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Prediction of the toxic impact on the freshwater microalgae *Scenedesmus intermedius* produced by the interaction of copper sulfate and copper oxychloride in a binary mixture with glyphosate

Solís-González Gerardo[1], Cortés-Téllez Alondra Alelie[1], Chacon-Garcia Luis[2], García-Pérez Martha-Estrella[3], Martínez-Flores Héctor E.[3] and Bartolomé Camacho María Carmen[3,*]

[1] Environmental Toxicology Laboratory, Faculty of Chemical Pharmacobiology, Universidad Michoacana de San Nicolás de Hidalgo, 403 Santiago Tapia Street, CTR, Morelia, Michoacan, Mexico. 58000.
[2] Chemical-Biological Sciences Institute, Universidad Michoacana de San Nicolás de Hidalgo, 403 Santiago Tapia Street, Morelia, Michoacan, Mexico. 58000.
[3] Faculty of Chemical-Pharmacobiology. Universidad Michoacana de San Nicolás de Hidalgo (UMSNH) Tzintzuntzan Ave. #173 Matamoros NBHD, Morelia, Michoacan, Mexico. 58240.

[*] Corresponding author: carmen.bartolome@umich.mx

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ABSTRACT

Prediction of the toxic impact on the freshwater microalgae *Scenedesmus intermedius* produced by the interaction of copper sulfate and copper oxychloride in a binary mixture with glyphosate

The high population growth worldwide causes a high demand for food with an increase in the use of different agrochemicals, with pesticides and herbicides being the primary pollutants of anthropogenic origin in the environment. One of the critical aims of ecotoxicology is the evaluation of toxic effects of mixtures of chemical substances that can have additive, synergistics or antagonistics effects in different ecosystems. For this reason, the aims of this research were to: 1) estimate the individual subchronic toxicity of agrochemicals copper oxychloride [$Cu_2(OH)_3Cl$], copper sulfate ($CuSO_4$), and glyphosate (Gly-BH) on the inhibition of population growth of the freshwater chlorophyte *Scenedesmus intermedius*, and 2) predict the antagonistic, synergistic, or additive behaviour of binary mixtures of Gly-BH with $Cu_2(OH)_3Cl$ and $CuSO_4$ through the Combination Index Equation (CIE). The individual toxicity order at 7 days of exposure in *S. intermedius* was $CuSO_4$ (IC_{50} 2.70 mg/l) > Gly-BH (IC_{50} 4.03 mg/l) with no statistical differences between both agrochemicals and > $Cu_2(OH)_3Cl$ (IC_{50} 25.59 mg/l) with a major tolerance if it compared with the other chemicals. For the binary mixture of $CuSO_4$/Gly-BH, an antagonistic effect was observed (combination index CI > 1), but with lower toxicity (IC_{50} 7.40 mg/l) when compared with the individual responses of these compounds. However, in the mixture, $Cu_2(OH)_3Cl$/Gly-BH was more toxic with a synergistic response (IC_{50} 0.85 mg/l) even between 5 and 30 times higher. These results highlight the importance of studying interactions of chemical substances in ecosystems to establish a better evaluation and regulation of their environmental impact.

Key words: copper, glyphosate, toxicity, binary mixtures, combination index

RESUMEN

Predicción del impacto tóxico sobre la microalga de agua dulce* Scenedesmus intermedius *producido por la interacción de sulfato de cobre y oxicloruro de cobre en una mezcla binaria con glifosato

El elevado crecimiento de la población mundial provoca una gran demanda de alimentos con un aumento del uso de diferentes productos agroquímicos, siendo los plaguicidas y herbicidas los principales contaminantes de origen antropogénico en el medio ambiente. Uno de los objetivos críticos de la ecotoxicología es la evaluación de mezclas de sustancias químicas que pueden tener efectos aditivos, sinérgicos o antagónicos en diferentes ecosistemas. Por ello, en este estudio, los objetos de esta investigación fueron: 1) estimar la toxicidad subcrónica individual de los agroquímicos oxicloruro de cobre [$Cu_2(OH)_3Cl$], *sulfato de cobre ($CuSO_4$) y glifosato (Gly-BH) sobre la inhibición del crecimiento poblacional en la clorofita de agua dulce* Scenedesmus intermedius *y, 2) predecir el comportamiento antagónico, sinérgico o aditivo en mezclas binarias de Gly-BH con $Cu_2(OH)_3Cl$ y $CuSO_4$ a través de la ecuación del Índice de Combinación (CIE). El orden de toxicidad individual a los 7 días de exposición sobre* S. intermedius *fue $CuSO_4$ (IC_{50} 2.70 mg/l) > Gly-BH (IC_{50} 4.03 mg/l) sin diferencias estadísticas entre ambos agroquímicos, y > $Cu_2(OH)_3Cl$ (IC_{50} 25.59 mg/l) con una mayor tolerancia si se compara con los otros químicos. Para la mezcla binaria de $CuSO_4$/Gly-BH, se presentó un efecto antagónico (índice de combinación CI > 1), pero con una menor toxicidad (IC_{50} 7.40 mg/l) si se compara con las respuestas individuales de estos compuestos. Sin embargo, la mezcla $Cu_2(OH)_3Cl$/Gly-BH fue más tóxica con una respuesta sinérgica (IC_{50} 0.85 mg/l) entre 5 y 30 veces más tóxica. Estos resultados destacan la importancia de estudiar las interacciones de las sustancias químicas en los ecosistemas para establecer una mejor evaluación y regulación del impacto ambiental.*

Palabras clave: *cobre, glifosato, toxicidad, mezclas binarias, índice de combinación*

INTRODUCTION

The high population growth worldwide causes a high rate of food demand with an increase in the use of different agrochemicals, with pesticides and herbicides being the major contaminants of anthropogenic sources in the environment (Konstantinou, 2006). Such agrochemicals, according to their chemical structure and physical properties, are persistent, show bioaccumulation where they are applied, and consequently biomagnify along the trophic chains (Jaramillo-Juárez et al., 2009).

Therefore, various conventions such as the Rotterdam Convention, which limits and regulates the international import of highly hazardous substances such as pesticides, or the Code of Conduct of the Food and Agriculture Organization of the United Nations (FAO) on pesticides, call on international authorities to review and regulate the use of pesticides worldwide (Del Puerto et al., 2014). Among the most widely used pesticides due to their chemical persistence are cupric fungicides. The most widely used is the agrochemical copper oxychloride ($Cu_2(OH)_3Cl$), which is used to control and prevent fungal and bacterial diseases in plants. Another agrochemical is copper sulphate ($CuSO_4$), which is used in the fungal control of plants and fruit trees and is a micronutrient at substrate concentrations ranging from 0.05 to 10 mg/l (PROMIX, 2022). However, both cupric agrochemicals are important environmental contaminants, and their toxicity is a problem of growing importance for ecological, evolutionary, nutritional, and environmental causes (Jaishankar et al., 2014). Cu is certainly less toxic compared to other heavy metals such as Cd, Cr, Pb, and Hg; however, toxicity can occur if its concentration significantly exceeds the normal range. For example, copper concentrations above 20 µg/g can be toxic and induce oxidative stress and DNA damage and reduce cell proliferation (Bradl, 2005; Oe et al., 2016).

In contrast to the above, the most widely used pesticide is Gly-BH and its formulations [N-(phosphonomethyl) glycine; $C_3H_8NO_5P$]. It is a broad-spectrum systemic herbicide that inhibits a pathway of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Alcántara-de la Cruz et al., 2021). It is widely applied for weed control in annual and perennial crops, in non-agricultural use (e.g., roadsides and urban areas), and in dewatering to ease grain harvesting (e.g., wheat). However, it is used intensively in 80 % of transgenic crops such as transgenic soybeans and maize (GMOs) due to high resistance to glyphosate (ISAAA, 2022). Without considering the aquatic toxic risk associated with the excessive use of these agrochemicals, the aquatic ecosystem as the final receptor of such spills and agricultural leachates is of urgent importance of toxicological studies due to the vast variety of chemicals and complex mixtures found in the aquatic ecosystems (Iannacone & Alvariño, 2011). Therefore, the assessment of toxic effects and quantification of risks associated with exposure to chemical mixtures is an important challenge and key to environmental toxicology and regulatory agencies (Schwarzenbach et al., 2006; Teuschler, 2007).

Thus, Chou (2010), proposed to evaluate the toxicity interaction of mixtures using the Combination Index (CI) based on the 50 % effective concentration without depending on the mode of action. Thus, CI has been widely used in the evaluation of the toxicity of drug combinations, attracting the attention of researchers in environmental sciences (Rodea-Palomares et al., 2010; Rodea-Palomares et al., 2012). Consequently, it is necessary to set up methodologies to evaluate the toxicity of xenobiotics *in vitro* to keep the safety and guarantee the integrity of freshwater ecosystems. Thus, to determine the destination of the release of pesticides residues, it is intended to implement the application of ecotoxicological bioassays using phytoplankton bioindicators representing the ecosystem receptor, since they are key elements, as primary producers that provide oxygen and energy transfer along the aquatic trophic chain with which the results can be extrapolated to other levels of the trophic chain (Bonnaffé et al., 2021).

Microalgae such as *S. intermedius* are important biological models for the regulatory evaluation of xenobiotics. They are overly sensitive to the presence of contaminants and are therefore an interesting alternative for ecotoxicological tests due to their key position as primary producers in aquatic ecological systems (Beardall & Raven, 2004; Levy et al., 2007). Hence, the assessment and prediction of the joint effects of environmental pollutants is an important topic of research in ecotoxicology (Backhaus & Faust, 2012).

Therefore, the aims of our study were to: 1) estimate the individual subchronic toxicity of agrochemicals $Cu_2(OH)_3Cl$, $CuSO_4$, and Gly-BH on the inhibition of population growth on the freshwater chlorophyte *Scenedesmus intermedius*, and 2) predict the antagonistic, synergistic, or additive behavior in binary mixtures of Gly-BH with $Cu_2(OH)_3Cl$ and $CuSO_4$ through the Combination Index (CI) method. We expect the combined exposure of $Cu_2(OH)_3Cl$ and $CuSO_4$ with Gly-BH will have a greater toxic impact on *Scenedesmus intermedius* compared to the individual exposure to each agrochemical.

MATERIAL AND METHODS

Experimental organisms and culture conditions

Laboratory experiments were performed using the Chlorophyta *Scenedesmus intermedius* (Chodat) obtained from the Environmental Toxicology Laboratory of the Chemical-Pharmacobiology Faculty, Universidad Michoacana de San Nicolás

Table 1. Physicochemical properties, crop application doses and aquatic environmental concentrations of copper agrochemicals and glyphosate. *Propiedades fisicoquímicas, dosis de aplicación al cultivo y concentraciones ambientales acuáticas de los agroquímicos cúpricos y el herbicida glifosato.*

Agrochemical	Physicochemical properties	Application rates	Environmental concentrations
Copper sulphate pentahydrate $CuSO_4 \cdot 5H_2O$	980 g/kg (98 %) Solubility 22 g/100g of H_2O at 25 °C 252 g Cu^{2+}/kg MW 249.68 g/mol	30-120 g/hl (300-1200 mg/l)	7.4 to 35 µg/l (ECHA, 2023)
Copper oxychloride $Cu_2(OH)_3Cl$	500 g/kg (85%) MW 213.57 g/mol Low solubility in water	3.0-400 g/100l H_2O	7.4 to 35 µg/l (ECHA, 2023)
Glyphosate (2-phosphonomethylamin acetate)	487 g/l (74 %) MW 228.2 g/mol Solubility 120000 mg/L at 25 °C	300-400 l/ha.	1.0 to 700 µg/l (Ruiz-Toledo et al., 2014)

de Hidalgo, Mexico. The strain of *S. intermedius* grew in culture flasks (Greiner, Bio-One, Longwood, NJ, USA) with 20 ml of BG-11 medium at pH 7.1 (Sigma, Aldrich Chemie, Taufkirchen, Germany), and was maintained at 21 °C under continuous light of 60 $\mu molm^{-2}s^{-1}$ over the 400–700 nm waveband in a 12:12 h light-dark photoperiod to ensure exponential algal growth in a thermostatically controlled chamber (Thermo Fisher Scientific Inc., Waltham, MA, USA). Cells were maintained in mid-log exponential growth by serial transfers of one-cell inoculum in a fresh medium once a fortnight. All tests were performed while the microalgal strain was in the exponential growth phase.

Chemicals

In the present study chemicals used were copper sulfate ($CuSO_4$) purchased from Agrocopper® SP Bayer CropScience, Mexico, copper oxychloride ($Cu_2(OH)_3Cl$) obtained from Agrocuper Ecovert, Agrochemical S. A. de C. V., Mexico, and the glyphosate-based herbicide ($C_3H_8NO_5P$) purchased from La FAM® S.G., Mexico. The physicochemical properties of the substances are presented in Table 1. By static assay, the agrochemical metals and the herbicide were dissolved using 100 mg to make stock solutions in triple-distilled water (Thermo Fisher Scientific Inc, USA) at exponential concentrations of each compound to reach concentrations 1 to 100 mg/l, the dilutions prepared daily for each assay.

Population growth inhibition of the agrochemical compounds

The population growth inhibition estimate is based on the OECD 201 guideline (OECD, 2011). First, the cell densities were adjusted to 1 x 10^5 cells/ml by count on a Neubauer chamber (Brand GmbH + CO KG, Germany) in an optical microscope (Zeiss, Carl Zeiss Microscopy GmbH, Germany) at a total volume of 10 ml of BG-11 medium. Preliminary toxicity tests were performed to define the range of concentrations that included 0 % and 100 % of growth inhibition. Test concentrations, chosen based on preliminary range finding tests, covered the range of 1-100 mg/l for all assays. With the results obtained through these previous assays, two copper-based agrochemical compounds and the glyphosate-based herbicide concentrations, as well as controls, were established and evaluated.

The estimation was made from the Inhibitory Concentration to 50 % (IC_{50}) at 7d of exposure individually in a range of concentrations of 1–100 mg/l in the *Scenedesmus intermedius* strain. All the cultures (control and treatments) were incubated for seven days (7 d) at 21 ºC in a thermostatically controlled chamber (Gilson Inc., Middleton, WI USA) at 60 $\mu molm^2/s$ to ensure exponential algal growth. Every 24 h the microalgal density was quantified. To unify the experimentation criteria, all tests were performed while green microalgae were in the exponential growth phase. Microalgal density was measured by the Neubauer chamber. Each assay was repeated eight times (n = 8).

Binary metal mixture design

The binary mixtures were formulated by taking account the toxicity levels of individual agrochemical on *Scenedesmus intermedius*, as determined by their respective Inhibitory Concentrations values to 50 % at 7 d of exposure (IC_{50}). The toxicity concentrations of the mixtures were arranged in the range of 1-100 mg/l, based on the observed toxicity results.

The estimation of the synergistic, antagonistic, or additive responses of the binary interactions between Gly-BH with the copper-based pesticides [$Cu_2(OH)_3Cl$ and $CuSO_4$] was based on the *in-silico* model described by Chou & Talalay, (1984) and Chou, (2006) through the "Mass-Action Law" founded on models of enzyme kinetics and receptor binding theories. We used processor simulations of synergism and antagonism involving the Median Effect Principle (MEP), Fraction affected (Fa), and the Combination Index (CI) according to the following equation:

Combination Index (CI) equation (1)

$$^{n}CI_x = \sum_{j=1}^{n} \frac{(D)_j}{[\![(D]\!]_x)_j} = \sum_{j=1}^{n} \frac{(D_x)_{1-n}\{[D]_j/\sum_i^n[D]\}}{[\![(D]\!]_m)_j\left\{(fa_x)/[1-(fa_x)_j]^{1/mj}\right\}}$$

Where ${}^{n}(CI)_x$ corresponds to the Combination Index for *n* substances at *x* % effect (fa), $(D)_j$ belongs to the concentration of each substance in *x* % (fa) inhibition in combination, and $(D_x)_j$ fits the concentration of each substance individually in *x* % inhibition). $[D]_j/\sum^{n}l$ is the proportionality of the individual concentration of *n* substances causing *x* % effect (fa) in combination, $(D_m)_j \{(fa_x)_j/ [1- (fa_x)_j]^{1/mj}$ is the concentration of individual metals causing *x* % effect (fa), fa_x is the fractional effect (fa) at *x* %, D_m is the antilog of the x-intercept, and m is the slope of the median-effect plot. If two substances present similar mechanisms of action present a CI < 1 indicates synergism, in contrast, show different mechanisms and are independent and have a CI > 1; they will exhibit antagonism. When CI ≈ 1 addition is indicated.

For the binary interactions $CuSO_4$/Gly-BH and $Cu_2(OH)_3Cl$/Gly-BH, the Maximum Permissible Limits (MPL) present in wastewater and surface water in mg/l by the Maximum Contaminant Level by the Environmental Protection Agency (U.S. EPA, 2014, 2015) were established at 1.3 mg/l for total copper and 0.7 mg/l for Gly-BH. CI values were measured from the percentages of

Table 2. IC_{50} values in mg/l at 7 days of exposure on *Scenedesmus intermedius* strain individually of copper sulfate ($CuSO_4$), copper oxychloride ($Cu_2(OH)_3Cl$), glyphosate-based herbicide (Gly-BH), and the binary mixtures of $CuSO_4$/Gly-BH and $Cu_2(OH)_3Cl$/Gly-BH, n = 8. *Valores de la IC_{50} en mg/l a los 7 días de exposición sobre la cepa* Scenedesmus intermedius *individualmente de sulfato de cobre ($CuSO_4$), oxicloruro de cobre ($Cu_2(OH)_3Cl$), herbicida glifosato (Gly-BH), y las mezclas binarias de $CuSO_4$/Gly-BH y $Cu_2(OH)_3Cl$/Gly-BH, n = 8.*

Agrochemicals/Mixture	$IC_{50(7d)}$ mg L^{-1} (C.L. 95 %)
$CuSO_4$	**2.78** (2.10-3.50)
$Cu_2(OH)_3Cl$	**25.59 ***** (14.06-37.24)
Gly-BH	**4.03** (3.20-5.11)
$CuSO_4$/Gly-BH	**7.40** (4.95-10.84)
$Cu_2(OH)_3Cl$/Gly-BH	**0.85 ***** (0.82-0.87)

***: One-way ANOVA (Tukey's Test), significant differences ($p < 0.0001$)

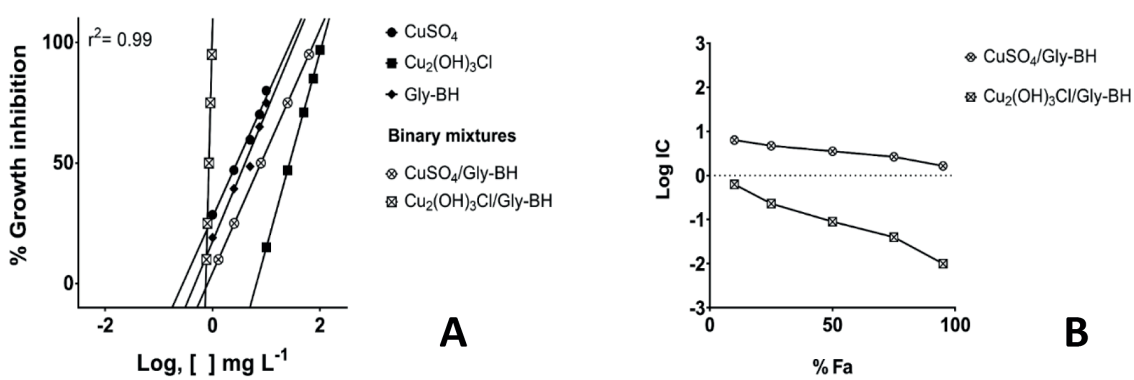

Figure 1. A) Median Effect Principle (MEP) plot of the individual responses of $CuSO_4$, $Cu_2(OH)_3Cl$, Gly-BH, and the binary combinations of $CuSO_4$/Gly-BH and $Cu_2(OH)_3Cl$/Gly-BH. B) Combination Index (Log CI) vs. Percentage of Fraction affected (% Fa) plot of binary interactions of $CuSO_4$/Gly-BH and $Cu_2(OH)_3Cl$/Gly-BH. The binary interactions were calculated from the use of the Maximum Contaminant Level by the Environmental Protection Agency (U.S. EPA, 2014; US EPA, 2015) for metal compounds and Gly-BH herbicide in water, by the Compusyn v. 1.0 pharmacological software package, on *Scenedesmus intermedius* at 7d of exposure, n = 8. *Gráfica del Principio del Efecto Medio (PEM) de las respuestas individuales de $CuSO_4$, $Cu_2(OH)_3Cl$, Gly-BH, y las combinaciones binarias de $CuSO_4$/Gly-BH y $Cu_2(OH)_3Cl$/Gly-BH. B) Índice de Combinación (Log CI) vs. porcentaje de Fracción afectada (% Fa) de interacciones binarias de $CuSO_4$/Gly-BH y $Cu_2(OH)_3Cl$/Gly-BH. Las interacciones binarias se calcularon a partir del Nivel Máximo de Contaminante de la Agencia de Protección Ambiental (U.S. EPA, 2014; US EPA, 2015) para compuestos metálicos y herbicida Gly-BH en agua, por el paquete de software farmacológico Compusyn v. 1.0, sobre* Scenedesmus intermedius *a los 7d de exposición, n = 8.*

Fraction affected (Fa) at 10, 25, 50, 75, and 95 % in *Scenedesmus intermedius*. The analysis was performed through the pharmacological software package CompuSyn Version 1.0 (ComboSyn Inc., Paramus, NJ, USA) by Chou (2010).

Experimental data analysis

The estimation of each IC_{50} value of the individual agrochemical compounds in *Scenedesmus intermedius* was done through linear regression with their respective 95 % confidence limits, showing their mean and standard deviation of each value (mean ± SD) with eight replicates (n = 8). D'Agostino-Pearson test assessed data normality. Multiple comparisons between the IC_{50} values individually and the binary interactions by One-way ANOVA through Tukey's Test. The binary interaction differences were examined by unpaired t-test through the correction by the Welch test.

The differences were considered significant at $p < 0.05$ and the analysis of all data is established through the statistical software GraphPad Prism Version 7.0 (GraphPad Software Inc., USA).

RESULTS

According to the IC_{50} values obtained after 7 days of exposure (subchronic) of *Scenedesmus intermedius* in individual exposure, the order of toxicity for agrochemicals was: $CuSO_4$ (IC_{50} 2.78 mg/l) > Gly-BH (IC_{50} of 4.03 mg/l) >> $Cu_2(OH)_3Cl$ (IC_{50} 25.59 mg/l).

Therefore, *S. intermedius* shows a clear tolerance to $Cu_2(OH)_3Cl$ with a reduction in toxicity up to 9 times what is needed to reduce the population growth as shown in Table 2 and Fig. 1A. Nevertheless, when the binary interaction of $CuSO_4$/Gly-BH and $Cu_2(OH)_3Cl$/Gly-BH was carried out through the *in-silico* model according to the MPL (U.S. EPA, 2014; US EPA, 2015), it was estimated that the mixture of $Cu_2(OH)_3Cl$/Gly-BH was more toxic with an IC_{50} of 0.85 mg/l, if we compare this value with the individual IC_{50} of both compounds that there were clear statistically different ($p < 0.0001$). Therefore, this mixture turns out to be highly toxic for *S. intermedius* at 7 d of exposure, exceeding the IC_{50} value of $Cu_2(OH)_3Cl$ individually up to 30 times and of Gly-BH almost 5 times (Table 2 and Fig. 1A). Hence, this response translates into synergism, and it intensifies at higher concentrations of both agrochemical compounds as shown by the Combination Index (CI < 1) values (Table 3 and Fig. 1B).

On the other hand, the estimated response in the binary mixture of $CuSO_4$/Gly-BH resulted in antagonism (CI > 1) as shown in Fig. 1B and Table 3, so the degree of toxicity was lower compared to the individual IC_{50} values values for $CuSO_4$ and Gly-BH (Table 2). However, this antagonistic effect was reduced in high concentrations of both chemicals, so it is also suggested

Table 3. Combination Index (CI) with the percentage of Fraction affected (% Fa) values in binary mixtures of $CuSO_4$/Gly-BH and $Cu_2(OH)_3Cl$. The binary interactions were calculated from the use of the Maximum Contaminant Level by the Environmental Protection Agency (US EPA, 2014; US EPA, 2015) for metal compounds and Gly-BH herbicide in water, by the Compusyn v. 1.0 pharmacological software package, on *Scenedesmus intermedius*, n = 8. *Índice de Combinación (CI) con el porcentaje de Fracción afectada (% Fa) valores en mezclas binarias de $CuSO_4$/Gly-BH y $Cu_2(OH)_3Cl$. Las interacciones binarias se calcularon a partir del uso del Nivel Máximo de Contaminante de la agencia de Protección Ambiental (US EPA, 2014; US EPA, 2015) para compuestos metálicos y el herbicida Gly-BH en agua, por el paquete de software farmacológico Compusyn v. 1.0, sobre* Scenedesmus intermedius, *n = 8.*

		$CuSO_4$/Gly-BH	**$Cu_2(OH)_3Cl$/Gly-BH**
% Fa	**n**	**CI ± SD**	**CI ± SD**
10	8	6.35 ± 0.81	0.63 ± 0.03***
25	8	4.73 ± 0.35	0.23 ± 0.01***
50	8	3.54 ± 0.30	0.09 ± 0.03***
75	8	2.65 ± 0.41	0.04 ± 0.01***
95	8	1.65 ± 0.50	0.01 ± 0.01***

*****:** Unpaired t-test, significant differences ($p < 0.0001$) in the binary mixtures of $CuSO_4$/Gly-BH compared with $Cu_2(OH)_3Cl$/Gly-BH that at some point it could be additive (Fig. 1B) if the IC_{50} value of 7.40 mg/l is considered.

DISCUSSION

One of the environmental problems worldwide is the aquatic impact derived from the inappropriate use of agrochemicals. In this context, multiple chemicals coexist in aquatic environments and effects of mixtures can be difficult to predict. Thus, estimating the effects of mixtures is vital for risk assessment and environmental monitoring. Aquatic organisms are often used in toxicology and ecotoxicology risk assessment studies because of their sensitivity, ubiquity, and simplicity of culture. Due to their ecological importance, microalgae are widely distributed among aquatic organisms and represent the base of the aquatic food chain as primary photosynthetic producers. Any process and substance that affects its population can have consequences at higher trophic levels, so it is important to investigate the effects of toxicity on phytoplanktonic microorganisms (Pikula et al., 2020).

Among the most widely used agrochemicals are cupric fungicides. Copper is an essential metal commonly found in aquatic ecosystems, due to its wide and diverse use (Gheorghe et al., 2017). The use of copper in agriculture is widespread, both at the phytosanitary and fertilizer levels. Although it is an essential metal for plants and animals, in high concentrations it exerts a wide range of adverse effects on aquatic organisms. Cu^{2+}, due to its high chemical persistence, produces oxidative stress by Reactive Oxygen Species (ROS) production and membrane lipid peroxidation, inactivation of key enzymes and proteins, alterations in mitochondrial respiration and alterations on the electron transport chain, photosynthetic activity and affect the homeostasis of Ca^{2+}, Mg^{2+} cations (Khangarot & Rathore, 2003; Pane et al., 2003).

Kiaune & Singhasemanon, (2011) found in bioassays on *Artemia* that copper inhibited the activity of the enzymes $Na+/K^{+}$-ATPase and Mg^{2+}-ATPase, and report that the main copper toxicity pathways involve the inhibition of ATP-driven pumps and ion channels. Besides, there are different commercial copper salts, among them is $Cu_2(OH)_3Cl$ with low solubility, so Cu^{2+} is released more slowly and acts longer, in addition to having a high ionization potential. On the other hand, sulfates have the fastest availability of Cu^{2+}, but their effect is very short since they have a lower ionization potential. This could explain why in this study the sensitivity of *S. intermedius* to $CuSO_4$ was higher with an $IC_{50(96h)}$ of 2.78 mg/l, while exposure to $Cu_2(OH)_3Cl$ had a major tolerance with an $IC_{50(96h)}$ value of 25.59 mg/l. Some factors influence the toxicity response of metals in aquatic organisms. Not only their concentration effects, but also the time of exposure to the biotic and abiotic factors are relevant (Castañé et al., 2003). In contrast, due to the widespread use of herbicides, Gly-BH is found in the aquatic environment in a concentration range from 1 to 700 µg/l (Ruiz-Toledo et al., 2014), where heavy metals are often found at elevated levels (Peterson et al., 1994), and the interaction between these substances is expected.

In this context, Mesnage et al. (2015), estimated the potential effects of Gly-BH and its formulations through an exhaustive review of several studies, finding that it is teratogenic and carcinogenic that contradicts what was established by the US EPA (lack of scientific evidence as a carcinogenic and endocrine disruptor), the IARC (International Agency of Research on Cancer) classifies Gly-BH in group 2A and the EFSA (European Food Safety Agency) defines it as a toxic cardiovascular, hepatotoxic, produces oxidative stress and is a powerful endocrine disruptor, where such effects are even detected in the acceptable daily intake range.

Gly-BH is an organophosphorus compound that contains three functional groups; a carboxylate, an amine, and a phosphonate group; these can react and form covalent bonds, serving as a chelating agent that conforms to stable complexes with divalent metal ions, including Cu^{2+}, although little is known about the bioavailability and ecotoxicity of Gly-Cu^{2+} complexes for aquatic organisms (Hansen & Roslev, 2016). A study on *Daphnia magna* showed that exposure to mixtures of Gly-BH and Cu^{2+} attenuated the acute toxicity of the metal but increased the toxicity of Gly-BH due to the formation of complexes with Cu^{2+} (Hansen & Roslev, 2016). Comparing our results suggests that Gly-BH is a mediator of aquatic metal toxicity.

This could have important ecological consequences, especially in agricultural areas where agrochemicals are regularly applied (Costas-Ferreira et al., 2022). However, few bibliographic data appear on the responses of this in microalgae. There is evidence that Gly-BH and its formulations Roundup® and Atanor® affect the structure and function of phytoplankton community (Pizarro et al., 2016; González-Pleiter et al., 2019). Previously, our research team evaluated Gly-BH toxicity in *Microcystis aeruginosa* with an IC_{50} of 53.95 mg/l (Solís-González et al., 2019) contrasting with the current work on *Scenedesmus intermedius* where after seven days of exposure, this algae showed a higher sensitivity, perhaps due to the exposure time or the type of bioindicator used, since *Scenedesmus intermedius*, unlike the cyanobacteria *Microcystis aeruginosa*, cannot use phosphorus (P), product of glyphosate degradation, in low concentrations (from 0.01 mgP/l to 5.00 mgP/l) as a nutrient (Qiu et al., 2013).

On the other hand, the toxicological effects of pollutants can manifest themselves differently when they are in a single or mixed form (Pavlaki et al., 2011). The Environmental Risk Assessment (ERA) of a single chemical and a single application may underestimate the impact on aquatic and terrestrial environments, where mixtures of multiple agrochemical stressors and various pesticide applications have been commonly documented, producing biochemical, physiological, and behavioural changes in species (Golombieski et al., 2008). Hence, predictive modelling applications are needed to estimate the environmental/ecological impact of pesticide mixtures. Recently, there have been scientific contradictions and uncertainties about the Concentration Addition (CA) and Independent Action (IA) models. Using the Combination Index (CIE) model, it is not necessary to know the action mechanism to evaluate the integral joint effects of mixtures. In addition, the CIE method also allows the simultaneous effects of compounds to be analyzed considering the different proportions of the chemicals (Yang et al., 2017; Tóth et al., 2019).

The comparative study of our bioassay using the CIE model of Chou and Talalay (2006) showed us that the mixture of $Cu_2(OH)_3Cl$/Gly-BH has greater toxicity than the individual response of both pesticides on the green microalgae *S. intermedius* with an IC_{50} value of 0.85 mg/l. Tsui et al. (2005) reported a contrasting finding where the addition of Gly-BH resulted in a significant reduction in the acute toxicity of copper and other heavy metals on *Ceriodaphnia dubia*. The IC_{50} of a mixture of $CuCl_2{\cdot}2H_2O$ and Gly-BH was found to be 7.40 mg/l, which is less toxic than the individual IC_{50} values (Gly-BH IC_{50} 5.39 mg/l and $CuCl_2{\cdot}2H_2O$ IC_{50} 0.01 mg/l). This suggest that Gly-BH has a protective effect against the toxic effects of heavy metals on *C. dubia*. Besides, Zhou et al. (2013), reported that the inhibition of the growth and reproduction of *Eisenia fetida* improved as the total absorption of Cu was decreased in the presence of Gly-BH. In addition, Zhou et al. (2013) observed that the contents of superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) were restored to a certain extent in the presence of this herbicide. The marked decrease in Cu toxicity and accumulation by Gly-BH may be related to the ability of Gly-BH to form stable compounds with Cu.

The free ion activity model implies that only free ions are expected to be toxic or bioavailable. Due to the complex capacity of ionization with metals, Gly-BH could affect the bioavailability, toxicity, and bioaccumulation of metals when it is released into aquatic ecosystems that receive both chemical groups (Tsui et al., 2005). However, the results of this study suggest that the combined toxic effects of the binary mixture of $Cu_2(OH)_3Cl$/Gly-BH (IC_{50} 0.85 mg/l) are greater than the toxicity of individual responses of $CuSO_4$ (IC_{50} 2.78 mg/l), $Cu_2(OH)_3Cl$ (IC_{50} 25.59 mg/l) and Gly-BH (IC_{50} 4.03 mg/l), which confirms the need for studies on the interactions of pollutants that coexist in the environment. Previously, the Combination Index equation (CIE) had already been used for the evaluation of mixture effects in ecotoxicology, such as the study carried out by Wang et al. (2015) applied the CI model to determine the nature of ecotoxicological interactions of two pesticides λ-cyhalothrin, imidacloprid, and the heavy metal Cd towards earthworm *E. fetida* with slightly synergistic at low effect levels, antagonism above of 60 %. Later, Cortés et al. (2018) evaluated the toxicity of binary mixtures between inorganic substances via CIE ($CuSO_4$, $KMnO_4$, NaClO,

$FeCl_3$) and organic substances (Glutaraldehyde) in LC_{50} on *Artemia franciscana naupli*i at 24h of exposure, indicating a high antagonistic effect.

So, the Chou's Combination Index (CIE) model evaluate the interaction between the tested substances shows that the $Cu_2(OH)_3Cl$/Gly-BH mixture with CI < 1 indicates a synergistic relationship between the components of the mixture depending on the concentration of both agrochemicals. This is confirmed in a study conducted by Cristofano et al. (2021), where, using *Raphidocelis subcapitata* the binary mixtures of Cu/Gly-BH (ATANOR®), Zn/Gly-BH (ATANOR®) and Cu/Zn show values of CI > 1 with an antagonistic effect that decreases with decreasing concentrations and tends towards an additive effect. The synergistic effect is a specific concern in joint toxicity studies because of the potential for individual chemicals to increase toxicity in a mixture (Uwizeyimana et al., 2018).

The new challenges of ecotoxicology imply the need for constant development and improvement of methods to predict and evaluate the impact of chemical pollutants their interactions in chemical mixtures. Besides, multiple organic and inorganic pesticides can interact with each other according to the chemical composition itself, the doses or concentration required or chemical persistence, the action mechanisms, and environmental factors like pH, temperature, and organic matter. So, the complexity of ecotoxicological agrochemical interactions can give rise to unpredictable effects of mixtures as they are determined by toxicokinetics and toxicodynamics involving several complex metabolic, molecular, genetic and epigenetic pathways.

In this context, the Combination Index equation (CIE) has some advantages: 1) the CIE is based on the unified theory of the Median-Effect Equation (MEE) proposed by Chou & Talalay (1984) and Chou (2006) in the prediction of two or more substances, through computer simulations by algorithms based on the Mass-Action Law (MAL) via mathematical induction/deduction of equations in specific action mechanisms; 2) is an analysis easily applicable in the prediction of different interactions in complex chemical mixtures by extrapolation of *in vitro* and *in vivo* responses in the graphical representation to polygonograms; 3) the model presents a high efficiency by reducing bioassay times; 4) it is an economic model by reducing the costs in the use of animal and/or cell populations, reagents, etc.; 5) The CIE model allows quantitative determinations of chemical interactions at different concentrations and effect levels. Besides, knowledge of the component–component type of interaction is not required to assess the overall interaction of the mixture. Therefore, the CIE model is an excellent predictive method to interpret the nature of the chemical interactions in the dynamics of the phytoplankton community, to extrapolate such effects to higher levels of the trophic food chain and protect public health.

CONCLUSIONS

In summary, the evaluation of the toxicity of *S. intermedius* through the inhibition of its growth rate at 7 days carried out in this study, indicates that the metallic compounds $Cu_2(OH)_3Cl$ and $CuSO_4$ and the herbicide Gly-BH, had different toxic effects either individually or in mixture. Results showed that *S. intermedius* presents the following sensitivities from highest to lowest in terms of its IC_{50}: $Cu_2(OH)_3Cl$/Gly-BH > $CuSO_4$ > Gly-BH > $CuSO_4$/Gly-BH > $Cu_2(OH)_3Cl$. For the binary mixture of $CuSO_4$/Gly-BH, an antagonistic effect was observed but with lower toxicity when compared with the individual responses of individual compounds. However, in the mixture, $Cu_2(OH)_3Cl$/Gly-BH was 5 and 30 times more toxic, respectively, with a synergistic response. The Combinations Index proposed by Chou is positioned as an *in-silico* method in ecotoxicology to define how potential pollutants that may exist in an environmental sample interact and can be particularly useful for defining risk assessment strategies. Therefore, more research on the different effects of mixtures is needed to explain the mechanistic basis for the toxic effects. This study establishes solid scientific basics when considering the assessment of agrochemical mixtures in aquatic ecosystems and their potential in the evaluation of hazards and risks to environmental health.

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