Desalination and Water Treatment
www.deswater.com
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doi: 10/5004/dwt.2012.2922

Taylor & Francis
Taylor & Francis Group

44 (2012) 334–339
June

Physiological responses of *Ankistrodesmus acicularis* to diuron

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Received 15 April 2011; Accepted 8 February 2012

ABSTRACT

The impact of diuron on the fresh water alga *Ankistrodesmus acicularis* was investigated. Parameters studies were the effect on photosynthetic pigments, growth rate, ^{14}C-photoassimilation, carbohydrate, protein, and free amino acids contents, adenosine triphosphate (ATP), uptake of nitrate and diuron by algal cells. Photosynthetic pigments [chlorophylls (a) and (b) contents and total carotenoids] of diuron—treated algae were significantly decreased compared to control. Carbohydrate, protein, and free amino acids contents of treated algae were significantly decreased in response to diuron treatments. The values of EC_{50}, which exert 50% inhibition, were calculated by Probit test which varied with respect to the exposure time. Decreased in ^{14}C-photoassimilation and ATP were in harmony with the decrease of other determined metabolic activities.

Keywords: Algae; Toxicity; Diuron; Herbicide; Photosynthesis; Uptake

1. Introduction

Phenylurea herbicides are widely used throughout the world for the protection of several crops and consequently, can have residual effects on crops, soil and surface waters [1].

The herbicide diuron [3-(3,4-dichlorophenyl)-1, 1-dimethylurea] inhibits photosynthesis via blockage of the electron transport in the photosystem II (PSII) [2], chlorophyll content and affect growth, cell organelles and metabolism of photosynthetic plants [3,4]. Moreover, the herbicide diuron is commonly incorporated into antifouling paints to boost the efficacy of the compound towards alga [5]. Several studies have reported the presence of diuron in surface waters [3,6].

Studies on the toxic effects of herbicides on algae are essential for the assessment of the potential environmental impact of these compounds on aquatic ecosystems. Algae play an important role in aquatic environment as the primary procedures of organic matter which is the first step in the food chain [7]. The use of photosynthetic organisms in toxicity test is very appropriate because more than 65% pesticides are herbicide [8]. Furthermore, about 50% of these herbicides act via inhibition of photosynthesis at the photosystem-I (PSI) and PSII level by replacing PSI's ultimate electron acceptor [9]. The response of algae is subject to variation according to the chemical nature of the herbicide, exposed algal species and the exposure periods [10,11]. The present study aims to investigate the major impacts of the herbicide diuron on *Ankistrodesmus acicularis* as representative of the green algae to be found in River Nile water. Parameters which reflect the impact of diuron on the alga include photosynthetic pigments, growth rate, carbohydrate, protein and free amino acids contents. In addition, effect of diuron on nitrate uptake, CO_2 photoassimilation, ATP content and uptake of diuron by algal cells were assessed.

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2. Materials and methods

The herbicide diuron of 98% purity was supplied by sigma. Chemically, diuron is *N*-(3,4-dichlorophenyl) *N*,*N*-dimethylurea. Extraction of diuron from aqueous media was carried out using chloroform [12]. Residues were identified and determined by gas chromatography (GC), Hp 6890 (USA) equipped with electron capture detector (Ni^{63}) and capillary column Hp. The temperature of the column was raised from 100°C to 180°C with rate 5°C min^{-1} the injector and detector temperature were 250°C and 280°C, respectively. Nitrogen was used as a carrier gas at a flow rate of 2 ml min^{-1}.

Ankistrodesmus acicularis was isolated from River Nile water and cultivated in a standard algal media, namely algal assay procedure bottle test [13]. The test organism was in the logarithmic phase of growth when introduced to the standard algal culture medium. Bioassay flasks were incubated at 24°C ± 2°C under continuous illumination (≈2500 lux). Prior to conducting the bioassay, the organism was separated from the medium by centrifugation at a temperature of 22–25°C for 5 min using a speed of 2000 rpm and washed with distilled water [14]. The amount of algae inoculated in bioassay culture flasks was determined at the beginning of the experiments using Chl(a) measurement. Diuron solutions was prepared in ethanol and the applied concentrations ranged from 0.01 to 0.016 mg l^{-1}. Each dose tested was represented by three replicates for each run, three control flasks free from diuron, represented the normal order of growth. Algal growth was determined by measuring chlorophylls (a) and (b) contents. Chl(a) and (b) contents were calculated according to American Public Health Association [15]. Also total carotenoids concentrations were determined according to Timothy et al. [16].

Growth rate was determined by the following equation:

$$\mu/d = (\ln \text{Chl (a) at T2} - \ln \text{Chl (a) at T1})/(T2–T1)$$

where T was time in day.

At maximum growth of algae, algal mass of each treatment was collected and used for carbohydrate determination according to Dubois et al. [17], protein was determined according to Quian and Wang [18], and free amino acids were determined calorimetrically as given by Lee and Takahashi [19] using glycine as standard. Results were subjected to statistical analysis by Duncan's multiple range test [20]. Means followed the same alphabetical in every column are not significant at 5% level. EC_{50} was also determined by Probit test according to Finney [21]. ATP measurement took place using Backman liquid scintillation counter model L.S. 700 as given by American Public Health Association [15].

Photosynthetic activity was measured with respect to the effect of diuron. For each treatment 60 ml bottles were filled with algal suspension plus various concentrations of diuron. The bottles were exposed to continuous illumination for 2 h in optimum growth conditions for radioactive treatment. At the end of the incubation period 10 μCi of labeled sodium bicarbonate solution were injected in each algal suspension and then it was left for 2 h at optimum conditions. The algal culture was transferred for 30 min in the dark followed by quantitative separation of cells by centrifugation and the cells were extracted by 80% hot ethanol. Duplicate samples from each treatment were taken to evaluate the $^{14}CO_2$ uptake. The activity of ^{14}C in samples was evaluated using liquid scintillation counting technique [22].

The rate of uptake of diuron from solutions was assessed by Meites [23] in term of the rate of first order reaction applying the equation:

$$\text{Log } C - \text{Log} C_0 = kt/2.303$$

where C is the concentration at time t, C_0 is the initial concentration at t=0 and k is the specific reaction rate constant.

3. Result and discussion

3.1. Changes in photosynthetic pigments

The concentration level of Chl(a) content of *Ankistrodesmus acicularis* treated with different doses of diuron are given in Table 1. Chl(a) of control culture progressively increased up to the end of seventh day of incubation, with a maximum value of 609.9 μg l^{-1}. In case of the algae treated with 0.01 mg l^{-1} diuron, Chl(a) content significantly decreased from the control, though it increased by the end of 10th day and its value exceeded that of the control. However, in all other diuron treatments, progressive significant decrease in Chl(a) content was recorded as the diuron concentration was increased.

Change in Chl(b) content tends to be similar to that obtained by Chl(a) content. Variation in Chl(b) values were significant compared to the control culture. Treatment with 0.01 mg l^{-1} diuron showed higher significant level compared to all other treatments (only on day 10) (Table 2).

Results of carotenoids content of algae are given in Table 3. In case of the 0.01 and 0.02 mg l^{-1} treatments the carotenoids content progressively increased and their level exceeded that of the control culture by the end of the run.

Increase in the concentration of photosynthetic pigments in presence of low concentration of organic herbicide was also recorded by other investigators [3,24,25].

Table 1
Changes in chlorophyll (a) content[1] (μg l^{-1}) of *Ankistrodesmus acicularis* in presence of diuron[2]

Concentration of diuron (mg l^{-1})	Time (d^{-1})						
	First	Second	Third	Fifth	Sixth	Seventh	Tenth
0.0	47.4[b]	97.8[f]	182.8[f]	348.1[f]	459.8[f]	609.9[f]	599.5[d]
0.01	40.2[a]	74.2[e]	116.7[e]	289.9[e]	400.5[e]	459.3[e]	647.8[e]
0.02	35.4[a]	63.5[d]	90.9[d]	186.9[d]	256.9[d]	311.7[d]	567.1[d]
0.04	32.0[a]	51.4[e]	67.3[e]	137.4[e]	191.3[c]	214.7[c]	354.2[c]
0.08	30.1[a]	43.9[b]	43.4[b]	84.3	105.7[b]	143.8[b]	187.5[b]
0.16	25.3[a]	28.9[a]	28.5[a]	49.6[a]	48.05[a]	66.8[a]	78.8[a]

Initial Chl(a) content = 28.3 μg l^{-1}.
[1]Average of three replicates.
[2]Mean followed by the same alphabetical in every column are not significant at 5% level, according to Duncan's multiple range tests.

Table 2
Changes in chlorophyll (b) content[1] (μg l^{-1}) of *Ankistrodesmus acicularis* in presence of diuron[2]

Concentration of diuron (mg l^{-1})	Time (d^{-1})					
	First	Second	Third	Fifth	Seventh	Tenth
0.0	12.2[b]	24.4[e]	51.6[f]	95.4[f]	124.4[f]	129.5[e]
0.01	11.6[b]	18.4[d]	36.3[e]	87.1[e]	116.9[e]	143.8[f]
0.02	11.4[b]	14.9[c]	25.8[d]	41.6[d]	756.9[d]	114.6[d]
0.04	8.2[a]	13.2[c]	20.2[c]	29.7[c]	49.2[c]	101.9[c]
0.08	7.2[a]	9.9[b]	15.4[b]	23.9[b]	27.4[b]	40.1[b]
0.16	6.12[a]	7.56[a]	9.8[a]	11.9[a]	16.9[a]	16.3[a]

Initial Chl(a) content = 4.16 μg l^{-1}.
[1]Average of three replicates.
[2]Mean followed by the same alphabetical in every column are not significant at 5% level, according to Duncan's multiple range test.

Table 3
Changes in carotenoids content[1] (μg l^{-1}) of *Ankistrodesmus acicularis* in presence of diuron[2]

Concentration of diuron (mg l^{-1})	Time (d^{-1})					
	First	Second	Third	Fifth	Seventh	Tenth
0.0	23.6[c]	55.9[f]	102.8[f]	207.4[f]	373.9[e]	350.7[c]
0.01	18.2[b]	43.6[e]	77.8[e]	170.3[e]	322.2[d]	467.6[e]
0.02	16.8[b]	38.2[d]	59.5[d]	106.8[d]	198.7[c]	402.8[d]
0.04	14.4[a]	26.8[c]	40.9[c]	73.9[c]	143.9[b]	190.2[c]
0.08	14.1[a]	20.7[b]	28.7[b]	52.1[b]	87.4[a]	107.6[a]
0.16	13.8[a]	10.9[a]	20.1[a]	31.5[a]	47.9[a]	60.4[a]

Initial Chl(a) content = 14.8 μg l^{-1}.
[1]Average of three replicates.
[2]Mean followed by the same alphabetical in every column are not significant at 5% level, according to Duncan's multiple range test.

Previous studies on the effects of diuron at the community level have described decreases in algal community photosynthesis [26]. According to Ashton and Graft [27] herbicides which inhibit photosynthesis interfere with the light reaction and carbon dioxide fixation is subsequently reduced. Compounds which decrease Chl(a) content of algae, will decrease their light harvesting efficiency leading to decrease in carbohydrate content of algal cells which coincide with the obtainable results. Previous study by Fayez and Abd-ElFattah [9]

reported that the growth of *Chlorella vulgaris* was seriously affected even with lowest diuron dose (0.1 μM). Photosynthetic pigment contents (Chl-a, Chl-b and carotenoid) of diuron-treated algae, was more sharply decreased as compared to control.

3.2. Variation in growth rate and EC_{50} values

Growth rate of *Ankistrodesmus* derived from Chl(a) content reflected the daily changes in growth rate (Table 4). Maximum growth rates (μ max) of the control and most of diuron treatments were attained by the end of the second day of incubation. However, maximum growth rate of algae treated with 0.16 mg l^{-1} diuron was recorded after 6–7 d of incubation. The correlation between the applied doses of diuron and growth rates was not significant in several periods. This may be linked with the ability of algal cells to adapt.

The values of EC_{50}, which exert 50% inhibition compared with control, were calculated by probits analysis. EC_{50} for the treated cultures varied with respect to the exposure time and are represented by the values 20, 26 and 25 μg l^{-1} diuron at the third, fifth and seventh days of the experimental run, respectively. The values of EC_{50} at the fifth and seventh days approached each other indicating the ability of algal cells to resist the toxic effect of diuron at these concentrations. The current finding of EC_{50} matches the data registered in the US EPA data base (i.e., Gatidou and Thomaidis [28], EC_{50} = 27 μg l^{-1} *Navicula forcipata* growth.

3.3. Changes in carbohydrate, protein and free amino acids contents

Build up of carbohydrate, protein and amino acids by plants and algae are a good indicator of normal photosynthesis activity and metabolic functions. The stress imposed by diuron on algal cells was reflected on the carbohydrate, protein and free amino acids content (Table 5).

In general, the previously given parameters progressively decreased as the applied diuron doses were increased. Statistical analysis of results indicated that carbohydrate, protein and free amino acids contents of algal control culture were significantly higher compared to the treated ones. Previous study by Fayez and

Table 4
Growth rates of *Ankistrodesmus acicularis* treated with diuron

Concentration of diuron (mg l^{-1})	Growth rates over time (d)					
	0–1	1–2	2–3	3–5	5–6	6–7
0.00	0.516	0.724[1]	0.625	0.322	0.278	0.283
0.01	0.351	0.613[1]	0.453	0.455	0.323	0.137
0.02	0.224	0.584[1]	0.359	0.360	0.318	0.193
0.04	0.123	0.474[1]	0.269	0.357	0.331	0.115
0.08	0.062	0.377[1]	−0.011	0.332	0.226	0.308
0.16	−0.112	0.133	−0.014	0.277	−0.032	0.329[a]
r^2	0.82[(s)]	0.97[(s)]	0.77[(s)]	0.43[(N.s)]	0.86[(s)]	0.36[(N.s)]

[1]Maximum growth rates (measured as Chl(a) content).
r^2 = Correlation coefficient of growth rates vs. treatment concentration.
(s) = Significant at 5% level.
(N.s) = Not significant at 5% level.

Table 5
Effect of diuron on carbohydrate, protein and amino acids of *Ankistrodesmus acicularis* at maximum growth

Concentration of diuron (mg l^{-1})	Parameters		
	Carbohydrate (mg g^{-1} d^{-1} wt^{-1})	Protein (mg g^{-1}.d.wt)	Amino acids (mg g^{-1} d^{-1} wt^{-1})
0.0	144.5[f]	4.5[f]	5.65[f]
0.01	115.0[e]	4.1[e]	4.2[e]
0.02	96.3[d]	3.5[d]	3.55[d]
0.04	79.2[e]	2.45[c]	2.95[c]
0.08	55.6[b]	1.38[b]	2.4[b]
0.16	28.4[a]	0.57[a]	1.7[a]

Mean followed by the same alphabetical in every column are not significant at 5% level, according to Duncan's multiple range test.

Abd-ElFattah [9] reported that Total amino acids, carbohydrate and protein contents of algal suspension decreased significantly in response to diuron treatments. Similarly, Ashton and Grafts [27] reported that sub-lethal concentration of cyanazine herbicide, dosed to *Anabaena flos aquae* and *Scenedesmus obliquus* induced general inhibition on growth, photosynthetic and reduction on protein and amino acids synthesis, on increasing the herbicide concentration.

3.4. Change in $^{14}CO_2$- photoassimilation and ATP content

Treatment of the studied algae with increasing doses of diuron leads to corresponding decrease in photosynthetic activity compared to the control (Table 6). That trend matched the effect of diuron treatments on carbohydrate, protein and amino acids content of the algal cells.

Compounds which inhibit photosynthesis (herbicides) interfere with light reaction [27]. Carbon dioxide fixation was subsequently reduced. Consequently, herbicides which decrease Chl(a) content of algae will decrease light harvesting efficiency leading to decrease in carbohydrate content of algae.

Meanwhile, sharp decrease was recorded in ATP content when diuron was dosed in the nutrient solution with an increasing concentration (Table 6). Percentage reduction increased from 71% to 95% as the herbicide concentration was increased from 0.02 to 0.08 mg l^{-1}. Change in ATP content of the algae coincide with the general trend exhibited by the growth rate and EC_{50} values.

3.5. Effect of diuron on nitrate uptake

Nitrate concentrations in algal cultures were measured at various time intervals and the results are given in Fig. 1. Nitrate concentration in the aqueous media progressively decreased as all exposure periods were extended. In general, the rate of nitrate uptake decreased as the concentration of diuron was increased. However, algal culture treated with 0.01 mg l^{-1} diuron showed high percentage uptake that exceeded the control by

Table 6
Effect of diuron on $^{14}CO_2$ Photo-assimilation and ATP content of *Ankistrodesmus acicularis*

Concentration of diuron (mg l^{-1})	$^{14}CO_2$ photo-assimilation	ATP % of control
0.00	100	100
0.01	76.7	97
0.02	72	27
0.08	61.7	5

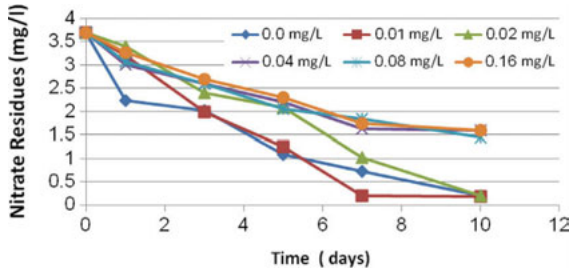

Fig. 1. Effect of diuron on uptake of nitrate by *Ankistrodesmus acicularis*.

the end of the run (10 d). Nitrogenous compounds are essential nutrient for growth of green algae which is not able to fix nitrogen as in the case of blue green algae. Hence decrease in nitrogenous compound will interfere with metabolite activities based on nitrate. Decrease in uptake of nitrate coincides with the progressive decrease in protein content and other growth parameters as diuron concentration in algal cultures was increased.

3.6. Uptake of diuron by ankistrodesmus cells

Available results revealed that the concentration of diuron in the culture media decrease as the period of algae exposure was extended. High uptake by algal cells was attained in the presence of the low diuron concentration (0.01 mg l^{-1}). However, the rate of uptake by algal cells decreased in presences of the high concentration level (0.08 mg l^{-1}) of diuron (Table 7). Such a result coincides with that obtained by Abd El-Aty and El Dib [25]. Application of the first order rate kinetics to the uptake of diuron by algal cells, yielded straight lines as Log residual concentration was plotted against contact time (Fig. 2). The specific rate constant (K) progressively decreased from 0.275 to 0.05 d^{-1} as the concentration of diuron was increased from 0.01 to 0.16 mg l^{-1}. Meanwhile, the high growth rate attainted at low herbicide concentration coincides with the shorter half-life time to which algal cells will be exposed to the inhibitory effects of the herbicide (Table 7).

Table 7
Kinetic data for the uptake of diuron by *Ankistrodesmus acicularis*

Concentration of diuron (mg l^{-1})	Rate constant (K d^{-1})	$t_{0.5}$ (d)
0.01	0.275	2.52
0.02	0.20	3.47
0.04	0.12	5.80
0.08	0.07	9.80
0.16	0.05	13.80

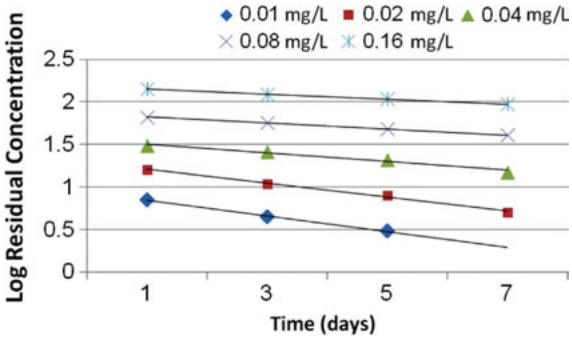

Fig. 2. Kinetic plot for absorption of diuron by *Ankistrodesmus acicularis*.

The increased uptake levels of herbicides by algae at low concentration may be attributed to the corresponding increase in growth rate and algal biomass.

We conclude that changes in growth, pigment levels and metabolite constituents in *Ankistrodesmus acicularis* can be used effectively as tools to evaluate the toxicity effect of other photosynthetic inhibitors.

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