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RESEARCH ARTICLE

Auramine dyes induce toxic effects to aquatic organisms from different trophic levels: an application of predicted non-effect concentration (PNEC)

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Abstract

The dyes Auramine and Auramine O are used in several industrial products, despite the scarce information regarding their ecotoxicity. The aim of the present study was to assess the acute and chronic toxicity of both dyes to aquatic organisms from different trophic levels (*Raphidocelis subcapitata*, *Daphnia similis*, *Hydra attenuata*, and *Danio rerio*) and calculate their predicted non-effect concentrations (PNEC). Auramine and Auramine O induced toxicity to all selected test organisms with L(E)C50 values ranging from 300 to 4800 ug/L. Both dyes induced inhibition in the growth rate of exposed algae, negatively affecting the reproduction of *D. similis* and induced deformities in *H. attenuata* (clubbed tentacles and shortened tentacles) and *D. rerio* (edemas, tail malformation and delay in yolk sac absorption). PNEC values of 0.92 μg/L and 4.0 μg/L were obtained for Auramine and Auramine O, respectively, based on results of the most sensitive test system (algae). Test results were analyzed using the Criteria of Reporting and Evaluating Ecotoxicity Data (CRED), confirming their reliability and relevance. Thus, PNEC values can be used in future risk assessments of those substances in freshwater systems.

Keywords Solvent Yellow 34 · Basic Yellow 2 · *Raphidocelis subcapitata* · *Dapnhia similis* · *Danio rerio* · *Hydra attenuata* · CRED analysis

Highlights

- Assessment of aquatic toxicity of auramine dyes using organisms from different trophic levels, *Raphidocelis subcapitata* (autotrophs), *Dapnhia similis* (primary consumers), and *Hydra attenuata* and *Danio rerio* (secondary consumers).
- Lethality for *D. similis*, *H. attenuata*, and *D. rerio* in concentrations below of 4800 μg/L.
- Induced inhibition in the growth rate of exposed algae, negatively affecting the reproduction of *D. similis* and induced deformities in *H. attenuata* (clubbed tentacles and shortened tentacles) and *D. rerio* (edemas, tail malformation and delay in yolk sac absorption) in sublethal concentrations.
- PNEC values of 0.92 μg/L (Auramine) and 4.6 μg/L (Auramine O) were derived.

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Introduction

Dyes are widely used in several industrial products, including textiles, paper, plastic, leather, food, cosmetics, and household products (Guaratini and Zanoni 2000; Bafana et al. 2011). In general, the conventional wastewater treatment processes are not efficient in their removal, and it is estimated that approximately 10–15% of dyes used in textile industries might be lost into the environment (Leite et al. 2016; USEPA 1990).

Several authors reported the occurrence of dyes in water and sediments from rivers located under the influence of textile industries discharges. In Brazil, dyes have been found in the aquatic environment with concentrations ranging from 0.0118 to 6.81 μg/L (Carneiro et al. 2010; de Aragão Umbuzeiro et al. 2005; Vacchi et al. 2016, 2017; Zocolo et al. 2015). In Canada, in a study conducted on the Yamaska River, dyes were detected in water samples, with concentrations ranging from 3 to 17 μg/L and in sediments in the range of 400–1500 (μg/Kg) (Maguire 1992). Rajaguru et al. (2002) registered a contamination by genotoxic compounds in groundwater, collected in an area under the

influence of textile dyeing and bleaching industries in Tirupur, India. According to the results obtained by these authors, after chemical characterization, aromatic amines probably derived from discharges of textile effluents may be responsible for the DNA damaging activity of the water samples. Those studies suggest a high potential of contamination of textile dyes into the aquatic environment worldwide. Therefore, ecotoxicological studies are necessary to elucidate the possible adverse effects of this contamination on aquatic biota.

Several dyes have been tested and found to be toxic to aquatic organisms (Table 1). However, there are still few studies that evaluated the toxicity of the same dye with organisms representing three trophic levels or more (Vacchi et al. 2016; Martínez-jerónimo 2019; Hernández-Zamora and Martínez-Jerónimo 2019). Providing toxicity data for different organisms is necessary to derive reliable and relevant criteria for assessing the risk of dyes when it is desired to preserve aquatic life.

The dyes Auramine and Auramine O (also referred as Solvent Yellow 34 and Basic Yellow 2) are used for dyeing leather, jute, cotton, and paper (International Agency for Research on Cancer - IARC 2010, Gessner and Mayer 2000). The diphenylmethane dyes are usually grouped with the triarylmethane dyes (IARC 2010), which comprises synthetic colorants, widely used due to their versatility and bright colors (IARC 2010; Gessner and Mayer 2000). However, there are only few articles regarding the toxicity of triarylmethane's dyes group to aquatic organisms (Table 1). According to the IARC, Auramine dyes are classified as Group 1 (carcinogenic to humans) with respect to production and as 2B (possibly carcinogenic to humans) with respect to their use. Despite that, these dyes are still being used, and were found even in food products in India and China (Tripathi et al. 2007; Lin 2007; Li et al. 2013; Tatebe et al. 2014). Information regarding auramine dyes toxicity in aquatic organisms is scarce. Moreover, it is expected to find auramine dyes in the aquatic environment because of their use in textile industries and high solubility when compared to other group of colorants, such as disperse dyes.

For the safety threshold, i.e., the predicted no-effect concentration (PNEC), the ideal is to have toxicity data on organisms representing three trophic levels (EUROPEAN COMMISSION 2011). PNEC values can be calculated based on the methodology adopted by the European Union, which uses toxicity data from a test substance and assessment factors, ranging from 10 to 1000, depending on the quality and quantity of available ecotoxicological data. Thus, the greater the set and/or quality of toxicity data, the greater the confidence to derive a criterion, and the lower the applied factor. PNEC values, derived from toxicological tests, can support an assessment of the toxicant's environmental risk and assist in regulations aiming at ensuring water quality for the protection of aquatic life (EUROPEAN COMMISSION 2011). Thus, PNEC derivation processes should be based on detailed, transparent, and unbiased assessments. Moreover, the aquatic ecotoxicity studies used for this purpose must be reliable and relevant (Moermond et al. 2016).

To calculate PNEC values for aquatic toxicity, algae, microcrustaceans, hydras, and fish can be used as test organisms. Algae are oxygen producers and staple food for primary consumer organisms. An imbalance at this basic trophic level could lead to imbalance throughout the trophic chain. Thus, adverse effects of both dyes on aquatic primary producers cannot be neglected.

Microcrustaceans are one of the most widely used organisms to evaluate the toxic potential of dyes in acute tests, and daphnids are a critical species in the aquatic food chain as an important dietary component of fish and invertebrate predators (Tatarazako and Oda 2007).

Hydras are well-established test organisms, although not often being used in toxicity assessment of aquatic pollutants. Since they represent secondary consumers in the trophic level (Suares-Ruppert 2005), the inclusion of this test system in ecotoxicological assessment of potential aquatic pollutants may be useful in the understanding of the toxic burden of determined compounds, such as dyes and other emerging contaminants, and the possible ecological effects that this toxicity may represent in an aquatic environment.

D. rerio is a vertebrate also inserted in the second level of the trophic chain and has increasingly being use in ecotoxicological assessments, especially with embryo exposure (Suares-Rocha et al. 2011).

Moreover, to evaluate the reliability and relevance of toxicological tests, a method called "Criteria for Reporting and Evaluating Ecotoxicity Data" (CRED) was developed (Moermond et al. 2016). In this method, key information need to be provided, e.g., CAS number, purity and source of the substances used for testing, and detailed information on the test organisms (scientific name, life stage, strain and source) as well as information on exposure conditions, including the real concentrations of the selected substances during the tests to assure a reliable PNEC derivation.

Thus, the aim of this study was to assess the ecotoxicity of two auramine dyes to aquatic organisms from three different trophic levels (*Raphidocelis subcapitata*, representing autotrophs, *Dapnhia similis* representing primary consumers, and *Hydra attenuata* and *Danio rerio* representing secondary consumers), providing highly reliable ecotoxicological data for PNEC derivation that can be used in future risk assessments.

Materials and methods

Chemicals and stock solutions

C.I. Auramine (CAS number 492-80-8, Color Index (C.I.) 41000B) and Auramine O (CAS number 2465-27-2, Color

Table 1 Ecotoxicity data of dyes retrieved from the literature for algae, crustacean, cnidarian, and fish

Taxonomic group Species	Dyes	Toxicity range L(E)50 µg/L	References
Algae *Raphidocelis subcapitata*	AZO: Acid Orange 7, Direct Red 28 (Congo Red), Disperse Red 1, Food Red 17, Food Yellow 3, Reactive Black 5, Reactive Orange 16, Vat Green 3, Acid Black 1, Acid Black 24, Acid Black 26, Acid Black 94, Acid Blue 113, Acid Blue 324, Acid Blue 80, Acid Brown 235, Acid Brown 354, Acid Green 111, Acid Green 68, Acid Orange 33, Acid Orange 7, Acid Orange 95, Acid Red 119, Acid Red 131, Acid Red 266, Acid Red 374, Acid Violet 48, Acid Yellow 42, Acid Yellow 49, Acid Yellow 61, Direct Blue 293, Direct Blue 71, Direct Green 26, Direct Red 227, Direct Red 23, Direct Red 81, Disperse Blue 79 Br, Disperse Blue 79 Cl, Disperse Orange 30, Disperse Orange 61, Mordant Yellow 10, Mordant Black 11, Reactive Black 5, Reactive Red 120, Reactive Red 195, Reactive Red 83, Reactive Yellow 15, Sulphur Black 1, Vat Blue 20, Vat Green 1, Vat Green 9 and Direct Blue 15.	1100–152.7 × 10^3	Luna et al. 2014; Novotný et al. 2006; Wong et al. 2007; Vacchi et al. 2016; Croce et al. 2017; Martínez-jerónimo 2019; Hernández-Zamora and Martínez-Jerónimo 2019.
	Anthraquinones: Disperse Blue 3 and Remazol Brilliant Blue.	500 and 81.1 × 10^3	Novotný et al. 2006.
Crustacean *Daphnia similis*	Azo: Acid Orange 7, Disperse Red 1, Disperse Red 13, Food Red 17, Food Yellow 3, Reactive Black 5, Vat Green 3 and Acid Black 210.	18–> 1000 × 10^3	Luna et al. 2014; Vacchi et al. 2016; Ferraz et al. 2011; Rocha et al. 2017.
Crustacean *Daphnia magna*	Azo: Direct Blue 218, Disperse Red 1, Remazol Golden Yellow, Remazol Parrot Green, Congo Red, Acid Black 1, Acid Black 24, Acid Black 26, Acid Black 94, Acid Blue 113, Acid Blue 324, Acid Blue 80, Acid Brown 235, Acid Brown 354, Acid Green 111, Acid Green 68, Acid Orange 33, Acid Orange 7, Acid Orange 95, Acid Red 119, Acid Red 131, Acid Red 266, Acid Red 374, Acid Violet 48, Acid Yellow 42, Acid Yellow 49, Acid Yellow 61, Direct Blue 293, Direct Blue 71, Direct Green 26, Direct Red 227, Direct Red 23, Direct Red 81, Disperse Blue 79 Br, Disperse Blue 79 Cl, Disperse Orange 30, Disperse Orange 61, Mordant Yellow 10, Mordant Black 11, Reactive Black 5, Reactive Red 120, Reactive Red 195, Reactive Red 83, Reactive Yellow 15, Sulphur Black 1, Vat Blue 20, Vat Green 1 and Vat Green 9.	550–322.9 × 10^3	Bae and Freeman 2007; Vacchi et al. 2016; Verma 2008; Martínez-jerónimo 2019.
	Phenol: HC Orange 1.	1540	Liu et al. 2007.
Cnidarian *Hydra attenuata*	Azo: Disperse Red 1.	48 × 10^3–75 × 10^3	Vacchi et al. 2016; Jong et al. 2016.
Fish *Danio rerio*	Azo: Acid Red 26, Direct Red 28 (Congo Red), Disperse Red 1, Acid Black 210, Basic Red 51, Reactive Red 239, Acid Yellow 23, Acid Blue 113, Allura Red, Amaranth, Direct Blue 15, Disperse Red 65, Reactive Black 5, Sunset Yellow and Tartrazine.	517–15710 × 10^3	Shen et al. 2015; Vacchi et al. 2016; Rocha et al. 2017; Abe et al. 2017; Jungtanasombut and Preeprem 2014; Joshi and Pancharatna 2019; Tippabathan et al. 2020; Jiang et al. 2020; Martínez-jerónimo 2019; Hernández-Zamora and Martínez-Jerónimo 2019; Ozmen et al. 2018; Manimaran et al. 2018.
	Triarylmethane: Basic Violet 14, Malachite Green, Malachite Green Chloride and Malachite Green Oxalate.	57–60630	Shen et al. 2015; White et al. 2012.
	Xhantane: Erythrosine B.	223 × 10^3	Manimaran et al. 2018.
	Coumarin: 7-Diethylamino-4-methylcoumarin.	959	Jung et al. 2012.
	Phenol: HC Orange 1.	190	Liu et al. 2007.
Fish *Pimephales promelas*	Azo: Disperse Yellow 7, Sudan Red G.	25.4 and 16.7	Parrot et al. 2016.
	Anthraquinones: Acid Blue 80, Acid Blue 129.	> 6700	Parrot et al. 2016.

Index (C.I.) 41000) were purchased from Sigma-Aldrich, with 98 and 87% of purity, respectively. Stock solutions for Auramine O were prepared dissolving the dyes in the appropriate test medium for each organism. Because Auramine was less soluble than Auramine O, it was necessary to use dimethyl sulfoxide (DMSO) to prepare the stock solutions. However, the maximum final concentration of DMSO in the experiments was 0.01%. In all tests using DMSO, the solvent was added to the negative controls. This concentration was defined in previous tests in our laboratory as a maximum concentration causing no adverse effects in any of the selected organisms.

Chemical analysis

Chemical analysis was performed with aliquots of the test solutions to confirm the nominal concentration of dyes in ecotoxicity tests using high-performance liquid chromatography with diode array detection (HPLC-UV/DAD) (Shimadzu Corporation, Kyoto, Japan) using a Shim-pack G-ODS (4) 4-mm internal diameter guard column and a Capcell Pack C18 AG120 S-5 (Shiseido Co) 250 mm long 4.6 mm internal diameter separation column. The methanol and water acidified with formic acid (0.1%) (50/50) were used as the mobile phase, with isocratic elution at a flow rate of 0.8 mL/min and running time of 10 min. The quantitative determination was achieved at 437 nm for both dyes. The limit of detection (LOD) = 3.3 s/S and limit of quantification (LOQ) = 10 s/S for Auramine and Auramine O were determined using a calibration curve, with s = the estimate of the standard deviation of the blank samples (n = 10) and S = the slope of the analytical curve obtained from 15 to 200 μg/L. The lowest, middle, and highest concentrations were analyzed at the end of each test with selected aquatic organisms. In order to measure the exposure concentrations, extra replicates without the organisms were prepared. No extraction was required and the samples were diluted in the mobile phase of the analytical method (50/50 MeOH/H_20) before being quantified by HPLC-UV/DAD (Azevedo et al. 2020). The analytical results are described in details in the Supplementary Material (SM1).

Ecotoxicity tests

All organisms used in this study were provided by the Laboratory of Ecotoxicology and Genotoxicity (LAEG), State University of Campinas (Limeira, Brazil), except the zebrafish embryos which were tested in cooperation with the Laboratory of Genetic Toxicology, University of Brasilia, (Brasilia, Brazil). The sensitivity of the test organisms was monitored by sensitivity tests and internal control charts, to warrant the use of only adequate cultures. Test concentrations were selected according to range finding tests, previously performed, in order to reach a range of concentrations inducing 0 to 100% effects in the exposed organisms.

Raw data from toxicity assays are presented in the Supplementary Material (SM2). Stock and exposure solutions for each dye tested were carefully prepared in adequate medium for each organism. No significant alterations in physical and chemical parameters (pH, conductivity, and dissolved oxygen) were observed after the addition of dyes to the media.

R. subcapitata

Algae *R. subcapitata* were maintained in a supplemented medium. Chronic toxicity tests using the freshwater algae were performed according to OECD guideline 201 (OECD 2011). Algae population was exposed to different dyes concentrations: 20, 60, 200, 600, and 2000 μg/L for Auramine and 40, 120, 400, 1200, and 4000 μg/L for Auramine O. Three replicates at each test concentration (treatments) were used. The inoculum was composed of algae harvested from a liquid stock algal culture, in an exponential growth phase of 3-day-old culture. The initial cell density was 10 000 ± 1000 cells/mL. The final volume of 50 mL, composed by algal inoculum and test dye in supplemented medium, was placed in an Erlenmeyer. The test was performed under static conditions for 72 h, at 24 ± 2 °C under continuous fluorescent light (4000 ± 400 lux), in a rotatory shaker with 150 revolutions per minute. To validate the test, the biomass in the control cultures should have increased exponentially by a factor of at least 16 within the end of the test.

Colored test chemicals can absorb photosynthetically active light and hence limit growth of algal cultures. Experiments were conducted with the highest concentration tested (4000 μg/L) to confirm that the toxic effects observed for the algae were not relate to inhibition of the photosynthesis caused by the color of the dye solutions. For that, solutions of highest concentrations of each dye were prepared and placed each in a beaker. Then, Erlenmeyers containing only algae in supplemented medium (10 mL) were placed into those beakers, in a way that the algae were totally covered by the dyes, but not in contact to them. Thus, the physical effect was evaluated without chemical interference. A control was performed at the same conditions, using only culture medium in the control beaker. Each independent test was performed in triplicate. At the end of 72 h, the growth inhibition of algae exposed to dye solution was compared to a control and the effective concentration inducing 50% growth inhibition (EC_{50}) was calculated. Algae cell number was counted using a Neubauer chamber with optical microscope MB-E-200 (Nikon).

D. similis

D. similis culture was cultivated in synthetic medium (MS), with conductivity of 200 ± 20 mS/cm, hardness of 40–48 mg/L $CaCO_3$, at 20 ± 2 °C and under a photoperiod of 16:8 h light:dark, according to ABNT NBR 12713 (ABNT 2016).

The organisms were fed five times a week with the algae *R. subcapitata*. Neonates less than 24 h old, from a healthy culture, were used for the experiments.

Acute toxicity tests with *D. similis* were performed according to OECD guideline 202 (OECD 2004). Concentrations of Auramine (1000, 1500, 2000, 3000, and 4000 μg/L) and Auramine O (1000, 3000, 4500, 7000, and 10000 μg/L) were prepared in MS. Twenty neonates (< 24 h old) were placed in acrylic tubes with 10 mL of dyes solutions. Acute assays were performed in three replicates, at 21 ± 0.3 °C and photoperiod of 16:8 h light:dark. After 48 h, the number of immobilized daphnids was recorded, and the effective concentrations inducing 10 and 50% immobilization (EC_{10} and EC_{50}) were recorded.

D. similis chronic toxicity tests were performed according to the OECD guideline 211 (OECD 2012) with adaptations described by Vacchi et al. (2016). This method measures the chronic toxicity using less than 24-h-old neonates exposed during 14 days to the chemical, with renewal of test medium every two days. Neonates < 24 h old were individually transferred to a 50-mL vessel containing 40 mL of treatments, in the following concentrations: 100, 200, 500, 1000, and 2000 μg/L for Auramine and 250, 500, 750, 1000, 1500, and 2000 μg/L of Auramine O. Ten control and treatment replicates were used for each tested concentration. The test organisms were fed daily with *R. subcapitata* at 20 ± 2 °C and under a photoperiod of 16:8 h light:dark. Chronic assays were performed in two replicates. After exposure period, total number of neonates per test concentrations was observed, and the effective concentrations inducing 10 and 50% reproduction inhibition (EC_{10} and EC_{50}) were recorded.

H. attenuata

H. attenuata were cultivated in Hydra medium, pH 7.0 ± 0.1, maintained at 22 ± 2 °C, under a 16:8 h light:dark photoperiod, according to Trottier et al. (1997). The organisms were fed three times a week with newly hatched nauplii of *Artemia salina*. In *Hydra* sp., it is known that progressive morphological changes are indicative of increased toxicity. Here, the test was based on Trottier et al. (1997) that determines five stages of morphological changes: (A) hydras in their normal stage, with extended body and tentacles; (B) the appearance of bulbs at the tips of the tentacles, indicating the first sign of intoxication; (C) the second sign of intoxication, with the shortening of tentacles and body, (D) tulip stage, with tentacles and body dramatically reduced; and (E) the last phase of intoxication, leading to death by disintegration. Stages B and C are reversible and are considered sub-lethal effects, while stages D and E are irreversible and indicate lethal endpoints.

The organisms were exposed for 96 h to different concentrations of Auramine (500, 1000, 2000, 3500, and 7000 μg/L) and Auramine O (100, 500, 1000, 1500, 2000, and 3000 μg/L), dissolved in Hydra medium. The organisms were placed in 12-well microplates (three organism per well with 5 mL test solution), in triplicate (totalizing 9 organisms per concentrations and control). Three independent investigations were done.

The same temperature and photoperiod were used for cultivation and the tests. During exposure, animals were not fed. At the end of exposure, lethal (stages D and E) and sublethal effects (stages B and C) were observed under a stereomicroscope and LC/EC_{10} and LC/EC_{50} were calculated. Photographs of morphological changes of organisms were taken.

D. rerio embryo

D. rerio adults were maintained in aquariums with reverse osmosis and activated carbon filtered water, with a photoperiod cycle of 12:12 h (light:dark), temperature of 27 ± 1 °C, conductivity of 650 ± 100 μS/cm, pH of 7.0 ± 0.5, and dissolved oxygen ≥ 95% saturation. Fish embryo toxicity test was based on the OECD guideline 236 (OECD 2013). Zebrafish eggs were collected immediately after natural mating, rinsed in water, and checked for egg viability under a stereomicroscope (Stereoscopic Zoom Microscope—Stemi 2000, Zeiss, Germany). The unfertilized eggs and those showing cleavage irregularities or injuries were discarded. Experiments were initiated immediately after fertilization using 60 eggs per treatment, divided in 3 replicates, selected, and distributed in 24-well microplates in the climate chamber (SL-24 Solab Científica, Brazil). Embryos were exposed to different concentrations of Auramine (1000, 1900, 3700, 7100, and 13600 μg/L) and Auramine O (300, 600, 1000, 1900, 3700, 7100, and 13600 μg/L) for 96 h under static conditions and observed daily, under a stereomicroscope. Developmental parameters were evaluated in embryos over the test period, using a × 70 magnification for eggs and × 40 magnification for hatched embryos. Lethality and malformations were evaluated: lack of otolith formation, general delay in development, lack of eye and body pigmentation, lack or delay in somite formation, edemas, non-detachment of the tail-bud from the yolk sac, non-absorption of yolk sac, lack or delay in hatching, and mortality. All parameters were registered as presence and absence of effect. Three independents experiments were done.

Statistical analysis

The effective concentrations $L(E)C_{10}$ and $L(E)C_{50}$ with the corresponding 95% confidence intervals were calculated via the logistic model, using the package drc in R software (Christian Ritz 2005).

Evaluation of the quality of the ecotoxicity test

The CRED method (Moermond et al. 2016) was introduced to assess the reliability and relevance of the ecotoxicity data used

in the derivation of PNEC, applying a set of 20 reliability and 13 relevance criteria. The CRED aimed at providing a complete report on the methodology performed during our tests, presenting unbiased and transparent results. Tables of the CRED are presented in the Supplementary Material (SM3 A and B).

PNEC derivation

PNEC values for protecting freshwater pelagic community from adverse effects of dyes Auramine and Auramine O were derived according the Technical Guidance for Deriving Environmental Quality Standards (TGD EQS) on the Water Framework Directive (2000/60/EC) (European Commission 2011) by using the deterministic approach, applying an adequate assessment factor (AF) to the lowest relevant EC_{10}/NOEC value from the available dataset, to extrapolate to an environmentally protective concentration.

Results

Nominal and real concentrations

No alterations in concentrations of Auramine and Auramine O were observed during the test performed with algae, daphnia, and hydra (final concentrations remaining within 80–120% of nominal concentrations; see Supplementary Material, SM1). Therefore, it is possible to affirm that the test substances remained stable in the test solutions, during the exposure periods. Thus, the expression of results for all experiments was based on nominal values. Nominal concentrations were also considered for the experiments using *D. rerio*, because test conditions and duration were similar to those performed with *H. attenuata*. Therefore, no changes in concentrations were expected.

Ecotoxicity tests

For all experiments, the effects of dyes followed a dose response curve (Fig. 1), and L(E)C10 and L(E)C50 values with confidence intervals (95%) were calculated for both dyes (Table 2). Tables with toxicity tests data are presented in the Supplementary Material (SM2).

R. subcapitata

All tests performed with algae were considered valid. The biomass in the control increased exponentially by a factor greater than 16 at the end of the test. The EC_{10} values for Auramine and Auramine O were 46 and 200 μg/L, and EC_{50} were 300 and 800 μg/L after 72 h of exposure, respectively (Table 2).

D. similis

In the acute immobilization test, no mortality was observed in the control group. Regarding the treatments, EC_{10} value of 1800 μg/L was registered for Auramine and of 1900 μg/L for Auramine O, while EC_{50} values of 2900 and 4300 μg/L were registered after 48 h of exposure, for each dye respectively. In the chronic test, after 14 days of exposure, similar results were obtained for both dyes, with EC_{10} value of 500 μg/L for Auramine and of 400 μg/L for Auramine O, while EC_{50} values of 900 and 800 μg/L were recorded at the end of the exposure period, respectively (Table 2).

H. attenuata

No mortality was observed in the control group, for the tests with *H. attenuata*.

The five stages of morphological alteration described in section 2.3.3 were observed for both dyes, in different concentrations (Fig. 2). In the assays with Auramine dye, in

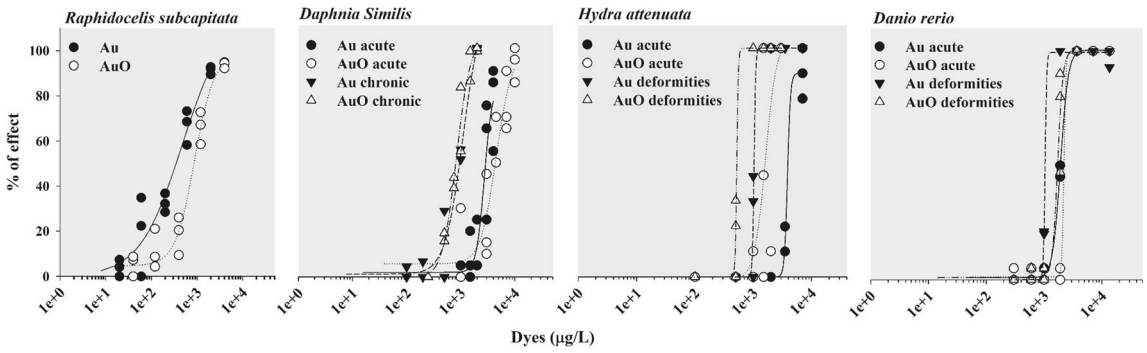

Fig. 1 Dose-response curves for effects data obtained to aquatic organisms belonging to different trophic levels exposed to Auramine (AU) and Auramine O (AUO). *Raphidocelis subcapitata*, chronic test (4 days). *Dapnhia similis*, acute test (2 days), and chronic test (14 days). *Hydra attenuata*, acute test (4 days) with lethal and deformities (morphological changes). *Danio rerio*, acute test (4 days) with lethal and deformities (edema, tail malformation, and delay in yolk absorption)

Table 2 Lethal and effective concentration values (LCx and ECx) obtained for the aquatic organisms used in this work in acute and chronic tests with Auramine and Auramine O

	Time of exposure days	Endpoint	L(E)C10 μg/L	L(E)C50 μg/L
Auramine				
R. subcapitata	3	Growth inhibition	46 (20–70)*	300 (200–400)
D. similis	2	Immobilization	1800 (1300–2200)	2900 (2600–3300)
D. similis	14	Reproduction	500 (300–700)	900 (800–1000)
H. attenuata	4	Lethality	3200 (2900–3500)	4800 (4500–5100)
H. attenuata	4	Morphological changes	Not determined	1100 (500–2200)
D. rerio	4	Lethality	1300 (1200–1500)	1900 (1800–1900)
D. rerio	4	All deformities	900 (800–1000)	1100 (900–1300)
D. rerio	4	Tail malformation	950 (900–1000)	1100 (960–1300)
D. rerio	4	Edema	1000 (970–1000)	1200 (870–1500)
D. rerio	4	Delay in yolk absorption	1250 (1220–1270)	1380 (1360–1400)
Auramine O				
R. subcapitata	3	Growth inhibition	200 (100–300)	800 (700–1000)
D. similis	2	Immobilization	1900 (1100–2700)	4300 (3700–5000)
D. similis	14	Reproduction	400 (400–500)	800 (700–900)
H. attenuata	4	Lethality	1000 (600–1400)	1600 (1300–1900)
H. attenuata	4	Morphological changes	500 (400–600)	600 (300–800)
D. rerio	4	Lethality	2100 (1800–2300)	2400 (1600–3100)
D. rerio	4	All deformities	900 (700–1100)	1300 (1100–1500)
D. rerio	4	Tail malformation	1270 (749–1806)	1690 (1500–1900)
D. rerio	4	Edema	1500 (1000–3900)	1900 (1700–2000)

*95% confidence limits between parenthesis

concentrations of 1000 μg/L, the first stage of morphological alteration (stage A) was observed in approximately 60% of organisms, while from concentrations of 2000 μg/L, all organisms already presented alterations in the formation of the tentacles (stages B and C). In the highest tested concentration (7000 μg/L), most of the organisms were in stages D or E, which are irreversible morphological effects.

For Auramine O, the first signals of toxicity were observed in concentration of 500 μg/L, while in concentration of 1000 μg/L, all organisms presented some morphological alteration (stages B and C). In the highest tested concentration (3000 μg/L), all organisms were in stages D or E.

When considering lethality (stages D and E), $LC_{10/50}$ values of 3200 and 4800 μg/L were recorded for Auramine

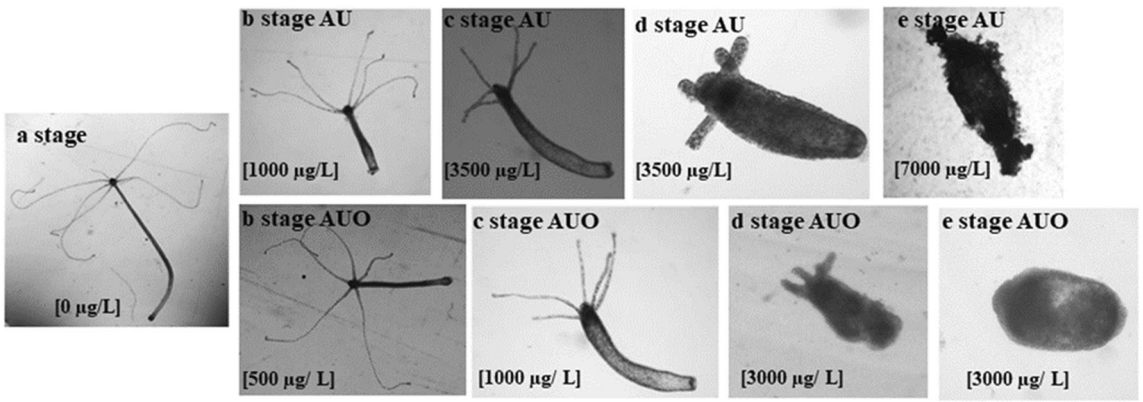

Fig. 2 Morphological stages in *Hydra attenuata* after 96 h exposure to increased concentrations of Auramine (AU) and Auramine O (AUO) illustrating stages from healthy to disintegrated polyp: a stage: (normal Hydra); b stage: minimal expression of toxicity, i.e., clubbed tentacles; c stage: shortened tentacles; d stage: tulip stage and e stage: disintegration. Magnification used for all each picture × 2.5

and of 1000 and 1600 μg/L were recorded for Auramine O. Considering morphological changes (stages B and C), EC_{50} values of 1100 μg/L and $EC_{10}/_{50}$ of 500 and 600 μg/L were recorded for each dye, respectively (Table 2, Fig. 2).

D. rerio embryos

After 96 h exposure, no significant mortality was observed in the control group.

LC_{10}/LC_{50} were calculated for lethality, with values of 1300 and 1900 μg/L for Auramine and of 2100 and 2400 μg/L for Auramine O.

Development deformities as edema and tail malformation were observed for both dyes at similar concentrations range, and delay in yolk absorption was observed more frequently in organisms exposed to Auramine. After 96 h, EC10/50 values of 900 and 1100 μg/L and of 900 and 1300 μg/L were obtained for each dye, respectively, considering all deformities. EC10/50 values were also calculated for each deformity (Table 2, Fig. 3).

PNEC calculation

PNEC calculations were performed by the deterministic method according to the guidelines of the European Commission (2011).

The ecotoxicity tests performed in this work were considered reliable and relevant for PNEC derivation (Supplementary Material, SM3) according to the CRED method. *R. subpcapitata* showed to be the most sensitive species to both dyes (Table 2); therefore, their EC10 values were selected for PNEC calculation. Because chronic data was available for two trophic levels, an AF of 50 was applied and PNECs of 0.92 μg/L and 4.6 μg/L were derived for Auramine and Auramine O, respectively.

Discussion

Solvent Yellow 34 Auramine and Auramine O dyes are toxic for the selected aquatic test organisms. The algae were the most sensitive organism for both Auramine and Auramine O dyes with IC_{10} of 46 and 200 μg/L, respectively. Auramine is up to 4 times more toxic to this test system when compared to Auramine O. In general, more water-soluble compounds tend to induce less toxicity, because they are less absorbed by cell membranes, while less soluble compounds tend to be more absorbed by organisms, and consequently are more likely to induce toxicity (Klassen et al. 2013). The higher maximum solubilities of Auramine (53.5 mg/L) in relation to Auramine O (10000 mg/L) may explain the higher toxicity observed for Auramine.

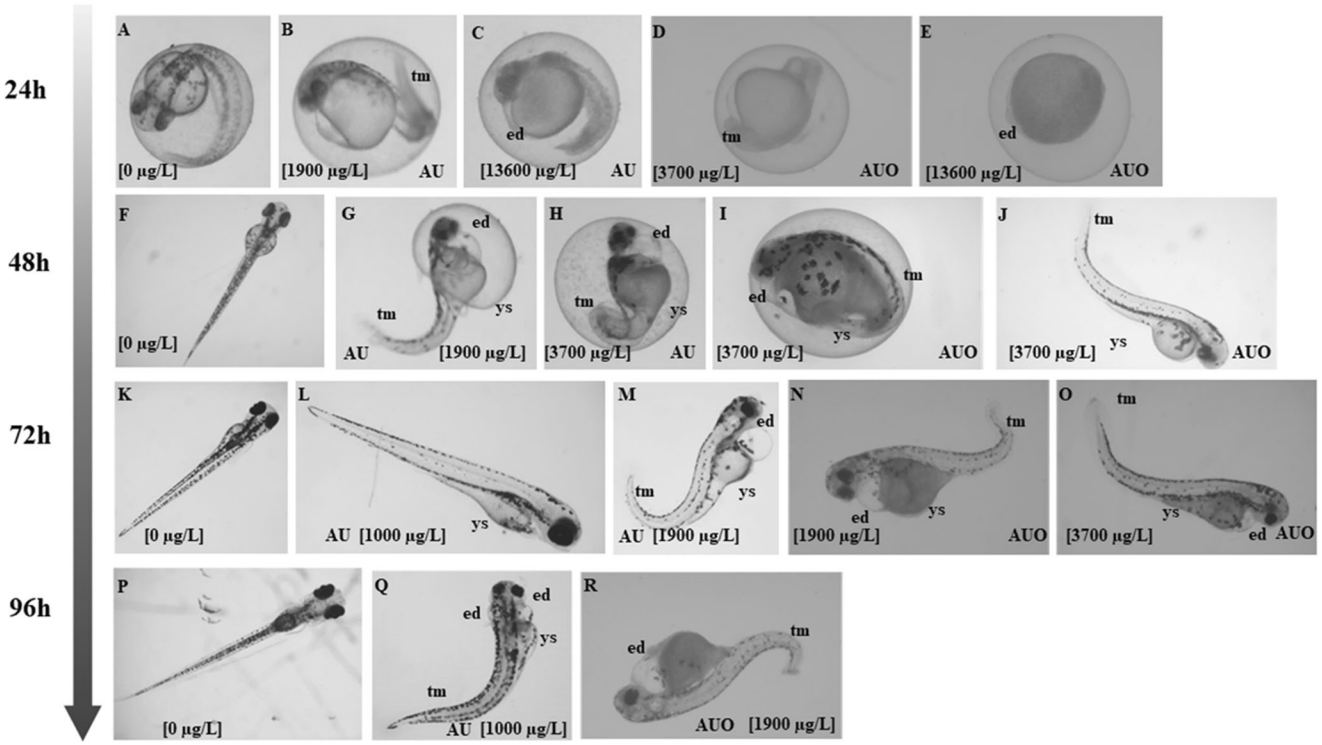

Fig. 3 *Danio rerio* embryo malformations due to Auramine (AU) and Auramine O (AUO) exposure during 24 h (**a**–**e**), 48 h (**f**–**j**), 72 h (**k**–**o**), and 96 h (**p**–**r**). tm tail malformation, ed edema, and ys delay in yolk absorption. Magnification used for all each picture × 3

EC_{50} values of 300 and 800 μg/L were determined for Auramine and Auramine O, respectively, for algae. In general, algae has been shown to be very sensitive to dyes, since most of the dyes tested for these organisms are considered very toxic or toxic according to some authors (Croce et al. 2017b; Hernández-Zamora and Martínez-Jerónimo 2019; Novotný et al. 2006). Novotný et al. (2006) found values similar to those registered in this study, for the algae exposed for 96 h to Disperse Blue 3 (EC_{50} of 500 μg/L). Hernández-Zamora and Martínez-Jerónimo (2019) evaluated the toxicity of Congo Red dye for algae, microcrustaceans, and fish, and found that algae was the most sensitive organism for this compound, with EC_{50} of 3110 μg/L. Other authors registered EC_{50} values in the same order of magnitude, for algae exposed to several dyes, for instance: Novotný et al. (2006) reported EC_{50} of 7800 μg/L for Reactive Orange 16 after 96 h of exposure and Luna et al. (2014) EC_{50} of 5600 μg/L to Vat Green 3 in a 72-h test. Croce et al. (2017) investigated the toxicity of 42 dyes belonging to different chemical classes, and found that 30 of them had EC_{50} values lower than 100 000 μg/L, while 12 presented EC_{50} values ranging from 102,400 to 152,800 μg/L, after 72 h of exposure. Vacchi et al. (2016) recorded EC_{50} values of 102,000 μg/L for algae exposed to Disperse Red 1. According to those data, it is possible to conclude that Auramine and Auramine O presented similar or higher toxicity to these aquatic organisms, when compared to several other dyes.

Some studies have indicated that the algae sensitivity to dyes is related to the ability of these substances to inhibit or block light, making it less accessible to aquatic organisms. In experiments using algae as test system, high concentrations of dyes may result in inhibitory effects that can be comparable to the effects of the internal toxicity induced by those dyes (Øllgaard et al. 1998). In addition, in some cases, the inhibition of light can represent up to 50% of the observed grown inhibition (ETAD 1994 in Croce et al. 2017). To verify the possible effects related to inhibition of the photosynthesis caused by light-blocking, due to the color of the dye solutions, we performed preliminary tests with the highest concentration of each tested dye. Apparently, the observed toxicity was related to dyes themselves, and not to the light-blocking, because no statistically significant difference in algal growth was observed when compared to the control experiments (see Supplementary 3, Fig. S1). However, testing with more replicates and longer exposure periods would be required to confirm this hypothesis.

For *D. similis*, it was observed a small difference between the results of the acute tests, with a higher toxicity for Auramine (Table 2). *D. similis* and *D. magna* are the most used cladocerans to assess the toxicity of dyes. Around 50 dyes have already been tested for these species and the levels of the acute toxicity varied significantly in the available literature (Table 2).

Auramine and Auramine O showed similar results for the chronic tests with *D. similis*, (inhibition of reproduction) (Table 1). It means 6- to 10-fold increase in toxicity from the acute test to the chronic test. Vacchi et al. (2016) observed an almost 50-fold increase in toxicity from the acute test (EC50 = 0.13 μg/L) to the chronic test (NOEC = 0.003 μg/L) with *D. similis* exposed to Disperse Red 1. These results confirmed the relevance of sublethal effects evaluation in toxicity assessments. However, there is still a lack of information in the literature related to chronic toxicity of dyes to microcrustaceans, such as daphnids.

Both dyes induced lethal (stages D and E) and sublethal (stages B and C) effects in *H. attenuata* (Fig. 2). For lethality and morphological changes, the toxicity of Auramine O was approximately 2 times higher than the toxicity recorded for Auramine. (Table 2). *H. attenuata* was the only organism that showed greater sensitivity to Auramine O. *Hydra* has shown high sensitivity to chlorinated compounds (Vacchi et al. 2013). Maybe the fact that Auramine O is chlorinated salt ($C_{17}H_{21}N_3$.HCl) could explain its higher toxicity in relation to Auramine.

The toxicity of dyes for *H. attenuata* is not well known, only Disperse Red 1 was investigated using this test organism. Vacchi et al. (2016) recorded an LC_{50} of 48000 μg/L (lethal effects) and a NOEC of 1000 μg/L (sublethal effects). Jong et al. (2016) also observed sublethal effects for *H. attenuata* even with lower concentrations (< 100 μg/L). Therefore, it is possible to conclude that dyes may induce toxic lethal and sublethal effects in *H. attenuata*.

Fish embryos are often used to assess dye toxicity and, in general, effects on organism survival are observed in the mg/L concentration range, in line with the results obtained to both Auramines in the present study (Table 1). Auramine and Auramine O caused similar sublethal and teratogenic effects in fish embryos, including edemas, tail malformation, and delay in yolk sac absorption (Fig. 3). Such effects occurred already in the first day of exposure, remaining until the end of the test. In the first 24 h, those effects were observed only in the highest concentrations. However, with the increasing of exposure period, they were recorded even at the lowest concentrations. No studies were found in the literature with Auramine and fish but Dach et al. (2019) studied the toxicity Auramine O on zebrafish development. They observed developmental effects such as alteration in craniofacial and body axis morphology and the presence of yolk-sac and pericardial edema after at 3.04 mg/L of Auramine O. Furthermore, teratological effects to developing *Microhyla ornata* embryos, such as malformation tail and eyes, were observed by Ghate and Mulherkar (1978) after the exposure of these organisms to effluents from a textile industry that used several dyes, including Auramine. To the best of our knowledge, no mechanistic explanation can be found in the current literature to explain the malformations induced by auramine in fish embryos. Auramine O caused DNA damage (comet assay) in human hepatocytes (Martelli et al. 1998). Auramine O was also

positive in the Salmonella/microsome mutagenicity test (TA98, TA1535, TA1538, and YG1024 strains), in the presence of metabolic activation (IARC 2010).

The Globally Harmonized System of Classification and Labelling of Chemicals (GHS 2009) classify the chemical compounds by criteria as very toxic (LC_{50} < 1000 μg/L), toxic (LC_{50} 1000–10000 μg/L), or harmful (LC_{50} 10000–100000 μg/L). According to this classification and based on the organisms and endpoints tested, the two dyes evaluated in this study can be considered toxic or very toxic to these organisms.

PNEC values of 0.92 μg/L for Auramine and 4.6 μg/L for Auramine O were derived based on the most sensitive organism in the chronic tests (algae *R. subcapitata*) divided by an assessment factor of 50. A PNEC of 2.3 μg/L was derived by the Canadian Government representing all azo dyes using the deterministic approach (Environment Canada 2016). Vacchi et al. (2016) determined a PNEC for the dye Disperse Red 1 of 0.06 μg/L and 1.8. The PNECs derived in this study for Auramine and Auramine O can be considered environmentally relevant because they are in the same order of magnitude of the concentration of other dyes found in surface waters (Carneiro et al. 2010; Zocolo et al. 2015; Vacchi et al. 2017).

Conclusions

We concluded that both synthetic dyes, Auramine and Auramine O, are toxic to aquatic organisms from different trophic levels, which could lead to an imbalance in an ecosystem contaminated with these substances. All 50% effective concentrations values were below 5000 μg/L. Concentrations ranging from 600 to 1300 μg/L negatively affected daphnids' reproduction and induced deformities in hydra and fish embryos, representing a risk to their populations when exposed to those concentrations in the field.

From all tested organisms, algae were the most sensitive, and 50% inhibition growth was observed in concentrations equal or lower than 800 μg/L. Because they are in the first trophic level, an imbalance in algae population in the field could lead to an imbalance throughout the trophic chain. The toxicity observed to algae does not seem to be related to the blockage of light caused by the dye solution color.

PNEC values based on algae chronic effects were derived (0.92 μg/L for Auramine and 4.6 μg/L for Auramine O), CRED showed the reliable values and, may be useful in future risk assessments related to the presence of these dyes in aquatic environments.

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Compliance with ethical standards

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

Ethical standards The experiments are in accordance with the current laws of the country in which they were performed. The study was approved by the Ethics Committee at the University of Brasilia (reference no 100226/2014).

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