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The contribution of azo dyes to the mutagenic activity of the Cristais River

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Abstract

To verify whether dyes emitted within the discharge of a dye processing plant were contributing to the mutagenicity repeatedly found in the Cristais River, São Paulo, Brazil, we chemically characterized the following mutagenic samples: the treated industrial effluent, raw and treated water, and the sludge produced by a Drinking Water Treatment Plant (DWTP) located ~6 km from the industrial discharge. Considering that 20% of the dyes used for coloring activities might be lost to wastewaters and knowing that several dyes have mutagenic activity, we decided to analyze the samples for the presence of dyes. Thin layer chromatographic analysis indicated the presence of three prevalent dyes in all samples, except for the drinking water. This combination of dyes corresponded to a commercial product used by the industry, and it tested positive in the Salmonella assay. The structures of the dye components were determined using proton magnetic resonance and mass spectrometric (MS) methods, and the dyes were tested for mutagenicity. The blue component was identified as the C.I. Disperse Blue 373, the violet as C.I. Disperse Violet 93, and the orange as C.I. Disperse Orange 37. The dyes showed mutagenic responses of 6300, 4600, and 280 revertants/μg for YG1041 with S9 respectively. A bioassay-directed fractionation/chemical analysis showed that the C.I. Disperse Blue 373 contributed 55% of the mutagenic activity of the DWTP sludge. We showed that these dyes contributed to the mutagenic activity found

Abbreviations: ANOVA = analysis of variance; BDCP = black dye commercial product; CYP = cytochrome P450 isozymes; DMSO = dimethylsulfoxide; DWTP = drinking water treatment plant; HPLC = high performance liquid chromatography; NMR = nuclear magnetic resonance; non-Cl-PBTAs = non-chlorinated 2-phenyltriazoles; PBTAs = chlorinated 2-phenylbenzotriazoles; Rf = reference factor; TLC = thin layer chromatography; UV = ultraviolet; XAD4 = copolymer of styrene divinyl benzene.

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in the Cristais River environmental samples analyzed and are indirectly affecting the quality of the related drinking water. Therefore, we believe that this type of discharge should be more thoroughly characterized chemically and toxicologically. Additionally, human and ecological risks associated with the release of dye processing plant effluents should be more fully investigated, especially where the resultant water is taken for human consumption.
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1. Introduction

A review of the mutagenicity of effluents showed that textile and other dye-related industries produce consistently genotoxic effluents that are in some cases potent when compared to other industrial discharges (Houk, 1992). In the metropolitan area of São Paulo, Brazil, Sanchez et al. (1988) found that wastewater from several textile industries gave the most positive mutagenic responses (nine positive results in 12 samples) among several types of industrial effluents analyzed. Likewise, Coelho et al. (1992) showed that a dye processing plant discharge was one of the major contributors to the mutagenic activity found in the Paraiba River in São Paulo State (Valent et al., 1993). In this study, the authors speculated that dyes could be causing the mutagenic activity although analysis for the presence of dyes was not performed. More recently, others found that untreated textile wastewater was contributing to the contamination of ground water in India (Rajaguru et al., 2002). In this case, dyes and/or their cleavage products as well as impurities and auxiliaries associated with textile wet processing were also speculated to be responsible for the observed mutagenicity. Unfortunately, none of the studies cited above chemically characterized the mutagenic samples so as to prove which compounds were causing the observed mutagenicity.

When a dye is used in the textile dyeing process, a portion does not attach to the fibers thus remaining in the dye baths. The resultant wastewater is usually treated in an activated sludge plant, and the liquid effluent is released to adjacent surface waters. A study performed in 1989 showed that the commercial aminoazobenzene dye, C.I. Disperse Blue 79, is not degraded in a conventionally operated activated sludge process and 85% of the dye remains in the system. Of this 85%, 3% is retained by the primary sludge, 62% by the activated sludge and 20% is found in the final, environmentally released liquid effluent (US EPA, 1989). If an anaerobic system is employed before the activated sludge treatment, cleavage of azo bonds can occur, and the release of the related aromatic amines is observed. Ekici et al. (2001) tested the stability of selected azo dye metabolites in activated sludge and in water. They concluded that these dye metabolites were relatively stable in the aquatic environment and could not be efficiently degraded under the wastewater plant conditions. In regard to mutagenicity, Fracasso et al. (1992) showed that dye factory effluents from primary and secondary biological treatment exhibited increased levels of mutagenic activity when compared with the raw (untreated) effluent. The use of activated carbon filtration was beneficial but did not completely remove the mutagenic activity of the final effluent.

According to Arslan et al. (1999), 20% of the dyes used for coloring activities might be lost to wastewater, but only two published studies were found that demonstrated the presence of dyes in the aquatic environment. Garrison and Hill (1972) found three anthraquinone blue disperse dyes associated with textile discharges in the Savannah River (USA). A study of the Yamaska River in Quebec, Canada detected 15 dyes in the river water, in suspended solids, and in sediments downstream of textile mills (Maguire, 1992). The identified dyes included C.I. Disperse Red 60, C.I. Disperse Blue 26, and C.I. Disperse Blue 79. In addition, a mutagenic reductive cleavage product and synthetic precursor of C.I. Disperse Blue 79 (2-bromo-4,6-dinitroaniline) was identified in the sediment at concentrations of 0.1–1.9 mg/kg, about 6 km downstream of the discharge. In the aquatic environment, because azo disperse dyes are hydrophobic compounds, they are expected to be adsorbed on the sediment and not be found in the water column. However, because they are combined with dispersing agents, commercial disperse dye products are water-soluble. For this reason, the hydrophilic properties of the commercial products will be much higher than the dyes themselves. This allows an increased presence of dyes in waters that receive effluents from textile processing plants employing such dyes.

Michaels and Lewis (1985) recognized that studies about the chemical characterization of textile dye industrial wastes are scarce even though dyes are present in significant amounts in the aquatic environment. According to the authors, this is because the dyes are often composed of mixtures of compounds with unknown or unpublished chemical structures.

The Salmonella microsome mutagenicity assay is one of the protocols routinely used in the São Paulo State

(Brazil) surface water quality monitoring program (CETESB, 2003). In this program, Cristais River, where water is treated for drinking water purposes, has repeatedly revealed low to moderate levels of mutagenic activity according to the classification of Umbuzeiro et al. (2001). Results of the Salmonella assay, using standard as well as nitroreductase and *O*-acetyltransferase overproducing strains, combined with different extraction procedures indicated that nitroaromatics and/or aromatic amines were causing the mutagenicity of the environmental samples analyzed. The origin of the contamination could be attributed to a textile wet processing plant that discharged its dye effluent into the local river (Umbuzeiro et al., 2004).

Therefore, the objective of the present work was to verify the possible contribution of dyes used by the textile plant to the mutagenic activity already detected in the Cristais River using chemical analysis and mutagenicity assays. Accordingly, we used the following strategy:

- verification of the presence of representative dyes in environmental samples collected in the Cristais River using thin layer chromatography;
- evaluation of the mutagenic activity of the dyes found in the environmental samples using the Salmonella/microsome assay;
- determination of the chemical structures of the dyes found in the samples, using nuclear magnetic resonance (MNR) and mass spectrometry; and
- independent confirmation and quantification of the dyes' contribution to the mutagenic activity of the environmental samples tested using bioassay-directed fractionation/chemical analysis.

2. Materials and methods

2.1. Salmonella/microsome mutagenicity assay

We collected the following samples: the azo dye processing plant treated effluent, untreated Cristais River water, treated water from the DWTP (located ~6 km after the industrial effluent discharge), and the DWTP sludge. For the liquid samples, natural pH and acidic pH organic extracts were prepared using XAD4 (Sigma–Aldrich, St. Louis, MO, USA) as described by Umbuzeiro et al. (2004) and for the DWTP sludge, the extraction was performed by ultrasonication, also as described by Umbuzeiro et al. (2004). Those extracts were assayed for mutagenicity using metabolic activation with the strain YG1041 (HisD3052, rfa, Δbio, ΔuvrB, pKM101), a nitroreductase and *O*-acetyltransferase overproducer (Hagiwara et al., 1983). This strain is derived from the Salmonella strain TA98 (Maron and Ames, 1983). The method employed was the microsuspension assay (Kado et al., 1983). Previous tests indicated that this strain and condition was the most sensitive for the detection of the mutagenic activity of similar samples (Umbuzeiro et al., 2004). The metabolic activation was provided by Aroclor 1254 induced Sprague Dawley rat liver S9 mix (MolTox, Boone, NC) which was prepared at a concentration of 4% v/v. The positive controls were 3 μg/plate of 2-nitrofluorene (Sigma–Aldrich, St. Louis, MO, USA) and 0.5 μg/plate of 2-aminoanthracene (Sigma–Aldrich, St. Louis, MO, USA), both dissolved in dimethylsulfoxide (DMSO) (Sigma–Aldrich, St. Louis, MO, USA). Samples were considered positive when a significant ANOVA and significant positive dose response were obtained. To calculate the potency in number of revertants per μg of compound, liter-equivalent of water, or per gram-equivalent of sludge, we used the Bernstein model (Bernstein et al., 1982).

The black dye commercial product (BDCP), as well as the three dye BDCP's components were tested for mutagenicity. This product was provided by the industry and was chosen because of its wide use in the cited processing plant process. The BDCP and its dye components' solutions were prepared at 1 mg/ml concentration and the doses tested were 200 μg, 100 μg, 20 μg, 2 μg, and 0.2 μg per plate in DMSO. The strains used were TA98 (HisD3052, rfa, Δbio, ΔuvrB, pKM101), TA100 (HisG46, rfa, Δbio, ΔuvrB, pKM101), and YG1041 in the presence and absence of S9. In this case, we used the plate incorporation method (Maron and Ames, 1983).

2.2. Chemical analysis

2.2.1. Thin layer chromatography analysis

Initially, we performed a thin layer chromatography (TLC) on the BDCP. The product (1 mg) was dissolved in methanol (Merck KgaA, Darmstadt, Germany) (0.2 ml) and an aliquot of the solution was spotted onto TLC plates (Whatman PE SIL G/UV, cat #4410222, 250 μm layer, 20 × 20 cm) about 0.5 cm from the lower edge of the TLC plate. The plate was developed in a standard TLC chamber containing about 4.5 ml of toluene (Sigma–Aldrich, St. Louis, MO, USA):ethyl acetate (Merck KgaA, Darmstadt, Germany) (8:1).

The presence of the BDCP was investigated in the mutagenic organic extracts of the treated dye processing plant effluent, raw river water at the drinking water treatment plant (DWTP), drinking water, and the sludge generated in the DWTP using TLC. As chromatographic controls, we used 1 mg of the BDCP dissolved in 0.2 ml of ethyl acetate. Volumes of 10 μl of the BDCP solution and methanol extracts of the environmental samples were spotted onto TLC plates. This volume contained the equivalent amounts of: 0.05 mg of BDCP,

100 ml of treated effluent, 500 ml of raw and treated water, and 200 mg of the sludge. To develop the TLC plates we used 2 ml of a mixture of toluene:ethyl acetate (10:1). After development of the plates, the dyes were detected by visual inspection and reference factor (Rf) values were recorded. For the evaluation of the presence of fluorescent compounds we irradiated the developed plates with short wave UV light (254 nm) and visually observed the spots that showed fluorescence.

2.2.2. Nuclear magnetic resonance and mass spectrometry analysis

The colored components of BDCP were separated using a 2.5×120 cm nylon membrane column packed with silica gel (Fisher Scientific Company, USA). To achieve the separation, 3.5 g of the commercial dye was applied to the top of the column and developed using toluene:ethyl acetate (8:1). After complete development, the column was sliced into sections to isolate the different colored bands. The silica gel sections from individual bands were extracted in a Soxhlet apparatus using methylene chloride and the resultant solids were dried in vacuum at 40 °C. The identification of the components of BDCP was performed using ^{1}H NMR and chemical ionization (CI) mass spectrometric analyses (MS). ^{1}H NMR spectra were recorded on a Bruker 500 MHz spectrometer. M^+ ions were produced by high-resolution mass measurement using 70 eV electron impact (EI) ionization. Mass spectra were recorded using a Jeol HX110 double-focusing mass spectrometer (Foris, 1977; Van Breemen, 1995).

2.3. Bioassay-directed fractionation/chemical analysis

This part of the study was performed in order to confirm and quantify which compounds were the major contributors to the mutagenic activity found in the environmental assays, in an independent experiment. The group that developed this part of the research was unaware of our findings about the presence of the dyes using the TLC methodology at the time they started the work. Unfortunately, only the extract of the sludge generated by the Cristais River DWTP was concentrated enough to be analyzed by a bioassay-directed fractionation/chemical analysis technique, because this technique employs subsequent fractionation procedures where the loss of material is quite substantial. The mutagenicity assays were performed with the Salmonella strain YG1024 (HisD3052, rfa, Δbio, ΔuvrB, pKM10, *O*-acetyltransferase overproducer) (Watanabe et al., 1990) in the presence of S9. YG1024 is very similar to YG1041, except it lacks the overproduction of nitroreductase, containing the same nitroreductase levels of the parental strain TA98. The strain YG1024 was chosen because this methodology was previously validated with this strain and the mutagenicity results compared well with that of YG1041 (results not shown). The chemical 2-[(2-bromo-4,6-dinitrophenyl)azo]-5-(diallylamino)-4-methoxyacetanilide (C.I. Disperse Blue 373, CAS registry no. 51868-46-3), used for the confirmation step of the compound found in the samples analyzed, was synthesized by the method reported previously (Watanabe et al., 2002) and used as a standard in the chemical analysis.

The sludge extract was fractionated into 25 fractions (A1–A25) using a Sephadex LH-20 column (20×400 mm) and a mixture of methanol:chloroform (1:1) at 10 ml/fraction was used for elution (Fig. 1). The most mutagenic fractions (A8–A10) were fractionated into 104 fractions (B1–B104) using Ultra Pack ODS column (26×300 mm). For the fractions B1–B81 the elution was done with 75% acetonitrile and for the fractions B82–B104, 100% acetonitrile, both at 6 ml/fraction. For the most mutagenic fraction (B101) an YMC-Pack ODS-AM324 column was used, and 91 fractions were obtained (C1–C91), with 90% methanol for 0–20 min, 90–100% methanol for 20–60 min, and then 100% methanol (3 ml/fraction). Fractions C39 and C40 were fractionated into 91 fractions (D1–D91) on a Luna 5 m phenyl–hexyl column (10×250 mm): 0–50 min 70% acetonitrile; 50–70 min 70–100% acetonitrile and 70 min 100% acetonitrile, at 3 ml/fraction. Fig. 1 summarizes the steps of this technique. The UV-absorption spectrum of the compound in the most mutagenic fractions, D31 and D32, was recorded using a Shimadzu SPD-M10Avp photodiode array detector. Moreover, the compound in D31 and D32 was analyzed using an Agilent 1100 HPLC system coupled with electrospray ionization-mass spectrometry (ESI-MS) and a LCQ Advantage instrument (Thermo Electron Company). The aliquot was injected into a HY-Purity C18 column (2.1×150 mm) and eluted with 80% aq. methanol in 1% acetic acid at a flow rate of 0.35 ml/min. Negative ion mode (spray voltage: 4.5 kV; sheath gas: 50 arb, auxiliary gas: 20 arb; capillary temperature 230 °C) was employed for the MS analysis. Mass spectra and retention times for the compound in the fractions D31 and D32 were compared to those of C.I. Disperse Blue 373. In addition, the presence of PBTAs (chlorinated 2-phenylbenzotriazoles) and non-Cl-PBTAs (non-chlorinated 2-phenyltriazoles) was evaluated in the most mutagenic fractions by comparison of UV absorption spectra peaks.

3. Results and discussion

3.1. Salmonella/microsome mutagenicity assay

3.1.1. Environmental samples

The results for the strain YG1041 in the presence of S9 were very similar to the ones obtained by Umbuzeiro et al. (2004): 3×10^5 revertants/l-equivalent for the treated

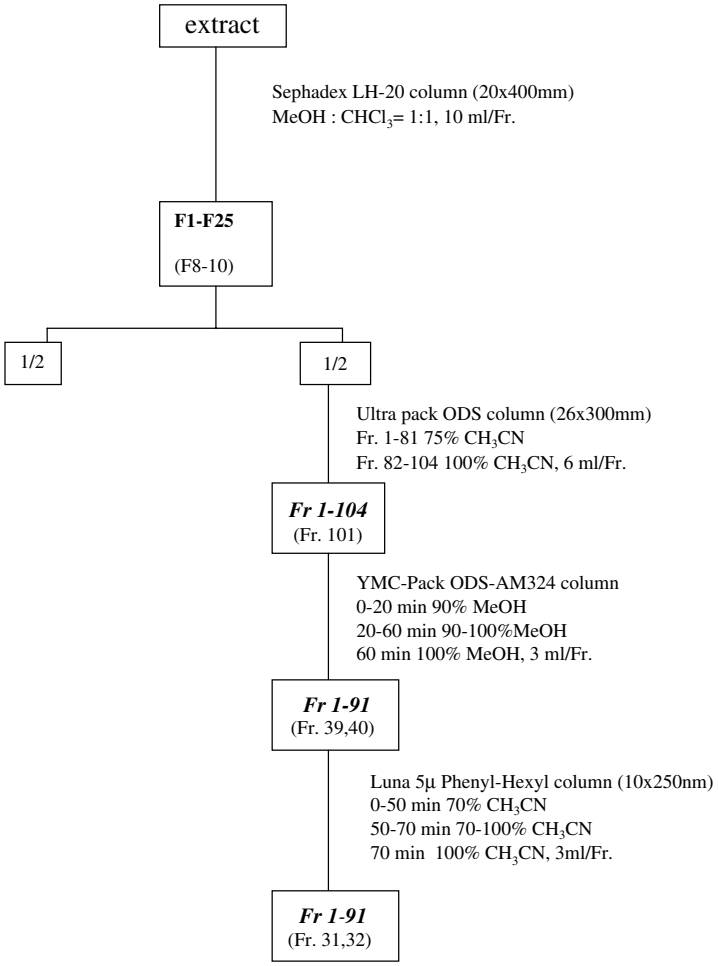


Fig. 1. Scheme for the bioassay-directed fractionation/chemical analyses performed with the sludge extract.

effluent, 2×10^4 revertants/l-equivalent for the raw water, and 7×10^3 revertants/l-equivalent for the drinking water produced by the DWTP. The sludge collected from the DWTP gave 4.7×10^4 revertants/g-equivalent.

3.1.2. BDCP and its three dye components

The BDCP, which is a mixture of three dyes (Fig. 2), an aromatic amine, and unidentified dispersing agents showed mutagenic activity with TA98 and YG1041 in the presence and absence of S9. We observed a ~20 fold increase in the mutagenic response with YG1041 when compared with TA98, both with and without S9. The addition of S9 also increased the mutagenicity ~20 fold over the levels observed without S9, for both strains (Fig. 3A). The three isolated dye components of BDCP were tested for mutagenicity using the same conditions described above, and they presented a similar mutagenicity pattern as the BDCP (Fig. 3B–D). When S9 metabolic activation was used, the mutagenic activities of the blue and violet components exhibited more pronounced increases than the orange component. For all three components, the use of YG1041 when compared to the results obtained for TA98 revealed the involvement of the nitroreductase and/or *O*-acetyltransferase activity in the activation of these compounds (Fig. 3B–D). We can conclude that the S9-containing P-450 CYP isozymes besides the nitroreductase and *O*-acetyltransferase are involved in the activation of this commercial product as well as its dye components. In comparison to the mutagenicity of the C.I. Disperse Blue 291 commercial product, we observed that the BDCP and the blue and the violet components are more mutagenic than the C.I. Disperse Blue 291 especially for the YG1041 in the presence of S9. The mutagenicity of the orange component was similar to the C.I. Disperse Blue 291. The role of the nitroreductase and *O*-acetyltransferase obtained with the C.I. Disperse Blue 291 was similar to the results obtained in this study. When examining mutagenicity dependence on S9 metabolism, the C.I. Disperse Blue 291 was less activated by the

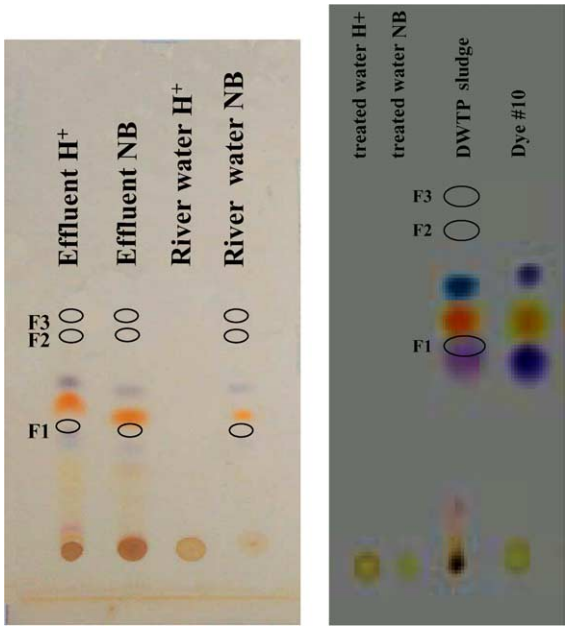


Fig. 2. Chemical analysis of environmental samples using the TLC method for the presence of dyes. NB = natural pH XAD4 extract; H^+ = acid pH XAD4 extract; F1, F2 and F3 = fluorescent compounds; DWTP—Drinking Water Treatment Plant. Dye 10 = BDCP.

CYP isozymes (Umbuzeiro et al., 2005) than the BDCP and its components. No additional toxicological information about the BDCP components was found in the literature.

Comparing the mutagenicity of the commercial product with its components, we can suggest that the dispersing agents and other non-dye components do not affect the mutagenicity of the mixture, because the mixture shows the same mutagenicity pattern as the individual compounds and the potency seems to be a sum of the responses of its dye constituents.

From these data, it is clear that the mutagenicity of the treated effluent could arise from the presence of the three components of BDCP, because they are all mutagenic and the potencies are sufficient enough (6300; 4600, and 280 revertants/μg for the blue, violet, and orange components respectively) (Fig. 3B–D) to cause the observed effects. Also, the mutagenicity of the raw water and sludge samples could be related to the presence of the same dyes (Fig. 2).

3.2. Chemical analysis

3.2.1. Evaluation of the presence of dyes in the environmental samples using thin layer chromatography

Using TLC analysis, we observed Rf values and colors comparable to the BDCP in both natural and acid pH extracts of the treated industrial effluent, in the natural pH extract of the river water samples, and in the DWTP sludge extract (Fig. 2). Only the drinking water did not contain dyes at concentrations above 0.02 μg/l, which was the lowest observed limit (LOL) of the technique. This limit was calculated by spotting 1:10 serial dilutions of a 100 μg/ml solution of BDCP.

BDCP is composed of three different dye constituents: a violet (Rf = 0.43), an orange (Rf = 0.48), and a blue (Rf = 0.59). We also observed a presence of a non-dye yellow impurity (Rf = 0.66) (Fig. 2). We found that this impurity had the same Rf value as the aromatic amine 2,6-dichloro-4-nitroaniline (DCNA) previously detected by GC/MS in the industrial effluent, river water, and drinking water samples from the same location (Umbuzeiro et al., 2004). It is relevant to point out that other unknown dyes appear to be present in the industrial effluent sample (Fig. 2). In addition, TLC results from the environmental sample extracts, excluding the drinking water, showed three fluorescent compounds designated here as FC1, FC2, and FC3 with Rfs 0.45, 0.72, and 0.76, respectively. The chemical structures of these compounds were not determined. Further studies should be performed in order to characterize the chemical and mutagenic characteristics of these compounds and to determine whether or not they are also involved in the mutagenic activity of those environmental samples.

It is clear from these results that the activated sludge treatment system used by the textile processing plant did not provide efficient removal of dyes. Also, the treatment was not effective in removing components responsible for the mutagenic activity. This is indicated by the high levels (3×10^5 revertants per liter) of mutagenic activity detected in the treated effluent samples analyzed in this study. The low efficiency of the removal of dyes in activated sludge systems was demonstrated by an US EPA study (1989) in which 20% of a C.I. Disperse Blue 79 sample remained in the liquid effluent after aerobic treatment. The same type of treatment did not remove mutagenicity with or without tertiary treatment that used activated carbon (Fracasso et al., 1992). Other procedures that combine anaerobic, with aerobic treatments (An et al., 1996; Dubrow, 1996; O'Neill et al., 2000; Rajaguru et al., 2000; Cruz and Buitron, 2001) and strong oxidation processes like ozonation (Gähr et al., 1994) seem to be more efficient in the removal of dyes; but unfortunately in those studies, the mutagenic activity of the raw effluent and treated effluent was not evaluated.

3.2.2. Identification of the structures of the components of the BDCP using NMR

The separation and purification of the components were performed as described in Section 2, and the chemical structures were identified using nuclear NMR and

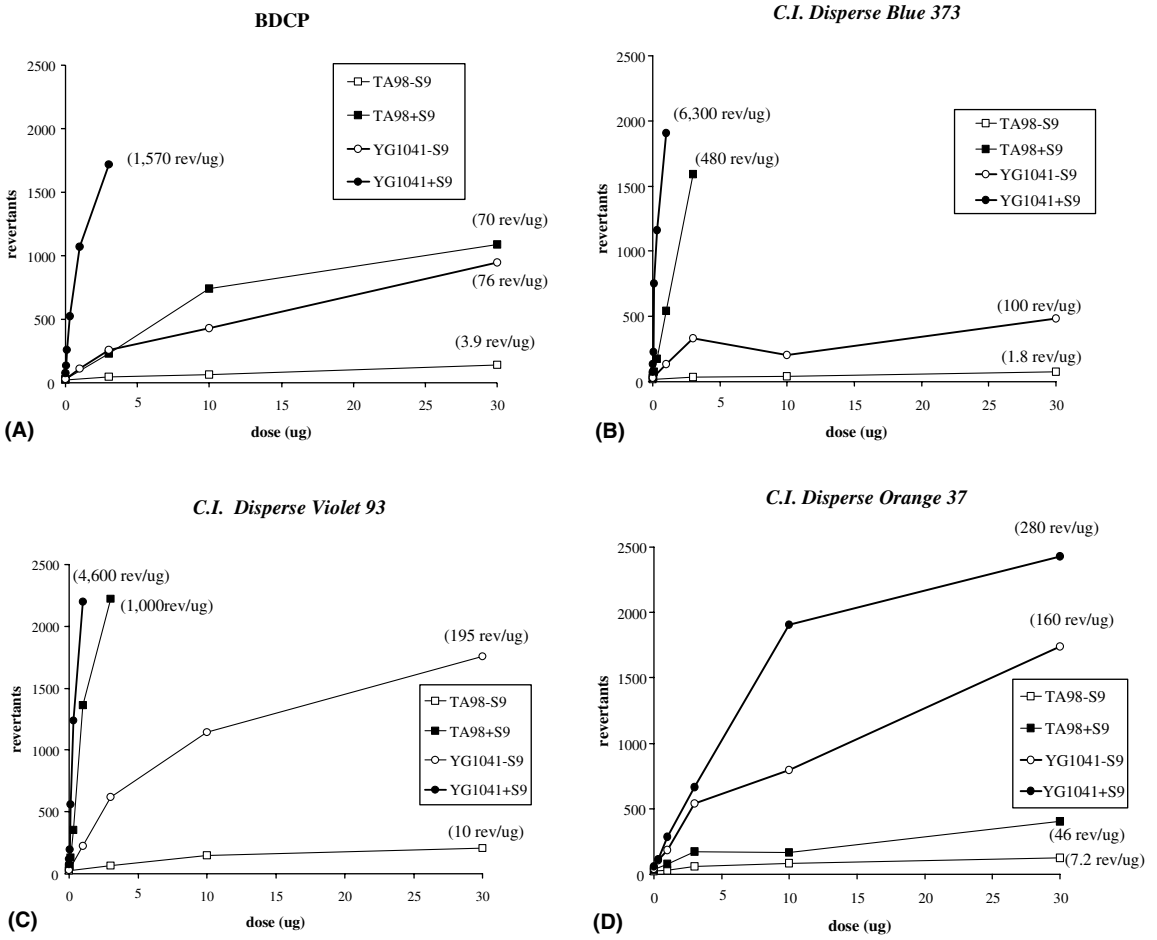


Fig. 3. Salmonella mutagenicity results for BDCP and its components with different strains (TA98 and YG1041) showing the effects of nitroreduction and/or *O*-acetylation as well as the CYP isozymes (S9) in the observed mutagenic activity.

MS analysis. The results of the NMR experiments are shown below and the structures are shown in Fig. 4.

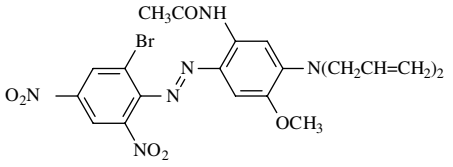
Orange dye: ^{1}H NMR ($CDCl_3$) δ: 8.30 (2H, s), 7.97 (2H, d, $J = 9.1$ Hz), 6.80 (2H, d, $J = 9.1$ Hz), 3.80 (2H, t), 3.64 (2H, q), 2.73 (2h, t), 1.32 (3H, t). CIMS *m/z*: 393 (M + H, $^{37}Cl)^+$, 391 (M + H, $^{35}Cl)^+$. HRMS (EI) confirmed $C_{17}H_{15}Cl_2N_5O_2$ as the elemental composition, which is identical to C.I. Disperse Orange 37. The CAS register number is 13301-61-6.

Blue dye: ^{1}H NMR ($CDCl_3$) δ: 8.68 (1H, d, $J = 2.2$ Hz), 8.32 (1H, d, $J = 2.2$ Hz), 8.20 (1H, s), 7.29 (1H, s), 5.93 (2H, m), 5.29 (4H, dd), 4.15 (4H, d), 3.88 (3H, s), 2.30 (3H, s). CIMS *m/z*: 535 (M + H^+, ^{81}Br), 533 (M + H^+, ^{79}Br). HRMS confirmed $C_{21}H_{21}BrN_6O_6$ as the elemental composition, identical to C.I. Disperse Blue 373. The CAS registry number is 51868-46-3.

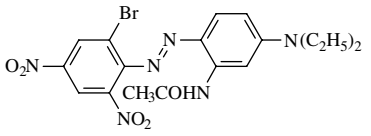
Violet dye: ^{1}H NMR ($CDCl_3$) δ: 8.65 (1H, s), 8.33 (1H, s), 8.15 (1H, d), 7.73 (1H, bs), 6.50 (1H, d), 3.55 (4H, q), 2.30 (3H, s), 1.32 (6H, t). CIMS *m/z*: 480 ($M^{+\cdot}$, ^{81}Br), 478 ($M^{+\cdot}$, ^{79}Br), 465 (M − CH_3, ^{81}Br), 463 (M − CH_3, ^{79}Br). HRMS (EI) confirmed $C_{18}H_{19}BrN_6O_5$ as the elemental composition, is identical to C.I. Disperse Violet 93. The CAS register number is 268221-71-2.

The pattern of mutagenicity observed for the BDCP and its components (Fig. 3) can be explained by the chemical structures identified (Fig. 4). The increase of the mutagenic response with the YG1041 in relation to TA98 (Section 3.1) is probably due to the presence of the NO_2 groups in the dye components of the BDCP. The other substituents of the molecule (e.g., $N(CH_2CH{=}CH_2)_2$, OCH_3) could explain the increase in the mutagenicity in the presence of S9 (Section 3.1). The mutagenic pattern of the BDCP and its components is also similar to the mutagenic activity of the related environmental samples collected in the Cristais River as previously reported by Umbuzeiro et al. (2004).

Blue Component of BDCP (C.I. Disperse Blue 373)



Violet Component of BDCP (C.I. Disperse Violet 93)



Orange component of BDCP (C.I. Disperse Orange 37)

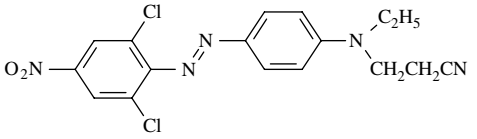


Fig. 4. The chemical structures determined for the dye components of BDCP using NMR and MS.

3.3. Confirmation and quantification of the major mutagen using bioassay-directed fractionation/chemical analysis

Among the different samples analyzed in this study, only the extract of the DWTP sludge was concentrated enough to be analyzed by this technique (see Section 2.3). After several fractionation steps and mutagenic evaluation with YG1024 in the presence of S9, the major mutagen found in the water treatment plant sludge extract was the dye C.I. Disperse Blue 373. The structure of the compound was confirmed by MS spectra, UV absorption spectra, and retention times of the mutagen isolated from the sample using authentic C.I. Disperse Blue 373. This dye accounted for 55% of the total mutagenicity of the extract when it was tested with YG1024 in the presence of S9. This result is an independent confirmation that this dye was an important contributor to the mutagenic activity of the samples collected in the Cristais River because we verified the presence of this dye in all the samples analyzed in this study, except the drinking water (Fig. 2).

Because C.I. Disperse Blue 373 was present in the river water and it is known to generate non-Cl-PBTA8 or PBTA8 by reduction reactions and subsequent chlorination (Watanabe et al., 2002), one could anticipate their presence in the sludge samples. The C.I. Disperse Blue 373 could be reduced under unknown circumstances and subsequently chlorinated during the treatment of the river water at the DWTP, generating such compounds in the sludge. The PBTA compounds were found as an important source of mutagenic activity in several Japanese Rivers that receive discharges from wastewater treatment plants that treat effluents from textile dyeing operations (Nukaya et al., 1997). The PBTA compounds were not detected in sludge sample evaluated in this study probably because the reduction step was not occurring or they were generated in amounts not detected by the applied methodology.

4. Final considerations

We can conclude that the components of the black dye commercial product, BDCP (C.I. Disperse Blue 373, C.I. Disperse Violet 93, and C.I. Disperse Orange 37), are present in the environmental samples arising from discharges at a local textile wet processing plant except for the drinking water sample analyzed. Additionally they are contributing to the mutagenic activity detected in the same samples. This is the first study that directly relates the mutagenic activity of a river to the presence of azo-dye compounds.

The various dye components and the associated mutagenicity were not efficiently removed by the industrial effluent treatment and persist in the environment. Consequently, there is a need to evaluate alternative treatment processes at azo dye processing plants in order to reduce the levels of mutagenicity and amounts of dyes present in receiving waters and sediments, e.g., in the Cristais River.

We showed that the use of both chromatographic methods like TLC and the Salmonella assay can complement the evaluation of the water quality of samples under the influence of dye processing plants discharges, as well as the current protocols (e.g., COD, BOD, suspended solids) used to test the efficiency of effluent treatment processes.

The conventional treatment employed by the Drinking Water Treatment Plant (DWTP) seemed to be efficient for the removal of dyes and fluorescent compounds, because they were not detected in the drinking water samples analyzed and were detected in the sludge extracts. On the other hand, the treatment is not removing the mutagenicity related to the presence of nitro compounds detected in the natural pH XAD extracts and blue rayon extracts of drinking water from the Cristais River (Umbuzeiro et al., 2004). We suspect that some uncolored mutagenic nitro-polycyclic compounds are being formed in the drinking water treatment process and not being efficiently removed. Experiments involving chlorination of this BDCP are in progress in order to determine which compounds are formed and if they are related to this mutagenic activity. Another possibility is that some other mutagenic nitro compound related to the dye processing plant is causing this activity. Efforts are also currently targeted toward analysis of blue rayon extracts of the

drinking water using LC/MS and other chemical techniques to try to identify the compounds as well as their structures.

Because the discharge of the azo dye processing plant analyzed in this study seems to be indirectly affecting the quality of the drinking water, mutagenic dyes and possibly other genotoxic substances present in this type of effluent should be better characterized toxicologically before released into the aquatic environment in areas where water is taken for human consumption. Other human (via consumption of organisms) and ecological risks also should be considered and evaluated in further studies before considering the discharge of mutagenic effluent containing azo dyes in water bodies as a safe activity.

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