Toxicology 376 (2017) 113–119

Contents lists available at ScienceDirect

Toxicology

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

Ecotoxicological risk assessment of the "Acid Black 210" dye

Otávio Pelegrino Rocha[a,*], Cibele Aparecida Cesila[a], Elisa Maria Christovam[b], Sílvia Berlanga de Moraes Barros[c], Maria Valnice Boldrin Zanoni[b], Danielle Palma de Oliveira[a]

[a] Laboratory of Environmental Toxicology, School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Ribeirão Preto-SP 14040-903, Brazil
[b] Institute of Chemistry, Universidade Estadual Paulista, Araraquara-SP 14800-060, Brazil
[c] Laboratory of Skin Biology, School of Pharmaceutical Sciences, University of São Paulo, São Paulo-SP 05508-000, Brazil

ARTICLE INFO

Article history:
Received 3 December 2015
Received in revised form 11 March 2016
Accepted 13 April 2016
Available online 19 April 2016

Keywords:
Acid Black 210
Dye
Ecotoxicological risk assessment

ABSTRACT

The "Acid Black 210" dye is one of the most used black dyes by the leather industry. This compound contains three azo groups in its chemical structure, and has been quoted as a non-regulated dye with toxicological concern, since it could generate carcinogenic aromatic amines. The objective of this study was to perform the ecotoxicological risk assessment of this dye through testing its toxicity *in vitro* and *in vivo* with the Ames test, the Comet assay, the *Daphnia similis* test, and the zebrafish embryo acute toxicity test. Moreover, we evaluated the presence of this dye in environmental samples related with a tannery industry. All the tests performed were negative, with the exception of the Ames test with the *Salmonella typhimurium* TA98 strain, which resulted in a low mutagenic potency. Due to the low concentrations of the "Acid Black 210" dye found in tannery effluents, and the high concentrations where any toxic activity is occasionally described, we concluded that this dye is safe from the ecotoxicological point of view in the areas evaluated and in the light of the current knowledge.

1. Introduction

Dyes are widely used in textile dyeing, paper printing, color photography, pharmaceutical, food, cosmetic, and other industries (Bafana et al., 2011). The world market of dyes, pigments and intermediates is valued at billions of dollars. Dyes must have a high degree of chemical and photolytic stability to be useful, and consequently, they can be resistant to degradation, and considered persistent bioaccumulative toxic (PBTs) substances.

The acute toxicity of azo dyes, as defined by the European Union criteria for classification of dangerous substances, is rather low. Azo dyes can also cause a variety of chronic effects, and carcinogenicity of many of the azo dyes has been demonstrated experimentally (Bafana et al., 2011). Since dye-containing effluents are normally discharged into water bodies, toxicity of dyes has mainly been studied against aquatic organisms. Azo dyes have been found to be toxic towards several aquatic organisms including algae, fish, and crustaceans (Bafana et al., 2011).

Acid Black 210 (AB210) is an azo (metal complex) dye containing sulphonyl and amino groups used in cotton, leather and woolen dyeing worldwide (Mohan et al., 2007). During a search for publicly available toxicity data on non-regulated aromatic amines, it was observed that the toxicity database was found to be insufficient or non-existent (Brüschweiler et al., 2014). AB210 was pointed to have the cleavage products 4-nitroaniline, which the Dutch Health Council recommended to classify as suspected human carcinogen (Dutch Health Council, 2008), and 4-aminobenzene sulfonic acid, which may cause sensitization by skin contact (Brüschweiler et al., 2014).

The only available data in the literature about the toxicity of AB210 are microbial toxicity test and comet assay with *Allium cepa* roots employing 1500 mg/L of AB210 (Agrawal et al., 2014). Due to the lack of toxicological data about the AB210, the aim of this work was to evaluate some *in vivo* and *in vitro* ecotoxicological endpoints. Also the presence of AB210 in environmental samples from effluent discharge from a tannery industry was measured. With these results we performed an ecotoxicological risk assessment of the AB210 in the area studied.

* Corresponding author.
E-mail addresses: otavio@fcfrp.usp.br, otavio_pelegrino@yahoo.com.br (O.P. Rocha), dpalma@usp.br (D.P. de Oliveira).

http://dx.doi.org/10.1016/j.tox.2016.04.002
0300-483X/

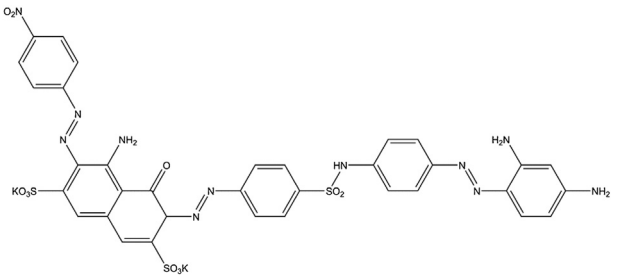

Fig. 1. Chemical structure of the "Acid Black 210" dye.

2. Material and methods

2.1. Chemicals

Commercial grade triazo Acid Black 210 (CAS #99576-15-5; 4-amino-6-((4-(((4-((2,4-diaminophenyl)azo)phenyl)amino)sulphonyl)phenyl)azo)-5-hydroxy-3-((4-nitrophenyl)azo)-2,7-naphthalenedisulphonic) (Fig. 1) was generously provided by Eco Vita Indústrias Química LTDA. All chemicals used were of analytical grade.

2.2. Mutagenicity assay

Salmonella typhimurium TA98 and TA100 strains were cultured overnight in Oxoid Nutrient Broth at 37 °C and 120 rpm in a shaking incubator. The tests were performed according to Maron and Ames (1983), and Mortelmans and Zeiger (2000), with (+S9) and without (−S9) metabolization using the pre-incubation method (Maron and Ames, 1983). Different concentrations of the AB210 diluted in water were used in the tests (0.5–5000 μg/plate). Briefly, tubes containing the dye, *Salmonella* strain and phosphate buffer 0.2 M pH 7.4 or S9 mix were incubated at 37 °C for 20 min. Then, 2 mL of top agar were added to the each tube and the mixture was poured onto minimal agar plates. After the incubation at 37 °C for 66 h, the number of His^+ revertant colonies was counted manually. The results were analyzed with the statistical software Salanal 1.0 (U.S. Environmental Protection Agency, Las Vegas, USA), adopting the Bernstein et al. (1982) model. 2-Aminoanthracene (2-AA) 2.5 μg/plate and 4-nitroquinoline-*N*-oxide (4-NQO) 0.5 μg/plate were used as positive controls in the experiments with and without S9 mix, respectively. The water used in the dilutions was used as negative controls. Viability tests were also performed to ensure that the bacteria concentrations were between 1.0 and 2.0×10^9 cells/mL prior to the tests. The background was carefully evaluated.

2.3. Genotoxicity assay

The Comet Assay was performed with HepG2 cells (American Type Culture Collection, No. HB 8065) cultured in DMEM medium (Gibco, Grand Island, USA) supplemented with 10% bovine fetal serum at 37 °C flushed with 5% CO_2 in air, according to Östling and Johanson (1984), with the modifications described by Singh et al. (1988), and the protocol described by Tice et al. (2000). Briefly, the cells (2×10^5 cells/mL) were exposed to different concentrations of the AB210 dissolved in water (0.5–5000 μg/mL) for 4 h. The cells were trypsinized, suspended and centrifuged (10 min, 212*g*), following the addition of 140 μL of low melting agarose, spread onto pre-coated slides and lysis (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, 1% Triton X-100, 10% DMSO, 1% SDS, pH 10) for 1.75 h. The slides were placed in the electrophoresis solution (1 mM EDTA, 300 mM 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 (0,4 M Tris-HCl, pH 7.5) for 20 min, stained with ethidium bromide (10 μg/mL), examined under a fluorescence microscope (Eclipse, Model E200-LED, Nikon), and counted by the video imaging system Comet Assay IV™ version 4.3 (Perceptive Instruments, United Kingdom). All experiments were carried out in triplicate and the tail moment of DNA migration was measured in 100 cells per treatment. The cell viability was measured by the Trypan Blue method.

2.4. Aquatic acute toxicity assay

Daphnia similis obtained from the CETESB Ecotoxicology Laboratory were maintained at 20 ± 2 °C, with light intensity of 1000 Lux, under 16:8 h (light/dark) photoperiod. The *D. similis* were fed daily with the green algae *Selenastrum capricornutum* and a mixture of trout food and yeast (Buratini et al., 2004). For each replicate five young organisms (6–24 h old) were exposed to the AB210 for 48 h in glass tubes in concentrations between 0.1 and 4000 μg/mL. After that, immobilized *Daphnia* were counted. As described in the OECD no. 202 guideline the immobilization rate was less than 10% in the negative control group (OECD, 2004). The results were statistically analyzed using the Spearman trimmed method (Hamilton et al., 1977). The sensibility of *D. similis* organisms is monthly monitored using potassium chlorate (KCl) at 370, 480, 620, 800 e 1000 mg/L.

2.5. Fish embryo acute toxicity assay

This assay was done according to OECD no. 236 guideline (OECD, 2013) and Nagel (2002). Newly fertilized zebrafish eggs were exposed to different concentrations of the AB210 (0.1–100 μg/mL) for a period of 144 h in 24 well-plates. After 8 h and every 24 h, up to four apical observations were recorded as indicators of lethality: coagulation, lack of somite formation, lack of detachment of the tail-bud from yolk sac, and lack of heartbeat. Other endpoins were analyzed, as the sublethal endpoints development of eyes, spontaneous movement, blood circulation, pigmentation, and oedema, and the teratogenic endpoints malformation of head, otoliths, tail and heart, as well as scoliosis, growth-retardation, length of tail, and swin bladder formation. 3,4-Dichloroaniline 4 μg/mL was used as positive control.

2.6. Chromatographic analysis of tannery samples

Samples (raw and treated effluent, samples 1 and 2, respectively) were collected from a tannery industry located in São Sebastião do Paraíso, Minas Gerais state, Brazil, which routinely

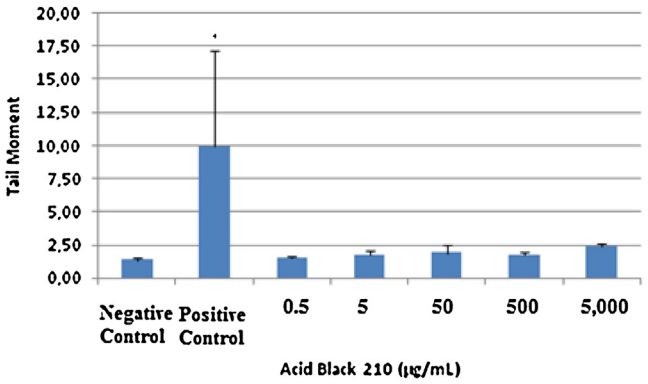

Fig. 2. Genotoxicity results using the Comet assay in HepG2 exposed to the AB210. The negative control was water, and the positive control methyl methanesulfonate (MMS) 15 mg/mL.

Table 1
Effect of the "Acid Black 210" dye in *S. typhimurium* TA98 and TA100 strains.

Concentration (μg/plate)	Average of the number of revertants/plate ± SD			
	S. typhimurium TA98		*S. typhimurium* TA100	
	Without S9 mix	With S9 mix	Without S9 mix	With S9 mix
Negative Control	37.00 ± 4.64	32.20 ± 3.11	223.60 ± 14.67	189.60 ± 19.18
Positive Control[a]	540.50 ± 19.09**	2,564 ± 356.38**	2,522.00 ± 381.84**	3,488.00 ± 565.69**
0.5	38.67 ± 6.51	35.00 ± 5.29	238.67 ± 21.59	––
5	43.00 ± 7.21	31.50 ± 4.95	226.67 ± 0.58	––
50	40.00 ± 6.93	44.67 ± 1.53**	227.00 ± 19.29	221.00 ± 17.73
100	––	––	––	215.67 ± 20.11
500	55.00 ± 8.72	94.33 ± 11.06**	258.33 ± 13.58	184.67 ± 19.40
1000	69.33 ± 9.24*	––	––	189.00 ± 31.58
2000	62.50 ± 0.71**	––	––	––
3000	84.67 ± 8.62**	––	––	––
4000	132.67 ± 10.02**	––	––	––
5000	116.67 ± 22.81**	263.33 ± 22.37**	234.33 ± 15.70	216.33 ± 17.95
Potency (revertants/μg)	0.017	0.124	––	––

The results are expressed as average of the number of revertants of 3 repetitions ± SD. *$p < 0.05$ and **$p < 0.01$ statistically different in relation to the negative control.
[a] 4-Nitroquinoline-*N*-oxide 0.5 μg/plate.

uses this dye. Also samples from the upstream and downstream of the disposal site (samples 3 and 4) were collected, corresponding to surface water samples in which tannery effluent is discarded. Collection of samples was performed as described in Standard Methods for the Examination of Water and Wastewater (APHA, 1998).

HPLC-DAD measurements were performed on a Shimatzu chromatograph, LC-10AT model (USA) coupled with a diode array detector (SPD-M10AVP model). The mobile phase was: 0.1% formic acid in water + acetonitrile mixed at different ratios. A Kinetix C18 5 μm column (150 × 4.6 mm) (Phenomenex, Torrance, CA, USA) was used in an Agilent 1200 Autosampler HPLC and a pump (Agilent Technologies, USA). A gradient elution system with 0.1% formic acid in water (solvent A) and acetonitrile (solvent B) was employed under the following conditions: 0.00–3.00 min (10%B), 3.00–13.00 min (10–100%B), 13.00–14.00 min (100%B), 14.00–15.00 min (100–10%B), and 15.00–20.00 min (10%B). Sample injection volume was 20 μL, time analysis was 20 min, and flow rate 0.5 mL/min. Solid phase extraction using Strata-X cartridge (Agilent, USA) was performed with 6 mL of methanol and deionized water. AB210 was also analyzed by LC-ESI–MS/MS QTRAP for low limit detection (below the detection limit of the HPLC described above). The ionization conditions selected were: Gas cone flow (150 L/h), gas flow of dessolvation (350 L/h), polarity (ESI^+), capillary energy (2900 V), power example cone (30 V), the power extraction thimble (2.0 V), solvation temperature (350 °C), source temperature (120 °C), the ionization energy (2.0 V), the energy collision (4 V), and energy multi-channel plate detector (2700 V).

3. Results

3.1. Comet assay

No genotoxic effect was observed for HepG2 cells treated with Acid Black 210 up to 5000 μg/mL (Fig. 2). Cell viability was higher than 92% for all concentrations assayed (0.5–5000 μg/mL), measured by the Trypan Blue method.

3.2. Mutagenicity assay

Acid Black 210 showed mutagenic effect only for the *S. typhimurium* TA98 strain in the higher concentrations tested, and this effect increased after S9 treatment. Under the condition of the test, the dye tested negative for TA100 (with and without S9) (Table 1).

3.3. Aquatic acute toxicity assay

Treatment with Acid Black 210 did not induced significant toxic effect on *D. similis* after 24 and 48 h (Tables 2 and 3). Only with the highest concentrations we observed a slight effect, 15 and 25% of immobility after 48 h treatment with 75 μg/mL and 100 μg/mL of the dye, respectively. The EC_{50} value of the sensibility of *D. similis* was 799.54 μg/mL at the moment of the assay. When continuing the evaluation through 4,000 μg/mL, the EC_{50} for this assay was 2999.73 μg/mL (Fig. 3).

Table 2
Immobility of the *D. similis* treated with the "Acid Black 210" dye for 24 h.

	Number of immobilized organisms/replicate				Immobility (%)	
Concentrations (μg/mL)					Total	
Negative Control	0	0	0	0	0/20	0
0.1	0	0	0	0	0/20	0
1.0	0	0	0	0	0/20	0
10	0	0	0	0	0/20	0
50	0	0	0	0	0/20	0
75	0	0	1	0	1/20	5
100	0	0	0	0	0/20	0

Table 3
Immobility of the *D. similis* treated with the "Acid Black 210" dye for 48 h.

	Number of immobilized organisms/replicate				Immobility (%)	
Concentrations (μg/mL)					Total	
Negative Control	0	0	0	0	0/20	0
0.1	0	0	0	0	0/20	0
1.0	0	0	0	1	1/20	5
10	0	0	0	0	0/20	0
50	0	0	0	0	0/20	0
75	2	0	1	0	3/20	15
100	1	2	1	1	5/20	25

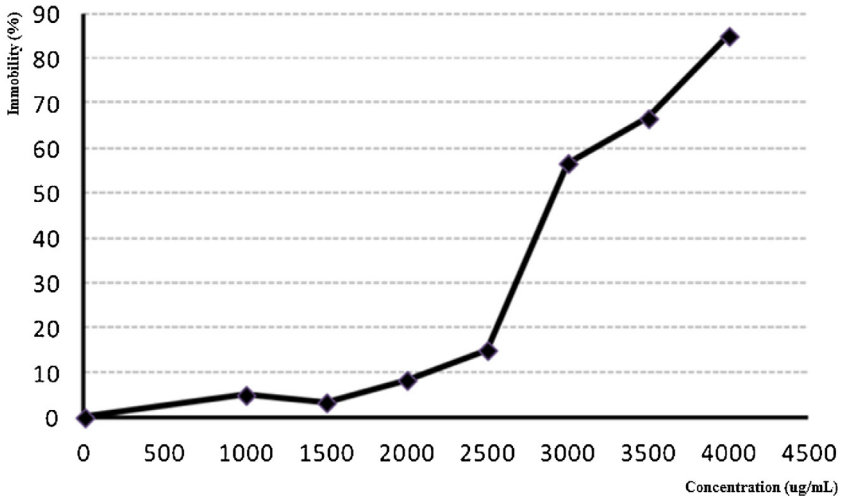

Fig. 3. EC_{50} of Acid Black 210 for the aquatic acute toxicity assay with *D. similis*.

3.4. Fish embryo acute toxicity assay

All assays performed with Acid Black 210 were negative after 144 h of treatment up to 100 μg/mL.

3.5. HPLC-DAD determination of Acid Black 210

Using some chromatographic parameters such as retention time (t_r), retention constant factor (k), selectivity and resolution between peaks (r), it was concluded that the best condition for the AB210 separation was obtained with a mixture of acetonitrile + water containing formic acid 0.1% in a gradient mode at ratios of 20% (0.01–10 min), 100% (10–11.50 min) and 20% (11.50–16.00 min). The respective chromatogram CLAE-DAD is shown in Fig. 4.

Good chromatographic separation of AB210 dye was obtained at retention time of 4.80 ± 0.17 min. Analytical curves were constructed from 10 to 100 ppm of AB210.

A linear relationship was obtained following the equation: Area (UA) = 25421.31 + 2267459.89C (ppm), R = 0.9993, n = 6. Limit of detection (LOD) evaluated as the signal-to-noise ratio equal to 3:1 reaches values of around 7.39 ppm. Limit of quantification (LOQ) determined as the signal-to-noise ratio equal to 10:1 was calculated (LOQ = 10 × (SD/B)) and the values are around 12.4 ppm. The repeatability of the proposed method, evaluated in terms of relative standard deviation, was measured as 3.2% over 10 experiments measuring samples containing 50 ppm of AB210 dye.

A blank sample (n = 3) of water was spyked with 10 ppm of AB210 to evaluate the recovery of the extraction method proposed. Recoveries between 95% and 110% was obtained indicating the accuracy of the method for the purpose of the analysis. Employing the *t*-test, we demonstrated that there were no significant differences between calculated and added concentrations at the 95% confidence level, indicating that the method can be applied for the determination of AB210 in environmental samples.

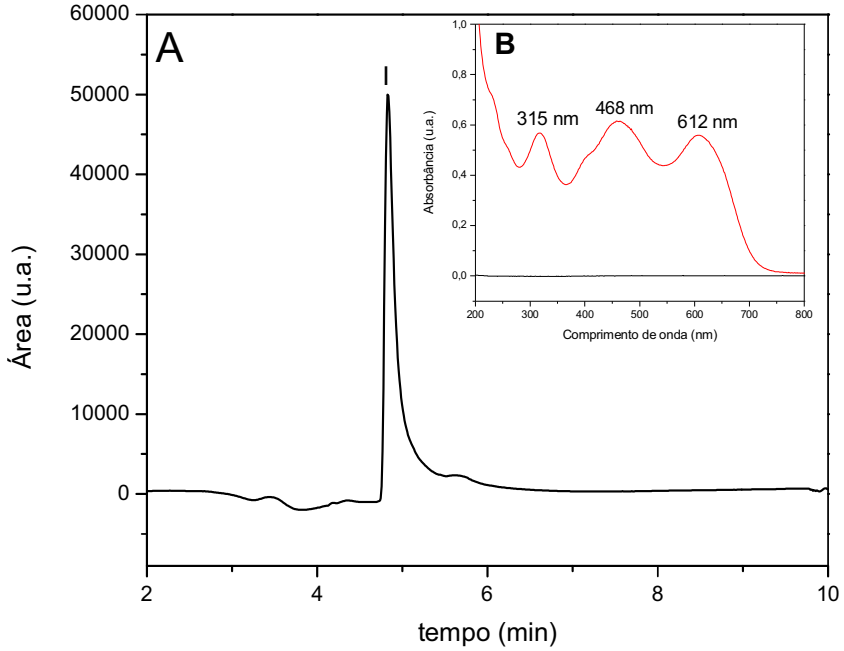

Fig. 4. (A) CLAE-DAD chromatogram obtained for 50 ppm of Acid Black 210 dye in aqueous solution in gradient mode. T = 30 °C, flow rate = 1.5 mL/min. Detection: λ = 612 nm. (B) UV spectrum of the peak detected in the chromatogram.

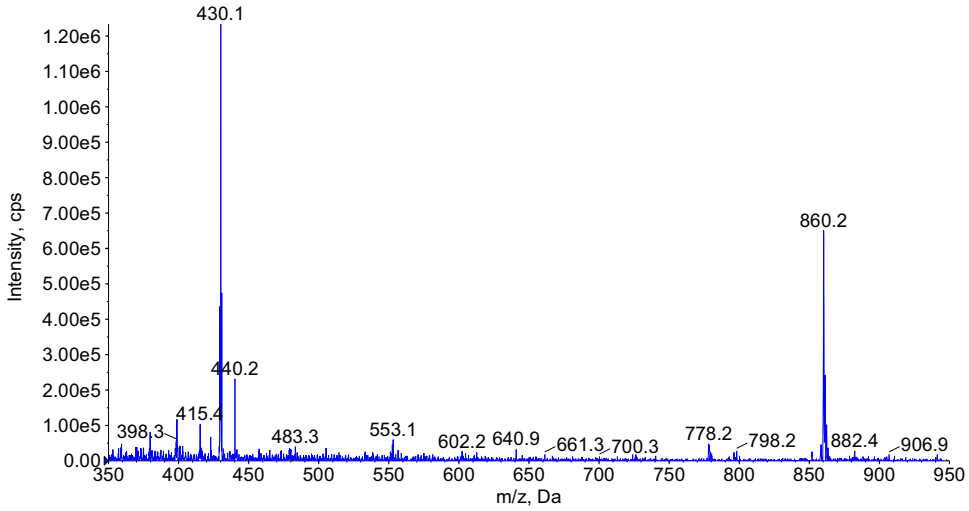

Fig. 5. Mass spectra obtained for 200 ppb of AB210 dye (negative mode).

3.6. Detection and quantification of AB210 dye in wastewater samples by LC–MS/MS

In order to develop a method for low limit detection of AB210 dye, new studies were carried out evaluating a low concentration of this dye (200 ppb) by LC–MS/MS, using optimized conditions shown previously in the experimental section. Standard solution of AB210 in water:methanol (1:1 v/v + ammonium acetate 5 mmol/L) full scan spectra is shown in Fig. 5.

The dye can be detected by the peaks with *m*/*z* 860 $[M-2K^{+}+1H^{+}]^{-}$ and *m*/*z* 430 $[M-2K^{+}]^{2-}$. AB210 chromatogram presented a retention time of 9.41 min as demonstrated in Fig. 6.

The chromatogram obtained for the raw water collected in the leather industry is presented in Fig. 7. A very small peak at 9.53 min indicates the occurrence of AB210 dye in the sample at very low concentration, checked with *m*/*z* ratio 860.

Thus, the method was applied in the analysis of samples 2 (treated watewater), 3 (upstream in the river) and 4 (upstream in the river). These samples were subjected to solid phase extraction (SPE) as described in the experimental session. After elution and drying, samples were redissolved in acetonitrile. There were no signals of AB210 sample in any other sample analysed, indicating that AB210 sample is present at only very small quantity in the raw water, but even the LC–MS/MS able to detect the AB210 dye at 1 ppm do not shown any quantitative signal of the dye in the effluent.

4. Discussion

The comet assay is used routinely for the detection of DNA damage using environmental samples (Avishai et al., 2003). A recent bibliometric study showed that the comet assay and environment health are nowadays closely related (Neri et al., 2015). Moreover, *in vivo* and *in vitro* comet assay has recently been implemented into regulatory genotoxicity testing of pharmaceuticals with the inclusion into the ICH 22R1 guidance (which also includes the Ames test) (Frötschl, 2015). In our study Acid Black 210 showed no genotoxic effects on the HepG2 cells at concentrations between 0.5 and 5000 μg/mL by comet assay. Matsumoto et al. (2003) showed the genotoxic potential of the water of a river receiving tannery effluents by the comet assay and Manzano et al. (2015) showed the genotoxicity of waters impacted by domestic and industrial effluents. Both authors suggest that the genotoxicity could be associated with the metals discharged by the tanneries industries, especially chromium. The mutagenicities of different aromatic amines and acetamides by the Ames test with the

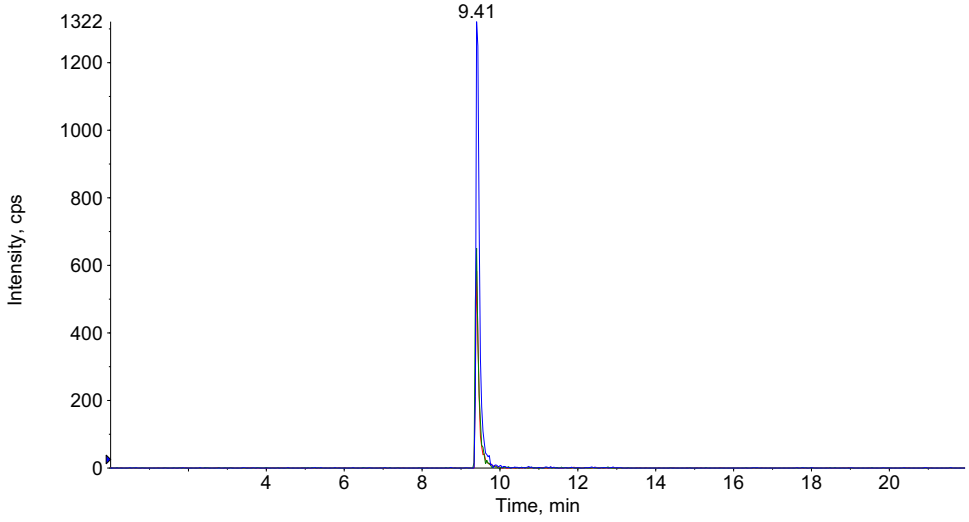

Fig. 6. LC–MS/MS for extract ion (XIC) obtained for standard solution of Acid Black 210.

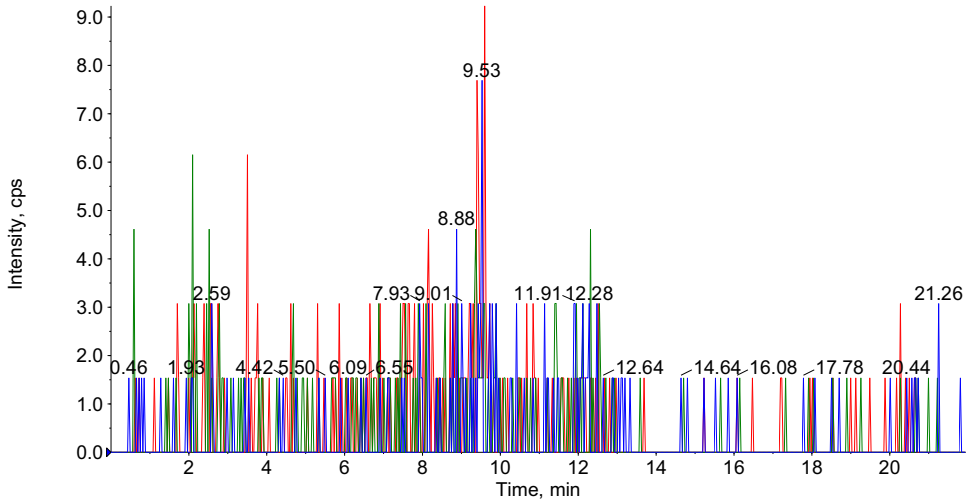

Fig. 7. XIC of raw sample collected in the leather industry.

TA98 and TA100 *Salmonella* strains were demonstrated in the past (Connor et al., 1983). The formation of these aromatic amines still concern the researchers worldwide nowadays, and Brüschweiler et al. (2014) pointed the Acid Black 210 as a non-regulated dye that could generate aromatic amines of toxicological concern. The Ames test showed mutagenic activity for the TA98 strain only in higher concentrations (generally not observed in the environment), even after metabolization by the S9. The mutagenic potencies observed for this strain were 0.0167 and 0.1245 revertants/μg, which we can be considered extremely low mutagenic potencies (Claxton et al., 1991).

It is important to point out that when the degradation of this dye occurs by the bacterium *Providencia* sp. SRS82, benzene, naphthalene and 4-aminophenyl-*N*-(4-aminophenyl) benzene sulphonamide are formed, reducing the toxicity of the dye in microbial toxicity test and *A. cepa* roots comet assay (Agrawal et al., 2014).

Different molecules can affect the pigmentation pathways in zebrafish embryos, and pigment cells are very conserved between zebrafish and other vertebrates (Colanesi et al., 2012), but no effects were observed for this or the other endpoints analyzed in the present study. The bioconcentration, metabolism and toxicity of different anilines in zebrafish have already been analyzed, and the formation of their corresponding acetanilides was observed (Zok et al., 1991).

Acid Black 210 dye did not cause acute toxicity to *D. similis*. Our study showed that the EC50 for this test was 2993.73 μg/mL. According to the classification proposed by Zucker (1985), only EC50 less than 100 μg/mL are of toxicological concern.

Blaauboer (2015) highlighted the relevance of the development of *in vitro* toxicology assays towards its implementation in toxicological risk assessment. Different authors used these tests to perform the risk assessment of different wastewaters in the last years. Tigini et al. (2011) pointed out that the interaction effect between dyes (including the Acid Black 210) and salts resulted in a synergistic effect of simulated wastewater toxicity with a battery of biotests. Lemos et al. (2011) identified the presence of substances that induce chromosome breakage or loss by the micronuclei assay of surface water under the influence of tanneries in Brazil. Masood and Malik (2013) evaluated the cytotoxic and genotoxic potential of tannery waste contaminated soils in India with the Ames test. Júnior et al. (2007) demonstrated the influence of tannery industries in ecotoxicity tests with *Daphnia magna* and *Ceriodaphnia dubia* using water and sediment samples.

The dyes have been pointed as environmental hazard (Kant, 2012), and studies in this area are new and important to guide legislations worldwide (Hessel et al., 2007). Leme et al. (2014) showed that dyes could migrate from cotton fibers to sweat solutions, and a weak mutagenic potential was detected by the Ames test for the dyes Reactive Blue 2 and Reactive Green 19. The azo dye Sudan III reacts with CYP-450 isoenzymes with the generation of hazardous aromatic amines (Zanoni et al., 2013). Ferraz et al. (2012) described the impact of aromatic amines on the environment. The cytotoxicity for hepatic cells were demonstrated for the disperse dyes Orange 1, Red 1, and Red 13 (Ferraz et al., 2012), and the Disperse Orange 1 also induced DNA damage, but did not cause toxicity to *D. similis* and *Vibrio fischeri* (Ferraz et al., 2011). However, considering the results that we obtained with all the assays, as well as the extreme low concentrations of AB210 in the samples related to the discharge of the tannery industry, we hypothesized that the use of this dye is not an ecotoxicological concern at least in the conditions of this study.

5. Conclusions

This is the first time that an ecotoxicological risk assessment with authentic water samples is reported for the Acid Black 210 dye, pointed as a non-regulated dye that could generate aromatic amines of toxicological concern (Brüschweiler et al., 2014). We can concluded that this dye is safe from the ecotoxicological point of view in the light of the current knowledge considering the acute toxicity tests done, since this dye is found in low concentrations in the environment, and higher concentrations of this dye are necessary to induce toxicity in the tests performed.

Conflict of interest

None.

Acknowledgements

This study was supported by grants (process #2008/10449-7) and scholarships from the São Paulo Research Foundation (Fundação de Amparo à Pesquisa do Estado de São Paulo—FAPESP) (processes #2013/09317-7, #2013/06172-8, and #2013/24340-5); and the National Council of Technological and Scientific Development (Conselho Nacional de Desenvolvimento Científico e Tecnológico—CNPq/CAPES).

References

APHA—American Public Health Association, American Water Works Association, Water Environment Federation, 1998. Standard Methods for the Examination of Water and Wastewater, 20th ed. APHA, Washington, USA.

Agrawal, S., Tipre, D., Patel, B., Dave, S., 2014. Optimization of triazo Acid black 210 dye degradation by *Providencia* sp. SRS82 and elucidation of degradation pathway. Process. Biochem. 49, 110–119.

Avishai, N., Rabinowitz, C., Rinkevich, B., 2003. Use of the comet assay for studying environmental genotoxicity: comparison between visual and image analyses. Environ. Mol. Mutagen. 42, 155–165.

Bafana, A., Devi, S.S., Chakrabarti, T., 2011. Azo dyes: past, present and the future. Environ. Rev. 19, 350–370.

Bernstein, L., Kaldor, J., McCann, J., Pike, M.C., 1982. An empirical approach to the statistical analysis of mutagenesis data from the *Salmonella* test. Mutat. Res. 97, 267–281.

Blaauboer, B.J., 2015. The long and winding road of progress in the use of *in vitro* data for risk assessment purposes: from carnation test to integrated testing strategies. Toxicology 332, 4–7.

Brüschweiler, B.J., Küng, S., Bürgi, D., Muralt, L., Nyfeler, E., 2014. Identification of non-regulated aromatic amines of toxicological concern which can be cleaved from azo dyes used in clothing textiles. Regul. Toxicol. Pharm. 69, 263–272.

Buratini, S.V., Bertoletti, E., Zagatto, P.A., 2004. Evaluation of *Dapnhia similis* as a test species in ecotoxicological assays. Bull. Environ. Contam. Toxicol. 73, 878–882.

Claxton, L.D., Houk, V.S., Allison, J.C., Creason, J., 1991. Evaluating the relationship of metabolic-activation system concentrations and chemical dose concentrations for the *Salmonella* spiral and plate assays. Mutat. Res. 253, 127–136.

Colanesi, S., Taylor, K.L., Temperley, N.D., Lundegaard, P.R., Liu, D., North, T.E., Ishizaki, H., Kelsh, R.N., Patton, E.E., 2012. Small molecule screening identifies targetable zebrafish pigmentation pathways. Pigm. Cell Melanoma Res. 25, 131–143.

Connor, T.H., Ramanujam, V.M.S., Rinkus, S.J., Legator, M.S., Trieff, N.M., 1983. The evaluation of mutagenicities of 19 structurally related aromatic amines and acetamides in *Salmonella typhimurium* TA98 and TA100. Mutat. Res. 118, 49–59.

Dutch Health Council, 2008. p-Nitroaniline: Evaluation of the Carcinogenicity and Genotoxicity. Health Council of the Netherlands, The Hague.

Ferraz, E.R.A., Grando, M.D., Oliveira, D.P., 2011. The azo dye disperse orange 1 induces DNA damage and cytotoxic effects but does not cause ecotoxic effects in *Daphnia similis* and *Vibrio fischeri*. J. Hazard. Mater. 192, 628–633.

Ferraz, E.R.A., Li, Z., Boubriak, O., Oliveira, D.P., 2012. Hepatotoxicity assessment of the azo dyes disperse orange 1 (DO1), disperse red 1 (DR1), and disperse red 13 (DR13) in HepG2 cells. J. Toxicol. Environ. Health. A 75, 991–999.

Frötschl, R., 2015. Experiences with the *in vivo* and *in vitro* comet assay in regulatory testing. Mutagenesis 30, 51–57.

Hamilton, M.A., Russo, R.C., Thurston, R.V., 1977. Trimmed Spearman-Karber method for stimating median letal concentrations in toxicity bioassays. Environ. Sci. Technol. 11, 714–719.

Hessel, C., Allegre, C., Maisseu, M., Charbit, F., Moulin, P., 2007. Guidelines and legislation for dye house effluents. J. Environ. Manage. 83, 171–180.

Júnior, H.M., Silva, J., Arezon, A., Portela, C.S., Ferreira, I.C.F.S., Henriques, J.A.P., 2007. Evaluation of genotoxicity and toxicity of water and sediment samples from a Brazilian stream influenced by tannery industries. Chemosphere 67, 1211–1217.

Kant, R., 2012. Textile dyeing industry: an environmental hazard. Nat. Sci. 4, 22–26.

Leme, D.M., Oliveira, G.A.R., Meireles, G., Santos, T.C., Zanoni, M.V.B., Oliveira, D.P., 2014. Genotoxicological assessment of two reactive dyes extracted from cotton fibres using artificial sweat. Toxicol. in Vitro 28, 31–38.

Lemos, A.O., Oliveira, N.C.D., Lemos, C.T., 2011. In vitro micronuclei tests to evaluate the genotoxicity of surface water under the influence of tanneries. Toxicol. in Vitro 25, 761–766.

Manzano, B.C., Roberto, M.M., Hoshina, M.M., Menegário, A.A., Marin-Morales, M.A., 2015. Evaluation of the genotoxicity of waters impacted by domestic and industrial effluents of a highly industrialized region of São Paulo State Brazil, by the comet assay in HTC cells. Environ. Sci. Pollut. Res. 22, 1399–1407.

Maron, D.M., Ames, B.N., 1983. Revised methods for the *Salmonella* mutagenicity test. Mutat. Res. 113, 173–215.

Masood, F., Malik, A., 2013. Cytotoxic and genotoxic potential of tannery waste contaminated soils. Sci. Total Environ. 444, 153–160.

Matsumoto, S.T., Mantovani, M.S., Mallaguti, M.I., Marin-Morales, M.A., 2003. Investigation of the genotoxic potential of the waters of a river receiving tannery effluents by means of the *in vitro* comet assay. Cytologia (Tokyo) 68, 395–401.

Mohan, S.V., Rao, N.C., Sarma, P.N., 2007. Simulated acid azo dye (Acid black 210) wastewater treatment by periodic discontinuous batch mode operation under anoxic-aerobic-anoxic microenvironment conditions. Ecol. Eng. 31, 242–250.

Mortelmans, K., Zeiger, E., 2000. The ames *Salmonella*/microsome mutagenicity assay. Mutat. Res. 455, 29–60.

Nagel, R., 2002. DarT: the embryo test with the zebrafish *Danio rerio*: a general model in ecotoxicology and toxicology. Altex 1, 38–48.

Neri, M., Milazzo, D., Ugolini, D., Milic, M., Campolongo, A., Pasquatti, P., Bonassi, S., 2015. Worldwide interest in the comet assay: a bibliometric study. Mutagenesis 30, 155–163.

OECD, 2004. Test no. 202: *Daphnia* sp. acute immobilization test. Guidelines for the Testing of Chemicals: Effects on Biotic Systems, Section 2. OECD doi:http://dx.doi.org/10.1787/9789264069947-en.

OECD, 2013. Test no. 236: Fish embryo acute toxicity (FET) test. Guidelines for the Testing of Chemicals: Effects on Biotic Systems, Section 2. OECD doi:http://dx.doi.org/10.1787/9789264203709-en.

Östling, O., Johanson, K.J., 1984. Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. Biochem. Biophys. Res. Commun. 123, 291–298.

Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantification of low levels of DNA damage in individual cells. Exp. Cell Res. 175, 184–191.

Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C., Sasaki, Y.F., 2000. Single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. Environ. Mol. Mutagen. 35, 206–221.

Tigini, V., Giansanti, P., Mangiavillano, A., Pannocchia, A., Varese, G.C., 2011. Evaluation of toxicity, genotoxicity and environmental risk of simulated textile and tannery wastewaters with a battery of biotests. Ecotoxicol. Environ. Safety 74, 866–873.

Zanoni, T.B., Lizier, T.M., Assis, M.D., Zanoni, M.V.B., Oliveira, D.P., 2013. CYP-450 isoenzymes catalyze the generation of hazardous aromatic amines after reaction with the azo dye Sudan III. Food Chem. Toxicol. 57, 217–226.

Zok, S., Gorge, G., Kalsch, W., Nagel, R., 1991. Bioconcentration, metabolism and toxicity of substituted anilines in the zebrafish (*Brachydanio rerio*). Sci. Total Environ. 109, 411–421.

Zucker, E., 1985. Hazard Evaluation Division—Standard Evaluation Procedure: Acute Toxicity Test for Freshwater Fish. USEPA Publication, Washington, USA (540/9-85-006).