

NASA Contractor NCC 2-501

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**Characterization of *Spirulina* Biomass  
for CELSS Diet Potential**

AMES GRANT  
IN-51CR

217270

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(NASA-CR-185329) CHARACTERIZATION OF  
SPIRULINA BIOMASS FOR CELSS DIET POTENTIAL  
(Alabama A & M Univ.) 53 p CSCI 06C

N89-25561

Unclas  
G3/51 0217270

**October, 1988**

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for CELSS Diet Potential**

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**Period Covered**

**11/01/87 - 10/31/88**

**NASA Cooperative Agreement CELSS Program**

**October 1988**

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## **ACKNOWLEDGEMENT**

*Appreciation is extended to NASA-AMES for their support of this project.*

*As the NASA Technical Monitor, Dr. Robert MacElroy has made invaluable guidance and assistance to the project. To him, we are especially grateful.*

## **PERSONNEL**

*The following personnel has been employed on this contract.*

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## ABSTRACT

Spirulina sp. as a bioregenerative photosynthetic and an edible alga for space craft crew in a CELSS, was characterized for growth rate and biomass yield in batch cultures, under various environmental conditions. The cell characteristics were identified for two strains of Spirulina: S. maxima and S. platensis. Fast growth rate and high yield of both strains were obtained under the following conditions: temperature (30°C-35°C), light irradiance 60-100  $\mu\text{E m}^{-2} \text{s}^{-1}$ , nitrate 30 mM, phosphate 2 mM, aeration 300 ml/min, and pH 9-10. The partitioning of the assimilatory products (proteins, carbohydrates, lipids) were manipulated by varying the environmental growth conditions. Our experiments with Spirulina have demonstrated that under "stress" conditions (i.e. high light 120  $\mu\text{E m}^{-2} \text{s}^{-1}$ , temperature 38°C, nitrogen or phosphate limitation; 0.1 M sodium chloride) carbohydrate increased at the expense of protein. In other experiments, where the growth media were sufficient in nutrients and incubated under optimum growth conditions, the total proteins were increased up to almost 70% of the organic weight.

In other words the nutritional quality of the alga could be manipulated by growth conditions. These results support the feasibility of considering Spirulina as a subsystem in CELSS because of the ease with which its nutrient content can be manipulated.

## INTRODUCTION AND BACKGROUND

Certain basic physiological needs must be met in order for human beings to stay alive. On earth, these needs are met by other life forms in conjunction with geochemical processes that effectively use human waste products in conjunction with energy from the sun to produce fresh supplies of food, oxygen and clean water. In the artificial environment of a spacecraft, these materials must be provided, and human wastes removed, without relying on the natural resources of the earth's biosphere.

Pursuit of our national goals in space exploration will eventually require man's long-duration tenancy of celestial vehicles and planetary bases. Requirements for life support could be met through expenditure of stored supplies and by regeneration and reuse of the waste products of human metabolism. The logistics necessary of regeneration for extended space missions are well documented.

The use of biological components Controlled Ecological Life Support System (CELSS) program as subsystems for the revitalization of air, waste processing, and for the production of food has been proposed for the long term - space flight (MacElroy, Bredt, 1985). Studies of biogenerative life support systems for use in space indicated that they are scientifically feasible. Support of a crew in space, whether in an orbiter or on the surface of a planetary body requires that oxygen, potable water and food be supplied and that waste material be removed. Employment of photosynthetic organisms (higher plants, green algae, cyanobacteria) allows biomass production from relatively simple components which are readily recycled in a CELSS system, namely carbon dioxide, minerals ( $\text{NO}_3^-$ ,  $\text{PO}_4^{3-}$ ,  $\text{K}^+$ ,  $\text{Na}^+$ , etc.) and micronutrients.

The primary source of all man's food and organic raw materials is solar energy. Conventional food sources consist of higher plants and animals. Unconventional food sources for human consumption are photosynthetic algae and bacteria and non-photosynthetic bacteria, yeasts and fungi. Conventional food sources are highly palatable, but require a long time to produce. The photosynthetic energy efficiency of higher plants is less than 3%. Algae, on the other hand, grow rapidly; their metabolism can be controlled; they produce a high ratio of edible to nonedible biomass; and their gas-exchange characteristics are compatible with human requirements.

The semi-microscopic blue-green algae (Cyanophyta; Cyanobacteria) occupy a unique taxonomic position, since they combine an autotrophic mode of growth that is common to eukaryotic plant cells with a metabolic system that is generally regarded as bacterial, rather than plant-like.



Cyanobacteria single cell protein (SCP) has been used as a food source in various parts of the world (e.g. Mexico, China and Africa) since ancient times; in fact, dried cyanobacteria and cyanobacterial tablets are now sold in health food stores in Japan, North America and Europe because they are recognized for their nutritional value. The nutritional quality of all cyanobacteria which have been tested (See Appendix) appears to be very high. For example, Spirulina, in addition to being the richest known source of vitamin B<sub>12</sub>, also contains significant amounts of vitamins B<sub>1</sub> and B<sub>2</sub>. Similarly, one gram of Spirulina contains one-half of the adult daily requirements of vitamin A (B-carotene). The trace elements and iodine found in cyanobacteria are also important when considering the nutritional quality cyanobacteria. The protein of S. maxima and Anabaena cylindrica is easily digestible and approximately 65% of the protein is assimilatable.

Changes in the supply of consumption of metabolites may have considerable effects on metabolic patterns. The accumulation of photosynthetic products in algae can be induced by manipulating the environmental conditions under which the algae are grown (Fogg, 1956). Physiological changes have been indicative of particular changes in nutrient deficiency (Healey, 1975). Studies have shown that limitation of nitrogen, phosphorus, and iron in culture media, affects the growth and physiology of cyanobacteria. Agitation of the culture with air leads to biomass increase. Enrichment with 5% CO<sub>2</sub> in the bubbling air was an efficient way of obtaining a good productivity (DE la Noue et. al., 1984). Packer, et. al., (1986), have shown that with proper manipulation of the osmotic environment, macromolecules of carbohydrates can be produced by N<sub>2</sub>-fixing cyanobacteria.

The most difficult problem in using algae as food is the conversion of algal biomass into products that a space crew could actually eat over a long period of time. If algae are to be considered as a primary food source, it will be necessary to determine that they can be converted into a wide enough range of a palatable complete diet. Therefore, Spirulina, an edible alga with less nucleic acids and no cell walls, offers a good prospect for further studies by manipulating growth parameters.

In order to evaluate the potential of Spirulina for a CELSS diet, it is essential to have background information on the environmental tolerances of the species and eventually the responses of physiological characteristics. This background will be obtained from studying the species in batch and continuous cultures.

The purpose of this project was to evaluate the growth and chemical composition of two strains of Spirulina under different growth conditions.

## **OBJECTIVES**

To characterize batch cultures of Spirulina: Growth, Biomass Yield, and Chemical Composition under varying:

- . Temperature
- . Light intensity
- . Aeration rate
- . Nutrient concentrations

## **SIGNIFICANCE:**

- . Development of CELSS relies, in part, on the ability to manipulate and control the organisms which are a part of the system.
- . Biological regeneration of supplies consumed in CELSS.
- . Direct utilization of algae in space craft crew diet.

This project started in November 1987. The accomplishments in the period November 1987 to October 1988 are described in this report.

## MATERIALS AND METHODS

### Culturing:

Organism: Two strains of cyanobacteria Spirulina:

- . S. Maxima: (UTEXLB 2342) was obtained from UTEX Algal Collection
- . S. Platensis: was obtained from Dr. Becker W. Germany
- . S. Maxima filaments have turns, while S. Platensis filaments are straight ones.

Growth Medium: Zarrouk medium (1966) was used as follows:

NaHCO<sub>3</sub> 16.0g; K<sub>2</sub>HPO<sub>4</sub> 0.5g; NaNO<sub>3</sub> 2.5g; K<sub>2</sub>SO<sub>4</sub> 1.0g; NaCl 1.0g; MgSO<sub>4</sub>·7H<sub>2</sub>O 0.2g; CaCl<sub>2</sub> 0.4g; FeSO<sub>4</sub> 0.01g; EDTA 0.08g; Solution A<sub>5</sub> 1mL; Solution B<sub>6</sub> 1mL; in 1L distilled water.

Solution A<sub>5</sub> in grams per litre of distilled water: H<sub>3</sub>BO<sub>3</sub>, 2.86; MnCl<sub>2</sub>·4H<sub>2</sub>O, 1.81; ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.222; CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.079; and MoO<sub>3</sub>, 0.015.

Solution B<sub>6</sub>, in milligrams per litre of distilled water: NH<sub>4</sub>VO<sub>3</sub>, 22.96; KCr(SO<sub>4</sub>)<sub>2</sub>·12H<sub>2</sub>O, 192.0; NiSO<sub>4</sub>·6H<sub>2</sub>O, 44.8; Na<sub>2</sub>WO<sub>4</sub>·2H<sub>2</sub>O, 17.94; TiOSO<sub>4</sub>·H<sub>2</sub>SO<sub>4</sub>·8H<sub>2</sub>O, 61.1; and Co(NO<sub>3</sub>)<sub>2</sub>·6H<sub>2</sub>O, 43.98.

The medium was autoclaved without the bicarbonate salts. The bicarbonate solutions were sterilized by filtration through 0.2 mm pore size filters.

The culture medium was modified for nutrient limitation studies:

- . For N<sub>2</sub> limited cultures, NaNO<sub>3</sub> was replaced by KCl, and nitrate ammonia, and urea were tested in different concentrations as nitrogen sources.
- . For P-limited medium the P was replaced by NaCl and H<sub>3</sub>PO<sub>4</sub> was used as P-source, in different concentrations.
- . For salinity studies, NaCl was used in different concentrations.

- . FeSO<sub>4</sub> was used in different concentrations.
- . pH was maintained in all cases at 9 with 2N NaOH

Contamination: The standard plate-count method was used to determine the number of bacteria present in the culture. Aliquots were plated using a bent glass rod on an agar medium, which is prepared from Zarrouk (1966) medium (algae medium mentioned previously) enriched with the following ingredients:

Tryptone glucose yeast agar: Tryptone, 5.0g; Yeast extract, 2.5g; Glucose, 1.0g; Agar, 15.0g; in 1L distilled water, pH 7.

0.1 ml filtrate was spread on the agar surface and incubated. Colonies were counted, dilution was made if necessary. Plates were incubated at 30°C and counted after 48 hours or longer to detect all organisms.

Purification of Spirulina Culture: The original cultures of Spirulina were contaminated with bacteria. Different procedures were used to purify the cultures. However, the following procedure was the most successful one:

Cells were collected, filtered with 8mm filter (Gelman), washed with basal medium and homogenized. Cells were spread in a plate, exposed to UV 5 min (20 W UV lamp, 30 cm distance) and inoculated in test tube cultures containing the basal medium. One drop size, inoculum per tube. From 100 culture tubes, 10 tubes were bacteria free.

#### **Growth Conditions:**

Culture Room: A small room (3m D x 2.45m W2 x 2.1m H), was available for this project. It was provided with shelves, which have been illuminated with cool white fluorescent tubes. Light intensity varied from 80-100 uE m<sup>-2</sup> s<sup>-1</sup> on the shelves. The room temperature was kept at 25°C.

Light Measurements: Light irradiation measurements were made with a Li-Cor Model Li-185 M (Lambda Instruments) Meter equipped with a spherical quantum sensor.

All experiments were incubated in continuous light.

#### **Culturing Techniques:**

- Culturing bottles: Small bottles (60 ml capacity) containing 30 ml growth medium were inoculated from stock culture in the

exponential phase and bubbled with air. These cultures were used for evaluating the growth parameters of the alga.

- b. Roux bottles: Experimental amounts of algal cells were grown in roux bottles, containing 800 ml sterile growth medium, by inoculating them with 50 ml of preadapted rapidly growing culture in a 125 ml erlenmeyer flask. Cultures were illuminated continuously by placing them in front of a bank of two cool white fluorescent lamps (40 W). Light irradiation, measured at the surface of culture bottles was  $80 \text{ uE m}^{-2} \text{ s}^{-1}$ . The cultures were grown in a water bath kept at 29-30°C by the use of a heater-thermostat combination.
- c. Aeration: The cultures were aerated with air (0.03% CO<sub>2</sub>) or air enriched with carbon dioxide. The air was delivered by an oil-less compressor. The air was passed first through concentrated sulfuric acid, a saturated ZnCl<sub>2</sub> solution and then distilled water. The air was then passed through a cotton filled erlenmeyer followed by a glass wool filled erlenmeyer prefilter. Finally, the air was sterilized by flowing through sterile filter 0.22 um (Gelman). The air flow rate was monitored by a flow meter. The source of carbon dioxide was from a pressurized tank (50 lb) which was provided with a regulator and solenoid valve to shut off the gas automatically through an electric timer. The carbon dioxide flow was monitored by a flow meter and was sterilized by passing through sterile 0.22 um filter (Gelman). Mixtures of air (0.36% CO<sub>2</sub>) and carbon dioxide were obtained by blending gases to a desired mixture in a two-gas proportioner. The flow rate of the mixed gas delivered to the culture was maintained at 300 ml/min.

### **Analysis:**

Growth Rate: Growth was measured by monitoring change in absorbance (O.D.) at 560 nm with spectrophotometer (Perkin Elmer Lambda I) and expressed as doublings day<sup>-1</sup>. The mean daily division rate t, K, is calculated from:

$$\bar{K} = \frac{3.32}{t} (\log_{10} OD_t - \log_{10} OD_0)$$

Where, t = days since inoculation

OD<sub>t</sub> = optical density after t days

OD<sub>0</sub> = optical density when t = 0.

Harvesting of Cells: Cells were collected by filtration using filter paper 10 mm pore size (Gelman). Cells were washed with buffer solution (pH 8), diluted to known volume and processed for further analysis. Cultures were harvested at O.D. 0.1 units, to avoid light limitation.

Triplicate samples of the algal suspension were taken for each determination. The mean value of these triplicates was recorded. The following determinations were carried out:

Total Chlorophyll: An aliquot from the culture was centrifuged for 2 min at 2000g. The precipitate was suspended in 5 ml methanol for 5 min in a water bath at 70°C, and thereafter centrifuged. The optical density of the supernatant was determined 655 nm.

Dry Weight Measurements (DW): A volume from the culture was filtered through a filter 10 um pore size, dried in previously dried, pre-weighed filter paper for 4 h at 80°C, and then weighed after cooling in a desiccator.

Ash-Free Dry Weight (AFDW): After recording the dryweight, the dried cells were ashed at 500°C for 2hrs. Then the ash wt. was recorded. The difference between dry weight and ash weight gave the organic weight of the sample.

Total Carbohydrates: The anthrone sulphuric acid method was followed (Strickland, Pearsons, 1972). The principle of this method is the formation of a blue-green color which is the product of anthrone and the furfural derivatives produced by acid decomposition of the sugar. The anthrone reagent consists of 0.2g anthrone, 8 ml ethyl alcohol, 10 ml distilled water. Then ml of the anthrone reagent was added to one ml of algal suspension (containing known weight of alga) and heated in a water bath for seven minutes and cooled.

The blue-green color was measured by a spectrophotometer at wave length of 620 nm. The value of the reading was calculated as micrograms of glucose from a standard curve for glucose which has been prepared by the same method.

Total lipids: Cellular lipids were solubilized by repeated extraction with methanol and methanol-chloroform (1:1), then phase separated after adjustment of the solvent ratios to 10:10:9 (methanol: chloroform: water, v/v) (Bligh and Dyer 1959). The chloroform phase was collected, evaporated to dryness under N<sub>2</sub>, and the weight of the lipid was determined. Lipid content was calculated as the weight of the lipid extract divided by the ash free dry weight of the original sample.

Total Nitrogen and Protein (Kjeldahl): One ml of algal suspension containing a given weight was digested in a Kjeldahl digestion flask containing 0.3g selenium mixture and one ml sulfuric acid. When the contents became colorless, they were transferred to the Kjeldahl apparatus with 10 ml of 50% sodium hydroxide solution. A strong current of steam was passed for 7 minutes during which the liberated ammonia was received in a 100 ml flask containing 5 ml of 2% boric acid solution and 4 drops of indicator. The indicator was composed of 0.016g methyl red and 0.83g bromocresol green in 100 ml of alcohol. The distillate in the boric acid solution was back titrated with 0.1 N sulphuric acid, until the color of the indicator turned pale pink. A blank sample was done for each series of nitrogen estimation, using D.W. The value of the readings was calculated in ug N, from a standard curve for nitrogen source as ammonium sulfate, which has been treated by the same method. Total protein was calculated from total N x 6.25.

## EXPERIMENTAL DESIGN

**Protocol:** Flow diagram for the experimental design was followed (Fig. 1).

### A. Growth parameters characterization:

#### I. Temperature, Light:

The algal growth was evaluated for temperature and light tolerance on a gradient plate. Temperature could be adjusted in range from 10°C to 50°C. Illumination was provided by eight cool white fluorescent tubes (40 W). Different light intensities were obtained by varying the distance between the cultures and light source. The algal species were cultured in small bottles (60 ml capacity) containing 30 ml growth medium. Triplicate cultures were placed on the gradient plate, at temperatures: 20°C, 25°C, 35°C and 40°C. The cultures were exposed to two light intensities and were aerated with air (0.03% CO<sub>2</sub>).

#### II. pH Effect:

The alga was incubated in small bottles as described in section A(I) at 35°C on a temperature gradient plate and 80 uE m<sup>-1</sup> s<sup>-2</sup> irradiance. The original medium was used for culturing, except the pH used for culturing was varied by using NaOH or HCl. The pH of cultures was adjusted daily to the original pH. The cultures were aerated with air (0.03% CO<sub>2</sub>).

#### III. Aeration Rate, Carbon Dioxide Enrichment, Bicarbonate Concentration:

The alga was incubated in small bottles described in Section A(I) at 35°C on a temperature gradient plate and 80 uE m<sup>-1</sup> s<sup>-2</sup> irradiance. Three sets of cultures were treated differently:

- a. Cultures were aerated with different flow rates (air 0.03% CO<sub>2</sub>).
- b. The flow rate which gave the best growth rate, was selected from "a". The cultures were aerated with air enriched with carbon dioxide in different concentrations 1%, 3%, 5%, 10%.
- c. Cultures were treated with different bicarbonate



# SPIRULINA FOR CELSS DIET

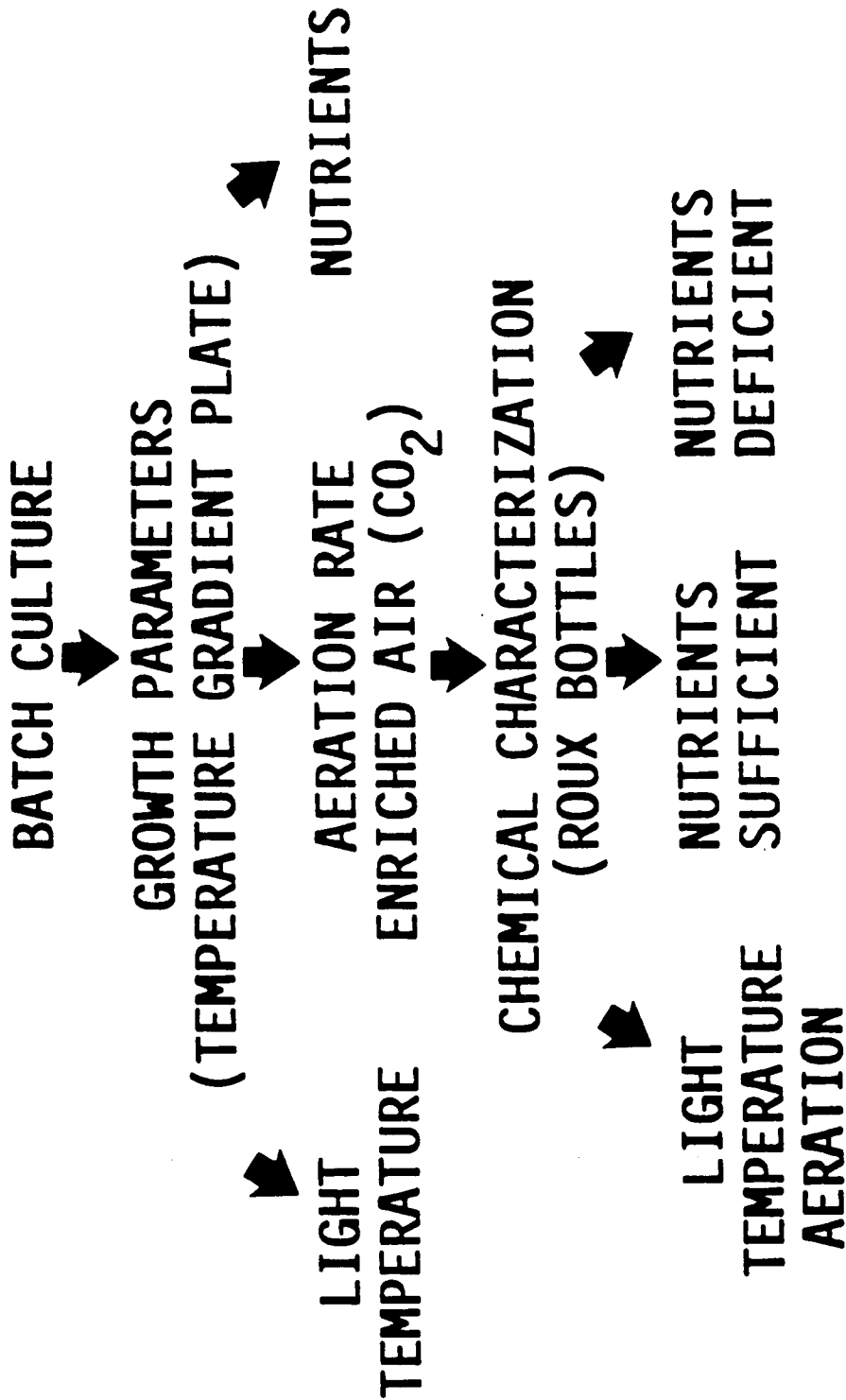


Figure 1. Flow Diagram for Experimental Design

concentrations in which one set was aerated with air (0.03% CO<sub>2</sub>) and other set was aerated with air containing 1% CO<sub>2</sub>.

The pH of all cultures was adjusted twice daily.

#### IV. Nutrient Requirements:

Cultures were incubated in small bottles under the same conditions as described in Section A(I). The original growth medium was modified by changing the concentration of one nutrient. Nitrogen, phosphorus, iron, bicarbonate and sodium chloride were studied in sufficient and limiting concentrations. The bicarbonate effect was studied together with the aeration effect "III".

In all experiments triplicate culture bottles were inoculated from stock cultures in the exponential phase. Growth response was measured as optical density and the growth rate was expressed as doublings per day. The yield of cultures was expressed as the total dry weight after 5 days of growth. The total dry weight was determined by harvesting the cells and drying it (see Methods).

#### B. Physiological Characterization of *Spirulina* in Batch Cultures:

For this experiment, the alga was grown in batch cultures (Roux bottles) as mentioned in "Methods". The cultures were maintained under optimum growth conditions and monitored in the exponential phase by the absorption measurement (see Methods).

I. O.D. of Cell Suspension versus D.W. and Chlorophyll: Both species grown in triplicate Roux bottles under the same conditions described before (see Methods). Twenty ml of culture samples were taken daily for measurements of the D.W., and chlorophyll. The experiment was continued for one week, and 20 ml of fresh culture medium were added to the Roux bottles immediately after each sampling in order to maintain the same volume of the culture medium during the cultivation.

#### II. Under Optimum Growth Conditions:

Both species were grown in duplicate Roux bottles under the same conditions described before (see Methods). Cultures were analyzed for growth parameters during the eight days.

### III. Various Stress Conditions:

#### Light and Temperature:

Batch cultures were incubated under high light irradiation and others at high temperature (38°C) in water bath.

#### Nutrients:

Both species were grown in Roux bottles under the same conditions described previously. Batch cultures were grown in duplicate until the exponential phase was reached at 0.1 OD, to avoid light limitation. One batch was analyzed and represented the culture sufficient in nutrients. The exponential phase lasted three to five days. Batch cultures were concentrated and diluted to the original batch volume but with a new medium modified in one element. The nitrogen limited batch received 0.05 mM nitrate; the phosphate limited batch received 0.01 mM phosphate; the bicarbonate limited batch received 4g/L bicarbonate; and sodium chloride was added in two concentrations; 0.1 M and 0.5 M. When one element was limited, the others were in sufficient concentrations. The cultures were incubated under stressed conditions for two days and then harvested for analysis.

## RESULTS AND DISCUSSION

### Temperature and Light:

Figure 2 (a and b) depict the growth and yield of both strains of Spirulina at two light irradiations and different temperatures ranging from 20°C to 40°C. Neither strains grew at 20°C but they started to grow at 25°C at very slow rate. Temperatures 30 and 35°C enabled the algal fastest growth rate and highest yield of cells. When the temperature was raised to 40°C, the algal cells turned yellow and gave a lower yield. S. platensis, on the other hand gave optimum yield at light irradiation  $80 \text{ uE m}^{-2} \text{ s}^{-1}$  while S. maxima tolerated light irradiance  $120 \text{ uE m}^{-2} \text{ s}^{-1}$ .

### Aeration Rate:

The effects of air agitation rate on the growth rate and cell yield are depicted in Figure 3. The growth rate of both Spirulina strains increased with increasing the flow rate of air in range of 150 ml/min and 500 ml/min. When the flow rate of aeration was increased to 2000 ml/min, the growth rate started to decline and cells turned yellow. On the other hand the cell yield in terms of dry weight was not affected. The pH of all cultures increased to 11. The cell yield of both strains showed parallel fluctuation to the growth rate of the alga.

*SPIRULINA maxima*

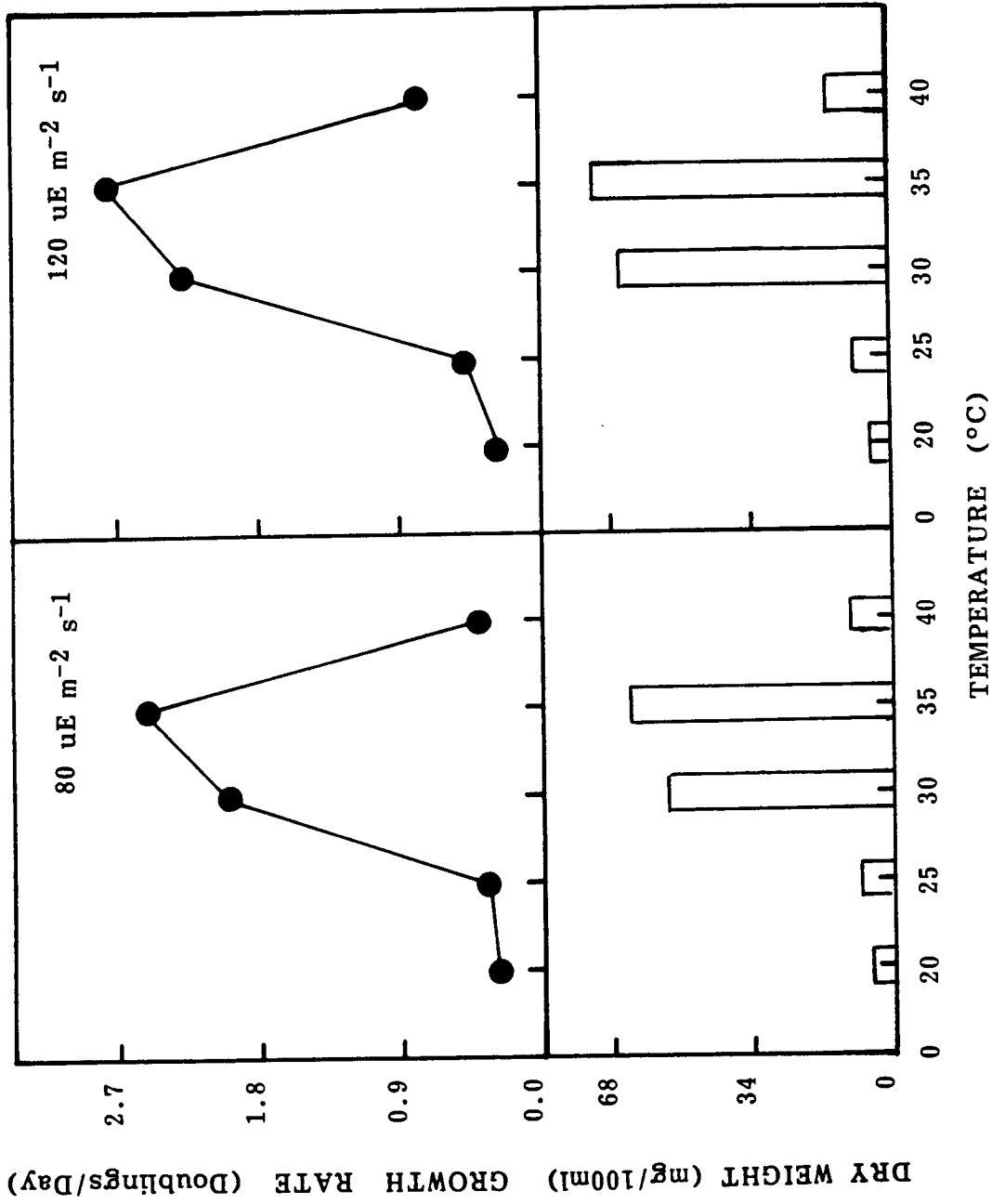


Figure 2a. Growth Rate and Yield of *S. maxima* as a Function of Temperature and Light Irradiance

*SPIRULINA platensis*

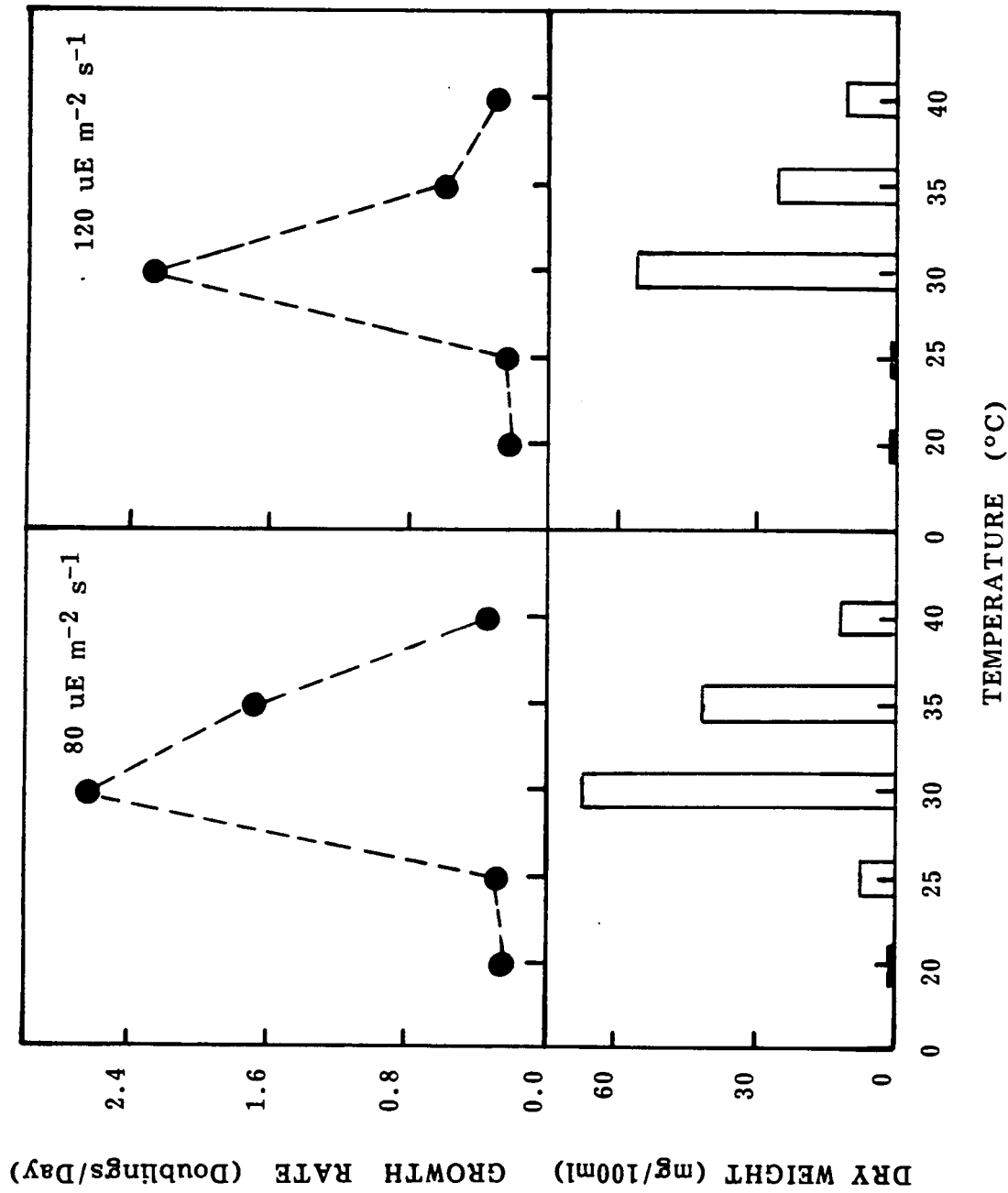


Figure 2b. Growth Rate and Yield of *S. platensis* as a Function of Temperature and Light Irradiance

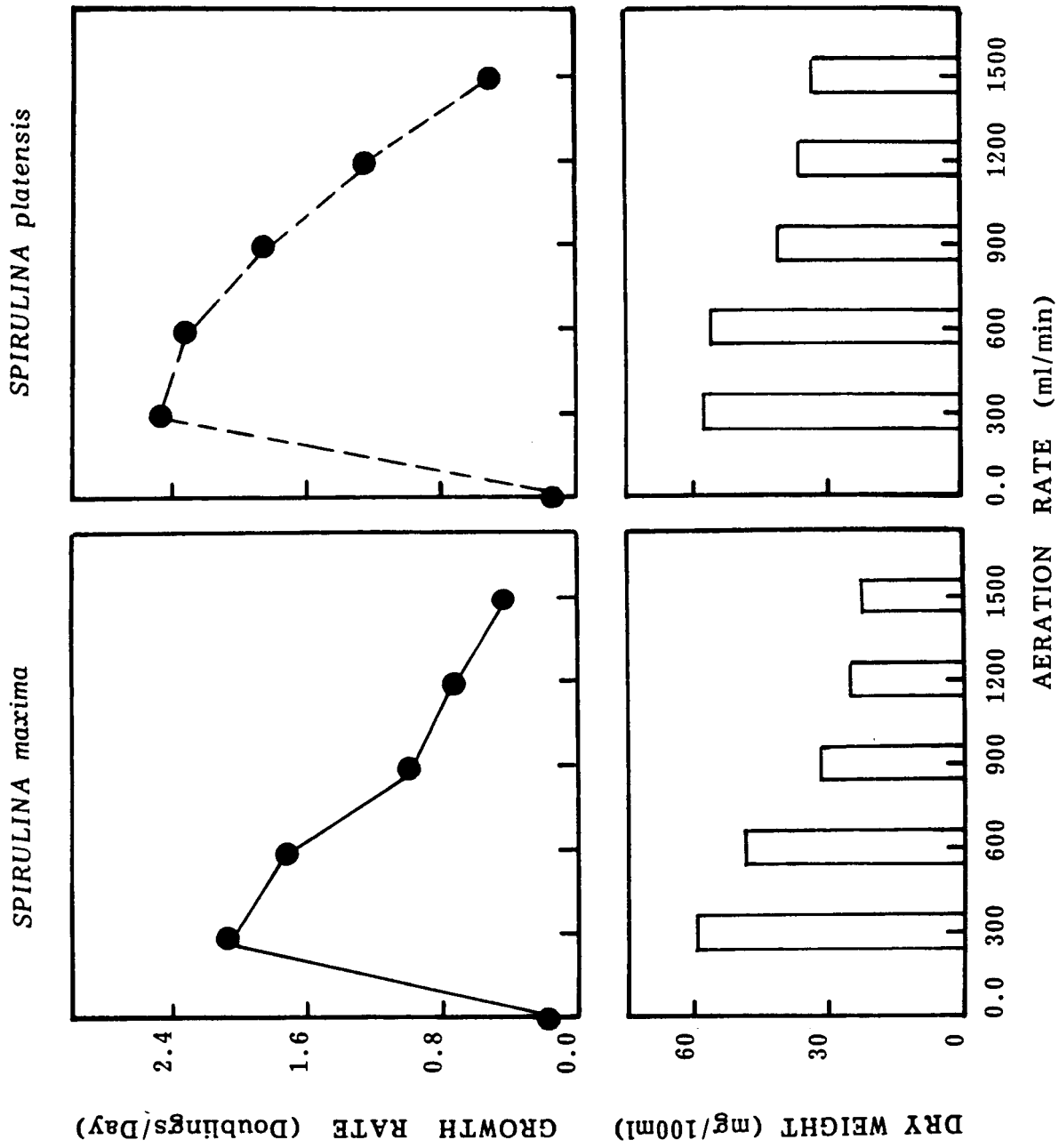


Figure 3. Growth Rate and Yield of *S. maxima* and *S. platensis* as a Function of Aeration Rate

### **Air Enrichment with Carbon Dioxide:**

Figure 4 shows the effect of air enriched with different concentrations of carbon dioxide on the growth rate and yield of both Spirulina strains:

- . Cultures aerated with 10% CO<sub>2</sub> - in air, did not grow and turned yellow. The pH dropped rapidly, within 3 hours, from 9.4 to 7.
- . When the CO<sub>2</sub> - concentration in air was decreased to 5% or 3% the cultures started to grow. The pH of the cultures were maintained at 9.4 by the addition of sodium hydroxide. However, the pH of cultures aerated with 1% CO<sub>2</sub> - enriched air was maintained stable.
- . Cultures aerated with air (0.3% CO<sub>2</sub>) grew at more or less the same growth rate of those aerated with 1% CO<sub>2</sub> - enriched air. The yield of cultures treated with different CO<sub>2</sub> concentrations, in terms of dry weight, was equivalent to the growth rate.

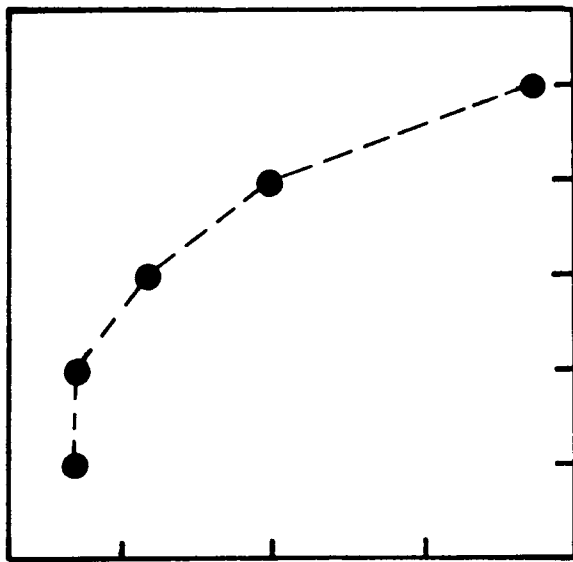
The results of this experiment are in agreement with those of Faucher and Coupal (1979). They reported that sparging 1% CO<sub>2</sub> - air in Spirulina cultures could maintain a constant pH of the culture medium, and at the same time generate HCO<sub>3</sub> ions which were used as carbon source for S. maxima. In a similar study with green algae, Goldman and Graham (1981), reported that in batch cultures, maximum growth rates were achieved at the CO<sub>2</sub> levels present in atmospheric air and at HCO<sub>3</sub> concentrations of 3 mM.

### **pH Effect:**

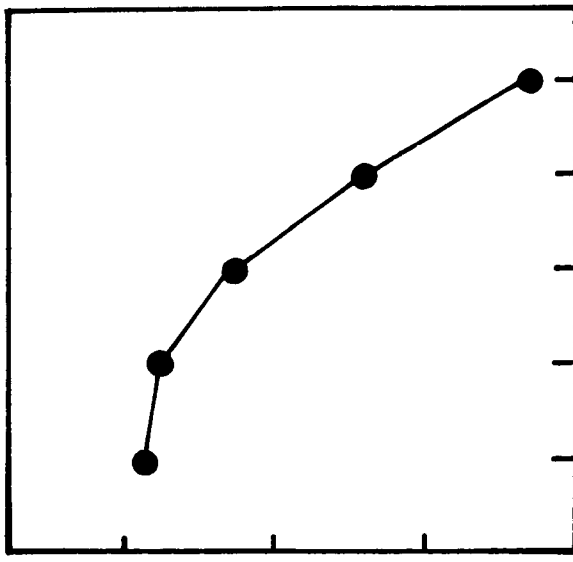
The growth rate of both Spirulina strains is clearly affected by the pH of the growth medium as in shown in Figure 5. Both strains exhibited higher growth rate in media of pH range of 9 to 10. The growth rate decreased with increasing pH above 10 and the cells turned yellow in case of S. maxima while in S. platensis the cells remained in bluish green in color. The cell concentration increased when increasing the pH of the medium from 8 to 10 and then decreased above pH 10.



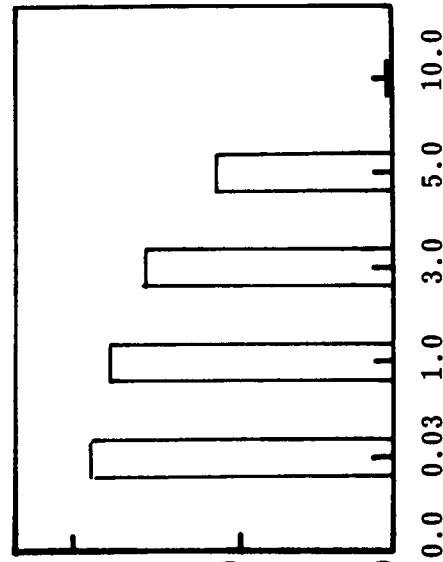
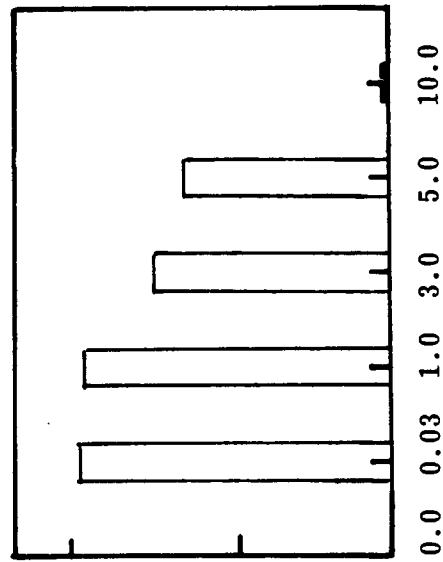
*SPIRULINA platensis*



*SPIRULINA maxima*



GROWTH RATE (Doublings/Day)

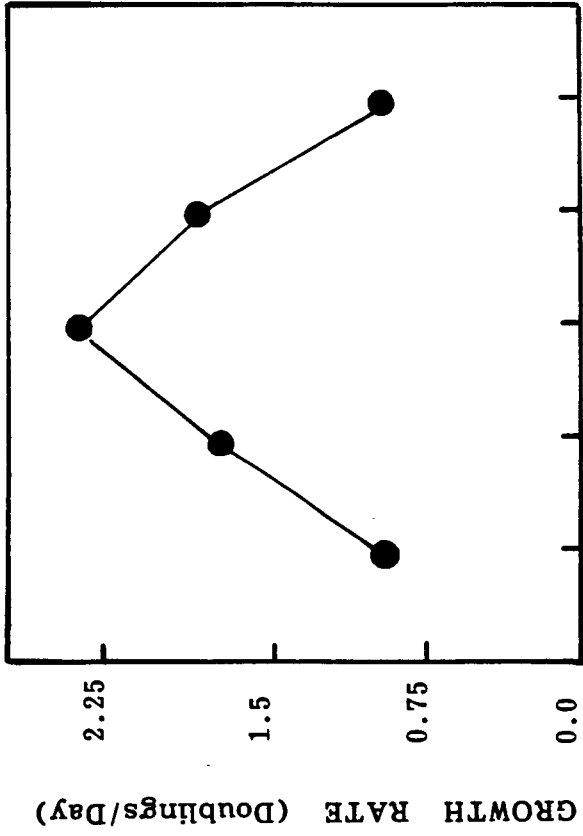


DRY WEIGHT (mg/100ml)

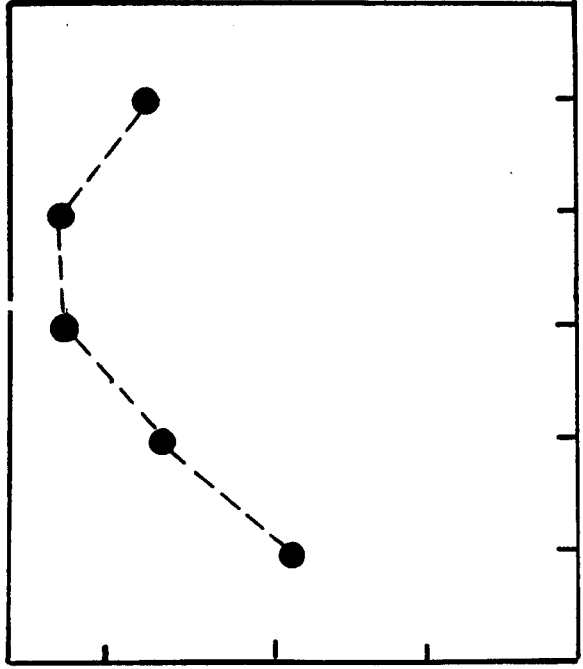
PERCENTAGE OF CO<sub>2</sub> IN AIR

Figure 4. Growth Rate and Yield of *S. maxima* and *S. platensis* as a Function of Carbon Dioxide Concentration in Air

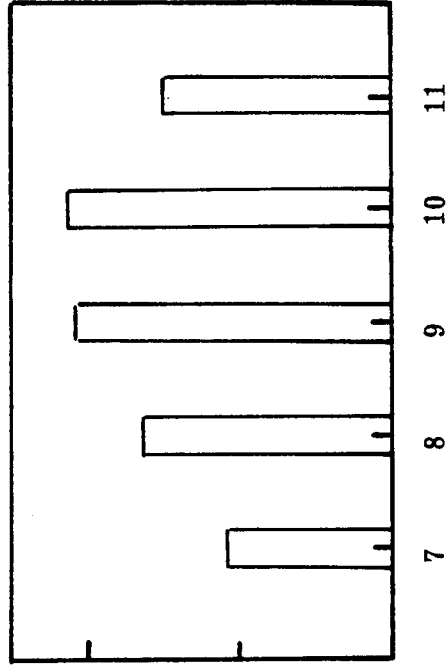
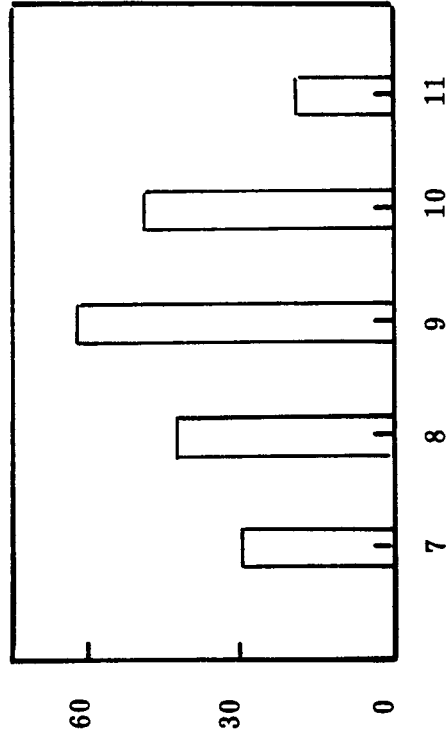
*SPIRULINA maxima*



*SPIRULINA platensis*



DRY WEIGHT (mg/100ml)



pH

Figure 5. Growth Rate and Yield of *S. maxima* and *S. platensis* as a Function of pH.

## Nutrient Requirement:

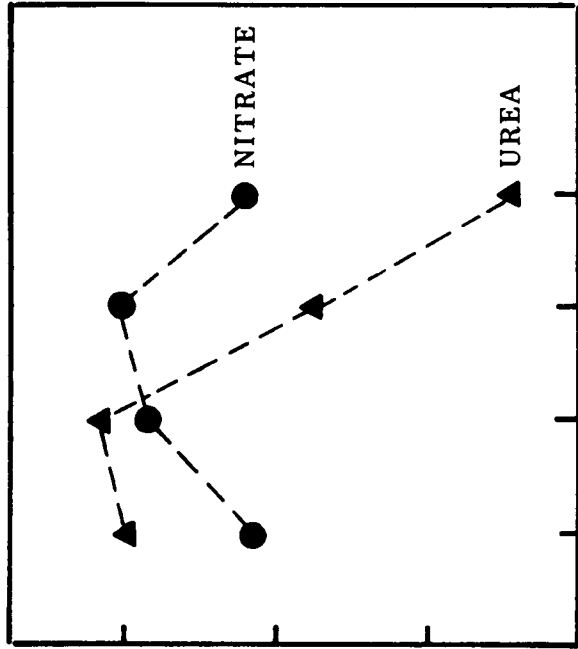
### Nitrogen:

Nitrogen sources in the form of nitrate, urea and ammonia were tested in different concentrations in order to determine their effectiveness as N-sources. Ammonia inhibited the growth of both strains and therefore the data were deleted. The results of nitrate-N and urea-N are represented (Figure 6). The growth rate of both Spirulina strains was enhanced with increasing the concentration of urea-N and nitrate-N. The urea-N at 20 mM concentration enhanced the growth rate, while further increase in its concentration limited the growth of both strains. On the other hand, nitrate-N at concentration 30 mM, enabled both strains to reach fast growth rate and high yield in terms of dry weight. Although lower concentration of nitrate-N (10 mM) supported the growth of both strains to some extent, the strains bleached and lost their pigments. This experiment demonstrated that the least amount of nitrate-N necessary to maintain the growth of Spirulina in culture was 10 mM. Microscopically, the trichomes became shorter in both strains and with average 6 turns/trichome, in media limited in nitrogen concentration. In agreement with our results, Faucher et. al. (1979), reported that urea-N in low concentration could support the growth of S. maxima, at high concentration of nitrate-N.

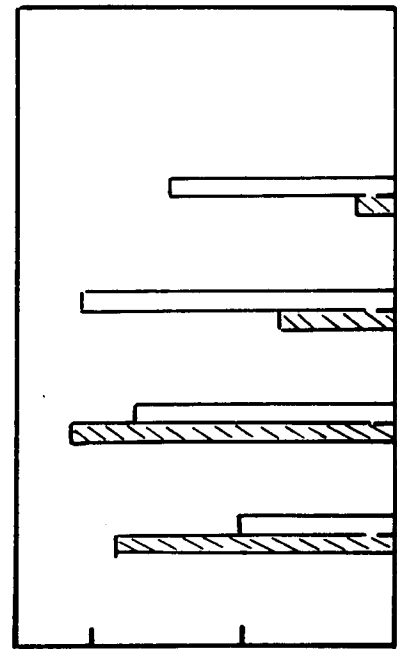
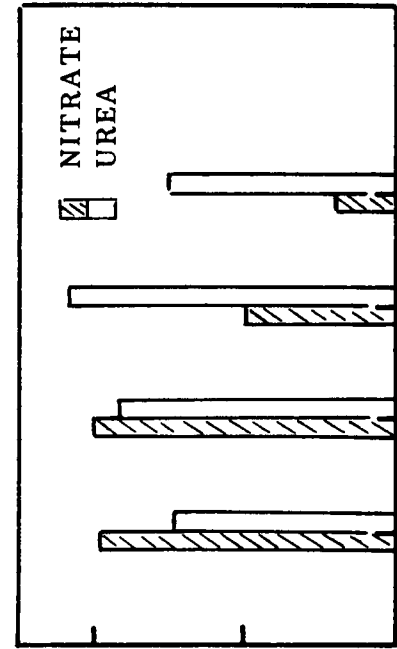
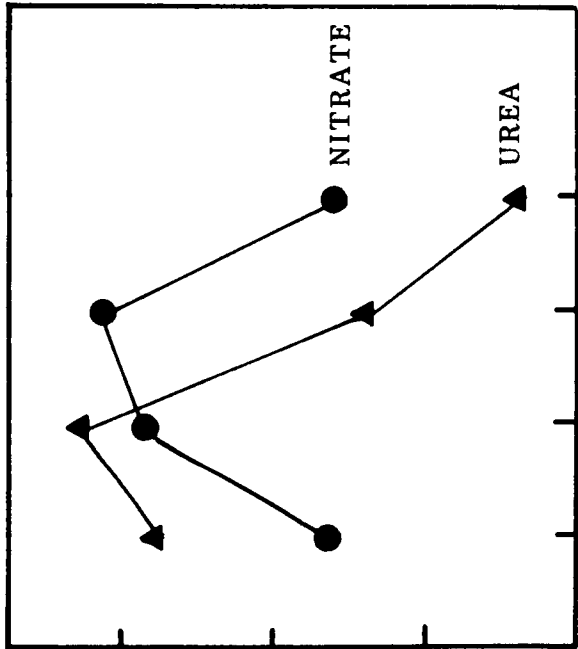
### Phosphate:

Increasing the phosphate-P concentration in the culturing media to 1 mM and 5 mM, enhanced the growth rate of both strains (Figure 7). But as the concentration increased to 10 mM, the growth rate of both strains declined. The mass yield of both strains showed similar responses coinciding with their growth rate. Microscopically, the trichomes became shorter in media of phosphate-P concentration below 1 mM and with few number of turns in case of S. maxima (5 turns/trichome). Generally, cyanobacteria require small concentrations of phosphate-P for growth. They can grow in phosphorus-limited media (Lang and Brown, 1981).

*SPIRULINA platensis*



*SPIRULINA maxima*



CONCENTRATION (mM)

NITROGEN

Figure 6. Growth Rate and Yield of *S. maxima* and *S. platensis* as a Function of Nitrogen Concentration

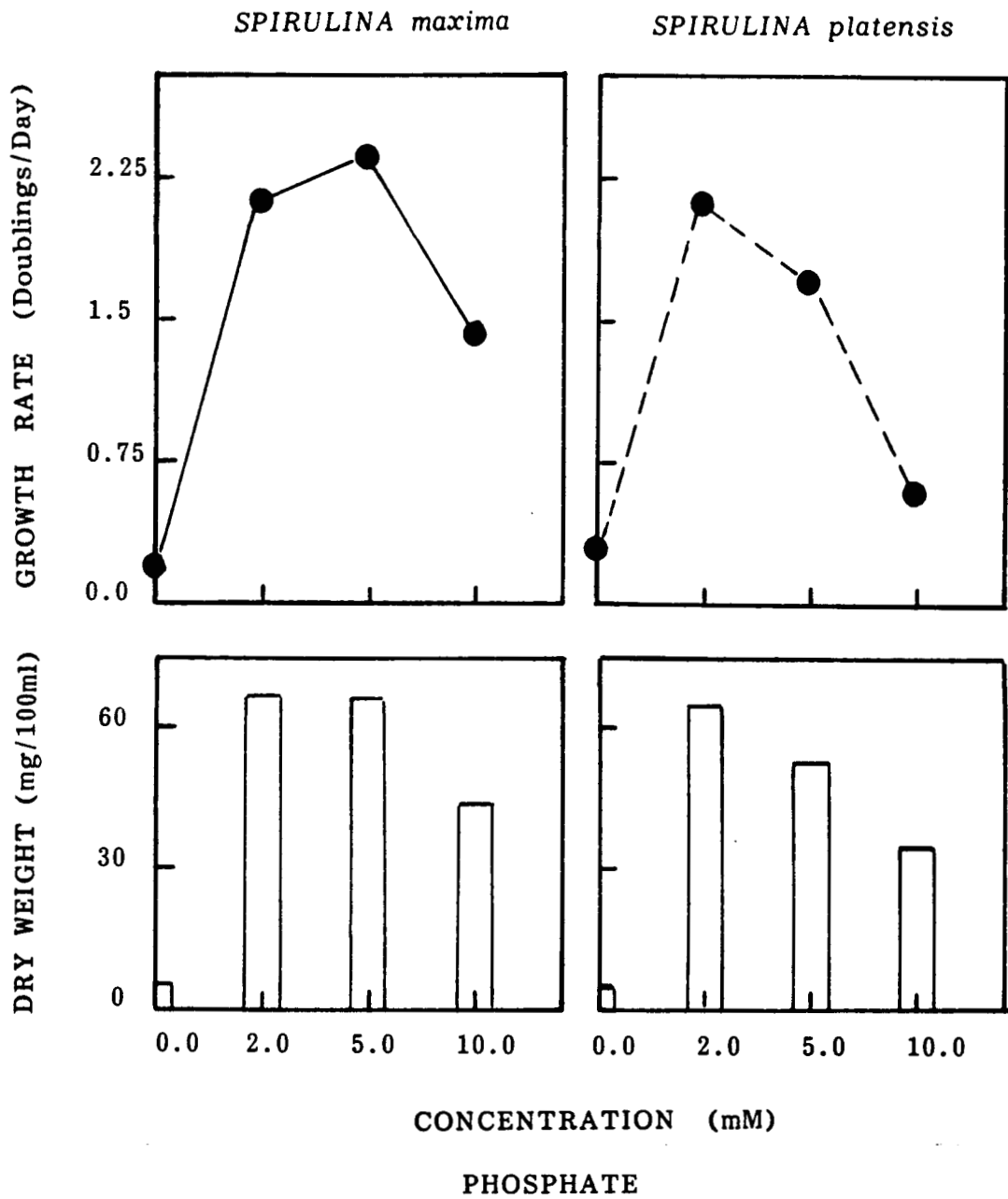


Figure 7. Growth Rate and Yield of *S. maxima* and *S. platensis* as a Function of Phosphate Concentration

### **Sodium Chloride:**

Both Spirulina strains grew in media lacking sodium chloride (Figure 8). They showed response in growth rate as the sodium chloride concentration increased to 10 mM. Further increase in sodium chloride concentration (100 mM) affected the growth rate of both strains and resulted in lower yield of cells. In addition, microscopic examination of both strains indicated that in media treated with a high concentration of sodium chloride 100 mM, the trichomes were short and with less turns in case of S. maxima (6 turns/trichome). The results of this experiment, indicate that Spirulina tolerate increases in sodium chloride concentration up to 100 mM. Spirulina tolerance to salt had been previously reported (Faucher, et. al., 1979).

### **Iron:**

Iron concentrations ( $\text{FeSO}_4$ ) influenced the growth and yield of both strains (Fig. 9). Concentration of 0.05 mM was sufficient for the growth of both strains, although media deficient in iron did not show any growth response. Increasing the concentration of iron beyond 0.1 mM lowered the yield of the alga and cells turned yellow.

### **Bicarbonate Concentration:**

Figure 10 "a" shows that S. maxima grows in the medium even without bicarbonate salt, providing that the culture was aerated with air (0.03%  $\text{CO}_2$ ). As the bicarbonate concentration increased, the growth rate as well as productivity increased. Further increase in bicarbonate concentration above 16g/L (190 mM) did not affect the growth rate. When the carbon dioxide concentration in the air increased from 0.03% to 1%, as shown in Figure 10 "b", the growth rate increased remarkably by decreasing the bicarbonate concentration in the medium as low as 4g/L (48 mM) i.e. one quarter of the concentration in the Zarrouk medium (see Methods). Spirulina platensis, does not show much variation in its response to increasing  $\text{CO}_2$  concentration in air, when compared to S. maxima (Figure 10 "b"). The cell concentration based on dry/weight measurement was related to the growth rate in both strains, in all treatments. The results of this experiment indicate that both strains can utilize atmospheric carbon dioxide when the media bicarbonate concentration is minimum in the culture medium. The pH of all cultures was adjusted daily to 9.4.

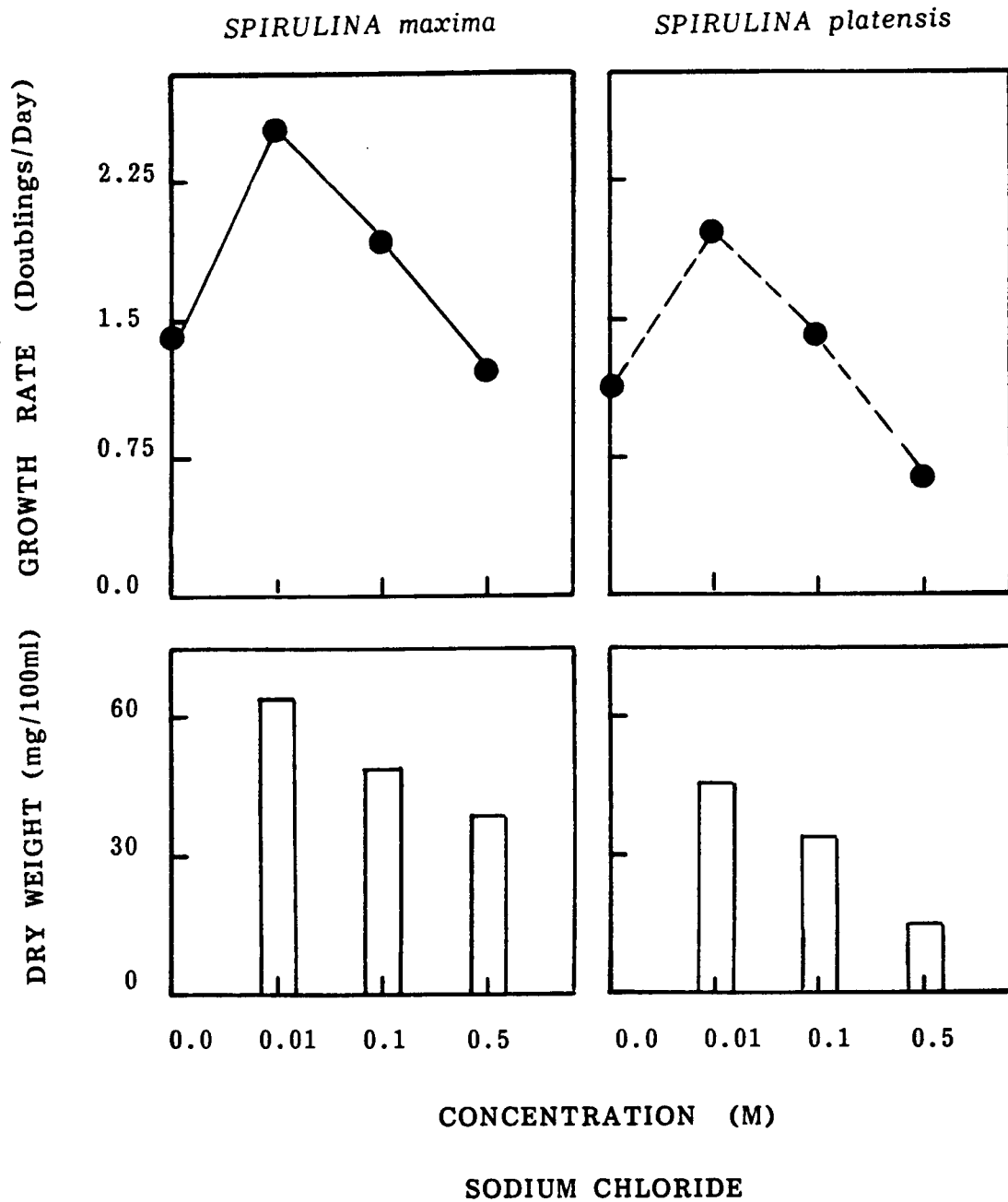
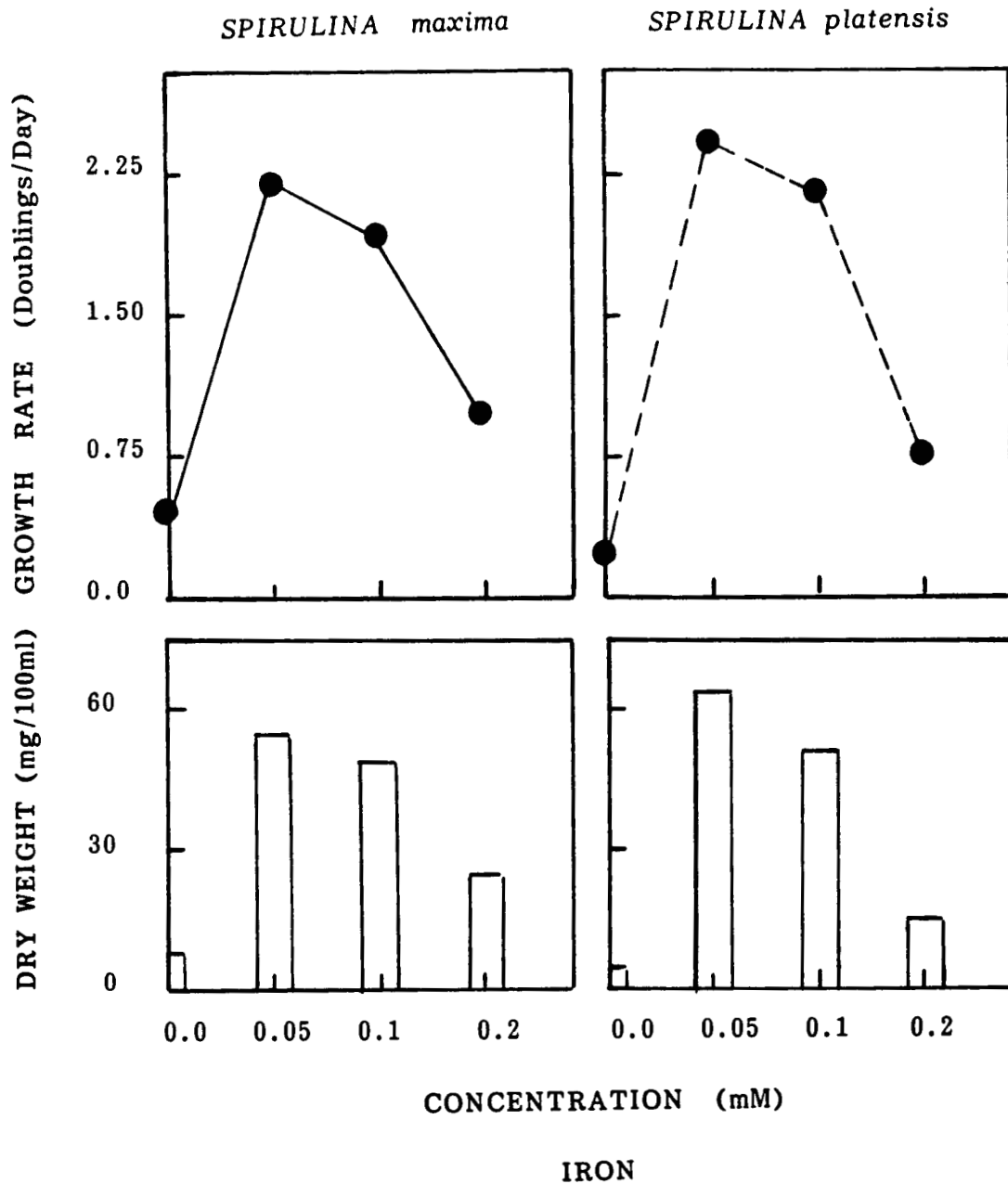


Figure 8. Growth Rate and Yield of *S. maxima* and *S. platensis* as a Function of Sodium Chloride Concentration



**Figure 9. Growth Rate and Yield of *S. maxima* and *S. platensis* as a Function of Iron Concentration**



*SPIRULINA maxima*

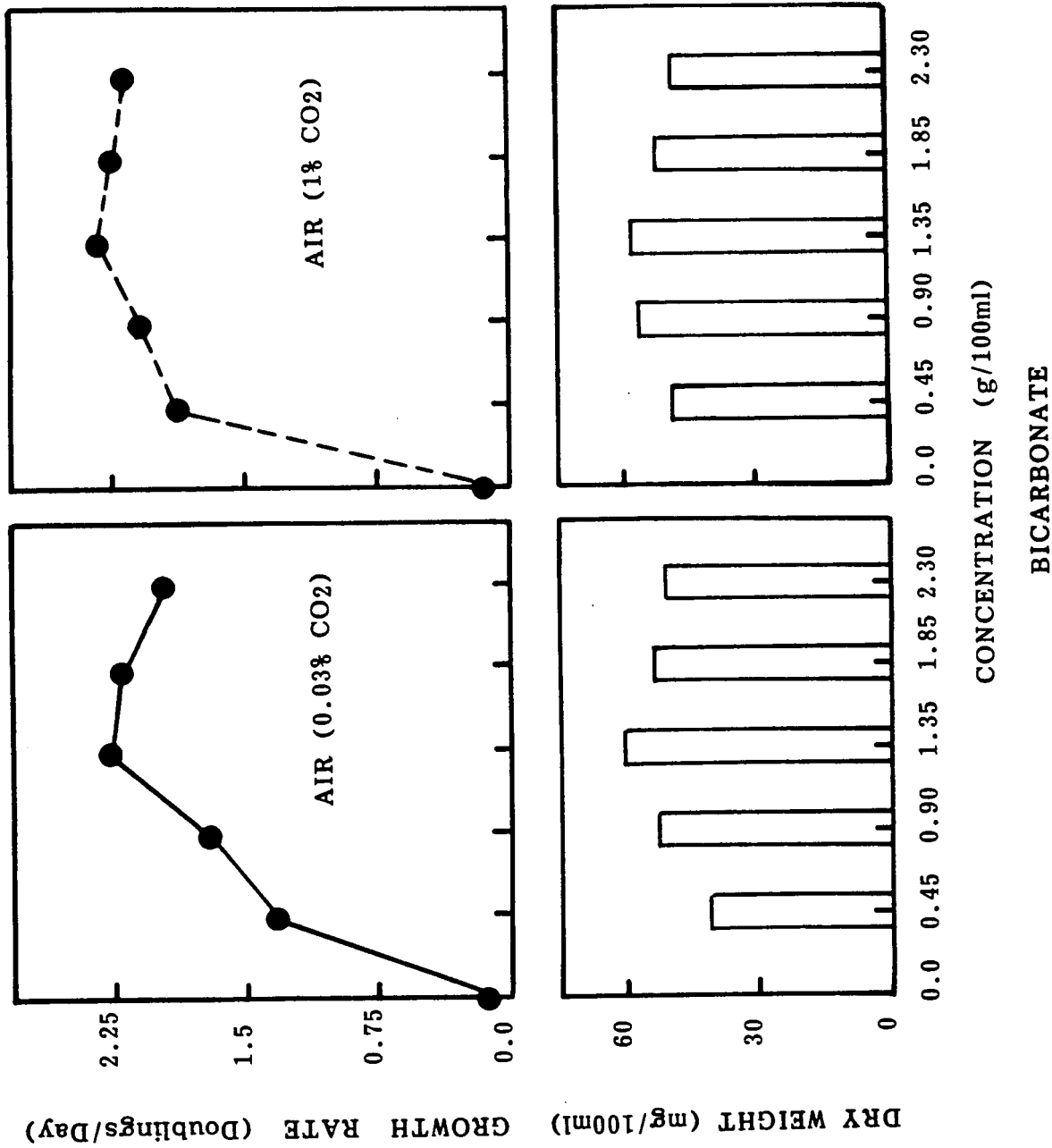


Figure 10a. Growth Rate and Yield of *S. maxima* as a Function of Bicarbonate Concentration

*SPIRULINA platensis*

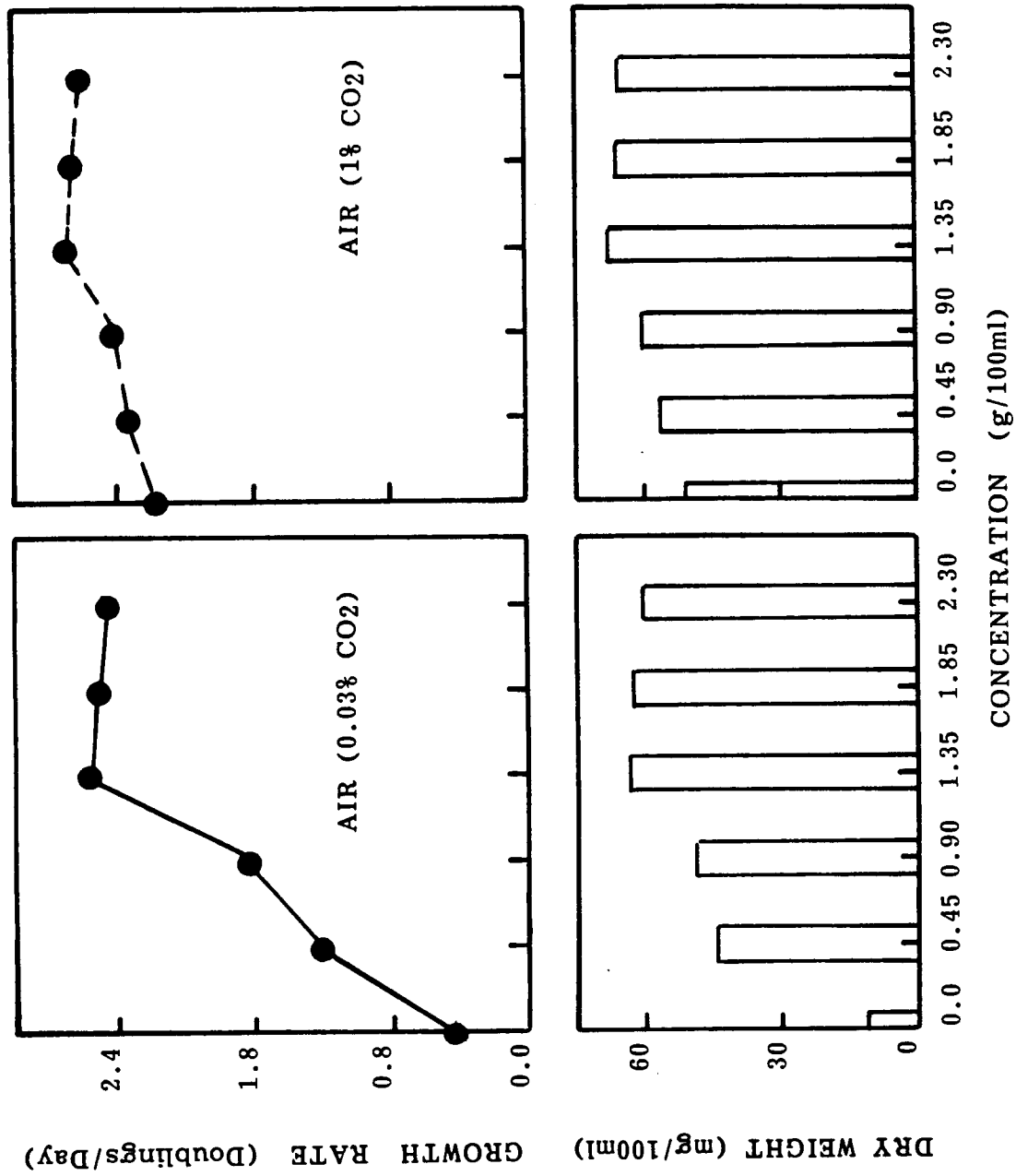


Figure 10b. Growth Rate and Yield of *S. platensis* as a Function of Bicarbonate Concentration

## Physiological Characterization of Spirulina in Batch Cultures:

### Batch Cultures:

#### I. Optical Density (O.D.) of Cell Suspension versus Dry Weight (D.W.) and Chlorophyll:

Growth can be expressed as growth rate or as yield. Yield, as an expression of organic production, is usually given in terms of dry weight of the organic mass produced over a period of time per unit volume. A relationship between optical density, dry weight and chlorophyll was established for both strains of Spirulina.

Results are presented in Figure 11. For all samples within the first three days of cultivation, which contain relatively small concentrations of biomass (400 mg DW/L or less), readings fell within the accurate range of the O.D. scale and they could be read directly from the spectrophotometer without dilution. However, for all samples during the later cultivation periods which contained high concentration of biomass (500 mg DW/L), dilution of the samples with distilled water was necessary prior to OD readings. The graphs show linearity between OD and dry weight. Each OD unit is equivalent to a concentration of 700 mg/L in the case of S. maxima and to 750 mg/L in the case of S. platensis. It is obvious from this experiment that other reliable indicators of estimating algal productivity can be computed from OD measurements. Therefore, OD measurements can be translated into biomass yield in terms of dry weight or chlorophyll.

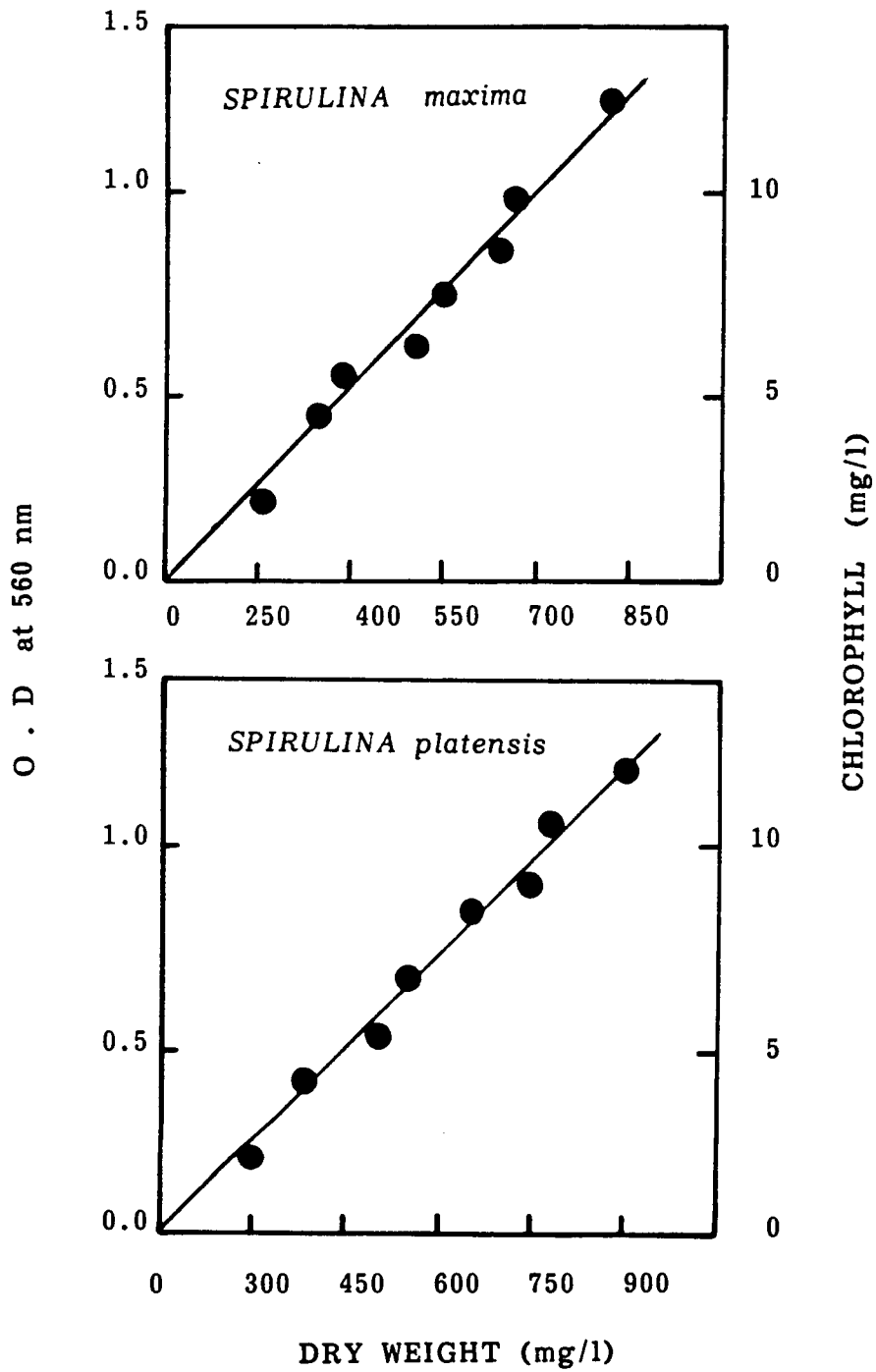


Figure 11. Optical Density versus Dry Weight *S. maxima* (a) and *S. platensis* (b)

## II. Physiological Characteristics of Culture, under Optimum Growth Conditions:

Both species were grown in duplicate Roux bottles under the same conditions described before (see Methods). Cultures were assayed for growth parameters during the eight days. (Fig. 12 "a and b"). Increments of carbohydrates, proteins, dry weight and chlorophyll are expressed as ug/ml culture. The results show that increases in the synthesis of chlorophyll, protein and yield of the culture are correlated. Growth parameters of cultures analyzed after 8 days started to level off, due the nutrient exhaustion and light limitation caused by increasing cell concentration.

## III. Physiological Characterization of Cultures, under Stress Conditions:

The results of analysis were expressed on the basis of organic weight (Ash Free Dry Weight: AFDW) and represented in Table 1 (Figure 13). Results of cultures grown under optimum conditions (II) were used as control for all experiments incubated under various growth conditions.

**Light Irradiance and Temperature:** Increasing the light irradiance to  $120 \text{ uE m}^{-2} \text{ s}^{-1}$ , led to an increase in the total carbohydrate content and a decrease in total protein content: S. maxima 19.58%, 29.06%; S. plantensis 15.22%, 27.18%. Increasing the temperature of culture incubation to  $38^\circ\text{C}$ , influenced the composition of both strains, in a similar manner to the light irradiation experiment: S. maxima, 18.75%, 45.28%; S. platensis, 13.12%, 35.32%; for protein and carbohydrates, respectively. Both strains produced a low percentage of lipids, when grown in high temperature experiments. Cells turned yellow green in color. Studies with light-limited cyanobacteria showed a high level of polysaccharide formation when they were exposed to high light intensities (Konopka, et. al., 1987). Cohen et. al., (1987) also reported a reduction in fat content of 19 strains of Spirulina by temperature and high light intensity.

**Nutrient Limitation:** Media limited in nitrate-N and phosphate-P, favored the accumulation of carbohydrate rather than protein. In nitrate and phosphate limited cultures: S. maxima had 37.52%, 35.21% carbohydrate and 21.56%, 41.25% protein, while S. platensis had 30.31%, 31.87% carbohydrate, and 32.81%, 34.68% protein. When the cultures were transferred to media limited in nitrogen and phosphate, cultures changed in color from blue to yellow-green. N-limited cultures of Anacystis nidulans (Lehman and Wober, 1978), Anabaena variabilis (Ernst and Boger, 1985) and P-limited cultures

*SPIRULINA maxima*

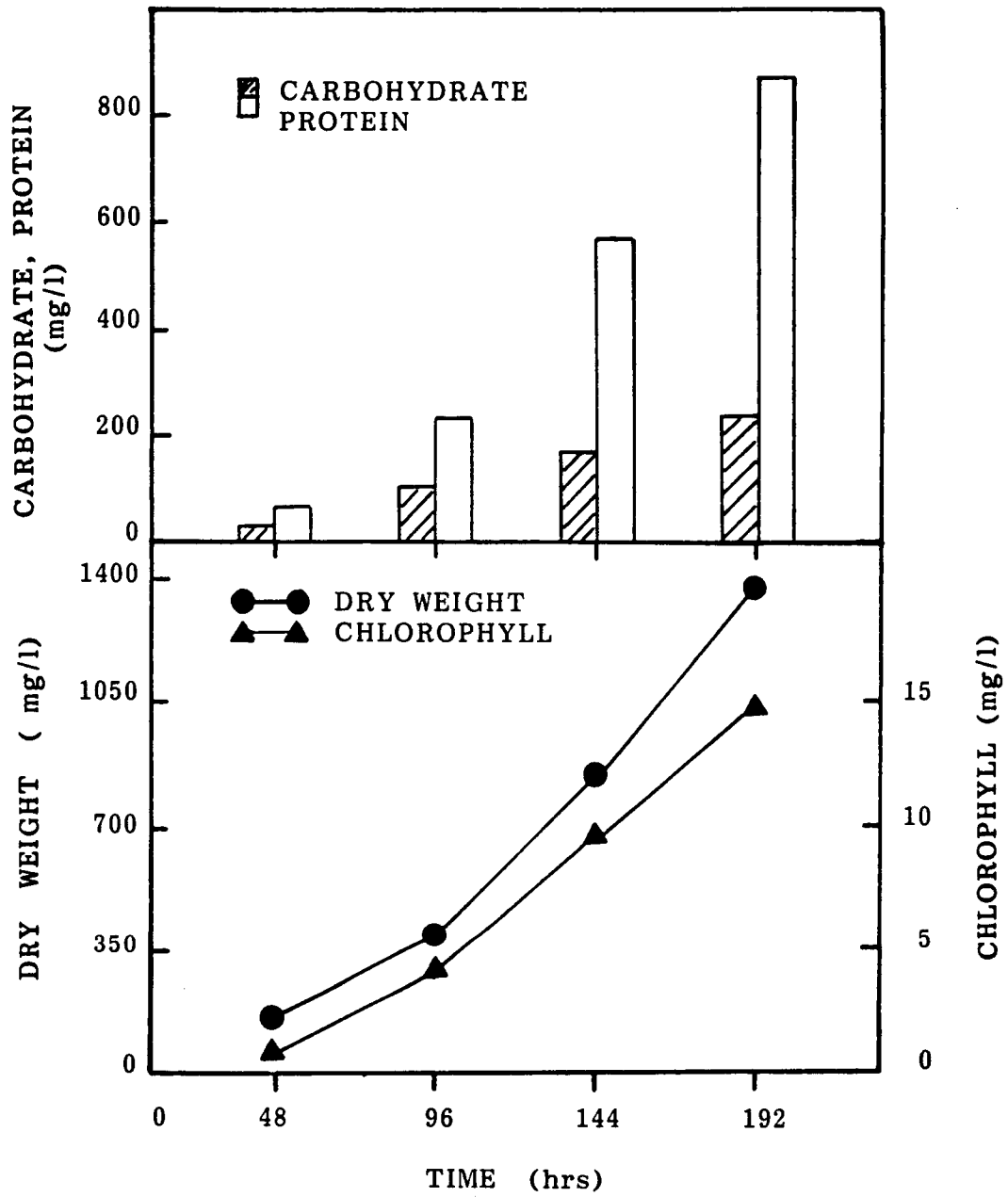


Figure 12a. Physiological Characteristics of *S. maxima* under Optimum Growth Conditions

*SPIRULINA platensis*

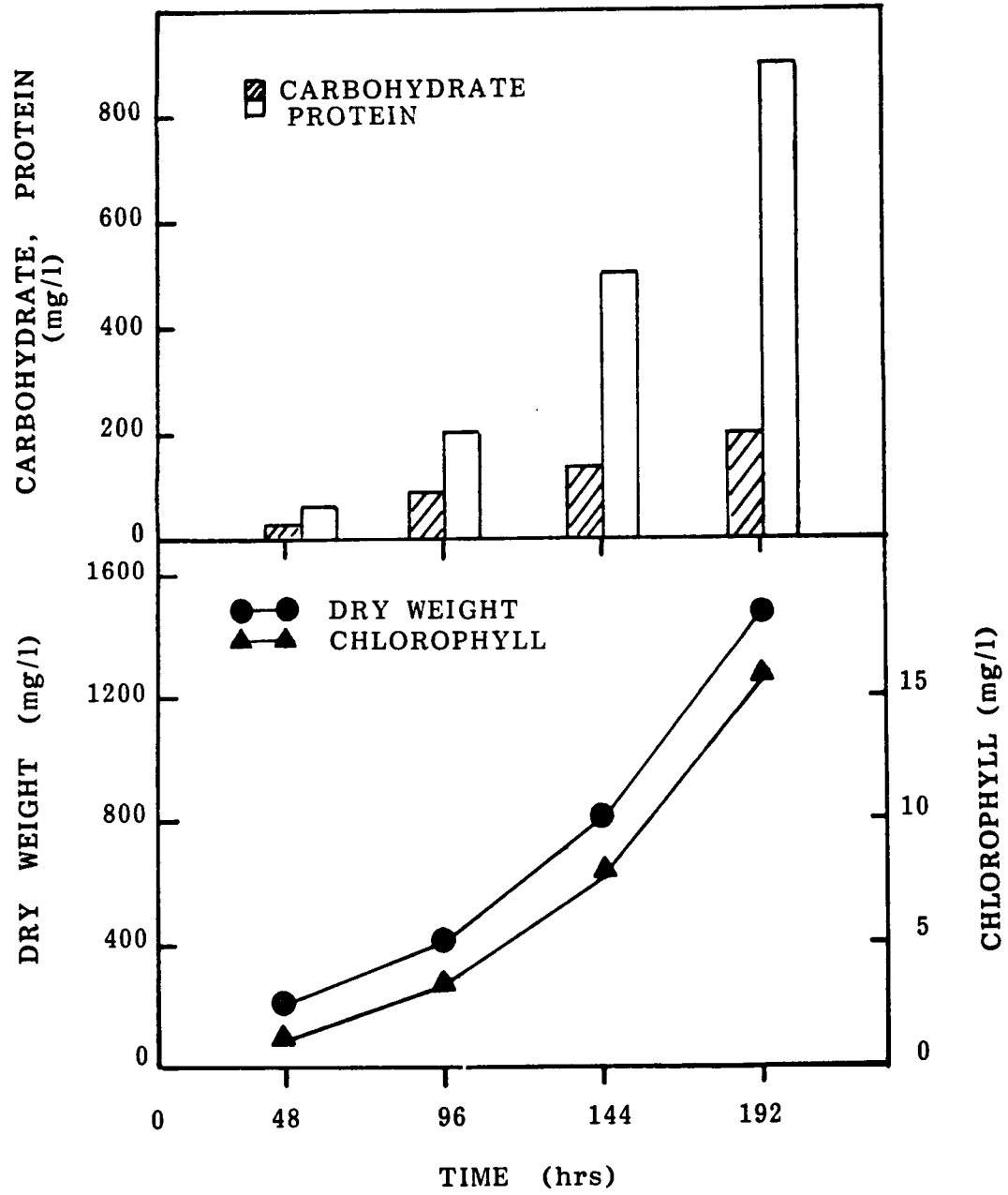


Figure 12b. Physiological Characteristics of S. platensis under Optimum Growth Conditions

Table 1. Molecular Composition of Spirulina strains

Species	Growth Conditions	% Organic Wt. (AFDW)		
		Protein	Carbohydrate	Lipid
<u>S. maxima</u>	* Sufficient Nutrients	69.75	11.5	4.68
	High Light (120 uE m <sup>-2</sup> s <sup>-1</sup> )	29.06	19.58	3.56
	High Temperature (38°C)	45.28	18.75	3.75
	N-limited	21.56	37.52	4.68
	P-limited	41.25	35.21	5.20
	Sodium Chloride 0.1M	52.62	26.25	4.68
	0.5M	45.64	36.73	7.52
	Bicarbonate (4g/L)	52.54	15.68	6.53
	<u>S. platensis</u>	* Sufficient Nutrients	65.12	9.37
High Light (120 uE m <sup>-2</sup> s <sup>-1</sup> )		27.18	15.22	3.65
High Temperature (38°C)		35.32	13.12	3.75
N-limited		32.81	30.31	5.43
P-limited		34.68	31.87	4.68
Sodium Chloride 0.1M		51.56	22.52	8.43
0.5M		37.50	32.10	10.31
Bicarbonate (4g/L)		41.25	13.25	5.62

\*Experimental conditions were:

temperature 30°C; light irradiance 80 uE m<sup>-2</sup> s<sup>-1</sup>;  
air flow rate 300 ml/min;

The values shown are averages of four independent determinations.



of Oscillatoria agardhii (Riegman et. al., 1985), showed elevated levels of polysaccharide storage.

Depletion of dissolved phosphate-phosphorus from the culture medium of Anabaena was accompanied by a decline in chlorophyll "a", protein, RNA and an increase in carbohydrate per unit dry weight (Healey, 1973).

**Sodium Chloride, concentration influenced the storage of carbohydrates and proteins of both strains.** As media (Zarrouk, see "Methods"), were enriched with 0.1 M and 0.5 M NaCl, the carbohydrate content of the cells increased, when compared to that of the control (Zarrouk: 0.01 M NaCl), to 26.25%, 36.73% in S. maxima and to 22.52%, 32.10% in S. platensis. On the other hand the total protein decreased respectively to: 52.62% 45.64% in S. maxima and 51.56%, 37.50%, in S. platensis. The lipid percentages showed little increase when compared to those of complete media (control). Many cyanobacteria are capable of adapting to a range of salinity in the environment by synthesizing internal osmotic support in the form of carbohydrates (Packer et. al., 1986).

**Bicarbonate:** When bicarbonate concentration of Zarrouch media was reduced to one quarter (4.g/L), neither strains showed much difference in the chemical composition as compared with the control media except their yield was somewhat below the control.

*SPIRULINA maxima*

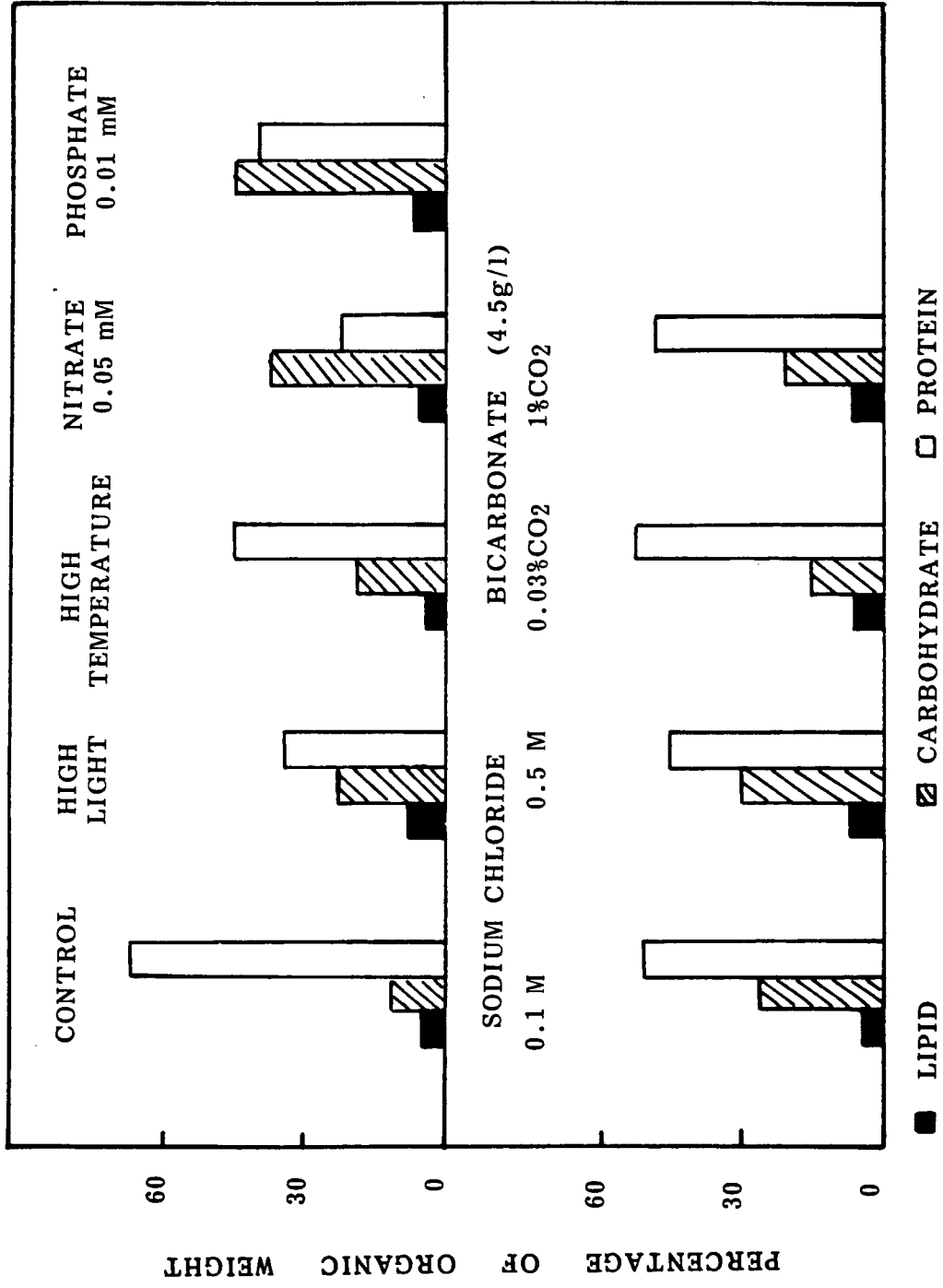


Figure 13a. Physiological Characterization of Cultures, *S. maxima* Cultures, under Stress Conditions

SPIRULINA platensis

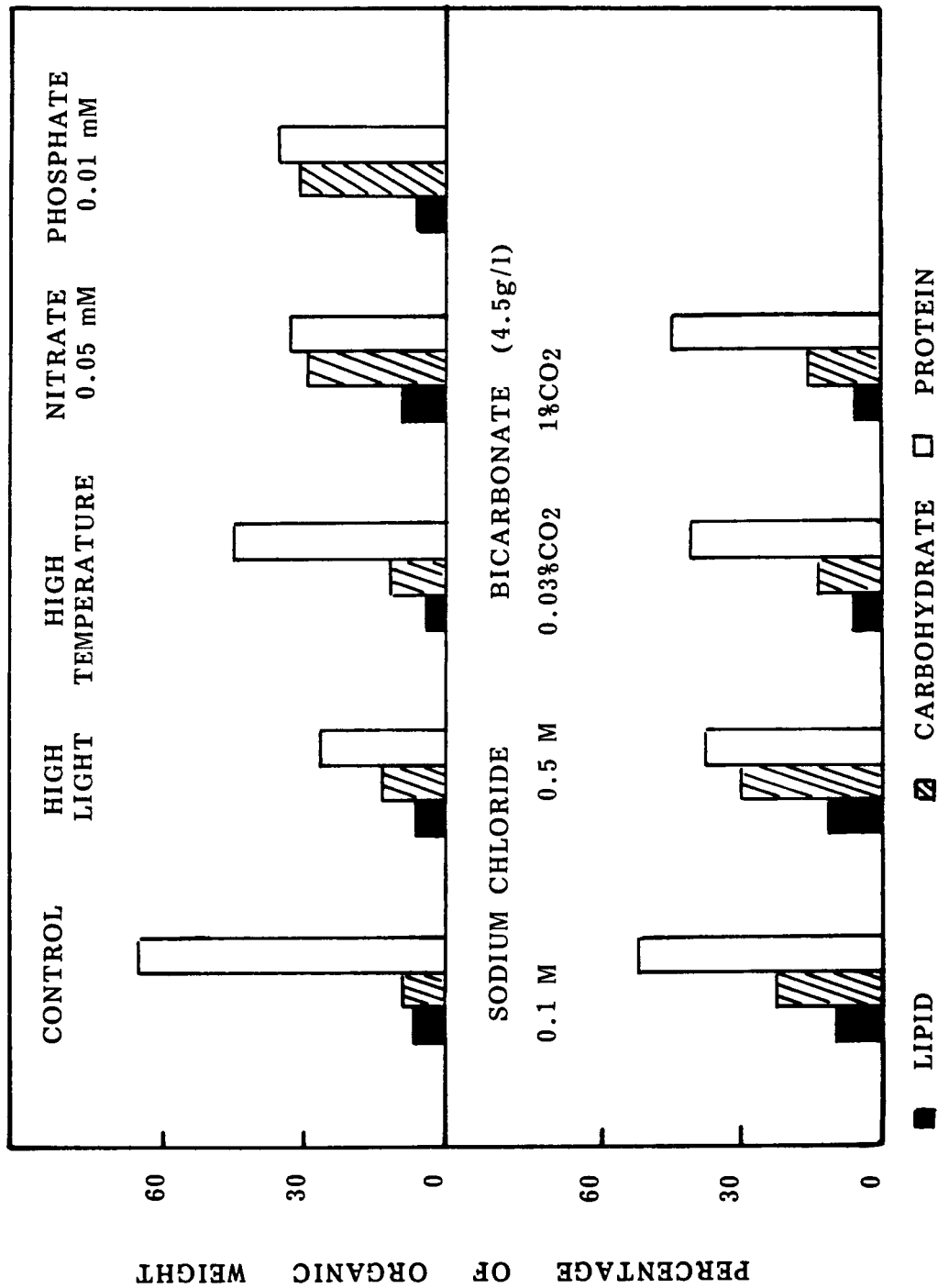


Figure 13b. Physiological Characterization of Cultures, *S. platensis* Cultures, under Stress Conditions

## CONCLUSIONS:

In an attempt to optimize the biomass of cyanobacteria "Spirulina", the growth parameters of two strains were characterized in batch cultures to gain basic information to be considered in a continuous culture system.

Conclusions of the first year study are summarized as following:

- . From the environmental variables studied, optimum growth temperature was clearly species specific. For S. maxima, 35°C and for S. platensis, 30°C.
- . Optimum light irradiation for both strains was 80  $\mu\text{E m}^{-2} \text{s}^{-1}$ .
- . Both strains exhibited a tolerance for a wide range of pH, from 8 to 11 with optimum pH in range of 9 to 10.
- . When studying the effect of flow rate of aeration and percentage of CO<sub>2</sub> present in the air on the growth rate and yield of the alga; it was concluded that the 500 ml/min for aeration rate and 1% CO<sub>2</sub> gave optimum conditions of growth. It was concluded also that cultures supplied with air (0.03% CO<sub>2</sub>) gave the same response in terms of productivity as well as those supplied with 1% CO<sub>2</sub> enriched air, providing that the bicarbonate concentration present in the medium was reduced to 4 g/L instead of 16 g/L (Zarrouk medium).
- . Variations in supply of nutrients: Nitrate-N, phosphate-P, sodium chloride, bicarbonate, affected the productivity rate of the alga. They not only influenced the production rates, but also the quality of the produced biomass as measured by the carbohydrate and total protein. In most of the cases the carbohydrate content increased when nutrients were limiting or in excess as sodium chloride concentration of 0.1 M and 0.5 M (see Table 1).
- . The lipid percentage, in particular, did not show much increase in different culture treatments. But, increasing the temperature of culturing to 38°C or the light irradiance to 120  $\mu\text{E m}^{-2} \text{s}^{-1}$ , reduced the total lipids drastically. However, increasing sodium chloride to 0.1 M in the culturing media, the lipids increased somewhat higher than in the control media.
- . The ability of the alga to utilize macroelements and microelements, and to convert it into biomass.

- . Variation of single environmental regulants such as light intensities or temperature, during the present study also revealed their detrimental effect even when the cultures contain sufficient nutrients. Cultures whose growth rates and productivities were reduced by any factor, became progressively more yellow (light, temperature, nutrient limitation, pH) and changed in morphology (Fig. 14).
- . A slight inverse relationship was observed between the protein content and carbohydrates which means that one increased at the expenses of the other. This suggests that quality of biomass may be manipulated for dietary purposes. An adequate supply of nutrients is therefore a pre-requisite for producing a uniform quality of biomass, which in turn could then be used in the formulation of diets. (see Sufficient Nutrients). The possibility of manipulating the quality of the biomass could have potential for the NASA/CELSS Program, when specific diet formulation is needed (e.g. low protein content).
- . Overall algal productivity and quality could be manipulated by means of varying nutrient concentrations or temperature and light irradiance.

Further work is needed to characterize the efficiency of the algal cells under such environmental conditions in terms of gas exchange and energy loss or gain in steady state.

"It can be concluded that through manipulating environmental conditions of the algal growth, one can modify the photosynthetic products. Thus, Spirulina can be, through manipulating growth factors, used as palatable diet comparable to higher plants (see Appendix)."

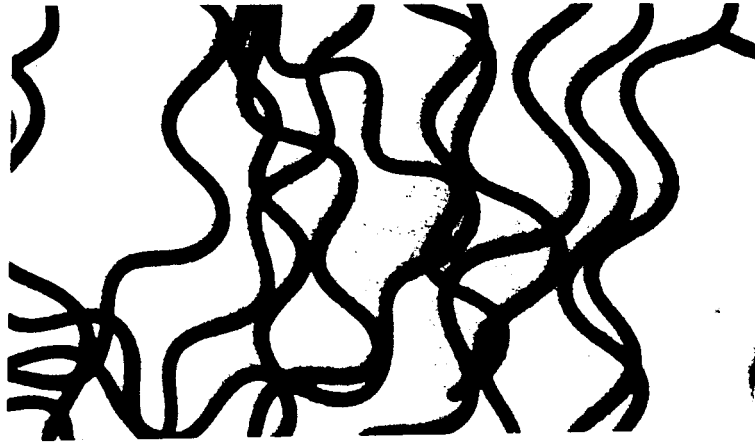


Figure 14a. Cells of S. maxima grown under Optimum Conditions. Scale: 1 cm = 25 u

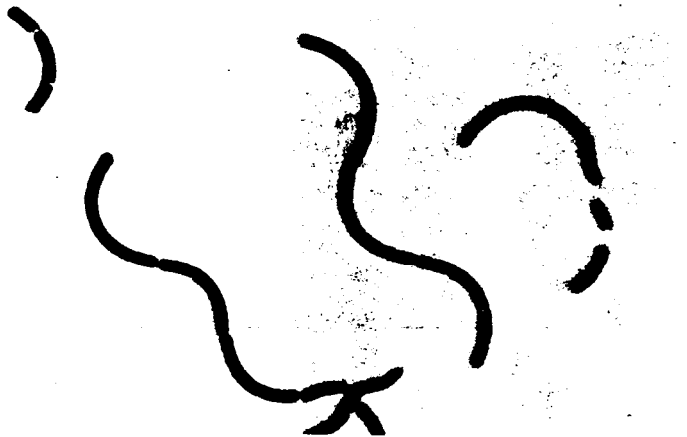


Figure 14b. Cells of S. maxima grown under Stress Conditions. Scale: 1 cm = 40 u

## **FUTURE RESEARCH (1988/1989)**

We plan to investigate the effects of: light irradiation, temperature, carbon dioxide on partitioning of macromolecules (protein, carbohydrates, lipids) and on elemental composition (carbon, hydrogen, oxygen) of Spirulina maxima. The manipulation of composition of Spirulina maxima by the environmental factors will be investigated at steady state.

### **Tasks:**

- I. Biomass (Dry Weight and Chlorophyll) and Cultivation Time
- II. Dilution Rate and Dry Weight
- III. Productivity and Dry Weight
- IV. Effect of Light Intensity:

This experiment will be performed using the best temperature and aeration obtained from batch culturing. The growth rate as a function of light intensity will be determined at: 30, 60, 100  $\mu\text{E m}^{-2} \text{s}^{-1}$  and as well as 35°C temperatures in relation to the productivity and efficiency of the alga.

- V. Aeration and Carbon Dioxide Concentrations

The following experiments will be performed at steady state:

- . Bubbling with air (0.03% CO<sub>2</sub>)
- . Bubbling with air (0.1% CO<sub>2</sub>)
- . Bubbling with air (1% CO<sub>2</sub>)
- . Bubbling with air (5% CO<sub>2</sub>)

Experiments IV and V will be analyzed for the following:

- . Dry Weight
- . Chlorophyll
- . Productivity
- . Light efficiency
- . Particulate carbon
- . Particulate nitrogen
- . Inorganic nitrogen and carbon
- . Total phosphorus
- . Proteins
- . Carbohydrates
- . O<sub>2</sub> - evolution
- . CO<sub>2</sub> - measured in flux in and out of culture

## EXPECTED OUTCOMES

- . Relationship between dilution rate (growth rate) and dry weight
- . Growth rate as function of light intensity e.g. 30, 60, 100  $\mu\text{E m}^{-2} \text{s}^{-1}$
- . Productivity and chemical analysis in relation to three light intensities will be determined in order to determine efficiency of the alga
- . Relationship between  $\text{CO}_2$  mass flux in ( $\text{mg C. day}^{-1}$ ) and algal carbon mass flux out ( $\text{mg C. day}^{-1}$ ) at fixed dilution rate.
- . Relationship between  $\text{CO}_2$  mass flux (in-out) at different concentrations and algal carbon flux out ( $\text{mg C. day}^{-1}$ ) at fixed dilution rate
- . Relationship between  $\text{CO}_2$  mass flux in ( $\text{mg C. day}^{-1}$ ) and algal carbon:nitrogen mass flux out ( $\text{mg C. day}^{-1}$ )
- . Relationship between  $\text{CO}_2$  mass flux in ( $\text{mg. C. day}^{-1}$ ) and algal composition (carbohydrate, protein, lipid)
- . Relationship between  $\text{O}_2$  evolved by the culture and  $\text{CO}_2$  flux
- . Efficiency evaluation of Spirulina biomass for CELSS, in terms of light,  $\text{CO}_2$  and nutrient parameters.



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A P P E N D I X

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**SPIRU•LEAN** contains the essential amino acid, **L-Phenylalanine** which enables the body to produce the Neural Transmitters, dopamine and norepinephrine. Nutritional doctors have determined that the increased production of these Neural Transmitters in the brain will result in increased energy, a feeling of well-being, and a decrease in appetite and cravings for food.

**SPIRU•LEAN** also contains the renowned and successful **Lecithin, Kelp, Apple Cider Vinegar and B-6 formula**. **Lecithin** is of course known as nature's most powerful emulsifier, helping to reduce fatty deposits and cholesterol in the body. **Vitamin B-6** has been used as an effective diuretic. Together this special combination will effectively aid in diet control by breaking down fatty tissues and reducing water retention.

**SPIRU•LEAN** is the most complete, effective, natural aid to weight control available. **SPIRU•LEAN** may be taken along with any sensible diet plan, complementing your diet's nutritional value with the finest quality, naturally balanced vitamins, minerals, and amino acids which are often sacrificed when food intake is limited.

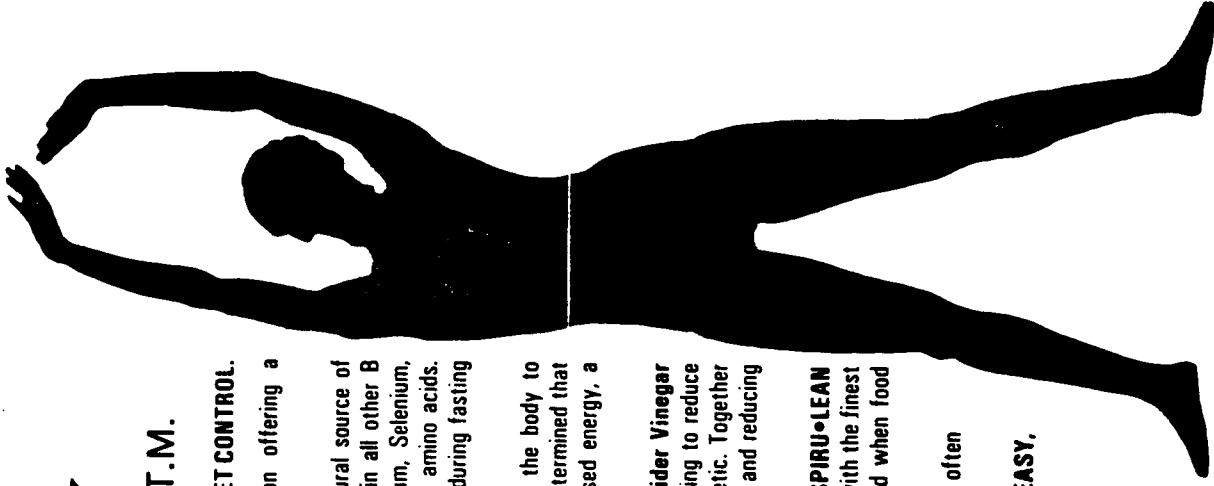
**SPIRU•LEAN** will naturally aid in controlling your appetite with no side effects often experienced when taking over the counter diet pills and prescription drugs.

**FINALLY....WITH NATURE'S PLUS SPIRU•LEAN ANY DIET CAN BECOME AN EASY, ENJOYABLE EXPERIENCE, OFFERING FAST AND REWARDING RESULTS!**

**PRODUCT # 4697 90 TABLETS**

**PRODUCT # 4698 180 TABLETS**

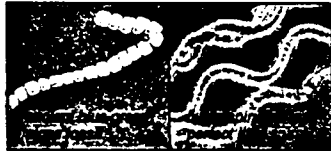
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ORIGINAL PAGE IS  
OF POOR QUALITY

# Three billion year old expert grants immunity

**"I am the immortal descendant of the original life form.** Over 3 billion years ago, blue-green algae produced our oxygen atmosphere so life could evolve. Today, I offer your life timely health benefits.



Your own technology and life-style threaten you. Pollution, toxic chemicals, radiation, disease, stress, drugs and processed foods attack your immune system. Damage to your body and its cells can cause premature aging and cancer.

## Natural Life Preserver

Vitamin supplements help out, but with two limitations. First, synthetic mega-doses are wasted if your body can't absorb them and may even be toxic. But my whole food spirulina vitamins and minerals are easily absorbed.

Second, because your body is not simply a mechanism, it needs more than isolated nutrients. Living cells need specific information to remember their function. DNA molecular codes in natural foods contain genetic memories of successful life forms for millions of years. I have rejuvenated myself since the beginning. My 3 billion years of cell memories can help your body remember its powers and renew itself. When you need a natural life preserver, put me to work inside your body.

## Unusually Concentrated

Within me is a powerful concentration of nutrients, unlike any other single plant, grain, food or herb. I flourished in the nutrients of the original primordial soup. Many of these nutrients are strongly recommended by scientists to build the immune system. New medical research has focused on my effects on cholesterol reduction, cancer and immune system response.

## Protective Nutrients

I have more beta carotene than chlorella algae and ten times more than any other food. Beta carotene is known to lower the incidence of cancer and protect against UV radiation. I am the only food that contains significant amounts of gamma linolenic acid (GLA), the beneficial nutrient in evening primrose oil. I am the best source of vitamin B-12, sometimes deficient in vegetarian diets. I am rich in iron, essential for healthy red blood cells and a strong immune system.

I have the highest protein content (60-70%), all the essential amino acids, and RNA and DNA nucleic acids. I'm 1% cleansing chlorophyll and 15% strengthening phycocyanin, a unique blue pigment found only in blue-green algae.

## Naturally Digestible

I am so old that I evolved before hard cellulose cell walls. My cell walls are naturally soft proteins. My younger cousin, chlorella, requires additional factory processing to break down its hard cell walls. But I'm already perfect - 95% digestible. The most digestible food. Many people say they feel my energy within minutes.



## Earthrise Quality Standard

Just in the past 20 years I've come to your awareness-a very short time in history for a new food. Rediscovered in the 1960's, I was introduced as a health food supplement in 1979 by Earthrise. Now I'm enjoyed by millions world wide-athletes, vegetarians, dieters, health practitioners, and people of all ages.

Earthrise is one of my most advanced farms. Here, I'm grown free of pesticides, herbicides, additives and preservatives, and 100% pure.

Extra iron and trace minerals added to my ponds make Earthrise Spirulina the best iron food.

My living cells are dehydrated in seconds by low temperature spray drying, best preserving valuable nutrients. Then I'm freshly sealed in anti-oxidant containers. Earthrise is the only company that can guarantee quality from my living ponds to my consumers.



Earthrise Spirulina is the highest quality

## Clean Green Energy

Enjoy my energy every day- great between meals and before strenuous activity for quick nutrition without feeling too full. I'm also helpful for dieting and cleansing your body.

Once known as a *food of the future*, I am already growing in villages in the developing world. I'm called 'green medicine' food by the children.

So, partake of my immortal body each day. Eat 3 billion years of cell memory and a concentration of protective nutrients. Renew your own health, renew your connection with your sisters and brothers in the third world, and with the origins of life."

You can find best selling Earthrise Spirulina at health food stores or order it direct from Earthrise.

Please send me free literature on:

- Earthrise Tablets and Powder
- New Medical Research with Spirulina
- Spirulina in Third World Villages

Name \_\_\_\_\_

Address \_\_\_\_\_

City, State, Zip \_\_\_\_\_



Earthrise Company, PO Box 1196-VT  
San Rafael, CA 94915

Earthrise® Spirulina - nature's most protective food