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Use of protista and algae to study toxicity of dye compounds

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Summary

Comparative assessment of the toxicological effects of two azo dyes [Reactive Orange 16 (RO16); Congo Red (CR)] and two anthraquinone dyes [Remazol Brilliant Blue R (RBBR); Disperse Blue 3 (DB3)] was performed on two *in vitro* cell models, the ciliate protozoa *Tetrahymena pyriformis* and the microalga *Selenastrum capricornutum*. Growth impairment, viability assay using calcein AM and ethidium homodimer-1 (CAM/EthD-1 assay), grazing and morphometric assay were the tests performed on *T. pyriformis* using 48 h-tests. They represented simple and fast bioassays providing overall information on the morphological and physiological state of the cells exposed to different dyes. The algal test measured growth inhibition after a 96-h exposure.

The anthraquinone dye DB3 was found to be the most toxic dye among all the dyes tested. The EC50 value of 0.5 ± 0.0 mg/l detected in the algal test was 10 to 100-fold lower compared to other dyes tested.

Keywords: Textile dyes; Toxicity bioassays; *Tetrahymena pyriformis*; *Selenastrum capricornutum*

Introduction

Dyes are used in a wide variety of materials including, paper, ink, lacquers, varnishes, plastics, cosmetics, some food items, and textiles. Among the chemical classes of dyes the azo group are the largest and most versatile. Azo dyes are often used for dyeing natural and synthetic substrates. Therefore a broad range of colours can be produced

with excellent fastness properties (Gomes, 2001). Anthraquinone dyes are also a wide group that includes vat dyes, which can be used on cotton and cellulose fibres, and disperse anthraquinones, often applied to coloration of hydrophobic, synthetic materials such as cellulose acetate, nylon, polyesters, and acrylics (Kirk and Othmer, 1993).

Most dyes used in textile industry are synthesized from aromatic amines that have often been shown to have a carcinogenic effect. This effect is, at present, the far most important factor considering the manufacture of colorants and their intermediates; this is why there has been a great awareness about the toxicity hazards associated with this industry. These hazards are directly or indirectly related to acute or chronic toxicities and depend on the nature of the corresponding molecule.

Reduced use of experimental animals, low cost and rapid performance are the benefits that make the *in vitro* cytotoxicity techniques increasingly used as the alternatives to whole animal testing of environmental pollutants (Olabarrieta *et al.*, 2001). Microorganisms are the lowest biological level affected by the discharge of pollutants in an ecosystem and the employment of *in vitro* methods using these organisms is of particular interest (Repetto *et al.*, 2001).

Among protozoa, *Tetrahymena pyriformis* is one of the most commonly used ciliated protozoa for laboratory research. In this ciliate various endpoints can be used to evaluate the cytotoxic effects of xenobiotics (Sauvant *et al.*, 1999). Growth rates and morphological changes are thought to be manageable toxicological assays and have been used for decades (Meyer *et al.*, 1971; Sauvant *et al.*, 1994; Larsen *et al.*, 1997; Kovács *et al.*, 1999; Nilsson, 1999) because of their simplicity and reproducibility. Population growth impairment is an often-used sublethal toxic endpoint which does not require special technical expertise (Nicolau *et al.*, 1999). Other parameters such as phagocytosis rate (Nilsson, 1981; Stefanidou *et al.*, 1990; Darvas *et al.*, 1999; Stefanidou *et al.*, 1999) can be assessed and have been proposed to determine the physiological and energetic state of *T. pyriformis* when in contact with pollutants. Calcein AM/ethidium homodimere-1 (CAM/EthD-1) test has also been used as a viability test to assess *T. pyriformis* membrane damage (Dias and Lima, 2002; Dias *et al.*, 2003).

Standard algal tests used for the estimation of biological toxicity are usually based on a 3-4-day exposure of a rapidly growing algal population to a particular toxicant present in the growth medium at effective concentrations (OECD, 1984; Environment Canada, 1992). *Selenastrum capricornutum* and *Scenedesmus subspicatus* are the most often used algal species. New modifications of the method have recently been developed including flow cytometry, use of microplates and immobilized algae for inoculation (Janssen *et al.*, 2000). In addition to the amount of cells, cellular ATP content or chlorophyll fluorescence can also be used to measure growth inhibition (Blaise *et al.*, 1986; Caux *et al.*, 1992).

S. capricornutum has been found to be sensitive to metals, herbicides and a number of organic chemicals (Thomas *et al.*, 1986; St-Laurent *et al.*, 1992; Klaine and Lewis, 1995). According to U.S. EPA ECOTOX database, this algal organism is more sensitive than other standard test organisms to many common compounds (Geis *et al.*, 2000).

In this work, a comparative assessment of the effects of two azo and two anthraquinone dyes was performed using two *in vitro* cell models, the ciliate protozoa *T. pyriformis* and the microalgae *Selenastrum capricornutum*. Growth impairment, viability assay using CAM/EthD-1 assay, grazing and morphometric assay were the tests performed with *T. pyriformis* on a 48-h-tests. The algal organism was used in a 72-h growth inhibition, acute toxicity test. With this approach we aimed at the comparison of two different microbes in order to find the best model microorganism to study the toxicity of textile dyes.

Material and Methods

Cell cultures and dye exposure

All toxicological assays used axenic 18-24-hour cultures of *Tetrahymena pyriformis* strain GL, ref. CCAP/1630/1F from Strains of Culture Collection of Algae and Protozoa, U.K. The cells were grown to exponential phase at the room temperature in Proteose Peptone Yeast Extract Medium (PPY), 2 % proteose peptone and 0.25 % yeast extract at pH 7.0-7.5. The density of *T. pyriformis* cultures was adjusted in fresh PPY in order to obtain 1×10^4 cells/ml.

The dyes used in this work were:

Congo Red (CR) (C.I. 22120, Direct Red 28),

Reactive Orange 16 (RO16) (C.I. 17757, Remazol Brilliant Orange 3R),

Remazol Brilliant Blue R (RBBR) (C.I. 61200, Reactive Blue 19),

Disperse Blue 3 (DB3) (C.I. 61505),

CR and RO16 are disazo and monoazo dyes, respectively. RBBR and DB3 are anthraquinone dyes. All dyes were used at a concentration of 500 mg/l and were previously filtered with a 0.45 µm membrane before adding to the cells. Untreated cells were used as the control. Untreated and treated *T. pyriformis* were incubated in 2-ml Eppendorf tubes in a total volume of 1.5 ml. Samples from two independent assays were taken at 1, 24 and 48 hours.

In vitro toxicity tests with *Tetrahymena pyriformis*

Population growth impairment and generation time determination were performed from aliquots of 100 µl, which were immediately taken (T_0) from the control and the treated cultures, and then subsequently at 24 and 48 hours. The samples were properly diluted in distilled water and fixed with Neutral Buffered Formalin (NBF) at a final concentration of 2-5 %, for 1 hour. NBF was made with 10 % (v/v) formalin in phosphate buffer saline (PBS) pH 7.0. The cell number was determined by counting every cell present in each of two 30 µl sub-samples with an inverted optical microscope (Nikon Diaphot 300) at 100× magnification.

The populations were characterized by their generation time (g) required for doubling the population. Generation time was calculated by the following formulas (1), (2).

Number of generations (*n*)   Generations time (*g*)

$$n = \frac{\log N_1 - \log N_0}{\log 2} \quad (1) \qquad g = \frac{\text{Time of growth}}{\text{Number of generations}} \quad (2)$$

Where N_1 is the number of cells at 24 h, N_0 is the number of cells at T_0, and Time of growth = 24 h.

The viability of control and exposed cells was assessed by the CAM/EthD-1 assay. Cells were labeled with freshly prepared solutions from the LIVE/DEAD® Viability/Cytotoxicity Assay Kit (Molecular Probes Europe, BV) at final concentrations of 10 µM calcein AM [4'5'-bis (N'N'-bis (carboxymethyl) aminomethyl fluorescein acetoxymethyl ester)] and 2.5 µM EthD-1 (ethidium homodimer-1) in PBS. No cytotoxic effects of calcein AM and no protozoan autofluorescence were observed under the experimental conditions. Storage and handling of reagents were performed as recommended by the supplier.

Morphometric analysis was performed by fixing control and exposed cells for one hour in NBF solution, washing and staining with 0.01 % toluidine blue. A drop of suspended culture was added onto microscopic slides and was analyzed with an inverted optical microscope (Nikon Diaphot 300) at 100× magnification. Images were taken with a CCD monochrome video camera (Sony AVCD5CE), binarized and the data analyzed by the MATLAB 5.1 software package (The Mathworks Inc., Natick) in order to determine the area and the ratio (W/L) of the shortest (W) and the longest (L) axis of cells. The morphological parameters were then determined from the final binary image. A previous calibration was performed through a slide engraved with a grid-ruler fitted to 1 mm. At 100× magnification, 0.5 mm corresponded to an average value of 459 pixels in the computer screen. Following the same conditions for image capture, the pixel to µm ratio was calculated following the equation: 1 pixel = 1.089 µm. In each sub-sample 100 randomly chosen cells were analyzed.

The grazing assay was performed in 2-ml Eppendorf test tubes, by adding yellow-green fluorescent latex Fluorospheres (0.5 µm diameter, Molecular Probes Europe BV) both to the treated and control cells, considering that ciliates have no prey selection in their feeding process (Fenchel, 1980). A final concentration of 1.05 x 10⁶ beads/ml in PPY medium was used according to Nicolau et al. (1999). After 20 minutes of incubation in the dark, the organisms were killed and fixed by the addition of NBF solution for one hour. The cells were then washed and resuspended in fresh 0.1 % (w/v) sodium azide in PBS and stored in the dark until subsequent analysis with an epifluorescence microscope (Leitz Laborlux S) at UV light (EX 450-490 nm excitation filter). The number of beads ingested by individual cells was counted at a

magnification of 1000×. In each sub-sample 50 randomly chosen cells were analyzed.

Statistical data analysis was performed by representing data as the mean of two independent assays. ANOVA test was used for significance calculations of growth and grazing tests calculations. t-student test was used in morphometry and calcein AM/EthD-1 tests. $P < 0.05$ and $P < 0.01$ were accepted as levels of statistical significance between groups.

The standard algal toxicity test on microplates followed the OECD Guideline No. 201 and the Environment Canada biological test method (OECD, 1984; Environment Canada, 1992). Algal cells were cultivated for 4 days in an incubation chamber (4000 lux, light/dark cycle of 13/11 h, 24 ± 2 °C, ATCC No. 625 medium). After dilution with $NaHCO_3$ buffer (15 mg/l) the cell amount was counted and adjusted to inoculate each well with 1×10^4 cells/ml. Sterile 96-well microtiter plates covered with lids were used wherein 36 peripheral wells were filled with 220 µl distilled water to minimize evaporation in the plate. The test system consisted of 60 inner wells containing 205 µl of a sample (or distilled water as the control), 5 µl of 50× diluted ATCC no. 625 medium and 10 µl of diluted algal culture for inoculation. The microplate was incubated for 96 h in the light chamber. The number of cells was counted after a 96-h exposure in a Neubauer counting chamber (100× magnification) and EC50 values were calculated. Each sample was evaluated using 18 replicates and the values were expressed as the mean value ± confidence interval. The test validity criteria were as described in Blaise et al. (2000).

Results

Addition of any of the dyes affected both growth (Fig. 1) and generation time (Table 1). However, the anthraquinone dye DB3 was the only dye to cause a significant decrease ($P < 0.01$) in T. pyriformis growth in a time-dependent manner. None of the used dyes inhibited completely the culture growth.

Intracellular esterases of living cells hydrolyze the non-fluorescent cell permeant CAM to the green (530 nm) fluorescent calcein, which is retained in viable cells. The addition of EthD-1, which binds to DNA, stained nuclei of dead cells in red. No significant viability alterations
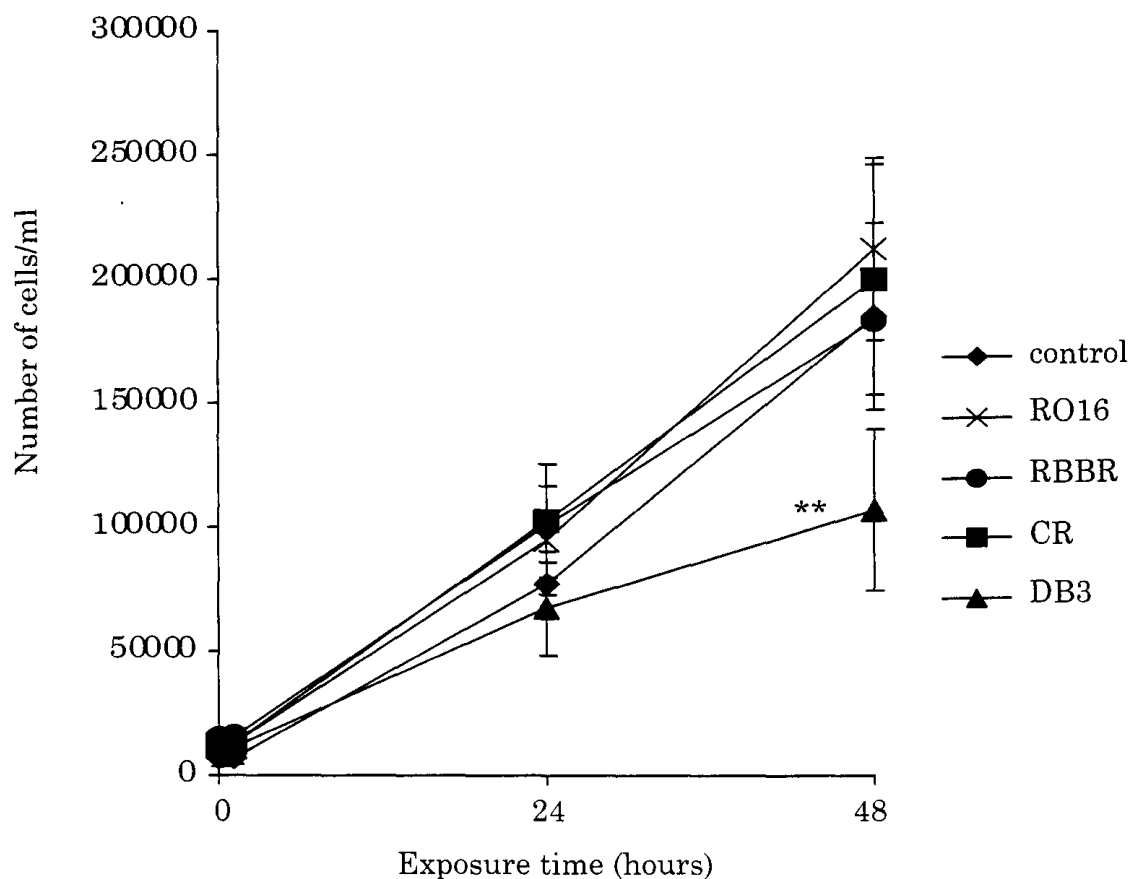
Figure 1. Dye effect on *T. pyriformis* population growth. Data are mean of two independent assays and bars represent standard deviations. ** Indicates significant differences from control values ($P < 0.01$).

Table 1. Generation time obtained in control and exposed *Tetrahymena pyriformis* cultures

Cultures	Generation time (hours)
control	6.85 ± 1.59
RO16 exposed	7.73 ± 1.25
RBBR exposed	7.95 ± 1.95
CR exposed	7.58 ± 1.46
DB3 exposed	10.61 ± 1.17**

Each value is the mean of two independent assays ± standard deviation
** Indicates significant differences from control values ($P < 0.01$)

were found, with the CR and RO16 azo dyes or RBBR anthraquinone dye, but a 48-h exposure to the anthraquinone DB3 dye significantly altered cell viability suggesting a time-dependent effect of the dye (Fig. 2). Although the cells had not suffered visible membrane damage, a slight decrease in the fluorescence was observed by epifluorescence microscopy in cultures exposed to all the dyes, compared with control cells. The fluorescence was strongly decreased in the culture exposed to DB3.

The results of two independent assays on the cell area and the cell axes ratio (W/L) are shown in Figure 3A and 3B, respectively. The dyes, CR, RBBR and DB3 caused significant ($P < 0.01$) differences in the cell area values after one hour of treatment. DB3 significantly influenced both the area and W/L ratio during the complete experimental period of 48 h by displaying a time-dependent effect.
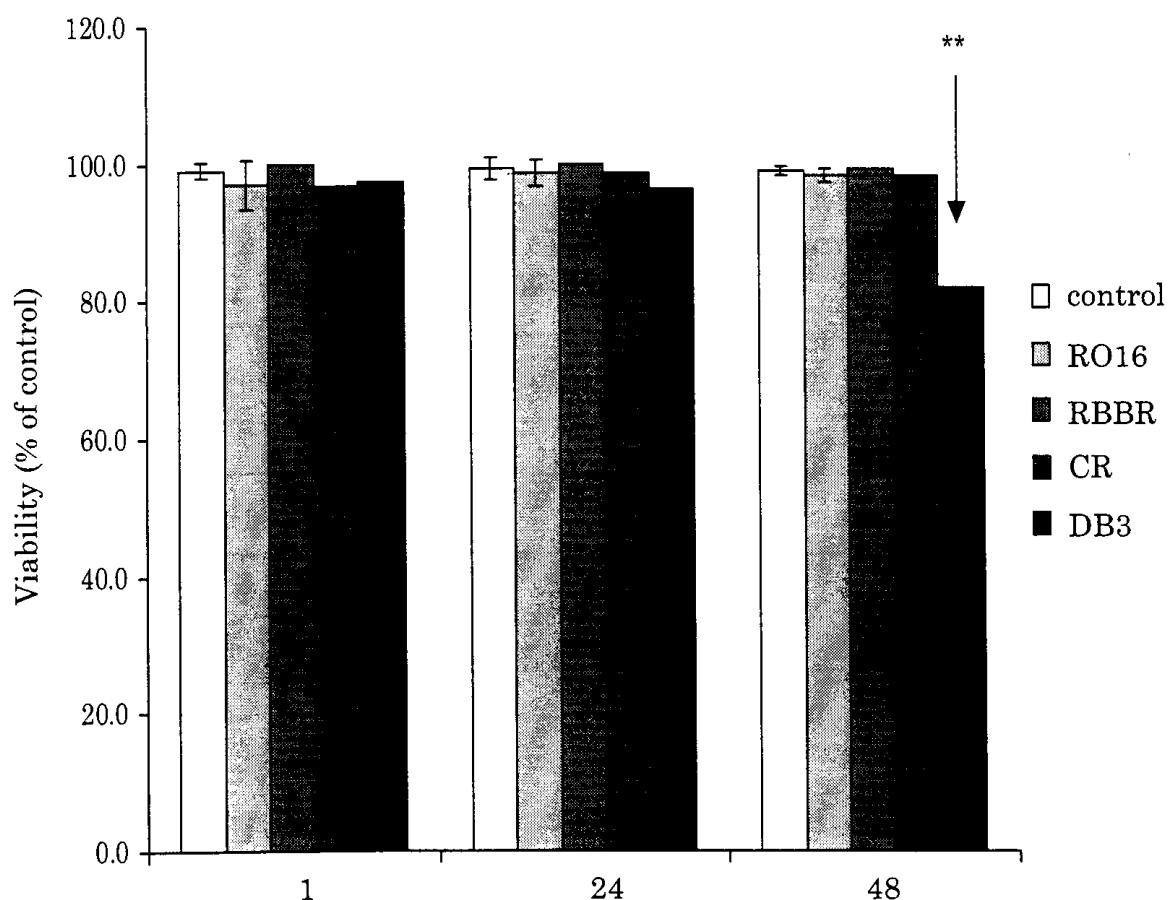

Figure 2. Dye effect on *T. pyriformis* population viability, using 1-48 h exposure time. Data are mean of two independent assays and bars represent standard deviations. ** Indicates significant differences from control values ($P < 0.01$).
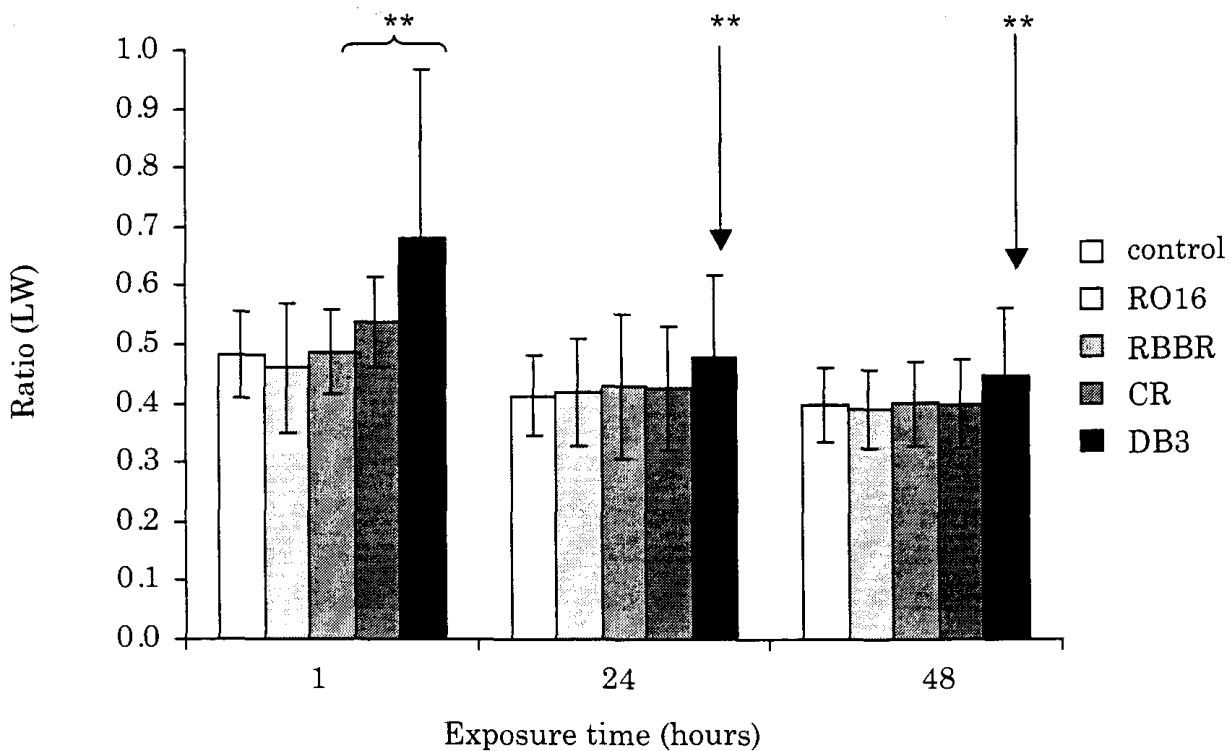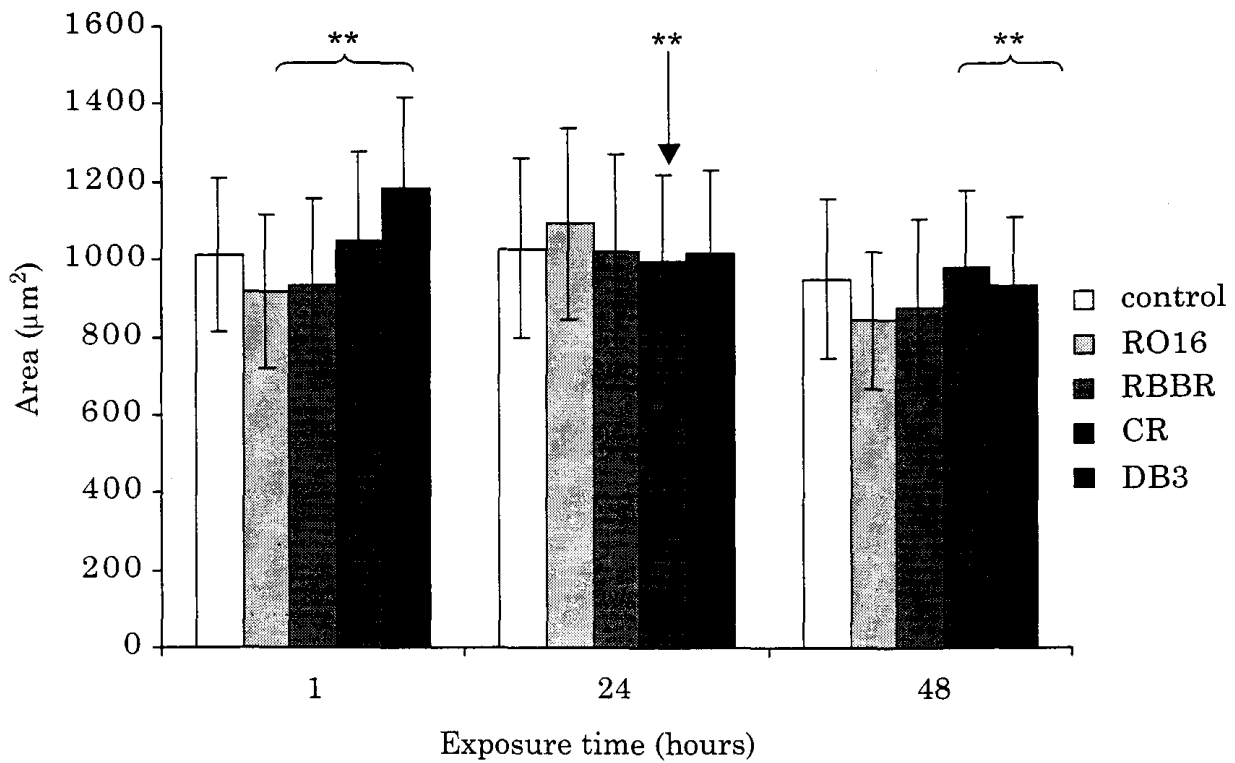
Figure 3. Dye effect on *T. pyriformis* population cell area (A) and the ratio between cell's shortest and longest axis (W/L) (B), using 1-48 h exposure time. Data are mean of two independent assays and bars represent standard deviations. * and ** Indicates significant differences from control values of $P < 0.05$ and $P < 0.01$, respectively.

The effects of the dyes on grazing capacity of *T. pyriformis* are shown in Fig. 4. A 24-h exposure to all dyes resulted in a decrease in the ingestion capability of *T. pyriformis*. This effect was shown to be time dependent since, after a 1-h exposure, this effect was observable only with CR and DB3. The anthraquinone dye DB3 reduced cell ingestion capability by 70 % compared to the control after 24 h of exposition.
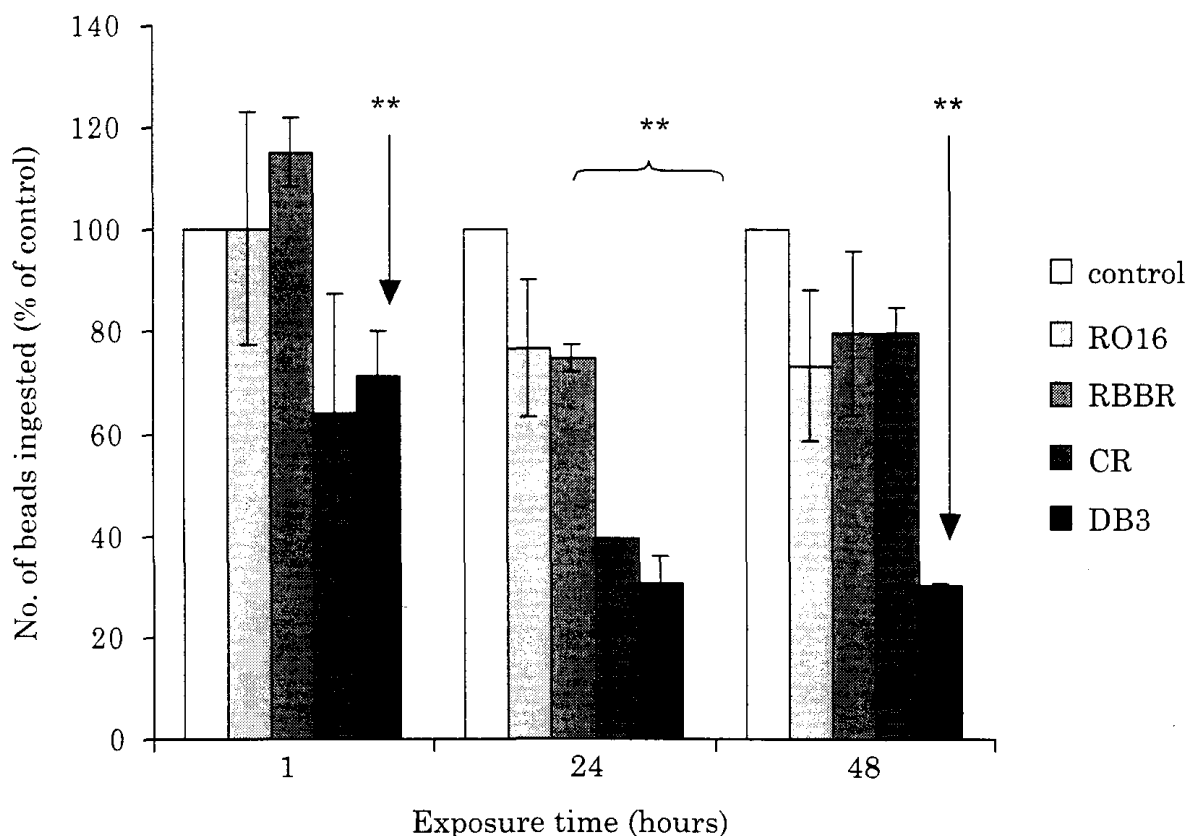
Figure 4. Dye effect on ingestion of microspheres by *T. pyriformis*, using 1-48 h exposure time. Data are mean of two independent assays and bars represent standard deviations. * and ** Indicates significant differences from control values of $P < 0.05$ and $P < 0.01$, respectively.

In biological toxicity experiments with the microalgae *S. capricornutum*, the anthraquinone dye DB3 efficiently inhibited algal growth, the EC50 value of 0.5 ± 0.0 mg/l being comparable to that of the standard toxicant $K_2Cr_2O_7$ (EC50 = 0.3 ± 0.1 mg/l) (Table 2). A medium growth inhibition efficiency was observed with the two azo dyes, CR and RO16, whose EC50 concentrations were about 10× higher. A weak growth inhibition was obtained with the anthraquinone dye RBBR where the EC50 concentration was more than 100× higher than that of DB3.

Table 2. Biological toxicity of dyes measured by the inhibition of growth of the alga *Selenastrum capricornutum*

Dye	Toxicity (EC50 ± Conf. Int., mg/l)[1]
Congo Red (CR)	4.8 ± 1.0
Reactive Orange 16 (RO16)	7.8 ± 1.8
Remazol Brilliant Blue R (RBBR)	81.1 ± 3.5
Disperse Blue 3 (DB3)	0.5 ± 0.0

EC50 value for potassium dichromate was 0.3 ± 0.1 mg/l.
* Conf. Int., confidence interval.

Discussion

There has been a great awareness about the toxicity hazards associated with dye manufacturing and processing, since it was suspected that some of the components of the dyes could have carcinogenic effects. A great effort has been made in order to investigate the biodegradation of textile azo dyes (Martins *et al.*, 1999; Martins *et al.*, 2001) their breakdown products (Martins *et al.*, 2003) and their toxic effect on the environment (Kopponen *et al.*, 1997).

In all the tests performed with the ciliated protozoa *Tetrahymena pyriformis* the anthraquinone dye DB3 showed to be the most toxic dye of all the compounds tested. Cell cycle was delayed by about 4 hours in the cultures exposed to DB3 compared to the control. Green fluorescence reduction of cells exposed to DB3 suggested that the dye affected the esterase activity and the ingestion capability of the protozoa. The cell size decrease and W/L ratio increase suggested a rounding off of the cells. Nevertheless DB3 did not seem to be a very good lytic agent, since less than 20 % of red (non-viable) cells were found after a 48-h exposure.

With the algal test no problems were found in measuring EC50 values as it can be the case with the bacterial luminiscence test (Dodard *et al.*, 1999; Wang *et al.*, 2002). The toxicity of the nonreactive dye DB3 by far exceeded the toxicity of usual reactive textile dyes (Heinfling *et al.*, 1997; Wang *et al.*, 2002).

Conclusion

Both *S. capricornutum* and *T. pyriformis* were found to be suitable organisms for testing biological toxicity of synthetic dyes. The former organism was more sensitive and the growth inhibitory effect could be observed at dye concentrations inferior to a concentration of 500 mg/l that was required to observe the toxicity effects with *T. pyriformis*. A high toxicity of the anthraquinone dye DB3, compared to the other dyes, was prominent to be observed using both the algal test and all the *T. pyriformis* tests. In contrast, a 10-fold difference in toxicity between RBBR and the azo dyes, observable using the algal test, could not be distinguished with any of the *T. pyriformis* tests.

Acknowledgments

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