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Aquatic toxicity evaluation of new direct dyes to the *Daphnia magna*

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Abstract

The aquatic toxicity of a series of new direct dyes based on benzidine congeners, 2,2′-dimethyl-5,5′-dipropoxybenzidine and 5,5′-dipropoxybenzidine, and a commercial dye (C.I. Direct Blue 218) were evaluated in acute toxicity studies using *Daphnia magna*. The purpose of the research described in this paper was to use bioassays with daphnids to determine the aquatic toxicity of new direct dyes synthesized. The results clearly show that C.I. Direct Blue 218 examined was highly toxic to daphnids and more toxic than unmetallized new direct dyes as expected. The study also suggested that the assay with *D. magna* was an excellent method for evaluation of dyes for aquatic toxicity.

Keywords: Direct dyes; Aquatic toxicity; *Daphnia magna*; Genotoxicity; Benzidine; C.I. Direct Blue 218

1. Introduction

Direct dyes have been used to dye cellulose for over 100 years. Owing to the ease of their application and the wide gamut of colors available at a modest cost, direct dyes are still a popular dye class [1]. Most direct dyes have disazo and trisazo structures, with each color dominated by unmetallized structures [2]. Azo dyes consist of a diazotized amine coupled to an amine or a phenol, and contain one or more azo linkages. They are the largest class (60–70%) of dyes with the greatest variety of colors [3].

Approximately 10–15% of the dyes are released into the environment during dyeing of different substrates, such as synthetic and natural textile fibers, plastics, leather, paper, mineral oils, waxes and even (with selected types) foodstuffs and cosmetics [4]. Even at very low concentrations (10–50 mg/L) water soluble azo dyes can cause waste streams to become highly colored. Aside from their negative aesthetic effects certain azo dyes and biotransformation products have been shown to be toxic, and in some cases these compounds are carcinogenic and mutagenic [5–11]. Approximately, it was determined that 130 of 3200 azo dyes in use have produced carcinogenic aromatic amines because of reductive degradation [12].

The commercial utility of benzidine-based azo colorants and concern over their potential health risks have caused the search for viable nonmutagenic analogs of benzidines to be an important research problem in the past [13–19]. However, researches concerning the aquatic toxicity of azo dyes were not performed seriously by textile chemists. Textile plants are very important sources of toxic discharges [20,21]. They usually employ cotton and synthetic fibers and include integrated printing and dyeing operations, applying a wide variety of organic dyes and full range stages of fabric processes [22–28]. Therefore, the aquatic toxicological investigation of azo dyes can be very beneficial to the further study of textile effluents.

Acute toxicity can be defined as toxicity elicited immediately following short-term exposure to a chemical. In accordance with this definition, two components comprise acute toxicity: acute exposure and acute effect. In contrast to acute toxicity, chronic toxicity is characterized by prolonged exposure and lethal effects elicited through mechanisms that are distinct from those that cause acute toxicity. Typically, acute toxicity and chronic toxicity of a chemical are easily distinguished. For example, mortality occurring on the second day

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of continuous exposure to the chemical would typically be considered acute toxicity. Similarly, reduced fecundity resulting from continuous exposure of organisms throughout their life cycle would be indicative of chronic toxicity. Thus, acute toxicity may result in chronic toxicity [29,30].

All chemicals elicit acute toxicity at a sufficiently high dose, whereas, all chemicals do not elicit chronic toxicity. Paracelsus often cited phrase ''all things are poison…the dose determines… a poison'' is clearly in reference to acute toxicity. Even the most benign substances will elicit acute toxicity if administered at a sufficiently high dose. However, raising the dose of a chemical does not ensure that chronic toxicity will ultimately be attained. Since, chronic toxicity typically occurs at dosages below those that elicit acute toxicity, toxicity observed at the higher dosage may simply reflect acute, and not chronic, toxicity [31].

Effects encountered with acute toxicity commonly consist of mortality or morbidity. From a quantitative standpoint these effects are measured as the LC_{50}, EC_{50}, LD_{50}, or ED_{50}. The LC_{50} and EC_{50} values represent the concentration of the material to which the organisms were exposed that causes mortality (LC_{50}) or some other defined effect (EC_{50}) in 50% of an exposed population. The LD_{50} and ED_{50} represent the dose of the material that causes mortality (LD_{50}) or some other defined effect (ED_{50}) in 50% of a treated population. The LD_{50} and ED_{50} are normalized to the weight of the animal (i.e., mg chemical/kg body weight); whereas, LC_{50} and EC_{50} are normalized to the environment in which the organisms were exposed (i.e., mg chemical/L water). Since ecotoxicology focuses upon the adverse effects of chemicals in the environmental, acute toxicity in this discipline is more commonly described by the LC_{50} or EC_{50}. LD_{50} and ED_{50} values are more commonly used when evaluating toxicity from a human health perspective [32,33].

Clearly, the LC_{50} value is not indicative of an acceptable level of the chemical in the environment. Allowing an environmental concentration of chemical that is predicted to kill 50% of the exposed organisms is hardly an example of good environmental stewardship. Rather the LC_{50} is used as an indicator of relative acute toxicity. The LC_{50} is used to this end rather than a more relevant descriptor of an environmentally suitable environmental concentration (i.e., LC_{05}) because the LC_{50} value has the greatest level of confidence associated with it due to its central location on the concentration–response line. LC_{50} and LD_{50} values are often interpreted as given in Table 1 [32,33].

In the present study, the acute toxicity of new non-genotoxic direct dyes was evaluated using *D. magna* to investigate the aquatic toxicity of azo dyes. Also, C.I. Direct Blue 218 was tested to compare the aquatic toxicity with new non-genotoxic direct dyes. These new direct dyes based on benzidine congeners, 2,2′-dimethyl-5,5′-dipropoxybenzidine (**1**) and 5,5′-dipropoxybenzidine (**2**) (Fig. 1), were synthesized and reported in our previous papers [34,35].

Table 1
The relationship between LC_{50}, LD_{50} and toxicity rating

LD_{50} (mg/kg)	LC_{50} (mg/L)	Toxicity rating
>5000	>100	Relatively non-toxic
500–5000	10–100	Moderately toxic
50–500	1–10	Very toxic
<50	<1	Extremely toxic

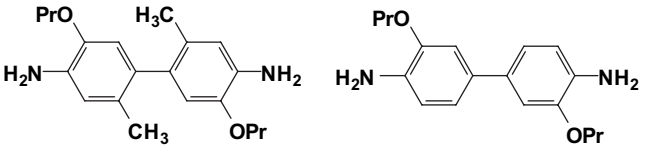

Fig. 1. Structures of 2,2′-dimethyl-5,5′-dipropoxybenzidine (**1**) and 5,5′-dipropoxybenzidine (**2**).

2. Materials and methods

2.1. Organisms

Daphnids (*D. magna*) (Fig. 2) were obtained from stocks that have been maintained at North Carolina State University for over 10 years. Daphnids were cultured and used experimentally in deionized water reconstituted with 192 mg/L $CaSO_4 \cdot 2H_2O$, 192 mg/L $NaHCO_3$, 120 mg/L $MgSO_4$, 8.0 mg/L KCl, 1.0 μg/L selenium and 1.0 μg/L vitamin B_{12}. Cultures were maintained at a density of 40–50 brood daphnids/L culture medium. Culture medium was renewed and offsprings were discarded three times weekly. Brood daphnids were discarded after 3 weeks in the culture and replaced with neonatal organisms. Cultured daphnids were fed twice daily with 1.0 mL (~4 mg dry weight) of Tetrafin® fish food suspension (Pet International, Chesterfill, New South Wales, Australia) and 2.0 mL (1.4×10^8 cells) of a suspension of unicellular green algae, *Selenastrum capricornutum*. The algae were cultured in Bold's basal medium. Culture and experimental solutions were maintained at 20 °C under a 16 h photoperiod. These culture conditions maintained the daphnids in the parthenogenic reproductive phase with the production of all-female broods of offspring [36,37].

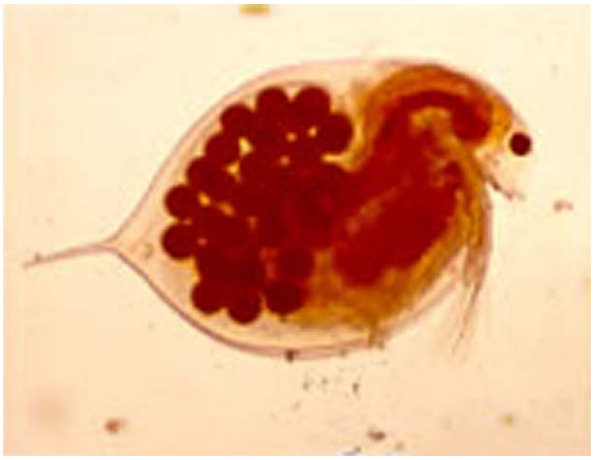

Fig. 2. A picture of *Daphnia magna*.

2.2. Chemicals

All dyes tested are novel and were synthesized in our laboratory. Figs. 3 and 4 show the structures of all 12 direct dyes (**3**–**14**) and C.I. Direct Blue 218 tested. The structure of each dye was confirmed by Electro Spray Mass Spectrometry (ESMS), the details of which are shown in other publications [34,35]. The purity of the novel dyes was confirmed by thin-layer chromatography (TLC).

2.3. Methods

Initially a 24-h preliminary test carried out at exposure concentrations of 100, 10, and 1.0 mg/L to determine if the dye solution was toxic and to define the concentration range to be employed in the definitive tests. If no toxicity is observed, material is considered to be non-toxic and no further testing required. If toxicity is observed, a more definitive experiment needs to be performed to define the concentration–response relationship [38,39].

The standard for a valid bioassay was no-movement rate of less than 10% in the control group. In the definitive test, the minimum number of dilutions was five plus the control group. Immobile organisms were counted to calculate the 24-h LC_{50} and 48-h LC_{50}. All assays were done in duplicate for each concentration [36,37].

For the toxicity tests, daphnid neonates less than 24 h old were used. Ten neonates were placed in individual 50 mL

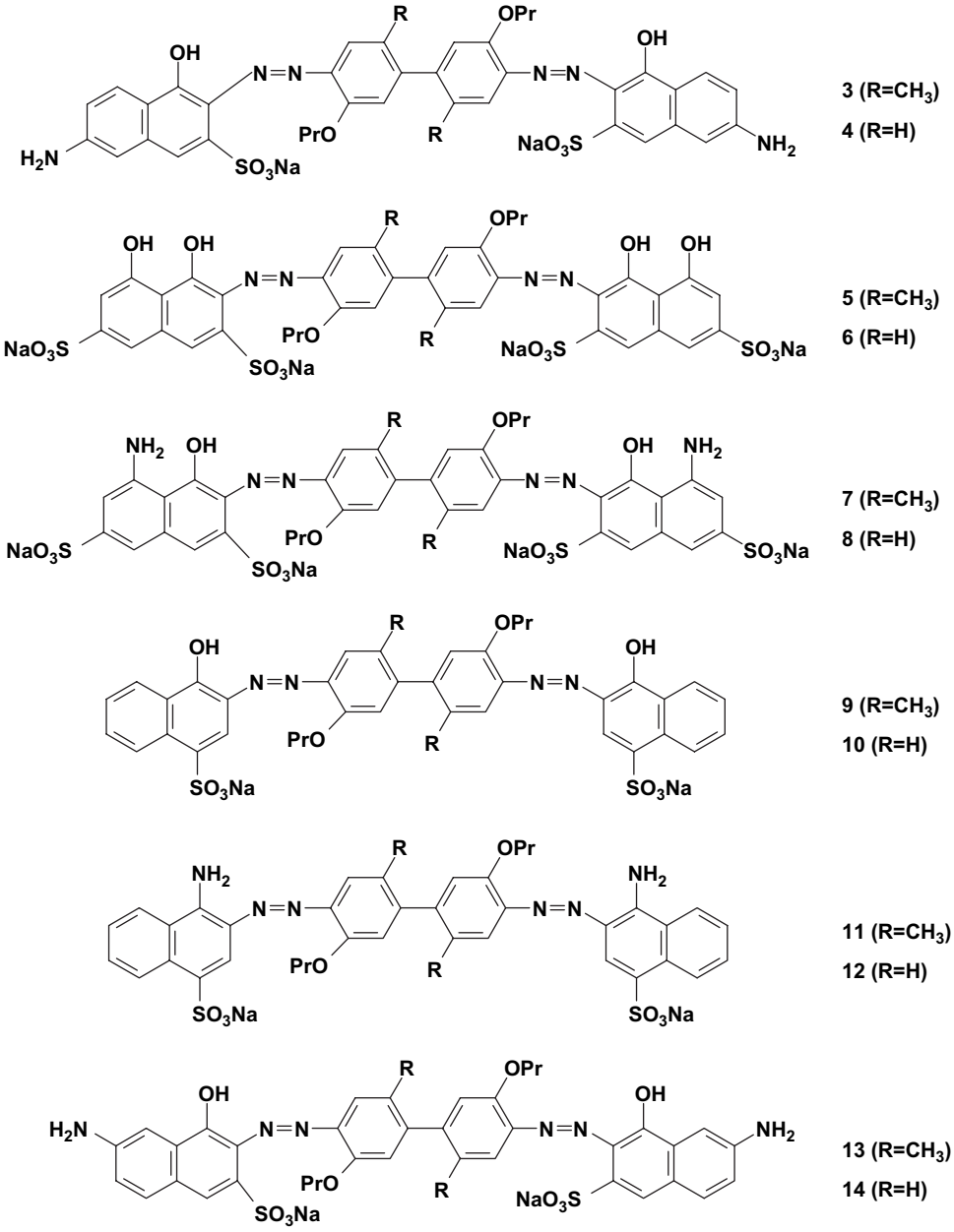

Fig. 3. Structures of 12 unmetallized direct dyes tested.

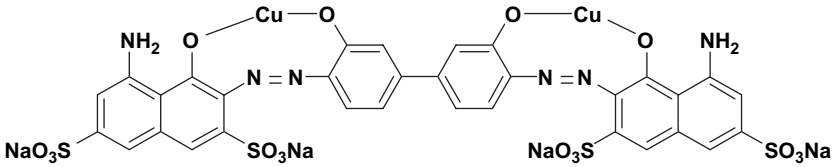

Fig. 4. Structure of C.I. Direct Blue 218.

containers, with 40 mL of the sample solution, diluted or undiluted as required with reconstituted water. Two sample solutions were prepared for each concentration. Algae (100 μL) and food (50 μL) were supplied to feed the neonates at the beginning of the test. Concentrations selected were 0.8, 1.3, 2.2, 3.6, 6.0, and 10.0 mg/L. Typically each treatment level is 60% of the next higher level to allow LC_{50} with a high degree of confidence. The test dye solutions containing *D. magna* were placed in upright incubator (cycle 16 h on/8 h off) and covered loosely with parafilm to prevent evaporation. The mortality of daphnids was observed at 24 and 48 h from initiation of the test.

3. Results and discussions

Acute toxicity of new non-genotoxic direct dyes and C.I. Direct Blue 218 to *D. magna* is summarized in Tables 2–4. In the present study, toxicity was evaluated at concentrations of 0.8–100 mg/L for four new direct dyes (**3**, **4**, **5**, **6**) and 0.8–10.0 mg/L for C.I. Direct Blue 218 to determine LC_{50} ranges. Also, control solutions (concentration of 0.0 mg/L) were conducted to confirm the accuracy of the test.

Tables 2–4 show the number of dead *D. magna* after 24- and 48-h aquatic toxicity tests. The results indicate that the LC_{50} of C.I. Direct Blue 218 for *D. magna* is about 6.0 mg/L at 24-h and 3.6–6.0 mg/L at 48-h tests. No mortality or lethal effects were observed at 0.8–2.2 mg/L and 100% mortality was observed at 48-h in 10.0 mg/L, the highest concentration tested. This means that 50% of daphnids were dead at between 3.6 and 6.0 mg/L after 48-h test. For four new non-genotoxic direct dyes, the LC_{50} is more than 100 mg/L at both 24- and 48-h tests. No mortality or lethal effects were observed at 0.8–22 mg/L for dye **3** and 0.8–36 mg/L for dyes **4**, **5** and **6** after the tests. This means that more than 50% of daphnids were still alive at 100 mg/L after 48-h test.

From the general concept of aquatic toxicity in Table 1, C.I. Direct Blue 218 was very toxic to daphnids, with a 48-h LC_{50} of between 1.0 and 10.0 mg/L whereas four new non-genotoxic direct dyes were relatively non-toxic to daphnids, with a 48-h LC_{50} of more than 100 mg/L.

The main difference between these two groups tested is the presence of copper in the dye structures. While C.I. Direct Blue 218 has two copper molecules inside the structure, four new direct dyes do not have any metal in their structures. Some heavy metals including copper are essential for many organisms, but also very toxic. Copper is the third most used metal in the world [40] and known to have a number of negative effects both on crops [41] and the microorganisms in the soil, which could have a negative effect on the fertility of the soil [42]. Bioavailability and toxicity of most metals, and certainly of copper, are controlled by the speciation in the water, and therefore it is crucial to test the toxicity of the metal complexed dye solution [43].

4. Conclusions

The results using *D. magna* have been used only as a model to extrapolate the toxicological implications that may result from azo direct dyes in the aquatic environment, but these results are not sufficient to assess the holistic health risk for a receptor aquatic ecosystem. However, the toxicity to daphnids is enough to suggest potential damage to every receptor ecosystem and emphasizes the need for the toxicological study of dye synthesis industry. The main object of this study was to demonstrate biological toxicity of azo direct dyes in textile industry. The results indicate that C.I. Direct Blue 218 was very toxic to daphnids, with a 48-h LC_{50} of between 1.0 and 10.0 mg/L while four new non-genotoxic direct dyes were relatively non-toxic to daphnids, with a 48-h LC_{50} of more than 100 mg/L, suggesting

Table 2
The number of dead daphnids for dyes **3**–**6** at 0.8–10.0 mg/L

Dye	Duration of exposure (h)	0.8	1.3	2.2	3.6	6.0	10.0
3	24	0	0	0	0	0	0
	48	0	0	0	0	0	0
4	24	0	0	0	0	0	0
	48	0	0	0	0	0	0
5	24	0	0	0	0	0	0
	48	0	0	0	0	0	0
6	24	0	0	0	0	0	0
	48	0	0	0	0	0	0

Table 3
The number of dead daphnids for dyes **3**–**6** at 8–100 mg/L

Dye	Duration of exposure (h)	8	13	22	36	60	100
3	24	0	0	0	1	2	4
	48	0	0	0	2	3	5
4	24	0	0	0	0	2	2
	48	0	0	0	0	3	2
5	24	0	0	0	0	0	2
	48	0	0	0	0	1	3
6	24	0	0	0	0	1	1
	48	0	0	0	0	2	4

Table 4
The number of dead daphnids for C.I. Direct Blue 218 at 0.8–10.0 mg/L

Dye	Duration of exposure (h)	0.8	1.3	2.2	3.6	6.0	10.0
C.I. Direct Blue 218	24	0	0	0	6	10	17
	48	0	0	0	7	18	20

copper molecules inside dye structures play an important role for the evaluation of aquatic toxicity of dye solutions.

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