AN ABSTRACT OF THE THESIS OF

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Title: THE EFFECT OF VARIATION OF SOME PHYSICAL AND CHEMICAL FACOTRS ON THE RATE OF GROWTH OF BOTRYOCOCCUS BRAUNII (CHLOROPHYTA, CHLOROCOCCALES)

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The culture of the green alga Botryococcus braunii Kütz. obtained for use in this study was contaminated with bacteria and fungi. Algal colonies from this culture sonicated, rinsed and treated with penicillin (500 µg/ml), gentamicin sulfate (50 µg/ml) and amphotericin B (5 µg/ml) were found to be axenic when incubated in ten microbiological media.

The density of the cultures was determined as the dry weight of the alga. The loss of hydrocarbon during this procedure was examined and small losses were found. The minimum size of algal sample needed to accurately determine the density of a culture was estimated to be 0.16 mg of the alga.

The increase in density of the axenic B. braunii culture grown in a modified Chu no. 10 medium over an extended period of time was monitored. Logarithmic growth was observed for the first 15 days with an approximate 3-day generation time. The rate of growth of the alga decreased greatly after 20 days.

The effect of media containing various concentrations of nitrogen on the growth of the alga was observed. The rate of growth of the alga increased with increasing concentrations of nitrogen up to 6.6 p.p.m. Above this concentration no further increase or decrease in the rate of growth was observed.

The effect on growth of varying concentrations of phosphorus was investigated and a relatively small influence was observed with the concentrations of phosphorus used.

Two experiments were performed on the effect of varying the H^+ -ion concentration on the growth of the B. braunii. The alga was observed to grow at a rate relatively unaffected by variation of H^+ -ion concentrations ranging from pH 5.5 to 9.0. At a pH above or below this range there appeared to be a decrease in rate of growth.

The effect on growth of various temperatures was also investigated. The rate of growth of the alga at 12 C and 25 C was relatively similar. At 29 C no increase was observed.

The Effect of Variation of Some Physical and Chemical Factors on the Rate of Growth of *Botyrococcus braunii* (Chlorophyta, Chlorococcales)

by

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INTRODUCTION AND LITERATURE REVIEW

Botryococcus braunii Kütz. has been the object of considerable geological and chemical interest. Much of the geological interest centers around the finding of Botryococcus in a bituminous shale boghead coal (torbanite) and a naturally-occurring rubber-like deposit coorongite. Some controversy has been raised concerning the role of Botryococcus in forming these substances and good reviews of past reports and findings are available (Thiessen 1925, Blackburn 1936 Temperley 1936, Traverse 1955, Douglas et al. 1968, Cane 1977).

Hydrocarbon production of B. braunii has been the center of the chemical interest in the alga. Brown et al. (1969) have reported three physiological states of the alga which are characterized by their hydrocarbon content. One of the states (brown, resting state) is characterized by a high concentration of two isomeric hydrocarbons: botryococcene and isobotryococcene. Maxwell et al. (1968) reported finding the hydrocarbon fraction of B. braunii, in the brown, resting state grown in nature, to comprise 76% of the dry weight of the alga. In actively growing cultures as used in this study, other hydrocarbons are found of smaller molecular weight and in a much smaller percentage (ca. 20).

Despite the interest in the hydrocarbon production of

Botryococcus and the alga's occurrence in geological sediments, few studies of the alga's response to culture conditions have been performed. There has been some culture work performed by investigators of the hydrocarbons produced by B. braunii. Brown et al. (1969) investigated the effect of varying the culture conditions upon the hydrocarbon synthesis of the alga. By using a high light intensity and reduced concentration of nitrogen, the characteristic hydrocarbon production of colonies was routinely altered. Wolf et al. (1980) reported the rate of hydrocarbon production increasing due to high intensity light regimes and certain nutrient deficiencies. Wolf (personal communication) has investigated variation of hydrocarbon production in 40 to 50 different culture media.

The only extensive work in culturing B. braunii has been performed by Chu (1942, 1943) and Belcher (1968). Chu investigated the effect of the chemical composition of media on the growth of B. braunii and other planktonic algae. Botryococcus, isolated by Chu, was grown in various media containing nitrogen as ammonia and/or nitrate and was found to grow best, as determined by turbidity measurements, in media containing nitrate as the only nitrogen source. Suitable concentrations of different salts and elements commonly found in culture media (e.g. KNO_3, K_2HPO_4, Ca, Mg) were estimated and compared with available ranges reported in nature. In the second part of Chu's (1943) study, the effect of various concentrations of phosphorus and nitrogen on growth were investigated. These findings are reported further in Discussion. The changes in color and morphology of the alga due to the different phosphorus and nitrogen concentrations were described.

In the study of B. braunii by Belcher (1968), the alga was cultured in a modification of a medium (no. 10) developed by Chu. The growth cycle of Botryococcus, which contained bacterial contamination, was investigated by measuring the increase in growth as colony number and dry weight. The changes in chemical composition of colonies in aging cultures were monitored. During separate experiments, the pigment content of Botryococcus was found to have increased with increasing light intensities or temperatures. Belcher also observed low concentrations of nitrogen increasing pigment and lipoid accumulation. The respiration of Botryococcus was studied by manometric methods in the dark and with various organic compounds.

If B. braunii was cultured in such a way as to produce hydrocarbons in high percentages, as found in the brown, resting state colonies grown in nature, the alga could be a possible source of hydrocarbon. Little success has been achieved in culturing the alga at conditions suitable for this high percentage of hydrocarbon production. Even if the colonies did have high percentages of hydrocarbon, using the alga for large scale production of hydrocarbons would not be feasible, unless it would grow at a substantial rate.

The objectives of this study are to investigate the effect of varying certain factors on the rate of growth of B. braunii and to determine the ranges of those factors which support optimal growth. It is hoped that information here will be useful for increasing the alga's rate of growth.

MATERIALS AND METHODS

Culture Methods

A culture of Botryococcus braunii Kütz. was obtained from the University of Indiana Culture Collection of Algae at Bloomington, Indiana (now at the University of Texas, Austin Texas). This algal culture, no. LB572, had both fungal and bacterial contaminants.

The alga was cultured in a modification (Table 1) of Chu no. 10 medium (Chu 1942). The sodium silicate has been omitted, sodium carbonate replaced with sodium bicarbonate and iron added in the form of ferric citrate at an equivalent concentration of iron to that found in the original Chu no. 10 medium. The micronutrients were the same as those used in the Woods Hole MBL medium (Nichols 1973) with the exclusion of ferric chloride and the chelate (Na_2EDTA). The Tris [tris (hydroxymethyl) aminomethane] buffer was added to prevent precipitation. Further modifications of the medium necessary for some of the experiments are listed with those experiments. Separate stocks of each macronutrient, the buffer and the iron sequestrine solution were prepared. A single stock solution was made for the combined micronutrients. One ml of each stock was added to 1 liter of glass distilled water and the resulting medium was autoclaved in individual culture containers. The chemicals used for culturing the alga were reagent grade as recieved from either Mallinckrodt, Inc., or J.T. Baker Chemical Co., or their purity and source were as shown in Table 2.

All the culture flasks used in this study initially were acid washed with concentrated H_2SO_4 containing NOCHROMIX®. The acid

Table 1. Composition of Chu no. 10 medium and modification

	Chu no. 10	Modification
Macronutrients	mg/l	mg/l
$Ca(NO_3)_2$	40	
$Ca(NO_3)_2 \cdot 4H_2O$		57.6
K_2HPO_4	10	10
$MgSO_4 \cdot 7H_2O$	25	25
Na_2CO_3	20	
$NaHCO_3$		15.8
Na_2SiO_3	25	
Iron Source		
Fe Citrate		1.47
Na_2EDTA		4.5
$FeCl_3$	0.8	
Micronutrients		
$CuSO_4 \cdot 5H_2O$		0.01
$ZnSO_4 \cdot 7H_2O$		0.022
$CoCl_2 \cdot 6H_2O$		0.01
$McCl_2 \cdot 4H_2O$		0.18
$Na_2MoO_4 \cdot 2H_2O$		0.006
Buffer		
Tris		80
		pH=7.3

Table 2. Purity and source of chemicals. Chemicals not listed here that were used for culturing the alga were reagent grade as received from either Mallinckrodt, Inc., or J.T. Baker Chemical Co.

Chemical	Purity	Source
$MgSO_4 \cdot 7H_2O$	U.S.P.	Mallinckrodt, Inc.
$NaHCO_3$	reagent grade	campus chemical store
Fe Citrate	U.S.P.	Fisher Scientific Co.
FeEDDHA	tech. grade	CIBA GEIGY Corp.
$CuSO_4 \cdot 5H_2O$	C.P.	campus chemical store
KCI	reagent grade	Baker & Adamson
$Na_2MoO_4 \cdot 2H_2O$	a	campus chemical store
Tris	buffer grade	Nutritional Biochemicals Corp.
Mes		U.S. Biochemical Corp.
Ches		U.S. Biochemical Corp.
Hepes		Sigma Chemical Co.
Pipes		Sigma Chemical Co.
Pencillin G		Sigma Chemical Co.
Gentamicin Sulfate		Sigma Chemical Co.
Amphotericin B (Fungizone®)		Grand Island Biological Co.
Thiamine		Sigma Chemical Co.
Cyanocobalamin		Sigma Chemical Co.
Biotin		Sigma Chemical Co.
Octacosane	97%m m.p.=61-63C	Aldrich Chemical Co.

[a]Purity was not known, but it was thought to be reagent grade.

washed flasks were then washed in hot tap water containing Labtone®, as they were after each subsequent use. The flasks were then rinsed with hot tap water and distilled water.

Stocks of the axenic algal culture (see Procedure for Obtaining Axenic Cultures) were maintained in petri plates containing 1.5% agar in the modified Chu's medium. Algal material from these stock cultures was used to inoculate axenic static cultures in 50 ml of liquid medium in 250 ml flasks with plastic foam stoppers. After suitable growth occurred, 10 ml of inoculum from the 50 ml culture was transferred aseptically to each of three 250 ml static cultures in 1 liter flasks plugged with cotton or plastic foam. The closure of the flask was in all cases covered with aluminum foil or with a stainless steel closure to reduce evaporation and to prevent contamination. "Cool White" fluorescent tubes on a 16-hour light (8-hour dark) cycle provided the stock cultures on agar with a light intensity of approximately 130-150 foot candles and a light intensity of approximately 200-350 foot candles for the 50 ml and 250 ml stock cultures. All the algal stock cultures were maintained at 25 C.

The cultures used in the algal growth experiments were inoculated from the 250 ml stock cultures. The experimental cultures were grown in 250 ml cotton stoppered flasks capped with aluminum foil and containing 50 ml of culture medium.

A Gyrotory® shaker (Model VS New Brunswick Scientific) was used for all the culture experiments, except Experiment 8. An 86 cm x 81 cm plywood sheet with a 90 position grid for 250 ml flasks was attached to the shaker's platform. The culture flasks were secured with double-coated tape to their assigned positions. The shaker was operated

at approximately 210-230 revolutions per minute during the course of the study. Continuous illumination for the cultures on the shaker was provided by arranging three "Cool White" and three "Warm White" fluorescent tubes in alternating order above the shaker. Aluminum foil reflectors were used to distribute the light uniformly. Light intensities ranged between 660 and 840 foot candles[1] during the course of the study, with no greater range than ± 50 foot candles during any one experiment. A fan dissipated the heat from the lights. The temperature of the cultures on the shaker was maintained at 23 ± 1 C, by adjusting the temperature of the air conditioned room. One or more 250 ml flasks containing approximately 50 ml of water on the shaker were used to determine the experimental cultures' temperature.

Four growth chambers, each at a different temperature, were used in Experiment 8. The static cultures were maintained at 6 ± 2 C, 12 ± 1 C, 25 ± 1 C and 29 ± 1 C in the growth chambers. "Cool White" fluorescent tubes supplied light intensities of approximately 150-170[2], 250-350, 280-320 and 260-380 foot candles, respectively. These cultures were provided with a 16-hour light (8-hour dark) cycle.

Procedure for Obtaining Axenic Cultures

In order to obtain single algal cells and small portions of algal

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[1]See Appendix Table I for a partial record of light intensities in microeinsteins/(m^2) (sec).

[2]Light intensity at 6 C is significantly lower than at other temperatures due to an error. The initial light intensity reading was taken at a temperature (estimated to be ca. 15 C) higher than the 6 C culture temperature. The light intensity decreased considerably as the temperature decreased. The final estimate of light intensity (ca. 150-170 ft-c) was based on light intensity readings at both 15 and 6 C taken later.

colonies, the colonies were sonicated with a BIOSONIC IV® (Brownwill) sonicator for five 1-minute intervals with a 2-minute period between each sonication. To help remove fungal hypae and larger colony fragments, the sonicated algal cells were gravity filtered through sterile cheesecloth, and then rinsed through the cheesecloth with sterile culture medium into a sterile flask. The resulting cell suspension was filtered onto a sterile 1.2 µm Millipore® filter (25 mm diameter), the cells collecting on the filter. The cells were then rinsed with 45 ml of sterile culture medium, using 15 ml per rinse. The filter with adhering algae was placed into 23 ml of sterile culture medium, which was then swirled. Approximately 0.5 ml of the suspension of sonicated, rinsed cells was pipetted onto 1.5% agar prepared with the modified Chu no. 10 medium containing penicillin G (500 µg/ml), gentamicin sulfate (50 µg/ml) and amphotericin B (5 µg/ml). The inoculcated agar plates were placed for 48 hours at the standard culture conditions for the 50 ml and 250 ml stock cultures. Some of the treated algae were then transferred with an inoculating loop to agar plates containing only the nutrients of the algal culture medium, and cultured under the conditions of the liquid stock cultures.

Sterility tests: Colonies, which appeared to be free of fungal or bacterial contamination by observations with a stereomicroscope, were incubated in ten microbiological media (Table 3) at 25 and 30 C for 1-3 weeks to check for contamination. Colonies from a culture of B. braunii known to be contaminated were incubated in the same microbiological media at 30 C for 6 to 13 days or until contamination was observed to aid in the recognition of contamination in the test media. The inoculated test media were observed with the unaided eye and with

Table 3. Microbiological media. Colonies treated with antibiotics and amphotericin B incubated for 1-3 weeks at 25 and 30 C. Colonies of contaminated culture incubated 6-13 days at 30 C.

1. Nutrient agar (Difco).
2. Potatoe dextrose agar (Difco).
3. Cornmeal agar (Difco).
4. Nutritive caseinate agar (Difco).
5. Modified Chu no. 10 medium plus proteose peptone (Difco) (5 mg/ml), tryptone (Difco) (5 mg/ml), thiamine (0.2 µg/ml), cyanocobalamin (0.001 µg/ml) and biotin (0.001 µg/ml).
6. Above medium plus 5% soil extract.[a]
7. Fluid thioglycollate medium (Difco).
8. Nutrient broth: peptone (Difco) (5 mg/ml) and beef extract (Difco) (3 mg/ml) in glass distilled water.
9. SST: glucose (10 mg/ml), tryptone (Difco) (10 mg/ml) and yeast extract (Difco) (5.0 mg/ml) in glass distilled water (Hoshaw and Rosowski 1973).
10. Peptone-glucose: glucose (1 mg/ml) and peptone (Difco) (1 mg/ml) in glass distilled water (Hoshaw and Rosowski 1973).

[a]Soil extract prepared as follows: 1.0 kg potting soil containing Sphagnum in 1.0 liter of glass distilled water was steamed for 1 hour on two consecutive days. The extract used in the above medium was filtered sterilized (.45 µm Millipore® filter).

a stereomicroscope. A final check for contamination was made by streaking cells from the nutrient broth used for determining sterility of the treated algae on a nutrient agar plate. The nutrient agar plates were incubated for 2 weeks at 30 C.

A culture of B. braunii inoculated with algae treated with antibiotics and amphotericin B and which had been checked for contamination as described above was also checked for bacterial contamination by direct microscopic examination at 400X. This culture then was used to inoculate the stock cultures for the algal growth experiments. The stocks were checked routinely for contaminants by incubation on nutrient agar at 30 C for 2 weeks.

Determination of Dry Weight of The Alga

Nuclepore® filters 47 mm diam., 8.0 μm pore size were exposed to α - radiation from polonium (Staticmaster®, Nuclear Products Co.) to eliminate static charges and dried at approximately 70 C. After 24 hours the filters were cooled in a desicator for a minimum of 10 minutes, then weighed to the nearest μg on an electric Perkin Elmer Autobalance® Model AD-2. Aliquants of uniform suspensions of the alga or entire cultures were then filtered onto individual prepared filter discs and dried at approximately 70 C for 48 hours, cooled in a desiccator and weighed as before. The dry weight of algal material was determined by subtracting the initial dry weight of the filter disc from the dry weight of the filter bearing algal material.

Experimental Procedures

Preliminary Experiments

Experiment 1: Loss of Hydrocarbon on Drying One experiment performed before the algal growth experiments checked for loss of hydrocarbon during drying. Samples of octacosane, which has a similar chemical structure to the predominant hydrocarbons reported in the green form of B. braunii (Brown et al. 1969, Gelpi et al. 1968), were initially weighed in tared, aluminum foil weighing pans approximately 1.5 cm in diameter, then dried at 75 C. Three samples were dried for 24 hours, allowed to cool, then weighed to the nearest 0.01 mg. These samples were again weighed after an additional 24 hours of drying. One other sample was weighed after drying for 4 days and weighed again after drying for 3 days. The weight changes during the drying periods were determined.[3]

Experiment 2: Determination of Minimum Size of Algal Samples A second preliminary experiment was performed to determine the minimum dry weight of Botryococcus sample required to estimate the density of a culture when determining the dry weight by the procedure used in this study. The density of a 250 ml stock culture was estimated from a variety of sample sizes. Each of three 10 ml samples from the culture were diluted with algal culture medium to final volumes of 100 ml. One half (50 ml) of each of the three well mixed suspensions was sampled, then one half of the remaining suspensions was sampled. This process was repeated until samples containing 1/2; 1/4; 1/8; 1/16; and 1/32 of the original algal mass in 10 ml were obtained. Three undiluted 10 ml samples from the 250 ml stock culture were also taken. All

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[3]During the hydrocarbon drying period, the sample usually emigrated to the underside of the weighing pan, then adhered there when cooled to be weighed. Once out of the weighing pan, no attempt was made to place the sample back into the pan before an additional drying period.

the samples were filtered onto tared 8.0 μm Nuclepore® filters (47 mm diameter), dried at 75 C for 72 hours and weighed. The mean dry weight of alga from each set of three samples having equal quantities of the alga was used to estimate the density of the 250 ml stock culture. By comparing the estimates of culture density and the dry weights of alga used to calculate those estimates, a minimum dry weight of algal sample needed to determine the density of a culture was established. This estimated minimum dry weight of algal sample was used as the minimum dry weight of inoculum for the experimental cultures.

Culture Experiments

Six of the major experiments performed in this study, involving the culturing of B. braunii, are reported here. One culture experiment investigated the alga's growth rate over an extended period of time. Other experiments examined the effect on the alga's growth rate of varying individually the concentrations of nitrogen and phosphorus in the culture medium. Separate experiments investigated the effect of variation of H^+ -ion concentration and temperature on the rate of algal growth.

Prior to starting a culture experiment, the mg dry weight of alga/liter was estimated for the one or the two pooled 250 ml stock cultures used to inoculate the experimental cultures. The density of a 250 ml stock culture was monitored before an experiment by periodically taking a 10 or 20 ml sample and determining the dry weight of the alga. The 250 ml stock cultures were started in sets of three, and each of the three cultures were inoculated with identical amounts of the alga (see

Culture Methods). The algal density determined for the one 250 ml stock cultures was used as an estimate of the density of the other cultures in its set. Aliquants from these cultures were used to inoculate the experimental cultures.

The volume of inoculum used in the culture experiments varied from 2 to 5 ml, depending on the estimated density of inoculum. Each culture in any one experiment was inoculated aseptically with an equal volume of algal suspension. After inoculating the experimental cultures, three or four 20 ml samples were taken from the remaining suspension of inoculum. The dry weights of these samples were determined to calculate the actual inoculum density, from which the initial density of the experimental cultures was computed.

For all the culture experiments, except Experiment 8, the inoculated cultures were attached to the surface of the shaker at positions assigned by the use of a random numbers table. In Experiments 8 the static cultures were rotated daily to new positions.

When the density of an experimental culture was determined, the entire 50 ml culture was filtered onto one or more tared filters. The combined dry weight of the alga on all the filters from one culture was used to calculate the culture's density as mg dry weight of alga/50 ml, which was converted to mg dry weight of alga/liter. The mean density of each set of replicate cultures was determined and the standard error of the mean was computed.

The generation time (days per dry weight doubling of alga) was calculated for the alga during various growing periods for each culture experiment. To calculate the generation time, first the doublings in dry weight of alga per day (D) was calculated by the

equation:

$$D(\text{doubling/day}) = \frac{\log_2(W_1/W_0)}{t_1-t_0} = 3.322\ \frac{\log_{10}(W_1/W_0)}{t_1-t_0}\ ,^4$$

Where W_0 and W_1 are the mean dry weights of alga at the beginning and end of the period during which the number of doublings per day was calculated and t_0 and t_1 are the corresponding times (days) at which W_0 and W_1 were determined. The generation time was determined by 1/D.

Experiment 3: Rate of Growth Over an Extended Period of Time The growth of the alga was monitored over a 30-day period. Thirty identical algal cultures were inoculated and placed on the shaker. Five[5] of the cultures were harvested every five days to determine the densities of the cultures. The experimental cultures were grown under the culture conditions reported for the shakers surface (see Culture Methods). The culture medium was modified[6] in this experiment by replacing the $Ca(NO_3)_2 \cdot 4H_2O$ with 40 p.p.m. $NaNO_3$ and 35.8 p.p.m. $CaCl_2 \cdot 2\ H_2O$ which provided equivalent concentrations of nitrogen and calcium.

The initial pH of the medium was measured with an Orion model 801 digital pH/mv meter and changes were recorded over the course of this experiment. The pH of one of the cultures being harvested was measured on each sampling day.

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[4]This equation is based on Guillard's equations to calculate growth rates of algae as divisions per day (Guillard 1973).

[5]One of the cultures intended to be sampled on day 30 was lost.

[6]$MgSO_4$ concentration in culture medium during Experiment 3 was 31 p.p.m. instead of the usual 25 p.p.m., due to an error in $MgSO_4$ stock solution preparation.

Experiment 4: Effect on Growth of Varying the Concentration of Nitrogen To investigate the effect of variation in the availability of nitrogen on the growth rate of B. braunii, media were prepared having five different concentrations of nitrogen. The $Ca(NO_3)_2 \cdot 4H_2O$ (6.8 p.p.m. N) in the algal culture media was replaced by $NaNO_3$, which was added at concentrations of 0.40, 4.0, 40, 80 and 400 p.p.m., corresponding to 0.066, 0.66, 6.6, 13.2 and 66 p.p.m. nitrogen, respectively. To replace the calcium, 35.8 p.p.m. $CaCl_2 \cdot 2H_2O$ was added to the different media to provide a concentration of calcium equivalent to that found in the modified Chu no. 10 medium. Five replicate cultures grown in each concentration of nitrogen were harvested every five days during the 15-day experiment.

Experiment 5: Effect on Growth of Varying the Concentration of Phosphorus The influence of eight different concentrations of phosphorus on the alga's growth rate was examined. The K_2HPO_4 (1.8 p.p.m. P) in the algal culture medium was replaced by Na_2HPO_4 and KCl. Media in the experiment had concentrations of Na_2HPO_4 of 0.10, 0.50, 1.0, 5.0, 10, 50, 100 and 500 p.p.m., which corresponded to 0.022, 0.109, 0.218, 1.09, 2.18, 10.9, 21.8 and 109 p.p.m. phosphorus, respectively. To all the media 8.56 p.p.m. KCl was added, providing potassium at a concentration equal to that previously supplied by the K_2HPO_4. To prevent precipitation at the higher phosphate concentrations, stock solutions of Na_2HPO were autocalved separately from the rest of the medium and added aseptically after cooling. Five replicates were grown at each concentration of phosphorus and harvested after 15 days.

Experiment 6: Effect on Growth of Varying the H^+ -ion Concentration, pH 5.0 - 10.0 Two experiments investigated the effect of variation in

H^+ -ion concentration on the rate of growth of the alga. In the first experiment the alga was cultured in nine media of different H^+ -ion concentrations ranging from pH 5.0 to 10.0. Ten treatments (two at pH 8.0) were obtained by replacing the 0.66 mM Tris in the algal culture medium with 10 mM of the following buffers:

	pH
Mes [2-(N-morpholino)ethanesufonic acid]	5.0 5.5 6.0
Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)	7.0 8.0
Tris	8.0
Ches (cyclohexylaminoethanesulfonic acid)	8.5 9.0 9.5 10.0

The iron was added to the media as 4.58 p.p.m. FeEDDHA (ferric ethylenediamine di-o-hydroxyphenyl acetate) (0.28 p.p.m. Fe), replacing the ferric citrate and Na_2EDTA, to better keep the iron in solution in the cultures at a high pH. The $Ca(NO_3)_2 \cdot 4H_2O$ and K_2HPO_4 were added aseptically to the media having a pH of 8.5 to 10.0, after the media had been autoclaved and cooled to prevent precipitation due to the presence of calcium or phosphorus. Five replicates were inoculated for each treatment. After a 15-day culture period, the pH of each treatment was checked, and the cultures were harvested.

Experiment 7: Effect on Growth of Varying the H^+ -ion Concentration, pH 6.0-7.9 In the second H^+ -ion concentration experiment, B. braunii was cultured at pH 6.0, 7.0, 7.5 and 7.9 in media buffered with 10 mM Pipes [piperazine-N,N'-bis(2-ethanesulfonic acid)]. Replacement of the Tris buffer with the Pipes buffer is the one modification of the

medium in this experiment. There were five replicates for each treatment in the 15-day experiment. The final pH of the cultures of each treatment was determined; then the cultures were harvested.

Experiment 8: Effect on Growth of Variation in Temperature In this experiment static algal cultures were grown at 6, 12, 25, and 29 C, at the culture conditions explained in the Culture Methods section. One change in the algal culture medium was made for this experiment by replacing the Tris buffer with 10 mM Mes at a pH of 6.0. Five replicates were grown at each temperature for 21 days. The cultures were swirled twice per day. At the end of the experiment, the pH of the cultures at each temperature was determined and the cultures were harvested and their densities determined.

RESULTS

Sterility tests: No contamination was found in the B. braunii culture used to inoculate the stock cultures for this study. Subsequent checks for contamination in the stocks used to inoculate the experimental cultures indicated that there were no bacterial or fungal contaminants present.

The microbiological media (Table 3). inoculated with the contaminated culture, all showed signs of contamination except the potato dextrose agar, nutritive agar and fluid thioglycollate medium.

Experiment 1: Loss of Hydrocarbon on Drying The loss in weight of octacosane samples dried at 75 C are shown in Table 4. Two (#1 and #3) of the three samples after drying 48 hours showed losses of approximately 3 and 4%, whereas the other sample (#2) showed a weight loss of 23%. The one sample (#4) weighed after drying 7 days had a total loss of approximately 4%.

Experiment 2: Determination of Minimum Size of Algal Samples The mean dry weights of alga (Table 5) are reported for fractions of the algal mass in 10 ml of a 250 ml stock culture. The estimates of culture density from these mean dry weights of alga are also reported. The estimates of culture density were approximately 130 mg/1 for algal samples fractions 1 - 1/8. Estimates of density of the smaller fractions were considerably below 130 mg/1. The smallest mean dry weight of alga (from the 1/8 fraction) which gave a culture density estimate of 130 mg/1 was 0.16 mg. This weight (0.16 mg) was considered an

Table 4. Experiment 1. Loss of octacosane (mg and %) reported for samples dried at 75 C.

Sample Number	Initial Weight Octacosane	Total Loss of Octacosane			
	mg	day 1	day 2	day 4	day 7
1	8.89	0.13(1.5%)	0.38(4.3%)		
2	16.15	0.11(0.7%)	3.77(23.3%)		
3	13.62	0.19(1.4%)	0.40(2.9%)		
4	18.39			0.31(1.7%)	0.76(4.1%)

Table 5. Experiment 2. Mean dry weight of alga (mg) of algal sample fractions and estimates of their culture density (mg/l). The algal samples were fractions of algal mass in 10 mls of a 250 ml stock culture. There were three replicates of each fraction. The algal samples were filtered and dried at 75 C for 75 hours prior to weighing.

Algal Sample Fraction	Mean Dry Weight of Alga mg	Estimate of Culture Density mg/l
1	1.26	126
1/2	.661	132
1/4	.327	131
1/8	.163	131
1/16	.066	105
1/32	.021	64

estimate of the minimum dry weight of an algal sample needed to determine a culture's density and it was considered the minimum dry weight of inoculum for the culture experiments.

Experiment 3: Rate of Growth Over and Extended Period of Time The increase in density of B. braunii cultures during a 30-day growing period is shown in Figure 1 and Appendix Table II. There was a steady increase in (mean) density up to day 20, after which the densities leveled off a little above 200 mg/1.

The generation time (Table 6) of each 5-day growing period up to day 15 was approximately 3 days. The generation time increased approximately two-fold during the following growing period (day 15 - 20), then greatly increased after day 20.

The pH of the cultures (Table 7) increased from the initial 7.3 to a pH of 8.1 by day 15, then decreased slightly to 7.8 by day 30.

Experiment 4: Effect on Growth of Varying the Concentration of Nitrogen After five days of growth, the B. braunii cultures at each nitrogen concentration had similar densities (Figure 2 and Appendix Table III). By day 10 the cultures at the two lower nitrogen concentrations (0.066 and 0.66 p.p.m.) had substantially lower densities of the alga than the cultures at the three highest nitrogen concentrations (6.6, 13.2 and 66 p.p.m.). This difference increased greatly by day 15, with the cultures having the lowest nitrogen concentration (0.066 p.p.m.) having the lowest mean density. The cultures grown at the three highest concentrations, by day 15, still had very similar densities.

The generation times (Table 8) for the cultures grown at the three highest nitrogen concentrations, during the day 0 - 15 time period, were approximately equal (2.9 - 3.0 days). During this same time period, the generation times of the cultures grown at 0.066 and 0.66 p.p.m. N

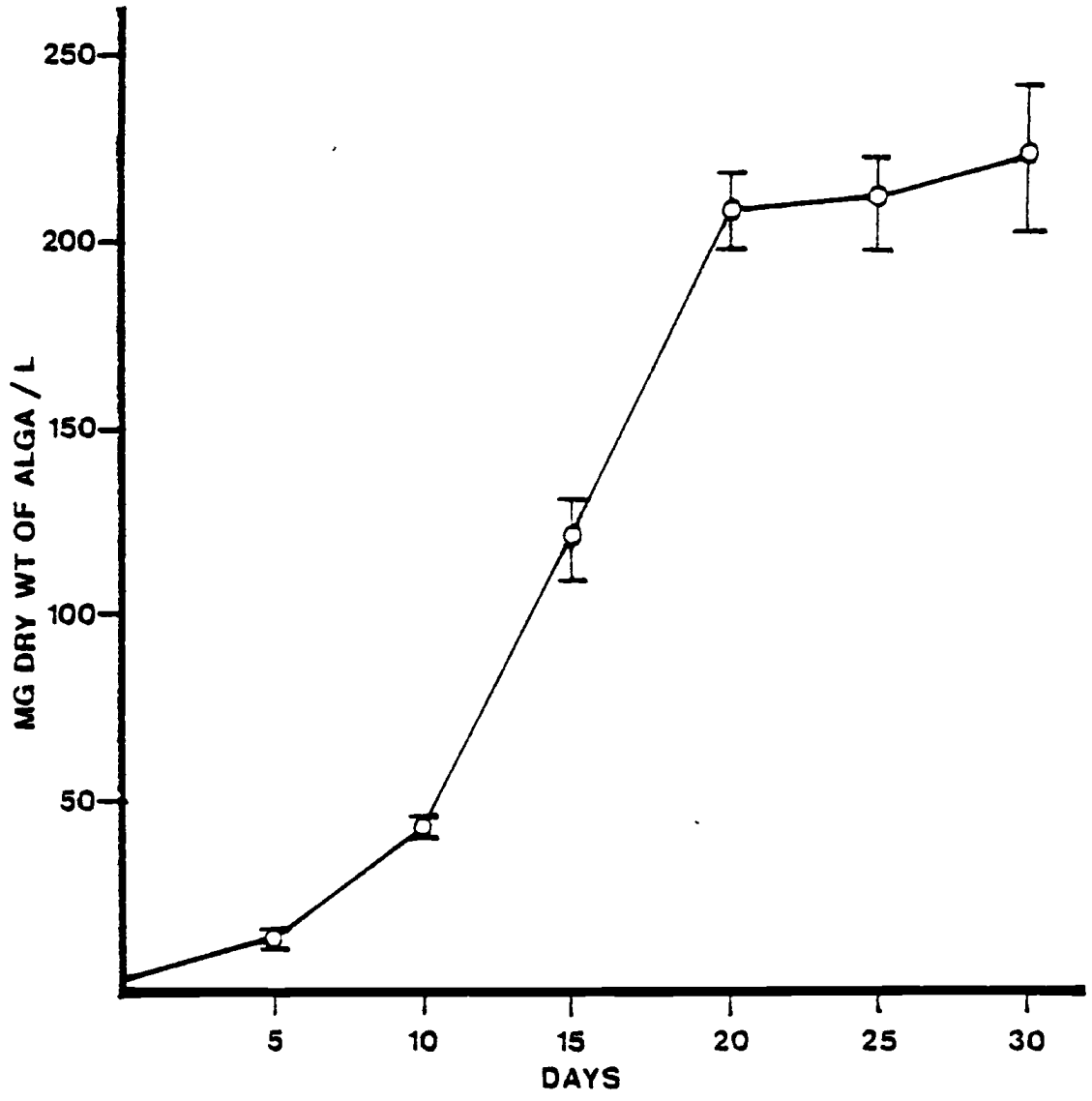

Figure 1. Experiment 3. Increase in density (mg dry weight of alga/liter of B. braunii grown over a 30-day period in a modified Chu no. 10 medium at 23 ± 1 C, at a light intensity of 800 ± 50 ft-c and shaken at ca. 225 rev/min. Five replicates harvested at five day intervals except four on day 30. Vertical bars represent ± standard error of the mean.

Table 6. Experiment 3. Generation times (days per doubling of dry weight of alga) of B. braunii during various periods of 30 days of growth. Method of calculation of generation time explained in Materials and Methods.

Growing Period (days)	0-5	5-10	10-15	15-20	20-25	25-30	0-30	0-15
Generation Time	3.04	2.82	3.38	6.29	385	57.8	5.18	3.06

Table 7. Experiment 3. Variation in H^+ -ion concentration of medium, measured at the time of harvest during 30 days of growth. Cultures were buffered with 0.66 mM Tris to an initial pH of 7.3.

Harvest Day	5	10	15	20	25	30
pH	7.5	7.7	8.1	7.9	7.9	7.8

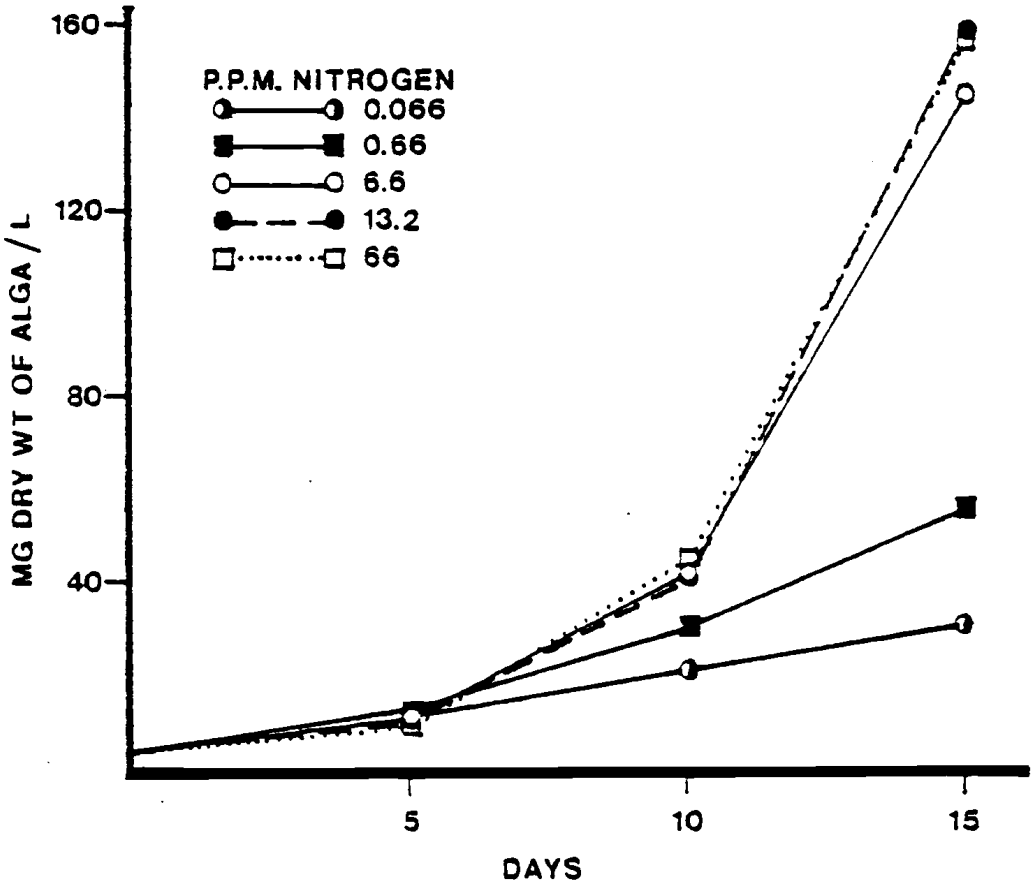

Figure 2. Experiment 4. Increase in density (mg dry weight of alga/liter) of B. braunii grown in various concentrations of 0.066, 0.66, 6.6, 13.2, and 66 p.p.m. of nitrogen provided by $NaNO_3$ at concentrations of 0.40, 4.0, 40, 80 and 400 p.p.m., respectively. Cultures were grown in a modified Chu no. 10 medium at 23 ± 1 C, at a light intensity of 750 ± 30 ft-c and shaken at ca. 225 rev/min. Five replicates of each treatment harvested every five days. The standard error of the means are reported in Appendix Table III.

Table 8. Experiment 4. Generation time (days per doubling of dry weight of alga) of B. braunii during various growing periods at various concentrations of nitrogen. Method of calculation of generation time explained in Materials and Methods.

Concentration of Nitrogen p.p.m.	Generation Time			
	day 0-5	day 5-10	day 10-15	day 0-15
0.066	3.58	6.38	8.72	5.45
0.66	3.57	3.68	5.69	4.13
6.6	3.79	2.64	2.77	2.99
13.2	3.91	2.62	2.56	2.92
66	4.39	2.31	2.74	2.92

were 5.5 and 4.1 days respectively, which indicates their slower growth rates.

Experiment 5: Effect on Growth of Varying the Concentration of Phosphorus The density of B. braunii cultures grown 15 days at eight different phosphorus concentrations are shown in Figure 3 and Appendix Table IV. The (mean) culture densities ranged from between 61 and 92 mg/1. The lowest density observed was in the culture grown at the greatest phosphorus concentration (109 p.p.m. P), while the highest density was found in the cultures with the next to the least phosphorus concentration (0.109 p.p.m. P). All the other densities, except for the lowest and highest densities, had a relatively small range of 75 to 86 mg/1.

The generation times (Table 9) of the cultures ranged between 3.4 and 3.6 days with the exception of the cultures grown at 109 p.p.m. P (the greatest phosphorus concentration), which had a generation time of 3.9 days.

Experiment 6: Effect on Growth of Varying of the H^+ -ion Concentration, pH 5.0 - 10.0 The densities were determined for B. braunii cultures grown 15 days at nine different H^+ -ion concentrations as provided by four different buffers. The densities (Figure 4 and Appendix Table V) ranged from 30 to 137 mg/1. There was a marked increase in the densities as the pH increased to pH 6.0 and a steady decrease in the densities as the pH increased after pH 8.5. While the densities of the cultures grown at pH 6.0 and 8.5 were relatively close (137 and 122 mg/l respectively), the densities were less for the cultures grown at intermediate pH values. The density at pH 8.0 (Hepes buffer) was only slightly less than at pH 8.5, but the densities were greatly reduced at pH 7.0 and pH 8.0 (Tris buffer).

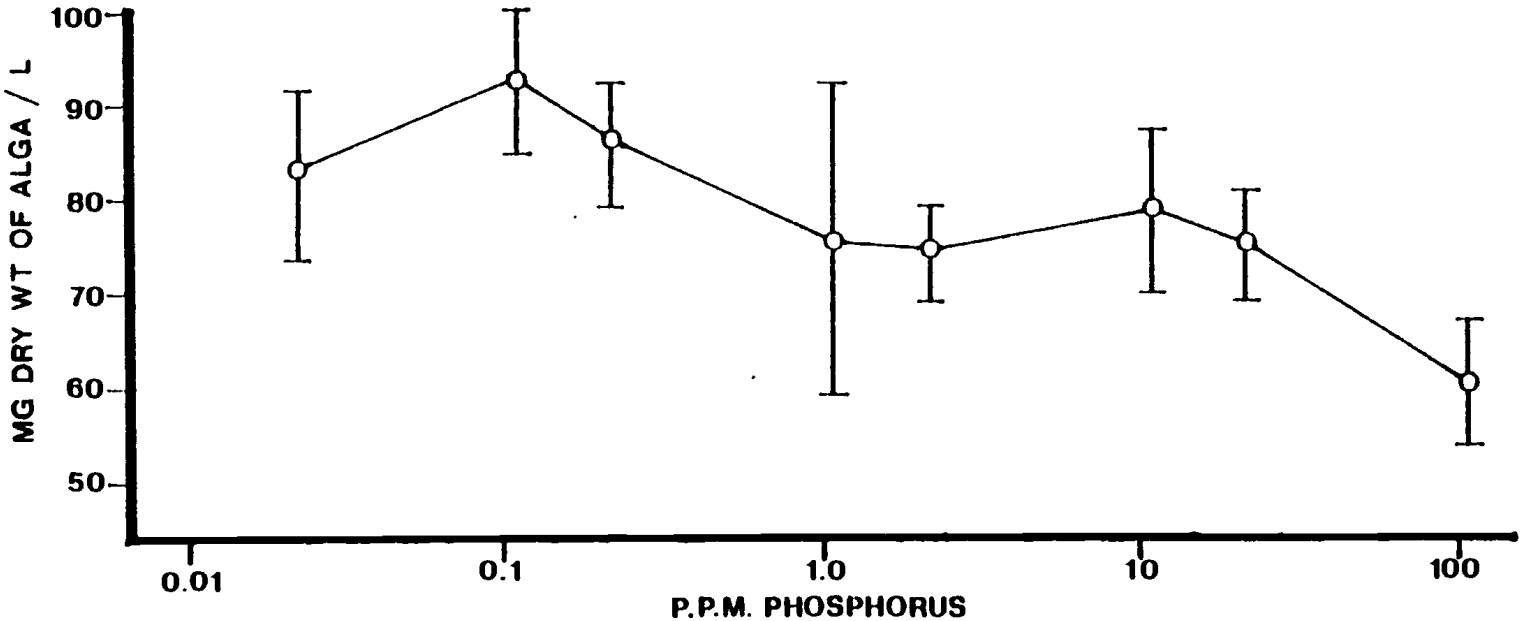

Figure 3. Experiment 5. Densities (mg dry weight of alga/liter) of B. braunii cultures grown 15 days at various concentrations of 0.022, 0.109, 0.218, 1.09, 2.18, 10.9, 21.8 and 109 p.p.m. of phosphorus provided by Na_2HPO_4 in concentrations of 0.1, 0.5, 1.0, 5.0, 10, 50, 100 and 500 p.p.m., respectively. Cultures were grown in a modified Chu no. 10 medium at 23 ± 1 C, at a light intensity of 800 ± 50 ft-c and shaken at ca. 225 rev/min. Five replicates of each treatment were harvested. Vertical bars represent ± standard error of the mean.

Table 9. Experiment 5. Generation times (days per doubling dry weight of alga) of *B. braunii* at various concentrations of phosphorus during 15 days of growth. Method of calculation of generation time explained in Materials and Methods.

Concentration of Phosphorus (p.p.m.)	0.022	0.109	0.218	1.09	2.18	10.9	21.8	109
Generation Time	3.49	3.36	3.44	3.59	3.61	3.54	3.59	3.89

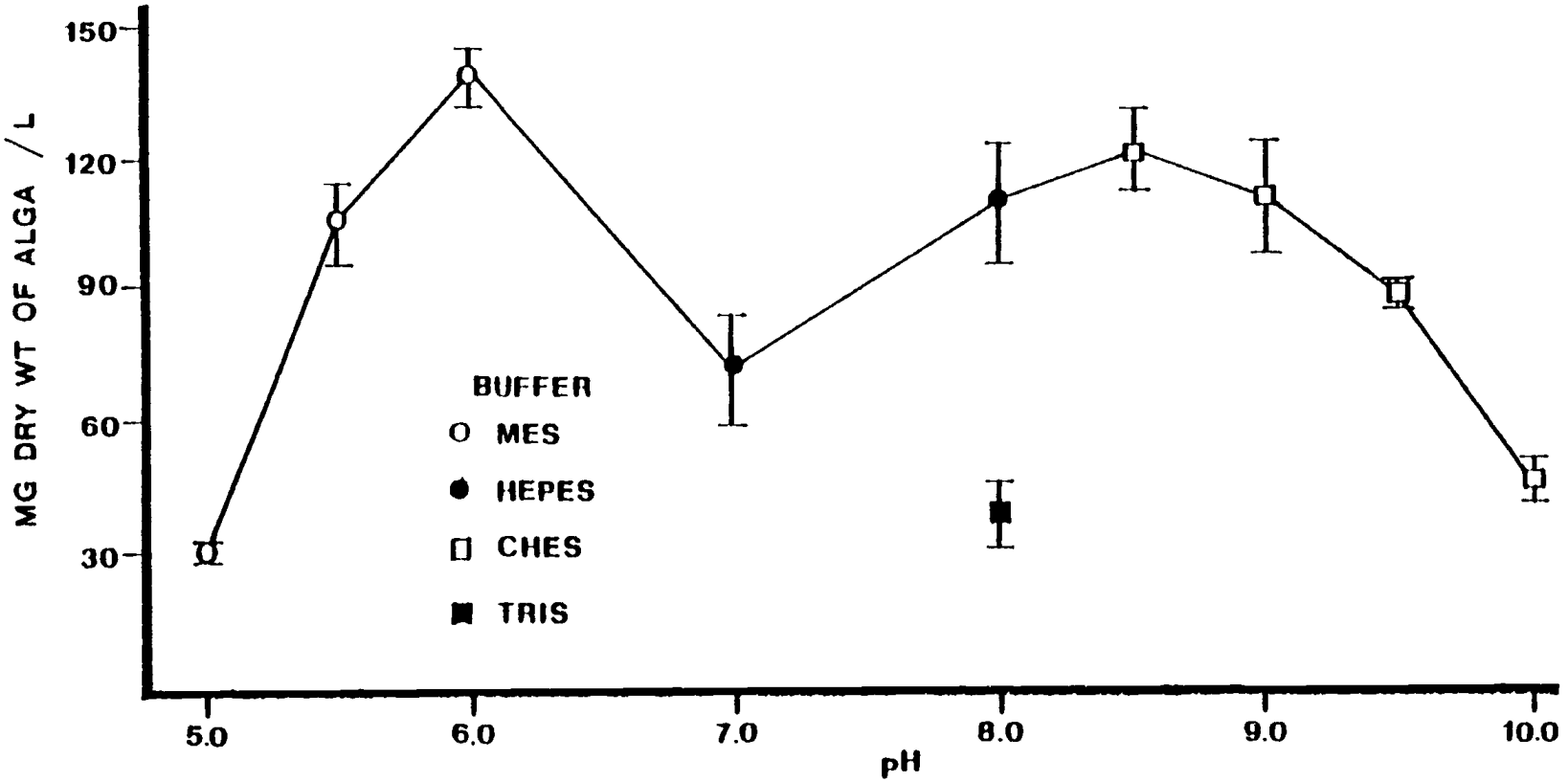

Figure 4. Experiment 6. Densities (mg dry weight of alga/liter) of B. braunii cultures grown 15 days at nine different H^+ -ion concentrations provided by four buffers [Mes (pH = 5.0, 5.5, and 6.0), Hepes (pH = 7.0 and 8.0), Tris (pH = 8.0) and Ches (pH = 8.5, 9.0, 9.5, and 10.0)] having a 10 mM concentration. Cultures were grown in a modified Chu no. 10 medium at 23 ± 1 C at a light intensity of 750 ± 50 ft-c and shaken at ca. 210 rev/min. Five replicates of each treatment were harvested. Vertical bars represent ± standard error of the mean.

The generation times (Table 10) of the cultures ranged from 2.9 to 4.9 days.

The difference between the initial and final H^+ -ion concentrations of the cultures in each treatment (Table 11) was 0.1 pH unit or less, except for the cultures buffered with Ches (pH 8.5, 9.0, 9.5 and 10.0). The pH in these cultures decreased during the course of the experiment. The most pronounced changes occurred in the cultures with the initial H^+ -ion concentrations of pH 9.5 and 10.0, which had decreases of 0.6 and 1.0 pH units, respectively.

Experiment 7: Effect on Growth of Varying the H^+ -ion Concentration, pH 6.0 - 7.9 The densities of B. braunii cultures grown 15 days in culture media buffered with Pipes at four different H^+ -ion concentrations are shown in Figure 5 and Appendix Table VI. The range of densities was relatively small (ca. 145 to 165 mg/1), with the low being at pH 7.9 and the high pH 6.0. The range of generation times (Table 12) was also small, 2.7 to 2.8 days.

The pH of the cultures of each treatment (Table 13) increased slightly (0.2 to 0.4 pH units) during the experiment.

Experiment 8: Effect on Growth of Variation in Temperature The densities of static cultures of B. braunii grown 21 days at four different temperatures are shown in Figure 6 and Appendix Table VII. The highest density (13 mg/1) was at 12 C. The density of 25 C was slightly less. The cultures grown at 6 and 29 C had densities considerably lower than those of the cultures grown at 12 and 25 C.

Since there was no increase in the mean density of the 29 C cultures there was no generation time (Table 14) computed for those cultures. The cultures grown at the other temperatures had generation

Table 10. Experiment 6. Generation times (days per doubling dry weight of alga) of B. braunii at various H^+ -ion concentrations during 15 days of growth. Method of calculation of generation time explained in Materials and Methods.

Buffer	Mes	Mes	Mes	Hepes	Hepes	Tris	Ches	Ches	Ches	Ches
pH	5.0	5.5	6.0	7.0	8.0	8.0	8.5	9.0	9.5	10.0
Generation Time	4.90	3.10	2.86	3.49	3.06	4.40	2.96	3.04	3.26	4.08

Table 11. Experiment 6. Initial and final H^+ -ion concentration of culture medium. The buffers were at a concentration of 10 mM.

Buffer	Mes	Mes	Mes	Hepes	Hepes	Tris	Ches	Ches	Ches	Ches
Initial pH	5.0	5.5	6.0	7.0	8.0	8.0	8.5	9.0	9.5	10.0
Final pH	4.9	5.5	6.0	7.0	8.1	7.9	8.3	8.7	8.9	9.0

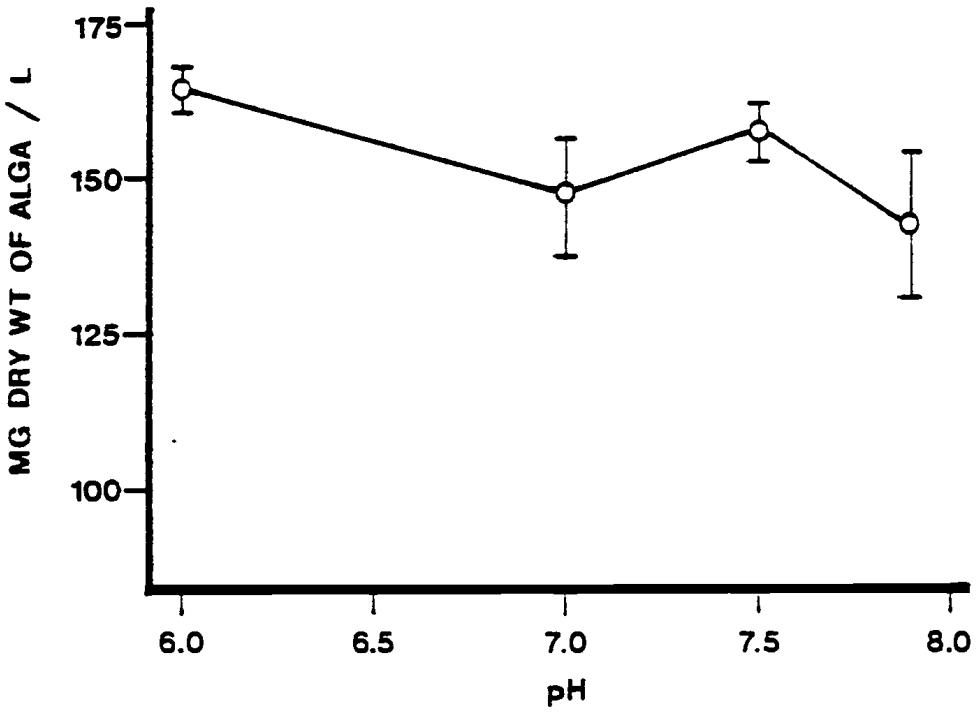

Figure 5. Experiment 7. Densities (mg dry weight of alga/liter) of B. braunii cultures grown 15 days at four different H^+ -ion concentrations (pH 6.0, 7.0, 7.5, and 7.9) in a modified Chu no. 10 medium buffered with 10 mM Pipes. Cultures were grown at 23 ± 1 C, at a light intensity of 700 ± 50 ft-c and shaken at ca. 230 rev/min. Five replicates of each treatment were harvested. Vertical bars represent ± standard error of the mean.

Table 12. Experiment 7. Generation times (days per doubling of dry weight of alga) of B. braunii at various H^+ -ion concentrations during 15 days of growth. Method of calculation of generation time explained in Materials and Methods.

pH	6.0	7.0	7.5	7.9
Generation Time	2.68	2.76	2.71	2.78

Table 13. Experiment 7. Initial and final H^+ -ion concentration of culture medium. Cultures were buffered with 10 mM Pipes.

Initial pH	6.0	7.0	7.5	7.9
Final pH	6.2	7.2	7.8	8.3

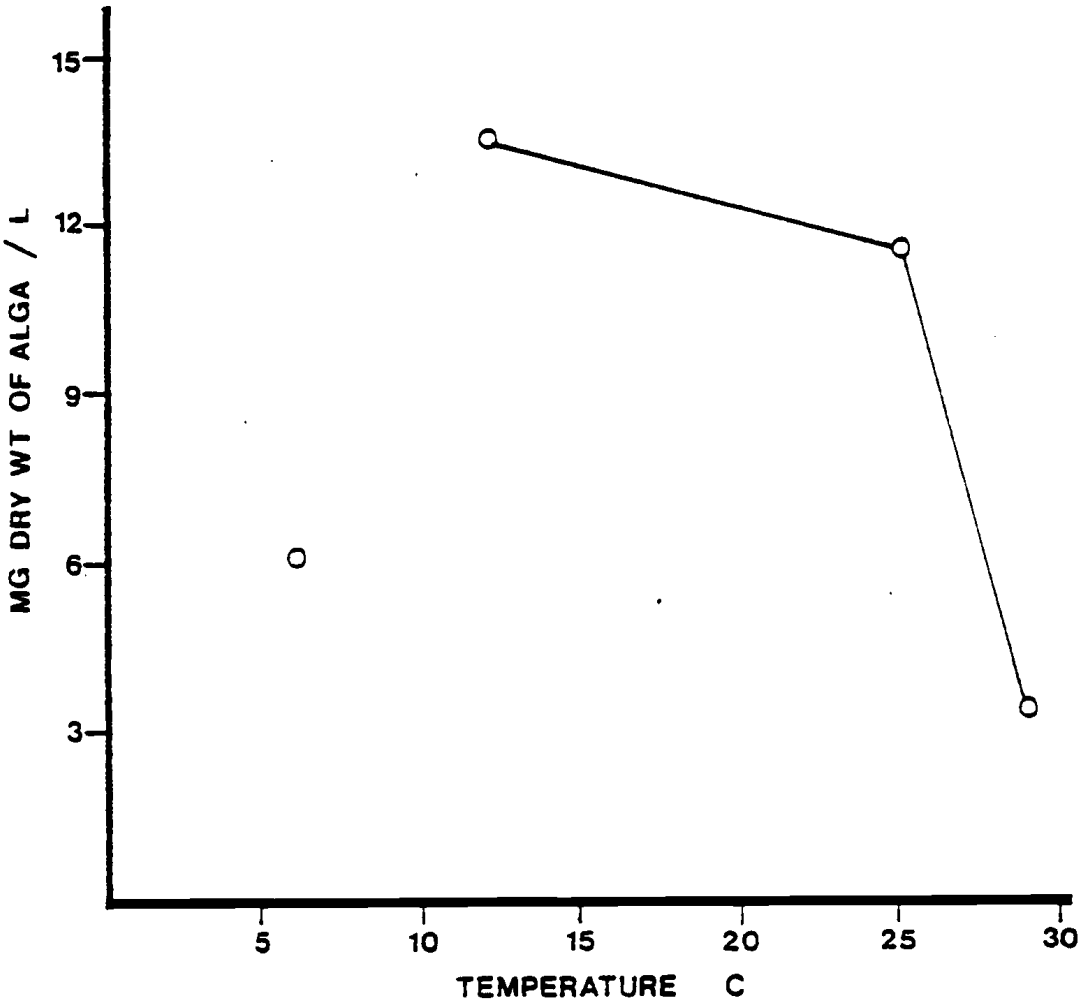

Figure 6. Experiment 8. Densities (mg dry weight of alga/liter) of B. braunii grown 21 days at various temperatures of 6 ± 2 C, 12 ± 1 C, 25 ± 1 C, and 29 ± 1 C, at light intensities (16-hour day) of 150 - 170, 250 -350, 280 - 320 and 260 - 380 ft-c, respectively. The static cultures were grown in a modified Chu no. 10 medium buffered with 10 mM Mes at a pH of 6.0. Five replicates of each treatment were harvested. Density at 6 C is not connected with other densities, because of the significantly lower light intensity at that temperature. The standard error of the means are reported in Appendix Table VII (too small to graph).

Table 14. Experiment 8. Generation times (days per doubling of dry weight of alga) of B. braunii at various temperatures during 21 days of growth. Method of calculation of generation time explained in Materials and Methods.

Temperature (C)	6	12	25	29
Generation Time	24.7	10.6	11.9	a

[a]No increase in culture density was observed.

Table 15. Experiment 8. Final H^+ -ion concentration of culture medium. Cultures were buffered with 10 mM Mes to an initial pH of 6.0.

Temperature (C)	6	12	25	29
Final pH	6.1	6.0	6.0	6.0

times ranging from 11 to 25 days.

There was no change in the cultures' H^+ -ion concentrations during the experiment (Table 15) with the exception of a slight increase in pH (0.1) in the cultures at 6 C.

DISCUSSION

Axenic algal cultures are needed for definitive physiological or biochemical studies. An alga's response to alterations in medium or culture conditions can be greatly affected by the presence of bacteria or other contaminants. The influence of bacteria is especially pronounced when organic compounds are present that they can metabolize. Since this study investigated the effect of culture conditions on the rate of growth of B. braunii, axenic cultures were deemed necessary to insure that the response observed only would be that of the alga. Sterility Tests: Inoculation of the sterility test media with material from cultures known to be contaminated was helpful in identifying those media that would support growth of the contaminating organisms. These cultures also provided proof that in fact the sterility test media, or at least some of them, would support sufficient growth of the contaminating organisms and that the experimental cultures could be assumed to be axenic if growth was absent in the test media.

No contamination was found in the B. braunii culture by incubation in the microbiological media or by direct microscopic observation. The culture was considered axenic and used to inoculate stock cultures. Finding no contamination in the stock cultures during the course of the experiment gave further support to the conclusion that the experimental cultures were axenic.

The Botryococcus culture used in Chu's (1942) experiments was apparently not bacteria free, though the colonies isolated to start the

culture were taken through an extensive sterile washing process to remove foreign organisms.

Belcher's (1968) attempts to obtain axenic cultures of Botryococcus were unsuccessful. The presence of bacteria was demonstrated by plating colonies on test media.

In an investigation of the hydrocarbons in B. braunii, Gelpi et al. (1968) stated that the cultures used were bacteria free, but reported nothing concerning how the axenic cultures were obtained or how they were checked for contamination. In a later related study (Gelpi et al. 1970), no mention was made about having bacteria free B. braunii cultures. The only checks for bacterial contamination reported in the study were made by microscopic examination.

Brown et al. (1969) in a study of the hydrocarbon content of B. braunii stated that it was considered necessary to produce an axenic culture to study the biosynthesis of the hydrocarbons in the alga. In an abstract of a paper presented by Brown (1969) dealing with hydrocarbon formation in B. braunii, axenic cultures were reported as being used, but there was no indication of how the culture was obtained or what tests were used to check for contamination.

Murray and Thomson (1977) claimed to have prepared an axenic B. braunii culture from single cell isolates for investigation of hydrocarbon production in B. braunii. The alga was first exposed to 1% phenol for 10 minutes, then single cell isolates were obtained by micromanipulation techniques using an agar block method. These single cell isolates, which had lost their ability to form colonies, were reported as being axenic. The cultures grown from the isolates were periodically tested for contamination by incubating cells on

nutrient agar. No other test media were indicated as being used. The cells were also examined microscopically. More rigorous testing would have been helpful to insure that the culture was actually axenic. From this report, it would appear that Murray and Thompson's experiments were performed using a strain of B. braunii damaged genetically during their efforts to obtain an axenic culture.

The axenic B. braunii culture obtained in the present study continued to grow as a colonial alga and will be useful for future studies. The culture would be especially valuable in investigations using media containing organic compounds or studies of hydrocarbon synthesis.

Experiment 1: Loss of Hydrocarbon on Drying Samples of octacosane were used in this experiment since the predominant hydrocarbons reported in the green form of B. braunii (Brown et al. 1969, Gelpi et al. 1968) were not available in a purified form for use in this study and octacosane has a chemical structure compariable to the hydrocarbons of the alga. The loss of octacosane on drying should be a reliable estimate of the loss of the hydrocarbons in Botryococcus under the same conditions.

Relatively small amounts of octacosane were lost from the samples with the exception of #2 (day 2). The high loss of this sample was probably due to part of the hydrocarbon, that emigrated to the underside of the weighing pan, not attaching to the weighing pan when the sample was cooled. It is likely that some of the octacosane lost by the other samples was lost in the same manner. If some of the impurities in the octacosane samples (97% pure, Table 2) were volitile at 75 C, then part of the weight losses of the samples could be due to loss

of impurities.

Heptacosane, which has a similar structure and comparable vapor pressure both to octacosane and the predominant hydrocarbons reported in the green form of B. braunii has a vapor pressure of only 2.76 x 10^{-05} mm Hg at 78.1 C (Morecraft 1964). This low vapor pressure would indicate that very little hydrocarbon in the alga would be volatilized during the two days at 70 C in the procedure used to determine the dry weight of the alga.

Experiment 2: Determination of Minimum Size of Algal Samples The minimum dry weight that would reliably reflect the density of the alga in the stock culture was determined to be 0.16 mg. This weight was chosen since the weights of smaller sample fractions did not give accurate estimates of the density of the stock culture. The usable minimum dry weight may indeed have been less than 0.16 mg, but intermediate fractions between 1/8 and 1/16 would need to have been taken. The smaller fractions may not have given accurate estimates due to inadequate mixing of the algal suspension.

Determining the minimum dry weight of an algal sample was helpful in developing the scheme for inoculating and sampling used in the culture experiments. The minimum dry weight was especially useful since it was used as the minimum dry weight of inoculum. Should the density of a culture not increase, as in Experiment 8 (29 C), the density could still be determined.

Experiment 3: Rate of Growth Over an Extended Period of Time In this experiment, there was no evidence of a lag phase in the alga's growth. The generation time (Table 6) being approximately equal (ca. 3 days) for each five day growing period up to day 15 indicated logarithmic

growth occurred during that time. The pH of the cultures increased slightly during this period of rapid growth. In this experiment, the rate of growth of B. braunii during the 15-day exponential phase is considerably lower than that found in most freshwater algae. Talling (1962) reported 1 to 3 cell divisions per day (1 to 0.33 days generation times respectively) are typical of many freshwater species during exponential growth. Though the generation times in this experiment are based on dry weight doublings and can not be directly compared with those based on cell divisions, the comparison is still useful.

A 15-day growing period was considered the best time in which to perform the other culture experiments on the shaker. The colonies are growing most actively during this time and the possibility increased of observing the alga's response to the variable of interest.

Belcher (1968) also observed no lag phase when measuring the growth of Botryococcus by colony number. Belcher reported colony counts showing an intitial exponential increase that gradually gave way to a stationary phase in about 5 weeks.

The rate of growth of Botryococcus in nature has been reported as being low. Swale (1968) reported that 24 days was the average time needed for doubling the number of colonies during a large bloom of Botryococcus, with a period of 6 weeks of more or less steady increase in population.

Both Chu (1943) and Belcher (1968) found Botryococcus grew slowly in culture. In static cultures at a light intensity of 2000 lux (185 ft-c), Belcher reported a 7-day mean generation time based on colony counts during exponential growth. Belcher also reported

increases in dry weight of Botryococcus, but only at monthly intervals. After the first month of active growth, the estimates of dry weight showed a slow steady increase until coming to a constant weight of approximately 250 mg/l after the fourth month. This constant dry weight is somewhat higher than the dry weight of the alga found at day 30 (221 mg/l) in this experiment.

The shaking and higher light intensity used in this study may be factors responsible for the rate of growth higher than that reported by Belcher. The culture medium (a modified Chu no. 10 medium) and temperature (20 C) in Belcher's experiments are similar to those used in this study. There was a substantial difference in rate of growth between the static cultures in Experiment 8 grown at 25 C with a 16-hour light (8-hour dark) cycle (300 ft-c) and the shaken cultures grown in this experiment at a similar temperature and in a similar medium under continuous illumination (800 ft-c). Even considering the difference in time of illumination, the large difference in growth rate indicates that light intensity or shaking, or both, greatly influence the alga's rate of growth.

Experiment 4: Effect on Growth of Varying the Concentration of Nitrogen All the cultures initially (day 5) appeared to have an adequate supply of nitrogen since they had similar growth rates. As growth continued, the growth rates of the culture with the two lowest nitrogen concentrations became lower than that of the other cultures. That the difference in growth rates were due to the difference in availability of nitrogen was also indicated by the fact that the set of cultures with the least available nitrogen had the lowest growth rate. The similarity of growth rates of the cultures with nitrogen concentrations ranging between 6.6 and 66 p.p.m. suggest that the alga's

growth rate is unaffected by increasing the nitrogen concentration above approximately 6.5 p.p.m., at least in the concentrations used in this experiment. If growth of these cultures had been allowed to continue and other nutrients did not become limiting, a decline in growth rates could occur as the nitrogen was finally depleted from the medium

In Part II of a two part study, Chu (1943) investigated the influence of nitrogen concentration on the growth of B. braunii by culturing the alga in 11 media with concentrations of nitrogen ranging from 0.014 to 69.3 p.p.m. supplied by KNO_3. The cultures were allowed to grow as long as 232 days in static 50 ml cultures at a light intensity of 3800 lux (335 ft-c), with turbidity being the measure of growth. Chu reported the optimum nitrogen concentration as being approximately from 0.35 to nearly 6.9 p.p.m. The cultures grown at nitrogen concentrations in this range had similar initial growth rates, with the growth at 6.9 p.p.m. being slightly less. As growth continued, the cultures in this range with lower nitrogen concentrations showed progressively less growth. Chu reported results similar to the above in Part I (1942) of his two part study. The rates of growth initially being the same, then changing with the least growth occurring in the cultures with the lowest initial nitrogen concentration is consistent with the results of this present study and appear to be simply a result of limiting nitrogen availability.

Chu found an inhibiting effect on the growth of the alga in the media with nitrogen concentrations higher than the optimal range. This inhibiting effect, which decreased as the experiment progressed, was particularly evident at the highest nitrogen concentration (69.3 p.p.m.). In the present study when cultures were grown at a very

similar nitrogen concentration (66 p.p.m.), no inhibition was observed. The difference can possibly be explained by potassium inhibition in Chu's medium. Chu (1942) reported a very harmful effect on the growth of Botryococcus in a particular medium by potassium at a concentration of 58 p.p.m. In the medium which Chu reported that greatest inhibition by nitrogen, the potassium concentration was 193 p.p.m. as supplied by 500 p.p.m. KNO_3. This was well above the concentration of potassium reported to be harmful. However, Chu also reported that in another medium at potassium concentrations of 56 to 274 p.p.m. growth of Botryococcus was better than at lower concentrations.

Experiment 5: Effect on Growth of Varying the Concentration of Phosphorus The three cultures with the lowest concentrations of phosphorus appear to show no sign of phosphorus depletion since their growth rates were somewhat higher than all the other cultures. The highest concentration of phosphorus may have caused an inhibitory effect since the cultures at that concentration have a lower final density than do the other cultures. However, the existence of inhibition is made questionable by the variability of growth in the various cultures.

Concentrations of phosphorus similar to those found in the present study were used by Chu (1943) to investigate the influence of the concentration of phosphorus on the growth of B. braunii. In Chu's study, two series of eight 50 ml static cultures were grown at phosphorus concentrations ranging from 0.018 to 89 p.p.m., with one series illuminated for 7 and the other 24 hours per day. The cultures were grown at 3800 lux (335 ft-c) with their growth being measured (turbidity) after 34, 74 and 187 days of growth.

The cultures in both series responded similarly to the different phosphorus concentrations, differing in that the cultures in the series having the short period of illumination showed a slower rate of growth. All the cultures except those grown at the lowest and highest phosphorus concentrations had similar initial rates of growth. Of the group of cultures having similar initial growth rates, only one culture in each series, did not continue to show a similar growth rate throughout the course of the experiment due to phosphorus depletion. Of all the cultures, the one in each series with the lowest phosphorus concentration in the entire experiment had poor growth throughout the study. The culture in each series at the highest phosphorus concentration of the experiment also had a lower growth rate, but by the end of the experiment it had a turbidity similar to the other cultures in its series that had grown well. This lower growth rate may be due to inhibition by potassium which in this medium had a concentration of 224 p.p.m. as supplied by K_2HPO_4. In Part I of Chu's (1942) study, similar results were reported, except the growth was not allowed to continue as long. Although the concentrations of phosphorus in the present study and in Chu's investigation are similar, the lower rate of growth at the lower concentrations reported by Chu was not observed here. Bacterial utilization of phosphorus at the lower concentrations may have contributed to the lower rate of growth seen in Chu's study.

Experiment 6: Effect on Growth of Varying the H^+ -ion Concentration pH 5.0 - 10.0 In the experiment, growth of B. braunii appears to be inhibited at a pH below 5.5 and above 9.5. The growth of the alga was greatly inhibited in the medium buffered with the 10 mM

Tris buffer at pH 8.0. This inhibition is clearly not due to the H^+ -ion concentration since B. braunii grew well at pH 8.0 when 10 mM Hepes was used to buffer the medium. From other experiments in this study (e.g. Experiment 3), it has been shown that in medium buffered with Tris, the alga can grow at rates similar to the better rates observed. The concentration of Tris used in this experiment (10 mM) is approximately 15 times the concentration (0.66 mM) used in the other experiments, indicating the inhibition of B. braunii seen in this experiment at pH 8.0 was due to the concentration of the buffer.

There is no obvious explanation for the substantially lower pH of the cultures buffered with Ches (Table 11) by the end of the experiment. Even though the pH of the media did not stay constant, the results are still useful. The pH range for each culture density is known and the growth rate decreased as the pH increased.

The density of B. braunii at pH 7.0 was considerably lower than at pH 6.0 and 8.0. This indicated the alga grows more slowly at pH 7.0 than at the higher or lower pH. Since the alga grew well at pH 8.0, in media with the same buffers (Hepes) and concentration (10 mM) used at pH 7.0, it is doubtful that the decreased growth was due just to the buffer or its concentration. The lower growth rate at pH 7.0 as compared to the growth at pH 6.0 cannot be simply explained as a result of the difference in pH since the buffers were also different.

Experiment 7: Effect on the Growth of Varying the H^+ -ion Concentration, pH 6.0 - 7.9 To investigate more closely this response of B. Braunii to pH ranging between 6.0 and 8.0, the second H^+ -ion concentration experiment was performed. In this experiment all the media

were buffered with 10 mM Pipes so the difference in growth rates could only be due to H^+ -ion concentration and not to differences in buffers. The rates of growth of B. braunii were similar at pH ranging from 6.0 to slightly less than 8.0, indicating that the lower growth seen at pH 7.0 in the first H^+ -ion concentration experiment was due to something other than the alga's response to H^+ -ion concentration.

From the results of both H^+ -ion concentration experiments, B. braunii appears to be able to grow equally well at a wide range of H^+ -ion concentrations. However, the alga's growth seems to be affected by different buffers and their concentrations.

Belcher (1968) stated that Botryococcus grew well in a modified Chu no. 10 medium buffered with a 10 mM phosphate buffer at pH 6.0. In the same medium at pH 8.0, the alga's growth rate was stated to be double that found at pH 6.0, with growth at pH 7.0 being intermediate. The results from the H^+ -ion concentration experiments in this present study do not support Belcher's findings. The differences in the results may be due to using different buffers. The 10 mM phosphate buffer added by Belcher provided the medium with a concentration of phosphorus of 310 p.p.m. The phosphorus at this high concentration would be available to be metabolizied and could affect the rate of growth of the alga.

Experiment 8: Effect on Growth of Variation in Temperature The similar densities of the cultures grown at 12 and 25 C (Figure 6) indicate a possible range at which the growth rate of B. braunii is relatively independent of temperature. Above 25 C the growth of the alga appears to be greatly inhibited as indicated by the lack of

increase in density of the cultures grown at 29 C. Some inhibiting effect by temperature may be responsible for the culture density at 25 C being somewhat less than that at 12 C. At temperatures below 12 C, little can be concluded from this experiment about the alga's response to temperature, because of the low light intensity supplied to the cultures grown at 6 C. It is noteworthy that despite the lower light intensity, the cultures at 6 C had a significantly higher final density than the cultures grown at 29 C. This indicates the temperature range between 6 and 12 C is more suitable for the growth of B. braunii than a temperature of 29.C.

SUMMARY AND CONCLUSIONS

This study investigates the growth of the green alga Botryococcus braunii in culture and reports the response of the alga's growth to variation of certain factors. To insure that only the alga's response would be observed in the growth experiments, axenic cultures were deemed necessary. To obtain axenic cultures, colonies from a contaminated B. braunii culture were sonicated, rinsed and treated with penicillin G (500 μg/ml), gentamicin sulfate (50 μg/ml) and amphotericin B (5 μg/ml). Some of the colonies, which were so treated, were found to be axenic when incubated in ten microbiological media.

The axenic cultures were maintained in a modified Chu no. 10 medium. Further modifications of the medium were made for individual growth experiments.

During this study, the density of the cultures was expressed as the dry weight of the alga. Under conditions similar to those used for this drying procedure, samples of octacosane, which has a chemical structure comparible to the predominant hydrocarbons reported in the green form of B. braunii, were dried to estimate the loss of hydrocarbon during this drying procedure; only inconsequential losses were found.

By estimating the density of a stock culture with progressively smaller samples of the alga, the minimum size of algal sample needed to accurately determine the density of a culture was approximated as being 0.16 mg of the alga.

Six experiments were performed involving the culturing of B. braunii. One experiment monitored the growth of the alga over a 30-day period. The density of the cultures increased exponentially during the first 15 days of growth and they had an approximate 3-day generation time. The rate of growth of the alga decreased greatly after 20 days. The rate of growth of B. braunii achieved during the exponential phase of these cultures is approximately double that reported previously by other workers and is thought to be the result of shaking the cultures or growing them at a higher light intensity or that both of these factors were influential. This rate of growth, however, is slow compared to that of many other freshwater algae.

The effect of various concentrations of nitrogen ranging from 0.066 to 66 p.p.m. on the rate of growth of B. braunii was investigated. The rate of growth of the alga increased with increasing concentrations of nitrogen up to 6.6 p.p.m. The cultures had similar rates of growth above this concentration suggesting that the rate of growth is unaffected by increasing the nitrogen concentration above approximately 6.5 p.p.m., at least in the concentrations used in this experiment.

The effect of various concentrations of phosphorus in the medium on the rate of growth of the alga was also investigated. With the exception of a possible inhibitory effect at the highest phophorus concentration, the rate of growth of the alga appeared to be relatively unaffected by varying the concentration of phosphorus from 0.022 to 109 p.p.m.

Two experiments investigated the effect of various H^+ -ion

concentrations on the rate or growth of B. braunii. One of the experiments studied the alga's growth in H^+ -ion concentrations ranging from pH 5.0 to 10.0 and the other experiment, using a single buffer, looked specifically at the alga's growth in the range pH 6.0 to 7.9. From the results of the two experiments, it was concluded that varying the H^+ -ion concentration between pH 5.5 and 9.0 had little influence on the rate of growth of the alga. Lowering of the alga's rate of growth was observed at H^+ -ion concentrations outside of this range. The rate of growth of the alga was inhibited by 10 mM Tris (pH 8.0), though at a lower concentration of Tris (0.66 mM, pH 7.3), in other experiments in this study, the alga grew well.

The effect of varying the temperature on the rate of growth of the alga was studied. The alga's rate of growth at 12 C was comparable, though slightly higher, than its rate of growth at 25 C. No increase in density was observed in cultures grown at 29 C.

In conclusion, rather broad optimal ranges were found for the factors examined in this study for their effect on the rate of growth of B. braunii. Outside of the optimal range the primary effect appeared simply to be a lower growth rate. Since the rate of growth remains relatively low even under optimal conditions, further research is needed on the factors studied here and on other factors that might contribute to an increase in the rate, if Botryococcus is to be used as a source of hydrocarbon.

Since the higher light intensity or shaking or both in this study (Experiment 3) were thought to have increased the rate of growth of B. braunii over that previously reported for the alga,

future research on the effect of these factors could be valuable. If shaking affects the alga's rate of growth, then the effect of various levels of oxygen and carbon dioxide in the culture medium should also be investigated. Further useful information could be obtained by investigating the effect on growth of various temperatures, 12 C and below, which would supplement findings in this study (Experiment 8). In future research, the factors that have previously been varied individually could be varied together in single experiments to investigate their effect in combination on the rate of growth of B. braunii.

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APPENDIX

Appendix Table I. Light intensities in microeinsteins/(m^2) (sec) (partial list).

		Light Intensity μein/(m^2) (sec)
Experiment 3		110 - 130
Experiment 5		100 - 130
Experiment 6		100 - 120
Experiment 7		105 - 115
Experiment 8	6 C	20 - 22
	12 C	35 - 49
	25 C	37 - 42
	29 C	32 - 47
Stock Cultures		
Agar stocks		13 - 17b
50 & 250 ml stocks		21 - 43b

[a]No light intensity readings in μein/(m^2) (sec) were taken for Expereiment 4. The appropriate meter was not available at that time.

[b]Light intensity readings in μein/(m^2) (sec) were recorded for the stock cultures at the conclusion of the experiments, and do not show the drop in light intensity during the course of the experiments.

Appendix Table II. Experiment 3. Mean densities (mg dry weight of alga/liter of B. braunii cultures at each sampling day during 30-day growing period.[a] Cultures were grown in a modified Chu no. 10 medium at 23 ± 1 C, at a light intensity of 800 ± 50 ft-c and shaken at ca. 225 rev/min. Five replicates harvested at five day intervals except four replicates at day 30.

Sampling Day	5	10	15	20	25	30
Mean Density (mg dry wt/l)	12.44	42.54	118.76	206.2	208.1	220.9
Standard Error	0.46	0.88	10.9	10.2	12.4	20.0

[a]Initial density of the experimental cultures was calculated to be 3.98 mg dry weight of alga/liter. Inoculum volume was 2.5 ml. Determination of the initial density of experimental cultures is explained in Materials and Methods.

Appendix Table III. Experiment 4. Mean densities (mg dry weight of alga/liter) at each harvest day of B. braunii cultures grown in concentrations of 0.066, 0.66, 6.6, 13.2 and 66 p.p.m. of nitrogen provided by $NaNO_3$ at concentrations of 0.40, 4.0, 40, 80 and 400 p.p.m., respectively.[a] Cultures were grown in a modified Chu no. 10 medium at 23 ± 1 C, at a light intensity of 750 ± 30 ft-c and shaken at ca. 225 rev/min. Five replicates of each treatment were harvested every five days.

Concentration of Nitrogen p.p.m.	Mean Density (mg dry wt/l)		
	day 5	day 10	day 15
0.066	11.80 ± 0.25	20.31 ± 0.71	30.22 ± 3.34
0.66	11.82 ± 0.24	30.28 ± 2.41	55.66 ± 0.88
6.6	11.19 ± 0.85	41.68 ± 1.54	145.6 ± 3.1
13.2	10.88 ± 0.53	40.80 ± 2.53	158.4 ± 11.0
66	9.87 ± 0.49	44.33 ± 1.09	157.1 ± 11.4

[a]Initial density of the experimental cultures was calculated to be 4.48 mg dry weight of alga/liter. Inoculum volume was 3.0 ml. Determination of the initial density of experimental cultures is explained in Materials and Methods.

Appendix Table IV. Experiment 5. Mean densities (mg dry weight of alga/liter) of B. braunii cultures grown 15 days in concentrations of 0.022, 0.109, 0.218, 1.09, 2.18, 10.9, 21.8 and 109 p.p.m. of phosphorus as provided by Na_2HPO_4 at concentrations of 0.1, 0.5, 1.0, 5.0, 10, 50, 100 and 500 p.p.m., respectively.[a] Cultures were grown in a modified Chu no. 10 medium at 23 ± 1 C, at a light intensity of 800 ± 50 ft-c and shaken at ca. 225 rev/min. Five replicates of each treatment were harvested.

Concentration of Phosphorus (p.p.m.)	0.022	0.109	0.218	1.09	2.18	10.9	21.8	109
Mean Denstiy (mg dry wt/l)	82.78	92.80	85.97	75.93	74.56	78.74	75.71	60.84
Standard Error	9.03	7.37	6.54	16.53	5.08	8.67	6.05	6.62

[a]Initial density of the experimental cultures was calculated to be 4.19 mg dry weight of alga/liter. Inoculum volume was 4.0 ml. Determination of the initial density of experimental cultures is explained in Materials and Methods.

Appendix Table V. Experiment 6. Mean densities (mg dry weight of alga/liter) of B. braunii cultures grown 15 days at nine different H^+ -ion concentrations provided by four buffers at a 10 mM concentration.[a] Cultures were grown in a modified Chu no. 10 medium at 23 ± 1 C, at a light intensity of 750 ± 50 ft-c and shaken at ca. 210 rev/min. Five replicates of each treatment were harvested.

Buffer	Mes	Mes	Mes	Hepes	Hepes	Tris	Ches	Ches	Ches	Ches
pH	5.0	5.5	6.0	7.0	8.0	8.0	8.5	9.0	9.5	10.0
Mean Density (mg dry wt/l)	30.40	104.7	137.4	71.72	109.4	38.58	121.9	111.2	88.27	46.54
Standard Error	0.97	9.7	6.1	11.84	14.0	7.16	9.6	12.6	2.79	3.96

[a]Initial density of experimental cultures was calculated to be 3.64 mg dry weight of alga/liter. Inoculum volume was 3.0 ml. Determination of the initial density of experimental cultures is explained in Materials and Methods.

Appendix Table VI. Experiment 7. Mean density (mg dry weight of alga/liter) of B. braunii cultures grown 15 days at four different H^+ -ion concentrations in a modified Chu no. 10 medium buffered with 10 mM Pipes.[a] Cultures were grown at 23 ± 1 C, at a light intensity of 700 ± 50 ft-c and shaken at ca. 230 rev/min. Five replicates of each treatment were harvested.

pH	6.0	7.0	7.5	7.9
Mean Density (mg dry wt/l)	164.1	146.5	157.8	142.9
Standard Error	3.3	9.7	4.7	11.7

[a]Initial density of experimental cultures was calculated to be 3.39 mg dry weight of alga/liter. Inoculum volume was 2.0 ml. Determination of the initial density of experimental cultures is explained in Materials and Methods.

Appendix Table VII. Experiment 8. Mean densities (mg dry weight of alga/liter) of B. braunii cultures grown 21 days at various temperatures (16-hour day).[a] The static cultures were grown in a modified Chu no. 10 medium buffered with 10 mM Mes at a pH of 6.0. Five replicates of each treatment were harvested.

Temperature (C)	6 ± 2	12 ± 1	25 ± 1	29 ± 1
Light Intensity (ft-c)	150-170	250-350	280-320	260-380
Mean Density (mg dry wt/l)	6.12	13.37	11.55	3.35
Standard Error	0.22	0.17	0.21	0.15

[a]Initial density of experimental cultures was calculated to be 3.39 mg dry weight of alga/liter. Inoculum volume was 2.0 ml. Determination of the initial density of experimental cultures is explained in Materials and Methods.