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Assessment of joint toxicity effects of heavy metals (Cu^{2+}, Cd^{2+}, Zn^{2+}, Pb^{2+}, Cr^{3+}, Co^{2+}, Ni^{2+} and Sr^{2+}) on photobacterium phosphoreum

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ABSTRACT

In this study, four different assessment methods, Toxic Unit (TU), Additive Index (AI), Mixture Toxicity Index (MTI) and Similarity Parameter (λ), were used to evaluate the joint toxic effect of heavy metal hybrid systems (Cu^{2+}, Cd^{2+}, Zn^{2+}, Pb^{2+}, Cr^{3+}, Co^{2+}, Ni^{2+} and Sr^{2+}) on photobacterium phosphoreum in three groups—EC30 mixture (Mix-R1), EC50 mixture (Mix-R2) and the equimolar ratio mixture (Mix-R3)—planned by the fixed ratio method. An orthogonal experiment was designed to evaluate and screen out the heavy metals that have significant influence on joint toxicity of the hybrid system. The results indicated that joint toxicities of the EC30 mixed-system (Mix-R1) and the EC50 mixed-system (Mix-R2) were analogous and differed from the equimolar-mixed system (Mix-R3), and joint toxic effects were not consistent in the same mixed system by the four evaluation methods. The joint toxicity of both the EC30 mixed system and the EC50 mixed system evaluated by the four evaluation methods (TU, AI, MTI and Similarity Parameter-λ) exhibited partly additive effect and antagonism effect and partly additive effect and antagonism effect separately. In the equimolar mixed system, only the antagonism effect was shown. The orthogonal experiment demonstrated that the joint toxic effect of the hybrid system was not dependent on a single metal. This study confirmed that varying the ratios of the mixture has an impact on the results of the evaluation.

Keywords: heavy metals, joint toxicity evaluation, photobacterium phosphoreum, mixed-system, orthogonal experiment

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INTRODUCTION

Heavy metals pollution, being widely distributed, environmentally persistent and conducive to bioaccumulation, has become increasingly serious in recent years [1-2]. Thus, study of the toxicity of heavy metals is critical in ecological environmental protection. However, heavy metals in the environment do not exist singly, but usually appear as mixtures [3]. Therefore, joint toxicity, which characterizes the hazards associated with heavy metals more succinctly, is of increasing significance in the study of heavy metals toxicity assessment. There are many effective methods for evaluating joint toxicity of combined heavy metals, especially the following four methods: Toxic Unit (TU), Additive Index (AI), Mixture Toxicity Index (MTI) and Similarity Parameter (λ). These methods were often used, and have produced significant achievements in previous use and show much potential for further study [4-7]. However, there are few examples available to clearly show whether one method could produce different results than another in the same assessment, and the toxicity of mixtures of 4 or more heavy metal components are not well documented at present.

Luminescent bacteria are used in heavy metal toxicity tests as a biological indicator. The rate of change in luminous intensity and the toxicant concentration have a linear dependence. Additionally, this method is verified as being rapid, sensitive, convenient and low cost [8].

The aim of this study is to study the joint toxic effect of component systems with more heavy metals, by different evaluation methods. In this paper, 8 heavy metals are used as the research object, photobacterium phosphoreum (T3) is used as the biological indicator, and the nonlinear least squares fitting technique is used to simulate the dose effect relationship and observe the toxicity of heavy metal mixture changes. The toxic unit (TU), additive index (AI), mixture toxicity index (MTI) and Similarity Parameter methods (λ) are applied to provide a qualitative analysis of joint toxic effect of the heavy metal hybrid systems. Furthermore, an orthogonal experiment is designed to evaluate and screen out the heavy metals that have significant influence on the joint toxicity of the hybrid system.

EXPERIMENTAL SECTION

Apparatus and Chemicals

A biological toxicity test instrument (DXY-2, Nanjing Soil Research Institute of the Chinese Academy of Sciences) and a cyclotron oscillator (HY-5, Jintan Province Jiangsu Ronghua Instrument Manufacturing Co Ltd) were used for toxicity testing in this study. Photobacterium phosphoreum lyophilized powder (Nanjing Soil Research Institute of the Chinese Academy of Sciences) was used as a biological indicator. We choose 8 heavy metals as hybrid system components, including $Cu(NO_3)_2 \cdot 3H_2O$, $Cd(NO_3)_2 \cdot 4H_2O$, $Zn(NO_3)_2 \cdot 6H_2O$, $Pb(NO_3)_2$, $Cr(NO_3)_3 \cdot 9H_2O$, $Ni(NO_3)_2 \cdot 6H_2O$, $Co(NO_3)_2 \cdot 6H_2O$, and $Sr(NO_3)_2$, all of which were obtained as analytically pure reagents.

Photobacterium Toxicity Test

The acute toxicity of tested compounds on luminescent bacteria was determined using methods in accordance with published sources [9]. The photobacterium phosphoreum lyophilized powder must be revived first, added to the reviving solution of 2%NaCl, and then oscillated for 2 min on the cyclotron oscillator (HY-5). Before the toxicity test, the light unit of photobacterium phosphoreum exposed to controls should be determined between (600-1900) mV by power shifting, and new lyophilized powder must be revived again.

First, eight heavy metal nitrates were prepared to test stock solutions as water extracts of sediments. The concentration gradients of three hybrid systems were separately prepared according to concentrations in the table, with a background liquid of sediment water extract, and a blank sediment water extract as control. An aliquot of 2 ml of 3% NaCl solution was added to each test solution. There were two parallels of each concentration gradient, and each parallel contained 3 parallel samples and 3 control samples. Upon instillation of 10 μL bacteria liquid, relative light intensity was determined by the biological toxicity test instrument after 15 min [9]. EC50 and the 95% confidence interval of three groups of single heavy metal ions in the mixed system were calculated.

Mixture Experimental Design

The fixed ratio experimental method [10] was used for joint toxicity evaluation in this study, with the eight heavy metals. The basic principle of this method is that the total concentration of a mixture is constantly changing, and the ratio of each pair of substances is fixed. Three mixed systems of this experiment were designed based on the responses of the concentrations of single compounds in previous studies [11] (Table 1). The first and second mixed systems were designed according to the concentration ratios of 30% and 50%, respectively, and the third hybrid system is based on the single mixture components in equimolar concentration ratios (the experimental concentration gradient is shown in Tables 2, 3, and 4). In this way, the three hybrid systems are defined as: EC30 (Mix-R1), EC50 (Mix-R2), and the equimolar mixture (Mix-R3). According to the experimental results, to select a concentration gradient suitable for luminescent bacteria, the determination of the joint toxicity of the hybrid system was made according to acute toxicity tests.

Table 1 Single toxicities of 8 heavy metals on Photobacterium Phosphoreum (×10^3 μmol/L)

Heavy metals	EC20	EC30	EC40	EC50	EC60	EC70	EC90
Cu^{2+}	[0.0026]	[0.0061]	[0.0118]	[0.0208]	[0.0348]	[0.0576]	[0.1903]
Cd^{2+}	[0.0006]	[0.0017]	[0.0038]	[0.0074]	[0.0135]	[0.0245]	[0.1003]
Zn^{2+}	[0.0032]	[0.0043]	[0.0055]	[0.0067]	[0.0081]	[0.0097]	[0.0150]
Pb^{2+}	[0.0004]	[0.0012]	[0.0026]	[0.0049]	[0.0089]	[0.0160]	[0.0638]
Cr^{3+}	[0.0757]	[0.1075]	[0.1405]	[0.1765]	[0.2174]	[0.2666]	[0.4327]
Co^{2+}	[0.0017]	[0.0095]	[0.0347]	[0.1044]	[0.2859]	[0.7660]	[7.9528]
Ni^{2+}	[0.1094]	[0.3552]	[0.8752]	[1.8828]	[3.7937]	[7.5289]	[38.3350]
Sr^{2+}	[31.9920]	[61.7460]	[127.5800]	[166.7000]	[212.8800]	[270.4200]	[477.3018]

Table 2 Concentration ratios of 8 heavy metals in the mixed system EC30 (Mix-R1) (×10^3 μmol/L)

Heavy metal	1	2	3	4	5	6	7	8	9	10	11
Cu^{2+}	[0.0305]	[0.0610]	[0.3050]	[0.9150]	[1.8300]	[2.7450]	[3.6600]	[4.5750]	[5.4900]	[6.1000]	[8.5400]
Cd^{2+}	[0.0085]	[0.0170]	[0.0850]	[0.2550]	[0.5100]	[0.7650]	[1.0200]	[1.2750]	[1.5300]	[1.7000]	[2.3800]
Zn^{2+}	[0.0215]	[0.0430]	[0.2150]	[0.6450]	[1.2900]	[1.9350]	[2.5800]	[3.2250]	[3.8700]	[4.3000]	[6.0200]
Pb^{2+}	[0.0060]	[0.0120]	[0.0600]	[0.1800]	[0.3600]	[0.5400]	[0 7200]	[0.9000]	[1.0800]	[1.2000]	[1.6800]
Cr^{3+}	[0.0005]	[0.0011]	[0.0054]	[0.0161]	[0.0323]	[0.0484]	[0.0645]	[0.0806]	[0.0968]	[0.1070]	[0.1500]
Co^{2+}	[0.0475]	[0.0950]	[0.4750]	[1.4250]	[2.8500]	[4.2750]	[5.7000]	[7.1250]	[8.5500]	[9.5000]	[13.3000]
Ni^{2+}	[0.0018]	[0.0036]	[0.0178	[0.0533]	[0.1066]	[0.1598]	[0.2130]	[0.2664]	[0.3200]	[0.3550]	[0.4970]
Sr^{2+}	[0.4656]	[0.9313]	[4.6565]	[13.9795]	[27.9389]	[41.9084]	[55.9000]	[69.8473]	[83.8167]	[93.1297]	[130.000]

Table 3 Concentration ratios of 8 heavy metals in the mixed system EC50 (Mix-R2) ($\times 10^3$ μmol/L)

Heavy metal	1	2	3	4	5	6	7	8	9	10	11
Cu^{2+}	[0.0210]	[0.1040]	[0.2080]	[1.0400]	[3.1200]	[6.2400]	[8.3200]	[10.4000]	[12.5000]	[14.5600]	[18.7200]
Cd^{2+}	[0.0070]	[0.0370]	[0.0740]	[0.3700]	[1.1100]	[2.2200]	[2.9600]	[3.7000]	[4.4400]	[5.1800]	[6.6600]
Zn^{2+}	[0.0070]	[0.0340]	[0.0670]	[0.3350]	[1.0050]	[2.0100]	[2.6800]	[3.3500]	[4.0200]	[4.6900]	[6.0300]
Pb^{2+}	[0.0050]	[0.0240]	[0.0490]	[0.2450]	[0.7350]	[1.4700]	[1.9600]	[2.4500]	[2.9400]	[3.4300]	[4.4100]
Cr^{3+}	[0.0002]	[0.0009]	[0.0018]	[0.0088	[0.0265]	[0.0530]	[0.0706]	[0.0883]	[0.1060]	[0.1235]	[0.1589]
Co^{2+}	[0.1040]	[0.5220]	[1.0440]	[5.2200]	[15.6600]	[31.3200]	[41.8000]	[52.2000]	[62.6000]	[73.0800]	[93.9600]
Ni^{2+}	[0.0019]	[0.0094]	[0.0188]	[0.0941]	[0.2824]	[0.5648]	[0.7530]	[0.9410]	[1.1290]	[1.3180]	[1.6950]
Sr^{2+}	[0.1670]	[0.8330]	[1.6670]	[8.3340]	[25.0000]	[50.0100]	[66.7000]	[83.4000]	[100.0000]	[116.7000]	[150.0000]

Table 4 Concentrations of 8 heavy metals mixed in the same molar ratio (Mix-R3) ($\times 10^3$ μmol/L)

Heavy metal	1	2	3	4	5	6	7	8	9	10
M_i	[0.1000]	[0.5000]	[1.0000]	[3.0000]	[6.0000]	[9.0000]	[12.0000]	[15.0000]	[18.0000]	[20.0000]

Orthogonal Experimental Design

The orthogonal experiment was designed in terms of the equivalent-toxicity concentration ratio method as $L_{27}(3^8)$. The effective concentrations of EC10, EC20 and EC30 (the concentrations that produce the effects of 10%, 20% and 30%, respectively) were selected as three levels of eight heavy metals. Detailed design formulae are listed in Table 5.

Table 5 Orthogonal experimental design formulae of mixtures of heavy metals in terms of the equivalent-toxicity concentration ratio method

Groups	Cu^{2+}	Cd^{2+}	Zn^{2+}	Pb^{2+}	Cr^{3+}	Co^{2+}	Ni^{2+}	Sr^{2+}
1	EC10	EC10	EC10	EC10	EC10	EC10	EC10	EC10
2	EC10	EC10	EC10	EC10	EC20	EC20	EC20	EC20
3	EC10	EC10	EC10	EC10	EC30	EC30	EC30	EC30
4	EC10	EC20	EC20	EC20	EC10	EC10	EC10	EC20
5	EC10	EC20	EC20	EC20	EC20	EC20	EC20	EC30
6	EC10	EC20	EC20	EC20	EC30	EC30	EC30	EC10
7	EC10	EC30	EC30	EC30	EC10	EC10	EC10	EC30
8	EC10	EC30	EC30	EC30	EC20	EC20	EC20	EC10
9	EC10	EC30	EC30	EC30	EC30	EC30	EC30	EC20
10	EC20	EC10	EC20	EC30	EC10	EC20	EC30	EC10
11	EC20	EC10	EC20	EC30	EC20	EC30	EC10	EC20
12	EC20	EC10	EC20	EC30	EC30	EC10	EC20	EC30
13	EC20	EC20	EC30	EC10	EC10	EC20	EC30	EC20
14	EC20	EC20	EC30	EC10	EC20	EC30	EC10	EC30
15	EC20	EC20	EC30	EC10	EC30	EC10	EC20	EC10
16	EC20	EC30	EC10	EC20	EC10	EC20	EC30	EC30
17	EC20	EC30	EC10	EC20	EC20	EC30	EC10	EC10
18	EC20	EC30	EC10	EC20	EC30	EC10	EC20	EC20
19	EC30	EC10	EC30	EC20	EC10	EC30	EC20	EC10
20	EC30	EC10	EC30	EC20	EC20	EC10	EC30	EC20
21	EC30	EC10	EC30	EC20	EC30	EC20	EC10	EC30
22	EC30	EC20	EC10	EC30	EC10	EC30	EC20	EC20
23	EC30	EC20	EC10	EC30	EC20	EC10	EC30	EC30
24	EC30	EC20	EC10	EC30	EC30	EC20	EC10	EC10
25	EC30	EC30	EC30	EC20	EC10	EC30	EC20	EC30
26	EC30	EC30	EC30	EC20	EC20	EC10	EC30	EC10
27	EC30	EC30	EC30	EC20	EC30	EC20	EC10	EC20

Joint Toxicity Evaluation

In this study, we used toxic unit (TU), additive index method (AI), mixture toxicity index (MTI) and the similarity parameter method (λ) (Table 6) to evaluate the combined toxic effects on the three different groups of hybrid systems and to discriminate combined effects of mixtures.

Table 6 Type of joint toxicity action and evaluation index

Evaluation methodology	formula	scope	Type of action
Toxic Unit [12] (TU)	$M=\Sigma TU_i=\Sigma C_i/IC_{50,i}$ $M_0=M/TU_{imax}$ C_i is the concentration of component I in a mixed system $IC_{50,}$ is the EC_{50} value of component I for a single action	$M=1$ $M>M_0$ $M<1$ $M=M_0$ $M_0>M>1$	Additive effect Antagonism Aynergistic effect Independent effect Partial additive effect
Additive index [13] (AI)	$M=1$，$AI=M–1$；$M<1$，$AI=1/M–1$ $M>1$，$AI=1–M$ The meaning of M is as above	$AI=0$ $AI<0$ $AI>0$	Additive effect Antagonism Aynergistic effect
Mixture toxicity index [14] (MTI)	$MTI=(\log M_0-\log M)/\log M_0$ The meaning of M and M_0 are as above	$MTI=1$ $MTI<0$ $MTI=0$ $0<MTI<1$ $MTI>1$	Additive effect Antagonism Independent effect Partial additive effect Aynergistic effect
Similarity parameter [15] (λ)	$\Sigma(TU_i)^{1/\lambda}=1$ The TU_i is as above	$\lambda=0$ $0<\lambda<1$ $\lambda=1$ $\lambda>1$	Independent effect Antagonism Additive effect Aynergistic effect

RESULTS AND DISCUSSION

Dose Effect Curve of a Mixed System

According to the test results of the toxic effects on luminous bacteria in the three hybrid systems, eight heavy metal mixture experimental dose-effect curves were plotted using Origin 8.0. The principle of the optimal fitting selection model was used to fit the experimental data, thus obtaining the optimal dose-effect curve (DRC) [16-17]. The results show that the DoseResp function, the BiDoseResp function and the Hill function are good descriptions of dose-effect of the three groups of the mixed system. The dose-effect diagram of the poison was worked out during the process.

DoseResp

$$y = A_1 + \left(A_2 - A_1\right) \Big/ \left(1 + 10^{\left(\log x_0 - x\right)p}\right) \quad (1)$$

BiDoseResp

$$y = A_1 + \left(A_2 - A_1\right)\left[p \Big/ \left(1 + 10^{\left(\log x_{01} - x\right)h_1}\right) + \left(1 - p\right) \Big/ \left(1 + 10^{\left(\log x_{02} - x\right)h_2}\right)\right] \quad (2)$$

Hill

$$y = V_{\max} \times x^n \Big/ \left(k^n + x^n\right) \quad (3)$$

Where A2 and A1 denote the upper and lower dose effect curves, respectively; P is the slope of the curve; x0 is the point of tangency abscissa value; and H1 and H2 are the cut-off point heights.

Three hybrid system dose effect parameters, the correlation coefficient R as reported in Table 7, and the EC50 values with 95% confidence intervals of all single components in the mixed system were shown in Table 8. The results show that the correlation coefficient value of the dose effect fitting with the experimental data is approximately 0.99; thus, the quality of the fit is remarkable.

Table 7 Fitted model and correlation coefficient (R) for the dose-response function of the three mixed systems to Photobacterium Phosphoreum

Mixture	Model	A_1	A_2	V_{max}	k	R
Mix-R1	DoseResp	6.7429	97.874	-	-	0.997
Mix-R2	BiDoseResp	28009	100.58	-	-	0.994
Mix-R3	Hill	-	-	91.487	9.93×10^{-4}	0.980

Table 8 Effect concentrations and the 95% confidence intervals of the three mixed systems

Mixed Components	Mix-R1	Mix-R2	Mix-R3
	EC50 and the 95% confidence intervals [μmol/L]		
Cu^{2+}	2.96(2.89, 3.03)	4.81(2.26, 7.35)	1.15(0.93, 1.44)
Cd^{2+}	0.83(0.80, 0.85)	1.71(0.81, 2.62)	1.15(0.93, 1.44)
Zn^{2+}	2.08(2.03, 2.13)	1.55(0.73, 2.37)	1.15(0.93, 1.44)
Pb^{2+}	0. 58(0.56, 0.60)	1.13(0.53, 1.73)	1.15(0.93, 1.44)
Cr^{3+}	52.17(50.89, 53.44)	40.85(19.22, 62.38)	1.15(0.93, 1.44)
Co^{2+}	4.61(4.50, 4.73)	24.16(11.37, 36.90)	1.15(0.93, 1.44)
Ni^{2+}	172.38(168.15, 176.59)	435.75(205.02, 665.46)	1.15(0.93, 1.44)
Sr^{2+}	45.196(44.089, 46.303)	38.581(18.152, 58.918)	1.15(0.93, 1.44)

Joint Toxicity of Mixed Systems

The toxic unit, additive index method, mixture toxicity index and similarity parameter method were applied to evaluate the combined toxicity of three groups of hybrid systems, and the results are listed in Table 9.

Table 9 Assessment of joint toxicity to mixed heavy metal systems

Hybrid system	Toxic unit method			Additive index method		Mixture toxicity index		Similarity parameter method	
	M	M_0	Type of action	AI	Type of action	MTI	Type of action	λ	Type of action
Mix-R1	1.38	4.46	Partial additive	-0.39	Antagonism	0.78	Partial additive	0.83	Antagonism
Mix-R2	1.93	7.99	Partial additive	-0.93	Antagonism	0.68	Partial additive	0.68	Antagonism
Mix-R3	0.64	2.71	Synergy	0.58	Synergy	1.46	Synergy	1.31	Synergy

From Table 9, we can find that, in each group of hybrid systems, the evaluation results by four types of evaluation methods were not exactly the same. Three groups of hybrid systems were evaluated. The result showed that two sets of hybrid systems (Mix-R1, Mix-R2) of equal toxicity ratio had the same combined effects, and they differed from the equal mole ratio mixed system (Mix-R3).

Equal toxicity ratio mixed systems (Mix-R1, Mix-R2) determined by the toxic unit and mixture toxicity index methods were found to have a partial additive effect; but the mixed system determined by the additive index method and the Similarity parameter method (Mix-R1, Mix-R2) resulted in antagonism. The equal mole ratio hybrid system (Mix-R3) was different from the previous two, and exhibited synergy in three toxicity evaluation patterns. This phenomenon may be due to the substantial toxicity difference among the toxicants (shown in Table 1). After equimolar ratio mixing, Cd^{2+}, Zn^{2+}, Pb^{2+} and Cu^{2+} play the main part in the toxic effect, whereas Ni^{2+} and Sr^{2+} hardly exerted toxic effects or have an enhanced effect on the toxicity of other ions because of their small sizes. Morley [18] found that an equal concentrations mixture of Zn and Cd showed a synergistic effect with low concentrations and an antagonistic effect with high concentrations; Wang Zhizeng [19] found that high toxicity intensities of Cr^{3+} and Zn^{2+} have an additive effect; Wu Shuhang [20] found that Cr^{3+} and Cd^{2+} exerted an additive effect; eight metals in an equimolar mixture had a more complex mode of action such that the mixture toxicity demonstrated enhancement. Heavy metals of different toxicities would exhibit various mechanisms of toxicity within a mixture, which will cause the poison to inhibit or enhance the toxicity, and metal concentrations and organisms will affect the toxicity performance.

Significance of Analysis of Heavy Metals to Joint Toxicity

From Table 10, the results of the orthogonal experiment show that Ni^{2+}, Co^{2+} and Cr^{3+} are highly significant to joint toxic effects of heavy metal mixtures. Pb^{2+} has a relatively significant influence compared with the other 4 heavy metals. In the single toxicity experiments of 8 heavy metals, Pb^{2+} and Co^{2+} exhibit higher toxic effects than Ni^{2+} and Cr^{3+}, revealing that the joint toxic effect of mixtures does not depend only on a single factor. Adding a highly toxic substance into certain mixtures may have little impact on joint toxicity of a hybrid system, and vice versa.

Table 10 Variance analysis of orthogonal experiment for joint toxic effects of 8 heavy metals

Heavy metal	sum of squares	mean square	F value	significance
Cu^{2+}	0.0137	0.0068	3.18	
Cd^{2+}	0.0064	0.0032	1.50	
Zn^{2+}	0.0012	0.0006	0.27	
Pb^{2+}	0.0005	0.0106	4.91	*
Cr^{3+}	0.0211	0.0389	18.08	**
Co^{2+}	0.1388	0.0694	32.24	**
Ni^{2+}	0.0417	0.0208	9.68	**
Sr^{2+}	0.0042	0.0021	0.92	

CONCLUSION

Joint toxicities of 8 heavy metal ions were determined, and the effects of hybrid systems were assessed by toxic unit, additive index method, and mixture toxicity index and similarity parameters. The results show that the combined effects of judging a mixture by four different evaluation methods are sometimes inconsistent. Comparing the four toxicity evaluations, results of toxic unit (TU) and mixture toxicity index (MTI) showed uniformity, and additive index (AI) and the similarity parameter method (λ) showed consistent evaluation results.

The experiments show that the joint toxicity effects of the eight heavy metal ions with equal toxicity ratios were consistent. The joint toxic effects of equimolar ratio mixtures were synergistic and different from the equal toxicity ratio mixtures, demonstrating that the heavy metal composition has a certain influence on the joint action.

Orthogonal experiments showed that the joint toxic effect of heavy metals in a hybrid system was not depended on a certain element. Rather, it was highly influenced by the interactions of the components of the system.

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