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Analysis of culturable and unculturable microbial community in bensulfuron-methyl contaminated paddy soils

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Abstract

To investigate the influence of bensulfuron-methyl (BSM) on culturable microbial quantities and unculturable microbial community structures, conventional and molecular biological methods were employed in five BSM treated soils with three replications, respectively. The results obtained with traditional culture-dependent methods showed that a low-level of BSM had slight and transient effects on culturable microorganisms; nevertheless, high concentration of BSM resulted in a dramatic decrease in bacterial colony forming units (cfus). The result obtained using denaturing gradient gel electrophoresis (DGGE) revealed that more than 17 bands were observed in low BSM contaminated soil samples and only 10 bands were detected in samples with high BSM contamination. In other words, the diversity of soil community structure is related to the concentration of BSM. Cluster analysis showed that the community structure under low level of contamination was more similar to that of the control, while heavy contaminated amendments were far away from the above group. In a sense, the cooperation of the traditional method and the molecular biological method is more powerful to study the soil microbial information in contaminated ecosystem.

Key words: bensulfuron-methyl (BSM); microbial quantities; community structures; DGGE

Introduction

The sulfonylurea herbicides are a relatively new group of compounds, which control broadleaf weeds and some grasses in cereal crops (Blair and Martin, 1988) at very low application rates (from 2 g/hm^2 of active principle (per year) of metsulfuronmethyl to 800 g/hm^2 of sulfometuronmethyl) (Brown, 1990; Blair and Martin, 1988). Bensulfuron-methyl, chemical IUPAC name: methyl alpha-(4,6-dimethoxypyrimidin-2-ylcarbamoyl sulfamoyl)-*o*-toluate (BSM), a relatively new kind of sulfonylurea herbicide, is one of the most used herbicides to control weeds in the paddy soils of California, Japan, and many regions of the world (Beyer *et al*., 1988; Okamoto *et al*., 1998). Its mode of action is to inhibit acetolactate synthase, and then to block the biosynthesis of the branched-chain amino acids, namely, valine, leucine, and isoleucine (Blair and Martin, 1988; Brown, 1990; Saeki and Toyota, 2004).

Extensive study concerning the toxicity of sulfonylurea herbicides on weeds and crops has been reported (Fletcher *et al*., 1993); however, only a few reports of their direct effects on soil microbial biomass, activities, or community structures, especially the influences of BSM, have been made. Gigliotti *et al*. (1998), using different selected media to study the effects of BSM on soil microbial communities of aerobic bacteria, anaerobic bacteria, nitrogen-fixing bacteria, nitrifying bacteria, and cellulose-degrading bacteria, revealed that after addition of BSM at a ten-fold field rate, only the number of cellulose-degrading bacteria decreased. Yamamoto *et al*. (1998) reported a negative effect on nitrification in their study with BSM and microbial biomass, nitrification, and nitrogen mineralization in flooded conditions. Contrary to the above findings, El-Ghamry *et al*. (2002) determined that with the addition of BSM at the field rate, microbial biomass and nitrogen mineralization were not affected, and the inhibitory effects on microbial biomass were observed only the first few days after the application. Later, Xie *et al*. (2004) studied the dynamics of soil microbial activity and biochemical characteristics in waterlogged paddy soils. They found that with an increase of herbicide BSM levels, the soil microbial biomass (phospholipid content) decreased. Meanwhile, with increasing BSM levels, especially at higher levels (375 and 750 g/hm^2), the abundance of heterotrophic bacteria rapidly decreased.

The above results are all based on culturable microorganisms. We know that only 0.01%–10.0% microbes on

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earth can be cultured and traditional pure-culture methods can identify only a few microbes, which cannot reflect their real existent conditions efficiently (Ward *et al*., 1990). Recently, the culture-independent molecular biological methods, such as denaturing (temperature) gradient gel electrophoresis (D(T)GGE), fluorescent *in situ* hybridization (FISH) etc., have been used in reference to conventional isolation techniques for the variation analysis of microbial community structures. So far, there has been no report about the co-analysis of the soil microbial community using both traditional and molecular biological methods in BSM-contaminated paddy soils in the previous studies. Thus, the aim of this study is to analyze the effects of the application of BSM on the variation of soil culturable microbial quantities and diversity of unculturable microbial community structures in field simulative paddy soils, and to provide the useful data for the prediction of potential changes in the BSM-contaminated soil ecosystem.

1 Materials and methods

1.1 Herbicide and chemicals

Bensulfuron-methyl (98.5% pure) was obtained from Hangzhou Tianyi Pesticide Manufactory, Zhejiang, China. All other chemicals used in the study were of analytical grade.

1.2 Soils

Soils were collected from the upper 5–15 cm layer of the experimental rice field at Huajiachi Campus, Zhejiang University, Hangzhou, China, where BSM was not used previously. The soil samples were air-dried at room temperature and sieved at 2 mm to remove plant materials, soil micro fauna, stones, and other extraneous matter. Then, the samples were thoroughly mixed in a rotary cylinder and stored at 4°C until use. The main soil physical and chemical characteristics were described using physiochemical analysis in Lü *et al*. (2004) (Table 1).

1.3 Soil treatment and procedures

There were five treatments – four with BSM in concentrations of 0.067, 0.355, 1.780, and 3.553 μg/g dry soil, and one without BSM as a control. For each treatment, 1,500 g of dry soil was put in a plastic pot (15 cm in diameter and 13 cm in depth) with 3 replications (total 15 pots). Then 1,000 ml of sterile distilled water was added to submerge the soil. All pots were incubated in the dark at 28°C till they were used for soil microbiological analysis. Sterile distilled water was added to keep the soils flooded periodically. At each time interval (e.g., 0, 7, 14, 23, 30, 40, 50, 65, 80 d for fungi), soil samples were taken from five treatments and put into flasks with beads shaking for 2 h. Then, the suspensions were used directly or diluted for microbe counting.

Table 1 Properties of the soil used in this study

Soil type	Organic matter (g/kg)	Total N (g/kg)	C/N	pH
General ferric-agric anthropic soil	16.70	1.43	13.20	7.20

Data are the means of three replications (Lü *et al*., 2004).

1.4 Incubation and counting of aerobic microorganisms

Colony forming units (cfus) of fungi, aerobic heterotrophic bacteria (AHB), and actinomycetes were determined using a modified ten-fold serial dilution plate technique on Martin's agar, meat-peptone agar, Gauses starch agar (Carter, 1993; Li *et al*., 1996). The determination of aerobic nitrogen-fixing bacteria was performed as described by Xu and Zheng (1986). Ammonifier, thiobacillus, and cellulose decomposing bacteria were counted by a ten-fold serial dilution test tube technique (Lü *et al*., 2004). The temperature for all microorganisms was 28°C. The incubation durations were 3–5 d for fungi, 36–72 h for AHB, 7–10 d for actinomycetes, 7 d for nitrogen-fixing bacteria, 7 d for ammonifiers, 15–30 d for thiobacillus, and 2 weeks for cellulose decomposing bacteria (Institute of Soil Science, 1985).

1.5 Incubation and counting of anaerobic bacteria

The number of anaerobic nitrogen-fixing bacteria and sulfate-reducing bacteria were determined by the rolling tube method in triplicate and the three-tube anaerobic most-probable-number (MPN) method with anaerobic liquid enrichment media (Xu and Zheng, 1986). Medium preparation was carried out using the Hungate anaerobic technique. The composition of anaerobic nitrogen-fixing bacteria medium (g/L) was: K_2HPO_4 (1.0); $MgSO_4{\cdot}7H_2O$ (0.5); NaCl (0.25); $FeSO_4$ (0.01); $MnSO_4{\cdot}4H_2O$ (0.01); glucose (20); and agar (18). 1,000 ml of distilled water was also added. After incubation at 28°C for 5 d, the number of anaerobic nitrogen-fixing bacteria was counted, and bubbles in a reverse Durham tube were checked as described by Min *et al*. (2001). The composition of sulfate-reducing bacteria medium (g/L) was as follows: sodium lactate (5.0); asparagine (2.0); $FeSO_4{\cdot}7H_2O$ (0.01); K_2HPO_4 (1.0); and $MgSO_4{\cdot}7H_2O$ (1.2). Distilled water, 1,000 ml, was added, and the pH was 7.2–7.5. The number of sulfate-reducing bacteria cfus (colony forming units) was numerated after incubation at 28°C for 2 weeks.

1.6 Statistical analysis

Unless specified, all the reported results were averages of three replications. Data were compared statistically by ANOVA with SPSS 13.0 for windows. Differences between values at $P < 0.05$ were considered statistically significant. Regression curve fitting of dose-effect curves for the logistics models was performed according to Haanstra and Doelman (1985).

1.7 Molecular biological analysis

The soil samples from five treatments were subjected to total DNA extraction, PCR, and DGGE as described previously (Tsai and Olson, 1992; Abed and Garcia-Pichel, 2001). The variable V3 region of 16S rDNA was

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enzymatically amplified in the PCR with the bacterial primers (338F: 5'-CGC CCG CCG CGC GCG GGC GGG GCG GGG GCA CGG GGG GAC T CC TAC GGG AGG CAG CAG-3'; 518R: 5'-ATT ACC GCG GCT GCT GG-3') to the conserved regions of the 16S rRNA genes. The DGGE gels were made from 8% (*W/V*) acrylamide stock solutions (acrylamide: bis acrylamide solution, 37.5:1) containing 0% and 100% denaturant (7 mol/L urea and 40% (*V/V*) formamide). The DGGE gradient concentrations were 35% to 65% for bacteria DNA. PCR-amplified material was loaded on the DGGE gel, which was subsequently electrophoresed at 60°C at a constant voltage of 150 V for 330 min with a Dcode™ Universal Mutation Detection System, Bio-Rad Laboratories (Hercules, California, USA). The DGGE bands were photographed with a scanner and analysis using the software Quantity One V4[1]4.0.0 (Bio-Rad, USA). The dendrogram was constructed automatically using UPGMA (unweighted pair-group method with mathematical averages) (Sokal and Michener, 1958).

2 Results

2.1 Effect of bensulfuron-methyl on soil aerobic microbial quantities

The cfu of fungi (Fig.1a) in control paddy soil was low and showed slight changes during the whole period of sampling time except day 7. The BSM treatments indicated that BSM or its metabolites could stimulate the growth of fungi 3–4 weeks after application with a nonlinear regression dose-effect equation of $y = 19.788\ e^{-0.4956x}$ ($R^2 = 0.9937$). The cfu of AHB (Fig.1b) varied between 4.8×10^4–8.7×10^4 cfu/g dry soil in the control during the 80 d incubation. There were significant differences between the control and BSM treatments at 14, 23, 30, 40, and 50 d ($P < 0.01$, respectively). The cfu of AHB in control soil was significantly higher than that of all BSM treated soils. 3.553 μg/g of BSM seriously inhibited the growth of AHB. During the incubation, the cfu of actinomycetes (Fig.1c) in control soil fluctuated from 10×10^2 to 240×10^2 cfu/g dry soil. The actinomycetes in soil treatments with 0.067 and 0.355 μg/g of BSM were significantly higher than the control ($P < 0.01$) at 14 d. However, at 21 d, the trend was reversed ($P < 0.01$). 3.553 μg/g of BSM significantly inhibited the growth of actinomycetes on day 14 and 21 ($P < 0.01$; $P < 0.001$).

For the response of aerobic nitrogen-fixing bacteria (Fig.2a), 3.553 μg/g dry soil of BSM inhibited the growth of nitrogen-fixing bacteria at 7, 14, 21, 30, and 40 d, but was not lethal and did not cause any significant differences at 65 and 80 d. At 14 d, the numbers of nitrogen-fixing bacteria in soil with 0.067 and 3.553 μg/g dry soil BSM were similar, while the reasons were not the same. For the former, BSM itself influences the growth of nitrogen-fixing bacteria, whereas for the latter, the metabolites of BSM may control the growth of nitrogen-fixing bacteria. Compared with the control, no significant differences ($P < 0.05$) were found after 21 d except 3.553 μg/g dry soil.

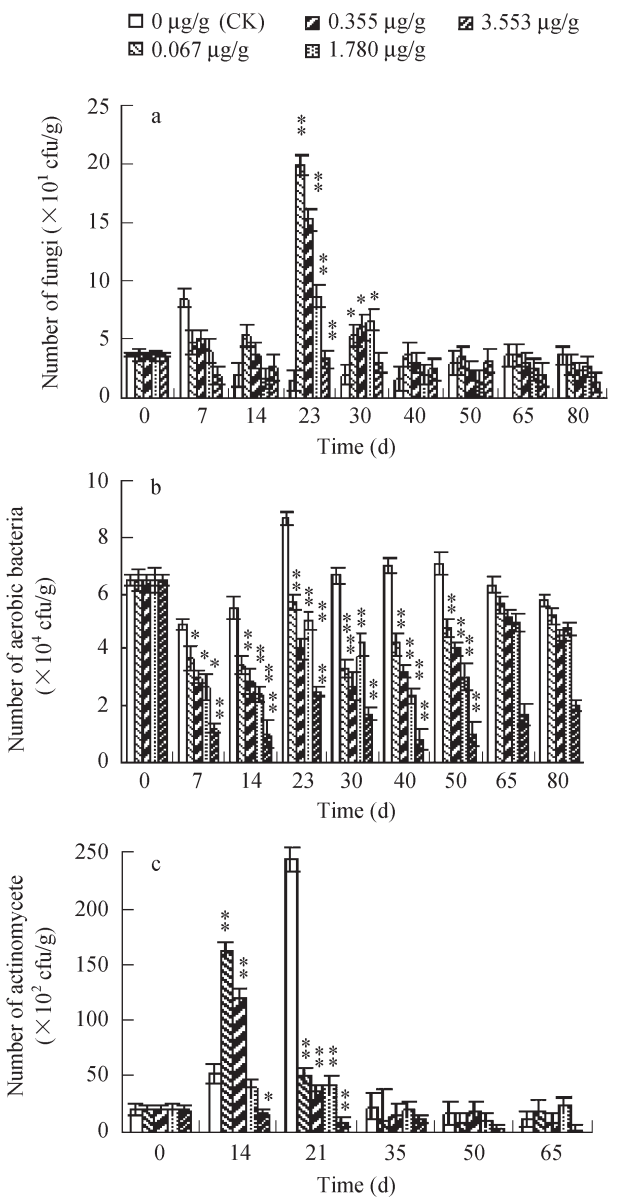

Fig. 1 Total cfus of soil microorganisms in different BSM treatments. (a) fungi; (b) soil aerobic bacteria (AHB); (c) actinomycetes.

At 7 and 14 d, the response of aerobic ammonifiers to BSM showed significant differences ($P < 0.05$; $P < 0.001$) compared to the control (Fig.2b). However, at 14 d, compared to the control, the growth of aerobic ammonifiers with 3.553 μg/g dry soil BSM was significantly lesser ($P < 0.05$), whereas that of the other BSM treatments were significantly greater ($P < 0.01$). Thus, ammonifiers were considerably higher at 14 d than at 7 d and decreased sharply at 28 d. At 45 d and after, there were no significant differences between the treatments and control, that is, the influence of BSM to ammonifiers was transient and could be recovered.

For cellulose decomposing bacteria (Fig.2c), the growth at 7 d with BSM treatments of 0.067, 0.355, and 1.780 μg/g dry soil was significantly greater ($P < 0.001$; $P < 0.01$; $P < 0.05$) than the control. Regression curve fitting of dose-effect curves for the logistics models at 7 d for all BSM treatments was $y = -2.0829\ln(x) + 4.4136$ (R^2=0.9907). The cellulose decomposing bacterial number in soil treated

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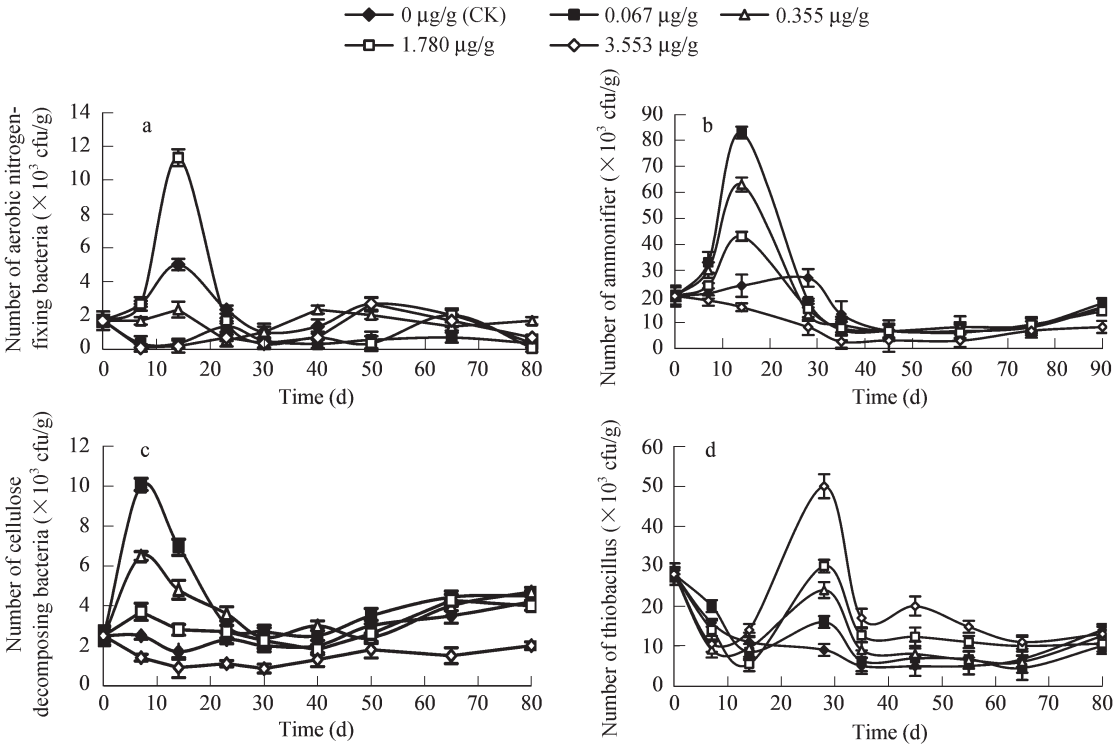

Fig. 2 Total cfus of soil aerobic bacteria in differet BSM treatments. (a) aerobic nitrogen-fixing bacteria; (b) aerobic ammonifiers; (c) aerobic cellulose decomposing bacteria; (d) aerobic thiobacillus.

with BSM of 3.553 μg/g dry soil was suppressed from 7 to 80 d. However, from day 30 on , there were no significant differences between the control and the three lower BSM treatments.

The growth of thiobacillus was stimulated at 28, 35, 45, and 55 d (Fig.2d). At 80 d, there were no differences between treatments with BSM and control. At 28 d, it could be seen that the higher the concentration of BSM, the greater the number of thiobacillus.

2.2 Effect of bensulfuron-methyl on soil anaerobic microbial quantities

Anaerobic nitrogen-fixing bacteria (Fig.3a) at 7 and 14 d were significantly less ($P < 0.05$) for the four BSM treatments compared to the control. After 21 d, the number of anaerobic nitrogen-fixing bacteria started to increase. At 40 d, the number of anaerobic nitrogen-fixing bacteria reached the peak; after that, it started to decline; until 80 d, the growth of anaerobic nitrogen-fixing bacteria with BSM treatment were higher than control, except 3.553 μg/g dry soil treatment.

Figure 3b shows the effects of BSM on sulfate-reducing bacteria. When compared to the control, the growth of sulfate-reducing bacteria with 1.780 μg/g BSM in dry soil was significantly higher ($P < 0.05$) at 30, and 40 d; probably, a certain concentration of BSM could increase the growth of sulfate-reducing bacteria. At concentrations of 0.067, 0.355, and 3.553 μg/g dry soil, the growth was suppressed on day 21, 30, and 40. However, compared to the control, soil with supplementation of 3.553 μg/g dry soil BSM had lower number of sulfate-reducing bacteria, except day 7 and 14. In other words, high dosage (3.553 μg/g) of BSM seriously restrained the growth of sulfate-reducing bacteria. At 80 d, except the treatment of 3.553 μg/g dry soil BSM, the number of sulfate-reducing bacteria was not significantly different from the control.

2.3 PCR-DGGE analysis of soil bacterial community structures

Bacterial community structures in the control and treated paddy soils were investigated by the PCR-DGGE method, and the results are shown in Fig.4. Fig.4A depicts the number and intensity of migrating bands of the DNA profiles of all samples. Fig.4B depicts the analysis results of lane comparison from the DGGE gels using Quantity One V4 [1] 4.0.0 software.

From the DGGE profiles (Fig.4), it can be found that more bands were observed in the control (lanes 1–2) and lower concentration (< 3.553 μg/g dry soil) of BSM treatments (lanes 3–8), respectively. Least bands appeared in the treatment with 3.553 μg/g dry soil of BSM. It was seen that the numbers of bands were related to the contaminated concentration of BSM. Four bands (band a, b, c, and d, Fig.4) were shared by all profiles, and they appeared more clearly in lanes 3–8, whereas other bands were faint in several lanes or more varied across profiles. The four main bands in lanes 9–10 were faint, while they were existent. The reason for the main bands in lanes 9–10 being faint may be that the bacteria stored the nutrition for living (Hu *et al*., 2007). Bands e and f appeared only in lanes 1–6 in correspondence to untreated soil samples (control) and 0.067, 0.355 μg/g dry soil samples.

The cluster analysis of DGGE profiles in different treatments is shown in Fig.5. Bacteria in the five treatments of

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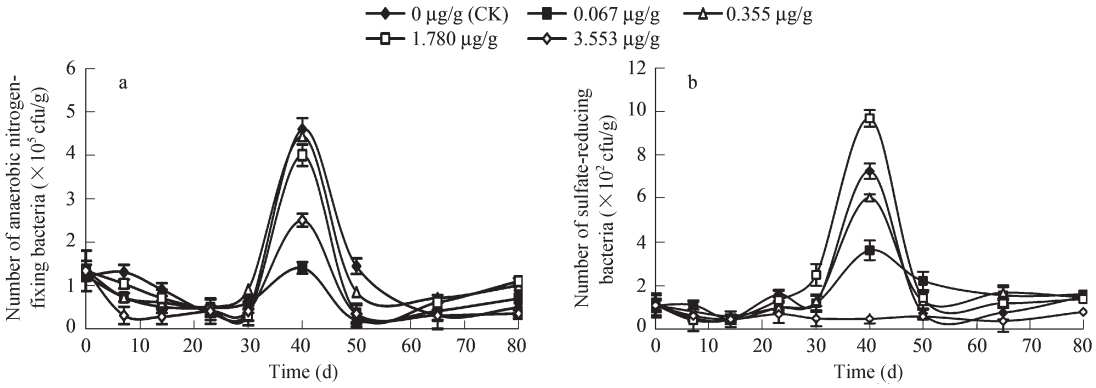

Fig. 3 Total cfus of soil anaerobic bacteria in different BSM treatments. (a) anaerobic nitrogen-fixing bacteria; (b) anaerobic sulfate-reducing bacteria.

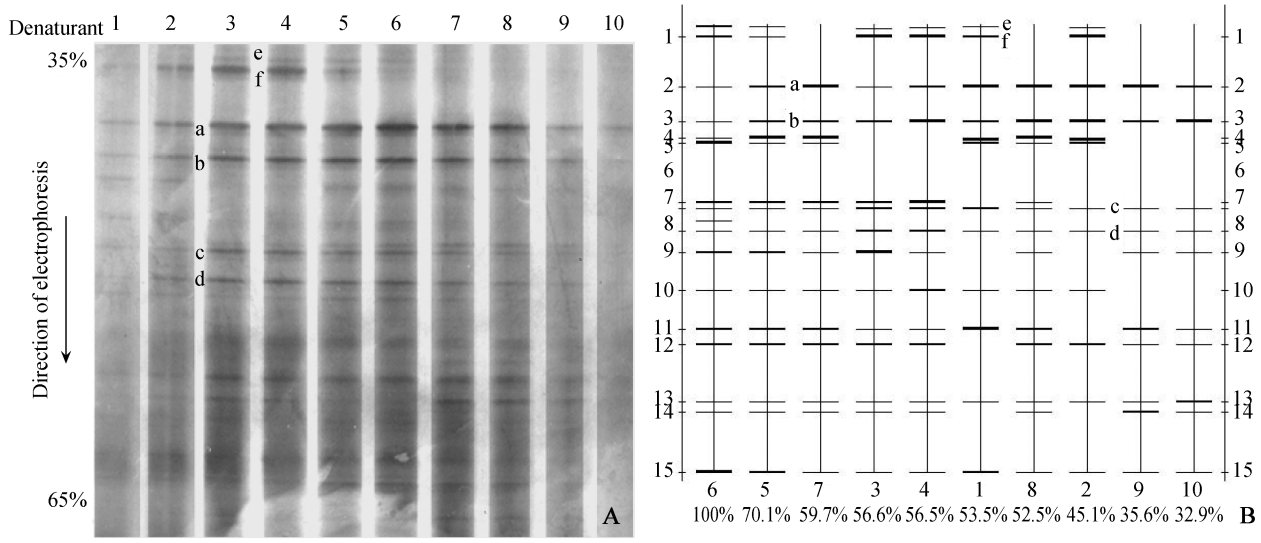

Fig. 4 Comparison of bacterial communities between the control and the four treatments. (A) the DGGE fingerprints of 16S rDNA genes (V3 region) by 338F and 518R. Each sample has two replications. Lanes 1–2, control; lanes 3–4, 0.067 μg/g dry soil; lanes 5–6, 0.355 μg/g dry soil; lanes 7–8, 1.780 μg/g dry soil; lanes 9–10, 3.553 μg/g dry soil. (B) the analysis results of lane comparison from the DGGE gels using Quantity One V4 4.0.0 software.

soils belonged to two clusters. The bacterial communities in control soils (lanes 1–2) and two slightly contaminated soils with 0.067 (lanes 3–4) and 0.355 (lanes 5–6) μg/g dry soil of BSM belonged to a separate cluster. Two BSM treated samples (lane 3–4 and lane 5–6) were merged into a single cluster. Two heavy contaminations (1.780 and 3.553 μg/g dry soil) established a new cluster far away from the clean control soil because of the stress of BSM contamination. There was a decrease in the number of bands with the increase of BSM concentration.

3 Discussion

The results displayed through the traditional method indicated that the application of BSM can influence the soil culturable microbial activities; however, the degree depended on the application concentrations. High dosage (3.553 μg/g) of BSM seriously restrained the growth of aerobic bacteria, actinomycetes, aerobic nitrogen-fixing bacteria, ammonifiers, and cellulose decomposing bacteria. The different kinds of aerobic bacteria differed markedly in their response to BSM.

Although there were some significant differences in the number of different microorganisms between the BSM

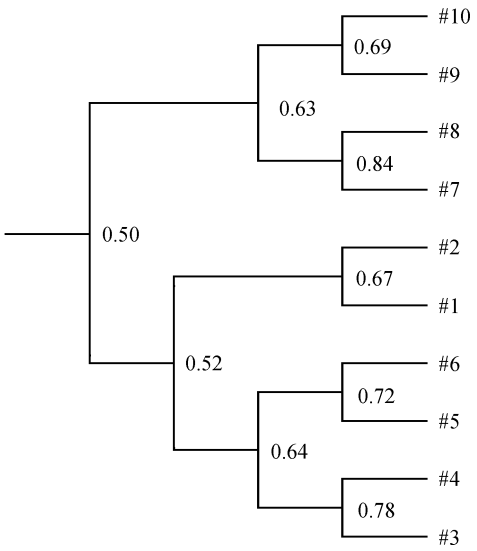

Fig. 5 Cluster analysis of 16S rDNA band profiles for communities from different soil samples. The numbers in the cluster trees refer to different amendments as described in the legend are the same as Fig.4.

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treated samples and the control at the early stage of incubation, the effects of BSM on soil culturable microbial populations, except aerobic bacteria and cellulose decomposing bacteria, were not persistent. Therefore, BSM at a normal dosage (0.067 μg/g) could be safe for these kinds of soil culturable microorganisms in this study, whereas at high concentrations (3.553 μg/g), BSM could have some negative influences on these microbes. In the field conditions, the amount of herbicide entering the soil also depended on the dosage, the application method, and the chemical properties. In general, BSM applied in the field is about 30–100 g/hm^2 (Sabater *et al*., 2002). With a short half-life in the soil of 7–21 d (Gigliotti *et al*., 1998), the effect of BSM will be minimal. If there are any effects, they will tend to disappear quickly. For the microorganisms, it will not cause a deadly ecological problem. In this experiment, except for the BSM concentration of 3.553 μg/g dry soil, the BSM applications were thought to be safe for the recovery of the tested soil microbial numbers at the end of the experiment (about 80 d). The above results indicated that low-level contamination only had a slight and short time influence on the growth of soil culturable microorganisms. However, the significant decrease in bacteria numbers at the level of 3.553 μg/g dry soil suggested that heavy BSM contamination resulted in the dramatic drop in bacterial cfus in BSM treated soils.

In the literature, there is limited study on the effects of BSM on soil microorganisms. The results of this study were neither identical to those obtained by Brusa *et al*. (2001) nor to those obtained by Gigliotti *et al*. (1998). They found that in all the tested soil microbial communities, only the number of cellulose degrading bacteria decreased. However, in this study, negative influences existed not only in cellulose-degrading bacteria, but also existed in other microbial communities at times. The growth of microbes was tested in this study for 80 d or considerably longer (except actinomycetes), which was a relatively longer time than the other studies in the references. As a herbicide, BSM not only brings inhibition, but also stimulates the growth of culturable soil microorganisms. Soil adsorption and desorption may reduce the concentrations of BSM, which will affect the growth of microorganisms. While BSM may serve as carbon and energy source for some strains, the possibility of decrease in repression by competing flora may be entertained. It is also possible that BSM residue concentrations affect the growth of soil microorganisms. In addition, the influence of herbicides on soils continues over a long time span. Thus, with complex field conditions data from field-simulated experiments will show greater fluctuation.

The changes in community structures in simulated laboratory microcosms during the experiment were documented. More than 17 bands were detected in low BSM concentration soils and only 10 bands were observed in the highest (3.553 μg/g dry soil) contaminated soil (Fig.4). Also, there were dissimilar intensities on different bands. The disappearance of bands e and f (Fig.4) revealed that bacterial community structures and genetic diversity were badly affected by the increase in the concentrations of BSM. Cluster analysis (Fig.5) revealed that the DGGE profiles of lower contaminations ($\leqslant$ 0.355 μg/g dry soil) were more similar to the clean control soils, and grouped away the other two heavily contaminated soils (1.780 and 3.553 μg/g dry soil). Besides, bands on DGGE represented microbial species (Ercolini, 2004), therefore, the four dominate bands in the five treatments may be considered to be the domain strains. The DGGE study also suggested potential strains with higher BSM resistance in the highly contaminated soils. In the future research, the prominent bands in DGGE gels can be excised and sequenced, and then their phylogenetic tree can be constructed using a BLAST search of the GenBank database. It will prove helpful for us to understand the variation of soil community structure and diversity and to study the changes of the whole ecosystem undergoing various contaminations.

4 Conclusions

This study investigated the microbial cfus variation of several typical culturable aerobic and anaerobic microorganisms through the traditional method and the changes of microbial community structures using the PCR-DGGE method in field-simulated paddy soils with five BSM treatments. The effects of BSM on the soil culturable microbial populations tested above varied with the concentration of BSM. Low-level BSM contamination had only a slight and short time influence on culturable microorganisms, whereas high level of BSM led to the dramatic decrease in microbial cfu in BSM treated soils. When the diversity of the soil microbial community structure was studied, the molecular biological method proved better than the conventional method, whereas, the culture-dependent method could be used to study the BSM degradation characteristics of bacteria in detail. The cooperation of the traditional method and the molecular biological method is necessary to compare the soil microbial information in contamination to some degree.

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