Comparison of the sensitivity of different toxicity test endpoints in a microalga exposed to the herbicide paraquat

R. Prado, R. García, C. Rioboo, C. Herrero, J. Abalde, A. Cid[1],

Environment International, Volume 35, Issue 2, February 2009, Pages 240–247
Received 18 April 2008, Accepted 22 June 2008, Available online 13 August 2008

DOI: 10.1016/j.envint.2008.06.012

Abstract

The use of herbicides constitutes the principal method of weed control but the introduction of these compounds into the aquatic environment can provoke severe consequences for non-target organisms such as microalgae. Toxic effects of these pollutants on microalgae are generally evaluated using phytotoxicity tests based on growth inhibition, a population-based parameter. However, physiological cellular endpoints could allow early detection of cell stress and elucidate underlying toxicity mechanisms. Effects of the herbicide paraquat on the freshwater microalga *Chlamydomonas moewusii* were studied to evaluate growth rate and cellular parameters such as cellular viability and metabolic activity assayed by flow cytometry and DNA damage assayed by the comet assay. Sensitivity of growth and parameters assayed by flow cytometry were similar, showing a significant effect in cultures exposed to a paraquat concentration of 0.1 μM or higher, although in cultures exposed during 48 h to 0.05 μM, a significant stimulation of cellular fluorescein fluorescence was observed, related to cellular metabolic activity. After only 24 h of herbicide exposure significant DNA damage was observed in microalgal cells exposed to all paraquat concentrations assayed, with a 23.67% of comets in cultures exposed to 0.05 μM, revealing the genotoxicity of this herbicide. Taking into account the results obtained, comet assay provides a sensitive and rapid system for measuring primary DNA damage in *Chlamydomonas moewusii*, which could be an important aspect of environmental genotoxicity monitoring in surface waters.

Keywords

Microalga; Toxicity; Paraquat; Cell viability; Comet assay

1. Introduction

Aquatic environments, including freshwater, estuaries and coastal marine waters, are often contaminated with numerous organic and inorganic compounds, such as pesticides (Steen et al., 1999, Fatokio and Awofolu, 2004, Hela et al., 2005 and Kumari et al., 2007). As much as ca.

[1] Laboratorio de Microbiología, Facultad de Ciencias, Universidad de A Coruña. C/ Alejandro de la Sota n° 1. 15008 A Coruña, Spain

99.7% of the applied load of pesticides is dispersed in the environment, not reaching the target pests. In this way, pesticides enter into aquatic ecosystems from agricultural runoff or leaching and, as a consequence, have become some of the most frequent organic pollutants in these ecosystems (Pimentel, 1995 and Ibáñez et al., 1996). The increasing occurrence of pollutants in general, and herbicides in particular, has stimulated many studies related to their toxicity on aquatic microorganisms, and the need for convenient methods to allow assaying pollutants toxicity has become evident.

Most phytotoxicological research with herbicides has been conducted on target plants. The sensitivity of algae to many herbicides is very high, and a better understanding of their environmental effects may be acquired by using test species representing non-target groups (Haglund, 1997). Because of their short generation times, microalgae respond rapidly to environmental changes and, since these organisms are an important part of the base of the aquatic food web, any effect on them could affect higher trophic levels (De Lorenzo et al., 2002 and Rioboo et al., 2007) with organisms that generally respond on longer time scales (McCormick and Cairns, 1994). Furthermore, microalgal tests are generally sensitive, rapid and cost-effective (Sosak-Swiderska et al., 1998). For these reasons, the use of microalgal toxicity tests is increasing, and today these tests are frequently required by authorities for notifications of chemicals and are also increasingly being used to manage chemical discharges (Mayer et al., 1997).

The response of microalgae to a toxicant is typically measured using population-based parameters, such as specific growth rate, biomass, cell yield, chlorophyll fluorescence and primary production. Flow cytometry is an alternative to the standard algal population-based endpoints, since it allows a rapid and quantitative measurement of responses of individual algal cells to toxic stress. Using this technique, simultaneous measurements of multiple cellular parameters are made separately on each cell within the suspension, in near in vivo conditions, and not just as average values for the whole population (Carter and Meyer, 1990).

Several compounds of polluted water are reported to be capable of interacting with the DNA of living cells, and therefore cause genotoxic effects (Galloway et al., 2002 and Mouchet et al., 2007). Sources of genotoxins are agricultural drainage containing DNA-damaging pesticides (Plewa et al., 1984 and Kale et al., 1995). The assessment of genotoxic potential in surface water is one of the main tasks of environmental monitoring for the control of pollution. Animal organisms, such as fish or mussels, have been examined intensively (Stahl, 1991, Wilson et al., 1998, Large et al., 2002, Avishai et al., 2003, Laffon et al., 2006 and Sharma et al., 2007), whereas relatively little information is available on the susceptibility of water plants and plankton organisms (Majer et al., 2005). Alkaline single-cell gel electrophoresis, also known as the comet assay, is reported as a useful and sensitive technique for genotoxicity monitoring (Cotelle and Férard, 1999 and Tice et al., 2000). Because of its simple procedure, high sensitivity, short response time and the requirement of relatively small number of cells and test substances, it has been a powerful tool for the determination of genotoxicity (Aoyama et al., 2003 and Avishai et al., 2003). Furthermore, this test method is applicable to any kind of eukaryotic cell and is

independent of proliferation or cell cycle status, reported as being a suitable tool for measuring primary DNA damage in microalgae (Erbes et al., 1997).

Paraquat (1,1′-dimethyl-4,4′-bipyridilium dichloride) is a non-selective herbicide widely used to prevent the growth of broad leaf weeds and grasses, mainly in railways and roadsides. Information about the influence of these chemicals on the lowest level of the food chain is scarce (Ibrahim, 1996, Bray et al., 1993 and Sáenz et al., 1997).

In the present study, paraquat toxicity assessment was conducted in the laboratory on the freshwater green microalga *Chlamydomonas moewusii*, to determine the effect of this herbicide on microalgal growth, the most used parameter in microalgal toxicity assays, as well as the effect of paraquat on cell viability and metabolic activity using protocols of flow cytometry; the potential genotoxicity of the herbicide was also assayed using the alkaline single-cell gel electrophoresis or comet assay.

2. Materials and methods

2.1. Microalgal cultures

C. moewusii Gerloff (*Chlamydomonadaceae*) was obtained from the Culture Collection of Algae and Protozoa of Dunstaffnage Marine Laboratory (Scotland, UK) (strain CCAP 11/5B) and was maintained in autoclaved (121 °C, 20 min) Bristol medium (Brown et al., 1967). The inoculum was taken from a 3-day-old culture, with the aim of using cells growing in a logarithmic phase for all experiments. Cultures were grown in autoclaved 500 ml Pyrex glass bottles containing 300 ml of sterile medium. Microalgal cultures were maintained at 18 ± 1 °C, illuminated with 68.25 μmol photon m^{-2} s^{-1}, with a dark:light cycle of 12:12 h, and continuously aerated, for 48 h. Initial cell density for each experiment was 4×10^5 cells ml^{-1}.

The paraquat concentrations used in the present work were selected in function of the observed effect on growth in previous studies carried out during 96 h; these concentrations were 0.05 μM (lower than the EC_{50}), 0.1 μM (ca. the EC_{50}) and 0.2 μM (higher than the EC_{50}). Herbicide stock solution (1 mM) was prepared by dissolving granulated herbicide paraquat (Sigma; MW: 257.2) in distilled and sterilized water. In addition to these, cultures without paraquat were also included as a control. All experiments were carried out in duplicate.

2.2. Growth measurement

Growth of microalgal cultures was measured by counting daily culture aliquots in a Coulter Epics XL4 flow cytometer equipped with an argon-ion excitation laser (488 nm). To exclude non-algal particles, chlorophyll-*a*fluorescence was used, and red fluorescence histograms (> 645 nm) were used as a gate. Total cells present in a known volume of culture were counted and then cell density was calculated.

Growth rates (μ) expressed as day^{-1} were calculated using the following formula:

$\mu = [\ln(Nt) - \ln(N_0)] / \ln 2 (t - t_0)$ where N_t is the cell density at time t and N_0 is the cell density at time 0.

2.3. Cell viability determination

Cell viability was analysed by flow cytometry using the fluorescent emission of cells stained with propidium iodide (PI). PI is a fluorescent dye that intercalates with double-stranded nucleic acids to produce red fluorescence when excited by blue light. Because of its polarity, it is unable to pass through intact cell membranes; however, when the cell dies the integrity of the cell membrane fails, and PI is able to enter and stain the nucleic acids (Ormerod, 1990). In this way, PI can be used to discriminate between viable non-fluorescent cells and non-viable fluorescent cells (Cid et al., 1996 and Franqueira et al., 2000).

Aliquots of 2×10^5 cells ml^{-1} resuspended in phosphate buffered saline solution were stained with PI at a final concentration of 2.5 μg ml^{-1} (3.74 μM), during an incubation period of 10 min, at room temperature and in darkness conditions. This fluorochrome concentration was established to allow the stain of 100% of non-viable cells, not affecting fluorescence or toxic to the viable cells. At least 10^4 cells were analysed per culture. Results are expressed as the percentage of non-viable cells vs. the total amount of cells analysed by flow cytometry.

2.4. Metabolic activity determination

Cytometric quantification of cellular fluorescence upon cleavage of fluorescein diacetate (FDA) was reported as a sensitive and rapid technique to assess phytoplankton metabolic activity(Jochem, 1999). Bentley-Mowat (1982) first reported that the intensity of fluorescence derived from the cleavage of FDA appeared to depend on the “metabolic vigour” of the cells. FDA is a non-polar, non-fluorescent lipophilic molecule that diffuses across cell membranes freely. Inside the cell, non-specific esterases break the FDA molecule into one brightly fluorescing fluorescein and two acetates. Since its high polarity, the fluorescein is trapped within cells exhibiting cell membrane integrity, and the fluorescence intensity will therefore increase over time depending on the metabolic activity of those esterases.

Many authors have used the FDA cytometric assay as an indicator of viability in microalgal cells exposed to cytotoxic conditions. After entering the cell, its acetate residues are cleaved off by non-specific esterases and the polar hydrophilic fluorescent product fluorescein is retained by cells with intact plasma membranes. In this way, cells with altered membranes rapidly leak the dye, even if they retain some residual esterase activity, and remain non-fluorescent (Lage et al., 2001). Since fluorescein is accumulated by active cells, metabolic activity can be measured by means of the fluorescent intensity signal emitted by cells, which is proportional to the amount of accumulated fluorescein and the time elapsed.

Proportion of metabolic active cells in the population were analysed by a single-staining FDA cytometric assay after 24 and after 48 h of herbicide exposure. A stock solution of FDA was prepared at a concentration of 100 μg ml^{-1} in dimethylsulfoxide (0.24 mM) and stored at − 20 °

C. Aliquots of 2×10^5 cells ml^{-1} resuspended in phosphate buffered saline solution were incubated with FDA at a final concentration of 0.05 µg ml^{-1} (0.12 µM), during 15 min at room temperature and in darkness conditions. FDA concentration and incubation time were established so that the fluorescence originated in the cell is significant and stable. At least 10^4 cells were analysed per culture. FDA-derived fluorescence was detected in FL1 detector (488–550 nm; voltage: 560, gane: 1) in a logarithmic scale. Results are expressed as percentage of metabolically non-active cells vs. the total amount of cells analysed by flow cytometry. Furthermore, we examined the effect of paraquat on the fluorescein fluorescence intensity generated in cells, indicative of the metabolic activity level, taking into account only active cells, and the results are expressed in percentage of mean fluorescence of cultures exposed to paraquat compared with control cultures at each point in time.

2.5. Comet assay

The alkaline single-cell gel electrophoresis or comet assay was applied to detect the DNA damage induced after 24 h of exposure to the herbicide paraquat on *C. moewusii*.

The comet assay protocol used is a modification of the original protocol (Singh et al., 1988) adapted to planktonic algae by Erbes et al. (1997), with an additional modification that is the DNA staining by adding 50 µl SYBR Green (SYBR Green I Nucleic acid Gel Stain) 100× to each slide, and not the conventional DAPI or ethidium bromide staining.

Two replicate slides were prepared for each treatment culture and negative control. Slides were observed at 400× magnification using an epifluorescence microscope Nikon Eclipse E400 equipped with a mercury lamp, with blue light as an excitation light, since SYBR Green emits green fluorescence when excited with blue light. The number of normal nuclei and number of comets were counted, converted into percentages and tabulated. At least 100 randomly chosen cells from duplicate slides were examined for each culture (at least 50 cells per slide).

Photographs of normal nuclei and comets were taken with a high-definition cooled colour camera Nikon DS-5Mc, and images were treated with the imaging software Nis-Elements.

2.6. Statistical analysis

Mean and standard deviation values were calculated for each treatment from two independent replicate cultures. To determine significant differences among test concentrations, data were statistically analysed by an overall one-way analysis of variance (ANOVA) using the SPSS 14.0 software. When significant differences ($P < 0.05$) were observed, Duncan's multiple-range test was used to isolate the treatment or treatments that differed from the control.

Paraquat concentrations which decreased by 50% (EC_{50}) the number of viable cells, metabolically active cells and cells with intact DNA were calculated using the Probit analysis, since these parameters are not continuous variables. This statistical method was carried out using the SPSS software. The EC_{50} for growth were obtained by graphic interpolation from

concentration-response curves. Growth rate data analysis was carried out using the Regression Wizard (SigmaPlot 9.0) software.

3. Results

3.1. Growth

Growth of microalgal cultures exposed to paraquat concentrations of 0.1 μM or higher was affected after only 24 h of herbicide exposure. These cultures exhibited a growth rate (μ) at 24 h significantly ($P < 0.05$) lower than control ones, and this growth rate was reduced as paraquat concentration increased (Table 1, Fig. 1). This growth pattern is maintained after 48 h of herbicide exposure; after 48 h of exposure to a paraquat concentration of 0.2 μM, *C. moewusii* growth was almost completely arrested, showing a growth rate of 0.18 day^{-1} (Table 1), near 25% of control cultures growth rate (Fig. 1).

Table 1.

Effect of paraquat on growth rate, cell viability and metabolic activity of *C. moewusii*

Paraquat concentration (μM)	Growth rate (μ) (day^{-1})		Non-viable cells (%)		Metabolically non-active cells (%)	
	24 h	48 h	24 h	48 h	24 h	48 h
0	0.64 ± 0.02	0.70 ± 0.01	3.50 ± 0.07	5.34 ± 0.24	1.88 ± 0.10	0.78 ± 0.13
0.05	0.60 ± 0.03	0.70 ± 0.00	3.05 ± 0.03	5.89 ± 2.41	3.64 ± 0.74	1.71 ± 0.14
0.1	0.46 ± 0.01 *	0.50 ± 0.00 *	7.80 ± 0.64 *	6.12 ± 0.13	4.40 ± 0.17*	13.31 ± 2.24 *
0.2	0.31 ± 0.02 *	0.18 ± 0.01 *	9.82 ± 0.28 *	25.92 ± 1.14 *	19.28 ± 0.65 *	87.28 ± 1.58 *

Herbicide was added to the culture medium at the concentrations indicated. Values shown correspond to determinations performed 24 and 48 h after herbicide addition. Significant differences with respect to control at a level of significance of 0.05 ($P < 0.05$) are represented by an asterisk (☐). Data are given as mean values ± standard error of the means.

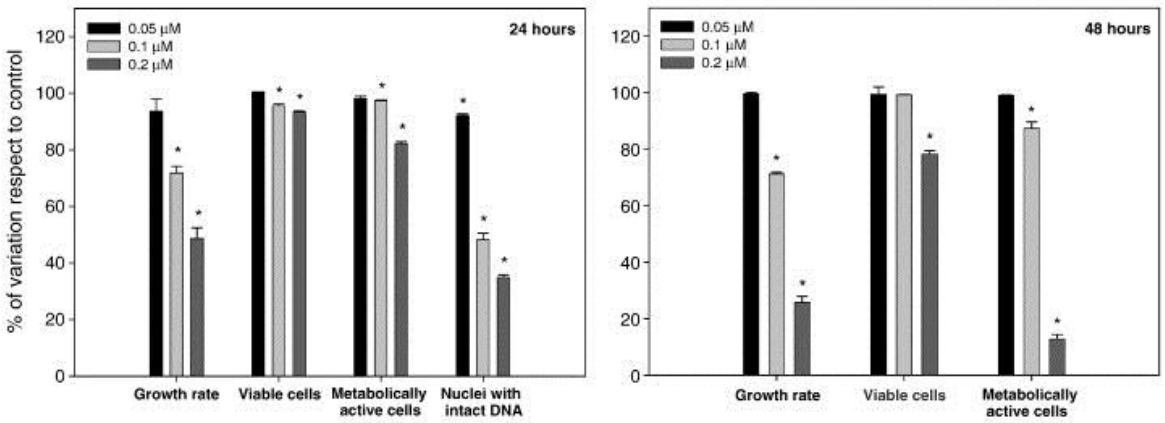

Fig. 1.

Percentage of variation of different toxicity endpoints assayed with respect to their respective control condition, considered as 100%, in function of the paraquat concentration in microalgal culture. Significant differences with respect to control at a level of significance of 0.05 ($P < 0.05$) are represented by an asterisk (☐). Data are given as mean values ± standard error of the means.

EC_{50} values after 24 and 48 h were 0.17 and 0.11 μM paraquat, respectively (Table 2).

Table 2.

Calculated EC_{50} values (μM) for paraquat on the parameters indicated at each point in time (n.a. means non-analyzed)

Time (h)	Growth rate	Cell viability	Metabolically active cells	Cells with intact DNA
24	0.17	0.49	0.3	0.15
48	0.11	0.26	0.15	n.a.

3.2. Cell viability

Cell viability analysed by flow cytometry using propidium iodide was affected after 24 h of exposure to herbicide concentrations of 0.1 μM or higher, so that microalgal cultures exposed to these paraquat concentrations showed, at that moment, a percentage of non-viable cells significantly higher ($P < 0.05$) in comparison with control cultures (Table 1, Fig. 1). After 48 h of herbicide exposure, a severe decrease in the cell viability was detected in cultures exposed to 0.2 μM of paraquat where about 26% of cells were non-viable, while lower paraquat concentrations did not induce significant differences ($P > 0.05$) in cell viability compared to control cultures (Table 1).

3.3. Cell metabolic activity

Percentage of metabolically non-active cells, determined by flow cytometry using fluorescein diacetate (FDA) increased significantly ($P < 0.05$) in cultures exposed 24 h to paraquat concentrations of 0.1 μM or higher respect to control cultures (Fig. 1 and Fig. 2A, Table 1). This percentage rose as the paraquat concentration increased, so that cultures exposed to 0.2 μM of paraquat showed a percentage of metabolically non-active cells significantly ($P < 0.05$) higher than that in cultures exposed to following lower paraquat concentration assayed (0.1 μM). After 48 h of herbicide exposure, metabolically active population was also significantly ($P < 0.05$) reduced in cultures exposed to paraquat concentrations of 0.1 μM or higher respect to control ones (Fig. 1 and Fig. 2B); percentage of metabolically non-active cells in cultures with 0.2 μM increased drastically to 87% (Fig. 2B, Table 1).

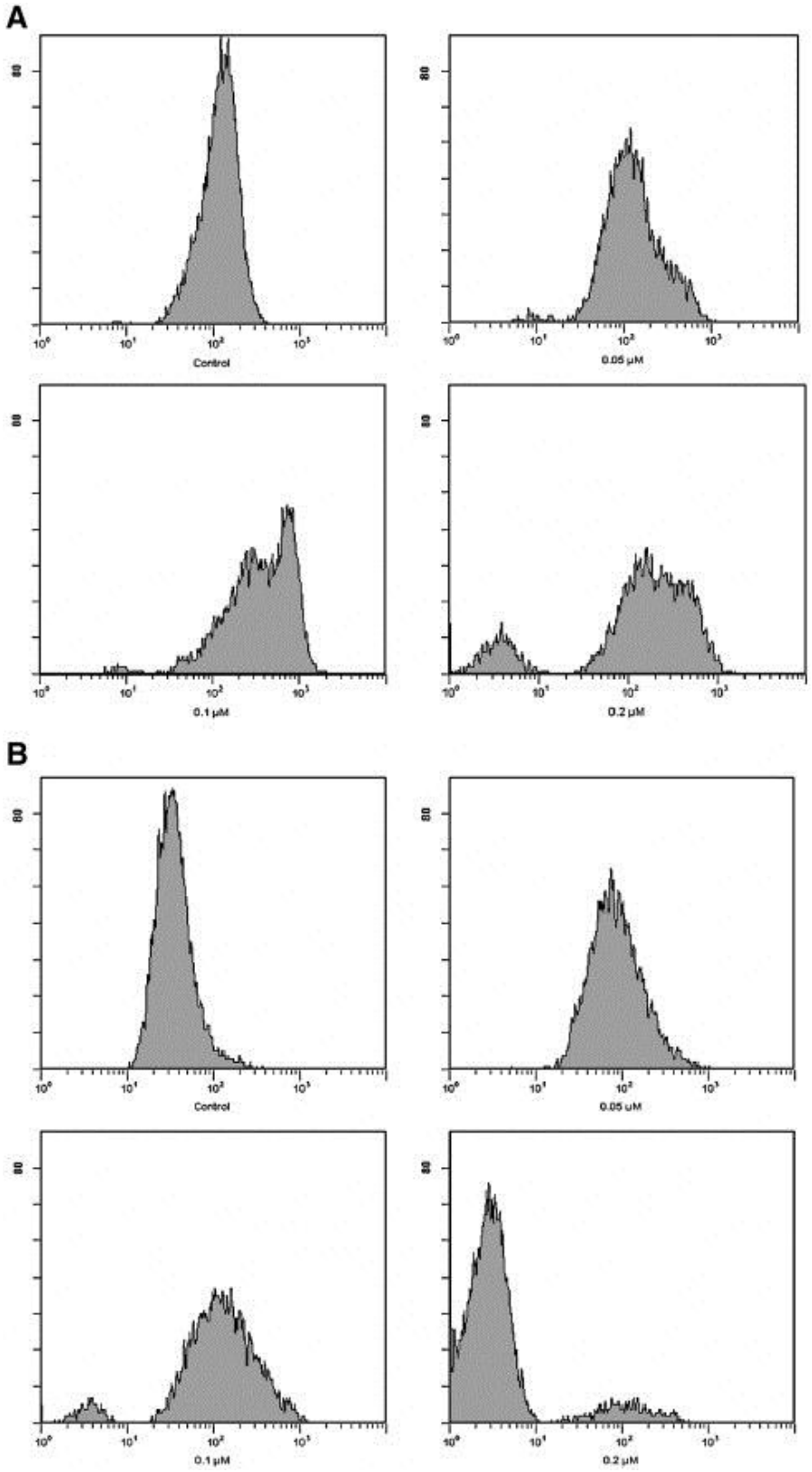

Fig. 2.

Flow cytometric histograms showing shifts in esterase activity (FL1 fluorescence) of *C. moewusii* exposed to paraquat. Herbicide was added to the culture medium at the concentrations indicated. *X* and *Y* histogram axes represent green fluorescence (a. u.) and number of cells, respectively. Values shown correspond to determinations 24 (A) and 48 (B) h after the addition of herbicide.

Regarding the activity level of metabolically active population, the pattern is the same after 24 and after 48 h of paraquat exposure. Active population in cultures exposed to paraquat showed a higher activity level in comparison with control cultures, reaching a maximum in cultures with an herbicide concentration of 0.1 μM (Fig. 2, Table 3). In spite of this, there are differences because at 24 h only the cultures exposed to 0.1 μM of paraquat showed a significantly ($P < 0.05$) higher metabolic activity compared to control cultures. However, after 48 h of herbicide exposure, all cultures exposed to the herbicide showed a significantly ($P < 0.05$) higher activity level in comparison with control cultures. In this way, metabolic activity exhibited by active population in cultures exposed to the lowest paraquat concentration assayed (0.05 μM) is twice the exhibited by control cultures; activity level of cultures exposed to 0.1 μM is four times that presented by the controls, while the level of metabolic activity displayed by cultures exposed to the highest concentration assayed (0.2 μM) is three times higher compared to that displayed by control cultures (Table 3).

Table 3.

Effect of paraquat on fluorescein fluorescence intensity of *C. moewusii*

Paraquat concentration (μM)	Fluorescein fluorescence	
	24 h	48 h
0.05	110.20 ± 4.72	238.69 ± 39.33*
0.1	273.42 ± 25.40*	400.22 ± 29.74*
0.2	168.10 ± 17.20	301.62 ± 43.00*

Data are expressed as percentage of variation in comparison with control cells. Herbicide was added to the culture medium at the concentrations indicated. Values shown correspond to determinations performed 24 and 48 h after the addition of herbicide. Significant differences with respect to control at a level of significance of 0.05 ($P < 0.05$) are represented by an asterisk (*). Data are given as mean values ± standard error of the means.

3.4. Comet assay

Under the applied experimental conditions, cell nuclei from untreated *Chlamydomonas* cells appeared as diffusely spread DNA fragments in the former nucleus area. Thus, unaffected control cells form comets with a head region and with a small or without a clearly fragmented tail (Fig. 3A). Genotoxically damaged *Chlamydomonas* cells resulted in comets consisting of a head region and a tail of DNA fragments (Fig. 3B). With increasing DNA damage, fragmentation became more apparent and the morphology of the head region was not so clear (Fig. 3C), decreasing the fluorescence intensity of head DNA. This effect could result in the complete disintegration of the head region, while the tail area appeared as a “cloud” of DNA fragments, culminating finally in the formation of comets without any visible area, consisting of long, extended and scattered DNA fragments. These comet shapes were difficult to recognize with the naked eye because the weak fluorescence intensity did not allow for sufficient contrast to the slide background, and they are often known as “ghost comets”. The small DNA content of the algal cells probably causes this effect. In preliminary experiments we observed that, after

48 h of exposure to assayed paraquat concentrations, slides corresponding to high concentrations showed few comets and barely perceptible ghosts, probably due to the strong DNA damage in cells exposed to the herbicide. Therefore, we decided to analyse paraquat genotoxicity on *C. moewusii* only after 24 h of exposure to these paraquat concentrations.

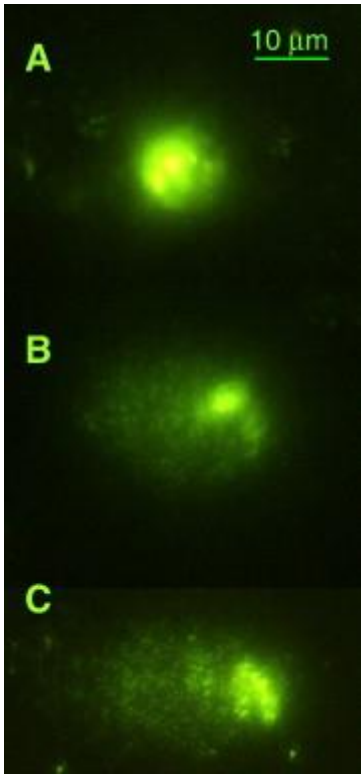

Fig. 3.
Nuclei after electrophoresis of lysed *C. moewusii* cells from control cultures (A) (normal nucleus) and cultures treated with paraquat (B, C) (comets).

Results obtained in this assay showed that paraquat induces DNA damage in cells of *C. moewusii,* after only 24 h exposed to paraquat concentrations of 0.05 µM or higher (Fig. 1, Table 4). The percentage of nuclei exhibiting comet tail was increased as paraquat concentration increased (Fig. 1), so that all cultures exposed to paraquat showed a percentage of comets significantly ($P < 0.05$) higher in comparison with control ones; in cultures exposed to the highest concentration assayed (0.2 µM) this percentage reached 71% (Table 4), although cultures exposed to the lowest concentration assayed (0.05 µM) showed a significant ($P < 0.05$) percentage of comets (23.67%).

Table 4.
DNA damage expressed as percentage of nuclei with comet of *C. moewusii*

Paraquat concentration (μM)	Nuclei with comet (%)
0	17.13 ± 1.20
0.05	23.67 ± 1.42*
0.1	59.99 ± 2.36*
0.2	71.04 ± 3.47*

Herbicide was added to the culture medium at the concentrations indicated. Values shown correspond to determinations 24 h after the addition of herbicide. Significant differences with respect to control at a level of significance of 0.05 ($P < 0.05$) are represented by an asterisk (☐). Data are given as mean values ± standard error of the means.

4. Discussion

There are different reports using microalgal growth in order to measure the toxicity of pesticides. Toxic action is measured depending on the growth rate change in cultures exposed to the pesticide in comparison with control cultures without pesticide (Schrader et al., 1997, Wong, 2000 and Oliveira et al., 2007). Algae show varied responses to pesticides from complete inhibition to initial suppression followed by gradual recovery and satisfactory growth (Mohapatra and Mohanty, 1992, Geoffroy et al., 2003, Tukaj and Pokora, 2006 and Rajendran et al., 2007).

In the present work, microalgal growth was significantly affected by paraquat in a dose-dependent manner. Cultures exposed to the highest paraquat concentration assayed (0.2 μM) showed a growth rate at 24 h that was higher than that at 48 h. This growth inhibition effect has already been observed in *Chlorella vulgaris*cultures exposed to high concentrations of the herbicides isoproturon and terbutryn(Rioboo et al., 2002) and in *C. vulgaris* and *Anabaena doliolum* cultures exposed to the herbicides dimethoate and endosulfan (Mohapatra and Mohanty, 1992).

Results of cell viability analysed by flow cytometry, both with PI and FDA assay, showed a significant reduction of viable microalgal population in cultures exposed to paraquat concentrations of 0.1 μM or higher, after only 24 h of herbicide exposure. The FDA assay led to results where this viability reduction compared to control cultures is more significant than in results obtained after IP assay, especially in cultures exposed to the highest paraquat concentration assayed. This can be explained, since both assay procedures can distinguish between cells with an intact plasmatic membrane (viable cells) and cells with a damaged membrane (non-viable), but the FDA assay may include as non-viable cells those cells with an intact membrane but without esterase activity. Due to the biochemical mechanism of paraquat toxicity, oxygen free radicals are generated, and hydroxyl radicals have been implicated in the initiation of membrane damaging by lipid peroxidation (Yu, 1994). This agrees with the results showing that there is a significant higher percentage of cells which have lost its membrane integrity in cultures exposed to high paraquat concentrations in comparison with control cultures without herbicide.

Flow cytometry combined with staining with fluorescent dyes such as PI or FDA is a technique increasingly being used to probe microalgal cell membrane integrity for the assessment of the environmental impact of toxic contaminants in aquatic systems. Toxic agents such as copper or pesticides have been reported as damaging to the membrane integrity of microalgal cells indicating a reduction in the percentage of viable cells exposed to that agent (Cid et al., 1997, Franqueira et al., 1999, Lage et al., 2001 and Nancharaiah et al., 2007).

Enzyme inhibition measurements in microalgae are becoming increasingly popular indicators of environmental stress because they offer a rapid and sensitive endpoint. Esterase activity, in particular, has been proven useful in a variety of cell types (Berglund and Eversman, 1988, Kaprelyants and Kell, 1992,Humphreys et al., 1994 and Breeuwer et al., 1995) and has been shown to relate well to general metabolic activity (Dorsey et al., 1989, Regel et al., 2002 and

Regel et al., 2004). In this way, FDA assay has been reported as being not only helpful to discriminate between “healthy” and “stressed” cells, but also to quantify subtle responses to environmental impacts. Furthermore, the advantage of cytometric measurements over fluorescence microscopy or bulk estimates by fluorometry lies in the assessment of minor changes in metabolic activity by the detection of changes in the fluorescence intensity exhibited on a single-cell basis (Jochem, 1999).

In the present study, both after 24 and 48 h viable microalgal population in cultures exposed to paraquat show a mean fluorescein fluorescence that is higher than in the control cultures, indicating a stimulation of the esterase enzyme activity, especially in cultures exposed to 0.1 µM of paraquat after 48 h. Esterases are essential to the phospholipid turnover in membranes. Due to membrane destruction induced by the oxidative damage caused by paraquat, it may be that intracellular esterases are stimulated in order to repair membranes, in cells still intact. This enzyme activity stimulation may also be a result of a general metabolic stimulation induced by the toxic agent in microalgal cells in order to adapt their physiology to adverse environmental conditions; esterase activity enhancement is lower in cultures exposed to the highest paraquat concentration assayed, which can be due to high levels of stress. Growth stimulation effects have been recorded in algal bioassays with different herbicides at low concentrations (Franqueira et al., 1999 and Rioboo et al., 2002). It has been reported that esterase activity in *Selenastrum capricornutum* and *Entomoneis* cf. *punctulata* decreased in a concentration-dependent manner as copper concentration increased (Franklin et al., 2001a), but stimulation of this enzyme activity has also been reported in microalgae exposed to certain copper concentrations (Franklin et al., 2001a, Lage et al., 2001 and Yu et al., 2007), to nutrient-enriched waters associated to sewage effluents (Regel et al., 2002) and to the biocide chlorine (Nancharaiah et al., 2007). It was proposed that this increase in FDA fluorescence could be either due to a simple increase in the uptake of FDA as consequence of cell membrane hyperpolarization or changes in cell membrane permeability (Franklin et al., 2001b and Nancharaiah et al., 2007), or due to increased esterase activity as consequence of an effect of copper on intracellular ionic regulation leading to increased intracellular pH (Lage et al., 2001).

Although the comet assay methodology is straightforward and does not require sophisticated equipment, the analysis of comet images is not so simple. In this work a visual analysis was used, classifying nuclei on the basis of their morphology in two groups: comets and normal nuclei. DNA damage is evaluated as an increase in the percentage of cells with comets (Devaux et al., 1997, Avishai et al., 2002 and Desai et al., 2006). Visual analysis is reliable and fast but, to a certain extent, the results rely on subjective decisions made by the investigator.

Results obtained showed that exposure of microalgal cells to paraquat for 24 h increased comet percentage, and thus primary DNA damage, in a dose-related manner at the concentration range assayed. In spite of this, damaged cells were also found in control samples, and in heavily treated samples undamaged cells could be found to a certain extent. The finding of damaged cells in control samples may be due to artificial DNA damage, which can be caused by inhomogeneities in the electric field or the formation of radicals during electrophoresis (Singh

et al., 1994). Further possible explanations for the heterogeneity of DNA damage in control and treated samples are variabilities in the age of cells or cell cycle status (Erbes et al., 1997 and Lah et al., 2004), since physiologically different cells were present in control and in treatment cultures which was shown by the heterogeneity observed in the response of the population analysed by flow cytometry. However, there is a study with other microalga, *Closterium ehrenbergii*, exposed to the disinfectant triclosan, on which comets in untreated cells were observed only occasionally (Ciniglia et al., 2005).

Results obtained from different studies suggest the capability of pesticides in significantly increasing DNA fragmentation (Ribas et al., 1995, Clements et al., 1997, Sasaki et al., 1997a, Sasaki et al., 1997b, Garaj-Vrhovac and Zeljezic, 2000 and Vindas et al., 2004). Paraquat genotoxicity may be related to the mode of action exhibited by this herbicide. Paraquat acts on photosynthesis, as a competitive inhibitor of photosynthetic electron transport at photosystem I level (Summers, 1980 and Devine et al., 1993). In this way, paraquat molecule experiments a single electron reduction and an oxidation reaction with molecular oxygen, leading to the generation of free oxygen radicals that could affect cellular DNA (Kaloyanova and El Batawi, 1991). Reactive oxygen species (ROS) have been shown to be active genotoxic agents (Gaivao et al., 1999), since a great number of studies show that ROS are major sources of DNA damage by causing strand breaks, removal of nucleotides and a variety of modifications of the bases of the nucleotides (Cooke et al., 2003). Furthermore, paraquat is very effective in generating free radicals and cell antioxidant mechanisms, as free radical scavengers, are rapidly overwhelmed. This may explain why microalgal cultures exposed to the lowest paraquat concentration assayed showed a percentage of comets significantly higher in comparison with cultures without herbicide, only after 24 h of exposure. Other pesticides, including some herbicides, have been reported as damaging DNA through the generation of ROS (Bagchi et al., 1995,Melchiorri et al., 1998, Cicchetti and Argentin, 2003, Bajpayee et al., 2006, Liu et al., 2007 and González et al., 2007), or through the interaction of pesticide or its metabolites with DNA (Fairbairn et al., 1995 and Sharma et al., 2007).

Comets of microalgal cells are essentially composed of nuclear DNA and, possibly, of chloroplast DNA to a minor extent, but comet images are not probably influenced by mitochondrial DNA (Erbes et al., 1997). DNA damage in microalgae evaluated by the comet assay has been observed previously in the marine diatom *Chaetoceros tenuissimus* exposed to cadmium, in a dose-related manner too (Desai et al., 2006), and this genotoxicity could be also related to oxidative stress (Schützendübel and Polle, 2002, Horvat et al., 2007 and Lin et al., 2007). The dose-dependent effect observed in paraquat-treated *C. moewusii* cells using the comet assay, was also found on *Euglena gracilis* with several mutagenic compounds causing DNA damage (Aoyama et al., 2003).

5. Conclusions

Algal growth measurements are the most frequently used methods of herbicide toxicity assessment. In the present work with *C. moewusii*, sensitivity of growth, cell viability and metabolic activity to the herbicide paraquat were similar, but the use of flow cytometry in combination with biochemically specific fluorescent dyes can also provide information about the physiological status of cells and the mechanisms of action of toxicants. Based on the EC_{50} values calculated for the different assayed parameters (Table 2), DNA damage was the most sensitive toxicity test endpoint, revealing that comet assay provides a sensitive and rapid system for measuring primary DNA damage in *C. moewusii*, which could be an important aspect of environmental genotoxicity monitoring in surface waters.

Acknowledgements

This work was carried out with the financial support of the Spanish Ministerio de Educación y Ciencia (CGL 2004/02037 BOS), and Xunta de Galicia (PGIDIT05PXIC10303PN). R. P. acknowledges a F.P.U. fellowship from the Spanish Ministerio de Educación y Ciencia.

References

K. Aoyama, K. Iwahori, N. Miyata
Application of Euglena gracilis cells to comet assay: evaluation of DNA damage and repair
Mutat Res, 538 (2003), pp. 155–162

N. Avishai, C. Rabinowitz, E. Moiseeva, B. Rinkevich
Genotoxicity of the Kishon River, Israel: the application of an in vitro cellular assay
Mutat Res, 518 (2002), pp. 21–37

N. Avishai, C. Rabinowitz, B. Rinkevich
Use of the comet assay for studying environmental genotoxicity
Environ Mol Mutagen, 42 (2003), pp. 155–165

D. Bagchi, M. Bagchi, E.A. Hassoun, S.J. Stohs
In vitro and in vivo generation of reactive oxygen species, DNA damage and lactate dehydrogenase leakage by selected pesticides
Toxicology, 104 (1995), pp. 129–140

M. Bajpayee, A.K. Pandey, S. Zaidi, J. Musarrat, D. Parmar, N. Mathur, P.K. Seth, A. Dhawan
DNA damage and mutagenicity induced by endosulfan and its metabolites
Environ Mol Mutagen, 47 (2006), pp. 682–692

J.A. Bentley-Mowat
Application of fluorescence microscopy to pollution studies on marine phytoplankton
Bot Mar, 25 (1982), pp. 203–204

D.F. Bray, J.R. Bagu, K. Nakamura
Ultrastructure of Chlamydomonas reinhardtii following exposure to paraquat: comparison of wild type and a paraquat-resistant mutant
Can J Bot, 71 (1993), pp. 174–182

D.L. Berglund, S. Eversman
Flow cytometric measurements of pollutant stresses on algal cells
Cytometry, 9 (1988), pp. 150–155

P. Breeuwer, J.L. Drocourt, N. Bunschoten, M.H. Zwietering, F.M. Rombouts, T. Abee
Characterisation of uptake and hydrolysis of fluorescein diacetate and carboxyfluorescein diacetate by intracellular esterases in Saccharomyces cerevisiae, which result in accumulation of fluorescent product
Appl Environ Microbiol, 61 (1995), pp. 1614–1619

T.E. Brown, F.L. Richardson, M.L. Vaughn
Development of red pigmentation in Chlorococcum wimmeri (Chlorophyta: Chlorococcales)
Phycologia, 6 (1967), pp. 167–184

N.P. Carter, E.W. Meyer
Introduction to the principles of flow cytometry
M.G. Ormerod (Ed.), Flow Cytometry. A Practical Approach, Oxford University Press, Oxford (1990), pp. 1–28

R. Cicchetti, G. Argentin
The role of oxidative stress in the in vitro induction of micronuclei by pesticides in mouse lung fibroblasts
Mutagenesis, 18 (2003), pp. 127–132

A. Cid, P. Fidalgo, C. Herrero, J. Abalde
Toxic action of copper on the membrane system of a marine diatom measured by flow cytometry
Cytometry, 25 (1996), pp. 32–36

A. Cid, E. Torres, C. Herrero, J. Abalde

Disorders provoked by copper in the marine diatom Phaeodactylum tricornutum in short-time exposure assays
Cah Biol Mar, 38 (1997), pp. 201–206

C. Ciniglia, C. Cascone, R.L. Giudice, G. Pinto, A. Pollio
Application of methods for assessing the geno- and cytotoxicity of Triclosan to C. ehrenbergii
J Hazard Mater, 122 (2005), pp. 227–232

C. Clements, S. Ralph, M. Petras
Genotoxicity of selected herbicides in Rana catesbeiana tadpoles using alkaline single-cell gel DNA electrophoresis
Environ Mol Mutagen, 29 (1997), pp. 277–288

M.S. Cooke, M.D. Evans, M. Dizdaroglu, J. Lunec
Oxidative DNA damage: mechanisms, mutation, and disease
FASEB J, 17 (2003), pp. 1195–1214

S. Cotelle, J.F. Férard
Comet assay in genetic ecotoxicology: a review
Environ Mol Mutagen, 34 (1999), pp. 246–255

M.E. De Lorenzo, L.A. Taylor, S.A. Lund, P.L. Pennington, E.D. Strozier, M.H. Fulton
Toxicity and bioconcentration potential of the agricultural pesticide endosulfan in phytoplankton and zooplankton
Arch Environ Contam Toxicol, 42 (2002), pp. 173–181

S.R. Desai, X.N. Verlecar, Nagarajappa, U. Goswami
Genotoxicity of cadmium in marine diatom Chaetoceros tenuissimus using the alkaline Comet assay
Ecotoxicology, 15 (2006), pp. 359–363

A. Devaux, M. Pesonen, G. Monod
Alkaline comet assay in rainbow trout hepatocytes
Toxicol In Vitro, 11 (1997), pp. 71–73

M. Devine, S.O. Duke, C. Fedtke
Physiology of herbicide action
PTR Prentice-Hall, Englewood Cliffs (1993)

J. Dorsey, C.M. Yentsch, S. Mayo, C. McKenna
Rapid analytical technique for the assessment of cell metabolic activity in marine microalgae
Cytometry, 10 (1989), pp. 622–628

M. Erbes, A. Webler, U. Obst, A. Wild
Detection of primary DNA damage in Chlamydomonas reinhardtii by means of modified microgel electrophoresis
Environ Mol Mutagen, 30 (1997), pp. 448–458

D.W. Fairbairn, P.L. Olive, L. ONK
The comet assay: comprehensive review
Mutat Res, 399 (1995), pp. 37–59

S. Fatokio, O.R. Awofolu
Levels of organochlorine pesticide residues in marine-, surface-, ground-, and drinking waters from the Eastern Cape Province of South Africa
J Environ Sci Health Part B, 39 (2004), pp. 101–114

N.M. Franklin, M.S. Adams, J.L. Stauber, R.P. Lim
Development of an improved rapid enzyme inhibition bioassay with marine and freshwater microalgae using flow cytometry
Arch Environ Contam Toxicol, 40 (2001), pp. 469–480

N.M. Franklin, J.L. Stauber, P. Lim
Development of flow cytometry-based algal bioassays for assessing toxicity of copper in natural waters
Environ Toxicol Chem, 20 (2001), pp. 160–170

D. Franqueira, A. Cid, E. Torres, M. Orosa, C. Herrero
A comparison of the relative sensitivity of structural and functional cellular responses in the alga Chlamydomonas eugametos exposed to the herbicide paraquat
Arch Environ Contam Toxicol, 36 (1999), pp. 264–269

D. Franqueira, M. Orosa, E. Torres, C. Herrero, A. Cid
Potential use of flow cytometry in toxicity studies with microalgae
Sci Total Environ, 247 (2000), pp. 119–126

I. Gaivao, L.M. Sierra, M.A. Comendador
The w/w+ SMART assay of Drosophila melanogaster detects the genotoxic effects of reactive oxygen species inducing compounds
Mutat Res, 440 (1999), pp. 139–145

T.S. Galloway, R.C. Sanger, K.L. Smith, G. Fillmann, J.W. Readman
Rapid assessment of marine pollution using multiple biomarkers and chemical immunoassays
Environ Sci Technol, 36 (2002), pp. 2219–2226

V. Garaj-Vrhovac, D. Zeljezic
Evaluation of DNA damage in workers occupationally exposed to pesticides using single-cell gel electrophoresis (SCGE) assay. Pesticide genotoxicity revealed by comet assay
Mutat Res, 469 (2000), pp. 279–285

L. Geoffroy, D. Dewez, G. Vernet, R. Popovic
Oxyfluorfen toxic effect on S. obliquus evaluated by different photosynthetic and enzymatic biomarkers
Arch Environ Contam Toxicol, 45 (2003), pp. 445–452

N.V. González, S. Soloneski, M.L. Larramendy
The chlorophenoxy herbicide dicamba and its commercial formulation banvel induce genotoxicity and cytotoxicity in Chinese hamster ovary (CHO) cells
Mutat Res, 634 (2007), pp. 60–68

K. Haglund
The use of algae in aquatic toxicity assessment
,in: F.E. Round, D.J. Chapman (Eds.), Progress in Phycological Research, vol. 12Biopress Lts, Bristol (England) (1997), pp. 181–212

D.G. Hela, D.A. Lambropoulou, K. KA, T.A. Albanis
Environmental monitoring and ecological risk assessment for pesticide contamination and effects in Lake Pamvotis, northwestern Greece
Environ Toxicol Chem, 24 (2005), pp. 1548–1556

T. Horvat, V. Vidakovic-Cifrek, V. Orescanin, M. Tkalec, B. Pevalek-Kozlina
Toxicity assessment of heavy metal mixtures by Lemna minor L
Sci Total Environ, 384 (2007), pp. 229–238

M.J. Humphreys, R. Allman, D. Lloyd
Determination of the viability of Trichomonas vaginalis using flow cytometry
Cytometry, 15 (1994), pp. 343–348

M. Ibáñez, Y. Picó, J. Mañes
Influence of organic matter and surfactans on solid-phase extraction of diquat, paraquat and difenzopuat from waters
J Chromatogr A, 727 (1996), pp. 245–252

E.A. Ibrahim
The influence of the herbicide paraquat "gramoxon" on growth and metabolic activity of three chlorophytes
Water Air Soil Pollut, 51 (1990), pp. 89–93

F.J. Jochem
Dark survival strategies in marine phytoplankton assessed by cytometric measurement of metabolic activity with fluorescein diacetate
Mar Biol, 135 (1999), pp. 721–728

P.G. Kale, B.T. Petty, S. Walker, B.J. Ford, N. Dehkordi, S. Tarasia, et al.
Mutagenicity testing of nine herbicides and pesticides currently used in agriculture
Environ Mol Mutagen, 25 (1995), pp. 148–153

F.P. Kaloyanova, M.A. El Batawi
Dipyridiliums
F.P. Kaloyanova, M.A. El Batawi (Eds.), Human Toxicology of Pesticides, CRC Press, Boca Raton (1991), pp. 147–158

A.S. Kaprelyants, D.B. Kell
Rapid toxicity assessment of bacterial viability and vitality by rhodamine
J Appl Bacteriol, 72 (1992), pp. 410–422

B. Kumari, V.K. Madan, T.S. Kathpal
Pesticide residues in rain water from Hisar, India
Environ Monit Assess, 133 (2007), pp. 467–471

B. Laffon, T. Rábade, E. Pásaro, J. Méndez
Monitoring of the impact of Prestige oil spill on Mytilus galloprovincialis from Galician coast

Environ Int, 32 (2006), pp. 342–348

O.M. Lage, F. Sansonetty, J.E. O'Connor, A.M. Parente
Flow cytometric analysis of chronic and acute toxicity of copper (II) on the marine dinoflagellate Amphidinium carterae
Cytometry, 44 (2001), pp. 226–235

B. Lah, S. Malovrh, M. Narat, T. Cepeljnik, R. Marinsek-Logar
Detection and quantification of genotoxicity in wastewater-treated Tetrahymena thermophila using the comet assay
Environ Toxicol, 19 (2004), pp. 545–553

A.T. Large, J.P. Shaw, L.D. Peters, A.D. McIntosh, L. Webster, A. Mally, et al.
Different levels of mussel (Mytilus edulis) DNA strand breaks following chronic field and acute laboratory exposure to polycyclic aromatic hydrocarbons
Mar Environ Res, 54 (2002), pp. 493–497

A.J. Lin, X.H. Zhang, M.M. Chen, Q. Cao
Oxidative stress and DNA damages induced by cadmium accumulation
J Environ Sci China, 19 (2007), pp. 596–602

Y. Liu, Y. Zhang, J. Liu, D. Huang
The role of reactive oxygen species in the herbicide acetochlor-induced DNA damage on Bufo raddei tadpole live
Aquat Toxicol, 78 (2006), pp. 21–26

B.J. Majer, T. Grummt, M. Uhl, S. Knasmüller
Use of plant bioassays for the detection of genotoxins in the aquatic environment
Acta Hydroch Hydrobiol, 33 (2005), pp. 45–55

P. Mayer, R. Cuhel, N. Nyholm
A simple in vitro fluorescence method for biomass measurements in algal growth inhibition tests
Water Res, 31 (1997), pp. 2525–2531

P.V. McCormick, J.J. Cairns
Algae as indicators of environmental change
J Appl Phycol, 6 (1994), pp. 509–526

O. Melchiorri, G.G. Ortiz, R.J. Reiter, E. Sewerynek, W.M. Daniels, M.I. Pablos, G. Nistico
Melatonin reduces paraquat-induced genotoxicity in mice
BioMetals, 95 (1998), pp. 103–108

P.K. Mohapatra, R.C. Mohanty
Growth pattern changes of Chlorella vulgaris and Anabaena doliolum due to toxicity of dimethoate and endosulfan
Bull Environ Contam Toxicol, 49 (1992), pp. 576–581

F. Mouchet, S. Cren, C. Cunienq, E. Deydier, R. Guilet, L. Gauthier
Assessment of lead ecotoxicity in water using the amphibian larvae (Xenopus laevis) and preliminary study of its immobilization in meat and bone meal combustion residues
BioMetals, 20 (2007), pp. 113–127

Y.W. Nancharaiah, M. Rajadurai, V.P. Venugopalan
Single cell level microalgal ecotoxicity assessment by confocal microscopy and digital image analysis
Environ Sci Technol, 41 (2007), pp. 2617–2621

J.B. Oliveira, A.M.M. Goncalves, F. Goncalves, M.J. Pereira
Growth inhibition of algae exposed to paraquat
Fresenius Environ Bull, 16 (2007), pp. 621–625

M.G. Ormerod
Analysis of DNA. General methods
M.G. Ormerod (Ed.), Flow Cytometry. A Practical Approach, Oxford University Press, Oxford (1990), pp. 69–87

D. Pimentel
Amounts of pesticides reaching target pests: environmental impacts and ethics
J Agric Environ Ethics, 8 (1995), pp. 17–29

M.J. Plewa, E.D. Wagner, G.J. Gentile, J.M. Gentile
An evaluation of the genotoxic properties of herbicides following plant and animal activation
Mutat Res, 136 (1984), pp. 233–245

U.M. Rajendran, E. Kathirvel, A. Narayanaswamy

Effects of a fungicide, an insecticide, and a biopesticide on Tolypothrix scytonemoides
Pest Biochem Physiol, 87 (2007), pp. 164–171

R.H. Regel, J.D. Brookes, G.G. Ganf, R.W. Griffiths
The influence of experimentally generated turbulence on the Mash01 unicellular Microcystis aeruginosa strain
Hydrobiologia, 517 (2004), pp. 107–120

R.H. Regel, J.M. Ferris, G.G. Ganf, J.D. Brookes
Algal esterase activity as a biomeasure of environmental degradation in a freshwater creek
Aquat Toxicol, 59 (2002), pp. 209–223

G. Ribas, G. Frenzilli, R. Barale, R. Marcos
Herbicide-induced DNA damage in human lymphocytes evaluated by the single-cell gel electrophoresis (SCGE) assay
Mutat Res, 344 (1995), pp. 41–54

C. Rioboo, O. González, C. Herrero, A. Cid
Physiological response of freshwater microalga (Chlorella vulgaris) to triazine and phenylurea herbicides
Aquat Toxicol, 59 (2002), pp. 225–235

C. Rioboo, R. Prado, C. Herrero, A. Cid
Population growth study of the rotifer Brachionus sp. fed with triazine-exposed microalgae
Aquat Toxicol, 83 (2007), pp. 247–253

M.E. Sáenz, J.L. Alberdi, W.D. Di Marzio, J. Accorinti, M.C. Tortorelli
Paraquat toxicity to different green algae
Bull Environ Contam Toxicol, 58 (1997), pp. 922–928

Y.F. Sasaki, F. Izumiyama, E. Nishidate, N. Matsusaka, S. Tsuda
Detection of rodent liver carcinogen genotoxicity by alkaline single-cell gel electrophoresis (comet) assay in multiple mouse organs (liver, lung, spleen, kidney, and bone marrow)
Mutat Res, 391 (1997), pp. 201–214

Y.F. Sasaki, E. Nishidate, F. Izumiyama, N. Matsusaka, S. Tsuda
Simple detection of chemical mutagens by alkaline single-cell gel electrophoresis (comet) assay in multiple mouse organs (liver, lung, spleen, kidney, and bone marrow)

Mutat Res, 391 (1997), pp. 215–231

K.K. Schrader, M.Q. DeRegt, C.S. Tucker, S.O. Duke
A rapid bioassay for selective algicides
Weed Technol, 11 (1997), pp. 767–774

A. Schützendübel, A. Polle
Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization
J Exp Bot, 53 (2002), pp. 1351–1365

S. Sharma, N.S. Nagpure, R. Kumar, S. Pandey, S.K. Srivastava, P.J. Singh, et al.
Studies on the genotoxicity of endosulfan in different tissues of fresh water fish Mystus vittatus using the comet assay
Arch Environ Contam Toxicol, 53 (2007), pp. 617–623

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

N.P. Singh, R.E. Stephens, E.L. Schneider
Modifications of alkaline microgel electrophoresis for sensitive detection of DNA damage
Int J Radiat Biol, 66 (1994), pp. 23–28

B. Sosak-Swiderska, D. Tyrawska, B. Maslikowska
Microalgal ecotoxicity test with 3,4-dichloroaniline
Chemosphere, 37 (1998), pp. 2975–2982

R.G.J. Stahl
The genetic toxicology of organic compounds in natural waters and wastewaters
Ecotoxicol Environ Saf, 22 (1991), pp. 94–125

R.J.C.A. Steen, P.E.G. Leonards, U.A.T. Brinkman, D. Barcelo, J. Tronczynski, T.A. Albanis, et al.
Ecological risk assessment of agrochemicals in European estuaries
Environ Toxicol Chem, 18 (1999), pp. 1574–1581

L.A. Summers
The Bipyridinium Herbicides

Academic Press, New York (1980)

R.R. Tice, E. Agurell, D. Anderson, B. Burlinson, A. Hartmann, H. Kobayashi, et al.
Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing
Environ Mol Mutagen, 35 (2000), pp. 206–221

Z. Tukaj, W. Pokora
Individual and combined effect of anthracene, cadmium and chloridazone on growth and activity of SOD izoformes in three Scenedesmus species
Ecotoxicol Environ Saf, 65 (2006), pp. 323–331

R. Vindas, F. Ortiz, V. Ramírez, P. Cuenca
Genotoxicity of three pesticides used in Costa Rican banana plantations
Rev Biol Trop, 52 (2004), pp. 601–609

J.T. Wilson, P.L. Pascoe, J.M. Parry, D.R. Dixon
Evaluation of the comet assay as a method for the detection of DNA damage in the cells of a marine invertebrate, Mytilus edulis L
(Mollusca: Pelecypoda). Mutat Res, 399 (1998), pp. 87–95

P.K. Wong
Effects of 2,4-D, glyphosate and paraquat on growth, photosynthesis and chlorophyll-a synthesis of Scenedesmus quadricauda Berb 614
Chemosphere, 41 (2000), pp. 177–182

B.P. Yu
Cellular defenses against damage from reactive oxygen species
Physiol Rev, 74 (1994), pp. 139–162

Y. Yu, F. Kong, M. Wang, L. Qian, X. Shi
Determination of short-term copper toxicity in a multispecies microalgal population using flow cytometry
Ecotoxicol Environ Saf, 66 (2007), pp. 49–56

Corresponding author. Tel.: +34 981 167000; fax: +34 981 167065.