CYTOTOXIC EFFECTS OF PESTICIDES ON MICROALGAE DETERMINED BY FLOW CYTOMETRY

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Citation:

Rioboo C, Prado R, Herrero C, Cid A (2009) Cytotoxic effects of pesticides on microalgae determined by flow cytometry. In *Progress in pesticides research*, Kanzantzakis CM (Ed.). Nova Science Publishers, Hauppauge, NY

ABSTRACT

As much as *ca*. 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 frequently ocurring organic pollutants in aquatic ecosystems.

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 is probably acquired by using test species representing non-target groups. Because of their short generation times, microalgae respond rapidly to environmental changes. Furthermore, microalgal tests are generally sensitive, rapid and low-cost effective. 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.

Cytotoxic effects of aquatic pollutants on microalgae are very heterogeneous, and they are influenced by the environmental conditions and the test species. Growth, photosynthesis, chlorophyll fluorescence and others parameters reflect the toxic effects of pollutants on microalgae; however, other relevant endpoints are less known because experimental difficulties, especially under *in vivo* conditions.

Flow cytometry offers multiparametric analysis of cells on a cell-by-cell basis in near *in vivo* conditions. Applied in the study of the aquatic environments since the 80's, this technique has achieved extensive use for the study of microalgae and has been introduced as an alternative to more traditional techniques of analyzing cells in culture and from natural populations.

Several parameters determined by flow cytometry, such as cell volume and granularity, chlorophyll *a* fluorescence, cell viability, cell proliferation, oxidative stress, membrane potential or intracellular calcium level, were employed to assess changes in the physiological status of different microalgae as a consequence of the toxic action of herbicides. The variety of results obtained in the present study reveals that flow cytometry is a useful tool in the toxicity tests with microalgae.

INTRODUCTION

Aquatic environments, including freshwater, estuaries, and coastal marine waters, are often contaminated with numerous organic and inorganic compounds, including pesticides. As much as *ca.* 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 aquatic ecosystems. The increasing occurrence of pollutants in general, and herbicides in particular, has stimulated many studies on their toxicity to aquatic microorganisms, and the need for convenient methods for assayed pollutants toxicity has become evident.

Microalgae as Environmental Monitoring Markers

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 is probably acquired by using test species representing non-target groups. Because of their short generation times, microalgae respond rapidly to environmental changes. Furthermore, microalgal tests are generally sensitive, rapid and low-cost effective. 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.

Growth, photosynthesis, chlorophyll fluorescence and others parameters reflect the toxic effects of pollutants on microalgae; however, other relevant endpoints are less known because experimental difficulties, especially under *in vivo* conditions. Cytotoxic effects of aquatic

pollutants on microalgae are very heterogeneous, and they are influenced by the environmental conditions and the test species.

Flow Cytometry Technique

Flow cytometry (FCM) offers multiparameter analysis of cells on a cell-by-cell basis in near *in vivo* conditions [1]. Since the overview of FCM in biological oceanography publishedby Legendre and Yentsch in 1989 [2], the aquatic environmental research has changed quite drastically. In a first period, the use of FCM was focused to fundamental ecological questions, mainly abundance and distribution of phytoplankton in the ecosystems. In the 1990s, the flow cytometric studies becoming progressively involved in programs to address some of the major socioecological problems that threaten human societies, such as the release of contaminants in aquatic systems.

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. The bulk population based endpoints used in algal toxicity tests did not supply information on the distribution of responses among the individual cells within the population. Flow cytometry is an alternative to the standard algal population-based endpoints, since it allows the rapid and quantitative measurement of responses of individual algal cells to a toxic stress. The development and use of fluorescent probes and dyes allow researches to assess changes in their physiological *status,* however their application is still far from routine.

Microalgae are ideal for flow cytometric analysis as they are unicellular and contain photosynthetic pigments such as chlorophyll *a* which emits fluorescence when excited by blue light [3]. FCM has the advantage in that it can perform multi-parameter analysis on a wide range of cell properties by measurement of algal cell densities, light scatter signals and chlorophyll autofluorescence, after excitation at 488 run.

The aim of this chapter is demonstrate the usefulness of the flow cytometry technique combined with microalgae to the assessment of potential cytotoxic effects of different herbicides to non-target species. Here we show some experimental results obtained in the study of toxic effects of paraquat on *Chlamydomonas moewusii* and terbutryn on *Chlorella vulgaris.* For this, data results presented in this chapter were obtained using a Coulter EPICS XL4 (Beckman Coulter Inc.) flow cytometer, equipped with an argon ion laser (15 mW) as light source with an emission wavelength of 488 nm (blue light), and the analysis software EXPO 32 ADC 1.1C (Beckman Coulter Inc.). At least 10.000 cells were analyzed for each parameter. Fluorescent

emission ranged the visible spectrum (400-700 run) detected by 4 different photomultipliers (FLl: <550 nm; FL2: 550-600 nm; FL3: 600-645 run; FL4: >650 nm).

EFFECTS OF PARAQUAT ON *CHLAMYDOMONAS MOEWUSII*

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 [4]. This quaternary ammonium compound is a cationic species in aqueous solution, and after application, this chemical can be adsorbed into the soil or transported to the aquatic environment by runoff or leaching [5]. Paraquat in cells leads to the generation of free oxygen radicals, leading to lipid peroxidation damaging cell membranes and cell death [6, 7]. Information on the influence of these chemicals on the lowest levels of the food chain is scarce [8, 9, 10].

Chlamydomonas moewusii cultures were carried out during 96 hours as described in Franqueira *et al.* (1999) [7J, using a paraquat concentration of 200 nM. Changes in cell volume, internal granularity, chlorophyll *a* fluorescence intensity and the evaluation of cell viability were analyzed by flow cytometry in cells after 96 hours of exposure to paraquat, respect to control cells cultured without paraquat. Aliquots of the different cultures were centrifuged and cells were resuspended in 0.5x PBS buffer (pH 7.2) to obtain cell densities of 2 x 10^5 cells ml^{-1} for their analysis by FCM. Shifts in forward scatter light (<20°) and side scatter light (90°) indicate cell size and internal granularity, respectively. Similarly, chlorophyll *a* fluorescence is detected at FL4 log channel (>650 mn). The fluorescence of cells stained with propidium iodide (PI; Sigma Chemical Co.) was measured to study cell viability. PI is a fluorescent dye that intercalates with double-stranded nucleic acids to produce red fluorescence when excited by blue light. It is unable to pass through intact cell membranes; however, when the cell dies the integrity of the cell membrane fails, and then PI is able to enter and stain the nucleic acids [11]. In this way, PI can be used to discriminate between live non-fluorescent cells and non-viable fluorescent cells; the orange fluorescent emission of this compound was collected in the FL3 channel (605-635 nm). Aliquots of 1 x $l0^6$ cells were stained with PI dissolved in PBS buffer (pH 7.4) to a final concentration of 2.5 µg ml^{-1} during an incubation period of 10 min, in darkness and room temperature. For each cytometric parameter investigated, at least 10^4 cells were analyzed per culture and fluorescence measurements were expressed in a logarithmic scale.

Since an increase in the forward light scatter (FSC) signal can be correlated with an increase in cell volume [12], paraquat concentration assayed caused a slight increase in the cell volume of

C. *moewusii*, respect to control cells (Figure 1). Increases of cell volume were reported for different microalgal species exposed to high concentrations of different pollutants [7, 13, 14]. Variations in the side light scatter (SSC) signal indicate variations in the internal granularity of cells [12]. A paraquat concentration of 200 nM provoked an important increase of the internal granularity of microalgal cells (Figure 2).

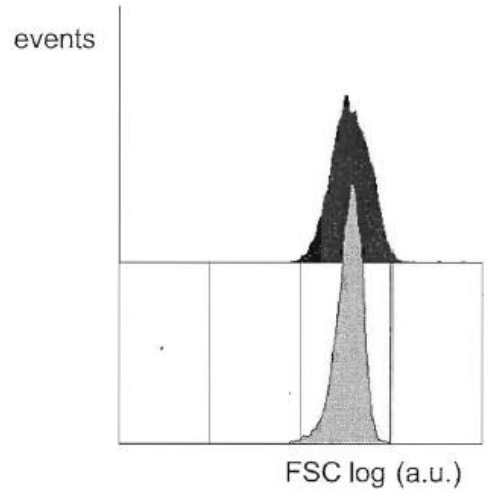

Figure 1. Overlay of FSC signal (a.u., arbitrary units) histograms showing the profiles of *Chlamydomonas moewusii* cells of control cultures (grey histogram) and cells exposed to 200 nM paraquat (black histogram) after 96 h of culture.

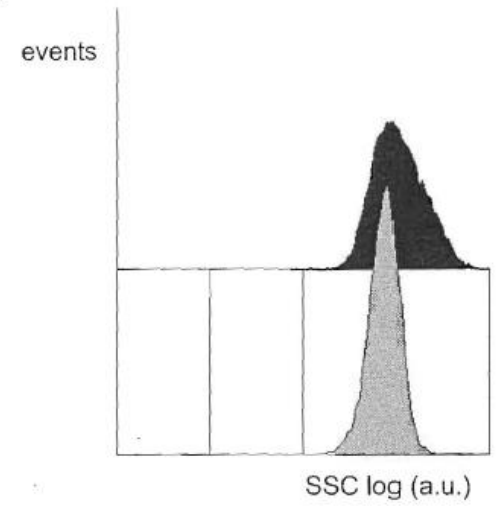

Figure 2. Overlay of SSC signal (a.u., arbitrary units) histograms showing the profiles of *Chlamydomonas moewusii* cells of control cultures (grey histogram) and cells exposed to 200 nM paraquat (black histogram) after 96 h of culture.

In cultures exposed to paraquat, cells formed palmelloid colonies (clusters of non-flagellated cells closed in a common wall), observed by light microscopy. The volume and complexity increases detected could be related to palmelloid colony formation, probably due to the incapacity to finish cell division as well as failures of regulation of cell volume because of the attack of oxidative radicals formed to membranes. Bray *et al.* [9], using transmission electron microscopy techniques, detected that *Chlamydomonas* cultures with 0.075 µM paraquat showed a small proportion of cells swollen and that they were less electron-dense than the normal cells, suggesting an increase in the cell moisture. At 0.15 µM paraquat, the proportion of cells with lower electrondensity had increased considerably and at 0.30 µM, all the cells appeared swollen

and were of low electron-density. These authors stated that in addition to the swelling and the low cellular electrondensity observed as a consequence of paraquat addition, other ultrastructural alterations were present that could explain the increase in the granularity that was detected using FCM at 96 h of culture.

Fluorescence of chlorophyll *a* was also affected after 96 hours of paraquat exposure (Figure 3). After 96 hours of culture, control cells showed a single and well defined peak of chlorophyll *a* fluorescence. However, *Chlamydomonas* cultures exposed to 200 nM of paraquat presented a bimodal distribution of chlorophyll *a* fluorescence, showing a differential cell response pattern. In the fluorescence histogram (Figure 3), we can distinguish two subpopulations of cells: one of them with a chlorophyll fluorescence signal close to that observed for control cells, and a second subpopulation showing an important decrease of chlorophyll fluorescence signal. Bulk measurements of fluorescence could not permit this assessment of a different behaviour of cells respect to this parameter, only achieved by a cell-to-cell analysis. The decrease of chlorophyll fluorescence observed for C *moewusii* cells exposed to paraquat could be related to an important decrease of the chlorophyll cellular content (unpublished data).

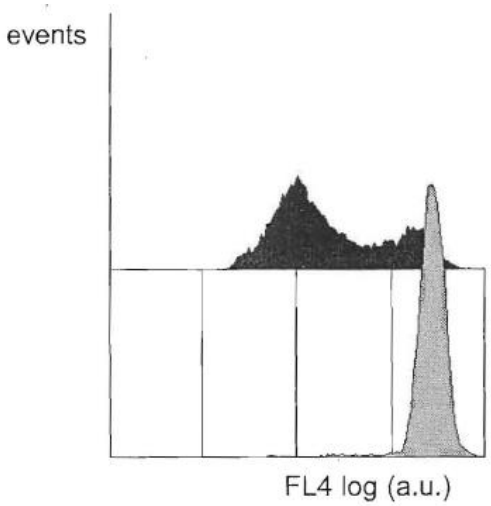

Figure 3. Overlay of chlorophyll *a* fluorescence (a.u., arbitrary units) histograms showing the profiles of *Chlamydomonas moewusii* cells of control cultures (grey histogram) and cells exposed to 200 nM paraquat (black histogram) after 96 h of culture.

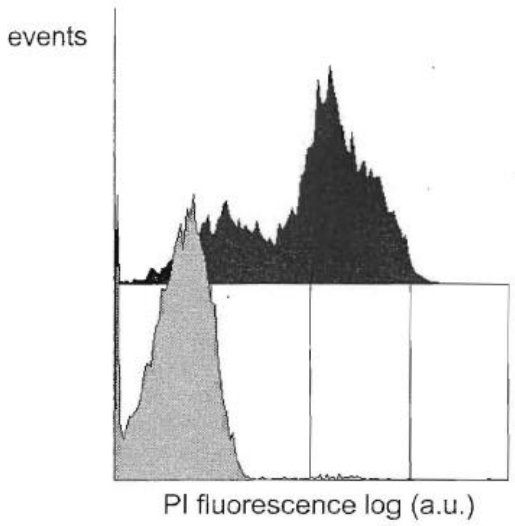

Figure 4. Overlay of propidium iodide (PI) fluorescence (a.u., arbitrary units) histograms showing the profiles of *Chlamydomonas moewusii* cells of control cultures (grey histogram) and cells exposed to 200 nM paraquat (black histogram) after 96 h of culture.

After 96 hours of culture, control cultures showed a 98.75% of viable cells, represented by non-fluorescent cells in the histogram (Figure 4), while the percentage of cell viability was only 26.09% cultures exposed to 200 nM of paraquat. Cellular membranes are selective, dynamic barriers that play an essential role in regulating biochemical and physiological events. The cell viability decrease after 96 h of paraquat exposure (Figure 4), showing a loss of their membrane integrity, like occurs in the degenerative processes driven by exogenous factors [15]. Standard microalgal bioassays are based in bulk population endpoints, as we indicated above, sacrificing information regarding the distribution of responses among individual cells within a population by providing the average response to a toxicant. Here we can distinguish between live of non viable cells using the flow cytometry technique which has the ability to analyze the real heterogeneous microalgal population.

EFFECTS OF A TRIAZINE ON *CHLORELLA VULGARIS*

Triazines are widely used herbicides that are highly toxic and frequently appear in natural watercourses. Terbutryn (N^2-tert-butyl-N-isopropyl-1,3,5-triazine-2,4-diamine) is a pre- or post-emergence systemic triazine herbicide, used to control most grasses and many annual broad-leaved weeds in winter cereals, potatoes, legumes, sunflowers, maize, sugar cane and citrus fruit, and as an aquatic herbicide for controlling submerged and free floating weeds and algae in watercourses [16, 17]. This herbicide affects the main energy production process in green cells, the photosynthesis [18], and this mode of action makes them potentially lethal to a wide variety of non-target species of primary producers, including phytoplankton and benthic algae.

The aim of this study is to assess the effect of terbutryn on the freshwater microalga *Chlorella vulgaris* from different flow cytometric approaches. The parameters used to monitor the herbicide effect are: the measurement of the evolution of cell progeny, the analysis of the oxidative stress, the changes in cell membrane potential and the intracellular calcium level.

Microalgal cultures were carried out during 96 hours as described in. *Rioboo et at. (2002)* [19], using a terbutryn concentration of 500 nM. Cultures without herbicide were also included as control. Red fluorescence of chlorophyll *a* (FL4; 645-675 nm) was used to exclude non-algal particles, to allow the analysis of *C. vulgaris* growth. Aliquots of the different cultures were centrifuged and cells were resuspended in 0.5x PBS buffer (pH 7.2) to obtain cell densities of 1 x 10^6 cells ml- l for their analysis by FCM. For each cytometric parameter investigated, at least 10^4 cells were analyzed per culture and fluorescence measurements were expressed in a logarithmic scale.

Evolution of Cell Progeny

During the last ten years, the stable intra-cytoplasmatic dye, 5-,6-carboxyfluorescein diacetate succimidyl ester (CFSE) has been widely used as a quantitative method to measure cell divisions in analysis of immunological lymphocyte proliferation. However, no data exists about its aplication in vegetable cells as microalgae are. CFSE allowed the identification of cell progeny and tracking and analysis of the division history of individual cells that had undergone multiple rounds of division. When incubated with cells, CFSE crosses the cell membrane and attaches to free amines groups of cytoplasmic cell proteins where it is transformed in fluorescein by intracellular esterases. Upon subsequent cell division, cytoplasnlic fluorescence per cell is equally redistributed among daughter cells at mitosis, providing an ideal tool for monitoring cell proliferation [20, 21].

The rapid and precise determination of proliferation rates and generation times by CSFE staining allow us to develop a model for assessing *in vivo* the effects of pollutants at microalgal cell level, non reached by radiolabeled techniques used until now in this kind of studies.

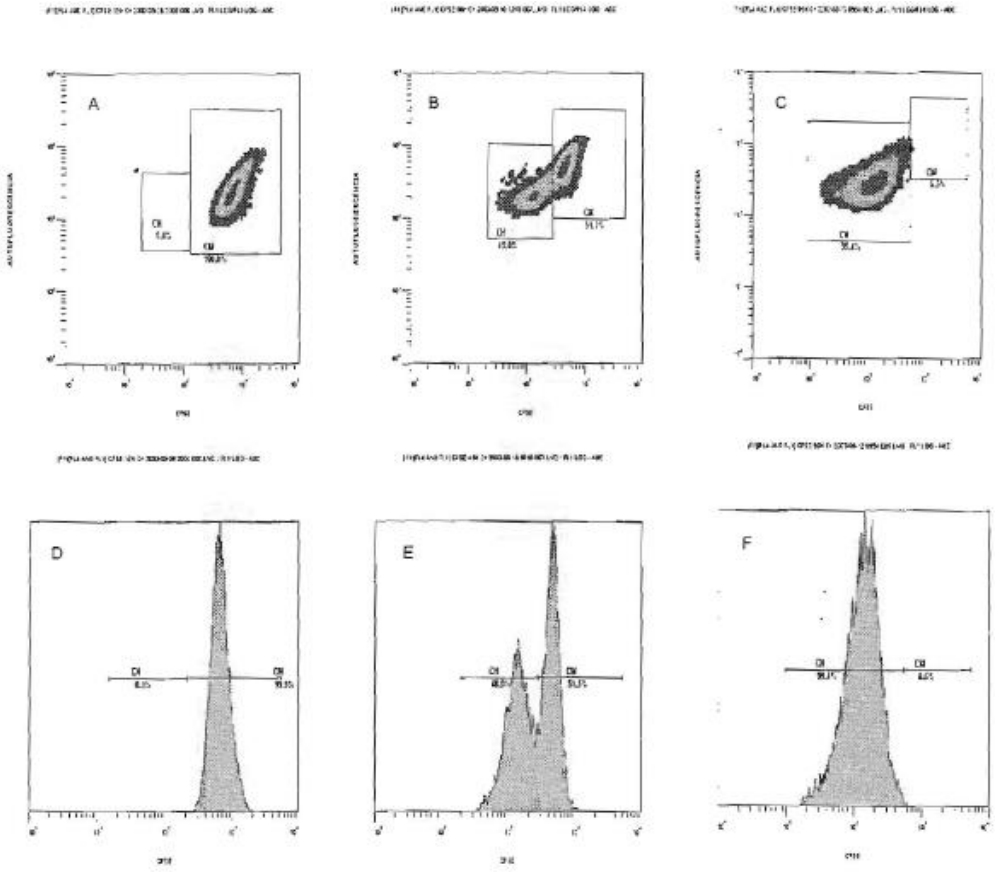

Figure 5. Cytograms of CFSE fluorescence *vs.* autofluorescence of *Chiarella vulgaris* cells cultured in control conditions at different times (12, 48 and 96 h; A, Band C, respectively). The histograms (D, E, F) show the CFSE fluorescence signal (a.u., arbitrary units). CH: daughter cells; CM: mother cells.

Cells were stained with CFSE dissolved in DMSO, at a final concentration of 32 µg CFSE per 150 x 10^6 cells per ml, in darkness at 20°C during 30 min before culture. Aliquots of cultures were analyzed by FCM after 12, 48 and 96 hours, when the green fluorescent emission of this compound was collected in the FL1 channel (<550 urn) (Figure 5 and 6). Figure 5 shows the evolution of cell progeny of a control culture of *Chlorella vulgaris.* After 48 hours of culture (Figure 5B, E) we can distinguish 2 subpopulations corresponding to cells that have not yet divided (CM; >50%) and daughter cells (CH); after 96 hours (Figure 5C, F), only one population appeared, being all of them daughter cells, with a minor level of CFSE fluorescence. However, cells exposed to 500 nM of terbutryn are not able to divide during this period (Figure 6), remaining constant the CFSE fluorescence. The CFSE results on the growth kinetics were consistent with those determined by cell counting [19].

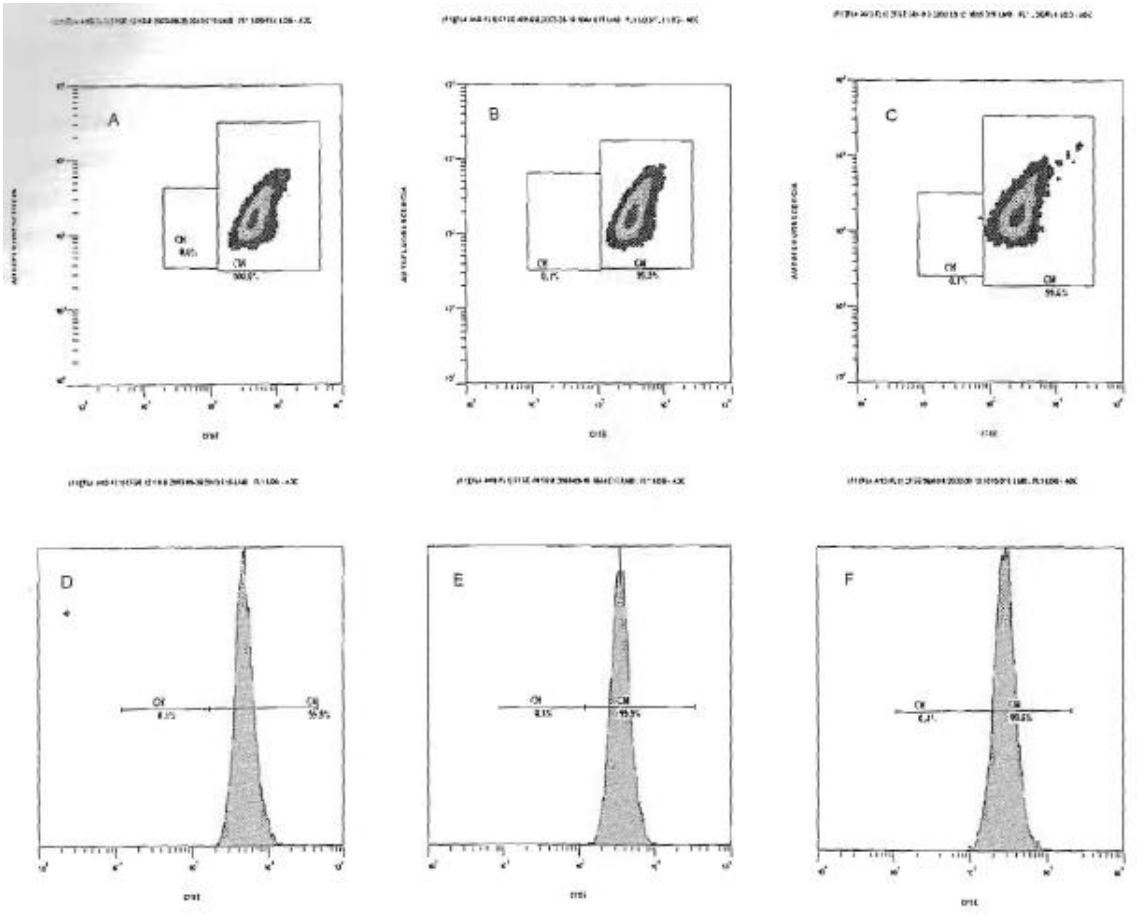

Figure 6, Cytograms of CFSE fluorescence *vs*, autofluorescence of *Chlorella vulgaris* cells exposed to 500 nM terbutryn at different times (12 (A), 48 (B) and 96 (C) hours). The histograms (D, E, F) showthe CFSE fluorescence signal (a,u., arbitrary units). CH: daughter cells; CM: mother cells.

Oxidative Stress

Aerobic organisms produce reactive oxygen species (ROS) in their metabolic process, as oxygen peroxide o superoxide. The level of these ROS increases during several cytotoxic process, leading to the named oxidative stress. The fluorogenic oxidation of hydroethidine (dihydroethidium; HE) to ethidium has been used as a measure of O_2^-. Microalgal suspensions were stained with hydroethidine (HE). HE is a chemically reduced fluorophore able to cross the cell membrane and it is oxidized by superoxide ion in the cytoplasm of cells to a red product (ethidium) [22]. Ethidium binds to the DNA inside cells and has a red fluorescence when is excited with blue light [12].

Aliquots of cells were stained with HE dissolved in DMSO, at a final concentration of 2.5 µg ml^{-1} in darkness at 20°C during 30 min. The red fluorescent emission of this compound was collected in the FL3 channel (600-645 nm).

After 96 hours of exposure to 500 nM of terbutryn, an important increase of the oxidative stress level, respect to control cells, can be observed (Figure 7). Membranes could be expected to be highly prone to free radical attack inasmuch as unsaturated fatty acids are major components of most membrane lipid bilayers [14]. The consequences of free radical attack on membranes are numerous and include the induction of lipid peroxidation [23], lysis [24], and fatty acid deesterification [25]. Senescence is an active process initiated by some combination of internal and environmental triggers, and membrane deterioration is an early and fundamental feature of this process.

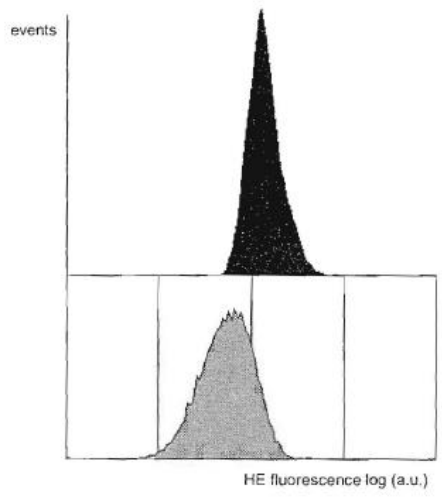

Figure 7. Overlay of hydroethidine (HE) fluorescence (a.u., arbitrary units) histograms showing the profiles of *Chlorella vulgaris* cells of control cultures (grey histogram) and cells exposed to 500 nM terbutryn (black histogram) after 96 h of culture.

Intracellular Calcium Level

Calcium ion plays an important role as a mediator in the transmembrane signal transduction, being its intracellular concentration increase part of the regulation of several cell processes [26].

The development of fluorescent probes that show a spectral response upon binding Ca^{2+} have enabled researchers to investigate changes in intracellular free Ca^{2+} concentrations [27]. These fluorescent indicators are derivatives of Ca^{2+} chelators. Fluo-3 is a Ca^{2+} fluorescent indicator excited with visible light and it is essentially non-fluorescent unless bound to Ca2+. After its union to Ca^{2+} Fluo-3 increases its fluorescence between 100 and 200 times [28].

Aliquots of cells were stained with Fluo-3 pentammonium salt dissolved in DMSO, at a final concentration of 5 μg ml^{-1} in presence of 0.1 % β-escine, in darkness at 20°C during 30 min. The green fluorescent emission of this compound was collected in the FL1 channel (505-545 nm).

The intracellular calcium level is closely related with the photosynthetic metabolism of control cultures cells, since the $[Ca^{2+}]_i$ increases in darkness and decrease in the light period (Figure 9A). However, those cells treated with 500 nM of terbutryn showed a drastically different pattern, remaining more or less constant the level of calcium after the first light period (Figure 9B). This terbutryn effect confirms that changes in the intracellular calcium level in this kind of cells are a consequence of the alteration in the photosynthetic process, and an increase in the $[Ca^{2+}]_i$ can be interpreted as an early signal of cell stress. Cessation of photosynthetic electron transport by light-off or by inhibitors (DCMO, DBMIB) causes the Ca^{2+} release from the chloroplast [29].

Changes in Cell Membrane Potential

Microorganisms, and microalgae in particular, are the first organisms affected by pesticides discharges in aquatic environments because they are directly *in* contact with the medium, separated only by the cytoplasmic membrane and the cell wall. Cellular membranes are selective, dynamic barriers that play an essential role *in* regulating biochemical and physiological events, so any alteration produced in the environment provokes changes in microorganisms membranes.

Potentiometric optical probes enable researchers to perform membrane potential measurements in cells (or organelles) too small for microelectrodes. The plasma membrane of a cell typically has a transmebrane protential as a consequence of K+, Na + and Cl^- concentration gradients that are maintained by active transport processes. Potentiometric probes offer an indirect method of detecting the translocation of these ions. Increases and decreases in membrane potential - referred to as membrane hyperpolarization and depolarization, respectively- playa central role in many physiological processes, including cell signalling.

$DiBAC_4(3)$ (bis-(1,3-dibutylbarbituric acid trimethine oxonol) is a slow-response probe, which exhibits potential-dependent changes in its transmembrane distribution that is accompanied by a fluorescence change. The dye enters depolarized cells where it binds to intracellular lipids. Increased depolarization results in more influx of the anionic dye and thus an increase in fluorescence. Conversely, hyperpolarization is indicated by a decrease in fluorescence. This dye is excluded from mitochondria because its overall negative charge, simplifying the membrane potential measurement in eukaryotic organisms [30].

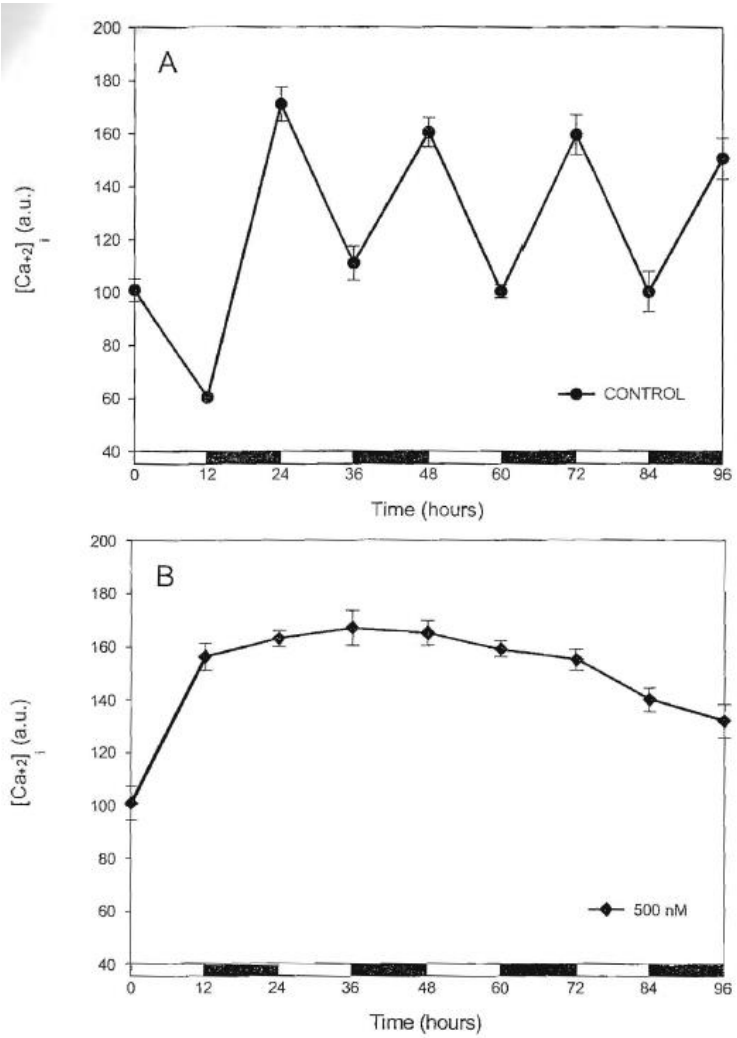

Figure 9. Evolution of the intracellular calcium level inferred from Fluo-3 fluorescence emission of *Chlorella vulgaris* cells of control cultures (A) and cells exposed to 500 nM terbutryn (B) after 96 h of culture. *X* axis represents time of culture, being white the light period and black the dark period.

Aliquots of cells were stained with $DiBAC_4(3)$ dissolved in DMSO, at a final concentration of 2.5 µg ml^{-1} in darkness at 20°C during 30 min. The green fluorescent emission of this compound was collected in the FU channel (505-545 nm).

A decrease of $DiBAC_4(3)$ fluorescence respect to control cells can be observed in *Chlorella vulgaris* cells exposed to 500 nM terbutryn after 96 hours (Figure 8), indicating the hyperpolarization of the cell membrane of these affected cells. Using eloctrophysiological and microfluorimetric techniques, it has been reported that increases in the intracellular calcium level can be accompanied by a hyperpolarization of cytoplasmic membrane in vegetal cells [29], being in agreement with our results obtained by flow cytometry and showed here.

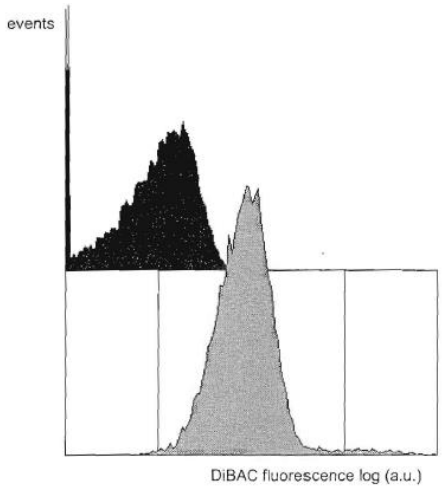

Figure 8. Overlay of $DiBAC_4(3)$ fluorescence (a.u., arbitrary units) histograms showing the profiles of *Chlorella vulgaris* cells of control cultures (grey histogram) and cells exposed to 500 nM terbutryn (black histogram) after 96 h of culture.

CONCLUSION

The potential for flow cytometry as a tool in ecotoxicological studies has not been yet fully explored. The variety of results showed in the present study confirms that flow cytometry is very useful in the toxicity tests carried out with microalgae to assess the potential cytotoxicity of herbicides at very low concentrations. Changes in environmental conditions are often most easily and rapidly recognized by the reaction of unicellular organisms like microalgae. Then, taking into account the obtained results, it is expected that FCM could improve the ability of the researchers to address the major environmental challenges that are confronting human societies.

ACKNOWLEDGEMENT

Results showed in this chapter were carried out with the financial support of Xunta de Galicia (PGIDIT04RFO103946PR) and the Spanish Ministerio de Educación y Ciencia (CGL2004-02037BOS). R.P. acknowledges a F.P.U from the Spanish Ministerio de Educación y Ciencia.

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