

Effect of tetraconazole application on the soil microbial community

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Abstract Tetraconazole is one of the most commonly used triazole fungicides in agricultural practice, and its continuous application poses a potential risk for non-target soil microorganisms. Therefore, the objective of this study was to evaluate the effect of tetraconazole at the field rate (T1, 0.33 mg kg⁻¹ of soil), three times the field rate (T3, 1.00 mg kg⁻¹ of soil) and 10 times the field rate (T10, 3.33 mg kg⁻¹ of soil) on the soil microorganisms. To ascertain this effect, the tetraconazole concentration and the microbial properties with potential as bioindicators of soil health (i.e. microbial biomass C, basal respiration, substrate-induced respiration, structure diversity and functional community profiling) were determined. The results showed that the degradation half-lives of tetraconazole varied from 69 to 87 days, depending on the three application concentrations. The microbial biomass C, basal respiration and substrate-induced respiration were inhibited, but they tended to recover at the end of the incubation when tetraconazole was applied at the recommended field rate. The ratios of the gram-negative to gram-positive (GN to GP) bacteria decreased, and the fungi to bacteria ratio increased after a temporal decrease on the seventh day. A principal component analysis of the PLFAs showed that tetraconazole application significantly shifted the microbial community structure on day 7. Different functional community profiles

were observed, depending on the tetraconazole application rates. It was concluded that tetraconazole application decreases the soil microbial biomass and activity and changes the structures of the soil microbial community.

Keywords Tetraconazole · PLFA · Biolog EcoplatesTM · Soil health · Soil quality

Introduction

In recent decades, the requirement to control diseases in fruits, vegetables and cereal grains has led to a marked increase in the use of fungicides (Smith et al. 2000; Zhang et al. 2013). Although the use of pesticides has led to significant economic profits in agriculture, it has also caused serious environmental pollution. A large proportion of any fungicide application reaches the soil where it interacts with organic and mineral constituents and undergoes biological and chemical transformations. Microbial degradation is the primary route for these transformations; therefore, it is the key process affecting the dynamics of fungicide residues in the environment, including their persistence in soil and their susceptibility to leaching (Aislabie and Lloyd-Jones 1995). However, pesticides can exert non-target effects on soil microbial communities, negatively affecting soil health (Lupwayi et al. 2010). Some soil microorganisms are very sensitive to pesticide inputs, thus soil microbial properties, particularly those related to the biomass, activity, structure and functional diversity of soil microbial communities, could be the most useful indicators of the effect of the pesticide disturbances on soil health.

Tetraconazole is an important triazole fungicide synthesised as a novel sterol demethylation inhibitor (DMI) fungicide with protective, curative and eradicant actions (Cairoli et al. 1996; Khalfallah et al. 1998). Tetraconazole is effective in controlling a broad spectrum of diseases, such as powdery

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mildew and rust on fruits, vegetables and cereals worldwide (Menkissoglu-Spiroudi et al. 1998). Tetraconazole has a relatively high soil organic carbon binding coefficient (Koc values ranging from 531–1922), as reported in the EPA Pesticide Fact Sheet (2005), and it is very persistent in water ($t_{1/2}$ =107–215 days) (Alam et al. 2013). Additionally, several studies have reported that the pesticides having the most significant effect on the soil microbial community were broad-spectrum killing and inhibiting fungicides (Bending et al. 2007; Chen and Edwards 2001; Wu et al. 2012). To prevent potential tetraconazole-induced adverse effects on the soil ecosystem, this important research is required to gain a better understanding of its degradation in soil and to provide a more accurate evaluation of its effect on soil microbial communities.

The primary goals of this study were the following: (i) to quantify the dissipation kinetics of tetraconazole when added at three different rates (0.33, 1.00 and 3.33 mg kg⁻¹) to soil samples with no previous known history of pesticide application, (ii) to evaluate the tetraconazole effect on soil microbial communities and (iii) to monitor the recovery of soil health after tetraconazole application. To our knowledge, this is the first evaluation of the effect of tetraconazole on soil microbial communities and soil health using simultaneous measurements of microbial biomass, activity, structure and functional diversity.

Materials and methods

Soil collection

Soil was collected from an area with no previous known history of pesticide application at the Shang Zhuang farm located in Beijing, China. The selected soil is a silty loam soil, and its properties are as follows: sand 35.6 %, clay 3.1 %, silt 61.3 %, organic matter content 17.5 g/kg, ammonium nitrogen 9.1 mg/kg, nitrate nitrogen 7.9 mg/kg, available phosphorus 11.3 mg/kg, rapid available potassium 239.0 mg/kg and pH 7.78. This soil was chosen because it is commonly encountered in mid-eastern China and is widely used in land application programs. An aggregate sample was generated by collecting soil from six separate cores with diameters of 10 cm from the surface soil (the depth was 0–15 cm) across the field. The soil samples were well pooled, sieved to 2 mm, adjusted to a soil moisture of 40 % water-holding capacity (WHC) and then incubated at 25 °C for 10 days. Plant residues and visible animals (e.g. earthworms and nematodes) in the soil were removed manually.

Soil incubation experiment

A series of pots (7×10 cm) containing 150 g DW soil was prepared. Tetraconazole (0.1 ml) (purity ≥98.47 %, Italy Isagro

Co., Ltd, Shanghai, China) dissolved in acetone was added to each pot at three application levels, i.e. the recommended dosage (T1, 50 g a.i./ha, registered amount on strawberries in China, 0.33 mg tetraconazole/kg DW soil), three-fold (T3, 1.00 mg/kg) and 10-fold (T10, 3.33 mg/kg) the recommended dosage. The control pot received 0.1 ml acetone. Each pot was well homogenised, and the soil moisture was maintained at 40 % WHC with deionised water. Each experiment was conducted in triplicate. The pots were covered with perforated polypropylene sheets and incubated in the dark at 25±2 °C. Throughout the incubation period, sterile deionised water was added to the soil to compensate for any water loss that exceeded 5 % of the initial amount added. The pots were randomly removed from the environmental chamber after various incubation time intervals (7, 15, 30, 45, 60 and 90 days), and the soils were then analysed for biochemical and microbial properties and the concentrations of tetraconazole as described below.

Determination of tetraconazole

The soil samples (10 g DW) were extracted by ethyl acetate and subsequently cleaned up using primary secondary amine (PSA) absorbent (Agela, Beijing, China) prior to GC-MS/MS analysis (Xu et al. 2011). Chromatographic separation of tetraconazole was performed on a Varian 450 GC system with an 1177 Series split-splitless auto-injector and an 8400 Series autosampler. The column temperature was initiated at 100 °C and held for 5 min and was then increased to 280 °C and held for 3 min at the rate of 15 °C/min. The temperature of the injector port was 250 °C, and a volume of 1 µL was injected in the splitless mode.

A 300 triple-quadrupole mass spectrometer (Varian, USA) operating in the electron ionisation mode at 70 eV was used for MS/MS detection. The temperature of the transfer line, manifold and ionisation source were set to 280, 40 and 250 °C, respectively. The electron multiplier voltage was set to 1,200 V to perform multiple reaction monitoring, and the solvent delay was set to 10.0 min. The scan time was 0.7 s. The precursor ion was m/z 336, its product quantitative ion was m/z 204 and its qualitative ions were m/z 155 when the collision energy was set to 35 V. Under these conditions, the total running time was 20.0 min, and the retention time of tetraconazole was 14.61 min.

Microbial biomass C and soil respiration experiment

The microbial biomass C (C_{mic}) was determined by the chloroform fumigation-extraction method, as reported by Lin et al. (Lin and Liu 1999). A fresh soil sample (20 g DW) was exposed to ethanol-free chloroform for 24 h and extracted with 0.5 M K₂SO₄ (40 ml). The other soil sample of equal weight was not fumigated but was extracted under the same

conditions. The microbial biomass C was calculated by the equation $C_{mic}=2.64 E_C$, where $E_C=(C \text{ extracted from fumigated soil})-(C \text{ extracted from non-fumigated soil})$, with 2.64 being a conversion factor. For determination of the basal respiration (R_B , which is an indicator of overall microbial activity), a beaker containing 10 mL of 0.2 M NaOH was placed into each 1,600 ml airtight plastic drum with the soil sample (20 g DW) to capture the evolved CO_2 ; the drums were incubated for 24 h at 25 °C. The soil controls with vials containing 10 ml of 0.2 M NaOH only were also included along with a no-soil blank. The CO_2 released from the incubated soil samples was trapped in 10 mL of 0.2 M NaOH and determined by titration with HCl. The substrate-induced respiration (SIR, which is an indicator of potentially active microbial biomass) was determined by adding 10,000 mg carbon (glucose)/kg DW soil, as reported by Muñoz-Leoz B et al. (Muñoz-Leoz B et al. 2011) and Epelde et al. (Epelde et al. 2008), to the soil samples. Subsequently, the CO_2 evolution was measured after 6 h of incubation, as previously mentioned for the determination of the basal respiration. The experiments to determine these parameters were performed in triplicate.

From these three parameters (basal respiration, substrate-induced respiration and microbial biomass C), the ecophysiological indices, which reflect the environmental stress in soil microbial populations and communities (Anderson and Domsch 1985), and concomitantly, the soil health were calculated, i.e. the microbial metabolic quotient (qCO_2) or the ratio of basal respiration to microbial biomass C ($qCO_2=R_B/C_{mic}$) and the respiratory quotient Q_R or the ratio of basal respiration to substrate-induced respiration ($Q_R=R_B/SIR$).

Phospholipid fatty acid experiment

The phospholipid fatty acid (PLFA) experiment was conducted based on the method described by Bossio et al. with minor modifications (Bossio and Scow 1998). The freeze-dried soil samples (5 g) were extracted using a one-phase mixture of $CHCl_3/CH_3OH/citric \text{ acid buffer}$ (0.15 mol L^{-1} , pH 4) (1:2:0.8, v/v/v). The $CHCl_3$ layer was collected and dried under N_2 for lipid fractionation. The extracted lipids were separated on silica gel columns (Supelco, Inc. USA) into glycol lipids, neutral lipids and polar lipids. The polar lipids were then transesterified with methanolic KOH to recover the PLFAs as methyl esters through methanolysis in hexane. Finally, the hexane supernatant containing the resultant fatty acid methyl esters (FAMES) was separated, quantified and identified by gas chromatography mass spectrometry (GC-MS).

A Polaris Q ion-trap GC-MS (Thermo Fisher Scientific, Inc. USA) with an HP-5 ms column (60-m \times 0.25-mm inner diameter, 0.25-m film thickness) was used for FAME identification. A 1- μ l injection with a 1:50 split was analysed at an

initial temperature of 140 °C held for 3 min, then ramped to 190 °C at 4 °C/min, held for 1 min, ramped to 230 °C at 3 °C/min, held for 1 min, ramped to 250 °C at 2 °C/min, held for 1 min, ramped to 280 °C at 10 °C/min and held for 5 min at a constant flow rate of 1 ml/min. The peaks were identified using bacterial fatty acid standards and NIST MS Search 2.0 software. The microbial biomass was evaluated using the following 17 fatty acids: 14:0, i15:0, a15:0, 15:0, 16:0, i16:0, 16:1 ω 7c, 17:0, i17:0, cy17:0, 18:1 ω 9c, 18:1 ω 9t, 18:2 ω 6, 9, 18:0, 10Me18:0, cy19:0 and 20:0. The branched phospholipids i15:0, a15:0, i16:0 and i17:0 were used as indicators of gram-positive (GP) bacteria, whereas the phospholipids 16:1 ω 7c, cy17:0 and cy19:0 were indicative of gram-negative (GN) bacteria. The fungal biomass was evaluated by quantifying 18:1 ω 9c, 18:1 ω 9t and 18:2 ω 6, 9 (Zak and Kling 2006). The total PLFAs (a good indicator of the living microbial biomass) (Baath and Anderson 2003) were the sum of all of the microbial phospholipids mentioned. The ratios of GN/GP and fungal/bacterial fatty acids were used to study changes in the microbial community structure for the different treatments (Bardgett et al. 1996). Prior to being subjected to principal component analysis (PCA), the results were expressed as a percentage of the total PLFAs.

Community level physiological profiles experiment

The community level physiological profiles (CLPPs) of potential metabolic functions were evaluated using Biolog Eco microplates (BIOLOG, Hayward, USA), as reported by Govaerts et al. (Govaerts et al. 2007). A soil sample (10 g DW) was extracted using sterile saline solutions (0.85 %, m/v). The supernatant was serially diluted to the 10^{-3} dilution. The addition of 150 μ L of the suspension to each well of the Biolog Ecoplates was performed, and the microplates were then incubated at 25 ± 2 °C in the dark. The colour development in the plates was measured every 24 h at 590 nm for 7 days using a BIO-TEK Elx808 automated microplate reader (Biolog, Hayward, CA, USA). The average well colour development (AWCD) and the Shannon, Simpson and McIntosh indices were determined by calculating the mean absorbance value of each well after 96 h of incubation, which corresponded to the time of maximal microbial growth in the Biolog EcoPlates. The absorbance values were calculated as follows: $AWCD=\sum OD_i/31$, where OD_i is the optical density value of each well after the water blank subtraction. The Shannon index (H'), which was used as a measure of richness and evenness, was calculated as follows: $H'=-\sum P_i \times \ln(P_i)$, where P_i is the ratio of the activity of each substrate (OD_i) to the sum of the activities of all of the substrates $\sum OD_i$. The Simpson index (D), which was used as a measure of the dominance index sensitive to the abundances of the most common species, was calculated as follows: $D=1-\sum(P_i)^2$, where P_i is the ratio of the activity of each substrate (OD_i) to the sum of the

activities of all of the substrates $\sum OD_i$. The McIntosh index (U), which was used as a measure of diversity based on the Euclidian distance of the community of the assemblage in an S dimensional hypervolume, was calculated as follows:

$U = \sqrt{\sum (n_i^2)}$, where n_i is the absorbance value of the i th well (Staddon et al. 1997).

Data analyses

Significant differences were accepted at $p < 0.05$, $p < 0.01$ or $p < 0.001$. The data on the soil properties were analysed using a two-way analysis of variance (ANOVA) with the tetraconazole concentration and the incubation time as the factors. The values were considered to be significantly different at a 95 % confidence level. The values in the figures and tables correspond to the average of triplicate data ($n=3$) \pm standard deviations (SD). The principal component analysis (PCA) was applied to examine the PLFAs among the different samples containing multiple variables. The statistics were calculated using the SAS 9.1 software.

Results

Degradation of tetraconazole

The degradation process of tetraconazole followed the classical first-order kinetic reaction, $C_t = C_0 e^{-kt}$, with a correlation coefficient (R^2) higher than 0.9033, based on the measurement of the analyte peak areas (Table 1). The degradation dynamic curve of tetraconazole in the soils showed the correlation between the tetraconazole residue concentration and the incubation time (Fig. 1). The degradation rates of tetraconazole were dependent on its application concentration, with half-lives of 69, 77 and 87 days for T1, T3 and T10, respectively. After the 90-day incubation, 39, 47 and 54 % of the initial tetraconazole concentration remained in the soil for T1, T3 and T10, respectively.

Microbial biomass C and respiration activity analysis

The microbial biomass C in all of the treatments varied significantly with different incubation times ($F_{\text{time}} = 181.2$,

Table 1 Regression equation of tetraconazole degradation dynamics in soils

| Treatment | Regression equation | Coefficient of correlation (R^2) | $T_{1/2}$ (day) |
|-----------|-------------------------|--------------------------------------|-----------------|
| T1 | $y = 0.2949e^{-0.01x}$ | 0.9382 | 69 |
| T3 | $y = 1.0947e^{-0.009x}$ | 0.9176 | 77 |
| T10 | $y = 3.5555e^{-0.008x}$ | 0.9033 | 87 |

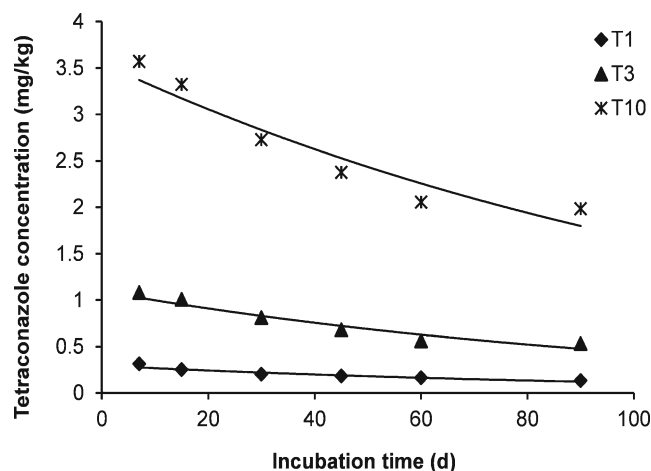


Fig. 1 Degradation dynamic curves of tetraconazole in soils

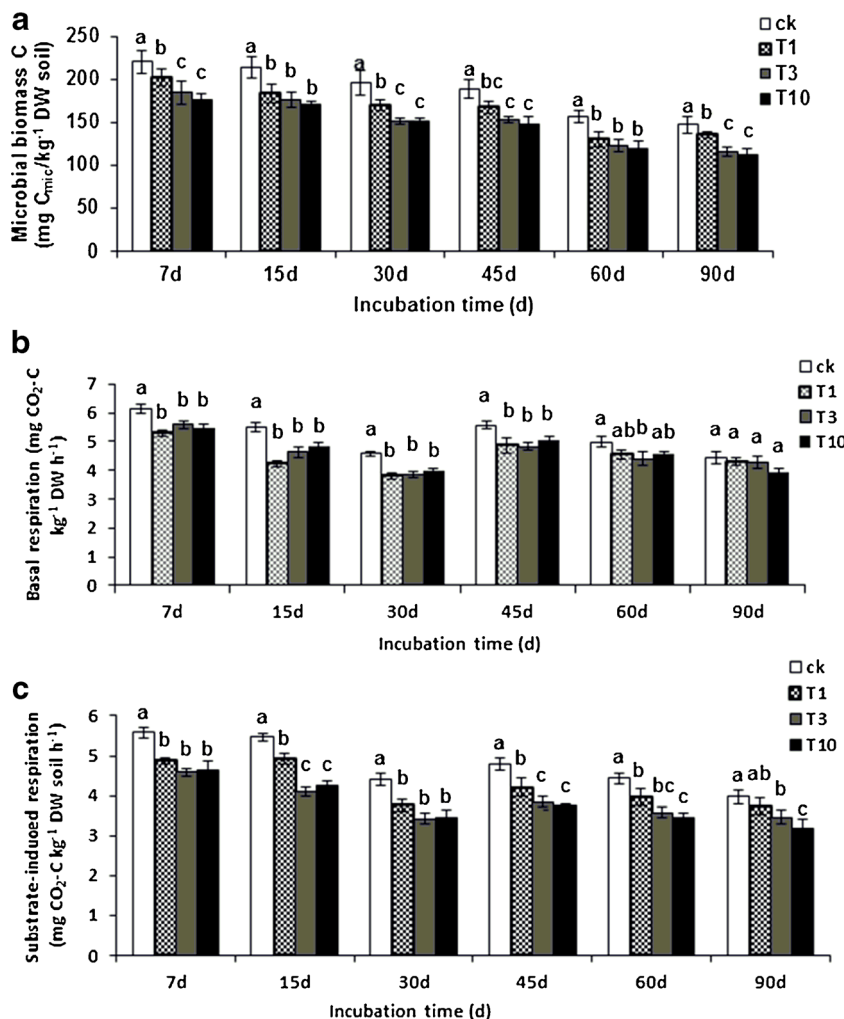
$p < 0.001$) and tetraconazole treatments ($F_{\text{treatment}} = 139.7$, $p < 0.001$). At day 30, the values of the microbial biomass C in the T10 soils were 65.9 % lower than in the control soils. Compared with the control, the microbial biomass C decreased in all of the tetraconazole treatments over the entire incubation period (Fig. 2a). A significant decrease in soil respiration was observed in the three tetraconazole treatments compared with the controls up to the 45th day (on average, the values were 17.4 and 12.0 % lower in the tetraconazole treatments than in the controls at day 15 and day 45, respectively, Fig. 2b). The basal respiration of the tetraconazole treatments recovered at the 90th day. The substrate-induced respiration decreased in all of the treatments, and the T1 treatments showed no difference compared with the controls until the 90th day (Fig. 2c). At day 15, it was found that a higher tetraconazole concentration caused a lower substrate-induced respiration (4.9, 4.3 and 4.2 mg $\text{CO}_2\text{-C kg}^{-1}$ DW soil h^{-1} at T1, T3 and T10, respectively).

The microbial metabolic quotient ($q\text{CO}_2$) is the ratio of basal respiration to microbial biomass C ($q\text{CO}_2 = R_B / C_{\text{mic}}$) and is used as an indicator of microbial contamination stress and a change in the soil microbial community. Figure 3a shows that the value of $q\text{CO}_2$ in the T3 and T10 soil treatments was higher than the controls over the entire period. Similarly, the respiratory quotient Q_R , the ratio of basal respiration to substrate-induced respiration ($Q_R = R_B / \text{SIR}$), was also calculated to evaluate the soil health. Again, a higher tetraconazole concentration had a significant effect on the Q_R values (Fig. 3b) compared with the control up to the 90th day.

Analysis of the PLFA experiments

The total PLFAs decreased significantly ($F_{\text{treatment}} = 47.3$, $p < 0.001$) in the tetraconazole-treated soils (T1, T3 and T10) compared with the controls over the entire incubation period (Tables 3, and 4). At day 90, the values of the total PLFAs in the T3 and T10 soils were 22.9 and 30.1 % lower than in the

Fig. 2 Effect of tetraconazole on soil microbial biomass carbon (a), basal respiration (b) and substrate-induced respiration (c); the mean values ($n=3$)±SD followed by different letters indicate significantly different results according to ANOVA with Duncan’s multiple-range test ($p<0.001$)



control soils, respectively. Generally, the values of the total PLFAs in the higher tetraconazole-treated soils (T3 and T10) did not recover at the end of the incubation time.

The amount of total fungi decreased temporarily and then increased, whereas the amount of total bacteria decreased over the entire incubation period of the tetraconazole treatments compared with the controls. Both values of the GN and GP bacteria in the tetraconazole-treated soils were lower than the controls. The ratios of the GN/GP bacteria in the tetraconazole treatments ($F_{\text{treatment}}=4.5, p<0.01$) tended to decrease compared with the controls during the incubation period. The ratios of fungi to bacteria significantly ($F_{\text{treatment}}=13.3, p<0.001$, Tables 3 and 4) increased after a temporal decrease on the seventh day.

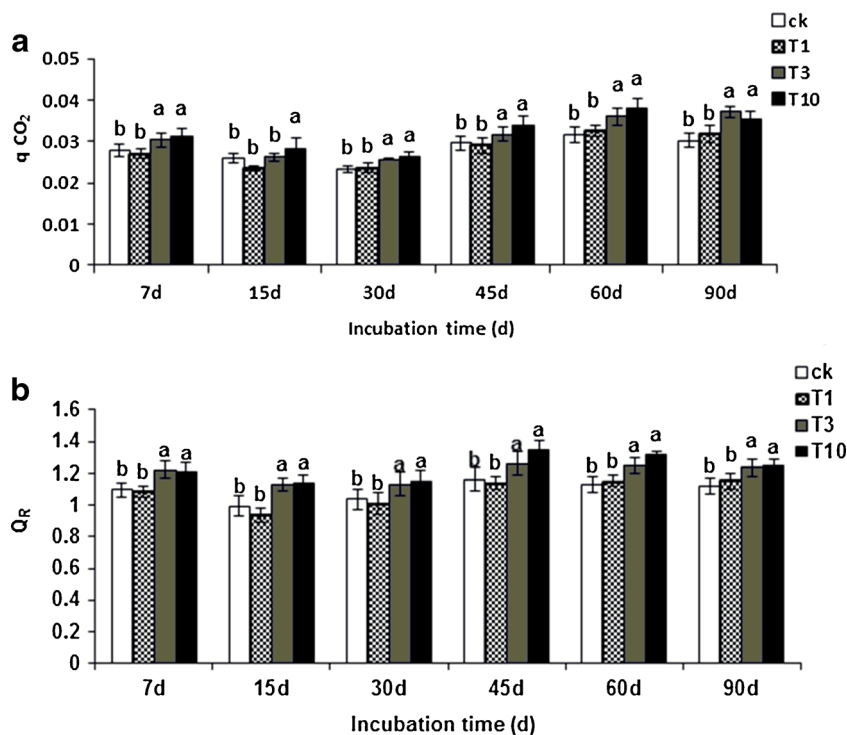
The PCA showed that the differences in the community structure of the treated and untreated soils were observed on successive sampling days (Fig. 4). However, the primary differences in the PLFA profiles were due to the incubation time. In our studies, the most significant differences between the microbial populations in both the treated and control soils were observed on day 7. At the end of the experimental

period, the microbial communities were only slightly affected by tetraconazole. The first principal component (PC1= 41.85 %) and the second principal component (PC2= 21.44 %) explained 63.29 % of the variation and discriminated between the samples treated with fungicide and the controls. Eight PLFAs were strongly correlated ($|r|\geq 0.6$, where $|r|$ is the absolute value of the correlation coefficient) with the PC1, including three gram-positive bacteria (i15:0, a15:0 and i17:0), two gram-negative bacteria (cy17:0 and cy19:0) and three saturated fatty acids (16:0, 17:0 and 20:0). Two PLFAs were strongly correlated with the PC2, including a gram-negative bacteria (16:1 ω 7c) and a fungal signature (18:2 ω 6, 9). The structures of the microbial communities in the control samples were distinct compared with the tetraconazole-treated samples on the 90th day.

Community level physiological profiles analysis

The AWCD, Shannon, Simpson and McIntosh indices were calculated from the Biolog EcoplatesTM data from the 96- h

Fig. 3 Effect of tetraconazole on the metabolic quotient (a) and the respiratory quotient (b); the mean values ($n=3$) \pm SD followed by different letters indicate significantly different results according to ANOVA with Duncan's multiple-range test ($p<0.001$)



incubation. The values of the AWCD in the T1 treatment soils had no significant changes compared with the control soils at the end of the incubation time, but the higher tetraconazole treatments (T3 and T10) displayed lower AWCD values than the control soil and did not recover after 90 days (Table 2). The values of the Shannon (H'), Simpson (D) and McIntosh (U) indices tended to be consistent with the AWCD.

Discussion

In this study, tetraconazole was slowly degraded, with half-lives ranging from 69 to 87 days, depending on the three application concentrations, and the kinetics were largely dependent on the applied tetraconazole concentrations. The higher concentration of tetraconazole-treated samples showed a lower degradation rate. Our result was consistent with other

Fig. 4 A principal component plot generated from the phospholipid fatty acid profiles obtained from the control (C), T1 (0.33 mg kg^{-1} of soil), T3 (1.00 mg kg^{-1} of soil) and T10 (3.33 mg kg^{-1} of soil) treated soils on days 7, 15, 30, 45, 60 and 90

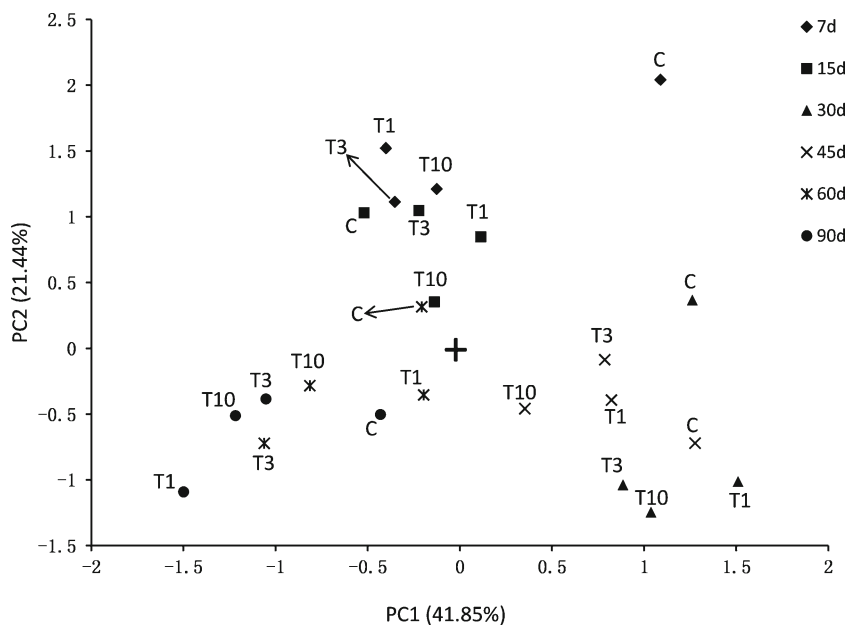


Table 2 Total PLFAs, bacterial and fungal biomass expressed as the amount of marker phospholipid fatty acids (nmol g⁻¹ DW soil) in tetraconazole-treated soil

| Time (day) | Treatment | Total PLFAs | Total fungi | Total bacteria | Gram-negative bacteria (GN) | Gram-positive bacteria (GP) | GN/GP | Fungi/bacteria |
|------------|-----------|--------------|-------------|----------------|-----------------------------|-----------------------------|-------------|----------------|
| 7 | CK | 26.42±1.30a | 5.85±0.06a | 14.36±1.23a | 3.97±0.21a | 6.15±0.23a | 0.65±0.01a | 0.41±0.01a |
| | T1 | 22.96±0.42b | 5.37±0.06b | 14.00±0.41b | 3.43±0.08b | 5.61±0.27b | 0.61±0.01b | 0.38±0.01b |
| | T3 | 19.04±1.02b | 5.25±0.04b | 12.99±0.99c | 3.32±0.17b | 5.61±0.32b | 0.59±0.01b | 0.40±0.01a |
| | T10 | 18.37±1.00b | 5.11±0.01b | 12.82±0.41c | 3.25±0.19b | 5.44±0.45b | 0.60±0.02b | 0.40±0.02a |
| 15 | CK | 24.24±0.36a | 4.48±0.04a | 12.67±0.60a | 3.62±0.29a | 5.35±0.14a | 0.68±0.01a | 0.35±0.01b |
| | T1 | 23.16±0.66a | 4.39±0.07ab | 12.34±0.60b | 3.16±0.12b | 5.32±0.25a | 0.59±0.02b | 0.36±0.02b |
| | T3 | 22.08±0.82ab | 4.35±0.07b | 12.19±0.74b | 3.09±0.05b | 5.27±0.15ab | 0.59±0.01b | 0.36±0.01b |
| | T10 | 21.40±0.37b | 4.38±0.07ab | 11.58±0.74c | 2.97±0.05b | 4.93±0.04b | 0.60±0.01b | 0.38±0.01a |
| 30 | CK | 23.40±0.93a | 1.96±0.00b | 20.24±0.35a | 3.81±0.05a | 11.92±0.15a | 0.32±0.01a | 0.10±0.01b |
| | T1 | 19.61±0.92b | 2.06±0.02b | 19.27±0.44b | 3.11±0.06b | 11.18±0.07b | 0.28±0.01b | 0.11±0.01b |
| | T3 | 15.35±0.93c | 2.42±0.02a | 18.48±0.93c | 2.88±0.17b | 10.77±0.30b | 0.27±0.01b | 0.13±0.01a |
| | T10 | 14.32±0.92c | 2.37±0.02a | 18.46±0.94c | 2.83±0.43b | 10.60±0.22b | 0.27±0.03b | 0.13±0.03a |
| 45 | CK | 23.78±1.2a | 1.07±0.03b | 18.53±1.18a | 2.93±0.27a | 11.12±0.50a | 0.26±0.00a | 0.06±0.00b |
| | T1 | 21.91±1.13b | 2.06±0.02a | 18.39±1.08b | 2.50±0.23b | 11.05±0.50a | 0.23±0.01b | 0.11±0.01a |
| | T3 | 14.07±0.25c | 1.99±0.01a | 17.97±0.27b | 2.40±0.23b | 10.85±0.12b | 0.22±0.03b | 0.11±0.01a |
| | T10 | 13.23±0.61c | 1.85±0.04a | 17.90±0.55b | 2.37±0.14b | 10.61±0.20b | 0.22±0.00b | 0.10±0.00a |
| 60 | CK | 22.19±0.93a | 4.06±0.00b | 13.72±0.35a | 3.23±0.05a | 6.75±0.15a | 0.48±0.01a | 0.30±0.00b |
| | T1 | 22.08±0.92a | 4.10±0.00b | 13.39±0.44b | 3.02±0.06a | 6.53±0.07a | 0.46±0.01ab | 0.31±0.01b |
| | T3 | 20.96±0.93ab | 4.15±0.02b | 12.58±0.93c | 2.32±0.17b | 6.02±0.30b | 0.39±0.01b | 0.33±0.01b |
| | T10 | 20.54±0.92b | 4.36±0.02a | 11.19±0.94d | 2.31±0.43b | 5.85±0.22b | 0.39±0.03b | 0.39±0.01a |
| 90 | CK | 21.80±1.2a | 3.48±0.03b | 12.76±1.18a | 5.04±0.27a | 6.30±0.50a | 0.80±0.00a | 0.27±0.03b |
| | T1 | 20.80±0.25a | 3.74±0.02a | 12.38±1.08b | 4.54±0.23b | 6.12±0.50ab | 0.74±0.01b | 0.30±0.00ab |
| | T3 | 16.81±0.25b | 3.68±0.01a | 11.64±0.27c | 4.30±0.23b | 5.88±0.12b | 0.73±0.03b | 0.32±0.01a |
| | T10 | 15.24±0.61b | 3.82±0.04a | 11.61±0.55c | 4.28±0.14b | 5.76±0.20b | 0.74±0.00b | 0.33±0.03a |

Different letters indicate significant differences ($p < 0.05$) among treatments at a given incubation time (ANOVA with Duncan’s multiple range test). Mean values ($n = 3$) ± S.E

triazole fungicide experiments conducted by Jui-Hung Yen et al. (Yen et al. 2009). Jui-Hung Yen et al. have proven that microorganisms play an important role in triadimefon dissipation, and soil microbes were inhibited by a higher concentration of propiconazole.

In our experiment, tetraconazole application decreased the microbial biomass C over the entire incubation period. The result showed that tetraconazole inputs have a significantly negative effect on soil microbial biomass. A similar result has been found with other triazole fungicides (Gonçalves et al. 2009; Hart and Brookes 1996; Muñoz-Leoz et al. 2011). This adverse effect on soil microbial communities was also reflected by the observed reduction in basal respiration and substrate-induced respiration. Ecophysiological indices, such as qCO_2 and Q_R , have been applied for the quantification of environmental effects on the microbial community in soils (Anderson and Domsch 1985; Anderson and Domsch 2010). For the high concentration treatments (T3 and T10), an increase in the Q_R or qCO_2 values was observed, which can represent a harmful effect of tetraconazole on microorganisms, and the negative effect on the soil quality

was not recovered during the incubation time. A higher respiratory activity for the same microbial biomass is indicative of higher levels of maintenance energy and may be an indication of a lower metabolic efficiency due to a fungicide-induced inhibitory/stressing effect (Gomez et al. 2009).

Dighton has argued that to gain a true sense of the functions of a microbial community, the evaluation must include the microbial communities and their population compositions (Dighton 1997). PLFA is a rapid, accurate and efficient biochemical method that can provide detailed information about the structure of the active microbial community and a measurement of its biomass (Kaur et al. 2005). In our experiment, the total PLFA decreased compared with the control was closely correlated with the microbial biomass C and had a significant difference with the controls up to the 90th day. The total fungi tended to increase following a temporary decrease on the 7th day, which is consistent with the results from soil samples treated with napropamide (Cycoñ et al. 2013).

The PLFA analysis showed an interesting phenomenon in which the total PLFA decreased in the tetraconazole

Table 3 Effect of tetraconazole on soil community-level physiological profiles from Biolog data at 96 h incubation, after tetraconazole application for all treatments

| Incubation time (day) | Tetraconazole concentration | | | |
|-----------------------|-----------------------------|-------------|-------------|-------------|
| | Control | T1 | T3 | T10 |
| AWCD | | | | |
| 7 | 0.35±0.05a | 0.27±0.03b | 0.20±0.01c | 0.19±0.04c |
| 15 | 0.17±0.03a | 0.14±0.01b | 0.13±0.02b | 0.14±0.04b |
| 30 | 0.15±0.04a | 0.14±0.03a | 0.10±0.01b | 0.11±0.02b |
| 45 | 0.13±0.02a | 0.12±0.01a | 0.10±0.01b | 0.09±0.01b |
| 60 | 0.14±0.01a | 0.13±0.01a | 0.10±0.02b | 0.09±0.01b |
| 90 | 0.13±0.03a | 0.12±0.02ab | 0.10±0.01b | 0.11±0.01b |
| Shannon (<i>H'</i>) | | | | |
| 7 | 2.91±0.03a | 2.71±0.04b | 2.70±0.03b | 2.62±0.02b |
| 15 | 2.82±0.05a | 2.60±0.07b | 2.59±0.01b | 2.57±0.06b |
| 30 | 2.61±0.04a | 2.55±0.05a | 2.47±0.08bc | 2.32±0.04c |
| 45 | 2.66±0.03a | 2.61±0.01a | 2.21±0.05c | 2.26±0.02c |
| 60 | 2.61±0.01a | 2.58±0.03a | 2.48±0.05b | 2.31±0.04c |
| 90 | 2.65±0.04a | 2.61±0.03a | 2.26±0.03b | 2.30±0.01b |
| Simpson (<i>D</i>) | | | | |
| 7 | 13.47±0.07a | 11.08±0.09b | 11.20±0.09b | 11.11±0.05b |
| 15 | 12.64±0.07a | 12.35±0.02a | 9.98±0.06c | 10.66±0.08c |
| 30 | 12.52±0.02a | 11.95±0.09a | 10.15±0.05b | 10.52±0.05b |
| 45 | 11.10±0.04a | 10.90±0.06a | 6.65±0.01c | 8.73±0.06b |
| 60 | 11.41±0.03a | 11.48±0.05a | 10.73±0.05b | 8.85±0.06c |
| 90 | 12.36±0.06a | 12.31±0.02a | 11.20±0.07b | 11.10±0.04b |
| McIntosh (<i>U</i>) | | | | |
| 7 | 1.84±0.01a | 1.67±0.04b | 1.68±0.03b | 1.65±0.04b |
| 15 | 1.64±0.05a | 1.34±0.05b | 1.25±0.01b | 1.22±0.06b |
| 30 | 1.51±0.04a | 1.47±0.03a | 0.97±0.05b | 1.02±0.03b |
| 45 | 1.43±0.04a | 1.35±0.05a | 1.09±0.02b | 0.90±0.01b |
| 60 | 1.27±0.05a | 1.18±0.02a | 0.89±0.02b | 0.94±0.01b |
| 90 | 1.10±0.03a | 0.93±0.01ab | 0.89±0.01ab | 0.79±0.01b |

Different letters indicate significant differences ($p < 0.05$) among treatments at a given incubation time (ANOVA with Duncan's multiple range test). Mean values ($n = 3$) ± S.E

treatments compared with the controls, but the total fungi decreased temporarily before 30 days and increased up to day 90. This result might be explained by a finding by Bending et al. that microbial death caused by pesticide effects can be followed by the growth of organisms using the killed biomass as a substrate (Bending et al. 2007). The results suggested that in addition to its direct effect on fungal populations, tetraconazole caused non-target effects on bacterial communities, as reported in the literature (Jackson et al. 2000). The GN bacteria tended to be fewer than the GP bacteria of the tetraconazole treatments compared with the controls, possibly because the GN bacteria were more sensitive to additional chemical inputs than the GP bacteria because the GP bacteria possess a specific structure of the cell wall and

Table 4 Multivariate analysis of variance by two-way ANOVA for soil microbial properties as affected by tetraconazole concentration (treatment), incubation time (time) and their interaction (treatment × time)

| Factor | R_B | SIR | C_{mic} | qCO ₂ | Q_R | AWCD | <i>H'</i> | <i>U</i> | PLFA _{tot} | F_{tot} | B_{tot} | GN | GP | GN/GP | F/B |
|------------------|---------|----------|-----------|------------------|---------|----------|-----------|----------|---------------------|-----------|-----------|---------|----------|----------|----------|
| Treatment | 12.5*** | 13.7*** | 139.7*** | 33.2*** | 17.6*** | 99.0*** | 12.5*** | 3.5* | 47.3*** | 3.1* | 3.0* | 2.9* | 3.4* | 4.5** | 13.3*** |
| Time | 79.8*** | 145.0*** | 181.2*** | 54.2*** | 17.5*** | 273.1*** | 25.9*** | 4.1** | 60.0*** | 16.3*** | 149.6*** | 36.0*** | 272.2*** | 195.6*** | 153.2*** |
| Treatment × time | 6.7*** | 13.5*** | 5.9*** | 6.6*** | 8.2*** | 16.7*** | 5.1*** | 0.1 | 6.7*** | 4.3** | 1.6 | 2.1 | 1.3 | 2.6 | 1.9 |

R_B basal respiration, SIR: substrate-induced respiration, C_{mic} microbial biomass C, qCO₂ metabolic quotient, Q_R respiratory quotient, AWCD average well color development, *H'* Shannon index, *D* Simpson index, *U* McIntosh index; PLFA_{tot} total PLFA; F_{tot} total fungi, B_{tot} total bacteria, GN gram-negative bacteria, GP gram-positive bacteria; F/B: fungi to bacteria

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

have an increased resistance to environmental stresses (Byss et al. 2008; Huang et al. 2009). Both of the ratio results of GN/GP and fungi/bacteria showed that the tetraconazole changed the soil community structure and composition.

The PCA of the PLFA analysis results suggested that different community structures were identified in soils subjected to different treatments or incubation periods. Several studies have reported a more significant influence of incubation time versus treatment (Böhme et al. 2005; Hund-Rinke et al. 2004). In our study, the structure of the microbial communities in the tetraconazole-treated soils displayed clear differences with the control soils during an earlier application period (day 7). The same correlation between napropamide application and the PLFA patterns has been noticed by Guo et al. (Guo et al. 2009). Using PCR-DGGE profiling of 16S rDNA, Guo et al. have revealed that napropamide significantly increased the number of bands that represented the dominant microbial populations on days 7 and 14 after application. According to the authors, the results suggested that some particular bacteria might adapt to the pesticide applied, hence dominating in the obtained DGGE patterns.

The BIOLOGTM system is the most commonly used method for measuring substrate utilisation patterns, and it is used to monitor the ability of inoculated populations to utilise substrates over time and the speed at which the substrates are utilised (Staddon et al. 1997). Although the Biolog data does not precisely describe the community level physiological profile (CLPP) with limited incubated bacteria, it is a good method for early warning of the functional (catabolic) diversity changes of heterotrophic cultivable bacteria caused by environmental fluctuations and pollution (Ball 2005). The AWCD of the T1 treatments showed no difference compared with the controls on the 90th day, which indicated that tetraconazole applied at the recommend field rate is innocuous to the soil community. The higher tetraconazole concentration (T3 and T10) treatments inhibited the metabolic activity of the soil microbes, as indicated by the AWCD. A similar decrease was observed in the H' , D and U values in the control soils over time (Table 3). Although the Shannon, Simpson and McIntosh indices showed similar trends, they deviated in their ability to resolve statistically significant differences. For example, the Simpson index is weighted toward the abundances of the most common species, whereas the Shannon index is influenced more by the species richness (Magurran and Magurran 1988). In contrast, the McIntosh index is a measure of uniformity (Atlas 1984). Therefore, it is prudent to examine the data with more than one index. Thus, this shift caused by higher tetraconazole concentrations in the functional diversity of the soil bacteria is a long-time response and was not recovered as the fungicide dissipated during the incubation time.

According to two-way ANOVA, several soil properties (R_B , SIR, C_{mic} , qCO_2 , Q_R , AWCD, PLFA_{tot}, among others) were significantly affected by the tetraconazole concentration

(Table 4). A minimal recovery of soil health (as reflected by the values of the soil microbial properties, except for the basal respiration measured herein) was observed in the higher tetraconazole-treated soils (T3 and T10) at the end of our experiment. These persistent harmful effects were acute for soil microbial mass, activity, microbial community structure and functional diversity, indicating that these parameters are the most useful indicators of the short-term adverse effect of tetraconazole on the soil microbial communities, providing a response gradient to a concentration gradient.

Conclusion

This study is the first to use microbial biomass C, soil respiration, PLFA and BIOLOG analysis to investigate the effects of tetraconazole application on the soil microbial community. The results indicated that the higher concentration tetraconazole application (T3 and T10) has a significant effect on the soil microbial biomass, microbial activity, GN bacterial and fungal abundance and microbial functional diversity. Similarly, the ecophysiological indices qCO_2 and Q_R of the higher concentration treatments showed a significant difference compared with the controls, indicating that the application of the high tetraconazole concentration presented a stressful condition, but the effects of tetraconazole applied at the recommended field rate on the function of the soil microbial community were minor. The soil microbial properties (except for the basal respiration) determined using a three-fold and a 10-fold recommended field rate in the tetraconazole-treated soils were not recovered during the entire experiment. These findings suggest that, although the residual pesticide concentration in soil degrades with an extensive incubation, its non-target effects on the soil microbial communities and soil health may persist for significantly longer periods (in our study, >90 days). This fact highlights the requirement to include ecologically relevant measurements (complementary to classical toxicological bioassays), such as the soil microbial properties determined in this study, when evaluating the environmental effect and possible non-target effects of triazole fungicides.

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References

- Aislabie J, Lloyd-Jones G (1995) A review of bacterial-degradation of pesticides. *Soil Res* 33:925–942
- Alam S, Sengupta D, Kole RK, Bhattacharyya A (2013) Dissipation kinetics of tetraconazole in three types of soil and water under laboratory condition. *Environmental monitoring and assessment*, 1–6

- Anderson T-H, Domsch K (1985) Determination of ecophysiological maintenance carbon requirements of soil microorganisms in a dormant state. *Biol Fertil Soils* 1:81–89
- Anderson T-H, Domsch KH (2010) Soil microbial biomass: the ecophysiological approach. *Soil Biol Biochem* 42:2039–2043
- Atlas R (1984) Diversity of microbial communities. *Adv Microb Ecol* 7: 1–47
- Baath E, Anderson TH (2003) Comparison of soil fungi/bacterial ratios in a pH gradient using physiological and PLFA-based techniques. *Soil Biol Biochem* 35:955–963
- Ball AS (2005) Soil health—a new challenge for microbiologists and chemists. *Int Microbiol* 8:13–21
- Bardgett RD, Hobbs PJ, Frostegård Å (1996) Changes in soil fungal: bacterial biomass ratios following reductions in the intensity of management of an upland grassland. *Biol Fertil Soils* 22:261–264
- Bending GD, Rodriguez-Cruz MS, Lincoln SD (2007) Fungicide impacts on microbial communities in soils with contrasting management histories. *Chemosphere* 69:82–88
- Böhme L, Langer U, Böhme F (2005) Microbial biomass, enzyme activities and microbial community structure in two European long-term field experiments. *Agric Ecosyst Environ* 109:141–152
- Bossio D, Scow K (1998) Impacts of carbon and flooding on soil microbial communities: phospholipid fatty acid profiles and substrate utilization patterns. *Microb Ecol* 35:265–278
- Byss M, Elhottová D, Tríska J, Baldrian P (2008) Fungal bioremediation of the creosote-contaminated soil: influence of *Pleurotus ostreatus* and *Irpex lacteus* on polycyclic aromatic hydrocarbons removal and soil microbial community composition in the laboratory-scale study. *Chemosphere* 73:1518–1523
- Cairolì S, Arnoldi A, Pagani S (1996) Enzyme-linked immunosorbent assay for the quantitation of the fungicide tetraconazole in fruits and fruit juices. *J Agric Food Chem* 44:3849–3854
- Chen S-K, Edwards C (2001) A microcosm approach to assess the effects of fungicides on soil ecological processes and plant growth: comparisons of two soil types. *Soil Biol Biochem* 33:1981–1991
- Cycoń M, Wójcik M, Borymski S, Piotrowska-Seget Z (2013) Short-term effects of the herbicide napropamide on the activity and structure of the soil microbial community assessed by the multi-approach analysis. *Appl Soil Ecol* 66:8–18
- Dighton J (1997) Is it possible to develop microbial test systems to evaluate pollution effects on soil nutrient cycling?. *Ecological Risk Assessment of Contaminants in Soil*. Springer, pp. 51–69
- Epelde L, Hernández-Allica J, Becerril JM, Blanco F, Garbisa C (2008) Effects of chelates on plants and soil microbial community: comparison of EDTA and EDDS for lead phytoextraction. *Sci Total Environ* 401:21–28
- Gomez E, Ferreras L, Lovotti L, Fernandez E (2009) Impact of glyphosate application on microbial biomass and metabolic activity in a Vertic Argiudoll from Argentina. *Eur J Soil Biol* 45:163–167
- Gonçalves ICR, Araújo ASF, Carvalho EMS, Carneiro RFV (2009) Effect of paclobutrazol on microbial biomass, respiration and cellulose decomposition in soil. *Eur J Soil Biol* 45:235–238
- Govaerts B, Mezzalama M, Unno Y, Sayre KD, Luna-Guido M, Vanherck K, Dendooven L, Deckers J (2007) Influence of tillage, residue management, and crop rotation on soil microbial biomass and catabolic diversity. *Appl Soil Ecol* 37:18–30
- Guo H, Chen G, Lv Z, Zhao H, Yang H (2009) Alteration of microbial properties and community structure in soils exposed to napropamide. *J Environ Sci* 21:494–502
- Hart M, Brookes P (1996) Effects of two ergosterol-inhibiting fungicides on soil ergosterol and microbial biomass. *Soil Biol Biochem* 28: 885–892
- Huang H, Zhang S, Wu N, Luo L, Christie P (2009) Influence of *Glomus etunicatum* *Zea mays* mycorrhiza on atrazine degradation, soil phosphatase and dehydrogenase activities, and soil microbial community structure. *Soil Biol Biochem* 41:726–734
- Hund-Rinke K, Simon M, Lukow T (2004) Effects of tetracycline on the soil microflora: function, diversity, resistance. *J Soils Sediments* 4: 11–16
- Jackson CJ, Lamb DC, Kelly DE, Kelly SL (2000) Bactericidal and inhibitory effects of azole antifungal compounds on *Mycobacterium smegmatis*. *FEMS Microbiol Lett* 192:159–162
- Kaur A, Chaudhary A, Kaur A, Choudhary R, Kaushik R (2005) Phospholipid fatty acid-A bioindicator of environment monitoring and assessment in soil ecosystem. *Curr Sci* 89:1103
- Khalfallah S, Menkissoglu-Spiroudi U, Constantinidou HA (1998) Dissipation study of the fungicide tetraconazole in greenhouse-grown cucumbers. *J Agric Food Chem* 46:1614–1617
- Lin Q-M, Liu H-L (1999) Modification of fumigation extraction method for measuring soil microbial biomass carbon. *Chin J Ecol* 18:63–66
- Lupwayi NZ, Brandt SA, Harker KN, O'Donovan JT, Clayton GW, Turkington TK (2010) Contrasting soil microbial responses to fertilizers and herbicides in a canola–barley rotation. *Soil Biol Biochem* 42:1997–2004
- Magurran AE, Magurran AE (1988) *Ecological diversity and its measurement*, 179. Princeton university press Princeton
- Menkissoglu-Spiroudi U, Xanthopoulou NJ, Ioannidis PM (1998) Dissipation of the fungicide tetraconazole from field-sprayed sugar beets. *J Agric Food Chem* 46:5342–5346
- Muñoz-Leoz B, Ruiz-Romera E, Antigüedad I, Garbisa C (2011) Tebuconazole application decreases soil microbial biomass and activity. *Soil Biol Biochem* 43:2176–2183
- Smith M, Hartnett D, Rice C (2000) Effects of long-term fungicide applications on microbial properties in tallgrass prairie soil. *Soil Biol Biochem* 32:935–946
- Staddon W, Duchesne L, Trevors J (1997) Microbial diversity and community structure of postdisturbance forest soils as determined by sole-carbon-source utilization patterns. *Microb Ecol* 34:125–130
- Wu X, Cheng L, Cao Z, Yu Y (2012) Accumulation of chlorothalonil successively applied to soil and its effect on microbial activity in soil. *Ecotoxicol Environ Saf* 81:65–69
- Xu J, Dong F, Liu X, Li J, Li Y, Shan W, Zheng Y (2011) Rapid analysis of tetraconazole residues in fruits and vegetables using ethyl acetate extraction and gas chromatography-tandem mass spectrometry. *Bull Kor Chem Soc* 32:4265–4269
- Yen J-H, Chang J-S, Huang P-J, Wang Y-S (2009) Effects of fungicides triadimefon and propiconazole on soil bacterial communities. *J Environ Sci Health B* 44:681–689
- Zak DR, Kling GW (2006) Microbial community composition and function across an arctic tundra landscape. *Ecology* 87:1659–1670
- Zhang W, Xu J, Dong F, Liu X, Zhang Y, Tao Y, Wu X, Zheng Y (2013) Simultaneous determination of three strobilurin fungicide residues in fruits, vegetables and soil by a modified quick, easy, cheap, effective, rugged (QuEChERS) method coupled with gas chromatography-tandem mass spectrometry. *Anal Methods* 5: 7102–7109