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Alteration of photosynthesis related parameters of *Chlamydomonas moewusii* stressed by paraquat

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ABSTRACT

Paraquat toxic action on the freshwater microalga *Chlamydomonas moewusii* was determined by several physiological parameters. ATP content seems to be the most sensitive parameter analyzed; it was reduced by the lowest paraquat concentration assayed (0,037 µM), because of the high energy consumption provoked by the stress. Nevertheless, a paraquat concentration 0,30 µM was necessary to alter microalgal growth significantly. Photosynthesis rate and chlorophyll *a* fluorescence were also reduced by a paraquat concentration lower than 0,30 µM. HPLC chromatograms of photosynthetic pigments showed also the paraquat toxicity on *C. moewusii*, since in chromatograms of cultures with 0,30 and 0,60 µM of this herbicide the peak of ß-carotene was not detected. Our results show the necessary use of microalgae in ecotoxicity studies; measurements of new sensitive physiological parameters contribute to improve earlier detection of damages in the first level of aquatic food chain provoked by low concentrations of pesticides.

KEYWORDS: microalga, toxicity, paraquat, ATP, photosynthesis, pigments

INTRODUCTION

Aquatic ecosystems are exposed to an increasing contamination of pesticides through water runoff. Among pesticides, herbicides are the most dangerous for aquatic contamination both of the sea and of freshwater [1, 2], since they are used on or near the soil, and, in many instances, in the water for aquatic weed control [3]. Herbicides are also used for controlling weeds in ponds, reservoirs, and irrigation canals.

Dibipyridilium herbicides are widely used in agriculture because they are inexpensive and highly effective against a wide variety of weeds. Paraquat (1-1´dimethyl 4-4´ bipyridylium dichloride) is included in this group of herbicides; it was first synthesized in 1955 and commercialized in 1962 [4], and it is one of the most highly toxic herbicides marketed and is the third most widely used herbicide in the world [5, 6].

Paraquat induces phytotoxic effects in chloroplast membranes via induction by photosystem I and auto-oxidation yielding superoxide radicals; additionally, further reactive oxygen species such as the highly toxic hydroxyl radical can be formed [7]. This mechanism of action and its lack of selectivity make paraquat a potentially lethal substance for many non-target species of primary producers, as the phytoplanktonic species are.

Paraquat is adsorbed very fast and strongly on any type of soil particles and becomes inactive. In this form, paraquat does not represent any risk of fresh or waste water contamination; however, the water solutions produced during the formulation, dilution, mixing, transfer and application of commercial pesticides may pollute the waste water lines and may reach the sources of freshwater [8]. After application, these chemicals can be adsorbed

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into the soil or transported to the aquatic environment by runoff or leaching [9].

In spite of an enormous amount of scientific literature on pesticides, the available information on the effects of these substances on microalgae is insufficient for an accurate prediction of the ecological consequences of pesticide application. The use of algae in bioassays has an acknowledged value in the estimation of the toxicity of pollutants [10] because they play a key role in the functioning of the entire ecosystems.

Based in this fact, the present work studies the physiological response to paraquat of the freshwater microalga *Chlamydomonas moewusii* mainly based in the measure of parameters closely related with photosynthesis, such as radioactive carbon assimilation, ATP content and photosynthetic pigment pattern.

MATERIALS AND METHODS

Chlamydomonas moewusii Gerloff (Chlamydomonadaceae) was obtained from the Culture Collection of Algae and Protozoa of Dunstaffnage Marine Laboratory (Scotland, UK) (strain CCAP 11/5B) and maintained as described before [11]. Initial density was 10×10^4 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.

Growth of the microalgal cultures was measured daily by counting culture aliquots in a Neubauer haemocytometer and growth rates (µ are expressed in day^{-1}, calculated using the formula:

$$\mu = (\ln N_t - \ln N_0)/ \ln 2\ (t-t_0)$$

where N_t is the cell density at time t and N_0 is the cell density at time 0.

The dry weight of cells was determined after 96 hours of culture according to Utting (1985) [12]. Culture aliquots (10 ml) were filtered through previously dried and weighed Whatman GF/C filters. Thereafter, the filters were dried in an oven at 80ºC for 48 hours.

The effect of the herbicide on photosynthesis was determined after 96 h of paraquat exposure, by estimation of carbon fixation, recording the uptake of ^{14}C from $NaH^{14}CO_3$.^{14}C-bicarbonate (Amersham, 674 µCi mg^{-1}) was added to the microalgal cultures to give an activity of 0,01 µCi ml^{-1}. After 2 h of incubation, the photosynthetically fixed radioactivity in microalgal cells was counted in a LKB scintillation counter with Readysafe (Beckman) as scintillator.

ATP extraction was carried out using the method of Holm-Hansen, as described by Larsson and Olsson (1979) [13], after 96 h of paraquat exposure. ATP concentration was determined using a commercial bioluminiscence assay ("ATP bioluminiscence CLS", Boehringer-Mannheim) based in the luciferin-luciferase reaction. The constant light signal produced in this reaction was measured in a LKB scintillation counter.

The analysis of photosynthetic pigments was carried out after 96 h of paraquat exposure. Pigments were extracted in 90% acetone at 4ºC, in dark conditions, and concentrated by evaporation of the solvent. Dry extracts of pigments were resuspended in 100 ml of methanol (HPLC grade). The HPLC analysis of photosynthetic pigments was made as described Wright (1991) [14].

Chlorophyll *a* fluorescence of cultures was determined by flow cytometry (FCM) [15], using a FACScan flow cytometer (Becton Dickinson Instruments), equipped with an argon ion excitation laser (488 nm); aliquots of microalgal cultures were directly analyzed in the flow cytometer. The chlorophyll *a* fluorescent emission was collected in the FL3 channel (>660 nm). For the cytometric parameter investigated, at least 10^4 cells were analyzed per condition and fluorescence measurements were in the logarithmic scale.

The mean of fluorescence for any given population was provided by the instrument software (LYSIS II program; Becton Dickinson Instruments). Data on chlorophyll *a* fluorescence of *C. moewusii,* were expressed as a percentage (%) of the fluorescence of control cells according to the following equation [16]:

$$\%F = 100 - [100(F_c-F_t)/F_c]$$

where %F is the percentage of fluorescence of *C. moewusii* cells; F_c the mean fluorescence of control cells; and F_t mean fluorescence of paraquat-treated cells.

Data were statiscally 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

Paraquat affected the growth of the freshwater microalga *Chlamydomonas moewusii* (Table 1). Significant differences were not observed between control and cultures treated with paraquat concentrations lower than 0,30 µM, with growth rates between 0,71 and 0,84 day^{-1}; paraquat concentrations 0,30 and 0,60 µM inhibited the growth of this microalga and cell death was detected in cultures with paraquat concentrations of 0,60 µM, with growth rates close to 0 or negative (0,36 and -0,22 day^{-1}, respectively). These cultures where growth were affected by the highest concentrations of paraquat assayed, also showed an acute increase of the cell size, observed by optical microscopy. This increase correlated well with the values of dry weight (Table 1). Maximum values of dry weight were reached in those cells exposed to 0,30 and 0,60 µM, being five times higher than the value determined for the control cells. Taking into account this result, the following results determined as a bulk measurement (e.g. photosynthesis and ATP content), will be expressed as a function of the dry weight, and not as a function of the volume of culture or per cell, as usually appears in the literature.

HPLC was used in order to obtain a qualitative determination of photosynthetic pigments after96 hours of exposure to paraquat. In these results the main photosynthetic pigments (chlorophyll *a*, chlorophyll *b* and ß-carotene) decreased with paraquat concentration (Fig. 1). The highest concentrations of paraquat assayed (0,30 and 0,60 µM) provoked the disappearance of ß-carotene in the chromatograms (Fig. 1).

Obtained results for photosynthesis rate by ^{14}C-bicarbonate technique after 96 h of culture are showed in Figure 2. Photosynthesis rate expressed as µg of per mg of dry weight and hour did not present important variations at the paraquat concentrations added; no significant differences were observed between control cultures (0,26 µg C mg^{-1} DW h^{-1}) and those cultures exposed to concentrations lower than 0,30 µM. Only *C. moewusii* cultures exposed to 0,60 µM showed a significant increase of this photosynthetic rate (0,36 µg C mg^{-1} DW h^{-1}) (Fig. 2).

Paraquat toxicity on microalgae cells becomes clear in cellular ATP content (Fig. 2). Maximum values of ATP content were reached in control cultures (57 pmoles ATP μg^{-1} DW). An important decrease of this ATP content was observed for all the cultures exposed to paraquat, reaching minimum values at 0,15 µM (21 pmoles ATP μg^{-1} DW) (Fig. 2).

Autofluorescence of chlorophyll *a*, analyzed by means of flow cytometry technique, was measured with the aim to determine its utility as a possible endpoint measurement in toxicity assays. This parameter showed a decrease when paraquat concentration increased; this decrease was up to 61% respect to control in cultures with paraquat 0,60 µM (100% in control cultures and 39% in cultures with a paraquat concentration 0,60 µM) (Table 1).

Table 1. Growth rates (µ) (day^{-1}), dry weight of biomass (ng $cell^{-1}$) and percentage respect to control chlorophyll *a* fluorescence signal (arbitrary units) of *Chlamydomonas moewusii* cells exposed to different paraquat concentrations (µM) after 96 hours. Data are given as mean values ± standard errors.

Paraquat	Growth rate	Dry weight	Chl *a* fluorescence
0	0,77 ± 0,06	0,12 ± 0,001	100
0,037	0,84 ± 0,02	0,11 ± 0,001	90
0,075	0,81 ± 0,01	0,10 ± 0,001	81
0,15	0,71 ± 0,03	0,10 ± 0,019	66
0,30	0,36 ± 0,05	0,20 ± 0,004	65
0,60	-0,22 ± 0,06	0,55 ± 0,001	39

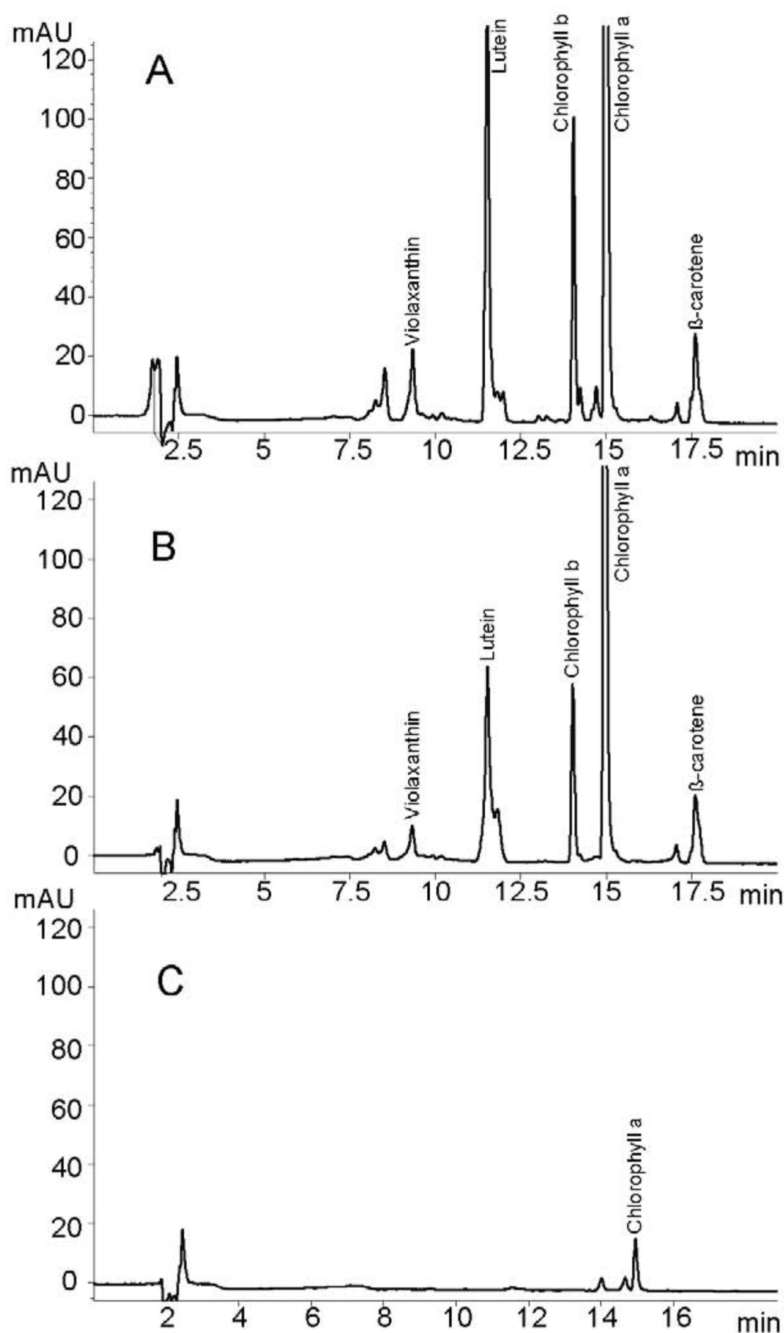

Figure 1. Chromatograms of cellular pigments in *Chlamydomonas moewusii* cells after 96 hours of culture. (A): control cultures non-exposed to paraquat, (B): cultures exposed to paraquat 0,15 µM, (C): cultures exposed to paraquat 0,60 µM.

DISCUSSION

Presence of chemical contaminants has become a serious threat for many natural aquatic ecosystems. From a historical point of view, pesticides have been one of the most frequent contaminants of freshwater ecosystems because of their wide use in agriculture, in order to increase and improve agricultural production; nevertheless, the use of pesticides in agriculture inevitably leads to exposure of non target organisms

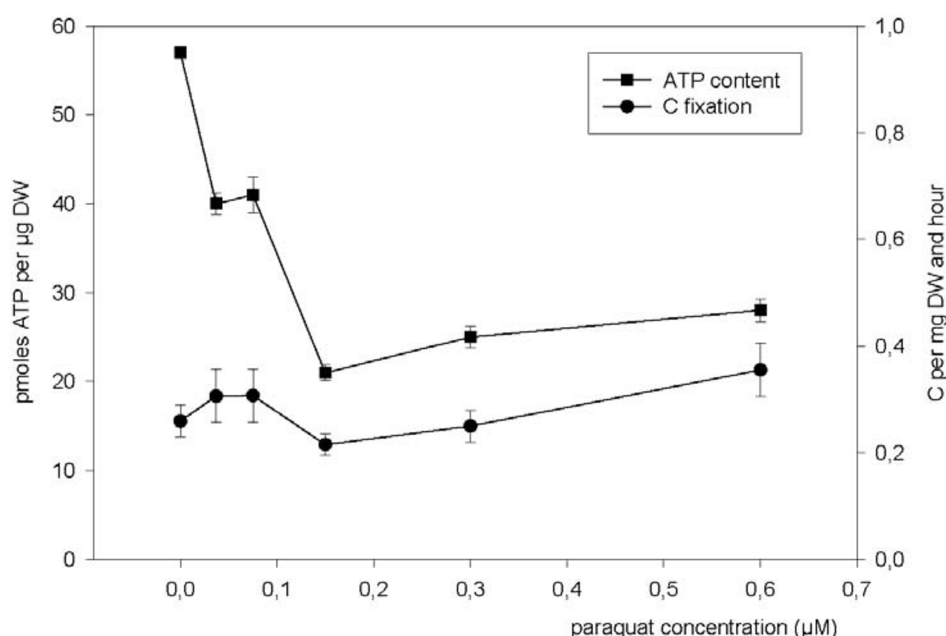

Figure 2. Carbon fixation rate (●) and ATP content (■) of *Chlamydomonas moewusii* cultures cells exposed to paraquat. Carbon fixation rate was expressed as µg C per mg of dry weight of biomass per hour. ATP content was expressed as pmoles per µg of dry weight of biomass. Data are given as mean values ± standard errors.

(including humans) undesirable effects may occur on some species, communities or on ecosystems as a whole [17].

Methods to assess the aquatic toxicity of pesticides use mainly fishes and crustaceans; but data on microalgae and pesticides are very interesting to expand the knowledge about harmful effects of toxic substances, since the planktonic algae are an important component of aquatic systems.

Paraquat dichloride is an electron acceptor at photosystem I; one of the most highly toxic herbicides to be marketed and the third most widely used herbicide in the world.

Photosynthetic electron transport is a universal feature of cyanobacteria, algae and higher plants. This essential process is sensitive to many classes of environmental contaminants, including herbicides [18], metals [19] and organic contaminants [20]. A common mechanism of toxic action of contaminants is inhibition of biological processes such as photosynthesis and mitochondrial electron transport [21]. If the photosynthetic apparatus is disturbed by environmental contaminants, changes in their cell physiology, growth and biomass yield are inevitable. Growth, dry weight, chlorophyll *a* fluorescence, ATP and pigments content of the microalga *C. moewusii* showed disturbances respect to control in cultures with paraquat concentrations lower than legally recommended for macrophyta control [22].

Growth is the most used parameter to study toxic effect of pollutants on cells. In many assays, toxicity is measured depending on a reduction of growth rate, or final biomass, in relation to control [23].

In the present study it was observed that paraquat altered the growth of *C. moewusii,* leading to a significant reduction of the growth rate in cultures with a paraquat concentration of 0,30 and 0,60 µM (Table 1). Paraquat is a redox-cycling compound capable of generating reactive oxygen species under aerobic conditions [24]. Normally, cells are well protected by the cellular antioxidant systems; however, when the production of reactive oxygen species overwhelms the cellular antioxidant defences, oxidative damages are inevitable [25]. Results obtained would indicate that when the highest concentrations of paraquat are added to cultures, these systems would be incapable of

protecting to *C. moewusii* cells of the oxidative stress provoked by this herbicide.

Therefore, the use of paraquat at recommended concentrations for controlling of aquatic weeds, 0,1-2 mg l^{-1} (0,38-7,7 μM) [26], could cause irreversible damages in natural populations, at least, of several microalgae species usually present in freshwater ecosystems.

Because of paraquat exerts its toxic action at level of chloroplast, where it can accept an electron of photosystem I [27, 28, 29], these organelles result clearly affected by paraquat. It has been observed several classes of damages in chloroplast provoked by paraquat addition. Organisms have evolved a variety of responses that help compensate for the physiological impact of environmental contaminants. The antioxidant defence mechanism forms the crux of this whole system, being the carotenoids one of these mechanisms. So, with respect to pigment content, its measure by mean of HPLC techniques showed a reduction in the area of peaks corresponding to the main photosynthetic pigments (chlorophyll *a* and chlorophyll *b*) and the disappearance of ß-carotene in cultures with the highest paraquat concentrations (0,30 and 0,60 μM). Reduction in such areas, directly proportional to these pigment concentrations, agrees to the inhibition effect provoked by paraquat on photosynthetic pigments in several assays using spectrophotometric techniques [11, 30]. ß-carotene is one of the non-enzymatic defensive mechanisms that cells have against oxidative stress; carotenoids are potent quenchers of reactive oxygen species such as singlet molecular oxygen and peroxyl radicals, thus acting as deactivators of excited molecules or as chain-breacking agents, respectively [31]. These mechanisms exist in cells because of chloroplast and other cellular organelles produce, in normal conditions, tolerable levels of active oxygen through auto-oxidation of components of the PSI electronic transport chain.

Under light conditions, paraquat can accelerate the production of these oxygen species [32]. ß-carotene, by auto-oxidation, protects other vital cellular components such as chlorophylls. A sharp fall of this protector pigment at the highest paraquat concentrations assayed could be, as it has previously reported, in the origin of other more evident cellular damages: growth decrease and cellular death, observed at same concentrations (Fig. 1).

Further damages in photosynthetic pigments, other disturbs provoked by paraquat on chloroplast level would be closely connected with photosynthesis activity or photosynthesis rate; however, ^{14}C-bicarbonate assimilation per unit of dry weight remained constant as the paraquat concentration increased in the cultures (Fig. 2), being this an unexpected result, since photosynthesis is the molecular target of this herbicide. Decline of photosynthetic pigment content because of their oxidative breakdown [33] could be also related with decrease of photosynthesis rate.

Cellular autofluorescence can be measured directly by flow cytometry, without fluorochromes; measurement of chlorophyll *a* fluorescence gives information about absorption, distribution and used of energy in photosynthesis [34]. In the present study, autofluorescence of chlorophyll *a* was measured with the aim to determine its utility as a possible endpoint measurement in toxicity assays. *Chlamydomonas moewusii* cells showed a decrease in chlorophyll *a* fluorescence when paraquat concentration increased (Table 1). Changes in chlorophyll *a* fluorescence at room temperature are closely connected with PSII activity and reveal redox state of primary electron acceptor of this photosystem, the Q_A. So, the decrease observed in this parameter would indicate the photosynthetic blocking leaded by paraquat in *Chlamydomonas* cells, by dissording in their membranes where PS are located and provoking a strong decrease in chlorophylls content with the highest concentrations assayed (0,30 and 0,60 μM).

Photosynthesis is the process of energetic production in vegetable cells. Energy obtained is stored in ATP form; so, any agent which affects photosynthetic process, would also be expected to alter the ATP cellular content [15]. ATP content per unit of dry weight decrease significantly in all of paraquat treated cultures (Fig. 2), in spite of the no differences found for the photosynthetic activity of these cultures. A possible explanation of this could be an increase of the ATP

consumption of cells with the aim of repair the stress provoked by the herbicide.

Stress is able to induce or accelerate numerous characteristic changes of ageing process. Stress breaks the cellular ability to maintain homeostasis [35, 36] and interfere with their self-maintenance systems, not only by producing damages, but by inhibiting their reparation and, possibly, upping energetic cost of this reparation [37].

According to the European Union, threshold values for herbicides in water intended for human consumption are 0.1 $\mu g\ l^{-1}$ for an individual herbicide and 0.5 $\mu g\ l^{-1}$ for total herbicides [22]. Concentrations of paraquat around this range and lower than necessary for provoking growth inhibition would affect fundamental microalgal process (e.g. photosynthesis) that are basic in function of all ecosystems. However, obtained results indicated that the photosynthetic rate determination could undervalue the effects of this herbicide, at least in the case of this microalgal species, being the ATP content or the autofluorescence of chlorophyll *a* the most sensitive parameters analyzed.

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