Chemico-Biological Interactions 292 (2018) 94–100

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Red disperse dyes (DR 60, DR 73 and DR 78) at environmentally realistic concentrations impact biochemical profile of early life stages of zebrafish (*Danio rerio*)

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ARTICLE INFO

Keywords:
Textile dyes
Oxidative stress
Neurotoxicity
Cellular energy allocation

ABSTRACT

Dyes have been used for more than twenty thousand years in textile, pharmaceutical, food, cosmetic, and photographic industries, among others. Despite their importance in these applications, dyes can be toxic and resistant to many degradation processes used in wastewater treatment plants. Although a large number of dyes have been released in the environments in high amounts, studies into the environmental toxicity of these substances are still scarce. The aim of this study was to evaluate the potential toxic effects of textile dyes Disperse Red 60, Disperse Red 73 and Disperse Red 78 in zebrafish early life stages. To this end, biochemical biomarkers were selected to evaluate non-enzimatic antioxidant (Total Glutathione), antioxidant enzymes (Glutathione S-transferase and Catalase), oxidative stress (lipid peroxidation), neurotransmission (acetylcholinesterase) and energetic metabolism parameters (energy available and energy consumed) after 96 h exposure to these dyes. Our results demonstrated that these disperse dyes induce biochemical alterations in zebrafish embryos at environmental realistic concentrations and that the discharge of these disperse dyes into water bodies should be carefully evaluated. The selected biomarkers were sensitive as early-warning endpoints of disperse dyes toxicity on zebrafish embryos. Implications for risk assessment and indications for future research are discussed.

1. Introduction

The aquatic environment is the major target of contaminants from industrial effluents [1–3]. The textile industry, for example, releases approximately 15–50% of the total amount of dyes used during manufacturing into the aquatic environment [3]. Due to their high persistence and general poor removal by conventional industrial wastewater treatments, dyes have been considered one of the most problematic water pollutants worldwide [4]. It has been estimated that approximately 10,000 different dyes and pigments are currently in industrial use, totalling an annual consumption of around 700 thousand tonnes worldwide [5,6]. In textile industry, up to 200 thousand tonnes of these dyes are lost to effluents every year during dyeing and finishing due to the inefficiency on the process [6]. To date, few studies have measured dye concentrations in water bodies [7]. In a study conducted on the Yamaska River (Quebec, Canada), Maguire and Tkacz [8] detected 15 dyes in water, sediment and suspended solids samples, and reported Disperse Blue 26, Disperse Blue 79 and Disperse Red 60 concentrations ranging from 3 to 17 μg/L. In Brazil, dispersed dyes have also been detected, such as Disperse Red 1, Disperse Blue 373 and Disperse Violet 93, in samples from the Ribeirão dos Cristais and Piracicaba rivers, with dye concentration ranging from 0.002 to 3.5 μg/L [9,10].

Relatively few studies have been conducted so far on biochemical damages of dyes to aquatic organisms [11–14]. In addition, since

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https://doi.org/10.1016/j.cbi.2018.07.007
Received 5 March 2018; Received in revised form 28 June 2018; Accepted 9 July 2018
Available online 10 July 2018
0009-2797/

environmental dye concentrations are often found at sublethal levels, sensitive methods are required to determine early-warning signs of adverse effects such as molecular, biochemical, or physiological responses rather than traditional acute LC_{50} (lethal concentration affecting 50% of the test population) bioassays [13,15]. Conjugation and antioxidant enzymes, such as, glutathione S-transferase and catalase have been considered effective indicators for the response of organisms to pollutant-induced oxidative stress [11,16]. Inhibition of acetylcholinesterase activity enzyme, involved in the breakdown of the neurotransmitter acetylcholine, has been proven to be a good indicator of chemical-induces neurotoxicity [17,18]. Energy budget-related parameters are also of great biological and ecotoxicological importance since they provide information about the available and consumed energy, as well as energy sources (proteins, lipids, carbohydrates) being used [14,19,20]. Detoxification responses induced by chemical stress in exposed organisms are energetically costly and may change the energy distribution between the physiological processes, which may alter their functioning [21].

Zebrafish (*Danio rerio*) early life stages have been commonly used as an alternative test method for conventional acute fish toxicity assays [22,23]. Several studies have used zebrafish early life stages to evaluate the toxicity of pollutants and emergent contaminants [24,25]. Few studies, however, have so far been conducted evaluating the effects of dyes on biochemical biomarkers in early life stages of zebrafish [13,14].

The assumption that substances with high molecular weight do not have access to the embryo due to the protective role of the chorion is one of the objections that have been made to the use of the fish embryo test (FET) [26]. However, disperse dyes are widely used in several industrial applications and have a low molecular weight, making them the smallest dye molecules among the dyes [27,28]. The aim of the present study was therefore to evaluate the toxicity of DR60, DR73 and DR78 on biochemical processes of early-life stages of zebrafish. To this end, a zebrafish early life stage test was conducted to evaluate the effects of these dyes on detoxification and antioxidant processes, oxidative damage, acetylcholinesterase activity and cellular energy allocation.

2. Materials and methods

2.1. Test chemicals

DR 60 was purchased from Huntsman Textile Effects (USA), whereas DR 73 and DR 78 were obtained from Classic Dyestuff INC (USA). Their main characteristics are provided in Fig. 1. Considering that these dyes are insoluble in pure water [27,28], stock solutions of the test concentrations were prepared in culture medium containing 0.01% dimethyl sulfoxide (DMSO).

2.2. Test organisms

All embryos used in the present study were provided by the zebrafish facility established at the Department of Biology of the University of Aveiro (Portugal). In this facility, adult organisms are kept under controlled conditions in a ZebTEC (Tecniplast) recirculating system. Culture water in this test system is purified by reverse osmosis and activated carbon filtered tap water, complemented with salt "Instant Ocean Synthetic Sea Salt" (Spectrum Brands, USA) and automatically adjusted for pH and conductivity. Culture conditions are: temperature = 26.0 ± 1 °C; conductivity = 750 ± 50 µS; pH = 7.5 ± 0.5; salinity = 0.35 ppt and dissolved oxygen = equal or above 95% saturation, with a photoperiod of 14 h light - 10 h dark. Nitrogen compounds are regularly monitored to keep concentrations always below 0.1 mg/L (nitrate) and 0.01 mg/L (nitrite and ammonia). Adult fish are fed twice a day with commercially available artificial diet (ZM-400 fish food; Zebrafish Management Ltd).

Zebrafish eggs were obtained by breeding of fish in aquaria with marbles on the bottom to protect eggs from predation by adult fish. The eggs were collected, rinsed in water, and checked under a stereomicroscope (Stereoscopic Zoom Microscope-SMZ 1500, Nikon). Eggs with cleavage irregularities, injuries, or other kind of malformations were discarded.

2.3. Experimental design

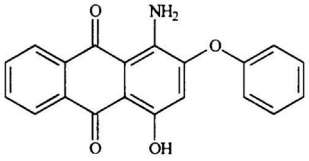
The eggs obtained as described in section 2.2 were transferred to petri dishes containing 15 mL test solution each (0.001; 0.01; 0.1 and 1 mg/L to DR 60 and DR73 and 0.001; 0.01; 0.1; 1; and 10 mg/L to DR 78) using twenty embryos per dish. Negative control (reverse osmosis water and 0.01% DMSO in reverse osmosis water v/v) were also analysed. The test was conducted under the same temperature (26.0 ± 1 °C) and light (14 h light: 10 h dark) conditions as those in the adult fish culture. After 96 h post-fertilization (hpf) 8 clusters of 20 embryos each were collected per concentration and transferred to 2-mL microtubes after 96 h of exposure to dyes and submersed in liquid nitrogen and subsequently kept in a freezer at −80 °C until further analysis.

Disperse Red 60

Molecular formula: $C_{20}H_{13}NO_4$

Molecular weigth: 331.32 g/mol

Purity: 60-80%

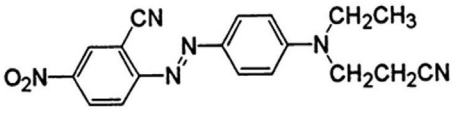


Disperse Red 73

Molecular formula: $C_{18}H_{16}N_6O_2$

Molecular weigth: 348.46 g/mol

Purity: 60-80%



Disperse Red 78

Molecular formula: $C_{17}H_{16}ClN_5O_2$

Molecular weigth: 357.71 g/mol

Purity: 60-80%

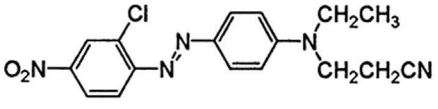


Fig. 1. Selected chemical characteristic and molecular structures of the three dyes tested.

2.4. Biochemical analysis

On the day of biochemical analysis, 1.6 mL ultrapure water was added to the content of each microtube on ice, and subsequently homogenized using a sonicator (Ystral GmbH D-7801 Dottingen). Subsequently, the sample volume was subdivided as follows to analyse the different biochemical parameters:

- 300 µL for lipid content;
- 300 µL for protein reserves and carbohydrates content;
- 300 µL electron transport system (ETS), which was mixed with 150 µL ETS standard solution and centrifuged (1000 g; 4 °C; 10 min) for further ETS determination in the supernatant fraction;
- 200 µL for lipid peroxidation to which 4 µL 2,6-di-*tert*-butyl-4-methylphenol solution (4% v/v in methanol) was added;
- 500 µL for total glutathione (TG), glutathione-S-transferase (GST), catalase (CAT), acetylcholinesterase (AChE) and the protein content determination, which was mixed with 500 µL phosphate standard solution (0.2 M; pH = 7.4) and subsequently centrifuged (9000 g; 4 °C; 20 min) in order to isolate the post-mitochondrial supernatant (PMS). The obtained supernatant fraction was distributed in new microtubes for determination of tGSH (250 µL), GST (250 µL), CAT (60 µL), AChE (250 µL) and proteins (60 µL).

Those PMS samples were stored at −80 °C until biochemical determinations, which were conducted in quadruplicate at 25 °C with a Labsystem Multiskan EX microplate reader.

2.4.1. Available energy reserves (Ea)

The Ea was estimated according to De Coen and Janssen [19] by the sum of lipids, carbohydrates and proteins content, transforming them into energetic equivalents by enthalpy of combustion (39.5 kJ/g lipids, 17.5 kJ/g carbohydrates and 24 kJ/g proteins).

2.4.2. Lipids

The homogenate for lipid quantification was mixed with chloroform, methanol and water and centrifuged at 1000x*g* at 25 °C for 5 min. The organic phase was removed and transferred to a new microtube. Subsequently, sulfuric acid was added and incubated at 200 °C for 15 min. After cooling the samples, water was added and the contents transferred to the microplates for spectrophotometer reading at 375 nm. Tripalmitin was used as the standard.

2.4.3. Carbohydrates and proteins

The homogenate was added with 15% trichloroacetic acid and incubated at −20 °C for 10 min. Subsequently, as samples were centrifuged at 1000x*g* at −4 °C for 10 min. The supernatant was removed and transferred to a new microtube for carbohydrate analysis, while the pellet was resuspended with sodium hydroxide and heated at 60 °C for 30 min. Then, hydrochloric acid was added for neutralization and a phase was obtained for protein analysis.

Total carbohydrate content was determined with phenol and sulfuric acid. The samples were incubated at room temperature for 30 min. After incubation, the absorbance was measured at 492 nm, using glucose as the standard. The protein content were determined using Bradford reagente. The absorbance was read at 592 nm and bovine γ-globulin was used as standard. The final value was expressed in mJ/embryo.

2.4.4. Energy consumption (Ec)

Ec was measured based on the electron transport system (ETS) activity, according to Coen and Janssen [19]. The absorbance was monitored at 490 nm for 3 min. The cellular consumed oxygen rate was determined using a stoichiometrical relationship (2 µmol INT-formazan formed to 1 µmol oxygen consumed). The activity was expressed in mJ/organism/hour.

2.4.5. Cellular energy allocation (CEA)

Cellular energy allocation is relation between available energy and energy consumption (CEA = Ea/Ec), as described by Verslycke et al. [29]. Values were expressed in mJ/organism.

2.4.6. Lipid peroxidation (LPO)

Lipid peroxidation (LPO) was determined by Ohkawa et al. [30] and Bird and Draper [31], adapted by Filho et al. [32] and Torres et al. [33]. The homogenate was separated with 2,6-ditert-butyl-4-methylphenol 4% in methanol (v/v). The LPO products were determined at 535 nm as thiobarbituric acidreactive substances per embryo (picomol TBARS/embryo).

2.4.7. Total glutathione (TG)

Concentration of total glutathione (GSH and oxidized glutathione - GSSG) was determined according to Tietze [34] and Baker et al. [35], through the reaction between a GSH and 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) in the presence of glutathione reductase (GR). Absorbance was monitored at 412 nm for 3 min and values expressed as µM/mg protein.

2.4.8. Glutathione S-transferase (GST)

GST activity was determined according to Habig et al. [36] and adapted to microplates by Frasco and Guilhermino [37], quantifying the conjugation of glutathione to the substrate 1-chloro-2,4-dinitrobenzene (CDNB). The measurement was performed at 340 nm for 5 min. The enzymatic activity was expressed in nmol of GSH/min/mg protein.

2.4.9. Catalase (CAT)

Catalase activity was determined according to Clairborne [38] by the absorbance resulting from the consumption of hydrogen. The activity was monitored for 2 min at 240 nm (ultraviolet light) and expressed in µmol of degraded peroxide/min/mg protein.

2.4.10. Acetylcholinesterase (AChE)

AChE activity was determined according to Ellman et al. [39], adapted for microplate as described in Guilhermino et al. [40], based on the velocity of thiocholine production by the hydrolysis of the acetylcholine. The activity was monitored at 414 nm for 5 min and expressed as nmol/min/mg protein.

2.4.11. Protein quantification

Protein quantification was performed as described by Bradford [41] and adapted to microplates according to Guilhermino et al. [40]. Bovine γ-globulin was used as standard and the spectrophotometric measurement was performed at 600 nm.

2.5. Data analysis

The biochemical dataset was first evaluated for normality and homogeneity using the Kolmogorov-Smirnov and Bartlett tests, respectively. Since all data fulfilled both criteria, a one-way ANOVA was used to detect differences between the treatments for normally distributed data sets. If significant differences were found, the post hoc Dunnetts' test was used to discriminate differences between the control with DMSO and dye treatments. For the nonparametric data Kruskall-Wallis test were performed. All statistical analyses were performed using GraphPad Prism® (Ver 5.0, GraphPad Software) and statistical significance was accepted at $p < 0.05$.

3. Results and discussion

The dyes did not exert any effect on survival or hatching rate, nor did they cause any embryos malformations at any of the sublethal concentrations tested in this study. The statistical significant effects of

Table 1
Summary of the statistical significant effects of the three different dyes evaluated on the different biochemical parameters. TG = Total Glutathione; GST = Glutathione S-transferase; CAT = Catalase; LPO = lipid peroxidation; AChE = acetylcholinesterase; LIP = lipids; CARB = carbohydrates; PROT = proteins; EA = available energy; EC = consumed energy; CEA = cellular energy allocation. The symbols " > " indicates no differences between the treatments ($p > 0.05$), whereas a "↓" and "↑" indicate a statistical significant decrease and increase, respectively, at the indicated test concentrations (in mg/L).

Group	Test parameter	DR 60	DR 73	DR 78	Figure/ Table
Non enzymatic antioxidant	TG	0.01 and 0.1 ↑; 1 ↓	>	1 ↑	Fig. 2
Antioxidant enzymes	GST	1 ↓	>	0.01 ↓	Fig. 2
	CAT	>	0.001 ↑	0.1 ↑; 1 and 10 ↓	Fig. 2
Oxidative stress	LPO	>	>	>	Fig. 3
Neurotoxicity	AChE	>	0.01 ↑; 1 ↓	10 ↓	Fig. 4
Energy sources	LIP	>	>	1 and 10 ↓	Table 2
	CARB	>	>	0.01–10 ↑	Table 2
	PROT	>	>	10 ↑	Table 2
Energy	EA	>	>	0.01–10 ↓	Table 2
	EC	>	1 ↑	1 and 10 ↑	Fig. 5
	CEA	>	>	0.01–10 ↓	Fig. 5

the three dyes on the biochemical parameters evaluated are scrutinized in Table 1.

These effects are further discussed in the following subsections on conjugation, antioxidant process and oxidative stress (3.1), neurotoxicity (section 3.2), and cellular energy allocation and consumption (section 3.3). The implications of study findings for the environmental risk assessment of dyes and concluding remarks are discussed in subsection 3.4.

3.1. Conjugation, antioxidant processes and oxidative stress

Intermediate DR 60 concentrations (0.01 and 0.1 mg/L) led to increased TG levels as compared to controls (Table 1; Fig. 2). TG is known to play a critical role in the detoxification of reactive oxygen species (ROS) due to its GSH sulfhydryl-nucleophilic site that directly reacts with ROS or electrophilic substances or donates electron for enzymatic antioxidants like GST [42]. Given the absence of increased GST at these DR 60 concentrations, it appears that TG was activated for direct action or that it triggered other enzymatic antioxidants not measured in the present study, such as glutathione peroxidase or glutathione reductase [42]. The decreased TG levels in the highest DR 60 treatment, however, was accompanied with decreased GST levels (Table 1; Fig. 2), which is indicative of an impairment of conjugation and antioxidant processes that may lead to oxidative damage [43]. It hence appears that the detoxification mechanism was unable to cope with this DR 60 concentration, although no effects were noted on LPO (Table 1; Fig. 3).

Only in the lowest DR 73 concentration, an increase in CAT activity was found (Table 1; Fig. 2). CAT activity is associated with higher capacity to degrade hydrogen peroxide to avoid LPO in aquatic organisms [11,12]. This hence also appears to be the case in the present study since indeed no effects of DR 73 on LPO were noted (Table 1; Fig. 3). In line with this, Oropesa et al. [44] also reported increased CAT levels and absence of effects in LPO in *Daphnia magna* after exposure to effluents spiked with emerging contaminants.

DR 78 showed increased TG level and decreased GST activity in intermediate concentrations and decreased CAT activity in higher test concentrations (Table 1; Fig. 2). As to whether these increased TG levels were induced as a direct detoxification mechanism or due to a decreased GST activity requires further study. Despite that all three dyes had significant effects on at least one of the enzymatic activities or TG levels assessed (section 3.1; Table 1), no significant effects were noted on LPO level at any of the dye concentrations tested (Fig. 3).

3.2. Neurotoxicity

Decreased AChE activity was only noted at the highest test concentration of DR 73 and DR 78 (Fig. 4), indicating a low neurotoxic potential. The neurotoxic potential of dyes depends on the compound, test species and concentrations evaluated. Abe et al. [14], for example, did not encounter neurotoxic activity of the synthetic azo dye Basic Red 51 in zebrafish larvae at the concentrations evaluated. The same study, however, did demonstrate a significant reduction in AChE activity in

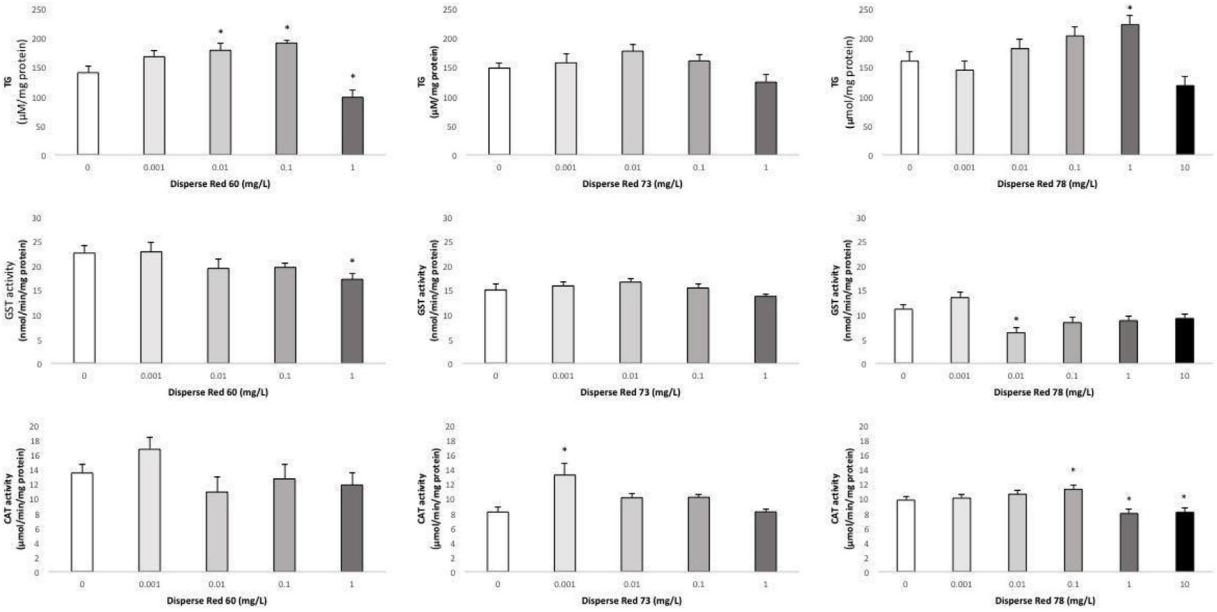


Fig. 2. Levels of total glutathione; activity of glutathione S-transferase and catalase in zebrafish embryos 96 h after the start of the exposure to the three dyes evaluated. The bars indicate mean ± SE (n = 8 clusters of 20 embryos each). *$p < 0.05$.

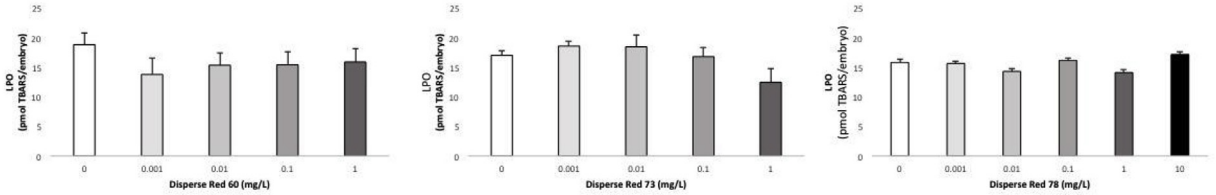


Fig. 3. Levels of lipid peroxidation in zebrafish embryos 96 h after the start of the exposure to the three dyes evaluated. The bars indicate mean ± SE (n = 8 clusters of 20 embryos each). *p < 0.05.

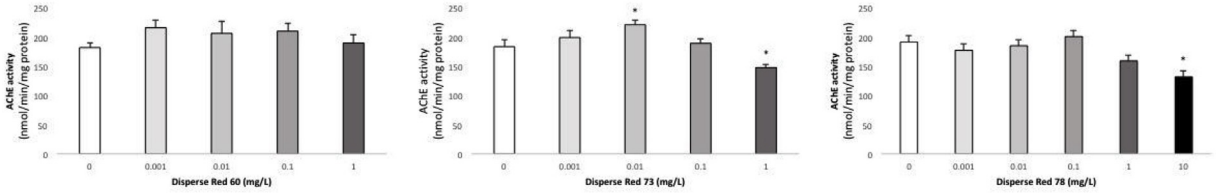


Fig. 4. Acetylcholinesterase activity in zebrafish embryos 96 h after the start of the exposure to the three dyes evaluated. The bars indicate mean ± SE (n = 8 clusters of 20 embryos each). *p < 0.05.

Table 2
Available energy (EA; mean ± SE) and their fractions in the zebrafish embryos 96 h after exposure to the different dye treatments (n = 8 clusters of 20 embryos each). Asterisks indicate statistical significant differences from the control (Dunnett or Kruskal Wallis test p < 0.05).

Treatment	Fractions of EA (%)			EA (mJ/embryo)
	Lipids	Carbohydrates	Proteins	
DR60				
0	94 ± 0,9	3.8 ± 0,3	2.4 ± 0,6	1908 ± 215
0.001	95 ± 0,6	3.1 ± 0,4	1.9 ± 0,3	2463 ± 252
0.01	94 ± 0,8	3.7 ± 0,5	1.9 ± 0,2	1728 ± 156
0.1	94 ± 1,2	3.9 ± 0,7	2.8 ± 0,5	1891 ± 367
1.0	93 ± 0,8	3.6 ± 0,4	3.1 ± 0,5	1979 ± 126
DR73				
0	95 ± 0,9	3.0 ± 0,4	2.6 ± 0,4	1745 ± 111
0.001	94 ± 0,7	3.3 ± 0,4	2.9 ± 0,3	1927 ± 216
0.01	95 ± 0,5	3.4 ± 0,7	2.8 ± 0,1	2416 ± 378
0.1	94 ± 0,8	2.8 ± 0,4	3.3 ± 0,5	2398 ± 281
1.0	94 ± 0,8	2.5 ± 0,4	3.0 ± 0,4	2748 ± 352
DR78				
0	96 ± 0,7	2.0 ± 0,3	1.9 ± 0,3	3660 ± 503
0.001	96 ± 1,1	2.1 ± 0,4	1.1 ± 0,2	2999 ± 457
0.01	94 ± 0,9	4.3 ± 0,4*	2.6 ± 0,5	1391 ± 102*
0.1	93 ± 0,8	5.0 ± 0,5*	2.1 ± 0,4	1409 ± 97*
1.0	90 ± 0,7*	5.3 ± 0,3*	4.6 ± 0,4*	1384 ± 58*
10	86 ± 1,9*	6.7 ± 0,8*	5.7 ± 0,8*	987 ± 116*

zebrafish larvae of the natural naphthoquinone dye erythrostominone [14].

Interestingly, embryos exposed to 0.01 mg/L of the dye DR 73 were noted to have increased AChE activity (Table 1; Fig. 4). Calabrese and Baldwin [45] discussed that an increase in AChE activity may be explained as a compensatory mechanism after homeostasis alteration, and hence indicates an initial neurotoxic effect. Alternatively, this increase might be attributed to an increase of cholinergic transmission resultant from apoptotic stimulus or cell apoptosis, given its demonstrated increase in apoptotic scenarios as a potential biomarker and regulator of apoptosis [46,47].

3.3. Cellular energy allocation and consumption

Under stress conditions, effects on energetic biomarkers have been related to the induction of detoxification processes and increased respiration, changing energy consumption, reserves and allocation [21,48]. In line with this, the greatest effects on energy-related biomarkers in the present study were noted for DR 78, which also showed the greatest effects on antioxidant enzymes. Besides increased energy consumption that was also noted for DR 73, the available energy was also decreased in zebrafish embryos exposed to higher DR 78 concentrations (Table 2; Fig. 5). Given that cellular energy allocation is defined as the ration between energy reserve and energy consumption [29], this test parameter was also decreased in these treatments. This increase in energy consumption for detoxification purposes indicates

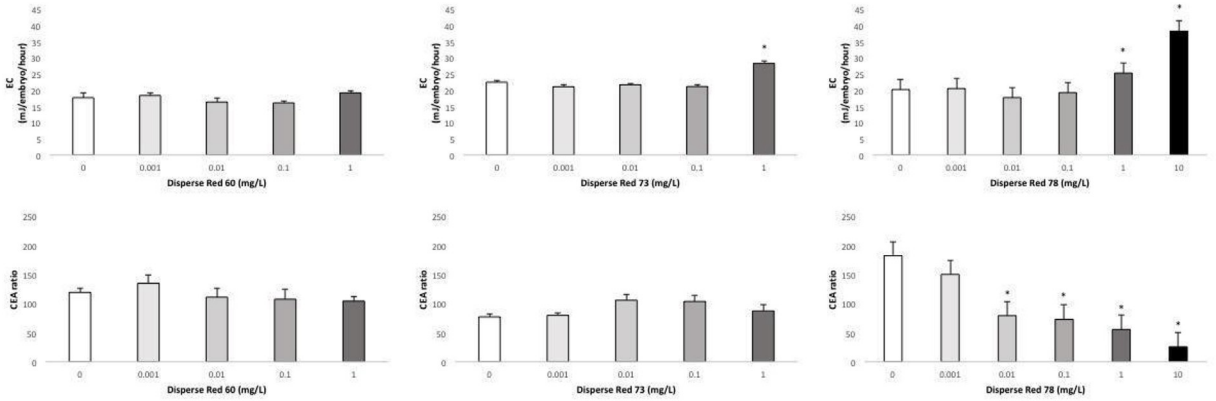


Fig. 5. Energy consumed and cellular energy allocation in zebrafish embryos 96 h after the start of the exposure to the evaluated dyes. Values represented in the Figure indicate mean ± SE (n = 8 clusters of 20 embryos each). *p < 0.05.

that less energy remains available for growth and reproduction, which may lead to long-term effects on the fitness on both the individual and population level.

As can be noted from Table 2, lipids were the principle energy source in control and unaffected zebrafish embryos. In line with this, lipids in yolk reserves represent the main energy source for fish embryos [20,49]. On the other hand, proteins had the lowest fractions in available energy reserves, and they are indeed known to be the last choice as energy source and are hence only mobilized under severe conditions [50]. In zebrafish embryos exposed to the higher DR 78 concentrations, the increased energy consumption was accompanied with a decrease in the lipid fraction of the available energy (Table 2). In line with this, lipids have been discussed to represent the first energy source that is mobilized when organisms are exposed to chemicals, as reported for *Daphnia magna* [19]. The increased fraction of proteins in these treatments may be related with the increased protein synthesis utilized in the induced detoxification mechanism [20,51].

3.4. Implications for risk assessment and concluding remarks

Different effects on the selected oxidative stress, cellular energy allocation and neurotoxicity parameters were obtained for the different dyes, and even for different concentrations of the same dye (Table 1). Depending of the contaminant, specific antioxidant enzyme activities are indeed known to be triggered to counteract oxidative stress [13,52]. Dye concentrations may be detected from traces (μg/L) up to 500 mg/L, depending upon the dyes and analytical methods used [3]. This indicates that the dye concentrations evaluated in the present study may exist under natural conditions and hence that they may exert toxic effects.

To ensure robustness of clothing colour and their resistance to different processes like transpiration, washing and exposure to sun, clothing dyes are designed to be persistent. Their high stability also dictates that they are relatively persistent pollutants [53,54]. For example, the half-life of the hydrolysed form of Reactive Blue 19 dye has been estimated to be 46 years [55]. Future studies are hence also needed to evaluate the chronic toxicity of dyes, the more since several studies demonstrated that dyes may need a prolonged exposure period to exert toxic side-effects [20,56].

The biochemical parameters evaluated in the present study allowed assessing the cellular toxic effects induced by the dyes tested. For most chemical compounds occurring in the environment, there is often little information about their modes of action [57]. Studies at the molecular level are needed to deduct the mode of action of chemicals and hence their exact underlying toxic mechanisms. Such studies could hence also aid in developing toxicokinetic-toxicodynamic (TKTD) models that are absolutely essential for a more mechanistic understanding of chemical stress [58].

Our results clearly demonstrated that the release of the dyes DR60, DR73 and DR78 into water bodies should be carefully evaluate, taking in account the final concentration in the aquatic environmental.

Funding sources

This work was supported by National Institute of Science and Technology for Detection, Toxicological Evaluation and Removal of Emerging and Radioactive Contaminants (INCT-DATREM); São Paulo Research Foundation (FAPESP – grant number 2016/03281-9). Scholarships were provided by National Council for Scientific and Technological Development (CNPq - grant number: 142379/2014-0), Coordination for the Improvement of Higher Education Personnel (CAPES – grant number 88881.134597/2016-01) through a doctoral grant for Gabriela Meireles and São Paulo Research Foundation through a doctoral grant for Ana Letícia Madeira Sanches (FAPESP – grant number 2017/05543-3 and 2014/14621-0). Special Visiting Researcher program (MEC/MCTI/CAPES/CNPq/FAPs reference 402392/2013-2) and Portuguese government (FCT) through a postdoc grant for Michiel A. Daam (SFRH/BPD/109199/2015) and the research unit UID/AMB/04085/2013 (CENSE). We also acknowledge FCT for Carlos Gravato contract (IF/01401/2014) and the funding support to CESAM (UID/AMB/50017) and co-funding by FEDER, within the PT2020 Partnership Agreement and Compete 2020.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.cbi.2018.07.007.

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