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Toxicity of dyes to zebrafish at the biochemical level: Cellular energy allocation and neurotoxicity☆

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ABSTRACT

Dyes are widely distributed worldwide, and can be found in wastewaters resulting from industrial or urban effluents. Dyes are of particular concern as contaminants of the aquatic environment, since their toxicity remain poorly understood. Thus, the current study was designed to assess the effects induced by the synthetic azo dye Basic Red 51 (BR51) and by the natural naphthoquinone dye erythrostominone (ERY) on zebrafish early life stages (*Danio rerio*) at different biological organization levels, i.e., studying how changes in biochemical parameters of important physiological functions (neurotransmission and cellular energy allocation) may be associated with behavior alterations (swimming activity). This approach was also used to assess the effects of ERY after its photodegradation resulting in a colorless product(s) (DERY). Results showed that after 96 h exposure to BR51 and Ery, zebrafish embryos consumed less energy (LOEC = 7.5 mg/L), despite the unaltered levels of available energy (carbohydrates, lipids and proteins). Hence, cellular energy allocation (CEA) was significantly increased. On the other hand, only ERY decreased the acetylcholinesterase activity (LOEC = 15 mg/L). Despite that, zebrafish larvae exposed to both dyes until 144 h were less active. In contrast, DERY did not affect any parameter measured. These results indicate an association between a decrease consumption of energy and decrease swimming activity resulting from an environmental stress condition, independently of the neurotoxicity of the dyes. Degradation of ERY by light prevented all toxic effects previously observed, suggesting a cheap, fast and easy alternative treatment of effluents containing this natural dye. All tools assessed in our current study were sensitive as early-warning endpoints of dyes toxicity on zebrafish early life stages, and suggest that the CEA assay might be useful to predict effects on locomotor activity when cholinergic damage is absent.

1. Introdution

Synthetic dyes containing the azo-aromatic chromophore group comprise the largest chemical class of dyes used worldwide (Guaratini and Zanoni, 2000), but these are relatively persistent pollutants due to their high stability (O'Neill et al., 1999; Chung, 2000), hindering the removal or reduction of toxicity of dye-containing industrial wastewaters by conventional treatments. It is known that textile industries release approximately 15–50% of the total amount of dyes used during manufacturing into the aquatic environment (Nojavan et al., 2013), and concentrations may be detected from traces (μg/L) up to 500 mg/L, depending upon the dyes and processes used (Nojavan et al., 2013; O'Neill et al., 1999). Few studies reported that azo dyes are toxic to freshwater organisms, such as the microcrustacean *Daphnia similis* (Luna et al., 2014), cnidarian *Hydra attenuata* (Jong et al., 2016) and fish *Labeo rohita* (Kaur and Kaur, 2015). Moreover, it is well known that azo dyes cause genetic damage, including mutagenicity and/or carcinogenicity (Reid et al., 1984; Chung, 2000; Umbuzeiro et al., 2005; Ferraz et al., 2011; Chequer et al., 2011; Leme et al., 2015).

Among these, Basic Red 51 (BR51) is an azo dye used by cosmetic and textile industries, and reaches a production of up to 0.5 t per year only for cosmetic purposes (IARC, 2010). The European regulatory data regarding BR51 clearly evidenced that this dye showed

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mutagenic activity after reductive metabolic activation on *Salmonella* test (EU SCCS/1436/11). However, available data about BR51 is still scarce and has been focused on its effects on humans, where it induces *in vitro* DNA damage in skin and liver cells (Zanoni et al. 2014; Tafurt-Cardona et al., 2015).

More recently, pigments and bioactive compounds derived from natural sources have been used to cosmetics, drugs, foods and textiles as promising alternatives to toxic synthetic dyes as the natural dyes are expected to be less harmful to humans and environment (Chigurupati et al., 2002; Prabhakara Rao et al., 2005; Mongkholrattanasit et al., 2010; Caro et al., 2012; Boonsong et al., 2012; Boga et al., 2013; Shahid et al., 2013; Punrattanasin et al., 2013; Lopes et al., 2013). For instance, the natural red dye erythrostominone (ERY), a naphthoquinone compound extracted from fungi (Kittakoop et al., 1999; Unagul et al., 2005; Prathumpai et al., 2007), is cytotoxic only at high concentrations (348 mg/L) (Abe and Oliveira, 2014) and is not genotoxic to human cells (Abe et al., 2015), and has high bioactivity potential as an antimalarial and antioxidant compound (Kittakoop et al., 1999). Natural dyes also have the advantage of being easily degraded before their release to the environment in of industrial and urban wastewater (Velmurugan et al., 2010; Zhou et al., 2012). However, for ERY or any other natural dye, there is no available data about their ecotoxicity other than Abe et al. (2017, 2017b).

There is an urgent need to gather scientific knowledge on the potential ecotoxicological effects of synthetic dyes, as well as of the new generation of natural dyes, to aquatic species. Since environmental concentrations of dyes are often at sub-lethal concentrations due to a high dilution factor on water bodies receiving wastewater effluents, the development of a new and more sensitive approach is still required to provide a battery of useful tools that can be used in a more realistic scenario. It has been reported that energy budget-related biomarkers have high ecological relevance (De Coen and Janssen, 1997; Smolders et al., 2003, 2004; Vasseur and Cossu-Leguille, 2003; Smolders et al., 2004; Lam, 2009), as chemical stressors induce detoxification responses (Abe et al., 2017) that are energetically costly to exposed organisms and the energy allocation to other physiological processes might be diminished or compromised (Sokolova et al., 2012). Energy metabolism plays a central role in physiological and behavior functions of organisms and provides information to extrapolate the responses towards biochemical, cellular, growth and reproductive functions as long-term effects on supra-individual levels of biological organization (McKee and Knowles, 1986; De Coen and Janssen, 1997; Smolders et al., 2003, 2004). Moreover, several studies have shown the association among energetic biochemical alterations and impairment of other physiological or behavior function in non-target organisms exposed to pollutants (De Coen and Janssen, 1997; Ribeiro et al., 2001; Gravato et al., 2014; Rodrigues et al., 2015).

Therefore, our current research aimed to assess the potential effects of BR51 and ERY on energy metabolism parameters and cholinergic activity on early life stages of the model organism zebrafish (*Danio rerio*). Furthermore, to simulate an easy effluent treatment that can be performed by industries, we also photodegraded the natural dye ERY (as described by Abe et al., 2017) and assessed the toxic effects of the resulting colorless products(s). The energetic metabolism parameters assessed comprised the energy available (Ea) and energy consumed (Ec). For that, Ea was measured as the sum of total lipid, carbohydrate and protein levels, whereas Ec was measured as oxygen uptake rate within the mitochondrial electron transport system activity (ETS), enabling the cellular energy allocation (CEA) calculation. Moreover, anaerobic metabolism was measured by lactate dehydrogenase activity (LDH) to assess if additional anaerobic energy is needed to overcome dyes-induced stress. In addition, larval behavior was monitored over 50 min to assess their swimming activity.

2. Material and methods

2.1. Chemicals

Basic Red 51 (BR51) (2-(((4-dimethyllamino) phenyl) azo)-1,3-dimethyl-1H-imidazolium chloride, CAS No. 77061-58-6, MW: 279.6 g/mol, purity 99% a.i.) (Fig. 1a) was purchased from LCW Import and Export Ltd (São Paulo, Brazil). BR51 stock solutions were directly prepared in reverse osmosis water at 100 mg/L final concentration. The endophytic fungus LC01-A strain (in identification phase) was kindly provided from Coleção de Microrganismos de Importância Agrícola e Ambiental (CCMA, EMBRAPA, Brazil). Erythrostominone (ERY) (CAS No. 26153-04-8, MW: 348.08 g/mol, purity 85–92% a.i.) (Fig. 1b) was extracted, purified and characterized by the group of Professor Dr. Luis Alberto Beraldo de Moraes from the Faculty of Philosophy, Sciences and Literature of Ribeirão Preto, University of São Paulo, Brazil (see Abe et al., 2017). ERY stock solutions were dissolved in DMSO (150,000 mg/L) and further diluted in reverse osmosis water to prepare final working solutions (DMSO 0.01%, v/v). Reverse osmosis water and 0.01% DMSO in reverse osmosis water were used as controls.

2.2. Dye photodegradation

Each ERY working solution (DMSO 0.01%, v/v) was placed in 50 mL tubes inside a light room (250 lx) along 7 d until the red solutions became colorless. Thereafter, the degraded ERY (DERY) solutions were directly used in bioassays. The structural degradation of ERY is described in Abe et al., (2017). The BR51 degradation was previously performed, but as BR51 solutions remained unchanged (see more details in Abe et al., 2017), so we only used DERY in our bioassays.

2.3. Zebrafish culture

Zebrafish (*Danio rerio*) was obtained from the facility at Biology Department from University of Aveiro (Aveiro, Portugal). Adults were kept in a flow-through system carbon-filtered water (Zebra-Tec, Tecniplast, Italy), at pH 7.5 ± 0.5, temperature $26 \pm 1\,^{\circ}C$, dissolved oxygen at 95% saturation and conductivity $750 \pm 50\,\mu s/cm$, 16 h:8 h light/darkness photoperiod. Fishes were fed twice a day with commercial feed (ZM400 Granular).

Breeding stocks of males and females were placed (2:1) into an 8 L aquarium and once the light onset in the morning, they spawned during 1 h. Batches of eggs were collected and rinsed in autoclaved osmotic water. Fertilized eggs in cleavage period until blastula stage (4–64-cell) were selected in stereomicroscope (SMZ800, Nikon) for subsequent experiments.

2.4. Exposure of early-life stages of zebrafish

Sub-lethal concentrations were selected from our previous studies (Abe et al., 2017) to perform these experiments (1.875, 3.75, 7.5 and 15 mg/L to BR51, ERY and DERY). Viable eggs were carefully placed into Petri dishes containing 50 mL dyes solutions each, placing 25 eggs per dish. Tests were performed at $26 \pm 1\,^{\circ}C$ in the dark to avoid further degradation of dyes. After 96 h post-fertilization (hpf), 7 clusters of 19 hatched embryos per treatment and controls were collected in 2 mL ice-covered microtubes and immediately stored at -80° until further biochemical analyses. Behavior analyses were performed on 144 hpf larvae, totaling 20 replicates per treatment (1 larvae per replicate individually placed in 96-well plates). Until 144 hpf, larvae still feed from their yolk sac,