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Research Article

Combined Toxicity of Copper, Cadmium and Lead Toward *Daphnia magna*: Recommendation for Bioassay-Based Whole Effluent Toxicity (WET) Testing in China

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ABSTRACT

Metals cause popular attention worldwide due to their non-degradability and universal distribution in the aquatic ecosystem. In this study, the single, binary, and ternary combined toxicity of copper, cadmium, and lead toward survival rate of *Daphnia magna* were investigated based on different level fixed equivalent-effect concentration ratios. Furthermore, the combined toxicity was predicted by concentration addition and independent action models based on an established concentration-response relationship of a single toxicant and compared with the experimental data. The results indicated both binary and ternary mixture of the three metals had a strongly synergistic effect ($EC_{50mix} < 1$ TU) on the survival of *Daphnia magna* in all designed mixture ratios, which is meaning that there may be potential risks even when the single toxicant meets the discharged standards just based on chemical analysis. So, it is suggested that biological testing of whole effluents toxicity based on aquatic organisms should be applied in environment and risk management in China.

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Introduction

Metal pollution is a worldwide serious environmental problem as they are non-biodegradable pollutants. They are commonly determined in aquatic, terrestrial, and aerial environments. Among them, copper (Cu), cadmium (Cd) and lead (Pb) are three of the most common and important metals in anthropogenic activities. They were normally determined in industrial, municipal wastewater and urban stormwater, which may be directly or indirectly released into aquatic ecosystems and pose a threat to environmental health [1-3]. Especially in China, Cu, Cd and Pb were commonly co-determined in soils, air dusts, sediments, and rivers, even in tissues of organisms [4-13]. The concentrations of Cu, Cd and Pb were about 7.91 µg/L, 0.08 µg/L and 15.7 µg/L in Yangtze River Basin, respectively; the maximum mean concentrations of Cu, Cd and Pb

reached 46.35 µg/L, 5.89 µg/L and 26.12 µg/L in Upper Han River in 2005 and 2006, respectively; in the Yellow River, the concentrations of metals varied within a range of 1.5-5.0 µg/L for Cu, 0.1-20 µg/L for Pb, and 0.2-1.1 µg/L for Cd, respectively [11-13]. Their discharge is regulated by environmental laws, but the laws are just based on chemical monitoring of single toxicant and ignore the interactions of coexisting toxicants in China, which may not provide sufficient protection for the health of the ecosystem due to additive or synergistic effects [14].

Several studies have indicated that toxicant mixtures may cause significant toxic below NOECs (No Observed Effective Concentrations) of a single toxicant [15-17]. Therefore, single toxicant discharge standard may not be protective for the safety of the aquatic ecosystem; biological testing of whole effluents toxicity (WET) is indispensable in

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environmental and risk management [18]. Moreover, with respect to biological testing, it is known that organisms within the same and in different trophic levels respond differently to a range of toxicants, both as single or complex mixtures, and there is, therefore, a need to develop toxicity bioassays using a suite of organisms [19–21]. This is important because of the complexity in terms of organism diversity present in natural environments and differences in physiological status [18].

The assessment of combined effects has become a research focus due to their significance to risk assessment and the establishment of water quality criteria. In recent years, several theoretical and experimental studies were done to explore joint toxicity or called combined toxicity [15, 22, 23]. Among them, Concentration addition (CA) and Independent action (IA) models are the basis of model development and widely applied. For example, a new two-step prediction model combined CA and IA models was developed and applied; a theory and mathematical description of combined toxicity, an approach to classifying types of action of three-factorial combinations, was further developed [23, 24]. CA and IA could predict binary and ternary mixtures with similar or dissimilar modes of action on *Daphnia magna* equally well [25]. Other researchers found that CA and IA models might be promising tools for the risk assessment of pollutant mixtures [26, 27]. Therefore, mathematical models may be a good useful tool in environmental management because it is easy to predict the combined toxicity of known mixture components based on the toxicity database of a single toxicant, which would save both some money and time.

In the present study, the acute toxicity of single, binary, and ternary mixtures of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{CdCl}_2 \cdot 2.5\text{H}_2\text{O}$ and PbCl_2 was investigated by *Daphnia magna*. Three fixed equivalent-effect concentration mixture ratios based on single toxicant EC_{20} , EC_{50} and EC_{80} values (effective concentration value that affects 20%, 50% and 80% of the test population reflecting three different concentration levels, respectively) for binary and ternary mixture were designed. For the binary and ternary mixtures, concentration-response relationships were established based on the toxic units (TU) approach. All the experimental results of the mixture were compared with the predictions estimated by the CA, IA models, respectively, which would provide an important reference of Cu, Cd and Pb co-exposure for possible disease diagnosis and risk based on single toxicant discharged standard. Furthermore, the results will offer an insight into the possible gap between the “real risk” and “thinkable risk” based on the present environment and risk management.

Materials and Methods

I Test Chemicals

$\text{CdCl}_2 \cdot 2.5\text{H}_2\text{O}$ ($\geq 99.0\%$ purity, CAS [7790-78-5]), $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ ($\geq 99.0\%$ purity, CAS [10125-13-10]) and PbCl_2 ($\geq 99.0\%$ purity, CAS [7758-95-4]) were purchased from Nanjing Chemical Reagent Co., LTD.

II Test Organism

The *Daphnia magna*, from a single clone, was cultured and kept in our laboratory for many years at Nanjing University. The cultural and experimental approaches have been described in our previous studies

[22, 28]. In brief, the clones, fed daily with a suspension of the green alga *Scenedesmus obliquus*, were kept in a controlled temperature at $23 \pm 1^\circ\text{C}$ with a light: dark cycle of 16:8 h photoperiod. Same temperature and light conditions were used in all experiments. Tap water (pH: 8.12 ± 0.11 , dissolved oxygen (DO): 6.07 ± 0.24 mg/L, conductivity: 319 ± 9.1 $\mu\text{S}/\text{cm}$, alkalinity: 95.48 ± 4.64 mg/L as CaCO_3 , and hardness: 125.5 ± 4.95 mg/L as CaCO_3) was used as medium in all controls and tests.

III Experimental Design and Statistical Analysis

Three-group experiments were set: (1) single toxicity tests, (2) binary toxicity tests, (3) ternary toxicity tests. Five less than 24 h old neonates were put in a 50 ml glass beaker with 30 ml of every test concentration medium. Four replicates were operated in every set concentration according to the OECD Guideline and others [22, 29, 30]. The test exposures were 48 hours without renewal of the test solutions. In order to ensure proper physiological conditions for the experiments of *Daphnia magna*, tests with the reference chemical $\text{K}_2\text{Cr}_2\text{O}_7$, as the positive control, were run simultaneously. For single group tests, each toxicant with seven different concentrations (3–300 $\mu\text{g}/\text{L}$ for Cu, 100–2500 $\mu\text{g}/\text{L}$ for Pb, 12.5–300 $\mu\text{g}/\text{L}$ for Cd) was used to obtain accurate dose-response relationship curves, which were used to define the studied mixtures and predict combined toxicity response. A fixed mixture ratio was designed for binary and tertiary mixture [22, 26, 31]. In this study, the fixed mixture ratios were EC_{20} -value, EC_{50} -value and EC_{80} -value derived from the single toxicant, representing three mixture levels. The survivability of the neonate, as the endpoint, was judged by the ability to occupy the water column within 10 seconds after exposure for 48 hours. The EC_{20} , EC_{50} and EC_{80} values with 95% confidence intervals were calculated by a three-parameter log-logistic regression model (Equation (1)) [22, 24, 25]:

$$f(x) = \frac{d}{1 + \exp\{b \cdot (\log(x) - \log(e))\}} \quad (1)$$

Where b is proportional to the slope at e , d is the upper limit, e is the EC_{50} value of the dose-response curve; $f(x)$ is the function of chemical concentration x , here it stands for survival percent. All statistical analyses were done by using the statistical computing software R (R version 2.15.2, R Development Core Team, Link).

IV Calculation of Predicted Mixture Effects

Based on the three-parameter log-logistic regression model (Equation (1)) derived from a single toxicant, the combined effects of mixtures with known composition could be predicted by using CA or IA models [24]. The predicted effect concentrations for mixtures by CA were calculated according to the Loewe equation:

$$\text{EC}_{x\text{mix}} = \sum_{i=1}^n \left(\frac{P_i}{\text{EC}_{xi}} \right)^{-1} \quad (2)$$

Where $\text{EC}_{x\text{mix}}$ is the predicted effect concentration of mixture causing effect E such as EC_{50} , P_i is the fraction of compound i in the mixture, EC_{xi} is the concentration of i compound causing effect E on its own. The predicted effects of a mixture with the known composition by the model of IA were calculated by using the following equation:

$$E(c_{\text{mix}}) = 1 - \prod_{i=1}^n (1 - E(c_i)) \quad (3)$$

Where $E(c_{\text{mix}})$ is the whole effect, expressed as a fraction of a maximum possible effect of a mixture composed of i toxicants, c_i is the

concentration of i toxicant in the mixture, and $E(c_i)$ is the effect of toxicant i caused on its own. The toxic units (TU) approach was used to assess mixture toxicity for the binary and tertiary mixtures of three metals. In this model, concentrations were expressed as TUs that were fractions of the EC_{50} values of the individual toxicants:

$$TU = \frac{C}{EC_{50}}; TU_{\text{mixture}} = TU_1 + TU_2 + \dots + TU_n \quad (4)$$

Here, the EC_{50} equals to 1 TU; C is the concentration of every single toxicant; n is the total number of mixture components. Then, the sum of

TU causing 50% inhibition for the mixture ($EC_{50\text{mix}}$) was derived from the TU-response relationship. According to the calculated TU, three type interactions were classified: concentration additive ($EC_{50\text{mix}} = 1$ TU), synergistic ($EC_{50\text{mix}} < 1$ TU), and antagonistic ($EC_{50\text{mix}} > 1$ TU). Before and after the neonates exposed to media for 48h, concentrations of Pb (II), Cu (II), Cd (II) (expressed in units of $\mu\text{g/L}$) in all samples were determined using AAS (iCE 3500, Thermo Scientific, America), the results were in (Table 1).

Table 1: Results of measured initial and final concentrations in water samples ($x \pm s.e$) compared to nominal concentrations.

Metals names	Nominal concentrations ($\mu\text{g/L}$)	Chemical-analytical concentrations	
		C_{initial} ($\mu\text{g/L}$)	C_{final} ($\mu\text{g/L}$)
Cu	3	3.33 $\pm$ 0.09	3.60 $\pm$ 0.06
	7.5	7.63 $\pm$ 0.29	8.33 $\pm$ 0.09
	20	20.17 $\pm$ 1.27	21.10 $\pm$ 0.93
	50	50.07 $\pm$ 0.77	55.43 $\pm$ 1.43
	120	130.70 $\pm$ 2.19	136.96 $\pm$ 3.80
	240	274.13 $\pm$ 3.62	272.03 $\pm$ 5.24
	300	314.37 $\pm$ 4.21	328.03 $\pm$ 5.43
Pb	100	96.33 $\pm$ 3.84	105.33 $\pm$ 5.70
	125	124.00 $\pm$ 6.93	125.33 $\pm$ 3.48
	200	197.67 $\pm$ 7.51	204.67 $\pm$ 4.06
	400	394.33 $\pm$ 14.50	428.67 $\pm$ 2.33
	800	917.67 $\pm$ 18.53	886.00 $\pm$ 26.08
	1600	1761.67 $\pm$ 41.60	1839.67 $\pm$ 44.60
	2500	2759.00 $\pm$ 52.85	2674.67 $\pm$ 74.98
Cd	12.5	14.27 $\pm$ 0.71	14.37 $\pm$ 0.44
	25	21.90 $\pm$ 0.1	26.37 $\pm$ 3.34
	50	58.10 $\pm$ 1.97	55.00 $\pm$ 4.50
	75	87.93 $\pm$ 2.22	72.03 $\pm$ 1.83
	100	104.70 $\pm$ 4.41	120.10 $\pm$ 1.90
	150	171.43 $\pm$ 2.37	156.73 $\pm$ 2.70
	300	301.07 $\pm$ 7.90	330.97 $\pm$ 9.44

Exposure period was 48 h; the values are the mean of four replicates; standard error in parentheses.

Results

I Single Chemical Toxicity

From the measured results of samples in (Table 1), there was little to no significance between nominal metal concentration and analysed metal concentrations, and the range of concentrations was generally consistent with the nominal concentration after 48 hours of exposure. So, the

nominal concentrations were used in further analysis. From the result of positive control of $K_2Cr_2O_7$, the *Daphnia magna* colony was in healthy condition according to OECD 202 [29]. Significant concentration-response relationships were observed with exposure concentrations leading to 0-100% effects (Figure 1) in all three single experiments. The parameters of the concentration-response model for the three single toxicants utilized in our studies are reported in (Table 2).

Table 2: Parameter values from the three-parameter log logistic curves fitted from the preliminary single substance experiments (Equation (1) in text).

	Cu	Cd	Pb
b	1.45 $\pm$ 0.24	1.18 $\pm$ 0.39	2.76 $\pm$ 0.54
d	97.90 $\pm$ 4.11	98.93 $\pm$ 4.42	94.84 $\pm$ 3.08
e	63.63 $\pm$ 9.84	75.20 $\pm$ 6.51	1034.81 $\pm$ 90.51
R^2	0.9836	0.9882	0.9781

Parameter values are given $\pm$ standard error.

The determination coefficients ($R^2 > 0.97$) of each simulated model indicated goodness of fit. Through the known fitting equations, the

effects at any concentrations could be calculated. The 48-h EC_{20} , 48-h EC_{50} and 48-h EC_{80} values with 95% confidence intervals (CI) were

39.78 (25.13-54.43) $\mu\text{g/L}$, 75.20 (61.32-89.07) $\mu\text{g/L}$ and 142.14 (110.23-174.05) $\mu\text{g/L}$ for Cd, 24.51 (10.38-38.65) $\mu\text{g/L}$, 63.63 (42.65-84.60) $\mu\text{g/L}$ and 165.15 (112.34-217.97) $\mu\text{g/L}$ for Cu, and 626.59 (420.14-833.05) $\mu\text{g/L}$, 1034.81 (841.89-1227.73) $\mu\text{g/L}$ and 1708.97 (1329.52-2088.43) $\mu\text{g/L}$ for Pb, respectively. Cd and Cu had a lower EC_{50} value (i.e., highest toxicity), indicating more toxic to *Daphnia magna* than Pb. Most of the environmentally relevant concentrations are lower than the EC_{20} for Cd, Cu and Pb, except the maximum of mean concentrations of Cu (46.35 $\mu\text{g/L}$) in Upper Han River [12].

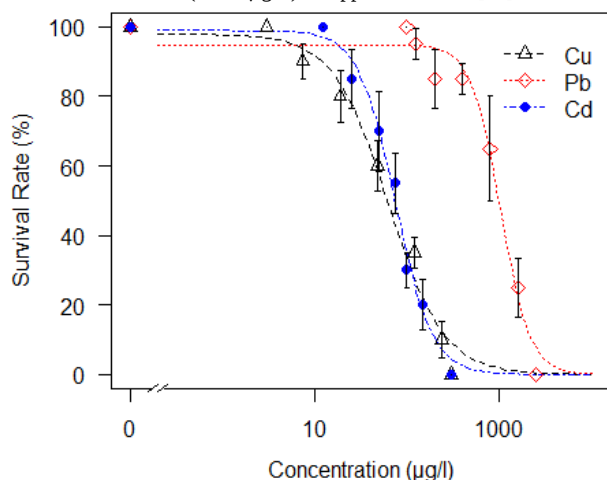


Figure 1: Concentration-response relationship for Cu, Cd, and Pb. Experimental effect data mean (Δ , $\bullet$, $\diamond$) with standard error ($n=4$), with the regression model (sigmoidal dash line).

II Binary and Ternary Mixtures

The concentration-response relationships of binary and ternary mixtures were well described by Equation (1) based on the TU approach (Figures 2 & 3). Simultaneously, the prediction of mixture effects was conducted by CA and IA models (Figures 2 & 3) to show the interaction relationship. Moreover, the mixture data were further explored synergism or antagonism by whether the toxicants induced a 50% survivability reduction of the cumulative effect at 1 TU.

From the (Figures 2 & 3), the combined effects predicted by CA or IA models deviated from the regression model simulated from the experimental data for both binary and ternary mixtures. In addition, the calculated EC_{50} from the regression models were all lower than any one of 1 TU, ones calculated by CA or IA models, which indicated a synergism for every two metals or all three metals (Table 3). The $\text{EC}_{50\text{mix}}$ with 95% CI values for Cu + Cd combinations were 0.091 (0.017-0.17), 0.28 (0.057-0.50) and 0.48 (0.24-0.71) based on EC_{20} -value, EC_{50} -value and EC_{80} -value mixture ratio by TU approach, respectively; Cu + Pb combinations were 0.084 (0.0060-0.16), 0.27 (0.11-0.43) and 0.20 (0.037-0.36) based on EC_{20} -value, EC_{50} -value and EC_{80} -value mixture ratio by TU approach, respectively; Cd + Pb combinations were 0.17 (0.075-0.27), 0.11 (0.047-0.18) and 0.27 (0.16-0.39) based on EC_{20} -value, EC_{50} -value and EC_{80} -value mixture ratio by TU approach, respectively. The $\text{EC}_{50\text{mix}}$ values for the ternary mixture were 0.13 (0.00-0.28), 0.16 (0.061-0.26) and 0.17 (0.0048-0.34) based on EC_{20} -value, EC_{50} -value, and EC_{80} -value mixture ratio by TU approach, respectively.

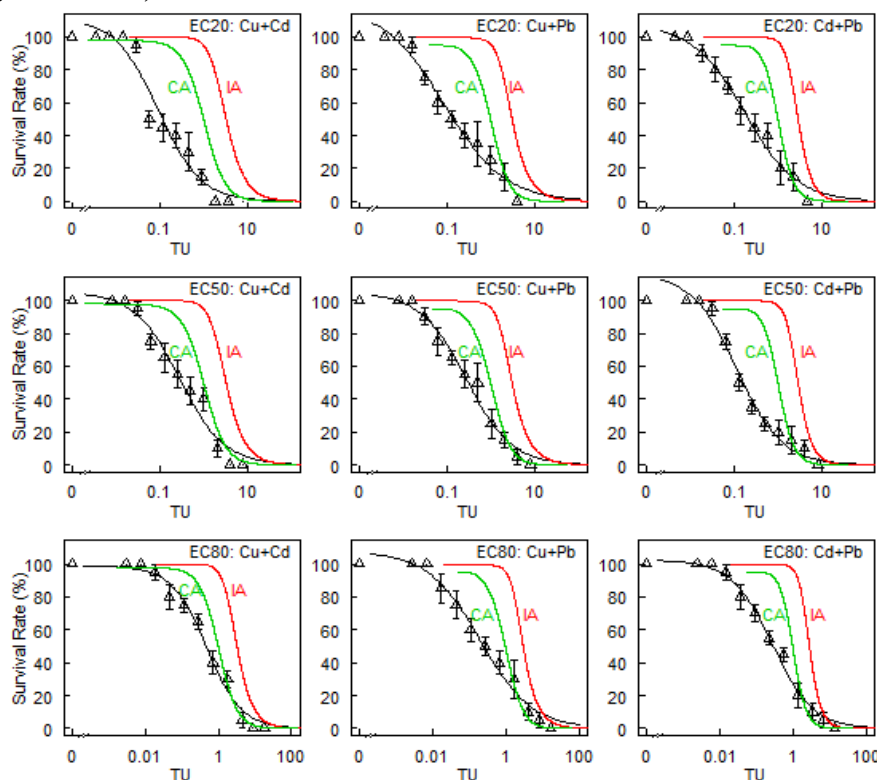


Figure 2: Predicted and observed effects of a mixture of all binary mixtures. Experimental effect data with standard error (solid circles, $n=4$) with the regression model (black solid sigmoidal line). The green and red solid lines show the predicted combination effects derived from CA and IA, respectively. Each point stands for the mean of four replicates with standard error ($n=4$) based on EC_{20} , EC_{50} or EC_{80} mixture ratios between every two metals.

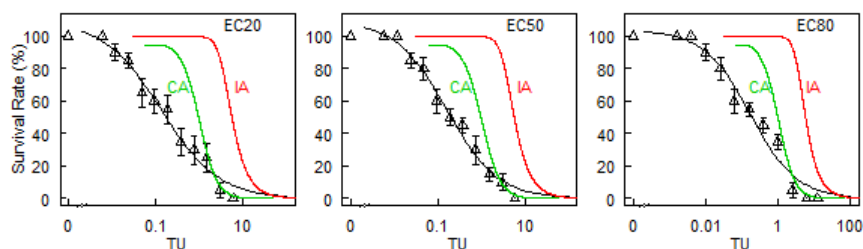


Figure 3: Predicted and observed effects of a mixture of all ternary mixtures. Experimental effect data with standard error (solid circles, $n=4$) with the regression model (black solid sigmoidal line). The green and red solid lines show the predicted combination effects derived from CA and IA, respectively. Each point stands the mean of four replicates with standard error ($n=4$) based on EC_{20} , EC_{50} or EC_{80} mixture ratio among three metals.

Table 3: The EC_{50mix} with 95% CI calculated through regression curves based on the TU approach from the three-parameter log-logistic curves.

Mixture ratio	Cu+Cd	Cu+Pb	Cd+Pb	Cu+Cd+Pb
EC_{20}	0.091(0.017-0.17)	0.084(0.0060-0.16)	0.17(0.075-0.27)	0.13(0.00-0.28)
EC_{50}	0.28(0.057-0.50)	0.27(0.11-0.43)	0.11(0.047-0.18)	0.16(0.061-0.26)
EC_{80}	0.48(0.24-0.71)	0.20(0.037-0.36)	0.27(0.16-0.39)	0.17(0.0048-0.34)

Discussion

In this study, the ranking order of three metals toxicity to survivability of *Daphnia magna* was $Cu > Cd > Pb$, which agrees with the findings of the previous study [32, 33]. However, some researchers indicated that the rank of toxicity might be different for different tested species (e.g., sea urchin, insert and shrimp) [34-38]. This also shows the different sensitivity of species to different toxicants, and the demands of at least three test species for WET testing in the United States are very reasonable and important for aquatic ecosystem safety.

The binary and ternary metals mixture toxicity with three different ratios (EC_{20} , EC_{50} and EC_{80}) of Cu, Cd and Pb to survivability of *Daphnia magna* was studied and was further predicted by CA and IA models based on the TU approach. Three-parameter log-logistic regression model could describe the experimental observations very well ($R^2 > 0.97$). Generally, CA and IA models are very useful tools and can be applied in most cases to predict mixture toxicity [25, 27]. However, lower toxicity was predicted by both models in all fixed ratios in this study, especially in the high effective area (low survival rate). It is likely that there may be a mechanism of synergism ($EC_{50mix} < 1$ TU) both in binary and ternary mixtures of Cu, Cd and Pb [39]. In addition, high mixture toxicity was observed at low ratios in most cases. That is maybe due to a different number of mixture components, concentration ratios and species [23, 40, 41]. According to previous studies, different acting mechanisms may be exhibited due to different types of exposure and target protein (acute vs chronic) [22, 42, 43].

When metals coexist in the environment, their interactions, including different metals and environmental factors, are very complex for the bioaccumulation processes in organisms and toxicological effects on different biological levels [14, 43, 44]. They may even show different interaction in some cases: concentration additive for a binary mixture of Cu and Cd on *Cucumis sativus*, synergistic responses for a binary mixture of Cu and Pb on *Cucumis sativus*, antagonistic responses for a binary mixture of Cd and Pb or a ternary mixture of Cu, Cd and Pb on *Cucumis sativus*, respectively, synergistic toxicological effects on Chinese cabbage [45, 46]. Interestingly, our results indicated that both the binary and ternary mixtures of the three metals showed a synergism

($EC_{50mix} < 1$ TU) on the survivability of *Daphnia magna*. As a matter of fact, the effects of mixtures are not only related to test species, mixture components, test duration, concentration ratios and concentration levels, but also endpoints [17, 40, 43, 44]. As a result, the discharge standards just based on chemicals in China may cause possible risk, which is the gap between real risk and thinkable risk.

Additionally, the single toxicity of the three investigated metals seems to be safe due to its lower environmentally relevant concentration than the EC_{20} , but the combined toxicity is considerable. Through the regression equation of ternary toxicity based on experimental data on EC_{20} -value mixture ratio, 46.02%, 74.59% and 63.38% of *Daphnia magna* would be affected according to the monitoring data of Yangtze River Basin, Upper Han River and Yellow River, respectively; based on experimental data on EC_{50} -value mixture ratio, 42.56%, 74.94% and 62.28% of *Daphnia magna* would be affected according to the monitoring data of Yangtze River Basin, Upper Han River and Yellow River, respectively; based on experimental data on EC_{80} -value mixture ratio, 44.66%, 73.44% and 61.91% of *Daphnia magna* would be affected according to the monitoring data of Yangtze River Basin, Upper Han River and Yellow River, respectively [11-13].

As the TU approach, however, only simply evaluation of the nature of the joint effects (antagonism, additive, synergism) is allowed, further additional characterization of combined effects has not been quantitatively described. For example, the detail mode of action, target organ/gene and toxicity pathway are not yet very clear. Moreover, CA model is generally considered suitable and proper for similar chemicals and IA model is believed to be applicable for dissimilar chemicals, but they actually both underestimate the toxicity of mixtures with synergism. In addition, the real exposed characteristics of the environment are frequently complex, including varied pH, dissolved oxygen, dissolved organic carbon and hardness, etc. [32, 47-51], which may affect the toxicity complicatedly. Therefore, it seems to be more reasonable to carry out the WET testing in the United States, especially for the industrial wastewater effluent into the aquatic environment, which is very significant for the safety of the ecosystem and sustainable development.

In spite of some disadvantages and uncertainties now, WET testing is a good complement to chemical analysis and model prediction in environmental management and risk assessment. With the emerging and maturity of on-line automatic monitoring devices of toxicity based on aquatic organisms (e.g., zebrafish and daphnia), WET testing would play a great role in risk management in the near future.

Conclusion

All binary and ternary toxicity of Cu, Cd and Pb showed a strongly synergetic effect on *Daphnia magna*, which offers an insight into the lack of risk management of mixture wastewater (e.g., industrial wastewater effluents) only based on chemical monitoring. It is strongly suggested the embarking of WET testing in China just as the development of its own national water quality criteria system.

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Conflicts of Interest

None.

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