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FLUOMETURON* INDUCED GROWTH, CHEMICAL AND PHOTOSYNTHETIC CHANGES IN *CHLORELLA VULGARIS*

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ABSTRACT

Presence of fluometuron at different concentrations in the culture medium of *Chlorella vulgaris* induced pronounced inhibition in the growth rate and dry weight of the alga throughout the experimental period. Furthermore, fluometuron variably reduced the photosynthetic pigment contents (chlorophyll a, chlorophyll b and carotenoids). At 1 and 2 ppm, fluometuron induced culture bleaching up to 3 and 5 days respectively. Thereafter, progressive marked increase in chlorophyll contents was observed accompanying culture regreening.

Supplemental addition of fluometuron to the culture media reduced the level of reducing sugars, sucrose, polysaccharides and total sugars. The reductions in the different carbohydrate fractions were associated with marked increases in the different nitrogen components (amino-, protein - and total soluble -N).

$^{14}CO_2$ - light fixation was significantly inhibited in the treated cultures. The incorporation of $^{14}CO_2$ into various photosynthetic intermediates was also, in general, markedly lowered below the control values and was found to vary with the concentration of fluometuron fed to the algal culture. The results are discussed in relation to the phytotoxicity of the herbicide used.

INTRODUCTION

Although the toxic action of herbicides on higher plants is usually studied and documented prior to the initial release of a herbicide to commercial use, no satisfactory studies have been carried out concerning the effects of these toxic substances on algal metabolism. However, the effects of some herbicides on growth and photosynthetic oxygen evolution by algae have been studied by many workers

*1, 1- dimethyl - 3 - (α, α, α,-trifluoro -m-tolyl) urea

(Sikka and Pramer, 1968; Ruben and Eshel, 1971; Tchan *et al.*, 1975; Richardson *et al.*, 1979 and Osman *et al.*, 1983 and 1984). They reported significant reduction in the growth rate and photosynthetic activity of the algae used as a result of herbicide treatment.

The substituted urea group of herbicides is considered to be one of the most important groups of herbicides, which seriously impair the photosynthesis of algae and aquatic plants (Audus, 1964 and Ashton and Crafts, 1981). Fluometuron is a member of the urea herbicides which is currently used in Egypt for weed control in cotton fields, and fear will arise from its adverse effects on the growth of algae which play an important role in the gas exchange of the biosphere.

This object of study was to add information on the responses of *Chlorella vulgaris*, which represents one of the most dominant spieces of algae in the river Nile, to various concentrations of fluometuron. Three different types of experiments were carried out to determine changes in growth parameters, pigment, carbohydrate and nitrogen constituents and to examine changes in photosynthesis and incorporation of ^{14}C into various metabolites.

MATERIALS AND METHODS

Chlorella vulgaris (Beij. var. Vulgaris, Fott.) was isolated from water samples collected from the Damietta branch of river Nile. A pure culture of the organism was obtained by repeated subculturing. Thus a mixture of streptomycin (30 ppm) and tetracyclin (30 ppm) was added to the culture medium for 20 minutes to obtain a bacterial and fungal free culture (Venkataraman, 1969) and the mass culture technique of Lorenzen (1964) was used for cultivation. As recommended by Kuhl (1962), the fresh water algal medium was used and the culture conditions followed by Ahmed and Osman (1973) and Osman *et al.* (1984) were adopted in this investigation.

The growth rate was measured as described by Zielinski and Price (1978). Dry weight determinations were made as adopted by Ahmed and Osman (1973). The photosynthetic pigments (Chlorophyll a, chlorophyll b and carotenoids) were determined by the method recommended by Metzner *et al.* (1965)

Determination of carbohydrates

A known volume of algal suspension was dried in an oven at 80°C to constant dry weight which was then ground to a fine powder. Sugars were extracted by the method of Younis (1963). The direct reducing value (D R V), which was considered to represent the reducing sugars, was estimated by the modified Nelson's Method (Naguib, 1964). Determination of the total reducing value (T R V) was carried out by measuring the optical density after hydrolysis with an adequate amount of invertase. The sucrose content was calculated from the

difference between T R V and D R V. Polysaccharides were determined in the dry residue left after alcohol extraction of soluble carbohydrates as described by Younis *et al.* (1969).

Estimation of nitrogenous components

The nitrogenous constituents were extracted by the method adopted by Yemm and Willis (1956). Total-N was estimated in the the dried ground algae and the total soluble-N was estimated in the extracts by the conventional micro-kjeldahl method (Pirie, 1955). Determination of amino-N was achieved by the method described by Muting and Kaiser (1963).

Determination of photosynthetic activity

Methods of determination of photosynthetic activity as well as ^{14}C incorporation into metabolic intermediates were patterned after those methods described by Bassham and Calvin (1957 and 1963) and Ahmed and Reis (1969).

(^{14}C) bicarbonate was obtained from the Radiochemical centre, Amersham, England. After 10 days incubation, the differently treated algal cells were precipitated by centrifugation and then suspended in 5 cc of 1/15 M phosphate buffer; pH 7.2 (Tsay *et al.*, 1966). After that, 0.2 cc of ^{14}C-aqueous sodium bicarbonate solution of known activity (100 uCi/cc) were added to the algal suspension. The sample was then subjected to an illumination source for 3 minutes. The light intensity at the sample level was 10 kilo-lux. At the end of the fixation time (3 minutes), the algal suspension was rapidly transferred to boiling 85% methanol in which algal cells were killed and extracted. Then the insoluble fraction was separated from the soluble one by centrifugation and the supernatant was made up to volume. An aliquot was evaporated and then few drops of 10% formic acid were added to transform the unused ^{14}C-bicarbonate into $^{14}CO_2$. The contents were resuspended in 2 cc methanol and radioactivity measurements were carried out by using Packard Scintillation Counter Model 526. The radioactivities measured are directly proportional to the amounts of $^{14}CO_2$ fixed in the organic compounds, which could be calculated as cpm/cc algal suspension.

The radioactivity of the insoluble material was determined by suspending the residue in an aliquot of 85% methanol; 1 cc of this suspension being transferred into a beaker, evaporated and then treated with formic acid as mentioned above. This was followed by suspending the contents in 2 cc methanol and the radioactivity determined.

To follow the pattern of ^{14}C-incorporation into the soluble metabolic intermediates, the autoradiography technique described by Ahmed and Reis (1969) was applied. Thus the soluble extract was evaporated *in vacuo* to 2 cc syrup at 45°C, and an aliquot was spotted onto a silica gel thin layer plate. Plates were developed using

two dimensional technique with n-propanol: ammonia: water (6: 3: 1) and phenol: water (75:25) as developing solvents. Plates were exposed to x-ray films and the dark spots that appeared after film developments and fixation, gave marks for the positions of radioactive compounds on the plate.

Amino acids were located onto the chromatograms by spraying with a ninhydrin reagent and heating at 110°C for colour development. Identification of developed spots was performed by comparing their locations with those on a reference chromatogram made with authentic compounds.

Organic acids after being scratched and eluted from the silica layer, were then identified by co-chromatography with authentic samples. The phosphorylated sugars and related compounds generally remained around the start (Ahmed and Reis 1969).

The radioactive spots, after being located onto the chromatograms, were scratched out of the plate and suspended in methanol, centrifuged and the radioactivity of each fraction was then determined using liquid scintillation counter. Radioactivity of each spot (corresponding to a compound) was comapred as percentage of the total radioactivity on the whole plate of each treatment.
Variation between 4 samples was about 5%; mean values are listed.

RESULTS AND DISCUSSION

Figure 1 shows that fluometuron reduced the growth of *Chlorella vulgaris* during the experimental period; the magnitude of reduction was more pronounced as the concentration of fluometuron was increased. Thus at 10 days the rate of reduction exceeded 80% in response to application of fluometuron at 2 ppm. These observations are in agreement with those obtained by Sikka and Pramer (1968) who found that fluometuron, at 10 ppm, reduced the growth of *Euglena* by 52%. Figure 2 illustrates varied reductions in dry weight as in the case of growth rate in response to fluometuron treatment. Thus close parallelism appeared to exist between the growth pattern and dry weight accumulation throughout the experimental period. This result suggests an inhibition of photosynthesis induced by fluometuron treatment. Chlorophyll a and b contents of cultures treated with 0.25 ppm and 0.5 ppm of fluometuron showed progressive increases up to the 7th and the 9th day of incubation respectively. Thereafter, these contents were found to decrease (Table 1). On the other hand, chlorophyll a and b contents of cultures treated with 1 and 2 ppm of fluometuron showed a considerable decrease up to 5 days of incubation; inducing culture bleaching. Thereafter, progressive marked increase was observed accompanying culture regreening.
With respect to carotenoids, table 1 shows that they exhibited the same pattern of changes as in the case of chlorophylls in response to fluometuron treatment. Fluometuron thus appeared to exert a destructive effect on both chlorophylls and

carotenoids. This may be attributed to degradation of chloroplast ribosomes and/or to destruction of chloroplast structure as reported by various workers (Bartels and Pegelow, 1967; Bartels and Hyde, 1970; Bartels and Watson, 1978; Feierabend and Schubert, 1978; Blume and McClure, 1980) for phenylpyridazinone, metafluorazon and norfluorazon herbicides. Chlorophyll bleaching may be attributed to its photooxidation induced by the inhibition of the electron transport system in thylakoids as a result of treatment with the herbicide as reported by Swader and Howe (1979).

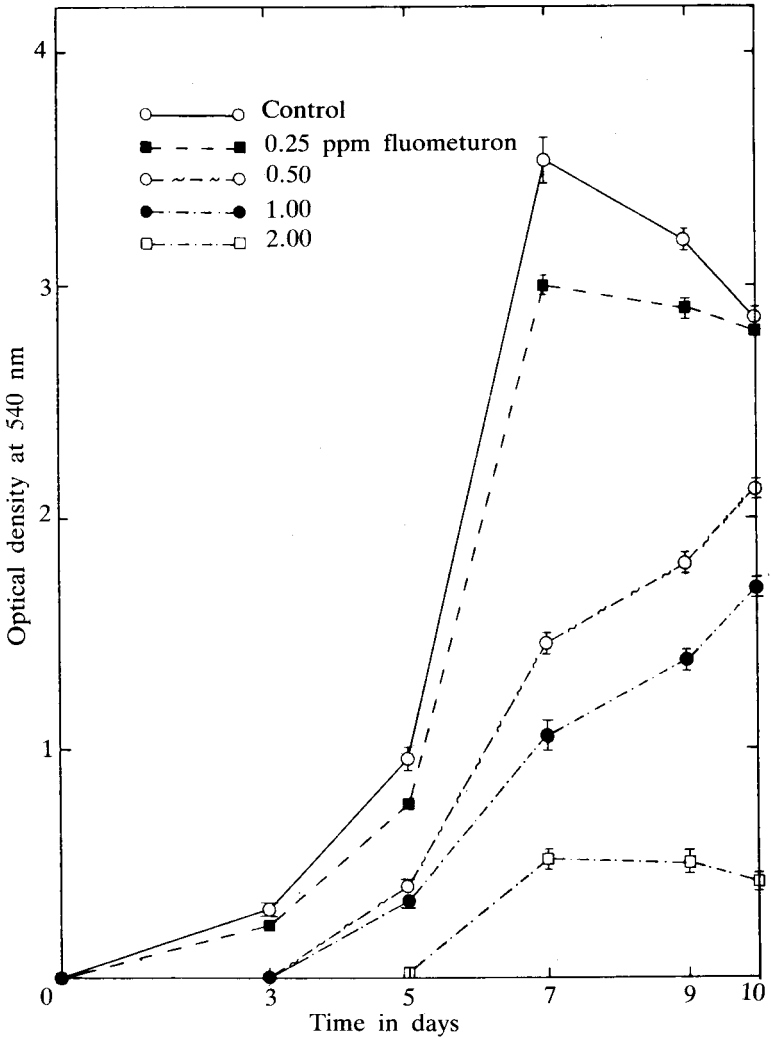

Fig. 1: Effect of different concentrations of fluometuron in the culture media on the growth of *Chlorella vulgaris*. Each value is the mean of 4 determinations.

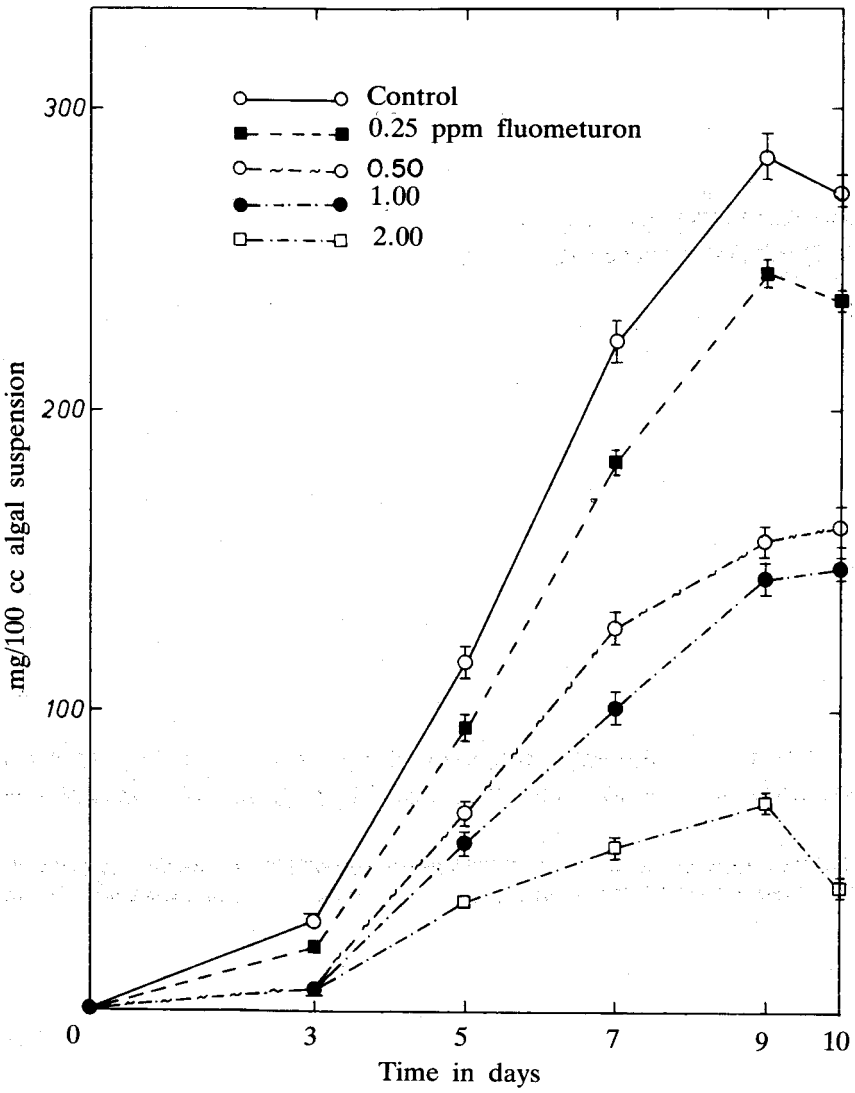

Fig. 2: Effect of different concentrations of fluometuron in the culture media on dry weight of *Chlorella vulgaris*. Each value is the mean of 4 determinations.

Table 1

Effects of different concentrations of fluometuron in the culture media of *Chlorella vulgaris* on the pigment contents at 25°C. Each value is the mean of four determinations calculated as ug/cc algal suspension.

Herbicide ppm	Age in days	Chlorophyll a	Chlorophyll b	Carotenoids	Chl.a/ Chl.b ratio	Chl.a+Chl.b/ Carot. ratio
Control	3	1.1	0.2	0.8	5.5	1.6
	5	4.8	1.7	2.8	2.8	2.3
	7	10.6	3.7	10.0	2.9	1.4
	9	8.6	4.2	3.3	2.1	3.9
	10	6.2	2.8	2.6	2.2	3.5
0.25	3	1.4 V.H.S.	0.2 N.S.	0.8 N.S.	7.0 S.	2.0 H.S.
	5	5.9 V.H.S.	1.9 V.H.S.	2.9 N.S.	3.1 N.S.	2.7 N.S.
	7	10.4 N.S.	4.5 V.H.S.	10.2 N.S.	2.3 N.S.	1.5 N.S.
	9	9.7 N.S.	3.4 S.	6.2 V.H.S.	2.9 H.S.	2.1 V.H.S.
	10	6.8 N.S.	3.2 N.S.	2.9 N.S.	2.1 N.S.	3.5 N.S.
0.50	3	—	—	—	—	—
	5	3.3 V.H.S.	0.9 V.H.S.	1.8 V.H.S.	3.6 H.S.	2.3 N.S.
	7	8.1 V.H.S.	2.9 V.H.S.	5.9 V.H.S.	2.8 N.S.	1.8 H.S.
	9	10.1 S.	4.8 N.S.	7.0 V.H.S.	2.1 N.S.	2.1 V.H.S.
	10	8.6 V.H.S.	3.9 V.H.S.	3.4 V.H.S.	2.2 N.S.	3.7 N.S.
1.00	3	—	—	—	—	—
	5	1.9 V.H.S.	0.5 V.H.S.	1.2 V.H.S.	3.8 .S.	2.0 N.S.
	7	5.9 V.H.S.	1.9 V.H.S.	4.7 V.H.S.	3.1 N.S.	1.7 N.S.
	9	7.9 N.S.	3.1 H.S.	4.5 V.H.S.	2.5 N.S.	2.4 V.H.S.
	10	8.0 H.S.	3.9 V.H.S.	3.4 V.H.S.	2.0 N.S.	3.5 N.S.
2.00	3	—	—	—	—	—
	5	—	—	—	—	—
	7	1.2 V.H.S.	0.3 V.H.S.	1.0 V.H.S.	4.0 V.H.S.	1.5 N.S.
	9	3.5 V.H.S.	1.6 V.H.S.	2.7 V.H.S.	2.2 N.S.	1.9 V.H.S.
	10	4.6 H.S.	2.3 S.	1.5 V.H.S.	2.0 N.S.	4.6 H.S.

S. = significant, H.S. = highly significant, V.H.S. = very highly significant, N.S. = not significant

As recorded in table 1, the ratios of chlorophyll a/chlorophyll b as well as those of chlorophyll a + chlorophyll b/carotenoids did not, in general, change significantly in the fluometuron - treated culture from those of untreated ones. Thus, fluometuron appeared to exert a similar effect on both chlorophylls and carotenoids in *Chlorella vulgaris.* The regreening of the high concentration of fluometuron - treated culture after 7 days may indicate that fluometuron has a reversible toxic action on the photosynthetic apparatus or on the reactions leading to its formation. Almost the same conclusion was cited by Boger and Schule (1976) for diuron, atrazine and mitribuzine herbicides which were added to the culture medium of *Bumelleriopsis.* Moreover, the regreening phenomenon was reported by Tantawy (1982), who observed that bleaching of *Chlorella fusca* cultures induced by metfluorzon disappeared with regeneration of green cultures within four days of treatment.

After 10 days of cultivation, progressively greater reductions in reducing sugars, sucrose, polysaccharides and total sugars were observed with an increase in concentration of fluometuron (Fig. 3). These reductions could be accounted for by an inhibitory effect of the herbicide on the photosynthetic activity as mentioned above and as realized from this study (see Fig. 5 and table 2) and/or by an increase in respiration as reported by many workers (Palmer and Allen, 1962; Funderburk and Davis, 1963; Davis *et al.*, 1976 and Richardson *et al.*, 1979). However, the role of utilization of carbohydrates as substrates in increased nitrogen metabolism cannot be ruled out. Thus, the present results (see Fig. 4) as well as the work of Zhirmunskaya (1966) well support this conclusion.

Figure 4 illustrates that rising of fluometuron concentration in the culture media was accompanied by significant increases in the content of the different nitrogen fractions determined at the end of the experimental period. The increase in these nitrogen fractions was most pronounced with the high concentration of the herbicide. As can be seen from figures 3 and 4, the reduction in the different carbohydrate contents was associated with a significant increase in the nitrogen fractions. This result may indicate that carbohydrates were utilized as substrates in increased nitrogen metabolism under the influence of the herbicide as earlier suggested by Zhirmunskaya (1966). He found that *Sonchus arvensis* treated with 2, 3, 6-TBA (2, 3, 6-trichlorobenzoic acid) contained 2- to 3- fold increase in total nitrogen. This was accompanied by an intensive synthesis of protein and accumulation of non-protein forms of nitrogen at the expense of the amides.

Treatment of *Chlorella vulgaris* cells with different concentrations of fluometuron induced considerable reductions in the labelled soluble and insoluble photosynthetic products (Fig. 5). There appeared to be progressively greater reduction of labelling in these fractions as the concentration of fluometuron was increased. These results give a good indication that fluometuron inhibits photosynthesis

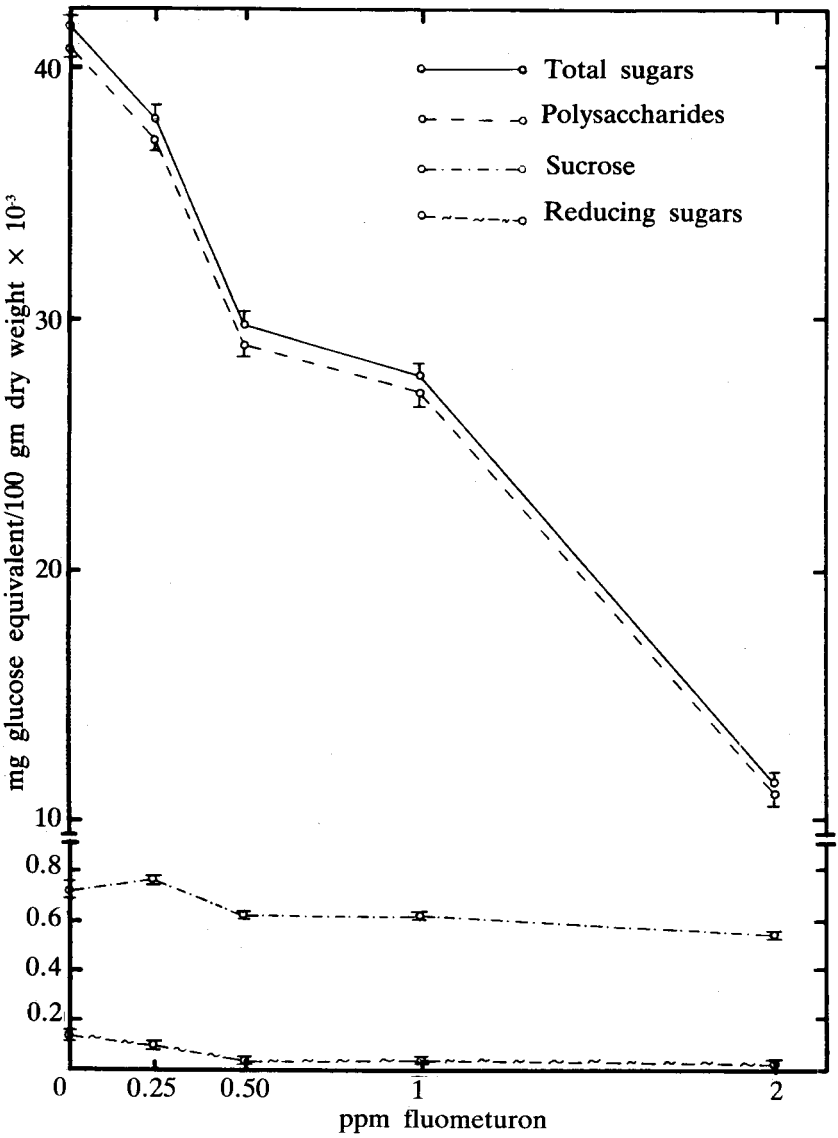

Fig. 3: Analysis of carbohydrate content of *Chlorella vulgaris* after 10 days of incubation at 25°C in culture media supplied with different concentrations of fluometuron. Each value is the mean of 4 determinations.

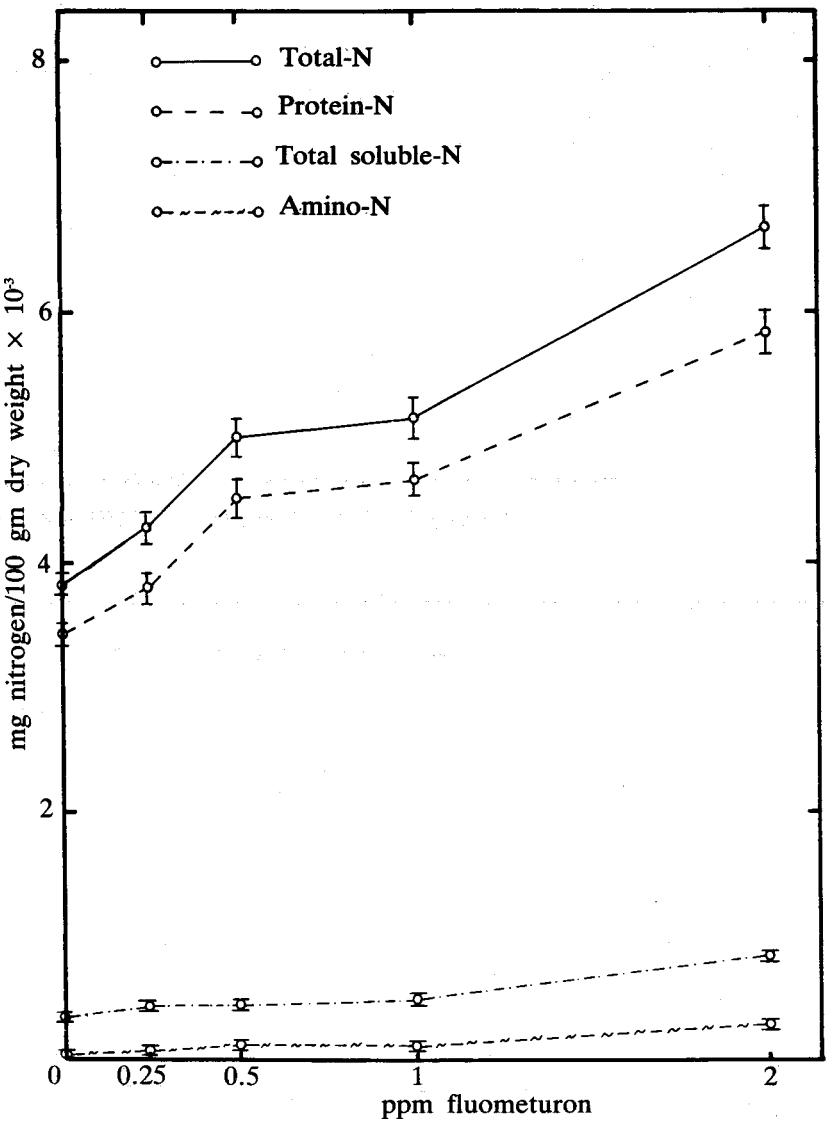

Fig. 4: Analysis of nitrogen content of *Chlorella vulgaris* after 10 days of incubation at 25°C in culture media supplied with different concentrations of fluometuron. Each value is the mean of 4 determinations.

process. Moreover, the inhibition of photosynthetic oxygen evolution in algae treated with fluometuron was recorded by many workers (Sikka and Pramer, 1968; Rubin and Eshel, 1971; Richardson *et al.*, 1979). This inhibition may be attributed to the blockage of electron transport between Q (primary electron acceptor of photosystem II) and PQ (Plastoquinone). This prevents the formation of ATP and NADPH, which are required for carbon dioxide fixation as suggested by Ashton and Crafts (1981).

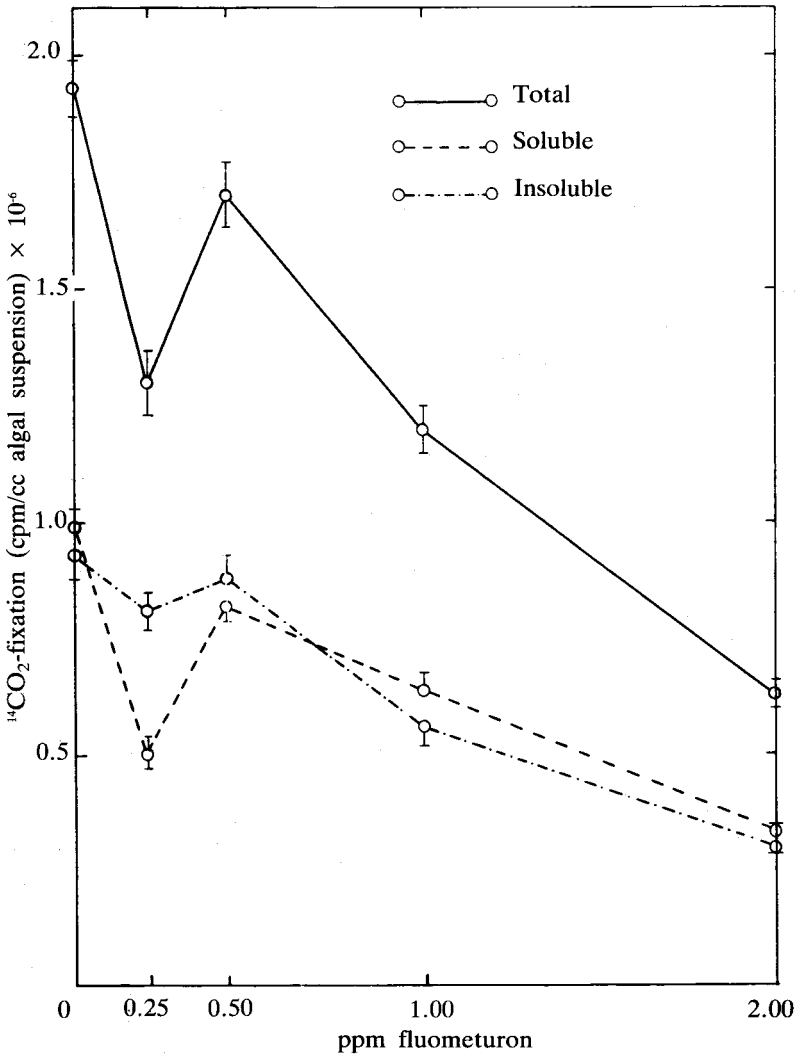

Fig. 5: Effect of different concentrations of fluometuron in the culture media of *Chlorella vulgaris* on the photosynthetic activity at 25°C after 10 days of treatment. Each value is the mean of 4 determinations.

Data presented in Table 2 show that different concentrations of fluometuron elicited various degrees of reduction in the labelling percentage of the phosphorylated sugars and related compounds. Methionine and arginine represented relatively high labelling percentage in the control cultures and supplemental addition of fluometuron to the culture media led to marked reduction in the labelling percentage of these fractions. The amino acids aspartic, glutamic, glutamine, serine and glycine exhibited relatively low labelling percentage in the control culture and this was, in general, decreased with rising fluometuron concentration. On the other hand, the low labelling percentage of phenylalanine and proline detected in the control culture was considerably increased upon

Table 2

Percentage labellings of photosynthetic intermediates of fluometuron treated and untreated cultures of *Chlorella vulgaris* after 10 days of incubation.

Intermediates	fluometuron (ppm)				
	control	0.25	0.5	1.0	2.0
Phosphorylated sugars	0.4	0.2	0.13	0.2	0.2
Arginine	14.6	2.1	0.4	1.7	1.1
Aspartic acid	1.3	0.4	0.2	0.3	0.9
Asparagine	—	—	0.1	0.2	0.8
Glutamic acid	2.5	1.3	0.2	0.6	1.1
Glutamine	4.6	0.5	2.1	5.2	3.2
Glycine	0.9	0.1	0.1	0.2	1.2
Methionine	28.9	25.0	22.7	20.0	18.9
Phenylalanine	1.3	11.0	10.5	18.4	17.1
Proline	1.5	11.9	13.0	32.2	19.8
Serine	1.0	0.5	0.3	0.5	0.8
Threonine	—	0.9	0.7	—	—
Citric acid	2.3	0.2	0.04	0.2	1.1
Malic acid	0.7	0.1	0.14	0.22	0.3
Fumaric acid	0.2	0.2	2.3	1.0	—
X_5 (unidentified)	—	—	3.3	0.3	—
X_6	—	—	0.2	—	—
X_7	—	—	10.3	—	—
X_8	—	1.2	—	0.03	—
Lipoid fractions	39.7	44.4	33.3	18.7	33.4
Total	100	100	100	100	100

application of different concentrations of fluometuron to the culture media. These results can be accounted for by a stimulatory effect of this herbicide on the transamination reactions and/or by an inhibition of *de novo* synthesis of amino acids. Our results concerning the effect of fluometuron on the levels of the different nitrogen fractions (amino-, total soluble-, protein- and total-N) in the tested alga strongly support the first interpretation. In accord with this conclusion, Ekanagake *et al.* (1979) reported an increase in the tyrosine content associated with reduction in phenylalanine of glyphosate treated leaves of *Panicum repens.*

Organic acids of fluometuron treated algae showed variable labelling percentages without a regular pattern. Thus, it seems that fluometuron inhibited photosynthesis but did not affect the pathway of utilization of the fixed ^{14}C in the tested algae. In this connection, Ashton and Uribe (1962) studied the metabolism of ^{14}C-sucrose by excised leaves of beans treated with atrazine and found that malic and citric acids were unaffected by the atrazine treatment.

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التغـــيرات في النمـــو
والتركيب الكيميائي والبناء الضوئي المستحثة
بالمبيد العشبي فلوميترون في طحلب كلوريلا فولجاريس

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أدت معاملة طحلب **كلوريلا فولجاريس** بتركيزات مختلفة من الفلوميترون إلى نقص ملحوظ في معدلات النمو والوزن الجاف والتي تناسبت قيمتها طردياً مع تركيز المبيد ، ولقد اقترنت تلك التغيرات بانخفاض محتوى الأصباغ البنائية الضوئية انخفاضاً إحصائياً ، هذا بالاضافة إلى أن تركيزات ١ ،٢ جزء في المليون من الفلوميترون استحثت شحوباً في المزارع في فترة تصل إلى ثلاثة وخمسة أيام على التوالي ، ومن ثم لوحظ إعادة إخضرار ملحوظ في هذه المزارع .

ولقد أظهر التحليل الكيميائي نقصاناً ملحوظاً في المحتوى الكلي والجزئي للكربوهيدراتات ، وكان ذلك مقروناً بزيادة في محتوى النيتروجين الأميني والبروتيني والنيتروجين الكلي الذائب . ولقد كان حجم النقصان أو الزيادة في هذه المكونات من البروز بمكان مع التركيزات العالية من المبيد العشبي .

أما فيما يتعلق بالنشاط البنائي (تثبيت الكربون المشع ضوئياً) ، فقد أدت المعالجة بالفلوميترون إلى نقص ملحوظ ، وبالنسبة للمركبات الوسيطة المشعة والتي تكونت أثناء عملية البناء الضوئي فقد لوحظ تبايناً كيفياً وكمياً في طحلب **كلوريلا فولجاريس** تحت التركيزات المختلفة .