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Toxicity of binary mixture of heavy metals and 2,4-dichlorophenoxyacetic acid on phosphatase activity of *Chlorella vulgaris*

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Abstract
The influence of mixtures of metals and pesticide on the activity of algal hydrolytic enzyme was investigated. 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 via inhibition of phosphatase enzyme activity of Chlorella vulgaris. 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 Chlorella vulgaris was assessed by inhibition of conversion of p-nitrophenylphosphate to p-Nitrophenol at optical density (OD) of 410nm. Results obtained 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 order: Cr > Cu > zn > Pb > Cd > 2,4-D. The threshold inhibitory concentration ($1C_{50}$) of Cr^{2+} at 0.19±0.01mM and Cu^{2+} at 0.28 ±0.0mM were most toxic against C. vulgaris. However, the pesticide 2,4-D exhibited low inhibition of phosphatase activity with only 20% inhibition. The effects of the amalgamated mixture 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+} (6.50±1.41):, 2,4-D/Zn^{2+} (4.97±0.52):, 2,4-D/Cr^{2+} (4.97±0.52):, 2,4-D/Cd^{2+}(6.78±0.25) (mM) while $IC_{50(s)}$ for 60:40 binary mixture ratio were: 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±0.08), 2,4-D/Cd^{2+} (6.74±0.08) (mM). For toxicant mixture ratio 50:50, the 2,4-D/Cu^{2+} was more toxic, followed by: 2,4-D/Cr^{2+}:, 2,4-D/Zn^{2+}:, 2,4-D/Pb^{2+} and2,4-D/Cd^{2+} been the least toxic. Similarly, 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 drop in the toxicity effect was probably due to the increase in the pesticide concentration. However, others demonstrated the propitiousness for an additive effect. The binary ratio of 60:40 components tend towards antagonistic effect due to the presence of 2,4 dichlorophenoxycetic acid. Generally, model predicted dose-response data fitted well in sigmoid 3, 4, 5 parameters and statistically significant difference ($p<0.05$) between $IC_{50(s)}$ of unary and binary mixtures. However, 2,4-D became more 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 individual toxicants.

Keywords: *Toxicity, phosphatase, 2,4 dichlorophenoxycetic acid, heavy metals, Chlorella vulgaris*

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I. Introduction

Among synthetic organic compounds such as polychlorinatedbiphenyls (PCBs), microplastics, pharmaceuticals, polycyclicaromatichydrocarbons (PAHs); heavy metals and pesticides are among the list of persistent organic pollutants (POPs) that have been detected in aquatic environment (Vagi *et al.*, 2021). From these organic compounds, a substantial amount of heavy metals discharged from different types of anthropogenic activities enter water systems either directly through waste water discharge or indirectly through rainfall and atmospheric deposition (Chen *et al.*,2016). However, several ecotoxicological studies have revealed the direct and indirect effects of these anthropogenic toxicants on marine biota (Vagi *et al.,* 2021). Heavy metals are dangerous due to its recalcitrant nature; tendency to accumulate in water column, sediments, in snow cover, and in aquatic organisms, which leads to the most undetermined pollution in the aquatic system (Amira and Leghouchi, 2018; Zhang *et al.,* 2021). Industrial applications and intensive agricultural practices have also

contributed to the pollution of freshwater habitats thereby causing harmful effects on aquatic organisms (Mohammad *et al.*,2021). In predicting the risk assessment of heavy metals, the effect of only single metal pollutants to aquatic biota has been argued. Hence, toxicological studies disclose greater toxicity interactions evoked by heavy metals on interactions in mixtures with other constituents rather than individually (Adnan *et al.*, 2021). These cocktails of toxicants are always in variable concentrations. As a fundamental part of ecotoxicology, microorganisms are used as models in aquatic toxicology, against doses of single heavy metals and mixture of heavy metals at similar levels as those found in natural rivers (Expósito *et al.*, 2021). Scientific study on TU (Toxicity Unit) and MTI (Mixture Toxic Index) approach have shown that toxicants can act not only individually and cumulatively but also collectively and even synergistically (Robert and Devon, 2021; Adnan *et al.*, 2021). The *in-vivo* introduction of biomarkers is an exceptional tool to assess the vulnerability, stimulatory and negative effect of environmental agents on a bioindicator. Numerous biomarkers have been used to demonstrate the biological effects of several chemical compounds on aquatic organisms, in both trial and normal conditions. Biochemical and physiological parameters such as inhibition of hydrolytic phosphatase enzyme activities is used as significant tools to determine the effect of environmental pollutants. Therefore, early analytical biomarkers are necessary for predicting the health of the aquatic environment and the bio-indicator itself. Thus, as a bio-indicator, the enzymatic biomarker in the microalgae; *Chlorella vulgaris*, which belong to the order: Chlorococcales, and of the genus Chlorella, is used, due its characteristics and metabolic adaptations, which make them ideal sources for risk assessment of aquatic environmental (Jesús Alberto *et al.*, 2021; Hernández *et al.*, 2017).The alteration of other physiologic functions like chlorophyll, intracellular proteins, carbohydrates, lipids, vitamin C, enzymes; carbonic anhydrase, hydrogenase, lipoxygenase, nitrilase, nitrogenase, phosphatase, and thiolase β-carotenes and B vitamins (B1, B2, B6 and B12), can be used as biomarkers (Mohammad *et al.*, 2021). Finally, toxicity interaction of binary mixture can be determined by comparing the observed toxicity data with the reference model of concentration Addition (CA) and independent Action (IA). (Ling-Yun *et al.*, 2019; Kilpi-Koski *et al.*, 2020). In binary mixture, the combination index can be

used to analyse the dose of components in a mixture. $\mathrm{CI} = \frac{a1}{A} + \frac{b1}{B}$ where, a1 and b1 are the dose of components

in the mixture, A and B are the dosed of the individual agents giving rise to the same effects as the mixtures.

II. Materials and Methods

Sample area

Ihiagwa is a town in Owerri West Local Government Area of Imo State, south-eastern Nigeria. It is located 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 depleted rain forest vegetation, with mean temperatures of 27 °C (81 °F) throughout the year. The river is polluted by organic 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 system fails. 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 - Ihiagwa stretch. Figure 1, shows the geographical map of the study area.

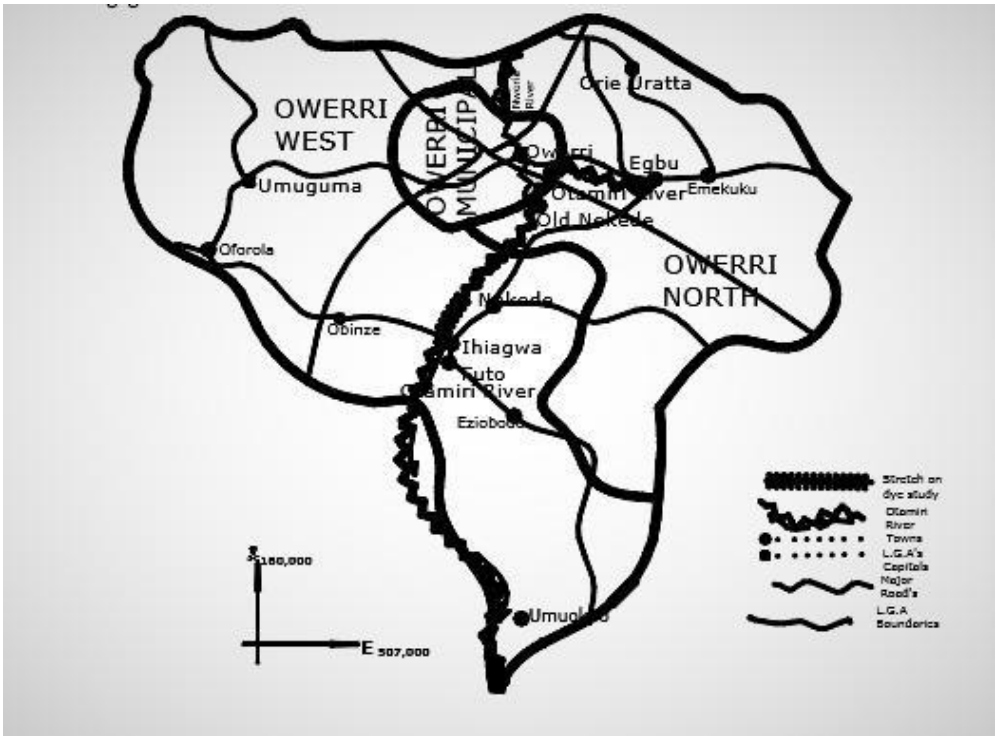

Figure 1, Geographical map of Otamiri River along the Nekede -. Ihiagwa stretch

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 160^0C, 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.

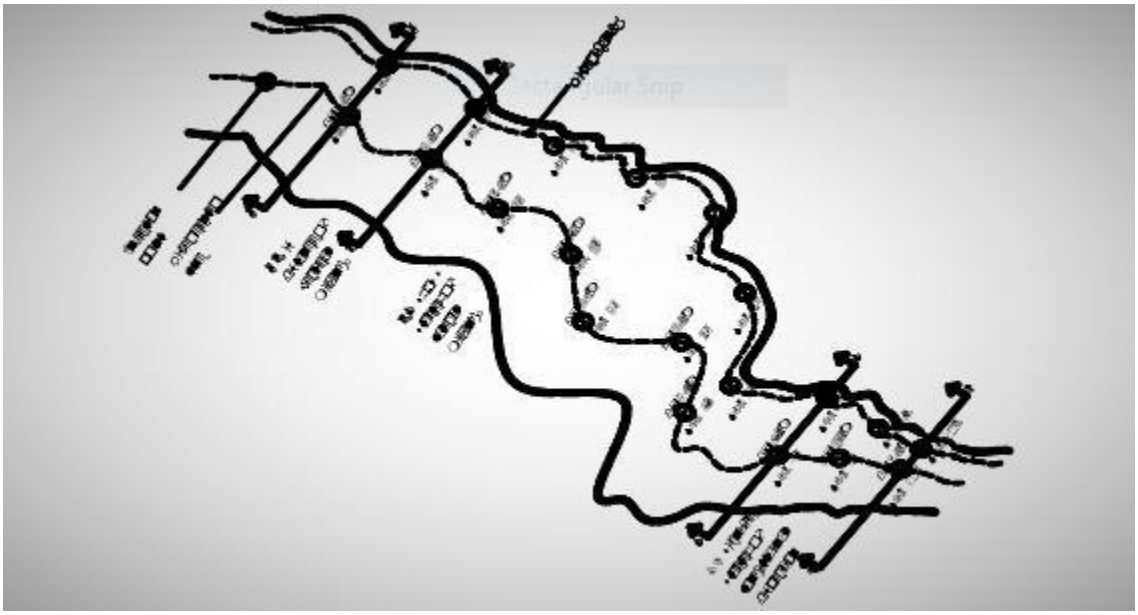

Figure 2, Volumetric flow of a small section of the river.

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.

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).

ALGOLOGICAL ANALYSIS

Preparation of agar plate: Agar plates were prepared by dissolving 2% agar (w/v) in BBM. The plates were autoclaved at 126^{o}C 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); KH2PO 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), H_2SO_4 (concentrated) 1ml/L, Trace Metal Solution 1ml, H_3BO_3 5.75g/500ml (0.7), F/2 Vitamin Solution 1ml (optional: cyanocobalamin, biotin, thiamine) (Nichols, and Bold, 1965).

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±2^{o}C). Thereafter, the culture was transferred into a broth inoculated with bold basal medium contained in 250ml conical flask. Continuous culture was maintained to sustain algal growth at exponential phase.

Subculture of isolate

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

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 5×10^4 cells ml^{-1}, (Jonsson and Aoyama, 2007).

Preparation and standardization of inoculum

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^{0}C), for 24hrours. After incubation, the cells were harvested by centrifugation at 3000rpm for 10minutes, the 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.

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).

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.

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, 100 microlitre 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.

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.

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.

Sequencing
Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, 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.

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).

Phosphatase Extraction
The method of Jonsson and Aoyama (2007), was used for this study. Algal pellets were Freezed with liquid nitrogen (N_2) 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.

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; toxicity was 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 (37^0C). 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.

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 fitted models are also elaborated with their equations below.

(2) Four parameter logistics

$$Y = \frac{a}{1+\left(\frac{x}{x_0}\right)^b}$$

(3) Four parameter sigmoid

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

(4) Three parameter logistics

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

(5) Hormetic model

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

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).

Statistical Analysis

The experimental data was fitted and median inhibitory concentrations (IC_{50}) of individual and mixtures of pesticides and metals were evaluated using Sigma plot software (10.0). Statistical analysis of IC_{50} values was computed using one-way analysis of variance ($p<0.05$) in statistical package for the social sciences (IBM, SPSS software 22).

III. Results And Discussion

Morphological and biochemical identification of the isolate.

The morphological traits of isolate R_1(AY591506) under the electron microscope comprises of a spherical microscopic cell with 2-10µm diameter. In morphometric observations, cell diameter was 3-12 µm. Chloroplast was parietal and cup-shaped with a single pyrenoid the isolate showed many structural elements similar to plants. It has a unilaminar cell wall with subspherical or ellipsoidal shape without flagella. The isolate R_1(AY591506) also comprises of a single chloroplast with a double enveloping membrane composed of phospholipids. The singly, stranded-shaped chloroplast with pyrenoids, contains a cluster of fused thylakoids of green chlorophyll pigment. It contains a gel-like substance confined within the barrier of the cell membrane, a double-layer membrane that resembles the mitochondrion and a dense circular patch (fig 5). Under the light microscopy it appeared green, unicellular, and spherical (coccoid) or subspherical. Chloroplast was parietal and cup-shaped with a single pyrenoid.

All morphological descriptions of R_1(AY591506 (fig 6), was tentatively identified as *Chlorella sp* following the works of Tomaselli, (2004); Borowitzka, (2018); Safi, *et al.,* (2014); Krienitz, *et al.,* (2015); Yamamoto, *et al., (*2005); Champenois, *et al.,* (2015); Garcia, (2012); Beijerinck, *et al.,* (1890); Yamamoto, *et al., (*2004). All morphological and biochemical screening are presented in table 1.

Molecular (Genome) Identification of the Isolates R_1(AY591506).

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 isolates (**R_1(AY591506)** 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 *Chlorella* sp and revealed a closely relatedness to *Chlorella vulgaris* respectively (Fig. 6).

Figure 6: Phylogenetic tree showing *Chlorella vulgaris* isolate

Table 1. Morphological and biochemical characteristics of the isolates.

Parameters	Results
Shape	spherical/cup-shaped pyrenoid
Form	ellipsoidal
Chloroplast	(singly stranded) present
Colour	green thylakoids pigment
Type	unilaminar
Cell diameter	2-12um
Phosphatase test:	++
Nitrogenase test:	++
Chlorophyll analysis:	++
Nitriles test:	++
Lipoxygenases	++
Amylase	+
Lipase test	++
Urease test:	++
Thiolase/gelatinase test:	++
Catalase:	++
Hydrogenases:	++
Carbonic anhydrase:	++
Chlorella vulgaris	

Table 2. Median inhibitory concentration (IC_{50}) of the effect of Unary toxicity of (metal/pesticide) to phosphatase enzyme activity from *Chlorella vulgaris*

Toxicant	IC_{50}(mM)
Cu^{2+}	0.28 ±0.01
Zn^{2+}	0.31 ±0.02
Pb^{2+}	1.86±0.76
Cr^{2+}	0.19±0.01
Cd^{2+}	2.59±0.32
2,4-D	16.82±1.47

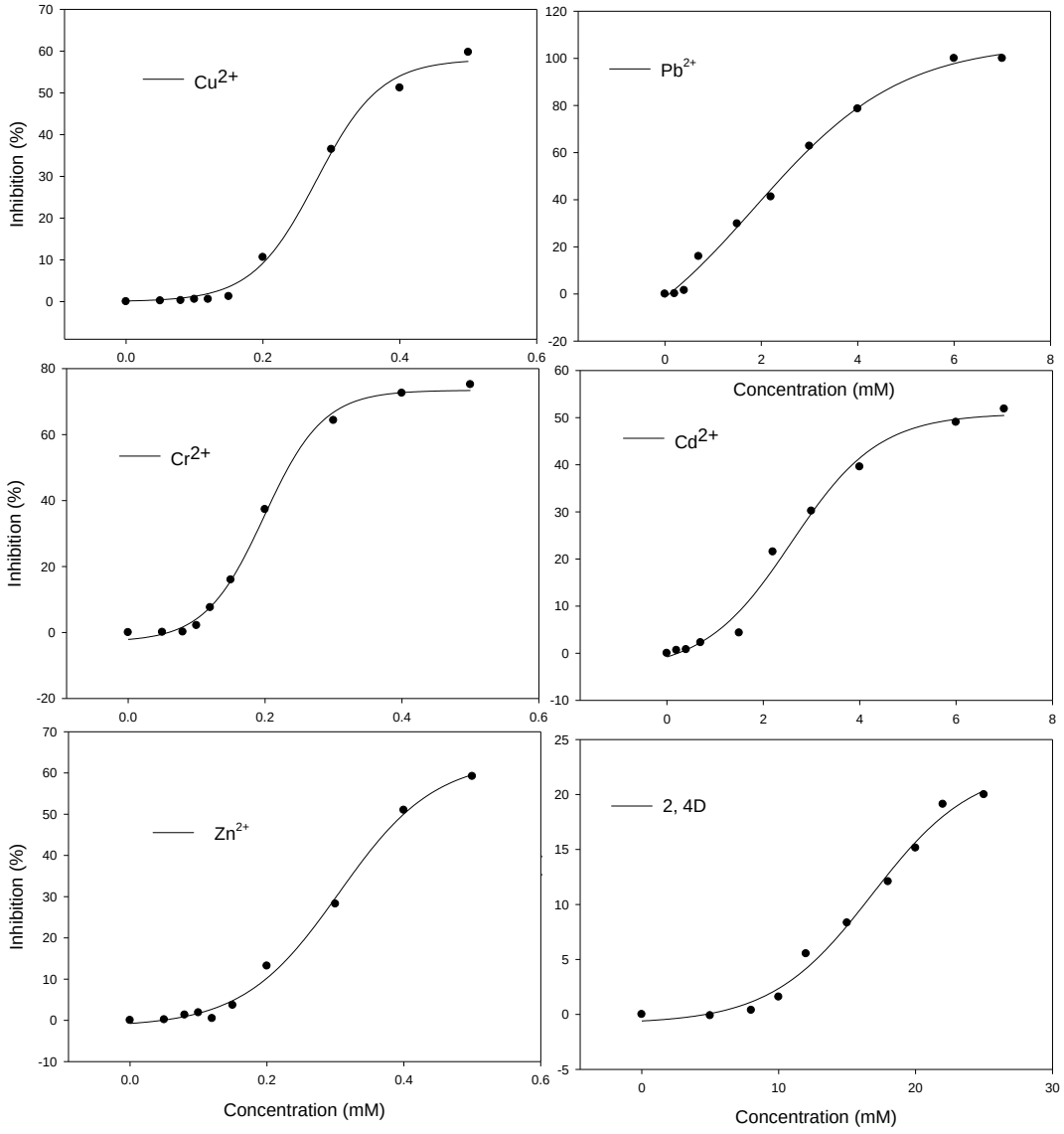

Figure 7: Experimental (data points) and model-predicted dose-response data for unary inhibition of phosphatase activity of *Chlorella vulgaris* by copper, lead, chromium, cadmium, zinc ion and 2,4-D.using Sigmoidal, Sigmoid, 3 Parameter model.

Table 3. Median inhibitory concentration (IC_{50}) of the effect of Binary mixture of (metal/pesticide) to phosphatase enzyme activity from *Chlorella vulgaris.*

Toxicant Mixture 50:50	**IC_{50} (mM)**
2,4-D/Cu^{2+}	4.32±0.55
2,4-D/Pb^{2+}	6.50±1.41
2,4-D/Zn^{2+}	4.97±0.52
2,4-D/Cr^{2+}	4.97±0.52
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±0.08
2,4-D/Cd^{2+}	6.74±0.08

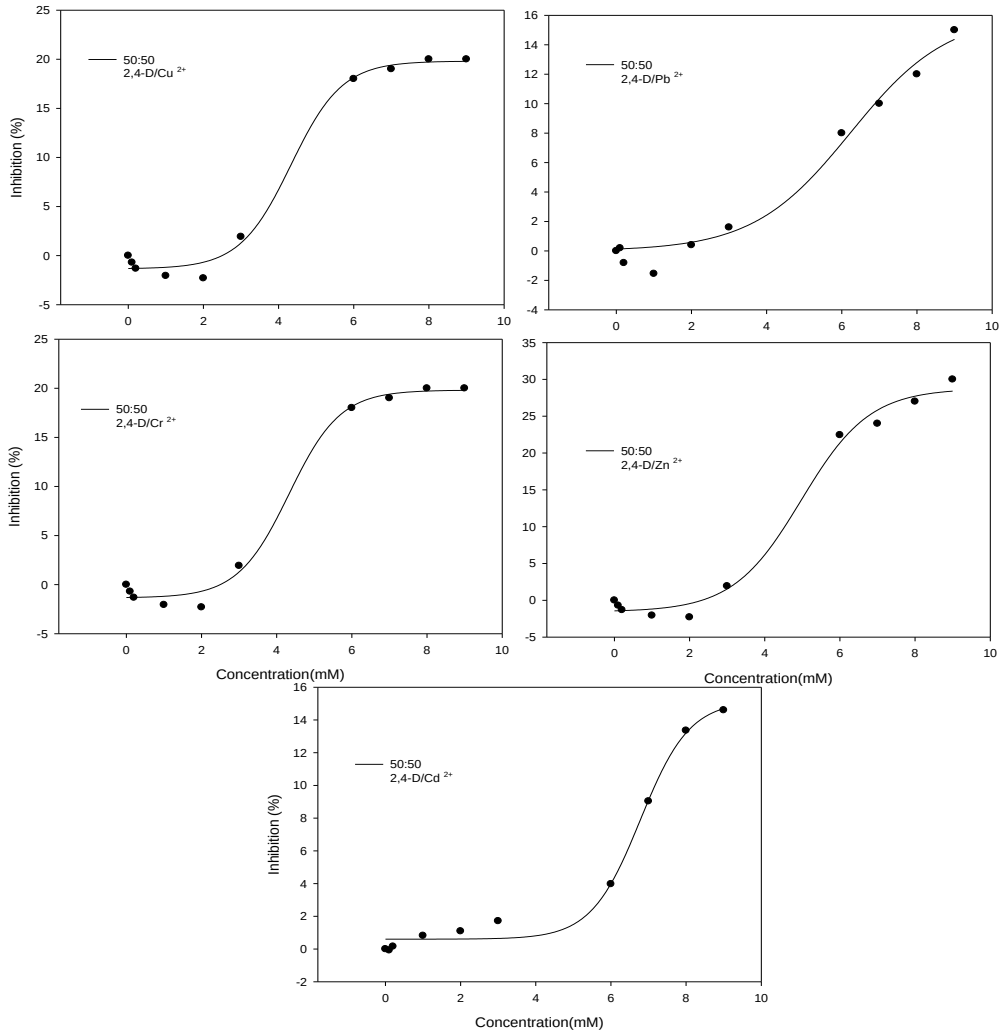

Figure: 8: Experimental (data points) and model-predicted dose-response of *C. vulgaris* phosphatase activity to Binary mixtures (50:50) % ratios of **2,4-D/Cu^{2+}**,. **2,4-D/Pb^{2+} , 2,4-D/Zn^{2+}, 2,4-D/Cr^{2+}, 2,4-D/Cd^{2+} .** The solid lines represent Sigmoidal, Sigmoid, 4 Parameter model

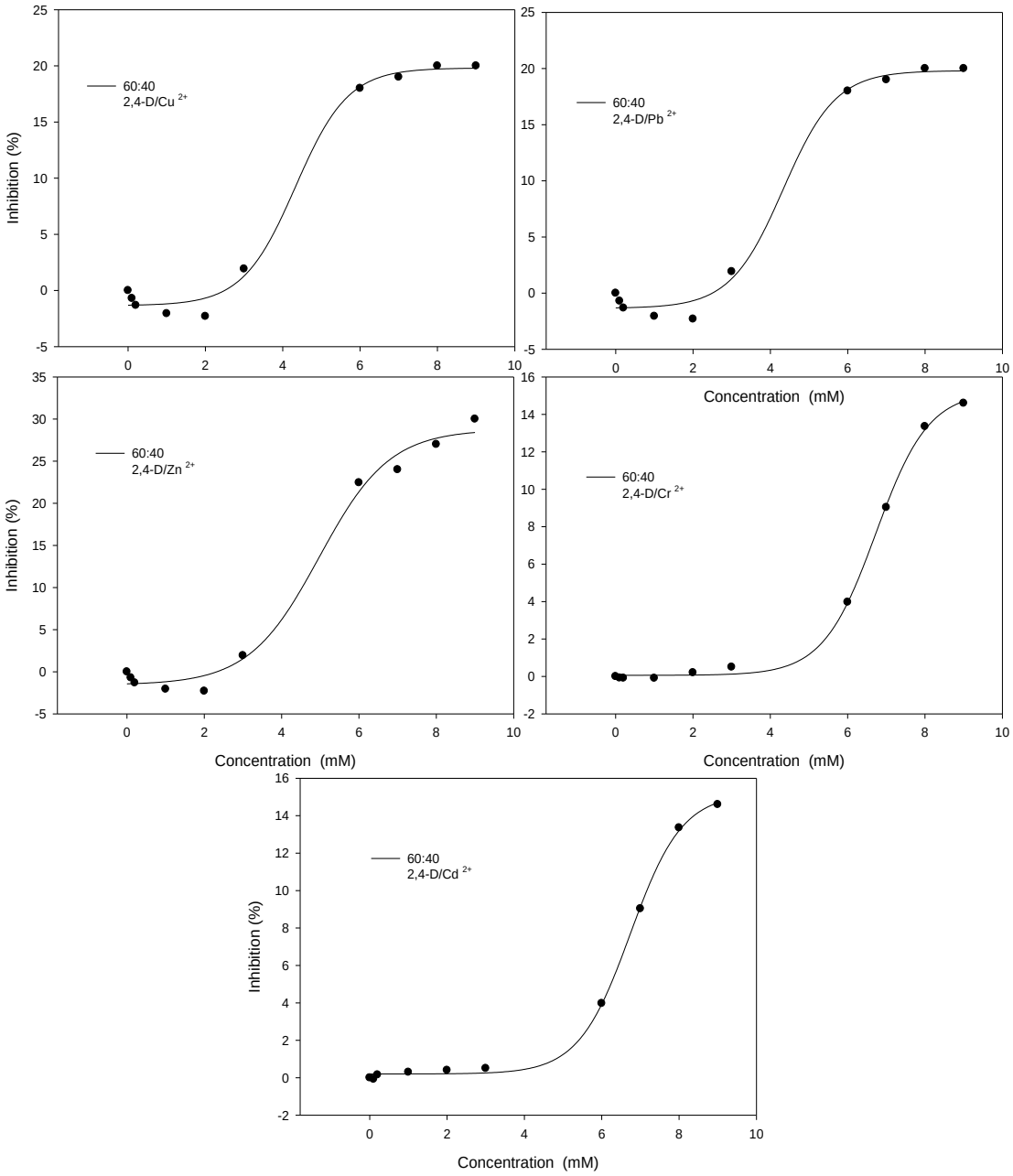

Figure 9: Experimental (data points) and model-predicted dose-response of *C. vulgaris* phosphatase activity to Binary mixtures (60:40) % ratios of **2,4-D/Cu $^{2+}$,. 2,4-D/Pb $^{2+}$, 2,4-D/Zn^{2+}, 2,4-D/Cr $^{2+}$, 2,4-D/Cd $^{2+}$** . The solid lines represent Sigmoidal, Sigmoid, 4 Parameter model.

The result of the morphological and biochemical characteristics of *Chlorella vulgaris* showed 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); Yamamoto, *et al., (*2004); Champenois, *et al.,* (2015); Garcia,(2012); Hegewald, 2000;). From the molecular (Genome) identification of the Isolate: *Chlorella vulgaris* **R_1(AY591506), t**he 18S of the isolate showed a percentage similarity to other families of related specie 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 *Chlorella* species and revealed a closely relatedness

*Chlorella vulgaris***.** The result of the Phylogenetic tree showing the evolutionary of the isolate is shown in figure 6.

The result of the effect of unary toxicity of (metal/pesticide) and binary mixture of (metal/pesticide) to phosphatase enzyme activity from *Chlorella vulgaris* 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 *Chlorella vulgaris* demonstrates the heavy metal ions toxicity was in the order Cr > Cu > zn > Pb > Cd > 2,4-D. Chromium ion was found to be highly toxic while 2,4-D was the least toxic. The median inhibitory toxicity value IC_{50} of Chromium showed the highest toxicity of 0.19±0.01mM as shown in the graphical interpolation of dose-response curve in figure 7. The experimental data points and model predicted dose-response data for inhibition of phosphatase activity of *Chlorella vulgaris* showed few outliers and the enzyme was sensitive to chromium. The findings of Al-Hasawi *et al.,* (2020) supported the reported sigmoidal trend of inhibition of chromium. Among the metals which were considered as the greatest risk to freshwater ecosystem in Bohai region of Chain, chromium was reported as one of the top five metal of concern (Su, *et al.*, 2017). The report on agro ecological responses of heavy metal pollution, indicated that chromium causes significant toxicity (Srivastava, *et al.*, 2017). Chromium toxicity has direct effect on flora that forms an integral component of the ecosystem. The presence of chromium (VI) reduced reactive oxygen species (ROS) from 1.6 fold to 1.1 fold at 0.5-5mMoIL^{-1}. Also, exposure of the phosphatase enzyme from *Chlorella vulgaris* to copper and zinc ions zinc resulted in significant inhibition of phosphatase activity with $1C_{50}$ of 0.28 0.01mM. and 0.31 0.02mM. respectively. Inhibitory effect of copper on growth rate, Chlorophyll-a content, superoxide dismutase (SOD) activity, SOD MRNA gene expression and frustule morphology of a benthic freshwater diatom *Halamphora veneta,* has been reported in the work of Mu, *et al*., 2017. Copper was deemed to represent the highest toxicity risk to freshwater ecosystem in Bolai region of China (Su, *et al.,* 2017). The study showed that freshwater organisms like *Chlorella vulgaris* or *Halamphora Veneta* are all potential candidates for the toxicological assessment of copper. The result on unary toxicity of copper strongly agrees with the works of Mohy-Eldim and Abeel-Kareen, (2020). Their work suggested that copper may induce some genotoxic influence in *Chlorella salina* and *Nannochloropsis isalina.*. Wang, *et al.,* (2020) used quotient and probabilistic methods to rank ecological risk of metals to freshwater organisms in lake Tashu, China. Based on the probabilistic method, copper posed the highest risk amongst Nikel and Zinc. The results of the physiological effects of copper on the freshwater alga *Closterium ehrenbergii meneghini* (Conjugtophyceae), indicated that copper induces oxidative stress in cellular metabolic processes and caused severe physiological damage within the cells (Wang, *et al.*, 2017). *Chlorella vulgaris and C. ehrenbergii* represented a potentially powerful test model for use in aquatic toxicity assessment (Wang, *et al*., 2017).

Wang, *et al.,* (2020), ranked ecological risk of metals to freshwater organism in Lake Taihu, China, showing that Zinc poses a considerable risk to fresh water organisms. Similar growth inhibitory test of *Chlorella vulgaris* was observed by Exposito, *et al.,* (2021). Furthermore, lead ion (fig.7) was the fourth metal ion in toxicity ranking to phosphatase activity. The moderate response of *C. vulgaris* to lead ions is consistent with the work of Al-Hasawi, *et al.,* (2020). Cadmium ion was the least toxic among the heavy metals with IC_{50} value of 2.59 0.32mM;

Cadmium indicated a progressive inhibition of *C. vulgaris* phosphatase activity. A similar inhibitory effect of cadmium on *Chlorella vulgaris* was reported by Cheng, *et al.,* (2016). Their results proved that cadmium influenced the physiological functions such as assimilation of pigment composition, soluble protein, oxidative status (production of hydrogen peroxide and superoxide anion), antioxidant enzymes (such as superoxide dismutase, peroxidase, catalase and glutathione reductase enzyme) in *Chlorella vulgaris.* Bellini, *et al.,* (2021) also described that cadmium ions can influence cells of charophytes and bryophytes through vacuolar sequestration and cell wall immobilization.

In figure (7), the dose-response curve of 2,4-D showed the lowest toxicity to phosphatase activity with IC_{50} of 16.82 1.47mM. Similar inhibitory toxicity effect was reported by (Mathieu- Houssou, *et al.,* 2020). However, in experimental exposures of ten herbicides to tropical marine microalgae *Rhodomonas salina* by Thomas, *et al.,* (2020), they reported a low or no-toxic responses to the function of the individual photosystem II (PSII) by 2,4-D, due to low inhibitory dose-response, less can be more (Schirrmacher, 2021).

Furthermore, binary mixture of pesticide/metals studied in fixed percentage ratio of 50:50 inhibited phosphatase activity with IC_{50} of 4.32±0.55mM, 6.50±1.41mM, 4.97±0.52mM, 4.97±0.52mM and 6.78±0.25mM for the mixtures of 2,4-D/Cu $^{2+}$, 2, 4-D/Pb $^{2+}$, 2,4-D/Zn^{2+}, 2,4-D/Cr $^{2+}$ and 2,4-D/Cd $^{2+}$ respectively. The graphical interpolation of the dose-response curve of 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+}:, 2,4-D/Pb $^{2+}$ and 2,4-D/Cd^{2+} being 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 C*hlorella vulgaris* exhibited largely sigmoidal relationship.

However, the evaluated 60:40 percentage ratio of metal/pesticides mixture was exhibited toxicity to

phosphatase activity with IC_{50} values of 4.33±0.55mM, 4.33±0.55mM, 4.97±0.52mM, 6.73±0.08mM and 6.74±0.08mM (figure 9), for the mixtures **2,4-D/Cu $^{2+}$**, **2,4-D/Pb $^{2+}$**, **2,4-D/Zn^{2+}**, **2,4-D/Cr $^{2+}$** and **2,4-D/Cd $^{2+}$**.respectively. From the 60:40 mixtures, the 2,4-D/Cu $^{2+}$ and 2,4-D/Pb $^{2+}$ mixtures were most toxic, followed by 2,4-D/Zn^{2+}:, 2,4-D/Cr $^{2+}$ and 2,4-D/Cd $^{2+}$.

The combination of 2,4D with the heavy metals, showed a drastic reduction in the toxicity of the heavy metals; evidenced in an increased inhibitory threshold compared to the toxicity of heavy metals alone. This general trend in all the mixture ratios maybe attributed to the influence due to the mixture of a non-metallic pollutant and a metallic pollutant. Therefore, while their joint action exhibits antagonistic toxicity to the heavy metals ions; it was an additive effect for for 2,4-D (Weissmannova, *et al*., 2018). Consequently, the general trend of the inhibition of phosphates activity in the microalgae by the mixture ratios of 50:50 and 60:40 demonstrated a progressive concentration dependent inhibition of enzyme activity.

IV. 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 binay mixtures with 2,4-D in 50:50 and 60:40 binary mixtures exhibited a strong inhibitory effect oh C. vulgaris phosphatase enzyme activity. The presence of 2,4-D in both 50:50 and 60:40 mixtures led to a decrease in the propensity of the toxicity of the heavy metals. However, 2,4-D became more toxic due to its amalgamation with the heavy metals ions. Thus, 2,4-D as an agro- friendly pesticide may become toxic when its in cocktails with diverse heavy metals ions.

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