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Carbendazim induces a temporary change in soil bacterial community structure

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Abstract

The effect of carbendazim applications on the diversity and structure of a soil bacterial community was studied under field conditions using temperature gradient gel electrophoresis (TGGE) and partial sequence analysis of PCR-amplified 16S rRNA gene. After four successive introductions of carbendazim at a level of 0.94 kg active ingredient (a.i.)/ha, the genetic diversity (expressed as Shannon index, H') decreased from 1.43 in the control to 1.29 in treated soil. This harmful effect seems to increase with the concentration of carbendazim. The value of H' in the soil treated with carbendazim at 4.70 kg a.i./ha was reduced to 1.05 ($P \leqslant 0.05$). The structure of soil bacterial community was also affected after four repeated applications of carbendazim at levels of 0.94, 1.88 and 4.70 kg a.i./ha, as seen in the relative intensities of the individual band. However, the bacterial community in carbendazim-treated soil recovered to that in the control 360 d after the first treatment. The results indicated that repeated applications of carbendazim could reduce soil microbial diversity and alter the bacterial community structure temporarily.

Key words: carbendazim; bacterial diversity; temperature gradient gel electrophoresis; phylogenetic analysis
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Introduction

Fungicides are designed for the control of pathogenic organisms and frequently applied in the agricultural system. Since they may affect non-target soil-inhabiting microorganisms, the assessment of the harmful effects of fungicides on soil microbial communities has been of great concern. Microbial processes such as carbon mineralization and nitrification have usually been regarded as sensitive indexes for this assessment (Bischoff *et al*., 2005; Colores and Schmidt, 2005). The short-term adverse effects of fungicides on soil biological processes were frequently observed. However, most of these results were obtained under laboratory conditions with short-term treatment of fungicides (Thirup *et al*., 2001; Liebich *et al*., 2003; Wang *et al*., 2004; Widenfalk *et al*., 2004). These harmful effects on soil microorganisms might be amplified by repeated applications of the chemicals. There is, therefore, an increasing concern about the effect of frequent applications of fungicides on the soil microbial community and health.

Carbendazim is a broad-spectrum fungicide and widely used for the control of plant fungal diseases on arable crops, fruits, vegetables and ornamentals (Anastassiades and Schwack, 1998). The behavior of carbendazim in the soil environment and its effect on soil microbial activities have been well investigated (Burrows and Edwards, 2004; Sousa *et al*., 2004). The reported results have indicated that carbendazim lasted 9 months and had a half-life of 4–6 months (Solel *et al*., 1979), which induced a negative effect on nitrogenase and hydrogen photoproduction (Chalam *et al*., 1996), substrate-induced respiration, and dehydrogenase and phosphatase activities (Sousa *et al*., 2004). In most cases, the soil microbial activities were reduced temporarily and recovered with the disappearance of fungicides. However, the soil microbial community structure may be altered even if the soil microbial activities appear to be unaffected by fungicides (Giller *et al*., 1997; Andrén and Balandreau, 1999). The change in soil microbial community may result in an alteration of soil activities and thus the stability and productivity of the soil ecosystem in the long term. Considering that carbendazim is usually recommended for frequent application over a growing season (Anastassiades and Schwack, 1998; Shi, 2007), the effect of repeated carbendazim applications on soil microbial community must be investigated.

In the present study, the soil bacterial diversity was analyzed before and after repeated applications of carbendazim at different levels. The purpose of this study was to assess the possible impact of carbendazim applications on the soil microbial community under field conditions.

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1 Materials and methods

1.1 Soils and treatments

The experiments were carried out on the Huajiachi campus, Zhejiang University, Hangzhou, China. The field studied had no history of carbendazim applications. The physical and chemical properties of the silt loam soil in this field are presented in Table 1. The field (90 m^2) was divided into 12 plots. The recommended dose of carbendazim is 0.75–1.13 kg a.i./ha in China, but it is usually misused at much higher dose. In this experiment, carbendazim (50% wettable powder, Zhenjiang Pesticide Co., Ltd., Jiangsu, China) was sprayed with an appropriate amount of water at levels of 0.94, 1.88, 4.70 kg a.i./ha, corresponding to the recommended dose, double recommended dose, and five times recommended dose, respectively. Carbendazim at these three levels was repeatedly applied at 15, 30 and 45 d after the first introduction. The controls received the same amount of water without carbendazim. Each treatment was performed in triplicates. The surface soil (0–10 cm) of each plot was collected with a 2.5-cm diameter augur before the first treatment (0 d), and 15 and 315 d after the fourth treatment (60 and 360 d after the first treatment). The soil samples were immediately transferred to the laboratory, passed through a 2-mm sieve, and then stored at –20°C for temperature gradient gel electrophoresis (TGGE) analysis.

1.2 Total DNA extraction from soils

The total soil DNA was extracted with the FastPrep FP120 cell disrupter (Qbiogene, Carlsbad, CA, USA). About 500 mg soil was placed in a FastPrep tube (Qbiogene, Carlsbad, CA, USA) containing lysing matrix, and the tube was shaken for 30 s. Isolation of the total DNA was accomplished with a FastDNA Spin Kit for Soil according to the manufacturer's protocol (Qbiogene, Carlsbad, CA, USA). The crude extract was further purified with the WizardR DNA CleanUp Kit (Promega, USA), according to the manufacturer's instructions. The crude extract was then analyzed by electrophoresis in a 1% (*W/V*) agarose gel in Tris-Acetate-EDTA buffer, and it was also analyzed by a spectrophotometer at 260 nm absorbance (Beckman DU-600, Beckman Coulter, USA) to check the amount, purity and molecular size. The final DNA obtained from the soil sample was amplified by PCR using 16S rRNA gene based primers.

Table 1 Physical and chemical properties of the soil

Property	Value
Water holding capacity (%)	39.4
Sand (%)	21.5
Silt (%)	71.1
Clay (%)	7.4
Texture	Silt loam
CEC (cmol/kg)	10.6
Organic matter (%)	3.1
Total N (%)	0.14
pH	6.8

CEC: cation exchange capacity.

1.3 PCR amplification of 16S rRNA V_3 region

The eubacterial primer set, GC341F and 518R (Muyzer *et al*., 1993), was used to amplify the V_3 region of the 16S rRNA genes. An adjoining 40-bp guanine-cytosine (GC) clamp was attached to the 5' end of the primer 341F (Muyzer *et al*., 1993) to prevent complete separation of the DNA strands during denaturing electrophoresis. The DNA was amplified in a TPersonal Thermalcycler (Whatman Biometra, Goettingen, Germany) with the following amplification program: 94°C for 5 min, 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 45 s and 72°C for 10 min. The 50-μL PCR mixture consisted of 1.0 U Taq DNA polymerase (TaKaRa, China), 5 μL of 10×buffer, 1.5 mmol/L $MgCl_2$, 100 μmol/L each of dATP, dCTP, dGTP, and dTTP, 0.5 μmol/L of primer GC341F and 518R (as above), and ultrapure water to a final volume of 50 μL. The PCR products were visualized under UV light after agarose (1.0%, *W/V*) gel electrophoresis with TAE buffer containing 1.5 mg/mL ethidium bromide to verify the size of the amplified DNA.

1.4 Temperature gradient gel electrophoresis (TGGE)

The PCR product was examined using the TGGE Mini system (Whatman Biometra, Goettingen, Germany). The 8% (*W/V*) polyacrylamide gel (37.5:1 of acrylamide:bisacrylamide) was prepared with 8 mol/L urea, 20% formamide and 2% glycerol. Aliquots of 3 μL of PCR products were loaded onto the gel and run at a constant voltage of 200 V for 3.5 h in TAE buffer (40 mmol/L Tris-Acetate, pH 8.0). A temperature gradient of 42–53°C parallel with the direction of electrophoresis was used. After electrophoresis, the gel was stained with silver, as described by the manufacturer (Biometra, Germany), and then photographed using a digital camera, DSC-F717 (Sony, Japan).

1.5 Recovery of bands from TGGE gel and sequence analysis

The bands of interest in the TGGE gel were excised manually and eluted in 30 μL of ultrapure water at 4°C overnight. Three microliters of the eluate was then used as the template for PCR amplification under the same conditions described above. The re-amplified PCR product was run on the TGGE gel to confirm the positions related to the excised bands. The PCR product was further purified with the WizardR DNA CleanUp kit (Promega, USA), ligated into a pMD19-T vector (TaKaRa, Dalian, China) and then cloned into *Escherichia coli* DH5α according to the manufacturer's recommendations. The partial 16S rRNA gene sequences were compared with the sequences in GenBank/EMBL by the BLAST program. A phylogenetic tree of the aligned sequences was constructed by using the neighbor-joining method of Saitou and Nei (1987) provided in the MEGA4 (Tamura *et al*., 2007) with Aquifex pyrophilus used as the outgroup.

1.6 Statistical analysis

The TGGE banding patterns were statistically compared using the quantity-one image analysis software (version 4.62; Bio-Rad Laboratories, Hercules, CA). The genetic diversity was calculated by the Shannon diversity index (H'):

$$H' = -\sum p_i \ln p_i \quad (1)$$

where, p_i is the relative abundance of bacterium i within the community. Principal component analysis (PCA) was performed using SPSS 13.0 based on the correlation matrix, which estimated the number and abundance of bacteria shared (or not shared) in carbendazim-treated soils. Statistical significance was assessed based on triplicate samples by analysing the variance (ANOVA) to determine the significant differences between samples.

2 Results and discussion

2.1 Effect of carbendazim applications on the structure and genetic diversity of bacterial community

The TGGE profiles of the 16S rRNA gene sequences amplified from the carbendazim-treated and control soils are shown in Fig. 1. Although the soil sample replicates were initially run in gels and analyzed individually, to facilitate banding pattern analysis, the PCR products from triplicate soil samples were pooled, and the products of all treatments at all sampling time were run on a single gel (Girvan *et al*., 2005). No detectable difference in relative band intensity was observed among the soils before carbendazim application. This indicated that after long-term adaptation to the environment, the composition of a bacterial community in soil is stable.

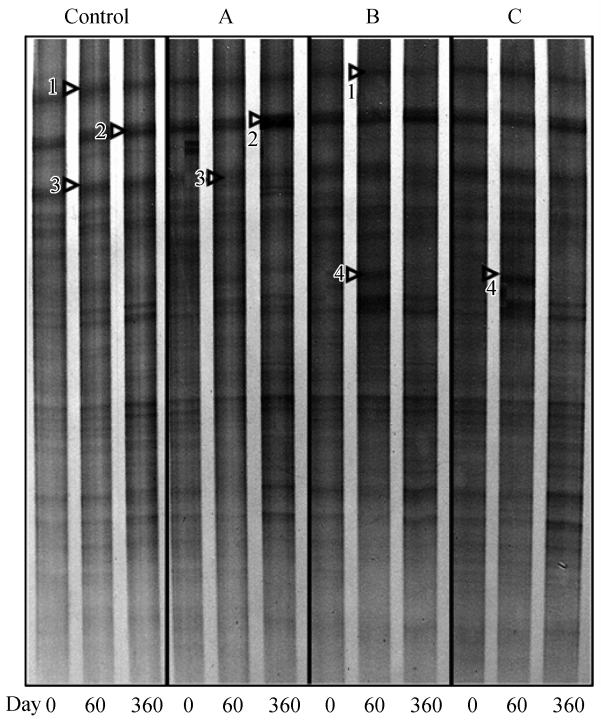

Fig. 1 TGGE analysis of 16S rRNA genes from the control and carbendazim-treated soils at 0, 60 and 360 d. The soils received four successive treatments of carbendazim at dosages of 0 (control), 0.94 (A), 1.88 (B) and 4.70 (C) kg a.i./ha during the first 60 d. Arrows indicate bands that were subsequently excised and sequenced.

After four successive applications of carbendazim, the soil bacterial community was remarkably affected. The TGGE profiles of soils treated with carbendazim at the three levels (0.94, 1.88 and 4.70 kg a.i./ha) were quite different compared to the profile of the control soil at 60 d, with a reduction in the number of bands (Table 2) and the intensive appearance of bands 2 and 4 (Fig. 1). This might suggest that the species of bacteria corresponding to bands 2 and 4 were enriched by the applications of carbendazim. Although the introduction of a pesticide may inhibit some microorganisms, some other microbes may gradually adapt to the pesticide under its selective pressure (Atlas *et al*., 1991; Bischoff *et al*., 2005; Baxter and Cummings, 2008). In this case, the soil bacteria corresponding to bands 2 and 4 might adapt to carbendazim with its successive applications, and thus their band intensity increased at 60 d. With the disappearance of the selective pressure caused by carbendazim, the soil bacterial community recovered at 360 d. This result implies that the effect of carbendazim on the soil bacterial community is temporary and will disappear with its dissipation in soil.

To understand the effect of carbendazim on the soil bacterial diversity, the Shannon-Wiener diversity index (H') was calculated from the number and relative intensity of the bands (Fig. 1). The values of H' indicated that the soil bacterial genetic diversity was significantly decreased by the application of carbendazim (Table 3). The value of H' decreased from 1.43 in the control soil to 1.29 at 60 d after successive treatment of carbendazim at a level of 0.94 kg a.i./ha. This inhibitory effect was enhanced with the increasing carbendazim concentration. The H' value in the soil treated with 4.70 kg a.i./ha of carbendazim decreased to 1.05 at 60 d ($P \leqslant 0.05$), which is much lower than the value in the soil treated with 0.94 kg a.i./ha of

Table 2 Number of TGGE bands detected in profiles from carbendazim-treated and control soils

Carbendazim treatment (kg a.i./ha)	Number of TGGE bands		
	0-d soil	60-d soil	360-d soil
Control	23 ± 1.7 a	26 ± 1.0 a	22 ± 1.7 a
0.94	23 ± 2.6 a	23 ± 2.0 ab	22 ± 1.0 a
1.88	21 ± 2.6 a	21 ± 2.6 ab	21 ± 3.6 a
4.70	22 ± 2.0 a	20 ± 1.7 b	21 ± 2.0 a

[a] Data are shown as mean ± SD (n = 3). Values followed by different letters within a column are significantly different ($P \leqslant 0.05$).

Table 3 Effect of carbendazim applications on the Shannon index of diversity (H')

Carbendazim treatment (kg a.i./ha)	Time (d)		
	0	60	360
Control	1.36 ± 0.02 a	1.43 ± 0.04 a	1.42 ± 0.07 a
0.94	1.37 ± 0.08 a	1.29 ± 0.10 b	1.47 ± 0.04 a
1.88	1.36 ± 0.11 a	1.16 ± 0.13 bc	1.41 ± 0.13 a
4.70	1.33 ± 0.07 a	1.05 ± 0.12 c	1.37 ± 0.02 a

Data are presented as mean ± SD (n = 3). Data followed by the same letters within a column are not significantly different ($P \leqslant 0.05$).

carbendazim. This observation is in agreement with the results of Ibekwe *et al*. (2001). At day 360, the *H′* value was measured to be 1.37, indicating that the soil bacterial genetic diversity recovered to a similar level as the control. In agreement with this observation, the recovery of the soil bacterial diversity with disappearance of tylosin has previously been observed, and its degradation products have also been found (Westergaard *et al*., 2001).

The PCA analysis of the TGGE profiles obtained (Fig. 2) showed that the soil community structure was altered after the carbendazim application. The bacterial communities of soils treated with 0.94, 1.88, 4.70 kg a.i./ha of carbendazim clustered away from the control soil community at 60 d, with a large separation on both axes ($P \leqslant 0.05$). All of the bacterial communities from soils treated with carbendazim at different levels recovered from the initial effects and clustered closer to the control at 360 d. This observation was in agreement with previous study (McNaughton, 1994). Wang *et al*. (2004) also found that soil bacterial communities were changed in soil treated with iprodione at the level of 5 μg/g, and these bacterial communities returned to their original status after 23 d.

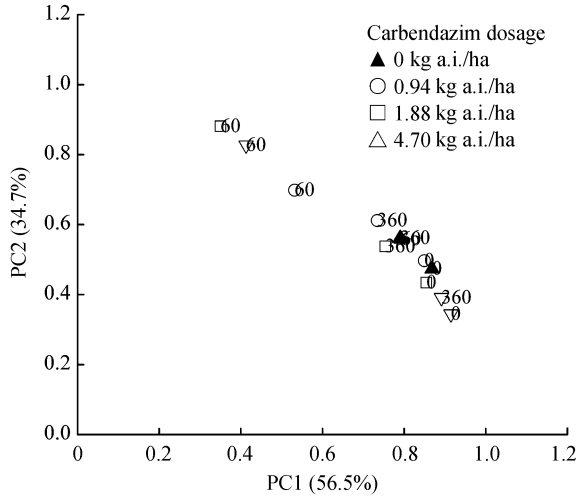

Fig. 2 Principal component analysis of the TGGE profiles from the control and carbendazim-treated soils at 0, 60 and 360 d. The soils received four successive applications of carbendazim at dosages of 0.94, 1.88 and 4.70 kg a.i./ha every 15 d during the first 60 d. PC1 and PC2 represent the first and second principal components in the PCA profiles, respectively.

2.2 Composition of bacterial community in soil

Approximately 8 dominant bands were excised from the TGGE gel and sequenced. Only 4 of the 8 sequences were distinctively different, and deposited in the GenBank database under accession numbers EU935848, EU935849, EU935850 and EU935854. The phylogenetic relationships between these four sequences and 16S rRNA gene sequences retrieved from the database are shown in Fig. 3. The phylogenetic analysis showed that the dominant bacteria in the soil were affiliated with three phyla: γ-subdivision of the *Proteobacteria*, low G+C Gram-positive bacteria, and *Cytophaga-Flavobacterium-Bacteroides* (CFB). The sequence of band 1 that clustered within the CFB group was closely affiliated with *Flavobacterium johnsoniae* (99% similarity). The band 3 sequence belonged to the gamma subclass of the *Proteobacteria* and was most closely related to *Luteibacter rhizovicinus* (97% similarity).

Of the bands sequenced, the band 4 sequence was the most intriguing. The species corresponding to band 4 was feeble in the control but dominant in soils treated with four successive applications of carbendazim (Fig. 1). The sequence of band 4 was clustered within the low G+C group of Gram-positive bacteria and most closely related to *Paenibacillus* sp. Eurl 9.39 (97% similarity). Interestingly, a similar trend was observed in the relative intensity of band 2. The microbe corresponding to band 2 was also categorized as a member of the low G+C Gram-positive bacteria. Previous studies have shown that the low G+C group of Gram-positive bacteria could survive in an unfavorable environment by spore formation (Zelles *et al*., 1997). Several bacteria belonging to this group have been successfully isolated from pesticide-contaminated

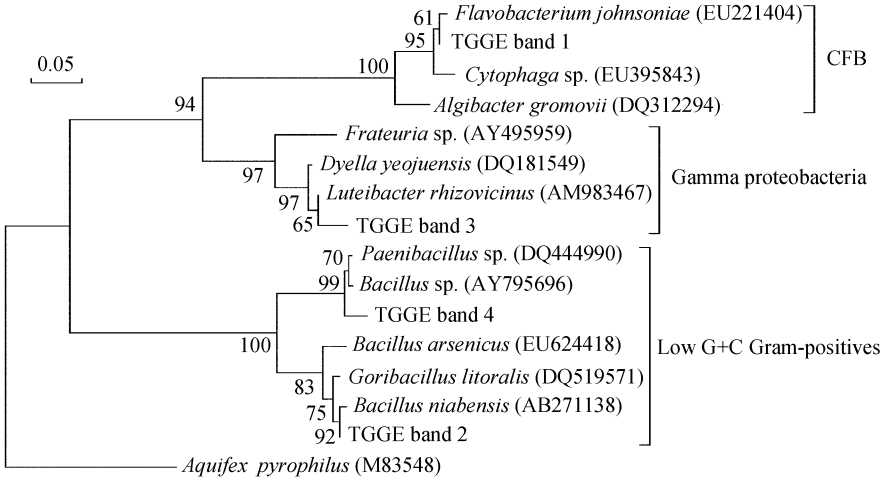

Fig. 3 Neighbor-joining tree showing the phylogenetic affiliations of the partial 16S rRNA gene sequences obtained from TGGE bands. All sequences are available from the NCBI gene bank. The tree was rooted with the partial 16S rRNA gene sequence of an *Aquificae* bacterium, *Aquifex pyrophilus*, as an outgroup. Values at the nodes represent the percentage of 1000 bootstrap replicates. The scale bar indicates an estimated change of 5%.

environments and identified as pesticide-degraders (Ou and Sharma, 1989; López *et al*., 2005). In our study, as the control soil bacterial communities were similar to each other at all sampling time, the dominance of these two species corresponding to bands 2 and 4 in carbendazim-treated soil may be induced by carbendazim. However, no carbendazim-degrading bacterium belonging to the low G+C group of Gram-positive bacteria has been isolated from tested soils. Thus, the role of the species in carbendazim-treated soil should be evaluated further.

3 Conclusions

The results from this study indicated that the soil bacterial community diversity was decreased after four successive treatments of carbendazim. This inhibitory effect increased with the concentration of carbendazim, and was measured to be transient and became undetectable at day 360 after the first carbendazim treatment. Similar alterations in the structure of bacterial community were also observed in soil frequently treated with carbendazim. Although no significant change in the bacterial composition was found, two species belonging to the low G+C group of Gram-positive bacteria were found to be capable of surviving in carbendazim-treated soil.

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References

Anastassiades M, Schwack W, 1998. Analysis of carbendazim, benomyl, thiophanate methyl and 2,4-dichlorophenoxyacetic acid in fruits and vegetables after supercritical fluid extraction. *Journal of Chromatography A*, 825(1): 45–54.

Andrén O, Balandreau J, 1999. Biodiversity and soil functioning-from black box to can of worms? *Applied Soil Ecology*, 13(2): 105–108.

Atlas R, Horowitz A, Krichevsky M, Bej A, 1991. Response of microbial populations to environmental disturbance. *Microbial Ecology*, 22(1): 249–256.

Baxter J, Cummings S P, 2008. The degradation of the herbicide bromoxynil and its impact on bacterial diversity in a top soil. *Journal of Applied Microbiology*, 104(6): 1605–1616.

Bischoff M, Lee L S, Turco R F, 2005. Accelerated degradation of N, N′ (DBU) upon repeated application. *Biodegradation*, 16(3): 265–273.

Burrows L A, Edwards C A, 2004. The use of integrated soil microcosms to assess the impact of carbendazim on soil ecosystems. *Ecotoxicology*, 13(1): 143–161.

Chalam A V, Sasikala C, Ramana C V, Rao P R, 1996. Effect of pesticides on hydrogen metabolism of *Rhodobacter sphaeroides* and *Rhodopseudomonas palustris*. *FEMS Microbiology Ecology*, 19(1): 1–4.

Colores G M, Schmidt S K, 2005. Recovery of microbially mediated processes in soil augmented with a pentachlorophenol-mineralizing bacterium. *Environmental Toxicology and Chemistry*, 24(8): 1912–1917.

Cox L, Walker A, Welch S J, 1996. Evidence for the accelerated degradation of isoproturon in soils. *Pesticide Science*, 48(3): 253–260.

Giller K E, Beare M H, Lavelle P, Izac A M N, Swift M J, 1997. Agricultural intensification, soil biodiversity and agroecosystem function. *Applied Soil Ecology*, 6(1): 3–16.

Girvan M S, Campbell C D, Killham K, Prosser J I, Glover L A, 2005. Bacterial diversity promotes community stability and functional resilience after perturbation. *Environmental Microbiology*, 7(3): 301–313.

Ibekwe A M, Papiernik S K, Gan J, Yates S R, Yang C H, Crowley D E, 2001. Impact of fumigants on soil microbial communities. *Applied and Environmental Microbiology*, 67(7): 3245–3257.

López L, Pozo C, Rodelas B, Calvo C, Juárez B, Martínez-Toledo M V *et al.*, 2005. Identification of bacteria isolated from an oligotrophic lake with pesticide removal capacities. *Ecotoxicology*, 14(3): 299–312.

Liebich J, Schäffer A, Burauel P, 2003. Structural and functional approach to studying pesticide side-effects on specific soil functions. *Environmental Toxicology and Chemistry*, 22(4): 784–790.

McNaughton S J, 1994. Biodiversity and function of grazing ecosystems. In: Biodiversity and Ecosystem Function (Schulze E D, Mooney H A, eds.). New York: Springer-Verlag Press. 361–383.

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. *Applied and Environmental Microbiology*, 59(3): 695–700.

Ou L T, Sharma A, 1989. Degradation of methyl parathion by a mixed bacterial culture and a *Bacillus* sp. isolated from different soils. *Journal of Agricultural and Food Chemistry*, 37(6): 1514–1518.

Saitou N, Nei M, 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, 4(4): 406–425.

Shi H Y, 2007. 20 vegetable diseases and their control. *Pesticide Market News*, 8: 36–36.

Solel Z, Sandler D, Dinoor A, 1979. Mobility and persistence of carbendazim and thiabendazole applied to soil via drip irrigation. *Phytopathology*, 69: 1273–1277.

Sousa P J, Rodrigues J M L, Loureiro S, Soares A M V M, Jones S E, Förster B *et al*., 2004. Ring-testing and field-validation of a terrestrial model ecosystem (TME) – an instrument for testing potentially harmful substances: Effects of carbendazim on soil microbial parameters. *Ecotoxicology*, 13(1): 43–60.

Tamura K, Dudley J, Nei M, Kumar S, 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, 24(8): 1596–1599.

Thirup L, Johnsen K, Torsvik V, Spliid N H, Jacobsen C S, 2001. Effects of fenpropimorph on bacteria and fungi during decomposition of barley roots. *Soil Biology and Biochemistry*, 33(11): 1517–1524.

Wang Y S, Wen C Y, Chiu T C, Yen J H, 2004. Effect of fungicide iprodione on soil bacterial community. *Ecotoxicology and Environmental Safety*, 59(1): 127–132.

Westergaard K, Muller A K, Christensen S, Bloem J, Sorensen S J, 2001. Effects of tylosin as a disturbance on the soil microbial community. *Soil Biology and Biochemistry*, 33(15): 2061–2071.

Widenfalk A, Svensson J M, Goedkoop W, 2004. Effects of the pesticides captan, deltamethrin, isoproturon, and pirimicarb on the microbial community of a freshwater sediment. *Environmental Toxicology and Chemistry*, 23(8): 1920–1927.

Zelles L, Palojärvi A, Kandeler E, Lutzow M V, Winter K, Bai Q Y, 1997. Changes in soil microbial properties and phospholipid fatty acid fractions after chloroform fumigation. *Soil Biology and Biochemistry*, 29(9-10): 1325–1336.