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A MICROCALORIMETRIC ASSAY OF *TETRAHYMENA THERMOPHILA* FOR ASSESSING TRIBUTYLTIN ACUTE TOXICITY

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The power–time curves of *Tetrahymena thermophila* exposed to tributyltin (TBT) were detected by microcalorimetry. Metabolic rate (*r*) decreased significantly while peak time (*PT*) increased with the enhancement of TBT level. Compared with the measured multibiomarker including catalase, lactate dehydrogenase, glutathione *S*-transferase, ATPase and membrane fluidity, *PT* and *r* could be sensitive biomarkers for assessing TBT toxicity at cellular level. The effective concentrations obtained by them were consistent to those obtained by the protozoan community toxicity test. As a result, the microcalorimetric assay of *T. thermophila* had a great potential in assessing TBT acute toxicity and monitoring TBT pollution in the freshwater ecosystem.

Keywords: *metabolic parameters, microcalorimetry, multibiomarker, protozoan community toxicity test, Tetrahymena thermophila, tributyltin*

Introduction

Organotin compounds have been used commercially for many years in a variety of applications such as polyvinyl chloride (PVC) stabilizers, industrial catalysts, wood preservatives, and biocides [1]. Among these compounds is tributyltin (TBT) that has been used since 1960s not only as an effective antifouling agent added in marine paint formulations but also as lumber preservatives and slimicides in cooling system since 1960s. The aquatic pollution by use of organotin compounds mainly arises from the direct exposure of TBT and its dramatic threat to non-target organisms in the ecosystem. It is widely acknowledged that triorganotins and specifically TBTs are 'probably the most toxic substances ever introduced deliberately into the marine environment' [2]. Due to its persistency and biological effects on various organisms, many developed countries have banned and/or restricted TBT usage for boating and aquaculture purposes from early 1980s [2, 3]. Even with the ban, TBT still exists in aquatic environments because it is difficult to be decomposed and can be highly accumulated by organisms [4]. Organotin compounds, particularly TBT, have also been found in the rivers and lakes [5]. The adverse effects of TBT on marine organisms have been widely recognized, but there is a lack of toxicity data for TBT and freshwater organisms used for in ecological risk assessments [6].

As the most common protozoan model in the toxicological studies, *Tetrahymena* presents many good features [7, 8]: (1) their ubiquitous distribution and ecological significance place them at the front rank of ideal early-warning indicators of freshwater ecosystem deterioration; (2) they perform key functions in energy flow and elementary cycling; (3) they are eukaryotic unicellular organisms which make them sensitive to the pollutants; (4) they grow rapidly and robustly in axenic condition. Therefore, *Tetrahymena* is well suited for toxicant screening studies and detecting water quality [9]. However, the toxic effects of TBT on *Tetrahymena* have scarcely been reported.

An effective biological toxicity assay should measure the physiological parameters related to overall metabolism. Because the thermokinetic parameters obtained by microcalorimetry are physiological parameters that result from a variety of cellular metabolic pathways, microcalorimetry is useful in quantitatively measuring toxic effects of various substances on the metabolism of the whole living systems. Microcalorimetry has demonstrated its power as a universal, integral, non-destructive, good reproducibility and highly sensitive tool for solving many environmental problems [10–12]. It also has been used for the rapid evaluation of the toxic effects of nonylphenol, chlorobenzene *p*-phenylene and ytterbium ion *vs. Tetrahymena* [13, 14]. In this paper, the power–time curves of *Tetrahymena thermophila* BF_5 exposed to different TBT levels were studied by ampoule method of microcalorimetry at 28°C in order to assay TBT toxicity. In addition, in order to determine

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the sensitivity of this assay for TBT impacts on freshwater ecosystem, multibiomarker at cellular level including several enzyme activity and membrane fluidity of *T. thermophila* BF_5 was evaluated under the stress of TBT, and its toxicity was also investigated on the PFU microbial community.

Experimental

Species, culture medium and chemicals

Tetrahymena thermophila BF_5 was provided by East China Normal University. The cells were cultured at 28°C in a liquid medium containing 2% (*w*/*v*) proteose peptone (Oxoid), 0.1% yeast extract (Oxoid) and 0.5 mM $FeCl_3$.

Tributyltin chloride (Acros Organic) with a purity of greater than 97% was dissolved in DMSO (Sigma).

Microcalorimetric measurements

The microcalorimeter is an eight-channel TAM Air isothermal heat conduction calorimeter 3114/3236 (Thermometric AB, Sweden). The microcalorimetric channels are in a single removable block contained in an air thermostat that keeps the temperature within ±0.02°C. Each channel consists of a sample and a reference vessel. The limit of detection is 2 μW and the baseline deviation over 24 h is ±5 μW. All the microcalorimetric measurements were performed in 20 cm^3 glass ampoules at 28°C.

Having measured the stationary-stage *Tetrahymena* cells, appropriate amount of cell suspension, TBT solution and sterilized culture medium were added into the sterilized ampoules. Thus, the samples contained different concentrations of TBT with the same volume of the solvent DMSO (5.56 μL). Note that the final volumes of the samples were 5 mL, and the cell densities were about 1000 per mL. All of the reference ampoules were added into 5 mL free-bacteria culture medium. Then, the sample and reference ampoules were hermetically sealed and put into the different channels. Finally, the power-time curves of *T. thermophila* at 28°C were recorded every minute by use of the Picolog software supplied with TAM Air.

Cellular enzyme activity measurement

The stationary-stage *Tetrahymena* cells (about $1 \cdot 10^6$ mL^{-1}) were under the stress of different concentrations of TBT for 24 h. Afterwards, cells were harvested by centrifugation and washed twice with 10 mM Tris-HCl (pH 7.4) at room temperature.

The following experimental procedures were carried out at 0–4°C. The suspending cells were homogenized using Branson digital sonifier cell disruptor. The homogenate was centrifuged at 10400 rpm for 20 min, and the supernatant was used as the cell extract. The activities of cellular enzymes including catalase (CAT), lactate dehydrogenase (LDH), glutathione *S*-transferase (GST) and ATPase were respectively measured by the assay kits produced by Nanjing Jiancheng Biotechnique Institute. Protein content was routinely determined by Bradford method using bovine serum albumin as standard. Spectrophotometric measurements were carried out on Sunrise Remote/Touch Screen, Tecan. Austria GmbH, Grödig, Austria.

Cell membrane fluidity determination

The *Tetrahymena* cells under the stress of different concentrations of TBT for 24 h were prepared for cell membrane fluidity determination. Fluorescence probe was 1,6-diphenyl-1,3,5-hexatriene (Sigma, USA). Fluorescence polarization method was used to measure values of fluorescence polarization by PerkinElmer LS 55 luminescence spectrometer [15].

TBT toxicity assessment on the PFU microbial community

The national standard biomonitoring method (Polyurethane Foam Units method) was applied to colonize the microbial communities in fresh water [16]. Ecotoxicological impact of TBT was evaluated by using PFU microbial community toxicity tests [17].

Statistical analyses

The data were given as the arithmetic mean ±standard derivation. The one-way ANOVA statistical method was used to assess the significance of differences in measured parameters among the samples at $P \leq 0.05$. Correlations among these parameters were also analyzed at $P \leq 0.05$.

Results

Power-time curves and metabolic properties of T. thermophila exposed to TBT

The power–time curves of *T. thermophila* BF_5 exposed to different TBT levels at 28°C were shown in Fig. 1. The differences of these curves showed that TBT had influenced the metabolism of the cells. As for the growth metabolism of *Tetrahymena*, the ki-

netic process in the increasing period of the curves follows the classical logistic model:

$$\ln\left(\frac{P_{max}}{P_t}-1\right)=\alpha-rt$$

where P_t is the power output at time t, r is metabolic rate, and P_{max} is the potential maximum power output, which means the power output when cell number gets to environmental carrying capacity (maximum number in specific environment). α is a constant which stands for the orientation of logistic curves relative to origin.

From Table 1, it was apparent that all of the correlation coefficients, R, were greater than 0.99, indicating a good correlation relationship. The values of metabolic properties were revealed in Table 2. P_{max} and Q_T showed no significant change. Likewise, there were no significant difference in P_m except for the cases with the effects of 20 and 25 μg L^{-1} TBT. However, metabolic rate r, on the other side, declined significantly under the influence of TBT. Meanwhile, *PT* showed significant increase with exception of those cases that were under the effects of 2.5 and 5 μg L^{-1} TBT. It was also found that r and *PT* had both strong correlations with the concentration of TBT. It should be noted that the effective concentrations of TBT on the metabolism of *T. thermophila* BF_5 could be calculated by the correlation equations. Shown in Table 3 were their results. The values of EC_5 obtained from r were lower than those obtained by *PT*, but EC_{50} appeared higher, showing that r was more sensitive to the lower concentration TBT.

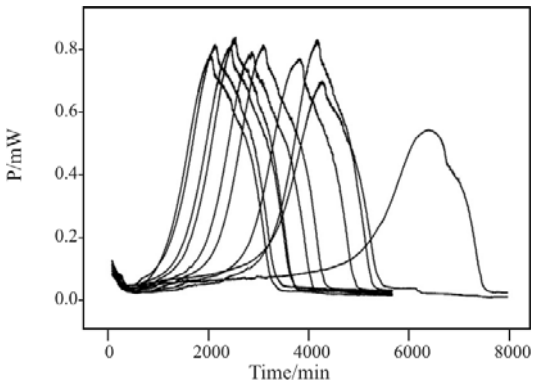

Fig. 1 Power–time curves of *Tetrahymena thermophila* BF_5 under the effects of TBT (0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20 and 25 μg L^{-1} from left to right)

Table 1 Logistic equations and coefficients of *Tetrahymena thermophila* BF_5 at 28°C

Sample	Logistic equation	Coefficient
Control 1	$\ln(1.01/P_t-1)=4.10-0.00339t$	0.999
Control 2	$\ln(1.41/P_t-1)=4.70-0.00299t$	0.998
Control 3	$\ln(0.96/P_t-1)=5.60-0.00353t$	0.999

Multibiomarker in Tetrahymena for assessment of TBT toxicity

Multibiomarker in *Tetrahymena* were determined for assessment of TBT toxicity, including LDH, GST,

Table 3 Correlation equations and effect concentrations

EC_5/μg L^{-1}	EC_{50}/μg L^{-1}	Equation	R
3.52	8.16	$PT=172.1C+1277.9$	0.95
1.81	18.32	$r=-9\cdot10^{-5}C+0.0033$	0.98
3.56	32.61	$PS=-0.1538C+0.016$	0.98
0.47	22.30	$CS=-0.3298C+5.354$	0.97
1.23	25.69	$TS=-0.6255C+33.07$	0.99

r is metabolic rate, *PT* is peak time, the time when the power output is P_m. PS is phytomastigophora species number, CS is ciliate species number, TS is total protozoa species number, C is the concentration of TBT, R is correlation coefficient

Table 2 Metabolic parameters of *T. thermophila* BF_5 under the effects of TBT

TBT/μg L^{-1}	$r/10^{-3}$ min	P_{max}/mW	Q_T/J	Q_{Log}/J	P_m/mW	*PT*/min
0.0	3.30±0.28	1.13±0.25	70.33±3.29	28.56±0.77	0.80±0.03	1794±209
2.5	2.85±0.01*	1.35±0.23	74.53±0.26	30.24±3.17	0.83±0.02	1978±213
5.0	2.90±0.18*	1.18±0.20	68.99±4.99	30.72±3.18	0.79±0.05	2089±357
7.5	2.75±0.16*	1.45±0.38	72.83±0.01	32.64±3.55	0.85±0.02	2445±146*
10.0	2.36±0.20*	1.83±0.63	73.04±1.45	32.85±2.29	0.82±0.05	2467±371*
12.5	2.36±0.15*	1.72±0.53	73.32±0.85	33.28±2.58	0.84±0.04	3052±66*
15.0	2.07±0.03*	1.56±0.21	72.55±1.14	37.64±3.06*	0.80±0.05	3851±37*
17.5	1.47±0.02*	1.87±0.35	70.74±2.38	38.47±0.69*	0.81±0.02	4200±22*
20.0	1.34±0.07*	1.64±0.01	68.35±6.80	38.96±3.28*	0.68±0.01*	4242±59*
25.0	1.16±0.03*	0.88±0.03	70.71±3.71	47.69±0.51*	0.53±0.01*	6451±69*

P_{max} is potential maximum power output, r is metabolic rate, Q_T is total heat, Q_{Log} is total heat in the increasing period, and P_m is measured maximum power output. *PT* is peak time, the time when the power output is P_m. The values under the stresses of 0, 5, 10 μg L^{-1} TBT are given as mean ±S.D. (n=3), and the other values are given as mean ±S.D. (n=2). The values marked with '*'are significant at $p<0.05$ compared with control group

Na^+–K^+–ATPase, CAT and membrane fluidity. The results were shown in Table 4. The TBT-mediated inhibition of LDH activity was demonstrated in *Tetrahymena*. Likewise, ATPase activity in *Tetrahymena* decreased after exposure to TBT. However, as for GST (a marker for phase-II metabolism) and CAT (an oxidative stress marker) activities, no significant changes were both observed in the cells exposed to TBT. Using DPH as a fluorescence probe, it was found that TBT accounted for an increase in the fluorescence polarization of DPH, reflecting a decrease in membrane fluidity of *Tetrahymena* exposed to 15 and 25 μg L^{-1} TBT.

Effects of TBT on the protozoa community

The toxicological impacts of TBT on ecosystem were evaluated by using protozoa community toxicity test. Species composition and number of protozoa were shown in Table 5. It was found that phytomastigophora, ciliate and total species number decreased significantly with the increase of TBT concentrations. It was also found that they had significant correlations with TBT concentrations. So the effective concentrations of TBT on the protozoa community could be derived and their results were shown in Table 3. The effective concentrations obtained by ciliate were the lowest and those by phytomastigophora were the highest.

Discussion

Microcalorimetry has proven a useful tool for measuring the energy. Its advantage is the capability to measure the total energy flow. Any changes in environment, such as pollutants of different biological toxicity that are distributed to the environment, can influence the living activities of organisms, which in turn lead to the changes of the heat produced by metabolism. These changes can be easily detected by microcalorimetry [18]. In our studies, the power–time curves showed that the metabolic process of *Tetrahymena* cells was influenced by TBT. Among the metabolic parameters, *r* and *PT* had significant changes. Analyses of their values revealed that TBT delayed the growth metabolism of cells. *PT* and *r* were both sensitive to TBT. EC_{50} of TBT obtained by them were much lower than those of TBT on five aquatic invertebrates including protozoa (*Paramecium caudatum*), which ranged from 25.65 to 355.63 μg L^{-1} by counting the living cell number [19]. Generally, the growth of protozoa population is studied by cell counts. It is worth pointing out here that cell counting is inconvenient for operators due to requirement to count the cells at each sampling time. It is usually accompanied with relatively high experimental errors as a result of uneven sampling and counting some dead cells. In addition to these, growth curves by cell counting only accounts for the cell number changes caused by toxic substances, not ev-

Table 4 Enzyme activities and fluorescence depolarization values of cell membrane of *T. thermophila* BF_5 exposed to TBT

TBT/μg L^{-1}	LDH	GST	Na^+–K^+–ATPase	CAT	P
0	4.81±1.21	2.94±0.55	1.22±0.88	59.36±18.07	0.31±0.02
5	2.86±1.21*	4.49±4.66	0.44±0.15*	42.42±0.44	0.32±0.02
15	1.44±0.82*	5.16±0.98	0.70±0.28*	50.22±6.51	0.36±0.02*
25	3.40±0.61	5.19±2.62	0.50±0.59*	89.24±51.99	0.37±0.01*

P is fluorescence polarization values. Values are given as mean ±S.D. (n=3). The values marked with '*' are significant at p<0.05 compared with control group

Table 5 Species composition and number of protozoa under the effects of TBT

TBT/μg L^{-1}	Species number				Total species number
	phytomastigophora	zoomastigophora	sarcodina	ciliate	
0	9.5±0.7	4.0±1.4	4.5±0.7	16.0±2.8	34.0±2.8
0.5	10.0±0.0	3.0±0.0	5.5±0.7	15.0±2.8	33.5±2.1
1	10.0±0.0	3.0±0.0	3.5±0.7	16.0±1.4	32.5±2.1
10	9.0±1.4	3.5±0.7	4.0±4.2	9.5±3.5*	26.0±1.4*
20	6.5±0.7*	2.5±0.7	0.0±0.0*	10.0±1.4*	19.0±2.8*
30	6.0±2.8*	2.0±0.0	0.0±0.0*	5.0±0.0*	13.0±2.8*
40	3.5±2.1*	3.5±2.1	0.5±0.7	2.5±2.1*	10.0±7.1*

Values are given as mean ±S.D. (n=2). The values marked with '*' are significant at p<0.05 compared with control group

ery cell physiological level. On the other hand, microcalorimetry can monitor metabolism of the living cells automatically, in which each power obtained by microcalorimetry depends on the total cell number and metabolic level of every cell. Therefore, the power–time curve is relatively accurate and easy to use. Most importantly, it can capture the complete information about the effects of the toxic substances on the cells.

The use of biomarkers measured at the molecular or cellular level have been proposed as sensitive 'early warning' tools for biological effect measurement in the environmental-quality assessment [20]. Then, could metabolic characteristics be sensitive biomarkers for TBT toxicity? In the present paper, multibiomarker were detected that used usually for toxic studies. As one enzyme of the intermediary metabolism, a significant reduction in LDH was found, which was consistent with the results in catfish and carp spermatozoa when they are exposed to TBT [21]. Reduced activity of LDH could be due to a direct interaction of TBT with the enzyme protein, as added albumin was able to protect LDH activity from TBT [22]. It was also surprisingly found that LDH activity recovered to the control level when exposed to 25 μg L^{-1} TBT. The cytotoxic effects of TBT on Leydig cells also showed no significant change in LDH activity at 3 μM TBT [23]. No significant change was also found in CAT and GST. This was probably because they were not sensitive to the environmental TBT contamination, as supported in the blue mussel *Mytilus edulis*, or TBT exposure time was too short to register the two biomarkers responses [24]. As for the ATPase activity in *Tetrahymena*, its decrease after exposure to TBT was consistent with the responses in the gill of *Meretrix meretrix* [25]. The ATPase system has long been identified as a target for toxic compounds and the inhibition of the enzyme caused by heavy metals or toxic organic compounds could alter the cellular membrane configuration by binding to the phospholipids portion of the membrane, altering its active site [26]. TBT can enter eukaryotic cell membranes by diffusion as a consequence of its lipophilic nature. Therefore, the inhibition of the Na^+–K^+–ATPase indicated that TBT had bind to phospholipids and led to the change of cellular membrane configuration. As a result, TBT should be able to alter the membrane fluidity. The reduction of cell membrane fluidity demonstrated it, which had already been confirmed in *Candida maltosa* [27]. All these findings indicated that presence of TBT in the aquatic environments not only could inhibit some enzyme activity, but also acted as a membrane active molecule with its effect on cell membranes as fundamental aspect of its toxicity. Although these biomarkers were responsive to TBT, they had no significant correlation relationships with TBT levels, and the changes of biomarkers were also not consistent with those of *r* and *PT*. This could be explained by that these biomarkers could only provide the responses of one enzyme system or cell structure. Microcalorimetry could monitor the whole metabolic processes presented the reactions of the whole enzyme system and the whole cell organisms. Therefore, *r* and *PT* were more sensitive to pollutants and could be useful biomarkers that reveal the TBT pollution level on the overall cell. In addition, the microcalorimetry of *T. thermophila* was very simple and reproducible so that this assay would be useful for assessing TBT acute toxicity.

Microcalorimetry of *Tetrahymena* was a sensitive assay to evaluate TBT toxicity at the population level, and metabolic parameters (*r* and *PT*) were also useful biomarkers for TBT toxicity assessment at the cellular level. Then, could the results of this assay represent the biological effects of TBT on the freshwater ecosystem? In order to answer this question, the TBT toxicity test was determined at the community level. The results of the protozoa community toxicity test demonstrated that TBT had greatly destroyed the structure of the microbial community. Among protozoan organisms, ciliate were the most sensitive to TBT because the value of EC_5 obtained by ciliate species was the lowest, which was similar to the criterion (0.46 μg L^{-1}) to protect freshwater aquatic life from acute toxic effects [28]. These results indicated that the changes of species diversity in the protozoan community could be used to accurately monitor the stress effects of pollutants on the ecosystem. The results acquired by the community toxicity test had the environmental dependability. However, protozoa species identification is very difficult in practice. Therefore, it is much desirable to develop a simple test for TBT toxicity assessment in freshwater ecosystem. In our study, microcalorimetry of *Tetrahymena* was used to evaluate TBT toxicity at the population level. The similar EC_5 values were obtained by PT and phytomastigophora species number. However, EC_{50} value obtained by *PT* was the lowest, which showed that *PT* was the most sensitive to the higher TBT level. The value of EC_5 obtained from *r* was a little higher than that obtained by ciliate and total protozoa species number, but EC_{50} was lower than that the results of the community toxicity test. As a whole, *r* presented approximately the same sensitivity to TBT as ciliate and protozoan species did, and more importantly, in comparison with protozoan species identification, microcalorimetry was simple and automatic. Therefore, the microcalorimetric assay of *T. thermophila* was convenient for us to early monitor

the TBT toxicity on the freshwater ecosystem, even for the operators without the protozoan taxonomic knowledge.

Conclusions

TBT toxicity was assessed by using microcalorimetry of *Tetrahymena thermophila*. The power–time curves of *T. thermophila* showed that heat production of cells had been inhibited by TBT. Metabolic rate (*r*) and peak time (*PT*) quantified the changes of the whole living cells influenced by TBT. They showed significant changes and correlation relationships with TBT levels.

The measured multibiomarker also offered biologically relevant information on the potential impact of TBT on *Tetrahymena* cells. Although the present results were essentially derived from the acute effects at the restricted TBT levels, its potential to inhibit some proteins should not be overlooked. However, compared with them, *PT* and *r* were more sensitive and useful biomarkers revealing TBT pollution level.

From the effective concentrations of TBT, *PT* and *r* showed the similar sensitivity to TBT with the protozoan community. However, microcalorimetric assay of *T. thermophila* was simple and automatic. Therefore, this assay was sensitive and convenient in assessing TBT acute toxicity and early monitoring TBT pollution in the freshwater ecosystem.

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