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Effects of copper Sulphate and Direct Blue-218 on growth, morphology and reproduction of *Daphnia magna*

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Abstract

The present study is aimed to evaluate toxic effects stimulated by copper-complexed dye DB-218 and CuSO₄ on *D. magna*. Acute toxicity tests and Chronic toxicity tests were performed in accordance with OECD guidelines no 202 and 211 respectively. Atomic adsorption spectroscopy revealed presence of 0.421mg of copper in per gram of Direct Blue-218. The LC₅₀ values of DB-218 and CuSO₄ were found to be 1mg^{-L} and 0.0008 mg^{-L} respectively at 48 hours. Morphological deformity in daphnids as swelling in carapace, distortion of gut was highly pronounced in higher concentrations of the test substances. Long term exposure affected all aspects of reproduction in *D. magna* at environmental concentrations higher than 0.1mg^{-L} and 0.0001mg^{-L} for DB-218 and CuSO₄ respectively. However, the overall effects of DB-218 on reproduction were lower than CuSO₄ which unquestionably indicate CuSO₄ is more lethal than DB-218.

Keywords: Direct blue-218 (db-218), copper sulphate (cuso₄), *Daphnia magna*, toxicity

Introduction

During the onset of nineteenth century, infusion of dyes exclusively occurred from natural sources, (animals or vegetables). With the passage of time the dye extraction from natural sources came to halt and was overtaken completely by synthetic sources in twentieth century (de campos *et al.* 2013) [12]. Rapid industrialization and market demands being the major causes for the substitution. Among dyes azo dyes form the largest class used in varied domains (such as textile industry, leather tanning industry, hair colourings, wood staining, agricultural, biological, paper production, food, colour photography, pharmaceuticals and medicine, cosmetic, and chemical research) (Jagruiti, 2015) [20]. About 60% to 80% of azo dyes have been found carcinogenic to humans including Direct Black 38 (Benkhaya *et al.* 2020; Berradi *et al.* 2019) [7, 8]. Being inexpensive direct dyes are applied on wide range of materials including cellulose paper, leather, polyamide (Benkhaya *et al.* 2020) [7]. Seventy five percent of direct dyes are used to dye cotton or viscose alone (Ouslimani and Boureghda, 2018) [28]. On the other hand, there is the growth in diminution of water resources (Vakili *et al.* 2019) [37]. In aquatic bodies, more than 100 tons of dyes are discharged every year (Yagub *et al.* 2012) [40]. The prominent contributors to water pollution also include heavy metals contamination which occurs through various man-made activities such as mining and smelting operations besides natural phenomena (weathering and volcanic eruptions). Some well-known toxic heavy metals are cadmium, lead, copper, mercury, zinc, arsenic, and aluminium (Verma and Dwivedi, 2013) [39]. Heavy metals are non- biodegradable in nature and gradually pile up in living beings eventually become toxic (Fu and Wang, 2011) [17]. The inappropriate treatment of toxic effluents that are discharged from textile industries into natural water bodies raise havoc among various ecological receptors. Relatively less data is available of certain selected dyes only concerning their toxicological/ecotoxicological studies which demands further assessment of dyes for the betterment of ecological receptors. Moreover, little information is available for the intercomparison of dye and heavy metal toxicities which also demands test results under unified conditions. The primary objective of this investigation is to assess the environmental safety of CuSO₄ and DB-218 with respect to *D. magna* which is utilized in toxicity bioassays.

Materials and methods

Chemicals: Analytical grade of dye Direct Blue-218 (DB-218) was purchased from Karsandas Mavji Dye Manufacturer (India) as fine dark blue pure powder. Copper sulphate pentahydrate was purchased from Thermo Fisher Scientific (India) as bright blue small crystals. Yeast Extract Powder was purchased from Molychem (India).

Preparation of *Daphnia magna* culture

Commonly known as Water flea, *D. magna* -a freshwater Zooplanktonic organisms belonging to subphylum crustacea of phylum Arthropoda were obtained from Dal Lake of Srinagar, Jammu and Kashmir. Daphnids were collected in early morning to ensure maximum catch (Munirasu *et al.* 2016) [24] with the help of zooplankton net of 0.04mm (Khan *et al.*, 2020) [30]. After isolating Daphnids from other zooplanktons, *D. magna* was subjected to species identification on the basis of specific morphological characteristics. Cultures of *Daphnia magna*, each containing ten adult females were maintained in 400ml of double distilled water. The pH of the cultures was maintained and at 22±2°C under cool light with 12-h light:12-h dark photoperiod (Table-1). *D. magna* was given yeast extract as feed regularly (Turcihan *et al.* 2022) [36] (0.1 and 0.2 mg C/Daphnia/day OECD-2012) [25, 26]. Dead daphnids or impurities if any settled at the bottom of the culture chambers were removed carefully.

Physico-Chemical parameters of Cultures and Data Analysis

Water quality parameters such Temperature, pH and Conductivity were monitored regularly using digital thermometer (HTC DT-1), digital pH meter (Systronics-802) and digital conductivity meter (Systronics-307). Other parameters such as, Dissolved oxygen, Total alkalinity, Hardness (as CaCO₃) were monitored every third day before medium renewal using titration method as described in APHA 2005. LC₅₀ Values were computed by Probit analysis. One-way ANOVA was used to analyze the statistical difference between the controls and test substance concentrations at $p < 0.05$ followed by Tukey's Multiple Comparison test by using GraphPad Prism 8 software. Intrinsic rate of population increase (rm) was determined by Euler's Equation given below;

$$\sum_{x=0}^n e^{-rm_x} l_x m_x = 1$$

Where r is the population increase rate (per day), x is the age in days (0 ... n), l_x is the survival probability in the age x, and m_x is the fecundity in the age x

Acute exposure

Initially the amount of copper present in one-gram powdered sample of direct blue-218 was estimated by using atomic adsorption spectroscopy (Agilent technologies AA-200) (Bae *et al.* 2003, Garduño *et al.* 2020, Bagherian *et al.* 2019) [4, 38, 5]. Subsequently the working concentrations of the test substances were conceptualized on its basis. The acute test was executed by following OECD Guideline 202 [25, 26]. Twenty-four-hour old *Daphnia magna* from healthy stock were used for the test. The different concentrations of DB-218 and CuSO₄ solutions were prepared by serial dilution

from fresh stock solutions in geometric series (with a factor of two). Seven different Concentrations of DB-218 (1, 2, 4, 8, 16, 32 and 64 mg⁻¹) and CuSO₄ (0.000420, 0.00084, 0.00168, 0.0036, 0.00672, 0.001344 and 0.002688 mg⁻¹) and three replicates for each treatment and a blank control group for each test substance to resolute accuracy of test were conducted. The test was conducted in vessels containing 100ml test solution and a total of ten neonates were placed in each test concentration. Feeding during the test was omitted (in both control and test series). The test was performed at 20±1°C under 12-h light:12-h dark photoperiod, the pH of the test solution for DB-218 ranged from 6.82-7.12 and for CuSO₄ the pH ranged from 6.78-7.05 from lowest to highest concentration of solutions. The medium of the test was not renewed and number of immobile and mobile daphnids were recorded at 24 and 48 hours after the beginning of the test. Immobility was determined by gently stirring the test vessel and registering the specimen that were unable to swim within 15 seconds. The structural deformity in *Daphnia magna* was visualized under Optical Microscope (Labomed 40X and 100X) at 48hours.

Full life-cycle test

To estimate the effect of DB-218 and CuSO₄ on the reproductive output of *Daphnia magna*, the 21-days chronic toxicity tests were performed in accordance with guideline no. 211 of OECD [25, 26]. The analysed endpoints were: fecundity (number of neonates produced), growth (body length of organisms) and longevity (surviving organisms). The wellfed healthy neonates less than 24 hours old from the maintained cultural stocks were used for the test. Five different concentrations of DB-218 (0.5,0.1,0.2,0.4,0.8 mg⁻¹) and CuSO₄ test solutions (0.00005, 0.0001,0.0002,0.0004 and 0.0008 mg⁻¹) were used based on the outcome of acute toxicity test in a geometric concentration of 2 and three replicates for each treatment and two blank controls to resolute accuracy of test were conducted. The test was conducted in vessels containing 100ml test solution and a total of ten neonates were placed in each test concentration. Feeding in test vessels was regularized and the test substances were renewed thrice in a week. Temperature, and pH values were measure weekly; the test was carried out at 20±2°C under 12-h light:12-h dark photoperiod, the pH of test solution ranged from 6.84-7.05 (DB-218) and 6.78-7.0 (CuSO₄) from lowest to highest concentration of solutions. Finally, micrometry was performed at the end of experiment to measure body length (from the helmet to the front end of the tail spine, excluding the tail spine) of organisms. Each test organism was measured thrice (Li *et al.* 2023) [23].

Results and Discussion

Table 1: Showing Physico-Chemical values of *D. magna* cultures

pH	Temperature (°C)	Conductivity (mS)	Dissolved Oxygen(mg ⁻¹)	Total hardness (mg ⁻¹)
7-8	22±2	0-0.06	7-7.8	130-150

Atomic absorption analysis

To quantify the amount of copper present in the dye, atomic adsorption spectroscopy was employed which revealed presence of 0.421mg of copper in per gram of dye.

Acute Toxicity Assay

Effect on Mobility of *D. magna*

During the acute toxicity test of the dye DB-218 no lethal effects were observed in concentrations $1\text{mg}^{-\text{L}}$, $2\text{mg}^{-\text{L}}$ and 100% immobility or mortality were observed in concentrations $8\text{mg}^{-\text{L}}$, $32\text{mg}^{-\text{L}}$, $64\text{mg}^{-\text{L}}$, whereas 83.3% and 16.6% immobility or mortality were observed in concentrations $16\text{mg}^{-\text{L}}$ and $4\text{mg}^{-\text{L}}$ respectively. Also, 0% mortality in control group confirmed accuracy of test. Immobility was induced in *D. magna* in dose-dependent manner which could be attributed to the geometric increase in the concentration of copper in the dye. The results indicate that the LC_{50} of Direct Blue-218 for *D. magna* is $7\text{mg}^{-\text{L}}$ at 24 h and $1\text{mg}^{-\text{L}}$ at 48 h. The azo dye-induced toxicity is significantly influenced by the chemical makeup of the dye, commercial formulations, impurities, physical interactions, breakdown of byproducts that have the potential to cause cancer (Øllgaard *et al.* 1999) [27] and light penetration (Baumer, 2018) [6]. In parallel investigation of acute toxicity on *D. magna* using Copper sulphate of five different concentrations, the LC_{50} values for copper sulphate ranged from $2.5\text{mg}^{-\text{L}}$ at 24 h to $0.0008\text{mg}^{-\text{L}}$ at 48 h. Mortality was observed in wide range of concentrations ranging from lowest ($0.000420\text{mg}^{-\text{L}}$) to highest ($0.002688\text{mg}^{-\text{L}}$). Apparently, a decrease in temperature render *D. magna* more sensitive to Cu exposure (Bae *et al.* 2016) [3] which resulted in widened mortality across all test series. Furthermore, conspicuously nether LC_{50} values classify each test substances very toxic which resulted in inevitable mortality of *D. magna* (Kahru and Dubourguier, 2010) [21].

Effect on morphology of *Daphnia magna*

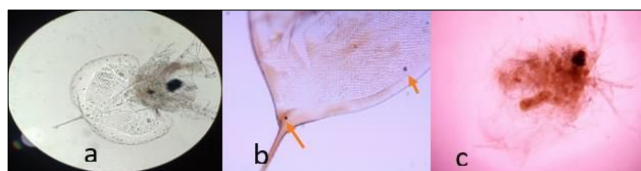


Fig 1: (a) Carapace swelling in *D. magna* on exposure to DB-218 and CuSO_4 (b)-Accumulation of lipid droplets under carapace of *D. magna*. (c)-Distortion of gut in *D. magna* on exposure to DB-218 and CuSO_4

The structural deformity in *D. magna* as a result of acute exposure to DB-218 and CuSO_4 such as swelling in carapace,

gut distortion was evident in higher concentrations of the direct blue-218 (Fig-1a) which exhibits the high intensity of toxin in higher concentration ($32\text{mg}^{-\text{L}}$). Moreover, swelling in carapace and distortion in the gut of *D. magna* was evident in all concentrations of copper sulphate solution beyond $0.0008\text{mg}^{-\text{L}}$, (Fig-1a,c) further supporting the interpretation that copper sulphate in pure form is highly toxic to *D. magna*. Various important key organismic processes such as embryo production, development, and growth actively involve stored lipids in *D. magna* (Fuentes *et al.* 2018) [18]. These lipid droplets are spherical in appearance (Cui *et al.* 2017) [11]. One of the causes of the carapace swelling could be due to the accumulation of lipid droplets under carapace which the organism displays in hostile environment (such as toxicity) (Asghari *et al.* 2012) [2]. The small bubblelike structures or lipids under the carapace of *D. magna* has been reported by Fuentes *et al.* 2018 [18]. Typically, they are neutral lipids such as cholesteryl esters and triacylglycerol encompassed by cholesterol and phospholipid. The appearance of lipid droplets is a sensitive characteristic that indicates the organism may be in a hazardous state. As a result, this issue may have a deleterious impact on *D. magna* development, molting regulation, and reproduction (Khoshnamvand *et al.* 2020) [22]. An increase in size of lipid droplet while encountering chemical stress in *D. magna* was evident similar to observations made by (Artal *et al.* 2013) [1], Tavares *et al.*, 2014 [35], Cui *et al.* 2017 [11] in their study. The distortion of gut can be attributed to accumulation of dye metabolites the gut of *D. magna*. Various other metabolites such as Copper ions can reach the cytoplasm after passing through cell membranes. This passage can generate reactive oxidative species (ROS), leading to oxidative stress (Rasera *et al.* 2019) [32]. This in turn can induce damage to biological structures, such as DNA, lipoproteins, organelles (Sadeq and Beckerman 2019) [31] mitochondrial damage, and inevitably cell apoptosis. Moreover, the higher toxicity could be ascribed to their greater solubility, which increased Cu availability in the media (Rasera *et al.* 2019) [32]. Previous studies have shown that *D. magna* tissues may absorb nano- and microplastics from the gastrointestinal tract (Schür *et al.* 2019) [33]. Similarly, the obstruction of gut cells on account of positive charge of gold nanoparticles at molecular level in *D. magna* has been reported (Dominguez *et al.* 2015) [15]. Hence the overall mechanism of toxicity is clearly intracellular.

Table 2: Showing Effects of DB-218 on reproduction, longevity and growth of *Daphnia magna* during 21 days of chronic assay (Results are expressed as means $\pm$ standard deviation).

Parameter	control	$0.05\text{mg}^{-\text{L}}$	$0.1\text{mg}^{-\text{L}}$	$0.2\text{mg}^{-\text{L}}$	$0.4\text{mg}^{-\text{L}}$	$0.8\text{mg}^{-\text{L}}$
Time of first reproduction	9 ± 1	9.3 ± 0.57	9.3 ± 0.57	9 ± 0	9.3 ± 0.57	9 ± 1
Maximum growth (mm)	4.45 ± 0.30	4.31 ± 0.07	4.18 ± 0.16	4.16 ± 0.11	4.06 ± 0.05	3.76 ± 0.152
Survival	9.3 ± 1.52	9.3 ± 0.57	8.6 ± 1.52	8 ± 3	8 ± 1	6.3 ± 1.52
Total reproduction	74.3 ± 4.16	70.6 ± 4.72	54.3 ± 13.86	50.3 ± 7.37	48.3 ± 2.88	33.6 ± 0.57

Table 3: Showing ANOVA for DB-216 at $P < 0.05$

ANOVA	SS	DF	MS	F (DFn, DFd)	P Value
Treatment (between columns)	3422	5	684.3	F (5,12) = 13.92	0.0001
Residual (within columns)	590.0	12	49.17		
Total	4012	17			

Table 4: showing Effects of Copper sulphate on reproduction, longevity and growth of *Daphnia magna* during 21 days of chronic assay (Results are expressed as means $\pm$ standard deviation).

Parameter	Control	0.00005 mg ^{-L}	0.0001 mg ^{-L}	0.0002 mg ^{-L}	0.0004 mg ^{-L}	0.0008mg ^{-L}
Time of first reproduction	9 $\pm$ 0	9.3 $\pm$ 0.57	10 $\pm$ 1	9.3 $\pm$ 0.57	12 $\pm$ 1.52	16 $\pm$ 0.57
Maximum growth (mm)	4.56 $\pm$ 0.32	4.5 $\pm$ 0.26	4.46 $\pm$ 0.25	4.23 $\pm$ 0.25	3.53 $\pm$ 0.15	3.16 $\pm$ 0.20
Survival	9 $\pm$ 1	7.3 $\pm$ 2.08	6.3 $\pm$ 0.57	4.6 $\pm$ 1.52	3.3 $\pm$ 2.08	1.3 $\pm$ 1.52
Total reproduction	69.3 $\pm$ 5.13	62.3 $\pm$ 2.51	38 $\pm$ 2	31.3 $\pm$ 10.26	12 $\pm$ 6	8 $\pm$ 2

Table-5: Showing ANOVA for Copper sulphate at $P < 0.05$

ANOVA	SS	DF	MS	F (DFn, DFd)	P Value
Treatment (between columns)	9559	5	1912	F (5, 12) = 63.02	$P < 0.0001$
Residual (within columns)	364	12	30.33		
Total	9923	17			

Chronic Assay

Effect on Reproduction

D. magna from all treatments and controls had varying broods, with the first reproduction occurring when the animals were 9 to 10 days old in DB-218 and 9 to 16 days old in CuSO₄ (Fig-2a) (Table 2 and 4). *D. magna* in CuSO₄ generated five broods in the control and 0.00005mg^{-L} concentrations, four broods in 0.0001 mg^{-L}, one brood in 0.0008mg^{-L}, and three broods in the other concentrations. Whereas *D. magna* generated four broods in all concentrations of DB-218 except for the lowest concentration (0.5 mg^{-L}) and control which generated five broods. During the chronic toxicity test an increase in the number of first brood neonates and total offspring produced by *D. magna* was found followed by a decrease with increasing concentration of both DB-218 and CuSO₄. The lowest concentration of DB-218 (0.05 mg^{-L}) and CuSO₄ (0.00005 mg^{-L}) exhibited highest number of total offspring produced (70.6 $\pm$ 4.72 in DB-218 and 62.3 $\pm$ 2.51 in copper sulphate) which were not statistically different from controls ($p < 0.05$) (Table-3, 5) thus these concentrations had no effect on *Daphnia magna* reproduction. The NOEC values were 0.05 mg^{-L} and 0.00005 mg^{-L} for DB-218 and CuSO₄ respectively (Table 2 and 4). On the other hand, in highest concentration of DB-218 (0.8 mg^{-L}) and copper sulphate (0.0008 mg^{-L}) lowest number of total offspring produced (33.6 $\pm$ 0.57 in DB-218 and 8 $\pm$ 2) was observed which varied significantly from the control group and other exposure groups ($p < 0.05$) (Fig-2b). Upon comparing the data, the overall reproductive performance of *Daphnia magna* was more affected by copper sulphate exposure. The intrinsic growth rate showed an increasing trend in control and 0.05 mg^{-L} followed by a sharp decrease in 0.1 mg^{-L} and 0.2 mg^{-L} which further slightly increased in 0.4 mg^{-L} thereafter remain unchanged in 0.8 mg^{-L} of DB-218, whereas the intrinsic growth rate of *D. magna* in copper sulphate was higher in groups 0.00005 mg^{-L}, 0.0004 mg^{-L} and lower in groups 0.0001 mg^{-L}, 0.0002 mg^{-L} and 0.0008 mg^{-L} (Fig-3a, b). Long-term exposure resulted in strong, contrasted impacts, with daphnids subjected to sub-lethal concentrations of DB -218 and Copper sulphate displaying delayed maturation, less neonates produced, and consequently considerable reductions in intrinsic rate of natural increase(r), when compared to control daphnids (Pestana *et al.* 2010) [29]. As the concentration of DB-218 increased, a decrease in the number of neonates produced, smaller brood size, and reduced neonate number were observed compared to the control group. These disparate effects point to the involvement of distinct toxicity mechanisms, such as increased metabolic cost, reduced energy acquisition (potentially via inhibition of digestive enzyme activity), and targeted inhibition of reproduction

(apparently via inhibition of vitellogenesis) (De Schamphelaere, 2007) [14]. Moreover, the decreased number of neonates produced, reduced brood size, and smaller neonate size compared to the control group observed in our study indicate that DB-218 exposure can lead to population decline. These disparate effects point to the involvement of distinct toxicity mechanisms, such as increased metabolic cost, reduced energy acquisition (potentially via inhibition of digestive enzyme activity), and targeted inhibition of reproduction (apparently via inhibition of vitellogenesis) (De Schamphelaere, 2007) [14]. Furthermore, the overall decreased reproductive output can also be explained by the presence of Cu²⁺ in dye whose exposure even at lowest concentration (0.006 mg^{-L}) causes immunological suppression in *D. magna* (Heinlaan *et al.* 2008) [19]. In case of daphnids exposed to various concentrations of copper sulphate. It is evident as the concentration of copper sulphate increased, a decrease in the number of neonates produced, smaller brood size, and reduced neonate number were observed compared to the control group. Smaller Cladocera's may absorb particles and ions more quickly than larger animals due to their increased respiration and circulation rates (Song *et al.* 2015) [34]. The reduction in reproduction of *D. magna* with increasing concentrations of copper is in agreement with outcome Sadeq and Beckerman (2019) [31]. These findings also imply that *D. magna* during long term exposure responded to varying temperatures by directing more energy into defence systems rather than growth and reproduction. Additionally, oxidative damage at high temperatures increases the possibility of decreased offspring fitness (Bae *et al.* 2016) [3]. Furthermore, the occurrence of increased total reproduction value in initial sub-lethal doses of CuSO₄ may be classified as hormesis i.e. an effect which occurs when toxic substances have a stimulatory effect on organisms at sub-lethal concentrations (Bodar *et al.* 1988) [9].

Effect on Survival

DB-218 mortality did not exceed 19.3% in all test series and 6.6% in control, whereas in CuSO₄ 33.3% mortality was observed in all test series and 10% in control (Fig-2c). The test was performed at 22 $\pm$ 2°C under 12-h light:12-h dark photoperiod, the pH of the test solution for DB-218 ranged from 6.84-7.05 and for CuSO₄ the pH ranged from 6.78-7.0 from lowest to highest concentration of solutions. The results clearly indicate that with an increase in toxin level the survival of the parent decreases. Mortality results of direct blue-218 and copper sulphate clearly indicate that with an increase in toxin level the survival of the parent decreases. The formation of hazardous chemicals, primarily aromatic amines, during the enzymatic breakdown of azo dyes is the primary issue of toxicity as they are mutagenic (Brüschweiler and Merlot, 2017) [10]. Whereas Cu ions could be introduced

into cells and stored in the soft sections of daphnids particularly intestine and appendages resulting in an intracellular toxicity mechanism (Rasera *et al.* 2019) [32]. It was validated through RT-PCR, an exposure to copper decreased the expression of genes namely DV437823 and DV437821 in *D. magna*, which may have a potential role in generating immunological response. Both these genes code for the proteins that are related to α -1, 3-glucan binding proteins and lectins. These proteins take charge in recognizing infections and act initially in a chain reaction that activates two important systems (prophenoloxidase system as well as complement-like system) which are implicated in the arthropod immune response (innate). In order to comprehend completely how copper impacts the immune system of *D. magna* which affects its survival more evidence of this linkage is required.

Effect on Body Length

The final body length measured at the end of experiment showed variations in all test series of DB-218 and copper

sulphate was observed to be shorter in higher concentrations of DB-218 (3.76 ± 0.152 in 0.8 mg^{-1}) and copper sulphate (3.16 ± 0.20 in 0.0008 mg^{-1}) than the controls (4.45 ± 0.30 in DB-218, 4.56 ± 0.32 in copper sulphate) (Table 2 and 4) (Fig-2d). In chronic toxicity assessments, various toxicity pathways can be linked to biological properties including impacts on digestive enzyme activity may be associated to suppression of energy consumption and acquisition, altering the amount of food consumed and, as a result, interfering with adult organism length (Flohr *et al.* 2012) [16]. Additionally, digestion is intimately connected to storage cells, which generate lipid and carbohydrate reserves, potentially impacting energy metabolism. The midgut of *Daphnia magna* plays a pivotal role in copper retention and metabolism. Toxic heavy metals that enter aquatic animals through their alimentary canal can lead to decreased nutrient transport across epithelial cells, thereby exacerbating their detrimental effects, thereby exacerbating their detrimental effects (De Coen *et al.* 1997) [13].

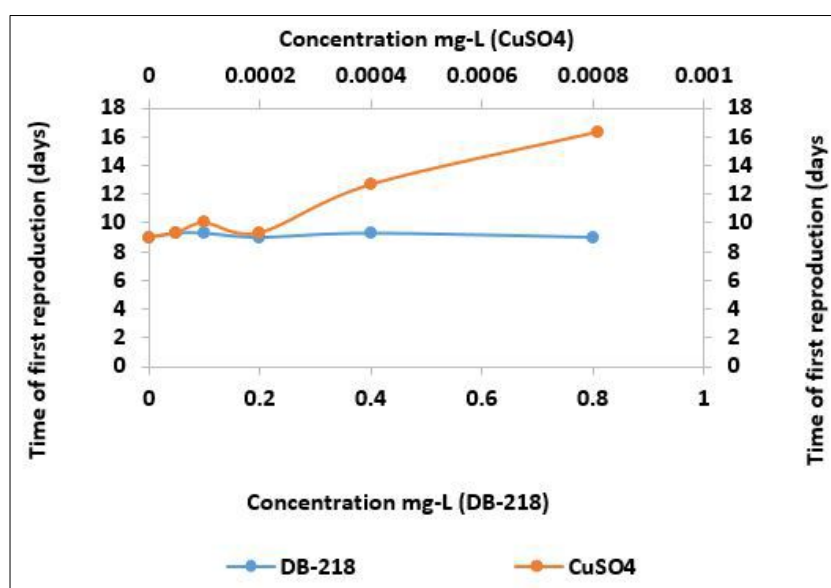


Fig 2 (a): Effect of DB-218 and CuSO₄ on Time of first reproduction

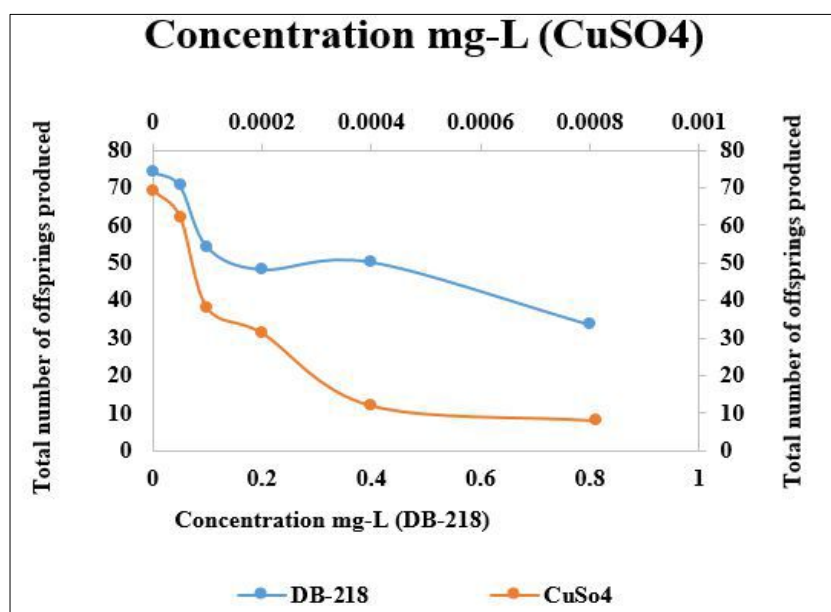


Fig 2 (b): Effect of DB-218 and CuSO₄ on Total number of offsprings produced

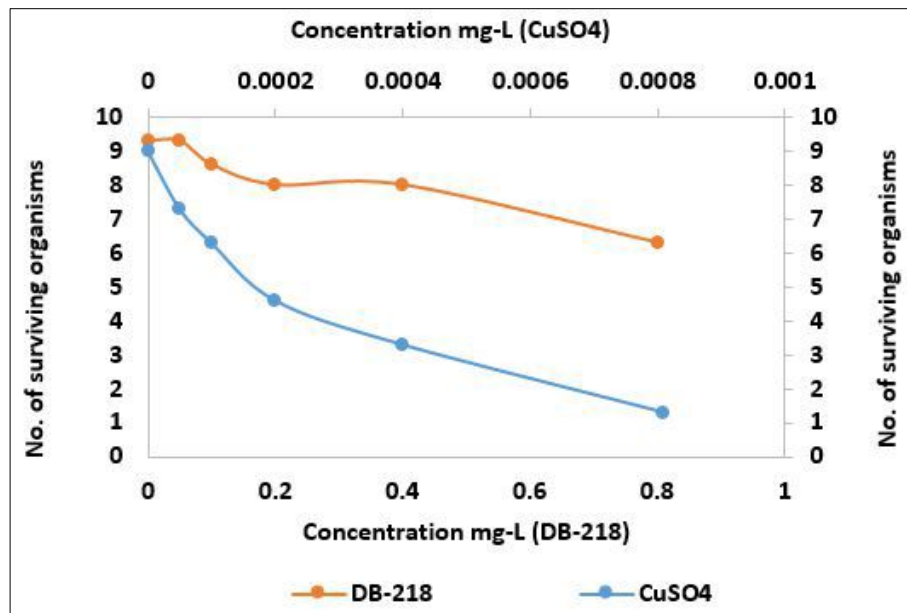


Fig 2 (c): Effect of DB-218 and CuSO₄ on No. of Surviving organisms

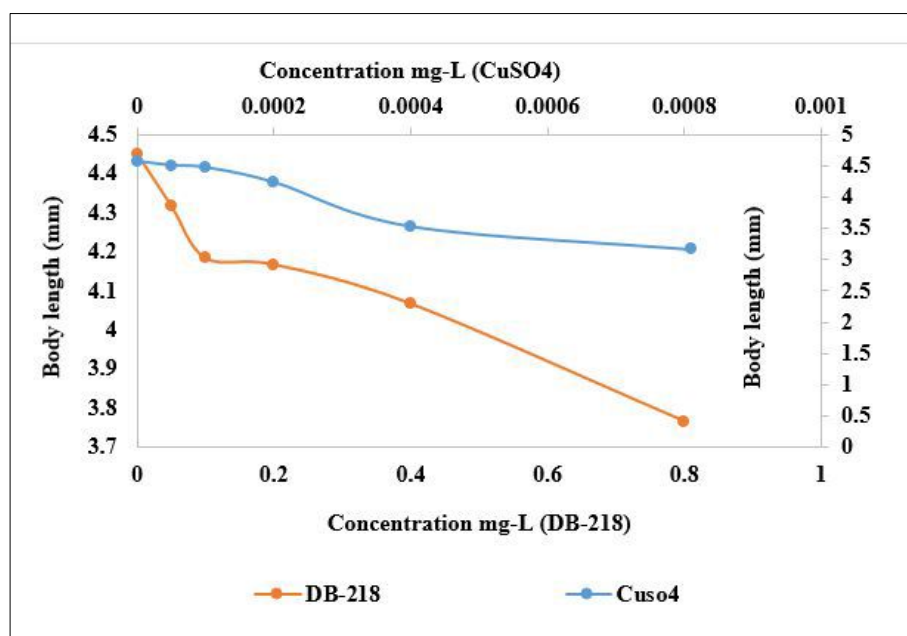
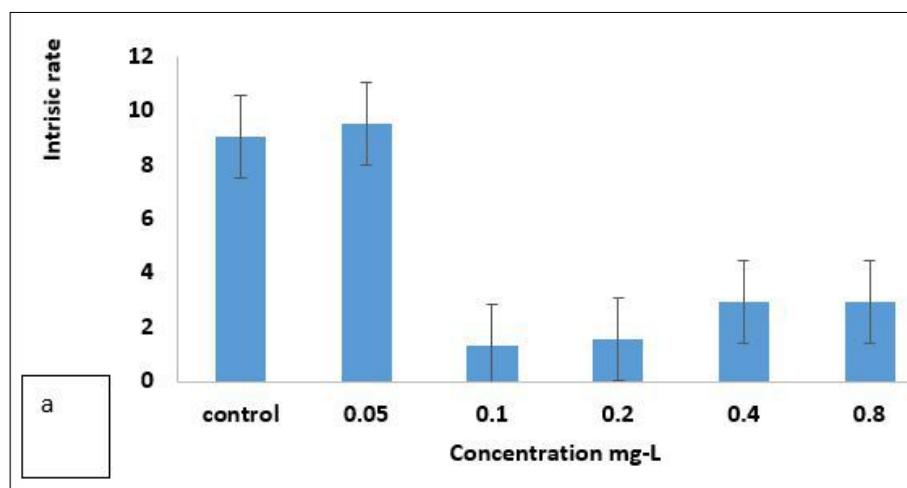


Fig 2 (d): Effect of DB-218 and CuSO₄ on Body Length (mm)



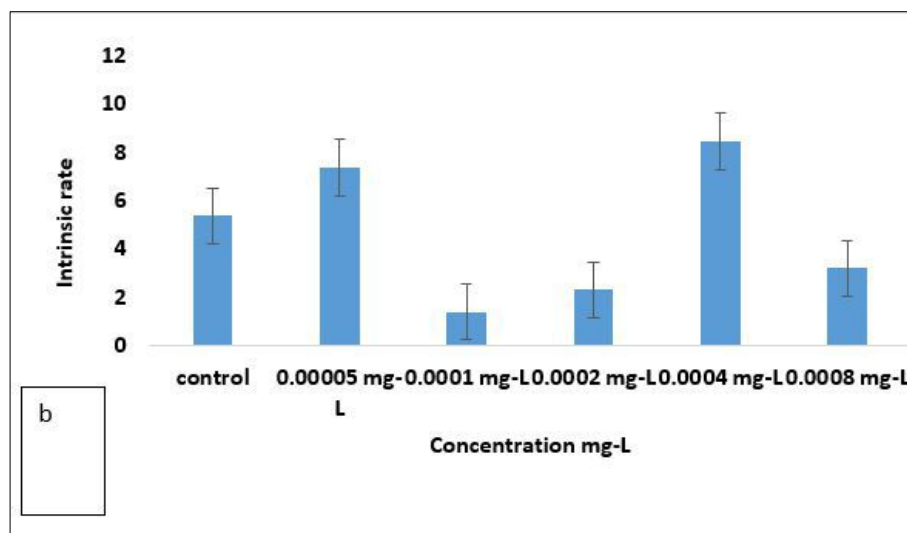


Fig 3: Intrinsic rate of population increase of *D. magna* (a) DB-218, (b) copper sulphate

Author Contributions

Taiba Bashir: Investigation, Methodology, Writing - original draft, Formal analysis; Joydeep Dutta: Investigation, Methodology, Writing - original draft, Formal analysis, Supervision; Shaista Masarat & Akshay Garg: Editing the original draft. All authors read and approved the final manuscript.

Conflicts of Interest: The authors declare no conflicts of interest.

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