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Toxicities of senary and septenary mixtures of five metals and two phenols to *Pseudomonas fluorescens*

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Abstract

Toxicities of senary (Ni(II) + Co(II) + Zn(II) + Cd(II) + Pb(II) + 2-Chlorophenol and Ni(II) + Co(II) + Zn(II) + Cd(II) + Pb(II) + Phenol) and septenary (Ni(II) + Co(II) + Zn(II) + Cd(II) + Pb(II) + Phenol + 2-Chlorophenol) mixtures of five metals and two phenols to Pseudomonas fluorescens were assessed through dehydrogenase inhibition. Fixed ratio mixtures comprising equieffect concentration ratio (EECR) mixtures and arbitrarily chosen mixture ratios (ABCR) were used to determine the joint effects of these heavy metals in mixture with either 2-chlorophenol or phenol or both. The concentration-response relationships of all the toxicants and their mixtures were describable by logistic model. Generally, concentration addition (CA) predicted higher toxicities than independent action (IA) models. The mixture toxicities were generally synergistic. This is of environmental concern and underlines the hazard in discharging effluents containing phenols and heavy metals.

Keywords: Heavy metals, phenol, 2-chlorophenol, dehydrogenase activity, synergy.

Introduction

Heavy metals and phenolic compounds are widespread environmental pollutants of great concern emanating from human activities. Owing to their non-biodegradability and cellular toxicity, heavy metals persist in the environment[1,2]. At relatively high concentrations, heavy metals including trace elements exert toxic effects to microorganisms via a number of mechanisms[3,4]. Metal pollutants rarely occur in isolation within the ecosystem[5]. Cadmium, zinc, cobalt and nickel have many industrial applications and thus exist as environmental co-contaminants[6].

Phenol and its derivatives are introduced into the environment through natural and anthropogenic processes. Some phenols like phenol, p-cresol and chlorophenols are produced during biodegradation of organic matter or synthesized by microorganisms[7]. In addition, phenolic compounds gets into the environment as a component of effluents from many industrial processes such as pulp bleaching with chlorine, disinfection of water, petroleum refining, coal gasification, incineration of waste, combustion of fossil fuel, etc. In addition, application of herbicides for enhanced agricultural output has resulted in the environmental contamination with phenols. Phenoxy herbicides are degraded to toxic phenolic intermediates[8-10].

Industrial effluents may contain phenols and heavy metals. The petroleum refining industry wastewater has been reported to contain phenol and metals[11]. Produced water from petroleum industry contains heavy metals including zinc, cadmium, cobalt, copper, lead and nickel[12]. Wastewater from coking, electroplating, dyeing, oil and gas industries contain both organic compounds and heavy metals including copper, mercury, chromium, zinc, cadmium and lead[13]. Thus, phenolic compounds and heavy metals may often coexist in natural environment to exert joint toxicity to microorganisms. It is therefore vital to study the interactive effects of phenol and metals on environmental microorganisms.

The assessment of ecotoxicological effects of metal and phenol co-contamination of the natural environment, required the evaluation of the combined actions of metal and phenol mixtures against environmental microorganisms. In ecotoxicology, joint action of chemical mixtures is an important concern because mixtures of chemicals can potentially elicit synergistic interactive toxicity[14]. Although the toxicity of metal mixtures has been widely studied, there has not been much study on the combined toxicity of metals and phenols. The few investigations on the combined toxicity of metals and phenol were limited to one or two metals. In this study, the joint toxicities of five metals [Cd(II), Zn(II), Co(II), Ni(II) and Pb(Ii)] and two phenols (phenol and 2-chlorophenol) in senary and septenary mixtures was assessed on the basis of total dehydrogenase activity inhibition in *Pseudomonas fluorescens*.

Materials and methods

Reagents: The metal ions [Ni(II), Cd(II), Co(II), Zn(II) and Pb(II)], were applied as $NiSO_4.6H_2O$, $CdSO_4.8/3H_2O$, $CoCl_2$, $ZnSO_4.7H_2O$ and $Pb(NO_3)_2$ respectively. The individual metal ions were prepared as 10mM working stock solutions in deionized distilled water. A 1/10 dilution of the 10mM stock

solution of Cd(II) was made to obtain 1mM working stock solution. Analytical grade of phenol, 2-chlorophenol (2-CP), 2,3,5-triphenyltetrazolium chloride (TTC) and salts of heavy metals were purchased from Sigma (Germany).

Design of mixture experiments: The bioassays were designed to assess the toxicities of senary (Cd(II) + Zn(II) + Pb(II) + Co(II) + Ni(II) + 2- Chlorophenol and Cd(II) + Zn(II) + Pb(II) + Co(II) + Ni(II) + phenol) and septenary (Cd(II) + Zn(II) + Pb(II) + Co(II) + Ni(II) + 2-chlorophenol + phenol) mixtures of five metals with 2-chlorophenol and phenol using fixed ratio designs. Three equieffect concentration ratios (EECR) based on toxicity thresholds of the individual toxicant and three arbitrary mixture ratios were evaluated. The equieffect mixture ratios were constituted on the basis of the EC_{20} (EECR-20 mixture), EC_{50} (EECR-50 mixture) and EC_{80} (EECR-80 mixture) of the individual chemicals. The specific ratios of each chemical in the equieffect mixtures and the arbitrary mixture ratios (ABCR-1, ABCR-2 and ABCR-3) are shown in Table-1. The total concentrations of the mixture were varied at a fixed mixture ratio to obtain a full concentration-response curve with maximum percent inhibition of greater than 90%. The toxicants were prepared as 50mM aqueous solutions and the necessary volumes of each solution were mixed to obtain a particular mixture ratio. Each mixture was used as if it is a solution of a single component during the bioassay.

Table-1: Equi-effect concentration ratio (EECR) and arbitrary concentration ratio (ABCR) mixtures.

Mixtures	Mixture ratio (%)						
	Cd(II)	Zn(II)	Pb(II)	Co(II)	Ni(II)	2-CP	Phenol
Metals + 2-chlorophenol							
EECR 20	4.21	30.18	21.75	11.93	8.42	23.51	-
EECR 50	3.78	33.84	13.80	17.20	16.26	15.12	-
EECR 80	3.23	32.49	7.49	21.60	26.83	8.36	-
ABCR 1	5.00	40.00	10.00	10.00	15.00	20.00	-
ABCR 2	10.00	20.00	20.00	20.00	20.00	10.00	-
ABCR 3	20.00	10.00	10.00	10.00	20.00	30.00	-
Metals + phenol							
EECR 20	0.16	1.17	0.84	0.46	0.33	-	97.04
EECR 50	0.20	1.82	0.74	0.93	0.87	-	95.44
EECR 80	0.28	2.79	0.64	1.86	2.30	-	92.13
ABCR 1	2.00	2.00	2.00	2.00	2.00	-	90.00
ABCR 2	1.00	1.00	1.00	1.00	4.00	-	92.00
ABCR 3	0.50	2.00	0.50	1.00	2.00	-	94.00
Metals + 2-chlorophenol + phenol							
EECR 20	0.16	1.16	0.83	0.46	0.32	0.90	96.17
EECR 50	0.20	1.80	0.73	0.92	0.87	0.81	94.67
EECR 80	0.27	2.77	0.64	1.84	2.29	0.71	91.48
ABCR 1	1.00	1.00	1.00	1.00	1.00	1.00	94.00
ABCR 2	1.00	2.00	1.00	2.00	2.00	2.00	90.00
ABCR 3	2.00	0.50	2.00	0.50	2.00	1.00	92.00

Laboratory cultures: *P. fluorescens* was isolated from soil and identified as described elsewhere[15]. The bacterium was grown in nutrient broth (Lab M) on a rotary shaker incubator (150rpm) at 28±2°C for 24h. The cells were harvested by centrifugation at 3000 rpm for 10min. Harvested cells were washed twice in sterile deionized distilled water and re-suspended in the water. The cell density was adjusted to 1.06x 10^8cells/ml based on Mcfarland turbidity standards and used as inoculum.

Toxicity tests: The toxicities of individual toxicant and the mixtures were determined in triplicates using same experimental procedure. Preliminary range finding experiments were performed for each toxicant. The range of concentration of a toxicant to produce toxicity ranging from 0% to 90% or greater inhibition on *P. fluorescens* was identified. Using the identified range of concentrations, toxicity of the individual toxicant was evaluated in a TTC-dehydrogenase activity inhibition test to obtain full concentration-response profile of each toxicant. The toxicity thresholds (EC_{20}, EC_{50} and EC_{80}) were determined by non-linear regression of the concentration-response data with logistic model and used to design the mixture concentration ratios.

The assay for dehydrogenase activity in *P. fluorescens* in response to the toxicants or their mixtures was modified from Nweke *et al*.[15]. The 2-ml reaction mixture contained 0.1% nutrient broth amended with graded concentrations of metal ions, 2-chlorophenol or phenol as well as their mixtures.

Determination of EC_{50}: The toxicities of the individual toxicants and their mixtures to *P. fluorescens* were normalized relative to the mean control (Equation-1).

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

In Equation-1, R is the inhibition of dehydrogenase activity (%), C_A is the mean absorbance of triphenyl formazan (TPF) extract in the control experiment and T_A is absorbance of TPF extract in the test experiments with varying concentrations of individual toxicants or their mixtures.

The concentration-inhibition data were fitted with 2-parameter logistic model (Equation-2).

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

In Equation-2, x is the concentration of individual toxicants or their mixtures. EC_{50} is the median inhibitory concentration, indicating the concentration of the individual toxicant or the mixture that produced 50% inhibition of dehydrogenase activity and b is the slope of the concentration-inhibition curve at EC_{50}.

In the case of hormetic concentration-inhibition relationship, EC_{50} (p = 50) was determined by fitting the data to the Brain-Cousens model[16] as reparameterized by Schabenberger et al.[17] and applied to U-shaped concentration-inhibition curve based on Equation 3 as described by Nweke et al.[18].

$$R = 100 - \frac{100 + fx}{1 + \left[\frac{p}{100 - p} + \left\{\left(\frac{100}{100 - p}\right)\frac{fEC_p}{100}\right\}\right]\left(\frac{x}{EC_p}\right)^b} \quad (3)$$

In Equation 3, f describes the degree of hormesis, p is the percent decrease in the response and EC_p is the concentration of the toxicant that inhibited dehydrogenase activity by p%.

Prediction of mixture toxicities: Based on concentration addition (CA) model, the mixture toxicities were predicted from the toxicities of the individual components (Equation 4).

$$EC_{x(mix)} = \left(\sum_{i=1}^{n} \frac{\pi_i}{EC_{xi}}\right)^{-1} \quad (4)$$

In Equation 4, $ECx_{(mix)}$ is the total concentration of the mixture that produced x% effect, EC_{xi} is the concentration of individual component that produced x% effect when tested as an individual, n is the number of components, π_i is the relative proportion of individual component in the mixture. By using Equation 4, the mixture toxicities were predicted as described elsewhere[15].

In addition, the toxicities of the mixtures were predicted with the independent action (IA) model which assumes that the components of the mixture acts dissimilarly. The IA model can be expressed generally as shown in Equation 5[19].

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

In Equation 5, $E(c_{mix})$ is the total predicted effect of an n-component mixture, c_i is the concentration of the individual component and $E(c_i)$ is the effect of the individual component. Based on the 2-parameter logistic model, Equation 5 can be simplified as Equation 6[15].

$$E(c_{mix}) = \left[1 - \prod_{i=1}^{n}\left[1 - \frac{1}{1 + \left(\frac{\pi_i x}{EC_{50i}}\right)^{b_i}}\right]\right] \times 100 \quad (6)$$

In Equation-6, $\pi_i x$ is the concentration of individual component in the mixture. EC_{50i} and b_i are the EC_{50} of the individual component and the slope at EC_{50} respectively obtained by fitting the concentration-inhibition data of the individual component to 2-parameter logistic function. By encoding Equation 6 in Microsoft Excel 2003, the percent inhibitions of dehydrogenase activity were estimated for total concentrations of the mixtures ranging from 0.004 mM to 1.0 mM.

The EC_{50} of the mixtures were predicted based on IA model using Microsoft Excel 2003. By using Equation 6 coded in Excel software, the value of x in each mixture that will give $E(c_{mix})$ of 50% was estimated iteratively. The EC_{50} of the mixtures based on CA model were simply computed using Equation 4 based on the proportion and EC_{50} of the individual component. The EC_{50} values derived from CA and IA models were correlated with the observed EC_{50}s by using simple linear regression implemented with Microsoft Excel 2003. The Duncan post-hoc tests to compare the observed and predicted EC_{50} of the individual metals and phenol as well as the different mixture ratios were implemented with IBM SPSS Statistics version 21.

The toxic index (TI): The Toxic Index (TI) of each mixture was determined as the sum of toxic units for all of the mixture components (Equation 7).

$$TI = \sum_{i=1}^{n} \frac{C_i}{EC_{50i}} \qquad (7)$$

In Equation 7, C_i is the concentration of the individual components in the mixture at the EC_{50} of the mixture (EC_{50mix}) and EC_{50i} is the concentration of the individual component that produced 50% inhibition when tested as an individual, n is the number of mixture components. TI = 1 indicates additivity, TI > 1 and TI < 1 indicates antagonistic and synergistic interactions respectively[20].

Deviations from additivity: The degree of deviations of the predicted effect of the mixtures from the observed effect were calculated for each mixture as the model deviation ratio (MDR) at EC_{50}, the ratio of the predicted EC_{50} to the observed EC_{50}, as shown in Equation 8. MDR of greater than 1 or less than 1 indicated synergism or antagonism respectively. Also, the percent deviations of the predicted effect from the observed effect for each mixture were calculated from the model deviation ratio as shown in Equation 9. A positive deviation indicates synergism while negative deviation indicates antagonism[21].

$$MDR = \frac{\text{Predicted } EC_{50}}{\text{Observed } EC_{50}} \qquad (8)$$

$$\text{Deviation } (\%) = (MDR - 1) \times 100 \qquad (9)$$

Results and discussion

Toxicity of individual chemical: The concentration-dependent effect of the individual metal ion and phenolic compounds are shown in Figure-1. The dehydrogenase activity inhibition in *P. fluorescens* by metal ions increases with concentration resulting in inhibitions above 90% at 0.4mM [Cd(II), Zn(II) and Pb(II)] and 0.6mM [Co(II) and Ni(II)]. Similarly, the phenolic compounds exhibited increasing inhibition of dehydrogenase activity with increase in concentration reaching percent inhibition greater than 90% at 0.4mM and 15mM of 2-chlorophenol and phenol respectively. All the concentration-response relationships could be described with logistic function. The EC_{50} of the metals varied from 0.02mM for Cd(II) to 0.201 mM for Zn(II) (Table-2). The order of toxicity for the metals was Cd(II) > Ni(II) > Co(II) > Pb(II) > Zn(II). In the case of the phenols, 2-chlorophenol with EC_{50} of 0.112 ± 0.008mM was significantly ($p<0.05$) more toxic than phenol with EC_{50} of 9.372±0.112mM (Table-2). The Duncan post hoc test indicated that the EC_{50} of the metal ions differed significantly from each other.

Toxicity of mixtures: ***Toxicity of metals + 2-CP mixtures:*** The experimentally-derived and predicted concentration-response relationships of the senary mixtures of the five metals and 2-CP are shown in Figure-2. Like the individual toxicants, as the concentration increases, the senary mixtures increasingly inhibited the dehydrogenase activity of *P. fluorescens* producing sigmoid curves that are describable with 2-parameter logistic function. The observed and predicted EC_{50} of the senary mixture ratios as well as their statistical associations are shown in Table-2. The CA-predicted EC_{50} of the equieffect concentration ratios of the metals+2-CP senary mixtures are not statistically different from each other. In contrast, the observed and IA-predicted EC_{50} of the equieffect concentration ratios of the senary mixture were statistically different from each other. The observed EC_{50} of the arbitrary concentration ratios of the senary mixture are not significantly different from each other (Table-2). Statistically different EC_{50} among the arbitrary concentration ratios were observed in the case of CA and IA predictions (Table-2). Generally, IA model predicted significantly lower toxicity than CA model in all the senary mixture ratios. Both CA- and IA-predicted mixture toxicities were significantly lower than the observed toxicities. This indicates synergistic interaction of the toxicants against *P. fluorescens*.

Toxicity of metals + phenol mixtures: The observed and predicted toxicities of the metals + phenol senary mixtures are shown in Figure-3. A concentration-dependent effect of the mixtures that could be described with logistic function was observed. The EECR 20 and EECR 50 equieffect mixtures produced similar effect against the test organism. Similarly, the arbitrary concentration ratios, ABCR 1 and ABCR 2, had similar toxicities on the organism. This similarity was also observed in the CA- and IA-predicted EC_{50} (Table-2). In all the mixture ratios, the IA model generally predicted lower toxicities

than the CA model. The observed toxicities of the metals + phenol senary mixtures were higher than the predictions of CA and IA models. This underestimation of toxicity by CA and IA models suggested synergistic interaction of the metals and phenol.

Table-2: Observed and predicted toxicity thresholds (EC_{50}) of individual chemical and their mixtures.

Individual metals and phenols	EC_{50} (mM) †		
	Observed	Predicted	
		CA	IA
Cd(II)	0.023 ± 0.003 a	-	-
Zn(II)	0.184 ± 0.017 e	-	-
Pb(II)	0.135 ± 0.007 d	-	-
Co(II)	0.099 ± 0.006 c	-	-
Ni(II)	0.080 ± 0.006 b	-	-
2-Chlorophenol	0.112 ± 0.008 c	-	-
Phenol	9.372 ± 0.112 f	-	-
Metals + 2-Chlorophenol			
EECR 20	0.078 ± 0.007 c	0.106 ± 0.009 c	0.192 ± 0.011 e
EECR 50	0.025 ± 0.003 a	0.104 ± 0.009 c	0.167 ± 0.010 d
EECR 80	0.054 ± 0.002 b	0.100 ± 0.009 c	0.139 ± 0.007 c
ABCR 1	0.020 ± 0.003 a	0.102 ± 0.009 c	0.166 ± 0.009 d
ABCR 2	0.020 ± 0.002 a	0.081 ± 0.008 b	0.114 ± 0.004 b
ABCR 3	0.023 ± 0.001 a	0.061 ± 0.007 a	0.081 ± 0.003 a
Metals + Phenol			
EECR 20	0.922 ± 0.074 c	2.578 ± 0.175 d	5.083 ± 0.282 d
EECR 50	0.898 ± 0.052 c	1.829 ± 0.134 c	3.127 ± 0.179 c
EECR 80	0.341 ± 0.017 b	1.116 ± 0.087 b	1.619 ± 0.079 b
ABCR 1	0.260 ± 0.014 a	0.592 ± 0.061 a	0.767 ± 0.017 a
ABCR 2	0.247 ± 0.010 a	0.788 ± 0.074 a	0.999 ± 0.021 a
ABCR 3	0.410 ± 0.024 b	1.223 ± 0.105 b	1.756 ± 0.076 b
Metals + 2-CP + Phenol			
EECR 20	1.298 ± 0.055 e	2.151 ± 0.149 d	4.916 ± 0.287 e
EECR 50	1.176 ± 0.038 d	1.625 ± 0.120 c	3.116 ± 0.186 d
EECR 80	0.717 ± 0.059 c	1.052 ± 0.082 b	1.636 ± 0.080 c
ABCR 1	0.442 ± 0.072 b	1.013 ± 0.098 b	1.531 ± 0.032 c
ABCR 2	0.278 ± 0.017 a	0.740 ± 0.067 a	1.149 ± 0.036 b
ABCR 3	0.330 ± 0.013 a	0.646 ± 0.069 a	0.835 ± 0.037 a

†In each set of observed EC_{50} for the individual toxicants and the mixtures, values with same alphabet are not significantly different from each other ($p > 0.05$). In each set of the mixtures, the CA-predicted EC_{50} with same alphabet are not significantly different from each other ($p > 0.05$). In each set of the mixtures, the IA-predicted EC_{50} with same alphabet are not significantly different from each other ($p > 0.05$). Within rows, for each mixture, the observed and the predicted EC_{50} (CA- and IA-) are significantly different from each other ($p < 0.05$).

Toxicity of metals + 2-CP + phenol mixtures: Figure-4 showed the observed and predicted toxicities of septenary mixtures of five metals with 2-chlorophenol and phenol. In all mixture ratios except ABCR 2, the dehydrogenase activity of *P. fluorescens* was progressively inhibited as the concentration increased resulting to concentration-response relationships that could be described with logistic function. The ABCR 2 mixture had biphasic effect, stimulated dehydrogenase activity at low concentrations up to 0.1mM and progressively inhibited it at higher concentrations. The EC_{50} of the mixture ratios are shown in Table-2. The EC_{50} of the equieffect mixtures varied significantly among each other for the experimentally observed and predicted effects. However, the observed and CA-predicted EC_{50} for the arbitrary concentration ratios, ABCR 1 and ABCR 2, did not vary significantly among each other in each case. Generally, the IA model predicted lower toxicity of the mixtures than CA model (Figure-4). In comparison with the experimental observations, lower mixture toxicities were predicted by CA and IA models indicating synergistic interaction between the mixture components.

Correlations between observed and predicted EC_{50}: The correlations between the observed and predicted EC_{50} of the mixtures are shown in Figures-5 and 6. Generally, there were no good correlation between the experimentally-derived EC_{50} and EC_{50} predicted from both CA and IA models for the metals+2-CP senary mixtures. However, there were good positive correlation between the observed EC_{50} and predicted EC_{50} for senary mixtures of metals and phenol (R^2=0.8832 (CA); R^2= 0.8632 (IA)). Similarly, there were good positive correlation between the observed EC_{50} and EC_{50} derived from CA model (R^2=0.9166) and IA model (R^2 =0.8689) for septenary mixtures of metals with 2-CP and phenol. In comparison, there were good positive correlation between EC_{50} predicted from CA and IA models for the metals + 2-CP mixtures (R^2=0.8486), metals +`phenol mixtures (R^2=0.9851) and metals + 2-CP + phenol mixtures (R^2=0.9784).

Deviations from additivity: The toxic indices, model deviation ratios and percent deviations from additivity based on CA and IA models are shown in Table-3. The toxic index values ranged from 0.184 to 0.741 (metals + 2-CP mixtures), 0.297 to 0.499 (metals + phenol mixtures) and 0.414 to 0.757 (metals + 2-CP + phenol mixtures). Generally, the MDR ranged from 1.359± 0.007 to 5.132±0.323 for CA model and from 2.283±0.072 to 8.381±0.816 for IA model. Thus, the senary and septenary mixtures are generally synergistic.

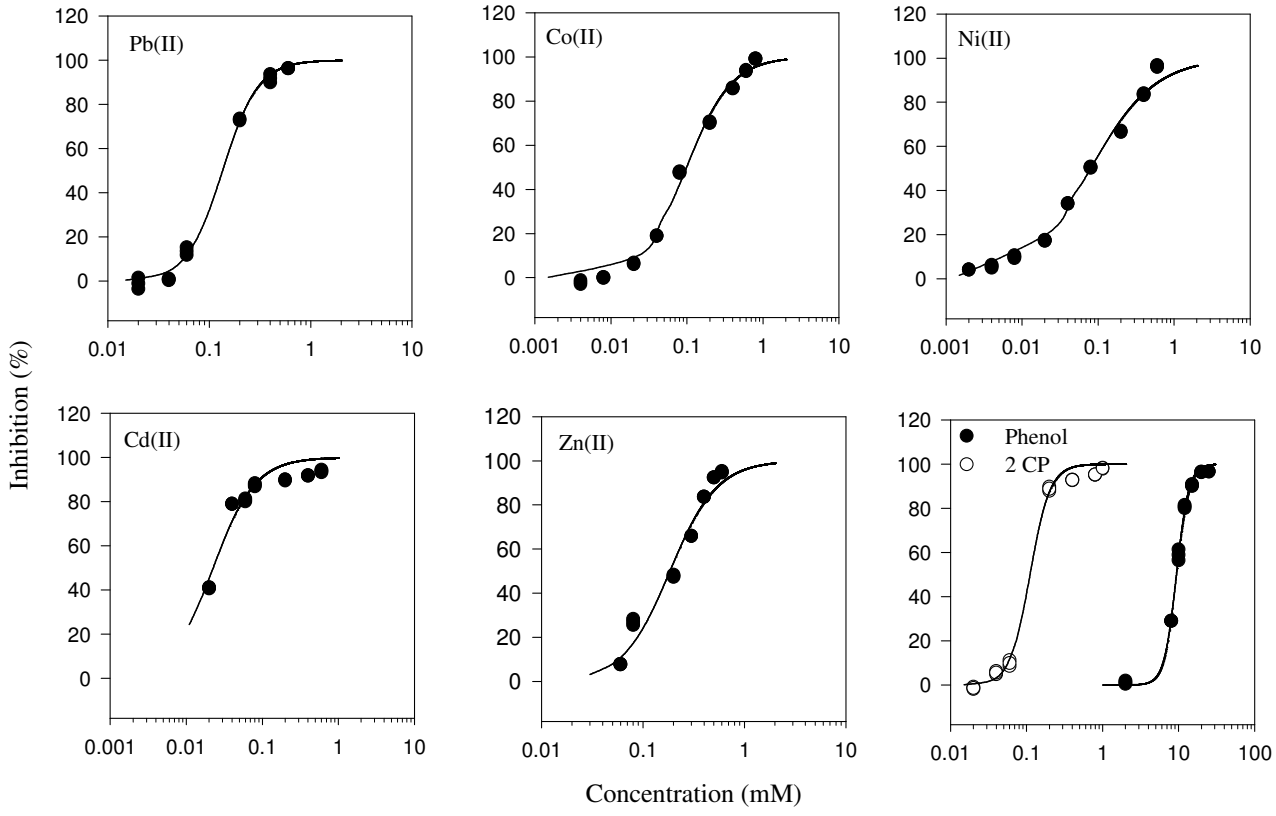

Figure-1: Observed (data points) and logistic model-predicted inhibitions (lines) of dehydrogenase activity in *P. fluorescens* by the individual metal and phenolic compound.

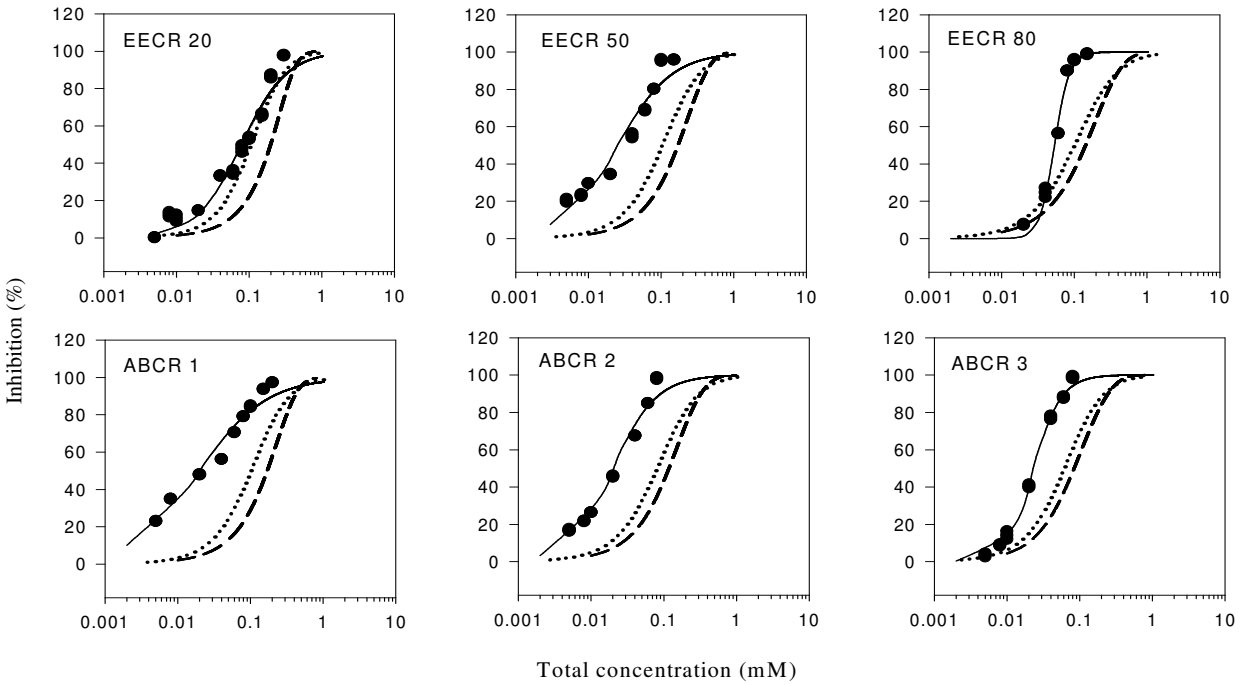

Figure-2: Observed (data points) and predicted (lines) inhibitions of dehydrogenase activity in *P. fluorescens* by senary mixtures of five metals and 2-chlorophenol. The solid lines represent logistic model (equation 2) fitting to the observed data. The dotted and dashed lines represent toxicities predicted from the CA and IA models respectively.

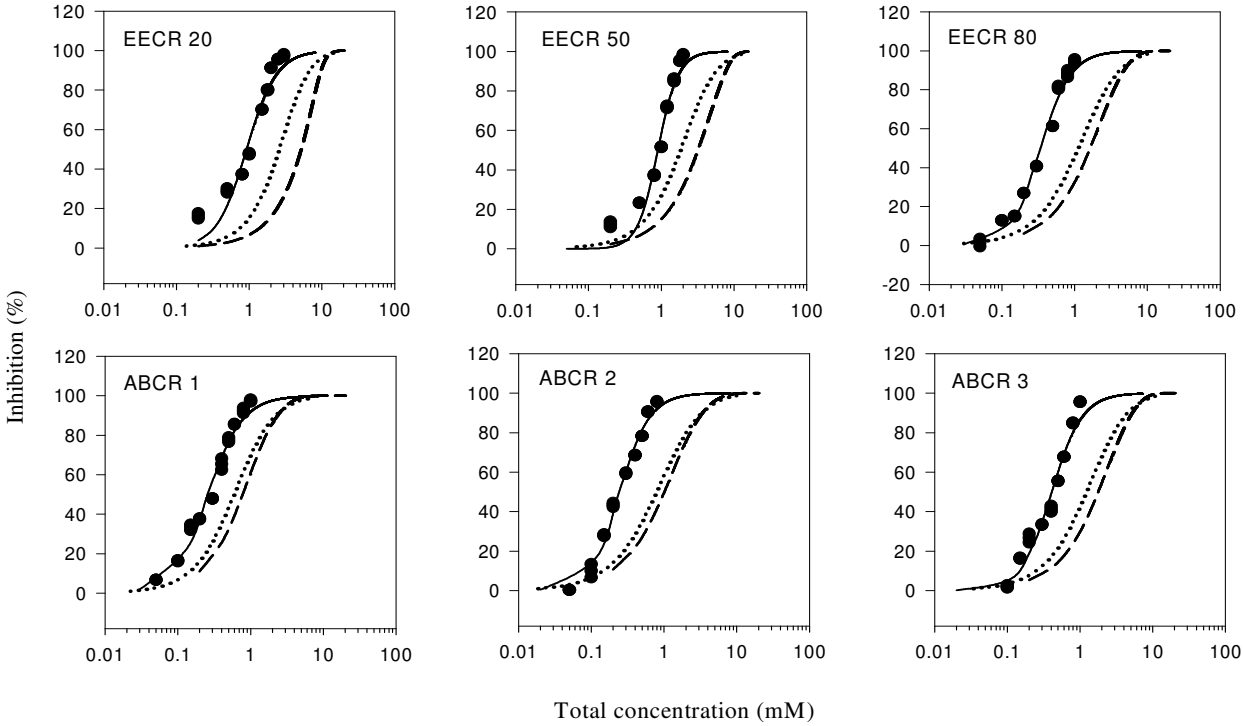

Figure-3: Observed (data points) and predicted (lines) inhibitions of dehydrogenase activity in *P. fluorescens* by senary mixtures of five metals and phenol. The solid lines represent logistic model (equation 2) fitting to the observed data. The dotted and dashed lines represent toxicities predicted from the CA and IA models respectively.

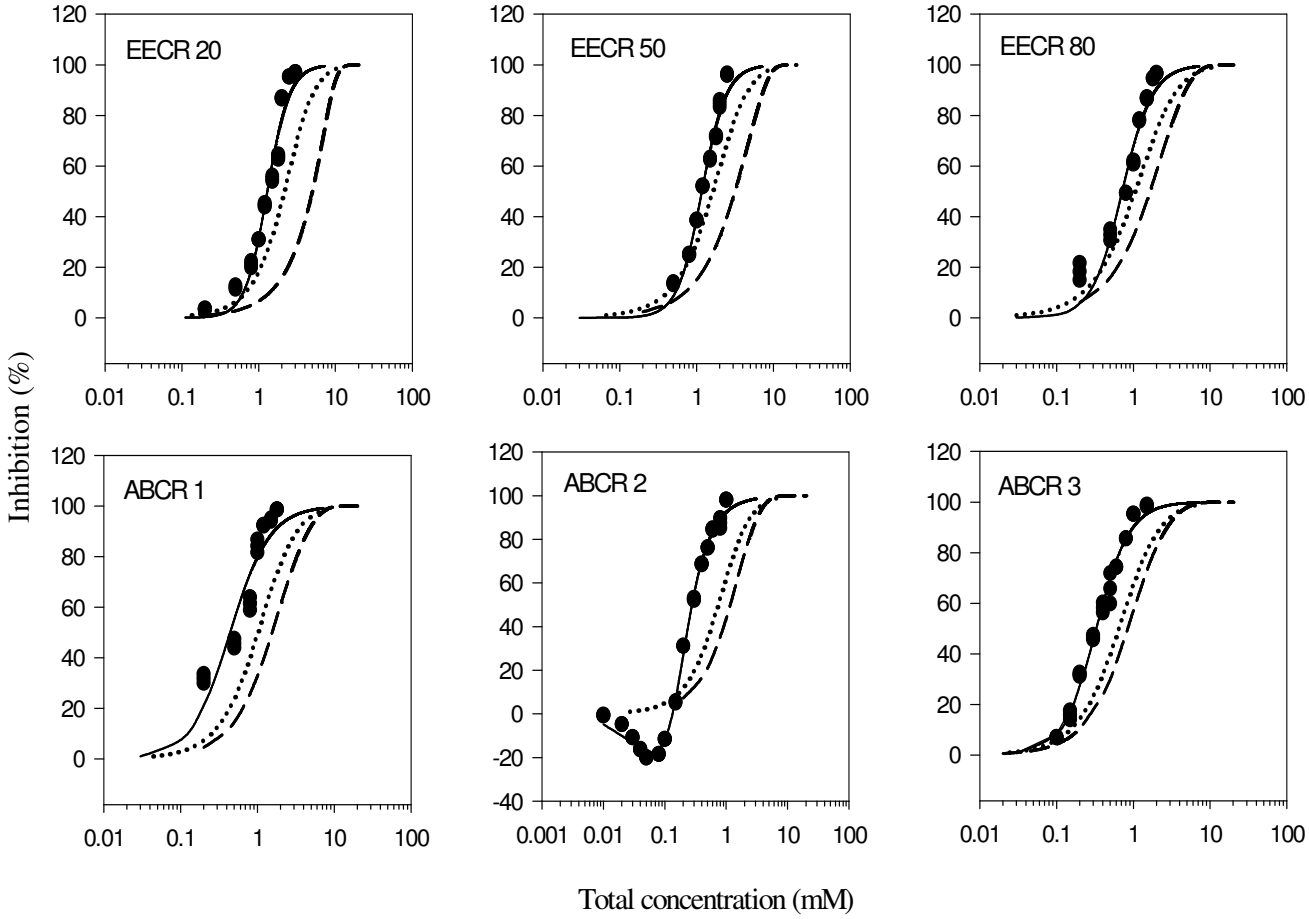

Figure-4: Observed (data points) and predicted (lines) inhibitions of dehydrogenase activity in *P. fluorescens* by septenary mixtures of five metals, 2-chlorophenol and phenol. The solid lines represent logistic model (Equation 2) fitting to the observed data. The dotted and dashed lines represent toxicities predicted from the CA and IA models respectively.

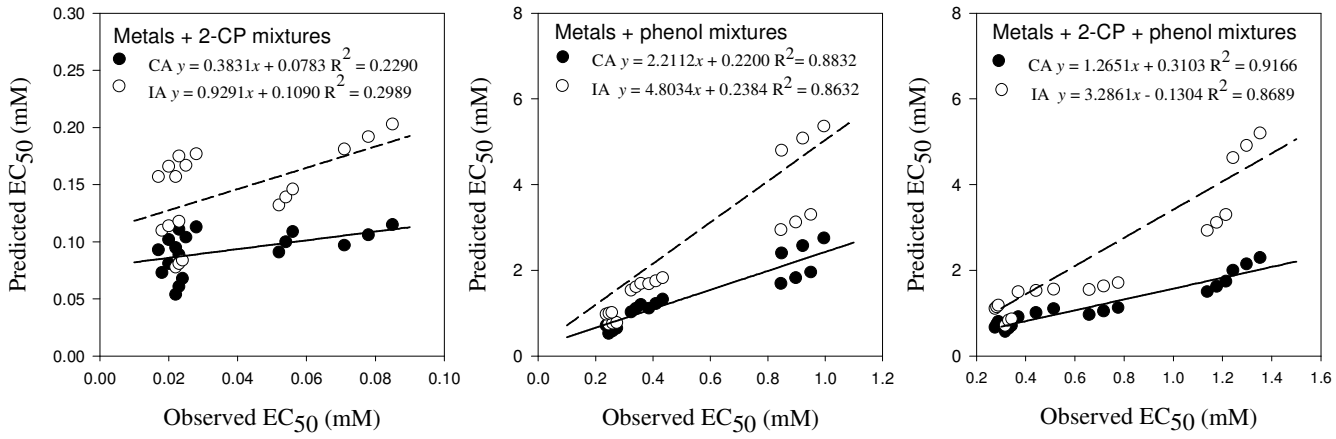

Figure-5: Correlation between the observed EC_{50} and predicted EC_{50} based on CA and IA models in the toxicity tests of the senary and septenary mixtures. The solid and dashed lines are the linear regression lines for CA and IA data respectively.

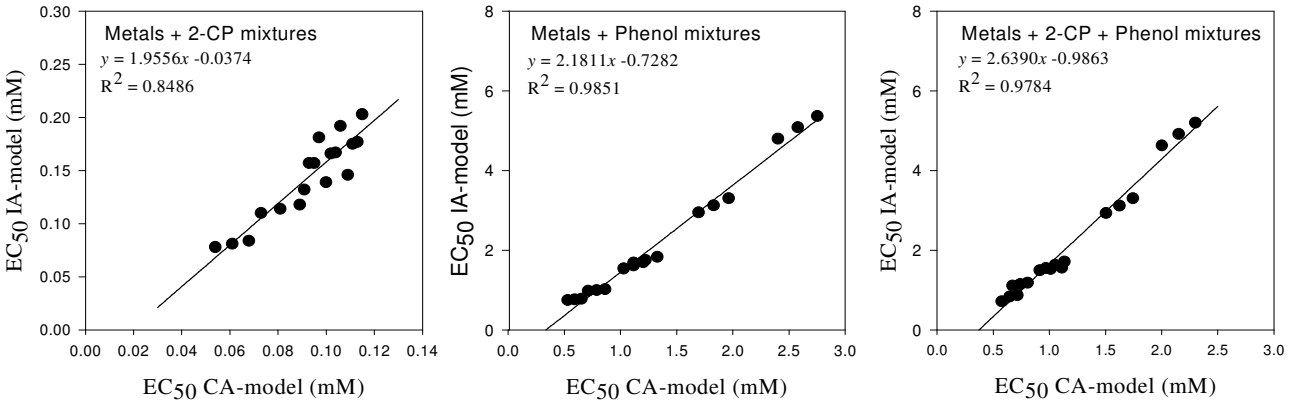

Figure-6: Correlation between CA and IA model predicted EC_{50} values for the senary and septenary mixtures.

Table-3: Toxic index and deviations of the predicted from observed toxicities.

Metal mixtures	Toxic Index (TI)*	MDR based on:		Deviation (%) based on:	
		CA model*	IA model*	CA model*	IA model*
Metals + 2-CP					
EECR 20	0.739 ± 0.002	1.359 ± 0.007	2.466 ± 0.081	35.937 ± 0.664	146.636 ± 8.064
EECR 50	0.244 ± 0.013	4.171 ± 0.142	6.713 ± 0.408	317.130 ± 14.157	571.260 ± 40.844
EECR 80	0.543 ± 0.024	1.849 ± 0.098	2.573 ± 0.034	84.943 ± 9.824	157.323 ± 3.435
ABCR 1	0.195 ± 0.011	5.132 ± 0.323	8.381 ± 0.816	413.223 ± 32.346	738.133 ± 81.634
ABCR 2	0.247 ± 0.012	3.992 ± 0.106	5.647 ± 0.492	299.171 ± 10.581	464.718 ± 49.247
ABCR 3	0.357 ± 0.028	2.647 ± 0.189	3.522 ± 0.023	164.668 ± 18.945	252.240 ± 2.273
Metals + Phenol					
EECR 20	0.357 ± 0.004	2.798 ± 0.035	5.520 ± 0.138	179.796 ± 3.487	454.037 ± 13.765
EECR 50	0.491 ± 0.008	2.036 ± 0.031	3.484 ± 0.002	103.554 ± 3.140	248.227 ± 0.232
EECR 80	0.306 ± 0.009	3.270 ± 0.092	4.748 ± 0.005	2226.966 ± 9.224	374.797 ± 0.504
ABCR 1	0.441 ± 0.026	2.273 ± 0.112	2.953 ± 0.094	127.289 ± 11.239	195.336 ± 9.378
ABCR 2	0.314 ± 0.019	3.186 ± 0.171	4.047 ± 0.079	218.568 ± 17.076	304.666 ± 7.888
ABCR 3	0.336 ± 0.010	2.980 ± 0.082	4.294 ± 0.079	197.974 ± 8.181	329.412 ± 7.912
Metals + 2-CP + Phenol					
EECR 20	0.604 ± 0.018	1.656 ± 0.045	3.786 ± 0.061	65.590 ± 4.467	278.565 ± 6.076
EECR 50	0.725 ± 0.032	1.381 ± 0.057	2.648 ± 0.073	38.057 ± 5.746	164.810 ± 7.263
EECR 80	0.681 ± 0.002	1.468 ± 0.006	2.283 ± 0.072	46.758 ± 0.642	128.290 ± 7.215
ABCR 1	0.434 ± 0.027	2.309 ± 0.156	3.519 ± 0.507	130.877 ± 15.643	251.867 ± 50.748
ABCR 2	0.383 ± 0.028	2.659 ± 0.174	4.133 ± 0.025	165.895 ± 17.411	313.267 ± 2.544
ABCR 3	0.514 ± 0.040	1.954 ± 0.132	2.446 ± 0.157	95.410 ± 13.221	144.585 ± 15.675

* All mixture ration are synergistic according to the TI, MDR and Deviation values

Discussion: Some heavy metals including zinc, nickel and cobalt are essential for normal metabolic activities in cells. However, they are toxic to microorganisms at increased concentrations. Lead and cadmium have no known metabolic role and are toxic to microorganisms at low concentrations[22]. Toxicities of both trace elements and toxic heavy metals to microorganisms have been widely studied. Inhibitory effects of heavy metals to *Pseudomonas* species have been assessed using dehydrogenase enzyme activity as a response. Based on inhibition of total dehydrogenase activity, IC_{50} ranging from 0,277mM to 0,729mM (Zn), 0,026mM to 0,340mM (Cd) and 0.065mM to 0.347mM (Co) was reported against *Pseudomonas* species[23-25]. Nwanyanwu et al.[26] reported IC_{50} of 0.232±0.008 mM Cd(II) and 0.328±0.015mM Zn(II) against dehydrogenase activity of a consortium of *Pseudomonas*, *Micrococcus*, *Bacillus* and *Staphylococcus* species. These reports are based on normal strength of rich medium that tend to chelate metal ions[27]. More recently, Nweke et al.[15] assessed inhibition of dehydrogenase activity in *P. fluorescens* by Zn(II), Co(II), Ni(II) and Cd(II) ions using a low strength (0.2%) nutrient broth. In the study, EC_{50} of 0.356±0.028mM (Ni), 0.123±0.006mM (Co), 0.180± 0.010mM (Zn) and 0.022±0.001mM (Cd) was reported. In the present study, working with a lower strength nutrient broth (0.1%), we estimated EC_{50} at 0.080±0.006mM, 0.099±0,006 mM, 0.184±0.017mM and 0.023±0.003mM for Ni(II), Co(II), Zn(II) and Cd(II) respectively. This indicated that the medium chelated Ni(II) ion more than other metal ions and could significantly reduce nickel toxicity against *P. fluorescens*. In the present study, the order of increasing toxicity is Cd(II) > Ni(II) > Co(II) > Pb(II) > Zn(II). Cadmium has no physiological function and is typically more toxic than the essential elements. Interestingly, Pb(II) is less toxic than the trace element, Ni(II) and Co(II). This could be a case of lead tolerance in *P. fluorescens*. Lead tolerance by *P. fluorescens* have been reported[28,29]. Lead resistance by microorganisms including species of *Pseudomonas* have been reviewed by Jarosławiecka and Piotrowska-Seget[30].

Phenol and phenolic compounds damage cell membranes and disrupts its physiological functions[31,32]. Dehydrogenase enzymes are membrane-associated in bacterial cells and thus are subject to phenol toxicity. Inhibitory effects of phenols on bacterial dehydrogenase activity have been assessed by many workers. IC_{50} ranging from 243.715mg/l to 378.441mg/l against *P*seudomonas species was reported by Nweke and Okpokwasili[33]. Based on dehydrogenase activity inhibition in *P. fluorescens*, IC_{50} of 210mg/l phenol was reported by Abbondanzi et al.[34]. At concentration ranging from 25.458mg/l to 39.344mg/l, 2-Chlorophenol inhibited dehydrogenase activity in *Pseudomonas* species by 50%[33].

In the natural environment, pollutants occur as mixtures of chemicals. Thus there is need for assessment of toxicity of chemical mixtures to microbial populations. Toxicity of heavy metal mixtures have been studied via a number of responses in microorganisms. Nweke et al.[15] assessed toxicity of Ni(II),Co(II), Zn(II) and Cd(II) mixtures against *P. fluorescens* dehydrogenase activity. In the study, additive, antagonistic and synergistic effects of metal mixtures were reported for various metal ion combinations. In a similar study, possible additive, antagonistic and synergistic effects of Ni(II) + Co(II) + Zn(II) + Cd(II) quaternary mixtures on the dehydrogenase activity of river water microbial community was reported[35]. In the present study, toxicity of five metals [Ni(II), Co(II), Zn(II), Cd(II) and Pb(II)], phenol and 2-CP in senary and septenary mixtures were investigated. The toxicants interacted synergistically to increase the inhibitory effects on the dehydrogenase activity. Information on the interactive effects of the five heavy metals and the phenols on microbial dehydrogenase activity is scarce. Nwanyanwu et al.[26] assessed toxicity of Zn(II), Cd(II), 4-chlorophenol (4-CP) and 2,4-dichlorophenol (2,4-DCP) in binary and quaternary mixtures based on inhibition of dehydrogenase activity in bacterial consortium. Both antagonistic and synergistic interactions of Zn(II) + 4-CP, Zn(II) + 2,4-DCP, Cd(II) + 4-CP and Cd(II) + 2,4-DCP binary mixtures were reported. However, only synergistic interactions was reported for the quaternary mixtures of Zn(ii), Cd(II), 4-CP and 2,4-DCP[26]. Synergistic interaction of copper and pentachlorophenol combinations on the growth of *Escherichia coli* was reported by Zhu et al.[36]. Antagonistic interaction of phenol and copper due to Cu-phenol complexation was documented[37,38].

Although the individual toxicants in this study did not have significant hormetic effects on dehydrogenase activity, it is important to note that one of the septenary mixture of metals, phenol and 2-CP (ABCR 2) exhibited significant hormetic effect on the dehydrogenase activity. Hormesis is a biphasic dose-response phenomenon characterized by low dose stimulatory and high dose inhibitory effects of chemicals and other agents on organisms. This phenomenon has been widely reported in many microorganisms and chemicals including phenols. Phenolic compounds have been reported to exert hormetic effect on bacterial dehydrogenase activity[18,26,33,39] and bioluminescence[40-43].

The CA-model predicted significantly higher toxicities of the mixtures than the IA-model. This observation corroborates the reports of Backhaus et al.[44] and Faust et al.[45] but is contrary to the reports of Mo et al[46] and Nweke et al.[15]. The CA and IA models were used to predict chemical mixture toxicities on the basis of the dose- responses of the individual components in the mixture. The CA model assumes that the mixture components act similarly by binding to the same active site, while IA model assumes that the components act dissimilarly by binding to different active sites[47]. The components of the senary and septenary mixtures in this study have different mechanisms of action. Ni(II), Co(II) and Zn(II) are essential heavy metals involved in many physiological functions while Pb(II) and Cd(II) are non-essential metals that are toxic to organisms. Phenolic compounds impair membrane functions by causing loss of cell membrane integrity. Thus, it was expected that IA

model would predict the toxicity of the mixtures accurately. Nevertheless, there were deviations from additivity. Both models underestimated the toxicities of the mixtures. This may be due to the complex nature of the biological system and the different mechanisms of action of the heavy metals and phenolic compounds. In most cases, the MDR values are greater than 2 and outside the expected inter-laboratory/inter-experiment benchmark, thus suggesting synergistic interactions of metals and phenols. It is of environmental concern that metals could interact with phenol and 2-CP to increase toxicity in bacterial model. Given that metals and phenols are present in industrial effluents, it is worthwhile to consider the toxicity of metals and phenols in toxicity assessment program.

Conclusion

The toxicities of senary and septenary mixtures of Ni(II), Co(II), Zn(II), Cd(II), Pb(II), phenol and 2-CP were assessed on the basis of dehydrogenase activity inhibition in *P. fluorescens*. The bacterium was exposed to varying concentrations of the components in the mixture which varied along fixed ratio ray based on 20%, 50% and 80% equieffect and arbitrary concentration ratios. The data analyses showed that the mixtures had synergistic toxic effects on the activity of dehydrogenase. Thus, the CA and IA models failed in predicting the mixture toxicities of the heavy metals and phenols. This raises concern about discharge of effluents containing phenols and heavy metals into the environment and emphasizes the need to consider the toxicity of metal-phenol mixtures in ecotoxicological tests by environmental protection agencies. It is not easy to extrapolate the findings described here to other organisms. However, the results described here provides information about the possible interactive toxicity of metals and phenols against bacteria. Further research is therefore necessary to study the effects of mixtures of metals and phenolic compounds on the biota of aquatic and terrestrial ecosystems.

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