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Assessment of the cytotoxic, genotoxic and mutagenic effects of the commercial black dye in *Allium cepa* cells before and after bacterial biodegradation treatment

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HIGHLIGHTS

- BDCP metabolites generated by bacterial biodegradation induce cellular alterations.
- BDCP products generated by bacterial degradation are more toxic than the original BDCP.
- Nuclear buds and binucleated cells indicate genotoxic effects.
- BDCP and its metabolites induce high rates of cell death on *Allium cepa* cells.

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ABSTRACT

The present study evaluated the cytotoxic, genotoxic and mutagenic actions of different concentrations (50 and 200 μg/L) of BDCP (Black Dye Commercial Product) used by textile industries, before and after bacterial biodegradation, by the conventional staining cytogenetic technique and NOR-banding in *Allium cepa* cells. Differences in the chromosomal and nuclear aberrations and alterations in the number of nucleoli were observed in cells exposed to BDCP with and without the microbial treatment. The significant frequencies of chromosome and nuclear aberrations noted in the tests with bacterially biodegraded BDCP indicate that the metabolites generated by degradation are more genotoxic than the chemical itself. Losses of genetic material characterize a type of alteration that was mainly associated with the action of the original BDCP, whereas chromosome stickiness, nuclear buds and binucleated cells were the aberrations that were preferentially induced by BDCP metabolites after biodegradation. The significant frequencies of cell death observed in the tests with biodegraded BDCP also show the cytotoxic effects of the BDCP metabolites. The reduction in the total frequency of altered cells after the recovery treatments showed that the test organism *A. cepa* has the ability to recover from damage induced by BDCP and its metabolites after the exposure conditions are normalized.

1. Introduction

Among the different classes of synthetic dyes, the azo dyes have been most widely used, particularly in the textile sector (Kunz et al., 2002). These dyes have one or more azo groups (−N=N−) associated with one or more aromatic rings in their structures (Wong and Yuen, 1996).

The pollution caused by effluents that contain azo dyes can cause serious impacts in the environment (Hao et al., 2000). Organisms exposed to azo dyes have shown different cellular alterations, such as chromosome aberrations and micronucleated cells, which demonstrate the genotoxic and mutagenic effects of the product (Caritá and Marin-Morales, 2008; Phugare et al., 2011; Ventura-Camargo et al., 2011; Fernandes et al., 2015). According to Umbuzeiro et al. (2005), assays performed with *Salmonella* using water samples from a river that receives effluent from a textile

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industry that uses BDCP point to a mutagenic action of this dye. Alves de Lima et al. (2007) showed the mutagenic and carcinogenic potential of a textile azo dye processing plant effluent.

According to Jadhav et al. (2007), Chengalroyen and Dabbs (2013) and Ventura-Camargo and Marin-Morales (2013), different methods for treating effluents that contain azo dyes have been used in an attempt to minimize the problems associated with this type of contamination. The use of biodegradation for the treatment of azo dyes is a process that has been indicated as having great potential for success. However, there is a need to establish an efficient biological treatment that considers the types of enzymes that are able to degrade certain azo dyes (Kunz et al., 2002; Jadhav et al., 2007; Saratale et al., 2011) because they are relatively resistant to biological degradation processes (Martins et al., 2003; Oliveira et al., 2007) due to their complex chemical structure and high light stability (Kim and Shoda, 1999).

Several bacterial species are used for the degradation of azo dyes (Hu, 1998; Saratale et al., 2011; Singh et al., 2015). Under anaerobic conditions, azo dyes can be degraded into recalcitrant aromatic amines (Weber and Adams, 1995). Treatment with bacteria under aerobic conditions is generally efficient at completely mineralizing aromatic amines (Isik and Sponza, 2003; Saratale et al., 2011), although some studies have shown that some azo dyes are more resistant to bacterial attack under aerobiosis conditions (Hu, 1998). Oliveira et al. (2007) showed that BDCP is recalcitrant even under aerobic conditions because components of this compound were detected in effluent samples that were treated by activated sludge systems.

Higher plants are recognized as important biological materials for detecting genotoxic aberrations caused by environmental pollutants (Yi and Meng, 2003). The species *Allium cepa* constitutes one of the pioneer materials in the study of chromosomal aberrations caused by the action of chemical agents (Levan, 1938) and has been used as an efficient test organism because it presents a genetic pattern that is suitable for genotoxicity tests (Fiskesjö, 1985; Fernandes et al., 2009; Leme et al., 2008; Hoshina and Marin-Morales, 2009; Ventura-Camargo et al., 2011). A study performed by Caritá and Marin-Morales (2008) showed the mutagenic potential of certain samples of industrial effluents contaminated by BDCP as well as other pollutants by applying the chromosomal aberration test in *A. cepa*. Another study (Ventura-Camargo et al., 2011) showed the genotoxic potential of different concentrations of BDCP on *A. cepa* meristematic cells.

Chromosomal staining techniques have been successfully applied to chromosomal analyses in plants (Tuna et al., 2004) and may provide information about the DNA composition and its arrangement along chromosomes, enabling a more detailed analysis of the structural organization of chromosomes (Kim et al., 2002). Staining with silver nitrate has been used to identify the number of nucleoli and possible active sites of NOR in different plant species (Carvalho and Guerra, 2002; Ventura-Camargo et al., 2011; Mazzeo and Marin-Morales, 2015). The positions of NORs helps researchers gain a better understanding of the chromosomal alterations that were established in each karyotype (Mazzeo and Marin-Morales, 2015). Thus, in cases in which chromosomal aberrations are induced by genotoxic agents, this technique may provide particular sensitivity by easily diagnosing the probable sites of action of these agents and determining the cell aberrations they caused. Ventura-Camargo et al. (2011) showed that BDCP can act in chromosomal regions of *A. cepa* that were or were not associated with rDNA sites using the NOR-banding technique.

Considering that azo dyes and their related products generally have the potential to cause serious damage in the genetic material of exposed organisms and that their mechanisms of action on DNA need to be further investigated, the aim of this study was to analyze the cytotoxic, genotoxic and mutagenic potential of the commercial black dye in root cells of *A. cepa* before and after the bacterial biodegradation treatment using the conventional cytogenetic staining technique (chromosomal and nuclear aberrations test) and AgNOR-banding.

2. Material and methods

2.1. Chemicals tested

We tested the commercial black dye (BDCP - Black Dye Commercial Product), which is an azo dye composed of 3 dyes that belong to the nitro-aminobenzene group (Umbuzeiro et al., 2005): C.I. Disperse Blue 373 ($C_{21}H_{21}BrN_6O_6$; CAS n° 51868-46-3), C.I. Disperse Violet 93 ($C_{18}H_{19}BrN_6O_5$; CAS n° 268221-71-2) and C.I. Disperse Orange 37 ($C_{17}H_{15}Cl_2N_5O_2$; CAS n° 13301-61-6).

2.2. Treatment solutions

The concentrations of the commercial black dye (50 μg/l and 200 μg/l) were determined by pilot tests to allow viable development of the cytogenetic techniques used in this study. It is important to consider that the concentrations tested are close to those detected in environmental samples derived from a textile industry that used BDCP, as studied by Oliveira et al. (2007).

2.3. Test organism

The assays were performed using seeds of a single variety of *A. cepa*.

2.4. Bacterial biodegradation treatments

The biodegradation treatments were performed using a pool of heterotrophic bacteria obtained from a biological wastewater treatment plant. This bacterial pool was added to the Sabouraud liquid culture medium. The microorganisms were previously incubated in a 35 °C incubator for 3 days. Then, tests were performed in sterilized test tubes, in which the final volume was of 10 mL for each tube, as follows:

- *Blank Tests* - Blank 1: 8.0 mL of culture medium + 2.0 mL of distilled water; Blank 2: 7.9 mL of culture medium + 100 μL of bacterial suspension + 2.0 mL of distilled water.
- *Tests with the commercial black dye* - Concentration of 50 μg/L: 7.9 mL of culture medium + 100 μL of bacterial suspension + 1.5 mL of distilled water + 0.5 mL of the stock solution of 100 mg/L BDCP; concentration of 200 μg/L: 7.9 mL of culture medium + 100 μL of bacterial suspension + 2.0 mL of the stock solution of 100 mg/L BDCP. The stock solution was prepared in distilled water.

The microorganisms were incubated at 35 °C for four more days. Subsequently, all of the tubes were autoclaved at 1 atm for 10 min. After this period, the germination assay was performed using *A. cepa* seeds. All tests were performed in duplicate.

2.5. A. cepa bioassays

A. cepa seeds (100 per dish) were germinated at room temperature (21 ± 4 °C) in Petri dishes covered with filter paper soaked in ultrapure water. When the roots reached 1.5 cm in length, they were transferred to Petri dishes covered with filter paper soaked in 50 μg/l and 200 μg/l BDCP. Tests with and without the bacterial treatment were conducted. The roots of the negative control

(ultrapure water, blank 1 and blank 2) and positive (9×10^{-5} M methyl methanesulfonate) (MMS, Sigma-Aldrich, CAS 66-27-3) control and those exposed to the dye remained in the dishes for a period of 20 h. Then, some roots from each dish were collected, and the rest of them were transferred to dishes covered with filter paper soaked in ultrapure water for periods of 48 and 72 h for the recovery treatments. After the recovery treatments, the remaining roots were collected.

All collected roots were fixed in a mixture of ethanol and acetic acid (3:1 – v/v) for 18 h at room temperature. Then, the roots were stored in a freshly made fixation solution at 4 °C before slides were prepared for the conventional staining technique or AgNOR-banding.

2.5.1. Conventional staining assay

Slides with meristematic root cells were prepared according to the procedure described by Leme and Marin-Morales (2008).

When the slides were analyzed, some cellular abnormalities were observed, such as chromosomal and nuclear aberrations (CNAs). These CNAs were classified as a genotoxicity endpoint (Leme and Marin-Morales, 2009). The presence of micronuclei and chromosome breaks (MN/B) in the meristematic cells of *A. cepa* was considered to be a mutagenic endpoint that was separate from that of the CNAs (Leme et al., 2008). Apoptotic and necrotic cells (AP/NE) were considered cytotoxicity endpoints (Leme et al., 2008). All of the experiments were performed in duplicate, and a total of 10,000 cells were counted per concentration tested per treatment (20, 48 and 72 h) by counting 500 cells per slide from a total of 20 slides evaluated.

A set of slides for the non-meristematic regions (F1 cells) of the *A. cepa* roots was prepared using the procedure described by Leme and Marin-Morales (2008) to verify the frequencies of cells with MN. These experiments were also performed in duplicate, and 2000 cells were counted per concentration tested and per treatment (20, 48 and 72 h) by counting 200 cells per slide from a total of 10 slides evaluated.

2.5.2. AgNOR-banding

The slides for AgNOR-banding were prepared after the radicular meristems were pretreated with an enzymatic solution of cellulase/pectinase (2:20 – v/v) for 60 min. After a bath of 2 min in distilled water, the meristems were sectioned, placed on slides, covered with coverslips and gently mounted in a drop of 45% acetic acid. Then, the slides were frozen in liquid nitrogen and the coverslips were removed. The slides were stained using the protocol proposed by Hizume et al. (1980).

Twenty slides per treatment were prepared to evaluate both cell damage and the variations in the number of nucleoli in interphase cells. Approximately 500 cells were analyzed per slide for a total of 10,000 cells per test for each treatment. All of the experiments were performed in duplicate. Comparisons were performed based on the means of the values for the different tests and treatments.

2.5.3. Statistical analyses

Statistical analyses were performed using the Kruskal-Wallis test, and a probability of 0.05 was accepted to indicate a significant effect.

3. Results and discussion

3.1. Conventional staining assay

The significant occurrence of cell death in the meristematic cells of *A. cepa* indicated that after bacterial biodegradation, BDCP was capable of causing cytotoxic effects in this test organism (Table 1). Studies performed by Bafana et al. (2009) also showed that the metabolites of an azo dye (Direct Red 28) exhibited greater cytotoxic action after bacterial degradation, as these intermediate compounds induced a significant frequency of HL-60 human cells to undergo cell death. On the other hand, a study conducted by Jadhav et al. (2011) showed that a textile azo dye (remazol red) was capable of reducing the cell viability of meristems of *A. cepa*, but this cytotoxicity was diminished after this dye was treated with the bacterium *Pseudomonas aeruginosa*. According to Hoshina and Marin-Morales (2009), the disturbance of the cells by chemicals may lead to a complex sequence of events that can result in cell death, whose main morphological features are nuclear fragmentation, increased cytoplasmic volume, vacuolated cytoplasm, and/or the presence of a heteropyknotic nucleus. Furthermore, according to Donelli et al. (2003), changes in the cell volume are a direct consequence of the alterations in cell membrane permeability, which also seem to be associated with changes in the cytoskeleton. However, the significant reduction in the frequencies of cell death observed after the recovery treatments in ultrapure water (Table 1) indicates that the meristems of *A. cepa* can recover from the cytotoxic damage resulting from both the action of the original BDCP and the metabolites generated after bacterial biodegradation.

The CNAs observed in this study were related to genotoxic events because they represent the damage observed in the genetic material of the cells, but there was no effective proof of fixation in the organism, either because they can eventually be repaired or because they are characterized as injuries that can lead to cell death and are not fixed in the subsequent cellular generations after the damage is established. The high and significant frequencies of CNAs (Table 1) observed in the roots exposed to the different tests and treatments with BDCP with and without microbial action showed the genotoxic action of this chemical and corroborated the data presented by Ventura-Camargo et al. (2011) and Caritá and Marin-Morales (2008), who observed some chromosomal aberrations in meristematic cells of *A. cepa* resulting from the action of BDCP and of effluents containing BDCP, respectively. Moreover, the highest and significant frequencies of CNAs that were observed in the tests with BDCP treated with bacteria indicate that the metabolites generated by the biodegradation of BDCP are more genotoxic than the original commercial black dye. According to Chung and Stevens (1993), the formation of metabolites by microbial action, which resulted from the reduction of the azo bond of the dyes, is associated with genotoxic events. These metabolites could react with DNA in different manners (Bartsch, 1981; Umbuzeiro et al., 2005). According to Levine (1991), they may interfere with the genetic material of the organisms by forming DNA adducts.

The present study disagrees with some studies that showed that the metabolites generated after the bacterial biodegradation of azo dyes are less genotoxic than the original dye. The AMES test verified complete detoxification of the sulfonated azo dye red HE7B by *Bacillus* sp. VUS (Dawkar et al., 2010). Jadhav et al. (2011) showed that the sulfonated azo dye remazol red (500 mg/l) that had been biodegraded by the bacterium *Pseudomonas aeruginosa* was less genotoxic than the original compound because the frequency of chromosomal aberrations was significantly reduced in the meristematic cells of *A. cepa* bulb roots of that had been treated with the biodegraded dye. A concentration of 500 mg/l of the textile azo dye redHE3B (reactive red 120) that had been previously treated with a bacterial consortium (*Providencia* sp. and *Pseudomonas aeruginosa*) was also proven to be less genotoxic toward *A. cepa* cells, showing that the azo dye subjected to bacterial treatment significantly decreased the frequencies of chromosomal aberrations (chromosomal aberration assay) and DNA damage (comet assay) compared to the original dye (Phugare et al., 2011). Prasad and Rao (2013)

Table 1
Frequency (%) of alterations (CNA, MN/B and AP/NE) observed in *A. cepa* meristematic cells exposed to the different concentrations of the BDCP, without and with bacterial biodegradation, before and after recovery treatments.

Treatment period		NC	PC	BDCP concentration (μg/L)		Culture medium	Bacterial biodegradation		
				50	200		B	B + A50	B + A200
20 h									
CNA	GML	0.170 ± 0.016	1.040 ± 0.012[a]	1.310 ± 0.021[a]	1.390 ± 0.020[a]	0.330 ± 0.015	0.210 ± 0.011	0.330 ± 0.013	0.250 ± 0.016[b]
	CL	0.020 ± 0.010	0.130 ± 0.013	0.130 ± 0.013	0.390 ± 0.019[a]	0.100 ± 0.021	0.040 ± 0.009	0.120 ± 0.016	0.380 ± 0.016[a]
	CA	0.020 ± 0.010	0.180 ± 0.010	0.290 ± 0.018[a]	0.340 ± 0.018[a]	0.170 ± 0.016	0.490 ± 0.026[a]	1.800 ± 0.018[ab]	0.670 ± 0.019[a]
	NB	0	0.090 ± 0.011	0.060 ± 0.014	0.070 ± 0.021	0.410 ± 0.017[a]	0.450 ± 0.012[a]	0.900 ± 0.024[ab]	1.820 ± 0.024[ab]
	CM	0.050 ± 0.010	0.050 ± 0.010	0.050 ± 0.010	0.300 ± 0.015[a]	0.110 ± 0.014	0.250 ± 0.014	0.250 ± 0.024	0.740 ± 0.026[a]
	PM	0.010 ± 0.011	0.120 ± 0.013	0.120 ± 0.014	0.160 ± 0.021[a]	0	0	0	0.400 ± 0.018[a]
	PI	0.010 ± 0.011	0.050 ± 0.015	0.120 ± 0.020	0	0.020 ± 0.022	0.040 ± 0.025	0.020 ± 0.016	0.250 ± 0.020
	CB	0.030 ± 0.010	0.170 ± 0.014	0.110 ± 0.013	0.130 ± 0.016	0.060 ± 0.009	0.110 ± 0.024	0.080 ± 0.009	0.040 ± 0.009
	MU	0.020 ± 0.010	0.080 ± 0.013	0.110 ± 0.013	0.140 ± 0.016	0.040 ± 0.009	0.020 ± 0.009	0.040 ± 0.009	0.110 ± 0.014
	BI	0	0.190 ± 0.012[a]	0.080 ± 0.016	0.060 ± 0.009	0	0.020 ± 0.014	0.320 ± 0.021[a]	0.480 ± 0.013[ab]
	CNA	0.330 ± 0.015	2.100 ± 0.015[a]	2.380 ± 0.021[a]	2.980 ± 0.016[a]	1.240 ± 0.014	1.630 ± 0.013	3.860 ± 0.013[a]	5.140 ± 0.012[a]
MNB	MN	0.210 ± 0.018	2.220 ± 0.011[a]	1.170 ± 0.013[a]	1.060 ± 0.027[a]	0.480 ± 0.021	0.230 ± 0.015	0.290 ± 0.013	0.510 ± 0.019
	CBr	0.050 ± 0.019	0.440 ± 0.028	0.220 ± 0.019	0.280 ± 0.020	0.140 ± 0.020	0.040 ± 0.017	0.070 ± 0.019	0
	MNB	0.260 ± 0.010	2.660 ± 0.022[a]	1.390 ± 0.036[a]	1.340 ± 0.018[a]	0.620 ± 0.019	0.270 ± 0.021	0.360 ± 0.024	0.510 ± 0.019
APNE	AP	0	0.030 ± 0.014	0	0.110 ± 0.000	0	0	0	0
	NE	0	0.780 ± 0.033[a]	0.990 ± 0.051[a]	1.190 ± 0.038[a]	0.110 ± 0.021	0.210 ± 0.033	1.820 ± 0.045[a]	2.190 ± 0.056[a]
	APNE	0	0.810 ± 0.044[a]	0.990 ± 0.051[a]	1.300 ± 0.055[a]	0.110 ± 0.021	0.210 ± 0.033	1.820 ± 0.045[a]	2.190 ± 0.056[a]
Total		**0.590 ± 0.043**	**5.570 ± 0.026**	**4.760 ± 0.057**	**5.620 ± 0.062**	**1.970 ± 0.034**	**2.110 ± 0.036**	**6.040 ± 0.038**[a]	**7.840 ± 0.041**[a]
Recovery-48 h									
CNA	GML	0.240 ± 0.018	1.800 ± 0.014[ac]	0.810 ± 0.034	0.920 ± 0.031	0.110 ± 0.020	0.110 ± 0.019	0.110 ± 0.024[c]	0.250 ± 0.015
	CL	0.080 ± 0.010	0.250 ± 0.014	0.070 ± 0.010	0.160 ± 0.013	0.060 ± 0.009	0.080 ± 0.013	0.130 ± 0.018	0.160 ± 0.018
	CA	0.070 ± 0.010	0.130 ± 0.013	0.280 ± 0.019	0.140 ± 0.014	0.080 ± 0.014	0.110 ± 0.019	0.990 ± 0.026[a]	0.720 ± 0.021[a]
	NB	0.010 ± 0.014	0.080 ± 0.016	0.060 ± 0.019	0.060 ± 0.020	0.600 ± 0.014[a]	0.750 ± 0.024[a]	0.500 ± 0.036[abc]	0.660 ± 0.018[ab]
	CM	0.020 ± 0.010	0.250 ± 0.015[ac]	0.080 ± 0.010	0.100 ± 0.012[c]	0.150 ± 0.018	0.300 ± 0.019[a]	0.150 ± 0.018	0.190 ± 0.014
	PM	0.010 ± 0.011	0[c]	0.060 ± 0.014	0.100 ± 0.022	0	0	0	0.040 ± 0.012[c]
	PI	0	0.100 ± 0.017	0.070 ± 0.018	0.150 ± 0.019[ac]	0.040 ± 0.020	0	0.060 ± 0.010	0.130 ± 0.019
	CB	0	0.310 ± 0.021[a]	0.150 ± 0.017[a]	0.100 ± 0.022	0.020 ± 0.015	0.020 ± 0.010	0.120 ± 0.005	0.210 ± 0.014[a]
	MU	0.020 ± 0.010	0.170 ± 0.012[a]	0.060 ± 0.010	0.100 ± 0.014	0	0.040 ± 0.013	0.100 ± 0.015	0.080 ± 0.012
	BI	0	0.100 ± 0.014	0.060 ± 0.009	0.070 ± 0.009	0.080 ± 0.009	0	0.080 ± 0.012[c]	0.140 ± 0.010[ac]
	CNA	0.450 ± 0.018	3.190 ± 0.013[ac]	1.700 ± 0.022	1.900 ± 0.016[a]	1.140 ± 0.020	1.410 ± 0.013	2.240 ± 0.019[a]	2.580 ± 0.013[ac]
MNB	MN	0.210 ± 0.017	2.450 ± 0.018[a]	0.890 ± 0.019	1.110 ± 0.017[a]	0.230 ± 0.021	0.15 ± 0.014	0.210 ± 0.023	0.420 ± 0.020
	CBr	0.010 ± 0.013	0.300 ± 0.015[a]	0.130 ± 0.012	0.150 ± 0.021	0	0.02 ± 0.024	0.020 ± 0.023	0.020 ± 0.020
	MNB	0.220 ± 0.012	2.750 ± 0.049[a]	1.020 ± 0.025	1.260 ± 0.068[a]	0.230 ± 0.063	0.17 ± 0.022	0.230 ± 0.036	0.440 ± 0.045
APNE	AP	0	0	0	0	0	0	0	0.040 ± 0.022
	NE	0	0.060 ± 0.027[c]	0.050 ± 0.022[c]	0.460 ± 0.039[ac]	0.290 ± 0.025	0.73 ± 0.015[a]	0.500 ± 0.044[abc]	0.270 ± 0.033[c]
	APNE	0	0.060 ± 0.027[c]	0.050 ± 0.022[c]	0.460 ± 0.039[ac]	0.290 ± 0.025	0.73 ± 0.015[a]	0.500 ± 0.044[abc]	0.310 ± 0.047[c]
Total		**0.670 ± 0.045**	**6.000 ± 0.042**[a]	**2.770 ± 0.037**	**3.620 ± 0.056**	**1.660 ± 0.023**	**2.31 ± 0.041**	**2.970 ± 0.025**	**3.330 ± 0.036**
Recovery-72 h									
CNA	GML	0.190 ± 0.013	1.190 ± 0.012[a]	0.370 ± 0.021[c]	0.690 ± 0.027	0.020 ± 0.010	0.120 ± 0.016	0.150 ± 0.015	0.200 ± 0.014
	CL	0.040 ± 0.014	0.390 ± 0.012[ac]	0.140 ± 0.014	0.130 ± 0.016	0.020 ± 0.010	0.130 ± 0.016	0.020 ± 0.009[c]	0.140 ± 0.012
	CA	0.070 ± 0.010	0.090 ± 0.012	0.270 ± 0.020	0.220 ± 0.015	0.080 ± 0.018	0.060 ± 0.009[c]	0.270 ± 0.015[ac]	0.920 ± 0.018[a]
	NB	0	0.080 ± 0.015	0.070 ± 0.013	0.070 ± 0.021	0.350 ± 0.022[a]	0.330 ± 0.011[a]	0.210 ± 0.016[ac]	0.470 ± 0.028[abc]
	CM	0.030 ± 0.010	0.150 ± 0.018	0.090 ± 0.010	0.130 ± 0.015	0.040 ± 0.010	0.150 ± 0.018	0.100 ± 0.013	0.330 ± 0.024[a]
	PM	0	0.030 ± 0.012	0.090 ± 0.014	0.090 ± 0.025	0	0	0.040 ± 0.012	0.020 ± 0.022[c]
	PI	0	0.080 ± 0.020	0.120 ± 0.014	0.160 ± 0.022[ac]	0.120 ± 0.020	0.040 ± 0.016	0.040 ± 0.016	0.210 ± 0.030[a]
	CB	0.010 ± 0.013	0.110 ± 0.021	0.080 ± 0.015	0.130 ± 0.021[a]	0.040 ± 0.014	0.060 ± 0.021	0.080 ± 0.023	0.140 ± 0.022[a]
	MU	0.040 ± 0.012	0.020 ± 0.015	0.080 ± 0.016	0.140 ± 0.022	0	0.020 ± 0.009	0	0.100 ± 0.008
	BI	0	0.040 ± 0.014[c]	0.060 ± 0.019	0.090 ± 0.015	0	0	0.080 ± 0.013[c]	0.120 ± 0.017[c]
	CNA	0.380 ± 0.014	2.180 ± 0.011[a]	1.370 ± 0.018[c]	1.850 ± 0.017[ac]	0.670 ± 0.016[c]	0.910 ± 0.011[c]	0.990 ± 0.013[c]	2.650 ± 0.011[ac]
MNB	MN	0.250 ± 0.018	2.060 ± 0.016[a]	0.500 ± 0.011[c]	1.040 ± 0.017	0.040 ± 0.010[c]	0.080 ± 0.009[c]	0.250 ± 0.011	0.310 ± 0.018
	CBr	0.010 ± 0.020	0.130 ± 0.017[c]	0.080 ± 0.015	0.130 ± 0.004	0.020 ± 0.024	0	0	0.020 ± 0.010
	MNB	0.260 ± 0.014	2.190 ± 0.047[a]	0.580 ± 0.045[c]	1.170 ± 0.039	0.060 ± 0.009[c]	0.080 ± 0.016[c]	0.250 ± 0.033	0.330 ± 0.054[c]
APNE	AP	0	0.020 ± 0.024	0.050 ± 0.019	0.020 ± 0.025	0	0	0	0.140 ± 0.037
	NE	0.050 ± 0.034	0.300 ± 0.019	0.240 ± 0.026[c]	0.460 ± 0.033[ac]	0.150 ± 0.024	0	0.090 ± 0.039[c]	0.390 ± 0.028[ac]
	APNE	0.050 ± 0.034	0.320 ± 0.046	0.290 ± 0.055[c]	0.480 ± 0.061[ac]	0.150 ± 0.024	0	0.090 ± 0.039[c]	0.530 ± 0.029[ac]
Total		**0.690 ± 0.024**	**4.690 ± 0.039**[a]	**2.240 ± 0.044**[c]	**3.500 ± 0.051**	**0.880 ± 0.019**	**0.990 ± 0.014**	**1.330 ± 0.026**[c]	**3.510 ± 0.029**

Note. NC. Negative Control; **PC.** Positive Control; **B.** Bacterial suspension; **B + A50.** Bacteria associated to the culture medium and 50 μg/L azodye; **B + A200.** Bacteria associated to the culture medium and 200 μg/L azodye; **CNA.** Chromosome and nuclear aberrations; **GML.** Genetic material losses; **CL.** Chromosome losses; **CA.** Chromosome adherences; **NB.** Nuclear buds; **CM.** C-metaphases; **PM.** Polyploidized metaphases; **PI.** Polyploidized interphases; **CB.** Chromosome bridges; **MU.** Multipolarities; **BI.** Binucleated cells; **MN/B.** Micronuclei and chromosome breaks; **MN.** Micronuclei; **CBr.** Chromosome breaks; **APNE.** Apoptotic and necrotic cells; **AP.** Apoptotic cells; **NE.** Necrotic cells; **Total.** Total of CNA, MNB and APNE.
10,000 cells analyzed for each concentration and treatment. Average ± Standard Deviation.
[a] Significantly different from the NC ($p < 0.05$), [b] Significantly different from the tests without biodegradation ($p < 0.05$), and [c] Significantly different from the 20 h treatment ($p < 0.05$), according to the Kruskal-Wallis test.

showed that the metabolites of the azo dye direct red-22 derived from aerobic biodegradation by *Bacillus cohnii* MTCC 3616 had negligible cytotoxic and genotoxic effects on *A. cepa* meristematic root tip cells. After the recovery treatments, there was evidence of a reduction in CNA frequencies, as was observed for all of the tests with BDCP (Table 1), which indicates a that *A. cepa* cells may have been able to reverse the genotoxic damage induced by the commercial black dye.

By considering each type of CNA separately, it was also evident in the 20 h treatment (Table 1) that higher concentrations of the commercial dye induced a significant increase in the CNAs. In addition, significant frequencies of chromosome loss, chromosome adherence, C-metaphase and polyploid metaphases were registered for the tests performed with BDCP. The greater induction of alterations, such as nuclear buds and binucleated cells, for the assays performed after bacterial biodegradation of the highest concentrations of BDCP confirms a more pronounced genotoxic action for this concentration after biodegradation. Regarding the types of CNAs, the most frequent abnormalities caused by the original BDCP were losses of genetic material, whereas the most evident types of cellular alterations after biodegradation were chromosome adherences, nuclear buds and binucleated cells. In the latter case, the types of CNAs must have preferentially originated from the BDCP metabolites. Furthermore, the persistence of the high and significant values of chromosome adherences in the test with the highest concentration of the commercial black dye after biodegradation and recovery reinforces the evidence that shows that the metabolites of this chemical promoted greater genotoxic damage in *A. cepa* meristematic cells than the commercial black dye that was not bioremediated with these microorganisms.

According to Leme and Marin-Morales (2008), chromosome adherences are common signs of the toxic action of a chemical on genetic material. The effects of this type of alteration are irreversible for cells and can even result in cell death. As shown in Table 1, these alterations (death) were also registered in our analyses. This effect could be induced by BDCP or its metabolites, which prevent chromosomes from migrating to the poles due to their aneugenic action. This action blocks cell division in metaphase following chromosome adherences. For Marcano et al. (2004), these alterations produce bridges and, consequently, chromosome breaks. Chromosome bridges can also result from cohesive chromosomal endings or structural rearrangements (Fiskesjö, 1993). Moreover, chromosome adherences can also lead to chromosome losses (Marcano et al., 2004), which are also considered to be potential causes of aneuploid and polyploid cells. The significant presence of losses of genetic material in prophase in the tests with the non-biodegraded dye and chromosome losses in metaphase, anaphase and telophase for the highest concentration of BDCP with and without bacterial degradation (20 h treatments) (Table 1) again suggest that both BDCP and its metabolites display aneugenic activity.

C-metaphase can be produced by aneugenic agents, which promote the complete inactivation of the mitotic spindle of the cell (Fiskesjö, 1985, 1993), generating other types of alterations, such as binucleated cells, cells with micronuclei (Fernandes et al., 2007), and polyploid cells (Fernandes et al., 2009). In the present study, c-metaphase was observed at significant frequencies after a 20 h treatment with the highest concentration of BDCP without bacterial biodegradation and with biodegraded BDCP after 72 h of recovery (Table 1), corroborating the studies of Caritá and Marin-Morales (2008), who showed the presence of c-metaphase and cells with micronuclei after *A. cepa* seeds were exposed to effluents contaminated with the BDCP. These results show that both BDCP and its metabolites from bacterial degradation can interfere with the formation of the mitotic spindle by first inhibiting the organization of the equatorial plate and, consequently, the division of the centromeres, demonstrating proving their aneugenic actions. It is important to also consider that the c-metaphases induced by the treatment with the commercial black dye that had been treated with the microorganisms showed more packed and shorter chromosomes than those induced by the treatment with the dye without bacterial biodegradation (Fig. 1A–B). The presence of this abnormality could be related to the significant frequencies of binucleated and polyploid cells in the roots exposed to BDCP after bacterial degradation (Table 1).

According to Fernandes et al. (2007), nuclear buds may be derived from c-metaphase and are alterations that can lead to polyploidization and, consequently, to the formation of micronuclei. According to Shimizu et al. (1998), nuclear buds may also be the result of cellular activities that promote the elimination of the amplified genetic material. Based on analyses in human lymphocytes (Shimizu et al., 1998) and in *A. cepa* (Fernandes et al., 2007), these authors suggest that amplified DNA from polyploid cells form buds and micronuclei, which may be expelled in the form of "mini cells". In our experiments, significant frequencies of nuclear buds were observed in meristematic cells of *A. cepa* exposed to BDCP after bacterial biodegradation (Table 1; Fig. 1C). We believe that these nuclear buds will be later eliminated in the form of micronuclei, which might be formed by the expulsion of the amplified genetic material and by the additional chromosomes derived from aneuploid cells. Finally, the significant occurrence of nuclear buds could be related to the higher intensity of BDCP aneugenic action after bacterial biodegradation treatment, which proves the more drastic action of its metabolites on the exposed cells.

The mutagenicity of BDCP was estimated by recording the induction of MN/B in meristematic cells of *A. cepa* after exposure to different concentrations of BDCP (Table 1). These alterations are closely associated with mutagenic events because they are alterations in chromosomes or DNA that can be passed on to new cellular generations and can thus be fixed in the organisms (Ventura et al., 2008). The present study showed significant frequencies of MN/B in meristematic cells that were exposed to BDCP for 20 h before the bacterial biodegradation process, as well as in cells that were exposed to the highest concentration of the commercial black dye that was not treated with these microorganisms and then subjected to the 48 h recovery treatment (Table 1). These data show that this black dye is mutagenic at these concentrations and under these conditions, corroborating the studies performed by Ventura-Camargo et al. (2011). As the chromosome breaks were not significant alterations, the mutagenic effect of BDCP was assigned to the high and significant frequency of micronucleated cells. Oliveira et al. (2007) associated the high values of mutagenic activity in *Salmonella* to the presence of the commercial black dye in environmental samples of Ribeirão dos Cristais. Studies conducted by Tsuboy et al. (2007) and Chequer et al. (2009) associated the high frequencies of azo dye-induced MN in animal cells to the mutagenic action of these dyes. Our data show a decrease in the frequencies of MN/B after the recovery treatments, which were only significant at the lower concentration of BDCP in the 72 h treatment (Table 1), indicating that the mutagenic action of the highest concentration of this chemical has not completely ceased, even after the test organism is no longer exposed to this dosage of the BDCP.

The absence of significant numbers of meristematic cells bearing MN/B, which were verified in the tests with commercial black dye that had been biodegraded by bacteria (Table 1), suggest that the metabolites of this chemical were associated more with the induction of genotoxicity than with mutagenic alterations in these cells. However, due to the high frequencies of MN recorded for the F_1 cells that were subjected to a 20 h exposure to BDCP that had been biodegraded by bacteria followed by 48 h of recovery (Table 2), it can be inferred that the metabolites of this azo dye promote genotoxic alterations in the meristematic cells that are fixed in the form of cells bearing MN in the F_1 generation, thus confirming the mutagenic effect of these metabolites on *A. cepa* cells. The reduction in the frequencies of these alterations after the 72 h recovery period (Table 2) suggests that the effects of the BDCP metabolites may not be cumulative after the exposure conditions

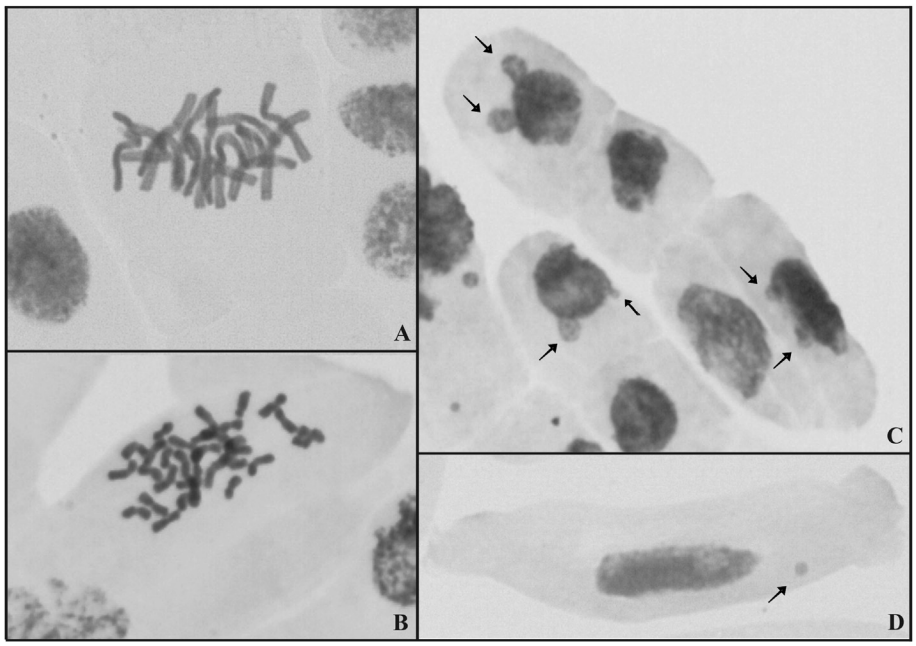

Fig. 1. Cellular abnormalities observed in *Allium cepa* cells exposed to BDCP (Black Dye Commercial Product). **A**. C-metaphase (without bacterial biodegradation); **B**. C-metaphase (with bacterial biodegradation); **C**. Interphasic cell with nuclear buds (arrows); **D**. Micronuclei in F1 cell (arrow).

Table 2
Frequency (%) of MN observed in F_1 cells of *A. cepa* roots exposed to the different concentrations of the BDCP, without and with bacterial biodegradation, before and after recovery treatments.

Treatment period	NC	PC	BDCP concentration (μg/L)		Culture medium	Bacterial biodegradation		
			50	200		B	B + A50	B + A200
20 h	0.390 ± 0.069	9.310 ± 0.072[a]	5.020 ± 0.061[a]	5.680 ± 0.057[a]	0.960 ± 0.070	2.050 ± 0.071	4.380 ± 0.056[a]	4.610 ± 0.052[a]
Recovery-48 h	0.290 ± 0.073	8.070 ± 0.066[a]	2.060 ± 0.054[c]	9.380 ± 0.057[ac]	0.790 ± 0.071	0.890 ± 0.066	2.790 ± 0.058[ac]	3.420 ± 0.057[a]
Recovery-72 h	0.290 ± 0.073	4.820 ± 0.056[ac]	1.480 ± 0.062[c]	5.910 ± 0.054[a]	0.780 ± 0.088	0.390 ± 0.049[c]	1.470 ± 0.085[c]	1.180 ± 0.077[bc]

Note. NC. Negative Control; **PC.** Positive Control; **B.** Bacterial suspension; **B + A50.** Bacteria associated to the culture medium and 50 μg/L azodye; **B + A200.** Bacteria associated to the culture medium and 200 μg/L azodye.
2000 cells analyzed for each concentration and treatment. Average ± Standard Deviation.
[a] Significantly different from the NC ($p < 0.05$), [b] Significantly different from the tests without biodegradation ($p < 0.05$), and [c] Significantly different from the 20 h treatment ($p < 0.05$), according to the Kruskal-Wallis test.

are normalized by placing the roots in ultrapure water.

The present study could also confirm the mutagenic action of BDCP by revealing high frequencies of MN in F_1 cells submitted to a 20 h BDCP treatment without biodegradation by microorganisms (Table 2; Fig. 1D). The persistence of significant and even higher frequencies of MN in the F1 cells after recovery from the 200 μg/L BDCP treatments (Table 2; Fig. 1D) indicated that the harmful effects observed in the meristematic cells were transferred or fixed to the F1 cells. These mutagenic effects indicate a potential danger of this chemical to the environment because this dosage is probably available in aquatic ecosystems, as it is close to the values that Oliveira et al. reported in environmental samples of industrial effluents (2007).

Overall, based on the cellular abnormalities (CNA, MN/B and AP/NE) observed after the 20 h treatment (Table 1) with BDCP before and after biodegradation, it was possible to observe a greater toxic potential of the commercial black dye after bacterial action. This result reinforces a more drastic action of the metabolites (including the aromatic amines that were produced from the reduction of the azo bonds) than the dye itself, although both are involved in the induction of the cellular alterations described above. The present study confirms the results reported by Bell et al. (2000), who stated that the increased danger of the secondary compounds generated by microbial degradation results from their higher toxic, mutagenic and carcinogenic potentials compared to the original compound. The reduction in the total frequency of altered cells after the recovery treatments (Table 1) shows that the meristems of *A. cepa* are able to recover from the damage induced by the BDCP and its metabolites.

3.2. AgNOR-banding

According to Mazzeo and Marin-Morales (2015), nucleolar alterations resulting from the action of either chemical or physical agents can serve as important biomarkers of genotoxicity. After the meristematic cells of *A. cepa* were exposed to BDCP before and after bacterial biodegradation, it was verified that most of the micronuclei presented a nucleolus organizer region. Studies performed by Cermeño et al. (1984) showed that the loss or inactivation of NORs can be related to deletions or translocations. The absence or presence of NORs in the micronuclei of *A. cepa* meristematic cells shows that the dye and its metabolites can act randomly on chromosomes, and therefore may be directly involved at DNAr sites, which are indispensable in transcription and protein translation. These results corroborate studies performed by Ventura-Camargo et al. (2011), which showed that different concentrations of the

Table 3
Frequency (%) of nucleolus quantity observed in *A. cepa* meristematic cells exposed to the different concentrations of the BDCP, without and with bacterial biodegradation, before and after recovery treatments.

Treatment period	Nucleolus per cell	NC	PC	BDCP concentration (μg/L)		Culture medium	Bacterial biodegradation		
				50	200		B	B + A50	B + A200
20 h	1	10.570 ± 0.011	12.410 ± 0.011	13.200 ± 0.011	13.430 ± 0.012	14.220 ± 0.010[a]	13.600 ± 0.010	15.190 ± 0.010[a]	13.060 ± 0.011
	2	43.620 ± 0.011	45.370 ± 0.010	40.930 ± 0.010	43.090 ± 0.010	40.100 ± 0.010	38.570 ± 0.010	39.310 ± 0.010	32.900 ± 0.010[ab]
	3	43.320 ± 0.010	36.420 ± 0.010[a]	37.860 ± 0.010	36.070 ± 0.011[a]	39.940 ± 0.010	42.040 ± 0.010	35.590 ± 0.010[a]	40.440 ± 0.010
	4	1.920 ± 0.014	4.580 ± 0.031	6.820 ± 0.024[a]	5.910 ± 0.012[a]	4.580 ± 0.010	4.020 ± 0.010	8.080 ± 0.011[a]	10.180 ± 0.011[a]
	5	0.570 ± 0.014	0.780 ± 0.012	1.070 ± 0.024	1.000 ± 0.016	0.810 ± 0.014	1.250 ± 0.011	1.250 ± 0.016	2.980 ± 0.012[ab]
	6	0	0.280 ± 0.010[a]	0.100 ± 0.012	0.460 ± 0.009[a]	0.340 ± 0.010[a]	0.510 ± 0.010[a]	0.400 ± 0.012[ab]	0.460 ± 0.010[a]
	7	0	0.130 ± 0.012[a]	0.020 ± 0.010	0.030 ± 0.011	0	0	0.180 ± 0.010[a]	0
	8	0	0.030 ± 0.020	0	0.010 ± 0.011	0	0	0	0
Recovery-48 h	1	10.740 ± 0.012	14.270 ± 0.012	10.690 ± 0.012[c]	12.630 ± 0.012	10.080 ± 0.011[c]	12.720 ± 0.010	12.710 ± 0.011[c]	12.380 ± 0.010
	2	39.580 ± 0.010[c]	46.470 ± 0.011[a]	36.140 ± 0.011	34.910 ± 0.011	33.490 ± 0.011[c]	42.880 ± 0.010[c]	44.790 ± 0.010[abc]	42.410 ± 0.010[c]
	3	37.280 ± 0.010[c]	25.990 ± 0.012[ac]	39.420 ± 0.010	37.960 ± 0.009[c]	45.040 ± 0.010[ac]	35.660 ± 0.010[c]	33.990 ± 0.010[b]	36.250 ± 0.010[c]
	4	11.560 ± 0.014[c]	9.910 ± 0.012[c]	9.780 ± 0.018	10.690 ± 0.017	10.040 ± 0.015[c]	6.400 ± 0.010[ac]	7.330 ± 0.011	8.280 ± 0.010[c]
	5	0.780 ± 0.012	2.860 ± 0.025[ac]	2.870 ± 0.015[ac]	2.410 ± 0.018[ac]	1.130 ± 0.017	1.620 ± 0.015	0.850 ± 0.024[b]	0.680 ± 0.019[bc]
	6	0.060 ± 0.010	0.490 ± 0.014[a]	1.050 ± 0.010[a]	1.370 ± 0.012[a]	0.220 ± 0.014	0.730 ± 0.011[a]	0.340 ± 0.014	0[bc]
	7	0	0[c]	0.050 ± 0.011	0.030 ± 0.010	0	0	0[c]	0
	8	0	0	0	0	0	0	0	0
Recovery-72 h	1	11.340 ± 0.012	16.350 ± 0.010[ac]	12.970 ± 0.010	12.750 ± 0.011	12.110 ± 0.010	13.920 ± 0.010	12.030 ± 0.011[c]	12.600 ± 0.011
	2	42.580 ± 0.010	43.030 ± 0.011	34.550 ± 0.012[a]	36.210 ± 0.012[c]	38.360 ± 0.011	36.130 ± 0.010[a]	30.190 ± 0.010[ac]	38.190 ± 0.011
	3	40.690 ± 0.010	34.070 ± 0.011[a]	38.950 ± 0.010	37.400 ± 0.010	40.130 ± 0.010	36.730 ± 0.011[c]	41.550 ± 0.010[c]	37.190 ± 0.010[c]
	4	4.930 ± 0.013[c]	6.130 ± 0.012	11.430 ± 0.021	11.840 ± 0.019	8.280 ± 0.013[c]	11.750 ± 0.011[ac]	13.680 ± 0.010[ac]	10.470 ± 0.012[a]
	5	0.300 ± 0.014[c]	0.260 ± 0.022[c]	1.920 ± 0.011[ac]	1.480 ± 0.013[a]	0.990 ± 0.016	1.290 ± 0.012	2.140 ± 0.011[ac]	1.410 ± 0.012[a]
	6	0.160 ± 0.010[c]	0.160 ± 0.012	0.150 ± 0.015	0.270 ± 0.020	0.140 ± 0.009[c]	0.180 ± 0.011[c]	0.400 ± 0.014[ab]	0.140 ± 0.010[c]
	7	0	0.020 ± 0.010[c]	0.050 ± 0.009	0	0	0	0[c]	0
	8	0	0	0.010 ± 0.011	0	0	0	0	0

Note. NC. Negative Control; **PC.** Positive Control; **B.** Bacterial suspension; **B + A50.** Bacteria associated to the culture medium and 50 μg/L azodye; **B + A200.** Bacteria associated to the culture medium and 200 μg/L azodye.
10,000 cells analyzed for each concentration and treatment. Average ± Standard Deviation.
[a] Significantly different from the NC ($p < 0.05$), [b] Significantly different from the tests without biodegradation ($p < 0.05$), and [c] Significantly different from the 20 h treatment ($p < 0.05$), according to the Kruskal-Wallis test.

original dye act in several chromosomal regions, whether or not they contain DNAr sites. Based on the significant numbers of dead cells and micronuclei bearing NORs observed in the tests performed with the dye before and after bacterial biodegradation, it could be inferred that the loss and, consequently, the lack of chromosomes bearing NORs in the main nucleus can inhibit the physiological activities of the cell, thus leading to death.

According to Arkhipchuk et al. (2000), Ventura-Camargo et al. (2011) and Mazzeo and Marin-Morales (2015), the variation in the number of nucleoli in plants is a result of the action of genotoxic agents. In the present study, the analysis of the number of nucleoli in the *A. cepa* meristematic cells treated with BDCP (Table 3) showed that the test was an efficient assessment of the genotoxicity of BDCP. A significantly increased number of nucleoli per cell was observed in the cells treated with BDCP compared to the negative control (ultrapure water). Treatments with 200 μg/L non-bioremediated BDCP or the dye that had been bioremediated with bacteria produced significant increases in the frequencies of cells with 6 nucleoli after a 20 h treatment. Moreover, treatment with 50 μg/L bacterially bioremediated BDCP produced a significant increase in the number of cells with 7 nucleoli. These data suggest that the number of nucleoli observed in this study resulted from the action of the BDCP and its bacterial biodegradation products on cells, confirming the genotoxic action of BDCP and its metabolites, which has already been described for other tests. The recovery treatments minimized the genotoxic effects of these compounds on cells because a significant decrease in the number of cells with 6 nucleoli (treated with 200 μg/L bioremediated BDCP) and 7 nucleoli (treated with 50 μg/L bacterially bioremediated BDCP) was observed after the recovery period (Table 3).

4. Conclusions

The increased cytotoxic, genotoxic and mutagenic potential of BDCP after bacterial biodegradation are probably due to the formation of aromatic amines resulting from the cleavage of azo bonds.

Although the recovery treatments reduced the frequencies of cytotoxic, genotoxic and mutagenic damage, the effects of the dye and its metabolites were not completely eliminated. These results are a cause for concern, particularly the high rates of cell death promoted by the dye and its metabolites, as well as the proven fixation of the damages caused in cells.

The chromosomal and nuclear aberration assays were very useful in providing information about the different mechanisms of action of BDCP before and after bacterial degradation. The AgNOR-banding technique provided some additional information about the genotoxic action of the dye and its metabolites by showing a higher variation in the number of nucleoli in cells treated with BDCP compared with the negative control. Thus, the AgNOR-banding technique is indicated as a complementary methodology for analyzing the toxicity of environmental chemicals.

Our data show that the bacterial biodegradation process is not always efficient and that it can even be more detrimental to the environment by generating toxic metabolites, thus indicating the urgent need to evaluate the effectiveness of biological treatments for industrial effluents.

Conflict of interest

The authors declare that they have no conflict of interest.

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