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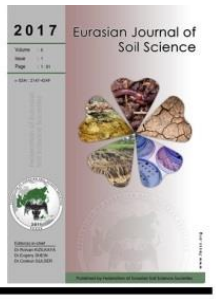

Persistence of myclobutanil and its impact on soil microbial biomass C and dehydrogenase enzyme activity in tea orchard soils

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Abstract

Persistence of the fungicide myclobutanil in three tea orchard soils with different cultivating ages, neighboring wasteland and forest soils, and its influence on microbial activities in 2- and 50-year-oldtea orchard soils at three rates were studied in the laboratory. Dissipation data fitted well to first-order kinetic equation, except for sterilized treatments, in which neglected dissipation of myclobutanil was observed. At 25°C, the dissipation half-lives (DT_{50}) at level of 1mg kg^{-1} were in the range of 15.07-69.32 days under non-flooded condition, significantly lower than flooded condition ($p < 0.05$), indicating that dissipation of myclobutanil was mainly driven by soil microorganisms under aerobic condition. Dissipation rate was significantly increased at 40°C compared to those at 4°C and 25°C for all five soils ($p < 0.05$). Under all incubation conditions, DT_{50} were lowest in 50-year-old tea orchard soil ($p < 0.01$). Correlation analysis between DT_{50} in tea orchard soils and soil properties showed that soil microbial biomass carbon was negatively correlated with DT_{50} under 25°C and 60% water holding capacity ($p < 0.05$). In general, soil microbial biomass carbon and dehydrogenase activity decreased as the concentration of myclobutanil and incubation time increased except 0.1 mg kg^{-1} spiked soils, in which soil dehydrogenase activity was stimulated after 10 days incubation.

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Introduction

Myclobutanil, an important triazole fungicide used against powdery mildew of cereal, vegetables, and fruits, is registered in more than 40 countries worldwide (Anon., 1993; Kemmitt et al., 2008). It is also widely applied to tea orchard to prevent brown spot disease. The amended European Union legislation has set the maximum residue limits (MRLs) for myclobutanil in different agricultural products and the MRL for tea is 0.05 mg kg^{-1} (Regulation EU, 567/2016). Residues of myclobutanil in some crops and the soils in which they were grown have been found (Athanasopoulos et al., 2003). It was also detected in agricultural head water streams (Smiley et al., 2014) and rain (Vogel et al., 2008) in the US.

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Persistence of myclobutanil in different soils has been studied in laboratory and field conditions. The reported dissipation half-life (DT_{50}) values were from 11.0-19.2 days (Liu et al., 2009; Han et al., 2009; Wang et al., 2012) up to 574 days in anaerobic soil (WHO/FAO, 2014).The reasons to the varying DT_{50} values in soils need to be addressed. Study on composting and digestion at full-scale plants revealed that myclobutanil was moderately persistent to persistent (DT_{50} > 70 days) in aerobic soils and persistent in anaerobic soils (Kupper et al., 2008). Organic residue amendments increased persistence and significant relationship was observed between the sorption of myclobutanil by the soils and DT_{50} values ($p < 0.05$) (Marín-Benito et al., 2014). Laboratory studies on the adsorption/desorption to soils indicated a low to moderate potential for vertical mobility (PPDB, 2011).

Tea (*Camellia sinensis*) is cultivated widely on acid red soils in the tropical and subtropical zones in China as an important economic crop (Wang et al., 2014) and also in more than 50 countries in the world with an annual production of approximately 4.7 million tons (Fang et al., 2014). Xue et al. (2006) reported that the pH value of tea orchard soil gradually decreases, and soil organic matter, soil N and P contents incease with the increase of cultivating ages. Therefore, tea orchard soils with different cultivating ages provide good samples to study the influence of soil physicochemical and biological properties on myclobutanil persistence.

Previous papers reported that triazole fungicides can have non-target effects on soil microbial communities (Yen et al., 2009; Muñoz-Leoz et al., 2011; Zhang et al., 2014). However, there are few studies on the impact of myclobuanil on soil microbial biomass and activity (Marín-Benito et al., 2014). To ascertain myclobutanil persistence and its impact soil microorganisms, 2-, 50-, and 100-year-old tea orchard soils, and neighboring wasteland and forest soils were collected. The two main objectives of this study were to: (1) evaluate the persistence of myclobutanil in tea orchard soils and the effect of soil properties and incubation conditions, i.e., water content and temperature, on its persistence, (2) investigate the influence of the fungicide spiked at three levels on soil microbial biomass carbon (C_{mic}) and dehydrogenase activity (DHA).

Material and Methods

Soil samples

The soil samples were collected from five sampling plots that were randomly chosen within a 2-year-oldtea orchard, a 50-year-old tea orchard and a 100-year-old tea orchard in Hangzhou, China. To evaluate soil biochemistry and microbial properties as a function of land-use change and management practice, neighboring wasteland and forest were also chosen as study sites. The wasteland in this red soil area was covered with sparse grasses. The 100-year-old forest, established on wasteland in 1914, was a mixed-conifer forest. From each sampling plot, 20 cores (5 cm in diameter × 20 cm in length) were taken and mixed. All soils investigated were classified as red soils by the China Classification System (Ultisols in USA soil taxonomy). Soil samples transported on ice to the laboratory and passed through a 2 mm sieve to remove rocks and plant debris. Each bulked sample was then separated into two parts. One part was air-dried for chemical analysis (except that mineral-N was immediately analyzed), and another was stored at 4°C until the incubation experiment.

Soil pH was determined in a 1:2.5 soil/water ratio. Soil organic matter was measured according to the method of dichromate oxidation (Nelson and Sommers, 1982). Total nitrogen was determined by Kjeldahl digestion (Keeney and Nelson, 1982), and ammonium and nitrate was extracted with 2 mol L^{-1} KCl and determined colorimetrically in a continuous flow analyzer (SA5000, Skalar Inc., the Netherlands).

Available phosphorus analysis was undertaken following the method by Olsen and Sommers (Olsen and Sommers, 1982). Heavy metal analysis: Soils were digested with nitric (HNO_3), hydrofluoric (HF), and hydrochloric (HCl) acid. Heavy metals (Cr, Ni, Cu, Zn, Cd, and Pb) were analyzed with Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) (Agilent 7700X, USA).

The selected soil characteristics and heavy metal contents are summarized in Table 1. With the increase of tea-orchard age, the pH value decreases, and SOM, nitrogen and phosphorous contents increase, except that C_{mic} is highest in 50-year tea orchard soil. According to the Environmental Quality Standard for Soils of China (GB 15618-1995), the concentrations of Cr, Cu, and Ni were generally at low levels. While in 50-year tea orchard soil and wasteland soil, the concentrations of Cd were 0.29 and 0.24 mg kg^{-1}, respectively, and over Grade I. In 100-year tea orchard soil and forest soil, Pb levels were over Grade I. The concentrations of Zn in all soil samples were over Grade II.

Table 1.Physico-chemical and biological properties parameters and heavy metal contents in five studied soils

Soil samples	pH (1:2.5)	SOM (g kg^{-1})	NO_3^--N	NH_4^+-N	TN	C_{mic}	A-P	Heavy metal contents（mg kg^{-1} dry weight）					
			(mg kg^{-1})					Cr	Cu	Ni	Zn	Cd	Pb
2-year	4.57	11.26	11.72	5.49	1.31	244.66	3.71	40.74	10.24	11.00	316.11	ND	29.24
50-year	4.00	33.26	13.54	16.83	2.93	565.78	56.68	31.06	15.28	10.89	287.77	0.29	31.35
100-year	3.52	61.13	57.96	22.06	5.61	341.44	246.02	38.09	20.34	10.98	298.74	0.18	36.35
Forest	4.11	76.51	17.18	19.49	5.11	349.56	22.96	37.62	11.05	9.41	211.76	ND	42.08
Wasteland	5.34	8.09	5.76	3.08	0.86	121.66	5.10	41.01	12.50	11.55	254.19	0.24	33.14

Persistence of myclobutanil in soils

Chromatography grade acetone and n-hexane were supplied by Sigma-Aldrich (Steinheim, Germany). An analytical standard of myclobutanil (> 99%) was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). The incubation experiments are based on a modification of the reported procedures (Singh and Dureja, 2000; Dong et al., 2013). To test water content on persistence of myclobutanil, each soil sample (5 g) was adjusted to 60% and 125% water holding capacity (WHC), and pre-incubated for 48 h in the dark at 25^{o}C. Soil samples (1 g) was artificially spiked by 1 mL of 5 μg mL^{-1} myclobutanil solution and set for 30 min under the hood until n-hexane was evaporated completely, and then added the rest soil to give a final concentration of 1 mg kg^{-1} soil. The sterilized controls (60% WHC), unflooded (60% WHC), and flooded (125% WHC) treatments were then incubated at 25^{o}C in the dark.- To test the season effect on persistence of myclobutanil, unflooded treatments were also incubated at 4^{o}C and 40^{o}C, respectively. Each treatment contained triplicate replicates. Soil moisture was regulated daily with sterile deionized water. Sampling was carried out after 0, 2 h, 1, 3, 5, and 10 days of incubation, and then stored at -20^{o}C for myclobutanil determination. For myclobutanil extraction, 25 mL of acetone and water (v:v/5:3) was added and shaked for 2 h at the speed of 200 r min^{-1} at 20 ± 2^{o}C, and then centrifuged at 7000 r min^{-1} for 5 min. This step was repeated twice by extraction with 10 mL of acetone and water. The extracts were concentrated until acetone was evaporated in a rotary evaporator, and the aqueous phase was extracted three times by ethyl acetate. The extracts were combined and dried over anhydrous Na_2SO_4, and then concentrated to near dryness in a rotary evaporator. The residue was dissolved again in 15 mL of n-hexane and transferred into 50 mL K-D tube, adjusted the volume to 20 mL. Sample was passed through 0.22 μm filter before GC analysis.

The concentration of myclobutanil was determined by gas chromatography equipped with Ni^{63} electron capture detector (Agilent 7890N, USA) and a HP-5 column (30 m × 0.25 mm i.d. × 0.25 μm film thickness) with ultrapure nitrogen as carrier gas and make-up gas at the flow rate of 1.0 mL min^{-1}. The injector and detector temperature were 260^{o}C and 300^{o}C, respectively. The oven temperature was programmed from 160^{o}C (held for 1 min) to 250^{o}C (held for 3 min) at 23^{o}C min^{-1}, and then increased to 280^{o}C at 4^{o}C min^{-1} (held for 10 min). The injection volume was 1 μL. Compounds were identified by retention time using external standard and quantified using peak area integration. For every set of 10 samples, a procedural blank consisting of all reagents was run to check for interference and cross contamination. The recovery study was carried out three replicates at two spiked levels (0.1 and 1.0 mg kg^{-1}). Table 2 listed the mean recovery and relative standard deviation (RSD) of the method. The data confirmed the practicability of the analytical protocols herein in the determination of myclobutanil residues in all soil samples.

Table 2. Recoveries of myclobutanil in five studied soils at two spiking levels (n=3)

Soil samples	Spiking levels (mg kg^{-1})	Recovery /%				RSD/%
		1	2	3	average	
2-year	0.1	86.4	94.7	91.9	91	4.640
	1.0	99.3	90.1	98.6	96	5.335
50-year	0.1	115.7	101.8	118.5	112	7.985
	1.0	105.5	118.1	127.4	117	9.394
100-year	0.1	86.3	95.4	85.3	89	6.253
	1.0	87.2	91.3	97.5	92	5.636
Forest	0.1	87.8	95.2	81.0	88	8.071
	1.0	87.6	95.4	99.0	94	6.199
Wasteland	0.1	113.2	110.7	121.1	115	4.721
	1.0	109.4	117.6	127.0	118	7.463

Soil microbial activities

Myclobutanil was applied to 2- and 50-year-old tea orchard soils at three rates (0.1, 1, and 10 mg kg^{-1}, respectively). Soils unexposed to myclobutanil were used as controls. Each treatment contained triplicate replicates. The soil samples were prepared as described above. After 0, 5 and 10 days of incubation, soil samples were transferred at 4°C for microbial biomass C and dehydrogenase activity analysis.

Soil C_{mic} was determined by the chloroform fumigation-extraction method (Vance et al., 1987). Moist soil was fumigated with ethanol-free $CHCl_3$ for 24 h at 25°C in sealed desiccators and extracted by shaking for 30 min with 0.5 M K_2SO_4 (40 mL). The other soil of equal weight was not fumigated but extracted under the same condition. Soil C_{mic} was calculated by the equation: C_{mic} = 2.64 E_C, where E_C = (C extracted from fumigated soil) – (C extracted from non-fumigated soil), with 2.64 being a conversion factor.

Soil dehydrogenase activity was measured according to the method described by Tabatabai (Tabatabai, 1994). Five grams of soil sample was incubated in 5 mL of 0.1% 1, 3, 5-triphenyltetrazolium chloride (TTC) solution for 24 h at 37°C in the dark. Two drops of concentrated H_2SO_4 were added to stop the reaction. After incubation, triphenylformazan (TPF) was formed by the reduction of TTC. TPF was extracted with 5 mL of toluene and determined by spectrophotometry at 492 nm. Blanks without soil were processed in the same manner.

Statistical analysis was performed by SPSS 16.0 (SPSS Inc., USA) using a one-way analysis of variance (ANOVA). The values were considered to be significantly different at a 95% confidence level. The values in the figures and tables are the average of triplicate data (n = 3) ± standard deviations. All data are based on soil dry weight (DW).

Results and Discussion

Persistence of myclobutanil

Persistence of myclobutanil in three tea orchard soils with different cultivating ages, adjunct waste and forest soils under 4°C, 25°C, 40°C, flooded and non-flooded conditions are presented in Table 3 and Figure 1.

Table3. Kinetic parameters of myclobutanil in soils under two WHC (60% and 125%) and three incubation temperatures (4, 25, and 40°C, respectively).

Soil samples	WHC (%)	Temperature	k (d^{-1})	R^2	DT_{50} (d)	Degradation (%)*
2-year	60	4°C	0.029	0.9550	$23.90^{a, A}$	23.84
		25°C	0.031	0.9633	$22.36^{a, A}$	26.47
		40°C	0.044	0.9717	$15.75^{b, A}$	36.59
	125	25°C	0.014	0.9665	$49.51^{***, A}$	12.66
50-year	60	4°C	0.045	0.9520	$15.40^{a, AB}$	34.80
		25°C	0.046	0.9816	$15.07^{a, B}$	35.44
		40°C	0.069	0.9556	$10.05^{b, AB}$	49.08
	125	25°C	0.022	0.9543	$31.23^{***, A}$	19.21
100-year	60	4°C	0.034	0.9627	$20.39^{a, B}$	27.27
		25°C	0.035	0.9509	$19.81^{a, C}$	28.49
		40°C	0.052	0.9602	$13.33^{b, B}$	40.70
	125	25°C	0.018	0.9612	$38.51^{***, A}$	17.02
Forest	60	4°C	0.020	0.9539	$34.66^{a, C}$	17.16
		25°C	0.020	0.9758	$34.66^{a, D}$	17.25
		40°C	0.030	0.9515	$23.11^{b, C}$	26.90
	125	25°C	0.011	0.9647	$63.02^{***, A}$	10.55
Wasteland	60	4°C	0.010	0.9449	$69.32^{a, D}$	9.57
		25°C	0.010	0.9356	$69.30^{a, E}$	9.44
		40°C	0.016	0.9502	$43.33^{b, D}$	15.21
	125	25°C	0.005	9376	$138.64^{***, B}$	4.90

Lower case letters indicate significant difference among different incubation temperatures under 60% WHCin the same soil ($p < 0.001$). Upper case letters indicate significant difference among different soils under the same incubation conditions ($p < 0.001$). *** indicates significant difference between 60% WHC and 125% WHC under 25°C ($p < 0.001$).

Dissipation data under different incubation conditions fitted well to first-order kinetic equation, $C_t = C_0 e^{-kt}$, with correlation coefficient (R^2) higher than 0.936, except for sterilized treatments, in which only 0.08-1.41% of myclobutanil were dissipated after 10 days incubation. The degradation (D) was calculated

according to the following equation: D = $(C_0 - C_t)/C_0 \times 100\%$. In non-sterile soils, 4.90-49.08% of myclobutanil was degraded. Neglected dissipation was observed in sterilized treatments indicated microbial decomposition played a critical role in myclobutanil dissipation in studied soils. At 25°C, the DT_{50} of myclobutanil at level of 1 mg kg^{-1} were in the range of 15.07-69.32 days under non-flooded condition, while under flooded condition, DT_{50} were from 31.23 days in 50-year-old tea orchard soil to 138.64 days in wasteland soil. The degradation rates decreased about 2-fold as the WHC increased from 60% to 125% for all soils, which indicated that dissipation of myclobutanil was mainly driven by aerobic biotransformation. Our results are consistent with other triazole fungicides, hexaconazole (Singh and Dureja, 2000), penconazole and propiconazole (Singh and Dureja, 2009), which were found more persistent in soils under flooded than non-flooded conditions. However, a minor role of microorganisms in hexaconazole degradation was found (Singh and Dureja, 2000). Another study showed that the two soil water contents did not cause significant differences in dissipation rates between two triazole fungicides, triadimefon and propiconazole (Yen et al., 2009).

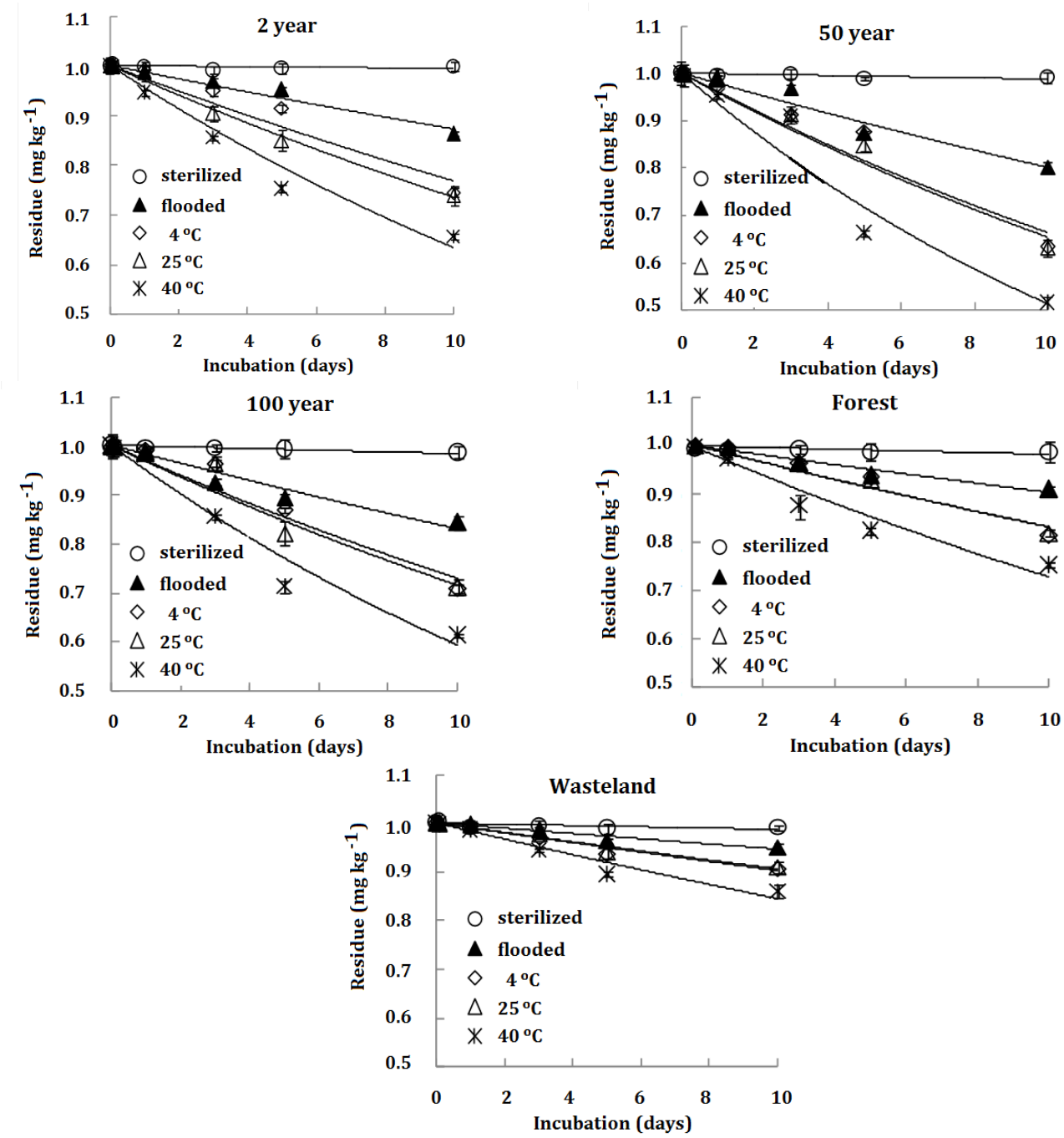

Figure 1. The dissipation of myclobutanil in five soils under sterilized (25°C, 60% WHC), unflooded (60% WHC)(4°C, 25°C, and 40°C, respectively) and flooded (25°C, 125% WHC) conditions.

Highest dissipation rate was observed in 50-year-old tea orchard soil, followed by 100- and 2-year tea orchard, and forest and wasteland soils, under all incubation conditions. Our result is in agreement with the findings on soil net nitrification study that the highest soil net nitrification was found in 50-year-old tea orchard, followed by 90- and 8-year-old tea orchard, and was significantly higher in the tea orchards compared to the wasteland and forest soils (Xue et al., 2006). Simple and multiple correlation analyses between DT_{50} and soil properties and heavy metal contents showed that C_{mic} was negatively correlated with DT_{50} ($p < 0.05$) in three tea orchard soils under 25°C and 60% WHC. Previous paper also showed that degradation of three fungicides (azoxystrobin, tebuconazole, and chlorothalonil) was fastest in the high microbial biomass soil (Bending et al., 2007).

Influences of temperature and water content–on myclobutanil degradation are similar in five soils. Dissipation rate was significantly increased at 40°C compared to those at 4°C and 25°C for all soils ($p < 0.05$). The effects of temperature on the persistence on myclobutanil are in agreement with other triazole fungicides. Degradation of hexaconazole (Singh and Dureja, 2000) and triadimefon (Singh, 2005) were faster at 35°C than at 27°C. Degradation rates of five triazole fungicides in two soils increased about 3-fold as the temperature was increased from 5 to 18°C (Bromilow et al., 1999). The faster degradation of myclobutanil at higher temperature could be mainly due to a higher microbial activity. Wang et al. (2014) found that high temperature significantly increased fungal abundance from 86 to 274% under 55% water holding capacity in a tea orchard soil. The volatilization in high temperature may have an insignificant effect on dissipation of myclobutanil, for myclobutanil is nonvolatile and has low mobility in soil. Myclobutanil exhibited negligible volatilization loss from golf course turfs (Wong et al., 2013).

Soil microbial activities

To assess the impact of application levels of myclobutanil on microorganisms in tea orchard soil, C_{mic} and DHA were analyzed in 2- and 50-year tea orchard soils at three incubation time (Figure 2).

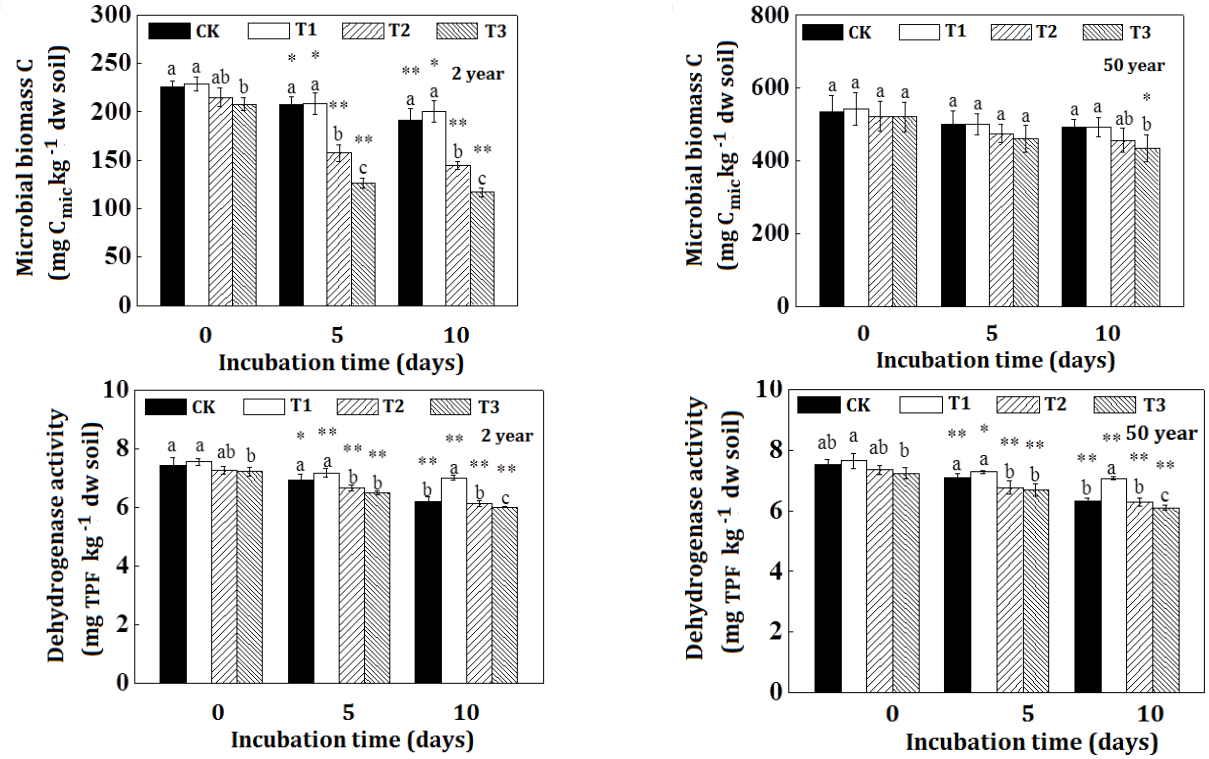

Figure 2. Effect of myclobutanil on soil microbial biomass C and dehydrogenase activity. Lower case letters indicate significantly difference among different spiking levels at the same incubation day ($p < 0.05$). ** ($p < 0.01$) and * ($p < 0.05$) indicate significant difference compared with day 0 at the same spiking levels of myclobutanil. CK, T1, T2, and T3 representing myclobutanil application levels at 0, 0.1, 1, and 10 mg kg^{-1}, respectively.

The C_{mic} values ranged from 116.90 to 228.83 mg kg^{-1} and from 461.27 to 542.59 mg kg^{-1} in 2- and 50-year-old tea orchard soils, respectively. Similar results have been found in forest soils that C_{mic} contents were higher in old-age forest soils compared to younger ones (Yan et al., 2009). No significant difference of C_{mic} was observed in 50-year old tea orchard soil among different spiking levels at all incubation times. The C_{mic} content only at 10 mg kg^{-1} spiked microcosms significantly decreased at day 10 compared with day 0 ($p < 0.05$). Inhibitory effect of myclobutanilon C_{mic} in 2-year old tea orchard soil was aggravated with the increase

of myclobutanil concentration and incubation time. Myclobutanil at 0.1 mg kg^{-1} level showed no significant impact on C_{mic} during the entire incubation period, while the C_{mic} values in 1 and 10 mg kg^{-1} spiked soils were significantly lower than controls at day 5 and 10 ($p < 0.05$). Compared with day 0, both 1 and 10 mg kg^{-1} of myclobutanil addition significantly inhibited C_{mic} at day 5 and 10 ($p < 0.01$). At day 10, the C_{mic} value in10 mg kg^{-1} of myclobutanil spiked 2-year old tea orchard microcosms was 43.8% lower than that in control soils, whereas in 50-year old tea orchard the C_{mic} were only 16.52% lower. The results indicate that 50-year old tea orchard soil is more resistant to myclobutanil than 2-year old one. Previous papers on other triazole fungicides showed that tebuconazole application decreasedC_{mic} (Muñoz-Leoz et al., 2011; Zhang et al., 2014), but tended to recover at the end of the incubation when tetraconazole was applied at the recommended field rate (Zhang et al., 2014). In contrary, no significant impact on total microbial biomass was observed after tebuconazole addition in either the low or high OM/biomass soils (Bending et al., 2007).

The DHA values and inhibitory effect were similar in two soils. DHA values were both from 6.0 to 7.7 mg TPF kg^{-1} DW soil. DHA increased at 0.1 mg kg^{-1} application rate and inhibited at 1 and 10 mg kg^{-1} levels in both soils. In the 0.1 mg kg^{-1} amended soils, a significant increase of DHA was observed at day 10. The maximum inhibition of DHA was observed in 1 and 10 mg kg^{-1} spiked soils at day 5 and tended to recover at day 10 compared with controls. Compared with day 0, DHA values were significantly lower in day 5 and 10 microcosms. Stimulation effect of myclobutanil on soil DHA has been reported at the application rate of 2 mg kg^{-1} (Marín-Benito et al., 2014). For other triazole fungicides, DHA were significantly reduced by the addition of triadimefon at 1 mg kg^{-1} rate (Singh, 2005) and tebuconazole (Muñoz-Leoz et al., 2011). Therefore, as Hussain pointed out that a number of factors, such as, chemical nature and application levels of pesticides, soil microbial community structure, soil properties and conditions can contribute to divergent research findings (Hussain et al., 2009).

Our samples were incubated for only 10 days. The effect of myclobutanil on soil microbial biomass and activity could be long, as Yen's study found that after two fungicides were applied for 60 days and longer, the compositions of microbial communities were not recovered (Yen et al., 2009). Further work should be carried out with longer observation time to study the resistance and resilience of soil microbial communities to the fungicide application and especially the influence on narrow niche functions of fungal community.

Conclusion

Myclobutanil was readily aerobic degraded in studied soils with DT_{50} about 2-10 weeks. The dissipation rates of myclobutanil were clearly influenced by soil properties, temperature, and water content. Myclobutanil degradation was fastest in 50-year old tea orchard soil with the highest C_{mic} content. Low myclobutanil level (0.1 mg kg^{-1}) stimulated soil dehydrogenase activity, whereas high concentration (1 and 10 mg kg^{-1}) had negative effect on soil quality indicators which decreased significantly as myclobutanil concentration and incubation time increased. This study gains a better understanding of myclobutanil dissipation and its effect on microbial activities of this unique soil ecosystem, and helps management practices in tea orchard soils with different cultivating age.

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