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Toxicity of quaternary mixtures of metals to aquatic microbial community

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Abstract

The toxicities of quaternary mixtures of metal ions [Cd(II), Co(II), Zn(II) and Ni(II)] against microbial community of river water were assessed using inhibition of INT-dehydrogenase activity as endpoint and uniform design concentration ratios. The effective concentrations (EC_{50}) were estimated using logistic concentration-response model. The toxicity of the individual metal ion was ranked as Cd(II) > Co(II) > Zn(II) > Ni(II). In comparison to observed toxicities, the concentration addition (CA) and independent action (IA) models predicted the combined toxicities of the mixtures with varying accuracy. The deviations from accurate prediction of the mixture toxicities indicate possible synergistic and antagonistic effects of the mixtures. However, the model deviation ratios (MDR) based on 50% effective concentrations (EC_{50}s) for most mixtures lie between 0.5 and 2.0. Thus, the combined action of the mixtures were considered to be additive.

Keywords: Metal ions, Prediction of toxicity, Chemical mixtures, Dehydrogenase activity.

Introduction

Although metals are naturally occurring in the aquatic and terrestrial environments, significant levels of metals are introduced into the environment by human activities. Heavy metals are of primary concern since they are non biodegradable and persistent. Heavy metals are bioaccumulative and are biomagnified through the food chain. These metals are equally toxic to microorganisms via a number of mechanisms. Heavy metals bind to cellular molecules and displace essential metals from their normal binding sites[1]. They also disrupt protein and DNA functions and may affect oxidative phosphorylation[1]. Metals negatively affect the physiology of microbes resulting in decreased biomass and diversity.

Some heavy metals (such as Fe, Cu, Co, Ni, Zn) are required for microbial growth, whilst others (like Cd, Hg, As, Ag, Au) have no biochemical function. The non-essential elements are toxic[2,3]. The essential heavy metals are usually protein stabilizers, biochemical catalysts, regulators of gene expression and osmotic balance controllers in microbial membranes[4]. As an essential element, zinc plays catalytic, structural and regulatory roles in living systems[5]. Zinc is also a component of many microbial enzymes where it is necessary for their catalytic function and structural stability[6]. However, zinc can become toxic to cells at high concentrations. For instance, zinc is known to be inhibitory to respiratory electron transport system of bacteria and eukaryotic organisms[7-9]. Cadmium competes with cellular zinc for binding sites and bind non-specifically to DNA, inducing single strand breaks[1]. Although nickel and cobalt are microelements, they are both microbial growth inhibitors, at relatively high concentrations[10,11].

In our laboratory, we have conducted investigations on the inhibitory activities of individual metals on pure cultures of bacteria, microbial communities of soil and river water[9,12-17]. However, in the environment heavy metals do not usually exist as individuals but as mixtures arising from many natural and anthropogenic sources. Thus, environmental microorganisms are exposed to multiple mixtures of metals which may have antagonistic, synergistic or additive effect. In order to truly evaluate the ecotoxicological implications of these metals, it is important to assess the interactive effects of the mixtures on environmental microbial assemblages. Toxicity assessment of mixtures of pollutants requires application of an ecotoxicity test that is cost effective, rapid, sensitive and reliable. Investigations on toxicity of metal mixtures have mainly based on inhibition of bacterial bioluminescence.

This study aimed at assessing the toxicity of quaternary mixtures of cadmium (II), nickel (II), zinc (II) and cobalt (II) to microbial community of pristine river water based on inhibition of dehydrogenase activity. The method of evaluating the interactive toxicity of quaternary metal combinations in this study involved the following steps: i. testing toxicity of individual metal ions in increasing concentrations, followed by normalizing the concentration-response relationships to percent inhibition and estimating the EC_{50}s, ii. toxicity testing of various quaternary combinations prepared by using uniform design concentration ratios where the components of each mixture is added in a specified percent, iii. determination of the EC_{50}s of the mixtures, iv. predicting the toxicity of the mixtures and assessing the interactive effects of the metal mixtures using concentration addition and independent action models, v. statistical testing and comparison of the experimental and predicted toxicities of the mixtures.

Materials and methods

Riverwater sample: River water was collected from Otamiri River at Ihiagwa, Imo State, south-eastern Nigeria at three points (5◦24.25′0.32″ N, 7◦0.36′0.036″ E; 5◦24.28′0.55″ N, 7◦0.38′0.36″ E and 5◦23.55′0.20″ N, 6◦59.46′0.39″ E) and processed as described by Nweke *et al.*[18]. The total viable bacterial population of the water sample was estimated at 1.32 x 10^{10} CFU/ml on nutrient agar plate using standard microbiological methods.

Quaternary mixture ratios: The quaternary mixtures of cadmium (II), nickel (II), zinc (II) and cobalt (II) ions were studied using fixed ratio ray designs. The toxicity tests determined the toxicity of individual metals and their quaternary mixtures. The concentration ratio of the mixtures are shown in Table-1. The mixtures were prepared as 8 mM stock solutions by mixing requisite volumes of the individual metal ion solutions to give a specific concentration ratio. Each mixture was treated as single metal ion solution during toxicity testing.

Table-1: Quaternary mixture ratios of the metal ions.

Mixture	Mixture ratio (%) Cd^{2+}: Ni^{2+} Zn^{2+}: Co^{2+}
1	60:20:10:10
2	40:20:20:20
3	10:60:10:20
4	10:10:20:60
5	25:25:25:25
6	20:10:60:10

Toxicity assay: The toxicity assessment was carried out as described by Nweke *et al.*[18]. The tests were done in triplicate 15 ml test tubes containing a total of 2 ml reaction mixture for metal ion concentrations ranging from 0.1 to 2 mM. Triplicate control tubes without the metal ions were prepared for each test toxicant, giving a total of 12 controls. Each tube contained 0.5 ml of x4-strenght nutrient broth, requisite volumes of distilled water and stock solutions (8 mM) of the individual metal ion or the mixture, 0.1 ml of 0.1% aqueous solution of INT and 0.5 ml of the river water as inoculums. The tubes were incubated at room temperature (28 ± 2°C) for 24 hr. Extraction and determination of the INT-formazan produced in each tube were done as described elsewhere[18].

Data analysis: The response for each test concentration was normalized relative to the mean of controls (with standard deviation < 5% of mean) as percent inhibition which ranged from 0 to 100% as shown in equation 1. The mean and standard deviations of percent inhibitions were generated from triplicate determinations.

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

Where: R is the inhibition (%) of dehydrogenase activity (the response), C_A is the absorbance of INT-formazan extract in the control experiment and T_A is absorbance of INT-formazan extract in the test experiment with different concentrations of metal ions.

The concentration-response relationship of the individual metals and the mixtures were fitted with 2-parameter logistic function (Equation-2), with the maximum inhibition fixed at 100%, to obtain the 50% effective concentration (EC_{50}).

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

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

The toxic index (TI): The TI of each mixture was calculated as sum of the toxic units of all the components of the mixture (Equation-3).

$$TI = \sum_{i=1}^{n} \frac{C_i}{EC_{50i}} \quad (3)$$

Where: n is the number of components in the mixture, C_i is the concentration of ith component in the mixture (at the EC_{50} of the mixture) and EC_{50i} is the concentration of the i^{th} component that elicited 50% decrease in dehydrogenase activity when tested as an individual. TI = 1 indicates additive effect, TI > 1 indicates antagonistic interaction and TI < 1 indicates synergistic interaction.

Prediction of mixture toxicities: The joint effects of the quaternary mixtures of metal ions on the dehydrogenase activity of river water microbial community were predicted according to concentration addition (CA) and independent action (IA) models. In each case, the predicted EC_{50s} were compared with the observed EC_{50s} using Duncan post-hoc test implemented with IBM SPSS Statistics 21.

CA model: Based on concentration addition, the EC_{50} of the mixture can be estimated from the equation:

$$EC_{50(mix)} = \left(\sum_{i=1}^{n} \frac{\pi_i}{EC_{50i}}\right)^{-1} \quad (4)$$

Where: n is the number of components, π_i is the proportion of i^{th} component in the mixture (sum of π_i = 1), EC_{50i} is the

concentration of i^{th} component that gave 50% effect when tested as an individual.

The concentration-response relationships of the microbial community to toxicity of quaternary mixtures of metal ions were predicted based on concentration addition using average slope approach as shown in equation 5[19,20].

$$R = \frac{100}{1 + \left(\sum_{i=1}^{n} \frac{\pi_i x}{EC_{50i}} \right)^{b}} \quad (5)$$

Where: x is the total concentration of all the components in the mixture, b is the average slope of the components. Other variables are as defined in equation 4.

IA model: The IA model assumes that the components of a given mixture have different mode of action. The mathematical expression is as follows:

$$E(c_{mix}) = 1 - \prod_{i=1}^{n} [1 - E(c_i)] \quad (6)$$

Where: $E(c_{mix})$ is the total effect or response (scaled from 0 – 1) of the n-component mixture, c_i is the concentration of the i^{th} component and $E(c_i)$ is the effect or response of the i^{th} component in the mixture.

Model deviation Ratios (MDR): The MDR values were calculated as shown in equation 7 using the predicted and observed EC_{50}[21,22]. MDR > 1 indicated that the model underestimated toxicity, while MDR < 1 indicated that the model overestimated toxicity.

$$MDR = \frac{\text{Predicted } EC_{50}}{\text{Observed } EC_{50}} \quad (7)$$

Results and discussion

Effects of the metal ions on the dehydrogenase activity of the microbial community are shown in Figure-1. The metal ions progressively inhibited the dehydrogenase activity reaching 99.13%, 98.74%, 90.14% and 98.58% inhibition for Ni(II), Co(II), Zn(II) and Cd(II) respectively at 1.2 mM. The concentration-response relationships were sigmoidal and describable with 3-parameter logistic model. The EC_{50}s obtained are shown in Table-2. Ni(II) with the EC_{50} of 0.265 ± 0.015 mM was the least toxic while Cd(II) with the EC_{50} of 0.109 ± 0.011 was the most toxic. 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). There were no significant increases in toxicity of the metal ions at concentrations greater than 0.8 mM.

Table-2: Median inhibitory concentrations of the individual metals.

Metals	EC_{50} (mM) †	R^2
Cd^{2+}	0.109 ± 0.011a	0.983
Ni^{2+}	0.265 ± 0.015b	0.991
Zn^{2+}	0.240 ± 0.012c	0.994
Co^{2+}	0.142 ± 0.010d	0.993

†Within column, EC_{50} values with different letters are statistically different ($p < 0.05$) from each other.

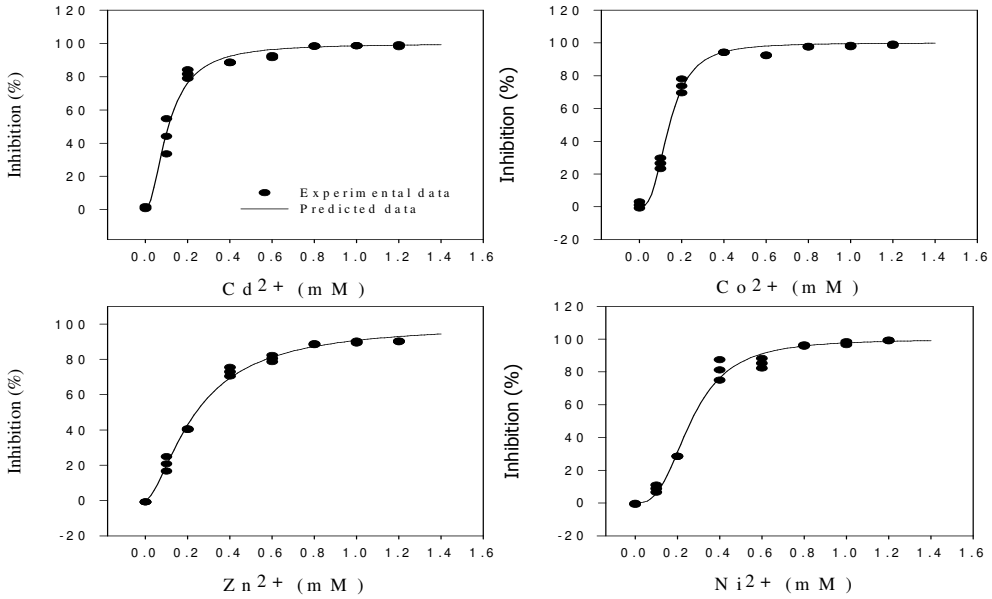

Figure-1: Effects of individual metal ions on the dehydrogenase activity in river water microbial community.

The toxicity of the quaternary mixture of metals was assessed using six different concentration ratios in a fixed ray uniform design. The responses of the quaternary mixtures of Cd(II), Co(II), Zn(II) and Ni(II) are shown in Figure-2. As was the case with individual metals, the mixtures progressively inhibited the enzyme activity as the concentration increases and seemingly reaching saturation at 0.8 mM. The inhibition of dehydrogenase activity was more abrupt in mixtures 1, 2, 4 and 5 at low concentrations. The inhibition was more gradual with mixtures 3 and 6. The concentration-response relationships produced by the mixtures were also described with 3-parameter logistic model. The EC_{50}s obtained are shown in Table-2. With the exception of mixtures 3 and 6, the EC_{50}s of the mixtures were lower than the EC_{50} of the most toxic metal (Cd(II), EC_{50} = 0.109 ± 0.011 mM). All the mixtures had EC_{50} lower than that of the least toxic metal (Ni(II), EC_{50} = 0.265 ± 0.015 mM). The order of toxicity of the mixture is mixture 4 > mixture 5 > mixture 1 > mixture 2 > mixture 6 > mixture 3. The statistical comparisons between the toxicities of the mixtures as shown in Table-3 indicated that the toxicities of mixtures 1, 2, 4 and 5 are not statistically different from each other and that toxicity of mixture 6 was significantly higher than that of mixture 3. The CA and IA models were used to predict toxicities of the mixtures. The predicted EC_{50}s and their statistical associations are shown in Table-3. There were no significant difference between the experimentally derived EC_{50} and the EC_{50} predicted from CA model for mixtures 1 and 6. Significantly higher EC_{50} were predicted from CA model when compared with the experimental EC_{50} for the mixtures 2, 3, 4 and 5. The toxic index of the mixtures varied from 0.431 ± 0.139 in mixture 4 to 1.218 ± 0.008 in mixture 3 (Table-2). In all the mixtures, EC_{50}s predicted from IA models are significantly higher than values derived experimentally or predicted from CA models. The relationship between the observed and predicted EC_{50}s are shown in Figure-3.

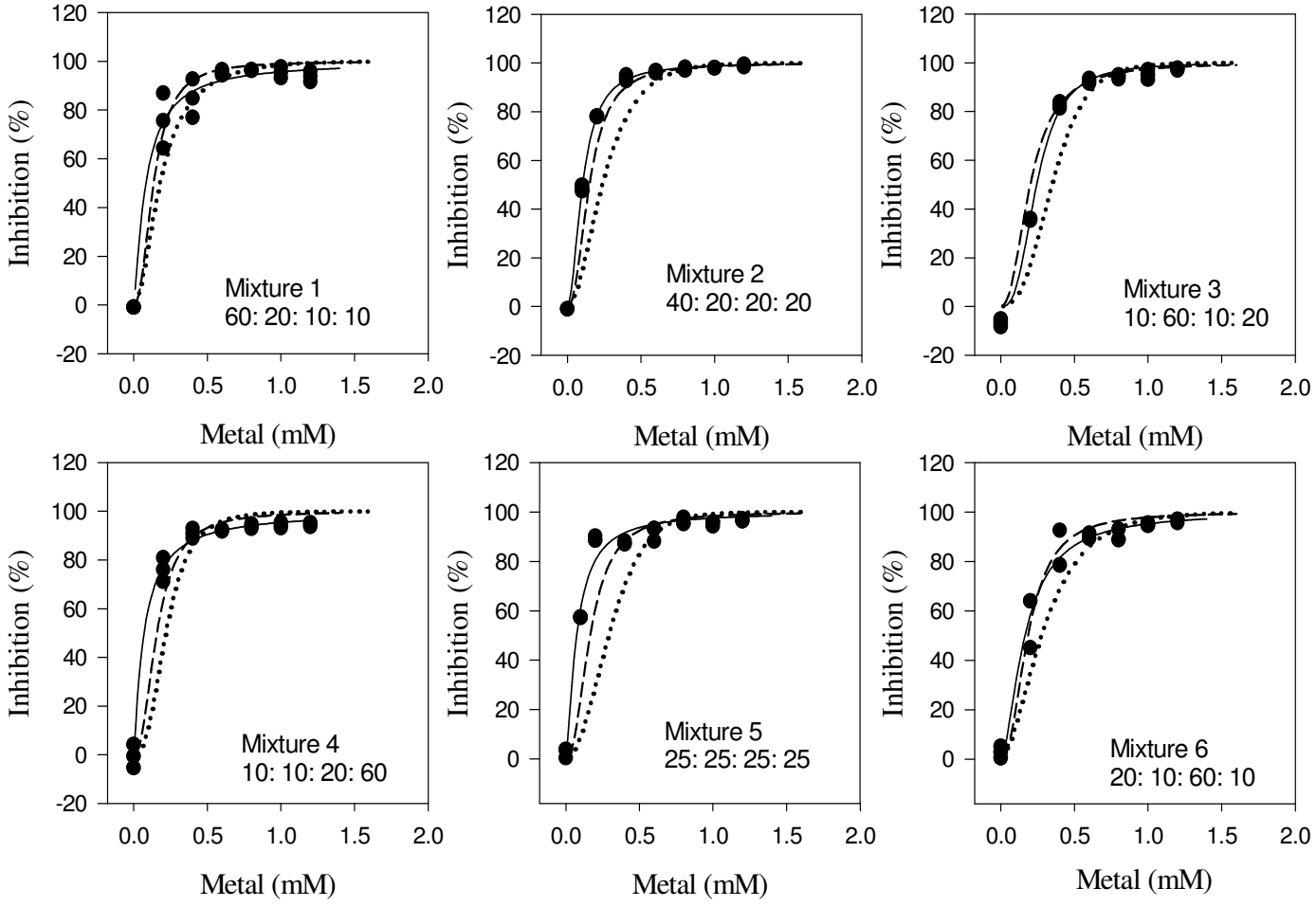

Figure-2: Observed and predicted concentration-response relationships for the inhibition of dehydrogenase activity in riverwater microbial community by mixtures of metal ions (in the ratio: Cd^{2+}: Ni^{2+} Zn^{2+}: Co^{2+}). Data points are observed values. The solid lines are the logistic model (Equation 2) fit to the observed data; the dashed lines are values predicted from CA model (Equation 5) and the dotted lines are values predicted from IA model (Equation 6).

Table-3: Observed and predicted toxicity thresholds and toxicity indices of the microbial community.

Metals Mixtures Cd^{2+}: Ni^{2+}: Zn^{2+}: Co^{2+}	Experimental†		Predicted†			
			CA		IA	
	EC_{50} (mM)	Toxic Index	EC_{50} (mM)	MDR	EC_{50} (mM)	MDR
Mixture 1 (60:20:10:10)	0.082 ± 0.045a	0.588 ± 0.281	0.135 ± 0.011a	2.051 ± 1.141	0.177 ± 0.016a	2.762 ± 1.787
Mixture 2 (40:20:20:20)	0.103 ± 0.002a	0.690 ± 0.039	0.150 ± 0.011a,b	1.453 ± 0.082	0.233 ± 0.009c	2.259 ± 0.084
Mixture 3 (10:60:10:20)	0.243 ± 0.013c	1.218 ± 0.008	0.200 ± 0.011d	0.821 ± 0.005	0.342 ± 0.006e	1.410 ± 0.098
Mixture 4 (10:10:20:60)	0.069 ± 0.025a	0.431 ± 0.139	0.158 ± 0.009b	2.505 ± 0.871	0.213 ± 0.005b	3.441 ± 1.396
Mixture 5 (25:25:25:25)	0.073 ± 0.014a	0.462 ± 0.032	0.165 ± 0.011b,c	2.169 ± 0.143	0.286 ± 0.003d	3.773 ± 0.494
Mixture 6 (20:10:60:10)	0.163 ± 0.029b	0.881 ± 0.097	0.184 ± 0.012c,d	1.144 ± 0.127	0.273 ± 0.003d	1.709 ± 0.320

Data are expressed as mean ± S.D., †within column, EC_{50} values with same letters are not statistically different ($p > 0.05$) from each other.

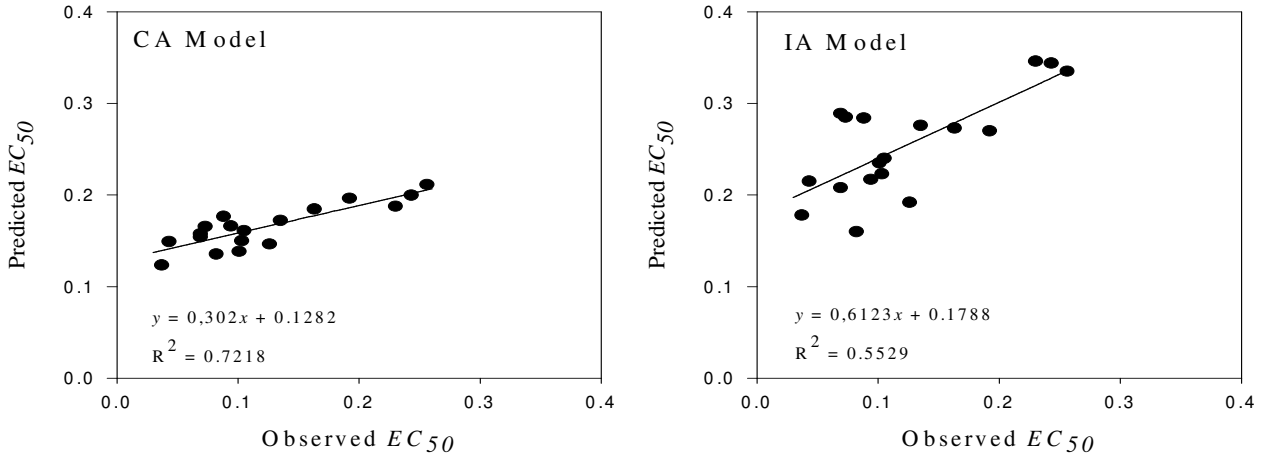

Figure-3: Relationships between the observed and predicted EC_{50} of the metal ion mixtures.

Heavy metal contamination of aquatic environment has been a serious problem because of their persistence and toxicity to aquatic organisms[23]. Heavy metals are deposited into the aquatic ecosystems from myriad of industrial activities. Cadmium, cobalt, nickel and zinc have many industrial applications and thus co-contaminate soil and aquatic habitats[24]. Majority of published articles regarding toxicity of heavy metals focused on individual metal effects. However, aquatic organisms are generally exposed to mixtures of metals. It is therefore important to evaluate toxicity of metal mixtures to microbial community of natural habitat. Different microbial responses have been used to assess metal toxicity to microorganisms. Among these responses is the dehydrogenase activity of the microorganisms. Microbial dehydrogenases are intracellular, rapidly degraded after cell death and are common to all microorganisms[25]. Thus, their activity could be used to evaluate toxicity of metals to microbial viability in response to toxicity of chemical substances. Dehydrogenase activity has been used to assess toxicity of metals to microbial community of soil and aquatic habitats. Nweke and Orji[9] assessed the toxicity of heavy metals to microbial community of New Calabar River water and reported IC_{50} of Cd(II), Ni(II) and Zn(II) to be 0.59 mM, 2.47 mM and 0.91 mM respectively. The IC_{50} of Cd(II), Co(II), Ni(II) and Zn(II) were 0.258 ± 0.018 mM, 0.239 ± 0.031 mM, 2.122 ± 0.245 mM and 0.257 ± 0.075 mM respectively for microbial community extracted from the root surface of *Vigna unguiculata*[17]. In the present study, cadmium was the most toxic metal. This corroborates the observations with pure cultures of *Pseudomonas* species[15,16]. Cadmium is known to have no physiological function and is toxic even at low concentration[26]. Cd(II) displaces Ca(II) and Zn(II) in proteins and cause oxidative stress[27,28]. In addition, Cd(II) could disrupt the integrity of microbial cell membrane and disturb the proton flux through the membrane[29]. Although cobalt, nickel and zinc are trace elements, they are toxic at high concentration, inhibiting dehydrogenases of the microbial community. This is in line with their reported toxicities. Zn(II) inhibited dehydrogenase activity in planktonic bacteria isolated from New Calabar River by 50%

at concentrations ranging from 0.192 mM for *Escherichia coli* to 1.002 mM for *Micrococcus* species[12]. In a similar experiment, the median inhibitory concentration of Zn(II) against sediment bacteria of New Calabar River ranged from 0.166 mM for *Bacillus* species to 0.873 mM for *Micrococus* species[13]. Dehydrogenase activity in microbial community of New Calabar river water was inhibited by 50% at 0.91 mM Zn(II) [9]. Ni(II) and Co(II) toxicity to microorganism have been widely reported and have been critically reviewed by Gikas[11]. The microbial community in the present study appeared to be more sensitive to the heavy metals. This difference may be attributed to the variation in microbial composition of the riverwater microbial community.

There is paucity of information regarding the effects of mixtures of metals to natural microbial communities particularly with the use of dehydrogenase enzyme activity as endpoint. However toxicities of binary and ternary mixtures of metals against luminescent bacteria have been reported[30-32]. Based on TI analysis, the joint action of the quaternary mixtures were generally synergistic. However, mixture 3 indicated antagonistic interaction of the metal ions against the dehydrogenase activity of the microbial community. The EC_{50} of the mixtures indicated that Cd(II) and Co(II) exerted greater toxicity in the mixture. The most toxic metal ions, Cd(II) and Co(II) made up to 50% or greater of the metal ions in mixtures 1, 2, 4 and 5. Cadmium, the best known toxic heavy metal exerts toxicity to microorganisms by denaturing proteins, cell membrane damage and interfering with zinc metabolism[26].

The CA and IA models have been used to predict toxicity of chemical mixtures based on the concentration-response relationship of the components of the mixture. The CA model is based on the assumption that the components of the mixture acts similarly while IA models assumes that the components acts dissimilarly. Some studies have reported underestimation of the combined toxicity of chemical mixtures by CA model[33]. Others have found that CA model overestimated the toxicity of chemical mixtures[34,35]. In the present study, the CA model overestimated toxicity of mixture 3 and underestimated the toxicities of the other mixtures. However, it is important to note that there are no significant differences between the observed EC_{50} and the CA-predicted EC_{50} for mixtures 1 and 6, indicating additive effect of the mixture components. The toxicity of mixtures 2, 4 and 5 were significantly underestimated indicating synergistic interaction. The reason for this departure from additivity could be attributed to the high ratios of the most toxic metals in the mixtures. Similar observation was made by Liu *et al*. for *Vibrio qinghaiensis* Q67 responding to toxicity of diquat[36]. In addition, CA model significantly overestimated the EC_{50} of mixture 3, indicating antagonism. The IA model significantly underestimated the toxicities of all the mixtures. Mixtures 1 and 6 are shown to be additive, having insignificant difference between the observed and predicted toxicities. In this study, CA model generally predicted higher toxicity than IA model. This observation has been made by other authors, leading to suggestions that CA model may be used as a worst case approach for the hazard analysis of mixtures[35,37]. However, a simple ratio, the MDR was also used to express the deviation of experimentally-observed toxicity from the toxicity predicted by CA and IA models. The MDR values between 0.5 and 2 ($0.5 \leq MDR \leq 2$) defines the mixture that deviated less than two-fold from the predictions of the models. Mixtures with MDR values outside this range are either antagonistic ($MDR < 0.5$) or synergistic ($MDR > 2$)[38]. MDR value within a factor of two ($0.5 \leq MDR \leq 2$) indicated that the mixture was likely to be additive as the ratio is within the expected inter-laboratory/inter-experiment deviation for most species[22,39]. Based on this principle, mixtures 1, 2, 3 and 6 are generally considered to be additive while mixtures 4 and 6 are considered to be synergistic. Information on the interaction of the four metals used in this study is scarce. However, there have been reports on the interactive toxicities of binary mixtures involving these metals. Toxicity of binary mixture of Zn(II) and Cd(II) to *lux*-marked *Escherichia coli* HB 101 was shown to be synergistic. An overall additive effect of Zn(II) and Cd(II) mixtures on luminescent *Pseudomonas fluorescens* was reported by Preston *et al*.[31]. Antagonistic interaction was reported for binary mixtures of Co(II) and Cd(II) as well as Zn(II) and Cd(II) against *Vibrio fischeri*[32]. However, the binary mixture of cobalt and zinc was shown to be additive[32]. Zeb *et al*.[40] reported antagonistic effect of Cd + Cu, Cd + Pb and Cu + Pb binary mixtures and partly additive effect of Cd + Cu + Pb ternary mixtures on *Photobacterium phosphoreum* T3S.

The relatively good correlation between observed and predicted toxicity based on CA model indicated that the metals may have similar or related mode of action. The metals Zn(II), Co(II) and Ni(II) are essential divalent metals required for normal physiological functions in cells[24,26] and Cd(II) is a non-essential toxic metal. In addition, from the analysis of the CA- and IA-derived equation, we found that the predicted mixture toxicity increased with increasing observed mixture toxicity. However, both CA and IA models did not predict the mixture toxicities accurately, based on the individual metal toxicity data. The reason for this inaccurate prediction might be due to complexity of biological system and the multiple modes of action of heavy metals[41-44].

Conclusion

In the present study, the toxicities of individual and quaternary mixtures of four metals (Cd, Ni, Zn and Co) to microbial community of river water were assessed using inhibition of dehydrogenase activity as response. Toxic index analysis indicated that the combined effect could be additive, antagonistic or synergistic. Nevertheless, the model deviation ratios (MDRs) indicated that the deviations from additivity are within the expected interlaboratory deviations for most organisms and the combined effects are likely to be additive. However, from the viewpoint of these analyses, synergistic effect of the mixture of these metals is a possibility. Thus, the combined effect of these metals should be considered in the risk assessment of heavy metal pollution in river water.

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