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Kinetics of soil dehydrogenase in response to exogenous Cd toxicity

Xiangping Tan[a,b], Ziquan Wang[a], Guannan Lu[a], Wenxiang He[a,d,*], Gehong Wei[c], Feng Huang[b], Xinlan Xu[b], Weijun Shen[b]

[a] College of Natural Resources and Environment, Northwest A&F University, Yangling, 712100, Shaanxi, China
[b] Key Laboratory of Vegetation Restoration and Management of Degraded Ecosystems, South China Botanical Garden, Chinese Academy of Sciences, CAS 723 Xingke Rd., Tianhe District, Guangzhou 510650, China
[c] College of Life Sciences, Northwest A&F University, Yangling, 712100, Shaanxi, China
[d] Key Laboratory of Plant Nutrition and Agro-environment in Northwest China, Ministry of Agriculture, Northwest A&F University, Yangling, 712100, Shaanxi, China

HIGHLIGHTS

- pH explained 30–45% of the dehydrogenase activity (DHA), V_{max}, and K_m variations across soils.
- Different inhibition mechanism of Cd to DHA varied soil types.
- Soil properties and inhibition constant affect the toxicity of Cd.
- Reaction constant (k) could indicate sensitively the toxicity of Cd to DHA.

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ABSTRACT

Soil dehydrogenase plays a role in the biological oxidation of soil organic matter and can be considered a good measure of the change of microbial oxidative activity under environmental pollutions. However, the kinetic characteristic of soil dehydrogenase under heavy metal stresses has not been investigated thoroughly. In this study, we characterized the kinetic characteristic of soil dehydrogenase in 14 soil types, and investigated how kinetic parameters changed under spiked with different concentrations of cadmium (Cd). The results showed that the K_m and V_{max} values of soil dehydrogenase was among 1.4–7.3 mM and 15.9–235.2 $\mu M\ h^{-1}$ in uncontaminated soils, respectively. In latosolic red soil and brown soil, the inhibitory kinetic mechanism of Cd to soil dehydrogenase was anticompetitive inhibition with inhibition constants (K_i) of 12 and 4.7 mM, respectively; in other soils belonged to linear mixed inhibition, the values of K_i were between 0.7–4.2 mM. Soil total organic carbon and K_i were the major factors affecting the toxicity of Cd to dehydrogenase activity. In addition, the velocity constant (k) was more sensitive to Cd contamination compared to V_{max} and K_m, which was established as an early indicator of gross changes in soil microbial oxidative activity caused by Cd contamination.

1. Introduction

Contamination of soil with heavy metals is of considerable concern due to the detrimental effects on soil environments and human health. Cadmium (Cd) is one of the most toxic heavy metals that may impose adverse impacts on nearly all biological processes [1,2]. A certain amount of Cd may reduce photosynthesis and protein synthesis rates, interfere stomatal opening, and therefore affect the growth of sensitive plants [3]. Furthermore, Cd in contaminated soils may be taken up by plant roots, exposed to humans through food chains, and cause many hazards such as kidney disease, skeletal damage, and cancers [4,5]. It is estimated that about $9.9–45 \times 10^6$ kg of Cd is introduced into terrestrial soils annually through fertilizer application, sewage irrigation, atmospheric precipitation, and industrial and mining waste emissions [6,7]. Cd concentration in contaminated soils ($0.6–1781\ mg\ kg^{-1}$) is now much higher than the background value ($0.41\ mg\ kg^{-1}$) of the world [8,9]. Understanding the mechanisms of Cd pollution on soil biochemical processes is helpful for identifying their environmental exposure risks and providing important information for the remediation of contaminated soils [10].

* Corresponding author at: College of Natural Resources and Environment, Northwest A&F University, Yangling, 712100, Shaanxi, China.
E-mail addresses: wenxianghe@nwafu.edu.cn, wxhe1986@163.com (W. He).

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Soil enzymes have been found to be sensitive to heavy metal pollution and frequently been used as bio-indicators for detecting and quantifying the toxicity of targeted heavy metals [11,12]. Numerous studies have been devoted to assessing the adverse impacts of heavy metal on soil enzyme activities under both field and laboratory conditions [2,13]. It is well-established that soil enzyme activity decreases exponentially or logistically with increasing heavy metal concentration [14,15]. The ecological dose (EDx) index, i.e., the concentration of the heavy metal at which enzyme activity is reduced by x% relative to its initial values, is often used as an indication of the heavy metal toxicity [14,15]. However, the EDx value based on enzyme activity measurements may vary with the substrate type and concentration used to measure the enzyme activity, and the ecological dose-response model used to describe responsive behavior [14,16]. Enzyme kinetics can indicate the catalytic ability (V_{max}) and substrate affinity (K_m) of an enzyme under a heavy metal stress, which can be further used to calculate the inhibition constant (K_i) that reflects the intrinsic property of interaction between an inhibitor and the target enzyme [17,18]. These enzyme kinetic-based parameters do not vary with the substrate concentration and therefore provide a more direct and realistic measure for the strength of an inhibitor [16,17].

Mechanistically, heavy metals inhibit enzymatic activities mainly through competing for the active sites of enzyme with the substrate, denaturing the enzyme protein, and/or forming a covalent bond with the enzyme-substrate complexes [2]. These different inhibition mechanisms are responsible under different circumstances depending on the heavy metal species, pH of the reaction system, and the state of soil enzymes [17,19]. For example, the inhibition mechanism of Zn for acid phosphatase (from wheat germ) bound to Latosol clay was anticompetitive inhibition at pH 5.0 of the reaction system [20]. As pH increased to 6.0, the inhibition type changed to line mixed inhibition [20]. However, An anticompetitive inhibition and line mixed inhibition patterns of the Goethite- and Kaolin-bounded acid phosphatase by Zn were obtained at both pH levels respectively [20]. These findings suggest that the inhibition mechanism of enzyme by inhibitor depends not only on the pH of the reaction system but also on the immobilized carrier. In natural soils, enzymes usually are adsorbed onto various soil particle surfaces such as mineral, humus, or organo-mineral complexes, which may result in differed affinity with the metal ion or substrate of mineral-enzyme complexes [21,22]. These different states of soil enzymes and soil pH may give rise to various inhibition mechanisms for one heavy metal species. It is therefore advisable to study the effects of heavy metals on the kinetic characteristic of enzyme in native soils [23].

Soil oxidation-reduction enzymes (e.g., dehydrogenase, peroxidase, and polyphenoase) are functionally important in degrading pollutants, transforming organic matter, and maintaining metabolism of microorganisms [24,25]. However, their kinetic characteristics are poorly understood [26–28]. The dehydrogenases (EC 1.1.1) are a kind of intracellular oxidoreductases and play an essential role in the initial oxidation stages of soil organic matter by transferring electrons or hydrogen from substrates to acceptors [29]. Moreover, their routine measurement is simple and low-cost under laboratory condition. Dehydrogenase activity is one of the most adequate, important and sensitive bio-indicators, relating to soil heavy metal pollution [30]. To our knowledge, the kinetic characteristics of soil dehydrogenase-catalyzed reaction in response to Cd pollution have not been characterized. A better understanding of the kinetic characteristics of dehydrogenase could help resolve the microbiological redox systems responses to Cd pollution, and provide important basis for evaluation and monitoring on Cd pollution by soil microorganisms and enzymes [30,31].

In this study, we investigated the kinetic characteristics of soil dehydrogenase under exogenous Cd stress using soil samples representing 14 different agriculture soil types in China, where 19.4% of the arable land soils have been contaminated and the primary inorganic pollutant is Cd [32]. Specifically, we addressed the following questions: (i) how does Cd contamination affect the kinetic characteristics of dehydrogenase? (ii) what is the responsible mechanism explaining the inhibition effects of Cd on dehydrogenase activity (DHA)? (iii) which soil properties influence the inhibition effects of Cd on dehydrogenase kinetics?

2. Materials and methods

2.1. Soil samples

A total of 15 soils covering a wide range of soil properties were collected throughout China (Fig. 1). The soils were selected to be representative of the major soil types and the distribution of soil pH of agricultural soils in China. At five subsites of each site, soil samples were taken from the surface (0–20 cm topsoil) of an uncontaminated farmland using a stainless steel spade and then were mixed thoroughly to form a composite sample for analysis. The coordinates of the sampling sites were recorded with GPS (GARMIN GPS72, Taiwan). The samples were air-dried at room temperature, homogenized, and passed through a 1-mm sieve to remove plant debris and large stones prior to use.

2.2. Experimental design

One gram of air-dried soil was spiked with different concentrations of $3CdSO_4{\cdot}8H_2O$ (AR, Xilong, China) solutions to result in final soil Cd concentrations of 0, 0.6, 5, 25, 50, 100, 200, 300, and 500 mg kg^{-1} soil (soil: Cd solution ratio = 1: 1). The solution was added dropwise to moisten the whole soil sample. After 30 min of equilibration, the soil DHA was measured. All experiments did in triplicate.

2.3. Soil assays

2.3.1. Soil physicochemical properties and dehydrogenase activities

The measurement methods of soil physicochemical properties were described [33]. Soil DHA was determined as previously described [31]. The unit of dehydrogenase is μg INF (iodonitrotetrazolium formazan) $g^{-1}\,h^{-1}$. The national standard soil samples, Rhodi-Udic Ferralosols (GBW07407(GSS-7), Cd 0.08 ± 0.033 mg kg^{-1}) and Loessi-Orthic Primosols (GBW07407(GSS-8), Cd 0.13 ± 0.05 mg kg^{-1}), were measured for quality control of the analytical results, and the Cd contents of the two standard soils were 0.07 and 0.11 mg kg^{-1}, respectively. The main physicochemical properties and DHA of the soils are showed in Table 1. Soil pH ranged from 5.7 to 8.8. The total organic carbon (TOC) content varied a great deal (4.9–27.4 mg kg^{-1}) and averaged 13.4 mg kg^{-1}. Clay content ranged from 6.7% to 45.9% and CEC ranged from 8.1 to 31.1 cmol kg^{-1}. Soil total Cd contents ranged from 0.1–0.2 mg kg^{-1}, which are less than the class II Soil Environmental Quality standard in China (0.3 mg kg^{-1}, GB 15618-1995).

2.3.2. Soil dehydrogenase kinetic parameters measurements

One mL of different concentration of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5- phenyltetrazolium Chloride (INT) (HPLC, TCI, Japan) was added to spiked soil samples. The concentrations of INT were 1.98, 3.95, 5.93, and 9.89 mM. Then the mixtures were static incubated at 40 °C (relative humidity of 70%) for 1, 2, 3, and 4 h, respectively. A substrate control (without soil) and an optional abiotic control (without INT) were simultaneously

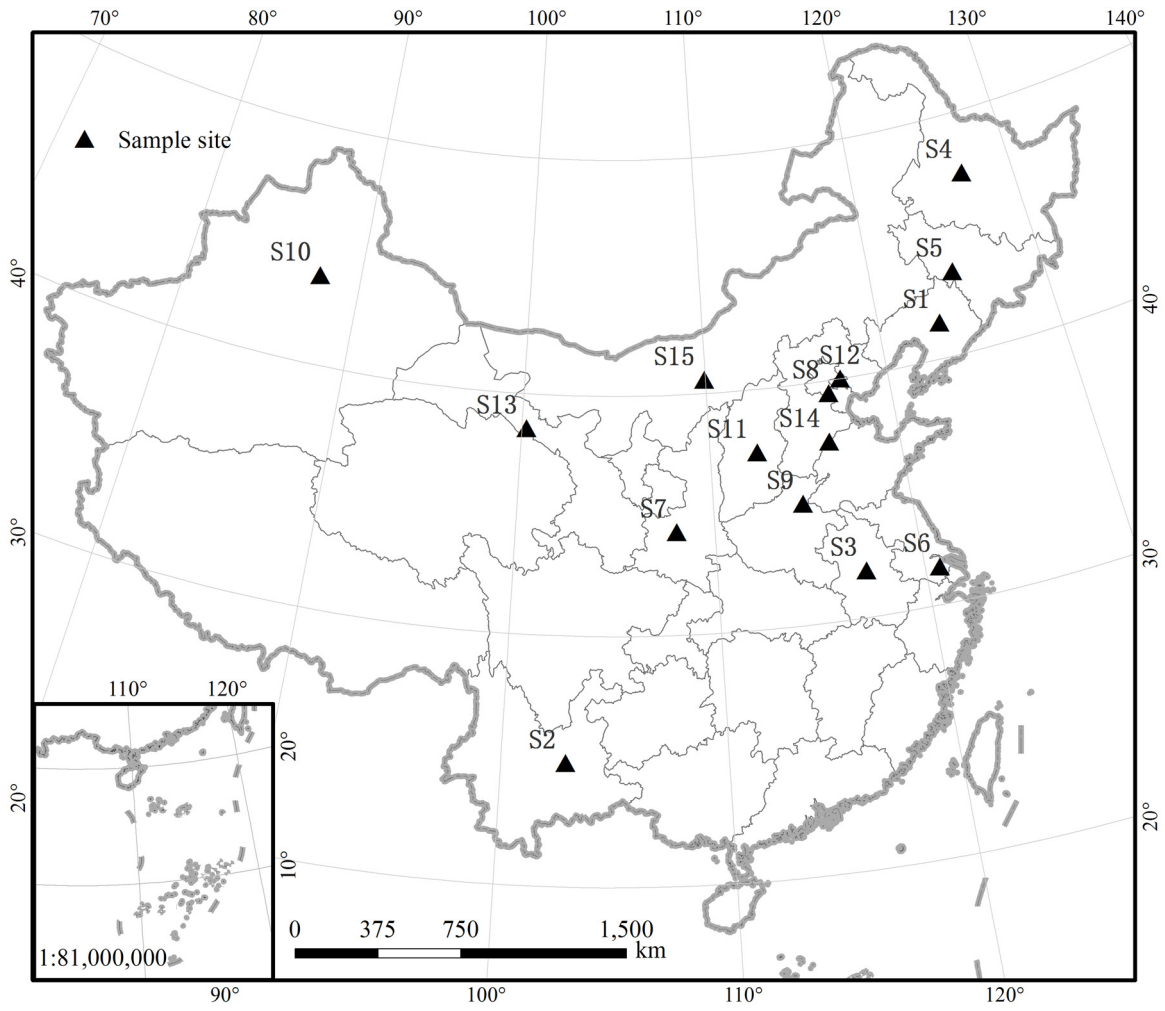

Fig. 1. Map of the soil sampling sites in China.

incubated. After incubation, 10 mL of extractant (ethanol and N, N-Dimethylformamide mixed at 1: 1 ratio) were added and all tubes were shaken for 5 min followed by centrifugation at 4000 r min^{-1} for 5 min. The supernatant was obtained and analyzed using spectrophotometer (722, Jingke, Shanghai) at 485 nm [28,31].

2.4. Data analysis

2.4.1. Calculation of soil enzyme kinetic parameters without Cd treatment

The characteristics of enzyme catalytic activity (V) is described by the Michaelis-Menten kinetics, and the kinetic constants are defined as V_{max} and K_m [26].

$$V = \frac{-d[S]}{dt} = \frac{V_{max} \times [S]}{K_m + [S]} \quad (1)$$

where, the K_m value is also equal to the ratio of the rate of dissociation of enzyme-substrate complex to the rate of association of enzyme and substrate [34,35].

The values of K_m and V_{max} are estimated by the integral Michaelis-Menten equation:

$$\frac{1}{t} \times \ln\left(\frac{[S_0]}{[S_t]}\right) = -\frac{1}{K_m} \times \frac{[S_0] - [S_t]}{t} + \frac{V_{max}}{K_m} \quad (2)$$

where t is reaction time (h), $[S_0]$ is initial substrate concentration, $[S_t]$ is substrate concentration at time t [36].

According to equation 2, $1/t \times \ln([S_0]/[S_t])$ is a linear function of $1/t \times ([S_0\text{-}S_t])$, then K_m and V_{max} can be estimated by the slope and intercept, with the units being mM and μM h^{-1}, respectively. Soil dehydrogenase enzymatic reaction rate constant k (h^{-1}) can be calculated by a linear fitting of t with $\ln([S_0]/[S_t])$. Reaction rate constant (k) is a quantitative representation of the chemical reaction rate in dependence of substrate concentration.

2.4.2. Calculation of soil enzyme kinetic parameters with Cd treatment

Four inhibition types have been identified to describe the responsive behaviors of the two kinetic parameters (V_{max} and K_m) to changes in the concentration of an inhibitor: competitive inhibition (unchanged V_{max} but increased K_m), noncompetitive inhibition (decreased V_{max} but unchanged K_m), liner mixed inhibition (decreased V_{max} and increased K_m), and anticompetitive inhibition (both decreased V_{max} and K_m) [37].

2.4.2.1. Kinetic parameters of an anticompetitive inhibition. The mathematical expression of an anticompetitive inhibition is [36]:

$$V = \frac{V^*_{max}[S]}{K^*_s + [S]} = \frac{(V_{max}/\alpha)[S]}{(K_s/\alpha) + [S]} \quad (3)$$

where V^*_{max} is the apparent maximum rate of enzyme activity in the presence of inhibitor, V_{max} is the maximum rate in the absence of

Table 1
Physicochemical properties of the 15 soils sampled across China.

Soil code	Location	Soil type (Genealogical classification)	Clay	TOC	pH	CEC	Cd
S1	Liaoning	Brown soil (Hapli-Udic Argosols)	17.3	14.8	5.7	12.2	0.16
S2	Yunnan	Latosolic red soil (Hapli-Udic Ferralosols)	27.5	19.7	5.9	11.1	0.26
S3	Anhui	Yellow brown soil (Ferri-Udic Argosols)	16.8	11.5	6.3	19.1	0.11
S4	Heilongjiang	Black soil (Hapli-Udic Isohumosols)	19.3	20.5	6.3	28.6	0.18
S5	Jilin	Black soil (Hapli-Udic Isohumosols)	30.2	18.8	6.8	31.1	0.13
S6	Jiangsu	Paddy soil (Gleyi-Stagnic Anthrosols)	45.9	27.4	6.9	26.2	0.15
S7	Shaanxi	Lou soil (Earth-cumuli-Orthic Anthrosols)	26.0	9.5	7.9	22.4	0.24
S8	Hebei	Fluvo-aquic soil (Ochri-Aquic Cambosols)	10.5	4.9	8.0	8.1	0.19
S9	Henan	Fluvo-aquic soil (Ochri-Aquic Cambosols)	18.2	10.2	8.1	16.0	0.19
S10	Xinjiang	Gray desert soil (Calci-Orthic Aridosols)	9.6	11.2	8.1	25.3	0.16
S11	Shanxi	Cinnamon soil (Hapli-Ustic Argosols)	17.7	13.3	8.2	16.8	0.20
S12	Tianjin	Coastal saline soil (Marinic Aqui-Orthic Halosols)	7.6	12.6	8.3	24.7	0.19
S13	Gansu	Anthropogenic-alluvial soil (Typic Siltigi-Orthic Anthrosols)	6.7	11.1	8.4	11.2	0.20
S14	Shandong	Fluvo-aquic soil (Ochri-Aquic Cambosols)	17.1	6.8	8.6	13.1	0.18
S15	Inner Mongolia	Chestnut coloured soil (Typic Calci-Ustic Isohumosols)	10.5	9.4	8.8	11.6	0.21
Mean			18.7	13.4	7.5	18.5	0.18
CV%			55.3	44.0	14.0	39.8	21.1

Clay, clay content (%); TOC, total organic carbon (g kg^{-1}); CEC, cation exchange capacity (cmol kg^{-1}); Cd, cadmium concentration (mg kg^{-1}); CV, coefficient of variation.

inhibitor. In an anticompetitive inhibition, $V^*_{max} = V_{max}/\alpha$, $K^*_s = K_s/\alpha$, and $\alpha = 1 + \frac{[I]}{K_i}$

The inhibition constant K_i (mM) is calculated by the following equation:

$$\frac{1}{V^*_{max}} = \frac{1}{V_{max}} + \frac{1}{V_{max}K_i} \times [I] \quad (4)$$

where [I] is the concentration of the inhibitor Cd (mg kg^{-1}).

2.4.2.2. Kinetic parameters of a linear mixed inhibition. In a linear mixed inhibition, an inhibitor can combine with either enzyme active sites or non-active sites of an enzyme-substrate complex [36]:

$$V = \frac{V^*_{max}[S]}{K^*_s + [S]} = \frac{(V_{max}/\beta)[S]}{(\alpha/\beta)K_s + [S]} \quad (5)$$

where V^*_{max} and K^*_s are apparent maximum rate and apparent dissociation constant of an enzyme-substrate complex in the presence of inhibitor, $Vmax* = V_{max}/\beta$, $Ks* = (\alpha/\beta) \times K_s$,

$$\alpha = 1 + \frac{[I]}{K_i} \quad (6)$$

$$\beta = 1 + \frac{[I]}{\delta K_i} \quad (7)$$

where K_i is the inhibition constant (mM); δK_i is the enzyme-substrate-inhibition dissociation constant (mM); δ represents the affinity of enzyme-inhibitor for substrate (dimensionless), and also could be considered as a calibration factor when the inhibitor takes place of enzyme active site.

2.4.3. Quantification of Cd inhibition effect

The degree of inhibition or activation by Cd was calculated from the follow equation:

$$\text{Inhibition} = ((y - y_c)/y_c) \times 100 \quad (8)$$

where y is the value of dehydrogenase kinetic parameters (y) under Cd treatment, y_c is the value of dehydrogenase kinetic parameter without Cd treatment.

2.4.4. Calculation of ecological dose

Log-logistic model is used to fitting the dose-response relationship between enzyme kinetic parameter (y) and Cd concentration (x) [38]:

$$y = \frac{a}{1 + e^{(b \times (x-c))}} \quad (9)$$

where a, b, and c are three parameters. Parameter a is the value of uninhibited kinetic parameter, and b is a slope parameter indicating the inhibition rate. The parameter c is equal to the logarithm of inhibitor concentration at which the value of uninhibited kinetic parameter falls to half. We calculated the ecological dose (ED_{25}) which is considered to be more suitable criteria for assessing the sensitivity of soil enzymes to heavy metal pollution and sever as an index for the slightly polluted soils [39]. ED_{25} represents the Cd concentration at which the kinetic parameter is reduced by 25%.

2.4.5. Statistical analysis

The way of F-test was used to estimate the goodness-of-fit of Log-logistic model. Correlations of kinetic parameters and soil properties were calculated and the significance level reported was based on the Pearson's coefficient. Effects of Cd concentrations on the kinetic parameters were analyzed using the ANOVA. Stepwise regression analysis was carried out to investigate the relationships between soil properties and ED_{25} values. Soil properties data were log-transformed (except for pH) prior to analysis and were eliminated from the multiple regression equation if they were not statistically significant ($P > 0.05$). All statistical analyses and calculation were conducted with the SPSS 22.0 software (SPSS Inc., Chicago, USA) and Excel 2010 (Microsoft Corporation, Redmond, USA).

3. Results

3.1. Soil dehydrogenase kinetic characteristics in unspiked soils

Dehydrogenase activity (DHA), as well as the kinetic parameters (V_{max}, K_m and k) derived from DHA based on Eq. (1)–(2) (Fig. A1), all varied across the 15 soils, with the coefficient of variation (CV%) ranging from 51.7% for K_m to 79% for V_{max} (Table 2). For DHA, V_{max} and k, the minimum values occurred in soil S1 with the lowest pH and the maximum in soil S15 with the maximum pH (Table 2). Soil pH was the most significant variable explaining about 45.6%, 35.2% and 48.4% of the variations in DHA, V_{max}, and k among the 15 soils, respectively (Fig. 2). K_m was highest in soil S4 with the highest CEC and was lowest in soil S13 with the lowest Clay content (Table 1). No clear relationships were found between K_m and either of the physicochemical variables.

3.2. Soil dehydrogenase kinetic characteristics in Cd-spiked soils

In Cd-spiked soils, the K_m for soils S1 and S2 showed a slight decreasing trend with increasing Cd concentrations, but the

Table 2
Kinetic characteristics of soil dehydrogenase in the 15 unspiked soils.

Soil code	DHA($\mu g\,g^{-1}\,h^{-1}$)	K_m(mM)	V_{max}($\mu M\,h^{-1}$)	[a] R^2	[a] F value	$k(\times10^{-3}\,h^{-1})$	[b] R^2	[b] F value
S1	6.8	4.9	15.9	0.836	33.1	2.7	0.942	16.2
S2	10.0	2.9	18.2	0.802	26.3	3.8	0.530	1.1
S3	13.1	2.3	18.7	0.684	14.1	6.7	0.868	6.6
S4	21.5	7.3	60.7	0.973	234.2	7.5	0.969	31.2
S5	25.1	5.1	59.3	0.976	264.3	7.8	0.980	49.3
S6	27.7	1.7	42.7	0.924	79.0	16.7	0.957	22.3
S7	56.4	2.5	92.0	0.838	33.6	27.2	0.998	499.0
S8	57.7	1.7	79.6	0.598	9.7	24.3	0.947	18.0
S9	54.0	2.6	86.1	0.815	28.6	20.6	0.938	15.2
S10	40.3	5.8	94.4	0.875	45.5	13.0	0.976	40.3
S11	37.2	3.0	68.1	0.943	107.5	15.6	0.997	302.0
S12	48.3	3.6	94.7	0.941	103.7	20.5	0.997	293.1
S13	15.9	1.4	21.1	0.672	13.3	7.8	0.841	5.3
S14	34.8	1.6	46.1	0.610	10.2	14.6	0.963	25.9
S15	122.7	4.3	235.2	0.811	27.9	42.0	0.993	150.5
Mean	38.1	3.4	68.8			15.4		
CV%	75.8	51.7	79.0			68.1		

DHA, dehydrogenase activity; [a] Coefficient of determination (R^2) and F value for K_m and V_{max} derived using Eq. (1)–(2) and the original data for regression are given in Fig. A1, $F_{0.05}$ (2, 13) = 3.63. [b] Coefficient of determination (R^2) and F value for k calculated by a linear fitting of t with ln ($[S_0]/[S_t]$) in Eq. (2) and the original data for regression are shown in Fig. A2, $F_{0.05}$ (1, 2) = 18.51.

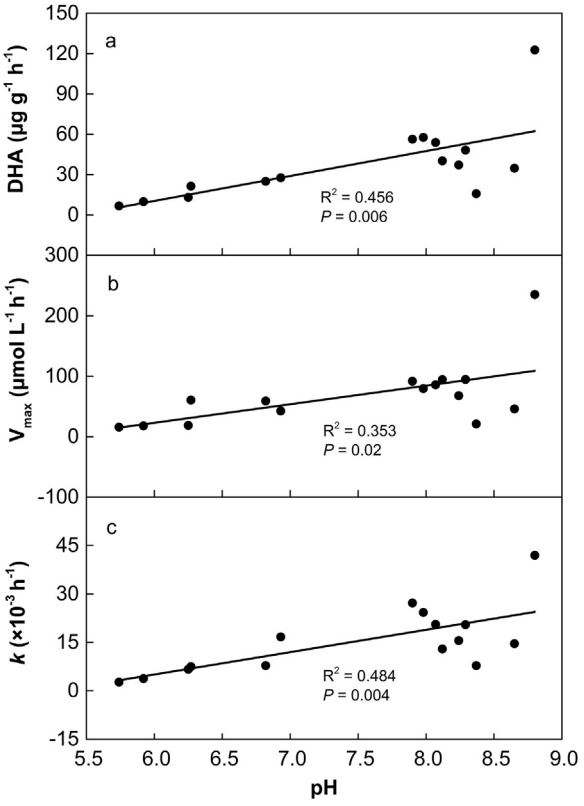

Fig. 2. Relationships among soil dehydrogenase activity, kinetic parameters, and soil pH.

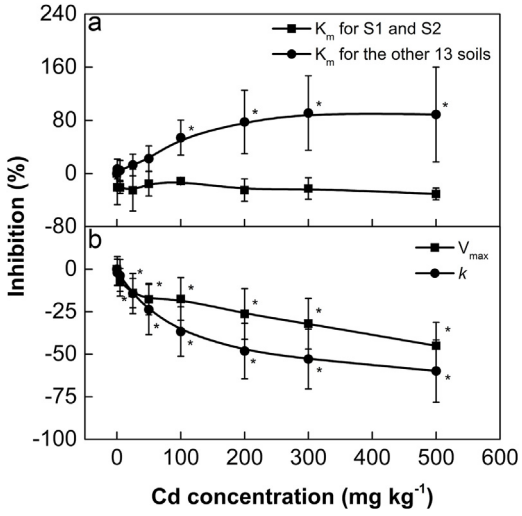

Fig. 3. Inhibition of soil dehydrogenase kinetic parameters by Cd. In panel a, samples size n = 2 for data points represented by solid squares; n = 13 for data points represented by solid circles. In panel b, sample size n = 15 for all data points. Asterisks denote significant difference ($P < 0.05$) between Cd treatments and control.

decrease was not significantly different ($P > 0.05$) from the control (Fig. 3a, Fig. A2). The K_m for the other 13 soils increased monotonically with Cd concentration ($P < 0.05$), especially at the Cd treatments less than 100 mg kg^{-1} the increase was apparently faster (Fig. 3a). Both V_{max} and k showed a clear decreasing trend with increasing Cd concentrations ($P < 0.05$; Fig. 3b). When the Cd concentration reached at 500 mg kg^{-1}, V_{max} and k was reduced by 46% and 61% on average for the 15 soils, respectively.

3.3. Mechanism of Cd inhibition to soil dehydrogenase

Based on the responsive patterns of kinetic parameters to Cd concentration showing in Fig. A2, two inhibition mechanisms could be identified to describe the inhibition of Cd on DHA in the studied soils: an anticompetitive inhibition for soils S1 and S2 characterized by the decreased K_m and V_{max}, and a linear mixed inhibition for the other 13 soils characterized by the increased K_m but decreased V_{max}. The inhibition constant K_i was much higher for soils S1 (12.2 mM) and S2 (4.7 mM) than for the other 13 soils (<4.6 mM). In general, K_i decreased with pH, with the mean K_i being 5.7, 2.8 and 1.4 mM for acidic (pH < 6.5), neutral (pH = 6.5–7.5), and alkaline (pH > 7.5) soils, respectively (Table 3), suggesting that the affinity of dehydrogenase for Cd in alkaline soils was greater than that in neutral and acidic soils. The values of parameter δ were all larger than 1 (Table 3), indicating that the affinity of enzyme-inhibitor complex for substrate was lower than the affinity of enzyme for substrate (Table 2).

The dose-response relationships between kinetic parameters (V_{max} and k) and Cd concentration were well fitted by the Logistic

Table 3
Inhibition constant and ED_{25} values of dehydrogenase kinetic parameters in the 15 Cd-spiked soils.

Soilcode	K_i(mM)	R^2	F value	δ	R^2	F value	[a]ED_{25}-V_{max}	R^2	[b]F value	ED_{25}-k	R^2	F value
S1	12.2	0.758	22				218.2	0.639	9	70.3	0.806	29
S2	4.7	0.967	202				182.9	0.949	112	321.7	0.915	75
S3	1.3	0.911	72	–[c]	–	–	–	–	–	11.9	0.864	44
S4	4.6	0.925	86	1.5	0.919	79	80.0	0.879	51	162.5	0.964	187
S5	3.4	0.920	80	3.2	0.900	63	427.8	0.879	51	248.7	0.904	66
S6	2.1	0.981	352	6.6	0.955	150	727.2	0.904	66	74.4	0.989	629
S7	1.4	0.930	93	6.6	0.759	22	295.7	0.860	37	50.6	0.972	243
S8	0.7	0.913	73	3.8	0.892	58	26.4	0.988	576	15.0	0.997	2326
S9	1.3	0.908	69	2.4	0.954	146	101.4	0.946	123	33.6	0.991	771
S10	1.4	0.978	308	3.0	0.931	94	290.5	0.959	140	36.1	0.992	868
S11	1.3	0.950	132	2.0	0.983	400	63.6	0.959	140	7.9	0.971	234
S12	2.3	0.910	71	2.2	0.813	31	96.2	0.966	199	35.3	0.979	326
S13	1.5	0.933	987	2.1	0.931	94	51.7	0.950	133	25.0	0.992	868
S14	1.9	0.948	127	1.5	0.906	68	39.8	0.944	118	18.3	0.992	868
S15	1.2	0.973	250	6.7	0.804	29	61.5	0.969	188	46.7	0.993	993
Mean	2.7			3.6			190.2			77.2		
CV%	105.3			55.1			102.8			121.2		

[a] The unit of ED_{25} is mg kg^{-1}.
[b] $F_{0.05}$ (1, 7) = 5.59.
[c] Not applicable.

model (Fig. 3 and Fig. A2). The regression determination coefficient (R^2) all passed the significance test except those for soils S1 and S3 (F test, $P < 0.05$; Table 3). To quantify the toxicity of Cd to the kinetics of dehydrogenase, ED_{25} for V_{max} (ED_{25}-V_{max}) and ED_{25} for k (ED_{25}-k) were calculated based on Eq. (9). The coefficient of determination (R^2) for ED_{25}-k was always larger than that for ED_{25}-V_{max} (Table 3), indicating that ED_{25}-k better reflects the inhibition effect of Cd on dehydrogenase kinetics. Among the 15 soils, ED_{25}-k had lower mean values (77.2 mg kg^{-1}) but larger CV% (121%) than those of ED_{25}-V_{max} (190.2 mg kg^{-1} and 103%).

3.4. Influence of soil properties and enzyme-Cd affinity on Cd toxicity

For all the 15 soils, ED_{25}-V_{max} was positively correlated to TOC, CEC, and clay, while ED_{25}-k was significantly correlated to pH and TOC ($P < 0.05$; Fig. 4), indicating that the Cd toxicity to dehydrogenase decreased with increasing TOC, CEC, and clay content. However, ED_{25}-k was negatively correlated to pH, indicating that Cd toxicity increased with pH. Our stepwise regression analysis further revealed that TOC content was the major factor affecting Cd toxicity to dehydrogenase kinetics, explaining 50% and 43% of the total variation in ED_{25}-V_{max} and ED_{25}-k, respectively (Fig. 4b, f).

For the 12 soils exhibiting linear mixed inhibition, we found that the affinity of enzyme-Cd also affected ED_{25}-k significantly ($P < 0.05$; Fig. 5). The K_i and the affinity of enzyme-inhibitor for substrate (δ) together explained 80% of total variation in ED_{25}-k (Fig. 5), indicating that the Cd toxicity to dehydrogenase decreased with increasing K_i.

4. Discussion

4.1. Soil dehydrogenase kinetic properties of different soil types

By analyzing the kinetic characteristics of dehydrogenase in different soil types without Cd treatment, we concluded that kinetic parameters (K_m, V_{max}, and k) varied according to soil types and properties. Though the types and properties of tested soil samples differed significantly (Table 2), the K_m values of soil dehydrogenase were kept in the same order of magnitude (1.4–7.3 mM), and showed a lowest value of CV%. In another research of sandy loam soils, the K_m values of dehydrogenase with different fertilization treatments were between 4.0–7.9 mM [28]. Since the K_m, an essential enzyme property, is used to measure the affinity of the enzymes to their relative substrates, i.e. the smaller the K_m, the greater the affinity. There were some soil enzymes existing forms of low and high affinity which could catalyze the same chemical reaction. For example, the K_m values of low affinity forms of arylsulfatase and phoshpomonoesterase were more 10 times higher than that of their high affinity forms when exposed to the same substrate, respectively [40]. In the soil, the enzyme-substrate interactions could be affected by the conformational alteration of enzyme protein sorbed by clay or covalently bound to soil particles (humic and clay) [20,21]. Unlike the extracellular enzymes (such as arylsulfatase and phoshpomonoesterase), dehydrogenase usually exists within all microbial cells [29]. We infer their affinity is not easily affected by soil properties, but mainly due to differences in the dehydrogenase isoenzymes presented in cell.

Compared with previous study, we found the V_{max} varied significantly in the same soil type or among various soil types. The maximun/minimun ratio of V_{max} in sandy loam soils and brown soils were 0.4/1.3 μg g^{-1} h^{-1} and 1.7/3.0 mg kg^{-1} 24 h^{-1}, respectively [28,41]. The catalytic reaction rate of soil enzyme is largely determined by soil properties, soil types, and climate [27]. In specific, the correlations between soil DHA and microflora potential activity, C and N turnover, pH, and OM content have been revealed in several works [30,42]. In this study, we found that soil dehydrogenase V_{max} and k were mostly affected by soil pH, which indicated the catalysis reaction of dehydrogenase to its substrate (INT) was mainly affected by soil pH. Soil pH caused the change of catalytic efficiency not only by affecting the three-dimensional conformation of enzymes and the catalytic function of active sites, but also by affecting the dissociation of enzyme-substrate complex [43]. Under this condition, soil pH is considered to be a well predictor of DHA in the soil environment [30].

This study provided the first systematic synthesis on the kinetic characteristics of dehydrogenase, and tried to clarify the effect of soil properties on its kinetic parameters. The results provided in this study could be outstretched and further applied in several aspects. First, it provided the background values of K_m, V_{max} and k of dehydrogenase in 14 different soils types. Because of its important roles in maintaining microbial respiratory processes, dehydrogenase is generally considered as an indicator of overall microbial activity [28]. Therefore, the kinetic parameters of dehydrogenase would be useful for modeling and qualitative analysis of soil microbial metabolic processes. Second, the quantitative relationships between kinetic parameters (V_{max} and k) of dehydrogenase and environmental pH provided a better understanding of its activity

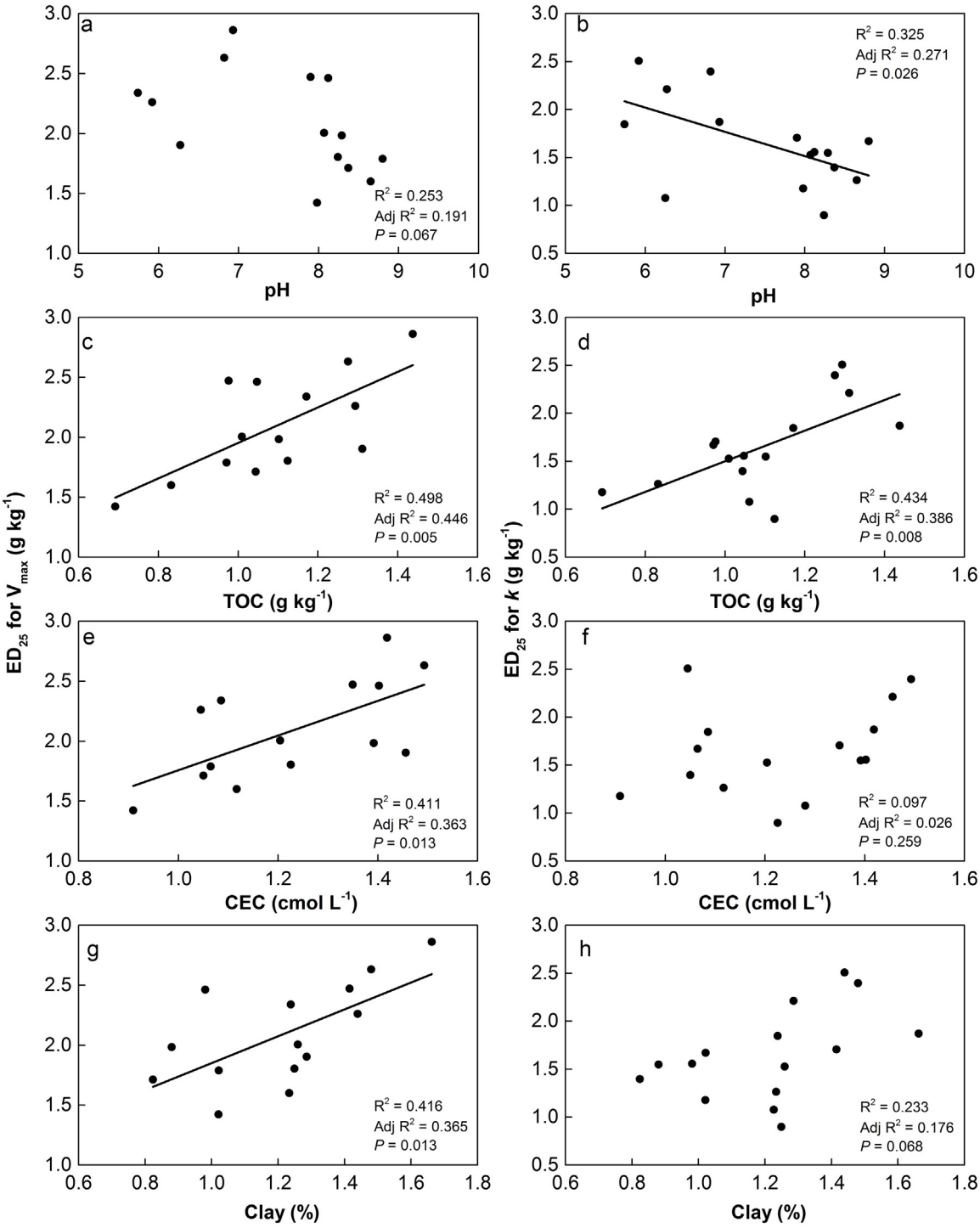

Fig. 4. Correlations between ED_{25} values and soil properties. Data were log-transformed (except for pH) prior to analysis.

respond to varied soil pH. Third, considering soil dehydrogenase activities significantly correlated to V_{max} ($r = 0.967$, $n = 15$, $P < 0.01$), it might be possible to predict V_{max} based on soil dehydrogenase activities. Moreover, this correlation indicated that the difference of apparent dehydrogenase activities were major determined by V_{max}, i.e. the enzyme contents [28].

4.2. Inhibition mechanisms of Cd to soil dehydrogenase

Heavy metals could affect the enzyme activity through several ways including: altering the affinity of enzyme to its substrate, denaturing of the enzyme protein, and influencing the synthesis of enzyme [44]. Known that Cd could alter enzyme activity by binding to its functional groups (sulfhydryl, carboxyl, imidazole, etc.) and/or by displacing other metal ions associated with it, resulting in the complex mechanisms of enzyme in response to Cd stress [19]. By analyzing the variations of kinetic parameters of soil dehydrogenase under different Cd concentrations, the inhibition mechanisms of Cd to soil dehydrogenase could be further deduced. For example, an anticompetitive inhibition type of mechanism in brown soil (S1) and latosolic red soil (S2) was found in this study. This type of mechanism showed that Cd pollution had stimulated the affinity of

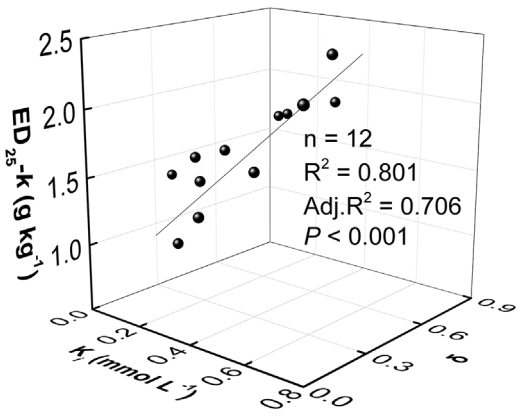

Fig. 5. Multiple linear regression between ED_{25} for *k* and inhibition constants (K_i and δ) from 12 soils for the linear mixed inhibition. Data were log-transformed (except for pH) prior to analysis.

dehydrogenase to its substrates, but it also weakened the decomposition of enzyme-substrate complex, thus in total the catalytic rate of enzyme was decreased. In addition, this deleterious effect on dehydrogenase may also be generated by binding of Cd to the cysteinyl and histidel groups of enzymatic proteins [2].

There was a linear mixed inhibition in other soil types. Along with this kind of inhibition, the K_m of soil dehydrogenase tended to increase which suggested that exogenous Cd decreased the affinity of soil dehydrogenase to its substrate and slowed down the rate of formation of enzyme-substrate complex, thus inhibited soil DHA. Meanwhile, V_{max} was decreased along with Cd concentrations increased. The values of parameter δ were bigger than 1, which further indicated the competitive inhibition was the dominated inhibition mechanism in the linear mixed inhibition [36]. In addition, when Cd binds to the active sites of carbonic anhydrase from gills of gilthead sea bream (*Sparus aurata*), its manner of toxicity was also belong to competitive inhibition [19].

The simultaneous change of K_m and V_{max} caused the sharply decreasing of *k* in linear mixed inhibition which implied that Cd might be more toxic in this type of inhibition. Actually, the inhibition constant (K_i) of linear mixed inhibition was smaller than that of anticompetitive inhibition type. The K_i reflects the affinity of enzyme to its inhibitor, the lower K_i is, the easier for inhibitor to combine with enzyme. Thus in the linear mixed inhibition, the combination of enzyme with its substrate was more restricted by inhibitors compared to in anticompetitive inhibition type.

4.3. Soil properties and inhibition constant affected ecology dose of Cd in different soil types

Some researches indicated that Cd was more toxic to DHA in alkaline soils [33,45]. In this study, the ED_{25} values in acid and neutral soil were generally higher than those in alkaline soil (except the soil S3), also implying the dehydrogenase in alkaline soils was more vulnerable to Cd toxicity. This could be explained by two reasons. On the one hand, Cd ion was prone to form $Cd(OH)^+$ which was easy to interact with enzymes in alkaline solution [45]; on the other hand, the K_i of Cd to dehydrogenase in alkaline soil was lower than that in acid and neutral soils, resulting in much easier combination of dehydrogenase and Cd.

The ecology dose of Cd reported in the vast number of literatures concerning this topic varied among a wide concentration range. For example, ED_{50} values in the range 90–5555 mg kg^{-1} were reported for DHA [2,14,33]. This may be explained by the fact that the toxicity of heavy metal in soils depends on several soil properties such as TOC, CEC, pH, metal ionic properties, and pollution time [14,33]. Often, the highly fertile soil had huge buffer capacity to protect soil dehydrogenase against different interferences. Firstly, soil with high TOC content provided a better nutritional condition that supplied sufficient carbon and nitrogen sources for microorganisms to resist ambient environmental changes [30]. Secondly, soil aeration parameters such as oxygen diffusion rate (ORD), redox potential (Eh), concentration of Fe^{2+}, water content, and bulk density had significant effects on DHA in soils [46]. Finally, higher contents of TOC, clay, and $CaCO_3$ might absorb more Cd in soils, resulting in alleviation of Cd bioavailability and toxicity [47].

Inferred from the regression results between ED_{25} based on V_{max} and soil properties, increased TOC content could alleviate the toxicity of Cd to soil dehydrogenase. However, it lowly explained the total variation of Cd toxicity. These results indicated that soil properties actually affected the toxicity of Cd to soil DHA, but their contributions were limited. In the linear mixed inhibition, K_i and δ could explain 80% of total variation in the toxicity of Cd, so we suggested that the affinity between enzyme and Cd (inhibition type and K_i) differentiated in different soil types might function as another important factor affecting the toxicity of Cd.

4.4. ED_{25} for parameters as indicator of Cd toxicity

In previous ecotoxicological studies, soil enzyme has been utilized as a sensitive indicator to define the impact of contaminants such as metals on soil biological functions [11]. Results from the present study showed the values of V_{max} and *k* decreased markedly along with the increasing Cd concentrations and an obvious dose-response relationship was found. ED_{25}-V_{max} (mean 190.2 mg kg^{-1}) had larger mean value than those of ED_{25}-*k* (mean 77.2 mg kg^{-1}), which indicated a significant difference in the sensitivity of kinetic parameters to Cd (Table 3). Due to the ED_{25}-DHA for Cd in the soils of this study had a mean value of 95.6 mg kg^{-1} [48], it could be seen that *k* was particularly sensitive to the toxicity of Cd.

In most studies, measuring activity and V_{max} based on the substrate saturation had the advantage of decreasing competitive inhibition by naturally occurring substrates within the soil matrix [49]. However, enzymes may operate under non-saturation conditions in soils [34,49], and the Michaelis-Menten function can be transformed to pseudo-first-order kinetic function. The first-order kinetic constant (*k*) became an important parameter, because *k* is a quantitative value of the rate of chemical reaction in dependence of substrate concentration. Judging from these results, *k* appeared to be a sensitive indicator for description of the toxicity of Cd to the soil DHA, which was well established as an early indicator of gross changes in soil microbial metabolism caused by Cd contamination.

5. Conclusion

The kinetic parameters of dehydrogenase in unspiked soils varied with soil types. Using a linear function, the effect of pH on the V_{max} and *k* could be described. When exposed to Cd, the K_m in latosolic red soil and brown soil were decreased, and V_{max} were increased. These indicate that the toxicity of Cd to dehydrogenase belongs to the uncompetitive inhibition mechanism. However, in the other soils the K_m were increased and V_{max} were decreased, which belongs to the linear mixed inhibition mechanism. These two inhibition types were existed to illuminate the toxicity of Cd to dehydrogenase. In addition, multiple stepwise regression analysis between ED_{25} and soil properties showed soil TOC content and K_i were major factors influencing Cd toxicity to DHA. Inferred from this study, *k* was suggested to be a sensitive indicator for the acute toxicity of Cd in soil and the threshold for slightly Cd-contaminated soil was considered at 7.9 mg kg^{-1}. In order to use soil enzyme as a biomarker of soil heavy metal pollution, the kinetic studies of

enzyme were necessary. These measurements could give more subtle information about enzyme activity changes in respond to the pollution of heavy metals. Furthermore, the kinetic characteristics of dehydrogenase involving different soil types along the natural Cd contamination gradients are needed to be studied.

Acknowledgements

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Appendix A.

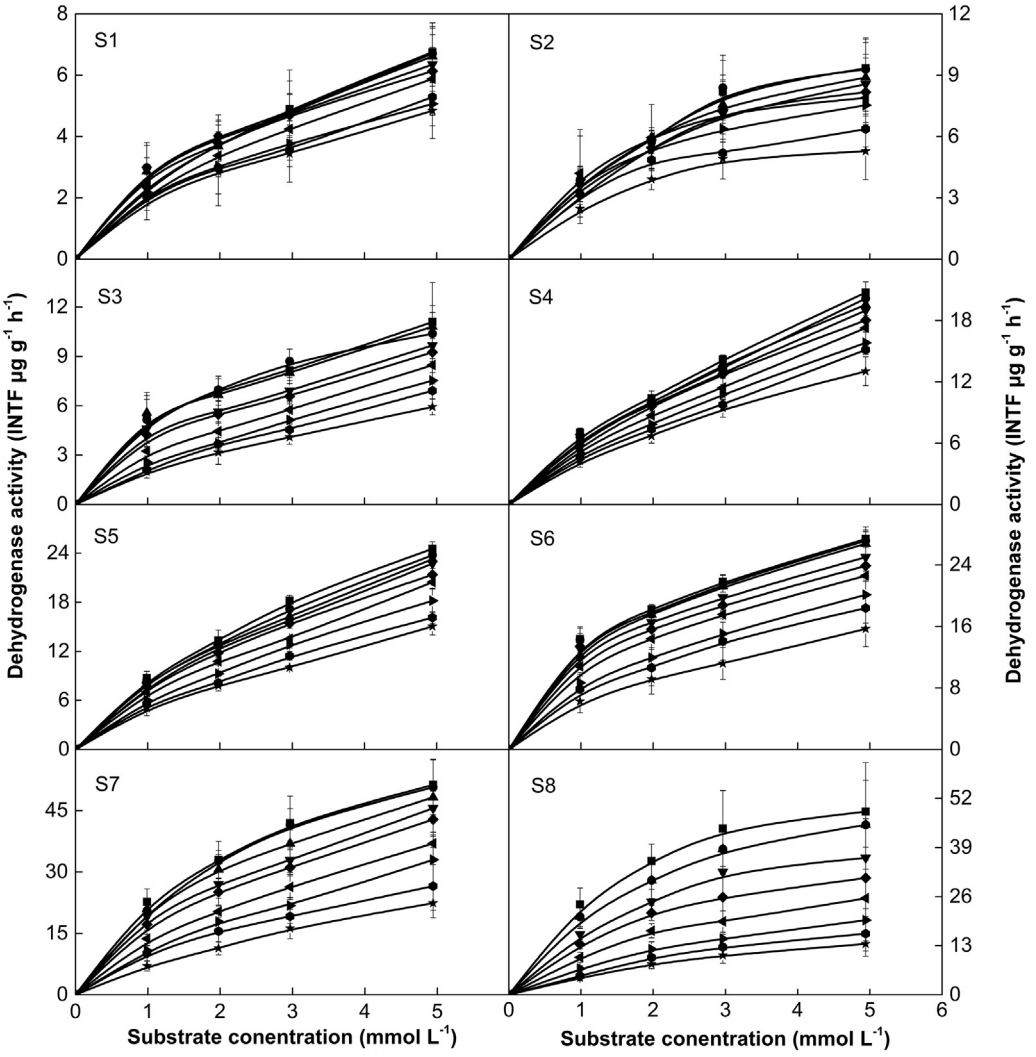

Fig. A1. Effect of Cd on dehydrogenase activities in different substrate concentration in 15 soils. The numbers S1 to S15 denote the soil samples and the number 0–500 denote that the samples were analyzed under different Cd concentrations (mg kg^{-1}).

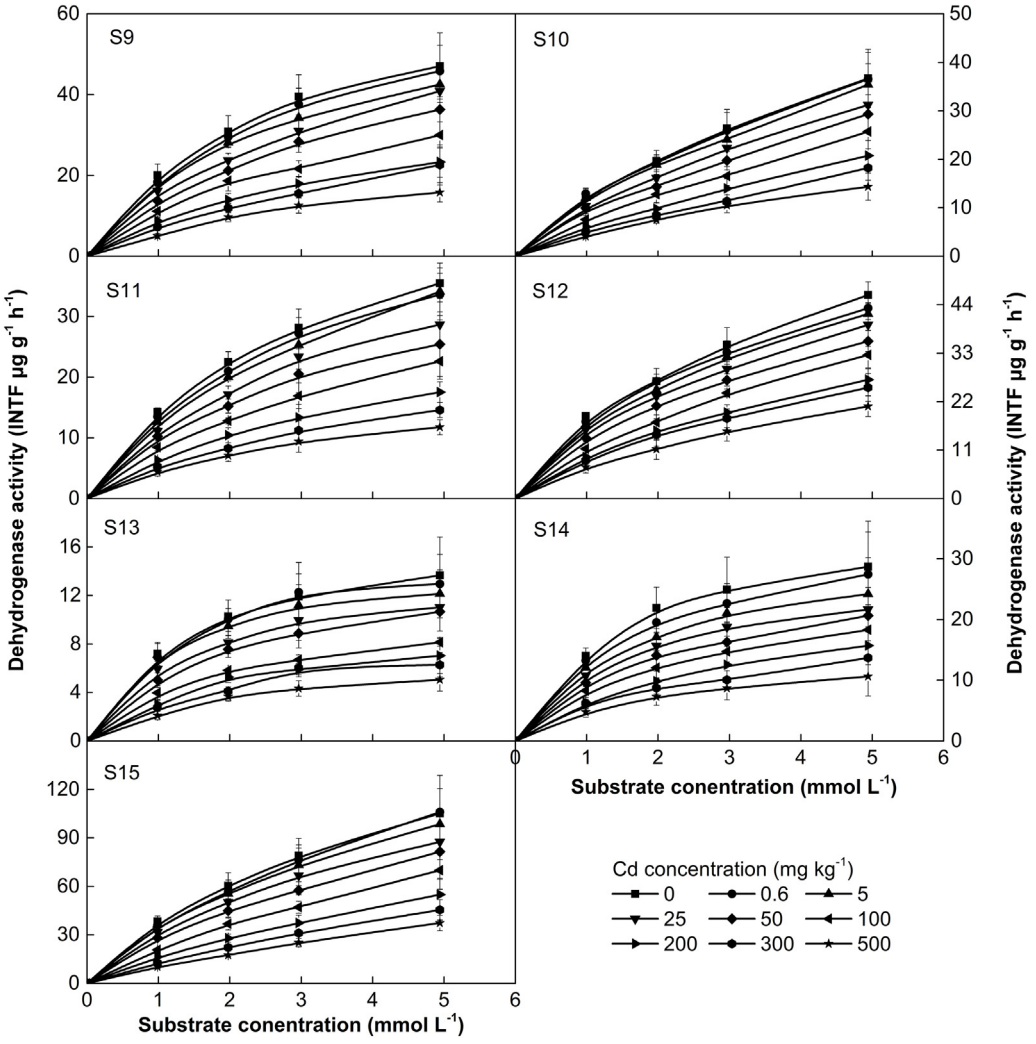

Fig. A1. (*Continued*)

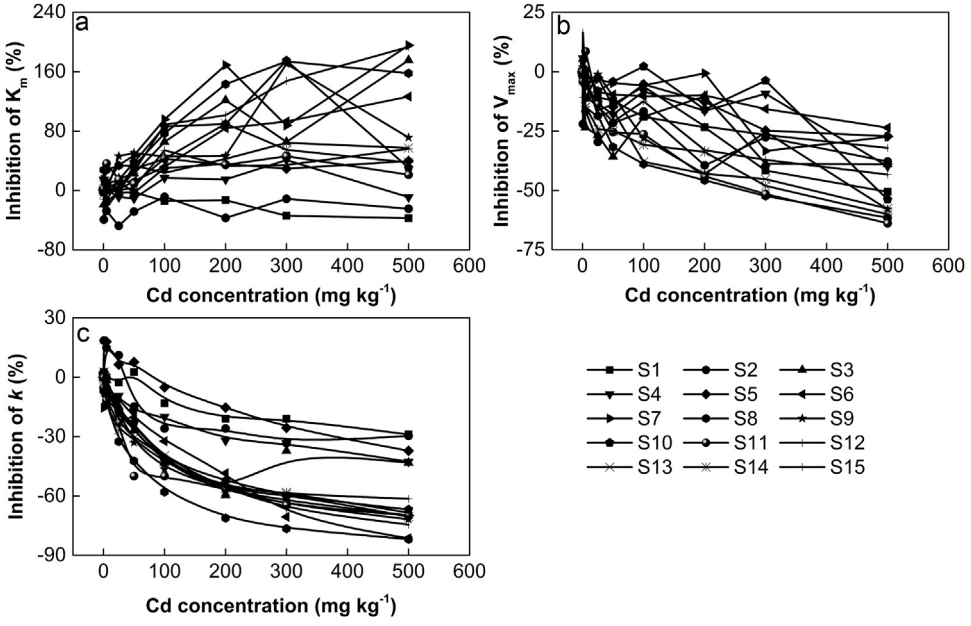

Fig. A2. Effect of Cd on kinetic parameters of dehydrogenase in 15 soils. The letters a, b, and c represent the inhibition of K_m, V_{max}, and *k* by Cd, respectively.

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