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Assessment of toxic interactions of heavy metals in multi-component mixtures using sea urchin embryo-larval bioassay

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ABSTRACT

The toxicities of copper, lead, zinc and cadmium ions and various concentrations of mixtures of them were studied using sea urchin (*Strongylocentyotus intermedius*) embryo-larval bioassay. Toxic unit analysis was used to determine type of joint action for each mixture combination (binary, ternary and quaternary). For the majority of the binary combinations, the interactions were of synergistic nature, but in ternary or quaternary mixtures, the joint action was mainly concentration additive, while antagonism was only observed for two mixtures (Cu + Pb and Zn + Cd) among all the 11 combinations. Two prevailing theoretical models: the concentration addition (CA) model and the independent action (IA) model were used to predict the mixture toxicities. The weak correlation obtained ($R \simeq 0.55$) indicated that the hypotheses of mode of action involved in the two models to some extent failed to describe the behavior of the mixture system. Then a novel bio-concentration factor-based model was developed and was successful to predict the toxicities of mixtures, with an obtained R of 0.92. This model indicated that in a mixture system of heavy metals, the joint toxicity was mainly determined by the combined action of bio-concentrations of metals other than the simply similar (CA) or dissimilar (IA) modes of action of the mixture components.

1. Introduction

In recent years, industrial activities, vehicle emissions, agricultural activities and shipping traffics especially in and close to harbors have created serious risks to human health associated with exposure to heavy metals including copper, lead and, to a lesser extent, zinc and cadmium (Reddy et al., 2005). The environmental contamination caused by heavy metals has led to serious problems involving organic morphological abnormalities, neurophysiological disturbances, genetic alteration of cells (mutation), teratogenesis and carcinogenesis (Rainbow, 1995; Waalkes, 2003). Due to the detrimental effects of the heavy metals, increasing concerns have been raised about monitoring and researching the metal pollution in ecological environment (Balogh and Salanki, 1987).

Some toxic heavy metals including Cu, Pb, Zn, and Cd are well known to exist in high concentrations in many marine areas, such as Thames Estuary of UK Turkey (Attrill and Thomes, 1995), Atlantic coast of southwestern Spain (Morillo et al., 2004), Izmir Bay of western Turkey (Kucuksezgin et al., 2006) and Bohai Bay of China (Meng et al., 2008). For detecting the toxicity of the metal pollutants in marine waters, several living systems like fish (Hogstrand and Haux, 1990), nematodes (Bongers and Ferris, 1999), oyster (Rainbow, 1995) and sea urchin (Coteur et al., 2003) were applied as bio-monitors to follow metal contamination of the marine waters.

Since 1972, sea urchin embryo-larval bioassay has been recommended as a simple, sensitive and reliable tool for assessing marine pollution (Kobayashi, 1972), and this perspective has been repeatedly proved in recent 30 years (Bay et al., 1983; Marin et al., 2007). Our recent study has also demonstrated its efficiency in evaluation of the individual and combined toxicity of pesticides by sea urchin *Strongylocentrotus nudus* (Sun et al., 2009). In this work, the embryo-larval bioassay with the sea urchin *Strongylocentyotus intermedius* (*S. intermedius*) was applied to assess the joint-action toxicity of binary or multiple mixtures of heavy metal compounds.

As an echinoderm of the class Echinoidea, the *S. intermedius*, is widely distributed in Hokkaido, northern coast of Japan and Liaodong Peninsula and Shandong Peninsula of China. Most individuals are mature between 50 and 55 mm in diameter and sexual reproduction may happen in spring (from May to June) and autumn (from September to November). Application of this particular ecological indicator species to aquatic toxicology has not been reported too much, leading to a desirable necessity to measure its performance for risk assessment of toxic heavy metals in the environment.

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Up to now, the aquatic toxicology research using sea urchin has mainly focused on investigating individual heavy metal toxicity on fertilization, embryo-larval development, survival and growth (Bitton et al., 1994; Warnau and Pagano, 1994). Few data are available on the effects of contamination by joint-action of multiple heavy metals at cellular level, although some work has attempted to investigate the toxicity of heavy metals in binary mixtures against shrimp (Vanegas et al., 1997), earthworm (Weltje, 1998) and fish (Svecevičius, 2001). Hence, in this work, this typical marine invertebrate, *S. intermedius*, was selected to evaluate the joint toxicity of multiple heavy metals to the marine environment.

For further exploring the toxic mechanism of the joint action of heavy metal compounds, two theoretical models, i.e., concentration addition (CA) and independent action (IA) models were introduced in the present study (Parvez et al., 2009). CA model assumes that the individual toxicant acts upon a similar pharmacological system, and thus is effective for toxic substances that have the same molecular target site (Pöch, 1993). Alternatively, IA model supposes that all the components in a given mixture have dissimilar mode of action and interact with different target sites, resulting in a common toxicological end point depending on a distinct chain of reactions within an organism (Pöch, 1993). Based on these assumptions, the interaction of ingredients in a mixture might be explored to describe the toxic mechanism of the mixture (Backhaus et al., 2000).

However, information concerning the use of the two models has been reported in succession that both concepts generally failed to detect the subtle action elicited by mixtures of components, and were only partially suitable to describe the mixture toxicity (Bellas, 2008; Parvez et al., 2009; Schnell et al., 2009). Therefore in this work, another novel bio-concentration factor (BF)-based model was developed for the mixture system of heavy metals and applied to evaluate the combined toxic effects of metal contaminants on the early development stage of sea urchin.

This study was designed to provide basic information on utilizing the sea urchin embryo-larval bioassay for assessment of joint-action toxicity of heavy metals, which mainly included two parts: (1) evaluating of toxicities of the four metals, i.e., Cu, Pb, Zn and Cd and their binary, ternary and quaternary joint-actions to the embryonic and sperm development of sea urchin; and (2) investigating of mechanism of mixture toxicity by the three theoretical models, i.e., CA, IA, as well as the newly developed BF-based models, and comparisons of performance of the three models were also made in order to present an more effective way to describe the joint toxicity of heavy metals.

2. Materials and methods

2.1. Biological material

Experiments were carried out from June 20th to 30th, 2009. The mature *S. intermedius* (⩾5 cm in diameter) were collected from the *Heishijiao* coastal area (121°44′–121°49′ east longitude, 39°01′–39°04′ north latitude) and immediately transported to our laboratory. The sea urchins were maintained in the large aquaria of seawater (300 × 100 × 55 cm) and fed with fresh seaweed *Laminaria japonica* daily with flowing natural seawater filtered through 0.22 μM filters for 5 days until the experiments. Tank holding conditions were 8 ± 1 mg/L dissolved oxygen, salinity of 34 ± 1 ppt, pH of 7.9 ± 0.1, and temperature of 20 ± 1 °C in 53 h darkness.

2.2. Metal solutions

Analytical grade copper sulfate ($CuSO_4{\cdot}5H_2O$), lead nitrate ($(Pb(NO_3)_2)$), zinc chloride ($ZnCl_2$) and chromium chloride ($CdCl_2{\cdot}2\,1/2H_2O$) were bought from Riedel-de Haën, Sigma–Aldrich. Metal solutions were obtained by dissolving them in the double-distilled water within 4 h before the beginning of the experiments. And three control cultures were supplied with similar amounts of filtered seawater for the subsequently individual, combined toxicity tests of the embryos and spermiotoxicity tests.

2.3. Toxicity of the four heavy metals

For the embryonic toxicity tests, gametes of sea urchin were harvested by injecting 1.0 ml of 0.5 M potassium chloride (KCl) solution into the perivisceral cavity of one male and one female sea urchin for each experiment and hence fertilized the eggs in the solution by adding 1 ml of sperm stock (0.05 ml of concentrated sperm plus 2.45 ml of filtered sea water (FSW)) into 100 ml FSW. The eggs were washed three times in FSW in order to remove the jelly coat. Sterile polystyrene 24-well tissue culture plates (2.5 cm^3/well) with lids (TPP, Switzerland) were used as test chambers at a cell density of 200 embryos/mL (Nilin et al., 2008). All oocytes were checked three times for maturity, fertilization and embryonic development.

To evaluate the single toxicity of a metal, nine concentrations of each metal were prepared by diluting the stock solutions into FSW. The final concentrations were: 0.09, 0.31, 0.72, 1.3, 1.9, 2.2, 2.6, 2.9, and 3.1 μM for Cu, 0.58, 0.95, 1.5, 1.8, 2.9, 3.7, 3.2, 3.6, 4, and 4.8 μM for Pb, 0.2, 0.42, 1.09, 2.02, 2.5, 3.6, 6.9, 7.9, and 8.9 μM for Zn, and 2, 3.5, 7, 15, 22, 34, 36, 39, and 42 μM for Cd, which were determined in the pre-experiment. Then the 53-h acute toxicity studies were carried out in triplicate in parallel to each metal and the combinations. The larvae (at 4-arm pluteu stage) were fixed in 10% formalin and counted at the collection well, based on which the abnormal rates were calculated.

2.4. Spermiotoxicity test

To study the effect of Cu, Pb, Zn and Cd on the viability of sperm cells, 20 μl of diluted sperm at appropriate concentrations was added to each well in the 24-well tissue culture plates. The sperms were exposed to the metals at the concentrations as those for embryonic toxicity test for 30 min before being used for further experiments. Two milliliters of untreated eggs (about 200 eggs) were added to each 40 μl of metal-sperm solution in the culture plates and then were incubated at 22 ± 1 °C. After 20 min a few drops of 10% formalin were added to preserve the samples, and the fertilization success was scored by counting the number of the fertilized eggs. The fertilized eggs were checked under microscope for the fertilization membrane elevation.

2.5. Metal interactions

For the joint-action toxicity tests, stock solutions of each metal were mixed just prior to renewals to give desired concentrations. The analyses of the heavy metal interactions, i.e., Cu + Pb, Cu + Zn, Cu + Cd, Pb + Zn, Pb + Cd, Zn + Cd for the binary combinations, Cu + Pb + Zn, Cu + Pb + Cd, Cu + Zn + Cd, Pb + Zn + Cd for the ternary combinations, and Cu + Pb + Zn + Cd for the quaternary combination, were carried out using a toxic unit (TU) approach (van der Geest et al., 2000). Based on the 53 h EC_{50} values of the single heavy metals, toxicity of the mixtures was determined using a fixed ratio design. While keeping the mixture ratio constant, the total concentration was varied so that a complete concentration–response relationship of the mixture could be expressed experimentally. In the TU model, concentrations in the mixtures were expressed as TU. The sum of TU was shown as:

$$\sum \text{TU}_i = \sum_{i=1}^{n} \frac{c_i}{\text{EC}_{50i}} \quad (1)$$

where n was the number of the mixture components, c_i was the individual concentration of the ith substance in a mixture, and EC_{50i} was the effective concentration of ith mixture substance causing 50% toxic effect (53 h EC_{50}). The combined effect was then defined as being concentration additive (EC_{50mix} = 1 TU), synergistic (EC_{50mix} < 1 TU) or antagonistic (EC_{50mix} or >1 TU) (van der Geest et al., 2000).

2.6. Modeling toxicity of mixtures

Once the concentration–response curves for Cu, Pb, Zn and Cd have been established; the toxic effects of the binary, ternary and quaternary mixtures of the four heavy metals were subsequently predicted using the concentration additive (CA) and the independent addition (IA) models, respectively. In the CA model, the mixture toxicities were predicted based on the toxic data of mixtures independently derived from the dose–response profile of each substance. The predicted EC_{50mix} values were calculated according to Eq. (2):

$$\text{EC}_{50\text{mix,p}} = \frac{1}{\sum \text{TU}_i} \quad (2)$$

where $EC_{50mix,\ p}$ was the predicted EC_{50mix} of the mixture, TU_i denoted the toxic unit of the ith component, and $\sum TU_i$ was the summation of the toxic units.

Alternatively, the expected response ($EC_{50mix,\ p}$) of an n-compound mixture by the IA model could be obtained by Eq. (3),

$$\text{EC}_{50\text{mix,p}} = E(c1 + c2 + c3) = 1 - \prod_{i=1}^{n} [1 - E(c_i)] \quad (3)$$

where c_i was the concentration of the ith component and $E(c_i)$ was the effect generated by the ith component. Clearly in this equation, $E(c_i)$ did not exceed 1.

As a comparison with the above two widely used models, a novel bio-concentration factor-based model was developed in this work. Here the expected joint toxicity was calculated based on the bio-concentration factor (BCF) of each component in a mixture and the model was:

$$\text{EC}_{50\text{mix,p}} = \sum_{i=1}^{n} a_i \log \text{BCF}_i \quad (4)$$

where a_i was the weight of equation; BCF_i represented the bio-concentration factor of a metal in a mixture. The logBCF (base 10 logarithm) values were 3.10 for Cu, 3.49 for Pb, 2.73 for Zn and 1.64 for Cd (Radenac et al., 2001).

2.7. Statistical analyses

All statistical analyses were conducted using the R statistical software (http://www.r-project.org/). Differences between treatments were tested for significance by means of one-way analysis of variance (ANOVA). All experiments were replicated three times (n = 3) and data were expressed as mean ± standard deviation (SD). The EC_{50} and their 95% confidence intervals (95% CI) in acute toxicity assays were calculated according to the probit method (Bliss, 1935).

3. Results

3.1. Individual effects of Cu, Pb, Zn and Cd

Individual embryonic toxicity was observed for the four heavy metals on the embryos of sea urchin *S. intermedius* with "4-arm pluteu" rate of 85 ± 5% in the control culture, and the obtained EC_{50} for each metal was further used to calculate the TU for the joint toxicity of a mixture. All the obtained EC_{50} values are summarized in Table 1 and the dose–response curves of each metal are depicted in Figs. 1–3. The results showed that among the four metals, Cu was the most toxic, as judged by its EC_{50} value (1.32 μM), Cd revealed the lowest potential for the embryonic toxicity with an EC_{50} of 13.05 μM. And the EC_{50} values for Pb and Zn were 1.95 and 3.09 μM, respectively. Thus, the toxic effects of these heavy metals in sea urchin embryos decreased in the order: Cu > Pb > Zn > Cd.

For the spermiotoxicity with the successfully fertilized rate of 87 ± 3% in the control culture, Cu was still very toxic (EC_{50} = 6.40 μM), which displayed only a relatively modest increase of EC_{50} (fivefold), as compared with its embryonic toxicity. Compared to Cu, Pb (EC_{50} = 31.80 μM) was approximately fivefold less spermiotoxic, while Zn had a slightly stronger effect (EC_{50} = 5.55 μM). Among the four heavy metals, Cd posed the lowest toxic potential with an EC_{50} of 82.13 μM. Thus the ranking of the sperm toxicity was Zn > Cu > Pb > Cd based on the EC_{50} values. When compared with the EC_{50} data of the four metals between the above two different bioassays, an interesting finding was that the embryonic toxicity tests were revealed to be more sensitive than the spermiotoxicity for these heavy metals (Table 1).

3.2. Analysis of joint-action of mixture by TU

The "4-arm pluteu" rate was 83 ± 3% for the control group. For the binary mixtures, the obtained TU values (the upper and lower limits of the 95% confidence interval) for all the combinations: Cu + Pb, Cu + Zn, Cu + Cd, Pb + Zn, Pb + Cd and Zn + Cd were 1.19, 0.86, 0.93, 0.67, 0.74 and 2.18, respectively. The mixture Zn + Cd showed a strong antagonistic effect to the embryos since its TU value was significantly larger than 1, while the combination Cu + Pb exhibited a weak antagonistic effect revealed by the small increase in TU value compared to 1. The combination of Cu + Cd showed an additive effect on embryonic toxicity as its TU value approaching to 1. However, synergistic effects were found for the remaining three mixtures (Cu + Zn, Pb + Zn and Pb + Cd), since their TU values were all less than 1.

With respect to the ternary mixtures, the effects of the Cu + Pb + Zn and Cu + Zn + Cd were found to be synergistic, as their TU values were 0.68 and 0.89, respectively. And the other two mixtures Pb + Cd + Cu and Pb + Cd + Zn showed additive effects indicated by their respective TU values of 0.93 and 0.96. Interestingly, the quaternary mixture of Cu + Pb + Zn + Cd had a strictly additive effect on the embryonic toxicity as demonstrated by its TU value of 0.98, which was almost equal to 1. The three types of joint-action for all the mixtures are shown in Fig. 4 (antagonistic (An), addictive (Ad) and synergistic (S)).

Table 1
The median effective concentrations (EC_{50} μM) of embryonic toxicity and spermiotoxicity for *S. intermedius* exposed to Cu, Pb, Zn and Cd for 53 h.

	Cu	Pb	Zn	Cd
EC_{50} – embryo	1.32 (0.64–2.07)	1.95 (1.56–2.37)	3.09 (1.71–6.43)	13.05 (10.57–15.87)
EC_{50} – sperm	6.4 (6.07–6.75)	31.8 (27.29–61.52)	5.55 (2.68–14.38)	82.13 (55.90–149.90)

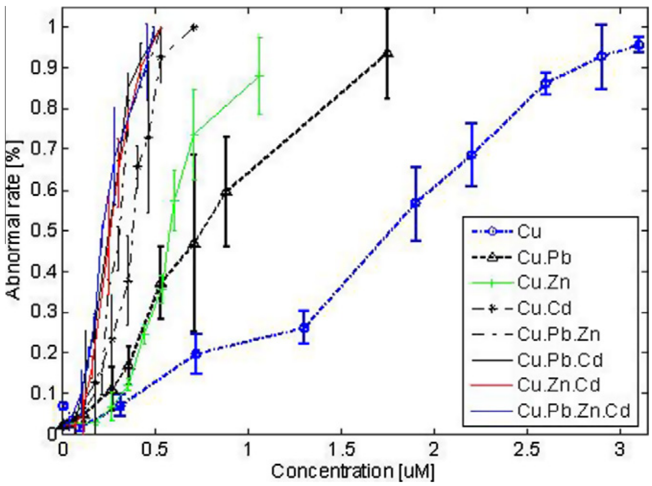

Fig. 1. Concentration–response curves for individual and mixture toxicity of Cu and compounds including Cu on embryos of sea urchin *S. intermedius*.

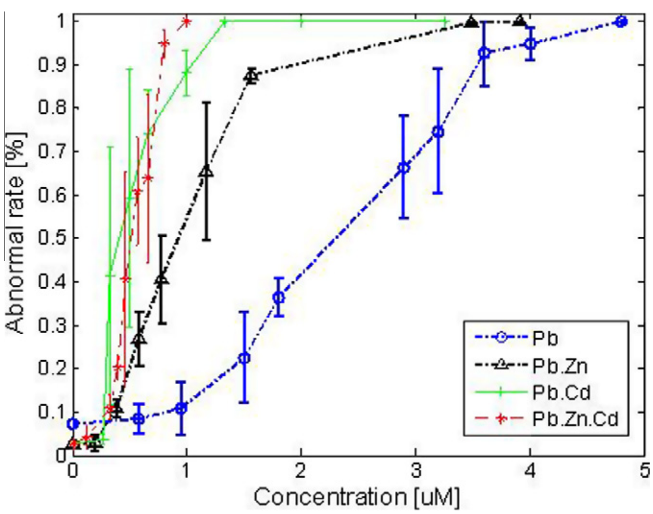

Fig. 2. Concentration–response curves for individual and mixture toxicity of Pb and compounds including Pb on embryos of sea urchin *S. intermedius*.

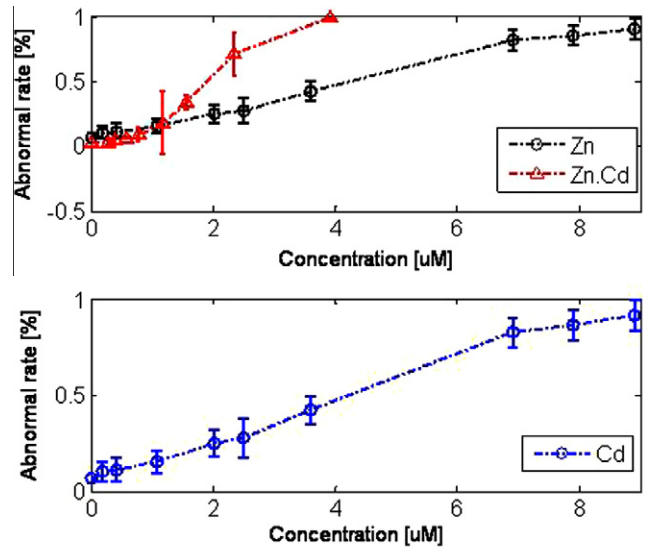

Fig. 3. Concentration–response curves for individual and mixture toxicity of Cd, Zn and Zn + Cd on embryos of sea urchin *S. intermedius*.

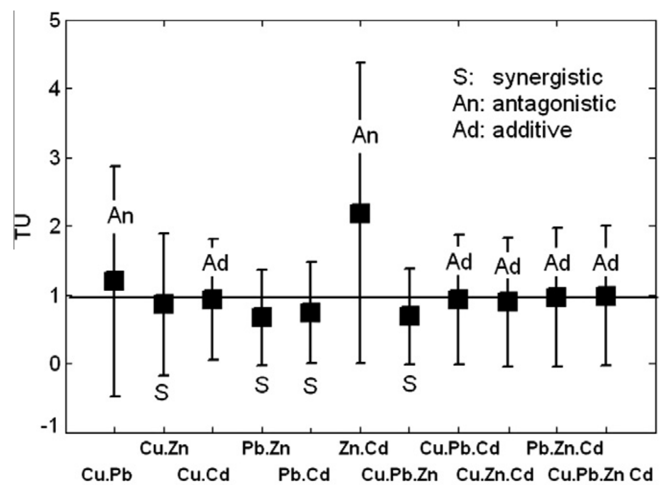

Fig. 4. TU values together with error bars of binary, ternary and quaternary mixtures of four heavy metals (Cu, Pb, Zn and Cd) on embryos of sea urchin *S. intermedius*.

3.3. Prediction of toxicity by theoretical models

The toxic effects of all mixtures on the embryos were predicted by using the three theoretical models: CA, IA and BF models, and the results are shown in Fig. 5. Based on the CA model (Fig. 5A), a relatively poor correlation ($R = 0.52$) was found between the predicted and measured EC_{50mix} of the mixtures. Fig. 5B shows the results of the IA model and a slightly better correlation ($R = 0.59$) was obtained.

In order to accurately predict the mixture toxicity, we proposed a novel model, i.e., the BF model to predict the toxicity of the mixtures. Eqs. (5)–(7) depicted relationships between the logBCF vs. predicted toxicity for the individual and mixtures of the four heavy metals, and significant correlations were observed for all cases with all the R values >0.90 and standard error less than 0.20

$$EC_{50mix,1} = -0.945 \times \log BCF_1 \quad (n = 1,\ R = 0.95) \tag{5}$$

$$EC_{50mix,2} = -0.611 \times \log BCF_1 - 0.614 \times \log BCF_2 \quad (n = 2,\ R = 0.92) \tag{6}$$

$$EC_{50mix,3} = -0.016 \times \log BCF_1 - 0.047 \times \log BCF_2 - 0.967 \times \log BCF_3 \quad (n = 2,\ R = 0.92) \tag{7}$$

where n was the number of the metal species in a mixture. No model was developed for the quaternary mixture ($n = 4$), since the least squares regression method could not be used for such a case with only one input sample. For building each BF model, the arrangement of $\log BCF_i$ in an equation was followed by the magnitude of the bio-concentration property of a heavy metal. In order to make it clear, an example for building the model for a binary combination like Cu + Pb was presented here. In this case, the $\log BCF_1$ was logBCF for Cu and the $\log BCF_2$ for Pb, since Cu was more bio-accumulative than Pb.

4. Discussion

The present study investigated the individual and joint action of the four toxic metals (Cu, Pb, Zn and Cd) frequently existing in the contaminated areas on the early developmental stage of the sea

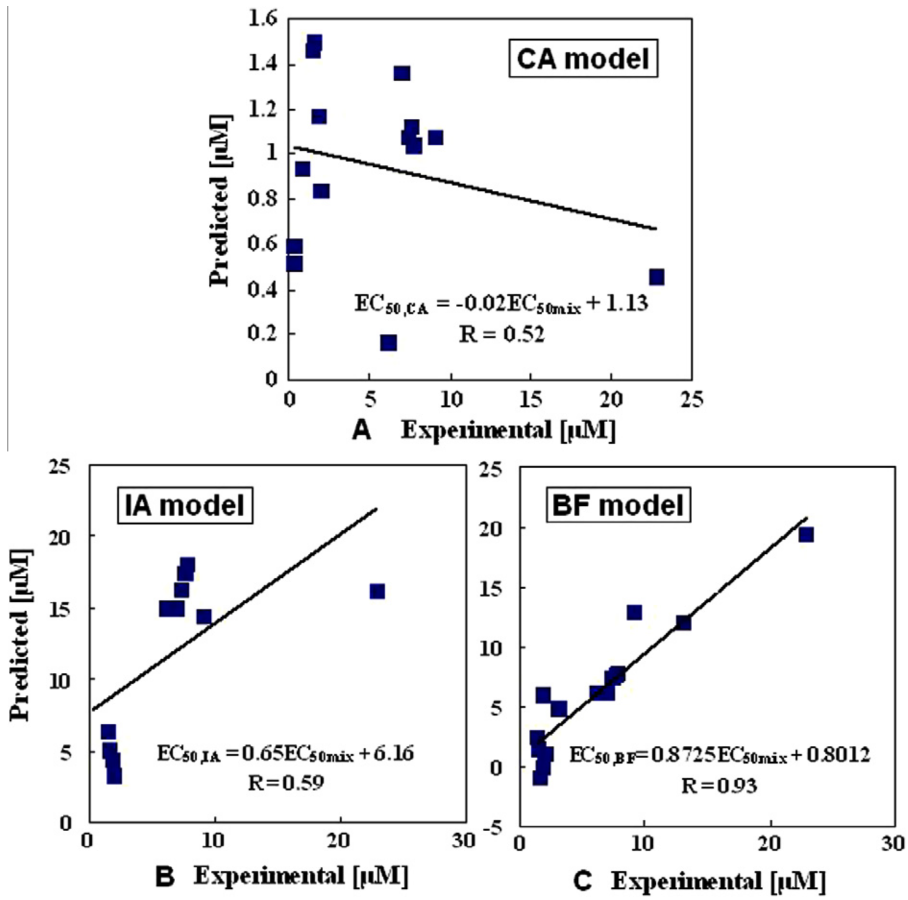

Fig. 5. Correlations between the measured and predicted EC_{50mix} values by CA, IA and BF model.

Table 2
Median effective concentration (EC_{50}) data of heavy metals (Cu, Pb, Zn, and Cd) for the embryonic toxicity from literature and this work.

Heavy metal	Species	Test duration and end-point	Embryonic toxicity EC_{50}	Reference
Cu	*S. purpuratus*	48 h	0.09	Jop (1989)
	P. lividus	48 h	1.1	Fernández and Beiras (2001)
	S. internedius	53 h	1.32	This work
Pb	*S. purpuratus*	48 h	1.13	Jop (1989)
	P. lividus	48 h	2.5	Fernández and Beiras (2001)
	S. internedius	53 h	1.95	This work
Zn	*S. purpuratus*	48 h	0.35	Jop, 1989
	S. internedius	53 h	3.09	This work
Cd	*S. purpuratus*	48 h	4.55	Jop (1989)
	P. lividus	48 h	82	Fernández and Beiras (2001)
	S. internedius	53 h	13.05	This work

urchin *S. intermedius*. In addition, different theoretical models, i.e., CA, IA and BF, were also applied on the data to explore the possible mechanism of the interactions of the heavy metals.

4.1. Individual toxicity

The individual toxicity of the four metals to different marine invertebrates has been investigated previously (Table 2), and in general, the data obtained for the embryonic toxicity and spermiotoxicity of the sea urchin fell within the range of the published data for different sea urchin species.

The EC_{50} value (1.32 μM) of Cu in this study was similar to the data for *S. purpuratus* (0.09 μM) (Jop, 1989) and *P. lividus* (1.1 μM) (Fernández and Beiras, 2001). Pb also exhibited similarly toxic effect with other sea urchin species, e.g., 1.13 μM for *S. purpuratus* (Jop, 1989). The effect of Zn on the embryos of *S. intermedius* was seven times less sensitive than that on *S. purpuratus* (Jop, 1989). The toxicity of Cd in this work was comparable to that on *S. purpuratus* (Jop, 1989) but was six times more toxic than that on *P. lividus* (Fernández and Beiras, 2001). These comparisons showed that, the three sea urchin species: *S. purpuratus*, *P. lividus* and *S. internedius*, might have similar sensitivity to certain toxic metals such as Cu, or different sensitivity to metals like Zn to a very large extent, indicating that carefulness should be taken while using these models to assess the risks of heavy metal contaminants. Additionally, the toxicity of the four heavy metals to sperms was approximately sixfold less sensitive than that to the embryos of sea urchin, showing that the sea urchin sperms were less sensitive to the heavy metals, which was also supported by the finding for another sea urchin species (Bellas et al., 2001).

4.2. Toxic effects of mixtures

Based on the TU analysis, the joint action of the mixture Cu + Pb was antagonistic to the embryos of *S. intermedius*, which might be explained by the fact that Cu could decrease the absorption of the mixture thus leading to reduction of the toxicity of the mixture (Alimohamadi et al., 2005). In addition, the antagonistic effect was also observed for the mixture Zn + Cd. It has been suggested that Cd could induce oxidative damage (Hassoun and Stohs, 1996) by causing the intercellular accumulation of reactive oxygen species (ROS), like. O_2^- and NO, thus increasing the sensitivity of cell to toxicants (Chowdhury et al., 1987; Brzo'ska et al., 2008). The synergism of the mixture Cu + Zn was evident as the redox-active metal Cu could enhance the zinc absorption in a living system (Flemming and Trevors, 1989; Stauber and Florence, 1990). And this combination of Cu + Zn might also play a main role in the two ternary mixtures Cu + Zn + Pb and Cu + Zn + Cd, since synergistic interactions were found for them. In addition, we also found an interesting phenomenon that most ternary and quaternary combinations (4/5) were concentration additive, particularly for the combination of Cu + Pb + Zn + Cd, showing a strictly additive effect on embryonic toxicity (TU = 0.98). This might indicate that the more the number of components included in a mixture, the less the joint effect of interaction of components could exert, thus leading to an average behavior of the whole components in the mixture.

4.3. Prediction of toxicity

The CA and IA models were proposed for the purpose of the prediction of toxicity of a mixture based on the concentration–response curve of the individual component in the mixture. The CA model assumes that the mixture components act in a similar way and bind to a commonly active site while IA model proposes that the components act on different mechanisms and involve dissimilar site of action (Pöch, 1993). Some work has found that the CA model overestimated the combined toxicity of mixtures (Niederlehner et al., 1998; Faust et al., 2003), which also supported our results as all the binary, ternary and quaternary mixtures were overestimated.

The poor correlation between the observed and predicted toxicity might be because that the four metals possessed different action mechanism on organisms. As we know, copper acts as a cofactor for over 30 enzymes in biology, all of which are redox catalysts (e.g., cytochrome oxidase, nitrate reductase) or dioxygen carriers (e.g., haemocyanin) (Flemming and Trevors, 1989). Zinc plays as a structural role in a large number of zinc finger proteins (Salgueiro et al., 2000). While Cd and Pb are non-essential metals, leading to toxicity for organisms mainly caused by high environmental persistence and bio-accumulation potential (Satarug et al., 2003). Clearly, the different action modes of the heavy metals would lead to the failure of performance of the CA in the heavy metal mixture system (Backhaus et al., 2000; Overmyer et al., 2010). Therefore, it was no doubt that special attention must be paid to use this CA to predict mixture toxicity.

In addition, from the analysis of CA-derived equation ($EC_{50,CA} = -0.02EC_{50mix} + 1.13$, Fig. 5A), we could find that the predicted mixture toxicity ($EC_{50,CA}$) decreased with the increasing of the experimental EC_{50mix}. The unreasonable performance revealed by the negative correlation between the predicted and experimental toxicity also demonstrated that the application of CA model in metal mixtures should be careful. Interestingly, the IA model presented a slightly better correlation ($R = 0.58$) compared to that by the CA model, which might also support that the four heavy metals have different toxic mechanisms. The obtained equation ($EC_{50,IA} = 0.65EC_{50mix} + 6.16$, Fig. 5B) indicated that the predicted values ($EC_{50,IA}$) increased following with the increasing of the experimental toxicities (EC_{50mix}). This was quite rational since the positive correlation was found between the predicted and observed EC_{50} values.

But in general, both CA and IA models were not valid enough to predict the mixture toxicity based on the single metal toxicity data, and were also not capable of deeply elucidating the toxicological mechanism of the mixtures. The reason for the inaccurate prediction by the two models might be due to the neglect of the complexity of biological system and the different action mechanisms of heavy metals as mentioned above (Flemming and Trevors, 1989; Salgueiro et al., 2000; Satarug et al., 2003).

Over the last two decades, the toxicity of a chemical has been found to be closely related to its bio-accumulation property in organisms (Radenac et al., 2001; Wang et al., 2008; Sun et al., 2008). The bio-accumulation/bio-concentration could be expressed in terms of bio-concentration factor (BCF) defined as the concentration ratio of a chemical in an organism to the chemical concentration in water. Therefore, BCF has been used as an easy tool to describe a compound with bio-concentration property and its toxic effect. The present study attempted to establish a new model to predict the mixture toxicity of the heavy metals by means of the BCF. And a good relationship between the logBCFs and EC_{50mix} of heavy metals was obtained for all the mixtures.

From the analysis of Eq. (5), we could find that the mixture toxicity decreased with the increasing of the logBCF values. It was reasonable because for the metal with large BCF value it should be more easily accumulated and thus more toxic. For example, Cu had a logBCF of 3.1, which was more toxic than Cd (logBCF = 1.64) as shown in this work. The similar weights (0.611 for BCF_1 vs. 0.614 for BCF_2) in the Eq. (6) also presented interesting indication that the two groups (more toxic vs. less toxic) of metals contributed equally to the toxicity of a binary mixture. Thus the interaction of components in the binary mixture tended to be concentration addictive. Regarding with the ternary mixture, Eq. (7) showed that the $\log BCF_3$ was the most important factor contributing to the toxicity of the combined metals, $\log BCF_2$ the second, and $\log BCF_1$ the third. Therefore, we might conclude that less toxic metals (Zn and Cd) contributed more to the toxicity of mixture than those more toxic ones (Cu and Pb) did. The BF models have presented us with important insights into the mechanism of mixture toxicities why they were affected by the bio-concentration property of each component in a mixture, which showed priority to the conventional methods including CA and IA models in modeling mixture system.

5. Conclusion

In the present study, the individual, binary, ternary and quaternary mixture toxicity of four heavy metals (Cu, Pb, Zn and Cd) on embryos of sea urchin *S.intermedius* was determined. Toxic unit analysis showed that in most of the binary combinations, the interactions were synergistic, but in the ternary or quaternary mixtures, the joint action was mainly concentration additive, while antagonism was only observed for two mixtures (Cu + Pb and Zn + Cd) among all the 11 combinations. Therefore the joint effects of heavy metals should be taken into account in the risk assessment of heavy metal pollution in the aquatic environment.

To some extent, the widely used concentration addition model and independent action model failed in predicting the mixture toxicity of heavy metals. As an alternative to the two models, a bio-concentration-based model was proposed, which was successfully evaluated the combined toxicities of the heavy metals. This BF model also explained that the mixture toxicity was mainly determined by the joint-bio-concentration property of heavy metals in a mixture.

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