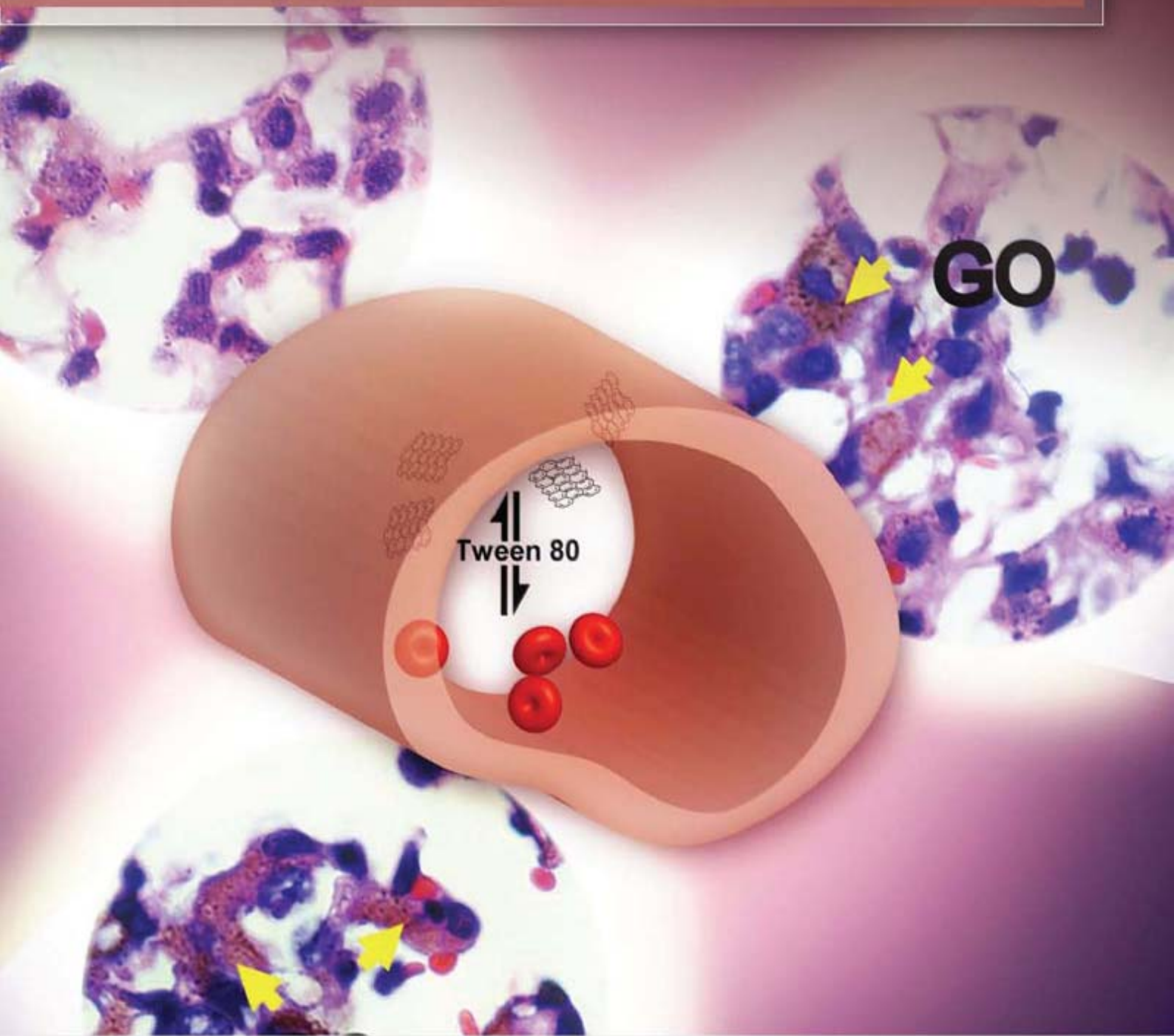


JES

JOURNAL OF
ENVIRONMENTAL
SCIENCES

ISSN 1001-0742
CN 11-2629/X

May 1, 2013 Volume 25 Number 5
www.jesc.ac.cn



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Joint effects of heavy metal binary mixtures on seed germination, root and shoot growth, bacterial bioluminescence, and gene mutation

In Chul Kong

Department of Environmental Engineering, Yeungnam University, Kyungbuk 712-749, Korea. E-mail: ickong@ynu.ac.kr

Received 18 September 2012; revised 12 November 2012; accepted 24 January 2013

Abstract

This investigation was to assess the joint effects of metal binary mixtures on seed germination, root and shoot growth, bacterial bioluminescence, and gene mutation based on the one toxic unit (1 TU) approach. Different sensitivities and orders of toxicity of metal mixtures were observed among the bioassays. In general, mostly additive or antagonistic effects were observed, while almost no synergistic effects by the binary metal mixtures in all bioassays. Therefore, the combined effects of heavy metals in the different bioassays were difficult to generalize since they were dependent on both chemical type and the organism used in each bioassay. However, these results indicate that a battery of bioassays with mixture chemicals as opposed to just a single assay with single metal is a better strategy for the bioassessment of environmental pollutants.

Key words: binary mixture; bioluminescence; heavy metal; revertant mutation; seed germination; toxicity

DOI: 10.1016/S1001-0742(12)60174-0

Introduction

Heavy metals are common non-biodegradable pollutants reported at elevated concentrations in many parts of the world (Horvat et al., 2007). Due to their non-biodegradability and high toxicity, heavy metals are considered to be one of the most resistant pollutants affecting environments. For example, arsenic (metalloid), which emanates from several industrial activities, is one of the most toxic and common pollutants found in soils (Ravenscroft et al., 2009; Yi et al., 2007). The majority of risk assessment and ecotoxicological studies involving metals have mainly focused on the toxicity of single chemicals under controlled conditions (Pavlaki et al., 2011). However, the natural environment is continuously exposed to complex mixtures of contaminants. Therefore, studies on the combined effects of mixtures more realistically reflect pollution of ecosystems compared to those in which toxicants are tested individually and could assist in identifying ecologically relevant criteria. Unfortunately, predicting the response of organisms simultaneously exposed to more than one potentially toxic chemical is one of the most difficult tasks in environmental toxicology and risk assessment (Norwood et al., 2003). To solve this, mixture models can be broadly classified into two basic types: concentration addition models and response (ef-

fects) addition models. The theoretically expected effects of binary mixtures on test organisms can be evaluated using a simple mathematical model based on the theory of probabilities (Kungolos et al., 2009) or by using the toxic unit (TU) approach (Horvat et al., 2007), which is mostly used to examine mixtures (An et al., 2004; van der Geest et al., 2000). Despite the limitations of the TU approach, it is useful as a means of comparing the likely relative toxicity induced by heavy metals and for understanding the major contributors to adverse effects. The mixture effect is thus defined as being either concentration additive or as greater (synergistic) or less (antagonistic) than additive based on the effects of sole and chemical mixtures at the same TU.

Use of bioassays for ecotoxicity evaluation of contaminated environments has gained widespread attention over the past two decades (Banks and Schultz, 2005). There are various test species used to examine the toxicity of chemicals, including bacteria, algae, protozoa, plants, and fish (US EPA, 1993). In addition to using whole organisms, several key metabolic processes of organisms, specifically enzyme activity, enzyme biosynthesis, and bioluminescence, have been used to assess toxicity in environmental systems (Bitton, 1999). Since metals are important global environmental contaminants, it is important to choose appropriate bioassay species as well as end points for any risk assessment. However, just one bioassay cannot

provide a true estimation of chemical toxicity since no single bioassay shows uniform toxicity and sensitivity to all pollutants. Therefore, the ranking of metals according to individual toxicity level is of limited benefit unless it can be correlated with other toxicity data.

There have been several types of toxicity studies performed involving plant processes in environmental biomonitoring (Di Salvatore et al., 2008; Wang, 1991). Plant toxicity assays are particularly relevant when phytotoxic contaminants are present in soil (Boutin et al., 2004). Germination studies primarily assess acute toxic effects and are considered to be short-term, while long-term studies designed to evaluate plant biomass and root elongation over a 2–8 week period can assess both acute and chronic toxicities (Wang and Liu, 2001). The Ames test is a short-term bacterial reverse mutation assay specifically designed to detect a wide range of chemical substances capable of inducing genetic damage and gene mutation (Mortelmans and Zeiger, 2000). This specific test utilizes a number of mutant *Salmonella typhimurium* strains that are unable to synthesize histidine, which is required for growth. Possible gene mutation upon chemical exposure is observed based on a reverse mutation ($his^- \rightarrow his^+$). Further, bioluminescence assay is a time-saving and cost-effective test that is widely used as a reproducible and sensitive screening method for determining the acute toxicities of different sample types (Wang et al., 2002).

The purpose of this investigation was to assess the joint effects of heavy metal binary mixtures based on the results of a battery of three bioassays focusing on seed germination and growth, bacterial bioluminescence, and gene mutation.

1 Materials and methods

1.1 Metal toxicity with bioluminescence bioassay

Acute toxicities of samples were determined using a bioluminescent mutant strain, *Escherichia coli* DH5 RB1436. This mutant contained a spontaneously deleted pUCD615 plasmid, which resulted in the translocation of a constitutive promoter of the plasmid to the proximity of the *lux*-genes (Kong et al., 2007). This *E. coli* strain (obtained from Dr. R. S. Burlage of the University of Milwaukee, USA) has the ability to release luminescent light during its growth phase. Light output of this strain decreases (inhibited) in proportion to the level of toxicity in solution.

Strains were stored at -70°C until needed, at which time they were grown overnight in Luria-Bertani^{ka} (LB^{ka}) medium (tryptone 10 g, yeast extract 5 g, NaCl 5 g, 2 mol/L NaOH 0.5 mL, kanamycin 50 mg/L) at 27°C with shaking (130 r/min). The strains were then diluted 1:30 into LB^{ka} medium and allowed to grow until the optical density (OD_{600}) was approximately 0.6. This culture was appropriately diluted with minimum salt medium ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2 g, CaCl_2 0.1 g, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05 mg,

$\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ 0.05 mg, K_2HPO_4 0.43 g, KH_2PO_4 0.23 g), with the final density for the toxicity test at $\text{OD}_{600} = 0.2$. For the bioassay, 1 mL of the bacterial strain was mixed with 9 mL of sample and incubated. An average of two measurements (1.0 and 1.5 hr) was used as the toxicity result. Bioluminescence was measured using a Turner 20/20 luminometer (Turner Design Inc., USA), where the maximum detection limits was 9999 relative light units (RLU).

1.2 Metal toxicity with seed germination assay

Seeds of lettuce (*Lactuca sativa* L.) were selected as recommended by many previous methods as well as due to its importance as a food crop. Prior to the germination test, all seeds were surface-sterilized in 3% H_2O_2 and then rinsed with distilled water. Filter paper was placed on a Petri dish and moistened with 5 mL of the metal solution. Controls were maintained by moistening the filter paper with 5 mL of distilled water. Twenty seeds of each species were then placed on a dish, which was covered by a lid and incubated in the dark at $(23 \pm 2)^\circ\text{C}$. Germinated seeds were counted after 3 days of incubation. When both the plumule and radical extended more than 2 cm from their junctions, germination was confirmed. Triplicate sets were performed for each treatment.

1.3 Metal toxicity with gene mutation bioassay

Mutagenicity test was performed using *Salmonella typhimurium* TA 98 by following the plate incorporation procedure as described by Maron and Ames (1983). Strains were maintained and stored according to standard methods (Mortelmans and Zeiger, 2000). A 0.05 mL sample of DMSO was plated onto a GM agar plate (minimal glucose agar plate with ampicillin 24 $\mu\text{g}/\text{mL}$: VB salts (Vogel-Bonner) medium (50 \times) ($\text{MgSO}_4 \cdot \text{H}_2\text{O}$ 10 g, citric acid monohydrate 100 g, K_2HPO_4 500 g, $\text{Na}_2\text{NH}_2\text{PO}_4 \cdot 4\text{H}_2\text{O}$ 175 g per water 650 mL) containing a mixture of 2 mL of top agar (NaCl 6 g, agar 6 g, 0.5 mmol/L histidine/biotin solution 100 mL, water 900 mL), 0.1 mL of culture, and 0.5 mL of buffer. Each sample was plated in triplicate, and his^+ revertants were counted after 48 hr of incubation at 37°C . The positive control used for TA 98 was 2-nitrofluorene (2.5 $\mu\text{g}/\text{plate}$) in order to monitor sensitivity of the bacterial strain.

1.4 Toxicity evaluation of mixture metals

Prior to toxicity evaluation of the binary mixtures, EC_{50} values of all single metals in each bioassay were determined using the Spearman computer program distributed by the US EPA's Center for Exposure Assessment Modeling (CEAM) (US EPA, 1999; An, 2004). In the mixture toxicity test, the toxic unit (TU = concentrations/ EC_{50}) approach was used, where 1 TU is equal to the EC_{50} concentration of each metal. All tests were performed using 1 TU of the binary equal mixtures. In the mutagenicity test,

a concentration range from 1 to 500 mg/L (corresponds to 0.1 to 50 $\mu\text{g}/\text{plate}$) was adopted for both tests of single and binary mixture metals.

2 Results and discussion

Studying the toxic effects of a single metal in a single bioassay is of limited benefit unless the results can be correlated with other toxicity data on metal mixtures. No single bioassay shows uniform toxicity and sensitivity to pollutants. Therefore, studies on the combined effects of mixtures more realistically reflect pollution of ecosystems compared to those in which toxicants are tested individually. In the present study, the toxicities of binary metal mixtures of arsenite, chromate, cadmium, and copper were assessed in a battery of different bioassays. Prior to the mixture investigation, the approximate concentration of 1 TU of each metal, which corresponds to the EC_{50} value, was determined. Of the EC_{50} values, the toxicity ranking of metals varied depending on the bioassay (Ko et al., 2012). The EC_{50} concentration of each metal was used as 1 TU for investigating the combined effects of the metal mixtures. With respect to the toxicity of sole metals, bioassays did not show consistent results. However, arsenite was the most inhibitory in the seed germination and bioluminescence tests, and the entire tested range of arsenite showed a mutagenicity ratio higher than 2.0, indicating positive mutagenicity. To investigate the effects of the binary mixture, two metals each at this concentration were mixed equally, resulting in a 1 TU mixture solution. In case of the mixture effects on mutation, both single and metal mixtures were tested at concentrations in the range of 1–500 mg/L.

2.1 Mixture effects on bioluminescence activity

Of the tested conditions, representative results of the bioluminescence activities of the metal mixtures are shown in Fig. 1. In general, slight rapid inhibition was observed during the first 1 hr of exposure. Control (no metal amendment) produced a mean bioluminescence of approximately

(736 \pm 103.7) RLU after 1 and 1.5 hr of incubation, whereas 1 TU of As(V), As(III), and the binary mixture of these two metals showed inhibitory activities with bioluminescence levels of (318 \pm 63.8), (292 \pm 59.8), and (313 \pm 61.8) RLU, respectively. Further, 1 TU of Cu, Cd, and the binary mixture of these two metals showed inhibitory activities with bioluminescence levels of (246 \pm 64.5), (448 \pm 144.4), and (490 \pm 151.8) RLU, respectively. The arsenic mixtures exhibited higher inhibitory activities compared to sole metals, whereas the Cu and Cd binary mixture showed lower inhibitory activity.

Therefore, the observed effects of the binary equal mixtures on a test organism produced different types of effects, such as antagonistic (less than additive), synergistic (greater than additive), and additive effects. The observed bioluminescence toxicity of the binary mixture was in the range of 31%–53% of control (no metal amendment), whereas expected bioluminescence toxicity was in the range of 43%–52% of control. Expected mixture effects were determined based on the concentration addition model, where the concentrations of all toxic constituents of the binary mixture are added together to predict toxicity (Norwood et al., 2003). Comparisons between expected and observed effects are shown in Fig. 2. Statistically significant differences were not observed in most cases (p -values ranging from 0.212 to 1.0), indicating additive effects of the binary mixtures. Although there were no statistically significant differences among the tested conditions, there is a trend that the mean observed toxicities of all binary mixtures of Cu showed lower (1.30–1.45 times) than those its expected activity due to an antagonism mechanism. This antagonism phenomenon (less additive) could be attributed to the formation of less bioavailable metal complexes (An, 2004). Based on these test results, all combinations are close to additive type effects.

2.2 Toxicity on seed germination and root and shoot growth

Preliminary experiments were conducted to determine the appropriate seed and sensitivity ranges for the tested

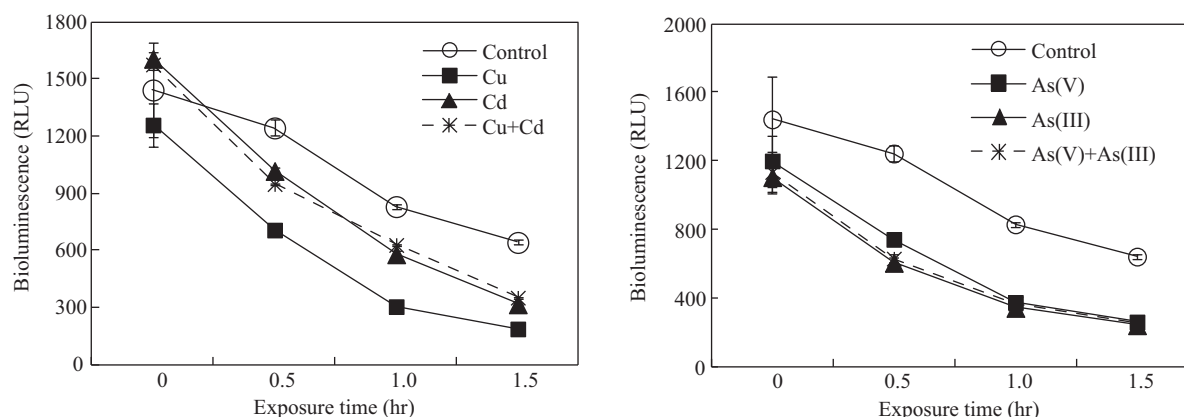


Fig. 1 Representative results of bioluminescence activity in the presence of no metal, single metals, and binary mixture metals.

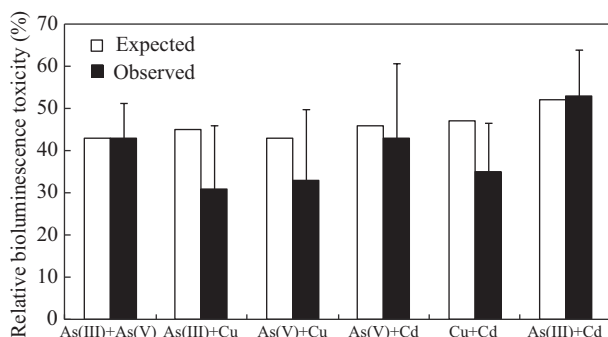


Fig. 2 Toxicity of binary mixtures of Cu, Cd, As(III), and As(V) on bioluminescence activity of RB1436.

plant species. Among the four tested species (*Lactuca*, *Cardamine*, *Raphanus*, and *Cucumis*), the sensitive species *Lactuca* was chosen for detailed investigation. Sensitive plant species can serve as indicators for assessing the ecotoxicity of soils since they respond rapidly to the toxic effects of pollutants. Seed germination relies almost exclusively on seed reserves for a supply of metabolites for respiration as well as other anabolic reactions (Liu et al., 2005). Starch is quantitatively the most abundant storage material in seeds and is degraded by various enzymes such as amylase for germination. Therefore, the inhibition of specific enzymatic reactions by metals is one of the main mechanisms behind metal toxicity.

Upon no metal treatment (control), an average of (16 ± 3) seeds per batch of 20 seeds was germinated (greater than 2 cm growth) during the 3-day incubation period. Unlike the bioluminescence test showing a wide range of sensitivities, all EC_{50} values for each metal were approximately in the range from 1–5 mg/L. In general, binary metal mixtures showed two types of interactions as shown in **Fig. 3**: additive and antagonistic (less than additive, more germination and less toxic) responses. Based on statistical differences, nine (toxicity 15%–46% of control; p -value 0.0001–0.0464) out of ten sets showed an antagonistic mode of action and one additive mode (toxicity $63\% \pm 26.2\%$ of the control; p -value 0.499). The most pronounced antagonistic effects on seed germination were seen in the binary mixture of As(III) and Cd, which showed 85% relative germination (15% toxicity). This antagonism phenomenon (less additive) could be attributed to the

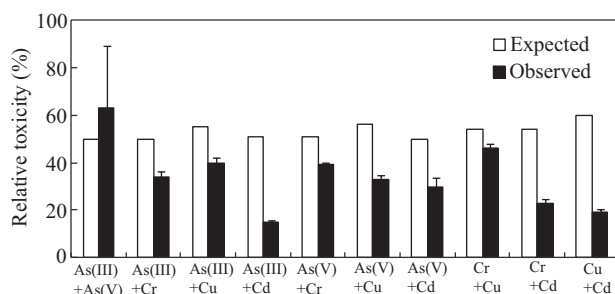


Fig. 3 Comparison of relative toxicities of binary mixtures of Cu, Cd, As(III), and As(V) on seed germination of *Lactuca*.

interactions among the metals, resulting the formation of less bioavailable metal complexes. Some studies also reported that one metal in mixture influenced uptake of other metal (Peralta-Videa et al., 2002). The toxicity of As(III) is related to its high affinity for the sulfhydryl groups of biomolecules such as glutathione (GSH) and lipoic acid as well as the cysteinyl residues of many enzymes (Aposhian and Aposhian, 2006). Formation of As(III)-sulfur bonds mediates various harmful effects by inhibiting the activities of enzymes such as glutathione reductase, glutathione peroxidases, thioredoxin reductase, and thioredoxin peroxidase (Sharma and Sohn, 2009). Shri et al. (2009) reported that induction of oxidative stress is the main process underlying arsenic toxicity in plants. Like the bioluminescence test, all combinations with Cu also showed lower toxicity than expected. As many reports have shown that seed germination is differentially affected according to the type of seed or metal, there is little agreement on the subject of metal mixture toxicity (Kraak et al., 1994). Studies also reported that the presence of one metal influenced uptake of other metal (Peralta-Videa et al., 2002).

Other vegetative response endpoints, such as root and shoot growth, were examined to investigate binary metal toxicity. Significantly different effects on root growth were observed compared to other endpoints (**Fig. 4**). Average toxicity to root ($71\% \pm 21.3\%$) was nearly two times higher than that to endpoints of shoot or seed germination ($30\% \pm 15.2\%$ and $34\% \pm 13.4\%$, respectively). Differences in mixture toxicity might be attributed to the mobility of metals from roots to shoots and direct contact with root surface. Heavy metals tends to retain in the root tissues, therefore, the mixture effects are generally greater in roots than in shoots (An et al., 2004). Liu et al. (2007) reported the degree of inhibition as following orders: root length > shoot height > biomass > germination frequency. Different mixture toxicity may be closely related to the bioaccumulation pattern within plants (An et al., 2004). In most cases, no significant differences were observed between either expected or observed toxicity of root and shoot, indicating an additive response. However, considerable significant antagonistic results for shoot were observed using the As(III) and Cd and As(V) and Cd binary mixtures, which showed 15% and 7% expected toxicity, respectively. Therefore, assessment using just one seed species and endpoint might not provide a representative result of binary metal toxicity.

2.3 Toxicity on gene mutation

The spontaneous reversion rate for TA 98 was ranged from 21 to 106 colonies, which was similar to previously reported results (Mathur et al., 2007; Mortelmans and Zeiger, 2000). The concentration of the positive control (2.5 $\mu\text{g/L}$ of 2-nitrofluorene) was determined for strain TA 98 prior to the experiment. In a previous investigation using single metals, ratios higher than mutation ratio (MR)

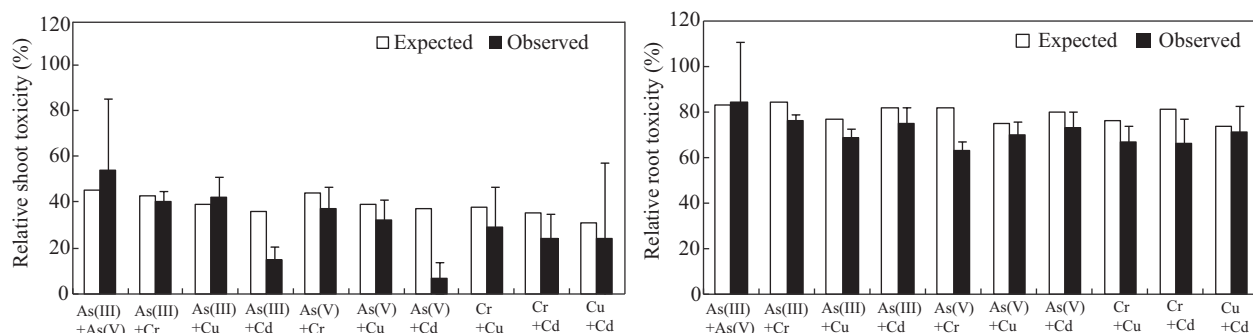


Fig. 4 Comparison between expected and observed effects of binary metal mixtures on root and shoot growth of *Lactuca* seeds during germination period.

2.0 (indications of positive mutagenicity) were observed for all doses of arsenite tested (1–500 mg/L), whereas no mutagenic activity was apparent with Cu or arsenate (Ko et al., 2012; **Table 1**). Therefore, arsenite was the most toxic metal with respect to mutagenic activity. It has been well documented that chromates are mutagenic in a number of bacterial systems (De Flora et al., 1990). This result also indicates a moderately high mutagenic ability for chromate compared to that of Cu or Cd. In terms of mutagenic activity, toxicity could be ordered as follows: arsenite \gg chromate $>$ cadmium \geq arsenate, copper. However, this order does not exactly correspond to previously reported methods.

For the effects of binary metal mixtures on gene mutation, the TU approach could not be applied in the same manner as the other assays. Therefore, tests on binary equal mixtures were performed at 1, 10, 100, and 500 mg/L. Most binary mixtures showed no mutagenic activity (MR lower than 2) (**Fig. 5**). However, the binary mixture of As(III) and Cr, which are prominent mutagens as single metals, showed high MR values compared to those of other mixtures. The most pronounced effects on gene mutation were seen in the binary mixture of As(III) and Cr (500 mg/L), which showed a MR of 3.9. Therefore, most binary mixtures produced significant antagonistic responses (especially, binary mixture of As(III) or Cr) and additive rather than synergistic ones.

3 Conclusions

This article presents useful data on the effects of metal mixtures in different bioassays compared to those of single metals. The observed effects of the equal binary mixtures

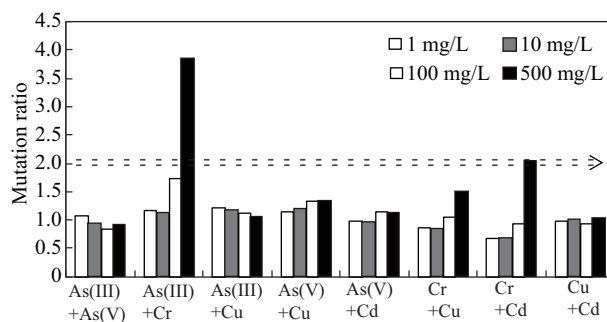


Fig. 5 Effects of binary metal mixtures on gene mutation. Dot line indicates the threshold value (mutation ratio = 2) for positive mutagenicity.

on the tested organism could be categorized as antagonistic (less than additive), synergistic (greater than additive), or additive. However, mostly additive or antagonistic effects were observed as followings: mostly additive effects on bioluminescence and root growth, mostly antagonistic effects on seed germination and gene mutation, both additive and antagonistic effects on shoot growth. Almost no synergistic effects by the binary metal mixtures were observed in all bioassays. With respect to the battery of bioassays in this study, one bioassay could not provide results equivalent to those of another bioassay. Overall, the mixture effects of heavy metals were difficult to generalize since they were dependent on both chemical type and the organism used in each bioassay. However, this result indicates that a battery of bioassays as opposed to just a single assay in the presence of mixture compounds is a better strategy for the bioassessment of environmental pollutants. Further detailed mechanisms of action of mixture metals have to be investigated in terms of mixture components and different bioassays.

Table 1 Effects of single metals on gene mutation of strain TA 98: adapted from Ko et al., 2012

Metal	Mutation ratio (sample/negative control)					
	1 mg/L	5 mg/L	10 mg/L	50 mg/L	100 mg/L	500 mg/L
As(III)	5.1	4.5	6.8	6.5	8.1	10.4
As(V)	1.4	1.3	1.5	1.4	1.6	2.0
Cr	1.3	1.9	2.4	6.3	6.8	7.0
Cu	0.7	0.8	0.9	1.1	1.1	1.5
Cd	1.4	1.4	1.5	1.4	1.6	1.2

Acknowledgments

This work was supported by the Basic Science Research Project through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (No. 2012-0007703).

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Journal of Environmental Sciences (Established in 1989)

Vol. 25 No. 5 2013

Supervised by	Chinese Academy of Sciences	Published by	Science Press, Beijing, China
Sponsored by	Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences	Distributed by	Elsevier Limited, The Netherlands
Edited by	Editorial Office of Journal of Environmental Sciences P. O. Box 2871, Beijing 100085, China Tel: 86-10-62920553; http://www.jesc.ac.cn E-mail: jesc@263.net , jesc@rcees.ac.cn	Domestic	Science Press, 16 Donghuangchenggen North Street, Beijing 100717, China Local Post Offices through China
Editor-in-chief	Hongxiao Tang	Foreign	Elsevier Limited http://www.elsevier.com/locate/jes
CN 11-2629/X	Domestic postcode: 2-580	Printed by	Beijing Beilin Printing House, 100083, China
		Domestic price per issue	RMB ¥ 110.00

ISSN 1001-0742

