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Some observations on the green planktonic alga, *Botryococcus braunii* and its bloom form

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Abstract. The cells and colonies of *Botryococcus braunii* laboratory cultures were examined by interference and scanning electron microcopy and compared with cells isolated from a bloom in Lake Kinneret, Israel. The green cells were located in cups representing the parental cell wall; the cell walls of successive daughter cells appeared to be fused to the parent and daughter cells where they were contiguous. The arrangement of green cells in laboratory cultures was consistent; groups of 2 or 4 cells formed a unit and these units appeared joined together at their bases to form clusters. Green cells from the lake bloom appeared in irregular clusters of units of 2, 3 or more cells. Red bloom cells appeared to be dead with each cup compressed and empty with a hole showing at the cell surface. A hypothesis describing the role of senescence and nitrogen depletion, based on cytological, ecological, and chemical data, is invoked to explain the change of the bloom cells from green relatively low lipid to orange (or red) high lipid cells.

Introduction

Botryococcus braunii Kutzing, an unusually high lipid-producing green alga, appears to be distributed in oligotrophic and in some eutrophic fresh water, brackish and saline ponds, lakes or reservoirs on all continents (Table I). *B. braunii* has been found in lakes near the Equator in Nicaragua close to sea level (Lake Nicaragua) and Managua (Swain and Gilby, 1964) and in West Cameroon, Africa (Lake Debundsha) (Green *et al.*, 1974) and in alpine lakes up to 4680 m above sea level, in Peru, Sweden and Central Europe (Thomasson, 1956). Fossil *B. braunii* were also found in boghead coal, lignite, torbanite, coorongite, N'hangellite, etc. ranging in geological time from the Precambrian and Cambrian to the present and recent past (Table I). The fossil forms are even richer in lipids, especially hydrocarbons, than the living forms and in at least one instance may have been a source for petroleum (Moldowan and Seifert, 1980).

In many of the contemporary lakes, as in the past, *B. braunii* formed significant blooms at specific times of the year. These blooms appear to be somewhat variable in color and in lipid content: according to Blackburn (1936) and Maxwell *et al.* (1968), the bloom consisted of globular clusters of algae which appeared at first to be green in color and relatively low in lipids and hydrocarbons (~50% and 20% of the cell weight, respectively) but with time these algal clusters became orange or red and unusually rich in lipids and hydrocarbons (up to 83% and 76%

Table I. Distribution of *B. braunii* from fossil and living records.

Fossil records

Continent	Type of fossil material	Selected references
Europe	Lignite (Denmark)	Ingwersen, 1854
	Boghead coal (France)	Frémy and Dangeard, 1938
		Temperly, 1936
	(USSR)	Stadnikov and Vozzhinska, 1930
Africa	N'Hangellite (East Africa)	Redwood, 1907
Asia	Lignite (India)	Rao and Misra, 1949; Vimal, 1953
	Boghead coal (Pakistan)	Nagappa, 1957
	Coorongite (Siberia)	Morgan, 1921
	Petroleum (Indonesia)	Moldowan and Seifert, 1980
Australia	Coorongite, torbanite	Cane and Albion, 1973
North America	Boghead coal (Alaska)	Dietz, 1957; Gray, 1960
	Lignite (Oregon, Texas, Vermont)	Gray, 1960; Traverse and Barghorn, 1953
South America	Marahunite (Brazil)	Dietz, 1957)

Living records

Continent	Site	Type of water[a]	Selected references
Europe	Lakes, ponds	f.w.	Gams-Wasserburg, 1922 (Austria); Jessen and Milthers, 1928 (Denmark); Blackburn, 1936 (UK)
Africa	Lakes	f.w.	Green *et al.*, 1974 (West Cameroon); Boodle, 1907; Redwood, 1907 (Mozambique)
Asia	Lakes	f.w.	Ruttner, 1952 (Sumatra); Litinsky, 1921; Zalessky, 1917 (Siberia)
		sal.	Zalessky, 1914; Morgan, 1921 (Siberia)
Australia	Lakes, reservoirs	f.w.	Cane and Albion, 1973; Wake and Hillen, 1980
North America	Lakes, ponds, reservoirs	f.w.	Button and Blinn, 1973 (Arizona); Wolf and Cox, 1981 (Oklahoma); Paterson, 1962 (Maryland)
		sal.	Masters, 1971 (Canada)
South America	Lakes	f.w.	Dietz, 1957 (Brazil); Swain and Colby, 1964 (Nicaragua)
		br.	Gilson, 1964 (Bolivia, Peru)

[a]f.w., fresh water; sal, saline water; br, brackish water.

of the cell dry weight, respectively. Wake and Hillen (1980) found that the algal clusters they isolated from an Australian reservoir remained green but contained low percentages of red, yellow or white cells. The growth of axenic *B. braunii* in

large laboratory cultures permitted us to examine the cytology and lipid content of *in vitro* algae with bloom algae from Lake Kinneret in Israel. Furthermore, no one has consistently produced an *in vitro* orange culture from green cultures and no explanation exists for the conversion of the green actively growing form to the orange, presumably nongrowing form.

Materials and Methods

Organism and growth

An axenic culture of *B. braunii* Kutzing No. 572 was obtained from the University of Texas Algal Collection. Cultures were grown in a modified Chu 13 medium (Chu, 1942) with the following composition (amounts in g l^{-1}): KNO_3, 0.2; K_2HPO_4, 0.04; $MgSO_4.7H_2O$, 0.1; $CaCl_2.6H_2O$, 0.08; ferric citrate, 0.02 and citric acid, 0.2. The pH was adjusted to 9.0 with NaOH and the medium was autoclaved at 121°C for 15 min. Cultures for the growth curve were incubated in 10 l of the above medium in a 14 l jar in a New Brunswick Microferm laboratory fermentor at 20°C and 250 μ-Einstein's m^{-2} s^{-1} and aerated at 51 cm^3 s^{-1}. An aliquot of 1 l was removed weekly, examined microsopically, centrifuged and the pellet lyophilized for lipid analysis. Biomass was determined gravimetrically.

Scanning electron and light microscopy

Cells were examined with a Nikon research microscope equipped with interference optics. *In vitro*-grown cells for scanning electron microscopy were fixed in 3% (v/v) glutaraldehyde, desiccated by the critical point method in CO_2, and sputter-coated with gold-palladium (60:40) alloy. They were examined in a JEOL JSM-35U scanning electric microscope.

Collection and scanning electron microscopy of bloom cells

Bloom cells of *B. braunii* were collected in a plankton net in Lake Kinneret, Israel in September, 1980 and placed in a glass container. The cells rose to the surface of the container and were concentrated by flotation and skimming. Cells were air dried to avoid squeezing out the cells, shadowed with gold, and examined with a scanning electron microscope.

Lipid analysis

Total lipids were extracted by refluxing $\sim 0.1-0.2$ g of cells for 6 h in a Soxhlet extractor with 175 ml of $CHCl_3$:MeOH (2:1, v/v) mixture. Total lipids from two growth curve experiments were extracted by the Bligh and Dyer (1959) method using $CHCl_3$:MeOH (2:1, v/v) mixture and then cleaned on a dextran column (Siakotos and Rouser, 1965). Lipid extracts were reduced in volume under N_2 and reduced pressure and redissolved in a known volume of $CHCl_3$ and aliquots taken for gravimetric determination by drying samples to constant weight at 55°C. The lipid samples were fractionated by column chromatography on silica gel to give neutral, glycolipid and phospholipid fractions (Rouser *et al.*, 1967). All chemicals were purchased from commercial sources as reagent grade. All solvents were redistilled before use.

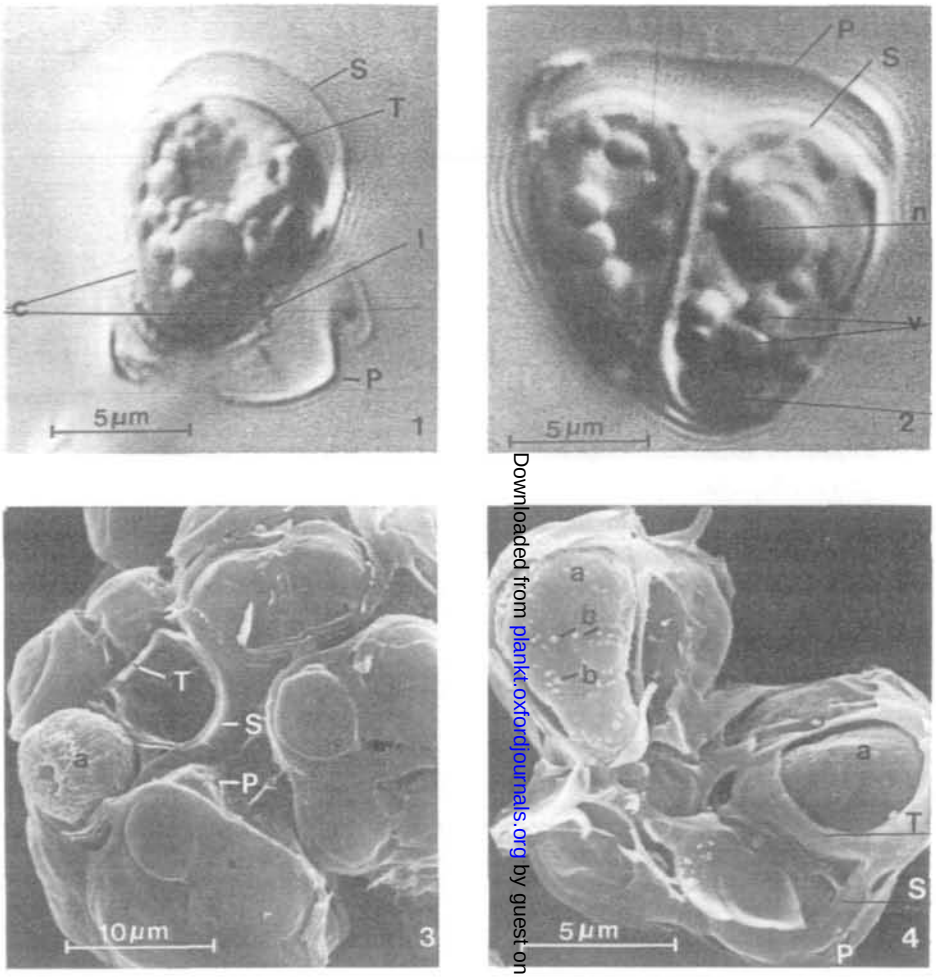

Fig. 1. Interference micrograph of *in vitro*-grown *B. braunii* cells. 3990 x. Abbreviations: a, algal protoplast; b, vesicles; c, chloroplast; l, lipid drops; n, nucleus; v, vacuoles; P, primary cell wall; S, secondary cell wall; T, tertiary cell wall.

Fig. 2. Interference micrograph of *in vitro*-grown cells. 3990 x. Abbreviations as in Figure 1.

Fig. 3. SEM of *in vitro*-grown cells. 2240 x. Abbreviations as in Figure 1.

Fig. 4. SEM of *in vitro*-grown cells. 3420 x. Abbreviations as in Figure 1.

Results and Discussion

Interference and scanning electron microscopy (SEM) were consistent with the light microscopic work of Blackburn (1936) and the transmission electron microscopy (TEM) of Schnepf and Koch (1978), Largeau *et al.* (1980) and Wolf and Cox (1981). Cells were enclosed in a cup or cell wall (primary wall) (Figures 1 and 2). Each cell divided into 2 daughter cells, each in its own cell wall (secondary wall) (Figure 3); each daughter cell produced 2 daughter cells, each with its own cell wall (tertiary wall) (Figure 3). Where these walls came in contact with each

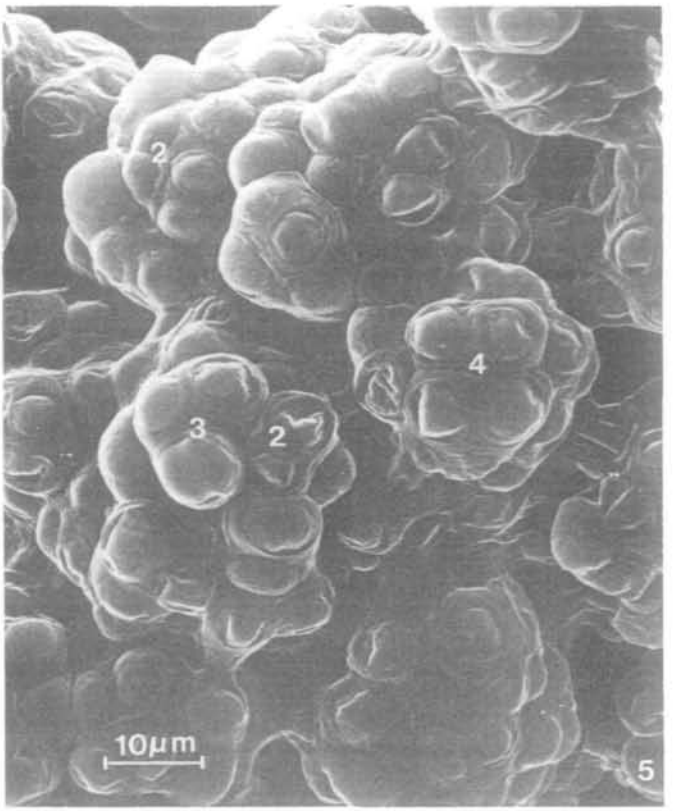

Fig. 5. SEM of green Lake Kinneret bloom cells. 1120 x. Abbreviations as in Figure 1.

other, they appeared to fuse (Figures 3 and 4). Interference microscopy revealed a row of lipid globules outside of and lining the secondary cell wall. The globules were held in place by the appressed primary cell wall and were adjacent to the chloroplast which was on the other side of the cell membrane (Figure 1) as first suggested by Schnepf and Koch (1978). *B. braunii* cells *in vitro* grew in clusters in even numbers (2, 4, etc.). The unit group of each cluster was 4 cells (Figure 3). Transparent adhesive tape was used on specimens affixed to SEM stubs to remove portions of the cell wall thereby revealing the cell in the cup (Figure 4). The arrangement of the primary secondary and tertiary walls is shown in Figures 3 and 4. The cells in the tetrad were about the same size and joined at the base with the other tetrads to form larger clusters. Cells eventually fall out of the cluster to form new clusters which were attached to the original cluster by fibrous materials. *In vitro*, individual clusters did not appear to increase in size beyond the 32–64 cell stage. Samples of green cells from Lake Kinneret examined by SEM showed that the clusters of green cells in the bloom were irregular in number. Groups of 2 or 3 or 4 cells were present with apparent discontinuities in the time of division of daughter cells (Figure 5). Clusters from the red bloom showed compression of individual cups, a large opening in each cell and ap-

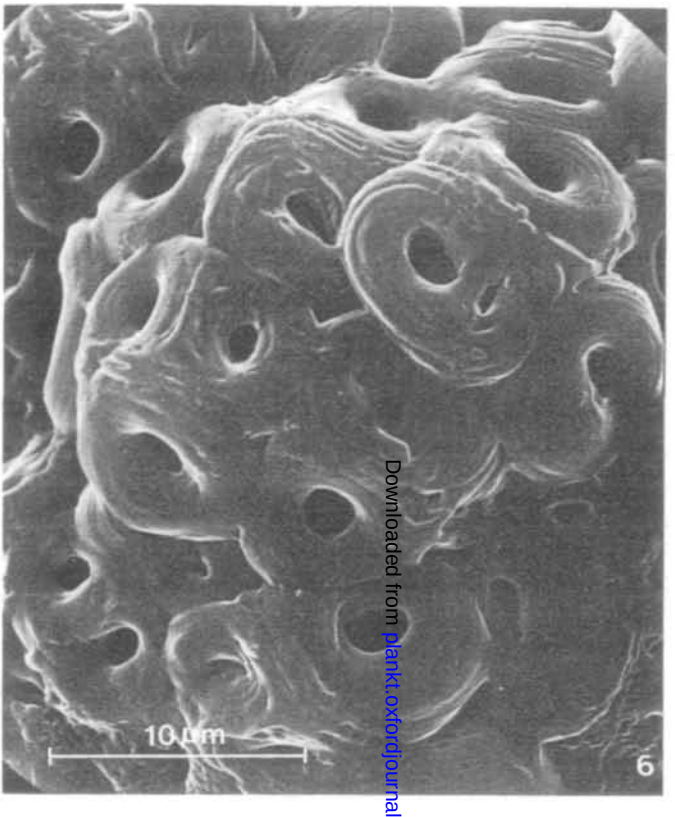

Fig. 6. SEM of red Lake Kinneret bloom cells. 3360 ×.

Table II. Biomass and lipid yield as a function of growth phase[a].

Incubation (days)	Biomass (mg/l)	Color of cells	Total lipid (% dry weight)	Neutral lipid (% dry weight)
0	25	Green	–	–
7	102	Green	–	–
12	231	Green	48.7	35.2
27	663	Light green	53.2	25.7
42	1061	Light green	50.9	32.0
57	1177	Light green	57.1	42.1
71	355	Green orange	54.0	43.5

[a]See Patni *et al.* (in preparation) for experimental details.

parently empty space where the protoplast or cell had been (Figure 6).

Light microscopic examination of orange *B. braunii* cells from fermentor cultures showed cell debris and apparently dead cells. Attempts to culture orange cell clusters were unsuccessful. The change in color of the cells in the fermentor

cultures from green to orange and the presumed autolysis inferred from the drop in biomass at the end of the stationary growth phase, are viewed here as evidence of a senescent culture (Table II). The total lipid of senescent cells rose slightly but the neutral lipid (mostly hydrocarbon) content of the *in vitro* senescent cells increased significantly (11% and 24%, respectively) over the neutral lipids of log-phase cells. The rise was not as high, however, as that reported in orange cells in natural blooms where as much as 83% and 76% of the cell dry weight was lipid and hydrocarbon, respectively (Belcher, 1957; Maxwell *et al.* 1968). In contrast to bloom cells which were collected by flotation and skimming, and were only slightly green, *in vitro* culture cells never floated on the surface of their medium and were mainly green in color. However, the numbers of reddish orange cells that appeared on the walls of the culture vessels at the meniscus increased with culture age. *In vitro* cells depleted of nitrogen (KNO_3) or grown on low nitrogen (0.1 mM KNO_3) appeared green and never floated on the medium. These cells contained 64–73% of total lipids and 49–55% of hydrocarbon by cell dry weight. Details of these experiments will be published elsewhere. From the observations on the laboratory cultures and natural blooms, we suggest that the orange (or red) floating bloom found in nature with its high lipid and hydrocarbon content results when *Botryococcus* blooms begin to run out of nitrogen, stop growing, and enter a senescent stage. This hypothesis is supported by the appearance of red *Botryococcus* blooms in Lake Kinneret in late September at a time when the nitrogen content of the hypolimnion had been significantly depleted by earlier algal blooms, especially that of *Peridinium* (Berman, 1973). Wake and Hillen (1980) made a similar observation on a *Botryococcus* bloom in the Darwin river reservoir in Australia. Apparently these high lipid and hydrocarbon-containing bloom cells are not always biodegraded; they may accumulate on the shores of lakes where blooms occur and become fossilized in time as boghead, coal, coorongite, etc.

Acknowledgement

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