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Comparison of organics and heavy metals acute toxicities to *Vibrio fischeri*

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Abstract: *Vibrio fischeri* bioluminescence inhibition has been widely used to test acute toxicities of metals and organics contaminants. However, the differences of metals and organics acute toxicities to *V. fischeri* have not been compared. Here, four heavy metals (Zn^{2+}, Cu^{2+}, Cd^{2+} and Cr^{6+}) and five organics (phenol, benzoic acid, *p*-hydroxybenzoic acid, nitro-benzene and benzene) acute toxicities to *V. fischeri* were investigated. Heavy metals toxicities to *V. fischeri* were increased along with the reaction time, while the organics toxicities kept the same level in different reaction times. In order to explain the difference, the relative cell death rate of *V. fischeri* was detected. In metals toxicities tests, the bioluminescence inhibition rate of *V. fischeri* was found to be significantly higher than the relative cell death rate ($P < 0.05$), while for the organics toxicities tests, the cell death rate was similar to the bioluminescence inhibition rate. These results indicated that organics acute toxicities to *V. fischeri* could reflect the death of cell, but metals acute toxicities to *V. fischeri* may not lead to the death of cell, just represent the bioluminescence inhibition.

Keywords: reaction time; luminescent bacteria; cell death; bioluminescence inhibition; cell death rate; Microtox® test.

INTRODUCTION

The luminescent bacterium, *Vibrio fischeri* has been widely applied for the toxicity detection of chemicals, including organics and metals.[1–3] Due to its advantages of ease use, low cost and high reproducibility, this biotest has been accepted as a quick method of chemicals toxicities assessment in the environment (named as Microtox® test), including wastewater effluent,[4] sediment extracts[5] and contaminated groundwater.[6]

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The Microtox® test is based on the luminescence response of the bacterium *V. fischeri.* The light emission of *V. fischeri* is an enzyme catalysis reaction, and the process could be concluded as Eq. (1) and (2).[7] Given the principle of this bioprocess, toxics that do harmful to any of the factors (O_2, aldehyde, flavin reductase, luciferase and NAD(P)H) attending in this process could induce luminescence inhibition, and the toxicities to *V. fischeri* could be assessed by the luminescence inhibition rate. In the other hand, cells death caused by toxics will also cause the luminescence inhibition. Thus, sporadically studies have issued that chemicals acute toxicities detected by the Microtox® test could either reflect disturbances of biosynthetic pathways[8] or death of *V. fischeri.*[9]

$$\mathrm{NAD(P)H + H + FMN \xrightarrow{\text{Flavin reductase}} NAD(P) + FMNH_2} \quad (1)$$

$$\mathrm{FMNH_2 + O_2 + R - CHO \xrightarrow{\text{Luciferase}} \rightarrow FMN + H_2O + R - COOH + hv(490nm)} \quad (2)$$

Nevertheless, there have been relatively few researches comparing the difference of heavy metals and organics acute toxicities to *V. fischeri*. When assessing the toxicities of metals and organics by Microtox® test, different reaction times were used by experienced-based,[10] but the reasons remain unclear. Based on the Microtox® test, a small quantity of studies had demonstrated that heavy metals toxicities to *V. fischeri* were fickle at different reaction times,[11,12] while the organics toxicities kept relatively stable.[13] These results in some extent explain the reasonable discrepancy reaction time for detecting heavy metals and organics acute toxicities to *V. fischeri*. However, systematic comparison of heavy metals and organics acute toxicities to *V. fischeri* has not been carried out, and the reasons of using different reaction times still need to be further elucidated.

In this study, heavy metals and organics acute toxicities to *V. fischeri* at various reaction times were determined by the Microtox® test. The relative cell death rate was detected following the Microtox® test to explain the possible reasons of the difference between metals and organics.

MATERIALS AND METHODS

Toxicity assay

The bacterium, *V. fischeri*, used in this study was purchased from the Institute of Soil Science, Chinese Academy of Sciences, Nanjing, China. The toxicity assay was performed according to national standard method of China (Water quality – Determination of the acute toxicity – Luminescent bacteria test. GB/T 15441-1995). Briefly, 5 mg freeze-dry powder of *V. fischeri* was revived in 1 ml chilled 3 % NaCl, followed by adding 4 ml 3 % NaCl. This solution was served as the working fluid for subsequent test. The toxicity assay was carried out by mixing 1.9 ml testing/control samples (solution only containing 3 % NaCl) and 0.1 ml working fluid of *V. fischeri* solution, and the light was recorded by the model DXY-2 luminometer after different incubation times.

According to the Microtox® test, four kinds of chemicals ($K_2Cr_2O_7$, $ZnSO_4·7H_2O$, $CuSO_4$ and $CdCl_2$) were selected as represent metals ions, Cr^{6+}, Zn^{2+}, Cu^{2+} and Cd^{2+}, respectively. Five organics were also tested: phenol, benzoic acid, p-hydroxy benzoic acid, nitro-benzene and benzene. These chemicals were purchased from Aladdin (http://www.aladdin-e.com/). These chemicals were selected as common contaminants in the water environment. The EC50 (chemicals concentrations that cause 50 % luminescence inhibition rate) was calculated according to previous research.[14] Dimethylsulfoxide (DMSO) was used as the co-solvent for organics according to the method reported in the reference[15]. Three different reaction times for metals (15, 30 and 45 min) and organics (5, 10 and 15 min) were tested in this study according to the preliminary experiments.

Relative cell death rate detection

In order to explain the different acute toxicities of metals and organics to *V. fischeri*, relative cell death rate detection was conducted after the toxicity assay. Following toxicity assay, the bacteria were spread on 12 cm plates containing LB medium with the modification of containing 3 % NaCl and cultivated at 20±1 °C for 48 h. The colony-forming unit (CFU) was used to enumerate the viable cell number. The cell death rate (*DR*) was calculated with Eq. (3):

$$DR = 100(1 - \frac{C_S}{C_C}) \quad (3)$$

C_S is the CFU of testing sample, and C_C is the CFU of the control sample without contacting with the toxics. All testing and control samples were carried out in quintuplicate.

Statistical analysis

All toxicity assays were performed in quintuplicate. The statistical significant of values were calculated by Student's *t*-test (mean analysis) at 5 % level of probability using the SPSS 19.0 package software (SPSS International, Chicago, IL, USA). Statistical significant difference was reported when the probability of the result assuming the null hypothesis (*P*) is less than 0.05.

RESULTS AND DISCUSSION

Heavy metals toxicities

The toxicities of the four kinds of metals (Zn^{2+}, Cu^{2+}, Cd^{2+} and Cr^{6+}) were detected, as they are common contamination in the environment.[16,17] The toxicities of four kinds of metals were showed in Fig. 1. Not surprising, the lumenescence inhibition rate was increased along with the concentration of each metal. Worth to note that, under a certain concentration of metals, luminescence inhibition rate was also increased along with the reaction times. EC_{50} was calculated by the linearity relationship of luminescence inhibition rate and the logarithm of concentration.[18] The EC_{50} at different reaction times were summarized in Table I. From 15 to 45 min, the EC_{50} of each metal was decreased significantly ($P < 0.05$).

As showed in Fig. 1, the luminescence inhibition rate increased gradually along with the increasing of reaction times. Metals toxicities to *V. fischeri* were attributed to the metals ions,[19] and earlier study demonstrated that the metals toxicities were mostly contributed by the ions that affected the luciferase in the

luminescence bioprocess.[20] Results in the present study further supported this idea. The toxicities of these heavy metals were closely in agreement with the previous reports.[21,22] It was reported that Cd^{2+} could be adsorbed and trapped to the exo-polysaccharides on the outer layer of the luminescent bacteria.[23] This may prevent the interaction between Cd^{2+} and key enzymes, which resulted in low toxicity of Cd^{2+} detected by only evaluating luminescence decrease in the present study. Meanwhile, the lower toxicity of Cr^{6+} in this study could result from Cr^{6+} being reduced to Cr^{3+}, which has less toxic to the bioenzymes.[24] Thus, different luminescence inhibition rate at different exposure times with these metals could partly demonstrates that the decrease of light emission in *V. fischeri* was due to the enzyme disturbance by heavy metals, and the toxicities were related with how they affect the enzymes rather than cause the cell death.

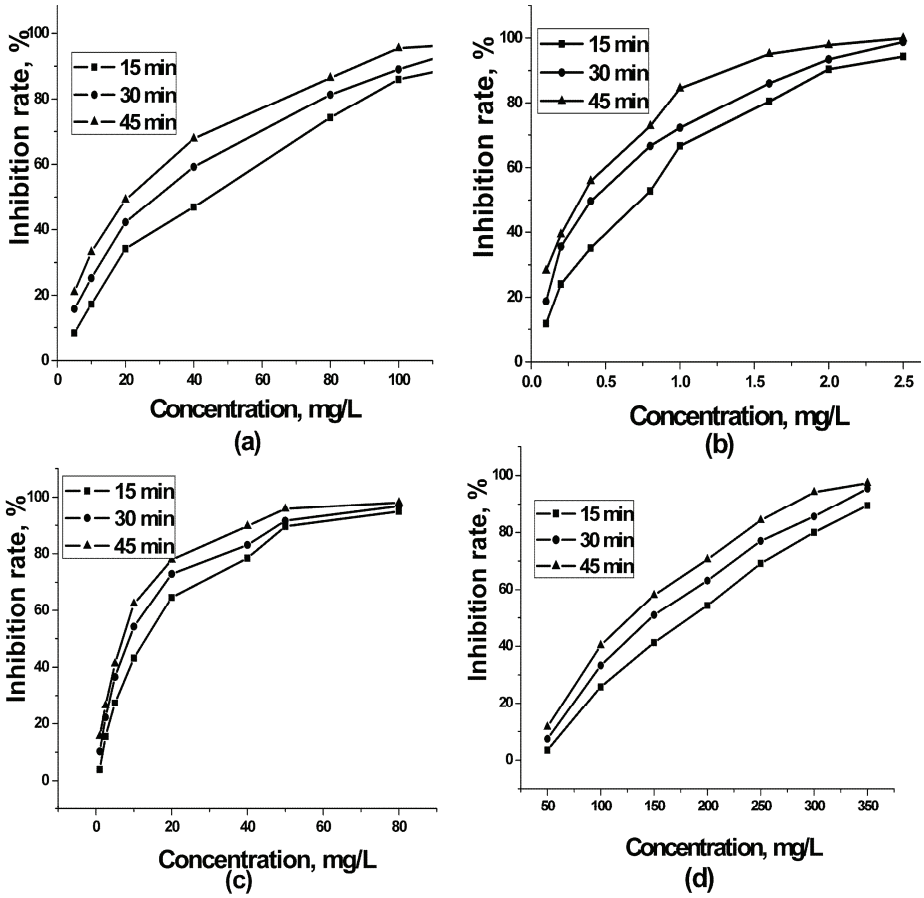

Fig. 1. The luminescence inhibition rate by four different kinds of metals under various concentrations: a), $ZnSO_4$, b) $CuSO_4$, c) $CdCl_2$ and d) $K_2Cr_2O_4$.

TABLE I. Toxicities of heavy metals at different contact times

Chemical	Contact time, min				
	15	30		45	
	EC_{50} / mg L^{-1}	EC_{50} / mg L^{-1}	P^a	EC_{50} / mg L^{-1}	P^a
(Zn) $ZnSO_4$	0.5±0.02	0.4±0.01	8.5×10^{-6}	0.3±0.01	4.1×10^{-8}
(Cu) $CuSO_4$	10.8±0.5	8.0±0.4	1.0×10^{-5}	6.2±0.3	1.1×10^{-7}
(Cd) $CdCl_2$	31.9±1.6	23.6±1.2	1.5×10^{-5}	18.4±0.9	1.9×10^{-7}
(Cr) $K_2Cr_2O_4$	160.3±8.0	138.3±6.9	0.002	121.3±6.1	2.4×10^{-5}

[a]*P* value was compared to that of 15 min, represent that whether the toxicities of chemicals were significant different between different contact times. The results represent the mean ± *SD*

Organics toxicity

Five organics toxicities to *V. fischeri* were showed in Fig. 2. Similarly, the luminescence inhibition rate increased along with the concentration increasing of organics. The EC_{50} of all organics at different reaction times were summarized in Table II. Data analysis showed that the EC_{50} of organics were close at different reaction times ($P > 0.153$).

Unlike heavy metals, reaction time was not a factor for organics acute toxicities to *V. fischeri*. Organics toxicities to *V. fischeri* could due to different ionization constant,[25] distinctive chemical–physical potential, chemical group, ionization, logarithm of the 1-octanol/water partition coefficient.[26] Previous researches had showed that the different atom and structure can result in much difference in the toxicities of the organics.[26–28] But how they affect the *V. fischeri* cells has been rarely interpreted. Sporadically studies issued that organics involved in the interaction with cell surface receptors, disruption of cell membrane function, or reaction with cellular components could be the reason for the luminescence inhibition of *V. fischeri*.[29,30] The harmful that organics do to *V. fischeri* seem to be irreversible, and could cause the cell dead in short time. Combined with results in this study, the reason of luminescence inhibition by organics could attribute to the cells death.

The relative cell death detection

In order to identify whether the luminescence inhibition was induced by the cells death, the relative cell death detection was carried out after Microtox® test. Table III showed the relatively death rate of *V. fischeri* at different luminescence inhibition rate (20, 50 and 90 %). At luminescence inhibition of 20 and 50 %, the cell death rate was close to the luminescence inhibition rate after exposure to organics. The results suggested that the organics acute toxicities to *V. fischeri* could reflect the death of cells. However, to heavy metals, the relatively death rate of *V. fischeri* was significantly lower than the luminescence inhibition rate, indicated that heavy metals acute toxicities to *V. fischeri* may not reflect the cell death, and only caused partly cell death after contacting with the bacteria.

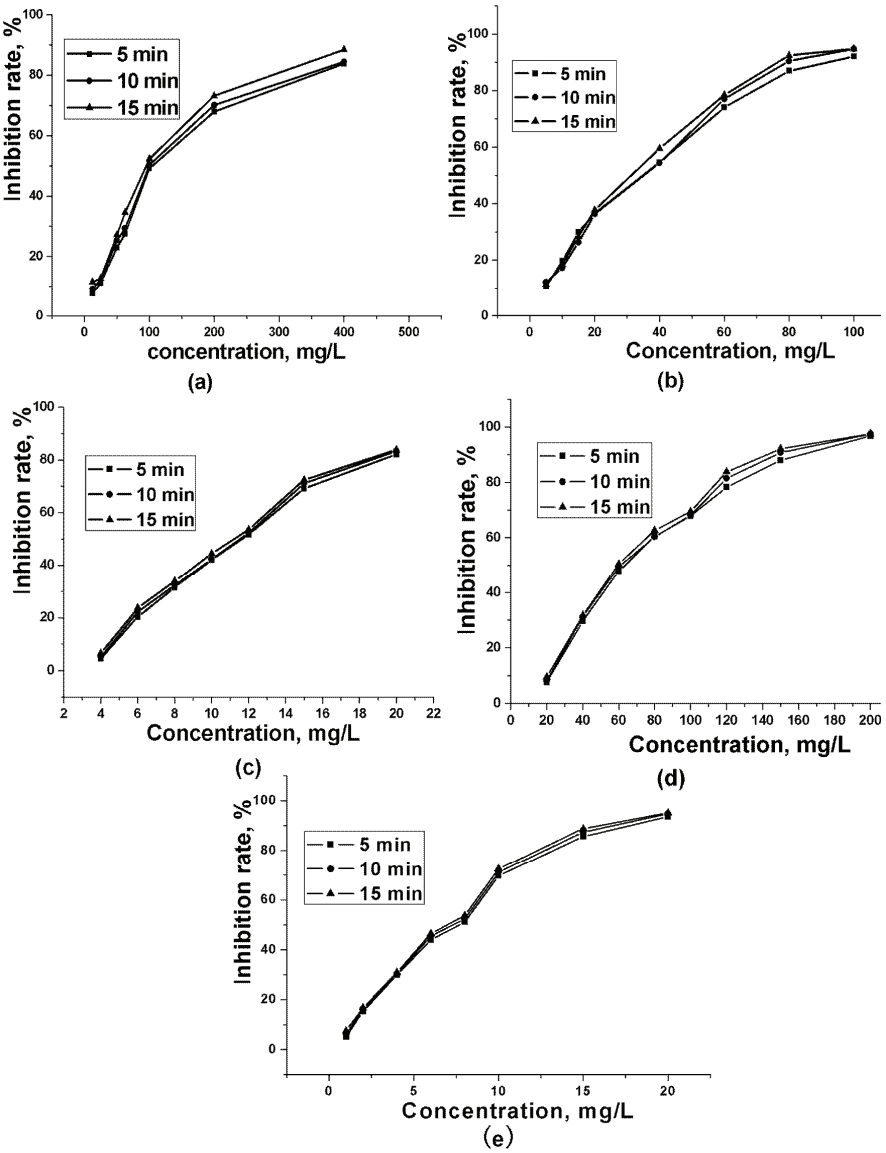

Fig. 2. The luminescence inhibition rate by five different kinds of organics under various concentrations: a), phenol, b) benzene, c) benzoic acid, d) nitrobenzene and e) *p*-hydroxy benzoic acid.

The different reasons of luminescence inhibition between heavy metals and organics could partly explain these results. The heavy metals may combine with some important enzymes involved in the luminescence bioprocess without causing the cells death. Organics may cause damage to the cells membrane and

induce cells death after contacting for a certain time. Of course, after contacting with higher concentrations of organics and heavy metals, such as at the concentrations of chemicals that caused 90 % luminescence inhibition, some irreversible effect on bacteria growth happened and the cell death rate was almost 90 % for both organics and heavy metals (Table III).

TABLE II. Toxicities of aromatics at different contact times

Chemical	Contact time, min				
	15	30		45	
	EC_{50} / mg L^{-1}	EC_{50} / mg L^{-1}	P^a	EC_{50} / mg L^{-1}	P^a
Phenol	111.5±5.8	110.9±5.5	0.871	107.9±5.0	0.324
Benzene	26.5±1.3	26.1±1.3	0.640	25.8±1.2	0.402
Benzoic acid	10.9±0.5	10.7±0.5	0.545	10.5± 0.5	0.241
Nitro-benzene	61.5±3.1	59.7±2.9	0.371	57.8±5.7	0.238
p-Hydroxy benzoic acid	6.3±0.3	6.1±0.5	0.931	6.0±0.3	0.153

[a]*P* value was compared to that of 5 min, represent that whether the toxicities of chemicals were significant different between different contact times. The results represent the mean ±*SD*

TABLE III. The relative cell death rate (*DR* / %) of *V. fischeri* at different luminescence inhibition rate; The results represent the mean ±*SD*

Chemical	Luminescence inhibition rate, %		
	20	50	90
Zn ($ZnSO_4$)	8.9±0.2	33.8±0.3	91.3±0.6
Cu ($CuSO_4$)	7.6±0.1	30.1±1.2	90.7±1.1
Cd ($CdCl_2$)	10.5±0.4	32.2±0.7	91.5±1.2
Cr ($K_2Cr_2O_4$)	7.9±0.1	29.8±0.6	92.6±2.2
Phenol	17.5±1.5	45.6±1.2	89.9±1.4
Benzoic acid	18.6±1.1	47.6±2.3	93.5±3.2
Nitrobenzene	19.7±3.2	46.5±0.8	92.6±1.3
p-Hydroxybezoic acid	17.5±1.4	48.1±1.4	91.5±0.9
Benzene	16.8±0.9	50.2±1.1	91.4±1.2

CONCLUSION

In summary, systematic comparison of heavy metals and organics acute toxicities to *V. fischeri* is reported in this study. Metals toxicities detected by luminescence inhibition rate was increased along with the reaction time and was significant higher than the cell death rate, while organics toxicities kept similar at different reaction times and were consistent with the cell death rate. The discrepancy results indicated that organics acute toxicities to *V. fischeri* could represent the cell death, but luminescence inhibition by metals could due to the disturbing of luminescence bioprocess and may not necessary cause the cell death.

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ИЗВОД

ПОРЕЂЕЊЕ ТОКСИЧНОСТИ ОРГАНСКИХ ЈЕДИЊЕЊА И МЕТАЛА ПРЕМА *Vibrio fischeri*

XUEPENG YANG[1], JI YAN[1], FANGFANG WANG[1], JIA XU[1], XIANGZHEN LIU[2], KE MA[1], XIANGMEI HU[1] и JIANBIN YE[1]

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Инхибиција биолуминисценције *Vibrio fischeri* широко се примењује за испитивање акутних токсичности метала и органских загађујућих материја. Међутим, до сада нису поређене разлике у токсичности ових материја према *V. fischeri*. У овом раду су испитане акутне токсичности четири тешка метала (Zn^{2+}, Cu^{2+}, Cd^{2+} и Cr^{6+}) и пет органских једињења (фенол, бензоева киселина, *p*-хидроксибензоева киселина, нитробензен и бензен) према *V. fischeri*. Токсичности тешких метала су се повећавале са реакционим временом, док је токсичност органских једињења остајала на истом нивоу при различитим реакционим временима. Како би се објаснила ова разлика, мерена је релативна брзина умирања ћелија *V. fischeri*. У тестовима токсичности метала, брзина инхибиције биолуминисценције код *V. fischeri* била је значајно већа него релативна смртност ћелија ($p < 0,05$), док је у тестовима токсичности органских једињења, смртност ћелија била слична брзини инхибиције биолуминисценције. Ови резултати дају индикацију да акутне токсичности органских једињења према *V. fischeri* одражавају смрт ћелије, али акутне токсичности метала не морају водити до смрти ћелије, већ само представљају инхибицију биолуминисценције.

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