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Toxicity of four metals and their mixtures to *Pseudomonas fluorescens*: An assessment using fixed ratio ray design

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Abstract

The toxicities of binary (Ni(II) + Co(II) and Zn(II) + Cd(II)), ternary (Zn(II) + Cd(II) + Ni(II) and Ni(II) + Co(II) + Cd(II)) and quaternary (Ni(II) + Co(II) + Zn(II) + Cd(II)) mixtures of metal ions to *Pseudomonas fluorescens* were assessed by using inhibition of dehydrogenase activity as an endpoint. Uniform design concentration ratio (UDCR) and equieffect concentration ratio (EECR) mixtures were designed to evaluate the combined toxicities of these heavy metal ions. All the dose-response relationships of the UDCR and EECR mixtures and the individual metals could be described by logistic function. Toxicities predicted by concentration addition (CA) and independent action (IA) models were compared with the observed toxicities. The CA and IA models predicted similar toxicities of the binary and Zn(II) + Cd(II) + Ni(II) ternary mixtures. Generally, Zn(II) + Cd(II) + Ni(II) ternary mixtures were synergistic while Ni(II) + Co(II) + Cd(II) ternary mixtures were antagonistic. The equieffect and quaternary mixtures were generally synergistic. Synergistic, antagonistic and additive effects of the mixtures are possible from the viewpoint of these analyses.

Keywords: Heavy metals, dehydrogenase activity, concentration addition model, independent action model.

INTRODUCTION

Heavy metals are widespread pollutants of great concern because they are non-degradable and thus persistent (Jansen *et al*., 1994). Although heavy metals are naturally occurring elements, most environmental contaminations result from human activities such as mining and smelting operations, industrial production, domestic and agricultural use of metals. Industrial effluents are discharged into the environment and thus exposing humans, animals and microorganisms to the harmful effects of metal pollutants. Some heavy metals (such as Zn, Co, Cu and Ni) are essential trace elements required for normal physiological function of microorganisms. They serve as micronutrients and are used for redox processes to stabilize molecules, as components of enzymes and for regulation of osmotic pressure (Bruins *et al*., 2000). However, at high concentrations, these essential metals can be inhibitory to microbial processes. Other heavy metals (such as Cd, Hg and Pb) have no physiological function and are toxic even at low concentrations.

Heavy metals generally exert toxicity in microorganisms by blocking essential functional groups, displacing essential metal ions or modifying the active conformation of biomolecules (Wood *et al*., 1983), denaturing and inactivating enzymes and disrupting cell membrane integrity (Gadd, 1993). Given that microorganisms respond promptly to environmental pollution, factors affecting their metabolism and diversity are of great importance. Therefore, toxicity tests using living organisms constitute a vital part of environmental monitoring (Awasthi, 2012).

Metals as contaminants rarely occur in isolation (Gikas, 2008). Cadmium, zinc, cobalt and nickel are widely used in many industrial processes and thus exist as co-contaminants in the environment (Nies, 1992). It is more suitable and advantageous to study their interactive effects in the environment. The combined toxic effects of multiple chemicals are recognized as an important consideration in ecotoxicology because mixtures of chemicals can possibly induce synergistic effects (McCarty & Borgert, 2006). Thus, in order to determine the ecotoxicological implications of

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metal contamination of natural environment, it is important to evaluate the joint effects of metal mixtures on natural biota. Such assessment requires sensitive, rapid, cheap and reliable toxicity test. In this regard, different microbial parameters such as growth rate (Juliastuti *et al*., 2003), biomass measurement (Guckert, 1996), inhibition of bioluminescence (Ren & Frymier, 2003; Ince *et al*., 1999; Preston *et al*., 2000; Fulladosa *et al*., 2005) and activity of enzymes (Bitton *et al*., 1992) have been adopted to evaluate toxic effects of metals on microbial population. Investigations on the toxicity of metal mixtures have mainly based on inhibition of bacterial bioluminescence.

In this study, fixed ratio ray design was used to evaluate the interactive effects of binary, ternary and quaternary mixtures of Cd(II), Zn(II), Co(II) and Ni(II) ions on *Pseudomonas fluorescens* based on inhibition of dehydrogenase activity. *P. fluorescens* is an environmentally-important bacterium that is ubiquitously distributed in soil and water. It has diverse metabolic capabilities and grows in minimal media supplemented with wide range of carbon sources and has found a number of applications in the area of bioremediation (Richard & Vogel, 1999; Bugg *et al*., 2000), control of plant pathogens and plant growth promotion (Ganeshan & Kumar, 2005; Sivasakthi *et al*., 2013). Thus, studying the factors that influence the metabolic activities of the organism is important. Such factors include many inorganic and organic pollutants that found their way into the environment via anthropogenic and natural sources.

MATERIALS AND METHODS

Metal ions and reagents

The Ni(II), Cd(II), Co(II) and Zn(II) ions were used as $NiSO_4.6H_2O$, $CdSO4.8/3H_2O$, $CoCl_2$ and $ZnSO_4.7H_2O$. Stock solutions of 10 mM of the individual metal ion were prepared in deionized distilled water. Working stock solution of 1mM Cd(II) was prepared by diluting the 10 mM stock 1/10 with deionized distilled water. The 2,3,5-triphenyltetrazolium chloride (TTC) was purchased from Sigma (Germany). All reagents are of analytical grade.

Test organism

The test bacterium *Pseudomonas fluorescens* was isolated from soil on *Pseudomonas* agar for fluorescein (Sisco Research Laboratories (SRL), Mumbai, India). The organism fluoresces green under ultraviolet light on *Pseudomonas* agar. The identity of the bacterium was further confirmed by oxidase production, oxidative fermentation, growth at 4 ^{o}C, no growth at 42 ^{o}C, nitrate reduction, phosphate solubilization (using Pikovskaya's broth, SRL, India), acid production from mannitol and maltose.

The bacterium was grown to mid exponential phase in nutrient broth (Lab M) on a rotary incubator (150 rpm) at room temperature (28 ± 2°C) and the cells harvested by centrifugation at 3000 rpm for 10 min. Harvested cells were washed twice in sterile deionized distilled water to avoid any nutrient carryover. Washed cells were re-suspended in sterile deionized distilled water and the optical density adjusted to 0.1 at 540 nm. The cell suspensions were used as inoculum in the toxicity assay.

Design of mixture experiments

The toxicity of the binary mixtures of Ni(II) and Co(II) as well as Zn(II) and Cd(II) were determined using fixed ratio designs, uniform design concentration ratios (UDCR) and equieffect concentration ratios (EECR). While keeping the mixture ratio constant, the total concentrations of the mixture were varied in order to obtain a complete dose-response relationship of the mixture. The concentration range of the mixtures was based on the concentration range of the individual metals that gave percent inhibitions ranging from 0% to 100% or near 100%. The concentrations of Ni(II), Co(II) and Zn(II) ranged from 0 to 2 mM while the concentrations of Cd(II) ranged from 0 to 0.2 mM such that the concentration series of Cd(II) was one tenth concentration series of Ni(II), Co(II) or Zn(II). The binary mixtures of Ni(II) and Co(II) were studied as a function of the following weight to weight ratios: p (%) = 80, 50 and 20, of Ni(II), and 100-p (%) of Co(II) corresponding to Ni(II): Co(II) ratios of: 80:20 %, 50:50 % and 20:80 %. Similarly, the binary mixtures of Zn(II) and Cd(II) were studied as a function of the following weight to weight ratios: p (%) = 80, 50 and 20 of the concentration series of Zn(II), and 100-p (%) of the concentration series of Cd(II) corresponding to Zn(II): Cd(II) ratios of: 80:20 %, 50:50 % and 20:80 % based on their respective concentration series. The effective Zn(II):Cd(II) fixed ratios were thus 97.56% Zn(II):2.44% Cd(II), 90.91% Zn(II):9.09% Cd(II) and 71.43% Zn(II):28.57% Cd(II) respectively. Additional two different mixture ratios were examined based on the relative toxicity of the individual metals. First, a mixture in which components was mixed in the ratio of the EC_{20} of the individual metals (referred to as EECR-20 mixture). Second, the toxicity of the mixtures based on EC_{50} (EECR-50 mixture) of the individual metals were assessed. The EECR-20 and EECR-50 equieffect mixtures of Ni(II) and Co(II) yielded effective ratios of 66.06% Ni(II):33.94% Co(II) and 74.32% Ni(II):25.68% Co(II) respectively. Similarly, the EECR-20 and EECR-50 equitoxic mixtures of Zn(II) and Cd(II) yielded effective ratios of 86.62% Zn(II):13.38% Cd(II) and 89.11% Zn(II):10.89% Cd(II) respectively.

Using the concentration ranges as described for the binary mixtures, the ternary mixtures, Zn(II) + Cd(II) + Ni(II) and Ni(II) + Co(II) + Cd(II) were also designed along fixed ratio rays. Three uniform design concentration ratios (UDCR) and two equieffect concentration ratios (EECR-20 and EECR-50) were evaluated. The quaternary mixtures of the four metals ions [Ni(II) + Co(II) + Zn(II) + Cd(II)] were also designed along fixed ratio rays. The specific ratios of metal ions in the ternary and quaternary mixtures are as shown in Table 1. The mixtures were prepared as 10 mM stock solutions by mixing requisite volumes of the individual metal ion solution to give a specific concentration ratio. Each mixture was treated as single metal ion solution.

Acute toxicity assay

The assay for dehydrogenase activity in the presence of metal ions was modified from Nweke *et al*. (2014). 2,3,5-triphenyltetrazolium chloride (TTC) was used as an artificial electron acceptor instead of 2-(p-Iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) in a lower strength culture medium. The reaction mixture consisted of 2-ml final volumes of low-strength nutrient broth supplemented with varying concentrations of metal ions (pH 7.2). Into each tube containing 0.5 ml portion of X4-strength nutrient broth, requisite volumes of distilled water and stock solutions of respective metal ion(s) were added. The final amount of nutrient broth in the reaction mixture was 0.2% w/v. Thereafter, 0.1ml of 0.1% aqueous solution of TTC and 0.1 ml of the standardized bacterial suspension were added into each tube to obtain varying concentrations of metal ions for the individual metals and the mixture ratios. Each concentration of the binary mixture and the individual metals were prepared in triplicates. Controls were prepared

Table 1: Experimental (observed) and predicted toxicity thresholds (EC_{50}) of individual metals and their mixtures

Metals and mixtures	EC_{50} (mM)		
	Experimental	Predicted	
		CA	IA
Ni(II)	0.356 ± 0.028	-	-
Co(II)	0.123 ± 0.006	-	-
Zn(II)	0.180 ± 0.010	-	-
Cd(II)	0.022 ± 0.001	-	-
NI(II) + Co(II) mixtures			
Ni(II) 20% + Co(II) 80%	0.305 ± 0.033	0.139 ± 0.004	0.144 ± 0.004
Ni(II) 50% + Co(II) 50%	0.205 ± 0.022*	0.179 ± 0.006*	0.191 ± 0.001*
Ni(II) 80% + Co(II) 20%	0.273 ± 0.020*	0.253 ± 0.010*	0.283 ± 0.002*
Ni(II) 66.06% + Co(II) 33.94% (EECR 20)	0.138 ± 0.021	0.212 ± 0.008	0.232 ± 0.001
Ni(II) 74.32% + Co(II) 25.68% (EECR 50)	0.141 ± 0.024	0.239 ± 0.015	0.260 ± 0.001
Zn(II) + Cd(II) mixtures			
Zn(II) 71.43% + Cd(II) 28.57%	0.056 ± 0.008*	0.060 ± 0.004*	0.062 ± 0.001*
Zn(II) 90.91% + Cd(II) 9.09%	0.117 ± 0.017*	0.110 ± 0.007*	0.117 ± 0.001*
Zn(II) 97.56% + Cd(II) 2.44%	0.078 ± 0.018	0.154 ± 0.009	0.165 ± 0.004
Zn(II) 86.62% + Cd(II) 13.38% (EECR-20)	0.044 ± 0.008	0.093 ± 0.006	0.098 ± 0.000
Zn(II) 89.11% + Cd(II) 10.89% (EECR-50)	0.051 ± 0.011	0.102 ± 0.006	0.108 ± 0.001
Zn(II) + Cd(II) + Ni(II) Ternary Mixtures			
Zn(II) 68.49% + Cd(II) 4.11% + Ni(II) 27.40%	0.068 ± 0.007	0.154 ± 0.012	0.169 ± 0.004
Zn(II) 36.585% + Cd(II) 2.439% + Ni(II) 60.976%	0.110 ± 0.012	0.207 ± 0.014	0.221 ± 0.006
Zn(II) 43.48% + Cd(II) 13.04% + Ni(II) 43.48%	0.040 ± 0.005	0.106 ± 0.007	0.112 ± 0.002
Zn(II) 33.70% + Cd(II) 5.20% + Ni(II) 61.10% (EECR-20)	0.070 ± 0.008	0.169 ± 0.012	0.182 ± 0.006
Zn(II) 32.30% + Cd(II) 3.90% + Ni(II) 63.80% (EECR-50)	0.055 ± 0.007	0.188 ± 0.013	0.202 ± 0.007
Ni(II) + Co(II) + Cd(II) Ternary Mixtures			
Ni(II) 36.58% + Co(II) 60.58% + Cd(II) 2.44%	0.225 ± 0.019	0.139 ± 0.005	0.160 ± 0.001
Ni(II) 32.57% + Co(II) 65.93% + Cd(II) 1.10%	0.401 ± 0.026	0.145 ± 0.004	0.161 ± 0.002
Ni(II) 60.98% + Co(II) 36.58% + Cd(II) 2.44%	0.222 ± 0.018	0.170 ± 0.006	0.203 ± 0.005
Ni(II) 62.54% + Co(II) 32.13% + Cd(II) 5.33% (EECR-20)	0.333 ± 0.026	0.146 ± 0.007	0.178 ± 0.006
Ni(II) 71.08% + Co(II) 24.55% + Cd(II) 4.39% (EECR-50)	0.235 ± 0.019	0.165 ± 0.008	0.201 ± 0.007
Ni(II) + Co(II) + Zn(II) + Cd(II) Quaternary Mixtures			
Ni(II) 60.98% + Co(II) 24.39% + Zn(II) 12.19% + Cd(II) 2.43%	0.102 ± 0.016	0.183 ± 0.012	0.224 ± 0.010
Ni(II) 32.258% + Co(II) 32.258% + Zn(II) 32.258% + Cd(II) 3.226%	0.068 ± 0.017	0.148 ± 0.009	0.182 ± 0.009
Ni(II) 46.48% + Co(II) 23.88% + Zn(II) 25.67% + Cd(II) 3.97% (EECR-20)	0.095 ± 0.012	0.155 ± 0.010	0.192 ± 0.010
Ni(II) 52.28% + Co(II) 18.06% + Zn(II) 26.43% + Cd(II) 3.23% (EECR-50)	0.147 ± 0.022	0.171 ± 0.011	0.209 ± 0.011

*EC_{50} predicted from either CA or IA were not statistically different from observed EC_{50} ($p > 0.05$)

without the toxicants. Triplicate control tubes were prepared for each metal ion, giving a total of 12 controls. The cultures were incubated at room temperature (28 ± 2°C) for 24 h. The extraction and quantification of TTC-formazan were done as described by Nweke *et al*. (2016).

Data analysis

The inhibition of dehydrogenase activity from each toxicity assessment was transformed relative to the mean control (SD < 5%) to a 0 to 100% scale as shown in equation 1. The normalized responses were generated as mean and their standard deviations from triplicate determinations.

$$R=\left(1-\frac{T_A}{C_A}\right)\times 100 \qquad (1)$$

Where R is the inhibition (%) of dehydrogenase activity, C_A is the absorbance of TPF extract in the control experiment and T_A is absorbance of TPF extract in the test experiment with different concentrations of metal ion(s).

The dose-response data of the single substances as well as the mixtures were then plotted and fitted with 2-parameter logistic function (equation 2).

$$R=\frac{100}{1+\left(\frac{x}{EC_{50}}\right)^{b}} \qquad (2)$$

Where x is the concentration of metal ion(s), EC_{50} is the concentration of metal ion(s), that inhibited dehydrogenase activity by 50% and b is the slope at EC_{50}.

Prediction of mixture toxicities

The toxicities of the mixtures can be determined from the toxicity of the individual component based on concentration addition (CA) model if the relative composition of each component is quantitatively known. The concept of concentration addition assumes that the components of the mixture acts similarly against the test organism. The CA model can be written as (Berenbaum, 1985):

$$EC_{x(mix)}=\left(\sum_{i=1}^{n}\frac{\pi_i}{EC_{xi}}\right)^{-1} \qquad (3)$$

Where $ECx_{(mix)}$ is the total concentration of the mixture that elicited x% effect, EC_{xi} is the concentration of ith component that gave x effect when tested as an individual, n is the number of components, π_i is the proportion of ith component in the mixture, such that the sum of π_i = 1. Using equation 3, the toxicities of the mixtures were predicted as described elsewhere (Altenburger *et al*., 2000; Backhaus *et al*., 2000). The total concentration of each mixture that elicited 1 – 99% effects were calculated in steps of 1%. The resulting 99 concentration/effect pairs were plotted as a line chart giving a visualization of the predicted dose-response curve. First, the EC_x for 1 – 99% was calculated for each component from the logistic dose-response model that fitted the individual dose-response data. Secondly, the EC_x values were substituted in equation 3 to obtain the 1 – 99% $ECx_{(mix)}$ values for each mixture. These calculations were done with Microsoft Excel 2003 and the data obtained were plotted using Sigmaplot 10.

The independent action (IA) or response addition model assumes that the components of a given mixture have different mode of action. The mathematical expression is as follows (Altenburger *et al*., 2000; Faust *et al*., 2003):

$$E(c_{mix})=1-\prod_{i=1}^{n}\left[1-E(c_i)\right] \qquad (4)$$

Where $E(c_{mix})$ represents the total effect or response (scaled from 0 to 1) of an n-component mixture, c_i is the concentration of the ith component and $E(c_i)$ is the effect or response of the individual component. The dose-response relationships F_i of the individual components were used to calculate their effects $E(c_i)$ as shown below (Backhaus *et al*., 2000):

$$E(c_{mix})=1-\prod_{i=1}^{n}\left[1-E(c_i)\right]=1-\prod_{i=1}^{n}\left[1-F_i(c_i)\right] \qquad (5)$$

By expressing the concentrations of the individual components as fractions, π_i, of the total concentration, c_{mix}, the overall effect of any given mixture concentration can be calculated as:

$$E(c_{mix})=1-\prod_{i=1}^{n}\left[1-E(c_i)\right]=1-\prod_{i=1}^{n}\left[1-F(\pi_i.c_{mix})\right] \qquad (6)$$

The total effect $E(c_{mix})$ of each mixture were calculated for c_{mix} values ranging from 0.004 – 4 mM and multiplied by 100, as shown in equation 7, to rescale the effect from 0 to 100%.

$$E(c_{mix})=\left[1-\prod_{i=1}^{n}\left[1-F(\pi_i.c_{mix})\right]\right]\times 100 \qquad (7)$$

To implement this, equation 2 was substituted into equation 7 for each metal ion as:

$$R=\frac{1}{1+\left(\frac{\pi_i x}{EC_{50}}\right)^{b}} \qquad (8)$$

Thus, the simplified expression of the independent action model is given as:

$$E(c_{mix})=\left[1-\prod_{i=1}^{n}\left[1-\frac{1}{1+\left(\frac{\pi_i x}{EC_{50i}}\right)^{b_i}}\right]\right]\times 100 \qquad (9)$$

Where $\pi_i x$ is the concentration of *i*th component in the mixture. The values of EC_{50i} and b_i as generated from equation 2 for individual metal ion were used.

The resulting concentration/effect pairs were plotted as a line chart giving a visualization of the dose-response curve predicted from the independent action model.

The toxic index (TI)

The Toxic Index (TI) of each mixture was calculated as sum of toxic units for all the components of the mixture (equation 10).

$$TI = \sum_{i=1}^{n} \frac{C_i}{EC_{50i}} = \sum_{i=1}^{n} \frac{\pi_i EC_{50mix}}{EC_{50i}} \qquad (10)$$

Where C_i is the concentration of the *i*th component in the mixture at the EC_{50} of the mixture (EC_{50mix}) and EC_{50i} is the concentration of the *i*th component that elicited 50% decrease in dehydrogenase activity when tested as an individual, *n* is the number of components in the mixture and π_i is the proportion of *i*th component in the mixture. TI = 1 describes additivity, TI > 1 describes antagonistic interaction and TI < 1 describes synergistic interaction (Boillot & Perrodin, 2008).

Model deviation ratios

The model deviation ratios (MDR) were calculated as the ratio of the predicted EC_{50} to the experimentally-derived EC_{50}. MDR greater than 1 indicated that the model underestimated toxicity, while a value of less than 1 indicated that the model overestimated toxicity. MDR values ranging from 0.5 to 2 ($0.5 \leq MDR \leq 2$) indicated that the mixture was most likely to be additive (Petersen & Tollefsen, 2011; Li *et al*., 2014).

Isobolographic analysis

The estimated EC_{50} were used in subsequent determination of isoboles and isobolographic analysis of the binary mixture toxicity as described by Nweke *et al*. (2014). The concentrations of each component at EC_{50} (C_i) were calculated and used to compute the isoboles. The C_i values (C_{iA} and C_{iB}) for the components can be calculated by multiplying the proportion of individual component in the mixture by the EC_{50} of the mixture as in the numerator of equation 10. Triplicate isoboles were generated and plotted in an isobologram as described elsewhere (Boillot & Perrodin, 2008; Nweke *et al*., 2014). The straight line joining the EC_{50} of component A on one axis and EC_{50} of component B on the other axis is the line of additivity representing the additive effect of the mixture. When an isobole plotted in the isobologram is below or above the additivity line, the interaction is taken to be synergistic or antagonistic respectively.

RESULTS

Toxicity of individual metal ion

The effects of the individual metal ion on the dehydrogenase activity of the *P. fluorescens* are shown in Figure 1. The responses of the organism to the toxicity of the metal ions was dose-dependent. The metal ions progressively inhibited the dehydrogenase activity as the concentrations were increased, giving percent inhibitions greater than 95% at 2 mM Ni(II) and Zn(ii), 1.5 mM Co(II) and 0.2 mM Cd(II). The EC_{50} values of the metals ranged from 0.022 mM for Cd(II) to 0.356 mM for Ni(II) (Table 1). The experimental data for all the metals were fitted with 2-parameter logistic model to obtain the predicted dose-response curve. The shapes of the dose-response curves are rather similar, indicating the similarity of the molecular mechanisms of the actions of the metal ions. The Duncan test indicates that the EC_{50} of the metals were significantly different from each other and the order of toxicity is Cd(II) > Co(II) > Zn(II) > Ni(II).

Toxicity of mixtures

The experimental dose-response relationships of the binary mixtures as well as the predictions made from CA and IA models are shown in Figures 2 and 3. The concentration addition and independent action concepts predicted similar toxicities for the binary mixtures, especially for Zn(II) and Cd(II) mixtures, and their dose-response curves were almost superimposed. The EC_{50} values predicted from the concepts for Zn(II) + Cd(II) mixtures were not statistically different from each other. As seen from Figure 2, both CA and IA models predicted higher toxicity for 20% Ni(II): 80% Co(II) mixture than suggested by the experimental data. As shown in Figure 2, both models predicted lower toxicities of equieffect mixtures (EECR-20 and EECR-50) of Ni(II) and Co(II) at low concentrations of the metal ions. Both models predicted accurately the toxicities of 50% Ni(II) + 50% Co(II) and 80%Ni(II) + 20% Co(II) mixtures.

In the case of Zn(II) and Cd(II) mixtures, the concepts of concentration addition and independent action predicted lower toxicities of EECR-20, EECR-50 and 97.56% Zn(II) + 2.44% Cd(II) mixtures than the observed toxicities (Figure 3). Toxicities similar to the experimentally derived values were predicted by both concepts for 90.91% Zn(II) + 9.09% Cd(II) and 71.43% Zn(II) + 28.57% Cd(II) mixtures (Figure 3).

The EC_{50} of the binary mixtures as derived from logistic model regression of the experimental data and as predicted from concentration addition and independent action models are shown in Table 2. Statistical analysis indicated that there were no significant differences ($p > 0.05$) between the EC_{50} values estimated from both models for all the Zn(II) + Cd(II) mixtures. Lack of significant difference between the EC_{50} values predicted by both models only occurred with 50% Ni(II) + 50% Co(II) and EECR 50 mixtures of Ni(II) and Co(II). The EC_{50} derived experimentally were not significantly different from the EC50 values predicted by both models for 80% Ni(II) + 20% Co(II) and 50% Ni(II) + 50% Co(II) mixtures. Similarly, for the mixtures 71.43% Zn(II) + 28.57% Cd(II) and 90.91% Zn(II) + 9.09% Cd(II), the EC_{50} values predicted by both concepts were not statistically different from the values obtained experimentally.

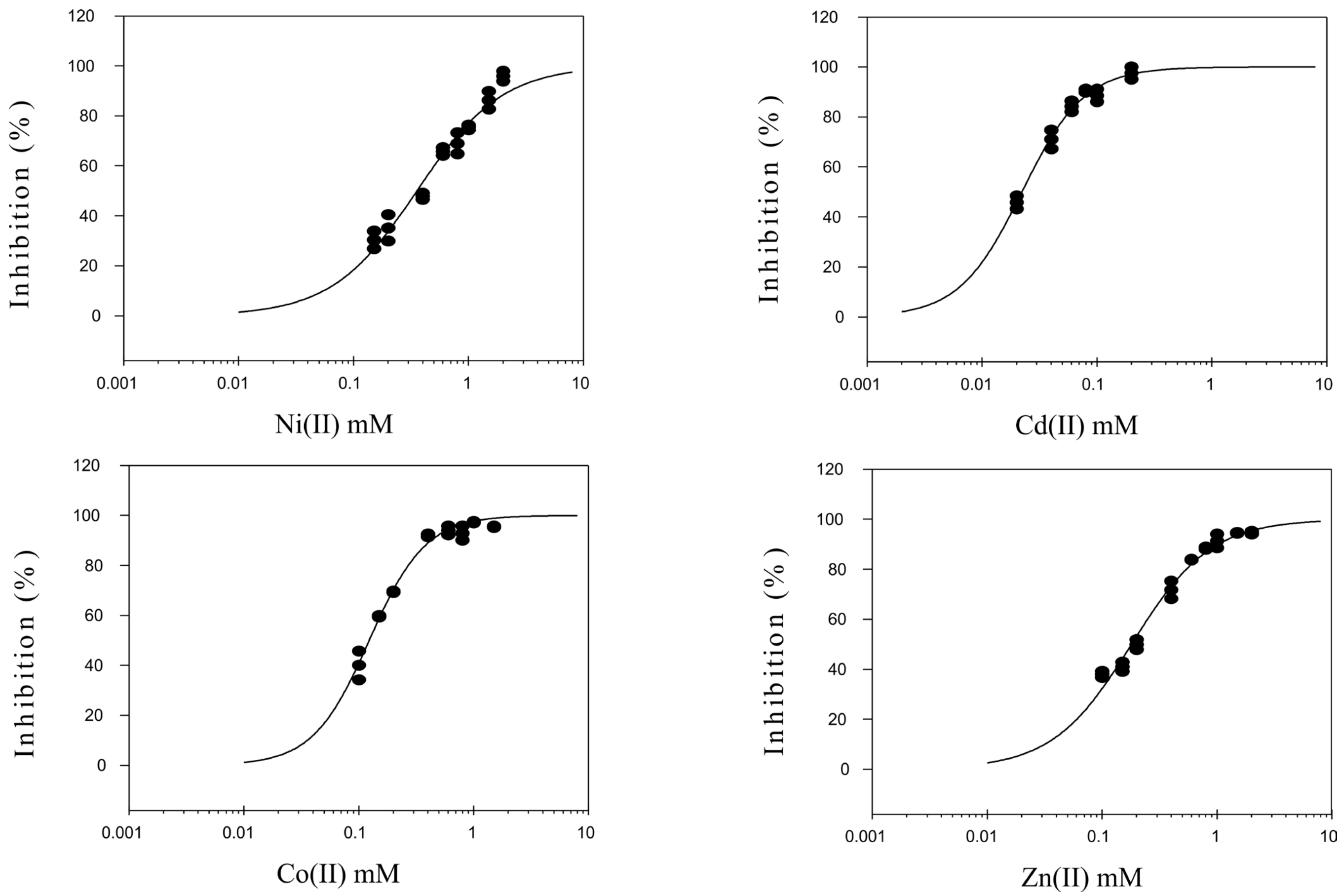

Figure 1: Inhibition of dehydrogenase activity in *Pseudomonas fluorescens* by the individual metal ions

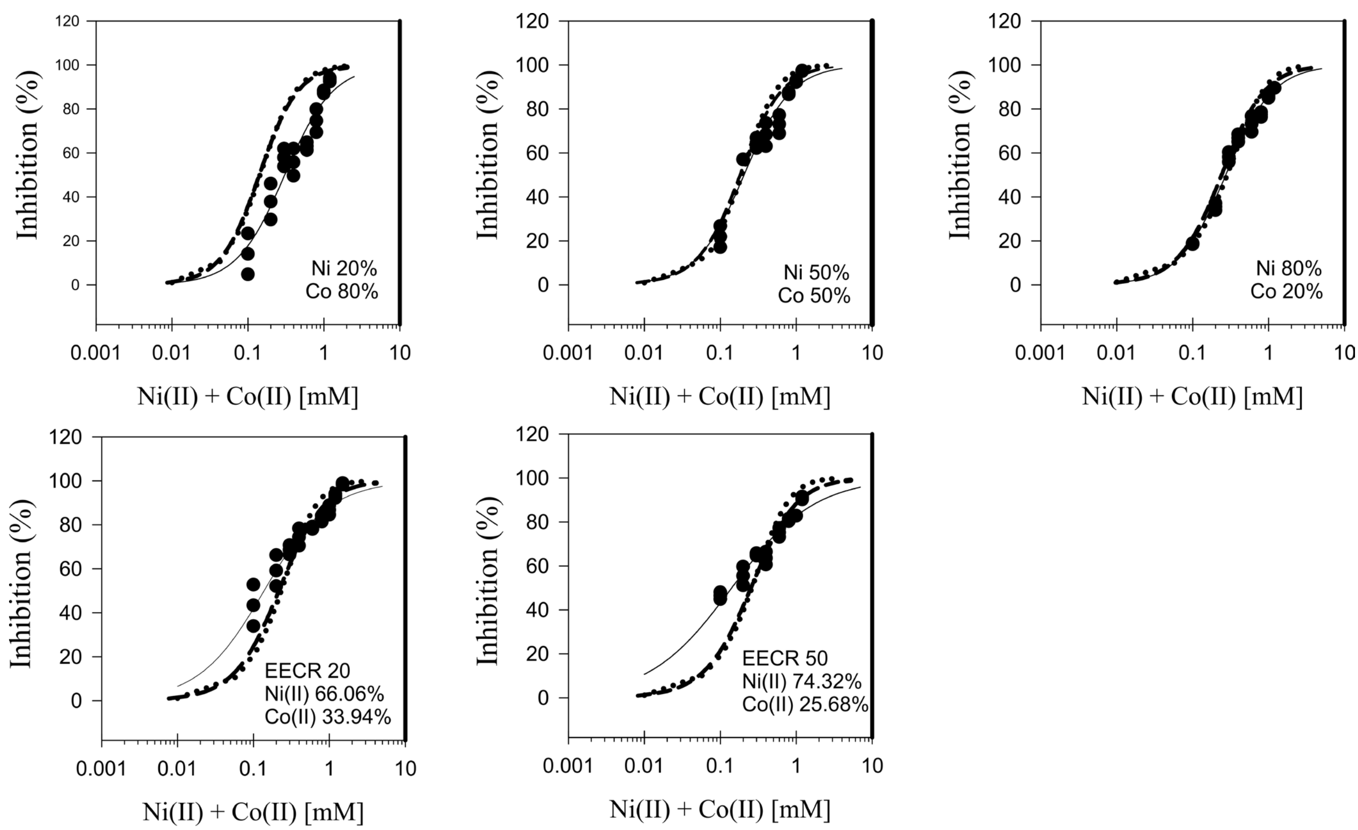

Figure 2: Observed and predicted inhibitions of *Pseudomonas fluorescens* dehydrogenase activity by binary mixtures of nickel and cobalt ions. The data points represent experimental dose-response data. The solid lines represent toxicities obtained from fitting experimental data to logistic model (equation 2). Dashed lines represent toxicities predicted from the concentration addition model while dotted lines represent toxicities predicted from the independent action model

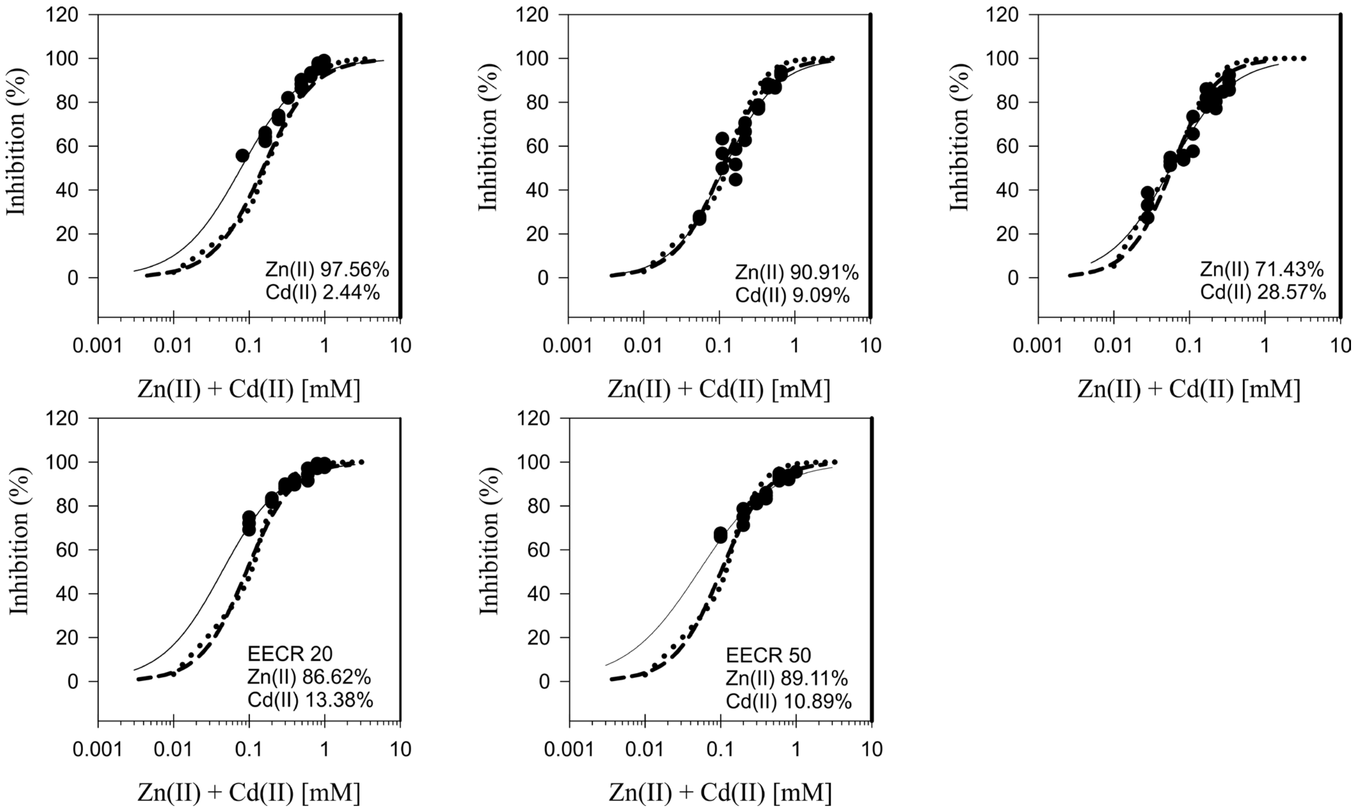

Figure 3: Observed and predicted inhibitions of *Pseudomonas fluorescens* dehydrogenase activity by binary mixtures of zinc and cadmium ions. The data points represent experimental dose-response data. The solid lines represent toxicities obtained from fitting experimental data to logistic model (equation 2). The Dashed lines represent toxicities predicted from the concentration addition model while the dotted lines represent toxicities predicted from independent action model.

The isobolographic analyses of the binary mixtures based on the EC_{50} values are shown in Figure 4. The isobologram indicated synergistic effect of the EECR-20 and EECR-50 equieffect mixtures, additive effect of 50% Ni(II) + 50% Co(II) and 80% Ni(II) + 20% Co(II) and antagonistic effect of 20% Ni(II) + 80% Co(II) for Ni(II) + Co(II) mixtures. In addition, for the Zn(II) + Cd(II) mixtures, the isobologram depicts synergistic effect of both equieffect mixtures and 97.56% Zn(II) + 2.44% Cd(II) as well as additive effects of 71.43% Zn(II) + 28.57% Cd(II) and 90.91% Zn(II): 9.09% Cd(II) mixtures. These observations were corroborated by the toxic index values as shown in Table 2.

The dose-response relationships for ternary and quaternary mixtures of metal ions are shown in Figures 5 - 7. The dose-response patterns are similar to those of binary mixtures and were describable with the 2-parameter logistic model. The EC_{50} of the mixtures are shown in Table 1. The EC_{50} of the Zn(II) + Cd(II) + Ni(II) mixtures ranged from 0.035 mM to 0.122 mM and seem to be dependent on the relative amount of Cd(II) in the mixture. Similar observation was made for Ni(II) + Co(II) + Cd(II) mixtures with EC_{50} ranging from 0.204 mM to 0.427 mM. The EC_{50} of the quaternary mixtures ranged from 0.051 mM to 0.169 mM. The toxicities of the ternary and quaternary mixtures as predicted from CA and IA models are shown in Table 1. Generally, CA and IA models predicted lower toxicities of Zn(II) + Cd(II) + Ni(II) mixtures than the experiment would suggest. However, the reverse was the case with Ni(II) + Co(II) + Cd(II) mixtures where the models predicted higher toxicities. The EC_{50} predicted by CA and IA models in all the Zn(II) + Cd(II) + Ni(II) mixtures were not significantly different from each other. As shown in Figure 7, CA and IA models predicted lower toxicities of the quaternary mixtures than were experimentally observed. However, the IA model predicted higher toxicities than CA model in all the quaternary mixtures.

The toxic indices and the effect of the mixtures on dehydrogenase activities are shown in Table 2. Generally, the ternary mixtures of Zn(II), Cd(II) and Ni(II) are synergistic while the ternary mixture of Ni(II), Co(II) and Cd(II) are antagonistic. The quaternary mixtures are synergistic except the EECR-50 mixture.

The relationship between the observed and predicted EC_{50} of the metal ion mixtures are shown in Figure 8. There were generally no good correlation between the observed EC_{50} and the EC_{50} predicted from either CA or IA model. However, there was fairly good correlation between observed and predicted EC_{50} for Zn(II) + Cd(II) + Ni(II) ternary mixtures (R^2 = 0.632 (CA); R^2 = 0.509 (IA)) Generally, observed EC_{50} decreased with increase in predicted EC_{50} for the binary mixtures of Ni(II) + Co(II)

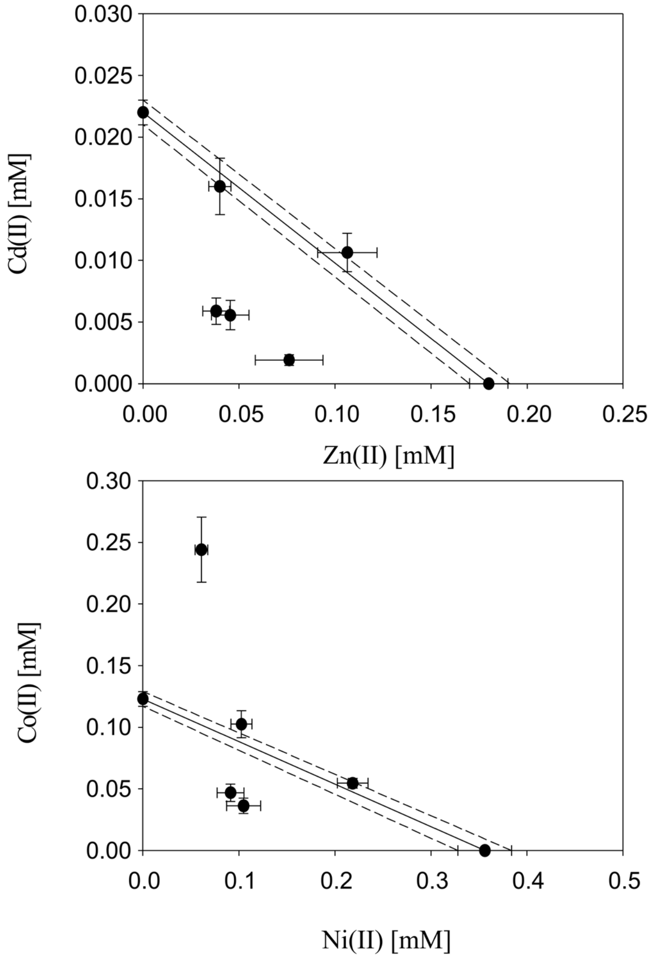

Figure 4. The EC_{50} isobole representations for metal ions as individual and mixtures tested against dehydrogenase activity of *Pseudomonas fluorescens*. The bars represent the standard deviations of the 95% confidence interval of the values. The solid and dotted lines represents additivity line and its 95% confidence belt

and ternary misture of Ni(II) + Co(II) + Cd(II). Conversely, the observed EC_{50} generally increased with increase in the predicted EC_{50} for Zn(II) + Cd(II), Zn(II) + Cd(II) + Ni(II) and Ni(II) + Co(II) + Zn(II) + Cd(II) mixtures.

DISCUSSION

Although Zn, Ni and Co are trace elements, they are toxic to microorganisms at elevated concentrations. Heavy metal inhibition of dehydrogenase enzymes have been reported by Nweke and co-workers for pure cultures of bacteria and microbial community of soil and river water (Nweke *et al*., 2006; 2007a,b; Orji *et al*., 2008; Nweke & Orji, 2009; Nweke & Okpokwasili, 2012). Cadmium has no physiological function and strongly inhibit microbial metabolism even at low concentrations.

A 50% effective concentration (EC_{50}) of zinc against sediment communities and natural microbial community in compost was reported as 0.92 mM and 0.05 mM respectively using acetate incorporation (Barnhart & Vestal, 1983). Minimum inhibitory concentration (MIC) of 1 mM Zn (II) was reported by Nies (1999) for *Escherichia coli* growing in TRIS-buffered mineral salt medium supplemented with yeast extract and sodium gluconate. The MIC is equivalent to the concentration of zinc ion that would inhibit dehydrogenase activity by 100% in this study. We estimated the IC_{100} at 1.483 mM Zn(II) in this study. Like zinc, nickel and cadmium ions were inhibitory to microbial activities at high concentrations. Codina *et al*. (1994) reported the EC_{50} of 763 mg/l Ni(II) (≈ 13 mM) and 1301.2 mg/l Ni(II) (≈ 22.17 mM) for *P. fluorescens* growing in buffer and wastewater respectively. Cobet *et al*. (1970) reported total inhibition (100%) of *Arthrobacter marinus* growth at 0.5 mM Ni(II). Chen *et al*. (2005) estimated EC_{50} of Co(II) at 150 mg/l (≈ 2.55 mM) for *Pseudomonas aeruginosa* growing in a batch system. According to Nweke and Okpokwasili (2012), Co(II) inhibited 50% of dehydrogenase activity in *Pseudomonas* species from petroleum refinery wastewater at Co(II) concentrations ranging from 0.065 mM to 0.347 mM. Cadmium ion is known to be more toxic than zinc, cobalt and nickel ions. Median inhibitory concentrations (IC_{50}s) ranging from 0.237 mM to 0.275 mM Cd(II) was reported by Nweke *et al*. (2007b) for the inhibition of dehydrogenase activity in microbial community extracted from soil. An IC_{50} ranging from 0.199 mM to 0.239 mM Cd(II) against bioluminescence in photobacterium Q67 was reported by Ge *et al*. (2014). IC_{50} ranging from 0.026 mM to 0.340 mM Cd(II) was reported for *Pseudomonas* species by Nweke and Okpokwasili (2012). The order of toxicity in the present study is Cd(II) > Zn(II) > Co(II) > Ni(II). This order seemingly agreed with other reports in literature. Cd(II) is a non essential element and is generally more toxic than the essential elements. Co(II) has been found to be more toxic than Ni(II) to *Pseudomonas* species (Chandy, 1999). It is important to note that in this study, there was no stimulation of dehydrogenase activity even at the lowest concentration (0.1 mM) of the tested metal ions. Stimulatory effects of metal ions have been reported for many microbial processes including dehydrogenase activity (Nweke *et al*., 2006; 2007a,b; Orji *et al*., 2008; Nweke & Orji, 2009), growth (Visca *et al*., 1992; Osman *et al*., 2004; Rai & Raizada, 1986; Gikas 2007) and bioluminescence (Christofi *et al*., 2002; Rodea-Palomares *et al*., 2009; Fulladosa *et al*., 2007). The absence of stimulatory effect in the present study may be attributed to the sensitivity of *P. fluorescens* to the metal ions or the fact that low concentration of nutrient broth was used which must have increased the bioavailability of the metal ions by reducing the tendency of the ions to bind to organic components of the medium.

Information on the effects of metal mixtures on microbial dehydrogenase activity is scarce. Toxicity of metal mixtures using other microbial responses has been reported by many researchers. However, little reports have been published on the combined effects of Ni(II) + Co(II), Zn(II) +Cd(II), Ni(II) + Co(II) + Cd(II), Zn(II) +Cd(II) + Ni(II) and Ni(II) + Co(II) + Zn(II) + Cd(II) mixtures on microorganisms. Using isobolographic representation, Gikas (2007) reported synergistic toxicity of binary mixtures of Ni(II) and Co(II)

Table 2: Toxic index and effect of binary mixtures of metal ions

Metal mixtures	Toxic Index (TI)	MDR*	Effect
NI(II) + Co(II) Binary Mixtures			
Ni(II) 20% + Co(II) 80%	2.153 ± 0.127	0.458 ± 0.037	Antagonistic
Ni(II) 50% + Co(II) 50%	1.119 ± 0.057	0.878 ± 0.065	Additive
Ni(II) 80% + Co(II) 20%	1.057 ± 0.008	0.928 ± 0.031	Additive
Ni(II) 66.06% + Co(II) 33.94% (EECR-20)	0.635 ± 0.058	1.554 ± 0.181	Synergistic
Ni(II) 74.32% + Co(II) 25.68% (EECR-50)	0.586 ± 0.063	1.716 ± 0.188	Synergistic
Zn(II) + Cd(II) Binary Mixtures			
Zn(II) 71.43% + Cd(II) 28.57%	0.940 ± 0.081	1.079 ± 0.084	Additive
Zn(II) 90.91% + Cd(II) 9.09%	1.062 ± 0.089	0.948 ± 0.079	Addiitive
Zn(II) 97.56% + Cd(II) 2.44%	0.474 ± 0.052	2.030 ± 0.363	Synergistic
Zn(II) 86.62% + Cd(II) 13.38% (EECR-20)	0.469 ±0.052	2.145 + 0.258	Synergistic
Zn(II) 89.11% + Cd(II) 10.89% (EECR-50)	0.498 ± 0.078	2.047 ± 0.372	Synergistic
Zn(II) + Cd(II) + Ni(II) Ternary Mixtures			
Zn(II) 68.49% + Cd(II) 4.11% + Ni(II) 27.40%	0.558 ± 0.022	2.269 ± 0.057	Synergistic
Zn(II) 36.585% + Cd(II) 2.439% + Ni(II) 60.976%	0.635 ± 0.031	1.888 ± 0.079	Synergistic
Zn(II) 43.48% + Cd(II) 13.04% +Ni(II) 43.48%	0.423 ± 0.027	2.663 ± 0.159	Synergistic
Zn(II) 33.70% + Cd(II) 5.20% + Ni(II) 61.10% (EECR-20)	0.472 ± 0.022	2.422 ± 0.106	Synergistic
Zn(II) 32.30% + Cd(II) 3.90% + Ni(II) 63.80% (EECR-50)	0.340 ± 0.022	3.435 ± 0.202	Synergistic
Ni(II) + Co(II) + Cd(II) Ternary Mixtures			
Ni(II) 36.58% + Co(II) 60.58% + Cd(II) 2.44%	1.237 ± 0.026	0.619 ± 0.030	Antagonistic
Ni(II) 32.57% + Co(II) 65.93% + Cd(II) 1.10%	2.033 ± 0.007	0.362 ± 0.014	Antagonistic
Ni(II) 60.98% + Co(II) 36.58% + Cd(II) 2.44%	1.071 ± 0.014	0.768 ± 0.035	Antagonistic
Ni(II) 62.54% + Co(II) 32.13% + Cd(II) 5.33% (EECR-20)	1.974 ± 0.020	0.439 ± 0.013	Antagonistic
Ni(II) 71.08% + Co(II) 24.55% + Cd(II) 4.39% (EECR-50)	1.250 ± 0.016	0.703 ± 0.023	Antagonistic
Ni(II) + Co(II) + Zn(II) + Cd(II) Quaternary Mixtures			
Ni(II) 60.98% + Co(II) 24.39% + Zn(II) 12.19% + Cd(II) 2.43%	0.525± 0.046	1.812 ± 0.169	Synergistic
Ni(II) 32.258% + Co(II) 32.258% + Zn(II) 32.258% + Cd(II) 3.226%	0.517 ± 0.047	2.250 ± 0.444	Synergistic
Ni(II) 46.48% + Co(II) 23.88% + Zn(II) 25.67% + Cd(II) 3.97% (EECR-20)	0.618 ± 0.037	1.640 ± 0.103	Synergistic
Ni(II) 52.28% + Co(II) 18.06% + Zn(II) 26.43% + Cd(II) 3.23% (EECR-50)	0.887 ± 0.076	1.173 ± 0.102	Additive

*MDR values are based CA predictions

against growth of activated sludge microbial community. However, in the same study, Ni(II) and Co(II) mixture was antagonistic at the zone of decreasing stimulation. This observation indicated that the overall effect of metal ion mixture may vary with the threshold under consideration. Similar observation was made in the present study where the CA and IA models underestimated toxicity of a specific mixture ratio at low doses and overestimated toxicity at high doses. The joint effect of Ni(II) and Co(II) on the growth of *Klebsiella pneumoniae* was characterized as additive (Ainswort et al., 1980). Synergistic interaction of Ni(II) and Co(II) against *Escherichia coli* and *Alcaligenes eutrophus* have also been reported (Barabasz *et al*., 1990). Using bioluminescent *Vibrio fischeri*, Fulladosa *et al*. (2005) reported antagonistic effect of equitoxic mixture of Zn(II) and Cd(II). Antagonistic effect of Zn(II) and Cd(II) was reported by Xu *et al*. (2011) using sea urchin embryo-larval assay. The toxicity of Zn(II) + Cd(II) combinations was reported to be synergistic to a bioluminescent construct, *Escherichia coli* HB101 pUCD607. On the contrary, Chu and co-workers reported that Zn(II) could neutralize the toxicity of many metals (Chu *et al*., 2005; Chu & Chow, 2002).

In the present study, isobolographic analysis and toxic index were used to characterize the joint effect of Ni(II) + Co(II) and Zn(II) + Cd(II) binary mixture of. Both approaches indicated synergistic, antagonistic and additive effects for different ratios of the mixtures. These ratio-dependent effects have been reported for binary mixtures of heavy metal ions against *Vibrio fischeri* and *Lemna minor* (Ince *et al*., 1999). The CA and IA models were used to predict the joint action of the binary mixtures. Both model made good predictions for 50% Ni(II) + 50% Co(II), 80% Ni(II) + 20% Co(II), 71.43% Zn(II) + 28.57% Cd(II) and 90.91% Zn(II) + 9.09% Cd(II) mixtures. However, in other cases, the model either overestimated or underestimated the joint toxicity of the metal mixture. This indicated the possibility of antagonistic and synergistic effects

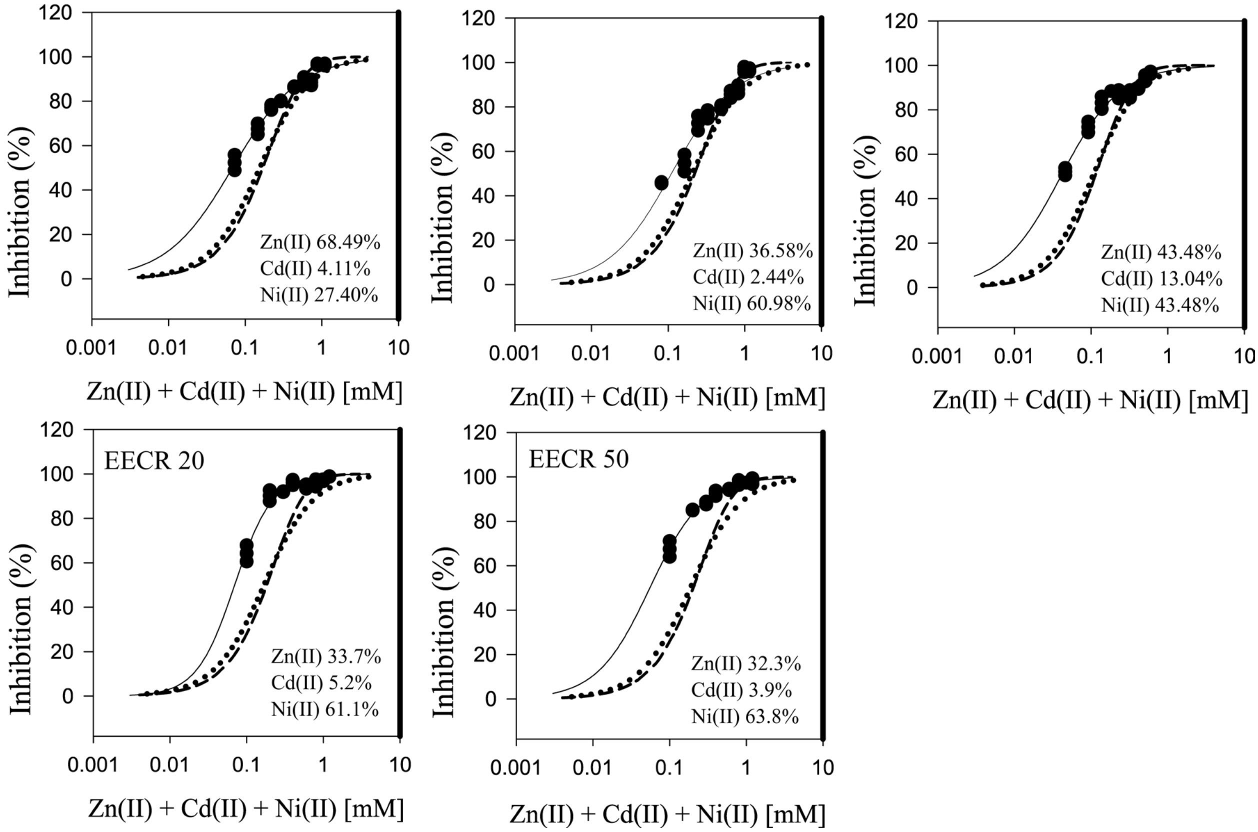

Figure 5: Observed and predicted inhibitions of *Pseudomonas fluorescens* dehydrogenase activity by ternary mixtures of zinc, cadmium and nickel ions. The data points represent experimental dose-response data. Solid lines represent toxicities obtained from fitting experimental data to logistic model (equation 2). The dashed lines represent toxicities predicted from the concentration addition model and the dotted lines represent toxicities predicted from the independent action model.

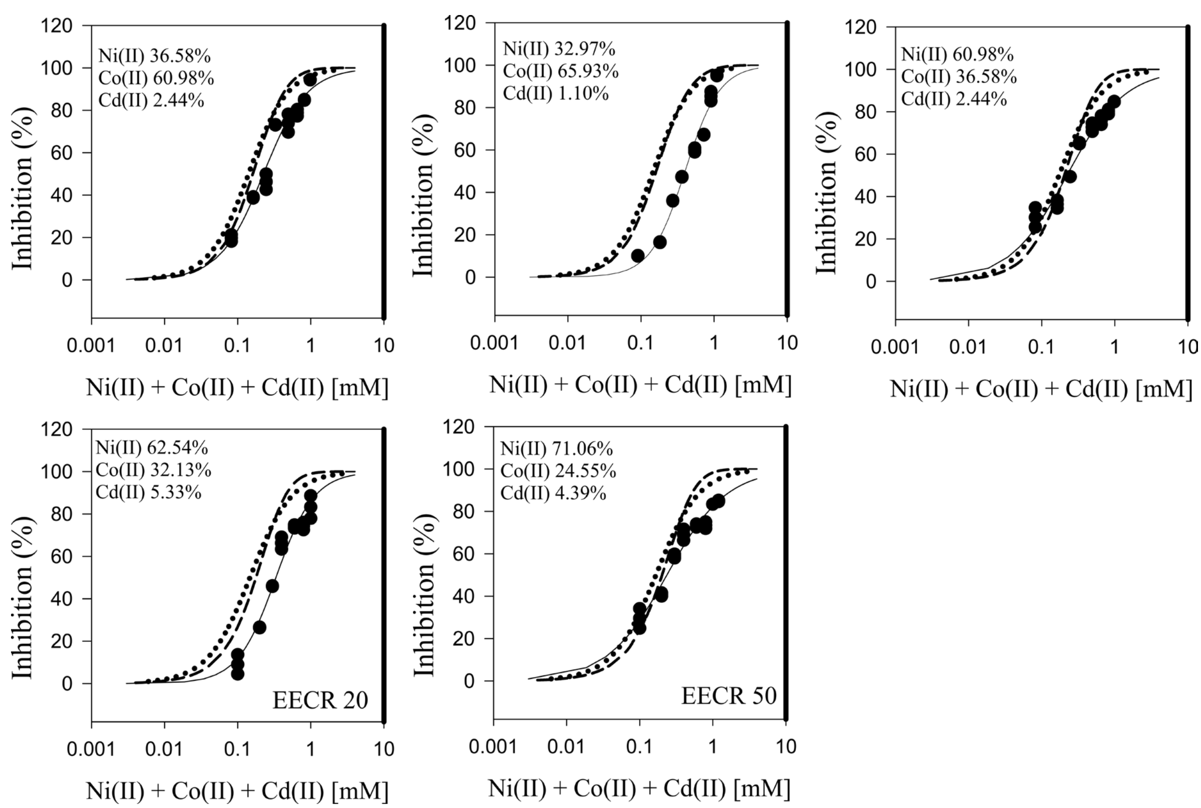

Figure 6: Observed and predicted inhibitions of *Pseudomonas fluorescens* dehydrogenase activity by ternary mixtures of nickel, cobalt and cadmium ions. The data points represent experimental dose-response data. Solid lines represent toxicities obtained from fitting experimental data to logistic model (equation 2). The dashed lines represent toxicities predicted from the concentration addition model and the dotted lines represent toxicities predicted from the independent action model.

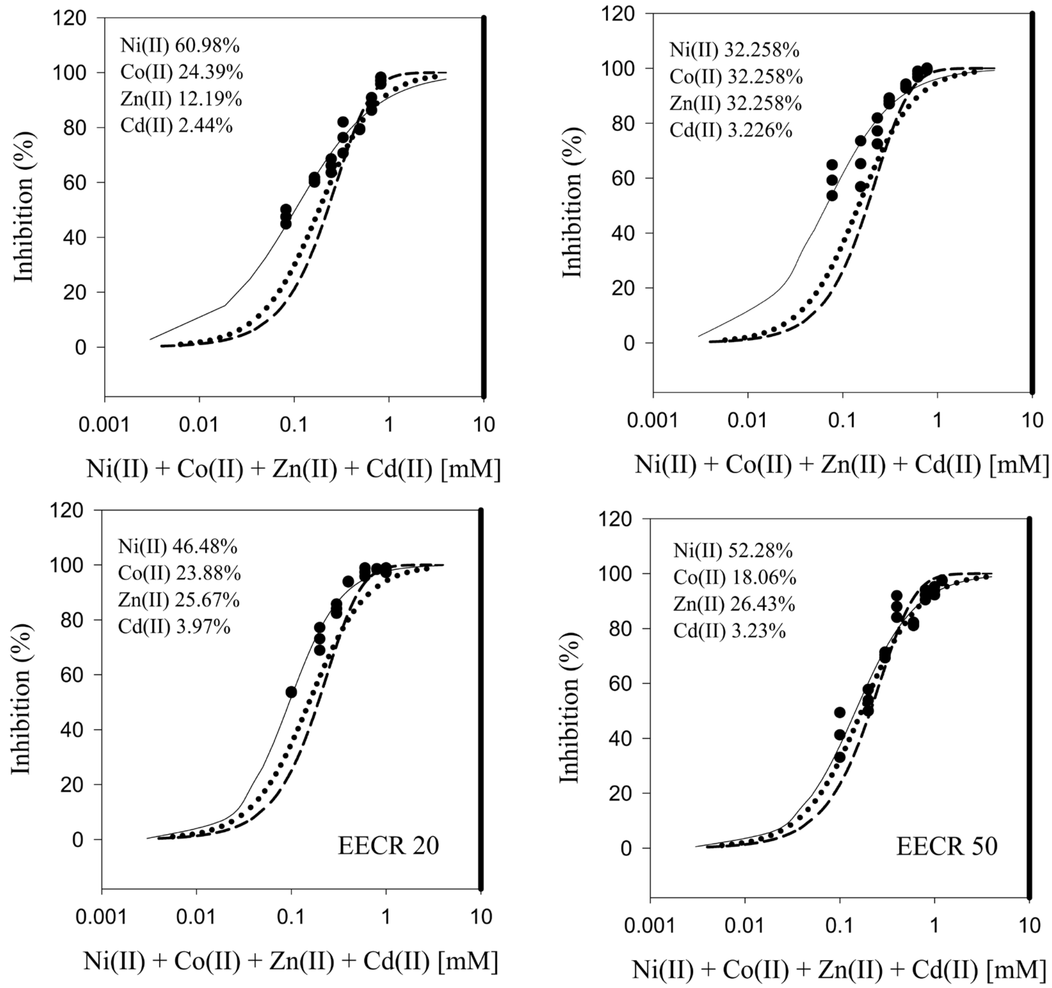

Figure 7: Observed and predicted inhibitions of *Pseudomonas fluorescens* dehydrogenase activity by quaternary mixtures of nickel, cobalt, zinc and cadmium ions. The data points represent experimental dose-response data. Solid lines represent toxicities obtained from fitting experimental data to logistic model (equation 2). The dashed lines represent toxicities predicted from the concentration addition model and the dotted lines represent toxicities predicted from the independent action model.

of the mixtures respectively. It is worthy of mention that in this study, the CA and IA models predicted identical toxicities of the binary mixtures. Studies have shown that under certain conditions, the toxicity thresholds (EC_x values) predicted by both models may be identical (Boedeker *et al*., 1993; Drescher & Boedeker, 1995; Chen *et al*., 2013; Backhaus *et al*., 2004; Zhang *et al*., 2008; Huang, 2011). According to Chen *et al*. (2013), equal predictions can be produced by CA and IA models when the dose-response relationship of every individual mixture component can be described by two-parameter Weibull function, the curves are strictly parallel and the slope parameter β equals 2.3. Depending on the slope of the individual dose-response relationships, both CA and IA may provide identical prediction (Drescher & Boedeker, 1995). However, it is important to note also, that according to reports of Cedergreen and co-workers, for binary mixtures of chemicals exhibiting dose-response curves with log-logistic slope parameters around 1, IA and CA predictions are similar (Cedergreen & Streibig, 2005; Cedergreen *et al*., 2007; 2008). This seems to be the case in the present study. We used two-parameter logistic model to describe the dose-response relationship of the individual metal ion with slope parameters of 1.172 ± 0.120, 1.263 ± 0.093, 1.770 ± 0.176 and 1.596 ± 0.149 for nickel, zinc, cobalt and cadmium ions respectively. These slope parameters are close to 1 and possibly explained the observed similarity between toxicities predicted from CA and IA models for Ni(II) + Co(II) and Zn(II) + Cd(II) binary mixtures. Similar observation was reported in our previous study involving binary mixtures of phenolic compounds and formulated glyphosate (Nweke *et al*., 2015). Barata *et al*. (2006) reported similar predictions by CA and IA models for binary mixtures of metal and pyrethroid insecticides against *Daphnia magna*. Thus it appears that this behaviour could be extended to mixtures of more than two components. This was also verified in the present study. The values of EC_{50} predicted for ternary and quaternary mixtures from CA model are not too far from EC_{50} predicted from IA model. The ratio of CA-EC_{50} to IA-EC_{50} varied between 0.911 and 0.946 with an average of 0.931 and standard deviation of 0.013 for Ni(II) + Co(II) + Cd(II) ternary mixture. Similarly, the Zn(II) + Cd(II) + Ni(II)

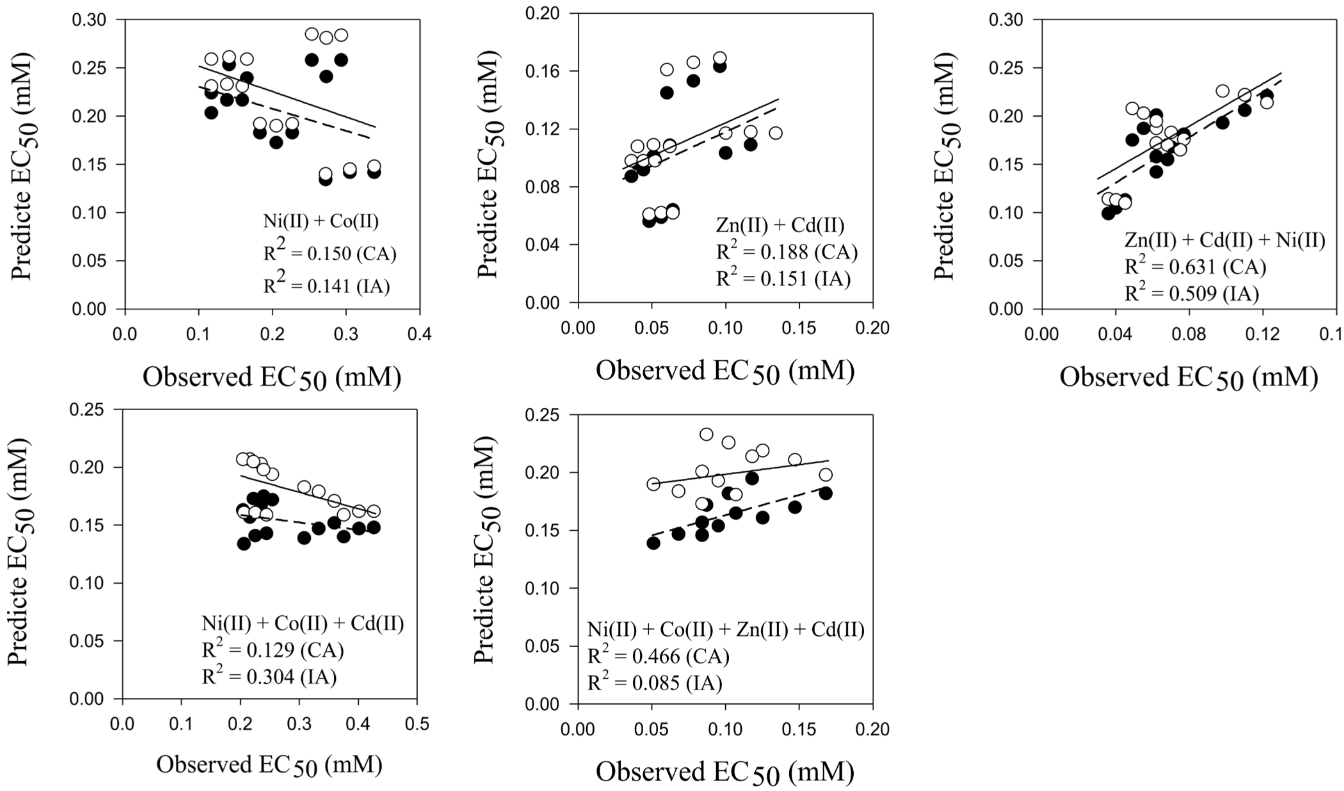

Figure 8: Correlation between the observed and predicted EC_{50}s of the metal ion mixtures. The closed circle represents CA and the open circle represents the IA data. The dashed and solid lines represent the linear regression of the CA and IA data respectively.

ternary mixture and Ni(II) + Co(II) + Zn(II) + Cd(II) quaternary mixture had average ratio of 0.850 and 0.821 respectively and corresponding standard deviation of 0.035 and 0.017. This indicated that CA and IA models may have similar capability in predicting toxicity of the metal mixtures. Insignificant difference between CA- and IA-predicted toxicity of mixtures of phenolic compounds with similar and dissimilar mode of action was reported by Huang *et al*. (2011). From the view point of the analyses in this study, synergistic, antagonistic and additive effect of binary, ternary and quaternary mixtures of metal ions against *P. fluorescens* could be possible. This underlines the ecological risk of environmental contamination with the mixtures. Synergistic interaction of these metal ions could be of serious concern.

CONCLUSION

Inhibition of dehydrogenase activity was used to assess toxicity of binary, ternary and quaternary combinations of nickel, cobalt, zinc and cadmium ions against *P. fluorescens*. The experiments were designed to expose the bacterium to varying doses of metal ions, which increased along fixed ratio ray based on uniform design and equieffect concentration ratios. The analyses of the experimental data indicated that the metal mixtures exhibited additive and interactive effects. The CA and IA models predicted identical or nearly identical toxicities. Generally, CA model predicted higher toxicities than IA predictions would suggest, which were not greater than 1.3 fold. However, most toxic index (TI) and model deviation ratios (MDR) between predicted and experimentally observed effect concentrations were between 0.5 and 2.0 and were within the expected inter-laboratory/inter-experiment deviation for most species. Although the results reported in this study cannot be easily extended to other organisms, it provides information on the potential synergistic toxicity of metal mixtures to bacteria. Further research is recommended to extend this investigation to natural microbial communities of aquatic and terrestrial ecosystems.

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