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Ratio-Dependent Inhibition of Alkaline Phosphatase by Heavy Metals and 2,4-D in Freshwater Microalgae

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Abstract

This study examined the combined toxic effects of heavy metals and the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) on alkaline hydrolytic phosphatase activity in the freshwater microalga Chlorella vulgaris. Toxicity was assessed by measuring inhibition of the enzyme's ability to convert p-nitrophenyl phosphate to p-nitrophenol at an optical density of 410 nm. Unary and senary mixtures of Copper (Cu^{2+}), Zinc (Zn^{2+}), Lead (Pb^{2+}), Chromium (Cr^{2+}), Cadmium (Cd^{2+}), and 2,4-D were tested at fixed molar ratios. All single metal ions caused concentration-dependent inhibition, with toxicity ranking as Cr^{2+} > Cu^{2+} > Zn^{2+} > Pb^{2+} > Cd^{2+} > 2,4-D. The most potent inhibitors were Cr^{2+} and Cu^{2+}, were the most toxic, with IC50 values of 0.19 ± 0.01 mM and 0.28 ± 0.00 mM, respectively. In contrast, 2,4-D alone showed a relatively low inhibitory effect, with only 20% suppression of enzyme activity. Senary mixtures prepared at two fixed molar ratios (10:18:18: 18:18:18 and 50:10:10: 10:10:10 for 2,4-D, Cu^{2+}, Pb^{2+}, Zn^{2+}, Cr^{2+}, Cd^{2+}) revealed variable toxicity, with the first ratio showing higher inhibition concentration indicating ratio-dependent combinatorial effects. The mixtures exhibited mainly additive and antagonistic toxicity interactions. Toxicity index values above 1 indicate antagonism, meaning the combined toxicity was less than expected. The presence of 2,4-D reduced the heavy metals' toxicity but increased its own toxicity, demonstrating complex modulation effects within the mixture. This suggests distinct molecular pathways by which metals and 2,4-D interact with biological targets, influencing overall toxic outcomes. This is likely due to complex interactions affecting modes of action and target receptors. Overall, the study shows that combined exposure can pose ecological risks to freshwater microalgae in a mixture composition ratio.

Keywords: Unary-Senary Mixtures; Combinatorial Effect; Modulation; Microalgae; Ecological Risks Alkaline Hydrolytic Phosphatase; Ecotoxicology

1. Introduction

The toxicological effects of chemical contaminants on algal populations critically modulate ecosystem structure and function, precipitating hypoxic conditions and diminished primary production (Bashir, et al., 2020). Due to their

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heightened susceptibility to xenobiotics, algae serve as sensitive bioindicators, sentinel species, and biomarkers for assessing ecotoxicological, genotoxic, and environmental risk profiles of pollutants in both terrestrial and aquatic matrices (Bashir, et al., 2020). Environmental risk assessments in aquatic systems integrate qualitative and quantitative analyses of individual contaminants to elucidate ecological perturbations (O'Brien and Keough, 2014; Schuijt et al., 2021; Jaegwon et al., 2022). The prevalence of complex chemical mixtures in aquatic milieus, resulting from industrial effluents, municipal waste discharges, and agrochemical runoff, necessitates mechanistic evaluation of mixture toxicity effects, including synergism, antagonism, additive, or reductive interactions which is relative to single-compound toxicodynamic (Carole et al., 2018; Nunes et al., 2018; Onyeukwu and Edna, 2022a, b). Agrochemicals employed for aquaculture disease management are often co-present with multiple xenobiotics, underscoring the need for integrated ecotoxicological assessments of such chemical conglomerates to elucidate combined toxicity mechanisms (Vanessa et al., 2023). These Argo-pesticides predominantly infiltrate environmental compartments via agricultural and horticultural applications. Comprehensive toxicity profiling of all mixture permutations is prohibitively complex, exacerbated by the capacity of some constituents to potentiate mixture toxicity at concentrations below their no observed effect concentration (NOEC) thresholds (Jong Woon et al., 2022; Altenburger, Nendza, and Schüürmann, 2003). As a result, microorganisms inhabiting aquatic ecosystems face chronic co-exposure to diverse organic and inorganic pollutants rather than isolated chemicals (Aaronson, Peluso and Coll, 2020; Kovalevas et al., 2020; Topaz et al., 2020; Zhang et al., 2021). Microalgal species, as principal producers within primary trophic levels, exhibit acute sensitivity to chemical pollutants and associated physicochemical perturbations, making them robust toxicity bioassay models due to their rapid response dynamics (Martínez-Ruiz and Martínez-Jerónimo, 2015; Nguyen, Moon and Lee, 2020; Nunes et al., 2018). Beyond inhibiting phosphatase activity, chronic exposure to complex pollutant mixtures can induce genotoxic, mutagenic, carcinogenic, teratogenic, immunosuppressive, and endocrine-disrupting effects. The pervasive presence of pesticides, heavy metals, and pharmaceutical residues demands focused investigation due to their multifaceted biological impacts (Göbölös et al., 2024). Biochemical and physiological biomarkers such as the inhibition of hydrolytic alkaline phosphatase represent critical endpoints for ecotoxicological evaluation (Onyeukwu and Edna, 2022a, b; Wenq et al., 2021). Given the exponential complexity inherent in experimentally testing all potential chemical mixture toxicity ratios, computational modelling emerges as an indispensable tool for elucidating mixture toxicodynamic. However, extant models exhibit limitations in predictive accuracy and practical applicability. This investigation evaluates the acute toxicity of individual and quaternary mixtures of Cu^{2+}, Zn^{2+}, Pb^{2+}, Cr^{2+} and Cd^{2+} ions, combined with 2,4-dichlorophenoxyacetic acid, on alkaline phosphatase activity inhibition in the microalga Desmosomes abundant. Toxicological impacts were quantified via hydrolytic alkaline phosphatase inhibition bioassays. Interaction effects such as synergistic, additive, or antagonistic across different effective concentrations were elucidated using the Combination Index (CI) methodology alongside complementary analytical approaches (Kahatagahawatte and Hara-Yamamura, 2020; Roell et al., 2017). The contributory effects of each constituent within mixtures were further dissected through sigma statistical metrics. Dose-response data for single compounds and mixtures were derived using relative response estimation, median inhibitory concentration (MIC_{50}), and effective concentration (EC_{50}) metrics. Predictive toxicological modeling employed statistical and Mattox frameworks incorporating an interaction parameter 'a', where $a<0$ denotes synergism and $a>0$ indicates antagonism (Onyeukwu and Edna, 2022a, b; Greco et al.,1992; Greco, Bravo and Parsons, 1995; Jonker et al.,2005). These models advance mixture modulation and mechanistic understanding of chemical interaction patterns across complex mixture scenarios (Kolmar and Grulke, 2021; Belfield et al., 2023).

2. Material and Methods

2.1. Sample Area

The study area encompasses a section of the Tamira River within the Owerri West Local Government Area of Imo State, southeastern Nigeria. Located approximately 12 kilometers south of Owerri, the state capital, the river passes through the community of Phagwa. Geospatial coordinates for the sampling site are approximately 4°54'14" N latitude and 7°08'30" E longitude. The Tamira River basin covers an expanse near 10,000 km^2, experiencing annual precipitation between 2,250 and 2,500 mm. The regional climate maintains a steady mean temperature around 27°C, characterized by a predominantly degraded rainforest biome. Anthropogenic influences heavily impact the river's water quality, with pollutants originating from domestic discharges, agricultural runoff, and industrial effluents. These contaminants manifest as elevated levels of phosphates, nitrates, heavy metals, pesticides, and herbicides, which collectively degrade the river's ecological integrity. The Tamira River is also a crucial supplementary water supply during interruptions in municipal water delivery, amplifying concerns about potential human health risks linked to contaminant exposure. Notably, inadequate waste management practices in the adjacent urban area of Owerri contribute significantly to river pollution. Accordingly, this investigation targeted the Tamira River segment extending from Nakade to Phagwa to assess the environmental impact of these combined stressors. A cartographic representation of the study zone is provided in Figure 1.

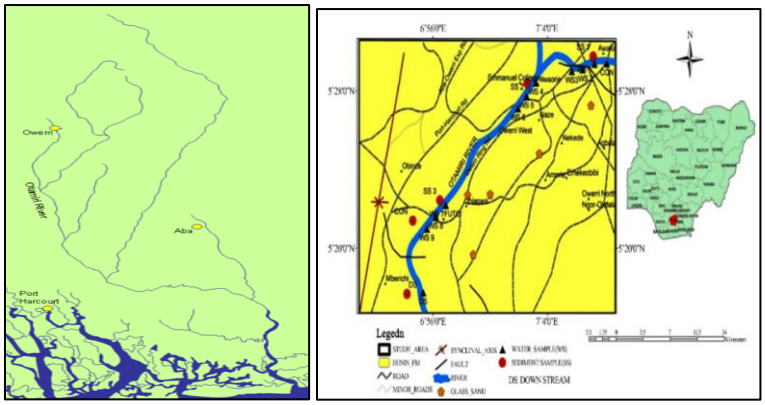

Figure 1 Cartographic representation of the study zone of Tamira River

2.2. Sampling Protocol

Sterile borosilicate glass bottles (≥200 mL) fitted with ground-glass or screw caps lined with silicone rubber resistant to sterilization at 160°C were employed for water sample collection. To prevent contamination, bottle necks and closures were wrapped with sterilized aluminum foil. Following autoclaving, bottles were aseptically sealed and stored until use. Sampling was performed at 10-minute intervals per site, monitored with a stopwatch by a field assistant. Figure one illustrates volumetric flow for the sampled river section. According to the Imo State Water Corporation, intake for treatment occurs upstream, whereas downstream activities include fishing and sand mining.

2.3. Sample Collection and Transport

Water samples were collected aseptically from midstream locations along the Tamira River at upper (5°24'0.32"N, 7°0'0.036"E), middle (5°24'0.55"N, 7°0'0.36"E), and lower (5°23'0.20"N, 6°59'0.39"E) points at a depth of 30 cm. Samples were pooled into sterile 1 L plastic or 200 mL glass bottles, placed immediately in insulated coolers, and transported to the laboratory for analysis within six hours to preserve sample integrity.

2.4. Isolation and Cultivation of Algal Strains

Collected samples were refrigerated during transport and processed within six hours. Algal cells were concentrated through successive washing or centrifugation and inoculated onto agar plates. Pure cultures were isolated and maintained as axenic stocks under standard microbiological conditions over 2–3 weeks prior to toxicity assays. Algal abundance was quantified in colony-forming units per milliliter (CFU/mL).

2.5. Algological Procedures

2.5.1. Agar Medium Preparation

Bold's Basal Medium (BBM) supplemented with 2% (w/v) agar was autoclaved at 126°C for 15 minutes. After cooling, plates were poured with 5 mL medium, inverted, and dried for 72 hours before usage. The medium was enriched with trace metal and F/2 vitamin solutions as described by Stein (1973) and Nichols and Bold (1965).

2.5.2. Algal Culture Maintenance

A 12 mL aliquot of washed, centrifuged algal samples was centrifuged at 3000 rpm for 15 min, resuspended in sterile water with vertexing, and this washing cycle was repeated six times to minimize contaminants (Parvin and Habib, 2007). Isolates were streaked onto BBM agar supplemented with antibacterial and antifungal agents, incubated at 28±2°C under fluorescent light for two weeks, then transferred to liquid BBM for continuous exponential growth maintenance.

2.5.3. Subculture and Stock Preservation

Axenic cultures were sub cultured in OECD (1981); recommended inorganic liquid medium. Broth cultures were incubated at 20±2°C under white fluorescent light (3000-4000 lux) on a rotary shaker. Stock cultures were refreshed every 40-60 days by inoculating approximately 5×104 cells mL−1 and stored at 4°C in darkness (Jonsson and Aoyama, 2007).

2.5.4. Inoculum Standardization

A loopful from BBM slants was inoculated into 200 mL BBM broth, incubated at 28±2°C with 150 rpm shaking for 24 h. Cells were harvested by centrifugation (3000 rpm, 10 min), washed twice with sterile distilled water, and standardized spectrophotometrically to an optical density of 1.8 at 600 nm.

2.6. Morphological Characterization

Colony morphology (color, form, transparency, diameter), pigmentation (chlorophyll a/b), and physiological traits including catalase, phosphatase, starch, and lipase activities were assessed following Stein (1973).

2.7. Molecular Identification Procedures

2.7.1. DNA Extraction

Genomic DNA was isolated using the ZR fungal/algal/bacterial DNA MiniPrep kit (Inqaba, South Africa) via bead-beating for cell lysis, followed by spin column purification. DNA was eluted in 100 µL buffer and stored at -20°C.

2.7.2. DNA Quantification

DNA concentration was measured using a NanoDrop 1000 spectrophotometer with 2 µL samples after blanking with sterile distilled water and normal saline.

2.7.3. 18S rRNA Gene Amplification

PCR amplification of the 18S rDNA utilized primers 18S C-2 b (5‘-ATTGGAGGGCAAGTCTGGT-3‘) and 18S D-2 b (5'-ACTAAGAACGGCCATGCAC-3'). Cycling parameters included initial denaturation at 95°C for 5 min; 35 cycles of 95°C for 30 s, 53°C for 30 s, 72°C for 30 s; and a final extension at 72°C for 5 min. Amplicons were visualized on 1% agarose gels under blue light.

2.7.4. Sequencing

Sanger sequencing was performed using the BigDye Terminator v1.1/v3.1 kit on an ABI 3510 sequencer (Inqaba Biotechnological, Pretoria). Reaction volumes were 10 µL, with cycling at 96°C (10 s), 55°C (5 s), and 60°C (4 min) for 32 cycles.

2.7.5. Phylogenetic Analysis

Sequences were edited with Trace Edit software and subjected to BLASTN searches against NCBI databases. Alignments were conducted using MAFFT, and phylogenies inferred by the Neighbor-Joining method in MEGA 6.0 with bootstrap support (500 replicates). Evolutionary distances employed the Jukes-Cantor substitution model.

2.7.6. Phosphatase Enzyme Extraction

Following Jonsson and Aoyama (2007), algal pellets were flash-frozen in liquid nitrogen, macerated with acetate buffer, freeze-thawed, and probe-sonicated on ice (3 cycles: 50 s sonication, 20 s rest, amplitude 70). After centrifugation (10,000 rpm, 20 min), supernatants containing alkaline phosphatase were collected for assay.

2.8. Toxicity Assessment of Unary and Senary Mixtures on Chlorella vulgaris Alkaline Phosphatase Activity

Graded concentrations of Cu2+, Zn2+, Cr2+ (0–0.5 mM), Pb2+, Cd2+ (0-7.0 mM), and 2,4-dichlorophenoxyacetic acid (2,4-D; 0–25 mM) prepared from 10 mM stocks were tested individually. Senary mixtures were formulated at fixed ratios (50:10:10: 10:10:10 and 10:18:18: 18:18:18) and assayed at total concentrations up to 9.0 mm Alkaline phosphatase inhibition was evaluated in 2 mL reaction mixtures containing substrate (p-nitrophenyl phosphate, p-NPP), enzyme extract, and toxicants, incubated at 37°C for 30-40 min. Reactions were stopped with 0.1 mL NaOH, centrifuged, and absorbance of the supernatant measured spectrophotometrically at 410 nm. Controls without toxicants were included. All assays were performed in triplicate.

2.9. Statistical and Dose-Response Modeling

Relative inhibition responses were calculated as percentages relative to control and fitted to hermetic dose-response models (Cedergreen et al., 2005) using Sigma Plot 10.0 software. Median inhibitory concentrations (EC50) were derived for individual and mixture toxicants. One-way ANOVA ($p < 0.05$) assessed statistical significance using IBM SPSS v25.

2.10. Toxicity Interaction Analysis via Toxicity Index Model

From equation one, Interaction effects within mixtures were quantified using the Toxicity Index (TI), defined as

$$TI = \sum_{i=1}^{n} \frac{C_{\text{mix},i}}{EC50_i} \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Figure 2 Toxicity index model

where $C_{\text{mix},i}$ is the concentration of component i in the mixture, and $EC50_i$ is its individual EC50. Interpretation: TI = 1 (additive), TI > 1 (antagonism), TI < 1 (synergism) (Boillot and Perrodin, 2008; Cedergreen et al., 2008).

Additional analytical frameworks employed include logistic dose-response curves, linear low-concentration approximations, Concentration Addition (CA), Independent Action (Bliss Independence), isobologram analyses, interaction coefficient modeling, dose-response surface approaches, fractional inhibition models for senary mixtures, and mixed-effects models incorporating modulation by 2,4-D to comprehensively describe enzymatic inhibition dynamics in Chlorella vulgaris (Durieux et al., 2002; Espósito et al., 2022). The toxicity interaction of the mixtures was compared using similar toxic index (TI), which sum the components toxic unit (Boillot and Perrodin, 2008). The TU values were calculated using the expression

$$TU_i = \frac{Cmix_i}{IC_{50I}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

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

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

$$Cmix_i = \frac{Ratio}{100} IC_{50I}mix \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (4)$$

Figure 3 Toxicity index model in terms of TU/TI

Where Cixi is the concentration of the itch toxicant in the mixture and EC50i is the EC50 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).

2.10.1. Concentration–Response Curve (Logistic Model) for Unary Toxicity

$$E(C) = \frac{E_{\max}}{1 + \left(\frac{IC_{50}}{C}\right)^n} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (5)$$

Figure 4 Unary Toxicity index model

where $E(C)$ is enzyme inhibition at concentration C, IC_{50} is the half-maximal inhibitory concentration, n is the Hill coefficient, and $E_{\max}$ is maximum effect.

2.10.2. Inhibition Percentage (Linear Approximation at Low Concentrations)

$$\%\text{Inhibition} = k \times C \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (6)$$

Figure 5 Inhibition Toxicity index model

where k is the inhibition constant for the toxicant.

2.10.3. Additive Toxicity Model (Concentration Addition, CA)

$$\sum_{i=1}^{n} \frac{C_i}{IC_{50,i}} = 1 \quad \ldots\ldots (7)$$

Figure 6 Additive Inhibition Toxicity index model

where C_i and $IC_{50,i}$ are concentration and half-max inhibitory concentration of component i.

2.10.4. Independent Action Model (Bliss Independence)

$$E_{mix} = 1 - \prod_{i=1}^{n} (1 - E_i) \quad \ldots\ldots (8)$$

Figure 7 Independent Inhibition Toxicity index model

where E_i is the individual toxic effect of component i.

2.10.5. Toxicity Index (TI) for Interaction Type

$$TI = \frac{Observed}{Expected} \quad \ldots\ldots (9)$$

Figure 8 Interaction Inhibition Toxicity index model

$TI > 1$ indicates antagonism; $TI = 1$ additive; $TI < 1$ synergism.

2.10.6. Isobologram Equation for Binary Mixtures

$$\frac{C_1}{IC_{50,1}} + \frac{C_2}{IC_{50,2}} = 1 \quad \ldots\ldots (10)$$

Figure 9 Binary Inhibition Toxicity index model

The equation used to examine interaction effects at fixed effect levels.

2.10.7. Interaction Coefficient Model

$$E_{mix} = \sum C_i^{p_i} \times \beta \quad \ldots\ldots (11)$$

Figure 10 Interaction Inhibition Toxicity index model

where p_i is potency parameter, and β measures synergistic or antagonistic interaction.

2.10.8. Dose-Response Surface Model for Mixtures

$$E(C_1, C_2, \ldots, C_n) = \frac{E_{\max}}{1 + \exp\left[-(a_0 + \sum a_i C_i + \sum a_{ij} C_i C_j)\right]} \quad \ldots\ldots (12)$$

Figure 11 Surface Inhibition Toxicity index model

The model captures nonlinear interactions including pairwise effects.

2.10.9. Fractional Inhibition Model for Senary Mixtures

$$E_{total} = \sum_{i=1}^{6} f_i E_i \quad \ldots\ldots (13)$$

Figure 12 Senary Inhibition Toxicity index model

with f_i being the fraction of each component in fixed ratio.

2.10.10. Mixed-Effects Model Incorporating Modulation by 2,4-D

$$E_{mix} = \frac{E_{\max}}{1 + \left(\frac{\sum \alpha_i C_i}{IC_{50,mod}}\right)^n} \quad \ldots\ldots (14)$$

Figure 13 Modulated Inhibition Toxicity index model

where α_i are weighting factors adjusting metal toxicity modulated by 2,4-D interaction; $IC_{50,mod}$ is modulated inhibitory concentration.

2.11. Relative responses

The relative responses were evaluated as percentage relative to control due to inhibition of the toxicants and fitted model equations

$$\text{Relative response \% Inhibition} = \frac{R_C - R_T}{R_C} \times 100 \quad \ldots\ldots (15)$$

Figure 14 Independent Inhibition Toxicity index model

In equation: (15), RC is the response of the control and RT is the response in the tests (at different concentrations of the toxicant). The data generated from relative inhibition responses due unary and Senary of metals and pesticide were fitted into dose-response models (Cedergreen, et.al., 2005) using sigma plot (ver:10). These models collectively allow quantification of unary toxicity, prediction of mixture effects at fixed ratios, and elucidation of additive, antagonistic, and synergistic interactions affecting enzyme inhibition in Chlorella vulgaris. (Durieux et al.,2002; Espósito et al.,2022).

3. Results

3.1. Morphological and Biochemical Characterization of Isolate R1 (Accession No. AY591506)

The isolate R1 (AY591506) was characterized morphologically using electron microscopy, revealing spherical unicellular cells with diameters ranging from 2 to 12 µm, averaging 3–12 µm in morphometric analysis. The cells exhibited a unilinear, ellipsoidal to subspherical cell wall lacking flagella. Chloroplast morphology was parietal and cup-shaped, containing a single pyrenoid and a cluster of fused thylakoids rich in green chlorophyll pigment. The chloroplast was bounded by a double membrane of phospholipid composition. Intracellular observations identified a gel-like cytoplasmic matrix enclosed by the plasma membrane, along with mitochondrial-like double membranes and electron-dense circular structures (figure 15). Under light microscopy, the isolate exhibited a green coccoid phenotype consistent with unicellular chlorophyte morphology. Based on morphological and ultrastructural features, isolate R1 (AY591506) was taxonomically assigned to the genus Chlorella, supported by comparative analyses with existing literature (Tomaselli, 2004; Borowitz, 2018; Safi et al., 2014; Kreitz et al., 2015; Yamamoto et al., 2004, 2005; Champions et al., 2015; Garcia, 2012; Baek, et al., 2019; Belden, Gillion and Lydy,2007). Biochemical assays further corroborated the identification, demonstrating positive reactivity across multiple enzyme activities and metabolic markers (Table 1). The isolate tested positive for phosphatase, nitrogenase, chlorophyll content, nitriles, lipoxygenases, lipase, urease, thiolase/gelatinase, catalase, hydrogenases, and carbonic anhydrase. Lower activity was noted for amylase. biochemical screening data are summarized in Table 1.

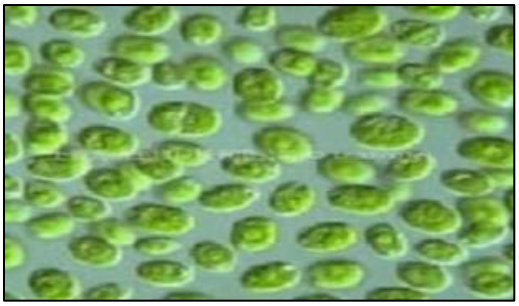

Figure 15 Schematic Microscopic structure of Chlorella vulgaris

Table 1 Morphological and biochemical screening assay of Chlorella vulgaris

Parameters	Results
Shape	Spherical/cup-shaped pyrenoid.
Form	Ellipsoidal
Chloroplast	(Single stranded) present
Colour	Green thylakoid 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

3.2. Molecular (Genomic) Identification of Isolate R1 (AY591506)

Molecular identification of isolate R1 (AY591506) was performed through comprehensive genomic analyses, including DNA extraction, quantification, 18S rRNA gene amplification, sequence assembly, annotation, and phylogenetic inference. The 18S rRNA gene sequence obtained from the isolate was subjected to a MEGABLAST search against the NCBI non-redundant nucleotide (nr/nt) database, yielding a 99–100% sequence identity with closely related Chlorella species. Phylogenetic analysis using evolutionary distances calculated by the Jukes-Cantor model corroborated the taxonomic placement of isolate R1 within the Chlorella clade. The isolate demonstrated strong phylogenetic affinity and close genetic relatedness to Chlorella vulgaris, confirming its species-level identity.

Figure 16 Phylogenetic tree showing the isolate Chlorella vulgaris

3.3. Toxicity of Individual Metals and 2,4-D on Chlorella vulgaris Phosphatase Activity

Figures17 illustrates the experimentally derived dose-response data (data points) alongside model predictions for the unary toxic effects of heavy metals: Copper (Cu2+), Lead (Pb2+), Chromium (Cr2+), Cadmium (Cd2+), Zinc (Zn2+) ions and 2,4-dichlorophenoxyacetic acid (2,4-D) on the alkaline phosphatase activity of Chlorella vulgaris. The inhibition

responses conformed well to a sigmoidal three-parameter dose-response model, with goodness-of-fit coefficients (R2) ranging from 0.98 to 0.99, indicating strong predictive accuracy. The median effective concentrations (EC50 values) for each individual compound are detailed in Table 2.

Table 2 Median inhibitory concentration (IC50) of the effect of Unary toxicity of (metal/pesticide) to phosphatase enzyme activity from Chlorella vulgaris

Unary Toxicants:	**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

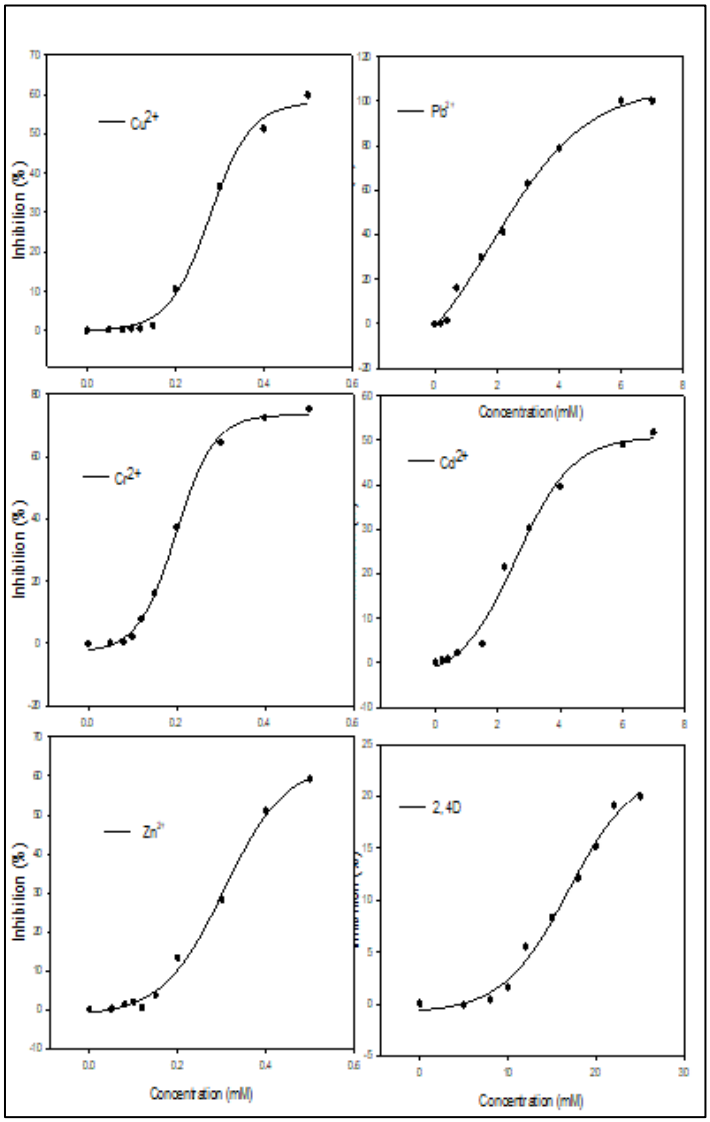

Figure 17 Experimental (data points) and model-predicted dose-response data for unary inhibition of phosphatase activity of Chlorella vulgaris by copper, lead, chromium, cadmium, zinc ion and 2,4-D., using Sigmoidal, Sigmoid, 3 Parameter model

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

The result presented in figure 18 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 Senary mixtures closely fitted into a Sigmoidal 4 Parameter model; with R2 values ranging from 0.96-0.99.

Table 3 Median inhibitory concentration (IC50) of the effect of Senary toxicity of (metal/pesticide) to phosphatase enzyme activity from Chlorella vulgaris

Toxicant	**EC50 (mM)**	**Toxic Index**	**Interaction**
Cu2+	0.28 ±0.01	_	Toxic
Zn2+	0.31±0.02	_	Toxic
Pb2+	1.86±0.76	_	Toxic
Cr2+	0.19±0.01	_	Toxic
Cd2+	2.59±0.32	_	Toxic
2,4-D	16.82±1.47	_	Toxic
Toxicant mixture	IC50(mM)	Toxicity index	Interaction
Senary mixture ratio (50:10:10: 10:10:10)			
{2,4-D, Cu2+, Pb2+, Zn2+, Cr2+, Cd2+}	6.98±0.06c	9.26±0.46c	Antagonism
Senary mixture ratio (10:18:18:18: 18:18)			
{2,4-D, Cu2+, Pb2+, Zn2+, Cr2+, Cd2+}	2.68±0.09c	6.27±0.31c	Antagonism

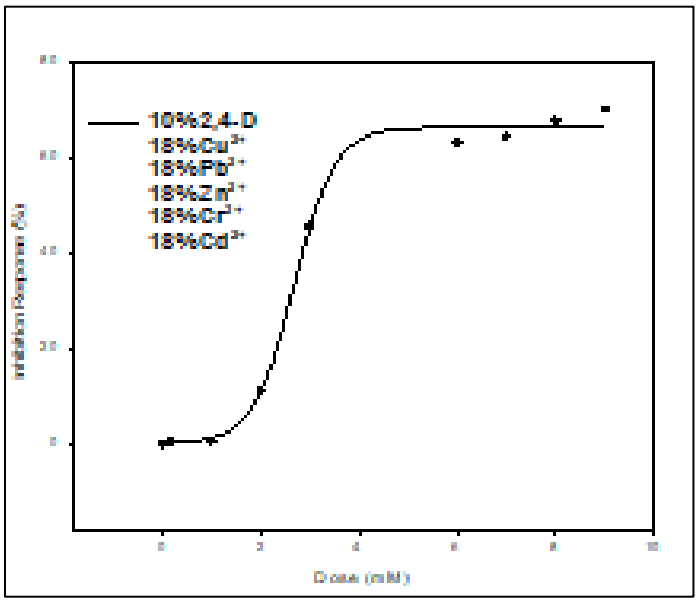

Figure 18 Experimental (data points) and model-predicted dose-response of Chlorella vulgaris phosphatase activity to Senary mixture toxicity ratios 10:18:18: 18:18:18 of 2,4-D, Cu2+, Pb2+, Zn2+, Cr2+, Cd2+. The solid lines represent sigmoidal, sigmoid 4 Parameter model

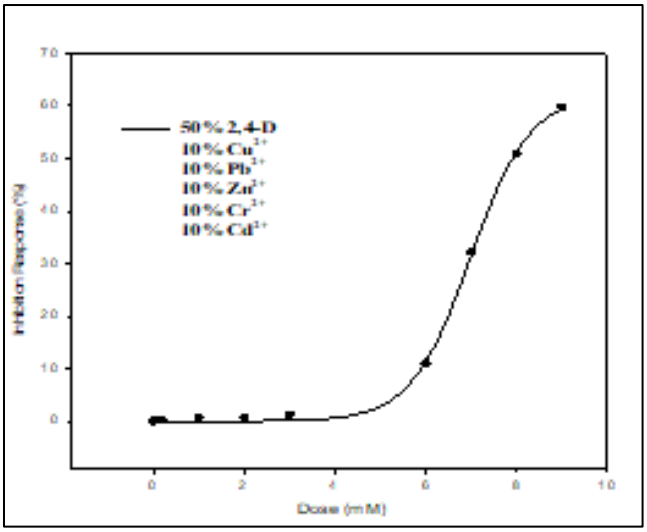

Figure 19 Experimental (data points) and model-predicted dose-response of Chlorella vulgaris phosphatase activity to Senary mixture toxicity ratios 50:10:10: 10:10:10 of 2,4-D, Cu2+, Pb2+, Zn2+, Cr2+, Cd2+. The solid lines represent sigmoidal, sigmoid 4 Parameter model

4. Discussion

4.1. Morphological and Biochemical Identification of Isolate R1 (AY591506)

The morphological and biochemical characterization of Chlorella vulgaris isolate R1 (AY591506) (Table 1) aligns closely with previous studies (Yamamoto et al., 2004; Illman et al., 2002; Champions et al., 2015; Garcia, 2012; Tomaselli, 2004; Borowitz, 2018; Safi et al., 2014; Kreitz et al., 2015; Hegewald, 2000; Felsenstein 1985; Jukes and Cantor 1969). The isolate exhibited plant-like structural features such as a unilinear cell wall, subspherical to ellipsoidal shape, and absence of flagella. A single chloroplast enveloped by a double phospholipid membrane was observed, consistent with Yamamoto et al., (2004).

4.2. Molecular (Genomic) Identification

Molecular analysis of isolate R1 via 18S rRNA gene sequencing demonstrated 99–100% sequence similarity with related Chlorella species. Phylogenetic inference using the Jukes-Cantor model further substantiated the isolate's close genetic affiliation to Chlorella vulgaris, confirming species-level identification.

4.3. Toxicity of Unary and Senary Metal/Pesticide Mixtures to Chlorella vulgaris Phosphatase Activity

Unary dose-response analyses revealed differential toxicity of heavy metals and 2,4-dichlorophenoxyacetic acid (2,4-D) towards phosphatase activity in C. vulgaris. The ecological risk ranking based on inhibitory concentration 50 (IC50) values was established as Cr2+ > Cu2+ > Zn2+ > Pb2+ > Cd2+ > 2,4-D, with chromium being the most potent inhibitor (IC50 = 0.19 ± 0.01 mM) and 2,4-D the least toxic (IC50 = 16.82 ± 1.47 mM) (Figure 17, Table 3). These results correspond with literature reports indicating chromium's high toxicity and ecological concern, particularly in freshwater ecosystems (Al-Haawa et al., 2020; Su et al., 2017; Srivastava et al., 2017).

Copper and zinc also exhibited significant phosphatase inhibition with IC50 values of 0.28 ± 0.01 mM and 0.31 ± 0.02 mM, respectively. Copper's toxicity has been linked to oxidative stress, genotoxicity, and adverse effects on algal physiology in multiple studies (Mu et al., 2017; Mohy-Eldim and Abeel-Kareen, 2020; Wang et al., 2017, 2020). Zinc's ecotoxicological significance aligns with findings by Espósito et al. (2021) and Wang et al., (2020).

Lead exhibited moderate toxicity (IC50 = 1.86 ± 0.76 mM), which is consistent with previous observations of C. vulgaris (Al-Haawa et al., 2020), while cadmium presented the lowest toxicity among the metals tested (IC50 = 2.59 ± 0.32 mM). Cadmium's inhibitory effects on pigment assimilation, protein content, and antioxidant enzyme activity in C. vulgaris have been previously documented (Cheng et al., 2016; Bellini et al., 2021).

Toxicity assessment of senary mixtures at ratios of 10:18:18: 18:18:18 and 50:10:10: 10:10:10 (2,4-D, Cu2+, Pb2+, Zn2+, Cr2+, Cd2+) revealed median EC50 values of 2.68 ± 0.09 mM and 6.98 ± 0.06 mM, respectively (Table 3). Interaction

analysis using the Toxicity Index (TI) indicated antagonistic effects (TI >> 1) in both mixture ratios. Notably, the antagonism was associated with the presence of 2,4-D, suggesting its potential role as a modulating chelating agent, consistent with observations by Nikiforov-Nikishin et al., (2021).

These findings corroborate previous studies demonstrating complex interactions in metal-pesticide mixtures and their cumulative ecotoxicological impacts on microalgae (Houssou et al., 2020; Zeb et al., 2017; Su, et al., 2017; Silva, et al., 2018).

5. Conclusion

Senary mixtures of 2,4-dichlorophenoxyacetic acid (2,4-D) and heavy metals at ratios 10:18:18:18:18:18 and 50:10:10:10:10:10 caused substantial inhibition of Chlorella vulgaris alkaline phosphatase activity. The presence of 2,4-D reduced the overall toxicity of metal mixtures, yet simultaneously exhibited enhanced toxicity when combined with heavy metals. These results highlight the dual role of 2,4-D as both a moderating and potentiating agent in agrochemical-metal mixtures, emphasizing the need to consider mixture effects in ecotoxicological risk assessments.

Compliance with ethical standards

Acknowledgments

I acknowledge the staff of Silvercrest Laboratories, Owerri, Imo State, Nigeria.

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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