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Impact of beta-cypermethrin on soil microbial community associated with its bioavailability: A combined study by isothermal microcalorimetry and enzyme assay techniques

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ABSTRACT

In this study, an isothermal microcalorimetric technique has been used to show that beta-cypermethrin (CYP) had no significant effect ($p > 0.05$) on soil microbial activity at 80 $\mu g\,g^{-1}$ soil. Our soil enzyme data indicated that beta-CYP ranging 10–80 $\mu g\,g^{-1}$ soil had no significant effect ($p > 0.05$) on soil enzyme activities such as β-glucosidase, urease, acid-phosphatase, and dehydrogenase. Therefore, our results infer that beta-CYP would not pose severe toxicity to soil microbial community, but its toxic level may vary greatly with environment that associates with its increase in bioavailability: the level in soil (at $\mu g\,g^{-1}$) < the level in sediment (varying from $\mu g\,g^{-1}$ to $\mu g\,L^{-1}$) < the level in water (at $\mu g\,L^{-1}$). The comparison of the results of solvent volatilization on soil microbial activity has shown that the acetone-treated sample had no significant difference with the control ($p > 0.05$). These results suggest that the heavy application of beta-CYP may not cause damage to soil microbial community which is very different from its high toxicity to the aquatic organism.

1. Introduction

Pyrethroid insecticides have been used in agricultural and home formulations for more than 30 years, accounting for approximately 25% of the worldwide insecticide market [1], and its application is anticipated to further increase because of the reduction in the use of organophosphate insecticide. Beta-cypermethrin (beta-CYP) is a synthetic pyrethroid containing four out of the eight isomers that constitutes technical cypermethrin (CYP), and it has been produced and applied in agricultural pest control in China since 1988, occupying more than 50% of the total production of pyrethroid market [2]. Such extensive use of beta-CYP may potentially cause damage to soils due to toxic effects on non-target soil dwelling organisms. For instance, CYP has shown to induce ecdysis in non-target arthropods [3], and its effect on non-target soil microorganisms and respiratory activity was also explored [4]. In addition, the toxicity of alpha-cypermethrin (alpha-CYP) to soil nontarget invertebrates has been reported recently [5–7]. However, little is known on the impact of beta-CYP on soil microbial and enzymatic activity [8].

There is concern about the impact of beta-CYP on soil microbial activity because the soil structure and stability depends strictly on the presence and activity of soil microorganisms [9]. For this reason, an isothermal microcalorimetry was performed to investigate the impact of beta-CYP on soil microbial activity. Microcalorimetric technique is a powerful tool for evaluating the metabolism of microbial biomass in soil because the heat produced in the various processes depends solely on the initial and final energy states of the system, is independent of the types of microorganisms and their form of evolution, and permits the continuous monitoring of the activity of a living process in situ over a prolonged period without disturbing the system [10,11]. In particular, a recent study reveals that the temperature may influence the toxicity of pyrethroids that are more toxic at a relatively low temperature [12], and an isothermal microcalorimeter can maintain a constant temperature, which just avoids the problem of temperature change. Through employing the microcalorimetric technique, our research group has conducted an extensive work on the effects of xenobiotics on soil microbial activities in recent years, such as the impacts of various heavy metals [13], and organic pollutants [14,15]. Alternatively, soil enzyme activities may behave as sensitive indicators in the

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evaluation of the extent of soil degradation degree in natural and agro-ecosystems, being easy to measure and rapidly responding to the changes caused by both natural and anthropogenic factors [9,16].

In this work, we assessed the activities of enzymes such as acid-phosphatase, β-glucosidase, dehydrogenase, and urease via the exposure of soils to various concentrations of beta-CYP. These enzyme activities are excellent indicators of soil microbial function, are key components in nutrient cycling, and have been applied in studies assessing soil quality and management [16]. Soil microcosms were initially treated with different concentrations of beta-CYP. Afterwards, the soil microbial and enzyme activities were assessed for up to six days. Finally, the toxicity of beta-CYP associated with its concentration and bioavailability were discussed. If beta-CYP is not bioavailable in soil, then beta-CYP mass will be left behind without creating much toxicity to the soil microbes [17]. The current study is intended to investigate the impact of beta-CYP on soil microbial and enzymatic activity through isothermal microcalorimetry and enzyme assay. It is anticipated that our results will help locate which soil microbial portion will be mostly affected after the use of pyrethroid insecticides.

2. Experimental

2.1. Materials

Technical grade (97%) beta-cypermethrin (beta-CYP) was obtained from the State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University (Wuhan, PR China). The composition of beta-CYP (molecular formula: $C_{22}H_{19}Cl_2NO_3$; molecular weight: 416.32; CAS number: 65731-84-2) is a reaction mixture comprising (1*R cis*)*S*+(1*S cis*)*R* and (1*R trans*)*S*+(1*S trans*)*R* enantiomer pairs of CYP in an approximately 2:3 ratio. A stock solution of beta-CYP (5.0 g L^{-1}) was prepared by dissolving an appropriate amount of beta-CYP in acetone and this solution was kept at 4 °C before use. Treatment solutions were prepared from this stock by diluting it with acetone to give the dosing concentrations of 0.25, 0.5, 1.0, and 2.0 g L^{-1}, respectively [18]. Analytical grade glucose (purity 99.5%) and ammonium sulfate (purity 99%) were purchased from Guangdong Guanghua Chemical Factory Co., Ltd. (Guangzhou, China) and Guangdong Shantou Xilong Chemical Factory (Shantou, China), respectively. A nutrient solution was constituted by 8.3 g glucose plus 8.3 g ammonium sulfate in a 1:1 proportion in 1 L of sterilized deionized water [14]. All other reagents of analytical grade or above were used as received from supplier.

2.2. Soil collection

The soil samples were collected using method of Tong et al. [16]. The soil (silty clay loam, 18.61 ± 1.52 g kg^{-1} of organic matter, 10.81 ± 1.61 g kg^{-1} of carbon, 1.19 ± 0.01 g kg^{-1} of nitrogen, and pH 6.71) was collected from continuous corn no-till plots located at the city of Wuhan, Central China, at a depth of 5–15 cm after the removal of surface layer. In this study, such soil was selected as it is mostly encountered in the Central China. An aggregate sample was generated by collecting soil from ten separate locations across the field. Soil samples were well mixed upon arrival at the laboratory. Pebbles, large plant residues and macrobiota were removed, and the samples were sieved through a 4-mm mesh screen. The soil sample was stored in polyethylene bags at 4 °C for up to three months before being used for calorimetric experiments to ensure the reproducibility of required measurements [10].

2.3. Calorimetric measurement

An isothermal multi-channel microcalorimeter TAM III (Thermometric, Järfälla, Sweden) was used for the microcalorimetric measurement. The procedure was operated using the method of Chen et al. [14]. The calorimetric curves were obtained by the 4-mL stainless steel ampoules that were hermetically closed by Teflon sealing discs to prevent evaporation and energy loss. Soil (1.2 g with moisture content 25%) was equilibrated to the temperature (28 °C) of the microcalorimetric measurement over a period of 6 h. The soil supplemented with 0.2 mL of a nutrient solution to avoid soil sample submergence, stimulate soil microbial activity, and provide the nitrogen and sulfur needed by the microorganisms to synthesize amino acids [15], was used as the control. The other soils were treated with 48 μL of prepared dosing solutions where different amounts of beta-CYP dissolved, in order to give final concentrations 10, 20, 40, and 80 μg g^{-1} soil, respectively. Soil samples were fully mixed by vortexing to achieve a homogeneous mixture of soil and beta-CYP. Then the samples were left in a fume cupboard for 3–4 h for the complete evaporation of acetone, and 0.2 mL of a nutrient solution was then added to adjust the moisture content to 35.7% of the maximum water-holding capacity [19,20]. In order to assess the possible impact of solvent volatilization on soil microbial activity, various organic solvents were examined. Soils were treated with 48 μL of *n*-hexane, methylene chloride, isopropanol, acetone, and methanol, respectively [21], then left in a fume cupboard for 3–4 h for the solvent to evaporate [19,20], and were finally supplemented with 0.2 mL of a nutrient solution. The viability of the soil microbial populations was continuously monitored over time and the power–time curves were recorded by a computer.

2.4. Enzyme assay

Enzyme activities in soil were evaluated using five treatments as described above (0–80 μg g^{-1} soil of beta-CYP), and separate subsamples were collected after two, four, and six days of incubation. The assay methods for β-glucosidase, phosphatase, and dehydrogenase activities in soil were strictly carried out as described by Tong et al. [16]. The assay method for urease activity was modified from Gianfreda et al. [22]. Soil (5 g) was mixed with 10 mL of 10% urea substrate solution and 20 mL of 0.1 M citrate buffer (pH 6.7) after contacting with 1.0 mL methylbenzene for 15 min. The samples were mixed on a vortex and incubated in the dark for 24 h at 37 °C. The mixture was filtered by Fisherbrand Q8 paper, and 3.0 mL of filtrate was diluted to 20 mL with distilled water, then 4.0 mL of sodium phenolate (12.5% (w/v) phenol + 5.4% (w/v) NaOH) and 3.0 mL of 0.9% sodium hypochlorite were added. The released ammonium was determined spectrophotometrically at 578 nm after a 20-min color development period. The enzyme assays were carried out in triplicate for each treatment.

2.5. Data analysis

The values of peak height (P_{peak}) and corresponding time (t_{peak}) of each thermokinetic curve were directly obtained from the power–time curve. Q_{peak} was calculated by the integration of the curve from time 0 to t_{peak}. It is well known that the cell number grows exponentially in the exponential phase and so the kinetic equation is $N_t = N_0 \exp(kt)$ or $\ln N_t = \ln N_0 + kt$. If the heat output power is P_0 at time 0 and P_t at time t, then

$$P_t = P_0 \quad \exp(kt) \text{ or } \ln \quad P_t = \ln \quad P_0 + k_t. \tag{1}$$

The power–time curves (see Fig. 1) in the exponential phase correspond to Eq. (1). Using the data $\ln P_t$ and t taken from the curves to fit a linear equation, the growth rate constant k of the

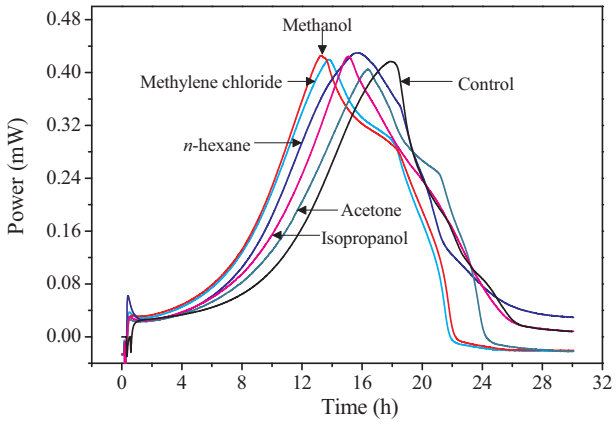

Fig. 1. Power–time curves of soil microbial activity treated with different organic solvents.

soil microorganisms was obtained [23]. The inhibitory ratio I was obtained from the following equation:

$$I\ (\%) = \frac{k_0 - k_C}{k_0} \times 100, \quad (2)$$

where k_0 is the rate constant of the control, and k_C is the rate constant for soil microbial activity inhibited by beta-CYP with concentration C. Since the heat evolution is proportional to the amount of glucose degraded by soil microorganisms, the metabolic enthalpy change per mole of glucose, ΔH_{met}, can be calculated from the equation [24]:

$$Q_t = \Delta H_{met}(S_0 - S_t), \quad (3)$$

where Q_t is the total heat evolved to time t; S_0 is the initial quantity of glucose; and S_t is the quantity of glucose at time t. Since it has been reported that the added glucose is practically exhausted (>99%) at the peak time [25], Q_t can be replaced by Q_{peak}. As such, it is possible to quantify ΔH_{met} from the equation [15]:

$$\Delta H_{met} = \frac{Q_{peak}}{S_0}. \quad (4)$$

Treatment effects and significant differences were assessed using one-way Analysis of Variance (ANOVA) with SPSS for thermokinetic parameters and enzyme activity, and significance was expressed at $p < 0.05$. The hierarchical clustering analysis (HCA) of solvents *n*-hexane, methylene chloride, isopropanol, acetone, methanol, and control was performed using SPSS statistical software (SPSS for Windows 13.0, Chicago, IL, USA). A method called "Between-Groups Linkage" was applied.

3. Results and discussion

3.1. Impact of solvent volatilization on the soil microbial activity

Organic solvent was used to dissolve beta-CYP because of its large hydrophobicity. Fig. 1 displays the power–time curves of soils treated with different organic solvents and compares with the control. There were obvious differences between these curves. The thermokinetic parameters are determined and summarized in Table 1 to compare the differences between in the solvent-treated soils and the control. Parameters collected from each solvent-treated soil were subjected to the Tukey's test for the treatment effects, indicating the significant differences ($p < 0.05$) of the t_{peak} and Q_{peak} between in the methanol-treated soil and the control. However, no significant differences ($p > 0.05$) of the thermokinetic parameters were observed between in the other solvent-treated soils and the control. As P_{peak} indicates the maximum activity of

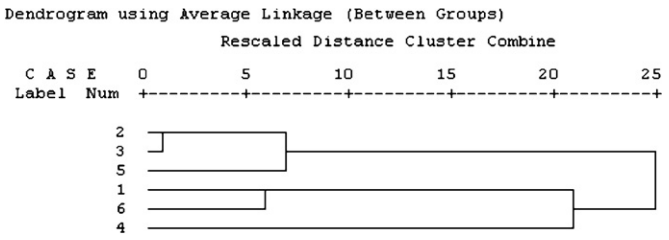

Fig. 2. Hierarchical clustering analysis of solvent-treated samples: (1) the control sample, (2) methanol, (3) methylene chloride, (4) *n*-hexane, (5) isopropanol, and (6) acetone.

the soil microbial community, k represents the growth rate of soil microorganisms, and Q_{total} expresses the soil microbial activity during the whole course of the experiment, all these parameters suggest that there is very little impact of solvent on soil microbial activity after they have been evaporated.

To select the most appropriate solvent, we performed an HCA as shown in Fig. 2. The HCA provides a qualitative comparison of the samples. It was found that the samples could be divided into two clusters, and the acetone-treated sample had the smallest difference with the control, possibly attributing to its extremely high volatility so that the contact time between the solvent and soil microbial community is very short. Therefore, acetone was used as the solvent to prepare the beta-CYP solution, which was comparable with the studies of Polat et al. [18] and Zhang et al. [2].

3.2. Power–time curves in the absence and presence of beta-CYP

Fig. 3 depicts the power of the microbial community generated in soil in the absence and presence of various amounts of beta-CYP against time. All of them exhibited lag, log, stationary and death phases. The profiles of these power–time curves before their peak times were very similar but a small inflection as well as a second peak when beta-CYP was present. Since Barja and Núñez [25] demonstrated that the added glucose has been exhausted (>99%) at the peak time, three causes may contribute to the inflection or the second peak [26]: (1) the decomposition of dead sensitive organisms which has the same stimulatory effect on respiration as glucose, (2) the usage of residual ammonium sulfate by some autotrophic microorganisms after the exhaustion of glucose, and (3) the growth of surviving fungus because fungi normally grow slower than bacteria. In general, these power–time curves suggested that there was very little impact of beta-CYP on soil microbial activity because their profiles showed very small

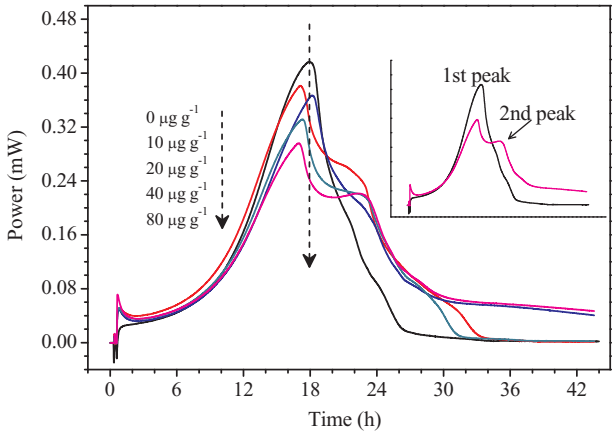

Fig. 3. Power–time curves of soil microbial activity as a function of concentration of beta-CYP. The inset displays the enlarged view of the power–time curves at 0.0 and 80 μg beta-CYP per gram of soil.

Table 1
Mean values of the thermokinetic parameters with on exposure to different organic solvents.

Solvent	P_{peak} (mW)	t_{peak} (h)	Q_{peak} (J g^{-1} soil)	Q_{total} (J g^{-1} soil)	k (h^{-1})
Control	0.417	17.892	7.515	12.123	0.225
Methanol	0.425	13.267*	5.684*	12.319	0.257
Methylene chloride	0.419	13.804	6.172	11.925	0.249
n-Hexane	0.429	15.661	7.531	14.633	0.259
Isopropanol	0.424	15.087	6.075	13.198	0.219
Acetone	0.405	16.384	6.607	12.029	0.213

The mean values were determined from three replicates.
* Significantly different from the control at $p < 0.05$.

differences. These results are different from our findings of soils contaminated with diphenol, chlorpyrifos and its oxon derivative where the profiles of the power–time curves changed remarkably [14,15]. It is possible that the exposure concentration of beta-CYP is much lower than diphenol and its affinity to the test soil is smaller, resulting in lower bioavailability as well as toxicity.

3.3. Thermokinetic parameters in the presence and absence of beta-CYP

Table 2 summarizes the thermokinetic parameters in the absence and presence of various amounts of beta-CYP. It is obvious that there are some differences between these parameters with the increase in the concentration of beta-CYP. The P_{peak}, Q_{peak}, ΔH_{met}, and k values decreased with the increase of the concentration of beta-CYP. There were significant decrease in P_{peak}, Q_{peak}, and ΔH_{met} compared to the control at $p < 0.05$ when the beta-CYP concentrations were 10–80 μg g^{-1} soil. However, no significant differences ($p > 0.05$) of t_{peak} and Q_{total} were observed between the treated soils and the control. As mentioned above, Q_{total} represents the soil microbial activity during the whole course of the experiment, suggesting that the soil microbial activity was not adversely affected. The results obtained from the study are congruent to the observations made by Vig et al. [4] where no significant changes were observed in the bacteria, fungus, actinomycete, and azotobacter numbers after CYP treatment in most cases. It has also been reported by Xie et al. [27] that the influence of 10 μg g^{-1} of CYP on the soil microbial diversity was slight.

In order to compare all of the thermokinetic parameters of the beta-CYP treated soil samples with the control, these parameters were expressed in terms of % relative to the control (*i.e.*, the control is 100%) as shown in Fig. 4. The change in the thermokinetic parameters (except Q_{total}) varied with the concentration of beta-CYP and these parameters were almost smaller that of the control. Among these thermokinetic parameters, the growth rate constant k can be used to express the glucose [25] as well as the soil microbial respiratory activity [16]. The k value of each beta-CYP concentration was subjected to the Tukey's test which showed no significant decreases ($p > 0.05$) between any treatment and the control, suggesting little impact of beta-CYP on soil microbial respiratory activity. Moreover, the inhibition ratio I also concurred in this suggestion since the highest inhibition ratio was only 10.67% at 80 μg g^{-1} soil of beta-CYP. In essence, beta-CYP only induced some increases or decreases of the thermokinetic parameters but the Q_{total} and k did not change significantly ($p > 0.05$), inferring that beta-CYP has minimum effect on the soil microbial and respiratory activity. Our findings are in complete agreement with those of Vig et al. [4] that CYP causes no adverse effect on the soil basal respiration as well as the substrate-induced respiration.

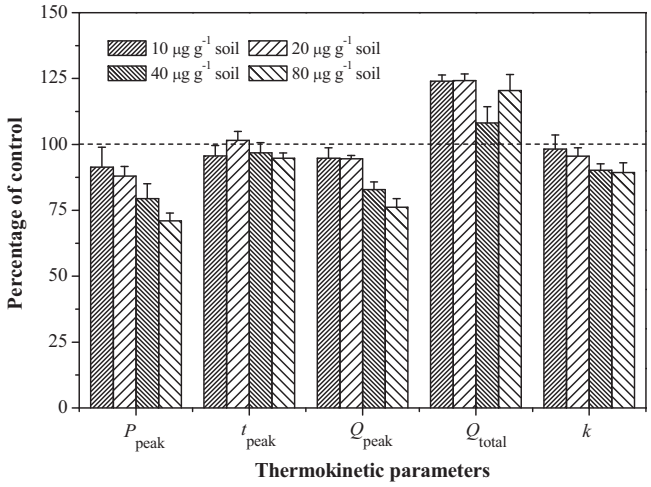

Fig. 4. Percentages of the thermokinetic parameters of soil control. * Significantly different from the control at $p < 0.05$. The bars indicate the standard deviation of the mean.

3.4. Soil enzyme activity

The results of enzyme assays for soils incubated in the presence of distinct amounts of beta-CYP for up to six days are summarized in Fig. 5. The results were expressed as an average of three parallel determinations and the enzyme activities were expressed as relative activities with reference to the control. Except for urease and acid phosphatase in soils treated with 10 μg g^{-1} of beta-CYP after the third 2-day incubation (urease: 101% of the control, acid-phosphatase: 108% of the control), the enzyme activities of the other treatments were all below the control. Moreover, all the enzyme activities could resume to the same values of the control when contact time increased. A possible mechanism to explain this situation was the tolerance and adaptation of soil microorganisms to pollutants [28]. The soil enzyme activities at each time point were subjected to the Tukey's test which showed that there were no significant differences ($p > 0.05$) between any treatment and the control, suggesting that beta-CYP has very little impact on soil enzyme activities. These results are different from those of Xie et al. [27] that CYP of 10 μg g^{-1} soil can significantly ($p < 0.05$) improve the activity of dehydrogenase, probably attributing to the different physicochemical properties of the test soils.

3.5. Comparison of beta-CYP toxicity in different environments

The toxic level of beta-CYP may vary greatly with the environments. The most notable is beta-CYP's high aquatic toxicity, up to ppb (μg L^{-1}) level [18,29–31]. In sediment, the toxicity of beta-CYP is regulated by phase distribution among the sediment, dissolved organic matter, and water phases [32], and its toxic level varies considerably from ppb (μg L^{-1}) to ppm (μg g^{-1}) level [33,34]. In soil, beta-CYP toxicity is controlled by the disparities of soil type and organic matter content, and its toxic level remains stable at the ppm (μg g^{-1}) level [5–7], which agrees well with the results in the present study. Under the assumption that only the freely dissolved fraction is more bioavailable [35], we deduce that the high

Table 2
Mean values of the thermokinetic parameters on exposure to different concentrations of beta-CYP.

C (μg g^{-1} soil)	P_{peak} (mW)	t_{peak} (h)	Q_{peak} (J g^{-1} soil)	ΔH_{met} (kJ mol^{-1})	Q_{total} (J g^{-1} soil)	k (h^{-1})	I (%)
0	0.417	17.892	7.515	977.855	12.123	0.225	0.00
10	0.381	17.117	7.125	927.109	15.035	0.221	1.78
20	0.367	18.175	7.104	924.376	15.059	0.215	4.44
40	0.331	17.317	6.227	810.260	13.111	0.203	9.78
80	0.296*	16.958	5.727*	745.200*	14.604	0.201	10.67

The mean values were determined from three replicates.
* Significantly different from the control at $p < 0.05$.

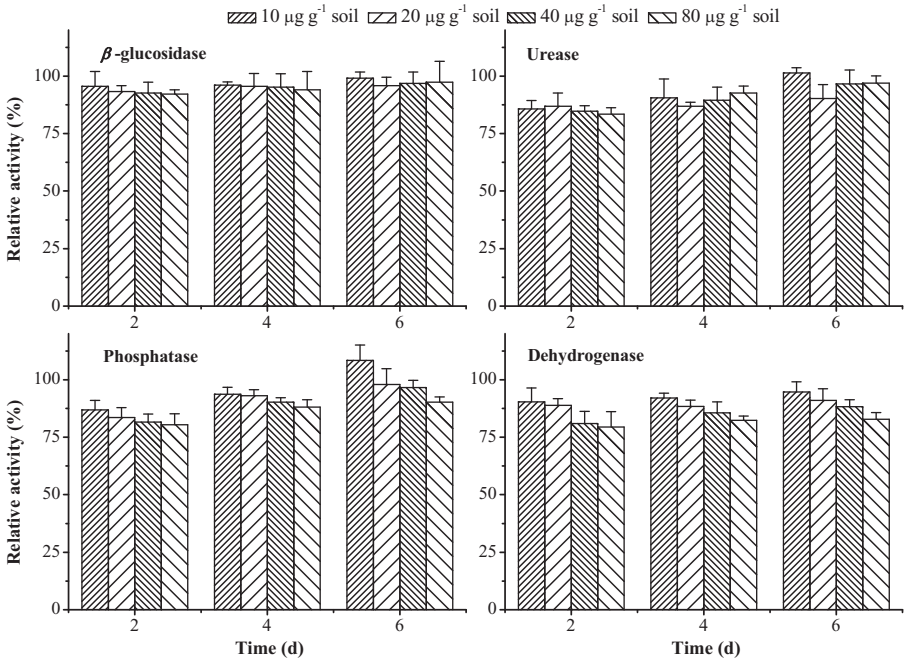

Fig. 5. Relative activities of urease, acid-phosphatase, β-glucosidase, and dehydrogenase in soil samples treated with beta-CYP compared with soil control soils (set to 100%) at times up to 6 days. The bars indicate the standard deviation of the mean.

aquatic toxicity of pyrethroids was due to the higher percentage of the total concentration that is more biologically available in water, followed by sediment and then soil.

4. Conclusion

In summary, this study successfully evaluates the effect of beta-CYP on soil microbial and enzymatic activity by isothermal microcalorimetry and enzyme assay. The thermokinetic parameters obtained from the metabolic power–time curves can be used quantitatively to indicate the toxic effect of beta-CYP to soil microbial activity. Our work revealed the very small impact of beta-CYP at high concentrations in soil, although other previous reports indicate that beta-CYP shows particularly high aquatic toxicity. Furthermore, the thermokinetic parameters obtained by microcalorimetry are in good agreement with the activities of the soil enzymes. Especially in certain situations, the effect of solvent volatilization like methanol on the soil microbial activity should be considered.

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