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3 **Textile dyes induce toxicity on zebrafish early life stages/early developmental**
4 **stages**

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6 Running title: Textile dyes induce toxicity on zebrafish early life stages
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Abstract

Textile manufacturing is one of the most polluting industrial activities not only because the large amount of water used during the process, but also due to the release of potentially toxic compounds, such as synthetic dyes. Due to consumer's desire for color stability of the fibers during washing, perspiration and sun exposure, textile dyes have very stable chemical structure, thus they are difficult to be degraded. Depending on the class of the dyes, their loss in wastewaters can range from 2% of the original concentration for basic dyes to as high as 50% for reactive dyes. Therefore, unlimited and uncontrolled use of such dyes can lead to serious consequences for human health and ecological balance. This study assessed the lethal and sublethal effects of the textile dyes Direct Black 38 (DB38), Reactive Blue 15 (RB15), Reactive Orange 16 (RO16), and Vat Green 3 (VG3) on zebrafish embryos (*Danio rerio*) for 144 h post-fertilization (hpf). Neither of the studied dyes induced significant mortality at 8, 24, and 48 hpf. The highest RO16 dose significantly delayed or inhibited the ability of zebrafish embryos to hatch from the chorion after 96 hpf. All dyes caused defects in the gas bladder inflation of zebrafish larvae from 120 to 144 hpf. In this exposure period, higher doses of DB38 also induced curved spine, whereas VG3 led to yolk sac edema in zebrafish larvae. Based on the present data, we concluded that the textile dyes DB38, RB15, RO16, and VG3 induce malformation during the embryonic and larval development of zebrafish, thus effluents discharge containing these compounds into water bodies should be prevented.

Keywords: aquatic toxicology, contaminants of emerging concern, developmental toxicity, ecotoxicology, ecological risk assessment, azo dyes, malformations, alternative to animal testing.

1. INTRODUCTION

Synthetic dyes have increasingly been used in the textile and dyeing industries because of their ease and cost-effectiveness in synthesis, firmness, high stability to light, temperature, detergent and microbial attack and variety in color, when compared with natural dyes [1]. Based on the chemical structure of the chromophoric group, synthetic dyes are classified into azo, nitro, anthraquinone, triarylmethane, xanthene, heterocyclic, or indigo dyes. According to the means of application in the fiber, they can belong to the classes of reactive, acid, direct, basic, mordant, disperse, pigment, vat, anionic and ingrain, sulphur, solvent, disperse and azoic dye [2,3].

In recent years, textile dyes are widely used all over the world, and textile industry plays an important role in nation's economy [4]. However, dyes have generated much concern regarding their use, due to the reported toxic effects. Additionally, textile manufacturing consumes a considerable amount of water during the industrial processes, generating large quantities of textile wastewaters, which are discharged with dyes and other undesirable chemicals [5,6]. The amount of dye directly lost to the wastewater is dependent on the class of dye applied, varying from only 2% loss when using basic dyes to 50% loss when certain reactive dyes are used [6-8].

Due to their chemical structure, dyes are resistant to chemical, physical, and biological treatments. In fact, many dyes are difficult to decolorize due to their complex structure and synthetic origin [6,9]. Thus, the discharge of these compounds into the environment is undesirable not only for esthetic reasons but also because many dyes and their byproducts are toxic to both aquatic life and humans [4,10-14].

Considering the environmental impact caused by the discharge of these compounds, this study investigated the dyes Direct Black 38, Reactive Blue 15,

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3 Reactive Orange 16, and Vat Green 3, as models to evaluate aquatic toxicity induced by
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5 synthetic dyes.
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8 Direct dyes are the most popular class of dyes with easy application, wide color
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10 range, and available low cost. Direct Black 38 (DB38) is a benzidine-based azo dye,
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12 classified as carcinogenic by the International Agency for Research on Cancer (IARC)
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14 [15,16] due to its biotransformation to benzidine [17]. Most direct dyes have disazo and
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16 trisazo structures. Azo dyes are the largest class (60–70%) of dyes with the greatest
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18 variety of colors. The ubiquitous use makes them widely spread in aquatic systems, with
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20 great potential to bring adverse effects on aquatic environment [4].
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24 Reactive dyes are frequently used in textile industries, specifically in cotton
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26 dyeing [6]. According to literature, Reactive Blue 15 (RB15), a copper-phthalocyanine
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28 dye, is moderately genotoxic for tadpoles *Rana hexadactyla* [18] and toxic for *Vibrio*
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30 *fischeri* [19]. Novotny et al. [20] have also observed that monoazo Reactive Orange 16
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32 (RO16) dye induces point mutations in TA98 and TA100 strains of *Salmonella*
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34 *typhimurium* after metabolic activation.
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38 Vat dyes, which include indigo and anthraquinone-based dyes, are chemically
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40 complex compounds insoluble in water, which can be reduced into their leuco form by
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42 alkaline solution of sodium hydrosulfite before application to cotton and polymeric
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44 fibers [21,22]. Vat Green 3 (VG3), an anthraquinone dye, is extremely toxic to the
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46 aquatic organisms *Daphnia similis*, *Ceriodaphnia dubia* and *Pseudokirchneriella*
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48 *subcapitata* [23].
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52 Therefore, according to the toxicological studies, several textile dyes and textile
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54 effluents may affect the aquatic ecosystem, due to their toxic effects on *Artemia sp*
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56 [24,25] *Vibrio fischeri* [20,24,26], green algae [20, 23], *Daphnia magna* [24,27,28],
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3 *Daphnia similis* [14,23], and fish [4,24,29,30]. However, to date, there is no information
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5 about the effects of these compounds on fish embryos.
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8 This is the first study on the effects of textile dyes on zebrafish early life stages.
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10 Zebrafish (*Danio rerio*) is a small freshwater tropical fish native to the rivers of India
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12 and South Asia, with a short lifecycle, high fecundity, short reproduction cycle, and
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14 with high number of eggs per spawning. Additionally, it has a rapid development and
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16 transparent eggs, which allow visualizing its development [31-33]. This kind of fish has
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18 become one of the most important model organisms in developmental biology of
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20 vertebrates and toxicology studies. Moreover, experiments with embryos are considered
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22 as refinement to animal experiments, and offer a multicellular system integrating the
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24 interaction of various tissues and differentiation processes, when compared to
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26 experiments with cell lines [32,34].
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31 In this context, the developmental toxicity of textile dyes in zebrafish early life
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33 stages was investigated. For that, zebrafish embryos/larvae were exposed to different
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35 concentrations of Direct Black 38, Reactive Blue 15, Reactive Orange 16, and Vat
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37 Green 3 dye, to clarify any differences in sensitivity to the adverse effects of these
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39 classes of dye during development. Mortality, hatching rates, and malformations at
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41 different developmental stages attributed to the dyes exposure were observed and
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43 analyzed.
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47 2. MATERIAL AND METHODS

48 2.1 Test compounds

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50 The dyes Direct Black 38 (DB38; Chlorazol Black E; purity \geq 45%; CAS No.
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52 1937-37-7), Reactive Blue 15 (RB15; Turquoise Blue; purity 35%; CAS No.: 12225-39-
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54 7), Reactive Orange 16 (RO16; Remazol Brilliant Orange 3R; purity 50%; CAS No.:
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3 12225-83-1) were purchased from Sigma (St Louis, MO, USA) and Vat Green 3 (VG3;
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5 Indanthrene Olive Green B; CAS No.: 69500) was gently donated by Dystar (Brazil).
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7 The chemical structure of each dye is presented in Figure 1. In this study, dyes will be
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9 grouped according to their application class.
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11 **2.2 Embryo-larval toxicity test**

12 **2.2.1 Maintenance of zebrafish and acquisition of eggs**

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15 Adult male and female zebrafish (*Danio rerio*) were obtained from a commercial
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17 supplier (Pisciber, Barcelona) and kept separately in a closed flow-through system in
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19 reconstituted water in accordance with ISO standard 7346-1 [35] (2 mM CaCl₂·2H₂O;
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21 0.5 mM MgSO₄·7H₂O; 0.75 mM NaHCO₃; 0.07 mM KCl). The criterium for fish
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23 keeping was temperature of 26 ± 1°C at a 14/10 h light/dark photoperiod. Fish were fed
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25 with commercial dry flake food and live brine shrimp.
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32 For the acquisition of eggs, adult zebrafish male:female ratio of 2:1 was placed in
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34 breeding tanks (Aquaneering, San Diego, California) the day before spawning. Plastic
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36 plants served as a spawning substrate and were fixed to the bottom grid to prevent
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38 predation of eggs by adult zebrafish. About 30 minutes after beginning of spawning
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40 (triggered by sudden illumination of the aquaria on the next morning), the spawn traps
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42 were removed and the eggs were collected using plastic disposable pipettes. Eggs were
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44 transferred immediately to Petri dishes and successively cleaned with water dilution
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46 corresponding to the reconstituted water, according to ISO-standard [35], which was
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48 diluted 1:5 with deionized water. All eggs were examined under a dissecting
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50 microscope. Then, the unfertilized eggs were discarded and those embryos exhibiting
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52 normal development that had reached the blastula stage were selected for subsequent
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54 experiments.
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2.2.2 *Embryo exposure*

Direct Black 38, Reactive Blue 15, and Reactive Orange 16 dyes were prepared with water dilution corresponding to the reconstituted water, according to ISO-standard [35]. Vat Green 3 dye was dissolved in reconstituted water ISO-standard [35] 1:5 using deionized water. The tests were carried out using five different concentrations of each dye and positive or solvent controls. Doses were established according to OECD draft guideline on Fish Embryo Toxicity (FET) Test [36], and determined in at least one previous range-finding assay or lethality test with a limit concentration at 100 mg/L (data not shown).

At 4 hours post-fertilization (hpf), 10 fertilized eggs per concentration were randomly selected and carefully distributed to a 24-well plate, filled with 2 mL of each dye solution as follows: Direct Black 38 (1.56, 3.12, 6.25, 12.5 and 25 mg/L), Reactive Blue 15, Reactive Orange 16 and Vat Green 3 at 6.25, 12.5, 25, 50, and 100 mg/L. The tests were performed in triplicate in a climate chamber at $27 \pm 1^\circ\text{C}$ on a 14-h light and 10-h dark cycle. The exposure of embryos was static. Neither food nor aeration was provided during the bioassays.

2.2.3 *Selected endpoints*

Fish embryo test was carried out according to the protocols described by Nagel [37] and OECD Guideline TG 212 [38], with slight modifications. The embryo test procedure was prolonged until 144 hpf to evaluate the hatching rate, besides the evidence of delayed effects found in the initial-range finding experiments. Embryo development was assessed at 8, 24, 72, 96, 120, and 144 hpf, using a stereomicroscope (SMZ-168, Motic). The evaluated endpoints in this study are shown in Table 1. Mortality was identified by coagulation of embryos, no somite formation, non-

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3 detachment of the tail, as well as lack of heart-beat. Survival and malformation of larvae
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5 were observed and recorded every day after hatching. Sublethal endpoints used for
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7 assessing the developmental effects of dyes included embryo malformations (yolk sac
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9 alterations, no rupture of the eggs membrane, defects in gas bladder inflation and
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11 skeletal deformities) and hatching success. Hatching was defined as the rupture of the
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13 egg membrane, and fully as well as partially hatched larvae were counted as hatched
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15 embryos. The distinction between normal and abnormal development of embryos was
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17 established according to the zebrafish development descriptions reported by Kimmel et
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19 al. [31]. Images of zebrafish at different post-fertilization times were captured using a
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21 microscope with digital camera (Moticam 2000, Motic).
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25 26 *2.3 Criteria validity*

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28 After egg collection, fertilization success was checked and only batches of eggs
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30 with a minimum fertilization rate of 80% were used. Control solutions were also made
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32 to confirm the accuracy of the test. 3,4 - Dichloroaniline (3.7 mg/L) was used as
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34 positive control, resulting in 100% mortality, while more than 90% survival was
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36 observed in the control group (data not shown).
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40 41 *2.4 Statistical analysis*

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43 One-way ANOVA followed by Dunnett's multiple comparison test was
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45 performed, using software package GraphPad Prism 5.0[®] (version 5.0, GraphPad
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47 Software, San Diego, CA, USA). Each experimental value was compared to its
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49 corresponding control. Statistical significance was accepted when the probability of the
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51 result assuming the null hypothesis (p) was less than 0.05.
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3. RESULTS

3.1 Effects on survival and hatching rate

No significant mortality of the fertilized zebrafish eggs exposed to DB38, RB15, RO16 and VG3 for 8 to 48 hpf was observed in the tested conditions.

No significant differences were observed between the frequencies of hatching induced by the DB38, RB15, and VG3 dyes and the negative control. Only RO16 caused delay or inhibition on the ability of zebrafish embryos to hatch from the chorion, which was observed after 96 hpf at 100 mg/L, and remained significant after 120 and 144 hpf (Figure 2).

3.2 Malformations

Embryo malformations such as defects in the gas bladder inflation (Figure 3) have become apparent from the onset of hatching (96 hpf) in embryos exposed to DB38, RO16, and VG3 dyes. In contrast, for RB15 this developmental effect occurred only after 120 hpf (Figure 4).

Larvae from the control groups presented no significant abnormality and had a oval shaped, transparent, and gas-filled bladder, which was easily detected under the microscope (Figure 3A), while embryos exposed to dyes DB38, RO16, RB15, and VG3 presented non-inflated swim bladder, as shown in Figure 3B, using a larvae exposed to DB38 as example.

According to Figure 4, larvae exposed to the highest doses of textile dyes showed defects in gas bladder inflation mainly after 120 hpf. However, larvae exposed to DB38 dye presented a failure in the gas bladder inflation from the concentration of

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3 6.25 mg/L at 96 hpf. More than 90% of larvae exposed to DB38 dye at 25 mg/L
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5 presented non-inflated swim bladder.
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8 In addition, other embryo malformations, such as yolk sac alteration and skeletal
9 deformity were observed after exposure of embryos to DB38 and VG3 dyes. DB38
10 exposure induced curved tail on embryos from 120 hpf in a concentration-dependent
11 way (Figure 5A), whereas VG3 dye caused yolk sac edema (Figure 5B) in zebrafish
12 larvae from 120 hpf.
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19 4. DISCUSSION

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22 Previous studies have mainly focused on the decolorization of textile dye
23 wastewater [26,39,40], evaluation of genotoxicity/mutagenicity of parent compound and
24 by-products *in vivo* [7] or *in vitro* [12-14], as well as toxicity to aquatic organisms such
25 as bioluminescent bacterium, algae, microcrustaceans and fish [4,14,20,23-30]. Once
26 there is no information about the effects of dyes on fish embryos, the toxicity of DB38,
27 RB15, RO16, and VG3 dyes in zebrafish early life stages was determined by the
28 hatching rate, embryo malformations, including defects in the gas bladder inflation,
29 yolk sac alteration and skeletal deformity. Table 1 shows these toxicological endpoints
30 for the evaluation of lethal and sublethal effects of the dyes on zebrafish at 8, 24, 72, 96,
31 120, and 144 hpf.
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45 Despite the great public demand for replacement of animal experiments for
46 ethical reasons, industry has searched for alternative testing methods that are less cost-
47 intensive and less time- and space-consuming [32]. Zebrafish embryos represent an
48 attractive model for studies of developmental toxicity of chemicals both for human and
49 environmental risk assessment, due to its rapid development, easy maintenance in the
50 laboratory, large number of offspring, transparency of embryos and access to
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3 experimental manipulation [32,41]. Additionally, according to current European Union
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5 legislation [42] for the protection of animals used for experimental and other scientific
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7 purposes, the use of vertebrate embryo is not regulated, and fish embryos are considered
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9 as non-protected life stages and similar to *in vitro* assays - an alternative to animal
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11 testing, despite using whole organisms [43,44]. Zebrafish embryos are also well suited
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13 for studies of sublethal effects and toxicity mechanisms. Inclusion of sublethal
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15 endpoints in ecotoxicity testing, in addition to lethality, is of great importance since
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17 such effects may occur in non-target organisms, with significant impacts on populations
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19 or ecosystems [41].
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24 According to the literature, LC₅₀ values of many commercial dyes have been
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26 estimated in adult fish at different time intervals. Hormazabal et al. [45] showed that
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28 malachite green (MG), a triarylmethane dye, is highly toxic to freshwater fish, in both
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30 acute and chronic exposures. Others authors also observed changes in LC₅₀ values of
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32 MG in the freshwater catfish *Heteropneustes fossilis*, at different exposure times, and
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34 stated that toxicity increased with exposure time [46]. The results of the present study
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36 demonstrated no significant mortality or lethal effect of DB38, RB15, RO16, and VG3
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38 dyes on zebrafish embryos at 8 to 48 hpf in the tested conditions.
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42 Figure 2 shows the effects of RO16 on hatching success of zebrafish embryo.
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44 Delayed hatching may be due to an inability of embryos to break the chorion. This
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46 result indicates that the RO16 dye significantly reduced the hatching rate at 100 mg/L at
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48 96, 120, and 144 hpf ($p < 0.05$). Recent studies have shown that other environmental
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50 contaminants including hexabromocyclododecanes diastereoisomers (α -HBCD and β -
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52 HBCD) also caused hatching delay in zebrafish embryos [47].
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3 Fish are exposed to aquatic toxicants via the delicate respiratory surface of the
4 gills, which comprise over half of the body surface area, and make intimate contact with
5 the surrounding water. Usually, low pollutant concentrations may not result in fish
6 mortality, but may still be toxic to this species [30]. In this study, the most considerable
7 developmental defect caused by textile dyes was the failure of the gas bladder to inflate.
8 The gas bladder, commonly referred as the swim bladder, is a vascularized internal
9 organ found in almost all teleosts. It controls the buoyancy and locomotory movements
10 of fish [48]. The failure in the gas bladder inflation of zebrafish larvae can cause
11 impaired motility, which can ultimately lead to death.
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24 As can be seen in Figure 3, all dyes have induced defects in the swim bladder
25 inflation of zebrafish larvae at 120 and 144 hpf ($p < 0.05$). Larvae exposed to DB38 dye
26 exhibited a morphological abnormality, which included the development of an abnormal
27 dorsally curved tail. This tail curvature was first observed at 120 hpf in fish exposed to
28 the highest dose of DB38 (25.0 mg/L), and remained significant at 144 hpf (Figure 5A).
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35 Direct Black 38 is a triazo dye based on benzidine, an aromatic amine, classified
36 as a human carcinogen by the IARC [17]. Although the European Community has
37 banned the use of benzidine-based dyes since 2003 [49], these compounds have been
38 detected in several countries, including Mexico [50], India [17], and Brazil [51].
39 According to Dapson [52], the use of benzidine-based dyes is very common in
40 underdeveloped countries, where ambitions in relation to international trade outweigh
41 concerns about the risks to health and environment. Similar fact was observed with the
42 MG, which has been banned in several countries and was not approved by US Food and
43 Drug Administration, but it is still used in many parts of the world due to its low cost,
44 ready availability and efficacy [29].
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3 Yolk sac was another toxicological endpoint analyzed in this study, once it
4 provides nutritive material vital for the movement of larvae, and plays an important role
5 in the early development of zebrafish [48]. Figure 5B shows that the VG3 dye at 100
6 mg/L also induced yolk sac edema in larvae at 120 and 144 hpf ($p < 0.05$). These results
7 are similar to the findings of Du et al. [47], who found that HBCD diastereoisomers,
8 substances detected in practically all environmental media, caused malformation in
9 zebrafish embryos including yolk sac edema.
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22 **5. CONCLUSION**

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24 In summary, the present results clearly show that the textile dyes DB38, RB15,
25 RO16, and VG3 can affect the embryonic and larval development of zebrafish (*Danio*
26 *rerio*) in a dose-dependent manner, based on the delayed hatching of eggs, presence of
27 malformation including defects in gas bladder inflation, yolk sac alteration and skeletal
28 deformity, and thus decreased survivorship. The four textile dyes exhibited different
29 developmental toxicity to zebrafish embryos, and the order of the toxicity was DB38 >
30 VG3 > RO16 > RB15.
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41 It is important to evaluate the toxicity at various trophic levels, in particular with
42 fish. Due to its similarities of vertebrate embryos, zebrafish is used as a model for
43 human and environmental sciences. Therefore, the bioassay with zebrafish embryos can
44 be used as an initial screening test to analyze the toxicity of dyes and to manage dye-
45 containing effluents, in order to prevent human health risks and minimize impacts on
46 aquatic environments.
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3 **CONFLICT OF INTEREST STATEMENT**
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5 The authors declare there were no conflicting interests.
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FIGURE LEGENDS

Figure 1: The chemical structures of the dyes Direct Black 38 (A), Reactive Blue 15 (B), Reactive Orange 16 (C) and Vat Green 3 (D).

Figure 2. Effects of Reactive Orange 16 on hatching success of zebrafish embryo. Bars represent the mean \pm SEM (n=3). *p<0.05 statistically different from the respective negative control.

Figure 3. Effects of textile dyes on swim bladder morphology during development of zebrafish larvae. (A) Photomicrographs of left lateral views of larvae with inflated swim bladder from control group (B) larvae with uninflated swim bladder from experimental group (Direct Black 38) at 144 hpf.

Figure 4. Effects of the Direct Black 38, Reactive Blue 15, Reactive Orange 16 and Vat Green 3 dyes on gas bladder of zebrafish larvae. Bar represent the mean \pm SEM (n=3). *p<0.05 and **p<0.01 statistically different from the respective negative control.

Figure 5. Skeletal deformity and yolk sac alteration caused by Direct Black 38 (A) and Vat Green 3 dyes (B) on zebrafish larvae, respectively. Bar represent the mean \pm SEM of three tests. *p<0.05 statistically different from the respective negative control.

Table 1. Toxicological endpoints for the evaluation of lethal and sublethal effects of the textile dyes on zebrafish embryos, adapted from Hagenaaers et al. [34] and Nagel [37].

Toxicological Endpoints	Exposure time (hpf)						
	8	24	48	72	96	120	144
<i>Lethal</i>							
Coagulation	X	X	X	X	X	X	X
Tail detachment		X	X	X	X	X	X
No somites		X	X	X	X	X	X
No heart beat			X	X	X	X	X
<i>Sublethal</i>							
Yolk sac edema				X	X	X	X
Gas bladder deffect				X	X	X	X
Skeletal deformities				X	X	X	X
Hatching rate				X	X	X	X

Figure 1.

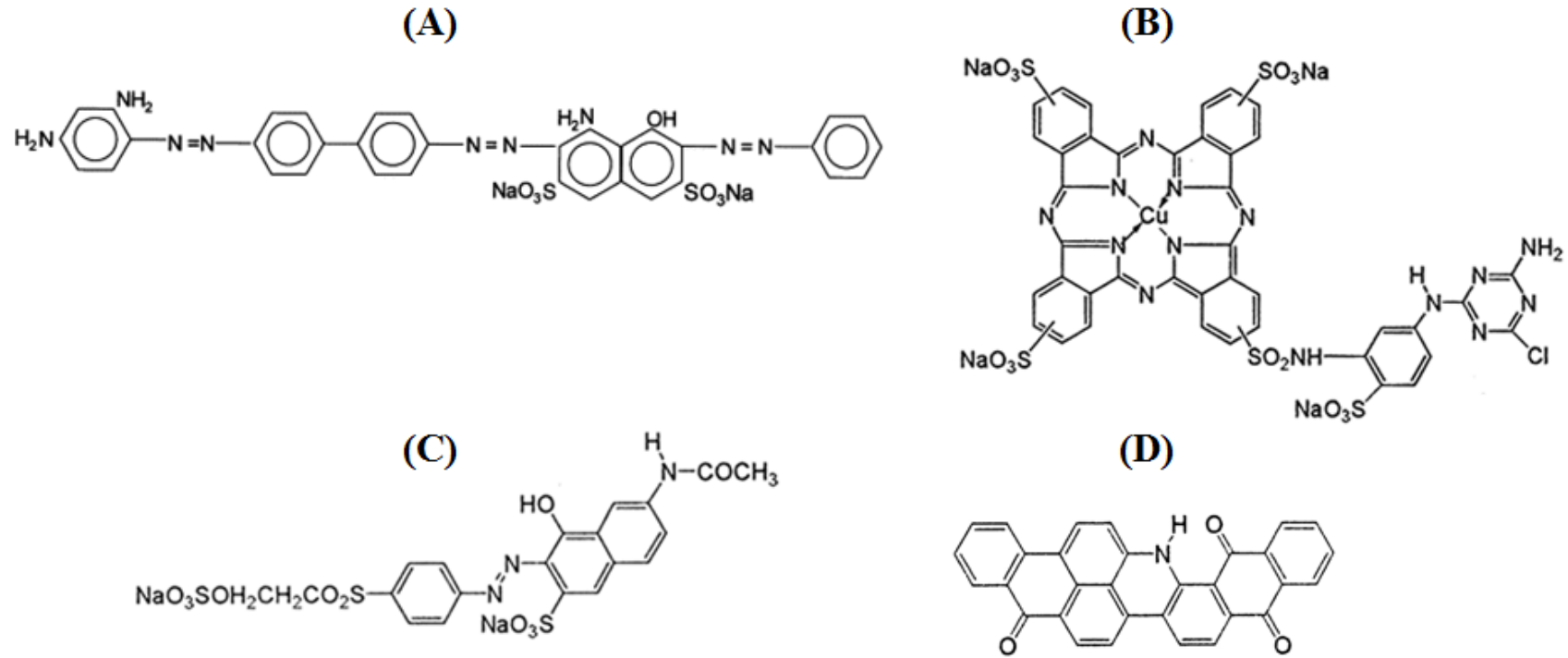
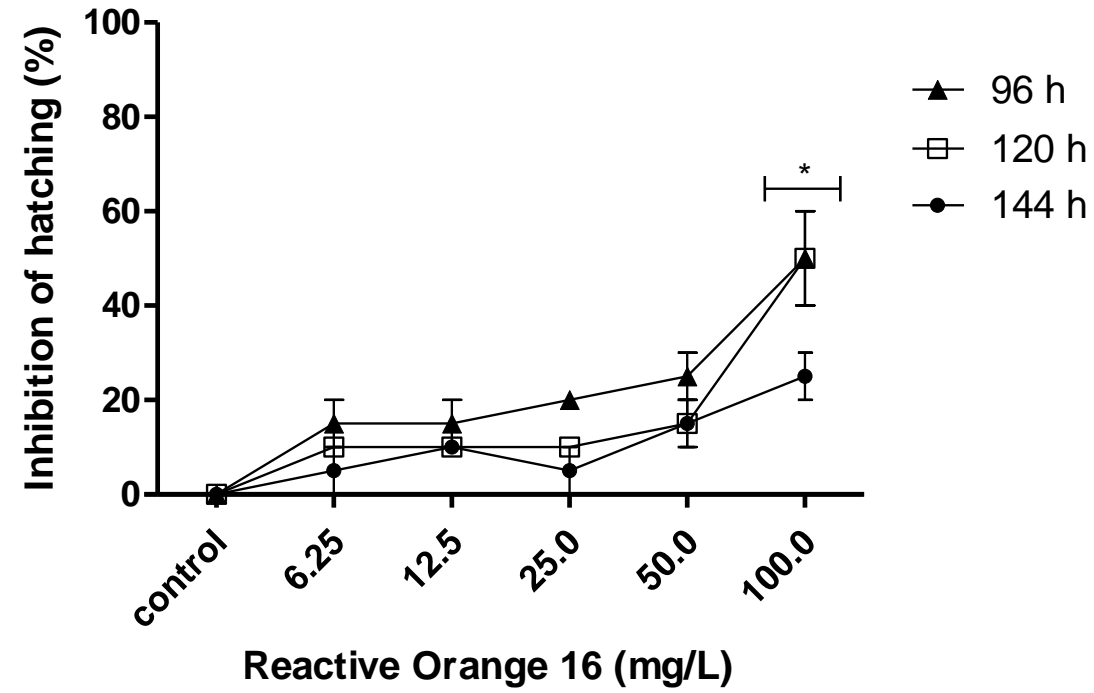


Figure 2.



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Figure 3.

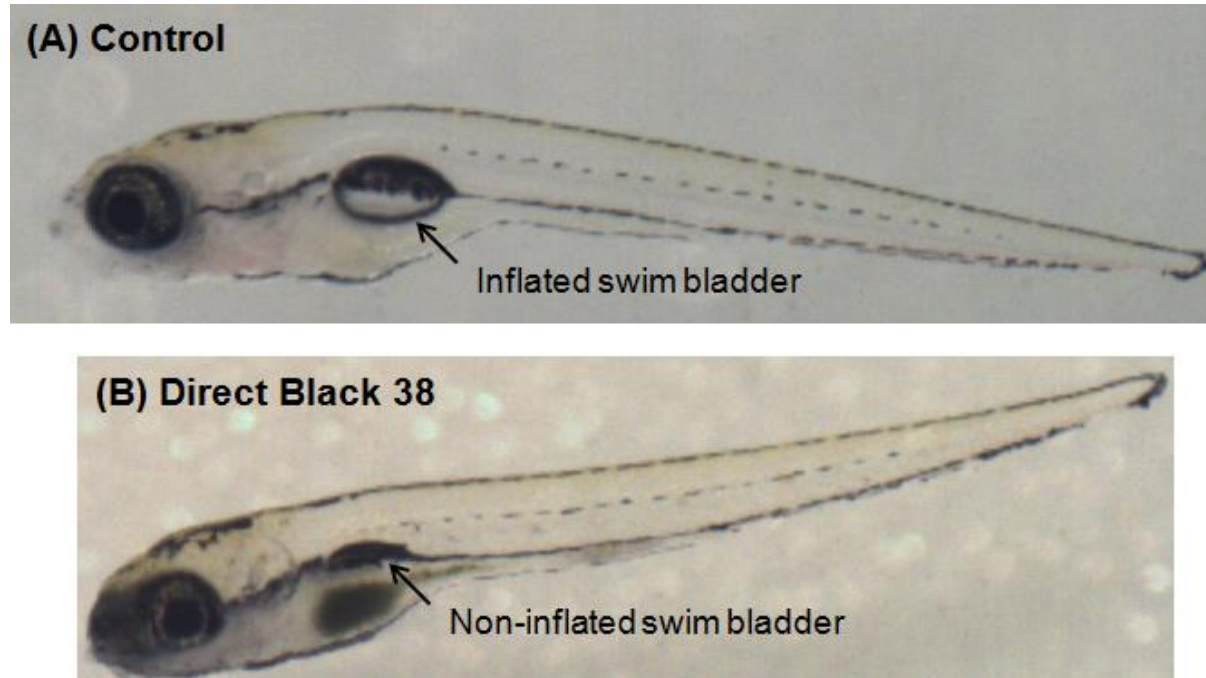
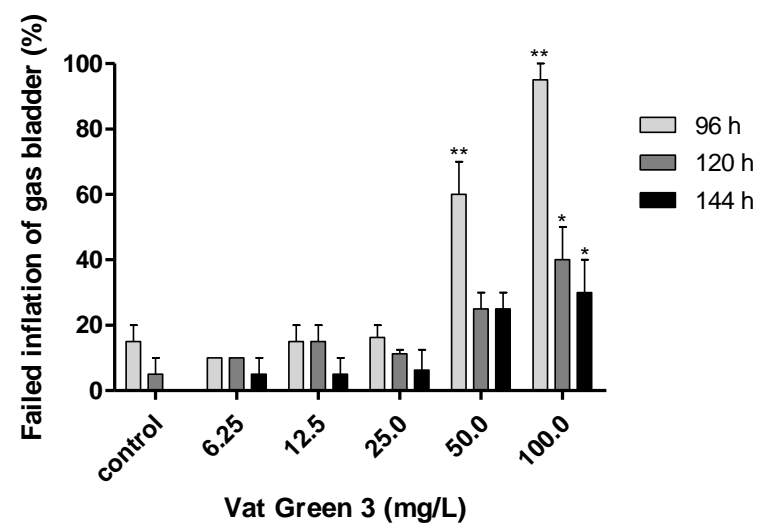
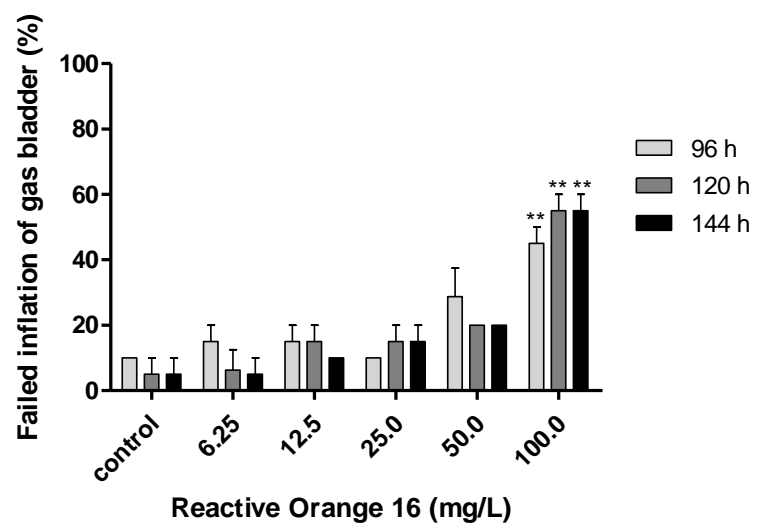
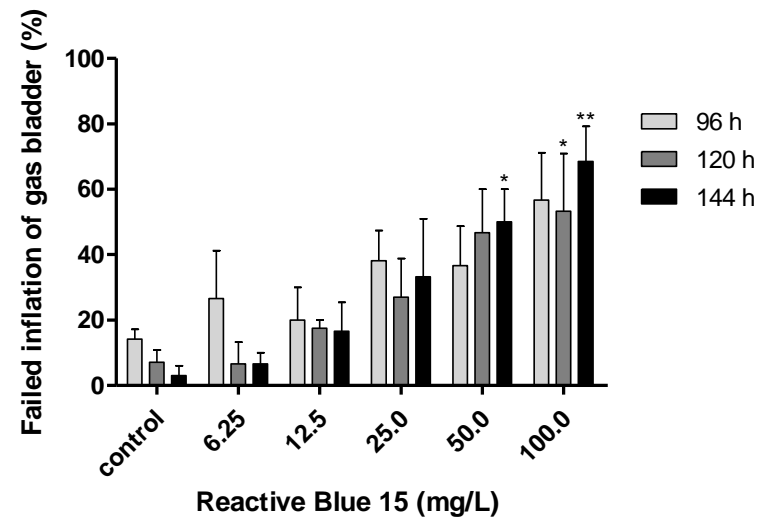
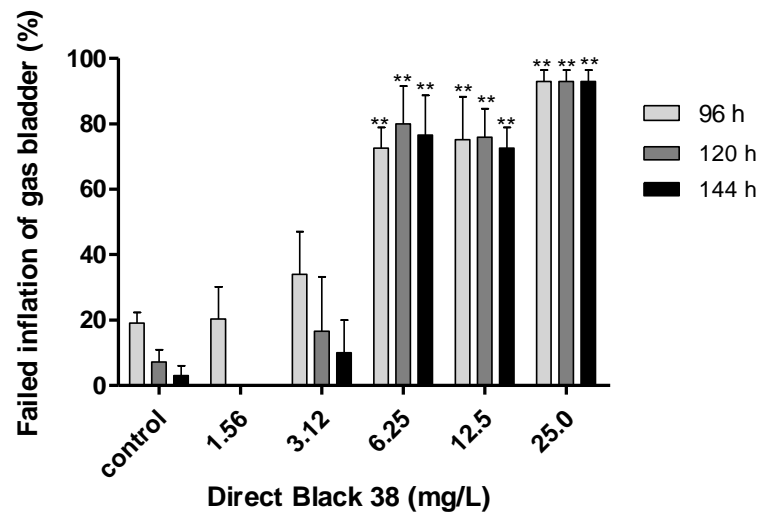


Figure 4.



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Figure 5.

