ECOTOXICOLOGICAL EFFECT OF MESOTRIONE ON ENZYME ACTIVITY AND MICROBIAL COMMUNITY IN AGRICULTURAL SOILS

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Abstract. Multiple experiments were performed to evaluate the ecotoxicological effect of mesotrione on soil enzymatic activity, functional diversity, and genetic microbial biodiversity. The results showed that catalase and invertase activity initially increased with higher mesotrione concentration and then declined slightly. However, urease activity experienced a 11.9–23.2% reduction in comparison to control soil. The average well-color development (AWCD) was positively affected by the mesotrione treatment, the amount of carbohydrate, amino acid, and phenolic acid increased by 1.7–2.1, 1.1–1.6, and 1.7–2.5 times, respectively, when compared with the blank treatment. However, the utilization of carboxylic and acid amine was prohibited after applying 50–100 mg kg^{-1} and 20–100 mg kg^{-1} mesotrione. Denaturing gradient gel electrophoresis (DGGE) analysis showed that the shift in the bacterial community structure for different mesotrione treatments can be mainly attributed to an increment in band intensity, while the dissimilarity in the bacterial genetic structure decreased with increasing mesotrione content in the soil. Sequencing and phylogenetic analyses showed that the four bands in the denaturing gradient gel electrophoresis results were closely related to *Bacillus subterraneus*, *Clostridium sp.*, and *Bacillus sp.* Obtained results show that abuse of mesotrione may pose a potential risk for soil microbial functioning.
Keywords: *mesotrione, Biolog EcoPlates, denaturing gradient gel electrophoresis, eco-environmental impact*

Introduction

Soil microbes are fundamental components of a soil's ecosystem playing an important role in many metabolic processes, e.g. the biogeochemical cycling of nutrients, decomposition of organic matter, formation of structural and hydrological properties, and various other biological–physical–chemical processes in the soil (Hu et al., 2011; Liu et al., 2015). Microorganisms can also serve as environmental bioindicators of quality due to their rapid and sensitive response to small environmental disturbances (Zhang et al., 2010; Cycoń et al., 2013b; Gryta et al., 2014). Thus, the microbial properties of the soil, and particularly those related to the diversity and activity of the soil's microbial communities, can be a most useful predictor of the fluctuation in soil health (Zhang et al., 2010; Chen et al., 2014; Allegrini et al., 2015). Soil enzyme also plays an important role in the microbial soil ecology by catalyzing various reactions, including those involved in the decomposition of organic residues and

nutrient cycling in soil–plant systems (Bhattacharyya et al., 2008; Sun et al., 2013a; Giacometti et al., 2014). In general, microbial activity and soil enzyme activity are usually influenced by various soil management practices, e.g. crop rotation, mulching, tillage, and the application of fertilizers and herbicides (Johnsen et al., 2001; Hua et al., 2009; Lupwayi et al., 2010). Therefore, these can all be used as potential indicators of microbial activity, soil fertility, and land quality (Ciarkowska et al., 2014).

Mesotrione [2-(4-methylsulfonyl-2-nitrobenzoyl)-1,3-cyclohexanedione] is a triketone herbicide that has recently been registered for the pre- and post-emergence control of annual broadleaved weeds in maize fields, and constitutes a replacement for atrazine (Sun et al., 2013b; Pose-Juan et al., 2015). Armel et al. (2009) reported that a dose equal to 235 g ai ha^{-1} was required to provide effective ($\geq 80\%$) control of some common weeds (lamb's quarters, smooth pigweed, and common ragweed). Vyn et al. (2006) documented successful control ($\geq 90\%$) of another *Amaranthus* species (*Amaranthus tuberculatus* var. rubis) at mesotrione doses of 175 (pre-emergence) and 100 g ai ha^{-1} (post-emergence). However, our previous studies found that the adsorption isotherms of mesotrione fit well into first-order and Freundlich equations. The hysteresis indices in phaeozem and red soil indicated that it had weak–moderate soil retention properties (Sun et al., 2015) and its degradation time (DT$_{50}$) varies from 6 to 34 d (Crouzet et al., 2010). Therefore, the continuous and extensive use of the herbicides would pose side-effects on the function and fertility of agricultural soils. A few studies have attempted to determine the effects of mesotrione on the nitrogen cycle, enzyme activity, and microbes in soil (Crouzet et al., 2010, 2013; Cycoń et al., 2013a; Pose-Juan et al., 2015). Crouzet et al. (2010) found that mesotrione applied at a dose of 0.45 mg kg^{-1} did not have any effect on the substrate-induced respiration, regardless of the post-treatment exposure time. However, higher doses (45 and 450 mg kg^{-1}) of mesotrione induced significant increases. Similarly, mesotrione had a strong negative impact on soil chlorophyll concentration and cyanobacterial genetic structure and diversity when its content was up to 450 mg kg^{-1} (Crouzet et al., 2013).

Non-targeted effects of herbicides on soil microorganisms can reduce microbial diversity but increase functional diversity. There may even be a tendency towards reversible stimulatory/inhibitory effects on microbial community structure and activity (Zabaloy et al., 2010; Pose-Juan et al., 2015). It has been reported that herbicides are adsorbed by organic matter in the soil to form bound or recalcitrant states, which, in turn, reduces the possibility of microbial attack (Crouzet et al., 2010). This might also account for the insignificant effect (or masking) of the impact of herbicides on the activity of enzymes (Cai et al., 2015). These effects depend on the herbicide's properties and environmental chemical behavior, soil type, environmental conditions, and functioning of the soil's microorganisms (Lupwayi et al., 2010; Pose-Juan et al., 2015). It is therefore essential to assess the ecological and environmental impact of newly marketed xenobiotics such as mesotrione in China. Unfortunately, information on the environmental impact of mesotrione in the soil (on enzyme activity and diversity of the microbial communities) is lacking. Therefore, the experiments reported here are the first aimed at extending our knowledge of these aspects. The objectives of the present study are to determine mesotrione's effects on: (1) soil enzymatic activity (via a microcosm approach), (2) functional diversity (using the Biolog method), and (3) genetic microbial biodiversity (using a method based on denaturing gradient gel electrophoresis, DGGE).

Materials and methods

Test chemicals

The mesotrione ($C_{14}H_{13}NO_7S$) was purchased from Sigma-Aldrich (St. Louis, MO, USA) in the form of dispersible granules (high performance liquid chromatography (HPLC) grade with a purity of 99.9%). The solubility of mesotrione in water increases with pH value (being 2.2, 15, and 22 g L^{-1} at pH 4.8, 6.9, and 9.0, respectively), and the stability stable to hydrolysis is at pH 4–9 (pKa 3.12).

Experimental design and treatment regime

The present research was conducted in Agro-environmental Protection Institute, Ministry of Agriculture and Rural Affairs, China. 10 kg soil samples were collected from 0-20 cm layer of agricultural fields in Changsha City, China (28º03' N and 113º 11' E) and then passed through a 20 mesh sieve for the analysis of physical and chemical fraction. The basic characteristics of this ferralsol soil are that it consists of 44.9% clay, 8.6% silt, and 46.5% sand. The amount of organic matter, total N, pH, available P, and available K equate to 1.03%, 0.84 g kg^{-1}, 4.92, 3.95 mg kg^{-1}, and 40 mg kg^{-1}, respectively.

Weighed amounts (500 g) of dried and sieved soil were placed in plastic pots and preincubated under controlled experimental conditions for two weeks. The effect of mesotrione on soil enzymatic activity and the microbial communities was investigated using 4 different doses of 0, 20, 50 and 100 mg kg^{-1}, respectively. After mixing thoroughly, the treated soils were incubated in a dark experimental chamber at $21 \pm 1°C$ for 30 d, and changed randomly every week. To make moisture conditions comparable to those in the field, the soil's moisture content was adjusted to 40% of the maximum (saturated) value. This was subsequently maintained throughout the experiment by weighing the microcosms on a weekly basis and adding sterile distilled water as needed.

Enzyme assay

Catalase activity was assayed according to the method used by Sun et al. (2015). The measured enzymatic activities were then calculated and expressed using units of mg g^{-1} h^{-1}. Urease activity was measured using the method adopted by Chen et al. (2014) with slight modification. The enzymatic activity was calculated and expressed as NH_4–N mg g^{-1} h^{-1}. Invertase activity was determined using an 8% sucrose solution as substrate and incubation at 37°C for 24 h, after which the glucose produced was measured at 508 nm using a colorimetric method. The enzymatic activity was finally expressed as mg g^{-1} h^{-1} (Kandeler et al., 1999).

Microbiological analyses

Functional diversity determination

The community-level physiological profiles (CLPPs) of the bacteria in the soil samples were evaluated using a Biolog EcoPlate™ system (Biolog Inc., CA, USA). This procedure tests the ability of a microbial community to utilize different carbon substrates contained in microplates. The soil samples (10 g) were mixed with 0.85% NaCl and then shaken for 1 h. After this time, aliquots (150 μL) of the soil suspension were added to the Biolog EcoPlate® microplates containing 31 sole carbon sources and

a water control. The plates were incubated at 24°C in the dark. Color development in each well was recorded in terms of the optical density (OD) at 590 nm (color development + turbidity) and 750 nm (turbidity). This was carried out after inoculation and at 24 h intervals for 168 h using a microplate reader (Victor TM X5 multilabel plate reader, PerkinElmer) (Osem et al., 2007; Cycoń et al., 2013b). Negative readings, after correction, were adjusted to zero.

Analysis of the microbial structure diversity

The total bacterial DNA in the test soils was extracted and purified according to previously published methods (Qing et al., 2007; Huang et al., 2008). It was then amplified via PCR using forward primer F_{357} (5'-CCT ACG GGA GGC AGC AG-3') and reverse primer R_{518} (5'-ATT ACC GCG GCT GCT GG-3'). As for the forward primer, a 40-base GC clamp (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3') was added to the 5' end to stabilize the melting behavior of the DNA fragments. The gradient concentrations used in the denaturing gradient gel electrophoresis (DGGE) analysis ranged from 35% to 65% for bacterial DNA. Initial denaturation was carried out at 94°C for 4 min. Amplification was carried out using 35 cycles including denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and DNA extension at 72°C for 1 min, followed by a final extension cycle at 72°C for 10 min.

PCR products from the soils were characterized using a DGGE run on a vertical acrylamide gel in a D-Code System (Bio-Rad Laboratories Ltd., Hertfordshire, UK). The DGGE gels were made from 8% (w/v) acrylamide stock solutions (37.5:1 of acrylamide:bis acrylamide solution) containing 100% denaturant (7 mol L^{-1} urea and 40% (v/v) formamide). The DGGE bands of interest were cut from the gel and soaked overnight at 4°C in 50 μL TAE buffer (10 mmol L^{-1} tris-acetate, pH 7.5, 1 mmol L^{-1} EDTA). The gel was stained for 15 min with ethidium bromide after electrophoresis and visualized using a UV transilluminator table using a gel documentation system (Bio-Rad, Hercules, CA, USA).

The 16S rDNA gene sequences of the excised DGGE bands were compared with existing sequences in the GenBank database using the BLAST 2.0 program (http:/www.ncbi.nlm.nih.gov/BLAST) to determine the nearest matches. The GenBank sequences were added to the data set for CLUSTAL W multiple sequence alignment and the phylogenetic distance tree was constructed using MEGA 4.0.

Statistical analysis

All of the experiments were replicated three times. The means and standard deviations (SDs) of the replicated data were calculated using Microsoft Office Excel 2010. One-way analysis of variance was performed using the SPSS (v10.0) software package. When a significant difference ($P < 0.05$ or $P < 0.01$) was observed between the treatments, multiple comparisons were made by performing an LSD test. Principal component analysis (PCA) was used to identify distinct differences in the soil microbial community functions under different mesotrione treatments.

Average well-color development

The average well-color development (AWCD) can be calculated to reflect the sole-carbon utilization of the soil microorganisms via *Equation 1*:

$$AWCD = \sum (C_i - R)/31 \qquad \text{(Eq.1)}$$

where C_i is OD in each carbon source well and R is OD of the control. When $C_i - R < 0$, the value is considered to be zero.

Microbial functional diversity indices

Several diversity indices can be calculated using the equations that follow. These are referred to as the Shannon index (H), Simpson index (D), Shannon evenness index (E), McIntosh index (U), and Dice index (C_s), and are given by *Equations 2-6*:

$$H = -\sum P_i \ln(P_i) \qquad \text{(Eq.2)}$$

$$D = 1 - \sum P_i^2 \qquad \text{(Eq.3)}$$

$$E = \frac{H}{H_{max}} = \frac{H}{\ln S_i} \qquad \text{(Eq.4)}$$

$$U = \left(\sum N_i^2 \right)^{1/2} \qquad \text{(Eq.5)}$$

$$C_s = \frac{2j}{a+b} \qquad \text{(Eq.6)}$$

In these expressions, N_i is the metabolic activity on each substrate, P_i is the ratio of n_i to the sum of the activities on all substrates, S_i is the natural logarithm of the number of species, j is the number of bands common to samples A and B, and a and b are the number of bands in samples A and B, respectively.

Results and discussion

Soil enzymatic activity

In the present study, the catalase activity initially increased and then declined as the amount of mesotrione increased. Under the application of 20 and 50 mg kg^{-1} mesotrione, the increase was amounted to 11.2% and 12.6%, respectively (*Fig. 1*). However, catalase activity fell by 2.7% when the concentration of mesotrione was increased up to 100 mg kg^{-1}. There was no significant difference in catalase activity across the 20–100 mg kg^{-1} mesotrione treatment range ($P > 0.05$). Similar results have been obtained by Baćmaga et al. (2014), who found that metazachlor applied at doses of 6.7–106.7 mg kg^{-1} stimulated the activity of catalase in the range of 7.5–92.5%. Moreover, Liu (2000) reported that imidacloprid can also stimulate catalase activity, and that the higher the concentration used, the stronger the observed effect on soil catalase for imidacloprid doses in the range 1–40 mg kg^{-1}. However, there was no obvious effect on catalase activity after application of 0.5 to 50 mg kg^{-1} of acetamiprid for 14 days (Yao et al., 2006).
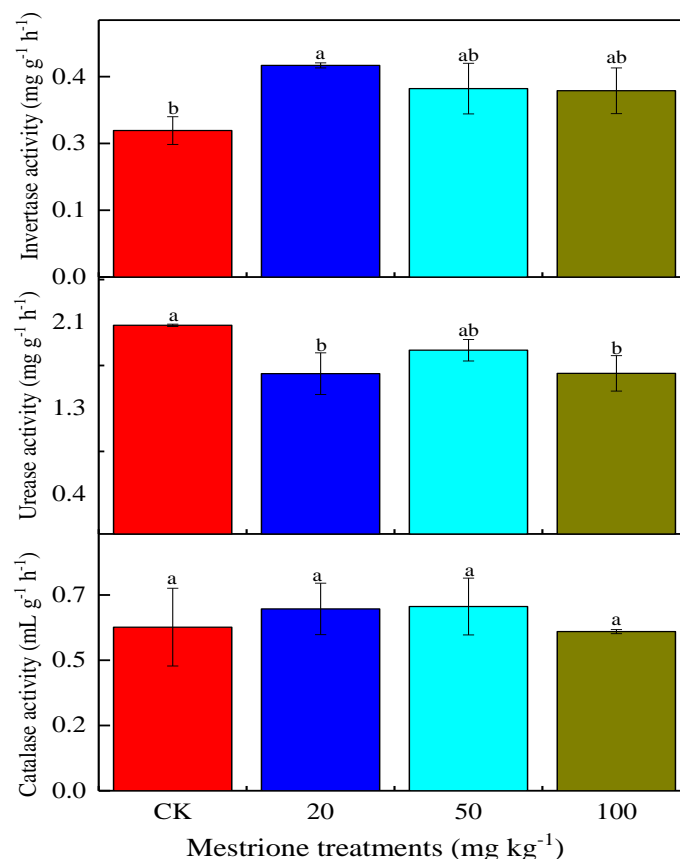
Figure 1. *Effects of mesotrione on soil enzyme activities. Letters above the bar diagram refer to the difference at significance level P < 0.05 among different treatments of mesotrione. The same as below*

Urease, which involves in the hydrolysis of urea-type substrates, is an enzyme that quickly responds to changes in the soil environment (Yao et al., 2006). Furthermore, contamination of soil with mesotrione has a destructive influence on the activity of urease. The application of mesotrione caused a 11.9–23.2% reduction in urease activity compared with control soil (*Fig. 1*), and amending the soil with 20 and 100 mg kg^{-1} mesotrione resulted in a significant decrease in urease activity ($P < 0.05$). Results similar to ours concerning the inhibitory effects of herbicides on urease activity have been reported by Baćmaga et al. (2014) who investigated metazachlor and by Sukul (2006) who tested metalaxyl. In turn, Yao et al. (2006), who investigated the eco-toxicology of the herbicide acetamiprid, did not detect any obvious influence on urease activity.

Another enzyme used in assays evaluating the quality of soil is invertase, which is involved in the hydrolysis of sucrose to glucose and fructose. Invertase is more efficient than other enzymes in reflecting soil fertility and biological activity levels (Cang et al., 2009). *Fig. 1* exhibits the promotion effect of mesotrione on invertase activity in the soil, which increased at first and then decreases with increasing concentration. It was, however, always 27.1–44.4% greater than that of the control soil over the 20-100 mg kg^{-1} mesotrione range. In particular, there was a significant increase using 20 mg kg^{-1} of mesotrione ($P < 0.05$). Positive effects of buprofezin and acephate on invertase had already been noted by Raju and Venkateswarlu (2014).

The results above clearly indicate that soil enzyme activity differs in sensitivity upon application of herbicide. Ye et al. (2003) likewise found that mefenacet strongly prohibited soil dehydrogenase activity in a concentration-dependent manner, but stimulated phosphatase activity. Indeed, both significantly positive and negative, as well as neutral, responses have been recorded compared to untreated soils (Floch et al., 2011; Baćmaga et al., 2014). Herbicides may change enzyme protein or interact directly by binding with protein-active groups which subsequently affects their catalytic activity. Another possible reason is that a herbicide may indirectly affect soil enzyme activity by acting on soil microorganisms (Johnsen et al., 2001; Floch et al., 2011; Baćmaga et al., 2014; Jacobsen and Hjelmsø, 2014). Baćmaga et al. (2014) demonstrated that in soil contaminated with excessive quantities of metazachlor there are significant effects on the biochemical activity of the soil (with correlation coefficients that are negative). In another report, no significant differences ($P > 0.05$) were found in enzyme activity in treated soil compared to a control in the initial 2 weeks, while enzyme activity was stimulated from the third week on (Yao et al., 2006). Moreover, the influence of pesticide on soil was generally a long-term process and closely related to the soil's characteristics (Yao et al., 2006).

Microbial community substrate utilization profile

Color development in the Biolog EcoPlates was analyzed to qualitatively and quantitatively assess the community-level physiological profile of the microbe communities in treated soils (Osem et al., 2007; Gryta et al., 2014). The average well-color development in the Biolog generally followed similar patterns (*Fig. 2a*), i.e. an apparent lag phase was experienced during the first 48 h. After this time, the AWCD values increased rapidly with incubation time for all treatments which is attributable to more rapid bacterial growth taking place. The period selected for metabolic activity analysis was 120 h as this period corresponded to the occurrence of the largest AWCD change. The slope of the AWCD curve at this time can be used to represent the average metabolic activity of a microbial sample (Kong et al., 2013). Thus, the slopes of the AWCD curves after 120 h were selected for use in the metabolic activity analysis. The analysis of the variation in AWCD in the stressed soils over the incubation time (during the period 0–120 h), showed that the AWCD values using 20–100 mg kg^{-1} of mesotrione were all higher than those of the control. Strong oxidation of the supplied carbon sources (high AWCD) usually reflects an increase in bacterial density (Loranger-Merciris et al., 2006). A possible reason for this might be that soil microbes tolerate and adapt to the herbicide after repeated applications, and/or degrade the herbicide as a source of carbon and energy (Yao et al., 2006; Xu et al., 2014). However, after an incubation time of 168 h, the AWCD values decreased with an increase in mesotrione, and a dose–effect relationship could be discerned.

The substrate utilization patterns currently established via the Biolog Ecoplates can be used to determine the changes in the functioning of the soil microbial communities (Floch et al., 2011). As shown in *Fig. 2b*, there were remarkable differences in the substrate utilization profiles of the mesotrione treated soil samples. Carbon source metabolism by the microbial population decreased in the following order: polymer > amino acid > carbohydrate > phenolic acid > acid amine > carboxylic acid. This suggested that the carboxylic acid could be more easily utilized by the microbes subjected to the different mesotrione treatments. Although the variation in the carbon sources appeared to be irregular under the series of mesotrione treatments, the increase

in the amount and rate of carbohydrate, amino acid, and phenolic acid utilization observed in the soils subjected to different mesotrione treatments corresponded to increases of 1.7–2.1, 1.1–1.6, and 1.7–2.5 times, respectively (compared to the control). Significant increases ($P < 0.05$) in substrate utilization were observed with a mesotrione concentration of 20 mg kg^{-1} for carbohydrate and amino acid, and 100 mg kg^{-1} for polymer and phenolic acid. However, there appeared a decrease in polymer, carboxylic, and acid amine by 3.0%, 12.5–17.1%, and 13.3–48.1%, respectively, in mesotrione treated soil of 50, 50–100, and 20–500 mg kg^{-1}, respectively, and the amount of acid amine decreased sharply at a mesotrione content of 20 mg kg^{-1} ($P < 0.05$).
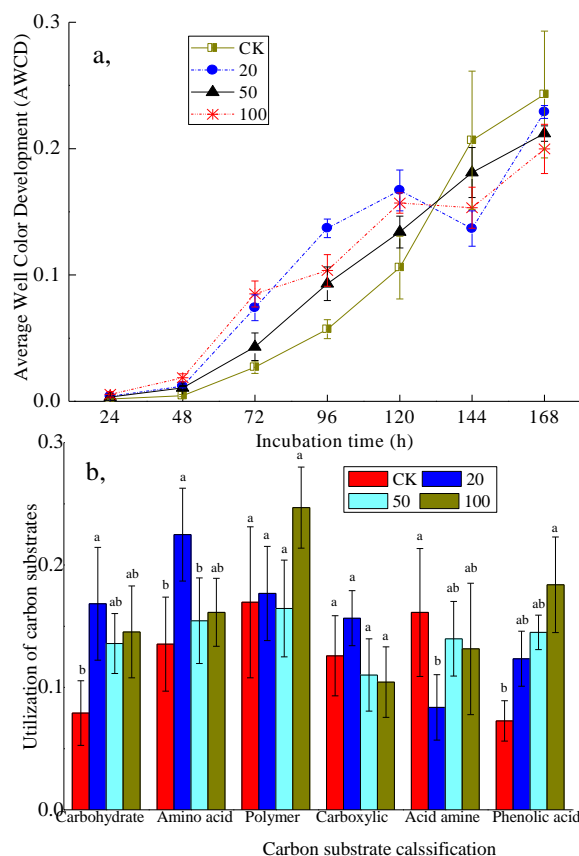

Figure 2. Microbial community level physiological profiles (CLPP) under different treatments of mesotrione. Note: (a) and (b) refers the variations in AWCD over time and utilization of 6 groups carbon sources, respectively

Principal component analysis (PCA) was used to assess the microbial communities in the stressed-soil environment (Kong et al., 2013; Shrestha et al., 2015). The PCA results from the Biolog Ecoplates data explained 67.3% of the total variance and components, and the first principal components (PC1) and the second principal components (PC2) accounted for 37.1% and 30.2%, respectively (*Fig. 3a*). Therefore, the first two principal components (PC1 and PC2) were used to describe the effects of the different treatments of mesotrione (0–200 mg kg^{-1}) on the utilization of 31 carbon sources. PC1 and PC2 are plotted against each other in *Fig. 3* for illustration purposes. The PCA plots indicated that bacterial functional diversity changes with the level of mesotrione used.

Most of the points were clustered in the first and second quadrants of the loading plot, and the plots amended with 20–100 mg kg^{-1} mesotrione were clustered together and are differentiated from the control. *Fig. 3b* also compares the Biolog Ecoplates utilization patterns for each substrate guild (i.e. carbohydrate, amino acid, polymer, carboxylic, acid amine, and phenolic acid) for different mesotrione treatments, using PCA. The first variable (PC1) accounted for 54.8% and the second variable (PC2) accounted for 28.7% of the total variance in the data. Rotation of the matrix revealed that the substrate utilization can be separated into three groups: (a) amino acid and carboxylic (relatively negatively related with PC2), (b) carbohydrate and phenolic acid (positively associated with values of PC1 and PC2), and (c) polymer and acid amine (associated with negative values of PC1 and PC2). The PCA plots indicate that the bacterial communities originally present in the tested soil were shifted after application of mesotrione.
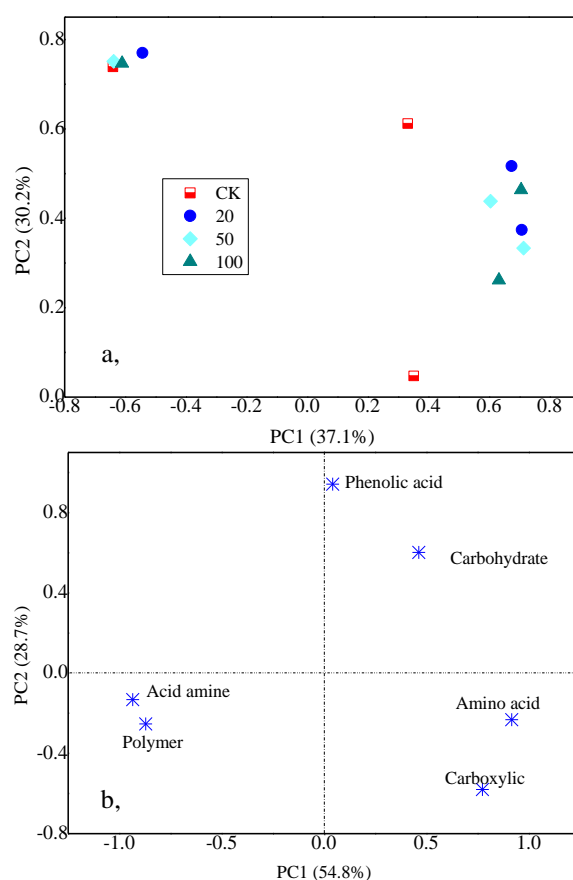

Figure 3. Scatterplots of principal components scores for soil samples based on (a) different treatments of mesotrione and (b) 6 carbon sources. Numbers in parentheses on axis labels is the percentage of the variation according to principal components analysis (PCA)

To further investigate the effect of mesotrione on the bacterial properties of the soil, the diversity indices of *H*, *D*, *E*, and *U* are calculated to assess the soil's microbial functional diversity (Gomez et al., 2006), *H* and *E* values indicate the richness and evenness of the soil microorganisms (Zhou et al., 2008), index *D* is often used to emphasize the dominant microbe population, and while *U* (which is based on Euclidean distance) indicated the evenness or homogeneity of the microbial community (Zhou et

al., 2008). The calculated values of the diversity indices of H, D, E, and U are presented in *Table 1*. In general, the H, D, and U values gradually increased with incubation time and then fell. However, the amount of H, D, and U was still 6.6–36.1%, 2.0–12.2%, and 70.1–894.0% greater than those of the control, respectively. Index E decreased by 1.6-14.1%, 18.1–32.1%, 7.6–14.6%, and 12.9–27.8%, under mesotrione treatments of 0, 20, 50, and 100 mg kg^{-1}, respectively, when compared with the control. The microbial community analysis was consistent with substantial increases in microbe diversity in the treated soil. The H, E, D, and U values were stimulated by the various mesotrione treatments, corresponding to increases of 2.7–3.4%, 1.3–10.2%, 1.4–2.2%, and 6.6-20.0%, respectively (compared to the control). The increase in the microbial activity in the soil may have been higher and the microorganisms more active if the mesotrione was used as an energy source (Crouzet et al., 2010; Zabaloy et al., 2010). The regression coefficients between the richness and Shannon–Weaver indices and organic carbon in the soil suggested that the increase in microbial community functional potential may be explained by the increase in carbon availability resulting from incorporation of the amendment (Gomez et al., 2006). The microorganism communities may have been indirectly stimulated by the dead biomass arising from more sensitive organisms together with a concomitant stimulation of the resistant microbial populations resulting from the loss of protozoan grazers or competitors (Crouzet et al., 2010). The number of live bacteria present was generally higher ($P < 0.01$) in linuron amended soil than in unamended soil (Grenni et al., 2009). However, there were no significant differences observed in the functional diversity parameters H, E, and U among the different mesotrione treatments ($P > 0.05$).

Table 1. *Diversity indices of Biolog in soils treated with mesotrione*

Diversity indices	Mesotrione (mg kg⁻¹)	Time (h)							Mean + S.D.
		24	48	72	96	120	144	168	
Shannon index (*H*)	0	1.94±0.08c	2.61±0.17a	2.56±0.15a	2.65±0.11a	2.63±0.10a	2.64±0.07a	2.64±0.11a	2.52±0.26a
	20	2.37±0.01a	2.94±0.12a	2.53±0.12a	2.57±0.10a	2.59±0.09a	2.55±0.07a	2.71±0.05a	2.61±0.18a
	50	2.15±0.11b	2.91±0.18a	2.59±0.19a	2.68±0.26a	2.63±0.14a	2.61±0.15a	2.57±0.13a	2.59±0.26a
	100	2.24±0.04a	2.56±0.34a	2.74±0.17a	2.66±0.15a	2.73±0.12a	2.61±0.13a	2.68±0.15a	2.60±0.22a
		P < 0.01	*P* > 0.05	*P* > 0.05	*P* > 0.05	*P* > 0.05	*P* > 0.05	*P* > 0.05	*P* > 0.05
Shannon evenness index (*E*)	0	2.80±0.12c	2.81±0.64a	2.76±0.64	2.41±0.10a	2.39±0.09a	2.40±0.06a	2.41±0.10a	2.57±0.36b
	20	3.42±0.01a	2.67±0.11a	2.73±0.63	2.78±0.68a	2.80±0.69a	2.32±0.06a	2.47±0.05a	2.74±0.49ab
	50	3.10±0.16b	2.65±0.17a	2.79±0.59	2.86±0.54a	2.84±0.63a	2.81±0.62a	2.77±0.63a	2.83±0.45a
	100	3.23±0.06ab	2.33±0.31a	2.49±0.15	2.42±0.14a	2.49±0.11a	2.81±0.64a	2.44±0.14a	2.60±0.38ab
		P < 0.01	*P* > 0.05	*P* > 0.05	*P* > 0.05	*P* > 0.05	*P* > 0.05	*P* > 0.05	*P* > 0.05
Simpson index (*D*)	0	0.81±0.02b	0.91±0.02ab	0.86±0.04b	0.89±0.03a	0.90±0.02a	0.91±0.00a	0.91±0.00a	0.89±0.04b
	20	0.89±0.00a	0.94±0.00a	0.90±0.00a	0.91±0.01a	0.91±0.00a	0.88±0.02b	0.91±0.01ab	0.90±0.02a
	50	0.87±0.01a	0.94±0.00a	0.90±0.00a	0.91±0.02a	0.91±0.00a	0.90±0.01a	0.89±0.01b	0.90±0.02a
	100	0.87±0.11a	0.87±0.05b	0.91±0.01a	0.91±0.01a	0.92±0.01a	0.90±0.01ab	0.90±0.01ab	0.90±0.03ab
		P < 0.01	*P* < 0.05	*P* < 0.05	*P* > 0.05	*P* > 0.05	*P* < 0.05	*P* > 0.05	*P* < 0.05
McIntosh index (*U*)	0	0.02±0.00b	0.04±0.00b	0.32±0.09b	0.59±0.14c	1.03±0.31b	1.92±0.05a	2.22±0.47a	0.88±0.88a
	20	0.04±0.00ab	0.09±0.01b	0.72±0.11a	1.27±0.11a	1.59±0.17a	1.47±0.25a	2.17±0.11sa	1.05±0.76a
	50	0.04±0.00b	0.08±0.01b	0.42±0.12b	0.85±0.19bc	1.25±0.10ab	1.74±0.13a	2.16±0.10a	0.93±0.79a
	100	0.06±0.03a	0.21±0.07a	0.78±0.14a	0.97±.17b	1.42±0.15a	1.51±0.23a	1.91±0.28a	0.98±0.67a
		P < 0.05	*P* < 0.01	*P* < 0.01	*P* < 0.01	*P* < 0.05	*P* > 0.05	*P* > 0.05	*P* > 0.05

The same letters are not significantly at *P*=0.05 (n=3) between different treatments of mesotrione according to the LSD test

Effect of mesotrione on microbial community structure diversity

The changes in the bacterial structures of the tested soils were elucidated according to PCR–DGGE phylotype. The H and D indices were calculated to estimate the bacterial diversities in the mesotrione-amended and control soils (*Table 2*). The value of H increased after different amounts of mesotrione were applied to the soil, being 0.7-3.6% higher than the H value of the control group. However, differences in the D index were negligible for the different mesotrione treatments. This indicated the stimulation effect or toxicity of the mesotrione on the soil microbial community was small (Hu et al., 2007).

Table 2. *Diversity indices of DGGE in soils treated with mesotrione*

Treatment of mesotrione (mg kg⁻¹)	Shannon index (H)	Shannon evenness index (E)
0	3.02±0.78	0.93±0.13
20	3.13±1.02	0.96±0.24
50	3.04±0.81	0.95±0.34
100	3.12±0.93	0.95±0.29

The bacterial DGGE profiles generated using universal bacterial primers (F357 and R518) revealed the structural compositions of the communities in the soil samples (*Fig. 4a*). The results showed that there are certain differences in the bacterial community diversities in the soils – some bands disappeared and several new bands appeared – following mesotrione application. The disappearance of bands A and B revealed that the bacterial community structures and genetic diversity were affected by the increase in mesotrione concentration. It appeared that the number of bands is related to the mesotrione content. Nine bands (labeled a–i in *Fig. 4a*) were shared in all profiles. This suggested that the bacterial groups represented by these bands may play an important role in mesotrione ecology (Gu et al., 2010). However, the number of bands in the soil samples initially increased with increasing mesotrione content, and then decreased slightly. Significant increases in microbial biomass were found afterwards (compared to the biomass values in unamended soil) in all cases. The microbial biomass was thus significantly increased. Moreover, the similarity indices decreased with increasing mesotrione content (varying from 68.0% to 47.6%). Similarly, the maximum dissimilarity in the bacterial and fungal genetic structures between the control and 100-fold field rate (FR) treated soil did not exceed 12% and 28%, respectively (Crouzet et al., 2010). This may be due to excessive stimulation of cellular redox processes which reflects an unspecified stress response of the microorganisms in the soil.

Clustering of the DGGE profiles was performed (to elucidate the similarities among the different banding patterns) and the results presented using a dendrogram (Zhang and Fang, 2000). The advantage of this method of presentation was that the coherence in the fingerprinting patterns can be rapidly assessed (Fromin et al., 2002). It can be seen that the 20–100 mg kg⁻¹ mesotrione treatments belong to a common cluster which had a similarity among them of over 60%, and 53% difference with the control group profiles (*Fig. 4a*). This showed that the bacterial communities in the soil changed upon addition of herbicide. El Fantroussi et al. (1999) compared the microbial communities in soil treated with three phenylurea herbicides (diuron, linuron, and chlorotoluron) using cluster analysis. They found that the microbial communities were significantly different compared to untreated soil.
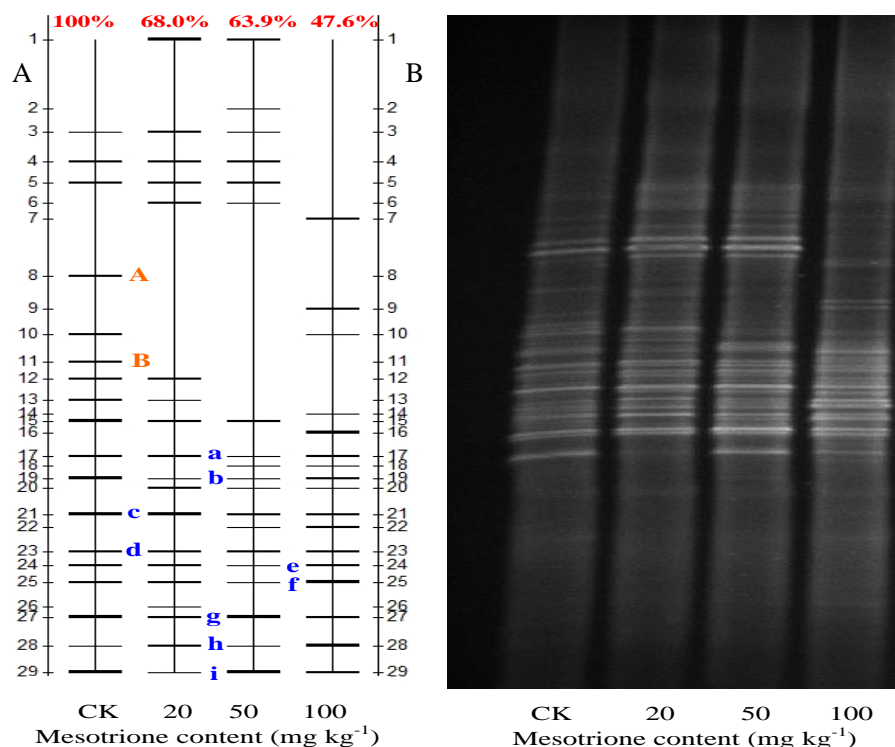
Figure 4. *Comparison of bacterial communities between the control and the three treatments. Note: (A) the analysis results of lane comparison from the DGGE gels using Quantity One V4 4.0.0 software, and (B) DGGE profiles for PCR-amplified fragments of 16 sDNA. A, B, and a-i refer the bands of bacterial DGGE profiles*

To obtain insight into the identities of the major bacterial populations, the discernible DGGE bands in the different mesotrione-treated samples (*Fig. 4b*) were all excised and used for nucleotide sequence analysis. Then, a BLAST search was conducted using the GenBank database to determine their phylogenetic types (*Table 3*). The sequences from bands a, c, and d were found to be closely related to members of the genus *Desulfitobacterium sp*. These are sulfate-reducing bacteria that can grow using meta-chlorinated benzoic acids as electron acceptors and are known to dechlorinate chlorinated phenols and other chlorinated compounds (Yoshida et al., 2007; Jing et al., 2013). The 16S rDNA of the isolated band b closely matched that of *Herbaspirillum sp.* (98%). Bacteria of this genus fall into the β-*Proteobacteria* class and include known nitrogen-fixing endophytes (Doty et al., 2009) and can enhance the phytoremediation of volatile organics and herbicides (Ryan et al., 2008). Band e can be identified (99%) with *Burkholderia caledonica* which had been found to degrade compounds such as herbicides (Hunter and Shaner, 2012), pentachlorophenols (Caliz et al., 2011), and a variety of aromatic hydrocarbons (Stopnisek et al., 2015). Band f showed 100% sequence similarity to a taxonomically unidentified bacterium. Bands g and h were identical to those from strains *Bacillus subterraneus* and *Clostridium sp.*, with 100% similarity. This also showed the capacity of these strains to acclimatize to organic compound pollution. The 16S rDNA sequences of band i were 100% similar to the 16S rDNA sequences of *Bacillus sp*. These bacteria commonly occurred in mesotrione-treated soil and was able to completely and rapidly biotransform mesotrione (Batisson et al., 2009). Biodegradation assays showed that *only* the *Bacillus sp*. strain

was able to completely and rapidly bio-transform mesotrione (Durand et al., 2006a, 2006b; Batisson et al., 2009). This is because of the bacterial reductase that is involved in the enzymatic step in which the nitro group of mesotrione is reduced to an hydroxylamine moiety during the degradation process (Durand et al., 2010).

Table 3. *Sequence analysis of bands excised from DGGE gels derived from bacterial 16S rDNA fragments*

Band	Closest organisms in the GenBank database	Accession no.	Similarity (%)
a	*Desulfitobacterium sp.*	AB596883.1	99
b	*Herbaspirillum sp.*	AB769218.1	98
c	*Desulfitobacterium sp.*	AB596883.1	98
d	*Desulfitobacterium sp.*	AB596883.1	97
e	*Burkholderia caledonica*	KM019850.1	99
f	*Uncultured bacterium*	KM153196.1	100
g	*Bacillus subterraneus*	LN774202.1	100
h	*Clostridium sp.*	JQ420069.1	100
i	*Bacillus sp.*	KJ769178.1	100

Conclusions

The results described in this study show that the treatment of soil with mesotrione was able to stimulate 7.5–92.5% and 27.1–44.4% increase for catalase and invertase activity, while urease activity was inhibited by 11.9–23.2%. CLPPs were obtained that reflect the increase in the amount and rate of carbon utilization of carbohydrate, amino acid polymer and phenolic acid observed in soil subjected to different mesotrione treatments. In contrast, the AWCD of carboxylic and acid amine was inhibited after the application of 50–100 mg kg^{-1} and 20–100 mg kg^{-1} mesotrione, and principal component analysis plots showed that microbial function diversity changed with mesotrione level and each substrate guild. The resulting diversity indices (*H*, *D*, *E*, and *U*) indicate that mesotrione-treated soil had a greater number of more active microorganisms. Our DGGE analysis showed that mesotrione caused slight changes to be made to the biodiversity of the soil bacteria. However, the appearance of some new bands in the DGGE profiles suggests that mesotrione treatment does not have significantly toxic effects on the soil function. In future, it would be useful if further research on the impact of mesotrione could be more integrated with the farming procedures employed, especially herbicide continuous and extensive use. This should provide a more comprehensive understanding of the response of the microbial community in the soil.

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