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INTERACTIVE EFFECT OF COPPER AND DICLOFENAC ON *Planktothrix* sp.

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ABSTRACT

The non-steroidal anti-inflammatory drug diclofenac (DCF) has a harmful effect on plants and algae by several studies. It is one of the most often used and found pharmaceuticals in water bodies, as the total biomass of phytoplankton and many phytoplankton are used as indicators in the aquatic habitat, their communities are sensitive to changes in their environment. The aim of this study is to assess the effect the interactive effect of copper and diclofenac on *Planktothrix* sp. Data provided by this research shows information on the interactive effect of copper and diclofenac on *Planktothrix* sp. At 500µg/L of diclofenac and 2.5µg/L of copper using the experimental design; Dry weight/cell density, pigment content, enzyme activity (POD, MDA, ROS, GST) and total protein content. Dry weight/cell density was recorded highest in the control treatment (CoR) with total weight of 2.14g, showing that diclofenac and copper inhibits the growth of *Planktothrix* sp. Reactive oxygen species ROS was highest in the control treatment (CoR) on day 7 (0.075µg/ml), glutathione S transferase (GST) was recorded highest on day 14 of Diclofenac and copper treatment (DiRCuR) having 37.04 µg/ml, peroxidase activity POD was highest on day 14 of copper (CuR) and diclofenac + copper (DiRCuR) treatment with 0.122µg/ml showing that diclofenac and copper singly and in combined form affects the antioxidant enzymes of *Planktothrix* sp. from the results of these study, it shows that there is a synergistic effect of copper and diclofenac on the growth of *Planktothrix* sp.

Keywords: Phytoplankton, Diclofenac, Copper, Biochemical composition, Toxicity

INTRODUCTION

The aquatic environment provides many ecosystem services to humans, yet they often receive high levels of pollutants from both direct and indirect sources. As analytical testing of chemical pollutants is time-consuming and expensive, there is great interest in finding environmental monitoring methods that are inexpensive, fast and easy (Raskin *et al*., 1997, Lovett-Doust *et al*., 1994a, Lovett-Doust and Biernacki, 1994b). Hence, measuring concentrations of pollutants in water and sediments alone does not provide information on the potential impact of pollution on resident organisms (Lovett-Doust *et al*., 1994b). Heavy metals are added to fresh water streams and ponds, deliberately as a herbicide (Bowmer *et al*., 1995, Brown and Rattigan, 1979) or as a byproduct of different human activities (Vesk and Allaway, 1997, Brown and Rattigan, 1979). Thus, getting to a threshold, they may become toxic to aquatic organisms.

Pharmaceuticals in the environment have attracted extensive concern worldwide due to their potential risks to ecosystems and human health (Tang *et al.*, 2015; Desbiolles *et al*., 2018; Sehonova *et al*., 2018; Patel *et al.*, 2019). Diclofenac (DCF) is among the most widely used nonsteroidal anti-inflammatory drugs worldwide (Acuña *et al*., 2015). The annual global consumption of DCF has exceeded 1000 tons (Acuña *et al.*, 2015). Due to the widespread usage and generally poor elimination of DCF in sewage treatment plants, DCF has been frequently found in sewage, surface water, drinking water and aquatic bodies/organisms (Liu *et al.*, 2015; Xie *et al*., 2015b; Xie *et al.*, 2017; Tran *et al*., 2018; Praveena *et al*., 2019). In China, DCF has been frequently detected in surface waters of Liao River, Pearl River, Yellow River, Taihu Lake and Dongting Lake with the maximum concentration of 717ng/L (Zhao *et al.*, 2009; Wang *et al*., 2010; Ma *et al.*, 2016; Xie *et al.*, 2017). DCF has also been found in surface waters in many countries in America, Africa, Asia and Europe (Sathishkumar *et al*., 2020). The maximum concentrations measured in surface waters in Nigeria, Germany, Pakistan, Spain and Portugal reached 57.16,15.03, 8.5, 3.5 and 3.2 µg L−1, respectively (Jux *et al*., 2002; Scheurell *et al.*, 2009; Carmona *et al.*, 2014; Olaitan *et al.*, 2014; Sousa *et al.*, 2019).

The global distribution of DCF in surface water has increased concerns about its possible toxic effects on aquatic organisms (Lonappan *et al.*, 2016). Growing bodies of studies have revealed toxic effects of DCF on aquatic organisms even at environmentally relevant concentrations. Previous studies of DCF have mostly focused on exposure in isolation to aquatic organisms with little attention given to the interactive effects of DCF and other contaminants (Lonappan *et al.*, 2016; González-Ortegón *et al*., 2016). However, DCF has been found to co-occur with many other contaminants in aquatic environments, such as organic compounds and heavy metals (Xie *et al.*, 2015b; Andreu *et al*., 2016). To fully understand the actual environmental impacts of DCF, it is necessary to investigate the joint effects of DCF and other contaminants.

Copper (Cu) is a common heavy metal pollutant in aquatic environments (Naser, 2013; Wang*et al*., 2013; Islam *et al.*, 2015; Coynel *et al.*, 2016; Qu *et al.*, 2018). Although Cu is an essential element in the biological processes of various organisms, it becomes toxic when present in excessive concentrations (Gaetke and Chow, 2003). In addition, absorbed Cu in aquatic organisms can induce genetic toxicity, endocrine disorders, oxidative stress, cell apoptosis, osmotic pressure disorders and oxidative metabolic enzyme inhibition (Gauthier *et al*., 2014). In aquatic environments, Cu can coexist with DCF at various concentrations. Cu and DCF have both been detected in some large rivers and shallow lakes in some parts of the world (Zhao *et al.*, 2009; Wang *et al*., 2010; Cui *et al.*, 2011; Tao *et al*., 2012; Wang *et al*., 2012; Xie *et al.*, 2015b). Previous studies showed that Cu could form complexes with antibiotics (ciprofloxacin, oxytetracycline and sulfamerazine) and the binding affinity of Cu was generally stronger than that of other heavy metals, such as zinc, cadmium and lead (Zhang *et al.*, 2012; Tong *et al.*, 2014). Similar to antibiotics, DCF also has amino, hydroxyl, carbonyl and carboxyl functional groups, which may enhance

the complications of DCF with Cu (Kovala-Demertzi, 2000; Lonappan *et al*., 2016). Hence, the co-existence of DCF and Cu in aquatic environments may alter their bioaccumulation and toxic effects in aquatic organisms in comparison with the effects of these compounds individually.

Pharmaceuticals in the aquatic environment are related to the discharge of wastewaters from hospitals and pharmaceutical plants, coupled with the excretion of unmetabolized drugs in the urine and feces of animals into the aquatic environment (Pereira *et al*. 2020). Studies on the potential effects of antibiotics and analgesics on non-target species have been receiving attention lately. More so, changes in the physicochemical structure of water bodies as a result of different pharmaceuticals such as enrofloxacin, ciprofloxacin, ibuprofen, paracetamol, tylosin, and lincomycin have often resulted in significant changes in the primary productivity of organisms such as green algae and cyanobacteria species (Ebert *et al*. 2011). The aim of this study is to check if there is an interactive of copper and diclofenac on *Planktothrix* sp.

MATERIALS AND METHODS

Sample collection

A pure culture and sub culture of *Planktothrix* sp. was obtained from the phycology laboratory, Department of Botany, Ahmadu Bello University Zaria.

Experimental design

The BG 11 medium was sterilized by autoclaving (121ºC, 30 min) and stored for 24 hours before use. Culture experiments were carried out in triplicates using 500ml conical flasks containing 250 ml of BG-11. The assays were initiated with dry weight of 0.75g of *Planktothrix* sp. The cultures were incubated for fourteen days at 12:12 h light and dark cycles photoperiod, and 21±2°C temperature. Aliquot of 0.05g was taken after 2 days for pigment content; also samples were harvested at day 7 and 14 of culture for antioxidant/oxidative, stress markers analysis, biomolecule and experiment terminated on day 14.

Maintenance of culture of *Planktothrix* sp.

Experimental cultures were maintained in blue green 11 (BG-11) medium (pH 7.4) inside an Erlenmeyer conical flask (500ml) containing 300ml of the medium and kept under controlled laboratory conditions (Light intensity, photoperiod 12:12 light:darkness and a temperature of 23ºC ± 1ºC) for the *Planktothrix* sp. to acclimate. The BG-11 medium was sterilized by autoclaving at 121ºC for 15 minutes and left for 24 hours before use. Experimental conditions were kept constant throughout the experiment.

Table 1: Medium composition (BG-11)

Stocks	Quantity (g)
$NaNO_3$	30.0
K_2HPO_4	8.0
$MgSO_4.7H_20$	15.0
$CaCl_2.2H_2O$	7.2
$C_6H_8O_7.H_2O$	1.2
$C_{12}H_{22}FeN_3O_{14}$	1.2
EDTA Na_2	0.2
Na_2CO_3	4.0
TRACE METALS	
H_3BO_3	1.43
$MnCl_2.4H_2O$	0.905
$ZnSO_4.7H_2O$	0.11
$Na_2MoO_4.2H_2O$	0.195
$Cu_2SO_4.5H_2O$	0.04
$Co(NO_3)_2.6H_2O$	0.025

Preparation of diclofenac stock

To prepare diclofenac stock solution, diclofenac was purchased from a licensed pharmaceutical store within Samaru community. 50mg of diclofenac with molecular formula $C_{14}H_{10}Cl_2NO_2$, was dissolved in 1000ml of distilled water.

Sample collection

Samples were collected five times for pigment and two times for antioxidant/oxidative stress
markers analysis and biomolecule.

Antioxidant analysis

Peroxidase (POD)

The activity of peroxidase in the algal samples was assessed employing the method described by Reddy *et al.* (1995) with some modifications.

Peroxidase catalyzes the conversion of H_2O_2 to H_2O and O_2, in the presence of the hydrogen donor pyrogallol. The oxidation of pyrogallol to a coloured product called purpurogallin can be measured spectrophotometrically at 430 nm with the specified time interval of 30s for 3mins. The intensity of the product is proportional to the activity of the enzyme.

Reagents

Pyrogallol (0.05 M in 0.1 M phosphate buffer, pH 6.5)
H_2O_2 (1% in 0.1M phosphate buffer, pH 6.5)

Procedure

3.0ml of Pyrogallol solution, 0.1ml enzyme extract was pipetted out into a cuvette. The spectrophotometer was adjusted to read zero at 430nm (used as blank), it was then followed by addition of 0.5 ml of 1% H_2O_2 and mixed. The change in absorbance was recorded every 30 seconds for 3 minutes.

The activity of enzyme was then calculated as follows:

$$\text{Peroxidase activity (units/mg)} = \frac{\Delta A_{510}/\text{min}}{6.58 \times \text{ml enzyme/ml reaction mixture}} \quad (1)$$

Where A = Absorbance

Reactive Oxygen Species (ROS): Internal Hydrogen Peroxide (H_2O_2) formation.

For measuring internal H_2O_2 formation of phytoplankton, the method of Jana and Choudhuri (1982) was adopted with some modifications. The sample biomass (cell pellet) obtained by centrifuging 40 ml of the samples at 4000 rpm, was homogenized in 3ml of 0.1 M phosphate buffer (pH 6.5) to extract internal H_2O_2. Afterwards the homogenate was centrifuged at 4000 rpm for 10 min, and the resultant supernatant (extract) was used for H_2O_2 determination. 133µl of 0.1% titanium chloride (in 20% H_2SO_4) was added to 400 µl algal extract of the supernatant, and the mixture was measured at 410 nm with a Spectrum lab 752s UV-VIS spectrophotometer.

Lipid peroxidation (MDA)

The Malondialdehyde (MDA) concentration was measured according to the method described by Heath and Packer (1968). MDA is a product of lipid peroxidation and an indicator of free radical production and possible tissue damage.

10% (w/v) trichloroacetic acid (TCA)

0.5% (w/v) thiobarbituric acid (TBA)

To 1ml of the algal extract, 2ml of 10% trichloroacetic acid (TCA) containing 0.5% thiobarbituric acid (TBA) was added, then the mixture was heated in a boiling water bath for 15 minutes and quickly cooled in an ice-bath, and the absorbance of the supernatant was read at 532 nm and 600nm with a spectrophotometer. The blank used contained the mixture without the enzyme extract (TCA + TBA + Distilled water).

The concentration of MDA was calculated, using an extinction coefficient of 155 mM−1cm−1.

$$\text{MDA (mM)} = \frac{\text{Abs } 532 - \text{Abs } 600}{155} \qquad (2)$$

Glutathione S-Transferase (GST)

Glutathione S-transferase activity was performed adopting the method of Habig et al. (1974) with some modification. Glutathione-S-transferase conjugates reduced glutathione (GSH) with 1-Chloro-2, 4-dinitrobenzene (CDNB) and the extent of conjugation is used as a measure of enzyme activity from the proportionate change in the absorption at 340 nm.

1-Chloro-2, 4-dinitrobenzene (CDNB) (1mM in ethanol)

Reduced glutathione (1mM in distilled water)

Phosphate buffer (0.1M, pH 6.5)

The assay mixture contains 10µl of GSH, 10µl of CDNB, and phosphate buffer in a total of 90µl. The reaction was then started by the addition of 10µl enzyme extract to the mixture and the readings were recorded against buffer blank after every one minute for a minimum of five minutes. The complete assay mixture without the enzyme served as the control to monitor non-specific binding of the substrates. The enzyme activity was determined by monitoring the change in absorbance at 340nm using a spectrophotometer (UV–VIS 722 N). One unit of GST activity is defined as the n moles of CDNB conjugated per minute.

The change in absorbance is directly proportional to the GST activity

$$\Delta A_{340} = \frac{A_{340}\ \text{(final reading)} - A_{340}\ \text{(initial reading)}}{\text{Reaction time (mins)}} \qquad (3)$$

Total protein

Determination of total protein was carried out using the procedure of Bradford (1976). The protein standard bovine serum albumin (BSA) was used as standard. The Bradford reagent constituent will consist of 4.7 % methanol, 8.5 % phosphoric acid and 0.01 % comasin blue. To every 0.5ml of the supernatant extract, 2.5 ml of Bradford reagent was added and incubated for 5 minutes at an ambient temperature after which the sample was read at an absorbance wavelength of 595 nm. The protein standard curve was plotted with the values obtained.

Statistical analysis

The significant difference among the different treatments would be determined by two-way analysis of variance (ANOVA) and Turkey's HSD post-hoc test will be used to separate the means, and the analyses were done at a 5% significance level using R software version 4.05 for macOS

RESULTS AND DISCUSSION

Results

Table 2 shows the growth response of *Planktothrix* expose to individual and combined copper and diclofenac recorded lowest for Chl.a (0.238 ± 0.167^{a}) and highest (5.808 ± 3.669^{a}). Chl.b recorded lowest (-0.029 ± 0.17^{b}) and highest (10.38 ± 1.427^{ab}) while T.Chl and Car recorded lowest and highest at (1.443 ± 1.049^{a}, 15.664 ± 13.641^{a}) and (0.577 ± 0.357^{b}, 9.851 ± 9.489^{a}).

Table 2: Growth Response of Planktothrix Expose to Individual and Combined Copper and Diclofenac (mean ± s.d)

Treatment	Day	N	Chl.A.µg.mL.	Chl.B.µg.mL.	T.Chl.µg.mL.	Car.
CoR	1	3	2.049 ± 0.263^{a}	-0.377 ± 0.086b	1.67 ± 0.177^{a}	0.58 ± 0.12^{b}
CoR	4	3	2.004 ± 0.725^{a}	-0.132 ± 0.058b	1.871 ± 0.67^{a}	0.799 ± 0.491^{b}
CoR	7	3	5.102 ± 3.883^{a}	-0.767 ± 0.779^{b}	4.331 ± 3.1^{a}	1.348 ± 1.105^{ab}
CoR	10	3	5.798 ± 2.519^{a}	-1.165 ± 0.58^{b}	4.627 ± 1.937^{a}	1.521 ± 0.818^{ab}
CoR	13	3	5.808 ± 3.669^{a}	-0.818 ± 0.762^{bb}	4.985 ± 2.902^{a}	1.729 ± 0.876^{ab}
CuR	1	3	3.32 ± 2.151^{a}	4.871 ± 4.427^{ab}	8.227 ± 6.61^{a}	4.379 ± 3.753^{ab}
CuR	4	3	1.16 ± 0.677^{a}	14.4 ± 13.09^{a}	15.664 ± 13.641^{a}	9.851 ± 9.489^{a}
CuR	7	3	0.507 ± 0.258^{a}	7.847 ± 5.849^{ab}	8.411 ± 6.149^{a}	4.648 ± 3.629^{ab}
CuR	10	3	0.683 ± 0.332^{a}	10.38 ± 1.427^{ab}	11.138 ± 1.105^{a}	5.944 ± 0.802^{ab}
CuR	13	3	0.238 ± 0.167^{a}	4.634 ± 2.299^{ab}	4.905 ± 2.355^{a}	2.565 ± 1.26^{ab}
DiCuR	1	3	3.773 ± 3.096^{a}	10.14 ± 4.412^{ab}	13.987 ± 6.92^{a}	8.853 ± 4.063^{ab}
DiCuR	4	3	0.298 ± 0.085^{a}	$5.26 \pm 2.29^{a}b^{ab}$	5.596 ± 2.248^{a}	3.073 ± 1.422^{ab}
DiCuR	7	3	0.354 ± 0.22^{a}	6.987 ± 2.361^{ab}	7.391 ± 2.596^{a}	4.008 ± 1.355^{ab}
DiCuR	10	3	0.829 ± 0.696^{a}	9.325 ± 5.856^{ab}	10.221 ± 6.565^{a}	5.369 ± 3.568^{ab}
DiCuR	13	3	0.368 ± 0.082^{a}	5.564 ± 0.658^{ab}	5.972 ± 0.627^{a}	3.026 ± 0.353^{ab}
DiR	1	3	2.677 ± 0.904^{a}	-0.461 ± 0.119^{b}	2.214 ± 0.786^{a}	0.882 ± 0.426^{b}
DiR	4	3	1.472 ± 1.217^{a}	-0.029 ± 0.17^{b}	1.443 ± 1.049^{a}	0.577 ± 0.357^{b}

Treatment	Day	N	Chl.A.µg.mL.	Chl.B.µg.mL.	T.Chl.µg.mL.	Car.
DiR	7	3	5.675 ± 6.217[a]	-0.747 ± 0.944[b]	4.924 ± 5.269[a]	1.491 ± 1.559[ab]
DiR	10	3	2.758 ± 1.96[a]	-0.562 ± 0.41[b]	2.192 ± 1.548[a]	0.649 ± 0.529[b]
DiR	13	3	3.557 ± 1.901[a]	-0.362 ± 0.161[b]	3.193 ± 1.741[a]	0.993 ± 0.517[b]

The effect of copper and diclofenac on the growth of *Planktothrix* sp. shows a decrease in growth as highest growth of 2.14g was recorded in the control treatment (CoR) and the lowest 0.82g was recorded in the combined treatment of diclofenac and copper (DiRCuR) on day 14 of the experiment as shown in figure 1. The effect of copper and diclofenac on chlorophyll a content of *Planktothrix* sp. compared to the control treatment shows that copper and diclofenac in their combined form inhibits the production of chlorophyll a in *Planktothrix* sp. as shown in figure 2. The impact of copper and diclofenac on chlorophyll b pigment of *Planktothrix* sp. demonstrates a rise in chlorophyll b production with a positive correlation, as illustrated in figure 3. Diclofenac and copper treatment had a negative effect on total chlorophyll as day 4 of the diclofenac treatment (DiR) produced the lowest output, 1.443 g/l, whereas day 4 of the copper treatment (CuR) produced the highest total chlorophyll, 15.66 g/ml (Figure 4). According to the results of the experiment, day 4 of the copper treatment (CuR) generated the maximum carotenoid content (9.851µg/ml), whereas day 4 of the diclofenac treatment produced the lowest amount (0.577µg/ml), Figure 5 illustrates this. Figure 6 show that the control treatment (CoR) has the highest reactive species (ROS) of 0.075µg/ml on day 7, while the lowest was recorded on day 14 of the same treatment. Copper and diclofenac in their combined form inhibits protein production, as lowest total protein of 4888.89mg/ml in *Planktothrix* sp. was recorded on day 7 and highest 15155mg/ml was recorded on day 14 of DiR treatment as shown in figure 7.

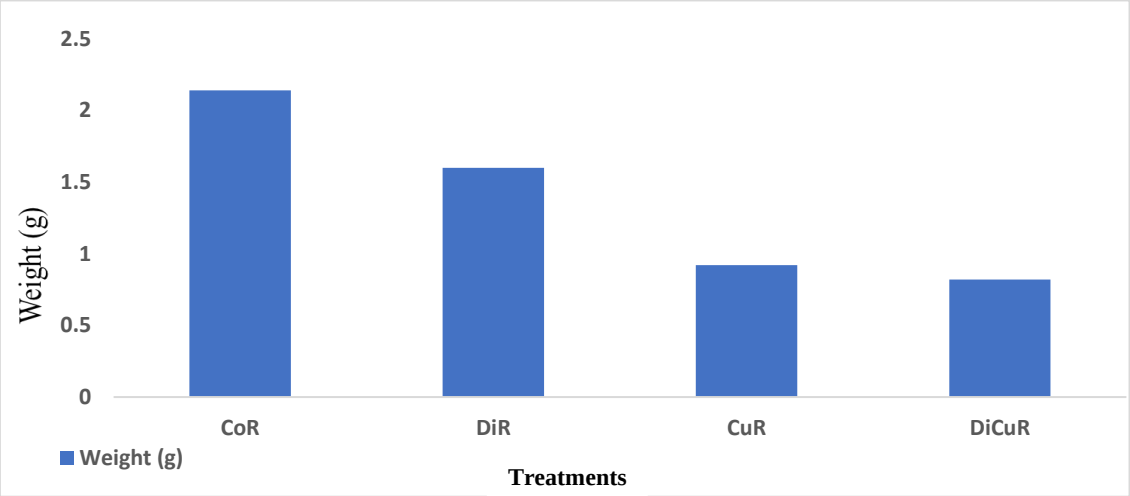

Figure 1: Growth and dry weight of *Planktothrix* sp.
Keys: CoR= Control, CuR= Copper, DiR= Diclofenac, DiCuR= Diclofenac + Copper

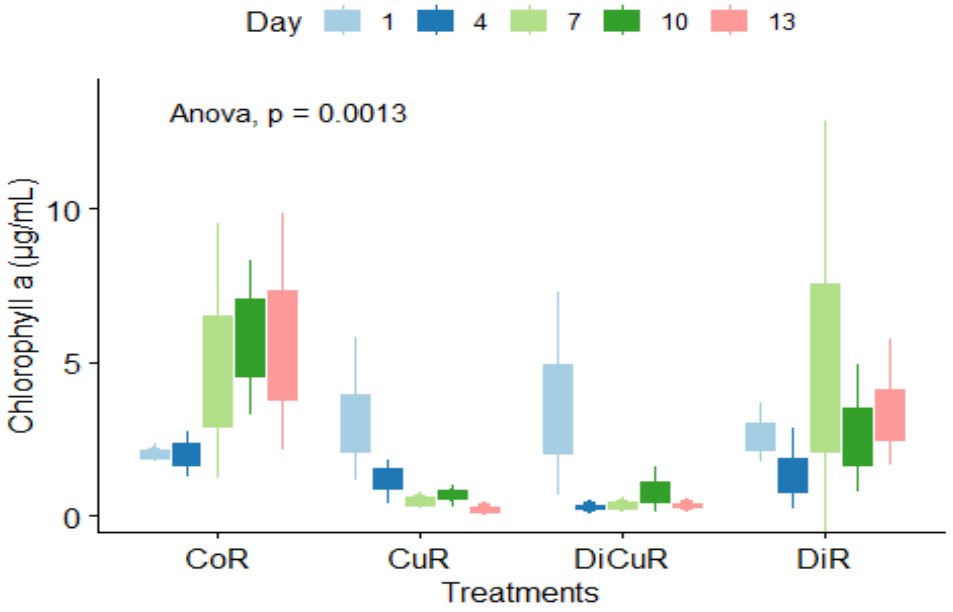

Figure 2: Chlorophyll a content of *Planktothrix* sp. under different treatments condition for 14 days
Keys: CoR= Control, CuR= Copper, DiR= Diclofenac, DiCuR= Diclofenac + Copper

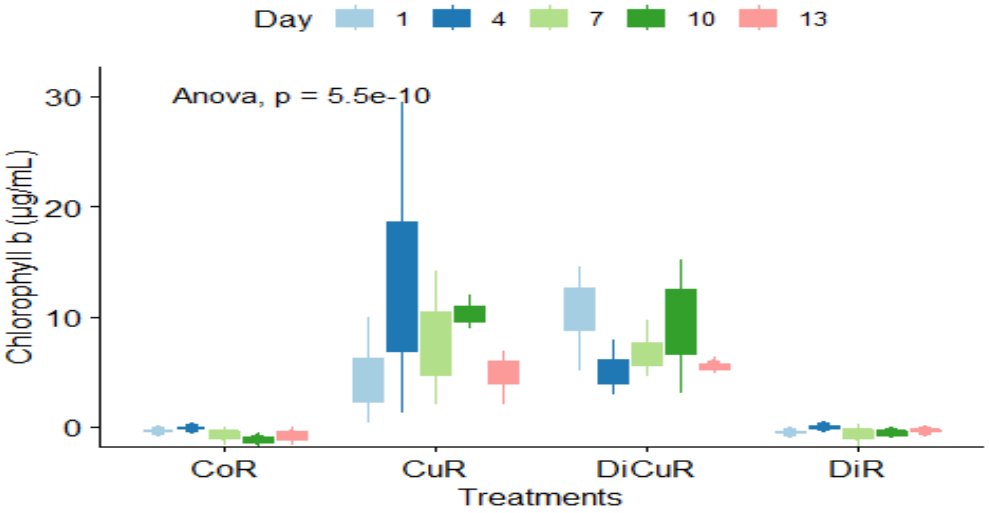

Figure 3: Chlorophyll b content of *Planktothrix* sp. under different treatments
Keys: CoR= Control, CuR= Copper, DiR= Diclofenac, DiCuR= Diclofenac + Copper

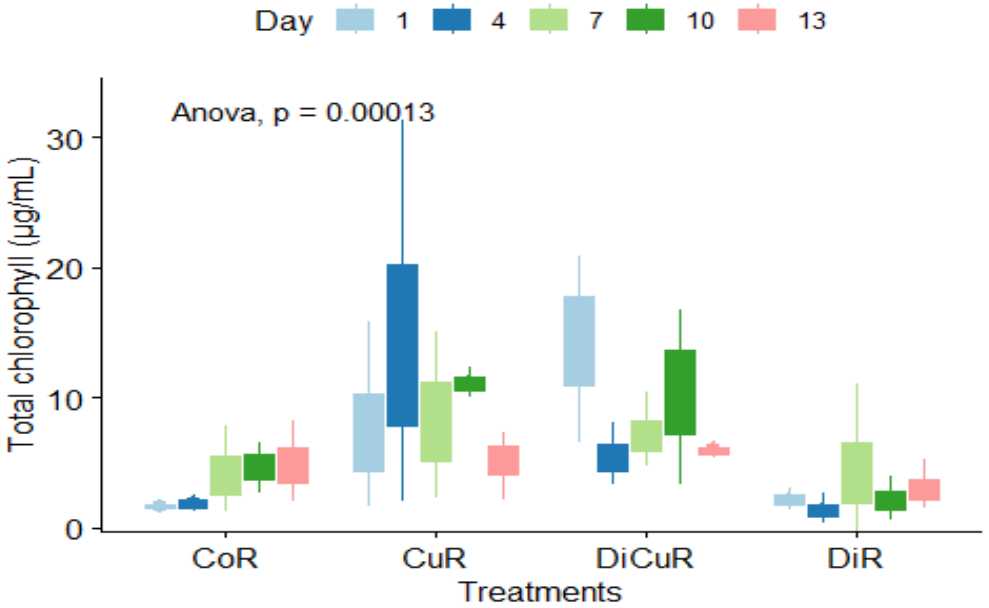

Figure 4: Total Chlorophyll content *Planktothrix* sp. under different treatments
Keys: CoR= Control, CuR= Copper, DiR= Diclofenac, DiCuR= Diclofenac + Copper

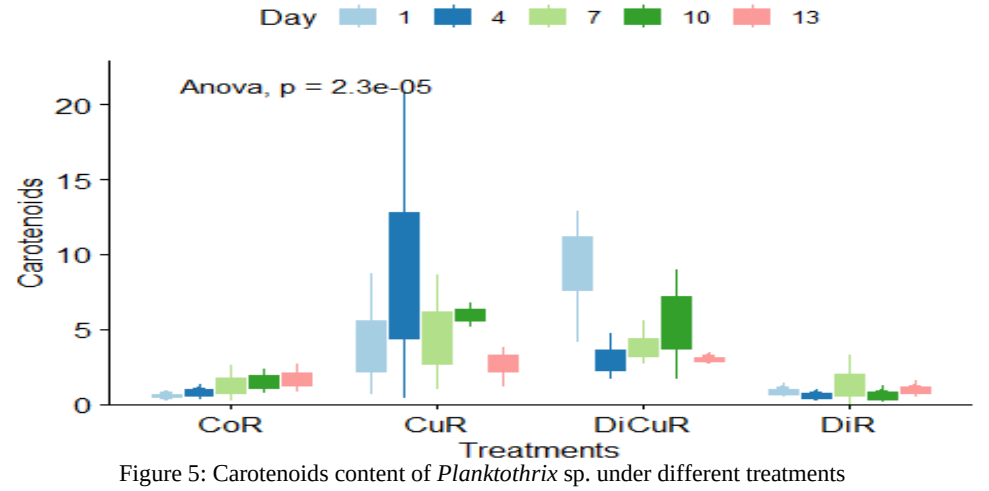

Figure 5: Carotenoids content of *Planktothrix* sp. under different treatments
Keys: CoR= Control, CuR= Copper, DiR= Diclofenac, DiCuR= Diclofenac + Copper

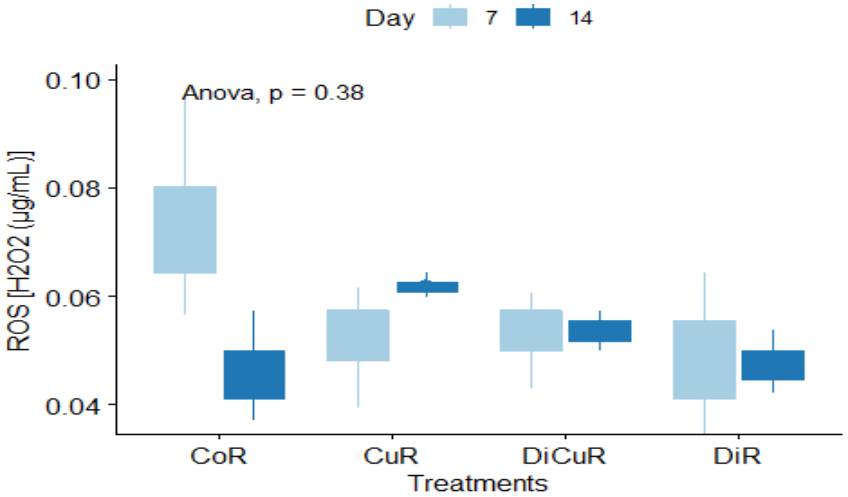

Figure 6: Reactive Oxygen Species activity of *Planktothrix sp.* Under different treatments
Keys: CoR= Control, CuR= Copper, DiR= Diclofenac, DiCuR= Diclofenac + Copper

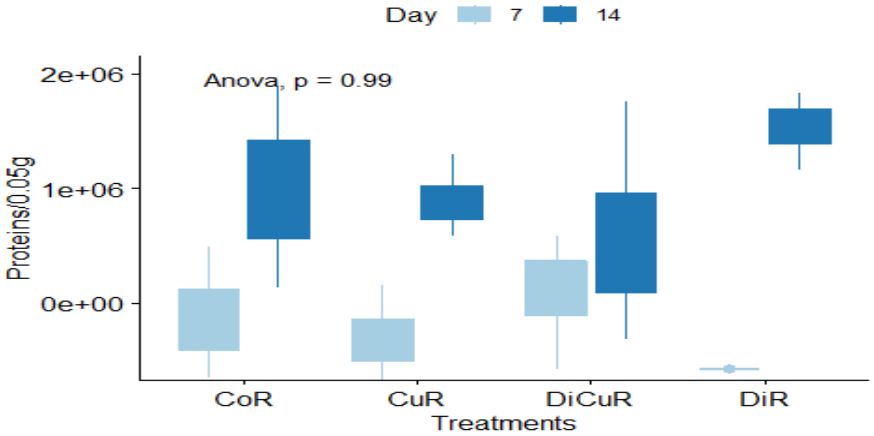

Figure 7: Total protein content of *Planktothrix sp*. Under different treatments
Keys: CoR= Control, CuR= Copper, DiR= Diclofenac, DiCuR= Diclofenac + Copper

The highest production of 0.008µg/ml of malondialdehyde which is an oxidative stress marker was produced on day 7 of the copper treatment (CuR) and lowest of 0.001µg/ml on day 7 of the control treatment as shown in figure 8. From figure 9, it was shown that the highest GST of 39.35µg/ml was produced on say 7 of the control treatment (CoR) while the lowest of 6.94µg/ml was produced on day 7 of diclofenac treatment (DiR) in *Planktothrix* sp. The effect of copper and diclofenac on peroxidase activity of *Planktothrix* sp. shows an increase in peroxidase activity 0.122µg/ml as compared to the control 0.041µg/ml which is as a result of induced stress it was exposed to in experiment as shown in figure 10.

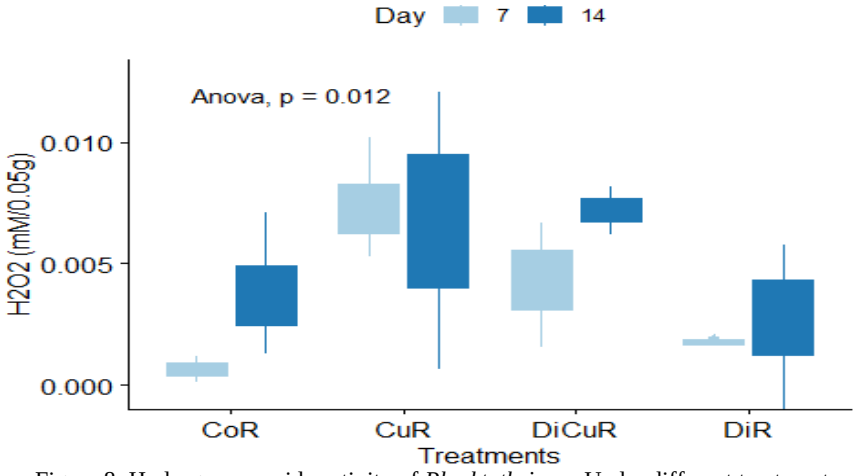

Figure 8: Hydrogen peroxide activity of *Planktothrix sp*. Under different treatments
Keys: CoR= Control, CuR= Copper, DiR= Diclofenac, DiCuR= Diclofenac + Copper

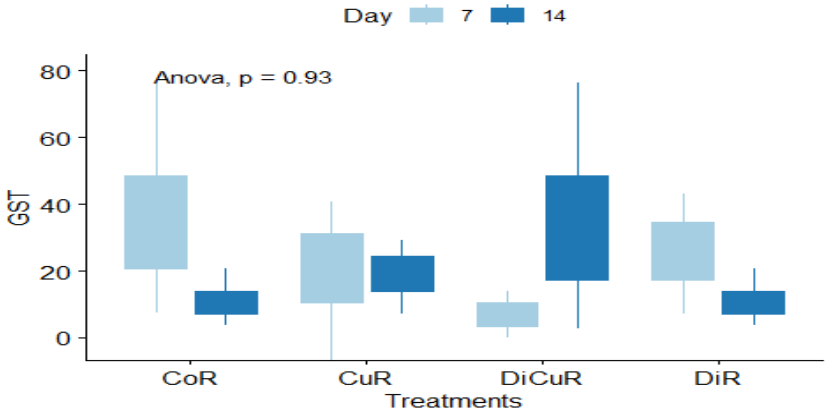

Figure 9: Glutathione S- transferase activity of *Planktothrix sp.* Under different treatments
Keys: CoR= Control, CuR= Copper, DiR= Diclofenac, DiCuR= Diclofenac + Copper

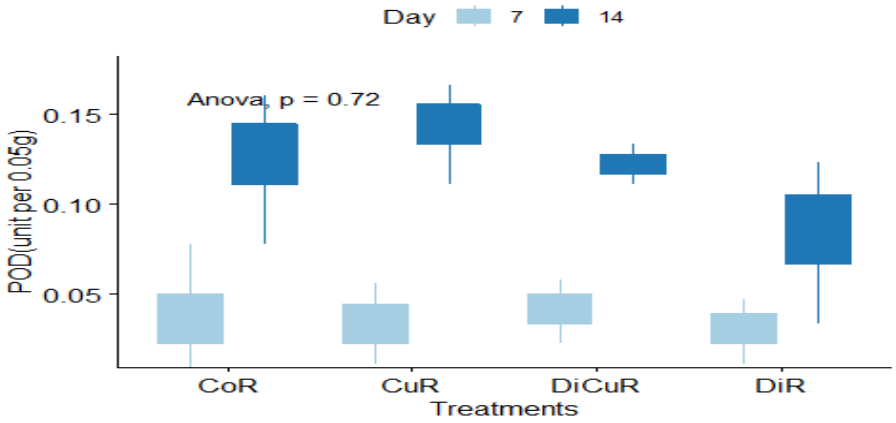

Figure 10: Peroxidase activity of *Planktothrix sp*. Under different treatments
Keys: CoR= Control, CuR= Copper, DiR= Diclofenac, DiCuR= Diclofenac + Copper

This study shows biomolecule and biochemical composition of *Planktothrix* exposed to individual and combined Copper and Diclofenac in Table 3 to have recorded lowest in day 7 for ROS, Protein, MDA, GST and POD (0.049 ± 0.014^{a}, -106666.67 ± 545690.19^{b}, 0.001 ± 0.001^{a}, 6.94 ± 6.94^{a}, 0.03 ± 0.017) and highest at (0.075 ± 0.019^{a}, 1004444.44 ± 867520.95^{ab}, 0.008 ± 0.002^{a}, 39.35 ± 32.08^{a}, 0.141 ± 0.026^{a}) respectively.

Table 3: Biomolecule and biochemical composition of Planktothrix eexposed to Individual and Combined Copper and Diclofenac (mean ± sd)

TREATMENT	DAY	N	ROS	PROTEIN	MDA.0.05G.	GST	POD.
CoR	7	3	0.075 ± 0.019^{a}	-106666.67 ± 545690.19^{b}	0.001 ± 0.001^{a}	39.35 ± 32.08^{a}	0.041 ± 0.032^{b}
CoR	14	3	0.046 ± 0.009^{a}	1004444.44 ± 867520.95^{ab}	0.004 ± 0.003^{a}	11.57 ± 8.02^{a}	0.122 ± 0.038^{a}
CuR	7	3	0.051 ± 0.01^{a}	-284444.44 ± 390631.02b	0.008 ± 0.002^{a}	18.52 ± 22.32^{a}	0.033 ± 0.022^{b}
CuR	14	3	0.062 ± 0.002^{a}	915555.56 ± 328858.86^{ab}	0.007 ± 0.006^{a}	18.52 ± 10.61^{a}	0.141 ± 0.026^{a}
DiCuR	7	3	0.052 ± 0.008^{a}	48888.89 ± 538860.25^{ab}	0.004 ± 0.002^{a}	6.94 ± 6.94^{a}	0.041 ± 0.017^{b}
DiCuR	14	3	0.054 ± 0.004^{a}	648888.89 ± 964557.09^{ab}	0.007 ± 0.001^{a}	37.04 ± 34.26^{a}	0.122 ± 0.011^{a}
DiR	7	3	0.049 ± 0.014^{a}	-573333.33 ± 0.000001^{b}	0.002 ± 0^{a}	25.46 ± 17.48^{a}	0.03 ± 0.017^{b}
DiR	14	3	0.048 ± 0.005^{a}	1515555.56 ± 315054.38^{a}	0.003 ± 0.003^{a}	11.57 ± 8.02^{a}	0.081 ± 0.042^{ab}

Discussion

Increasing levels of active pharmaceutical ingredients are changing the physicochemical characteristics of water bodies. Consequently, these compounds are altering the growth patterns and community structure of aquatic organisms (Brodin *et al*., 2014; Daughton and Brooks, 2011; Guo *et al*., 2015). The growth of the *Planktothrix* sp. went along the treatment gradient, as control without contamination and presence of high nutrient stimulated its growth, while diclofenac and copper treatment recorded the lowest growth because generally plants, animals, microorganism and specifically cyanobacteria grows better when there is more nutrient and less stress and these nutrients are highly needed to enhance biomass production for the organism which in turn enables other secondary processes (Yadav *et al.*, 2022)

Chlorophyll a, as the primary pigment in algae, can act as a standard to reflect the growth and proliferation of algae (Fan *et al*., 2018). From the study conducted above, Copper at 2.5g/L negatively affected the production of chlorophyll a which is the primary photosynthetic pigment of *Planktothrix* sp. this is because high level of copper can damage photosynthesis and cellular redox balance, and can change the ultrastructure of cell that usually leads to death (Nagalakshmi, 2001). Significant levels of chlorophyll a are an indication of *Planktothrix* to the treatments is a sign of acclimatization. In addition, the excess Cu can disturb the photosynthesis and

redox equilibrium of the cells, disrupting the cells' ultrastructure and, finally, leading to death (Wang *et al.*, 2010).

Diclofenac induces excessive ROS generation in aquatic systems, which damages phytoplankton through oxidative stress, according to Chia *et al*., 2018. Due to the high quantities of internal hydrogen peroxide, the phytoplankton experienced oxidative stress. In stressed phytoplankton, GST and POD enzymes remove ROS (Chia *et al*.2019).

Reactive oxygen species (ROS) are a group of highly reactive molecules containing oxygen that are formed as natural byproducts of cellular metabolism. They include free radicals such as superoxide anion (O2•−), hydroxyl radical (•OH), and non-radical species like hydrogen peroxide (H2O2) and singlet oxygen (^1O2). While ROS play important roles in various physiological processes, their excessive production can lead to oxidative stress, causing damage to cellular components such as DNA, proteins, and lipids. Concentration of ROS was highest in the control treatment and lowest in diclofenac treatment which is a result of increased oxidative stress and the ability of cyanobacteria to scavenging of reactive oxygen species (ROS) (Rezayian *et al.*, 2016)

Malondialdehyde (MDA) is a naturally occurring organic compound that is formed as a byproduct of lipid peroxidation, a process that occurs when free radicals attack polyunsaturated fatty acids in cell membranes. It is one of the most studied and frequently used biomarkers for assessing oxidative stress and lipid peroxidation levels in various biological systems. MDA is a reactive aldehyde that can interact with various cellular components, such as proteins, nucleic acids, and phospholipids, leading to the formation of adducts that can disrupt normal cellular functions. The highest was recorded in the copper treatment (CuR) led to the disruption of the cell membrane. Enzymes are proteins in nature; therefore, the highest production protein was recorded on day 14 of diclofenac treatment.

CONCLUSION

This study showed that the individual and interactive effect of copper and diclofenac had a significant effect on the growth rate of *Planktothrix*, where highest growth (dry weight) of 2.14g was recorded in the control treatment (CoR) and lowest growth of 0.82g was recorded in the diclofenac and copper treatment (DiRCuR). This implies that copper and diclofenac may have a toxic or inhibitory effect on the growth of *Planktothrix*, especially when combined. This could have implications for aquatic ecosystems where these substances are present, potentially disrupting the growth of algae and affecting the food web and overall water quality. Given the negative impact of copper and diclofenac on *Planktothrix* growth, it is recommended that efforts be made to reduce the concentration of these substances in aquatic environments. This can be done by monitoring and regulating the discharge of pharmaceutical and industrial waste into water bodies.

There was a significant effect on some antioxidant activities exhibited by *Planktothrix* sp. in all treatments (MDA) $p<0.05$ and no significant effect on ROS, GST and POD where $p>0.05$. This could imply that while the treatments might cause oxidative stress in *Planktothrix sp.*, the organism's mechanisms to neutralize ROS (such as GST and POD) may not be sufficiently affected under the conditions tested. It is recommended that further studies be conducted to explore why only MDA levels were significantly affected and not other antioxidant markers like ROS, GST, and POD.

There was a synergistic effect of copper and diclofenac on the growth and malondialdehyde (MDA) content of *Planktothrix* sp. A synergistic effect suggests that the interaction between copper and diclofenac produced a greater impact than the individual effects of each substance alone. Given the synergistic effect observed, it is recommended that the discharge of copper and diclofenac into aquatic environments be carefully regulated and reduced. Environmental monitoring programs should focus on detecting both substances in water bodies to prevent potential toxic effects on algae and other aquatic organisms.

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