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Exposure to the azo dye Direct blue 15 produces toxic effects on microalgae, cladocerans, and zebrafish embryos

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Abstract

Aquatic pollution caused by dyes has increased together with the growth of activities using colorants such as the textile, leather, food, and agrochemicals industries. Because most popular azo dyes are synthesized from benzidine, a carcinogenic compound, a threat to aquatic biota could be expected. The use of single species for toxicity assessment provides limited data, so a battery of test organisms, including representatives of different trophic levels such as algae, zooplankters, and fish, could undoubtedly provide more information. Therefore, our study was aimed at evaluating the toxic effect of the azo dye Direct blue 15 (DB15) on a battery of bioassays using a primary producer (*Pseudokirchneriella subcapitata*), a primary consumer (*Ceriodaphnia dubia*), and a secondary consumer (*Danio rerio*). *P. subcapitata* was more sensitive to DB15 (IC_{50} = 15.99 mg L^{-1}) than *C. dubia* (LC_{50}: 450 mg L^{-1}). In the algae exposed to DB15, chlorophyll-*a* and -*b* were significantly increased, and carotenoids were reduced. The concentrations of protein, carbohydrates, and lipids per cell in *P. subcapitata* exposed to all DB15 concentrations were significantly higher than that measured in control. At 25 mg L^{-1} of DB15, survival, total progeny, and the number of released clutches were significantly decreased, and the start of reproduction was delayed in *C. dubia*. DB15 did not induce lethal or sublethal effects in *D. rerio* embryos at any of the tested concentrations from 24 to 72 h post-fertilization (hpf), but from 96 to 144 hpf, the larvae exposed to 100 and 500 mg L^{-1} developed yolk sac edema, curved tail, and skeletal deformations. After 144 hpf, DB15 produced a significant increase in embryos without a heartbeat, as the concentration of dye raised. The textile-used, azo dye DB15, caused toxic effects of different magnitude on microalgae, cladocerans, and zebrafish embryos; for this reason, the discharge of this colorant into waterbodies should be regulated to prevent environmental impacts.

Keywords Azo dyes · Cladocerans · Zebrafish embryos · Microalgae · Toxicity

Introduction

The dyeing process of fabrics produces approximately 90% of the total textile industry wastewaters containing significant concentrations of dyes (Zaharia and Suteau 2013). The main synthetic colorants are azo dyes (Balapure et al. 2015), being the most important producers USA, China, India, and Middle East countries (Zaharia and Suteu 2012).

Effluents containing dyes reduce light penetration in waterbodies and affect the photosynthesis of phytoplankton (Hernández-Zamora et al. 2014). Azo dyes (such as Direct Blue 15, DB15), are made from benzidine, a carcinogenic compound (Golka et al. 2004). DB15 has a strong affinity to cellulose fibers and is, thus, employed in textile industries (Rehman et al. 2017). According to European regulations (SCCNFP 2002), DB15, when degraded, produces carcinogenic amines. Its use has been banned in India since 1997 (Ministry of Environment and Forest 1997); however, it is manufactured in India for exportation and consumption in other countries.

The environmental impact caused by the discharge of textile dyes effluents has been scarcely studied. Even at low concentration (1 mg L^{-1}), dyes could be highly perceptible in waterbodies, causing not only aesthetic pollution but disturbing aquatic ecosystems and water resources (Vimonses et al. 2009). In recent years, information about concentrations of azo dyes in the aquatic environment has been published. In the rivers Piracicaba and Quilombo

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(Brazil), Vacchi et al. (2016) reported that Disperse Red 1 was detected in a range of 50 to 500 ng L^{-1}. Zocolo et al. (2015) estimated that the concentrations of the dyes Disperse 1, Disperse blue 373, and Disperse violet 93 ranged from 84.4 to 3453.3 ng L^{-1} in samples from the Piracicaba and Cristais Rivers, in the state of Sao Paulo, Brazil. In North America, Environment Canada and Health Canada (2009, 2013a, 2013b) estimated concentrations of 3.7, 0.40–10.8, and 0.12–102.7 µg L^{-1} for disazo dyes, azo solvents, and azo disperse dyes, respectively in Canadian waters. Disperse blue 79, Disperse blue 26, and Disperse red 60 were detected in samples of water, suspended solids, and sediments in a river in Canada (Maguire and Tkacz 1991).

Not enough data is available for Mexico; however, Villegas-Navarro et al. (1999) reported that in the State of Puebla, Mexico, were 353 textile industries that use great quantities of water. From this number, 53 facilities are for dyeing of textiles, and they discharge polluted colored effluents to water bodies.

The toxic impact of dyes has been demonstrated on aquatic and terrestrial organisms and in humans (Umbuzeiro et al. 2005; Puvaneswari et al. 2006; Copaciu et al. 2013). Toxicity of some azo dyes has been studied in aquatic organisms including algae, crustaceans, and fish (Bafana et al. 2011; Hernández-Zamora et al. 2016); nevertheless, some of these dyes have been studied only with a reduced number of test organisms.

Due to their importance as primary producers and their sensitivity to chemical stressors, microalgae have been used to assess environmental perturbations and as indicators of water quality (Mohamed 1994). Microalgae have been used to evaluate the toxicity of chemical pollutants as metals, herbicides, insecticides, and, recently, dyes (Levy et al. 2007; Wen et al. 2011; Jena et al. 2012; Hernández-Zamora et al. 2016).

Cladocerans are one of the main components of the zooplankton community and are important inhabitants of most water bodies (Wong et al. 2006). *Daphnia magna* and *Ceriodaphnia dubia* are reference test organisms for ecotoxicological studies (Blaise and Férard 2005). Although information about the effects of dyes to aquatic invertebrates is scarce, some studies have determined the toxicity of azo dyes to cladocerans (Villegas-Navarro et al. 1999; Bae and Freeman 2007; Ferraz et al. 2011; Malik et al. 2018; Oliveira et al. 2018).

Zebrafish embryos have been widely used as a model for toxicity studies of chemicals due to their rapid development and easy identification of different endpoints, easy maintenance in the laboratory and large fecundity; in addition, the high transparency of the chorion allow elucidating toxic effects on embryogenesis and larval development (Kimmel et al. 1995; Hill et al. 2005; Scholz et al. 2008; Lammer et al. 2009). Recently, zebrafish have been used to determine the toxicity of some dyes (Parrott et al. 2016; Rodrigues de Oliveira et al. 2016; Abe et al. 2017; Meireles et al. 2018).

The environmental impact caused by the discharge of textile dyes into water bodies and the toxicity to aquatic biota have not been fully documented. Regarding the toxic effects of azo dyes, most of the few available studies have been done with single-species tests, thus, no complete scenario can be elucidated to establish the expected, variable damages, when diverse organisms are exposed to these potential pollutants. With this aim, we studied the toxic effect of the azo dye Direct blue 15 on a battery of bioassays, including microalgae (*Pseudokirchneriella subcapitata*), a cladoceran (*Ceriodaphnia dubia*), and a fish fed on zooplankton (*Danio rerio*).

Materials and methods

Direct blue 15

This anionic diazo direct dye was obtained from Sigma-Aldrich® ($C_{34}H_{24}N_6O_6S_4Na_4$; molecular weight: 992.80 g mol^{-1}; CAS No: 2429-74-5). For all experiments, the dye was prepared as a stock solution at 1000 mg L^{-1}. This compound is a deep purple to dark blue microcrystalline powder that decomposes when exposed to air. It is highly soluble in water (60 g L^{-1} at 85 °C), and insoluble in most organic solvents. It is also called Direct sky blue A and Direct sky blue 5B (Rehman et al. 2017).

Test organisms

The test organisms (*Pseudokirchneriella subcapitata*, *Ceriodaphnia dubia*, and *Danio rerio* embryos) were obtained from the collections of the *Laboratorio de Hidrobiología Experimental*, *Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional*, México.

The microalga was grown in Bold's basal mineral medium (Stein 1973) using flat-sided glass bottles with a total capacity of 0.5 L and a test volume of 0.25 L. Incubation conditions were: temperature: 24 ± 1 °C, light intensity: 120 µmol m^{-2} s^{-1}, constant illumination, and continuous bubbling with air flow of 200 mL min^{-1}.

Ceriodaphnia dubia was cultured in ISO medium (2 mM $CaCl_2 \cdot 2H_2O$, 0.5 mM $MgSO_4 \cdot 7H_2O$, 0.75 mM $NaHCO_3$, 0.07 mM KCl) (ISO 1996) and deionized water 1:5. Cultures were maintained at 25 ± 1 °C, and 16:8 h (light: darkness photoperiod). The cladocerans were fed with the microalga *P. subcapitata* at a concentration of 1×10^6 cells mL^{-1}. For neonate production, 24 h before the experiments, adult parthenogenetic females were separated and neonates

produced from these adults were used in the study (USEPA 2002).

Adult zebrafish males and females have been successfully cultured under controlled conditions for over eight years. Fish are maintained in 40-L glass aquaria at 26 ± 1 °C and photoperiod 16:8 h (light: darkness). Fish are fed with commercial micropellets (Azoo® Plus Ultra Fresh Tropical Bits) 3-times daily at regular intervals, complemented sporadically with cultured live food (small daphnids). One week before spawning, reproducers were fed daily with daphnids to promote optimal mating.

Direct blue 15 toxicity to microalga *P. subcapitata*

Tha algal growth inhibition test was performed according to the OECD protocol 201 (OECD 2011) to determine the toxic effects of DB15. For the bioassays, vials with inocula of *P. subcapitata* (1×10^4 cells mL^{-1}) were exposed to various concentrations of DB15 (4, 8, 16, 32, and 64 mg L^{-1}) in OECD medium (pH 8.1). Incubation conditions for the tests were 24 ± 1 °C, and continuous fluorescent light (120 µmoles m^{-2} s^{-1}). Algal growth was determined in the samples with a Neubauer hemocytometer every 24 h for four days. The median inhibitory concentration (IC_{50}) was obtained from three independent assays, with three replicates for each bioassay.

Sub-inhibitory toxicity test in *P. subcapitata*

Experiments were performed in flat-sided glass bottles with a total capacity of 0.5 L and a test volume of 0.25 L of OECD medium, inocula of *P. subcapitata* (1×10^4 cells mL^{-1}) and the concentrations of 1.63, 3.57, 6.29, 10.19, and 15.99 mg L^{-1} of DB15 equivalent to the IC_{10}, IC_{20}, IC_{30}, IC_{40}, and IC_{50}, respectively. Test organisms were incubated at 24 ± 1 °C, continuous illumination of 120 µmol m^{-2} s^{-1}, and constant bubbling with an air flow of 200 mL min^{-1}. The cell density was determined daily with a Neubauer chamber. After 96 h of exposure, the microalgae pellet was washed three times using OECD medium to eliminate all the dye remnants and the dye possibly adhered to the cell wall, following the centrifugation at 3500 rpm for 10 min. Finally, the cells were resuspended in OECD medium and stored in darkness at 4 °C to determine photosynthetic pigments and macromolecules.

Photosynthetic pigments

Photosynthetic pigments of the cells contained in 500 µL sample were extracted with 600 µL dimethyl sulfoxide. Absorbance was read at 665, 649, and 470 nm. The concentration of Chl-*a*, Chl-*b*, and total carotenoids was determined according to the equations of Wellburn (1994):

$$\text{Chlorophyll } a(\text{Chl } a) = 12.19\,A_{665} - 3.45\,A_{649}$$

$$\text{Chlorophyll } b(\text{Chl } b) = 21.99\,A_{649} - 5.32\,A_{665}$$

$$\text{Carotenoids} = \frac{1000\,A_{470} - 2.86\,\text{Chl } a - 129.2\,\text{Chl } b}{221}$$

Protein content in *P. subcapitata*

Proteins were quantified according to Lowry et al. (1951) using a calibration curve of albumin in a standard solution; 500 µL of *P. subcapitata* cells was extracted with 300 µL of a 1 N solution of NaOH at 90 °C for 15 min to extract soluble protein. After this, the sample was centrifuged at 14,000 rpm for 5 min, and 100 µL of supernatant was used to determine the amount of protein. Absorbance of samples and standard solution was read at 590 nm.

Carbohydrates content in *P. subcapitata*

Cells contained in 500 µL were pretreated with 200 µL of 2 N HCl at 90 °C for 1 h. Total carbohydrates were determined according to Dubois et al. (1951). Carbohydrates quantitation was based on calibration curves using glucose as standard. Absorbance of samples and standard solution was read at 490 nm.

Lipids

Lipids of algal cells (500 µL) were extracted with a solution of chloroform: methanol (2:1) and, subsequently, evaporated at 80 °C. Lipids were determined according to Zöllner and Kirsch (1962). Cholesterol was used as standard solution. Absorbance of samples and standard solution was read at 525 nm.

Direct blue 15 toxicity to *Ceriodaphnia dubia:* acute and chronic toxic effects

The acute toxic effects of DB15 dye on *C. dubia* were determined according to USEPA (2002) guidelines. For the bioassays, we used different concentrations of DB15 (25, 50, 100, 200, 300, and 400 mg L^{-1}) diluted in ISO medium and deionized water (1:5); test volume was 30 mL in each of three replicates per test. Thereafter, 10 neonates were transferred to each test vessel and incubated in an environmental chamber at 24 ± 1 °C, with a photoperiod of 16:8 h (light: darkness). No food was supplied during the assays. The number of affected, immobilized, and dead (no heartbeat, through observation with the stereomicroscope)

organisms were recorded at 24 and 48 h. Three independent bioassays were carried out to calculate the average LC_{50}.

The chronic toxicity of DB15 was determined following Method 1002.0 Daphnid, *Ceriodaphnia dubia*, survival, and reproduction test (USEPA 2002). The bioassays were started with neonates, and each experiment had 10 replicates. Neonates were individually placed in recipients containing 25 mL of test solution (ISO medium and deionized water 1:5) and different concentration of DB15 (5, 10, 15, 20, and 25 mg L^{-1}). *C. dubia* organisms were fed daily the microalga *P. subcapitata* (1×10^6 cells mL^{-1}). The test solutions and food were renewed daily.

Bioassays were incubated in an environmental chamber at 24 ± 1 °C and 16:8 h (light: darkness) photoperiod. Survival, accumulated progeny, age at first reproduction, and the number of clutches were daily recorded for seven days.

Direct blue 15 toxicity to zebrafish embryos: lethal and sublethal endpoints

To obtain fertilized zebrafish eggs, we followed the protocol proposed by the OECD Guideline 236 (OECD 2013). Briefly, the day before the test, adult, mature zebrafish males and females (ratio 2:1) were placed in spawning tanks in a room with controlled temperature and photoperiod (16:8, light: darkness). After the light turned on the next morning, spawn was promoted, and fertilized eggs were obtained by natural mating. After spawning, the eggs were collected by hand, rinsed with ISO medium, and checked under a stereomicroscope; unfertilized eggs were discarded, and live embryos were used for the toxicity tests.

We followed the procedure described in the OECD guideline 236 (OECD 2013) and by Busquet et al. (2014). Two sets of DB15 concentrations were tested: (1) 6.25, 12.5, 25, 50, 100 mg L^{-1} and (2) 100, 200, 300, 400, and 500 mg L^{-1}. 3, 4-Dichloroaniline (4 mg L^{-1}) was used as positive control, and ISO medium: deionized water (1:5) was the negative control and dilution water.

Viable eggs were individually placed into 24-well plates, each well containing 2 mL of test solution. For all experiments, 20 eggs for each concentration of DB15 (one plate for each concentration) and four eggs as the internal negative control (ISO medium: deionized water 1:5) were used, as well as one plate with 20 eggs as the positive control and 24 eggs as negative external control. The samples were incubated at 26 ± 1 °C for 144 h with a photoperiod of 16:8 h (light: darkness) in an environmental chamber. Test solutions were fully renewed at 24, 72, and 120 h, as indicated in the guideline.

Toxic effects of DB15 on early life stages (ELS) of zebrafish were determined according to the criteria described in the OECD guideline 236 through observations under a stereomicroscope at 24, 48, 72, and 96 h, but extended observations were performed at 120 and 144 h post fertilization (hpf). Lethal endpoints assessed were: coagulation, no tail detachment, no somite formation, and no heartbeat (this last one measured as presence and absence after 48 h). Larval survival and malformations were documented and photographed every day after hatching. Sublethal endpoints used to assess the effects of DB15 on zebrafish development included the presence of yolk sac edema, pericardial sac edema, skeletal deformities (curved tail) and delayed pulse. These endpoints were qualitatively evaluated as presence or absence; the affected embryos and larvae were also counted.

Statistical analysis

The median inhibitory concentration (IC_{50}) and the median lethal concentration (LC_{50}) with corresponding 95% confidence intervals were calculated via the Probit method, using RA software (Risk Assessment. Hazard Assessment Tools, v. 1.0).

In *P. subcapitata* and *C. dubia*, an analysis of variance (ANOVA) was performed using Dunnett's comparison test to identify significant differences between the concentrations of DB15 and the control. Tukey's pairwise multiple comparisons test was carried out to identify significant differences among all the treatments, including the control. Kruskal–Wallis test was used to analyze the toxicity of Direct blue 15 on zebrafish, followed by Dunnett's and Tukey's pairwise tests. All statistical analyses were completed using Statistica ver. 10.0 and SigmaPlot ver. 12.0.

Results

Direct blue 15 effects on the growth of *P. subcapitata*

The 96-h growth inhibition trend lines of algae exposed to DB15 are shown in Fig. 1a, b. *P. subcapitata* growth was affected by exposure to the dye at all tested concentrations. ANOVA test demonstrated significant differences ($P < 0.05$) for population density values among treatments, determined at 96 h. Cell density of microalgae in the control group was significantly higher than at all the tested DB15 concentrations (Dunnett's test, Fig. 1a).

The 96-h growth inhibition at the concentrations of 4, 8, 16, 32, and 64 mg L^{-1} was 29%, 41%, 48%, 68%, and 80%, respectively, compared to the control (Fig. 1b). The determined median inhibition concentration (IC_{50}) for DB15 was 15.99 mg L^{-1} (95% CI limits: 12.94–19.75 mg L^{-1}).

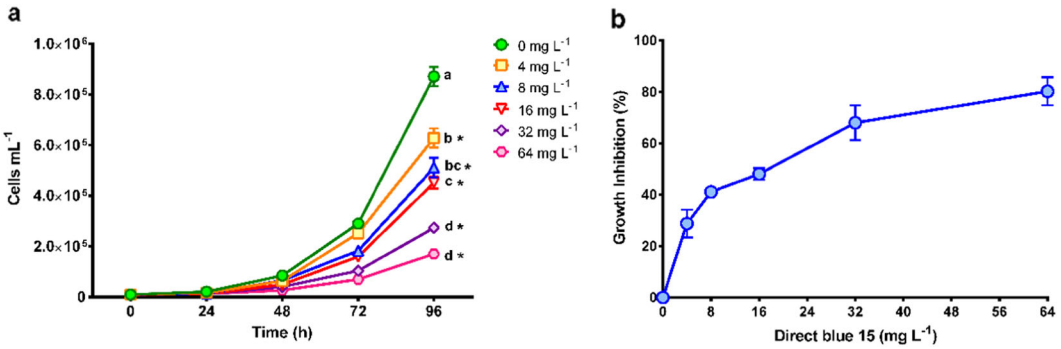

Fig. 1 Population growth (**a**) and growth inhibition (**b**) of *P. subcapitata* exposed during 96 h to different concentrations of Direct Blue 15. Mean values ± standard error. * Indicates significant differences ($P < 0.05$) with respect to the control (Dunnett's test). Different letters indicate significant differences ($P < 0.05$) after Tukey's pairwise comparisons

Direct blue 15 effects on the pigments and macromolecules content of *P. subcapitata*

Figure 2a shows the concentration of photosynthetic pigments quantified at the end of the test (96 h). Chlorophyll *a* in cells exposed to 3.57, 10.19, and 15.99 mg L^{-1} DB15 was significantly higher than in the control (Dunnett's test, $P < 0.05$). Chlorophyll *b* content was significantly lower in the control that at all the dye concentrations, but carotenoids content displayed an inverse situation. Tukey's differences among treatments and the control are shown in Fig. 2a.

Figure 2b shows significant differences in the concentration of macromolecules (protein, carbohydrates, and lipids) at the end of the sub-inhibitory assay. The concentration of protein per cell in *P. subcapitata* exposed to any of the dye concentrations was significantly higher than that measured in the control (Dunnet's test, $P < 0.05$), and was significantly different among them (Tukey's test, $P < 0.05$). The highest protein concentration was observed in cells exposed to 6.29 mg L^{-1} of dye.

The carbohydrate concentrations in cells exposed to any of the DB15 concentrations were significantly higher, according to the Dunnett's test ($P < 0.05$), than that measured in the control. Figure 2b shows that, among treatments (1.63, 13.57, 10.19, and 15.99 mg L^{-1}), there were no significant differences according to Tukey's test ($P < 0.05$). The highest carbohydrate content was measured in cells exposed to 6.29 mg L^{-1} of dye.

DB15 exposure significantly augmented lipids concentration in cells of microalgae respect to the control (Dunnett's test). The concentration of lipids per cell was not significantly different among treatments, except for 6.29 mg L^{-1} according to Tukey's test ($P < 0.05$), as shown in Fig. 2b.

Effect of Direct blue 15 on cladocerans

Acute toxicity test results demonstrated that the median lethal concentration (LC_{50}) for *C. dubia* was 450 mg L^{-1} of DB15 (95% limits: 422.65–476.34 mg L^{-1}). Although the LC_{50} is not an environmentally relevant concentration for cladocerans, we observed that the digestive tract of test organisms was remarkably blue colored, hence toxic effects could be expected at chronic exposures.

Figure 3a–d show the effect of chronic DB15 exposures on survival, accumulated progeny, age at first reproduction, and the number of clutches of *C. dubia*. The percentage of survival was 100% after seven days of exposure at all concentrations of DB15, except for 25 mg L^{-1} (Fig. 3a). Accumulated progeny decreased significantly (Fig. 3b, $P < 0.05$) in organisms exposed to 20 and 25 mg L^{-1} respect to the control (according to Dunnett's test). As observed in Fig. 3c, the age at first reproduction was significantly higher ($P < 0.05$) at the 25 mg L^{-1} concentration respect to the control and the other dye concentrations. The two highest concentrations of DB15 (20 and 25 mg L^{-1}) caused the reduction of clutches in 37 and 70%, respectively, compared to the control (Dunnett's test, Fig. 3d).

Effect of Direct blue 15 on zebrafish embryos

Fish embryos were exposed to two different DB15 concentration ranges (0–100 and 0–500 mg L^{-1}) to determine their toxic effects. In the first assay, hatching started at 72 h in the controls and at all DB15 concentrations (Fig. 4a), and 100% hatched larvae were observed at 96 h. However, in the experiment where the range of 0–500 mg L^{-1} was tested, hatching was significantly reduced in all the DB15 concentrations, except in 100 mg L^{-1}; at 144 h hatching percentage was 35%, 35%, 10% and 5%, respectively in the

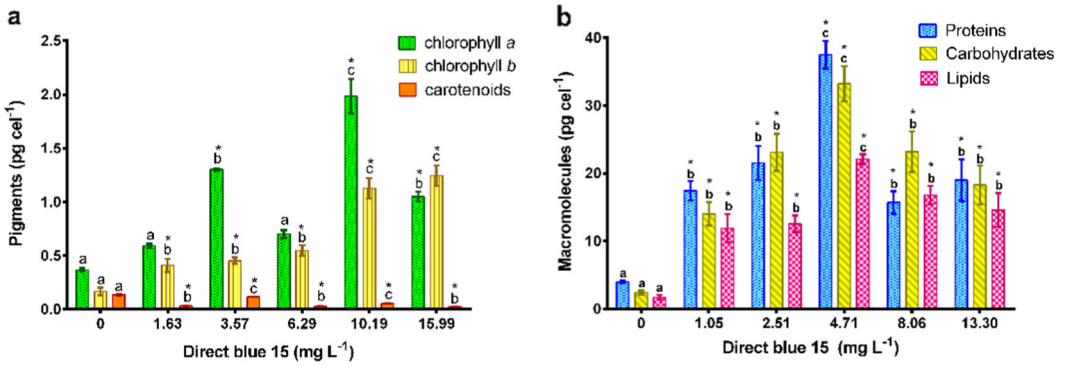

Fig. 2 Biomarkers in cells of *P. subcapitata* exposed to sub-inhibitory concentrations of Direct Blue 15 during 96 h: (**a**) concentration of photosynthetic pigments, and (**b**) concentration of macromolecules. Mean values ± standard error bars. *Indicates significant differences ($P < 0.05$), comparing with the control (Dunnett's test). Different letters above the bars indicate significant differences ($P < 0.05$) for each set of pigments or macromolecules (proteins, carbohydrates, and lipids) data, after Tukey's pairwise comparisons

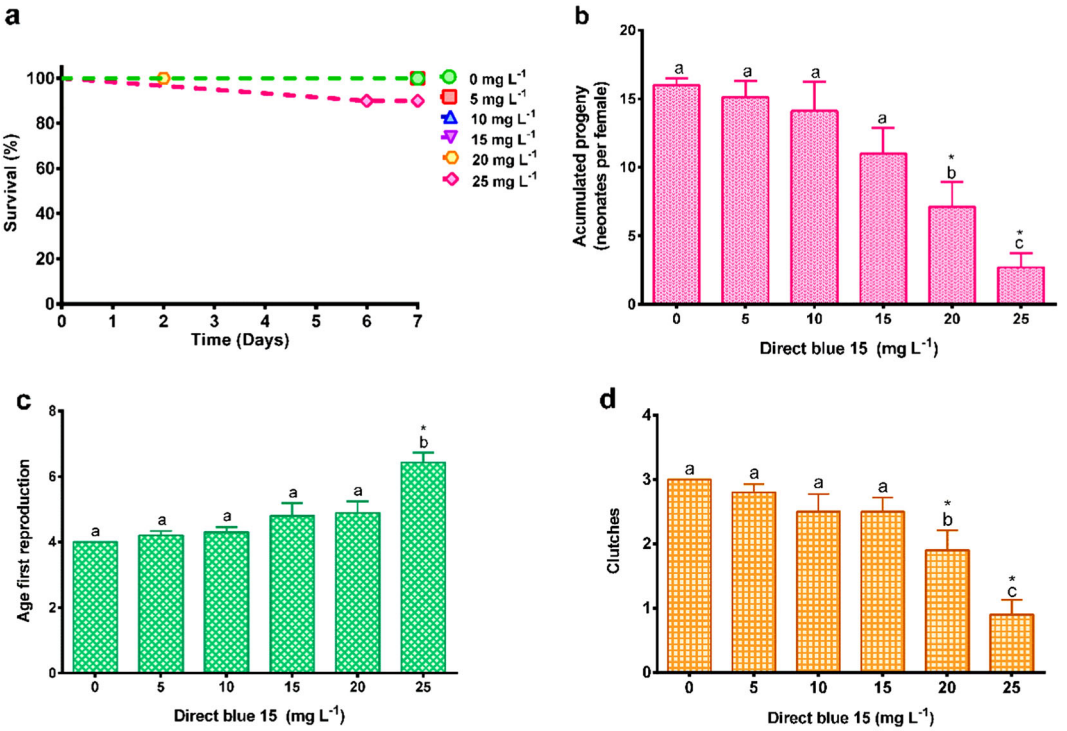

Fig. 3 Effect of Direct Blue 15 on *Ceriodaphnia dubia* (**a**) survival, (**b**) accumulated progeny, (**c**) age at first reproduction and (**d**) number of clutches. Mean values ± standard error. *Indicates significant difference ($P < 0.05$) compared with the control (Dunnett's test). Different letters above the bars indicate significant differences ($P < 0.05$), after Tukey's pairwise comparisons

concentrations of 200, 300, 400 and 500 mg L^{-1} (Dunnett's test) (Fig. 4b, $P < 0.05$).

Figure 5 shows the development of the zebrafish embryos exposed to 6.25, 100, and 500 mg L^{-1} DB15. Exposure to the dye from 24 to 72 hpf did not induce lethal effects (coagulation, no tail detachment, no heartbeat, or no somite formation) or sublethal effects (skeletal deformations and yolk sac edema) at all tested concentrations (Fig. 5a–d). However, from 96 to 144 hpf, the organisms exposed to 100 and 500 mg L^{-1} showed yolk sac edema, curved and deformed tail (Fig. 5c, d).

The percentage of lethal (no heartbeat) and sublethal effects (delayed pulse, curved tail, and yolk sac edema) observed at 144 hpf in early life stages of zebrafish are shown in Fig. 6. Exposure to DB15 produced a significant increase in no heartbeat frequency, as the dye concentration increased ($P < 0.05$), however, yolk sac edema only increased significantly in the organisms exposed to 100, 200, and 300 mg L^{-1}, according to Tukey's test. The percentage of larvae with delayed pulse and curved tail did not show significant differences at DB15 concentrations from 100 to 500 mg L^{-1} ($P < 0.05$).

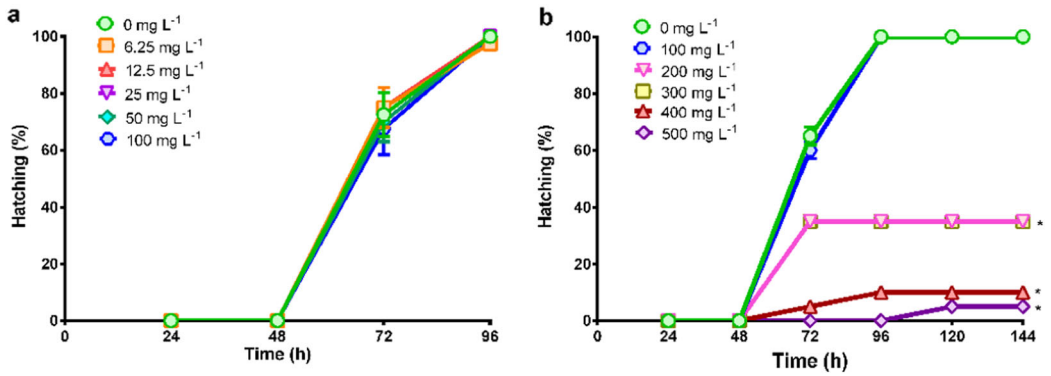

Fig. 4 Hatching of zebrafish embryos exposed to different concentrations of Direct blue 15 (**a**) 0–100 mg L^{-1}; (**b**) 0–500 mg L^{-1}. Mean values ± standard error. *Indicates significant differences ($P < 0.05$) after Dunnett's test

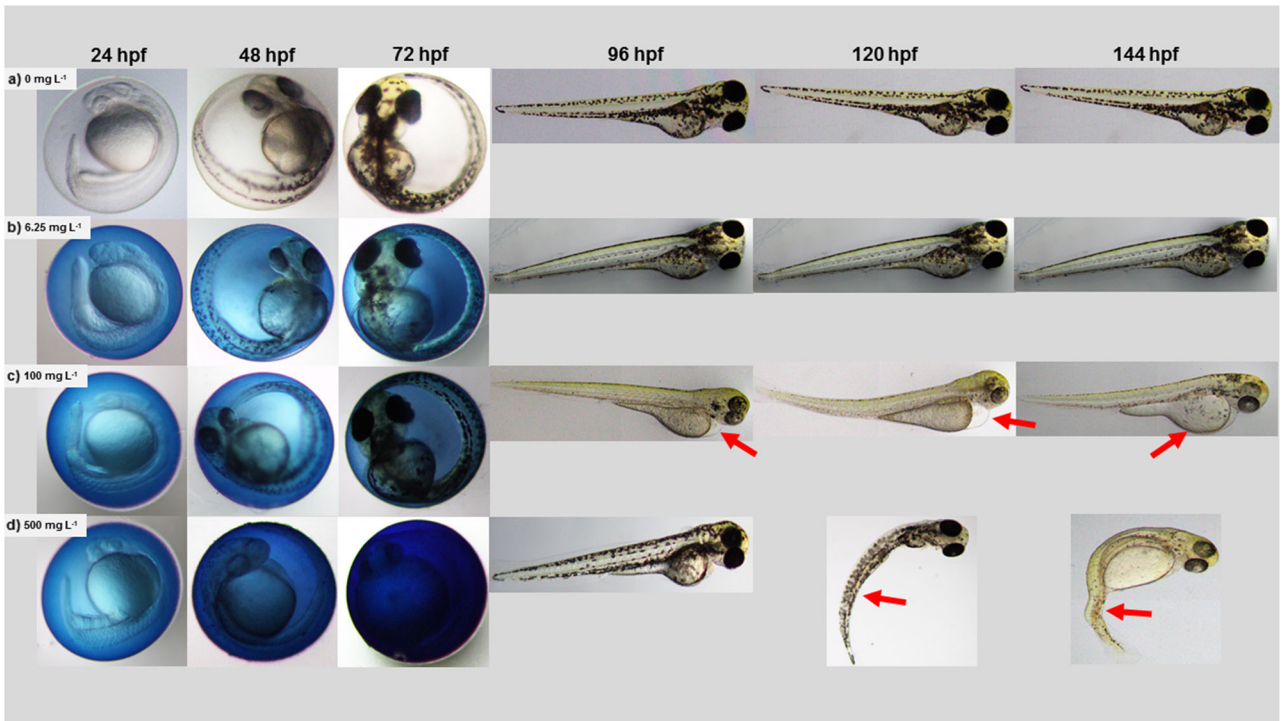

Fig. 5 Toxic effects of Direct blue 15 on the development of zebrafish embryos. (**a**) Normal embryo and larva at 144 hpf, (**b**) Normal embryo in 6.25 mg L^{-1} of dye at 144 hpf, (**c**) Larvae of 96–144 hpf with pericardial sac edema and yolk sac edema (arrows) exposed to 100 mg L^{-1} of dye, (**d**) Larvae of 120–144 hpf with tail deformation (arrows), observed at 500 mg L^{-1} of DB15

Discussion

The green alga *P. subcapitata* is ubiquitous in freshwater ecosystems and is frequently used as test organism due to its high sensitivity to toxicants (Greene and Baughman 1996; Croce et al. 2017). Results demonstrated that the growth of the microalga *P. subcapitata* exposed to DB15 was inhibited as the dye concentration increased (Fig. 1a, b). Similar results were reported by Lim et al. (2010) when the microalga *Chlorella vulgaris* was grown on textile wastewaters containing azo dyes. Chia and Musa (2014) showed that effluents containing indigo dye were toxic to *Scenedesmus quadricauda*, having negative effects on growth, biomass production, and the number of cells per coenobium. Moreover, Greene and Baughman (1996) demonstrated that six dyes inhibited population growth in more than 20% in *Selenastrum capricornutum*; however, seven basic textile dyes stimulated growth by >20% compared to control cultures. These opposite results can be related to the exposure conditions and the species-specific effects; this

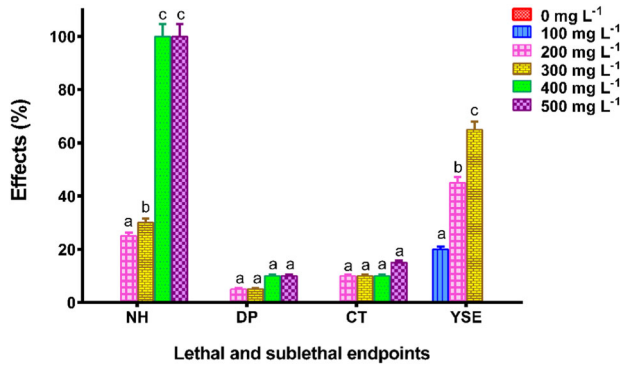

Fig. 6 Effects on zebrafish embryos after 144 hpf exposure to different concentrations of Direct Blue 15. The effects included no heartbeat (NH), delayed pulse (DP), curved tail (CT), and yolk sac edema (YSE). Mean values ± standard error bars ($n = 20$). Different letters above the bars indicate significant differences ($P < 0.05$) for each endpoint after Kruskal-Wallis's test followed by Tukey's pairwise comparisons

should be taken with care to discard toxic effects, because many others results, including ours, have demonstrated inhibitory effects in the population growth of microalgae.

Øllgaard et al. (1998) mentioned that when the dye is present at high concentrations, the resulting growth inhibition in microalgae is produced by toxicity inside the cell. Roy et al. (2010) showed that textile dyes produced growth inhibition in freshwater microalgae because of the reduction in cell division and photosynthetic activity. Hernández-Zamora et al. (2014) reported that the alteration of photosynthetic activity at the electron donor stage of photosystem II was one of the possible causes of the growth inhibition in the microalga *C. vulgaris* exposed to Conge Red dye.

The sensitivity of *P. subcapitata* to DB15 was demonstrated as the mean inhibitory concentration (IC50 = 15.99 mg L^{-1}, 95% confidence limits: 12.94–19.75 mg L^{-1}). Greene and Baughman (1996) reported that *P. subcapitata* had EC_{50} values of 0.025 and 0.247 mg L^{-1} for dyes 28 and 41, respectively. The 96-h EC_{50} values of Reactive Orange 16, Congo Red, Remazol Brillant Blue R, and Disperse Blue 3 reported for *P. subcapitata* are 7.8, 4.8, 81.1, and 0.5 mg L^{-1}, respectively (Novotný et al. 2006); some of these values are lower than the one determined in the present study for DB15, but differences could be related to the conditions used for their determination.

Moreover, Tigini et al. (2011) demonstrated that *P. subcapitata* was sensitive to dyes such as Acid Bath for wool (EC_{50} 18.8%), Acid Bath for leather (EC_{50} 17.7%), Reactive Bath for cotton (EC_{50} 2.2%), and Direct Bath for cotton (EC_{50} 31.7%). Vacchi et al. (2016) and de Luna et al. (2014) reported comparatively higher values of EC_{50} for *P. subcapitata* exposed to Disperse Red and Acid Orange 7 (102 and >100 mg L^{-1}, respectively). Similarly, another study with *P. subcapitata* demonstrated that Direct Blue 71 and Direct Blue 293 produced EC_{50} values of 62.260 and 137.975 mg L^{-1}, respectively (Croce et al. 2017). However, other studies showed algae susceptibility below 100 mg L^{-1} for different azo dyes, in the following order: mordant > basic/acid/disperse > direct > hydrolyzed > reactive dyes (ETAD 1992; Øllgaard et al. 1998).

Based on the presented information, we can infer that the sensitivity of *P. subcapitata* to dyes could be related mainly to the chemical structure of colorants, the exposure time, and the exposure conditions, but invariably toxic effects on the growth of microalgae have been documented. Most of the textile dyes are not available as pure reagents; DB15 contains other components, such as organic compounds related to the synthesis of the dye, and inorganic salts (sodium chloride, sodium sulfate); these impurities are present in the product sold as a chemical compound (from Sigma-Aldrich and other suppliers), and in the commercial form (Shan and Harbin 1988). The dye and all these accompanying impurities are used as a whole during the dyeing process and could contribute to the toxic effects produced, but also could be related with the variable effects reported in the growth of *P. subcapitata*.

The photosynthetic pigments content has been used as a sensitive parameter for microalgae under environmental stress conditions caused by heavy metals and dyes (Hernández-Zamora et al. 2014; Martínez-Ruiz and Martínez-Jerónimo 2015). Toxic compounds could affect the synthesis of chlorophyll-*a*, -*b* and carotenoids in algae. DB15 exposure stimulated the content of chlorophylls in *P. subcapitata*; however, the carotenoids content was diminished, as observed in Fig. 2a. Similar effects were observed by Hernández-Zamora and Martínez-Jerónimo (2019) when *P. subcapitata* was exposed to Congo Red dye. The increase in photosynthetic pigments could be a response to compensate the chemical stress produced by the dye and to reduce the toxic effect; this reaction can also be related to adaptations to perform the photosynthesis in the colored culture media, after the addition of the dye.

Mahalakshmi et al. (2015) reported that when *Haematococcus* sp. was exposed to different concentrations of Congo Red, the content of chlorophyll-*a* showed the highest accumulation on the 5th day at 12 mg L^{-1}. In contrast, Gita et al. (2019) observed that the textile dyes Optilan yellow, Drimarene blue and Lanasyn brown, inhibited chlorophyll and carotenoids content in *Chlorella vulgaris*; in this case, the observed effects could be related to a toxic response. Nevertheless, in *Chlamydomonas reinhardtti* exposed to 10 and 20 mg L^{-1} of Malachite green and Crystal violet, Yadav et al. (2014) reported that the chlorophyll content was not significantly modified; this result could indicates that the concentration of these dyes was low enough to produce changes in this endpoint.

The concentration of proteins, carbohydrates, and lipids per cell increased significantly in *P. subcapitata* under

DB15 stress (Fig. 2b). The increase of proteins in *P. subcapitata* is an effect similar to that reported by Einicker-Lamas et al. (2002) with *Euglena gracilis* and by Rocha et al. (2016) with *Selenastrum gracile*; these authors suggested that the inability of cells to divide could be responsible for the increase in this biomolecule production. However, Gita et al. (2019) reported that comparatively higher concentrations of Optilan yellow, Drimarene blue, and Lanasyn brown reduce the content of protein in *Chlorella vulgaris*.

Algae use light and nutrients to produce proteins, carbohydrates, and lipids during photosynthesis; however, under unfavorable conditions, microalgal growth is arrested, and the energy in excess could be stored as carbohydrates and lipids (George et al. 2014). D'Alessandro and Antoniosi Filho (2016) reported that lipid metabolism could be modified in response to several factors that induced lipid accumulation, as a strategy to assure their growth and reproduction via cell division; this effect was observed in *P. subcapitata* exposed to DB15 as a reduction in the population growth (Fig. 1b), and increase in lipid content (Fig. 2b).

Seo et al. (2015) reported in *C. vulgaris* exposed to rhodamine 101 (red solution) and 9,10-diphenyl anthracene (blue solution) increases in lipid content of 1.4 and 1.6 times, respectively, compared with the control. Ruyters (1984) reported that blue light stimulates nitrogen metabolism and the activation of enzymes related to lipid synthesis. We can assume that the increase in lipid content we observed in *P. subcapitata* is produced by the color of the dye solution, in addition to the toxicity of DB15.

Hernández-Zamora and Martínez-Jerónimo (2019) reported that when *P. subcapitata* was exposed to Congo red, the proteins, carbohydrates and lipids content increased in almost all the tested concentrations, similar to the effects we observed in the present study. These changes, as a toxic response to DB15, could affect the nutrimental quality of microalgae, with consequences to filter-feeder consumers in natural conditions, but this issue must be further studied.

Direct Blue 15 did not induce significant acute toxic effects on *C. dubia* after 48 h (LC_{50} 450 mg L^{-1}). Similarly, Rocha et al. (2017) demonstrated that Acid Black 210 dye does not cause acute toxicity in the cladoceran *D. similis* (EC_{50} was 2993.73 mg L^{-1}). Croce et al. (2017) reported that the LC_{50} for *D. magna* exposed to Direct Blue 293, Direct Blue 71, and Direct Red 227 was higher than 100 mg L^{-1}. Dyes with a 48-h LC_{50} higher than 100 mg L^{-1} are considered as non-toxic, whereas those with 48-h LC_{50} values lower than 100 mg L^{-1} are classified as toxic and of toxicological concern for aquatic biota (Zucker 1985; OECD 2004). According to this classification, DB15 should be classified as non-toxic, but this classification is valid only for acute, lethal effects because chronic exposure to sublethal concentrations could produce toxic effects that can be a risk for aquatic organisms.

Survival, accumulated progeny, age at first reproduction, and the number of clutches were examined during chronic exposure of *C. dubia* to DB15 (Fig. 3a–d). Survival of *C. dubia* was 100% after seven days at almost all tested concentrations (Fig. 3a). For other cladocerans, Wong et al. (2006) observed that the survival of *Moina macrocopa* exposed to different concentrations of Procion Red, Procion Yellow, and Congo Red decreased with increasing dye concentration.

Walthall and Stark (1999) reported that *Daphnia pulex* exposed to up to 250 mg L^{-1} of fluorescein did not show a significant reduction in survivorship after ten days; however, a decrease of 80% was documented at 350 mg L^{-1}. In our study, DB15 tested concentrations were lower than the used by Walthall and Stark (1999), and no significant effects were observed in this endpoint.

Regarding reproductive effects, our results demonstrated that the accumulated progeny was significantly reduced in *C. dubia* exposed to 20 and 25 mg L^{-1} DB15 (Fig. 3b). Similarly, Wong et al. (2006) reported that the total number of neonates produced by *M. macrocopa* after seven days exposure to Procion Red, Procion Yellow, and Congo Red was reduced in 49.8, 44.5 and 69% respectively, compared with the control. In *D. pulex* exposed to fluorescein Walthall and Stark (1999) observed that reproduction in control was significantly higher ($P < 0.05$) than that observed in all the tested concentrations (200, 250, 350, 450, and 600 mg L^{-1}).

Reduction in cladoceran fecundity is the consequence of intoxication produced by dye exposure. Schroder et al. (1991) reported that municipal effluents, containing toxic wastes, were also extremely toxic to *C. dubia* reducing significantly survival and reproduction even at low concentrations (6.25% and 10%). Reproduction in cladocerans exposed to chemical stressors is negatively affected because energy is invested in the detoxification process, thus reducing the amount of energy allocated to growth and reproduction, which could explain our present results.

The first reproduction of control *C. dubia* was recorded at 4-d age; however, cladocerans exposed to 25 mg L^{-1} of DB15 delayed reproduction until the sixth day (Fig. 3c). Wong et al. (2006) reported that reproduction in *M. macrocopa* was delayed by 1–1.5 days in organisms exposed to diazo dyes; delay in reproduction began at 0.01 mg L^{-1} for all three dyes, and cladocerans exposed to Procion Red at 100 mg L^{-1}, and Congo Red at 1, 10, and 100 mg L^{-1} did not reproduce.

In our study, the number of clutches per female was significantly decreased in *C. dubia* by high DB15 concentrations (Fig. 3d). In *M. macrocopa*, Wong et al. (2006) reported that the onset of reproduction was delayed and the number of produced neonates was reduced at azo dye

concentrations as low as 0.01 mg L^{-1}. With other toxicants, similar results were reported by Souza et al. (2014) for the cladoceran *Simocephalus serrulatus* fed with cadmium-contaminated algae; they found significantly reduced clutches per female. Negative changes in accumulated progeny, age at first reproduction, and the number of released clutches by *C. dubia* exposed to DB15 can be regarded as a physiologically impaired response to DB15 stress.

The hatching rate of zebrafish embryos did not decrease at 100 mg L^{-1} of DB15 (Fig. 4a); however, from 200 to 500 mg L^{-1} hatching was diminished as the concentration of dye increased (Fig. 4b). Similarly, Manjunatha et al. (2014) reported that hatching of zebrafish embryos was affected by henna dye proportionally to its concentration. Parrott et al. (2016) reported that Acid Blue 80 and Acid Blue 129 caused no effects on larval fish at the highest measured concentrations they tested (7700 and 6700 µg L^{-1}, respectively); however, Disperse Yellow 7 and Sudan Red G decreased survival of larval fish at 25.4 and 16.7 µg L^{-1}, respectively. One probable explanation for the delayed hatching in our results could be due to the inability of the embryos to break the chorion, which was observed in some eggs. Another reason could be the inhibition of enzymes involved in hatching provoked by the dye (Du et al. 2012; Manjunatha et al. 2014).

After 72 hpf, DB15 did not induce lethal or sublethal effects in the range of 6.25 to 500 mg L^{-1}. At 96 hpf abnormalities originated since the early exposure stages were now visible in the embryos, such as yolk sac edema, curved tail, and tail deformities (Fig. 5c, d). Joshi and Katti (2018) observed that 50% of hatched larvae exhibited tail distortion, yolk sac edema, and cardiac edema with 20 to 30 mM tartrazine (a synthetic colorant used in the food industry). Abe et al. (2017) reported that the synthetic dye Basic Red 51 and the natural dye, erythrostominone, induced in zebrafish embryos a large yolk sac that is related to deformities or delayed yolk resorption. Yolk sac malformation might be associated also with the hypoactivity of zebrafish larvae. Yolk sac plays a fundamental role in growth, associated with secretion of growth factors, cytokines, and proteins (Herbomel et al. 1999). Moreover, pericardial edema in zebrafish embryos has been associated with an abnormal intracellular Ca^{2+} signaling pattern (Tsuruwaka et al. 2015).

Lethal and sublethal effects such as no heartbeat, delayed pulse, curved tail, and yolk sac edema were observed with different DB15 concentrations in zebrafish larvae at 144 hpf (Fig. 6). Joshi and Katti (2018) observed that from 40 to 50 mM tartrazine, the larvae exhibited a reduction in heartbeat frequency followed by mortality within 96 to 144 hpf. Rodrigues de Oliveira et al. (2016) reported that Direct Black 38 and Vat Green 3 induced curved tail and yolk sac edema in zebrafish embryos at 25 and 100 mg L^{-1}, respectively. Jang et al. (2009) observed blood circulation defects, including slow heartbeat, heart edema, blood accumulation near the heart region, and a big yolk in *D. rerio* embryos exposed to malachite green (150 mg L^{-1}) at 72 hpf. According to our results, we could suppose that DB15 dye crossed the chorion barrier and for this reason, zebrafish had sublethal effects in embryos, evidenced after larvae hatched (Fig. 5c, d).

In contrast to our results, Abe et al. (2017) demonstrated that zebrafish embryos exposed to Basic Red 51 and erythrostominone did not present lethal or sublethal effects such as coagulation, not detached tail, malformation of somites, changes in the development of eyes, alterations in the pigmentation patterns, malformation of head, tail and otoliths, scoliosis, and retarded growth. These variable reports indicate that different synthetic dyes provoke diverse acute and chronic effects in early life stages of fish; these effects could be related with the chemical structure and purity of dyes, but differences in test conditions must be considered also. It is important to mention that some azo dyes are toxic, and some of the observed sublethal effects are difficult to quantify and hinder inferring their consequences in the future development of fish.

Conclusions

The magnitude of toxic effects in organisms exposed to chemical stressors is species-specific. In our study, the green microalga *P. subcapitata* was the most sensitive organism to Direct blue 15, because this azo dye inhibited population growth, altered the photosynthetic pigments concentration, and modified the content of proteins, lipids, and carbohydrates. Toxic damages to primary photosynthetic producers are relevant because a general disruption in aquatic food webs could be expected when compounds like this azo dye are discharged in waterbodies.

Despite the acute effects of DB15 on the planktonic crustacean *Ceriodaphnia dubia* were comparatively slight, this cladoceran was significantly sensitive in chronic exposure, displaying affectations in survival, fecundity, the beginning of the reproductive process, and a reduction in the number of released clutches.

Direct blue 15 can be an environmental toxic compound with the potential to provoke lethal and sublethal effects in early life stages of the *Danio rerio* fish.

The results obtained in these experiments confirmed that a battery of toxicity tests undoubtedly provide more information on the effects of emergent pollutants (like the azo dyes), than the use of single-species toxicity tests. *P. subcapitata*, *C. dubia*, and *D. rerio* can be used as test organisms to establish safe levels of dyes in the aquatic environment.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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