Short Communication

TEXTILE DYES INDUCE TOXICITY ON ZEBRAFISH EARLY LIFE STAGES

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Abstract: Textile manufacturing is one of the most polluting industrial sectors because of the release of potentially toxic compounds, such as synthetic dyes, into the environment. Depending on the class of the dyes, their loss in wastewaters can range from 2% to 50% of the original dye concentration. Consequently, uncontrolled use of such dyes can negatively affect human health and the ecological balance. The present study assessed the toxicity of the textile dyes Direct Black 38 (DB38), Reactive Blue 15 (RB15), Reactive Orange 16 (RO16), and Vat Green 3 (VG3) using zebrafish (Danio rerio) embryos for 144 h postfertilization (hpf). At the tested conditions, none of the dyes caused significant mortality. The highest RO16 dose significantly delayed or inhibited the ability of zebrafish embryos to hatch from the chorion after 96 hpf. From 120 hpf to 144 hpf, all the dyes impaired the gas bladder inflation of zebrafish larvae. DB38 also induced curved tail, and VG3 led to yolk sac edema in zebrafish larvae. Based on these data, DB38, RB15, RO16, and VG3 can induce malformations during embryonic and larval development of zebrafish. Therefore, it is essential to remove these compounds from wastewater or reduce their concentrations to safe levels before discharging textile industry effluents into the aquatic environment.

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INTRODUCTION

The textile industry plays an important role in the economy of many countries [1]. However, this activity releases textile dyes into water bodies, which reduces light penetration and photosynthetic activity, causes oxygen deficiency, and changes the life cycle of aquatic organisms [2]. The amount of dye that is directly lost in wastewater depends on the class applied—it varies from only 2% of the initial dye concentration in the case of basic dyes to 50% for certain reactive dyes [2–4]. Color is usually noticeable at concentrations above 1 mg/L, and an average dye concentration of 300 mg/L has been detected in effluents from textile manufacturing processes [5,6].

The complex chemical structure of synthetic dyes renders them resistant to chemical, physical, and biological treatment [2,7]. Hence, discharge of these compounds into water bodies is undesirable not only for aesthetic reasons but also because many dyes and their by-products are toxic to both aquatic and human life [2,8–13].

Direct Black 38 (DB38) is a benzidine-based azo dye and has been classified as carcinogenic by the International Agency for Research on Cancer [14,15] because of its biotransformation to benzidine [16]. Reactive Blue 15 (RB15), a copper-plthalocyanine dye, is moderately genotoxic to tadpoles (Rana hexadactyla) [17] and toxic to Vibrio fischeri [18].

Novotný et al. [19] observed that the monoazo dye Reactive Orange 16 (RO16) induces point mutations in Salmonella typhimurium TA98 and TA100 strains after metabolic activation. Considering 50% effect (immobilization) concentration (EC50) or 50% inhibition (reproduction) concentration (IC50), the anthraquinone dye Vat Green 3 (VG3) is extremely toxic to the aquatic organisms Daphnia similis (EC50 48 h 6.9 mg/L), Ceriodaphnia dubia (IC50 8 d 0.5 mg/L), and Pseudokirchneriella subcapitata (IC50 72 h 5.6 mg/L) [20].

Acute toxicity tests on vertebrates are an integral part of environmental hazard identification and risk assessment of chemicals and effluents [21,22]. Zebrafish embryos represent an attractive model for studies of developmental toxicity of chemicals both for human and for environmental risk assessment because of their rapid development, easy maintenance in the laboratory, large number of offspring, embryo transparency, and access to experimental manipulation [23–25].

Considering the environmental impact caused by the discharge of textile dyes into water bodies, the present study investigated the toxicity of the dyes DB38, RB15, RO16, and VG3 to the embryonic and larval development of zebrafish and compared the toxicity of these different classes of dyes.

MATERIALS AND METHODS

Test compounds

The dyes DB38 (Chlorazol Black E, purity ≥45%; Chemical Abstracts Service [CAS] no. 1937-37-7), RB15 (Turquoise Blue, purity 35%; CAS no. 12225-39-7), and RO16 (Remazol Brilliant Orange 3R, purity 50%; CAS no. 12225-83-1) were purchased from Sigma. Dystar (Brazil) donated the dye VG3 (Indanthrene Olive Green B; CAS no. 69500). Figure 1 illustrates the chemical structure of each dye.

Embryo larval toxicity test

Zebrafish maintenance and egg acquisition. Adult male and female zebrafish (Danio rerio) were obtained from a commercial supplier (Pisciber) and individually kept in a closed flow-through system in reconstituted water in accordance with
International Organization for Standardization (ISO) standard 7346-1 [26] (2 mM CaCl2/C2H2O, 0.5 mM MgSO4/C2H7H2O, 0.75 mM NaHCO3, 0.07 mM KCl). Fish were kept at 26 ± 1°C, in a 14:10-h light:dark photoperiod, and fed with commercial dry flake food (TetraMin Tropical Flakes) and live brine shrimp. Zebrafish eggs were collected approximately 30 min after natural mating (adult zebrafish male to female ratio 2:1) and cleaned with low hardness reconstituted water. This water was obtained by 1:5 dilution with deionized water, resulting in a hardness of 30 mg/L to 35 mg/L CaCO3 [27,28]. All eggs were examined under a stereomicroscope (SMZ-168; Motic). Unfertilized or injured eggs were discarded. The fertilization success was checked, and only batches of eggs with a minimum fertilization rate of 90% were used.

**Embryo exposure to tested dyes.** The dyes DB38, RB15, RO16, and VG3 were dissolved in water reconstituted by 1:5 dilution in deionized water, to achieve hardness levels typical of surface waters and to prevent excessive calcium carbonate precipitation. For each dye, the tested doses were established according to the fish acute toxicity test (Organisation for Economic Co-operation and Development [OECD] 203) [29] and determined in at least one previous range-finding assay or lethality test with a limit concentration of 100 mg/L (data not shown). Reconstituted water, which provided >90% survival, was the negative control. 3,4-Dichloroaniline (3.7 mg/L), which resulted in 100% mortality, was the positive control.

At 4 h postfertilization (hpf), fertilized eggs were randomly selected and carefully distributed into the wells of a 24-well microplate [30]. For each substance and concentration, 10 fertilized eggs were exposed to 2 mL of the tested dye solution, as follows: DB38 at 1.56 mg/L, 3.12 mg/L, 6.25 mg/L, 12.5 mg/L, and 25 mg/L; RB15, RO16, and VG3 at 6.25 mg/L, 12.5 mg/L, 25 mg/L, 50 mg/L, and 100 mg/L. The tests were performed in a climate chamber at 26 ± 1°C on a 14:10-h light:dark cycle, in triplicate. The exposure of embryos was static. Neither food nor aeration was provided during the bioassays.

**Selected endpoints.** Fish embryo tests were carried out according to the protocols described by Lammer et al. [30] and OECD 212 [31], with slight modifications. Embryonic development was assessed at 8 hpf, 24 hpf, 48 hpf, 72 hpf, 96 hpf, 120 hpf, and 144 hpf, under a stereomicroscope. Table 1 lists the endpoints evaluated in the present study, according to Nagel [32]. Embryonic coagulation, absence of somite formation, nondetachment of the tail, and lack of heartbeat characterized mortality. Larval survival and malformation were observed and recorded every day after hatching. The sublethal endpoints used to assess the effects of the dyes on zebrafish development included embryo malformations (yolk sac alterations, no rupture of egg membrane, gas bladder inflation defects, and skeletal deformities) and hatching success. The distinction between normal and abnormal embryonic development was established according to the zebrafish development parameters described by Kimmel et al. [23].

**Statistical analysis**

One-way analysis of variance followed by Dunnett’s multiple comparison test was performed, using the software package GraphPad Prism 5.0 (Ver 5.0, GraphPad Software). Each experimental value was compared with its corresponding control. Statistical significance was accepted when the probability of the result assuming the null hypothesis (p) was <0.05.

### RESULTS

**DB38**

Mortality of zebrafish embryos and larvae was recorded at different times (8 hpf, 24 hpf, 48 hpf, 72 hpf, 96 hpf, 120 hpf, and 144 hpf). Figure 2 shows the mortality rate at 96 hpf, 120 hpf, and

| Toxicological endpoints | Exposure time (hours postfertilization) |
|-------------------------|----------------------------------------|
| Lethal                  |                                        |
| Coagulation             | X X X X X X X X                       |
| Tail detachment         | X X X X X X X X                       |
| No somites              | X X X X X X X X                       |
| No heartbeat            | X X X X X X X X                       |
| Sublethal               |                                        |
| Yolk sac edema          | X X X X                               |
| Gas bladder defect      | X X X X                               |
| Skeletal deformities    | X X X X                               |
| Hatching rate           | X X X X                               |

*Adapted from Nagel [32].

![Figure 1. Chemical structures of the dyes (A) Direct Black 38, (B) Reactive Blue 15, (C) Reactive Orange 16, and (D) Vat Green 3.](image-url)
144 hpf. Although exposure to DB38 did not induce lethal effects in zebrafish embryos in the tested conditions, developmental malformations like noninfated swim bladder (Figure 3B) and skeletal deformity were observed at 120 hpf and 144 hpf. The control groups showed no significant effects on survival or malformation rates (Figure 2A–C). The most frequent morphological alteration in embryos exposed to DB38 was noninfated swim bladder. From a concentration of 6.25 mg/L, DB38 significantly induced swim bladder deflation at 96 hpf ($p < 0.01$) in a dose-dependent manner (Figure 2A); >90% of the larvae exposed to DB38 at 25 mg/L presented noninfated gas bladder at 120 hpf and 144 hpf (Figure 2B,C). In addition, a curved tail was observed in larvae exposed to DB38 dye from 120 hpf in a dose-dependent manner, but this effect was significant only for the highest concentration (25 mg/L, $p < 0.05$; Figure 2B,C).

**RO16**

Hatching of the zebrafish embryos and larvae was recorded from 48 hpf. Figure 4 shows the hatching rate registered at 96 hpf, 120 hpf, and 144 hpf. Considering the hatched embryos, there was no significant mortality in the group exposed to RO16 up to 144 hpf (Figure 4A–C). From 96 hpf to 120 hpf, RO16 significantly delayed or inhibited the ability of zebrafish embryos to hatch from the chorion at the highest tested concentration (100 mg/L, $p < 0.01$; Figure 4A,B), but this effect was reversed at 144 hpf (Figure 4C). The highest concentration of RO16 also impaired gas bladder inflation significantly ($p < 0.01$) from 96 hpf (Figure 4A–C).

**VG3**

As in the case of DB38 and RO16, VG3 did not induce significant mortality. A nonlinear dose–response behavior for this endpoint was observed at 120 hpf and 144 hpf because the effect induced by the highest tested concentration (100 mg/mL) was less intense than the effect elicited by the lowest tested concentration (6.25 mg/mL; Figure 5). Although exposure to VG3 did not cause lethal effects in zebrafish embryos, sublethal effects like yolk sac edema and swim bladder deflation occurred from 120 hpf for VG3 at 100 mg/L ($p < 0.05$; Figure 5A,B).

**RB15**

RB15 also exhibited a nonlinear dose–response behavior for mortality. Larvae from the control groups presented no significant abnormalities and had an oval-shaped, transparent, and gas-filled bladder, as detected by microscopy (Figure 3A). Embryos exposed to RB15 presented noninfated swim bladder from 120 hpf, in a dose-dependent manner ($p < 0.05$; Figure 6A,B).

**DISCUSSION**

Previous investigations into the environmental effects of dyes have focused on decolorization of textile dye wastewater [33–35], in vivo [3] or in vitro [8–11] genotoxicity/mutagenicity of parent compounds and by-products, and toxicity to aquatic organisms such as bioluminescent bacteria, algae, microcrustaceans, and fish [11,19,20,36]. However, only...
one study has reported on the impact of textile dyes on zebrafish early life stages [37].

Many commercial dyes have had their 50% lethal concentration (LC50) values estimated in adult fish, at different time intervals. Hormazabal et al. [36] showed that both acute and chronic exposure to the triarylmethane dye malachite green is highly toxic to freshwater fish. The zebrafish embryo test can replace the acute tests performed on juvenile or adult fish and represents a refinement of the 3 Rs principle (reduction, refinement, and replacement) [22]. In the present study, we determined the toxicity of DB38, RB15, RO16, and VG3 to zebrafish by assessing the effects of these dyes at developmental

Figure 4. Mortality, gas bladder deflation, and hatching inhibition induced by Reactive Orange 16 at (A) 96 h postfertilization (hpf), (B) 120 hpf, and (C) 144 hpf. Bars represent the mean ± standard error of the mean of 3 tests. **p < 0.01 statistically different from the negative control. NC = negative control (reconstituted water); PC = positive control (3,4-dichloroaniline).

Figure 5. Mortality, gas bladder deflation, and yolk sac edema induced by Vat Green 3 at (A) 120 h postfertilization (hpf) and (B) 144 hpf. Bars represent the mean ± standard error of the mean of 3 tests. *p < 0.05, **p < 0.01 statistically different from the respective negative control. NC = negative control (reconstituted water); PC = positive control (3,4-dichloroaniline).

Figure 6. Mortality and gas bladder deflation induced by Reactive Blue 15. Bars represent the mean ± standard error of the mean of 3 tests. *p < 0.05, **p < 0.01 statistically different from the respective negative control. NC = negative control (reconstituted water); PC = positive control (3,4-dichloroaniline).
levels up to 144 hpf. The addition of sublethal endpoints in the ecotoxicity tests is of great importance because such effects may occur in nontarget organisms, with significant impacts on populations or ecosystems [30].

In the present study, mortality results were not significant for DB38, RO16, VG3, or RB15. The dyes VG3 and RB15 presented a nonlinear concentration–response behavior the highest concentration induced a less intense effect than the lower concentrations. Fish exposure to aquatic toxicants takes place via the delicate respiratory surface of the gills, which comprise over half of the body surface area and make intimate contact with the surrounding water. Although low pollutant concentrations do not usually result in fish mortality, they may still be toxic to these organisms [38].

The hatching rate determined by counting of the zebrafish larvae outside the eggshell, only changed with exposure to RO16, at the highest tested concentration (100 mg/L) from 96 hpf to 120 hpf, This delayed hatching may have resulted from inability of the embryos to break the chorion [39]. In addition, the gas bladder could be a primary target organ for all the tested textile dyes: the most prominent malformation in exposed larvae was the presence of a noninflated swim bladder. Shen et al. [37] demonstrated a similar effect for Basic Violet 14, Direct Red 28, and Acid Red 26, which induced noninflated swim bladder in a dose-dependent manner at concentrations ranging from 3.3 mg/L to 2500 mg/L.

Swim bladder formation in zebrafish begins during the pharyngula period (30–42 hpf); its initial inflation at approximately 72 hpf contributes to feeding efficiency at the beginning of the freely swimming phase of teleost larval forms [40]. Therefore, defects in the inflation of zebrafish swim bladder impair important functions like swimming behavior and buoyancy. These defects negatively affect the ability of the fish to capture food and escape from predators, which can ultimately lead to death [41,42].

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The dye DB38 is a triazo dye based on benzidine, an aromatic amine. The International Agency for Research on Cancer has classified it as a human carcinogen [16]. Although the European Community has banned the use of benzidine-based dyes since 2003 [43], these compounds have been detected in several countries, including Mexico [44], India [16], and Brazil [45]. According to Dapson [46], the use of benzidine-based dyes is very common in underdeveloped countries, where ambitions related to international trade outweigh concerns about environmental and health hazards.

The diazo dye Direct Red 28 has also been classified as carcinogenic by the European textile ecology standard but is not acutely toxic to zebrafish embryos (LC50 of 476.84 mg/L), but it induces swimming bladder deflation at concentrations ranging from 28.8 mg/L to 351 mg/L [37]. In the present study, lower DB38 concentrations (6.25–25 mg/L) also elicited noninflated gas bladder from 96 hpf to 144 hpf.

Moreover, tail curvature and yolk edema occurred in zebrafish larvae exposed to DB38 and VG3, respectively. Birhanli and Ozmen [47] evaluated the potential developmental toxicity of 6 different textile dyes (Basic Red 46, Basic Blue 159, Remazol Red RR, Reactive Blue 21, Cibacron Red FN-3G, and Cibacron Blue FNR) to Xenopus laevis embryos. They observed that low concentrations of all of these dyes generally produced tail flexure as well as yolk and head edemas.

At the highest tested concentration, DB38 (25 mg/L) and VG3 (100 mg/L) also induced curved tail and yolk sac edema in zebrafish embryos, respectively, from 120 hpf to 144 hpf. Yolk sac edema is an important toxicological endpoint because it provides vital nutritive material for larval movement and plays an important role in the early development of zebrafish [42].

**CONCLUSION**

In summary, all the tested dyes exhibited developmental toxicity to zebrafish embryos, which could decrease survival. Potential developmental toxicity of dyes occurred in the following order: DB38 > RO16 > VG3 > RB15. The swim bladder is an important organ to assess the toxic effects of dyes, and it could constitute an endpoint in toxicity testing protocols involving zebrafish embryos. Because of its similarities with vertebrate embryos, the zebrafish is used as a model in human and environmental sciences. Therefore, bioassays on zebrafish embryos could be applied to establish safe levels of dyes in aquatic environments.

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**Conflict of interest**—The authors declare there are no conflicting interests.

**Data availability**—The data are available on request. Please contact giselle25.rodrigues@hotmail.com.

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