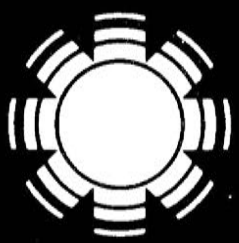

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Aquatic Species Program Review

Proceedings of the March 1985 Principal Investigators Meeting

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PREFACE

This volume contains progress reports presented by the Aquatic Species Program subcontractors and SERI researchers at the SERI Aquatic Species Review held at SERI, March 20 and 21, 1985. These reports present and discuss research advances achieved by the program participants during the preceding year. The SERI Aquatic Species Program receives its funding through the Biomass Energy Technology Division of the Department of Energy (Beverly Berger, Director).

Robins P. McIntosh
Aquatic Species Program

Approved for

SOLAR ENERGY RESEARCH INSTITUTE

Eric H. Dunlop, Director
Solar Fuels Research Division

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SERI

INTRODUCTION

The worldwide energy shortage and Arab oil embargo of the early 1970s encouraged many nations to look for new sources of oil, electricity, and gas. Renewable resources such as biomass were often highlighted as a long-term solution to the energy problem because of their nondepletable, renewable nature. While the first biomass sources considered were the readily available ones such as corn or wood, it was apparent that new biomass sources should also be developed, among them aquatic species.

The purpose of the Aquatic Species Program is to improve the productivity, conversion to fuels, and cost efficiency of aquatic plant culture technologies. The emphasis of the program is on developing a mass culture technology for cultivating oil-yielding microalgae in the American Southwest. A technical and economic analysis indicated that such a concept would be feasible if (1) lipid yields from microalgae are improved, (2) there is sufficient saline water for large-scale development, and (3) microalgal lipids can be eonomically converted to conventional fuels. It was determined that fuels from microalgal lipids presented better options than converting the microalgal biomass to either alcohols or methane. All lipids can potentially be catalytically converted to gasoline, or the fatty acids can be converted to substitute diesel fuels. The Southwest has the necessary low, flat, underutilized land, and carbon dioxide is available from either natural deposits or flue gas from industrial plants. The amount of saline water available will probably determine how much fuel can be produced from aquatic species, and this question should be answered during 1985.

The largest constraint to this technology is the economical production of an oil-rich microalgal feedstock. In addressing this constraint, the Aquatic Species Program is concentrating research on (1) initially selecting the best species, (2) developing the best culture and management methodologies, and (3) improving the most promising species. An improved screening program has allowed for more efficient selection of species. A total of 46 new, fast-growing strains were added to the program this year for further characterization and future improvement. Outdoor studies resulted in a 40% improvement in biomass yields. Annualized yields of 70 tons/ac/yr were sustained for one month in Hawaii. This is a 35% improvement from the previous year. Major cost reductions resulted from recycling culture media and reducing inputs of costly trace elements.

The 1985 annual research review of the Aquatic Species Program was held March 20-21 at the Solar Energy Research Institute in Golden, Colorado. In attendance were 65 participants, which included 16 principal investigators and 5 advisers. The advisory panel included Drs. Jack Myers, Ian Morris, Richard Radmer, Michael Neushul, and Robert Krauss.

The agenda for the review was divided into four sections: species selection and characterization, applied physiological studies, outdoor mass cultivation, and systems design and analysis. Papers from these presentations are included in these proceedings. Program advances were reported in the areas of species collection and selection, modulated light physiology, mass culture yields, harvesting of microalgae, mass culture facility design and analysis, and assessments on fuel options from microalgae.

SPECIES SCREENING AND CHARACTERIZATION

SELECTION OF DESERT SALINE MICROALGAE FOR HIGH YIELDS AT ELEVATED TEMPERATURES AND LIGHT INTENSITIES AND IN SERI STANDARD ARTIFICIAL MEDIA

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ABSTRACT

This work continues and supplements that concerning cultural requirements, yields, and photosynthetic efficiences of some desert saline microalgae reported in 1984. However, our selection process has become more rigorous in that high temperatures and light intensities have been used and the algae have been cultured in SERI standard artificial salts media that approximate the composition of saline groundwaters in New Mexico. Forty-one new strains have been isolated under these more rigorous conditions. The enrichment selection procedure was shown to select a marine "weed" strain, *Platymonas* in competition with natural marine microalgae. Forty-three strains (new isolates plus some previously isolated) were grown in SERI standard media at 24 ° and 30 ℃ and 40% La Jolla sunlight. These preliminary experiments separated 33 strains that were "good" or "intermediate" growers from 10 stains that grew poorly. Twelve good growers were tested in all five SERI media and the best medium was chosen for further studies of the temperature and salinity requirements of eight strains. Four strains were identified as being tolerant of high temperatures and salinities. One of these, a *Chlorella* from Salt Creek, California grew well even at 40 ℃. Two strains were grown in mass culture under the "best" conditions found in the above studies to determine yields and efficiencies of light utilization (PAR only). Yields ranged from 31.9-55.5 gm $m^{-2}day^{-1}$ and efficiencies ranged from 7.7-17.6%. We conclude that we have been able to isolate and identify high-yielding strains of desert saline microalgae that show promise for future outdoor culture, particularly with regard to their tolerance of wide ranges of temperatures and salinity.

INTRODUCTION

This report extends that presented at the last SERI Aquatic Species Program Review Meeting in April 1984 (Thomas et al, 1984). However, new procedures have been applied to the problem of selecting high-yielding strains of desert saline microalgae in that 1) the algae have been subjected to high temperatures and light intensities during the initial screening process; and 2) standard SERI artificial media have been used in preliminary searches for high-yielding strains; in studies of their temperature/salinity requirements; and in mass cultures set up to determine maximum yields. Through this new selection process we have obtained high-yielding desert microalgal strains that grow well at elevated temperatures, light intensities, and salinities in standard SERI media.

MATERIALS AND METHODS

Special Equipment

We have designed and constructed two reciprocal shaker tables that shake water baths containing 65-ml culture bottles with 50 ml of medium. The water baths have glass bottoms so that the cultures are illuminated from below with 2000-watt incandescent stage lamps that provide the cultures with a light intensity of 40% of the PAR sunlight that is found maximally at La Jolla. The temperatures of the water baths were 25 ° and 30 °C. 65-ml bottle cultures were used for initial enrichment experiments to grow up algae in their natural water, in preliminary tests of growth in SERI media, and in tests of growth in all five SERI standard media.

The temperature/salinity gradient block described previously (Thomas, et al, 1984) has continued to be used with 65-ml bottle cultures and SERI standard media. Growth in these cultures and in shaker table bottles has been assessed by non-invasive optical density measurements as described previously.

We have also set some lighted Percival incubators at 30 °C and 15% sunlight (fluorescent lamps) for additional enrichment experiments. Cultures in these incubators were contained in 125-ml flasks and were not shaken.

Field Trips and Isolations

Two short field trips were carried out (May, 1984 and July-August, 1984) to the deserts of eastern California and western Nevada. The sites which were sampled were the same as those described in our previous report (Thomas et al, 1984). Water and soil samples were brought back to our La Jolla laboratory, enriched with nutrients (NH_4^+ and urea used as N sources), and incubated on the shaker tables and in the Percival incubators. When growth was apparent, cells were isolated using micropipetting and agar plating. After 2-3 weeks of additional incubation of isolates they were examined for culture purity and transferred into artificial salts media (Thomas et al, 1984) and into our culture collection. The collection was incubated in fluorescent light or in natural light (north window).

Media

The major salts for five SERI Standard Media (B. Barclay, SERI) are shown in Table 1. Ammonium (400 μ mole liter^{-1}), phosphate (40 μ mole liter^{-1}), and Chapman trace metals (Thomas et al, 1984) were added as nutrient enrichments in preliminary and media experiments. The ammonium and phosphate levels were increased to 20 M mole liter^{-1} and 2 M mole liter^{-1}, respectively, in mass culture experiments on yield.

These media were based on the composite chemical composition of saline ground waters in New Mexico and SERI specified that we carry out our experiments with them. Nevertheless, they are not ideal media in that precipitate was formed when they were made up. This precipitate probably consisted of insoluble salts of calcium and magnesium. It was removed when the media were filter-sterilized and thus there is some uncertainty regarding the exact chemical composition of these media as used in the experiments. To use these media with NH_4^+ as an N source it was necessary to adjust their pH to pH 8. This did not reduce the amount of precipitate in them.

Preliminary Experiments

Over 40 algal strains (those isolated in the 1984 field trips and some isolated previously) were tested for growth in 65-ml bottle culture incubated on the shaker tables at 25 ° and 30 °C and at 40% sunlight. Standard media were used that most closely approximated the composition of the water from which each strain was isolated. Growth was assessed by daily non-invasive optical density measurements in the bottles and exponential rates of growth and maximum optical densities were determined. These experiments identified "good", "intermediate", and "poor" growing species.

Media Experiments

Good growing species were tested further in all five SERI media on the shaker tables at the temperatures (25 ° or 30 °) at which they did best in preliminary experiments. Again growth was

Table 1. Formulas for SERI Standard Media

mg liter^{-1}			
Low Salinity			
	Type I	*Type II*	
$CaSO_4$	140	$CaSO_4$	877
$CaCl_2$	3425	NA_2SO_4	1045
$MgSO_{4R}$	2216	$MgSO_4$	853
KCl	90	KCl	394
$NaHCO_2$	671	$NaHCO_3$	2129
NaCl	1376	NaCl	2599
Moderate Salinity			
	Type I	*Type II*	
$CaSO_4$	2097	$CaCl_2$	245
$CaCl_2$	316	$MgCl_2 \cdot 6H_2O$	3169
$MgSO_4$	1619	Na_2SO_4	1195
KCl	18.4	KCl	369
$NaHCO_3$	1111	$NaHCO_3$	2902
NaCl	8967	NaCl	8768
High Salinity			
	Type I		
$CaCl_2$	1755		
$MgSO_4$	1240		
Na_2SO_4	5524		
KCl	773		
$NaHCO_3$	2206		
NaCl	13482		

assessed by daily non-invasive optical density measurements in the 65-ml bottles. Maximum experiential growth rates and optical densities were determined.

Temperature/Salinity Requirement Experiments

Once a good medium was defined in the media experiments, it was used in experiments to test the response of good growing strains to varying temperatures (15-40 ℃) and salinities (0-40 gm liter^{-1}) in the temperature gradient block. Daily optical density measurements were carried out until a consistent response to temperature and salinity was obtained. These final optical densities (yields) were plotted as a function of temperature and salinity. In some instances the culture bottles were placed on a chart of salinity and temperature and photographed with color Polaroid film to delineate further the response of the given algal strain.

Mass Cultures

Once the proper salinities and temperatures were known, good growing species were grown up in 12-liter carboys in their best SERI media using fluorescent light (5-10% sunlight) and a temperature of 25 ℃. Once these cultures became dense (300-500 mg dry weight liter^{-1}) they were poured into the 15-cm, 12-liter mass culture apparatus described previously (Thomas et al, 1984) and grown at a light intensity of 50% La Jolla sunlight provided by a 2000-watt tungsten-halide lamp. These cultures were mixed with 1-2% CO_2-in-air. Measurements of dry weight liter^{-1} and optical density (at 750 μm in 1 cm cells) were made daily and yields and photosynthetic efficiencies were determined as previously described. After several days of growth the light intensity was increased to 70% La Jolla sunlight. When each cultures reached 2000 mg dry weight liter^{-1}, half of it (6 liters) was harvested by centrifugation washed with isotonic ammonium formate, and recentrifuged. The resulting paste was freeze-dried and sent to Dr. Tom Tornabene of Georgia Institute of Technology for chemical analyses of the cells. The remaining culture was diluted back to 12 liters with fresh medium containing no added N and P and was allowed to grow for a further 10 days as a nutrient-deficient crop. The culture was then harvested and sent to Dr. Tornabene for analyses of deficient cells. The disappearance of NH_4^+ from the culture as nutrient deficiency progressed was measured with an ammonium electrode.

RESULTS AND DISCUSSION

New Cultures

Cultures that were obtained from the 1984 field trips are listed in Table 2. Forty-one new strains were isolated and added to our culture collection. We are carrying another 86 cultures isolated in 1982-83. A good many of these are strains of *Dunaliella, Chaetoceros* and *Nitzschia* that SERI has requested us to keep. Most are maintained on agar slants and therefore only have to be transferred every three months rather than our usual transferring of liquid cultures once a month.

Test of Selection- by- Enrichment Procedure

Following enrichment, while these new strains were growing up, we tested our procedure to see if an alga known to be hardy would be selected. Varying concentrations of the Hawaiian strain of *Platymonas*, a "weed species", was added to Scripps Pier seawater containing natural phytoplankton. The dominant algae in the initial sample were a cryptomonad sp. (41 cells ml^{-1}); a dinoflagellate (22 cells ml^{-1}); and a 10 μm naked flagellate (24 cells ml^{-1}). Other algae -- *Anabaena, Nitzschia, Oscillatoria, Mallomonas, Navicula* and an unidentified centric diatom-- made up an additional 17 cells ml^{-1}. *Platymonas* was added at 10, 100, 1000, and 10000 cells ml^{-1} and the cultures were enriched with inorganic nutrients (NH_4^+,PO_4^-3, Si, and trace metals). The cultures were incubated for 8 days at 40% La Jolla sunlight and at 24 ℃ and 30 ℃ on two shaker tables. At the end of the experiment all cultures were visibly green. Phytoplankton were then counted in those cultures having an initial ratio of *Platymonas*: natural phytoplankton of 0.1 and 1.0 . In 24 ℃ cultures *Platymonas* counts were 3.7x10^4 cells ml^{-1} (initial ratio 1.0) and no other algae were seen. At the same temperature and at an initial ratio of 0.1, the final *Platymonas* concentration was 3 x10^3 cells ml^{-1} and the final concentration of a centric diatom as 3x10^2 cells ml^{-1} (no other algae were seen). At 30 ℃ and an initial ratio of 0.1, the

Table 2. Cultures that were obtained by enriching saline waters and soils from the 1984 field trips.

Algal Strain	Date Sampled	Water Temperature (^{o}C)	Salinity ($gm\ liter^{-1}$)
CALIFORNIA			
Owens Lake			
OL-10 *Nitzschia*	May 16, 1984	27	15.4
OL-11 *Cymbella*	May 16, 1984	27	15.4
OL-12 *Chaetoceros*	May 16, 1984	27	15.4
OL-13 Unidentified yellow round cells	Aug. 2, 1984	30	2.9
Black Lake			
BL-4 *Chaetoceros*	May 16, 1984	15	9.3
BL-5 *Nitzschia*	May 16, 1984	15	9.3
BL-6 *Chlorella ellipsoidea*	May 16, 1984	15	9.3
BL-7 *Cymbella*	May 16, 1984	15	9.3
BL-8 *Chaetoceros*	Aug. 1, 1984	26.2	13
Harper Lake			
HL-7 *Cymbella*	May 14, 1984	18	4.9
HL-8 Unidentified pennate diatom	May 14, 1984	18	4.9
HL-9 *Chlamydomonas*	May 14, 1984	18	4.9
HL-10 *Cyclotella*	May 14, 1984	18	4.9
HL-11 Unidentified pennate diatom	May 14, 1984	18	4.9

Table 2. (continued)

Algal Strain	Date Sampled	Water Temperature (°C)	Salinity (g liter^{-1})
CALIFORNIA			
Salt Creek			
SC-2 *Chlorella*	July 30, 1984	38	13.5
SC-3 *Gloeocystis*	July 30, 1984	38	13.5
SC-4 *Nitzschia*	July 30, 1984	38	13.5
Salton Sea			
SS-12 Unidentified pennate diatom	Aug. 10, 1984	35.6	--
SS-13 Unidentified green oval cell	Aug. 10, 1984	35.6	—
SS-14 *Chaetoceros*	Aug. 10, 1984	35.6	—
SS-15 *Nitzschia closterium*	Aug. 10, 1984	35.6	—
NEVADA			
Walker Lake			
WL-9 unidentified yellow-green round cells	May 15, 1984	14	7.4
WL-10 *Chaetoceros*	May 15, 1984	14	7.4
WL-11 *Chlorococcum*	May 15, 1984	14	7.4
WL-12 unidentified yellow round cells	May 15, 1984	14	7.4
WL-13 *Nitzschia*	May 15, 1984	14	7.4
WL-14 *Nannochloris*	May 15, 1984	14	7.4

Table 2. (continued)

Algal Strain	Date Sampled	Water Temperature (°C)	Salinity (gm liter^{-1})
NEVADA			
Columbus Salt Marsh			
CSM-3 unidentified green round cells	May 15, 1984	--	--
CSM-4 Chlorella	May 15, 1984	--	--
CSM-5 Selenastrum	July 31, 1984	26	4.7
Milepost 56 off Highway 80			
56R80-1 unidentified green flagellate	July 31, 1984	26.4	21.8
56R80-2 Dunaliella	July 31, 1984	26.4	21.8
56R80-2 unidentified green flagellate	July 31, 1984	26.4	21.8
56R80-4 Dunaliella	July 31, 1984	26.4	21.8
56R80-5 unidentified green round cells	July 31, 1984	26.4	21.8
Big Soda Lake			
BS-6 unidentified yellow-green round cells	May 15, 1984	15.5	16.6
BS-8 Cymbella	July 31, 1984	28.8	18.6
BS-9 unidentified green flagellate	July 31, 1984	28.8	18.6
BS-10 unidentified green colonies	July 31, 1984	28.8	18.6
Silver Peak Salt Marsh			
SP-4 Chlorella	May 15, 1984	--	--
SP-5 Nitzschia	July 31, 1984	--	--

Platymonas count was $9x10^4$ cells ml^{-1} and no other algae were seen. At an initial ratio of 1.0 and 30 °C, the *Platymonas* count was 10^6 cells ml^{-1} and no other algae were seen. We conclude that *Platymonas*, a "weed species," outcompeted the naturally-occurring algae under the conditions that we used for selecting desert algae strains and particularly at 30 °C.

Preliminary Cultures in SERI Media

Forty-two strains have been grown in SERI media most closely approximating the composition of the natural waters from which the algae were isolated. These cultures were grown at 24 °C and 30 °C on the shaker tables and at a light intensity of 40% La Jolla sunlight. Growth was monitored by daily optical density measurements from which exponential growth rates were calculated. We have attempted to set up criteria for "good", "intermediate", and "poor" growing cultures, but this is rather arbitrary and subjective. Good growers have experimental growth rates > 0.5 and/or maximum optical density > 0.5; intermediate growers have rates of 0.3-0.5 doublings day^{-1} and/or maximum optical densities from 0.25 to 0.5; poor growers have rates < 0.3 doublings day^{-1} and/or maximum optical density < 0.25. Another, but subjective, way to establish these criteria is to rate the cultures visually. Good growers would be rated "+ + + +" or "+ + +", intermediate ones "+ +", and poor growers "+", "±", or "–".

Figure 1 shows a growth curve for the 1984 Black Lake *Chlorella ellipsoidea*. It is a typical good grower. A growth curve for an intermediate grower is shown in Figure 2, and that for a poor grower is shown in Figure 3.

Table 3 presents the growth data in preliminary culture for 33 strains that grew at "good" or "intermediate" levels. Some of these grew very well; for example, the BL-6 *Chlorella ellipsoidea* strain from Black Lake had a growth rate of 2.48 doublings day^{-1} at 30 °C. An additional 10 strains were tested, but grew "poorly" if at all and the growth data are not presented. Some of these "poor" growers grew well (visual observations) in SERI media at a lower light intensity supplied by fluorescent lamps. We repeated the experiment using the shaker tables and high light intensity with two of these "poor" cultures, and again the growth was "poor" under these conditions.

Growth of "Good" Cultures in All Five SERI Media

We tested some of the "good" or "intermediate" growers in all five SERI standard media so that we could choose the best medium for a particular alga. Figure 4 shows the results of one of these experiments with the Black Lake *Chlorella ellipsoidea*, BL-6. This strain grew best in SERI standard medium II at a low or moderate salinity.

Table 4 summarizes growth data in all five SRI standard media for 12 strains. For any given culture either type I or type II media was best and certain salinity patterns were also in evidence. For instance, the *Chlorella* we obtained from Joel Weismann grew best at moderate salinities in either medium type. The Salton Sea *Nitzschia* (SS-1A) grew best at a high salinity in type I water or at a moderate salinity in type II water. This probably reflects the fact that the Salton Sea is a highly saline environment (TDS 39 gm $liter^{-1}$). A similar pattern was also observed for the Mono Lake *Nannochloris* (MO-2A) and Mono Lake is also highly saline.

Growth of Marine Species in SERI Media

Four marine species were cultured on the shaker tables at 25 ° or 30 °C in all five standard SERI media and at a light intensity of 40% sunlight. The results are shown in Table 5. *Phaeodactylum* grew well in all media; our strain of *Chaetoceros gracilis* grew well in four media; and *Platymonas* grew well in two media. *Isochrysis* did not grow under these conditions but grew in standard medium I, high salinity at 30 °C in fluorescent light. In the shaker table bottles, *Isochrysis* did not die since motile cells were seen at the end of the experiment, but it did not grow and probably the light intensity and/or the turbulence due to shaking were inhibitory on the shaker table.

Responses to Temperature and Salinity

Once the best medium was defined for a good growing strain, we could test the response of given algae to varying temperatures and salinities in our temperature/salinity gradient block

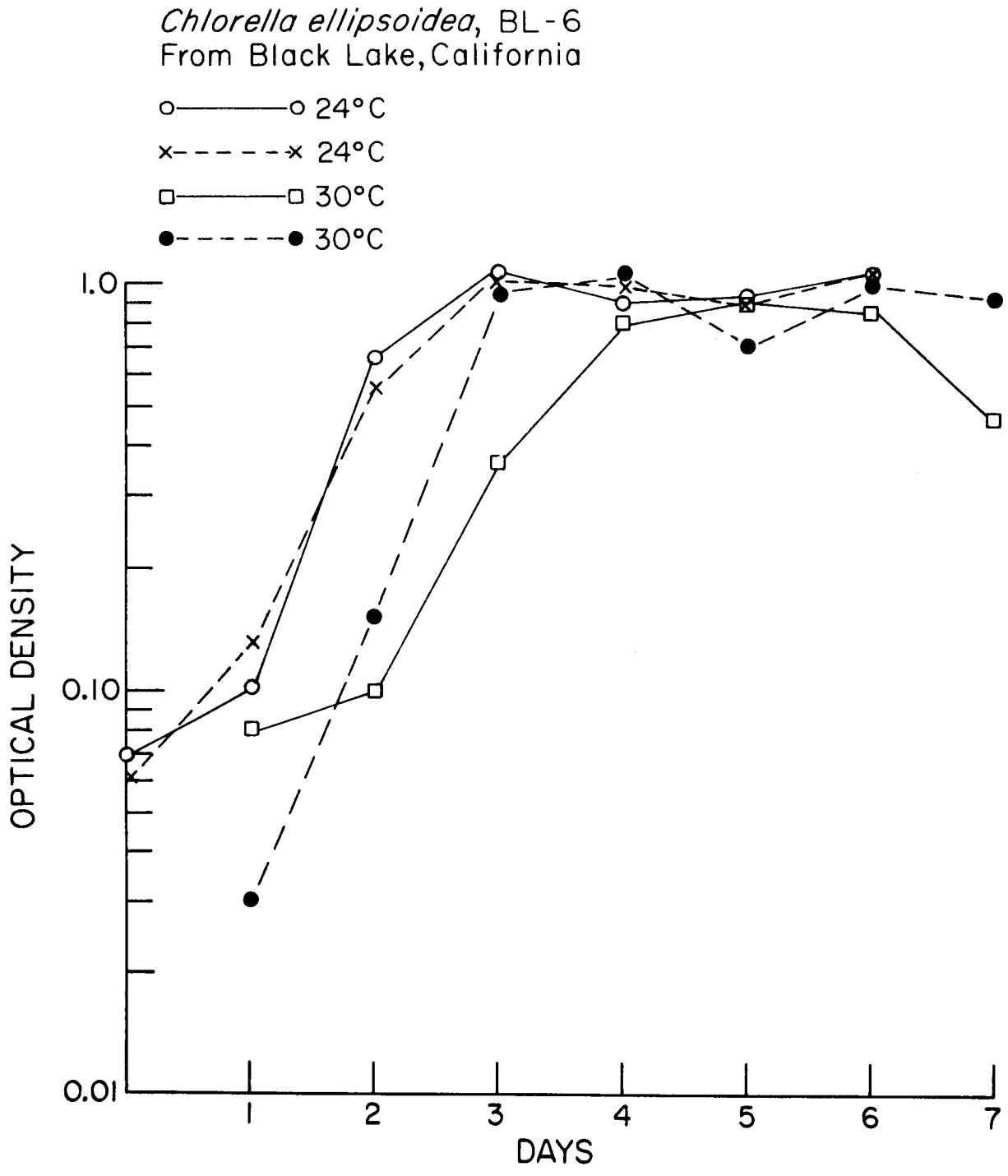

Figure 1. Growth of the Black Lake *Chlorella ellipsoidea* strain BL-6 in SERI standard medium II, low salinity at 24 ° and 30 °C and 40% La Jolla sunlight. The 30 °C growth curves have been displaced for clarity. This strain is a "good" grower.

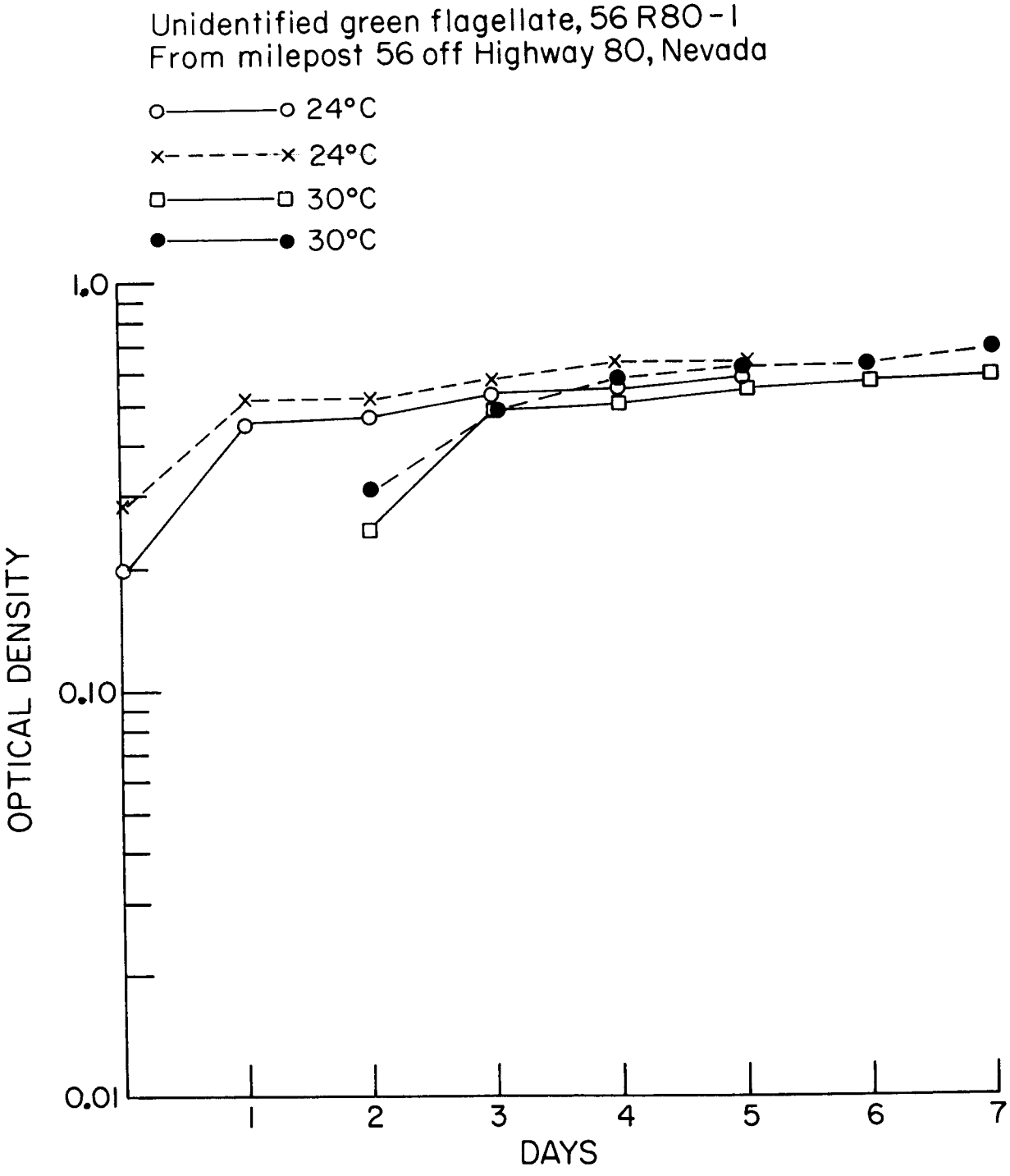

Figure 2. Growth of an unidentified green flagellate strain 56R80-1 in SERI standard medium I, moderate salinity at 24 °C and 30 °C and 40% La Jolla sunlight. The 30 °C growth curves have been displaced for clarity. This is an "intermediate" grower.

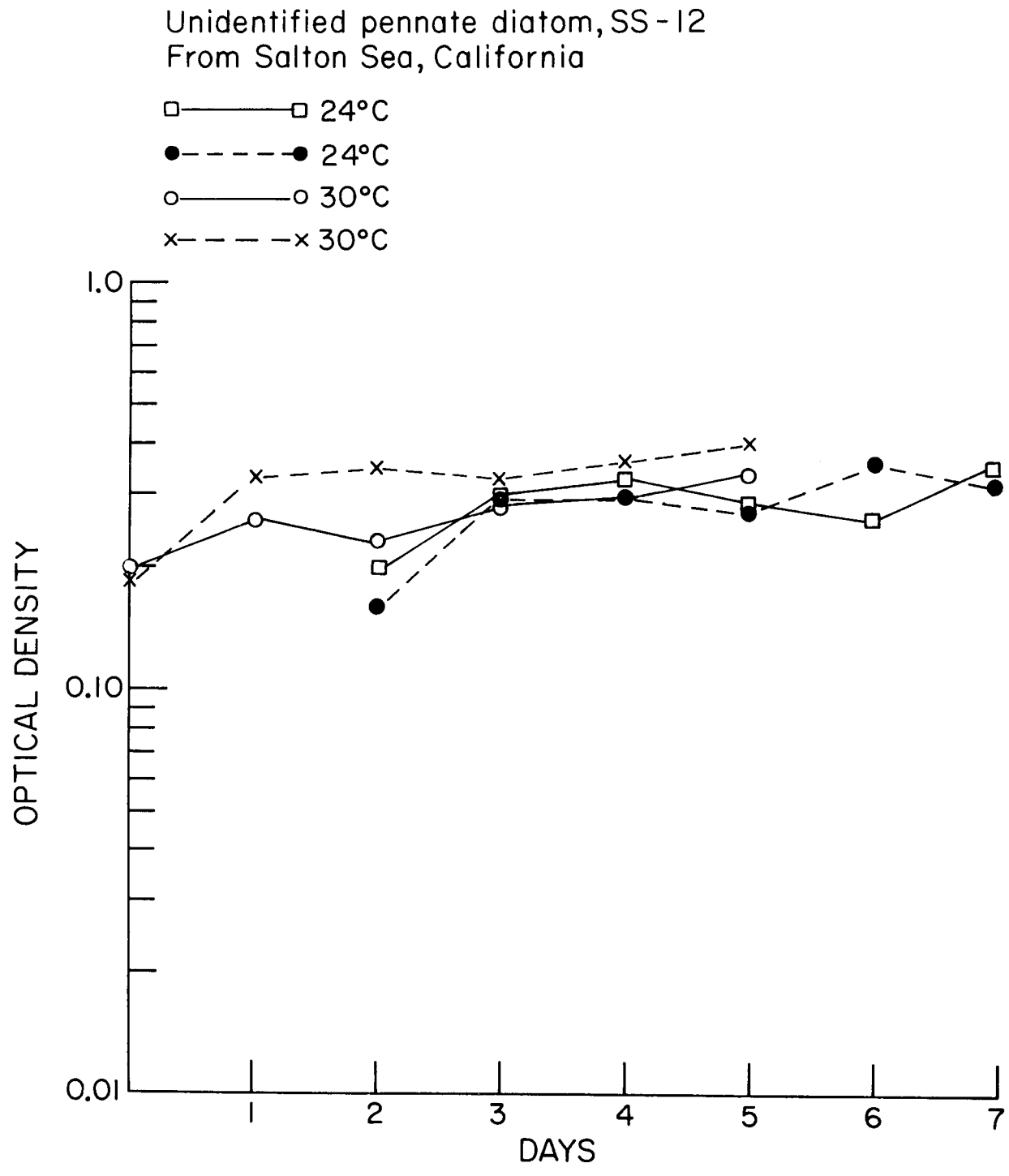

Figure 3. Growth of an unidentified pennate diatom SS-12 in SERI standard medium I, high salinity at 24 ° and 30 °C and 40% La Jolla sunlight. The 30 °C growth curves have been displaced for clarity. This is a "poor" grower.

Table 3. Preliminary growth in SERI standard media using shaker tables and 40% La Jolla sunlight. Good and intermediate growing strains.

No.	Strain	From	Date	Medium/ Salinity	Temp. °C	Exponential Growth Rate (doublings day^{-1})	Maximum Optical Density	Rating
PA-2	Oocystis	Pond A	4/83	II, low	24	0.43	0.74	+++/Good
					30	0.21	0.52	++/Good
PB-4	Nannochloris	Pond B	7/83	II, low	24	0.50	1.02	++++/Good
					30	0.28	0.72	+++/Good
Rt6-2	Nannochloris	Rt 6 Spring	9/83	II, low	24	0.89	0.64	++/Good
					30	1.08	0.62	+++/Good
CSM-3	Green round Cells	Columbus Salt Marsh	5/84	II, low	24	0.24	0.48	++/Inter
B-2	Nannochloris	Badwater	9/83	II, low	24	0.17	0.52	++/Inter
BL-6	Chlorella ellipsoidea	Black Lake	5/84	II, low	24	1.31	1.07	+++/Good
					30	2.48	1.02	++++/Good
MO-2A	Nannochloris	Mono Lake	--	I, high	24	0.26	0.30	++/Inter
					30	0.88	0.76	+++/Good
--	Chinese Chlamydomonas euryale	Chinese Salt Pond (Lewin)	--	I, high	24	1.05	0.92	+++/Good
					30	0.58	1.07	++/Good
HL-9	Chlamydomonas	Harper Lake	5/84	I, moderate	24	0.27	1.10	+++/Good
					30	0.26	1.02	++/Good
OL-12	Chaetoceros	Owens Lake	5/84	II, moderate	24	1.23	0.30	++/Good
					30	1.29	0.44	++/Good
SP-4	Chlorella	Silver Peak Marsh	5/84	II, moderate	24	0.24	0.44	++/Inter
					30	0.21	0.53	++/Good

Table 3. (continued)

No.	Strain	From	Date	Medium/ Salinity	Temp. °C	Exponential Growth Rate (doublings day^{-1})	Maximum Optical Density	Rating
SV-10-2	Dunaliella	Saline Valley	10/82	I, high	24	0.48	0.23	++/Inter
					30	0.59	0.33	++/Good
--	Platymonas	Hawaii	--	I, high	24	0.54	0.56	++/Good
					30	0.49	0.74	+++/Good
OL-10	Nitzschia	Owens Lake	5/84	II, moderate	24	0.73	0.21	++/Good
					30	1.51	0.46	++/Good
SS-1A	Nitzschia	Salton Sea	4/83	I, high	24	0.88	0.62	++/Good
					30	0.39	0.40	++/Inter
SS-13	Green oval	Salton Sea	8/84	I, high	24	0.98	0.60	++/Good
					30	0.84	0.60	++/Good
OL-13	Chlorella ellipsoidea	Owens Lake	8/84	I, moderate	24	0.11	0.87	++/Good
					30	0.13	0.89	++/Good
SC-2	Chlorella	Salt Creek	7/84	I, moderate	24	0.28	1.02	+++/Good
					30	0.20	1.06	+++/Good
JW-1	Chlorella	Fairfield (J.Weismann)	--	II, low	24	0.83	1.04	++++/Good
					30	0.93	1.20	++++/Good
BS-6	Yellow-green round cells	Big Soda Lake	5/84	I, moderate	24	0.06	0.54	++/Inter
					30	0.11	0.68	++/Inter
56R80-1	Green Flagellate	Rt 80 Spring	7/84	I, moderate	24	0.12	0.62	++/Inter
					30	0.10	0.68	++/Inter
PB84-1	Yellow-green cells	Pond B	9/82	II, low	24	0.33	0.37	++/Inter
					30	0.42	0.60	++/Inter
SS-4	Green cells	Salton Sea	4/83	I, high	24	0.46	0.50	++/Good
					30	0.65	0.33	+++/Good

Table 3. (continued)

No.	Strain	From	Date	Medium/ Salinity	Temp. °C	Exponential Growth Rate (doublings day^{-1})	Maximum Optical Density	Rating
SS-5	Green cells	Salton Sea	4/83	I, high	24	0.98	0.60	+++/Good
					30	0.84	0.60	++/Good
SS-14	Chaetoceros	Salton Sea	8/84	I, high	24	1.14	0.61	+++/Good
					30	1.03	0.61	+++/Good
ML S-9	Cocomyxa	Mono Lake	--	I, high	24	0.50	0.40	+++/Inter
					30	0.62	0.37	++/Good
BS-9	Green Flagellate	Big Soda Lake	7/84	I, high	24	0.61	0.52	++/Good
					30	0.60	0.58	++/Good
PA-7	Green Flagellate	Pond A	7/83	II, low	24	0.85	0.39	++/Good
					30	1.08	0.41	+++/Good
BL-7	Cymbella	Black Lake	5/84	I, moderate	24	0.33	0.33	++/Inter
					30	0.57	0.39	++/Good
HL-1	Cryptomonas	Harper Lake	2/83	I, moderate	24	1.16	0.53	+++/Good
					30	0.54	0.83	++/Good
56R80-3	Green Flagellate	Rt 80 Spring	7/84	I, moderate	24	1.04	0.49	+++/Good
					30	0.91	0.65	++/Good
SC-3	Gloeocystis	Salt Creek	7/84	II, low	24	0.37	0.44	++/Inter
					30	0.77	0.37	++/Good
BL-8	Chaetoceros	Black Lake	8/84	I, high	24	0.26	0.33	++/Inter
					30	0.62	0.30	++/Inter

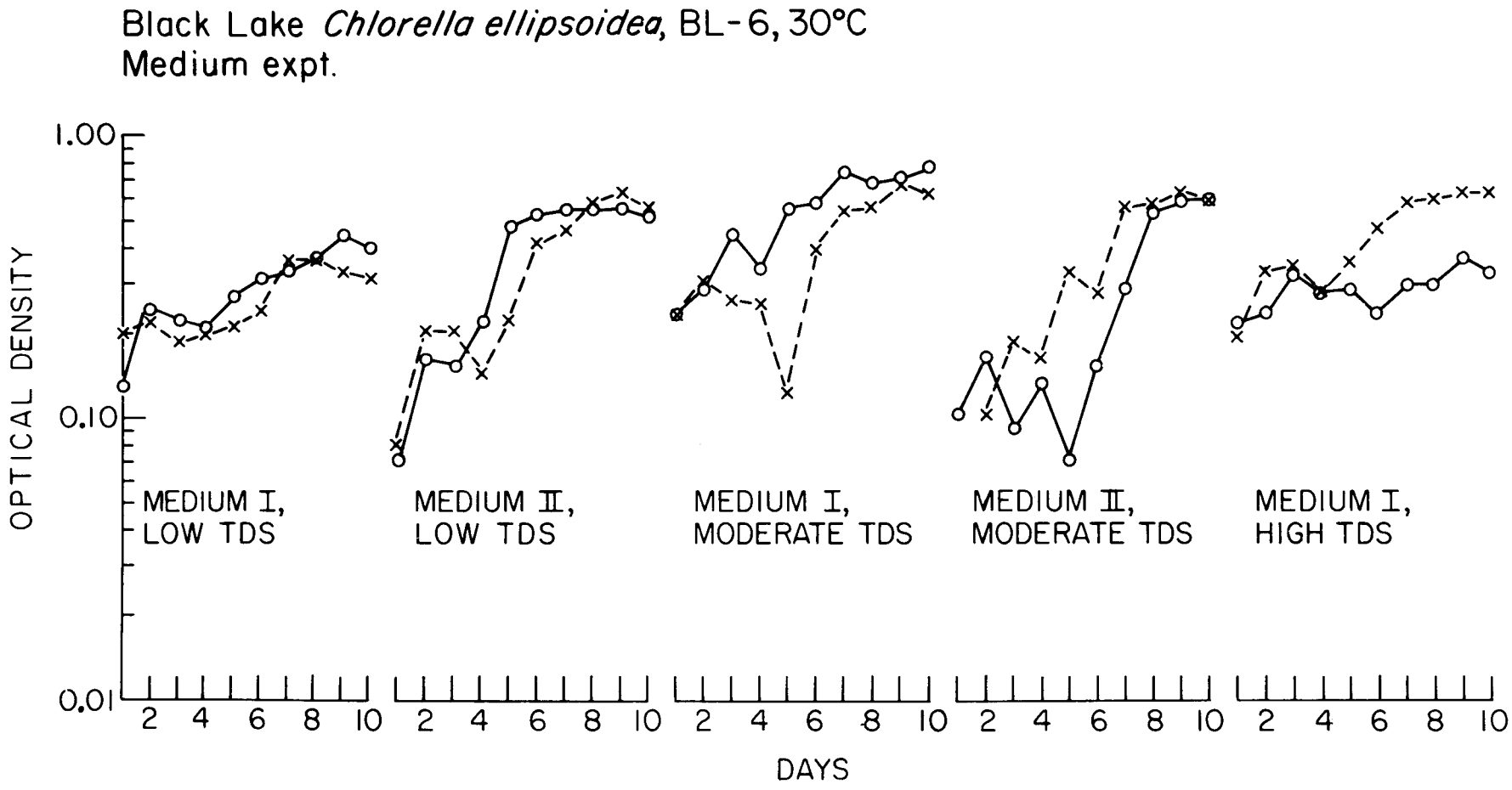

Figure 4. Growth of the Black Lake *Chlorella ellipsoidea* BL-6 strain in all five SERI standard media at 30 °C and 40% La Jolla sunlight.

Table 4. Media tests of some good growing desert microalgae.

Culture		Temp. °C	Medium: Standard I Low TDS	Standard I Moderate TDS	Standard I High TDS	Standard II Low TDS	Standard II Moderate TDS
			Exponential Growth Rate (Doublings day^{-1}) / Maximum Optical Density				
PB-4	Nannochloris	25	0.76/0.35	1.87/0.28	0.58/0.30	0.62/0.34	1.20/0.34
BL-6	Chlorella ellipsoidea	30	0.15/0.44	0.23/0.75	0.20/0.62	0.40/0.61	0.35/0.61
MO-2A	Nannochloris	30	0.00/0.38	0.11/0.50	0.21/0.95	0.20/0.59	0.32/0.76
	Chlamydomonas euryale (Chinese)	25	0.32/0.51	0.32/0.62	0.40/0.59	0.37/0.60	0.66/0.57
HL-9	Chlamydomonas	25	3.89/0.57	1.56/0.63	1.47/0.85	3.41/0.61	3.90/0.63
OL-12	Chaetoceros	30	0.00/0.13	1.53/0.26	0.62/0.20	0.00/0.18	1.72/0.76
SS-1A	Nitzschia	25	0.00/0.07	0.00/0.08	1.30/0.16	0.32/0.16	1.78/0.25
OL-13	Chlorella ellipsoidea	25	0.83/0.36	0.85/0.47	0.63/0.41	0.79/0.46	1.12/0.51
SC-2	Chlorella	30	1.83/0.46	1.23/0.50	1.61/0.28	1.88/0.48	1.32/0.63
JW-1	Chlorella	30	0.77/0.48	1.49/0.36	0.56/0.29	1.34/0.40	0.63/0.40
SS-14	Chaetoceros	30	0.57/0.19	0.81/0.37	0.39/0.26	0.00/0.10	1.26/0.36
HL-1	Cryptomonas	25	0.77/0.38	1.09/0.48	0.65/0.39	2.46/0.58	1.79/0.68

Table 5. Media tests of some marine microalgae

Culture	Temp. °C	Medium: Standard I Low TDS	Standard I Moderate TDS	Standard I High TDS	Standard II Low TDS	Standard II Moderate TDS
		Exponential Growth Rate (doublings day^{-1}) / Maximum Optical Density				
Phaeodactylum Thomas strain	25	1.42/0.34	1.24/0.43	1.25/0.45	1.24/0.43	1.21/0.39
Chaetoceros gracilis Thomas strain	25	0.93/0.25	0.75/0.32	0.75/0.31	no growth	1.25/0.40
Platymonas Hawaiian strain	30	no growth	1.26/0.29	0.82/0.24	no growth	no growth
Isochrysis Tahaitian strain	30	← no growth →				

using that medium. Figure 5 shows the response of the Black Lake *Chlorella ellipsoidea* (BL-6) to temperature and salinity. No growth occurred at 15 °or 40 °C and the best growth occurred from 25-35 °C. The best salinities were 20-40 gm liter^{-1} with reduced growth at 10 gm liter^{-1} and no growth at 0 gm liter^{-1}. Another strain showing a wide temperature response was the Salt Creek *Chlorella* (SC-2) --see Figure 6. This alga was unusual in that it grew well at 40 °C. The best salinity range was 10-20 gm liter^{-1}. Growth was reduced at 0 and 30 gm liter^{-1} and no growth occurred at 40 gm liter^{-1}.

Table 6 summarizes all of the temperature/salinity requirement data from eight strains using SERI standard media. Half of these are truly high temperature strains with best growth occurring up to 35 °C and in one case to 40 °C. They vary greatly in their salinity responses and these generally reflect the salinity of the natural waters from which they were isolated. For instance, the Mono Lake *Nannochloris* (MO-2a) grows best at 30-40 gm liter^{-1} and the marine species *Platymonas* grows best at 40 gm liter^{-1}. Mono Lake and the Sea are both highly saline environments.

Measurements of Yields and Photosynthetic Efficiencies in Dense Mass Cultures of Desert Algae

We measured the growth of two good growing strains in our 12 liter mass culture apparatus at 50-70% sunlight and at the best temperature range found for each strain. After dense growth was achieved, half the culture was harvested for cell analyses by Dr. Tom Tornabene at Georgia Institute of Technology. The remaining culture was diluted with fresh medium containing trace metals, Fe, and vitamins but no N or P. The diluted culture then grew up again and depleted the NH_4^+-N to an undetectable amount. After 10 further days of growth, cells were again harvested for analyses by Dr. Tornabene.

Figure 7 shows the growth curves for the Black Lake *Chlorella ellipsoidea* (BL-6) cultured under these conditions. (27-32 °C, 50-70% sunlight). The yield before dilution was 35.5 gm m^{-2}day^{-1} and the efficiency of light utilization was 11.3%. After dilution these values were 55.5 gm m^{-2}day^{-1} and 17.6% respectively. Figure 8 shows similar data for the Mono Lake *Nannochloris* (MO-2A) grown under the same conditions. The yield before dilution was 31.9 gm m^{-2}day^{-1} and the efficiency was 10.1%. After dilution the yield was reduced to 24.2 gm m^{-2}day^{-1} with a corresponding efficiency of 7.7%.

These yields and efficiencies were similar to those found previously with *Ankistrodesmus falcatus* (Thomas et al, 1984) and are comparable to the best values found in outdoor cultures.

CONCLUSIONS

This work has been a step-wise selection process to find strains that grow well in SERI media that simulate saline desert waters in New Mexico and also that grow well at high temperatures and light intensities. Many cultures have been isolated that grow up under these extreme conditions and that grow well ("good growing cultures") in preliminary tests with the media. Some of these strains have been tested for growth in all five SERI standard media and their temperature and salinity responses have been assessed. Two strains have been grown in mass culture and their yields and efficiencies have been determined. Both of these strains seem promising for further work on their yields and efficiencies in outdoor pond or raceway mass cultures.

ACKNOWLEDGEMENT

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Thomas, W.H., D.L.R. Seibert, M. Alden, and P. Eldridge, (1984). Cultural requirements, yields and light utilization efficiencies of some desert saline microalgae. Proc. April 1984 Principal Investigators Meeting, Aquatic Species Program Review, Solar Energy Research Institute. SERI/CP-2313-2341, DE 83012000 Rept., pp. 7-63.

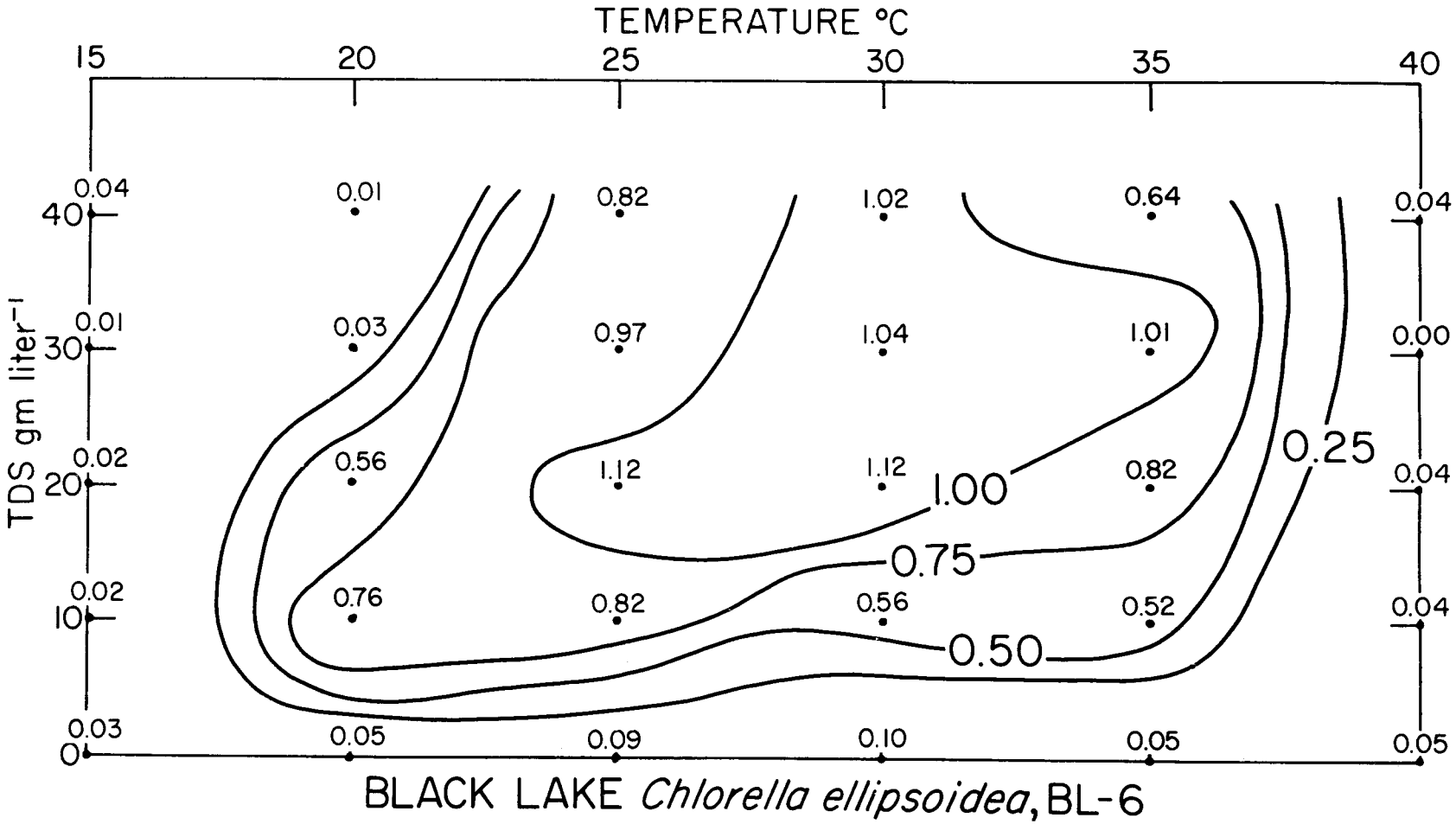

Figure 5. Response of the Black Lake *Chlorella ellipsoidea* strain BL-6 to temperature and salinity. The numbers are final optical densities in each culture.

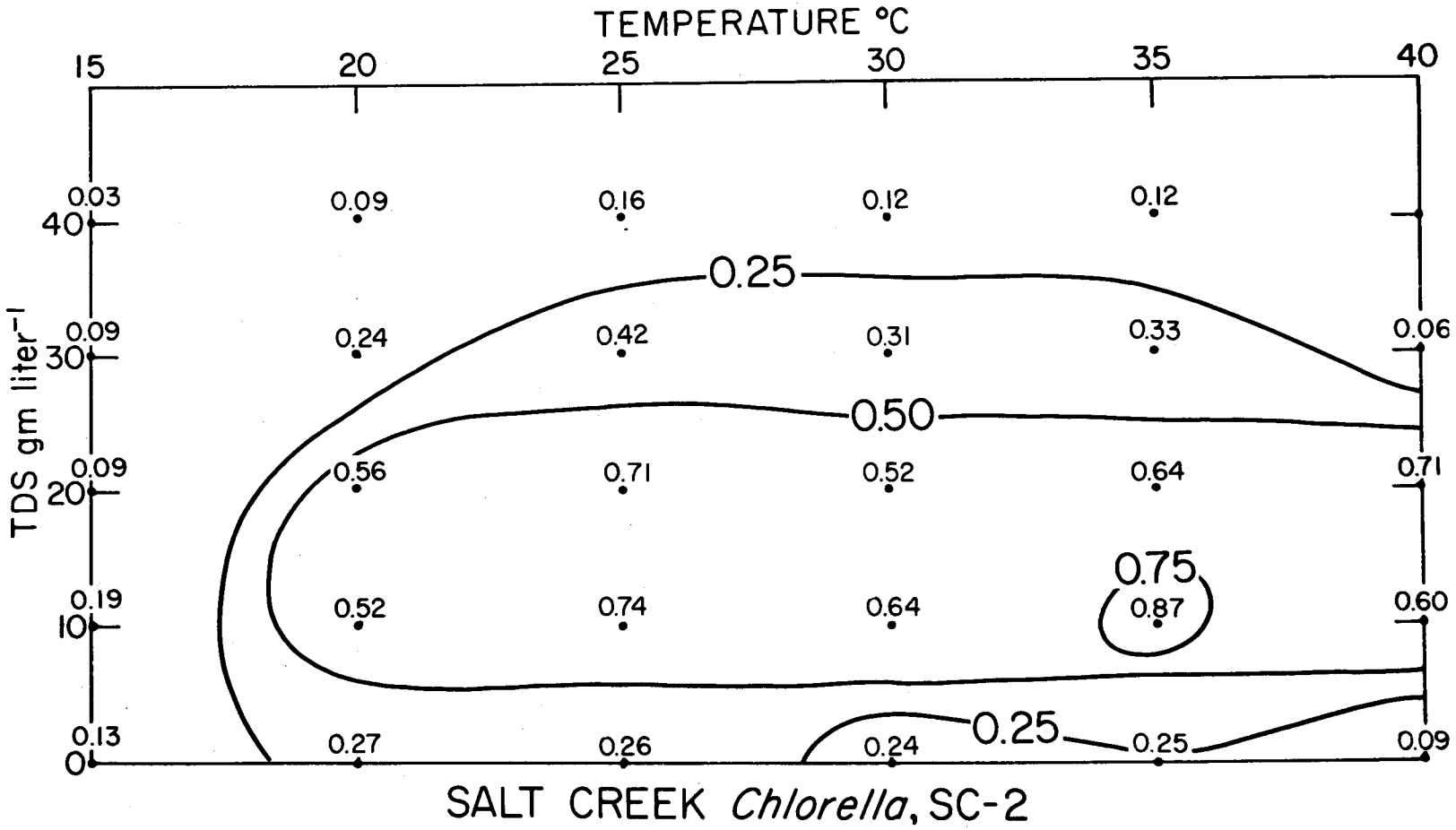

Figure 6. Response of the Salt Creek *Chlorella* strain SC-2 to temperature and salinity. The numbers are final optical densities in each culture.

Table 6. Temperature and salinity responses of some desert saline microalgae.

Culture		Temperature (°C)			Salinity (gm liter^{-1})		
		Best Growth	Some Growth	No Growth	Best Growth	Some Growth	No Growth
PB-4	Nannochloris	15-25	--	30,40	10-30	--	0,40
BL-6	Chlorella ellipsoidea	25-35	20	15,40	20-40	10	0
MO-2A	Nannochloris	25-35	--	15,20,40	30-40	10-20	0
	Chlamydomonas euryale (Chinese)	20-25	15,30	35-40	30-40	20-10	0-10
HL-9	Chlamydomonas	20-25	15,30	35-40	20-30	10,40	0
JW-1	Chlorella (Weismann)	25-35	20,40	15	10-30	0,40	--
	Platymonas Hawaii	30	15-25	35-40	40	20-30	0-10
SC-2	Chlorella	20-40	--	15	10-30	0	40

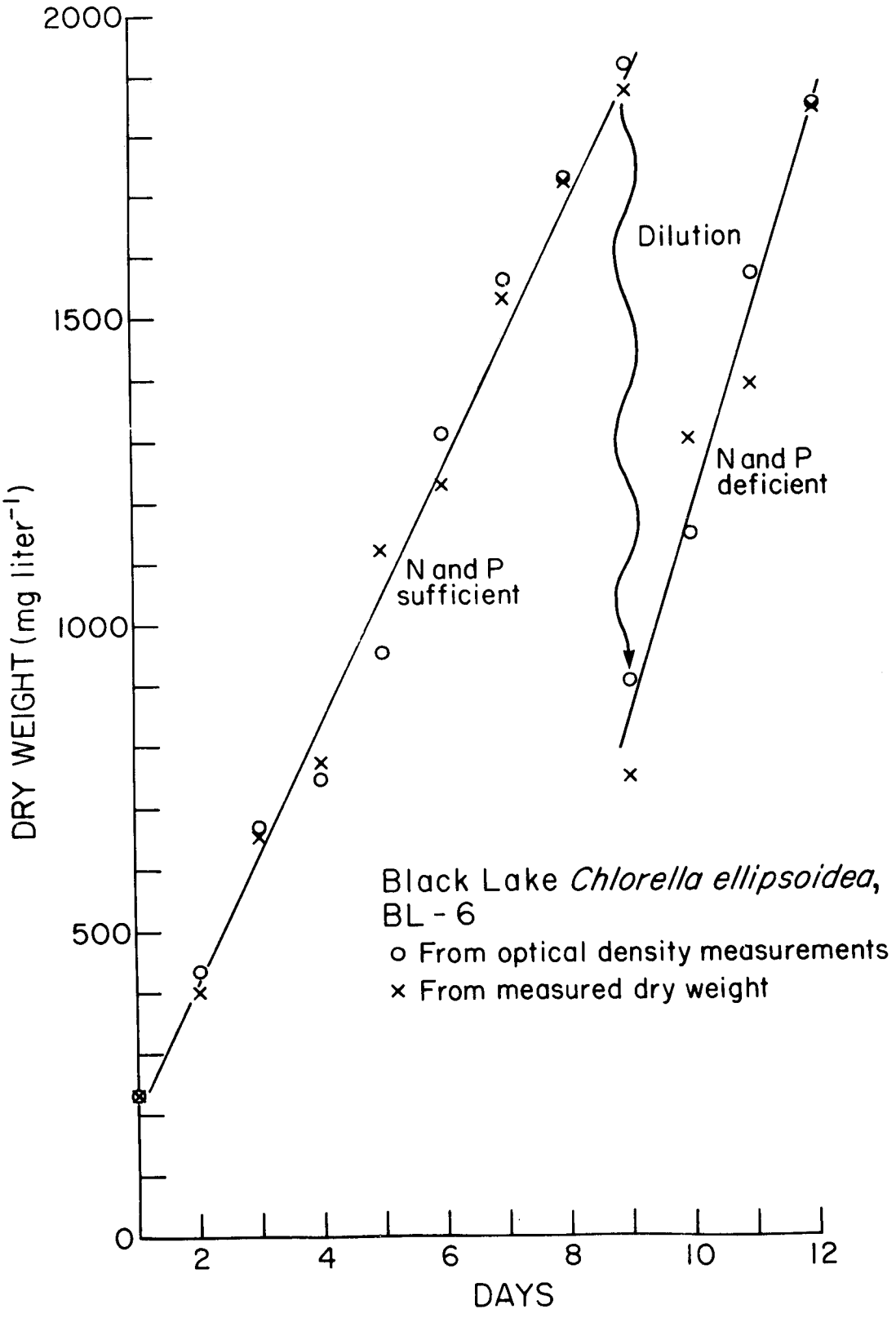

Figure 7. Growth of the Black Lake *Chlorella ellipsoidea* strain BL-6 in a 12-liter mass culture at 27-32 °C and 50-70% La Jolla sunlight.

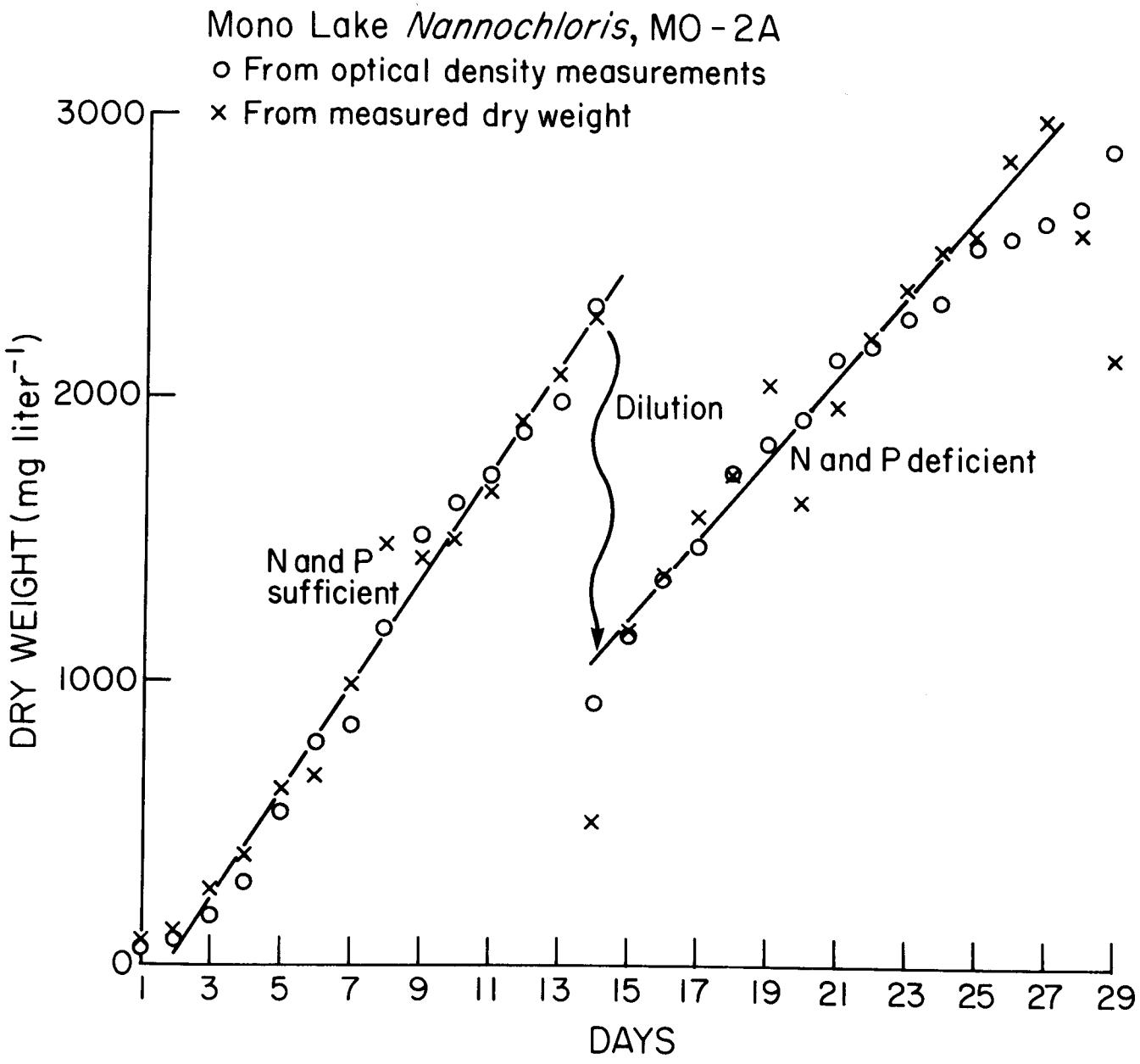

Figure 8. Growth of the Mono Lake *Nannochloris* strain MO-2A in a 12-liter mass culture at 27-32 °C and 50-70% La Jolla sunlight.

SCREENING AND CHARACTERIZING OLEAGINOUS MICROALGAL SPECIES FROM THE SOUTHEASTERN UNITED STATES

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ABSTRACT

The purpose of this study was to select and characterize promising algal species which tolerate high light intensities, temperature variations and accumulate lipids. Samples have been collected from freshwater and saltwater locations in the State of Alabama and intertidal regions of the Gulf of Mexico. Samples were screened through a multi-step process. Selected species: Cyclotella, Nitzschia, and Scenedesmus have been examined for growth requirements. Approximate cellular composition of these species was determined.

INTRODUCTION

In recent years, interest in microbial lipids has been renewed because of an urgent need for utilization of alternative renewable resources as carbon sources for production of hydrocarbons and lipids. Since 1980, SERI/DOE has been interested in microalgal liquid fuels (McIntosh, 1984).

Microalgal species are capable of producing biomass yields containing high percentages of oils (Aaronson, et. al., 1980). Microalgal systems can use low value natural resources, such as saline water and arid lands, therefore offering the potential for large biomass energy contributions without competition for prime agricultural or forest land. The growth of microalgal biomass and lipid harvesting represent potential for harvesting solar energy products. One of the methods by which the energy storage capacity of photosynthesis can be maximized is by controlling the metabolism of the organism. Tuning the metabolism of algae can lead to enhanced production of energy-rich compounds such as fatty acids and glycerol. A single algal species may show remarkable variation in its metabolism, according to the conditions to which it is exposed, such as carbon dioxide supply, light intensity, temperature, nutrient concentrations, and salinity (Holm-Hansen, et. al., 1959). Changes in the supply or consumption of metabolites may have considerable effects on metabolic patterns. The accumulation of energy storage compounds in algae, such as fats and oils, can be induced by manipulating the environmental conditions under which the algae are grown (Shifrin and Chisholm, 1981). Nutrient deficiencies generally lead to a decrease in protein and photosynthetic pigments and an increase in energy-rich products such as carbohydrates and lipids (Healey, 1973).

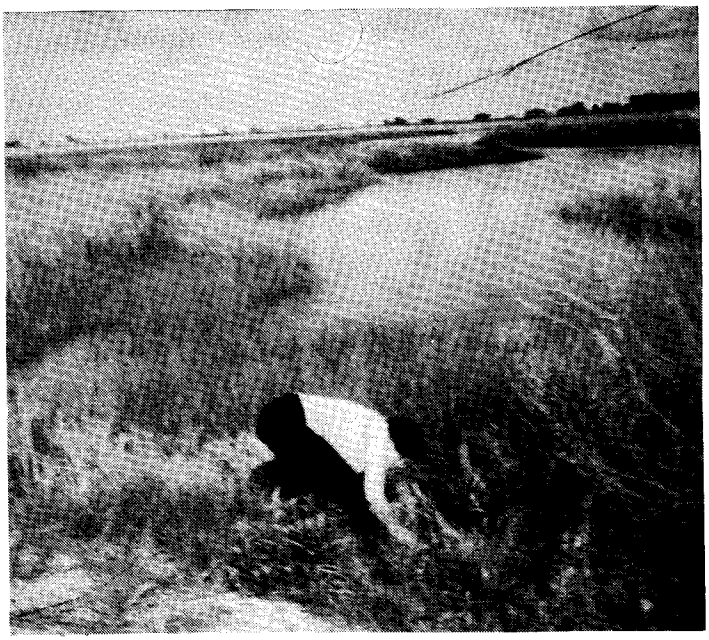
Nitrogen starvation in particular can lead to remarkable changes in algal cell composition (Fogg, 1959). Opute (1974) demonstrated that lipids accumulate in the diatom Nitzschia palea, under N-deficient conditions. Cyclotella cryptica produces high lipids in N-deficient media (Werner, 1966). Diatoms (Bacillariophyceae), mostly in their stationary growth phase, accumulate fats. Chlorella pyrenoidosa (Chlorophyceae), produces high concentrations of lipids, from 28% to 70% dry weight (Fogg, 1959) when grown in N-starved cultures. Even during silicon starvation, the marine diatom, Navicula pelliculosa, accumulates lipids (Coombs, et. al., 1967). With aging, nutrients become exhausted; this is then reflected in lipid content increase and changes of fatty acid composition e.g., Euglena gracilis, (Gomez, et. al., 1974), and Nitzschia, (Opute, 1974; Badour and Tadros, 1965). Fat production is also stimulated by light. Spoehr and Milner (1948), showed that N-deficient Chlorella pyrenoidosa attains a greater lipid content at high light intensity than at low light intensity. The length of light period as well affects the content and composition of fatty acids of Nitzschia palea (Opute, 1974) and Nitzschia closterium (Orcutt and Patterson, 1974). Ochromonas danica (Chlorophyceae) accumulated lipids up to 53% dry weight when cultured at high temperature (Aaronson, 1973). The fatty acid composition of the diatom Coscinodiscus eccentricus (Pugh, 1971) showed variations in different salinity media. In a better characterized system, the diatom Phaeodactylum tricornutum growing in sea water produced high yields of lipids (Raymond, 1981).

From previous reports it is evident that hydrocarbon production by algae is occurring in natural systems and can be maximized by the application of a variety of environmental conditions (Shifrin and Chisholm, 1981). In order to select promising algal species as potential producers of oils for energy technology, these growth conditions need to be identified.

The long-range goal of this project is to establish an adequate biological resource pool of oil-producing microalgal species from the southeastern United States. The specific objectives of the research reported herein were:

- To collect algal samples from freshwater and saltwater sources in the State of Alabama.
- To isolate oleaginous algal species capable of growth under high temperature and light intensity.
- To characterize oleaginous species, for their temperature, salinity and light tolerances.
- To determine the nitrogen source requirements for the selected species.
- To quantify the lipid accumulated by the selected strains, under nitrogen deficiency and nitrogen sufficiency.

This paper describes some of the results accomplished in the period February 1984 - January 1985.



Collections of Microalgae Being Made from Estuarine Habitats in Southern Alabama

Characterization of Growth Response at Different Light Intensities, Salinities, and Temperatures

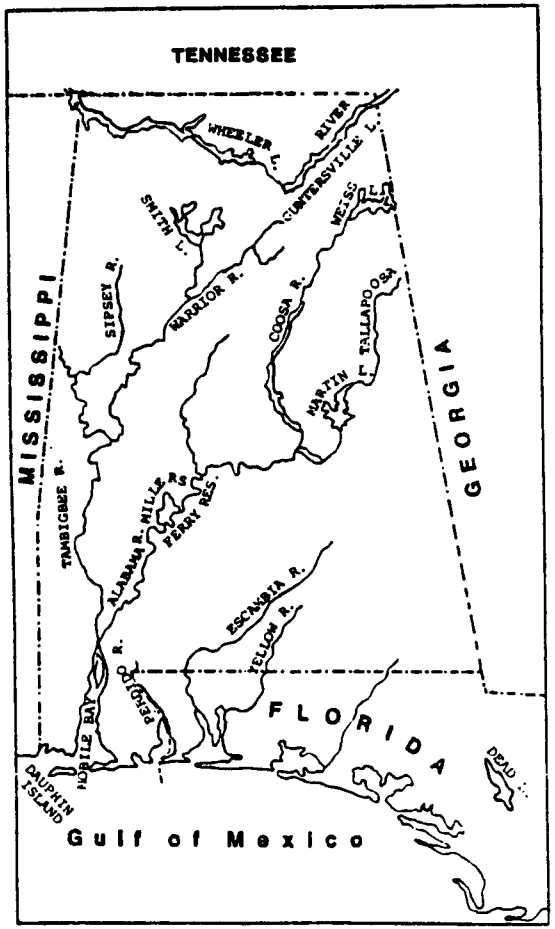


Figure 1. Locations of Sampling Sites in Alabama

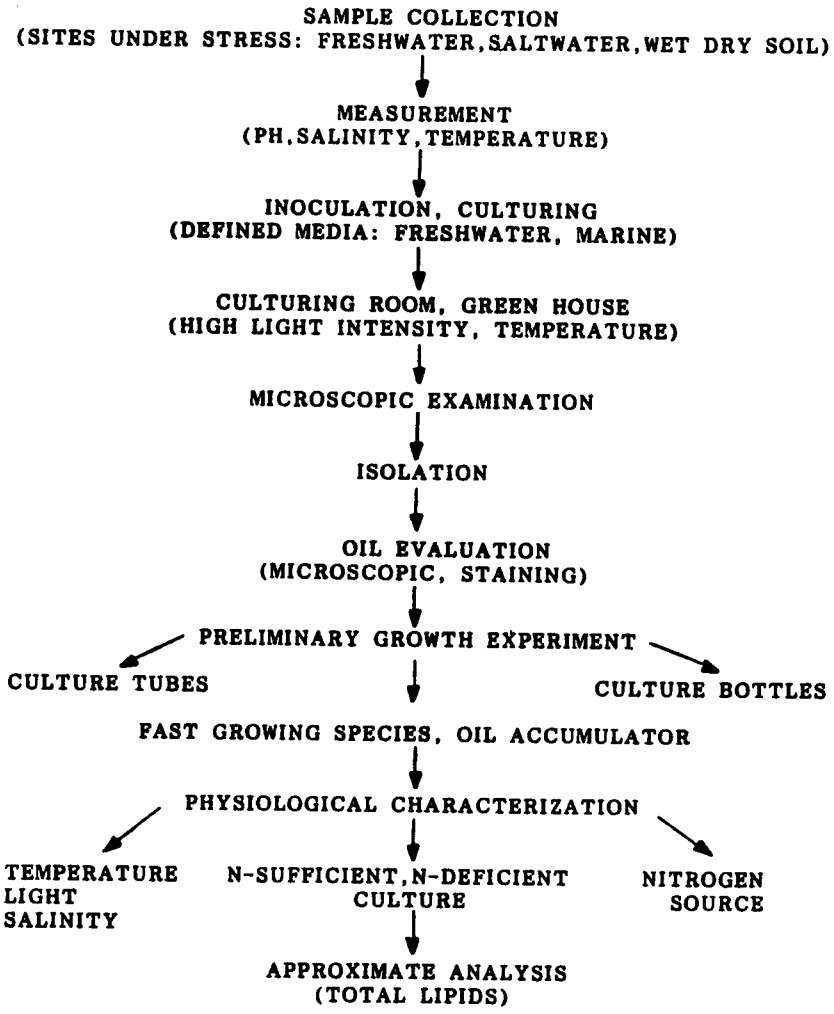


Figure 2. Screening Procedure of Oleaginous Algal Species

MATERIALS AND METHODS

The methods have been previously described in detail (Tadros, 1985). Field trips have been conducted to Alabama water resources (Fig. 1). Fresh water and marine algal samples were collected from habitats under stress. Temperature, pH, and salinity were recorded. A multi-step screening process has been designed for the selection of oleaginous species tolerant of high temperature and light intensity (Fig. 2). Screening and growth experiments were carried in a culture room provided with shelves, which have been illuminated with cool white light fluorescent tubes. Light intensity varied from 400 to 500 foot candles on the shelves. Intermittent illumination was used for culturing (14 h : 10 h light dark cycle). Temperature ranged from 29-30°C. Growth media for freshwater and marine algal species were used for isolation. Defined media were supplemented with artificial sea salts, at different strengths. Among the principal basic media are Bold Basal (Nichols and Bold, 1965); Chu no 10 (Chu, 1942) and "f/2" (Guillard and Ryther, 1962). The diatoms and unicellular green species were cultured in "f/2" (triple strength) and BBL media. Algal species were isolated by streaking enriched agar plates or by micropipetting under a light microscope. Oil accumulation was identified by microscopic examination. Preliminary cultures were performed in shaked test tubes and aerated small culture bottles. Strains were selected on the basis of rapidity of growth and dominance. Selected strains were characterized for temperature, salinity and light tolerance, on a gradient plate. Algal species were cultured in small culture bottles. Nitrogen requirements for selected species were determined by enriching culturing flasks with different concentrations of N-source as nitrate or urea and yields were measured by cell counting. For proximate analysis, batch cultures (800 ml) were aerated with air mixed with 3% CO_2 and grown in a water bath. Batch cultures were illuminated continuously by placing them in front of a bank of six white fluorescent lamps (32 W.). Cultures were analyzed for: total dry weight, total carbohydrates, total proteins and total lipids (see Methods: Tadros, 1985). All determinations were run in triplicate.

RESULTS AND DISCUSSIONS

Sample Collection and Preliminary Growth Experiments

Data from sample collections, field trips, and processing according to the methods, are summarized in Table 1. Following the use of the schematic diagram (Fig. 2) for screening the samples for oleaginous algal strains, the growth of the isolates in preliminary experiments was evaluated visually and represented by signs (+) in Table 1. The preliminary growth experiments were designed to test the growth of the isolates in liquid media. Strains were selected for characterization on the basis of fast growth rate and microscopic identification of oil accumulation in the cells.

Temperature, Light, Salinity Requirements

The growth parameters for the selected species were examined under different combinations of light intensity, temperature and salinity, in order to define the optimal growth conditions as well as high and low limits. The gradient block was used for this experiment. The maximum densities reached by each species was determined by evaluating cell counts in the stationary phase.

Table 1. Algal Species Isolated From Field Trips; Water Characteristics; Preliminary Growth and Evaluation

No.	Species	Date of Collection	Temp.	pH	Salinity ppt.	Growth Condition	Preliminary Growth, Eval.
AR[1]-47	Nitzschia sp.	October 1983	26°C	8.0	0	FW	+
AR-67	Nitzschia sp.	October 1983	26°C	8.0	0	FW	++
AR-68	Navicula sp.	October 1983	26°C	8.0	0	FW	++
MB-63a	Chlorella sp.	October 1983	28°C	7.2	0	FW	+
MB-63b	Flagellate	October 1983	28°C	7.2	0	FW	+
MF-7B	Motile (green)	November 1983	24°C	7.8	0	FW	++
MF-8	Motile (green)	November 1983	24°C	7.8	0	FW	++
DI-31	Diatoms, Chlorella	November 1983	23°C	7.6	12	SW	+
DI-35	Cyclotella	November 1983	22°C	7.5	15	SW	++++
DI-34	Navicula	November 1983	22°C	7.5	15	SW	+
DI-32	Achnanthes	November 1983	22°C	7.5	15	SW	+
DI-42	Melosira	November 1983	22°C	7.5	15	SW	++
DI-51	Melosira, Phaeodactylum	November 1983	23°C	7.2	20	SW	+
DI-31	Navicula, Phaeodactylum	November 1983	23°C	7.2	20	SW	+
DI-34	Navicula, Nitzschia	November 1983	24°C	7.5	18	SW	++
DI-38	Rhizosolenium	November 1983	24°C	7.5	18	SW	+
DI-38a	Phaeodactylum, Nitzschia, Cocconeis	November 1983	24°C	7.5	18	SW	+
BW-46	Scenedesmus	November 1983	24°C	7.5	0	FW	+++
BW-52	Scenedesmus, Ankistrodesmus	November 1983	22°C	7.5	0	FW	+++
SL-1	Flagellate (green)	November 1983	25°C	6.8	0	FW	+++
SL-2	Chamydomonas	November 1983	25°C	6.8	0	FW	+++
SL-3	Motile (green)	November 1983	25°C	6.8	0	FW	++
SR-2	Chlorella	November 1983	22°C	6.5	0	FW	+
SR-3	Chlorella	November 1983	22°C	6.5	0	FW	+
MB-31	Chlorella	June 1984	30°C	7.2	0	FW	++++
MB-81	Motile (green)	June 1984	30°C	7.2	0	FW	++
DI-34	Chlorococcum	June 1984	28°C	7.8	18	SW	+++
DI-160	Hantzschia	June 1984	29°C	8.0	26	SW	++++
TR-67	Scenedesmus	June 1984	29°C	7.6	0	FW	++
TR-84	Scenedesmus	June 1984	29°C	7.6	0	FW	++++
TR-87	Ankistrodesmus	June 1984	29°C	7.6	0	FW	++++
TR-114	Nitzschia	June 1984	32°C	7.4	0	FW	++++
TL-24	Ankistrodesmus	July 1984	32°C	7.4	0	FW	++
TR-40	Chlorella, Flagellate	July 1984	30°C	7.6	0	FW	+
TR-43	Selenestrum, Closterium	July 1984	30°C	7.6	0	FW	++
TR-45	Scenedesmus, Closterium	July 1984	30°C	7.6	0	FW	++
UP-61	Unicellular (green)	July 1984	32°C	7.4	0	FW	+
UP-62	Motile (green)	July 1984	32°C	7.4	0	FW	+
UP-87	Filamentous (green), Unicellular (green)	July 1984	32°C	7.4	0	FW	+

1. Site Collection

AR = Alabama River
BW = Black Warrior River
DI = Dauphine Island
MB = Mobile Bay
MF = Miller Ferry Reservoir
SL = Smith Lake
SR = Spicey River
TL = Tuscaloosa Lake
TR = Tombigbee River
UP = University Pond

2. Growth Condition

FW = Freshwater
SW = Saltwater

Cyclotella sp.: (Fig. 3)

An increase in cell number could not be detected at 0.0 ppt. salinity; however increasing the salinity to 15 ppt. resulted in an increase in final culture density. At 32 ppt. salinity, the maximum yield of cells declined and reached about 75% that at 15 ppt. salinity. At both light intensities, 400 ft-C and 800 ft-C, the yield decreased in higher salinities. High temperatures, 30°C and 35°C under 400 ft-C and 800 ft-C light intensities, favored maximum yield of cells. On the other hand, at lower temperatures (15°C and 20°C) cell division completely ceased. It is clear from these results that Cyclotella sp. DI-35 tolerates high temperatures (30°C, 35°C) and salinities (32 ppt.) at high light intensity (800 ft-C).

Nitzschia sp. TR-114: (Fig. 3)

The optimum yield of cell division was obtained at 15 ppt. salinity and 30°C and 400 ft-C. At 25°C, no obvious effect of light intensity was observed. Lower temperatures (20°C and 15°C) arrested the growth of the diatom.

Scenedesmus sp. TR-84: (Fig. 4)

The final cell concentration was highest at 400 ft-C light intensity and 25°C. It decreased as the temperature increased to 30°C and 35°C. Increasing sodium chloride concentration in the growth medium produced an inhibitory effect at both light intensities.

From the results reported in this experiment, it is evident that estuarine species as Cyclotella sp.; Nitzschia sp. have the ability to tolerate wide salinities from 15 ppt. to 32 ppt. and temperatures from 20°C to 35°C. On the other hand, the freshwater species such as Scenedesmus sp. responds significantly to sodium chloride concentrations and the yield drops drastically.

Nitrogen Source Requirements

The selected strains were treated with different concentrations of urea and nitrate (see Methods). The growth response of the strains, was determined by cell counting.

In the diatom (Cyclotella sp. DI-35 (Fig. 6), the cell number increased with increasing the urea - N (1mM). As the urea - N was increased to 5mM, the cell number dropped. Similar results were obtained with nitrate-N. It enhanced the cell number at 2 mM and inhibited it at 5 mM concentrations. In the case of Nitzschia sp. TR-114 (Fig. 6), cultures containing 0.01 mM nitrate-N reached greater cell densities than those containing 2 mM nitrate-N. Urea - N, compared to nitrate-N, produced no significant effect on the growth of Nitzschia sp.

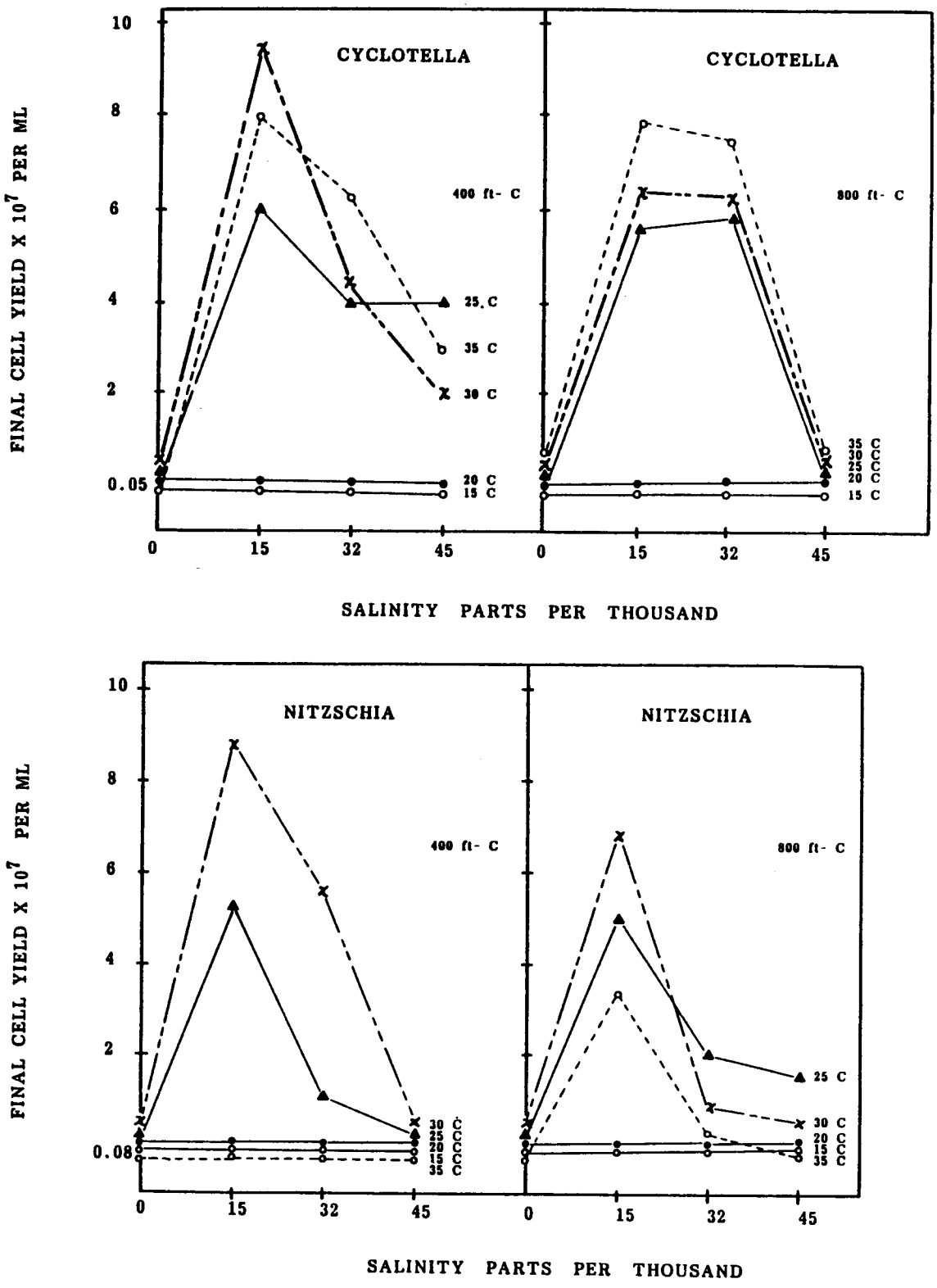


Figure 3. Final Yield of Cyclotella sp. and Nitzschia sp. as a Function of Temperatures, Light Intensity and Salinity, after 12 Days Growth Period

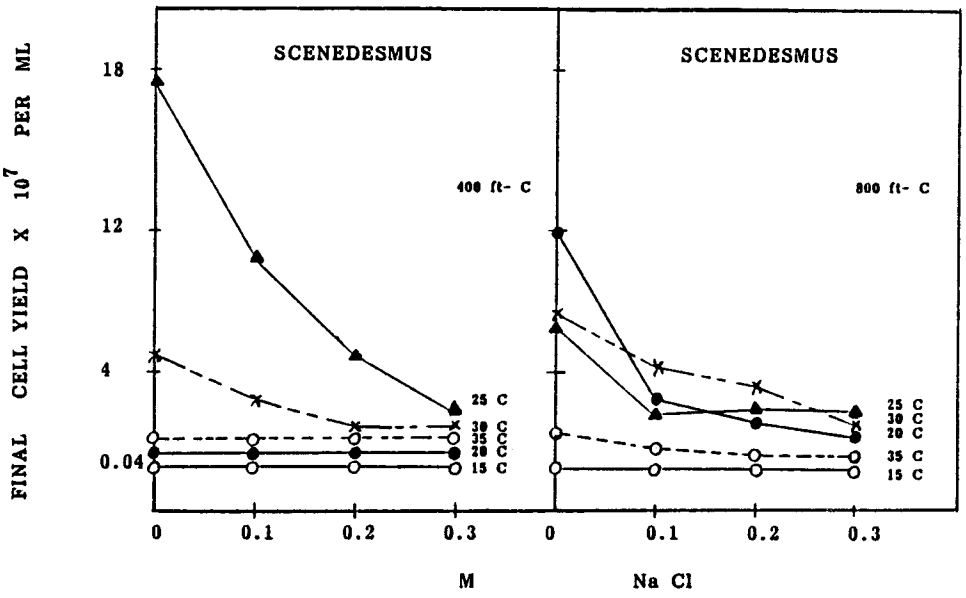


Figure 4. Final Yield of Scenedesmus sp. as a Function of Temperature, Light Intensity and Sodium Chloride Concentration, after 12 Days Growth Period

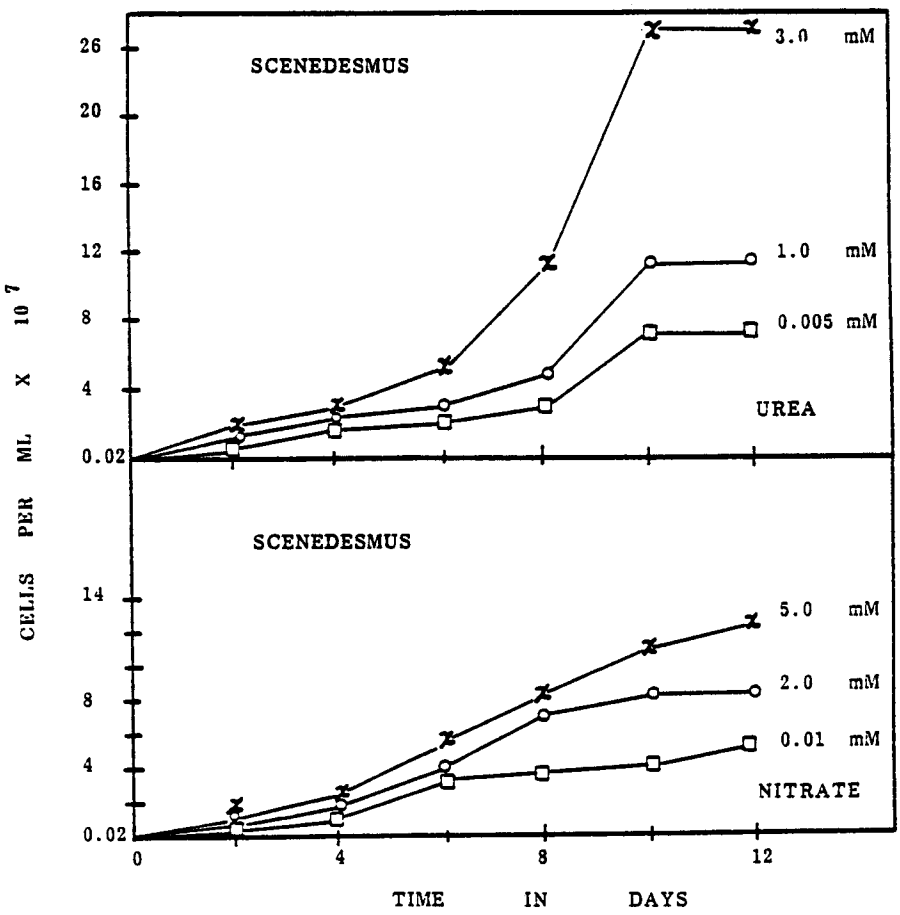


Figure 5. Growth Curve of Scenedesmus sp. in a Series of Media Containing Different Concentrations of Urea or Nitrate (as N-source)

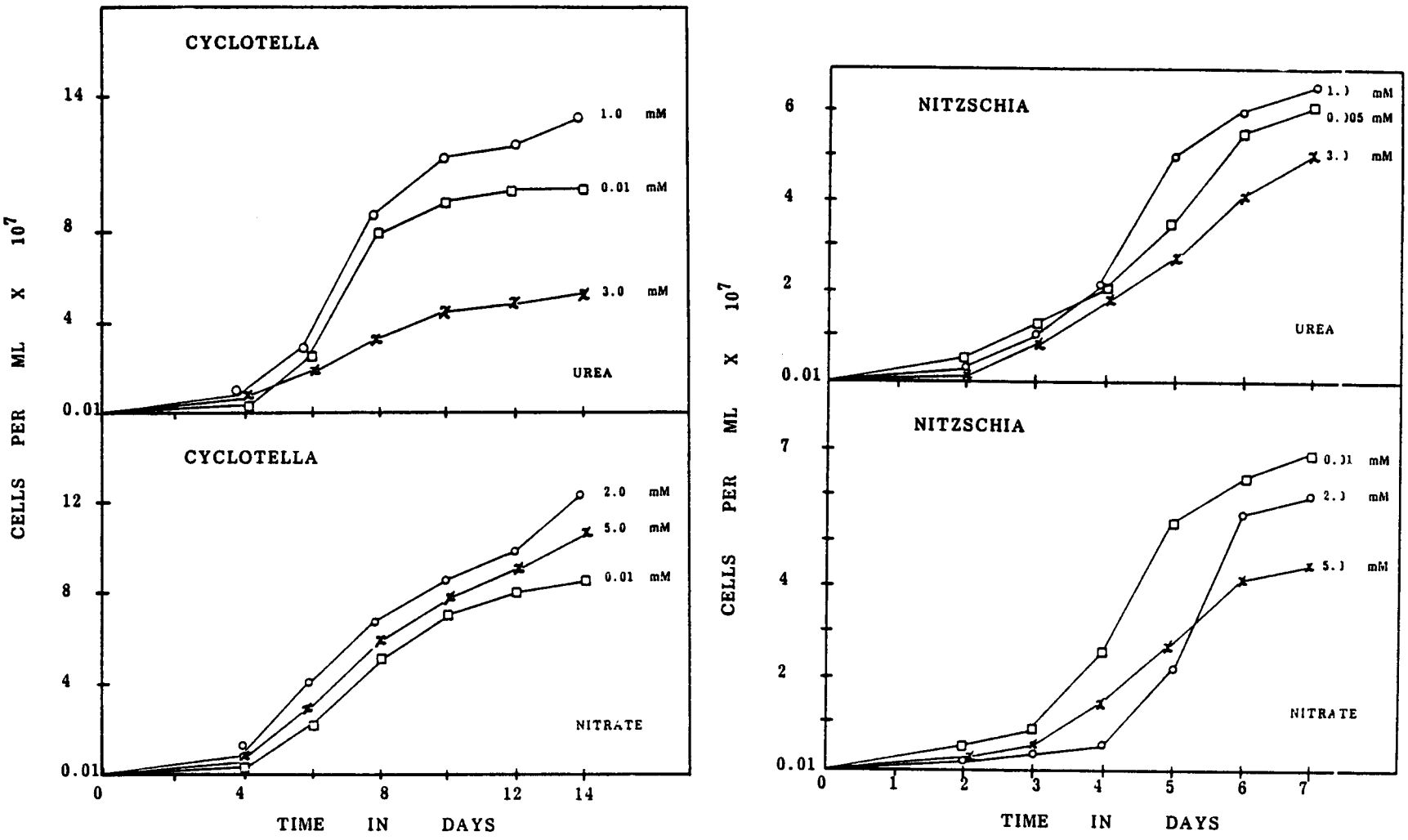


Figure 6. Growth Curve of Cyclotella sp. and Nitzschia sp. in a Series of Media Containing Different Concentrations of Urea or Nitrate (as N-source).

For the Chlorophyceae: Scenedesmus sp. TR-84 (Fig. 5), increasing urea and nitrate concentrations in the medium resulted in higher cell densities. However, the final yield of cell number was still limited by N concentration in the medium. Increasing the urea and nitrate concentrations could produce higher cell numbers.

It can be concluded that N-source requirements vary according to the species. Diatoms utilize nitrate rather than urea, while the growth of unicellular green species enhanced more by urea. This is in agreement with Reimann, et. al. (1963) who reported that Cyclotella cryptica species were unable to grow on urea. In addition, growth of diatoms was not limited by N concentration. By varying N concentrations in the nutrient medium, the green algal species investigated could be manipulated with respect to biomass production. In order to obtain higher yields, the green algal species could presumably be grown at still greater N concentrations.

Approximate Analysis for the Selected Species

In this experiment, all the selected species were grown in duplicate batch cultures. The cultures were maintained under similar temperatures and light intensities. One batch of each species was analyzed in the exponential growth phase (5 days old), when the cells were actively dividing and sufficient nitrogen was available in the medium. The second batch was analyzed in the stationary phase (14 days old), when the cells ceased dividing and the medium had become N-depleted. Data for approximate cellular compositions were expressed on the basis of organic weight and represented in Table 2. In Cyclotella, N-deficient cells contained 42.1% total lipids. Nitzschia, on the other hand, under N-stress contained 28.1% lipids. Chlorella, whether in freshwater or saline medium, did not show a clear difference in composition. Nevertheless, N-starved cells contained more lipids 28.6% (freshwater) and 32.4% (saline) than N-sufficient cells which contained 15.3% and 26.5% respectively. Proteins decreased on the expense of carbohydrates which increased relatively nitrogen deficient cells of Scenedesmus synthesized almost 44.7% total lipids, almost double that of the young cells. In addition, N-exhausted cells developed an orange color resulting from the accumulation carotenoid pigments. In experiments with the Ankistrodesmus sp. the total lipids reached up to 28.1% in N-depleted cultures. An interesting diatom, identified as Hantzschia sp. DI-160 by Dr. Barclay at SERI, was collected from the Dauphin Island. Although the culture was not unialgal, it grew very well in enriched saltwater (salinity 45 ppt.). Growth requirements of this species have not yet been identified. However, approximate analysis (Table 2) revealed the presence of high amounts of lipids (66%). See Figure 7 for oil evaluation.

It should be mentioned that in case of diatoms, the cells accumulate oil droplets as a result of N-deficiency. Silicon efficiency was studied independently for these strains to assure that oil formation was due to N-deficiency and not to silicate depletion. In case of the green algal strains, the cells changed in color to orange or yellow in the stationary phase, as a result of N-depletion. The most general effect of a nitrogen

deficiency on the composition of the selected strains is a decrease in protein and an increase in the storage products, lipids and carbohydrates. The results are in agreement with those of Fogg, 1953 and Healy, 1973.

FUTURE PLANS

Specific research plans for FY 85 - FY 86 will follow the research objectives as explained above in this report, with special emphasis:
- Sample collections from saline habitats in the Gulf of Mexico
- Screening of the samples under higher light intensities (20 - 50% of sunlight).

ACKNOWLEDGEMENT

This work was supported by Subcontract number XK-3-03-50-1 from the Solar Energy Research Institute.

Table 2. Approximate Cellular Composition of Selected Algal Species

Species	Cell Size (μM^3)	Growth Rate	Growth Conditions	% Organic Wt. Protein	Carbohydrate	Lipid
Cyclotella sp.			SW, NE	12.2	37.5	13.2
DI-35	3-5	1.37	SW, ND	16.4	10.2	42.1
Nitzschia sp.						
TR-114	10-15	0.84	SW, NE	25.7	18.8	15.2
			SW,ND	7.2	13.2	28.1
Chlorella sp.	2-3	0.92	FW, NE	51.2	12.3	15.3
MB-31			FW, ND	25.4	26.2	28.6
			SW, NE	23.5	24.7	26.5
			SW, ND	18.3	29.6	32.4
Scenedesmus sp.	5-6	1.79	FW, NE	30.2	29.8	20.3
TR-84			FW, ND	11.4	32.3	44.7
Ankistrodesmus sp.	5-7	1.11	FW, NE	35.3	32.5	16.9
TR-87			FW, ND	24.5	38.2	28.11
Hantzschia sp.	15-35	1.32	SW, NE	20.2	29.4	26.3
DI-60			SW, ND	12.6	9.3	66.0

FW = Freshwater SW = Saltwater NE = Nitrogen Sufficient ND = Nitrogen Deficient

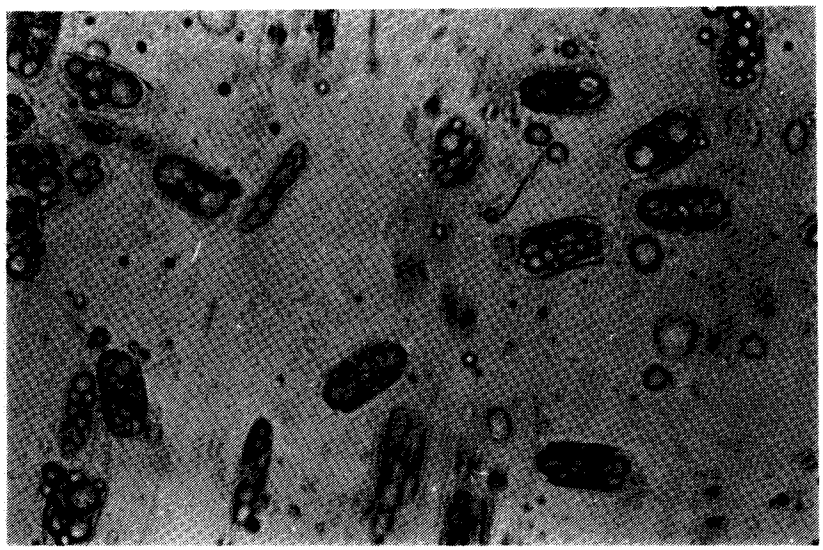


Figure 7. Hantzschia sp. Cells Showing Oil Droplets (Scale: 1 cm = 20 μm)

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PRODUCTION OF HYDROCARBONS BY MICRO-ALGAE; ISOLATION AND CHARACTERIZATION OF NEW AND POTENTIALLY USEFUL ALGAL STRAINS

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ABSTRACT

Species of the little-known genus Nannochloropsis (small, non-motile algae common in marine plankton) contain abundant lipids when grown under suitable conditions. N. marina has been studied by A. Ben Amotz. A newly isolated strain ("Nanno-Q") from China may contain up to 50% by weight of lipid, of which half may consist of hydrocarbons. We have undertaken to study this strain and to seek others with similar or improved physiological and biochemical features.

Recent articles on the subject (with references to the earlier literature) are given below. (Note: some "Nannochloris" spp. = Nannochloropsis spp.)

RESULTS

Preliminary Results with Nannochloropsis sp. ("Nanno-Q") as reported 16 Jan 85

This strain grows in nutrient media with salinities ranging from those of sea water down to brackish water only one-tenth as saline. In addition to Na^+ it seems to require divalent cations, e.g. Ca^{++} in the range 2-20 mM.

It grows well in media with nitrate, as sole N source, in concentrations up to 1 mM, but when cultured with so much available N the lipid yield is comparatively low. When the N is reduced to 0.1 - 0.2 mM, lipid production is abundant, and in old cultures (ca. 1 mo) the cells float as an orange scum.

On agar media, colonies grow to recognizable size and form in 7 days, so it should be possible to distinguish and isolate mutants with distinct cell-aggregation features after growth for 2 weeks. Irradiation with u-v light (10 cm from a "Sterilamp") is lethal at doses exceeding 15 sec, so lower doses will be used for mutagenesis.

Growth of various strains at various temperatures

The results of one experiment are summarized in the graphs appended.

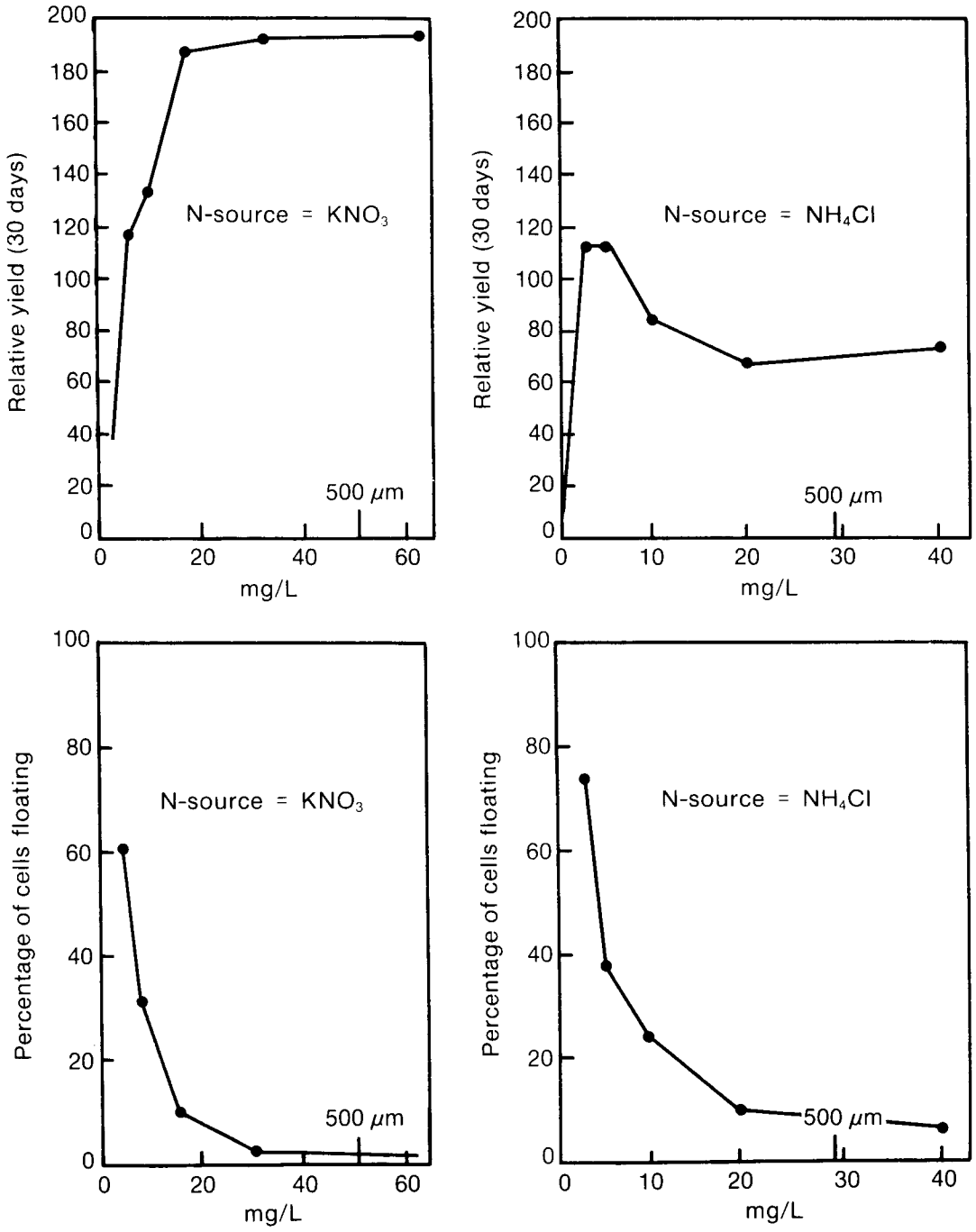

Figure 1. Yields and Percentages of Floating Cells of Nanno Q

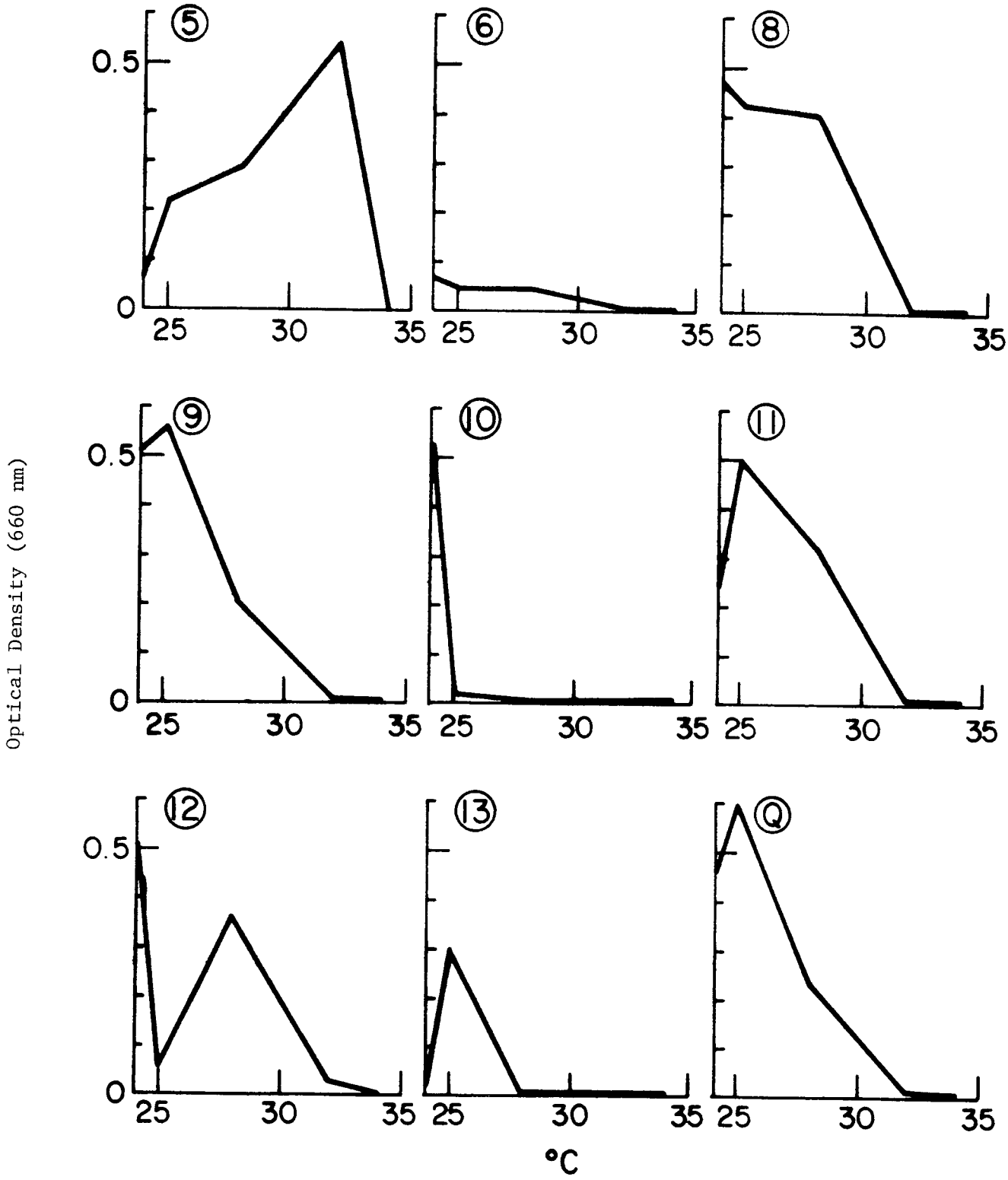

Figure 2. Growth Response of Nannochloropsis Strains to Temperature

Legend for Figures

Yields (optical density at 660 nm) of Nannochloropsis spp. strains grown for 4 weeks with continuous illumination (ca. 2000 lux) at various temperatures. Most strains from C.C.M.P. (Bigelow Laboratory), provided by Dr. R. Guillard. Sources of original cultures:

5. Great South Bay, L.I.
6. Pamlico Sound, N.C.
8. Milford Harbor, Conn.
9. Continental shelf, 39 N, 70 W.
10. Millport, Argyll, Scotland
11. Sayville, N.Y.
12. Sayville, N.Y.
13. Tunis, North Africa
Q. Qingdao, China

Lipid Contents

Samples grown in various conditions have been sent to Dr. T. Tornabene for analysis.

Survey of Sub-tropical Waters for New, Thermo-tolerant Oil-producing Strains of Algae — February-March 1985

Some 132 water samples were collected from inshore, lagoon, mangrove swamp and saline pool sites on the coasts of 13 Caribbean islands and from the North American mainland (Florida, Yucatan, Pacific and Atlantic coasts of Panama). A list of sites is appended. Apart from 5 samples of almost fresh water, salinities ranged from 26.4 to 68.8 parts per thousand: most were close to normal sea water, ca. 35 p.p.t. They were pre-filtered, to eliminate larger organisms and dross, and re-filtered through 0.45 micrometer Millipore filters to retain organisms in the range of 1-3 micrometers. The Millipore filters, with 1 ml of filtrate, were returned to S.I.O. Here they have been sub-cultured to mineral sea-water medium (SWM) and illuminated continuously at ca. 2 klux at 25°C. So far (after about 3 weeks), 110* have yielded viable cultures of algae, of which 85 contain or comprise only non-motile cells in the desired size range (1-3 micrometers, cf. Nannochloropsis). Subcultures have been set up in a medium with a reduced N content (10 mg/l $Ca(NO_3)_2 \cdot 4H_2O$) to encourage lipid production. From among the cells that ultimately float we shall isolate, by streaking on nutrient 1% agar, putative new Nannochloropsis strains for further physiological and eventual biochemical characterization.

*125 (18 Mar 85)

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ACKNOWLEDGEMENTS

...To Dr. L. Cheng and to C. Burrascano for assistance.

Table 1. SERI - Caribbean Samples 1985

No.	Date	Locality
1	26.I	California, Del Mar Lagoon
2	"	" " "
3	01.II	St. Thomas, dock of College of Virgin Islands
4	"	" " " " " "
5	"	Beach by dock, CVI
6	"	" " "
7	01.II	St. John, Lameshur Bay near dock
8	"	" " " "
9	02.II	" Lameshur Bay mangrove
10	"	" " "
11	"	" " "
12	02.II	St. Thomas, Vessup Bay Red Hook
13	"	" Compass Pt. Marina, Avicennia pool
14	"	" " " " boat dock
15	"	" Benner Bay, Nadir Rd.
16	"	" Coki Pt. seagrass bed
17	"	" " sand beach
18	"	" Mandal Bay, open sea
19	"	" " lagoon
20	Not used	
21	02.II	St. Thomas, Hull Bay
22	03.II	" French Town boat lagoon
23	"	" Magens beach, landlocked mangrove
24	"	" " open water
25	04.II	Tortola, Road Town, mangrove inlet
26	"	" " " "
27	"	" " Harbour
28	"	" Brewes Bay, Atlantic side
29	"	" Mangrove stream estuary
30	"	" Cane Garden Bay, mangrove
31	"	" " " open water
32	"	" Carrot Bay, rocky reef
33	"	" Frenchman's Cay, mangrove
34	05.II	St. Croix, Coakley Bay, open water
35	"	" Tamarind Reef Club Marina, mangrove
36	"	" Great Salt Pond, open water
37	"	" " " small mangrove pool
38	"	" Great Salt Pond proper
39	"	" Robin Bay open water
40	"	" " mangrove pond

SERI - Caribbean Samples 1985

No.	Date	Locality
41	05.II	St. Croix, Grapeten Bay, open water
42	"	" Boiler Bay nearshore water
43	"	" Salt River, mangrove lagoon
44	"	"
45	"	" Teague Bay, NIL Pier
46	06.II	" Salt River, Hydrolab pier
47	07.II	Puerto Rico, Loquillo Beach, nearshore
48	"	" " " mangrove, brackish
49	"	" Loizo Ferry, Rio Grande, brackish
50	"	" Piñones Beach
51	"	" " " behind breakwater
52	"	" Boca de Congrejos harbor
53	"	" San Juan, Condado Lagoon
54	"	" " " Escambron rock pool
55	"	" " " " outdoor pool
56	08.II	" Aguadilla
57	"	" "
58	"	" Mayaguez Harbor
59	"	" "
60	"	" Guanajibo
61	"	" "
62	"	" Pt. Arenas wet lab
63	"	" " " " "
64	"	" Joyuda Lagoon
65	"	" " "
66	09.II	Curaçao, Spaansch Water, inner lagoon
67	"	" Schottegat (100 ml)
68	"	" Piscadera Bay, CARMABI jetty
69	"	" St. Willebrodus (50 ml) (Hypersaline)
70	"	" Boca St. Martha, mangrove pool
71	"	" " " " open sea
72	"	" " " " inner bay
73	"	" Willemsted, Avila Beach Hotel
74	10.II	" " "Coney Island," mangrove
75	12.II	Panama, Naos, Inlet west of causeway
76	"	" " (Windward) east of causeway
77	"	" " west of causeway
78	"	" " STRI, raw seawater pipe
79	"	" " " filtered seawater pipe
80	"	" " Top water from 10 m tank (kept 2 mo. in darkness)

SERI - Caribbean Samples 1985

No.	Date	Locality
81	12.II	Panama, Diablo Hts., mangrove boat ramp
82	"	" " " mangrove creek (sewage?)
83	"	" " " mangrove channel (100 ml)
84	"	" Perico Is., west side bay
85	"	" " " east side bay
86	13.II	" Mangrove under Thatcher Ferry Bridge
87	"	" Cocoli, steep-sided dike, mangrove
88	"	" Naos, STRI sea-snake tank
89	14.II	" Galeta, mangrove pool at post 13
90	"	" " bay near STRI Lab., mangrove
91	"	" " open water, STRI Lab.
92	"	" " mangrove back water
93	"	" " open water eddy
94	"	" " STRI Penicillus tank
95	"	" " " shallow Sargassum tank
96	"	" " " indoor aquarium
97	"	" " " exposed SW tank
98	"	" " " foam sea surface
99	"	" " " " " "
100	"	" " open reef
101	"	" " mangrove by roadside
102	"	" " mangrove-lined channel
103	"	" " mangrove under bridge
104	"	" Limon Bay, Isla Margarita
105	"	" Lagoon, east of Isla Margarita
106	"	" French Canal diversion Bridge 15-11
107	"	" Colon Cristobal Yacht Club Pier
108	15.II	" Barro Colorado, boat dock (FW)
109	"	" Frijoles, boat dock (FW)
110	16.II	Miami, Mashta Is. Causeway by boat harbour
111	"	" Mashta Pt. open ocean
112	"	" " " enclosed pool
113	"	" " " mangrove
114	"	" Cape Florida, Pines Canal
115	"	" Stagnant water, " "
116	"	" RSMAS Pier
117	17. II	Miami, mangrove ditch (US 1), Florida Key
118	"	" mangrove area (brackish)
119	"	" Florida Key, boat ramp
120	"	" Mile 108, US 1, shallow mangrove

SERI - Caribbean samples 1985

No.	Date	Locality
121	16.II	Miami, Key Largo, boat canal, Gilberts Hotel
122	"	" " " Rowel's Marina
123	"	" " " " " , standing pool
124	"	" Plantation Key, standing water
125	"	" " " open water canal
126	"	" Key Largo Lagoon "Sea Castle Restaurant" (60 ml)
127	"	" " " enclosed lagoon near above
128	"	" " " boat lagoon, ocean side
129	"	" Lake Surprise
130	"	" Mile 110, US 1, mangrove ditch
131	~23.I	Cancun, Mexico (per W.A.N.)
132	"	" " " "

COLLECTING AND SCREENING MICROALGAE FROM SHALLOW, INLAND SALINE HABITATS

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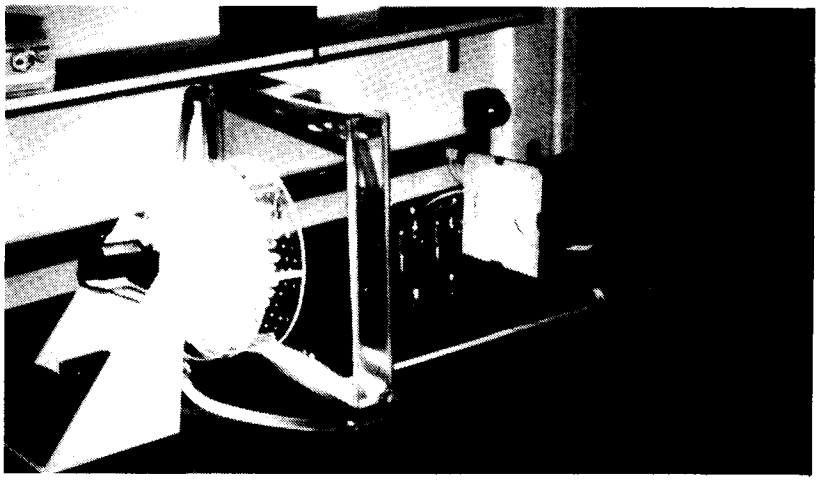
Introduction

Over the past four decades, systems have been researched and developed to culture microalgae outdoors as sources of food, chemicals and fuels (Goldman 1979). In most of these cases, "weed" species of microalgae have been utilized as production organisms. In many of the cultivation systems designed for protein production, the operators have been content to cultivate those species from the local flora which invade and become dominant (Yang et al. 1980, Castillo et al. 1980). Very few attempts have been made to collect and screen microalgal species from nature for specific product content or physiological attributes which would identify them as valuable and easily cultivatible organisms. More effort has been devoted to collecting and screening macroalgal species or strains, for example for improved polysaccharide or hydrocolloid production potential (van der Meer 1983).

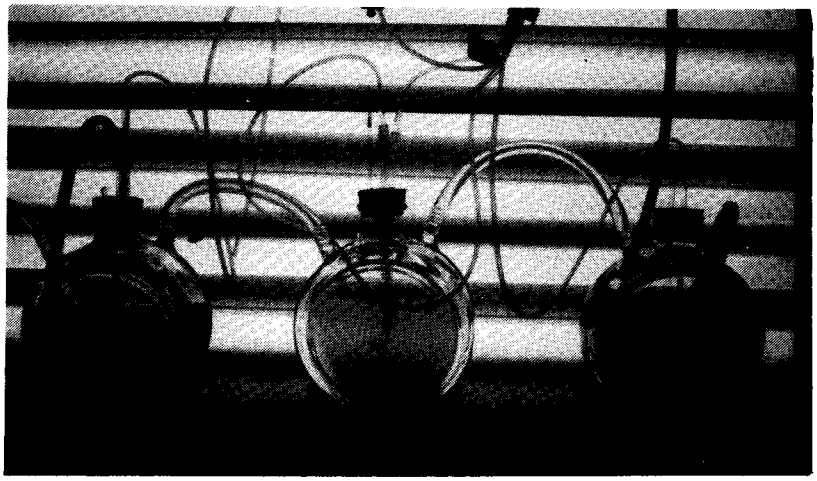
The efforts of the SERI Aquatic Species Program (ASP) to identify species for the production of cellular lipids therefore represents one of the first attempts at large scale screening of microalgae. The ASP has identified 5 desirable characteristics of microalgal strains for biomass fuel applications. In order of importance they are: 1) rapid growth in dense culture; 2) growth in unstable culture environments (fluctuating temperature and salinity); 3) growth in saline waters (marine waters and saline groundwater); 4) growth at high light intensities; and 5) the ability to accumulate large amounts of product (lipids). Early collection and screening activities in the ASP focused on species from marine environments and from large saline lakes in California and Nevada (Thomas et al. 1983 1984a,b). Environmental tolerances and lipid production capacity were not evaluated early in the selective process, and hence most of the species identified were poor performers in outdoor cultivation systems. Problems with these species included a low range of temperature tolerance, inhibition by high light intensities, and low lipid production potential.

A major effort in 1984 was directed toward the development of improved collection and screening protocols. The goal was to develop protocols with a sound theoretical basis, so that the program would be more successful in identifying and developing superior strains for biomass fuel applications. Two hypotheses were tested during the development and restructuring of the collection and screening protocol. The first hypothesis was that microalgal strains exhibiting the characteristics



A

B



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Protocol for Species Screening. (A) Initial Selection from Cultures Grown under High Light and Temperature; (B) Characterization of Growth Response at Different Salinities and Temperatures; (C) Maximization of Growth and Photosynthetic Efficiency in Dense Cultures

outlined above would most likely be found in shallow, unstable saline habitats such as temporary pools, playas and thermal springs. The small size of these habitats implies a low buffering capacity against changes in temperature, light and salinity. Species which grow well in these habitats are likely to be adapted to a wide range of environmental conditions. Additionally, the ecological literature suggests that many of the species in this class of habitats should employ strategies of lipid rather than carbohydrate accumulation for food storage products, in part because lipids are a more mass-efficient storage material and they may provide some protection from photooxidation and desiccation (Evans 1959, Davis 1972).

The second hypothesis tested was that a screening protocol would be more successful in identifying superior candidates if species possessing the five desirable physiological characteristics were identified as early in the protocol as possible. This would speed the screening process by eliminating undesirable species earlier and ultimately allowing the evaluation of more samples.

The research described here examines the validity of these hypotheses. Some of the results from the implementation of a revised collection and screening protocol in the field are presented.

Materials and Methods

Screening Protocol Development. After discussions with several phycologists and microbiologists, a rotary screening apparatus was designed that would allow for the simultaneous screening of numerous samples at elevated light intensities and temperatures. The rotary screening apparatus constructed is essentially a modified version of the common roller drum devices utilized by microbiologists. The modifications necessary for adapting this device for microalgae were suggested by Dr. Abe Flexer of Synergen, Inc. The rotary screening apparatus is a clear plexiglass wheel rotated at 20-40 rpm which holds up to 160, 16 x 150 mm culture tubes. The rotary wheel can be housed in a temperature controlled box and illuminated with a high intensity stage lamp capable of producing light intensities from 0-240 W m^{-2}. The rotary action of the wheel keeps the cultures suspended while they are exposed to the elevated temperatures and light intensities.

As a second improvement in the screening protocol, we determined the ionic characteristics of the major inland saline water types that are potentially available for use in algal biomass production systems. Maps of the southwestern U.S. depicting overall suitability of the land for algal biomass production systems were obtained from the SERI Resource Assessment Branch, and water quality data were compiled from state and federal reports on saline wells in those areas deemed suitable for biomass production systems. Data were only analyzed for wells with depths of 200 feet or less since waters from deeper aquifers may be unavailable due to high energy costs for pumping. These defined saline waters, rather than media based on

the salinity and ionic composition of the collection sites, were used to screen candidate species, in order to increase the probability that the species selected will grow in the sources of saline waters available in the southwest.

Analysis of the data compiled from New Mexico has been completed. The results indicate that 85% of the available saline water is in the 3,000 to 10,000 ppm total dissolved solids (TDS) range, with an average of 4116 ppm. Multivariate statistical analyses were performed on the water quality data in order to analyze relationships among the various ionic parameters. Correlation analysis and R-mode factor analysis indicated that two major factors accounted for 70% of the variance in the saline water ionic compositions: 1) sodium chloride concentration, and 2) calcium plus magnesium concentration. Scatter plots of the factor scores for each site suggest that there are two major saline water types available for use in producing microalgal biomass. These waters were termed Type I and Type II. Each water type was prepared at 4000 TDS and allowed to evaporate at 35°C (with stirring). During evaporation, samples were periodically removed and filtered, and conductivity, pH, Na, Ca, Mg, K, HCO_3, CO_3, SO_4, and Cl were determined. Cations were determined by inductively coupled plasma spectrometry (ICP) (Instrumentation Laboratory Model 100), anions by high pressure liquid chromatography (HPLC), and bicarbonate and carbonate by titration. Ion concentrations were plotted versus conductivity, and recipes were derived for preparing waters at 10, 25, 40, 55, and 70 mmho cm^{-1} (Tables 1 and 2). These waters form no precipitates between 10 and 35°C. It is believed that these waters give a good approximation of the conditions which algae in culture systems in the southwest will experience, where high salinities will be achieved through evaporation of moderately saline sourcewaters.

Table 1. Formulas for Preparing Type I Inland Saline Water at Various Conductivities (Evaporation Stages). All concentrations are mg L^{-1}.

Salt	Conductivity (mmho cm^{-1})				
	10	25	40	55	70
$CaCl_2$	-----	3,932	5,618	7,610	8,430
$MgCl_2 \cdot 6H_2O$	4,114	11,844	22,789	35,305	42,230
Na_2SO_4	-----	2,925	3,310	3,705	3,620
KCl	194	407	662	960	1,186
$NaHCO_3$	184	168	168	168	168
NaCl	2,118	3,845	14,132	13,023	16,039
$CaSO_4$	1,686	-----	-----	-----	-----

Table 2. Formulas for Preparing Type II Inland Saline Waters at Various Conductivities (Evaporation Stages). All Concentrations are mg L^{-1}.

Salt	Conductivity (mmho cm^{-1})				
	10	25	40	55	70
$CaCl_2$	28	28	28	28	28
$MgCl_2 \cdot 6H_2O$	1,953	3,026	3,920	4,362	4,230
Na_2SO_4	2,671	5,870	15,720	23,305	28,360
KCl	466	965	2,028	3,044	3,673
$NaHCO_3$	1,208	2,315	2,855	3,234	3,245
Na_2CO_3	231	876	1,234	1,492	1,527
NaCl	1,511	8,078	12,963	20,588	26,075

The screening and characterization protocol that was developed employing the rotary screening apparatus in conjunction with the standard inland saline waters is outlined in Figure 1. This protocol is described in greater detail below.

Collection Activities. Microalgal collection activities during the 1984 season concentrated on small saline environments including temporary ponds, playas and springs in the arid regions of Utah and Colorado. The locations of the sampling sites are depicted in Figure 2. Water and sediment samples containing algae were collected in polyethlyene containers and kept cool and darkened in ice chests until returned to the laboratory (usually 1-3 days after collection). At each site, temperature, pH and redox potential were measured with a Markson Model 76 Temp/pH/mv meter. Conductivity was measured with a Markson Model 16 conductivity meter, and alkalinity was determined by potentiometric titration. Upon return to the laboratory, portions of the water samples were filtered through 0.2 µm filters for determination of cation and anion concentrations. Cation concentrations were determined by ICP, sulfate and chloride by HPLC.

Species Enrichment. Step II of the screening process involved sample enrichment incubations on a rotary screening apparatus at elevated temperatures (25°C and 30°C) and light intensity (80 to 200 W m^{-2}). Collected site water containing algae was placed in 16 x 150 mm culture test tubes and enriched with urea (300 µM), PO_4^{3-} (30 µM), silica (36 µM), NaFeEDTA (3 µM), and 5 mL L^{-1} PII trace elements stock and vitamins. The

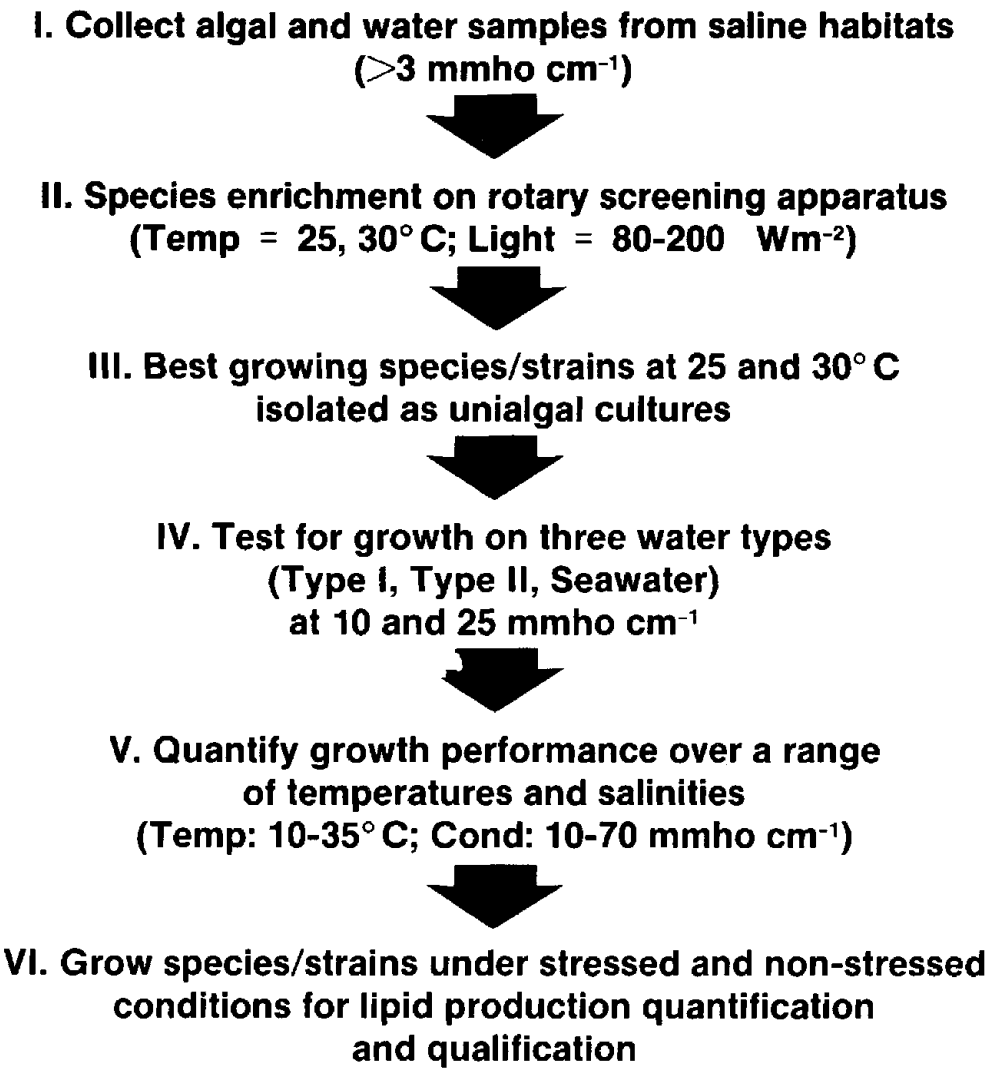


Figure 1. Screening Protocol

tubes were then placed in the rotary screening apparatus and light intensities were slowly increased over a 5 day period from 80-200 W m^{-2}. At the end of this time, the tubes were removed and examined for algae.

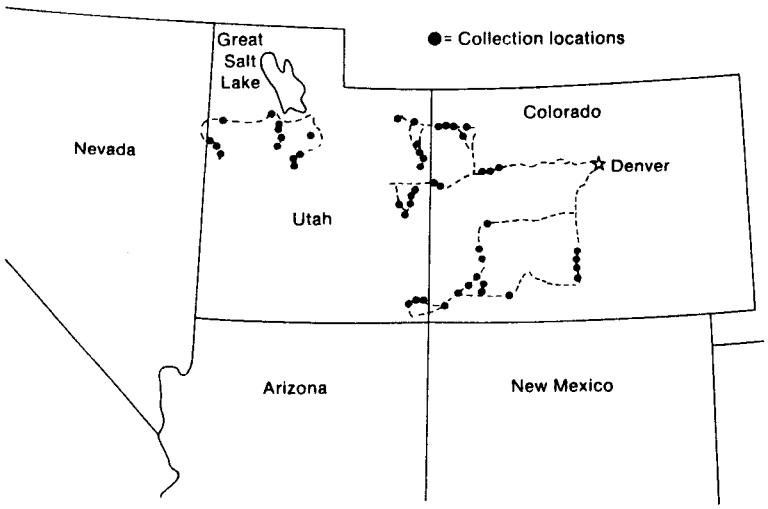


Figure 2. Collection Trips Made During the 1984-85 Collection Season. X = Location of saline habitat where samples were collected.

Species Isolation and Growth Performance Quantification. The best growing strains were isolated as unialgal cultures on agar or in liquid cultures (Step III). In Step IV of the screening protocol, these strains were run through a preliminary growth evaluation on three water types; Type I, Type II, and artificial seawater at conductivities of 10 mmho cm^{-1} and 25 mmho cm^{-1} in a light (30-40 W m^{-2}) temperature-controlled (25°C) incubator. Artificial seawater was prepared with Rila Marine Mix (Rila Products, Teaneck, New Jersery). Species exhibiting good growth on at least one of these water types were selected for quantification of their growth performance in a temperature-salinity cross gradient (Step V). The temperature gradient apparatus employed was modified from the design described by Siver (1983). Illumination from a bank of twelve 40 W fluorescent lamps suspended over the table was adjusted to 40 W m^{-2}.

The growth responses of the selected species to 30 combinations of temperature and salinity were evaluated in each of the three water types (Type I, Type II, and seawater). Duplicate 25 ml cultures were grown in 50 ml Erlenmeyer flasks over a matrix of 5 salinities (10, 25, 40, 55 and 70 mmho cm^{-1}) by 6 temperatures (10, 15, 20, 25, 30 and 35°C), for a total of 60 experimental cultures for each water type. Each treatment was enriched to 300 µM urea, 30 µM phosphate, 36 µM silicate, and 3 µM iron, and also received trace element and vitamin enrichments (see Table 1). Prior to the initiation of the experiments, stock cultures were preconditioned on the gradient table at 17 and 27°C in the appropriate water type at each of the 5 experimental salinities, and these populations were used to inoculate the experimental cultures. The experimental cultures were grown in either batch or semicontinuous mode. For batch cultures, optical density (O.D.)

of the cultures (750 nm) was determined twice daily for five days. The exponential growth rate was determined as the slope of a log-linear plot of O.D. versus time during the exponential phase of growth. The semicontinuous cultures were inoculated at an initial O.D. of 0.03. O.D. was measured daily and the culture diluted to 0.03 with fresh medium when an O.D. greater than 0.06 was reached. Growth rates were calculated from the O.D. after dilution (where necessary) and the O.D. before dilution the following day; these values were averaged over the five day duration of the experiments. This technique yields improved results by decreasing variability between samples, and provides a more objective estimate of growth rate.

Lipid Production. For lipid production quantification under stressed and nonstressed conditions (Step VI) the algae were grown at the optimal conditions determined in Step V in 2 liter bottles in a growth chamber at 40 W m^{-2} on a 18:6 light:dark cycle. Two treatments were employed, one with 600 µM urea and 60 µM PO_4 and the other with 300 µM urea and 30 µM PO_4. The other nutrients were maintained at the concentrations described above in the growth performance evaluation experiments. The cultures were inoculated, and the O.D. monitored daily. On the second day after the 300 µM urea culture reached stationary phase, all of the 600 µM culture and one-half of the 300 µM culture was harvested and freeze dried. Seven days later the remaining volume in the 300 µM culture was harvested and freeze dried. This provided 3 samples for lipid determination: 1) nitrogen sufficient, 2) start of nitrogen depletion, and 3) nitrogen depleted for 10 days.

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) (Tornabene 1984). The chloroform phase was collected, evaporated to dryness under N_2, and the weight of the lipids determined. Lipid content was calculated as the weight of the lipid extract divided by the ash free dry weight of the original sample.

Results

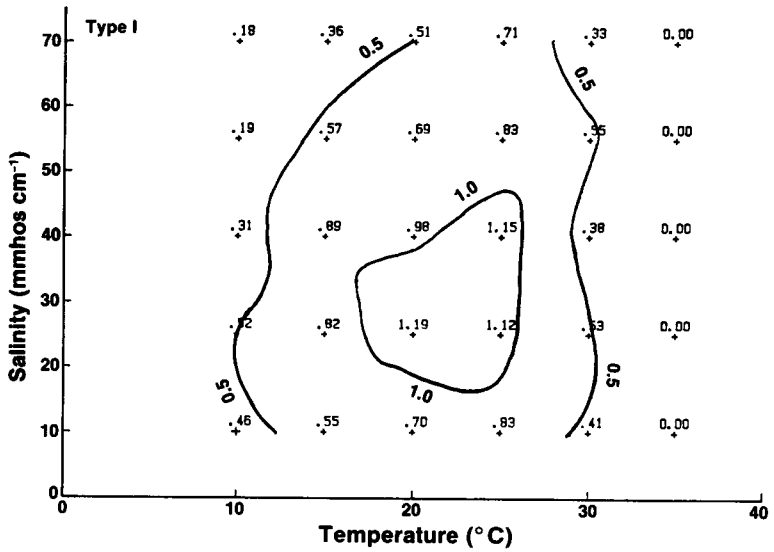
Collection Activities. Five percent (15 out of 300) of the saline water samples collected in Utah and Colorado produced potentially promising microalgal species. These promising species from the 1984 collecting season are listed in Table 3. All of these strains grow well at salinities greater than 5 mmho cm^{-1} and temperatures of at least 30°C. A smaller number of particularly attractive species will be selected from this list based on their performance through the remainder of the screening process.

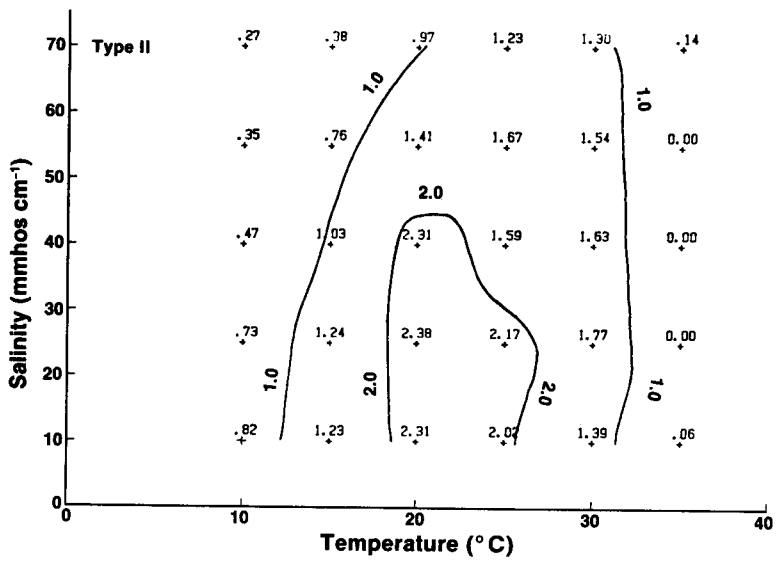
Table 3. Promising species from the 1984 collecting season.

Genus	Number of strains collected
Chryso/F1	1
Amphora	2
Cymbella	2
Amphipleura	1
Chaetoceros	1
Nitzschia	2
Hantzschia	1
Diploneis	1
Chlorella	1
Scenedesmus	1
Ankistrodesmus	2
Chlorococcum	1

Analysis of the physical and chemical characteristics of both the productive and nonproductive 1984 collection sites is in progress. The goal of this activity is to develop an environmental profile of collection sites most likely to yield promising strains. Such a profile would allow us to identify and focus our collection efforts on the types of collection sites with the greatest promise for yielding species of interest to the ASP. Table 4 presents a preliminary summary analysis of 33 of the 1984 collection sites. Of the 11 site characteristics quantified, depth was the only variable which was significantly different between sites yielding promising strains and sites with less productive strains. Sites yielding highly productive strains tended to be very shallow, averaging 6.5 cm as opposed to the other sites which averaged 27.6 cm. These data support our hypothesis that species with physiological characteristics desirable for outdoor biomass fuel production will be found in shallow saline habitats. Additional multivariate analyses of these data are in progress and may identify other site parameters which will be useful in the selection of collection sites for the 1985 season.

Species screening and characterization. Determinations of the temperature and salinity tolerance of two of the species isolated in 1984 have been completed. The growth rates of these species, Chryso/F1 and Ankistrodesmus sp., are presented in Figures 3 and 4. Dr. Rick Meyer of the University of Arkansas has tentatively identified Chryso/F1 as a flagellated chrysophyte of the genus Boekelovia. Chryso/F1 exhibited a very wide range of





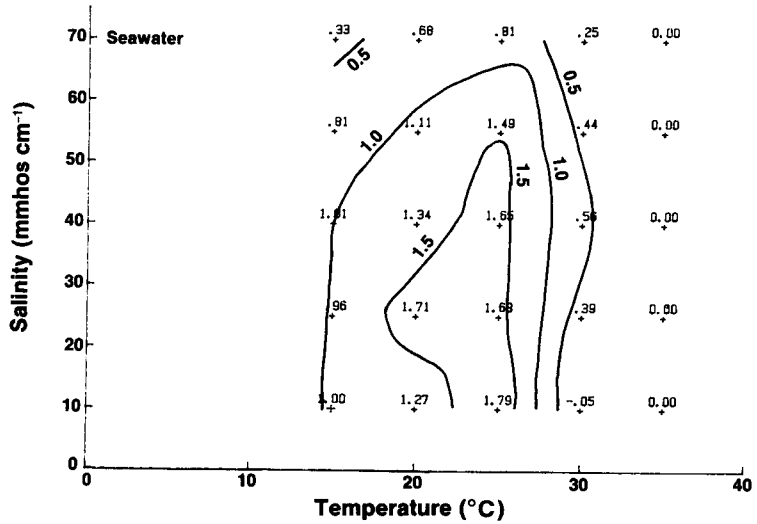
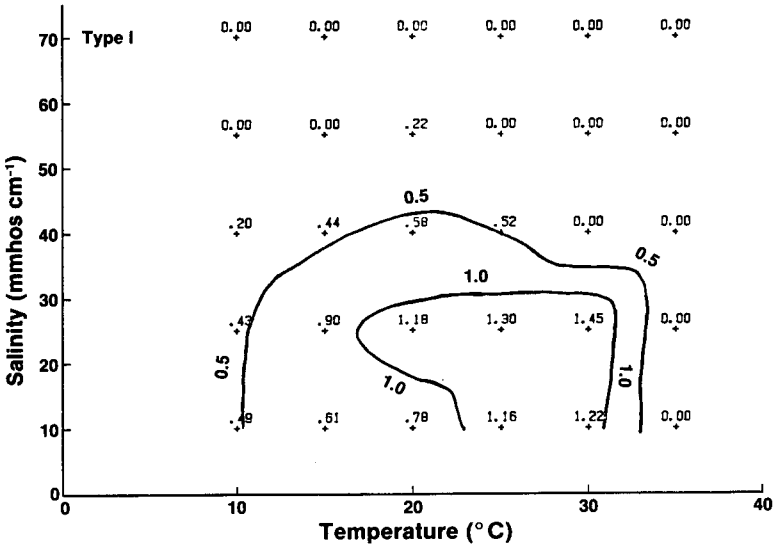
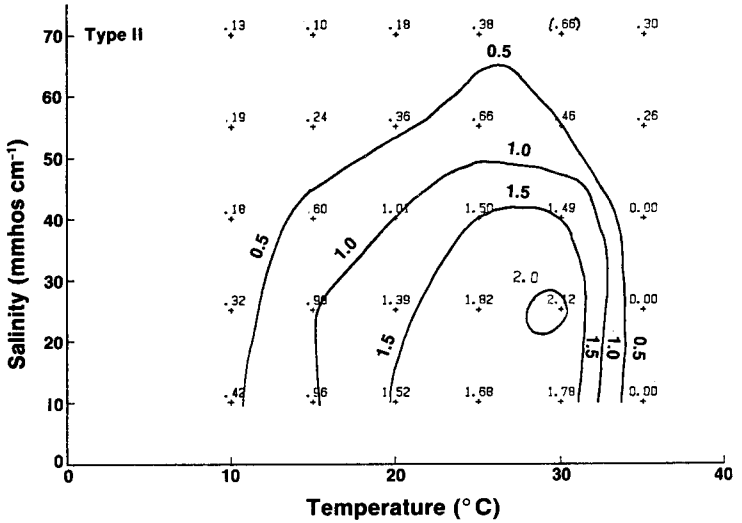


Figure 3. Exponential growth rate (day^{-1}) of Chryso/Fl in batch culture. Each point represents the mean of two replicate determinations. A = Type I inland saline water; B = Type II inland saline water; C = Seawater.





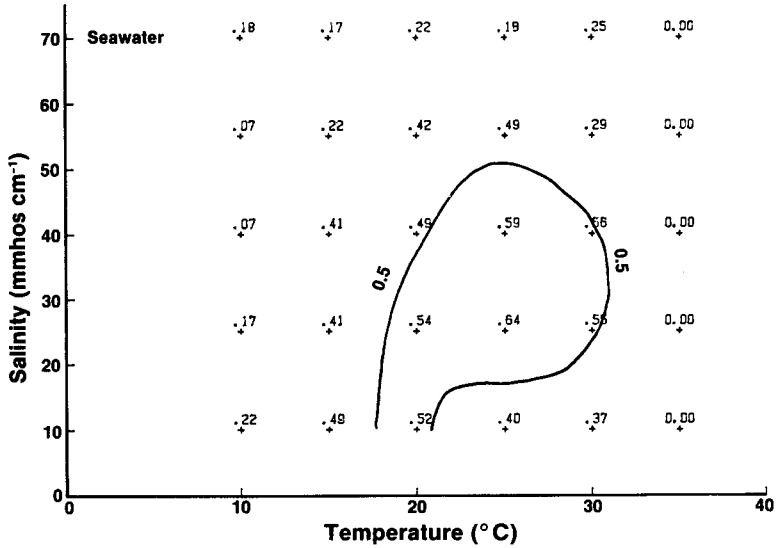


Figure 4. Exponential growth rate (day^{-1}) of Ankistrodesmus sp. in semicontinuous culture. Each point represents the mean of two replicate determinations. A = Type I inland saline water; B = Type II inland saline water; C = Seawater.

Table 4. Physical and chemical characteristics of 1984 collection sites yielding strains with good biomass production potential as compared to those sites yielding slow growing strains or strains with narrow environ mental tolerances. Number of sites with productive strains = 6, number of sites without highly productive strains = 27. X = mean, s.d. = standard deviation, t = t test.

Collection Site Characteristic	Sites Yielding Highly Productive Strains			Sites Not Yielding Highly Productive Strains			
	$\bar{X}$	±	s.d.	$\bar{X}$	±	s.d.	t statistic
Depth (cm)	6.5	±	1.9	27.6	±	24.8	2.06**
Temp. (^{o}C)	31.3	±	3.7	29.9	±	7.0	0.72
pH	8.3	±	1.1	8.0	±	0.7	0.80
Cond. (mmho)	14.5	±	11.6	9.1	±	9.4	1.22
Na (meq/L)	131.98	±	88.5	78.7	±	87.7	1.34
K (meq/L)	2.5	±	2.3	3.4	±	7.2	0.28
mg (meq/L)	9.2	±	9.3	20.3	±	43.5	0.61
Ca (meq/L)	9.8	±	10.2	10.5	±	11.4	0.14
Cl (meq/L)	119.9	±	115.2	65.9	±	82.6	1.34
SO_4 (meq/L)	16.9	±	17.0	25.7	±	30.9	0.67
ALK* (meq/L)	19.8	±	38.0	9.3	±	22.9	0.89

* Total Alkalinity
** Significant at L.05 level

temperature and salinity tolerance. It grows at rates of 1 doubling day^{-1} or more over a salinity range of 10-70 mmho cm^{-1} and temperatures of 10-32^{o}C. At its optimum temperature and salinity in Type II water, this species grows at a rate of ~ 3.5 doublings per day (2.3 day^{-1}).

Chryso/Fl grew most rapidly in Type II water, followed by seawater and Type I water in that order. The maximum growth rate for Chryso/Fl in seawater was 1.79 day^{-1} and in Type I 1.19 day^{-1}. Ankistrodesmus sp. also preferred Type II inland saline water, but it exhibited higher growth rates in Type I water than in seawater. Its growth rate in seawater was particularly poor in that growth rates at all temperature and salinity combinations were less than 1 doubling day^{-1}. The Ankistrodesmus sp. exhibited its fastest growth

rate at a higher temperature than Chryso/F1. The optimum temperature for Ankistrodesmus sp. in Type II was 30°C (2.12 day^{-1}), while that for Chryso/F1 was 25°C (2.17 day^{-1}).

The growth performance of the two species is summarized in Table 5. The temperature/salinity ranges selected for the gradient experiments (10-70 mmho cm^{-1} and 10-35°C) represent estimates of the operational ranges of mass algal cultivation systems. As can be seen in Table 5, Chryso/F1 exhibits growth rates greater than 1 doubling/day over 82% of the range in Type II water and 63% of the range in seawater. Ankistrodesmus sp. also showed a wide range of tolerance in Type II water, exhibiting growth rates greater than 1 doubling/day over 51% of the temperature/salinity range.

A hot springs diatom of the genus Amphora is now being evaluated for temperature and salinity tolerance. This species grows poorly in Type II water, and appears to prefer seawater and Type I water. Results with this species should provide an interesting comparison with the data for Chryso/F1 and Ankistrodesmus sp.

Table 5. Summary of the Growth Performance of Chryso/F1 and Ankistrodesmus sp. in the three Standard Water Types. Results are presented as the area within each of three growth rate contours. Numbers in parenthesis indicate the percentage of the experimental range enclosed by the contour.

Species	Water Type	Contour Area (mmho °C) for Growth Rate (doublings day^{-1})		
		1 (.693 day^{-1})	1.44 (1.0 day^{-1})	2 (1.386 day^{-1})
Chryso/F-1	Type I	651 (43)	186 (12)	0
	Type II	> 1231 (82)[2]	>1009 (67)[2]	722 (48)
	Seawater[1]	> 760 (63)[2]	548 (46)	205 (17)
Ankistrodesmus sp.	Type I	474 (32)	226 (15)	6 (0.5)
	Type II	770 (51)	556 (37)	320 (21)
	Seawater	0	0	0

[1] Reduced range (1200 mmho °C versus 1500 for remaining experiments).
[2] Open contours at salinity maximum; tolerates higher salinities than were tested.

Lipid production quantification and qualification experiments have been carried out for the same three species. Only Chryso/F1 exhibited significant induction of lipid production under nitrogen limitation (Table 6). Lipid content in this species increased from 26.6% AFDW in exponential phase to 58.5% AFDW in late stationary phase. Both Amphora and Ankistrodesmus exhibited maximum lipid contents below 30% AFDW under nitrogen limitation. Chryso/F1 is also unique in that lipid induction appears to occur relatively rapidly at the start of stationary phase. The

lipid content of this species had risen from 26.6 to 42.4% after the algae had been in the stationary phase for only 2 days.

Table 6. Lipid content at various growth phases of three highly productive species screened in 1984. A = nitrogen sufficient cells. B = 2nd day of stationary phase of growth. C = 10 days of stationary phase of growth.

Strain	Growth Phase A	B	C
Chryso/Fl sp.	26.6	42.4*	58.5
Amphora sp.	11.7	15.4	25.7
Ankistrodesmus sp.	23.4	24.4	29.4

*s.d. = ± 2.2

Discussion

Two of the species, Chryso/Fl and Ankistrodesmus sp., for which the screening process has been completed this year exhibit excellent biomass production potential. Both species exhibit maximum growth rates (3.5 doublings day^{-1}) equal to the highest growth rates reported for any marine species of microalgae (Thomas, 1966). These species are also the fastest growing microalgae isolated by the ASP to date. Both Chryso/Fl and Ankistrodesmus sp. grow well at high densities. In greenhouse cultures, we have been able to easily achieve cell densities of 1.3 g L^{-1} for Ankistrodesmus sp. and 1.2 g L^{-1} for Chryso/Fl. The growth potential and physiological characteristics of both species suggest that the restructured screening protocol has the potential for identifying superior strains for biomass fuel applications. Of the two species, Chryso/Fl has the greatest potential in biomass fuels applications because it produces large amounts of lipids, up to 58% AFDW. This is very close to the ASP goal of obtaining strains which produce lipid contents of 60% AFDW (Hill et al., 1985). It is also able to grow at 1 doubling day^{-1} over 82% of the expected operational range of temperature and salinity for outdoor cultivation systems.

The concept of employing standard inland saline water types in the screening protocol appears successful, and the results have important implications for mass algal cultivation technologies. The data presented here indicate that there is considerable variability in growth responses to the different water types and that species for a given system will probably need to be selected based on the chemistry of the sourcewater available at that cultivation site. When more of these salinity/temperature evaluations

have been completed for other species, we plan to statistically analyze the data using factorial designs in order to determine which ions or ionic ratios are causing the variability in growth responses.

Of the collection site characteristics we measured, depth appears to be the most significant variable of saline habitats with highly productive species. This will prove advantageous in selecting the 1985 collection sites, because depth is one of the easiest environmental parameters to measure. Shallow sites can be easily detected and focused on in the field. Collections can still occur at larger saline habitats, but collection efforts will focus on saline microhabitats surrounding or on the fringe of these larger water bodies. Employing these results, we plan to establish artificial saline microhabitats at some locations, add nutrients to them, and collect strains which invade these systems. This may provide an additional, and possibly simpler, method of obtaining strains which are adapted to shallow saline environments.

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Addendum

The SERI Microalgal Culture Collection (B. Barclay, K. Terry, N. Nagle and S. Hock).

The culture collection acts as a repository for the most promising species identified by various investigators within the Aquatic Species subprogram. These species are made available to investigators within and outside of the program in order to encourage research with species which are of particular interest for fuel production. These species are selected from among organisms which are collected by Aquatic Species researchers or which are available from culture collections and have demonstrated potential for outdoor fuel production. A steering committee meets yearly to aid in the selection of appropriate species for inclusion in the culture collection. The culture collection presently includes the strains listed in Table 7.

The organisms in the culture collection are listed in a catalog which is issued yearly and distributed to researchers with interests in basic and applied phycology. In addition to listing the species, this catalog provides physiological information about each species and indicates the extent of a literature data base which is available upon request with any culture shipment. Physiological data which are presented in the catalog include size, growth form, maximum growth rate, cultural requirements, temperature and salinity tolerance, light curve of growth, chemical composition, lipid composition, life cycle, and outdoor culture history. Not all data are currently available for each species listed, but as time permits, characterizations similar to those employed for newly isolated species will be

applied retroactively to species in the culture collection for which the necessary data have not yet been collected.

Table 7. The SERI Microalgae Culture Collection

Species	Source	Collection Site
Ankistrodesmus falcatus	W.H. Thomas	Pyramid Lake, Nevada
Botryococcus braunii	UTEX[1]	
Chaetoceros gracilis	R. York	
Chlorella sp. (S01)	S. Lien	Golden, Colorado
Isochrysis aff. *galbana*	R. York	Tahiti
Nannochloropsis salina		Great South Bay, NY
Nitzschia sp.	W.H. Thomas	Mono Lake, Calif.
Oocystis pusilla	W.H. Thomas	Walker Lake, Calif.
Phaeodactylum tricornutum		Woods Hole, Mass.
Phaeodactylum tricornutum	W.H. Thomas	
Platymonas sp.	E.A. Laws	Honolulu, Hawaii

[1]UTEX: University of Texas Culture Collection

APPLIED PHYSIOLOGY

ULTRASTRUCTURE EVALUATION OF LIPID ACCUMULATION IN MICROALGAE

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ABSTRACT

To increase our understanding of the cellular changes involved in lipid accumulation, the ultrastructure of three microalgal species is being evaluated. As a necessary first step, appropriate preparative techniques are being developed for electron microscopy. At least one of five different fixation and embedding techniques has been applied to each of the species, yielding quite different results. Fixation is adequate for Ankistrodesmus falcatus, nearly adequate for Isochrysis aff. galbana and inadequate for Nannochloropsis salina. Once adequate preparation methodologies are available, ultrastructural changes in the species will be examined during logarithmic growth and at regular intervals after the initiation of nitrogen deficiency. In addition, the feasibility of using flow cytometry to separate an algal subpopulation with high lipid-producing capabilities will be investigated.

OBJECTIVES

Lipid accumulation in microalgae can be readily detected at the light microscopic level. Morphological changes observable at this level include variations in refractive properties of the cell, an increase in the size and/or number of visible inclusions, and changes in the color of the cells. This information, while useful as an indication of the metabolic state of the cells, reveals little about the exact intracellular location of the lipids in relation to other cell organelles, or about changes occurring in those other organelles concomitant with lipid accumulation. Such information will be most useful in understanding and controlling the physiological changes involved in switching from the rapid cellular growth phase to that of rapid lipid accumulation.

The primary objective of this study is to identify and evaluate the ultrastructural changes occurring in three species of oleaginous algae as they proceed from rapid, unstressed growth to the massive build-up of lipid reserves which often results from nitrogen deprivation. Specifically, morphological variation in chloroplasts, endoplasmic reticulum, mitochondria, membranes, lipid storage bodies, and microbodies are of interest.

A secondary objective of this study is to explore the feasibility of using flow cytometry to separate, from cultures of known lipid-producing microalgae, subpopulations of cells having exceptionally high

lipid-producing capabilities. Such a method of genetic selection could rapidly yield superstrains of oleaginous algae in a straightforward manner, and could well be applied to many of the strains already under development.

APPROACH

Task 1

The ultrastructure of three species of microalgae known to be good lipid producers is being studied: Ankistrodesmus falcatus (S/ANKIS-1), Isochrysis aff. galbana Green (S/ISOCH-1), and Nannochloropsis salina Hibberd (S/NANNO-1). Ultrastructural information is obtained by transmission electron microscopy of thin sections of cells. In order to cut the necessary 100 nm thick sections, the cells must be fixed to preserve cellular structure and dehydrated to remove water prior to embedding in an epoxy resin. The resin is polymerized to a hard block which can be cut with a diamond knife on an ultramicrotome. Initial work thus involves the development of adequate fixation and embedding procedures. Once these have been established, cells in known stages of growth (logarithmic growth, and at regular intervals following the onset of nitrogen deprivation) will be prepared for electron microscopy and examined. The lipid, protein, and carbohydrate content of the cells will also be determined. Evaluation of cytological detail will concentrate on changes in cell components known to be involved in energy storage and conversion (e.g., chloroplasts, mitochondria, microbodies, lipid bodies, pyrenoids, and various other food storage bodies) as well as those possibly involved in enzymatic regulation of the process (e.g., ribosomes, endoplasmic reticulum, Golgi apparatus).

Task 2

Batch cultures of the three microalgal species used in Task 1 will be evaluated in the flow cytometer to determine, first, whether their morphologies are appropriate to this technology and, second, whether discrimination among subpopulations is possible based solely on natural autofluorescence. There are indications that, at least in Nannochloropsis, high chlorophyll content correlates with high lipid content (Ben Gurion 1985). If cells with high chlorophyll content can be separated from the rest of the population, they would also be expected to have a high lipid content. Flow cytometry, using the natural fluorescence from chlorophyll, might be used to effectively discriminate and separate a high lipid subpopulation of cells. To make this determination, cells from both ends of the chlorophyll-content spectrum will be separated using flow cytometry and will then be cultured, to obtain enough material for analysis of lipid, protein, and carbohydrate content. If the capacity for accumulating large amounts of lipid is genetically determined, and if the relationship between chlorophyll and lipids holds, it should thus be possible to evaluate whether such a separation has indeed been accomplished.

An effort will also be made to identify and obtain a vital (non-toxic) lipid-specific fluorescent stain, which would allow cell separation directly on the basis of lipid content. Validation of the effectiveness of separations by means of this stain will be accomplished as outlined above.

PROGRESS

Establishment of Cultures

Cultures of all three microalgal species from the Solar Energy Research Institute (SERI 1984) collection have been established and are now growing well. Ankistrodesmus is being maintained in a Pyramid Lake medium (SERI 1984) in an environmental growth chamber at 25°C under a 16:8 light:dark regime. The light intensity has not been determined, but the species does best away from a direct light source. It is not being shaken, because of its tendency to clump when agitated. Nannochloropsis and Isochrysis are both being grown in an f/2 medium (SERI 1984), using pasteurized sea water obtained from the sea water tables at the Duke Marine Lab, Beaufort, North Carolina. They are being grown under the same environmental conditions as Ankistrodesmus, except that they are exposed to much higher light levels and are being agitated on an orbital shaker, approximating natural conditions more closely.

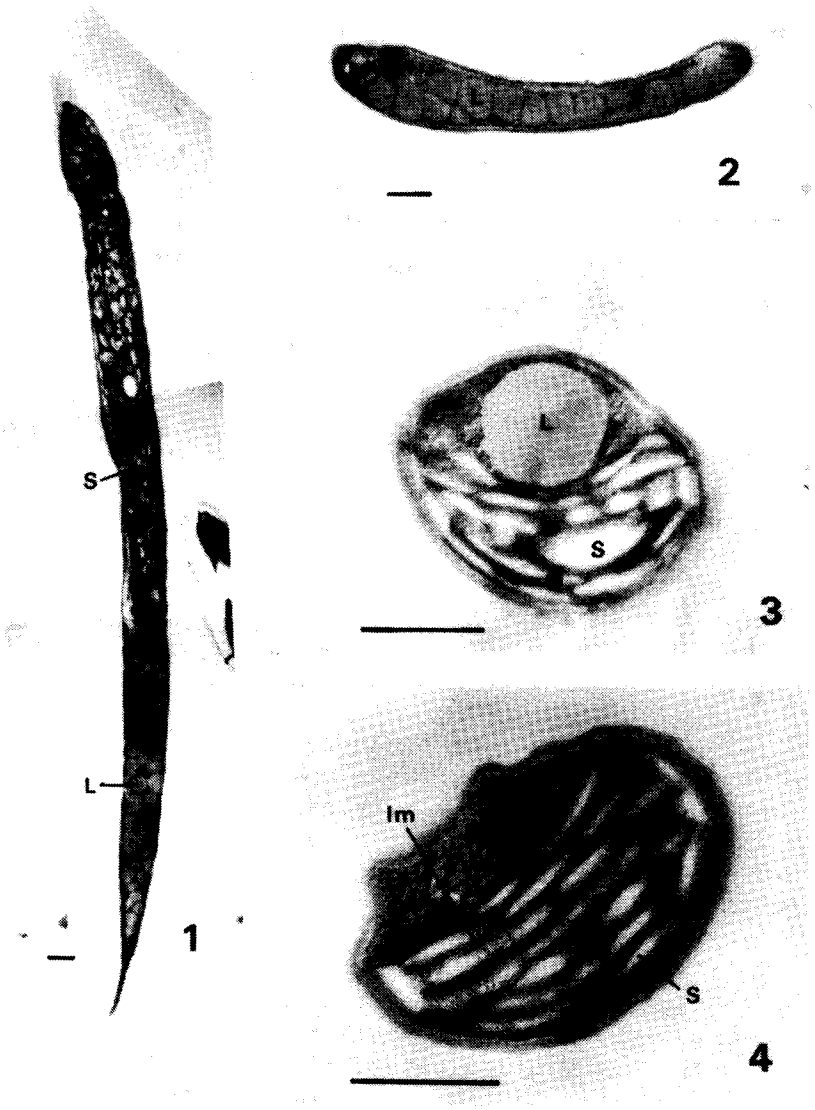
Preparative Procedures

Five different fixation and embedding procedures have been used (Table 1) with varying degrees of success, depending on the algal species. Thin sections (80 to 100 nm thick) were cut on a Sorval MT-2 ultramicrotome, using a Dupont diamond knife. Sections were poststained with either aqueous or methanolic uranyl acetate and lead citrate and were examined with a Zeiss EM 9S-2 (60 kV operating voltage), Hitachi H-600 (100 kV) or, in the majority of cases, a JEOL JEM 100CX (100 kV) transmission electron microscope.

Ankistrodesmus. Procedures 2 and 3 (Table 1) were used for this organism, with rather good results from procedure 2. The results of fixation 3 (Table 1) have not yet been evaluated. Figures 1-4 show several views of Ankistrodesmus fixed using procedure 2. Lipid bodies are plentiful, although, judging from the lack of electron density, much of the lipid has been removed, probably during the dehydration procedures. The cytoplasm is rather uniform and dense and the chloroplast lamellae are orderly, although distended by starch granules (Fig. 4). Membranes surrounding these starch granules appear intact (Fig. 4). Overall, the preservation seems adequate, particularly if lipid retention can be increased.

Table 1. Summary of Preparative Procedures Used

Procedure number	Primary fixation	Duration	Secondary fixation	Duration	Temperature	Dehydration	Embedding
1	Glutaraldehyde added directly to culture medium, 1% final concentration	1.3 h	1% OsO_4 in sea water	1 h	Room	Acetone series on ice	Spurr's low viscosity resin
2	4% glutaraldehyde with 1% OsO_4 in phosphate buffer, pH 6.8	10 min	none		Room	Acetone series on ice	Spurr's low viscosity resin
3	2% glutaraldehyde with 2% OsO_4	15 min	0.5% OsO_4 in cacodylate buffer, pH 7.3	35 min	Room	Acetone series on ice	Spurr's low viscosity resin
4	2% glutaraldehyde in phosphate buffer, pH 8.0	45 min	0.5% OsO_4 in phosphate buffer, pH 8.0	1 h	4°C	Ethanol, propylene oxide on ice	Araldite/ EMbed 812
5	2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3, with 0.05% $CaCl_2$	45 min	0.5% OsO_4 in 0.1 M cacodylate buffer, pH 7.3	1 h	4°C	Ethanol, propylene oxide on ice	Araldite/ EMbed 812



Figures 1–4. Ankistrodesmus falcatus prepared by procedure 2.
Fig. 1. Longitudinal section (L.S.) of cell with abundant starch granules (S) and lipid bodies (L). Scale = 1.0 µm. Fig. 2. L.S. through lipid area, showing close packing of lipid globules (L). Scale = 1.0 µm. Fig. 3. Transverse section (T.S.) showing relationship of lipid (L) body to choloroplast with its enclosed starch granules (S). Scale = 0.5 µm. Fig. 4. T.S. showing fixation of cytoplasm, chloroplast lamellae (lm), and membranes around starch granules (S). Scale = 0.5 µm.

Isochrysis. Fixation protocols 1, 2 and 3 (Table 1) have been used for Isochrysis. Procedures 2 and 3, the simultaneous glutaraldehyde and osmium fixations, showed the best results (Figs. 5-11), with excellent preservation of pyrenoids, nucleus, mitochondria, Golgi apparatus, and membranes in general. The few lipid bodies found were also quite well defined in both procedures, but the cytoplasm was dense and uniform only with procedure 2. Considerable disorganization appears to be present in the chloroplasts (Figs. 5, 6, and 10), however, and in favorable sections, swelling of the plasmalemma is evident (Fig. 10). Procedure 1 yielded good preservation and spreading of the external layer of scales, but very poor preservation of cellular detail, particularly of the membranes (Fig. 12).

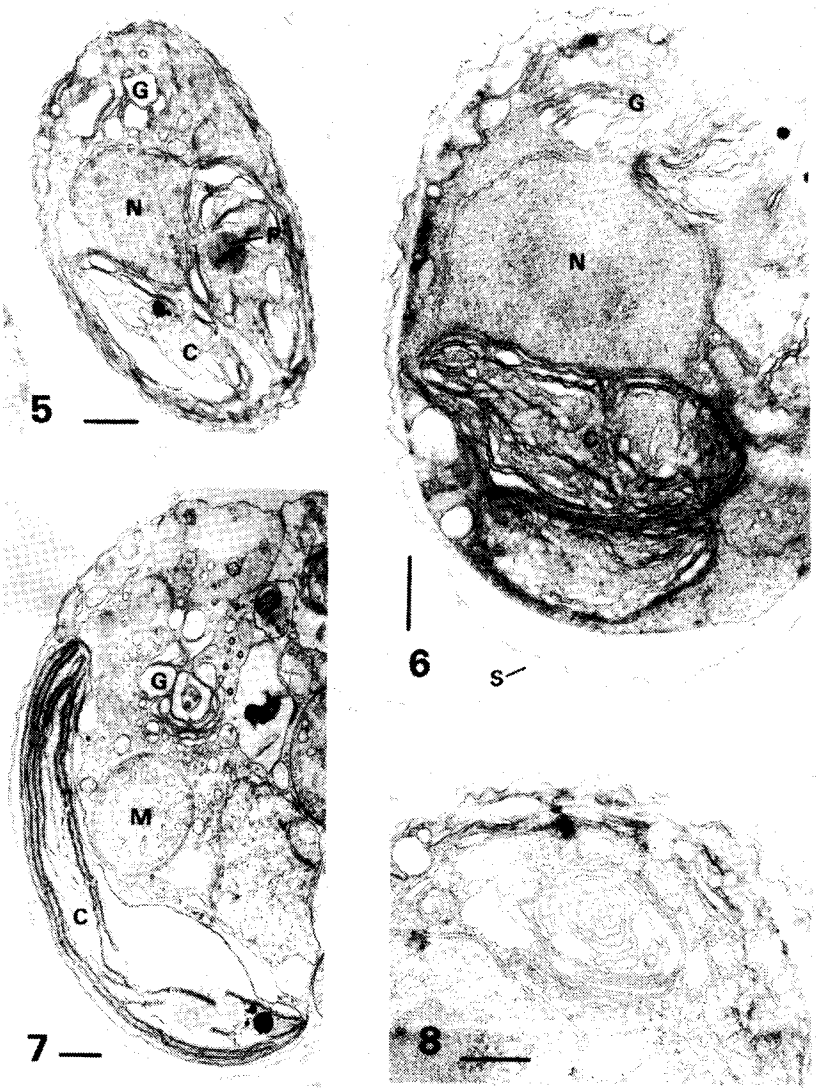
This poor preservation obtained using procedure 1 is probably due to the long duration of the fixation with lack of buffering. The occasional bulging of the plasmalemma and the disorganization of the chloroplast with the other two procedures could well be osmotic damage, because no attempt was made in the fixing procedures to duplicate the natural osmotic effect of the sea water in which they were cultured.

Nannochloropsis. All five procedures (Table 1) have been utilized for Nannochloropsis, but none has yielded acceptable results (Figs. 13-16). Other workers have noted similar difficulties in fixing Nannochloropsis, and indeed all of the Monodopsidaceae (Hibberd 1981, Lubian 1982). The most intractable problem at this point is the inadequacy of embedding. The cells seem to be very hard and were easily pulled from the epoxy resin. An occasional well-fixed cell (Fig. 16) was retained in the sections, but these were very few.

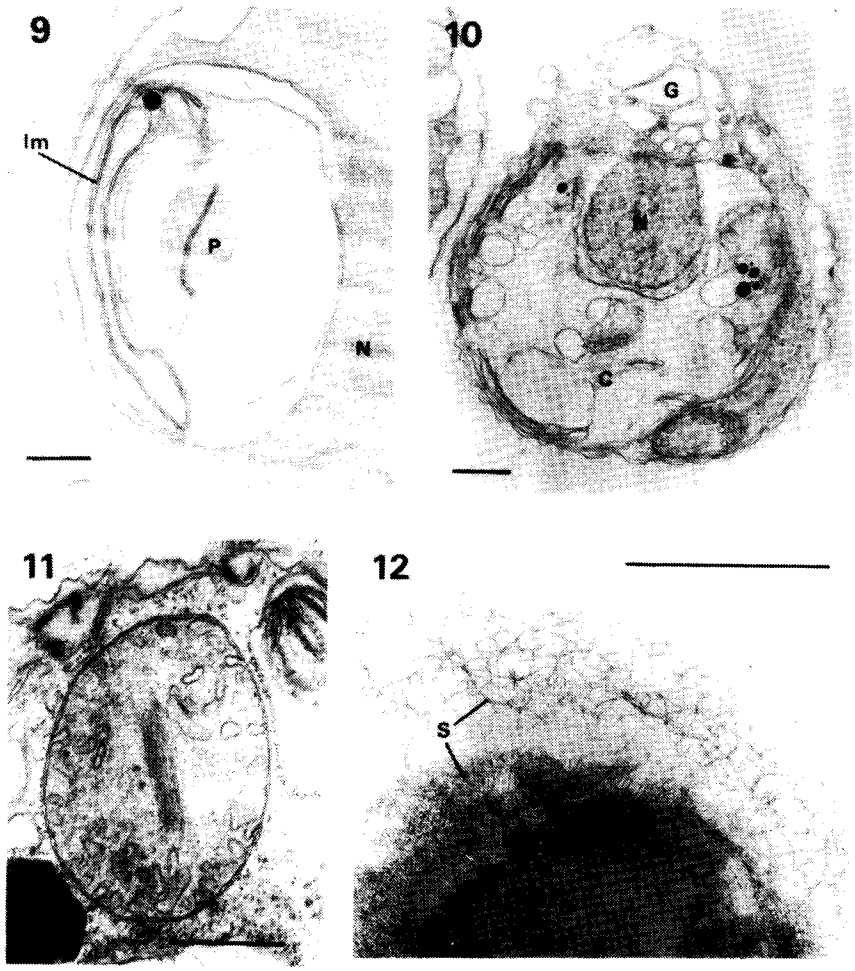
Fixations 4 and 5 (Table 1) were sectioned twice, with very different results. When sectioned two days after the completion of embedding, intact sections were obtained rather easily and many cells were retained in the plastic. Upon repeated sectioning several weeks later, however, the resin shredded and became completely unusable. Sectioning as soon as possible after polymerization may solve some of these problems.

Cells occasionally retained within the epoxy resin permitted some evaluation of the thoroughness of the fixation itself. Fixation 1 could not be evaluated because of the severe sectioning difficulty discussed above. The second procedure yielded exceedingly poor results, with sparse cytoplasm and few other organelles remaining within the cell. Procedures 3, 4 and 5 produced somewhat better results (Figs. 13-16). Lipid bodies can be seen packing the cells, although their limits are ill defined and much lipid has been extracted. In contrast to Isochrysis choloroplasts, however, the Nannochloropsis chloroplasts appear relatively well preserved, as do the mitochondria, but significant amounts of cytoplasm were lost in fixation 3.

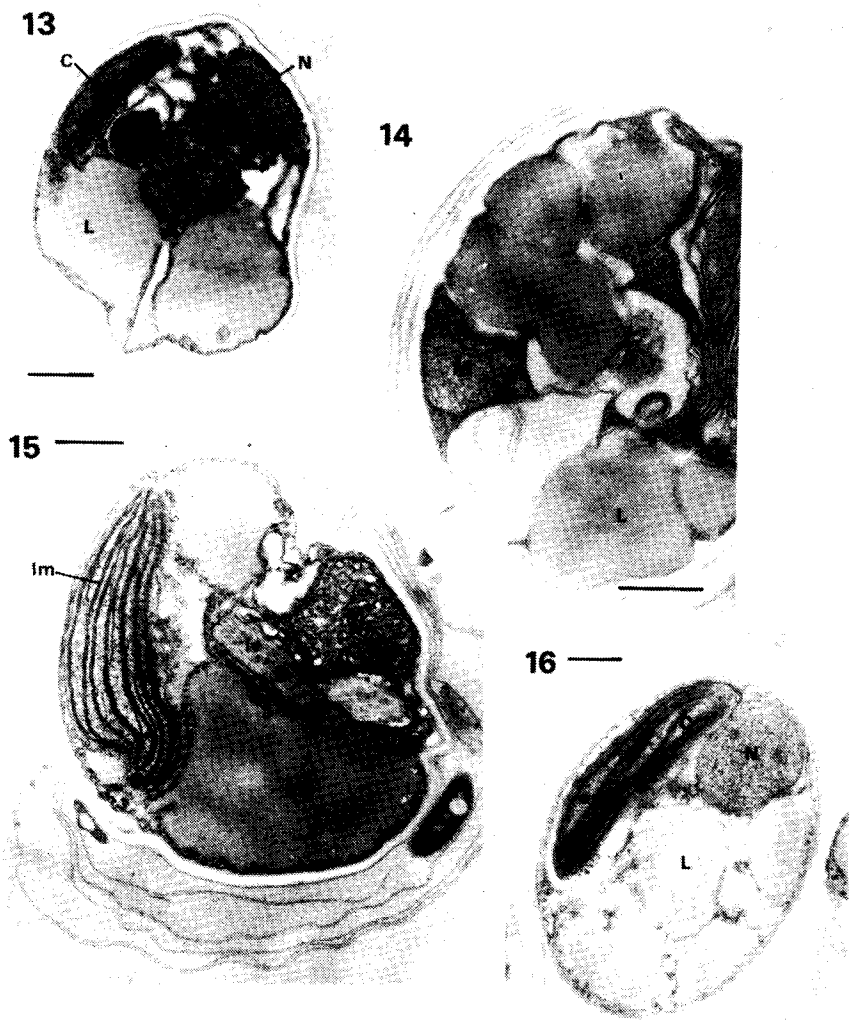
The majority of Nannochloropsis cells assumed an angular, presumably shrunken, shape (Figs. 13 and 15). This might indicate a problem in the



Figures 5-12. Isochrysis aff. galbana prepared by Procedure 2. Scale = 0.5 µm. Fig. 5. L.S. of cell showing good preservation of chloroplast (C) with pyrenoid (P), nucleus (N), Golgi apparatus (G), and cytoplasm. Fig. 6. L.S. of cell. Lamellae in chloroplast (C) appear disrupted. Golgi apparatus (G) and nucleus (N) are well preserved. Fig. 7. Although the mitochondrion (M), Golgi apparatus (G) and chloroplast (C) appear well preserved, the distended area (top) may indicate osmotic damage. Fig. 8. Golgi apparatus in face view.



Figures 9-12. Isochrysis aff. galbana prepared by procedure 2. Scale = 0.5 μm. Fig. 9. Pyrenoid (P) traversed by a two-thylakoid lamella. Three-thylakoid lamellae (lm) of chloroplast are well preserved. Fig. 10. L.S. showing osmotic damage at anterior end of cell containing Golgi apparatus (G). Chloroplast (C), partially enclosing nucleus (N), appears disrupted. Fig. 11. Mitochondrion, showing good preservation of detail. Fig. 12. The cell body has suffered severe damage, but detail of the scales (S) is clear, both in face view in transverse section.



Figures 13-16. Nannochloropsis salina. Scale = 0.5 μm.
Fig. 13. Although cell shape is distorted, lipid bodies (L), nucleus (N), and chloroplast (C) are intact. Procedure 5. Fig. 14. Portion of cell illustrating good chloroplast (C) and mitochondrion (M) preservation. Much lipid (L) has been retained. Procedure 4. Fig. 15. Angularly deformed cell with large lipid body (L) and well-preserved mitochondrion (M), chloroplast lamellae (lm), and lamellate food storage vesicle (lv). Procedure 3. Fig. 16. One of very few cells with reasonably good fixation. Procedure 5.

dehydration series, during which water is removed from the cells and may not be replaced with solvent. Careful monitoring of the cells by light microscopy at each step of the fixation, dehydration, and embedding procedure might provide insight into the cause of this shrinkage.

Other Progress. I have identified several steps that can be taken to improve the retention of lipids. Inclusion of 0.05% $CaCl_2$ is mentioned by Hayat (1981) as being useful, and both Boshier et al. (1984) and Boyles (1984) found that adding p-phenylenediamine in the 70% ethanol step of dehydration was the most effective of several treatments. Since most lipid extraction occurs during dehydration, particularly in the final stages (Hayat 1981), the duration of these last dehydration steps can be shortened or some steps can be eliminated. Many of these refinements were included in fixations 4 and 5 (Table 1), but unfortunately the embedding was inadequate to permit a full evaluation of their impact. These remedies will be included as appropriate in future work.

Interpretation of Ultrastructure

Cytological interpretation of the micrographs obtained, particularly when the cells are distorted by inadequate preservation, is facilitated by comparison with ultrastructural studies already available in the open literature. I have surveyed the literature and find, unfortunately, that few cytological studies are available on any of these organisms. Pertinent publications of which I am aware include Antia et al. (1975), Lubian (1982) and Pickett-Heaps (1972). I am currently broadening my search to include closely related organisms.

FUTURE RESEARCH

Task 1

Once adequate fixation procedures have been identified, work will begin on evaluating ultrastructural changes as lipid accumulation proceeds. This will be done in collaboration with the Georgia Institute of Technology (GIT), where chemostat culturing methods have been developed and can be used to trigger lipid accumulation in a dependable manner (T. G. Tornabene, Georgia Institute of Technology, personal communication to J. A. Solomon, Nov. 26, 1984). GIT personnel will perform the fixation and embedding of cells in known stages of lipid accumulation, and the polymerized blocks will be sent to Oak Ridge for sectioning, staining, and examination. At the time of sampling, additional samples should be taken for the determination of lipid, protein, and carbohydrate content, so that ultrastructure can be correlated accurately with metabolic state.

Currently, fixation procedures are adequate for Ankistrodesmus, nearly adequate for Isochrysis, and in much doubt for Nannochloropsis.

Task 2

In cooperation with Dr. Reinhold Mann of the Biology Division, Oak Ridge National Laboratory, I will apply flow cytometry technology to nitrogen-deficient cultures of the three species of microalgae mentioned above. Due to its greatly elongated morphology, Ankistrodesmus is the least promising of the three species, since the methodology is most appropriate for spherical particles. If any separation can be achieved based solely on chlorophyll autofluorescence, the resulting cells will be cultured and then sent to GIT for determination of lipid content. In addition, I will continue to seek an appropriate lipid-specific vital stain for use with this method.

ACKNOWLEDGMENT

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CHEMICAL PROFILES ON MICROALGAE WITH EMPHASIS ON LIPIDS

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ABSTRACT

Both suspended culture and immobilized cell reactors were constructed during this project. In the suspended culture reactor a batch growth experiment followed cellular lipid, protein and carbohydrate accumulation during nitrogen limitation of an Ankistrodesmus culture as a function of light intensity and CO_2 tensions (0.5%, 0.04% and zero) in the gas phase. Highest lipid content (24%; dry weight basis) was observed with the zero CO_2 culture. Low carbohydrate content, low cell density and higher protein content of the culture suggests that the increased lipid content was not due simply to higher lipid productivity.

The principal lipid of nitrogen starved Isochrysis spp. was identified as C35 - C39 alkenones. Other algae being cultivated for studies of lipid biosynthesis, and progress in analyses of chemical contents of samples submitted by other investigators in the Aquatic Species Program are described.

INTRODUCTION

The purpose of the SERI/DOE program is to identify algae that have the highest lipid (oil) production capabilities and to evaluate the economics of the microalgal products as a fuel. The research project at the Georgia Institute of Technology in the SERI/DOE program is to provide analytical services for determining the specific chemical compositions of the micro- and macro-algae identified by other researchers in this program. The program at Georgia Tech is in its third year. Results of the research have been published in Enz. Microb. Technol. (1), Eur. J. Biochem. (2), J. Phycology (3), and Marine Ecology (4). In addition to providing analytical services, the research for this year is directed towards developing cultivation strategies for lipid production and for investigating the metabolic relationships between the protein, carbohydrate and lipid biosynthesis during nitrogen or silicon deprivation.

OBJECTIVES

The specific objectives are:

- Identify the structure of the C-37 compound(s) of Isochrysis.
- Identify the major lipid fractions of "Nanno Q".
- Determine relationship between protein-carbohydrate-lipid biosynthesis of microalgae.

- Culture 5-6 promising microalgae and identify lipid compositions.
- Identify and quantify the chemical composition of micro-and macro-algae submitted by other members of Aquatic Species Program.

MATERIALS AND METHODS

Organisms

Algal strains and their origin are summarized in Table 1.

Cultivation

Ankistrodesmus was cultivated in the growth medium given in Table 2. All other organisms were cultivated in GPM medium as described in SERI culture collection catalog supplemented with 6mM $NaHCO_3$. Stock cultures were maintained in 50 ml of media in 125 ml or in 1 liter quantities in shallow culture flask on a shaker table in an environmental room held at 25°C and continuous illumination. The shaker table cultures were transferred to Roux bottles (800 ml medium) kept in the dark overnight and then subjected to a 14 hr:10 hr light:dark cycle. Light intensities were 800 $\mu E/M^2$ sec for a high light experiment or 300 $\mu E/M^2$ sec for low light experiments.

Cultures were mixed by magnetic stirring and air enriched with 1% CO_2. Temperature in the cultures cycled between 28-29°C in the light period and 24-25°C in the dark period. All nitrogen deficient cultures initially contained 0.5 mM NO_3 unless otherwise indicated and were inoculated with exponential phase cultures from Roux bottles.

Table 1. Stock Cultures

Organism	Date Received	Stock Culture	Shake Culture	Source
Chyrsochro-mulina strobilus	10-22-84	no	no	UTEX
LB 981	11-2-84	no	no	UTEX
Pavlova lutheri	10-22-84	no	no	UTEX
LB1293	11-2-84	yes	yes	UTEX
Chroomonas sp.	10-22-84	no	no	UTEX
LB 2007	11-2-84	no	no	UTEX
Chattonella japonica LB2162	10-22-84	no	no	UTEX
	11-2-84	no	no	UTEX

Roux Bottles Used in Lipid Induction Studies

Table 1. cont'd.

Organism	Date Received	Stock Culture	Shake Culture	Source
Dicratoria inornata LB 988	10-22-84	no	no	UTEX
	11-2-84	no	no	UTEX
Pseudoisochrysis paradoxa LB 1988	10-22-84	yes	yes	UTEX
Pymnesium parva LB 995	10-22-84	yes	yes	UTEX
Chlorosarcinopsis halophila 2078	10-24-84	yes	yes	UTEX
Isochrysis galbana LB 987	10-22-84	yes	yes	UTEX
Rhodomonas sp. 2163	10-22-84	no	no	UTEX
Tetraselmis seucica WTET	10-15-84	yes	yes	SERI
Thalassiosira pseudonana 3H	10-15-84	yes	yes	SERI
Nanno-Q				
0938	11-9-84	yes	yes	Dr. Lewin
0902	11-9-84	yes	yes	Dr. Lewin
0948	11-9-84	yes	yes	Dr. Lewin
0954	11-9-84	yes	yes	Dr. Lewin
Chlorella pyrenoidosa 26	11-20-84	yes	yes	Micro. Prod.
Scendesmus obliquus 1450	11-20-84	yes	yes	Micro. Prod.
Tahitian-Isochrysis	12-26-84	yes	yes	SERI
Chaetoceros gracilis	1-2-85	yes	yes	SERI

Table 1. cont'd.

Organism	Date Received	Stock Culture	Shake Culture	Source
Cyclotella DI-35	2-1-85	yes	yes	Dr. Tadros
Hantzschia DI-160	2-1-85	yes	yes	Dr. Tadros

Table 2. Ankistrodesmus Medium

Component	Concentration
NaCl	56.4 mM
$NaHCO_3$	20.0 mM
$CaCl_2$	0.25 mM
KCl	3.3 mM
$MgCl_2-6H_2O$	2.5 mM
Na_2SO_4	1.5 µM
$FeSO_4-7H_2O$	70.0 µM
Na_2EDTA	70.0 µM
K_2HPO_4	1.0 µM
Biotin	0.5 µg/ℓ
Vit. B_{12}	0.5 µg/ℓ
Thiamine	0.1 mg/ℓ
KNO_3	0.5-5.0 µM
Trace Metals Mixture (TMM)	0.5 ml/ℓ
TMM (concentration in media):	
Mn	0.50 ppm
Mo	0.10 ppm
Zn	0.05 ppm

Table 2. (cont'd)

Component	Concentration
Cu	0.02 ppm
B	0.50 ppm
V	0.01 ppm
Co	0.01 pm
Ni	0.01 ppm
Cr	0.01 ppm

Analytical

Cell suspensions were extracted for lipids by the modified method of Bligh and Dyer (5,6). Extracted cell debris was saponified in NaOH-MeOH, and extracted with petroleum ether. The nonpolar and polar lipids were separated by silicic acid column chromatography (7) with hexane, benzene, chloroform, acetone and methanol. Polysaccharides were extracted from dried cells by the phenol-water method (8) and isolated by isopycnic density gradient ultracentrifugation. Lipid components were deacylated by mild alkaline methanolysis (7). Samples were hydrolyzed with 2 N HC1 at 100°C for 2 h for neutral sugars; and 4 N HC1 for 6 h at 100°C for amino sugars. Lipids were analyzed by thin-layer chromatography in diethyl ether-benzene-ethanol-acetic acid (45:50:2:0.2) as first solvent and hexane-diethyl ether (96:4) as second solvent or chloroform-acetone-methanol-acetic acid-water (50:20:10:10:5). Lipids were detected by exposure I_2 vapor, acid charring, phosphate spray (10), ninhydrin, α-napthol for glycolipids (9), Draggendorff stain for quartenary amines (9), or H_2SO_4-acetic acid (1:1) for sterols (9). The deacylated water-soluble products were separated on cellulose-TLC plates (11). The samples were visualized by the o-tolidine staining method overstained with an acidic ammonium molybdate solution (7), or by ninhydrin. Total carbohydrates were determined by the phenol-sulphuric acid method after hydrolysis in 1 N HC1.. Nucleic acids were estimated spectrophotometrically after hydrolysis with 1N NaOH. Total protein was determined after hydrolysis in 1N NaOH at 100°C for 1 h (12). Chlorophyll and carotenoids were assayed as described by Jensen (13). Fatty acids were converted to the methyl ester form in a 2.5% anhydrous methanolic-HC1 (9). Aliquots of fatty acids were hydrogenated with H_2 and 10% Pd on activated charcoal. Free sugars were converted to alditol acetates (14). Derivatized components were analyzed on a Varian 3700 gas-liquid chromatograph (GLC) equipped with dual flame ionization detectors and a Varian Vista 402 data system. The analyses were as follows: 30 m x 0.252 mm fused quartz capillary column with 0.25 μm of OV 351 or 0.25 μm of DB-5 at 8 psi of He and 4°C/min from 125°C to 220°C and held isothermally; 2 m x 0.31 cm glass column packed with 10% SP2330 on 100/120 Gas chrom W AW at 27 psi of He and 4°C/min from 110°C to 250°C and held isothermally.

RESULTS

Culture Apparatus and Experimental Protocol

A culture apparatus was developed to culture species efficiently and for the purpose of determining the relationsip of carbohydrate accumulation and subsequent lipid synthesis when cells are placed under nitrogen deficient conditions. High productivity requires light limitation which precludes nitrogen limitation (only one nutrient can be limiting at one time). It is clear that in the past many experiments in which nitrogen limitation was to be achieved failed, within the time restraints imposed for productive cultures, due to the fact that dense (light limited) cultures were used. Such cultures continued to grow (arithmetically) and depleted media nitrogen and reduced cellular nitrogen, however, nitrogen limitation only slowly resulted in reducing pigments or in storing carbohydrates and/or lipids. Thus, nitrogen limitation must be induced under conditions of relatively high light which implies low density and, therefore, productivity. It is possible to separate the growth and induction (nutrition limitation) phases of the experiments by using different light intensities, densities and/or reactor designs for these phases. Thus, productivity can be maximized even with nutrient deficient cultures.

It was concluded that continuous cultures are not appropriate to maximize induction of products in response to nutrient limitations because of the requirement of long detention times that would be necessary for product formation by nutrient limitations. Simple batch cultures are the most effective way to study nutrient limitations. The effect of continuous cultures can be easily achieved in batch cultures by using a relatively concentrated nutrient feed - which would not significantly dilute the culture. Thus, in this project, only batch cultures are used.

The actual design of the laboratory reactor for growth of suspended cultures requires relatively little sophistication. Roux bottles are an effective way of operating batch cultures; they provide a relatively thin culture vessel (e.g. relative high light intensity) and a sufficient volume to supply adequate samples for analysis. Relatively high light intensities can be obtained by an assembly of fluorescent light boxes which provide the equivalent of about 35-40% of full sunlight Results of growth experiments with these vessels are reported in the next section.

For larger cultures, and to allow better control over mixing, a 1.8 liter culture apparatus was constructed containing a stirring rod with two sets of 4 glass paddles.

Immobilized algal cultures was proposed as an alternative to liquid cultures. The initial design carried out under this contract involved a flat plate system filled with porous plastic ("Porex") substratum. However, this design proved to be very difficulty to seal, with leaks developing repeatedly. Thus, the reactor was redesigned. It consisted of an array of parallel tubes loaded with alternating hydrophilic and

hydrophobic tubular substratum sections (approx. 10 cm in length) of various pore sizes. Ankistrodesmus was loaded on the reactor and attachment was visually observed. Only at the lower side of the substratum (shaded area) did algae cells accumulate. Neither pore size nor hydrophobic/hydrophilic properties had any pronounced effect. Photo-inhibition appeared to be a major design constraint and will need to be investigated. At present a simplified version consisting of two 103 cm x 3 cm (I.D.) glass tubes with a central core (approx 2.6 cm) of plastic substratum is being operated. The support consists of a hydrophobic plastic material (Porex, Corp.) with average pore dimensions of 100 μm. It is currently being tested with Chaetocerus.

The Roux bottle system was selected for time course relationship study of chemical class productivity (see below). The initial experimental protocol was to study Ankistrodesmus, because of the prior years reported data (see last years final report) which showed up to 40% lipid production. In the initial experiments, the protein, carbohydrate and lipid content was to be determined for up to one week following nitrogen starvation.

Relationship of growth stages and chemical composition

Studies have begun to determine the time course relationship between nitrogen deficiency, carbohydrate accumulation, and subsequent lipid synthesis in four representative strains of microalgae. The specific objective is to provide an understanding of the mechanism that leads to the triggering of lipid synthesis in microalgae.

The first experiment was with Ankistrodesmus. The analyses indicated that only 10 to 16% lipids on cell dry weight basis was produced and that there was no apparent correlation with nitrogen limitation. The relatively low levels of lipids even after nitrogen limitation were contrary to the 40% lipid content observed in previous work (see last year's annual report) with Ankistrodesmus grown in shaker cultures. Thus, the next experiment was designed to test some alternative parameters that could account for this difference. Light and carbon supply were considered possible factors since they were relatively limiting in shaker cultures. Conditions of low, high, and no carbon supply and high and low light were investigated.

Table 3. Chemical Composition of Ankistrodesmus in Nitrogen Limited Media

		Relative Percentage			
Treatment[1]	Dry Weight mg/	Protein	Carbohydrate	Total Lipid Extract[2]	Lipid[3]
HC, HL	750	10.8	26.0	26.0	11.2
AC, HL	630	10.1	46.1	26.3	12.0
AC, LL	580	11.4	41.4	29.8	16.0
NC, HL	390	14.8	28.9	37.7	23.9

1. HC=High CO_2 (1% in Air), AC = air
   NC=Negligible CO_2 (NaOH scrubbed)
   HL=High light (800 $\mu E/M^2 s$), LL = Low light (300 $\mu E/M^2 s$)
2. Fraction soluble in $CHCl_3$:MeOH:H_2O
3. Fraction soluble in chloroform

The results of this experiment are shown in Figure 1 and Table 3. While there was very little change in protein levels, both carbohydrate and lipid yields were altered. Cells grown on air at both high and low light intensities had elevated carbohydrate levels; while one culture grown in the absence of CO_2 (NaOH scrubbed air, pH 10) had a carbohydrate level comparable to a 1% CO_2 case, but yielded a higher lipid fraction. It is important, however, to consider these results in terms of overall culture productivity of lipid (Table 3). On this basis, the treatment effects are not that significant. This of course being due to the net reduction of biomass (particularly for the CO_2 scrubbed case - see Fig. 1). Thus, the almost 50% decrease in biomass in the absence of CO_2, as compared to the 1% CO_2 case, over the one week after nitrogen limitation sets in, would account for the two fold differential in lipid content between these cultures, without requiring additional lipid biosynthesis. If it is assumed that lipids are not significantly oxidized (respired) in this time frame, or, at least, that no net oxidation took place. Preferential respiration of carbohydrates, in absence of CO_2 would account for the relatively low value as well as the higher protein content and ash level (11.5% vs 5.5% for the other cultures, respectively). Thus, in conclusion, this experiment does not demonstrate that lipid biosynthesis has been "triggered" under these conditions.

The next experiment with Ankistrodesmus (currently in progress) involves the switching of the nitrogen limited cultures from low to high lights and the supply of low levels of nitrate to nitrogen limited cultures. The latter experiment is to determine whether biosynthesis of lipids could be restricted by enzyme biosynthesis. Small amounts of nitrate will not allow significant cell growth, but could allow biosynthesis of enzymes induced under nitrogen limitation.

Finally, Isochrysis, Tahitian strain, has also been grown in Roux bottles and subjected to nitrogen limitation. The nitrogen limited cultures produced large amounts of extracellular material as well as soluble pigmented compounds. Analysis are in progress.

Future work will concentrate on the diatoms isolated by Dr. Tadros, Cyclotella and Hantschia.

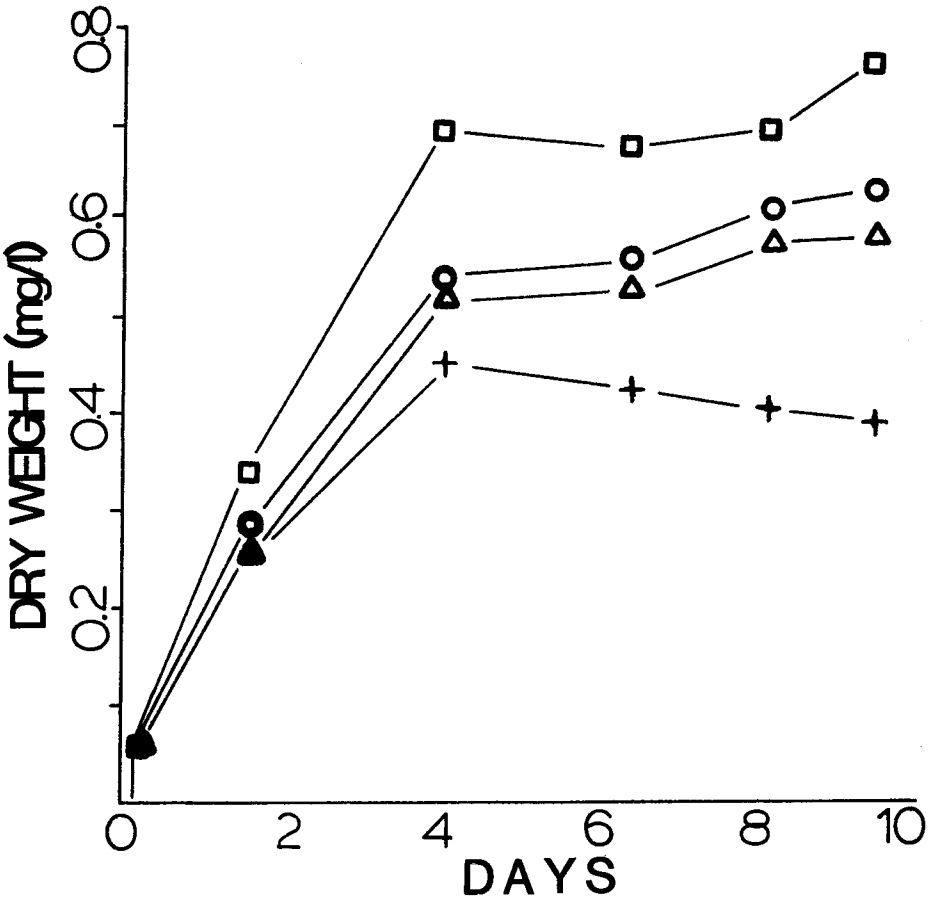


Figure 1

Nitrogen deficient batch cultures of Ankistrodesmus in Roux bottles.

Fluorescent light boxes provided 35-40% of full sunlight; ◘ = HC, HI; ● = AC, HI; ▲ = AC, LL; and + = NC, HI. See Table 1 for abbreviations.

Identification of the C37 Compounds of Isochrysis

A study on the chemical composition of representative species of freshwater, desert and marine unicellular eukaryotic microalgae grown under controlled conditions was initiated in August 1982 under the SERI contract. The research progress was first reported in the March contractor's review meeting (1983) in San Diego. We reported on the chemical composition of seven microalgae species of the genera Ankistrodesmus, Dunaliella, Nannochloris, Isochrysis, Botryococcus and Nitzchia. The final results of this study was relatively complete with the exception of the elucidation of the major lipid component of Isochrysis. The major component isolated in the benzene eluate collected from a Unisil (silicic acid) column represented 4% of organic cell weight and resulted in a single spot on a thin-layer chromatogram (Rf 0.27, with petroleum ether-diethyl ether-acetic acid, 90:10:1). The preliminary report to the March 1983 Contractor Review Meeting tentatively identified the unknown as "an oxygenated cyclic C-37 isoprenoid compound the exact identity of which had not been fully ellucidated". We can now report that the structure of the major benzene eluate component has been elucidated.

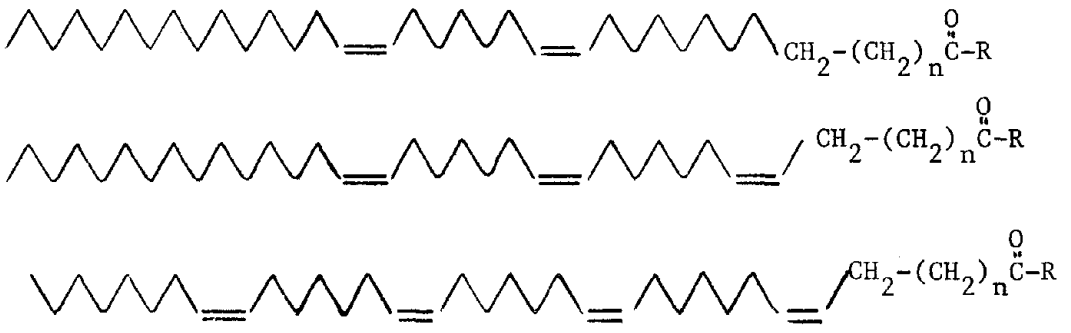
In the beginning of the analyses, it was observed that high-resolution capillary gas chromatography was unable to resolve what appeared to be a family of polyunsaturated isomers. Thus, the sample was hydrogenated with hydrogen gas and palladium on charcoal. The reduced sample then consisted of 3 major component types with molecular mass ions of M-506, M-534, and M-562. Each component had strong M-18 and m/e=59 identifying the components as C35, C37, and C39 alcohols, each with one unsaturated position. Because the sample was hydrogenated it appeared that the absence of 2 atomic mass units was not the result of incomplete saturation but an indication of a ring structure.

In an attempt to further reduce the samples, the components were hydrogenated with $H_2/PtO_2/HClO_4$. This condition should assure the reduction of the unsaturate position, if present, and deoxygenate the compounds to hydrocarbons. The following major compounds were obtained:

M=506--C35 alcohol with one unsaturation
M=534--C37 alcohol with one unsaturation
M=508--C35 alcohol
M=536--C37 alcohol
M=492--C35 hydrocarbon
M=520--C37 hydrocarbon
(C39 compounds in the preparation were in relatively small quantities and were not analysed).

The above mixture of compounds were obtained because the reductive reaction was not quantitative. The C37 components were still the major compounds in the sample. H-NMR analysis of the sample indicated one isopropyl group, a high proportion of CH_2 groups, and a low proportion of CH_3 groups indicative of a tertiary-hydroxyl group. Thus, this preliminary data supported a presumption that the princi-

pal compounds were unsaturated C35-C37 oxygenated cyclic hydrocarbons. Definitive identification was not possible until the various compounds in the original sample were obtained in purified forms and independently analyzed. This was recently accomplished with an experimental super critical fluid chromatography system. The analyses of the purified component clearly indicated that the principal compounds were not cyclic alcohols but instead acyclic unsaturated ketones of the following 3 types for each of the C35, C37, and C39 structures.



$$R = CH_3 \text{ or } CH_2\text{-}CH_3$$

Reevaluation of the previous mass spectral and NMR data obtained for unpurified samples supported the structures given above except for the interpretation of the data that indicated the presence of the OH group and ring structure. Ketones are reasonably stable and should not be reduced by H_2/Pt. Recent tests with standard ketones and especially unsaturated ketone forms showed that, in fact, ketones are converted to hydroxylated cyclic and acylic unsaturated hydrocarbons. Thus, our initial reduction experiments complicated our study by creating artifacts. After the identity of the compounds were established, a literature search was conducted for evidence of their novelty in algae and/or sediments.

In 1979, 1980, and 1981, Volkman, et al. (15-17) reported the results of their study on lipid composition of representatives of the Prymnesiophyceae (Haptophyceae). Their investigation was designed to provide information on the food sources of higher marine organisms and the origin of lipids in sediments. They found long chain alkenones, esters and sterols in Emiliania huxleyi and lipid distribution difference among the genera that distinguished the taxonomic orders in Prymnesiophyceae. The identity of the long chain alkenones, esters and sterols and their distribution in the taxonomic orders were published in September 1984 issue of Br. Phycol. J. by Marlowe et al. (18). The alkenones and esters occur in Chrysotila lamellosa, three species of Isochrysis, and Emiliania huxleyi. They were absent from 5 other members of the order Isochrysidales, and the orders Coccosphaerales, Prymnesiales and Pavlovales.

In addition to the reports of Marlowe et al. (18) that three species of Isochrysis contained C37 and C39 alkenones as the principal lipids, this study identified C35, C37 and C39 alkenones in both I. galbana and I. galbana Tahitian strain (Tiso) as the principal lipids in total lipid extracts from cell cultivated in limited nitrogen media.

Evaluation of Nanno Q as a potential oil Producer

It was purported that isolates of Nanno Q were seemingly prolific oil producers (R. Lewin, personal communication). The following lines describe the status of evaluating the fuel potential of this organism.

Cultivation of Nanno Q at Georgia Tech is underway in both shake culture flask and Roux bottles as described earlier in this text. None of the samples prepared in our laboratory have yet been analysed. The only analyses of lipid composition of Nanno Q performed thus far have been on cells obtained from nitrogen sufficient medium that were cultivated by Ralph Lewin. Quantitation on a cell dry weight basis was obtained only for the nitrogen efficient cultivated cells. Only the relative intensities of the lipid classes (polar/non-polar) was determined for the nitrogen deficient cultivated cell. Sufficient sample size was provided by William Barclay and quantitation was possible whereas the nitrogen deficient cultivated cells were provided as a small quantity of dried cells impregnated on a cellulose acetate filter. The nitrogen sufficient cultivated cells contained 19-27% lipids, on a cell dry weight basis, for three different sample preparations.

The results of preliminary lipid analyses for fatty acids and glyceride lipids are described in Tables 4-6. The hydrocarbon composition consists of both isoprenoid and nonisoprenoid hydrocarbons. The ratio of hydrocarbons to glyceride lipid in nitrogen deficient cultivated cells was estimated to be 1:1, a ratio comparable to laboratory cultivated Botryococcus. The difference between the two organism is the relatively much faster generation time of Nanno Q.

Further detailed analyses of the lipids has been delayed until controlled sample preparations (radioactively labeled with 32-P, 35-S and 14-C) from the in-house cultivated cells become available.

Table 4. Percentage Fatty Acids of Free Lipids of Nanno Q

Identity	Nitrogen Sufficient	Nitrogen Deficient
14:0	0.61	1.3
14:1	0.19	0.3
15:0	0.18	0.6
16:0	21.60	43.4
16:1	26.11	29.6

Table 4 (cont'd)

Identity	Nitrogen Sufficient	Nitrogen Deficient
Br-17	0.78	0.3
17:0	0.26	0.2
18:0	0.89	2.2
18:1	10.34	15.6
18:2	0.11	---
18:3	0.64	---
19:0	0.15	---
20:3	12.44	2.1
20:4	25.67	4.4

Chemical characterization of five Microalgae

To complete the taxonomic survey of chemical characteristics of selected microalgae, five potentially promising algae will be selected, cultivated, and chemically characterized at the Georgia Tech laboratories.

Microalgal cultures obtained thus far are listed in Table 1. Both their culturing status and source are designated. Development and cultivation parameters are being tested. As soon as larger scale cultures are in hand, test of more organisms will begin.

Culture Screening for Members of Aquatic Species Program

Micro- and macro-algae will be analyzed for the quantity and nature of the chemical constituents using samples submitted by other investigators in the Aquatic Species Program.

The following subcontractors are to submit samples for chemical analyses:

	# strains	# samples
William Thomas (Scripps)	10	20
William Barclay (SERI)	6	12
John Ryther (Ft. Pierce)	6 macroalgae	12
John Ryther (Ft. Pierce)	2 microalgae	2
Ralph Lewin (Scripps)	3	6

As of February 19, 1985, two microalgal strains (4 samples) and ten macroalgal samples were received from William Thomas and John Ryther, respectively. A small quantity of ANZAY (Chamydomonas sp.), isolated from desert soil was received from Ralph Lewin on February 4, 1985. He reported that "it produces spores full of an orange yellow oil." Because

Table 5. TLC of Neutral lipid components in Lipid Fractions of Nanno Q-Nitrogen Sufficient/Deficient

Hexane eluate	Benzene eluate
acyclic hydrocarbons (++++)	Carotenes +++ Steryl esters (++++) Ketones (?) (++) Triglycerides (+)
Chloroform eluate	**Acetone eluate**
Steryl Ester (+) Ketones (++) 1,3 Diglyceride (+) 1,2 Diglyceride (++)	Triglyceride 18 resolved pigments

Table 6. TLC of Polar lipid components in Lipid Fractions of Nanno Q-Nitrogen Sufficient/Deficient

Hex	Benz.	$CHCl_3$
None	None	None

Acetone eluate	Methanol eluate
5 resolved pigments	Phosphatidic Acid (+++)
Monogalactosyl diglyceride (++++)	Diphosphatidyl glycerol (++)
Diphosphatidyl diglyceride (++)	Phosphatidyl Ethanolamine (+++)
Phosphatidyl ethanolamine (+)	Phosphatidyl glycerol (+++)
Phosphatidyl glycerol (+)	Phosphatidyl choline (+++)
Sterol glycoside (++)	Digalactosyl digyceride (+++)
	Phosphatidyl Inositol (++)

Abbrev: ++++ major compound
+++ moderate
++ minor
+ trace

of limited sample size, only a chromatographic scan on a thin layer chromatogram was obtained. A pigmented neutral lipid was the principal compound in the lipid extract. Another cell preparation will have to be prepared before any further analyses can be performed. William Thomas' samples of Chlorella Ellipsoidea and Nannochloris sp. arrived on February 10, 1985. All of the C. ellipsoidea and Nannochloris sp. samples were greenish/black in appearance when they were harvested on December 6, 1984 (nitrogen sufficient) and December 14, 1984 (Nitrogen deficient) for Chlorella and January 17, 1985 (nitrogen sufficient) and February 1, 1985 (nitrogen deficient) for Nannochloris (Thomas, personnel communication). Based principally on the pigmentation of the samples it was presumed that the nutritional stress of the cells did not occur and that no carbon storage response was triggered. These samples were subjected to analysis to test this presumptive conclusion. No difference in lipid concentration on a cell dry weight bases was observed (Table 7) The C. ellipsoidea and Nannochloris sp. consisted of about 10% and 18% lipids, respectively. For nitrogen deficient cultivated cells; the protein dropped slightly with a concommitment rise in carbohydrate. Even though nitrogen depletion had been measured in the culture medium by Dr. Thomas, the cultures were apparently not yet in a "metabolic stressed" (nitrogen limited) state.

Table 7. Approximate Chemical Composition*

	Relative Percentage		
Samples	Protein	Carbohydrate	Lipid
Chlorella (N-suff)	34.2	20.5	10.9
Chlorella (N-def)	26.1	26.3	8.9
Nannochloris (N-suff)	15.2	28.6	19.7
Nannochloris (N-def)	9.8	38.1	16.9

*% of cell dry weight

John Ryther's ten samples were received on February 18, 1985. The samples are Gracilaria tikvahiae and Ulva lactuca (N-low), and Gracilaria, Ulva and Caulerpa prolifera grown at temperature of 10 C (low-temp.), and companion control samples (N-high, Normal temperature). Lipid composition studies of the three macroalgae and detailed carbohydrate analyses of two of the macroalgae had previously been performed. The samples were carbohydrate rich and lipid poor. These studies will be repeated on these recently received samples with emphasis on the carbohydrate moeities.

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THE ENERGETICS OF BIOMASS AND LIPID PRODUCTION BY LIPOGENIC MICROALGAE UNDER NITROGEN DEPRIVATION

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Introduction

A technical-economic analysis on the mass cultivation of microalgae for the production of liquid fuels has recently been completed (Hill et al. 1985). The results of this analysis indicate that a photosynthetic efficiency of 18% (based on photosynthetically active radiation, PAR) must be attained with 60% of the resulting biomass in the form of lipids in order for such microalgal systems to be considered as a cost effective fuel supply option. Although the projected values for energy efficiency and lipid content are subject to revision as new technical parameters and economic data become available, the current projection serves to emphasize the importance of maintaining a high overall biomass production efficiency. It also demonstrates the desirability to be able to selectively enhance the production efficiency of the primary end product, in this case, the storage lipids of the algal cells.

A maximum theoretical photosynthetic efficiency of 20-25% has been reported for microalgae (Goldman 1979) and actual production efficiencies of 8-12% have been reported for nutrient-sufficient dense cultures (Thomas et al. 1983). Under a nutrient-sufficient, exponential stage of growth, most species of microalgae do not exhibit high lipid contents. However, during the nutrient-limited, early stationary phase of growth, large increases in lipid content have been demonstrated. In several cases, the lipid content of nutrient-limited algae exceeds 50% of the total algal dry mass (Piorreck and Pohl 1984, Lien and Spencer 1983, Shifrin and Chisholm 1980). Frequently, a reduction in algal productivity has been noted for stationary phase cultures. However, a detailed examination of the energetic aspects of biomass and lipid production attending the transition from nutrient-sufficient to nutrient-deficient stages of growth has not been documented.

The objective of the research presented here was to determine the magnitude of change in the energy efficiency of microalgae as photosynthetically fixed energy was redirected from cell growth and reproduction to the synthesis of storage lipids in response to various lipogenic stresses. The energetic aspects of biomass and lipid production in three species of oleaginous microalgae, Chlorella sp. S01, a fresh water strain of Ankistrodesmus sp. and a newly isolated chrysophyte designated Chryso/F-1 were examined under laboratory conditions. Because of the profound impacts of the nitrogen supply on the physiological status and productivity of algal cells, the initial energetic analysis on the impacts of lipogenic stresses was concentrated on effects of nitrogen deprivation on these three oleaginous organisms.

Materials and Methods

Organisms and Growth Conditions. *Chlorella* sp. SO1 was grown in Bold's Basal Medium (BBM) containing 5 mM $NaNO_3$ and 0.1 M NaCl. NaCl was added to prevent cell clumping during logarithmic cell growth. *Ankistrodesmus* sp. was grown in BBM containing 5mM $NaNO_3$. Shaker cultures of these two green algae were maintained at 30°C under continuous light and were used as inocula for fermenter experiments. Chryso/F-1 was grown in Type II artificial inland saline water, 25 mmho cm^{-1} conductivity, supplemented with 1.7 mM urea as the sole nitrogen source under continuous illumination at 25°C.

Batch culture nitrogen depletion experiments were carried out in a 15 L New Brunswick Microferm fermenter equipped with a specially designed centrifugal-pump spray apparatus to prevent the accumulation of cells on the glass walls. Illumination around 360° of the cylindrical growth chamber was provided continuously (see below for details). Cultures were sparged with 2 to 5% CO_2 in air at 200 ml min^{-1} under constant and vigorous agitation by a stirring impeller operating at 360-400 revolutions min^{-1}.

Determination of Light Energy Input to the Culture. The light for the growth of the algal cultures used in these fermenter experiments was supplied by tubular fluorescent lamps (15 W, G.E. cool white). The spectral output of these lamps consists of 93.8% photosynthetically active radiation (PAR, 400-700 nm) (Figure 1). The light energy received by the culture was measured from the interior surface of the culture vessel with a calibrated Si-photodiode fitted with a cosine-correction dome. In the case of the fermenter experiments, the complex geometrical relationship between the light source and the culture volume requires a detailed mapping of the irradiance for the entire culture vessel (see Figure 1 for details). Figure 2A correlates irradiance as a function of culture volume. These data were used to calculate the total photon flux incident on the culture volume (Figure 2B), which decreased from a maximum of 13.5 L to a much smaller volume due to the withdrawl of samples for dry mass and lipid analyses. A time-dependent decay of the lamp output of the experiment was also corrected for using a previously determined lamp decay function. In addition, throughout the course of each experiment the penetration of light into the interior of the column was monitored with a fiber optic probe placed near the center of the fermenter vessel.

Analytical Methods. Dry cell mass was determined following filtration of culture samples (5-50 ml) onto 2.5 cm Whatman GF/C filters and drying to constant weight at 60° C. Subsequently, the same samples were heated at 500° C for 3-4 h to determine the ash content and the ash-free dry mass (AFD). Cell counting was performed on a Coulter model ZM electronic particle counter.

The chlorophyll and carotenoid content of *Chlorella* and *Ankistrodesmus* were

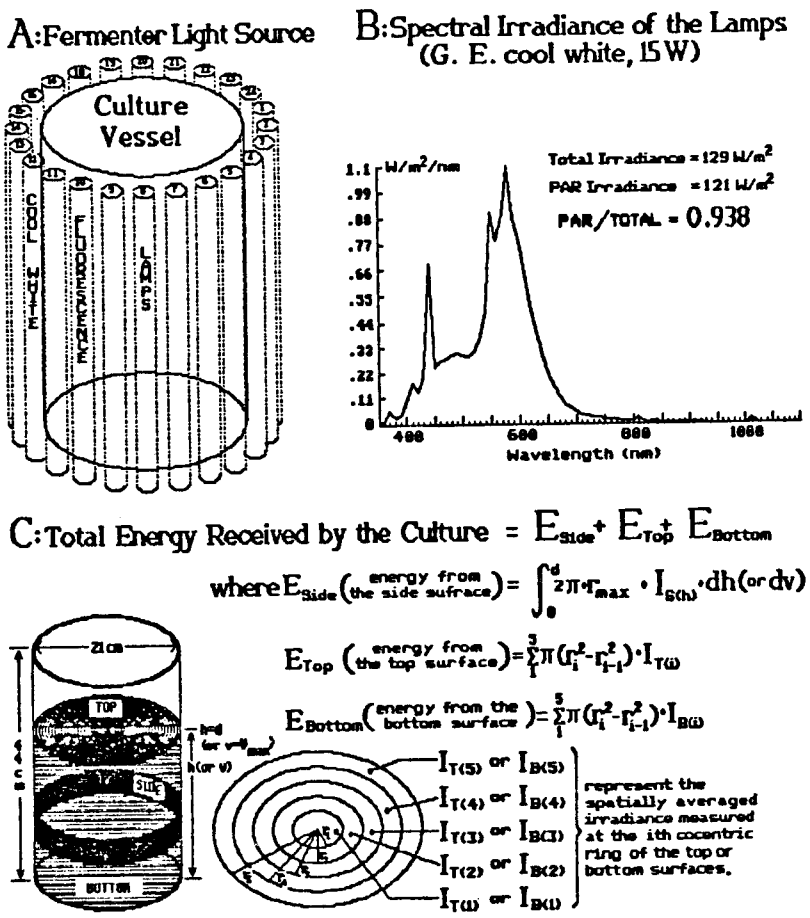


Figure 1. The light source and quantification of light input to fermenter cultures.
A. The spatial relation between the fermenter chamber and the light source. B. Spectral energy distribution of the fluorescent lamps showing that PAR contributes 93.8% of the total amount. C. Quantification of the light input to the culture is accomplished by summing the energy received from three surfaces of the culture: (1) the bottom (independent of culture volume), (2) the top (irradiance is dependent on the culture volume) and, (3) side surface (both irradiance and area are dependent functions of the culture volume).

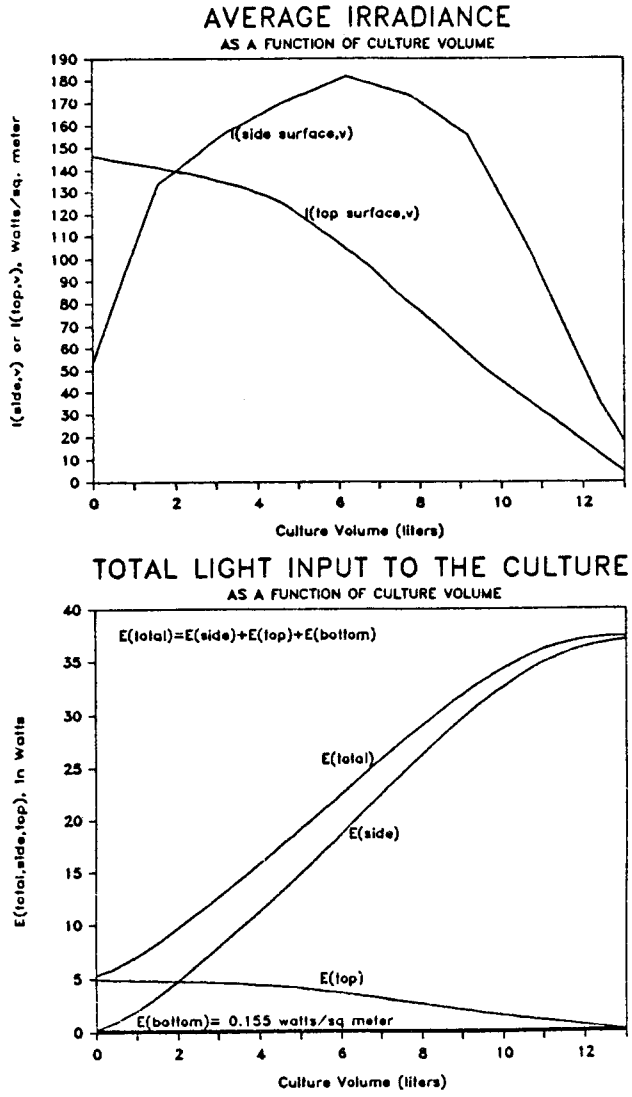


Figure 2. Spatially averaged irradiance and total light input to fermenter cultures.

determined after extraction into 95% ethanol. Spectra were obtained with a Hewlett-Packard 8450A or 8451A spectrophotometer. For chlorophylls *a* and *b*, the equations of Wintermans and DeMots (1965) were used. Pigments from Chryso/F-1 were extracted with 90% acetone. Chlorophylls *a* and *c1+c2* were estimated via the equation of Jeffrey (1972). For estimation of total carotenoids in the presence of chlorophylls, a normalized absorption spectrum of chlorophylls was subtracted from the absorption spectrum of the total algal lipid extract (Spencer and Lien 1985) and the extinction coefficients reported by Jensen (1978) were used to calculate the amounts of the extracted pigments.

Nitrate concentration was determined by ion chromatography of the culture filtrates. An anion exchange column (250 mm x 4.5 mm ID), was used in a Hewlett-Packard 1081 B liquid chromatograph with a Wescan conductivity detector. For nitrate determinations in the presence of high concentrations of NaCl, the sample was treated with $AgSO_4$ to precipitate chloride ions. Urea concentrations were determined colorimetrically with diacetyl monoxime according to the procedure of the Sigma Chemical Company (procedure 535).

For the extraction and quantitation of lipids, whole cells (20-150 mg dry mass) were harvested and washed by successive 5 min 300 rpm centrifugations. Alternatively, the cells were harvested and washed on Whatman GF/C filters. The pellets and/or filters were stored at -20° C. The samples were allowed to thaw at room temperature immediately prior to extraction with organic solvents. The samples were extracted twice with methanol (30 min at 60° C), followed by two additional extractions with chlorofrom-methanol (1:1 v/v). The crude lipid extracts were filtered through a silicic acid column or phase separated into the chloroform fraction from a chloroform-methanol-water mixture (1:1:0.9) and dried to constant weight for lipid mass determination. Neutral and polar lipids (including steroids) in the total algal lipid extracts were separated by chromatography on silicic acid columns with chloroform (Jensen 1978) for mass determination.

To determine the cellular content of storage carbohydrates (polysaccharides), cells from a known volume of culture samples were collected by centrifugation or filtration on GF/C filters and subjected to a mild acid hydrolysis (0.05 M H_2SO_4 at 60° C for 2 h). The mixtures were centrifuged for 10 min with a clinical centrifuge. Aliquots of clarified supernatant and of clarified culture medium from the same sample were treated with phenol-sulfuric acid to determine the amount of reducing sugar present using glucose as the calibration standard.

The calorific values of total algal dry mass, total algal lipids, polar and neutral algal lipids were expressed as the heat of combustion determined with a Parr oxygen bomb calorimeter. The algal materials used for the oxygen bomb calorimetry were obtained from axenic cultures of *Chlorella* sp. SOl in both nitrogen-sufficient and nitrogen-deficient media. The procedures approved by the American Society for Testing and Materials were followed: ANSI/ASTM D2382-76 (reapproved 1980) for liquid samples

(extracted total lipids and neutral lipids) and ANSI/ASTM D3286-77 for solid samples (dried algal cell mass and non-lipid algal materials obtained after extraction of lipids). In both cases, known quantities of Na-benzoate were used to standardize the energy equivalent of the calorimeter.

Determination of the Energy Efficiency. In batch-culture, nutrient-depletion experiments, the cultures were supplied with a limiting quantity of nitrate or urea as the sole source of nitrogen. After inoculating a 13 liter BBM medium (containing 5 mM of nitrate) with a 500-600 ml of log-phase culture of the indicated organism, aliquots of 200-500 ml were sampled at the indicated time. The experiments were terminated when the total mass density of the culture showed no increase over a 48 hour period. The nitrate concentration of the media, cell number, pigment concentration, total dry mass, ash content (and AFD), total lipids, and when indicated, neutral and polar lipids of these samples were analyzed. In addition the amount of nonlipoidal materials designated as carbohydrate + proteins (C+P) were estimated by subtracting the total lipid mass from the total dry algal mass of each sample. The amount of storage polysaccharides as well as the reducing sugar excreted into the medium were also monitored colorimetrically. A partial list of data is presented in Table 1 (collected from the fermenter experiment with C. sp. SOl) to illustrate the experimental protocols used to determine the productivity and energy efficiency of the culture. The data were used to calculate the total short-term production rates and cumulative amount of algal mass, in terms of ash free dry mass (AFD), lipids and other major cellular components (i.e. carbohydrates + proteins) of the culture. The energy content of the total algal mass was computed by summing the heat of combustion of the various cellular components using the values given in Table 2. Finally the short-term energy efficiency for the production of AFD and other cellular components was calculated by dividing energy associated with the net gain of each component material (of the entire culture corrected for the sampling loss) during each sampling period by the total light energy received by the culture over the same sampling interval.

Results and Discussion

General Culture Parameters and Mass Yields. The experimental data used in this report were derived from three separate runs of fermenter batch cultures of the oleaginous algae mentioned earlier. The time course of dry mass yield and lipid yield of the three species is depicted in Figure 3. For easy comparison, the relevant culture parameters as well as the final cell density, mass density, and the lipid contents of these three experiments (designated as experiments I to III) are presented in Table 3. All three organisms exhibited a substantial gain in both AFD and lipids after the nitrogen supply in the medium became completely exhausted. However, the final yield and post-N-exhaustion yield of AFD and lipids in the three experiments were quite different. In all three organisms, 2 to 3 fold increases in the lipid content were obtained

Table 1. Mass and Lipid Yields of *C.* sp. SO1 in N-Limited Batch Culture

Sample	El. Time Hr.	Cult Vol. L	Nitrate Conc. mM	Chl mg/L	E.flx(in) Watts	Yields/Culture (Corrected for Sampling Loss) AFD g	Total Lipids g	Neutral Lipids g	Polar Lipids g	C+P g
1	0.	12.6			16.80					
2	43.	12.4	4.98		16.78					
3	68.5	12.2	4.99	0.59	16.76					
4	92.	12.0	4.87	2.06	16.71					
5	104.	11.8	4.54	3.11	16.66	3.710	0.356	0.055	0.302	3.354
6	116.	11.6	4.29	4.14	16.58	4.754	0.395	0.057	0.338	4.359
7	128.	11.4	4.23	5.66	16.49	6.122	0.634	0.099	0.535	5.488
8	140.	11.2	3.75	9.44	16.39	7.466	0.852	0.133	0.719	6.614
9	152.	10.2	3.14	12.91	15.64	8.076	1.050	0.162	0.888	7.026
10	164.	11.0	2.42	23.22	16.27	12.027	1.837	0.286	1.551	10.190
11	170.	10.8	1.41	27.55	16.13	12.862				
12	175.	10.6	0.59	30.89	15.98	14.396				
13	179.	10.4	0.05	31.31	15.82	14.418	2.135	0.300	1.645	10.922
14	182.	10.0	0.005	33.22	15.46	15.447				
15	184.	9.8	0.0		15.25					
16	187.	9.6		32.28	15.04	16.299	2.445	0.419	2.026	13.854
17	194.	9.4		32.24	14.82					
18	200.	9.2		32.43	14.58	19.089				
19	212.	9.0		31.88	14.34	20.349				
20	224.	8.8		31.43	14.09	22.461	3.816	1.789	2.027	18.644
21	239.	8.6		30.66	13.83	23.923	4.325	2.436	1.890	19.597
22	260.	8.4		30.01	13.57	26.695	5.229	3.272	1.957	21.466
23	284.	8.2		28.03	13.30	28.562	6.380	4.382	1.998	22.182
24	308.	8.0		24.02	13.02	31.300	7.423	5.446	1.977	23.877
25	333.	7.8		23.71	12.74	32.782	8.161	6.171	1.990	24.621
26	380.	7.6		20.41	12.46	34.226	9.453	7.310	2.143	24.774
27	428.	7.4		17.06	12.17	35.279	10.812	8.471	2.341	24.467
28	480.	7.2		NA	11.89	36.847	12.307	9.962	2.344	24.540
29	500.	7.0		14.43	11.61	37.889	13.106	10.658	2.448	24.783
30	548.	6.8		10.59	11.32	38.372	13.747	11.287	2.459	24.626
31	596.	6.6		8.21	11.02	39.323	14.938	12.437	2.501	24.386

Table 2. Calorific Values of Algal Mass Lipids

Sample Materials *C.* sp. SO1	Heat of Combustion cal/g
Triglycerides stds (cal/g)	9450 ± 229
Total algal lipids (cal/g)	
From N-starved cells	9365 ± 208
From N-replete cells	7880 ± 167
Neutral algal lipids (cal/g)	
From N-starved cells	9680 ± 113
From N-replete cells	9520 ± 148
Polar algal lipids (cal/g)	
From N-starved cells	8300 ± 211
From N-replete cells	7768 ± 156
Non-lipid algal mass (cal/g)	4340 ± 181

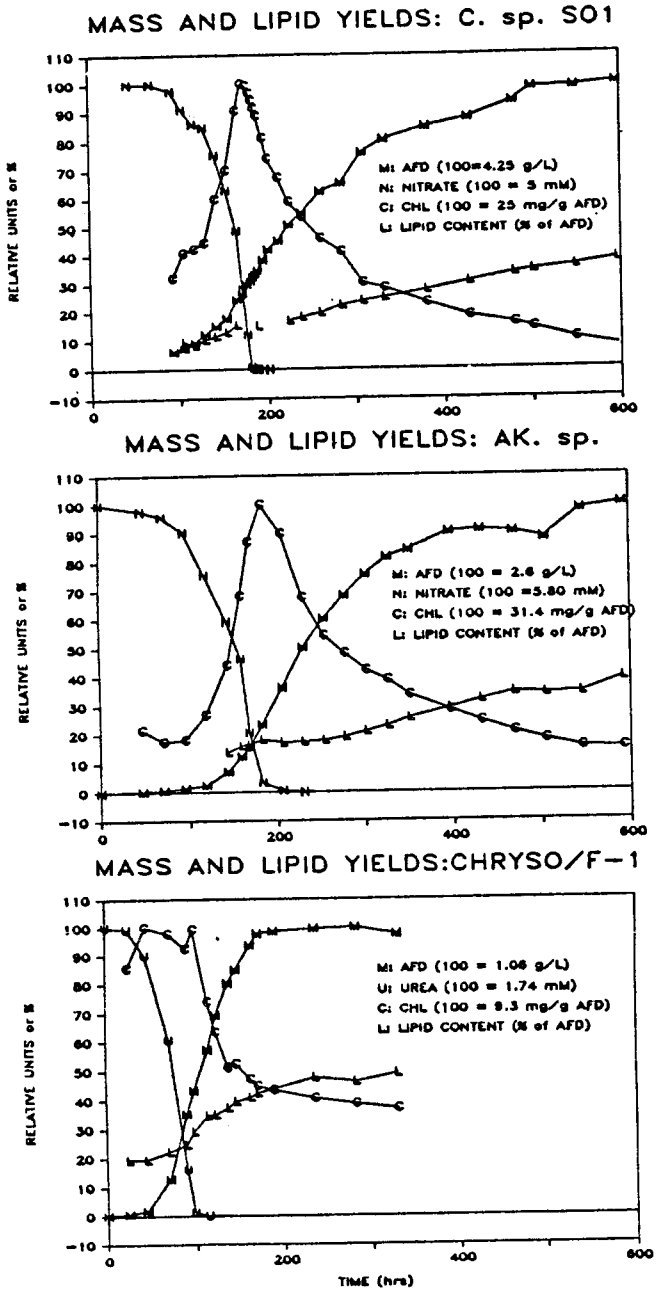


Figure 3. The time course of AFD-production lipid content and chlorophyll content in batch cultures of Chlorella, Ankistrodesmus and Chryso/F-1.

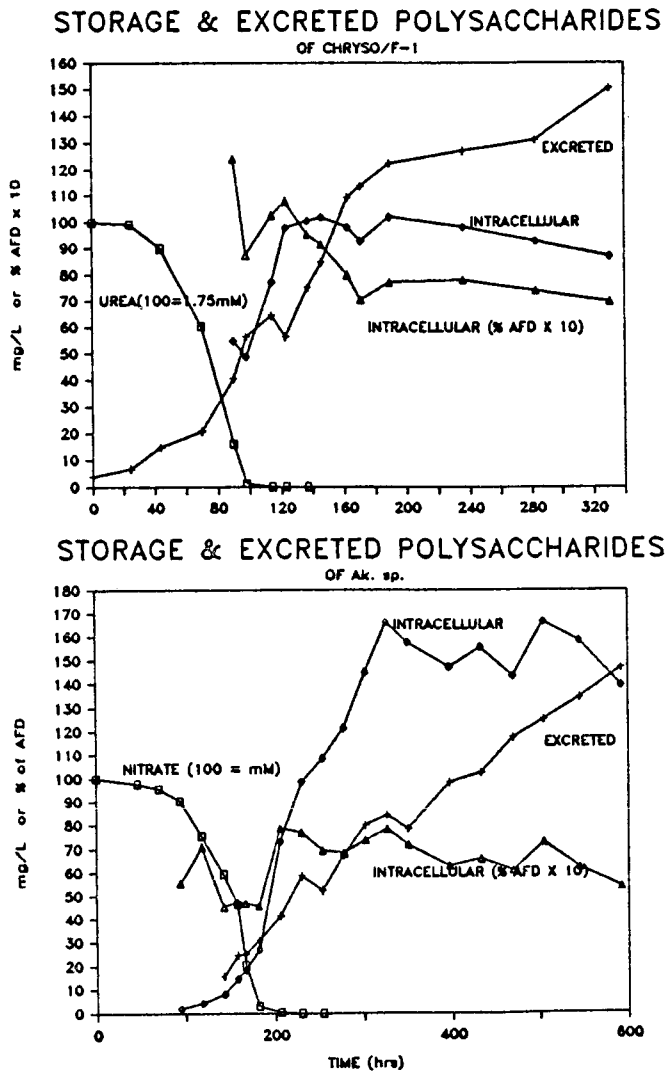


Figure 4. Intracellular and Excreted Polysaccharide of Ankistrodesmus and Chryso/F-1. The amount of both intracellular and excreted reducing sugar are expressed in unit of mg glucose equivalent per liter of the culture. For better clarity, the contents of intracellular polysaccharides were expressed as % of AFD x 10.

Table 3. Parameters of Three Fermenter Batch Experiments

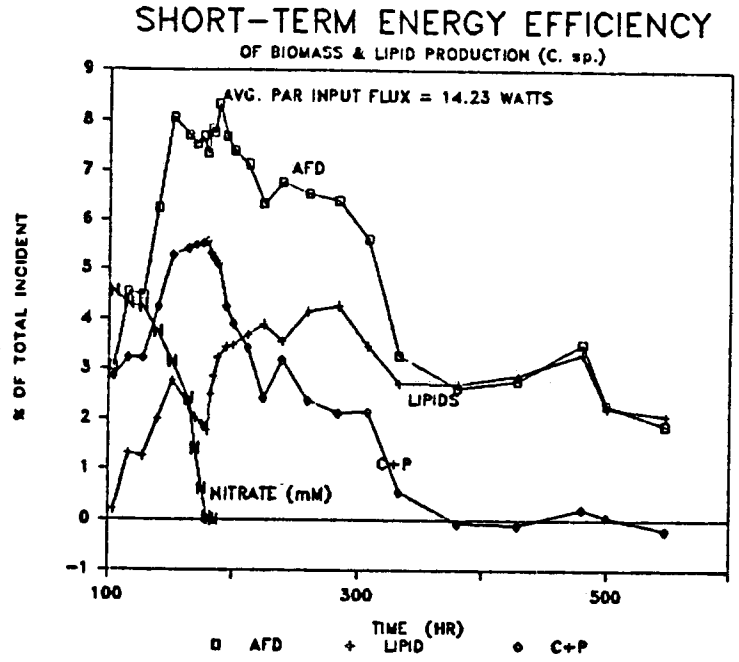
	Experiment I	Experiment II	Experiment III
Organism:	Chlorella sp. SOl	Ankistrodesmus sp.	Chryso/F-1*
Growth Medium:	BBM + 0.1 M NaCl	BBM	SERI Type II/25000
Nitrogen Source:	5 mM Sodium Nitrate	5.8 mM Sodium Nitrate	1.75 mM Urea
Temperature:	30 deg C	30 deg C	25 deg C
Avg. Input PAR Flux: (received by total culture volume)	14.21 watts	14.66 watts	26.6 watts
Culture Duration:	25 days	25 days	14 days
Mass Yield			
Final	4.24 g AFD/L	2.61 g AFD/L	1.06 g AFD/L
at N-depletion	1.38 g AFD/L	0.93 g AFD/L	0.63 g AFD/L
Final/N-depletion	3.07	2.81	1.68
Lipid Content			
Final	35.7% AFD	39.2% AFD	47.6% AFD
AT N-depletion	14.9% AFD	17.5% AFD	29.1% AFD
Final/N-depletion	2.40	2.24	1.64
Maximum Cellular Storage Carbohydrates	-----	8.4% AFD	12.4 AFD
Maximum Excreted Carbohydrates	-----	147 mg/L	150 mg/L
Maximum Carotenoids/Chl	21.2	16	-----

following an extended period of N-deprivation. In contrast, as shown in Figure 4 for Ankistrodesmus and Chryso/F-1, the intracellular storage polysaccharides appeared to be relatively insensitve to changes in nitrogen supply of the growth medium. The response of polysccharide production by Chlorella sp. SOl was not monitored. It should be pointed out that current data on the response of storage polysccharide production to N-deprivation in these two oleaginous species is quite different from the results obtained for non-lipogenic speices (see Thomas, et al. 1983) Thomas et al. (1983) reported that Tetraselmis and Isochrysis responded to N-depletion with an enhanced carbohydrate content while the lipid content remained unchanged or was reduced.

The Energy Efficiency of Nitrogen-sufficient Cells. The effects of N-deprivation on the energy efficiency of total cell mass and lipid production by each of the three species of algae are given Figures 5 to 7. During the initial stage of exponential growth the apparent efficiency was low because of incomplete absorption of the light energy by the dilute suspension of algal cells. As the culture became denser the efficiency increased with increasing cell density. For all three experiments reported here, a light probe placed at the center of the culture vessel indicated that near complete absorption of the light energy occurred when cell mass density of the culture exceeded 200-250 mg/L. Thus under our experimental conditions, the energy efficiency for the production of total algal mass (expressed as ash free dry mass, AFD) should reach its maximum at that mass density. Figures 5-7, however show a continuous increase in the efficiency until the nitrogen became totally exhausted. The the maximal energy efficiency was obtained just prior to nitrogen-exhaustion and ranged between 5 to 9% of the total incident light. The efficiency for lipid production throughout the nitrogen-sufficient phase of the experiment was approximately 30 to 60% of values obtained for AFD production.

Interestingly, while the efficiencies of AFD and lipid production for Chlorella sp. SOl were similar to those for Ankistrodesmus sp., the values obtained for Chryso/F-1 is considerably lower (see Figures 5-7). Although available experimental data does not allow us to exclude the possibility that the observed lower efficiency in Chryso/F-1 is intrinsic to this organism, it appears rather unlikely. It is possible that the lower efficiency for Chryso/F-1 reflects a higher mean light intensity experienced by the cells in experiment III as compared to that of experiments I and II as shown in Figure 3. The maximum chlorophyll content of Chryso/F-1 was approximately one-third of that for either Chlorella or Ankistrodesmus. In addition, the total mass density of Chryso/F-1 in experiment III was also lower. These differences, together with the higher light intensity used in experiment III, may be the major factors which contributed to the lower efficiency of Chryso/F-1.

The Energy Efficiency during the Early Stage of N-deprivation. Data on the effect of nitrogen supply on the energy efficiency for the



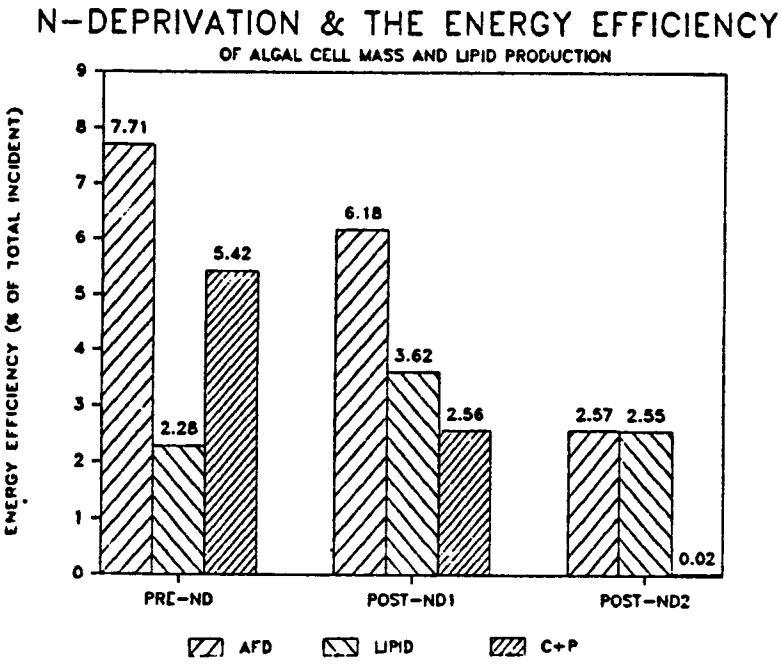
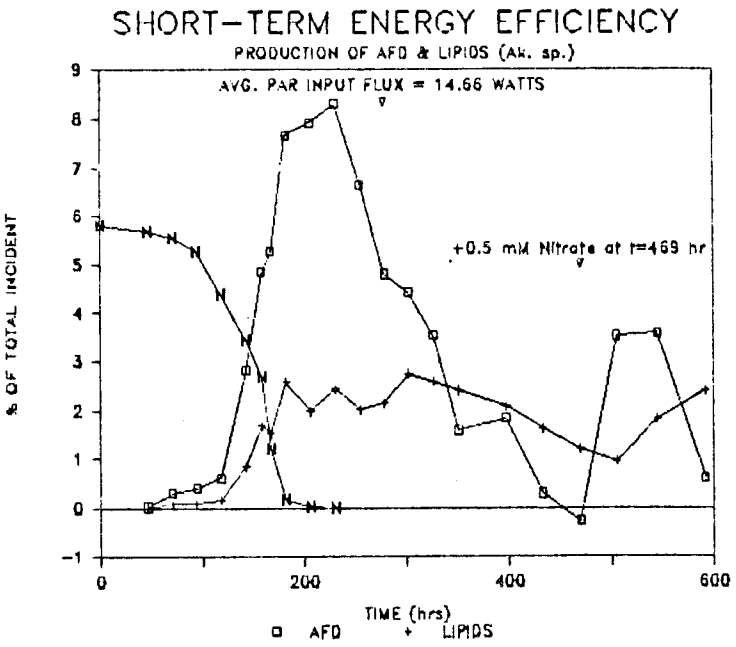


Figure 5. Energy efficiency of mass and lipid production in Chlorella sp. SO1.
AFD = Ash free dry mass, Lipid = Total algal lipids, C+P = Carbohydrates + proteins. Pre-ND = Pre-nitrogen depletion, POST-ND1 = 1st stage of nitrogen deprivation, Post-ND2 = Second stage of nitrogen deprivation.



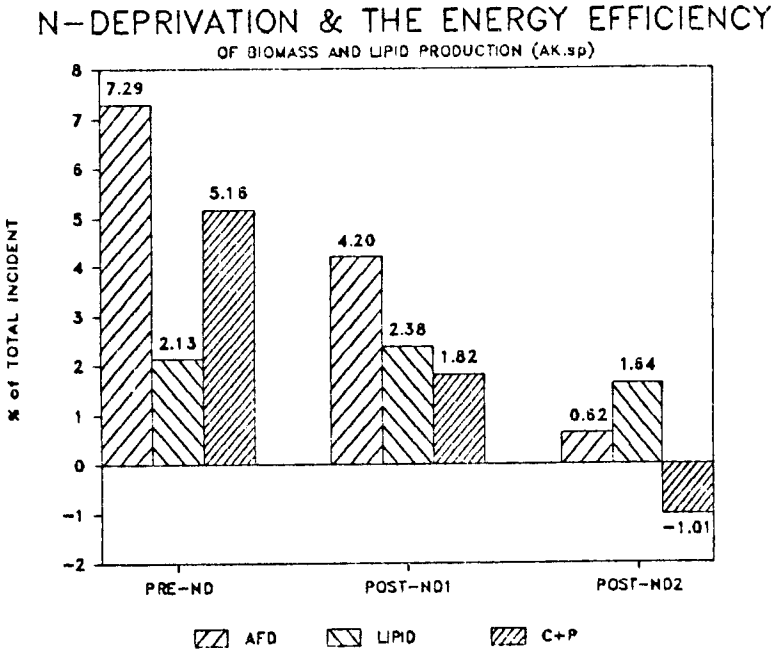


Figure 6. Energy efficiency of mass and lipid production in Ankistrodesmus sp.

AFD = ash free dry mass, Lipid = total algal lipids, C+P = carbohydrates + proteins. Pre-ND = pre-nitrogen depletion, Post-ND1 = 1st stage of nitrogen deprivation, and Post-ND2 = 2nd stage of nitrogen deprivation.

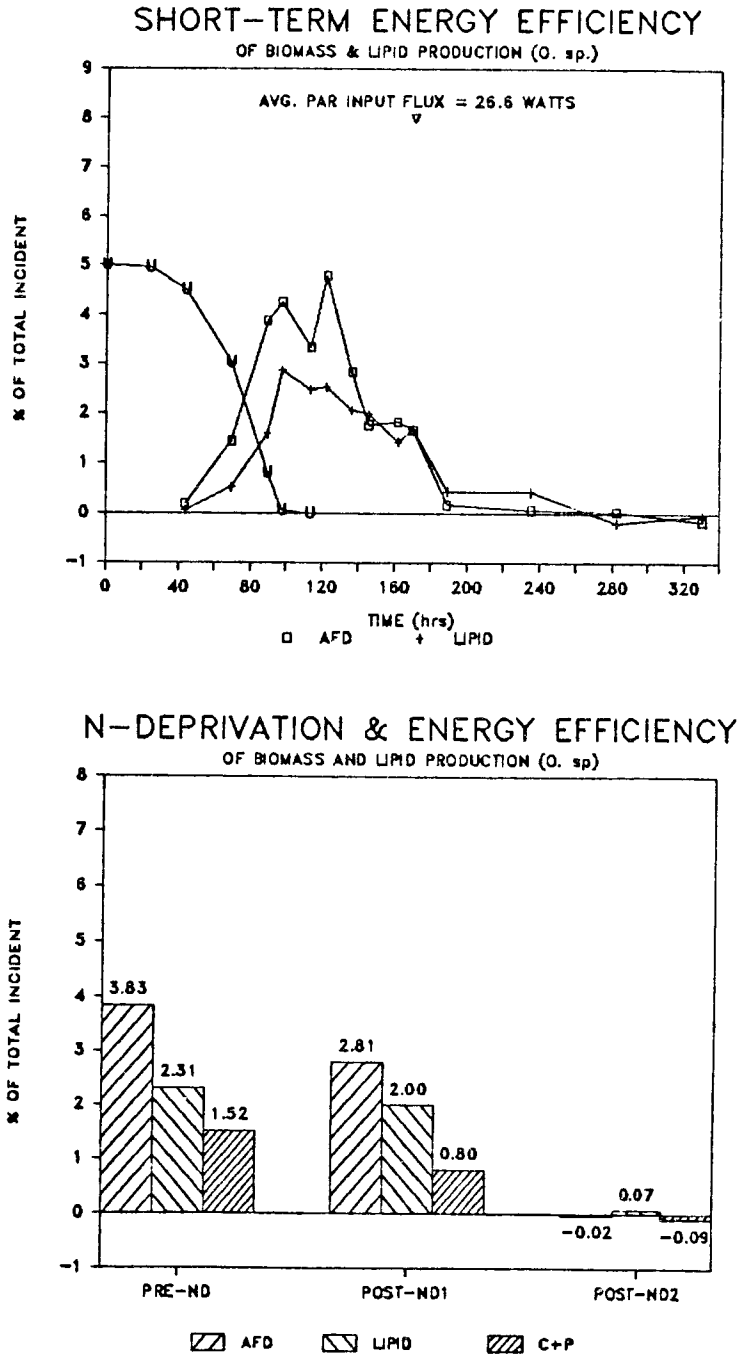


Figure 7. Energy efficiency of mass and lipid production in Chryso/F-1. AFD = ash free dry mass, Lipid = total algal lipids, and C+P = carbohydrates + proteins. Pre-ND = pre-nitrogen depletion, Post-ND1 = 1st stage of nitrogen deprivation, and Post-ND2 = 2nd stage of nitrogen deprivation.

production of storage (neutral) lipids and structural (polar) lipids are presented in Figure 8. During the first stage of N-deprivation (starting at the onset of N-exhaustion of and lasting for 2 to 5 days afterwards depending on the experimental conditions), a slight loss in the efficiency for AFD-production and a much sharper loss in the efficiency for the production of nonlipoidal materials (i.e. carbohydrates and proteins) was observed. In contrast, the energy efficiency of lipid production during nitrogen-sufficient growth was either maintained or significantly enhanced during the early stage of N-deprivation (designated as post-ND1 in Figures 5-7). Typically a value of 2 to 3% of the total incident PAR energy was stored as lipids by the three species of the algae examined to date. Interestingly the ratio of efficiency of lipid production to the efficiency for AFD production was highest in Chryso/F-1 among the three organisms.

The Energy Efficiency under Prolonged N-deprivation. As expected, prolonged nitrogen starvation led to a decline in the efficiency of all algal productivity (Figures 5-7 under POST-ND2), although the apparent efficiency for lipid production appeared to linger for a longer duration when compared to that of the AFD production. Whether these data indicate a reconversion of nonlipids into lipoidal materials during severe N-deprivation remains to be examined.

N-resupply and the Efficiency of N-starved Culture. Addition of a limiting amount of nitrogen (0.5 mM) to the culture of Ankistrodesmus during the late phase of N-deprivation (Fig. 6) led to a partial, but significant, restoration of energy efficiency for lipid and AFD production by Ankistrodesmus. (The response of the other two organisms to resupply the limiting nitrogen source has not yet been tested.)

Enhancement of the Energy Efficiency for Neutral Lipids by N-deprivation. When the effect of nitrogen deprivation on the composition of algal lipids was monitored during the transition from pre-N-deprivation to post-N-deprivation stage of growth, it revealed a dramatic and rapid inhibitory action on the synthesis of polar, structural lipids. This observation is consistent with the observed effect of N-deprivation on the cell division and structural differentiation. In contrast to the situation of polar lipids, the production of neutral (storage) lipids were strongly stimulated by nitrogen starvation. Data presented in Figure 8 indicate that a seven-fold increase in the efficiency of neutral lipids production was associated with the transition from pre-ND to post-ND1 period of experiment I. Similarly when the cells of Chlorella were removed from a nitrogen-rich medium and tranferred to a nitrogen-free medium, a similar enhancement of the efficiency of neutral lipid production occurred at the expense of polar lipid.

Conclusions

Energy efficiency for AFD production was optimal as batch cultures of algae

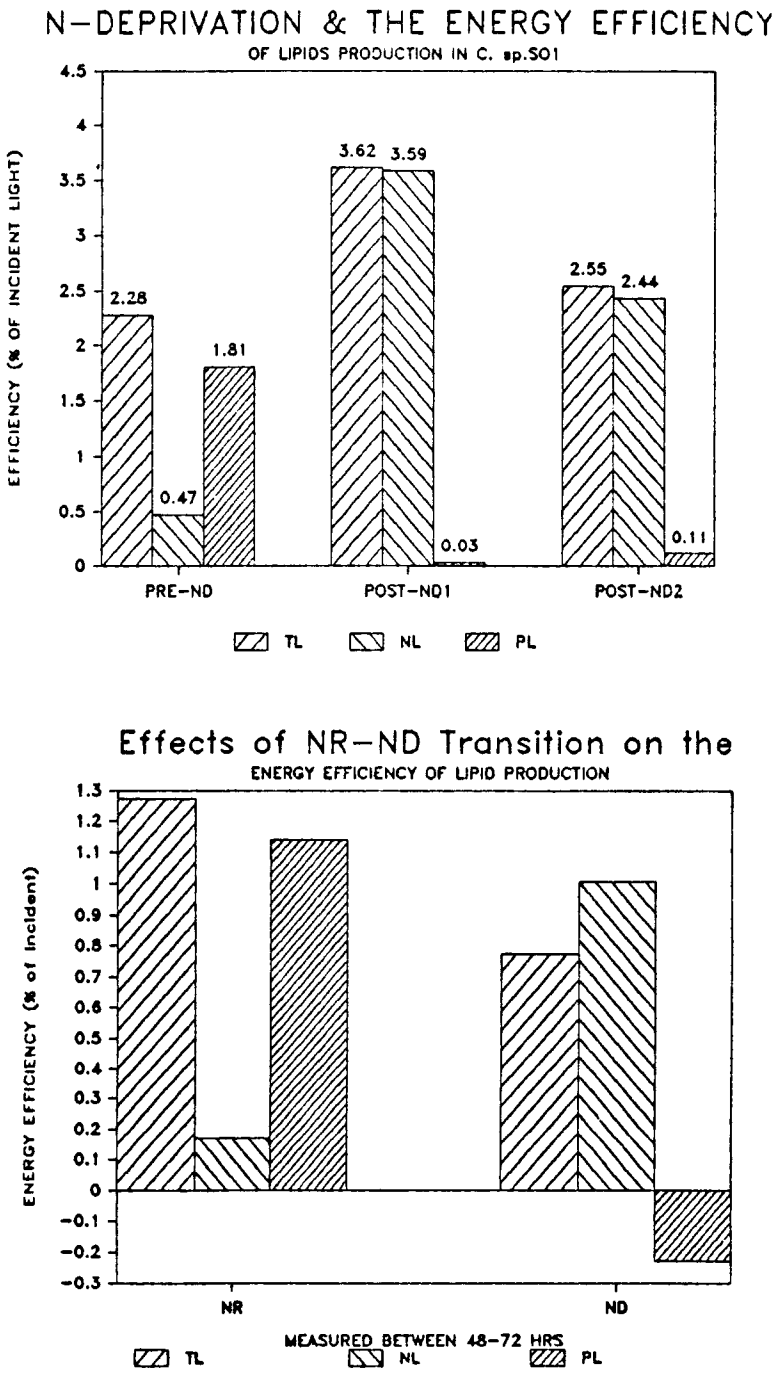


Figure 8. The energy efficiency for the production of total, neutral and polar lipids in Chlorella sp.- SO1. TL = total lipid, NL = neutral lipid, and PL = polar lipid.

approached N-exhaustion. In other words, the maximum energy efficiency for AFD production was attained when the pigment content of the cultures reached its maximal value and the mean light intensity experienced by the algal cells became minimal.

In the early stage of N-deficiency, the efficiency of the production of nonlipoidal materials decreased but the efficiency of lipid production remained unchanged or became stimulated. Lipid production efficiency of 2-3.6% could be maintained for a limited duration.

Extended N-deprivation led to a decline of all algal production efficiency, but the supply of nitrogen resulted in a partial restruction of production efficiency for AFD and lipids in Ankistrodesmus.

Nitrogen deprivation caused a strong increase in the efficiency of neutral, storage lipid production and suppressed the efficiency of the production of polar, structural lipids.

Continuing Research

Although the experiments with three species of oleaginous algae provided the initial set of energetics data for cell mass and lipid production, due to a limited scope of completed research, a quantitative assessment of the overall energetics of algal lipid production could not be made at the present time. Additional experiments are planned to extend the current energetic analysis to cover more organisms (including "promising" species of diatoms, chrysophytes and green algae in the SERI culture collection).

In order to extend the depth of our understanding on the energetics of N-deprivation, a series of experiments will be conducted to establish the relation between the concentration of initial nitrogen-supply and the efficiency and duration of post-N-exhaustion AFD and lipid production. Additional N-resupply experiments will be conducted to explore the possibility of enhancing the efficiency and extending the duration of lipid production by the oleaginous algae.

Finally, for comparative purposes, the energy efficiency of AFD and lipid production in steady-state continuous cultures will also be studied. The experiments on steady-state cultures will be carried in collaboration with Dr. Ken Terry of our group.

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Addendum

Effects of Silicon Deficiency on Lipid Metabolism in Diatoms (P. Roessler)

Diatoms require substantial quantities of silicon in order to synthesize the protoplast-enclosing frustule which characterizes members of this group. Thus, cell wall synthesis is inhibited in silicon-deficient diatoms, preventing cell division. A wide variety of other metabolic processes, seemingly unrelated to cell wall synthesis, are also strongly affected in silicon-deficient diatoms. Silicon deficiency induces a decrease in the biosynthesis of most cellular constitutents, but certain diatom species have been reported to contain elevated lipid levels when grown under silicon-deficient conditions. Studies which have investigated changes in diatom cell metabolism due to silicon deficiency have been

carried out primarily with Navicula pelliculosa, Cylindrotheca fusiformis, and Cyclotella cryptica.

Net protein synthesis is greatly reduced in silicon starved cultures of all three of the above species (Werner 1966, Coombs et al. 1967, Darley and Volcani 1969). In C. cryptica and N. pelliculosa, there is a decrease in cellular levels of glutamate, aspartate, and α-ketoglutarate associated with the decrease in protein synthesis rates (Coombs and Volcani 1968, Werner 1968). Using two-dimensional electrophoresis to separate ^{35}S-labeled polypeptides synthesized by silicon-replete and silicon-deficient C. fusiformis cells, Okita and Volcani (1978) demonstrated a role for silicon in the regulation of gene expression in this species, since the abundance of some polypeptides increased in response to silicon deficiency while other polypeptides decreased.

The net synthesis of storage carbohydrate (chrysolaminarin), nucleic acids, and most pigments is also greatly diminished in silicon-deficient diatoms (Werner 1966, Coombs et al. 1967, Healey et al. 1967, Coombs and Volcani 1968, Darley and Volcani 1969). A reduction in the rate of DNA synthesis in C. fusiformis is believed to be due to reduced DNA polymerase activity, possibly a result of post-transcriptional control of the synthesis of the polymerase (Okita and Volcani 1978).

In sharp contrast to the effects of silicon starvation on the synthesis of all other macromolecular components of diatoms, the biosynthesis of lipids is not inhibited. Lipids continue to increase at an exponential rate in log phase N. pelliculosa cultures which have depleted the medium of silicon (Coombs et al. 1967). During a 14 hour starvation period, the lipid mass per cell increased by approximately 60% in this species, during which time the carbohydrate mass per cell decreased by 8%. Lipid synthesis accounted for 72% of the total mass increase in these cells. Net lipid synthesis ceased immediately following the reintroduction of silicon into these starved cultures, but this may be attributable to a concomitant rapid increase in respiration. In a separate study, it was observed that the incorporation of $^{14}CO_2$ into lipids increased from 20 to 45% of the total when cells were starved for silicon (Coombs and Volcani 1968).

In studies involving C. cryptica, Werner (1966) observed that lipids increased from 16 to 38% of the dry weight after 24 hours in silicon-deficient conditions. Calculations indicated that the rate of lipid synthesis approximately doubled during the first six hours of silicon starvation in this species. Shifrin and Chisholm (1981) also reported rapid increases in the lipid content of silicon limited C. cryptica cells. These investigators reported that within 12 hours of silicon depletion, the lipid mass per cell doubled, corresponding to about 67% of the total cell mass increase.

The rapid switch from carbohydrate to lipid accumulation which occurs in silicon starved diatoms may prove to be an excellent model for studying the regulation of carbon metabolism in algae. Silicon, unlike nitrogen, phosphorous, and sulfur, is not a constituent of any of the macromolecular

components of diatoms, and therefore the control of carbon partitioning by silicon is not simply a manifestation of substrate concentration. Rather, the regulation is more likely to be the result of differential gene expression or allosteric modulation of the enzymes of carbon metabolism.

We have initiated a study designed to investigate further the role of silicon in diatom lipid metabolism. We will examine the kinetics and extent of lipid accumulation in several diatom species grown under silicon-deficient conditions, and will also determine the energetic efficiency of lipid production in silicon-stressed applications. The information obtained can be compared to data collected with other microalgal species grown under different types of stress, allowing a comparative evaluation of silicon-deficiency as a mechanism for enhancing lipid yields.

This study will also investigate the mechanisms by which silicon deficiency leads to lipid accumulation in diatoms. The activities of various key enzymes of carbon metabolism will be assayed in silicon-replete and silicon-deficient diatoms in order to identify the primary control points affected by silicon deficiency. This information may prove to have fundamental importance in future studies involving physiological and genetic manipulation of algal species aimed at increasing the yields of desired products.

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PHOTOSYNTHETIC EFFICIENCY EHHANCEMENT IN MODULATED LIGHT: DEPENDENCE ON THE FREQUENCY OF MODULATION

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Introduction

Beginning with some of the earliest efforts at microalgal mass culture under natural light, it was recognized that microalgae use high intensity light at a low efficiency and that this efficiency can be raised if light is supplied on an intermittent rather than on a continuous basis (Kok 1953, Davis et al. 1953). In most cases, efforts to create an intermittent light environment were based on mixing dense cultures of microalgae, taking advantage of the shading of cells at depth by cells at the surface. Since the 1950's, a number of culture devices have been proposed which would take advantage of this effect (Fredrickson et al. 1961, Phillips 1961, Miller et al. 1964, Howell et al. 1966, Fredrickson and Tsuchiya 1969, Setlik et al. 1970, Lee and Pirt 1981, Laws et al. 1983, Prokop and Fekri 1984). Many of these devices attempt to create ordered vertical mixing (vortex mixing) rather than purely turbulent mixing, in order to expose the cells to regular intervals of light and darkness. It has been argued that turbulent (random) mixing processes are inadequate to provide for photosynthetic enhancement (Powell et al. 1965), and recent experimental evidence seems to support this argument (Weissman & Goebel 1985).

Recently, Laws et al. (1983) introduced a device employing wing-like foils to generate vortex mixing in flowing cultures. An increase in productivity by a factor of about 2.2 was attributed to these devices, although it is not entirely clear that intermittent light effects were solely responsible for the productivity increase which was observed. Laws et al. (1983) measured the vortex rotation rate in their flowing cultures, and observed turnover times of 1-2 seconds (frequencies of 0.5-1 Hz).

There is a large body of literature on the rates of photosynthesis in flashing light. Most of these studies have been directed toward the separation of the reactions of photosynthesis, but some effort has been directed toward evaluating the potential of intermittent illumination to enhance the photosynthetic efficiency of algal cultures (Kok 1953, 1956, Phillips and Myers 1954, Powell et al. 1965). The most useful data on the subject were collected by Phillips and Myers (1954), who examined the growth of Chlorella pyrenoidosa under flashing light regimes with total cycle periods up to about 600 ms. Reanalysis of these data showed that factorial enhancement of photosynthetic efficiency varied as a function of the length of the flash period and the length of the intervening dark period (Terry 1984), but data were not available to cover the longer cycle times which are of interest for outdoor cultures.

The most consistent interpretation of the photosynthetic response to intermittent light environments was proposed by Weller and Franck (1941; Kok 1956, Rabinowitch 1956), who argued that the photosynthetic rate in flashing light should approach, with increasing flash rate, the rate in constant light of the same average intensity. A later reanalysis of much of the published data on the subject (Sager and Geiger 1980) was strongly supportive of this interpretation.

Studies of intermittent light have typically employed devices where light was turned on and off abruptly, frequently through the use of a sectored disk. These devices approximated a square-wave modulation of light over time (Figure 1), but many other wave forms are possible. Cells which are mixed vertically in outdoor cultures experience a smooth wave rather than a square wave modulation. Figure 1 shows the light environment experienced by cells following a circular path in a dense culture of microalgae where light intensity declines exponentially with depth.

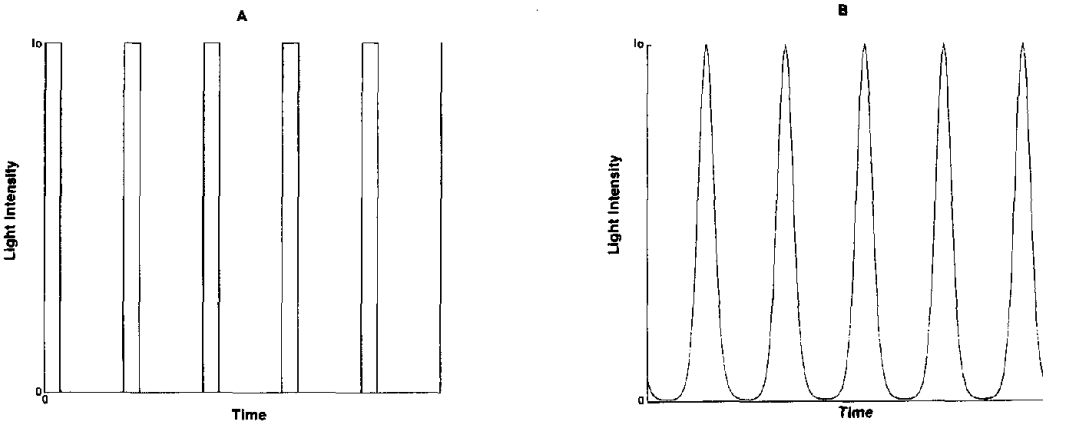

Figure 1. Light intensity as a function of time in square wave (A) and smooth wave (B) modulations for $\phi_t = 0.2$. The smooth wave modulation was calculated as $I = I_o e^{-k(\sin t + 1)/2}$ with k = 5.4. The time scale is determined by the cycle frequency.

The research described here was directed toward a quantification of the relationship between the frequency of light modulations similar to those experienced by cells in vortex mixed systems and the resultant enhancement of photosynthetic efficiency. The Weller and Franck hypothesis was examined experimentally and the results provide a bases for the description of a functional relationship between modulation frequency and photosynthetic efficiency enhancement. Additionally, the responses to cycle frequency during smooth wave and square wave modulation were compared. The resulting data were used to develop a plot of photosynthetic enhancement as affected by light:dark cycle times in a range of interest for outdoor cultures. The relationships which were observed permit an assessment of the potential contribution of modulated light effects to productivity increases such as those observed by Laws et al. (1983). These data provide a necessary input to the design requirements of systems which

may be proposed in the future to take advantage of these effects.

Theory. The advantage which is to be derived from light intensity integration can be readily inferred from an examination of the photosynthesis versus irradiance curve, which approaches a saturating value at relatively low light intensities (see, for example, Figure 3). The photosynthesis versus irradiance relationship can be described by a nyperbolic function such as

$$P_G(I) = \frac{P_G' I}{(K_I^{\,m} + I^m)^{(1/m)}} \tag{1}$$

where I is the light intensity, and P_G', K_I and m are constants (Smith 1936, Bannister 1979). In a modulated light environment, light intensity varies as a function I(t) of time. Where there is no light intensity integration, the cell responds at all times to the instantaneous light intensity, and the mean gross photosynthetic rate is

$$P_G^o = \frac{1}{t_1} \int_{t=0}^{t_1} P_G(I(t)) \tag{2}$$

while with complete light intensity integration

$$P_G^* = P_G(\frac{1}{t_1} \int_{t=0}^{t_1} I(t)) \ . \tag{3}$$

For a square wave modulation, the flash proportion ϕ can be defined as

$$\phi = t_L/(t_L + t_D) \tag{4}$$

$$= \bar{I}/I_o \tag{5}$$

where t_L and t_D are the durations of the flash period and the dark period, respectively, and $\bar{I}$ is the time average of the light intensity, and equations 2 and 3 can be reduced to

$$P_G^o = \phi P_G(I_o) \tag{6}$$

and

$$P_G^* = P_G(\phi I_o) \tag{7}$$

(Terry, 1985). Since the P versus I curve is convex upwards, for all physically meaningful values of ϕ and I_o, $P_G^* > P_G^o$; that is, light intensity integration leads to increased rates of gross photosynthesis.

The data analysis presented below is based on determinations of the efficacy of light intensity integration under various modulated light

regimes. Square wave light modulations can be characterized by three parameters: ϕ, as defined above (dimensionless), ν, the frequency of the flash (Hz)(= $1/(t_L + t_D)$), and I_o, the intensity of the flash (W m^{-2}). For smooth wave modulations, the same parameters can be employed if I_o is defined as the peak intensity and ϕ is defined by equation 5.

For any combination of ϕ, ν, and I_o the dimensionless index of proportional light intensity integration, Γ, can be calculated as

$$\Gamma = \frac{P_G(\phi, \nu, I_o) - P_G^o}{P_G^* - P_G^o} \tag{8}$$

where $P_G(\phi, \nu, I_o)$ is the observed rate under the experimental conditions specified. The results presented below describe Γ as a function of ν under a variety of conditions.

Materials and Methods

Preconditioning cultures. Populations of Phaeodactylum tricornutum Bohlin and an unidentified flagellated chrysophyte designated Chryso/F-1 (see section 2) were preconditioned in continuous cultures maintained at 20°C. These cultures were illuminated by a bank of eight 40 W fluorescent lamps, which provided approximately 48 W m^{-2} of photosynthetically active radiation (400-700 nm) at the external surface of the culture vessel. Culture densities were turbidostatically controlled based on the output of a LiCor quantum sensor mounted on the face of the flat-sided growth chamber farthest from the light source (10 cm path length). The output of this sensor was monitored by computer (see Addendum for details) and the nutrient supply pump turned on or off as needed to control culture turbidity. Formulas for the media employed appear in Table 1. In all cases, the populations employed were nutrient-sufficient and were growing at the rate determined by the available light. For some populations, the turbidity was sufficiently high that the average intensity in the growth chamber was significantly reduced due to self-shading. Population sizes and growth rates of the various preconditioning cultures are presented along with results in Table 4.

Light Modulation. Square wave modulations were achieved with sectored disks, adjustable from ϕ=0 to ϕ=0.5, rotated by a speed-controlled DC gearmotor. Smooth wave filters were generated by computer with transparency (T) decreasing exponentially from one edge, arbitrarily designated the top:

$$T = e^{-kZ} \tag{9}$$

where k is a constant and Z is the distance from the top in diameters of the circular filter. Filters were generated for k=1.75, 5.4 and 34 diameter^{-1}, although only the filter with k=5.4 received extensive use. The filters were reproduced photographically with a diameter of 25 cm, and the photographic transparencies rotated in front of a focused beam of light (see below). The transparencies were doubled to provide almost complete absorption of light over the range 200-800 nm where blackened. (A peak of 2.5% transparency appeared at a wavelength of 320 nm, with the peak covering the range 310-330 nm; the filter was effectively opaque to all other wavelengths. The transparent portions of the filter passed 70-80% of the incident light with approximately neutral density over the range 400-700 nm. However, there was a sharp cutoff at 310 nm, and shorter wavelengths were excluded.) Figure 2 shows light intensity as a function of time for the filter with k=5.4.

Table 1. Medium Composition

Phaeodactylum tricornutum Artificial Seawater Medium	*Chryso/P-1* Artificial Piceance Creek Medium	
Per liter dionized water	Per liter deionized water	8 mg KH_2PO_4
30 g RILA salts	0.35 g $MgCl_2 \cdot 6H_2O$	5 ml PII metal mix (SERI 1984)
0.05 g $NaHCO_3$	1.20 g NaCl	1 ml vitamin mix
0.5 ml NH_4Cl, 1M	.20 mg NaF	1 g/L thiamine
0.5 ml K_2HOP_4, 0.1 M	8 mg $BaCl_2 \cdot 2H_2O$	2 mg/L biotin
0.5 ml Trace Metal Mixture (Brune, et al. 1981)	45 mg $CaCl_2$	1 mg/L B_{12}
	19 mg KCl	1 ml 1% $Na_2SiO_3 \cdot 9H_2O$
	10.28 g $NaHCO_3$	1 ml FeEDTA solution
	15 mg Na_2SO_4	(26.1 g EDTA in 268 ml INKOH
	36 mg Urea	+ 24.9 g $FeSO_4 \cdot 7H_2O$
		up to 1 L)

The frequency of modulation ν was determined by placing a photodiode in the modulated beam, and routing the signal from this sensor through a frequency-to-voltage converter to the data acquisition system. This method of determining modulation frequency was not reliable below about 0.5 Hz; slower rates were determined by manual timing of the disk rotation rate.

Light was provided either by a tungsten halogen lamp or by a xenon arc lamp. In either case, the beam of light was focused through a convex lens to a circle of about 1 cm diameter at the rotating disk, then recollimated with a similar lens and directed downward into the oxygen electrode chamber (see below) by a mirror. For the experiments described here, all optical components were of common glass; both the lenses and the photographic transparencies effectively excluded ultraviolet radiation.

Light Measurements. Light intensities were determined with a LiCor quantum

sensor placed in the light path above the oxygen electrode chamber. The signal was passed through an operational amplifier assembly which permitted direct reading of the signal or integration with time constants of 1/60 s or 1 s and was recorded with the computerized data acquisition and control system. The correlation between the light intensity measured above the chamber and that received by the experimental sample was determined by replacing the teflon membrane and the platinum electrode at the bottom of the incubation chamber with a second light sensor; this correlation was employed in the computer conversion of voltage to light intensity.

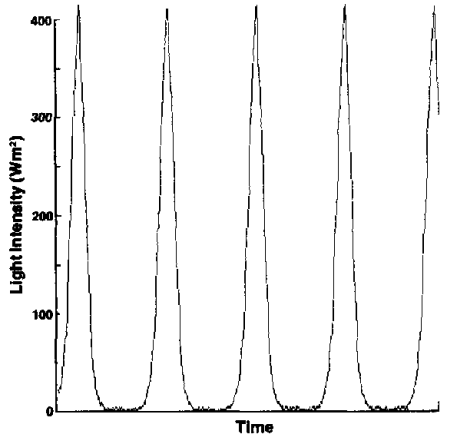

Figure 2. Light intensity as a function of time for modulations generated with a computer-constructed filter with k = 5.4 (equation 9). While the time scale is arbitrarily determined by the filter rotation rate, the readings shown here were taken over 24 s at 0.05 s intervals.

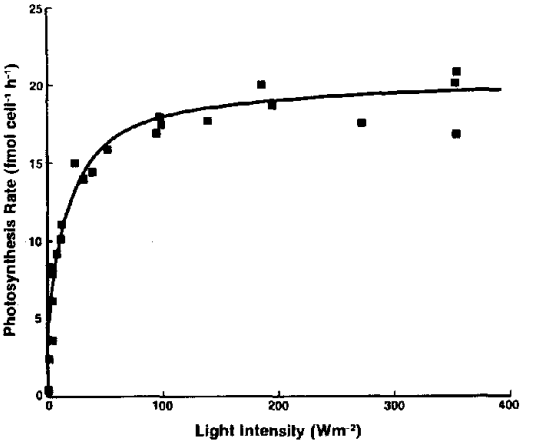

Figure 3. Photosynthesis versus light intensity for P. tricornutum, experiment 1. The curve represents equation 1 with the parameters given in Table 4.

Photosynthetic Rate Determinations. Photosynthetic rates were determined as oxygen evolution rates in an oxygen electrode chamber maintained at 20°C by water circulating through the outer jacket. The chamber was 1.6 cm in diameter and was filled to a depth of 0.5 cm with 1 ml of sample. The sample was illuminated from above through a clear glass plunger which prevented oxygen exchange with the atmosphere. The time course of oxygen evolution was recorded by the computerized data acquisition system, and the rate determined as a linear regression of oxygen concentration on time.

For each determination, a culture sample was placed in the electrode chamber in the dark for approximately 5 min before the experiment was started. During this period, the light intensity and the flashing rate were adjusted and measured. Oxygen concentrations were then monitored for 3 min with the chamber in darkness to determine the respiration rate, R. The light shield was then removed from the chamber and the net photosynthetic rate (P_N) determined, also over a period of 3 min, after a delay of 15 s to allow for electrode response. The gross photosynthetic rate was calculated as $P_G = P_N - R$ (recall that R is negative).

Experimental Design. Γ was determined as a function of ν under a variety of experimental conditions. For each experiment, it was necessary to determine photosynthetic rate as a function of light intensity in constant light in order to interpret the data obtained in modulated light.

Table 2. Light Intensity Integration by Phaeodactylum tricornutum in Flashing Light. Coefficients of least squares fits of equation 10 to values of Γ averaged over light intensity I_o (A) or flash proportion ϕ (B).

A.	ϕ	K_ν (Hz)	Γ_m
	.03	.53	.964
	.10	.91	.963
	.25	.90	1.19
	.50	.42	.807
	POOLED	.67	.972

B.	I_o ($W\ m^{-2}$) nominal	I_o ($W\ m^{-2}$) actual ± s.d.	K_ν (Hz)	Γ_m
	48.9	46.6 ± 3.1	.61	1.06
	97.8	90.0 ± 2.7	.61	1.05
	195.7	191.0 ± 5.1	1.32	1.02
	342.5	351.5 ± 9.2	.58	.815
	POOLED	-------	.67	.972

Nominal values of ν were 0.25, 0.50, 1.00, 3.00, 5.00, 7.50 Hz. Actual values were 0.27 ± 0.02, 0.51 ± 0.02, 1.02 ± 0.06, 3.00 ± 0.05, 5.02 ± 0.06, 7.53 ± 0.11 Hz (± s.d.).

Table 3. Percents of total sample variance explained by (1) hyperbolas fit to each set of 24 samples, (2) a single hyperbola fit to all 96 samples, (3) a linear dependence of Γ on I, and (4) a linear dependence of Γ on ϕ.

Data sorted by ϕ					Data sorted by I				
ϕ	n	(1)	(2)	(3)	I (μEinst $m^{-2}\ s^{-1}$)	n	(1)	(2)	(4)
0.03	24	49	48	25	48.9	24	60	56	27
0.10	24	70	61	5	97.8	24	53	49	0
0.25	24	79	69	0	195.7	24	78	66	3
0.50	24	41	31	3	342.5	24	52	37	1
TOTAL	96	62	56	9	TOTAL	96	64	56	2

[1] - nominal values

The effects of square-wave flash frequency (ν), flash intensity (I_o) and flash proportion (ϕ) on the photosynthetic rate of P. tricornutum cells preconditioned in a high density culture were determined in a full three-way factorial design of six by four by four values. The values of the parameters which were employed are shown in Table 2. The factorial comprised 96 measurements, and an additional 23 measurements were required to determine the P_G versus I curve and confirm stability of the experimental material over the period required to complete all experiments. (Table 4., Experiment 1)

The frequency response of P. tricornutum to square wave modulations was determined a second time with a population preconditioned at a low population density in order to explore effects of changing preconditioning environments on the light intensity integration response. For these experiments, ϕ was 0.2 and I_o 391 W m^{-2}. A disk which provided twelve cycles per rotation was utilized in these experiments to provide high frequency modulations (up to 80 Hz). (Table 4, Experiment 2)

The frequency response to smooth and square wave modulations was determined for Chryso/F-1 with ϕ=0.2 and I_o=365 and 403 W m^{-2} in two separate sets of experiments. The response of populations with different preconditioning experiences to light modulations were also compared. (Table 4, Experiments 3-8)

Results

Square Wave Modulations. The photosynthesis versus light intensity relationship for the cells employed in all experiments were well described by equation 1, with the parameters given in Table 4 (e.g. Figure 3). For each experimental determination of photosynthetic rate, Γ was calculated according to equation 10, with P_G^o and P_G^* calculated according to equations 6 and 7 for square wave modulations.

In a factorial experiment, both the first-order and higher order effects of the variables need to be assessed (Box et al. 1978). First order effects of an independent variable (the independent variables were ν, ϕ, and I_o) will act to bring about changes of a predictable magnitude in the dependent variable regardless of the values of the other independent variables. Second order effects occur when the effect of one independent variable is modified based on the value of another. Higher-order interactions are also possible, although often difficult to detect.

The first order effect of ν on Γ was examined for each ϕ with Γ averaged over all values of I_o, and for each I_o with Γ averaged over ϕ. In each case, Γ could be described as a function of ν by the equation

$$\Gamma = \frac{\Gamma_m \nu}{K_\nu + \nu} \qquad (10)$$

where Γ_m and K_ν are constants. The values of Γ_m and K_ν, determined by nonlinear least squares, did not depend strongly on ϕ or I_o (Table 2). These hyperbolas remove 62% of the variance of the original values (n=96) when the parameters are determined for values averaged over I_o, and 64% for values averaged over ϕ (Table 3). Where the parameters of the hyperbola were determined for all data pooled (Tables 2, 3, Figure 4), 56% of the variance was explained ($p<.001$, F test).

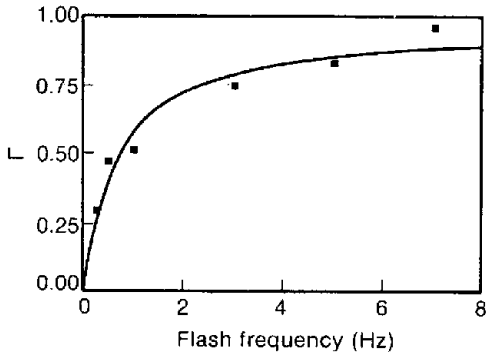

Figure 4. Proportional integration of light intensity, Γ, as a function of flash frequency ν for P. tricornutum experiment 1, with Γ averaged over both ϕ and I_o. Each point represents the mean of 16 determinations. The curve represents equation 10 $K\nu = 0.67$ and $\Gamma_m = 0.972$.

The possibility of first order contributions by the variables ϕ and I_o was examined by removing the contributions of ν to the observed values of Γ according to equation 11 and examining the dependence of the residuals on ϕ and I_o. The residuals showed a weak linear dependence on I ($r = -0.305$), but no significant dependence on ϕ. The inclusion of I in the prediction of Γ explained only an additional 9% of the total sample variance, and its maximum contribution to Γ over the range of interest was ± 0.07. Second-order effects of ϕ or I_o on the Γ versus ν relationship should be reflected in a dependence of the curve-fitting parameters Γ_m and K_ν on the variables ϕ and I_o. No such relationships were observed.

The results of these experiments are presented and discussed in more detail by Terry (1985).

Smooth Wave Modulations. The calculation of Γ for experiments performed with smooth wave modulations requires the determination of P_G^o according to equation 2, rather than with the simplified form in equation 6. In order to determine this value, the modulated light regime created by the filter rotating at a low frequency (<0.2 Hz) was monitored by computer, with data acquired at 50 ms intervals (Figure 2). Photosynthetic rate

Table 4. Modulated Light Experiments

	Expt. No.	Cells (10^9 L^{-2})	Growth Rate (day^{-1})	Wave Form[1]	ϕ[2]	I_o[3] (W m^{-2})	$\bar{I}$[4] (W m^{-2})	P_G'[5] (fmol O^2 $cell^{-1}$ h^{-1})	K_I[5] (W m^{-2})	m[5]	Photosynthetic Rates (fmol O_2 $cell^{-1}h^{-1}$) $\phi P_G(I_o)$[6]	P_G^o[7]	P_G^*	Γ_m[8]	H_ν (Hz)
P. tricornutum	1	120[11]	~0.1	F	- [12]	- [12]	- [12]	22	5.5	0.75	- [12]	- [12]	- [12]	0.97	0.67
	2	10	1.46	F	0.2	391	78.5	141	45.6	1.1	26	28	.95	0.89	6.82
				F[13]	0.23[13]		91.4[13]				30[13]	36[13]	100[13]		
Chryso/F-1	3	3.30	1.24	S	0.2	333	66.7	551	66.5	2.3	109	207	418	1.0[9]	16.7[9]
	4	3.34	1.24	S	0.2	403	80.6	609	64.0	2.3	127	242	490	1.0[9]	17.6[9]
	5	3.30	1.24	F	0.2	403	80.6	577	41.7	1.7	114	124	489	1.0[9]	10.0[9]
	6	n.a.	n.a.	S	0.2	318	63.6	1613[10]	66.7	1.0	267[10]	435[10]	787[10]	1.0[9]	48.2
	7	4.32	1.69	F	0.2	367	75.3	218	50.3	1.0	39	43	131	0.78	4.12
	8	4.32	1.69	S	0.2	362	72.4	218	50.3	1.0	38	69	126	1.09	3.05

Footnotes

1. F = square wave (flashing), S = smooth wave.
2. ϕ = flash proportion.
3. I_o = peak intensity during cycle, 400 - 700 nm.
4. $\bar{I}$ = mean intensity during cycle, 400 - 700 nm.
5. Parameters of equation 1, determined by non-linear least squares analysis of photosynthesis versus irradiance data.
6. Where $P_G(I_o)$ is calculated according to equation 1, with the parameters given.
7. Integrated productivity under modulated light, calculated from computer-acquired data for intensity versus time and equation 1, employing the parameters given.
8. Parameters of equation 10, determined by non-linear least squares from the Γ versus ν data.
9. Least squares fit for Γ_m fixed at 1.0, due to absence of data for $\Gamma > 0.5$.
10. μ mol O_2 L^{-1} h^{-1}.
11. Samples were diluted by a factor of 5 before experiments were run.
12. Various values of these factors were employed, as shown in Table 2.
13. Experiments were performed with a chopping disk having 12 sections over the circumference to permit determinations at high flash frequencies.

was calculated for each data point over 3 to 4 cycles (n= 300 to 450) from equation 1 with the appropriate parameters from Table 4 and averaged to provide the estimate of P_G^o .

For Chryso/F-1, the dependence of Γ on ν was described by equation 10 for smooth wave modulations as they were for square wave modulations (Figure 5). The parameters of equation 10 were similar in both cases (compare experiments 4 and 5, 7 and 8 in Table 4). There was sufficient scatter in the data to preclude the detection of small differences in K_ν between smooth and square wave modulations, but there did not appear to be major differences in these parameters.

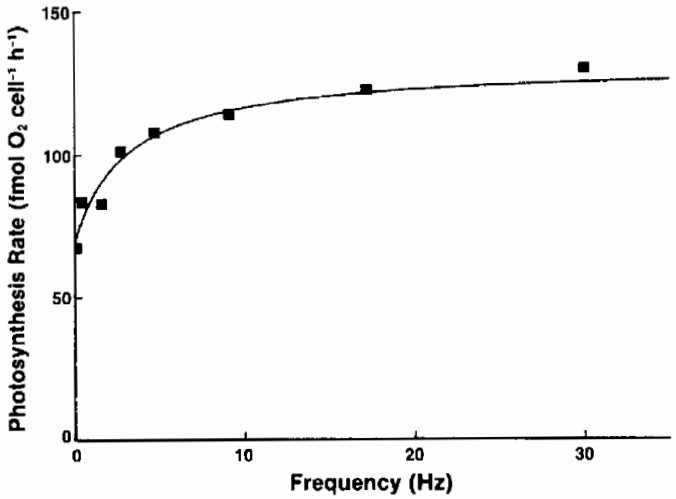

Figure 5. Photosynthetic rate as a function of modulation frequency for Chryso/F-1 (experiment 8). Each point represents the mean of three replicate determinations. The curve represents the equation

$$P_G = P_G^o + (P_G^* - P_G^o)\Gamma_m \nu / K_\nu = \nu)$$

employing the parameters listed in Table 4.

Preconditioning Effects. A population of Phaeodactylum tricornutum which was preconditioned at a low population density, and hence a relatively high average light intensity within the culture (experiment 2), showed a Γ versus ν relationship which differed significantly from that observed for a high density population (experiment 1, note that the high density population was diluted for photosynthesis measurements). Much higher frequencies were necessary for populations grown at low density to achieve significant light intensity integration than were required by high density populations; K_ν was 6.82 for the former compared with 0.67 for the latter. For the multisectored disk employed to generate high frequency modulations, the time required for passage of the sector edges across the

light beam occupied a significant fraction of the total cycle period; thus, P_G^o was calculated from computer acquired light data, as for the smooth wave experiments. (The same determinations with the single-sectored disk led to estimates of P_G^o which differed only slightly from $\phi P_G(I_o)$: Table 4).

The values of K_ν determined for Chryso/F-1 were also widely variable (experiments 3-8, Table 4). Differences in K_ν between sets of similarly preconditioned cells (3-5, 7-8) were greater than differences within these sets. In experiment 6, for which the preconditioning parameters were not measured, the value of K_ν=48.2 was particularly high, but the data appear to strongly support a value of this magnitude. Γ increased in a nearly linear fashion with frequency, and at 30 Hz, the highest frequency available from the experimental apparatus which was used, Γ was about 0.4. The value of P_G^o was confirmed at the conclusion of the experiment by a direct measurement in constant light of intensity $\bar{I}$.

Discussion

Light Intensity Integration. Equation 10 provides a concise empirical description of the light intensity integration response to light modulations of various frequencies. This equation describes the frequency response to both square wave and smooth wave modulations. For cells with a given preconditioning history the parameters of the equation do not appear to differ appreciably between wave forms, although the data obtained show sufficient scatter that small differences would not have been detected. The parameter Γ_m should be equal to 1 in order to be consistent with the hypothesis of Weller and Franck (1941) that the maximum effect of light modulation is light intensity averaging. The data from experiment 1, which is the most detailed determination available, are fully consistent with this hypothesis (Γ_m=0.972). In many of the other experiments, it was necessary to set Γ_m to 1 in order to estimate K_ν, due to limitations of the experimental device which prevented the use of freqencies high enough to yield Γ values in significant excess of 0.5. When these limitations were remedied, the additional values of Γ_m which were determined were also near to 1 (e.g. experiments 2 and 8).

While the value of Γ_m can reasonably be fixed at 1 (and hence eliminated from equation 10), there appears to be considerable variability in the value of K_ν with the preconditioning history of the algae. Populations preconditioned at low light intensities (lowered by self-shading of the cultures) exhibited low values of K_ν. However, since cells in heavily self-shaded cultures (e.g. experiment 1) experience light modulations as they are mixed through the culture, it is not possible to determine from the present data whether the low K_ν values for these cells reflect adaptation to a low mean light intensity or to a modulated light environment. Additional experimentation would be required to resolve these differences.

Surprisingly, there was little variability in K_ν with ϕ and I_o (experiment 1). The generality of this result, however, cannot be assumed without similar factorial analyses with different species under different preconditioning regimes.

Photosynthetic Efficiency Enhancement. While equation 10 provides a direct empirical description of light intensity integration data, applied interest in modulated light effects is based on a potential for productivity enhancement in outdoor algal cultures. The total amount of light available to outdoor cultures is fixed by geography and climate, and productivity in these cultures is determined by their photosynthetic efficiency. The relative gross photosynthetic efficiency of a culture which experiences a constant intensity I_o is

$$\varepsilon_G = \frac{P_G(I_o)}{I_o} \tag{11}$$

However, it is the net rather than the gross photosynthesis of algal cultures which is of interest. Net photosynthetic efficiency is

$$\varepsilon_N = \frac{P_G(I_o) + R}{I_o} . \tag{12}$$

In a flashing light regime with complete light intensity integration

$$\varepsilon_N' = \frac{P_G^* + R}{\bar{I}} \tag{13}$$

or for any proportional integration Γ $(0 < \Gamma < 1)$

$$\varepsilon_N' = \frac{P_G^o + \Gamma(P_G^* - P_G^o) + R}{\bar{I}} . \tag{14}$$

The enhancement of photosynthetic efficiency due to light modulation is

$$E = \frac{\varepsilon_N'}{\varepsilon_N} = \frac{P_G^o + \Gamma(P_G^* - P_G^o) + R}{\phi\, PG(I_o) + \phi\, R} \tag{15}$$

The enhancement of photosynthetic efficiency which is obtained with modulated light thus depends on ϕ, I, and Γ. E depends on ν as well, since Γ varies as a function of ν (equation 10).

Equation 15 can be used to generate a diagram similar to that shown by Terry (1984) for the data of Phillips and Myers (1954) showing photosynthetic efficiency enhancement contours in the t_L-t_D plane, but with

a greatly extended range (Figure 6). The values of the parameters of this relationship (which are K_I, m, R and K_ν; normally, Γ_m=1) will vary between species and between cells with different preconditioning experience.

A frequently applied rule of thumb has been that the optimum enhancement of photosynthetic efficiency can be achieved through the utilization of light:dark ratios of about 1:10 (Kok 1953, Phillips and Myers 1954). Optimum combinations of light and dark periods are shown in Figure 7. Calculated optimum t_L:t_D ratios vary with the length of the light and dark periods and with the intensity of the light source. The value of 1:10 represents a useful rule of thumb over part of the range, but detailed knowlege can lead to the selection of ratios which differ significantly from this value under some conditions.

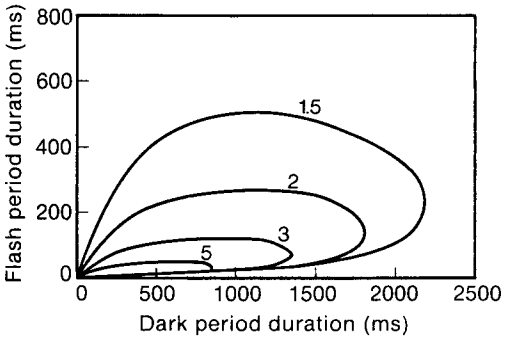

Figure 6. Values of the photosynthetic efficiency enhancement factor E, calculated from equation 15 with the parameters determined for P. tricornutum in experiment 1, plotted in the t_L - t_D plane.

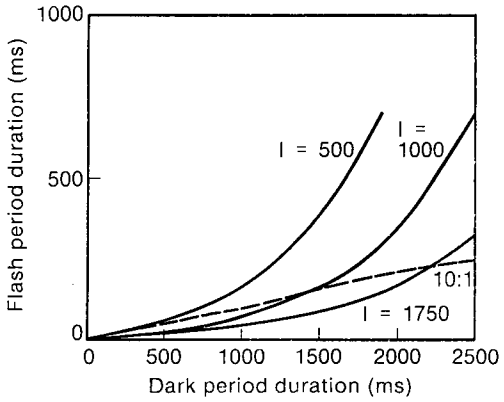

Figure 7. Optimum flash period length as a function of dark period length for P. tricornutum, based on data such as that shown in Figure 4. The three solid lines show optima for light intensities of 100, 200, and 350 W m^{-2}. The broken line represents a light:dark ratio of 1:10.

Growth in Modulated Light. The data presented above describe the modulated light response in terms of photosynthesis rates of cells preconditioned in various environments, but in no case were the cells preconditioned to the modulated light environments in which the rates were determined. Thus, these rates do not represent a fully adapted response. Adaptation to a specific modulated light environment would be expected to improve performance; thus, the enhancement profile presented here should be regarded as a conservative estimate of the enhancement potential. To examine this hypothesis, values of E calculated according to equation 15 with the parameters observed for P. tricornutum (experiment 1) were compared with observed growth efficiency enhancements (Terry and Hirata, unpublished data) (Table 5). In both cases, the fully adapted response was greater than the short-term response, consistent with the above

hypothesis. Thus, the values given in Figure 6 should be regarded as conservative estimates of the enhancement potential.

Table 5. *Phaeodactylum tricornutum* Growth in Flashing Light

I $W\ m^{-2}$	ν (Hz)	ϕ ---	t_L (s)	t_D (s)	E_{obs} ---	E_{pred} ---
117.4	1	0.2	0.2	0.8	2.66	1.86
72.4	1	0.5	0.5	0.5	1.35	1.26

Equation 15, with the parameters determined for *P. tricornutum* in experiment 1, was used to calculate the enhancement of productivity which would be predicted for an outdoor culture over a full diel cycle (including dark respiratory losses). For each time interval during the diel cycle, the attenutation of the incident light intensity with depth was calculated, and the depth integrated photosynthetic rate (P_G^o) and average light intensity over depth ($\bar{I}$) were estimated. Then, for a specified turnover frequency ν the value of Γ was calculated from equation 10, and the enhancement factor E was calculated from equation 15 (recall that $P_G^* = P_G(\bar{I})$). Photosynthetic rates with and without mixing were integrated numerically over the diel cycle and enhancement calculated as the rate with mixing divided by the rate without mixing. For each turnover frequency, the enhancement factor was dependent on the areal density of the population; the response at optimum population density is presented here (Figure 8). A detailed description of the model employed is beyond the scope of this paper, and will be presented elsewhere.

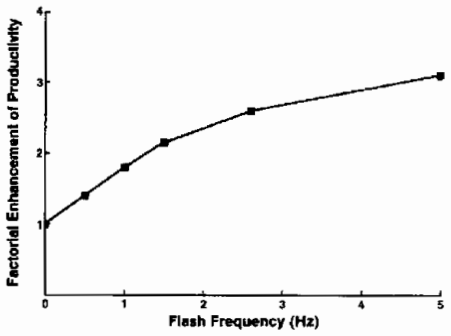

Figure 8. Factorial enhancement of productivity as a function of the frequency of light modulation, calculated for a hypothetical outdoor culture of *P. tricornutum* using the parameters given in Table 4. See text for details.

Application to Outdoor Cultures. The outdoor culture environment differs significantly from the laboratory environment with respect to a wide variety of features, but from the point of view of modulated light effects on photosynthetic efficiency the most important of these are diel fluctuations in the incident light intensity I_o and attenuation of light intensity with depth in the culture. These processes create an environment in which some cells at some times receive low light intensities which are utilized with good photosynthetic efficiency. Thus, the potential of light intensity integration to improve photosynthetic efficiency is somewhat less in outdoor cultures than in experimental cultures receiving constant high intensity light.

For cycle frequencies of 0.5 to 1.0 Hz, enhancement factors of 1.4 to 1.7 were predicted based on the data obtained for P. tricornutum. These values are somewhat lower than the enhancement factor of 2.2 observed by Laws et al. (1984). Adaptive responses of the cells to the modulated light environment were not included in our estimate, and may explain at least a part of the difference. The improvement in productivity observed by Laws et al. (1983) with the introduction of the mixing foils may not be entirely explained by modulated light effects, but the present data indicate that these effects may have been responsible for a significant portion of the enhancement observed.

Analysis of enhancement over the diel cycle also pointed out that the majority of the benefit obtained from intensive vertical mixing in outdoor cultures is obtained during the few hours of the day when the sunlight intensity is highest. Systems for mixing which are only active during these few hours of the day could attain most of the benefits of light modulation with considerable savings in energy costs. However, these systems must be designed in such a way that culture stability will be maintained during periods of reduced mixing intensity.

Conclusions

1. The effects of square wave or smooth wave light intensity modulations on gross photosynthetic rate can be empirically described by a hyperbolic relationship between the frequency of modulation and the degree of light intensity integration which is attained (equation 10).

2. Complete light intensity integration represents the limit of the modulated light effect (Γ_m=1); under a fully effective modulation regime, cells photosynthesize at a rate determined by the average intensity which they receive, further confirming the hypothesis originally proposed by Weller and Franck (1941).

3. The half integration constant (K_ν) of equation 10 varies widely with the preconditioning experience of the experimental population. The common observation that high frequencies are required for modulated light effects may reflect the preconditioning of the populations employed.

4. Significant enhancements of photosynthetic efficiency are possible with modulation periods on the order of seconds, although somewhat less enhancement can be expected under natural lighting conditions than in laboratory apparatus. A significant portion of the productivity enhancement which was observed upon the introduction of vortex mixing devices in to an outdoor system (Laws et al. 1983) can be explained on the basis modulated light effects.

Continuing Research

Most of the objectives of this research have been accomplished, and efforts in this area are being phased down. Further parameterization of the observed relationships will be required for specific systems and specific organisms, but does not appear to be required at this time. Future work, at a low level of effort, will include investigations of preconditioning effects on the value of K_v and of the possible significance of light modulations in reducing photoinhibition.

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Addendum

Laboratory Systems Development (S. Hock, K. Terry, S. Lien)

The development of a data acquisition and control system for laboratory continuous cultures and other laboratory experiments has significantly

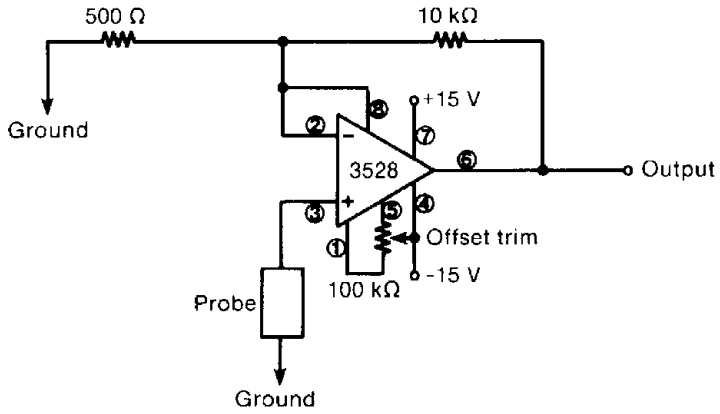

Figure 9. A high impedence ($10^{13}\Omega$) operational amplifier circuit for ion-selective electrodes. The millivolt signal from the electrode is converted to a voltage suitable for use with a low impedence A/D converter. The circuit also applies a gain of 20 to the input signal, which helps to significantly reduce the relative noise level. The circuit is based on a Burr-Brown 352B ultra low bias current FET operational amplifier, chosen for its operating features, availability (delivery less than two weeks), and low cost (about $25). The power supply was a Burr-Brown Model 551 (±15 V, ±50 mA); since each amplifier draws only about 1 ma, a number of these op amps can be driven by one supply. The cost of the power supply is $70, which can be spread over several amplification circuits to reduce per unit costs. Signal conversion to pH values are accomplished digitally.

broadened experimental capabilities. These systems have been developed primarily for use in our laboratories, but information concerning systems and monitoring components is also available to ASP subcontractors. The acquisition and control system employs a Keithley System 500 multiplexing unit interfaced with an IBM personal computer. Software was developed through the use of the Keithley/DAS Soft 500 command package. While system modifications will continue, the present system provides the necessary framework for the control of experimental cultures. An independent system for data acquisition employing an HP85 has been developed for use with batch culture experiments.

In some cases it has been necessary or desirable to develop sensing components for the data acquisition and control systems. A pH amplifier which is presently being employed in our monitoring and control system, at considerable cost savings over the dedication of commercially available pH meters, is shown in Figure 9. A flow-through turbidity sensor employing CO_2 gas flushing to retard fouling is in the preliminary stages of development. Such a sensor will be a necessary improvement over our present system of full-culture turbidometry if high-density cultures are to be accurately monitored and controlled.

OUTDOOR CULTIVATION STUDIES

PRODUCTION OF LIQUID FUELS AND CHEMICALS BY MICROALGAE

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ABSTRACT

With a two species rotation--a high temperature Chlorella sp. in the summer and a Scenedesmus sp. in fall, winter, and spring--a yearly average of 15 gm/m^2/day was obtained in sequential batch, nitrogen sufficient cultivation. At a location where maximum ambient temperatures are above 25°C for 10 months, and minimum ambient temperatures above 10-15°C, 25-30 gm/m^2/day could be achieved. Further increases in productivity may result using strains prescreened for tolerance to high DO, low respiratory rates at high temperature, tolerance to high irradiance, and broad temperature optimum.

Productivity was independent of mixing speed, for mixing regimes characterized by random turbulence, at least in the range from 1-30 cm/sec up to productivities of at least 25-30 gm/m^2/day. Mixing need only be sufficient to keep cells in suspension (15-20 cm/sec at most). Higher mixing speeds require substantially more power input and increase CO_2 losses due to outgassing.

Induction of storage products, under nitrogen-depleted conditions has little negative impact on productivity if accomplished within one to three days.

CO_2 was the largest cost factor. Culture operation must be optimized for high productivity (which increases with CO_2 level) and reduced CO_2 outgassing (which increases with CO_2 level and mixing speed). Sources of CO_2 must be found which are both plentiful and below present market prices. Media recycle is a feasible method for conserving water.

EXTENDED CULTIVATION RESULTS

Three species of algae were cultivated in 100 m^2 (and 200 m^2) ponds for at least one month. Two species were wild types that invaded the ponds during cultivation of inoculated species. One, a Scenedesmus quadricauda invaded during freshwater cultivation of a laboratory grown strain, S. obliquus 1450. The S. quadricauda was cultivated for thirteen months in freshwater and for three months in brackish water (4 ppt TDS). In freshwater it was grown both continuously (continuous dilution over daylight hours) and semicontinuously (sequential batches diluted every three to seven days). Another wild strain, Chlorella sp. arose during attempts to grow Oocystis (Walker Lake isolate) in brackish water (4.5 ppt TDS), during July 1984. It was cultivated for two months, Aug.-Sept. 1984, semicontinuously. The S. quadricauda provided both long-term productivity data and, due to its pronounced tendency to form large settleable clumps, data on water recycling and biomass removal. The Chlorella, on the other hand, grew as non-settleable single cells which afforded the opportunity to measure productivity as a function of channel mixing velocity even at the very low end of the scale.

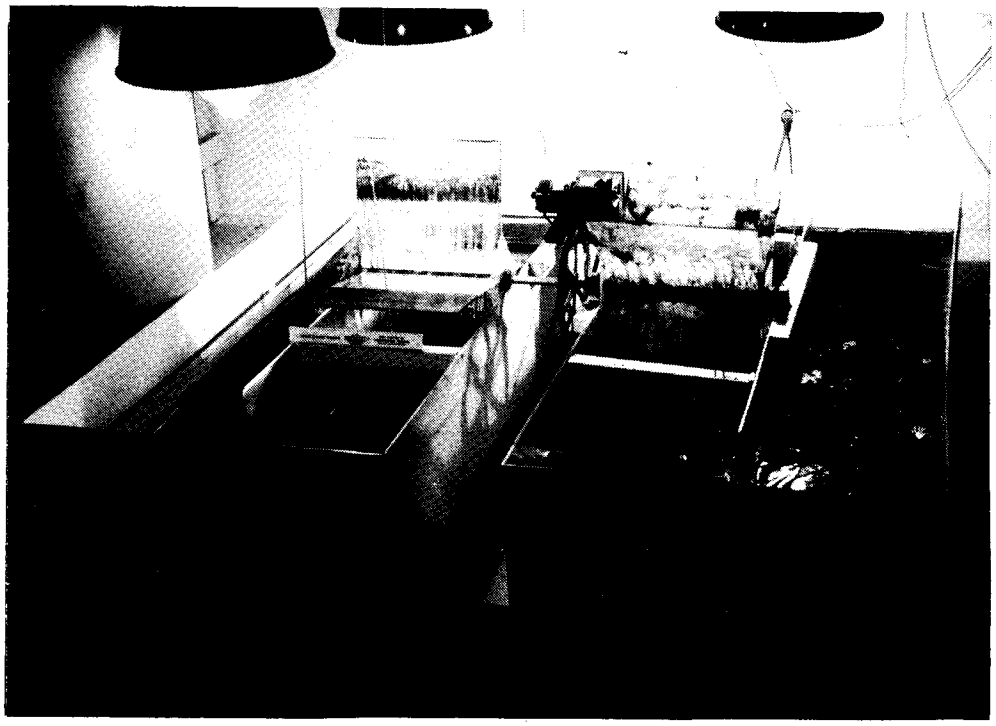
Two strains, provided by species screening efforts of other subcontractors, were also cultivated. Ankistrodesmus falcatus was grown for two months at a time in the Fall of 1983 and Spring of 1984 in brackish water (4-8 ppt TDS). A freshwater Scenedesmus sp, So2a was grown during October 1983 both continuously and semi-continuously for a short period of time. However, neither of these strains could outcompete wildtype organisms in the long run. Two other pre-screened strains, an Oocystis and Chlorella SOla were introduced into the outdoor environment but could not be grown long enough to inoculate the large-scale ($\geq 100 m^2$) ponds.

Ten months of productivity data obtained from semi-continuous cultivation of S. quadricauda in freshwater are shown in Figure 1. The sustained average productivity for nine months (excluding Nov. 1983-February 1984 when productivity was about 3-5 $gm/m^2/day$) was 14 $gm/m^2/day$. Exclusion of one more month, July 1984, during which time high temperatures eventually led to the death of the cultures, sustained productivity averaged almost 16 $gm/m^2/day$. Photosynthetic efficiencies, based on total insolation varied between 1.2 and 2.2%. The lowest efficiencies were recorded during the warmest months. Mid to late spring appeared to be optimal for cultivating this organism. The productivities from three months of continuous cultivation are compared to concurrent semi-continuous values in Table 1. The difference in productivity was greater during the summer. Maximum pond temperatures were also lower during continuous cultivation as expected due to the significant dilution with water at 20-25°C. Another important distinction is the maximal density achievable at harvest time. Here semi-continuous (batch) cultivation is advantageous since densities were twice as great when productivity was reduced only 25%. In 1984, typical batch densities reached 700-800 ppm at time of dilution.

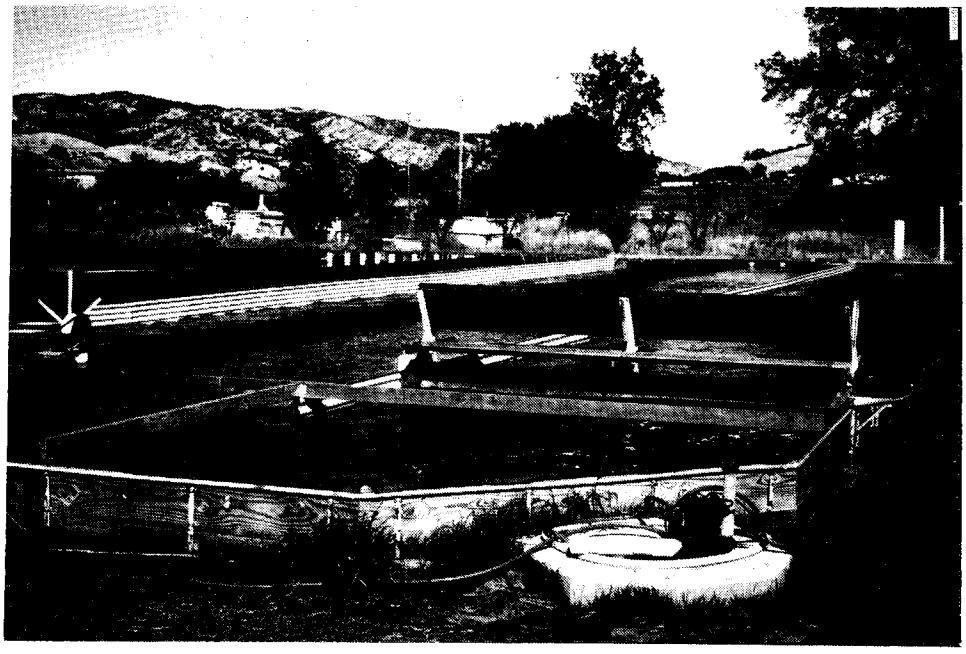
During continuous cultivation several colonies associated forming clumps of 20-100 μm in typical dimension. However, during batch growth, hundreds of colonies clumped into flocs of one hundred to several thousand microns. Except during July 1984 when growth of the strain was tentative, these clumps were dense in cells and sparse in cell debris and detritus. It was typical that as temperature exceeded the optimum of 25-30°C, more and more of the biomass of a clump was composed of cellular debris.

Table 1. S. quadricauda Continuous vs. Sequential Cultivation

Month/yr	Cont. Prod. ($gm/m^2/day$)	Typical Density (ppm)	Seq. Batch Production ($gm/m^2/day$)	Typical Max. Batch Density (ppm)	Productivity (% Increase)
7/83	18.6	170	14.8	275	26
8/83	18.8	150	14	350	34
9/83	15	140	14	350	7



Indoor 1 m^2 Tanks Used for Studies Conducted Indoors during Winter Months



Experimental (100 m^2) Pond System Operated in California

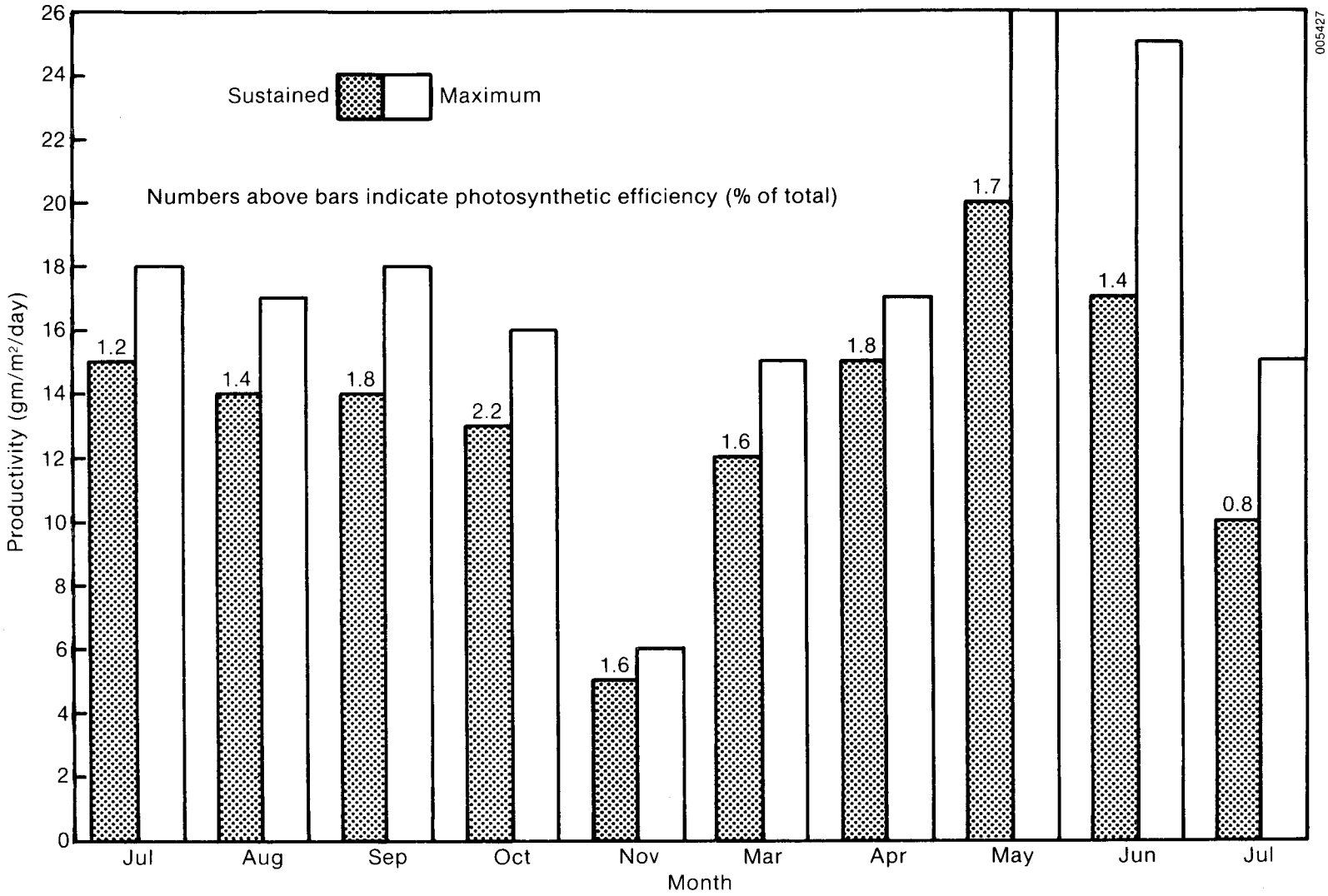


Figure 1. Longterm Productivity: S. quadricauda Freshwater

The wildtype Chlorella was the naturally dominant organism during the summer in medium containing 4.5 ppt TDS, 2.5 mM Mg^{2+} and 3 mM K^{+}. Ankistrodesmus and Oocystis were the organisms originally intended to be grown in this medium. However, the Chlorella was only sustainable during the warmest time of the year. As temperatures dropped in October 1984 to below 15°C minimum and 25°C maximum, the organism died. Table 2 shows average weekly productivities measured with the Chlorella sp. Sustained and maximum productivities were higher with Chlorella during the summer, than with S. quadricauda.

Ankistrodesmus falcatus was cultivated during the late summer and fall of 1983 and again during the spring of 1984. Although the original medium called for vitamin supplementation, indoor experimental results indicated that this was not necessary. Average production from this strain was only 12 gm/m^2/day. It was difficult to prevent contamination by S. quadricauda during April 1984 and impossible during May 1984. Increasing the salinity from 4 to 8.5 ppt in April did not lead to faster growth, higher productivity or increased competitiveness. During the summer months of 1984, growth of Chlorella prevented the establishment of even a small-scale outdoor Ankistrodesmus culture.

None of the strains tested in the outdoor system produced lipids in large amounts, either when N-sufficient or starved. Table 3 lists lipid content of the strains used outdoors.

Table 2. Chlorella Productivity Summary

Mo/Wk	Average Productivity gm/m^2/day	Lgly/d	Eff. % Tot	Maximum Productivity gm/m^2/day	Lgly/d	T,°C Min/Max	Typical Batch Ending Density ppm
8/1	23	641	2.0	30	641	19-32	560
2	22	610	2.0	31	552	21-32	390
3	18	601	1.6	23	615	21-32	325
4	22	525	2.3	26	536	19-30	625
Mo. ave.	21			27			
9/1	19	560	1.9	26	565	18-29	400
2	21	506	2.3	--	--	18-29	650
3	11	490	1.2	15	497	20-30	450
4	11	427	1.4	15	417	15-25	400
Mo. ave.	16			20			

Table 3. Lipid Content of Mass Cultures

Organism	N-Sufficient			N-Starved		
	# tests	% lipid	S.D.	# tests	% lipid	S.D.
S. quadricauda	8	20.2	2.4	2	13.8	0.1
Ankistrodesmus	9	26.3	3.2			
S. So2a	2	18.7	0.3			

PHYSIOLOGICAL FACTORS AFFECTING NET PHOTOSYNTHETIC YIELD

Experiments were conducted to try to ascertain the reasons for low productivity in specific cases, and the appearance and disappearance of particular strains at certain times of the year. The S. quadricauda faltered during the hottest months, but dominated ponds at other times. The results indicated that (1) this strain is respiratory at high temperatures, (2) that it is susceptible to grazing at high temperatures, i.e., when grazing activity increases, and (3) it is relatively tolerant to very high levels of dissolved oxygen.

In several instances productivity profiles were calculated in an attempt to determine how the biomass increase was distributed over the daytime. Table 4 summarizes the split day profiles from S. quadricauda cultures (and one Ankistrodesmus culture). An afternoon productivity depression was observed on 6/13 and 6/15. A disproportionate amount of total yield was obtained prior to 1300 hours. The effect was greater in heated ponds, and appears to correlate with pond temperature, with a greater decrease in efficiency occurring during warmest afternoons. Thus respiration is a possible cause.

Experiments were conducted in the laboratory in 1-L Roux flasks to test growth in terms of culture conditions. Both strains cultivated outdoors and strains of importance to the ASP were used. The results using S. quadricauda are presented in order of descending productivity relative to the control in Figure 2. Only small decreases in productivity were measured as salinity was increased, and/or as DO was made very high, and/or as temperature was increased from 25 to 30°C. The most relevant result (not shown in the figure) is that the strain would not grow at 35°C in freshwater, nor above 30°C in brackish water.

Figure 3 shows results from Chlorella cultures. The pH effects will be discussed later. With this organism, DO had a much greater effect, reducing productivity 50% during the first batch, 85% during growth of the culture after dilution, and (data not shown), killing the culture thereafter. However, switching back from O_2 to air, led to swift and almost total recovery. Figure 4 shows the same results with Ankistrodesmus. However, one important difference was the failure of this strain to recover when switched back to air. With both organisms, partial bleaching of pigmentation accompanied growth under pure oxygen. The Ankistrodesmus failed to "regreen" when switched back to air, as the Chlorella did. Figure 5 shows a similar experiment with Oocystis. O_2 inhibition of yield was faster. This organism grew at 35°C but not at reduced pCO_2.

Table 4. Split-Day Productivities

Date	T, °C			1st Half Photosynthesis			2nd Half Photosynthesis		
				I_T	P	$(P/I_T)x10^3$	I_T	P	$(P/I_T)x10^3$
	0700	1300	1900	lang.	gm/m^2	gm/m^2 lang.	lang.	gm/m^2	gm/m^2 lang.
4/25	8[a]	17	14.5	362	12.5	34±2	161	2.6	16±6
5/7	14	23	29	332	10.2	31	332	9.4	28
	14[b]	24	29	332	8.2	25	332	8.0	24
	14	24	29	330	10.0	30	332	6.4	19
6/13	12	27	26	426	11.7	27±4	267	3.7	14±9
	17[c]	31	29	426	14.5	34±4	267	3.9	15±5
6/15	14	27	27	319	10.6	33±4	347	6.1	18±4
	25[c]	31	29	319	10.9	34±7	347	4.5	13±8

[a]Cloudy afternoon
[b]Ankistrodesmus culture. All others S. quadricauda PE = (P/L x 10^3 x (0.055).
[c]Heated pond

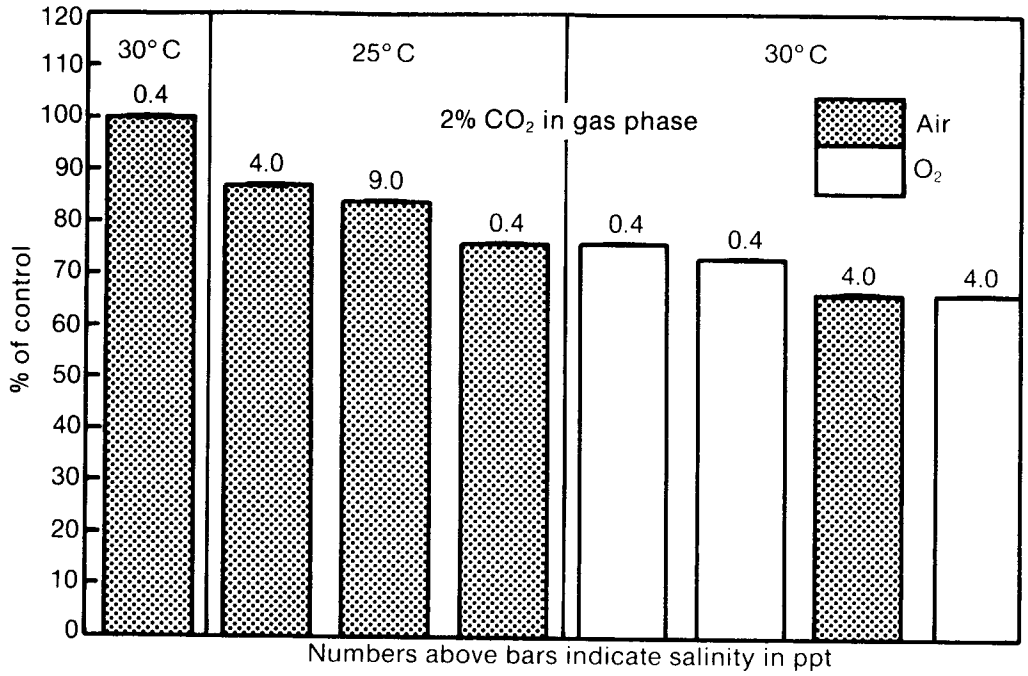


Figure 2. Scenedesmus: Productivity vs O_2, Temperature, TDS

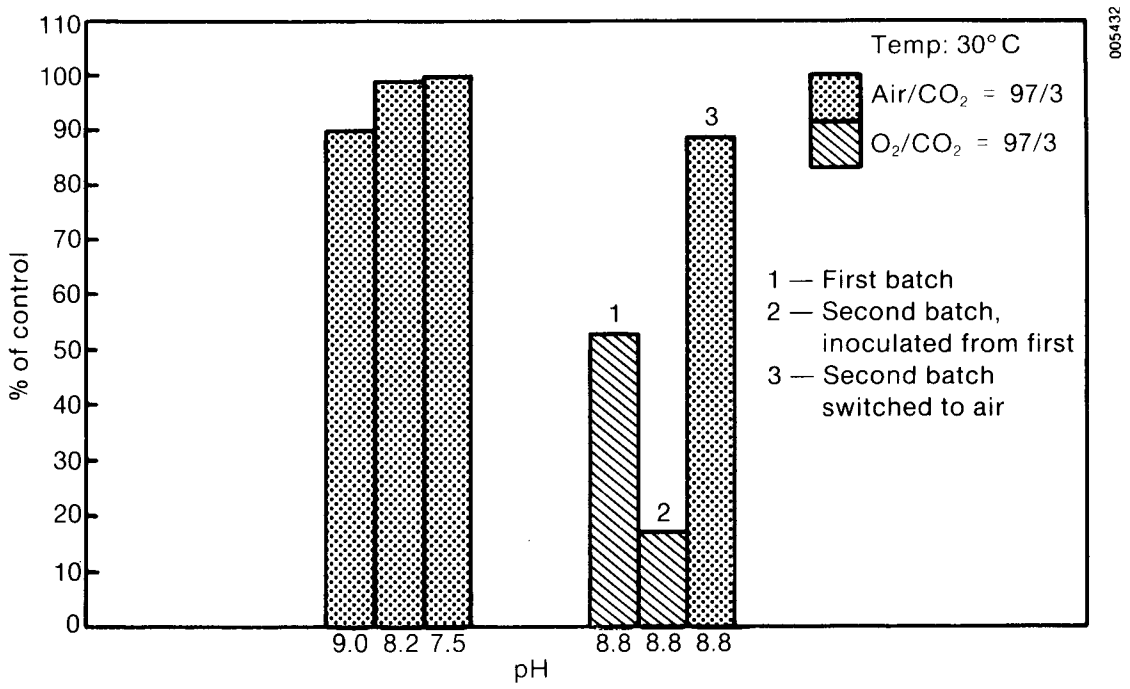


Figure 3. Chlorella: pO_2 - Productivity

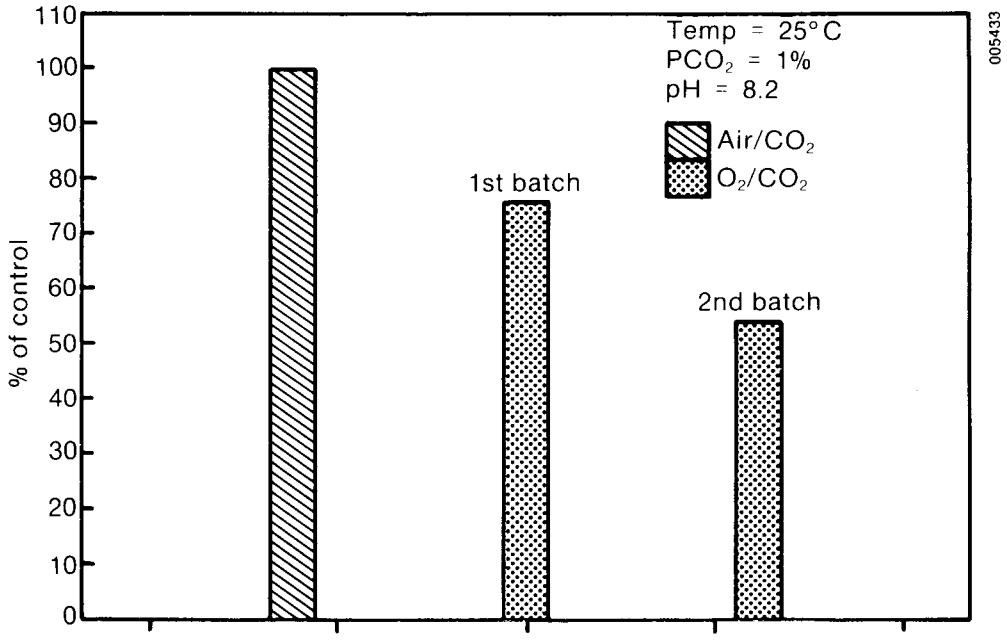


Figure 4. Ankistrodesmus: pO_2 - Productivity

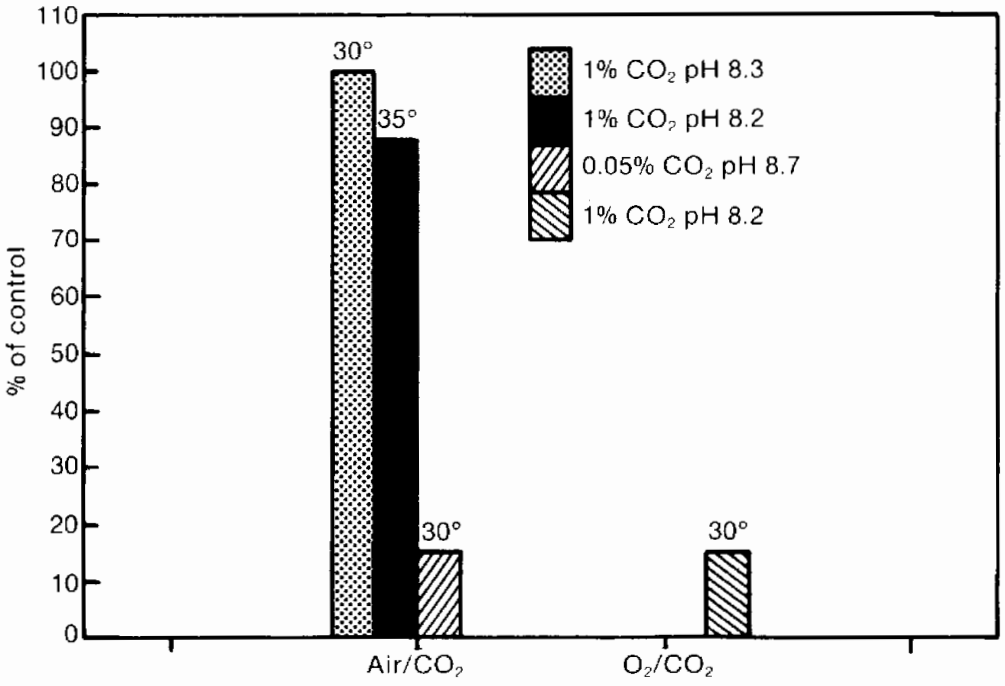


Figure 5. Oocystis: pO_2 - Productivity

RECYCLING CLARIFIED EFFLUENTS

Ankistrodesmus was grown in 1.4-m^2 ponds indoors with and without recycle of the medium. The pH was kept at 7.5 ± 0.3 and the illumination was diurnal (12:12) at 125 W m^{-2}. The effluent was clarified by centrifugation (Sharples T1, 20,000 rpm). On average 98% of the effluent was returned to the pond with fresh medium used as make-up. The culture volume was adjusted for evaporation with distilled water prior to removal of the effluent. The productivity from the recycled pond and a control pond, as a function of sequential batch (cycle) number, is shown graphically in Figure 6. As with all of the Ankistrodesmus cultures, productivity from the recycle and control ponds declined slowly with time. Pond characteristics are shown in Table 5. Even after eight cycles of recycling, little organic matter accumulated in the medium.

An effluent recycle experiment was performed with a mass culture of Scenedesmus for six weeks during June-July 1984. The experiment was terminated when this species failed to grow, in non-recycled ponds as well, during July.

In this experiment, pond effluents were clarified via sedimentation in 2m deep settling ponds. This could be done because of the large size of the flocs (1-2 mm) formed in the growth ponds. Settling ponds were filled by gravity flow from the growth pond. This took about 2 hrs. Sedimentation times varied from 6 to 12 hrs. Clarified supernatents were pumped back to the growth pond using a floating intake. The settled biomass compacted well enough to allow return of about 90% of the effluent. Fresh medium was used to make up the difference. Irrigation water, without mineral supplementation, was used for evaporative make up water. Urea,

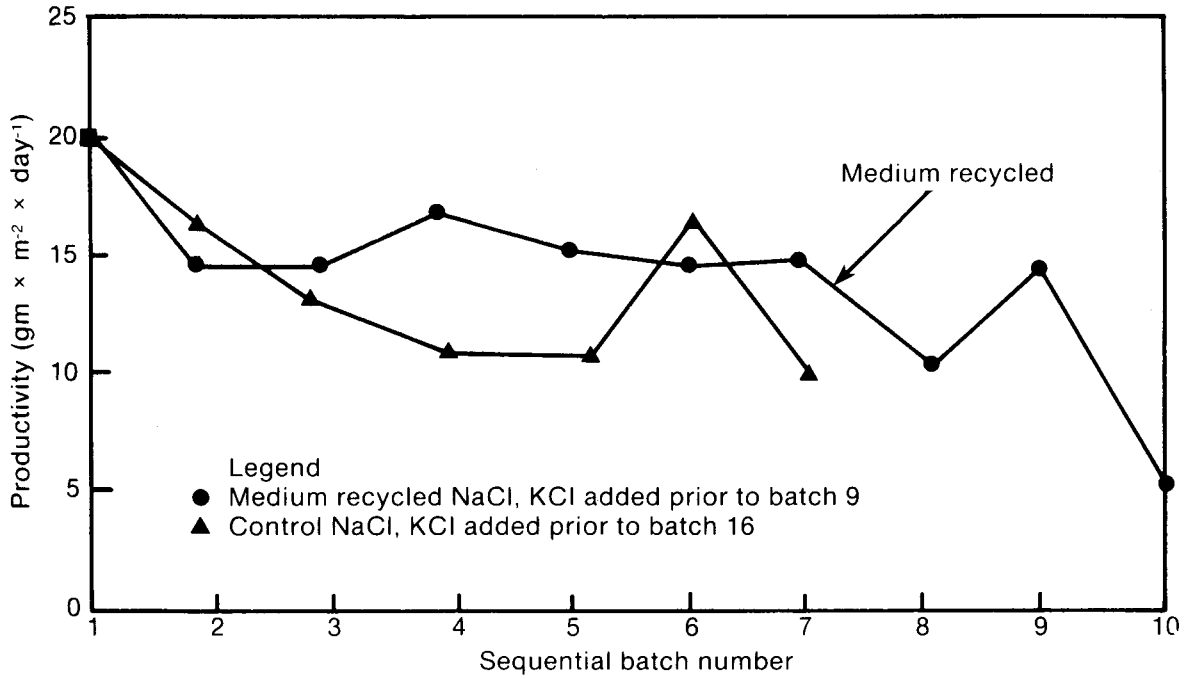


Figure 6. Sustained Sequential Batch Growth of Ankistrodesmus

phosphate, and iron were added to replenish nutrients taken up by algal biomass production. The operational data, productivity, harvested weight, and efficiencies of harvest are shown in Table 6.

MIXING VELOCITY EXPERIMENTS

Results of Hydraulic Measurements

The power and flow measurements for the 100 m^2 pond are summarized in Figure 7. Total power measurements were made over most of the motor's speed range,

Table 5. Media Recycle Dissolved Species

	Start (12-6-83)	Finish (1-13-84)	Units
Alkalinity	20	23	mM
Hardness	400	200	ppm $CaCO_3$
CHO	0	14	ppm glucose equiv.
Protein	0	14	ppm Folin-Lowry BSA equiv.
TOA	--	--	

Table 6. *S. quadricauda* Biomass Harvest--Effluent Recycle

200 m^2 Pond											
Cycle	Harv. Date	Harv. Eff. (%)	Sed. Time (hrs.)	Effl. Dens. (ppm)	Blowdown (%)	Evap. loss (%)	TDS (ppm)	Hd (ppm)	Alk (mM)	Ave. Prod. ($gm/m^2/d$)	Harv. wt. (kg)
0	5/27	99	9	9	12	22	--	--	--	20	25.1
1	6/2	98	8	21	6	16	270	--	--	24.5	24.6
2	6/10	99	12	5	6	25	300	206	7.3	13*	20.3
3	6/17	93	6	55	8	22	326	208	6.6	19	19.6
4	6/24	86	12	87	9	19	450	235	7.5	16.	18.3

*Rotifer bloom, pH raised from 8.3 to 9.3

TDS, Hd, Alk - initial values for succeeding cycle

Hd - ppm as $CaCO_3$

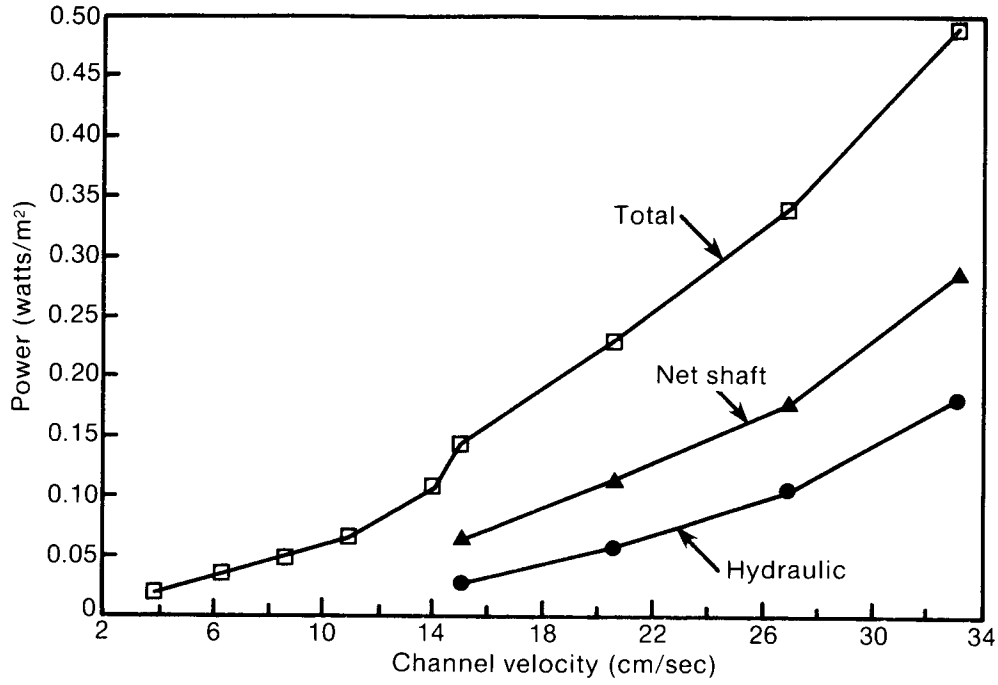


Figure 7. Mixing Power - 100 m^2 Pond

corresponding to channel velocities of 3.8 - 33.1 cm/sec. The cost of mixing is very low at low mixing speed, but increases quickly. At 15 cm/sec, mixing power required (at 100% efficiency) was 0.026 W m^{-2}. At 24 hr/day mixing and 20 gm/m^2/day algal productivity, this is equal to about 0.03 kw.hr/kg algae or about $0.003/kg algae produced. On an energy basis the mixing power required represents only 0.5% of the heat of combustion of the algae output. At 30 cm/sec these numbers changed to $0.02/kg algae and 3.3%.

Mixing - Productivity Experiments

A controlled mixing experiment was conducted in August and September 1984 when the Chlorella was being cultivated. This organism lent itself well to mixing experiments since its small size (3-5μ) precluded settling, even in practically still water. Thus mixing speeds from <1 to 60 cm/sec were tested. Also, the level of DO, which decreases as mixing speed is increased in this range, was kept constant. The major result, productivity vs. mixing speed is shown in Figure 8. It is clear that for productivity up to 25 gm/m^2/day and mixing speeds from 0 to 30 cm/sec there is no dependence of productivity on mixing speed. This result really is not surprising for slow uncorrelated movement of cells in the water column. Each cell responds to the slowly changing irradiance it is receiving. The turnover of cells even at the lowest mixing speed was apparently sufficient to prevent cell death due either to prolonged exposure to the highest irradiance or to total respiratory decay in the darkest zones at the pond bottom. These cultures grew to maximal densities of about 600 ppm.

Mass Transfer Through the Surface

The mass transfer coefficient, K_L, was measured at two mixing velocities: 15 and 30 cm/sec. The measurements were done by measuring the rate of decrease of total carbon from a 100-m^2 pond with known alkalinity. The carbon level was calculated from the carbonate equilibrium equations with constants adjusted for temperature and ionic strength. pH was measured using a Radiometer pH meter capable of accurately measuring .01 pH units. The pH measurements were monotonically decreasing. The range of pH, 7.4 to 7.2, was chosen to preclude effects from back pressure. In the given system, the driving force of CO_2 in the bulk liquid was about 0.61 mmolar. Air equilibrates to about 0.01 mmolar. The resistance coefficient was calculated as:

K_L = transfer rate/(610-10): meters per hour

for 30 cm/sec, K_L = 0.1 m/hr. For 15 cm/sec, K_L = 0.038 m/hr.

CARBONATION

The overall CO_2 utilization efficiency was measured by two methods. In the first method, the biomass production from 2 x 100 m^2 plus 1 x 200 m^2 was totalled over 42 days of operation (5/8-6/20) and then divided by the total CO_2 used, as measured by the difference in the CO_2 meter readings on the six ton CO_2 tank. The result, 3.3 kg CO_2/kg AFDW corresponds to a 30% efficiency,

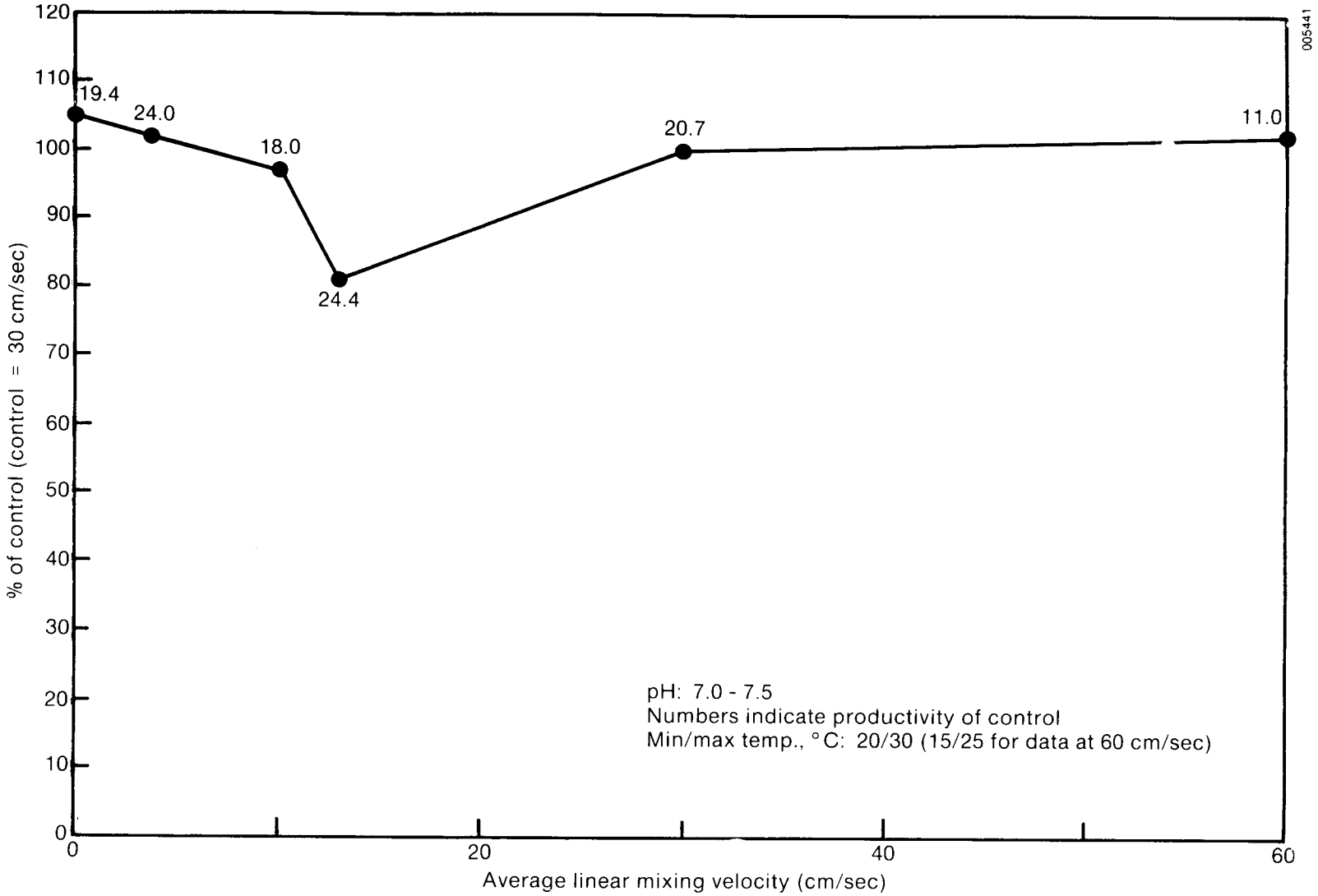


Figure 8. Chlorella Mixing Velocity Experiment

or 54% of that theoretically achievable. This includes injection losses and outgassing losses, but does not account for urea-derived CO_2. The latter only contributes 5% of the carbon input and thus could maximally reduce the efficiency to 29% overall or 52% of theoretical.

In the second method, CO_2 meters and timers, which were installed on the 100-m^2 ponds in August 1984, were used to tally the total CO_2 input to the ponds. The equivalent algal biomass that the total daily CO_2 input could have supported (based on 1.8 kg CO_2/kg algae) was calculated. The actual productivity was divided by this number. The overall CO_2 utilization efficiencies calculated varied from 43-65%, depending on mixing speed. Not unexpectedly, overall efficiencies were greatest at lower mixing speeds where outgassing is reduced. Thus the interplay between algal productivity, mixing velocity, and dissolved CO_2 concentration will determine the economics of CO_2 utilization.

Since outgassing of CO_2 is so dependent on dissolved CO_2 levels, and thus can be very high or very low, experiments were conducted to investigate the growth response of the Chlorella as a function of dissolved CO_2, pH, and alkalinity. In practice alkalinity is usually a given and the pH of operation will determine the CO_2 level. But experiments with differing alkalinities were required to yield specific CO_2 levels independent of pH. Fortunately this organism exhibited a very broad range of tolerance to alkalinity. The results of over 30 experimental runs are shown (averaged) in Figures 9 and 10. Reproducibility was good. Neither maximum specific growth rate nor average production depended on alkalinity. Both were dependent on pCO_2 and pH. However, production was more sensitive than U_{max}.

Average production was more dependent on CO_2 level than pH. At 2% CO_2, pH hardly mattered between 7.0 and 9.0. At 0.5% CO_2 there is some dropoff at pH $>$9.0. There is no significant difference between productivity at 0.5% and 2%. The lowest CO_2, 0.01% (one third air equilibrium) resulted in the lowest production at all pH, but still showed (as in the case of 0.5% CO_2) a 25% decrease at pH $>$9 relation to pH 7. An important conclusion is that pH cannot be used as a variable independent of pCO_2, unless care is taken to vary the two independently. This is not usually done in "pH optima" experiments. In this experiment the effect of pCO_2 on productivity is seen to be more pronounced than that of pH.

The same was found to be true of U_{max}, but to an even greater extent. There was no correlation between U_m and pH, so all of the data is grouped by pCO_2 only. Even the dependence on CO_2 level is less pronounced, with only a 25% reduction in U_{max} compared to a 40% reduction in productivity as pCO_2 is decreased. Maximum specific growth rate determinations are not good predictors of productivity.

Once the response of an alga to CO_2 level and pH is known (or better yet to cycles of changing CO_2 and pH) the specific economics of the use of that strain can be determined. Choice of an organism will depend on the trade-off between productivity at increased CO_2 level and CO_2 loss due to increased outgassing. As an example, the equivalent algal biomass production potential of outgassed CO_2 is plotted in Figures 11 and 12 as a function of pH and alkalinity (that is, dissolved CO_2 concentration) for two different types of waters: brackish water and saline water.

If a given medium, after evaporative concentration contains, for example, 20 mM alkalinity then the range of dissolved CO_2 (pH) at which outgassing is minimal is restricted. If productivity increases as CO_2 level increases, the increase must justify the larger CO_2 outgassing loss. At about 20 mM alkalinity losses are not too severe in low TDS waters down to pH 8 or so, in high TDS waters down to pH ~7.7.

The corresponding dissolved CO_2 concentration is about 0.3 mM (equivalent to equilibrium with a 1% CO_2 gas phase) which does not limit productivity of the Chlorella. pH would rise in between carbonation stations, lowering CO_2 to 0.07 mM (at pH 9.3 or 8.7) which might impact productivity. However, operating closer to pH 7 becomes uneconomical unless alkalinity is very low as in a seawater system without recycle. Even so, the lack of alkalinity lowers the carbon storage capacity so much that the distribution system for CO_2 injection would become prohibitively expensive.

The above discussion is based on a mixing speed of 15 cm/sec. If this is increased to 30 cm/sec, then operation below pH 8 becomes expensive, since outgassing increases 2.5 fold. If the pond is operated at 10 cm instead of 20 cm, the outgassing is increased another factor of about 1.5. In addition, any mechanism which increases the efficiency of vertical movement of water will further increase outgassing losses.

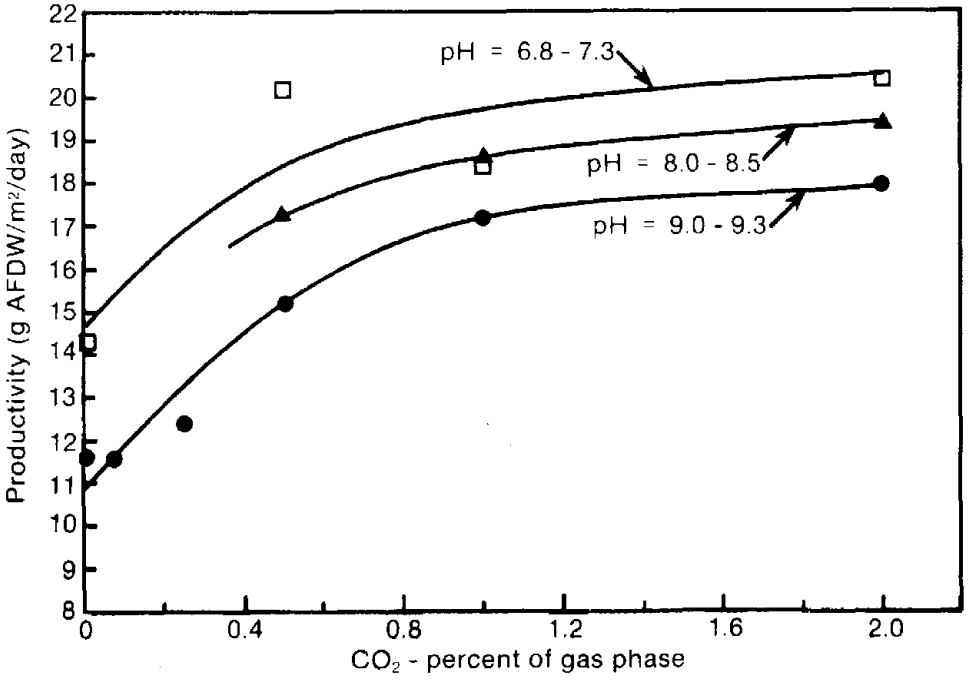


Figure 9. Chlorella: Productivity vs. pCO_2, pH

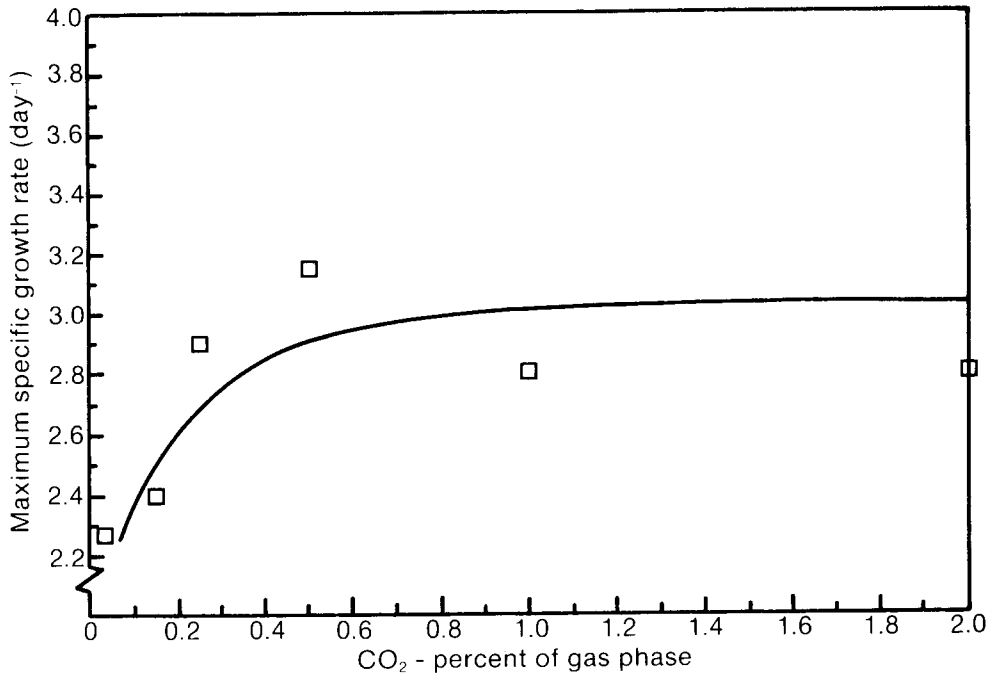


Figure 10. Chlorella: Maximum Specific Growth Rate vs. pCO_2

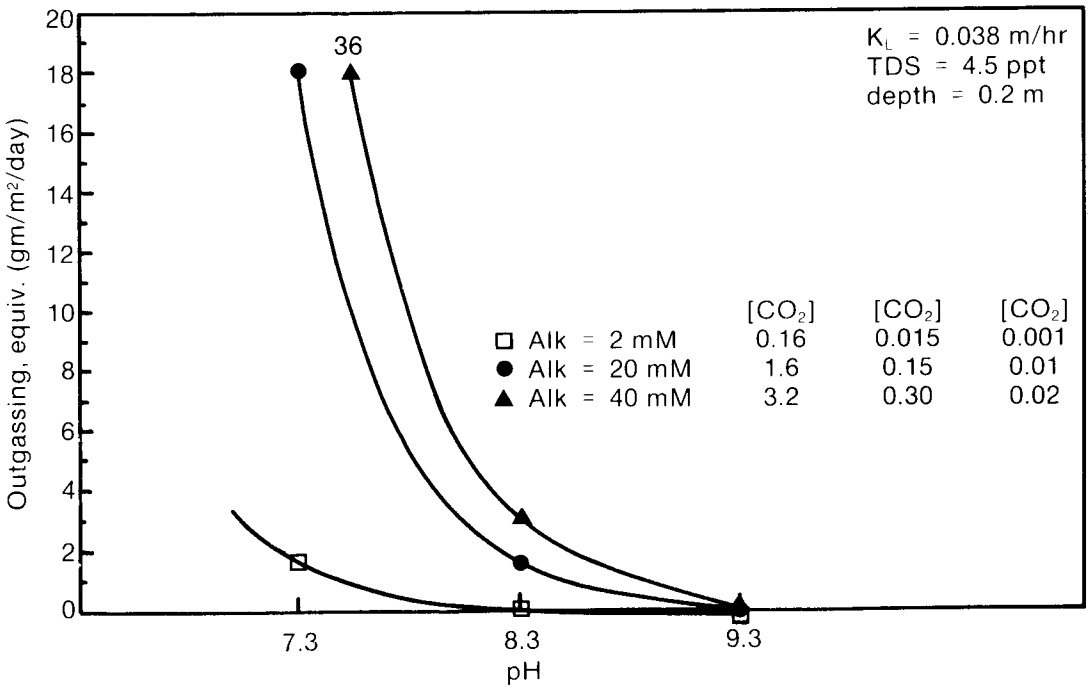


Figure 11. CO_2 Outgassing - 4.5 ppt TDS

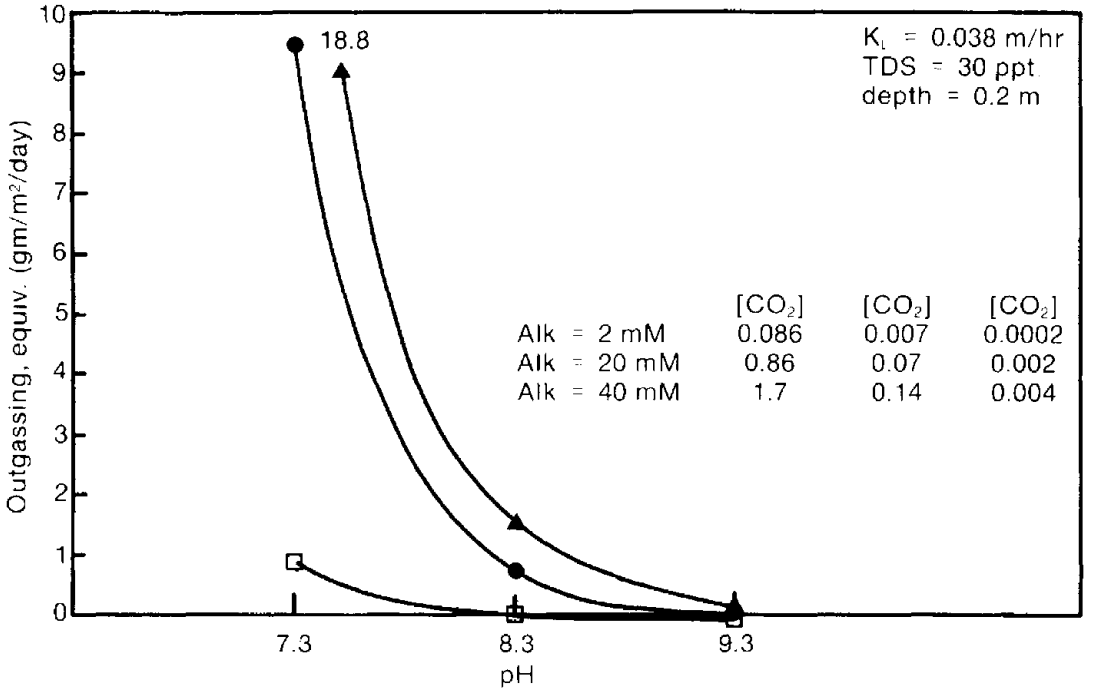


Figure 12. CO_2 Outgassing - 30 ppt TDS

OPERATING COSTS

During the recycling experiment described, the inputs to the system, i.e., nutrients, water, and power, were monitored for about a month, during which time harvested productivity also averaged 15 gm/m^2/day. Thus the cost per unit biomass, of these inputs, can be calculated. Annualized capital costs, costs of labor, and biomass processing are not included in the discussion below.

Table 7 summarizes the costs of inputs during the recycle experiment that is being taken as representative of system performance. During this experiment, the depth of the pond was 20 cm, the mixing speed was 30 cm/sec, and the loss of CO_2 at injection was about 34%. An overall input cost of $1.09/kg, AFDW resulted. Most of this was due to the high cost of CO_2 and its inefficient use. Nitrogen contributed 12% of the total cost, at a biomass N content of 12%. The other nutrient costs are higher than would be expected in a large-scale system since (1) many of these chemicals would have to be available in the water resource and (2) bulk purchasing would decrease unit costs of phosphate and iron. Mixing power cost is based on a high channel velocity that is not necessary when less than 25 gm/m^2/day are being produced.

Table 8, shows the costs of the same factors, but modified to take into account cost reduction possibilities. CO_2 is still the largest cost factor, and is, in fact, still too expensive even at the price of $.05/kg, a representative commercial price available now. An increased utilization efficiency is used: only 5% loss at injection and outgassing losses equivalent to 5 gm/m^2/day algal production. Both should be easily achieved when a sump is used for carbonation, when mixing speed is $\leq$ 20 cm/sec, and when the pH range

Table 7. Initial Algal Biomass Production Costs

Component	Unit Price,$	Amt. Used	Cost,$	% Total Cost
CO_2	0.275/kg	3 kg/kg alg.	68.31	75.9
Urea	0.51/kg	0.26 kg/kg alg.	10.78	12.0
Other Nutr.	--	--	6.71	7.4
Pump power	0.06/kW hr	5.2 kW hr	0.31	0.3
Mixing power	0.06/kW hr	0.42 W/m^2	3.38	3.8
Water	11.5/ac ft	91 m^2/day	0.48	0.5
		TOTAL	89.97	99.9

Notes: 28 days, S. quadricauda, effluent recycle, fresh water, 200m^2.
Total biomass harvested: 82.8 kg (15.0 gm/m^2/day av).
Cost: $1.09/kg AFDW = $0.49/lb.

Table 8. Cost Reduction Possibilities

Component	Cost Reduction	Cost,$	% Total	c/kg alg.	Projected c/kg alg.
CO_2	$.275--0.05/kg CO_2 3 kg/kg--2.5 kg/kg	10.35	51	12.5	6.2
Urea	0.26 kg/kg--0.13 kg/kg	5.39	26.6	6.5	3.3
Other chemicals	All but P, Fe available in water, bulk pricing	3.30	16.3	4.0	2.0
Pump Power	--	0.31	1.5	0.37	0.4
Mixing Power	0.42 W/m^2--0.14 W/m^2 Effic.: 50%	0.45	2.2	0.54	0.5
Water	--	0.48	2.4	0.69	0.6
	TOTAL	20.28	100	24.6	13.0

is chosen to avoid excessively high dissolved CO_2 levels. Even so, CO_2 comprises 50% of the total input costs, at $0.125/kg algae produced. Since this is about all that the biomass fuel precursor is worth, CO_2 cost must be cut further, at least 50% more. Finding large amounts of low cost CO_2 is probably the greatest single obstacle to lowest cost algal biomass production.

SUMMARY

The overall objective of the project was to conceptually determine if simple open pond systems have application for the production of fuels from microalgae. To demonstrate this objective, work concentrated on showing the potential microalgal yields that are possible from an open pond system on a sustained basis. Furthermore, problems (pond management, design) associated with this experimental system were documented and reported so that future endeavors shall benefit. Finally, operational costs were documented to permit preliminary economic analysis of the system.

All of the tasks outlined for this project were addressed during project performance. Of the strains of microalgae provided by other ASP subcontractors, none could outcompete invading wildtype algae. Nonetheless, two strains, Ankistrodesmus falcatus and Scenedesmus SO2a were grown outdoors in mass culture, one for two months, the other for one month. Results obtained using these strains did provide information useful to the screening program in the future. Two wildtype organisms, Scenedesmus quadricauda and Chlorella sp. provided most of the outdoor results. The Scenedesmus was maintained for thirteen months, averaging about 13 $gm/m^2/day$. It was used to compare batch vs. continuous cultivation, for mixing speed experiments, for media recycling, for storage product induction tests, and for the determination of operational costs. The Chlorella was grown for two months at over 20 $gm/m^2/day$, and was used to determine the relationship between mixing velocity and productivity.

The maintenance of a monoculture was not difficult when climatic conditions were relatively constant. Changes of species, from Scenedesmus to Chlorella, occurred when pond temperatures rose above 35°C and reversed when the temperatures dropped. Both wildtypes tolerated a range of TDS and mineral composition. Ankistrodesmus tolerated most medium composition variations, except low K^+, but was sensitive to high irradiance when in dilute suspension. None of the organisms required chelator, trace supplementation (beyond that available in the water used), or vitamin supplementation. The results indicate that a monoculture can be maintained if either the seasonal temperature variations are small or a competitive strain is used which has a broad temperature optimum. A competitive strain is one which does not exhibit significant photoinhibition, oxygen inhibition or respiratory losses. Of course, TDS will exert an overall selection criterion.

As mentioned above, the productivity results obtained with the "summer" Chlorella and the Scenedesmus during the remainder of the year, indicate that a year round average of 15-20 $gm/m^2/day$ is attainable in this northern California climate with the strains used. A yearly average of over 25-30 $gm/m^2/day$ could probably be obtained 500 miles south, where ambient temperatures are higher during spring, fall and winter. Continuous cultivation increased production relative to sequential batch operation, by 30% during summer. However, the typical density of the culture at harvest time was much lower under continuous cultivation. In addition, induction of storage products required one to three days of batch growth after a continuous or batch active growth stage.

Mixing velocity experiments revealed four factors of importance in considering optimum mixing speed: CO_2 outgassing which increases quickly with mixing

speed, power input which increases even more quickly, suspension of cells, and productivity response. It was found that 15-20 cm/sec mixing speed was sufficient to keep even the largest (1-2 mm) clumps of Scenedesmus suspended. It was also found that, from 1 to 60 cm/sec, mixing speed had no effect on productivity.

Experimental results indicated that organisms respond very differently to pH and CO_2 concentration, as well as to dissolved oxygen. The latter may turn out to be an important screening criterion, as high DO is endemic to large systems. The response to pH and CO_2 determines the range of pH (given water alkalinity) in which a system can be operated. The pH should be as low as possible to obtain the highest dissolved CO_2 required for maximizing average production. But this must be balanced against outgassing loss of CO_2 which increases with concentration. Mixing speed affects the surface mass transfer coefficient.

The data obtained from one month of operation of a Scenedesmus culture with effluent recycle was used to evaluate the cost of the inputs to the system. Cell harvesting was inexpensively achieved due to the very good sedimentation properties of this strain which grew in large clumps. The nutrient, CO_2, and power inputs were monitored. The harvested biomass output averaged 15 gm/m^2/day. Initial input costs were \$1.09/kg AFDW and were dominated by CO_2 costs (75% of the total). Realistic assumptions concerning prices of CO_2 and nutrients, bought in bulk, led to a 75% reduction in projected cost. Another 50% reduction would be necessary and would have to come from the assumption of a source of CO_2 at below present market price. Increased productivity, although important in terms of distributing annualized capital costs and labor costs, has little impact on the inputs considered here since most are nutrients that are proportional to biomass output.

The major conclusions of this project can be summarized as follows:

(1) Using two wildtype species in northern California a yearly average productivity of 15 gm/m^2/day, or 24 tons/acre/yr can be obtained in water with TDS = 4-8 ppt.

(2) This can probably be increased to 25-30 gm/m^2/day or 40-50 tons/acre/yr in southern California.

(3) Productivity can probably be further increased by using competitive strains screened for

   a) low respiration rates

   b) tolerances to high levels of dissolved oxygen

   c) broad temperature optima

   d) resistance to photoinhibition.

(4) In systems with randomized, turbulent mixing, productivity is independent of channel velocity at least for productivities up to 25-30 gm/m^2/day and velocities from 1-30 cm/sec.

(5) Storage product induction requires one to three days of growth in batch mode under N-depleted conditions.

(6) Critical cost centers include CO_2 input, harvesting and system capital cost. Increased mixing speed increases CO_2 losses, power input, and system costs and must be adequately offset by very large increases in productivity.

(7) Media recycling, necessary for water conservation, has no adverse effects, at least in the short term for strains which do not excrete organics, and when the harvesting method is at least moderately effective for all algal forms which may be present.

PRODUCTIVITY OPTIMIZATION OF SALINE MICROALGAE GROWN IN OUTDOOR MASS CULTURE

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PROJECT OBJECTIVES

Task I

Evaluate the growth response of at least three thermophilic marine microalgae in water types representative of saline waters found in the southwestern United States.

Task II

Optimize yields of three species in the outdoor raceways, at least two of the species selected from microalgae that have been collected from the Southwestern region of the United States. The third species shall be a marine thermophilic strain that has been shown to grow in Southwestern water types.

Task III

Evaluate management strategies that will improve the culture efficiency (lower costs or increase yields in the outdoor raceway systems.

Task IV (Optional)

Optimize the lipid yield from one strain of oil producing microalgae in the outdoor raceway.

PROGRESS TOWARD OBJECTIVES

Task I

Growth rate studies have been completed on a laboratory scale for four species at temperatures ranging from 25 - 32^{o}C in various water types characteristic of the Southwestern U.S. At the lower temperatures the best growth was achieved in low salinity type I water. At the higher temperatures the best growth was observed in higher salinity type I water or in type II water.

Task II

Production studies in 9.2 m^2 flumes have been completed with three species using Southwestern U.S. water and seawater of comparable salinity as a control. Under the experimental conditions Platymonas grew best in type I water, and Chaetoceros gracilis grew best in type II water. Optimum photosynthetic efficiencies for these two species ranged from 8 - 10% based on visible light. A green flagellate from the Southwestern U.S. grew best in type II water, but photosynthetic efficiencies for this species were less than 5%.

Task III

Optimization studies in the 48 m^2 flume indicated that completely removing the foil arrays reduced photosynthetic efficiencies by a factor of 1.5, but removing half the foil arrays (so that arrays were spaced eight feet apart) reduced phtosynthetic efficiency by a factor of only 1.04. Turning off the CO_2 supply altogether reduced photosynthetic efficiencies from 8 - 10% to less than 3%; turning on the CO_2 at a pH of 8.5 rather than 7.5 reduced photosynthetic efficiency to 6.2%.

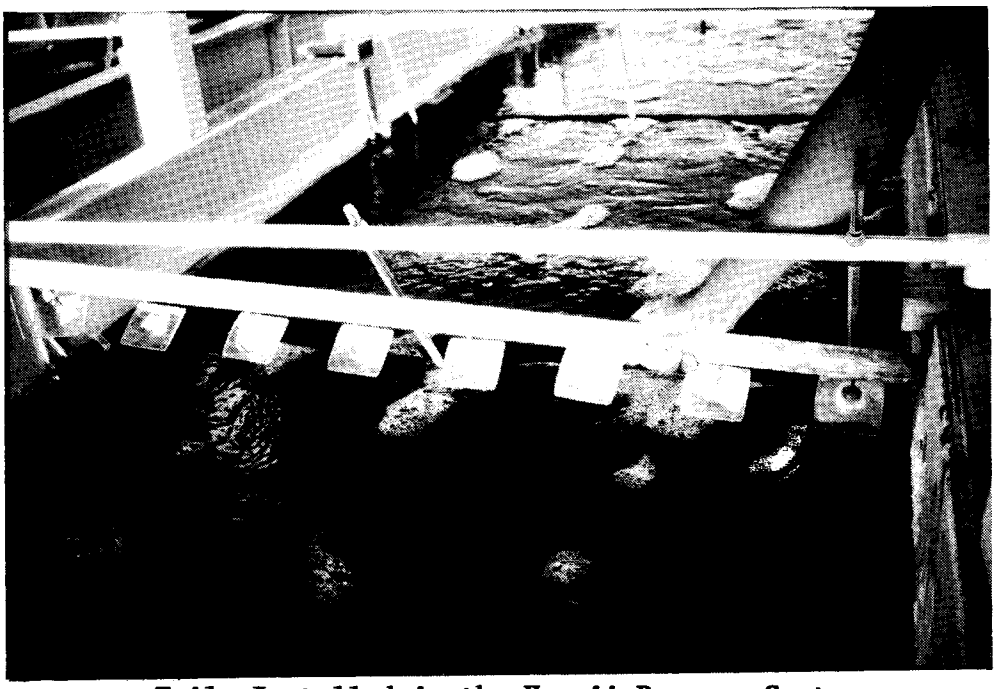
Task IV

Silicate starvation experiments with C. graciles showed that this species can convert over 50% of its carbon to lipid within 2 - 3 days of the onset of silicate limitation when provided with adequate light. Whether similar conversions can be achieved in the light-limited outdoor flumes remains to be seen. Studies of the physiology of Platymonas over the course of several three-day dilution cycles revealed a trend toward greater light limitation as the culture become more dense, but at no time did the culture show any evidence of approaching significant growth rate limitation due to a lack of light. This result may in part explain the remarkably high production rates which have been achieved with this species when grown on a three-day dilution cycle.

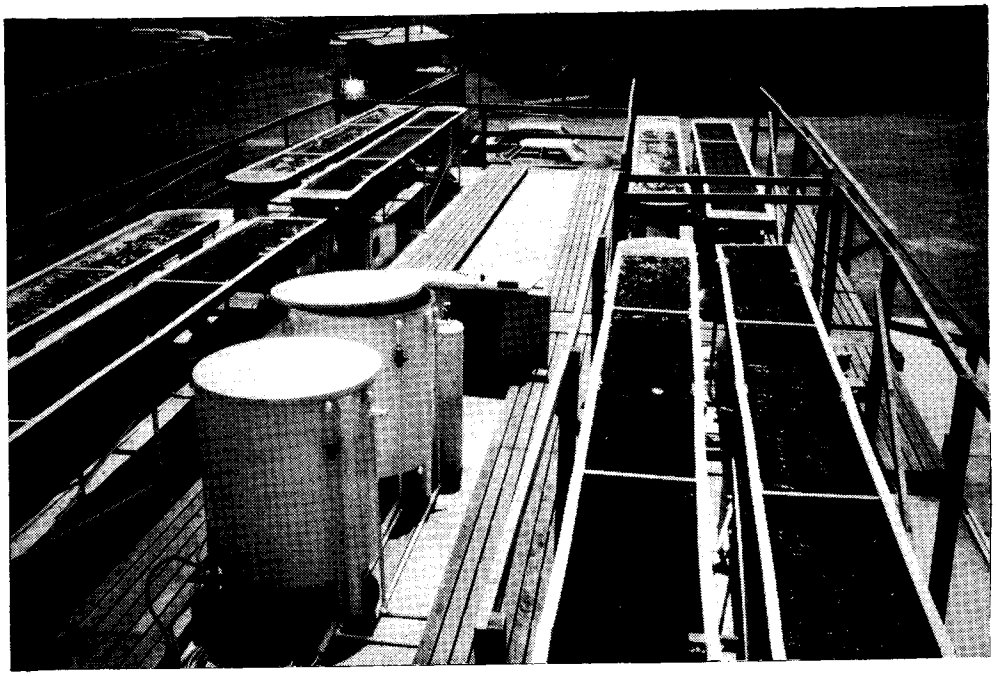
ACCOMPLISHMENTS AND ACTIVITIES

Task I

Laboratory-scale studies of the ability of four species to grow in Southwestern U.S. water types have been completed at temperatures from 25 to 32°C. The results are shown in Figures 1-4. At 25 and 28°C, the best growth was invariably obtained in type I water at 15°/oo. At 30 and 32°C the best growth was obtained either in type I at 25°/oo or in type II water. The chemical composition of the four water types is given in Table 1. All four species showed some tendency to go through a lag period prior to entering exponential growth at 32°C, and I suspect this observation reflects the fact that 32°C is close to the lethal limit for all of them. In general



Foils Installed in the Hawaii Raceway System



Small (8 m^2) Experimental Raceways Used in Factoral Experiments

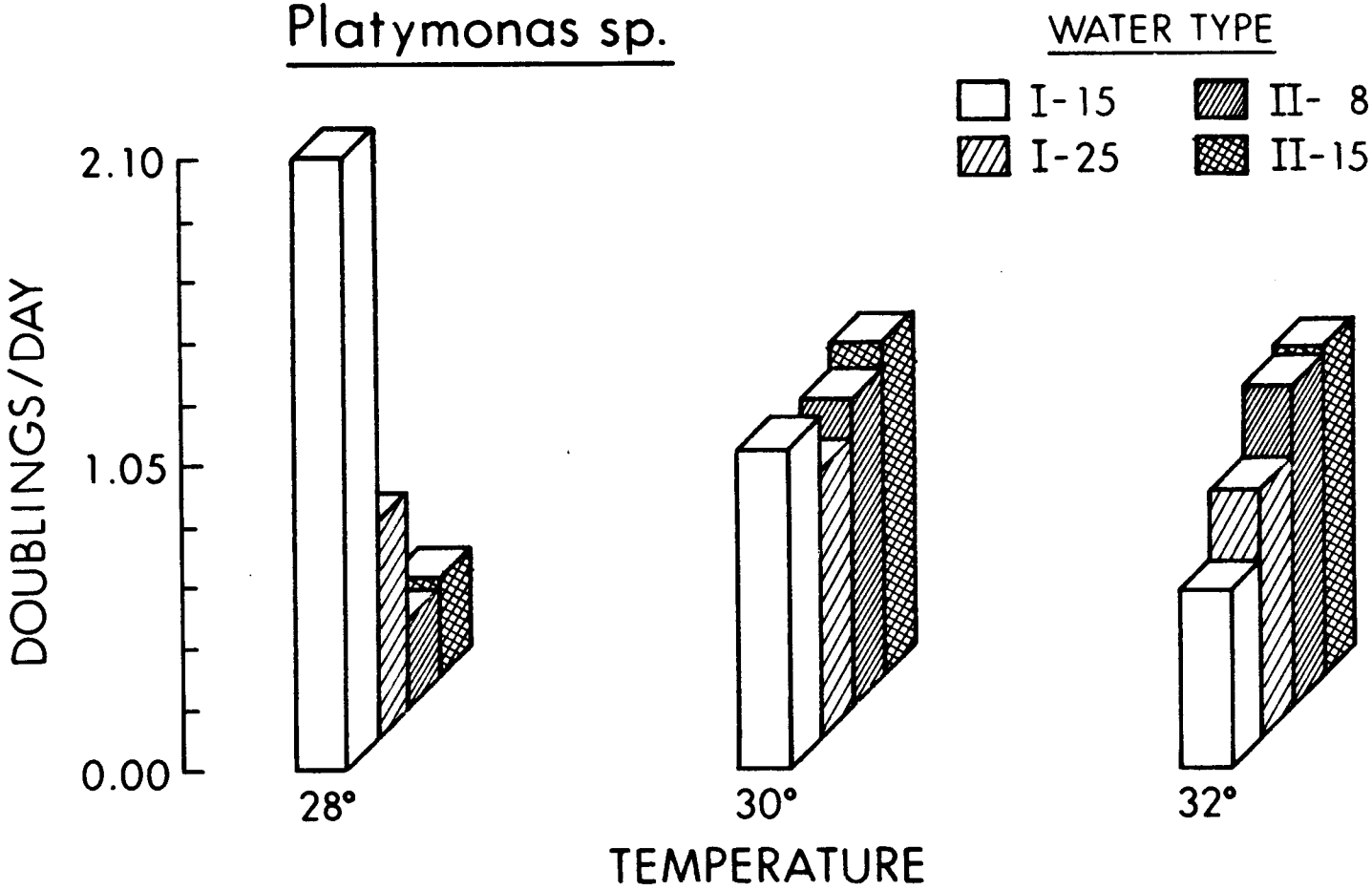


Figure 1. Growth rate of Platymonas sp. as a function of temperature and water type in laboratory-scale experiments.

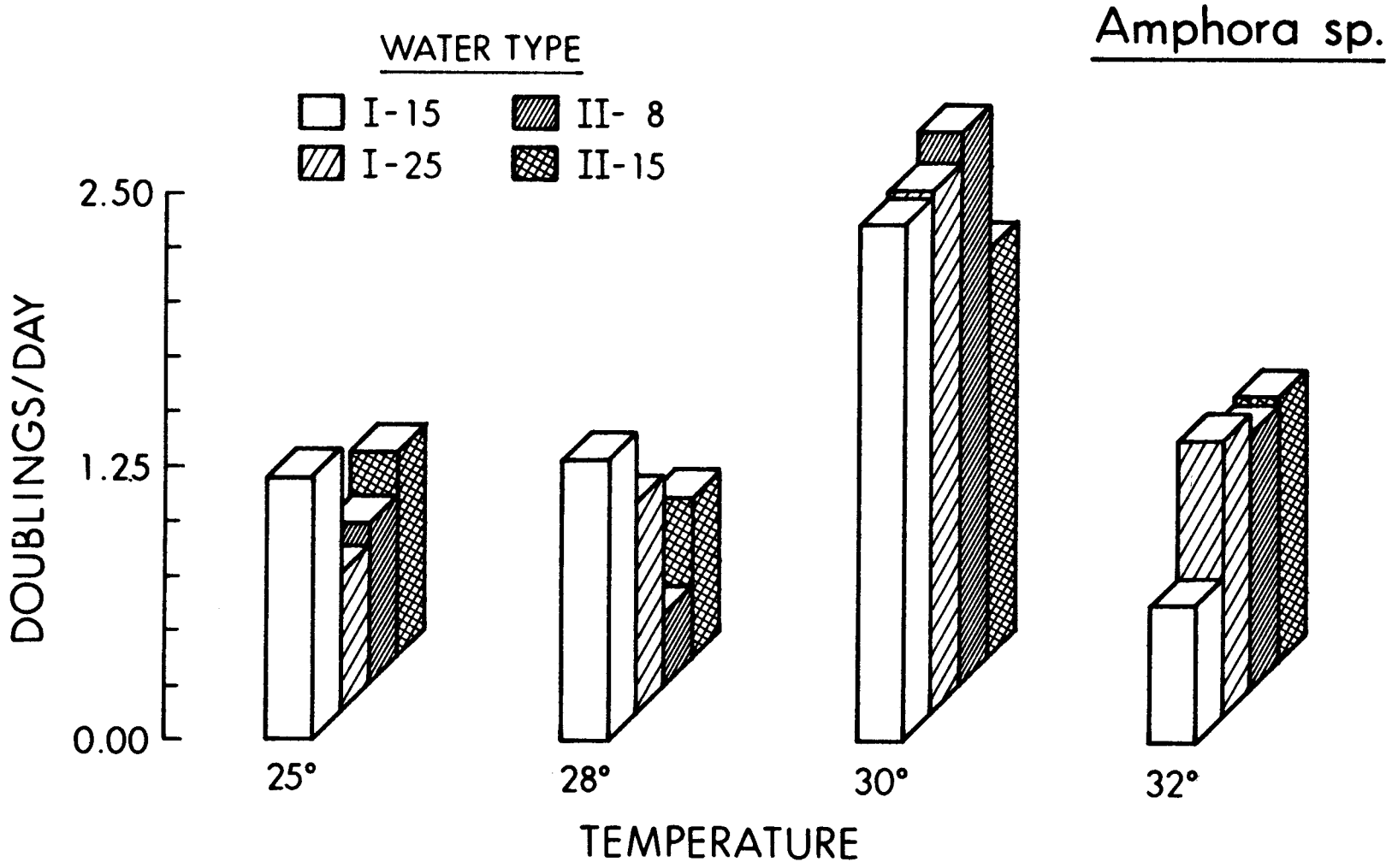


Figure 2. Growth rate of Amphora sp. as a function of temperature and water type in laboratory-scale experiments.

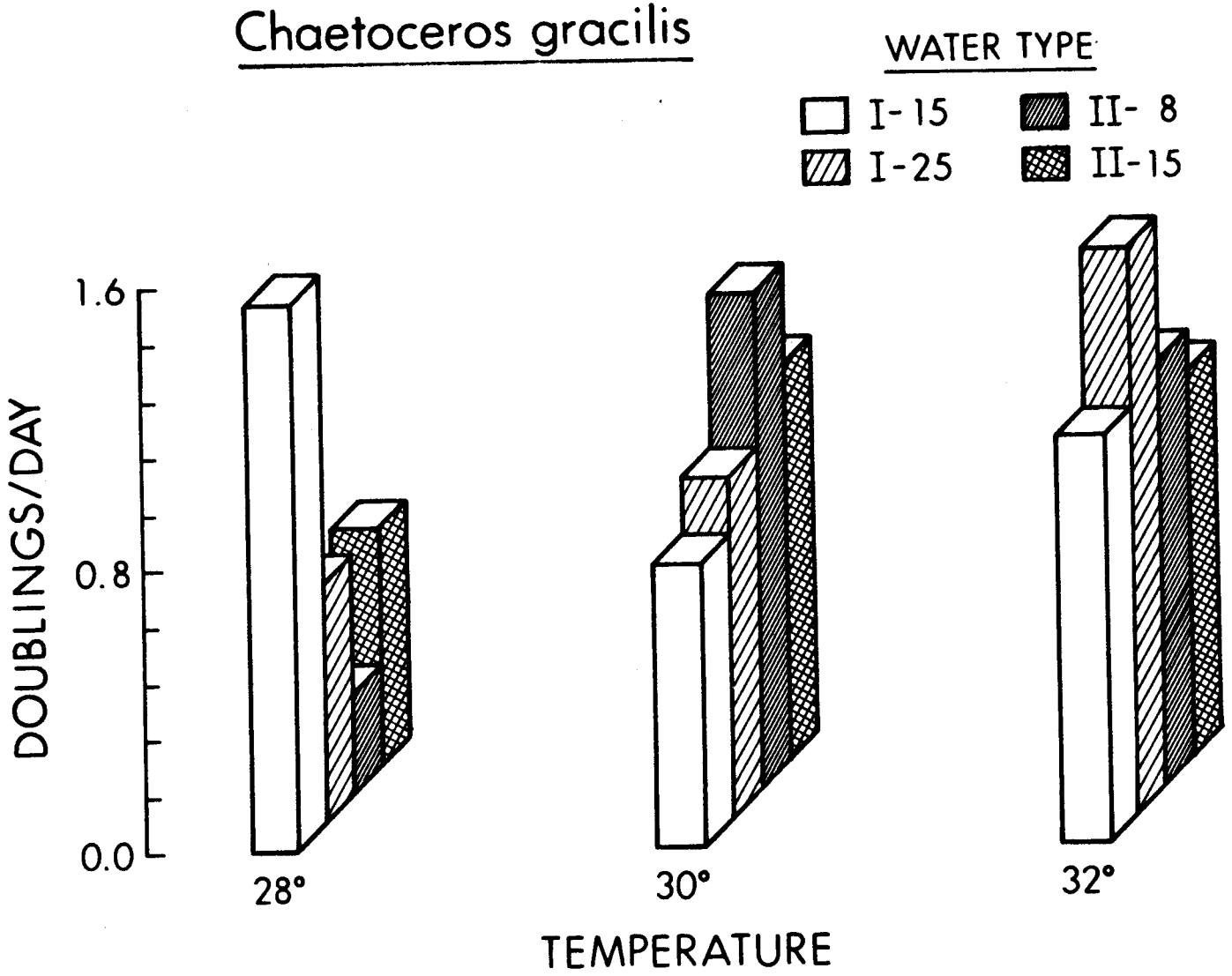


Figure 3. Growth rate of Chaetoceros gracilis as a function of temperature and water type in laboratory-scale experiments.

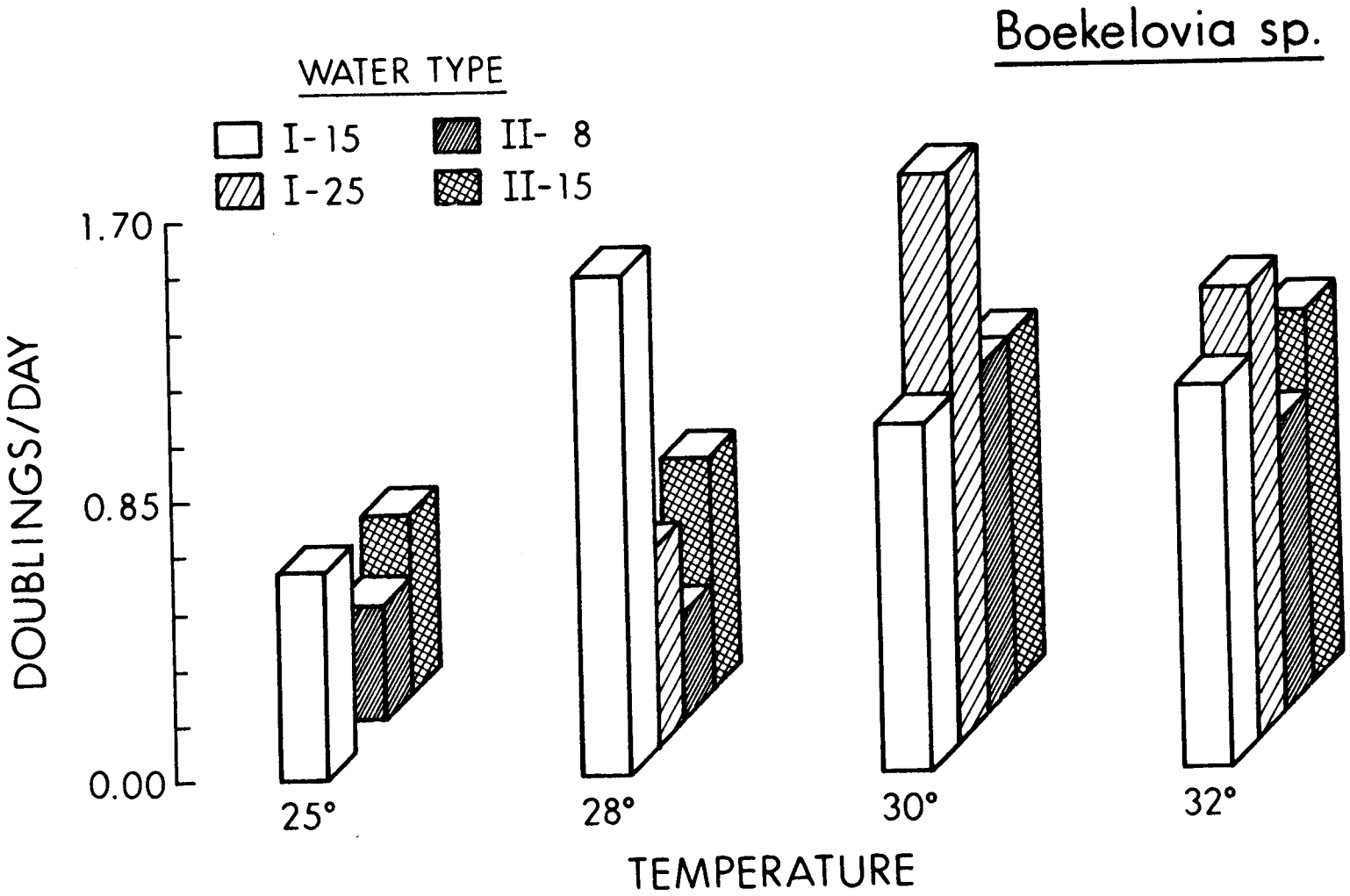


Figure 4. Growth rate of Boekelovia sp. as a function of temperature and water type in laboratory-scale experiments.

growth rate tends to peak at a temperature not much below the lethal temperature, and this fact probably explains why the growth rates have held up rather well even at 32°C. The shift of maximum growth rate to higher salinity or type II water at the higher temperatures is so consistent that I feel the observation is almost certainly not an experimental quirk, but I do not know the physiological explanation.

Table 1. Ionic Composition of Southwestern U.S. Water Types (ppm)

Ion Species	Type I		Type II	
	15°/oo	25°/oo	8°/oo	15°/oo
Na^+	3552	7692	1929	4646
K^+	278	405	205	194
Ca^{2+}	678	633	256	89
Mg^{2+}	303	250	171	392
HCO_3^-	867	1605	1622	2100
SO_4^{2-}	2973	4735	2025	805
Cl^-	6349	9680	1782	6775

Task II

Production studies in the four 9.2 m^2 flumes have been completed with three species. Two of the species, Platymonas sp. and Chaetoceros gracilis, are marine species which we have previously studied in the outdoor flumes in seawater medium. The third species is an unidentified green flagellate which appeared in a culture sent to us by Bill Barclay at SERI.

The results for Platymonas are shown in Figure 5. The culture was grown on a three-day dilution cycle in all cases and was diluted to a cell concentration of 2 x 10^6 ml^{-1}. In other words, every third day the culture was diluted to a concentration of 2 x 10^6 cells ml^{-1}. This management strategy had been found to be optimal for Platymonas in previous studies (Laws et al. 1985). Several aspects of the results are noteworthy. First, production efficiency was positively correlated with salinity in the range 8 - 25°/oo. The peak efficiency was a little over 10% based on visible light irradiance. Second, Platymonas performed best in type I water, and poorest in type II water. We have previously achieved photosynthetic efficiencies of 8 - 11% with Platymonas whenever it has been grown on a three-day dilution cycle (Laws et al. 1985), and the results obtained here are obviously consistent with those earlier studies.

C. gracilis was studied in the flumes only at 15°/oo, and the results are shown in Figure 6. C. gracilis was grown on a two-day dilution cycle in all cases, and was diluted so as to maintain a growth rate of one doubling

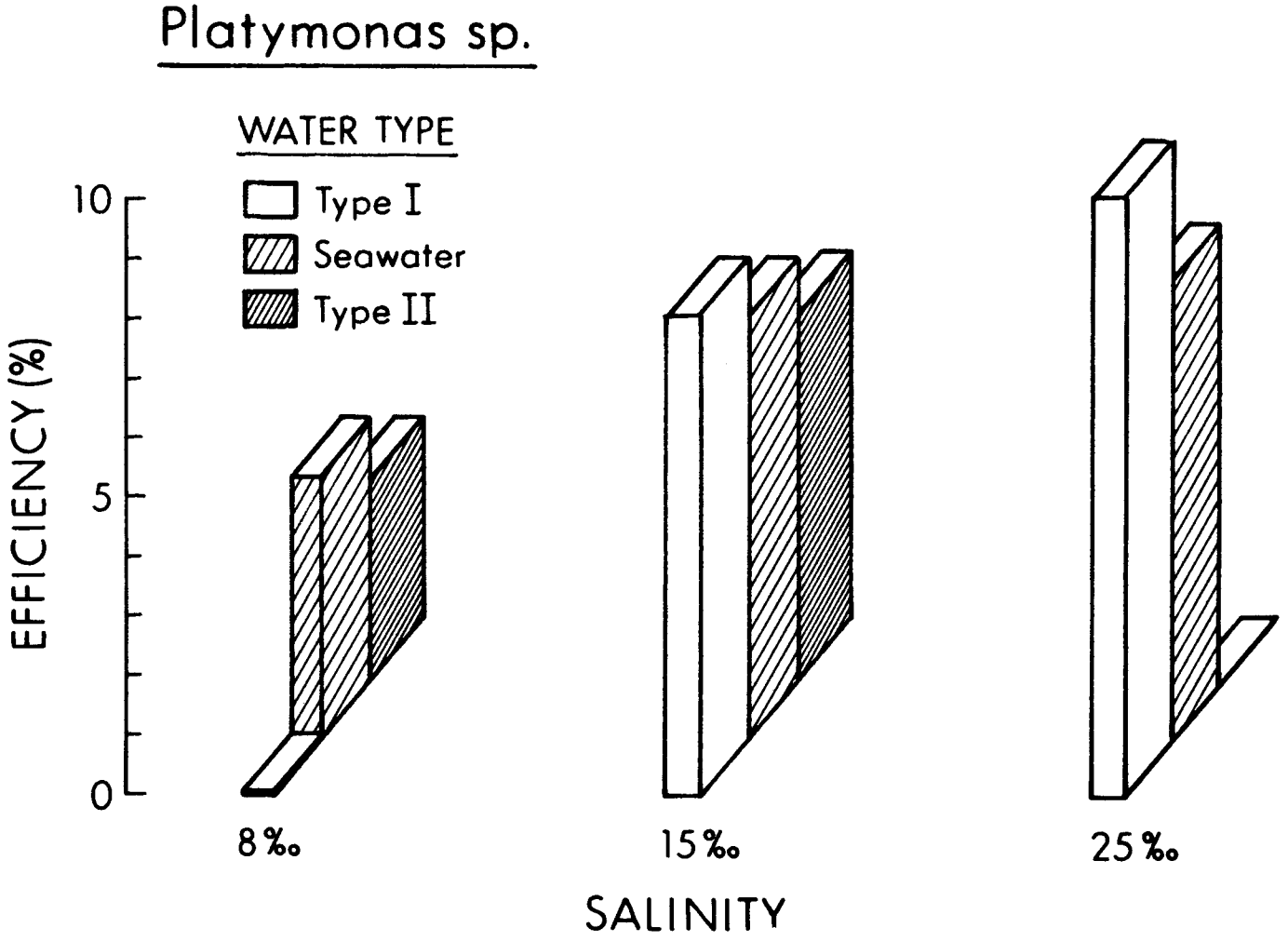


Figure 5. Photsynthetic efficiency as a $_2$function of water type for Platymonas sp. grown in 9.2 m^2 flumes over periods of 9 - 12 days.

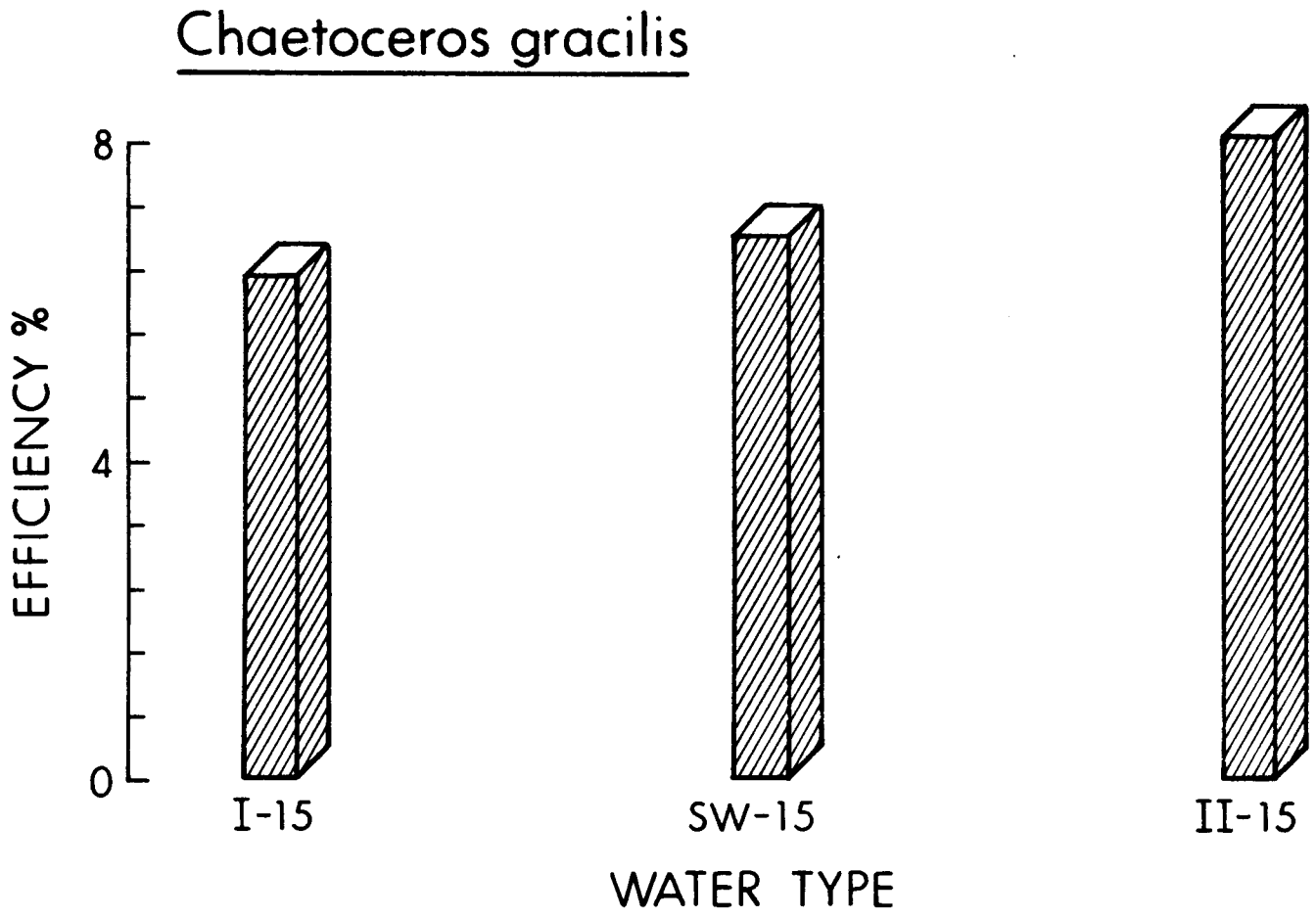


Figure 6. Photosynthetic efficiency as a function of water type for C. gracilis grown at 15°/oo in 9.2 m^2 flumes over periods of 8 - 12 days.

per day. This strategy had previously been found to yield the best production with C. gracilis in a seawater medium. In this case the best production was achieved in type II water, and the worst in type I water. The optimum photosynthetic efficiency was 8%. A comparison of these results with the Platymonas results obviously suggests that the species of choice for a given water type will probably vary from one water type to another. Platymonas seems to prefer type I water; Chaetoceros prefers type II. However, perhaps the most important result of the study is that both of these marine species grow well in at least one Southwestern U.S. water type, and in fact appear to grown even better in at least one of these water types than in seawater.

Production results with the green flagellate are shown in Figure 7. We had no previous experience culturing this species in the outdoor flumes, and therefore had no very clear idea of how often to dilute the culture or by how much. We decided to try a dilution scheme of 75% every three days, which was similar to the methods we employed with C. gracilis and Platymonas. This approach in effect forces the culture to grow at an average rate of two doublings every three days. We ran all the flumes at 15°/oo. In order to get some idea of the sensitivity of production to the dilution cycle, we ran two seawater controls, one diluted every three days by 87½% and the other by 75%. The other two flumes contained type I and type II water. Production was worst in the two seawater controls, and differed only slightly between the type I and type II flumes. However, the photosynthetic efficiencies were well below those achieved with Platymonas and C. gracilis. The change in dilution cycyle in the seawater cultures produced very little change in photosynthetic efficiency. Thus there is no evidence at this time to indicate that the green flagellate is competitive with Platymonas or C. gracilis either in seawater or in Southwestern U.S. water.

Task III

A number of experiments were conducted with the large 48 m^2 flume to explore management strategies which might lower production costs. First, we investigated the effect of removing half or all of the foil arrays. The results are shown in Table 2, and are the averages of production and photosynthetic efficiency over two-week periods. Although removing all the foil arrays greatly reduced photosynthetic efficiency, removing half the foil arrays reduced photosynthetic efficiency from only 8.0 to 7.7%. Since the foil arrays do exert a certain amount of drag, a reduction in the number of foil arrays could significantly reduce the energy inputs to the system.

Second, we investigated the effect of reducing the CO_2 inputs to the system. Standard procedure has been to hold the pH at 7.5 with additions of CO_2 during the day. We explored two alternate strategies. In one case we turned off the CO_2 altogether for one week. In another case we held the pH at 8.5 rather than 7.5. The results are shown in Table 3. Clearly turning off the CO_2 greatly reduces production and photosynthetic efficiency. Allowing the pH to rise to 8.5 reduced photosynthetic efficiencies from the range of 8 - 10% typically achieved with Platymonas to 6.2%. However, it is possible that this reduction in production will be cost effective,

because the CO_2 supply is turned on for only about one hour per day in order to hold the pH at 8.5. Thus the CO_2 utilization efficiency is much greater when the pH is held at 8.5 than at 7.5.

Table 2. Results of Growing Platymonas in 48 m^2 Flume to Determine Effect of Removing Foil Arrays.

	Production (g AFDW m^{-2} d^{-1})	Photosynthetic Efficiency %(based on visible light)
All Arrays in Place (4 feet apart)	18.8	8.0
Half Arrays Removed (arrays 8 feet apart)	17.1	7.7
All Arrays Removed	14.4	5.5

Table 3. Results of Growing Platymonas in 48 m^2 Flume at Reduced CO_2 Supply Rates

CO_2 Supply	Production (g AFDW m^{-2} d^{-1})	Photosynthetic Efficiency %(based on visible light)
no added CO_2	6.6	2.8
turned on at pH = 8.5	15.6	6.2
turned on at pH = 7.5	20 - 40	8 - 10

Task IV

Figure 8 summarizes the results of a silicate starvation experiment performed with C. gracilis on a laboratory scale. The culture was grown in batch mode and was uniformly labeled with C-14 to facilitate studies of carbon partitioning. The percentage of lipid in the cells began to increase when the silicate concentration dropped to about 5 μM, and over a period of four days rose from about 13% to over 50%. The average rate of lipid carbon production since day 1 peaked on day 8 and then slowly declined. These results clearly show that C. gracilis has the potential to store large amounts of lipid when silicate starved. The results are very much in contrast to those obtained previously with Platymonas, which stores carbohydrate rather than lipid when nitrogen or phosphorus starved. However, production of carbon storage products obviously requires light, and in the outdoor flumes light is the factor limiting production. Therefore these laboratory-scale experiments can indicate only the potential for lipid production. How fast and how much lipid can be produced in the outdoor flumes will depend on the culture density and irradiance, and can only be

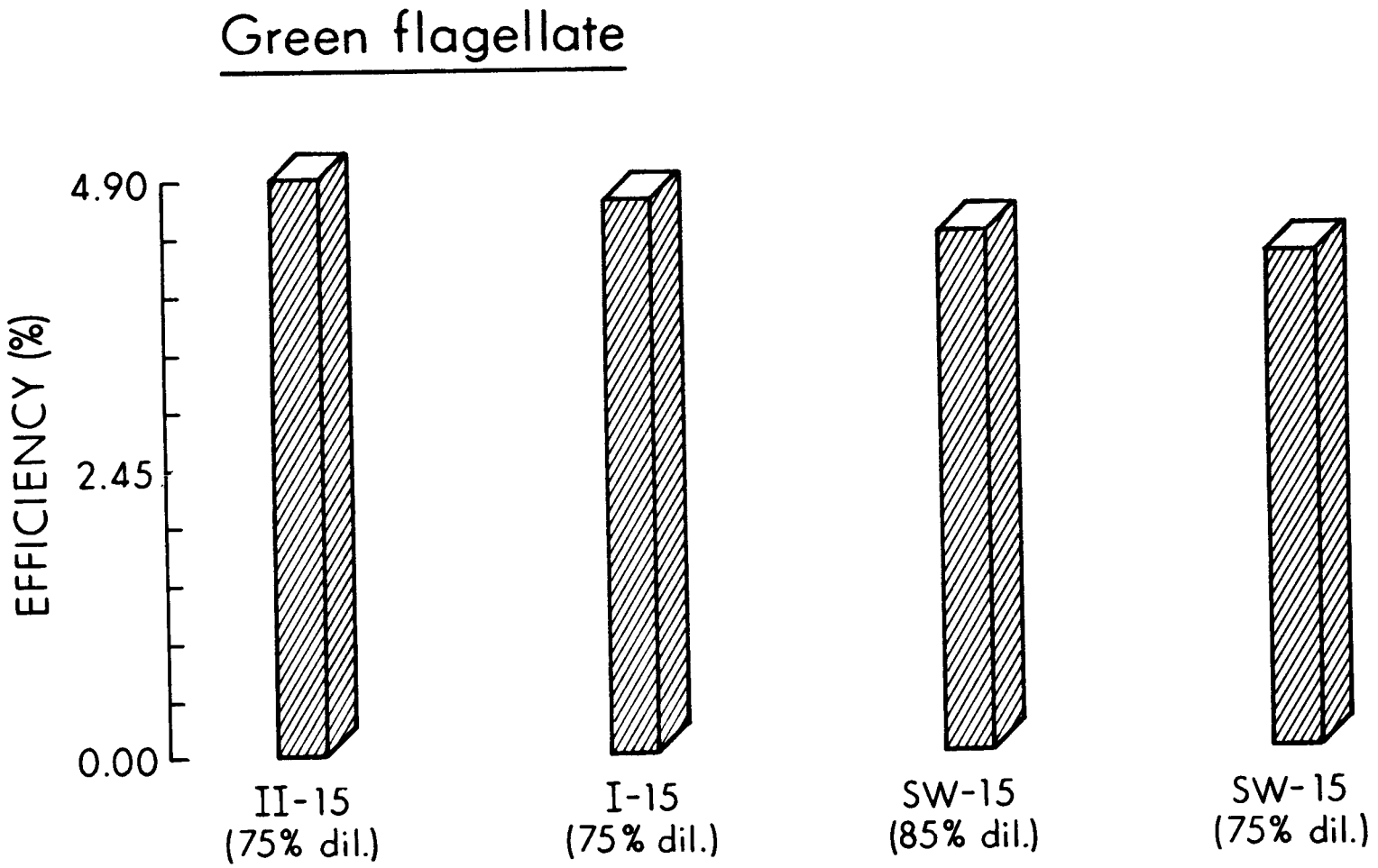


Figure 7. Photosynthetic efficiency as a function of water type for the green flagellate grown at 15°/oo in 9.2 m^2 flumes over periods of 9 - 12 days. Cultures were diluted every three days by either 75% or 85% as indicated.

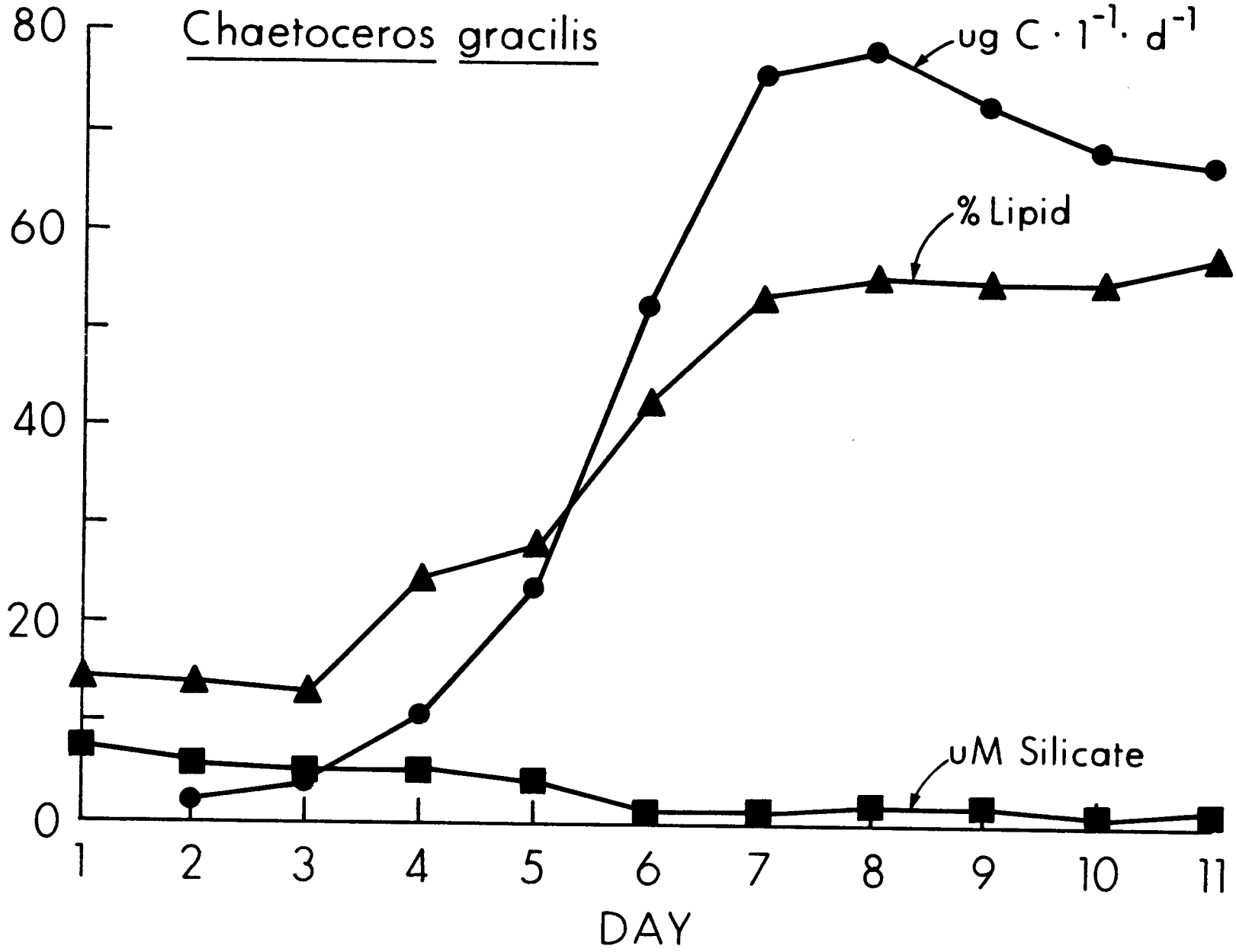


Figure 8. Effect of silicate starvation on lipid production in laboratory culture of C. gracilis. Lipid carbon production rates are the mean rate from day one until the indicated day (i.e. they are cumulative rather than rates on the given day).

determined by conducting an experiment in the outdoor flumes.

We conducted a nine-day study in the 48 m^2 flume with Platymonas in an effort to gain some insight as to why this species grows so well when diluted every third day. We suspected that adaptation to changing light conditions might play a role in the physiological response of the organism to the rapid changes in cell density and hence average irradiance. We measured light-saturated photosynthetic rates at noon over the course of three consecutive three-day dilution cycles, and the results are shown in Table 4. Although the light-saturated photosynthetic rate normalized to chlorophyll a (= assimilation number) did decrease as the culture became more dense, the change was only about 10%. Furthermore, the assimilation numbers are not as low as one would expect for a culture that was significantly light-limited (Laws and Bannister 1980). We suspect therefore that the key to obtaining high yields with Platymonas is to prevent the cells from becoming severaly light-limited and yet periodically (i.e. every third day) bring the culture to a high density so as to maximize the areal production rate.

Some question arose during the course of this research concerning the relationship between the cellular carbon content and ash-free dry weight (AFDW). We routinely measure particulate carbon concentrations in our flumes and convert these numbers to AFDW by multiplying by 1.9. Are production numbers calculated in this way really comparable to produciton results obtained from measurements of AFDW or dry weight (DW). Figure 9 shows the degree of correlation between particulate carbon (PC) and DW over the course of several weeks during our flume studies with C. gracilis. Obiviously the correlation is very good ($r = 0.987$), and the slope of the regression line implies that carbon accounts for 42.76% of the DW. If AFDW equals 1.9 times PC, then AFDW accounts for 81% of the DW, the remaining 19% being ash. These figures are quite reasonable for a diatom. We have obtained similar results with Platymonas, and the conversion factor of 1.9 between PC and AFDW was obtained from a correlation analysis of PC vs. AFDW for Platymonas.

FUTURE ANTICIPATED DIRECTIONS

A number of promising candidates have been sent to us from the mainland U.S., and we are continuing to screen them in the 9.2 m^2 flumes for photosynthetic efficiency. Nutrient starvation studies will be performed on the most promising species to see whether significant lipid production can be induced in a reasonably short time period under conditions in the flume.

We are continuing to use the large 48 m^2 flume to examine possible methods of reducing produciton costs and/or increase lipid production. Experiments in the immediate future call for reducing the flow rate in the system at night by cutting back on the supply of air to the air lift. The large flume may also be used to study the effectiveness of various pesticides in controlling predators. We also hope to be able to continue exploring the physiology of the species which give the best production to try to understand why certain dilution cycles are so effective in increasing yields.

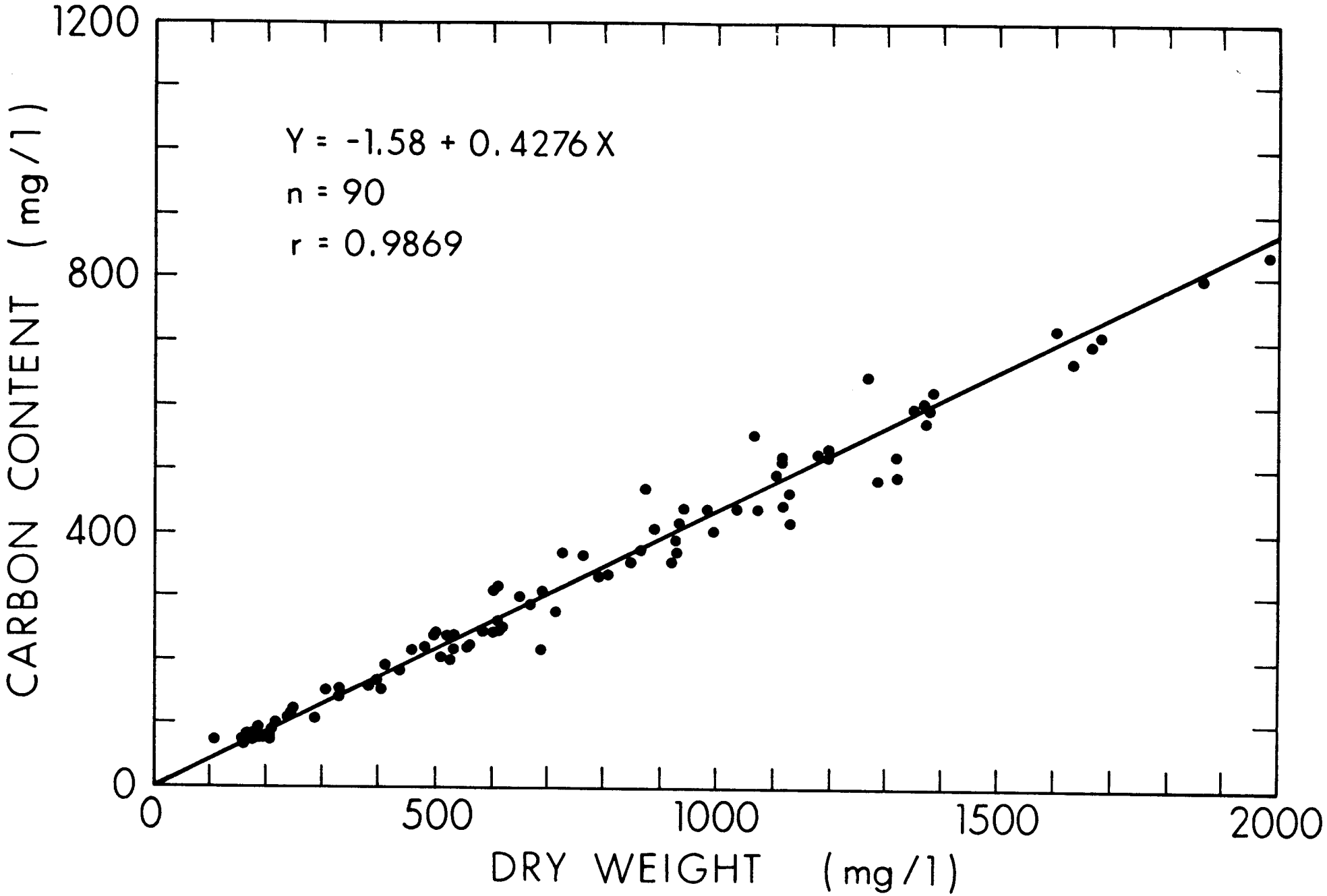


Figure 9. Correlation between carbon content and dry weight for C. gracilis grown in outdoor flumes.

Table 4. Light-saturated photosynthetic rates of a Platymonas culture grown on a three-day dilution cycle as a function of time.

Days after dilution	Light-saturated photosynthetic rate (g C g^{-1} chl *a* h^{-1})
0	7.9 ± 1.6
1	7.6 ± 1.1
2	7.1 ± 2.4

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STUDIES WITH MARINE MACROALGAE: SALINE DESERT WATER CULTIVATION AND EFFECTS OF ENVIRONMENTAL STRESS ON PROXIMATE COMPOSITION

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Introduction

Studies conducted in recent years have shown that saline waters of southwest (U.S.) desert lakes may be utilized as an alternative medium to seawater for land-based production of microalgae (Thomas et al., 1984). Such desert lakes may also be a promising, under-utilized resource for macroalgae production, as they occur in regions of high solar radiation inputs, and where land costs are low. Currently, the purchase of coastal land represents up to 30% of the total fixed cost of a land-based macroalgae biomass farm, depending on factors such as location and pond construction methods (Ryther et al., 1984).

The desert lakes under consideration for algae production exhibit a wide salinity range and a diverse ionic composition (Thomas et al., 1984). One goal of the present study is to determine whether waters from these saline desert lakes provide a suitable medium for cultivation of marine macroalgae, in particular Gracilaria tikvahiae and Ulva lactuca, two species for which high, sustained yields have been attained in seawater culture.

Macroalgae research conducted during the past seven years at the Harbor Branch Foundation has emphasized the development of culture techniques which maximize organic matter yields while minimizing the energy costs associated with cultivation. For purposes of energy production, however, it is the concentration of storage products such as carbohydrates (for methane, alcohol) and lipids (for hydrocarbons), rather than total organic matter content, which determines the value of the algal biomass which is produced. For example, Habig et al.(1984) found that methane yields (per g volatile solids) from the seaweed Ulva sp. increased 1.5-fold in response to a doubling in thallus carbohydrate content. Prior studies with micro and macroalgae have shown that the synthesis of certain storage products can be enhanced by subjecting algae to environmental stresses. Nitrogen deprivation is one such stress which commonly induces carbohydrate accumulation in macroalgae (Craigie, 1974). However, the effect of other environmental conditions on the synthesis of macroalgal storage products is not well known. The second task addressed in the present contract is to determine how certain environmental stresses (eg. nitrogen, salinity, temperature) affect the proximate composition of several marine macroalgae.

Different Growth Forms of the Alga Gracilaria tikvahiae

Caulerpa Growing on Aquaria System Used to Determine Environmental Effects on Chemical Composition

Task 1. Photosynthesis, growth and composition of marine macroalgae in saline desert waters.

Methods

Most of the algal photosynthesis and growth studies for this task were conducted on a light table located in a temperature controlled room (22 ± 2 C).This table provided 28 stirrer stations, each of which contained a recessed alnico magnet which was rotated at 60 rpm by belt driven pulleys. Light was provided by six 96" cool-white fluorescent lamps positioned as 3 banks of two lamps each. The maximum irradience at the midpoint of each stirrer station was 2.7×10^{16} quanta $cm^{-2} sec^{-1}$, or approximately 120 watts m^{-2}.

Measurements of growth and proximate composition of macroalgae in the saline desert waters were also conducted in 13 L aquaria in the same constant temperature room. Each aquarium was provided with gentle aeration, which kept the algae continuously in suspension. Light was provided from two fluorescent lamps, at a radiant flux density of approximately 72 watts m^{-2}. Water temperature in each aquarium was maintained at 22 ± 2 C. Detailed methods of the experiments conducted for this task are as follows.

A. Photosynthesis of macroalgae in saline desert water media

Experiment A1

Net oxygen production of Gracilaria tikvahiae and Ulva lactuca was measured in media simulating the waters of Black and Harper Lakes, two California desert lakes of widely different ionic composition. Black Lake is characterized by high pH (10.1), moderate salinity (21 ppt), low concentrations of Ca and Mg (relative to seawater), and very high levels of inorganic carbon(Table 1). In contrast, Harper Lake has a pH of 8.6, salinity of 14 ppt, and low levels of Mg, K and inorganic carbon (Table 1). Analyses of the lake waters were provided by Dr. W. H. Thomas.

For this experiment, Ulva and Gracilaria thalli (100-300 mg wet weight) were incubated in 300 ml glass bottles containing either ocean water (diluted to 25 ppt with deionized water), Black Lake, or Harper Lake media. Triplicate bottles were established for each treatment. The ocean water had a pH of 8.0, while the pH of the Harper and Black Lake media were adjusted with CO_2 to 7.7 and 7.8, respectively. Although nutrients were not added to the media, the algae were well-nourished by a high estuarine water flow and a pulse-feeding of nitrogen immediately prior to their use in the experiment. Photosynthesis incubations were conducted on the light table for four hours, with initial(0 hour), 2 hour, and 4 hour oxygen readings taken with a YSI model 51 B oxygen meter. Seaweed samples were strained from the bottles at the end of the experiment, rinsed in fresh water, dried (70 C for 48 hr), and weighed.

Experiment A2

Studies during the remainder of the contract were conducted with the type I and II media, solutions which are typical of saline New Mexico lake waters. Formulae (Table 2) for these lake types were provided by SERI. The

Table 1. Ionic composition (major cations and anions) of Black Lake, Harper Lake and seawater (35 ppt).

	Seawater[1]	Black Lake[2]	Harper Lake[2]
	------Concentration ($mg\ L^{-1}$)------		
Ca	412	4	435
Mg	1294	4	70
Na	10733	8800	6080
K	399	825	48
CO_3	-	5580	60
HCO_3	142	2526	61
SO_4	2712	3200	2800
Cl	19344	3200	7725

1 Riley and Chester (1971)
2 Thomas, et al. (1984)

Table 2. Ionic composition (major cations and anions) of seawater (15 ppt) and two saline media typical of New Mexico desert lakes.

	Seawater (at 15 ppt)	I M[1]	II M[1]
	------Concentration (Mg l^{-1})------		
Ca	177	678	89
Mg	555	303	392
Na	4600	3552	4646
K	171	278	194
HCO_3	61	867	2100
SO_4	1162	2973	805
Cl	8290	6349	6775

1 Ionic composition provided by Solar Energy Research Institute.

monovalent/divalent cation ratios of these media differ, although both are more alkaline than seawater (Table 2).

In this experiment, net photosynthesis of four common macroalgae (Ulva lactuca, Entermorpha sp., Acanthophora sp., and Agardhiella sp.) was examined in four media: ocean, artificial ocean, and I and IIM saline desert water media. The artificial seawater was utilized in addition to ocean water as a control since nutrients were not added to any of the media during these short (2-4 hr) incubations. This seawater medium was formulated with deionized water and the following salts, providing an ionic composition equal to that of 15 ppt seawater (Table 2): (in mg L^{-1}); KCl, 327; $CaCl_2 \cdot 2H_2O$, 648; $NaHCO_3$, 84; $MgCl_2 \cdot 6H_2O$, 4658; Na_2SO_4, 1711; and NaCl, 10185. The salinity of all media (including ocean water) was adjusted to ca. 15 ppt. Sections of thalli (discs for Ulva) approximately 100-300 mg in wet weight were stocked into duplicate 300 ml glass bottles of each medium. Initial oxygen levels in the bottles were lowered by bubbling N_2 gas prior to their placement on the light table. The plants were incubated for 4 hours, with oxygen levels and seaweed dry weights determined as previously described.

A second photosynthesis run was conducted using thalli/discs which were soaked in the four media for 1.5 days prior to the incubation, to determine the effect of "preconditioning" on oxygen production by these algae.

B. Proximate composition and short-term growth of macroalgae in saline desert water media

Experiment B1

Ulva and Gracilaria thalli were cultured in 300 ml glass bottles containing ocean, artificial ocean and I and IIM saline desert waters. The bottles were placed on the light table, which was adjusted to provide 14/10 hr light /dark photoperiod and intermittant stirring (ca 1 hr/day). The salinity of all media was adjusted to 15 ppt, and a complete nutrient solution (Table 3) was added to each bottle. Specific growth was calculated from the change in wet weight over one weeks time using the expression $\mu = \log (w_f / w_i) 3.32/t$.

Experiment B2

Gracilaria and Ulva thalli (170-360 mg) were cultured on the light table in 1 L bottles containing ocean, artificial ocean, and IM waters. Salinities of all media were adjusted to 15 ppt, and pH levels to 7.0-8.0. Each medium was also amended with a complete nutrient solution (Table 3). Triplicate bottles were utilized for each treatment. Unlike the preceding experiment, specific growth in this study (doublings day^{-1}) was calculated on a dry weight basis (final dry weight - initial dry weight). Dry matter (70 C for 48 hr) and ash contents (550 C for 4 hr) of the final samples were also measured.

Experiment B3

Separate 13 L aquaria were filled with ocean, artificial ocean, and I and IIM saline desert waters. A complete nutrient medium was added to each solution, salinities were adjusted to 15 ppt, and pH levels were normalized.

Table 3. Nutrient medium[1] utilized for task I and II experiments.

	Concentration (Mg L^{-1})
$NaNo_3$	46.67
Na_2 glycerophosphate	6.67
Fe Cl_3	1.08
Na_2EDTA	5.53
Fe $Cl_3.6H_2O$	0.33
$MnSO_4.4H_2O$	1.09
$ZnSO_4.7H_2O$	0.15
$CoSO_4.7H_2O$	0.03
H_3BO_3	3.80
HCl (3N)	0.81 (ml)
$NaHCO_3$	200.0

1 Modified from Provasolis' enriched seawater.

Irradience was provided as previously described. After 7 days time, growth and proximate composition of the seaweed in each treatment were determined using methods described in Task 2.

C. Long-term growth of macroalgae in saline desert media

Experiment C1

In order to examine the long-term growth of Ulva in the saline IM medium, small discs of algae were placed in 350 ml storage dishes containing ocean, artificial ocean, and IM waters. Light and stirring were provided on a 14:10 hour on:off basis. Plastic screens were placed just above the bottom of each dish to prevent the seaweed from snagging on the magnetic stirrers. Wet weight gains in each treatment were measured weekly, and new discs were re-cut from the thalli. The media were changed and amended with nutrients each week, and the discs restocked. Seaweed wet weights were converted to dry weight values using dry weight:wet weight ratios obtained from the previous experiment.

Experiment C2

This experiment was conducted to determine whether small increments of ocean water (diluted to 15 ppt), when added to the IM medium, would help support Ulva growth. Ulva discs were placed in 350 ml storage dishes containing the following solutions: ocean water, IM medium, and 1:9, 1:3 and 1:1 mixtures of ocean water and IM water. Growth measurements, media changes and nutrient additions were conducted weekly.

Results and Discussion

A. Photosynthesis of macroalgae in saline desert waters

Short-term oxygen production by Gracilaria and Ulva in Harper Lake water was equal to or slightly greater than that in ocean water (Fig 1). In contrast, oxygen production by these two algae in the Black Lake water was poor (Fig 1). Gracilaria in this latter medium appeared to lose its phycoerythrin pigments during the 4 hour long incubation.

It is likely that the low divalent cation levels (probably Ca) were responsible for the poor growth of Gracilaria in the Black Lake water. However, an attempt to stimulate oxygen production by Gracilaria in this medium by the addition of Ca and Mg was unsuccessful. Net photosynthesis was not stimulated by a ten-fold increase of Mg in the medium. Additions of Ca to the Black Lake medium resulted in the formation of $CaCO_3$ precipitate. Hardness determinations on the supernatent indicated that none of the added Ca remained in solution. Gracilaria photosynthesis in this "Ca-amended" medium was not significantly higher than that in the standard Black Lake medium.

Following this experiment, studies in this task were conducted with media formulations provided by SERI (Table 2) which are typical of New Mexico saline lake waters

Both the type I and IIM media supported rapid photosynthesis by the macroalgae Ulva lactuca, Enteromorpha sp, Acanthophora sp, and Agardhiella sp (Fig 2). Net photosynthesis by these algae in the IM medium was generally equal to that in ocean water, while net photosynthesis in the IIM waters was slightly higher, probably due to the high bicarbonate levels (15 times that of full strength seawater) in this water .

Similar trends in net photosynthesis were observed in these media even after the algal thalli were held, or "preconditioned" in the waters for 1.5 days. A slight decrease in oxygen production by Enteromorpha and Ulva in the IIM medium, relative to that in ocean and IM waters, was the only obvious effect of this conditioning period (Fig 3).

B. Proximate composition and short-term growth of macroalgae in saline desert waters

Although the photosynthesis experiments showed that both of the desert media supported rapid, short-term oxygen evolution by a number of algae, our week-long growth experiments with these solutions indicate that the IIM water is a much poorer growth medium than the IM water. In a comparison of Gracilaria growth in ocean, artificial ocean, IM and IIM waters, the maximum specific growth (on a wet weight basis) occurred in the two ocean water solutions (Fig 4). Although growth of this alga was not substantially lower in the IM water, the thalli became pale and brittle during the seven day incubation period. In the IIM water, the alga died during the latter part of the seven day incubation period.

In a companion experiment, Ulva grew at a slightly faster rate in the ocean water than in the artificial ocean water (Fig 4). However, the most

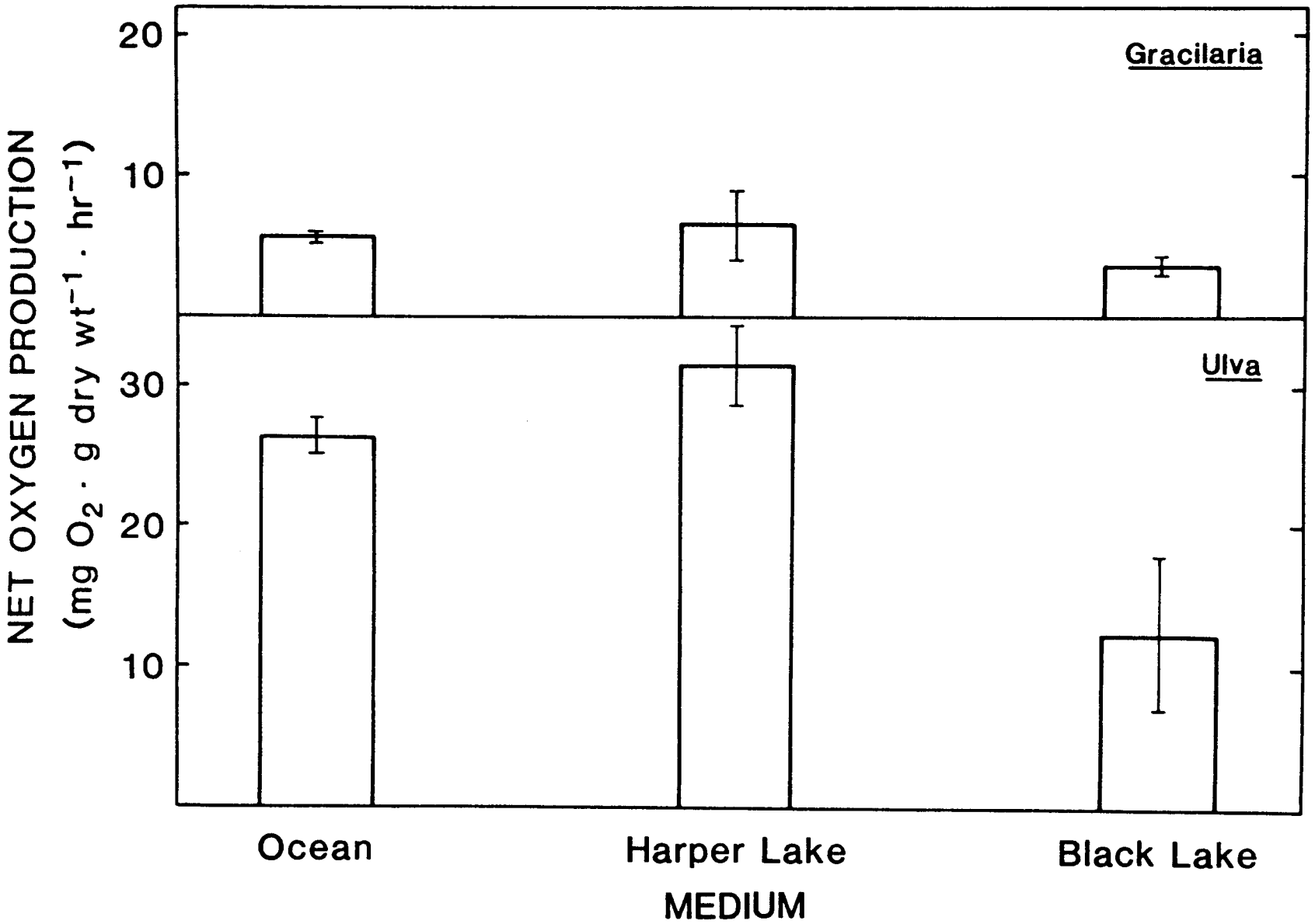

Figure 1. Net photosynthesis of Gracilaria and Ulva in ocean water, and in two artificial saline desert waters. Error bars denote ±1 s.d.

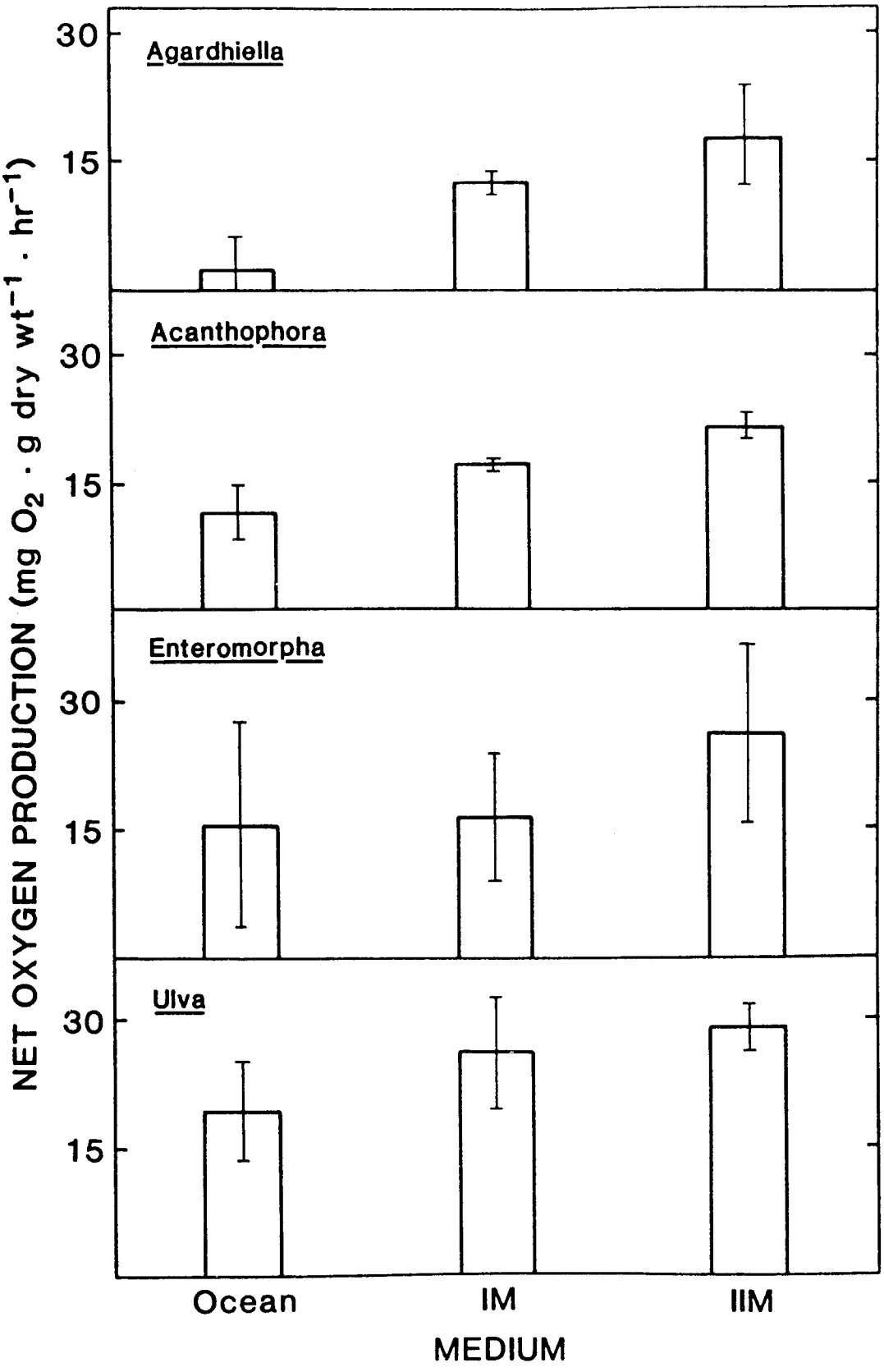

Figure 2. Net photosynthesis of four macroalgae in ocean water, and in the IM and IIM saline desert media. Error bars denote ±1 s.d.

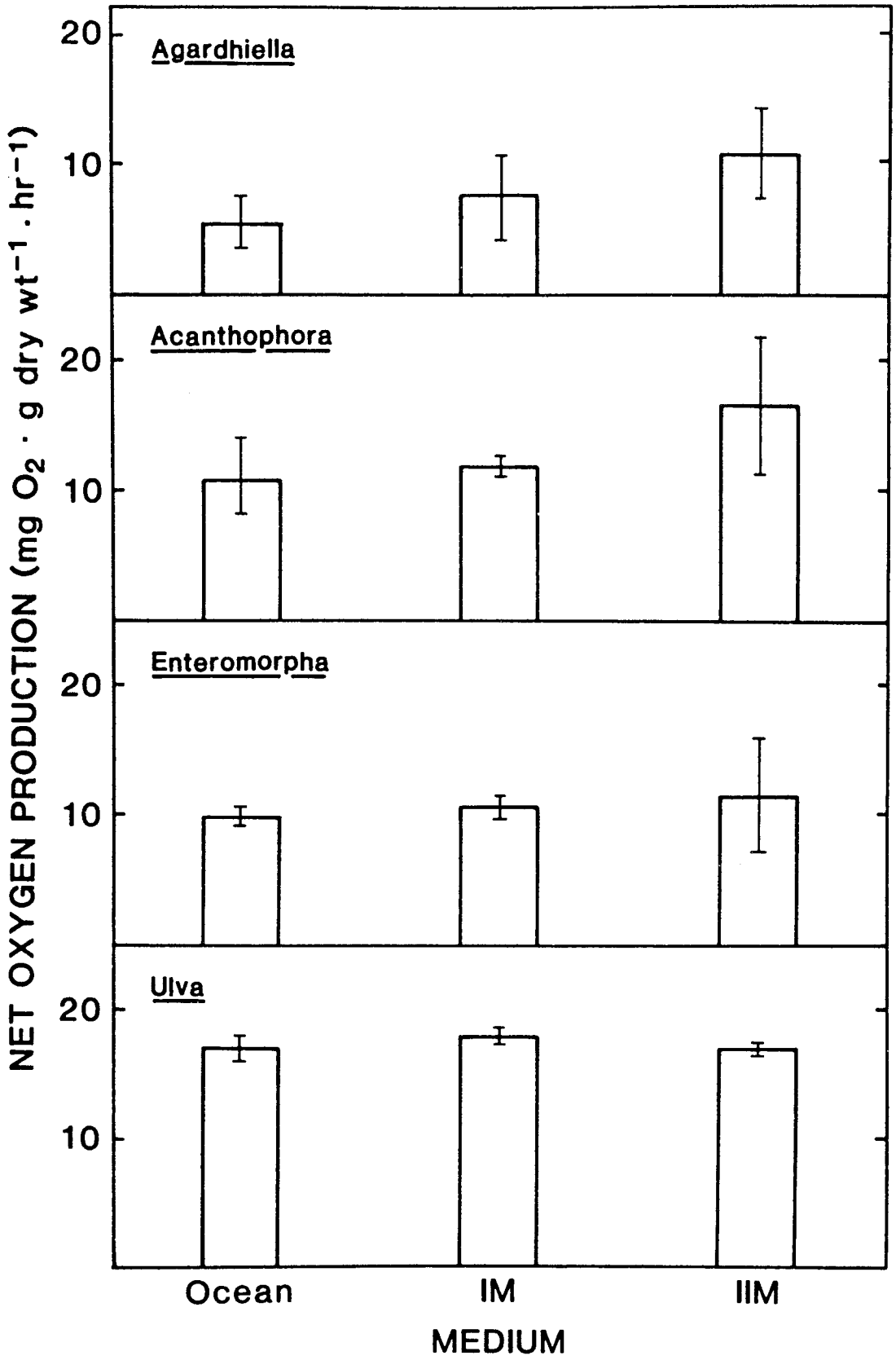

Figure 3. Net photosynthesis of four macroalgae in ocean water, and in the IM and IIM saline media. Algae were held in each respective medium for 1.5 days prior to the incubations. Error bars denote $\pm$1 s.d.

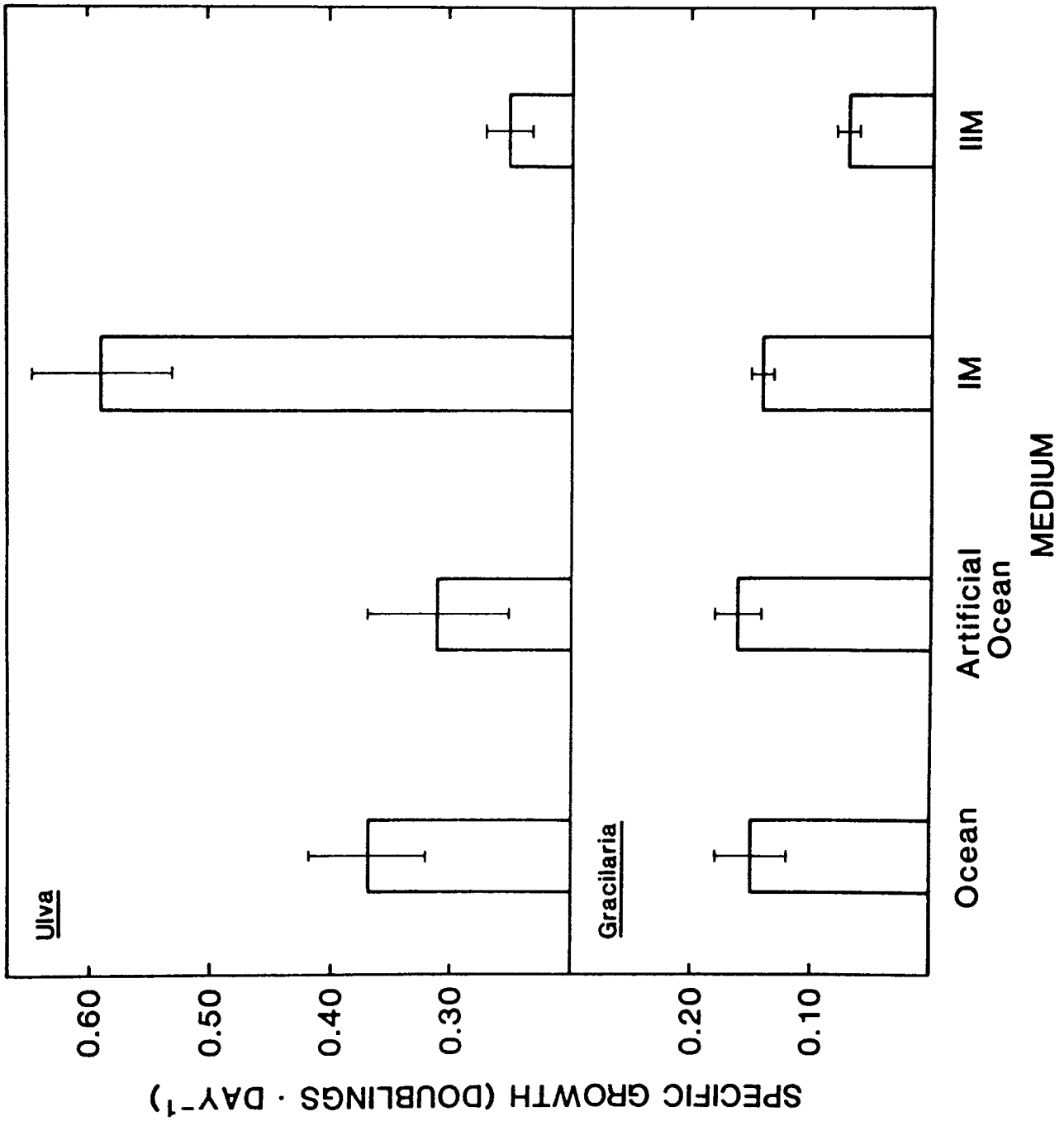

Figure 4. Specific growth of Ulva and Gracilaria in ocean water, artificial ocean water, and two saline desert water media.

rapid growth for this species occurred in the IM water, with the alga exhibiting a doubling time of less than one day. Relative to the seaweed in the ocean and artificial ocean water media, however, the Ulva in the IM and IIM waters became pale and soft textured during the incubation period. Ulva growth in the latter treatment (IIM) was relatively poor (Fig 4).

Because specific growth rates of Ulva and Gracilaria in this experiment were calculated based on changes in wet weight biomass, a followup experiment was conducted to determine whether the recorded growth rates (particularly that of Ulva in the IM medium) represented a true increase in dry matter, or merely an increase in thallus water (or ash) content.

On a dry weight basis, the specific growth rates of Gracilaria in the ocean and artificial ocean waters was found to be equal, at 0.1 doublings day^{-1} (Fig 5). Although a gain in Gracilaria wet weight was observed in the IM medium, the thallus dry matter content dropped 50% (initial % dry matter was 12.3), so net specific growth on a dry weight basis was zero (Fig 5). There were no between-media differences in ash content, although it is notable that this parameter decreased sharply (initial % ash was 44.7) after Gracilaria was transferred from high (32 ppt in the outdoor holding tanks) to low salinities (15 ppt for the experimental media) (Fig 5).

In contrast to Gracilaria, Ulva grew at a slower rate in the artificial ocean medium than in the ocean water (Fig 5). The reason for this reduced growth, which was also observed in the preceding experiment (Fig 4), is unknown. The weight increase of Ulva in the IM medium was 40% higher than that in ocean water (Fig 5). However, because of the high ash content of Ulva in the IM medium (Fig 5), the difference between the organic matter increase in ocean water and that in the IM medium on a volatile solids basis is narrower.

Because Ulva grew more rapidly in the artificial desert media than did Gracilaria, the former species was selected for an experiment to determine the effect of the saline desert waters on macroalgal proximate composition. This study was conducted in 13L aquaria with an algal stocking rate of 40g, so that plant samples of an adequate size for tissue analyses could be retrieved at the end of the study. During this one week incubation, productivity (on a volatile solids basis) of Ulva decreased among treatments in the following order: ocean, artificial ocean, IM and IIM (Fig 6). The following effects of water type on composition were also observed (Fig 6):

1) Ulva grown in the IM water had elevated dry matter and ash contents (relative to ocean water).
2) The carbohydrate composition of the slow growing Ulva in the IIM water was low.
3) The protein content of Ulva was equal among all water types.

These data show that over a short period of cultivation (one week), the IM medium supports moderate Ulva growth, and the seaweed produced is not atypical in composition.

C. Long-term growth of macroalgae in saline desert media.

Additional studies were conducted to determine how long the IM and IIM media could support continuous Ulva growth. Ulva discs were cultured in the two desert media and in control solutions of ocean and artificial ocean

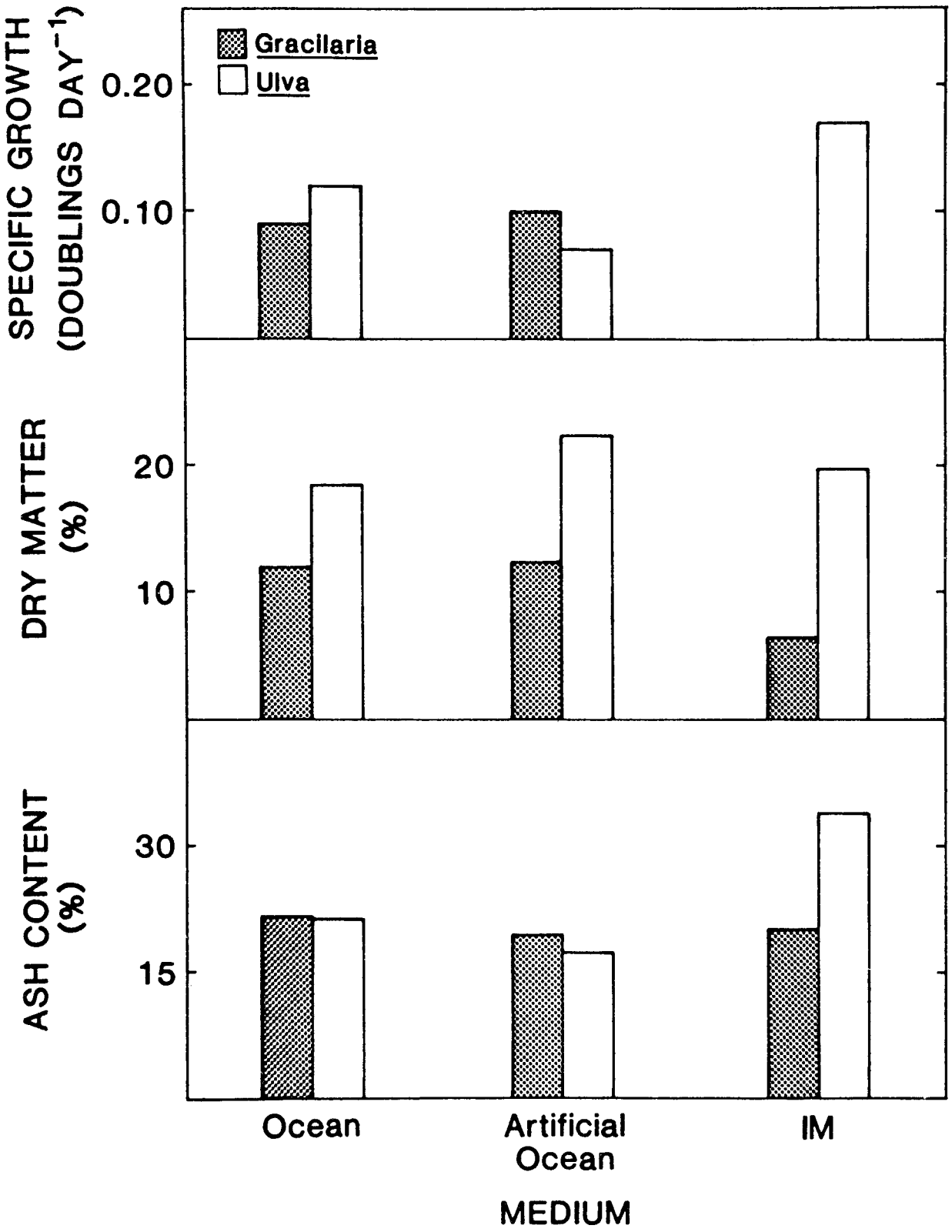

Figure 5. Specific growth, dry matter content and ash content of Gracilaria and Ulva cultured in ocean water and the artificial ocean and IM media.

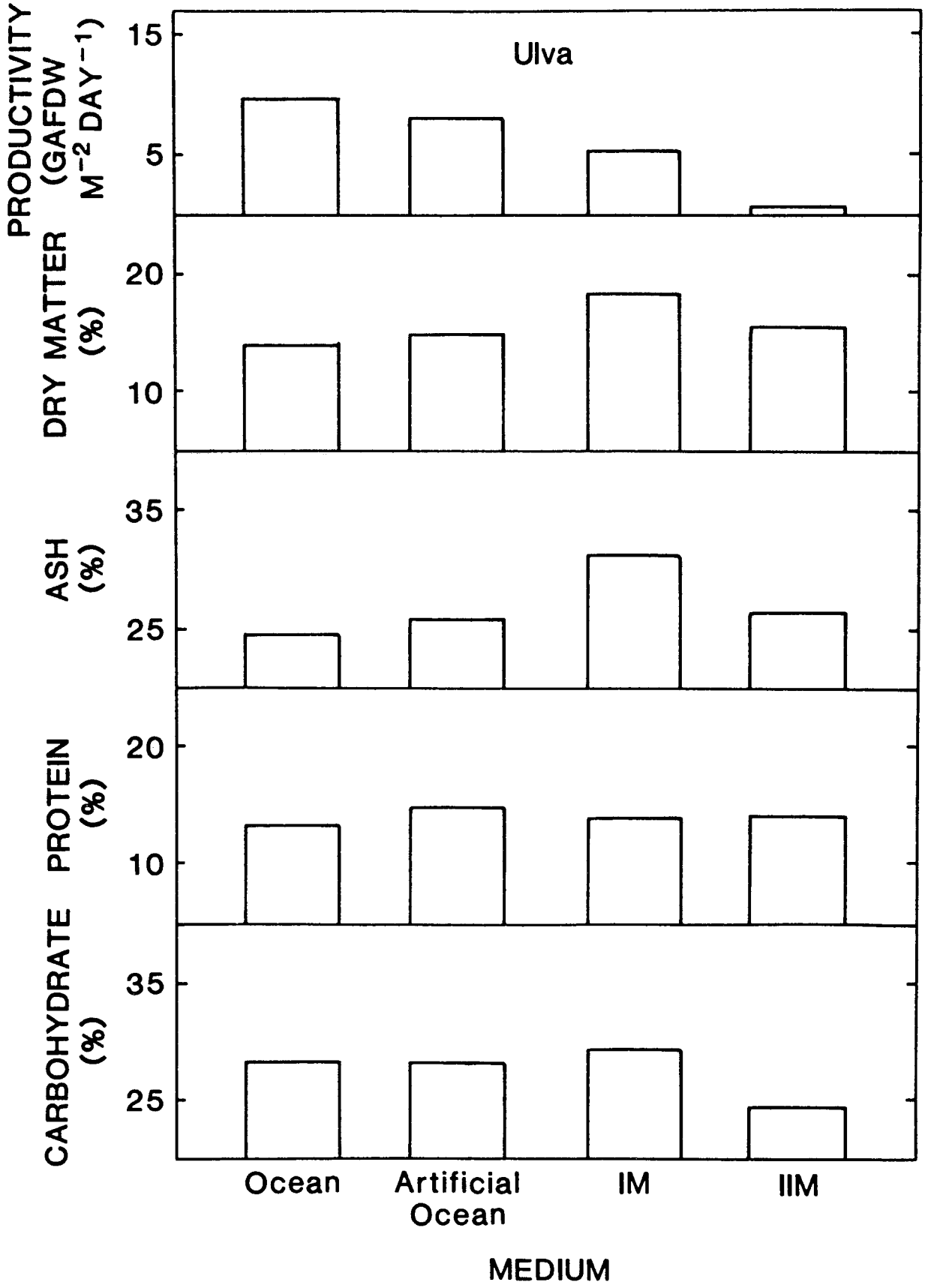

Figure 6. Productivity and proximate composition of Ulva cultured in ocean water, and in three artificial media.

water. As expected, growth in the IIM medium was poor, with the thalli deteriorating within the first week of the experiment. In the ocean and artificial ocean waters, growth was relatively constant both between treatments and over time (from weeks one through three). In the IM medium, Ulva growth was equal to that in the ocean waters in weeks one and two, but then dropped sharply during week three (Fig 7). The Ulva in this treatment fragmented when new discs were cut at the end of week three, so the experiment was terminated.

Although our experiments show that neither the I nor IIM media will support Ulva growth for longer than a few weeks, the reason for the gradual death of the alga in these media is unknown. Seaweed decline may have resulted from the lack of a particular ion or nutrient, or because of a general ionic imbalance of the medium. Relative to seawater at a salinity of 15 ppt, the IIM formula has an extremely high bicarbonate level, but is low in cations, particularly calcium. The IM formula is also high in bicarbonate and low in Mg, but unlike the IIM water, it has a high Ca concentration. However, because of precipitate formation, the true ionic composition of these media during this experimental work was unknown. The IM medium precipitated immediately upon mixing, whereas a precipitate would not generally form in IIM medium until day 2 or 3 of a growth experiment.

The precipitate which formed immediately in the IM waters was probably $CaCO_3$. Surface seawater is roughly 100-300% oversaturated with respect to both Ca^{++} and $CO_3^{=}$ (Riley and Chester, 1971), and the IM medium provides for even higher levels of both minerals. Attempts were made during media formulation to eliminate precipitation by altering both medium pH (to reduce $CO_3^{=}$ levels) and the order of salt additions (to eliminate heterogeneous substances which could act as nucleation sites [Stumm and Morgan, 1970]), but none of these procedures were successful.

A final experiment was conducted to determine whether various mixtures of ocean water and IM medium would support long-term Ulva growth. Maximum growth rates (doublings day^{-1}) during the two week study occurred in the 1:1 mixture of ocean water and IM medium (Fig 8). Growth rates declined among the remaining media in the following order: 1:3; ocean water; 1:9; and finally, IM. The high growth in the 1:1 mixture probably was a result of the high bicarbonate levels provided by the IM medium. During week two, Ulva growth in the IM medium amended with 25% ocean water was substantially higher than that in the IM medium alone (Fig 8). The relatively large percentage of ocean water required to improve Ulva growth in the artificial solution suggests that the IM water is an unbalanced medium for macroalgae growth.

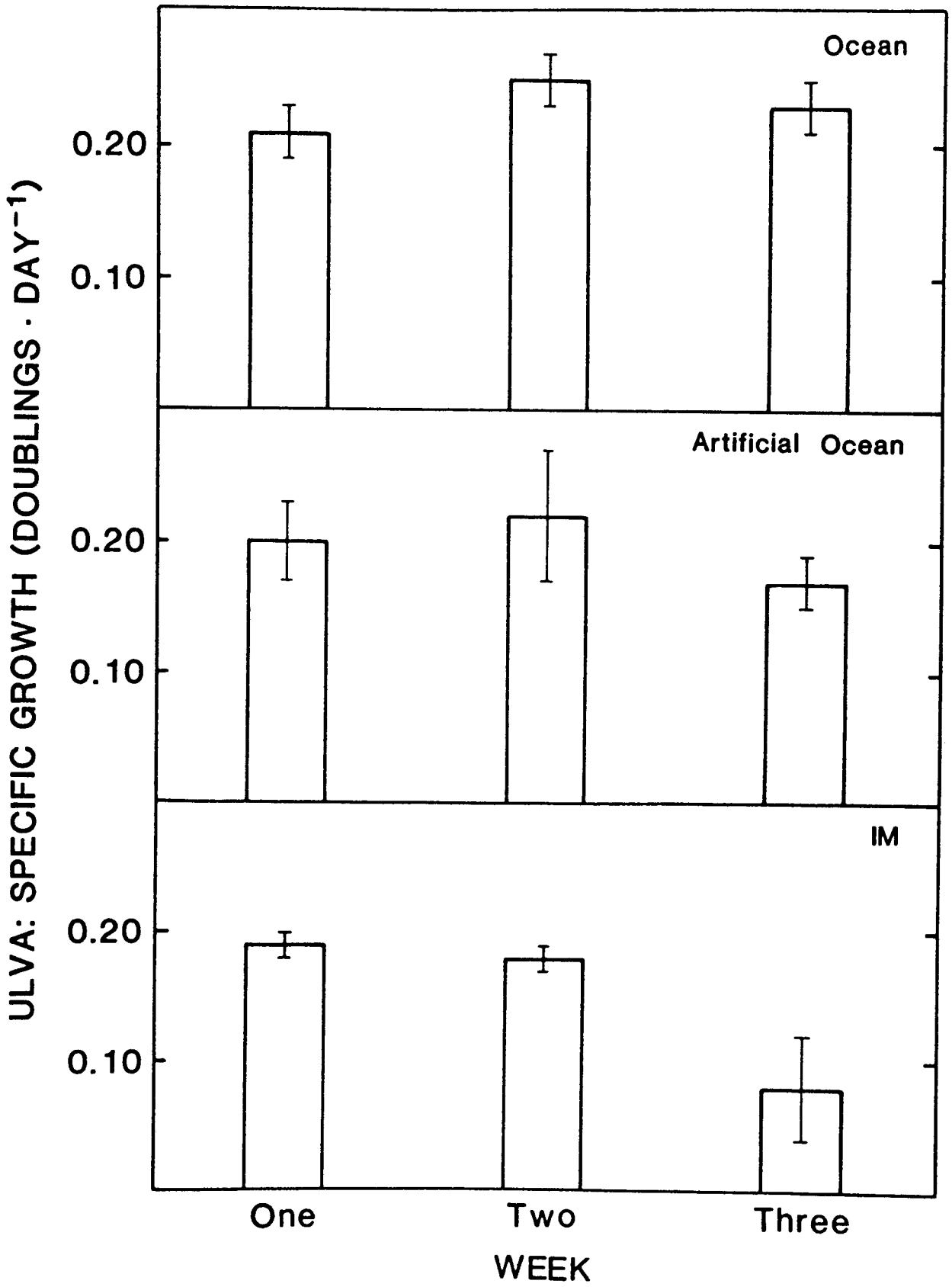

Figure 7. Specific growth of Ulva in ocean water and the artificial ocean and IM media. Each week, thalli were trimmed back to their original size and restocked.

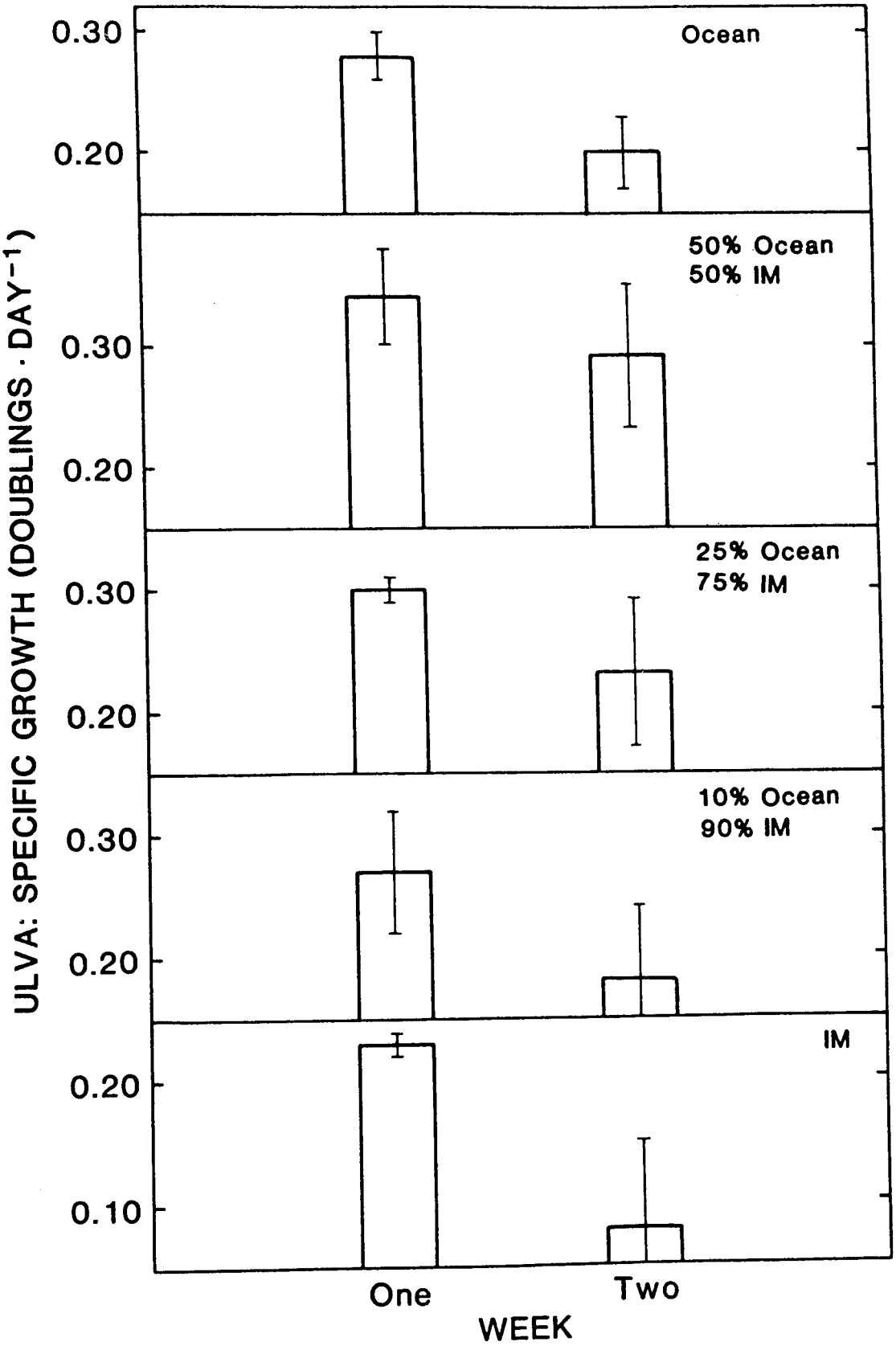

Figure 8. Specific growth of Ulva in ocean water, IM medium, and in three mixtures of these two solutions.

Task 2. Effect of environmental conditions on the proximate composition of marine macroalgae.

Methods

To date, the effect of the environmental parameters salinity, nitrogen availability, water temperature and irradiance on seaweed composition have been examined. Three macroalgae species were utilized for this study. Gracilaria tikvahiae and Ulva lactuca grow rapidly year-round in central Florida, and typically contain moderate to high levels of carbohydrates (Habig et al., 1984). These species were thus chosen as candidates for experiments on increasing the yields of carbohydrates and other storage products. In contrast with the carbohydrate fraction, lipid levels in macroalgae are generally quite low: the average lipid composition of 20 seaweeds examined by Ryther et al. (1984) was only 2-3%. One of the species found to contain highest lipid levels (6%) was Caulerpa prolifera, a green alga common to the Indian River. This species was also utilized for the experiments in this task.

These studies were conducted using a bank of eighteen 13 L aquaria held in a temperature-controlled room (23 ± 2 C). An air wand was placed inside each tank along the back wall to provide continuous mixing of the tank contents. A styrofoam cover was placed over each aquarium to prevent evaporation. Gracilaria and Ulva were grown loose in the aquaria, whereas Caulerpa was grown attached, or "rooted" in washed quartz sand. The following standard conditions were established and utilized as a control treatment in each experiment.

-Irradiance of 6 X 10^{15} quanta $cm^{-2} sec^{-1}$ (measured in situ) provided by two fluorescent lamps on a 14:10 hour light:dark photoperiod (ca 72 watts m^{-2}).

-Water temperature of 22 ± 2 C.

-A tank medium consisting of offshore ocean water, diluted to a salinity of 29 ppt with well water.

-A nutrient medium provided at the concentrations listed in table 3.

The Ulva and Gracilaria utilized for this study were each single clones, maintained in outdoor tanks under a "fixed" cultivation regime. Caulerpa prolifera was obtained on four dates from a natural population on Merritt Island, FL. The plant material was transplanted and held in 700 L outdoor tanks for at least one week prior to its use in the experiments. Detailed methods for the experiments are as follows.

Salinity

Based on data from prior studies with Gracilaria and Ulva (M.D. Hanisak, Harbor Branch Foundation, personal comm.), salinities of 8 and 50 ppt were selected to provide media which would stress, but not kill the three macroalgae during the incubation period. Gracilaria and Ulva were stocked at densities of 50 and 40 g, respectively, into aquaria containing a seawater solution at a salinity of 29 ppt. Triplicate tanks (for each species) with waters of low salinity (8 ppt) were obtained by gradual dilution (over 3 days time) with well water. A highly saline medium (50 ppt) was established in another set of replicate tanks by the gradual addition of a balanced salt mixture similar to that described in experiment A2. An additional group of

three tanks for each species was maintained at 29 ppt as a control. Nutrients (Table 3) were added to each tank after final salinity adjustments were made.

On day 11 of the experiment, the algae in the high and moderate salinity tanks were weighed, harvested back to initial stocking weights (to prevent overcrowding), and restocked. Nutrients in the media were replenished at this time with levels 0.6X those shown in table 3. Ulva and Gracilaria in the low salinity treatment were harvested on this date, but were not restocked because of the poor appearance of the plants. On day 13, the experiment was terminated and the algae (high and medium salinities) were rinsed and weighed. Dry matter production was calculated as the dry weight biomass increase from day 1 to day 13 (or 11).

In the salinity study with Caulerpa, duplicate, rather than triplicate tanks were utilized at each salinity. Salinity adjustments (to high and low levels) were made over a period of 4 days, and the total duration of the study was 14 days. Productivity was not measured because of the plants attached habit.

Nitrogen

Gracilaria and Ulva were cultured in seawater containing high, moderate and low levels of nitrogen. A medium containing the $NaNO_3$ concentrations shown in table 3 was established as the "moderate" nitrogen level. High nitrogen levels were established in a medium by elevating the preceding $NaNO_3$ concentration eight-fold. Nitrogen was omitted from the standard medium to create a solution deficient in nitrogen. Initial nitrogen concentrations in these treatments were 0, 7 and 54 mg L^{-1}, or approximately 0, 19 and 130 mg N per gram of dry seaweed stocked in the tanks.

Three aquaria were utilized for each species under each N-loading treatment. Gracilaria and Ulva were stocked at 50 and 40g wet weight, respectively. After seven days time, the algae were harvested back to their original densities and restocked. The water in the tanks was then changed and the nutrient media were replenished. On day 14 of the experiment, the algae were harvested, weighed, and subsamples were collected for tissue analyses. Water samples were collected from one tank in each treatment, filtered, and analyzed for inorganic N and P. Productivity of the algae under the three nitrogen loading regimes was calculated as the biomass increase between day 7 and day 14.

A similar experiment to determine the effect of nitrogen availability on the proximate composition of Caulerpa prolifera was conducted using only moderate and low nitrogen levels. High nitrogen concentrations were not attempted in the Caulerpa aquaria because of past experience under such conditions of severe epiphyte fouling. Caulerpa was cultured under the low and moderate nitrogen levels for 10 days, with nutrient media replenished on day 5.

Temperature

Gracilaria, Ulva and Caulerpa were cultivated under three temperature regimes in the 13 L aquaria. High water temperatures (31-34 C) were

maintained in one group of aquaria with 50 watt heaters. In another group of aquaria, low temperatures were maintained by passing chilled water through heat exchangers placed along an inside wall of each tank. Cold water was produced either in an ice bath or by using a commercial chiller (Aqua-chiller).

Experiments with Gracilaria, Ulva and Caulerpa were conducted separately. Gracilaria was stocked (40 g wet weight) into 9 aquaria containing water at the ambient room temperature (22 C). Temperatures were then slowly adjusted to the following levels: high, 34-35 C; moderate, 22-23 C; and low, 8.5-14 C. On day 6 of the experiment, the seaweed was harvested back to its original density, the water in each aquarium was changed, and the nutrient media were replenished. The experiment was terminated on day 10.

Ulva was stocked at 40 g into nine aquaria containing media at a temperature of 22 C. Temperature levels were changed over the course of 2 days to the following ranges: High, 34 C; moderate, 23 C; and low, 10 C. The Ulva was harvested and the experiment was terminated on day 7 due to the poor appearance of the seaweed in the high and low temperature treatments.

Caulerpa was stocked at 11 g into aquaria containing media at a temperature of 22 C. Three temperature levels were established in the tanks after 2 days: high, 31-32 C; moderate, 23 C; and low, 10 C. On day 10, the water in each aquarium was changed and nutrient levels were replenished. The experiment was terminated on day 14.

Light

The effects of light intensity on the proximate composition of the three macroalgae were studied in a glass greenhouse. Nine 13 L aquaria were partially submersed in small water baths which received a constant flow of well water (24 C). The resulting diel fluctuations in temperature within each aquarium during the study were slight (23-25.5 C).

All aquaria were covered with a sheet of clear polyethylene (4 mil) to prevent evaporation. Layers of neutral density screening were used to establish three light levels among the tanks. Total insolation was measured with an Epply pyroheliometer, while the extinction of PAR within each tank was determined with a Biospherical Instruments quantum sensor. Seaweeds cultured at the highest irradiance received approximately 150 g cal cm^{-2} day^{-1}, while those in the medium and low light treatments received approximately 0.2 and 0.02 times this amount, respectively.

Experiments on the three macroalgae were conducted at separate times. Gracilaria was stocked at 40 g per tank. Supplemental fertilization was provided on days 4 and 8. Water in each aquarium was changed on day 12, at which time nutrient solutions were also replenished. On days 9 and 12, the seaweed was harvested back to original densities and restocked. The experiment was terminated on day 15, with productivity calculated from biomass changes from days 1 through 15.

Ulva was also stocked at 40 g, and was harvested back and restocked on day 7. Supplemental fertilization was provided on days 4 and 7. The experiment was terminated on day 9, with productivity calculated as the biomass change from days 1-9.

Caulerpa was stocked at a density of 13 g per tank. On day 7, the water in each aquarium was changed and nutrients were replenished. The experiment was terminated on day 13.

Composition analyses

Algae harvested from the tanks were rinsed in freshwater, blotted dry and weighed. Productivity calculations were based on dry weight biomass changes of the seaweeds (final dry weight - initial dry weight) and thallus ash content. Two subsamples of algae were collected from each tank at the termination of all experiments. The first was lyophilized and stored in a freezer for lipid analyses. The second was placed on a tared square of foil, weighed, dried (70 C for 48 hr) and then reweighed, thus providing a measure of % dry weight. The sample was then ground and stored for later analyses.

The ash content of the oven-dried samples was determined gravimetrically following combustion in a muffle furnace (500 C for 4 hours). The protein content of ca. 20 mg dried algal samples was determined colorimetrically using a slight modification of the Lowry (Folin-Phenol) method. Following the addition of 5 ml of 1 N NaOH to the test tubes containing the samples, the extraction was conducted in a water bath at 50 C for 12 hours, rather than at room temperature for 24 hours.

The carbohydrate content of 10 mg portions of the dried samples was determined colormetrically by the Dubois Phenol-Sulfuric Acid method. Lipids were extracted from 1-3 g of freeze-dried algal samples by grinding (Virtis tissue homogenizer) in 100 ml of extraction solvent (methanol:methylene chloride:water, 10:5:4 by volume) (Tornabene et al, 1982). The methylene chloride fraction was seperated and evaporated just to dryness on a Buchler Evapomix. The remaining lipid fraction was measured gravimetrically.

Results and Discussion

Salinity

Gracilaria and *Ulva* exhibited different trends in productivity in response to salinity of the culture medium (Fig 9). *Ulva* grew well at both low and moderate salinities, but growth at the high salinity was poor. In contrast, organic matter production by *Gracilaria* at the low salinity was slight, but then increased with increasing salinity. These data suggest that *Gracilaria* is an oceanic species, whereas *Ulva* is more adapted to estuarine conditions.

Although salinity of the culture medium affected the dry matter content (% of wet wt.) of the three macroalgae, the effects were not consistent between species (Fig 9). The % dry weight of *Gracilaria* increased with increasing salinity, whereas that of *Ulva* decreased. Maximum % dry wt. of *Caulerpa* occurred at the medium salinity level. In contrast, the % ash content of all three macroalgae increased with increasing salinity. This increase was most pronounced in *Gracilaria* between the salinities of 8 and 29 ppt, where ash content increased from 28 to 48 %. Such a high thallus mineral content may be required at high salinities in order to maintain the osmotic balance between the cells and the culture medium.

Little difference was observed between salinities in the composition of protein, carbohydrate, and lipid for each species (Fig 9). A very gradual increase in protein (from 12.2 to 13.8%) in *Gracilaria* was observed with increasing salinity. The protein content of *Ulva* did not change with salinity, whereas the maximum protein content of *Caulerpa* (11.8%) occurred at the lowest salinity.

Highest soluble carbohydrate levels (60.0%) in *Gracilaria* were found at a salinity of 50 ppt. Elevated salinities have also been found to stimulate the production of the carbohydrates floridoside and isofloridoside in the red alga *Porphyra perforata* (Craigie, 1974). In contrast, salinity of the culture medium had little effect on carbohydrate levels in *Caulerpa*, and carbohydrate levels in *Ulva* actually declined with increasing salinity.

A slight increase in the lipid composition of *Gracilaria* and *Caulerpa* was observed with increasing salinity of the medium. The sharpest increase was observed in *Caulerpa*, the species with the highest lipid content, where lipid levels increased from 6.3-8.2%. Previous investigators have reported an even greater stimulation of lipid production in microalgae at high salinities. Craigie and McLachlan (1964) found that a hundred-fold increase in culture medium NaCl concentrations increased glycerol levels in *Dunaliella tertiolecta* by 133X.

The most prominent effect of salinity on relative concentrations of potentially valuable storage products was found in *Gracilaria*, where the carbohydrate content of thalli cultivated at 50 ppt was 34% higher than that of seaweed cultured at 29 ppt. High salinity also caused a slight increase in lipid synthesis in *Caulerpa*.

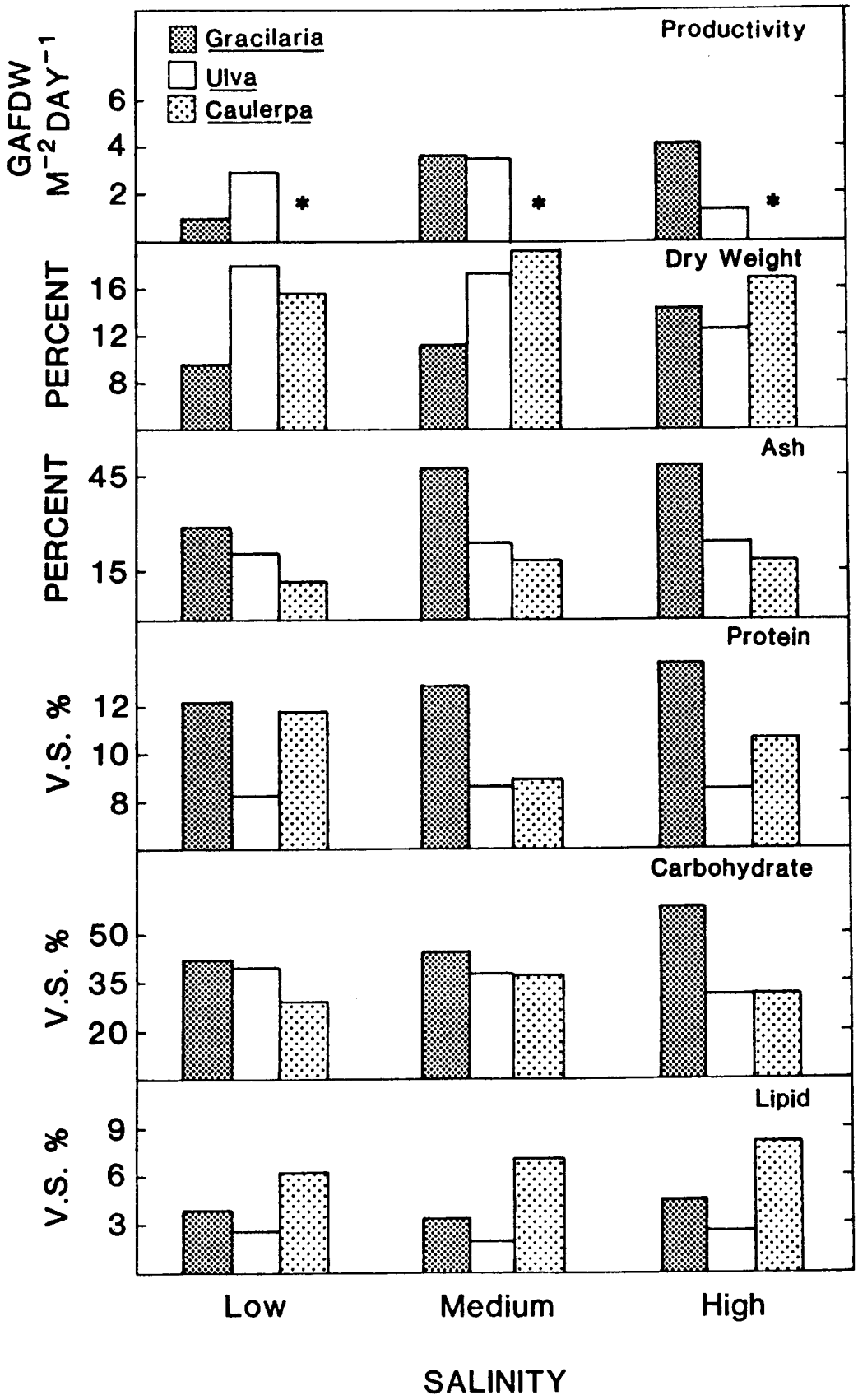

Figure 9. Productivity and proximate composition of three macroalgae cultured at low (ca 8 ppt), medium (29 ppt) and high (50 ppt) salinities. Parameters not measured for Caulerpa are denoted by an asterisk.

Nitrogen

Nitrogen availability in the medium markedly affected the productivity and composition of the three macroalgae (Fig 10). Productivity of both Ulva and Gracilaria increased with increasing nitrogen level in the medium. This stimulation in organic matter production was greatest between low and moderate nitrogen levels. Only a slight increase in productivity (4% for Gracilaria and 10% for Ulva) was observed between moderate and high nitrogen levels.

The observed trends in dry weight, ash, protein and carbohydrate composition among nitrogen levels were similar for each of the three macroalgae. With decreasing nitrogen availability, % dry weights increased, ash and protein contents decreased, and carbohydrate levels increased. Tissue nitrogen concentrations for Gracilaria were 1.0, 1.8 and 3.1% in the low, moderate and high nitrogen media at the end of the experiment. The respective tissue nitrogen concentrations for Ulva were 0.7, 2.1 and 2.6% in the three treatments. Amino acid synthesis, and hence, protein production, is limited when little nitrogen is available in the medium. Sugars produced in photosynthesis under n-deficient conditions are utilized primarily for the production of structural and non-structural (soluble) carbohydrates (Bird et al., 1982; Habig et al., 1984). Increased structural carbohydrate levels in the cells may in turn reduce the water content of the plant (Russell, 1971). The high ash content observed in algae cultured at elevated salinities may be related to an increase in mineral (cation) uptake which would accompany increased NO_3 assimilation.

Nitrogen availability in the medium had little effect on the lipid compostion of the three macroalgae. The lipid composition of Caulerpa was equal at low and moderate nitrogen levels, whereas the high nitrogen medium produced highest lipid levels in Gracilaria, but lowest lipid levels in Ulva. Prior studies have shown that prolonged n-starvation can stimulate the production of lipids in microalgae (Shifrin and Chisholm, 1981). Carbohydrate synthesis apparently occurs during the initial stages of N-deprivation , whereas after a prolonged period without nitrogen, lipid synthesis dominates. None of the macroalgae in the present study exhibited a substantial increase in lipid concentration after two weeks cultivation in a N deficient medium. However, a month long N deprivation study is now being conducted with Caulerpa in an attempt to increase lipid synthesis by this alga.

Temperature

Maximum growth of Ulva and Gracilaria in this experiment occurred in the media maintained at a moderate (21-24 C) temperature level (Fig 11). However, Gracilaria was more tolerant of the high and low temperature extremes than was Ulva. Growth of Ulva was less than 1 gafdw m^{-2} day^{-1} in both the 8 C and 34 C treatments during the one week study.

The macroalgae cultivated at 8-12 C had the highest % dry weight, while the maximum ash content for all species occurred at 22 C (Fig 11). It is unknown what effect temperature may have on dry matter content of algae. The reduced ash content of the plants cultured at the temperature extremes may

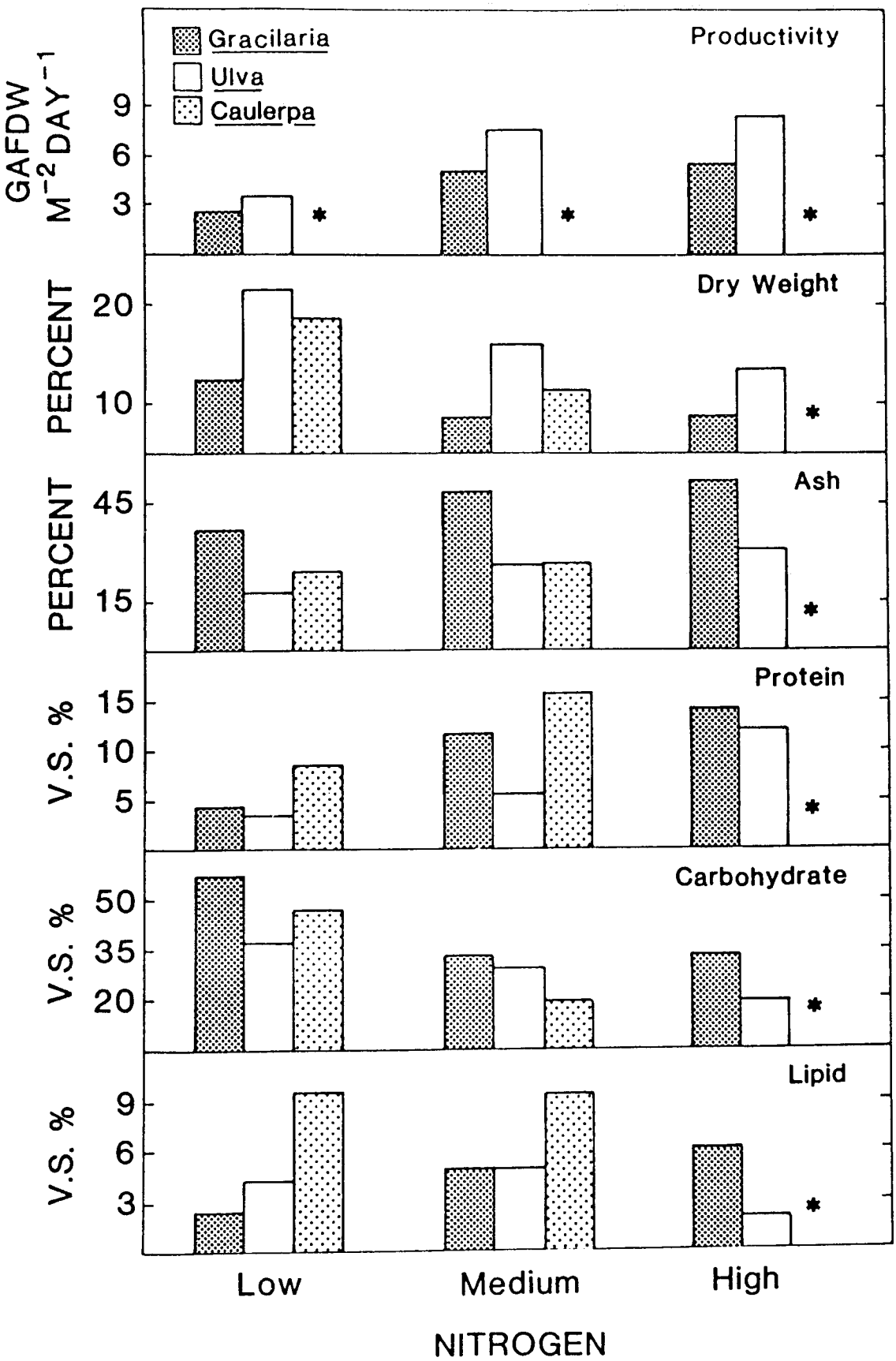

Figure 10. Productivity and proximate composition of three macroalgae cultured at low (0 mg L^{-1}), medium (7 mg L^{-1}) and high (54 mg L^{-1}) nitrogen levels. Parameters not measured for Caulerpa are denoted by an asterisk.

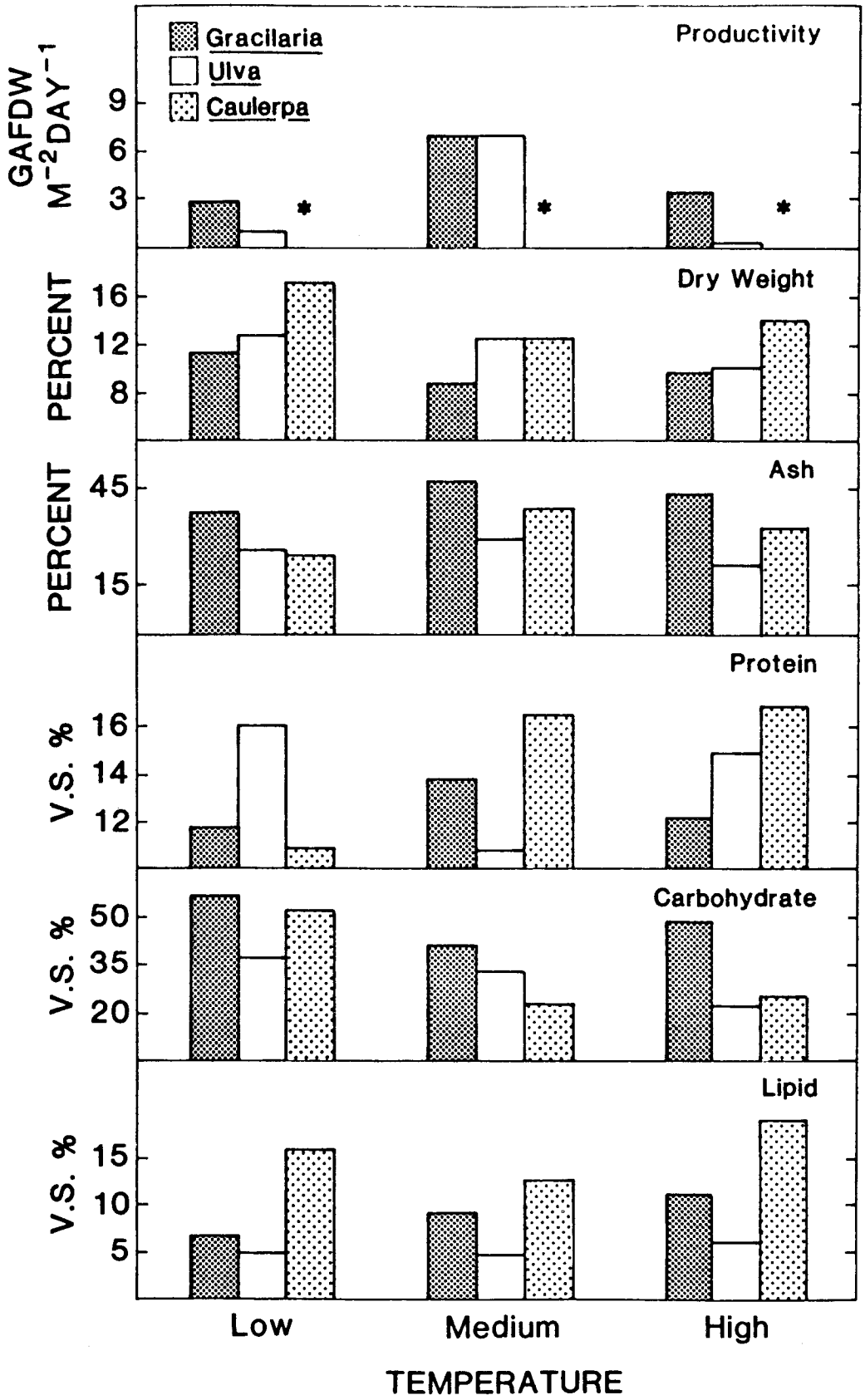

Figure 11. Productivity and proximate composition of three macroalgae cultured at low (ca 10°C), medium (22°C) and high (ca 33°C) water temperatures. Parameters not measured for Caulerpa are denoted by an asterisk.

have resulted from lower rates of nutrient assimilation by the relatively slow-growing plants in these treatments.

Gracilaria and Caulerpa cultivated at the lowest temperature contained the lowest levels of protein (Fig 11). This suggests a low temperature inhibition of either NO_3 uptake or protein synthesis, as the growth of the algae in this treatment was probably not sufficiently rapid to dilute protein reserves. In contrast, however, protein levels of Ulva cultured in the low temperature (8 C) treatment were quite high.

The carbohydrate content of the three macroalgae was greatest at the lowest cultivation temperatures (Fig 11). For Caulerpa, carbohydrate levels at 10 C were twice that in thalli cultured at 22 C. The increased levels of carbohydrate of these algae at 10 C may be explained by the differing effect of temperature on photosynthesis and respiration reactions. Q_{10} values for the collective reactions of photosynthesis, some of which are mediated photochemically, range from 1.0 - 2.7. Because the Q_{10} values for respiration are generally higher (2.0-2.5), the catalysis of sugars and carbohydrates for energy may not keep pace with their synthesis under conditions of low temperature. Hence, relative quantities of soluble carbohydrates in the algal cells may increase at low temperatures, particularly when other environmental factors (ie, light) favor rapid photosynthesis.

Although between treatment differences in lipid composition of these algae were not great, maximum lipid concentrations for all species were found in thalli cultured at 34 C.

Light

Irradiance strongly affected organic matter production by Gracilaria and Ulva (Fig 12). Productivity of both species was almost negligible at the lowest light level, but then increased sharply as culture tank irradiance was increased. Gracilaria apeared slightly more tolerant of low light levels than Ulva.

Light intensity had little effect on most compositional parameters of the three macroalgae (Fig 12). Dry weight, ash and carbohydrate contents were essentially equal among the high, medium and low light levels. However, the protein composition of Ulva and Caulerpa did vary inversely with irradiance. It is likely that nitrogen uptake by the "high light" algae could not keep up with tissue synthesis, or growth, and thus internal nitrogen pools were diluted. Lapointe (1981) observed this phenomenon in a rapidly growing culture of Gracilaria tikvahiae, even though the thalli were maintained in a medium containing high nitrogen levels.

Irradiance had little effect on the lipid composition of Ulva and Gracilaria (Fig 12). The highest lipid compostion for Ulva was found at the highest light level, whereas maximum lipid concentrations in Gracilaria occurred at the lowest light intensity. Caulerpa lipid analyses for this experiment have not yet been completed.

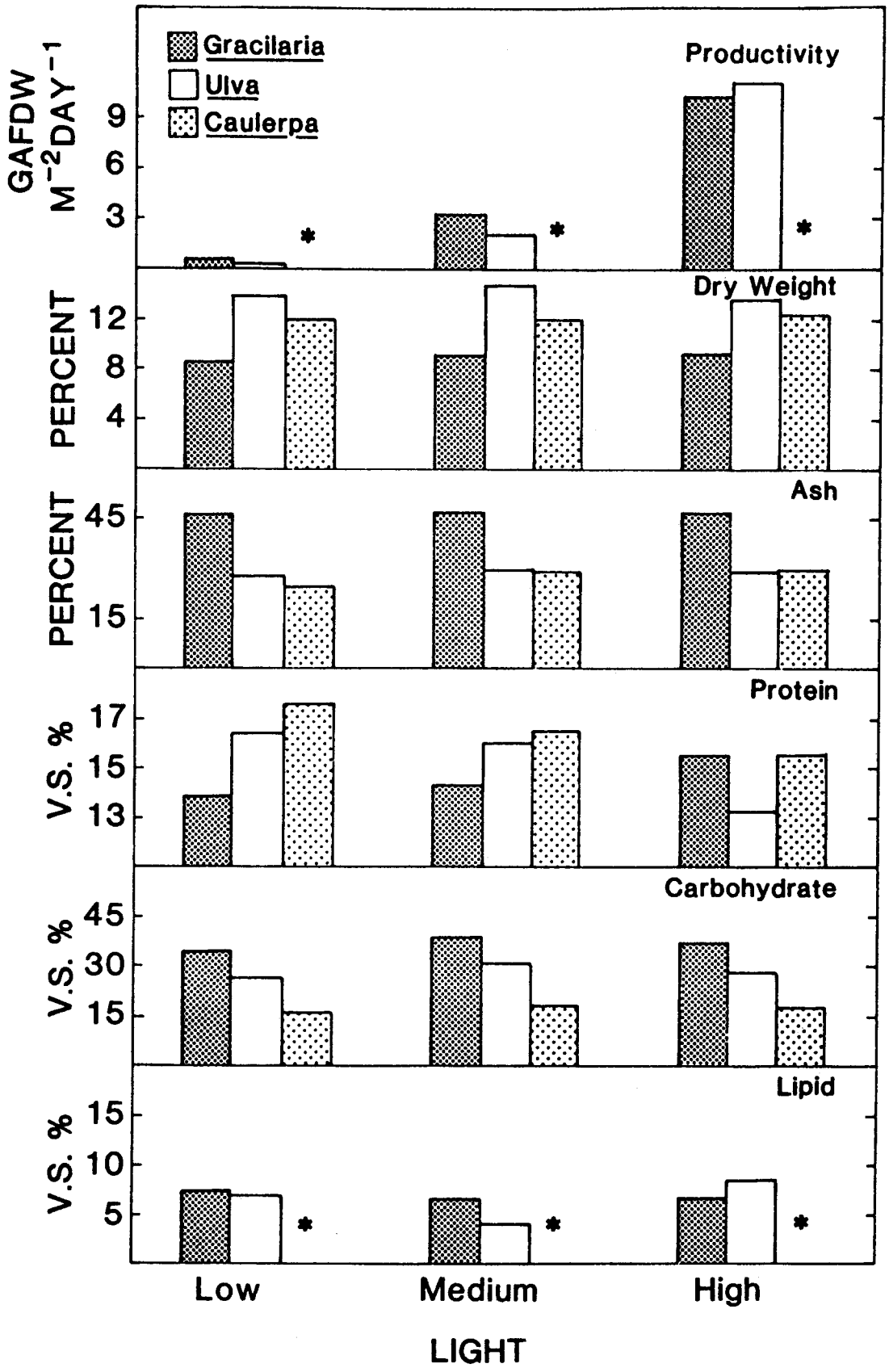

Figure 12. Producitivity and proximate composition of three macroalgae cultured at low (3 g cal cm^{-2}day^{-1}), medium (30 g cal cm^{-2}day^{-1}) and high (150 g cal cm^{-2}day^{-1}) levels of irradiance. Parameters not measured for Caulerpa are denoted by an asterisk.

Relationship between environmental conditions and proximate composition.

In figures 13-17, the effects of the four environmental parameters salinity, nitrogen availability, temperature and light intensity are considered for each individual storage product (ie, dry weight, carbohydrate, etc.). The relative importance of each environmental parameter to the synthesis of a particular storage product can thus be quickly determined. Note, however, that although identical environmental conditions were provided in the control (medium) treatment of each experiment, the composition of the algae under these standard conditions varied among experiments. Relative fluctuations were greater for some parameters (eg. lipid, dry weight) than for others (eg. carbohydrate). The algae used in these studies were obtained from outdoor cultures which were exposed to gradual (solar radiation and temperature) and short-term (salinity) fluctuations in environmental conditions. Hence, it is likely that the composition of the initial plant stock varied at least slightly among experiments.

Tables 4-9 were adapted from figures 13-17 in order to "standardize" plant composition values in the control (medium) treatment among experiments. Data in these tables represent the difference between proximate composition (or productivity) in the experimental treatment and that in the control treatment. The most prominent effects of environmental conditions on plant composition are described briefly in the following section.

Productivity of Gracilaria and Ulva increased sharply in response to high light conditions (Fig 18, Table 4). In contrast, the growth of these macroalgae was greatly reduced at the high and low temperature extremes, and under conditions of low nitrogen availability.

Nitrogen availability was the dominant factor affecting dry weights of Gracilaria, Ulva and Caulerpa, with highest % dry weights occurring under low nitrogen conditions (Fig 13, Table 5). Light intensity had almost no effect on the % dry weight of these algae, while the effects of temperature and salinity varied with species.

Wide fluctuations in ash content, particularly that of Gracilaria, were observed during this study (Fig 14, Table 6). Salinity was the environmental parameter which had the greatest effect on ash content, whereas ash composition remained relatively constant at different light intensities.

The protein composition of Gracilaria, Ulva and Caulerpa was sharply influenced by nitrogen availability, and to a lesser extent, water temperature (Fig 15, Table 7). Ulva cultured at high and low temperatures had a high protein content. In contrast, low and high temperature cultivation either decreased, or had no effect upon the protein levels of Caulerpa and Gracilaria.

The carbohydrate composition of Gracilaria was greatly increased by cultivation under conditions of high salinity, low nitrogen availability and low temperature (Fig 16, Table 8). Although carbohydrate levels of Ulva and Caulerpa declined at high salinity, carbohydrate compostion was increased by low temperature and low N cultivation.

Table 4. Effect of environmental factors in the productivity of Gracilaria tikvahiae and Ulva lactuca. Values represent difference in productivity (as g AFDW m^{-2} day^{-1}) between experimental and control conditions. See text for description of treatments.

	Gracilaria		Ulva	
	Low	High	Low	High
Salinity	-2.8	+0.4	-0.7	-2.2
Nitrogen availability	-2.7	+0.2	-4.1	+0.8
Temperature	-4.2	-3.8	-5.8	-6.8
Light intensity	-2.4	+7.0	-2.1	+9.4

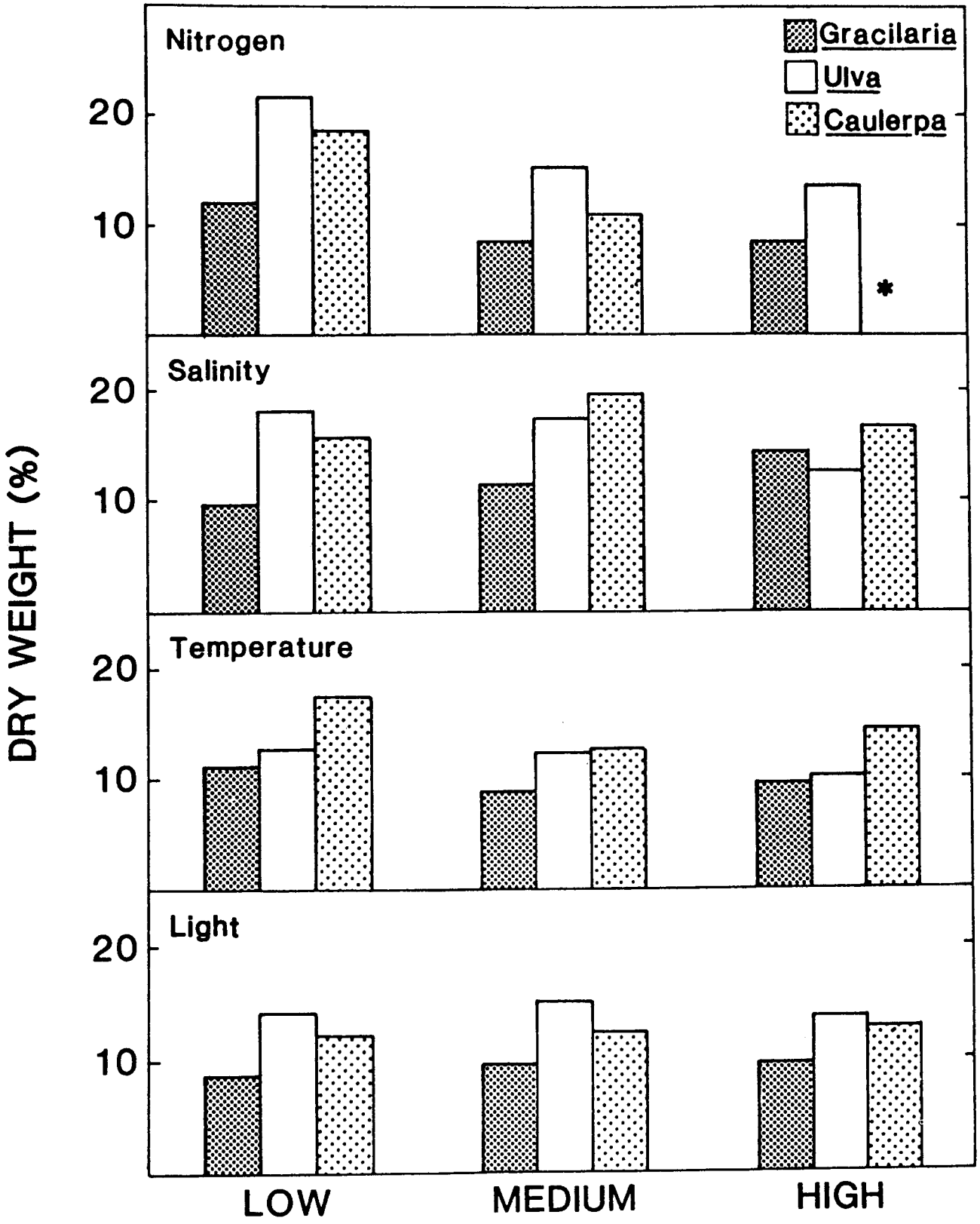

Figure 13. Effect of four environmental parameters on dry weight (as % of wet wt) of three macroalgae. Low, medium and high treatment regimes are described in text.

Table 5. Effect of environmental factors on the dry weight of three macroalgae. Values represent difference in dry weight (as % of wet weight) between experimental and control conditions. See text for description of treatments.

	Gracilaria		Ulva		Caulerpa	
	Low	High	Low	High	Low	High
Salinity	-1.7	+3.0	+0.7	-5.1	-3.8	-2.7
Nitrogen availability	+3.5	-0.1	+6.5	+1.7	+7.6	-
Temperature	+2.3	+0.6	+0.6	-2.2	+4.7	+1.5
Light intensity	-0.6	+0.1	-1.0	-1.4	-0.1	+0.2

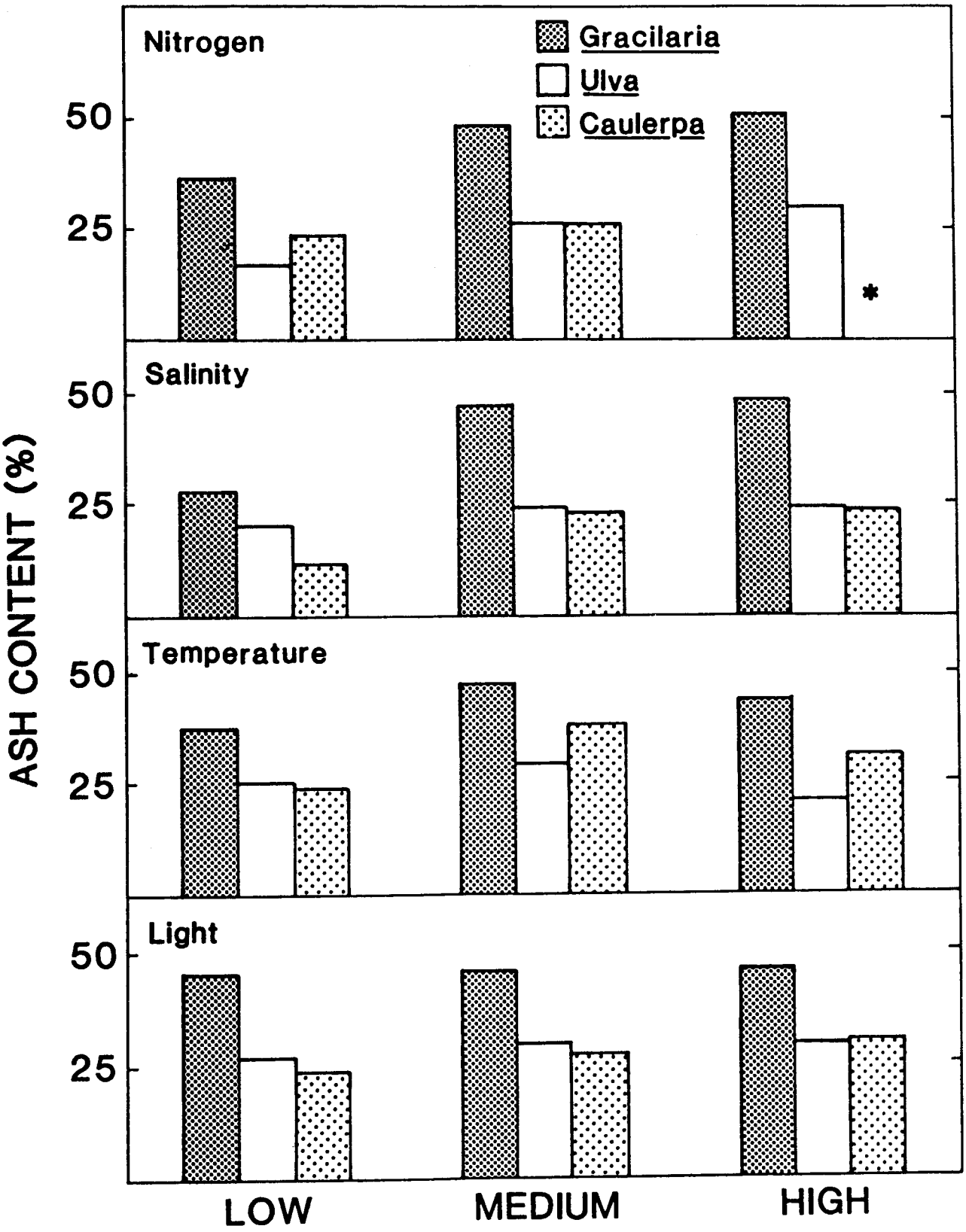

Figure 14. Effect of four environmental parameters on ash content (as % of dry weight) of three macroalgae. Low, medium and high treatment regimes are described in text.

Table 6. Effect of environmental factors on the ash content of three macroalgae. Values represent difference in ash content (as % of dry weight) between experimental and control conditions. See text for description of treatments.

	Gracilaria		Ulva		Caulerpa	
	Low	High	Low	High	Low	High
Salinity	-19.7	+1.3	-3.8	+0.3	-6.5	-0.2
Nitrogen availability	-11.5	+2.9	-8.3	+4.8	-2.6	-
Temperature	-10.0	-3.9	-3.6	-7.5	-14.0	-5.9
Light intensity	-0.4	+0.2	-2.0	-0.2	-3.9	+2.4

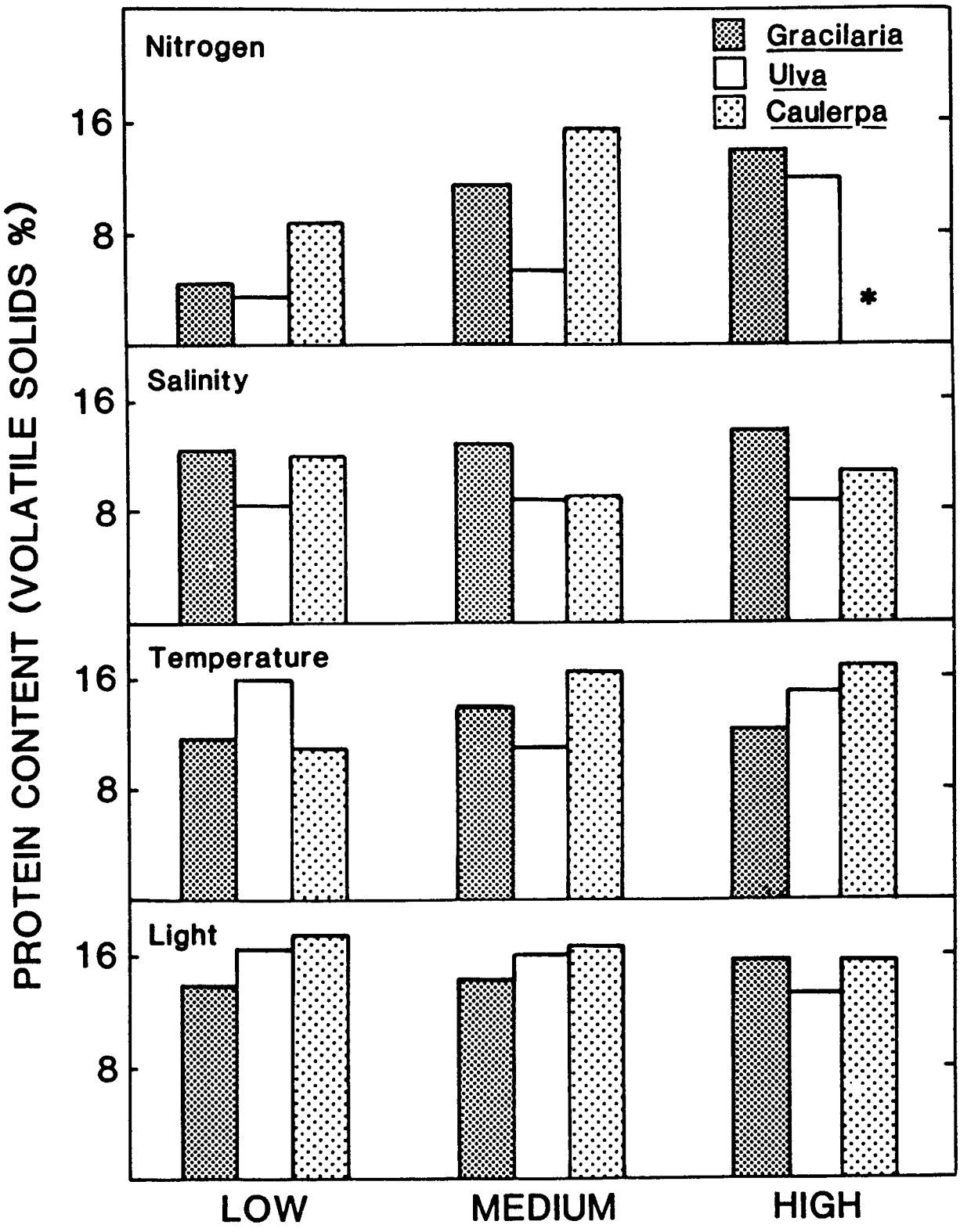

Figure 15. Effect of four environmental parameters on the protein content (as % of volatile solids) of three macroalgae. Low, medium and high treatment regimes are described in text.

Table 7. Effect of environmental factors on the protein content of three macroalgae. Values represent difference in protein content (as % of volatile solids) between experimental and control conditions. See text for description of treatments.

	Gracilaria		Ulva		Caulerpa	
	Low	High	Low	High	Low	High
Salinity	-0.6	+1.0	-0.5	-0.2	+2.9	+1.9
Nitrogen availability	-7.2	+2.3	-1.8	+6.7	-6.8	-
Temperature	-2.1	-1.7	+5.2	+4.1	-5.6	+0.4
Light intensity	-0.4	+1.2	+0.4	-2.8	+1.0	-0.9

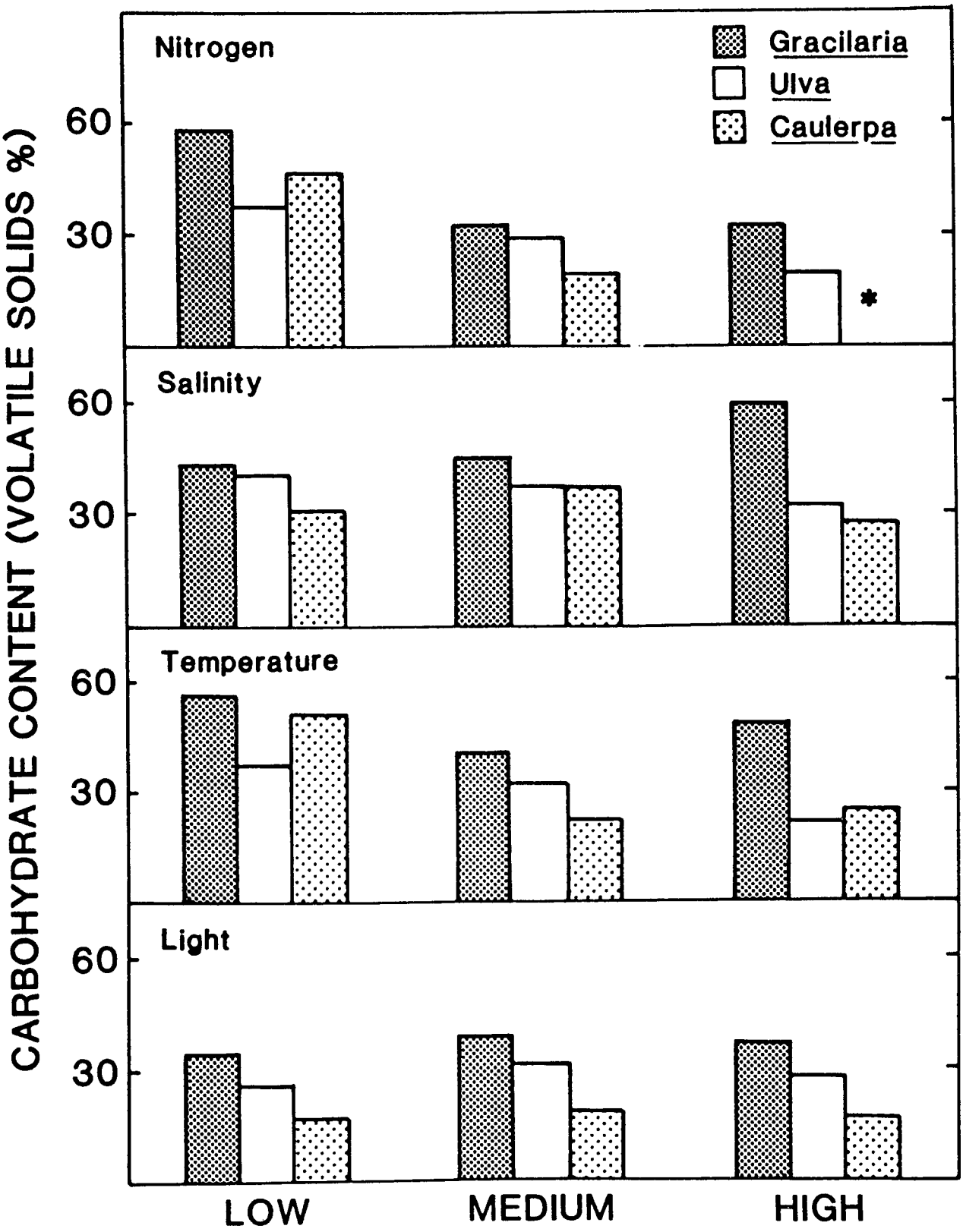

Figure 16. Effect of four environmental parameters on the carbohydrate content (as % of volatile solids) of three macroalgae. Low, medium and high treatment regimes are described in text.

Table 8. Effect of environmental factors on the carbohydrate content of three macroalgae. Values represent difference in carbohydrate content (as % of volatile solids) between experimental and control conditions. See text for description of treatments.

	Gracilaria		Ulva		Caulerpa	
	Low	High	Low	High	Low	High
Salinity	-2.1	+15.3	+2.4	-5.7	-6.2	-9.4
Nitrogen availability	+25.1	-0.6	+8.1	-9.5	+28.4	-
Temperature	+15.6	+7.9	+4.7	-11.6	+29.3	+2.6
Light intensity	-5.0	-1.8	-4.2	-2.7	-1.7	-1.5

Lipid levels in Gracilaria increased slightly in response to high nitrogen and high temperature cultivation (Fig 17, Table 9). Maximum lipid storage in Ulva occurred at high and low light intensities, whereas lipid levels in Caulerpa were increased by cultivation at the high and low temperature extremes.

Storage product yields.

For each experiment, protein, carbohydrate and lipid concentrations were multiplied by the rates of organic matter production, thus providing a measure of the daily production of these constituents by Gracilaria and Ulva.

Maximum protein production by these two algae occurred under conditions of high light intensity, and high nitrogen availability in the medium (Fig 19). Although maximum thallus protein levels were not found at the highest light intensity, the rapid productivity of the algae in this treatment resulted in high protein yields (ca 1.4 g $m^{-2}day^{-1}$). In the N availability experiment, the high nitrogen treatment resulted in highest thallus protein concentrations as well as rapid seaweed growth. Other conditions under which protein production by Gracilaria was maximized were high salinity, and medium temperature. The maximum protein production for Ulva also occurred at the medium temperature, but at the medium (29 ppt), rather than the high salinity.

Maximum carbohydrate production by Ulva and Gracilaria occurred under high light conditions (Fig 20), primarily because of the rapid productivity of the algae in this treatment. In contrast with protein, however, maximum carbohydrate production in the N availability experiment occurred at medium nitrogen levels. Conditions of medium salinity and temperature resulted in maximum carbohydrate production by Ulva, but for Gracilaria, medium temperature and high salinity were the environmental conditions which favored carbohydrate production.

Conditions of high light and medium temperature stimulated maximum lipid production by these macroalgae (Fig 21). For Gracilaria, high salinity and high nitrogen conditions also enhanced lipid production, whereas for Ulva, lipid production was maximized at medium nitrogen and medium salinity levels.

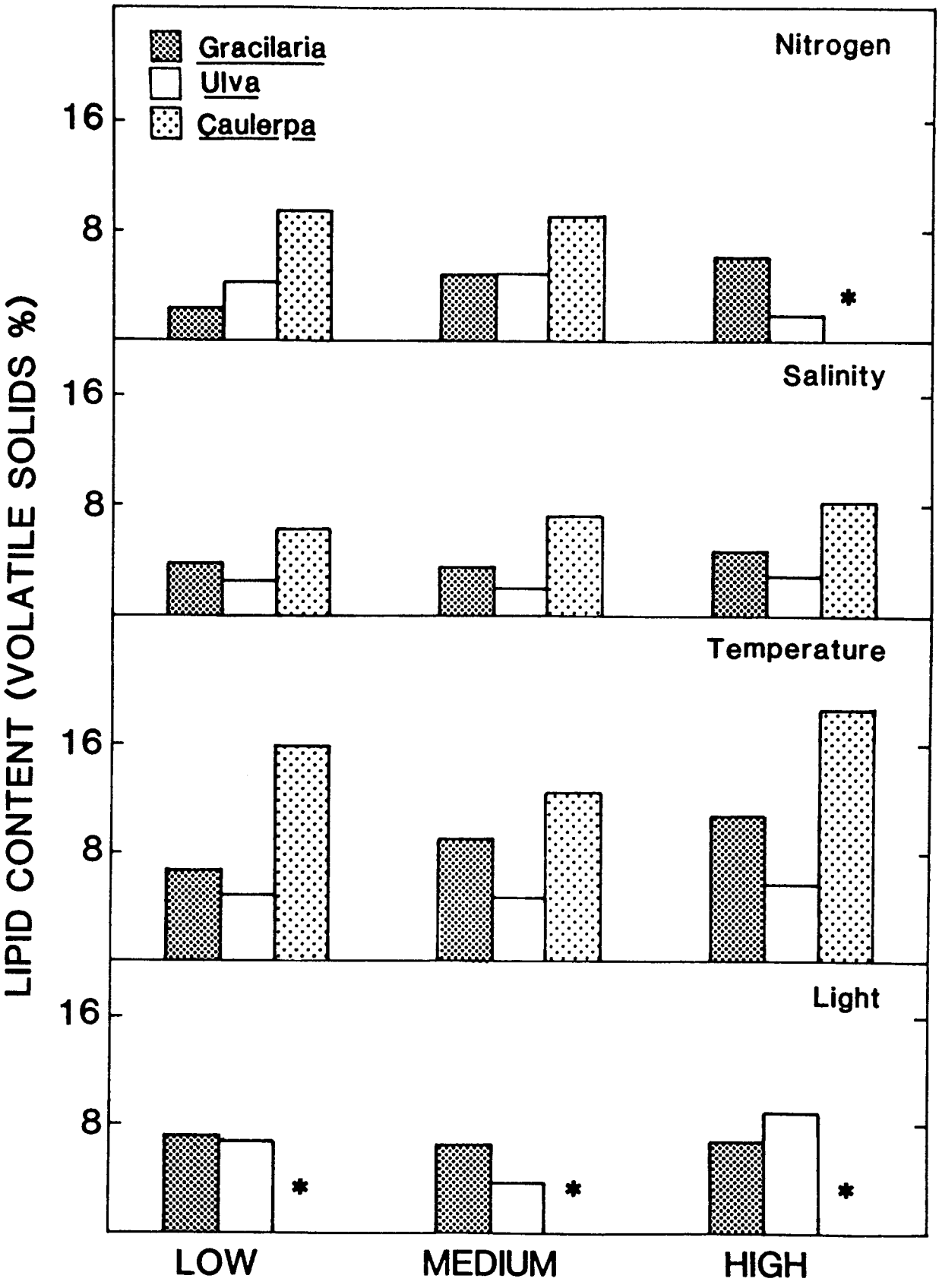

Figure 17. Effect of four environmental parameters on the lipid content (as % of volatile solids) of three macroalgae. Low, medium and high treatment regimes are described in text.

Table 9. Effect of environmental factors in the lipid content of three macroalgae. Values represent difference in lipid content (as % of volatile solids) between experimental and control conditions. See text for description of treatments.

	Gracilaria		Ulva		Caulerpa	
	Low	High	Low	High	Low	High
Salinity	+0.4	+0.9	+0.6	+0.7	-0.8	+1.1
Nitrogen availability	-2.4	+1.2	-0.6	-3.1	+0.4	-
Temperature	-2.3	+1.7	+0.3	+1.2	+3.6	+6.6
Light intensity	+0.9	+0.4	+3.0	+4.8	-	-

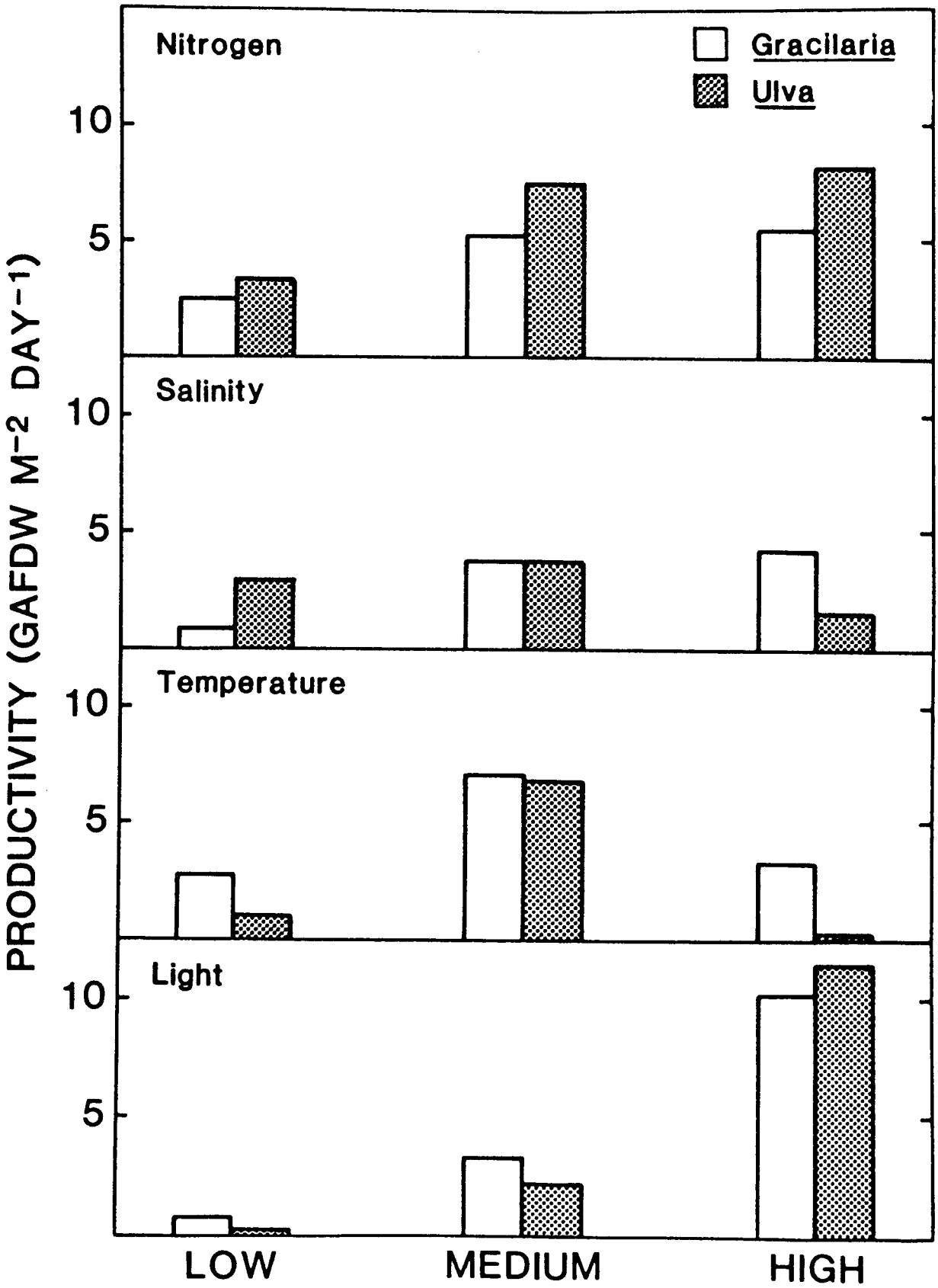

Figure 18. Effect of four environmental parameters on the productivity (on an ash-free dry weight basis) of Ulva and Gracilaria. Low, medium and high treatment regimes are described in text.

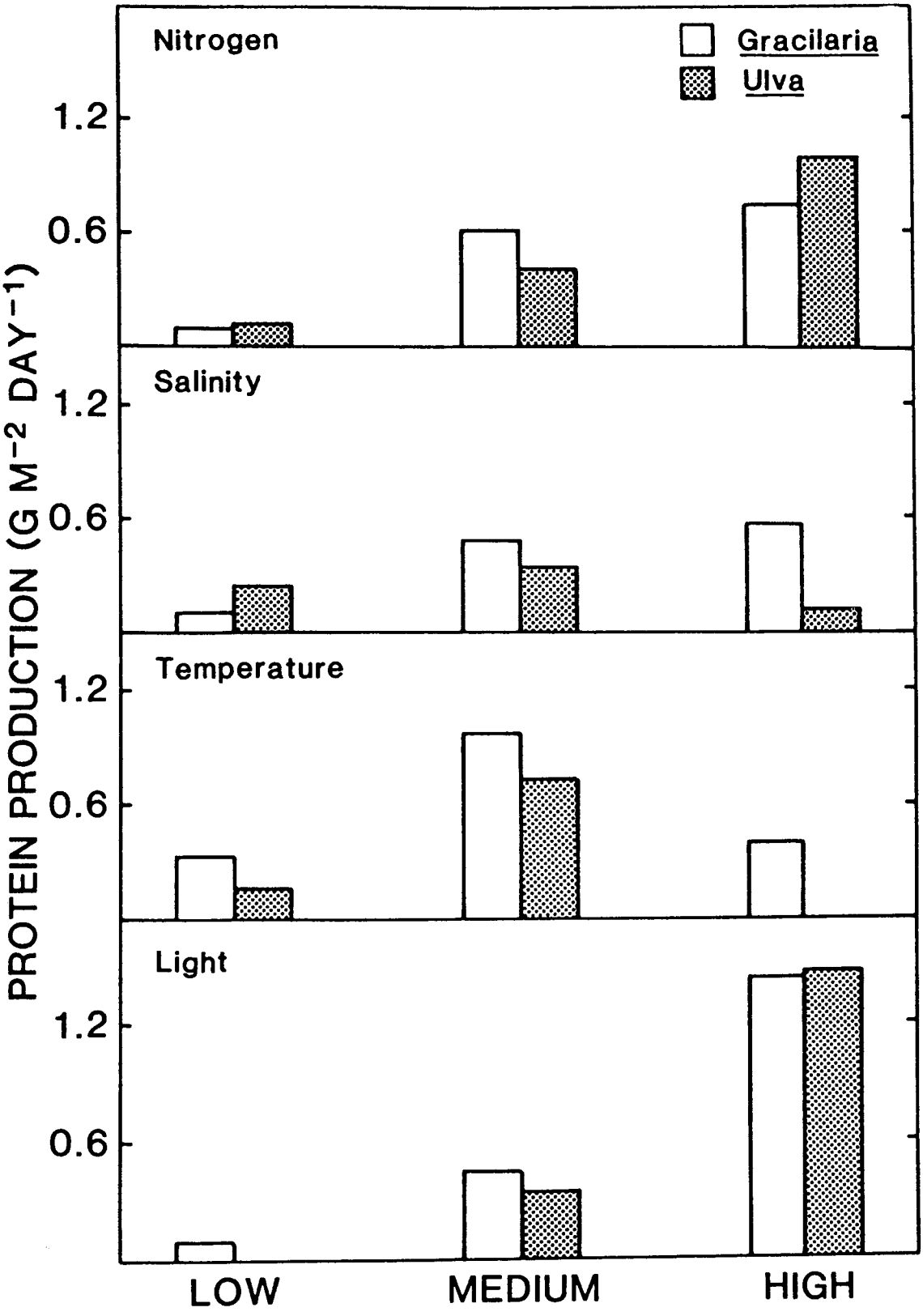

Figure 19. Daily protein production by Ulva and Gracilaria under a range of environmental conditions. Low, medium and high treatment regimes are described in text.

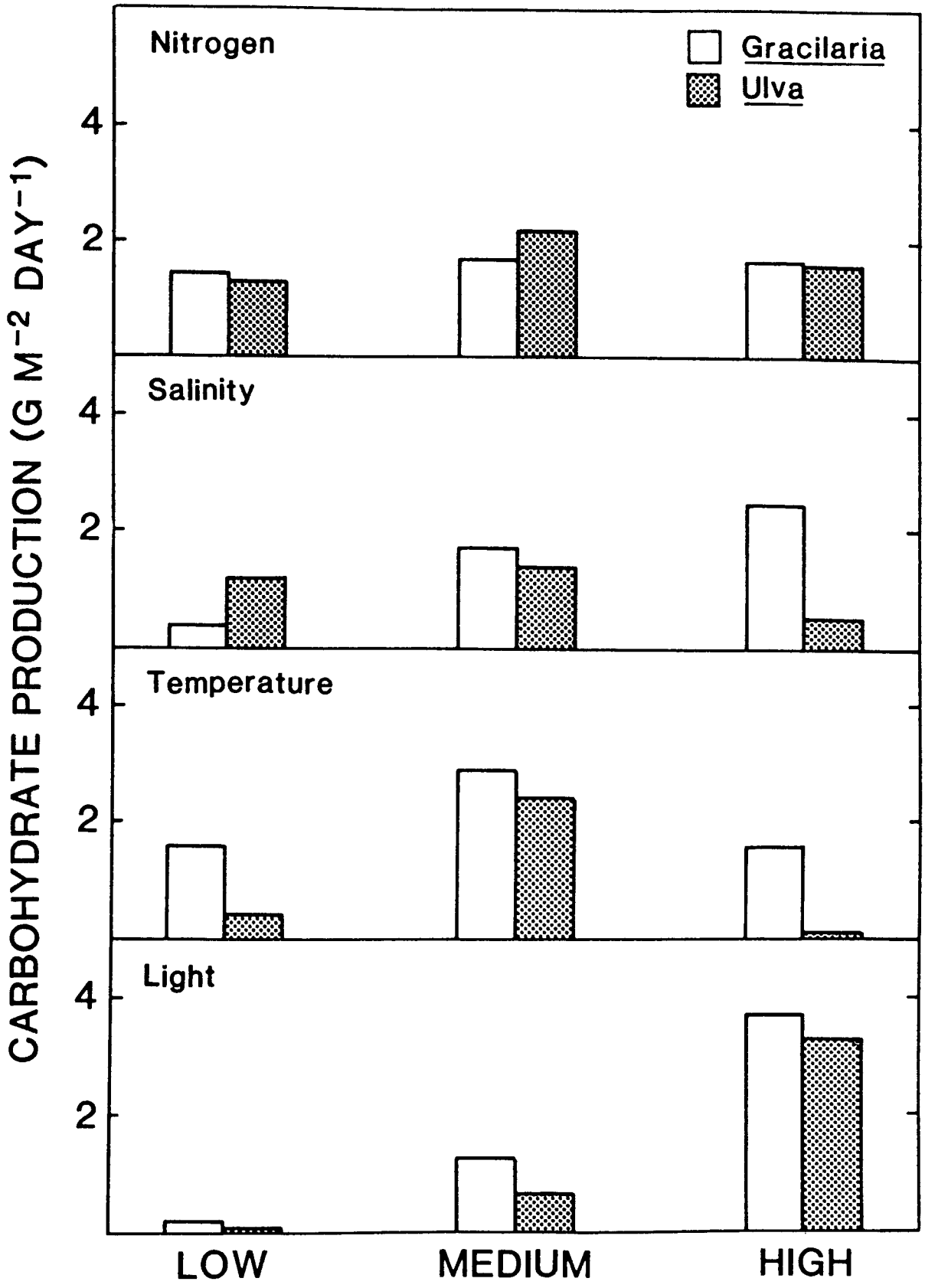

Figure 20. Daily carbohydrate production by Ulva and Gracilaria under a range of environmental conditions. Low, medium and high treatment regimes are described in text.

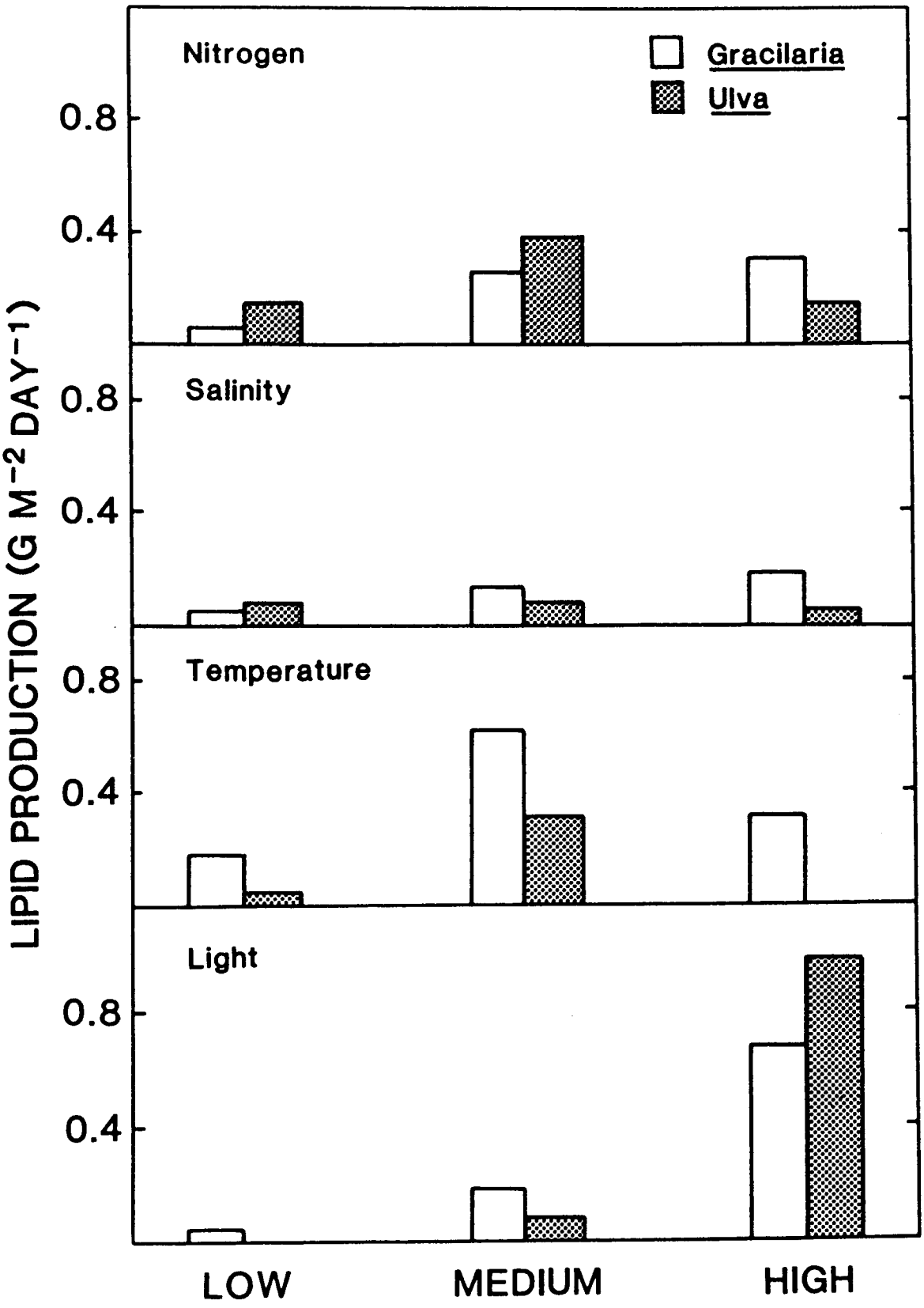

Figure 21. Daily lipid production by Ulva and Gracilaria under a range of environmental conditions. Low, medium and high treatment regimes are described in text.

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DEVELOPMENT OF OUTDOOR RACEWAY FOR PRODUCING OIL-RICH HALOTOLERANT MICROALGAE

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This project is being conducted by three main research groups in Israel: 1) one at the Israel Oceanographic and Limnological Research, headed by Dr. A. Ben-Amotz, 2) one group at the Desert Research Institute of Ben-Gurion University, led by Prof. A. Richmond, and 3) one at the Technion, headed by Prof. G. Shelef.

The general objectives of this project are to select lipid-producing halotolerant microalgae, to cultivate them outdoors on a large scale, to optimize their growth, and to compare various methods of harvesting. Started in June 1983, the project is now nearing the end of its second year. The specific objectives of the present stage of research are:

1) completion of the characterization of the growth of selected Israeli species;
2) identification of culture conditions (e.g. stress) that lead to an accumulation of lipids;
3) optimization of growth conditions for the outdoor culture of selected species;
4) economic evaluation of selected techniques for separation and harvesting.

Based on the literature survey prepared last year and presented to SERI, algae belonging to three genera were selected for further investigation: Isochrysis, Nanochloropsis, and Chlorella. They are considered to be model organisms for the present studies and not necessarily the final organisms of choice. These model organisms have been used for characterization of growth and for determination of productivity and lipid accumulation under controlled conditions, where the light intensity, pH, and salinity were varied. We should note here that, according to several physological and chemical characteristics, both Isochrysis and Nanochloropsis seem to meet many of the requirements of this project. We found that the levels of lipids and hydrocarbons in both algae were satisfactory, as compared with the levels in other algae.

These model organisms were also cultivated outdoors in 1-sq.m and 2.5-sq.m ponds under desert conditions. Some aspects of outdoor cultivation and problems encountered under these conditions are being studied. Data on biomass production were accumulated throughout the year. The algae were also used for harvesting experiments, in which the motile alga Isochrysis and the nonmotile alga Chlorella were compared using various harvesting techniques.

Although the model organisms might not be the ones finally chosen, the advantage of the present study is our gaining experience in operating a system with saline water under desert conditions with all its special constraints (e.g. high temperatures and strong solar radiation). Continuation of the study

with the same organisms will allow us to extend the know-how already accumulated and help us solve many problems that may arise in the future in the cultivation of other organisms as well. The study will thus contribute to the development of the biotechnology for production of lipids from halotolerant algae.

In addition, efforts are being made to isolate suitable algae from the Mediterranean and from small salt ponds in Israel. These algae should meet the project requirements for outdoor production, i.e. ability to thrive under conditions of high solar irradiation, high temperature, and high salinity.

During the next phase we should continue the efforts to select suitable algae producing lipids; to study in-depth their physiological characteristics and determine the chemical composition of the model organisms, using a factorial design system for understanding the specific physiological conditions for the induction of lipids; and to continue our outdoor experiments in 2.5-sq.m ponds in which the problems involved in large-scale open-air production of oil-producing algae in saline water will be defined and solved. In order to be able to recommend a harvesting technique, the results obtained on a small scale should be verified on a large scale, several methods should be compared, and only then should the results be subjected to thorough economic analysis.

For testing our results under real conditions, we propose to put into operation a demonstration plant of 100-sq.m pond in the desert at the end of summer 1985 for the cultivation of an alga that produces high amounts of lipids and hydrocarbons (Isochrysis may be a good candidate). The plant will be operated by the three Israeli groups on the basis of their results with respect to the suitable medium, mode of mixing, dilution rate, and harvesting technique. Several months of such operation should supply important data on the productivity of lipids and their composition in a desert environment.

DEVELOPMENT OF OUTDOOR RACEWAY CAPABLE OF YIELDING OIL-RICH HALOTOLERANT MICROALGAE

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ABSTRACT

Species of unicellular algae from throughout Israel and other origins were screened and cultivated under laboratory controlled conditions to identify culture requirements, optimal growth rate and algal lipid profile. Nannochloropsis spp., Phaeodactylum tricornutum and Chaetoceros gracilis were selected for intensive study by bifactorial design experiments.

The effect of nitrate and iron limitation and the response to light intensity, temperature, pH and salt concentration were tested with respect to algal production and cellular chemistry variation.

The lipid profile of most screened microalgae was studied with emphasis on hydrocarbons eluted with hexane and benzene on silicic acid columns. Significantly low amounts of acyclic hydrocarbons were detected only in Botryococcus braunii and not in the other algae. Botryococcus braunii, Isochrysis sp., Nannochloropsis salina and Cylindrotheca fusiformis accumulated high amounts of cyclic and polyunsaturated components which were identified as alkenones and various isoprenoid derivatives.

INTRODUCTION

Utilization of the photosynthetic machinery for the production of energy, chemicals and food has a particular appeal because it is the abundant energy-storing and life-supporting process on earth. Starting with the basic photosynthetic reaction converting carbon dioxide and water into organic carbon and oxygen with solar irradiation as the energy source, photosynthetic plants and algae utilize intricate biochemical pathways to produce a variety of organic products. For many years, phycologists have had a special interest in the possibilities of mass culturing of microalgae. The early trials of algal biomass production were aimed at single cell protein production, but in recent years many other potential applications for large-scale algal cultures have been advanced (Shelef and Soeder, 1980). Recently, work on lipid accumulation by certain microalgae has raised the idea of using the light harvesting machinery of the algae to produce photosynthetically large amounts of combustible oil. The research has been directed in two ways: (a) identification and isolation of natural microalgae capable of producing large amounts of lipids, and (b) physiological and environmental attempts to enrich the lipid fraction in fast growing marine and halotolerant microalgae in order to achieve fast growing species comprising high amounts of energy-rich lipids. The

research studies in Israel were aimed at identifying Israeli strains of oil yielding microalgae that are adapted to arid saline regions with high temperature and high light intensity, and in parallel at enhancing the accumulation of desirable lipid products by manipulation of environmental conditions of a few selected species.

MATERIALS AND METHODS

Algae

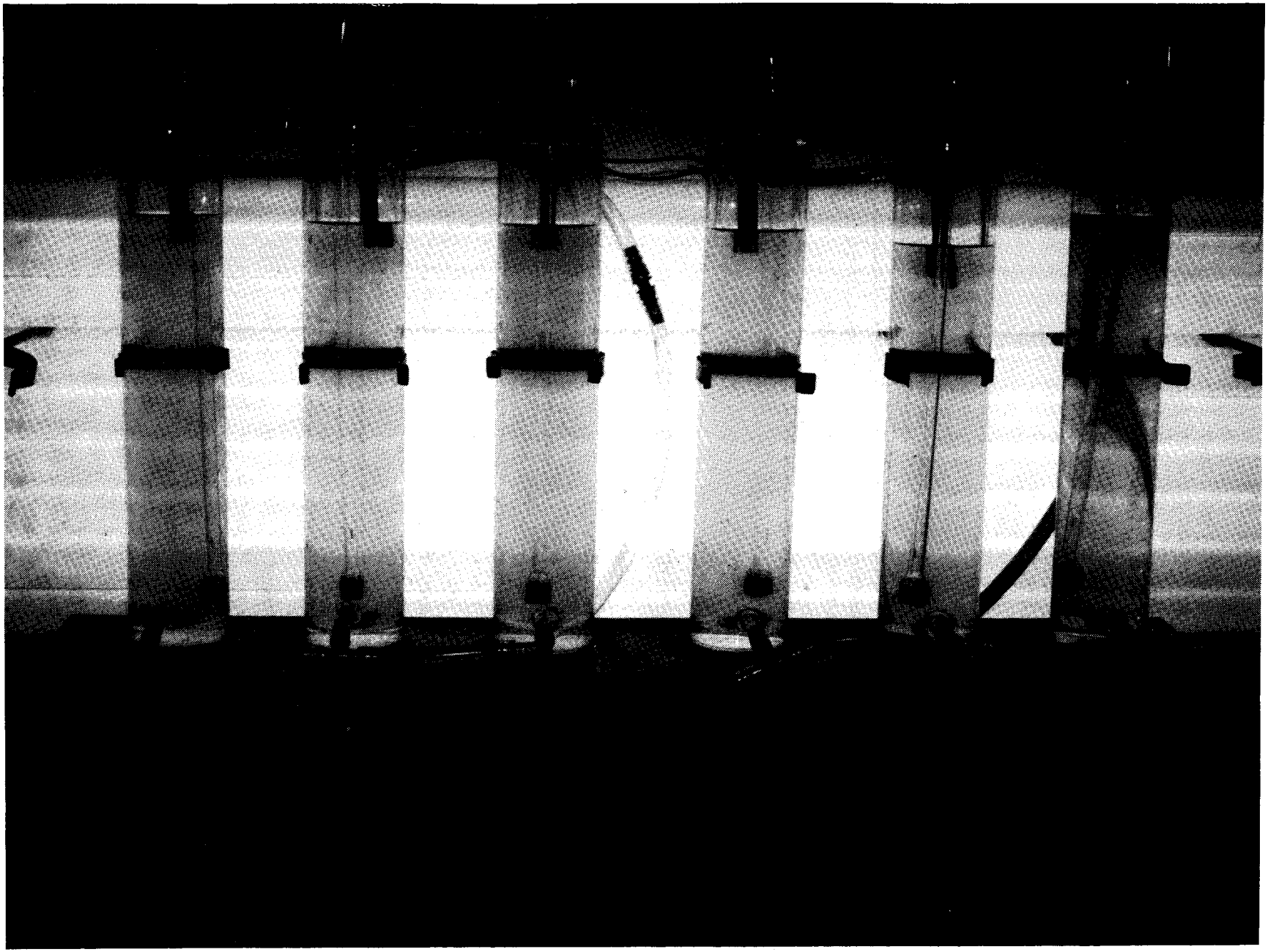
Table 1 presents a list of the algae surveyed in our study in the last two years.

Table 1. List of Algae Analyzed in Israel Oceanographic & Limnological Research, Haifa, Israel

Cyanophyceae:	
Rhodophyceae:	Porphyridium sp.
Cryptophyceae:	
Dinophyceae:	Amphidinium sp.
Haptophyceae:	Isochrysis galbana, Isochrysis sp.
Chrysophyceae:	Monochrysis sp.
Xantophyceae:	
Eustigmatophyceae:	Nannochloropsis oculata, Nannochloropsis salina
Phaeophyceae:	
Prasinophyceae:	Asteromonas gracilis, Platymonas sp.
Bacillariophyceae:	Asterionella sp., Chaetoceros gracilis, Cylindrotheca fusiformis, Navicula sp., Phaeodactylum sp., Skeletonema sp.
Chlorophyceae:	Botryococcus braunii, Chlamydomonas sp., Chlorella stigmatophora, Dunaliella bardawil, Dunaliella salina
Euglenophyceae:	
Charophyceae:	

Growth Conditions

All algae except Botryococcus braunii were grown in an enriched sea water medium containing 5 mM KNO_3 or as indicated, 0.4 mM KH_2PO_4, 1.5 μM $FeCl_3$, 30 μM EDTA, 2 mM $NaHCO_3$ or as indicated, 0.1 mM Na_2SiO_3, 0.1 mg/litre thiamine-HCl, 0.5 mg/litre biotin, 0.5 mg/litre B_{12} and trace metal mix as reported by McLachlan (1973). Algae were grown in two different ways: (a) in batch cultures with 10 mM $NaHCO_3$, or (b) in glass chemostats equipped for pH control by supply of CO_2 on demand. The pH controlled unit included a battery of eight 0.5 litre each glass chemostats with independent temperature control by circulating water from constant temperature baths through a glass jacket on the outside of each culture unit. Radiant energy incident to the outer surface of all cultures was



Chemostats Used in Growth and Lipid Physiology Studies

accomplished by a battery of ten Cool-White and Agro-Lite fluorescent lamps to provide different light intensities between 50 to 500 $\mu E\ m^{-2}\ sec^{-1}$. Algae were grown semi-continuously. Botryococcus braunii was grown in a fresh medium as previously described (Ben-Amotz et al., 1985). Daily sampling for growth measurements was done on aliquots of the culture suspension and included cell counting with a Coulter Counter Model ZB; chlorophyll assay was determined following Jensen (1978); organic weight was determined by freeze-drying samples for 24 hrs and ashing at about 600°C.

Extraction and Fractionation of Lipids

Total lipids were assayed by repeated extraction with methanol--chloroform-water (10:5:4, v/v) (Bligh and Dyer, 1959) modified as previously described (Kates, 1964) to complete visual extraction of chlorophyll and other pigments. The lipids were then phase separated by adjustment of the solvent ratios to 10:10:9 (methanol-chloroform-water, v/v). The chloroform phase was evaporated to dryness under a stream of N_2, dried under vacuum, and then the weight determined gravimetrically.

Total lipid extracts were then fractionated on heat activated silicic acid columns (Unisil, Clarkson Chemical Company, Williamsport, PA) with hexane, benzene, chloroform, acetone and methanol to improve the resolution of the lipid components by thin-layer and paper chromatography (Tornabene et al., 1969; Morrison et al., 1971). The following types of components were eluted: acyclic hydrocarbons (hexane); cyclic hydrocarbons, polyunsaturated acyclic hydrocarbons, fatty acid methyl esters, sterols, isoprenoid derivatives and carotenoids (benzene); tri-, di- and mono-glycerides, free fatty acids, and carotenoids (chloroform); glycolipids, chlorophylls a and b and carotenoids (acetone); phospholipids, and chlorophyll c (methanol). The fractions were reduced in volume by flash evaporation and taken to dryness under a stream of N_2, further dried under vacuum over KOH or P_2O_5, and weighed gravimetrically.

Thin Layer Chromatography

Column-fractionated lipids were studied by thin-layer chromatography on 20 cm x 20 cm glass plates with precoated hard-layered commercial TLC silica gel plates (DESAGA, Inc.). Chromatography was carried out in lined jars by the ascending method using solvent mixtures: a) hexane-benzene (9:1, by vol.); b) petroleum ether-diethyl ether-acetic acid (90:10:1, by vol.); c) diethyl ether-benzene-ethanol-acetic acid (40:50:2:0.2, by vol.) as first solvent and hexane-diethyl ether (96:4, by vol.) as second solvent for separating non-polar lipids; and d) chloroform-acetone--methanol-acetic acid-water (50:20:10:10:5, by vol.) for separation of polar lipids. Spots were visualized by exposure to I_2 vapors, acid charring, ninhydrin for amino acids, molybdate for phosphates, Draggendorff for quaternary amines, α-naptol solution for glycolipids, and sulfuric-acetic acid for sterols and sterol esters (Kates, 1972).

Analytical Method

Glycerol was determined by periodic oxidation followed by treatment with acetylacetone as previously described (Ben-Amotz and Avron, 1978). Protein was assayed as previously described by Lowery et al. (1951) or by Kochert (1978a) after hydrolysis in 1 N NaOH for 1 hr at 100°C. Total carbohydrates were analyzed by the phenol-sulfuric acid method following acid hydrolysis in 2 N HCl for 1 hr at 100°C (Kochert, 1978b). Extended hydrolysis of up to 8 hrs did not produce a detectable increase in the carbohydrate concentration.

RESULTS AND DISCUSSION

Growth and Yield of Algae

Growth and yield of the microalgae grown at the laboratories of Israel Oceanographic & Limnological Research in Israel are summarized in Table 2.

Table 2. Growth and Yield of Species Investigated

Species	Origin	Doubling time, hrs	Yield: cells ml^{-1}	Yield: µg organic weight, ml^{-1}
Amphidinium sp.	Israel	25	5.0×10^6	635
Asterionella sp.	Israel	24	3.0×10^5	215
Asteromonas gracilis	Israel	26	6.0×10^6	750
Botryococcus braunii	USA	45	-	900
Chaetoceros gracilis	USA	24	5.0×10^6	600
Chlorella stigmatophora	Israel	20	1.0×10^7	1010
Cylindrotheca fusiformis	Israel	26	6.0×10^6	650
Dunaliella salina	Israel	18	5.1×10^6	420
Isochrysis sp.	Israel	24	2.1×10^6	534
Monochrysis sp.	Israel	30	3.2×10^6	580
Nannochloropsis oculata	Israel	26	7.0×10^6	590
Nannochloropsis salina	Israel	30	8.0×10^6	580
Navicula sp.	Israel	30	1.2×10^6	720
Phaeodactylum tricornutum	Israel	18	3.0×10^7	1021
Platymonas sp.	Israel	24	9.2×10^6	920
Porphyridium sp.	Israel	24	3.0×10^6	820
Skeletonema sp.	Israel	24	2.6×10^5	200

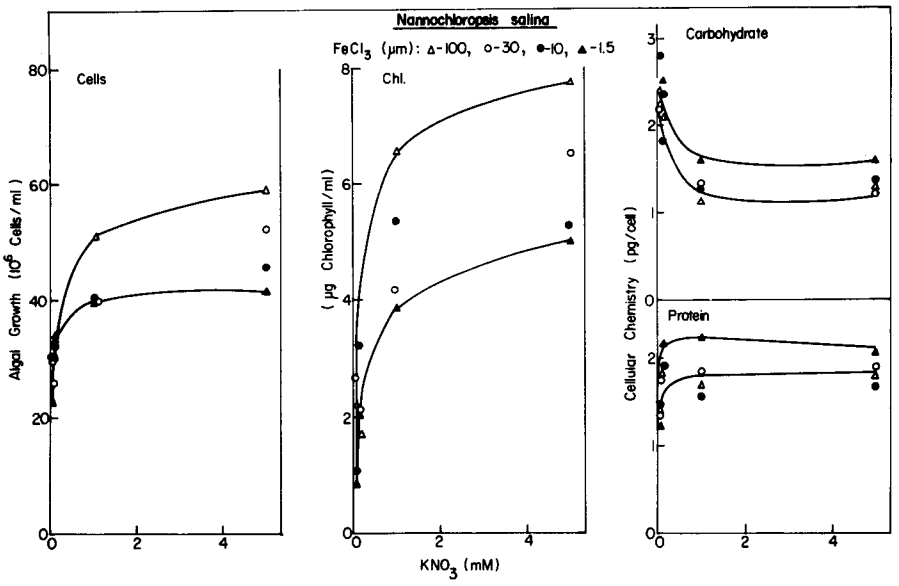
Algae were grown in batch culture at 22°C under a light intensity of 100 µE m^{-2} sec^{-1} with gentle shaking. For further details see MATERIALS AND METHODS.

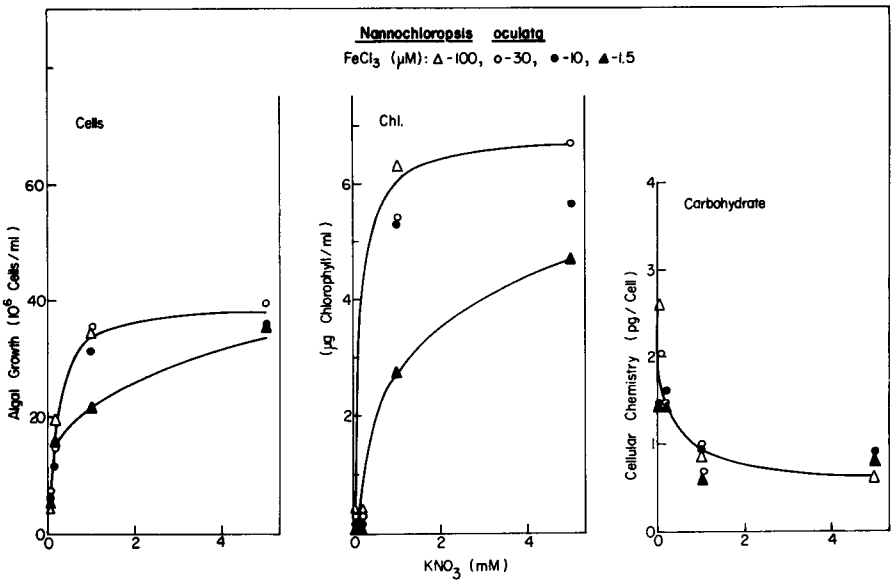
The fast growing species were Chlorella stigmatophora and Phaeodactylum tricornutum. The slow growing species was clearly Botryococcus braunii. Determination of growth yield and productivity cannot be deduced accurately from batch growth; the major limiting variable is obviously the pH rise due to photosynthetic CO_2 uptake. In order to attain growth limitation by light intensity, the culture has to be maintained under constant controlled conditions of temperature, mixing, light intensity, pH, salt concentration, nutrients, etc. For that purpose, we have built a set of glass chemostats in which the microalgae were grown under strict control of pH, temperature and light intensity. The unit has a set of 10 built-in pH controllers and 10 solenoids for flow of gas or liquid. The pH can be controlled within a small error of 0.1 pH units and thus avoid chemical modification in the culture medium due to pH rise. Most difficulties of algal flocculation by calcium phosphate and calcium carbonate precipitation, as well as by microelements' precipitation, at pH above 8.2 in marine cultures were thus eliminated on using the pH controlled chemostats. Examples included algae such as Dunaliella, Asterionella, Skeletonema and others, which exhibited fast initial growth rate but poor yield in uncontrolled batch culture, and high, light limited yield on growth in the pH controlled chemostats. The experiments described below on the culture conditions of the algae were initially conducted in batch cultures to assay growth characteristics and chemical composition and thereafter, selected species were grown in the chemostat, to determine growth yield and lipid productivity.

Culture Requirements

Nannochloropsis spp. The effect of nitrate versus iron on the growth and cellular composition of Nannochloropsis oculata and Nannochloropsis salina is illustrated in Figures 1-3. The optimal concentration of nitrate for growth is about 5 mM. The optimal amount of $FeCl_3$ is above 30 μM, higher than the iron concentration usually employed in microalgae cultures. The protein-to-carbohydrate ratio increased with nitrate concentration but was not affected by the iron concentration. The lipid concentration in Nannochloropsis spp. was not affected by the variations in nitrate and iron concentrations and remained constant at about 20% irrespective of the growth conditions employed. The relatively low level of lipids in Nannochloropsis cultivated under pH controlled nitrogen deficiency clearly shows that the accumulation of lipids in Nannochloropsis salina (SERI, Microalgae Culture Collection Publ. 1984-5) is not related to nitrogen starvation per se but is probably due to microelement deficiency on pH rise in the medium.

Phaeodactylum tricornutum (Israel). A series of experiments was conducted to determine the culture requirements and the optimal growth conditions of P. tricornutum. The effect of light intensity and temperature is illustrated in Table 3. Maximal algal production was obtained at 15°C under 200 $\mu E\ m^{-2}\ sec^{-1}$. The low temperature was favorable for cell





Figures 1-3. Synergistic Effect of Iron and Nitrate on Growth and Cellular Chemistry in Nannochloropsis salina (Upper) and in N. oculata (Lower)

division while the high temperature of 25°C was favorable for chlorophyll production. Pigment content per algal cell unit at 25°C increased to about twice its content at 15°C (Table 4).

Table 3. Phaeodactylum tricornutum: Average Daily Production in Semi-Continuous Cultures at Different Temperatures and Light Intensities

Temp. (°C)	Light intensity ($\mu E\ m^{-2}\ sec^{-1}$)	Cell number ($\times 10^6$/ml)	Chlorophyll a (μg/ml)	Carotenoids (μg/ml)
15	100	5.3±1.6	2.38±0.43	0.79±0.14
	200	10.6±3.1	2.68±0.42	1.06±0.31
	300	9.2±3.8	1.82±0.26	0.82±0.22
25	100	4.2±0.7	3.55±0.34	1.25±0.18
	200	7.9±0.6	3.11±0.05	1.22±0.01
	300	5.4±0.5	2.31±0.30	0.97±0.14

Table 4. Phaeodactylum tricornutum: Pigments/Cell Ratios at Different Temperatures and Light Intensities

Temp. (°C)	Light intensity ($\mu E\ m^{-2}\ sec^{-1}$)	Chlorophyll a (pg $cell^{-1}$)	Carotenoids (pg $cell^{-1}$)	Chlorophyll a/ carotenoids
15	100	0.46±0.07	0.16±0.02	3.0±0.1
	200	0.26±0.04	0.10±0.01	2.6±0.4
	300	0.21±0.05	0.09±0.01	2.3±0.3
25	100	0.81±0.14	0.31±0.07	2.7±0.2
	200	0.40±0.03	0.16±0.01	2.5±0.1
	300	0.44±0.02	0.18±0.02	2.4±0.1

The effect of pH and light intensity on growth of P. tricornutum is illustrated in Figure 4. Optimum pH for cell production was 8.0 at 200 $\mu E\ m^{-2}\ sec^{-1}$, and pH 8.5 and 100 $\mu E\ m^{-2}\ sec^{-1}$ for chlorophyll production. Light intensity, but not pH, had a marked impact on the pigment content with maximal content of about 0.2 pg Chl/cell at 100 $\mu E\ m^{-2}\ sec^{-1}$.

The effect of KNO_3 concentration at 20°C and at pH 8.0 is illustrated in Figure 5. Maximal cell production was observed at 5 mM KNO_3; 8 mM was slightly inhibitory, yet the cells accumulated more protein and chlorophyll. Under an optimal concentration of KNO_3, the cellular composition of P. tricornutum comprised about 40% carbohydrate, 30%

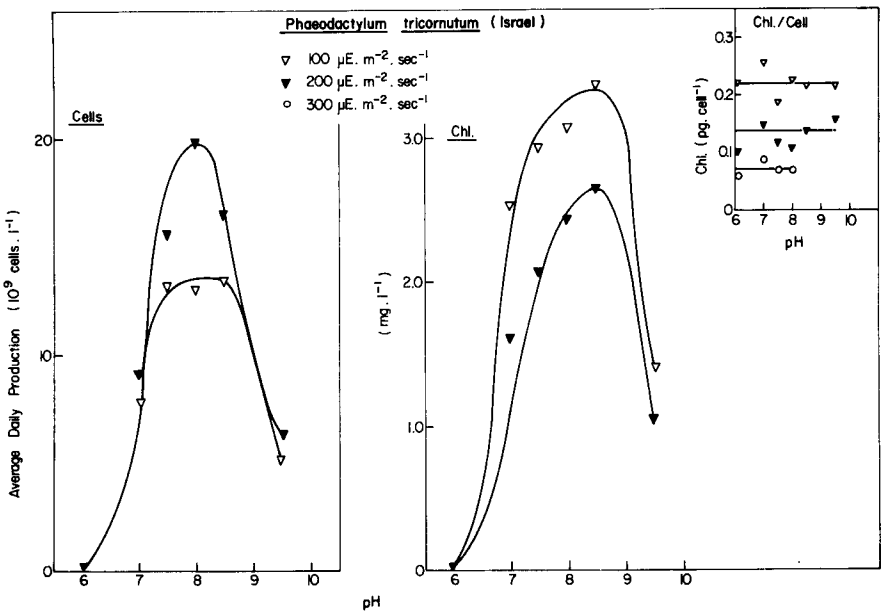


Figure 4. Average Daily Production of Phaeodactylum tricornutum in Relation to Light Intensity and pH

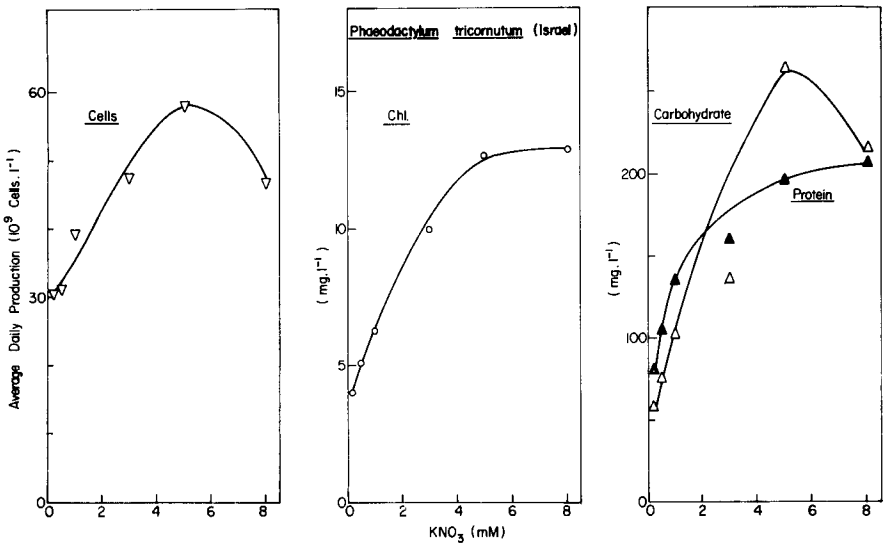
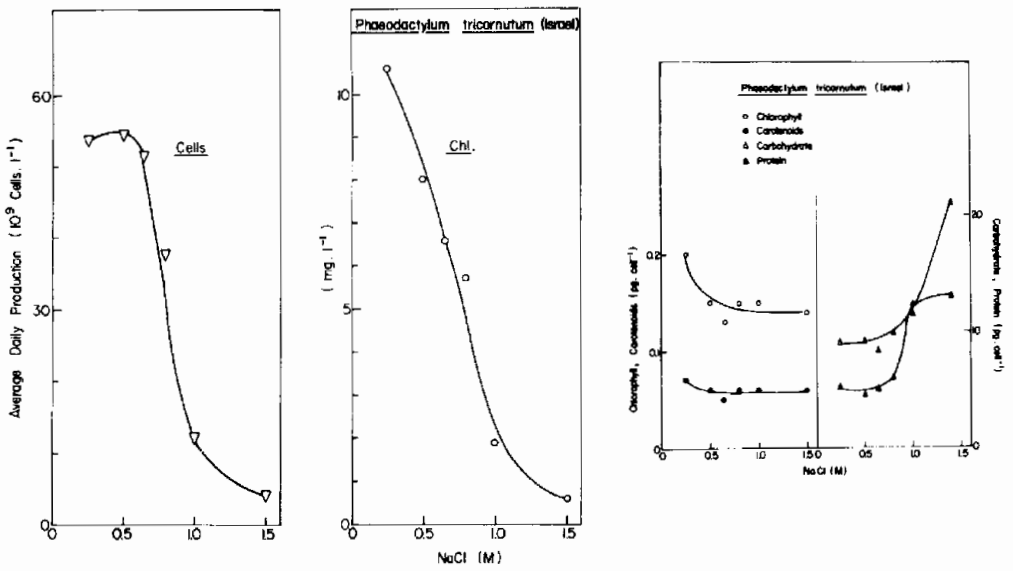


Figure 5. Effect of KNO_3 Concentration on the Average Daily Production of Phaeodactylum tricornutum

protein and 25% lipid. Under nitrogen limitation, the carbohydrate increased to 45%, protein decreased to 20%, and the lipid fraction increased to 35% of the cell organic weight. The effect of NaCl concentration at 20°C, pH 8.0 and 5 mM KNO_3 is illustrated in Figure 6. Maximal algal production of P. tricornutum as a function of salt is around 0.5 M NaCl. Higher concentrations were inhibitory and no growth was observed above 1.5 M NaCl. The effect of salt inhibition on cell growth was associated with the accumulation of protein and carbohydrate (Figure 7), and with a change in the cell volume and morphology from the oval form to the triradiate form. No significant change has been observed in the lipid concentration on growth at elevated salinities. Fawley (1984) recently attributed the cell size changes of P. tricornutum to the vacuole-to-chloroplast ratio, the size being larger when the ratio is smaller. Our observations confirm that assumption and complete it by indicating a parallel increase in the non-aqueous intracellular space (protein and carbohydrate space).



Figures 6 & 7. Effect of NaCl Concentration on the Average Daily Production (Left) and on Cellular Chemistry (Right) of Phaeodactylum tricornutum

Chaetoceros gracilis. Preliminary experiments showed high sensitivity of the diatom Chaetoceros gracilis to vigorous mixing and air bubbling as was expressed by clumping of the algae to the chemostat wall, formation of aggregates and collapse of the culture. We have replaced the CO_2 supply in the chemostats with a supply of diluted HCl on demand for pH control

and with $NaHCO_3$ in the medium for carbon supply. When the medium was mixed slowly, the culture grew well with no culture failure symptoms.

The effect of NaCl concentration at 20°C and 25°C at pH 8.0 and at a low light intensity of 100 $\mu E\ m^{-2}\ sec^{-1}$ is illustrated in Figure 8.

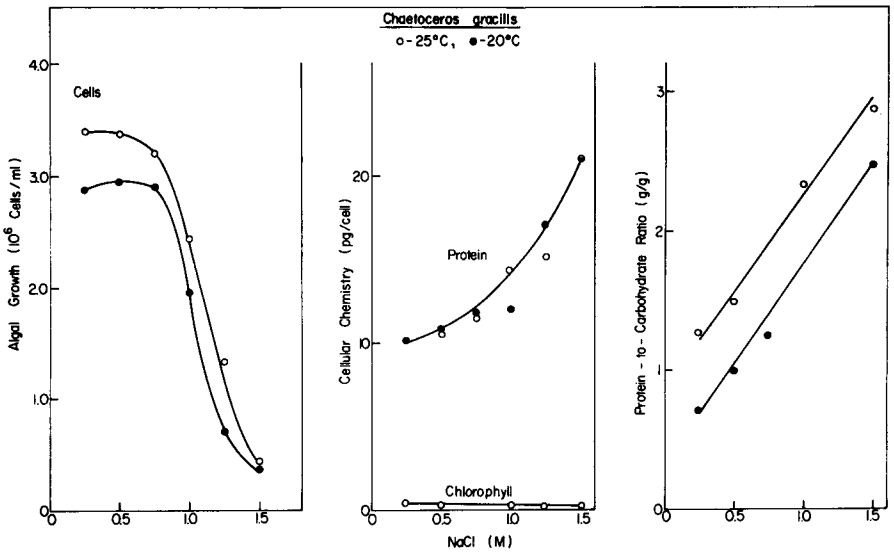


Figure 8. Effect of Temperature and NaCl Concentration on Chaetoceros gracilis

Maximal growth was obtained at 25°C on a wide range of salinities between 0.25 and 0.75 M NaCl. Above 0.75 M, growth was inhibited and at 1.5 M NaCl the algae survived but did not divide. The effect of NaCl on growth at 20°C was similar to that at 20°C with a lower cell productivity. The effect of salt inhibition on cell division was associated with the accumulation of protein. Since the carbohydrate level in C. gracilis was not affected by the salt concentration, the protein-to-carbohydrate ratio increased from about 1 to 3, being higher at 25°C (Figure 8). However, the cellular chemistry of C. gracilis was markedly influenced by the growth phase. Cells from the early logarithmic phase contained a low level of glucan, the amount of which increased in the stationary phase, yielding a low protein-to-carbohydrate ratio in late stationary phase cells. The lipid content in C. gracilis was usually higher (about 30%) in late logarithmic phase cells but was not affected by salt inhibition, remaining constant at about 20% of the algal organic weight.

Lipid Contents

Analysis of the microalgal lipids and their fractions as eluted on Unisil columns is illustrated in Table 5.

Table 5. Lipid Concentration and Fractionation of Microalgae Grown Under Optimal Conditions

Species	Lipid % of organic weight	Lipid fraction, % of total lipid				
		Hex.	Benz.	Chlo.	Acet.	Meth.
Amphidinium sp.	22	-	-	-	-	-
Asterionella sp.	23	-	-	-	-	-
Asteromonas gracilis	17	0.1	0.5	6.4	71.0	22.0
Botryococcus braunii	45	4.6	51.4	4.5	30.0	9.4
Chaetoceros gracilis	20	-	-	-	-	-
Chlamydomonas sp.	23	<0.1	<0.1	<0.1	85.6	14.4
Chlorella stigmatophora	20	<0.1	2.1	9.7	82.7	5.4
Cylindrotheca fusiformis	15	1.4	18.8	6.0	43.6	30.2
Dunaliella salina	15	<0.1	0.4	6.3	76.8	16.5
Isochrysis sp.	20	1.4	27.4	32.1	26.3	12.6
Monochrysis sp.	18	-	-	-	-	-
Nannochloropsis salina	21	<0.1	21.0	19.0	28.0	32.0
Navicula sp.	12	0.4	4.6	20.5	50.2	24.3
Phaeodactylum tricornutum	25	<0.1	5.0	24.5	41.5	29.0
Porphyridium sp.	17	<0.1	<0.1	15.7	48.7	35.6
Skeletonema sp.	21	<0.1	3.5	26.5	39.0	31.0

Hex. = Hexane, Benz.= Benzene, Chlo. = Chloroform, Acet. = Acetone, Meth. = Methanol.

Among the algae tested under optimal growth conditions, only Botryococcus braunii showed an outstanding photosynthetic capacity to accumulate large amounts of lipids. Other algae synthesized lipids to about 20% of the algal organic weight. Fractionation of the lipids on Unisil columns showed significant amounts of hydrocarbons (hexane and benzene fractions) in Botryococcus braunii, Isochrysis sp., Nannochloropsis salina, and Cylindrotheca fusiformis. The benzene eluates contained the major fraction of hydrocarbons in these four species. Analysis of the benzene components by thin layer chromatography identified different neutral lipids in each alga, with a most probable identification as long-chain (C30-C40) alkenones or isoprenoids (Wolf, 1983; Marlowe et al., 1984).

The occurrence of significant amounts of long-chain hydrocarbons in the microalgae under optimal growth conditions raises the possibility of regulation in the biosynthesis of hydrocarbons by physiological and

environmental manipulations. Previous SERI reports discussed ways to alter the algal chemistry by nitrogen deficiency as a means to increase the total amount of lipids at the expense of carbohydrate and/or protein. The present paper clearly shows that when algae are grown under careful pH control, nitrogen limitation does not induce the production and accumulation of lipids; the algae attain a low protein-to-carbohydrate ratio with no change in the lipid content.

The most probable speculative explanation for the accumulation of lipids in algae grown under nitrogen deficient conditions is not the nitrogen limitation but rather the deficiency of microelement(s) which tend to precipitate at high pH. The precipitation of calcium phosphate and calcium carbonate at pH above 8.2 may facilitate the removal of microelements and thus increase a microelement specific deficiency. The purpose of our forthcoming studies is to seek and identify oil producing microalgae as well as the lipid regulatory nutrient(s) with the aid of bifactorial design growth experiments under control of pH, temperature, light intensity, salinity and medium chemistry.

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SEPARATION AND HARVESTING OF MICROALGAE FROM SALINE MEDIA

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ABSTRACT

Separation of microalgae from saline media involves the concentration of algae from suspensions with 150-600 mg/L solids into slurries with 100-200 gr/L solids, or a concentrating factor of 160-600 fold. To achieve this concentration, sand filtration, filtration on fine weaves and dissolved air flotation, were tested with or without flocculation. Sand filtration results were discouraging. Only 40% removal was obtained on 0.55 mm sand particles. Removal efficiency improved by the addition of flocculant, but filtration cycles were short (30 mins). A Discostrainer with a weave of 50 was tested with fresh water Chlorella conditioned with alum. A 40-60% removal efficiency was obtained and a slurry with 3.6% solids was collected. Using a finer weave, of 21 nm, 95% of the algae were removed but the algae concentrate contained only 2-3% solids.

Flocculation followed by Dissolved Air Flotation (DAF) was tried on a continuous basis in a pilot plant. While a high algae removal efficiency (90%) and 5.6% solids in the concentrate were achieved when Chlorella was separated from freshwater, no consistant results were accomplished when separating marine microalgae from sea water. These variable results were related to the difficulties encountered in the flocculation of microalgae in sea water. Since flocculation is an essential step common to both, the Discostrainer and DAF, efforts were dedicated to a better understanding of the flocculation process in brackish and saline waters and to improving it. Lower doses of flocculant were required to achieve 90% removal from brackish water as compared to the dose required in sea water. No significant reduction of inorganic flocculant dose was obtained by the combined use with polyectrolytes, but a significant reduction was achieved when Chitosan was used together with the inorganic flocculant. A major breakthrough in the flocculation of microalgae in saline and brackish waters was achieved in laboratory experiments by the recycle of preformed flocs, resulting in a 60-80% reduction of the required dose of inorganic flocculant. The breakeven costs of flocculant were found for different flocculants, at USA and Israel prices, compared to the allowable cost of flocculant calculated for different combinations of lipids in algae, algae concentration in the culture, price of crude oil, prices for algae cultures produced on media with different salinities. Microalgae flocculation in brackish waters with salinities such as the Southwest USA lakes and underground water of the Arava, Israel, is economically feasible from cultures with 300 mg/l V.S.S. 30% oil, and at a crude oil price of 29 US $/bbl using the Enforced flocculation. When conventional flocculation is considered, the Algae by-product has to be taken into account to remove economically microalgae from medias with a salinity below 25 g/l.

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1. INTRODUCTION

The production of microalgae biomass has been studied for about four decades in terms of growth kinetics, culture reliability and biomass production rate. There is an increasing scientific and commercial interest in biomass production of various microalgae species as a source of human food, animal feed, biochemicals and energy (1). Most previous work dealt with freshwater algae but in recent years it became evident that the most preferred regions for large scale algae biomass schemes would be arid regions where land and sunlight are amply available, marine enclosures or even sea surface. In all these regions freshwater is scarce while only brackish or sea waters are available. Therefore a special emphasis was given in recent years in mass cultivation of algae species grown in various saline waters ranging from brackish water - Spirulina sp. (2,3), through marine microalgae - Phaeodactylum sp. (4) ending with halophytic species Dunaliella sp. (5) grown in hypersaline conditions. Furthermore, SERI Aquatic Species program is currently aimed at developing oil yeilding microalgae which will grow in the saline waters of the deserts in the American Southwest.

It is envisioned that further improvement in production rates and species control to produce desirable products, can be expected. Still, in order to bring about the recognition of microalgae production as a main stream biotechnological industry, the economical feasibility of separation technology, harvesting and processing of the algal biomass or its products should be established.

Most microalgae suspensions in outdoor facilities have a solids concentration that ranges between 0.015 (150 mg/l) and 0.06 percent (600 mg/l). For the extraction of lipids, a higher solids concentration is required. Even for the extraction from wet materials, a solids content of 10% - 20% is desirable.

Table 1 shows the effect of each stage of a train of processes on the algae biomass concentration and on the concentration factor.

Table 1. Solid Concentration & Total Bulk Weight in A Conventional Train of Algae Harvesting Process

Stage	Algal Biomass Conc. % Solids	Metric Tons of Bulk Weight per Metric Ton of Dry Biomass	Concentrating Factor
Pond Suspension	0.015-0.03-0.05	6700 - 3300 - 2000	0
Autoflocculation	0.5 - 1	200 - 100	20 - 66
Flocculation-Flotation	4 - 6	25 - 16.5	80 - 400
Post Flotation	8 - 9	12.5. - 11	160 - 600
Thermal Treatment	13.5	7.4	270 - 900
Centrifugation & Polyelectrolytes	25	4	500 - 1660
Press Filtration & Polyelectrolytes	33	3	660 - 2200
Drying (Commercial)	88	1.14	1760 -5860

The first stage of concentration is essential since it removes free water at concentration factors ranging from 80 to 400 fold with lower costs than subsequent stages.

Previously we reported (6) on our attempt to separate marine microalgae from the culture medium by autoflocculation as well as by chemical flocculation. It was shown that the high salinity of marine microalgae cultures required high dosages of inorganic flocculants (alum or ferric chloride) for optimal algae separation and harvesting, as compared to fresh water algae cultures. These high dosages were partly explained by the algae cell properties such as motility, and partly by the high salt concentrations which reduced the flocculant chemical activity and masked its active sites. Polymeric flocculation of microalgae in a marine system was completely inhibited, evidently due to reduction in polymer ionization degree which was caused by the medium high ionic strength. Contrary to sea water medium, such inhibition diminished in brackish waters as total dissolved solids were being reduced below 5 grams per liter (ionic strength of approximately 0.1M and below). It became clear that the lower the ionic strength, the flocculant dosage demand for optimal algae removal was lower and the process became more economically feasible.

If various water sources in Israel or in Southwest United States are evaluated for microalgae production, water salinity should be taken in account not only from the algae physiological stand point but also in terms of flocculation and separation efficiency. Table 2 summarizes the salinity and ionic strength of various water sources in Israel and in southwest deserts of the U.S. It is shown that huge amounts of waters which cannot be used for conventional agriculture are available for growing microalgae, and still those waters are of such salinity which do not greatly effect microalgae separation efficiency (ionic strength 0.15M) as compared to marine environment (ionic strength 0.7M).

Table 2. Salinity and Ionic Strength of Various Water Sources in Israel and in the Southwest Deserts, U.S.A.

Location	Chloride mg/L	Total Dissolved Solids TDS gram/L	Conductivity mmho/cm	Ionic Strength
Jezrael Valley, wells & springs (Israel)	290	0.90	1.37	0.020
Arava ground water (Israel)	600	2.50	4.5	0.062
Pyramid Lake (Nevada)	1,970	5.55	8.63	0.093
Walter Lake (Nevada)	2,200	7.76	12.1	0.178
Big Soda Lake (Nevada)	6,700	18.8	29.4	0.445
Big Alkali Lake (California)	180-570	1.02-2.9	1.59-4.57	0.03-0.10
Saline Valley (California)	169.10	270.0	190.0	5.64
Sea Water	194.10	34.0	39.0	0.71

(1) Thomas T.H. SERI Review Meeting 1984
(2) Stumm and Morgan. Aquatic Chemistry

Two different harvesting techniques were tried on a continuous pilot plant operation, following the flow-diagram seen in Fig 1.

One process which is based on the discostrainer, increased the content of solids of the pond from 0.02-0.06 % to 3.5% solids, provided that algae were flocculated previously. The second process which is based on flocculation - dissolved air flotation (DAF) increased the solids content from 0.02-0.06% to 4-6%. After a post-decantation the solids content would rise to 9-10% and a wet extraction of lipids is to be tried. Alternatively, the slurries of the discostrainer or of the DAF would be concentrated to 20-25% solids in a centrifuge. A semi-wet extraction of lipids is to be tried on the centrifuge cake.

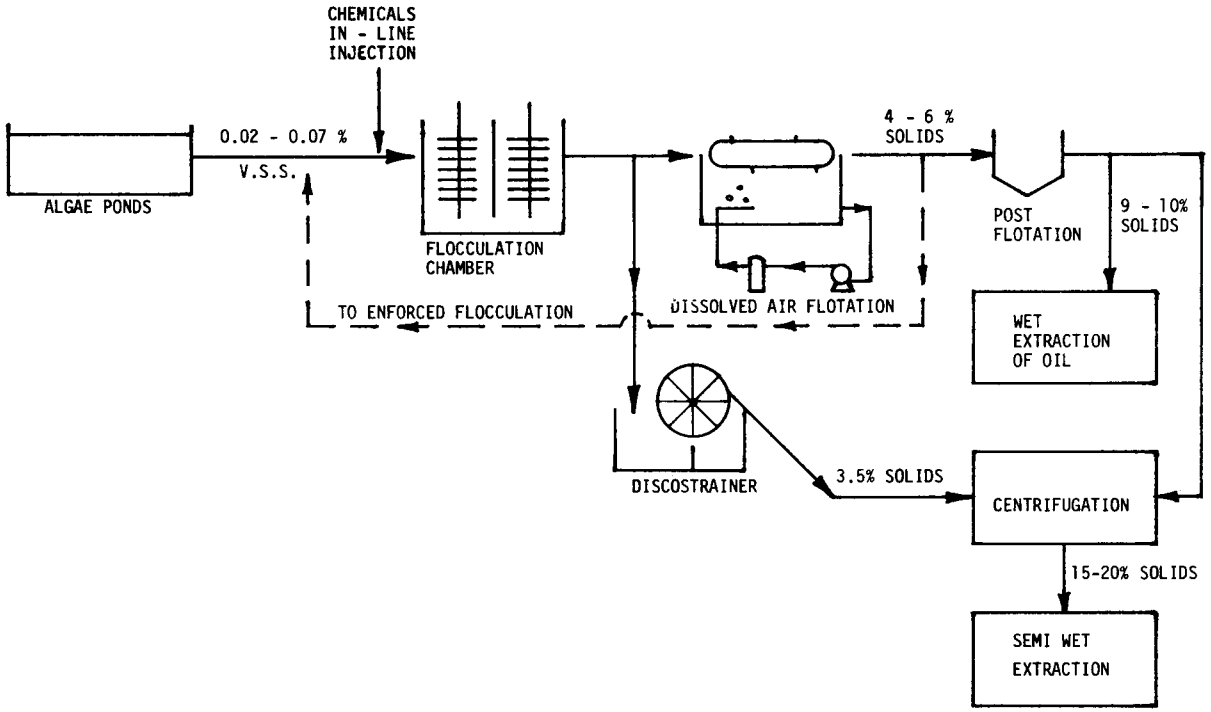

FIG. 1: FLOW-DIAGRAM OF PROCESS UNITS FOR MICROALGAE HARVESTING AND PROCESSING

Since flocculation is important for both methods of harvesting, on the second year of the SERI Project, our efforts were dedicated to understanding better the flocculation of microalgae in brackish and marine waters in order to reduce flocculant demands and to improve the separation process.

During this year no work was performed on the extraction of lipids and it is hoped that this task will be studied during next year.

FLOCCULATION OF MARINE MICROALGAE

The combination of a very small size and a marked surface electric charge of marine microalgae tend to keep the cells in a stable suspension and questions the use of direct physical separation techniques such as filtration, sedimentation or flotation. Preliminary experiments show that destabilization and flocculation of the marine motile chrysophyte Isochrysis galbana and the non motile green algae Chlorella stigmatophora is an important and essential procedure in the separation and harvesting process. Previously (6), the flocculation process in a marine environment was found to be difficult, evidently due to high salt content of the culture medium. Therefore, part of the research was dedicated to elucidate the effects of marine medium and algae cells motility on the flocculation process and to find out improved combined procedures in order to reduce the high inorganic flocculants demand.

The results of our investigation on microalgae flocculation during the last year are summarized in three articles which are currently processed to be submitted for publication.

1. Flocculation of Microalgae in Brackish and Sea Waters.
2. Salt concentration effect on polymeric flocculation of microalgae.
3. Effect of oxidants on microalgae flocculation.

Since the forecast vision of oil yielding microalgae production should include the use of various marginal waters including brackish, saline and sea waters, harvesting of microalgae from various salinity brackish waters was examined and evaluated. (Table 3).

Table 3. Flocculant Dose Required to Achieve 90% Removal of Isochrysis from Media with Different Sea Salt Concentrations.

Salt Concentration (g/l)	Alum Dose (mg/l)	$FeCl_3$ (mg/l)
5.0	30	15
10.0	75	30
20.0	100	60
30.0	150	--
36.0	225	125

As it is explained later, a novel technique has been developed lately at the Technion which substantially reduces (up to 5 fold and more) the above flocculant dosages. From Table 3 it is clear that growing algae on brackish waters of up to 10gr/liter salt (over 10 fold salinity as in regular fresh water) will substantially reduce the cost of flocculants, hence the cost of the overall separation as compared to seawater.

Once the practical ineffectiveness of the traditional fresh water inorganic flocculant or polyelectrolytes become evident, as far as marine medium is concerned, two venues which both still involve inorganic flocculants were suggested, namely: (a) combination of polyelectrolytes as "flocculant aids" and inorganic flocculants, and (b) pretreatment with relatively low ozone dosages followed by inorganic flocculants. Contrary to sea water medium, brackish water exhibits a lower demand for inorganic flocculants and polymeric flocculation inhibition is reduced. diminishing (brackish waters). Therefore simple flocculation processes with either low inorganic flocculant dosages or polyelectrolytes should be considered for separation of such microalgal cultures.

The effect of the combined use of FeCl3 and polyelectrolite is shown in Fig 2. When FeCl3 was added first, the polyelectrolyte did not have any effect, while it had some effect on removal when it was added before the FeCl3. However, less than 70% removal was achieved.

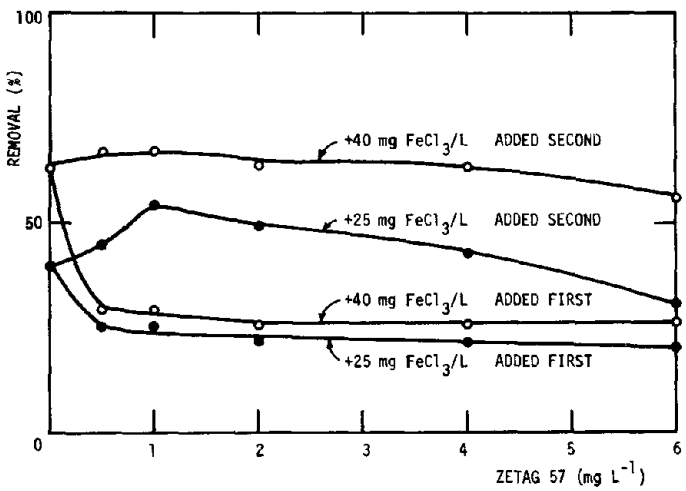

FIG. 2: EFFECT OF ZETAG 57 ON THE REMOVAL EFFICENCY OF $FeCl_3$ FOR FLOCCULATION OF A MARINE MICROALGAE

The effect of the combined use of Chitosan and FeCl3 (Fig 3) shows a marked positive effect on removal. More than 85% removal was attained with half the dose of FeCl3 replaced by 2mg/l Chitosan added first.

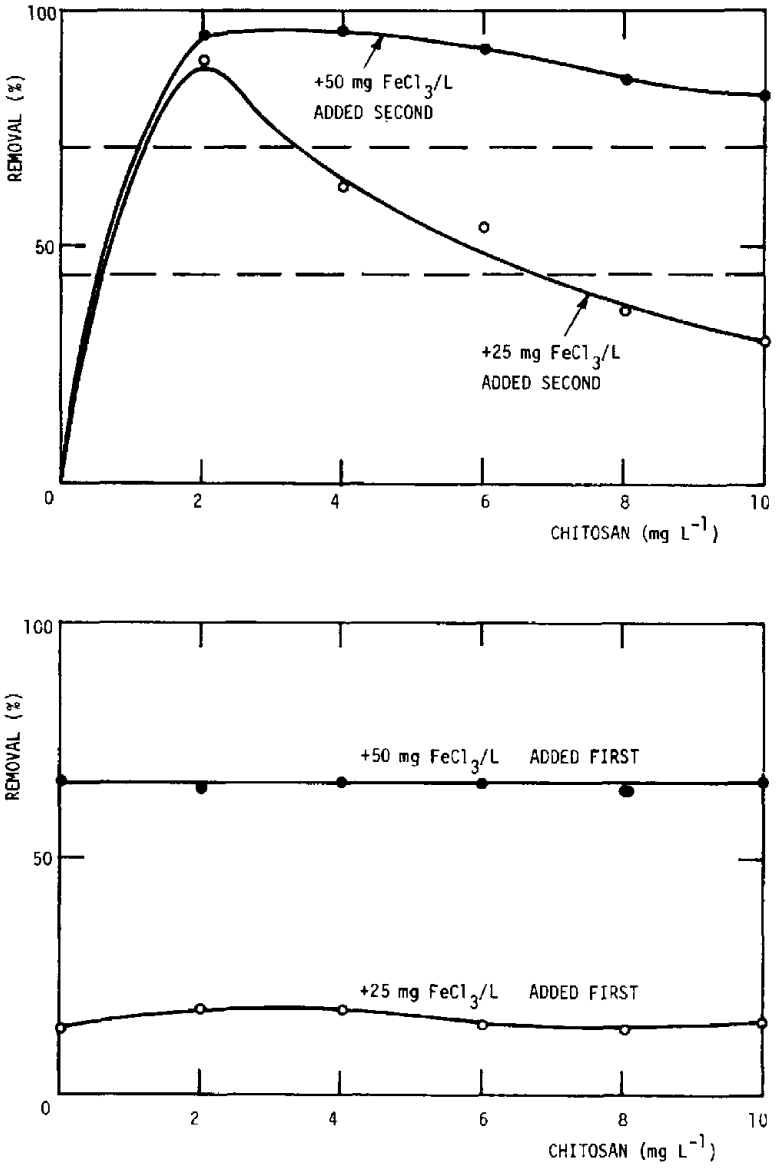

FIG. 3: EFFECT OF CHITOSAN ON ISOCHRYSIS FLOCCULATION WITH $FeCl_3$.

The effects of media salinity on microalgae flocculation by polyelectrolytes was studied with the non motile marine green algae Chlorella sygmatophora and was qualitatively related to both polymer configuration changes and molecule dehydration due to different ionic strength conditions. Furthermore, the results indicate that polymers which have higher rigid backbone are less affected by the salt concentration and are recommended as flocculants of microalgae in brackish water (8).

Previously, improvement of marine microalgae flocculation after treatment with relatively low ozone dosages was reported (6). The effect of three different oxidative agents (chlorine, ozone and chlorine dioxide) on the fate of algal cells as well as on flocculation effectiveness was elucidated with fresh water cultures of Scenedesmus sp. Low dosages of ozone or chlorine dioxide (about 3.0 mg/l) were found to be most effective as a flocculant aid of inorganic flocculation. Nevertheless pretreatment with ozone seems to be more promising since ozone does not cause severe damage to the algal cells as chlorine dioxide does.

Low to medium chlorine dosages do not improve and even deteriorate the algal flocculation efficiency, cause severe damage to algal cells and reduce their viability (9). Therefore this oxidant should be excluded when oxidative pretreatment is considered in a flocculation process.

3. SEPARATION AND HARVESTING

Two different solid liquid separation techniques were used to harvest marine microalgae in conjuction with a flocculation process:
a) Filtration and b) Flotation. Parts of the experimental work which required a large supply of algal cultures was performed with high rate oxidation pond (HROP) effluents.

3.1 Filtration

3.1.1 Sand Filtration

Sand filters were constructed and preliminary experiments were conducted in order to check algae filtrability with different types of sands. It is shown in Figure 4 that even beds of small sand particles (0.55mm) removed only about 40% of the algae from the cultures.

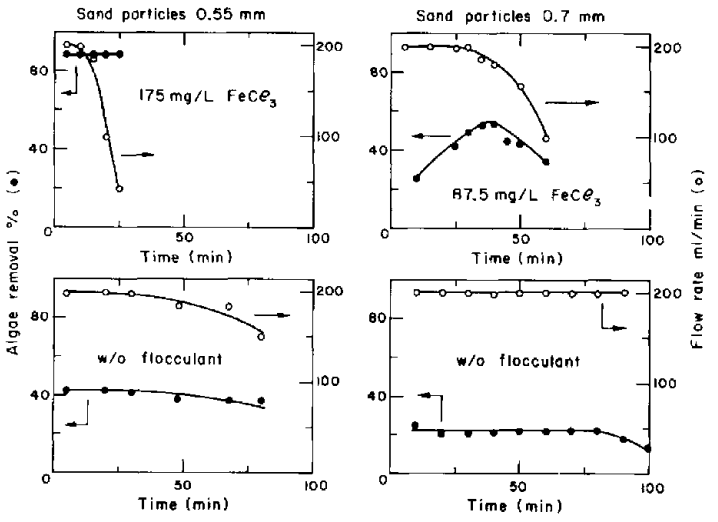

FIG.4 : MARINE MICROALGAE REMOVAL BY A SAND BED FILTRATION PROCESS.

Addition of flocculant (FeCl3 175 mg/L) improved significantly the removal efficiency, however, algae flocs were concentrated at the sand bed upper layer and clogged the filter rapidly (less than 15min). The addition of flocculant improved also the algae removal efficiency on beds of larger sand particles (0.7mm). But the filtration cycle was not long enough (39min). These results were discouraging and indicated that filtration took place preferentially at the sand filter surface. Therefore surface filtration on fine weaves was examined.

3.1.2 Microstraining

Algae filterability on different fine polyester weaves was tested. When no flocculant was added, algae removal efficiencies were zero (shown in Fig 5 as effluent to influent optical density ratio). Addition of flocculants to the algae cultures prior to the filtration improved significantly the filtration efficiency (Fig 5).

This experimental stage was followed by the construction of a microstrainer apparatus. The microstrainer unit consisted of a fine weave on a rotary drum (area of 0.32m) which was fed with algal culture after flocculatiion with ferric chloride in a flocculation vessel. Two kinds of micro-weaves were used: 21µm polyester weave and 50 µm stainless steel weave. The drum rotation rate was varied between 1.2 and 5 rpm. The results of various havesting runs with microstrainer are given in Table 4.

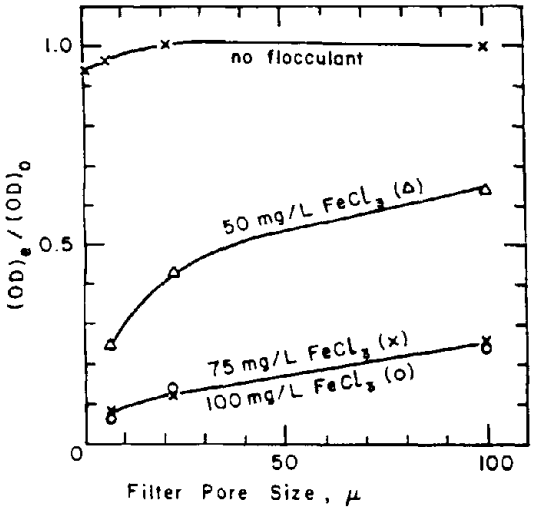

FIG. 5: THE EFFECT OF ALGAE FLOCCULATION ON REMOVAL EFFICIENCY IN FILTRATION LEAF TEST EXPERIMENTS.

Table 4 Marine microalgae removal by microstrainer

Weave type	Rotation Rate rpm	Algae Removal Efficiency %
21μm Polyester	1.2	85
	2.5	80
	5.0	92
50μm Stainless	1.2	50
	2.5	48
	5.0	67

The results indicate the effect of weave pore size and drum rotation rate on algal removal efficiency. No reduction in algae concentration was obtained without flocculation process prior to the filtration.

The microstrainer was not equipped with back-wash system and algal slurry was not collected, therefore no information on concentration factor was available.

3.1.1 Discostrainer

A pilot Discostrainer (Hycor Corporation II) was supplied by a local engineering company (Dagilio Ltd. Tel Aviv). This unit was provided with two stainless steel weaves of 50 μ and is shown schematically in Figure 6.

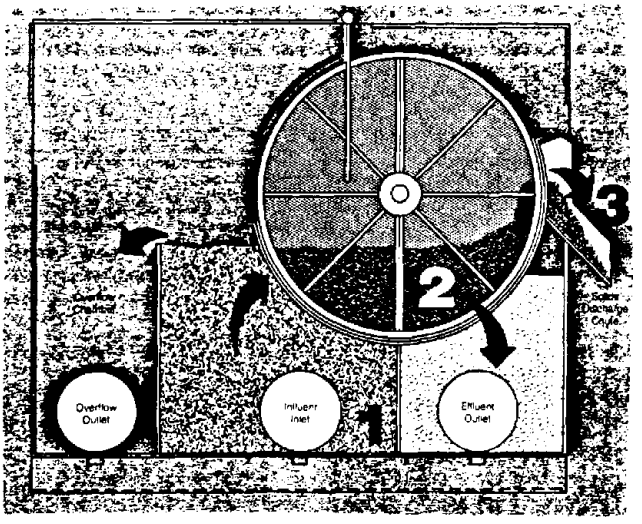

FIG. 6: SCHEMATIC PRESENTATION OF DISCOSTRAINER (HYCOR, IL).

In order to prevent solid sedimentation in the influent chamber (1 in Fig.6) and to shorten the weave precoat formation time, the connection between the influent chamber and the filtration chamber was closed and the inflow was fed directly into the filtration chamber. The Discostrainer was fed with HROP effluent containing 0.36g algae/ liter and dominated by Chlorella sp. The inflow was conditioned on line with Alum to a final concentration of 200 mg/L. The filtrate contained 130mg solid/L meaning an algae harvestability of 40 to 60%, depending on back-wash rate.

The solids concentration of the algal retentate as a function of filtration time and filtration rate is given in Figure 7.

As the feed volume increased, the solid concentration of the retantate increased up to a final concentration of 3.6% total solids after 3.7m^3 of HROP were fed to the Discostrainer (Fig.7).

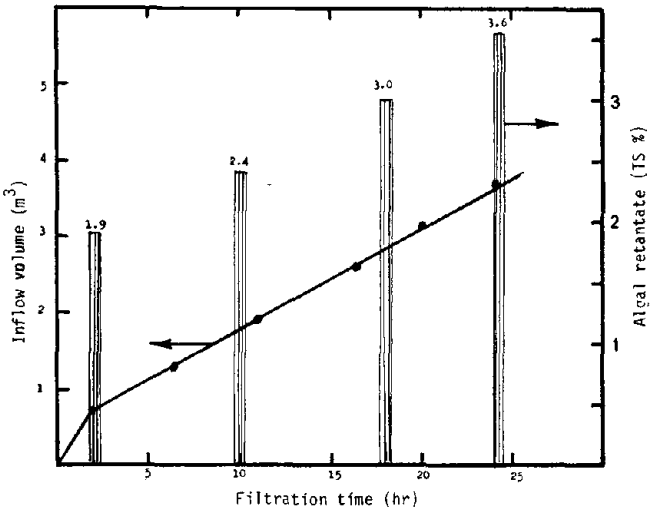

FIG. 7: DISCOSTRAINER PERFORMANCE WITH HROP EFFLUENTS.

When finer mesh weave (21 μm polyester weave) was used in the discostrainer, 95% algae removal was obtained. However, the collected algal concentrate contained only 2 to 3% solids at a feed rate of 160 to 100 liter/hr respectively.

3.2. Dissolved Air Flotation - DAF

The HR/SR-1 (Kromline Sonderson Eng. Corp. Ontario, Canada) pilot dissolved air flotation is a semi-automatic flotation unit in which the skimming must be done manually.

The DAF unit was fed with HROP effluents at three different feeding rates (150, 200 and 250 L/hr).

The algal cultures fed the DAF, were previously conditioned in line with ferric chloride and acid. The flow rate velocity (4 to 8 cm/s) in the feed line and the 1.5m pipe between the chemical introduction point and the flotator, provided 18 to 38 seconds mixing time which was sufficient for rapid mixing and for flocculation to occur in the feed line.

Table 5 summarizes the results of the DAF unit operation for microalgae harvesting from HROP effluents containing 0.280g solids/L. Ferric chloride was added to final concentration of 85 mg/L and acid ($1.0\ N\ H_2SO_4$) was added to final pH 5.0 using a pH controller.

Table 5. Harvesting of Microalgae From HROP Effluent By DAF Unit.

Feeding Rate L/min	Retention Time min.	Algae Harvestability* %	Solid Concentration of Algal Float
2.5	20	90.2	3.1
3.3	15	91.4	5.6
5.0	10	90	6.9

*Algae harvestability is defined as $\frac{TSS_{in}-TSS_{out}}{TSS_{in}} \times 100$

Algae harvestability was about the same for the three different feeding rates. The algal float of 10 min retention time operation contained the highest solid concentration. However, these results are misleading, since the algal float skimming was done manually. Under a continuous float skimming, an algal float of 4 to 5 percent solids is expected.

No significant difference in algal harvestability was found when the air/solid ratio was changed between 0.015 to 0.1. It means that once strong algal flocs were formed, they were not affected even by high air/solid ratio. On the other hand, it may indicate that the HROP effluents were supersaturated with photosynthetic oxygen. Therefore low air/solid ratio was sufficient for good solid liquid separation.

At this stage of the experimental work the HR/SR-1 pilot DAF unit was fed with marine microalgal culture at a rate of 2.5 L/min and a recycling rate which gave 0.05 air/solids ratio.

The results of the continuous flocculation flotation process are compared with these of a batch flocculation sedimentaion process and are given in Table 6.

It should be mentioned that the batch process included a flocculation vessel, while in the continuous process the flocculation took place in the feed pipe without any slow mixing chamber. In addition the formed algal flocs were tiny and consequently less sensitive to the flotation process than large flocs.

These two factors probably caused the poor results of the continuous flocculation process (Table 6). Therefore, a simple flocculation chamber was designed and constructed and is currently used in conjuction with the flotation unit.

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Table 6. Comparison of Marine Microalgae Harvesting by a Batch Flocculation Sedimentation process, to a Continuous Flocculation Flotation Process.

	Batch Flocculation Sedimentation	Continuous Flocculation Flotation
Flocculant dose FeCl mg/L	140	135
pH	4.2	4.5
Algae Harvestability %	97	48
Volatile Solids in Algal Concentrate %	N.D	1.5
Concentration Factor	N.D	60

The flocculation chamber had a rapid mixing zone and a slow mixing zone. When the DAF separation was operated with this set-up, no removal was obtained. The possibility that flocs preformed in the feed line would break in the rapid mixing zone, led to its elimination.

The effect of the slow mixing flocculation chamber following the in-line injection of flocculant was compared to the in-line injection and direct flow to the flotation unit, under equal operational conditions and a dose of 120 mg/l FeCl .

Table 7. Effect of Slow Mixing in Flocculation of Marine Microalgae

	Hr.	V.S.S.	% Harvestability
In-line injection + DAF	0 (POND)	155	
	0.5	73	52.9
	1.0	--	
	1.5	118	23.9
In-line injection + Slow Mixing + DAF	0 (POND)	229	
	0.5	85	62.9
	1.0	65	71.6
	1.5	56	75.5

Unlike what occurs in the separation of freshwater microalgae, where in-line injection is sufficient to obtain adequate flocs for flotation, in the separation of marine microalgae the slow mixing improves separation by DAF. However, the 70-75% harvestability from sea water could not be repeated consistently when using lower flocculant doses, which were effective in jar-tests. This indicates that further improvements in the pilot plant unit operation could be achieved. Apart, it is suspected that the physiological condition of the culture may affect flocculation and it would be convenient to have a large pond to supply the same culture, for different flotation runs.

Another continuous separation experiment was designed to evaluate the effect of salinity on the efficiency of removal of microalgae. The pH was adjusted to 5.5 previous to the in-line injection of $FeCl_3$ at a dose of 60 mg/l. After one hour of continuous operation of the DAF samples of the outflow were taken every half-hour and composited. Initial and final concentrations of suspended solids were determined and the percent removed was calculated for each salinity (Fig.8).

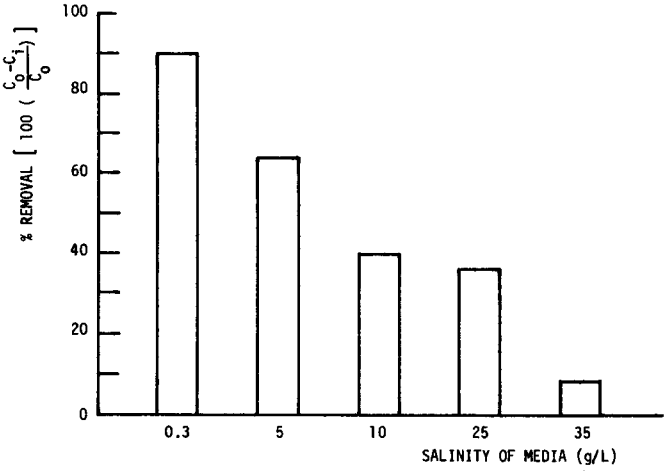

FIG. 8: EFFECT OF SALINITY ON THE EFFICIENCY OF MICROALGAE REMOVAL BY CONTINUOUS FLOCCULATION-FLOTATION IN A PILOT PLANT. (ISOCHRYSIS)

The different removal percentages obtained in this series of runs show that though flocculation-DAF, achieved above 90% removal efficiency from low salinity water, the removal efficiency decreased at increasing salinities. This is in accord with the results which showed the negative effect of ionic strength on flocculation. Since no flotation will occur without good flocculation, efforts are done to improve flocculation even at high ionic strengths.

3.3 Enforced Flocculation.

A marked reduction in flocculant dose was achived by recycling of the precipitant following flocculation back into the mixing-flocculation chamber.

Figures 9, 10 and 11 describe the algal removal efficiency as a function of flocculant net dosages under conditions of algal suspensions in tap water, brackish water (5g/l sea salt) and sea water respectively. With equilibrium multiple cycles reduction in net dosages of 75 percent and more were achieved. With sea water 3 cycles enforced flocculation not only caused a marked reduction of flocculant dosage but was vital for marine algae removal of 90 percent. It is assumed that the enforced flocculation is caused by a combination of nucleation, extra active charges and enmeshment.

The enforced flocculation (E.F) has been successful under laboratory conditions and should be optimized for outdoor pilot plant conditions. If so it will revolutionize the technology and economics of algae harvesting.

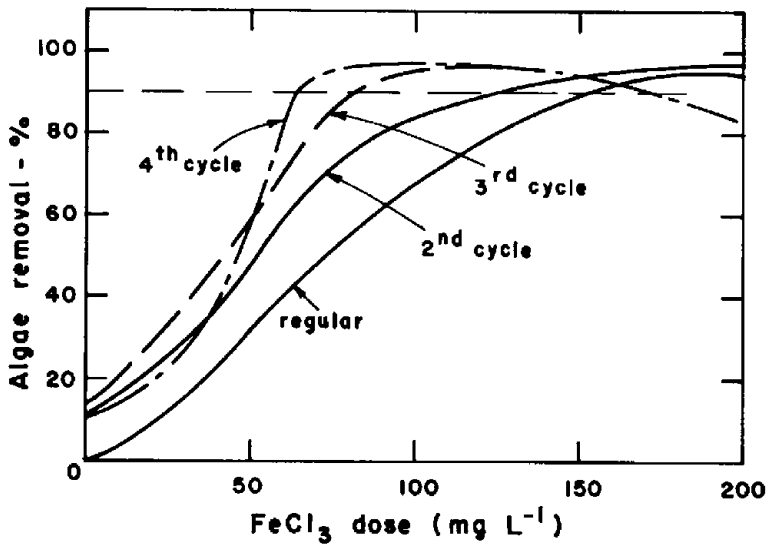

FIG. 9 ENFORCED FLOCCULATION IN TAP WATER, HARP OD_0 = 0.27

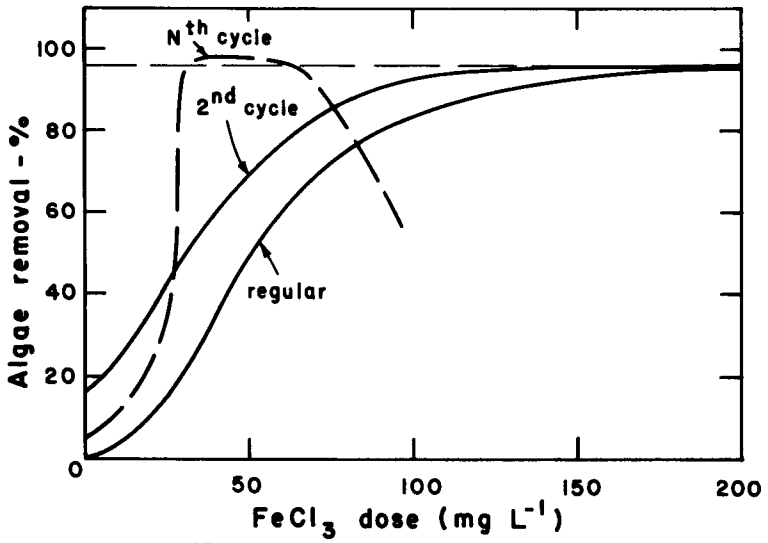

FIG.10 ENFORCED FLOCCULATION IN BRACKISH WATER, HRAP WITH 5g L^{-1} SEA SALT; OD_o = 0.21

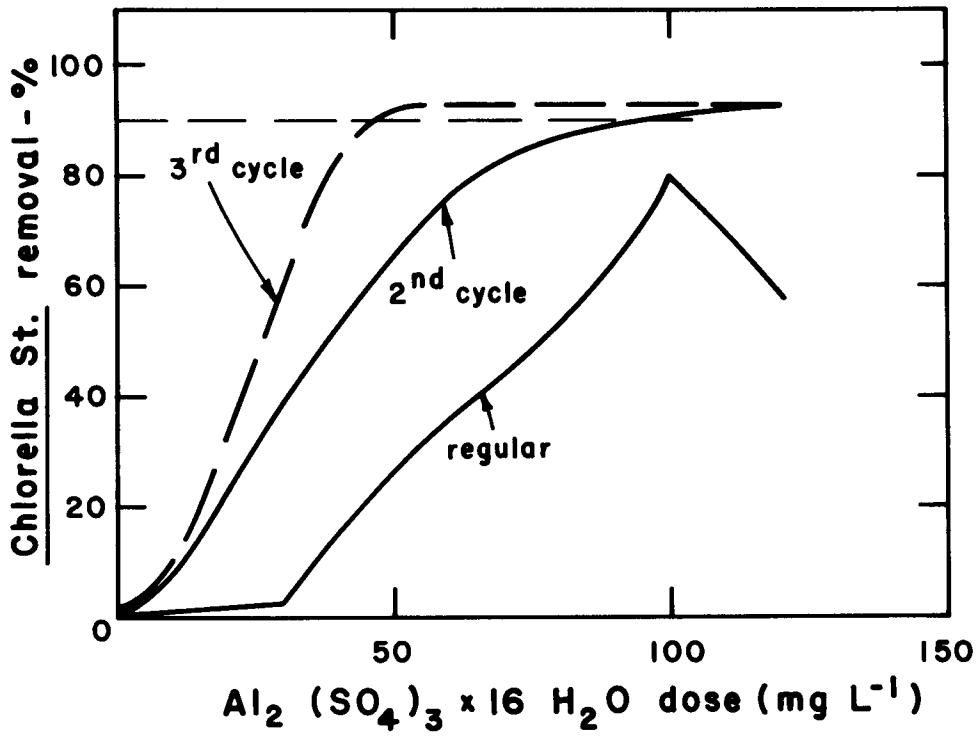

FIG.11 ENFORCED FLOCCULATION IN SEA WATER

4. ECONOMICS OF MARINE MICROALGAE FLOCCULATION

Economic analysis of two proven microalgae harvesting methods from HROP effluents centrifugation and alum flocculation followed by flotation, show that centrifugation was almost twice as costly as flocculation flotation process (Moraine et. al 1979). Both investment and operating (primarily power) costs were higher for centrifugation even though it did not require using chemicals as did alum flocculation.

The cost of separation (excluding drying) was estimated to be 25% of the overall cost of production and processing of freshwater algae (7).

Microstraining and Dissolved Air Flotation are the two methods selected for the separation of microalgae from marine and brackish water cultures for the first and crucial 150-250 fold concentration of the thin algae suspension of 0.02 to 0.04 percent solids to a 5 to 7 percent slurry. Previous experience shows that destabilization and flocculation is an essential procedure for harvesting microalgae by either method.

Flocculation was studied with two species of marine microalgae: Isochrysis galbana and Chlorella stigmatophora as model species of motile and non-motile algae. Flocculation of these algae in sea water reqiured high flocculant dosage and this was attributed mainly to the high ionic strength of the media, to the marked surface charge of marine microalgae, and in the case of I. galbana, also to motility. Brackish water provided a better media as far as flocculation dosage is concerned.

The total cost of separation (C_T) includes capital costs (C_c), and the recurrent operational costs,(equation (1))

$$C_T = C_c + C_o \qquad (1)$$

The operational cost can be broken down into: cost of maintenance (Cm), cost of flocculation (C_f), cost of acid (C_a), cost of labour (C_l) and cost of energy (C_e).

$$C_T = C_c + (C_m + C_f + C_a + C_l + C_e) \qquad (2)$$

More than 60% of the operational costs is the price of chemicals, (flocculation) therefore the economical estimation is focused on their cost. Experimental results of flocculation tests (Table 3) together with the price of chemicals in Israel and in the USA, were used to estimate the cost of flocculant required to remove 90% of the algae from cultures with different concentrations of salt.

Price of Chemical

Prices of chemicals in Israel were obtained from manufacturers and wholesalers. Prices in USA were estimated using 1976 bulk prices adjusted to 1984 with the Total Cost Index reported by Engineering News Record. (Table 8).

Table 8. Cost of Chemicals Used for Flocculation in Israel* and in USA**. (In U.S.$ per ton).

	Israel	USA
Alum 7.7% Al (solution)	193	
Alum, $Al_2(SO_4)_3.18H_2O$		133
SO_4H_2 98%***	82	93
Ferric chloride 99% technical (100kg barrels)Ferric chloride bulk	1042	185
Zetag 57	5000	5000
Chitosan	No commercial supply in bulk. Lab. supply	13,000

* Prices Dec. 1984, include 15% VAT and cost of containers refill.
** Prices Oct. 1984 (ENR index 4160) adjusted from prices in 1976 (index 2475).
*** Produced in Israel.
**** With increased use of chitosan, its prices should be substantially reduced.

Data from Tables 3 and Table 8 was used to calculate the cost of inorganic flocculant required to harvest a Kg of algae from cultures with different salt concentrations (Table 9). Although the cultures used experimentally contained relatively low concentrations of algae (75mg/l), since the algae requirement of flocculant is negligible as compared to the chemical requirement of the solutions, for the purpose of cost calculation, a 300 mg/l concentration of algae is assumed.

Table 9. Calculated cost of flocculant required to remove 1 Kg of algae from cultures with different salinites by Conventional Flocculation.

Sea Salt Concentration (g/l)	Cost of Flocculant (in US cents per Kg.) For Cultures with 300 mg/l VSS			
	U.S.A		ISRAEL	
	ALUM	$FeCl_3$	ALUM	$FeCl_3$
5	1.3	0.9	1.9	5.2
10	3.3	1.9	4.8	10.4
20	4.4	3.7	6.4	20.8
30	6.7	--	9.7	--
36	10.0	7.7	14.5	43.3

Flocculant costs of non-motile algae as Chlorella stygmatophora are reduced by 20-30 percent (6) since inorganic flocculant demand is reduced.

A novel and promising approach for flocculation, enforced flocculation decreased the dose of flocculant by 60-80%, in batch experiments, under laboratory conditions. Calculated costs of flocculation by this method are shown in Table 10 and they stress the effect on improvement of the technology of separation may have as compared to conventional flocculation (Table 9).

Table 10. Calculated Cost of Flocculant Required to Remove 1 Kg of Algae by ''Enforced Flocculation'' from Cultures With Different Salinites.

Sea Salt (g/l)	Cost of Flocculant (in US cents per Kg Algae) For Cultures With 300 mg/l VSS			
	U.S.A.		ISRAEL	
	Al	$FeCl_3$	Al	$FeCl_3$
5	0.26	0.18	0.38	1.09
10	0.66	0.38	0.9	2.08
20	0.88	0.74	1.28	4.16
30	1.34	--	1.94	--
36	2.0	1.54	2.9	8.66

Data on the cost of inorganic flocculants required to separate 90 percent of the microalgae from cultures with different salt concentrations were compared to the calculated allowable cost of flocculants in ¢/Kg algae, which take into account the culture density, the lipid content, the crude oil price and the following assumptions, which were used to calculate the breakeven cost.

1) The density of algae may vary between 100 to 600 mg/l V.S.S. dependent on culture depth and growth conditions. Most calculations were done with a realistic value of 300 mg/l.

2) The algal biomass could contain from 10% to 60% lipids.

3) The price of algal lipids is equal to crude oil price. Actual price 29 US$/bbl, future price, 50 US$/bbl with an upper price of 80 US$/bbl. (7bbl/Ton).

4) Algae are either used solely for energy or they are used for energy and the extracted cake is given a value of 0.3 US$/Kg (assumed price of proteinaceous feedstuff).

5) The cost of separation represents 25% of the total cost of producing algae.

6) The cost of flocculant represents 60% of the cost of separation up to solids concentrations which will allow wet extraction of about 9-10 percent solids (See Fig.1).

The actual costs of different flocculants (in US ¢/Kg algae) required to separate microalgae as a function of salinity, was calculated with data of Table 3 and experimental data shown in Table 7, and are represented graphically in Fig. 12.

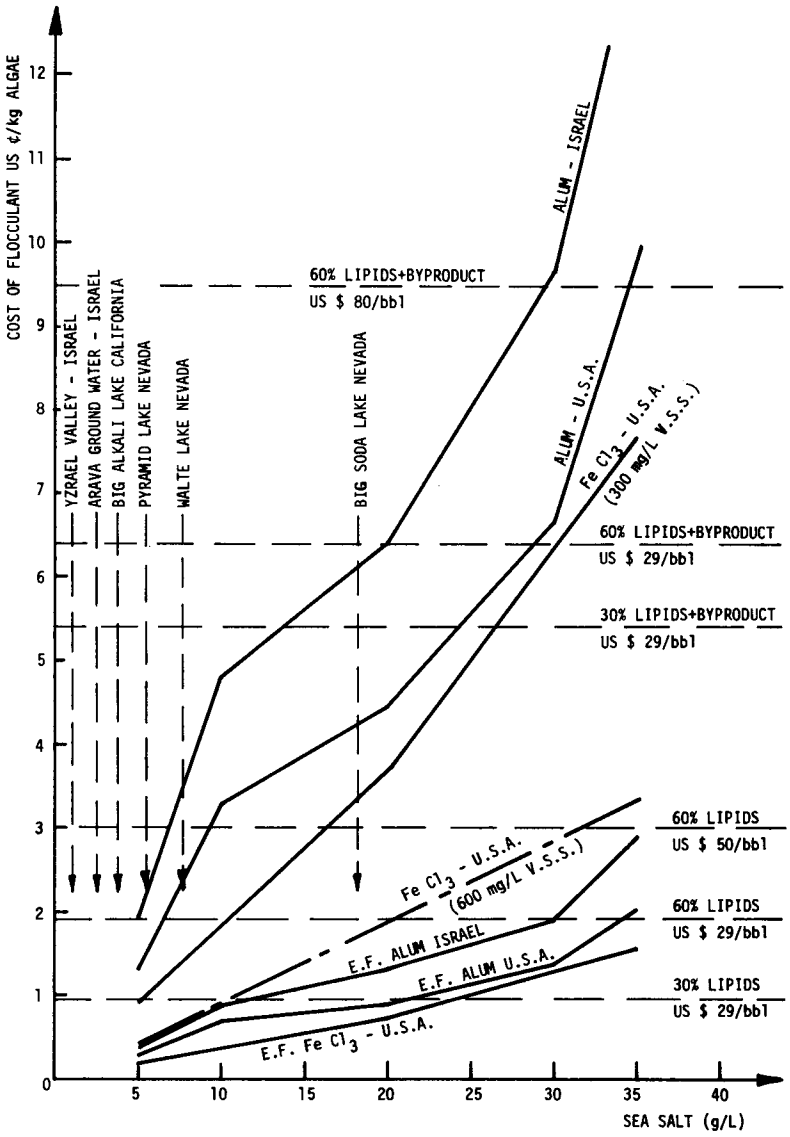

FIG. 12: COST OF FLOCCULANT REQUIRED TO REMOVAL 90% MICROALGAL BY CONVENTIONAL OR ENFORCED FLOCCULATION FROM CULTURES WITH DIFFERENT SALINITIES

The upper group of full line curves refers to conventional flocculation while the lower group of curves shows costs of Enforced Flocculation. In general, there is a fourfold reduction in the cost of flocculant required for the separation of microalgae from brackish or seawater by the use of Enforced Flocculation and if this reduction will be corroborated in the pilot plant, it is a major breakthrough in microalgae separation.

The obvious effect of an increase in the biomass of algae, on decreasing the cost of flocculant per Kg algae is shown in Fig. 12 by comparing the dotted curve which represents, 600 mg V.S.S./L, to the curve of 300 mg V.S.S/L, both calculated for $FeCl_3$ in USA. The dotted vertical lines in Fig. 12, are added as reference points, They show the salinities of brackish water from different locations in Israel and USA (From Table 2)

Separation of microalgae is economically feasible for the points of the cost curves below the breakeven point. The breakeven point is indicated by the intersection of the cost curves with the horizontal dotted lines, which represent the allowable cost of flocculant (from Fig. 13). These figures of allowable cost of flocculant were calculated for different combinations of % lipids in algae, oil prices and by taking or not taking into account the cost of the algae by-product after lipids extraction.

Two examples will illustrate the effect of these variables and will highlight which research efforts deserve priority towards the achievement of reductions in the cost of separation.

If we take a culture with 300 mg/l V.S.S, 60% lipids in algae, and 80 US$/bbl (Fig.11) the calculated allowable cost of flocculant is 5.0 US ¢/Kg algae without taking into account the cost of by product and 9.5 US ¢/Kg taking into account both the cost of oil and the cost of the by product.

Marking these allowable costs of flocculant in Fig. 10 as dotted horizontal lines it can be seen that when the by-product is also considered (300 mg/l; 60% lipids in algae, 80 $/bbl, by product value 0.3 $/Kg algae) at 9.5 c allowable cost of flocculants, it is feasible to separate algae by Enforced flotation and by conventional flotation from cultures with all salinity ranges (including sea water) with the exception of Alum-Israel which allows only for separation from cultures with 29 gr/L/salt. When the by product is not accounted for at 5.0 c/Kg algae allowable cost for flocculant, separation can be performed economically only from cultures with 25% salt (or lower), by conventional flocculation with Alum and $FeCl_3$ at USA prices, and of course from cultures of all salinities by Enforced Flocculation.

This example shows that by taking into account the value of the by product, the allowable cost of flocculant is higher, the range of salinities that can be dealt is larger and flocculation is feasible even by conventional flocculation.

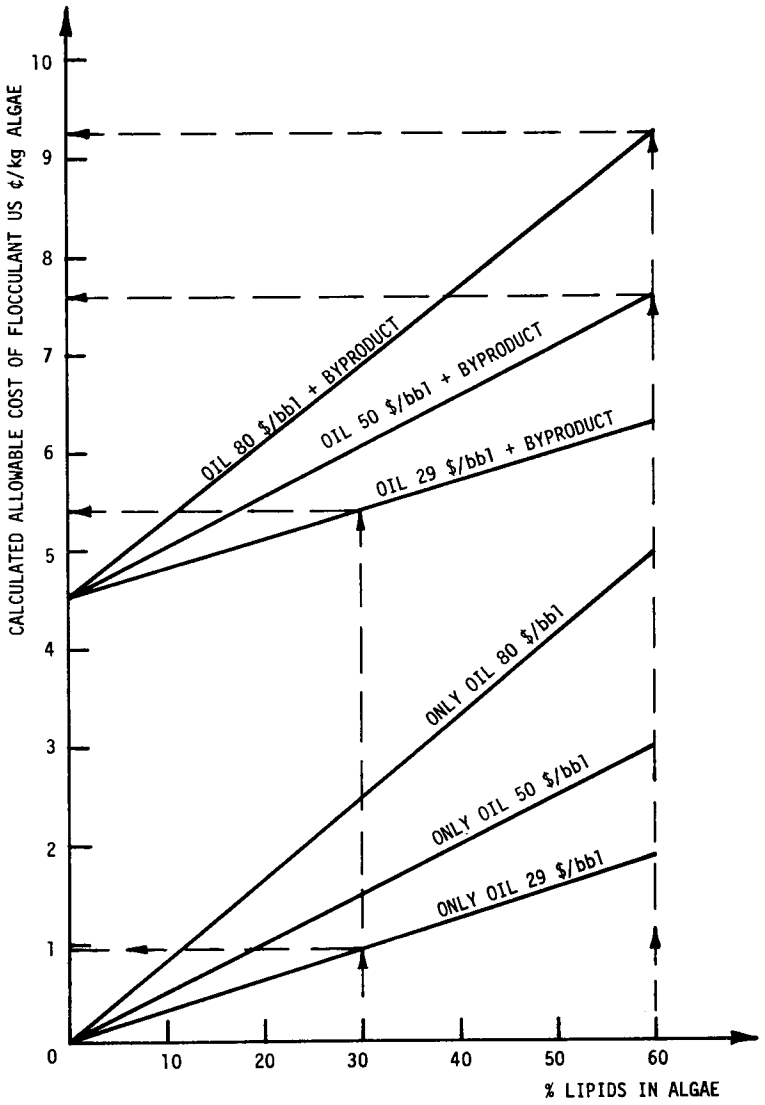

FIG.13 : EFFECT OF THE ALGAL LIPID PERCENTAGE ON THE ALLOWABLE COST OF FLOCCULANT FOR MICROALGAE SEPARATION. (300 mg/l VSS).

Another example. Taking in Fig. 13, more conservative figures: 300 mg/l V.S.S, 30% lipids in algae, 29 US $/bbl we obtain 0.9 C/Kg algae and 5.4 ¢/Kg algae allowable cost for flocculant without and with the value of the by-product, respectively. These values, as shown in Fig. 9 would allow the separation with FeCl (USA) from cultures with salinities below 25 g/L and with Alum-Israel from salinities below 10g/L, by Enforced Flocculation. It should be noted here, that with exception of the Big Soda Lake, Nevada, all the other lakes of the southwest USA and the Arava groundwater and the Jeezrael Valley (vertical dotted lines, Fig.12) could be harvested even with the conservative figures of this example.

In conclusion, it is desirable to bring to the separation stage algae cultures with the highest cell density as economically feasible, because doubling cell density, halves the cost of flocculant. Similarly an increase in lipids content from 30% to 60% would double the allowable cost of flocculants that can be used for separation. It is also important to take into account the intrinsic value of the by-product after lipid extraction, since it triples the allowable cost of flocculant. It is therefore worth to have an input of efforts for the experimental evaluation of the by-product.

Enforced flocculation reduced to one fourth the cost of flocculants in laboratory batch experiments, as compared to the cost of flocculants required by conventional flocculation. Therefore, major efforts should be invested in researching and scaling-up this breakthrough.

Future research should operate an existing 150m production pond facility to provide enough material for several runs of continuous operation of the pilot plant Dissolved Air Flotation during the same day. This would allow the evaluation of modifications in the flocculation flotation unit, in order to achieve high removal efficiencies from sea water cultures on continuous basis.

Extraction of lipids from semi-wet and wet algal products has to be tried experimentally. Economic evaluations of the whole process should be made including capital and energy costs.

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DEVELOPMENT OF OUTDOOR SYSTEM FOR PRODUCTION OF LIPID-RICH HALOTOLERANT MICROALGAE

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ABSTRACT

Under optimal laboratory continuous conditions, the doubling time of Isochrysis and Nannochloropsis cultures were determined to be 9.6 and 23 hr, respectively.

Outdoor Nannochloropsis cultures maintained at concentration of 5 mg l^{-1} chlorophyll produced a higher output rate than denser cultures (10-20 mg l^{-1} chlorophyll).

pH higher than the optimal (7.5) in Nannochloropsis resulted in a decrease in the algal biomass output rate, while growth media changes (Artificial Sea Water vs. Enriched Sea Water) did not effect this rate.

Nitrogen starvation caused an increase in the lipid content - from 20 to 25% of dry weight in Nannochloropsis and from 25 to 46% in Isochrysis. However, the lipid production rate was significantly lower in the nitrogen starved cultures.

A monoalgal culture of Nannochloropsis was maintained for more than six months by maintaining an optimal biomass concentration, while in Isochrysis, NH_4^+ and herbecides prevent the proliferation of some zooplankton.

A prototype of a data acquisition system that monitors pH, dissolved oxygen, optical density, light intensity, water and air temperatures was set up and performed favourably. An on-line estimation of photosynthetic activity is also possible.

INTRODUCTION

Our efforts in the second year of the project were directed towards the optimization of growth conditions for maximal production of biomass in two halotolerant microalgae, Nannochloropsis sp. and Isochrysis galbana which were cultivated outdoors.

The production of algal biomass is directly affected by three main factors: nutrients, temperature and light. A prerequisite for obtaining maximal productivity under outdoor conditions is to ensure that there is no nutritional limitation, and that culture growth is limited by light and temperature only. Ideally, light should be the sole factor limiting growth. Light utilization can be optimized by the proper selection of pond depth and cell concentration throughout the year.

The effect of light on the biomass output rate was studied by varying the cell concentrations.

The effect of nutrient concentration on growth and lipid production rate was also studied. N-starvation was specifically examined, since earlier reports (1) indicated a significant increase in total lipid cell content in response to this treatment.

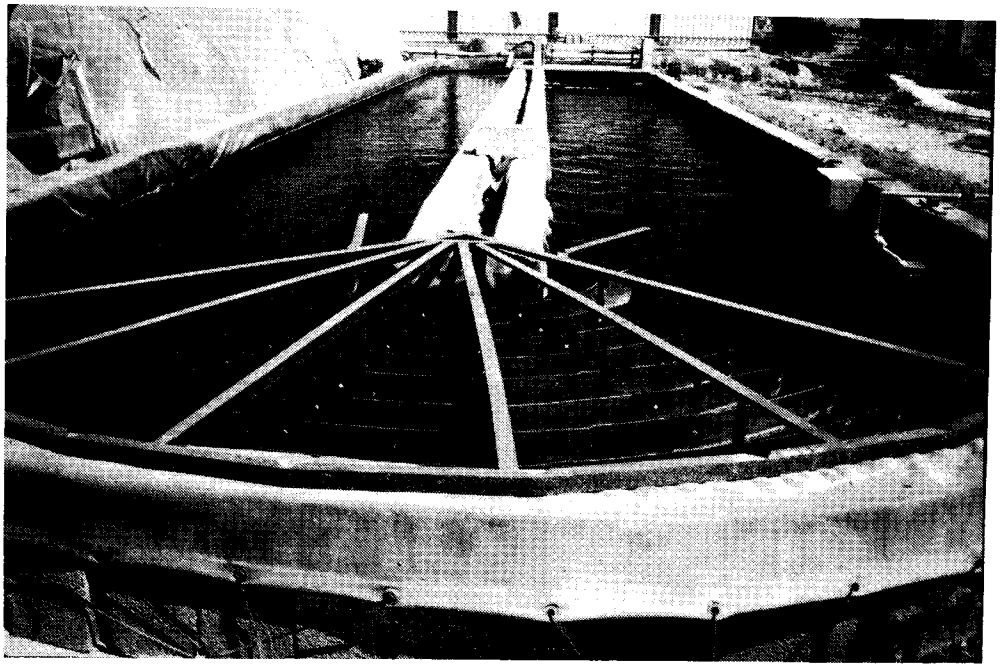
We also studied the prerequisites for maintenance of a monoalgal culture which, in our opinion, is a major challenge in large-scale production of microalgae.

Finally, we present preliminary data on the development of a data aquisition system which can automatically monitor different growth parameters such as pH, temperature and light intensity. According to our experience, a reliable data aquisition system will significantly increase the knowledge about the algal pond system. It will aid in maintaining a constant maximal output rate and will recognize the warning signs of a potentially disastrous situation, which could result in the total loss of culture.

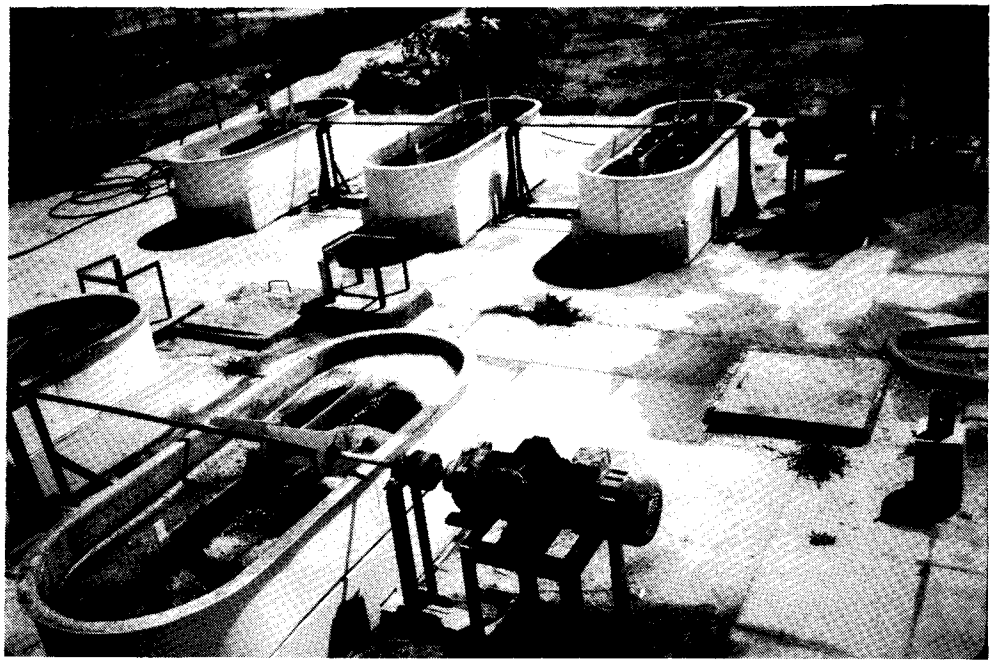
R E S U L T S A N D D I S C U S S I O N

DETERMINATION OF MAXIMAL SPECIFIC GROWTH RATE (μMAX) OF NANNOCHLOROPSIS AND ISOCHRYSIS UNDER LABORATORY CONDITIONS

One of the major purposes of our work in the laboratory was to define conditions in which μ_{max} could be acheived. As summarized in Table 1, μ_{max} for Isochrysis and Nannochloropsis was found to be 0.072 and 0.031 h^{-1}, corresponding to doubling times of 9.6 and 23 hr, respectively, which are in agreement with previous reports (2,3). By utilizing this parameter, the proper medium composition is defined for optimization of outdoor production.



A 100 m^2 Pond that Is Operated in Israel



Small Outdoor Ponds Used for Optimization Studies

Table 1
The specific growth rate of Nannochloropsis and Isochrysis under laboratory conditions

GROWTH CONDITIONS	GROWTH RATE Isochrysis (h^{-1})	d.t(h)	Nannochloropsis (h^{-1})	d.t(h)
a	0.023	30.0	0.030	23.1
b	0.072	9.6	0.029	24.0
c	0.082	8.5	0.031	23.1

μ - specific growth rate; d.t - doubling time

a - A batch culture of 250 ml flasks placed in a gyrotory shaker under light intensity of 75 µEinstein m^{-2} sec^{-1} at 29 $\pm$ 1°C (ASW or ESW medium).
b - A batch culture of 500 ml glass tubes bubbled with either air or 1.5% CO_2 in air. The light intensity at the surface was 185 µEinstein m^{-2} sec^{-1}, the temperature was 28°C.
c - Values of µmax and d.t previously reported (2,3).

EFFECT OF POPULATION DENSITY ON BIOMASS OUTPUT RATE

The population density is dependent upon the amount of light energy available for the individual cell in the culture. Clearly, the lower the population density, the higher the specific growth rate which is expected in a primarily light-limited system. In the winter, the major environmental factor limiting the growth and output rate is the temperature, while light is the dominant limiting factor in the summer. It is therefore expected that the effect of population density on the output rate will be more pronounced in the summer in comparision to the winter.

Table 2
The effect of population density on the output rate in Nannochloropsis (December 1984-Mid-February 1985).

Chlorophyll mg l^{-1}	Output rate gr m^{-2} day^{-1}	Photosynthetic Efficiency %	Lipids % of AFDW	Rate of lipid Production gr m^{-2} day^{-1}
5	7.1	3.1	15	1.1
10	5.3	2.4	17	0.9
20	4.1	1.8	20	0.82

Table 2 summarizes the effect of cell concentration on productivity and rate of lipid production in Nannochloropsis. Clearly, the decrease in cell concentration caused an increase in output rate up to 45% in comparision to the output obtained in highest cell concentration (20 mg l^{-1}).
A pattern of the effect of cell concentration on the growth and harvest regime is presented in Fig. 1.

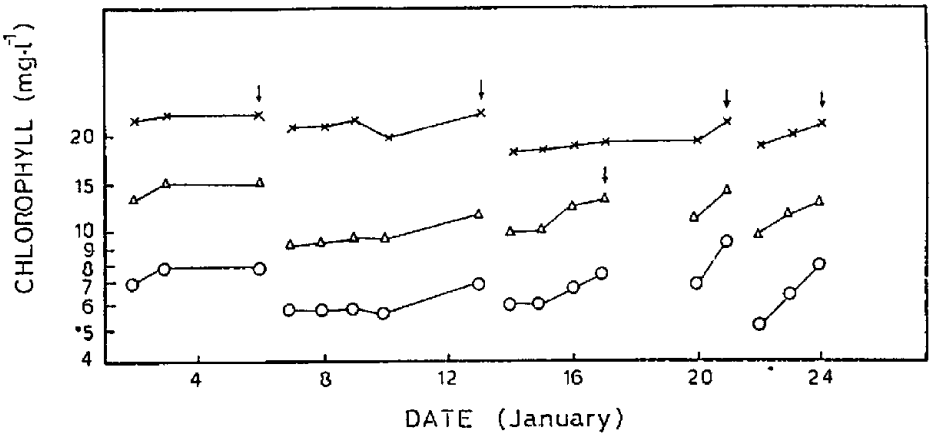


Fig 1: Effect of chlorophyll concentration on the growth of Nannochloropsis.
x-x 20=mg ml^{-1}; Δ-Δ=10 mg ml^{-1}; o-o=5 mg ml^{-1}

Of interest was the effect of cell concentration on lipid content observed in Nannochloropsis sp. and Isochrysis galbana under indoor conditions. Samples of both algae were taken during the growth period (Fig. 2).

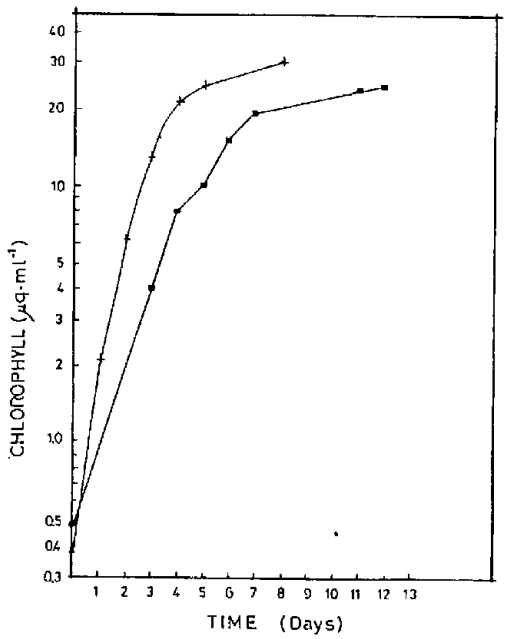


Fig. 2
Growth curve of two halotolerant microalgae
■-■ Nannochloropsis culture bubbled with air;
x-x=Isochrysis bubbled with 1.5% CO_2

In Nannochloropsis, the data (Table 3) indicates that the increase in cell concentration is accompanied with an increase in lipid content, while in Isochrysis lipid content was not affected by cell density. The same pattern was observed under outdoor conditions (Table 2, Table 4).

Table 3
Changes in lipid content in the course of growth

N A N N O C H L O R O P S I S		I S O C H R Y S I S	
Chlorophyll ($mg\ l^{-1}$)	Lipid content (% Of AFDW)	Chlorophyll ($mg\ l^{-1}$)	Lipid content (% of AFDW)
5	10.0	1.4	17.8
15	13.0	3.8	19.5
35	20.0	6.0	15.4
		18.8	18

Table 4
Changes in lipid content in the course of growth of Isochrysis cultivated outdoors

Days	Cell No. (Cell ml)	Lipid content % of(AFDW)
0	$7x10^6$	24.0
10	$1.6x10^7$	23.0
20	$4x10^7$	21.0

We intend to continue this experiment in order to define the optimum cell density for each season. This will indicate the maximal rate of production of both biomass and lipids.

The effect of cell concentration on the maintanence of monoalgal culture is discussed in Section VI.

EFFECTS OF MEDIA COMPOSITION ON THE RATE OF LIPID PRODUCTION

In order to maximize output rate of outdoor cultures, it is imperative that all nutrients should be in concentrations that do not limit growth. However, changes in nutrient level are used to modify cell composition. The increase in glycerol content in response to increasing the amount of salt in the medium in Dunaliella is an excellent example (4).

Changes in temperature, light intensity and medium composition may affect the lipid content of algal cells. In particular, nitrogen starvation caused the most massive increase (1). The effect of nitrogen starvation on the rate of lipid production was studied, as well as the effects of medium composition and pH.

A. Nitrogen starvation

1. Isochrysis: When Isochrysis was grown in a limited nitrogen medium, growth ceased after five days (Fig. 3). An increase in lipid content was observed soon as nitrogen was depleted (Fig. 4).

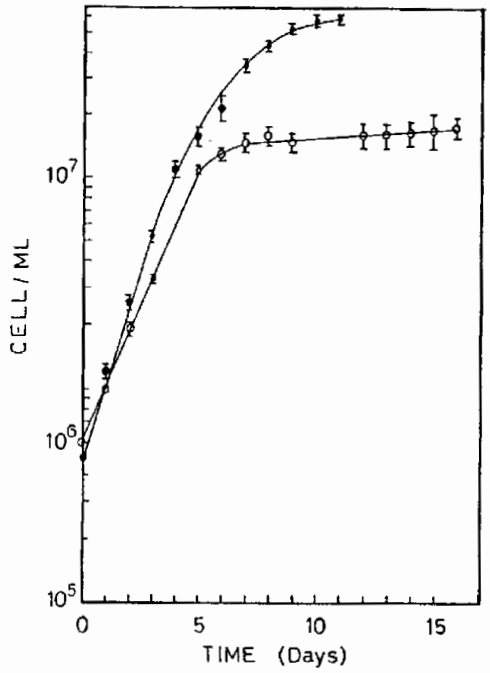


Fig. 3
Effect of NO_3^- concentration on the growth of Isochrysis
●-●=Control-1,000 mg l^{-1}; ○-○=N-deprivation-200 mg l^{-1}.

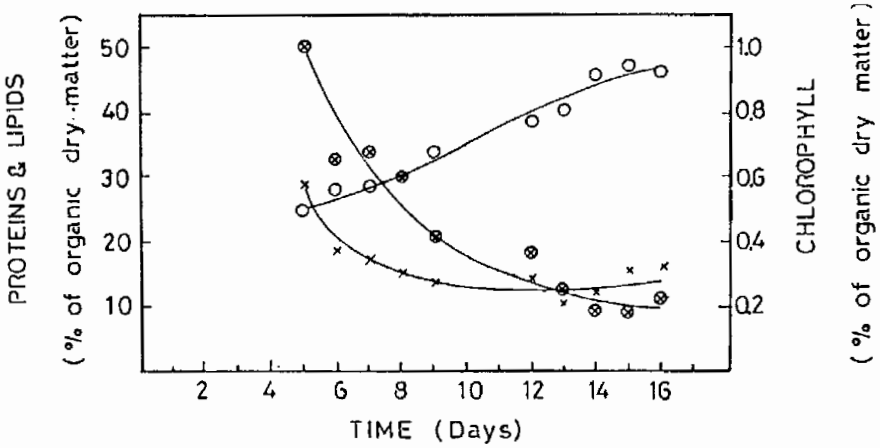


Fig. 4
Changes in lipid, protein and chlorophyll-a content in the course of nitrogen starvation.
o-o=lipid content; ⊗-⊗=chlorophyll; x-x=protein

By comparing lipid production rates of starved and control cultures, it is obvious that an increase in lipid production cannot be obtained by nitrogen starvation. When nitrogen starvation was introduced under outdoor conditions, no increase in lipid content was observed (Table 5). This phenomenon will be further studied.

Table 5
The effect of N-deprivation on lipid content in Isochrysis cultivated outdoors.

Days	Lipids (% AFDW) N-deprivation	Control
1	20.0	-
8	22.8	25
15	21.5	22
22	23.1	23

2. Nannochloropsis: A slight increase in lipid content of a nitrogen deprived Nannochloropsis culture was observed both indoors (Table 6) and outdoors (Table 7). A significant decrease in the overall biomass production rates was observed in the outdoor experiment (Fig. 5, Table 7).

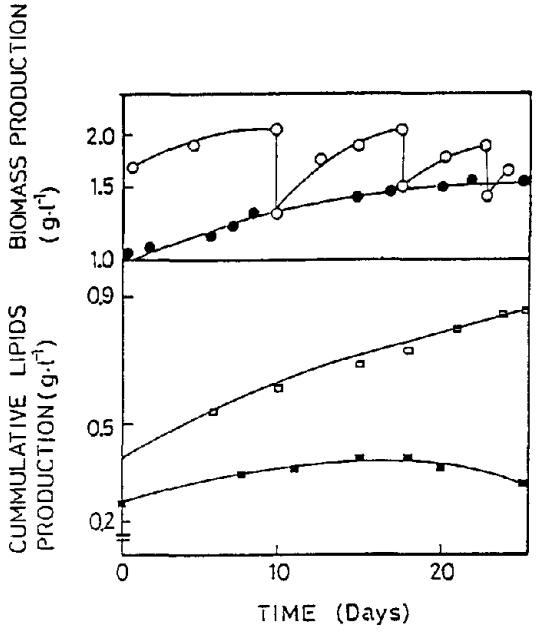


Fig. 5
The effect of nitrogen starvation on output rate and accumulative lipid production in Nannochloropsis
o-o=Control - biomass (g l^{-1})
●-●=Nitrogen starved - " " "
□-□=Control - lipid production
■-■=Nitrogen starved - " "

Table 6
Effect of N-deprivation on lipid content in Nannochloropsis cultivated indoors

N-deprivation (days)	Lipid Content (% of AFDW)
0	22.0
8	26.0
14	25.8
21	25.0

Table 7
Effect of N-deprivation on lipid production rate in Nannochloropsis

	Output rate $g\ m^{-2}\ day^{-1}$	Lipid content (% of AFDW)	rate of lipid production $g\ m^{-2}\ day^{-1}$
Control	13.5	22	3.0
N-deprivation	3.5	25	0.9

B. Effect of pH on lipid production in Nannochloropsis

In a long term outdoor experiment (50 days) pH changes had no significant effect on the rate of production (Table 8).

Table 8
Ifluence of pH on lipid production rate

pH	Output rate $g\ m^{-2}\ day^{-1}$	Lipid content (% Of AFDW)	Rate of lipid production $g\ m^{-2}\ day^{-1}$
7.0-7.5	15	24	3.6
8.0-8.5	11	23	2.5

C. Influence of growth media

The influence of two growth media composition on productivity and lipid content were compared. No effect was noted.

Table 9
Influence of growth media on the output rate in Nannochloropsis cultivated under outdoor conditions.

Medium	Output rate $g\ m^{-2}\ day^{-1}$	Lipid content (% of AFDW)	Rate of lipid production $g\ m^{-2}\ day^{-1}$
ASW	5.6	18	1.0
ESW	5.8	19	1.1

ASW - Artificial sea water
ESW - Enriched sea water

SEASONAL EFFECTS ON LIPID CONTENT

The lipid content of Nannochloropsis salina cultures grown outdoors was followed. Although lipid content varied, higher concentration was observed during the summer as compared to the winter (Fig. 6).

Laboratory experiments were conducted to assess the effects of temperature and light intensity on lipid content.

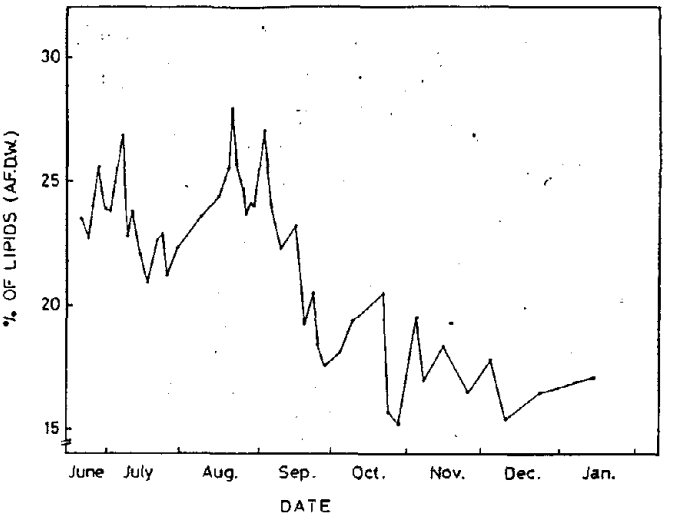


Fig. 6
Changes in lipid content in Nannochloropsis (6/84-1/85)

THE MAINTENANCE OF MONOALGAL CULTURE

The cultivation of monoalgal culture is frequently hampered by contamination with other algae and with zooplankton. It is therefore important to establish conditions which will selectively promote the growth of the alga of interest and/or inhibit the growth of other organisms. Different environmental factors were suggested as selective forces for algal species compositions and selectivity (5).

The following factors were found to affect the maintenance of Isochrysis and Nannochloropsis monoalgal cultures cultivated outdoors:

A. Effect of Ammonium ion

There are advantages for using NH_4^+ as a nitrogen source for mass production of Isochrysis. NH_4^+ was found to prevent proliferation of some contaminants.

Our suggested strategy for outdoor cultures is to supply them with NH_4^+ at concentration of ca. 2mM supplements with 5 mM NO_3^-.

B. The effect of herbicides

Diazinon - Diazinon (5 ppm) was found to be exterminating the paramecium developed in Isochrysis culture outdoors. An immediate effect was observed (within 30-60 min).

C. Effect of population density

Fig. 7 presents three photomicrographs of Nannochloropsis culture maintained at 3 different cell concentrations.

Monoalgal culture was preserved by maintaining high cell concentration (20 mg/l). In the lower concentration pond (5 mg/l), contaminants accumulated ca. 10-15% of the total biomass. When growth is limited by temperature (as is the case in our local winter), we suggest to maintain the culture at a density between 10-20 mg chl. l^{-1}, even at the cost of some productivity loss (Table 2).

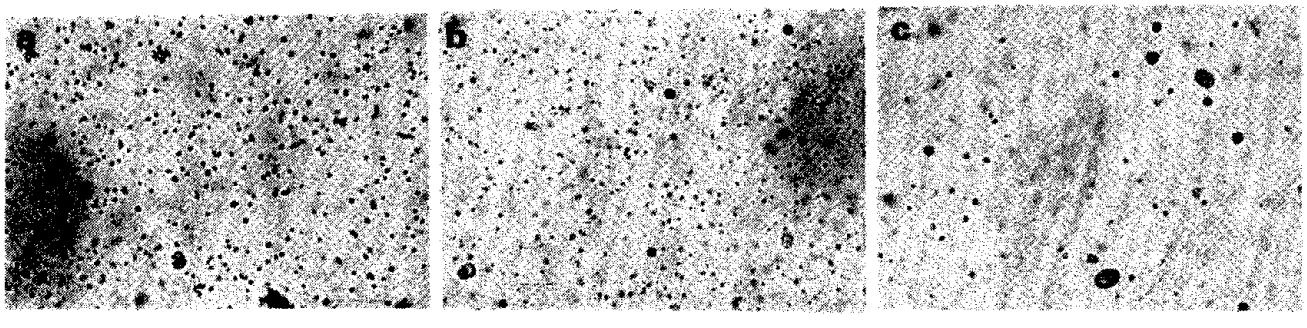


Fig. 7
Effect of population density on the maintenance of monoalgal culture in Nannochloropsis
a- 20 mg chl. l^{-1}
b- 10 " " "
c- 5 " " "

MONITORING OF CULTURE PERFORMANCE

A. Nannochloropsis - data collecting system

1. On-line monitoring of algal ponds: The need for a reliable data aquisition system for monitoring environmental parameters in algal ponds has already been pointed out. Through a cooperative work with Prof. Ben-Yaakov of the Department of Electrical Engineering, such a system is now in the process of being set-up. The design and development of the system are part of another research program, and our aim was to modify the system to meet the specific requirements of marine microalgal culture.

The project was divided into 3 phases:

Phase I - Set-up of the system and culture monitoring under laboratory conditions.

Phase II - Set-up of the system under outdoor conditions: checking the performance of the system's compatability to saline water.

Phase III - Debugging and improvement of the system based on Phases 1 and 2.

2. System design: The primary objective in the development of the microcomputer controlled system is to provide maximum flexibility in terms of electrode calibrations, sampling strategy and computational procedures. This was acheived by minimizing hardware and providing flexibility for computer control. Furthermore, an effort was made to design an all purpose sensor interface that can operate under a wide range of applications with practically any microcomputer.

The parameters measured were: pH, dissolved oxygen (DO), optical density (OD), light intensity, water and air temperature. The electrode signals were interfaced to a microcomputer via a general purpose interface/controller.

The interface also permits the control of outside devices, such as operation solutions feeding pumps and gas valves. Operation of interface/controller is software controlled and most monitoring and control programs are written in a high level language (BASIC). The application of a microcomputer with a BASIC interpreter simplifies and hence reduces the cost of programming, a subject that could prove to be a bottle-neck if frequent program changes are required - as often is the case in the research laboratory.

A complete description of the system is given elsewhere (6).

3. System performance evaluation: The prototype has been operated continuously for three months and final conclusions are given for the construction of the system.

Hardware: There were no problems with the computer system, the taperecorder or the monitor. The power supply system performed very well. Frequent power failures caused no damage to the system.

Electrodes:

Temperature and light - Results were satisfactory. No calibration problems occured in three months of operation.

pH - We found gel-filled electrodes to be most suitable, mainly because of their resistance to mechanical damage. In general, the electrode has to be cleaned once a day and calibrated once a week.

Oxygen - The home-made electrode was very reliable. Difficulties arose only when the membrane was mechanically damaged. The electrode has to be cleaned once a day and recalibrated once a week.

O.D. - The electrode we designed performed favourably under laboratory conditions. However, its performance under outdoor conditions is yet to be improved. A new model is now being constructed for further testing.

Software - The interactive mode of operation and calibration was user friendly. A few modifications must be made as follows:

I) Self calibration of electrodes: system calibration failure will result in a visual alarm that will indicate problems in any a specific parameter.

II) Data presentation: At present, only the last set of measurements are displayed. Modification will be made to allow a graphic display of measurements from the previous 48 hours.

System debugging and rebuilding has now started. We plan to operate the system again in the coming spring. Enclosed is the flow diagram of the computer program (Fig. 8) and some examples of the information obtained from the prototype (Fig. 9).

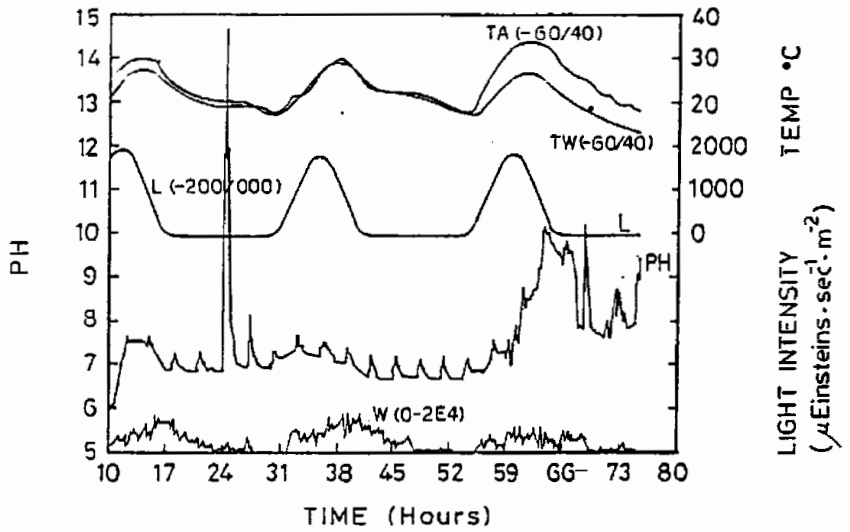


Fig. 8

Preliminary data obtained from a Nannochloropsis pond culture outdoors. TA- Air Temperature; TW- Water Temperature; L- Light intensity (μEinstein sec^{-1} m^{-2}); W- Wind velocity (relative units).

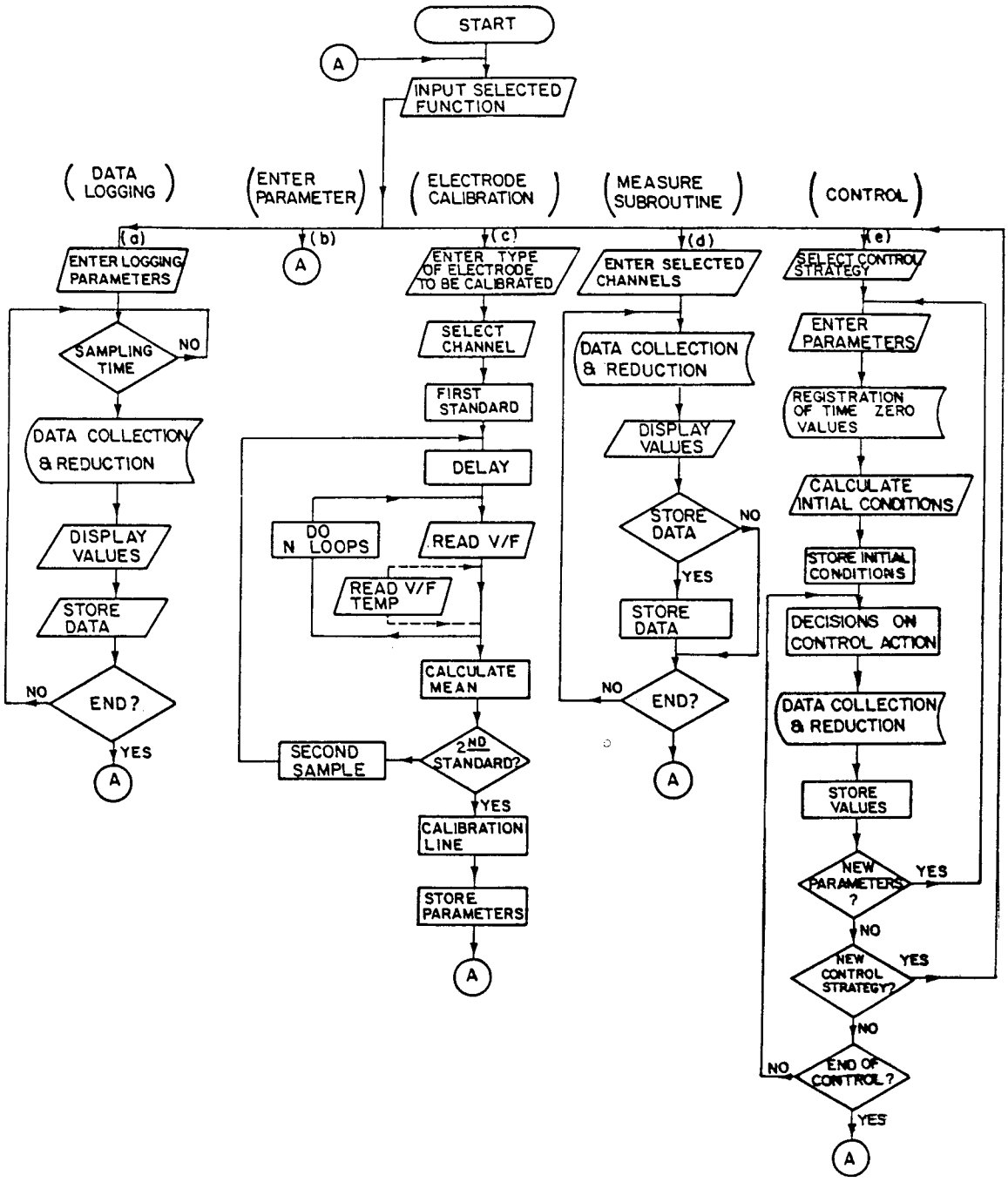


Fig. 9
Flow diagram of computure programs for real time monitoring and control of an algal pond including sensor calibration interactive routines.

SCREENING OF CHLORELLA STRAINS FOR HIGH LIPID PRODUCTION

A. Selection of Chlorella strains

Screening of Chlorella strains from marine habitats were carried out under three different culture conditions. A full list of code numbers and cultivation media is given in the following table.

Table 10
List of Chlorella strains obtained

Species	Code	Obtained from	Cultivation medium in culture collection
C. capsulata	LB2074	UTEX*	Enriched sea water
C. stigmatophora	LB993	"	Soil sea water
C. sp.	LB2070	"	Enriched sea water
C. sp.	LB2069	"	" " "
C. sp.	LB2068	"	" " "
C. minutissma	LB2341	"	Artificial sea water
C. luteoviridis	211-59	U. Gettigen**	" " "
" "	211-3	"	" " "
" "	211-2a	"	" " "
" "	211-2b	"	" " "
C. ovalis	211/2/B	CCAP***	M14 medium
C. marina	211/27	"	" "
C. ovalis	211/21B	"	" "
C. spaorchii	211/29a	"	" "
" "	211/29b	"	" "
C. salina	211/25	"	" "

* University of Texas Culture Collection, USA
** University of Gettigen, Germany
*** Culture Center of Algae and Protozoa, England

As a first approximation in identifying the growth medium in the screening procedure, the received cultures were transfered to culture tubes containing 5 ml of artificial sea water (ASW), or enriched sea water (ESW), and incubated on illuminated shelves for 5 days. The medium on which better growth was observed was used for further studies.

Optimal temperature: Culture tubes containing 3 ml of culture were incubated in a temperature gradient block illuminated from below with cool white fluorescent lights. The increase in biomass in each tube were monitored by cell counts and cholorophyll measurments. The responses of three different Chlorella strains to the temperture gradient are shown in Fig. 10.

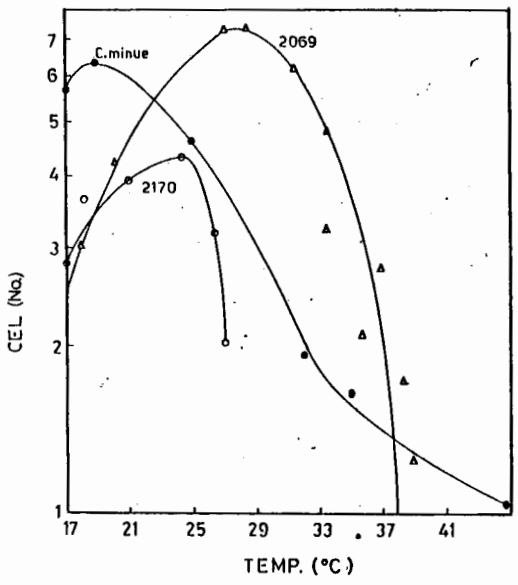


Fig. 10
The response of Chlorella strains to temperature

Strain	Code	Optimal temp.
Minue	= ●–●	19^0
2069	= Δ–Δ	28^0
2170	= o–o	25^0

Growth: Cells were grown in 500 ml erlenmeier flasks containing 100 ml of culture with continuous illumination under controlled temperature. Growth was monitored by daily chlorophyll and turbidity measurements. The growth of three Chlorella strains is shown in Fig. 11.

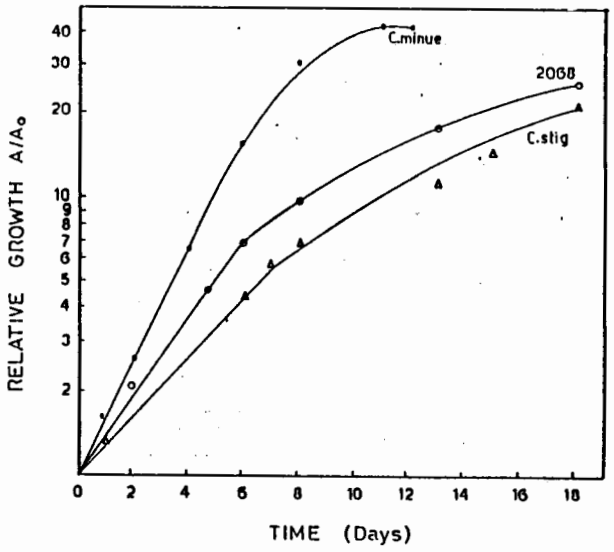


Fig. 11
Growth of three different strains of Chlorella

Nitrogen starvation and lipid content: Cultures grown at their optimal temperature were harvested and divided to two: 1) Control cultures were resuspended in fresh medium; 2) Test cultures were resuspended in nitrogen-free medium and illuminated for seven days. Cells were then harvested for lipid determination.

In Table 11, the lipid content of several Chlorella strains are given. It is evident that none of the strains has a very high level of lipids. Nevertheless, it is our recommendation that during the next year, one or two Chlorella strains with lipid content higher than 25% will be carefully studied under laboratory, as well as outdoor, conditions.

Table 11
The effect of nitrogen starvation on lipid content in Chlorella strains

Chlorella Strain	Lipid Content % of AFDW	
	+N	-N
C. marina	15.4	-
C. salina	15.5	-
C. sp.	16.0	-
C. luteoviridis	17.5	28.8
C. sp. 2068	18.2	27.6
C. stigmatophora	16.2	14.9
C. minutissima	27.4	26.4
C. capsulata	11.7	11.4

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TECHNOLOGY ANALYSIS AND DESIGN

FUEL PRODUCTION OPTIONS FROM AQUATIC SPECIES: TECHNICAL AND ECONOMIC CONSIDERATIONS

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INTRODUCTION

Aquatic species (microalgae and macroalgae) offer significant opportunities for renewable fuel production. Among the qualities that make aquatic species valuable energy feedstocks are high biomass productivities. For microalgae, long-term dry basis productivities of 25 T ac^{-1} yr^{-1} (60 t ha^{-1} yr^{-1}) have been achieved (Benemann et al. 1984), and annual yields up to 46 T ac^{-1} yr^{-1} (110 t ha^{-1} yr^{-1}) have been sustained over a one-month period (Laws 1984). Yields for macroalgae have been reported to be slightly lower: 10-15 T ac^{-1} yr^{-1} (24-36 DAFMT ha^{-1} yr^{-1}) on an ash-free basis (Bird, 1985). Considering the degree of development of this technology, the probability exists that higher yields could be achieved. While macroalgae contain primarily carbohydrates, microalgae have the ability to accumulate large quantities of storage lipids. A lipid content over 60% of ash-free dry weight (AFDW) has been found in some species (Tornabene et al. 1984). Some aquatic species can survive and grow well in waters of moderate to high salinity (waters which usually have low or even negative economic value compared with fresh water). Although macroalgae have lower yields than microalgae, they may also be cultivated with less intensive strategies in existing bodies of water such as lakes, oceans, and estuaries.

Technical and economic evaluations of fuel production options from both microalgae and macroalgae have been performed at the Solar Energy Research Institute (SERI). These evaluations were based on the results of economic analyses of mass culture production of algal feedstocks suitable for conversion to fuel products. This report summarizes the methodology and results of the SERI studies. Feedstock characteristics such as chemical composition, production strategies and economics are presented first. Promising conversion processes and their economics are then discussed. The ultimate potential of the various feedstocks and fuel options can then be assessed based the results of the fuel product cost estimates.

ALGAL FEEDSTOCKS: CHEMISTRY AND PRODUCTION ECONOMICS

In order to select the best algal feedstocks for energy production, as well as the best fuel options for a particular feedstock, an examination of proximate chemistry must first be made. A brief discussion of the strategies for cultivation of aquatic species on the scale required for fuel production is then presented. The Gas Research Institute (GRI) initiated the research and development of two nearshore concepts, while SERI/DOE continued investigations of concepts that cultivated benthic species of macroalgae. The discussion of algal chemistry and morphology provides a basis on which the most appropriate strategies for each algal species can be selected. In general, the variety of nearshore schemes, plus the land-based scheme, are the types which are felt to be applicable for cultivation of macroalgae feedstocks; the other option, offshore

cultivation, was not considered, as it was determined not to be economically favorable (Tompkins 1980). Finally, the resulting feedstock production economics are summarized. Details of these are discussed more fully elsewhere (Feinberg and Hock 1985; Hill 1984; Hill, et al. 1984).

Macroalgae

Macroalgae belong to one of three classes: red, green, or brown. Of the species listed in Table 1, Macrocystis is a large brown algae which grows attached off the coast of California; Sargassum, which grows floating in tropical waters, is brown; Gracilaria (red) and Ulva (green) both grow unattached and in the bottoms of shallow embayments. Kelp (Macrocystis) was originally harvested in the early 1900's for recovery of potash and iodine salts which are major constituents of the ash; sodium, magnesium, and bromine are also present (Jain 1983).

The carbohydrate fraction consists of a variety of components. A major one in the brown algae is mannitol, an alcohol derived from mannose, a hexose (six-carbon) sugar similar to glucose. Another is algin, a copolymer derived from mannose and gulose (another hexose). A major cell wall structural component of the brown algae, algin is converted into various gums used in the food, pharmaceutical, and textile industries. The red macroalgae have the polygalactans (polymers of galactose) agar and carrageenan, which are also recovered and used industrially. Other hexose-derived polysaccharides include fucoidan (from fucose), laminarin (from glucose), and cellulose (from glucose). Tompkins (1981) presents detailed analytical data on carbohydrate content of various algae, while Jain (1983) discusses commercial uses for some of the algal constituents. The fact that the great majority of carbohydrate is ultimately reducible to hexose will be of interest to the examination of ethanol production presented later.

Kelp/Nearshore

Kelp (Macrocystis) is a species of large brown algae that is differentiated into blades, stipes and a holdfast. Extensive natural kelp beds are present off the Coast of California. In the nearshore kelp production concept (Figure 1), juvenile plants would be fastened to anchor lines from tugboat barges. The plants could be cropped several times per year by kelp harvesting boats; the boats might also be capable of shredding and pumping the resulting kelp slurry to a transport barge, from which it would be transported to the fuel production (conversion) facility. Nearshore areas less than 60 feet in depth, out of shipping lanes, and with suitable water currents, would be potentially suitable. Feedstock production costs from this concept would range from a baseline value of $84/DAFMT (assuming current yields of 22 DAFMT ha^{-1} yr^{-1}) to an advanced value of $44/DAFMT (based on yields of 50 DAFMT ha^{-1} yr^{-1}).

Sargassum/floating-adjacent

In this concept, a floating species of macroalgae like Sargassum would be contained by large booms in open areas of the sea. A boom-winch harvestor system (Figure 2) would then be used to collect the biomass. If the area is close enough to the shoreline for the harvestor to be land-based, the concept is referred to as adjacent. Coastal areas off Florida, the Gulf states, California and Hawaii are potentially suitable for this type of macroalgal production. Feedstock production costs range from $54/DAFMT (based on yields of 22 DAFMT ha^{-1} yr^{-1}), to $27/DAFMT (based on yields of 34 DAFMT ha^{-1} yr^{-1}).

Table 1. Macroalgae: Composition and Biochemical Profiles

Species	Macrocystis	Sargassum	Ulva	Gracilaria	
				Baseline	Advanced
Ash (% of TS)	40	38	30	48	37
Volatile (% of TS)	60	62	70	52	63
(% of VS)					
Carbohydrate	80	89	85.7	80	82
Protein	15	8	8.6	14	12
Lipid	5	3	5.7	6	6
Energy Content (GJ/DAFMT)	19.6	18.7	19.3	19.7	19.6
Methane production (SCM/kg VS)	0.34	0.31	0.37	0.37	0.37
Solubles (% of CH_2O)	50	17.5	25	50	68
Fermentables (% of CH_2O)	75	75	75	75	75

[a]To obtain MJ/kg (or GJ/mt) divide Btu/lb by 430.9.

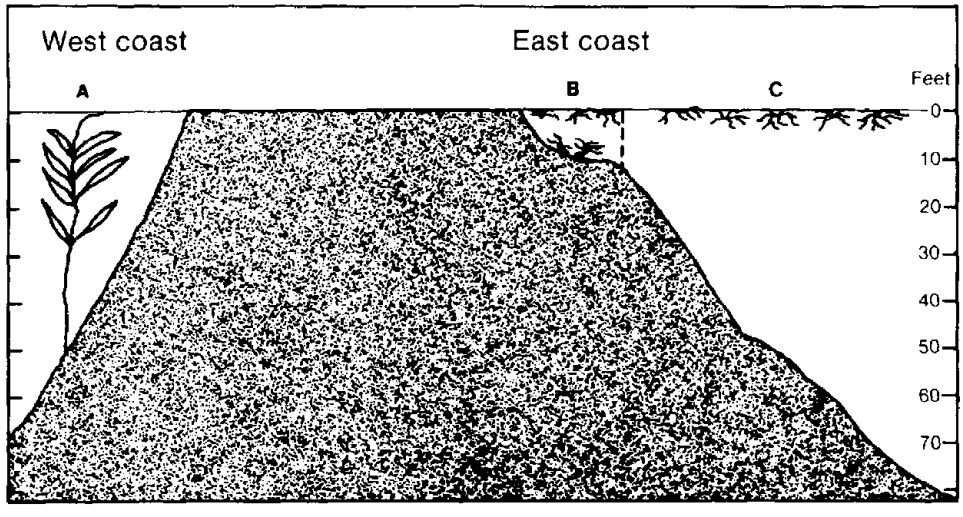
[b]To obtain MJ/L divide Btu/gal by 3,588.

[c]To obtain SCF/lb divide SCM/kg by 0.06.

Sources:
Brehany (1983), Habig, et al. (1983), Habig and Ryther (1984). Ryther (1985), Tompkins (1981).

Table 2. Macroalgae Feedstock Production

Feedstock	Kelp		Sargassum				Gracilaria/Ulva		Gracilaria/Ulva	
Concept	Nearshore		Adjacent		Nonadjacent		Estuarine		Land-Based	
	Base	Advanced	Base	Advanced	Base	Advanced	Base	Advanced	Base	Advanced
Facility Size (ha)	5340	5340	5340	5340	7460	7460	5340	5340	100	100
Annual Yield (DAFMT/ha)	22.4	50.4	22.4	44.8	22.4	44.8	22.4	33.6	54	78
Feedstock Cost (1984 $/DAFMT)	83.64	44.32	48.25	23.95	80.37	40.19	53.92	27.45	226.13	130.98



Production concepts are related to depth and the morphological form that can grow in the habitat.

Area:

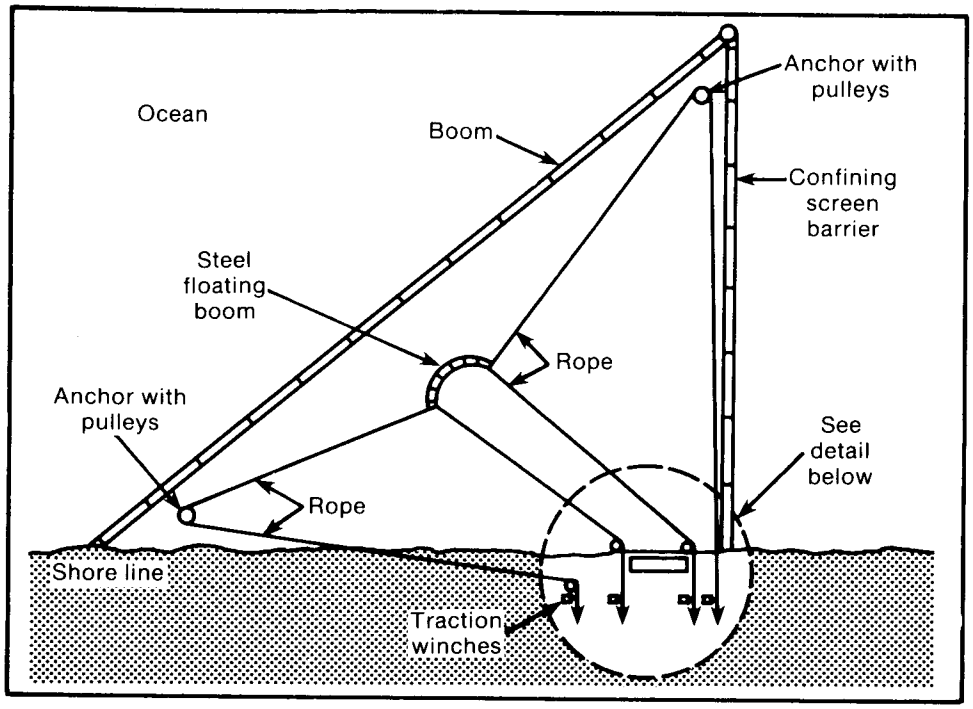
A - *Macrocystis*, an attached form, requires moving water, and suitable substrates of less than 60 feet.

B - The estuarine and adjacent concept, require protected, shallow areas of less than 10 feet for cultivation of non-attached benthic types.

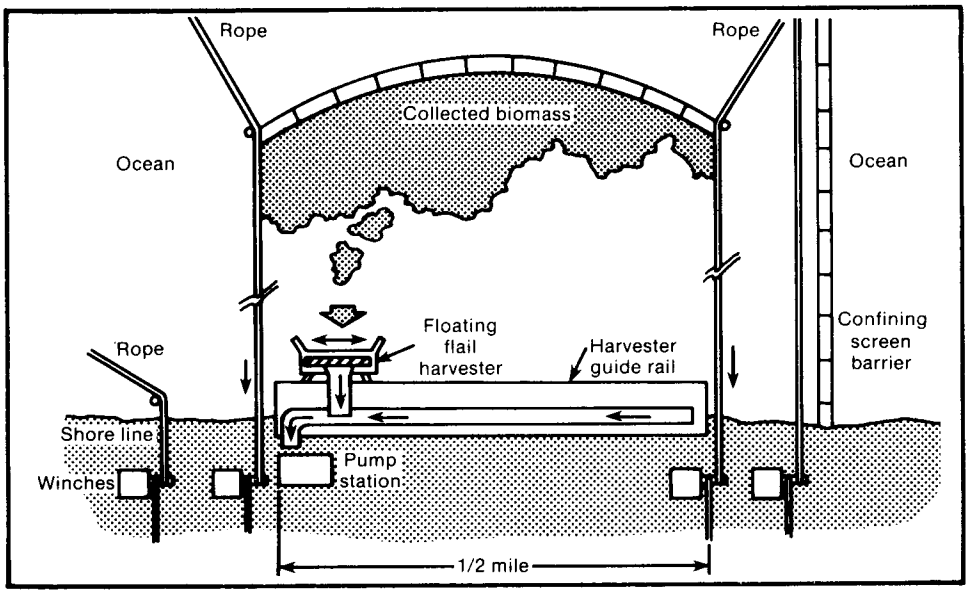
C - The nonadjacent floating concept can occupy open, deep areas of the continental shelf.

Figure 1. Macroalgae Production Concepts

Sargassum



Floating *Sargassum* is confined within a barrier for cultivation.



A boom-winch harvesting system is used to collect the floating biomass.

Source: GRI

Figure 2. Boom-Winch Harvesting System for Cultivation of Sargassum

Sargassum/floating-nonadjacent

If the production area is offshore where the harvestor would have to be located on a platform or barge, then the concept is known as nonadjacent. Such a facility would be larger than the adjacent type, but yields and other factors would remain constant. Because of the greater distances from shore, the capital and operating costs are considerably higher for the nonadjacent system: feedstock production costs vary from $80 to $40/DAFMT, based on identical yields as for the adjacent system.

Bay/estuarine culture

This type of culture would utilize protected areas of water less than 10 feet in depth, similar to shrimp cultivation in Central and South America. Benthic macroalgae such as Gracilaria or Ulva would be cultivated. Seeding would be accomplished by fragmenting and then distributing the thallus into the bay. When the plant biomass reached harvest size, barges would harvest the biomass and return a portion of the thallus as seed for the next production cycle. Potentially, areas in Florida, eastern coastal states, Gulf states and Hawaii would be applicable to this type of production. Production costs for either Gracilaria or Ulva would range from a baseline value of $54/DAFMT (based on a yield of 22 DAFMT ha^{-1} yr^{-1}) to an advanced value of $27/DAFMT (with yields of 34 DAFMT ha^{-1} yr^{-1}).

Land-based culture

This concept would require intensive cultivation in a channel or raceway system, with mixing accomplished either mechanically or through addition of pressurized gas (e.g., carbon dioxide, which is also required as a major nutrient). Intensive algae cultivation thus requires both energy and nutrient inputs, so higher yields would be required for competitive economics. The land-based production concept is applicable to both coastal areas and desert areas with available saline waters. Harvesting can be accomplished by filtration and centrifugation, with efficiencies varying greatly among species. Because of the intensive nature of this cultivation technique, yields are higher than for the nearshore strategies (54-78 DAFMT ha^{-1} yr^{-1}). However, the land-based system would utilize a much smaller facility size (100 ha); capital costs must be spread over much less total biomass production. For this reason, feedstock production costs range from a baseline of $226/DAFMT to an advanced value of $130/DAFMT, much higher than the less intensive culture systems.

Microalgae

Rather than choosing representative microalgal species for examination, a slightly different approach is employed. Based on the compositional variation within a species, it becomes difficult to fix representative compositions. Therefore, this analysis is based on three representative microalgae feedstocks: a current feedstock, and two reseach-improved feedstocks, one a high-lipid and the other a high-carbohydrate feedstock (Table 3). The economics of feedstock production were then estimated for each representative composition.

The algal culture facility is sized at 1000 ha (2500 ac), divided into forty-three 20-ha modules, or ponds. Mixing is accomplished by paddlewheels located at the end of each channel. The harvesting system consists of two stages: a microstrainer followed by a centrifuge. Carbon, the major nutrient, is supplied in the form of gaseous carbon dioxide, which is obtained from power plant flue gas. The CO_2 is scrubbed, compressed, and transported by pipeline to the culture facility. Delivered CO_2 cost is $0.14/$m^3$

Table 3. Microalgae: Composition and Biochemical Profiles

Composition	Reference	High-lipid	High-carbohydrate
Ash (% of TS)	8	8	8
Volatile (% of TS)	92	92	92
(% of VS)			
Carbohydrate	21.7	9.8	70.7
Protein	34.7	14.1	14.1
Lipid	32.6	65.2	9.8
Intermediate	10.9	10.9	10.9
Energy Content (GJ/DAFMT)	24.9	30.1	18.2
Methane production (SCM/kg VS)	0.30	0.49	0.25
Solubles (% of CH_2O)	100	100	100
Fermentables (% of CH_2O)	65	65	65

[a]To obtain MJ/kg (or GJ/mt) divide Btu/lb by 430.9.

[b]To obtain MJ/L divide Btu/gal by 3,588.

[c]To obtain SCF/lb divide SCM/kg by 0.06.

($4.00/10^3$ SCF) for a transport distance of 80 km. For the reference composition at 7% photosynthetic efficiency (based on PAR, the photosynthetically active radiation), CO_2 represents over half of the total feedstock production cost of $470/DAFMT (Table 4). Increases in photosynthetic efficiency and/or carbohydrate content decrease the production costs, while increases in lipid content cause slight increases in production costs.

Besides photosynthetic efficiency and lipid content, other parameters have some affect on feedstock production costs. Several of these other parameters could also be improved through continued research and development. Carbon dioxide cost could be reduced to $0.08/m^3$ ($2.30/10^3$ SCF) by locating the algal facility closer to a coal-fired electric power plant. This could also reduce the cost of electric power from $0.05 to $0.03/kWh. Other nutrient costs could be reduced by as much as 10% to reflect contract prices available to a large user. Higher source water salinity, wider salinity tolerances, and decreased outgassing losses are other parameters for which improved values could be chosen. The algal production costs could be reduced to $242/DAFMT using the high-lipid feedstock (based on a yield of 120 DAFMT ha^{-1} yr^{-1}) or $189/DAFMT using the high-carbohydrate feedstock (based on 200 DAFMT ha^{-1} yr^{-1}). These costs are substantially higher than even the most expensive macroalgae production options. It remains to be seen whether the potentially higher values of the lipid-based fuel products can compensate for these high production costs.

CONVERSION PROCESSES

Processing Options

The objective of the fuel products portion of this analysis is to determine how best to exploit the chemical composition of macro- and microalgae to produce fuels. This section examines a series of options for conversion of aquatic feedstocks. The overall goal is to arrive at an integrated scheme which maximizes the conversion of aquatic biomass to fuel products while maintaining a favorable economic picture.

The economic comparisons presented were developed from simple models and are subject to a good deal of uncertainty. One basic assumption is that the algal refinery is located near the algal culture facility and is sized to match its capacity. In addition, some cases are examined in which the refinery is sized to serve ten culture facilities, thereby realizing significant improvements in conversion costs.

Production of Methane

Through anaerobic digestion essentially all of the aquatic feedstock could be converted into a single fuel product. Methane, the primary constituent of natural gas, is widely used as both a fuel and as a chemical feedstock; natural gas boilers and combustion turbines are in wide use. Since methane is usually a gas and therefore bulky to handle, its use as a transportation fuel has been limited. However, liquefied natural gas (LNG), with increased density, has begun to be used as a transportation fuel.

An anaerobic digester typically contains microbial populations to convert a variety of organic substrates to methane and carbon dioxide. Few problems are expected in the adaptation of macroalgae or microalgae feedstocks to anaerobic digestion. The major technical concern is the continuing development of more efficient digesters. This analysis includes two reactor designs: the plug-flow reactor and the upflow solids reactor (USR). The USR has been developed more recently and might offer better conversion efficiencies and shorter residence times than the plug-flow digester.

Table 4. Microalgae Feedstock Production Options

Composition	Reference			High-Lipid				High-Carbohydrate
Photosynthetic efficiency (%)	7	12	18	7	12	18	18[a]	18[a]
Facility Size (ha)	1000	1000	1000	1000	1000	1000	1000	1000
Annual Yield (DAFMT/ha)	57	97	145	48	80	120	120	200
Feedstock Cost (1984 $/DAFMT)	470	341	273	503	364	284	242	189

[a] Includes other cost improvements (see text).

The primary digester product, usually referred to as biogas, is not pure methane, but contains large amounts (typically 40%) of carbon dioxide, plus trace amounts of water and hydrogen sulfide. The water, sulfur, and nitrogen components must be removed prior to use. Removal of the CO_2 is not required if the gas is to be burned on site, and direct use of this medium-Btu gas is probably cheaper than CO_2 removal in small-scale applications. However, when large volumes of gas are sold to a pipeline company or gas utility, CO_2 removal will be required. In addition, recycle of the CO_2 to land-based culture systems would be essential.

The organic fractions of the algae (all components except ash) are anaerobically digestible, so once the algae has been harvested, little if any pretreatment is required. For macroalgae, shredding or other size reduction may be required. The liquid effluent contains soluble nitrogen from the original algal proteins, which can also be recovered and recycled to the culture.

Production of Ethanol

Ethanol is the highest-volume product produced commercially by biological fermentation. Any carbohydrate source is suitable for conversion to sugars and subsequently to ethanol. Both macroalgae and microalgae will be examined as possible ethanol feedstocks, although macroalgae are probably less expensive.

One advantage microalgae offer over macroalgae is that typically their polysaccharides are in the form of starch, so the sugars are easily accessible for yeast fermentation. The fibrous and colloidal polysaccharides of macroalgae may not be so easily accessible. This analysis was based on the addition of a mild acid hydrolysis step, similar to one used for a lignocellulosic feedstock. First, a filtration or washing step would separate the soluble from the insoluble carbohydrates, after which the insolubles would be hydrolyzed. All sugars could then be treated as solubles and fermented together. Downstream processing would then be identical to the microalgal-based ethanol facility.

The major issues concerning ethanol production from aquatic species are the maximum carbohydrate content that can be achieved and the portion of the carbohydrate that could be fermented to ethanol. In the case of macroalgae, the carbohydrate content does not vary widely among species, but the soluble fraction does (e.g., 20% in Sargassum to almost 70% in some Gracilaria). The other parameter, the fermentable fraction of carbohydrate, can be estimated from the ratio of six-carbon to five-carbon sugars. Very small amounts of five-carbon sugars have been identified in macroalgae--about 75% to 90%. For microalgae, the C_6/C_5 ratio is about 2:1, yielding about 65% fermentable. Essentially all the carbohydrate is soluble and requires no additional hydrolysis.

Lipid-Based Processes

Pseudo Vegetable Oil (PVO). One way to use lipids, which represent the highest energy biochemical fraction available in aquatic species, is to extract them as pseudo vegetable oil (PVO). These refined oils consist of the glycerol esters (triglycerides) of a variety of fatty acids. PVO (and conventional seed oils) typically have lower fuel values and higher boiling ranges than diesel fuel; thus they would require a fuel preheater or precombustion chamber for reliable performance in diesel engines.

As microalgae allocate larger percentages of their cell mass to lipids, larger percentages in turn become storage lipids--those not associated with membranes. At 30% lipid content, about 40% of the total lipids will be in the form of triglyceride (and possibly phospholipid); at 60% lipids, this proportion will be as high as 50%. A modified allocation of carbon might increase the triglyceride fraction of total lipid to 75%.

No chemical reactions are involved in the production of PVO from microalgae. The success of the conversion depends on identification of a suitable solvent to extract a fuel from microalgae cellular lipids that has the desired properties. The lipids not extracted plus the remaining portions of the algae (carbohydrates and protein) may be anaerobically digested to produce methane and carbon dioxide.

Ester Fuel. Another option for utilization of algal lipids is the the conversion of triglycerides to methyl or ethyl fatty esters (transesterification). The transesterification process and its product, called ester fuel, have recently been the subject of extensive investigation as a substitute for petroleum-derived diesel fuels (Freedman and Pryde 1982; Kusy 1982; Clark et al. 1984).

Although the fuel value is about 10% lower than diesel fuel (5% lower than vegetable oils), ester fuel has a significant advantage over PVO in viscosity; it flows much more evenly, especially below 20°C. Ester fuels perform almost as well as No. 2-D in indirect-injection diesels, but not as well in direct-injection engines (Ryan et al. 1984). The distillation curve (boiling range) is between those of diesel and PVO.

The main drawbacks to the use of ester fuel are its tendency toward injector fouling (especially in direct-injection engines) and its relatively high pour point (the point at which it will no longer flow -- about 0°C). Use of ester fuel at or near this temperature would require some fuel or fuel system preheating, although a diesel-ester blend would minimize this problem. Solutions to the fouling problems, either through the development of additives or some modification to the fuels themselves, are also technically feasible as research and development continue.

Conversion from triglyceride to ester fuel is routinely achieved at 98%, and the process is slightly more complex than the conversion to PVO. The esterification reaction is accomplished by adding excess alcohol (generally double the stoichiometric minimum) plus an alkaline catalyst. One mole of glycerol is produced for each 3 moles of esters; the glycerol can be recovered and then purified for substantially less than its current market value of over 70¢/lb ($1540/t), thus providing an additional source of revenue for the production facility.

Catalytic Conversion. Mobil Research and Development Corporation has developed a thermal-catalytic process that converts methanol to light hydrocarbons in the gasoline range (Voltz et al. 1976). At a less advanced stage of development is the use of feedstocks similar to microalgal lipids (e. g., corn oil). The process itself is basically a catalytic reduction; most of the initial oxygen is removed to produce hydrocarbons, which, unlike ester fuel, are direct substitutes for petroleum-derived products. As in the other integrated facilities examined, lipid extraction precedes the main process and anaerobic digestion follows it. The efficiencies of lipid utilization used here are preliminary and depend on process optimization reactor studies; they could include more lipids than just the triglyceride component. In any case, algal lipids appear to be good candidates for catalytic conversion to high-value liquid fuels.

Conversion Process Economics

Cost goals were developed by the Department of Energy to compare the values of fuel products. The cost goal is the amount equal to the cost of the competing nonrenewable fuel product at a specified time. The cost goals for each fuel product are based on a series of energy price projections developed by DOE's Office of Policy, Planning and Analysis (1983) in support of the National Energy Policy Plan (NEPP). Based on the state of development of the technologies evaluated in this study, the time chosen for macro-

algae-derived fuel products is the year 1995, and for microalgae-derived products is the year 2000. The high- and low-range projections are presented in Table 5. The low-range projection represents essentially no net increase in crude oil price through 1995, while the high-range projection represents a doubling by 1995 and a net 170% increase by 2000.

Normalized costs are used to compare fuel production options. They are defined as estimated fuel production costs divided by the high cost goal (Table 5). Thus, an algae-derived fuel product with a cost goal below one is considered to have met its cost goal.

Macroalgae Production Options

The fuel production options for macroalgae are shown in Table 6; normalized costs are plotted in Figure 3). With the advanced strategy and kelp as a feedstock, 2.5×10^{15} J/yr of methane could be produced at a cost of $7.00/GJ. With the achievement of a proximate chemistry optimized for ethanol production (60% solubles with 90% fermentables), the ethanol cost would be $2.00/gal in the baseline case and $1.30 in the advanced case.

The yield from Sargassum grown under the adjacent production strategy is similar to the kelp baseline case, but the cost is substantially lower than for kelp. Lower methane costs are primarily the result of the lower feedstock cost: the lower digestibility of Sargassum is compensated by the lower feed cost. In the advanced case, with a doubled productivity, methane cost decreases to $4.80/GJ. The nonadjacent cultivation strategy for Sargassum uses higher production facility sizes, resulting in higher system yields but also incurring higher costs.

Of the macroalgae that can be cultivated using the estuarine techniques, Ulva can be used to produce up to 1.3×10^{15} J/yr of methane at a cost of $7.20/GJ from baseline strategy, while 1.9×10^{15} J/yr could be produced in the most advanced case at $4.60/GJ (Table 6). These are the lowest estimated methane costs in this study, primarily because of the higher digestibility of this species. Ethanol costs are among the lowest, but higher hydrolysis costs are required because of the 25% solubles concentration.

Methane production yields and costs are estimated to be equal for Gracilaria and Ulva grown under the estuarine production strategy (Table 6). However, Gracilaria has much higher solubles content, so that, although total carbohydrate is lower, ethanol yield is higher. Coupling the highest feedstock production yield with the highest fermentables content gives an ethanol production of 15.8 million gal/yr with a cost of $1.30/gal. If further improvement of Gracilaria's proximate chemistry could be achieved, the ethanol production potential would be further enhanced. The land-based option for Gracilaria was shown to be the most expensive option for macroalgae cultivation; Table 5 shows that this option is not economically feasible for fuel production, even with the most advanced production strategy.

Microalgae Production Options

Table 7 presents the fuel production options for microalgae, including the high-lipid feedstock and the high-carbohydrate feedstock. Normalized costs are shown in Figure 4. Since the reference feedstock contained 30% lipid and 20% carbohydrate, it was marginally acceptable for all options but not outstanding for any of them. Shown on Table 7 in order of increasing photosynthetic efficiency, methane production ranges from $31 to $17/GJ; the lipid-based diesel fuel substitutes are $6/gal ($50/GJ) at 18% photosynthetic efficiency. The gasoline product has a slightly higher cost ($6.30/gal), but on an energy basis it is slightly less expensive ($48/GJ). The lowest cost for ethanol from this feedstock would be $10.20/gal ($116/GJ).

Table 5. Fuel Cost Goals

YEAR	1995		2000	
	LOW	HIGH	LOW	HIGH
World Oil Price ($/BBL)	30.30	59.50	36.00	80.30
($/GJ)[1]	4.95	9.73	5.88	13.13
Natural Gas ($/GJ) Industrial Sector	4.40	7.08	5.26	10.40
Gasoline (Alcohols) ($/GJ)	10.27	15.54	11.31	19.30
($/gal)[1]	1.35	2.05	1.49	2.55
Ethanol ($/gal)[1]	0.91	1.38	1.00	1.71
Diesel (No. 2) ($/GJ)	7.24	12.99	8.38	17.09
($/gal)[1]	1.07	1.92	1.24	2.52
PVO/Ester fuel ($/gal)[1]	0.96	1.73	1.13	2.27

Notes:

1 Volumetric heating values are higher (gross) heating valves, per NEPP IV convention:
Crude Oil = 5.8 10^6 Btu/BBL
Gasoline = 125,000 Btu/gal
Ethanol = 84,000 Btu/gal
No. 2 Diesel = 140,000 Btu/gal
PVO/Ester fuel = 126,000 Btu/gal

2 Normalized cost = $\frac{\text{Estimated Fuel Production Cost}}{\text{Cost goal (High)}}$

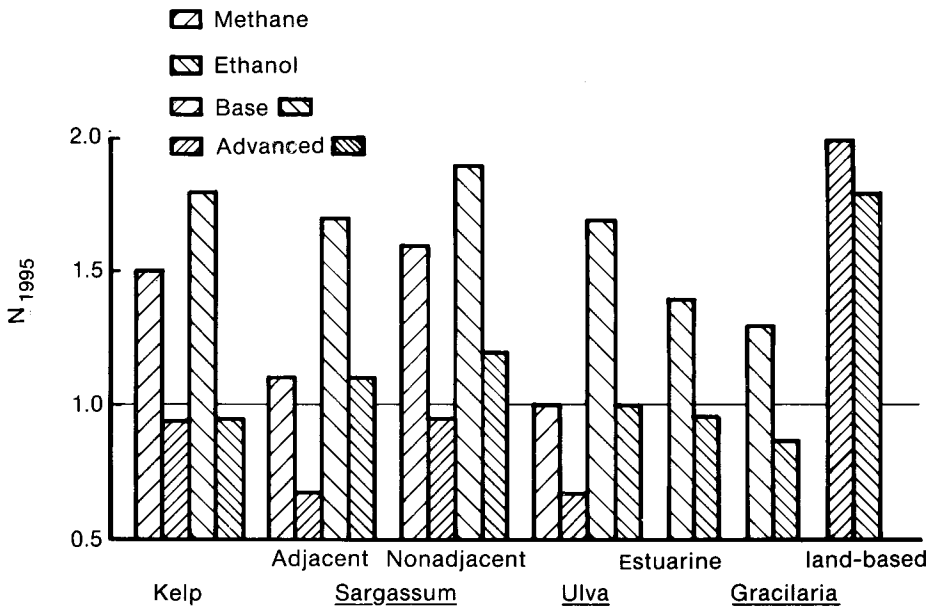
3 SI Conversions:
To obtain MJ/kg (or GJ/t) divide Btu/lb by 430.9.
To obtain MJ/L divide Btu/gal by 3,588.
To obtain $/GJ divide $/$10^6$ Btu by 1.055.

Source: Department of Energy 1983.

Table 6. Macroalgae Fuel Production Options

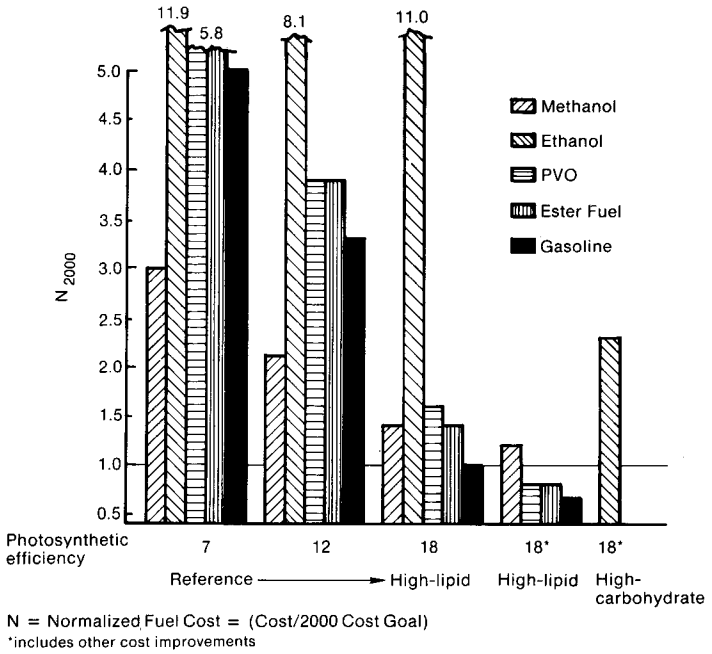
Concept	Kelp		Sargassum				Ulva		Gracilaria				Gracilaria
	Nearshore		Adjacent		Nonadjacent		Estuarine		Estuarine				Land-Based
									Baseline Composition		Improved Composition		Improved Composition
METHANE	Base	Advanced	Base	Advanced	Base	Advanced	Base	Advanced	Base	Advanced	Base	Advanced	Advanced[a]
10^{15} J/yr	1.2	2.6	1.1	2.1	1.5	2.9	1.3	1.9	1.3	1.9	1.3	1.9	0.75
Methane cost, $/GJ	10.70	6.60	7.60	4.80	11.20	6.60	7.20	4.60	7.20	4.60	7.20	4.6	14.30
Normalized cost	1.5	0.94	1.1	0.67	1.6	0.94	1.0	0.66	1.0	0.66	1.0	0.66	2.0
ETHANOL													
10^6 gal/yr	8.8	24.5	8.8	21.0	12.2	29.3	8.4	16.9	8.8	15.8	9.6	17.3	7.5
Ethanol cost, $/gal	2.40	1.30	2.40	1.60	2.70	1.70	2.40	1.40	2.00	1.30	1.75	1.20	2.50
$/GJ	27.5	15.6	26.60	17.70	30.00	18.80	26.80	15.70	22.20	15.00	19.80	13.40	28.30
Normalized cost	1.8	0.95	1.7	1.1	1.9	1.2	1.7	1.0	1.4	0.96	1.3	0.87	1.8

[a] Optimized scale: Conversion facility size = 10 x feedstock production facility



N = Normalized Fuel Cost = (Cost/1995 Cost Goal)

Figure 3. Macroalgae Fuel Product Normalized Costs



N = Normalized Fuel Cost = (Cost/2000 Cost Goal)
*includes other cost improvements

Figure 4. Microalgae Fuel Product Normalized Costs

Table 7. Microalgae Fuel Production Options

Composition	Reference			High-lipid			High-carbohydrate	
Photosynthetic efficiency (%)	7	12	18	7	12	18	18[a]	18[a]
METHANE								
10^{15} J/yr	0.9	1.4	2.1	0.9	1.5	2.2	0.2	0.2
Methane cost, $/GJ	31.30	21.80	17.20	25.00	18.00	14.00	12.70	26.10
Normalized cost	3.0	2.1	1.6	2.4	1.7	1.4	1.2	2.4
ETHANOL								
10^6 gal/yr	1.2	2.0	3.0	-	-	1.3	-	13.3
Ethanol cost, $/gal	20.30	13.80	10.20	-	-	18.70	-	4.00
$/GJ	230	155	116	-	-	211	-	45.10
Normalized cost	11.8	8.0	6.0	-	-	11.0	-	2.3
PVO								
Lipid available (% of lipid)	40	40	40	50	50	50	75	-
10^6 gal/yr	2.1	3.6	5.4	4.4	7.5	11.2	168	-
PVO cost, $/gal	11.80	7.90	6.05	5.60	4.00	3.10	2.05	-
$/GJ	89	59	45	42	30	23	15.40	-
Normalized cost	5.2	3.5	2.7	2.5	1.8	1.4	.90	-
ESTER FUEL								
Lipid available (% of lipid)	40	40	40	50	50	50	75	-
10^6 gal/yr	2.1	3.5	5.3	4.3	7.3	10.9	163	-
Ester fuel cost, $/gal	11.90	8.10	6.05	5.70	4.00	3.00	1.60	-
$/GJ	90	61	46	43	30	22	12.30	-
Normalized cost	5.2	3.6	2.7	2.5	1.8	1.3	.72	-
GASOLINE								
Lipid available (% of lipid)	50	50	50	80	80	80	90	-
10^6 gal/yr	1.9	3.2	4.8	4.2	7.1	10.6	120	-
Gasoline cost, $/gal	12.60	8.40	6.30	5.30	3.60	2.60	1.70	-
$/GJ	96	64	48	40	27	20	13.00	-
Normalized cost	5.0	3.3	2.5	2.1	1.4	1.0	.68	-

[a] Includes other cost improvements (see text)

The situation improves considerably for the high-lipid feedstock (Table 7). Even at the lowest photosynthetic efficiency, lipid-based liquid fuels are less expensive than the highest-efficiency cases using the reference feedstock. The methane and ethanol options are presented primarily for comparison with the reference cases. Methane costs are slightly lower than for the reference case, but the difference between methane and the liquid fuels is now much smaller than before. That the ethanol costs are higher should not be surprising, since the high-lipid feedstock is also a low-carbohydrate feedstock. Even in the most advanced case 13.3 million gal/yr of ethanol could be produced for $4.00/gal from high-carbohydrate feedstock. It is clear that microalgae will not be good feedstocks for ethanol production.

One important caveat to the discussion of algae-derived ester fuel is the sustainability of the market for by-product glycerol. In the reference case, the glycerol production is 760 t/yr, but in the advanced case it increases to 60,000 t/yr, which is 40%-50% of the current domestic market (International Trade Commission 1982). Clearly, a new 60,000-t glycerol facility would have a dramatic impact on the market price. The very first such glycerol-from-microalgae facility would be able to claim a credit of $1540/t by displacing the most expensive synthetic production, but it is doubtful that subsequent facilities could sustain a credit that large. The result is a negative effect on the economics of ester fuel from microalgae: each 10¢/lb ($220/t) decrease in the glycerol credit will further increase the ester fuel cost by $0.10/gal. A credit of only $880/t ($0.40/lb) thus increases the ester fuel cost $0.30 to $1.90/gal. Smaller facilities that produce 4000 t/yr or less should not suffer any adverse affects, at least for the first 10-20 facilities.

The cases which are consistently the best for microalgae are those which exploit high-lipid content to produce liquid fuels. For macroalgae, any fuel production schemes which exploit the low cost of the macroalgae feedstock are suitable for further development.

CONCLUSIONS

Both microalgae and macroalgae are unique feedstocks that could be competitive sources of renewable liquid or gaseous fuels by the early 21st century. Their most important attributes to be exploited are their potentially high biomass productivities, plus the higher lipid yields of microalgae.

Even at this preliminary stage of evaluation, two processes have been identified for each type of algae that offer potential for commercial development. Macroalgae could be anaerobically digested to produce methane, or their large store of carbohydrates could be aerobically fermented to produce ethyl alcohol. The two major fuel products which could be produced from microalgae are catalytically produced gasoline and ester fuel, an oxygenated diesel fuel substitute. Economic success of the transesterification process depends to a significant degree on the utilization of by-product glycerol, which could be produced in sufficiently large quantities to affect its market structure. There are no such problems with the other processes examined, since all major by-products are either fuels themselves (with no assumed upper market limit) or are returned directly to the algal culture facility.

The most promising fuel production option overall is anaerobic digestion of macroalgae to methane. Although none of the baseline production cases meet the methane cost goal, all the advanced cases do meet this goal; the 50-100% yield improvements required for success are potentially achievable in the time frame considered. The liquid fuel option from macroalgae, ethanol production, is successful only when solubles content as well as

yields are extremely favorable. The land-based option is clearly the least favorable of the macroalgae fuel options.

Examination of the microalgae results showed that the requirements for competitive fuel production requires high-lipid feedstocks (60% of dry weight) plus substantial improvements in photosynthetic efficiency (to 18% of PAR). Neither microalgal mass culture technology nor the processes for conversion of microalgae to high energy liquid fuels are in a mature stage of development. Both require substantial continued research efforts before commercialization can occur.

The technology improvements required for commercialization of macroalgal fuel production options are not as great as those required for commercialization of microalgae. In either case, achievement of cost-competitive renewable fuels from aquatic species requires research directed at species improvement and development of optimized, integrated conversion processes.

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DESIGN, FABRICATION, AND OPERATION OF INNOVATIVE MICROALGAE CULTURE EXPERIMENTS FOR THE PURPOSE OF PRODUCING FUELS

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Abstract

A conceptual design was developed for a 1000-acre (water surface) algae culture facility for the production of fuels. The system is modeled after the shallow raceway system with mixing foils that is now being operated at the University of Hawaii. The facility takes advantage of the high yield and high cell density achieved in the shallow raceway system, as well as the unusual settling behavior of the proposed alga, Platymonas. A computer economic model was created to calculate the discounted breakeven price of algae or fuels produced by the culture facility. A sensitivity analysis was done to estimate the impact of changes in important biological, engineering, and financial parameters on product price.

In the baseline case for a facility in Hawaii, the discounted breakeven algae price is $366/MT dry weight ($398/MT AFDW) in the form of a 10% solids slurry. A case using more optimistic, but probably achievable, parameters reduced the price to $229/MT dry weight. The discounted breakeven price of methane produced from the algal slurry is $36/MSCF for the baseline case and $23/MSCF for the more optimistic case. These prices are probably too high for the facility to be viable on methane sales alone. A case in which credits are taken for shellfish grown on unharvestable algae in the facility's effluent demonstrated that byproduct sales can make a very substantial contribution to the economics of a large algae culture system. The discounted breakeven price for algae was $61/MT dry weight after credit was taken for shellfish sales.

A similar conceptual facility near the Salton Sea in California was also modeled. Algae from the California facility was less expensive than from the Hawaii facility because of geological CO_2 available at the Salton Sea site. Methane produced at the California facility would have a price similar to that in Hawaii because of the additional heating requirements for a digester in California.

A scaled experiment was proposed to test the concept of the large shallow-raceway culture facility. The experiment would have two stages: one to optimize raceway design, the second to test production, costs, labor, and maintenance requirements in raceways close to full production scale. Concurrent experiments at a New Mexico site would screen species to find ones appropriate for a U.S. Southwest version of the shallow raceway system.

Introduction

Beginning with the "oil crisis" of the 1970's, microalgae have been under study as a source of renewable fuels. Microalgae in culture frequently have a higher efficiency of solar energy conversion than land plants. The ability of many microalgae to store a substantial proportion of cell mass as lipids has sparked interest in algae culture to replace liquid fuels, which are expected to become scarcer and more expensive.

In Hawaii, research on fuel production from algae began with Raymond (1978), who arrived at optimistic projections for oil production based on a small experimental system. Since 1980, SERI has funded a University of Hawaii study using a single 48 m^2 raceway and several smaller raceways to investigate the potential of a shallow raceway system. Distinctive features of this system besides the depth include high phytoplankton cell densities (>10^7 cells/ml), high water velocity (30 cm/sec), and mixing foil structures which create vortices in the flowing water. In dense cultures, the vortices expose algal cells to rapidly alternating light and dark periods, creating a "flashing light effect" that increases photosynthetic efficiency (Laws et al., 1983). The last year's research has increased production in the shallow raceway by the use of species adapted to the relatively high temperatures (30-35^{o}C) in the Hawaiian raceway and the discovery that the dilution schedule has a strong effect on algal production. The shallow raceway now achieves yields substantially higher than those attained in other systems using inorganic nutrients (Laws, 1984).

In 1983 SERI issued a Solicitation for Letter of Interest to design a large-scale algae culture system for fuel production, and to propose a scaled experiment to validate the proposed design. Aquaculture Associates, Inc. (AAI) responded with a proposal based on the University of Hawaii raceway system. In April 1984 AAI was awarded one of three competitive design contracts. This report is a summary of the work carried out under Phase I of that contract.

Objectives

The overall objective of the project, as stated by SERI, was to develop a cost-effective design for a saline-water microalgae culture facility producing lipid fuels in the U.S. Southwest. Specific objectives of the subcontract were to:

1. Develop a conceptualized design for a large-scale raceway system at a site in Hawaii.

2. Develop a detailed design of an experimental system that would be appropriate for the Hawaiian site.

3. Develop an operating plan for the experimental facility.

4. Extrapolate the results of the Hawaiian facility to a site in the American Southwest.

Shortly after the project contracts were awarded, the requirement that algal lipids be the fuel source was relaxed, and consideration of any fuel product was permitted. It was also agreed that each contractor would report the price to produce an algal slurry of 10% solids content in order to allow comparisons among systems that would produce different fuel products.

Preliminary Analysis and Species Selection

Before a baseline algae farm design could be attempted, it was necessary to determine which components of the farm were the most important cost drivers. Engineering aspects of a shallow raceway system with foils were studied to discover potential limitations on raceway length, width, and depth and to model water circulation costs as a function of raceway design. Flume studies were done to estimate what foil spacing might be adequate to produce the desired turbulence at minimum cost. Choices had to be made among different algal species, harvesting methods, and processing options.

Only four or five algal species have been cultured in the experimental shallow raceway system because it is a relatively new system. Of the algae that have been tried, two have been grown successfully at high production rates: Platymonas sp., a motile green alga, and Chaetoceros gracilis, a diatom. Both species have exhibited sustained production rates exceeding 40 g AFDW/m^2-day in the Hawaii experimental raceway (Laws, 1984; SERI Biomass Program Monthly Report, October 1984). Platymonas was chosen over Chaetoceros because it appeared to have the best combination of desirable traits:

1. Platymonas has exhibited slightly higher production rates in the experimental raceway.

2. Platymonas is a dominant species, resistant to competitors and predators in the Hawaii shallow raceway system.

3. Chaetoceros requires silicate and vitamin additions (Laws, 1984); Platymonas does not.

4. Platymonas apparently can be harvested simply and with little energy expenditure because of its settling behavior (see below); Chaetoceros does not appear to settle rapidly and would require more expensive means of harvesting.

5. Under optimal culture conditions as defined so far in the experimental raceway, Platymonas requires less total water exchange than Chaetoceros (Laws, 1984; SERI Biomass Program Monthly Report, October 1984).

The major disadvantage of Platymonas is that its storage product is carbohydrate rather than lipid. To date, the SERI fuels-from-microalgae program has focused strongly on lipid-derived fuels because of their relatively high value. However, when microalgae are growing rapidly (not nutrient-limited) they generally store little lipid (Goldman, 1980); both Platymonas and Chaetoceros in the shallow raceway system contain about 15-20% lipid. In order to induce microalgae to produce large amounts of storage lipids, it has been necessary to expose them to nutrient limitation for periods of several days. During this period, algal growth slows down; because of the growth slowdown, total energy production usually declines even though the energy content of the cells themselves rises (Lien and Spencer, 1984). It was decided to accept Platymonas because of its high rate of total energy fixation.

Harvest Experiments

Because of the small size of microalgal cells, harvesting has always been one of the major issues in algal mass culture technology (Mohn, 1980). A variety of harvest techniques has been tried over the past 30 to 40 years (e.g. Mohn, 1980; Shelef et al., 1984); most have proved inefficient or too expensive and energy-intensive for all but the most valuable algal products. Harvestability is therefore a major criterion for selection of an algal species for mass culture, although in practice species have usually been selected for their growth rate, production of valuable compounds, or resistance to competition and predators.

One of the reasons Platymonas was selected as the species to be cultured in the conceptual facility is its reported settling behavior in the experimental raceway in Hawaii. Technical personnel reported that Platymonas cells settle rapidly in flasks, and must be resuspended so that the cells can be counted. In addition, if a power failure occurs and raceway circulation stops for several hours, the cells settle to the bottom of the raceway (L. Pang, pers. comm.). Walne (1970) reported that species of Tetraselmis (= Platymonas) settled rapidly when placed in containers, but regained their swimming behavior in about 24 hours.

Settling experiments with Platymonas were conducted in Erlenmeyer flasks. Cell counts showed that 80-90% of Platymonas cells settled to the bottom within 24 hours. To confirm the harvestability of cultured Platymonas on a larger scale, a 55-gallon drum with one end removed was used as a settling tank. The tank was filled with a suspension of Platymonas (approx.10^7 cells/ml) harvested from the experimental raceway as a part of normal raceway operations. Approximately 24 hours later, samples were taken from the top, center, and near the bottom of the water column for cell counts. Then the water was drained slowly from the drum. When as much water as possible had been drained, the drum was tilted slightly to allow remaining water to

run off the viscous sludge at the bottom of the drum. A sample of the sludge was scraped from the bottom and weighed, then dried to determine solids content.

Two settling drum experiments were done. In the first trial, approximately 82% of the cells originally present in the harvest water settled to the bottom. The solids content of the harvested sludge (after subtracting the presumed weight of the salt present) was a surprisingly high 13.5%. In the second trial, approximately 90% of the cells settled out and the sludge solids content was 7.1%. The supernatant water from this trial had a pH of 7.3 at the end of the 24-hour period, suggesting that high-pH induction of flocculation (Arad et al., 1980) is not critical to the settling process.

Benemann et al. (1980) reported that under certain culture conditions, rapid bioflocculation occurs in Micractinium with similar settling time and removal efficiency to the settling exhibited by Platymonas in the recent experiment. However, the solids concentration of the settled Platymonas seems to be much higher than that of Micractinium. Electron micrographs indicated that Micractinium aggregates by means of extracellular filaments; micrographs of settled Platymonas might reveal whether or not its settling mechanism is similar. Koopman et al. (1980) suggest that bioflocculation is encouraged by high pH and low nitrogen levels, but these conditions are not normally present in the Hawaii experimental raceway.

In summary, Platymonas exhibits rapid settling which, in a properly designed settling tank or pond, may allow harvesting at minimal energy cost. Harvest efficiencies of 80-90% and solids concentrations exceeding 7% may be achieved with a 24-hour settling period. These few experiments do not demonstrate that rapid settling of Platymonas occurs consistently under all culture conditions, or that settling occurs as rapidly and efficiently in a large settling pond as in smaller containers. Experiments to answer these questions were proposed for the scaled experiment.

The Hawaii Conceptual Facility

The site selected for the Hawaii-based conceptual design is located near the southwest corner of the island of Oahu, west of Pearl Harbor, on land owned by the Campbell Estate and leased by the Oahu Sugar Company. Figure 1 illustrates the overall layout of the commercial facility design on the Oahu Sugar site. Seawater wells are located as close as possible to the seashore, limited only by the State shoreline setback line which prohibits construction directly on the ocean front. The coral caprock which can be found 2 to 10 feet below the soil surface in this area is reported to have very good communication with the sea. The Honolulu-based well drilling company, Roscoe Moss, Inc., reported that there should be little difficulty in obtaining 35-40 million gallons per minute from seven or eight 100-foot deep

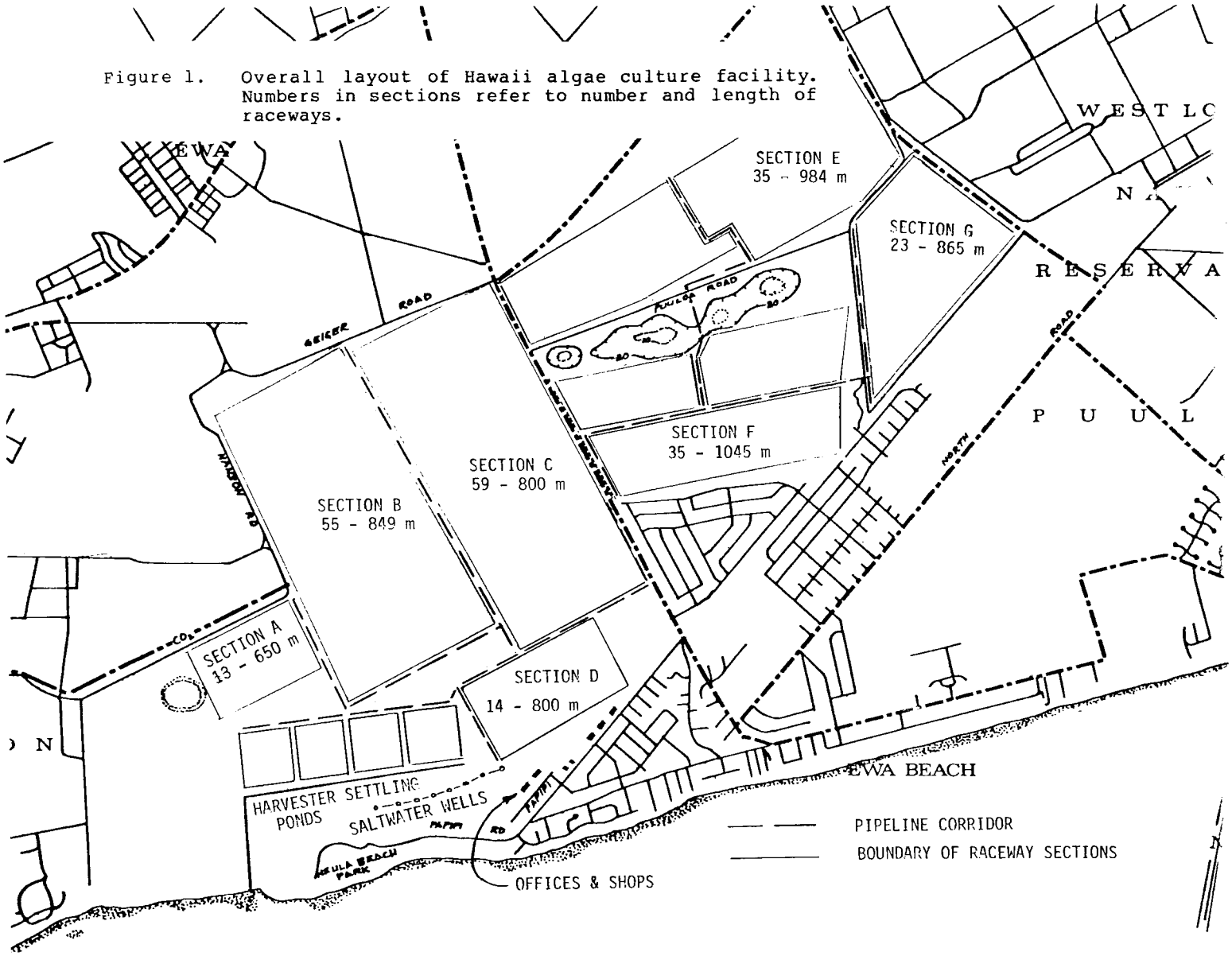

Figure 1. Overall layout of Hawaii algae culture facility. Numbers in sections refer to number and length of raceways.

by 20-inch diameter wells drilled in this area. Water is pumped from these wells through pipe corridors established between the production raceways. The facility harvesting system is also located as close as possible to the waterfront. This would enable a gravity feed, open channel flow system to transport the water with its suspended biomass from the raceways to the harvesters. This network of open channels runs parallel to the feed water pipes in the pipe corridor shown. Following removal of the biomass from the water, the effluent is discharged through another open channel leading directly to the ocean.

CO_2 is supplied by a pipeline that originates at the Hawaiian Independent Refinery, Inc. (HIRI), a subsidiary of Pacific Resources, Inc. (PRI). The refinery is located at Campbell Industrial Park, just west of the Barbers Point Naval Air Station. The refinery produces CO_2 as a byproduct of its oil refining and SNG production operations. Nearly pure (>98%) CO_2 would be purchased from HIRI and transported through a pipeline which traverses the Barbers Point Naval Air Station and then winds throughout the production facility, along the pipe corridors.

A separate set of pipes supplies non-carbon nutrients to the raceways. Nutrient mixing takes place near the seaward side of the facility because of its proximity to the water supply wells and the main buildings.

Harvesting is accomplished in four 286-m square settling ponds, taking advantage of the settling behavior of Platymonas. Each pond can hold one day's harvest water; three ponds can handle normal facility production but a fourth pond is provided as a backup. The algal cells are allowed to settle for 24 hours; the pond is drained in 6 hours. The algal slurry at the bottom is removed by suction into tank trucks which transport the biomass to the processing facility.

The raceways themselves are laid out approximately parallel to the elevation contours, and obvious physical landmarks such as large holes and borrow pits are avoided. All the facility offices, storehouses and employee space are located along Papipi Road just on the outskirts of Ewa Beach township. Major roadways such as Fort Weaver Road, Geiger Road and Puuloa Road would not be disturbed by the facility.

The facility has 234 raceways with a total effective culture area of 409 ha. Roads take up another 130 ha. Because of the irregularity of the site boundaries and the presence of areas unsuitable for raceway construction, another 143 ha are left open. Some of this area can be used for processing facilities and effluent utilization.

The basic characteristics of the raceway designed for the Hawaii commercial facility are shown in Figure 2 and listed below:

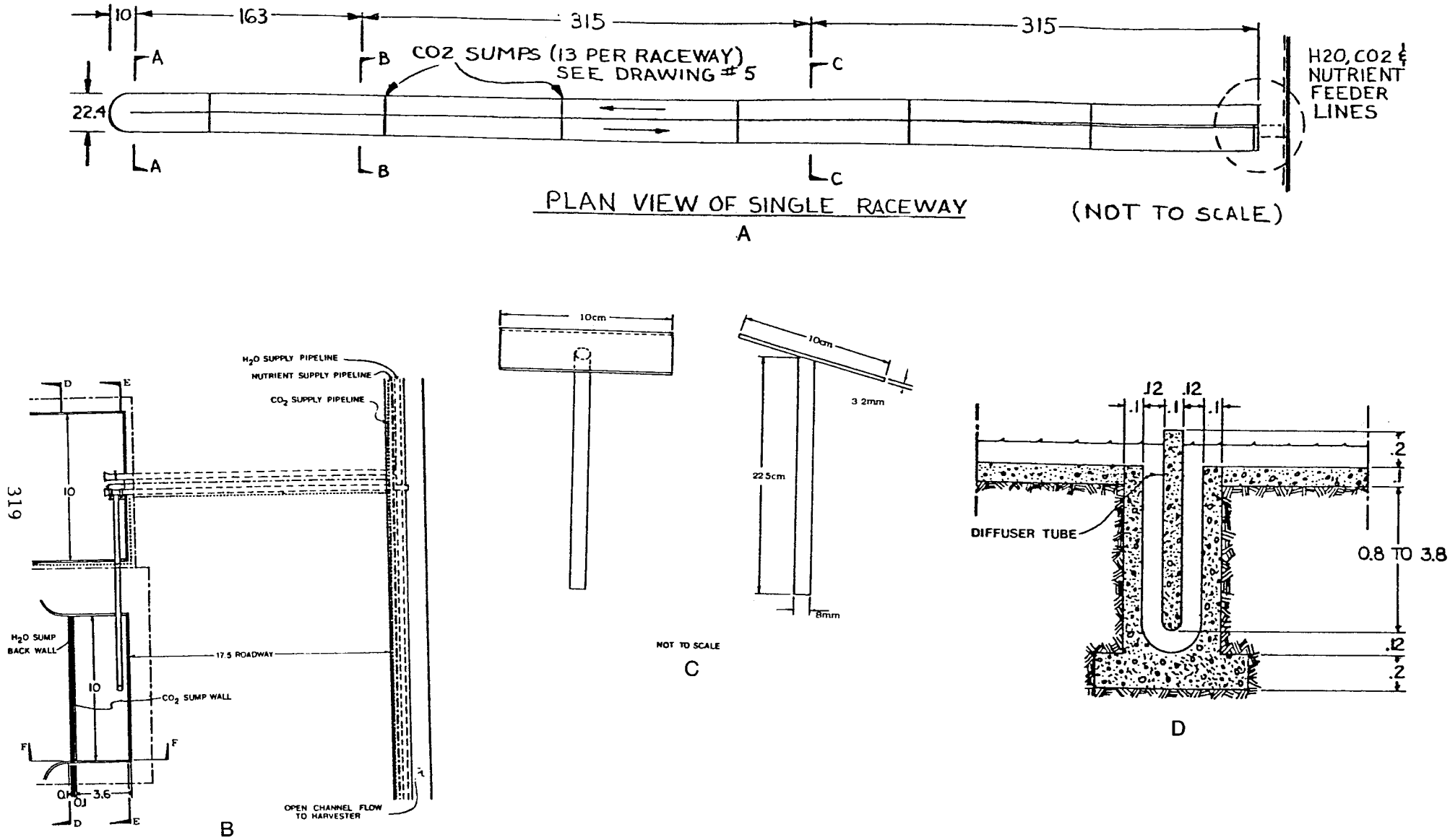

Figure 2. Characteristics of conceptual raceway design. (A) Overall raceway configuration. (B) Overhead view of pump sump, showing pump, supply pipes, and harvest channel. (C) Mixing foil design. (D) CO_2 delivery sump. Dimensions in meters unless otherwise noted.

1. The raceway channel width is 10 m and the depth is 12 cm.

2. Raceway length is variable from 650 to 1400 m depending on site topography and boundaries; 800 m is used for design purposes.

3. The raceway slope is 0.08%.

4. Raceway channels are constructed from earthen berms which are a minimum of 24 cm high over the first 900 running meters of the raceway. Over the last 700 meters these berms gradually increase in height to provide a water catchment area at the sump end of the raceway in the case of a pump failure or an electrical blackout.

5. The raceways are lined with 4" of lime-stabilized soil. The earthen berms are covered with 2" of lime-stabilized soil.

6. Mixing foils (10 cm square plates set at a 20^{o} angle) are placed 20 cm on center with respect to the raceway width and 3 m on center with respect to the raceway length.

7. Water circulation is accomplished by pumping with a 720-rpm wet pit propeller pump located in a pumping sump at one end of the raceway.

8. Raceway water supply, nutrient supply, and harvest drain are all located at the same end of the raceway as the pumping sump.

9. CO_2 distribution sumps are constructed every 124 m along the running length of the raceway.

10. A 5 m wide graded earth roadway runs the full length of the raceway on either side.

Economic Model Development

A spreadsheet-based computer model was developed to test the feasibility of the conceptual facility. The model combines biological, engineering, environmental, and cost parameters to calculate capital and operating costs for the conceptual facility design. The financial section of the model takes these costs and calculates the discounted breakeven price of the algae (or fuel product) as described below. The model is designed to be flexible; the spreadsheet design accepts parametric changes easily.

The financial model is designed to solve for a unit algae price ($/metric ton) that will be charged for all algae produced during the project lifetime. The calculated price affords just

enough revenue so that the present value of the stream of revenue inflows exactly equals the present value of all cost outflows, including costs associated with borrowed and equity capital. Alternatively stated, the price is just sufficient to keep the present value of net cash flow at zero when the discount rate equals the weighted cost of equity capital. Costs, including opportunity costs of capital, are just covered by revenue inflows and zero excess profit is generated. Furthermore, at this price, the internal rate of return exactly equals the weighted cost of equity capital. This price is called the discounted breakeven price.

Baseline Algae Cost

The cost of algae grown in the baseline culture facility is estimated to be $366/MT dry weight ($398/MT AFDW) on a net yearly production of 58,939 MT dry weight (54,224 MT AFDW). These cost calculations assume that the algal raceway and harvest experiments can be scaled up successfully to production scale, but that few changes are made to the system. The cost of the raceways dominates the facility capital cost, with the settling ponds next in magnitude (Figure 3A). Total facility capital cost is $66 million including 15% contingency and 5% engineering fee. The largest single cost is the foils; the liner cost is next largest (Figure 3B). CO_2 purchase is the largest operating cost; non-carbon nutrients, interest and depreciation, labor, utilities, and maintenance are other important costs (Figure 4).

Sensitivity Analysis

Following the baseline cost calculation, individual parameters were varied to determine their impact on algae cost. Table 1 lists the parameters tried, the ranges tested for each parameter, and the algae cost range produced. Some parameters (for example, dilution rate) were not varied because they were assumed to be fixed by the requirements of the chosen species. Other parameters (for example, minor cost items) were not tested because their impacts on total algae costs would have been minimal.

Several scenarios were modeled in addition to the baseline facility. One innovation included in the modeling studies was a mechanism under development by Makai Ocean Engineering for the extraction of CO_2 from atmospheric air. While the CO_2 content of air is low, the amount of CO_2 that would actually pass over a given area per day under normal wind conditions is large. A device that could extract the CO_2 efficiently could supply enough CO_2 to support high production levels in an algal raceway. The CO_2 collectors could be substituted for the CO_2 sumps in the raceways. Operating costs (electricity to run the CO_2 collectors) were estimated at $5/MT CO_2 under ideal conditions. Two cases were run: an "ideal" case in which collector cost = $2000 each and operating cost = $5/MT CO_2, and a much less

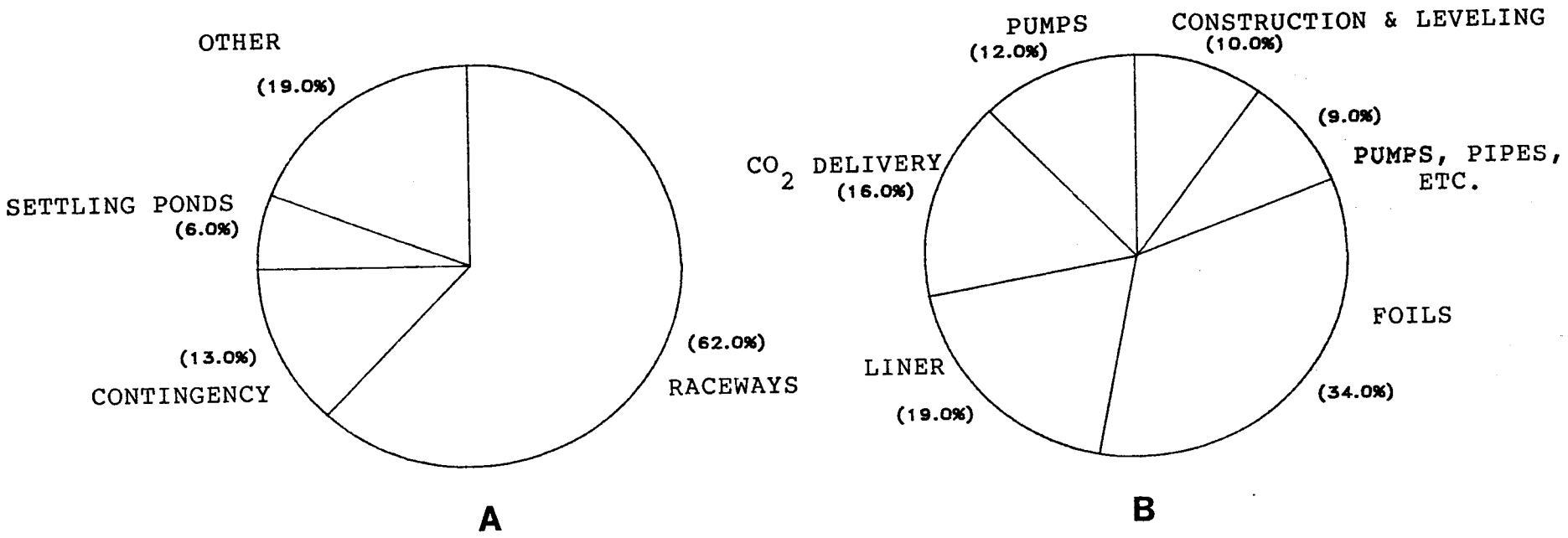

Figure 3. Relative cost contributions to Hawaii baseline capital costs (A) and raceway costs (B).

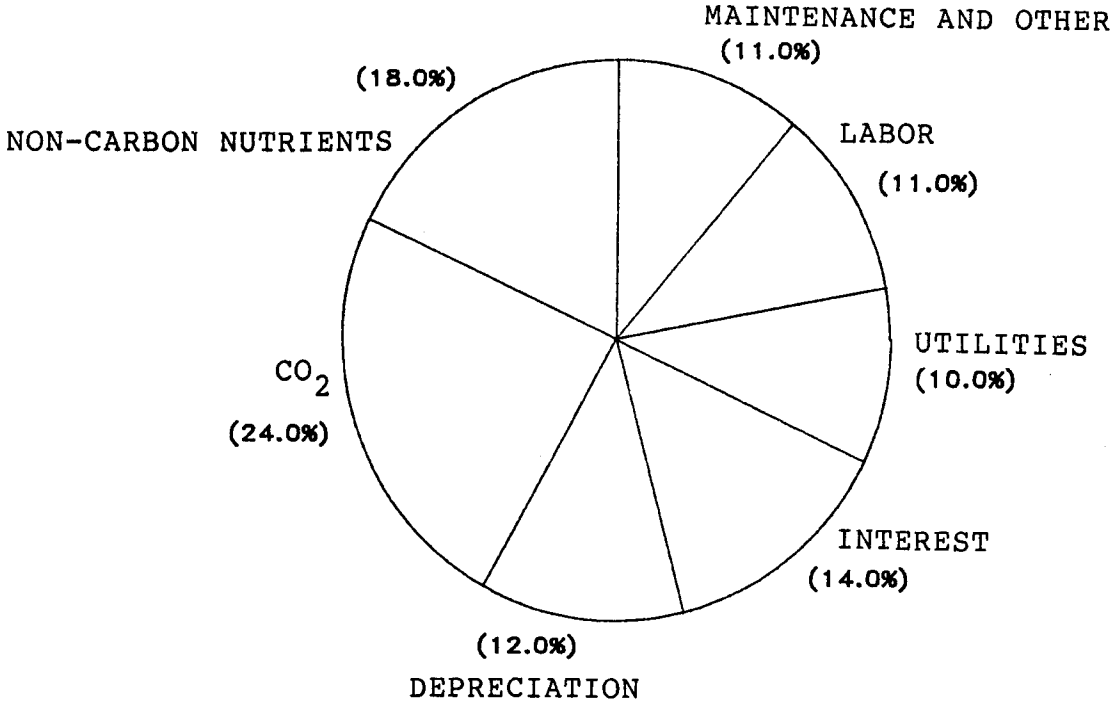

Figure 4. Relative contributions to Hawaii baseline operating costs.

PARAMETER	RANGE TESTED Low	High	ALGAE COST ($/MT) Low	High	RANGE
Photosynthetic efficiency (% PAR)	8	18	491	289	202
Harvest efficiency (%)	70	95	417	307	110
Foil cost ($/row)	25	200	338	399	61
Carbon dioxide cost ($/MT)	15	35	335	395	60
Distance between foils (m)	1.5	5	403	350	53
Carbon dioxide uptake efficiency (%)	0.5	0.9	394	350	44
Liner cost ($/running meter)	10	40	353	393	40
Carbon dioxide sump cost ($/unit)	1567	5000	365	402	37
Ammonia cost ($/MT)	250	500	346	373	27
Contingency allowance (%)	10	25	359	379	20
Harvest module cost ($/unit)	711867	2500000	365	384	19
Laborers/supervisors (# people)	55/9	110/18	355	374	19
Capacity factor (days/year)	330	360	375	357	18
Raceway cut & fill ($/running m)	3	15	361	377	16
Distance between CO_2 sumps (m)	124	1748	365	350	15
Electricity cost ($/kwh)	0.11	0.16	365	379	14
Water supply pumping head (m)	30	60	359	372	13
Mixing velocity (cm/sec)	15	40	360	373	13
Pump sump & supply pipe cost ($/rcwy)	10000	30000	362	374	12
Land rent ($/ha-yr)	200	1000	365	375	10
Raceway pump cost ($/unit)	20000	35000	365	374	9
Nitrogen conc. in effluent (mmole/l)	0	1	362	369	7
Raceway leveling ($/running meter)	1.5	6	361	367	6
Water supply pump efficiency (%)	0.6	0.8	369	363	6
Foil angle (degrees)	10	30	364	370	6
Engineering fee (%)	0.03	0.07	360	366	6
Raceway carbon dioxide delivery pipe	2500	10000	363	369	6
Effluent disposal capital cost ($)	530417	2000000	365	370	5
Road grading cost ($/sq m)	0.5	1	363	367	4
Phosphate fertilizer cost ($/MT)	300	400	363	367	4
Raceway roughness coefficient	0.013	0.02	365	368	3
Raceway pump efficiency (%)	0.65	0.8	367	365	2
Land preparation ($/ha)	250	1000	365	367	2
Evaporation rate (cm/yr)	160	200	365	366	1

Table 1. Parametric sensitivity analysis: effect of varying individual parameters over specified ranges.

optimistic case in which collector cost = $4000 and CO_2 production cost = $15/MT. The two cases gave algae prices of $298/MT and $349/MT, respectively.

Another case reexamined the benefits of foils as a mechanism for increasing production in light of the fact that they are the single largest capital cost in the baseline model. If the increase in photosynthetic efficiency does not outweigh the effects of foil cost and foil drag on the cost of algae produced in the raceways, then the foils are not worth installing. The analysis showed that despite the high cost of the foils, they are cost-effective.

An "optimistic" but reasonable case was modeled to give some idea of a minimum potential cost for algae produced by the culture facility. The assumptions in this case were:

1. CO_2 produced by MOE CO_2 collectors; collector cost = $2000 each and CO_2 production cost = $7/MT.

2. Harvest efficiency = 90% rather than the baseline 80%.

3. CO_2 uptake efficiency = 80% rather than 70%.

4. Foil cost = 1/2 baseline, foil lateral spacing = 1/2 baseline.

5. Capacity factor = 355 days/year instead of 345.

6. Ammonia cost = $375/MT instead of $425 (assumes lower shipping costs to Hawaii).

7. Labor requirements reduced: 70 laborers, 10 supervisors rather than 84 and 14.

All other parameters were held at their baseline values. All of the parameters that were varied in this case are subject to testing, or at least refinement, in the proposed experimental facility, or are cost estimates that can be refined with further investigation. It may not be possible to investigate the effect of CO_2 collectors in the scaled experiment, since the concept is still being developed, but their potential impact on system costs should be better known.

The algae cost predicted under the above assumptions is $229/MT. Yearly operating costs for this case were $13 million. A further, highly optimistic case was tested using the above assumptions, but with photosynthetic efficiency equal to 18%; the algae price for this case was $175/MT. This latter case assumes that additional breakthroughs in algae production are made; the former case requires no real breakthroughs, but does assume that small-scale experiments (such as the harvest experiments and bench-scale CO_2 collector experiments) can be scaled up very successfully. Barring major savings on facility construction costs, these estimates are probably close to the maximum

potential of the raceway system without including nutrient recycle from a processing facility.

Processing Options

Two processing options were considered: anaerobic digestion and combustion in a supercritical water reactor. If the algal biomass were used as feedstock for anaerobic digestion, the baseline system would produce 614,462 thousand standard cubic feet (MSCF) of methane, about 1/5 of present SNG consumption on Oahu (State of Hawaii Data Book, 1983). It would recycle 22,000 MT of CO_2, about 12% of total facility requirements. The discounted breakeven price for methane produced by this system is $36.43. This price is well above the SERI projected price goal for methane in the year 2000 of $7.40/$10^6$ BTU (Hill et al., 1984; 1 MSCF equals approximately 10^6 BTU). The "optimistic" assumptions listed in Section reduce the required gas price to $22.57 on sales of 711,307 MSCF. Finally, in the highly optimistic case where photosynthetic efficiency = 18%, the production is 1,049,469 MSCF and the breakeven price is $17.13. This price is still much higher than Year 2000 projections. Even in Hawaii, where the average retail price of SNG in 1982 was about $16.50/MSCF (State of Hawaii Data Book, 1983), it appears that methane sales alone (without byproduct credits) would not support the conceptual facility.

Supercritical water combustion is a process recently developed by Modar Corporation of Natick, Mass. (Modell, 1980). Supercritical water combustion involves the introduction of a slurry of organic material and a stream of air or oxygen into a reactor containing supercritical water at high temperature and pressure. The properties of supercritical water are different from those of ordinary liquid water; both the organic matter and the oxygen are highly soluble in supercritical water. The organic matter oxidizes rapidly and completely if enough oxygen is provided, releasing essentially all of its energy content to the surrounding water. In a well-insulated reactor, very little of the heat produced is wasted, as opposed to the inherent inefficiency of a boiler system.

Because of the potential for improved energy conversion with a supercritical water processing system, a simple computer model of such a system was added to the algal production model. The system was assumed to use #6 residual fuel oil (the fuel presently used by Hawaiian Electric Company in its power plants) to bring the heating value of the 10% algal slurry up to 4.9 MJ/kg water, which is required for most efficient net energy conversion of 40-45% (M. Modell, pers. comm.). A net energy production of 40% of algal energy content was assumed. In this type of system, the algae can be regarded as a fuel supplement, reducing the need for fossil fuel to produce a given amount of power. At 44.1 MJ/kg, 0.05 kg oil per kg water is required; approximately 45% of the total energy production is supplied by the oil and 55% by the algal biomass.

The baseline breakeven price of electricity produced by the supercritical water reactor was $0.113/kwh at a production level of about 28 MW. A credit of $105,000/yr was taken for water production. The "optimistic" case gave a price of $0.080/kwh. The present "avoided cost" rates paid by Hawaiian Electric Company for electricity from alternative energy projects are $0.059/kwh peak and $0.051/kwh off-peak; the peak rate may be lower than justified by present economic conditions (R. Neill, Hawaii Natural Energy Institute, pers. comm.). Conceivably algae culture could play a role where adequate supplies of fossil fuels are difficult to obtain and other biomass sources are limited, such as Pacific islands. In other regions, the cost of algae as a fuel for the supercritical water process would have to be considered in relation to the cost of other available fuel materials.

Byproduct Option: Shellfish Production

The economics of an algae culture facility cannot be judged by fuel sales alone, even if most of the algal production is used for fuel production. Byproducts of the algae facility will affect the overall revenues of the facility, improving its economic outlook and reducing the price that must be asked for the fuel product in order to achieve a specified return on investment. Potential byproducts of an algae culture system include herbivorous marine animals grown on unharvested algae in the facility's effluent. In the baseline conceptual culture system, 20% of the algal biomass (about 140,000 MT wet weight) is lost in the effluent if nothing is done to capture it.

In order to estimate the potential impact of food production on the conceptual facility, a section was added to the baseline model that modeled the addition of an intensive shellfish culture system to the facility's effluent stream. The shellfish was assumed to be the Eastern oyster, Crassostrea virginica, but other types of fish and shellfish could also be grown on the unharvested algae. Platymonas (as Tetraselmis) species have been tested successfully as food for juvenile bivalves, including oysters (Walne, 1970).

The total capital cost of the hypothetical oyster facility was $23.6 million. Operating costs were $12.7 million/yr. Farm revenues were $36.6 million on a production of 12,200 MT meat weight. Shellfish production could be a major revenue producer for the conceptual facility. Taking credit for oyster production, the resulting breakeven algae price is $61/MT in the baseline case. In other words, the shellfish supply most of the revenues required for the anticipated rate of return on facility investment, and the harvested algae can be sold at a much lower price than would be possible without byproduct credits.

The number of oysters such a farm could produce is quite large. Total U.S. production of oysters is 40,000-80,000 MT/yr meat weight (Fallon et al., 1984; Lee and Yamauchi, 1980). Most

likely a farm as large as the one modeled here would raise more than one product so as not to exceed market demand for any one.

U.S. Southwest Facility

As part of this conceptual design study, it was requested that an estimate be made to transfer the Hawaii commercial facility conceptual design to a site in the American Southwest. Such an estimate was prepared by comparing important site characteristics and analyzing their impact on important facility cost parameters.

The site selected in the American Southwest is located on the southeastern corner of the Salton Sea in the Imperial Valley of southern California. In Figure 5 the commercial facility has been plotted with respect to the Salton Sea and the towns of Niland and Calipatria, California. The microalgae production facility has been sited approximately 1-1/2 miles from the lake shore shown in this 1956 USGS map. The Salton Sea has continued to fill since 1956 and many of the CO_2 wells shown are now underwater (J. Kelly, Dept. of Planning, Imperial County, California). The siting of the facility as shown in Figure 5 is therefore a conservative prediction of where the facility might reasonably be located.

The Salton Sea site was selected because the necessary resources are available for a large algae production facility. In particular, saline water is abundant and significant unused deposits of geological CO_2 are available. Water would be obtained from salt water wells located within one-half mile of the Salton Sea shoreline. Shallow water wells of 50 to 150 ft deep will have very good communication with the Salton Sea water if located within one-half mile of the sea shore (F. Welsh, H & W Well Drilling Co., pers. comm.). Salton Sea water is salty (30-40 ppt), and well water in the area can be even more salty (up to 50 ppt for shallow wells). It is estimated that 8 to 10 wells, each supplying approximately 5,000 gpm, would be required to supply the entire commercial facility.

Effluent disposal is accomplished by direct discharge into the Salton Sea. Imperial County officials in El Centro, California reported that a past aquaculture facility had received permission to discharge effluent directly into the lake. It is necessary, however, to conduct an environmental impact study and to obtain the required permits before effluent discharge would be allowed. It was assumed, for this conceptual design, that the environmental impact statement would be positive, and the required permits could be obtained to allow such discharge.

There would be no difference between the raceway construction at the Hawaii site and the California site, with the exception that all the raceways in the California facility would be of equal size (1600 m running length), and 256 production raceways would be built. A separate soil analysis would be

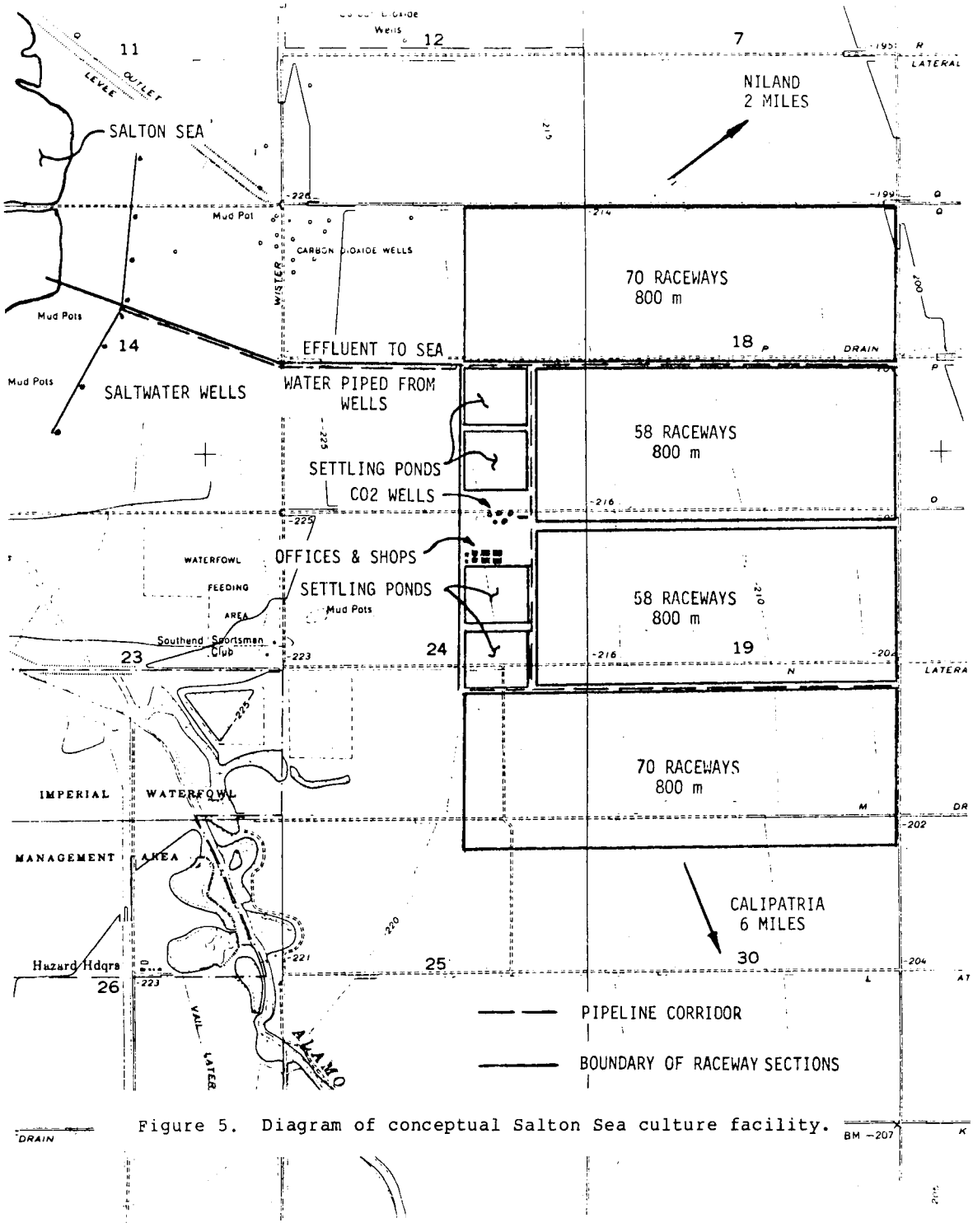

Figure 5. Diagram of conceptual Salton Sea culture facility.

required to determine the exact lime requirements in order to stabilize the soil in this area.

The winter climate near the Salton Sea appears mild enough to allow the facility to operate year-round. The average air temperature in January is 13 ^{o}C; on average there are 12 frost days per year. Only one snowfall has ever been recorded (Layton and Ermak, 1976). A system with flowing, saline water should be able to endure a small frost period without freezing over. The capacity factor for the California site is assumed to be 345 days/year, the same as for the Hawaii site. The combination of low light levels and low temperature during the winter could cause revenues to fall below operating costs for some period, so the facility might shut down seasonally for economic reasons.

Because of the less stable climatic conditions, the baseline California photosynthetic efficiency is assumed to be 11% of PAR (assuming PAR = 45% of total solar radiation) rather than the 12.2% in Hawaii.

For the purposes of this analysis, it was assumed that algae are found that grow well under conditions typical of the Salton Sea site and that can be harvested in a similar manner. Platymonas itself may succeed during summer in the Southwest, provided that salinity is not too high and that Platymonas will tolerate daily water temperature fluctuations. Platymonas has been grown in simulated Southwestern water types; simulated Type I water produced better growth than diluted seawater of the same salinity (SERI Biomass Program Monthly Report, January 1985). Also, other species of Platymonas may exhibit similar settling behavior (Walne, 1970). It is recommended as an adjunct to the scaled experiment that species screening trials be done in Hawaii-type shallow raceways at a Southwestern site.

Given various assumptions about capital and operating costs in California vs. Hawaii, the discounted baseline price of algae produced by the Salton Sea facility is $284/MT ($309/MT AFDW). The capital cost of the facility is roughly the same as that of the Hawaiian facility; slightly lower construction costs are balanced by the cost of land purchase and a longer effluent trench. Operating costs of the California facility are much lower ($11 million vs. almost $18 million in Hawaii), mainly because of the free CO_2 presumed to be available at the California site but also because of lower costs for non-carbon nutrients, electricity, and labor and the absence of land rent. An "optimistic" case was run using the same assumptions as the Hawaiian optimistic case, except that CO_2 collectors were not necessary and nutrient costs were not changed from the baseline case. The optimistic case gave a breakeven price of $217/MT. The "highly optimistic" case of 18% photosynthetic efficiency gave a price of $144/MT. This case is probably less likely for a Mainland site than for a Hawaiian one.

Breakeven costs were also calculated for methane produced by the California facility. Assumptions were the same as for the

Hawaii methane option, with the exception that 20% less methane production per unit algae was assumed because of the additional heating requirements for a digester sited in California. Maintaining the digester temperature at 30-35 ^{o}C may consume as much as 30% of total energy production if the incoming water temperature is 15^{o}C (Srivastava, 1984).

The breakeven price for methane produced by the California facility is $34.93/MSCF on production of 443,834 MSCF. The "optimistic" and 18% conversion efficiency cases gave prices of $26.01/MSCF and $17.72/MSCF, respectively. Although the discounted breakeven price of algae produced in the California facility is calculated to be lower than in Hawaii, methane produced by the California facility has little or no cost advantage over methane produced by the Hawaii facility because of the greater energy required to heat the California digesters. The California facility also benefits less from digester nutrient recycling than the Hawaii facility because of the lower nutrient costs in California.

Scaled Experiment

Task II of this study is to develop a facility design for a scaled experiment to validate the performance and the operating costs expected for a large commercial algae culture facility producing fuels. Emphasis is to be placed on the culture and harvest systems.

Both the biology and the engineering development of the Hawaii-type shallow raceway are still at a rudimentary stage. Many of the engineering parameters have not been optimized (i.e. raceway slope, foil design and placement, CO_2 sump design, settling pond design). The system is intended to be applicable to the U.S. Southwest, but it has not been tested even on a small scale under conditions truly representative of that region; the algal species that might do best in a Southwestern shallow raceway system are unknown. Because of the uncertainty of many design parameters, it is not appropriate to construct large production raceways immediately. The scaled experiment has therefore been broken down into two stages: an initial stage (Stage 1) to test biological and design parameters in a flexible experimental system, and a second stage (Stage 2) in which larger production-scale raceways are built and operated based on the results of the first several Stage 1 experiments. Because the project duration is limited to two years, the two stages overlap.

The design of the scaled experiment rests on the premise that, by the end of the experiment, costs and performance should be sufficiently well known to attract potential private investors to algae culture based on the proposed design. The Stage 2 raceways should therefore be of sufficient scale to estimate realistic construction costs, to demonstrate that algae grows as well in a production-scale raceway as in small experimental ones, and to demonstrate that operation of the raceway system is

realistic in terms of labor required for culture operations, harvesting, and maintenance. A true commercial pilot, in which at least several full-sized raceway modules would be constructed and operated and a fuel product would actually be produced in saleable quantities, would require more time (and presumably more funding) than has been allotted by SERI for the scaled experiment. A pilot system would probably be the next step after the scaled experiment and would presumably be privately funded.

Most of the Stage 1 experiments will be done in modular aboveground raceways constructed of plywood and lined with vinyl pond liner. The raceways will be built of sections (probably 8' x 10' to conform to readily available lumber sizes and give a 3 m width, which is desirable to avoid significant effects of the raceway sides on head loss). The system offers great flexibility in use; raceway size, number and slope can be varied as desired for each experiment. Enough sections will be provided to build a raceway of 500 m running length in order to provide sufficient length to determine an optimal distance between CO_2 diffusers. This number of sections will be enough (with appropriate end pieces and sumps) to construct as many as 10 50-m running length raceways for experiments to determine the effects of design parameters and operating strategies on production and harvestability of algae. Total surface area of the modular raceways will be 1500 m^2. It has been suggested that experimental algae culture ponds should be at least 100 m^2 in area if the results are to be extrapolated to other systems (Benemann et al., 1983). The minimum size of the modular raceways during the scheduled experiments will be 150 m^2.

After the modular raceway experiments have determined design characteristics such as proper pump type, slope/water velocity, head losses, and optimal distance between CO_2 sumps, two large (10 m wide x 400 m running length) soil cement raceways will be designed and constructed. The large raceways will be constructed in essentially the same manner as has been proposed for the conceptual production facility, as modified by the results of Phase 1 experiments. They will be operated as if they were modules of a real production system. Their purpose is to:

1. Test performance of full-width raceways relative to smaller experimental ones.

2. Refine construction cost estimates for large shallow raceways.

3. Determine maintenance requirements: labor, materials, costs.

4. Test culture stability and amount of "downtime".

The large raceways will be about 1/4 the length of the raceways proposed for the conceptual facility, based on the assumption that beyond a certain point, added length will not affect the

performance of a raceway.

One settling pond will also be constructed to test the mechanics of settling, draining, and harvesting the algae on a large scale and to determine the actual harvest efficiency and solids content of harvested material from the large raceways. The settling pond will be large enough to accept the harvested water from one large raceway (approx. 350 m^3). The pond should be large enough to allow tests of alternative methods for removing the harvested material.

Species screening experiments in the U.S. Southwest are proposed for the Roswell Test Facility in Roswell, New Mexico. Eastern New Mexico is a candidate region for large-scale algae culture because of a relatively mild climate, availability of saline water, and proximity to geological CO_2 resources (Maxwell et al., 1984). The Roswell Test Facility has the necessary facilities (including saline water at 14 ppt) and analytical equipment already on site.

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EVALUATION OF IMMOBILIZED CELL SYSTEMS FOR THE PRODUCTION OF FUELS FROM MICROALGAE

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GENERAL FEATURES OF IMMOBILIZED CELL SYSTEMS

Concept of Immobilized Cell Systems

A major development in the pharameutical and chemical processing industries in the past decade has been the introduction of large scale immobilized enzyme and immobilized cell reactor systems. Industrial use of such immobilized cell bioreactors suggests the possibility that this approach also could have applications for biotechnical processes involving algae. This paper examines the potential for using immobilized cell technology for large scale production of fuels from microalgae.

Immobilization techniques involve the physical confinement or localization of cells (or enzymes) in a particular region of space with retention of catalytic activity (and viability in some cases) in order to permit repeated or continuous use. The idea is to convert the reaction from homogeneous (i.e., freely suspended cells or dissolved enzymes) to a heterogeneous condition. This is done by developing a catalytically active solid phase of macroscopic dimensions to be placed in contact with a catalyst-free solution of reactants.

Two important characteristics of an immobilized biocatalyst are: (a) its level of catalytic activity, and (b) its stability in use and during storage. Stability generally is indicated in terms of half-life, the time for a 50% decrease in activity. Conditions for immobilizing cells with good retention of enzymatic function and viability have been devised, and in many cases, the stability of the immobilized cells is considerably greater than for free cells. Half-lives up to 12 months have been achieved.

Various methods for immobilizing cells are available and the appropriate procedure to use for any particular cell and process must be determined empirically; what is found suitable in one case may be unsuitable for another. Immobilization methods include:

(1) Physical entrapment of cells in a (porous) matrix, such as agarose, alginate, carageenan, polyacrylamide, polyurethane, etc.

(2) Enclosure or separation of the cells from the reaction mixture by encapsulation or thin films (dialysis membranes, etc.).

(3) Adsorption onto the surface of an insoluble material. This may

involve ionic or non-ionic interaction of cells with some substratum, such as synthetic ion exchange resins, dextran or porous glass beads, titanium oxide particles, wood chips, etc.

(4) Covalent bonding between the cell and a support surface, or direct covalent crosslinking of the cells without any carrier material.

A common practice in preparing immobilized cells is to first grow the cells in a fermenter under suitable conditions, harvest the cells by centrifugation and then immobilize them at a very high cell density. By use of pre-grown cells suspended (entrapped) in epoxy resin, cell densities of 0.7-1.0 g wet cells/ml of matrix can be obtained. Alternatively, a few cells may be entrapped in a matrix and then allowed to grow after they are immobilized. Another procedure is to inoculate the culture into a vessel containing liquid growth medium and suitable support materials or "carriers" (e.g., porous glass beads, diatomaceous earth, etc.) and allow the cells to colonize the carriers during growth. Recently, there has been interest in immobilizing living cells in the hollow fibers of ultradialysis apparatus.

Advantages of Immobilized Cell Reactors

Immobilized cell reactors are used in two different types of situations. One of these is the use of immobilized cells instead of an immobilized purified enzyme to catalyze a single-step enzymatic reaction. In this case, the whole cell is simply a convenient and less expensive source of (crude) enzyme. The second type of process is the substitution of immobilized cells for a conventional fermentation process (batch or continuous flow) that uses free cells.

Immobilized cells used as enzymes have the same advantages over free (soluble) enzymes as do immobilized purified enzymes. These include:

(1) Enhanced stability of the enzyme;

(2) Ability to recover and re-use the enzyme in batch operations;

(3) Ability to use the enzyme for continuous flow operation;

(4) Use of small simple reactor design;

(5) Product can be obtained in high yield, which simplifies purification.

Use of the whole cell instead of purified enzyme is particularly indicated when the enzyme involved is intracellular, or when the extracted enzyme is unstable during and after immobilization. Often enzymes retain better activity when present in the whole cell. Whole cells may be usable as an enzyme source provided the substrates and products are not high molecular weight compounds (cell wall and cell membrane are barriers to high molecular weight substances), and provided the cell does not have interfering enzyme reactions.

When compared to conventional fermentation processes using free cells, the immobilized cell systems have certain advantages. Among these are:

(1) It is possible to obtain high cell density without high viscosity. This affords better mass transfer and easier mixing.

(2) Immobilized cells often shown greater stability than free cells.

(3) With immobilized cells biomass can be retained in continuous flow operation. In conventional continuous fermenters the cells are lost with the outflowing spent medium.

(4) Immobilized cells can be used in a continuous flow system under non-growth conditions. In a conventional continuous fermenter non-growing cultures are washed out. Fermentation of some products requires conditions different from those needed for growth, so non-growing cells are needed. Use of non-growth conditions simplifies the purification of products by eliminating growth substrates from the reaction mixture.

Reactor Types

One advantage of cell immobilization is that it allows the selection of a number of different reactor configurations. These have different limitations and advantages for particular types of processes. The basic types of reactors used for immobilized cell processes include the following (see Figure 1):

(1) Packed bed reactors;

(2) Stirred tank reactors: batch and continuous;

(3) Fluidized bed reactors;

(4) Miscellaneous reactors: hollow fiber reactors; tubular reactors; dialysis fermenters; cell-based electrodes.

The packed bed reactor has the advantage of simple operation and it is inexpensive to construct. In one simple form the immobilized cells (cast as beads or chips) are placed in a length of glass tubing and confined by a mesh screen at either end. The substrate solution is pumped through the bed, often in an upward flow to counteract compression by gravity. Such an arrangement allows high mass transfer rates under conditions that approach plug-flow. For reactions that are not substrate-inhibited the packed bed reactor gives high reaction rates, and it is particularly advantageous for product-inhibited reactions. Packed bed reactions are prone to clogging with particulates or growth-associated products of cells (slime, capsules) and to compression effects (especially with non-rigid gels and small particles). Problems include difficulties of providing adequate oxygen and nutrients to the densely packed biomass, and efficient CO_2 removal.

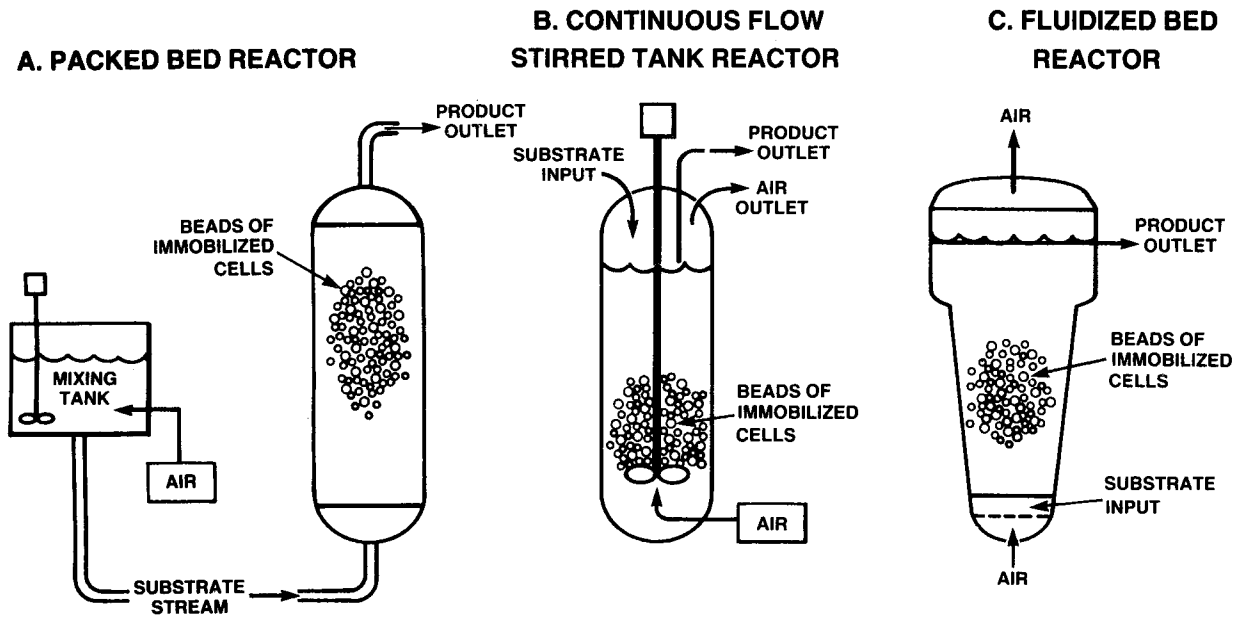

Figure 1. Basic Configurations Immobilized Cell Reactors

In a stirred tank reactor the immobilized cells (in bead form, etc.) are agitated to obtain a well-mixed condition. It is easier to obtain adequate aeration and pH control in such well-mixed vessels, but the shear forces from the agitations tend to disintegrate the immobilized cell preparations. Batch-type stirred tank reactors are seldom used for immobilized cells, and the continuous flow stirred tank reactors (CFSTR) also have limited uses. In the CFSTR the level of substrate remains relatively low, so this type of reactor is advantageous for a substrate-inhibited reaction. Product levels in a CFSTR are relatively high, so it is less suitable for product-inhibited reactions than a packed bed reactor.

A fluidized bed reactor uses an upward flow of liquid and air to keep the immobilized cells (beads, chips, etc.) from settling out, but the shear forces are less than with a stirred tank. A flared shape is employed to cause the particles to lose velocity as the rise to the top, thus preventing wash-out. This type of reactor is well suited for providing aeration and gas mixing for pH and temperature control, and for the use of high flow rates. Fluidized bed reactors are not particularly susceptible to plugging because of compression, use of viscous or particulate substrates or formation of capsular slimes.

CONSTRAINTS ON APPLICATION

Mechanical and Hydraulic Factors

In packed bed reactors there is a problem of compression of the immobilized cells, even if a relatively rigid matrix is used. In a CFSTR the shearing by the impellar blades tends to disintegrate the immobilized cell preparation. Both these effects are minimized in a fluidized bed reactor but precise control of flow rates is needed to balance the density of the immobilized cells. Such control is complicated by possible density changes over time due to gas bubble formation, lipid accumulation or other alterations.

Maintenance of Viability and/or Catalytic Activity

Some immobilizing techniques tend to cause loss of viability. This is particularly true of methods involving covalent bonding of cells to a support or to other cells. Chemicals used for covalent attachment or for polymerizing a matrix often are strongly inhibitory. If viability or half-life are unsatisfactory, less harsh methods may be possible. For some purposes it is sufficient that a particular enzyme or enzyme system remain active, even if the cells are no longer viable.

Gas Exchange

Gas exchange is a general problem in all aqueous systems due to the limited solubility of oxygen in water. Immobilization of cells at high

concentrations further complicates the problem by imposing additional diffusion barriers along with increased O_2 demand. Packed bed reactors are particularly ill-suited for dealing with the need for gas exchange (providing O_2 and removing CO_2 from respiring cells). When gas exchange is involved, the fluidized bed reactor is more appropriate.

Nutrient Supply

In a packed bed reactor nutrients may be used completely as the reaction mixture passes through the first part of the bed, leaving nothing for the rest.

General Constraints

Certain general problems are encountered with immobilized living cells that are not necessarily found with non-viable immobilized cells. These include pH changes, foaming, and problems with microbial contamination. Use of growth promoting nutrients in the reaction mixture requires use of sterile technique to avoid growth of contaminants. Live cells produce pH shifts due to fermation of acids (lactic, acetic, etc.) or ammonia. Aeration of live cultures in media containing proteins, peptone, or excreted biosurfactants leads to foaming whether the cells are free or immobilized.

For various reasons it is probably advantageous to limit the size of immobilized cell reactors and use several smaller reactors instead of a single larger one. The use of several reactors arranged in parallel would allow the shut-down or replacement of one without affecting the others.

Specific Constraints for Algae

In addition to the general consideration already described, there are two specific requirements for immobilized algae: the light supply and the CO_2 supply. A fluidized bed reactor should permit adequate amounts of CO_2 to be supplied, but it is not clear how it would be possible to supply sufficient light to a dense algal biomass to allow high photosynthetic efficiency. It appears that those conducting laboratory experiments with immobilized algae have been content to demonstrate that photosynthesis occurs, but there is no indication that it is occurring at a rate anywhere approaching maximum efficiency.

PRELIMINARY ECONOMIC ANALYSIS

Immobilizing photosynthetic algae and using them as a source of lipids for energy has many technical problems, most of which can be solved with research. However, even given that the technical problem of growing immobilized photosynthetic organisms can be mitigated or circumvented,

the question remains as to whether the economics of using immobilized algae for energy production is favorable. We have taken a broad-brush look at the economics of immobilizing algae for energy production with the following important constraint. We used the feasibility case inputs to the SERI economic model described in Fuels from Microalgae: Technology Status, Potential and Research Issues, Hill, Feinberg, McIntosh, Neeron, Terry, 1984, to evaluate our concepts. Any parameter not explicitly changed by introducing the immobilization process was used as entered in the SERI model. Parameters in our analysis that were affected by immobilization are noted.

To conduct the economic assessment, several technical concepts were first considered. There are clearly more possible schemes which could be defined and analyzed. Some are technically feasible, while others are only possible with the development of new materials or procedures. We have chosen one system for our first analysis and eliminated others for reasons to be discussed.

There are several generic advantages for using immobilization systems which apply to mass cultivation for energy. The most important is the ability to increase the yield of cells per unit of culture volume. Another advantage is the ability to easily harvest the larger immobilized particles and reduce the cost of harvesting. Introducing immobilization, coupled with reducing the size of process units, allows greater process control, an important but difficult concept to quantify economically.

The first step in assessing the economics of immobilized algae for fuel production was to develop a reasonable conceptual scheme. Concept development proceeded from traditional schemes to novel adaptations of immobilization techniques. The first conceptual system examined and eliminated from consideration was the extraction and immobilization of enzymes from algal cells. In general, immobilized enzyme systems are most economic and effective when they are used to catalyze a single reaction. However, lipid production involves many enzymatic reactions for a multi-step synthesis and, therefore, is not well suited as an immobilization system for the production of the vast quantities of lipid being proposed as an energy product.

The second system considered was eliminated on biological grounds. This system assumed that algae could be selected or genetically engineered to excrete lipids while still growing at 18 percent photosynthetic efficiency and storing 60 percent of the cell weight as lipids. This system assumes that such an organism can be obtained, that it can be grown vigorously in culture and that the excreted lipids are transported outside the cells and the immobilization matrix (if applicable). While it is not impossible that such a system could be developed, it will be difficult and is not technically feasible now. In this concept the leaky algae are immobilized by absorption on the surface of particles or by encapsulation in a translucent substance (i.e., carageenan or algin) and grown in a pond or in an above ground fluidized bed reactor. Carbon dioxide and recycled culture medium provide the necessary fluidization with light introduced at the pond surface or through transparent walls

and top of the tank. By adjusting the fluidization rate, a stream of older inactivated particles and culture medium could be continuously removed from the top of the reactor and fresh immobilized algae could be added. The major problem with this concept (as mentioned previously) is the limited likelihood that lipids would be excreted from a vigorously growing culture in the quantities necessary to justify the cost of immobilization. Also, from a physical property standpoint lipids are hydrophobic and prefer a non-aqueous environment and are, therefore, more likely to remain associated with the cell than with the culture medium.

The next concept considered was the use of cell immobilization as a part of the biomass production/harvesting process rather than for catalysis. In this case, immobilization is used to increase the culture density, improve process control, and facilitate harvesting. Algae are known to attach to many substrates and one example of an inexpensive and available substrate is anthracite coal. After the algae are immobilized they can be grown in ponds or in an above ground fluidized bed reactor. To harvest, a stream of lipid-rich algae particles are removed from the top by adjusting the rate of fluidization so that only the algae-rich particles reach the top due to the reduced density of the particle due to algal growth. Concentrated particles are centrifuged to shear the cells off and the anthracite particles are recycled. This approach provides an intensive continuous culture alternative to batch pond culture.

The above concept was modified for closer analysis. The process examined is a two-step process where two above ground reactors are operated in series, separating the growth and lipid production stages. In this process we assumed the algae would be grown in hollow cylindrical vertical tanks with transparent walls.

Above ground tanks were chosen over a pond system for two reasons. First, the culture depth for the SERI analysis ranges from .15 to .3 meters. Increasing the system's culture density without decreasing the amount of light reaching the system would result in a very shallow pond (i.e., increasing culture density by a factor of three would reduce the depth of the culture to a range of .05 - .1 meters). Second, above ground tanks reduce the surface area exposed to the atmosphere thus decreasing evaporation losses. The above ground tank system that was selected increased the culture density by a factor of three without a reduction in the sunlight reaching the system. This system also reduced evaporation losses by 99 percent.

The role of immobilization in this system is to increase cell yield and facilitate harvesting. The advantage of this system is several-fold. First growing algae in the tubes instead of ponds reduces the probability of mass infestation and predation. Contamination, when it does occur, should be easier to eliminate as only a portion of the tanks will be affected at any given time and because cleaning the tanks will be simpler than cleaning ponds with a granular cover over a clay bed. A smaller surface area also reduces the amount of dust and debris getting into the tank.

If the algae are immobilized on the surface of the substrate, the overall density of the algae-bound substrate will decrease as the algae grow and the older particles will rise to the top and can be separated by controlling the fluidization rate and reactor configuration. In this concept, after sufficient algae growth on the surface of the immobilization material, the particles will flow out of the first reactor into the second.

In the second reactor, a nitrogen-poor medium is used to promote lipid production. As lipid content increases, the particle density decreases further. With the same differential controls as those in the first reactor, the crop of lipid-rich algal particles can be selectively bled out of the second reactor and centrifuged to yield a lipid-rich product. This approach increases process control ability and allows a continuous step-wise process to be used as shown in Figure 2. The hollow cylindrical vertical tanks with transparent walls were chosen as culture vessels in an attempt to decrease water volume while maintaining the surface area exposed to light.

The outside diameter of the growth tubes is one foot with an inside diameter of a hollow column of 9 inches. Assuming that half of each tube is exposed to light, 5,242,000 tubes are needed for this system to obtain the same amount of light as the SERI ponds. However, the volume of water used by the above ground tanks is reduced by one-third which permits higher algal concentrations. The surface area exposed to the air is also reduced by 99 percent. The reduction in the system's water volume and evaporation losses result in lower salinity buildup and, thus, lowers energy and water costs. This system also eliminates the need for first stage harvestors as the immobilized system further concentrates lipid-rich algae.

Total capital costs of the immobilization system (Table 1) is approximately 8.5 times that of the SERI attainability case. The transparent cylindrical tanks represent the major cost increase over the SERI system. Based on current retail prices of these tanks (and assuming that the large quantities are available), less a 50 percent discount for a large order, each tank would run approximately $60 per tank. R&D on the use of low cost materials, however, could offer substantial cost reductions over time. The analysis assumed the same biological parameters as the SERI attainability case.

Capital cost reductions come from the elimination of first stage harvestors, mixing and culture systems, as well as a reduction in site preparation (i.e., laser grading no longer required). The land area needed to site the tanks so they do not shade each other is approximately the same as that needed for the SERI system.

The immobilized systems also realize operating savings (Table 2) from reduced water and pumping requirements as well as reduced carbon and nitrogen losses. The reduced carbon and nitrogen losses, however, would be somewhat lower as less nutrients are provided from reduced levels of make up water.

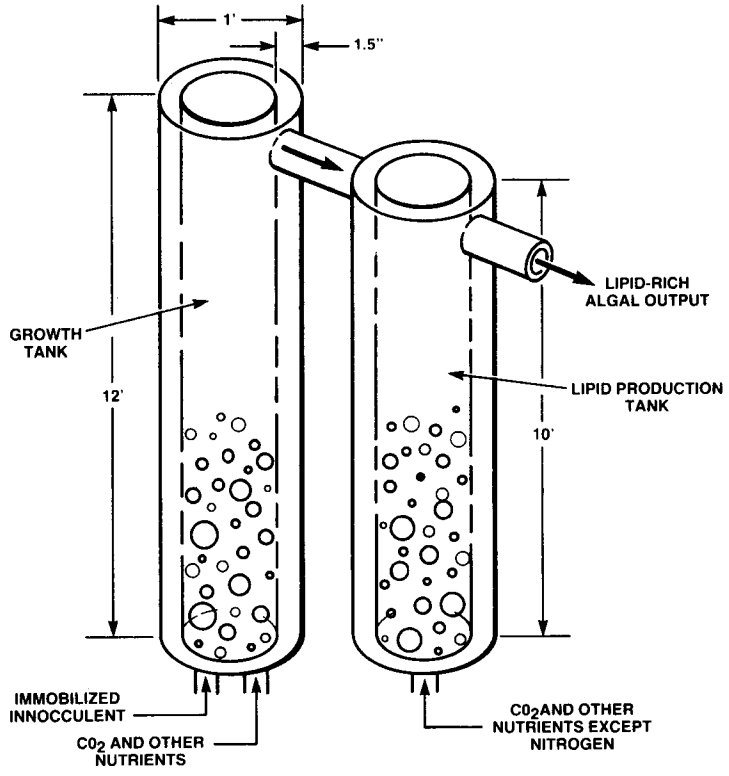

Figure 2. Two Staged Immobilized Algal System

Table 1: Comparison of Capital Costs
SERI Attainability Case vs Immobilized Case

CAPITAL COSTS	SERI CASE	IMMOBILIZED CASE
Capital Cost of Lining/tanks	4,300,000	314,520,000
Capital Cost of Mixing System	2,150,000	-----
Capital Cost of Culture System	2,308,431	-----
Capital Cost of Water/Nutrient System	987,847	987,847
Capital Cost of CO_2 System	690,066	690,066
Capital Cost of Buildings	291,767	291,769
Capital Cost of Electrical System	1,488,011	1,488,011
Cost of Immobilization Material	-----	94,400
Total Cost of Culture System	12,216,122	318,072,093
Capital Cost of 1st Harvestor System	5,355,504	-----
Capital Cost of 2nd Harvestor System	4,591,121	4,591,121
Total Depreciable Cost	22,162,747	322,663,214
Cost of Site Preparation and Survey	8,452,000	3,010,000
Engineering Fee	3,324,412	3,324,412
Contingence	5,097,432	5,097,432
Land cost	1,216,275	1,216,275
Total Non-Depreciable Cost	18,909,119	12,648,119
Total Capital Investment	40,252,866	335,311,333

Table 2: Comparison of Operation Costs
SERI Attainability Case vs Immobilized Case

OPERATING COSTS	SERI ATTAINABILITY CASE	IMMOBILIZED CASE
Direct Labor	1,345,000	1,345,000
Overhead	1,008,750	1,008,750
Utility Costs	462,291	184,916
Carbon Dioxide Costs	9,377,566	8,908,688
Nitrogen	853,092	810,437
Potassium	50,634	50,634
Phosphorous	84,634	84,634
Total Nutrient Cost	10,365,592	9,854,393
Total Water Costs	603,400	45,053
Other Operating Costs	1,108,138	1,108,137
Total Operating Costs	13,993,170	13,546,249

The major problem with immobilizing algae is the fact that sunlight drives the system. One of the major benefits of immobilization in other areas has been the ability to substantially increase culture density. However, this is difficult to achieve with a photosynthetic process. Increasing culture density by an order of magnitude while obtaining the same amount of light becomes extremely expensive and introduces other problems such as temperature control. The use of the hollow cylindrical tanks results in a three-fold increase in the algal culture density.

An advantage to above ground immobilized systems is that they can be scaled-down to smaller units and sited close to CO_2 sources. Used this way they mitigate adverse local environmental problems associated with a large scale pond system (i.e., reinjection of highly saline water).

At present, an immobilized algal system to mass produce lipids for use as a liquid fuel does not appear to be economically feasible. The major drawback is developing a low-cost system that obtains the same amount of solar energy as provided to a shallow 3 square mile pond while increasing the culture density by an order of magnitude. R&D to increase light availability and to develop low cost transparent tanks could increase the competitiveness of immobilized algal systems.

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