A Comparison of the Relative Sensitivity of Structural and Functional Cellular Responses in the Alga *Chlamydomonas eugametos* Exposed to the Herbicide Paraquat

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Abstract.

The effect of the herbicide paraquat on the freshwater microalga *Chlamydomonas eugametos* was studied in function of different parameters such as growth, elemental composition, total lipids, and photosynthetic pigments content and others assayed by flow cytometry (cell viability, cell volume, and granularity). The study reveals that paraquat concentrations above 0.15 µM are toxic for the microalga *C. eugametos,* inducing an inhibition of all the physiological parameters analyzed and strong structural changes. However, lower concentrations cause alterations in certain cellular components that are especially sensitive to the toxic action of the herbicide; so total lipids and photosynthetic pigments content are affected by concentrations such low as 0.037 µM. Taking into account these results, these parameters are better indicators of the cellular state than data on biomass or growth rate.

Currently, the most commonly used ecotoxicity test for screening aquatic pollutants is the short-term fish lethality test, which has been recently criticized (Fentem and Balls 1993); the alternatives to this test include the use of ‘‘lower’’ organisms such as bacteria and microalgae (the United Kingdom’s *Animals (Scientific Procedures) Act 1986* and EC *Directive 86/609/EEC* legislation). Microalgae have been recognized as useful indicators of environmental quality (Ho¨rnstro¨m 1990); simple bioassays conducted in the laboratory using microalgae can provide valuable information about the possible disruptive effects of pesticides on algal morphology and physiology (Blaise 1993).

Aquatic environments, including fresh water, estuaries, and coastal marine waters, are often contaminated with numerous organic and inorganic compounds, including herbicides. Paraquat (1,18-dimethyl-4,48-bipyridilium dichloride) is a nonselective herbicide widely used to prevent the growth of broad leaf weeds and grasses, mainly in railways and roadsides (Kalayanova and El Batawi 1991). 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 (Ibáñez *et al.* 1996). Information on the influence of this chemical on the lowest levels of the food chain is scarce (Ibrahim 1990; Bray *et al.* 1993; Saenz *et al.* 1997).

The mode of action of paraquat, as a competitive inhibitor of photosynthetic electron transport at PS I level (Summers 1980; Devine *et al.* 1993), makes it potentially lethal to a wide variety of nontarget species of primary producers, including phytoplankton and benthic algae. Paraquat in cells leads to the generation of free oxygen radicals. These free oxygen radicals cause lipid peroxidation damaging cell membranes and leading to cell death (Tissut *et al.* 1987). Free radical scavengers, such as carotenoids, are rapidly overwhelmed due to the efficiency of paraquat in generating free radicals.

The test species chosen for this study was the freshwater microalga *Chlamydomonas eugametos.* Different parameters for microalgal activity show widely different sensitivities to aquatic pollutants. The present work examines the effect of paraquat on parameters commonly used in microalgal toxicity assays, such as growth and biochemical composition. Since flow cytometry could be considered useful in the monitoring of the physiological state of microalgal cells (Cid *et al.* 1995, 1996), other parameters assayed by flow cytometry, such as cellular viability, volume, and granularity, were examined.

Materials and Methods

Chlamydomonas eugametos Moewus (Chlamydomonadaceae) was obtained from the Department of Pure and Applied Ecology of the University of Amsterdam (The Netherlands); it was cultured in Bristol medium (Brown *et al.* 1967). Cultures were carried out in Pyrex glass bottles containing 500 ml of medium, after having been autoclaved at 121°C for 20 min. Microalgal cultures were maintained at 18 ± 1°C and 68.25 µmol photon m^{-2} s^{-1}, with a dark:light cycle of 12:12 h. Initial density was 1 x 10^5 cells ml^{-1}.

Paraquat concentrations assayed were 0.037, 0.075, 0.15, 0.30, and 0.60 µM; stock solutions were prepared by dissolving granulated herbicide paraquat (Sigma, MW: 257.2) in distilled and sterilized water. In addition to these, control cultures without paraquat were also included. All experiments were carried out in triplicate for 96 h as has already been indicated for toxicity assays with microalgae (Walsh and Merril 1984).

Growth of the microalgal cultures was measured by counting daily culture aliquots in a Neubauer hemocytometer, and growth rates were expressed in doublings day^{-1}. The most common parameter used in toxicity assays is the EC_{50}, *i.e.,* the concentration of the tested substance that decreases the growth by 50%. In order to obtain a confidence interval for the EC_{50} value, probit analysis of the growth data was carried out using the SPSS-PC + software (SPSS Inc.).

Viability, cellular volume, and granularity were determined using flow cytometric techniques. Aliquots of microalgal cultures were analyzed in a FACScan flow cytometer (Becton Dickinson Instruments), equiped with an argon excitation laser (488 nm). Chlorophyll *a* red fluorescence was used to set the gating level, excluding particles without red fluorescence, which are obviously nonalgal particles. Viability was measured by incubation of culture aliquots for 5–10 min at room temperature with propidium iodide (PI) at a final concentration of 60 µM (Abalde *et al.* 1995); this compound stains nonviable cells and if excited by blue light, it gives off orange fluorescence (530–560 nm) that was measured in the flow cytometer. Since an increase in the forward light scatter (FSC) signal can be correlated with an increase in cell volume and an increase in side light scatter (SSC) signal can be correlated with an increase in cell granularity (revised by Shapiro [1995]), aliquots of microalgal cultures stained with PI were analyzed to study the possible changes in cell volume and granularity; only viable cells were analyzed for both cell parameters.

For each cytometric parameter investigated, at least 10^4 cells were analyzed per culture and fluorescence measurements were expressed in a logarithmic scale. Data collection was performed using the list mode. The means of forward and side scatter signal were provided by the instrument software (LYSIS II program; Becton Dickinson Instruments). Data on the forward and side scatter signal of the cells, related to cell volume and granularity, were expressed as a percentage of the control cells signal according to the equation of Reader *et al.* (1993):

$$\% P = 100 - [100(P_c - P_t)/P_c]$$

where % P is the percentage of the FSC or SSC signal of *C. eugametos* cells; P_c is the mean FSC or SSC signal of control cells; and P_t is the mean FSC or SSC signal of paraquat-treated cells.

Samples for biochemical analysis were taken after 96 h of paraquat exposure. The algal cells were harvested by centrifugation and frozen at -70°C prior to liophilization for 24 h. The dry weight was determined according to Vonshak (1986). Carbon and nitrogen content were determined with an elemental analyzer (Carlo Erba CHNS-O 1108). Total lipids were determined gravimetrically after chloroformmethanol (2:1) extraction using the Bligh and Dyer method (Bligh and Dyer 1959).

Photosynthetic pigments were determined from spectrophotometric measurements of 90% acetone extracts using the equations of Jeffrey and Humphrey (1975) for chlorophylls, and Strickland and Parsons (1972) for total carotenoids.

Data were statistically analyzed by an overall one-way analysis of variance (ANOVA), and, when differences observed were significant, means were compared by the multiple range Duncan test, at a level of significance of 0.05 ($p<0.05$).

Results

Growth

Paraquat affected the growth of the freshwater microalga *C. eugametos* (Figure 1, Table 1). There were significant differences in growth between control cultures and those cultures exposed to paraquat concentration higher than 0.075 µM ($p<0.05$), whereas lower concentrations did not provoke significant differences in comparison with control cultures (Figure 1).

Maximum growth rates (0.77–0.84 doublings day^{-1}) were obtained in cultures treated with paraquat doses lower than 0.15 µM; higher paraquat concentrations inhibited the growth of this microalga (Table 1). The EC_{50} of paraquat for growth, calculated by probit analysis, was 0.28 µM after 96 h of the herbicide exposure. Experimental confirmation of this theorical value was carried out, and the experimental results are into the confidence interval of the statistical analysis.

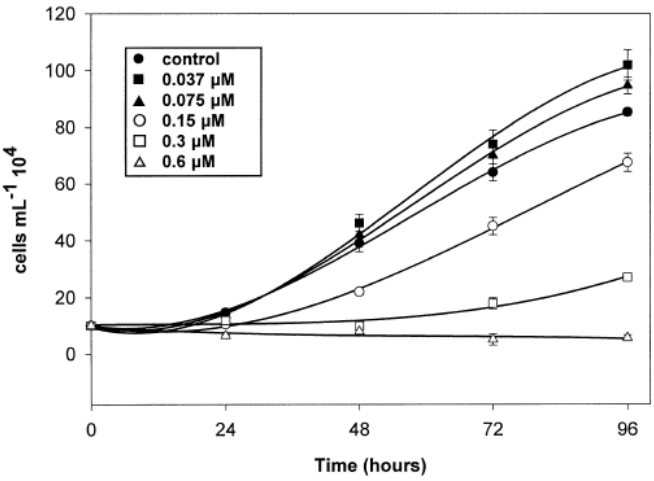


Fig. 1. Growth curves (expressed as cells 3 104 ml^{-1}) of cultures of *Chlamydomonas eugametos* exposed to different paraquat concentrations (expressed as µM)

Cellular Viability

After 96 h of paraquat exposure, the proportion of viable cells in control cultures was 99%; paraquat concentrations of 0.30 and 0.60 µM provoked a decrease of this parameter in comparison to the control (85% and 37% cellular viability, respectively) (Table 1). Cellular viability decrease observed in 0.60 µM cultures can be related to the strong inhibition observed in the growth data (Table 1).

Forward Scatter (FSC) and Side Scatter (SSC)

Paraquat provoked an increase in the FSC signal, related to an increase of cell volume, of *C. eugametos* cells after 96 h of exposure to this herbicide. The highest concentrations tested (0.30 and 0.60 µM) caused higher increases of this signal (47 and 74%, respectively) in comparison with control cultures (Figure 2).

An increase in the SSC signal, related to an increase in the internal cellular granularity was also observed (Figure 2). After 96 h of paraquat exposure, paraquat concentrations of 0.30 and 0.60 µM caused a drastic increase in the internal cellular granularity (63% and 309% respectively, with respect to the control) (Figure 2).

Dry Weight and Elemental Composition

Cellular dry weight (DW) after 96 h of paraquat exposure showed a significant increase at paraquat concentrations higher than 0.15 µM (p , 0.05), being 545.4 pg per cell in cultures exposed to a paraquat concentration of 0.60 µM, nearly five times greater than the cellular DWof the control cultures (117.8 pg $cell^{-1}$) (Table 1).

Carbon and nitrogen percentages in the dry biomass, determined after 96 h of culture, showed that the C/N ratio increased significantly (p , 0.05) in cultures with 0.15, 0.30, and 0.60 µM (5.10, 5.31, and 6.76, respectively) in comparison with the ratio obtained in control cultures (4.88) (Table 1).

Lipids and Pigments Content

Total lipid content in biomass presented a significant decrease, with respect to the lipid content in the control cultures (170.1 mg g^{-1} dry weight), in all cultures exposed to paraquat concentration higher than 0.037 µM; minimum lipid content was obtained in cultures with 0.60 µM paraquat (90 mg g^{-1} dry weight), which showed nearly a 50% decrease with respect to the controls (Figure 3).

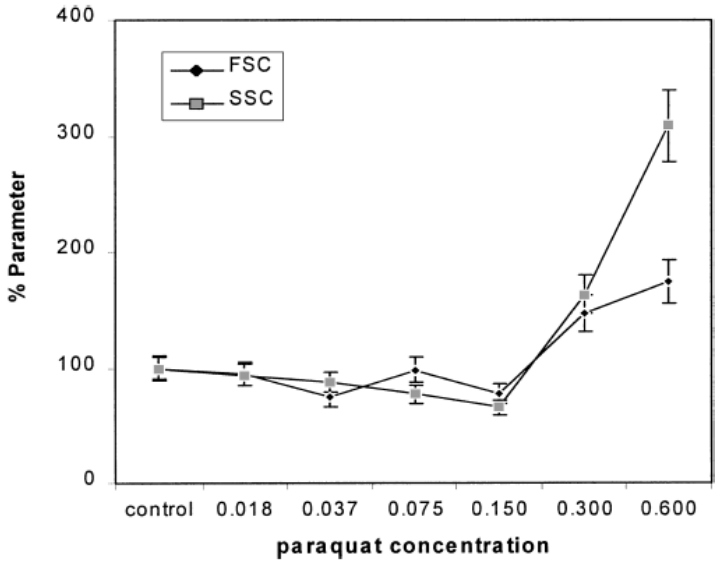
Photosynthetic pigment content (chlorophyll *a*, *b*, and total carotenoids) showed changes after 24 h of paraquat exposure, but maximum differences with respect to the control occurred after 96 h of treatment. Chlorophylls were more sensitive to paraquat toxicity than total carotenoid (Figure 4). After 96 h of treatment, chlorophyll content decreased as paraquat concentration increased, showing values of 33.2 mg of chlorophyll *a* and 8.2 mg of chlorophyll *b* per gram of dry weight in control cultures and values of 5.3 mg of chlorophyll *a* and 2.4 mg of chlorophyll *b* per gram of dry weight in cultures with 0.60 µM paraquat (Figure 4).

Similar pattern occurred in carotenoid content; control cultures showed a carotenoid content value of 9.8 mg g^{-1} dry weight. Paraquat concentrations of 0.30 and 0.60 µM reduced the carotenoid content significantly ($p<0.05$): 0.30 µM of paraquat reduced the carotenoid content by half, and 0.60 µM reduced the carotenoid content seven times compared to the control culture (Figure 4).

Table 1. Growth rate (expressed as doublings day^{-1}), percentage of cell viability, dry weight (expressed as pg $cell^{-1}$), and carbon/nitrogen ratio obtained in cultures of *Chlamydomonas eugametos* exposed to different paraquat concentrations (expressed as µM) after 96 h

Paraquat (µM)	Growth Rate (µ) (doublings day^{-1})	Cell Viability (%)	Dry Weight (pg $cell^{-1}$)	C/N
0	0.77 ± 0.06	99	117.80 ± 0.59	4.88 ± 0.02
0.037	0.84 ± 0.02	99	105.03 ± 3.93	4.82 ± 0.04
0.075	0.81 ± 0.01	98	104.10 ± 0.53	4.84 ± 0.02
0.15	0.69 ± 0.03	95	100.30 ± 9.26	5.10 ± 0.02
0.3	0.36 ± 0.05	85	200.90 ± 3.72	5.31 ± 0.05
0.6	−0.22 ± 0.06	37	545.40 ± 7.33	6.76 ± 0.15

Fig. 2. Forward and side scatter signal (FSC and SSC) of the *Chlamydomonas eugametos* cells at different paraquat concentrations (expressed as µM), after 96 h. Data are expressed as the percentage of the control cells signal (arbitrary units) according to the equation of Reader *et al.* (1993)



Discussion

Many classes of compounds can exhibit environmental toxicity. One of the most commonly discussed and researched are the pesticides, which are introduced into aquatic ecosystems from agricultural runoff (Landis and Yu 1995). Two bipyridyl herbicides, paraquat (methyl viologen) and diquat, have been in global use for over 30 years.

The results obtained in the present work indicate that paraquat affects the growth of *C. eugametos* at concentrations of 0.15 µM or higher. Other experiments performed on *Chlamydomonas reinhardtii* showed that there was an obvious growth reduction by 0.10 µM paraquat and that the growth was completely inhibited at concentrations of 0.30 µM (Bray *et al.* 1993). Therefore, the paraquat concentrations (1 mg L^{-1}) allowable for controlling of macrophytes in freshwater fish farms (Ibrahim 1990) are clearly growth inhibitory for the test photosynthetic microorganism *C. eugametos.*

The inhibitory effect of paraquat on growth only at the highest concentrations assayed could be explained on the existence of detoxifying mechanisms in the cells. Low paraquat concentrations could induce oxidative stress that would lead to an increase in the activity of specific enzymes involved in the detoxification (superoxide dismutase, glutation reductase) as observed in the microalgae *C. reinhardtii* (Bray *et al.* 1993) and *Chlorella* (Rabinowitch *et al.* 1983). The cells would be able to tolerate the presence of certain herbicide concentrations without significant effects on growth. However, these mechanisms would not be effective against the higher herbicide concentrations since paraquat, in the presence of light, accelerates the production of species of active oxygen (Bray *et al.* 1993). The defense mechanisms would be overcome, and the cells could undergo deleterious effects brought about by the high levels of superoxide radicals and the formation of hydrogene peroxide (Kirtikara and Talbot 1996).

The EC_{50} value for inhibition of growth obtained at 96 h of exposure to paraquat (0.28 µM) is in accordance with the measurements for cell viability using FCM techniques, which showed a decrease in cell viability in cultures with the highest doses of paraquat assayed (85% in cultures with 0.30 µM paraquat and only 37% of cell viability with 0.60 µM paraquat).

The calculated EC_{50} values for the reduction of growth of *Scenedesmus dimorphus* and *Ankistrodesmus falcatus* were 39.8 and 93.3 µg L^{-1}, respectively (Ibrahim 1990); the value that we have obtained for *C. eugametos* (72.0 µg L^{-1}, equivalent to 0.28 µM) indicates that *C. eugametos* is quite tolerant to the herbicide paraquat. The differences between results could be due to species differences. Different species can respond to the same pesticide in different ways (Ibrahim 1990).

The increase of cellular dry weight at the highest concentrations of paraquat assayed is related with the inhibitory effect on growth; in these cultures, cells formed palmelloid colonies (clusters of nonflagellated cells closed in a common wall), observed by light microscopy. Several factors can induce the formation of these structures in *Chlamydomonas* (Iwasa and Murakami 1968; 1969; Nakamura *et al.* 1976; Olsen *et al.* 1983). Palmelloid colonies became attached to the walls of the culture vessel and subsequently showed a much slower growth rate than nonpalmelloid vegetative cells.

In this way, the increase in the cellular volume of *C. eugametos* detected by FCM in the cultures treated with 0.30 and 0.60 µM paraquat (Figure 2) could be related to palmelloid colony formation, probably due to the incapacity to finish cell division as well as failures of regulation of cellular volume because of the of membranes as a consequence of the high levels of oxidative radicals formed. Bray *et al.* (1993), using transmission electron microscopy techniques, detected that 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. Among the alterations that were observed in cells containing severe damages, they indicated the presence of multivesicular bodies in the cytoplasm that seemed to originate from polyribosomal complexes and endoplasmic reticulum ribosomes; the free ribosomes would decrease in number and appear aggregated; the plasma membrane was often discontinous and separated from the cell wall, and so on. Using transmission electron microscopy (unpublished data) we can confirm the presence of palmelloid structures, as well as numerous structural changes.

Fig. 3. Total lipid content, expressed as mg per g of dry weight, extracted from cultures of *Chlamydomonas eugametos* exposed to different paraquat concentrations (expressed as µM), after 96 h

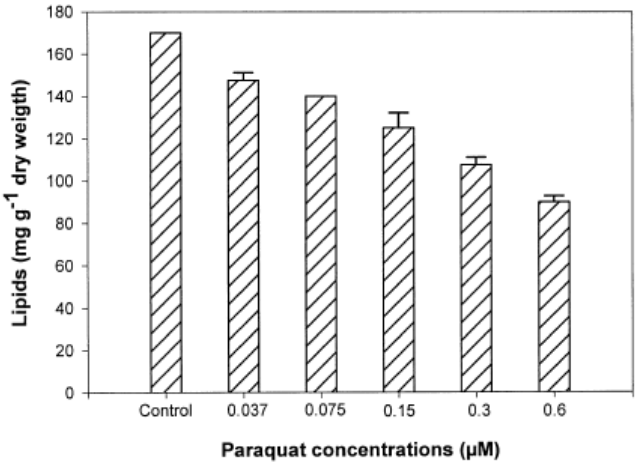
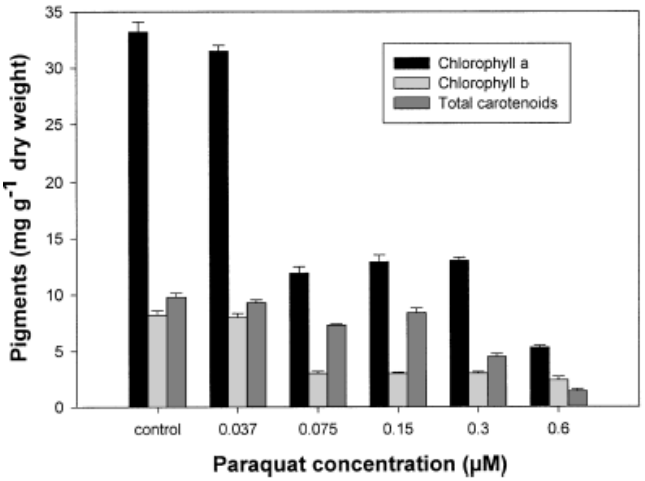


Fig. 4. Photosynthetic pigments (chlorophylls *a* and *b*, and total carotenoids), expressed as mg per g of dry weight, obtained from cultures of *Chlamydomonas eugametos* exposed to different paraquat concentrations (expressed as µM), after 96 h



The element composition (C, N) as well as the total lipid content of the cultures were also affected when the higher doses of paraquat being tested were added. The C:N ratio is a parameter inversely correlated with the growth rate (Laws and halup 1990). Our results agree, since the C:N ratio was significantly higher, as compared with the control (4.88), in cultures with paraquat concentrations of 0.15, 0.30, and 0.60 µM (Table 1).

Lipid content was particularly sensitive to paraquat, since lipid content was significantly affected at paraquat concentrations of 0.075 µM. The decrease in lipid content is due to lipid peroxidation, as well as to the decrease in the lipid synthesis, that is NADPH-dependent, initiated by the presence of paraquat into the cells, where this herbicide competes for the electrons of the transporting chain to the PS I level (Tissut *et al.* 1987; Brooks *et al.* 1988; Neuhaus and Stitt 1989; Mehlhron 1990; Bowler *et al.* 1991).

The photosynthetic pigments, chlorophyll *a* and *b*, were affected at 0.075 µM and higher paraquat concentrations. As with total lipids, the chlorophylls act as a sensitive indicator to paraquat toxicity for microalgal cells. It has been indicated that paraquat causes an oxidative degradation of chlorophyll (Kirtikara and Talbot 1996), and our results are in agreement with

data obtained for three different chlorophytes (*Scenedesmus dimorphus, Scenedesmus quadricauda,* and *Ankistrodesmus falcatus*) exposed to paraquat for 96 h, indicating that chlorophyll *a* was the most sensitive metabolic parameter assayed (Ibrahim 1990).

Cellular detoxification mechanisms involve enzymatic mechanisms (SOD, GR), as mentioned before, and nonenzymatic mechanisms; among the nonenzymatic mechanisms, vegetative cells contain substantial amounts of α-tocopherol and carotenoids that act as nonenzymatic ‘‘rubbish bins’’ for oxygen radicals (Young and Britton 1990; Fryer 1992). This carotenoid protective role could explain the differences in the inhibition pattern observed with the lipids and the photosynthetic pigments chlorophyll *a* and *b*; while the chlorophyll content shows a significant decrease at paraquat concentrations of 0.075 μM or higher, the total carotenoids only show a significant inhibition when the highest herbicide concentrations are added (0.30 and 0.60 μM) and the oxidative effects brought about by the paraquat overcome the response capacity of all the cellular detoxification mechanisms.

Our study reveals that paraquat concentrations above 0.15 μM are toxic for the microalga *C. eugametos,* inducing an inhibition of all the physiological parameters analyzed and strong structural changes. However, lower concentrations cause alterations in certain cellular components that are especially sensitive to the toxic action of the herbicide; so total lipids and photosynthetic pigments content are affected by concentrations such low as 0.037 μM. Having into account these results, these parameters are better indicators of the cellular state than data on biomass or growth rate.

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