation-independent methods were used to analyze the changes of the soil microbial community. In the present study, the results of decreased cultivable bacteria population in the first 14 days resulting from the second metalaxyl applications were similar to Monkiedje et al. (2002), probably due to stress of metalaxyl on sensitive bacteria. The increased soil bacterial number during the first 14 days in the third treatment of metalaxyl could be due to the fact that soil bacteria were able to use metalaxyl as carbon and energy sources to growth (Sukul et al. 2008). However, soil bacterial population recovered at the end of experiment despite of the application frequencies of metalaxyl, which indicated that the negative or positive effects of metalaxyl on soil bacteria were transient. The results of stimulated populations of soil actinomycetes with three repeated applications of metalaxyl were similar to studies of Sukul et al. (2008), in which the sum of three applications of the recommended dose of metalaxyl was a single use rate. This indicated that soil actinomycetes could use metalaxyl and/or its degraded products as carbon and energy sources to growth (Sukul et al. 2008). Fungal growth was inhibited within 14 days in the second metalaxyl treatment probably due to toxic effect exerted by metalaxyl, and the subsequent recovery in fungal

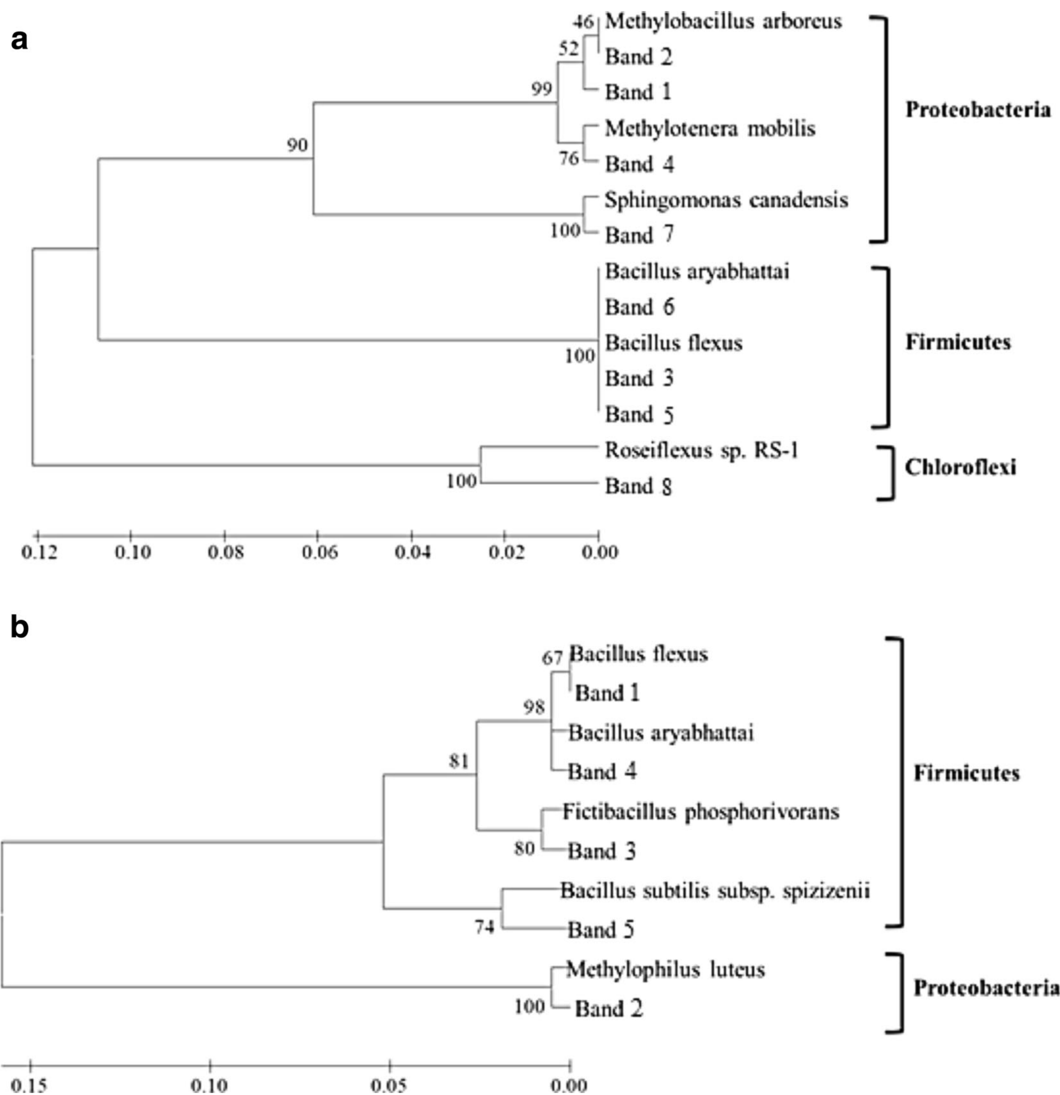


Fig. 5 Neighbor-joining tree showing phylogenetic affiliations of bacterial 16S rDNA gene sequences in metalaxyl treated and untreated soil. **a** Two repeated applications of metalaxyl; **b** three repeated applications of metalaxyl. The *scale bar* represents 5 % sequence divergence. *Numbers* indicate bootstrap values (percent confidence of 1000 replicate analyses)

population after 28 days of incubation might be due to reduced metalaxyl concentration in soil. However, soil fungi is tolerant to metalaxyl, so there were no obvious inhibition effects in the third metalaxyl treatment. As is known, only a minor fraction of the total microbial community could be cultured, and no information of nonculturable microorganisms could be provided by the plate counting technique on selective media (Müller et al. 2002). So, DGGE was also conducted to investigate the bacterial community affected by repeated metalaxyl applications.

4.3 Effects of Repeated Application of Metalaxyl on Soil Bacterial Communities

Soil bacterial community structure experienced an obvious alteration after two and three repeated metalaxyl applications in the current study, and some bands disappeared, indicating that metalaxyl selective pressures were being exerted (Macur et al. 2007). This was consistent with previous study (Triky-Dotan et al. 2010), which reported that three repeated applications of metam sodium caused decreased number of dominant bands than in nontreated ones. Some bands appeared

Table 3 Identity of excised DGGE bands by sequencing and BLAST analysis

Application frequencies	Band number	Genbank accession no	Closest match from GenBank	Similarity index (%)
Two repeated applications	Band 1	NR_108851.1	*Methylobacillus arboreus* strain *Iva*	98
	Band 2	NR_108851.1	*Methylobacillus arboreus* strain *Iva*	98
	Band 3	NR_113800.1	*Bacillus flexus* strain *NBRC 15715*	100
	Band 4	NR_102842.1	*Methylotenera mobilis* strain *JLW8*	98
	Band 5	NR_118442.1	*Bacillus aryabhattai* strain *B8W22*	99
	Band 6	NR_118442.1	*Bacillus aryabhattai* strain *B8W22*	99
	Band 7	NR_108892.1	*Sphingomonas canadensis* strain *FWC47*	99
	Band 8	NR_074197.1	*Roseiflexus* sp. *RS-1*	93
Three repeated applications	Band 1	NR_113800.1	*Bacillus flexus* strain *NBRC 15715*	100
	Band 2	NR_116847.1	*Methylophilus luteus* strain *Mim*	99
	Band 3	NR_118455.1	*Fictibacillus phosphorivorans* strain *Ca7*	98
	Band 4	NR_118442.1	*Bacillus aryabhattai* strain *B8W22*	100
	Band 5	NR_118591.1	*Bacillus subtilis* subsp. *spizizenii* strain *168*	96

indicating that specific populations were enriched. However, some gene bands did not alter throughout the experiment, and it is possible that some microbes developed tolerance and adapted to metalaxyl under selective pressure (Wang et al. 2012).

PCA profiles in this study showed that bacterial communities in metalaxyl treatments were separated from the controls on both axes. This result is in agreement with that of Wang et al. (2012), who reported that repeated applications of carbendazim resulted in significant differences between the treated samples and the controls. PCA profiles also showed the succession of soil bacterial community with different application frequencies, application rates of metalaxyl, and incubation time. Sample 2 with two metalaxyl repeated applications was close to the control, and sample 3 was separated from the control on day 7. However, the samples on day 14 were close together, as were the samples on days 28 and 56, suggesting the influence of different metalaxyl application rates on the bacterial community in the short term, but not in the long term. Samples with three successive metalaxyl applications on days 7 and 14 clustered together, but with the prolonged incubation time, the samples were separated from each other due to the dosage of metalaxyl. These results indicated that the bacterial communities were influenced by the dosage of metalaxyl not in the short term, but in the long term, which was consistent with Zhang et al. (2015).

Some bands existed throughout the whole experimental period, such as bands 3, 5, 6, and 7 in two repeated applications and band 4 in three repeated applications, respectively. Phylogenetic analysis showed that the sequence of band 3 was 100 % similar to 16S rRNA genes of *Bacillus flexus* strain *NBRC 15715*, bands 5 and 6 were 99 % similar to that of *Bacillus aryabhattai* strain B*8W22*, and band 4 in three repeated metalaxyl treatments was 100 % similar to *Bacillus aryabhattai* strain *B8W22*. It has been reported that *Bacillus flexus* would be able to degrade nitrobenzene, 2-nitrotoluene, 3-nitrobenzoate, and 4- nitrobenzoate (Mulla et al. 2011). Band 7 was found to be 100 % similar to 16S rRNA genes of *Sphingomonas Canadensis* strain *FWC47*, able to degrade polycyclic or monocyclic aromatic compounds and using them as carbon resources to grow (Story et al. 2004). Studies on species capable of degrading metalaxyl have been conducted; Bailey and Coffey (1986) isolated eight fungi and six bacteria capable of degrading metalaxyl from the liquid medium, and a *Trichoderma* sp. and *Bacillus* sp. from a soil with no applications of metalaxyl were also very active. Droby and Coffey (1991) found that *Trichoderma* spp., *Fusarium* spp., actinomycetes, and *Bacillus* spp. were the predominant microorganisms isolated from the soil solution systems and natural soils. Massoud et al. (2008) reported that *Pseudomonas sp.* (ER2) was the most effective isolate able to degrade metalaxyl, followed by fungi such as *Aspergillus niger* (ER6), *Cladosporium herbarum* (ER4), and *Penicilluim sp.* (ER3). There was a similar nitrobenzene in chemical structure of metalaxyl, so we

suggest that *Bacillus flexus* spp. and *Sphingomonas Canadensis* spp. play an important role in metalaxyl degradation. Metalaxyl could be degraded by bacteria, fungi, and actinomycetes, but in the present study, DGGE fingerprinting was performed only for bacteria, so further studies are needed to be investigated in DGGE fingerprinting for both fungi and actinomycetes.

5 Conclusions

In conclusion, this study demonstrated that different influences on soil cultivable microbial population and bacterial community structure were observed between two and three repeated inputs of metalaxyl. The third treatment of metalaxyl in soil led to an increase in the rate of degradation compared with the second application, but higher concentration of metalaxyl in soil inhibited the degradation of the fungicide. Soil bacteria were temporarily inhibited for the second metalaxyl application, but temporarily stimulated for the third application of metalaxyl, and 2×FR of metalaxyl had profounder inhibition or stimulation effect than that of 1×FR. Repeated metalaxyl application obviously inhibited soil fungi in the first 14 days, but no significant inhibition effect in the third treatments of metalaxyl. For actinomycetes, both the second and third input of metalaxyl increased the numbers of soil microorganism. Repeated metalaxyl applications led to significant changes in the bacterial community during the incubation time. *Bacillus flexus* spp. and *Sphingomonas Canadensis* spp. may play an important role in the degradation of metalaxyl with repeated applications.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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