# Potential use of flow cytometry in toxicity studies with microalgae

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## Abstract

Cytotoxic effects of aquatic pollutants on microalgae are very heterogeneous, and they are influenced by environmental conditions and the test species. Stress produced by copper or paraquat addition to the culture medium of two microalgae was analysed by flow cytometry. Parameters assayed were: cell volume, chlorophyll *a* fluorescence and cell viability. The variety of results obtained in the present study reveals that flow cytometry is a useful tool in the toxicity tests with microalgae, both marine and freshwater species, and for different kind of pollutants.

## Keywords

Microalgae; Aquatic toxicity test; Copper; Paraquat; Flow cytometry

## 1. Introduction

All types of pollutant provoke changes in the structure of ecosystems, affecting biotic communities. Microorganisms are the lowest biological level affected by the discharges of pollutants in an ecosystem, as they are directly in contact with the medium, only separated by a membrane and/or cell wall. Microalgae have already been used as biological indicators to assay pollutant toxicity in marine or freshwater environments (Hörnström, 1990).

It has been confirmed that inhibition of growth and photosynthesis, as well as other variables closely related to photosynthesis, such as ATP formation, radioactive carbon assimilation, oxygen evolution and algal fluorescence induction phenomena, reflect the toxic effects of pollutants on microalgae (Paau et al., 1978,Wong et al., 1980, Sicko-Goad, 1982, Ibrahim, 1990, Blaise, 1993, Cid et al., 1995 and Saenz et al., 1997). Nevertheless, other relevant endpoints are less known because experimental difficulties, especially under in vivo conditions. Flow cytometry (FCM) has been applied in the study of the aquatic environment since the 1980s (Yentsch and Pomponi, 1986); this technique has achieved extensive use in the study of microalgae and has been introduced as an alternative to the more traditional techniques of

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analysing cells in culture and from natural populations (Yentsch and Horan, 1989, Reiriz et al., 1994, Abalde et al., 1995 and Cid et al., 1996).

In the present study, some measurements obtained by flow cytometry, such as cellular volume, chlorophyll *a* fluorescence and cellular viability, were employed to assess the toxic action of two different pollutants on microalgae, analysing the effect of copper on the marine diatom *Phaeodactylum tricornutum* and the effect of the herbicide paraquat on the freshwater microalga *Chlamydomonas eugametos*.

## 2. Material and methods

### 2.1. Microalgal cultures

The marine microalga, *Phaeodactylum tricornutum* Bohlin (Bacillariophyceae) was cultured in raw seawater as described previously ( Cid et al., 1995), salinity 35‰, and initial pH 8.4. Initial cell density was $0.24\times10^6$ cells $ml^{-1}$. Copper concentration assayed was 1 mg Cu $l^{-1}$, added as copper chloride; control cultures without copper were also included. Cultures were illuminated with 140 μmol photon $m^{-2}$ $s^{-1}$.

The freshwater microalga, *Chlamydomonas eugametos* Moewus (Chlamydomonadaceae) was cultured in Bristol medium as described before ( Franqueira et al., 1999), supplemented with the herbicide paraquat at a final concentration of 0.6 μM. Initial cell density was $0.10\times10^6$ cells $ml^{-1}$. Cultures without paraquat were included as control cultures. Cultures were illuminated with 68.25 μmol photon $m^{-2}$ $s^{-1}$.

All experiments were carried out in triplicate. Cultures were maintained at 18±1°C, with a light–dark cycle of 12 h:12 h, during 96 h as has already been indicated for toxicity assays with microalgae (Walsh and Merril, 1984).

### 2.2. Growth measurement

Growth of the microalgal cultures was determined daily by counting culture aliquots in a Malassez or Neubauer haemocytometer. Growth rate (μ), expressed in $day^{-1}$ ($d^{-1}$) was calculated by the usual formula:

$$\mu = (\ln \quad N_t - \ln \quad N_0) \ / \ (t - t_0)$$

where *N* is cellular density at time *t* after pollutant exposure and time is expressed in days.

### 2.3. Flow cytometry analysis

Stress produced by the heavy metal or the pesticide addition was analysed by flow cytometry, using cell volume, chlorophyll *a* fluorescence and cell viability as toxicity parameters.

Aliquots of microalgal cultures were collected after 24 and 96 h of treatment; these aliquots were centrifuged and washed twice, and resuspended in buffered saline solution to be analysed in a FACScan flow cytometer (Becton Dickinson Instruments) equipped with an argon-ion laser

(blue light, 488 nm). Chlorophyll *a* red fluorescence histograms were used to set gating levels, excluding particles without red fluorescence, which are obviously non-algal particles.

The fluorescence of cells stained with propidium iodide (PI) was measured to study the 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 PI is able to enter and stain the nucleic acids (Ormerod, 1990). In this way, PI can be used to discriminate between live non-fluorescent cells and non-viable fluorescent cells.

#### 2.3.1. Staining protocol

Aliquots of cells were washed as indicated before and stained with PI at a final concentration of 60 μM, during an incubation period of 20 min, at room temperature and darkness conditions. The cell viability of the cultures is expressed as the percentage of viable cells vs. the total amount of cells analysed by flow cytometry.

Since the forward light scatter signal (FSC) can be correlated with the size of the cell (Shapiro, 1995), aliquots of microalgal cultures stained with PI, were analysed to study the possible changes in cell volume. Only viable cells were analysed.

For each cytometric parameter investigated, at least $10^4$ gated cells were analysed per culture and fluorescence measurements were expressed in a logarithmic scale. Data collection was performed using the list mode and statistically analysed by the instrument software (LYSIS II Program; Becton Dickinson Instruments).

## 3. Results

### 3.1. Effect of copper on *Phaeodactylum tricornutum*

The concentration of copper assayed (1 mg $l^{-1}$) inhibited the growth of the marine diatom *P. tricornutum* ( Fig. 1), with a growth rate close to 0 (0.001 $days^{-1}$), while in control cultures, without copper, growth rate was 1.16 $days^{-1}$. This copper concentration also provokes changes in cell viability, cellular volume and chlorophyll *a*fluorescence. Cell density after 96 h of copper exposure was $0.23\times10^6$ cells $ml^{-1}$ (S.D.=$0.03\times10^6$), while in control cultures the density was $1.49\times10^6$ cells $ml^{-1}$ (S.D.=$0.12\times10^6$) ( Fig. 1).

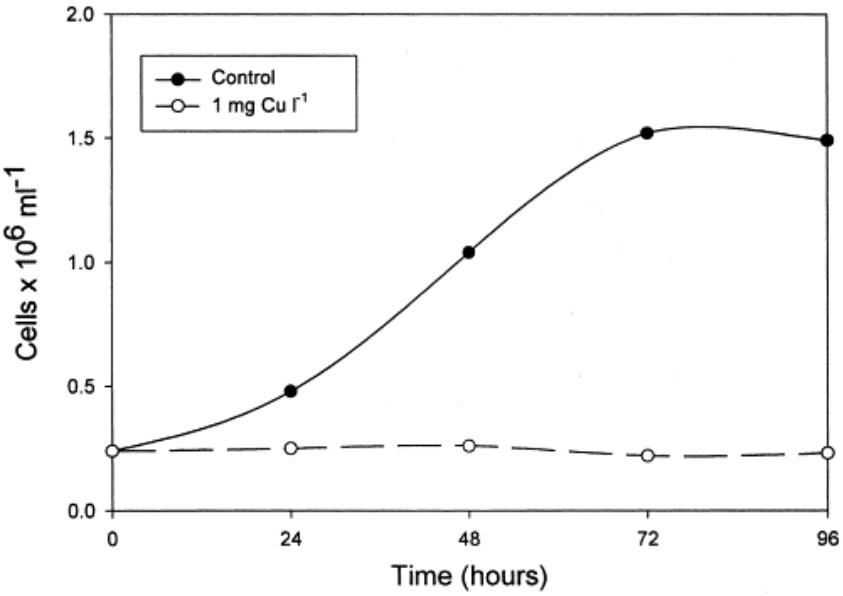


Fig. 1.

Growth curves of *P. tricornutum* cultures. Results are the means of three replicates.

In control cultures of *P. tricornutum*, cell viability remains constant near to 100% during the 96 h of the experiment; however, in cultures with copper the cell viability decrease over this time period ( Table 1). The copper concentration assayed (1 mg $l^{-1}$) reduced the cell viability of *P. tricornutum* to 8% after 96 h of treatment.

Table 1.

Percentage of viable cells (non-stained with propidium iodide) of *P. tricornutum* and *C. eugametos* after 24 and 96 h of culture, with respect to the total amount of analysed cells by flow cytometry

| | *P. tricornutum* | | | *C. eugametos* | |
|---|---|---|---|---|---|
| | 24 h | 96 h | | 24 h | 96 h |
| Control | 100% | 96% | Control | 100% | 99% |
| + Copper | 97% | 8% | +paraquat | 97% | 38% |

Copper provokes changes in the FSC signal, related to changes of cell volume, of *P. tricornutum* cells with respect to the volume seen in control cultures ( Fig. 2). Copper provokes an increase of the cell volume after 96 h of treatment, while this difference is not observable after 24 h ( Fig. 2). The treatment of the data obtained showed an increase of 49% in the FSC signal of cells exposed during 96 h to copper with respect to the control ones.

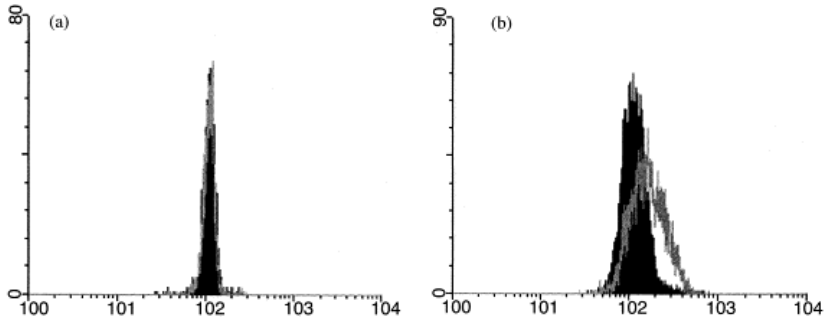


Fig. 2.

Overlay of FSC signal (related with cell volume) histograms showing the profiles of a *P. tricornutum* control culture sample (solid histogram) and a sample of a culture with a copper concentration of 1 mg $l^{-1}$, after 24 and 96 h of culture (a and b, respectively).

Chlorophyll *a* fluorescence of *P. tricornutum* cells was also affected by the copper concentration assayed. After 24 h of treatment, this chlorophyll *a* fluorescence detected by flow cytometry increased, but this increase is more important after 96 h of exposure to copper ( Fig. 3).

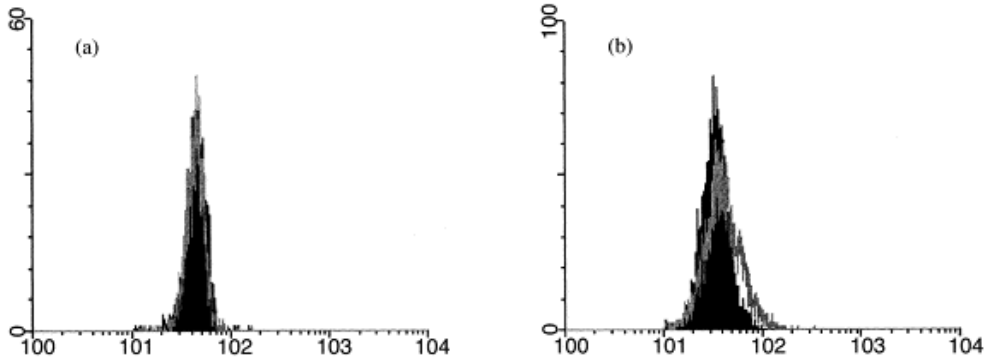


Fig. 3.

Overlay of chlorophyll *a* fluorescence histograms showing the profiles of a *P. tricornutum* control culture sample (solid histogram) and a sample of a culture with a copper concentration of 1 mg $l^{-1}$, after 24 and 96 h of culture (a and b, respectively).

## 3.2. Effect of paraquat on *Chlamydomonas eugametos*

Growth of *C. eugametos* cultures were totally inhibited by the paraquat concentration assayed and the cell density of paraquat exposed cultures decreased over the 96 h of the assay ( Fig. 4). After 96 h of culture, the cell density of control cultures was $0.85\times10^6$ cells $ml^{-1}$ (S.D.=$0.01\times10^6$), while cell density of paraquat exposed cultures was $0.05\times10^6$ cells $ml^{-1}$ (S.D.=$0.01\times10^6$) ( Fig. 4).

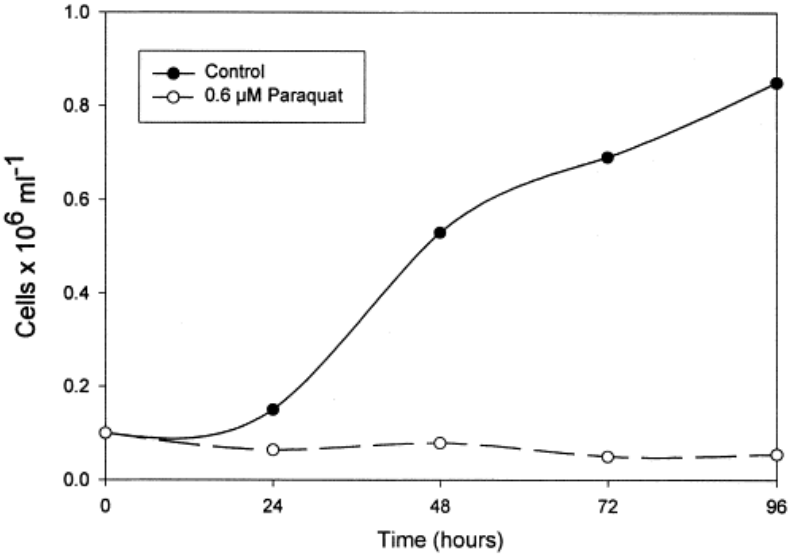


Fig. 4.

Growth curves of *C. eugametos* cultures. Results are the means of three replicates.

*C. eugametos* cultures with paraquat showed an important decrease in the percentage of viable cells after 96 h of exposure to the herbicide (38%) ( Table 1). The cell viability of control cultures, without paraquat, remains constant near to the 100% over the 96 h of the experiment.

The concentration assayed of the herbicide paraquat also provokes changes in the cellular volume of *C. eugametos* with respect to the volume shown in control cultures ( Fig. 5). After 24 h of treatment, the cell volume of this microalga showed alterations, but most cells of paraquat cultures showed an FSC signal similar to the control cells after 24 h. However, after 96 h of paraquat exposure, this FSC signal is more heterogeneous, having practically the same mode of distribution for control and paraquat cultures ( Fig. 5).

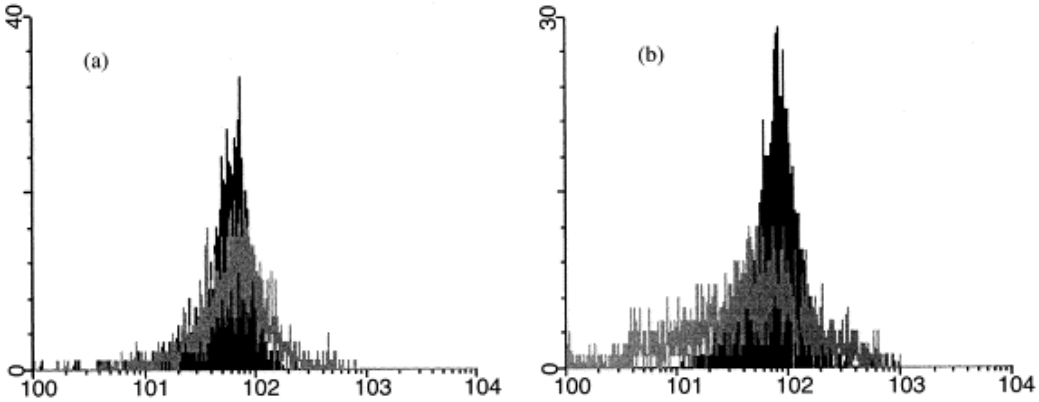


Fig. 5.

Overlay of FSC signal (related with cell volume) histograms showing the profiles of a *C. eugametos* control culture sample (solid histogram) and a sample of a culture with a paraquat concentration of 0.60 μM, after 24 and 96 h of culture (a and b, respectively).

Paraquat concentration assayed modified the chlorophyll *a* fluorescence ( Fig. 6). After 24 h of treatment the chlorophyll *a* fluorescence seems to be lower than the control cells, but only slightly lower; however, after 96 h the chlorophyll *a* fluorescence signal decreased strongly in comparison to the signal provided by control cultures cells ( Fig. 6).

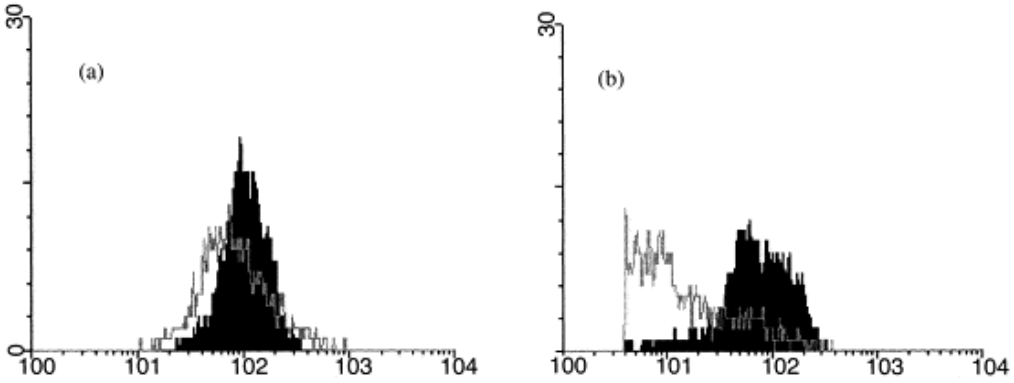


Fig. 6.

Overlay of chlorophyll *a* fluorescence histograms showing the profiles of *C. eugametos* control culture sample (solid histogram) and a sample of a culture with a paraquat concentration of 0.60 μM, after 24 and 96 h of culture (a and b, respectively).

## 4. Discussion

Cytotoxic effects of aquatic pollutants on microalgae are very heterogeneous, and they are influenced by the environmental conditions and the test species. The assayed copper and paraquat concentrations (1 mg $l^{-1}$ and 0.60 µM, respectively) provoked the total inhibition of growth (Fig. 1 and Fig. 4), this parameter being one of the most commonly used in toxicity tests. Copper is an essential micronutrient for growth, metabolism, and enzyme activities of algae, cyanobacteria, and other organisms; however, it is also a proven inhibitor of algal growth at high concentrations (Lustigman, 1986, Stauber and Florence, 1987 and Abalde et al., 1995). Microalgal cells would be able to tolerate the presence of certain herbicide concentrations without significant effects on growth (Rabinowitch et al., 1983 and Bray et al., 1993); however, their defense mechanisms would be overcome at higher exposure levels (Kirtikara and Talbot, 1996).

Flow cytometry allows the rapid analysis of structural characteristics, as the cell volume, of cells without staining. Both pollutants assayed, copper and paraquat, provoke changes in the cellular volume of *P. tricornutum* and *C. eugametos* with respect to the volume shown in control cultures ( Fig. 2 and Fig. 5). *P. tricornutum* cells showed an increase in the FSC signal when exposed to the copper concentration assayed (Fig. 2). Several authors, using optical or electron microscopy, have observed an increase in the cell volume of different microalgal species in response to toxic levels of heavy metals ( Fisher et al., 1981, Stauber and Florence, 1987 and Bolaños et al., 1992), and also using flow cytometry ( Abalde et al., 1995). Heavy metals change cell membrane permeability to small cations ( Overnell, 1975), and the increase in cell volume is probably due to the increase of the permeability of cell membrane to $Na^+$ ( Riisgård et al., 1980).

Bray et al. (1993) also observed an increase of the cellular volume of *Chlamydomonas reinhardtii* exposed to paraquat. Variations observed in the FSC signal detected by FCM in the *C. eugametos* cultures treated with 0.60 µM paraquat ( Fig. 5) could be related to palmeloid colony formation, since several factors can induce the formation of these structures in the genus *Chlamydomonas* ( Nakamura et al., 1976 and Olsen et al., 1983). This palmeloid structure appears probably due to the incapacity to finish cell division, as well as failures of regulation of cellular volume as a consequence of the high levels of oxidative radicals formed. However, an important proportion of the analysed cells presented a lower FSC signal also ( Fig. 5), and may be due to the inhibition of growth in these cultures ( Fig. 4).

Chlorophyll *a* fluorescence is determined by flow cytometry and it is a good indicator of the physiological state of this kind of photoautotrophic cells ( Cid et al., 1995). Measurement of the fluorescence of chlorophyll *a* in intact algal cells provides information on the absorption, distribution and utilisation of energy in photosynthesis ( Hawkins and Griffiths, 1982 and Phinney and Cucci, 1989). Algal fluorescence induction phenomena were used to study phytotoxicity ( Sicko-Goad, 1982 and Murthy et al., 1989). Chlorophyll *a* fluorescence monitored by the single-laser based flow cytometer is the maximum fluorescence when the photosystem II (PS II) reaction centers are locked in the $Q_A^-$ state ( Xu et al., 1990). The inhibition of the electron flow in the PS II reaction center in the donor side induces a decrease in

the chlorophyll *a*fluorescence ( Overnell, 1975, Butler, 1977 and Samson and Papovic, 1988) as occur in *C. eugametos* cells affected by the paraquat concentration assayed ( Fig. 6). However, when the inhibition of this electron flow is produced in the acceptor side of the PS II, an increase in the chlorophyll *a* fluorescence is observed (Overnell, 1975, Butler, 1977 and Samson and Papovic, 1988), as can be observed in *P. tricornutum* cultures with copper ( Fig. 3). According to other authors copper's inhibitory effect on PS II activity is located on its oxidising side ( Samson and Papovic, 1988), probably because copper inactivates some PS II reaction centers.

The variety of results obtained in the present study reveal that flow cytometry is a useful tool in toxicity tests with microalgae, both marine and freshwater species, and for different kinds of pollutants.

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