Screening acute cytotoxicity biomarkers using a microalga as test organism

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Abstract

The present study checked the suitability of the integration of flow cytometry (FCM) as technique and a freshwater microalga (*Chlamydomonas moewusii*) as cell system model for ecotoxicological studies, looking for sensitive biomarkers of acute cytotoxicity of potential contaminants in aquatic systems. The detection of the potential acute toxicity of a pollutant is of interest because pulse discharges of contaminants to natural waters could lead to high concentrations of these substances that are only present for short periods of time but can affect aquatic organisms such as microalgae. Physiological alterations in *C. moewusii* cells were analysed after 1 h of exposure to different concentrations of the herbicide paraquat. Cell viability was not affected, but the acute toxicity of paraquat was evident at other levels of cell physiology. Herbicide-treated cells showed lower autofluorescence and higher size and internal complexity, lower esterase activity and lower mitochondrial membrane potential. Paraquat induced the depolarisation of the plasma membrane and the increase of intracellular free calcium level and cytosolic pH in a concentration-dependent percentage of cells. All these effects can be related to the oxidative stress induced by the herbicide, as revealed the significantly increased intracellular levels of reactive oxygen species in cultures exposed to paraquat concentrations which induced the physiological alterations mentioned above. Excluding cell viability and mitochondrial membrane potential, these cytotoxicity endpoints could be considered sensitive biomarkers for the short-term exposure to pollutants such as herbicides.

Highlights

- This study examine the acute cytotoxicity of paraquat on microalgal physiology.
- Flow cytometric protocols assayed allow the screening of different toxicity cellular endpoints. ► Cell viability is not a sensitive biomarker.
- Short-term paraquat exposure induces alteration in the overall cellular ionic homeostasis.
- Most alterations observed could be related with the overproduction of ROS.

Keywords

Microalga; Flow cytometry; Cytotoxicity; Pollution; Biomarker

1. Introduction

Agricultural areas are generally pesticide-dependent and generate water pollution due to transfer of pesticide residues. Their ecological effects in aquatic ecosystems are broad-ranging owing to the variety of substances present (DeLorenzo et al., 2001). In particular, herbicides are widely used to protect crops against adventicious plants, but an excess of them can generate negative effects on the environment. Aquatic non-target organisms are of primary interest when addressing the potential adverse effects of these pollutants, microalgae an example of them. They are known comparatively sensitive to chemicals (Real et al., 2003) and, because of their short life cycle, often provide one of the first signals of ecosystem impacts. Their primary production and their essential roles in nitrogen and phosphorus cycling are critical to aquatic ecosystems (Källqvist and Svenson, 2003 and Sabater and Carrasco, 2001). Moreover, the alteration of species composition in aquatic community may affect the structure and function of the whole aquatic ecosystem (Rioboo et al., 2007).

The authorisation for pesticide commercialisation in Europe currently requires previous testing of potential negative effects of the active ingredients on non-target aquatic organisms (Pereira et al., 2009). It is necessary the development of methods for the detection of contaminants based on the physiological response of organisms to provide an early warning of sublethal pollution. It is also desirable being able to test the acute toxicity of chemicals on response variables that are sensitive to sublethal

concentrations. Algal growth bioassays remain the preferred technique for assessment of phytotoxic effects in most ecotoxicological studies (Tai et al., 2010) since growth is a population parameter that tends to integrate and reflect most sublethal effects and an effect at a population level is likely ecologically relevant. But these tests provide bulk measurements and results obtained do not offer information regarding mode of toxic action, as well as requiring between 3 and 7 days to determine growth rates based on an increase in cell number with adequate precision and the test duration can result in substantial losses of toxicant by volatilisation, adsorption to the test container and degradation, all of which might lead to an underestimation of toxicity. Flow cytometry (FCM) is an alternative to the standard population-based endpoints, allowing the rapid and quantitative measurement of functional responses of individual cells to a toxic stress (Cid et al., 1996 and Franqueira et al., 2000). Different studies with microalgae as test organisms have demonstrated that FCM combined with the use of different fluorochromes is a quick and convenient technique to assess toxic effects of contaminants (Adler et al., 2007; Jamers et al., 2009; Prado et al., 2011; Rioboo et al., 2009a,b).

The highest concentration of herbicides measured in flowing surface waters can be associated with pulse discharges (Cedergreen et al., 2005). A study of the potential toxic effects of short-term exposures to high but environmentally relevant concentrations of these pollutants may be of interest. In this work, changes on the physiological status of a microalgal species (*Chlamydomonas moewusii*) exposed during 1 h to a worldwide used herbicide (paraquat) were evaluated by FCM. Besides inherent cell properties (chlorophyll autofluorescence, size and intracellular complexity), cytomic indicators for assessing viability (membrane integrity), metabolic health (esterase activity), oxidative stress (superoxide anion and hydrogen peroxide levels), cytoplasmic and mitochondrial membrane potentials and ionic homeostasis (Ca^{2+} level and cytosolic pH), were also studied.

2. Materials and methods

2.1. Microalgal cultures

C. moewusii Gerloff (*Chlamydomonadaceae*) (strain CCAP 11/5B) was maintained in sterile Bristol medium (Brown et al., 1967).

All tests were carried out in an incubator under controlled conditions according to conditions established for maintaining stock cultures: 18±1 °C, illuminated with a

photon flux of 70 μmol m^{-2} s^{-1} under a dark:light cycle of 12:12 h. Microalgal cells in early exponential growth phase were used as inoculum. Initial cell density was 4×10^5 cells ml^{-1}. Paraquat stock solution (1 mM) was prepared by dissolving granulated pure herbicide in sterile distilled water. Cultures without paraquat were included as controls. All cultures were carried out in triplicate. Final paraquat concentrations assayed were 1, 5 and 10 μM. Actual paraquat concentrations in culture samples were determined by a HPLC-MS protocol (Castro et al., 1999), being close to nominal concentrations: 0.96, 4.80 and 9.60 μM.

2.2. Flow cytometric analysis of microalgal cells

Flow cytometric analysis of *C. moewusii* cells were performed after 1 h of culture in a Coulter Epics XL4 flow cytometer (Beckman Coulter Inc.) equipped with an argon-ion excitation laser (488 nm), detectors of forward (FS) and side (SS) light scatter and four fluorescence detectors corresponding to different wavelength intervals: 505–550 nm (FL1), 550–600 nm (FL2), 600–645 nm (FL3) and >645 nm (FL4). Forward scatter and red chlorophyll fluorescence histograms were used to characterise the microalgal population, setting gating levels in order to exclude non-microalgal particles.

Cell suspensions (2×10^5 cells ml^{-1}) were incubated with the appropriate fluorochrome at room temperature and darkness for the necessary time. The lowest fluorochrome concentration and the shortest incubation time were chosen in order to obtain significant and stable staining of cells without toxicity being developed.

For each cytometric parameter investigated, at least 10^4 gated cells were analysed per sample and fluorescence measurements were obtained in a logarithmic scale. Data were collected using listmode files and statistically analysed using the EXPO32 ADC software (Beckman Coulter Inc.). Results were expressed as mean values obtained from histograms in arbitrary units (a.u.), unless it was said otherwise.

2.2.1. Inherent cell properties

Aliquots of microalgal cultures were resuspended in phosphate buffered saline solution (PBS, pH 7.4) and analysed by FCM to study the potential alterations in the forward light scatter (FS), related to the cell size; the side light scatter (SS), related to the intracellular complexity; and the red autofluorescence (FL4), related to the chlorophyll *a* fluorescence emission, as described in a previous paper (Prado et al., 2011).

2.2.2. Cell viability

Propidium iodide (PI) can be used to discriminate between viable non-fluorescent cells and non-viable fluorescent cells, then variations on cell viability for *C. moewusii* cultures were studied staining cells with PI at a final concentration of 4 µM(Prado et al., 2009a).

2.2.3. Metabolic activity

Metabolic health was assessed using a fluorescein diacetate-based cell esterase activity assay, a sensitive and rapid technique to assess phytoplankton metabolic activity (Jochem, 1999 and Prado et al., 2009a).

A kinetic approach to the FDA assay (*in fluxo* analysis) was used in this work, recording the increase of the FDA-dependent fluorescence after FDA addition (final concentration: 0.2 µM), depending on time (Prado et al., 2012).

2.2.4. Oxidative stress

Oxidative stress in *C. moewusii* was evaluated based on FCM determinations of intracellular levels of superoxide anion radical ($O_{\bullet 2^-}$) and hydrogen peroxide (H_2O_2), using the fluorochromes hydroethidine (HE) (final concentration: 16 µM) and dihydrorhodamine 123 (DHR123) (final concentration: 29 µM), respectively (Prado et al., 2012).

To avoid the variability of data due to differences in cell size, fluorescence was corrected by cell size estimated using the FS.

2.2.5. Cytoplasmic membrane potential

This parameter was monitored using a slow-response potentiometric probe, the bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$), at a final concentration of 1 µ M(Prado et al., 2012). Cytoplasmic membrane depolarisation will be reflected in an increased intracellular anionic dye concentration, *i.e.,* by accumulation of dye in the cells, whilst decreased accumulation will reflect hyperpolarisation.

Results were expressed as the percentage of depolarised cells *vs*. the total amount of cells analysed per culture.

2.2.6. Mitochondrial membrane potential

The cationic fluorescent dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1) has been reported as a reliable probe for analysing mitochondrial membrane potential by FCM (Salvioli et al., 1997). JC-1 exhibits potential-dependent accumulation in respiring mitochondria that is detectable

by a fluorescence shift from green to orange, due to the concentration-dependent formation of orange fluorescent oligomers (Reers et al., 1991).

A JC-1 stock solution was prepared in dimethylsulfoxide (DMSO) at a final concentration of 1.5 mM. Cell suspensions were incubated with JC-1 (final concentration: 0.8 μM) for 15 min. The fluorescence intensities of both monomer and aggregated JC-1 molecules were recorded by the detectors FL1 and FL2, respectively. Results were expressed as the orange (JC-1 oligomers)/green (JC-1 monomers) fluorescence intensity ratio, a live-time software generated parameter, which is dependent only on the mitochondrial membrane potential (Cassart et al., 2007). This ratio was calculated directly during data acquisition by the cytometer data analysis system, and presented on a linear 1024-channel histogram. The ratio value is given by the mean channel number of this histogram.

To verify the specificity of JC-1 staining, cells were treated with the mitochondrial potential dissipator carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (final concentration of 49 μM during 15 min), and stained with JC-1 as described above.

2.2.7. Intracellular free calcium

Cytosolic Ca^{2+} was analysed by FCM staining cells with one of the most sensitive indicators, Calcium Green-1 acetoxymethyl ester (Calcium Green-1 AM) (Lee et al., 1999). The cell-permeant acetoxymethyl ester, non-fluorescent and Ca^{2+} insensitive, can be passively loaded into cells, where it is cleaved by ubiquitous intracellular esterases to the cell-impermeant fluorescent product Calcium Green-1, which exhibits an increase in fluorescent emission intensity (530 nm) upon binding Ca^{2+}.

A Calcium Green-1 AM stock solution was prepared in DMSO at a final concentration of 2 mM. Cell suspensions were incubated with the fluorochrome (final concentration: 8 μM) at 30 °C for 2 h, and the green fluorescent emission was collected by the FL1 detector. In order to avoid the variability due to differences in cell size, fluorescence was corrected by cell size estimated using the FS.

Validation of this staining protocol was carried out by incubating cells with the fluorochrome in the presence of mastoparan, a G-protein activator known to induce Ca^{2+} release from intracellular stores in *C. moewusii* cells (Kuin et al., 2000).

2.2.8. Intracellular pH (pH_i)

Changes in pH_i were evaluated using the non-fluorescent acetoxymethyl ester form of the fluorochrome 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF AM). BCECF AM diffuses through the cell membrane and intracellular esterases cleave the ester bond releasing BCECF, which fluoresces according to the pH_i. When excited by

blue light, this fluorochrome emits fluorescence with a maximum at 525 nm and, in the range of physiological pH, the emission intensity increases with increasing pH. The fluorescence emitted at 620 nm is not pH-dependent, in spite of this wavelength is not really an isosbestic point (Franck et al., 1996). Therefore, the ratio of the fluorescence emitted at 525 and 620 nm (green/red) was used to analyse pH_i in cells stained with BCECF, as reported by several authors (Corvini et al., 2000, Cherlet et al., 1999 and Koo et al., 2007). This ratiometric method allowed correcting for differences in fluorochrome uptake and cell size.

A BCECF AM stock solution was prepared in DMSO at a final concentration of 1.2 mM. Cell suspensions were incubated with BCECF (final concentration: 6 µM) for 40 min. The green and red fluorescences were collected by the FL1 and FL3 detectors, respectively. The ratio of the two signals was calculated directly during data acquisition by the cytometer data analysis system, and presented on a linear 1024-channel histogram.

Validation of this method was carried out by resuspending BCECF-labelled cells in high $[K^+]$ buffers prepared at increasing pH (6, 7 and 8) in the presence of 20 µM nigericin and 1 µM valinomycin for 10 min before FCM analysis, in order to equilibrate the pH_i with that of the surrounding buffer (Corvini et al., 2000).

2.3. Data analysis

Data are given as mean values±standard error (S.E.) of the mean. To determine significant differences among test concentrations, data were statistically analysed by overall one-way analysis of variance (ANOVA) using SPSS 16.0 software. A *p*-value<0.05 was considered statistically significant. When significant differences were observed, means were compared using the multiple-range Duncan test.

3. Results

3.1. Inherent cell properties

Cell size, intracellular complexity and chlorophyll *a* fluorescence were affected by a 1 h-exposure of *C. moewusii* to the assayed paraquat concentrations (Table 1). The most sensitive parameter was the intracellular complexity, which was significantly increased in all cultures exposed to the herbicide. An increase of cell size was also

observed in cultures exposed to paraquat although it was statistically significant in cultures exposed to paraquat concentrations of 5 μM or highe r (Table 1).

Chlorophyll *a* fluorescence was significantly reduced in cultures with a paraquat concentration of 5 μM or higher (Table 1).

Table 1.

Flow cytometric analysis of inherent cell properties (chlorophyll fluorescence, cell size and intracellular complexity) in *C. moewusii* cultures after 1 h of exposure to different paraquat concentrations. Data, expressed as arbitrary units (a.u.), are mean±S.E. Statistically significant differences with respect to control at $p<0.05$ are indicated by (□).

Paraquat (μM)	**Chlorophyll*a*fluorescence (a.u.)**	**Cell size (a.u.)**	**Intracellular complexity (a.u.)**
0	578±7	488±2	187±2
1	575±2	498±2	205±1□
5	550±6□	515±2□	285±3□
10	500±1□	522±4□	292±5□

3.2. Cell viability

Cell viability of *C. moewusii* was not significantly affected by the assayed paraquat concentrations. A small percentage of non-viable cells, about 5 percent, began to be apparent only in cultures exposed to the highest paraquat concentration (10 μM) (Fig. 1).

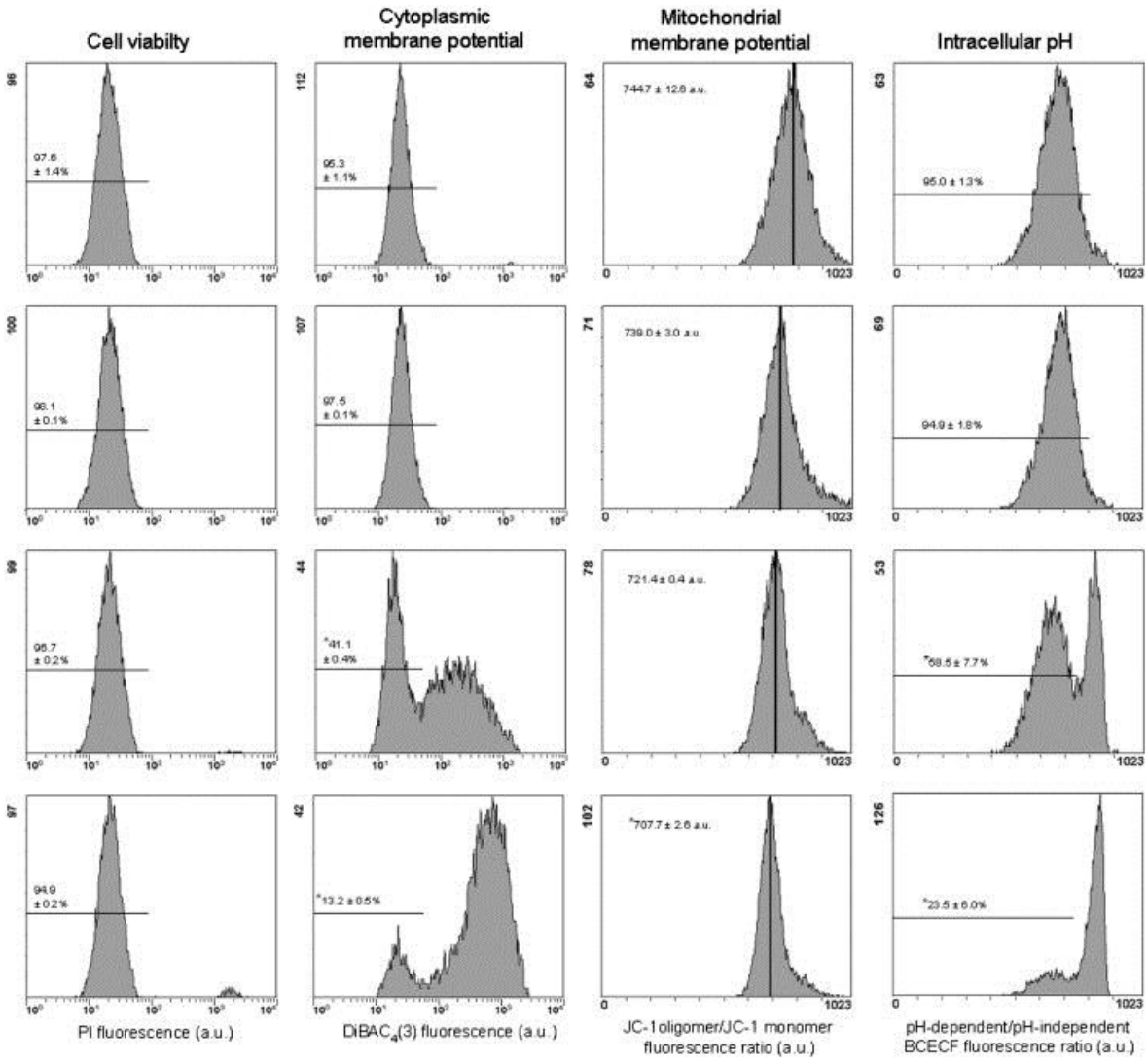

Fig. 1.

Representative histograms of flow cytometric analysis of cell viability, cytoplasmic membrane potential, pH_i and mitochondrial membrane potential in *C. moewusii* cells cultures after 1 h of exposure to different paraquat concentrations. Histograms show the distribution of fluorescence intensity of the fluorochrome, in logarithmic scale for PI and $DiBAC_4(3)$ and lineal scale for ratiometric measurements in case of BCECF and JC-1, among the cells analysed (*Y*-axis: cell number; *X*-axis: fluorescence intensity in arbitrary units, a.u.). Percentages of viable cells, normally polarized cells and non-affected pH_i cells are indicated in their respective histograms. Mean mitochondrial membrane potential (a.u.) is indicated for each culture. Data are expressed as mean values±S.E. (□) indicates significant differences with respect to control (p<0.05).

3.3. Cytoplasmic membrane potential

Results showed that the 1 h-exposure to paraquat lead to the depolarisation of the plasma membrane in a concentration-dependent percentage of microalgal population (Fig. 1). In cultures exposed to 5 μM, more than 50 percent of cells were depolarised cells, and only 13 percent of cells in cultures with 10 μM maintained a normal cytoplasmic membrane potential comparable to control cells (Fig. 1).

3.4. Mitochondrial membrane potential

Mitochondrial membrane potential of *C. moewusii* cells exposed to paraquat, measured as the orange (JC-1 oligomer)/green (JC-1 monomer) fluorescence ratio, was reduced in a concentration-dependent manner and this reduction was statistically significant in cultures exposed to 10 μM(Fig. 1).

3.5. Intracellular pH

C. moewusii pH_i, measured as the green/red BCECF fluorescence intensity ratio, was affected in cultures exposed to paraquat concentrations of 5 μM or higher (Fig.1). In these cultures, a significant percentage of cells showed a pH_i significantly higher with respect to the herbicide non-exposed cultures. In this way, nearly 80 percent of cells exposed to 10 μM of paraquat showed an alkalinisation of their cytoplasm (Fig. 1).

3.6. Metabolic activity

The esterase activity analysis revealed that the metabolic activity level of these cells was reduced by the herbicide, in a concentration-dependent manner (Fig. 2). It was already significantly reduced in cultures exposed to 5 μM, where cells showed an activity twice lower than control cells (Fig. 2).

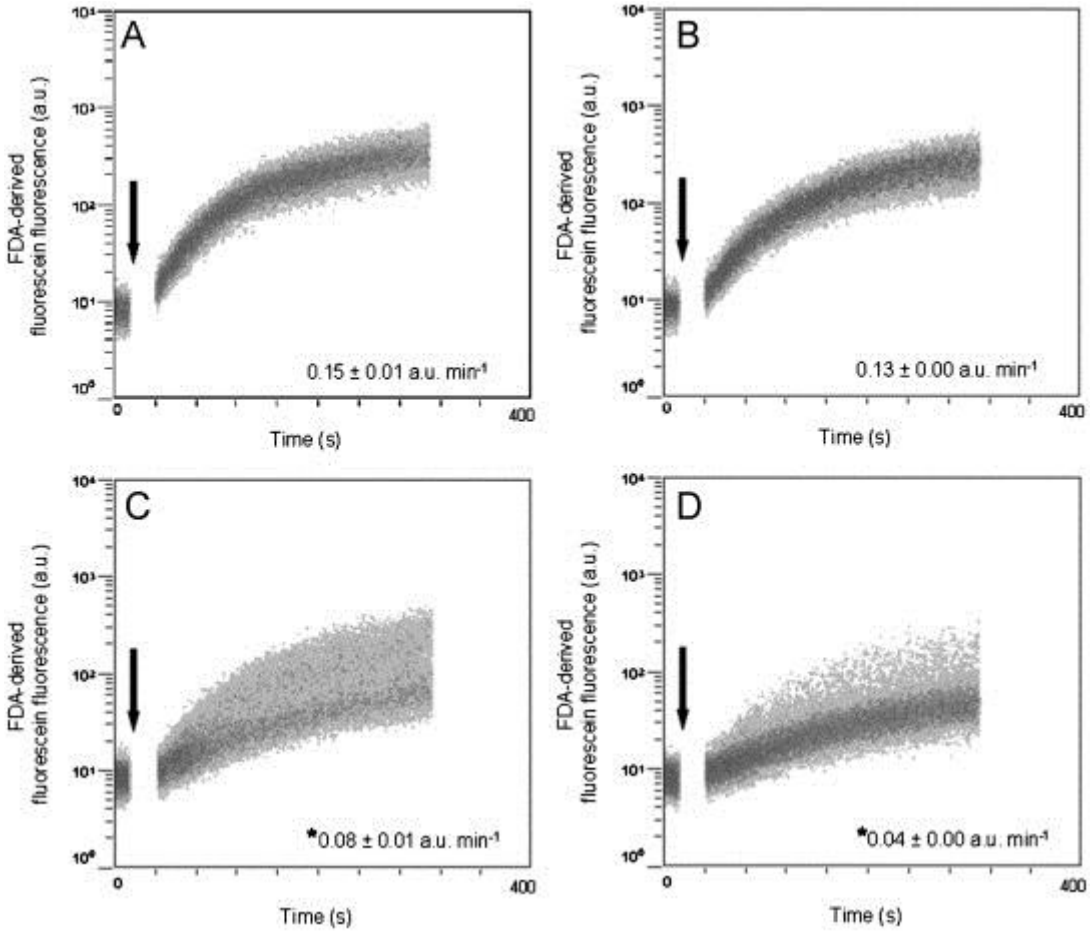

Fig. 2.

Analysis of esterase activity (as an index of metabolic activity) through kinetic plots showing the increase of the FDA-derived fluorescein fluorescence in function of time, for *C. moewusii* cells from control cultures (A) and cultures exposed to 1 (B), 5 (C) and 10 (D) μM of paraquat over 1 h. The arrow indicates the time of addition of FDA to unstained microalgal suspensions. Fluorescence generation rates are indicated in arbitrary units per minute (a.u. min^{-1}). Data are expressed as mean values±S.E. (□) indicates significant differences with respect to control (p<0.05).

3.7. Oxidative stress

A 1 h-exposure to paraquat concentrations of 5 μM, or higher, affected ROS intracellular levels. It was observed a heterogeneous cell response within the population (Fig. 3).

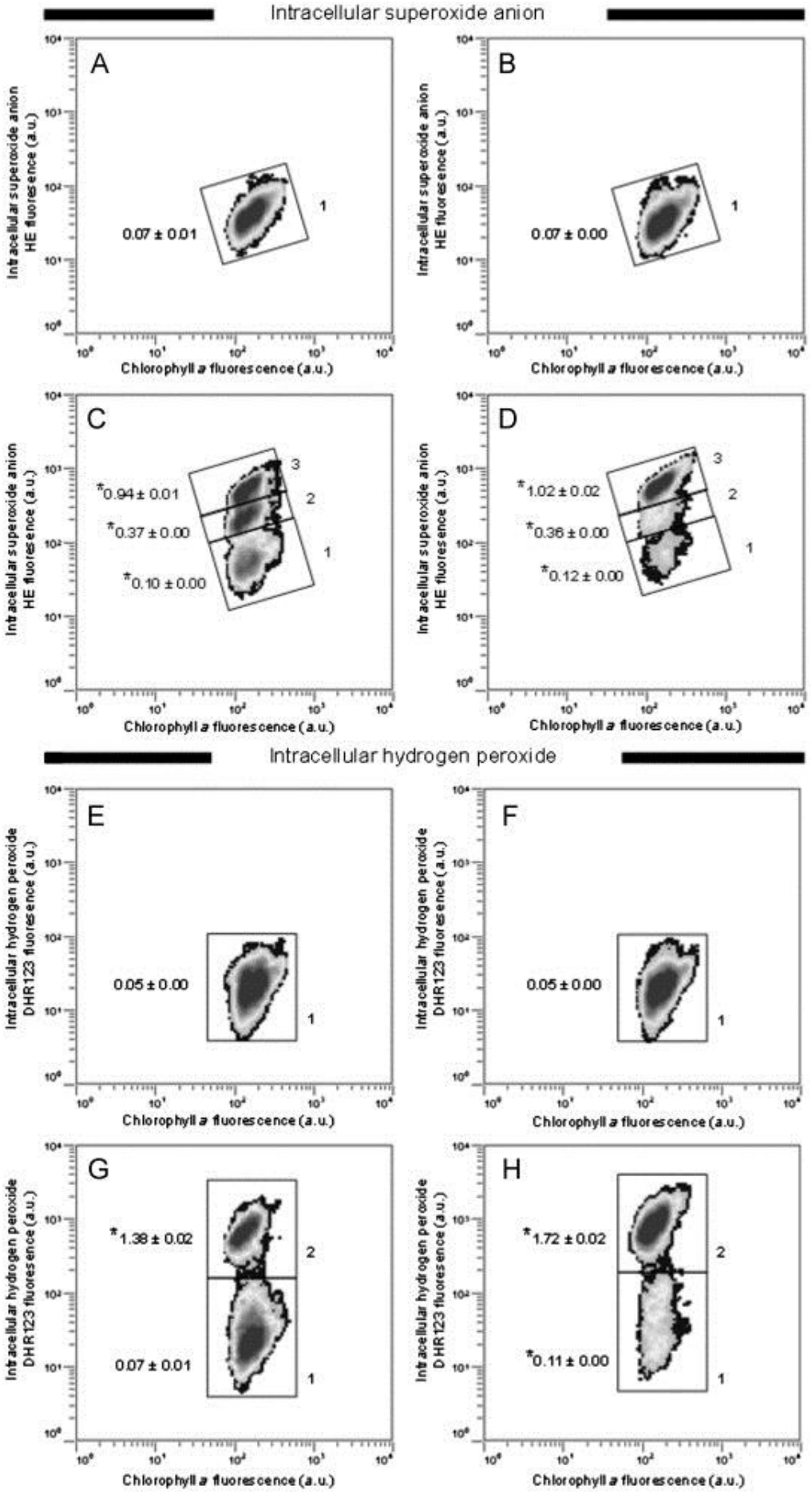
Intracellular superoxide anion
A
0.07 ± 0.01
1
B
0.07 ± 0.00
1
C
*0.94 ± 0.01
*0.37 ± 0.00
*0.10 ± 0.00
3
2
1
D
*1.02 ± 0.02
*0.36 ± 0.00
*0.12 ± 0.00
3
2
1
Intracellular hydrogen peroxide
E
0.05 ± 0.00
1
F
0.05 ± 0.00
1
G
*1.38 ± 0.02
0.07 ± 0.01
2
1
H
*1.72 ± 0.02
*0.11 ± 0.00
2
1
Intracellular superoxide anion HE fluorescence (a.u.)
Intracellular hydrogen peroxide DHR123 fluorescence (a.u.)
Chlorophyll a fluorescence (a.u.)

Fig. 3.

Biparametric histograms showing the intracellular levels of superoxide anion (A, B, C, D) and hydrogen peroxide (E, F, G, H), determined by flow cytometry using the fluorochromes HE and DHR123 respectively, *vs*. chlorophyll fluorescence, for control *C. moewusii* cultures (A, E) and cultures exposed to 1 (B, F), 5 (C, G) and 10 (D, H) μM of paraquat over 1 h. The regions correspond to each subpopulation differentiated. ROS intracellular level corrected by cell size is indicated for each subpopulation (mean±S.E.) and (□) indicates significant differences with respect to control (p<0.05).

In cultures exposed to 5 and 10 μM of paraquat, three cell subpopulations showing different intracellular $O_{\bullet 2}^-$levels could be distinguished, in any case a level significantly higher than that of control cells (Fig. 3A, C, D). In both cases, the highest percentage of cells (39 percent and 82 percent, respectively) match with the subpopulation showing the highest intracellular $O_{\bullet 2}^-$ level, which was more than ten times the level observed in control cells (Fig. 3A, C, D).

The intracellular H_2O_2 level was also increased in cultures exposed to 5 and 10 μM of paraquat (Fig. 3E, G, H), although the response was less sensitive. In cultures exposed to 5 μM only 37 percent of cells showed a H_2O_2 level significantly higher with respect to control. In cultures exposed to 10 μM, the two cell subpopulations differentiated showed an intracellular H_2O_2 level significantly higher with respect to control and, similarly to $O_{\bullet 2}^-$ level, 72 percent of cells belonged to the subpopulation showing the highest intracellular ROS level, which was more than thirty times the level observed in control cells (Fig. 3E, H).

3.8. Intracellular free Ca^{2+}

Two cell types could be differentiated in *C. moewusii* cultures: cells with a basal level of cytosolic Ca^{2+}(named 1 in Fig. 4), and cells with an increased Ca^{2+} level (named 2 in Fig. 4). In cultures exposed to herbicide concentrations of 5 μM or higher the percentage of cells with an increased cytosolic Ca^{2+} level was significantly increased with respect to control cultures, and this effect occurred in a concentration-dependent manner, so that after 1 h of exposure to 10 μM of paraquat 38 percent of cells showed this high intracellular free Ca^{2+} level, which was more than ten times the basal level observed in most cells of control cultures (Fig. 4).

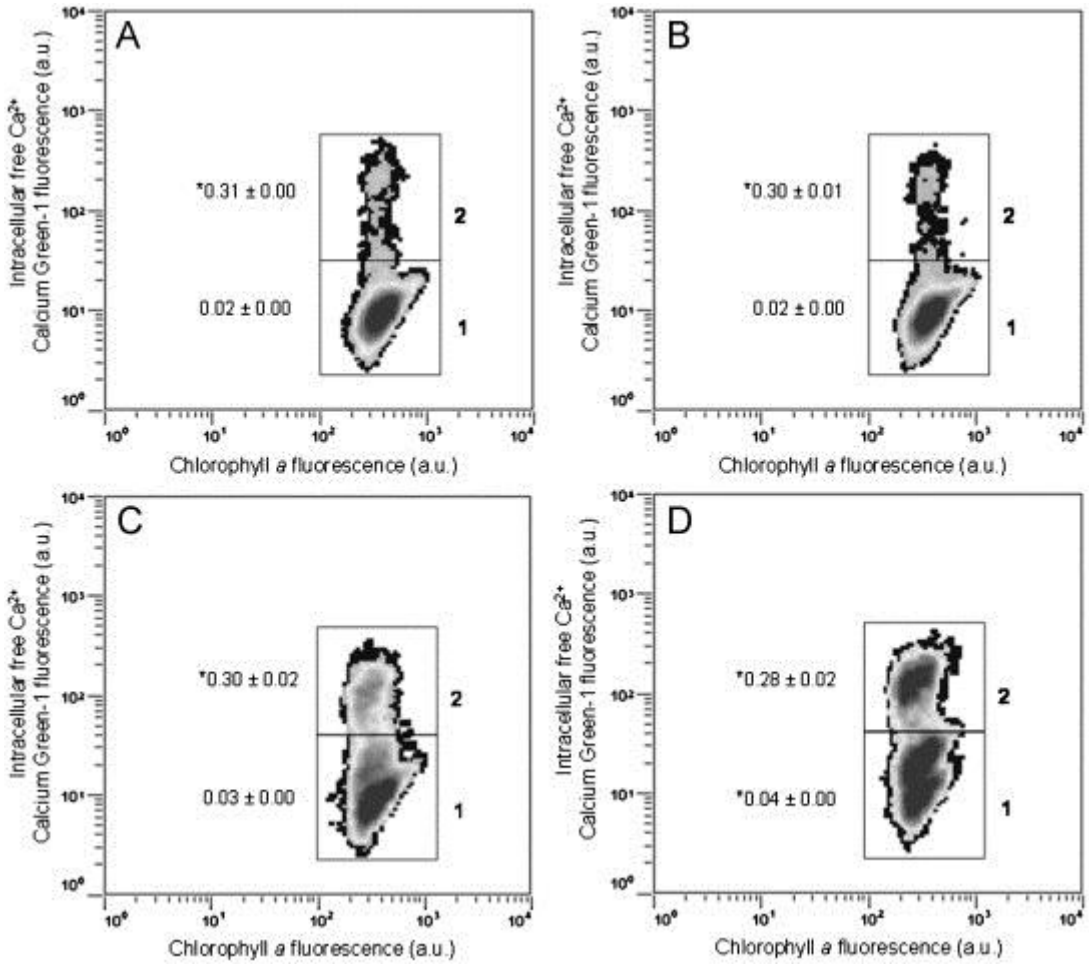

Fig. 4.

Biparametric histograms showing the intracellular free Ca^{2+} level, determined by flow cytometry using the fluorochrome Calcium Green-1 AM, *vs.* chlorophyll fluorescence, for control *C. moewusii* cultures (A) and cultures exposed to 1 (B), 5 (C) and 10 (D) μM of paraquat over 1 h. The regions correspond to each subpopulation differentiated. Ca^{2+} intracellular level corrected by cell size is indicated for each subpopulation (mean±S.E.) and (□) indicates significant differences with respect to the basal level showed by most cells in control cultures (p<0.05).

4. Discussion

Paraquat concentrations assayed here, being higher than those used in studies based on a more prolonged exposure to the herbicide (up to 96 h of exposure to concentrations up to 0.2 μM) (Prado et al., 2009a, Prado et al., 2009b and Prado et al., 2011), are still environmentally relevant, since they are within the range of concentrations applied for aquatic weed control (0.4–19 μM) (Bacchetta et al., 2002, Cochón et al., 2007 and Eisler, 1990) and within the range of concentrations found in some watershed areas (up to 11 μM) (Interface, 2008).

Biomarkers can be of great value in ecotoxicological studies, since they usually represent the first warning signals to predict changes at higher levels of organisation during environmental stress. In this study, the integration of cell physiology analysis by FCM, as technique, and a freshwater microalga, as cell system model, has allowed screening several cell biomarkers of acute toxicity, as a sensitive ecotoxicological tool to early detect toxic effects arising from pulse discharges of contaminants to the environment.

Inherent cell properties analysed were affected, being intracellular complexity the most sensitive parameter (Table 1). The use of chlorophyll fluorescence as an endpoint in common monoalgal toxicity assays has been described in several studies (Eisentraeger et al., 2003 and Geis et al., 2000). Fluorescence yield may be associated with the cellular pigment amount, and also with the current status of the cell and environmental conditions, *e.g.,* under a toxic stress (Gregor et al., 2008). The autofluorescence decrease can be related to oxidative damage to PSII since the main source for the *in vivo* emission of chlorophyll *a* fluorescence is PSII (Sobrino et al., 2005). On the other hand, ROS produced as a consequence of paraquat action (Fig. 3) are highly reactive and can cause chlorophyll bleaching (Ekmekci and Terzioglu, 2005). In fact, long-term exposures of this microalgal species to paraquat caused chlorosis in a concentration-dependent manner (Prado et al., 2011). The overproduction of ROS detected (Fig. 3) could also explain the increase of intracellular complexity in cultures exposed to paraquat, related to a degradation and disorganisation of cell organelles as a result of the interact of these highly toxic agents with unsaturated lipids of membranes (Bray et al., 1993 and Suntres, 2002).

Oxidative stress induced by paraquat in *C. moewusii* cells became patently clear by the increased intracellular levels of $O_{\bullet 2}^{-}$ and H_2O_2 observed in cultures exposed to the herbicide, being $O_{\bullet 2}^{-}$ level more sensitive (Fig. 3). Paraquat has been reported to be a potent redox-cycling chemical agent (Bus and Gibson, 1984 and Cochón et al., 2007). Excess $O_{\bullet 2}^{-}$ results in the production of hydroxyl radicals and $H_2O_2$by a variety of reactions (Winterbroun, 1981). In cultures exposed to the lowest paraquat concentration, ROS intracellular levels did not show differences with respect to control (Fig. 3), which may be related with the efficiency of cellular antioxidant mechanisms.

Enzyme inhibition measurements in microalgae are becoming increasingly popular indicators of environmental stress because they offer a rapid and sensitive endpoint (Blaise and Ménard, 1998 and Peterson and Stauber, 1996). In particular, esterase activity, has been proved a useful indicator of environmental stress (Hadjoudja et al., 2009, Jamers et al., 2009 and Yu et al., 2007), and usually a decline in enzyme activity

is considered indicative of the presence of stress (Franklin et al., 2001 and Regel et al., 2002). This is in accordance with our results, where a reduction of esterase activity is observed in a concentration-dependent manner (Fig. 2). FDA-derived fluorescein is a pH-sensitive probe and its fluorescence intensity is enhanced as pH increases (Lage et al., 2001, Slavík, 1982 and Visser et al., 1979), but FDA-derived fluorescein fluorescence generation was significantly reduced in cultures exposed to paraquat concentrations of 5 µM or higher despite the increase of pH_i (Fig. 1), confirming the negative effect of paraquat on esterase activity.

Perturbations of the cytoplasmic membrane potential have been reported to be sensitive and rapid indicators of physicochemical changes in the extracellular environment (Lloyd et al., 2004). The results obtained revealed that paraquat induces a depolarisation of the cytoplasmic

membrane in *C. moewusii* cells after 1 h of exposure at concentrations of 5 µM or higher (Fig. 1), but membrane integrity was not affected, so that it can be stated that this treatment damages microalgal plasma membrane mainly by permeabilisation rather than by disruption of membrane integrity. The use of membrane potential probes for viability measurements, in particular $DiBAC_4(3)$, has been reported (Jepras et al., 1995 and Papadimitriou et al., 2006), although results obtained in the present work can not conclude that depolarised cells were non-viable cells, but the decrease of cytoplasmic membrane potential match with the reduction of metabolic activity. Alterations of the membrane permeability properties can be related to the increase of cell size observed (Table 1), due to failures in the regulatory cell volume control process.

The increase in the cytosolic Ca^{2+} level has been usually observed in plant cells in response to different stress factors (Gong et al., 1998, Knight et al., 1997 and Sivaguru et al., 2005). The depolarisation of the cytoplasmic membrane of *C. moewusii* cells exposed to paraquat match with an increase in the intracellular free Ca^{2+} level and an alkalinisation of the cytosol (Fig. 1 and Fig. 4), suggesting a possible interaction between these parameters. Short-term exposures to paraquat induce substantial perturbations in the overall cellular ionic homeostasis of *C. moewusii*. Cytoplasmic membrane depolarisation and increases of cytosolic free Ca^{2+} has been related in several studies (Akiyoshi et al., 2007, Okazaki et al., 2002 and Sivaguru et al., 2005). A role of Ca^{2+} as a secondary messenger in the cell response to oxidative stress has been suggested (Price et al., 1994).

The alkalinisation of cytoplasm in *C. moewusii* cells exposed to high paraquat concentrations could be a consequence of the consumption of intracellular protons by

reactions implicated in ROS metabolism. A cytosolic alkalinisation has already been related to oxidative stress in microalgae (Cid et al., 1996).

Obtained results show that mitochondrial membrane potential is the less sensitive parameter among those analysed (Fig. 1). This could be indicative of mitochondria is not the main site of action of paraquat in *C. moewusii* cells, or this organelle has antioxidant mechanisms that neutralises paraquat radical toxicity, according to Vicente et al. (2001).

5. Conclusion

Sublethal paraquat concentrations early affects the physiological status of *C. moewusii* since all the cytotoxicity endpoints assayed showed a significant response after a short period (1 h) of herbicide exposure. In the present study, most alterations observed could be related with the overproduction of ROS at this early moment.

This microalgal species has shown to be very sensitive to this kind of pollutants and all the parameters assayed can be considered potential and sensitive biomarkers for the acute toxicity of several pollutants (pesticides, metals), except cell viability and the mitochondrial membrane potential. Furthermore, these protocols can be adapted to other sensitive microalgal species (freshwater or marine species) providing a useful technique for ecotoxicity studies.

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