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Effect of hardness on acute toxicity of metal mixtures using *Daphnia magna*: Prediction of acid mine drainage toxicity

Jin Hee Yim, Kyoung W. Kim, Sang D. Kim *

Department of Environmental Science and Engineering, Gwangju Institute of Science and Technology, 1 Oryong-dong, Buk-gu, Gwangju 500-712, Republic of Korea

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Abstract

In this study, the effect of hardness on the combined outcome of metal mixtures was investigated using *Daphnia magna*. The toxic unit (TU) was calculated using modified LC_{50} values based on the hardness (i.e., $LC_{50\text{-soft}}$ and $LC_{50\text{-hard}}$). From a bioassay test, the degree of sensitivity to hardness on the toxicity changes was in the order: Cd < Cu < Zn < Pb, with 25, 66, 77 and 88% decreases in the LC_{50} values, respectively, when the hard test solution was replaced with a soft test solution. In mixture toxicity tests, the difference in the test solution hardness was found to clearly cause different toxicities, as determined by the TU calculated by the $LC_{50\text{-hard}}$, using the toxicity of a standard culture medium as the reference. That is, approximately four to five times higher toxicity was observed in soft (i.e., 44 ± 4 mg/L as $CaCO_3$) rather than hard water (i.e., 150 ± 10 mg/L as $CaCO_3$) test solutions. In the tests where the modified reference toxicity values (i.e., $LC_{50\text{-soft}}$ and $LC_{50\text{-hard}}$ for soft and hard test solution, respectively) obtained from the individual metal toxicity tests with different hardness were used to calculate the TU, the results showed very similar *D. magna* toxicities to those of the TU from the mixture of soft and hard test solutions, regardless of the hardness. According to the toxicity results of the mixture, the aquatic toxic effects of the acid mine drainage (AMD) collected from mine areas that contained metal mixtures were investigated using *Daphnia magna* and the modified LC_{50} value of the TU hardness function calculated for varying solution hardness. The results of the biological WET test closely matched our overall prediction, with significant correlation, having a p-value of 0.513 in one way ANOVA test ($n = 19$). Therefore, this study revealed that the predicted toxicity of the metal mixture agreed well with the biological toxicity test when the modified LC_{50} value was employed as the basis of hardness in the TU calculation.

Keywords: Heavy metals; Mixture toxicity; *Daphnia magna*; Hardness; AMD

1. Introduction

Heavy metal release into the environment has occurred continuously through acid mine drainage (AMD), and is a severe water pollution problem associated with mining activity. High concentrations of trace metals and acidic pHs have been known to adversely affect aquatic ecosystems [1,2]. The exposure of pyrite materials or sulfide minerals to water and air results in a series of chemical and biological oxidation reactions, which forms a highly acidic effluent [3]. The oxidation of pyritic materials produces an acidic leachate (pH 2–4) that is rich in sulfate. At these low pH values, high concentrations of soluble and exchangeable metals are commonly present in the leachate. The elevated levels of metals in mine drainage are due to the natural abundance of metals in the mined materials and their microbially mediated solubilization in these forms. Thus, acid mine drainage is highly acidic and contain high concentrations of iron and other potentially toxic heavy metals such as Cd, Cu, Pb and Zn. However, a large number of abandoned mines remain without environmental consideration or further treatment, despite their potential danger.

Many studies have shown the toxicity with changes in the environmental parameters of metals as single components only. However, the toxicity of pollutants in aquatic systems does not occur due to individual compounds, but from mixtures within a real environment. The combined effect of chemical mixtures has been studied by several researchers to develop new methods, concentration addition with similar mode of action and independent action with dissimilar mode of action, for the prediction of mixture toxicities [4,5]. To evaluate the toxicity of heavy met-

* Corresponding author. Tel.: +82 62 970 2445; fax: +82 62 970 2434.
E-mail address: sdkim@gist.ac.kr (S.D. Kim).

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als in mixtures, the reference toxicities of heavy metals were determined in synthesized test media. Thereby, the toxic unit (TU), which is the ratio of the present chemical and reference toxic concentrations, such as LC_{50} or EC_{50}, is miscalculated and incorrectly applied between the lab and field due to the different environmental conditions. Furthermore, the toxicity of metals to aquatic organism is predicted by understanding the thermodynamic interaction between dissolved organic matter (DOM) and metals in the basis of biotic ligand model (BLM) [6,7]. In addition, many physiochemical factors, such as pH, hardness, temperature, dissolved oxygen and flow rates, affect the toxic properties of a compound toward aquatic species in freshwater [8–10]. The hardness of water is a major factor, which influences the toxic effects of heavy metals on fish. Generally, in fresh water as the hardness increases, the heavy metal toxicity decreases due to competition between the heavy metal and Ca^{2+} and Mg^{2+} ions for the uptake sites of organisms. Heavy metals are generally an order of magnitude more toxic in soft than in hard water [9]. In the natural environment, Ca and Mg are present at much higher concentrations than heavy metals. Therefore, by competing with heavy metals, and blocking their access to aquatic organisms, the Ca and Mg levels are important considerations with respect to the toxic effects of heavy metals on the biota in aquatic systems. Thus, the US EPA has formulated water quality criteria (WQC) for heavy metals, which are expressed by an equation with respect to water hardness. Therefore, it is essential to consider the changes in toxicity with hardness, and these modified toxic values must be applied to the calculation of TU to determine the toxicity of a mixture. In most parts, researchers use the LC_{50} values calculated in fixed water hardness. However, it is well known that the toxicity values vary according to hardness. If the LC_{50} values obtained from toxicity tests in hard water are applied to mixture toxicity tests in soft water, as present in real environments, there will be a misreading of the combined heavy metals effect.

In this study, we examined the feasibility to use a mixture toxicity model based on effect summation for assessing metal mixtures toxicity in acid mine drainage and investigated the effect of hardness on the acute toxicity of metal mixtures using *Daphnia magna*. The presented study also evaluated the relationship between chemical analysis and biological toxicity results and thereby the prediction of mixture toxicity was verified by biological observation.

2. Materials and methods

All reagents were of analytical grade, and used without further purification. All laboratory glassware, polyethylene and polypropylene were soaked in 10% HNO_3 (v/v) for at least 48 h and rinsed with distilled water more than three times prior to use. Deionized (DI) water from a Millipore Milli-Q^{TM} ultra pure (<18.2 MΩ/cm) water system was used throughout the study. Zinc ($ZnSO_4{\cdot}7H_2O$), copper ($CuSO_4{\cdot}5H_2O$), cadmium ($CdCl_2$) and lead ($Pb(NO_3)_2$) were purchased from Aldrich (Milwaukee, WI, USA). Stock solutions of the chemicals were prepared in deionized water and polyethylene or polypropylene test bottles and equipment mostly used to avoid the adsorption of metals. In order to analyze the selected metals by inductively coupled plasma mass spectrometry (Elan 6000 model, Perkin-Elmer Co.), the samples were filtered with a 0.45 μm filter and 1% of the sample volume of 65% HNO_3 added for acidification. The detector used for the ICP-MS was a discrete dynode electron multiplier tube. The detection limits of the heavy metals (Cd, Cu, As, Pd and Zn) ranged from 10 to 100 ng/L.

2.1. Description of sampling sites

Sampling stations were selected at abandoned mines located in Jeollanam-do and Gangwon-do, Korea. In the study areas included 19 AMD sampling stations from 6 non-operational gold (Au) mines: the Duckum (DU), Myungbong (MB), Gwangyang (GY), Gwangsun (GS), Deaduck (DD) and Nakdong (ND) mines. Most outcropped parts of the mines consisted of piled rock, similar to a dam. The effluent or leachate from the mines is discharged into reservoir waters and many residential areas. Water samples were returned to the laboratory within 24 h of sampling, and stored at 4 °C. The pH of the discharged waters was measured using an Orion 720A pH meter (Thermo Electron Inc.). The hardness was measured by EDTA titrimetric methods, according to the standard methods [11].

2.2. Test organisms and culture conditions

The test organism, *D. magna*, was obtained from the Korea Institute of Toxicology (Daejon, South Korea), and the food, *Selenastrum capricornutum* and yeast, trout chow and Cerophyll® (YTC) mixture were purchased from Aquatic Biosystem Inc. (Fort Collins, CO). The organisms were cultured and handled according to the procedures outlined in the US EPA manual [12]. Culture water for *D. magna* was reconstituted hard water ($CaSO_4{\cdot}H_2O$ 120 mg/L, $NaHCO_3$ 192 mg/L, $MgSO_4$ 120 mg/L and KCl 8 mg/L), with a hardness of 150 ± 10 mg/L and alkalinity of 121 ± 10 mg/L, both as $CaCO_3$, and a pH of 8.0 ± 0.2.

2.3. Bioassay of heavy metals

Acute 48 h toxicity tests for the heavy metals were examined under static non-renewal conditions, using *D. magna* at 25 ± 1 °C in a temperature controlled room, maintained with a 16 h light and 8 h dark photocycle. A minimum of 2 h prior to the test, the *D. magna* were fed on YTC mixture and green algae *Selenastrum carpricornutum*, and transferred them into test cups. Four replicates, holding five neonates of less than 24 h old, were set up for each heavy metal concentration in 25 mL of test water. Each set of tests was comprised of different concentrations and a control. The mortality was defined as the number of live organisms remaining after 48 h of exposure.

In this study, the experiments were performed with the organisms exposed to metal mixtures (i.e., Cd, Cu, Pb and Zn) as for individual metal toxicity. The synergistic or antagonistic effects of the combined metal mixtures were evaluated in media of different hardness. As noted, the TU is the ratio of the exposure concentration to the LC_{50} value for the test species, where the

LC_{50} value is generally obtained from standard test media that do not represent a real environment. Therefore, it is necessary to use the modified LC_{50} values when considering the hardness of site waters. In the presented study, the LC_{50} of a single compound was calculated in both soft and hard waters to compare the effects of a metal mixture to *D. magna*. From these data, the sum of the toxic units was calculated for each metal combined in the soft and hard test water solutions. For the first mixture set bioassay, the TU was calculated for each metal using the LC_{50} values from a hard water solution; for the second set, the LC_{50} value determined in soft water solution was employed to calculate the TU.

For a comparison of the toxic effects of heavy metals on *D. magna* on the basis of the hardness, bioassays were conducted in hard and soft waters. The hardness of the test media was controlled by varying the components, as described in the EPA manual. The conditions of the soft test water were described by a hardness of 44 ± 4 mg/L and an alkalinity of 30 ± 5 mg/L, both as $CaCO_3$ mg/L, and a pH of 7.8 ± 0.2. Basically, the total toxic unit is the summation of each TU for each chemical, divided by individual LC_{50} values. In order to prepare the quaternary metal mixture tests, the reference toxicity was used as the LC_{50} value from each single metal test in different media. The bioassays were conducted at various ranges of TU ($\sum$0.1 TU–$\sum$4.0 TU) and with a control. The target TUs of the heavy metals were prepared by an equitoxic combination, where each metal was present at a concentration showing equal individual 48-h LC_{50} values.

2.4. Toxicity of acid mine drainage

Water samples collected from the AMD sites were stored in a refrigerator at 4 °C for a day prior to use. The sample waters were filtered with a 0.45 μm nylon filter. Because it is assumed that the AMD is diluted in the stream and river water, if the sample pH is quite low, it is necessary to raise the pH (7.8 ± 0.2) to reduce the effects of the pH itself on the aquatic organisms. Organisms were exposed to 100, 50, 25, 12.5 and 6.25% of the samples, using a dilution factor of 0.5 with synthetic reconstituted water in 25 mL of the total test volume. The tests were accomplished according to the whole effluent toxicity (WET) test method, and the survival rate of the organisms was observed from four replicates containing five neonates, totally 20 neonates, for each concentration [12].

The toxic unit of AMD was determined by dividing 100 by LC_{50} values in percentage obtained from the whole effluent toxicity tests to *D. magna*. For example, the TU is 4 if 50% of the test organisms are killed in a 25% raw water sample (e.g., LC_{50} is 25%), where 75% synthetic reconstituted water has been used for the dilution. For the cases where the percentage mortality of organisms exposed to 100% raw sample was lower than 50%, the modified calculation method was used, which multiplies the percentage mortality of *D. magna* by 0.02 for the calculation of the TU. To take into consideration the effect of hardness on the metal toxicity, the hardness of each site was measured by standard EDTA titrimetric methods, and the reference toxicity value for the different hardness values used in this study was calculated by the relationship equations of hardness with LC_{50} values.

2.5. Data analysis

The mean LC_{50} values of the heavy metals were calculated by the Probit Program version 1.5, a parametric statistical method for the analysis of mortality data, associated with a 95% confidence limit, which was downloaded from the website of the US EPA. The experimental results were treated to produce regression and concentration–response curves using the SigmaPlot® software (SPSS Inc.).

3. Results and discussion

3.1. Individual toxicity of heavy metals

Toxicity tests for individual metals were conducted to find the changes in the LC_{50} values for different hardness of test waters. From the bioassay test using *D. magna*, the LC_{50} values were 3, 4, 95 and 300 μg/L for Cd, Cu, Pb and Zn in soft water, and 4, 12, 894 and 1290 μg/L in hard water, respectively (Table 1). These values are denoted as $LC_{50\text{-soft}}$ and $LC_{50\text{-hard}}$ as the toxicity values for soft and hard waters, respectively. The acute toxicity for 48 h exposure increased in the following order: Zn < Pb < Cu < Cd in both of hard and soft water solutions. The degree of sensitivity to hardness on the toxicity changes was in the order: Cd < Cu < Zn < Pb with 25, 65, 77 and 88% of decreases in the LC_{50} values, respectively, when hard test solutions were replaced with soft. As shown in Table 1, the results in soft water are shown to be more toxic than those in hard water. This means that the hardness of a test solution may affect the toxicity of heavy metals. This can be explained by the influence of biological membrane permeability to toxic metals due to the change of hardness, resulting in an increase of the passive flux of metal ions across the membrane as the calcium concentration decreases [13]. The uptake of calcium and magnesium ions by the cell membrane causes it to stabilize, which reduces its permeability to metal ions [14]. Part et al. [15] also demonstrated that a decrease in calcium ion led to an increase in the cadmium flux. A related study using *Ceriodaphnia dubia* showed that the effects of copper toxicity decreased with increasing water hardness [8].

Table 1
Comparison of LC_{50} values obtained from toxicity tests with *D. magna* in soft and hard standard waters for each metal

Metals	Tested LC_{50} (μg/L)	
	Soft water ($LC_{50\text{-soft}}$)	Hard water ($LC_{50\text{-hard}}$)
Cd	3	4
Cu	4	12
Pb	95	894
Zn	300	1290

The hardness was 44 ± 4 mg/L for soft water and 150 ± 10 mg/L for hard water as $CaCO_3$, respectively.

Table 2
The relationship of hardness (mg $CaCO_3$/L) and LC_{50} (μg/L) values to *D. magna*

Heavy metals	Equations	Correlation coefficient, r^2	Remark
Cd	$LC_{50} = 1.13$hardness $- 43.79$	0.70 ($n = 15$)	[16]
Cu	$LC_{50} = 0.28$hardness $+ 1.00$	0.87 ($n = 19$)	[17]
Pb	$LC_{50} = 9.93$hardness $- 92.48$	0.69 ($n = 5$)	[18]
Zn	$LC_{50} = 8.20$hardness $- 157.5$	0.84 ($n = 8$)	[18]

The data was provided from the ECOTOX data system and ambient water quality criteria of the US EPA.

To generalize the relationship between LC_{50} values of heavy metals on *D. magna* and the hardness, or confirm the effects of hardness on toxicity, their linear equations were computed using the literature [16,17] and ECOTOX results supplied from the US EPA database [18] (Table 2). Each equation for the LC_{50} value was calculated separately on the basis of the hardness for Cu, Cd, Pb and Zn, respectively. A high correlation coefficient for the value of LC_{50} versus hardness was observed. Consequently, the toxicity in a real environment, which has different water characteristics, such as hardness, can be evaluated from the formulated toxicity equation for hardness, with these results reflecting that the toxicity of heavy metals is governed by the water hardness.

3.2. Mixture toxicity of heavy metals

The effect of hardness on the mixture toxicity of metals to *D. magna*, relative to the response curve corresponding to the equitoxic mixtures of the four heavy metals, was investigated and is presented in Fig. 1(a). The similar mode of action was assumed in the toxicity tests of metal mixture with different hardness concentration. The results showed the combined effects of heavy metals in soft and hard test waters. For the calculation of TU, the $LC_{50\text{-hard}}$ value was used as the reference toxicity, as determined in hard water media, which is the standard culture media used for *D. magna*. As shown in the results, the exposure of *D. magna* to metal mixtures with variable hardness caused remarkable differences in the mortality. The toxicity was extremely increased in the soft test solution. For instance, the addition of a ∼1.4 TU mixture in hard test water resulted in the same 50% mortality of *D. magna* as the addition of a ∼0.25 TU mixture in soft test solution after a 48-h exposure period. Clearly, a difference in the hardness of a test solution would cause a different toxicity, that is, higher toxicity in a soft compared to a hard test solution. Conversely, the result showed that the toxicity data of the mixture in hard water closely matched the equality line, meaning there was no synergistic toxic effect of the metal mixtures on *D. magna*.

In the second set, the modified LC_{50} values obtained from individual metal toxicity tests with different hardness (i.e., $LC_{50\text{-soft}}$ and $LC_{50\text{-hard}}$) were used to calculate the sum of the toxic units (Fig. 1(b)). That is, the $LC_{50\text{-soft}}$ and $LC_{50\text{-hard}}$ values were used for the mixture toxicity tests in soft and hard test solutions, respectively. The results indicated very similar toxicities of *D. magna*, corresponding to the TU of the mixture regardless of its hardness. In addition, no combined effect on *D. magna* was found in heavy metal mixtures. All data points were closely placed to the equality line. Notably, there was no significant difference ($p = 0.268$ at one way ANOVA test) between the observed mixture toxicity and the calculated sum of individual toxicities of each metal, indicating no antagonistic or synergistic responses.

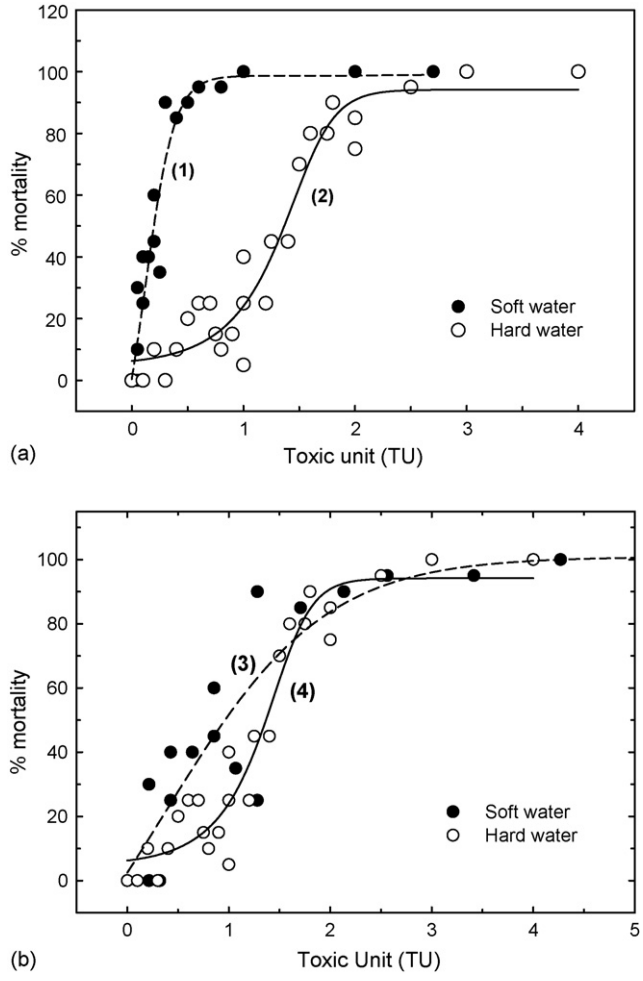

Fig. 1. Effect of hardness on % mortality toward *D. magna* in the heavy metal mixture toxicity tests. (a) The LC_{50} values for calculating the TU (toxic unit) were obtained from tests in synthesized hard water. (b) The LC_{50} values were determined on the basis of hardness. TU = concentration/LC_{50} value, where the LC_{50} value was determined as a function of hardness. Solid lines represent best-fit lines for combined effects of metal mixture in hard water. Dotted lines represent best-fit lines in soft water. The equations were as follows: $y = y_o + [a/(1 + \exp(-((x - x_o)/b)))^c]$ with five parameters of $a = 145.2$, $b = 0.13$, $c = 0.76$, $x_o = 0.16$, $y_o = -46.5$ and $r^2 = 0.91$ for curve (1), $a = 89.37$, $b = 0.17$, $c = 0.44$, $x_o = 1.59$, $y_o = 4.79$ and $r^2 = 0.94$ for curve (2), $a = 183.7$, $b = 0.74$, $c = 0.58$, $x_o = 0.75$, $y_o = -82.8$ and $r^2 = 0.83$ for curve (3), and $a = 89.37$, $b = 0.17$, $c = 0.44$, $x_o = 1.59$, $y_o = 4.79$ and $r^2 = 0.94$ for curve (4).

Many studies have shown that the toxicity of the constituent metals in a mixture was enhanced and affected by the interactions of mixtures with the binding sites of organisms [19,20]. A recent study has demonstrated an inverse relationship between copper toxicity of *D. magna* and hardness. Low concentrations of hardness, ranging from 7 to 50 mg/L as $CaCO_3$, significantly affected the copper toxicity [21]. Therefore, it is clear that hardness is a major factor affecting metal toxicity and should be considered in determining reference LC_{50} values when the toxicities of metal mixtures on aquatic species are evaluated.

3.3. Toxicity of acid mine drainage

The pH, hardness and concentration of major metals in the AMD samples collected from the 19 stations at 6 different mine sites are listed in Table 3. The characteristics of the AMD samples had pH values ranging from 3.2 to 9.8 and hardness ranging from 16 to 640 as $CaCO_3$ mg/L. The metals and other elements in the AMD samples were analyzed by inductively coupled plasma-mass spectrometry, and indicated a wide range of concentrations at all sites.

Based on the results of the analysis, the theoretical TU was calculated from the ratio of the measured concentration of contaminants to the reference toxicity value (i.e., LC_{50}). According to our results, the water hardness affected the metal toxicity, so the modified LC_{50} value was used when considering the hardness (Table 2). The $\sum$TU values of the 15 sampling sites were

Table 3
The chemical properties of the mine drainage in the study area

Sample	pH	Hardness[a]	Zn	Pb	Cu	Cd
GY1[b]	6.6	96	5.6	0.4	0.8	0.1
GY2	6.7	64	4.6	BDL[c]	1.6	BDL
GS1[b]	3.4	180	84.4	0.5	15.8	BDL
GS2	7.4	58	11.8	BDL	0.4	BDL
DD1[b]	4.4	32	1646	1541	3.4	10.7
DD2	6.1	16	156	40.2	BDL	1.3
DD3	6.8	25	11.8	2.8	BDL	0.05
DU1[b]	3.8	188	2346	610	114	25.5
DU2	3.2	640	7347	106	55.1	30.4
DU3	6.7	72	7.4	0.2	3.8	1.0
DU4	7.2	240	10.2	0.1	1.4	BDL
DU5	7.8	48	4.4	0.3	2.3	0.1
DU6	6.6	192	5.9	0.9	5.5	BDL
DU7	8.2	160	80.2	0.1	7.4	0.1
MB1[b]	6.9	17	238	0.5	11.6	1.6
MB2[b]	7.8	50	4797	1.5	29.6	17.6
MB3	7.1	36	51.5	0.4	6.0	0.1
ND1[b]	7.3	188	5.8	0.1	13.7	9.0
ND2	6.5	184	353	0.1	8.8	4.8

The concentrations of the heavy metals (in μg/L) were measured by ICP-MS. The names of the initial sample sites are indicated as follows: GY, Gwangyang (Gwangyang city, South Cholla province); GS, Gwangsun (Gwangyang city, SC province); DD, Daeduck (Damyang county, SC province); DU, Dukum (Naju city, SC province); MB, Myungbong (Boseong county, SC province) and ND, Nakdong (Jeongseon county, Gangwon province).

[a] Expressed as $CaCO_3$ mg/L.

[b] Indicates the site of mine gob piles. The other sites were small streams, wells and reservoirs, etc., around the mining sites.

[c] BDL indicates that the sample was below the detection limit.

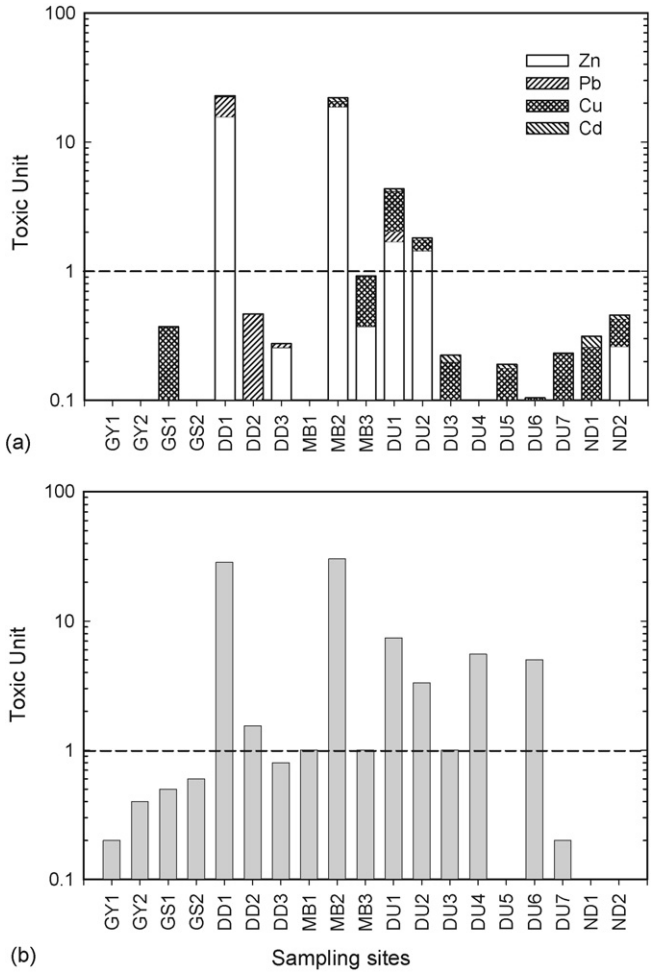

Fig. 2. Comparison of toxicity from the biological and predicted toxicity results in the AMD effluents. (a) Predicted toxicity values were calculated by the sum of the ratio of chemical analysis and the LC_{50} values. The LC_{50} values were obtained from an equation as a function of the hardness corresponding to the samples from the AMD sites. (b) Observed toxicity values were determined by the whole effluent toxicity (WET) test toward *D. magna* for 48 h.

less than 1 (0.1–0.6), indicating relatively no effect of the AMD sample metals to the aquatic ecosystem if 1 TU is considered as reference toxicity (Fig. 2(a)). The $\sum$TU at 4 sampling sites were higher than 1, with TUs ranging from 1.81 to 22.9. Of note: DD1 and MB2 showed extremely high $\sum$TU of 22 and 22.9, respectively, which will provoke much higher effect than reference effect level. As shown in Fig. 2(a), the source of toxicity at DD1 was mostly from Zn (15.7 TU) and Pb (6.8 TU), containing 1646 and 1540 μg/L, indicating 104.9 and 225 μg/L for the modified LC_{50} values, respectively, at a hardness of 32 mg/L as $CaCO_3$. The MB2 sampling point also showed high Zn toxicity (18.8 TU) from the ratio of the dissolved Zn concentration (4797 μg/L) and the modified LC_{50} values (255.8 μg/L) at a given water hardness of 50.4 mg/L as $CaCO_3$. Biological toxicity of the AMD samples using whole effluent toxicity (WET) test was also evaluated, as shown in Fig. 2(b), for comparison with the results of the theoretical prediction of Fig. 2(a). The results of biological WET test closely matched our overall prediction, with significant correlation at one way ANOVA test

($p = 0.513$, $n = 19$). The observed TU values for DD1 and MB2 were extremely high (28.6 and 30.3, respectively), as were the theoretical $\sum$TU from the chemical analysis. For most samples the results were closely related, except for sites DU4 and DU6, indicating big variations between the observed and predicted $\sum$TU values. Eleven of the 15 sites, these sites that showed non-toxic AMD effects in the theoretical $\sum$TU prediction, had no toxicity of the AMD samples (i.e., less than 1 TU) in the whole effluent toxicity tests on *D. magna*. This revealed that the prediction of toxicity in a metal mixture was well verified by the biological toxicity test when the modified LC_{50} value is applied as the basis of hardness in the calculation of TU. On the other hand, the sampling sites of GS1, DD1, DU1, and DU2 showed low pH values (3.2–4.4), which were adjusted to pH 8.0. The pH adjustment reduced free metal ion forms due to precipitation and complexation with hydroxide ion and thereby decreasing AMD toxicity. Therefore, considering the speciation of metals in different pH values, the original toxicity of AMD samples in GS1, DD1, DU1, and DU2 would be different from our measurements. In addition, one of important factors modifying metal toxicity in real environmental system (e.g., stream and river water) is dissolved organic carbon (DOC) that complexes with free metal forms, which is a major physicochemical form in metal bioavailability. The importance of DOC is explained by the competition between DOC and surface sites of aquatic organisms for metal binding. Biotic ligand model (BLM) was introduced to illustrate the competition and is necessary for more accurate prediction of metal toxicity in mixtures [6,7].

The toxic effects of chemicals on aquatic species generally occur in the form of mixtures, including various interactions with environmental parameters. Of these parameters, hardness is the primary factor changing the metal toxicity, which can be easily measured and monitored. Most studies have used the LC_{50} as a reference toxicity value, as calculated from standard test methods, but without considering the hardness of the test waters when determining the toxicity of metal mixtures. As shown in the presented study, the observed toxic effect of metals to *D. magna* were no different from the expected sum of the toxic effect of each metal, despite the different test water hardness (i.e., soft and hard water solution) when the modified LC_{50} value was used for considering the hardness of each sample in the calculation of the TU. The result of the WET in the AMD samples confirmed that the biological toxicity can be explained by the predicted $\sum$TU when considering the water hardness. Therefore, this study suggests the use of modified toxicity values when considering the hardness in the determination of the toxicity of metal mixtures for cases encountered in receiving waters that contain a variety of hardness concentrations.

4. Conclusions

This study demonstrated that the toxicity of heavy metals varies with changes in the hardness; thereby, the hardness should be considered in the calculation of TU in the mixture toxicity tests of heavy metals. In this study, the results indicated a very similar toxicity of *D. magna* corresponding to the TU of a mixture, regardless of its hardness when the modified LC_{50} values were used as a function of the hardness. Furthermore, in most samples the prediction of toxicity in a metal mixture was found to be well verified by the biological toxicity test when the modified LC_{50} value was employed for calculation of the TU. Therefore, this study strongly recommends the use of modified toxicity values when considering hardness in the determination of the toxicity of metal mixtures for cases encountered in receiving waters that contain a variety of hardness concentrations.

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