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Investigation of the toxic effect of cadmium on *Candida humicola* and *Bacillus subtilis* using a microcalorimetric method

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ABSTRACT

In this study, the technique of microcalorimetry based on heat-output by aerobic bacterial respiration was explored to evaluate the toxic effect of cadmium on *Candida humicola*, *Bacillus subtilis*, singularly or in a mixture of both. Power–time curves of the growth metabolism of *C. humicola* and *B. subtilis* and the effect of Cd^{2+} were studied using the TAM III (the third generation thermal activity monitor) multi-channel microcalorimetric system, isothermal mode, at 28 °C. The differences in shape of the power–time curves and the thermodynamic and kinetic characteristics of microorganisms growth were compared. The effect of cadmium added into microorganism would significantly reduce the life cycle and change the thermal effect of microbial metabolic process with different concentrations of Cd^{2+}. The experimental results revealed that at the same concentration, the sequence of inhibitory ratio (I) and maximum thermal power (P_{max}) of the Cd^{2+} was: mixed microorganisms > *C. humicola* > *B. subtilis*. The sequence of total thermal effect (Q_{total}) and growth rate constant (k) is mixed microorganisms > *B. subtilis* > *C. humicola*. These results are important to further studies of the physiology and pharmacology of *C. humicola* and *B. subtilis* and may support the theory of restoring contaminated soil.

1. Introduction

Techniques for assessing heavy metal toxicity to microbes have developed remarkably since the 1980s, the investigations deeply influencing the biochemical and physical chemistry field. Heavy metals are a common cause of environmental pollution and they often accumulate in soils [1,2] due to their affinity for soil organic matter in general and humic acids in particular [3,4]. Extensive data suggests that cadmium is the most toxic heavy metal and it is listed as a priority pollutant by the US Environmental Protection Agency [1,5].

Among heavy metals, cadmium (Cd), mercury (Hg), and copper (Cu) are known to have deleterious effects on the density, activity, and diversity of soil micro-flora. Heavy metal pollution (of Cd^{2+}, $Cd(NO_3)_2$) has been shown to affect plant and soil microbial function. The toxic effects of Cd^{2+} on plant growth, metabolism and enzyme activity are well documented: Cd^{2+} inhibits plant growth and also disturbs photosynthesis, sugar metabolism, sulphate assimilation, and several enzyme activities even at low concentrations [3]. In daily life, the main sources of cadmium are glaze, plastic, rubber, oil paint, printing and dyeing, nickel–cadmium batteries, electroplating and the liquid waste emissioned from the electroplating. If these liquid wastes contaminate a river, the pollutant emission will cause the contamination of the shellfish and fish, and also have toxic effect on the vegetables and rice which are irrigated by the river [6]. Chronic intoxication of cadmium will cause damage of renal cells, the impediment of liver function, recurrent abortions, and antecedent prostatic carcinoma, etc. Further more, older citizens have accumulated high contents of Cd^{2+} through their diet.

In determining microbial activities, radioisotopes, chemical measurements and microelectrodes were used frequently. However, most of the experiments dealing with the impact of metals on soil microorganisms have been field experiments, generally associated with inorganic nutrients and organic matter, which has made it difficult to distinguish the effect of one metal from the effect of another [7]. However, with the development of microcalorimetric techniques, the investigations of living materials have been improved during the past 25 years. A wide range of practical applications of such techniques have been envisioned [8]. In recent years, microcalorimetric techniques have been

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Nomenclature

C	concentration
Q_T	total thermal effect
P_M	Values of thermal power at the maximum of the peak
t_M	values of peak time
k	the microbial growth rate constant
t_G	generation time
I	inhibitory ratio
IC_{50}	half-inhibitory concentration

increasingly used to study microbial activities in soils. Microcalorimetry is a highly sensitive method to assess the overall biomass and activity of soil microorganisms. It can provide important quantitative indices of activities like microbial growth rate constant, heat evolution processes and heat yield of microbial growth. Another advantage of the calorimetric method is its simplicity and non-disturbance of samples. Ameliorating effects of organic matter on Cd toxicity have been reported for crops and herbaceous plants [3,9–11].

Soil microorganisms are essential to the environment due to their role in many reactions, such as in cycling mineral compounds, decomposing organic materials, promoting/suppressing plant growth and various soil biophysical processes. For studies of soil microbial activity, information on changes in microbial biomass is valuable, because it not only provides an indication of slower, less easily detectable soil organic matter changes, but also it represents an important labile pool of available nutrients to plants [12]. In addition, knowledge of the microbial processes in agricultural soils requires a great number of measurements at different conditions, as well as a great number of samples and accurate measurement methods [13]. So the microcalorimetric method has a large potential for investigating the toxic effect of heavy metal towards soil microorganisms.

In this study, using the TAM III (the third generation thermal activity monitor) multi-channel microcalorimeter we evaluated the short-term effects of cadmium at different doses in *Bacillus subtilis*, *Candida humicola* and mixed microorganisms under the closed, static experimental environment. The thermodynamic parameters, that is, growth rate constant (k), inhibitory ratio (I), half-inhibitory concentration (IC_{50}), the maximum thermal power (P_{max}), total thermal effect (Q_{total}), have been calculated. To the best of our knowledge, there are no reports on the study of the effect of Cd on the growth of *C. humicola* and *B. subtilis* by means of microcalorimetry. The aims of this study were to investigate the toxic effect of Cd on soil microbial activity measured by calorimetry, so the dosage infused in the microorganisms is higher and unrealistic than the background values for the environment, which experiments based on the pure and mixed culture is to test the toxic effect of Cd^{2+} on microorganisms. The fundamental power–time curves of *C. humicola*, *B. subtilis* and mixed microorganisms under different concentrations of Cd^{2+} were obtained. The microorganisms choosed in this experiments are the typical microorganisms blong to soil eukaryotic microorganisms (*C. humicola*) and Gram-negative bacteria of prokaryotic microorganisms (*B. subtilis*), so the toxic effect is representational in heavy metal's toxic study. According to the different curves, the thermodynamic parameters of the energy release by *C. humicola* and *B. subtilis* under different conditions have been calculated. These can help to elucidate the toxicological effect of Cd^{2+} on biological processes [14,15,3].

2. Experiments and materials

2.1. Materials

B. subtilis and *C. humicola* were provided by the China Center for Type Culture Collection, Huazhong Agricultural University (Wuhan, PR China).

Analytical-reagent grade $Cd(NO_3)_2{\cdot}4H_2O$ was obtained from Shanghai Tingxin Chemical Factory (Shanghai, PR China). The concentration of Cd^{2+} mentioned in the article was Cd ion concentration.

The peptone culture medium where *B. subtilis* were grown was prepared by dissolving 5 g peptone, 3 g beef extract and 5 g NaCl in 1000 mL of deionized water at pH 7.2. It was then sterilized with high-pressure steam at 120 °C for 30 min.

The potato culture medium where *C. humicola* was grown was prepared by dissolving 200 g potato in 1000 mL of deionized water at pH 7.1 boiled for 30 min, decanted through cotton-cloth, filtered through paper, volume added to 1000 mL, and then added 10 g glucose to the fluid. It was then sterilized with high-pressure steam at 120 °C for 30 min [16,17].

The mixed culture medium where mixed microorganisms grown was prepared by mixed potato culture medium and peptone culture medium together with cubic content proportion 1:1 to 500 mL, then adjusting the pH value of mixed culture medium at 7.0.

2.2. Instrument and microcalorimetric method

The microcalorimetric study was performed on a TAM III multi-channel thermal activity microcalorimeter, Thermometric AB, Sweden. TAM III is modular, comprising thermostats, calorimeters, sample handling systems and auxiliary equipment. It is designed to monitor a wide variety of processes and complex systems continuously such as the thermal activity of physical, chemical and biological processes in terms of heat, heat flow and heat capacity over a temperature range of 15–150 °C. There are three operating modes for the TAM III multi-channel thermal activity microcalorimeter: isothermal mode, step-isothermal mode and scanning mode. The performance of this instrument and the details of its construction have been described in the user manual. Microcalorimetry is used to test the thermodynamics curve and the toxic effect of heavy metal in soil microbes. Because of the low intensity of the febriferous effect and the long reaction cycle in the organism, we cannot get a satisfactory result from normal calorimetry. Therefore we use the microcalorimeter. The toxicity of the heavy metal to the microbes was investigated in this study [18]. The dosage of Cd^{2+} compounds ($Cd(NO_3)_2{\cdot}4H_2O$) used in experiments are 0, 200, 400, 800, and 1600 μg/mL to the *C. humicola* and mixed microorganisms, and 0, 30, 120, 240, and 480 μg/mL to the *B. subtilis*. Choosing same dosage of Cd^{2+} compounds ($Cd(NO_3)_2{\cdot}4H_2O$) to mixed microorganisms is to test how the sensitivity of two microorganisms contribute to the mixed microorganisms at higher level concentration of Cd.

In this experiment, Cd^{2+} compounds ($Cd(NO_3)_2{\cdot}4H_2O$) were dissolved in double distilled water. The Cd^{2+} solutions ($Cd(NO_3)_2{\cdot}4H_2O$) were diluted with the peptone and the potato culture mediums to obtain the final different concentration of Cd^{2+} in the culture medium. The 4 mL steel ampoules containing the *B. subtilis* and *C. humicola* were cleaned and sterilized. *B. subtilis* were inoculated in 2 mL peptone culture medium and the initial density of *B. subtilis* was 2×10^6 cells/mL. *C. humicola* were inoculated in 2 mL potato culture medium and the initial density of *C. humicola* was 2×10^6 cells/mL. Different concentrations of Cd^{2+} were added respectively to the peptone culture media and potato culture medium containing *B. subtilis* and *C. humicola* in the

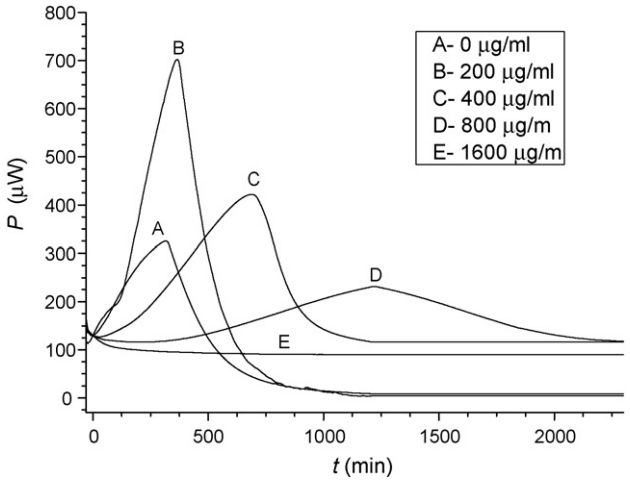

Fig. 1. The thermogenic curves for the growth of *Candida humicola* at 28–30 °C for the different concentrations of Cd^{2+}: (A) 0 μg/mL, (B) 200 μg/mL, (C) 400 μg/mL, (D) 800 μg/mL, and (E) 1600 μg/mL.

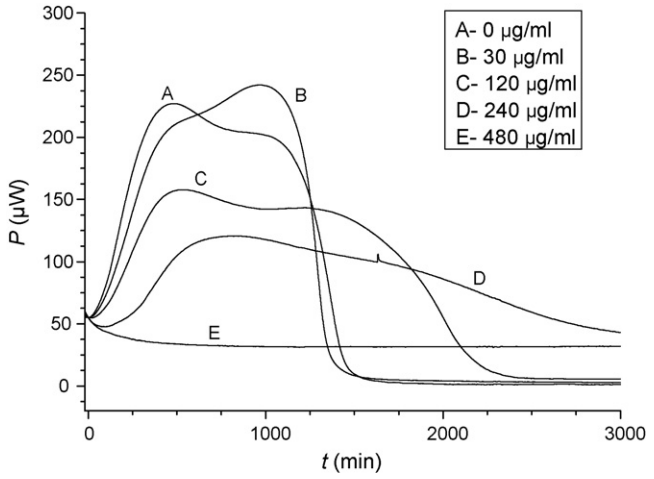

Fig. 2. The thermogenic curves for the growth of *Bacillus subtilis* at 28–30 °C for the different concentrations of Cd^{2+}: (A) 0 μg/mL, (B) 30 μg/mL, (C) 120 μg/mL, (D) 240 μg/mL, and (E) 480 μg/mL.

4-mL steel ampoules. The ampoules with samples were put into the microcalorimetric system. The temperatures of the calorimeter system and the isothermal box were controlled at 28 °C. At the same time, a computer was used to continuously record the power–time curves of the growth of *B. subtilis* and *C. humicola* growth [19,20].

3. Results and discussion

3.1. Application areas of microcalorimetry

The power–time curves for growth of *C. humicola* and *B. subtilis* and their mixture at 28 °C, in the presence of different Cd^{2+} concentrations are shown in Figs. 1–3. They can all be divided into four phases, that is, lag phase, log phase, stationary phase and decline phase. Fig. 3 shows the power–time curves for the growth of mixed microorganisms at 28 °C in the presence of Cd^{2+} liquor of different concentrations. The power–time curves can still be divided into four phases, in which log phase and decline phase are very similar to the former, but the stationary phases are significantly different from those of *C. humicola* and *B. subtilis*.

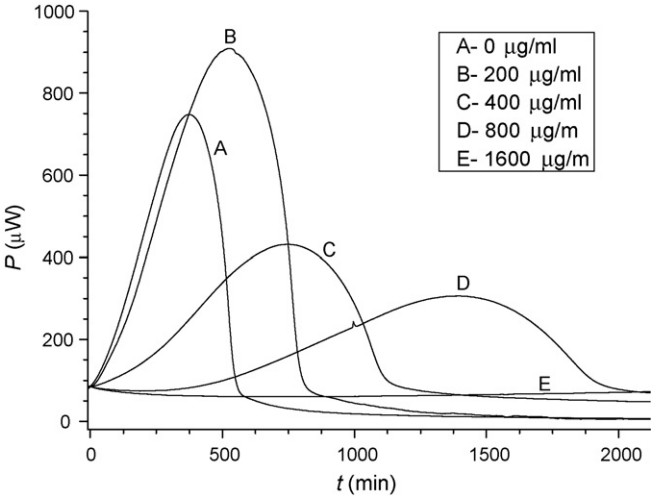

Fig. 3. The thermogenic curves for the growth of mixed microorganisms at 28–30 °C for the different concentrations of Cd^{2+}: (A) 0 μg/mL, (B) 200 μg/mL, (C) 400 μg/mL, (D) 800 μg/mL, and (E) 1600 μg/mL.

3.2. The important parameters from power–time curves

During the lag phase and the log phase for *C. humicola* and *B. subtilis*, Cd^{2+} inhibits their growth to different extents and

Table 1
Effect of Cd^{2+} on *Candida humicola*, *B. subtilis* and mixed microorganisms

Inhibitor	C (μg/ml)	Q_T (J)	P_M (μW)	t_M (min)	k ($\times 10^{-3}$ min^{-1})	t_G (min)	I (%)	IC_{50} (μg/ml)
Candida humicola	0	6.15	325.19	316.0	7.38	0.094	0.00	428.66
	200	10.06	702.04	365.0	5.77	0.120	21.82	
	400	10.01	421.64	689.5	3.80	0.182	48.51	
	800	11.86	230.92	1209.0	2.33	0.297	68.43	
	1600	ND	ND	ND	ND	ND	100.00	
Bacillus subtilis	0	13.75	227.28	478.5	10.91	0.064	0.00	310.37
	30	13.56	242.19	967.5	7.33	0.095	32.81	
	120	7.84	253.30	525.5	7.47	0.093	31.53	
	240	13.59	157.85	792.5	14.01	0.049	29.79	
	480	ND	ND	ND	ND	ND	100.00	
Mixed microorganisms	0	13.89	747.92	372.0	11.09	0.063	0.00	225.54
	200	24.92	908.81	528.5	5.69	0.122	48.69	
	400	15.91	430.85	742.5	4.54	0.153	59.06	
	800	16.54	305.9	1398.0	2.67	0.259	75.92	
	1600	ND	ND	ND	ND	ND	100.00	

Values in brackets are standard errors of the estimate ($p < 0.05$).

the inhibitory effects increase with the concentrations of Cd^{2+}. The time of the lag phase suggests that the retarding time of *C. humicola* and *B. subtilis* is longer with the increasing concentrations of Cd^{2+}. During the stationary phase of *C. humicola* and *B. subtilis* in the presence of Cd^{2+}, the heat generated exceeds that of *C. humicola* and *B. subtilis* without Cd^{2+}. From analysis of the power–time curves for *C. humicola* and *B. subtilis* in Figs. 1 and 2, it can be known that the heat generated increases exponentially during the log phases. The parameters are listed in Table 1, which indicate that Cd^{2+} has stimulatory effects in certain concentration ranges. The growth power–time curves of *C. humicola* and *B. subtilis* show that the log phases of growth obey the equation:

$$\ln P_t = \ln P_0 + kt \quad (1)$$

where t is the time, P the power output at time t, P_0 the power at time $t = 0$ and k is the growth rate constant. Using the equation, the growth rate constant (k) of the log phase of the first peak was calculated by the data obtained from the power–time curves. The generation times (t_G), which is $(\ln 2)/k$, were also obtained. The growth rate constants (k) and generation times (t_G) of *C. humicola* and *B. subtilis* growth are shown in Table 1. Where the relationship between inhibitory ratio I and C of the *C. humicola*, *B. subtilis* and microorganisms are shown in Fig. 4, and the relationship between the microbial growth rate constant k and C of the *B. subtilis* and *C. humicola* are shown in Fig. 5.

3.3. Dosage and data in the experiment

The relationship between total thermal effect Q_T and C of the *B. subtilis*, *C. humicola* and *mixed microorganisms* are shown in Figs. 6–8. Comparing the total thermal effect (Q_{total}) of *C. humicola* to the *B. subtilis*, the quantity of heat generated from the interaction of *C. humicola* and Cd^{2+} is significantly lower than the *B. subtilis*', but the maximum thermal power (P_{max}) of *C. humicola* is higher than *B. subtilis*, and the time of maximum thermal power

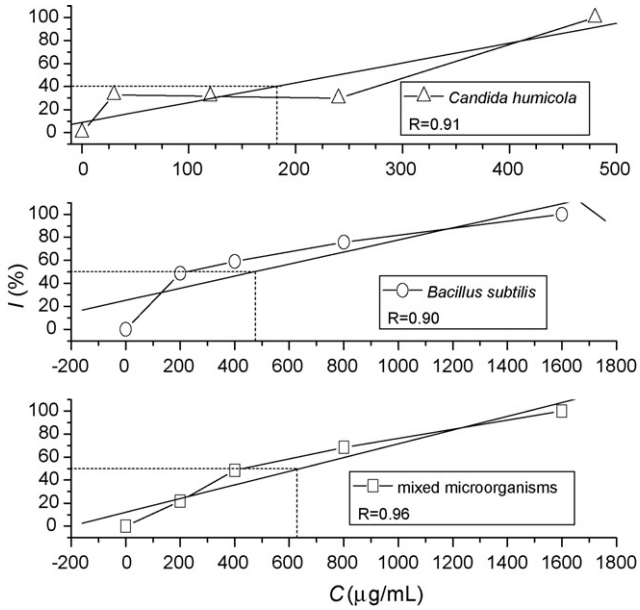

Fig. 4. The relationship between inhibitory ratio I and C of the *Candida humicola*, *B. subtilis* and microorganisms.

(T_{max}) of *B. subtilis* is later than the *C. humicola*, this phenomena shows that the life periods of *B. subtilis* is longer than *C. humicola*. This is because *B. subtilis* produces a large amount of antifungal substances, which strongly inhibits various plant pathogenic fungi, such as *Rhizoctonia solani*, *Fusarium graminearum*, etc. [21]. The data are listed in Table 1; the comparison of the statements is based on the parameters from the two experiments with single organisms. According to the data and curves, the Cd^{2+} has a positive effect on the *C. humicola* when the concentration is less than the 200 μg/mL, and when the dosage is 60 μg/mL, this positive effect also reflects on *B. subtilis*. Then the dosage was increasing contin-

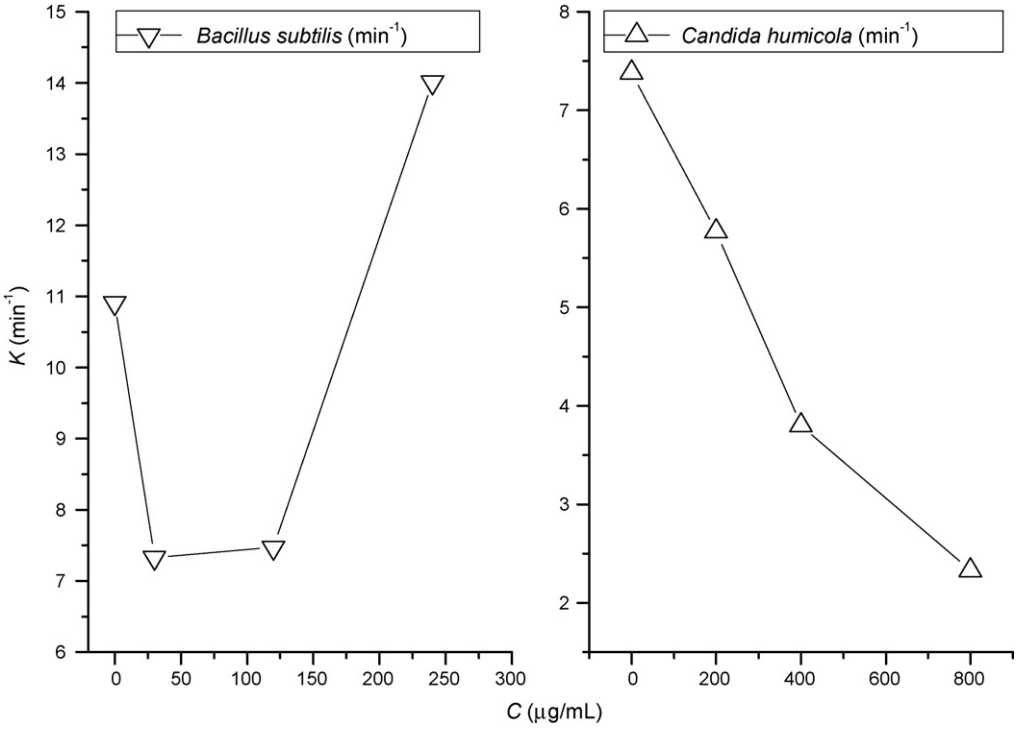

Fig. 5. The relationship between the microbial growth rate constant k and C of the *B. subtilis* and *Candida humicola*.

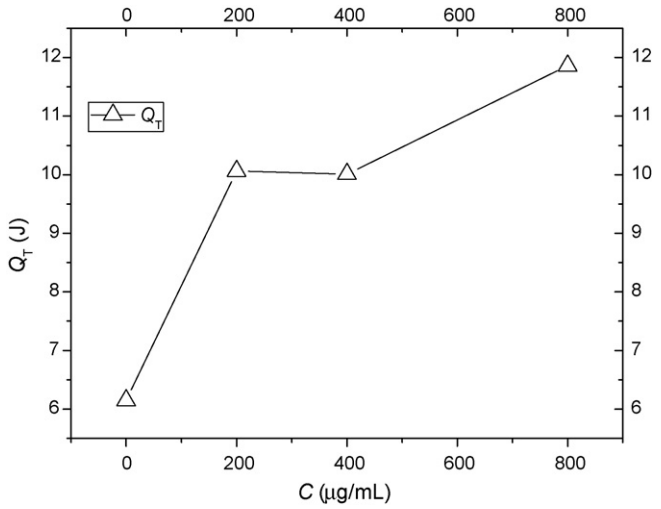

Fig. 6. The relationship between total thermal effect Q_T and C of the *Candida humicola*.

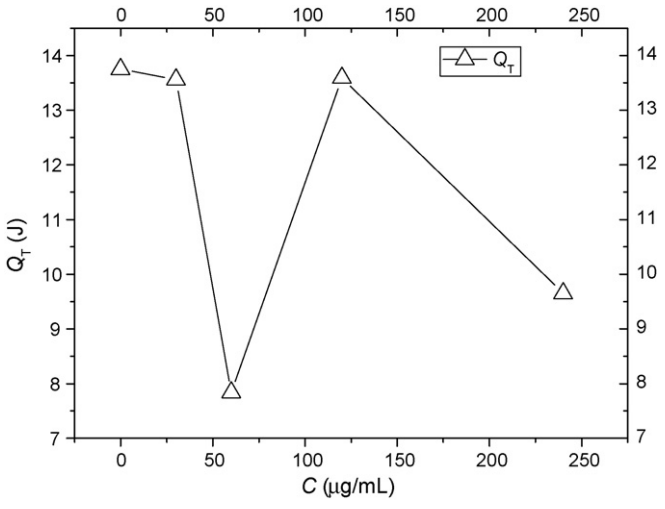

Fig. 7. The relationship between total thermal effect Q_T and C of the *B. subtilis*.

uously, the result of experiment showed that Cd^{2+} inhibited both microorganisms.

3.4. The idiosyncrasy of mixed microorganisms

In the experiment with the two microorganisms together, we choose the same concentration as for *C. humicola*.

Some similarities and differences can be observed from a qualitative point of view. Every parameter in Table 1 of the experiment with the mixed organisms is higher than the parameters from the experiments with a single organisms, this shows that the mixed microorganisms has a higher endurance than single microorganisms, because the microbial biodiversity influences the generation time (t_G) of mixed microorganisms. Comparing the experiments with a single organism to that of mixed organisms, we see that the inhibitory ratio (I) and the effect of restrain is different, because the concentrations of Cd^{2+} in *B. subtilis* and the *C. humicola* are different. I can be defined as:

$$I = \left[\frac{(k_0 - k_c)}{k_0}\right] \times 100\%. \quad (2)$$

where k_0 is the rate constant of the control and k_c is the rate constant for *B. subtilis* and *C. humicola* inhibited by an inhibitor with a concentration of c. When the inhibitory ratio I is equal to 50%, the corresponding concentration of the inhibitor is called the half-inhibitory concentration (IC_{50}). IC_{50} can be regarded as the inhibiting concentration of causing a 50% decrease of the *C. humicola* and *B. subtilis* growth rate constant. The values of I and IC_{50} are also depicted in the Table 1.

The value of P_{max} comes from the experiment showed that the mixed microorganisms are lower than *C. humicola*'s, but the P_{max} of mixed microorganisms are higher than *B. subtilis*'. It is because the activity of *B. subtilis* has a slightly negative effect on *C. humicola*. When the concentration of Cd^{2+} reached 1600 μg/mL, the growth of *C. humicola* is totally inhibited. When the concentration reaches 480 μg/mL, the growth of *B. subtilis* is totally inhibited. The endurance of *B. subtilis* to the heavy metal Cd^{2+} is obviously lower than *C. humicola*. If we put both microorganisms under the same situation in the laboratory, for instance, at the same concentration, the *B. subtilis*'s degree of endurance is lower than *C. humicola*'s. When the concentration is higher than 480 μg/mL, it has reached the dead dose of *B. subtilis*, under such conditions, the trend of the curve is only based on the *C. humicola* growth [22].

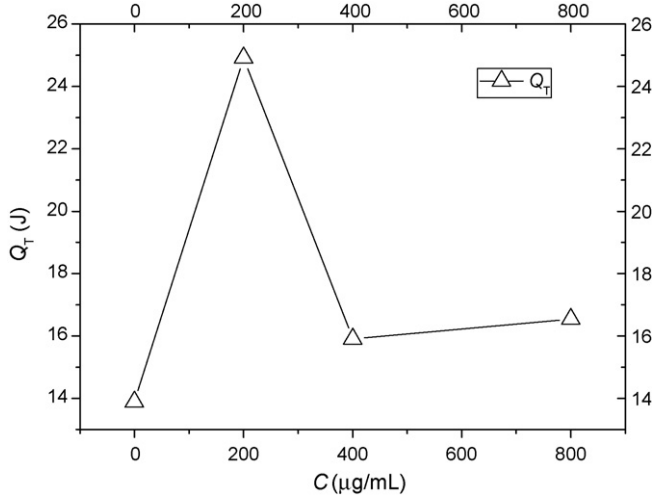

Fig. 8. The relationship between total thermal effect Q_T and C of the *mixed microorganisms*.

3.5. Difference of endurance among the microorganisms

Through the experiments, we have evaluated the endurance concerning Cd^{2+} of *B. subtilis* and *C. humicola*. The endurance of *C. humicola* is different from *B. subtilis*. The low dosage (0–60 μg/mL for *B. subtilis*, 0–200 μg/mL for *C. humicola*) of heavy metal Cd^{2+} will stimulate the growth of the two microorganisms in the short term, and the high concentration (60–240 μg/mL for *B. subtilis*, 200–800 μg/mL for *C. humicola*) will inhibit the growth of the two microorganisms. From the calculation of Table 1, The IC_{50} of *B. subtilis* is lower than *C. humicola*'s, higher than mixed microorganisms', which is support the result that the sensitivity of *B. subtilis* to Cd is higher than *C. humicola*'s. Whether or not the two microorganisms have a chemical reaction when put together, that is another academic question for discussion, and will be explored in further research.

4. Conclusion

C. humicola and *B. subtilis* are important microorganisms in the soil. The method of microcalorimetry is the advanced way to analyze the toxic effect of different amounts of Cd^{2+} on the growth of

microorganisms in soil, and in this paper especially in the investigation of *C. humicola* and *B. subtilis*. These two microorganisms have different reflections with Cd^{2+} in different dosages. *C. humicola* has the higher endurance of the heavy metal Cd than *B. subtilis*.

The curves of Cd^{2+} are expressed as T_{max} in this study. When *C. humicola* and *B. subtilis* reacted with Cd^{2+} from Figs. 1 and 2, it is observed that *C. humicola* has a larger range of distribution than *B. subtilis*. Compared to one another, the Q_{total} of the *C. humicola* is higher than *B. subtilis*' [23–25]. The toxic study value will provide the extremum of heavy metal used in isolating microorganisms from soil in liquid laboratory medium environment. So the values will useful in the toxic effect of heavy metal study field.

Nevertheless, new methods and approaches are needed for the development of toxicity test systems and studies. We anticipate that this will be one of the most important and powerful applications developed in the field of toxicology. In conclusion, microcalorimetric investigations on microorganisms are possible and promising. We believe that microcalorimetry is a useful and accurate system for studying the detailed mechanism of microorganisms and can provide important information for microbiology research [26].

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