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Effect of selected herbicides on growth and hydrocarbon content of *Botryococcus braunii* (Race B)

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ABSTRACT

The algae *Botryococcus braunii*, one of the potential renewable resources for production of liquid hydrocarbons, was used for testing the effect of selected herbicides on algal growth and hydrocarbon content. Twenty-two herbicides representing 14 modes of action were assayed. Accelerated solvent extraction (ASE) was used to extract hydrocarbon from algae and the results showed that ASE was an efficient method for algal hydrocarbon extraction at 50 °C extraction temperature, 10.3 MPa of pressure and 1 static cycle using n-hexane as extraction solvent. The photosystem II inhibitor diuron was the most toxic herbicide for algal growth, but hydrocarbon content increased from 34.9 to 42.4% of dry biomass in the presence of a 0.1 mg/L concentration of diuron. The photosystem I inhibitor diquat was inhibitory to growth of *B. braunii*, but hydrocarbon content increased to 43.3% of dry biomass when treated with 5 mg/L diquat. *S*-metalochlor, a mitosis inhibitor, reduced both algal growth and hydrocarbon content. Hydrocarbon content decreased to 16.4, 14.0 and 5.5% of dry biomass, respectively when treated with 0.1, 1, and 5 mg/L *S*-metalochlor. The carotenoid biosynthesis inhibitor fluridone did not affect algal growth at 0.1 mg/L, but decreased hydrocarbon content from 34.9 to 13.2%. The fatty acid and lipid biosynthesis inhibitor thiobencarb had no effect on algal growth or hydrocarbon content at 0.1 mg/L, but it inhibited algal growth and decreased hydrocarbon content from 34.9 to 7.8% at 1 mg/L. The oxidative phosphorylation uncoupler dinoterb at 0.1 mg/L reduced algal biomass by 52.8% and decreased hydrocarbon content from 34.9 to 30.0%. The use of *B. braunii* as a source of fuel will undoubtedly require the use of an outdoor open pond system. These results will be useful for improving algal oil production while developing management systems to control invasive algal species in outdoor open pond systems ultimately keeping the oil producing algae pure.

1. Introduction

Biomass is widely considered to be a major potential biofuel and renewable resource for the future (Şensöz and Kaynar, 2006). Microalgae have been suggested as very good candidates for fuel production because of their advantages of higher photosynthetic efficiency, higher biomass production, and faster growth compared to other energy crops (Banerjee et al., 2002; Miao and Wu, 2006; Li et al., 2007). *Botryococcus braunii* is a green colonial microalga that is widespread in freshwater and brackish lakes, reservoirs, and ponds. It is recognized as a potential renewable resource, for its production of large amounts of liquid hydrocarbons. *B. braunii* is classified into A, B and L races that are defined based on the type of hydrocarbons synthesized. Race A accumulates nonterpenoid alkadienes and alkatrienes derived from fatty acids (Templier et al., 1984, 1991; Metzger et al., 1985), while race L accumulates the tetraterpene lycopadiene (Metzger et al., 1990). The B race produces polyunsaturated and branched C_{30}–C_{37} terpenoid hydrocarbons referred to as polymethylated botryococcenes. These compounds are promising as a renewable energy source as they comprise 26–86% of dry biomass (Brown et al., 1969; Banerjee et al., 2002; Metzger and Largeau, 2005). Therefore, *B. braunii* has been identified as an untapped resource for production of renewable hydrocarbons. Successful use of this organism as an alternate source of energy depends on its growth rate, hydrocarbon productivity and fuel efficiency.

Herbicides are chemicals commonly used to control weeds in agricultural activities and are often discharged into aquatic environments through surface runoffs 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

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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 toward herbicides varies depending on the species and specific herbicide (DeNoyelles et al., 1982; Mayasich et al., 1986).

The use of *B. braunii* as a source of fuel will undoubtedly require the use of outdoor open pond systems 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. While there are many studies about the effect of herbicides on algal growth, biochemical composition, metabolic activities and the 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 *B. braunii*. Thus, it is important to determine the effect of herbicides on *B. braunii* growth and hydrocarbon production for potential use in open pond systems. The objective of this work was to examine the effect of selected herbicides on biomass and hydrocarbon accumulation of *B. braunii*.

2. Materials and methods

2.1. Algal culture

B. braunii Kützing Berkeley strain (Showa) B race (Nonomura, 1988) was cultured in modified Chu13 medium (Grung et al., 1989) and grown under illumination of 22 μmol m^{-2} s^{-1} by a 14-h light and 10-h dark cycle at 23 °C. The cultures were continuously aerated with filter-sterilized air containing 2% CO_2 (Weiss et al., 2010).

2.2. Herbicides

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

2.3. Experimental process

Algal cultures in the logarithmic phase of growth were inoculated in 200 mL autoclaved modified Chu13 medium. The herbicide solutions were added to the growth medium to give a final concentration of 0.1, 1 and 5 mg/L. Duplicated assays without herbicides were used as controls. *B. braunii* were cultured at 23 °C with fluorescent lights by a 14-h light and 10-h dark cycle, and aerated with filter-sterilized air. The experiment was designed as a completely randomized design with three replications. The growth rate was determined by measuring the absorbance at 680 nm using a Spectronic 21 UV-Visible spectrophotometer at 2 days intervals for 30 days (Lee et al., 1998; Sim et al., 2001).

2.4. Biomass estimation

At the end of each culture cycle, an aliquot of culture was filtered by pre-weighed Whatman GF/C filters. The accumulated biomass was rinsed with sterilized deionized H_2O, dried in a fume hood at room temperature for 48 h, and then weighed to determine the dry weight of algal biomass.

2.5. Hydrocarbon extraction

Hydrocarbons were extracted using an accelerated solvent extraction (ASE) method. An automated Dionex-200 ASE system was used for all the extractions. Algal samples (about 1–2 g dry weight) were mixed with 10 g Ottawa sand (20–30 mesh) prior to being loaded into 11-mL sample cells, to fill extra space in the cell. Extraction methods were optimized at different conditions. Different solvents (n-hexane, acetone, and ethyl acetate) and temperatures (50, 100 and 125 °C) were evaluated, and then extraction efficiencies were determined by using 1, 2 or 3 static cycles. The efficiency of hydrocarbon extraction by optimized ASE method was compared with Soxhlet method. Each extraction was repeated three times. The experiment was designed as a completely randomized design. Extraction cell pressure was maintained at 10.3 MPa during the experiments. The static cycle was 5 min, during which the 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 N_2 gas 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 and further purified by silica column with n-hexane as eluent. Hydrocarbon content was measured gravimetrically and expressed as the percentage of dry biomass (Sawayama et al., 1992; Toussaint et al., 2002; Dayananda et al., 2007).

2.6. Statistical analysis

Data were subjected to analysis of variance (ANOVA) by using SAS (Version 9.1) to evaluate the significance of any interactions and determine treatment means. The summary procedure in SAS was used to calculate the standard deviation of means. The different letters for each treatment in each column and row indicate significant difference ($P < 0.05$).

3. Results and discussion

3.1. Effect of herbicides on algal growth

Herbicides are liable to exert algistatic (prevent algal growth), inhibitory or lethal effects on algae. The magnitude of these effects depends on the concentration and chemical properties of the herbicide as well as various species exposed to it (Maly and Ruber, 1983). Growth of algae was monitored spectrophotometrically by measuring the optical density as well as the dry weight of algal biomass (Abou-Waly et al., 1991; El-Sheekh et al., 1994). Algal growth curves that were significantly affected by some herbicides are shown in Fig. 1. The photosystem II inhibitor diuron was the most toxic herbicide for algal growth. Algae essentially stopped growing in 1 mg/L diuron after 20 days of exposure. Also, diquat, *S*-metolachlor and bensulfuron-methyl provided strong inhibition of algal growth, and inhibition increased with increasing concentration. Algal growth slowed in 1 mg/L fluridone, while no inhibition was observed in 0.1 mg/L fluridone. Dinoterb at 0.1 mg/L inhibited algal growth. Simazine, thiobencarb, carfentrazone, clomazone,

Table 1
Mode of action and common names of herbicides used to treat *Botryococcus braunii*.

HRAC Code[a]	WSSA Code[b]	Mode of action	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

[a] HRAC Code: Herbicide Resistance Action Committee designation of herbicides.
[b] WSSA Code: Mode of Action Code designated by the Weed Science Society of America (Senseman, 2007).

Table 2
Analysis of variance for biomass yield of *B. braunii* at different herbicide groups based on herbicide solubility.

Herbicide group[a]	Source	DF	Anova SS	Mean square	*F* value	Pr > F
A	Herbicide	12	0.94	0.078	7.42	<.0001
	Concentration	2	0.12	0.059	5.61	0.0072
	Herbicide × Concentration	24	0.49	0.020	1.93	0.033
B	Herbicide	4	0.97	0.24	23.74	<.0001
	Concentration	1	0.13	0.13	13.03	0.0048
	Herbicide × Concentration	4	0.2	0.05	4.80	0.0202
C	Herbicide	3	0.23	0.059	35.25	0.0007

[a] Group A: The solubility of herbicides is higher than 100 mg/L in water at room temperature. The concentration of standard solutions of group A was 100 mg/L. The concentrations of herbicides used to treat algae were 0.1, 1 and 5 mg/L. Group B: The solubility of herbicides is 5–50 mg/L in water at room temperature. The concentrations of standard solutions were 5 and 10 mg/L. The concentrations of herbicides used to treat algae were 0.1 and 1 mg/L. Group C: The solubility of herbicide is less than 5 mg/L in water at room temperature. The concentrations of standard solutions were 0.2 and 1 mg/L. The concentration of herbicides used to treat algae was 0.1 mg/L.

mesotrione, ethofumesate, 2,4-D, and dichlobenil had no significant effect on algal growth at the tested concentrations after 30 days of exposure.

Similar inhibitory effects were also evaluated by measuring the dry weight of biomass at algal harvest after 30 days of incubation with herbicides (Table 2). The interaction between herbicide and concentration was significant for herbicides in groups A and B. Biomass as affected by selected herbicides at concentrations of 0.1, 1 and 5 mg/L in group A is shown in Table 3. The acetolactate synthase (ALS) or acetohydroxyacid synthase (AHAS) inhibitor, bensulfuron-methyl at 0.1, 1 and 5 mg/L, decreased biomass by 22.6, 42.5 and 57.8%, respectively. Sabater et al. (2002) studied the acute toxicity of sulfonylurea herbicides on four species of freshwater phytoplankton and bensulfuron-methyl concentrations eliciting a 50% growth reduction over 96 h (EC_{50}) ranged from 0.015 to 6.2 mg/L. Diquat inhibited algal growth and inhibition increased with increasing concentration. Algal growth was inhibited approximately 71.4% by 5 mg/L diquat. Many studies have assessed the ecological risk of the brominated herbicide diquat (Delorenzo et al., 2001). Holst et al. (1982) found that diquat at 0.1 mg/L partially inhibited *Azolla* sp. growth, and at 1 mg/L caused a total inhibition after 10 days. Peterson et al. (1994) compared nine algal species exposed to 0.73 mg/L diquat and found 53–69% inhibition of ^{14}C uptake in two green algal species, 99–100% inhibition in two

Table 3
Algal biomass of *B. braunii* as affected by selected herbicides with water solubilities > 100 mg/L (solubility group A[a]).

Herbicides	Biomass[b]		
	0.1 mg/L	1 mg/L	5 mg/L
		% of control	
Sethoxydim	66.8 Aa	65.4 Aa	58.1 ABCa
Bensulfuron-methyl	77.4 Aa	57.5 Ab	42.2 BCc
Halosulfuron-methyl	76.4 Aa	71.8 Aa	67.8 ABCa
Diquat	62.4 Aa	54.2 Aa	28.6 Cb
Mesotrione	66.4 Aa	89.0 Aa	83.7 ABa
Glyphosate	87.7 Aa	69.8 Aa	64.8 ABCa
Glufosinate	64.8 Aa	82.4 Aa	75.7 ABa
Asulam	77.1 Ab	80.4 Aab	97.0 Aa
Ethofumesate	91.7 Aa	91.7 Aa	90.4 Aa
2,4-D	80.4 Aa	91.7 Aa	67.8 ABCa
Clomazone	77.7 Aa	69.1 Aa	70.4 ABCa
Carfentrazone-ethyl	85.7 Aa	81.1 Aa	75.8 ABa
S-Metolachlor	81.7 Aa	63.8 Aa	55.5 ABCa

[a] The concentrations of herbicides used to treat algae were 0.1, 1 and 5 mg/L.
[b] Means in the same column followed by the same capital 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 small letter are not significantly different at the 5% level as determined by Tukey's studentized range test.

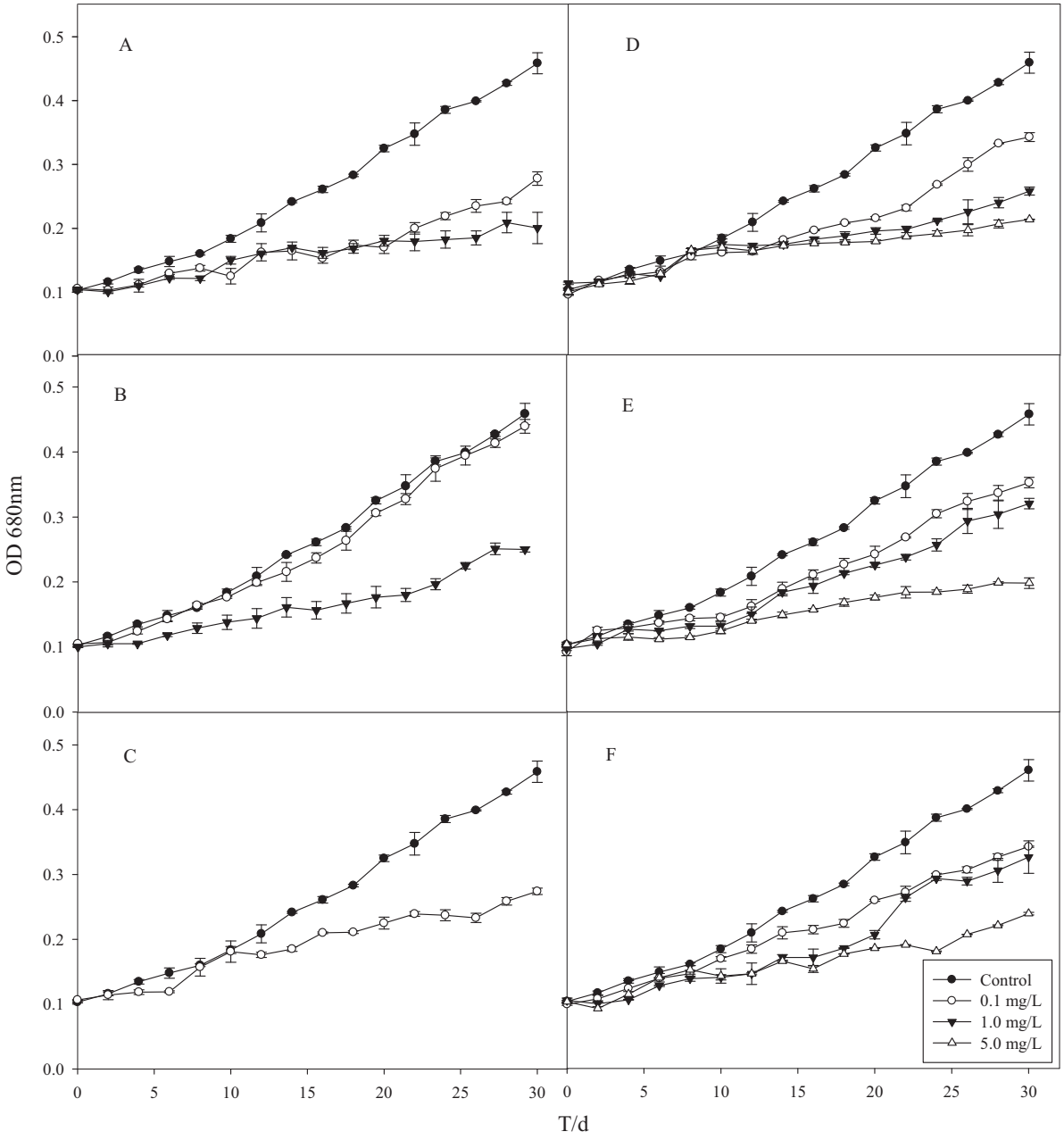

Fig. 1. Algal growth curves significantly affected by some selected herbicides. Herbicides and concentrations used to treat algae: (A) diuron: 0.1 and 1 mg/L; (B) fluridone: 0.1 and 1 mg/L; (C) dinoterb: 0.1 mg/L; (D) bensulfuron-methyl: 0.1, 1 and 5 mg/L; (E) diquat: 0.1, 1 and 5 mg/L; (F) *S*-metalochlor: 0.1, 1 and 5 mg/L.

diatom species, and 100% inhibition in five species of cyanobacteria. Diquat significantly altered the algal densities of naturally derived microbial communities tested in freshwater microcosms at concentrations greater than or equal to 0.3 mg/L, with no observable recovery after 21 days (Melendez et al., 1993). *S*-metolachlor had a significant effect on biomass, and 0.1, 1 and 5 mg/L concentrations decreased biomass 18.3, 36.2 and 44.5%, respectively. The result was consistent with growth inhibition of *Chlorella pyrenoidosa* treated with *S*-metolachlor (Liu and Xiong, 2009).

Biomass as affected by selected herbicides at 0.1 and 1 mg/L in group B is shown in Table 4. The photosystem II inhibitor diuron was the most toxic herbicide to *B. braunii*. Biomass yield was reduced to 30.6 and 25.2% of the control when treated with 0.1 and 1 mg/L diuron. Maule and Wright (1984) found that diuron was toxic to seven different green algal species. The anti-photosynthetic herbicides may cause plant death by the production of high-energy free

Table 4
Algal biomass of *B. braunii* as affected by selected herbicides with water solubilities between 5 and 50 mg/L (solubility group B[a]).

Herbicides	Biomass[b]	
	0.1 mg/L	1 mg/L
	% of control	
Diuron	30.6 Ba	25.2 Ca
Simazine	81.7 Aa	78.4 ABa
Fluridone	93.4 Aa	41.5 BCb
Thiobencarb	94.4 Aa	70.4 ABCa
Dichlobenil	88.7 Aa	91.4 Aa

[a] The concentrations of herbicides used to treat algae were 0.1 and 1 mg/L.
[b] Means in the same column followed by the same capital 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 small letter are not significantly different at the 5% level as determined by Tukey's studentized range test.

Table 5
Algal biomass of *B. braunii* as affected by selected herbicides with water solubilities < 5 mg/L (solubility group C[a]).

Herbicides	Biomass[b] % of control
Cyhalofop	87.7 AB
Pendimethalin	89.7 A
Dinoterb	47.2 C
Isoxaben	73.1 B

[a] The concentration of herbicides used to treat algae was 0.1 mg/L.
[b] Means in the same column followed by the same capital letter are not significantly different at the 5% level as determined by Tukey's studentized range test.

radicals which destroy cell membranes (Dodge, 1977). No significant effect was observed in 0.1 mg/L fluridone, while biomass yield decreased 58.5% in 1 mg/L fluridone. Millie et al. (1990) found that fluridone inhibited the growth of *Oscillatoria agardhii*, and biomass decreased with increasing fluridone concentration.

Table 5 shows biomass as affected by selected herbicides at 0.1 mg/L in group C. Dinoterb at 0.1 mg/L significantly reduced algal biomass compared with other herbicides in group C. Biomass yield decreased 52.8% in 0.1 mg/L dinoterb. Holst et al. (1982) found that 0.1 mg/L dinoterb partially inhibited growth of *Azolla* sp. and 1 mg/L caused complete inhibition *in vitro* after 10 days.

3.2. Hydrocarbon extraction

Hydrocarbons can be extracted from dry biomass of *B. braunii* with solvents (Metzger and Largeau, 2005). Although various methods have been used in extraction of algal lipids, accelerated solvent extraction (ASE) is a fully automated technique that uses conventional liquid solvents at elevated temperatures and pressures to achieve quantitative extraction from solid and semi-solid samples in a short time and with a relatively small volume of solvent (Conte et al., 1997; Peterson et al., 2007). Several papers have been published describing extraction of lipids from algae using ASE (Macnaughton et al., 1997; Zhuang et al., 2004). In this study, the effects of solvent, temperature and number of static cycles on hydrocarbon extraction efficiency were evaluated.

3.2.1. Effect of solvent

To determine the effect of solvent, extraction experiments were conducted using three non-polar solvents including n-hexane, acetone, and ethyl acetate under the same extraction conditions of 50 °C cell temperature, 10.3 MPa cell pressure, and 5-min static time (Fig. 2A). The results showed n-hexane was more efficient than acetone and ethyl acetate.

3.2.2. Effect of temperature

Using n-hexane as extraction solvent, hydrocarbon was extracted at three temperatures of 50, 100 and 125 °C to determine the most efficient extraction. The results showed the extraction efficiency was not enhanced by increasing temperature (Fig. 2 B). Therefore, 50 °C was chosen as the optimum temperature.

3.2.3. Effect of static cycles

Extraction efficiency was determined by using 1, 2, or 3 sequential static cycles at 50 °C extraction temperature and 10.3 MPa cell pressure using n-hexane as extraction solvent. The results showed that adding extraction cycles did not increase extraction efficiency (Fig. 2C). Average hydrocarbon extraction efficiency was 40.8% with one extraction cycle. One static cycle was enough for adequate and reproducible hydrocarbon extraction.

3.2.4. Comparison of ASE with Soxhlet method

The efficiency of hydrocarbon extraction by ASE and Soxhlet method was determined using n-hexane as solvent. ASE was operated at 50 °C, 10.3 MPa cell pressure and 1 static cycle. The hydrocarbon extracted by the optimized ASE method was 6.1% higher than the Soxhlet extraction method (Fig. 2D). Furthermore, ASE required only 12 min sample^{-1} and approximately 13 mL of solvent, while the Soxhlet method required 6 h and 60 mL of solvent. Thus, ASE saved time and solvent compared to the Soxhlet method. Shen and Shao (2005) compared ASE, Soxhlet extraction, and ultrasonic-assisted extraction for analysis of terpenoids and sterols in tobacco, and found that ASE was a promising alternative to classical methods since it was faster and used less solvent, especially when applied to extraction of large batch samples. Based on these experiments, ASE appears to be an effective extraction method for algal hydrocarbon extraction.

3.3. Effect of herbicides on hydrocarbon content

Analysis of variance for hydrocarbon content is shown in Table 6. Interaction effects were significant for herbicide groups A and B. Hydrocarbon content as affected by selected herbicides at 0.1, 1 and 5 mg/L is shown in Table 7. There were no significant differences between herbicide concentrations and the untreated samples for sethoxydim, bensulfuron-methyl, halosulfuron-methyl, glufosinate, asulam, ethofumesate, and 2,4-D. As concentration of *S*-metalochlor increased from 0.1, 1 to 5 mg/L, hydrocarbon content decreased to 16.4, 14.0 and 5.5% of dry biomass, respectively. *S*-metalochlor had a significant effect on both algal growth and algal hydrocarbon content. The herbicides like *S*-metalochlor within the K_3^{15} mode of action are currently thought to inhibit very long chain fatty acid (VLCFA) synthesis (Husted et al., 1966; Böger et al., 2000; Senseman, 2007). Although the photosystem I inhibitor diquat was inhibitory to growth of *B. braunii*, it resulted in increasing of hydrocarbon content to 43.3% of dry biomass when treated with 5 mg/L diquat. However, the hydrocarbon productivity was 0.18 g/L, which was 66.0% less than the productivity of the control (0.53 g/L).

Table 6
Analysis of variance for hydrocarbon content from *B. braunii* after exposure to herbicides of different solubility ranges.

Group[a]	Source	DF	Anova SS	Mean square	*F* value	Pr > F
A	Herbicide	13	0.28	0.022	77.51	<.0001
	Concentration	2	0.001	0.0006	2.30	0.1129
	Herbicide × Concentration	26	0.060	0.0023	8.10	<.0001
B	Herbicide	5	0.18	0.036	76.61	<.0001
	Concentration	1	0.012	0.012	26.09	0.0003
	Herbicide × Concentration	5	0.058	0.012	24.72	<.0001
C	Herbicide	4	0.0054	0.0014	21.12	0.0025

[a] Group A: The solubility of herbicides is higher than 100 mg/L in water at room temperature. The concentration of standard solutions of group A was 100 mg/L. The concentrations of herbicides used to treat algae were 0.1, 1 and 5 mg/L. Group B: The solubility of herbicides is 5 to 50 mg/L in water at room temperature. The concentrations of standard solutions were 5 and 10 mg/L. The concentrations of herbicides used to treat algae were 0.1 and 1 mg/L. Group C: The solubility of herbicides is less than 5 mg/L in water at room temperature. The concentrations of standard solutions were 0.2 and 1 mg/L. The concentration of herbicides used to treat algae was 0.1 mg/L.

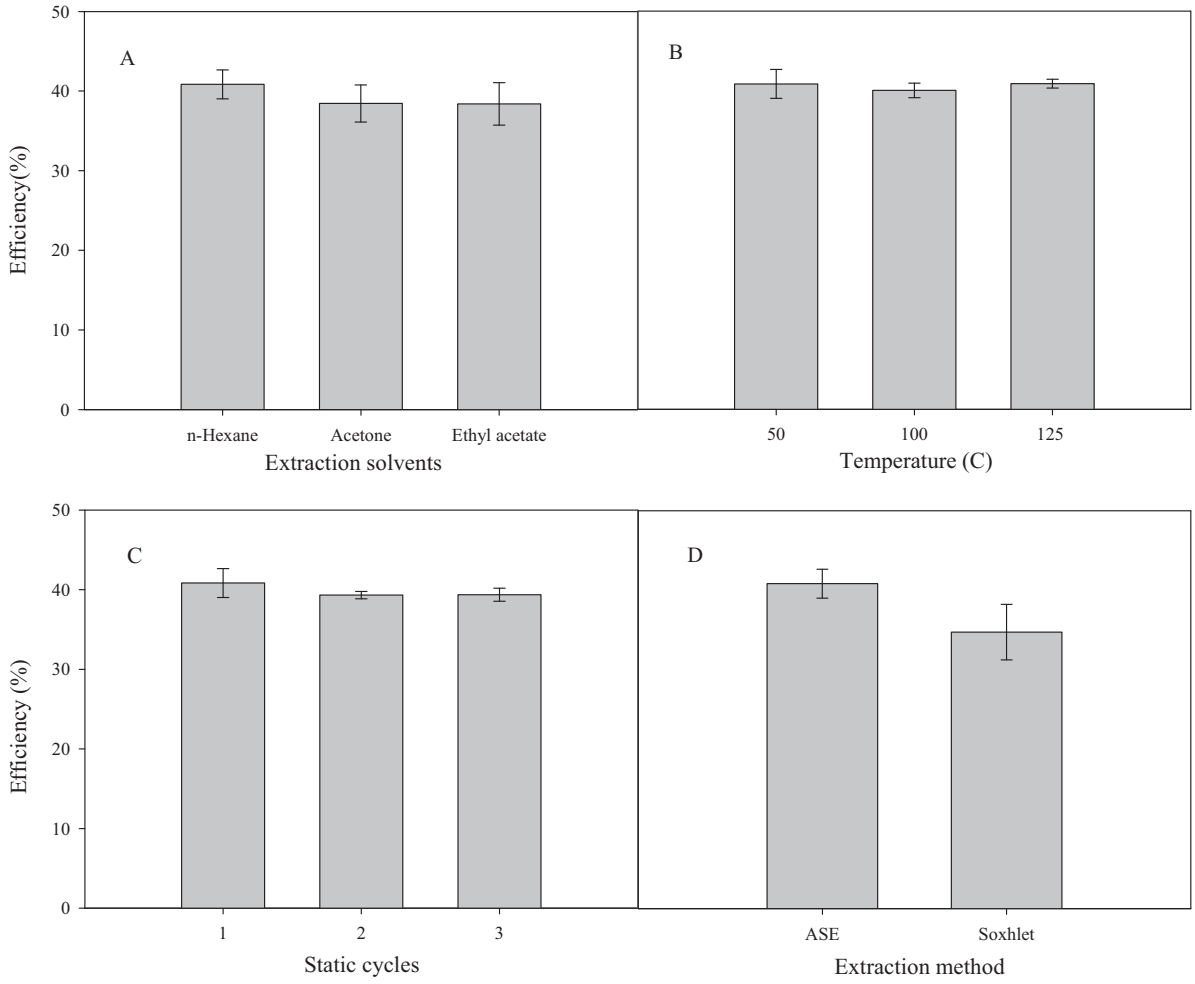

Fig. 2. Extraction efficiency of hydrocarbon using accelerated solvent extraction (ASE) at different conditions. (A) Comparing n-hexane, acetone, and ethyl acetate at 50 °C extraction temperature, 10.3 MPa pressure and one static cycle. (B) Comparing extraction temperatures of 50, 100, and 125 °C at 10.3 MPa pressure and one static cycle using n-hexane as extraction solvent. (C) Comparing the numbers of static cycles at 50 °C extraction temperature and 10.3 MPa pressure using n-hexane as extraction solvent. (D) Comparison of accelerated solvent extraction (ASE) with Soxhlet for extraction of hydrocarbon from algae.

Table 7
Hydrocarbon content from cultures of *B. braunii* as affected by selected herbicides with water solubilities >100 mg/L (solubility group A[a]).

Herbicides	Hydrocarbon content[b]		
	0.1 mg/L	1 mg/L	5 mg/L
		% of dry biomass	
Untreated	34.9 Aa	34.9 ABa	34.9 BCa
Sethoxydim	31.9 ABCa	30.9 BCa	33.1 Ba
Bensulfuron-methyl	31.8 ABCa	33.2 ABCa	35.6 ABCa
Halosulfuron-methyl	31.1 ABCa	32.8 BCa	34.1 BCa
Diquat	25.8 Cc	31.3 BCb	43.3 Aa
Mesotrione	33.1 ABa	30.9 BCa	28.4 Ca
Glyphosate	27.2 BCb	34.2 ABCa	29.2 BCab
Glufosinate	32.0 ABCa	32.1 BCa	35.1 BCa
Asulam	32.8 ABa	30.9 BCa	32.1 BCa
Ethofumesate	37.0 Aa	36.1 ABa	36.6 ABa
2,4-D	34.0 Aa	36.0 ABa	35.3 ABCa
Clomazone	33.7 Aa	29.1 Cc	31.0 BCb
Carfentrazone-ethyl	35.8 Aa	38.7 Aa	36.6 ABa
S-Metolachlor	16.4 Da	14.0 Da	5.5 Db

[a] The concentrations of herbicides used to treat algae were 0.1, 1 and 5 mg/L.
[b] Means in the same column followed by the same capital 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 small letter are not significantly different at the 5% level as determined by Tukey's studentized range test.

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).

Table 8 shows hydrocarbon content as affected by herbicides in group B. Diuron was the most toxic herbicide for algal growth. Biomass decreased to 30.6% of control, but hydrocarbon content increased to 42.4% of dry biomass when treated with 0.1 mg/L diuron. The hydrocarbon productivity was 0.20 g/L compared to 0.53 g/L of control, which was 62.3% less than the productivity of control. Fluridone at 0.1 mg/L did not affect algal growth, but decreased hydrocarbon content from 34.9 to 13.2% of dry biomass. Fluridone is a selective inhibitor of carotenoid synthesis in cells. 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 rRNA synthesis (Bartels and Watson, 1978; Reib et al., 1983), and also affects lipid composition (Lem and Williams, 1981). Thiobencarb at 0.1 mg/L did not affect algal growth and hydrocarbon content, but 1 mg/L thiobencarb inhibited algal growth slightly and decreased hydrocarbon content from 34.9 to 7.8% of dry biomass. Thiobencarb is a fatty acid and lipid biosynthesis inhibitor, which in turn can have many secondary effects on growth (Eladel

Table 8
Hydrocarbon content from cultures of *B. braunii* as affected by selected herbicides with water solubilities between 5 and 50 mg/L (solubility group B[a]).

Herbicides	Hydrocarbon content[b]	
	0.1 mg/L	1 mg/L
	% of dry biomass	
Untreated	34.9 Ba	34.9 Aa
Diuron	42.4 Aa	34.9 Aa
Simazine	34.2 Ba	38.5 Aa
Fluridone	13.2 Cb	18.7 BCa
Thiobencarb	31.5 Ba	7.8 Cb
Dichlobenil	35.4 Ba	29.7 ABa

[a] The concentrations of herbicides used to treat algae were 0.1 and 1 mg/L.
[b] Means in the same column followed by the same capital 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 small letter are not significantly different at the 5% level as determined by Tukey's studentized range test.

Table 9
Hydrocarbon content from cultures of *B. braunii* as affected by selected herbicides with water solubilities < 5 mg/L (solubility group C[a]).

Herbicides	Hydrocarbon content[b]
	% of dry biomass
Untreated	34.9 AB
Cyhalofop	32.6 BC
Pendimethalin	34.8 AB
Dinoterb	30.0 C
Isoxaben	36.8 A

[a] The concentration of herbicides used to treat algae was 0.1 mg/L.
[b] Means in the same column followed by the same capital letter are not significantly different at the 5% level as determined by Tukey's studentized range test.

et al., 1999; Eladel, 2010). Altered fatty acid synthesis may also indirectly alter photosynthesis and respiration (Wilkinson and Smith, 1975; Percival and Baker, 1991). Xia (2005) concluded that dry weight yield and protein content were not affected by thiobencarb exposure from 2 to 6 mg/L, whereas high thiobencarb concentration (10 mg/L) resulted in significant decreases in biomass yield, protein content and photosynthetic rate in *Nostoc sphaeroides* colonies. Bhunia et al. (1991) found that thiobencarb at concentrations ranging from 2 to 6 mg/L led to significant decreases in growth, DNA, RNA and total protein content of *Nostoc muscorum*. Further more, it has been shown that cellular chlorophyll A content was significantly reduced with thiobencarb treatments on *Protosiphon botryoides* (Eladel et al., 1999) and *Anabaena variabilis* (Battah et al., 2001).

Hydrocarbon content as affected by selected herbicides at 0.1 mg/L in group C is shown in Table 9. Dinoterb reduced hydrocarbon content from 34.9 to 30.0% of dry biomass. All other herbicides in group C did not significantly affect algal hydrocarbon content.

4. Conclusions

The effect of 22 selected herbicides on algal growth and hydrocarbon content was determined in this study. Diuron was the most toxic herbicide for algal growth. Biomass yield decreased to 30.6% of control, but hydrocarbon content increased from 34.9 to 42.4% of dry biomass in the presence of a 0.1 mg/L concentration of diuron after 30 days of exposure. Diquat, *S*-metolachlor and bensulfuron-methyl had strong inhibition of algal growth, and inhibition increased with increasing concentration. Although diquat was inhibitory to growth of *B. braunii*, it resulted in increasing hydrocarbon content to 43.3% of dry biomass when treated with 5 mg/L diquat. *S*-metalochlor had a significant effect on both algal growth and hydrocarbon content. Bensulfuron-methyl had no significantly effect on hydrocarbon content. Fluridone at 0.1 mg/L did not impact algal growth, but decreased hydrocarbon content from 34.9 to 13.2%. Thiobencarb at 1 mg/L inhibited algal growth slightly and decreased hydrocarbon content from 34.9 to 7.8%. Dinoterb at 0.1 mg/L had a significant effect on both algal biomass and hydrocarbon content. These results will be useful for developing *B. braunii* and other algae as a source of renewable fuel in outdoor open pond systems such that pure cultures of the oil producing algae may be more easily sustained. Further studies are needed toward a comprehensive understanding of herbicides mode of action and their effects on algal growth and hydrocarbon content.

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