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A test battery for assessing the ecotoxic effects of textile dyes

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A B S T R A C T

The textile dyeing industry is one of the main sectors contributing to environmental pollution, due to the generation of large amounts of wastewater loaded with dyes (ca. 2–50% of the initial amount of dyes used in the dye baths is lost), causing severe impacts on human health and the environment. In this context, an ecotoxicity testing battery was used to assess the acute toxicity and genotoxicity of the textile dyes Direct Black 38 (DB38; azo dye) and Reactive Blue 15 (RB15; copper phthalocyanine dye) on different trophic levels. Thus these dyes were tested using the following assays: Filter paper contact test with earthworms (*Eisenia foetida*); seed germination and root elongation toxicity test (*Cucumis sativus*, *Lactuca sativa* and *Lycopersicon esculentum*); acute immobilization test (*Daphnia magna* and *Artemia salina*); and the Comet assay with the rainbow trout gonad-2 cell fish line (RTG-2) and *D. magna*. Neither phytotoxicity nor significant effects on the survival of *E. foetida* were observed after exposure to DB38 and RB15. Both dyes were classified as relatively non-toxic to *D. magna* (LC_{50} > 100 mg/L), but DB38 was moderately toxic to *A. salina* with a LC_{50} of 20.7 mg/L. DB38 and RB15 induced significant effects on the DNA of *D. magna* but only DB38 caused direct (alkaline comet assay) and oxidative (hOGG1-modified alkaline comet assay) damage to RTG-2 cells in hormetic responses. Therefore, the present results emphasize that a test battery approach of bioassays representing multiple trophic levels is fundamental in predicting the toxicity of textile dyes, aside from providing the information required to define their safe levels for living organisms in the environment.

1. Introduction

The textile industry plays an important role in the economy of many countries, providing a vast range of colored fabrics for marketing, but its manufacturing activities pose challenges for environmental management, since large amounts of synthetic dyes, resistant to conventional wastewater treatments, are daily released into the environment [1–7]. The amount of dye that is released into the textile effluents may vary from 2% to 50% of the initial dye concentration [3,8].

Thus dye-based toxicity has been investigated over the years in order to identify hazardous dyes and, consequently, to protect human health and the environment [3–5,9–15]. However, most of the studies on dye toxicity have focused on humans, and safe thresholds to protect environmental organisms are not available for most of the dyes commonly used in textile dyeing [6,14]. For example, Direct Black 38 (DB38) is an azo dye classified as carcinogenic to humans due to its biotransformation to benzidine [16–18], but there is a lack of data on its impact to terrestrial and aquatic organisms. Recently, Oliveira and co-authors [5] pointed out that care should be taken in discharging DB38-containing wastewater, since this dye has been shown to be embryotoxic for the zebrafish model. Reactive Blue 15 (RB15), a copper-phthalocyanine dye, is toxic for the bacteria, tadpoles and embryo fish models [5,19,20]. The identification of its toxicity for species covering other trophic levels is relevant to gather additional

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ecotoxicological knowledge, and hence determine its safe level to protect the environment. Species-specific toxicity has already been demonstrated for Reactive Red 120, which reinforces the use of a multiple trophic levels eco-tests approach to accurately predict the ecotoxic effects of textile dyes [4].

One of the core missions of ecotoxicology is to understand the underlying mechanisms behind pollutants that can disturb the normal physiological condition of biological systems, in order to prevent adverse outcomes resulting from them [21]. Thus short-term assays are useful ecotoxicological tools for estimating the acute toxicity caused by environmental chemicals.

Plants are the foundation of terrestrial and aquatic ecosystems, acting as primary producers in the food chain [22]. Thus, phytotoxicity tests are a vital part of ecotoxicological assessment, the seed germination and root elongation test being the simplest one, and representative of the action of the toxicant at the first interface of the developing plant (seed) and its environment [23]. Earthworms (terrestrial invertebrate) are considered to be suitable indicator species for ecotoxicological assessment, since they may represent 60–80% of the total soil biomass and play crucial roles in soil functioning (*e.g.* aeration, moisture content, nutrient cycling), apart from being sensitive to low toxicant concentrations [24,25]. Thus the acute earthworm toxicity test using *Eisenia foetida* or *Eisenia andrei* has been recommended for detecting potential soil toxicants by both regulatory agencies and environmental monitoring programs [26].

Daphnia, also called “water flea”, is a planktonic invertebrate organism inhabiting freshwater ecosystems. Since *Daphnia* are sensitive to several chemicals and easily cultured under laboratory conditions, they are considered to be very useful bioindicators in ecotoxicology [27]. The acute immobilization test [28] with daphnids is used to detect water toxicants [29,30]. *Artemia* spp. (brine shrimp) is a major taxon in many hypersaline biotypes throughout the world, feeding primarily on phytoplankton and being an important primary consumer [31]. They present several advantages, such as a short life cycle and adaptability to wide ranges of salinity, which have contributed to increasing the use of brine shrimps in ecotoxicological studies [31,32].

Fish are also currently used in the assessment of chemical toxicity in aquatic environments, since they are the most diverse group of vertebrates found in this ecosystem [33]. Toxicological research on fish is largely based on *in vivo* studies. However, today there are a number of economic, scientific and ethical reasons for supporting efforts to develop and apply *in vitro* assays in aquatic ecotoxicology as alternative tools to animal testing [34,35]. Fish cell lines are of particular interest since they represent standardized systems that can be carried out in a controlled environment, giving fast, affordable and ethically eligible results [34–37]. Among the fish cell lines available so far, the RTG-2 cell line derived from rainbow trout (*Oncorhynchus mykiss*) gonadal tissue has been successfully used in assessing aquatic genotoxicants [38–43].

Considering that textile dyes are often discarded into aquatic environments and that their ecotoxicological effects are not completely known, the aim of this work was to assess the acute toxicity and genotoxicity of the textile dyes Direct Black 38 (DB38; azo dye) and Reactive Blue 15 (RB15; copper phthalocyanine dye). For this purpose, an ecotoxicity testing battery approach composed of multiple trophic levels test systems was used, since dyes, like other chemicals, can display species-specific toxicity.

2. Material and methods

2.1. Tested compounds

The dyes Direct Black 38 (DB38; Chlorazol Black E; purity ≥ 45%; CAS No. 1937-37-7) and Reactive Blue 15 (RB15; Turquoise Blue; purity 35%; CAS No.: 12225-39-7) were purchased from Sigma-Aldrich (St Louis, MO, USA). The chemical structure of each dye is presented in Fig. 1. For all ecotoxicity assays, the dye solutions were prepared in distilled water or test medium (*i.e.*, culture medium) used for culturing each test organism, and without addition of solvents.

2.2. Ecotoxicity endpoints

2.2.1. Seed germination and root elongation toxicity test

Lettuce (*Lactuca sativa*), cucumber (*Cucumis sativus*) and tomato (*Lycopersicon esculentum*) seeds were purchased from an agricultural supplies retailer. Selected species are recommended as standard species for ecotoxicological assessment by the US Environmental Protection Agency (USEPA) [44]. Prior to the test, the seeds were sterilized with ultraviolet light for 5 min and then rinsed several times in distilled water to prevent fungal growth. The seed germination and root elongation test on filter paper was carried out according to USEPA [44]. Ten seeds of each species were exposed on filter paper (Whatman 1) containing 3 mL of DB38 and RB15 at 62.5, 125, 250, 500 and 1000 mg/L in Petri dishes sealed with a plastic film. These concentrations were established after carrying out a preliminary test (range-finding test) in which the highest concentration tested, for water soluble compounds, should be the saturation concentration or 1000 mg/L. The other concentrations should be chosen in a geometric series ranging from 1.5 to 2.0 [44]. Distilled water was used as the negative control (NC) and zinc sulfate heptahydrate ($ZnSO_4{\cdot}7H_2O$; 3 mg/mL) as the positive control (PC). Three plates per concentration were prepared and incubated in complete darkness in a growth chamber at 20 ± 1 °C for 120 h. After this exposure period, the number of germinated seeds was counted, and the length of the root measured. The relative seed germination percentage was calculated by dividing the number of seeds germinated in the exposed groups by the number of seeds geminated in the NC. The criterion for test validation was that at least 65% of the seeds from the NC should germinate, and 5 mm of radicular protrusion was regarded as germinated.

2.2.2. Acute toxicity test with earthworms

E. foetida (Oligochaeta, Lumbricidae) earthworms were obtained from laboratory cultures using cow dung as the substrate and food. To void the gut contents, the earthworms were placed on moist filter paper for 3 h before testing. They were then washed and dried before use. Adult earthworms weighing about 300 mg (after voiding the gut contents) with well-developed clitella were selected. The acute toxicity assay was carried out according to the Organization for Economic Cooperation and Development (OECD) Guideline 207 by the filter paper contact test [45]. The filter paper was placed on the inside wall of a tube (3 × 8 cm) and 1 mL of DB38 or RB15 at 0.1; 1; 10; 100; and 1000 mg/L then added to each tube using a micropipette, and evenly distributed over the filter paper. For this bioassay when using water soluble substances, the maximum recommended concentration tested should be 1000 mg/L [45]. The earthworms were exposed to a series of widely spaced concentrations of the dyes in order to determine the maximum effect (100% of mortality) at the highest concentration and no observable effect at the lowest concentration. Distilled water was used as the NC and $ZnSO_4{\cdot}7H_2O$ (6 mg/mL) as the PC. Ten replicates per concentration were made, each consisting of one adult earthworm per tube. All tubes were sealed with a plastic film with small ventilation holes and maintained at 20 ± 2 °C and 60–80% relative humidity in the dark for 48 h. The earthworms were considered to be dead when there was no reaction to mechanical stimulation.

2.2.3. Daphnia magna acute immobilization test

The acute toxicity test using *D. magna* was carried out according to OECD Guideline 202 [28] with modifications. *D. magna* were maintained at 20 ± 1 °C under a 16:8 h light/dark photoperiod, and fed daily with the green alga *Chlorella sp*. The experiments were carried out with 4 concentrations of DB38 and RB15 (250; 500; 750 and 1000 mg/L) and NC (water culture). These concentrations were determined after