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Antagonistic and Additive Toxicity Assessment of Quinary Mixtures of Metal and 2,4-D Combinations on Algal Phosphatase Function

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Abstract

The present study comprehensively examined the impact of combined metal and pesticide mixtures on the activity of the hydrolytic phosphatase enzyme in the freshwater alga *Chlorella vulgaris*. Toxicological assessments entailed evaluating inhibition of phosphatase-mediated conversion of p-nitrophenylphosphate to p-nitrophenol, monitored spectrophotometrically at 410 nm. Both unary and quinary mixtures of Copper (Cu^{2+}), Zinc (Zn^{2+}), Lead (Pb^{2+}), Chromium (Cr^{2+}), Cadmium (Cd^{2+}), and 2,4-dichlorophenoxyacetic acid (2,4-D) were investigated at predetermined fixed molar ratios. Experimental and model-predicted dose-response analyses revealed that all tested heavy metals elicited concentration-dependent inhibitory effects on phosphatase activity. Notably, Cr^{2+} and Cu^{2+} demonstrated the highest toxicities with IC_{50} values of 0.19 ± 0.01 mM and 0.28 ± 0.00 mM, respectively, whereas 2,4-D manifested comparatively low inhibition, inducing approximately 20% enzymatic suppression. Quinary mixtures formulated at fixed ratios of 20:20:20:20:20 and 16:21:21:21:21 exhibited differential toxicological profiles wherein the 2,4-D/Pb^{2+}/Zn^{2+}/Cr^{2+}/Cd^{2+} mixture consistently presented the highest toxicity. Across the different mixtures, a general decline in toxicity was observed, yet toxicity saturation was reached, indicating uniform toxic effects at higher concentrations. Toxicity index evaluations predominantly signalled antagonistic interactions (TI >>1), aside from one mixture in the 20:20:20:20:20 ratio displaying additive interactions. The modulation of toxicity by 2,4-D within these cocktails notably attenuated metal toxicity yet concurrently amplified its own toxic effects. This dualistic modulation likely arises from differential modes of action, temporal exposure variances, and receptor site interactions among the toxicants. Collectively, the findings underscore the ecological risk posed by metal and pesticide co-exposures to freshwater microalgae, mediated through complex antagonistic and additive enzymatic inhibition mechanisms.

Keywords: Toxicological assessment; Enzymatic inhibition mechanisms; Dose-response analysis; IC_{50} values; Metal-pesticide interaction; Ecological risk to microalgae

1. Introduction

Chemical mixtures are combinations of two or more chemicals that maintain their individual chemical identities without alteration (European Commission, 2012). Despite most toxicity data used for chemical safety evaluations focusing on single compounds, real-world aquatic environments are typically exposed to mixtures rather than individual chemicals (Belfield *et al.,* 2023 in Yang *et al.,* 1998). Therefore, exposure to chemical mixtures is therefore the norm in environmental toxicology (Jongwoon *et al.,* 2022). Furthermore, traditional toxicity assessments may either evaluate

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the mixture as a whole (top-down approach) or analyze the individual components and their combined effects (bottom-up approach). Generally, chemical toxicity evaluations use the bottom-up method, where the toxicities of the separate components are modelled to predict the overall mixture effect (Hernandez, Gil, and Lacasana, 2017). A key challenge in assessing mixtures arises when individual chemicals are present at concentrations below their no-observed-effect level (NOEL), but in combination exhibit unexpected toxic effects (Jongwoon *et al.,* 2022; European Commission, 2012b). Predictive toxicity methods often rely on assumptions like concentration addition (CA) for chemicals with similar modes of action or independent action (IA) for those with different modes. In many aquatic ecosystems, organisms such as microalgae occupy an important position in energy trophic levels and are exposed to organic and inorganic chemical mixtures rather than single pollutants (Aronzon, Peluso, and Coll, 2020; Kovalakova *et al.,* 2020; Topaz *et al.,* 2020; Zhang *et al.,* 2021). The inhibition of phosphatase activity in these microalgae serves as a useful indicator for evaluating the effects of environmental toxicants (Nguyen, Moon, and Lee, 2020; Nunes *et al.,* 2018; Göbölös *et al.,* 2024; Onyeukwu and Edna, 2022a; 2022b; Wenq *et al.,* 2021). The effective concentrations of chemical mixtures can be quantified using the combination index (CI) method, and data analysis is often facilitated by sigma statistical plots. The responses for both individual compounds and mixtures are typically evaluated by metrics such as the median inhibitory concentration (MIC_{50}) or effective concentration (EC_{50}). Aside combination index method, quantitative structure property relationship (QSPR) model can be used to develop mathematical relationships that predict chemical's physical or chemical property based on its molecular descriptors. For example, QSPR models can used to predict thermodynamic properties such as thermal energy, entropy, and heat capacity of camptothecin drug derivatives, using molecular descriptors which is optimized by genetic algorithms and multiple linear regression methods. The model's predictive ability is said to be validated by cross-validation techniques to ensure accuracy in property prediction (Yue and Li, 2022; Ahmadinejad, *et al.,* 2018). Additionally, is the use of artificial neural networks combined with QSPR modelling to predict properties of chemical mixtures more accurately than experimental methods alone. These advanced QSPR models can help in predicting properties related to new biomass fuels, pollution analyses, or toxicological risks (Mu and Li, s2022). Quantitative structure-property relationship (QSPR) models serve as predictive tools to estimate the physicochemical properties of pure compounds and mixtures. To address gaps in QSPR predictions, new formulations and models have been developed to address some inconsistences in QSPR usage. Molecular descriptors used in these models can be constitutional, topological, geometric, or quantum chemical in nature. QSPR and QSAR models are increasingly applied in toxicology, ecotoxicology, pharmaceuticals, pharmacodynamics, pharmacokinetics, chemometrics, and physical-chemical property predictions (Hassold *et al.,* 2021; European Commission, 2020, 2012b; Hernandez *et al.,* 2017; WHO, 2017). A major difference between QSAR modelling of single chemicals and mixtures involves the types of molecular descriptors used, which are critical for accurately predicting toxicity, especially when dealing with nonlinear properties. Common mathematical techniques in QSAR/QSPR include Multiple Linear Regression (MLR), Partial Least Squares (PLS), Neural Networks (NN), and Support Vector Machines (SVM), which continue to be refined with advanced algorithms and hybrid methods. In this study, the focus is to evaluate both individual and combined effects of single and quinary mixtures of metal ions Copper (Cu^{2+}), Zinc (Zn^{2+}), Lead (Pb^{2+}), Chromium (Cr^{2+}), Cadmium (Cd^{2+}) alongside 2,4-dichlorophenoxyacetic acid on the alkaline phosphatase activity of *Chlorella vulgaris,* using a toxic index model. The interaction effects of mixtures based on concentration were assessed using the toxic index (TI), which sums the toxic units of each component (Boillot and Perrodin, 2008). The TU values were calculated using the expression: $TU_i = \frac{Cmix_i}{IC_{50I}}$ $TI = \sum_{i=1}^{n} TU_i$, While TI is the summation of TU for n toxicants in the mixture, where *Cmixi* is the concentration of the *i*th toxicant in the mixture and $EC_{50}i$ 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). The synergistic, additive, and antagonistic effects between the heavy chemicals and 2,4-dichlorophenoxyacetic acid in various mixtures at different effective concentrations were determined using the combination index (CI) method, aside other methods (Kahatagahawatte and Hara-Yamamura, 2020).

2. Material and methods

2.1. Sample area

Ihiagwa is a town situated within the Owerri West Local Government Area in Imo State, located in southeastern Nigeria. It lies approximately 12 kilometers south of Owerri, the state capital. The Otamiri River flows through Ihiagwa, with geographic coordinates at latitude 4°54'14.00" N and longitude 7°08'30.00" E. The river's watershed extends over roughly 10,000 square kilometers (3,900 square miles) and receives an annual rainfall ranging between 2,250 and 2,500 millimeters. The area is predominantly covered by depleted rainforest vegetation, with average temperatures hovering around 27°C (81°F) year-round. Unfortunately, the Otamiri River is subject to pollution from organic waste and chemicals, due to inefficient waste management system in Owerri which exacerbates the river's contamination. This pollution primarily results from intensive human activities, including household waste disposal, agricultural runoff,

failing drainage infrastructure, factory discharges, and various industrial operations. This has led to increased concentrations of pollutants such as phosphates, nitrates, metals, pesticides, and herbicides in the Otamiri River. Despite these challenges, the river serves as an important alternative source of drinking water when the public water supply is unavailable. To better understand the extent of pollution, the current study focuses on a large segment of the Otamiri River along the Nekede to Ihiagwa stretch. The geographical map of the study area is presented in Figure 1.

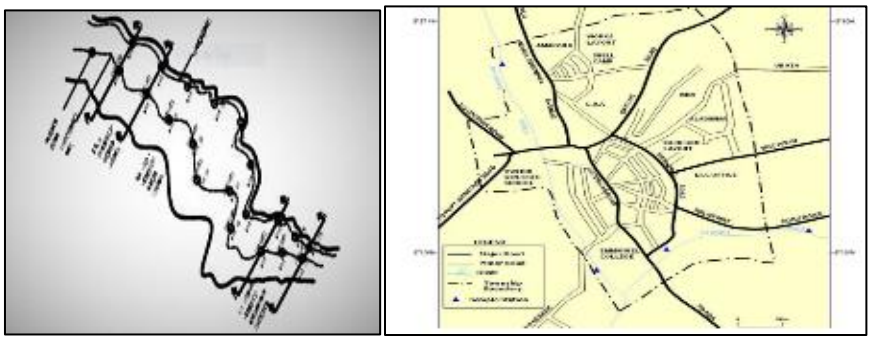

Figure 1 Digital Geographical map of Otamiri River along the Nekede -. Ihiagwa stretch

2.2. Sampling

2.2.1. Sampling Material

Sterile glass bottles, with capacity of 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. 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.

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 126^{0}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); KH_2PO: 48.75g/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: (optional: cyanocobalamin, biotin, thiamine) (Nichols and Bold, 1965).

2.5.2. Culture of isolate

An aliquot 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^{0}C). Thereafter, the culture was transferred into a broth inoculated with bold basal medium contained in 250 ml 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±2^{0}C), under white fluorescent light (3000-4000) lux, on a rotary shaker. New stock culture was initiated at 4^{0}C in the dark, in every 40-60 days, by inoculating approximately 5×104 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^{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.

2.6. Morphological Identification of Isolated organism

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 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 10,000x*g* for 1 minute. Four hundred (4s00) microlitre of supernatant was transferred to a Zymo Spin IV spin Filter (orange top) in a collection tube and centrifuged at 7000x*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 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.

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, 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.

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 96ºC for 10s, 55ºC for 5s and 60ºC 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

Phosphatase extraction followed the method described by Jonsson and Aoyama (2007). Algal pellets identified molecularly were frozen in liquid nitrogen and macerated in a mortar with acetate buffer. The mixture was subjected to freeze-thaw cycles by storing at −20°C and thawing at room temperature to produce a 1:4 (w/v) suspension. Cell disruption was achieved by probe sonication on ice (0°C) with 50 seconds sonication followed by 20 seconds intervals (1 cycle) at 70% amplitude; repeated twice. The lysate was centrifuged at 10,000 rpm for 20 min. Afterwards, the extract from supernatant was assayed for the conversion of colourless p-nitrophenylphosphate to yellow, p-nitrophenol. The supernatant containing the enzyme extract was identified as alkaline phosphate using litmus indicator and stored to be used for phosphatase assays.

2.8. Toxicity of Unary-Quinary mixtures of metals and pesticide to *Chlorella 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 from 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). Quinary mixtures of pesticide/metal were amalgamated in a simple percentage ratio of 20:20:20: 20:20 and 16:21:21: 21:21 for the quinary combinations' ratios. Their toxicities were determined at concentrations from 0-9.0mM. Inhibitory study on the alkaline phosphate (ALP) activity was determined in 3ml reaction final volume consisting of the graded concentration of single or quinary 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 consists of set-ups 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 (37ºC). 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. Statistical Analysis

2.9.1. Estimation of relative response, Median Inhibitory Concentration of Unary- Quinary Combinations of Metals and Pesticide and Modeling of Data from the Inhibition analysis.

The relative responses were evaluated as percentage relative test to control, due to inhibition of the toxicants and fitted model equations (Figure 2),

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

Figure 2 Relative responses

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-**quinary** 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 *Chlorella vulgaris* was generated from the models. The fitted models are also elaborated with their equations below (Figure 3).

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

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

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

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

Figure 3 Ppercentage relative control inhibition and fitted model equations

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 as shown in figure four:

$$TU_i = \frac{Cmix_i}{IC_{50I}} \quad (6)$$

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

$$TI = \sum_{i=1}^{n} TU_i \quad (7)$$

$$Cmix_i = \frac{Ratio}{100} IC_{50I}mix \quad (8)$$

$$y = \frac{a}{1 + \left(\frac{x}{b}\right)^c} \quad (9)$$

$$y = a + \frac{b}{1 + \left(\frac{x}{c}\right)^d} \quad (10)$$

$$y = a\left[1 - -exp\left[-\left[\frac{x + c\ (\ln 2)^{1/d} - b}{c}\right]^d\right]\right] \quad (11)$$

Figure 4 Toxicity exponent model equations

Where *Cmixi* is the concentration of the *i*th toxicant in the mixture and IC_{50I} or $EC_{50}i$ 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). 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 R1(AY591506).

The morphological traits of isolate R1(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 R1(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 and phylogenetic tracing with descriptions of R1(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

Table 1 Morphological and biochemical screening assay of *Chlorella vulgaris*

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	++
Species: Chlorella vulgaris	

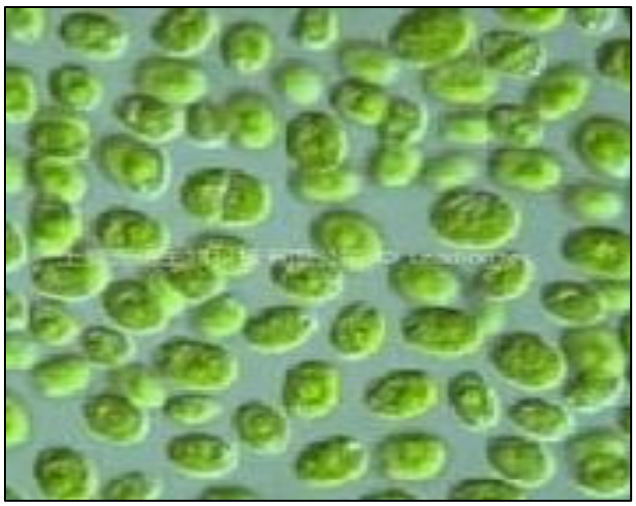

Figure 5 Schematic Microscopic structure of *Chlorella vulgaris*

3.2. Molecular (Genome) Identification of the Isolate R1(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 (**R1(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

Figure 6 Phylogenetic tree showing the isolate *Chlorella vulgaris*

3.3. Toxicity of Individual Metals and 2,4-D on *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 2.0.

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

Toxicants	IC_{50} (mM)	Toxicity index	Interaction
Unary (100%)			
Cu^{2+}	0.28 ± 0.01^c	-	Toxic
Zn^{2+}	0.31 ± 0.02^c	-	Toxic
Pb^{2+}	1.86 ± 0.76^c	-	Toxic
Cr^{2+}	0.19 ± 0.01^c	-	Toxic
Cd^{2+}	2.59 ± 0.32^c	-	Toxic
2,4-D	16.82 ± 1.47^c	-	Toxic

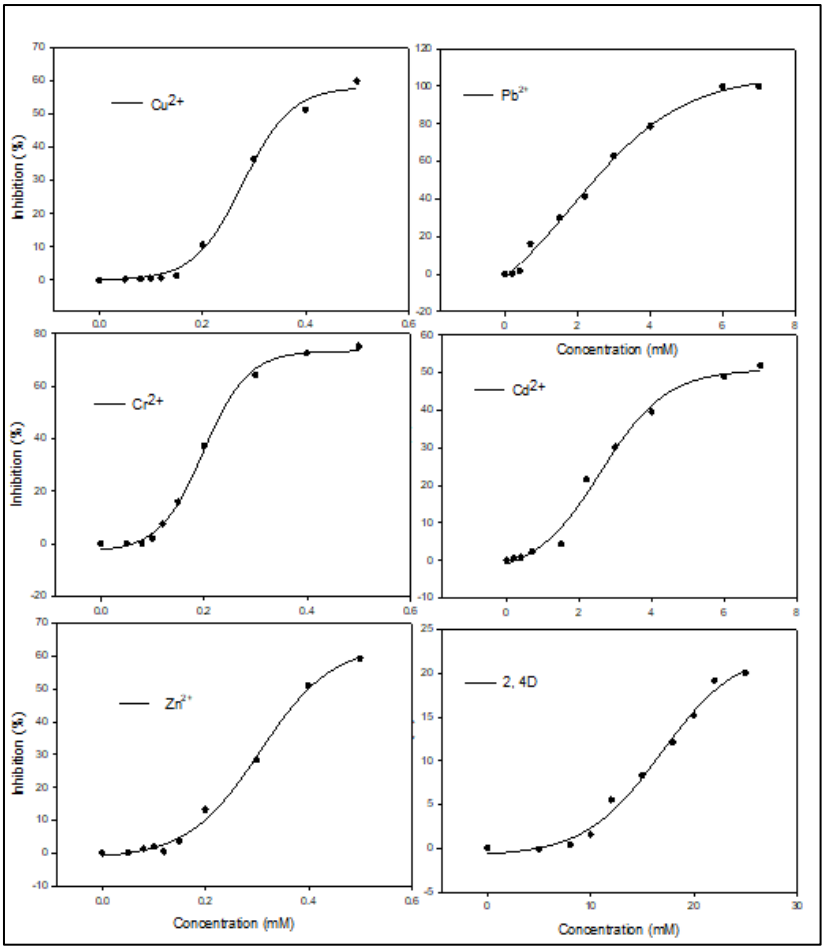

Figure 7 Experimental (data points) and model-predicted dose-response data for unary inhibition of phosphatase activity of *Chlorella vulgaris* by copper, lead, chromium, cadmium, zinc ion and 2,4-D.using Sigmoidal, Sigmoid, 3 Parameter model

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

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

Toxicant: mixture	IC_{50} (mM)	Toxicity index TI	Interaction a > 0
Quinary mixture ratio ((20:20:20: 20:20)			
2,4-D/Cu^{2+}/Pb^{2+}/Zn^{2}/Cr^{2+}	5.99±0.04^{c}	15.16±0.76^{a}	Antagonism
2,4-D/Pb^{2+}/Zn^{2+}/Cr^{2}/Cd^{2+}	5.29±0.22^{c}	13.39±0.69^{a}	Antagonism
2,4-D/Zn^{2+}/Cr^{2+}/Cd^{2}/Cu^{2+}	5.55±0.19^{c}	14.04±0.70^{a}	Antagonism
2,4-D/Cr^{2+}/Cd^{2+}/Cu^{2+}/Pb^{2+}	5.65±0.24^{c}	14.30±0.71^{a}	Antagonism
2,4-D/Cd^{2+}/Cu^{2+}/Pb^{2+}/Zn^{2+}	5.86±0.16^{c}	14.33±0.74^{a}	Antagonism
Quinary mixture ratio (16:21:21: 21:21)			
2,4-D/Cu^{2+}/Pb^{2+}/Zn^{2}/Cr^{2+}	5.95±0.13^{c}	15.79±0.78^{a}	Antagonism
2,4-D/Pb^{2+}/Zn^{2+}/Cr^{2}/Cd^{2+}	4.19±0.74^{c}	11.12±0.55^{a}	Antagonism
2,4-D/Zn^{2+}/Cr^{2+}/Cd^{2}/Cu^{2+}	6.84±0.22^{c}	18.16±0.90^{a}	Antagonism
2,4-D/Cr^{2+}/Cd^{2+}/Cu^{2+}/Pb^{2+}	5.80±0.13^{c}	15.36±0.76^{a}	Antagonism
2,4-D/Cd^{2+}/Cu^{2+}/Pb^{2+}/Zn^{2+}	6.11±0.07^{c}	16.22±0.81^{a}	Antagonism

The results presented in figure 6-7 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.

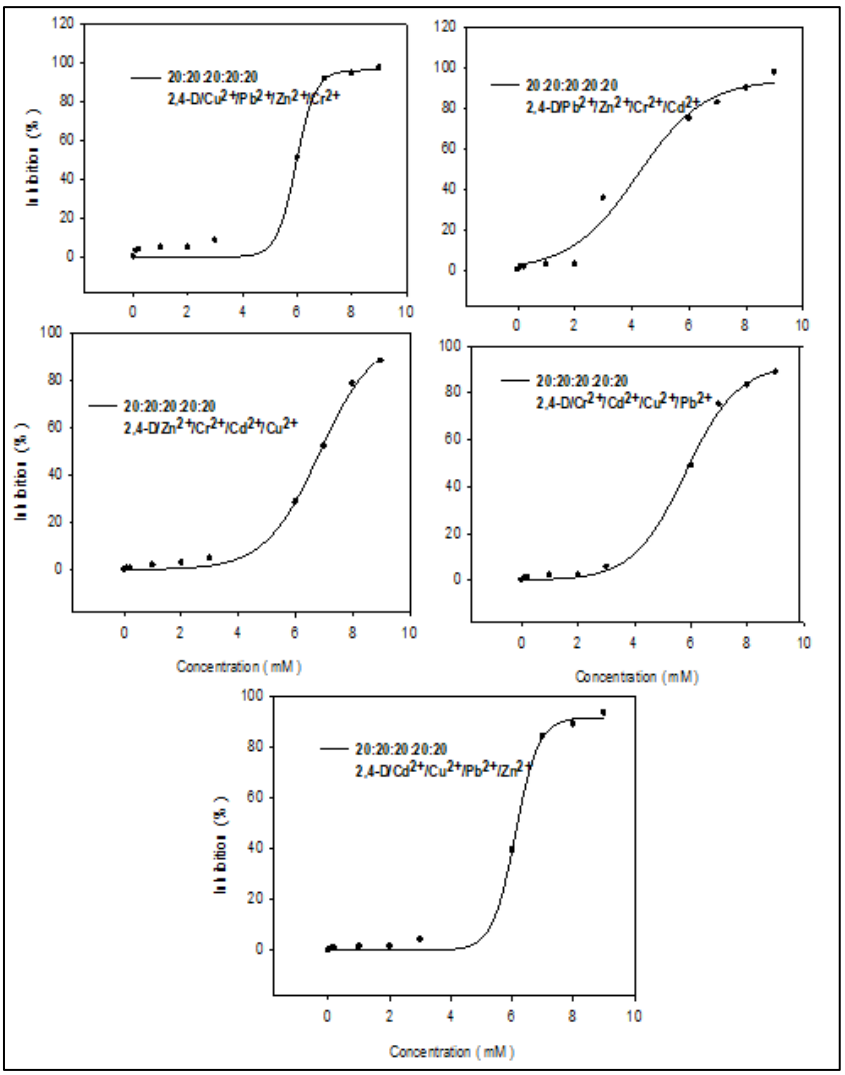

Figure 8 Experimental (data points) and model-predicted dose-response of *Chlorella vulgaris* phosphatase activity to Quinary mixture toxicity ratios 16:21:21:21:21 of 2,4-D/Cu^{2+}/Pb^{2+}/Zn^{2+}/ Cr^{2+}:2,4-D/Pb^{2+}/Zn^{2+}/Cr^{2+}/Cd^{2+}:2,4-D/Zn^{2+}/Cr^{2+}/Cd^{2+}/Cu^{2+}:2,4-D/Cr^{2+}/Cd^{2+}/Cu^{2+}/Pb^{2+}:2,4-D/Cd^{2+}/Cu^{2+}/Pb^{2+}/Zn^{2+}.The solid lines represent sigmoidal, sigmoid 4 Parameter model

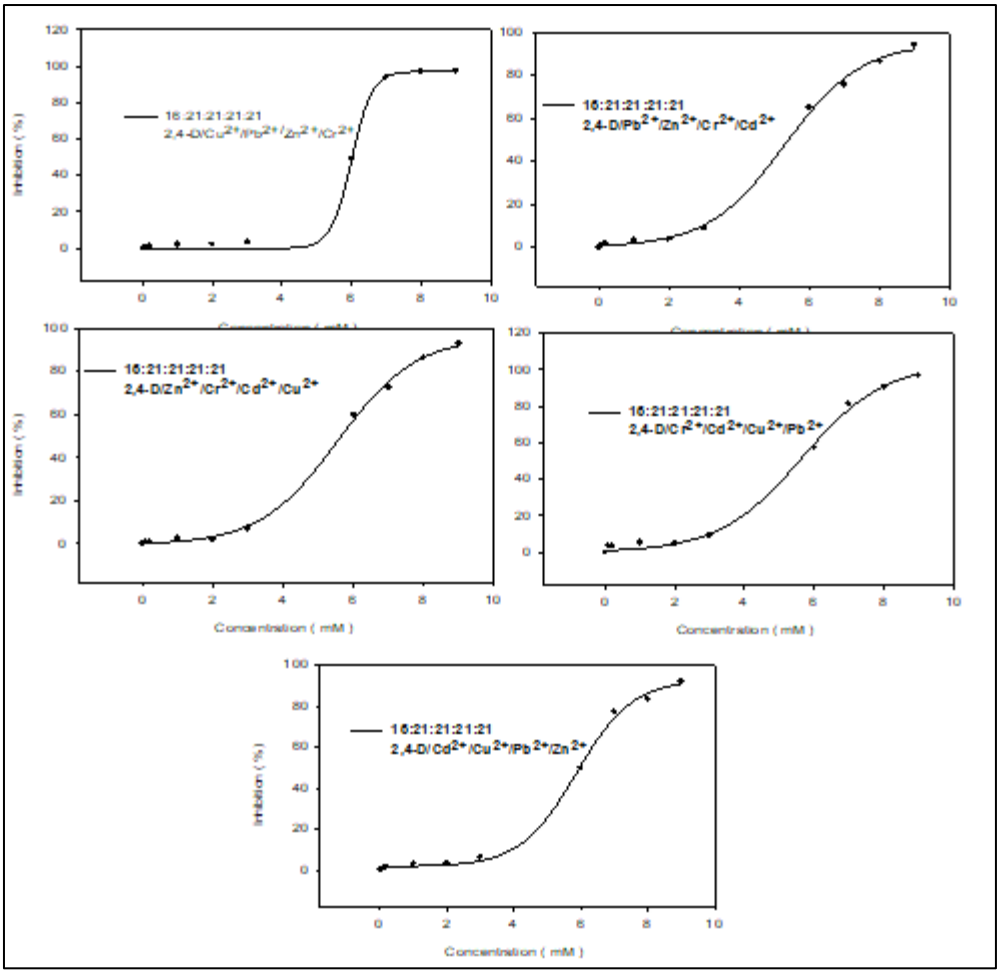

Figure 9 Experimental (data points) and model-predicted dose-response of *Chlorella vulgaris* phosphatase activity to Quinary mixture toxicity ratios 20:20:20:20:20 of 2,4-D/Cu^{2+}/Pb^{2+}/Zn^{2+} /Cr^{2+}:2,4-D/Pb^{2+}/Zn^{2+}/Cr^{2+}/Cd^{2+}:2,4-D/Zn^{2+}/Cr^{2+}/Cd^{2+}/Cu^{2+}:2,4-D/Cr^{2+}/Cd^{2+}/Cu^{2+}/Pb^{2+} :2,4-D/Cd^{2+}/Cu^{2+}/Pb^{2+}/Zn^{2+}.The solid lines represent sigmoidal, sigmoid 4 Parameter model

4. Discussion

4.1. Morphological and biochemical identification of the isolate R1(AY591506).

The numerated results of the morphological and biochemical characteristics of *Chlorella vulgaris* showed in table 1, 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;). The organism showed many structural elements similar to plants. It has a unilaminar cell wall with subspherical or ellipsoidal shape without flagella. The internal features of the organism also comprises of a single chloroplast with a double enveloping membrane composed of phospholipids, which is in line with the study by Yamamoto *et al.* (2004).

4.2. Molecular (Genome) identification of the isolate

From the molecular (Genome) identification of the Isolate: *Chlorella vulgaris* R1(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***.**

4.3. Unary-Quinary mixture toxicity of (metal/pesticide) to phosphatase enzyme activity *Chlorella vulgaris*

The result of the effect of unary toxicity 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 $Cr^{2+} > Cu^{2+} > Zn^{2+} > Pb^{2+} > Cd^{2+} >$ 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, which is also illustrated 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 China, 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 IC50 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).

The concentration values of the respective ratio of 16:21:21:21:21 Quinary metal/pesticides mixtures, showed the median ecotoxicity concentration EC_{50} effect to the hydrolytic alkaline phosphatase activity in *Chlorella vulgaris* were: (2,4-D/Cu^{2+}/Pb^{2+}/Zn^{2+}/Cr^{2+}:(5.99±0.04),(2,4-D/Pb^{2+}/Zn^{2+}/Cr^{2+}/Cd^{2+}:(5.29±0.22),(2,4-D/Z
n^{2+}/Cr^{2+}/Cd^{2}/Cu^{2+}:(5.55±0.19),(2,4-D/Cr^{2+}/Cd^{2+}/Cu^{2+}/Pb^{2+}:(5.65±0.24),(2,4-D/Cd^{2+}/ Cu^{2+}/Pb^{2+}/Zn^{2+}:(5.86±0.16)mM. Similarly, the various mixtures in the ratio of 20:20:20:20:20 Quinary metal/pesticides mixtures to phosphatase activity from *Chlorella vulgaris* were distinct: 2,4-D/Cu^{2+}/Pb^{2+}/Zn^{2+}/ Cr^{2+}:(5.95±0.13),2,4-D/Pb^{2+}/ Zn^{2+}/Cr^{2+}/Cd^{2+}:(4.19±0.74),2,4-D/Zn^{2+}/Cr^{2+}/Cd^{2+}/Cu^{2+}:(6.84±0.22),2,4-D/Cr^{2+}/Cd^{2+}/ Cu^{2+}/Pb^{2+}:(5.80±0.13),2,4-D/Cd^{2+}/Cu^{2+}/Pb^{2+}/Zn^{2+}:(6.11±0.07) and as shown in Table 3. The underlying analysis of the mixture interaction effect showed that the mixtures in fixed percentage ratio: 16:21:21:21:21 in *Chlorella vulgaris*, of heavy metals with 2,4-D were all antagonistic with toxic index (TI) >>1.The toxicity sequence due to quinary mixture in fixed percentage ratio: 16:21:21:21:21 of *Chlorella vulgaris*, exhibited the trend in the following order: 2,4-D/Pb^{2+}/Zn^{2+}/Cr^{2+}/ Cd^{2+} >2,4-D/Zn^{2+}/Cr^{2+}/Cd^{2+}/Cu^{2+} >2,4-D/Cr^{2+}/Cd^{2+}/Cu^{2+}/Pb^{2+} >2,4-D/Cd^{2+}/Cu^{2+}/Pb^{2+}/Zn^{2+}>2,4-D/Cu^{2+}/Pb^{2+}/Zn^{2+}/Cr^{2+}. In a similar setup, the computational effect due to the analysis of the mixture interaction demonstrated the toxicity mixtures in fixed percentage ratio: 20:20:20:20:20 against the hydrolytic alkaline phosphatase from *Chlorella vulgaris*, were

antagonistic in the following mixtures: 2,4-D/Cd^{2+}/Cu^{2+}/Pb^{2+}/Zn^{2+}, 2,4-D/Zn^{2+}/Cr^{2+}/Cd^{2+}/Cu^{2+} and 2,4-D/Cr^{2+}/Cd^{2+}/ Cu^{2+}/Pb^{2+} with toxic index (TI) >>1. The rest of the mixtures were antagonist in effect to the quinary combinations. The toxicity pattern of the antagonistic effect was traced with the amalgamation of 2,4-D. The toxicity due to mixture ranking in fixed percentage ratio: 20:20:20:20:20 of *Chlorella vulgaris*, as conducted in the analysis was recorded in the following order: 2,4-D/Pb^{2+}/Zn^{2+}/Cr^{2+}/ Cd^{2+}>2,4-D/Cr^{2+}/Cd^{2+}/Cu^{2+}/Pb^{2+}>2,4-D/ Cu^{2+}/Pb^{2+}/Zn^{2+}/Cr^{2+}>2,4-D/Cd^{2+}/Cu^{2+}/Pb^{2+}/Zn^{2+}>2,4-D/Zn^{2+}/Cr^{2+}/Cd^{2+}/Cu^{2+}. Hence, The effect of the quinary toxicity effects for the micro-alga was consistent with the works of (Adnan, *et al.,* 2021; Chauhan, *et al.,* 2021; Li *et al.,* 2020; Carterina, *et al.,* 2021 and Houssou, *et al.,* 2020). The results provides an undeviating relationship with the findings of Zeb, *et al.* (2017). Contrastingly, 2,4 dichlorophenoxyacetic acid acted as chelating agent to some of metal mixtures. It therefore influences an additive effect as opined by (Nikiforov-Nikishin, *et al.,* 2021).

5. Conclusion

The quinary mixtures of 2,4-dichlorophenoxyacetic acid (2,4-D) and metals in 16:21:21:21:21 / 20:20:20:20:20 exhibited a strong inhibitory effect against *Chlorella vulgaris* phosphatase enzyme activity. All mixtures were uniformly toxic. The increase in the cocktails, especially with 2,4-D plummeted the degree of toxicity. Probably a toxicity saturation point was attained. Therefore, 2,4-dichlorophen oxyacetic acid (2,4-D) in both ratios, 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, the use of 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

Acknowledgments

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Disclosure of conflict of interest

There was no conflict of interest.

Statement of Ethical approval

Although ethical approval was not required for this study because it involved minimal risk to participants, however, all procedures were conducted in accordance with ethical guidelines

Statement of informed consent

All participants were fully informed about the study's purpose, procedures, risks, and their rights before participation.

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