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Aquatic toxicity evaluation of copper-complexed direct dyes to the *Daphnia magna*

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Abstract

The aquatic toxicity of a series of copper-complexed direct dyes based on benzidine congeners, 2,2′-dimethyl-5,5′-dipropoxybenzidine and 5,5′-dipropoxybenzidine, 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 metallized direct dyes synthesized. The results clearly show that all of the copper-complexed dyes examined were 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; Copper complex dyes; Benzidine

1. Introduction

Copper is the third most used metal in the world [1] and is known to have a number of negative effects both on crops [2] and the microorganisms in the soil, which could have a negative effect on the fertility of the soil [3]. Bioavailability and toxicity of most metals, and certainly of copper, is controlled by the speciation in the pore water, and therefore it is crucial to test the toxicity of the solution. Heavy metals in general have a low solubility in water, and the concentration of metals in water depends on parameters such as pH, redox potential, organic matter content and the amount of metal present in the solution [4].

It has long been known that after-treatment with salts of metals, such as chromium, aluminum, iron, etc., can give not only varied shades, but can also improve the light and washfastness properties of many direct dyes [5–16]. Probably, in all cases, the dye is in a position to form a chelate with the metal ion, forming a large molecular complex, which might be less soluble in water (which is responsible for improved washfastness properties). Also, the newly formed complex may be more stable photolytically than the original dye. Thus there is an increase in the lightfastness of dyes with certain specific structures when complexing occurs. Many attempts [5–16] have been made in the past to improve the lightfastness properties of direct dyes on textile materials by after-treatment methods. Among the various after-treatments mentioned in the literature, treatment with metallic salts and particularly with copper sulfate is of commercial importance.

Metallization of dyes originally occurred during the mordanting process to help fix the dye to the substrate. Premetallized dyes are now used widely in various outlets to improve the properties of dyes, particularly its lightfastness. However, this is at the expense of brightness since metallized azo dyes are duller than nonmetallized dyes. A major application for copper complexes is in the prior metallization or after-treatment of direct dyes containing at least one *ortho*, *ortho′*-dihydroxyazo or *ortho*-methoxy-*ortho′*-hydroxyazo chromophoric system

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[9–16]. The best-known example is C.I. Direct Blue 76 and C.I. Direct Blue 218, a copper complex derived from C.I. Direct Blue 1 and 15 by metallization with cuprammonium sulfate in the presence of an alkanolamine (Fig. 1).

Most direct dyes have disazo and trisazo structures, with each color dominated by unmetallized structures [16,17]. 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 [18].

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 [19]. 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 [20–26]. Approximately, it was determined that 130 of 3200 azo dyes in use have produced carcinogenic aromatic amines because of reductive degradation [27].

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 [28–34]. However, researches concerning the aquatic toxicity of metallized azo dyes were not performed seriously by textile chemists. For dyes that contain metals as an integral part of its molecule, the metallic content is essential to the dye's performance as a textile colorant. The metals most commonly found in dyes as part of the dye structure are shown in Table 1.

Textile plants are very important sources of toxic discharges [35,36]. 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 [37–43]. Therefore, the aquatic toxicological investigation of metallized azo dyes can be very beneficial to the

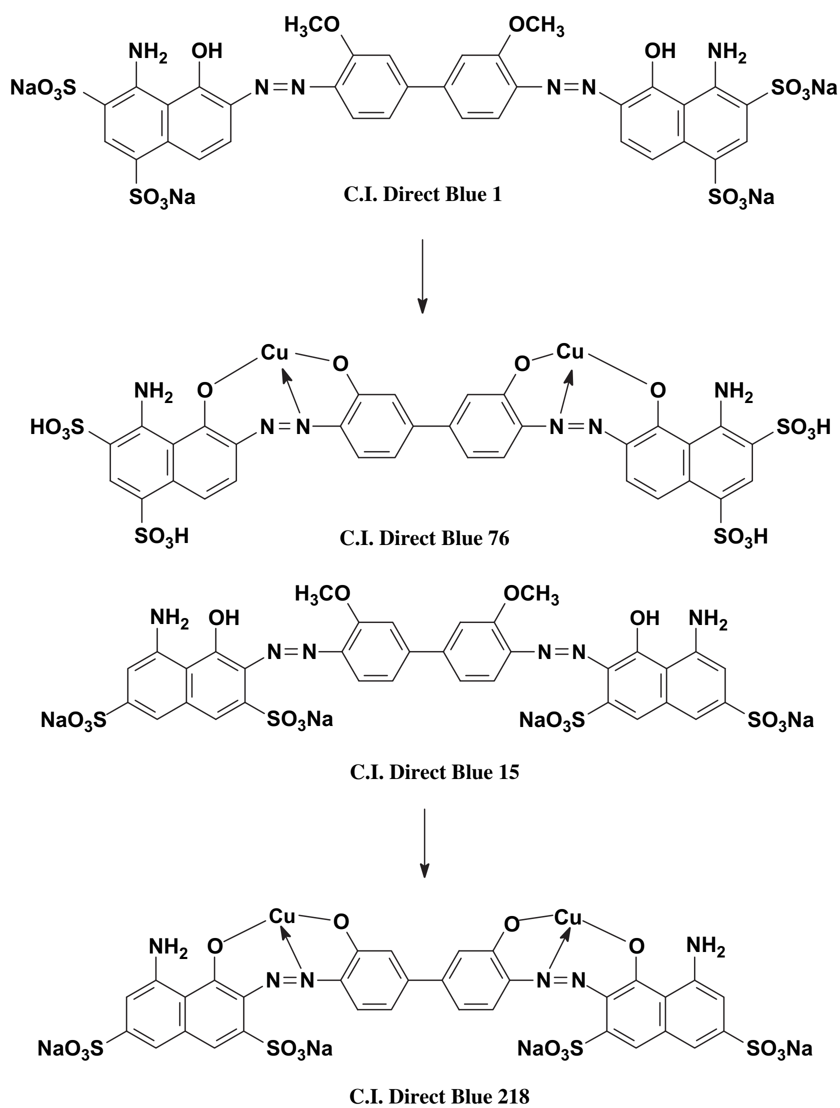


Fig. 1. Metallization of C.I. Direct Blue 1 and C.I. Direct Blue 15.

Table 1
Typical metals found in dyes by dye class

Dye class	Typical metals in structure
Direct	Copper
Fiber reactive	Copper and nickel
Vat	None
Disperse	None
Acid	Copper, chrome, cobalt
Premetallized	Copper, chrome, cobalt
Mordant	Chrome

further study of textile effluents. Table 2 shows the typical sources of metals in textile effluent.

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 of continuous exposure to the chemical would typically be considered acute toxicity. Similarly, reduced fecundity resulting from continuous exposure of organisms all through their life cycle would be indicative of chronic toxicity. Thus, acute toxicity may result in chronic toxicity [44,45].

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 [46].

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 environment, 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 [47,48].

Table 2
Typical sources of metals in textile effluent

Metals	Typical sources
Arsenic	Fibers, incoming water, fugitive, treated timber
Cadmium	Impurity in salt
Chrome	Dyes, laboratory
Cobalt	Dyes
Copper	Dyes, incoming water, fiber
Lead	Dyes, plumbing, shop
Manganese	Permanganate strip
Mercury	Dye/commodity chemical impurities
Nickel	Dyes
Silver	Photo-operations
Tin	Finishing chemicals, plumbing
Titanium	Fiber
Zinc	Dyes, impurities in commodity, chemicals, incoming water

Table 3
The relationship between LC_{50}, LD_{50} and toxicity rating

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

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 concentration 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 3 [47,48].

In the present study, the acute toxicity of copper-complexed direct dyes was evaluated using *Daphnia magna* to investigate the aquatic toxicity of azo dyes. Also, C.I. Direct Blue 218 as a commercial dye was tested to compare the aquatic toxicity with new metallized direct dyes. These new dyes based on benzidine congeners, 2,2′-dimethyl-5,5′-dipropoxybenzidine (**1**) and 5,5′-dipropoxybenzidine (**2**) (Fig. 2), were synthesized and reported in our previous papers [49,50].

2. Materials and methods

2.1. Organisms

Daphnids (*D. magna*) (Fig. 3) were obtained from stocks that have been maintained at North Carolina State University

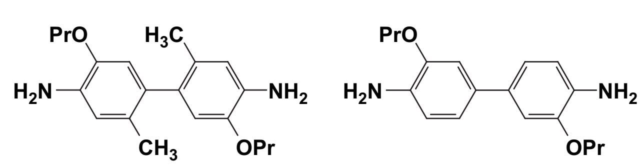


Fig. 2. Structures of 2,2′-dimethyl-5,5′-dipropoxybenzidine (**1**) and 5,5′-dipropoxybenzidine (**2**).

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 offspring were discarded three times a week. 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 [51,52].

2.2. Chemicals

All dyes tested are novel and were synthesized in our laboratory. Figs. 1 and 4 show the structures of all 12 copper-complexed dyes (**3–14**) and C.I. Direct Blue 218 tested. The structure of each dye was confirmed by atomic absorption and neutron activation analysis, the details of which are shown in other publications [49,50]. The purity of the novel dyes was confirmed by thin-layer chromatography (TLC).

2.3. Methods

Initially a 24-h preliminary test was 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 nontoxic and no further testing required. If toxicity is observed, a more definitive experiment need to be performed to define the concentration–response relationship [53,54].

The standard for a valid bioassay was a no-movement rate 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 [51,52].

For the toxicity tests, daphnid neonates less than 24 h old were used. Ten neonates were placed in individual 50 mL containers, with 40 mL of the sample solution, diluted or undiluted with reconstituted water, as required. 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 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.

Fig. 3. A picture of *Daphnia magna*.

3. Results and discussions

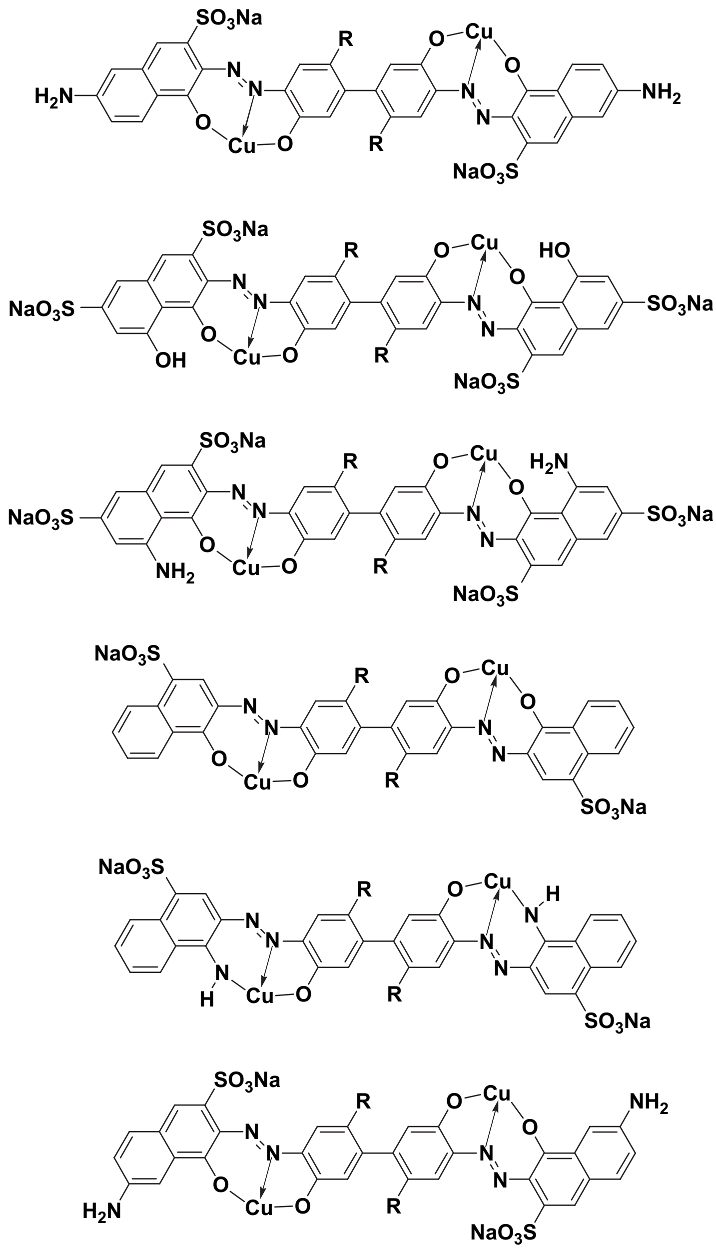
Acute toxicity of new metallized non-genotoxic direct dyes and C.I. Direct Blue 218 to *D. magna* is summarized in Tables 4 and 5. In the present study, toxicity was evaluated at concentrations of 0.8–10.0 mg/L for copper-complexed direct dyes (**3–14**) and C.I. Direct Blue 218 to determine LC_{50} ranges. Also, control solutions (concentration of 0.0 mg/L) were conducted to confirm the accuracy of the test.

Tables 4 and 5 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.

Out of 12 new non-genotoxic metallized direct dyes, only 10 dyes were evaluated for aquatic toxicity since dyes **11** and **12**, in which naphthionic acid was used as a coupler, could not be tested because of very poor water solubility. No mortality or lethal effects were observed at 0.8–1.3 mg/L for all dyes tested. Overall, the LC_{50} is 2.2–3.6 mg/L at both 24- and 48-h tests except dye **8** in which 5,5′-dipropoxybenzidine was coupled with H-acid and dye **13** in which 2,2′-dimethyl-5,5′-dipropoxybenzidine was coupled with gamma-acid. The most toxic dye was **8**, whose LC_{50} is 1.3–2.2 mg/L at both 24- and 48-h tests and the least toxic dye was **13**, whose LC_{50} is more than 10.0 mg/L at both 24- and 48-h tests.

From the general concept of aquatic toxicity in Table 1, C.I. Direct Blue 218 and nine (**3–10**, **14**) new non-genotoxic metallized direct dyes were very toxic to daphnids, with a 48-h LC_{50} of between 1.0 and 10.0 mg/L whereas dye **13** was moderately toxic to daphnids, with a 48-h LC_{50} of more than 10.0 mg/L.

Copper is an offensive pollutant and should receive maximum pollution prevention attention. In addition to dyestuff itself, other sources of copper in the dyehouse may include



3 (R=CH3)
4 (R=H)

5 (R=CH3)
6 (R=H)

7 (R=CH3)
8 (R=H)

9 (R=CH3)
10 (R=H)

11 (R=CH3)
12 (R=H)

13 (R=CH3)
14 (R=H)

Fig. 4. Structures of 12 copper-complexed direct dyes tested.

spillage from handling, implement cleanup, drum washing, and incorrect weighings as well as dyebath effluent. Generally, the amount of dyes still entering open water today is quite small. Because even small amounts strongly color an effluent, they are immediately noticeable in industrial wastewater as an unsightly stain and can be diluted, usually to below the limit of detection. As regards the heavy metal content in dyes, very low concentrations have been reached nowadays and it is expected that they would not cause insuperable pollution problems for the processing plants in the future. Exceptions are those dyes which are after-metallized with copper and chromium salts. If there are no alternatives but to use them, their effluents should be treated separately. In case of heavy metals including copper, not only essential for many organisms, but also very toxic to aquatic organisms even though small amounts are present. Therefore, dye chemists should understand the aquatic toxicity of the metals in dyestuffs and investigate how to reduce the toxicity in metal-complexed dyes. Pollution prevention measures also include special worker training, identification of problem dyes, better record keeping, and auditing of heavy metal use.

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

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

Table 5
The number of dead daphnids for copper-complexed direct dyes (**3–14**) at 0.8–10.0 mg/L

Dyes	Exposure time (h)	0.8	1.3	2.2	3.6	6.0	10.0
3	24	0	0	0	16	19	20
	48	0	0	1	20	20	20
4	24	0	0	1	3	16	20
	48	0	0	2	7	20	20
5	24	0	0	0	7	20	20
	48	0	0	2	11	20	20
6	24	0	0	2	17	20	20
	48	0	0	6	20	20	20
7	24	0	0	2	19	19	20
	48	0	0	5	20	20	20
8	24	0	0	13	20	20	20
	48	0	0	13	20	20	20
9	24	0	0	0	3	8	16
	48	0	0	0	3	16	19
10	24	0	0	0	5	18	19
	48	0	0	0	6	20	20
11	24	–	–	–	–	–	–
	48	–	–	–	–	–	–
12	24	–	–	–	–	–	–
	48	–	–	–	–	–	–
13	24	0	0	0	0	0	1
	48	0	0	0	0	0	6
14	24	0	0	0	0	7	19
	48	0	0	1	1	12	20

4. Conclusions

The results using *D. magna* have been used only as a model to extrapolate the toxicological implications that may result from metallized 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 objective of this study was to demonstrate biological toxicity of copper-complexed direct dyes in textile industry. The results indicate that copper-complexed dyes tested and C.I. Direct Blue 218 were very toxic to daphnids, with a 48-h LC_{50} between 1.0 and 10.0 mg/L, as expected. Comparing with the previous paper submitted, it suggests that heavy metals like copper molecules inside dye structure play an important role for the evaluation of aquatic toxicity of dye solutions.

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