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Interactions of Cd, Cr, Pb, Ni, and Hg in their effects on activated sludge bacteria by using two analytical methods

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Abstract Since trace metals rarely appear singly in industrial effluents, it is a major challenge to address combined effects of such toxicants on biological units of wastewater treatment plants. The aim of this study was interaction assessment of Cd, Cr, Pb, Ni, and Hg in their effects on activated sludge bacteria using analytical methods. Two mathematical models were used to determine the effect of binary mixtures of Hg, Cd, Cr, Pb, and Ni on activated sludge bacteria using a dehydrogenase enzyme assay. Calculated EC_{50} values were compared to experimentally observed values of mixtures. Interactive effects were counted to be antagonistic for Hg and Cd, Cd and Pb, Cd and Ni, and Cr and Pb, synergistic for Cd and Cr and Hg and Cr, and additive for other binary mixtures. Maximum toxicity was related to Hg and Cr, Cd and Cr, and Hg and Cd. Physicochemical monitoring of single metals may underestimate hazards arising from these pollutants in environmental samples. Therefore, any possible interaction between metals in such environments should be considered when establishing environmental safety standards.

Keywords Toxicity · EC_{50} · Modeling · Synergistic

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Introduction

Wastewater treatment plants (WWTPs) dealing with industrial effluents are often faced with the entrance of toxicants which may cause the complete or partial loss of the efficiency of biological treatment units. The activated sludge, as a common wastewater treatment technology, is based on bacterial ability to use organic pollutants as a source of food and energy. In WWTP influent, a high metal concentration often originates from by-products of industries which use heavy metals. A useful approach for toxicity assessment in such WWTPs—that could be manipulated by different microorganisms—is dehydrogenase enzyme assay (DHEA). This bioassay is relatively rapid, simple, and inexpensive method for toxicity assessment of chemical compounds and is based on the inhibition of bacteria dehydrogenase activity in the presence of toxicants (Rampersad 2012).

Combination of several toxicants may give rise to poisoning shocks in WWTPs. Although the toxicity aspects of chemicals in sediment or water in many environments have been investigated, combined effects of toxicant chemicals on WWTP microorganisms still remain poorly considered. To predict the combination effect of several toxicants, a variety of analytical algorithms can be used. In this regard, Koneman (1981) considered the toxicity of mixtures of more than two chemicals containing phenol and different chlorophenols on guppies and concluded that compounds with a good quantitative structure-activity relationship are expected to have a similar action and their concentrations can be added up to evaluate the mixture toxicity. Similar toxicity result proves that each toxic substance can be replaced by another toxicant without difference in their effects on the bio-indicator, meaning that they affect on creatures or cells via similar mechanisms. This effect is known as concentration-addition (Christen et al. 2014; Tachikawa and Yamanaka 2014). Ribo and Roger (1990) studied the toxicity of binary mixtures of different chlorinated phenols on *Vibrio fischeri* and proposed an analytical model which considered that the toxic mechanism of all studied toxicants is similar. Fernandez-Alba et al. (2001) studied the toxicity of pesticides in water singly and in mixtures on the *Daphnia magna* and *Vibrio fischeri* using Ribo and Roger's (1990) analytical model. In their study, antagonistic and synergistic effects were reported with pesticide cocktails; however, the predicted data failed to match the observed toxicity.

Metals are almost always present in environmental samples in mixtures. But few investigations have reported combined effects of heavy metals on community structure of living microorganisms such as nematode *C. elegans* (Chen et al. 2013), copepod *Amphiascus tenuiremis* (Hagopian-Schlekat et al. 2001), and human keratinocytes (Zhang et al. 2010). However, the combined toxicity of metals on microorganisms existing in the wastewater treatment plant biological units has not been investigated yet. In addition, it should be emphasized that each heavy metal may exhibit its specific toxicity through a variety of metabolic pathways. In this regard, no study has addressed the combined toxicity of heavy metals on dehydrogenase enzyme function of activated sludge bacteria. The short time and simplicity of dehydrogenase enzyme assay are the most positive points of this test. The weakness of the test can be its dependence on the laboratory environment and equipment (Zare et al. 2016).

In this study, joint effects of all Hg, Cd, Cr, Pb, and Ni binary mixtures on the dehydrogenase enzyme function of activated sludge bacteria were investigated using two different mathematical models that were proposed in previous studies (Garcia-Nino and Pedraza-Chaverri 2014; Ribo and Roger 1990).

Material and methods

Sampling and toxicity assays

Stock solutions of $CdCl_2$, $HgCl_2$, $K_2Cr_2O_7$, $NiCl_2$, and $PbCl_2$ were prepared in distilled water. The concentration of each heavy metal in stock solutions was determined by using an ICP-OES (Thermo-Electron Corporation, Dreieich, Germany). More information on the applied material and method is given by Alonso et al. (2000).

Wastewater samples were obtained from the aeration basins of a WWTP in Tehran, Iran. The dehydrogenase enzyme assay mixture was 500 μL phosphate-HCl buffer, 500 μL of nutrient broth (× 10), 100 μL of resazurin solution (0.5 g/L), and distilled water up to a volume of 4000 μL (Zare et al. 2015a, 2015b, 2016). By adding 1000 μL of freshly isolated bacteria, the bioassays initiated. In all experiments, bacteria-free tests were carried out as control. All assays in each concentration were performed in four replicates and their means were used for calculating the EC_{50} values.

Single metal toxicity and EC_{50} determination

Before the binary mixture tests, toxicity experiments were performed for Hg, Cd, Cr, Pb, and Ni, separately. Each toxicity experiment was carried out at least in four replicates.

All data were log-transformed before the analysis was set up. Probit analysis using the SPSS ver. 16.0 software was performed for determination of 30 min EC_{50} values. The 95% confidence intervals of the EC_{50} were also determined.

The joint toxic effects of binary mixtures

Concentration of heavy metals in binary mixtures for performing bioassays was determined according to the concentration of each heavy metal which produced a similar inhibition in resazurin reduction when being alone, i.e., the EC_{50} values which reduce a 25% or 50% resazurin reduction. According to the results of single metal toxicity, the concentrations of heavy metals in binary mixtures are showed in Table 1. The toxicity of all possible combinations of Cd, Hg, Cr, Ni, and Pb was measured. Each toxicity experiment was carried out at least in four replicates.

Two different analytical methods were performed to calculate the metal toxicity of binary compounds: model 1 assumes that the two heavy metals in the tested mixture act interchangeably on the similar biological target and that they do not react on each other.

The observed toxicity of binary mixtures ($\mathrm{EC}_{50\mathrm{Obs}}$) was compared with their expected toxicity ($\mathrm{EC}_{50\mathrm{Exp}}$). The expected mixture toxicity was calculated according to Eq. (1):

$$EC_{50\mathrm{Exp}} = C_m/\left(\left(C_x/EC_{50x}\right) + \left(C_y/EC_{50y}\right)\right) \tag{1}$$

where C_m is the total values of the heavy metals (μmol/L) in the mixture (sum of the C_x and C_y); C_x and C_y are the concentrations of two applied heavy metals; and the EC_{50y} and EC_{50x} are the effective concentrations (μmol/L) that calculated according to the observed toxicity of each toxicant.

Determination of binary effects of heavy metals involved: (a) computation of $\mathrm{EC}_{50\mathrm{Diff}}$ parameter, which calculated as difference between the expected toxicity (according to Eq. 1) and the observed toxicity of the binary mixture; (b) determination of P value for testing the significance of the differences for each $\mathrm{EC}_{50\ \mathrm{Diff}}$ via one sample T test using the ver. 16.0 of SPSS software; and (c) determination of the antagonistic, synergistic, or additive effects according to P value. In this regard, an increase or a decrease in the measured EC_{50} of the binary mixture as compared to the calculated one would represent either a synergistic or an antagonistic phenomenon, respectively. Not significant difference of analysis results between the expected and observed toxicity of the binary mixture (at 95% confidence level) would confirm that the effect is neutral or merely additive.

Table 1 Applied concentration of heavy metals in binary mixtures

Heavy metals	Concentration (μmol/L)
Hg	0.35
Cd	7.95
Cr	0.15
Pb	358.00
Ni	205.00

The second model is based on a theory which assumes that toxicant chemicals act independently by different mechanisms (Tachikawa and Yamanaka 2014). According to this model, the computed mixture EC_{50} values should be expressed as toxicity units (TU) to obtain clearer results. This parameter can be calculated according to Eq. (2).

$$TU = 100/EC_{50} \tag{2}$$

On the basis of this model, the combined effect of heavy metals was obtained by comparing the observed TU of the values of the null hypothesis (H_0) with the single metals. In this regard, the null hypothesis assumed as "the sum of the TU of the two single metals (x and y) when evaluated alone." For each binary mixture of metals, the null hypothesis was examined by adding up the TUs according to Eq. 3.

$$H_0 : \mathrm{TU}_{H0(x+y)} = \mathrm{TU}_x + \mathrm{TU}_y \tag{3}$$

where $\mathrm{TU}_{\mathrm{H0}(x+y)}$ include the TUs introducing a primary calculated toxicity of a binary metals ($x+y$) and TU_x and TU_y are TUs introducing the observed toxicity of each heavy metal.

Determination of binary effects of heavy metals involved examining the null hypothesis: (a) computation of $\mathrm{TU}_{\mathrm{Diff}}$ parameter, between the expected mortality as calculated from the null hypothesis (according to Eq. 3) and the observed mortality ($\mathrm{TU}_{\mathrm{Diff}} = \mathrm{TU}_{\mathrm{H0}} - \mathrm{TU}_{\mathrm{Obs}}$); (b) determination of P value for testing the significance of the differences for each $\mathrm{TU}_{\mathrm{Diff}}$ via one sample T test; and (c) determination of the antagonistic, synergistic, or merely additive according to P value (Ince et al. 1999). A negative $\mathrm{TU}_{\mathrm{Diff}}$ value indicates an antagonistic interaction and a positive $\mathrm{TU}_{\mathrm{Diff}}$ value indicates a synergistic interaction. Otherwise, the $\mathrm{TU}_{\mathrm{Diff}}$ was recognized statistically non-significant and the combined toxicity effect of heavy metals considered as additive (at a 95% confidence level).

Results and discussion

Single metal toxicity

Results of 30-min EC_{50} estimation after exposure of activated sludge bacteria to single heavy metals are showed in Table 2. The log scale graphs representing the reduction rate of dehydrogenase enzyme activity at different toxicant values (Fig. 1) make it easy to classify the heavy metals into two different groups. A group with high toxicity, including Cr, Hg, and Cd, had an EC_{50} ranging from 0.31 to 15.9 μmol/L and the less toxic class, including Ni and Pb, had an EC_{50} ranging from 410 to 716 μmol/L. In this regard, it seems that the EC_{50} values calculated in the present study are not in appropriate agreement with the previous studies (Das et al. 2014; Garcia-Nino and Pedraza-Chaverri 2014).

For the five tested heavy metals, the toxicity, in descending order, was as follows: Cr (VI) > Hg > Cd > Ni > Pb. It seems that the toxicity order obtained in the present study is similar to the study of Hsieh et al. (2004) that reported the effect of 13 heavy metals to *Vibrio fisheri*. However, this order was different in comparison with the some other studies (Ren and Frymier 2003). Such considerable differences in similar studies can be attributed to concentration of anions, differences in pH value, competition from other cations, differences in the media compositions, and concentration of chelating agents (Das et al. 2014; Garcia-Nino and Pedraza-Chaverri 2014).

The joint toxic effects of binary mixtures

Heavy metal species in the industrial effluents almost always exist in mixtures. Therefore, the results illustrated in previous section showed only a single metal assay without considering the actual conditions in the WWTPs or other environments. To study the potentially real situation, this study was carried out using pair-wise heavy metal assays with dehydrogenase enzyme of activated sludge bacteria. According to the results of single metal toxicity, the concentration of heavy metals in binary mixtures was the following: Hg, 0.35 μmol/L; Cd, 7.95 μmol/L; Cr, 0.15 μmol/L; Pb, 358.0 μmol/L; and Ni, 205.0 μmol/L. Although such concentrations for Pb, Ni, and Cd may seem rarely occur in the industrial effluents due to their high values, some study reported the concentration of 31.4 mg/L, 33.1 mg/L, and 38.1 mg/L for Pb, Zn, and Cd respectively (Lokhande et al. 2011; Zare et al. 2015c). Therefore, the results of our study are applicable in WWTP that accept such industrial effluents and expose their bacteria to such industrial effluents.

Toxicity of the binary mixtures of tested heavy metals in terms of observed and expected EC_{50} is presented in Table 3. In this table, predicted interaction effects (by using model 1) between the pair-wise metals are shown. Figure 2 shows relationship between observed and expected toxicity of heavy metals, using models 1 and 2. In this regard, good correlation was proved between the experimental and the expected data in both two models. However, the correlation coefficient of model 2 was more than that of model 1.

Table 4 shows the TU values and predicted interaction effects between the two metals, obtained using model 2. In this table, predicted interaction effects were calculated on the basis of toxicity units (observed $TU = 100/EC_{50}$).

Comparing the results of Tables 3 and 4 shows that the same interaction is obtained by both analytical models in spite of an insignificant difference that can

Table 2 EC_{50} and TU values after 30-min exposure of activated sludge bacteria to heavy metals at pH 7.0 according to the protocol of dehydrogenase enzyme assay

Heavy metals	EC_{50} (μmol/L)	95% confidence limits	TU
Hg	0.71	0.55–87	140.8451
Cd	15.9	12.7–19.3	6.2893
Cr	0.31	0.22–0.38	322.5806
Pb	716	517–864	0.1397
Ni	410	368–452	0.2439

EC_{50} = analytically derived estimate of a value of a toxicant resulting in 50% reduction in dehydrogenase enzyme activity within a specified time

TU = toxicity unit

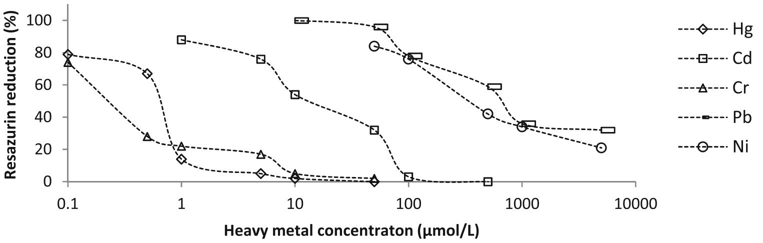


Fig. 1 Percent of resazurin reduction via activated sludge bacteria at the presence of different heavy metals (Hg, Cd, Cr, Pb, and Ni); incubation time, 30 min; inoculum cell density, 0.300 ± 0.01 at 540 nm

be related to the level of confidence of different statistical analysis: both analytical models 1 and 2 indicate that interaction between 4 over 10 of the studied metal mixtures was merely additive. In the toxicology terms, the consequence of interaction is called additive when the metals have similar nature, analogous structure, the same action mechanism or target organ. Such phenomenon means the effect of binary or multi-toxicant mixture is equal to the sum of individual toxicant effect. In fact, additive interaction is based on the basic theory that the expected reaction of a mixture of toxicant can be illustrated based on the sum of the toxic effects of each chemical alone. In present study, the joint effect of four binary mixtures was additive (Tables 3 and 4) that may be due to analogous structure and the similar action mechanism.

Toxicity results of four binary mixtures (Hg and Cd, Cd and Pb, Cd and Ni, and Cr and Pb) showed that their adverse effect is lower than the sum of the enzyme activity inhibition effects that can be introduced by each single metal. This phenomenon can be considered as an antagonism interaction between the two or more toxicants. Such effect between different chemicals considers that they affect on different levels of the similar energy-producing procedure. So, the toxic mechanism of one of the chemicals may be included on a pathway which has already been adversely influenced by the other chemical.

Results of this study concerning the Hg and Cd, Cd and Pb, and Cd and Ni mixture, as compared to other possible mixture (Tables 3 or 4) do propose that Cd may decrease the enzyme inhibition effects of Pb, Hg, and Ni

Table 3 Experimental versus expected EC_{50} values of all the possible binary mixtures of Hg, Cd, Cr, Pb, and Ni on activated sludge bacteria using the dehydrogenase enzyme assay. Results obtained by using analytical model 1

Binary mixtures	Experimental data		Estimated data		Interaction effect	*P* value
	Observed EC_{50}* (μmol/L)	Observed standard error	Expected EC_{50} (μmol/L)	$EC_{50\ (\mathrm{Diff})}$		
Hg and Cd	12.94	0.842	8.31	− 4.63	ANT*	0.008
Hg and Cr	0.23	0.018	0.53	+ 0.30	SYN**	0.001
Hg and Pb	341.82	48.564	358.44	+ 16.62	ADD***	0.614
Hg and Ni	195.9	23.101	205.32	+ 9.42	ADD***	0.312
Cd and Cr	7.12	0.551	8.15	+ 1.03	SYN**	0.031
Cd and Pb	479	71.003	366	− 113	ANT*	0.001
Cd and Ni	365	34.452	213	− 152	ANT*	0.001
Cr and Pb	495	53.307	358.1	− 136.9	ANT*	0.001
Cr and Ni	344.32	26.992	358.1	+ 13.78	ADD***	0.721
Pb and Ni	350.1	33.456	358.1	+ 8.00	ADD***	0.334

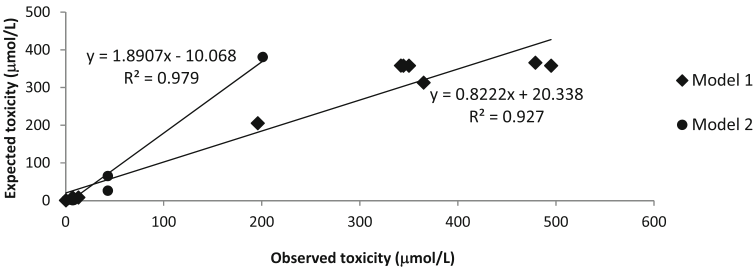
EC_{50} = effective concentrations of two combined metals that induce a 50% reduction in dehydrogenase enzyme activity after 30-min exposure

Expected EC_{50} = computed EC_{50} value $(C_m/((C_x/EC_{50x}) + (C_y/EC_{50y})))$

$EC_{50(\mathrm{Diff})}$ = expected EC_{50} − observed EC_{50}

**ANT*, antagonistic effect; ***SYN*, synergistic effect; ****ADD*, additive effect (synergistic or antagonistic effects are stated at $P < 0.05$)

Fig. 2 Relationship between observed and expected toxicity of heavy metals, using two different models



on activated sludge bacteria. The fact that Cd, under our experimental conditions, may reduce or suppress the toxicity of some other heavy metals is very puzzling. This effect, sometimes observed in other studies (Caballero Araúz et al. 2008; Mowat and Bundy 2002), is in all probability limited to the prokaryote bioassay but, anyway, deserves more complete studies.

Mowat and Bundy (2002) reported that joint effect of Pb and Cd can be considered an additive effect when they apply in equal concentration but they differently induce antagonistic interaction when in binary mixture Pb was used either 25% or 75%. Caballero Araúz et al. (2008), when studying toxicity interaction of Cd and Se on *Lactobacillus caseirhamnosus*, also reported an antagonistic effect. According to their results, in the presence of Cd and Se, the bacterial growth was motivated and lipid peroxidation was lower than the presence of Cd alone. They suggest that the antagonistic effect of Se can be related to lower reaction of Cd at a high molecular mass.

Regarding requirement in adequate environmental protection, antagonistic interactions cannot be considered in establishing standard limits and regulations because such interactions cannot be considered for all possible chemical or toxicants in real situations. However, any possible synergistic effects in such environments can be used.

When a toxic chemical can cause cells to accumulate and assimilate other toxic chemicals or prohibit its degradation and excretion, the interaction is known as synergistic. Synergistic term when used that the interaction result of multi-toxicant mixture is more toxic than the sum of their separate toxicity effect.

In two mixtures (Hg and Cr and Cd and Cr), the obtained result is more toxic than the sum of the effects

Table 4 Experimental versus expected TU values of all the possible binary mixtures of Hg, Cd, Cr, Pb, and Ni on activated sludge bacteria using the dehydrogenase enzyme assay. Results obtained by using analytical model 2

Binary mixtures	Experimental data		Estimated data		TU_{Diff}	Interaction effect	*P* value
	Observed TU	Observed standard error	Expected TU (TU_{H0})	Standard error of TU_{H0}			
Hg and Cd	43	3	26	1.6	− 17	ANT*	0.001
Hg and Cr	201	19	381	22	+ 180	SYN**	0.006
Hg and Pb	0.9	0.1	1.1	0.1	+ 0.2	ADD***	0.190
Hg and Ni	1	0.1	1.4	0.1	+ 0.4	ADD***	0.420
Cd and Cr	43	5.7	65	3.2	+ 22	SYN**	0.014
Cd and Pb	7	0.4	1	0.1	− 6.0	ANT*	0.001
Cd and Ni	8.1	0.9	1.2	0.1	− 6.9	ANT*	0.041
Cr and Pb	1.4	0.1	0.8	0.01	− 0.6	ANT*	0.032
Cr and Ni	0.6	0.1	0.7	0.01	+ 0.1	ADD***	0.570
Pb and Ni	0.6	0.01	0.8	0.01	+ 0.2	ADD***	0.410

TU = toxicity unit = $100/EC_{50}$

TU_{H0} = expected toxicity units of H_0 ($TU_{H0(x+y)} = TU_x + TU_y$)

TU_{Diff} = differential toxicity units ($TU_{H0} - TU_{Obs}$)

**ANT*, antagonistic effect; ***SYN*, synergistic effect; ****ADD*, additive effect (synergistic or antagonistic effects are stated at $P < 0.05$)

of the separate heavy metals, illustrating a synergistic effect. The synergism which is proved when Hg and Cr or Cd and Cr are mixed together is more complicated. In this regard, one of the proposed mechanisms is the early acting of the chemicals on the energy supplying system while the other toxicant affects later on (Tachikawa and Yamanaka 2014).

Using the *Caenorhabditis elegans*, a free-living nematode species, to evaluate metal toxicity in some binary mixtures, Chu and Chow (2002) found the interactions between Hg and Cd, Cd and Pb, and Ni and Pb to be synergistic, Zn and Cd to be antagonistic, and Cd and Ni, and Ni and Hg to be additive. In this regard, results of the present study were absolutely different (Tables 3 and 4) from previous study. However, comparison of the other similar mixtures showed our results concerning mixtures of Pb with Hg and also Ni and Hg are in agreement with those of Chu and Chow (2002). The most important difference between these studies is the use of different bioassay methods. With respect to the different results obtained in the previous investigations and in this study, one bioassay could not provide results equivalent to those of another bioassay. Overall, the interactions of heavy metals are complicated to generalize since they were dependent on both the organism used in each bioassay and chemical type.

Conclusions

The interaction of metals in the Hg and Cd, Cd and Pb, Cd and Ni, and Cr and Pb mixtures can be expressed as antagonistic effect while interaction in Hg and Cr and Cd and Cr mixtures can be considered as synergistic. Interaction of other studied mixtures including Hg and Pb, Hg and Ni, Cr and Ni, and Pb and Ni can be described as additive. Results of present study in comparison with the other studies revealed a complex pattern of possible interactions between heavy metals. Therefore, as a safer discharge practice, the standard value for each single heavy metal must consider the probable hazardous interactions, and all demonstrated synergistic interactions should be certainly manipulated.

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