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Influence of Binary Mixture of Heavy Metals and 2,4-Dichlorophenoxyacetic Acid on Phosphatase Activity of *Desmodesmus Abundans*

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Abstract:- The impart of mixtures of metals and pesticide on the activity of algal hydrolytic enzyme was examined. Toxicity of unary, and binary combinations of Copper (Cu^{2+}), Zinc (Zn^{2+}), Lead (Pb^{2+}), Chromium (Cr^{2+}), Cadmium (Cd^{2+}) ions and 2,4-dichlorophenoxyacetic acid (2,4-D) was assessed over inhibition of phosphatase enzyme activity of *Desmodesmus abundans*. The unary ions of Cu^{2+} , Zn^{2+}, Pb^{2+} , Cr^{2+}, Cd^{2+} and 2,4- dichlorophenoxyacetic acid (2,4-D) effect on phosphatase activity of *Desmodesmus abundans*was assessed by inhibition of conversion of p-nitrophenylphosphate to p-Nitrophenol at optical density (OD) of 410nm. The results gathered from the study for experimental data points and model-predicted dose-response data for inhibition of phosphatase enzymatic activity showed that all the metal ions were all toxic to algal phosphatase activity by the gradual reduction of the coloured p-Nitrophenol.The order of their toxicity ranking appeared in the following decreasing order: Zn^{2+}>Pb^{2+}>Cd^{2+}>Cr^{2+}>2,4-D>Cu^{2+}. The threshold inhibitory concentration ($1C_{50}$) of Zn^{2+}at 0.13±0.00, Pb^{2+} at 0.16±0.01 and Cd^{2+}at0.16±0.01(mM) were most toxic against *Desmodesmus abundans*. However, the pesticide: 2,4-D exhibited a low inhibition of phosphatase activity with only 15% inhibition. The effects of the amalgamation of different ratios assessed via binary mixture, was determined.The IC_{50} for 50:50 binary mixture ratio were: 2,4-D/Cu^{2+}(4.32±0.55):,2,4-D/Pb^{2+}(6.23±0.79):,2,4-D/Zn^{2+}(4.97±0.52):,2,4-D/Cr^{2+}(4.33±0.55):,2,4-D/Cd^{2+}(6.78±0.25) (mM), while IC_{50} for 60:40 binary mixture ratio : 2,4-D/Cu^{2+}(4.33±0.55):,2,4-D/Pb^{2+}(4.33±0.55):,2,4-D/Zn^{2+}(4.97±0.52),2,4-D/Cr^{2+}(6.73±0.07):,2,4-D/Cd^{2+}(6.78±0.25) (mM).For toxicant binary mixture ratio 50:50: 2,4-D/Cu^{2+} and 2,4-D/Cr^{2+}were more toxic than others, followed by 2,4-D/Zn^{2+},2,4-D/Pb^{2+} and 2,4-D/Cd^{2+}.Their toxicity ranking appeared in the following order: 2,4-D/Cu^{2+}>2,4-D/Cr^{2+}>2,4-D/Zn^{2+}>2,4-D/Pb^{2+}>2,4-D/Cd^{2+}. Similarly, from the 60:40 toxicant ratio:2,4-D/Cu^{2+}and2,4-D/Pb^{2+}were more toxic, followed by 2,4-D/Zn^{2+}. the least toxic mixtures were 2,4-D/Cr^{2+}and2,4-D/Cd^{2+}. Their ecological toxicity ranking appeared in the following order as well: 2,4-D/Cu^{2+}>2,4-D/Pb^{2+}>2,4-D/Zn^{2+}>2,4-D/Cr^{2+}>2,4-D/Cd^{2+}. The drop in the toxicity effect from unary state as compared in their mixture state, was probably due to the increase in the pesticide concentration. Consequently, the binary ratio of 60:40 components tend towards antagonistic effect due to the presence of 2,4-dichlorophenoxycetic acid. However, others showed the tendency for an additive effect.Generally, model predicted dose-response data fitted well in sigmoid 3, 4, 5 parameters and statistically significant difference ($p<0.05$) between IC_{50} of unary and binary mixtures. However, 2,4-D became less toxic due to its amalgamation with the heavy metals ions. These results indicate the risk which mixtures of pollutants represent in a freshwater environment as against their occurrence as unary toxicants.

Keyword:- *Toxicity: Desmodesmus abundans: 2,4 dichlorophenoxycetic acid:dose-response: p-Nitrophenol: Threshold inhibitory concentration ($1C_{50}$).*

I. INTRODUCTION

Instances have shown that, river water containing heavy metals such as iron, manganese, zinc, copper, nickel, lead, chromium and cadmium are capable of generating oxidative stress and DNA damage in fishes(Zhang *et al.*, 2021). These pollutants are of global concern due to their accumulation in the environment, even in cold regions and in the cells or tissues of aquatic organisms (Zhang *et al.*, 2021). Their presence is indicative of many industrial applications and intensive agricultural practices which has contributed to the pollution of freshwater habitats, thereby causing detrimental effects on aquatic organisms and the health of human beings at large. In other to measure their effects in aquatic systems, predictable diagnostic biomarkers for analyzing the health of anaquatic environment and the bio-indicator are essential for environmental risk assessment protocol. The pollutants are use asbio-markers to evaluate the interaction between a bioindicator and any environmental agent, which could be physical, chemical, and biological. The response of a bioindicator during and after the uptake of a stressor could lead to the alteration in biochemical level, molecular level, tissue level, cell structure, and physiologic function, and in the behavior of such aquatic organism. In other to evaluate the possible effect in natural systems, in natural conditions, the *in vivo* application of bio-indicators serves as an excellent tool to estimate the susceptibility and negative effect of environmental agents on organisms. Certain physiological parameters such as enzyme (leak on tissue damage) activities and biochemical end points like creatinine and enzyme activity (alkaline phosphatase, aspartate amino transferase, and alanine aminotransferase) , catalase, TLC, lymphocytes, respiratory burst, and nitric oxide synthase activity, superoxide dismutase, lipid peroxidation, glutathione S-transferase, and glutathione may be affected in varied capacity due to the effect of environmental pollutants (Mohammad *etal.*,2021). Therefore, responses such as cell multiplication, bioluminescence, enzyme activity and synthesis, nutrient uptake, CO_2 evolution, oxygen consumption and metabolite production can be used to

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measure the effect of a heavy metal on microbial population. However, predicting the effect of single metal pollutant against the aquatic ecosystem had been argued for a period of time. Hence, heavy metals may induce greater toxicity on interactions with other constituents rather than on individual level (Adnan *et al.*, 2021). The dose-responses from the pollutants and their endpoints relationship are usually shown in a dose-response curve, which is a sigmoidal 2-dimensional plot on an*x*-axis and *y*-axis. The sigmoidal dose-response curve can be fitted with a number of monophasic dose-response models such as logistic, Weibull, Logit, Probit, Gormpertz models and their modifications (Altenburger *et al.*, 2000; SYSTAT Software Inc., 2002, 2006). The use of algal phosphatase inhibition as physiological parameters is important for the assessment of the negative effects of pollutants in an aqueous environment. The alkaline phosphatase (ALP) catalyzes the hydrolysis of 4nitophenylphosphate (4-NPP) with the formation of free 4-nitrophenol and an inorganic phosphate. The reaction is monitored kinetically at 410nm by the rate of formation of the coloured 4-nitrophenol, which is proportion to the activity of ALP present in the sample.

II. MATERIALS AND METHODS

A. *Sample area*

Ihiagwa is a town in Owerri West Local Government Area of Imo State, south-eastern Nigeria. It islocated 12km south from the capital city of Owerri. The Otamiri River in Ihiagwa has coordinates with latitude:4° 54' 14.00" N and longitude: 7° 08' 30.00" E. Its watershed covers about 10,000 square kilometres(3,900 sq. mi), with annual rainfall of 2,250 to 2,500 millimetres. The watershed is mostly covered by depletedrain forest vegetation, with mean temperatures of 27 °C (81 °F) throughout the year. The river is polluted byorganic wastes and chemicals due to intensive human, household, agricultural, dilapidated drainage system, factory-runoffs, and industrial activities. It serves as the source of drinking water when the public water systemfails. The waste management system in Owerri is inefficient and contributes to the pollution of the river, which, however creates a high concentration of phosphate nitrate, metals pesticides and herbicide in the Otamiri river. Therefore, the present study was carried out on a small stretch of Otamiri River along the Nekede - Ihiagwastretch. Figure 1, shows the geographical map of the study area.

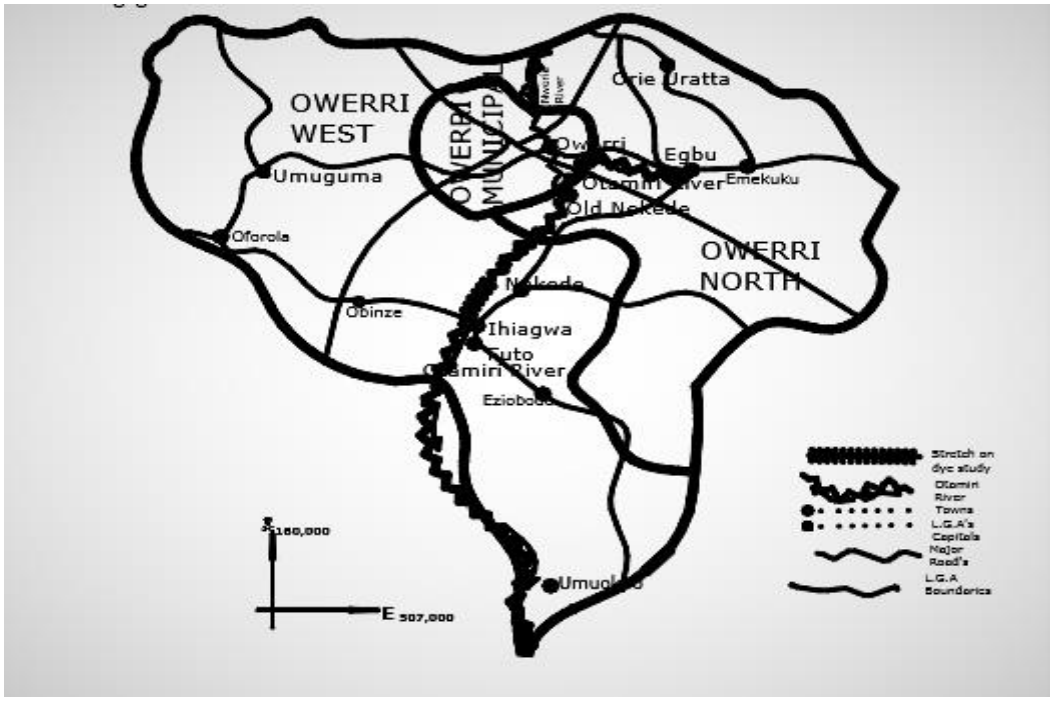

Fig. 1: Geographical map of Otamiri River along the Nekede -. Ihiagwa stretch

B. *Sampling*

- Sampling Bottles:
  Sterile glass bottles, with capacity of at least 200ml was used for water sampling collection. They were fitted with ground glass or screw caps. The stopper or cap and neck of the bottle were protected from contamination by suitable cover of sterilized thin aluminum foil. Silicon rubber lines, that could withstand repeated sterilization at 1600C, was used inside the screw caps. After being sterilized, the bottle was aseptically stored without opening the sterilized bottle. The samples were collected at a time interval of ten (10) minutes for each sampling points. The timing was regulated by the use of a stop watch by one of the field assistants. Figure 2, shows the volumetric flow of a small section of the river. The Imo State Water co-operation shows water for treatment in the upstream of this stretch and many activities such as fishing and sand mining that go on downstream.

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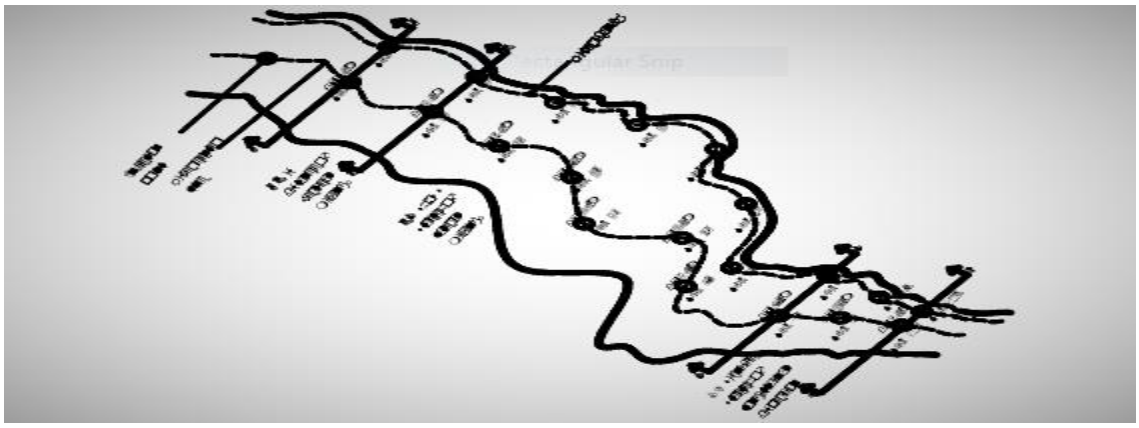

Fig. 2: Volumetric flow of a small section of the river.

C. Collecting Sample

- Sample collection and transportation
  Samples of water were collected in sterile bottles. Care was ensured to prevent accidental contamination of water during collection. The River water was collected from Otamiri River in Ihiagwa, Imo State south-eastern Nigeria. Water samples were collected midstream along the course of the river at three spots (upper, middle and lower-course) with different coordinates (a): (5◦24.25 ″ 0.32 ′ N, 7◦0.36 ″ 0.036 ′ E; (b): 5◦24.28 ″ 0.55 ′ N, 7◦0.38 ″ 0.36 ′ E and (c): 5◦23.55 ″ 0.20 ′ N, 6◦59.46 ″ 0.39 ′ E) from a depth of 30 cm and pooled in 1-litre sterile plastic bottle or 200 ml sterile glass bottle. Immediately after collection, samples were placed in an insulated cold box or cooler for transport to a water testing laboratory. Water samples were examined upon arrival, 6 hours of collection to ensure continual viability of cells.

D. Isolation

The pooled sample were stored in a cooler and taken to the laboratory. The algal load of the sample was determined by washing or centrifugation method and agar-plated within six hours of collection. Within the period the water sample was collected, pure cultures of the test organism(s) were isolated and 2-3weeks thereafter for axenic cultures, that was stored under standard microbiological conditions before toxicity assay. The algal load of the water sample was estimated at colony forming unit (CFU/ml).

III. ALGOLOGICAL ANALYSIS

A. Preparation of agar plate:

Agar plates were prepared by dissolving 2% agar (w/v) in BBM. The plates wereautoclaved at 126oC for 15 minutes. The plates were allowed to cool down and 5 ml warm agar medium was put in the plates. The plates were allowed to cool, kept in inverted position for complete removal of steam and drying at least 72 hrs before streaking in bold basal medium (modified with highly enriched trace metal solution and F/2 vitamin solution), (Stein, 1973). Modified bold basal media plates contained (g/ml); KH_2PO_4 8.75g/500(10ml), $CaCl_2$•$2H_2O$ 12.5g/500ml (1ml), $MgSO_4$•$7H_2O$ 37.5g/500ml (1ml), $NaNO_3$ 125g/500ml (1ml),K_2HPO 4.37.5g/500ml (1ml), NaCl 12.5g/500ml (1ml), Na_2EDTA•$2H_2O$ 10g/L (1ml), KOH 6.2g/L,$FeSO_4$•$7H_2O$ 4.98g/L (1ml), H2SO4 (concentrated) 1ml/L, Trace Metal Solution 1ml, H_3BO3 5.75g/500ml (0.7),F/2 Vitamin Solution 1ml (optional: cyanocobalamin, biotin, thiamine) (Nichols, and Bold, 1965).

B. Culture of isolate:

A volume of 12 ml of washed and centrifuged algal sample taken from transported pooled samples into the sterile tubes. The tubes were centrifuge at 3000 rpm for 15 minutes. The supernatant was removed.The cells were suspended in a fresh sterile water in each tube using vortex mixer (rotated at 1000-1500 rpm up to homogeneous suspension). Centrifugation and washing were repeated for six times to expel contaminants and most microorganisms present in the algal sample (Parvin and Habib, 2007). A loopful of each labeled isolate was streaked onto bold basal medium agar supplemented with antibacterial and anti-fungal drug. Culture plates were kept under fluorescent light. Culture plates were incubated for 2 weeks at room temperature (28±2oC).

Thereafter, the culture was transferred into a broth inoculated with bold basal medium contained in 250mlconical flask. Continuous culture was maintained to sustain algal growth at exponential phase.

C. Subculture of isolate:

Axenic culture of test organism was sub-cultured in an inorganic liquid medium prepared as recommended by OECD, 1981.

D. Storage of pure stock culture:

For optimal yield of test organism(s), axenic broth culture was incubated at temperature (20±2°C), under white fluorescent light (3000-4000) lux, on a rotary shaker. New stock culture was initiated at 4°c in the dark, in every 40-60 days, by inoculating approximately 5×104 cells ml^{-1} , (Jonsson and Aoyama, 2007).

E. Preparation and standardization of inoculums:

A loopful of the isolates, stored in bold basal medium slant in the refrigerator was inoculated into 200 ml of bold basal medium broth contained in 500 ml conical flask and incubated on a rotary shaker at 150rpm at room temperature (28 ± 2^0C), for 24hrours. After incubation, the cells were harvested by centrifugation at 3000rpm for 10minutes, the

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supernatants were discarded and the sediment which contained the green pigment cells was harvested. The harvested cells were washed twice in sterile distilled water. The cell extracts were standardized in a spectrophotometer to a density of 1.8 at 600nm.

F. Morphological Identification of Isolated organism(s):

The morphological traits evaluated comprised of colony morphology, green pigment and chlorophyll a/b production. Morphological analyses were based on type, elasticity and appearance, while colony morphology parameter was based on colour, form, transparency and diameter. The other tests carried out for identification of the isolate included; chlorophyll production, catalase, phosphatase, starch and lipase (Stein, 1973).

G. Molecular (Genome) Identification of the Isolated test organism

The following molecular identification were carried out: DNA extraction, DNA quantification, 18S sequencing Amplification, Assembly and Annotation and Phylogenetic analysis.

H. DNA extraction

Extraction was done using a ZR fungal/algal/bacterial DNA mini prep extraction kit supplied by Inqaba South Africa. A heavy growth of pure culture of the suspected isolates was suspended in 200 microlitre of isotonic buffer into a ZR Bashing Bead Lysis tubes, 750 microlitre of lysis solution was be added to the tube. The tubes were secured in a bead beater fitted with a 2ml tube holder assembly and processed at maximum speed for 5 minutes. The ZR bashing bead lysis tube was centrifuged at 10,000x*g* for 1 minute. Four hundred (400) microlitre of supernatant was be transferred to a Zymo-Spin IV spin Filter (orange top) in a collection tube and centrifuged at 7000 x*g* for 1 minute. One thousand two hundred (1200) microlitre of algal/fungal/bacterial DNA binding buffer was added to the filtrate in the collection tubes bringing the final volume to 1600 microlitre, 800 microlitre was then transferred to a Zymo-Spin IIC column in a collection tube and centrifuged at 10,000x*g* for 1 minute, the flow through was be discarded from the collection tube. The remaining volume was transferred to the same Zymo-spin and spun. Two hundred (200) microlitre of the DNA Pre-Wash buffer was be added to the Zymo-spin IIC in a new collection tube and spun at 10,000x*g* for 1 minute followed by the addition of 500 microlitre of algal/fungal/bacterial DNA Wash Buffer and centrifuged at 10,000x*g* for 1 minute. The Zymo-spin IIC column was transferred to a clean 1.5 microlitre centrifuge tube, 100microlitre of DNA elution buffer was added to the column matrix and centrifuged at 10,000x*g* microlitre for 30 seconds to elute the DNA. The ultra-pure DNA was then stored at -20 degree for other downstream reaction.

I. DNA quantification

The extracted genomic DNA was quantified using the Nano drop 1000 spectrophotometer. The software of the equipment was launched by double clicking on the Nanodrop icon. The equipment was initialized with 2ul of sterile distilled water and blanked using normal saline. Two microlitre of the extracted DNA was loaded onto the lower pedestal; the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the “measure” button.

J. 18S sequence Amplification

The 18S regions of the isolates was amplified using the 18S C-2 b: 5‘- ATTGGAGGGCAAGTCTGGT- 3‘' and 18S D-2 b: 5'- ACTAAGAACGGCCATGCAC-3, Primers on a ABI 9700 Applied Bio systems thermal cycler at a final volume of 30 micro litres for 35 cycles. The PCR mix included: The X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and the extracted DNA as template. The PCR conditions was maintained as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 53ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 15 minutes and visualized on a blue light trans illuminator.

K. Sequencing

Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by InqabaBiotechnological, Pretoria South Africa. The sequencing was done at a final volume of 10ul; the components includes: 0.25ul BigDye® terminator v1.1/v3.1, 2.25ul of 5 x BigDye sequencing buffer, 10uM Primer PCR primer, and 2-10ng PCR template per 100bp. The sequencing condition was maintained as follows 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4min.

L. Phylogenetic Analysis

Obtained sequences was edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using MAFFT. The evolutionary history was inferred using the Neighbour-Joining method in MEGA 6.0 (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) was taken to represent the evolutionary history of the taxa analysed. The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor, 1969; Saitou and Nei, 1987; and Felsenstein,1985).

M. Phosphatase Extraction

The method of Jonsson and Aoyama (2007), was used for this study. Algal pellets were Freezed with liquid nitrogen (N2) in a mortar, supplemented with acetate buffer and thereafter macerated by placing at -20°c by thawing at room temperature. The volume was adjusted in order to obtain a 1:4 (w/v) suspension. The suspension mixture was subjected to probe sonication at 0°c (ice bath) 50secs followed by 20secs interval (1 cycle) with amplitude at 70. The latter procedure was repeated twice. The disrupted cells suspension was decanted, and centrifugation of resultant cell disrupted suspension at 10,000 rpm for 20 minutes. The resulting supernatant fluid (extract) was stored as the enzyme and used for phosphatase.

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N. Toxicity of Unary and binary mixtures of metals and pesticide to C. vulgaris alkaline enzyme phosphatase activity

The individual or single dose of graded ionic concentrations of copper, zinc, chromium, lead, cadmium and 2,4-dichlorophenoxyacetic acid was assessed. The metals were each prepared in 10mM stock concentration. The concentration range for Copper (Cu^{2+}), Zinc (Zn^{2+}) and Chromium (Cr^{2+}) was (0-0.5mM) , Lead (Pb^{2+}) and Cadmium (Cd^{2+}) ions (0-7.0mM) and 2,4-dichlorophenoxyacetic acid (2,4-D) (0-25mM). Binary mixtures of pesticide/metal were amalgamated using simple percentage ratio of 50:50 and 60:40. Their toxicity were determined at concentration of 0-9.0mM. Inhibitory study on ALP activity was determined in 2ml reaction final volume consisting of the graded concentration of single toxicants or binary mixture, distilled water, buffered enzyme, and substrate (p-NPP), contained in 15 ml sterilized culture tubes in triplicates. The control consisted of set-up devoid of toxicants. The set-up contained graded concentrations of toxicants amended in requisite volume of distilled water, 0.5ml buffered substrate p-NPP (pH 8.0) and 0.5ml crude enzyme was added and the culture tubes were incubated for 30-40 minutes at room temperature (370C). A 0.1ml of sodium hydroxide was added to stop the reaction. Thereafter, the tubes were centrifuged and 2ml of the supernatants was spectrophotometrically measured at 410nm.

O. Estimation of relative response, Median Inhibitory Concentration of Unary and Binary Metals Mixtures of Metals and Pesticide and Modeling of Inhibition Data:

The relative responses were evaluated as percentage relative to control due to inhibition of the toxicants (figure 3).

$$\text{Relative response\% Inhibition} = \frac{R_C - R_T}{R_C} \times 100 \quad (1)$$

In equation: (1), R_C is the response of the control and $R_T$is the response in the tests (at different concentrations of the toxicant).The data generated from relative inhibition responses of unary and binary of metals and pesticide were fitted into dose-response models (Cedergreen, *et.al*., 2005) using sigma plot (version 10). The individual median inhibitory concentration (IC_{50}) of the individual toxicants and toxicants mixture to phosphatase enzymatic activity in *C. vulgaris* was generated from the models. The $Y = \frac{a}{1+\left(\frac{x}{x_0}\right)^b}$ fitted models are also elaborated with their equations below.

$$Y = y_0 + \frac{a}{e^{-\left(\frac{x-x_0}{b}\right)}}$$ (2) Four parameter logistics

$$Y = y_0 + \frac{a}{1+\left(\frac{x}{x_0}\right)^b}$$ (3) Four parameter sigmoid

$$Y = y_0 + \frac{a - y_0 + fx}{1+\left(\frac{x}{x_0}\right)^b}$$ (4) Three parameter logistics

(5)Hormetic model

Figure 4.is the relative response. **Where:**

y_0 is the response at infinite *x*.

b parameter determining the slope of the hormetic increase,

a is the Maximum Response.

f is the parameter describing the degree of hormetic increase.

x is the concentration of phenol.

x_0 is IC_{50}.

In addition, data from phenol dehydrogenase assay were described with a biphasic dose–response model for hormesis (Cedergreen, *et.al*., 2005).

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P. Statistical Analysis

The experimental data was fitted and median inhibitory concentrations (IC50) of individual and mixtures of pesticides and metals were evaluated using Sigma plot software (10.0). Statistical analysis of IC50 values was computed using one-way analysis of variance ($p<0.05$) in statistical package for the social sciences (IBM, SPSS software 22)

IV. RESULTS AND DISCUSSION

A. Morphological and biochemical identification of the isolate R_2-KJ676104.

The phenotypic features of the isolate labeled R_2-KJ676104,under the electron microscope (fig 5) comprises of cell dimensions with 11-15µm ± 0.48m in length and 4-6µm ± 0.5m in width. The morphological traits alsorevealed the presence of both unicellular and colonial forms of 2-4 celled with spiny projections. The isolate cell showed ellipsoidal or obovate, heavily-granulated and enclosed in mucilaginous sheath. The isolate cells were arranged in tetrads but single and double-celled forms were also observed. The colonial forms possessed spiny projections on the cells. All morphological descriptions of the isolate, R_2-KJ676104, (fig 5), was tentatively identified as *Desmodesmus sp* following the works of (Charmaine*et al*.,2021;Hegewald, 2000;Perumal, *et al*., 2012; Nichols and Bold,1965).The morphological and biochemical screening are presented in table 1.

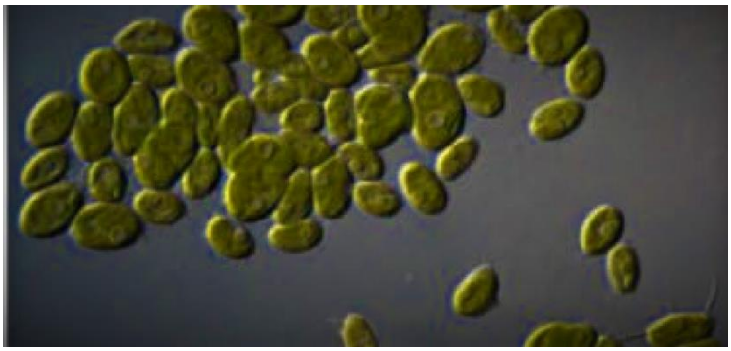

Fig 5: Schematic Microscopic structure of *D. abundans*

B. Molecular (Genome) Identification of the IsolateR_2-(KJ676104)

From the molecular identification to specie level using the process of DNA extraction, DNA quantification,18S sequencing Amplification, Assembly, Annotation and Phylogenetic analysis, the obtained 18S sequence from the isolate (**R_2(KJ676104)** produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. The 18S of the isolates showed a percentage similarity to other species at 99-100%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of 18S of the isolates within the *Desmodesmussp* and revealed a closely relatedness to *Desmodesmus abundans* (Fig. 6).

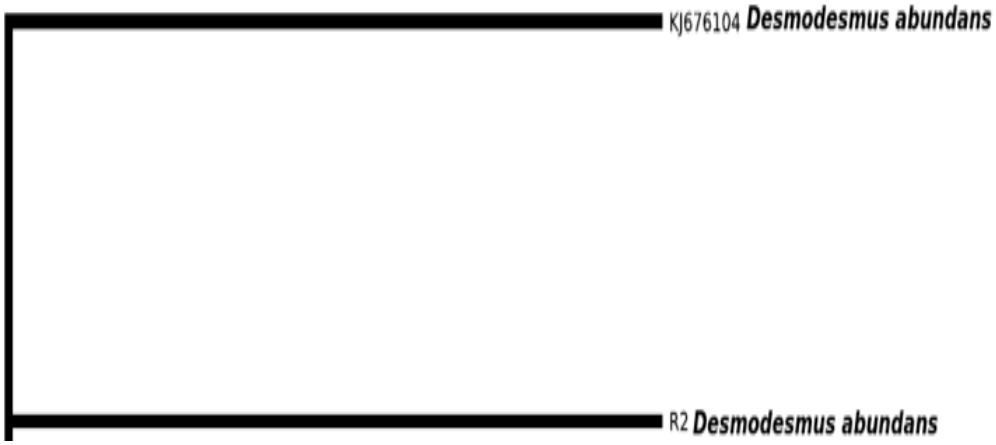

Fig 6: Phylogenetic tree showing *Desmodesmus abundans*isolate

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Parameters	Results
Shape Obovate / Ellipsoidal	
Form Tetrad / Mucilaginous	
Chloroplast Present	
Colour Green pigment	
Type Spiny projection	
Cell diameter 11-15um ±0.48m	
Phosphatase test:	++
Nitrogenase test:	++
Chlorophyll analysis:	++
Nitriles test:	++
Lipoxygenases	+
Amylase	+
Lipase test	+
Urease test:	++
Thiolase/gelatinase test:	+
Catalase:	+
Hydrogenases:	+
Carbonic anhydrase:	+
Desmodesmus abundans	

Table 1: Morphological and biochemical characteristics of the isolate.

Toxicant	IC_{50}(mM)
Cu^{2+}	2.18±0.47
Zn^{2+}	0.13±0.00
Pb^{2+}	0.16±0.01
Cr^{2+}	0.17±0.01
Cd^{2+}	0.16±0.01
2,4-D	0.26±0.01

Table 2: Median inhibitory concentration (IC50) of the effect of Unary toxicity of (metal/pesticide) tophosphatase enzyme activity from *Desmodesmus abundans.*

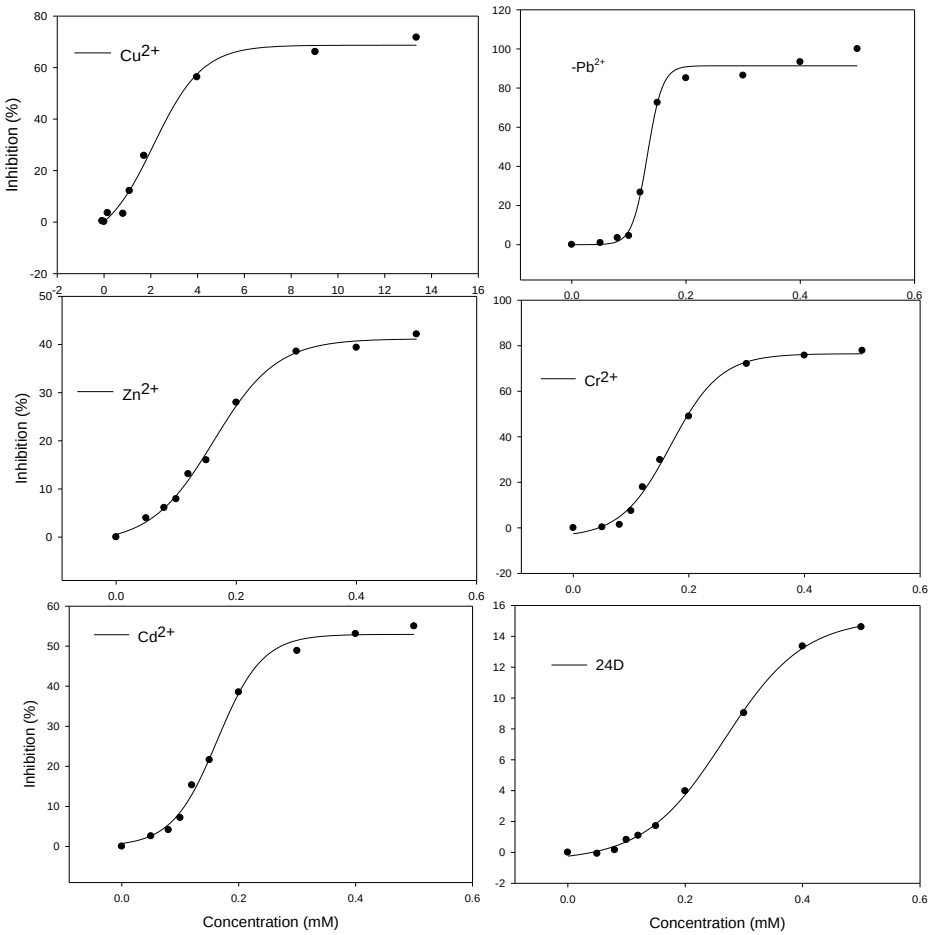

Fig. 7: Experimental (data points) and model-predicted dose-response data for unary inhibition of phosphatase activity of *Desmodesmus abundans*by Copper , Zinc, Lead, Chromium, Cadmium ions, and 2,4-D usingSigmoidal, Sigmoid, 3 and 4 Parameter model.

Toxicant Mixture 50:50	**IC_{50}(mM)**
2,4-D/Cu $^{2+}$	4.32±0.55
2,4-D/Pb $^{2+}$	6.23±0.79
2,4-D/Zn^{2+}	4.97±0.52
2,4-D/Cr $^{2+}$	4.33±0.55
2,4-D/Cd $^{2+}$	6.78±0.25
Toxicant Mixture 60:40	
2,4-D/Cu $^{2+}$	4.33±0.55
2,4-D/Pb $^{2+}$	4.33±0.55
2,4-D/Zn^{2+}	4.97±0.52
2,4-D/Cr $^{2+}$	6.73±0.07
2,4-D/Cd $^{2+}$	6.78±0.25

Table 3: Median inhibitory concentration (IC50) of the effect of Binary mixture of (metal/pesticide) tophosphatase enzyme activity from *Desmodesmus abundans*

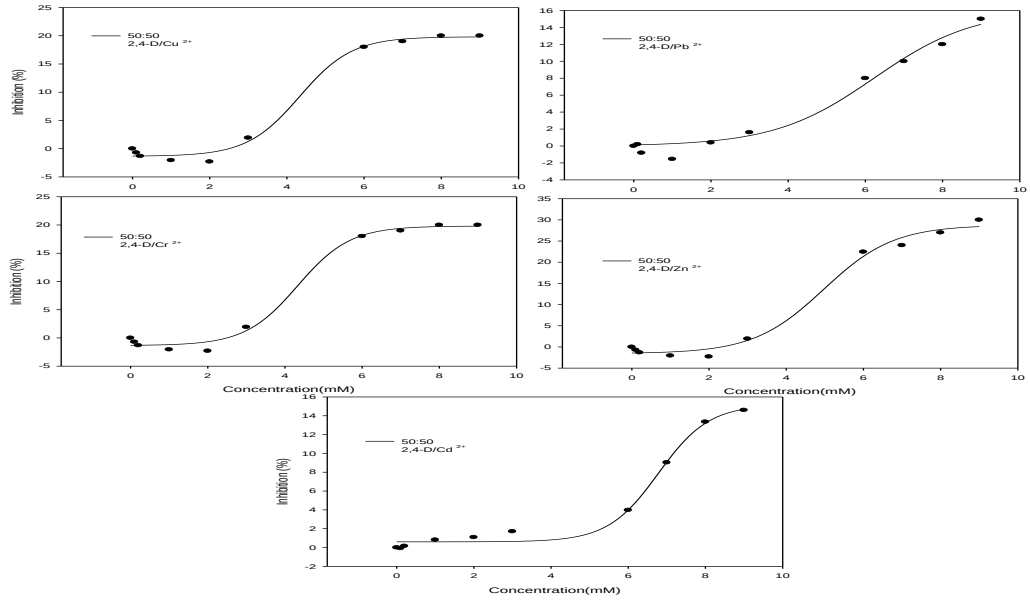

Fig. 8: Experimental (data points) and model-predicted dose-response data for Binary Toxicity ratio (50:50) inhibition of phosphatase activity of *Desmodesmus abundans* by 2,4-D/Cu^{2+}, 2,4-D/Pb^{2+}, 2,4-D/Zn^{2+}, 2,4-D/Cr^{2+}, 2,4-D/Cd^{2+}. The solid lines represent Sigmoidal, Sigmoid, 4 Parameter model.

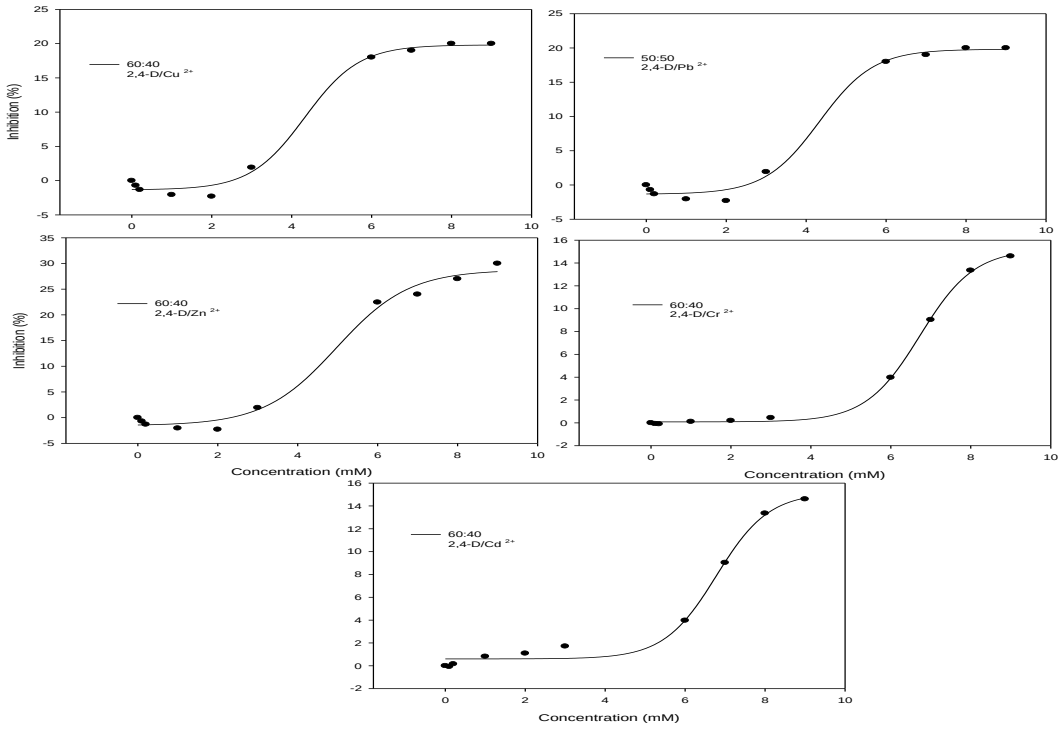

Fig. 9: Experimental (data points) and model-predicted dose-response data for Binary Toxicity ratio (60:40) inhibition of phosphatase activity of *Chlorella vulgaris* by(2,4-D/Cu^{2+},. 2,4-D/Pb^{2+}, 2,4-D/Zn^{2+},2,4-D/Cr^{2+},2,4-D/Cd^{2+}). The solid lines represent Sigmoidal, Sigmoid, 4 Parameter model

The result of the morphological and biochemical characteristics of *Desmodesmus abundans* shown in table 1, numerated were consistent with the report of other researchers (Yamamoto,*et al.,* 2004, Illman, *et al.,* 2002, Yamamoto, *et al.*, 2005; Champenois, Marfaing and Pierre, 2015; Garcia, 2012; Tomaselli, (2004); Borowitzka, (2018); Safi, *et al.*,(2014); Krienitz, *et al.*, (2015);Champenois, *et al.*, (2015); Hegewald, 2000;).From the molecular (Genome) identification of the isolate:*Desmodesmus abundans* **R_2 (KJ676104)**. The 18S of the isolate showed a percentage similarity to families of related species of *Desmodesmus* at 99-100%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of 18S of the isolate within the

Desmodesmus sp and revealed a closely relatedness to *Desmodesmus abundans.*The result of the phylogenetic tree showing the evolutionary relationship of the isolate is shown in figure6.The result of the effect of unary toxicity of (metal/pesticide) and binary mixture of (metal/pesticide) to phosphatase enzyme activity from *Desmodesmusabundans*showed different level of toxicity. From the graphical interpolation of the dose-response curve, the ranking of ecological risk of the heavy metals ions to phosphatase inhibition in *Desmodesmusabundans*demonstrates the heavy metal ions toxicity was in the order(zn > Pb(Cd) > Cr >2,4-D>Cu >). Zinc ion was found to be highly toxic while Copper was the least toxic. The median inhibitory toxicity value IC50 of Zinc ion showed the highest toxicity of 0.13±0.00mM as shown in the graphical interpolation of dose-response curve in figure 7. The experimental data points and model predicted doseresponse data for inhibition of phosphatase activity of *Desmodesmusabundans*showed few outliers and the enzyme was sensitive to Zinc ion.

The underlying unary toxicity results of the model predicted dose-response for inhibition of phosphates activity of *Desmodesmus abundans* by zinc in figure (7) was consistent with effects from nanoparticle of zinc oxide. Zinc oxide nanoparticles were found to be more toxic than bulk form to freshwater alga *Coelasterella terrestris* (Saxena and Harish, 2019: Vilela, 2020). Al-Hasawi *et al.,* (2020) cited a similar trend of inhibition toxicity of zinc to *Pseudokirchneriella subcapitata*. Nano-particles were revealed to influence the particle size, morphology density, surface area to growth inhibition and algal death (Adochite and Andronic, 2021; Andreani, *et al.*, 2021). Lead ion in figure 7,exhibited a considerable high inhibition response above 90% (ninety percent).The findings of Ahmadmahoodi, *et al.*,(2020) supported the reported sigmoidal trend of inhibition of Lead ion.They elaborated on the acute toxicity of lead to be about 2.7 times more toxic than Cadmium on marine micro alga, *Nannochloropsis oculata*. Al-Hasawi, *et al.*, (2020) reported on toxic inhibitory effect of lead to free and alginated immobilized cells of *P. subcapitata*. All their assertions were in-line with enzymatic activity inhibition of phosphate in *Desmodesmus abundans*, analyzed in the present study.The extent of the percentage inhibition of chromium ion (in figure 7) to enzymatic activity of phosphatase in *D. abundans* was compared with the results from ranking of ecological risk metals in fresh water in Taihu lake in China by (Wang, *et al.*, 2020). The findings from the present study held common inference. Other scholarly write-up that supported and provided consistent view include;(Su, *et al.*, 2017, and Al-Hasawi, 2020).A gradual inhibitory sigmoidal trend in figure 7, showed the effect of cadmium ion on enzymatic activity of phosphatase in *D. abundans*. The present study provides consistent views to the findings of (Mohy EI-Din and Abdel–Kareem, 2020). They reported the inhibition toxicity of cadmium as median lethal concentration.However, cadmium was not ranked amongst ecological risk metal of great concern (Wang, *et al.*, 2020). Inhibition of cell growth and pigment content of marine microalga, *Nannochloropsisoculata* by cadmium was reported by (Ahmadmahmoodi, *et al.*, 2020). Also, the toxicity of binary mixtures of diclofenac and cadmium ion to the algae *Desmodesmus subspicatus*using normalization method, supported the current report on cadmium ion toxicity (Weissmannová, 2018). Contrary to their assertion, cadmium was rated as the highest metal in the order Cd> $Co > Hg > Cu > Ni > Zn > Cr > Al > Se > As > Pb > Sr$ (Al-Hasawi, *et al.*, 2020).Results from the findings of Clement and Lamonica (2018) showed that cadmium toxicity to microalgae *P. subcapitata* could be correlated to the free divalent fraction of the metal. However, cadmium toxicity can be limited by the presence of EDTA. Generally, Heavy metals like cadmium showed negative effect both to the growth rates and nutrient removal capacity (Braglia, *et al.*, 2021:Silva, 2018). In comparison with other metallic ions except for copper, however, the pesticide, 2,4-D (fig. 7) had a percentage inhibitory toxicity lowered by 16 percent as compared with the other toxicants. This trend suggests the reason why the pesticide is agro-friendly.From the dose response curve in figure (7), copper wasn't as acute in toxicity degree like other toxicants. Wang, *et al.,* (2020) cited a similar trend of copper using probabilistic method to rank copper metal in Lake Taihu, of China. Exposure of *C. vulgaris* to photosynthesis inhibition test was more sensitive for copper (Expósito, *et al*, 2021). Similar inhibitory toxicity of copper was reported by (AL-Hasawi, *et al.*, 2020). Such phenomenon in phosphates inhibitory activity in *D. abundans*was consistent with the report of (Al-Hasawi 2020). Copper was ranked as one of the highest toxic metal to *Pseudokirchneriella subcapitata* in free and alginate immobilized cells. At nanoscale level, nano material such as nano-CuO(copper oxide) on different aquatic species *Raphidocelis subcapitata, Daphnia magna and Lemna minor* was toxic to *Raphidocelis subcapitata* and *D. magna* (Andreani, *et al.*, 2021). Nano copper oxide, CuO was very toxic to Caco-2 and HepG2 cell of humans (Andreani, *et al.*, 2021).The presence of copper as copper oxide nanoparticle caused a more potent inhibitory effect on microalgal growth and severely disrupted algal cells membrane (Adochite and Andronic 2021).However, whether as nanoparticle or bulk particle, this present study is consistent with the findings of inhibition of *Desmodesmus abundans* by copper (Braglia, *et al.*, 2021).

Furthermore, binary mixture of pesticide/metals studied in fixed percentage ratio of 50:50, inhibited phosphatase activity with IC50of 4.32±0.55mM, 4.33±0.55mM,4.97±0.52mM,6.23±0.79mM, , and 6.78±0.25mM , for mixtures of **2,4-D/Cu^{2+}, 2,4-D/Cr^{2+}, 2,4-D/Zn^{2+}, 2,4-D/Pb^{2+}, and2,4-D/Cd^{2+}**respectively. The graphical interpolation of the dose-response curve due to 50:50 toxicant ratio, shows that **2,4-D/Cu^{2+}**was more toxic, followed by: **2,4-D/Cr^{2+}** and 2,4-D/Zn^{2+} with 2,4-D/$Pb^{2+}$and 2,4-D/Cd^{2+}been the least toxic. The study of the binary mixture of experimental data points and model predicted dose-response data for inhibition of phosphates activity in *Desmodesmus abundans* exhibited a sigmoidal, sigmoid 4 parameters in all the mixtures. The decreasing order of their toxicity includes: **2,4-D/Cu^{2+}>2,4-D/Cr^{2+}>2,4-D/Zn^{2+}>2,4-D/Pb^{2+}>2,4-D/Cd^{2+}.**The effects of the median inhibitory concentrations (IC_{50}) due to 60:40 ratio mixture of 4.33±0.55mM, 4.33±0.55mM, 4.97±0.52mM, 6.73±0.07mM, and6.78±0.25mM for mixtures of **2,4-**

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D/Cu^{2+},2,4-D/Pb^{2+}, 2,4-D/Zn^{2+},2,4-D/Cr^{2+},2,4-D/Cd^{2+}respectively figure 9. From the 60:40 toxicant ratio, 2,4-D/Cu^{2+} and 2,4-D/Pb^{2+} were most toxic, followed by 2,4-D/Zn^{2+}:, 2,4-D/$Cr^{2+}$and 2,4-D/Cd^{2+}. The median inhibitory concentration 4.32±0.55mM was uniform in both ratio mixtures for **2,4-D/$Cu^{2+}$and 2,4-D/Pb^{2+}**.Their decreasing order of their toxicity includes :**2,4-D/Cu^{2+} [2,4-D/Pb^{2+}]>2,4-D/Zn^{2+}>2,4-D/Cr^{2+}>2,4-D/Cd^{2+}.** The joint effect of 2,4-D and the heavy metals, showed a major reduction in the toxicity inhibition of their binary mixtures in all the mixture ratios. Thus, the amalgamation with 2,4-D was antagonistic to phosphatase inhibition as compared to acute toxicity exhibited by the metal ions alone. This general trend in all the ratios was attributed to the influence due to the mixture of a non-metallic pollutant and a metallic pollutant. Their joint action exhibitedantagonistic toxicity to the heavy metals ions. (Weissmannova, *et al.*, 2018). Consequently, the general trend of the inhibition of phosphates activity in the microalgae at toxicity ratio index of 50:50 and 60:40, demonstrated a progressive decrease in the percentage inhibition rate as the concentration (dose) of the binary mixture increased. However, a possible synergistic effect maybe expected if different doses of the pollutants are used (Zeb, *et at.*, 2017).

V. CONCLUSION

The heavy metals, Copper (Cu^{2+}), Zinc (Zn^{2+}), Lead (Pb^{2+}), Chromium (Cr^{2+}), Cadmium (Cd^{2+}) ions and 2,4-dichlorophenoxyacetic acid (2,4-D) and their binary mixtures with 2,4-D in 50:50 and 60:40 binary mixtures exhibited a strong inhibitory effect on*Desmodesmus abundans* phosphatase enzyme activity. Generally, the presence of 2,4-D in both 50:50 and 60:40 toxicant ratios led to a decrease in the propensity of the toxicity of the heavy metals, vice verse. However, the toxicity of 2,4-D declined due to its amalgamation with the heavy metallic ions. Thus, 2,4-D as an agro-friendly pesticide can chelate toxicity effect when in cocktails with diverse heavy metals ions. Consequently, the general trend of the inhibition of phosphates activity in the microalgae*(Desmodesmus abundans)*in toxicity ratio index of 50:50 showed less significant effect, when compared to toxicity ratio index 60:40. However, at a higher concentration, more inhibitory significant effect may be expected. The equal percentage combinations in the 50:50 suggests either a synergistic or additive effect. Unlike the 60:40 mixture, where the the percentage ratio of the 2,4-D is higher (Zeb, *et at.*, 2016).

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