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ALLELOCHEMICALS OF *BOTRYOCOCCUS BRAUNII* (CHLOROPHYCEAE)[1]

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The alga *Botryococcus braunii* Kützing (Chlorophyceae) present in Liyu Lake (Huanlien County, Taiwan) has toxic effects on a variety of aquatic organisms. Blooms of this alga, which typically occur in autumn, are associated with fish deaths in this lake. Experiments using 15 phytoplankton and 5 zooplankton isolated from Liyu Lake indicate that these plankton exhibit various susceptibilities to *B. braunii*. A close correlation between the degree of susceptibility tested in the laboratory and the absence of certain phytoplankton during *B. braunii* blooms in the lake was observed, suggesting allelopathic effects. Isolation, identification, and verification with authentic compounds indicated that allelochemicals were a mixture of free fatty acids, including α-linolenic, oleic, linolic, and palmitic acids. Compared with other phytoplankton isolates, *B. braunii* produced significantly higher amounts of free fatty acids, particularly of oleic and α-linolenic acids. The role of these fatty acids in favoring dominance of *B. braunii* in the natural environment was elucidated.

Key index words: **allelochemical; allelopathy; *Botryococcus*; free fatty acid; Liyu lake; toxicity**

Harmful algal blooms are a worldwide problem, commonly associated with public health concerns. Bloom-forming algae in aquatic ecosystems include cyanobacteria, dinophytes, chrysophytes, diatoms, and chlorophytes. A number of these algae produce toxins, such as saxitoxin and microcystis, and some produce active compounds with allelopathic activity (Gleason and Baxa 1986, Bagchi et al. 1993, Wu et al. 1998, Jüttner and Wu 2000). Some active compounds have been isolated and identified (Gross et al. 1991, Bagchi 1995, Papke et al. 1997, Jüttner 2001, Mundt et al. 2003).

The green colonial alga *Botryococcus braunii* is characterized by its unusually high hydrocarbon content. The production of renewable hydrocarbon by this alga, such as botryococcene (Wake and Hillen 1981), has received great attention in the last two decades (Okada et al. 1997, Banerjee et al. 2002) because the hydrocarbon has the potential for use as an alternative fuel.

Massive growth of *B. braunii* can give rise to conspicuous blooms in a variety of aquatic environments worldwide. However, it has never been reported as toxic to aquatic organisms. In Liyu Lake (N 23°55.83', E 121°30.83'), a natural lake situated in eastern Taiwan, *B. braunii* blooms have occurred several times over the last decade. The occurrence of these blooms has caused massive death of fish in the lake, particularly of *Tilapia* sp. A preliminary study indicated that such events were possibly related to the toxic effects of *B. braunii*. In the present study, the toxicity of *B. braunii* to a variety of phytoplankton and zooplankton isolated from Liyu Lake was tested. Additionally, the compounds responsible for the toxicity were isolated and identified.

MATERIALS AND METHODS

Sampling of phytoplankton. Phytoplankton from Liyu Lake were collected from July 1998 to June 2002. Water samples were fixed with Lugol's iodine solution immediately after collection. Observation and identification of species were conducted under a light microscope (Leica DM-LB, Heidelberg, Germany). The abundance of each phytoplankton species was calculated on the basis of enumeration of at least 10,000 cells. Relative abundance, in terms of percentage, was used to designate the abundance of each species in phytoplankton assemblages. The Shannon index (Shannon and Weaver 1949) was used to display the diversity of phytoplankton assemblages. Samples of *B. braunii* were also obtained by harvesting cell colonies with a plankton net (20 μm) during algal blooms in autumn 1998 and 1999. The harvested cell mass was lyophilized and stored under –40° C before use.

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TABLE 1. The average density (cells · mL^{-1}) of phytoplankton species present in Liyu Lake during the autumns (from October to November) of 1998, 1999, and 2001 and their susceptibility (IC_{50}, mg · mL^{-1}) to the cell-free extract of *Botryococcus braunii*.

Phytoplankton	1998	1999	2001	IC_{50}[a]
Green algae				
Botryococcus braunii	11,981	23,675	155	nt
Chlorella sp.	—[b]	—	25	0.48 ± 0.02
Coelastrum astroideum	8	—	149	0.81 ± 0.04
Coelastrum polycordum	103	195	172	nt
Didymocystis sp.	—	—	—	0.43 ± 0.01
Eutetramorus fottii	1643	117	938	1.23 ± 0.12
Kirchneriella dianae	—	—	774	0.22 ± 0.02
Monoraphidium circinale	—	2	—	1.14 ± 0.04
Monoraphidium contortum	—	—	—	0.04 ± 0.00
Oocystis parva	274	273	94	9.00[c]
Pediastrum simplex	1130	429	2179	1.83 ± 0.14
Scenedesmus ecornis	34	39	27	1.09 ± 0.03
Scenedesmus quadricauda	—	4	12	0.48 ± 0.01
Cyanobacteria				
Anabaena spiroides	—	—	—	0.03 ± 0.00
Anabaena viguieri	—	—	274	0.05 ± 0.00
Aphanocapsa delicatissima	6093	8152	3495	nt
Chroococcus minutus	34	156	2150	nt
Microcystis flos-aquae	8386	4641	11,409	9.00[c]
Oscillatoria sp.	—	—	—	0.62 ± 0.02
Diatoms				
Aulacoseira granulata	377	234	907	nt
Nitzschia sp.	—	—	—	0.05 ± 0.00
Other species	477	1092	519	nt
Shannon diversity index of phytoplankton assemblages	1.91	1.64	2.48	

[a]Values are means ± SD ($n = 3$).
[b]Not detected.
[c]Approximate value.
nt, not tested

Algal strains and cultivation. In addition to *B. braunii*, 20 phytoplankton (Table 1) were isolated from Liyu Lake for this study. They were cultured in the mediums of Kuhl (1962) for chlorophytes, of BG11 (Rippka and Herdman 1992) for cyanobacteria, and of Schlösser (1994) for diatoms, with an illumination of approximately 300 μmol photons · m^{-2} · s^{-1} at 25° C in the laboratory. Cells grown in log-phase were used for toxicity tests.

Zooplankton. Three Cladocera and three Copepoda (Table 2) were isolated from Liyu Lake. They were used for the bioassay of toxicity immediately after isolation. *Thamnocephalus platyrus*, an anostraca purchased from Aboatox Oy, Turku, Finland, was used as a reference organism in the toxicity tests.

Toxicity tests. Procedures described by Todorova and Jüttner (1996) were used to test the toxicity of cell-free extract and each fraction during isolation of allelochemicals of *B. braunii*. Growth inhibition activity was used as a measure of the allelopathic effect on phytoplankton. Growth inhibition activity was assayed in the cultures containing approximately 0.04 μg chl *a* · mL^{-1} at time zero. The results were evaluated after incubation for 24 h at 25 ± 1° C under illumination at 300 μmol photons · m^{-2} · s^{-1}. Changes in chl *a* content in the cultures were used as a measure of growth rate.

Acute grazer toxicity was tested with zooplankton isolated from Liyu Lake. The methods described by Todorova and Jüttner (1996) were adopted for this purpose. Tested cultures were incubated for 24 h at 25 ± 1° C. The numbers of living and dead individuals were counted under a dissecting microscope. The median lethal concentration (LC_{50}), determined by a moving average method and calculated with Softtox (WindowChem Software™, Inc., Fairfield, CA, USA), was used to designate the toxicity to the zooplankton tested. For phytoplankton, the same method was adopted to determine the median inhibitory concentration for the growth rate (i.e. IC_{50}) of the tested species, which was calculated on the basis of the rate of increase of chl *a* content over times. The unit for LC_{50} and IC_{50}, mg · mL^{-1}, was given in terms of dry weight of *B. braunii*. Statistical calculation of these values was based on at least three repeated tests. Each test was conducted with three replicate cultures.

TABLE 2. Median lethal concentration (LC_{50}) of cell-free extract of *Botryococcus braunii* for various strains of zooplankton isolated from Liyu Lake.

Zooplankton tested	LC_{50} (mg · mL^{-1})
Cladocera	
Bosmina sp.	3.58 ± 0.48
Diaphanosoma leuchtengianum	5.00[a]
Monia macrocopa	5.00[a]
Copepoda	
Cyclopina sp.	1.56 ± 0.14
Eodiaptomus sp.	0.93 ± 0.12
Nauplius sp.	0.56 ± 0.03
Anostraca	
Thamnocephalus platyrus	0.61 ± 0.04

Values are means ± SD ($n = 3$).
[a]Approximate value.

Preparation of cell-free extract. Cell-free extract of *B. braunii* was obtained by extracting the lyophilized cells with either ethanol (90%) or distilled water at 4° C overnight. After removal of cell debris by centrifugation (10,000 *g* for 10 min), the extract was concentrated to dryness with an evaporator. The residue was then taken to a defined volume (5 mL) by adding ethanol (90%) or distilled water.

Isolation and identification of allelochemicals. The extraction of cell-free extract from lyophilized *B. braunii* cells was first done with 90% ethanol. The crude extract was partitioned by H_2O/t-butylmethylether. After discarding the water-soluble fraction, the ether-soluble fraction was loaded on a C_{18} RP column for solid phase extraction and eluted with 90% ethanol. The fractions with positive allelopathic activity were then separated by a Sephadex LH-20 column (Pharmacia Biotech, Uppsala, Sweden) for gel permeation chromatography and eluted with methanol (100%). Subsequently, the fractions with allelopathic activity were further purified by HPLC (Jasco, Hachioji, Japan) with a Hyperprep HS C8-RP column (Thermoquest, Runcom, UK), eluted with acetonitrile:H_2O (8:2, v:v) solution. Each fraction obtained was subjected to a bioassay of allelopathic activity, using six sensitive plankton species as the test organisms (Table 3).

For detection of fatty acids under HPLC analysis, a derivation reaction with additions of 2-bromo-2-acetonaphthone and 18-crown-6 to isolated compounds was done before analysis using the methods of Tsuchiya et al. (1984). A Hypersil HS 18C 5-μm column (250 × 4 mm) (Thermoquest) eluted with 93% methanol was used for quantitative and qualitative analysis of fatty acids. Margaric acid served as the internal standard. The identification of allelochemicals was conducted with a ^{1}H- and ^{13}C-NMR, gas chromatography-mass detector (HP 5890, Hewlett Packard, Palo Alto, CA, USA), Fourier transform infrared spectra (FTIR-410, Jasco), and UV-visible spectra (DU-640B, Beckman, Palo Alto, CA, USA). Authentic fatty acids purchased from Sigma (St. Louis, MO, USA) were used for the purpose of

TABLE 3. Median inhibitory concentration (IC_{50}, $mg \cdot mL^{-1}$) for growth of phytoplankton and median lethal concentration (LC_{50}, $mg \cdot mL^{-1}$) for zooplankton in response to four fatty acids isolated from *Botryococcus braunii*.

Organism tested	Palmitic acid	Oleic acid	Linoleic acid	α-Linolenic acid
Phytoplankton				
Pediastrum simplex	177.3 ± 14.8	19.8 ± 2.0	14.0 ± 0.1	15.8 ± 2.4
Chlorella sp.	59.1 ± 1.5	12.4 ± 0.3	9.4 ± 0.0	10.0 ± 0.4
Monoraphidium contortum	9.2 ± 0.6	12.1 ± 0.9	8.0 ± 0.0	14.5 ± 0.7
Zooplankton				
Eodiaptomus sp.	>200	>200	47.0 ± 3.4	19.6 ± 1.3
Monia macrocopa	>200	>200	>200	106.2 ± 9.8
Thamnocephalus platyrus	>200	>200	26.0 ± 0.2	13.3 ± 0.7

Values are means ± SD ($n = 3$).

verification. The amounts of each fatty acid on chromatogram were estimated by standard addition of authentic compounds.

RESULTS

Occurrence of phytoplankton in Liyu Lake. Liyu Lake is eutrophic, with phytoplankton assemblages characterized by the dominance of green algae and cyanobacteria. A pronounced seasonal succession of various phytoplankton groups occurred over the period of study. In general, cyanobacteria dominated in summer, whereas chlorophytes dominated in other seasons. In autumn 1998 and 1999, *B. branuii* formed a bloom. During those times, fewer phytoplankton species were found in the lake, giving rise to lower values of species diversity index, when compared with autumn 2001, when a bloom of *B. braunii* did not occur (Table 1).

Susceptibility of phytoplankton to Botryococcus braunii. Sixteen phytoplankton species, belonging to the chlorophytes, cyanobacteria, and diatoms, were isolated from Liyu Lake. A test of their susceptibility to *B. braunii* was conducted with the cell-free extract (Table 1). The growth of all tested phytoplankton species was inhibited to various degrees by exposure to *B. braunii* extract.

The magnitude of IC_{50} values of phytoplankton exhibited a wide range of variation. A plot of IC_{50} versus the relative abundance of each species, expressed by percentage abundance in the phytoplankton assemblages occurring during *B. braunii* blooms in

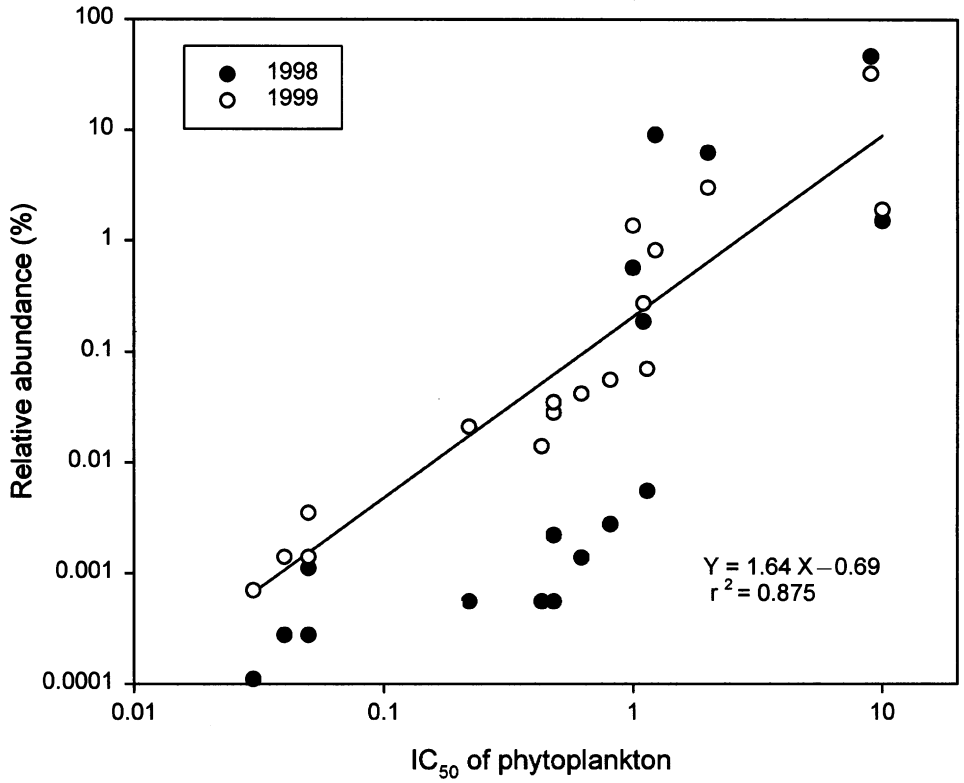

FIG. 1. Relative abundances of phytoplankton in Liyu Lake during November 1998 and 1999 as a function of their susceptibility (IC_{50}) to the extract of *Botryococcus braunii*.

November of 1998 and 1999, indicated a close correlation (Fig. 1). This suggests that the abundance of the phytoplankton in the lake was affected by the occurrence of *B. braunii*.

Susceptibility of zooplankton to Botryococcus braunii. In Liyu Lake, zooplankton assemblages were characterized by the dominance of Copepoda (such as *Nauplius* sp., *Eodiaptomus* sp., and *Cyclopina* sp.), Cladocera (such as *Bosmina* sp. and *Diaphanosoma* sp.), and Rotifera (*Keratella* sp.). Tests with six zooplankton species isolated from Liyu Lake indicate that *B. braunii* extract was toxic to all of them. However, the zooplankton exhibited various degrees of susceptibility. In general, copepods were more susceptible than cladocerans (Table 2).

For comparison, the toxicity of *B. braunii* was tested with *T. platyrus*, an anostracan commonly used for toxicity tests. This anostracan had about the same susceptibility as *Nauplius* sp. to the extract of *B. braunii*. However, it was more susceptible than the other zooplankton isolated from Liyu Lake.

Identification of allelochemicals. Positive allelopathic effects of *B. braunii* (inhibition of plankton growth) were found in the ethanol-soluble extract rather than in the water-soluble extract. A partitioning of the ethanol-soluble extract showed that active compounds were also ether soluble. Pure active compounds were obtained after further purification by gel permeation, followed by TLC and subsequent HPLC.

With the aids of NMR, gas chromatography-mass detector, infrared spectra, and IV-visible spectra, the purified compounds were identified to be free fatty acids. Qualitative analysis, by comparison with the authentic compounds and by co-chromatography, indicated that the purified compounds were α-linolenic acid (18:3), linoleic acid (18:2), oleic acid (18:1), and palmitic acid (16:0).

Toxicity of free fatty acids. Tests with three phyto- and three zooplankton taxa indicated that the four purified fatty acids have different toxicities (Table 3). The highest toxicity was observed with α-linolenic acid, followed by linoleic acid and oleic acid. With a very high IC_{50}, palmitic acid was relatively less toxic than C_{18} fatty acids to the organisms tested, except to *M. contortum*.

For zooplankton, the lowest LC_{50} values were obtained with α-linolenic acid, indicating the highest toxicity of this fatty acid. Linoleic acid was less toxic than α-linolenic acid to the three zooplankton tested. The LC_{50} values of other fatty acids, oleic and palmitic acids, were very high (i.e. $>200\,mg \cdot L^{-1}$), indicating that neither of them were toxic to zooplankton.

Amount of free fatty acids in Botryococcus braunii *cells.* Quantitative analysis with HPLC indicated that *B. braunii* cells cultured in the laboratory contained high amounts of oleic and α-linolenic acids and lesser amounts of palmitic and linoleic acids (Fig. 2). Similar results were obtained for cells cultured in the laboratory and those obtained from the natural system. The

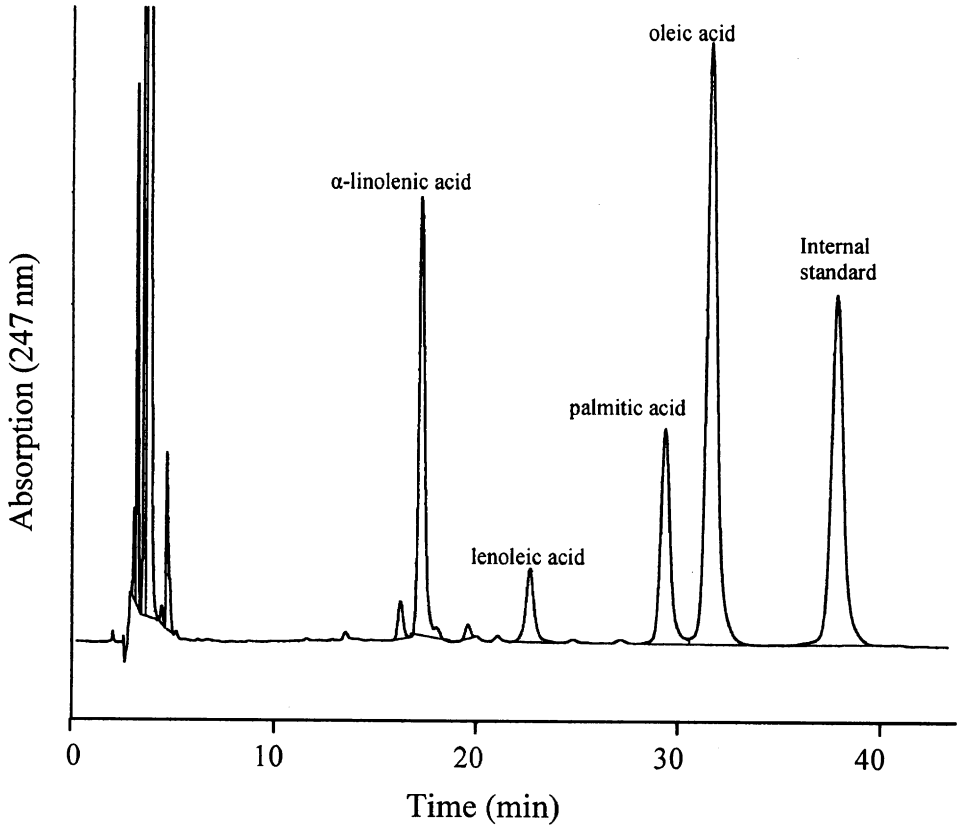

FIG. 2. HPLC chromatogram of the derivatives of free fatty acids in *Botryococcus braunii* cells detected at 247 nm. After a derivation reaction with 2-bromo-2-acetonaphthone and 18-crown-6, it was analyzed by a Hypersil HS 18C column (250 × 4 mm, 5 μm) and eluted with 93% methanol. Margaric acid served as an internal standard.

TABLE 4. Comparison of percentage composition of C_{18} free fatty acids (oleic acid, linoleic acid, and α-linolenic acid) in *Botryococcus braunii* cells from early and late stages of blooms and in five cultivated phytoplankton isolated from Liyu Lake.

Organism	$\mu g \cdot mg^{-1}$ dry weight of *B. braunii* cells			
	Oleic acid (18:1)	Linoleic acid (18:2)	α-Linolenic acid (18:3)	Total
B. braunii (from early stage of bloom)	11.5 ± 0.1	1.3 ± 0.0	5.5 ± 0.1	18.3 ± 0.2
B. braunii (from late stage of bloom)	4.9 ± 0.6	0.6 ± 0.1	4.3 ± 0.5	9.8 ± 1.2
Microcystis flos-aquae	0.2 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.3 ± 0.0
Chlorella sp.	0.1 ± 0.0	0.9 ± 0.1	2.4 ± 0.02	3.4 ± 0.2
Monoraphidium contortum	0.9 ± 0.2	0.7 ± 0.1	0.8 ± 0.1	2.4 ± 0.2
Scenedesmus quadricauda	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Gymnodinium sp.	1.1 ± 0.0	0.6 ± 0.1	0.5 ± 0.1	2.2 ± 0.1

Values are means ± SD ($n = 3$).

content of C_{18} fatty acids varied among cells, depending on the time of harvesting during a bloom (Table 4). Cells harvested from the early stage of blooming produced more C_{18} fatty acids than those from later stages. Moreover, the ratios of oleic to linoleic to α-linolenic acid varied. Proportionally, less oleic and linoleic acids were produced at the later bloom stage than at the early stage. As a result, α-linolenic acid became relatively more important as the bloom progressed.

The content of free fatty acids in *B. braunii* cells was compared with five cultured dominant phytoplankton species isolated from Liyu lake (Table 4). There were significant differences. In general, all the phytoplankton had a lower content of free fatty acids than *B. braunii*.

DISCUSSION

The present study showed that fewer phytoplankton species appeared in Liyu Lake during blooms of *B. braunii*. In addition, the abundance of phytoplankton co-occurring with *B. braunii* was negatively correlated with their susceptibility to *B. braunii* extract. This suggests that *B. braunii* may have exerted allelopathic effects on other phytoplankton.

Although Yamagichi et al. (1987) analyzed the lipid composition of two strains of *B. braunii*, nothing has been reported about the ecological role of fatty acids for this alga. Our study indicates that the strain of *B. braunii* growing in Liyu Lake has a significantly higher content of free C_{18} and C_{16} fatty acids than other phytoplankton isolates studied. The content of these acids in *B. braunii* cells is also higher than in *Pavlova* reported by Volkman et al. (1991) or 24 freshwater microalgae reported by Ahlgren et al. (1992). Possibly, the higher content of these free fatty acids in *B. braunii* cells might play a role in favoring its predominance over other phytoplankton in Liyu Lake.

The active compounds accounting for the toxic effects of *B. braunii* are four free fatty acids, including C_{18} and C_{16} fatty acids. C_{18} fatty acids, including linolenic, linoleic, and oleic acids, have been found to be major components of the toxin produced by *Chlamydomonas reinhardtii* and were shown to be toxic to a variety of algae (McCracken et al. 1980). Ikawa et al. (1996) reported that the growth of *Chlorella pyrenoidosa* was inhibited by fatty acid–containing fractions of *Microcystis aeruginosa*. The inhibitory activity was confirmed to be due to linoleic and linolenic acids. Linolenic acid from *Chlorococcum* and *Dunaliella* might act as an antibiotic against microorganisms (Ohta et al. 1995). C_{18} fatty acids were proven to be toxic to zooplankton (Spruell 1984). Thus, it is possible that, like many phytoplankton, *B. braunii* uses free fatty acids as an allelochemical to inhibit the growth of other plankton in its natural environment.

Douglas et al. (1969) reported that palmitic and oleic acids were the major components for a strain of *B. braunii* forming winter blooms. In contrast, oleic acid was found to be the major component of total acids in another strain of *B. braunii* isolated in Austin, Texas, USA (Yamagichi et al. 1987). In the present study, palmitic acid is shown to be less toxic to both the phytoplankton and zooplankton than oleic acid and two other C_{18} fatty acids. Thus, this fatty acid should play a less important role in the allelopathic activity of the strain of *B. braunii* found in Liyu Lake.

For the phytoplankton tested, about the same degree of toxicity was measured for all of three C_{18} fatty acids studied. For zooplankton, however, α-linolenic acid was more toxic than linoleic acid, whereas oleic acid was essentially nontoxic. Quantitative analysis demonstrated that the highest content was measured for oleic acid, followed by α-linolenic acid, regardless of the stage of bloom. Taking into account the specificity of toxicity and their content in the cells, oleic acid could be more important than other fatty acids for inhibiting the growth of other phytoplankton, and α-linolenic acid, in contrast, could be the major component of allelochemicals against zooplankton for *B. braunii* in Liyu Lake.

The amount of free fatty acids in *B. braunii* was found to vary when cells were harvested from different stages during blooms. It is known that the pigmentation, lipid, and hydrocarbon metabolism of *B. braunii* cells change with growth stage (Brown et al. 1969, Aaronson et al. 1983, Wolf et al. 1985). Fatty acids, particularly oleic acid, seem to be the key intermediate metabolites in the biosynthesis of certain hydrocarbons

(Laureillard et al. 1988). Presumably, the change in the amount of free fatty acids in *B. braunii* is related to the altered metabolism of hydrocarbons during blooming and, as a result, is associated with a change in its toxicity.

In principle, the free fatty acids produced by *B. braunii* are synthesized and stored in the cells. They become toxic to phytoplankton only when they are liberated by *B. braunii* from intra- to extracellular medium. This can happen when *B. braunii* cells disintegrate, particularly at the late stage of blooms. However, less is known about the relationship between the formation and disintegration of free fatty acids during bloom of *B. braunii* in the natural environment. To clarify this, further study is necessary.

In the present study, the free fatty acids of *B. braunii* cells were obtained by extraction with organic solvent. During extraction, an enzymatic hydrolysis of lipids or disintegration of cells might occur. These would give rise to an enhancement in the extracted amount of free fatty acids. As a result, the level of free fatty acids in *B. braunii* cells could be overestimated. However, the same extraction methods were used with five other dominant phytoplankton found in Liyu Lake. The results showed that all of them contained very low level in free fatty acids, compared with *B. braunii*. Thus, although the amount of extracted free fatty acids is not exactly the intracellular level, it appears that *B. braunii* cells have higher levels of free fatty acids than other dominant phytoplankton in Liyu Lake.

Our previous study showed that water in Liyu Lake was slightly alkaline, with pH usually between 8.0 and 9.0. Under such conditions, fatty acids are known to exist in a free form, namely $RCOO^-$. Fatty acids of this form are known to be more toxic to aquatic organisms than uncharged forms (Procter 1957). This was further verified by Vedediktov and Krivoshejeva (1983) by studying the inhibitory effects of fatty acids on photosynthesis. Apparently, the pH of Liyu Lake enhances the toxicity of free fatty acids and, as a result, favors the dominance of *B. braunii*.

It was observed that a number of fish died when *B. braunii* bloom occurred in Liyu Lake. However, no studies have been conducted to determine how *B. braunii* impacts fish. Because *B. braunii* is toxic to all the zooplankton tested in the present study, it seems possible that it is toxic to fish. However, an assay with fish is necessary to provide direct evidence for this hypothesis.

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