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Assessment of Experimental and Predicted Toxicity of Pesticides and Heavy Metals Binary Mixtures to *Rhizobium* Species

Gaius-Mbalisi, V. K.*[1], Asiwe, E. S.,[2] Nlemolisa, O. R.,[1] Kemka, U. N.,[1] Onyeukwu, U. R.[3]

[1]Department of Microbiology, School of Biological Sciences, Federal University of Technology Owerri, Imo State, Nigeria.
[2]Department of Biological Sciences, University of Agriculture and Environmental Sciences, Umuagwo, Imo State.
[3]Department of Industrial Microbiology and Brewing, NnamdiAzikiwe University, Awka, Anambra State.

Abstract:- The present study assessed the experimental and predicted toxicity of pesticides and heavy metals binary mixtures to *Rhizobium* species. The experimental toxicity response was assessed using the inhibitory effect of the individual and binary mixtures of glyphosate (Gly) with cadmium (Cd (II) and lead (Pb (II)); and 2, 2 Dichlorovinyl dimethyl phosphate (DDVP) with Cd (II) and Pb (II) to *Rhizobium* species total dehydrogenase activity using 2,3,5 triphenyltetrazolium chloride (TTC) as the artificial electron acceptor. The binary mixtures were composed using fixed percentage ratios of 50% : 50%, 60% : 40% and 80% : 20% mixtures of Gly + Cd (II), Gly + Pb (II) and mixtures of DDVP + Cd (II), DDVP + Pb (II). The Half maximal effective concentration (EC_{50}) of the toxicants were estimated using monotonic logistic dose-response model. Prediction of binary mixture toxicity effect was carried out using Concentration Addition (CA) and Independent Action (IA) models. Results obtained showed that Cd (II) and Pb (II) ions inhibited *Rhizobium* sp. dehydrogenase activity in a logistic concentration dependent manner; glyphosate and DDVP exhibited a biphasic toxicity effect to *Rhizobium* sp. Glyphosate and DDVP at concentrations below 5000mg/L and 1000mg/L respectively stimulated the total dehydrogenase activity of the isolate. Using EC_{50} ranking, the order of toxicity was Cd (II) >Pb (II) > DDVP > glyphosate. The binary mixtures of glyphosate and Pb (II) in ratios of Gly (50%) : Pb (II) (50%) and Gly (80%) : Pb (II) (20%) stimulated total dehydrogenase activity of *Rhizobium* sp. at concentrations below 1000 mg/L and 500mg/l for the respective mixtures. Prediction of binary mixtures toxicity using the CA and IA model differed in their outcome of toxicity interactions. The CA and IA model predicted additive interactions for the mixtures of Gly 50% + Cd (II) 50%, DDVP 60% + Cd (II) 40%, DDVP 50% + Cd (II) 50% and DDVP 80% + Cd (II) 20%, which differed from experimentally observed synergism. The CA model underestimated the toxicity of binary mixtures of DDVP + Cd (II) and glyphosate + Pb (II), while overestimating the toxicity of the DDVP + Pb (II) and glyphosate + Cd (II) binary mixtures. Furthermore, the IA model predicted mostly additive interaction for binary mixtures of glyphosate + Cd (II) and DDVP + Cd (II) ions mixture across the ratios; which was a gross overestimation of toxicity in most of the binary mixtures studied. The joint action of the mixtures on test organism predicted with concentration addition (CA) and independent action (IA) models may present varying interactions dependent on the relative amount of the heavy metals present in the binary mixtures and their relative mode of action.

Keywords*: Prediction, Toxicity, Pesticides, Heavy Metals, Concentration Addition, Independent Action.*

I. INTRODUCTION

Over the years, the use of Pesticides and other Agrochemicals has become an important practice in global agricultural system, allowing for increase in crop yields and food production. Due to the exponentially growing worldwide population expected to be 9.2 billion in 2050, the general perspective is that global agriculture production has to be increased by about 60-70% from the current levels to meet the increase of food demand in 2050 [1].

To this end, the modern day agricultural sector has relied greatly on the extensive use of pesticides which has played an effective and economical role towards enhanced quality as well as quantity of agricultural produce. The term pesticide covers a wide spectrum of compounds including herbicides, insecticides, fungicides, rodenticides, molluscicides, nematicides, plant growth regulators and others [2]; which are used for agricultural purposes or for public health protection programs in order to protect plants from pests, weeds or diseases, and humans from vector-borne diseases [3]. Despite the beneficial functions, the use of pesticides has been associated with negative health and environmental impacts. They contaminate air, soil, surface water bodies, underground water and vegetation and consequently exert their toxic effect on target and non-target organisms [4].

The toxicity of pesticides on most life forms has been reported [5, 6, 7] including microorganisms, with concentration of the pesticide and duration of exposure as the major determinant factors [8, 9]. Several studies have been conducted, demonstrating the negative effects of pesticides on soil microbial biomass, biochemical and enzymatic activities [10, 11], and others with contrasting result, showing a stimulatory effect due to the pesticides acting as carbon source [12].

Dichorvos, an organophosphate pesticide with the chemical name 2,2-dichlorovinyl dimethyl phosphate (DDVP) is widely used in the agricultural and industrial sector for the control of insect pest [13]. It is marketed with brand names such as Dedevap, Nogos, Nuvan, Phosvit, Vapona, Sniper and Daksh [14]. As an organophosphate, pesticides Dichlorvos lack target specificity and can cause severe, long lasting population effects on terrestrial and aquatic non-target species. It exerts its toxicity on both vertebrates and invertebrate by irreversibly inhibiting the cholinergic enzyme; acetylolinesterase, accumulating acetylcholine in synapses and consequently disrupts neural function [15]. Li. *et al*. [9] reported non-cholinergic neurotoxicity of DDVP leading to the inhibition of the proliferation of porcine kidney epithelial cells, suggestive of DDVP-induced apoptosis. Application of DDVP has shown to adversely affect cultivated plants by inhibiting soil microbial population responsible for soil biochemical processes such as nitrification and denitrification [16].

Glyphosate, another organophosphate compound is the most widely used herbicide with broad spectrum activities [17]. It exerts its lethal effects on some plants and microorganisms by inhibiting the 5-enolpyruvilshikimate-3-phosphate synthase enzyme of the shikimic acid pathway, a metabolic pathway involved in the biosynthesis of aromatic amino acids. The blockage of this pathway will lead to the reduction in protein synthesis and consequently the death of the organism in a few days [18].

In a study reported by Hadi *et al*. [19], the harmful impact of glyphosate was affirmed to alter the soil texture and microbial diversity by reducing the microbial richness and increasing the population of phytopathogenic fungi. In contrast to this, existing literatures have recorded the ability of glyphosate to significantly stimulate soil microbial activities [20] and the potential of some microbial species such as *Achromobacter sp. Comamonasodontotermitis, Ochrobactrum intermedium* Sq20, *Arthrobacter atrocyaneus, Alcaligenes sp. Arthrobacter sp*. and *Pseudomonas* as well as fungi and *Actinomycetes* to mineralize glyphosate; using it as carbon, nitrogen and phosphorus source [21, 22]. This indicates the capability of these organisms when grown under optimum conditions to be utilized in the bioremediation of glyphosate-polluted environment [23].

Over time, increasing heavy metal pollution due to intensive anthropogenic activities has been an issue of great concern especially in the agricultural sector; raising concern of plant uptake and bioaccumulation. Their accumulated presence in agricultural soil, arise from the use of fertilizers, pesticides, livestock manure and wastewater [24] and as a result exert their toxic effect on soil biota including microorganisms [25]. Although small amount of essential heavy metals such as Zinc, Copper and Iron are needed by all life forms including microorganism, for vital physiological and biochemical functions, others such as cadmium, lead, mercury do not have any biological role and are toxic to microorganism even at low concentration [26].

Chemical contaminants in the environment usually exert their toxicity on living organisms in combined form rather than individual compounds. Therefore assessing their potential combined toxicities is important as individual risk evaluation tend to underestimate the effects associated with toxic action of chemical mixtures [27]. Several studies have dealt on various toxicity mixtures of heavy metals, phenolic compounds, and pesticides on indigenous microorganisms, employing microbial parameters such biomass measurement, inhibition of bioluminescence, enzyme activity, growth rate, respiratory rate as endpoint [7, 28, 29]. These studies are very imperative in determining the ecotoxocological implications of chemical contaminants as their mixture exposure may lead to a worrisome toxicological interaction and response in the ecosystem. This study in its novelty, tries to assess the toxic pattern of the binary mixture of pesticides and heavy metals on *Rhizobium* species.

II. MATERIALS AND METHODS

➢ *Test Chemicals and Reagents*

In this study, the metal ions Pb(II) and Cd(II) ions were used as $Pb_2(NO)_3$ and $CdSO_4.8/3H_2O$ respectively. Formulated glyphosate (Banbino®) and DDVP (Sniper®) were the pesticides used. Reagents and media used include 2, 3, 5-triphenyltetrazolium chloride (TTC) (Sigma-Aldrich, USA), n-Butanol (BDH, England), distilled water, Nutrient agar (Titan Biotech, India), nutrient broth (Titan Biotech, India) and yeast extract mannitol salt agar. The entire reagents used were of analytical grade.

➢ *Isolation of Test Organism and Purification*

Using the method described by Tchounwou *et al.* [30], root nodules of young Arachis hypogeal plants were carefully collected, prepared and crushed with a sterilized glass rod to obtain a milky suspension and consequently release encapsulated *Rhizobium* species present in the nodules. The *Rhizobium* species was isolated by plating the suspension on yeast extract mannitol salt agar (YEMA) which was compounded with 4gm of mannitol salt agar, 0.4gm of Yeast extract powder, 8gm of agar powder, 0.08gm of $MgSO_4$, 0.2gm of K_2PO_4, 0.04gm of NaCl, 0.01gm of Congo red and 400ml of sterile distilled water [30, 31]. Pure cultures of organisms were then obtained by plating out the distinct colonies on nutrient agar plates. The isolates were stocked in YEM agar slants and stored at 4°C. The culture was characterized biochemically using standard microbiological methods.

➢ *Preparation of the Stock Solution*

For the individual metal ion, a 10mM working stock solution equivalent to 3312mg/L and 2565mg/L for Pb (II) and Cd (II) respectively was prepared, while a stock solution of 20,000mg/L was prepared for glyphosate and

5000mg/L for DDVP. The stock solution for the binary mixtures of the toxicants across all ratios studied were 6254mg/L, 5000mg/L, 2000mg/L and 500mg/L for Gly + Cd, Gly + Pb, DDVP + Cd and DDVP + Pb respectively. This was prepared by mixing the different concentration of each toxicant according to the desired ratio of the total stock solution.

➢ *Preparation of the Inoculums*

The test organism was grown to mid exponential phase in a nutrient broth on a rotary incubator at a room temperature and 150rpm. The cells were harvested by centrifugation at 3000rpm for 10mins. The harvested cells were washed twice and re-suspended in sterile deionised water. Using a spectrophotometer, the optical density of the re-suspended cells was adjusted to 0.1 at 540nm. An aliquot of 0.1 ml of the standardized cell suspension was used as inoculum in the dehydrogenase assay.

➢ *Design of Binary Mixture Ratios*

To determine the toxicity of the binary mixtures of the test chemicals, fixed ratio ray design was used. Here, mixture ratio of the constituting toxicants was kept constant while the total concentrations for each of the mixtures were varied so as to obtain a dose-response relationship of the mixture. The range of concentration used for the mixture was based on the concentration range of the individual toxicants that brought about a 0-100% or almost 100% dehydrogenase enzyme inhibition. The concentration for the metal ion Pb(II) ranged from 0 – 1656mg/L (0-5mM) while that of Cd(II) is from 0 – 770mg/L (0-3mM). The concentration range for glyphosate and DDVP was 0 – 15,000mg/L and 0- 4000mg/L respectively. The binary mixtures of glyphosate with Cd(II) and glyphosate with Pb (II) were studied as a function of the following fixed weight to weight ratios: Gly (50%) + Cd (II) (50%), Gly (60%) + Cd (II) (40%) and Gly (80%) + Cd (II) (20%); and Gly (50%) + Pb (II) (50%), Gly (60%) +Pb (II) (40%) and Gly (80%) + Pb (II) (20%). Similar ratios were applied in binary mixtures of DDVP and Cd(II) and DDVP and Pb(II). The concentrations for all the binary mixtures studied ranged from 0- 1000mg/l for glyphosate + Cd(II), glyphosate + Pb (II), DDVP + Cd(II), and DDVP + Pb(II) respectively. The stock solution for all the mixtures was prepared by mixing requisite volumes of equal concentration of individual test chemical solution to arrive at desired concentration ratio treating each mixture as an individual toxicant.

➢ *Acute Toxicity Assay*

The acute toxicity assay for dehydrogenase activity of *Rhizobium* in the presence of the test chemicals was carried out using 2,3,5-riphenyl tetrazolium chloride (TTC) as the artificial electron acceptor which was reduced to the red-coloured triphenyl-formazan (TPF). Each reaction tube of a 2ml final volume contained a particular volume of the toxicant in proportion to its final concentration and the requisite volume of sterile deionised water. This was supplemented with a 0.5ml portion of a quadruple strength nutrient broth. Into each tube was also added, a 0.1ml aliquot of the standardized *Rhizobium* cells and 0.1ml of 0.1% (w/v) TTC. Each concentration setup for the binary mixtures and individual toxicant was prepared in triplicates. Control reaction tubes without toxicants were also setup in triplicates. The reaction tubes were then incubated at room temperature (28±2ºC) for 24 h. The red triphenylformazan (TPF) formed after 24h, was extracted by the addition of 4ml of n-butanol to the tubes, followed by vigorous shaking. The formazan was quantified by measuring absorbance in a spectrophotometer at 500nm. The inhibition of the dehydrogenase activity of the test organism was determined in relation to the control.

➢ *Data Analysis*

The normalized responses of each toxicity assessment were generated as mean from the triplicate values. Then, the inhibition was transformed to a 0 -100% scale in relation to the mean value (SD < 5%) of the control as shown in equation 1.

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

Where R is the inhibition (%) of the dehydrogenase activity of the test organism, C_A is the absorbance of TPF extracted from the control experiment and T_A is the absorbance of the TPF extract from the test experiment with different toxicant concentration. The dose – response data obtained for the single toxicants and their mixture as well, were plotted and fitted with 2- parameter logistic function as described in equation 2.

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

Where x is the concentration of the toxicants or their mixtures, EC_{50} is concentration of the toxicants or the concentration of their mixture that elicited a 50% inhibition of the dehydrogenase enzyme activity and b is the slope at EC_{50}.

- *Toxic Index*

The interaction of the binary mixtures was determined using the toxic index (TI) model. The TI of each of the mixtures was calculated as the sum of toxic units of all the components of the mixture as indicated in equation (3)

$$TI = \sum_{i=1}^{n} \frac{C_i}{EC_{50i}} = \sum_{i=1}^{n} \frac{\pi_i EC_{50\,mix}}{EC_{50\,i}} \qquad (3)$$

Where C_i is the concentration of the ith component in the mixture at EC_{50} of the mixture (EC_{50mix}) and EC_{50i} is the concentration of the ith component that inhibited the dehydrogenase activity by 50% when tested as an individual toxicant, n is the number of components in the mixture and π_i is the proportion of ith component in the mixture. According to [32], TI value of 1 indicates an additive interaction; values greater than 1 indicate an antagonistic interaction while those less than 1 indicate a synergistic interaction.

- *Prediction of Binary Mixture Toxicities*

Prediction of binary mixture toxicity effect on *Rhizobium sp.* was carried out using concentration addition (CA) and independent action (IA) models. The CA model assumes similarity of the mixture components mode of action. If the mixture is additive, it obeys concentration addition model. The effective concentration of the mixture (*ECx (mix)*) can be predicted from the equation [33]:

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

Where *ECx* (mix) is the total concentration of the mixture that elicit x% effect, *ECxi* is the concentration of component that will produce x% effect when tested singly, n is the number of mixture components, πi is the fraction of i^{th} component in the mixture, Therefore, $\sum \pi i = 1$

The independent action model assumes dissimilarity in the mixture components mode of action; mathematically expressed as follows:

$$E(C_{mix}) = 1 - \prod_{i=1}^{n}[1 - E(C_i)] \quad (5)$$

Where $E(C_{mix})$ represents the total effect or response (scaled from 0 to 1) of an n-component mixture, C represents the concentration of the i^{th} component and $E(C_i)$ is the effect or response of the individual component [34, 35]

The effect of the individual component $E(C_i)$ was estimated from the 2-parameter logistic function (Eqn 2.) for the individual toxicant. Thus the IA model is simplified in Eqn 6 for mixtures scaled from 0-100% [7].

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

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

- *Model Deviation Ratio (MDR)*

The predicted EC_{50} of the mixtures based on CA and IA model were derived using Eqn. 4 and 6 respectively, based on the proportion of the EC_{50} of the individual component. The MDR were calculated as ratios of predicted EC_{50} to experimental EC_{50} (eqn7), using the CA and IA predicted EC_{50} and experimental-derived EC_{50} [7]. MDR values ranging from 0.5 -2.0 ($0.5 \leq MDR \leq 2$) predict that the mixture was most likely to be additive MDR > 1 and MDR < 1 predicts underestimation (synergism) and overestimation (antagonism) of toxicity respectively [7].

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

III. RESULTS AND DISCUSSION

The toxicity of the individual compounds glyphosate, DDVP, Cd (II) and Pb (II) to *Rhizobium* sp total dehydrogenase activity (figure 1), indicated varying responses of the isolate to herbicides and heavy metals exposure. Cd (II) and Pb (II) ions inhibited *Rhizobium* sp. dehydrogenase activity in a concentration dependent manner. The effects of Cd (II) and Pb (II) ions on the total dehydrogenase activity of *Rhizobium* sp largely fitted into a monotonic logistic model as shown in figure 1. However, the toxicity of glyphosate and DDVP were biphasic. Glyphosate and DDVP at concentrations below 5000mg/L and 1000mg/L respectively stimulated the total dehydrogenase activity of the isolate. Inhibitory effect of the toxicants on *Rhizobium* sp occurred beyond this hormetic zone. The threshold eco-toxic concentration of the compounds were 22.55 ± 2.50 mg/L, 489.29 ± 14.86 mg/L, 6716.73 ± 754.83 mg/L and 1712.23 ± 94.79 mg/L for cadmium, lead, glyphosate and DDVP respectively (table 1). Comparatively, the order of toxicity using the EC_{50} in the present study is Cd(II) >Pb (II) > DDVP > glyphosate. Heavy metal inhibition of microbial enzymes activities in vitro and in situ in soil have been reported [36, 37, 7]. The heavy metals inhibit microbial enzyme activity and modify microbial communities in the soil; their actions are mediated through blocking of enzyme-binding sites by combining with the active protein groups of enzymes and modifications of genomic DNA tem-plate stability [38, 36, 39]. These changes are significant in microflora composition and activity of individual enzymes which decreases organic matter decomposition. Our reported low dose stimulation of dehydrogenase activity by glyphosate and DDVP are consistent with the works of Nweke *et al.* [40] and Anuniru *et al.* [41]. Previous studies have described stimulation of microbial growth by pesticides, and a wide range of statistical models have been proposed for characterizing hormetic dose-response relationships in living organisms [42, 43, 44, 45]. Nweke *et al.* [40] reported glyphosate stimulation of dehydrogenase enzyme activity at concentrations up to 400 mg/l, and saturation of the inhibition of dehydrogenase activity of the *Rhizobium* species, at 1200 mg/l for glyphosate. The work of Anuniru *et al.* [41] reported glyphosate low dose of stimulation of dehydrogenase activity of *Pseudomonas* sp. and *Bacillus* sp. at the range of 0-800 mg/L. Tolerance to pesticides and heavy metals are linked to physiological changes that may lead to alternative metabolic pathways to bypass metabolic enzyme reactions inhibited by a specific toxicants; also tolerance to toxicity may depend on transmissible genetic modifications to microbial progeny [46, 47, 48].

Mathematical models are applied in description of biological phenomenon; resulting in improved understanding of ecology and environmental health. The CA and IA models are applied in the prediction of mixture toxicity (34, 32, 33, 49). The CA and IA model assumes similarity and dissimilarity in mixture component mode of action respectively. The results of the present study (figure 2) show the experimental and predicted toxicity of binary mixtures of glyphosate and cadmium in ratios of Gly (50%)

+ Cd (II) (50%), Gly (60%) + Cd (II) (40%) and Gly (80%) + Cd (II) (20%); and mixtures of glyphosate and lead in ratios of Gly (50%) + Pb (II) (50%), Gly (60%) + Pb (II) (40%) and Gly (80%) + Pb (II) (20%) to *Rhizobium sp.* Result show that the toxicity of the binary mixtures of glyphosate with the two heavy metals fitted into a monotonic logistic models. The threshold eco-toxic concentration (EC_{50}) values of the glyphosate and cadmium was 61.73 ± 3.49mg/L, 35.61 ± 8.06 mg/L, and 146.98 ± 5.95mg/L for Gly (50%) + Cd (II) (50%), Gly (60%) + Cd (II) (40%) and Gly (80%) + Cd (II) (20%) mixtures. However, result of the binary mixture of glyphosate and Pb (II) show that Gly (50%) +Pb (II) (50%), and Gly (80%) +Pb (II) (20%) mixtures were biphasic, stimulating total dehydrogenase activity of *Rhizobium* sp. at concentrations below 1000 mg/L and 500mg/l for the respective mixtures. The threshold eco-toxic concentration (EC_{50}) values of the glyphosate and lead binary mixtures was 986.18 ± 95.00 mg/L, 1097.42 ± 91.56 mg/L and 563.99 ± 35.10 mg/L for Gly (50%) +Pb (II) (50%),

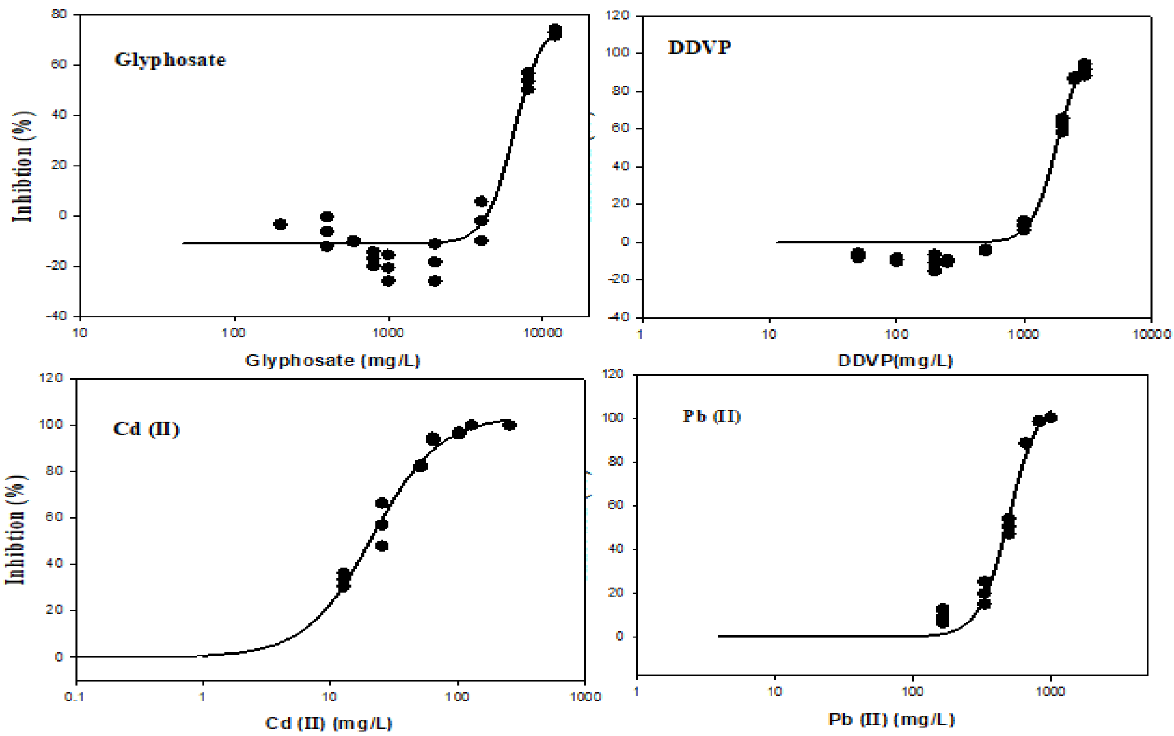

Fig 1 Inhibition of Dehydrogenase Activity of *Rhizobium Sp* by the Individual Toxicants

Gly (60%) +Pb (II) (40%) and Gly (80%) +Pb (II) (20%) respectively. The toxicity of the mixture was in the order Gly (50%) +Cd (II) (50%) >Gly (60%) + Cd (II) (40%) >Gly (80%) + Cd (II) (20%) for binary mixtures of glyphosate with Cd (II), while the order was seen to be the reverse for mixtures with Pb (II). Increasing concentration of Cd (II) ions in the mixture resulted in modulation of glyphosate to become more toxic; the reverse was the case for binary mixtures of glyphosate with Pb (II). The differences in their effect on glyphosate toxicity may be attributed to their mode of action on *Rhizobium* sp.

Also, the experimental and predicted toxicity of binary mixtures of glyphosate and cadmium in ratios of DDVP (50%) + Cd (II) (50%), DDVP (60%) + Cd (II) (40%) and DDVP (80%) + Cd (II) (20%); and mixtures of glyphosate and lead in ratios of DDVP (50%) +Pb (II) (50%), DDVP (60%) +Pb (II) (40%) and DDVP (80%) +Pb (II) (20%) to *Rhizobium* sp. presented in figure 3 show that the toxicity of the binary mixtures of glyphosate with the two heavy metals fitted into a monotonic logistic models. The threshold eco-toxic concentration (EC_{50}) values of the glyphosate and cadmium was 17.29 ± 1.04mg/L, 34.17 ± 1.91mg/L, and 42.33 ± 2.31mg/L for DDVP (50%) + Cd (II) (50%), DDVP (60%) + Cd (II) (40%) and DDVP (80%) + Cd (II) (20%) mixtures.

However, result of the binary mixture of glyphosate and lead show that Gly (50%) +Pb (II) (50%), and Gly (80%) +Pb (II) (20%) mixtures were biphasic, stimulating total dehydrogenase activity of *Rhizobium* sp. at concentrations below 1000 mg/L and 500mg/l for the respective mixtures. The threshold eco-toxic concentration (EC_{50}) values of the glyphosate and lead binary mixtures was 871.66 ± 38.85mg/L, 584.59 ± 56.87mg/L and 1371.23 ± 132.72mg/L for DDVP (50%) +Pb (II) (50%), DDVP (60%) + Pb (II) (40%) and DDVP (80%) + Pb (II) (20%) respectively. Notably, the toxicity of the heavy metals with DDVP varied in the order; DDVP (60%) + Cd (II) (40%) > DDVP(50%) + Cd (II) (50%) > DDVP (80%) + Cd (II) (20%) for mixtures with Cd (II) and DDVP(50%) + Pb (II) (50%) > DDVP (60%) +Pb (II) (40%) > DDVP (80%) +Pb (II) (20%) for mixtures with Pb (II) ions respectively. The binary mixtures of DDVP + Cd (II) in all the ratios produced toxic index values less than 1 and thus interacted synergistically to produce higher toxicity than that of the single compound. While the binary mixtures of

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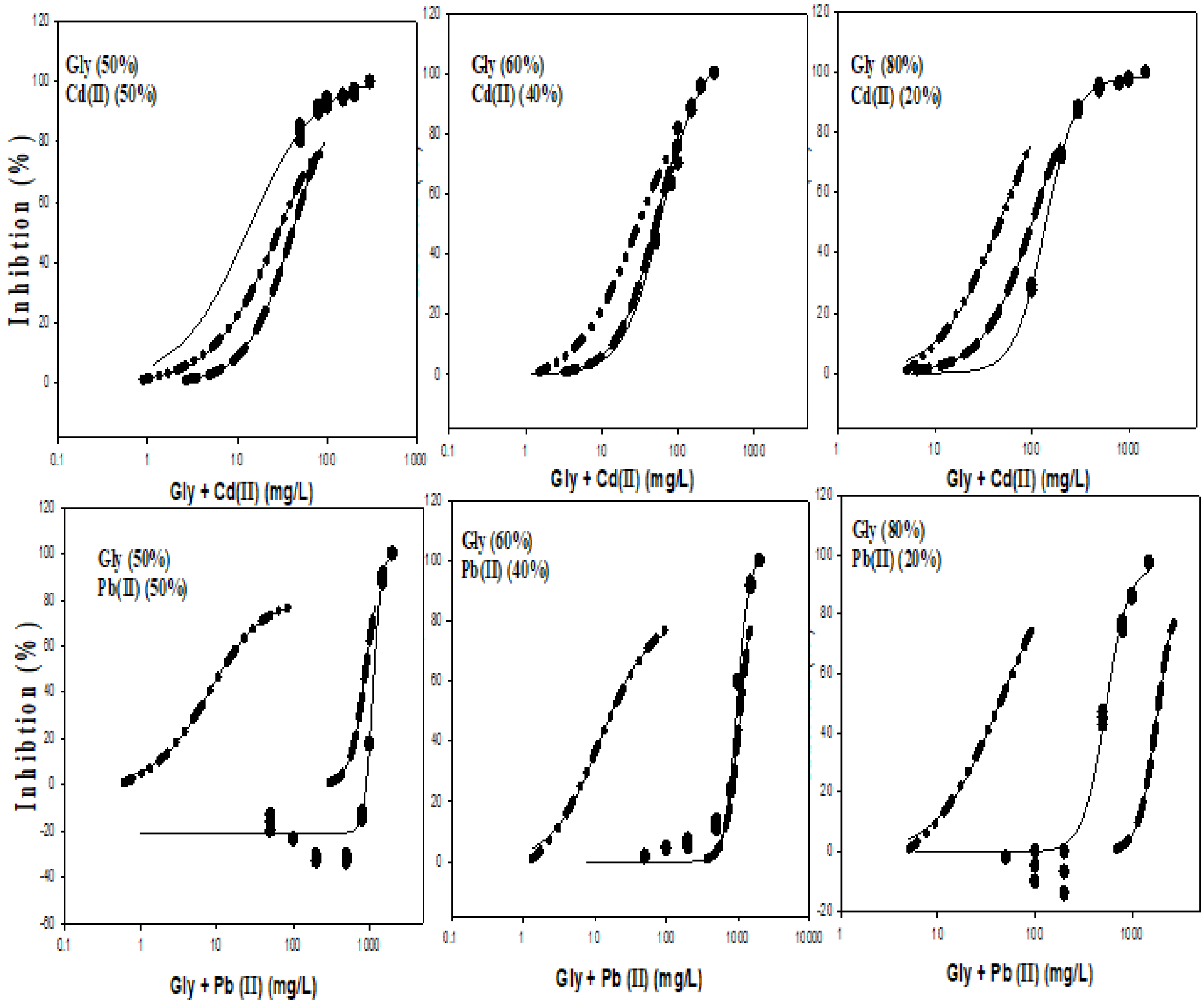

Fig 2 Toxicity of Glyphosate and Cadmium Ions Binary Mixtures, Glyphosate and Lead Ion to *Rhizobium Sp* Dehydrogenase Activity. The Data Points Represent Experimental Dose-Responses. The Solid Line Represents Estimated Toxicities by Fitting Experimental Data to Logistic Model (Eq. 2), the Long Dash and Dash/Dotted Lines Indicate the Predicted Toxicities from Concentration Addition and Independent Action Models Respectively

DDVP + Pb (II) mixtures produced a mix of additive, synergistic and antagonistic interaction for ratios DDVP 60% + Pb (II) 40%, DDVP 50% + Pb (II) 50%, and DDVP 80% + Pb (II) 20% respectively.

The increasing concentration of metal ions in the mixture at ratios of 20% to 40% shifted the toxicity interaction from antagonistic to additive and eventually synergistic at 50%. Increasing metal ion ratios, produced corresponding increase in toxicity. Similar trend was also seen in binary mixtures of glyphosate and Cd (II) ions. Assessment of toxic index has been applied to weigh the effect of single chemicals for the determination of mixture toxicity (40, 50, 51]. Nweke *et al.* [40] reported synergistic and additive interaction of formulated glyphosate with intermediates of 2, 4-D against the dehydrogenase activity of *Rhizobium* species; the modulation of toxic effect were largely depend on the relative amounts of the binary mixture components. The analysis of the CA predicted toxicity of the binary mixtures using MDR indicated that MDR values ranged from 0.5 to 2; ($0.5 \leq MDR \leq 2$) indicating that the binary mixtures of glyphosate with Cd (II) and Pb (II) ion was most likely additive. Also, similar prediction of additive interaction was seen for the binary mixtures of DDVP with Cd (II) and Pb (II) ions across the studied ratios, with the exception of the DDVP 50% + Pb (II) 50% which was predicted to be synergistic. The prediction result indicated that the CA model underestimated the toxicity of binary mixtures of DDVP + Cd (II) and glyphosate + Pb (II); however, overestimating the toxicity of the DDVP + Pb (II) and glyphosate + Cd (II) binary mixtures, with the exception of glyphosate 50% + Pb (II) 50% mixtures. On the contrary, the IA model predicted mostly additive interaction for binary mixtures of glyphosate + Cd (II) and DDVP + Cd (II) ions mixture across the ratios. However, the model predicted synergism for mixtures of Pb (II) with glyphosate and DDVP. Notably, toxicity prediction using IA model showed a gross overestimation of toxicity across all the binary mixtures with exception of the DDVP 60%: Cd (II) 40% mixture when compared to the experimentally-derived data.

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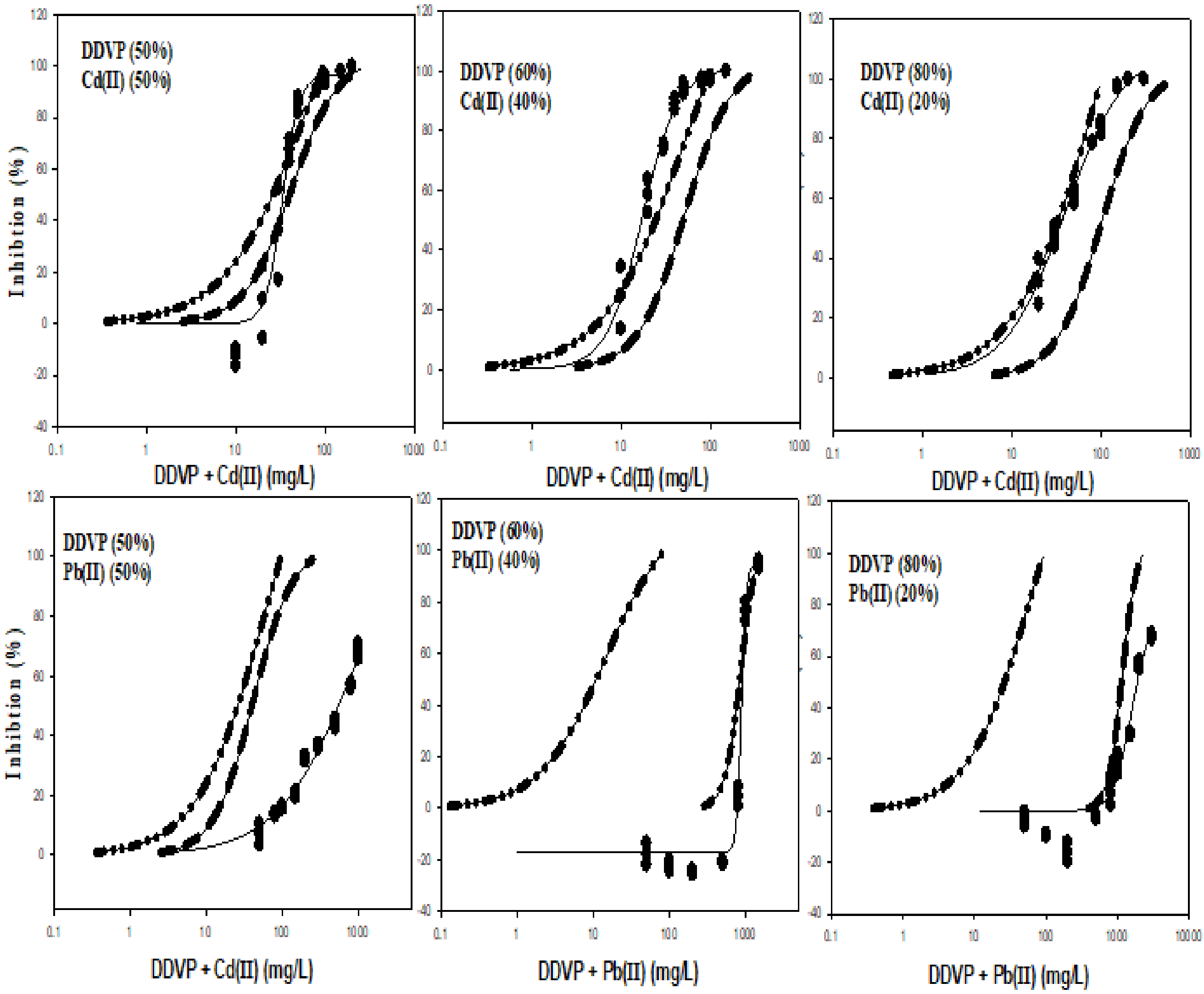

Fig 3 Toxicity of DDVP and Cadmium Ions Binary Mixtures and DDVP and Lead Ion Binary Mixtures to *Rhizobium Sp* Dehydrogenase Activity. The Data Points Represent Experimental Dose-Responses. The Solid Line Represents Estimated Toxicities by Fitting Experimental Data to Logistic Model (Eq. 2), the Long Dash and Dash/Dotted Lines Indicate the Predicted Toxicities from Concentration Addition and Independent Action Models Respectively

Comparative analysis of the experimental and predicted toxicities of the binary mixtures showed that the CA and IA model differed in their prediction of toxicity interactions of binary constituents. The CA and IA model predicted additive interactions for the mixtures Glyphosate 50% + Cd (II) 50%, DDVP 60% + Cd (II) 40%, DDVP 50% + Cd (II) 50% and DDVP 80% + Cd (II) 20%, however, experimentally derived toxic index indicated a synergistic interaction of mixture components. However, the CA model accurately predicted the toxicity interaction of the binary mixtures: glyphosate 60% + Cd (II) 40%, glyphosate 80% + Cd (II) 20%, DDVP 60% + Pb (II) 40%, DDVP 50% + Pb (II) 50%, DDVP 80% + Pb (II) 20%, Glyphosate 60% + Pb (II) 40%, and Glyphosate 50% + Pb (II) 50% to be additive. This strongly agrees with the experimental derived toxic index of the mixtures which were either marginally additive or out rightly antagonistic. In addition, the IA model could only accurately predict the toxicity of the Glyphosate 60% + Cd (II) 40%, DDVP 50% + Pb (II) 50% and Glyphosate 80% + Pb (II) 20% mixtures.

The classic CA and IA models for the prediction of mixture toxicity may underestimate or overestimate toxicity; CA can underestimate the actual toxicities of mixtures if they components are similar in their mode of action. Similarly, mixture toxicity decreases when mixture components follow the assumption of IA model [52]. The work of Feng *et al.* [52] reported that IA more accurately predicted the mixture toxicity of Cu–Zn and Cu–Cd to zebrafish larvae (Danio rerio) than CA. In the study of Nweke *et al.* [33], CA and IA model prediction of toxicity of most mixtures of glyphosate with 2,4-DCP or 4-CP against *Rhizobium sp* did not significantly vary. Underestimation of toxicity of heavy metals and ionic liquids on Photobacterium Q67 by CA models has also been reported [53]. In the study of Okechi *et al.* [49], CA and IA models greatly underestimated the joint effect of SDS and metal mixtures to *S. marcescens* (SerEW01) even at low concentrations.

IV. CONCLUSION

The present study has demonstrated that the CA and IA models may not adequately predict the toxicity of binary mixtures of glyphosate and DDVP with cadmium and lead to *Rhizobium* species. The CA model underestimated the toxicity of binary mixtures of DDVP + Cd (II) and glyphosate + Pb (II); while overestimating the toxicity of the DDVP + Pb (II) and glyphosate + Cd (II) binary mixtures

Table 1 Experimentally-Derived Toxicity Thresholds (EC_{50}), Predicted MDR and Toxic Interaction of Binary Mixtures of Herbicide and Metal on *Rhizobium Sp*

Toxicants	Mean EC_{50}	Mean Toxic Index	MDR		Toxic Effect
			CA	IA	
Single					
Cadmium	22.55 ± 2.50	-	-	-	-
Lead	489.29 ± 14.86	-	-	-	-
Glyphosate	6716.73 ±754.83	-	-	-	-
2,2-dichlorovinyl dimethylphosphate (DDVP)	1712.23 ± 94.79	-	-	-	-
Binary Mixtures					
Glyphosate 60% + Cd (II) 40%	61.73 ± 3.49[a]	1.19 ± 0.11	0.82	0.49	Additive
Glyphosate 50% + Cd (II) 50%	35.61 ± 8.06[b]	0.85 ± 0.14	1.14	0.80	Synergistic
Glyphosate 80% + Cd (II) 20%	146.98 ± 5.95[c]	1.33 ± 0.09	0.69	0.32	Antagonistic
DDVP 60% + Cd (II) 40%	17.29 ± 1.04[d]	0.31 ± 0.02	2.89	1.42	Synergistic
DDVP 50% + Cd (II) 50%	34.17 ± 1.91[b]	0.77 ± 0.04	1.18	0.76	Synergistic
DDVP 80% + Cd (II) 20%	42.33 ± 2.31[b]	0.40 ± 0.02	2.30	0.73	synergistic
DDVP 60% + Pb (II) 40%	871.66 ± 38.85[e]	1.10 ± 0.07	0.97	0.01	Additive
DDVP 50% + Pb (II) 50%	584.59 ± 56.87[f]	0.77 ±0.05	0.07	0.04	synergistic
DDVP 80% + Pb (II) 20%	1371.23 ±132.72[g]	1.20 ±0.06	0.83	0.02	Antagonistic
Glyphosate 60% + Pb (II) 40%	986.18 ± 95.00[e]	1.02 ±0.13	0.91	0.01	Additive
Glyphosate 50% + Pb (II) 50%	1097.42 ± 91.56[e]	1.20 ±0.06	1.73	0.04	Antagonistic
Glyphosate 80% + Pb (II) 20%	563.99 ± 35.10[f]	0.30 ±0.00	1.92	0.03	synergistic

Values with the same superscript across column are significantly different ($p<0.05$).

Furthermore, the IA model predicted mostly additive interaction for binary mixtures of glyphosate + Cd (II) and DDVP + Cd (II) ions mixture across the ratios. Notably, toxicity prediction using IA model grossly overestimated toxicity across most of the binary mixtures studied. The toxicity interaction of glyphosate mixture with cadmium and lead are dependent on the relative amount of the heavy metals present in the binary mixtures.

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