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Effect of selected herbicides on growth and lipid content of *Nannochloris oculata*

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ABSTRACT

Nannochloris oculata (Droop) D.J. Hibberd has attracted attention as a potential source of biofuels because of high oil content and rapid growth rate. The effect of selected herbicides on algal growth and total lipid content was examined. Twenty-two herbicides representing 14 modes of action were assayed. Photosystem II inhibitor diuron was the most toxic herbicide to algal growth. At low concentrations, sethoxydim was not toxic to algae but decreased its lipid content significantly. Photosystem I inhibitor diquat at low concentration did not affect algal growth, but algal growth inhibition increased with increasing concentration. Diquat at 0.1 and 1 mg L^{-1} decreased lipid content to 8.66 and 8.53%, respectively. At 5 mg L^{-1}, glyphosate stimulated algal growth but decreased lipid content to 5.60% of dry weight. *S*-metolachlor had a significant effect on both algal biomass and lipid content. *S*-metolachlor at 0.1 mg L^{-1} decreased algal biomass to 75% of untreated control and decreased lipid content from 13.33 to 6.21% of dry weight. Fluridone at 0.1 mg L^{-1} decreased algal biomass to 84% of untreated and completely killed algae when the concentration increased to 1 mg L^{-1}. Simazine at 0.1 mg L^{-1} had no significant effect on algal growth but decreased lipid content from 13.33 to 5.15% of dry weight. Pendimethalin and dinoterb had no significant effect on algal growth but reduced lipid content to 6.19 and 6.03%, respectively. The use of algae as a source of fuel will undoubtedly require the use of outdoor, open-pond systems. These results will be useful for developing management systems to control invasive algal species in outdoor, open-pond systems, ultimately keeping the oil-producing algae pure.

Key words: accelerated solvent extraction, biofuel, dinoterb, diquat, diuron, fluridone, glyphosate, pendimethalin, sethoxydim, simazine, *S*-metolachlor.

INTRODUCTION

Microalgae have been suggested as good candidates for fuel production because of their higher photosynthetic efficiency, higher biomass production, faster growth compared with other energy crops, and ability to be cultivated in nonarable land (Banerjee et al. 2002, Miao and Wu 2006, Li et al. 2007, Jones et al. 2012). Successful use of these organisms as alternate sources of energy depend on their growth rate, oil productivity, and fuel efficiency. *Nannochloris oculata* (Droop) D.J. Hibberd is a species of small, green, marine microalgae that belongs to the family of Eustigmatophyceae. *Nannochloris oculata* has attracted attention for potential biofuel production because of its high oil content and rapid growth rate (Gouveia and Oliveira 2009). The oil content of *N. oculata* may vary between 8 and 50% (Brown 1991, Chiu et al. 2009, Converti et al. 2009). However, no detailed data are available on practices that can affect the production of valuable compounds from *N. oculata*.

Herbicides are chemicals commonly used to control weeds in agricultural activities and are often discharged into aquatic environments through surface runoff and atmospheric deposition. Such discharge can lead to contaminated aquatic environments, which are hazardous to resident organisms (Fargasova 1994). Therefore, it is important to assess the adverse effect of herbicides on nontarget organisms in aquatic ecosystems (Peterson et al. 1994). Algae have frequently been the subject of these investigations because of their importance as primary producers in freshwater systems (Jurgensen and Hoagland 1990). Microalgae are quite sensitive to herbicides because they share many characteristics with higher plants. However, the sensitivity of algae to herbicides differs depending on the species and specific herbicide (Mayasich et al. 1986).

The use of *N. oculata* as a source of fuel will undoubtedly require the use of an outdoor, open-pond system, where it will be imperative to control the growth of unwanted algae. Herbicides may be ideal for controlling the growth of such invasive algal species while maintaining target species growth. Although there are many studies about the effect of herbicides on algal growth, biochemical composition, metabolic activities, and ultrastructure morphology (El-Sheekh et al. 1994, Caux et al. 1996, Kobbia et al. 2001, Rioboo et al. 2002, Gonzalez-Barreiro et al. 2006, Liu and Xiong 2009, Vendrell et al. 2009), little is known about the effect of herbicides on *N. oculata*. Therefore, it is important to determine the effect of herbicides on *N. oculata* growth and lipid production for potential use for controlling the growth of invasive algal species in the future. The objective of this work was to examine the effect of selected herbicides on algal growth and lipid content of *N. oculata*.

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MATERIALS AND METHODS

Algal culture

Nannochloris oculata (UTEX: LB 1998) was acquired from Lou Brown at the Texas Agrilife Research Station (Pecos, TX). *Nannochloris oculata* samples were inoculated in autoclaved, modified Erdschreiber's growth medium (Luedecke 2011). The medium contained the following ingredients (per liter): 0.75 g potassium chloride, 0.65 g calcium chloride dihydrate, 7.62 g magnesium sulfate heptahydrate, 0.85 g sodium nitrate, 0.034 g boric acid, 0.04 g sodium dihydrogen phosphate monohydrate, 21 g Tru-Soft (sodium chloride, 0.001%; sodium sulfate, 0.04%; and moisture 0.04%), 2 g sodium hydrogen carbonate, and 1 ml of trace metals. The trace elemental solution had the following composition: 3.15 g L^{-1} iron(III) chloride hexahydrate, 0.18 g L^{-1} manganese(II) chloride tetrahydrate, 0.022 g L^{-1} zinc sulfate heptahydrate, 0.01 g L^{-1} copper(II) sulfate pentahydrate, and 0.01 g L^{-1} cobalt(II) chloride hexahydrate. The algae cultures were maintain at a temperature of 25 C and a 14/10-h light/dark cycle. Filter-sterilized air was bubbled through the cultures.

Herbicides

Twenty-two herbicides[1,2] representing 14 modes of action were assayed (Table 1). The purities of herbicide standards ranged from 95 to 100%. Stock solutions were prepared by dissolving standards in deionized, distilled water (H_2O). Herbicides were divided into three groups according to solubility. For group A, herbicide solubilities were $>$ 100 mg L^{-1} in water at room temperature. The concentration of standard solutions of group A was 100 mg L^{-1}. The concentrations of herbicides used to treat algae were 0.1, 1, and 5 mg L^{-1}. For group B, solubilities ranged from 5 to 50 mg L^{-1} in water at room temperature. The concentrations of standard solutions were 5 and 10 mg L^{-1}. The concentrations of herbicides used to treat algae were 0.1 and 1 mg L^{-1}. For group C, solubilities were $<$ 5 mg L^{-1} in water at room temperature. The concentrations of standard solutions were 0.2 and 1 mg L^{-1}. The concentration of herbicides used to treat algae was 0.1 mg L^{-1}.

Experimental process

Algal culture in the logarithmic phase of growth was inoculated in a 200-ml autoclave modified Erdschreiber's growth medium as described above. The algae were cultured at 25 C with fluorescent lights and a 14/10-h light/dark cycle and were shaken every 2 h. The herbicide solutions were added to the growth medium to give a final concentration of 0.1, 1, and 5 mg L^{-1}. Duplicate assays without herbicide were used as controls. The experiment used a completely randomized design with three replications. The entire experiment was repeated, and the results of two repeat experiments in triplicates are shown. The growth rate was determined by measuring the absorbance at 660 nm with a Spectronic 21 ultraviolet-visible spectrophotometer[3] at 2-d intervals for 40 d (Hegde 2007).

Biomass estimation

Algal dry weights were used to determine the amount of biomass in the culture. The dry procedure was compared between oven and hood. The weight kept increasing after being removed from oven because the filter and the algae adsorbed the humidity from the air. Algae drying under a fume hood for 12, 24, 36, 48, and 72 h were observed, and the weight of the filter and algae stabilized after 48 h of fume-hood drying. The biomass was rinsed with sterilized, deionized H_2O, and the filters were dried in a fume hood at room temperature for 48 h and were then weighed to determine the dry weight of the algal biomass (Deng et al. 2012). The algal dry weights were determined by weighing the filters before and after the sampled algae had been filtered and dried (Davis 2011).

Lipid extraction

Total lipid was extracted with an accelerated solvent extraction (ASE) method (Macnaughton et al. 1997, Zhuang et al. 2004, Deng et al. 2012). An automated Dionex-200 ASE system[4] was used for all extractions. Algal samples (approximately 1 to 2 g dry weight) were mixed with 10 g Ottawa sand (20 to 30 mesh)[5] before being loaded into 11-ml sample cells to fill extra space in the cell. Extraction cell pressure was maintained at 10.3 MPa during the experiments. Total lipids were extracted using chloroform/methanol (2 : 1) (Bligh and Dyer 1959) as the extraction solvent at a temperature of 50 C for one static cycle. The static cycle lasted for 5 min; during which, cell contents were held at the desired temperature and pressure. Then, the cell was flushed with fresh solvent equal to 60% of the cell volume, which was purged from the cell by a stream of nitrogen gas (N_2) for 60 s and expelled into a 40-ml collection vial. Crude extracts were concentrated to near dryness by a stream of N_2 gas. The total lipid content was determined gravimetrically after the chloroform phase was removed (Toussaint et al. 2002).

Statistical analysis

Data were subjected to ANOVA using SAS software[6] to evaluate the significance of any interactions and to determine treatment means. The summary procedure in SAS was used to calculate the standard deviation of means (Tables 3–5 and 7–9). The different letters for each treatment in each column and row indicate significant differences ($P < 0.05$). Means in the same column followed by the same uppercase letter are not significantly different at the 5% level as determined by Tukey's studentized range test; means in the same row followed by the same lowercase letter are not significantly different at the 5% level as determined by Tukey's studentized range test.

RESULTS AND DISCUSSION

Effect of herbicides on algal growth

Growth of algae was monitored spectrophotometrically by measuring the optical density as well as the dry weight

Table 1. Mode of action and common names of herbicides used to treat *Nannochloris oculata*.

HRAC Code[1]	WSSA Code[2]	Mode of Action[3]	Common Names
A	1	ACCase inhibitor	Sethoxydim
A	1	ACCase inhibitor	Cyhalofop
B	2	ALS or AHAS inhibitor	Bensulfuron-methyl
B	2	ALS or AHAS inhibitor	Halosulfuron-methyl
C_1	5	Photosystem II inhibitor	Simazine
C_2	7	Photosystem II inhibitor	Diuron
D	22	Photosystem I inhibitor	Diquat
E	14	PPG or Protox inhibitor	Carfentrazone-ethyl
F_1	12	Carotenoid biosynthesis inhibitor	Fluridone
F_2	28	Carotenoid biosynthesis inhibitor	Mesotrione
F_3	13	Carotenoid biosynthesis inhibitor	Clomazone
G	9	EPSP synthase inhibitor	Glyphosate
H	10	Glutamine synthetase inhibitor	Glufosinate
I	18	Dihydropteroate synthetase inhibitor	Asulam
K_1	3	Mitosis inhibitor	Pendimethalin
K_3	15	Mitosis inhibitor	*S*-metolachlor
L	21	Cellulose inhibitor	Isoxaben
L	20	Cellulose inhibitor	Dichlobenil
M	24	Oxidative phosphorylation uncoupler	Dinoterb
N	8	Fatty acid and lipid biosynthesis inhibitor	Thiobencarb
N	16	Fatty acid and lipid biosynthesis inhibitor	Ethofumesate
O	4	Synthetic auxin	2,4-D

[1]HRAC Code: Herbicide Resistance Action Committee designation of herbicides.
[2]WSSA Code: Mode of Action Code designated by the Weed Science Society of America (Senseman, 2007).
[3]ACCase = acetyl coenzyme A carboxylase; AHAS = acetohydroxy acid synthase; ALS acetolactate synthase; EPSP = 5-enolpyruvylshikimate-3-phosphate; PPG = protoporphyrinogen.

(Abou-Wally et al. 1991, El-Sheekh et al. 1994). Algal growth curves that were greatly affected by some herbicides are shown in Figure 1. The photosystem II inhibitor (diuron) was the most toxic herbicide for algal growth. Algae were essentially killed by 0.1 mg L^{-1} diuron after 20 d of exposure. Also, fluridone and stereoisomeric acetamide provided strong inhibition on algal growth, and inhibition increased with increasing concentration. (diquat) and (simazine) at 0.1 mg L^{-1} demonstrated increases of algal growth slightly, whereas diquat at 5 mg L^{-1} and simazine at 1 mg L^{-1} inhibited algal growth significantly. At low concentrations, (glyphosate) inhibited algal growth, whereas 5 mg L^{-1} of glyphosate increased algal growth.

Inhibition effects were also evaluated by measuring dry weight of biomass at algal harvest after 40 d of herbicide interaction. The interaction between herbicide and concentration was significant for herbicides in groups A and B (Table 2). The biomass of treated algae exposed to selected herbicides at concentrations of 0.1, 1, and 5 mg L^{-1} in group A is shown in Table 3. At 0.1 and 1 mg L^{-1}, (bensulfuron-methyl) inhibited algal growth but increased algal biomass at a concentration of 5 mg L^{-1}. Biomass yield was 87, 98, and 113% of control, respectively, when treated with 0.1, 1, and 5 mg L^{-1} of bensulfuron-methyl. It is unknown why bensulfuron-methyl increased biomass at the 5 mg L^{-1} rate. One theory is that bensulfuron-methyl is broken down in the water and used as an energy source by the algae. Bensulfuron-methyl can be degraded rapidly in natural water under sunlight to methyl 2-(sulfomethyl) benzoate and (4,6-dimethoxypyrimidin2-yl)urea, with a DT_{50} of 3 to 4 days (Crosby 1989). However, further evaluation of this theory would be required to validate whether bensulfuron-methyl breakdown products could be used as an energy source. At 0.1, 1, and 5 mg L^{-1}, (halosulfuron-methyl) decreased algal biomass to 91, 87, and 77% of control, respectively. Halosulfuron-methyl inhibits branched chain amino acid production by inhibition of the enzyme acetolactate (ALS) synthase or acetohydroxy (AHAS) acid synthase (LaRossa and Schloss 1984, Senseman 2007). Diquat at 0.1 mg L^{-1} did not affect algal growth, whereas higher concentration inhibited algal growth significantly, and inhibition increased with increasing concentration. Holst et al. (1982) found that diquat at 0.1 mg L^{-1} partially inhibited *Azolla* Lam. sp. growth and, at 1 mg L^{-1}, caused a total inhibition after 10 d. Peterson et al. (1994) compared nine algal species exposed to 0.73 mg L^{-1} of diquat and found 53 to 69% inhibition of carbon 14 (^{14}C) uptake in two green algal species, 99 to 100% inhibition in two diatom species, and 100% inhibition in five species of cyanobacteria tested. Glyphosate at 0.1 and 1 mg L^{-1} inhibited algal growth but increased algal biomass to 111% at 5 mg L^{-1}. Glyphosate can be used as a sole source of phosphorus for algal growth (Lipok et al. 2007). Glyphosate can alter the composition of natural aquatic communities, potentially tipping the ecological balance and giving rise to harmful algal blooms. It can have profound effects on microorganisms, plankton, algae, and Amphibia at low concentrations: one study showed a 40% increase in algae. (Watts 2009). Glyphosate and 2-amino-4-(hydroxymethylphosphinyl)buta-

Table 2. ANOVA for biomass yield at different herbicide groups based on herbicide solubility.

Herbicide group[1]	Source	df[2]	ANOVA SS[2]	Mean Square	*F* Value	Pr > *F*
A	Herbicide	12	3.69	0.31	46.62	< 0.0001
	Concentration	2	0.17	0.09	12.97	< 0.0001
	Herbicide × concentration	24	2.54	0.11	16.04	< 0.0001
B	Herbicide	4	5.07	1.27	915.42	< 0.0001
	Concentration	1	0.95	0.95	683.93	< 0.0001
	Herbicide × concentration	4	1.83	0.46	330.72	< 0.0001
C	Herbicide	3	0.26	0.0089	1.71	0.24

[1]Group A: The solubility of herbicides is > 100 mg L^{-1} in water at room temperature. The concentration of standard solutions of Group A was 100 mg L^{-1}. The concentrations of herbicides used to treat algae were 0.1, 1, and 5 mg L^{-1}. Group B: The solubility of herbicides is 5 to 50 mg L^{-1} in water at room temperature. The concentrations of standard solutions were 5 and 10 mg L^{-1}. The concentrations of herbicides used to treat algae were 0.1 and 1 mg L^{-1}. Group C: The solubility of herbicide is < 5 mg L^{-1} in water at room temperature. The concentrations of standard solutions were 0.2 and 1 mg L^{-1}. The concentration of herbicides used to treat algae was 0.1 mg L^{-1}.
[2]df = degrees of freedom; SS = sum of squares.

Table 3. Algal biomass from *Nannochloris oculata* as affected by selected herbicides with water solubilities > 100 mg L^{-1}.[1]

Herbicides	Biomass[2] (% of Control) 0.1 mg L^{-1}	1 mg L^{-1}	5 mg L^{-1}
Sethoxydim	93 Aa	90 Aa	88 Aa
Bensulfuron-methyl	87 Ab	98 Ab	113 Aa
Halosulfuron-methyl	91 Aa	87 Aa	77 Ab
Diquat	104 Aa	89 Aa	25 Bb
Mesotrione	98 Aa	96 Aa	95 Aa
Glyphosate	85 Ab	95 Ab	111 Aa
Glufosinate	82 Ab	99 Aab	106 Aa
Asulam	88 Aa	89 Aa	76 Ab
Ethofumesate	92 Aa	91 Aa	90 Aa
2,4-D	101 Aa	85 Ab	81 Ab
Clomazone	84 Aa	88 Aa	88 Aa
Carfentrazone-ethyl	68 Aa	74 Aa	73 Aa
S-metolachlor	75 Aa	0 Bb	0 Bb

[1]The concentrations of herbicides used to treat algae were 0.1, 1, and 5 mg L^{-1}.
[2]Means in the same column followed by the same uppercase letter are not significantly different at the 5% level as determined by Tukey's studentized range test. Means in the same row followed by the same lowercase letter are not significantly different at the 5% level as determined by Tukey's studentized range test.

noic acid (glufosinate) had similar effects on algal growth. *S*-metolachlor at 1 and 5 mg L^{-1} had a significant effect on algal growth, and no algal biomass was obtained because algae were killed completely. *S*-metolachlor is a chloroacetanilide herbicide inhibiting the biosynthesis of several plant components, such as fatty acids, lipids, proteins, isoprenoids, and flavonoids (Böger et al. 2000, Senseman 2007). Deng et al. (2012) found that *S*-metolachlor at 0.1, 1, and 5 mg L^{-1} concentrations decreased *Botryococcus braunii* Kützing biomass 18, 36, and 44%, respectively. The same herbicide had a different effect on these algae, which means that it could be used for controlling the growth of invasive algal species while maintaining target species growth.

Biomass, as affected by selected herbicides at 0.1 and 1 mg L^{-1} in group B, is shown in Table 4. Of the tested herbicides, diuron was the most toxic herbicide to *N. oculata*. No biomass was obtained because algae were completely killed by diuron. Diuron has adverse effects on aquatic plants at low concentrations and has been used to prevent algal growth in home aquariums and ponds (Cox 2003). The photosynthetic inhibitor herbicides may cause plant death by the production of high-energy free radicals, which

Table 4. Algal biomass of *Nannochloris oculata* as affected by selected herbicides with water solubilities between 5 and 50 mg L^{-1} mg L^{-1}.[1]

Herbicides	Biomass[2] (% of Control) 0.1 mg L^{-1}	1 mg L^{-1}
Diuron	0 Ca	0 Ca
Simazine	109 Aa	0 Cb
Fluridone	84 Ba	0 Cb
Thiobencarb	109 Aa	94 Aa
Dichlobenil	102 Aa	105 Aa

[1]The concentrations of herbicides used to treat algae were 0.1 and 1 mg L^{-1}.
[2]Means in the same column followed by the same uppercase letter are not significantly different at the 5% level as determined by Tukey's studentized range test. Means in the same row followed by the same lowercase letter are not significantly different at the 5% level as determined by Tukey's studentized range test.

Table 5. Algal biomass of *Nannochloris oculata* as affected by selected herbicides with water solubilities < 5 mg L^{-1}.[1]

Herbicides	Biomass[2] (% of Control) 0.1 mg L^{-1}
Cyhalofop	89 A
Pendimethalin	104 A
Dinoterb	99 A
Isoxaben	119 A

[1]The concentration of herbicides used to treat algae was 0.1 mg L^{-1}.
[2]Means in the same column followed by the same uppercase letter are not significantly different at the 5% level as determined by Tukey's studentized range test.

destroy cell membranes (Dodge 1977). Maule and Wright (1984) found that diuron was toxic to seven different green algal species. Our previous research found that the biomass of *B. braunii* was reduced to 31 and 25% of the control when treated with 0.1 and 1 mg L^{-1} diuron (Deng et al. 2012). Simazine at 0.1 mg L^{-1} did not affect algal growth but completely killed *N. oculata* at 1 mg L^{-1}. Our previous study showed that simazine at 0.1 and 1 mg L^{-1} did not affect *B. braunii* (Deng et al. 2012). This result would be useful for purity of algae in an outdoor, open-pond system. According to available literature, simazine is effective at controlling unicellular and attached filamentous algae at a concentration of 0.5 to 1.0 mg L^{-1}. Based on studies in lakes and ponds, blue-green algae are the most sensitive to treatment by simazine, whereas diatoms and flagellates are the least sensitive. As a group, green algae are only moderately sensitive to simazine at recommended concentrations. Thus, the efficacy of the product depends on the type of algae present (Frank 1991). Fluridone at 0.1 mg L^{-1} decreased algal biomass to 84% of control and completely killed algae when the concentration increased to 1 mg L^{-1}. Deng et al. (2012) found that 0.1 mg L^{-1} fluridone had no significant effect on *B. braunii*, whereas biomass yield of *B. braunii* decreased 59% in 1 mg L^{-1} fluridone. Millie et al. (1990) found that fluridone inhibited the biomass of *Oscillatoria agardhii* (Gomont) Anagnostidis & Komárek, and biomass decreased with increasing fluridone concentration. Fluridone is a selective herbicide that inhibits carotenoid synthesis (Bartel and Watson 1978, Senseman 2007). Treatment of plants, algae, or cyanobacteria with fluridone leads to a decrease in photosynthesis (Lem and Williams 1981), chlorophyll (Vaisberg and Schiff 1976), and ribosome number per plastid and plastid ribosomal RNA synthesis (Bartels and Watson 1978, Reib et al. 1983) and also affects lipid composition (Lem and Williams 1981).

Table 5 shows biomass as affected by selected herbicides at 0.1 mg L^{-1} in group C. No significant difference was found among different herbicides at 0.1 mg L^{-1}.

Effect of herbicides on lipid content

ANOVA for lipid content is shown in Table 6. Interaction effects were significant for herbicide in groups A and B. Lipid content as affected by selected herbicides at 0.1, 1, and 5 mg L^{-1} is shown in Table 7. At low concentrations, (sethoxydim) was not toxic to the algae but decreased lipid content significantly. Zhekisheva et al. (2005) found that the

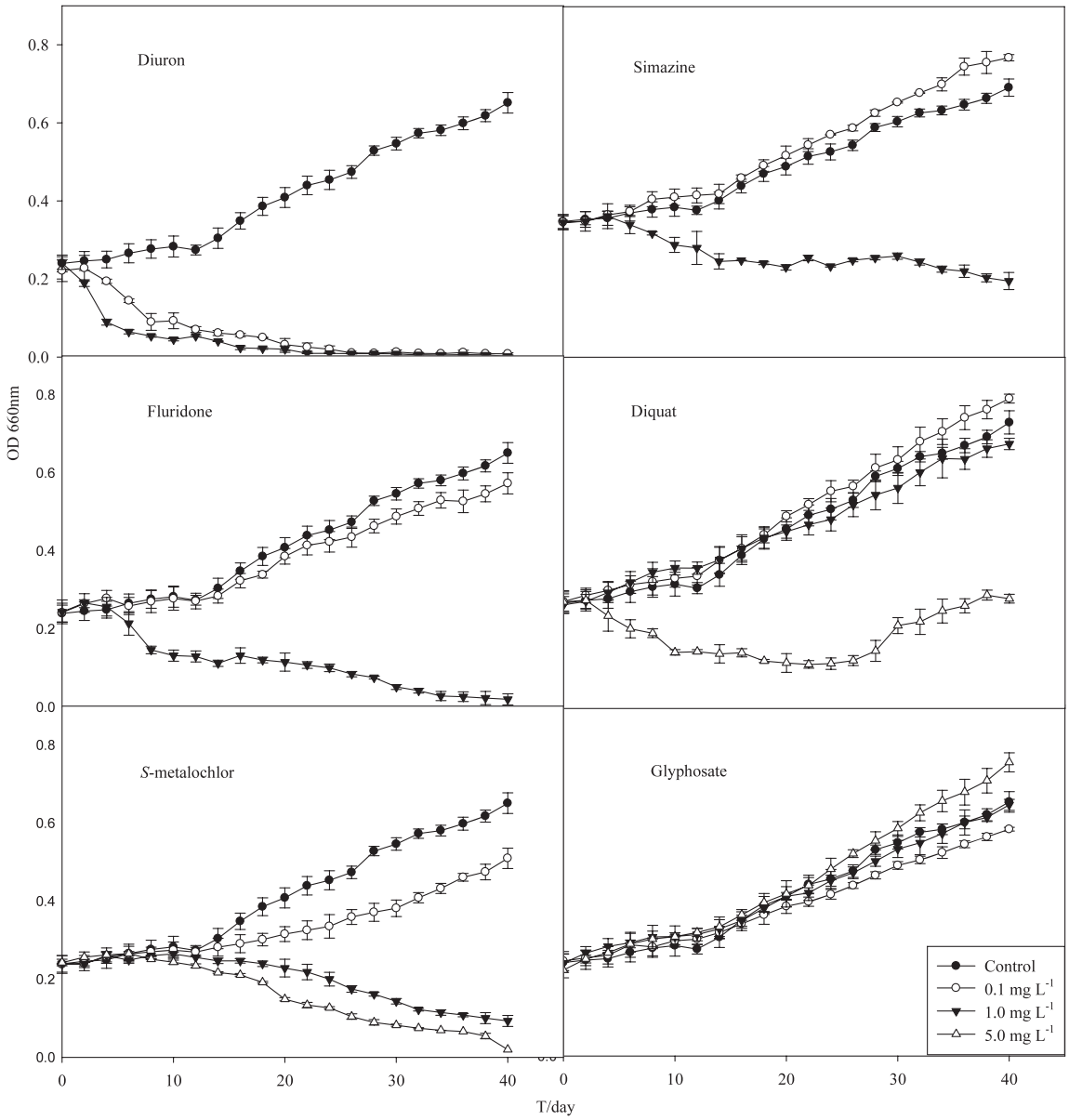

Figure 1. Algal growth curves significantly affected by selected herbicides. The growth rate was determined by measuring the absorbance at 660 nm. Herbicides and concentrations used to treat algae: (A) diuron, 0.1 and 1 mg L^{-1}; (B) fluridone, 0.1 and 1 mg L^{-1}; (C) *S*-metolachlor, 0.1, 1, and 5 mg L^{-1}; (D) simazine, 0.1 and 1 mg L^{-1}; (E) diquat, 0.1, 1, and 5 mg L^{-1}; (F) glyphosate, 0.1, 1, and 5 mg L^{-1}. The error bars showed standard deviations of the means.

lipid biosynthesis inhibitor, sethoxydim inhibited acetyl-coenzyme A carboxylase, significantly decreased *de novo* fatty acid synthesis and, ultimately, inhibited astaxanthin formation in the green algae *Haematococcus pluvialis* Flotow (Chlorophyceae).

Bensulfuron-methyl at 5 mg L^{-1} increased algal growth, but decreased lipid content from 13.33 to 7.37% of dry weight. Halosulfuron-methyl at 0.1, 1, and 5 mg L^{-1} decreased lipid content to 9.09, 6.62, and 6.39%, respectively. Bensulfuron and halosulfuron are herbicides that inhibit branched chain amino acid production by inhibition of the enzyme ALS or AHAS (LaRossa and Schloss 1984, Senseman 2007). Photosystem I inhibitor diquat at 0.1 and 1 mg L^{-1} decreased lipid content to 8.66 and 8.53%, respectively. According to its mode of action, diquat is reduced to its free radical, and reoxidation of the free radical gives rise to production of peroxides (Gregory 1968). Lipids and proteins are attacked and oxidized, resulting in loss of chlorophyll and carotenoids and in leaky membranes, which allow cells and cell organelles to dry and disintegrate rapidly (Devine et al. 1993). Glyphosate at 5 mg L^{-1} stimulated algal growth but decreased lipid content to 5.60%. Ho and Ching (2003) asserted that glyphosate has the potential to disrupt many important enzyme systems that use phosphoenolpyruvate, including energy metabolism and the synthesis of key membrane lipids required in nerve cells. *S*-metolachlor at 0.1 mg L^{-1} decreased algal biomass to 75% of control and decreased lipid content to 6.21% of dry

Table 6. ANOVA for hydrocarbon content from *Nannochloris oculata* after exposure to herbicides of different solubility ranges.

Herbicide Group[1]	Source	df[2]	ANOVA SS[2]	Mean Square	*F* Value	Pr > *F*
A	Herbicide	13	0.064	0.0049	25.46	< 0.0001
	Concentration	2	0.00012	0.000058	0.30	0.74
	Herbicide × concentration	26	0.033	0.0013	6.58	< 0.0001
B	Herbicide	5	0.051	0.010	106.43	< 0.0001
	Concentration	1	0.0072	0.0072	74.49	< 0.0001
	Herbicide × concentration	5	0.012	0.0023	24.36	< 0.0001
C	Herbicide	4	0.0092	0.0023	15.56	0.005

[1]Group A: The solubility of herbicides is > 100 mg L^{-1} in water at room temperature. The concentration of standard solutions of group A was 100 mg L^{-1}. The concentrations of herbicides used to treat algae were 0.1, 1, and 5 mg L^{-1}. Group B: The solubility of herbicides is 5 to 50 mg L^{-1} in water at room temperature. The concentrations of standard solutions were 5 and 10 mg L^{-1}. The concentrations of herbicides used to treat algae were 0.1 and 1 mg L^{-1}. Group C: The solubility of herbicides is < 5 mg L^{-1} in water at room temperature. The concentrations of standard solutions were 0.2 and 1 mg L^{-1}. The concentration of herbicides used to treat algae was 0.1 mg L^{-1}.
[2]df = degrees of freedom; SS = sum of squares.

weight. *S*-metolachlor had a significant effect on both algal growth and algal lipid content. The herbicides like *S*-metolachlor inhibit very long chain fatty acid (VLCFA) synthesis (Böger et al. 2000, Senseman 2007).

Table 8 shows lipid content as affected by herbicides in group B. No lipid was obtained when treated with diuron because algae were controlled by diuron. Simazine at 0.1 mg L^{-1} had no significant effect on algal growth but decreased lipid from 13.33 to 5.15%. Fournadzhieva et al. (1995) studied the influence of simazine on *in vitro* growth and carbohydrate, lipid, pigment, and protein content of *Chlorella vulgaris* Beyerinck [Beijerinck], *Scenedesmus acutus* Meyen (eukaryotes), and *Arthrospira fusiformis* (Voronikhin) Komárek & J.W.G. Lund (prokaryote). It was established that simazine, in concentrations of 0.1 to 1.0 mg L^{-1}, inhibited the photosynthetic activity 25% of the strains studied at 0.1 mg L^{-1} simazine and increasing to a maximum of 100% at concentrations > 1 mg L^{-1} simazine. The degree of growth inhibition depended on the algal species. The protein content of the simazine-treated cells of *Arthrospira fusiformis* decreased, whereas carbohydrates increased, but their lipid content did not change. The amount of γ-linolenic acid decreased 20-fold, compared with the untreated control, in the simazine-treated cells of *Arthrospira fusiformis*. This proved that the photosynthetic membranes and their physiological functions were adversely affected because γ-linoleic acid is important for photosynthetic membrane function (Strandberg and Scott-Fordsmand 2002). At 0.1 and 1 mg L^{-1}, 2,6-dichlorobenzonitrile (dichlobenil) had no significant effect on algal growth but decreased lipid content to 7.42 and 6.95%, respectively. Dichlobenil primarily inhibits cell (cellulose) wall biosynthesis. Cell plate formation is disrupted because cellulose is an important component of the plate (Heim et al. 1990). In addition to inhibition of cellulose synthetase, dichlobenil causes other effects because its breakdown products affect important physiological processes (Cox 1997).

Table 9 shows lipid content as affected by herbicides in group C. Pendimethalin and dinoterb at 0.1 mg L^{-1} had no significant effect on algal growth but reduced lipid content to 6.19 and 6.03%, respectively. Cyhalofof and Isoxaben did not affect algal growth and lipid content.

In summary, the effect of 22 selected herbicides on algal growth and lipid content of *N. oculata* was determined in this study. Of the tested herbicides, diuron was the most toxic herbicide for algal growth. Diquat at high concentration significantly reduced both algal growth and lipid content. Sethoxydim at low concentrations was not toxic to the alga but decreased lipid content significantly. Simazine at 0.1 mg L^{-1} did not affect algal growth but decreased lipid content to 5.15%. *S*-metolachlor had a significant effect on both algal growth and algal lipid content. Fluridone at 0.1 mg L^{-1} decreased algal growth but did not affect algal lipid content. Some low-solubility herbi-

Table 7. Lipid content from cultures of *Nannochloris oculata* as affected by selected herbicides with water solubilities > 100 mg L^{-1}.[1]

	Lipid content[2] (%)		
Herbicides	0.1 mg L^{-1}	1 mg L^{-1}	5 mg L^{-1}
Untreated	13.33 Aa	13.33 ABa	13.33 ABa
Sethoxydim	7.27 Ba	7.77 Da	7.77 BCa
Bensulfuron-methyl	11.53 ABa	10.83 BCDab	7.37 BCb
Halosulfuron-methyl	9.09 ABa	6.62 Db	6.39 Cb
Diquat	8.66 ABa	8.53 CDa	0 Db
Mesotrione	11.23 ABa	11.33 ABCa	14.57 Aa
Glyphosate	11.29 ABb	13.59 Aa	5.60 Cc
Glufosinate	9.04 ABb	9.50 BCDb	13.29 ABa
Asulam	8.83 ABb	10.54 BCDab	13.29 ABa
Ethofumesate	9.34 ABa	8.86 CDa	10.43 ABCa
2,4-D	9.61 ABa	8.39 CDa	11.55 ABCa
Clomazone	9.00 ABa	8.39 CDa	11.55 ABCa
Carfentrazone-ethyl	8.64 ABb	11.94 ABCa	11.99 ABCa
S-Metolachlor	6.21 Ba	0 Eb	0 Db

[1]The concentrations of herbicides used to treat algae were 0.1, 1, and 5 mg L^{-1}.
[2]Means in the same column followed by the same uppercase letter are not significantly different at the 5% level as determined by Tukey's studentized range test. Means in the same row followed by the same lowercase letter are not significantly different at the 5% level as determined by Tukey's studentized range test.

Table 8. Lipid content from cultures of *Nannochloris oculata* as affected by selected herbicides with water solubilities between 5 and 50 mg L^{-1}.[1]

	Lipid Content[2] (%)	
Herbicides	0.1 mg L^{-1}	1 mg L^{-1}
Untreated	13.33 Aa	13.33 Aa
Diuron	0 Ca	0 Ca
Simazine	5.15 Ba	0 Cb
Fluridone	12.44 Aa	0 Cb
Thiobencarb	12.68 Aa	10.01 Ba
Dichlobenil	7.42 Ba	6.95 Ba

[1]The concentrations of herbicides used to treat algae were 0.1 and 1 mg L^{-1}.
[2]Means in the same column followed by the same uppercase letter are not significantly different at the 5% level as determined by Tukey's studentized range test. Means in the same row followed by the same lowercase letter are not significantly different at the 5% level as determined by Tukey's studentized range test.

Table 9. Lipid content from cultures of *Nannochloris oculata* as affected by selected herbicides with water solubilities < 5 mg L^{-1} [1].

Herbicides	Lipid content[2] (%) 0.1 mg L^{-1}
Untreated	13.33 A
Cyhalofop	11.00 A
Pendimethalin	6.19 B
Dinoterb	6.03 B
Isoxaben	10.55 A

[1]The concentration of herbicides used to treat algae was 0.1 mg L^{-1}.
[2]Means in the same column followed by the same uppercase letter are not significantly different at the 5% level as determined by Tukey's studentized range test.

cides like pendimethalin and dinoterb had no significant effect on algal growth but decreased lipid content significantly. These results will be useful for developing *N. oculata* and other algae as sources of renewable fuel in outdoor, open-pond systems, such that pure cultures of oil-producing algae may be more easily sustained. Further studies are needed toward a comprehensive understanding of herbicide modes of action and their effects on algal growth and lipid content.

SOURCES OF MATERIALS

[1]Herbicide standards, AccuStardard, Inc., 125 Market Street, New Haven, CT 06513.

[2]Herbicide standards, Chem Service, Inc., 660 Tower Lane, West Chester, PA 19381.

[3]Spectronic 21 ultraviolet-visible spectrophotometer, Milton Roy Co., Analytical Products Division, 820 Linden Avenue, Rochester, NY 14625.

[4]Automated Dionex-200 ASE system, Dionex Co., 1228 Titan Way, Sunnyvale, CA 94088-3603.

[5]Ottawa sand, Catalogue No. S23-3, Fisher Scientific, 1 Reagent Lane, Fair Lawn, NJ 07410.

[6]SAS software, Version 9.1, SAS Institute Inc., 100 SAS Campus Drive, Cary, NC 27513.

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