(RESEARCH ARTICLE)

Toxicity of single and ternary mixtures of heavy metals and 2,4-dichlorophenoxyacetic acid to Chlorella vulgaris alkaline phosphatase activity

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Abstract

The influence of mixtures of metals and pesticide on the activity of algal hydrolytic enzyme alkaline phosphatase was investigated. Toxicity of unary and ternary 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 effects of the amalgamated ternary mixtures were studied in their fixed percentage ratio of 20:40:40 and 40:30:30. Results obtained showed that all the unary heavy metal ions were toxic to algal phosphatase activity, with a concentration dependent inhibition of alkaline phosphatase activity. The analysis of eco-toxicity concentration (EC_{50}) of toxicant ternary mixtures of metals/ pesticides mixtures in 20:40:40 ratios show that the ternary mixtures 2,4-D/Pb^{2+}/Cd^{2+} and 2,4-D/Zn^{2+}/Cd^{2+} were more toxic, followed by 2,4-D/Cu^{2+}/Cr^{2+}, 2,4-D/Pb^{2+}/Zn^{2+}, 2,4-D/Cu^{2+}/Zn^{2+} and 2,4-D/Cu^{2+}/Cd^{2+}. Whereas among the 40:30:30 mixture ratios 2,4-D/Pb^{2+}/Cd^{2+} and 2,4-D/Zn^{2+}/Cd^{2+} were most toxic. The decline in the trend of their toxicity strength was followed by 2,4-D/Cu^{2+}/Cr^{2+}, 2,4-D/Cu^{2+}/Zn^{2+}, 2,4-D/Cu^{2+}/Cd^{2+}, 2,4-D/Cu^{2+}/Pb^{2+} and 2,4-D/Pb^{2+}/Zn^{2+}. In addition, the least toxic amongst them were the 2,4-D/Pb^{2+}/Cr^{2+}, 2,4-D/Zn^{2+}/Cr^{2+} and 2,4-D/Cr^{2+}/Cd^{2+} ternary mixtures. Toxicity index analysis of the mixture interaction effect showed that the mixtures of heavy metals with 2,4, D were mostly antagonistic with toxic index (TI) >>1, However, exceptions were the 2,4-D/Pb^{2+}/Cd^{2+} and 2,4-D/Pb^{2+}/Cd^{2+}mixture which were additive and synergistic with TI=1 and TI<1 respectively. From the foregoing study, the toxicity of the mixtures exhibited largely sigmoidal relationship, increased composition of heavy metal in the mixture resulted in a potentiation of 2,4-D toxicity. These results indicate that mixtures of heavy metals with largely non-toxic 2,4-D may pose ecological risk to freshwater microalgae *Chlorella vulgaris,* presenting antagonistic, additive and synergistic toxicity interactions based on their relative occurrence in the mixture.

Keywords: Toxicity; Mixture; Alkaline phosphatase; 2,4-dichlorophenoxycetic acid; Heavy metals; *Chlorella vulgaris*

1. Introduction

Qualitative as well as quantitative ecological risk assessments no longer involve single or individual chemical pollutants (O'Brien and Keough, 2014; Schuijt *et al.,* 2021). Hence, the need to assess risks due to amalgamation of chemicals have become obvious, since micro-organisms are exposed to mixtures of chemical pollutants in natural environments rather than individual compounds, especially in aquatic ecosystems (Aronzon, Peluso and Coll, 2020; Kovalakova, *et al.,* 2020; Topaz *et al.,* 2020). Heavy metals and organic pollutants are two of the major kinds of toxic pollutants found in aquatic ecosystem (Shi *et al.,* 2021: Zhang *et al.,* 2021). In aquatic ecosystems, principal producers like microalgae are sensitive to physiochemical variations and chemical pollution. Furthermore, microalgae are considered as essential models in

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toxicity assessment (Martínez-Ruiz and Martínez-Jerónimo, 2015; Nguyen, Moon and Lee 2020; Nunes *et al.,* 2018). They amass chemical pollutants present in aquatic ecosystems and transfer them to other trophic levels (Chen, Chen and Dong, 2016). Pollutants mixtures may influence the aquatic ecosystem by affecting certain functions such as altering microalgal morphology, physiology, and genetics, photosynthesis, nitrogen metabolism, the biosynthesis of amino acids, as well the biosynthesis of secondary metabolites, oxoacid metabolism, organic acid metabolism, carboxylic acid metabolism, and oxidation-reduction processes (Chen, Chen and Dong, 2016; Shi *et al.*, 2021). However, biochemical and physiological parameters such as inhibition of hydrolytic phosphatase enzyme activities have been applied as significant indicator of the effect of environmental toxicants (Onyeukwu and Edna, 2022a; Onyeukwu and Edna, 2022b; Wenq *et al.*, 2021). Therefore, early analytical biomarkers are necessary for predicting the health of an aquatic environment and the bioindicator itself. Thus, as a bio-indicator (*Chlorella vulgaris)*, the enzymatic (phosphatase) biomarker in the microalgae of the genus *Chlorella* is used due its characteristics, and metabolic adaptations; which make them ideal indicators for risk assessment of aquatic environment (Onyeukwu and Edna, 2022ab; Jesús Alberto *et al.,* 2021; Hernández *et al.,* 2017). As a result, the cocktail of heavy metals and organic pollutants, in terms of unary and binary mixtures enhances either their additive, synergistic or antagonistic toxicity effect (Robert, and Devon, 2021: Zhang *et al.,* 2021: Onyeukwu and Edna, 2022b). To assess the effects of mixed toxicity treatments, values are obtained from each compound and the mixtures using the estimation of relative response, median inhibitory *(*EC$_{50}$*)* or effective concentration *(EC$_{50}$)* protocols. Concentration addition (CA) and independent addition (IA) models are supportive to evaluate interaction patterns of heavy metal such as in the 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). Predictive data are modelled using mathematical statistical tool or mixtox model can be used with the additional parameter '*a*' to evaluate potential interaction patterns (Onyeukwu and Edna, 2022a; 2022b; Greco, *et al.,* 1992; Greco, Bravo and Parsons 1995). The '*a*' parameter in mixtox tool indicates the synergism or antagonism of two chemical interactions, wherein *a* < 0 indicates synergism and *a* > 0 indicates antagonism. (Jonker *et al.,*2005). These models evaluate putative interaction patterns for combinations of chemicals. CA models are generally used to evaluate combinations of chemicals that share the same mechanism of action. In contrast, IA models are generally used for those that exhibit different mechanisms of action.

2. Material and methods

2.1. 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.
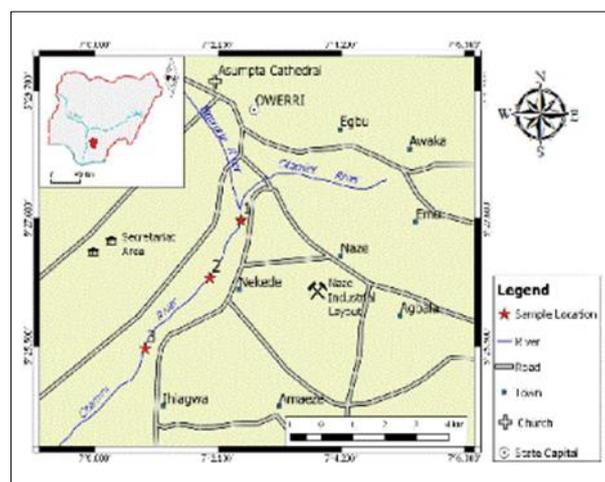
Figure 1 Digital Geographical map of Otamiri River along the Nekede -. Ihiagwa stretch

2.2. Sampling

2.2.1. Sampling Bottles

Sterile glass bottles, with capacity of at least 200 ml 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 ^{0}C, 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.
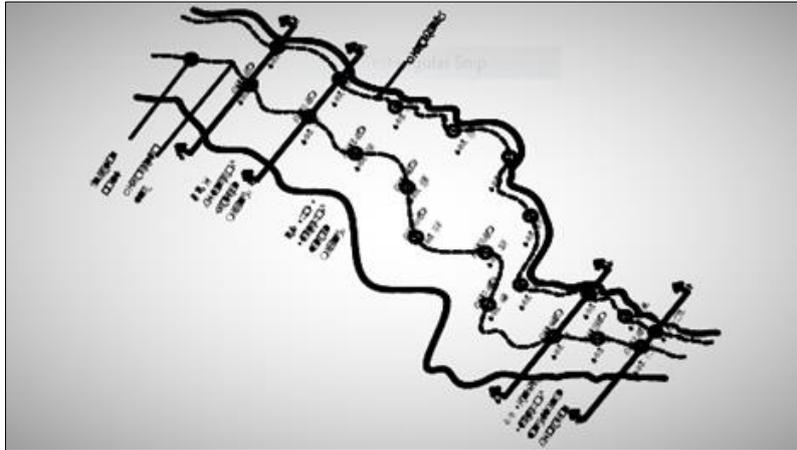
Figure 2 Volumetric flow of a small section of the river

2.3. Collecting Sample

2.3.1. 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.

2.4. 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 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).

2.5. Algological analysis

2.5.1. Preparation of agar plate

Agar plates were prepared by dissolving 2% agar (w/v) in BBM. The plates were autoclaved 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); KH2PO: 48.75g/500(10ml), CaCl$_2$•2H$_2$O: 12.5g/500ml (1ml), MgSO$_4$•7H$_2$O: 37.5g/500ml (1ml), NaNO$_3$: 125g/500ml (1ml), K$_2$HPO: 4.37.5g/500ml (1ml), NaCl: 12.5g/500ml (1ml),

$Na_2EDTA \cdot 2H_2O$: 10g/L (1ml), KOH: 6.2g/L, $FeSO_4 \cdot 7H_2O$: 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: (optional: cyanocobalamin, biotin, thiamine) (Nichols and Bold, 1965).

2.5.2. Culture of isolate

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

2.5.3. Subculture of isolate

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

2.5.4. Storage of pure stock culture

For optimal yield of test organism(s), axenic broth culture was incubated at temperature ($20\pm2°c$), 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).

2.5.5. Preparation and standardization of inoculum

A loopful of the isolates, stored in bold basal medium slant in the refrigerator was inoculated into 200ml 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 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.

2.6. 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).

2.7. 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.

2.7.1. 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 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 10,000xg for 1 minute. Four hundred (400) microlitre of supernatant was transferred to a Zymo-Spin IV spin Filter (orange top) in a collection tube and centrifuged at 7000xg 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 10,000xg 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 added to the Zymo-spin IIC in a new collection tube and spun at 10,000xg for 1 minute followed by the addition of 500 microlitre of algal/fungal/bacterial DNA Wash Buffer and centrifuged at 10,000xg 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 10,000xg microlitre for 30 seconds to elute the DNA. The ultra-pure DNA was then stored at -20 degree for other downstream reaction.

2.7.2. 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.

2.7.3. 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, 95 ^{0}C for 5 minutes; denaturation, 95 ^{0}C for 30 seconds; annealing, 53 ^{0}C for 30 seconds; extension, 72 ^{0}C for 30 seconds for 35 cycles and final extension, 72 ^{0}C 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.

2.7.4. 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 ^{0}C for 10s, 55 ^{0}C for 5s and 60 ^{0}C for 4min.

2.7.5. 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).

2.7.6. Phosphatase Extraction

The method of Jonsson and Aoyama (2007), was used for this study. Molecular identified 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.

2.8. Toxicity of unary and ternary mixtures of metals and pesticide to *C. vulgaris* alkaline 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 ranges 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). Ternary mixtures of pesticide/metal were amalgamated using simple percentage ratios of 20:40:40 and 40:30:30 for ternary mixtures toxicity ratios. Their toxicities were determined at concentrations from 0-9.0mM. Inhibitory study on ALP activity was determined in 2ml reaction final volume consisting of the graded concentration of single or ternary toxicants mixture, distilled water, buffered enzyme, and substrate (p-NPP), contained in 15ml sterilized culture tubes. The set-up was conducted 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^{0}C). 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.

2.9. Estimation of relative response, Median Inhibitory Concentration of Unary and Ternary 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),

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

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 (EC_{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.

$$Y = \frac{a}{1 + \left(\dfrac{x}{x_0}\right)^b} \qquad\qquad \text{(2) Four parameter logistics}$$

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

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

$$Y = y_0 + \frac{a - y_0 + fx}{1 + \left(\dfrac{x}{x_0}\right)^b} \qquad\qquad \text{(5) Hometic model}$$

Where:
y is the relative response.
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 EC_{50}.

2.10. Analysis of toxicity using toxic index model

The toxicity interaction of the mixtures was assessed using the toxic index (TI), which sum the components toxic unit (Boillot and Perrodin, 2008).

The TU values were calculated using the expression:

$$TU_i = \frac{Cmix_i}{IC_{50I}} \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots(4)$$

While TI is the summation of TU for n toxicants in the mixture

$$TI = \sum_{i=1}^{n} TU_i \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3)$$

Where $Cmix_i$ is the concentration of the ith toxicant in the mixture and EC_{50i} is the EC_{50} of the same toxicant when tested singly. TI=1 implies additive interaction, TI > 1 implies antagonistic interaction and TI < 1 implies a synergistic interaction (Boillot and Perrodin, 2008).

2.11. Statistical Analysis

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

3. Results

3.1. 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. The Chloroplast appeared 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. 1). 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.

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

From the molecular identification the isolate 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. 1).
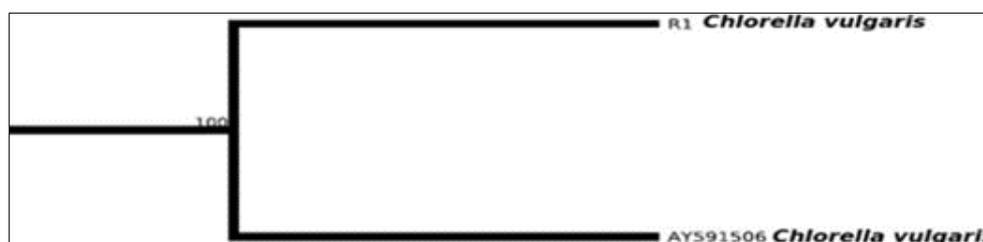
Figure 3 Phylogenetic tree showing *Chlorella vulgaris* isolate

3.3. Toxicity of single metals: copper, lead, chromium, cadmium, zinc ions and 2,4-D to Chlorella vulgaris phosphatase activity

Result presented in figure 4 shows the experimental (data points) and model-predicted dose-response data cure for unary toxicity of heavy metals: Copper, Lead, Chromium, Cadmium, Zinc ions and 2, 4-D to *Chlorella vulgaris* phosphatase activity. The results indicate that the inhibition of phosphatase activity closely fitted into a Sigmoidal 3 Parameter model; with R^2 values ranging from 0.98-0.99. Threshold inhibitory concentrations (EC_{50}) of the single compounds are presented in table 1.0.

3.4. Toxicity of ternary mixtures of copper, lead, chromium, cadmium, zinc ions with 2,4-D to *Chlorella vulgaris* phosphatase activity

Result presented in figure 5-6 shows the experimental (data points) and model-predicted dose-response data curve for ternary mixtures of heavy metals (Copper, Lead, Chromium, Cadmium and Zinc ions) with 2, 4-D to *Chlorella vulgaris* phosphatase activity. The results indicate that the inhibition of phosphatase activity by the ternary mixtures closely fitted into a Sigmoidal 4 Parameter model; with R^2 values ranging from 0.96-0.99.

Table 1 Morphological and biochemical characteristics of the isolates

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

Key; +: Low degree of reactivity, ++: High degree of reactivity

Table 1: Threshold inhibitory concentrations (EC_{50}) and toxicity Interaction analysis of single and ternary mixtures

Threshold inhibitory concentrations (EC_{50}) of the ternary mixture of pesticide/metals, studied in their corresponding fixed percentage ratio of 20:40:40, inhibited phosphatase activity showed EC_{50} of 5.27±0.27mM (2,4-D/Cu^{2+}/Pb^{2+}), 4.41±0.25mM (2,4-D/Cu^{2+}/Zn^{2+}), 4.14±0.19mM (2,4-D/Cu^{2+}/Cr^{2+}), 4.42±0.25mM (2,4-D/Cu^{2+}/Cd^{2+}), 4.37±0.25mM (2,4-D/Pb^{2+}/Zn^{2+}), 5.27±0.27mM (2,4-D/Pb^{2+}/Cr^{2+}), 2.77±0.03mM (2,4-D/Pb^{2+}/Cd^{2+}), 5.29±0.31mM (2,4-D/Zn^{2+}/Cr^{2+}), 2.77±0.03mM (2,4-D/Zn^{2+}/Cd^{2+}) and 5.29±0.30mM (2,4-D/Cr^{2+}/Cd^{2+}) (Table 1). Similarly, the evaluated median ecotoxicity concentration EC_{50} values of respective 40:30:30 ternary metal/pesticides mixtures to phosphatase activity were 4.42±0.25mM (2,4-D/Cu^{2+}/Pb^{2+}), 4.36±0.26mM (2,4-D/Cu^{2+}/Zn^{2+}), 4.28±0.20mM (2,4-D/Cu^{2+}/Cr^{2+}), 4.37±0.25mM (2,4-D/Cu^{2+}/Cd^{2+}), 4.42±0.25mM (2,4-D/Pb^{2+}/Zn^{2+}), 5.27±0.27mM (2,4-D/Pb^{2+}/Cr^{2+}), 2.77±0.03mM (2,4-D/Pb^{2+}/Cd^{2+}), 5.29±0.31mM (2,4-D/Zn^{2+}/Cr^{2+}), 2.77±0.03mM (2,4-D/Zn^{2+}/Cd^{2+}) and 5.29±0.30mM (2,4-D/Cr^{2+}/Cd^{2+}). The analysis of the mixture interaction effect showed that the mixtures of heavy metals with 2,4-D were mostly antagonistic with toxic index (TI) >>1, However, exceptions were the 2,4-D/Pb^{2+}/Cd^{2+} and 2,4-D/Zn^{2+}/Cd^{2+} mixture which were additive and synergistic with TI=1 and TI<1 respectively.
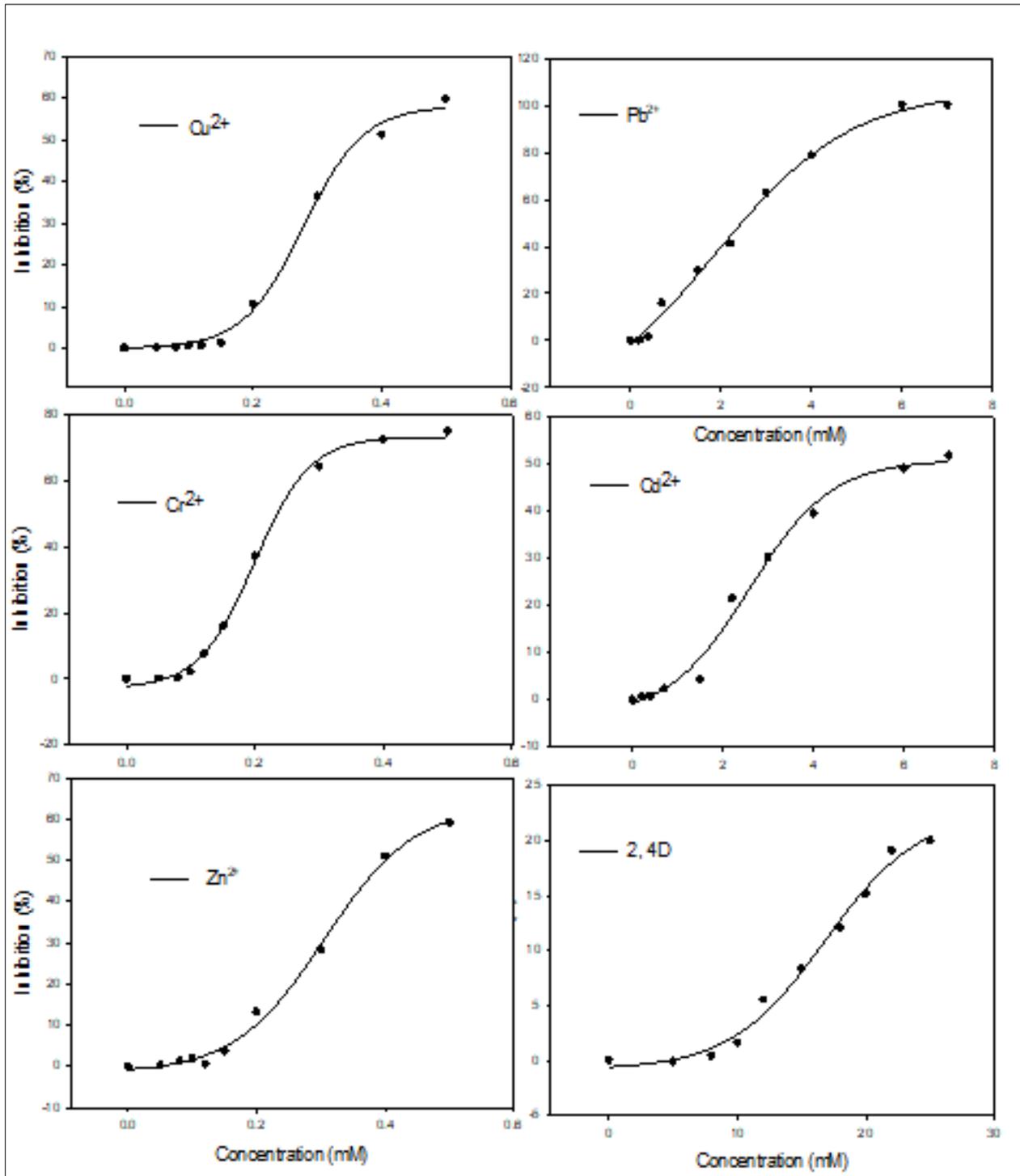
Figure 4 Experimental (data points) and model-predicted dose-response data for unary inhibition of phosphatase activity of *Chlorella vulgaris* by copper, lead, chromium, cadmium, zinc ions and 2,4-D using Sigmoidal, Sigmoid, 3 Parameter model
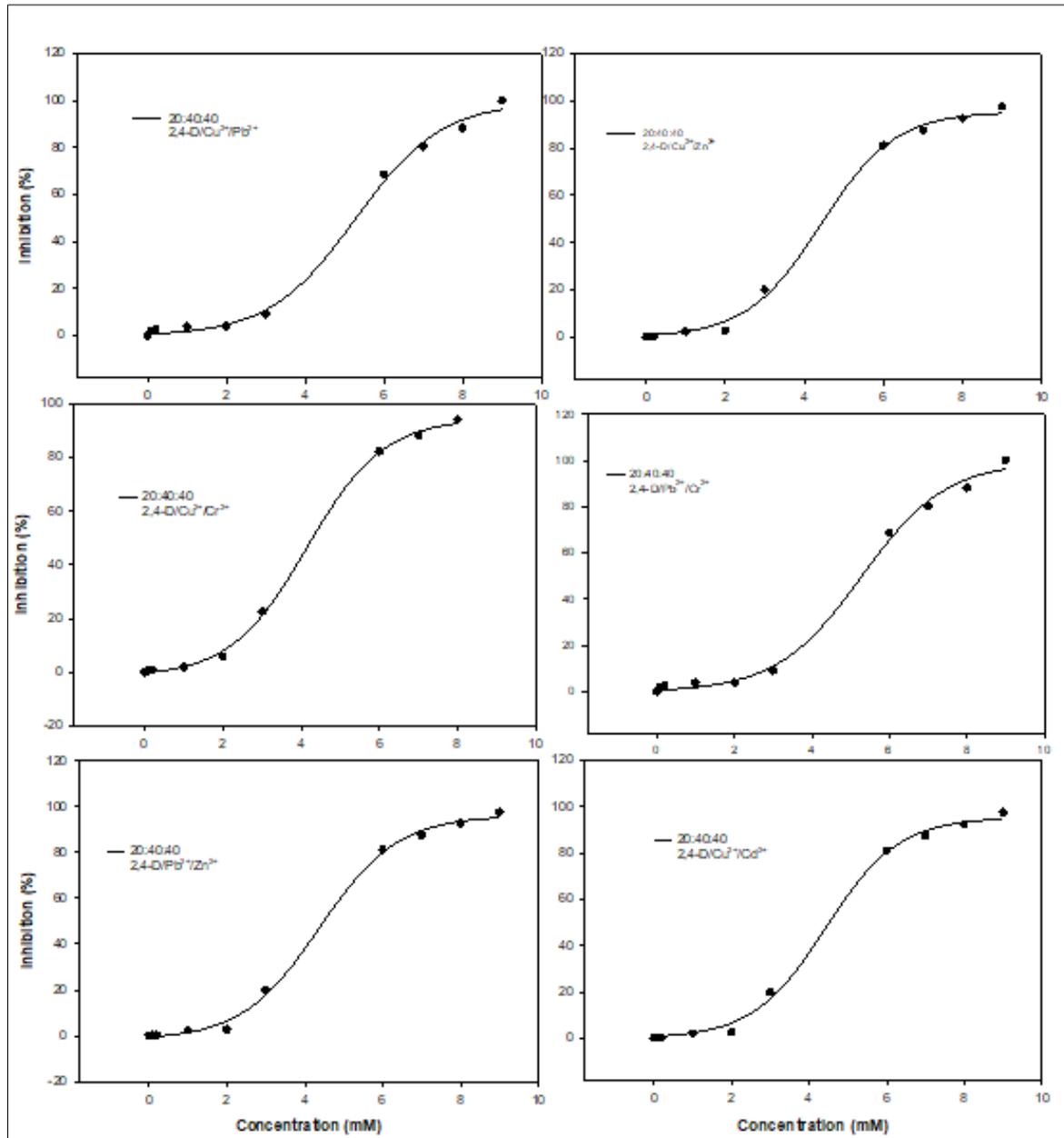
Figure 5 Experimental (data points) and model-predicted dose-response of *C. vulgaris* phosphatase activity to ternary mixture toxicity ratios 20:40:40 of 2,4-D/Cu^{2+}/Pb^{2+}, 2,4-D/Cu^{2+}/Zn^{2+}, 2,4-D/Cu^{2+}/Cr^{2+}, 2,4-D/Pb^{2+}/Cr^{2+}, 2,4-D/Cu^{2+}/Cd^{2+}, and 2,4-D/Pb^{2+}/Zn^{2+} The solid lines represent sigmoidal, sigmoid 4 Parameter model

4. Discussion

The results of the morphological and biochemical characteristics of *Chlorella vulgaris* shown in table 1, were consistent with the report earlier studies (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 to *Chlorella vulgaris***.**
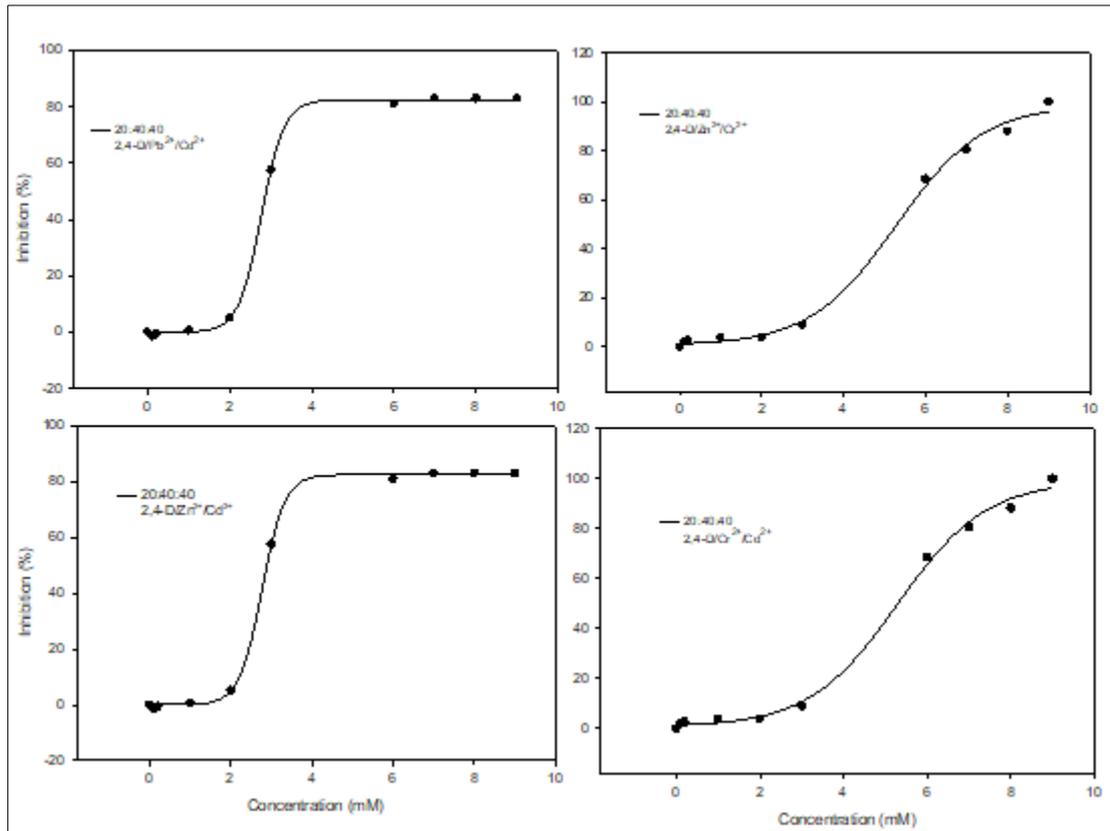
Figure 6 Experimental (data points) and model-predicted dose-response of *C. vulgaris* phosphatase activity to ternary mixture toxicity ratios 20:40:40 of 2,4-D/Pb^{2+}/Cd^{2+}, 2,4-D/Zn^{2+}/Cr^{2+}, 2,4-D/Zn^{2+}/Cd^{2+}, 2,4-D/Cr^{2+}/Cd^{2+}. The solid lines represent sigmoidal, sigmoid 4 Parameter model

The dose response curve for unary toxicants, show the ranking of ecological risk of the heavy metals ions to phosphatase activity of *Chlorella vulgaris* 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 EC$_{50}$ of Chromium ion showed the highest toxicity with EC$_{50}$ value of 0.19±0.01mM. The experimental data points and model predicted dose- response data for inhibition of phosphatase activity of *Chlorella vulgaris* showed the enzyme was sensitive to chromium. The findings of Al-Hasawi *et al.,* (2020) supported the reported sigmoidal response of *C. vulgaris* phosphatase enzyme to chromium toxicity. Also, exposure of the phosphatase enzyme from *Chlorella vulgaris* to copper and zinc ions resulted in significant inhibition of phosphatase activity with 1C$_{50}$ of 0.28 0.01mM and 0.31 0.02mM respectively. Chromium and copper has been reported among the top five metal of greatest risk to freshwater ecosystem in Bohai region of China (Su *et al.,* 2017). The report on agro ecological responses of heavy metal pollution indicated that chromium causes significant toxicity to flora that forms an integral component of the ecosystem (Srivastava *et al.,* 2017). The presence of chromium (VI) reduced reactive oxygen species (ROS) from 1.6 fold to 1.1 fold at 0.5-5mMoIL^{-1}. 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.
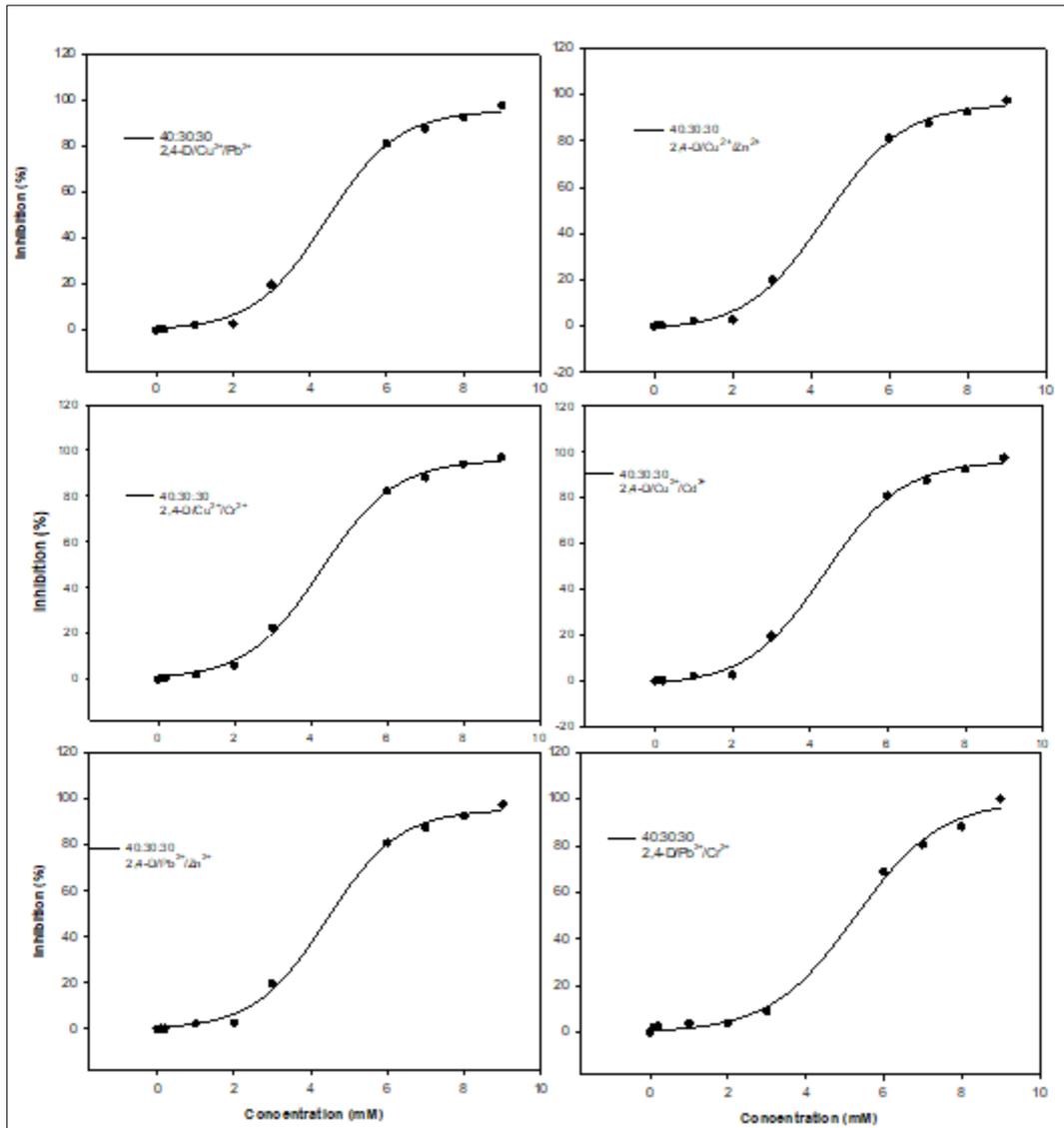
Figure 7 Experimental (data points) and model-predicted dose-response of *C. vulgaris* phosphatase activity to Ternary mixture toxicity ratio 40:30:30 of 2,4-D/Cu^{2+}/Pb^{2+},2,4-D/Cu^{2+}/Zn^{2+}, 2,4-D/Cu^{2+}/Cr^{2+},2,4-D/Cu^{2+}/Cd^{2+}, 2,4-D/Pb^{2+}/Zn^{2+} and 2,4-D/Pb^{2+}/Cr^{2+}. The solid lines represent Sigmoidal, Sigmoid 4 Parameter model
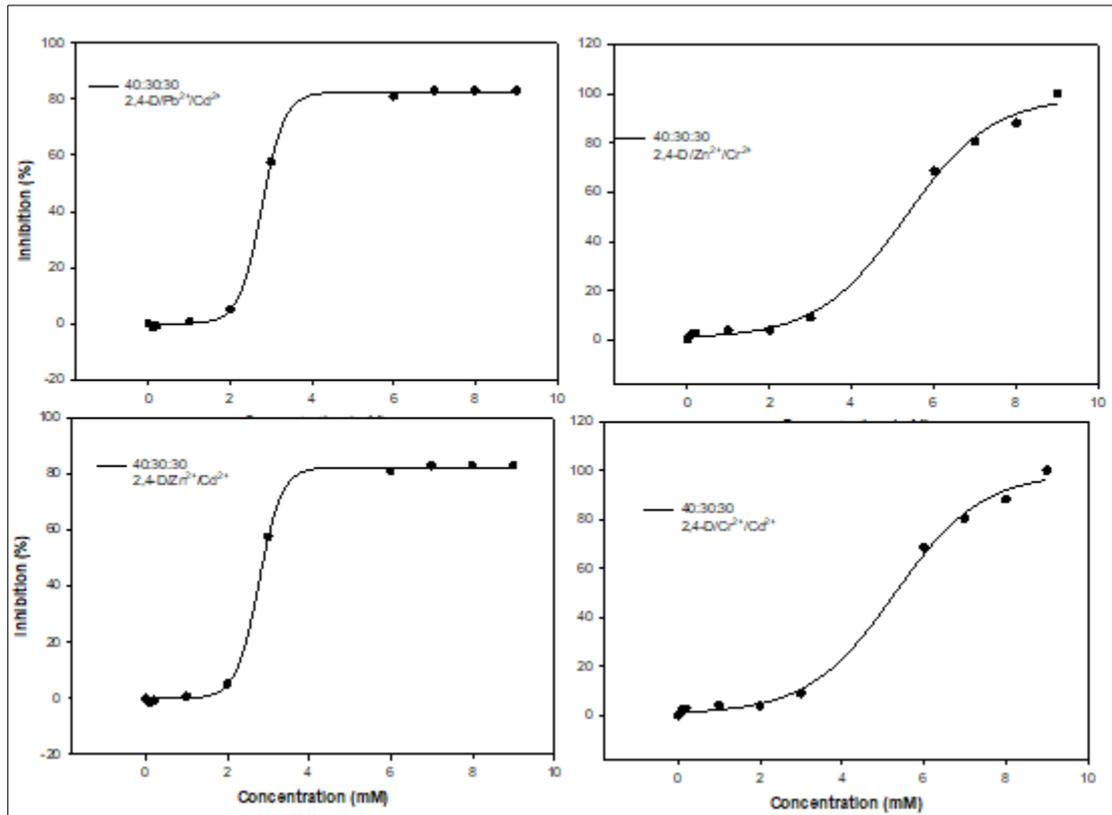
Figure 8 Experimental (data points) and model-predicted dose-response of *C. vulgaris* phosphatase activity to Ternary mixture toxicity ratio 40:30:30 of 2,4-D/Pb^{2+}/Cd^{2+}, 2,4-D/Zn^{2+}/Cr^{2+}, 2,4-D/Zn^{2+}/Cd^{2+}, 2,4-D/Cr^{2+}/Cd^{2+}. The solid lines represent Sigmoidal, Sigmoid 4 Parameter model

Table 2 Median inhibitory concentration (EC50) and toxic index of Unary and Ternary mixtures of metal/pesticide to phosphatase enzyme activity from Chlorella vulgaris

Toxicant	EC_{50} (mM)	Toxic Index	Interaction
Cu^{2+}	0.28 ±0.01[a]		
Zn^{2+}	0.31 ±0.02[a]		
Pb^{2+}	1.86±0.76[b]		
Cr^{2+}	0.19±0.01[a]		
Cd^{2+}	2.59±0.32[b]		
2,4-D	16.82±1.47[de]		
Toxicant Mixture (20:40:40)			
2,4-D/Cu^{2+}/Pb^{2+}	5.27±0.27[d]	8.72	Antagonism
2,4-D/Cu^{2+}/Zn^{2+}	4.41±0.25[cd]	12.04	Antagonism
2,4-D/Cu^{2+}/Cr^{2+}	4.14±0.19[c]	14.68	Antagonism
2,4-D/Cu^{2+}/Cd^{2+}	4.42±0.25[cd]	7.05	Antagonism
2,4-D/Pb^{2+}/Zn^{2+}	4.37±0.25[cd]	6.63	Antagonism
2,4-D/Pb^{2+}/Cr^{2+}	5.27±0.27[d]	12.29	Antagonism

2,4-D/Pb^{2+}/Cd^{2+}	2.77±0.03[b]	1.06	Additive
2,4-D/Zn^{2+}/Cr^{2+}	5.29±0.31[d]	18.03	Antagonism
2,4-D/Zn^{2+}/Cd^{2+}	2.77±0.03[b]	4.03	Antagonism
2,4-D/Cr^{2+}/Cd^{2+}	5.29±0.30[d]	12.02	Antagonism
Toxicant Mixture (40:30:30)			
2,4-D/Cu^{2+}/Pb^{2+}	4.42±0.25[c]	5.55	Antagonism
2,4-D/Cu^{2+}/Zn^{2+}	4.36±0.26[c]	8.99	Antagonism
2,4-D/Cu^{2+}/Cr^{2+}	4.28±0.20[c]	11.45	Antagonism
2,4-D/Cu^{2+}/Cd^{2+}	4.37±0.25[c]	5.29	Antagonism
2,4-D/Pb^{2+}/Zn^{2+}	4.42±0.25[c]	5.10	Antagonism
2,4-D/Pb^{2+}/Cr^{2+}	5.27±0.27[c]	9.30	Antagonism
2,4-D/Pb^{2+}/Cd^{2+}	2.77±0.03[b]	0.83	Synergism
2,4-D/Zn^{2+}/Cr^{2+}	5.29±0.31[c]	13.60	Antagonism
2,4-D/Zn^{2+}/Cd^{2+}	2.77±0.03[b]	3.07	Antagonism
2,4-D/Cr^{2+}/Cd^{2+}	5.29±0.30[c]	9.09	Antagonism

Mean with different superscript across columns are significant ($p < 0.05$).

The unary metal ions and pesticides with ternary mixtures of metal and pesticide to phosphatase enzyme activity of *Chlorella vulgaris* showed different levels of toxicity. The works of Su *et al.,* (2017) 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).

In a similar study, 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 to *Chlorella vulgaris* was observed by Exposito *et al.,* (2021). Furthermore, lead ion 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, cadmium indicated a progressive inhibition of *C. vulgaris* phosphatase activity; 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), and 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.

However the response curve of *C. vulgaris* phosphatase activity to 2,4-D toxicity showed the lowest toxicity with EC$_{50}$ 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, the ternary toxicity mixture of pesticide/metals, studied in their corresponding fixed percentage ratio of 20:40:40, inhibited phosphatase activity; the graphical interpolation of the dose-response curve of 20:40:40 toxicant ratio, shows that 2,4-D/Pb^{2+}/Cd^{2+} and 2,4-D/Zn^{2+}/Cd^{2+} were more toxic, followed by 2,4-D/Cu^{2+}/Cr^{2+}, 2,4-D/Pb^{2+}/Zn^{2+}, 2,4-D/Cu^{2+}/Zn^{2+} and 2,4-D/Cu^{2+}/Cd^{2+}. However, the remaining toxicant mixtures of 2,4-D/Cu^{2+}/Pb^{2+}, 2,4-D/Pb^{2+}/Cr^{2+},

2,4-D/Cr^{2+}/Cd^{2+} and 2,4-D/Zn^{2+}/Cr^{2+} were least toxic. The study of the ternary mixture experimental data points and model predicted dose-response data for inhibition of phosphates activity in C*hlorella vulgaris* exhibited largely sigmoidal relationship.

Similarly, the evaluated 40:30:30 ternary percentage ratio of metal/pesticides mixture displayed toxicity to phosphatase activity with 2,4-D/Pb^{2+}/Cd^{2+} and 2,4-D/Zn^{2+}/Cd^{2+} the most toxic. The decline in the trend in their toxicity strength was followed by 2,4-D/Cu^{2+}/Cr^{2+}, 2,4-D/Cu^{2+}/Zn^{2+}, 2,4-D/Cu^{2+}/Cd^{2+}, 2,4-D/Cu^{2+}/Pb^{2+} and 2,4-D/Pb^{2+}/Zn^{2+}; the least toxic were the 2,4-D/Pb^{2+}/Cr^{2+}, 2,4-D/Zn^{2+}/Cr^{2+}, 2,4-D/Cr^{2+}/Cd^{2+} ternary combinations. The analysis of the mixture interaction effect showed that the mixtures of heavy metals with 2,4, D were mostly antagonistic. The exceptions in toxicity trend seen in the 2,4-D/Pb^{2+}/Cd^{2+} and 2,4-D/Pb^{2+}/Cd^{2+} mixture may be attributed to the presence of two highly toxic heavy metals in the mixture. Toxic index analysis describes possible eco-toxicological risk of toxicants mixtures (Nwanyanwu *et al.,* 2017; Asiwe *et al.,* 2018; Nweke *et al.,* 2018, Nzeh *et al.,* 2019, Arua *et al.,* 2021). These studies described toxicity interaction of mixtures of chemicals, using toxic index, TI =1 describes additive interaction, TI > 1 describes antagonistic interaction and TI < 1 describes synergistic interaction.

The combination of 2,4D with the heavy metals showed a major diminution in the toxicity of the heavy metals; evidenced in an increased median inhibitory threshold concentration compared to the toxicity of heavy metals singly. This general trend in all the mixture ratios maybe attributed to differences in mode of action. While their joint action exhibits antagonistic toxicity to the heavy metals ions; it was an additive effect 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 20:40:40 and 40:30:30 demonstrated a progressive concentration dependent inhibition of enzyme activity.

5. Conclusion

The ternary mixtures of 2,4-D and metals in (20:40:40) / (40:30:30) exhibited a strong inhibitory effect against *Chlorella vulgaris* phosphatase enzyme activity. The presence of 2,4-D in both 20:40:40 and 40:30:30 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 it's in cocktails with diverse heavy metals ions.

Compliance with ethical standards

Disclosure of conflict of interest

There was no conflict of interest.

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