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EVALUATION OF BIOACCUMULATION AND TOXICITY OF CONGO RED ON *Pseudochloris wilhelmii* AND *Daphnia magna*

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Abstract

Congo Red, which may have allergic, carcinogenic and mutagenic effects for organisms used in textiles and in biochemistry and histology for dyeing microscopic media, is an organic pollutant that causes environmental concerns. This study shows that the acute toxic effects of Congo Red on two different aquatic organisms (Pseudochloris wilhelmii and Daphnia magna). The toxic effects of increasing dye concentrations on the growth of Pseudochloris wilhelmii were demonstrated by algal inhibition test. The maximum chlorophyll (a+b) concentration was determined as 0.445 µg/mL at a dye concentration of 5.38 mg/L after 72 hours of exposure. This value decreased to 0.218 µg/mL at 28.46 mg/L dye concentration, indicating a decrease of approximately 50%. For Daphnia magna, it was also demonstrated that acute toxic effects reached their highest level with increasing concentrations and duration (72h LC50: 89.91 mg/L). This study shows that the introduction of Congo red into ecosystems could cause stress on the environment and organisms.

1. INTRODUCTION

Water is the most basic resource for all organisms and the health of the ecosystem. However, in recent years, pollution of water resources have become a growing concern. Due to rapidly increasing urbanization and globalization, the demand for industrial products is also accelerating. Increasing demand brings along industrial wastes and thus water pollution [1]. Textile industries, which are responsible for about 75% of the global dye market, cause pollution of existing waters by causing excessive application of dyes or pigments [2]. Azo dyes represent approximately 60-70% of industrial production. These xenobiotic chemicals, which are widely used due to their low cost, permanence and diversity, can be identified by the presence of the azo group (-N=N-) [3]. The

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presence of these pollutants alters photosynthetic mechanisms by reducing light transmittance, thus altering O_2 concentration [4]. Some carcinogens such as benzidine, which may be present in the structure of these chemicals used for colouring, also raise concerns. Azo dyes are very difficult to degrade, so physical and chemical processes used in wastewater removal can be quite costly and limited [5]. Congo Red is one of the azo dyes with molecular formula $C_{32}H_{22}N_6Na_2O_6S_2$ and molecular weight = 696.68 g mol-1, which is widely used in the textile industry for dyeing paper, silk and wool due to its low cost. Congo Red, discovered by Paul Bottinger in 1884, is an anionic di-azo dye consisting of a sodium salt of benzidinedithiazo-bis-1-naphthylamine-4-sulfonic acid [6] (Figure 1. Congo Red chemical structure). This study aims to determine the toxic effects of Congo red using *P. wilhelmi* and *D. magna* (Figure 2).

P. wilhelmii is a less studied species than other microalgae. It is a species with richer biomass and chlorophyll concentration than *Chlorella sorokiniana* and *Tetraselmis obliquus* [7]. In addition, this species is introduced in the literature as a species with fast growth and a wide nutrient tolerance, including wastewater [8]. In another study investigating the effects of different iron concentrations on biomass and biofuel production on *P. wilhelmii*, it was stated that increasing the iron concentration led to an increase in biomass productivity [9]. Studies on this species are limited in the literature and more research is needed.

Daphnia is a genus of small planktonic crustaceans. They are known as "water fleas". They are classified as members of the Cladocera order within the Branchiopoda class. *Daphnia* have a large head, a simple compound eye, a double shell, and are relatively transparent. *Daphnia* generally live in stagnant freshwater. They are primary consumers, filtering small suspended particles found in lakes and ponds, especially microalgae. Therefore, they are important food sources for fish [10]. *Daphnia* continue to trend as frequently studied model organisms in the fields of ecology, environmental biotechnology and ecotoxicology [11, 12, 13].

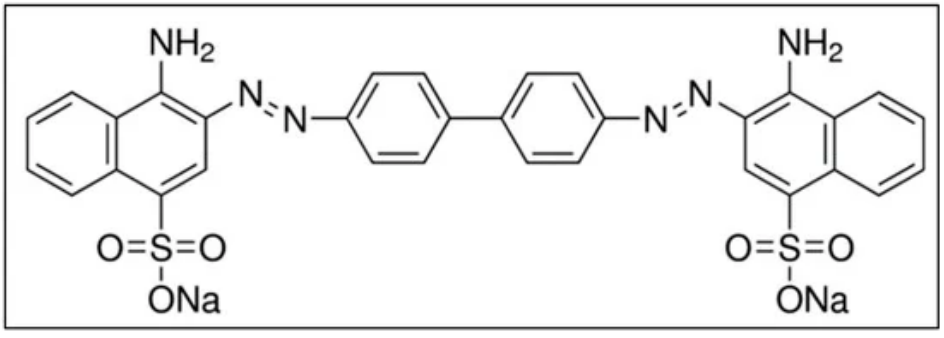

FIGURE 1. Congo Red chemical structure [14]

2. MATERIALS AND METHODS

2.1 Dye solution

Congo Red as an anionic di azo dye was obtained from Sigma (573-58-0), in pure form. Stock solution of congo red was prepared by dissolving the dye in dH_20 to obtain a concentration of 2% w/v. Stock solution of dye in relevant volumes were added in culture media.

2.2 Algal growth inhibition assay

The green algae *Pseudochloris wilhelmii* was isolataed from the spring water in Ankara, Turkey [15]. Microalgal incubation was performed in 100 mL BG11 culture medium in 250 mL Erlenmayer flasks at 25 ± 2 °C and 25 μmol/m^2s (1750 lx) under a 24:0 light:dark photoperiod. [16]. The algal growth inhibition test was performed in the BG11 culture medium according to the OECD 201 procedure [17]. Exponentially growing microalgae was inoculated in Erlenmeyer flasks containing Congo Red dye at 0, 5.38, 6.92, 10.00 and 28.46 mg/L of the BG11 media, respectively. Experimental sets without congo red were used as controls.

2.3 Acute toxicity test

Daphnia magna from Cladocera group was used for acute toxicity test trials [11]. *D. magna* culture conditions were set as 16: 8 hours light/dark cycle and constant temperature of 20 ± 1 ºC. Acute toxicity test was performed according to OECD 202 [18]. *D. magna* acute toxicity tests were carried out in the test medium specified by ISO by preparing increasing dye concentrations at 0, 5.38, 6.92, 10.00 and 28.46 mg/L. Each concentration was designed with 3 replicates and 10 *D. magna* were used for each concentration. Experimental periods were determined as 24, 48 and 72 hours and the toxic effects of Congo Red were analysed at the end of these periods. Control groups were studied simultaneously under the same conditions (Figure 2).

2.4 Bioremoval assay

Increasing concentrations of congo red dye were tested to determine algal bioremoval efficiency of *P. wilhelmii*, samples were incubated in BG11 culture media at 0, 5.38, 6.92, 10.00 and 28.46 mg/L of Congo Red dye concentrations. For analyses, 3 mL samples were taken from each experimental set at 24, 48 and 72 hours of incubation. Congo red absorbance at 498 nm was analyzed using Shimadzu UV 2001 spectrophotometer. Optical density, maximum dried cell mass and chlorophyll (a+b) concentrations are the parameters used to analyze *P. wilhelmii* growth. Optical density was determined at 600 nm, maximum dried cell mass, was determined by measuring the weights of *P. wilhelmii* pellets that were dried at 80 ºC for a night after centrifuging at 5000 rpm for 10 minutes after incubation, and chlorophyll (a + b) concentrations were determined spectrophotometrically at 646.6 nm and 663.6 nm for chlorophyll a and

chlorophyll b, respectively. Control and experimental sets were studied in 3 replicates (Figure 2).

Microalgal dye removal calculations were performed using Equation 1, which is formulated below [18]. Equation (1);

$$removal\ (\%) = \frac{(C_0 - C_t)}{C_0} \times 100 \qquad \text{Eq (1)}$$

In this equation, C_0 and C_t represent the initial and final concentrations of the Congo Red (mg/L), respectively.

Specific growth rate (μ) was calculated according to Equation (2). In this equation, X indicates the dry weight values recorded at the beginning and end of the incubation period, and t indicates the incubation period [19].

$$\mu = (\ln X_2 - \ln X_1) \div (t_2 - t_1) \qquad \text{Eq (2)}$$

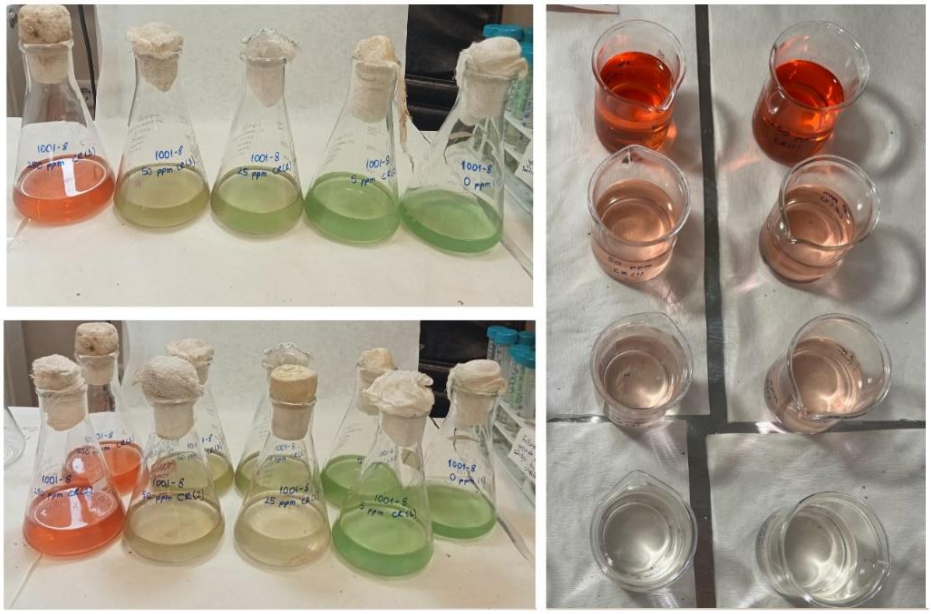

FIGURE 2. The study to determine the toxic effects of Congo red using *P. wilhelmi* and *D. magna*

3. RESULTS AND DISCUSSION

3.1 Bioremoval assay

P. wilhelmii was tested for its ability to removal of dye in BG11 culture media at increasing congo red dye concentrations. The highest bioremoval yield of congo red was 71.40 % at 5.38 mg/L dye concentration. The dry biomass concentration (X), Table 1 shows the μ value and chl(a+b) values at increasing congo red dye concentrations of 5.38-28.46 mg/L after 72 hours of incubation. It was observed that increasing congo red concentrations had a clear effect on microalgal dry weight. While the maximum biomass concentration of the control experimental set was 0.158 g/L, it decreased to 0.068 g/L in the experimental set where the dye concentration was 28.46 mg/L. Similiar results were obtained in a study that reveals the effect of dye and heavy metal ions on bioremoval effect of *A. versicolor*. Increasing remazol blue dye concentrations had a negative effect on fungal growth. As dye concentration increased, removal yield decreased [20]. In another study, *Gonium* sp. a green microalgae removed Reactive Blue 220 dye with the highest yield of 84.20% at 26.20 mg/L dye concentration at the end of 14 days of incubation period [21]. In our study *P. wilhelmii* removed 28.46 mg/L congo red dye with a yield of 29.72 % at 72 hours of incubation period. The highest chl (a+b) concentration was 0.507 μg/mL at the control group and decreased at about 12% when the dye concentration increased to 5.38 mg/L. Interestingly, the specific growth rate (μ) showed its effect up to 10 mg/L dye concentration. However, microalgal growth was not observed after 10 mg/L dye concentration. In this context, it can be considered that the dye removed after this concentration was retained by the biosorption mechanism [22].

TABLE 1. Comparison of the removal yields and X, chl (a+b) and μ values at different Congo red dye concentrations of *P. wilhelmii*

Congo red (mg/L)	**X (g/L)**	**Chl (a+b) (μg/mL)**	**μ (1/d)**	**Y (%)**
0	0.158± 0.001	0.507 ± 0.002	0.109± 0.001	0 ± 0
5.38	0.139± 0.014	0.445 ± 0.046	0.066± 0.038	71.40 ± 1.54
6.92	0.117± 0.033	0.377 ± 0.008	0.009± 0.010	55.53 ± 4.00
10.00	0.095± 0.028	0.305 ± 0.033	0	38.46 ± 4.44
28.46	0.068± 0.020	0.218 ± 0.025	0	29.72 ± 4.61

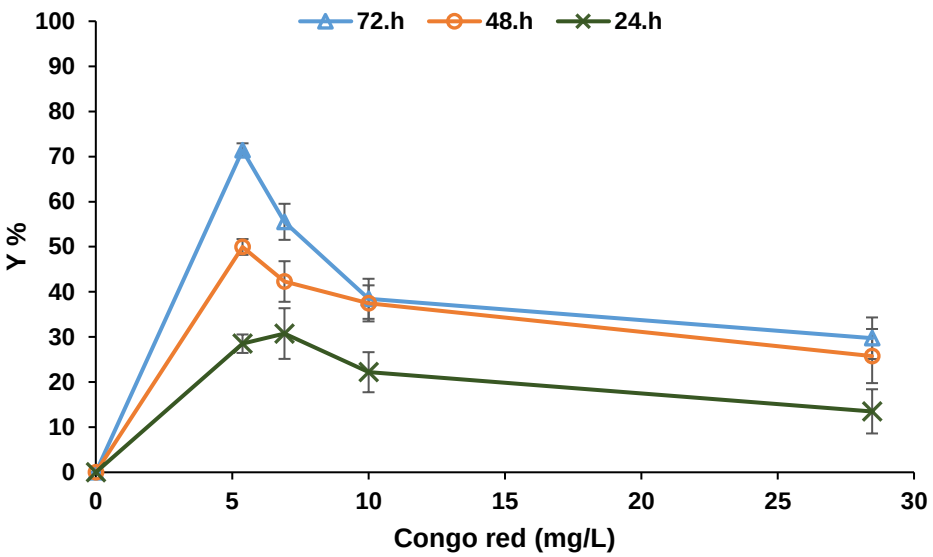

FIGURE 3. Effect of increasing Congo red dye concentrations on removal yield (Y %) of *P. wilhelmii* at 24, 48 and 72 hours

3.2 Algal growth inhibition assay

Figure 4 shows the effects of congo red dye on chl (a+b) concentrations and % dye removal of *P. wilhelmii* at the end of 72 hours. It was noted that microalgal growth decreased during the incubation period as the dye concentrations increased. The control experimental set reached the highest biomass concentration among all the sets studied (0.158 g/L at 72h). The lowest congo red concentration studied, 5.38 mg/L, had no toxic effect on *P. wilhelmii* and its biomass reached 0.139 g/L. When the concentration increased to 28.46 mg/L, *P. wilhelmii* had the lowest biomass amount (0.068 g/L) (Table 1). When the maximum specific growth rates were compared, the μ value of the control culture was recorded as 0.109 l/d, while no maximum specific growth value was recorded when the dye concentration was increased to 10 mg/L. In all experimental sets where congo red dye was applied, lower microalgal growth rates were observed than the control experimental set. When the total chlorophyll values were compared, 0.445 μg/mL was recorded at the lowest congo red concentration studied at 5.38 mg/L and approximately 50% lower chl (a+b) of 0.218 μg/ml was recorded at the highest congo red concentration studied at 28.46 mg/L (Fig. 4). As a result, it was recorded that increasing congo red concentrations had toxic effects on the growth of *P. wilhelmii* and chlorophyll (a+b) concentrations. In a study that was revealed the effect of a nanoparticle La_2O_3 on biomass of *Chlorella* sp. showed different results. The increasing La_2O_3 nanoparticle concentrations did not show an adverse effect on the biomass of *Chlorella* sp. [11]. It can be concluded from here that, Congo Red showed toxic effects of the growth of *P. wilhelmii* and the dye was more toxic than La_2O_3 nanoparticles.

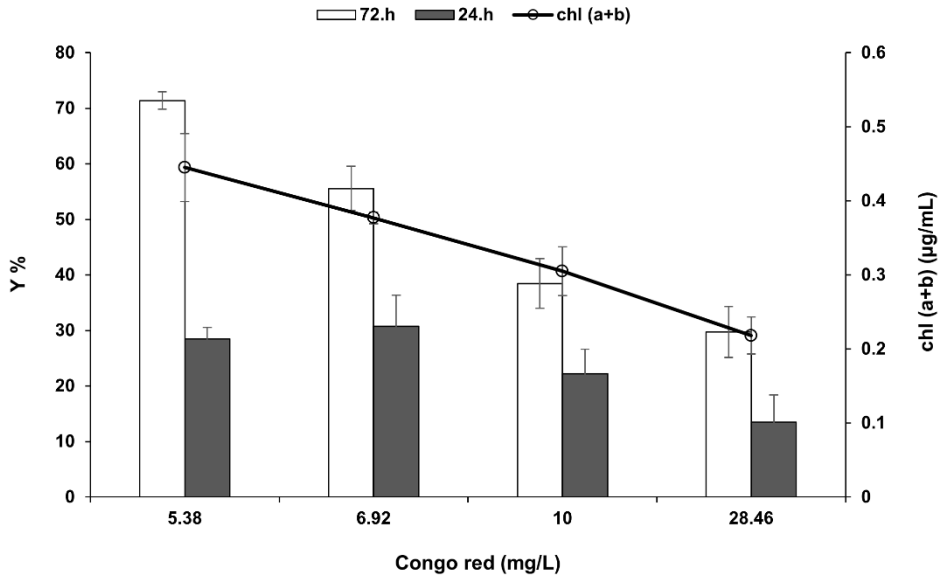

FIGURE 4. Chlorophyll (a + b) concentrations and removal yields of *P. wilhelmii* after 24 and 72h of incubation period

3.3 Acute toxicity test

Congo red, used as a colorant in the textile, dyeing, rubber, and printing industries, is an organic pollutant that raises environmental concerns. Water pollution occurs due to the input of environmental stressors at concentrations exceeding the permitted maximum levels, which restricts access to clean water [23]. *Daphnia magna* serves as a bioindicator organism for understanding the toxicity of chemicals and monitoring wastewater and contaminated waters [24,25,26]. For this reason, *Daphnia magna* was chosen in this bioassay to determine the lethal concentrations of Congo Red.

The acute toxic effects of congo red on *Daphnia magna* were studied at different time points (24, 48, and 72 hours). The highest toxic effect of Congo Red was observed at the 72 h (LC50: 89.91 mg/L) (Table 2). It was observed that the acute toxic effects of congo red on *D. magna* increased with increasing concentration and duration. Parallel to the conclusion that Congo red shows toxic effects on the growth of *P. wilhelmii* and that this dye is more toxic than La_2O_3 nanoparticles, it was also observed that Congo red had a more toxic effect on *D. magna* compared with La_2O_3 nanoparticles [17]. Congo Red is allergic, carcinogenic, and mutagenic for humans and animals and it can also cause infertility in water fleas (*Ceriodaphnia dubia*) [27,28,29,30]. Additionally, it showed phytotoxic effects on plants [31]. Due to being a di-azo dye, Congo Red appears red in basic medium and blue in acidic medium, and it can form an amine compound such as benzidine upon the cleavage of its azo groups [32]. Benzidine, a widespread carcinogen, was led to the ban on the use of Congo Red [33]. Acute toxicity studies on Congo Red were conducted on Cladocerans (*Daphnia magna*, LC_{50}:

322.9 mg/L), *Ceriodaphnia rigaudi*, LC_{50}: 62.92 mg/L) and zebra fish (*Danio rerio*, IC50: 3.11 mg/L) are found in the literature [34]. However, these studies are quite limited in number. Dose-mortality curves for Congo Red (Figure 5) were calculated using probit (Spss 22v.) analysis. Congo Red exhibited different trends characterized by an increase in daphnids mortality after the 24th hour of exposure. No deaths were observed in the control groups.

TABLE 2. Acute toxic effects of CR on *Daphnia magna* at 24, 48 and 72 hours

	LC50 (mg/L)	**Probit**	**Results**
Time	**24h**	**48h**	**72h**
Congo Red	133.096	94.921	89.913

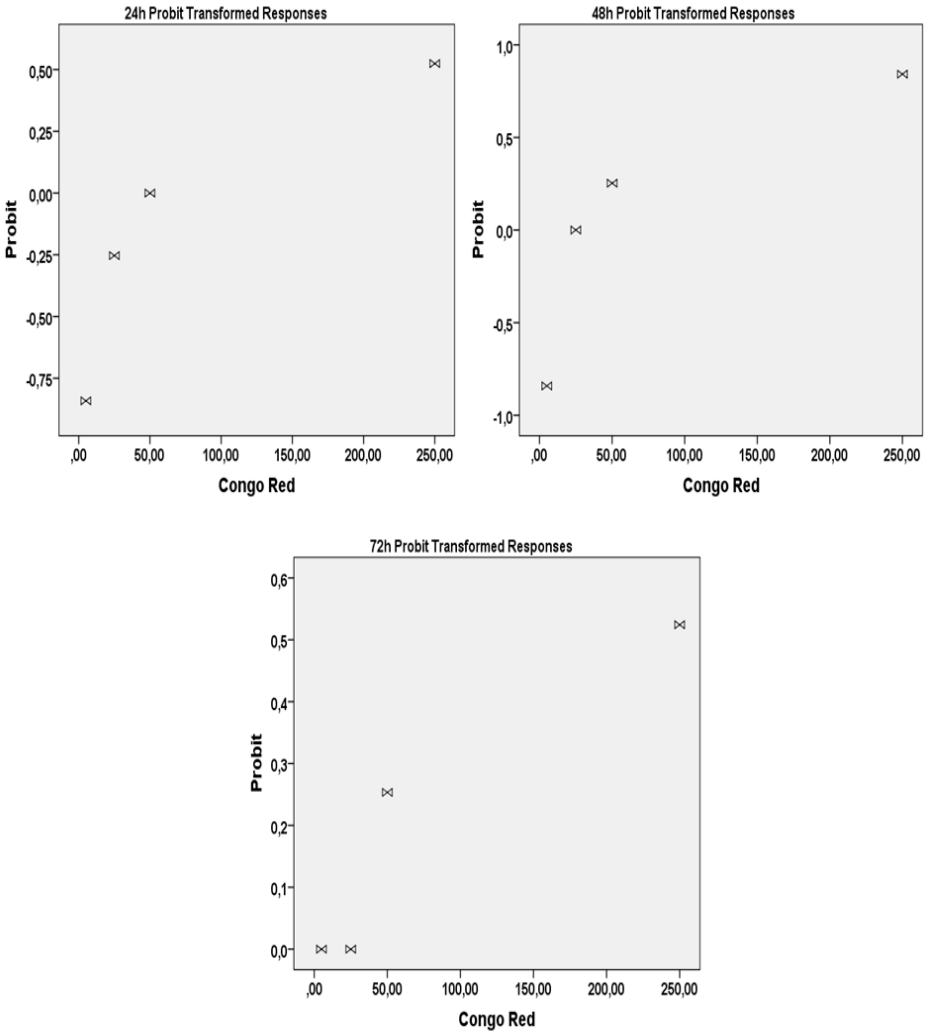

FIGURE 5. Mortality-dose curve of *D. magna* exposed to Congo Red at different durations (24, 48 and 72 hours)

4. CONCLUSIONS

The results of the study demonstrate that Congo red showed toxic effects on both the growth of *P. wilhelmii* and *D. magna*. In both test organisms, 72 hours was identified as a critical time, suggesting that an increase in exposure duration to Congo red is likely to lead to more pronounced negative effects on the organisms. There are very few studies in the literature that adequately address the stress that such dyes may impose on ecosystems. Therefore, more research is needed to elucidate the potential health risks associated with these dyes.

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Author Contribution Statements All authors contributed equally to the conduct of the experiments and the writing of the article.

Declaration of Competing Interests The authors declare no conflict of interest in relation to this particular article.

Ethical Statement This research did not involve human participants or animals. Therefore, no ethical approval was required.

Use of Artificial Intelligence No artificial intelligence-based tools or applications were used in the preparation of this study. The entire content of the study was produced by the author(s) in accordance with scientific research methods and academic ethical principles.

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