Journal of Hazardous Materials 192 (2011) 628–633

Contents lists available at ScienceDirect

Journal of Hazardous Materials

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

The azo dye Disperse Orange 1 induces DNA damage and cytotoxic effects but does not cause ecotoxic effects in *Daphnia similis* and *Vibrio fischeri*

Elisa R.A. Ferraz*, Marcella Daruge Grando, Danielle P. Oliveira

USP, Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Av. do Café, s/n, 14040-903 Ribeirão Preto, SP, Brazil

ARTICLE INFO

Article history:
Received 18 October 2010
Received in revised form 18 April 2011
Accepted 21 May 2011
Available online 27 May 2011

Keywords:
Disperse Orange 1
DNA damage
Ames test
Vibrio fischeri
Daphnia similis

ABSTRACT

Azo dyes constitute the largest group of colorants used in industry and can pass through municipal waste water plants nearly unchanged due to their resistance to aerobic treatment, which potentially exposes humans and local biota to adverse effects. Unfortunately, little is known about their environmental fate. Under anaerobic conditions, some azo dyes are cleaved by microorganisms forming potentially carcinogenic aromatic amines. In the present study, the azo dye Disperse Orange 1, widely used in textile dyeing, was tested using the comet, *Salmonella*/microsome mutagenicity, cell viability, *Daphnia similis* and Microtox® assays. The human hepatoma cell line (HepG2) was used in the comet assay and for cell viability. In the mutagenicity assay, *Salmonella typhimurium* strains with different levels of nitroreductase and *o*-acetyltransferase were used. The dye showed genotoxic effects with respect to HepG2 cells at concentrations of 0.2, 0.4, 1.0, 2.0 and 4.0 μg/mL. In the mutagenicity assay, greater responses were obtained with the strains TA98 and YG1041, suggesting that this compound mainly induces frameshift mutations. Moreover, the mutagenicity was greatly enhanced with the strains overproducing nitroreductase and *o*-acetyltransferase, showing the importance of these enzymes in the mutagenicity of this dye. In addition, the compound induced apoptosis after 72 h in contact with the HepG2 cells. No toxic effects were observed for either *D. similis* or *Vibrio fischeri*.

1. Introduction

Synthetic dyes are extensively used in textile dyeing, paper printing and photography, and in the pharmaceutical, food, cosmetics and petroleum products industries [1]. According to their chemical structure, dyes may be classified into several groups such as azo, anthraquinone, benzodifuranone, quinophthalones and others [2]. Of these, the azo compounds, characterized by the presence of one or more azo groups, are the most used [3,4].

Currently, there are at least 3000 azo dyes in use [5], representing about 60% of the dyes applied throughout the world [6]. In the textile industry, azo dyes are used for coloring wool, cotton, polyester and various other substrates [5].

Pollution of the environment with these compounds causes, apart from visual pollution, changes in the biological cycles mainly affecting processes of photosynthesis [7]. Moreover, non-ionic azo dyes are considered potentially toxic [8] not only because of the dye itself, but also because azo compounds can generate carcinogenic/mutagenic products, such as aromatic amines due to the metabolism of intestinal microflora and/or mammalian azo reduction and chemical reduction [1,5,9–15]. McCann et al. [11] reported a 90% correlation between carcinogenicity and mutagenicity for 61 aromatic amines and azo dyes tested using the *Salmonella*/microsome mutagenicity test [11].

It is important to point out that each dye should be tested individually because the potency of these compounds is strongly dependent on the nature and position of the substituents with respect to both the aromatic rings and the amino nitrogen atom. For example, 3-methoxy-4-aminoazobenzene (3-OMe-AAB) is a potent hepatocarcinogen in rats and a strong mutagen in bacteria, whereas 2-methoxy-4-aminoazobenzene (2-OMe-AAB) is apparently a non-carcinogen and an extremely weak mutagen under similar conditions [16].

The amount of azo dyes produced in the world per year is estimated to be over 10,000 tons [17], with about 2000 types of color available just for the textile industry [18]. About 12% of this amount is lost to waste streams, and of this percentage, 20% is released into the environment [19], since the traditional wastewater treatment technologies have been shown to be ineffective in removing these dyes because of the chemical stability of these pollutants [20]. In this context, it is important to evaluate the azo dyes not only from the human point of view, but also considering the ecotoxic effects.

Considering the lack of studies evaluating the toxic effects of Disperse Orange 1 (Fig. 1), in the present work the genotoxic,

* Corresponding author. Tel.: +55 16 3602 0663.
E-mail addresses: eraquel@fcfrp.usp.br, elisa.raquel@ig.com.br (E.R.A. Ferraz).

0304-3894/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jhazmat.2011.05.063

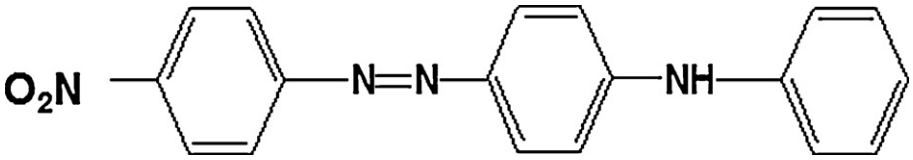

Fig. 1. Chemical structure of the dye Disperse Orange 1.

mutagenic, cytotoxic and ecotoxic effects of this chemical were evaluated using the comet, *Salmonella*/microsome mutagenicity, cell viability and *Daphnia similis* and Microtox® assays, respectively. In addition, the *Salmonella*/microsome mutagenicity assay was also used to study the role of the enzymes nitroreductase, *o*-acetyltransferase, and cytochrome P450 in the metabolic activation of this dye.

2. Materials and methods

2.1. Chemical

The dye Disperse Orange 1 (4-(4-nitrofenilazo)difenilamina); CAS registry no 2581-69-3 purchased from Sigma (St. Louis, USA) (Fig. 1), was analyzed.

2.2. Comet assay

The comet assay is used to detect genomic damage which sometimes can turn into mutation. It has been indicated as a method able to detect very small changes in DNA structure, such as repair activities, the manner of its packing and its integrity [21–23]. In the present work, the comet assay was used with HepG2 cells derived from human hepatoma [24]. This cell line was also used to evaluate the induction of cell death by the azo dye.

The HepG2 cells were obtained from the American Type Culture Collection, No HB 8065. The cell line was cultured for 24 h in Dulbecco's medium (Sigma, St. Louis, USA) supplemented with 10% bovine fetal serum, 100 IU/mL penicillin G, 100 mg/mL streptomycin and 1 μg/mL amphotericin, all from Gibco (Grand Island, USA) at 37 °C, flushed with 5% CO_2 in air.

The comet assay was carried out according to Tice et al. [23] and the concentrations were chosen based on a previous study performed by Chequer et al. [12], in which they used micronucleous assay in HepG2 cells to evaluate the same dye. Briefly, after 4 h of exposure of the HepG2 cells (2×10^5 cells/well) to the dye (0.05 μg/mL, 0.1 μg/mL, 0.2 μg/mL, 0.4 μg/mL, 1.0 μg/mL, 2.0 μg/mL and 4.0 μg/mL dissolved in PBS) or to PBS in different Petri-dishes (60 mm × 15 mm, Greiner), the cells were trypsinized (0.1%, 4 min), suspended, homogenized in 1 mL of medium and centrifuged (10 min, 212 g). Subsequently 100 μL of low melting agarose (Gibco, Grand Island, USA) was added to the cell suspension. They were then transferred to agarose-coated slides and lysed (2.5 M NaCl; 100 mM EDTA; 10 mM Tris–HCl, pH 10; 1% Triton X-100 and 10% DMSO) for 24 h. The slides were placed in the electrophoresis solution (200 mM EDTA + 10 M NaOH) for 20 min and submitted to electrophoresis (300 mA and 1.0 V/cm, 20 min). The slides were then transferred to the neutralization solution (4.85% Tris–HCl, pH 7.5) for 20 min, stained with ethidium bromide (10 μg/mL – Sigma, St. Louis, USA) and examined under a fluorescence microscope (Nikon, Model 027012). All experiments were carried out in triplicate and the length of DNA migration measured in 50 cells per slide (100 cells per treatment). The cells were scored visually into five classes according to tail intensity (from undamaged, 0, to maximum damaged, 4). The damage index (DI) was based on the length of migration in order to evaluate the overall damage caused by the compound, and to compare it with the positive (benzo[a]pyrene, 12.5 μg/mL (BZO), Sigma, St. Louis, USA) and negative (PBS) controls. The DI was calculated as follows:

$$DI = n1 + 2n2 + 3n3 + 4n4,$$

where $n1$ represents the number of cells with level 1 damage; $n2$ the number of cells with level 2 damage; $n3$ the number of cells with level 3 damage; and $n4$ the number of cells with level 4 damage.

In addition, Trypan Blue was used to verify cell viability and was >90% in all experiments.

The statistical analysis was performed using the analysis of variance (ANOVA), followed by Dunnett's Multiple Comparison Test according to Tsuboy et al. [25]. The differences were considered significant when $p < 0.05$.

2.3. Mutagenicity assay

The *Salmonella*/microsome mutagenicity assay or Ames test is widely used to detect chemical mutagens and potential carcinogens [26,27]. In the present work, this assay was also used to determine the role of metabolization on the mutagenic activity of Disperse Orange 1. For this purpose the traditional strains TA98, able to detect frameshift mutations (hisD3052, rfa, Δbio, ΔuvrB, pKM101), TA100, able to detect base pair substitution (hisG46, rfa, Δbio, ΔuvrB, pKM101), and the strains YG1041 and YG1042 (derived from TA98 and TA100, respectively), both of which overproduce nitroreductase and *o*-acetyltransferase, were employed [28]. The combination of these strains can help elucidate the contribution of these enzymes in the mutagenicity of nitroazo dyes.

For the Salmonella mutagenicity assay, the standard pre incubation procedure with and without exogenous metabolic activation (S9), according to Maron and Ames [26] and Mortelmans and Zeiger [27] was employed. The dose of Disperse Orange 1 ranged from 0.05 to 3000 μg/plate based on preliminary assays (data not shown). Briefly, 100 μL overnight cultures of each strain of *Salmonella typhimurium* (TA98, YG1041, TA100, YG1042) (about 10^9 cells/mL), 500 μL of 0.2 M sodium phosphate buffer or S9 mix and 100 μL of the dye concentration were incubated at 37 °C for 30 min without shaking. After incubation, 2 mL of molten top agar was added, the mixture poured onto a minimal agar plate, and the plates incubated at 37 °C for 66 h. Colonies were counted by hand and the background was carefully evaluated.

Dimethylsulfoxide (DMSO) (Merck, Darmstadt, Germany) was used as the negative control. For TA98 and TA100, the positive controls were 4-nitroquinoline-oxide – 4NQO (5 μg/plate, Sigma, St. Louis, USA) and 2-aminoanthracene – 2AA (25 μg/plate, Aldrich, Seelze, Germany). For YG1041, 4-nitro-*o*-phenylenediamine – 4NOP (100 μg/plate, Aldrich, Seelze, Germany) and 2-aminoanthracene – 2AA (0.625 μg/plate Aldrich, Seelze, German). For YG1042 the positive controls were 100 μg/plate of 2-nitrofluorene – 2NF (Aldrich, Seelze, Germany) and 2-aminoanthracene – 2AA (25 μg/plate, Aldrich, Seelze, Germany). The dye and the positive controls were dissolved in DMSO.

Metabolic activation was provided by Aroclor 1254 induced Sprague Dawley rat liver S9 mix (MolTox, Boone, USA), which was prepared at a concentration of 4% (v/v) according to Maron and Ames [26].

The samples were considered positive when a significant ANOVA and dose response was obtained from the Bernstein model [29]. We have chosen this model because the standard method for analyzing Salmonella mutagenicity data based on fold-increases may be too insensitive for Salmonella strains with relatively high reversion frequencies, such as TA100, TA97, and TA102 [30]. Moreover, several studies have used this model to analyze its data [14,31–35]. The results were expressed as the mean of number of revertants per plate ± standard deviation.

All the experiments were performed in triplicate.

2.4. Cell viability (cytotoxicity assay)

Cell viability was determined by the propidium iodide/annexin V assay using flow cytometry and HepG2 cells [36]. This assay assesses the toxicity for the cell and at the same time discriminates between necrotic and apoptotic cell death. The use of annexin V in this cytotoxicity assay offered the possibility of detecting early phases of apoptosis before the loss of cell membrane integrity, and permitted measurement of the kinetics of apoptotic death in relation to the cell cycle [36]. Concentrations of the dye were chosen based on the higher response obtained from Salmonella mutagenicity assay and we decided to test until 72 h of incubation in order to evaluate the cell death induction after longer period, since no effects were observed after 48 h. This exposure time is recommended by some authors in studies of cytotoxicity [37–39].

The cells (1×10^5 cells/well) were exposed to Disperse Orange 1 (2.5 μg/mL, 5.0 μg/mL and 10.0 μg/mL, dissolved in PBS) for 24–72 h at 37 °C and 5% CO_2. The cells were then harvested by trypsinization, washed twice with PBS, re-suspended in the working solution of 5 μg/mL propidium iodide and 0.25 μg/mL annexin V (Becton Dickinson, San Diego, USA), incubated for 15 min at 37 °C, and analyzed with a FACSCanto flow cytometer (Becton Dickinson, San Jose, USA) using Diva software. Approximately 10^5 cells were analyzed after each treatment. Dulbecco's medium was used as the negative control and 100 mM tert-butylhydroperoxide (tBOOH) as the positive control.

The statistical analysis was carried out using one-way ANOVA, assuming equality of variance with the Dunnett's post hoc test for pair-wise comparison. Results with $p < 0.05$ were considered statistically significant.

2.5. Aquatic acute toxicity assay

2.5.1. Aquatic organism cultures

D. similis, obtained from the CETESB Ecotoxicology Laboratory, were maintained at 20 ± 2 °C, with light intensity of 1000 lux, under a 16:8 h (light/dark) photoperiod. The Daphnias were fed daily with the green algae *Selenastrum capricornutum* and a mixture of trout food and yeast [40].

2.5.2. D. similis toxicity assay

The genus Daphnia is comprised of microcrustaceans extensively used in tests to evaluate the acute and chronic toxicity of chemical agents and effluents. Several authors have evaluated *D. similis* as a test species for use in ecotoxicological assays [41–43], showing that this species can be used in ecotoxicological assays as well as *Daphnia magna* [40].

The experiment was performed with 6 different doses (0.001–10 mg/L) based on cell viability assay so that the higher concentration in both assays was 10 μg/mL (equal 10 mg/L) with 4 replicates. For each replicate five young organisms (6–24 h old) were exposed to the dye for 48 h in a 10 mL glass tube. After the 48-h exposure period, the immobilized *Daphnia* were counted. The test was considered valid if the immobilization rate was less than 10% in the negative control group [44]. The results were statistically analyzed using the Spearman trimmed method, which has good statistical properties, is easy to use, and is recommended for accurate and precise calculation of EC50 values and their 95% confidence interval end points [45].

2.5.3. Microtox bioassay

Microtox® is an acute toxicity test based on inhibition of the bioluminescence of the marine bacterium *Vibrio fischeri* NRRL B 11 177 [46]. Since the bioluminescence of *V. fischeri* is directly linked to its respiratory activity, it provides a good indicator of metabolic activity and has been found to be well correlated with several in vivo toxicity tests [47–50].

The acute toxicity tests were performed using the Microtox procedure with the Microbics Model 500 analyzer (AZUR Environmental, Carlsbab, CA) and the procedures outlined in the Microtox Users Manual using the "81.9% Basic Test" protocol [51].

Freeze-dried luminescent bacteria, *V. fischeri*, were reconstituted and exposed to a series of nine diluted dye solutions, osmotically adjusted with 22% sodium chloride (NaCl). The resulting decrease in bioluminescence was measured after 5 and 15 min at a constant temperature of 15 °C. The data for fifteen minutes are here reported, and the toxicity was measured as percent inhibition of light emission of the dye treated aliquot according to Lin and Chao [50], corrected for loss of light in the control as follows:

$$\%\text{inhibition} = 100 \times \frac{I_0 \times I_t}{I_0}$$

where I_0 = initial bacterial luminescence and I_t = luminescence after introduction of the dye into the bacterial suspension. All the Microtox data were recorded and analyzed by MicrotoxOmni on-line software, which is able to calculate percent when only one usable gamma is calculated from data [51], and the results expressed as percent inhibition of light emission [50]. A 2% NaCl solution was used as the negative control and zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$) 100 mg/L as the positive control.

3. Results

3.1. Comet assay

The results of the dose response experiment are shown in Fig. 2. At the lower concentrations of Disperse Orange 1 (0.05 and 0.1 μg/mL), no genotoxic effect was observed for the HepG2 cells. However, a significant increase in the migration of DNA fragments

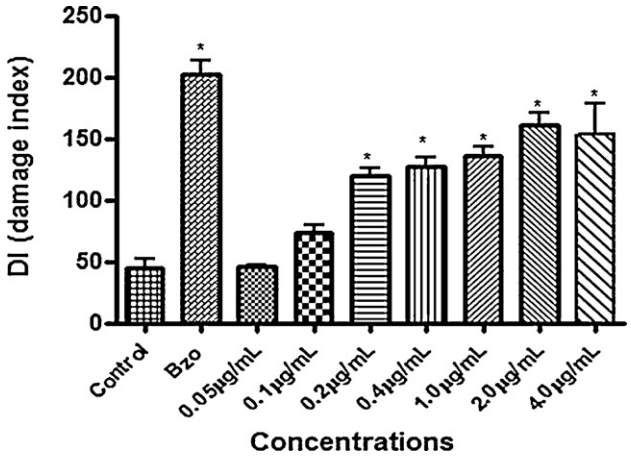

Fig. 2. Genotoxic effect of the dye Disperse Orange 1 for HepG2 cells, detected by comet assay. We analyzed 100 cells from each concentration. Bars represent median ± standard deviation of three repetitions. *Statistical significant $p < 0.01$.

Table 1
Results of the Salmonella/microsome pre incubation assay for the azo dye Disperse Orange 1.

Concentration (μg/plate)	Mean of number of revertants/plate ± standard deviation							
	TA98		YG1041		TA100		YG1042	
	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
0.05	–	–	92.0 ± 4	105.3 ± 5	–	–	–	–
0.1	–	–	115.7 ± 9	118.7 ± 21	–	–	–	–
0.5	–	–	144.0 ± 16*	121.7 ± 6	125.3 ± 8	177.5 ± 18	104.0 ± 16	–
1	–	–	207.3 ± 8**	–	–	–	112.0 ± 5	–
5	18.0 ± 3	–	385.0 ± 41**	238.0 ± 3**	138.3 ± 12	164.3 ± 45	145.3 ± 3**	89.3 ± 6
10	19.3 ± 2	–	–	437.7 ± 14**	–	–	154.7 ± 4**	103.0 ± 6
50	30.7 ± 6	24.7 ± 5	–	–	141.1 ± 14	185.0 ± 23	277.0 ± 13**	114.3 ± 3*
100	41.7 ± 3*	33.0 ± 4	–	–	–	–	297.3 ± 45**	128.3 ± 4**
500	102.3 ± 17**	45.0 ± 2**	–	–	178.7 ± 16*	155.5 ± 8	–	201.3 ± 8**
1000	–	78.0 ± 8**	–	–	–	–	–	–
3000	–	–	–	–	194.0 ± 7**	182.7 ± 22	–	–
Negative control	23.5 ± 6	25.8 ± 3	96.8 ± 11	106.8 ± 15	125.6 ± 14	142.2 ± 13	106.4 ± 7	98.6 ± 6
Positive control	400 ± 28[a]	630 ± 42[b]	846 ± 40[c]	615 ± 7[d]	1910 ± 28[a]	2080 ± 99[b]	1008 ± 18[e]	1535 ± 78[b]
Potency (rev/μg)	0.2	0.05	109	27	0.1	Negative	3.5	0.4

–Not tested.
[a] 4NQO (5 μg/plate).
[b] 2AA (25 μg/plate).
[c] 4NOP (100 μg/plate).
[d] 2AA (0.625 μg/plate).
[e] 2NF (100 μg/plate).
* Significant at 5%.
** Significant at 1%.

was detected for all the other concentrations tested (0.2, 0.4, 1.0, 2.0 and 4.0 μg/mL).

The cell viability in all the dye concentrations was higher than 90% (data not shown).

3.2. Mutagenicity assay

Disperse Orange 1 showed mutagenic activity for strains TA98, YG1041, TA100 and YG1042. The mutagenicity was greatly increased using strain YG1041 and S9 decreased the effect observed in all cases. Under the conditions tested, the dye tested negative for TA100 with S9 (Table 1).

3.3. Cell viability (cytotoxicity assay)

Fig. 3 shows cell death by apoptosis caused by Disperse Orange 1 after 72 h in contact with the HepG2 cells. In the period from 24 to 48 h of incubation, the dye did not induce cell death (data not shown).

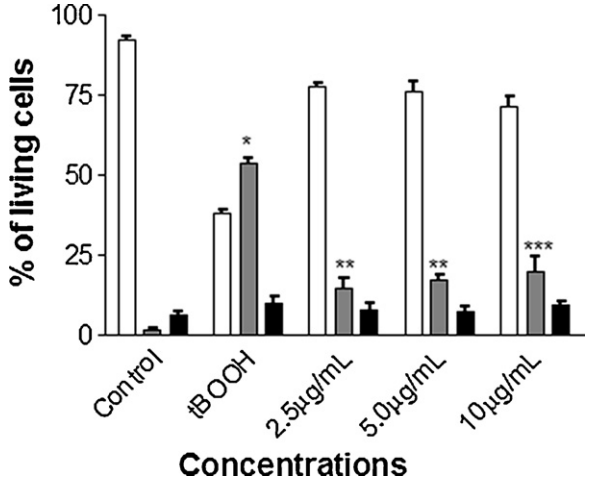

Fig. 3. Cell death induced by different doses of Disperse Orange 1 after 72 h of incubation at different concentrations. Bars represent median ± standard deviation of three repetitions. White bars represent viable cells, gray apoptotic cells and black necrotic ones. Statistically significant $^{*}p < 0.001$, $^{**}p < 0.05$, $^{***}p < 0.01$.

Table 2
Immobilization of *Daphnia similis* caused by Disperse Orange 1.

Disperse Orange 1	Number of immobilized organisms				Immobilization	
	1	2	3	4	Total	%
Control	0	0	0	0	0/20	0
0.001 mg/L	0	0	0	0	0/20	0
0.01 mg/L	0	0	0	0	0/20	0
0.05 mg/L	0	0	0	0	0/20	0
0.1 mg/L	0	0	0	0	0/20	0
1 mg/L	0	1	0	0	1/20	5
10 mg'L	1	1	2	1	5/20	25

3.4. Aquatic acute toxicity assay

3.4.1. D. similis

The dye Disperse Orange 1 did not cause a significant toxic effect on *D. similis*. Table 2 shows the results obtained for this experiment, based on the percent immobilization of these microorganisms as induced by the dye.

Monthly we test the sensibility of *D. similis* using the reference compound potassium chloride (KCl) at 250 mg/L, 500 mg/L, 1000 mg/L and 2000 mg/L. EC50 values are plotted in protocols and if they are out of the range (last EC50 value ± 2 times the standard deviation) the organisms are not used. At the moment of the assay, EC50 value was 792.17 mg/L.

3.4.2. Microtox bioassay

Similar to Daphnia, the dye did not cause significant toxicity with respect to *V. fischeri*. Table 3 shows the inhibition of light emission by *V. fischeri* in the presence of the dye studied for 15 min. A tendency for an increase in % inhibition of light emission was observed, but the effect was not sufficient to calculate the EC50 values. EC50 for positive control was 3.045 mg/L (95% confidence range: 1.952–4.750).

Table 3
Inhibition of light emission by *Vibrio fischeri* caused by the presence of the dye Disperse Orange 1 during 15 min of treatment.

Disperse Orange 1 concentration (mg/L)	Mean of % inhibition of light emission corrected for loss of light in the control
0.03	7.45 ± 2.37
0.06	14.85 ± 3.75
0.128	4.98 ± 2.32
0.21	14.21 ± 1.12
0.51	14.82 ± 0.26
1.02	15.35 ± 9.33
2.05	18.45 ± 4.41
4.1	22.73 ± 2.70
8.19	22.84 ± 0.47

4. Discussion

The comet assay is used routinely for the detection of DNA damage [23]. Disperse Orange 1 showed genotoxic effects on the HepG2 cells at concentrations of 0.2, 0.4, 1.0, 2.0 and 4.0 μg/mL, but it appears that at the highest concentration tested (4.0 μg/mL) the damage response had saturated despite cell viability being around 90%, which might be due to cytotoxic effects. During evaluation of the slides, an increase in completely fragmented cells was observed at the higher doses, which might suggest that the cytotoxic effects were starting to occur and the Trypan Blue assay was not sensitivity enough to detect it. Chequer et al. [12] observed a similar phenomenon for the micronucleus assay of azo dyes, using the same cell line.

This cytotoxic effect was confirmed by the results obtained in the cell death assay. The results showed that Disperse Orange 1 induced apoptosis after 72 h of exposure at doses above 2.5 μg/mL. It is important to point out that in the comet assay the cell exposure was 4 h, whilst in this assay, the exposure period was 72 h.

The dye Disperse Orange 1 was mutagenic for the strains TA98, YG1041, TA100 and YG1042 (Table 1). This suggests that the dye is able to induce both base pair substitutions and frameshift mutations. However, comparing the mutagenic potencies detected with the different strains tested, the hypothesis was raised that this dye induces mainly frameshift mutations.

Moreover, the mutagenic potency was about 545 times higher for the strain YG1041 than for TA98 (Table 1), clearly demonstrating the importance of nitroreduction and acetylation in the mutagenicity of this dye, since YG1041 is able to produce high levels of nitroreductase and *o*-acetyltransferase. The mechanism of mutagenicity of these products could be nitroreduction by nitroreductases forming *N*-hydroxylamines that could induce the DNA damage [52]. The role of nitroreductase in the metabolism and bioactivation of 1-nitropyrene (1-NP) by intestinal flora has been well established. Nitroreduction can occur as a two-electron transfer (nitro-nitroso-hydroxylamine-amino) or through one-electron transfers, forming an anion free radical intermediate [53]. Considering that the response detected for YG1041 was much higher than for TA98, the product of the nitroreductase (probably a hydroxylamine) may have been acetylated by the *o*-acetyltransferase, generating very reactive species. According to a study carried out by Watanabe et al. [54] using the Salmonella strain YG1024, *o*-acetyltransferase is not specific for oxygen and can act on the amine group leading to the production of reactive species.

With all the strains, the addition of exogenous metabolic activation (S9 mixture) decreased the mutagenicity of the dye. The action of the P450 isoforms probably generated more stable products, less likely to interact with DNA. According to Chung and Cerniglia [55], sulfonation, carboxylation, deamination or substitution of an ethyl alcohol or acetyl group for the hydrogen in the amino group will lead to a decrease or diminishing of mutagenic activity, which could explain the decrease in mutagenic activity after treatment with S9.

The present results show that Disperse Orange 1 is a mutagenic dye. Other authors have shown that azo dyes can cause harmful effects, mainly for the genetic material. The genotoxicity of 24 azo compounds selected from the IARC (International Agency for Research on Cancer), groups 2A, 2B, and 3 was determined by the comet assay in eight mouse organs. For 17 azo compounds, the assay was positive for at least one organ [56]. The azo dye Sudan I showed a genotoxic effect with HepG2 cells using the comet and micronucleous assays [57].

Disperse Orange 1 did not cause acute toxicity to both *D. similis* or *V. fischeri*, what can be attributed to its chemical structure, since some dyes have shown to be toxic to *D. magna*. In a study performed by Bae and Freeman [58], they found that copper-complexed dyes and Direct Blue 218 were very toxic to daphnids. The authors suggest that heavy metals like copper molecules inside dye structure play an important role for the evaluation of aquatic toxicity of dye solutions. In other study, Wang et al. [59] evaluated reactive dyestuffs and found that the most toxic azo dye was Ambifix yellow VRNL, and the least toxic one was Procion blue HERD.

5. Conclusions

The dye Disperse Orange 1 induces damage in DNA, mainly frameshift mutations. The enzymes nitroreductase, and *o*-acetyltransferase were shown to be very important in the mutagenicity of this dye. In addition, this compound causes cell death by apoptosis and DNA breakage in the human cell HepG2. No toxic effects were detected for *D. similis* and *V. fischeri*, but it is important to add that other ecotoxic tests should be done in order to evaluate the impact of this dye on the biota. Thus the results of this study emphasize the need for the development of non mutagenic dyes and for investment in new methods for the treatment of effluents, in order to prevent the possibly deleterious effects of these compounds for both humans and aquatic organisms.

Disclosure statement

The authors declare that there are no conflicts of interest.

Acknowledgments

The authors thank Célia Maria Rech, Ana Cristina Morseli Polizello, Maristela Rodini Luiz Ferraz, Eduardo Angelino Savazzi and CETESB for their technical assistance; and FAPESP and CAPES for the financial support of this study.

References

[1] H. Zollinger, Color-Chemistry-Synthesis: Properties and Applications of Organic Dyes and Pigments, VCH Publishers, New York, 1987.
[2] K. Othmer, Dyes and dye intermediates Encyclopedia of Chemical Technology, John Wiley, Sons, United States of America, 1993, pp. 542–753.
[3] A. Stolz, Basic applied aspects in the microbial degradation of azo dyes, Appl. Microbiol. Biotechnol. 56 (2001) 69–80.
[4] E.A. Sweeney, J.K. Chipman, S.J. Forsythe, Evidence for direct-acting oxidative genotoxicity by reduction products of azo dyes, Environ. Health Perspect. 102 (1994) 119–122.
[5] K. Hunger, On the toxicology and metabolism of azo dyes, Chimia 48 (1994) 520–522.
[6] C.M. Carliell, S.J. Barclay, N. Naidoo, C.A. Buckley, D.A. Mulholland, E. Senior, Microbial decolourisation of a reactive azo dye under anaerobic conditions, Water S.A. 21 (1995) 61–69.
[7] A. Kunz, P.P. Zamora, S.G. Moraes, N. Durán, Novas tendências no tratamento de efluentes têxteis, Quim. Nova 25 (2002) 78–82.
[8] C. Novotný, N. Dias, A. Kapanen, K. Malachová, M. Vándrovcová, M. Itävaara, N. Lima, Comparative use of bacterial, algal and protozoan tests to study toxicity of azo- and anthraquinone dyes, Chemosphere 63 (2006) 1436–1442.

[9] K.T. Chung, S.T. Stevens Jr., Degradation of azo dyes by environmental microorganisms and helminthes, Environ. Toxicol. Chem. 12 (1993) 2121–2132.
[10] T.F.X. Collins, J. McLaughlin, Teratology Studies on Food Colourings. Part I. Embryotoxicity of Amaranth (FD & C Red No. 2) in Rats, Food Cosmet. Toxicol. 10 (1972) 619–624.
[11] J. McCann, E. Choi, E. Yamasaki, B.N. Ames, Detection of carcinogens as mutagens in the Salmonella/microsome test: assay of 300 chemicals, Proc. Natl. Acad. Sci. U.S.A. 72 (1975) 5135–5139.
[12] F.M.D. Chequer, J.P.F. Angeli, E.R.A. Ferraz, M.S. Tsuboy, J.C. Marcarini, M.S. Mantovani, D.P. Oliveira, The azo dyes Disperse Red 1 and Disperse Orange 1 increase the micronuclei frequencies in human lymphocytes and in HepG2 cells, Mutat. Res. 676 (2009) 83–86.
[13] E.R.A. Ferraz, G.A. Umbuzeiro, G. De-Almeida, A. Catolo-Oliveira, F.M.D. Chequer, M.V.B. Zanoni, D.J. Dorta, D.P. Oliveira, Differential toxicity of Disperse Red 1 and Disperse Red 13 in the Ames test, HepG2 cytotoxicty assay, and Daphnia acute toxicity test, Environ. Toxicol., in press.
[14] G.A. Umbuzeiro, H. Freeman, S.H. Warren, F. Kummrow, L.D. Claxton, Mutagenicity evaluation of the commercial product C.I. Disperse Blue 291 using different protocols of the *Salmonella* assay, Food Chem. Toxicol. 43 (2005) 49–56.
[15] R. Carita, M.A.M. Morales, Induction of chromosome aberrations in the *Allium cepa* test system caused by the exposure of seeds to industrial effluents contaminated with azo dyes, Chemosphere 72 (2008) 722–725.
[16] M. Kojima, M. Degawa, Y. Hashimoto, M. Tada, Different effects of DNA adducts induced by carcinogenic and noncarcinogenic azo dyes on in vitro DNA synthesis, Biochem. Biophys. Res. Commun. 179 (1991) 817–823.
[17] E. Forgacs, T. Cserháti, G. Oros, Removal of synthetic dyes from wastewaters: a review, Environ. Int. 30 (2004) 953–971.
[18] C.C.I. Guaratini, M.V.B. Zanoni, Textile dyes, Quim. Nova 23 (2000) 71–78.
[19] M.P. Urquiza, R. Ferrer, J.L. Beltrán, Determination of sulfonated azo dyes in river water samples by capillary zone electrophoresis, J. Chromatogr. A 883 (2000) 277–283.
[20] G.M. Shaul, T.J. Holdsworth, C.R.K.A. Dempsey Dostal, Fate of water soluble azo dyes in the activated sludge process, Chemosphere 22 (1991) 1117–1191.
[21] A.M.M.C. Gontijo, R. Tice, Teste do cometa para detecção de dano no DNA e reparo em células individualizadas, in: L.R. Ribeiro, D.M.F. Salvadori, E.K. Marques (Eds.), Mutagênese Ambiental, Ulbra, Canoas, 2003, pp. 173–200.
[22] N.P. Singh, M.T. McCoy, R.R. Tice, E.L. Schneider, A simple technique for quantitation of low levels of DNA damage in individual cells, Exp. Cell Res. 175 (1988) 184–191.
[23] R.R. Tice, E. Agurell, D. Anderson, B. Burlnson, A. Hartmann, H. Kobayaashi, Y. Miyamae, E. Rojas, J.C. Ryu, Y.F. Sasaki, Single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing, Environ. Mol. Mutagen. 35 (2000) 206–221.
[24] S. Knasmüller, W. Parzefall, R. Sanyal, S. Ecker, C. Schwab, M. Uhl, V. Mersch-Sundermann, G. Williamson, G. Hietsch, T. Langer, F. Darroudi, A.T. Adayapalam, Use of metabolically competent human hepatoma cells for the detection of mutagens and antimutagens, Mutat. Res. 402 (1998) 185–202.
[25] M.S. Tsuboy, J.P.F. Angeli, M.S. Mantovani, S. Knasmuller, G.A. Umbuzeiro, L.R. Ribeiro, Genotoxic, mutagenic and cytotoxic effects of the commercial dye CI Disperse Blue 291 in the human hepatic cell line HepG2, Toxicol. In Vitro 21 (2007) 1650–1655.
[26] D.M. Maron, B.N. Ames, Revised methods for the *Salmonella* mutagenicity test, Mutat. Res. 113 (1983) 173–214.
[27] K. Mortelmans, E. Zeiger, The Ames *Salmonella*/microsome mutagenicity assay, Mutat. Res. 455 (2000) 29–60.
[28] Y. Hagiwara, M. Watanabe, Y. Oda, T. Sofuni, T. Nohmi, Specificity and sensitivity of *Salmonella typhimurium* YG1041 and YG1042 strains possessing elevated levels of both nitroreductase and acetyltransferase activity, Mutat. Res. 291 (1993) 171–180.
[29] L. Bernstein, J. Kaldor, J. McCann, M.C. Pike, An empirical approach to the statistical analysis of mutagenesis data from Salmonella test, Mutat. Res. 97 (1982) 267–281.
[30] N.F. Cariello, W.W. Piegorsch, The Ames test: the two-fold rule revisited, Mutat. Res. 369 (1996) 23–31.
[31] M. Cerná, D. Pochmanová, A. Pastorková, I. Benes, J. Lenıcek, J. Topinka, B. Binková, Genotoxicity of urban air pollutants in the Czech Republic. Part I. Bacterial mutagenic potencies of organic compounds adsorbed on PM10 particulates, Mutat. Res. 469 (2000) 71–82.
[32] K.T. Chung, T.J. Hughes, L.D. Claxton, Comparison of the mutagenic specificity induced by four nitro-group-containing aromatic amines in *Salmonella typhimurium* his genes, Mutat. Res. 465 (2000) 165–171.
[33] M.I.Z. Sato, G.U. Valent, C.A. Coirnbrão, M.C.L.S. Coelho, P.S. Sanchez, D.C. Alonso, M.T. Martins, Mutagenicity of airborne particulate organic material from urban and industrial areas of São Paulo, Brazil, Mutat. Res. 335 (1995) 317–330.
[34] F. Kummrow, C.M. Rech, C.A. Coimbrão, G.A. Umbuzeiro, Blue rayon-anchored technique/Salmonella microsome microsuspension assay as a tool to monitor for genotoxic polycyclic compounds in Santos estuary, Mutat. Res. 609 (2006) 60–67.
[35] R.O.A. Lima, A.P. Bazo, D.M.F. Salvadori, C.M. Rech, D.P. Oliveira, G.A. Umbuzeiro, Mutagenic and carcinogenic potential of a textile azo dye processing plant effluent that impacts a drinking water source, Mutat. Res. 626 (2007) 53–60.
[36] I. Vermes, C. Haanen, H. Steffens-Nakken, C. Reutelingsperger, A novel assay for apoptosis Flow cytometric detection of phosphatidylserine early apoptotic cells using fluorescein labeled expression on Annexin V, J. Immunol. Methods 184 (1995) 39–51.
[37] M.F.A. Bakar, M. Mohamad, A. Rahmat, S.A. Burr, J.R. Fry, Cytotoxicity, cell cycle arrest, and apoptosis in breast cancer cell lines exposed to an extract of the seed kernel of *Mangifera pajang* (bambangan), Food Chem. Toxicol. 48 (2010) 1688–1697.
[38] M. Gorduysus, N. Avcu, O. Gorduysus, A. Pekel, Y. Baran, F. Avcu, A.U. Ural, Cytotoxic effects of four different endodontic materials in human periodontal ligament fibroblasts, J. Endod. 33 (2007) 1450–1454.
[39] F.G.E. Perabo, G. Landwehrs, C. Frössler, D.H. Schmidt, S.C. Mueller, Antiproliferative and apoptosis inducing effects of indirubin-3-monoxime in renal cell cancer cells, Urol. Oncol., in press.
[40] S.V. Buratini, E. Bertoletti, P.A. Zagatto, Evaluation of *Daphnia similis* as a test species in ecotoxicological assays, Bull. Environ. Contam. Toxicol. 73 (2004) 878–882.
[41] P.S. De-Carvalho, E. Zanardini, S.V. Buratini, M.C. Lamparelli, M.C. Martins, Oxidizing effect on metal remobilization and *Daphnia similis* toxicity from a Brazilian reservoir sediment suspension, Water Res. 32 (1998) 193–199.
[42] M.C. Liu, C.M. Chen, H.Y. Cheng, H.Y. Chen, Y.C. Su, T.Y. Hung, Toxicity of different industrial effluents in Taiwan: a comparison of the sensitivity of *Daphnia similis* and Microtox®, Environ. Toxicol. 17 (2002) 93–97.
[43] R.B. Sotero-Santos, O. Rocha, J. Povinelli, Evaluation of water treatment sludges toxicity using the Daphnia bioassay, Water Res. 39 (2005) 3909–3917.
[44] OECD, 2004. Daphnia sp. Acute Immobilisation Test. OECD Guideline for testing of Chemicals, no. 202, adopted:13/04/2004.
[45] M.A. Hamilton, R.C. Russo, R.V. Thurston, Trimmed Spearman–Karber for estimating median lethal concentrations in toxicity bioassays, Environ. Sci. Technol. 11 (1977) 714–719.
[46] V.L.K. Jennings, M.H.R. Brandes, D. Bird, Assessing chemical with the bioluminescent photobacterium (*Vibrio fischeri*): a comparison of three commercial systems, Water Res. 35 (2001) 3448–3456.
[47] S.A. Buton, R.V. Petersen, S.N. Dickman, J.R. Nelson, Comparison of in vitro bacterial bioluminescence and tissue culture bioassays and in vivo tests for evaluating acute toxicity of biomaterials, J. Biomed. Mater. Res. 20 (1986) 827–838.
[48] A.A. Bulich, K.K. Tung, G. Scheibner, The luminescent bacteria toxicity test: it's potential as an in vitro alternative, J. Biolumin. Chemilumin. 5 (1990) 71–77.
[49] K.L.E. Kaiser, M.B. McKinnon, F.L. Fort, Interspecies toxicity correlations of rat, mouse and photobacterium phosphoreum, Environ. Toxicol. Chem. 13 (1994) 1060–1599.
[50] T.C Lin, M.R. Chao, Assessing the influence of methanol-containing additive on biological characteristics of diesel exhaust emissions using microtox and mutatox assays, Sci. Total Environ. 284 (2002) 61–74.
[51] Azur EnvironmentaL, Microtox Acute Toxicity Test, 1998, pp. 1–23.
[52] V.M. Arlt, H. Glatt, E. Muckel, U. Papel, B.L. Sorg, H. Schmeiser, D.H. Phillips, Metabolic activation of the environmental contaminant 3-nitrobenzanthrone by human acetyltransferases and sulfotransferase, Carcinogenesis 23 (2002) 1937–1945.
[53] R.W. Chadwick, S.E. George, L.D. Claxton, Role of the gastrointestinal mucosa and microflora in the bioactivation of dietary and environmental mutagens or carcinogens, Drug Metab. Rev. 24 (1992) 425–492.
[54] M. Watanabe, T. Nohmi, M. Ishidate Jr., New tester strains of *Salmonella typhimurium* highly sensitive to mutagenic nitroarenes, Biochem. Biophys. Res. Commun. 147 (2005) 974–979.
[55] K.T. Chung, C.E. Cerniglia, Mutagenicity of azo dyes: structure–activity relationships, Mutat. Res. 277 (1992) 201–220.
[56] S. Tsuda, N. Matsusaka, H. Madarame, S. Ueno, N. Susa, K. Ishida, N. Kawamura, K. Sekihashi, Y.F. Sasaki, The comet assay in eight mouse organs: results with 24 azo compounds, Mutat. Res. 465 (2000) 11–26.
[57] Y. An, L. Jiang, J. Cao, C. Geng, L. Zhong, Sudan I induces genotoxic effects and oxidative DNA damage in HepG2 cells, Mutat. Res. 627 (2007) 164–170.
[58] J.S. Bae, H.S. Freeman, Aquatic toxicity evaluation of copper-complexed direct dyes to the *Daphnia magna*, Dyes Pigments 73 (2007) 126–132.
[59] C. Wang, A. Yediler, D. Lienert, Z. Wang, A. Kettrup, Toxicity evaluation of reactive dyestuffs, auxiliares and selected effluents in textile finishing industry to luminescent bacteria *Vibrio fischeri*, Chemosphere 46 (2002) 339–344.