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PREFACE

This volume contains progress reports presented by the Aquatic Species Program subcontractors and SERI researchers at the SERI Biomass Program Review held in Boulder, Colorado, April 4, 1984. These reports present and discuss research advances achieved by the program participants during the preceding year. The SERI Biomass Program receives its funding through the Biomass Energy Technology Division of the Department of Energy.

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Approved for

SOLAR ENERGY RESEARCH INSTITUTE

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INTRODUCTION

The production of microalgal biomass has been studied for about 4 decades. Its potential to provide food, feed, and energy for mankind has been the reason for increasing interest in these organisms. Microalgae also contain high amounts of fats and oils (lipids). They are produced by direct use of the sun's energy via photosynthesis. In principle, lipids from microalgae are suitable for refining into conventional liquid fuels. Indeed, in the past, biological oils have been refined to fuels during shortages in petroleum supply. In 1980, Aaronson and Dubinsky proposed using saline water resources in arid lands to produce microalgae for oils. They also projected that these microalgal oils could be used as a source of fuel.

The purpose of the Aquatic Species program is to improve the productivity, conversion to fuels, and cost efficiency of aquatic plant species for energy. The emphasis of the program is on developing oil-yielding microalgae which will grow in the saline waters of the desert in the American Southwest. To meet this objective, research is carried out under three tasks: biological, engineering, and analysis. The principal objectives of the biological task are (1) to conduct a comprehensive and complete screening activity that will result in the selection of the best microalgae strains for production of fuels; (2) to establish a gene pool so that species can be improved through agronomic means; and (3) to develop techniques that will result in enhancing the yields of the desired fuel product. Engineering research is concerned with integrating biological concepts with engineering principals to develop the most cost-effective microalgal culture technology. Work is concentrated on developing culture management strategies and required subsystems such as harvesting technologies. Analysis supports the technology development through cost goal determination, resource assessment, and technology evaluation. The program also supports the development of macroalgae culture for methane gas or alcohols.

COMPARATIVE STUDIES ON THE CARBOHYDRATE COMPOSITION OF MARINE MACROALGAE

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INTRODUCTION

The objective of this subcontract is to evaluate carbohydrates from macroalgae common to the Gulf of Mexico. Information from these analyses shall be used to provide an indication of the feasability of fermenting macroalgae to ethanol. Knowledge of the carbohydrates and sugars shall allow assessment of required pretreatments and utilization efficiencies in converting algal feedstocks to ethanol.

The work under this subcontract shall provide an annotated bibliography to references on macroalgal carbohydrates and analytical techniques applicable to determination of these carbohydrates. A protocol shall be developed that will be a guide for analysis of these carbohydrates in a format that will allow evaluation of these carbohydrates for fermentation to ethanol. Finally, macroalgae common to the Gulf of Mexico shall be obtained and analyzed for chemical composition with an emphasis on carbohydrates.

METHODOLOGY

The macroalgae utilized in this investigation will be representative of the divisions Phaeophyta, Rhodophyta and Chlorophyta. Carbohydrates from each species will be extracted, fractionated and analyzed for chemical composition. Schemes for the total extraction of carbohydrates and selective fractionation of carbohydrates are as shown in figures 1 and 2, respectively. Spectrophotometry and chromotography will be the methods for chemical analyses of carbohydrate fractions.

PROGRESS AND PROJECTIONS

The initial three months (November 1983 - February 1984) of the project were relegated to the development of an annotated bibliography relative to carbohydrates of marine macroalgae. Presently (March - April 1984), various analytical protocols for the extraction, fractionation and chemical analyses of carbohydrates are being compared for efficiency. Subsequently, during the last phase of the subcontract (May - October 1984), the most appropriate protocol will be utilized to analyze at least six species of macroalgae.

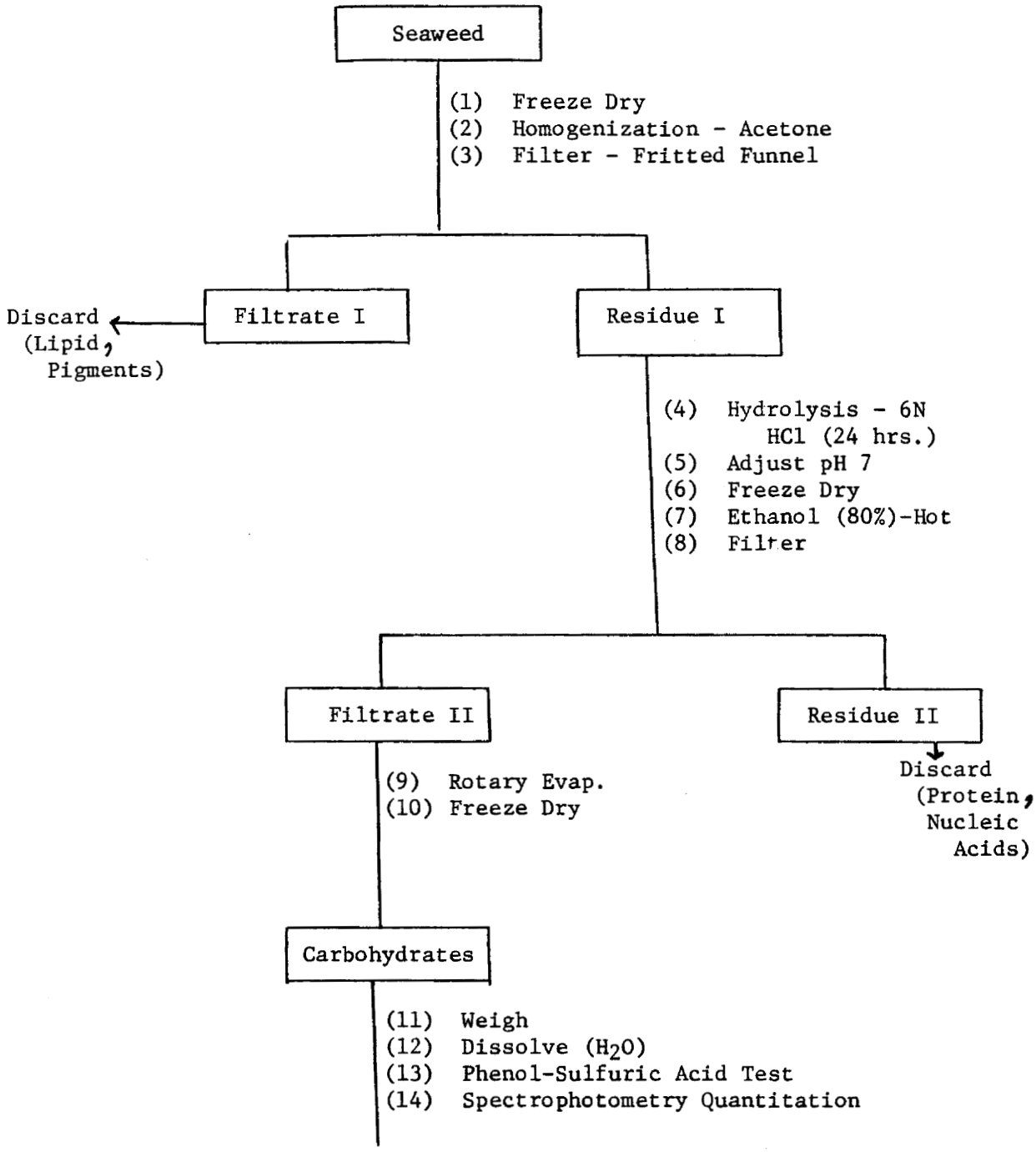

Fig. 1. Extraction of Seaweed Total Carbohydrate

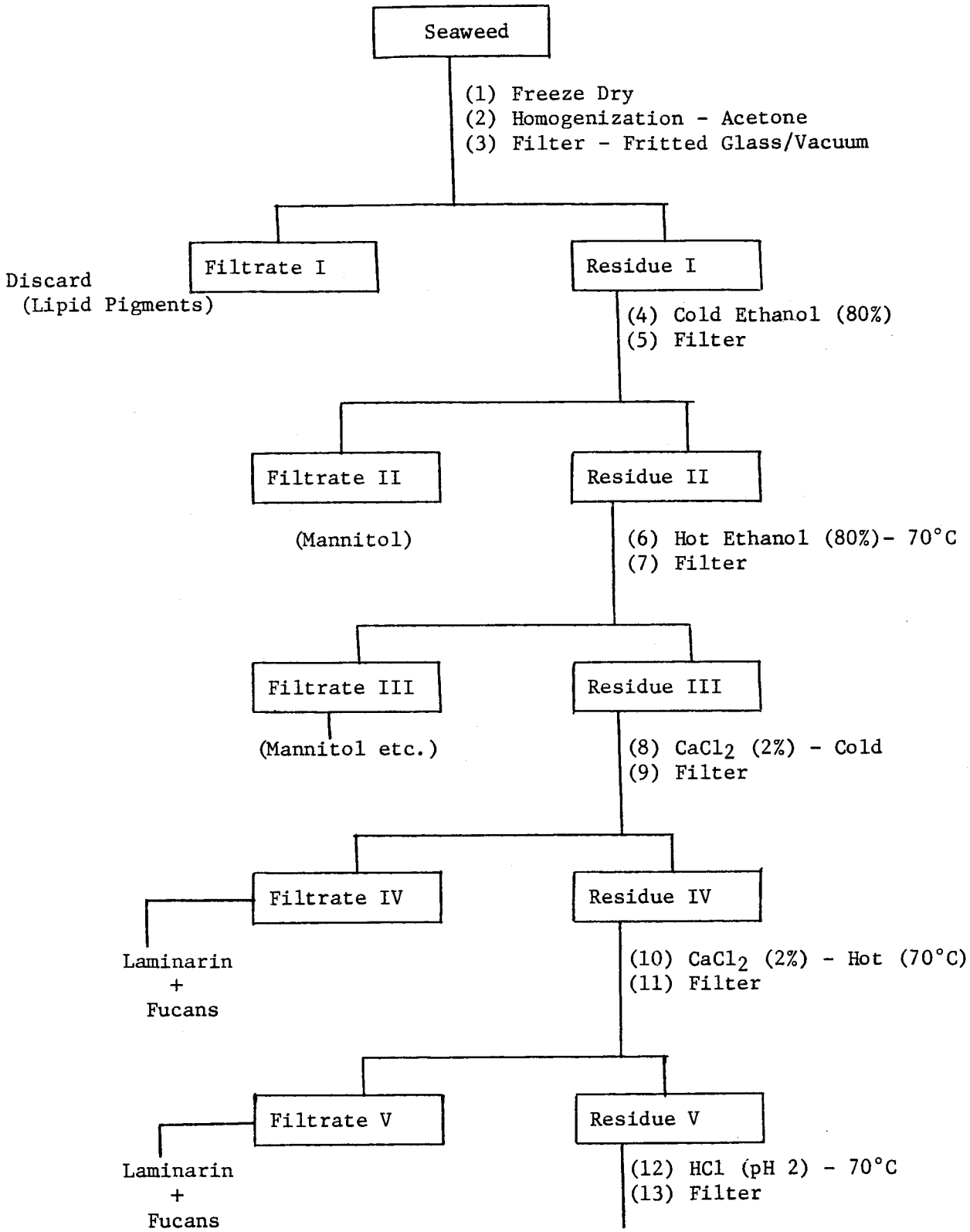

Fig. 2. Extraction and Fractionation of Seaweed Carbohydrates

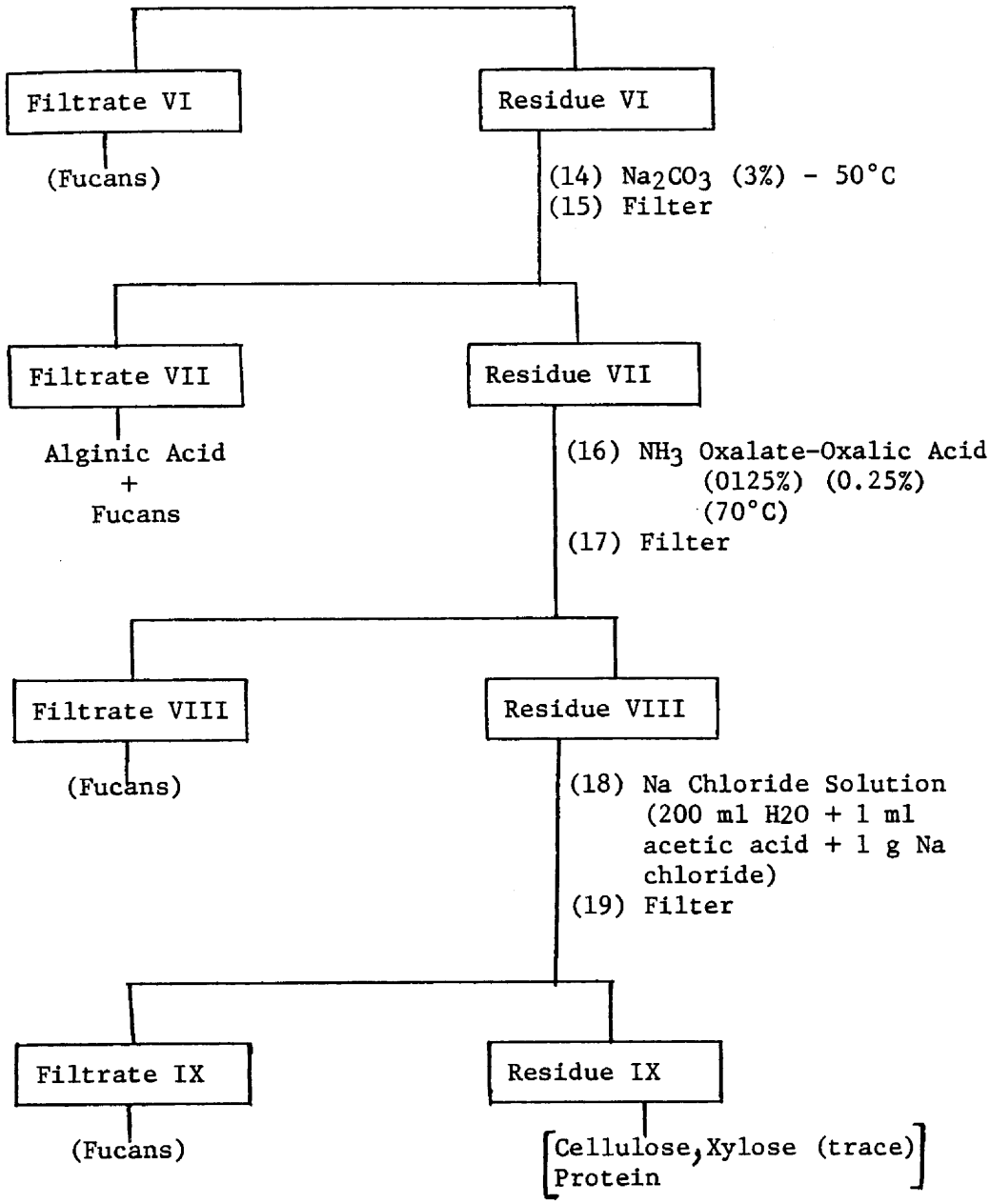

Fig. 2 (cont'd). Extraction and Fractionation of Seaweed Carbohydrates.

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CULTURAL REQUIREMENTS, YIELDS, AND LIGHT UTILIZATION EFFICIENCIES OF SOME DESERT SALINE MICROALGAE

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ABSTRACT

Approximately 100 strains of microalgae were isolated from saline waters of the deserts of easter California and western Nevada. The growth of 42 strains in artificial media was estimated visually. Thirty one isolates grew well; seven strains showed moderate growth; and the others grew only poorly. In 6-liter mass cultures, roughly calculated yields of 9 isolates ranged from 11.5 to 45.8 gm dry weight m^{-2} day^{-1} using either nitrate or urea as nitrogen sources. Most cultures did not grow on ammonium at the high pH levels of these media. Depending on the species tested, eight of these algae grew at temperatures ranging from 11-35°C, but best growth was at 20-25°C. Since the algae were isolated from waters of varying salinity, they also had varying salinity requirements, but generally grew best at the salinity prevailing in the waters from which they were taken. They grew poorly in completely fresh water. In two experiments testing growth at varying light intensities, Ankistrodesmus falcatus and Oocystis pusilla grew best at 50-60% of the maximum sunlight irradiance reported for La Jolla --225 to 270 cal cm^{-2} day^{-1}. At these light levels the yield of Ankistrodesmus was 21.9 gm m^{-2} day^{-1} and that of Oocystis was 25.7 gm m^{-2} day^{-1}. Corresponding efficiencies of utilization of light in the photosynthetically active spectral range were 4.9% and 5.7%, respectively. In one experiment to maximize yields of Ankistrodesmus using culture chambers of varying thickness, the yields in 5- and 10-cm thick cultures were 18.8 and 26.3 gm m^{-2} day^{-1}, respectively. In a second experiment, yields in 5-, 10- and 15-cm thick cultures were 41.1, 40.2, and 52.7 gm m^{-2} day^{-1}, respectively.

INTRODUCTION

Since they have abundant resources of land, sunlight, and saline water which cannot generally be used for conventional agriculture, the American deserts are an attractive area in which to locate outdoor facilities for growing microalgae as energy and/or food and fodder resources. The concept of utilizing desert areas for these purposes has been previously described (1).

Algal Yields

Yields of microalgal cultures both in the laboratory and in outdoor ponds have been summarized previously (2,3,4,5). Generally yields are reported as gm dry weight m^{-2} day^{-1} (multiply by 1.4 for tons $acre^{-1}$ $year^{-1}$ and by 3.7 for tons ha^{-1} $year^{-1}$).

Yields of 30-40 gm m^{-2} day^{-1} have been regularly achieved, but average yields over long periods were somewhat less. However, there is one report of research in which laboratory yields of > 700 gm m^{-2} day^{-1} were achieved with the high temperature strain of Chlorella grown at light intensities of about 7x that of full noon sunlight (6). Thus, with proper mixing, temperatures, adequate nutrients and CO_2 supplies, etc. it seems possible to "push" algal cultures to very high yields. One might expect that, with the development of the right technology and selection of the best strains of algae, yields in excess of 50 gm m^{-2} day^{-1} might be achieved in desert outdoor cultures.

Efficiencies of Light Utilization by Algae.

Efficiencies of light utilization are generally reported as the percentage of photosynthetically active radiation --light in the spectral region 400-700nm (PAR)-- that is fixed as cellular energy (calories).

Algae are attractive as a biomass source, not only because of anticipated high yields, but because they utilize light at efficiencies 5-7 times greater than higher plants (7). Efficiencies for various algae range from about 3% up to 20% (2,3,4,5,7,8,9).

Objectives

The objectives of the research reported herein were 1) to measure the abilities of desert saline algae to grow on artificial salts media simulating the water from which they were isolated; 2) to obtain rough data on yields in larger-scale cultures that were aerated and grown to very high densities; 3) to determine the best ranges of temperatures and salinities; 4) to measure the light intensity requirements of dense cultures; and 5) to maximize yields by varying culture thickness. This research is a process of selection of microalgae from desert saline environments which may be useful for outdoor mass cultures in the deserts of the southwestern United States.

MATERIALS AND METHODS

Field Trips

Seven field trips were completed to the deserts of eastern California and western Nevada. Figure 1 shows the general locations that were sampled. These trips were made to two established field stations: the Sierra Nevada Aquatic Research Laboratory (SNARL) run by the University of California near Mammoth Lakes, California and the Desert Studies Center (Zzyzx Springs) run by the California State University System near Baker, California. Various saline waters and soils were sampled from these field stations.

Microalgal Isolations

The growth of algae in water samples was stimulated by setting up enrichment cultures. Nitrate (10μM), phosphate (1μM), silica (10μM) and trace metals (Chapman formulation --see below) were added to 50 ml of natural waters contained in 125 ml flasks. Agar plates were also prepared from enriched natural water. At SNARL these cultures were incubated in a north window (natural diffuse light) or in a lighted incubator at the natural temperature and a 12:12 hr light:dark cycle. After growth was apparent the algae were isolated by micropipetting and washing of several cells of the same species under the inverted microscope. Further isolations were made at our La Jolla laboratory and all isolations from the Zzyzx locations were made at La Jolla. Soil algae were obtained from biphasic soil --Zzyzx medium enrichment cultures. Soil algae were also obtained from Winogradsky columns (10) incubated in a north window at La Jolla.

Chemical Analyses

Inorganic chemical analyses were carried out on these samples by a commercial laboratory, E. S. Babcock and Sons, Riverside, California. The data were used to formulate artificial media simulating each water source that was sampled and from which algae were isolated.

Media

The media formulations are presented in Table 1. Their pH was high due to high levels of carbonate and/or bicarbonate. Nitrogen sources, phosphate, and soluble silica were added as needed for each experiment. Vitamins (thiamin, biotin, and B_{12}) were sometimes added using the f/2 formulation given by Guillard (11) and trace metals and Fe were added in the concentrations suggested for the Mono Lake medium designed by D. Chapman (personal communication). These trace metal salt concentrations were $ZnSO_4 \cdot 7H_2O$, 84.4 μg $liter^{-1}$; H_3BO_3, 600 μg $liter^{-1}$; $CoCl_2 \cdot 6H_2O$, 150 μg $liter^{-1}$; $CuSO_4$, 37.5 μg $liter^{-1}$; $MnCl_2 \cdot 4H_2$), 10 mg $liter^{-1}$; $(NH_4)_2MoO_4$, 370 μg $liter^{-1}$; and NaFeEDTA, 10 mg $liter^{-1}$. All media were filter sterilized through Gelman 0.22 μm filters and major and minor nutrients were then added aseptically.

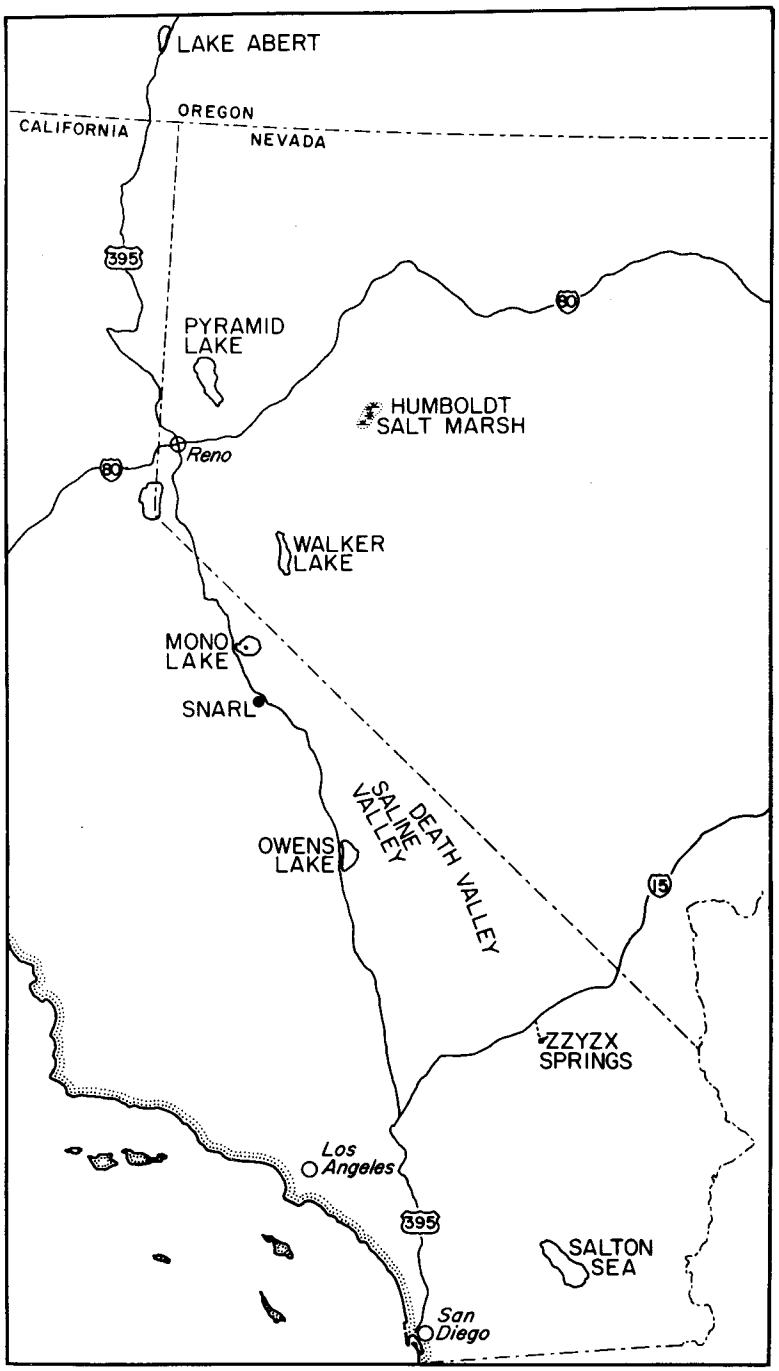

Figure 1. General location of areas sampled in the desert of eastern California and western Nevada.

TABLE 1. Desert Saline Water Media

	Location					
	Pond A	Pond B	Pyramid Lake	Black Lake	Owens Lake	Walker Lake
	mg liter^{-1}					
NaCl	443	793	3271	4031	2608	4075
$NaHCO_3$	370	1596	1176	3515	1332	2184
Na_2CO_3	423	741	392	10433	4932	1322
$CaCl_2$	13	17	28	22	42	28
$MgSO_4 \cdot 7H_2O$	104.4	57.3	--	90.2	194.5	790
KCl	82	237	246	1571	1614	430
Na_2SO_4	--	176	207	4740	2477	3392
$Na_2B_4O_7 \cdot 10H_2O$	68.2	176.3	9.48	87.2	528.9	168.7
NaF	9	22	11	42	11	9
$MgCl_2 \cdot 6H_2O$	--	--	508	--	--	--

TABLE 1. (Cont'd)

	Location					
	Saline Valley	ZZYZX	Armagosa River	Sperry River	Harper Lake	Salt Creek
	mg liter^{-1}					
NaCl	242790	1762	3174	1769	12,191.2	2905
$NaHCO_3$	202	382	1327	1008	84	327.6
Na_2CO_3	--	192	1185	666.5	105.8	--
$CaCl_2$	610	78	22.2	77.9	1207	360.7
$MgSO_4 \cdot 7H_2O$	4517	86	82.6	246.5	1435	2059
KCl	2871	38	183.4	137.9	91.7	99.2
Na_2SO_4	13494	615	3652	3112	3728.55	221.6
$Na_2B_4O_7 \cdot 10H_2O$	1369	53.1	339.4	171.52	213.6	34.3
NaF	5	29	17.6	12.6	3.36	17.6
$MgCl_2 \cdot 6H_2O$	--	--	--	--	--	--

Algal Strains

The various strains of desert microalgae that have been established are given in Table 2 along with temperature and salinity data for each location from which they were isolated. Not all of the strains are unialgal and these await further separation by micropipetting. Most of the strains have been identified to genus and only a few of the strains which have been extensively investigated have been identified to the species level. All of these strains are now maintained in artificial media.

Preliminary Cultures

Various strains were grown in artificial media to see which ones gave the best growth. These cultures were contained in 50 ml of media in 125-ml flasks. The cultures were incubated for one week at a constant light intensity of 0.05 ly min^{-1} (PAR) supplied by cool-white fluroescent lamps and at a temperature of 21°C. Light intensity was measured with a Li-Cor No. 185 meter and a No. 190-SEB flat-response sensor. Growth was then estimated visually and rated +, ++, +++, or ++++ depending on the growth that was observed. These ratings were always done by the same person to keep them less subjective and relatively uniform from culture to culture.

Mass Cultures

Larger scale cultures of the algae that showed the best growth in preliminary cultures were established in 6 liters of medium contained in 9-liter serum bottles. The medium was supplemented with 20 mg-at N mg $liter^{-1}$ as ammonium, nitrate, or urea and with 2 mg-at P mg $liter^{-1}$ as K_2HPO_4. Trace metals (Chapman formulation) were added along with vitamins (11). These cultures were incubated at 21-22°C in a water bath and were illuminated from below with cool white Powergroove fluorescent lamps at an intensity (PAR) of 0.056 ly min^{-1}. The light was on a 12:12 hr light:dark cycle. When the cultures became dense they were bubbled with 1% CO_2-in-air at approximately 2,000 ml min^{-1}. When growth slowed down, more nutrients were added at the original concentrations. Growth was estimated daily by optical density measurements at 750 nm and by filtering aliquots onto tared Whatman GF/C glass fiber filters for cellular dry weight determinations. The filters were washed with isotonic ammonium formate solution. Blank dry weight filters were prepared by filtering sterile media followed by washing with ammonium formate. The filters were dried overnight at 70°C and weighed to the nearest 0.01 mg on a Cahn microbalance. Dry weights generally correlated very well ($r > 0.9$) with optical densities and this regression relationship was used in subsequent light intensity experiments (see below) to obtain daily estimates of dry weight increases. Growth curves were plotted from dry weights and optical densities as a function of time in days. At high culture densities these plots were linear and regression analyses were used to obtain dry weight changes per day. These were then transformed to rough yield figures over the illuminated bottom area of the bottle. The yield data are rough because light was received through the sides of the bottles as well as through their bottoms. Rough light

Table 2. Cultures of Desert Algae Isolated and Maintained by the IMR Phytoplankton Resources Group.

Organism		Date Sampled	Water Temp.	TDS (gm liter^{-1})
CALIFORNIA (SNARL)				
Owens Lake Unialgal				
OL1-1	Blue-green	Oct 11 1982	27.5	11.9
6-1	Blue-green	Oct 11 1982	27.5	11.9
OL-10	Cymbella	May 04 1983	16	29.5
4-1	Blue-green	Oct 11 1982	27.5	11.9
Owens Lake Not Unialgal				
OL-7	Chaetoceros	Oct 11 1982	27.5	11.9
OL-3	Myremecia	Wayne Armstrong Sample		
OL-9	Chaetoceros	Oct 11 1982	27.5	11.9
	Chaetoceros	Oce 11 1982	27.5	11.9
Saline Valley Unialgal				
10-2	Dunaliella	Oct 11 1982	25.4	254.9
15-1	Dangeardinella	Oct 11 1982	25.4	254.9
10-1	Dangeardinella	Oct 11 1982	25.4	254.9
12-1	Dunaliella	Oct 11 1982	25.4	254.9
Pond A (Big Alkali Lake) Unialgal				
PA-3	Nitzschia	Oct 01 1982	9.6	1.4
PA-1	Pinnularia	Apr 30 1983	5.0	1.2
PA-5	Coelastrum	Jul 03 1983	29.5	5.0
PA-6	Nitzschia	Jul 03 1983	29.5	5.0
PA-7	Green flagellate	Jul 03 1983	29.5	5.0
PA-8	Nitzschia	Jul 03 1983	29.5	5.0
Pond A (Big Alkali Lake) Not Unialgal				
PA-2	Green flagellate	Apr 30 1983	5.0	1.2
87-1	Cymbella	Oct 01 1983	9.6	1.4
Pond B (Big Alkali Lake) Unialgal				
PB-1	Chlorella	Sep 29 1983	2.0	3.2
PB-8	Chlorella	Sep 29 1983	2.0	3.2
82-1	Cymbella	Sep 29 1983	2.0	3.2

84-1	Yellow flagellate	Sep 29 1983	2.0	3.2
PB-2	Microcystis	Apr 30 1983	6.5	1.0
PB-5	Chlorella	Jul 03 1983	29.5	5.0
PB-6	Oocystis	Jul 03 1983	29.5	5.0
PB-7	Cymbella	Jul 03 1983	29.5	5.0
PB-9	Nitzschia	Apr 30 1983		

Pond B (Big Alkali Lake) Not Unialgal

PB-4	Botrycoccus	Jul 03 1983	29.5	5.0

Spring 10/1 (Hot Spring nearr Big Alkali Lake) Unialgal

96-1	Chlorella	Oct 01 1982	*1.2	
97-1	Chlorella	Oct 01 1982	*1.2	

Black Lake Unialgal

78-1B	Green flagellate	Oct 03 1982	13.2	26.5
77-1	Chaetoceros	Oct 03 1982	13.2	26.5
BL-3	Nitzschia	Jul 02 1983	20.0	9.5

Black Lake Not Unialgal

78-1A	Cymbella	Oct 03 1983	13.2	26.5
11-15	Cymbella	Oct 03 1982	13.2	26.5
76-2	Chaetoceros	Oct 03 1982	13.2	26.5
	Yellow flagellate	Oct 03 1982	13.2	26.5
75-2	Mixture	Oct 03 1982	13.2	26.5
BL-1	Nitzschia	Apr 30 1983	14.0	7.1
BL-2	Achnanthes	Apr 30 1983	14.0	7.1

NEVADA (SNARL)

Pyramid Lake Unialgal

20-1	Yellow round	Oct 14 1982	17.4	4.9
24-1	Anacystis	Oct 14 1982	17.4	4.9
26-1B	Nannochloris	Oct 14 1982	17.4	4.9
91-1	Ankistrodesmus falcatus	Oct 14 1982	17.4	4.9
P-3B	Synechococcus	May 16 1983	15.0	4.7
PL-1	Nitzschia	May 16 1983	15.0	4.7
PL-5	Green flagellate	May 16 1983	15.0	4.7
26-1A	Nannochloris	Oct 14 1982	17.4	4.9
PL-2	Green round	May 16 1983	15.0	4.7
PL-6	Green ovoid	May 16 1983	15.0	4.7

Pyramid Lake Not Unialgal

C-1	Nannochloris	Oct 14 1982	17.4	4.9
PL-4	Coccoid green	May 16 1983	15.0	4.7

Walker Lake Unialgal

28-1	Cyclotella	Oct 05 1983	18.0	10.6
31-1	Anacystis	Oct 05 1982	18.0	10.6
32-1	Oocystis	Oct 05 1982	18.0	10.6
30-1	Yellow-green flagellate	Oct 05 1982	18.0	10.6
36-1	Yellow-green flagellate	Oct 05 1982	18.0	10.6
WL-1	Green ovoid	May 17 1983	13.5	10.3
WL-2	Green flagellate	May 17 1983	13.5	10.6
WL-3	Nitzschia	May 17 1982	13.5	10.6
WL-4	Nannochloris	May 17 1983	13.5	10.6
WL-5	Desmococcus	May 17 1983	13.5	10.6
WL-6	Cyclotella	May 17 1983	13.5	10.6
WL-8	Chlorococcum	May 17 1983	13.5	10.6

Walker Lake Not Unialgal

33-1	Cyclotella	Oct 05 1982	18.0	10.6
34-1	Blue-green	Oct 05 1982	18.0	10.6
35-1	Nannochloris	Oct 05 1982	18.0	10.6
WL-7	Chlorella	May 17 1983	13.5	10.3

Columbus Salt Marsh Unialgal

47-1	Chlorococcus	Oct 13 1982	11.2	*60.8

Pond #2 off Highway 80 Unialgal

2R80-1	Chlorella	May 17 1983	20.0	5.9
2R80-2	Navicula	May 17 1983	20.0	5.9
2R80-3	Chlorella	May 17 1983	20.0	5.9

Big Soda Lake Unialgal

BS-1	Nitzschia	May 17 1983	15.0	20.7
BS-3	Nitzschia	May 17 1983	15.0	20.7
BS-2	Desmococcus	May 17 1983	15.0	20.7
BS-4	Chlorella	May 17 1983	15.0	20.7
BS-5	Chlorella	May 17 1983	15.0	20.7

CALIFORNIA (ZZYZX)

Lake Tuendae Unialgal

49-1a	Chaetoceros	Oct 22 1982	25.0	*2.8
Z-2	Selenastrum	Aug 17 1983	27.5	2.7
Z-3	Chlorella	Aug 17 1983	27.5	2.7
50-1	Yellow round	Oct 22 1982	25.0	*2.8
54-1	Pennate diatom	Oct 22 1982	25.0	*2.8

NC Spring Unialgal

Z-1	Chlorella	Oct 22 1982	23.0	*4.8
64-1	Anacystis	Oct 22 1982	23.0	*4.8

NC Spring Not Unialgal

62-1	Green + flagellate	Oct 22 1982	23.0	*4.8

Harper Lake Unialgal

HL-2	Oocystis	Feb 03 1983	9.0	15.8
HL-6	Small green flagellate	Feb 03 1983	9.0	15.8

Harper Lake Not Unialgal

HL-1	Cryptomonas	Feb 03 1983	9.0	15.8
HL-3	Oocystis	Feb 03 1983	9.0	15.8
HL-4	Oocystis	Feb 03 1983	9.0	15.8

Sperry River Unialgal

SR-1	Coccomyxa	Feb 01 1983	14.0	5.5

3-BATS Unialgal

37-1	Cosmarium	Oct 22 1982	24.0	*4.7
39-2	Anacystis	Oct 22 1982	14.0	*4.7

3-BATS Not Unialgal

44-1	Chaetoceros	Oct 22 1982	24.0	*4.7

Armagosa River Unialgal

AR-1	Green flagellate	Feb 01 1983	8.4	8.4
AR-2	Nitzschia sigmoida	Aug 16 1983	24.0	2.2
AR-3	Nitzschia	Aug 16 1983	24.0	2.2a

Armagosa River Not Unialgal

AR-A	Cyclotella	Aug 16 1983	24.0	2.2

Salt Creek Unialgal

SC-1	Cyclotella	Aug 16 1983	27.0	4.4

MISCELLANEOUS CALIFORNIA CULTURES

Salton Sea Unialgal

SS-1	Chaetoceros	Apr 07 1983	20.7	39.0
SS-2	Dunaliella	Apr 07 1983	20.7	39.0
SS-3	Nitzschia	Apr 07 1983	20.7	39.0
SS-4	Dunaliella	Apr 07 1983	20.7	39.0
SS-6	Pennate (agar)	Apr 07 1983	20.7	39.0
SS-7	Flagellate	Apr 07 1983	20.7	39.0

SS-8	Cryptomonad	Apr 07 1983	20.7	39.0
SS-9	Botryococcus	Apr 07 1983	20.7	39.0
SS-10	Green oval	Apr 07 1983	20.7	39.0
SS-11	Dunaliella	Apr 07 1983	20.7	39.0

Salton Sea Not Unialgal

SS-1	Navicula + green alga	Apr 07 1983	20.7	39.0
SS-5	Green colony	Apr 07 1983	20.7	39.0

Mono Lake Unialgal

S-16	Nitzschia	David Chapman Sample
S-9	Coccomyxa	David Chapman Sample
MO-2	Ctenocladus circinnatis	David Herbst Sample

Soil Algae

RT6-1	Myrmecia (Rts. 295 + 6)	Sep 21 1983
RT6-2	Nannochloris (Rts. 295 + 6)	Sep 21 1983
SP-1	Dunaliella (Silver Peak)	Sep 21 1983
B-1	Chlamydomonas (Bad water)	Sep 21 1983
CSM-1	Chlorogonium (Columbus Salt Marsh)	Sept 21 1983

OREGON SAMPLES

Abert Lake Unialgal

AB-1	Ctenocladus circinnatis	David Herbst Sample

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*TDS calculated from conductivity measurements.

utilization efficiency values were also calculated from the dry weight changes assuming a cellular caloric value of 5,000 cal gm^{-1} dry weight. This estimation gives cellular calories formed per unit time and this was divided by the light in calories supplied per unit time to obtain the percent efficiency values.

Temperature - Salinity Experiments

The apparatus and methods of assessing the optimum temperatures and salinities for desert algal growth have been described previously (12) Algae were incubated at a continuous PAR of 0.156 ly min -1 supplied by Powergroove cool-white fluorescent lamps. Fifty-ml culture suspensions were contained in 65-ml Pyrex bottles which were placed in 50-cm diameter holes in the aluminum temperature gradient block A described previously (13). The block was maintained in a constant-temperature room and temperatures along one axis of the block ranged from 11-35 C. Salinity was varied in five increments along the other axis of the block. Thus there was an array of 30 different combinations of temperature and salinity. In each experiment a different set of salinities was used because the various algae were obtained from natural waters of varying salinity. The salinities of these artificial media ranged from 0-2x that of the natural waters.

Growth (optical density) was measured daily by placing each of the 30 bottles successively in a capped, opaque PVC tube, thus interrupting a light beam provided by a precision voltage-regulated incandescent lamp. The Li-Cor Model 190-SEB flat response sensor was mounted onto the PVC tube in a precise location across from the lamp to receive maximum intensity of the light beam. Each culture bottle was calibrated and marked prior to an experiment at the position giving the maximum light intensity when filled with sterile, deionized water. For the optical density equation ($-\log I_t/I_o$), the uninterrupted standard light value (I_o) was obtained from the mean of several measurements in the bottle containing sterile deionized water taken before and after processing the culture samples. These standard and treatment signals were either calculated manually or sent to a microcomputer. Optical densities were recorded daily as a function of salinity and temperature either manually or by the computer and the resulting plots were contoured by eye to determine the effects of temperature and salinity on growth. After a few days of each experiment, responses to these variables were obtained that were consistent from day to day and representative plots of these responses are given herein (see RESULTS).

Light Intensity Experiments

For these experiments cultures of a given strain were contained in a vertically mounted 5-cm thick transparent plexiglass culture vessel which was divided into five compartments that were 5 cm wide. The sides of the vessel and the compartment walls were opaque plastic so that light would not be received from the sides. The light source was directed laterally toward the front of the vessel and consisted of a 2000 watt tungsten-halide lamp mounted in a parabolic stage light reflector as previously described (2). The light was filtered through

7 cm of flowing tapwater to remove infra-red radiation. Various intensities --30, 40, 50, 60, 70% summer maximum sunlight at La Jolla (450 ly day^{-1}) as reported by Strickland (14)-- were achieved by the placement of the compartments away (50%, 60%) from the center of the light field (70%) and by filtering the light on two compartments away from the center one through Nitex netting (30%, 40%). Light received by each compartment was measured over its area with the LiCor flat response PAR sensor and the measurements were integrated by computer to determine that the nominal intensities were achieved. Each compartment was filled to 0.83 liters volume with the test culture grown up as reported above (see Mass Cultures) and diluted out to an optical density of 0.1 with sterile artificial medium. The medium was supplemented with nutrients at the above concentrations (see Mass Cultures) and either nitrate or urea were used as N sources. The cultures were mixed with air at 2,600 ml min^{-1} delivered through Pyrex tubes extending to the bottom of each compartment. When the cultures reached an optical density of about 0.3, 1% CO_2 was added to the airstream.

Growth was measured daily as optical density at 750 nm and by dry weight measurements (see Mass Cultures). After OD reached about 1.0, growth was linear. Precise yields were calculated from regressions of measured dry weight and that calculated from OD determinations versus days over the illuminated area of each compartment. Light utilization efficiencies were calculated as in the Mass Culture experiments, but are more precise since light did not pass through the sides of each compartment.

Maximizing Yields

Experiments to maximize yields by varying culture thickness were carried out in chambers having an illuminated area of 703.5 cm^2 that were previously described (2). These chambers had inner thicknesses of 5, 10, and 15 cm. *Ankistrodesmus falcatus* from Pyramid Lake was used as a test alga. The chambers were illuminated at 50% La Jolla sunlight intensity since this was found to be the best intensity in Light Intensity experiments (see Results and Discussion). The medium containing nitrate as an N source used in the Mass Culture and Light Intensity experiments was also used in these experiments. The cultures were mixed vigorously by aeration with 1% CO_2-in-air through holes in the bottoms of the chambers. The aeration rates in the cultures were 2.7, 7.2, and 14.5 liters min^{-1} in the 5- 10- and 15-cm cultures, respectively. Visual observation indicated that the turbulences in each culture were similar and high. Growth was measured by optical density at 750 nm and by dry weight measurements, and yields and efficiencies were calculated as in the Light Intensity experiments.

RESULTS AND DISCUSSION

Inorganic Chemical Composition of Desert Natural Water

Extensive data on the inorganic chemical composition of these waters are given in a series of Appendix tables. The salinity or total dissolved solids ranged from nearly freshwater (the Mohave River -- 705 mg $liter^{-1}$) to the saturated brine at the salt lake in the Saline Valley -- 79 to 323 x 10^3 mg $liter^{-1}$. At this location the salinity varied seasonally and was less in the spring than in the summer or fall; this effect is probably due to rainfall and runoff in the spring. Similar effects were observed for other locations -- Big Alkali Lake (Ponds A B) and Black Lake. Salinities were more stable in the large lakes (Mono, Pyramid, Walker, and Big Soda).

Most of these waters had high levels of carbonate and bicarbonate and thus their pH values were generally >9.0. Exceptions were the pH of 4.9 at Brady Hot Springs, 6.9 in the Salton Sea, and 7.1-7.5 in the Saline Valley salt lake. Thus most of these waters contain enough carbon as bicarbonate or carbonate that natural algal growth would not be limited by carbon deficiency. The principal major cation was sodium and these waters were high in the anions chloride and sulfate. They were uniformly low in nitrogenous and phosphoric nutrients. Most minor metals were present in very low or undetectable amounts except for Fe. These waters generally contained high amounts of B or Fe. Arsenic was high in Owens Lake water and in that of the Humboldt Salt Marsh.

Mason (15) has summarized the chemical composition of Mono Lake. His value for salinity is about 72 grams $liter^{-1}$. Our values are slightly higher. Our corresponding values for individual major and minor elements are also slightly higher that those reported by Mason. This may reflect increases due to diversion of input water and subsequent evaporation since the 1960's. Our salinity values for Pyramid Lake are similar to those reported by Galat et al. (16) for the years 1976-77 and the ionic compositions reported herein (see Appendix) are also similar to the earlier values. Koch et al (17) have summarized chemical data for Walker Lake from 1937-1975. In 1937 the salinity as 5430 mg $liter^{-1}$ and was 10,300 mg $liter^{-1}$ in 1975. We report one low value -- 4720 in July of 1983 -- but otherwise our values are similar to the 1975 values. Our low value may be in error, but the sodium and chloride values are also lower on that date than the values of Koch et al or our earlier ones. Otherwise the individual ionic concentrations for Walker Lake given in the appendix are similar to those reported by Koch et al for 1975. Big Soda Lake is meromictic in that relatively fresh, O_2-rich water overlies a layer of more saline anoxic water (18,19,20) that is also high in nutrients. We sampled only surface water and our values for surface chloride are almost identical to those reported earlier (18,19). Earlier complete chemical data are lacking for this lake. The salinity of the Salton Sea has increased since its formation in 1907. In 1955 the salinity was 33.7 gm $liter^{-1}$. Our value was 39 gm $liter^{-1}$. Compared to seawater the Sea is low in CO_3^{-2} and HCO_3^{-1} (21). Carbonate and bicarbonate are lower in the Salton Sea than in most of the other waters that we sampled and

this is reflected in the pH value -- 6.9 -- that we observed. Our values for the algal nutrients in the Sea -- NO_3^{-1}, NH_4^{+1}, and PO_4^{-3} are somewhat lower than those reported earlier (22).

The main reason for obtaining chemical data from these natural waters was to be able to formulate artificial media that were similar to their compositions. Such formulations were still not easy to achieve since the analyzed total cations in me $liter^{-1}$ were close to, but did not exactly equal, the analyzed total anions in me $liter^{-1}$. Table 3 compares the chemical composition of natural Pyramid Lake water with that of the Pyramid Lake artificial medium. It will be seen that the artificial medium is slightly higher in sodium and chloride than the natural water. Nevertheless, this medium supports the growth of algae from Pyramid Lake quite well (see below) when enriched with nitrogen, phosphorus, silica (for diatoms), trace metals and vitamins.

Growth in Preliminary Cultures

Table 4 shows visual estimations of growth in small scale cultures of 42 desert algal strains. Fifteen of these grew very well (++++); 16 grew well (+++); 7 grew moderately (++); and 4 grew only slightly (+). There seemed to be no pattern as to which kinds of algae from which locations succeeded or did not succeed. The algae were grown under standardized conditions and in media similar to the waters from which they were isolated. While these differences in growth may be genetic in origin, various algae may have different light and temperature optima and we used a standard set of physical conditions for all algae tested.

Growth in Mass Cultures

Certain of the better growing (++++ or +++) algae were tested in mass cultures which could be mixed by aeration. Three N sources were tested -- NO_3^-, urea, and NH_4^+. At the high pH values of most of these media, the latter source was lost as ammonia gas and in most cases no growth occurred. Rough yields and efficiencies of light utilization were calculated from the growth curves. Since dry weights generally correlated ($r > 0.95$) very well with optical density, data were obtained that could be used in calculating dry weights from OD measurements in subsequent experiments.

A typical growth curve for Nannochloris from Pyramid Lake is shown in Figure 2. Note that at OD values >0.8 growth was linear and not exponential. Such a linear relationship for algal growth in very dense cultures has been described previously (23) and was attributed to light limitation due to self-shading by the cells. Growth curves for Ankistrodesmus falcatus from Pyramid Lake -- the alga we have investigated most thoroughly -- are shown in Figure 3. Better growth was obtained using urea as an N source than with nitrate. In light intensity experiments (see below) this was not the case -- equivalent growth was obtained with each source.

Nine successful mass culture experiments were carried out. Two others were unsuccessful in that a Cryptomonas sp. from Harper Lake became

TABLE 3. Chemical Composition of Pyramid Lake Water and the Medium Derived from It.

	Natural Water Pyramid Lake	Pyramid Lake Medium
Date	10/4/82	
Ion (mg $liter^{-1}$)		
Ca	10	10.1
Mg	60	60.7
Na	1775	1909
K	128	129
CO_3	222	222
HCO_3	854	854
SO_4	140	140
Cl	2150	2279
F	5	5
B	11	10.7

TABLE 4. Growth in Preliminary Cultures of Desert Algae*

Location	Strain	Growth (Estimated Visually)
Owens Lake, California	Chactoceros	+++
	Myremecia	++++
Saline Valley, California	Dangeardinella	++, + (2)
Big Alkali Lake, California		
Pond A	Nitzschia	+
	Coelastrum	++
Pond B	Chlorella	++++, +++, ++ (3)
	Oocystis parva	+++
Zzyzx Springs, California	Unidentified (50-1)	+++
	Chlorella	++++, +++, +++ (3)
	Cosmarium	+++
Harper Lake, California	Oocystis	++++
	Unidentified flagellate	++++
Armagosa River, California	Unidentified flagellate	+++
	Nitzschia sigmoida	++
	Nitzschia sp.	+++
Salton Sea, California	Chaetoceros	+++
	Dunaliella	+++
	Nitzschia	++++
	Unidentified cryptomonad	++++
	Botryococcus	++
Mono Lake, California	Nitzschia	++++
	Coccomyxa	+++
Black Lake, California	Unidentified flagellate	+++
	Chaetoceros	+++
	Cymbella	++

TABLE 4. (Cont'd)

Location	Strain	Growth (Estimated Visually)
Pyramid Lake, Nevada	Unidentified (20-1)	+
	Nannochloris	++++, ++++ (2)
	Ankistrodesmus falcatus	+++
Walker Lake, Nevada	*Cyclotella*	++, + (2)
	Anacystis	++++
	Oocystis	++++
	Unidentified flagellates	++++, ++++ (2)
	Nannochloris	++++
Big Soda Lake, Nevada	*Desmococcus*	++++

*The numbers in parentheses indicate that multiple strains from the same source were tested and do not refer to separate experiments with the same strain.

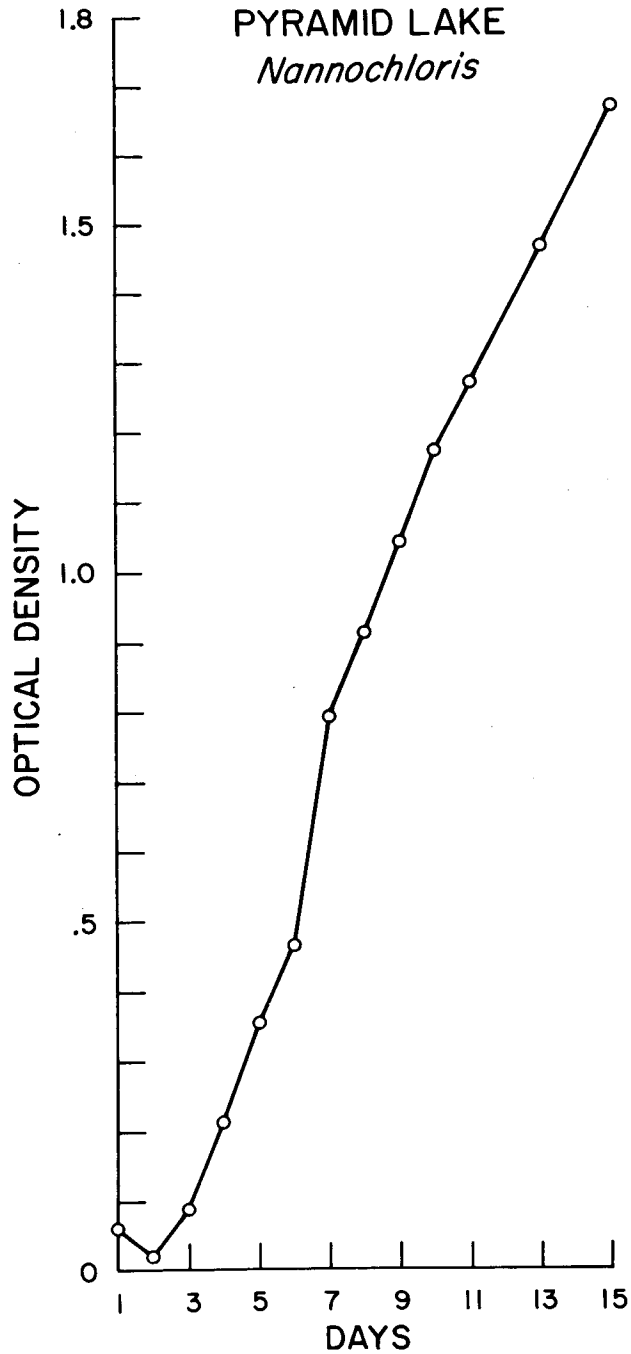

Figure 2. Increases in optical density of a Pyramid Lake *Nannochloris* culture grown on nitrate.

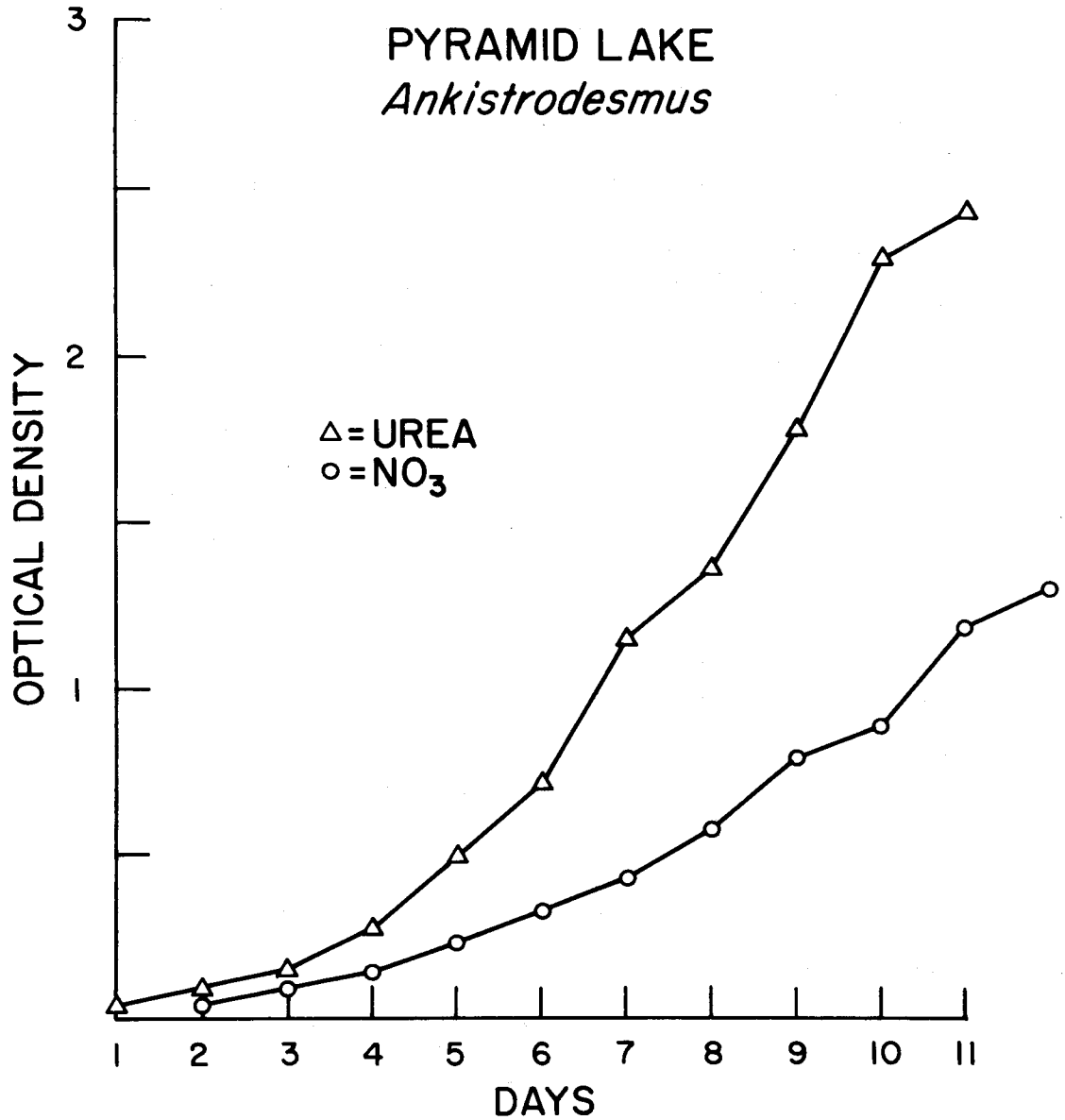

Figure 3. Increases in optical density of a Pyramid Lake *Ankistrodesmus falcatus* cultures grown on nitrate and urea.

contaminated and dry weight data from an experiment with Dangeardinella from the Saline Valley were erratic due to high salt residues on the dry weight filters. Biomass, yield, and efficiency data for nine experiments are summarized in Table 5. Yields generaly ranged from about 3 to 23 gm m^{-2} day^{-1} but the yields for Oocystis pusilla were exceptionally high. This result was not confirmed in a light intensity experiment with this species (see below) in which yields were estimated with more precision. Efficiencies of light utilization ranged from about 2% to 12% with the exception of those for Oocystis pusilla which ranged up to 28.3%. Maximum biomass figures ranged up to 2200 mg dry weight $liter^{-1}$.

Effects of Temperature and Salinity

Several temperature/salinity gradient experiments were carried out. Figures 4 and 5 show the results for Ankistrodesmus falcatus and Nannochloris sp. from Pyramid Lake. The optimum temperature for Ankistrodesmus was 26°C and was 16°C for Nannochloris. Optimal salinities for the two strains were 5.8-8.5 and 16 gm total dissolved solids $liter^{-1}$, respectively. Table 6 summarizes the data that were obtained for eight strains. Some strains grow well at high temperatures (> 30°C) and generally the range of optimal temperatures is 20-30°C. With the exception of the Mono Lake Nitzschia, none of our strains are truly high temperature species such as Spirulina or the high temperature strain of Chlorella (24) which has been used so widely in physiological studies. Salinity tolerances are similar to those from the waters from which these strains were isolated. None tolerated the lowest salinity tested.

Since most strains tolerated the highest salinity tested -- 2 times that of the natural water -- and did not grow well at the lowest salinity tested, these are truly halophilic algae. Previous studies of the Mono Lake Nitzschia showed that it would also not grow at salinities much higher that than presently in the lake (90 gm $liter^{-1}$) (25); we confirm this result. Dangeardinella and Dunaliella were isolated from saturated NaCl brine in the Saline Valley and thus would have a high tolerance of saline water. The latter species in probably the most salt-tolerant plant known -- it occurs in the Great Salt Lake, the Dead Sea, and other highly saline waters throughout the world.

Yields and Efficiencies of Dense Cultures at Various Light Intensities

Two experiments on the effects of high light intensities on yields and efficiencies of dense cultures were carried out. Figure 6 shows linear curves for Ankistrodesmus falcatus from Pyramid Lake at different light intensities. The duplicate points represent actual measured dry weights and those estimated from a regression of optical densities on measured dry weights. Yields and efficiencies were calculated from linear regressions of these growth curves. These values are shown in Figure 7. The highest yield was obtained at a light intensity 50% of the maximum summer daily light intensity measured in 1967 at La Jolla (14). Fifty percent of this maximum intensity is 225 cal cm^{-1} day^{-1}. Yields were reduced at lower intensities since light

TABLE 5. Mass Culture Biomass, Yield and Efficiency Data.

Strain	NO_3^-			Urea		
	Maximum Biomass (mg liter^{-1})	Yield (gm m^{-2}day^{-1})	Efficiency (% of Light Utilized)	Maximum Biomass	Yield (gm m^{-2}day^{-1})	Efficiency (% of Ligh Utilized)
Nitzschia, S-16 (Mono Lake)	640	12.4	7.7	848	8.8	5.4
Ankistrodesmus falcatus, 91-1 (Pyramid Lake)	700	11.5	7.2	1250	17.4	10.8
Nannochloris, 26-1A (Pyramid Lake)	1400	23.2	14.4	114	___**	___**
Oocystis pusilla, 32-1 (Walker Lake)	840	40.6	25.1	2290	45.8	28.3
Chlorella, Z-1 (Zzyzx - NC Spring)	650 550*	13.2 8.46*	8.2 5.2*	372	3.52	2.2
Chlorella, PB-1 (Big Alkali Lake - Pond B)	828	13.9	8.6	562	9.4	5.8
Chlorella, 96-1 (Zzyzx - NC Spring)	910	12.0	7.5	1100 790*	13.9 10.2*	8.6 6.3
Oocystis parva, PB-6 (Big Alkali Lake - Pond B)	897	21.7	13.5	162	___**	___**
Selenastrum, Z-2 (Zzyzx - Lake Tuendae)	1027	16.4	10.2	887	15.2	9.6

* At relatively low pH values in the Zzyzx media, growth occurred with ammonium as an N source an these values are reported.

** Little or no growth occurred using urea as an N source with these algae.

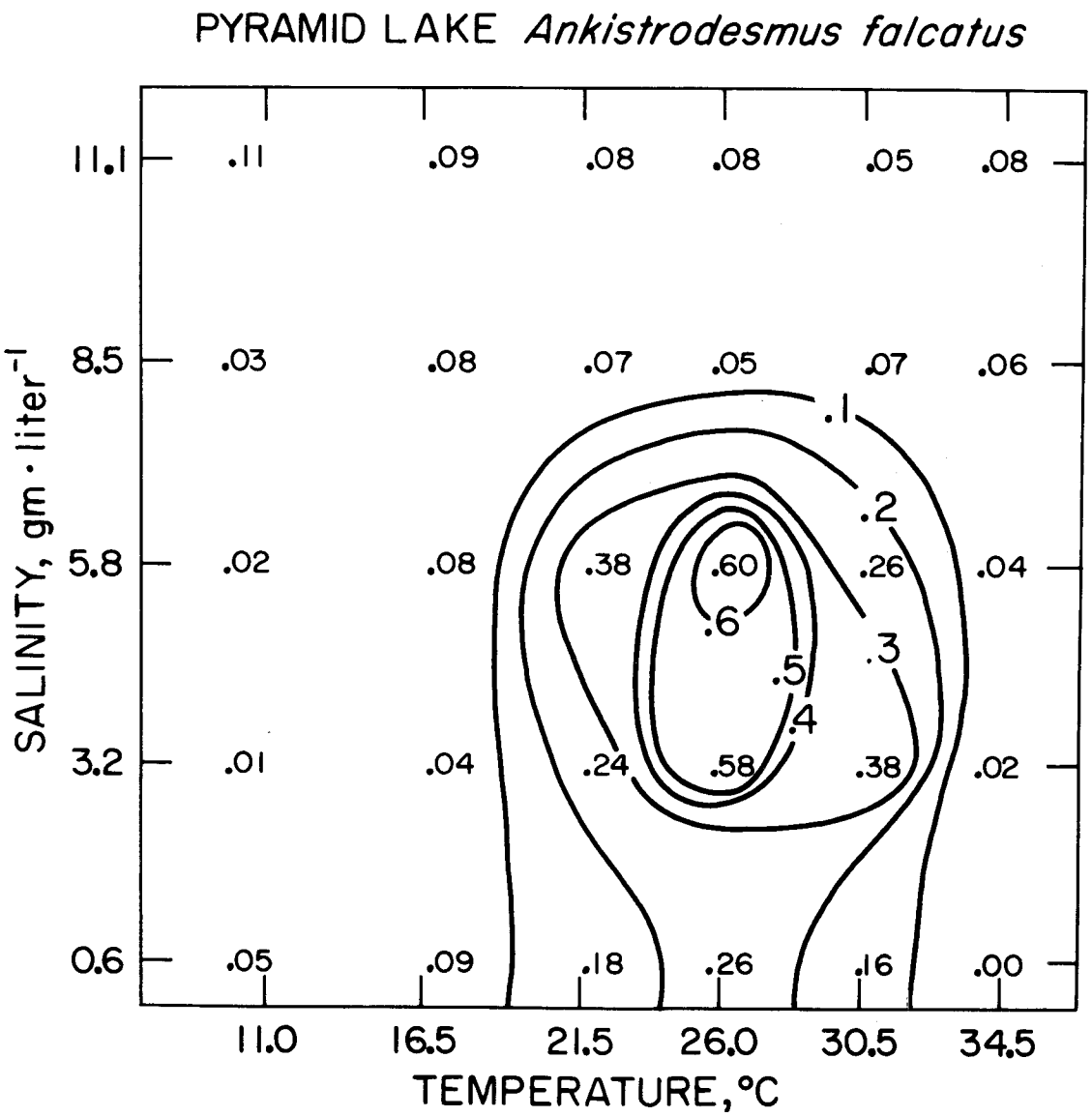

Figure 5. Optical densities of Pyramid Lake Nannochloris cultures as a function of temperature and salinity.

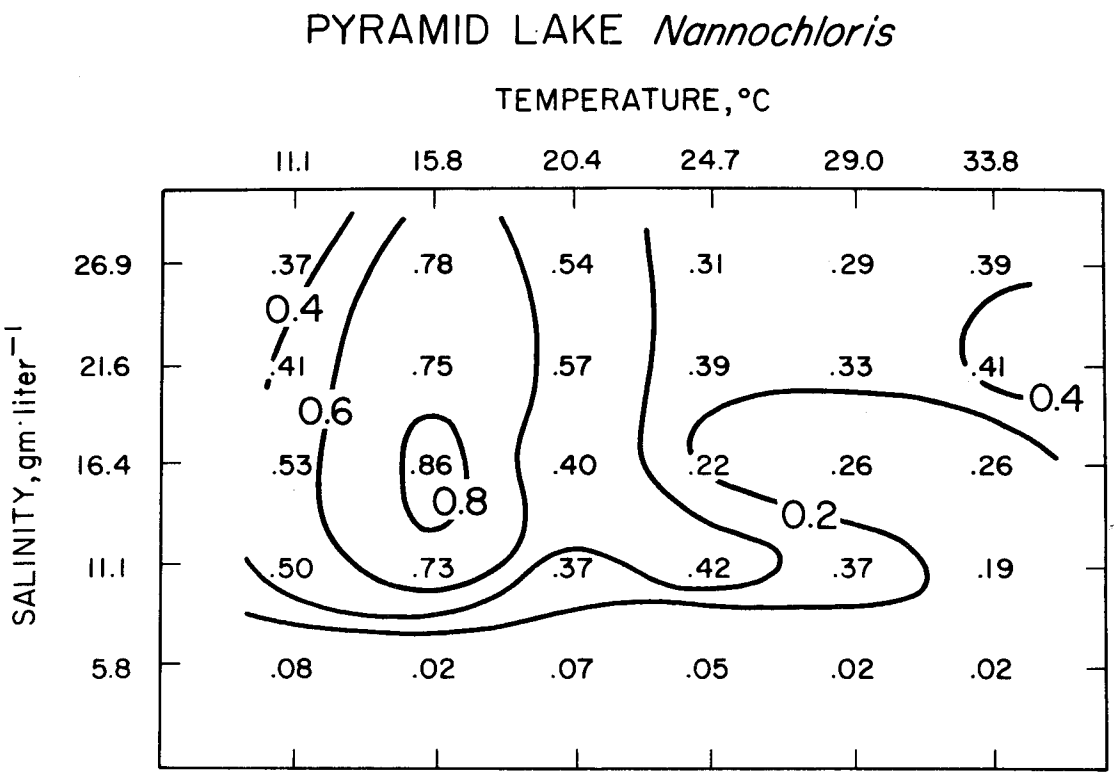

Figure 4. Optical densities of Pyramid Lake <u>Ankistrodesmus</u> <u>falcatus</u> cultures as a function of temperature and salinity.

TABLE 6. Summary of Temperature and Salinity Data.

Strain	Temperature, °C			Salinity, gm liter^{-1}		
	Best Growth	Some Growth	Litte or No Growth	Best Growth	Some Growth	Little or No Growth
Nitzschia, S-16 (Mono Lake)	30-36	15-37	10 41-44	50-70	28-86	9, 120
Ankistrodesmus falcatus, 91-1 (Pyramid Lake)	26	21-30	11-16 35	5.8-8.5	0.6-3.2	11
Nannochloris, 26-1A (Pyramid Lake)	16	11-34	--	16	11-27	5.8
Oocystis pusilla, 32-1 (Walker Lake)	20-25	16-33	11	18	12.4-24	1.2-6.8
Chlorella, Z-1 (Zzyzx - NC Spring)	15-25	30	9, 34	6.2-9	3.2, 12	0.3
Chlorella, 96-1 (Zzyzx)	20.5-26.5	30.5	34.5, 11.0	4.7-6.1	1.8-3.2	0.3
Oocystis parva, PB-6 (Big Alkali Lake - Pond B)	21-30	35	10-15.5	3.8-11	15	0.1
Selenastrum, Z-2 (Zzyzx - Lake Tuendae)	20-30	11-34.5	--	4.7-6.1	1.8-3.2	0.3

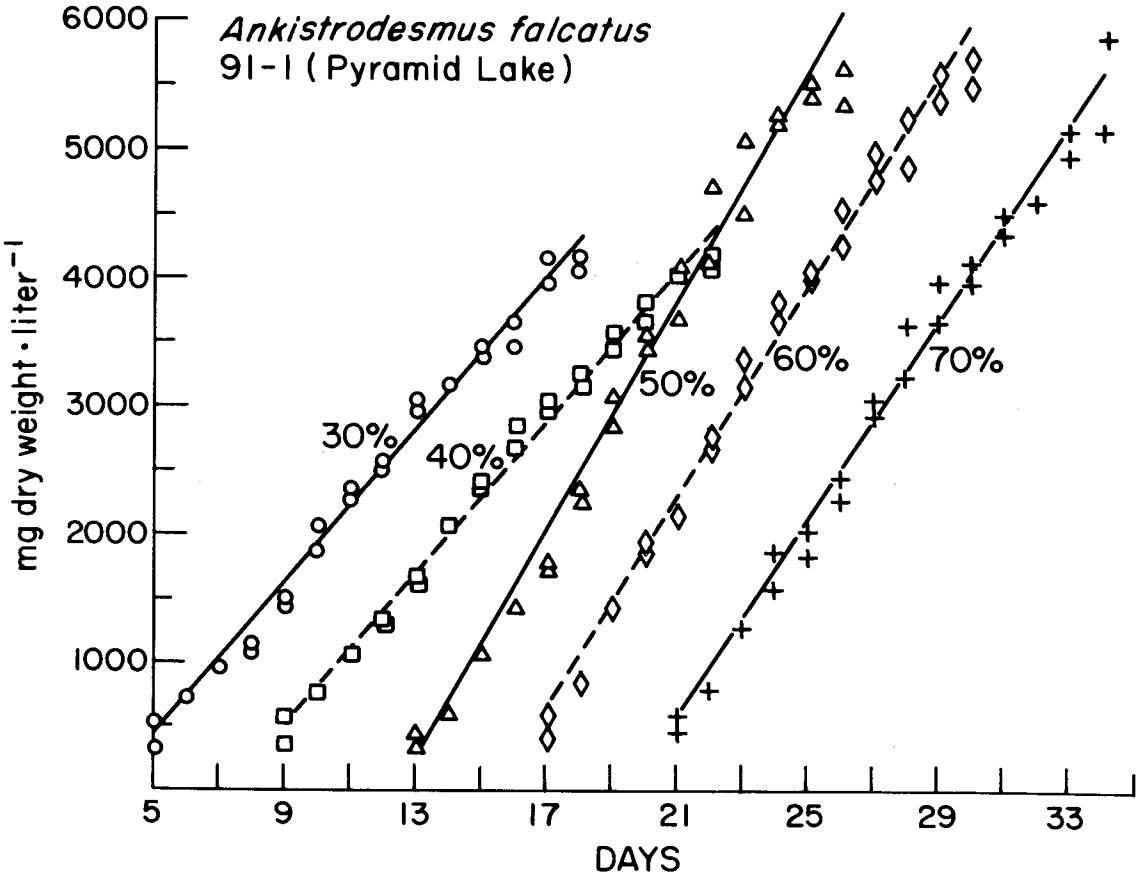

Figure 6. Increases in dry weight of Pyramid Lake Ankistrodesmus falcatus cultures at 30, 40, 50, 60, and 70% of maximum daily sunlight intensities at La Jolla, California. The growth curves are displaced for clarity.

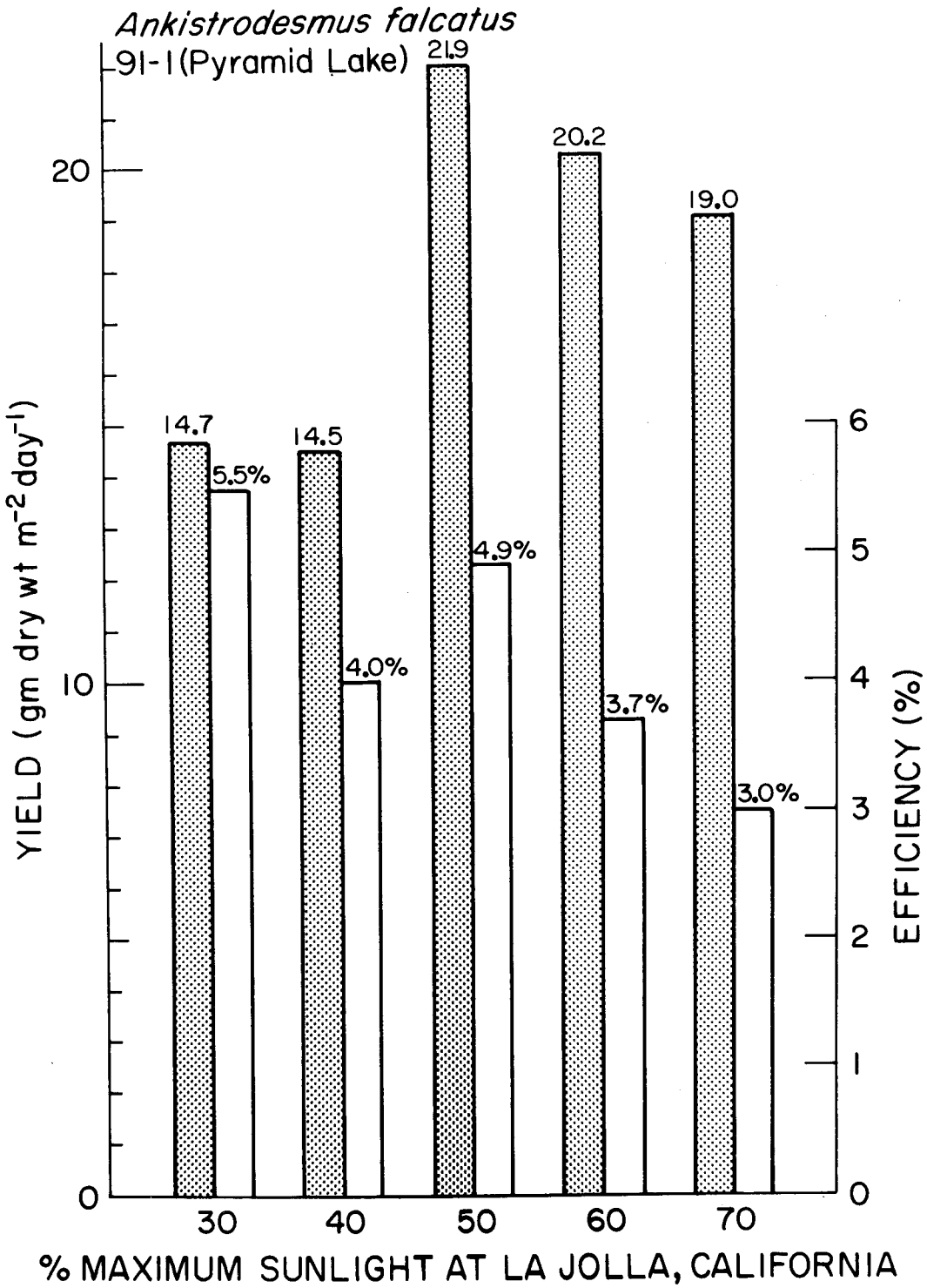

Figure 7. Yields and efficiencies of light utilization of Pyramid Lake *Ankistrodesmus falcatus* cultures grown at 30, 40, 50, 60, and 70% of maximum daily sunlight intensities at La Jolla, California.

was limiting. They were also reduced at higher intensities probably because of photoinhibition. The efficiency of light utilization was greatest at the lowest light level tested and was very much reduced at high intensities. These cultures were grown using nitrate as an N source. Contrary to data obtained in a previous mass culture experiment, yields were not increased when this experiment was repeated using urea as an N source. These yield data are more precise than those reported in Table 5 since no light came into the cultures through their sides.

Figures 8 and 9 show similar data for Oocystis pusilla from Walker Lake. The maximum yield was again found at 50% sunlight and was decreased at light levels greater or less than this value. Oocystis yields at each intensity were greater than those found for Ankistrodesmus. This result is similar to that given in Table 5, but Oocystis yields were less than the yields reported for mass cultures in bottles. The yields for this light intensity experiment again are more precisely estimated since light from the sides of the cultures was excluded. Efficiencies varied similarly to those in the Ankistrodesmus cultures.

In these dense cultures light only penetrated the first 5 mm of the culture and most of the cells were in a darkened layer in the remaining 45 mm thickness. Growth in this two-layered system then becomes dependent on proper turbulent mixing between the layers. Such mixing would result in a "flashing light" effect and growth was probably enhanced (26).

Photosynthesis and growth of various algae were previously reported to be saturated at somewhat lower intensities (29,28,29) but earlier data were not obtained with cultures as dense as ours. Thus, self-shading by the cells in our cultures probably accounts for this difference. With even greater densities than ours and intensities >> sunlight, much greater yields (approximately 700 gm m^{-2} day^{-1}) have been observed for Chlorella (6). Thus, in an outdoor pond, maximizing yields becomes a problem of providing the right culture density for the prevailing daily light intensity and mixing the pond properly so that the algae are in the lighted and dark layers for the best amounts of time.

Maximizing Ankistrodesmus Yields by Varying Culture Thickness

Yields are equivalent to growth rate (mg $liter^{-1}$ day^{-1}) x culture volume ÷ area illuminated (m^2). Growth rates at various light intensities are given for Ankistrodesmus falcatus and Oocystis pusilla in Table 7. The growth of Ankistrodesmus over a larger illuminated area was tested at a light intensity of 50% sunlight in three cultures of differing thicknesses (5, 10, and 15 cm) and accordingly different volumes (4, 8, and 12 liters). We hypothesized that the growth rates in the cultures would be similar provided mixing was similar but that yields would increase with thickness due to increasing culture volumes.

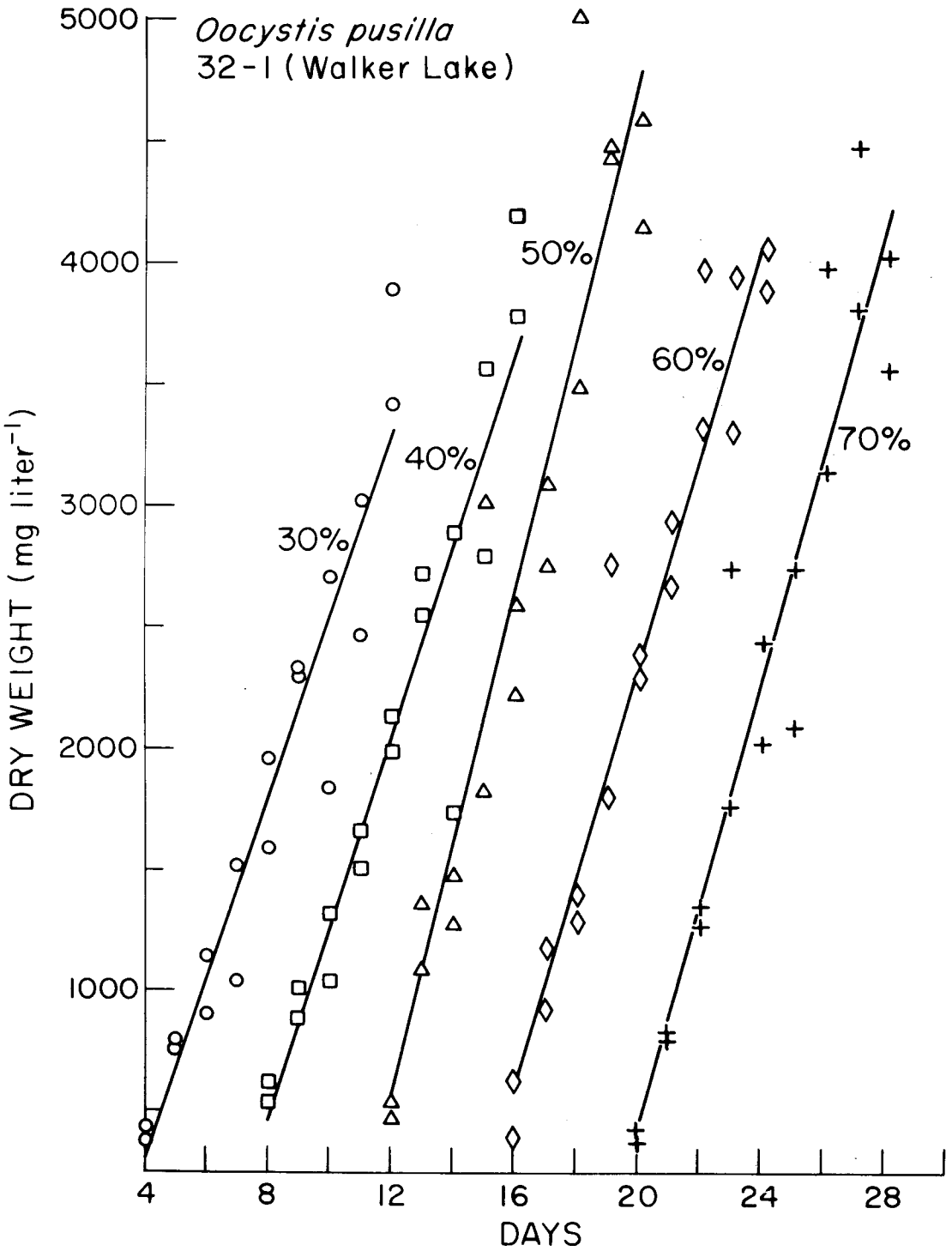

Figure 8. Increases in dry weight of Walker Lake <u>Oocystis pusilla</u> cultures grown at 30, 40, 50, 60, and 70% of maximum daily sunlight intensities at La Jolla, California. The growth curves are displaced for clarity.

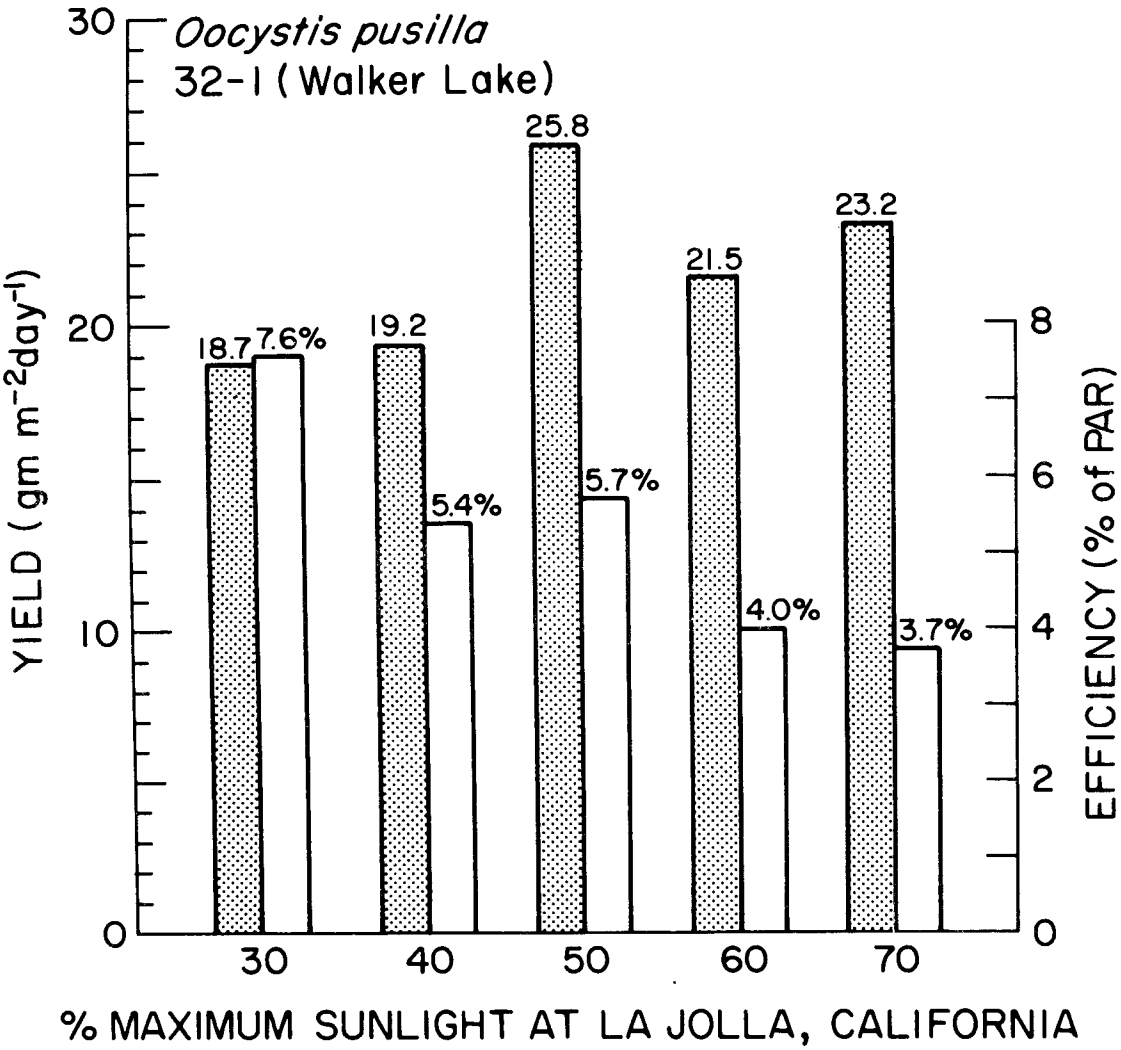

Figure 9. Yields and efficiencies of light utilization of Walker Lake *Oocystis pusilla* cultures grown at 30, 40, 50, 60, and 70% of maximum daily sunlight intensities at La Jolla, California.

Table 7. Growth Rates at Different Light Intensities.

	% of Maximum La Jolla Sunlight				
	30%	40%	50%	60%	70%
Alga	(mg liter^{-1} day^{-1})				
Ankistrodesmus falcatus	298.1	292.9	442.4	408.1	385.1
Oocystis pusilla	373.6	392.7	521.1	434.4	469.1

Two experiments were performed in which culture thickness was varied. In the first experiment only 5- and 10-cm thicknesses were employed. Figure 10 shows the growth curves at these thicknesses and Figure 11 shows the daily rates of growth. From days 2 to 6 growth was linear in these light-limited cultures and was relatively constant in each chamber. During this period the mean rate in the 5-cm culture was 463.7 mg dry weight liter^{-1} day^{-1} while that in the 10-cm culture was 259.7 mg liter^{-1} day^{-1}. Thus the rates were not similar despite intense mixing of the cultures. The mean yield in the 5-cm culture was 26.3 gm m^{-2} day^{-1} while that in the 10-cm culture was 29.5 gm m^{-2} day^{-1} (see Figure 12 for yield values over the whole experiment). Thus increased thickness did increase the yields.

After day 6, the rates slowed, particularly in the 5-cm culture. This was due to nutrient limitation since the 5-cm culture became pale. On day 7 a complete set of nutrients was added to both cultures at the initial concentrations. Growth was still low until day 9 when there was a strong spurt of growth as stored nutrients were assimilated into cell material. We attempted to avoid nutrient depletion thereafter by adding nutrients at their initial levels on day 10 and day 12.

The mean rates of growth during the whole experiment were 353.5 and 232.0 mg liter^{-1} day^{-1} in the 5- and 10-cm cultures, respectively. Again the overall rates were not similar. Corresponding efficiencies of light utilization were 4.5 and 5.9% in the 5- and 10-cm cultures, respectively. Corresponding yields over the whole experiment were 20.1 and 26.4 gm m^{-2} day^{-1} in the 5- and 10-cm cultures, respectively. Thus it was apparent that, despite greater light-limitation in the thicker culture which reduced the rate of growth, the yields were greater in that culture.

Toward the end of the experiment (days 14 and 15) samples were taken every two hours through two light and dark cycles to determine whether growth went on only during the light period and what the magnitudes of respiratory losses were during the dark period. Figure 13 shows changes in dry weight (from optical density measurements), pH, and temperature in both cultures. In the 5-cm culture, growth (mg liter^{-1}) occurred during the day, but barely kept up with respiration during the night. Due to nitrate uptake, pH went up during the day and decreased at night. During the second night, the CO_2 supply was turned off and pH increased only slightly. It decreased when the CO_2 was turned back on at 0600. Temperature increased during the day due to absorption of heat from the lamp and decreased when the lamp was turned off. Such a temperature cycle might occur in an outdoor pond, but the change would not be as great in the larger volume of the pond. The 10-cm culture grew more vigorously than the 5-cm culture and respiratory losses at night were only about 25% of the increases in biomass during the day. The pH cycle was similar to that in the 5-cm culture except a strong increase in pH occurred during the second dark period—probably because nitrate uptake occurred using energy assimilated during the previous light period. This pH change was especially apparent because the CO_2 was turned off. In a pond CO_2 could be conserved by turning it off at night. The temperature cycle in the 10-cm culture was similar to that in the 5-cm culture except that the

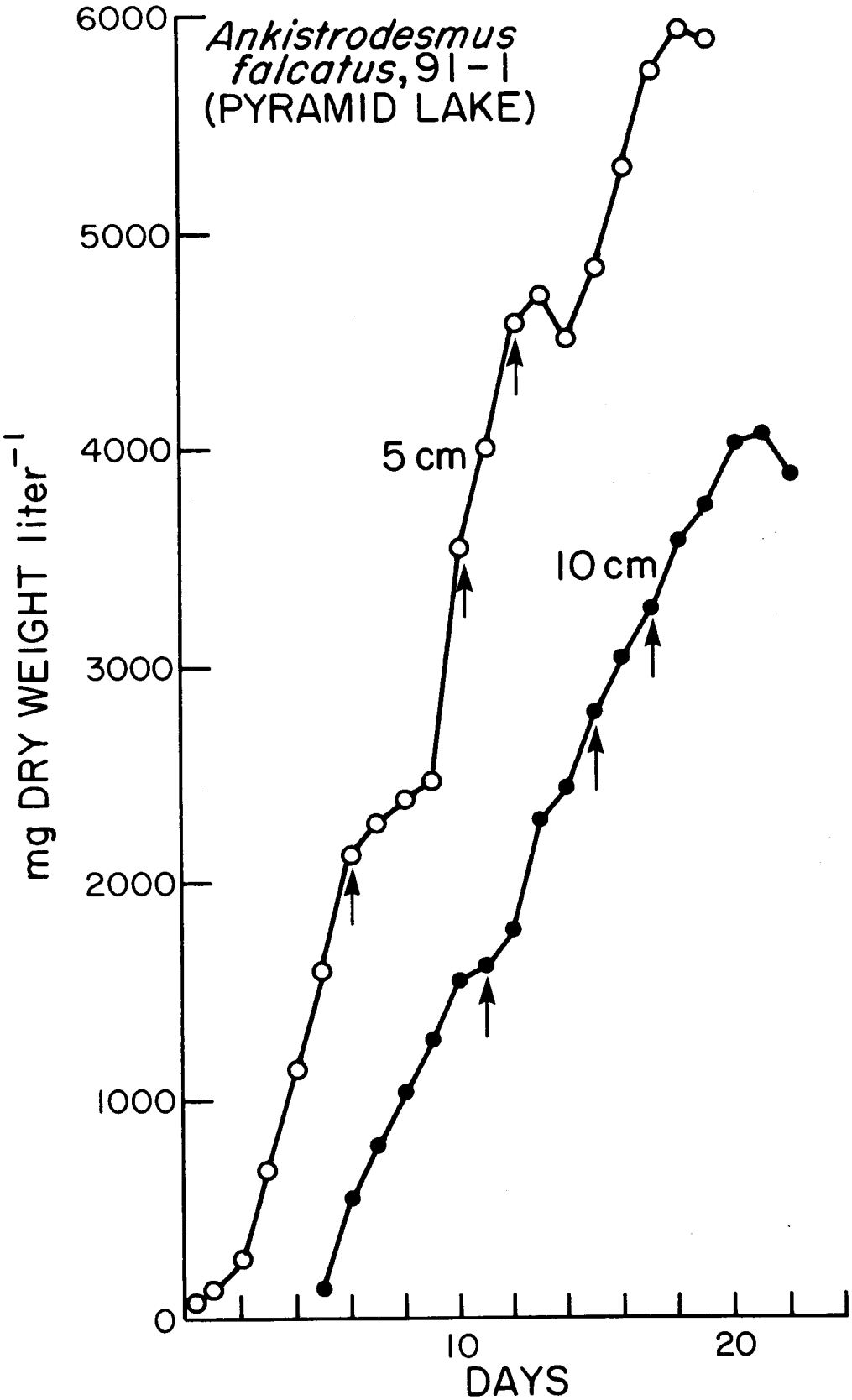

Figure 10. Increases in dry weight of Pyramid Lake Ankistrodesmus falcatus cultures of 5- and 10-cm thickness. The 10-cm growth curve is displaced for clarity. Arrows indicate when nutrients were added.

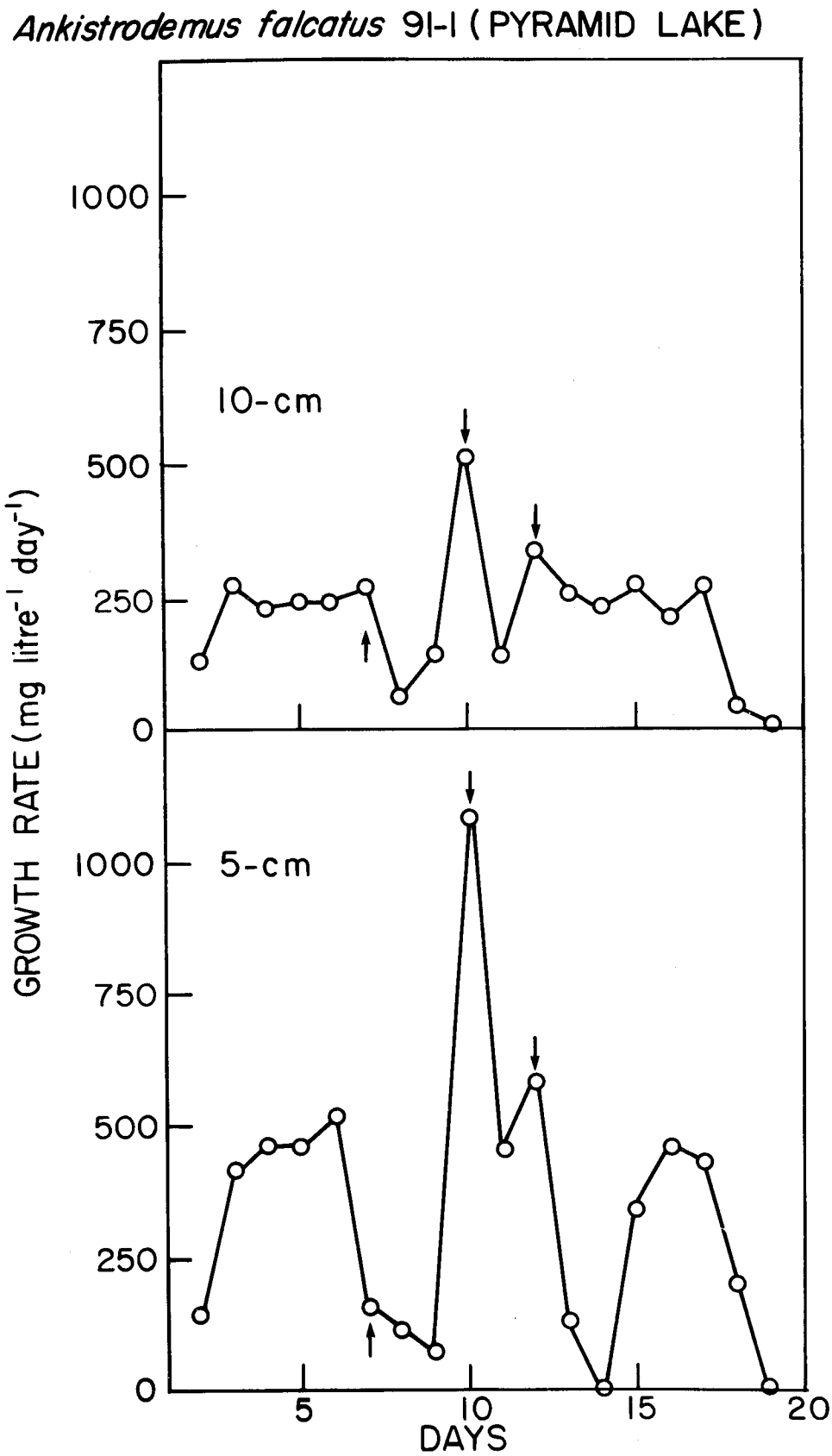

Figure 11. Rates of growth of Pyramid Lake Ankistrodesmus falcatus cultures of 5- and 10-cm thicknesses. Arrows indicate when nutrients were added.

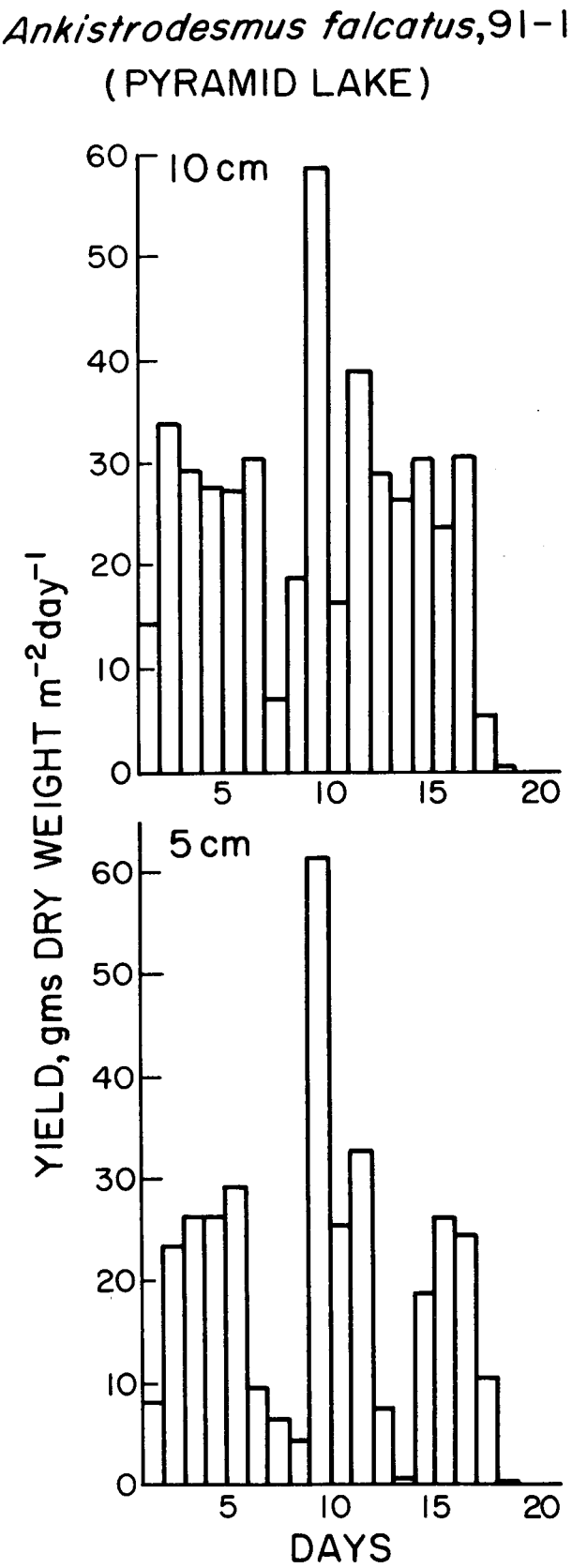

Figure 12. Daily yields of Pyramid Lake <u>Ankistrodesmus</u> <u>falcatus</u> cultures of 5- and 10-cm thicknesses.

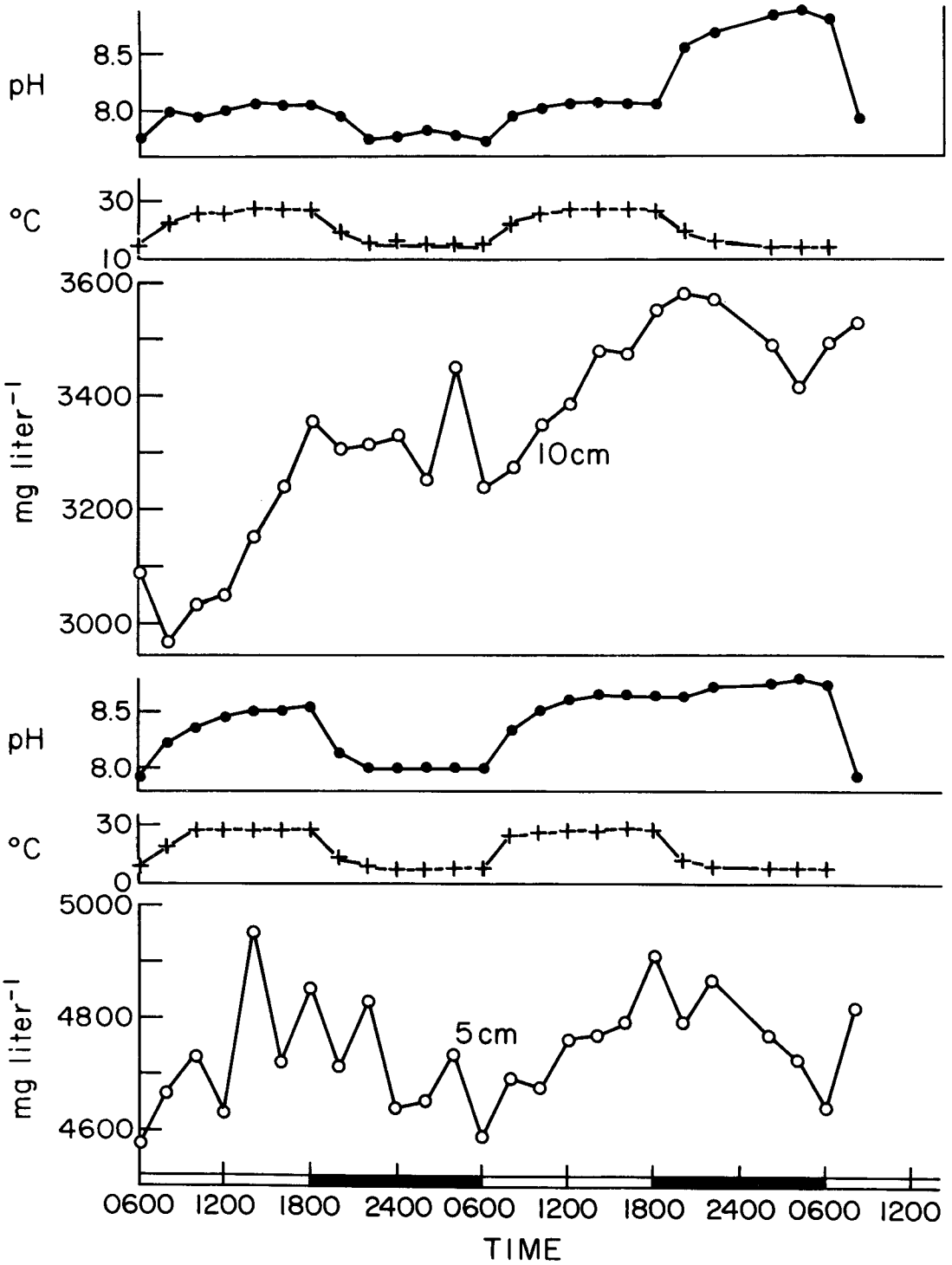

Figure 13. Changes in dry weight, pH, and temperature of Pyramid Lake *Ankistrodesmus falcatus* cultures over a 48 hour period.

temperature did not go up as much since the culture volume was greater in the 10 cm culture.

In the second experiment, three thicknesses were tested. These were 5, 10, and 15 cm and their volumes were 4, 8, and 12 liters, respectively. We hypothesized that the growth rates in the 15-cm culture would be so small due to light limations that the volume-dependent yields would be less than those in the 5- and 10-cm cultures. Figure 14 shows the growth curves for these three cultures and Figure 15 shows the daily rates of growth. In all three cultures there was a hiatus in growth from days 2 to 3 probably because of a strong decrease in pH (from 9.28-9.41 to 7.44-7.81) when aeration with 1% CO_2-in-air was commenced. The CO_2 level was then reduced to 0.5% until day 4. The growth rates increased and the pH was 7.91 to 8.81. On day 4 the full 1% CO_2-in-air addition was commenced. The pH was not excessively low thereafter and the growth rates were maximal and steady. We estimated that the initial nutrient levels would support about 2 gm dry weight liter^{-1} so nutrient levels were added at half-strength on days 5, 7, 9 and 11 to avoid nutrient limitation. The initial levels of KNO_3 were 20 mmole liter^{-1}, 2 mmole K_2HPO_4 1 ml liter^{-1} 1% NaFeEDTA, 1 ml liter^{-1} Chapman trace metals and f/2 vitamins; the additions were half of these levels. The mean rates of growth (days 3-12) in the 5-, 10- and 15-cm cultures were 723.2, 353.8, and 309.2 mg liter^{-1} day^{-1}, respectively. Corresponding efficiencies of light utilization were 9.1, 8.9 and 11.7%, respectively.

Despite the fact that the rates were less in the 15-cm culture than in the other two cultures, they were not low enough to result in a lower yield when the rates were multiplied by the increased volume in the 15-cm culture. The daily yields are shown in Figure 16. Yields were greatest in the 15-cm culture and reached as high as 71.2 gm m^{-2} day^{-1} (100 tons acre^{-1} year^{-1}) from days 7 to 8. The mean yields from days 3 to 12 in the 5-, 10-, and 15-cm cultures were 41.1, 40.2 and 52.7 gm m^{-2} day^{-1}, respectively. Thus the rate was not reduced enough in the 15-cm culture to reduce the overall yields as compared to the other two cultures, but the mean yield in that culture was only about 20% greater than those in the other two cultures.

It might be argued that dry weight values shown in Figures 10 and 14 might not be valid since they were based on optical density measurements. However some actual dry weights were determined. The regression of OD on dry weight was dry weight = (OD x 295.8)-18.5 (r=0.994) and this relationship was determined using dry weight values ranging from 125 to 1,650 mg liter^{-1} and ODs from 0.45 to 5.45. Since the spectrophotometer would not measure such high OD's, they were calculated from measured ODs that ranged from 0.4 to 0.8 in samples diluted with Pyramid Lake medium and these measured ODs were multiplied by a dilution factor to obtain the actual ODs in the original samples.

Yields were also not based on actual cell material harvested continuously or semi-continuously from the culture, but from changes in estimated dry weights during each day. Yields based on semi-continuous, daily sampling were measured by Krauss and Thomas (30) for Scenedesmus. In future work to maximize yields we plan to follow

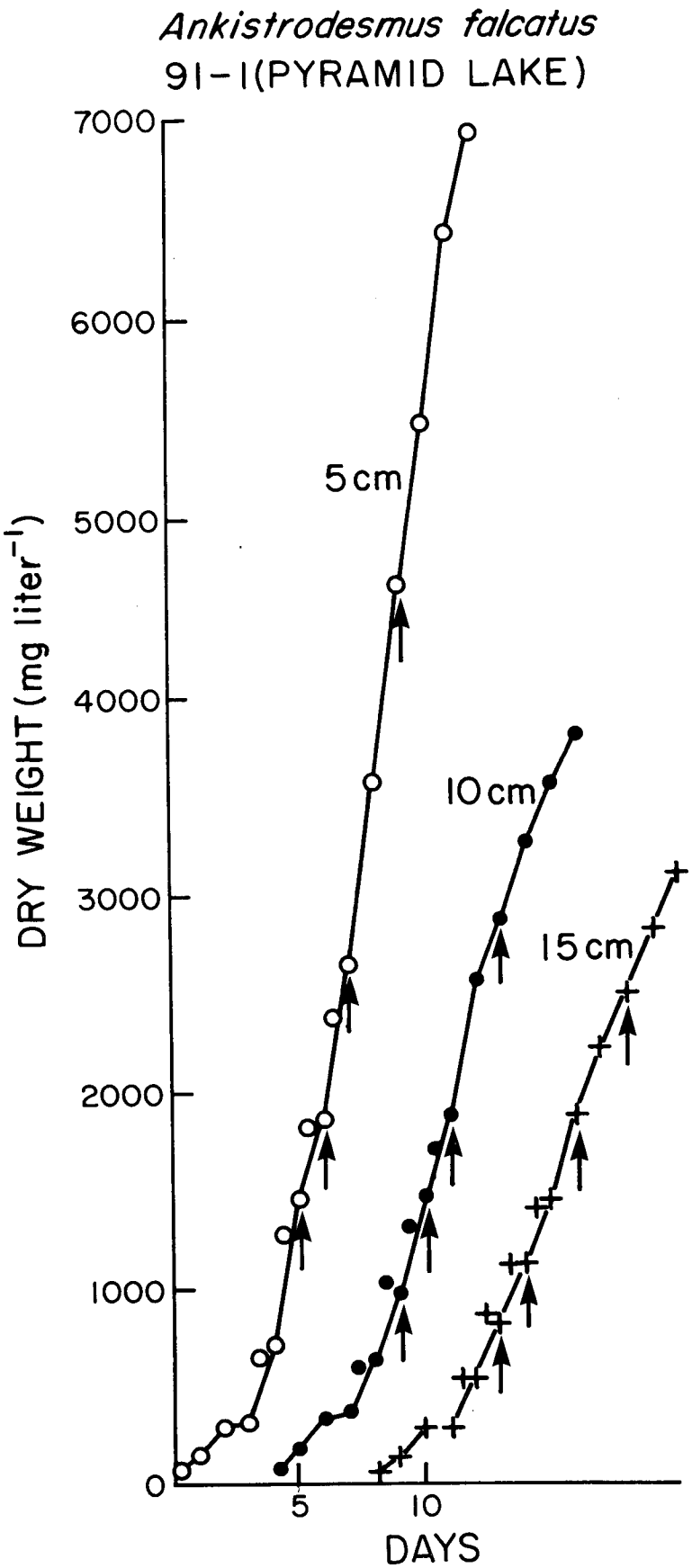

Figure 14. Increases in dry weight of Pyramid Lake Ankistrodesmus falcatus cultures of 5-, 10- and 15-cm thicknesses. The growth curves are displaced for clarity. The arrows indicate when nutrients were added.

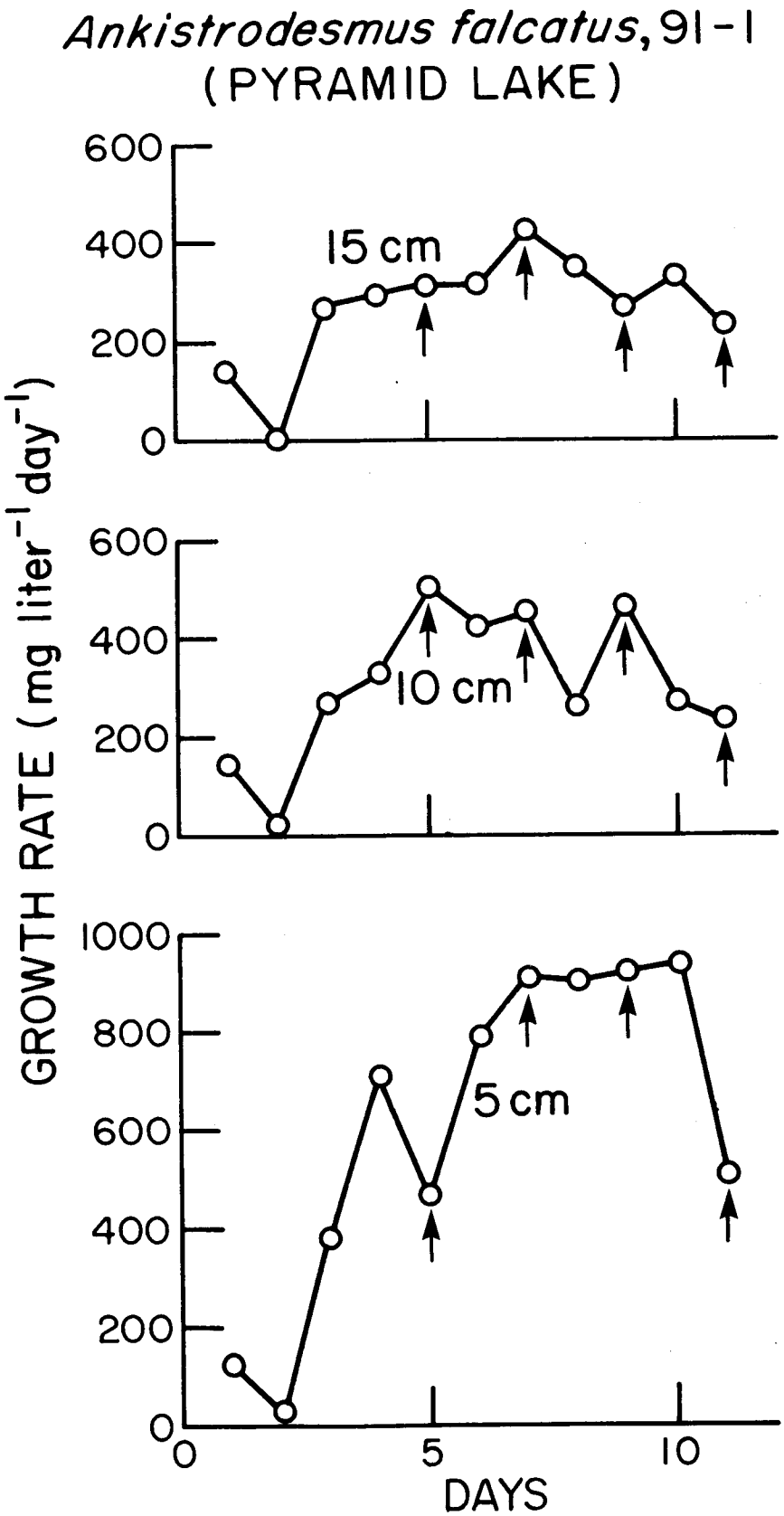

Figure 15. Rates of growth of Pyramid Lake Ankistrodesmus falcatus cultures of 5-, 10- and 15-cm thicknesses. The arrows indicate when nutrients were added.

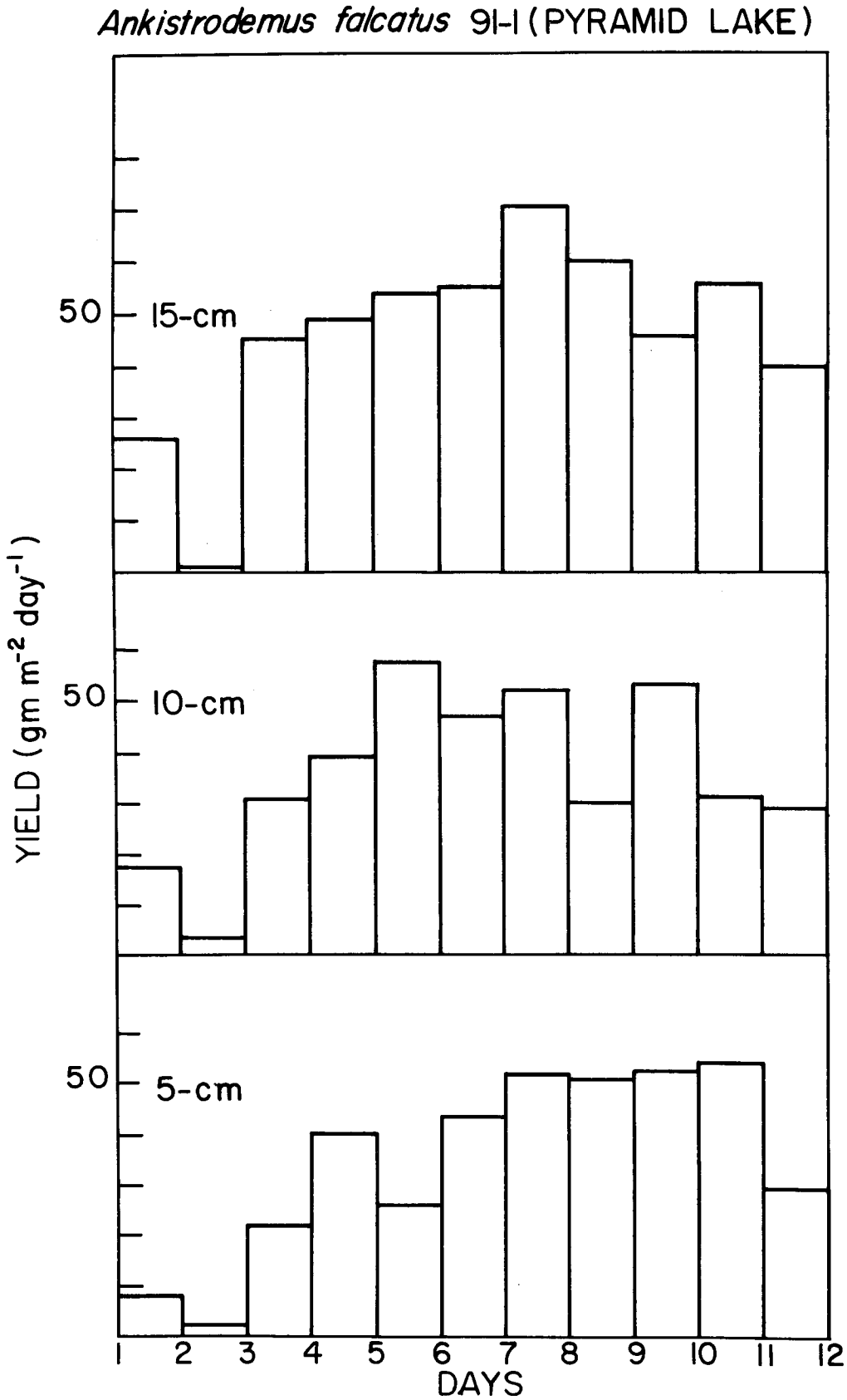

Figure 16. Daily yields of Pyramid Lake <u>Ankistrodesmus</u> <u>falcatus</u> cultures of 5-, 10-, and 15-cm thicknesses.

their experimental protocol since this might be the way harvests would be taken in an outdoor facility.

These yields are somewhat greater than those reported for other cultures using light intensities approaching or equivalent to full sunlight (2, 3, 4, 5, 26). Other values generally range up to 30 gm m^{-2} day^{-1}. We attribute our results to high turbulence and resultant proper mixing between the light and dark layers within the cultures and to judicious resupplying of nutrients. After the cultures became really dense (> 1,000 mg $liter^{-1}$), the two layered systems were readily apparent visually through the sides of the vessels. On day 8 the estimated thicknesses of the lighted layers were 4, 5, and 7 mm for the 5-, 10- and 15-cm cultures, respectively. The differences in the lighted layer thicknesses was due to differences in density of the cultures. On that day the densities were 3565, 2556, and 1881 mg $liter^{-1}$ in the 5-, 10-, and 15-cm cultures, respectively.

Ankistrodesmus contains about 27% lipid (Tornabene, personal communication). Therefore the mean lipid yields would 11.1, 10.9, and 14.2 gm m^{-2} day^{-1} or 15.5, 15.3, and 19.9 tons $acre^{-1}$ $year^{-1}$. At our highest yield (100 tons $acre^{-1}$ $year^{-1}$ extrapolated) the lipid yield would be 27 tons $acre^{-1}$ $year^{-1}$. Thus this alga has real potential for producing energy-related substances at high yields.

On day 12 we harvested the 5-cm culture and obtained 19.2 gm of dry material. This corresponds to 4.87 gm $liter^{-1}$ (compared with 6.9 gm $liter^{-1}$ measured by OD). This material will be analyzed for its inorganic nutrient and trace metal composition by Babcock Laboratories so that, as algae are produced in future experiments, enough nutrients can be added back daily to maintain the necessary cell quotas of each element. Such additions of necessary nutrients based on cellular composition to maintain high rates and yields were done previously by Krauss and Thomas (30) using Scenedesmus as the test alga.

CONCLUSIONS

These investigations have been a continuing selection process to find those saline desert microalgae that would be best for outdoor use. Best growing strains have been identified and grown in artificial media simulating the waters from which they were isolated. Temperature, salinity and light requirements for some strains have been established and utilized in experiments designed to maximize yields. Ankistrodesmus falcatus from Pyramid Lake has been studied most thoroughly and this alga is very promising as a high-yielding species that produces an accordingly high yield of lipid. Engineering and design studies are obviously needed to translate these biological results to outdoor facilities using natural sunlight.

ACKNOWLEDGEMENTS

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Appendix Table 1. Chemical Data on Major Constituents of Water Samples from East-Central California Desert Locations.

	Big Alkali Lake						Black Lake	Black Lake
	Pond A	Pond A	Pond A	Pond B	Pond B	Pond B		
Date	9/29/82	4/30/83	7/3/83	9/29/82	4/30/83	7/3/83	10/3/82	4/30/83
Temperature, C	4.0	5.0	20	2.0	6.5	29.5	13.2	14.0
Major Cations (mg liter^{-1})								
Ca	5	5	7	67	7	6	4	20
Mg	5	3	4	5	6	2	4	12
Na	450	460	140	1150	240	1940	8800	2650
K	43	52	16	124	24	180	825	275
NH_4	1	1	3	1	1	1	2	1.5
Total Cations (me liter^{-1})	21.40	21.87	7.35	53.96	11.96	89.70	404.4	124.3
Major Anions (mg liter^{-1})								
CO_3	240	186	117	420	45	924	5580	1680
HCO_3	268	515	98	1159	445	1806	2526	1525
SO_4	20	50	22	140	218	318	3200	1400
Cl	300	187	55	600	81	1020	3200	1063
Si			40			70	1	<1
NO_3	1	<1	1	1	<1	1	1	<1
Ortho PO_4	0.1	0.5	1.0	1.9	0.3	4.3	0.1	3.4
Total PO_4	1.2	0.7	6.5	1.9	0.7	7.5	18.5	7.4
Total Anions (me liter^{-1})	20.89	20.96	7.52	52.84	11.66	95.77	294.8	140.1
Electrical Conductivity (mmho cm^{-1})	1.857	1.980	0.94	4.710	1.210	7.80	30.00	10.60
Equivalent TDS (mg liter^{-1})	1200	1267	602	3014	774	4992	19200	6784
Total Dissolved Residue (mg liter^{-1})	1350	1195	495	3205	955	5015	26460	7100
pH	9.6	9.4	10.0	9.5	9.1	9.5	9.8	9.9

Appendix Table 1 Chemical Data on Major Constituents of Water Samples from East-Central California Desert Locations (Continued).

Black Lake	Mono Lake	Mono Lake	Owens Lake	Owens Lake	Owens Lake	Saline Valley	Saline Valley	Saline Valley
7/2/83	4/30/83	7/2/83	10/11/82	5/4/83	7/6/83	10/11/82	5/4/83	7/6/83
20.0	8.2	23.5	27.5	16.0	23.0	25.4	24.0	33.0
15	20	12	8	23	10	220	380	120
11	63	39	20	70	14	320	480	720
3500	20800	31600	4700	8100	6800	$100x10^3$	$120x10^3$	$128x10^3$
300	1800	1600	525	900	840	1500	3000	3800
1.5	1	1	0.6	1.5	10	0.6	2	1.5
161.6	956.8	1418.9	229.3	382.1	319.4	4424.0	5553.3	5128.7
1950	16860	8970	2640	4410	4740	0	0	0
2790	5307	1220	915	2349	1952	146	1049	994
1150	11500	10900	1600	2200	2000	$10.4x10^3$	$29x10^3$	$27.6x10^3$
2000	20040	20120	2250	3846	3800	$154x10^3$	$172x10^3$	$182x10^3$
110		<1			20			10
2	<1	3	1	<1	1	2	<1	1
4.5	92	1.5	3.8	8.4	3.5	0.8	1.4	0.8
15	104	85	7.9	10.2	15	3.0	4.9	2.0
191.8	945.0	1112.9	199.7	339.7	338.7	4557.1	5466.4	5718.1
13.30	86.90	64.50	15.60	26.40	23.40	209	220	141
8512	55616	41280	9984	16896	14976	$133.8x10^3$	$140.8x10^3$	$90.2x10^3$
9560	79600	80100	11870	20500	18400	$225x10^3$	$79x10^3$	$323x10^3$
9.6	9.8	9.5	9.8	9.9	10.0	7.5	7.5	7.1

Appendix Table 2. Chemical Data on Major Constituents of Water Samples from Western Nevada Desert Locations.

	Pyramid Lake	Pyramid Lake	Pyramid Lake	Walker Lake	Walker Lake
Date	10/4/82	5/16/83	7/12/83	10/5/82	5/18/83
Temperature, C	17.4	15.0	25.5	18.0	15.5
Major Cations (mg $liter^{-1}$)					
Ca	10	29	20	10	55
Mg	60	127	150	77	220
Na	1775	1600	1300	3900	3350
K	128	120	280	225	175
NH_4	1	1.5	3	2	4
Total Cations (ml $liter^{-1}$)	86.01	84.68	77.37	182.3	171.2
Major Anions (mg $liter^{-1}$)					
CO_3	222	321	270	750	600
HCO_3	854	695	622	1586	1626
SO_4	140	240	236	2600	2000
Cl	2150	2050	1713	2600	2530
Si	--	2	5	--	9
NO_3	1	<1	1	1	<1
Ortho PO_4	<0.1	0.3	0.4	<0.1	3.0
Total PO_4	0.7	0.5	1.5	2.3	3.5
Total Anions (ml $liter^{-1}$)	84.70	84.85	72.38	178.4	159.6
Electrical Conductivity (mmho cm^{-1})	8.030	9.240	8.740	15.00	13.90
Equivalent TDS (mg $liter^{-1}$)	5139	5914	5594	9600	8896
Total Dissolved Residue (mg $liter^{-1}$)	4900	4735	5550	10580	10340
pH	9.1	9.1	9.2	9.3	9.4

Appendix Table 2. Chemical Data on Major Constituents of Waters Samples from Western Nevada Desert Locations (Continued).

Walker Lake	Big Soda Lake	Big Soda Lake	Spring No. 1 off Rt. 80	Spring No. 2 off Rt. 80	Humboldt Salt Marsh	Brady Hot Spring
7/13/83	5/17/83	7/13/83	5/17/83	7/13/83		
31	15.0	22	20.0	28	--	42
20	65	42	200	140	50	54
70	190	240	160	140	5	4
1650	7600	8200	1800	3750	$122x10^3$	770
85	280	340	108	220	600	53
1	1.5	1.5	<0.2	1.5	<0.2	2
82.33	356.7	406.4	104.4	187.4	5325.7	37.98
315	1500	1290	0	0	3720	0
833	793	1310	268	677	1220	110
1150	4400	5400	370	740	7750	296
1400	6731	6673	3441	6300	$180x10^3$	1160
10	7	5	33	25	8	160
1	<1	1	<1	3	1	1
1.6	11.5	14	0.2	0.4	296	1.3
3.8	11.8	14	0.4	5.8	304	6.0
87.57	344.3	365.0	109.0	204.0	5375.9	40.66
7.490	29.70	29.10	10.30	17.70	231.0	4.370
4794	19008	18624	6592	11328	$147.8x10^3$	2794
4720	20700	21750	5915	10450	$122x10^3$	2675
9.4	9.6	9.5	7.8	7.9	8.9	4.9

Appendix Table 3. Chemical Data on Major Constituents of Water Samples from Southern California Desert Locations.

	Zzyzx Springs					
	Lake Tuendae	Lake Tuendae	3-BATS	3-BATS	NC Spring	Sperry River
Date	2/3/83	8/17/83	2/3/83	8/17/83	8/17/83	2/1/83
Temperature, C	9.0	27.5	8.8	27.0	25.0	14.0
Major Cations (mg liter^{-1})						
Ca	18	11	16	18	15	28
Mg	7	3	7	7	6	24
Na	920	1010	1650	2360	640	2250
K	20	16	36	42	15	72
NH_4	0.8	1	0.6	1	1	0.8
Total Cations (me liter^{-1})	42.03	--	74.07	--	--	103.1
Major Anions (mg liter^{-1})						
CO_3	57	156	81	288	0	384
HCO_3	210	0	522	744	244	732
SO_4	490	476	690	1030	296	2200
Cl	910	1080	1725	2410	685	1190
Si	20	75	3	95	65	40
NO_3	2.4	1	2.6	1	5	<1
Ortho PO_4	<0.1	1	0.1	3	2	1.2
Total PO_4	0.1	3	0.2	3	3	1.2
Total Anions (me liter^{-1})	42.43	--	74.26	--	--	104.2
Electrical Conductivity (mmho cm^{-1})	4.040	4.790	6.490	10.30	3.190	8.070
Equivalent TDS (mg liter^{-1})	2586	3066	4160	6592	2042	5165
Total Dissolved Residue (mg liter^{-1})	2290	2770	4225	6040	1820	5465
pH	9.1	9.8	9.1	9.4	7.9	9.0

California Desert Locations (Continued).

Armagosa River, Tecopa	Armagosa River, Tecopa	Salt Creek	Salt Creek	Harper Lake	Harper Spring	Mohave River, Afton	Salton Sea
2/1/83	8/16/83	2/1/83	8/16/83	2/3/83	8/18/83	2/3/83	4/7/83
8.4	29.5	19.4	27.0	9.0	20.0	14.0	20.7
8	9	130	136	435	296	14	1200
8	8	200	115	70	54	3	1160
3360	770	1270	1330	6080	1260	235	10600
96	31	52	50	48	4	10	300
1.0	1	0.06	1	0.8	1	0.4	3
149.8	--	79.75	--	293.2	--	11.45	625.4
672	45	0	0	60	0	72	0
964	689	238	247	61	586	192	220
2500	580	950	720	2800	980	116	8400
2030	405	1880	2050	7725	1450	131	15278
55	120	20	40	5	100	48	10
1.6	1	2.9	1	60	59	1.0	<1
2.4	4	<0.1	2	0.1	3	0.2	<0.1
2.5	7	0.1	2.4	0.2	4.3	0.3	0.4
147.5	--	76.81	--	279.9	--	11.68	605.37
12.30	3.420	7.180	7.640	23.50	6.840	1100	44.80
7872	2189	4595	4890	15040	4378	704	28672
8400	2200	4080	4440	15800	4490	705	39000
9.4	8.6	8.2	8.2	8.7	7.1	9.2	6.9

Appendix Table 4. Chemical Data on Minor Constituents of Water Samples from East-Central California Desert Locations.

	Big Alkali Lake						Black Lake	Black Lake
	Pond A	Pond A	Pond A	Pond B	Pond B	Pond B		
Date	9/29/82	4/30/83	7/3/83	9/29/82	4/30/83	7/3/83	10/3/82	4/30/83
Element								
				mg liter^{-1}				
As	0.2	0.3	0.1	1	0.2	1.5	0.63	0.2
Ba	<0.5	<0.5	<1	<0.5	<0.5	<1	<0.5	<0.5
B	8	8.6	2	20	5.0	31	21	6.3
Cd	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Cr	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Cu	<0.02	<0.01	<0.05	<0.02	<0.01	<0.05	<0.02	<0.01
F	4	5.2	2.1	10	3.3	25	20	8.1
Fe	0.15	0.04	<0.05	0.15	0.06	<0.05	0.15	0.37
Pb	<0.05	<0.1	<0.01	<0.05	<0.01	<0.1	<0.05	<0.1
Mn	0.04	0.01	<0.05	0.04	<0.01	<0.05	0.02	0.03
Hg	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Se	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Ag	<0.01	<0.01	<0.05	<0.01	<0.01	<0.05	0.01	<0.01
Zn	<0.01	0.01	<0.05	<0.01	0.02	<0.05	0.01	0.03

Appendix Table 4. Chemical Data on Minor Constituents of Water Samples from East-Central California Desert Locations (Continued).

Black Lake 7/2/83	Mono Lake 4/30/83	Mono Lake 7/2/83	Owens Lake 10/11/82	Owens Lake 5/4/83	Owens Lake 7/6/83	Saline Valley 10/11/82	Saline Valley 5/4/83	Saline Valley 7/6/83
				mg liter^{-1}				
0.3	13	11	5.9	8.5	7.5	0.25	<0.01	0.6
<1	<0.5	<1	<0.5	<0.5	<1	<0.5	<1.0	<1
7	480	500	60	110	80	155	590	410
<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.02	<0.04	<0.02
<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.2	0.10
<0.05	<0.01	<0.05	0.04	<0.01	<0.05	<0.04	<0.04	<0.05
12	50	58	5	5.8	7.7	2.5	1.8	1.2
<0.05	0.96	0.78	0.25	0.27	<0.05	0.20	<0.01	<0.06
<0.1	<0.1	<0.1	<0.05	<0.1	<0.1	<0.05	<0.4	<0.2
<0.05	0.04	0.06	<0.02	<0.01	<0.05	0.08	0.24	0.20
<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.01	<0.001
<0.01	<0.01	0.01	<0.01	<0.01	<0.01	0.01	<0.01	<0.01
<0.05	<0.01	<0.05	<0.01	<0.01	<0.05	0.04	<0.01	<0.05
<0.05	0.03	<0.05	0.05	0.02	<0.05	0.10	0.08	<0.05

Appendix Table 5. Chemical Data on Minor Constituents of Water Samples from Western Nevada Desert Locations.

	Pyramid Lake	Pyramid Lake	Pyramid Lake	Walker Lake	Walker Lake
Date	10/4/82	5/16/83	7/12/83	10/5/82	5/18/83
Element					
			mg liter^{-1}		
As	0.03	0.1	0.01	0.8	0.6
Ba	<0.5	<0.5	<1	<0.5	<0.5
B	11	11	6	19	24
Cd	<0.01	<0.01	<0.01	<0.01	<0.01
Cr	<0.05	<0.05	<0.05	<0.05	<0.05
Cu	<0.02	<0.01	<0.05	<0.02	<0.01
F	5	1.7	1.4	4	15
Fe	<0.05	0.03	<0.05	0.15	0.05
Pb	<0.05	<0.1	<0.01	0.05	<0.01
Mn	<0.02	<0.01	<0.05	0.02	<0.01
Hg	<0.001	<0.001	<0.001	<0.001	<0.001
Se	<0.01	0.01	<0.01	<0.01	<0.01
Ag	<0.01	<0.01	<0.05	<0.01	<0.01
Zn	<0.01	<0.01	<0.05	<0.01	0.01

Appendix Table 5. Chemical Data on Minor Constituents of Water Samples from Western Nevada Desert Locations (Continued).

Walker Lake	Big Soda Lake	Big Soda Lake	Spring No. 2 off Rt. 80	Spring No. 2 off Rt. 80	Humboldt Salt Marsh	Brady Hot Spring
7/13/83	5/17/83	7/13/83	5/17/83	7/13/83	5/17/83	7/13/83
			mg liter^{-1}			
0.4	0.5	1.0	0.06	0.4	7.5	0.1
<1	<0.5	<1	<0.5	<1	<1.0	<1
10	44	35	9.8	19	140	3
<0.01	<0.01	<0.01	<0.01	<0.01	<0.04	<0.01
<0.05	<0.05	<0.05	<0.05	<0.05	<0.02	<0.05
<0.05	<0.01	<0.05	<0.01	<0.05	<0.04	<0.05
9.2	5.8	6.5	2.2	4.6	15	29
<0.05	<0.01	<0.05	0.01	0.15	<0.04	0.63
<0.1	<0.1	<0.1	<0.01	<0.1	<0.04	<0.1
<0.05	0.03	<0.05	0.35	0.30	0.08	0.10
<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
<0.01	0.02	0.02	<0.01	<0.01	0.01	<0.01
<0.05	<0.01	<0.05	<0.01	<0.05	<0.04	<0.05
<0.05	<0.01	<0.05	0.01	<0.05	<0.04	<0.05

Appendix Table 6. Chemical Data on Minor Constituents of Water Samples from Southern California Desert Locations

	Zzyzx					
	Lake Tuendae	Lake Tuendae	3-BATS	3-BATS	NC Spring	Sperry River
Date	2/3/83	8/17/83	2/3/83	8/17/83	8/17/83	2/1/83
Element						
	mg liter^{-1}					
As	0.02	0.04	0.02	0.09	0.03	0.31
Ba	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
B	3.2	4.3	6.7	12	3.0	20
Cd	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Cr	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Cu	<0.02	0.01	<0.02	<0.01	<0.01	<0.02
F	10.4	12	20	24	7.7	5.8
Fe	0.15	0.12	0.70	0.66	0.08	0.95
Pb	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Mn	0.02	<0.01	0.02	0.07	<0.01	0.04
Hg	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Se	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Ag	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Zn	<0.01	0.01	0.01	0.03	<0.01	0.02

Appendix Table 6. Chemical Data on Minor Constituents of Water Samples from Southern California Desert Locations (Continued)

Armagosa River, Tecopa	Aragosa River, Tecopa	Salt Creek	Salt Creek	Harper lake	Harper Spring	Mohave River, Afton	Salton Sea
2/1/83	8/16/83	2/1/83	8/16/83	2/3/83	8/18/83	2/3/83	4/7/83
			mg liter^{-1}				
9	10	0.01	0.01	0.02	0.01	0.03	0.01
<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.05	<0.05
40	11	4.0	4.2	25	5.2	1.3	11
<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.005
<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
<0.02	<0.01	<0.02	<0.01	0.02	0.01	<0.02	<0.02
8.0	3.8	8.0	5.4	1.5	2.2	3.4	3.5
1.8	4.2	0.35	0.68	8.4	0.20	0.40	0.58
<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
0.08	0.20	0.14	0.34	0.24	0.01	0.04	0.05
<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
<0.01	<0.01	<0.01	<0.01	<0.05	<0.01	<0.01	<0.01
0.03	0.03	0.01	0.01	0.04	0.01	0.01	0.01

CHEMICAL PROFILE OF MICROALGAE WITH EMPHASIS ON LIPIDS

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PURPOSE

The research is directed towards identifying algae that have the highest lipid producing capacities. The laboratory at Georgia Tech has the responsibilities of providing to all members of the Aquatic Species Program (ASP) analytical expertise for determining the quantity and quality of algal lipids; assist in the identification of potential oil-producing algae; search for the trigger(s) that control the regulatory mechanisms within cells; and, to optimize productivity.

INTRODUCTION

This report is restricted to the current contract period which began in October 1983. Data collected prior to October has been included in the annual report and in two manuscripts submitted for publication. The titles of the manuscripts are: "Lipids and lipopolysaccharide constituents of a commercial preparation of Spirulina platensis", by T. G. Tornabene and A. Ben-Amotz (1), and "Chemical Profiles of selected species of Algae with emphasis on lipids" by A. Ben-Amotz, W. H. Thomas and T. G. Tornabene (2). Previous results were also described by T. G. Tornabene, G. Holzer, S. Lien and N. Burris, "Lipid composition of the nitrogen starved green alga Neochloris oleoabundans", in Enzyme Microb. Technol. 5, (1983) 435-440.

SPECIFIC OBJECTIVES

1) Provide analytical expertise to Aquatic Species Program for microalgae evaluation; 2) examine the influence of environmental parameters (nitrogen deficiency and temperature) on lipid production; 3) select for clones of test strains having the greatest potential for synthesizing lipids; and 4) study suspected inhibitors that will block the polyisoprenoid pathway and result in the overproduction of isoprenoid hydrocarbons and/or alcohols, preferably within the C_5-C_{15} range.

MATERIALS AND METHODS

Cultivation of organisms. Most organisms in this study were cultivated by members of the ASP and forwarded to us as frozen wet-packed cells or as freezed-dried cells. All algae received were cultivated in nitrogen sufficient media. For details of algal cultivation see SERI Annual Report for FY 83.

Selected algal cultures were cultivated at Georgia Tech in both nitrogen-sufficient and deficient media. Algae were cultivated in Mono Lake or Pyramid Lake artificial media as described by E. Thomas (Scripps Institute); natural and

synthetic sea water media fortified with Guillard trace metal mix; or, modified Provasoli medium. Cell cultivations in this and previous reports were conducted in flasks on a platform shaker under continuous illumination at 25°C. These systems are described in detail in the annual report (FY83). Cell suspensions were extracted for lipids by the modified method of Bligh and Dyer, (3, 4). 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 (5) with hexane, benzene, chloroform, acetone and methanol. Polysaccharides were extracted from dried cells (10 g) by the phenol-water method (6) and isolated by isopycnic density gradient ultracentrifugation. Lipid components were deacylated by mild alkaline methanolysis (7). Samples were hydrolyzed with 2 N HCl at 100°C for 2 h for neutral sugars; and 4 N HCl 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 to I_2 vapor, acid charring, phosphate spray (9), ninhydrin, α-napthol for glycolipids (8), Draggendorff stain for quartenary amines (8), or H_2SO_4- acetic acid (1:1) for sterols (8). The deacylated water-soluble products were separated on cellulose-TLC plates (10). The samples were visualized by the o-tolidine staining method overstained with an acidic ammonium molybdate solution (10), or by ninhydrin. Total carbohydrates were determined by the phenol-sulphuric acid method (11). Nucleic acids were estimated spectrophotometrically after hydrolysis with 0.1 N NaOH. Total protein was determined after hydrolysis in NaOH at 100°C for 1 h (12). Samples were assayed for 3-deoxy sugars (13), KDO (14), glucosamine (15), heptose (16), and phosphorous (17). The total fatty acid of LPS was determined gravimetrically (18). Chlorophyll and carotenoids were assayed as described by Jensen (19). Fatty acids were converted to the methyl ester form in 2.5% anhydrous methanolic-HCl (8). Aliquots of fatty acids were hydrogenated with H_2 and 10% Pd on activated charcoal. Free sugars were converted to alditol acetates (20). Derivatized components were analyzed on a Varian 3700 gas-liquid chromatograph (GLC) equipped with dual flame ionization detectors and a Varian Vista 401 data system. The analyses were as follows: 30 x 0.252mm fused quartz capillary column with 0.25 M of OV 351 or 0.25 M of DB-5 at 8 psi of He and 4°C/min from 125°C to 220°C and held isothermally; 2m 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 screening for members of ASP

Algal samples were analyzed for their approximate chemical composition. Five isolates were selected and cultivated, under various growth conditions, by Dr. Thomas. The organisms and general growth conditions are described in Table 1. Twenty-two samples comprised this set. One of each algal preparation was designated as "aged". These "aged" cultures were older cultures than the others but they are not to be confused with nitrogen-limited algal preparations as described in following sections. Five cultures were prepared by Microbial Products/J. Weisman (Table 1). No information was provided as to the identity or method

TABLE 1

PROXIMATE CHEMICAL COMPOSITION OF SELECTED MICROALGAE

Culture No.		Initial Dry Wt. cells (gm)	Lipids %	CHO %	Protein %
1	Flagellate, Walker L., Urea	0.86681	17.88	37.97	20.04
2	" " " (Aged)	1.10922	12.05	41.73	19.23
3	Cryptomonad, Harper L., NH_4^+	0.08323	15.83	9.33	48.78
4	" " (Aged)	0.75793	18.22	8.34	52.91
5	" " NO_3	0.68536	3.15	7.67	19.94
6	" " (Aged)	0.81458	5.02	9.66	26.84
7	" " Urea	0.42120	6.41	9.00	30.23
8	" " (Aged)	2.16098	10.59	9.37	43.61
9	Dangeardinella, Salina Valley, NO_3	0.56196	5.57	1.44	16.93
10	" " "	1.6220	5.46	3.38	16.61
11	" " Urea	0.38870	4.20	2.42	17.94
12	" " " (Aged)	0.22664	4.77	3.36	18.15
13	Chlorella/Pond B NO_3	0.57920	20.08	10.54	65.72
14	" " (Aged)	1.01492	18.04	13.84	44.96
15	" "	0.53883	20.68	9.71	46.94
16	" " (Aged)	0.94596	20.15	7.84	57.91
17	Chlorella/Spring 10/l NH_4^+	0.48283	17.53	11.90	41.95
18	" " (Aged)	1.83487	15.23	9.44	52.49
19	" " NO_3	0.81650	10.85	14.09	46.50
20	" " (Aged)	1.85140	2.95	14.72	46.07
21	" " Urea	0.71039	13.34	9.28	61.73
22	" " " (Aged)	2.59074	7.99	10.68	60.23
23	G1(1)	0.93062	9.55	38.13	18.18
24	G1 (2)	0.45224	8.92	18.66	4206
25	M3 (1)	0.38514	19.96	26.97	20.77
26	M3(2)	0.51865	8.96	10.91	73.96
27	M4	0.51195	22.23	33.62	34.67

*culture number 1-22 were from Thomas, Scripps Institute whereas 23-27 were from Microbial Products.

of preparation of the latter five algal samples (Table 1) which were in small quantities, relatively "unclean" as indicated by the diverse stratifications of the wet-packed cells. No direct ash content was determined for any of the 27 samples for several reasons: Sample weights for most samples were less than 1 gm; salt crystals and extrinsic materials were readily visible and estimated to be close to or greater than one-half of the sample and, the organisms prepared were apparently all green algae, cultivated in nonstress environments, some of which are recognizable algal "weeds" that have been studied in considerable detail in this laboratory and elsewhere. The ash content can be extrapolated from the result of the approximate organic contents. These results are given in Table 1. The relative distribution of nonpolar and polar lipids in total free lipid fractions of representative organism, as selected by elution from a silicic acid column, is given in Table 2. Each column eluate was assayed by TLC in mixtures of nonpolar and polar solvents to survey the number and nature of the lipid components. A range of 15-30 components as resolved in each of the silicic acid column eluates with the exception of the $CHCl_3$ eluates which had 5-10 resolved components. Many of the pigmented components in related eluates were duplicates. The composition of the phopholipids in all of the methanol eluates were similar. These lipids were the diacylglycerol phosphate type. The composition of these diacylglycerol derivatives in biomembranes is relatively uniform with regard to their overall stereostructures and they are generally of little value as chemotaxonomic markers. Each cell will make no less than the amount of them that is essential for membrane structure and function. The essential amount is generally less than one-half of the amount they normally produce. The minimal amount of phospholipids are generally produced when the cells are under "stress" and the metabolic flow of carbon is directed towards carbon storage. In terms of relative quantity, the acetone eluate is often the most concentrated eluate for unstressed algal cells. Glycolipids of the galactosyl diacylglyceride type are common in the acetone eluate. The chlorophylls and carotenoids also predominate in this fraction. The neutral lipid (hexane, benzene, $CHCl_3$ eluates) represent relatively small amounts of lipids. This is typical of virtually all algae. A decrease in the concentration of the chlorophyll contents and a rise in concentration of the carotenoid (isoprenoid) types (both polar and nonpolar) often occurs in cells cultivated under stress. Unfortunately, none of the cells studied in this group of organisms were challenged; therefore, it is not possible to determine if these organisms have a potential for high-lipid production and exactly what would happen to the lipid production in these algae under stress. The lipid concentration ranges determined in this laboratory for the test algae are rather typical of all algae cultivated in "sufficient" growth media.

Organism cultivation and analysis at GIT

Two organisms isolated by Dr. Thomas and evaluated in the previous year were selected for further studies. These two organisms were *Oocystis* and *Ankistrodesmus*. They had demonstrated no special lipid producing potential; however, they are easily cultivatable algae and they had never been tested for lipid production while cultivated under nitrogen deficient conditions. Two additional algae were included in this phase of the study: *Cylindrotheica fusiformis* and *Monallanthus salina*. All four organisms were cultivated in both nitrogen sufficient and deficient media as described in the following procedure.

The two cultures supplied by Bill Thomas were cultivated in Walker Lake medium for *Oocystis* and Pyramid Lake medium for *Ankistrodesmus* as he recom-

mended. All cultures were grown in shallow flask on a shaker under continuous illumination at 25°C.

I. Ankistrodesmus falcatus. One liter Pyramid Lake medium (1mM KNO_3) was inoculated with 20ml log phase Ankistrodemus. After five days, when the nitrate level was approximately 0.5mM, the growth was heavy and dark green. The culture was divided into four equal volumes, and diluted with one of the following:

1) 250ml Pyramid Lake medium, no nitrogen source;
2) 250ml Pyramid Lake medium, no nitrogen source, 10mM $NaHCO_3$;
3) 250ml Pyramid Lake medium, 2mM KNO_3, 10mM KNO_3;
4) 250ml Pyramid Lake medium, 2mM KNO_3 (no $NaHCO_3$)

After two weeks, nitrate levels of nos. 1 and 2 had been at background level for seven days (value determined by NO_3 assay with medium with no KNO_3 as a control), the cultures were then harvested by centrifugation.

Values obtained for protein, carbohydrate, ash and lipid levels are summarized in Table 3. Protein levels in the nitrogen deficient cultures were approximately one-half of that of the nitrogen sufficient cultures. Results of the carbohydrate assay indicated that total cellular carbohydrate increased with nitrogen deficiency. No trend was apparent in the ash weights.

Lipids comprised 40% of the total dry cell weight of culture no. 2; 27.24% of no. 3; and 19.5% of no. 4. There was not enough sample recovered for an adequate extraction and quantification of no. 1. A slight shift from polar to nonpolar lipids was seen in nos. 2 and 4--methanol fractions of these two are lower than no. 3, while the chloroform fractions of 2 and 4 hold a greater percentage of the total than no. 3. The preponderance of lipids, however, was in the acetone fraction. TLC of fractions in polar lipid solvent showed identical separation patterns for chloroform, acetone and methanol fractions of all three extracts, the major difference being quantitative rather then qualitative. In the nitrogen deficient culture, intense yellow to orange pigments predominated. The yellow pigments in benzene fraction of no. 2 (Table 3) were particularly sensitive to drying and illumination. When the benzene fraction of no. 2 was dried (at 40°C) under nitrogen and subdued illumination, it turned from a bright yellow to a bright orange color and the absorption spectrum in the region from 400nm to 490nm was increased by 50% with respect to a new peak absorption at 660nm. TLC of this oxidized benzene fraction revealed a separation pattern identical to that observed in the acetone fraction of no. 2. On refractionation on Unisil, all visible pigment was eluted by acetone. Re-extraction of another sample of no. 2 with no illumination produced a benzene fraction with the principal component having an R_f value corresponding to a β-carotene standard.

In both TLC solvents a and b all three treatments had a major spot that co-chromatographed with a triglyceride standard. Saponification of chloroform fractions resulted in the near-total disappearance of this spot in no. 3, with a corresponding increase in the size of a spot co-chromatograhing with a fatty acid standard.

TABLE 2

FRACTIONATION ON "UNISIL" SILIC ACID COLUMNS

Culture Number	Sample	μg loaded	% Eluted Hex.	Benz.	$CHCl_3$	Acet.	MeOH	Total
1	Flagellate Walker Lake	64.8	0.43	2.70	19.06	59.90	18.38	100.47
3	Cryptomonad, Harper Lake	8.0	0.03	0.08	0.15	100.40	0.93	101.59
11	Dangeardinella	13.8	0.65	17.85	0.43	76.45	6.72	102.10
15	Chlorella Pond B	97.6	0.08	0.56	1.72	61.63	22.10	85.93

II. Oocystis sp:

The analysis of Oocystis was complicated by the presence of extrinsic bacteria. Cultivating Oocystis on agar surfaces and the subculturing of clones greatly reduced the number of bacteria but it did not eliminate them as judged by their substantial increase in numbers relative to the number of successive cultivations. The algal cells excreted glycoproteins as soon as the cells approached late exponential growth phase. The bacterial population then prolifierated. We therefore found it impossible to conduct meaningful analyses on the Oocystis in nitrogen free media. Visual detection of bacterial activity in the Oocystis cultures was masked by the deep green pigments of the algal cells; low speed centrifugation of Oocystis in nitrogen sufficient medium, however, demonstrated strata in the wet pellet. When in nitrogen deficient media, the culture was pale green; as the bacterial population increased, the culture turned red in color. A new sample of Oocystis will be obtained from Dr. Thomas. The results of the study attempted is described in the following lines.

One liter of log-phase culture was centrifuged (100 x g), washed two times with an isoosmotic salt solution and divided into four equal parts to inoculate two flasks each containing one liter Walker Lake, 20mM $NaHCO_3$ with no nitrogen source, and two flasks each with one liter as above supplemented with 4mM KNO_3. Harvesting was at two day intervals beginning 24 hours after inoculation.

The addition or omission of a nitrogen source caused effects or changes with time which were discernible in protein (8.4% - 24%) carbohydrate (16.5% - 32.7%) or ash (23%-26%). In nitrogen-deficient cultures lipid levels apparently decreased with time to 4-9% of cell dry wt. These results were attributed to decrease in the algae and bacterial proliferation.

TABLE 3

Profile of *Ankistrodesmus falcatus*

	ASH %	PROT. %	CHO %	LIPID %	Silicic Acid Separation of lipids HEX.	BENZ.	$CHCl_3$	ACET.	MeOH	TOTAL
1) no KNO_3	7.0	13.3	18.3	insufficient sample						
2) no KNO_3, 10mM $NaHCO_3$	5.4	14.3	18.3	40.3	-	3.3	13.5	66.5	12.1	95.4
3) 1.5mM KNO_3 10mM $NaHCO_3$	7.0	34.4	11.4	27.2	-	2.5	9.8	72.6	19.5	104.4
4) 1.5mM KNO_3	13.6	28.6	9.2	19.5	-	5.8	16.4	69.2	12.0	103.4
2') re-extraction of 2				40.0	-	3.2	14.1	66.8	10.5	94.6

Lipids of nos. 2 and 4 appear to have changed in the same way--the methanol fraction is slightly lower in these two than in no. 3, and the chloroform fractions have a greater percentage of the total lipids in 2 and 4 than in 3. The protein level of no. 2 is exactly half of that of no. 4. The apparent increase in carbohydrate levels in nitrate-deficient cultures may be an indication of the production of different types of sugars, since only glucose was used as a standard in the carbohydrate assay.

III. Monallantus salina: Monallantus was grown in either a supplemented seawater media or a completely defined media based on that of Provasoli. A lipid yield of 60% was obtained on the supplemental seawater medium (Table 4). Attempts to establish axenic cultures of Monallantus met with little success; both antibiotic and physical selection methods were tried.

Nitrate deficient cultures began as cultures low in nitrate allowed to exhaust the nitrate as evidenced by colorimetric assay. Cultures were typically given 10 mM of sodium bicarbonate every 3 to 5 days.

Composition under Nitrate Deficiency: These cultures were 9 days old at harvest. Initial nitrate was 1 and 5 mM for the nitrate deficient and sufficient cultures, respectively. The nitrate was exhausted in the nitrate deficient culture on the seventh day of cultivation as determined by colorimetric assay.

TABLE 4

Approximate Chemical Composition of Monallantus salina

	Nitrate Sufficient		Nitrate Deficient	
Component	Dry Cell Wt. (%)	Organic Wt. (%)	Cell Dry Wt.	Organic (%)
Ash	18	--	24	--
Protein	43	55.8	20	24.3
Carbohydrate	12	15.6	13	15.9
Lipid	22	28.6	49	59.8

Several attempts were made to grow the alga under these same conditions for longer periods of time, but bacterial blooms occured at 11 to 12 days.

Fractionation of Lipid on Unisil: Lipid from both nitrate sufficient and deficient cultures was fractionated on unisil by standard procedure (Table 5).

TABLE 5

Percentage of Silicic Acid Fractionation of Monallantus salina lipids

	Cell Preparation	
Fraction	Nitrate Sufficient	Nitrate Deficient
Hexane	2	1
Benzene	5	5
Chloroform	5	25
Acetone	55	42
Methanol	33	27

Nitrate sufficient and deficient cells gave similar lipid patterns on TLC except as noted. Only the major compounds are indicated; most of the pigments are not indicated.

TABLE 6-A,-B,-C

Thin Layer Chromatography of *Monallantus* lipids

A. Rf	Silicic Acid Fraction Hex.	Benz.	$CHCl_3$	Acet.	Probable Identity
0.97	+	+			Neutral lipids
0.84			+	+	Triglyceride
0.79			+		Pigment
0.68			+		1,3-Diglyceride
0.62			+		1,2-Diglyceride

Solvent: Diethyl ether, benzene, ethanol, acetic acid (40:50:2:0.2).

B. Rf	Hexane	Benzene	Probable Identity
0.80	+		Hydrocarbons
0.77		+	Pigment
0.70		+	Steryl esters

Solvent: hexane, diethyl, ether, 96:4.

C. Rf	Acetone	Methanol	Identity
0.84	+		Monogalactosyl-diglyceride
0.51		+	Phosphatidyl ethanolamine
0.44	+	+	Digalactosyl-diglyceride
0.43		+	Phosphatidyl glycerol
0.34		+	Phosphatidyl choline
0.23		+	Phosphatidyl inositol

Solvents: Chloroform, acetone, methanol, acetic acid, water 50:20:10:10:5.

The major lipid shift, in the cells conversion from nitrogen sufficient to deficient states was observed in the pigment contents and the triglycerides. The latter were the major lipid component in the nitrogen-limited cells, accounting for 42% of the total extracted lipids. The elution of triglycerides into the acetone faction is not uncommon when the triglyceride content is relatively large. Triglycerides complex in a undefined architectural arrangement with the gylcolipids. Recycling the acetone eluate through a silicic column eventually strips most all of the triglycerides into the chloroform eluate.

The fatty acids were predominantly polyunsaturated straight chains with the major species being 16:0, 16;1, 18.1, 18:2, 18:3 and 18:4. There were no major qualitative differences in the fatty acid contents of the lipids of nitrogen-sufficient and -deficient cells.

IV. Cylindrotheica fusiforma: The agal was grown in a medium of seawater supplemented with trace elements, phosphate, iron, silicate, borate, and nitrate.

The cellular composition under conditions of nitrate sufficiency and deficiency is given in Table 7.

TABLE 7

Approximate composition of Cylindrotheica

	Nitrogen Sufficient		Nitrogen Deficient	
	Cell Dry Wt.(%)	Organic Wt. (%)	Cell Dry Wt. (%)	Organic Wt.(%)
Ash	51	--	67	--
Protein	10	20	4	12
Carbohydrate	8	16	5	15
Lipid	9	18	4	12
Unassigned	22	15	20	61

A significant proportion of the approximate chemical composition is unassigned. This unassigned fraction consists of predominantly amphilic compounds that are comprised of oligo- and poly-saccharides derivatized with fatty acids (glycolipids) and amino acids (glycolipoproteins). These complex "lipids" are demonstrable by acid hydrolysis, solvent partitioning, derivatization,and analyses.

Cylindrotheica, cultivated on nitrogen deficient medium, shifts towards complex polymer biosynthesis, which are designated in the unassigned category (Table 7). Fractionation of the lipids from nitrogen sufficient or deficient cells on a unisil column indicated no significnt changes in the distribution of nonpolar to polar lipid (Table 8).

TABLE 8

Silicic Acid Fractions of Lipids of *Cylindrotheica*

Fraction	Nitrate Sufficient	Nitrate Deficient
Hexane	3%	3%
Benzene	3%	3%
Chloroform	16%	16%
Acetone	55%	56%
Methanol	19%	21%

Also, TLC of the fractions also revealed no significant qualitatively difference between the lipids of nitrate sufficient and deficient cells.

Isoprenoid Biosynthesis Inhibitors

A specific objective of this years project is to test the effects of chemicals on deregulating the biochemical pathway for isoprenoid biosynthesis.

Preliminary to any investigation of these inhibitors it is necessary to establish bacteria free cultures; this was accomplished for both *Dunaliella salina* and *D. bardawil*.

Screening of several inhibitors for lethal dose was undertaken for *D. salina* *D. bardawil*, and *Isochyris galbana*. The results of two inhibitors are shown in Table 9.

TABLE 9

Toxicity Measurements on Selected Algae

Inhibitor	D. salina*	D. bardawil*	I. galbana
$Pb(NO_3)_2$:			
10mM	-	-	-
0.1mM	+	+	-
1 μM	+	+	+

Table 9 continued:

Inhibitor	D. salina*	D. bardawil*	I. galbana
Pyridine			
10mM	-	+	-
0.1mN	+	+	-
1μM	+	+	+

*response recorded as growth (+) or nongrowth (-).

8-Hydroxyquinone was also tested and it was demonstrated to be nontoxic in doses as high as 10mM. Diphenylamine was toxic to all three test organisms at 10mM.

D. salina was grown in 10 μM diphenylamine under light for six days. The lipid content as a percentage of organic weight was increased in the diphenylamine treated culture to 35% compared to 28% in the control. The lipid fractionation pattern on Unisil was essentially identical to the control.

D. salina was also cultivated in media containing 100 ppm CPTA (p-chlorophenylthiotriethylamine-HCl) for two days in both light and dark. Even though CPTA prevented replication, the cells remained viable at this concentration; however, only a small alteration in the lipid pattern was observed as indicated in Table 10.

TABLE 10

Effects of CPTA on fractionated lipids of Dunaliella salina*

Unisil Fraction	Light, Control	Light, CPTA (%)	Dark, Control	Dark, CPTA (%)
Hexane	5	3	4	0
Benzene	6	2	2	0
Chloroform	11	13	23	15
Acetone	56	55	46	57
Methanol	22	26	25	28

*data reported as percentage of the total.

The benzene fraction does decrease, possibly suggesting a minor decrease in caretenoid.

Currently,studies of inhibitors are being conducted with radioactive bicarbonate to label the lipids and for a more convenient tracing method for evaluating the systems.

Inhibitor studies were also conducted with Botryococcus braunii. B. braunii was cultivated in a airlift system pressurized with either 1 or 5% CO_2 in

air. The organism grew rapidly for two generations; the growth thereafter quickly declines. Renewal of a media was sufficient to stimulate additional growth, but even then the limit was rapidly reached. The growth limitation stage was visually indicated by cell clumping even in rapidly stirred cultures.

Preliminary experiments have been initiated to indicate conditions stimulatory for lipid synthesis. Using C-14 labelled glucose nitrate deficient B. braunii accumulated more ^{14}C-lipids than nitrate sufficient cells. These results are indicated in Table 11.

TABLE 11

^{14}C-glucose uptake studies in Botryococcus in Light

	Cell Preparation			
	Nitrate Sufficient		Nitrate Deficient	
Initial Counts	19,000	cpm	19,000	cpm
Counts remaining in media after two days	4,160	cpm	9,500	cpm
Chloroform lipids	2,570	cpm (17.3%)*	3,330	cpm (35.0)*
Methanol-water soluble	1,120	cpm (7.5%)*	1,280	cpm (13.5%)*

*% of total incorporation.

B. braunii also incorporated more of the label into lipid when grown in the dark (heterotrophically). Again, the carbon source was ^{14}C-glucose, uniformly labelled (Table 12).

TABLE 12

^{14}C-glucose uptake studies in Botryococcus in Dark

	Cell Preparation			
	Nitrate Sufficient		Nitrate Deficient	
Initial Counts	19,000	cpm	19,000	cpm
$^{14}CO_2$ evolved	7,780	cpm	1,430	cpm

Table 12 continued:

	Nitrate Sufficient		Nitrate Deficient	
Chloroform lipids	1,520	(0.19)*	1,550	(1.08)*
Methanol-water soluble	1,220	(0.16)*	650	(0.48)*

*the ratio of incorporated ^{14}C to respired ^{14}C.

Research Developments in progress

There are several areas that we are exploring for facilitating the studies being conducted. These are:

1. Designing and Testing bioreactors for conducting steady state cultivations.

2. Establishing parameters for effective immobilization of Algal Test strains.

3. Correlating tandem-bioreactors for rapid conversion of nitrogen-sufficient cultures to nitrogen-deficient cultures and for testing metabolic inhibitors.

4. Final testing of a system for effective, rapid and inexpensive concentration and/or recovery of intact algal cells.

General Comments

The lipids of different microalgal cultures cultivated in "sufficient" media in batch culture are fundamentally similar in both quantity and quality. The chemotaxonomic specificities of the lipids usually restricted to specific pigments, fatty acid, and/or metabolic accessory components. These specific component types usually represent a relatively minor part of the total lipid. The screening of microalgae for lipid production is, therefore, of little value unless the isolates are also challenged by some stress state, such as nitrogen starvation, to force the metabolic mechanisms into a carbon storage pathway. The specific character of lipid production of each microalgal isolate is then exemplified. Examples of productivities under induced metabolic triggers are Botryococcus (botryococcenes), Dunaliella bardawil (β-carotene), Neochloris (triglycerides), Isochrysis ($C_{37}H_{70}O$) and so forth. It would be impractical, however, to conduct such thorough studies on every isolate obtained and being screened by members of the ASP. Thus, a better selection model must be developed for screening microalgae to assure a proper sample of the diverse algal systems and to minimize unnecessary duplications.

Equally important to the development of a screening model is the cultivation of the algae. The testing of algae for lipid production, metabolic triggers and/or metabolic inhibitors by cultivating batch algae in flask with agitation is unworkable. To adequately study these parameters we must have bioreactor(s) that support continuous cultures with control strategies for manipulating the external physical and chemical environment of the cells so as to optimize productivity. For these reasons we are working towards developing innovative bioreactor

designs with the vision directed towards scale-up pilot plants. This aspect of the work is being conducted in addition to our specific objectives outline in our subcontract. Several combined immobilization-continuous tandem bioreactors being tested show promise for enhancing our efforts to better understand the metabolic regulatory trigger for lipid biosynthesis.

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1984 Aquatic Species Program Review: Physiology of Oil Producing Microalgae in Response to Stress Conditions

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I. Introduction

Accumulation of storage lipids has been noted in certain species of algae. The storage lipids from algae, like those of the oily seeds, consist mainly of neutral lipids (mostly,in the form of triacylglycerols). Thus, algal lipids may serve as renewable sources of highly reduced organic carbon suitable for conversion into liquid fuels and/or as feedstock materials for the petrochemical industries. Previously most investigation of storage lipid production have been done with higher plants, especially the tissue of oily seeds. The substrates for lipid production are organic carbons transported to the seeds from the photosynthetic tissues of the leaves. In this respect, the oleaginous microalgae are unique in that oil accumulation takes place in cells that are photosynthetically active and intercellular transport of the intermediate metabolites are not required. These oleaginous microalgae are ideal subjects for the studies on the regulation of photosynthetic reductant utilization and partitioning of photosynthetically fixed carbon substrates.

During the last 3 years, work on lipid production and oil accumulation in algae has led the identification and isolation of numerous species of microalgae capable of accumulating massive quantities of intracellular storage lipids. Table I summarize the taxonomical distribution of the oleaginous algae currently maintained in our laboratory.

Table I A Taxonomic List of Oleaginous Microalgae

Chlorophyceae
- Volvocales
  - *Heamatococcaus sp*
- Chlorococcales
  - *Botryococcus braunii* (Gottingen strain)
  - *Botryococcus braunii* 572
  - *Chlorococcum aureum* 1768
  - *C. citriforme* 1769
  - *C. croceum* 1770
  - *C. oleofaciens* 107
  - *C. pinguideum* 774
  - *C. refringens* 1783
  - *C. scabellum* 1233
  - *Neochloris oleoabundans* 1185
  - *N. pseudostigmata* 1249
  - *N. pyrenoidosa* 777
  - *N. texensis* 1980
  - *N.* sp. S11 *** (From M. L)?
  - *Spongiochloris spongiosa*
  - *Protosiphon botryoides*
- Chlorellales
  - *Chlorella pyrenoidosa*
  - *C.* sp S01
  - *Ankistrodesmus braunii* 750
  - *A.* sp 163 (UC Berkeley)
  - *Scenedesmus* sp S02
  - *S.* sp S08
  - *S.* sp 308
- Uncertain: S03, S04, S05, S07, S09, S10 (*** From Mexico by M. S.), S11(Neochloris?)

Chrysopphyceae
- Vancheriales
  - *Botrydium becherianum*
  - *B. stoloniferum*
- Pennales
  - *Nitschia palea*
  - *Nitschia* sp (Mono Lake)
- Unknown: S06

Based on the currently available information in the literature and also from our own research data (1,2), one of the major technical problem in adapting the oleaginous microalgae as a renewable source of lipids is that for most algal species studied so far, the cultivation conditions which are most conducive to high rates of vegetative reproduction of algal cells tends to suppress

the active production and accumulation of the storage lipids. As a result, massive amounts of oil are present only in the old, non-dividing cells which appears to have a diminished photosynthetic capacity and efficiency. The research on the physiological and biochemical aspects of the storage lipid accumulation in these oleaginous algae to be reported here is aimed at obtaining the fundamental information useful to the resolution of this important technical limitation in the production of storage lipids by microalgae.

II. Materials and Methods

Organisms and Growth conditions. For the majority of work described here,the 37 strains of oleaginous algae currently maintained in our laboratory (see Table I) were used as the experimental organisms. Four strains Chlorella sp. S01, Neochloris texensis, Neochloris oleoabundans and Scenedesmus 308 has been studied more extensively. All strains were grown on Bold's Basal Medium (BBM) supplemented with 3.0 mM $NaNO_3$ or with nitrate plus urea. Shake cultures were maintained at 30°C under continuous light. Fermenter growth experiments were carry out in a New Brunswick Microferm fermenter equipped with a specially designed centrifugal-pump spray apparatus to prevent accumulation of cells on the glass walls. Cultures were 14 L of BBM containing the indicated amount of $NaNO_3$ as the sole source of nitrogen and were supplemented with 0.1 M NaCl. The salt was added to prevent cell clumping during logarithmic cell growth. Illumination around 360° of the cylindrical growth vessel was provided continuously at intensities of 30 or 70 $W.m^{-2}$ as indicated. Cultures were sparged with 4% CO_2 in air at 150 ml per minute under constant and vigorous agitation by a stirring impeller operating at 360-400 rpm. For dark culturing cell suspensions were aseptically transferred to 1 L flasks, wrapped with foil and placed on a shaker with a 30°C, 1% atmosphere.

Cell Growth Parameters. Dry mass was determined following filtration of specific culture volumes onto 2.5 cm Whatman GF/C filters and drying overnight at 85°C (constant weight were reached within 3 hours at this temperature).Cell counting and sizing were done on a Coulter counter model ZM with Accucomp data processing. Viable counts and contamination checks were performed by plating dilutions onto BBM or BBM with 1% yeast extract agar media.

Pigment Determination. Chlorophyll and carotenoids were determined after extraction into 95% ethanol. Spectra were obtained on a Hewlett-Packard 8450A spectrophotometer. For chlorophyll the formula of Wintermans and DeMots was used (3). For carotenes the extinction coefficients reported by Jensen (4) were used. For estimation of total carotenoids in the presence of chlorophylls, a normalized absorption spectrum of chlorophylls was first subtracted from the absorption spectrum of the total algal lipid extract (2).

Ion Determination. Nitrate and sulfate levels were determined using ion chromatography of culture filtrates. A Vydak 302 anion exchange column (250 mm x 4.5 mm ID) was used in a Hewlett-Parkard 1081B liquid chromatograph with a Wescan conductivity detector. For nitrate determination in the presence of NaCl the sample was first treated with Ag_2SO_4 to precipitate the chloride ion (2).

Photosynthesis and Respiration Rates. Rates of $^{14}CO_2$ fixation were measured in 1 ml samples transferred directly from the fermenter into the assay tubes containing 0.05 ml of 0.2 M K-phosphate buffer (pH 6.8). After preincubation in the dark for 10 min by slowly bubbling with CO_2-free air, 0.1 ml of $NaHCO_3$ (0.04 mCi,1.680 mg/ml) were added immediately before illumination with saturating intensity of white light from 120 W tungsten floodlamp. 0.05 ml aliquats were sampled at 1,3,5 min. and transferred into scintillation vials containing 0.05 ml of formic acid to stop the reaction and drive out the remaining bicarbonate. The fixed carbon were determined with a Beckman LS9000 scintillation counter with automatic quench correction in Beckman Ready-Sol.V MP scintillation cocktail mixture. The rates of photosynthetic oxygen production and respiratory oxygen consumption were assayed polarographically with a YSI-5331 oxygen electrode in a water-jacketed reaction vessel (5). A

tungsten-halogen lamp with fiber optic was used to provide saturating white light illumination for photosynthetic assays.

Gravimetric Determination and Microquantitation of Lipids. Whole cells were harvested and washed by successive 5 min 3000 rpm centrifugations. The pellet was extracted with ethanol and chloroform-methanol (1:1, v/v) as described previously (2). The mass of filtered (through a silicic acid column) or phase separated total lipid extracts were dry to constant weight in a vacuum oven and determined by analytical balance.(2). Triacylglycerols were quantified following separation on silica gel G plates (Supelco) as described previously (2). For the separation of neutral lipids and polar lipids (including steroids) total algal lipid extracts were chromatographed on silicic acid column with chloroform (6). Quantification of microsample of lipids were performed by differential refractive index analysis in a HPLC (when applicable) or by the colorimetic assay of dichromate consumption (6).

Nitrate Reductase. The enzyme assay was performed by a modification of the nitrite colorimetric assay (7). Cells were resuspending at 8x concentration in 0.1 M K_2HPO4 (pH 7.5) and were disrupted by three passes through a French Press cell at 18,000 psi. A 0.5 aliquat of the cell-free extracts was added to a mixture of 0.4 ml 0.1 M KNO_3, 0.5 ml of 0.2 N K_2PO_4 (pH 7.5) and 2.5 ml glass distilled water. The reaction was initiated by the addition of 0.1 ml of 20 mM NADH. After incubation (10-20 min) the reaction were stopped by addition of 0.5 ml of 1 M Zn-acetate. The samples are centrifuged for 5 min at 3000 rpm in a clinical Centrifuge. The supernatant was used for the quantification of nitrite produced by nitrate reducatase.

III. Results and Discussion

A: The Physiological Responses of Nitrogen Deprivation in Chlorella sp S01.

On a chronological basis, many of the physiological and biochemical parameters studied and discussed in the following sections show changes correlating best with nitrogen depletion regardless of its starting concentrations (up to 10 mM). The major differences in the culture conditions and yields are summarized in Table II. Our preliminary experiments with this alga indicated that the mass yield with BBM media is nitrogen limited when the initial concentration of nitrate is below 10 mM. For Experiment 1, which contains the highest initial concentration of nitrate (5 mM), the level of sulfate in the medium is also monitored. This ion also became depleted, but not until day 21 of the culture (i.e., 14 days after nitrate has become depleted). At this time both mass and lipid yield of the culture were nearly maximal (see Fig. 5 in Lipid Synthesis and Yield section).

Effects on Growth Parameters and Mass Yields. In N-limited, batch cultures of Chlorella, the concentration of nitrate in the medium quickly become depleted (Fig. 1, trace 1) following a period of exponential growth. A rapid rate of cell division was maintained only prior to the depletion of nitrate (Fig. 1, trace 2). Although, in this experiment, the cells also continued to divide slowly for a time after nitrate exhaustion (see Table II, cell density for Experiment 1). It should be point out that, at the end of the culture period, total cell count does not decline perceptibly. In addition, the viable cell counts also remain constant during this phase of culture. In preliminary experiments we have observed that

the oleaginous cells can maintain their viability for more than six months in low light,liquid cultures. Therefore, the decline in biochemical and physiological parameters to be described in later sections is most likely not a related to the death of algal cells.

In contrast to cell division, a steady increase in the total dry mass of the alga was maintained long after the complete exhaustion of nitrate (Fig.1, trace 3). In fact, nearly two thirds of the total algal mass were produced after nitrogen depletion. Similar increases during nitrogen starvation have been reported for other strains (8,9). Assuming that the inorganic cellular constituents remain at a relatively fixed fraction of the total algal mass, this steady increase indicates that photosynthesis can continue at rates above the compensation point for a significant period of time after nitrogen runs out. The duration of this post nitrogen-exhaustion production appears to be a sensitive function of both the initial level nitrogen supply and light intensity. This is clearly demonstrated by the ratio of mass yields obtained at nitrate depletion to that obtained during the post-nitrate depletion period of these three experiments (Table II, rows 1 and 2 under DRY MASS YIELD). Interestingly, when dry mass yields of these experiments were normalized with respect to amount of total nitrate supplied, this difference in the pre-depletion and post-depletion productivity of the culture becomes even more apparent (Table II, rows 4 and 5 under DRY MASS YIELD)

Finally it should be mentioned that during the post nitrogen depletion, nonreproductive phase of the culture, the cell volume steadily increases reflecting the differential rates of mass

accumulation and cell division. The increased cell size of the aged nitrogen-starved cultures of this organism is also readily apparent under the microscope.The fluctuations in cell volume prior to nitrogen depletion (Fig. 1, trace 4) are, probably, due to semisynchronous production and release of autospores in this culture.

Table II. Culture Conditions and Yields from Fermenter Experiments of *Chlorella* sp. S01

Cultures were maintained at 30°, under continuous illumination, bubbled with 4% CO_2 in air, and continued until cell mass showed no measurable increase.

	Experiment		
	1	2	3
CULTURE CONDITIONS			
Initial Nitrate (mM)	5	1.5	1.5
Light Intensity (W/m^2)	30	30	70
Culture Duration (days)	25	9	6.5
CELL DENSITY (10^7 cell/L)			
At nitrate exhaustion	13	4.2	4.3
Final	21	4.9	4.6
PIGMENT CONTENT (mg/g dry mass)			
Chlorophylls (peak)	25	12	8.9
Chlorophylls (final)	1.9	0.89	0.95
Carotenoids (peak)	1.5	0.94	0.97
Carotenoids (final)	1.3	0.94	0.87
DRY MASS YIELD (g/L)			
At nitrate exhaustion	1.3	0.48	0.53
Post-nitrate exhaustion	3.0	0.41	0.19
Final	4.3	0.89	0.72
Dry Mass/Initial Nitrate (g/mmol.)			
At nitrate exhaustion	0.26	0.32	0.35
Post-nitrate exhaustion	0.60	0.27	0.13
Final	0.86	0.59	0.48
Total Dry Mass/Peak Chlorophylls (g/g)	126	143	151
LIPID YIELD (g/L)			
At nitrate exhaustion	0.17	(0.06)	(0.07)
Post nitrate exhaustion	1.37	(0.30)	(0.17)
Final	1.54	0.36	0.24
Lipid content (% of dry mass)	36	36	34

Pigments. In this and all other nitrogen depletion experiments conducted so far, the content of chlorophylls in Chlorella S01 was observed to decrease rapidly commencing at the time when nitrate in the medium dropped below the level of detection (c.a. 100 uM) as illustrated by traces 1 and 2 of Figure 2 (also see Table II,rows 1 and 2 under PIGMENT CONTENT and Fig.4, traces 1 and 3). The key role played by the chlorophylls in the production of algal mass is best illustrated by the data of Table II. In spite of the large differences in the level of initial nitrate and the light intensity received by these cultures, the final yield of total algal mass is fairly constant if it is normalized with respect to the peak amounts of chlorophylls present in the culture (TableII,row 7 under Mass Yield). These data can be interpreted to indicate that the effective catalytic life time of individual chlorophyll molecules stays approximately the same under the different culture conditions of these experiments. However, as will be discussed in the next section (**Photosynthesis**), the observed changes in the chlorophyll content of the algal cell was accompanied by a large alteration in the overall photosynthetic activity. Therefore, the mechanism via which chlorophyll degradation occurs in nitrogen deficient cultures was investigated further. Table III shows the results of an 84-hour dark period imposed shortly after nitrogen starvation (in Experiment 3).It is clear from the data presented in column 4 (chl,mg/ml), the loss of chlorophylls in nitrogen starved cells occurs only in the presence of light. The total preservation of chlorophylls in the dark also indicates that a photochemical process is required for chlorophyll degradation in Chlorella under nitrogen starvation.

In contrast, to the chlorophylls, the carotenoids show little change in in their relative amount regardless of the culture conditions (Fig. 2. trace 3 and Table II, rows 3 and 4 under Pigment Content). Carotenoid synthesis continues, keeping pace with the mass increase long after nitrogen deprivation. The different responses of carotenoids and chlorophylls lead to a greatly enhanced ratio of carotenoids to chlorophylls (Fig. 2, trace 4) and resulted in the bright orange appearance of aged cultures of this and other oleaginous algae. Comparison of successive experiments shows that total carotenoid content in algal cells is not affected significantly by light intensity or initial nitrate concentration. These results (Table II) differ from those of Iwamoto and Nagahashi (10) who found that carotenoids declined along with chlorophylls in Chlorella ellipsoidea in the light. Although individual pigments have not yet been quantified, it is apparent from the appearance of chromatographic separations, such as those shown in Fig. 6, that certain carotenoids appear and others disappear in response to nitrogen deprivation.

Photosynthesis. Pratt and Johnson (11) have stated that "lipid accumulation in excess of about 25% of total dry weight is indicative of waning vigor in cultures of Chlorella. . ." The decline in photosynthesis commencing immediately upon nitrogen deprivation tends to support this diagnosis for Chlorella sp. S01. Figure 3 (trace 2) shows that the overall measured photosynthetic capacity per unit culture volume drops as chlorophyll is degraded in the light. Similar photosynthetic changes over a somewhat longer period of the time were seen in the reduced light conditions of Experiment 2 (data not shown). The drop in the culture pH in

Experiment 3 (Fig.3, top trace) and the other experiments occurs within 24 hours after nitrate depletion also suggests a decline in photosynthesis.

In Experiment 3 the specific rate of CO_2 fixation reached a peak of 120 umol CO_2 ·mg $chl^{-1}\cdot h^{-1}$. Twenty-four hours later, during which time the nitrate supply was exhausted, this rate was only 40 umol CO^2 ·mg $chl^{-1}\cdot h^{-1}$ (Fig. 3, trace 3). Clearly the changes in photosynthesis cannot be explained by the degradation of chlorophyll alone. This change could reflect a real drop in the efficiency of photosynthesis and /or an increase in the photosynthetic quotient. Van Oorschot (cited in 12) found a drop in the quantum efficiency after nitrate deficiency in Chlorella. However, Fogg (12) reported a decline in the photosynthetic rate for Monodus only proportional to the loss of chlorophyll after transfer of cells to nitrogen-free medium. He observed no significant change in the photosynthetic quotient. Measurements of the CO_2 fixation following the prolonged dark period (Table III, last column) show that, like chlorophylls, photosynthetic competence remains intact in the dark. There may even be a partial restoration to the rate observed before nitrogen deprivation.

Nitrate Reductase Activity. As demonstrated in Figure 4 (traces 1 and 2), nearly 97% of the total nitrate reductase (NR) activity detectable in nitrogen-sufficient cells of Chlorella sp. S01 was lost within the first 6-hours after nitrogen exhaustion. An equally rapid decline of NR activity was also observed during an earlier experiment (Experiment 1, data not shown) responding to nitrogen deprivation. It is not yet known whether this change in level of NR activity is a reflection of a reversible inactivation or

of an actual degradation of the enzyme, in vivo. Since NR is the first enzyme of the nitrate utilization pathway, a better knowledge concerning the biochemical control of its activity and the regulation its biosynthesis may improve our understanding on the link between nitrogen metabolism and lipid metabolism in this organism.

Lipid Synthesis and Yield. As mentioned earlier in the introduction that cultures of Chlorella sp. S01 under nitorgen deprivation enter into a nonreproductive, oleaginous phase of growth during which synthesis and accumulation of storage lipids occurs. Although the experiments reported here were carried out under continuous light, it should be noted that cultivation of this organism in the similar nitrogen-limiting media under a 16:8 light:dark illumination cycle also leads to the same oleaginous condition according to the results obtained in an earlier fermenter experiment.

Spoehr and Milner (8) found that an external nitrate concentration of less than 1 mM was required for high lipid content in Chlorella pyrenoidosa. Richardson et al. (13) found no increased lipid synthesis in continuous cultures of C. sorokiniana and Oocystis polymorpha supplied with nearly limiting level of nitrogen. In our experiments accumulation of storage lipids does not commence until after the external nitrate drops below 100 uM. When calculated on a total dry mass basis, the lipid content of the algal cells rises steadily after nitrogen depletion (Fig. 5, ratio of trace 3 to trace 2). As seen in Table II,(rows 1 to 3 under Lipid Yield), the bulk of total lipid found at the end of the culture were of post

nitrate exhaustion origin. In Experiment 1, the rate of total lipid synthesis calculated for a 24-hour period preceding nitrate exhaustion is approximately 8 pg $\cdot cell^{-1} \cdot d^{-1}$. During this reproductive phase of growth the culture had already become light limited, therefore, the potential lipid synthesis rate is probably significantly higher. This same rate of total lipid synthesis calculated for a 36-hour period starting 12 hours after nitrate depletion is 2.8 pg$\cdot cell^{-1} \cdot d^{-1}$. This rate is also probably light-limited since 5.0 pg.$\cdot cell^{-1} \cdot d^{-1}$ of total lipid are synthesized after nitrogen deprivation under the brighter light of Experiment 3. Although it is not possible to determine the maximum potential rate of total lipid synthesis due to light limitation in these experiments, our current data illustrate that an increased rate of total lipid biosynthesis is not a prerequisite for the accumulation of storage lipid in this oleaginous alga.

Qualitatively, there is a marked shift in the lipid composition. Starting approximately 12 hours after the exhaustion of nitrate triacylglycerols progressively become the dominant lipid class. They reach a maximum at approximately 80% of the total lipid(Fig. 5, traces 4 and 5). A comparison of the slope for traces 3,and 4 of Figure 5 indicates that triacylglycerols alone can account for virtually all of the lipid produced after nitrogen starvation. We have calculated that, for a 36-hour period starting at 12th hour after nitrate depletion, the rate of total lipid synthesis is 2.8 pg$\cdot cell^{-1} \cdot d^{-1}$ in Experiment 1. During this same period the rate of triacylglycerols production corresponds to 2.7 pg$\cdot cell^{-1} \cdot d^{-1}$. Thus, under nitrogen deprivation 96% of newly synthesized lipids is triacylglycerol. Figure 6 shows a

chromatographic separation of the representative total lipids extracted from cells obtained at different time during the Experiment 1 (Figures 1, 2, and 5). Although other lipid classes have not been quantified, it is apparent that most of them decline in relation to the triacylglycerols. There is no apparent increase in the hydrocarbon fraction in this alga.

The Effect of Dark Period on Lipid and Mass Yields of Nitrogen-starved Chlorella The results in Table III show the basic effect of an extended dark period on algal dry mass and its lipid content. Under nitrogen starvation the lipid content (column A under Lipid) increases slightly in the dark but this is more a reflection of respiratory consumption of non-lipoidal material rather than any true increase in the actual amount of lipid (column B under Lipid). The simplest explanation for this result is that other cell materials are preferentially catabolized in the dark aerobic metabolism of the alga. In fact,under dark, nitrogen-limiting conditions the amount of lipid per unit volume of culture is so well conserved that there appears to be neither an active degradation nor a significant synthesis of lipids (from other cellular constituents). These observations agree with those of Spoehr and Milner (8) who found that lipid yield is proportional to the time in the light for cultures of C. pyrenoidosa.

Table III. Growth of *Chlorella* sp. S01 in Nitrogen-depleted Medium

The algal cells used in this experiment was obtained from fermenter Experiment 3 (see table II for details). Culture was grown in the light (70 $W \cdot m^{-2}$) until 12 hours past nitrogen starvation (= starting). A portion was then transferred to darkness and the remaining culture was maintained under illumination. Both were terminated for analysis after an additional 84 hours.

	Dry Mass	Lipid (A) Content g/g mass	Lipid (B) Amount g/L	Chl	Photosynthesis umol CO_2 fixed / mg chl·h
STARTING	0.71	0.21	0.15	4.4	38
AFTER 84 HOURS					
Light	0.71	0.34	0.24	0.7	34
Dark	0.55	0.24	0.13	4.5	58
% CHANGES					
Light:Starting	0	+62	+60	-84	-11
Dark:Starting	-23	+14	-17	+2	+53

Conclusions

1. The data on the physiological responses of *Chlorella* induced by nitrogen deprivation underscore the fact that storage triacylglycerol synthesis in this green alga is a stress response phenomenon.

2. The final mass yield can be affected by initial nitrate level and by light intensity. However, these conditions do not affect the percentage of lipid formed. On the other hand, they can affect the duration of post-nitrogen lipid production and thereby affecting the amount of lipid produced by a given volume of culture.

3. The rate of total lipid synthesis on a unit mass or per cell

basis does not increase upon nitrogen exhaustion. Thus it is likely that the observed increase in the lipid content of this oleaginous alga is a result of a substantial decline in the rate of other anabolic functions.

4. Independent of the initial nitrate concentration (up to 5 mM), the rate of photosynthetic carbon fixation begins to decline immediately upon nitrate depletion.

5. The light requirement for lipid synthesis along with the declining photosynthetic rate in the light after nitrogen depletion means that there is a limited time window during which it is possible to maintain a substantial rate of lipid synthesis.

6. greater understanding of the regulation of triacylglycerol synthesis and the nature of the concomitant changes in photosynthesis will be required before it will be known if storage triacylglycerol synthesis is fundamentally linked to a suboptimal photosynthetic rate in green algae.

B:Other Technical Accomplishments (FY83-84).

1). Observed an increase in the lipid content of *Chlorella* when the cells are subjected to a high salinity stress in nitrogen sufficient media. Preliminary results indicating that high salinity also leads to a quick reduction of the nitrate reductase activity.

2). Developed a sensitive microquantification method for the analysis of total lipids and neutral lipids from small samples (i.e. 2-5 mg total dry mass) of microalgae

3) Observed an increased extent of "Post-illumination Oxygen Gulp" under nitrogen deprivation.

IV. Future Research Plan. (FY1985-FY1986)

1. Photosynthesis. As mentioned under results and discussion, light is required for lipid synthesis in algae. But under nitrogen deprivation, light also leads to the destruction of chlorophylls and degradation of the photosynthetic capacity. These observations underscore the great need to conduct a detailed characterization of the structural and functional aspects of the photosynthetic apparatus of the algae under nitrogen deprivation and other stressful conditions in order to ascertain the nature of the light-dependent degradation of photosynthesis. Experiments will be conducted with the algal cells subjected to transitions from nitrogen-limited and nitrogen-sufficient media. The competence of the photosynthetic apparatus with respect to light harvesting and electron transport will be examined by correlating such parameters as the number of reaction centers, the kinetics of chlorophyll a fluorescence, and various partial reactions of the electron transport to the amount of chlorophylls present. Changes in the photosynthetic carbon metabolism will be examined by photosynthetic quotient measurements and by $^{14}CO_2$-fixation product analysis experiments. As the nature and the mechanism of the degradation of photosynthetic capacity become understood, additional experiments will be conducted to determine the degree to which this undesirable effect of nitrogen deprivation can be retarded and or reversed.

Parallel experiments concerning the above mentioned characterization of the photosynthetic parameters will also be conducted using diatoms subjected to silicon deficiency which has also been reported to induce active lipid synthesis.

2.Enzymology of Storage Lipid Biosynthesis. The modulation of the activities of the key enzymes in fatty acid synthesis, triacylglycerol synthesis,and lipid utilization enzymes by the nutritional stresses will be studied at the level of protein synthesis and modifications as well as at the level of metabolite feedback control. Key enzymes involved in the synthesis of carbohydrate in these algae will also be studied to see whether the level of enzymes or the mode of modulation of their catalytic activity shows any complementary relation with those of the lipid synthesis pathway.

3. Interaction between Nitrate metabolism and Lipid Metabolism. Lipid biosynthesis and nitrates assimilation represent two of the major consuming reaction for the photosynthetic reductant. Experiments will be conducted to evaluate the degree of competition for the available photosynthetic reductant between these two pathways under stressful and nonstressed conditions. The possibility that this competition for for a limiting quantity of reductant is one of the short term controlling factor in the regulation of the partitioning of the photosynthetically fixed carbon between the lipid synthesis and nonlipid pathways will be explored. On a longer term basis, the effect of substituting nitrate with ammonia on the lipid metabolism will be studied. The relation between nitrate assimilation and lipid synthesis will also be examined at enzymic level. Nitrate reductase activity of algae has been shown to be under the control of various chemical and physical parameters. The effect of lipid-promoting stress conditions on the level and activity of nitrate reductase will be examined.

4. The Effect of Lipid-promoting Stresses on "Post-illumination Oxygen Gulp" and Photorespiration. Preliminary results in our laboratory showed that oleaginous Chlorella under nitrogen deprivation exhibits a much more pronounced "post-illumination oxygen gulp". The nature of this phenomenon and its relationship to photorespiration in the oleaginous algae will be investigated. In addition, the impact of the "post-illumination oxygen gulp" on the energy efficiency of the algal mass and lipid production will be evaluated.

5. Isolation of Additional Oleaginous Strains and Mutants of Oleaginous Algae. Algal strains with the following phenotype will be sought:

a).Wild type and mutant strains with unimpaired photosynthetic capacity under lipid-inducing stresses.

b). Mutants with impaired lipid synthesis (useful for delineation of the biosynthesis pathway and regulatory mechanism).

c). Spontaneous and induced mutant strains with enhanced level of total lipid producing capacity will be isolated through a positive selection scheme taking the advantage of the higher buoyancy resulting from high lipid content of the desired mutants.

V. Publications.(FY83-84)

1) S.Lien and K.G. Spencer. 1983. "Microalgal Production of Oils and Lipids," in Proceedings of IGT Symposium on Energy from Biomass and Wastes, Vol. VII, pp.1107-1121.

2) S. Lien and K.G. Spencer. 1983. "Algal Oil Production and Lipid Metabolism Research," in Proceedings of Aquatic Species Program Principal Investigator's Meeting, SERI/CP231-1946, pp.3-18.

3) Tornabene, T.G., G. Holzer, S. Lien and N. Burris. 1983. "Lipid composition of the nitrogen starved green alga Neochloris oleoabundans," Enzyme Microb. Technol. 5:435-440

4) K.G. Spencer and S. Lien. 1983. "Utilization of Stored Oil in a Strain of Chlorella", Plant Physiology 72:170 Abstract #966.

5) K.G.Spencer and S. Lien. 1984. "The Synthesis of Storage Lipid by Green Algae. I. The Effects of Nitrogen Depletion on a Species of Chlorella", Plant Physiology (in press).

6) K.G. Spencer and S. Lien. 1984. "Extraction and Microquantification of Lipids from Green Microalgae." (Manuscript to be submitted for publication in Analytical Biochemistry).

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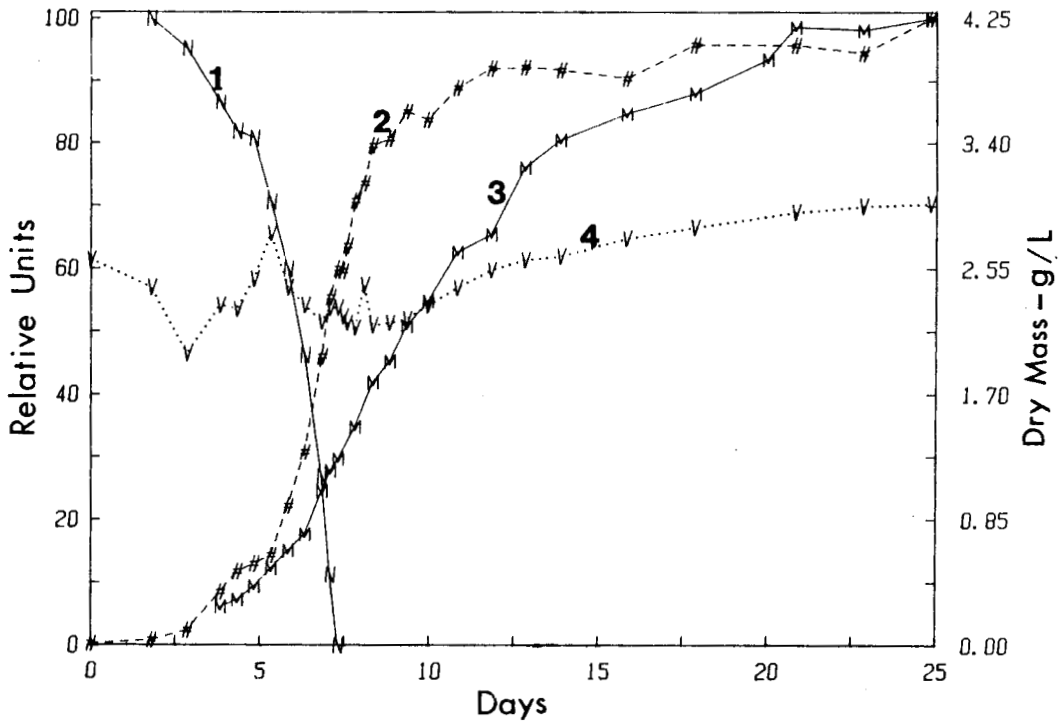

Fig. 1. Cell growth parameters of Chlorella sp. S01 during the complete time course of experiment 1. Trace 1: N is nitrate concentration (100 = 5.0 mM). Trace 2: # is cell number in relative scale (100 = 2.2×10^{10} cells/L). Trace 3: M is dry mass in the units of the right ordinate. Trace 4: V is average individual cell volume in relative scale (100 = 500 fl).

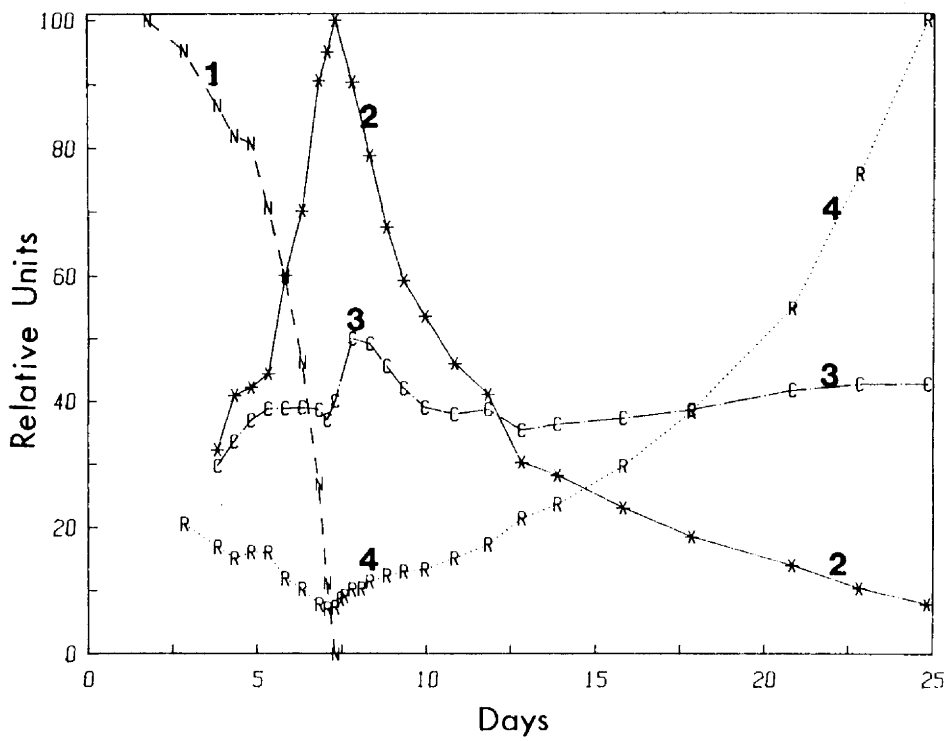

Fig. 2. Pigment content during experiment 1. Trace 1: N is nitrate concentration in relative scale (100 = 5.0 mM). Trace 2: * is chlorophyll content (100 = 22 mg per g dry mass). Trace 3: C is total carotenoid (100 = 2.5 mg per g dry mass). Trace 4: R is the mass ratio of carotenoid:chlorophyll (100 = 0.66 g/g).

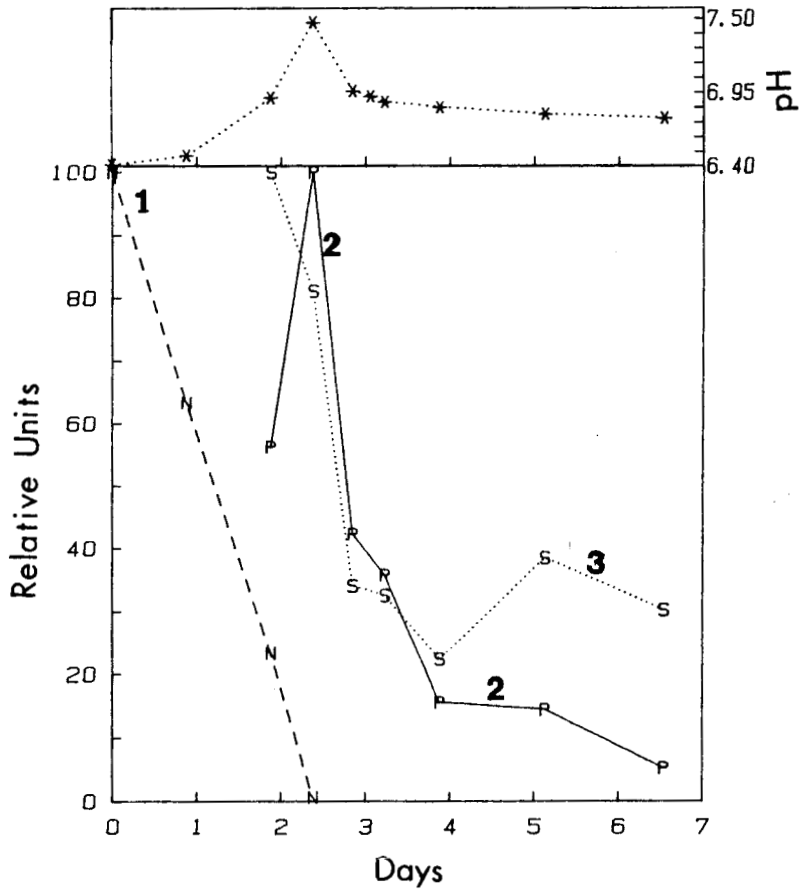

Fig. 3. Photosynthesis and culture pH during experiment 3. The top plot (*) is the change in the pH of the culture which was bubbled throughout with 4% CO_2 in air at 150 ml per min. In the bottom graph, Trace 1: N is nitrate concentration (100 = 1.5 mM). Trace 2: P is CO_2 fixation of samples directly from fermentor (100 = 0.46 $mmol \cdot L^{-1} \cdot h^{-1}$). Trace 3: S is a plot of the same data as $\mu mol \cdot Chl^{-1} \cdot per\ h^{-1}$.

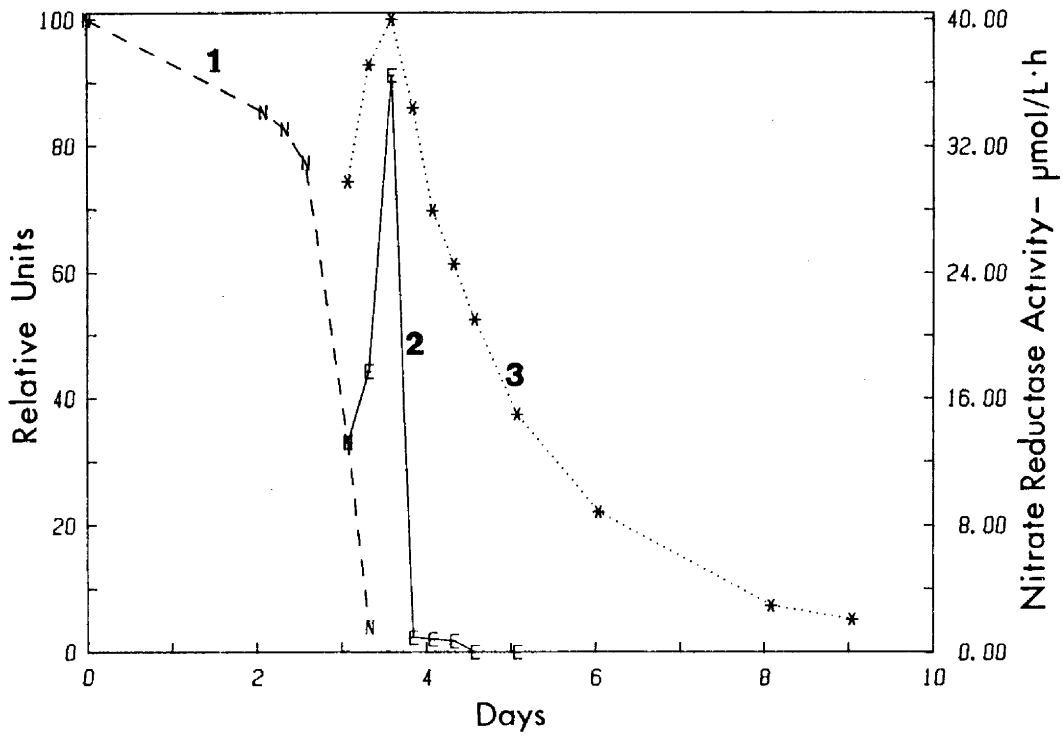

Fig. 4. Nitrate reductase and chlorophyll content durig experiment 2. Trace 1: N is the nitrate concentration (100 = .5 mM). Trace 2: E is the enzyme activity of crude extracts in the units of the right ordinate. Trace 3: * is the chlorophyll content (100 = 12 mg per g dry mass).

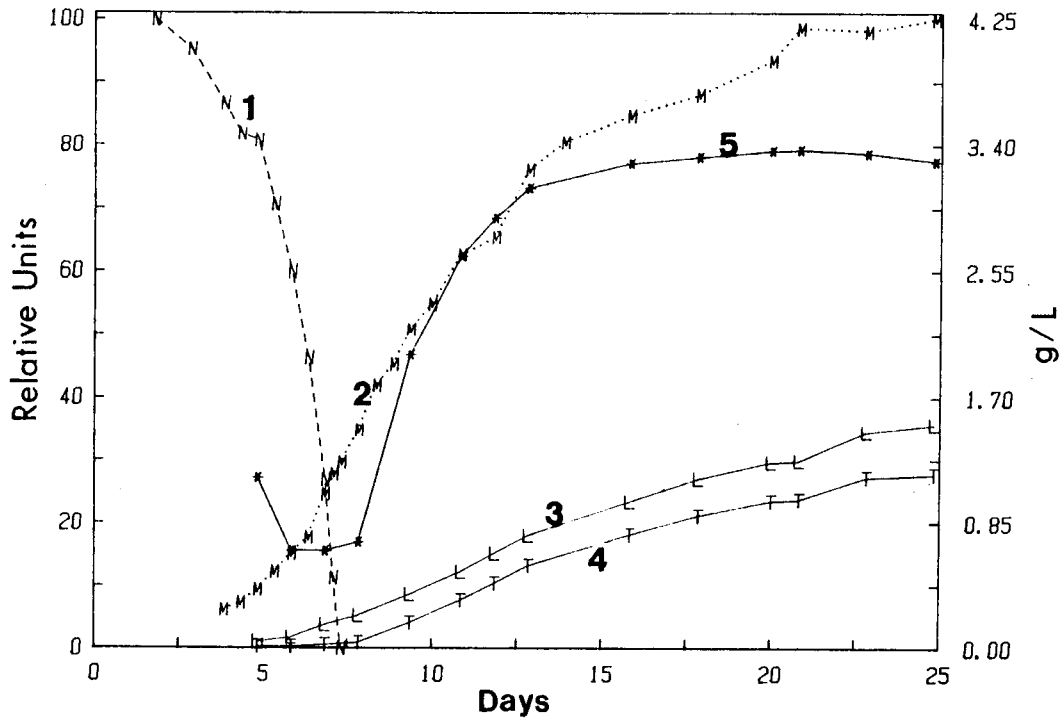

Fig. 5. Mass and lipid production during experiment 1. Trace 1: N is nitrate concentration (100 = 5.0 mM). Trace 2: M is dry mass in g/L. Trace 3: L is total lipid in g/L. Trace 4: T is triacylglycerol in g/L. Trace 5: * is the ratio of triacylglycerol/total lipid (100 = 1.0 g/g).

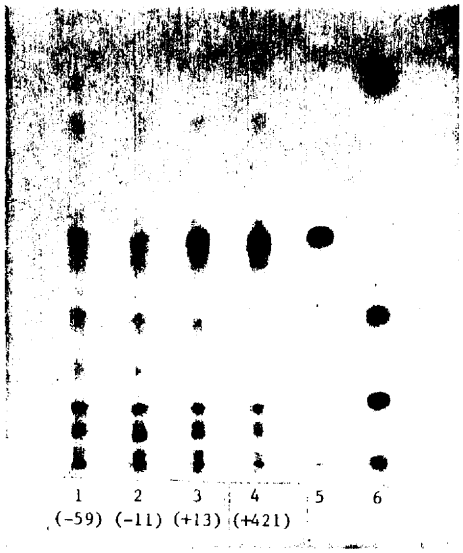

Fig. 6. TLC separation of total lipids from experiment 1. Separation on silica gel G is with hexane:ether:formic acid (75:25:1, v:v:v). Visualization is by charring. Forty µg of total lipid from selected samples are separated in lanes 1-4. The numbers in parentheses are the approximate time (in hours) before (-) or after (+) nitrogen depletion. Lane 5 is 25 µg of triolein. Lane 6 is the separation of lipid standards (25 µg each). From top to bottom they are a C18-C24 alkane mix, stearic acid, chloesterol and phosphatidyl choline dioleoyl.

OPTIMIZATION STUDIES IN SHALLOW ALGAL MASS CULTURE FLUMES

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INTRODUCTION

Historical Background

Microalgae are attractive as biomass producers because they generally exhibit higher yields and photosynthetic efficiencies than terrestrial plants. In addition, many species of microalgae produce high concentrations of intracellular oils as energy storage compounds. Microalgae are projected from laboratory scale experiments to be capable of producing over 100 barrels of liquid fuel per acre-year. Most pilot-scale mass culture systems, operated at depths of greater than 20 cm, have shown peak average yields of around 15 dry ash free grams $m^{-2}d^{-1}$. This project is based on the premise that operation at less than 15 cm depth will result in significantly higher productivities.

Research on this problem began at the University of Hawaii under SERI subcontract on February 15, 1980 under the title "Research, Development, and Demonstration of Algal Production Raceway (APR) Systems for the Production of Hydrocarbon Resources." Work during the first two and one-half years resulted in the collection of a great deal of laboratory data on the performance of *Phaeodactylum tricornutum*; the design, construction, and operation of one $48m^2$ continuous flume; the development of predator control techniques and baseline performance characteristics for the flume; and the construction of four $9.2m^2$ experimental flumes. During the next year these four flumes were operated in a factorial design experiment to determine the maximum sustainable biomass yields achievable with *P. tricornutum* cultured in shallow flume systems. The results of the factorial experiment showed that production of about 25 g ash-free dry wt $m^{-2}d^{-1}$ could be achieved in the flumes. This production corresponded to a photosynthetic efficiency of $\sim$5%. These figures are 50-100% better than the production rates achieved in earlier *P. tricornutum* cultures using conventional culture techniques.

An obvious problem with the use of *P. tricornutum* for energy production was the requirement to cool the culture flumes during most seasons of the year under the conditions prevailing in Hawaii. *P. tricornutum* grows poorly if at all when mean temperatures exceed 25°C, and during the summer in Hawaii flume temperatures were found to be no lower than about 24°C, even at night. The emphasis of research during the present contract year has been to identify microalgal species which can grow satisfactorily in the outdoor flumes without supplemental cooling, and to analyze the potential of these cultures for energy production. These goals are to be achieved through work on the following three tasks:

I Screening of candidate species in laboratory cultures
II Yield optimization of promising species in outdoor flumes
III Analysis of promising culture management strategies

Theoretical Background

It has been known for a number of years that the efficiency of light utilization by phytoplankton can be markedly increased by exposing the cells to alternating periods of light and dark [1-7]. More recently, a number of investigators have explored this phenomenon both experimentally and theoretically [8-15]. Although early work [2-4] indicated that flash periods as short as 10 μs were needed to take maximum advantage of the flashing light effect, it is now clear that flash periods as long as 10-70 ms may still result in significant enhancements of photosynthetic efficiencies [14,16], and Marra [13] has reported increases up to 87% in photosynthesis by simply modulating the irradiance on algal cells on a time scale ranging from minutes to hours. It seems reasonable to postulate that the physiological mechanisms responsible for these observed photosynthetic enhancements differ substantially over the vast range of time scales involved. However, regardless of the physiological mechanisms involved, it is clear that photosynthetic efficiencies may be enhanced by a factor of two or more by modulating or flashing the incident light on an appropriate time scale. For the case of flashing light, it is apparent that the maximum duration of the light pulse which produces the highest photosynthetic efficiency will be negatively correlated with light intensity at light intensities greater than or equal to I_K, the light intensity above which photosynthetic efficiency begins to decrease under constant illumination [17], and that the duration of the dark period must lie somewhere in the approximate range 0.1-10s [18].

A critical question in algal mass culture work is whether a physical mechanism exists for taking advantage of the flashing light effect in a practical way. As noted by Phillips and Myers [14], "Any attempt to grow algae in sunlight will experience some gain by turbulence. The feasability of increasing the turbulence will depend upon the extent of the gain in growth as compared to the increased power requirements of stirring or pumping the suspension." Unfortunately, it does not appear that merely producing random turbulence is sufficient to take advantage of the flashing light effect to any significant degree in a mass culture system. Powell, Chaddock, and Dixon [15] reached this conclusion based on theoretical calculations, and Miller et al. [19] commented that, "Utilization of the flashing light effect for improvement of photosynthetic efficiency of dense algal cultures requires a nonrandom mixing pattern - one in which cells are exposed to regular sequences of light and darkness..."

In the past two types of experimental systems have been studied as possible means of utilizing the flashing light effect. A so-called Couette device was used by Miller et al. [19], Davis et al. [20], and Howell, Fredrickson, and Tsuchiya [21]. The Couette device consists of two concentric, circular cylinders, the inner of which can be rotated at a selected speed. The system is illuminated either from the outside or inside, and the culture is grown in the gap between the cylinders. The flow pattern in the culture medium is

complex, but sufficiently nonrandom that a systematic flashing light effect is achieved. Howell, Fredrickson, and Tsuchiya [21] estimated the period of the light-dark cycles in their system to be about 40 msec. More recently, Oswald et al. [22] and Shelef, Sabanas, and Oswald [23] have described a chemostat-type system called an algatron, which consists of a drum that is rotated about its vertical axis. The algal culture is contained within the drum as a film a few centimeters thick on the inside wall of the drum, and is retained against the wall by centrifugal force. A light source is mounted inside the drum, and a row of steel strips extending to within 0.5-1.0mm of the inside wall produces highly turbulent wakes, which extend in spirals around the culture. The photosynthetic efficiencies which can be achieved with the Couette device and algatron are on the order of 10% [17].

It seems unlikely that either the Couette device or the algatron will prove to be a practical means of utilizing the flashing light effect. Both devices are mechanically complex, and the power requirements for operating them may well offset any increase in production, both from a financial and a energetic standpoint. As noted by Fredrickson and Tsuchiya [17], "Clearly some device that is mechanically more simple than the foregoing devices is required." The mechanism we have devised to produce systematic vertical mixing in our flume is illustrated in Figures 1 and 2. The device consists of a series of foils similar in design to airplane wings placed across the flume. As water flows over and under these foils, a pressure difference is created as illustrated in Figure 1. At the tips of the foil, the flow of water from the high pressure region below the foil to the low pressure region above the foil creates a vortex off each tip of the foil. If the foils are properly spaced along a suitable supporting structure (Figure 2), the vortices on adjacent foils rotate in opposite directions and thus reinforce each other. The width of each foil and the gap between foils are equal to the depth of the culture, so that circular vortices created by the foils effectively mix the culture from top to bottom (Figure 2). The system of vortices with rotational axes parallel to the direction of flow produces the sort of systematic mixing necessary to produce the flashing light effect.

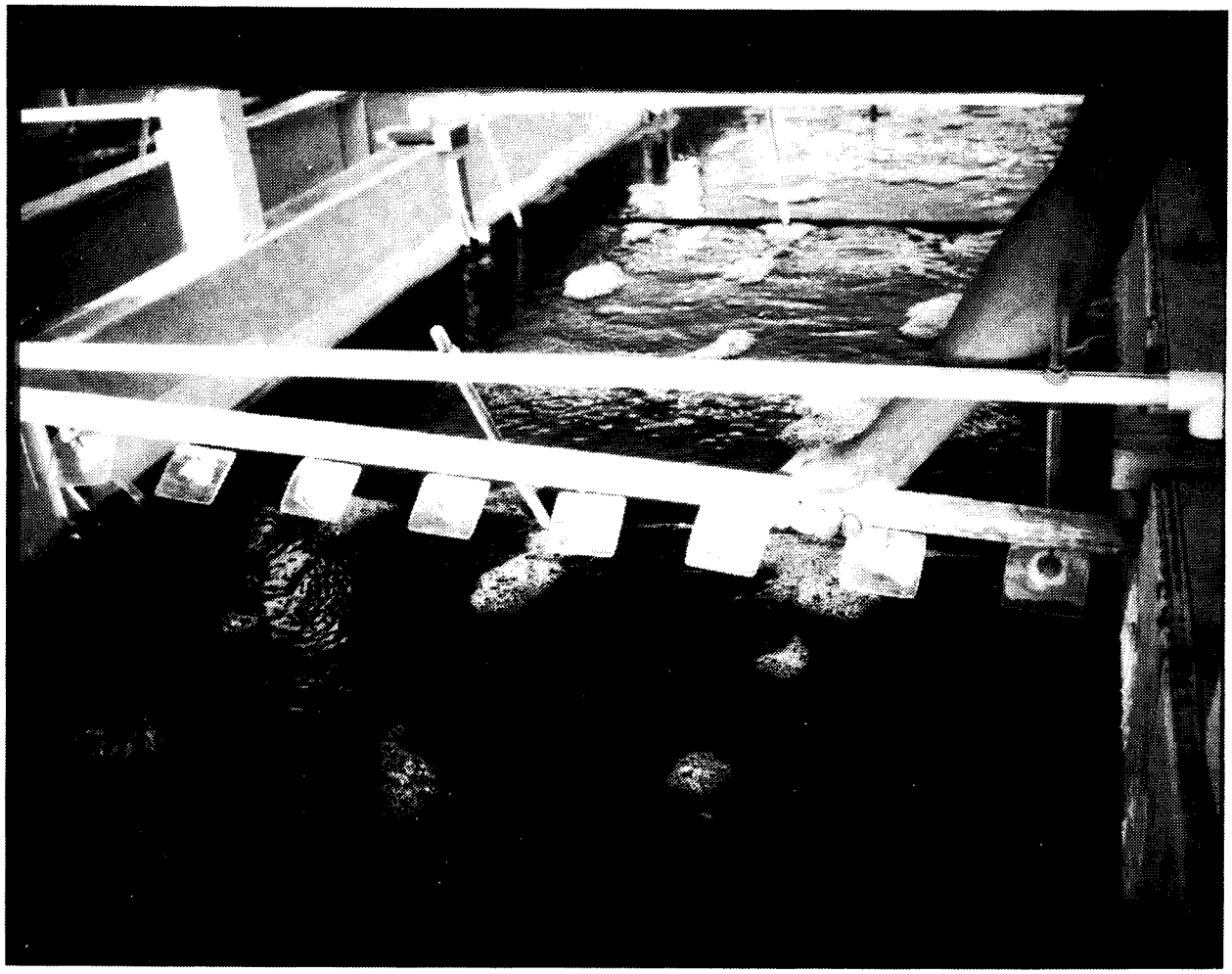
MATERIALS AND METHODS

The Culture System

The flumes utilized in our studies consisted of one $48m^2$ continuous flume and four $9.2m^2$ continuous flumes. The $48m^2$ flume is illustrated in Figure 3, and specifications for the $48m^2$ flume appear in Table 1. The flume is of a modular construction. The base channel module is a 1.2x2.4 molded fiberglass trough with 10 cm sides. The sides are raised an additional 23 cm with a plywood extension, coated with acrylic resin. All seams are covered with fiberglass cloth and resin. The channel modules are reinforced with split 7.5 cm diameter polyvinyl chloride (PVC) pipe, and are mounted on 5x10 cm wooden rails supported by concrete blocks.

In addition to the channel modules, there are two end-box modules and a

Four Small (9.2-m^2) Raceways Used to Run Factorial Design Experiments



Foil Array Used to Effect Systematic Vertical Mixing in the Culture

TABLE 1. FLUME SPECIFICATIONS.

Construction	Modular: 1 liftbox module 1 turning box/heat exchanger 1 drainbow 14 channel modules 3 recirculation pipes/airlifts
Centerline channel length	37.2 m
Area	48.39 m^2
Depth range	5-28 cm
Operating depth	7.75 cm (standard deviation 0.74 cm, 91 measurements), with upper level electrode set at 7.6 cm.
Volume at operating depth	4150 L
Flow rate	30 cm/s (circuit time 120 s)
Slope	0.16 cm/m

Module Specificiations

Liftbox	
Construction	2-cm plywood painted with fiberglas resin
Area	1.49 m^2
Volume at operating depth	115 L
Turning box/heat exchanger	
Construction	2-cm plywood painted with fiberglas resin 3.2 tubular titanium PVC fittings
Area	3.73 m^2
Volume at operating depth	290 L
Drainbox	
Construction	2-cm plywood painted with epoxy resin
Area	1.49 m^2
Volume at operating depth	220 L
Channel modules	
Construction	Spray-molded fiberglas PVC reinforcements 2-cm plywood painted with fiberglas resin
Area	2.98 m^2 each module, 41.67 m^2 total
Volume at operating depth	230 L each module, 3225 L total
Recirculation	
Construction	15 cm i.d. PVC pipe PVC fittings epoxy resin silicone glue (three separate pipes, nested, with separate airlifts)
Volume:	300 L
Airlift pumping rate:	690 liter min^{-1} each lift, 2070 liter min^{-1} tota
Airlift lift height:	163 cm (air outlet to water surface at operating depth)
Airlift air consumption rate:	550 liters min^{-1}

turning box module. One end-box serves as a drain box, the other as a lift box, and they are connected by three recirculation pipes with airlifts. The drain box includes a small 15-cm deep sump to provide for even flow. The airlifts discharge directly onto the level surface of the flume in the lift box. The recirculation pipes are 15 cm outside diameter PVC pipe turned in a U-shape using 45° PVC elbows to reduce flow resistance over that which would be introduced by 90° elbows. Each of the two inner recirculation pipes has a small drain port at the bottom of the U; there is a 15-cm valve and drain line located in the outermost recirculation pipe which allows the culture to be transfered to the culture storage tank (Figure 4). The airlifts are fed by 2.4 cm PVC pipes which discharge 1.4 m below the water surface. Oil-free air is supplied by an air blower driven by a 7.5 horsepower motor.

A seawater system (Figure 4) provides capabilities for adding seawater to the flume, obtaining seawater for laboratory use, partially or completely draining the flume, and storing the culture or seawater. Seawater is drawn from a 45m-deep well. The seawater is filtered through coral rubble, and is therefore very low in particulate organic matter. Levels of major inorganic nutrients are typically 50 μM ammonium and 0.8 μM phosphate. The inlet to the seawater pump is from the saltwater well or from the storage tanks. The outlet is to the flume, to the utility outlet, or to waste. The flume can be drained into the culture storage tank through the 15-cm PVC pipe using a manual valve, and pumped from that tank to any of the outlet ports, or discarded by gravity through the waste port. Wastewater is discharged into an underground sump excavated in the coral rubble.

The flume incorporates a computer-based system that monitors and controls the physical and chemical environment of the culture. The system controller is a Hewlett-Packard model 9845B computer, which acquires data from the sensors and manipulates the control devices based on the information received. The seawater pump is controlled on the basis of the operator input and the output of the level detector. Carbon dioxide gas addition is controlled on the basis of pH. Draining is controlled on a time-of-day basis.

The four $9.2m^2$ flumes are similar in design to the $48m^2$ flume, but consist of two channels 7.6m long by 0.6m wide by 0.15m deep. The channels are connected by 20 cm recirculation pipes; one of the two recirculation pipes of each culture contains an airlift pump which drives culture circulation. Each $9.2m^2$ flume is equipped with a CO_2 supply line. pH sensors in the cultures supply data to the Hewlett-Packard 9845B computer, which in turns controls the CO_2 supply solenoids.

Analytical Data

Production in the flume was calculated from daily changes in particulate carbon (PC) concentrations corrected for dilution due to drainage and introduction of fresh medium. Samples for PC analyses were collected on glass-fiber (GF/C) filters and analyzed on a Hewlett-Packard model 185B CHN analyzer following procedures recommended by Sharp [24]. These carbon production

numbers were converted to ash-free dry weight production by multiplying by the ratio of ash-free dry weight:PC. This ratio averages about 2.0±0.1 for marine microalgae [25]. Lipid contents were measured by a modified Bligh-Dyer extraction. Culture NH_4^+ concentrations were determined by the phenol-hypochlorite method. Urea concentrations were determined by the same method after the samples were digested with urease for 15 min.

Solar irradiance was measured with a Lambda model LI-1905B quantum sensor, which has a nearly flat response to light in the wavelength range 400-700 nm. Visible quantum fluxes were converted to energy fluxes using the spectral distribution of sunlight [26]. The conversion ratio was 19.5 μEinst per calorie. The caloric content of the product algae was calculated assuming an energy content of 8 kcal per gram carbon [27], and solar energy conversion efficiencies were calculated based on this caloric content and the incident energy flux of visible light.

RESULTS

Task I: Screening of Candidate Species in Laboratory Cultures

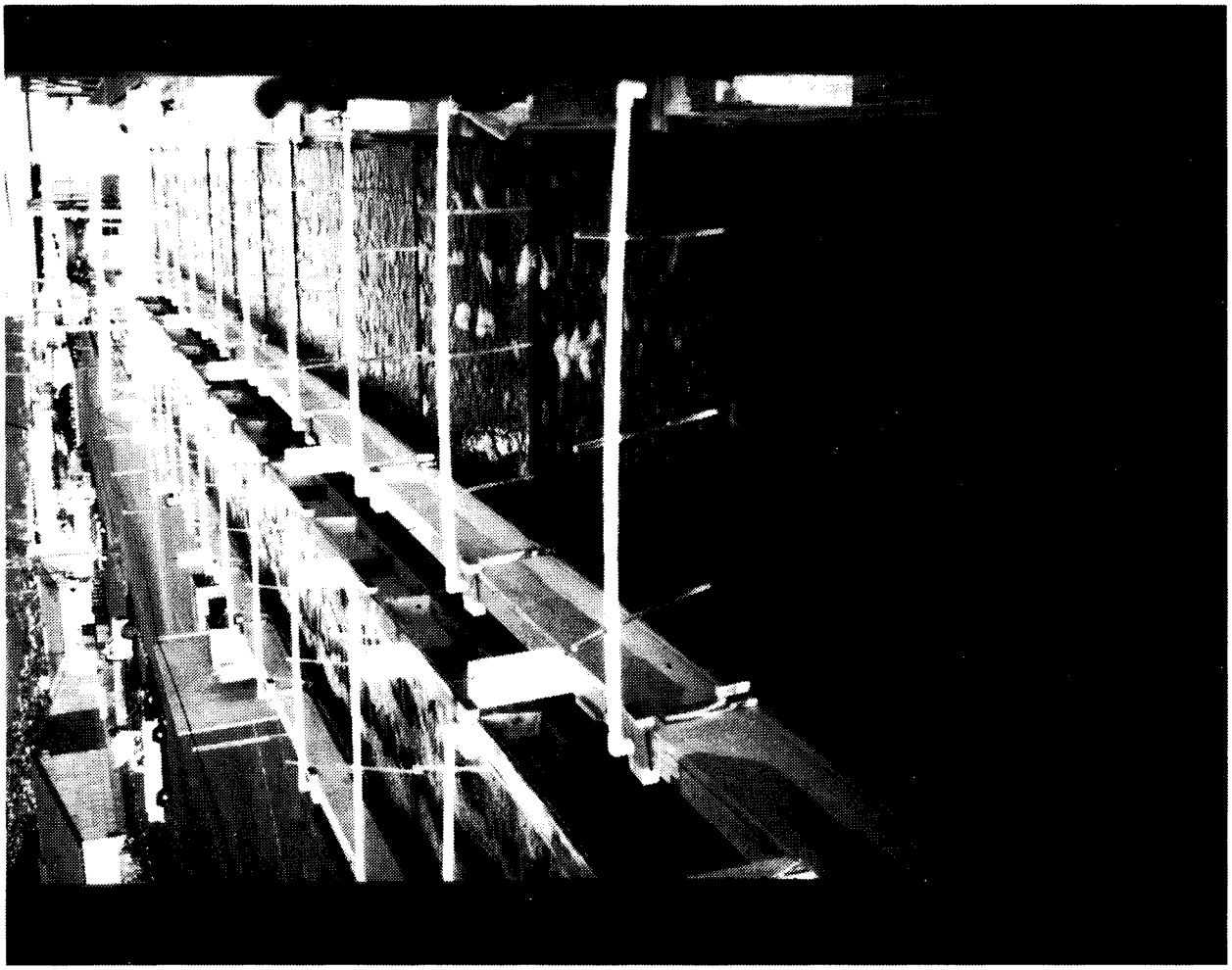
Objective
- To determine which microalgal species are most likely to grow well at elevated temperatures in culture systems, and to select three or more species for outdoor yield studies

Purpose
- To reduce the number of species which must be studied in the outdoor flume factorial experiments

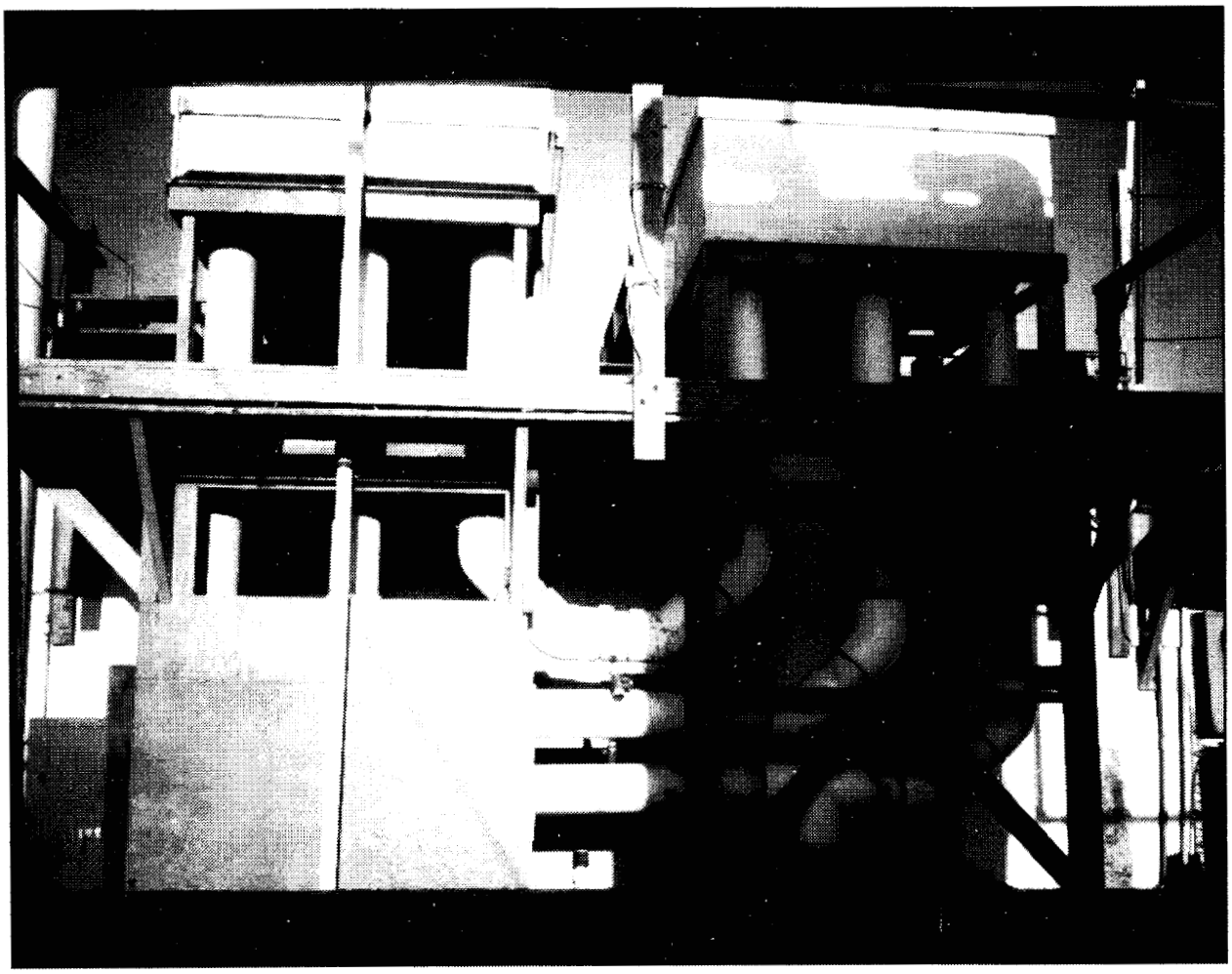
Candidate species have been screened based on their ability to grow in the temperature range 30-35°C and on their ability to produce high concentrations of cells. Figure 5 shows the results of some of the laboratory studies carried out at 34°C. The irradiance in all cases was approximately 150 μEinst $m^{-2}s^{-1}$ from a bank of cool white fluorescent lamps, and was provided on a 12:12 L:D cycle. Growth rates were estimated from regressions to the linear portion of the log-linear plots in Fig. 5. Based on these studies and similar results obtained at other temperatures, we tentatively selected three species for further study, *Platymonas sp.*, *Isochrysis sp.* (Tahitian), and *Chaetoceros gracilis*. Studies of this sort continue to be performed on promising microalgal species. However, the fact that a given species grows well in the laboratory is no guarantee that it will perform well in an outdoor culture system. Determination of performance characteristics for promising species in the outdoor flumes was therefore critical to our screening process.

Task II: Yield Optimization of Promising Species in Shallow Flumes

Objectives
- To determine how much algal production can be expected from shallow outdoor flumes without temperature control



View of the 50-m^2 Flume with Foils in Place



View of the Airlift End (left) and Return End (right) of the Large 50-m^2 Flume

- To determine the sensitivity of the production function to changes in design characteristics

Purpose
- To determine whether the production of shallow outdoor flumes can be maintained or even enhanced by foregoing temperature control and by growing thermophilic microalgae during the warm summer months

The species chosen for the initial experimental study was Platymonas sp. We performed a two-way factorial optimization with Platymonas, in which dilution rate and CO_2 cut-in pH were varied. These two parameters were chosen because they appeared to be the most important variables affecting production in our earlier work with Phaeodactylum. Table 2 summarizes the experimental results.

TABLE 2. PRODUCTION IN $gC \cdot m^{-2} \cdot d^{-1}$ IN EXPERIMENTAL FLUMES RUN AT A DEPTH OF 10 cm.

	CO_2 cut-in pH		
μ(%)	7	7.5	8
20			7.00
30	11.03		8.78
40	10.29,11.64	10.28	
50		11.32,11.68	10.17
60	12.34		10.98
70	9.38	10.10	9.50

Note that these dilution rates refer to once-a-day dilutions, not to continuous dilution rates. For example, our 50% dilution rate (requiring a doubling per day to maintain steady state cell numbers) would correspond to a continuous dilution rate of 69.3% per day, and our highest dilution rate of 70% per day (requiring a 3.3-fold increase in population numbers per day at steady state) would correspond to a continuous dilution rate of 120% per day.

We fit parabolas to the production data at each pH and obtained the optimum dilution rates shown in Table 3. The results indicate that the production

TABLE 3. OPTIMUM DILUTION RATES AT EACH pH.

pH	optimum μ (d^{-1})	production at maximum ($gC \cdot m^{-2} \cdot d^{-1}$)
7.0	48.2	11.8
7.5	51.7	10.7
8.0	53.7	10.6

maximum is fairly insensitive to pH in the range 7-8, and since pH = 7.0 corresponds to having the CO_2 bubbling all the time during the photoperiod, there was no reason to explore lower CO_2 cut-in pH's. Carbon accounts for about 47% of the ash-free dry weight (AFDW) of Platymonas [25], and the peak production estimated at pH = 7 therefore corresponds to 11.8/0.47 = 25.1 g AFDW $m^{-2}d^{-1}$. The observed peak production of 12.34 g C $m^{-2}d^{-1}$ corresponds to 26.3 g AFDW $m^{-2}d^{-1}$. This rate of production is slightly better than what we were able to achieve with Phaeodactylum, but was achieved without the benefit of temperature control. Removal of the temperature control constraint has been a big step towards making the operation energetically favorable.

We ran two experiments to check on the reproducability of flume performance. The results are shown in Table 4. These results indicate that the standard

TABLE 4. REPRODUCABILITY OF FLUME PRODUCTION RESULTS.

	pH = 8, μ = 50% $gC \cdot m^{-2} \cdot d^{-1}$	pH = 7.5, μ = 50% $gC \cdot m^{-2} \cdot d^{-1}$
flume 1	11.20	11.33
flume 2	9.14	11.37
flume 3	10.77	12.49
flume 4	9.57	11.53
mean ± SD	10.17 ± 0.97	11.68 ± 0.55

deviations are 5-10% of the means. As a further check we repeated certain conditions in individual flumes on different weeks, and obtained very similar results, i.e. at pH = 7, μ = 40%, production = 10.29 and 10.64 g C $m^{-2}d^{-1}$; at pH = 7.5, μ = 50%, production = 11.32 and 11.68 g C $m^{-2}d^{-1}$. Thus results were reasonably reproducible from week to week and from flume to flume.

After completing these experiments, we inoculated the $48m^2$ flume with Platymonas, and began an experiment to determine the rate of production that could be sustained on a long-term basis. At the same time, we decided to systematically alter the depth of the culture (the small flumes had been operated at a depth of 10 cm) to determine the optimum culture depth to greater accuracy. The culture depth was varied in 2.5 cm increments between 7.5 cm and 15.0 cm. The flume was operated at each depth for one week. The results indicated that the best production rates would be obtained at a depth of about 12 cm, and following completion of the depth variation experiments the culture has been operated at that depth for the past several weeks.

Production rates obtained in the large flume during the first two months of 1984 are shown in Fig. 6. The general upward trend in the data undoubtedly reflects in part a gradual increase in insolation, but probably also reflects gradual optimization of flume operating conditions (i.e. culture depth). The highest production that has been achieved on an extended basis in an algal culture growing solely on inorganic nutrients is 23 g dry wt $m^{-2}d^{-1}$ [29].

That production figure was achieved with a freshwater culture of Scenedesmus over a 62-day period. If we assume that about 10% of the dry weight of the Scenedesmus cells consisted of ash [25], then our average production rate of 23.1 g AFDW $m^{-2}d^{-1}$ is 10% better than the previous best long-term result. However, during January and February insolation in Hawaii is at a seasonal low. The average insolation during the course of the year is about 30% higher than the Jan-Feb mean [30]. The production rate of 23.1 g AFDW $m^{-2}d^{-1}$ translates into a photosynthetic efficiency of about 7.4%. If this efficiency can be maintained over the course of the year, the average production should be about 30 g AFDW $m^{-2}d^{-1}$, about a 45% improvement over the previous best long-term production figure.

Because Platymonas appeared to be a good producer and was easy to culture, we set up an experiment in the four small flumes to determine the effects of nutrient limitation on cellular composition. Flume 1 was run in a nutrient-saturated mode, flume 2 was P limited, flume 3 was N limited, and flume 4 was both N and P limited. Table 5 shows the carbon/nitrogen (C/N) ratios in the

TABLE 5. C/N RATIOS BY WEIGHT IN THE FOUR SMALL FLUMES

Date	Flume 1 (Nutrient saturated)	Flume 2 (P limited)	Flume 3 (N limited)	Flume 4 (N & P limited)
11/3	6.10	6.28	6.50	6.31
4	6.42	5.95	6.21	6.10
5	6.39	6.61	6.86	6.64
6	—	6.31	6.83	6.24
7	6.53	5.88	6.50	7.01
8	6.31	—	6.75	7.59
9	6.75	6.42	6.50	—
10	6.57	6.39	6.28	9.75
11	6.57	6.72	6.75	9.89
12	6.61	6.86	6.94	10.80
13	6.42	6.50	8.10	12.00
14	6.53	6.90	—	12.10
15	6.24	6.61	8.21	12.50
16	6.21	6.17	8.14	12.20

algae over the period from 11/3/83 to 11/16/83. The C/N ratios in the nutrient-saturated and P limited flumes changed very little over this two-week period. Hence there was no evidence of storage product accumulation in these flumes. The N-limited flume showed about a 30% increase in C/N ratio, and the N and P limited flume showed about a doubling in C/N ratio. These results are indicative of storage product accumulation. Evidently simultaneous N and P limitation was far more effective in triggering the manufacture of storage products than N limitation alone.

The flumes were examined for lipid content on four separate dates. Most

analyses were performed in duplicate. The results are shown in Table 6.

TABLE 6. LIPID ANALYSES ON THE FOUR SMALL FLUMES

	Date	Flume 1 (Nutrient saturated)	Flume 2 (P limited)	Flume 3 (N limited)	Flume 4 (N & P limited)
% Lipids	11/3	17.8	17.5	—	—
	11/7	15.3	20.8	16.0	18.4
		15.9	21.8	17.0	17.8
	11/14	18.9	18.3	15.9	14.9
		—	19.2	—	15.7
	11/21	15.4	16.9	13.1	13.8
		15.2	18.4	12.7	12.8
% Neutral Lipids	11/7	21.5	32.3	28.9	30.9
	11/14	25.0	25.3	25.9	36.3
	11/21	25.0	26.1	31.1	39.6

There was no change in the percentage of lipids in flumes 1 and 2, and there was a decrease in the percentage of lipids in flumes 3 and 4. We conclude from these results that the increase in C/N ratios in flumes 3 and 4 reflected carbohydrate storage rather than lipid storage. The increase in carbohydrate content presumably resulted in a decrease in percentage lipid. The lipids can be crudely broken down into a neutral and polar fraction; our results indicate no significant change in the relative amounts of these two fractions.

We are currently in the midst of performing a factorial design experiment with *Isochrysis sp.* (Tahitian) using the four small flumes. We chose this species as our second test organism because, unlike diatoms, it has no large requirement for silicate, because Chrysophyceae are known to be lipid storers [28], and because others have reported good success in growing this particular strain in outdoor cultures. At this time we are not in a position to say what level of production may be achieved with *Isochrysis*. The culture appears to be more sensitive to high temperature than *Platymonas*, but at the moment is growing well in the flumes.

Task III. Analysis of Promising Culture Management Strategies

Objective

- To prepare an evaluation of system inputs which quantifies operational parameters and determines the costs and benefits of operating the shallow outdoor flumes with and without temperature controls, in the latter case by substituting thermophilic microalgae for *P. tricornutum* during the warm months of the year.

Purpose • To provide the data required to guide this emerging technology toward effective application

We will not be in a position to provide a complete analysis of culture management strategies until we have completed a more thorough screening procedure, but it does seem possible to reach several tentative conclusions at this time. First, without temperature control it is probably possible to achieve annual production rates averaging 25-30 g AFDW $m^{-2}d^{-1}$ with Platymonas, and present indications are that the average will be closer to 30 than to 25. Thus we do not feel that temperature control will be necessary for the success of the culture operation. Second, the cost of cooling the flumes accounts for about 15% of the operating costs of the present facility. However, over half the operating costs of the present facility go to salaries. The cost of salaries would presumably decline appreciably in an actual production system, because relatively little scientific research would be carried out in a commercial facility. A tentative analysis indicates that removal of the requirement for cooling could reduce the operating costs of a commercial production facility by at least 30%. Third, the energy balance in a facility requiring cooling would almost certainly be unfavorable. From the standpoint of energy production, it therefore seems imperative that the flumes be operated without cooling. Our results indicate that high rates of production can be achieved without temperature control.

SUMMARY

The results obtained to date during the current contract year show that production rates in the range 25-30 g AFDW $m^{-2}d^{-1}$ can be achieved in a shallow outdoor algal mass culture system without temperature control. Production averaging 23 g AFDW $m^{-2}d^{-1}$ was obtained during the first two months of 1984. Insolation during these months is generally lower than during any other months of the year in Hawaii. If comparable photosynthetic efficiencies (7.4%) can be achieved throughout the year, then average production rates should be very close to 30 g AFDW $m^{-2}d^{-1}$. Elimination of the need for temperature control will greatly improve the economics and energy balance of the algal culture system.

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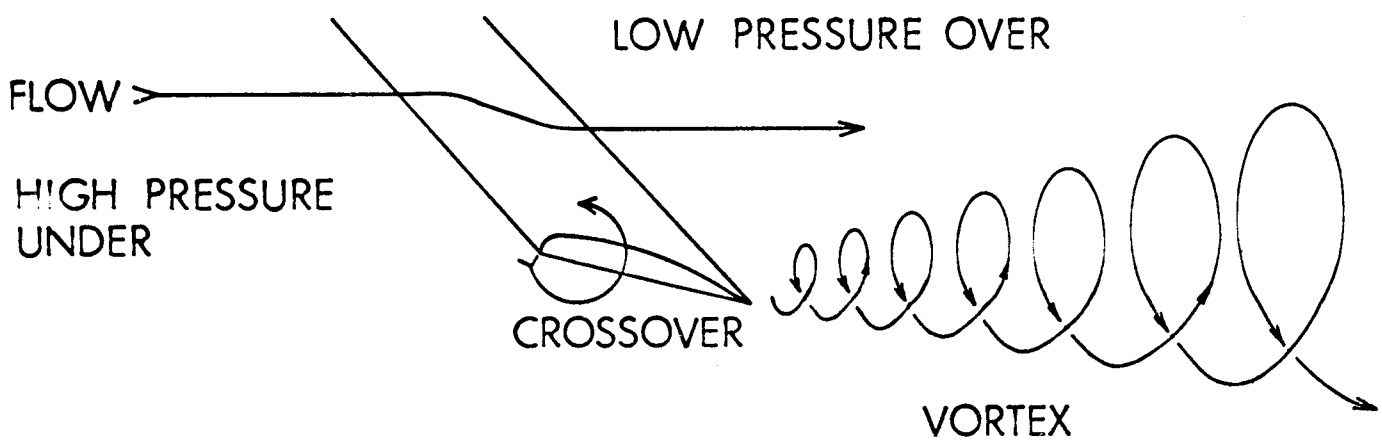

Fig. 1. Design of a single foil indicating mechanism of vortex production.

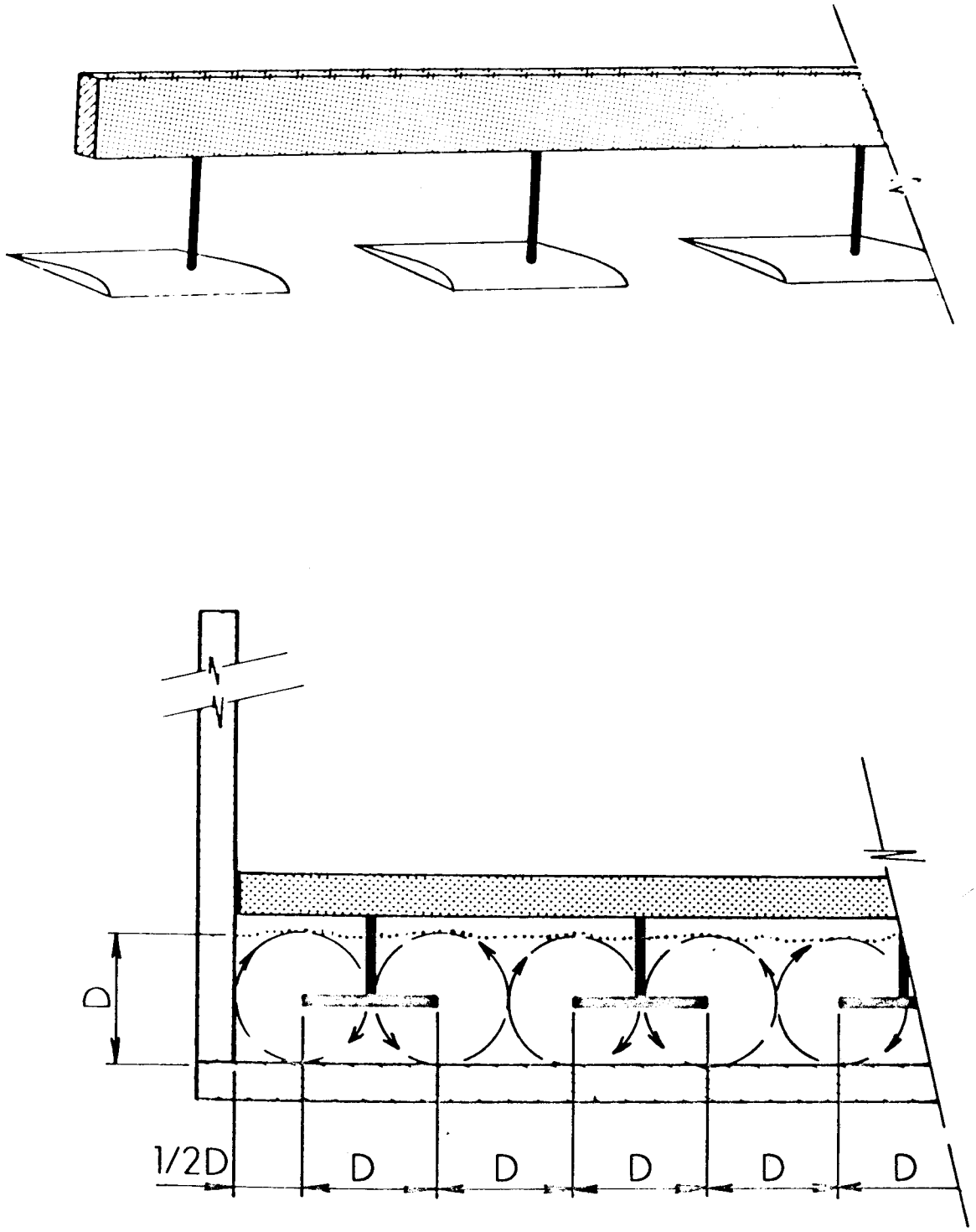

Fig. 2. Positioning of individual foils in foil array. Lower figure indicates positioning of array in flume. D is the depth of the water. Arrows indicate rotational direction of vortices.

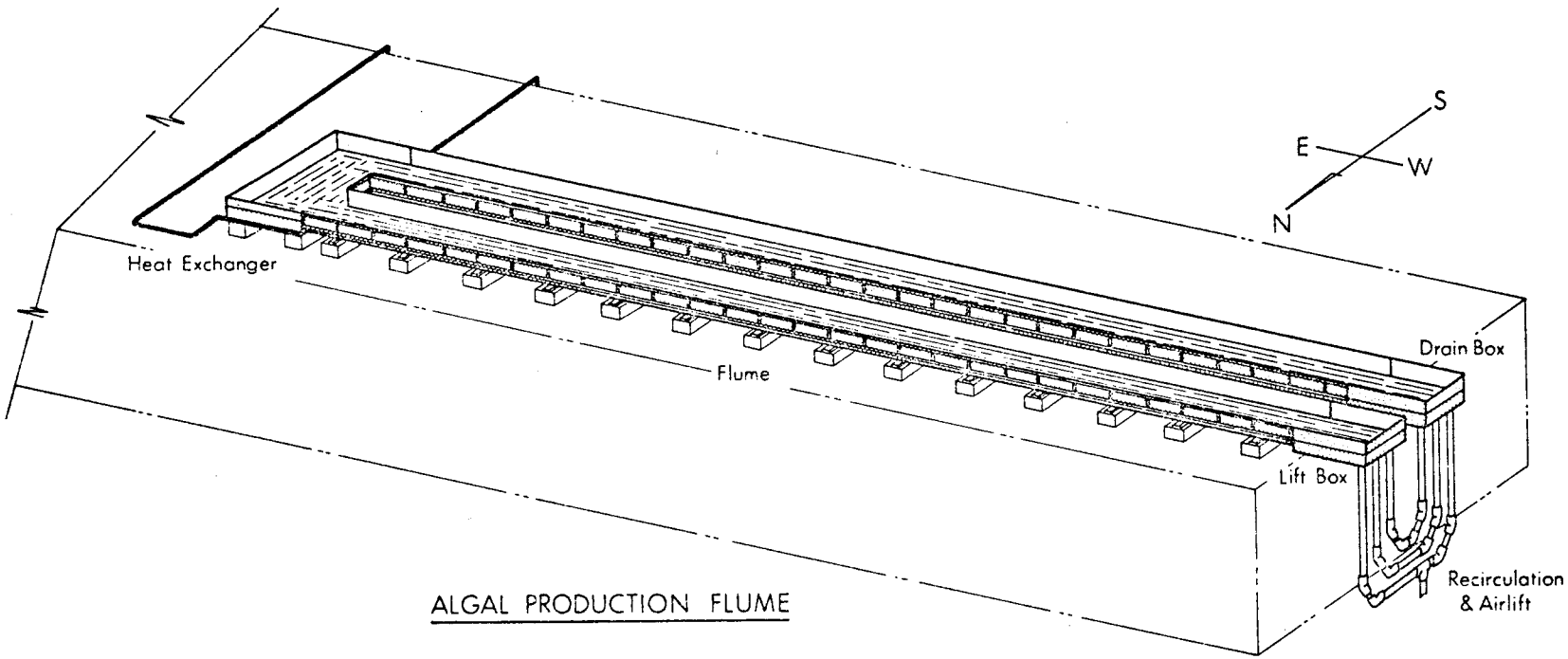

Fig. 3. Perspective of algal production flume showing location of lift box, drain box, and airlift system.

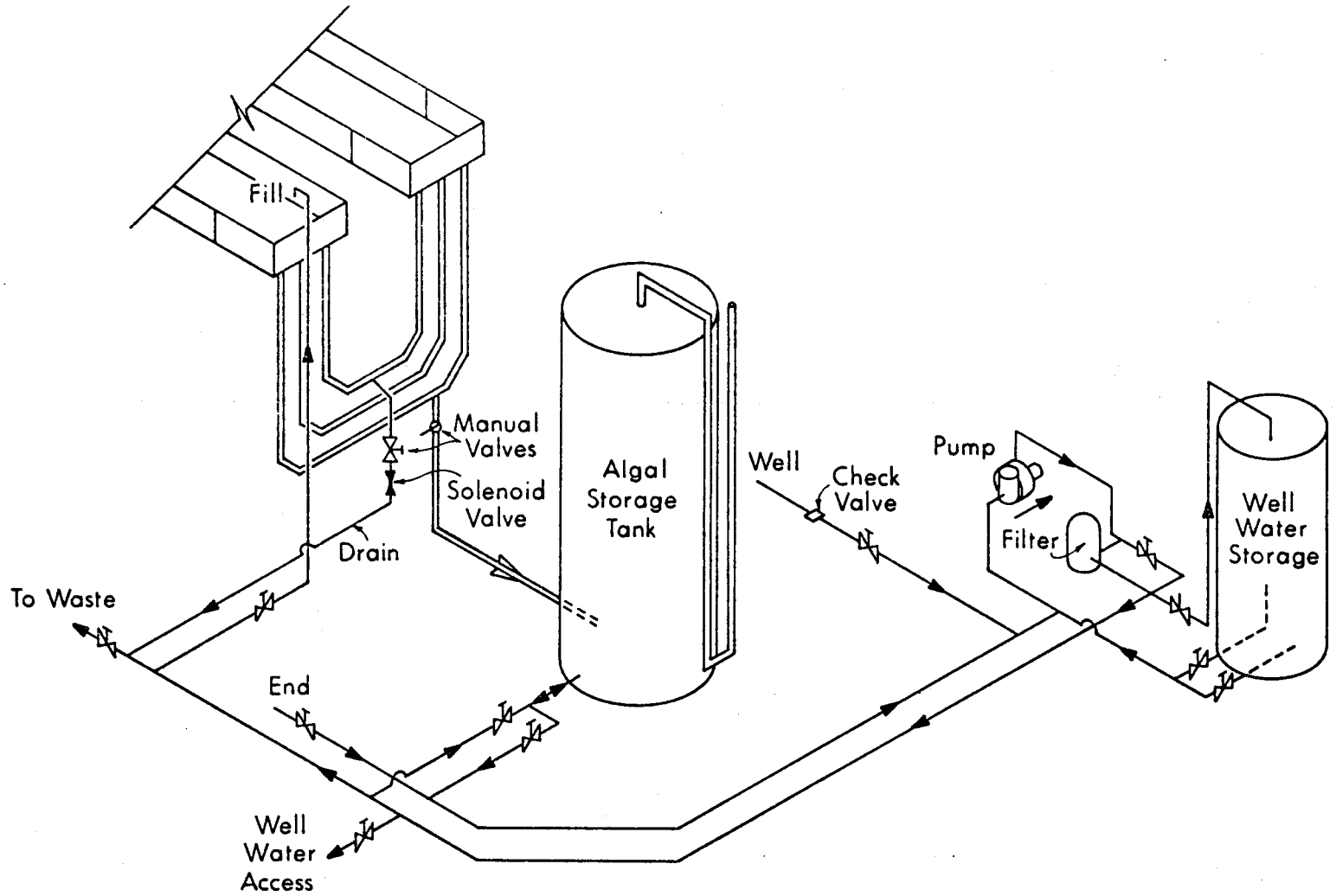

Fig. 4. Schematic of seawater supply system and algal storage tank.

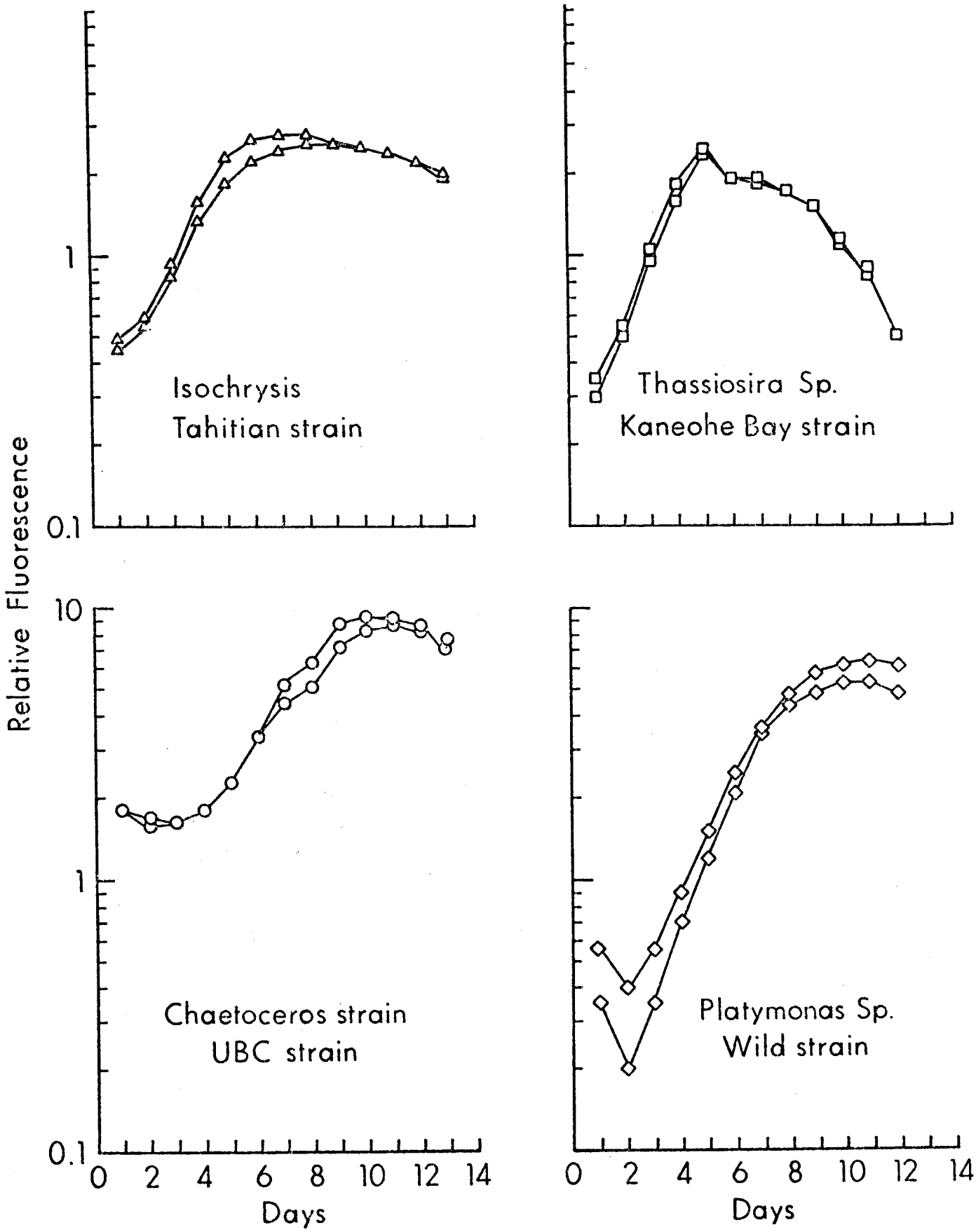

Fig. 5. Log-normal plot of relative fluorescence versus time of selected microalgal species grown at 34°C. Growth rates were estimated from slope of linear portion of curves during log phase.

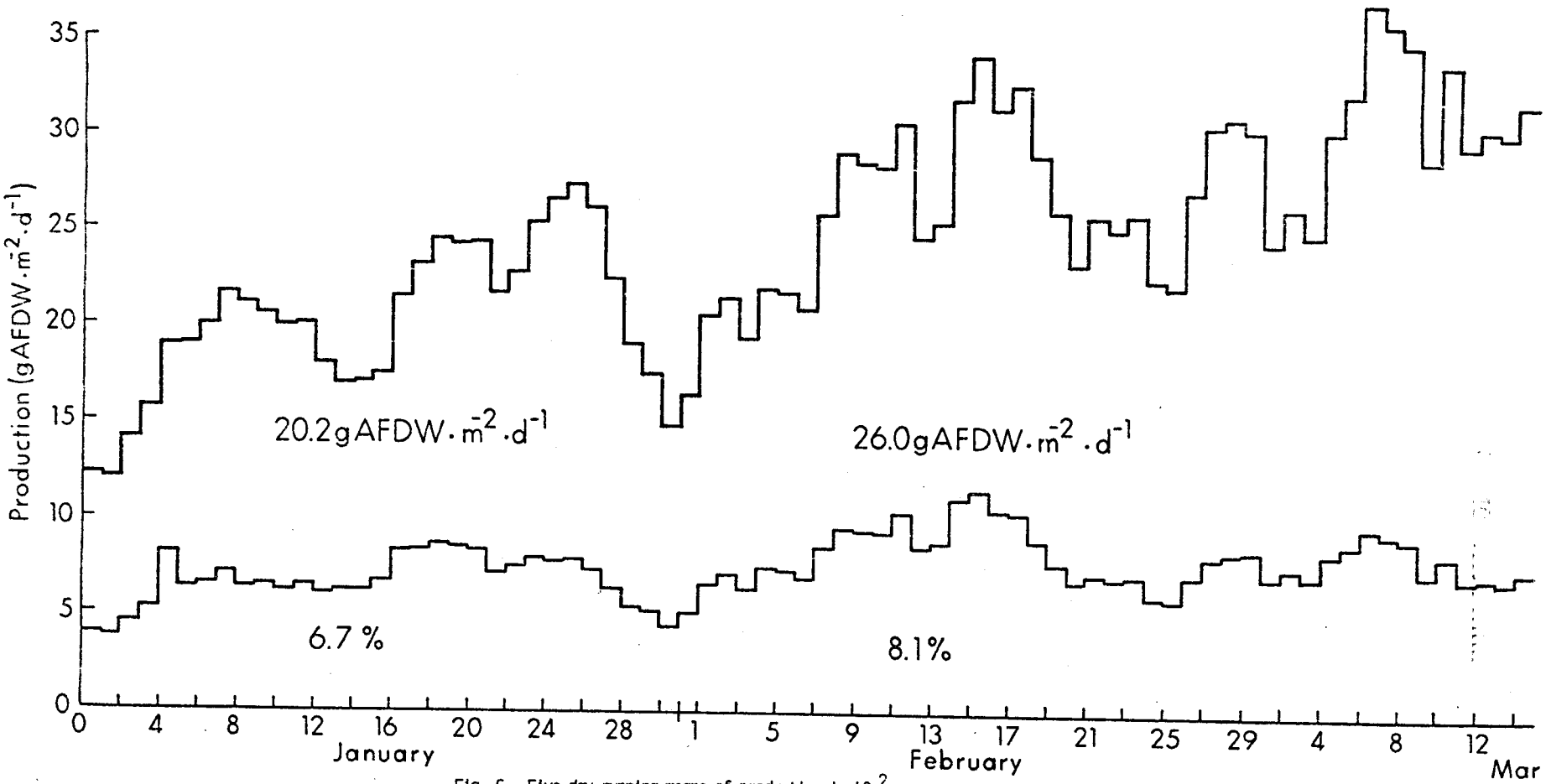

Fig. 6. Five-day running means of production in 48m^2 flume during first two months of 1984.

CULTIVATION OF MICROALGAE FOR LIQUID FUELS

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Fairfield, California

ABSTRACT

Three species of algae have been cultivated in an outdoor pond system at Vacaville, CA in 1983. Two of these organisms, an Ankistrodesmus sp. and a Scenedesmus sp. (SO2a) were recommended as good lipid producers. Another Scenedesmus sp. (tentatively identified as S. quadricauda) invaded a pond and was thereafter cultivated. Under nitrogen-sufficient growth conditions the strains contained, respectively, about 27%, 20% and 20% lipid. None of the organisms produced more lipid under nitrogen-deficient conditions. Both Scenedesmus strains doubled in carbohydrate content and decreased about 25% in lipid content. The Ankistrodesmus grew poorly when N-limited and, after readdition of nitrogen, did not fully regreen. The results of growth outdoors, after depletion of nitrogen from the growth medium, indicate that after about two (2) days productivity declines precipitously. Thus the induction of lipid (or carbohydrate) must be nearly completed by this time.

Growth of the Ankistrodesmus was studied indoors in 1 L bottles and 1.4 m^2 intensely illuminated tanks. The results indicate that (1) EDTA is probably not required, or at least not for iron availability at moderate rates of production; (2) trace metals available in most water resources are adequate; (3) nitrate or urea are adequate sources of nitrogen added incrementally or all at once; (4) salinity must be greater than 5-6 ppt but growth is unchanged at 12 ppt; (5) increasing B (0.5 to 5 ppm) or F (1 to 5 ppm) has no effect; (6) reducing alkalinity from 20 mM to 5 mM has no short term effect; (7) productivity is similar at pH 7 or cycled between 6.5-9.5, constant pH=8.7 reduces productivity; (8) media recycling (10 batch cycles, 98% media return) has positive effect on species dominance and no effect on productivity; and (9) prolonged semi-batch growth led to a deterioration of the culture, a decline in average and maximum productivity per batch, and reduced resistance to contamination.

Salinity tolerance was investigated for several strains with particular emphasis on Isochryses galbana (UTEX 987). Under low (30 Wm^{-2}) continuous illumination, growth and productivity were similar at TDS=28 ppt and 21 ppt. Productivity was reduced 35% at TDS=7 ppt. Under a twelve hour diurnal cycle at 100-150 Wm^{-2}, I. galbana grew poorly even at seawater salinity. Productivity was

only marginally greater at the higher light intensity. Excretion products (organic acids plus total carbohydrates) often equaled 40% or the particulate biomass. Lipid content was 30-40% of nitrogen-sufficient biomass and 40-60% of nitrogen-deficient biomass.

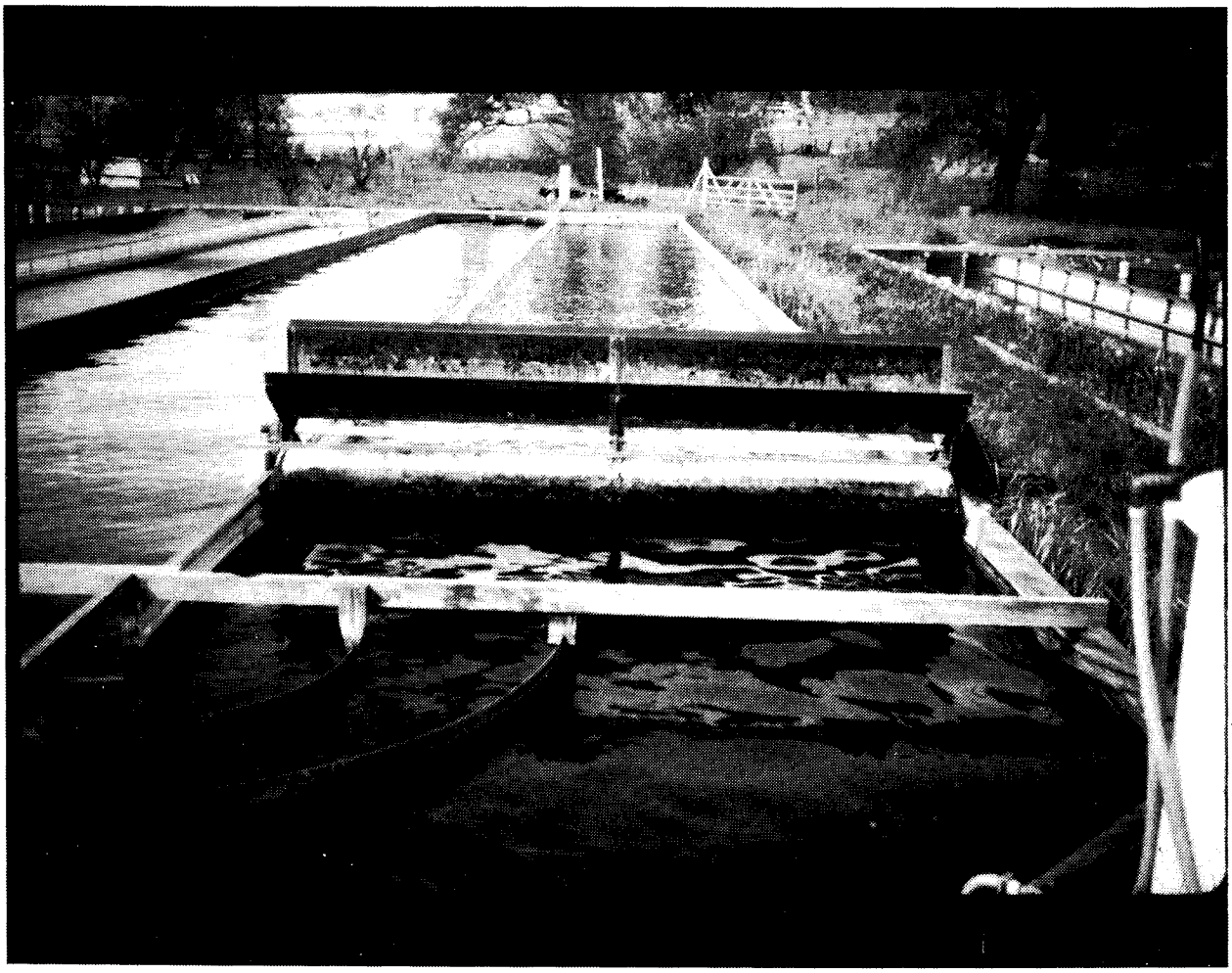
Sedimentation rates of algal biomass grown in the outdoor ponds were measured in the laboratory. The naturally occurring Scenedesmus strain spontaneously formed large clumps in batch culture. The rate of sedimentation was thus high (100-300 cm/hr) for over 70% of the biomass. Dispersed cultures of algae did not sediment quickly (<15 cm/hr). Nitrogen-starved dispersed cultures settled more readily than non-starved cultures, but not nearly as quickly as clumped cultures. Ankistrodesmus biomass settled rapidly when cells were aggregated.

SUMMARY OF OUTDOOR CULTIVATION

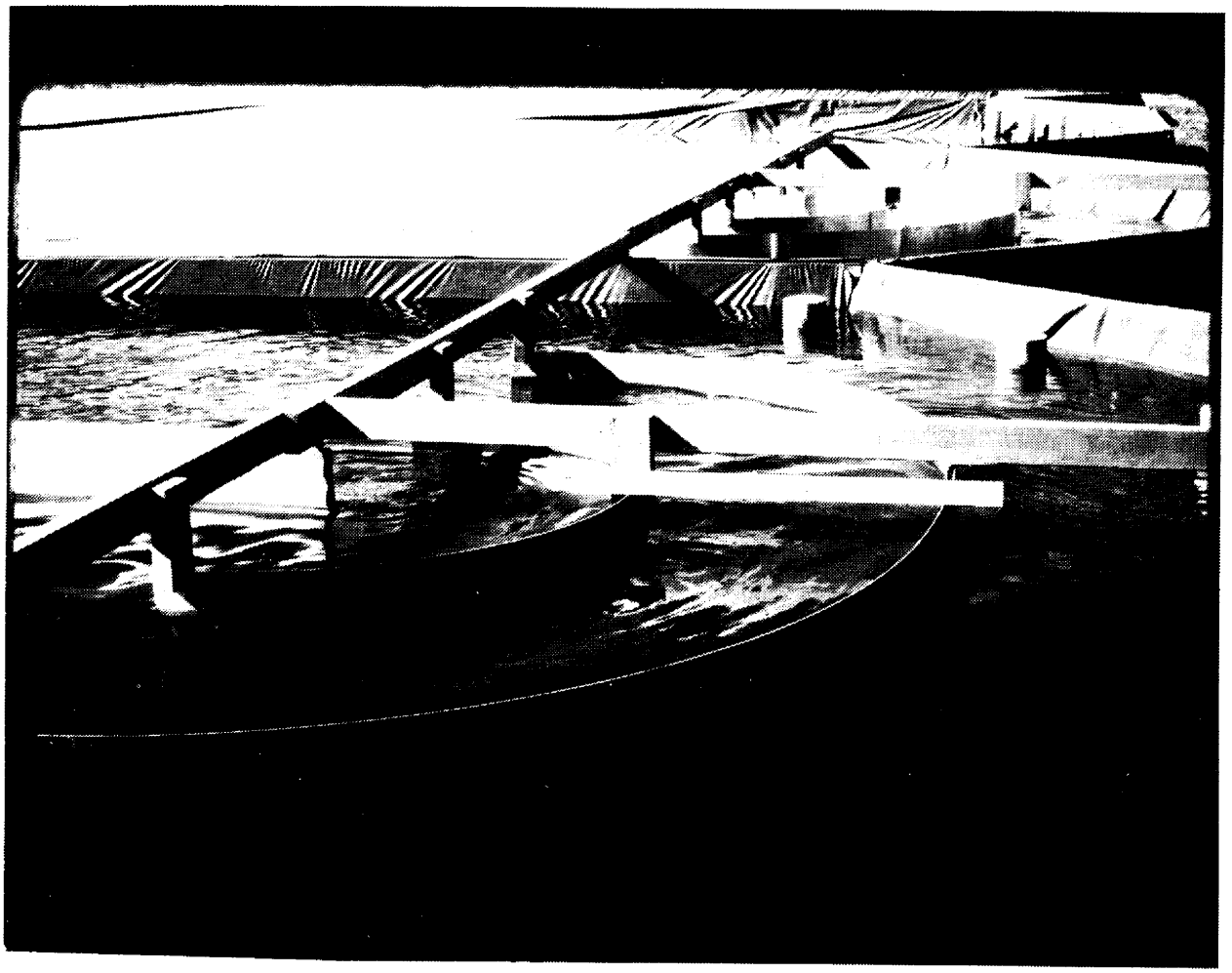
A summary of the average productivities from the outdoor work is shown in Table 1. The continuous cultures were more productive than the batch cultures on a sustained basis. However standing densities in the continuous ponds were 120-180 mgL^{-1}. Generally, highest productivity during batch growth occurred below 250 mgL^{-1} but there was a notable exception. The batch grown S. native was highly flocculant (floc size ranged from .1-3.0 mm within any culture) and grew, at higher productivity, to densities between 300-500 mgL^{-1}.

A 200 m^2 pond was operated with continuous dilution during the daytime, using S. quadricauda, from June through September 1983. Max/min temperatures, insolation, and daily productivities were measured. The highest productivities (and lowest standing densities) were obtained at detention times of 1.7-1.9 days. It may be that higher productivities could have been attained at shorter detention times. At the beginning of September the detention time was lengthened to avoid possible washout of the culture as daylength and insolation decreased. The culture density increased slowly in response to the change in detention time, resulting in low productivity for a week. When a steady higher density was achieved productivity leveled off also, at above 15 $gm/m^2/day$. The culture contained a stable mixture of at least two forms of a pleiomorphic colonial Scenedesmus. The dominant form resembled mature coenobia of S. quadricauda. It comprised about 80% of the populations and occurred in a large (5x13 μ) and small (3x10 μ) body size, both in two, four and eight cells per colony. Four-celled colonies predominated. The other form resembled reproducing S. quadricauda and also occurred in large (5x7 μ) and small (4x6 μ) cell sizes and in four and eight celled colonies.

The Pyramid Lake Ankistrodesmus was cultivated in the laboratory



View of a 200-m^2 Outdoor Pond Showing Paddlewheel and Flow Deflectors



Close-Up View of the Flow Deflectors Used to Prevent Algae from Settling out of Suspension

and inoculated outdoors in late July. Until late September the cultivation was characterized by a browning of the cultures after the first batch growth. It was determined (via bioassay) that insufficient potassium was the cause. Potassium deficient cultures when brought indoors and potassium added, greened quickly. Outdoor brown cultures, after supplementation with potassium, greened very slowly and incompletely. The same problem with regreening occurred after nitrogen starvation.

The growth of this organism, when healthy, is characterized by a deep green color, the appearance of swirls, and a complete dispersing of cells. As any one of a number of nutrient deficiencies set it, the cells clump (with scores of cells to a clump) and the swirls disappear. The cells themselves may be almost straight or sickle-shaped, with sizes varying between 12-40 μ in length and 2-4 μ in width.

The recommended medium for this organism (W.Thomas, personal communication) contains supplementation with vitamins: B_{12}, biotin, and thiamine. After 9/28/83 no vitamins have been added to outdoor cultures. The cultures have remained unialgal and appear healthy. Although productivities dropped off in October and November, photosynthetic efficiencies increased. The Ankistrodesmus was more productive than the Scenedesmus in November (and December-January) when insolation and temperature decreased greatly.

TABLE 1. PRODUCTIVITY SUMMARY ($gm/m^2/day$, average)

Month	Lgly/Day Total	S. native (quadricauda) Batch	S. native (quadricauda) Cont.	Ankistrodesmus Batch	S.SO2a Batch	S.SO2a Cont.
April(1/3)	335	8(9)	-	-	-	-
May	615	12(15)	-	-	-	-
June	630	13(15)	16	-	-	-
July	655	15(17)	20	-	-	-
August	580	14(17)	19	12(15)	-	-
September	480	14(18)	15	16(18)	10	-
October	380	13(16)	-	8(10)*	8	14
November	170	5(6)	-	8(11)	-	-

Numbers in ( ) indicate average of maximum daily production from each batch. Asterisk indicates data from a 12 m^2 pond receiving 70% of insolation.

CULTURE REQUIREMENTS FOR ANKISTRODESMUS

The Ankistrodesmus strain was grown in 1.4 m^2 tanks to determine the requirements for adequate growth and thus the cost - productivity trade-offs for media constituents. The data obtained does indicate that expensive media inclusions, such as trace metals,

EDTA, and alkalinity need not be added in large amounts. Indeed, trace requirements can probably, in most cases, be supplied by the water resource, EDTA is necessary in only small amounts ($<10\ \mu M$) or not at all, and alkalinity tolerance is broad (at least 5-20 mM). Two problems were encountered in attempting to study long-term growth of this organism: as semi-batch cultivation endured the culture degenerated, losing its productivity and competitiveness and as light intensity was raised to 40% solar, this deterioration accelerated.

The deterioration of the strain was characterized by increasing aggregation of cells into clumps, with more and more "debris" becoming evident in the clumps. Progression of this clumping to a point where 90-100% of the cells became aggregated, was soon followed by an increase in the contamination by various Chlorophytes (Chlamydomonas sp., Scenedesmus sp.) until over 50% of the biomass was contaminated. Initial healthy cultures of Ankistrodesmus were totally dispersed. It took over four months of intensive, semi-batch cultivation for the deterioration to begin. However, once started it progressed rapidly under high light intensity and frequent dilution. Outdoor cultures, growing more slowly under more intense illumination, deteriorated very slowly. Thus as time progressed, the source of inoculum for the 1.4 m^2 pond experiments was switched from 1 L low-light cultures to the outdoor pond cultures. The results to be discussed will have frequent reference to the "condition" of the cultures, in terms of per cent clumped, as it was found that this, rather than media changes, was most responsible for changes in productivity.

The first series of experiments compared growth at several light intensities and pH regimes. This was done to establish a good set of conditions for control. (Unfortunately the culture degeneration which later occurred interfered with this.) Table 2 shows productivity data vs. light input and pH for healthy, dispersed cultures. From the table it is evident that cultivation at pH 7 resulted in the highest productivity. However cycling pH between 6.5-9.5 was nearly as satisfactory. Productivity was lower at pH 8.7. Figure 1 illustrates productivity vs. light input for healthy, dispersed cultures at optimal pH and at temperatures equal to 26±2°C. The average productivities (averaged over the entire batch cycle) show the saturation that would be expected in a turbulent, but not intensely mixed system (mixing speed approximately 45 cm/sec, 1.5 fps). Maximal productivities (averages of the maximal production of each batch) show this to a lesser extent with basically a steeper linear increase at low light vs. high light. This leveling of productivity vs. light input became very pronounced for both average and maximum productivity as the irradiance was increased above 200 Wm^{-2}. In addition, clumped Ankidstrodesmus cells competed very poorly with contaminants at the higher light intensity (250 Wm^{-2}, data not shown).

TABLE 2. LIGHT INTENSITY-pH

n	Langleys /day	Avg Wm^{-2}	Avg. Prod.	Avg. Eff.	Max. Prod.	pH	Media	Cond.
4	80	60	12(10)	8.2	14(12)	6.5-9.5	Basal	D
3	80	60	8(7)	5.5	14(12)	8.7	Basal	D
4	120	100	16(14)	7.3	23(20)	6.5-9.5	Basal	D
1	120	100	19(16)	8.7	23(20)	7.0	Basal	D
4	120	100	14(12)	6.4	19(16)	8.7	Basal	D
2	125	125	15	6.6	20	7.0	Basal+	80% D
2	175	175	20	5.5	30	7.0	Basal	D
1	225	225	20	4.9	25	7.0	Basal+	D

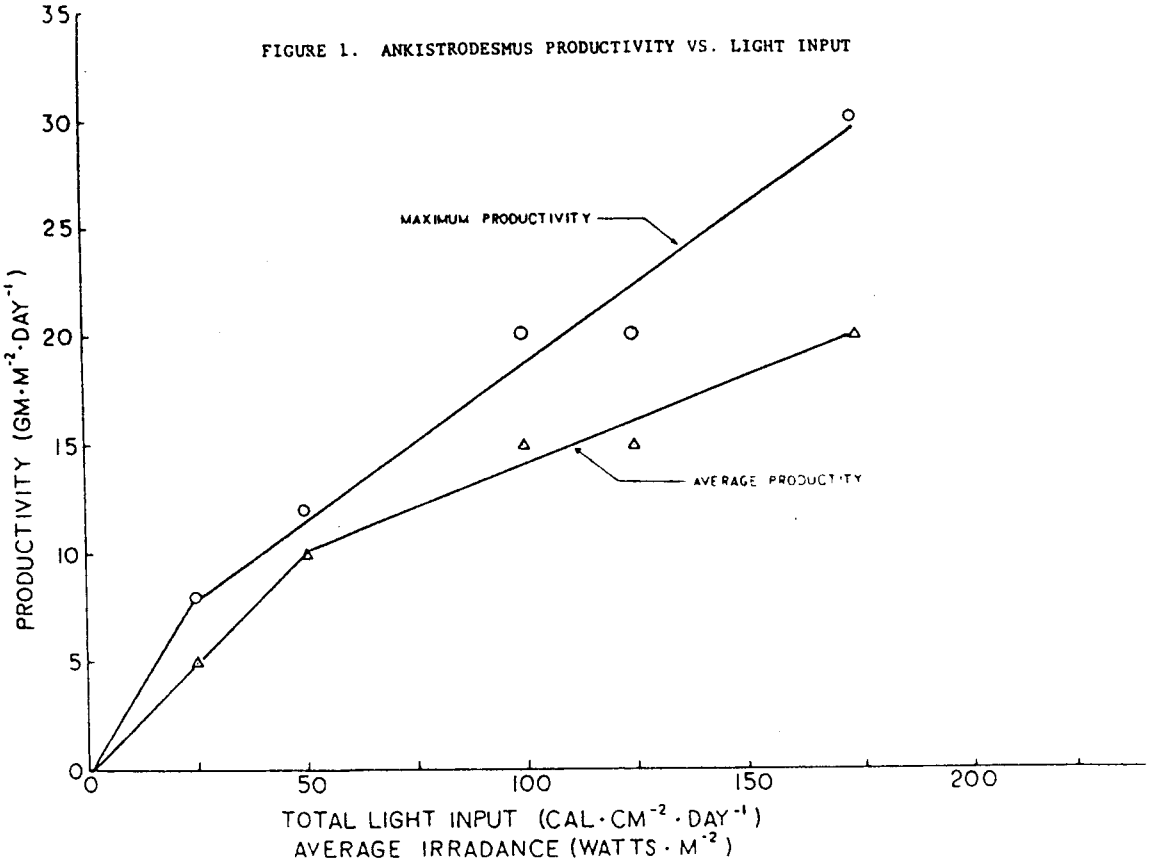
See Table 3 for media used. D = dispersed.
Values in ( ) were normalized from L:D cycle of 14:10 to 12:12.

Table 3 shows productivity data as a function of media composition. Lowering EDTA to 13 uM (.2 mole EDTA: 1 mole Fe) from 130 uM (2 mole EDTA:1 mole Fe) had little effect. It might not be expected that such a short-term lowering (1 sequential batch) would have much effect on availability of trace metals, but even over the long-term (5 sequential batches) lowering the EDTA did not lower productivity in comparison to a long-term control (Table 3, Figure 2). Since iron was near yield limiting levels in the medium (4 ppm as Fe for 1500 ppm biomass), even short-term reduction in EDTA, if important, should have had an effect.

Elevating boron and floride levels (to levels in Pyramid Lake from which the Ankistrodesmus was isolated) also showed no difference from the control. Bioassays also indicated that low levels of these elements were not responsible for culture deterioration. Adding urea incrementally (1-2 mM per day) was no better or worse than adding it all at once (10 mM urea-N initially). Nitrate could be substituted for urea. Doubling the levels of NaCl, KCl, $CaCl_2$ and $MgCl_2$ had no short-term effect. Growth of cells which were obtained from the outdoor ponds and thus grown for a prolonged period of time on trace metals available in the water supply only (10-50% of lab levels) was as good as controls. Reduction of alkalinity levels from 20 mM to 5 mM did not change productivity. In the short term Ankistrodesmus grew equally well over a range of salinity, alkalinity, trace metal concentration, and chelator concentration. In addition, growth in outdoor ponds and at low light (30 Wm^{-2}, continuous) with and without vitamin supplementation indicated that vitamins are not required for productivity up to 20 $gm/m^2/day$.

As culture degeneration appeared to be a serious problem, data was obtained to document it and bioassays were performed (under low lights in 1 L bottles) to determine whether it was reversible. Figure 2 shows data from three long series of semi-batch growth.

Small (1.4-m^2) Indoor Tanks Used for Definition of Media Requirements and Studies of Media Recycle



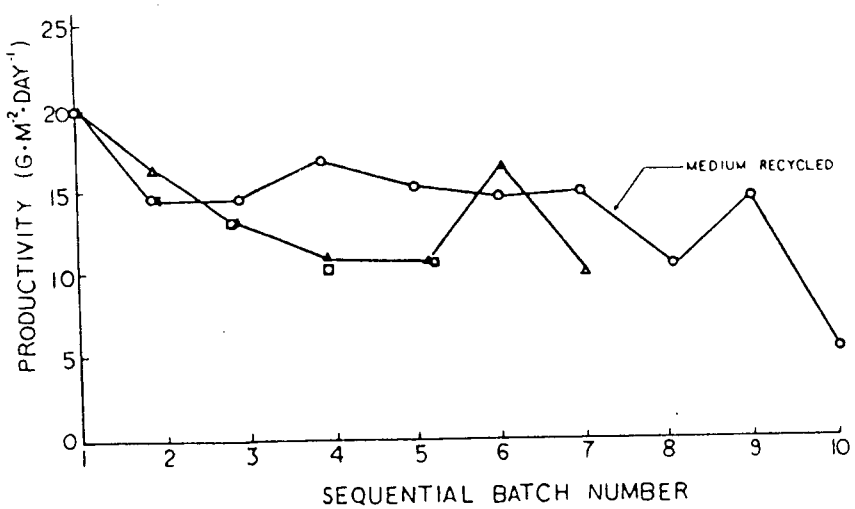


FIGURE 2. SUSTAINED SEQUENTIAL BATCH GROWTH OF ANKISTRODESMUS

Legend

○ Medium recycled: NaCl, KCl added prior to Batch #9
△ Control: NaCl, CKl added prior to Batch #6
□ 10% EDTA

TABLE 3. MEDIUM COMPOSITION EXPERIMENTS

n	Langleys /day (Wm^{-2})	Avg. Prod. $gm\ m^{-2}d^{-1}$	Avg. Eff. % vis.	Max. Prod. $gm\ m^{-2}d^{-1}$	Medium	Culture Condition
1	175	20	6.2	30	Basal	Dispersed
1	175	20	6.2	28	13 uM Na_2EDTA	Dispersed
5	175	14.2	4.4	20.8	Basal	75% Clumped
5	175	13.6	4.3	17.4	13 μM Na_2EDTA	75% Clumped
1	175	16	5.0	20	Basal +	25% Clumped
1	175	15	4.7	21	Basal +,dosed urea	25% Clumped
1	175	12	3.8	22	Basal +,dosed NO_3	25% Clumped
2	175	15	4.7	20	Basal +	90% Clumped
1	175	15	4.7	21	Basal+,2x salts	90% Clumped
1	175	12	3.8	22	Basal +,2x salts,NO_3	90% Clumped
2	125	15	6.6	20	Basal +	Dispersed
2	125	15.6	6.8	21	Basal +, 5mM Alk.	Dispersed
2	125	14.3	6.3	19	Basal +, 10% Trace	Dispersed
1	125	11	4.8	19	Basal+	90% Clumped
1	125	10	4.4	19	Basal +,50% Trace 50% EDTA	90% Clumped

n refers to number of sequential batches

Basal: in de-ionized water 20 mM $NaHCO_3$, 56.4 mM NaCl, 0.25 mM $CaCl_2$, 3.3 mM KCl, 2.5 mM $MgCl_2 \cdot 6H_2O$, 1.5 mM Na_2SO_4,70 uM $FeSO_4 \cdot 7H_2O$, 125 uM Na_2EDTA, 1.0 mM PO_4 buff(pH 7), Trace metals, 0.5 ug/L Biotin, B-12 0.1 mg/L thiamine, 10-20 mM Urea-N.

Basal +: Above plus 24 uM $Na_2B_4O_7 \cdot H_2O$(1.0 ppm B) and 0.26 mM NaF(5 ppm F)

2x Salts: Above but 113 mM NaCl, 6.6 mM KCl, 5.0 mM $MgCl_2 \cdot 6H_2O$, 0.5 mM $CaCl_2$

Trace Metals: 0.5 ppm Mn, 0.1 ppm Mo, 0.05 ppm Zn, 0.02 ppm Cu, 0.5 ppm B, 0.01 ppm V, Co, Ni, Cr.

Decreases in productivity were associated in every instance with cell aggregation into clumps. As indicated in Table 3, as clumping increased, productivities of control cultures decreased. Eventually the cultures failed, i.e., productivity declined precipitously and contaminants grew. These events became typical. No significant levels of extracellular products were measured. After ten batch cycles with 98% media recycling dissolved levels of organic acids, carbohydrates and proteins were 24 ppm, 14 ppm and 14 ppm respectively. Over 8000 ppm of particulate biomass was grown and harvested during the ten cycles.

A new culture of the Ankistrodesmus was obtained (from Dr. W. Thomas) and grown. It was, as before, initially mostly dispersed. When grown at 25 Wm^{-2} continuous illumination, the cultures became about 20-30% clumped. However, when grown at 150-250 Wm^{-2} with a 12 hr. light cycle, the cells slowly became more clumped as the number of sequential batches increased. The same medium was used in both cases. Thus, it appears that high light intensity may be responsible for deterioration of the culture. One low light culture was grown with 0.1 mM PO_4. It clumped 30% as well.

In order to determine if a nutrient deficiency was involved in the culture deterioration, bioassays were performed. The bioassays consisted of (1) growing healthy (dispersed) cells in basal laboratory medium, outdoor pond water, and clarified liquid from an unhealthy pond; (2) growing unhealthy (clumped) cells in the same media; (3) spiking the deteriorating culture liquid with several different nutrients; and (4) spiking a healthy outdoor culture liquor with the same set of nutrients. Detailed results of the bioassays can be found in our Interim Report to SERI. To summarize the following conclusions were drawn (1) iron was not growth-rate limiting in unhealthy indoor pond media, but it was the nutrient that first limited yield; (2) the healthy cells increased themselves 4-10 fold at similar rates in all media and remained 80% dispersed; (3) the unhealthy cells grew as well in all media tested, but remained aggregated; (4) no nutrient deficiency was evident from the nutrient-spiking experiments; (5) initially dispersed cells did form clumps during the nutrient bioassays, with more clumping evident (90% of the biomass) with added $CaCl_2$, but increased clumping (50%) under all treatments. Thus, under low light during short term assays (7 days), both dispersed and clumped cultures grew the same on all media. Furthermore clumping was not found to be reversible by adding any particular nutrient. (Basal medium contained 0.25 mM Ca, 1 mM P, 20 mM alkalinity, and pH was 7.2-7.8.)

Several factors may contribute to culture deterioration, including changes in salt balance especially with relation to KCl, NaCl, and $CaCl_2$, and photoinhibition. The development of clumps, whether induced by a chemical flocculation or photoinhibition or an excretion product, may itself be self-perpetuating once started, changing the chemical balance in the media. Chemical measurements made on

the media from the indoor pond after 8 cycles of growth showed no decrease in alkalinity but a substantial decrease in TDS (15% beyond dilution, 30% overall). Addition of NaCl and KCl did stimulate growth somewhat temporarily (Figure 2). It was also observed that Ankistrodesmus contaminating ponds with more saline media (and 10 mM Ca) grew entirely dispersed, regardless of light intensity. Productivities were not as high, however.

Reversible control over cell aggregation would be highly desirable since the clumped cultures sediment rapidly and the dispersed cultures grow well. At present such control has not been achieved.

CULTIVATION OF ISOCHRYSES GALBANA

I. galbana (987) was grown indoors to study productivity in brackish water (~7 ppt TDS) vs. seawater (28 ppt TDS). The alga was grown in 1 L Roux flasks under continuous illumination (25 and 40 Wm^{-2}) and in 1.4 m^2 tanks under a 12:12 dirunal cycle (125 Wm^{-2}).

Successive batches (diluted to 200 ppm every 5-10 days from 2000-7000 ppm) were grown using aspeptic technique in the 1L flasks for a six month period at 28, 21 and 7 ppt TDS. Average productivity was 0.45 gm/L/day (7.0 $gm/m^2/day$) and 0.63 gm/L/day (9.8 $gm/m^2/day$) at 25 and 40 Wm^{-2} at the two higher salinities. At TDS=7 ppt (NaCl reduced 75%, Mg reduced 75%) production was about 35% lower at both light levels, 4.6 and 6.2 $gm/m^2/day$ respectively. The cultures were often grown past the N-sufficient growth potential of about 4000 ppm biomass. Lipid content varied between 30-40% of the biomass regardless of alinity. Cultures which became N-deficient at high density (4000 ppm) contained about 40% lipids and maintained productivities similar to N-sufficient cultures. When N-deficiency occurred at low density (400-800 ppm) lipid content increased to over 60% but due to the long period of induction required, productivity was only 1 $gm/m^2/day$. Even under low light conditions lags upon dilution were frequent and prolonged (1 to 2 days), lowering productivities.

Inoculations of the 1.4 m^2 pond with I. galbana were attempted at the low salinity. However, the culture became contaminated quickly by the Ankistrodesmus which was being grown at a similar salinity in another pond. Doubling the salinity resulted in coexistence of the two organisms, but at a low overall productivity and with a 4:1 ratio of Ankistrodesmus to Isochryses. Subsequently, inoculation of a 1.4 m^2 tank was achieved, but at TDS=28 ppt. A lag of two days occurred before substantial growth ensued. Productivity was low, 5-10 $gm/m^2/day$ and ceased when cell density reached 600 ppm. Data was taken to measure loss of biomass during the twelve hour dark period, and excretion of products into the medium. Table 4 shows data from an I. galbana

culture (28 ppt TDS, 125 Wm^{-2} illumination), and a healthy Ankistrodesmus culture for comparison. Both nighttime respiration and excretion of metabolic products were much greater in the I. galbana culture. Both cultures were grown under N-sufficient conditions. When particulate biomass (ash-free) of I. galbana reached 400 ppm, the dissolved levels of protein, CHO and TOA (total organic acids) all together were more than 40% of that. During this time, along with production of 100 ppm of particulate biomass, almost 60 ppm of carbohydrate and organic acids were excreted into the medium. As shown in Table 4, no measurable protein was excreted by either organism. Although extracellular levels of carbohydrate, produced by I. galbana constantly increased, organic acid levels increased and decreased with time. Another 1.4 m^2 pond was inoculated with I. galbana but at TDS=7 ppt. Productivity averaged 8 $gm/m^2/day$ with a maximum of 10 $gm/m^2/day$, when density was kept between 100 and 500 ppm. Productivity was the same in a control pond at seawater salinity.

TABLE 4. LOSSES FROM 1.4 m^2 TANK CULTURES

	Ankistrodesmus (125 Wm^{-2})	I. galbana (125 Wm^{-2})
Production during light period	16.2	4.7
Loss during dark period*	-3,6	-2.2
Net 24 hr production*	12.6	2.5
Biomass density, VSS ppm	600	400
Dissolved protein, ppm	0	0
Dissolved CHO, ppm	2	60
Dissolved organic acids, ppm	2-30	90-115
ΔTOA/ Δ Biomass	.01	.40
ΔCHO/ Δ Biomass	.01	.20
ΔProt/ Δ Biomass	0	0

*$gm/m^2/day$

NITROGEN-STARVED GROWTH

Outdoor cultures were grown past their nitrogen-sufficient growth potential. Effluents from continuously operated ponds of both S. quadricauda and S. SO2a were diluted with nitrogen free medium and allowed to "induce" in batch. Batch cultures of both Scenedesmus strains and the Ankistrodesmus were grown on yield-limiting nitrogen levels. In general none of the organisms tested increased in lipid content after nitrogen-starved growth. Total carbohydrate content did increase. The S. quadricauda strain was productive after nitrogen depletion and after readdition of nitrogen. The S. SO2a grew after nitrogen depletion but productivity declined compared to N-sufficient growth. The Ankistrodesmus grew poorly when N-starved and did not recover; it did not resynthesize pigment and eventually died, after readdition of urea-nitrogen.

Induction of Continuous Pond Effluents

Effluents from continuously grown cultures of *S. quadricauda* were used for growth, in batch, on N-deficient media. Table 5 shows total, lipid, and carbohydrate productivities of those effluents that actually became nitrogen depleted. The effluents were diluted with nitrogen-free medium to lower initial nitrogen levels.

In general, productivities of nitrogen-starved cultures were as high as non-starved batch cultures operated at the same time. This was especially true if starvation did not proceed past 2-3 days. Severe drop offs in productivity occurred thereafter. There often was a day of lowered productivity as nitrogen depletion set in, although variable insolation make this uncertain. Carbohydrate content doubled and lipid content dropped 25%.

TABLE 5. BATCH INDUCTION OF CONTINUOUS POND EFFLUENTS

Date	Day	% Lipid	Lipid Prod.	% CHO	CHO Prod.	Total Prod.	Lgly /day Total	Density
S. quadricauda								
9/11	0	26.6	3.5	25	3.5	14.0	530	107-126
9/12	→1	-	-	-	-	7.4	514	126-213
9/13	2	16.3	(-.4)	42	(6.6)	10(8.7)	531	213-263
9/14	3	14.7	-.3	47	4.3	3.4	522	263-280
9/15	4	14.0	-.5	55	4.0	0	514	280-275
9/21	→0	20	2.5	25[1]	3.2	12.6	376	60-132
9/22	1	-	-	45	9.0	8.2	361	132-173
9/23	2	-	-	-	-	9.0	249	173-218
9/24	3	14.2	(3.2)	-	-	15.3	474	218-295
9/25	4	-	-	-	-	15.8	473	295-374
S. SO2a								
10/7	0	19.5	0	25	-	8.2	295	135-180
10/8	→1	15.9	0.6	42	12	14.0	230	180-250
10/9	2	15.9	0.2	51	5	1.0	40	250-255

[1]Initial values at beginning of batch growth.
( ) Indicate an average value.
→ Indicate depletion of N from medium.

Batch Growth and Batch Induction

Batch cultures of three strains were started with minimal levels of urea to result in depletion of nitrogen from the medium. The induced cultures of *S. quadricauda* were as productive as uninduced

cultures (Table 6). Again, lipid content dropped 25%. Carbohydrate content was not routinely measured in these experiments. The cells used in runs 9-11 were N-deficient before addition of 10 ppm urea-N at the start of each run. As well, during Run #10 100 ppm of urea-N was added after day 4. The culture increased in productivity (to 16 $gm/m^2/day$) over the next day and a half. These results indicate that this strain recovers quickly from N-starvation.

The nitrogen-starved cultures of S. SO2a increased from 25% to 60% carbohydrate content, but productivity was lowered. The Ankistrodesmus grew poorly after depletion of N from the medium (7 $gm/m^2/day$) and carbohydrate content only increased to 32%.

TABLE 6. S. QUADRICAUDA BATCH INDUCTION

	Prod. Before N-depletion $gm/m^2/day$		Prod. After N-depletion $gm/m^2/day$		
Run #	Max.	Avg.(Days)	Max.	Avg.(Days)	Avg.(Days)
9	17.6	13(2)	19.7	18(2)	12(5)
10	18.2	13(2)	18.0	13(2)	--
11	17.3	13(2)	17.6	15(3)	9(5)

Tests for storage product induction were performed in the lab with I. galbana, Ankistrodesmus, and S. quadricauda, to determine the potential for increases in fuel precursors under more easily controlled conditions. Batch cultures were grown in 1L Roux flasks, under continuous illumination of 25 Wm^{-2} at pH 7.5±.5 and T=28±2°C. The results are shown in Table 7, along with some data previously taken by the author for comparison.

TABLE 7. INDUCTION OF STORAGE PRODUCTS BY ALGAE

Alga	Illum Wm^{-2}	Productivity $gm/m^2/day$ N-suffic. Avg.	N-suffic. Max.	N-starved Avg.	N-starved Max.	Storage Product Type	Max. Cont. % VSS	Maximum Product. $gm/m^2/day$	Average Product. $gm/m^2/day$
Ankistrodesmus (Pyr.Lake Isolate)	25	10	14	8	10	CHO	50	--	4
S. quadricauda (Vacaville strain)	25	10	14	8	10	CHO	60	8	5
Scenedesmus acutus (U.C.Berkeley #207)	25	10	15	12	20	CHO	75	15	9
Spirulina platensis	25	9	10	11	15	CHO	80	15	9
D. tertiolecta (UTEX 999) Seawater	25	11	17	13	20	CHO	75	--	--
(UTEX 999) 2.4MNaCl	25	6	8	--	--	CHO	--	--	--
I. galbana (UTEX 987) Hi Density	25	7	10	7	9	Lipid	40	4	3
Lo Density	25	7	10	1	--	Lipid	63	--	0.6

SEDIMENTATION RATES

Samples from the outdoor ponds were taken to the laboratory to measure biomass sedimentation rates. The rates of clarification were based on turbidometric assays. The tests performed were not aided by chemically induced flocculation. Flocculants can lead to greatly increased rates of clarification, but must be evaluated in terms of cost per unit biomass concentrated. Also, the effects of residual flocculants in the media, which must be recycled many times, are unknown.

The data from sedimentation tests are summarized in Table 8. It is clear that the naturally flocculant culture, S. quadricauda, grown in batch, sedimented much more quickly and completely than any other culture. Nitrogen starvation of this clumped culture did not have much effect on clarification rates. When this organism was grown in continuous culture, it did not aggregate. The sedimentation rates reflect this with only 40-60% of the biomass settling faster than 15 cm/sec. Effluents from continuous ponds "induced" in batch of S. quadricauda settled somewhat faster than uninduced biomass with 65-75% sedimenting faster than 15 cm/sec. Sedimentation of Ankistrodesmus and S. SO2a cells was also not very rapid. Again, induction (nitrogen starvation) increased the rates somewhat.

TABLE 8. SEDIMENTATION RATES

% of Biomass Sedimenting at Rates >V cm/hr

	S.quadricauda (clumped)		S. quad 2stage	S.quad Cont.	S.SO2a Batch		Ankist. Batch
	Unind	Induc	Induc.	Unind	Unind	Induc	Unind.
V cm/hr	9-1	9-8	9-25	9-25	10-3	10-7	10-7
300	40	40	--	--	--	--	--
150	70	65	--	--	--	--	--
100	70	70	--	--	0	10	5
60	85	80	10	0	--	25	15
45	--	--	--	--	5	30	25
30	90	90	30	10	10	35	45
15	--	--	65	40	--	--	--

Slurry density is another sedimentation parameter that was measured in certain instances. Table 9 lists culture, sedimentation slurry and supernatent densities measured during some of the sedimentation tests. Concentration factors were 50-100 fold, indicating that compaction of the biomass was good.

TABLE 9. SEDIMENTED SLURRY BIOMASS DENSITIES

		V cm/sec	Cult. Dens. gm/L	Slur. Dens. gm/L	Super-natent Dens. gm/L
S. quadricauda batch					
Uninduced	9-1	60	0.275	25.2	.023
	9-14	60	0.680	38.5	.103
	2-13	60	0.405	25.0	.100
Induced	9-8	60	0.515	29.6	.070
S. quadricauda continuous					
	9-8	15	0.140	24.5	.080
	9-25	15	0.178	15.0	.073
	9-30	30	0.174	13.0	.105
S. quadricauda continuous effluent					
	9-15	15	0.325	22.5	.055
	9-25	15	0.398	14.0	.140
Ankistrodesmus	9-30	30	0.145	11.0	.05
	10-7	30	0.210	25.0	.09

CONCLUSIONS

Of the three species of algae that were cultivated in outdoor ponds (an Ankistrodesmus sp., a Scenedesmus sp. (SO2a), and an indigenous Scenedesmus quadricauda) none accumulated lipid in the short-term under nitrogen deficient conditions. The Scenedesmus strains doubled in carbohydrate content. In addition, the induction of storage products must be nearly completed after 2-3 days of N-deficient growth in order to avoid a large decrease in productivity. Although the conditions were probably not optimal in the experiments reported above, better productivities and larger increases in storage products will not increase this time frame. Unless a means can be found to promote increased lipid content in these strains, they are not good candidates for oil production. Any method developed for inducing lipid must be practical in the field where (1) high light intensities impose severe conditions on light sensitive strains, e.g. the Ankistrodesmus, and (2) the time frame for increasing lipid content is 2-3 days before productivity is depressed. Testing of strains for lipid production, in the laboratory under high intensity illumination, is more prognostic than testing under low light intensity.

The outdoor studies, combined with the high light intensity indoor tank experiments, indicate that some "saturation" phenomenon is limiting the productivities achieved. There may be several mechanisms involved including light inhibition, classical msec saturation, and longer time scale saturation. The last, and possibly the first, are amenable to solution by improving pond hydraulics. Strain screening could provide organisms that are not sensitive to high light intensity (many are) and strains with the highest I_k (saturating light intensity). But this, again, requires screening strains under high intensity experimental apparatus. All of the strains grew equally well under low light intensity (10% noon solar) in the lab. The highest productivities obtained, outdoors, during this work (20-30 $gm/m^2/day$) may be increased and sustained using organisms adapted for the high light environment. Since most organisms induce storage product accumulation (lipid or carbohydrate), under nitrogen limitation, some fuel precursor would be produced.

Laboratory studies were initiated to optimize the medium for Ankistrodesmus growth both from the biological and economical standpoints. So far no means has been discovered to increase productivity under high light intensity. A progressive deterioration of the cultures occurred. Interestingly, this did not occur under higher light intensity, in the outdoor ponds. This underscores the necessity of testing screened strains in the outdoor system. Indoors, under 40% noon solar intensity, the Ankistrodesmus grew well over a large range of salinities, pH (when cycled), with EDTA reduced 90%, lowered trace and alkalinity, or with recycling of the medium for ten batch growth cycles. The problems that need to be addressed are light sensitivity, lipid induction, harvesting, and long term species dominance. Up to now the Ankistrodesmus has maintained itself unialgal outdoors. However, it has been grown only half of a growth season.

The native Scenedesmus strain was more productive than the Ankistrodesmus, averaging 15-20 $gm/m^2/day$ during the summer months with no attempts to optimize the growth medium. It is not photosensitive. Its tendency to clump during batch growth makes harvesting relatively simple and inexpensive. However, it accumulates carbohydrate when nitrogen-starved, not lipid. Further work with this strain is needed to determine whether overall productivities can be improved and carbohydrate content increased to 70% of the ash-free biomass.

An analysis of the cost of algae biomass production in the experimental system showed that efficiency of CO_2 utilization is the most critical operational issue. CO_2 loss at high alkalinity and low pH is substantial. Thus, screening criteria should include good growth at high pH.

MICROALGAL TECHNOLOGY RESEARCH AT SERI

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INTRODUCTION

The SERI Microalgal Technology Research Group (MTG) was organized in 1983 as part of the Biotechnology Research Branch, Solar Fuels Division, to support the DOE Aquatic Species Program (ASP) and its efforts to enhance the efficiency of the development of microalgal technologies for the production of fuels. The MTG will provide a centralized technical resource which will help to bring a number of research organizations toward the common long-term objectives of the ASP. The research capabilities of the MTG will be available for the rapid resolution of technical questions of general programmatic interest. Members of the MTG will also be involved in research and service activities which will suggest and facilitate research within the ASP.

The research of the MTG in FY 1983-1984 has concentrated on three major areas:

- Technical literature reviews
- The SERI microalgal culture collection
- Experimental research on key technical issues of interest to the ASP

Four literature reviews have been written, and these documents are undergoing internal SERI review. They will then be reviewed externally and published. The four reviews cover the following subject areas:

An Engineering History of Outdoor Microalgal Production Systems summarizes the development of microalgal systems beginning in the 1940's. The evolution of these systems is traced for pond culture systems, deep channeled systems, and shallow circulating systems. Indoor systems which have been tested in concept, but not applied to outdoor production are also described.

Technical Considerations of the Outdoor Production of Microalgae addresses the requirements of microalgae in culture and the environmental constraints on their production. Topics discussed include light utilization and culture mixing, carbon dioxide supply and culture pH control, nutrients and nutrient sources, temperature responses, culture contamination, and the manipulation of cell chemistry through the manipulation of the cell environment.

Characteristics and Properties of Inland Saline-Adapted Chrysophyta addresses the research reported to date on these algae and suggests future research that could be beneficial in evaluating and developing the potential of these microalgae for biomass production.

Lipid Biosynthesis and Accumulation in Microalgae: Considerations of Pathways and Regulation addresses the current state of knowledge of lipid biosynthesis, focusing particularly on aspects of enzymology and metabolic regulation that may be associated with triggers of lipid systhesis. Topics discussed include the lipid composition of microalgae, important pathways of carbon metabolism and their relationships to lipid biosynthesis, general aspects of metabolic regulation, metabolic pathways of lipid biosynthesis in microalgae, and relationships between carbon flow and storage metabolites.

The SERI Microalgal Culture Collection will serve as a respository for the most promising species developed by the various microalgal projects within the ASP, and for centralized documentation of those species. These species, along with the avialable documentation, will be made available to researchers both within and outside of the ASP, with the intent of stimulating research on organisms of particular interest to the program. The culture collection is described in greater detail in the following section.

Experimental research during FY 1983-1984 is directed toward the resolution of several technical issues which are of immediate interest to the ASP.

- As a part of the overall species screening activity of the ASP, microalgal strains are being collected from the saline waters found in arid regions of the Rocky Mountain States and screened for their potential as producers of fuels in outdoor cultures.

- The photosynthesis and growth of microalgae in modulated light environments is being investigated, to permit an assessment of the "flashing light" effect.

- The enzymology of microalgal lipid production is being investigated to determine the enzymologic basis of lipid trigger mechanisms, and to provide the information necessary for the physiological, chemical, or genetic manipulation of these mechanisms.

- Monitoring and control components are being developed for automated laboratory culture units. These units will be used for MTG experimentation, but the components and routines which are developed will be available for use in experimental systems which are being operated elsewhere within the ASP.

These experimental programs are discussed in greater detail in the four sections which follow.

MICROALGAL TECHNOLOGY RESEARCH AT SERI: SPECIES COLLECTION AND CHARACTERIZATION

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INTRODUCTION

One of the primary goals of the Microalgal Technology Group (MTG) at SERI is to establish a culture collection of lipid/oil producing microalgae to foster research and development of these species by SERI researchers and other investigators throughout the world. Candidates for the collection are to be selected from species that have been collected and screened by SERI contractors and by the SERI MTG.

To compliment this collection and screening process, MTG collection efforts will focus on collecting species from saline aquatic habitats in Colorado, New Mexico and Utah. Arid region, saline aquatic environments are excellent sites for the collection of species which may be candidates for biomass production. These sites are very diverse in terms of their environmental characteristics (water chemistry, temperatures, etc.) They also tend to be isolated and island-like in their distribution allowing for the potential development of strains of species with a wide range of environmental tolerances. Species adapted to the environmental conditions in these arid regions may prove to be the easiest to develop for biomass production systems. Arid regions of the western United States have often been proposed as sites for algal cultivation systems. They are characterized by large areas of undeveloped land, high amounts of solar insolation, and abundant groundwater resources which are not in demand for other uses because of their salinity.

Algal populations in arid region saline habitats are generally dominated by members of the Cyanophyta and Chrysophyta [6, 7]. The Cyanophyta (blue-green algae) have received a great amount of research attention because of their ability to fix nitrogen and withstand relatively high temperatures [3]. This group of algae however are not known to be lipid accumulators.

The majority of research on the Chrysophyta to date has focused on those species occurring in marine environments, where they are usually the dominant element of the phytoplankton in estuaries and oceans [1]. Although there is some controversy surrounding the subject, most Chrysophyta occurring in inland saline envionments are considered to have evolved from freshwater ancestors [2]. This suggests that there may be considerable differences in their environmental tolerances, metabolic processes and other characteristics related to their potential for cultivation. Also, because the Chrysophyta are one of the major groups of algae known to produce oil as a food storage product [4], they appear to be excellent candidates for evaluation in biomass production systems for the purposes of producing oils.

The major algal culture collections in the United States are at the University of Texas, Austin, and Bigelow Marine Laboratories in Maine. The Texas collection contains marine and freshwater species while the Bigelow collection contains only marine species. Development of an inland saline species collection by SERI will serve as an important stimulus for research and development of these species for biomass production systems.

Purpose and Objectives

The species collection and screening effort has four main objectives. They are:

- Assemble and maintain a set of viable mono-specific algal cultures stored under conditions best suited to the maintenance of their original physiological and biochemical characteristics.
- Develop storage techniques which will help maintain the genetic variability and physiological adaptability of the species.
- Collect single species cultures of microalgae from the arid regions of Colorado, Utah, and New Mexico for product and performance screening, and to develop media which are suitable for their growth.
- Evaluate each of the collected species for their temperature and salinity tolerances, and quantify growth rates and proximate chemical composition for each species over the range of tolerated conditions.

Results and Methods

- Culture Collection

The first meeting of the Species Collection Steering Committee will be held April 4, 1984. At that time, the committee will select the initial set of species to be maintained in the collection. Candidate species will be selected from among those collected and screened by SERI contractors and the SERI Microalgae Technology Research Group (MTG). Growth chambers and refrigerators have been ordered to maintain the collection and should in place shortly after the April meeting. Viable culture samples are to be made available to SERI researchers and other investigations throughout the world to promote and encourage investigations of lipid-producing species.

In additional support of this effort, a catalogue listing all species in the culture collection and summary information for each species (information culture media, environmental tolerance, growth potential, lipid content, etc.) will be published in September 1984.

The initial storage approach employed will be to maintain the cultures at 5-15°C in low light as recommended by Gallagher [5]. An additional experiment will be conducted to determine the effect of storage under

Collecting Microalgae from a Saline Hot Spring in Western Colorado

Innoculating Microalgae on a Salinity, Temperature, Light Gradient Table for Determining Environmental Tolerances

fluctuating environmental conditions. Cultures maintained under both sets of conditions will be monitored periodically for several selected physiological and growth related parameters to determine if any changes have occurred during storage under the two types of conditions. Based on these findings storage environments for the collection will be recommended.

- Species Collection and Characterization

Two collecting trips were completed before the onset of winter snows. Collection efforts focused on the saline hot springs of western Colorado. During an initial survey of a series of these springs, five springs were selected for collection efforts based on their abundant diatom populations and different ionic compositions. Algae were collected and placed in tubes of enriched (NH_4/NO_3 + PO_4) filtered spring water and transported back to the laboratory. Water samples were also collected at each site and preserved for later analysis of their ionic composition. Nitrate/nitrate, phosphate, sulfate and ammonium ion concentrations were quantified within 24 hours of collection. Temperature and conductivity measurements were also made at several locations at each site. Water samples were sent to the University of Colorado, Denver for analysis on their Inductively Coupled Plasma unit. A brief summary of the environmental characteristics at each collection site is presented in Table 1. The conductivity of seawater is generally around 30,000 Mhos/cm^2 with a monovalent/divalent ion ratio of about 4. The hot spring collection sites represent environments with salinities and ion ratios above and below those found in marine environments. The temperatures at the hot spring collection sites however are much more similar to those expected in arid region biomass production systems.

In the laboratory several methods were evaluated for separating the diatoms from the blue-green algae and other organisms occuring in the spring water. Agar plating techniques proved to be the easiest and most efficient process. Approximately 125 strains were collected and processed in this manner to yield unialgal cultures. Initial identification of the collected diatoms indicated that the strains were from 12 main genera (Table 2). A preliminary survey of 25 of the strains under nitrogen limiting conditions indicate that members of the genera Amphora and Cymbella readily form lipid droplets in the cells which can be observed under a microscope. Blue-green algae have proved to be a persistent contaminent in a few of the cultures but treatments with antibiotics is underway to clean up these strains.

A one meter square temperature/light gradient table was constructed to provide a method for evaluating the growth rates and environmental tolerance of each collected strain. The apparatus was designed to provide a temperature gradient of 10-42°C and light gradient of 0-200 $E/m^{-2}/s^{-1}$. Salinity and/or ionic gradients can also be established in the apparatus perpendicular to the temperature gradient. Preliminary testing of the gradient table has been completed and design changes have been made to fix several operational problems that were identified including uneven light distribution and difficulties with procedures for monitoring growth

TABLE 1. ENVIRONMENTAL CHARACTERISTICS OF THE COLLECTION SITES

Site	Conductivity (Mhos/cm^2)	Monovalent / divalent ion ratio	Temp °C	Elevated ion Concentrations	Redox Potential (mv)
Glen-10 Spring	41,000	11.9	46	Cu	-177
Hobo Spring	34,500	8.3	36		- 60
Redstone Spring	21,300	8.6	26	Mn	-214
Piceance Spring	85,000	57.3	13	Ba,F	-275
Salt Creek Vernal Pond	3,500	0.4	11	Fe,SO_4	+174
South Canyon Spring	1,850	33.4	41		- 12

TABLE 2. MAJOR GENERA OF DIATOMS COLLECTED FROM THE HOT SPRINGS HABITATS

Achnanthes	Gyrosigma
Amphora	Melosira
Caloneis	Navicula
Camphylodiscus	Nitzchia
Cymbella	Pleurosigma
Entomoneis	Surirella

performance. Growth and environmental tolerance testing of the algal strains collected from the hot springs is just commencing and a summary of the data will be presented at the annual review meeting in April.

Chrysophyta, because they are a group of oil producing algae and are abundant in arid regions, have been identified by SERI as an important group of algae to evaluate for biomass production. Towards this effort, a literature survey of all published papers on arid region Chrysophyta was conducted and a summary review paper prepared entitled: "Characteristics and Properties of Inland, Saline Adapted Chrysophyta". The results of the review indicate that only floristic (species list) types of studies have been conducted on these inland saline species. In most cases little or no environmental data was collected during these studies and only one physiological study has been conducted to date on this entire group of algae. The paper suggested future areas of research that could be beneficial for evaluating and developing the potential of these algae for biomass production.

Plans for Continuing and Future Research

In order to enhance the availability of the SERI microalgal culture collection, a catalogue will be published, listing all species in the culture collection, including summary information for each species (preferred culture media, environmental tolerances, growth potential, lipid content, etc.). The physiological and growth performance data will be compiled from the evaluation and screening research performed by various SERI researchers and contractors. The first edition of the catalogue will be published in September, 1984.

In the spring, collection efforts will resume stressing other types of inland saline habitats including ephemeral ponds, playas and lakes. When laboratory testing of a sufficient number of collected strains is complete, environmental data from each collection site will be combined with the data on a strain growth potential and environmental tolerance and analyzed using multivariate statistical procedures. The purpose of these analyses will be to develop a theoretical basis for future collection efforts, both in identifying the most important environmental data to collect at each site, and to characterize the best sites at which to focus future collection efforts. This could help to greatly decrease the amount of data needed to be collected in the field and improve the efficiency of SERI collection and screening efforts.

To compliment the environmental tolerance and growth performance evaluation of collected species, experiments are planned to develop techniques for rapidly screening their lipid production potential. Initial experiments will evaluate several of the new fluorescent dyes developed for staining lipids, especially those which change their fluorescence characteristics as lipid chain length increases.

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MICROALGAL TECHNOLOGY RESEARCH AT SERI: MODULATED LIGHT PHOTOBIOLOGY

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INTRODUCTION

Many efforts at microalgal mass culture have attempted to take advantage of the "flashing light effect" to enhance the productivity of culture systems. Algal cells which are exposed to high intensity light on an intermittent basis can utilize the light more efficiently than cells which receive the same intensity on a continual basis (see Background, below). In algal mass cultures, the achievement of such an effect has been predicated on the rapid vertical mixing of very dense cultures, causing the the cells to be circulated from a lighted zone at the surface of the culture to a darkened zone at depth. The efficacy of such a regime for the enhancement of production is not well documented, although recent results with an outdoor system suggest that some such enhancement is possible [7], and similar results have been obtained with indoor systems [2, 4, 8, 9].

Purpose and Objectives

The objective of MTG research into modulated light effects is to evaluate the potential for enhanced photosynthetic efficiency through vertical mixing of outdoor cultures and to provide quantitative estimates of the amount and nature of the mixing which is required to achieve this enhancement. Variables which are expected to affect enhancement include the duration of the "flash", the duration of the dark period, the waveform of the light modulation, the intensity of the incident light, and the temperature of the solution. The first three of these variables will be determined by the culture density and the rate of mixing. A quantitative understanding of the effects of these variables on production rate is a necessary prerequisite to the engineering of mixing regimes in algal systems.

Background

The light environment experienced by cells in dense, vertically mixed cultures is considerably different from the flashing light environment normally used in laboratory experiments. In laboratory experiments, sectored-disc light chopping devices provide approximately square-wave light modulations, while the light environment experienced by cells mixed vertically in a dense culture is a smooth wave function of time (Figure 1). These smooth-wave light environments have not been investigated quantitatively. For the purposes of this discussion, the term "flashing light" will be used to refer to squarewave modulations, while "modulated

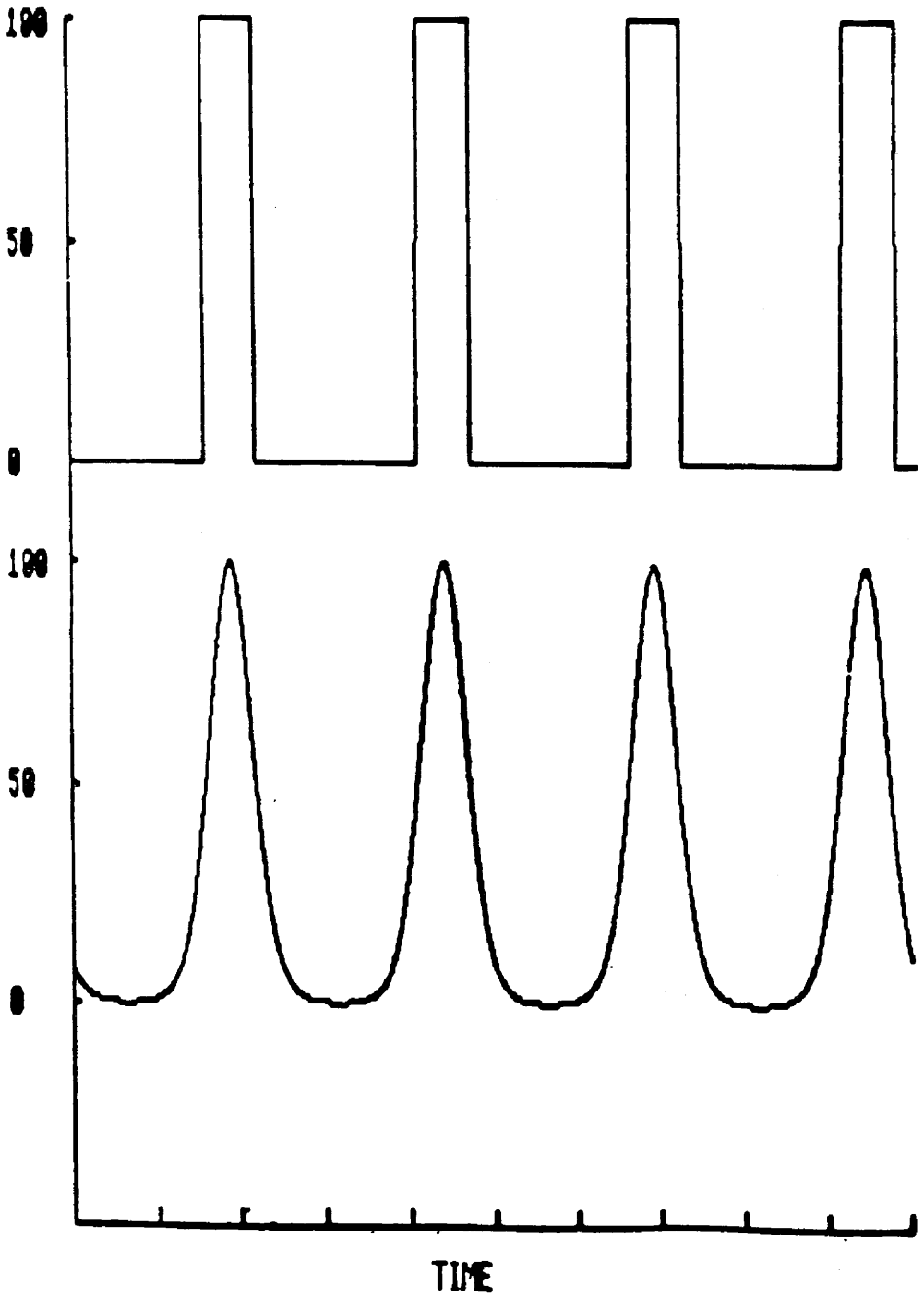

Figure 1. IRRADIANCE AS A FUNCTION OF TIME FOR A LABORATORY FLASHING LIGHT EXPERIMENT USING A SECTORED DISC (ABOVE) AND FOR A CELL FOLLOWING A CIRCULAR PATH IN A DENSE ALGAL CULTURE (BELOW).

light" will refer to the more general case of fluctuations in intensity over time.

In dense outdoor algal cultures, light is attenuated with depth very rapidly. Irradiance may fall to less than 1% of the surface irradiance within a few centimeters. However, the majority of the quanta which fall on the culture are absorbed in the upper layers of the culture, where the intensity is high and the photosynthetic efficiency is low. Thus, the average utilization efficiency remains low.

When algae are exposed to high light intensities intermittently rather than continuously, the photosynthetic efficiency is improved [3, 5, 6, 8, 10, 11, 15]. The preponderance of evidence suggests that an alga exposed to high light intensity on an intermittent basis can achieve, under an optimum intermittency regime, a photosynthetic efficiency similar to that attained under constant light of the same average intensity [6, 12, 13, 15]. Thus, within certain limits, algae can integrate light intensity when light is received on an intermittent basis, and respond only to the long-term average of the photon flux.

Studies of photosynthesis in flashing light began with the work of Brown and Escombe [1], who attempted to use a sectored, rotating disk as a neutral density filter for photosynthetic studies. Warburg [14] performed similar experiments with Chlorella. In both cases the intermittent application of high intensity light was as effective at promoting photosynthesis as continuous application, within certain limitations on the rate of flashing.

Weller and Franck [15] expressed photosynthetic rate as a function of the average light intensity received by the cells, and found that rates in intermittent light did not significantly exceed those in constant light of the same average intensity. There was a significant increase in the photosynthetic efficiency, since the light was utilized at the efficiency characteristic of lower intensities. Recently [13], the conclusions of Weller and Franck [15] have been confirmed by reanalysis of the data collected by many other investigators.

Work performed subsequent to that of Weller and Franck [15] embodied a growing understanding that the response to flashing light was dependent on a number of variables, including (1) flash length, (2) dark period length, (3) flash intensity, (4) temperature, and (5) the preconditioning history of the culture. Kok [5, 6], studied the effects of flash period, dark period, flash intensity and temperature on the rate of oxygen evolution by Chlorella cells. Coverage of these variables was limited, however, and much of the data collected apparently was never published. The preconditioning history of the algae does not seem to have been uniform between experiments, and thus little understanding can be gained regarding multivariate responses.

By far the most useful information on the response of algae to flashing light was gathered by Phillips and Myers [10, 11]. These authors observed the long-term (48 h) growth of Chlorella in various flashing light environments. As the authors point out, observations of growth rate are much more valuable

than observations of photosynthetic rate, since these observations include the adaptive response of the algae to the flashing light environment. Comparable short-term results are not available, so no assessment of the magnitude of the adaptive component of the response can be made.

The data of Phillips and Myers [11] can be reanalyzed to explicitly quantify the productivity enhancement which these data predict. Consider an algal culture receiving light from a source of intensity I with the beam broken by a sectored disk into a flash period of duration t_f and a dark period of duration t_d. Rather than being wasted, the light falling on the sectored disk during the dark period could be used to illuminate one or more additional cultures. The number of cultures which could be grown using the single light source, N, can be calculated as

$$N = (t_f + t_d)\ t_f^{-1} \tag{1}$$

and the total productivity possible with the light source is

$$P_t(I) = NP_f(I) \tag{2}$$

where $P_f(I)$ is the net productivity of the single experimental culture exposed to flashing light of a flash intensity I. The improvement in photosynthetic efficiency over that which would be obtained by a single culture receiving the same intensity on a continuous basis can be expressed as a factor

$$F = P_t(I) \cdot P(I)^{-1} \tag{3}$$

where P(I) is the productivity of the single experimental culture in light of constant intensity I. A value of F of 2, for example, implies that photosynthetic efficiency can be doubled by employing the particular flashing light regime for which the F value was obtained.

The data of Phillips and Myers [10, 11] permit the calculation of F as a function of t_f and t_d for Chlorella (Figure 2). Only a limited portion of the range of interest was covered by these measurements, but it is apparent that significant increases in productivity are attainable in this manner.

METHODS AND RESULTS

Initial experimental activity by the MTG will concentrate on relatively rapid data acquisition, investigating the short-term photosynthetic response to flashing and other modulated light environments. While growth measurements

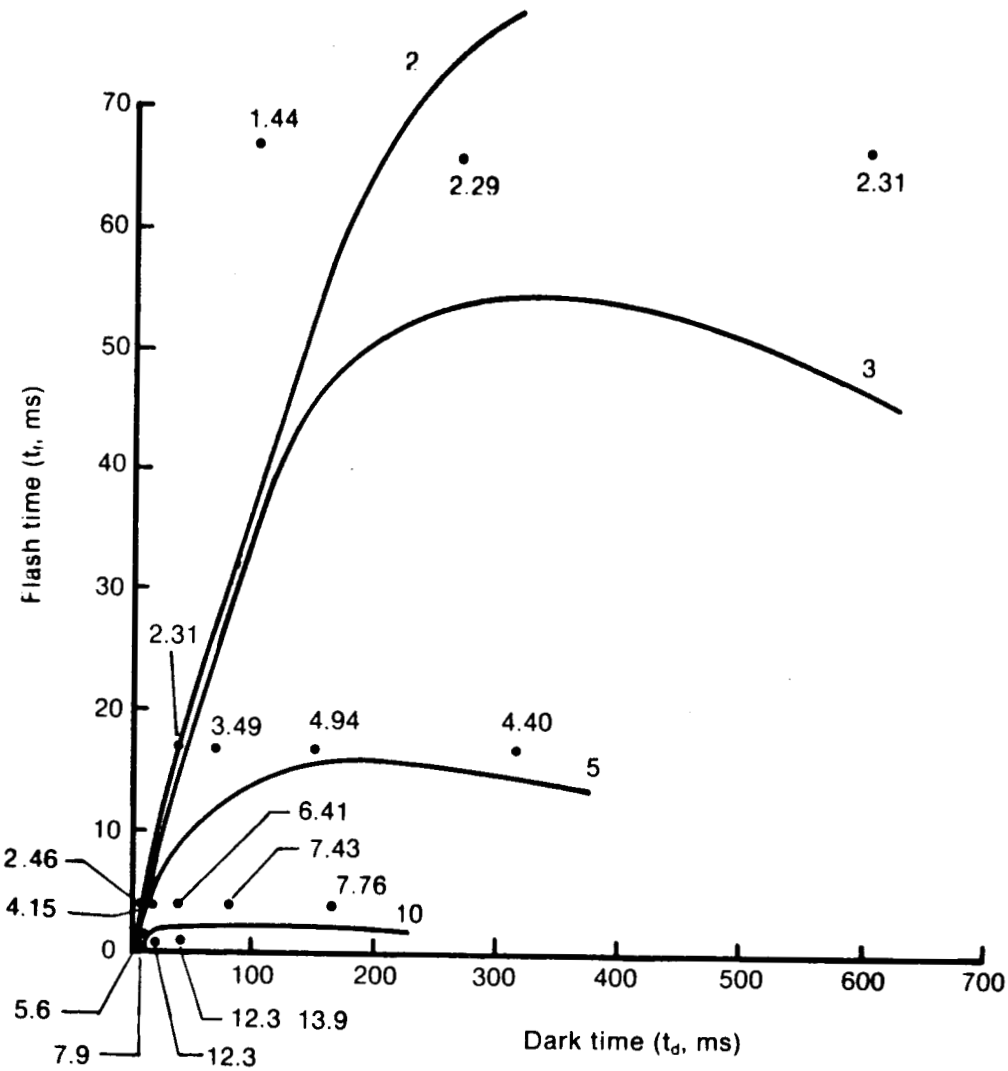

Figure 2. THE PHOTOSYNTHETIC ENHANCEMENT FACTOR F AS A FUNCTION OF FLASH TIME (t_f) AND DARK TIME (t_d) FOR THE GROWTH OF CHLORELLA PYRENOIDOSA IN A FLASHING LIGHT ENVIRONMENT. DATA FROM PHILLIPS & MYERS (1954b).

are of considerably greater interest than photosynthesis measurements, photosynthesis measurements can be obtained much more rapidly, and will aid in the selection of the conditions under which growth experiments will be run. If the relationship between photosynthetic and growth responses can be understood, then individual species can be characterized more rapidly on the basis of the photosynthetic response.

The research plan which will be followed can be roughly divided into four steps.

1. Photosynthesis measurements in flashing light will be made with the apparatus described below. A preliminary quantification of the multivariate response to light modulations will be obtained. The most thorough coverage of the independent variables will be available at this stage, due to relatively rapid data acquisition. Data obtained during this step will be useful in selecting values of the variables to be employed in later, more time consuming experiments.

2. Photosynthesis measurements in smooth-wave modulated light will be made by mechanically moving small enclosed samples of algal cells in circular paths through dense suspensions of algae. Photosynthetic rates will be determined by ^{14}C fixation measurements.

3. Growth rates will be determined in modulated light environments generated by external modification of the incident light (rather than by culture mixing). The nature of the adaptive component of the modulated light response will be evaluated in this way, and the quantitative model developed in steps 1 and 2 will be modified to reflect this adaptation.

4. Growth rates will be determined in laboratory cultures subjected to vortex mixing and illuminated from one surface to evaluate predictions based on 1-3 above.

Methods

Chlorella pyrenoidosa and *Phaeodactylum tricornutum* were grown in nutrient sufficient continuous cultures in 1.5 L growth chambers at dilution rates of 0.3-0.5 day^{-1}. The cultures were cooled by 20C water circulated through the outer jacket of the double-walled growth chamber. Illumination was provided by a bank of six 40 W cool white fluorescent lamps, which provided a quantum flux of approximately 250 uEinst m^{-2} s^{-1} to the surface of the culture facing the lamps. These cultures provided uniformly preconditioned cells for use in the photosynthesis experiments.

Photosynthetic rates were determined by oxygen evolution measurements made with a Rank Brothers (Cambridge, England) oxygen electrode system. The electrode chamber was cooled by water from the same constant temperature circulator which cooled the growth chambers. Algal samples of 1.2 mL in the electrode chamber were illuminated from above through a clear glass plunger.

For the preliminary experiments presented here, the light source was the tungsten lamp of a Kodak Carousel slide projector. The light was interrupted by a sectored disc driven by a speed-controlled DC gearmotor.

For each determination a fresh sample was removed from the continuous culture and added to the electrode chamber. It was necessary to dilute the samples by a factor of two to three with unpopulated medium in order to prevent self-shading effects within the electrode chamber. Samples were kept in the chamber in the dark for three minutes to estimate the population respiration rate, then illumination was begun under the desired illumination regime. Three to five minutes were required to determine the photosynthetic rate. Photosynthesis and respiration rates were corrected for oxygen consumption by the electrode, which was determined with samples of unpopulated medium. Controlled variables were the ratio of light to dark determined by the configuration of the sectored disc, and the frequency and intensity of the flash.

Knowlege of the P versus I relationship permits the calculation of the expected extremes of the flashing light response. If no flashing light response is achieved, photosynthesis will take place during the flash at the rate observed in constant light of the same intensity and will cease between flashes:

$$P_{min} = P(I)\cdot N^{-1} \tag{4}$$

with the terms defined as above. At the other extreme, if complete integration is achieved, the photosynthetic rate will be

$$P_{int} = P(I\cdot N^{-1}). \tag{5}$$

The extent to which a given modulated light regime leads to integration of the light intensity can be expressed as

$$\%\ \text{integration} = (P_f - P_{min})(P_{int} - P_{min})^{-1}\cdot 100. \tag{6}$$

Percent integration should vary between 0 and 100, and this predicted result was observed in the preliminary experiments which were performed (Figure 3). The data presented in Figure 3 show % integration as a function of the flash frequency and the proportion of the total time the culture was illuminated (N^{-1}). Percent integration was a saturating function of the flash frequency; that is, more rapid flashing led to greater integration. The shape of the saturation function changed as a function of N^{-1}. Primarily, the sharpness of curvature increased with decreasing values of N^{-1}. The asymptote of the curve also differed between these different flash regimes, but in no consistent manner. It seems likely that these differences can be explained by errors in estimating P_{int} for these preliminary data.

For N^{-1}=0.5, integration data were collected at two irradiances, 270 and 630 uEinst m^{-2} s^{-1}. Percent integration as a function of flash frequency did not noticeably vary between these cases. If this function proves to be

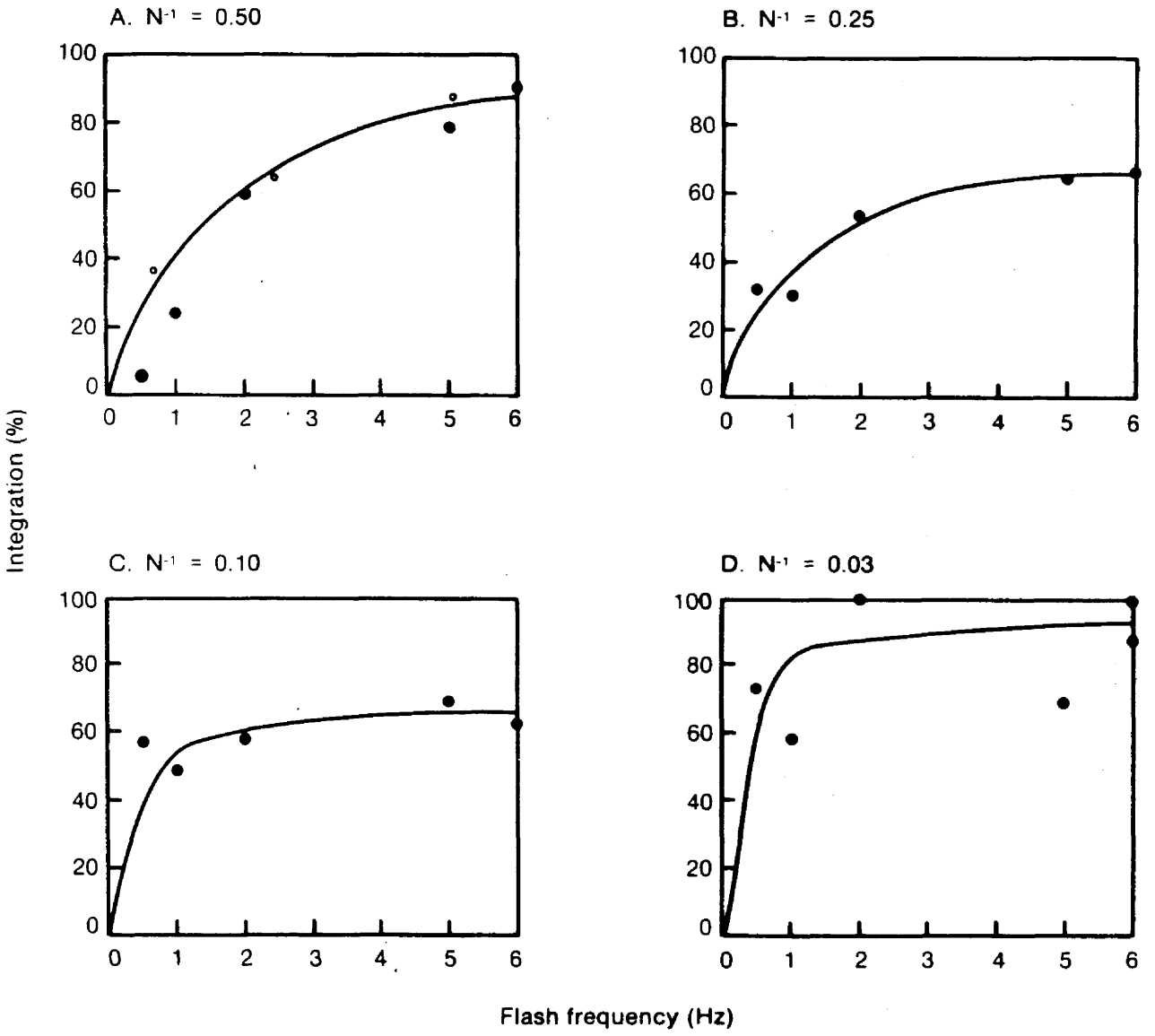

Figure 3. PERCENT INTEGRATION OF LIGHT INTENSITY AS A FUNCTION OF FLASH FREQUENCY FOR FOUR DIFFERENT PERCENTAGES OF THE TOTAL CYCLE TIME SPENT IN THE LIGHT. A. 50%. B. 25%. C. 10%. D. 3%. IN A, OPEN CIRCLES REPRESENT DATA TAKEN AT 270 uEinst m^{-1} s^{-1}, WHILE FILLED CIRCLES REPRESENT DATA TAKEN AT 630 uEinst m^{-2} s^{-1}. ALL OTHER DATA WERE TAKEN AT THE LATTER INTENSITY.

independent of irradiance, analysis of the total data set will be considerably simplified.

Discussion

The data presented by Laws et al.[7] on the achievement of vertical mixing in outdoor cultures through the use of vortex mixing suggest that cycle periods in the range of 0.5 - 1 Hz can be achieved. Unless cultures can be made considerably shallower, it seems unlikely that much more rapid cycle times will be achieved without inordinate energy costs. It is important to determine, then, whether significant enhancement of photosynthetic efficiency can be achieved with flashes of this frequency. The data of Phillips and Myers [10, 11] do not cover total cycle periods of this length, but visual extrapolation of the curves shown in Figure 2 suggests that enhancement might be available through the use of short flash periods and long dark periods, i.e., in the lower right hand portion of the figure. The preliminary data are consitent with this conclusion. They suggest that integration can be achieved at flash frequencies as low as 0.5 Hz, and that the greatest integration can be achieved at these frequencies when the relative proportion of the cycle for which the cells are lighted is small.

In outdoor cultures, cells spend the smallest proportion of their time in the lighted zone when the culture is very dense, so high culture densities would be required to achieve maximum integration. Thus, small values of N^{-1} can only be achieved at the expense of increased respiratory losses to the non-illuminated portions of the culture. The tradeoff between increased gross photosynthetic efficiency and the increased population respiration which is required to achieve this efficiency must be taken into consideration in choosing the optimum culture density for a given turnover rate.

Plans for Continuing and Future Research

The study of square wave modulations will be completed in the next two months (May 1984). The results obtained in this phase of the study will provide an initial indication of the range of interest of the independent variables, and will direct the choice of conditions for more time-consuming experiments in subsequent experimental phases. An apparatus for the study of smooth-wave modulations will then be constructed, and the relationship between square-wave effects and smooth wave effects will be explored. Growth experiments in modulated light will also commence prior to the next contractors' review.

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MICROALGAL TECHNOLOGY RESEARCH AT SERI: THE BIOCHEMISTRY OF LIPID TRIGGER MECHANISMS IN MICROALGAE -- ALTERATION OF LIPID METABOLISM DURING TRANSITION FROM RAPID GROWTH TO RAPID LIPID ACCUMULATION

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INTRODUCTION

Successful development of microalgal technologies for production of lipid-based fuels will require a thorough understanding of species physiology and lipid metabolism in order to promote maximum product yield while maintaining optimum growth conditions. Theoretically, this could be accomplished by selecting species which grow rapidly, divide rapidly, and channel a large percentage of reduced carbon towards the synthesis of lipids. Unfortunately, a general inverse relationship appears to exist between rate of growth and rate of lipid accumulation. That is, species which assimilate large proportions of carbon as lipid tend to be slow growers.

This has led some workers to suggest a two-stage growth process for the maximum production of lipid by microalgae. In the first stage, an ideal species would be grown under conditions which stimulate the rate of cell division. At some point, growth conditions would then be altered, initiating a second stage in which cell division becomes limited while synthesis of storage lipid is stimulated. In this way, rates of both growth and lipid production could conceivably be optimized, satisfying a major criterion for successful mass cultivation of microalgae for fuel production.

Alteration in growth conditions to promote lipid accumulation might be accomplished by several different methods. These include changes in nutrient balance, light intensity, cell density, cell age, culture pH, temperature, or introduction of a specific chemical agent intended to alter cellular metabolism. An environmental or chemical alteration which serves to stimulate lipid production (usually at the expense of rapid cell division) may be termed a <u>lipid trigger</u>.

Presently, this term is of limited usefulness, serving only to describe a phenomenon associated with the physiological response of a cell towards its environment. Information is currently limited concerning the metabolic regulation of this process. There is also a general lack of information as to why, for instance, nitrogen starvation might lead to lipid accumulation in one species, polysaccharide accumulation in a second, and accumulation of neither storage material in a third. In addition, it can be assumed that not all environmental or chemical alterations which serve to limit cell division in a given algal species, will bring about stimulated production of storage metabolites. Clearly, there is a great need to understand the metabolic regulation associated with the use of lipid triggers.

Purpose and Objectives

The biochemical investigations of the MTG are currently directed towards gaining an understanding of the metabolic process associated with operation of recognized lipid triggers. This information could then be used in efforts to develop new triggers, which are both effective and rapid in mass-culture applications. This will be accomplished through studies of carbon pathways associated with lipid synthesis in selected algal species during rapid and limited growth states. Emphasis will be placed on the following:

1) Analysis of factors regulating carbon flow towards the synthesis of lipid precursors, focusing on enzymes of acetate metabolism

2) Localizing the pathways for acetate formation within the cell

3) Determination of whether changes in the pathway of carbon flow occur as a result of transition from states of rapid growth to rapid lipid accumulation

4) Determine if specific enzyme levels can be used as accurate diagnostic tools for assessing the lipid production potential of a given algal species during screening activities.

Literature Review and Documentation

An extensive review of the literature pertaining to lipid biosynthesis and regulation in microalgae was performed to aid in formulating an effective experimental plan for analyzing lipid trigger mechanisms. This material has since been organized as a written technical review which is currently undergoing an internal peer review cycle. The salient features and conclusions of this report can be summarized as follows:

1) To a great extent, an understanding of lipid metabolism in microalgae must rely on an examination of comparative biochemistry, due to a specific lack of such information in the microalgal literature.

2) Lipid synthesis is generally subject to multiple regulatory constraints, including carbon precursor availability, allosteric regulation of enzyme activity, genetic regulation of enzyme expression, regulation by light and dark effects, and metabolic compartmentation.

3) The metabolic controls associated with lipid triggers in microalgae have never been studied in detail, particularly in relation to the regulation of carbon precursor availablilty for lipid synthesis.

4) An organized effort to define the spectrum of effective lipid triggers has not been carried out to date. There is no reason to believe that nitrogen deprivation represents an ideal trigger.

5) There are a limited number of reports in the literature that suggest a

shift in the intracellular site of lipid synthesis in microalgae as a result of exposure to environmental stress. This compartmentation may be important in determining whether membranous or storage lipid is being produced by the cell.

6) The exact origin of acetate for the synthesis of lipids in microalgae is unknown. A detailed investigation of this subject in higher plants, has only been carried out within the last three years.

Central Role of Acetate in Lipid Metabolism

Acetate (or its biologically activated thioester form, acetyl-CoA) is the ultimate precursor for lipid synthesis in microalgae. This two carbon building block may arise within the cell through a number of important metabolic pathways. Likewise, it has a number of important metabolic fates. Some of the major pathways for formation and utilization of acetyl-CoA, which might operate in microalgae, are shown in Fig 1. Acetyl-CoA obviously occupies a central role in cellular metabolism. Many major cell activities including synthesis of lipids, some amino acids, carbohydrate via the glyoxylate cycle, and energy metabolism (TCA cycle) are all tied intimately to the availablility of acetyl-CoA. Conditions of cellular metabolism which promote the formation of acetyl-CoA can also be expected to promote its utilization by one or more of these potential pathways. The metabolic fate of acetyl-CoA is determined by the instantaneous requirements of the cell. Regulation of this process is potentially very complex, with factors such as allosteric enzyme regulation, genetic regulation of enzyme expression, metabolic compartmentation, and effects of environmental and growth conditions being important considerations.

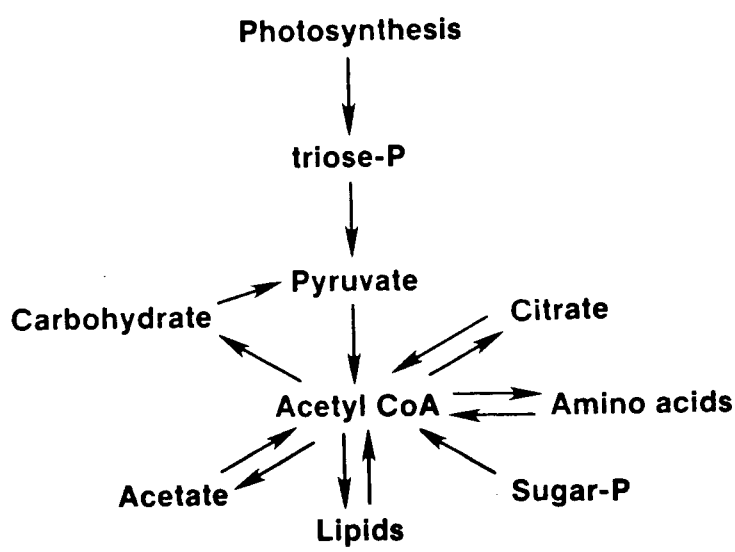

Figure 1. Potential pathways for formation and utilization of acetyl-CoA in microalgae

As a starting point for learning more about what controls carbon partitioning into lipids in microalgae, the question of acetyl-CoA availability can be addressed. Specifically, what is the metabolic origin of acetyl-CoA for lipid synthesis, and does this synthetic pathway change as a result of metabolic changes associated with lipid triggers.

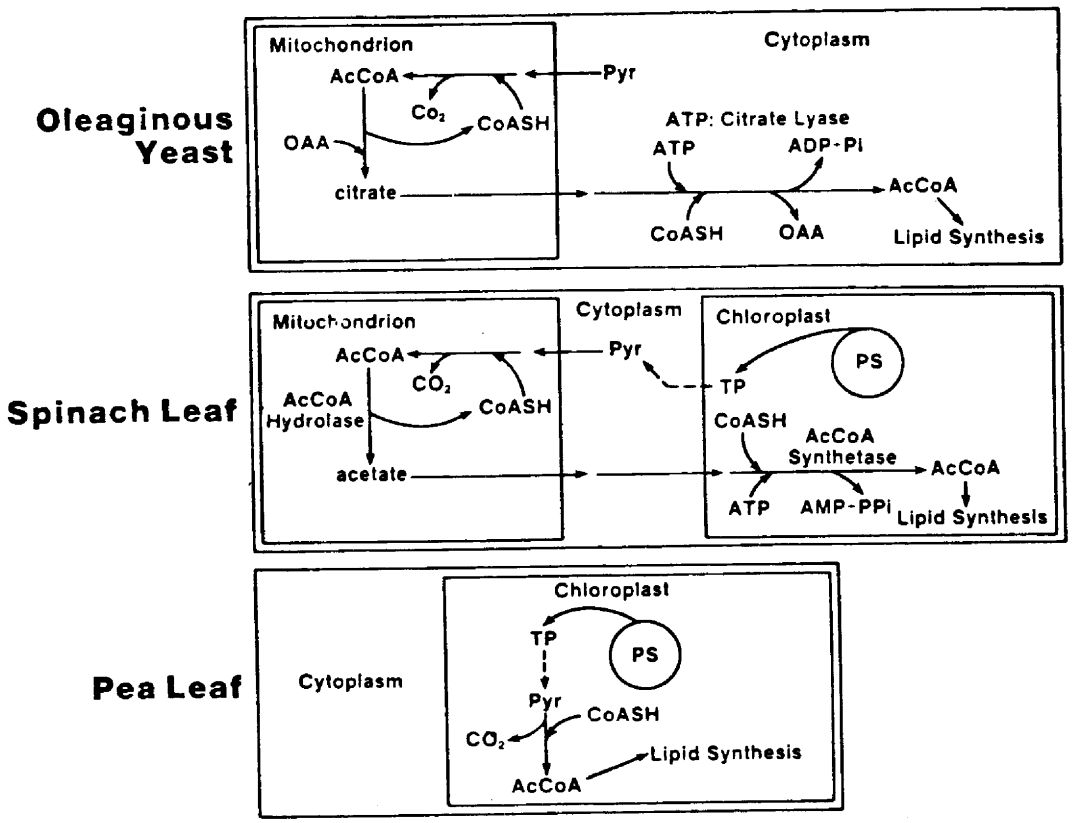

Figure 2. Proposed pathways for the generation of acetyl-CoA at the site of lipid synthesis in oleagenous yeast (a), spinach leaf (b), and pea leaf (c). Key enzymes catalyzing reactions in this figure are ATP:citrate lyase, mitochondrial acetyl-CoA hydrolase, and chloroplastic acetyl-CoA synthetase. Abbreviations are: pyruvate, Pyr; acetyl-CoA, AcCoA; free coenzyme A, CoASH; oxaloacetate, OAA; adenosine triphosphate, ATP; adenosine diphosphate, ADP; adenosine monophosphate, AMP; pyrophosphate, PPi; inorganic phosphate, Pi

A comparative biochemical examination reveals several potential pathways for acetyl-CoA production which might operate in microalgae. The top cell in Figure 2 represents the situation found in oleagenous yeast. The cytosolic enzyme, ATP:citrate lyase, catalyzes a key reaction in the pathway leading to synthesis of storage lipid under conditions of carbon or nitrogen-limited growth [1]. This enzyme has also been found in higher plant seeds undergoing active lipid accumulation [2,7]. The middle cell illustrates the reactions currently thought to occur in spinach leaf cells, in which shuttling of photoassimilated carbon from chloroplast to mitochondrion, and back to chloroplast, must occur before acetyl-CoA is made available for lipid synthesis within the chloroplast [6]. A key enzyme in this mechanism is mitochondrial acetyl-CoA hydrolase. This enzyme catalyzes the formation of free acetate, which can then pass freely through the mitochondrial membrane and into the chloroplast, where action by chloroplastic acetyl-CoA synthetase converts acetate into acetyl-CoA. These latter two enzymes are not required by the pathway represented in the top cell. The pathway currently thought to operate in pea leaf is illustrated in the bottom cell [3,4]. The primary feature of this pathway centers on the fact that photoassimilation, acetyl-CoA production, and lipid synthesis, all occur within the chloroplast. Enzymes such as ATP-citrate lyase, acetyl-CoA hydrolase, and acetyl-CoA synthetase are unnecessary in this metabolic scheme.

Laboratory Investigations

Materials and Methods

Euglena gracilis Z was grown photoautotrophically in defined media under continuous illumination. Heterotrophic cells were grown in the dark and supplimented with 1.8% glucose. Cells were harvested in late exponential growth. *Chlorella pyrenoidosa* was grown in nutrient sufficient media under two growth conditions; either batch or semi-continuous culture, under continuous illumination. Cells in batch culture were allowed to deplete their nutrients over several weeks before harvest. *Phaeodactylum tricornutum* was grown in nutrient sufficient media under continuous illumination in batch culture. A portion of the cells in exponential growth were transfered to nitrogen free media for three days before harvesting. In addition, another portion of cells in exponential growth were transferred to deaeriated media containing 10mM sodium acetate. The culture container was tightly stoppered and left for 48 hours at which time the cells were harvested.

The following procedure, with minor variation, was used to prepare cell extracts from all three organisms. All procedures were carried out at 4 degrees Centigrade. Cells were centrifuged (2000xg, 10 min.) and washed once in 10mM potassium phosphate buffer, 1mM dithiothreitol, pH 7.0. Cells were resuspended in the wash buffer and broken by two passages through a pre-cooled French press cell at 16,000 psi. The extent of cell disintegration was monitored by light microscopy. The cellular extract was centrifuged at 30,000xg for 20 min. and the pellet discarded. This produced a crude extract which was used for all enzyme assays. When extracts were being prepared for analysis of acetyl-CoA hydrolase activity, dithiothreitol

was not included in the extraction medium because of possible reaction with DTNB.

Spectrophotometric assays for ATP:citrate lyase (8), and acetyl-CoA hydrolase (5), were taken from the literature. The assay used for detection of acetyl-CoA synthetase activity was based on the formation of acetohydroxamate from newly formed acetyl-CoA (8).

Results

Laboratory studies in support of the proposed experimental plan for FY84 were begun on a very limited and preliminary basis in January 1984. Efforts to date have centered on determining the presence or absence of key enzymes which might be involved in pathways of acetyl-CoA formation in microalgae. Focus has been on analyzing whole cell extracts of selected species, grown under different growth conditions, for enzyme activities associated with ATP:citrate lyase, acetyl-CoA hydrolase, and acetyl-CoA synthetase. Table 1 gives the results of these very preliminary investigations.

Table 1[a]

	Euglena gracilis Z		Chlorella pyrenoidosa		Phaeodactylum tricornutum		
	Photoautotrophic	Heterotrophic[b]	Semi-continous Culture	Senescent[c] Batch Culture	Experimental Growth, Batch Culture	Nitrogen Limited Batch Culture	CO_2 Limited[d] Batch Culture
ATP: Citrate Lyase	-[e]	-	-	-	-	-	N.D.[f]
Acetyl Coenzyme A Hydrolase	N.D.	N.D.	-	+	-	-	N.D.
Acetyl Coenzyme A Synthetase	-	+	-	+	(-)[g]	-	+

[a] Results are very preliminary, pending reexamination using sensitive radiochemical analysis techniques
[b] Growth on glucose in the dark.
[c] CO_2 enriched culture; all other nutrients depleted.
[d] Culture supplemented with sodium acetate.
[e] Below level of detection by assay procedure employed
[f] Not determined.
[g] A small amount of activity detected, but below level of accurate determination.

Although it is inappropriate to reach any conclusions based on these data, certain interesting points emerge. It appears in some cases that enzyme expression may be tied to growth conditions while in others it is not. The

presence of acetyl-CoA hydrolase and acetyl-CoA synthetase in extracts of senescent Chlorella pyrenoidosa may indicate operation of a pathway similar to spinach leaf, while the absence of these enzymes in continuously cultured C. pyrenoidosa or exponentially grown Phaeodactylum tricornutum may represent a situation similar to pea leaf. The inability to demonstrate ATP:citrate lyase in any extract so far argues against operation of a system similar to oleagenous yeast in microalgae. However, this as well as other data, are subject to reexamination as assay conditions become refined.

Plans for Continuing and Future Research

Meeting the overall objectives of characterizing the mechanism and regulation of lipid triggers in microalgae, and developing improved triggers based upon this information, are beyond the scope of the current fiscal year. Even so, planned efforts for the remainder of this calender year should lay a substantial ground work towards this goal. These activities include:

1) Selection and culture of microalgal species which respond to various lipid triggers. Emphasis will be placed upon species showing promise towards fractionation of subcellular organelles.
2) Continued development of sensitive assays for organelle marker enzymes and key enzymes of lipid synthesis and acetate metabolism.

3) Comparative analysis of key enzyme activities in microalgal cells during states of active cell division, and active lipid accumulation.

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MICROALGAL TECHNOLOGY RESEARCH AT SERI: COMPONENT DEVELOPMENT

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INTRODUCTION

The long-term goal of fully automated outdoor cultures requires the use of an integrated monitoring and control system to maintain the culture conditions within specified limits to maximize yield.

This task involves the development of components that can ultimately be integrated into systems for the production of fuels and oils from microalgae. The ultimate objective is to improve the competitive postion of microalgal fuels and oils with other alternative energy sources. This task will support that effort by providing data acquisition and control systems for laboratory operations, developing the sensors required for continuous monitoring of culture condition and operations, and designing the software needed to integrate equipment, experimental operations, data processing and storage, and systems control.

Purpose and Objectives

The key objective of this effort is to develop components for monitoring cultures. Figure 1 shows the steps involved in achieving this objective. The algal test systems will require detailed monitoring of many parameters to establish an optimal growth environment and provide for a better understanding of their growth requirements. It will therefore be necessary to sense such parameters as light intensity, temperature, pH, salinity, nuturient concentration, population density and algal compositional characterisitics. A literature review and manufacturer's survey will be conducted to further define these monitioring requirements, and to identify those components that are commercially available. Most of these measurements can be made with existing sensors, which will be procured as needed. Additional sensor needs will be met by developing innovative sensing techniques. This process will involve theoretical design, prototype testing, and fabrication of the sensor components.

A computer and a data acquisition control system must also be obtained to receive and process information from the sensors, and to control the production environment of the microalgal systems in order to maximize yield. Commercially available systems will be selected for this purpose based on requirements such as analog to digital conversion capacities, precision, number of input and output channels, expandability, and ease-of-interfacing to the selected computer. The data acquisition and control system will be interfaced to the computer, and connected to the installed sensors. Software

Objective: Initial development of key components to monitor algal cultures.

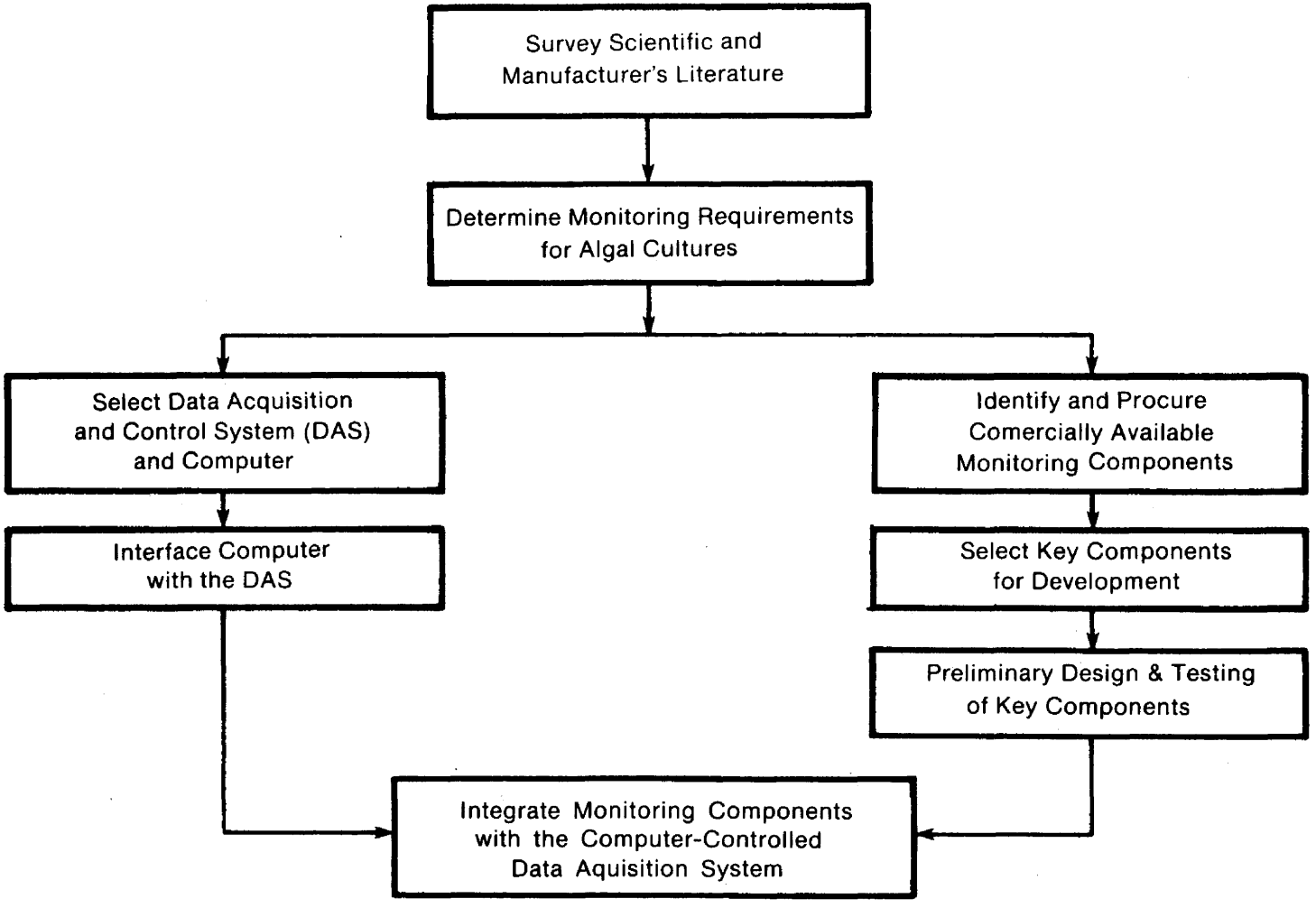

Figure 1. Component Development

Chemostats with Special Design for Photosynthetic Efficiency Measurements

will then be developed to direct the data acquisition and control process, interpret the data, and dictate appropriate control commands.

Current Status

The effort up to this point has been focused on the literature review, planning, and consulting manufacturer's information to select commercially available equipment. Some of the components that have already been acquired are listed below.

- IBM-PC (dual disk drive, 256K memory, graphics card) to interface with the data acquisition system and process data.
- Keithley/DAS data acquisition system 510 plus additional upgrade boards for a capacity of 24 input channels, and 16 output relays.
- Li-cor quantum sensors and underwater quantum sensors.

The Keithley/DAS system includes an interface card for the IBM-PC as well as measurement and control software to provide a relatively simple-to-use system. Both are expandable to allow for increased monitoring requirements.

Several other components have been designed and fabricated for use in the laboratory experiments. An innovative culture vessel was required for the chemostats, to provide a growth chamber that would be able to provide a controllable environment. The vessel (Figure 2) was designed to hold 1.5 liters, to have a water jacket for temperature control, to have optically flat windows for light attenuation measurements, and to allow for sensor probes to be inserted through a cork in the top. The vessels were then fabricated by a local glass blower.

The modulated light experiments required the measurement of the light flashing rate. An optically sensitive circuit was designed which displays the flashing rate on a meter and can be connected to the data acquisition system for future monitoring requirments.

Plans for Continuing and Future Research

This task will continue to provide engineering support to the experimental activities as required. The major effort, however, will be towards providing a complete culture monitoring and control system, as depicted in Figure 3.

The first component which has been selected for development work is a turbidity sensor, for continuous measurement of cell concentration in algal cultures. The turbidity sensor operation will be based on Beer's Law, which basically states that light transmission through a culture is a function of the culture concentration. Studies will also be initiated for the development of nutrient sensors.

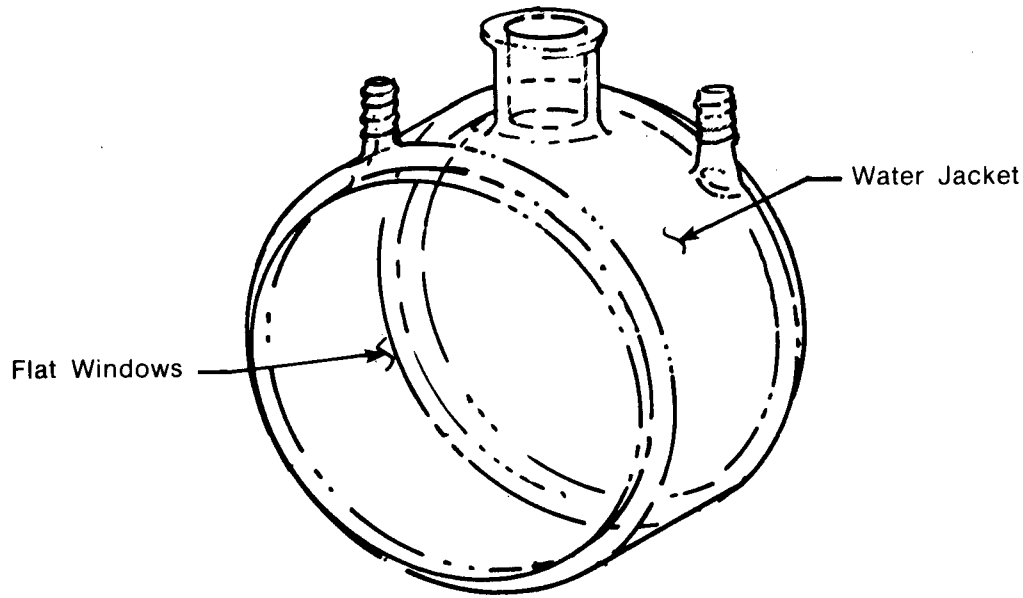

Figure 2. Chemostat.

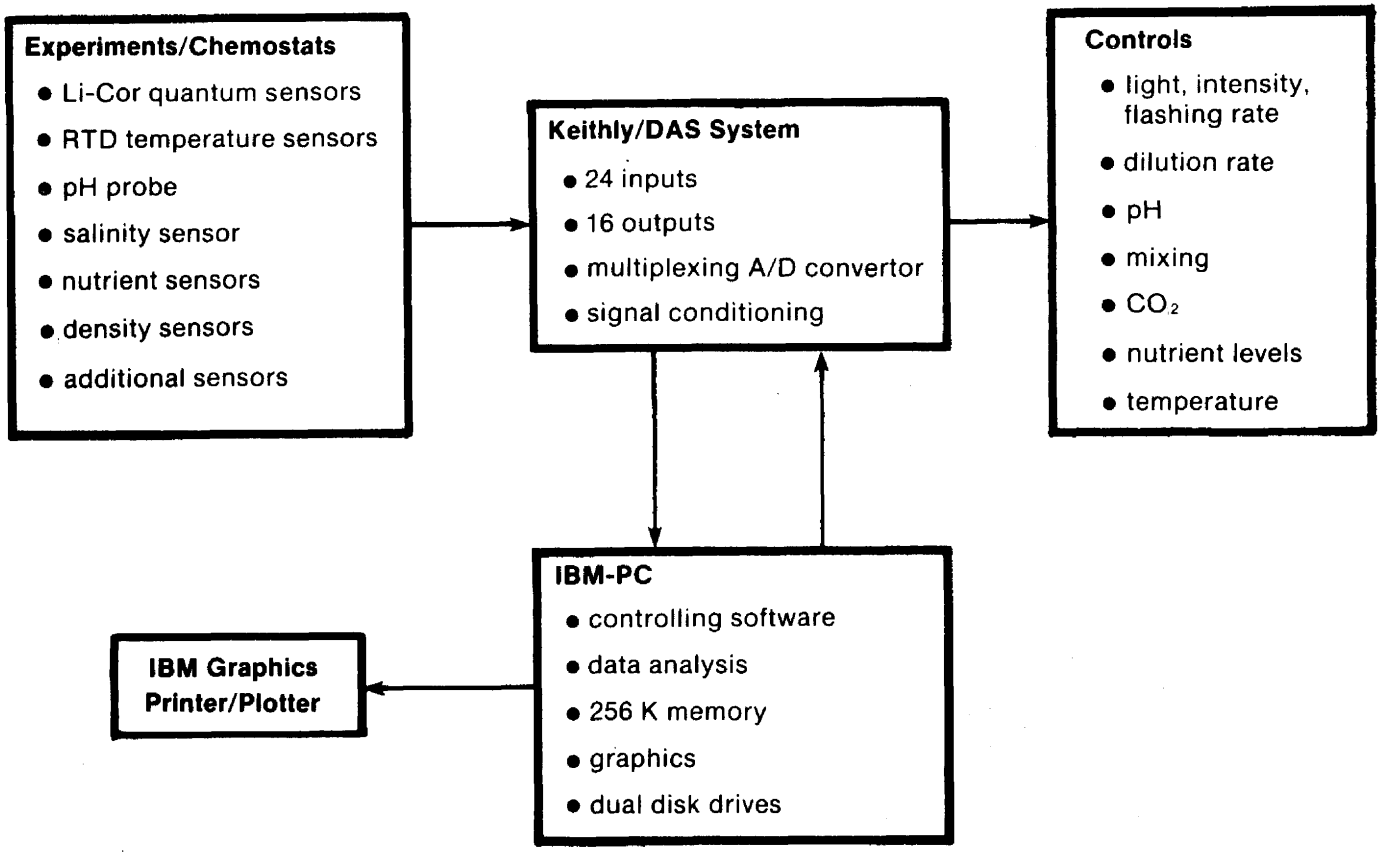

Figure 3. Integration of Monitoring Components.

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

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GENERAL OVERVIEW

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

The objectives of the first phase of the research are:-

a) to select lipid-producing halotolerant microalgae as test organisms

b) to identify facility design and nutrient requirements for cultivation of halotolerant microalgae

c) to incorporate the design features into an existing open raceway facilities for microalgae cultivation

d) to assess the harvesting system that provides efficient, low cost harvesting of microalgae

e) to select many species of halotolerant microalgae throughout Israel, mainly from the Mediterranean Sea;

f) to study the optimal conditions for their cultivation

g) to analyze the chemical composition of algae selected with an emphasis on their lipid quality and quantity.

We have started the project in June 1983. The first stage was a literature survey, especially that written in languages other than English. Two surveys were presented to SERI: 1) by Prof. Richmond and colleagues, on screening of growth conditions and physiology of halotolerant microalgae for mass cultivation: As a result of the literature survey three organisms were selected for further studies 2) by Prof. G. Shelef and colleagues on separation and processing methods of microalgae from pond effluent. Updated technologies for separation and processing of algae and other microorganisms were reviewed, and promising technologies are recommended.

STATE OF WORK

Dr. A. Ben Amotz has started screening and selecting Israeli microalgae, and is analyzing various lipid fractions. He is also studying the effect of some environmental conditions on lipid accumulation.

It should be mentioned again and emphasized that any attempt to select promising species of alga as potential producers of lipids or hydrocarbons for energy, based on the available published literature has to be concerned with 3 problems:

1) The quality and quantity of lipids (as well as other cell constituents) vary significantly with growth rate and culture age. In addition, environmental conditions, e.g. light intensity, temperature, and particularly nutritional conditions, exert a significant influence on lipid content. Comparison between different species has to be done under the same conditions. This is, however, rarely done 2) A unified classification system is not always available, thus causing significant confusion. For example, different strains of *Chlorella* are only marked *Chlorella*. 3) The capacity to grow as a unialgal culture in continuous operation throughout the year. This factor is most important for outdoor biomass production.

Based on the literature survey, three organisms were selected for further studies: *Isochrysis*, *Monolanthus* and *Chlorella*. They are considered as model organisms rather than a final recommendation. These model organisms will be used for studying productivity under outdoor conditions. Prof. A. Richmond and his group are optimizing growth conditions of these three organisms and already started their cultivation outdoors.

Prof. Shelef is studying one of the main problems in algal biomass production, i.e. harvesting of the algae. The term algae harvesting refers to the concentration of fairly dilute (0.02-0.06% TSS) algae suspension until a slurry or a paste containing 5-25% TSS is obtained. This aim can be achieved by two steps: a harvesting step, which brings the algal slurry to 2-7% TSS and a dewatering step yielding an algal paste of 15-25% TSS. It can also be achieved in a single step. The concentration of the resultant algal paste or slurry influences the next processing steps such as drying or organic extraction. The methods to be used for harvesting depend on the algal species, production system, and the nature of the final product. There is no single answer to the question which of the various methods and technologies of microalgae harvesting would be most suitable. The decision of the preferable harvesting technology depends on four variables: alga species (size, shape, mobility, oxygen release), growth medium (salts), algae concentration, and production cost benefit. Another basic criterion for selecting suitable harvesting technique is the final paste concentration required for the extraction.

The harvesting experiments are dealing with 2 model organisms: *Isochrysis* as a model for a flagellate, on which the major part of the work was performed, and *Chlorella* - an immobile alga

DEVELOPMENT OF OUTDOOR RACEWAY CAPABLE OF YIELDING OIL-RICH HALOTOLERANT MICROALGAE. IDENTIFICATION OF OIL-RICH STRAINS.

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SUMMARY

Ten species of unicellular algae from throughout Israel and other origins were cultivated under laboratory controlled conditions. The cultures were harvested semicontinuously with a yield of 0.4 to 0.5 g organic weight liter^{-1} day^{-1}. The proximate cellular composition of the unicellular algae and of seven Mediterranean macroalgae was measured with emphasis on the lipids. Botryococcus braunii and Nannochloropsis salina grown on low nitrate concentration contained the highest content of lipids, comprising about 50% of the algal weight. The lipid fraction of the other algae represented about 20% of the cellular composition. Aliphatic hydrocarbons isolated from the lipid fraction by hexane were found only in Botryococcus braunii. Nitrogen deficient Botryococcus braunii, Nannochloropsis salina, Isochrysis sp. and high salt cultivated Dunaliella bardawil consisted of relatively high concentrations of neutral lipids with multiple branched hydrocarbons predominating. The possible multiple relationship between photosynthetic carotenoid metabolism, environmental control and hydrocarbon biosynthesis will be discussed.

INTRODUCTION

In recent years algae have begun to be considered as a potential source for a variety of compounds of commercial application. Lewin [13] summarized the literature on the cellular composition of algae and focused his review on biochemical taxonomy. Aaronson et al. [1] have recently surveyed the literature with a general view on the potential use of algae for chemicals and valuable products. These two reviews emphasized the enormous potential existing in algae for the production of valuable products as an intrinsic part of the algal photosynthetic machinery. The production of lipids and hydrocarbons by a few algae and plants is of particular interest since this is the only photosynthetic catalytic reaction that converts carbon dioxide and water into oil with solar irradiation as the energy source [19,20].

Our study was devoted to collecting, isolating and screening a few algae from the Mediterranean area and other origins with an attempt to identify species rich in hydrocarbon. Similar attempts have been directed to enhancing physiologically the capacity of hydrocarbon production by a few selected species.

MATERIALS AND METHODS

Algae

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

TABLE 1. LIST OF ALGAE ANALYZED IN ISRAEL OCEANOGRAPHIC & LIMNOLOGICAL RESEARCH, HAIFA, ISRAEL

Cyanophyceae:	
Rhodophyceae:	Porphyridium sp., Pterocladia sp.
Cryptophyceae:	
Dinophyceae:	
Haptophyceae:	Isochrysis galbana, Isochrysis sp.
Chrysophyceae:	
Xantophyceae:	
Eustigmatophyceae:	Nannochloropsis salina
Phaeophyceae:	Halopteris sp., Padina sp., Sargassum sp., Taonia sp.
Prasinophyceae:	Asteromonas gracilis
Bacillariophyceae:	Cylindrotheca fusiformis, Navicula sp., Phaeodactylum sp.
Chlorophyceae:	Botryococcus braunii, Chlamydomonas sp., Chlorella stigmatophora, Codium sp., Dunaliella bardawil, Dunaliella salina, Ulva sp.
Euglenophyceae:	
Charophyceae:	

Growth Conditions

All algae except Botryococcus braunii, Dunaliella bardawil and the macroalgae were grown in enriched seawater containing 5 mM KNO_3 or as indicated, 0.2 mM KH_2PO_4, 1.5 μM $FeCl_3$, 30 μM EDTA, 2 mM $NaHCO_3$, 0.1 mM Na_2SiO_3, 0.1 mg/liter thiamine-HCl, 0.5 mg/liter biotin, 0.5 mg/liter B_{12} and trace metal mix as reported by Guillard [7]. Mixing and pH control was accomplished by bubbling 1% to 3% CO_2 in air at a pH of about 8. The growth system included a battery of 8 0.6-liter each glass chemostats. Constant temperature of 23 ± 0.1 °C was maintained by circulating water from a constant temperature bath through glass jackets on the outside of each culture unit. Radiant energy incident to the outer surface of all cultures was accomplished by Cool White and Agro-Lite fluorescent lamps (light intensity of about 20 W m^{-2}). Unicellular marine algae were cultivated and harvested semi-continuously. Batch cultures of Botryococcus braunii were grown in a fresh water medium as described in Tornabene et al. [18]. Dunaliella bardawil was grown in a seawater medium augmented with NaCl to 3 M and with 0.5 mM KNO_3, 0.2 mM KH_2PO_4, 1.5 μM $FeCl_2$ and 30 μM EDTA. Dunaliella bardawil was cultivated in open outdoor ponds with CO_2 to supply carbon and to control pH at 8.0.

Daily sampling for growth measurements was done on aliquots of the culture suspension and included cell counting microscopically and with Coulter Counter Model ZB; chlorophyll content was determined following extraction with acetone or methanol [8]; organic weight was determined by freeze drying samples for 24 hr and ashing at 600 °C. Macroalgae collected from the Mediterranean were lyophilized similarly prior to chemical analysis.

Extraction and Fractionation of Lipids

Total lipids were assayed by repeated extraction with methanol-chloroform-water (10:5:4, v/v) [5] modified as previously described [9] 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 [15,17]. The following types of components were eluted: acyclic hydrocarbons (hexane); cyclic hydrocarbons, polyunsaturated acyclic hydrocarbons, fatty acid methyl esters, sterols, 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 and acetic acid for sterols and sterol esters as previously described [10].

Analytical Method

Glycerol was determined by periodic oxidation followed by treatment with acetylacetone as previously described [3]. Protein was assayed as previously described by Lowery et al. [14] or by Kochert [11] 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 [12]. Extended hydrolysis of up to 8 hrs did not produce a detectable increase in the carbohydrate concentration.

RESULTS

Growth and Yield of Algae

The growth patterns and cellular yields of a few species grown semicontinuously are illustrated in Fig. 1.

Chlamydomonas sp. and *Phaeodactylum* sp. grew at the rate of about 0.4 g organic weight $liter^{-1}$ day^{-1}. Due to filtration difficulties of *Porphyridium* sp., the organic weight determination was calculated from the chlorophyll content and from cell number data yielding approximately 0.5 g $liter^{-1}$ day^{-1}. Assuming a depth of 10 cm, a 1 m^2 algal pond will yield about 18 g algal organic weight m^{-2} day^{-1}

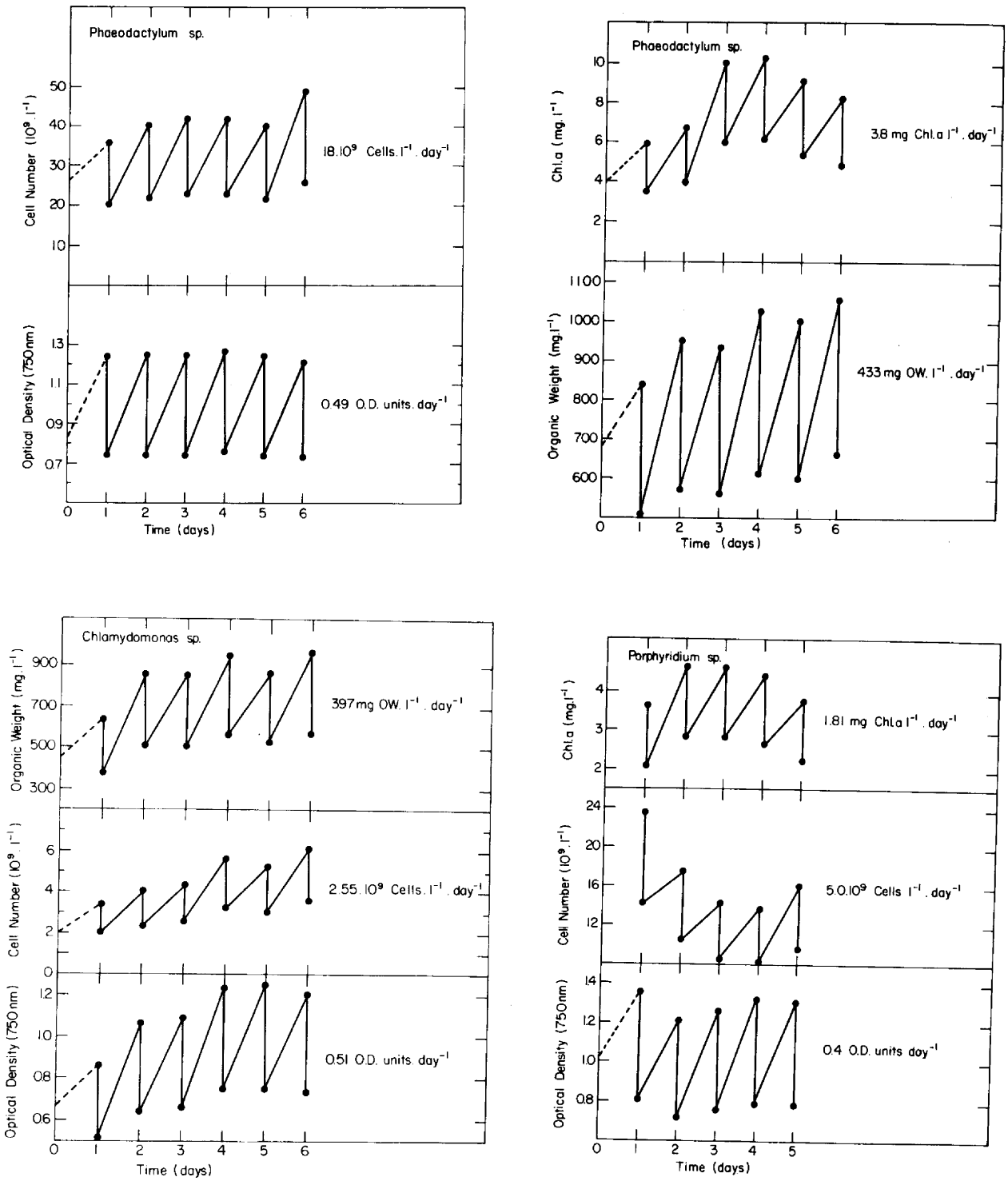

FIG. 1. SEMICONTINUOUS GROWTH OF CHLAMYDOMONAS SP., PHAEODACTYLUM SP. AND PORPHYRIDIUM SP. ALGAE WERE GROWN AS DESCRIBED UNDER MATERIALS AND METHODS.

under the indicated conditions and under a light intensity of 20 W m^{-2}. Calculated yields of the other algae tested were in the range of 10 to 20 g organic weight m^{-2} day^{-1}.

Proximate Cellular Composition

The results of the proximate chemistry analysis of the unicellular algae are summarized in Table 2. Most algae contained 20 to 30% protein, 10 to 25% carbohydrate and 15 to 25% lipid. Notable exceptions were very high carbohydrate content in *Chlamydomonas* sp. (59%) and in *Porphyridium* sp. (63%), high lipid content in nitrogen deficient *Botryococcus* sp. (45%) and in nitrogen deficient *Nannochloropsis* sp. (54%), high percentage of unknown cell constituent in *Isochrysis* sp., and high glycerol content as osmoticum in *Dunaliella bardawil* [2].

TABLE 2. PROXIMATE CELLULAR COMPOSITION OF UNICELLULAR ALGAE GROWN UNDER OPTIMAL OR NITRATE DEFICIENCY CONDITIONS

Species	Growth conditions	Protein	Carbo-hydrate	Lipid	Glycerol	Unknown
		% of organic weight				
Botryococcus braunii	FW, ND	19	9	45	-	27
Chlamydomonas sp.	SW, NE	17	59	23	-	1
Chlorella stigmatophora	SW, NE	31	21	20	-	29
Cylindrotheca fusiformis	SW, NE	30	12	15	-	33
Dunaliella bardawil	3 M NaCl, NE	30	24	17	25	4
Isochrysis sp.	SW, NE	21	14	20	-	45
Isochrysis sp.	SW, ND	12	25	19	-	44
Nannochloropsis salina	SW, NE	35	9	38	-	18
Nannochloropsis salina	SW, ND	23	7	54	-	16
Navicula sp.	SW, ND	30	12	23	-	35
Phaeodactylum sp.	SW, NE	33	28	21	-	18
Porphyridium sp.	SW, NE	20	63	17	-	-

FW - freshwater; SW - seawater; ND - nitrogen deficient; NE - nitrogen sufficient.

The results of the proximate chemistry analysis of a few macroalgae collected from the Mediterranean Sea are summarized in Table 3. All species contained low levels of lipids (2 to 12%) and a high level of unknown fraction, probably fibers. The protein and carbohydrate content in the macroalgae was equivalent to the published information [16].

TABLE 3. PROXIMATE CELLULAR COMPOSITION OF MACROALGAE COLLECTED FROM THE MEDITERRANEAN SEA.

Species	Protein	Carbo-hydrate	Lipid	Unknown
	% of organic weight			
Codium sp.	9.1	41.7	6.3	-
Halopteris sp.	14.5	18.5	7.6	59.4
Padina sp.	13.6	33.7	2.2	50.5
Pterocladia sp.	23.2	36.5	7.0	30.3
Sargassum sp.	16.1	12.6	2.6	68.6
Taonia sp.	14.3	19.5	7.5	58.7
Ulva sp.	20.0	24.4	11.9	43.7

Lipid Composition

The total lipid extracts of each alga were fractionated on silicic acid columns with hexane, benzene, chloroform, acetone and methanol. The distribution of the lipids is summarized in Table 4. High amounts of aliphatic hydrocarbons were found in the hexane fraction of Botryococcus braunii [6] and to a smaller extent in Nannochloropsis salina, Isochrysis sp. and Cylindrotheca fusiformis. The presence of these hydrocarbons was verified by TLC.

The benzene eluates contained the major fraction of neutral lipids of all species as well as the major fraction of the total lipids of Dunaliella bardawil, nitrogen deficient Nannochloropsis salina, nitrogen deficient Isochrysis sp. and nitrogen deficient Botryococcus braunii. The TLC distributions of the components comprising the benzene eluates (not shown) provided relative identification of the major spots: β-carotene in Dunaliella bardawil [4], oxygenated cyclic C-37 isoprenoid component in Isochrysis sp. [18], and a few unidentified neutral lipids with relative medium polarity in Nannochloropsis salina and in Botryococcus braunii.

TLC separations of the chloroform, acetone and methanol eluates identified the major spots in relation to authentic standards and by the aid of differential calorimetric stains. The chloroform eluate contained mainly tri-, di- and monoglycerides, sterols and free fatty acids. The acetone eluate contained mainly digalactosyldiglyceride, monogalactosyldiglyceride and pigments. The methanol eluate contained almost all of the phospholipids, the remaining glycolipids and the remaining pigments.

TABLE 4. FRACTIONATION OF ALGAL LIPIDS ON UNISIL COLUMNS

Species	Growth conditions	Total lipids, % organic weight	Lipid fraction, % total lipid weight				
			Hexane	Benzene	Chloro-form	Acetone	Methanol
Botryococcus braunii	FW, ND	44.5	18.8	31.7	24.9	11.5	13.1
Chlamydomonas sp.	SW, NE	22.8	0	0	0	85.6	14.4
Chlorella stigmatophora	SW, NE	20.1	0.1	2.1	9.7	82.7	5.4
Cylindrotheca fusiformis	SW, NE	24.5	1.4	18.8	6.0	43.6	30.2
Cylindrotheca fusiformis	SW, ND	20.0	1.9	5.5	2.2	48.3	42.1
Dunaliella bardawil	3 M NaCl, NE	17.0	1.2	50.0	24.3	10.9	13.6
Isochrysis sp.	SW, NE	28.1	1.5	15.2	13.5	31.6	38.2
Isochrysis sp.	SW, ND	29.3	2.5	35.6	12.7	28.0	21.2
Nannochloropsis salina	SW, NE	38.2	2.5	12.4	25.4	28.7	31.0
Nannochloropsis salina	SW, ND	54.3	4.0	40.2	35.5	16.0	4.3
Navicula sp.	SW, NE	23.1	0.4	4.6	20.5	50.2	24.3
Phaeodactylum sp.	SW, NE	21.2	0	5.0	26.0	41.8	27.2
Porphyridium sp.	SW, NE	17.5	0	0	15.7	48.7	35.6

Preparations of lipid eluates were determined gravimetrically. FW - freshwater; SW - seawater; ND - nitrogen deficient; NE - nitrogen sufficient.

DISCUSSION

The principal effort of this study was to screen a few strains of marine algae in an attempt to identify selected strains capable of producing a high content of hydrocarbon-oil. Selected strains may thereafter be manipulated to grow on different environmental conditions, and their hydrocarbon content can be maximized in relation to nutrient concentration, pH, salinity, temperature, etc. The oil-rich species identified in our study were nitrogen deficient Botryococcus braunii, Nannochloropsis salina, Isochrysis sp. and nitrogen sufficient Dunaliella bardawil. All these species contained a high content of neutral lipids, not predominantly straight chain saturated hydrocarbons, but rather multibranched and/or polyunsaturated components. These interesting lipids were found in the benzene eluates of the algae and most have not been identified yet. However, their behaviour on TLC, the inability to separate the fractions with gas chromatography, and preliminary analysis with mass spectrometry

showed that the benzene fraction is mainly composed of isoprenoid components. Nitrogen starvation clearly induced and enhanced isoprenoid biosynthesis in the indicated species, but it is probably not the only inducing factor. Nannochloropsis salina grown on a natural seawater medium was always enriched with a hydrocarbon fraction relative to Nannochloropsis salina grown on an artificial seawater medium. Enrichment of the hydrocarbon content in algae grown on natural seawater points to the presence of an unknown lipid trigger other than nitrogen starvation. Recently, Tornabene et al. [18] have speculated that the lipid components comprising the benzene eluate are intermediates, derivatives or homologues of the biosynthetic pathway of carotenoids. If that is the case, then multiple regulation sites on the carotenoid biosynthesis pathway may be affected and diverted to produce a variety of isoprenoid products. A comprehensive study of the metabolic pathway in algae capable of producing hydrocarbons may lead to optimization in photosynthetic production of the required oily end product.

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DEVELOPMENT OF OUTDOOR SYSTEM FOR PRODUCTION OF LIPID-RICH HALOTOLERANT MICROALGAE

A report for the period

June 1st, 1983 - March 31st, 1984

Edited by: Prof. Amos Richmond - Principal Investigator

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Introduction
Development of an outdoor system for production of lipid-rich halotolerant microalgae

A litrature survey was conducted in the first phase of our work, the objective of which was to screen reseach works on the growth and physiology of halotolerant microalgae so as to identify promising species suitable for commercial production on saline water of lipid-rich halotolerant algal mass.

Ten species which were considered to have a good potential for this purpose were identified, as follows: Chlorella sp. ; nannochloropsis salina (GSB) ; Isochrysis galbana ; Pheodactylum sp. ; Dunaliela sp. ; Nitzschia sp. ; Thalassiosira fluviatilis ; Prymnesium parvum and Chrysochromulina sp. .

From this group of microalgae, we suggested to foccus our attention first on three species - Isochrysis , Nannochloropsis and Chlorella , for the following reasons:

1 - Isochrysis sp. was chosen for:

a. In response to nitrogen deprivation, the lipid content in Isochrysis rises significantly.

b. There was evidence from preliminary observations that this species could grow rapidly as monoalgal cultures, yeilding high output.

2 - Nannochloropsis salina was chosen because of exceptionally high lipid content and because of evidence for a high output rate.

3 - Chlorella sp. was chosen for several reasons:

a. Many reports show that under nitrogen starvation, certain strains of Chlorella accumulate lipid to a very appreciable extent.

b. Chlorella is known to grow rapidly throughout the year, reaching record high output rates.

c. Chlorella can be readily cultivated as a monoalgal culture

Chapter 1
growth optimization studies
A. Isochrysis galbana

Isochrysis sp. is a marine uncellular free living motile phytoflagelate. Limited experience in growing this microalgae for feeding larvae of the American oyster proved very succesful.

The purpose of the present work on Isochrysis is to define optimal growth conditions for maximal production of biomass.

Isochrysis was grown using defined artificial sea water (ASW) in batch culture (100ml medium in 250ml erlenmayer flasks) on a shaker, under continuous light at 27^0 C. Under these conditions, cell number was dubbled in 42-30 hr., and reached maximal concentration of ca. 10^7 cells/ml.

1. Effect of temperature :

Optimal growth temperature, as well as high and low temperature limits for growth, were defined in cultures grown in a temperature gradient block ranging from 13 to 42^0C.

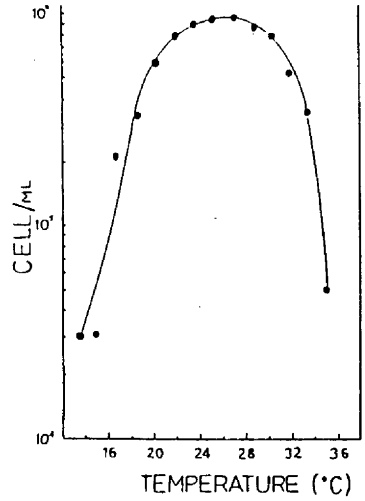

Fig. 1 : Effect of temperature on the growth of *Isochrysis sp.* .

Tubes containing 10ml of cell suspension were placed in the block and the growth rate as a function of temperature was followed by measuring the increase in cell number. Light intensity at the surface of the tubes placed was 30 μEinstein.m^{-2}. sec^{-1}.

The optimal temperature for the growth of *Isochrysis* was 28^0 C. At temperatures higher than 32^{0}C and lower than 19^{0}C, growth was arrested, and the alga died when temperature was kept at either 35^{0}C or 15^{0}C. (Fig. 1)

Effect of pH :

Different pH values were maintained in cultures grown in the presence of 42 mg/L $NaHCO_3$, by daily titration with HCl or NaOH. The pH range for optimal growth was 5.7-6.0. At higher pH, growth slowed down becoming totaly arrested at pH 9.0 (Fig. 2). At pH lower than 5.5, the growth rate steadily decreased. In the absence of $NaHCO_3$, the effect of the pH on growth was not as marked, yet the pH optima were not changed.

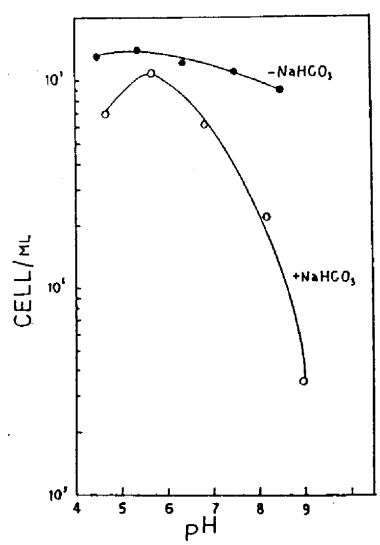

Fig. 2 : The effect of pH on the growth of *Isochrysis sp.*

Isochrysis was grown on defined artificial sea water (ASW) medium in batch cultures, (100ml medium in 250ml erlenmayer flasks placed in a rotary shaker), under continuous light of 150 $\mu E\ m^{-2}\ sec^{-1}$ at 27^{0}C. The pH was daily adjusted by addition of HCl or NaOH.

The effect of NaCl concentration :

The effect of various NaCl concentrations on the growth rate of *Isochrysis* under laboratory conditions was measured (Fig. 2).

This species was tolerant to a large range of NaCl concentrations. Growth decreased at a concentration of NaCl higher than 40g/l or lower than 5g/l, becoming totaly arrested at higher than 60g/l NaCl.

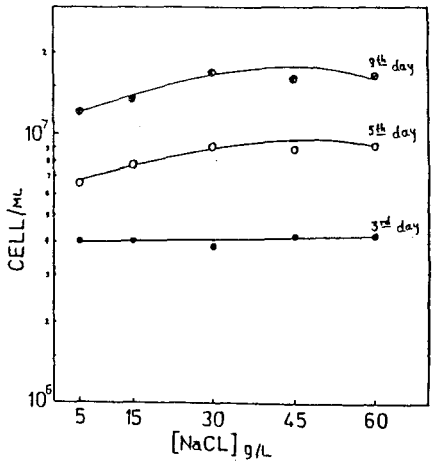

Fig. 3 : Effect of NaCl concentration on the growth of Isochrysis sp.

Nutritional studies :

Preliminary experiments revealed a marked response to addition of high concentration of Iron to the growth medium. In fact, a deceisive effect of added CO_2 on growth was exhibited only when the Fe^{+3} concentration was greatly enhanced (Fig. 4).

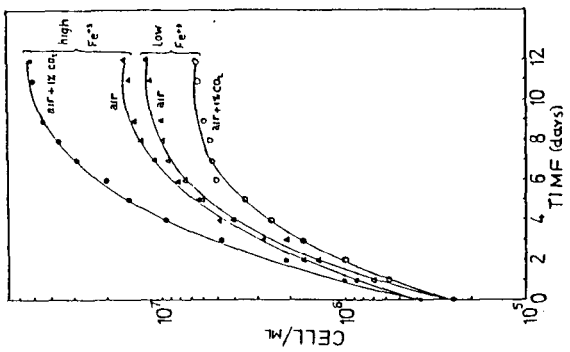

Fig. 4 : Effect of CO_2 and Fe on growth rate of Isochrysis sp.

Isochrysis was grown on ASW medium in batch cultures in 10L flasks at 27 ^{0}C, under continuous illumination of 300 µE m^{-2} sec^{-1} at the surface of the flasks. The Fe levels in the growth medium and the type of airation was as indicated in the figure.

The optimal growth conditions for Isochrysis sp. are summarized in Table 1.

Table 1: Optimal growth conditions for Isochrysis sp.

1. Temperature: 26-28^0 C.
2. pH: 5.5.-6.5 with KNO_3 as nitrogen source.

3. Nutrient medium:

macroelements g/l		microelements mg/L	
NaCl	32.0	$ZnCl_2$	0.020
$MgCl_2$ $7H_2O$	6.7	(NH_4) $M_{07}O_{24}.4H_2O$	0.185
$MgSO_4$ $6H_2O$	5.5	$CuCl_2$ $2H_2O$	0.170
$CaCl_2$	1.1	$MnCl_2$ $4H_2O$	0.200
* KNO_3	1.0	H_3BO_3	0.300
KH_2PO_4	0.07	$FeCl_3$ $6H_2O$	1.200
* or NH_4Cl	o.54	EDTA (dina salt)	0.500

vitamins mg/L	
Thiamine HCl	0.400
Biotin	0.100
B_{12}	0.010

Monoalgal cultures of Isochrysis sp. :

A test was developed to define the degree of possible contamination of the culture by chlorophytes and cyanobacteria (Chlorella sp., Chroococcus sp.). The test was based on the fact that Isochrysis, like other chrysophytes, contain fucoxanthin as a major xanthophyll.

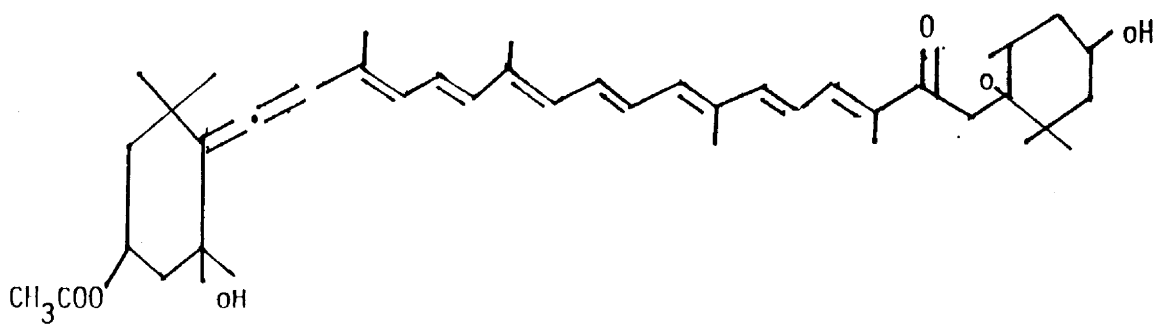

Fig. 5 : Fucoxunthin - $C_{42}H_{58}O_6$

When fucoxanthin is acidified and heated, the 5.6 expoxide undergoes isomerization to a 5,8 form with strong absorbance in the red. The sprectrum of total cartenoides isolated from Isochrysis galbana befor and after acidifying and heating, reveals that a shift in the peak from 445nm to 430nm takes place, typical for the rearangement of 5,6 expoxide to the 5,8 form. Absorbtion spectrum of the total pigments extracted from Isochrysis galbana is shown in Fig. 6. Upon acidification and heating, the shift at 445nm and a peak at 710nm appeared. These were missing when pigments extracted from Monallanthus sp. ; Scenedesmus obliquus ; Chroococcus sp. and Chlorella sp. were treated similarly.

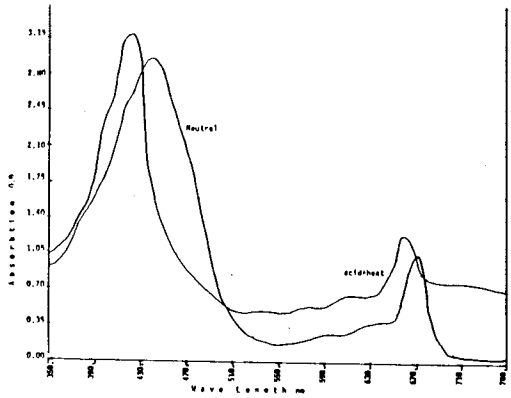

Fig. 6 : Absorbtion spectrum of pigments extracted from Isochrysis , after acidification and heating.

These changes in the ratio of absorbtion of chlorophyll a (670nm) to acidified and heated fucoxanthin (710nm) made it possible to set one component of a "purity profile" of Isochrysis - a task which is considered essential for the development of the biotechnology for its production. Of course, this criterion is not suitable to distinguish Isochrysis from other species containing fucoxanthin.

Chapter 2
Optimization of growth of Nannochloropsis salina grown under laboratory conditions

Cultures of Nannochloropsis salina were grown in glass tubes 40mm in diameter, carbon and stirring being provided by a stream of air+2.0% CO_2 which was bubbled through the culture at a rate of 10 lit. min^{-1} . Illumination was provided by cool white fluourescent light with intensity of 150$\mu E/m^{-2}/s^{-1}$ at 28°C. Under these conditions, generation time at log phase was 22h in a medium based on artificial sea water.

a. The growth of Nannochloropsis salina as a function of temperature.

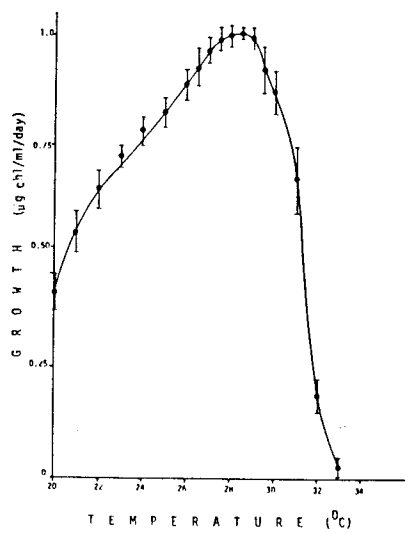

Fig. 7 : The effect of temperature on the growth of Nannochloropsis salina .

Nannochloropsis salina was grown in test tubes in a

temperature block on artificial sea water, containing (g/l): NaCl 27; $MgSO_4 \cdot 7H_2O$ 6.6; $MgCl_2 \cdot 6H_2O$ 5.6; $CaCl_2$ $2H_2O$ 1.5; KNO_3 1.0; KH_2PO_4 0.07; $NaHCO_3$ 0.04; $FeCl_3$ $4H_2O$ 2.4; NaEDTA 1.6 and the microelements (mg/l) ZnCl 40; H BO_3 600; $CoCl_3$ 1.5; $CuCl_2$ $2H_2O$ 40; $MnCl_2$ 400; $(NH_4)Mo_7O_{24} \cdot 4H_2O$ 370. Illumination was 150 $\mu E\ m^{-2}\ s^{-1}$ with cool white fluoures cent light and the pH kept of 7.6. Growth rate was measured in the logarithminc phase, error bars indicating data range.

b. Salt tolerance of Nannochloropsis salina

N. salina was grown on artificial sea water medium containing increasing amounts of NaCl in shaking batch cultures with 100 $\mu E.\ m^{-2}.\ sec^{-1}$ at 28°C. Maximal growth rate was obtained in 0.6M NaCl with higher or lower salinities affecting a marked decrease in growth (Fig. 8).

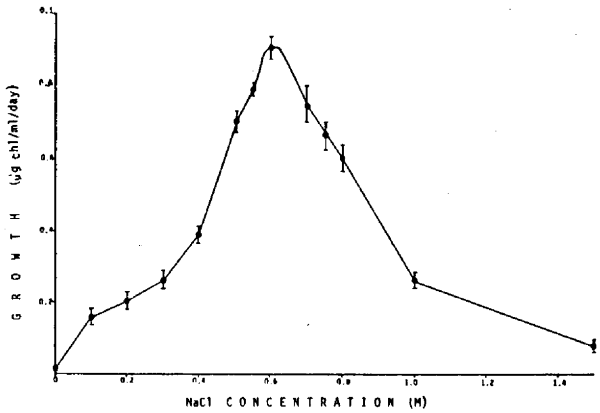

Fig. 8 : The effect of NaCl concentration on the growth of Nannochloropsis salina .

Nannochloropsis salina was grown on artificial sea water (see legend for Fig. 1) with the NaCl concentrations as indicated, on a rotary shaker (see legend for Fig. 2). Rate of growth was determined in the logarithmic phase, error bars indicating the range obtained in several replications.

c. Optimization of pH :

The growth response of Nannochloropsis salina to the pH is shown in Fig. 9.

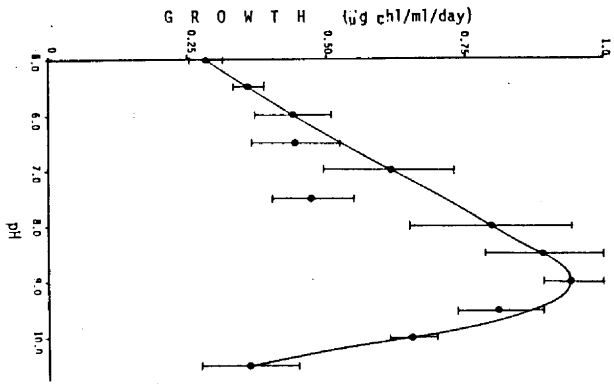

Fig. 9 : Growth of Nannochloropsis salina as a function of pH

Nannochloropsis salina cultures were grown on a rotary shaker at 180 r.p.m., being illuminated with cool white fluorescent light at 50 $\mu E.m^{-2}$ sec^{-1}. The pH of the artificial sea water medium (see Fig. 1) was adjusted twice daily, with 0.1 M NaOH or HCl. Growth rate was measured in the logarithmic phase. Error bars indicate data range.

Table 2: Summary of optimal growth conditions for Nannochloropsis salina under controlled conditions

Variable	Optimum	Range permiting growth
temperature	28 C	17 - 32 C
pH	9.0	5.0-10.5
NaCl Concentration	0.6M	0.1-1.0M
Nitrate concentration	10mM	3.0-100mM
Na-Bicarbonate	0.5mM	0.2-5mM

d. Maintaining monoalgal cultures :

N. salina is a small microalga, measuring 1μ in diameter. under regular light microscopy, it is very difficult to distinguish from small nondividing cells of Chlorella sp. which are suspected to be common contaminants in cultures of N. salina. Searching for distinguishing features, N. salina cells were found to fluoresce bright red with incident illumination of 450-490nm viewed with a filter transmitting light between 510-520nm. Under these conditions, the chloroplasts appear crescent shaped and flat in N. salina, occupying about 30-50% of the cell volume. Using phase and fluorescent microscopy, it is possible to readily distinguish between N. salina and Chlorella , diatoms, blue-green algae, and Porphyridium - all common contaminants in outdoor cultures of N. salina.

e. Outdoor cultures .

N. salina was cultured outdoors in 1m miniponds during the Fall of 1983, and in the following Spring (Fig. 10).

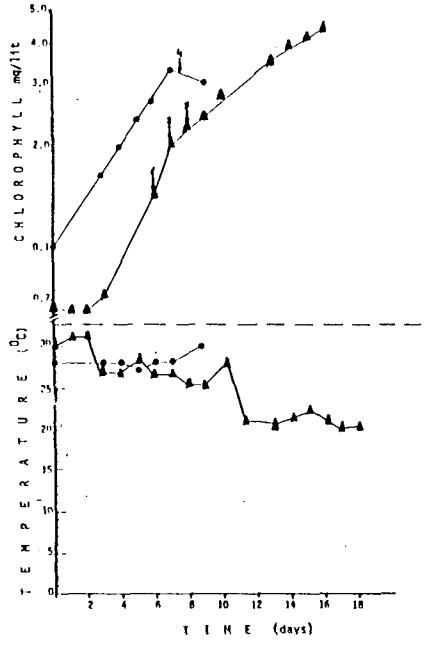

Fig. 10 : Growth of outdoor cultures of N. salina as related to the average daily temperature. ●— Fall 1983. ▲ — Spring 1984.

Arrows: 1 - culture diluted from 70 to 80 l.
2 - culture dilutde from 80 to 100 l.
3 - start of CO bubbling.
4 - failure of electricity - paddle not operating.

The ponds were glass covered in the Fall, and were uncovered but heated in the Spring. Under the cover, illumination was reduced by ca. 50% but the temperature was higher than anbient temperature by 6 to 10 C. In the spring, the pH was corrected each day for 9.0. Culture medium contained the macro and the microelements as detailed for Fig. 1. The salt content of the spring culture was eleveted to 0.6 M.

It was also attempted to grow N. salina in mid-winter. Growth was slow, but, initially, the culture remained remarkebly monoalgal. After nine days, however, a proliferation of contaminating algal species was evident, particularly of diatoms and chlorella-like spheres. We believe the decline of the culture grown in the open, unheated pond was due to the low temperature, and the proliferation of contaminating algal species seems to have reflected on the poor state of the culture.

Chapter 3

Screening Chlorella strains for high lipid production

Four Chlorella strains have benn tested so far, as follows:

1. Chlorella marina 211/27
2. Chlorella salina 211/25
3. Chlorella ovalis 211/21B
4. Chlorella sp. - isolated from a local source of alkaline brackish water.

The basic approach is to first screen a great number of strains for their potential of producing biomass of high lipid content without exerting special efforts, at this stage, in optimizing exact growth conditions and nutrients requirements for the tested types.

All strains were initially grown under the same conditions, i.e. - 30 C, enriched sea water, 2% CO_2 and 8000 Lux. Testing the temperature optima of the different strains revealed some differences, as shown in Fig. 11.

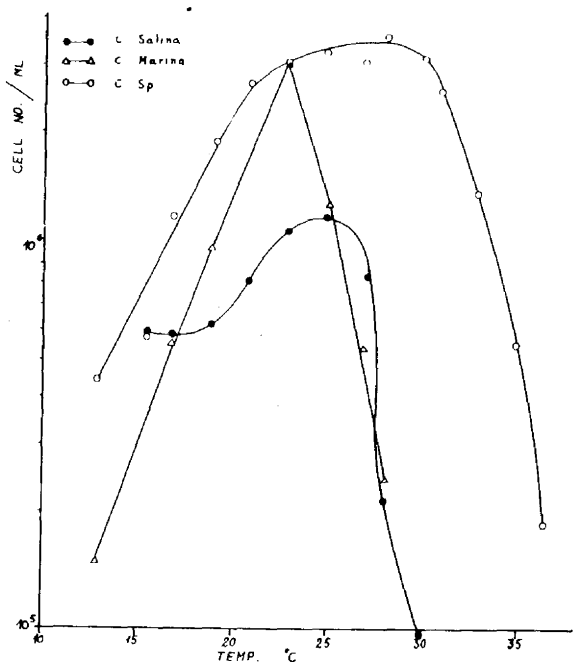

Fig. 11: The effect of temperature on the growth of C. salina, C. marina and C. sp.

2ml cultures of the different strains grown in ASW were incubated in a temperature gradient block, and illuminated from below by cool white fluorescent light. The cells were counted microscopically after 3 to 5 days of incubation.

To obtain the light saturation constans of two of the more promising Chlorella strains, the light response curve was dtermined (Fig. 12).

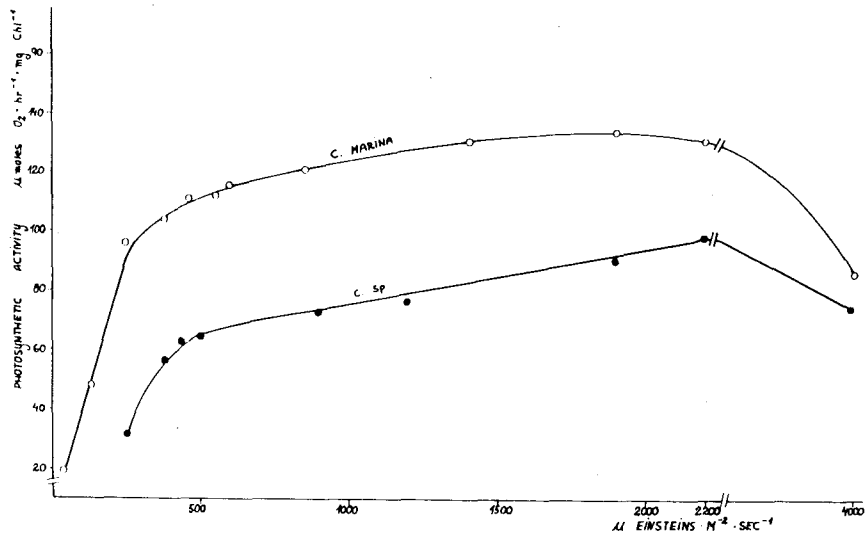

Fig. 12 : Light curves of C. marina o— and C. sp. •—. Cultures grown in enriched sea water were harvested and resuspend in fresh medium, containing 0.5mM of $NaHCO_3$. O_2 evolution was measured by clock type oxygen electrode in a termoregulated glass. Cells were kept at 25 C and light intensity was manipulated using natural density filters of different N.D.

Clearly, the two Chlorella strains exhibit a very good response to high light intensiities. Chlorella sp. in particular responded very well to increasing light intensity, and up to 2200 $\mu E.m^{-2}$ sec^{-1} , a radient flux which represents the peak of solar irradience during the summer, an increase in light intensity resulted in enhanced photosynthetic activity. Such a response to high light intensity makes this strain a promising candidate for outdoor production of biomass. This strain was isolated from our local Spirulina ponds and was compared to the marine species in its ability to grow in enriched sea water. Preliminary results suggest there was no significant effect of the salt concentration on the growth rate. Another reason why we believe this strain to be of particular potential for production of biomass relates to its ability to use bicarbonate and its proven tolerance to high pH. Under outdoor conditions, cultures growing at high pH medium buffer by the carbonate system would be advantegeous from stand point of carbon nutrition.

An additional advantage of this Chlorella strain is its lipid content, which was found to be 16% on dry weight basis. Since this lipid content was present prior to attempts to manipulate it by nutrient limitations, it is felt that this strain represents an interesting candidate for more detailed studies.

Chapter 4

Lipid determination

The procedure for determination of the lipid content was as foloows: washed and lyophylized algae samples were soxlet-extracted with chlorofom-methanol (2:1) for 2 hours. The solvent mixture was washed according to the Falch method, with 0.9% saline solution. The solvent was evaporated and the residue weighted.

A. Isochrysis :

Cultures in stationary phase were found to contain 18.5, 17.0 and 24.9% lipids (on dry weight basis), depending on growth conditions. Details of the experiment show that air-bubbled cultures contained the expected 19.6% lipids, while adding 1% CO_2 to the air stream resulted in increasing lipid content, up to 24.9%. In order to ascertain that no loss of volatile hydrocarbones occuured during lyophylization, the lipid extract of 2 samples was lyophylized under similar conditions, and the results showed that no more than 4% of the lipids in each sample was lost.

To further ascertain that no lipids were left after the methanol-chloroform extraction, the residue in some experiments was further extracted with chloroform-methanol-HCl (200:100:1) according to Dubinsky and Aaronson (Phytochem, 18:51-2, 1979) who claim to have recovered additional amounts of lipids using this treatment. The additional lipid that this treatment yeilded however was in the range of 1%, and even that could be partially atributed to solvent impurities.

Algae from iron deffecient culture were found to contain 13.4% lipids, in contrast to a control culture which contained under similar conditions, other than the iron content, 19.3% lipids.

B. Nannochloropsis salina

In the two cultures grown outdoors (covered and uncovered) the lipid content was 24.2%.

Following 3 days of withholding nitrogen, no significant effect on the lipid content could be resolved. In another experiment, different levels of several sources of nitrate nitrogen had no significant effect on the lipid content.

Thus under laboratory conditions, nitrogen deprivation in N. salina resulted in 25.8, 26.8, 26.5 and 25.3% of lipids, after 8, 14, and 21 days, respectively. Just recently, however, N. salina deprived of nitrogen and grown on sea water and not on ASW, was found to contain ca. 48% lipids.

C. The lipid content in Chlorella

Chlorella marina - 15.4% lipids
Chlorella salina - 15.5% lipids
Chlorella sp. - 16.0% lipids.

MARINE MICROALGAE SEPARATION AND HARVESTING

by

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1. INTRODUCTION

Important advances in mass culturing of microalgae on fresh water, wastewater and brackish water or saline media(seawater and or brines) have been accomplished in the past 35 years in terms of growth kinetics, culture reliability and biomass production rates. It is envisioned that further improvements in production rates (thus increasing solar conversion efficiency) and in species control to produce desirable products, can be expected. Still, in order to bring about the recognition of microalgae production as a mainstream biotechnological industry, optimal pond design and even more so, the economically feasible technology of separation,harvesting and processing of the algal biomass or its products should be established.

It is estimated that not less than a third and up to 80 percent of the total production costs of microalgal biomass or its products are due to harvesting and processing costs. The exact figure within this range depend on the size of the microalgae, its initial concentration, the type of growth media, the type and characteristics of the final products, etc.

Most microalgae solid concentration in the harvestable suspension in commercial scale outdoor facilities range between 0.015 (150 mg/l) and 0.06 percent (600mg/l). For most uses, the desired solids concentration of the biomass should be over 85 percent with the possible exception of liquid solvent extraction of lipids where solids concentration can be less but still over 25 percent, where higher solid concentration is more desirable. In-house uses such as wet-feeding are excluded from this consideration. Thus the solid concentrating factor from a photosynthetic pond to the final product is in the range of three orders of magnitude and can reach a factor of over 4000.

Concentrating the biomass solids involves liquid-solid separation (water constitutes the liquid in this case) in three major stages, namely: (a) removal of free water to a level of up to 10 percent solids; (b) removal of bound water (capillary, interstitial, etc). to levels of between 13 and 25 percent solids, and (c) removal of hydration and cellular waters up to full dryness or commercial dryness of over 85 percent solids. Since the cost for each percent of water removed is exponentially increased in each ascending stage, it is vastly uneconomical to "skip a stage". It will be very costly, for example, to dry slurry of two percent solids without applying thickening and dewatering techniques to produce a cake of at least 14 percent as it will be very expensive to directly centrifuge pond suspension containing 0.03 percent solids to produce a cake of 18 percent solids without first separating the biomass to produce a slurry of 4 to 8 percent solids.

The first stage of removing most of the free water from the pond suspension is therefore most essential as concentrating factors of between 100 and 300 are attainable at relatively reduces costs. Various techniques and considerations are shown and discussed in this paper, particularly with respect to micro-algae of smaller sizes that cannot be removed by vibrating screens and strainers. The attempt to separate marine microalgae by autoflocculation as well as by chemical flocculation followed by sedimentation and flotation is emphasized since the ionic strength of sea water greatly interefere with chemical coagulation and flocculation which have been commonly used in the separation of freshwater and wastewater borne microalgae. Experiments with other techniques such as ozonization and chlorination as well as sand filtration and microstraining are summarized and discussed.

2. EXPERIMENTAL WORK - CURRENT RESEARCH

The combination of a very small size and a marked surface electric charge of marine microalgae tend to keep the cells in stable suspensions, and questions the use of direct physical separation techniques such as filtration, sedimentation or flotation. Preliminary experiments showed that destabilization and flocculation of the marine microalgae Isochrysis galbana is an important and essential procedure in the separation and harvesting processes.

Organic polymers as well as inorganic flocculants were used in standard jar tests, in order to determine the algae flocculability potential and select the best flocculant for destabilization of I. galbana suspensions. The flocculation process in marine environment was found to be difficult, evidently due to high salt content of the culture medium, and the high motility of the marine microalgae I. galbana.

Therefore, part of the research was dedicated to elucidate the effects of marine media and algae cells motility on the flocculation process and to find out improved combined procedures in order to reduce the high inorganic flocculants demand. These procedures included the use of polymers in conjunction with inorganic flocculants and oxidation treatment prior to the flocculation process.

The results of the flocculation studies are being presently used in the selection of harvesting techniques and will be included in the forthcoming harvesting experimental program which will be based on bench scale experiments: Lamella settling separation, dissolved air flotation and microstraining.

2.1 Marine Microalgae Flocculation

2.1.1 Special Problems of Saline Media

The extreme chemical conditions of marine water (salinity upto 36 g/L, ionic strength of 0.7 and high concentrations of magnesium, calcium, and sulfate) impose special problems in the marine microalgae flocculation process. Studies of the salinity effects on microalgae flocculation are reported in this chapter and the nature of these effects is elucidated.

I. galbana cultures were grown batchwise in Thomas medium either under laboratory conditions or in outdoor ponds. Cultures of high cell densities were diluted with fresh media which contained different sea salt concentrations to obtain constant final cell density at different ionic strength conditions. These cultures were examined in flocculation tests using different flocculants (1).

Inorganic flocculants. Different ionic strength conditions were obtained by dissolving various amounts of sea salt in water. The ionic strength (I) of a given medium is calculated by the equation $I = \frac{1}{2}\Sigma CiZi^2$ where Ci is the molar concentration of the i-th ion and Zi its valency.

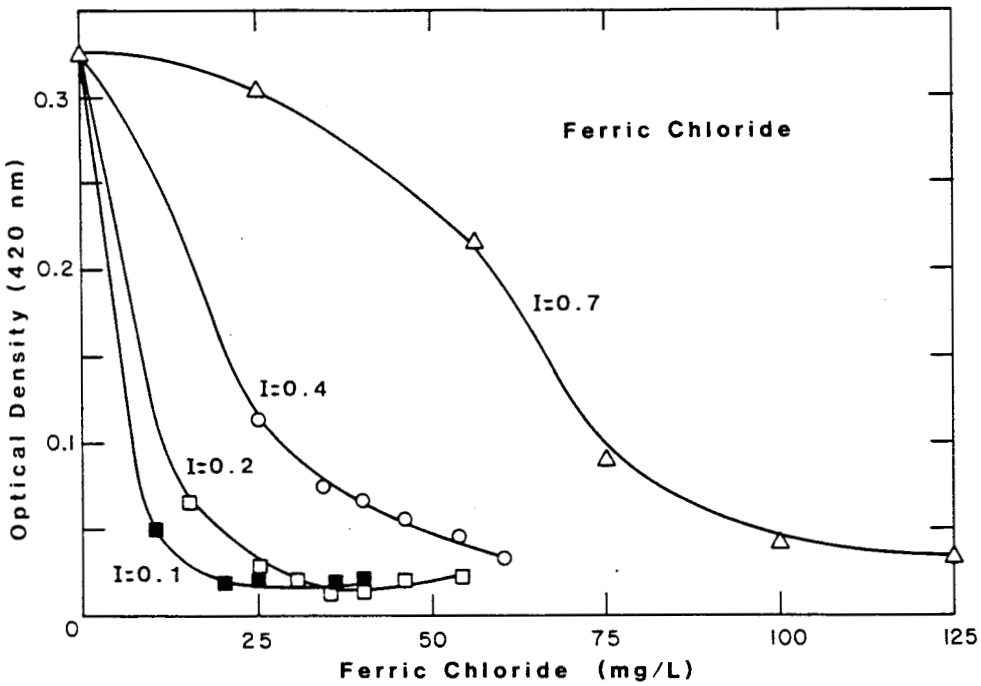
The effect of various ionic strength conditions (I) on I.galbana flocculation with alum ($Al_2(SO_4)_3 \cdot 18H_2O$) and ferric chloride ($FeCl_3$) at pH 5.5 is shown in Fig. 1. The dosages which are required for an optimal algae removal (final optical density below 0.03) at various ionic strength conditions are given in Table 1. Inorganic flocculants optimal dosages increase as the medium ionic strength increases (Table 1).

Table 1: The effect of various medium ionic strength conditions on optimal dosages for I. galbana flocculation by ferric chloride and alum

Medium Ionic Strength I	Optimal dosage for flocculation mM	
	Alum	Ferric Chloride
0.1	0.045	0.092
0.2	0.113	0.185
0.4	0.145	0.369
0.6	0.225	-
0.7	0.338	0.770

The algae flocs which are obtained by ferric chloride are much stronger and bigger than these obtained by alum. Ferric chloride gives yellow colour to the algae flocs and over dosages give such colour to the effluent. Ferric chloride and alum optimal dosages for I.galbana flocculation in sea water medium (I=0.7) are 0.77 mM and 0.34 mM respectively and are similar and even higher than the optimal dosages for wastewater systems where organic substances increase the flocculants dosages demand (2,3).

A linear relationship (r=0.968) was found between the medium ionic strength and alum optimal dosages for algae flocculation (Fig. 2). Activities of total aluminium ions were calculated for these alum optimal dosages and were exponentially related with the medium ionic strength (r=0.924). These results indicate that high salt concentrations inhibit the flocculation process by both reducing the chemical activity of the flocculant and by masking of its functional active sites. Consequently, the inorganic flocculant demands increase as the medium ionic strength is increased. The high flocculant demand of the marine system affect the quality of the algal final product or the processing technology (extraction, drying etc) and obviously will affect the overall cost of the algae separation, therefore some advanced methodology to reduce the flocculant dosage was considered and is reported later in this report.



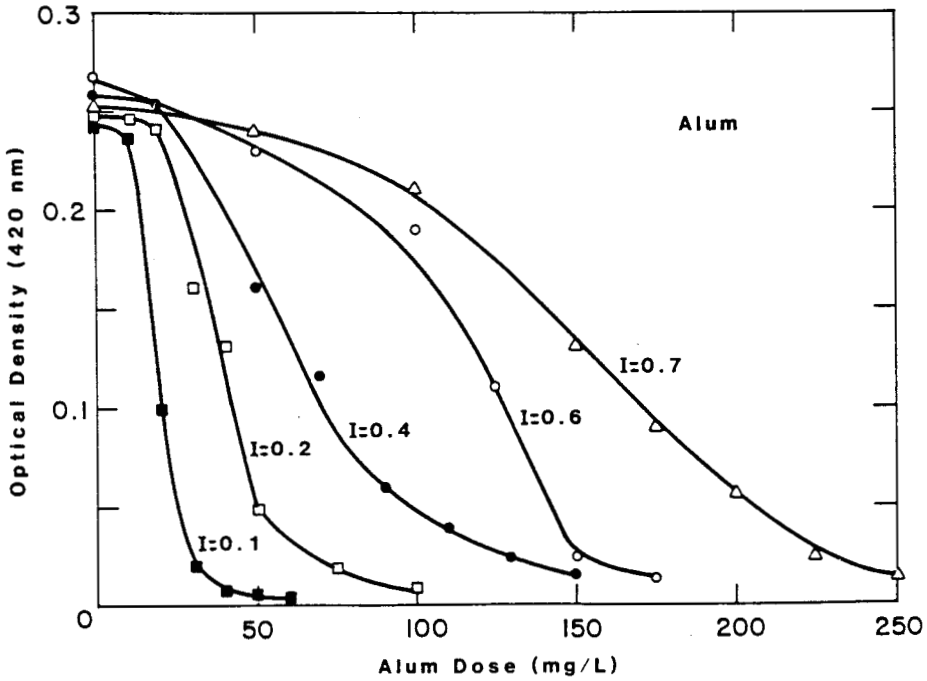


FIGURE 1: EFFECT OF MEDIUM IONIC STRENGTH ON *I. GALBANA* FLOCCULATION BY FERRIC CHLORIDE (ABOVE) AND ALUM (BELOW)

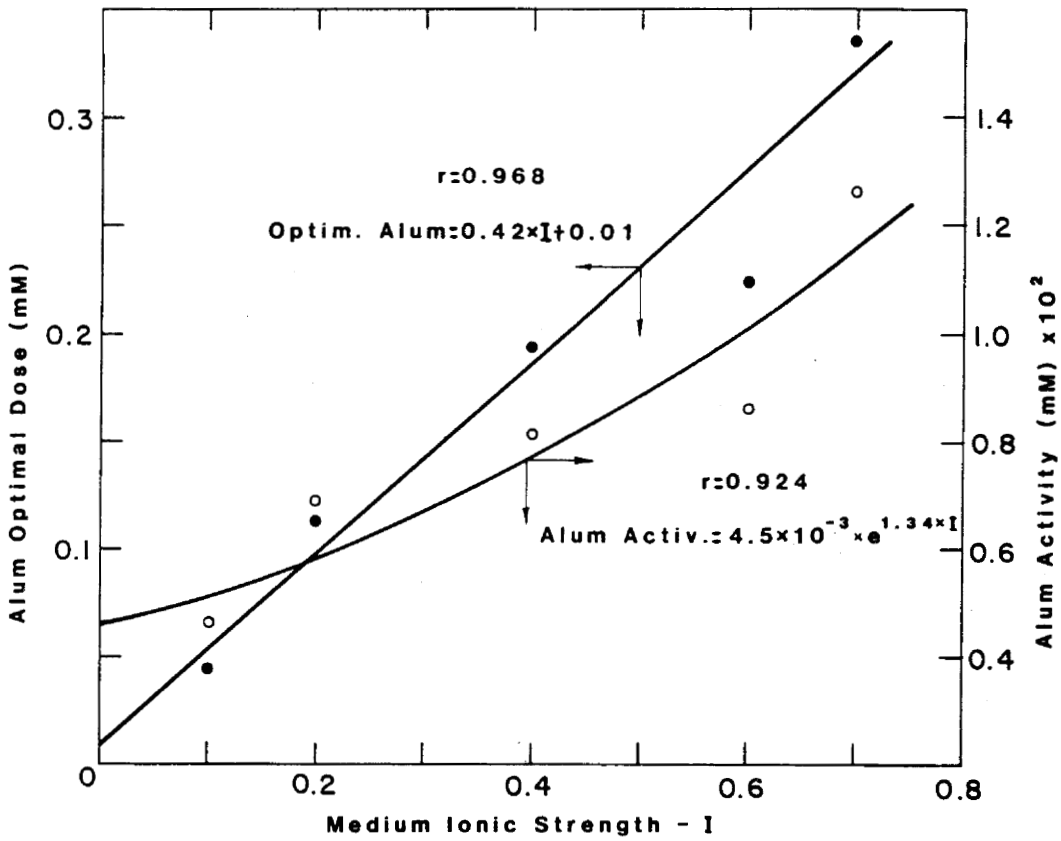


FIGURE 2: RELATIONSHIPS BETWEEN MEDIUM IONIC STRENGTH AND ALUM OPTIMAL DOSE FOR FLOCCULATION

Organic Polyelectrolytes. Several cationic polymers which were most efficient in fresh water, such as Zetag and Chitosan, were examined and found ineffective for flocculation of I. galbana in a marine system. Reduction of the sea salt concentration of the culture medium improved significantly the micro-algae flocculation. The effect of various sea salt concentrations on polymeric flocculation (Chitosan) is shown in Fig. 3a. Chitosan is effective as a flocculant only when the ionic strength of the culture medium is lower than 0.1. The intrinsic viscosity [η] of the chitosan at various ionic strength conditions were determined by Ostwald viscometer and are given in Fig. 3b. The intrinsic viscosity gives information about the chitosan polymer configuration. As the sea salt concentration increases (given as ionic strength) the intrinsic viscosity value decreases rapidly from 2000ml/gr to a constant value of 100 ml/gr. These changes of the intrinsic viscosity in conjunction with the flocculation tests results (Fig. 3) indicate that the polymer configuration is changed from extended linear configuration to a randomly coided polymer. These changes are evidently caused by masking and screening of the polymer functional groups by high salt concentrations. Consequently the polymer effectiveness in the flocculation is reduced and inhibited by high salinity.

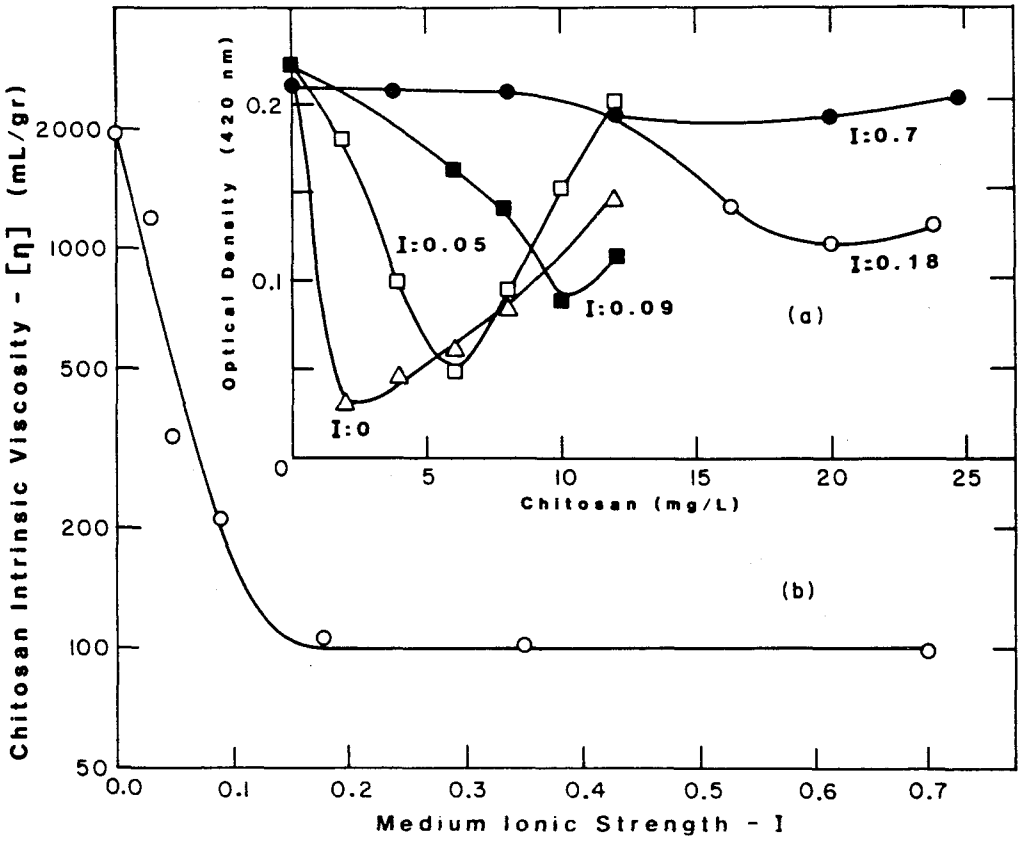


FIGURE 3: EFFECT OF MEDIUM IONIC STRENGTH ON I. GALBANA FLOCCULATION BY CHITOSAN POLYMER (A) AND ON CHITOSAN INTRINSIC VISCOSITY (B)

Autoflocculation. Finding the mechanism of autoflocculation and enhancing it in practical systems, thus reducing or eliminating the need of chemical flocculants has been long sought after. Algal autoflocculation process in freshwater system has been investigated to provide the baseline for further applications in higher salinity media (4,5). The spontaneous flocculation and rapid sedimentation of outdoor freshwater algae cultures is described in Figure 4. Algae were grown batchwise in outdoor ponds and carbon dioxide was added to the culture to keep a constant pH of 7.0. At the 8th day, carbon dioxide supply was stopped and the pond agitation ceased. The continuing photosynthetic carbon assimilation caused alkaline conditions which promote autoflocculation of the algae and subsequently rapid algal sedimentation (Figure 4). By using autoflocculation simulation system the phenomenon mechanism was elucidated and can be generally explained by algae biomass coprecipitation with calcium phosphate nuclei. The chemical precipitation is enhanced by the alkaline conditions which are obtained in intensively photosynthetic active algal cultures. The algae calcium phosphate interaction is explained as an electrostatic one (6).

Flocculation of marine I. galbana cultures in an autoflocculation simulation system was inhibited. The effect of various ionic strength conditions on microalgae removal by an autoflocculation simulation system is shown in Figure 5. Ionic strength conditions higher than 0.1 interfere with the

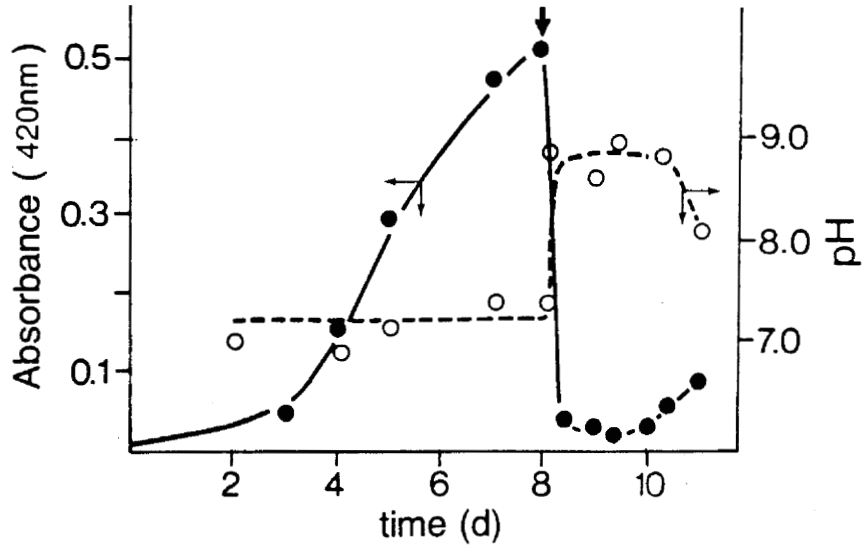


FIGURE 4: GROWTH AND AUTOFLOCCULATION OF FRESHWATER MICROALGAE IN OUTDOOR POND. (ARROW INDICATES POINT OF CEASING THE CO_2 SUPPLY AND POND AGITATION).

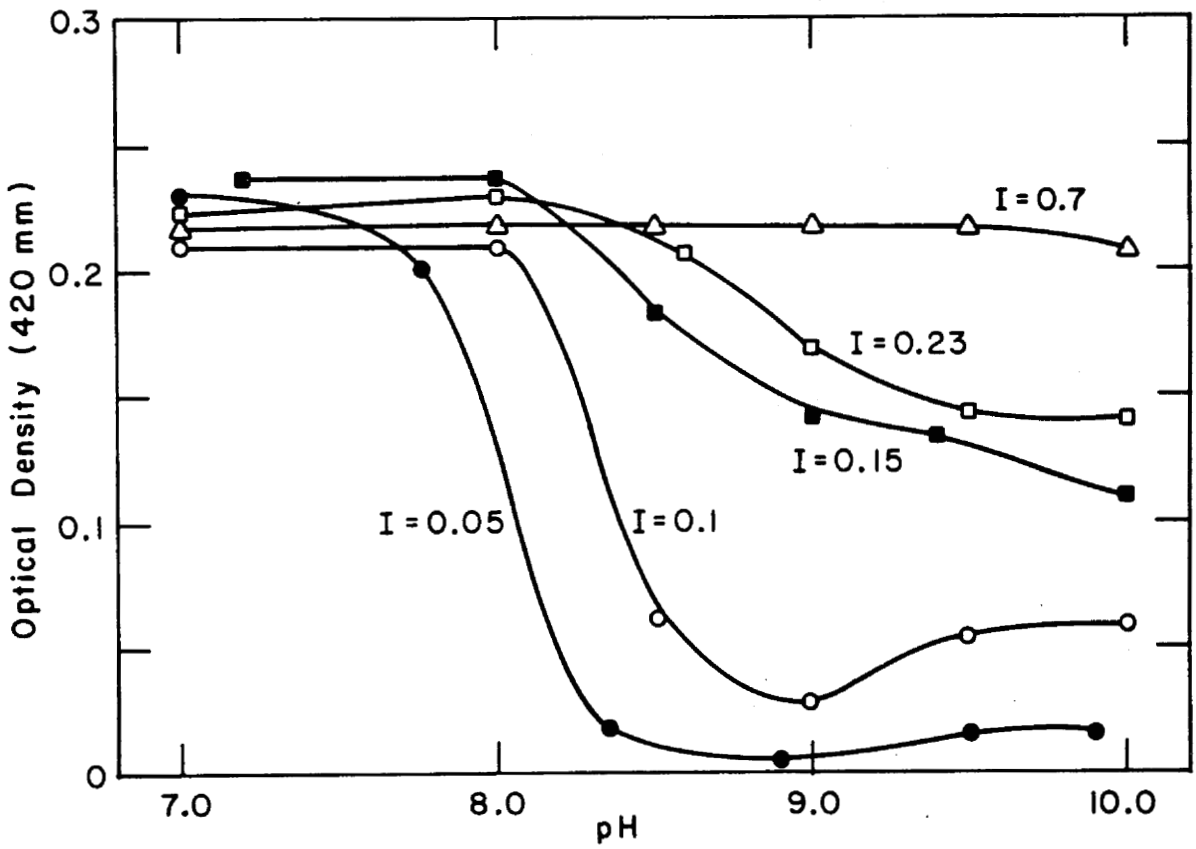


FIGURE 5: EFFECT OF VARIOUS IONIC STRENGTH CONDITIONS ON I. GALBANA FLOCCULATION IN AUTOFLOCCULATION SIMULATED SYSTEM

flocculation process in the autoflocculation simulation system. It is suggested that either calcium phosphate precipitation is inhibited by sea salt concentrations above 5 g/L or the precipitate surface electric charge is changed. Subsequently the electrostatic interaction between the algae biomass and the chemical precipitates is not obtained. The autoflocculation results indicate that this process is not applicable to a marine system. However, it should not be rejected out of hand as far as brackish water (up to 5 g/L salts) are considered as culture media. For marine systems, various modifications of the culture medium, as enrichment with phosphate salts, should be considered.

2.1.2 Combined Flocculation Process

In order to reduce the high inorganic flocculant demand of a marine microalgae system, combined flocculation processes were studied using two different approaches: 1) combination of polymeric flocculants with an inorganic flocculation process, 2) treatment of microalgae cultures with oxidants prior to the flocculation process.

Combination of Polymers in Inorganic Flocculation. When low concentrations of chitosan were used in conjunction with inorganic flocculant (ferric chloride or alum) the flocculation process was improved and the inorganic flocculant dosages were substantially reduced than when used alone. Figure 6 shows the effect of 2.5 mg/L chitosan addition to *I. galbana* culture on the flocculation with ferric chloride as compared with flocculation with ferric chloride alone. The ferric chloride dosage demand for 95% algae removal was reduced from 200 mg/L where no chitosan was added to only 50 mg/L when 2.5 mg/L chitosan was added to the culture prior to the addition of the inorganic flocculant (Figure 6).

It was found that higher chitosan concentrations upto 10 mg/L were somehow less effective than the lower chitosan dosage (2.5 mg/L), and that the order of the flocculants addition was of great importance. It is shown in Figure 7, that a removal of upto 95% of the algae was obtained when the chitosan was added to the flocculation process prior to the addition of 50 mg/L ferric chloride. When ferric chloride was added first chitosan addition did not improve the algae removal. Similar trend of the flocculation process improvement was obtained when chitosan was added prior to the addition of 25 mg/L ferric chloride (Figure 7).

These results suggest that polymers which are not effective for microalgae flocculation in marine system when used alone may be important as "flocculant aids" by evidently being adsorbed to the algae surface. When low amounts of polymer are used, its adsorbance reduces the algae surface electric charge, consequently improves inorganic flocculation process by both increasing the algae removal percentage and reducing the optimal inorganic flocculant

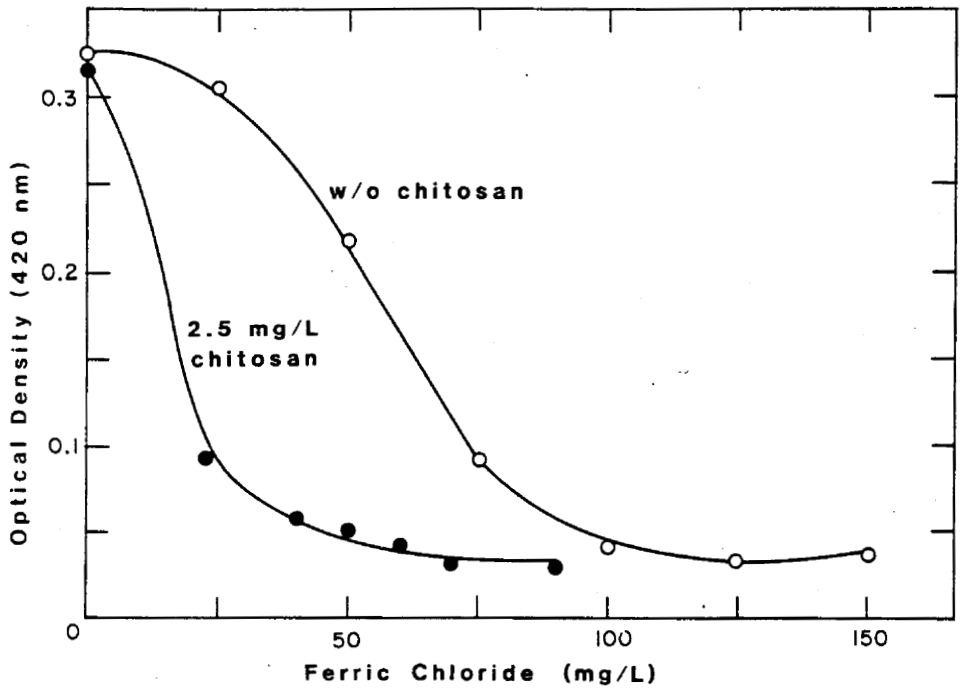


FIGURE 6: EFFECT OF CHITOSAN ADDITION ON I. GALBANA FLOCCULATION BY FERRIC CHLORIDE

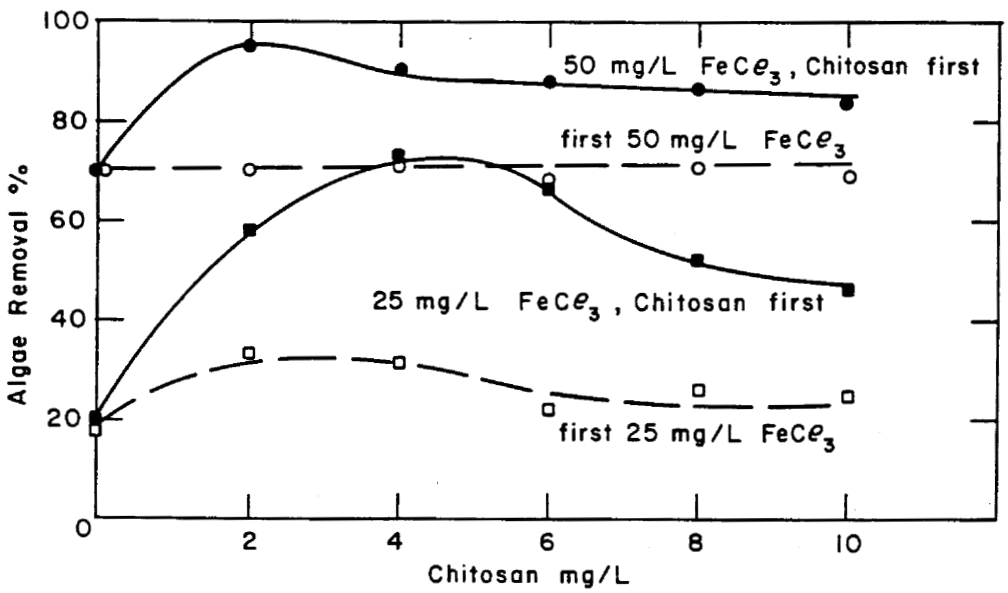


FIGURE 7: EFFECT OF POLYMER ADDITION ORDER ON I. GALBANA FLOCCULATION IN CONJUNCTION WITH FERRIC CHLORIDE

dosage. The superior results of the combined flocculation process should be included whenever microalgae harvesting techniques are considered for a marine system.

Oxidation Pretreatment. Due to the high motility of the microalgae I. galbana and the algae stability suspensions it was suggested that an oxidation pretreatment would reduce the algae motility, change its surface characteristics and improve its flocculability. Several oxidatns were used, namely: chlorine, hydrogen peroxide and ozone. The first two oxidants did not improve I. galbana flocculation, although, hydrogen peroxide at 25 mg/L stopped the algae motility. Pretreatment by ozone was most effective and improved the flocculation process by both increasing the algae removal efficiency and reducing the optimal flocculant dosage. Fig. 8 shows the effect of pretreatment by 2.5 and 4.5 mg O_3/L on I. galbana flocculation with ferric chloride. Optimal dosages of ferric chloride for the marine microalgae flocculation after treatment with different concentrations of ozone are given in Table 2.

TABLE 2: EFFECT OF OZONE PRETREATMENT ON FERRIC CHLORIDE OPTIMAL DOSAGE FOR FLOCCULATION OF I. GALBANA (pH = 7.0)

Ozone dose pretreatment mg O_3/L	Optimal dosage of $FeCl_3$ mM
0 (control)	1.4
2.5	0.3
4.5	0.3

Low ozone dosages reduced the optimal ferric chloride dose to 0.3mM as compared to 1.4mM, where no ozone was applied. Although the effect of low ozone doses on algae suspensions is still unclear, it is suggested that the ozone pretreatment causes some modifications of the algae surface characteristics. Some modification of the algae suspension light absorbance characteristics are caused by ozone, however, no severe leakage of cells cytoplasm to the culture media could be measured using organic carbon analyser (Table 3). The dissolved organic carbon (DOC) concentration in the culture media was increased by 8% after 5.4 mg O_3 were applied to a 200 mg/L I. galbana culture. Higher dosages of O_3 (10.5 mg O_3/L) reduced the DOC by 20%.

The results suggest that pretreatment by ozone is an effective alternative combined flocculation process which should be considered when microalgae harvesting techniques are to be selected. In addition, the effect of chlorine and other oxidation agents should be examined when new selected algae species are studied for their separability and harvestability.

TABLE 3: EFFECT OF OZONE TREATMENT ON THE DISSOLVED ORGANIC CARBON CONCENTRATION IN CULTURE MEDIUM OF I. GALBANA

Ozone dose mg O_3/liter	Dissolved organic carbon in culture medium mg C/l	Difference from control %
0 (control)	10.0	0
2.5	10.45	+4.5
5.4	10.8	+8.0
6.1	8.0	-20.0
10.5	7.8	-22.0

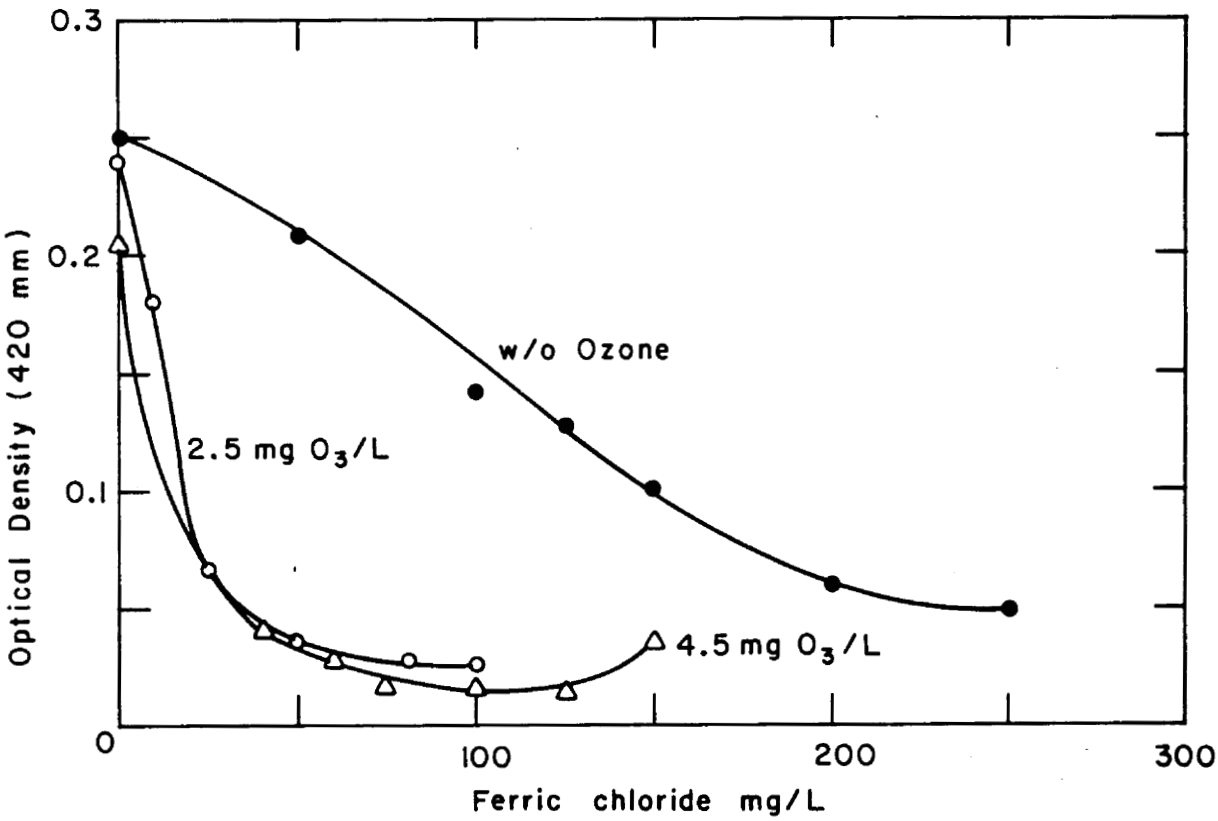


FIGURE 8: EFFECT OF OZONE PRETREATMENT ON THE IMPROVEMENT OF MARINE MICROALGAE FLOCCULATION BY FERRIC CHLORIDE

2.2 Microalgae Harvesting Techniques

2.2.1 Sedimentation

The marine microalgae I. galbana has a slow sedimentation rate and the sedimentation process is interefered by the algae motility. Therefore, a flocculation process is required in order to obtain algae separation and concentration within a reasonable and economical time and equipment. Our jar test experiments were based on sedimentation period not longer than ½ hr. When the residual optical density of cultures was not reduced by at least 85% after that sedimentation period, the whole process was considered as ineffective. Because of the small scale of the experiments we could not determine the algae concentration factor which was obtained by the flocculation sedimentation procedure. Our previous experiences (2) indicate that the conventional sedimentation techniques are inferior to flotation methods, however, we consider the construction and examination of lamella separator which will be combined with a flocculation equipment.

2.2.2 Flotation

Electroflotation. Rectifier and special electrodes were combined in a standard jar test apparatus in order to produce tiny hydrogen bubbles through water hydrolysis. This instrument was used in conjunction with flocculation experiments and the algae removal efficiencies were compared with parallel runs of flocculation sedimentation experiments.

The flocculation-flotation process was found superior to the sedimentation process in terms of algae removal efficiency only when large and strong flocs were obtained. Such flocs are obtainable with ferric chloride or with a combination of polymers with inorganic flocculants, including alum.

While performing electroflotation process of marine cultures, in addition to hydrogen, high amounts of chlorine is produced. Altough no effects of chlorine (up to 100 mg/L) on the I. galbana cultures were observed during short contact times (2 hr) chlorine is considered toxic to algae (7), and may impose technical complications when clarified algae free medium is recycled to the production unit. In addition ferric is oxidized during the hydrolysis process and the clarified effluent became greenish. Therefore dissolved air flotation (DAF) process seems to be most suitable for marine microalgae harvesting. This technique is much more complicated for operation in small bench scale equipment, however a small size (50L) continuous flocculation DAF unit is available and will be examined.

Ozone Flotation. Flotation of I. galbana by ozone treatment was characterized by foam production and algae concentration in to the foam. The flotation was operated batch wise in 3 L column which was fed with oxygen gas stream containing various ozone concentrations.

Figure 9 shows the relationships between the ozone concentrations in the inlet gas stream and the ozone doses demand for the beginning of algae separation from the liquid medium and that for the completion of the separation process. Doses of 10 to 15 mg O_3/L were required to start the separation process while the ozone dosages required to complete the separation process and to achieve clarity of the medium were depended on the ozone concentration in the inflow gas, and were up to 25 times higher than the doses required to start the separation (Fig. 9). These results are similar to those which were reported by Betzer et al (8) and the ozone flotation process seems to be a reliable one.

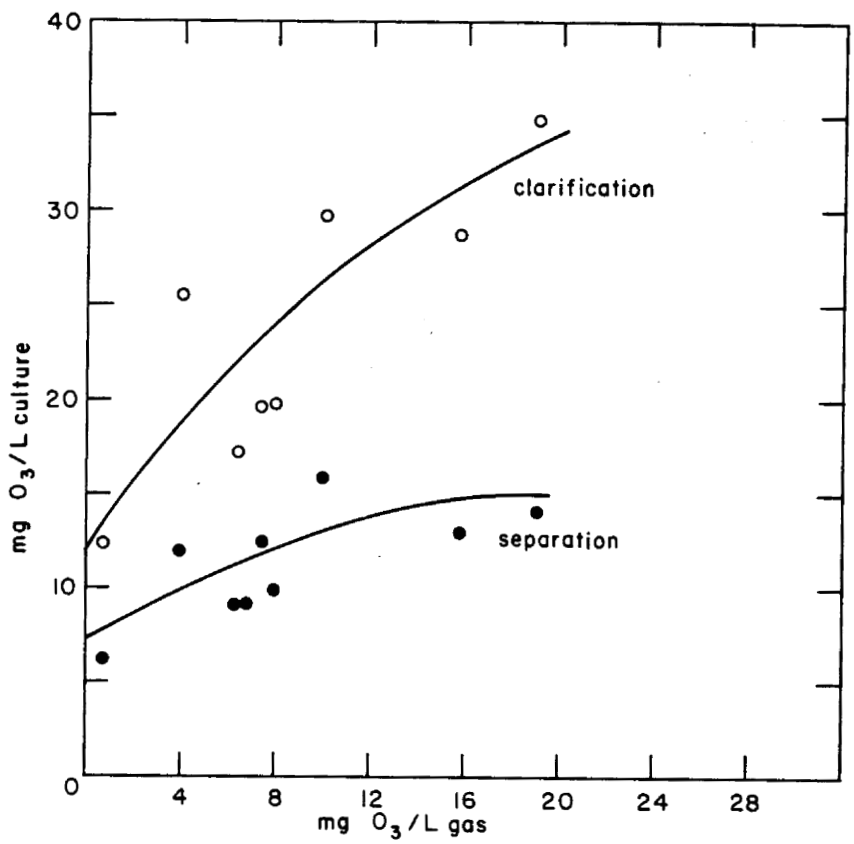


FIGURE 9: RELATIONSHIPS BETWEEN OZONE CONCENTRATION OF INLET GAS AND OZONE DOSE FOR MARINE MICROALGAE SEPARATION AND CULTURE CLARIFICATION.

2.2.3 Filtration

Filtrability of Isocrysis galbana was first studied using filter papers which showed that only GFA filter paper (particles size retention - 1.5μ) was able to remove most of the algae cells.

Filtrability of I. galbana was also studied using Filtester instrument (Mather & Platt Anti Pollution systems, London). The filtester is an instrument which simulates the microstraining process. Removal efficiency and the optimum grade of the microfabric can be established by analyses of filtrate samples from the instrument. Few types of different fine wave polyester mesh, 15μ, 6μ and 1μ were tested. Algae removal efficiencies were close to zero even with a mesh specified by the manufacturer to be of 1μ. A microspopic picture of the "1μ" polyester showed that a high percentage of the spaces enclosed by the microfabric threads were greater than 5μ.

Filtration experiments on I. galbana suspensions after flocculation, using fine wave polyester mesh are now in progress.

Sand Bed Filtration. The laboratory filter system is composed of two constant-head tanks, two filter beds, flowmeters and piezometers. The filters are made of 100cm long perspex column, with an inside diameter of 5.5cm. The constant head device is placed at a height of 150cm above the sand surface and this is approximately the gross available head for each experiment. A piezometer is adopted at the outlet of each filter. The clear water head is determined in the beginning of each run, and the head losses are calculated during the experiments as the difference between the clear water head and the head read at a specific time. Few types of different sand particles size were used as filter media in the experiments . In all the experiments, I. galbana suspensions of about 150 mg/L were used.

In the first stage of the experiments no flocculant was added to the algae suspension before filtration. In the second stage, $FeCl_3$ was used as the flocculant and was added to the algae suspensions in different concentrations.

I. Experiments without flocculation. Sand particles size of 0.55 and 0.70 mm were tested as filter media for a flow rate of 200 ml/min, filtration velocity of 5 m/h. Low algae removal efficiencies were achieved (less than 44% removal) and after 45 minutes the flow rate could not be maintained at 200 ml/min, in the filter media of 0.55 mm particles size (Table 4). Smaller sand particles size (<0.5) did not give the desired filtration velocity and the medium clogged after a very short run (less than 30 minutes).

II. Experiments with $FeCl_3$ as the flocculant. Few preliminary experiments were conducted using several doses of $FeCl_3$ and different size of sand particles (Table 5). Although the finer sand (0.55mm) gave very good clarification (89%) the filter clogged after 25 minutes and the desired flowrate could not be maintained after 15 minutes. Therefore it seems that coarse sand (>0.7mm a) and $FeCl_3$ dose of about half the dose required for flocculation-sedimentation/flotation (∿87mg/1) will give the best results although further improvement in suspended solids removal efficiency should be sought for. More filtration experiments on flocculated suspensions using the sand bed filter are now in progress.

TABLE 4: SAND BED FILTRATION RESULTS WITHOUT FLOCCULANT

Sand particles (mm)	Flowrate (ml/min)	TIME (minutes)	Algae removal (%)
0.55	200	0	24
	200	20	44
	200	30	42
	200	40	40
	180	48	37
	180	68	34
	150	80	36
	150	100	36
0.70	200	10	29
	200	20	20
	200	30	22
	200	40	23
	200	50	23
	200	60	23
	200	70	23
	200	80	23
	200	90	18
	200	105	14
	200	120	14

TABLE 5: SAND BED FILTRATION RESULTS WITH FLOCCULANT ($FeCl_3$)

$FeCl_3$ concentration (mg/l)	Sand particles size (mm)	Flowrate (ml/min)	Time (minutes)	Algae removal (%)
175	0.55	200	5	89
		200	10	89
		180	15	89
		100	20	89
		33	25	91
87.5	0.7	200	5	15
		200	15	25
		200	25	42
		200	30	49
		180	35	53
		170	40	53
		150	45	38
		100	60	35
44	0.7	200	5	15
		200	10	18
		200	20	21
		200	30	21
		200	40	23
		200	50	21
		200	60	21

SUMMARY

Marine microalgae separation imposes special problems when autoflocculation or chemical flocculation are considered due to seawater high ionic strength which masks active sites and charges both at the algal surface and binding agents.

The microalgae Isochrysis galbana which was selected as a test organism is of small size and is motile which bring about its separation as a challenging endeavor.

Following this research, two venues of destabilization of the marine algal suspension have been selected: (a) flocculation with a combination of organic polymer followed by inorganic flocculant, and (b) pretreatment by low ozone dosage followed by inorganic flocculant.

Autoflocculation is quite problematic because of the high ionic strength of seawater but conditions for its enhancement might be found.

Microstraining and sand filtration should also be considered, particularly for larger size microalgae or in combination with flocculation.

FORTHCOMING PROGRAM

Once the optimal flocculants combination (polymers + $FeCl_3$ or alum), their optimal dosages and ozone pretreatment are established, the program will focus on establishing design and operation parameters of dissolved air flotation and lamella type separation to perform the harvesting in pilot-plant units. The use of microstraining in followed flocculation will be examined as an alternative. The efficiency of harvestability of various marine and brackish water species besides I. galbana will be tested at both the laboratory scale and pilot plant scale units.

Algal harvestability, the cost of flocculants and ozone the separation efficiency and the concentrating factor will be weighted for the final selection of the recommended process for marine and brackish water algae.

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ANALYSIS AND EVALUATION OF THE ECONOMICS OF MICROALGAL FUEL PRODUCTION

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Objective

The overall objective of the task "Economic Evaluation of Microalgae to Fuel Production" is to develop an analytic framework for systematic evaluation of microalgae production. This analytical framework consists of modeling the production and conversion of microalgae into fuels and developing product costs based upon plant investment costs and operating expenses.

Accomplishments

Task activity over the last year has been the development of technology and market cost goals for microalgae to fuel technology. A comprehensive discussion of the methodology and preliminary estimates of these cost goals can be found in reports by Neenan and Hill (1982) and Neenan (1983). This paper presents current cost goals estimates for the microalgae program resulting from refinements in the methodology and input assumptions since the last published reports. A complete description of the methodology is presented, in addition to input assumptions, to permit outside evaluation and comment.

Program Milestones

Program milestones for the Aquatic Species Program were established in the multiyear program plan. Table 1 presents a summary of the projected achievements and schedule.

Table 1. Outyear Milestones for the Aquatic Species Program

Program Accomplishment	Year
Current year for planning (t_0)	1983
Component technical feasibility (t_{CTF})	1995
System feasibility (t_{SF})	1998

Of particular interest is that the ASP is based on a 15-year research and development schedule. By 1996, a substantial portion of the program will be devoted to technology transfer to the private sector. The three years between t_{CTF} and t_{SF} are for data validation and dissemination with cost-sharing demonstration projects. After 1998, the year of commercial readiness, the program will be completed according to assumed research progress and achievements and in accordance with overall program goals and objectives.

Market Cost Goal

The goal of the Aquatic Species Program is to develop the technology base for obtaining renewable liquid fuels from microalgae. Oil prices are the appropriate market criteria for measuring program success. Lipid oils from microalgae are a mixture of triglycerides and hydrocarbons which must be further refined to the final fuel products. Lipid oils from microalgae are assumed to be a perfect substitute for wellhead, crude oil; therefore, projected wellhead, crude oil prices are used to establish the market cost goal.

Figure 1 graphically illustrates oil price projections from government sources used to establish the competing crude oil price. The DOE Energy Information Administration's (EIA) most recent projections, presented in Table 2, were the basis for program cost goals. All monetary values are in 1983 dollars, developed by inflating the EIA 1982 dollar forecasts by 4.6%, according to the gross national project deflator index estimated by the Council of Economic Advisors (Economic Report of the President, Washington, DC, 1983).

The market cost goals to be met by 1998, corresonding to high, middle, and low price market forecast scenarios, are $82.0, $60.0, and $42.0 per barrel, respectively. As indicated in Figure 1, the real cost of crude petroleum is projected to decline through the mid-1980s, reflecting increased supply of crude petroleum and sluggish demand. However, toward the end of the decade, a real increase in oil prices is anticipated, continuing to the end of the forecast period.

The price forecasts in Table 2 represent the market constraints imposed on microalgae production technology. While specific production conditions required for cost competitiveness with conventional petroleum sources are not defined, the market cost goal imposes product cost constraints independent of the technology.

Table 2. World Oil Price Projections, 1983 to 2000[a]
(1983 dollars per barrel)

Year	Oil Price Scenario		
	Low	Middle	High
1983	29.0	31.0	33.0
1985	32.0	26.0	36.0
1990	29.0	39.0	50.0
1995	39.0	58.0	76.0
1998[b]	42.0	60.0	82.0
2000	44.0	62.0	86.0

[a] All prices reflect the average landed price of crude oil in the United States.

[b] SERI estimates for 1998 were obtained by interpolation of EIA projections.

Source: U.S. Department of Energy, EnergyInformation Administration, April 1983, Annual Energy Outlook with Projections to 1990, DOE/EIA-0383 (83).

Overview of Microalgal Analysis Model

The production model estimates gross biomass yields for a facility of a given size. The equation which follows was reported by Oswald and Benemann in "Biochemical and Photosynthetic Aspects of Energy Production," edited by Anthony San Pietro (1980). Providing the basis for estimating gross yields of algal biomass, the equation is most appropriate for steady-state continuous flow culture systems similar to chemostats. The input parameters of depth, detention time (or the inverse of dilution rate) and culture density are based on empirical data obtained from field experiments.

$$\text{Production} = \left(\frac{D}{\theta}\right) d\, C_f\, Z\, (.01)$$

Where:

Production	= Gross yield (dry ash-free metric tons per year)
D	= Culture depth (meters)
θ	= Retention time (days)
C_f	= Capacity factor (days per year)
Z	= Facility size (hectares)
d	= Culture density (mg/l)
.01	= Scaling constant

Photosynthetic efficiency is calculated from the gross biomass yield using average daily solar insolation representative of the Southwest United States (5000 Kcal/m^2/d) and the average total energy content of the algal biomass, (Kcal per gram) determined from user-defined input assumptions for gross lipid and carbohydrate content (heating value for lipid, carbohydrate and protein fractions are 9.3 Kcal/gm; 3.7 Kcal/gm and 5.6 Kcal/gm respectively; Milner; in Bulew, 1976). The ash content of the algae is fixed at 8% of dry cell weight.

$$\text{Photosynthetic Efficiency} = \frac{\text{total annual energy output}}{\text{total annual energy input}}$$

Where:

Total annual energy output = total algal yield x average heating content of algal biomass (Kcal/yr)

Total annual energy input = average daily solar radiation x fraction of total light spectrum available for photosynthesis x capacity factor x facility size (Kcal/yr).

Net biomass yield for the facility is calculated by multiplying gross production by user defined harvesting efficiency.

Lipid production is calculated from net biomass yield and lipid content (% of ash free dry weight), the latter parameter being user defined. The conversion of product tons to product barrels assumes 146.7 kilograms per barrel (Lipinsky, et. al. 1981).

Capital costs were derived from Benemann (1982) in which the author updated 12 previously published economic analyses of microalgae production and developed product costs. Because of inconsistency among the studies in facility size, type of harvesting system, land costs and indirect capital investment requirements (e.g., engineering fees and contingencies), SERI used only those costs associated with growth pond construction, pumps, piping and other fixed onsites (e.g, buildings, fences, etc.) to develop a relationship between facility size (acres) and pond construction costs ($ per acre). This relationship is presented in Figure 2 as a line entitled "Culture Systems Only." The correlation coefficient for the least squares regression line is .77, indicating a reasonable fit of the data.

Figure 2 suggests declining unit costs with increased facility size. The extent to which this relationship indicates real economies of scale is not certain in the absence of actual cost histories of large scale microalgal systems. It is expected that a large scale facility would be comprised of a number of individual modules linked together through nutrient, water and harvesting distribution subsystems. Whether declining unit costs for large scale systems are applicable to a single module or to the entire facility is uncertain; however, economics of scale have been noted in analogous systems such as EPA waste treatment systems and desalination ponds. The results presented in this paper are based on developing capital costs as a function of total facility size.

Total capital investment is estimated by including engineering fees, contingency, land costs and a harvesting subsystem defined by the user. Engineering fees are estimated at 22% of the capital investment for the culture system and harvesting system. Contingencies are estimated at 10% of the capital investment plus engineering fees. Land costs are user defined according to an assumed unit price ($ per hectare) and facility size.

Currently, the SERI model is capable of analyzing three harvesting subsystems; microstrainer, nozzle centrifuge and settling ponds. Capital costs for each system are normalized to a 35,000 gallon per hour harvesting system with a size-cost exponent of .796 (Intertechnology, 1978). Thus, the model calculates flow rate (culture volume/retention time) to the harvesting subsystem and sizes the harvester accordingly.

Operating costs for the microalgae production facility include direct labor, overhead, maintenance expenses, utility costs and nutrient expenses.

Direct labor is assumed to involve five men per 100 hectares having an annual salary of $20,000. Overhead costs are 75% of the direct labor expenses. Maintenance expenses are assumed to be 2% of the capital investment for the growth ponds and harvesting subsystem.

Utility expenses include the costs associated with operation of the harvester subsystem, mixing subsystem and pumping requirements (e.g., make-up, harvester, and recycle). The flowrate (gallons per hour) in each pumping subsystem determines the required power demand. Harvester total energy requirements are estimated according to unit energy use estimates (kWh per m^3) as reported by Mohn (1980) multiplied by the throughput of the harvesting subsystem. Mixing velocity energy requirements are estimated by calculating headloss through the culture system according to Benemann (1982). The model allows the user to define a geometric shape of the culture system (length, width and depth) in order to calculate appropriate headloss. Using assumptions for capacity factor of the facility, total kilowatthours required for the pumping subsystem, harvester subsystem and mixing subsystem are calculated and used to determine total energy costs according to user specified unit energy costs ($ per kilowatthour).

Nutrient expenses are derived from the gross production yield estimates based upon general elemental composition of microalgae. For example, if 50% of algae is carbon, then .5 metric tons of carbon are required per metric ton of algae gross yield. The amount of carbon dioxide (CO_2) as a carbon nutrient source, required to sustain a metric ton of algae is determined by the ratio of the molecular weight of carbon dioxide (44) to the atomic weight of carbon (12). Thus, one metric ton of algae would require 1.7 metric tons of CO_2. Nutrient expense for nitrogen, potassium and phosphorus are similarly determined according to this adjusted nutrient requirement multiplied by a nutrient supply price selected by the user. The major nutrients are supplied as indicated in

Table 3. The nutrient prices are based upon supplier quotes for spot purchases as listed in the Chemical Marketing Reporter, and do not reflect contracted prices that might be available to large users of industrial chemicals. Contract prices for chemicals for use in microalgae production can be significantly lower than the spot prices presented in Table 3.

Table 3. Major Nutrients Supplied To Microalgae Culture Facility and Related Supply Costs.

Nutrient	Supplied as	Market Price ($/metric ton)	(Refs)
Carbon	Carbon dioxide	$82.5	(1)
Nitrogen	Ammonia	$203.0	(2)
Potassium	Potassium Muriate	$102.0	(2)
Phosphorus	Superphosphate	$281.6	(2)

Notes:
(1) Argonne, (1983) estimate of current commercial CO_2 price.

(2) Chemical Marketing Reporter (Sept. 12, 1983).

Once the system specific operational parameters are calculated, the model determines the finances of the microalgal facility by utilizing a capital budgeting technique. Doane, et. al. (1978) reported a required revenue methodology for providing comparative evaluation of technologies using standard and consistant economic and financial parameters. The economic model determines the present value of capital investment costs (including interest during construction) and the present value of annually recurring costs over the system lifetime. This aggregated present value is distributed over the system lifetime in equal cash flows and then divided by the expected annual energy output. The result is a required unit price for the energy product, the revenues of which would exactly recover the full costs of the system over its lifetime, including a return on the investments of stockholders and creditors. This methodology specifies a unit product cost in constant dollars necessary for the net present value of revenues and costs for a production facility to equal zero. The weighted average after tax cost of capital internally computed by the economic model represents the internal rate of return for the facility.

Input Assumptions used to Calculate Production Costs

The operational and financial assumptions used to develop state-of-the-art and theoretical cost estimates for microalgae production are listed in Appendix A. To facilitate comparison between different sets of operational data, consistent supply cost and financial parameters are maintained. Where difference values between SOTA and theoretical parameters occur, the changes reflex a degree of optimistism appropriate with the definition of theoretical best. Carbon costs and real escalation in capital and

operating costs are lowered in the theoretical best case. Recycling is increased and the time frame for construction varies. The SOTA case presumes near-term application of currently available technology; whereas the theoretical best case presumes a long term improvement in biological and engineering parameters.

System specific parameters are presented in Table 4 for the SOTA and theoretical best design facilities. Since photosynthetic efficiency determines annual yield, the values presented in Table 4 for the theoretical example represent one set of values to obtain a PAR efficiency of 24%. These values are not meant to be considered optimal nor limiting conditions to attainability in large scale cultivation of microalgae. Yield, calculated from values for depth, detention time and density, was determined by increasing culture density while holding constant values for detention time and culture depth until PAR efficiency is 24%. Other combinations of values for depth, detention time and culture density can be used to estimate a theoretical yield based on a 24% PAR efficiency.

Table 4. Operational Input Parameters Used to Calculate Microalgal Production Costs

Parameter	SOTA	Theoretical
Density (mg/L)	800	2200
Retention time (days)	5	5
Depth (cm)	15.2	15.2
Mixing Velocity (m/sec)	.305	.305
Mixing System	Paddlewheel	
Efficiency (%)	56.0	67.5
Harvesting System	Settling Pond	
Efficiency (%)	95	100
Lipid Content (% dry ash-free weight)	30	70
Carbohydrate Content (% dry ash-free weight)	15	10
Non Carbon Nutrient Recycle	0	90

State of the Art Technology Costs

This section presents microalgal product costs. These costs (expressed in 1983 dollars) represent the revenue-required price allowing a specified return on investment over the lifetime of the facility. The cost estimates are based on a commitment to build a 405 hectare (1000 acre) facility in 1983 to be operational in 1985.

The microalgae production system is estimated to be capable of producing 134.7 barrels of lipid oils per hectare per year. This product yield is based on a total net biomass yield of 66.0 metric tons per hectare per year (gross productivity of 23.0 g/m^2-d). Overall photosynthetic efficiency for the system is 5.9% of the photosynthetically active region (PAR) based on insolation values representative of the U.S. southwest. Processing losses were assumed at 5% of gross yield.

Construction costs for the growth ponds, pipes, pumps, buildings, and offsites are estimated to be $37,600/hectare (in 1983 dollars), or $3.76/m^2. Including a harvesting system, the total depreciable capital investment is approximately $55,330 per hectare. Additional capital cost charges for engineering, contingencies, and land increase result in total fixed costs for the microalgae production facility to $75,560 per hectare. On an annualized cost basis, these fixed costs ($5,150 per hectare) represent 18.8% of the total product costs for microalgae production as indicated in Table 5.

Variable costs or costs associated with annual operation of the microalgae facility represent 81.2% of the total annualized costs and are directly proportional to production output. The major variable cost category is nutrient requirements to sustain the biomass production (54.5% of the total variable costs). Utilities requirements to run the pumping subsystems (e.g., recirculation) constitute the second largest cost category. Power for mixing — the amount of horsepower required to maintain a specific flow velocity along the channel length — requires the most energy. Of the total utility energy demand (22.9 x 10^6 kWh), 69.4% is required to maintain a 30.5 cm/s velocity. The settling pond harvesting system consumes 3.5 x 10^6 kWh (15.3 of the total utility energy requirements of the facility) with the balance attributed to recirculation requirements. The total annualized variable costs for the system are $8.9 million or $336.5 per net metric ton of biomass. The levelized product cost for microalgae lipids from this state-of-the-art facility is $203 per barrel of oil, based upon an average lipid content of 30%.

Table 5. Summary of Microalgae Production Costs for the State of the Art Design Systems.

	1983 $/per Barrel	% of Total
Annualized Capital Investment	38.2	18.8
Annual Operating Cost		
Utilities	27.4	13.5
Nutrients	110.8	54.5
O & M	10.1	4.9
Labor and Overhead	16.5	8.1
Total Production Cost (1983 $/BBL)	203.1	100.0

Theoretical Cost Estimates

Certain input parameters have potential for improvements through continued research efforts. To define the absolute "best" operational conditions for the microalgae systems, specific engineering and biological parameters were set at their maximum possible values, thus bounding the limits of attainability. These limits for biological and engineering parameters are presented in Tables 4 and Appendix A. Again, values for depth detention time and density are not representative of an optimal nor limiting condition, but rather, one set of biological and engieering parameters that result in a 24% efficiency on PAR.

For the theoretical best production facility net production yield of biomass is 232 metric tons per hectare-year resulting from a productivity of 63.7 grams per square meter-day. Lipid product yield is 1100 barrels per hectare-year assuming no harvesting losses.

Table 6 summarizes the distribution of annual costs for the theoretical best production. Compared with the SOTA cost summary presented in Table 5, the distribution of annual costs between the two cases is similar. Recycling of non carbon nutrients and a lower delivered price for carbon dioxide reduce the nutrient supply costs to 42.6% of the total costs. Annualized capital costs as a percentage of total product costs increase slightly from the SOTA estimate since no improvements in unit capital costs for the theoretical best facility were assumed, and other costs are reduced.

Table 6. Summary of Microalgae Production Costs for the Theoretical Best Design System

	1983 $/per Barrel	% of Total
Annualized Capital Investment	4.5	26.6
Annual Operating Cost		
Utilities	2.7	15.9
Nutrients	7.2	42.6
O&M	.9	5.3
Labor and Overhead	1.6	9.4
Total Production Cost (1983 $/BBL)	16.9	100.0

The cumulative effect of the biological and engineering improvements is to reduce unit costs to $16.9 per barrel. The theoretical best design system results in product costs substantially below the lowest 1998 market cost projection for crude wellhead petroleum of $42 per barrel (Table 2).

The significant changes in the operational and cost input parameters in going from SOTA to theoretical best are summarized below.

	Input Values	
Parameter	SOTA	Theoretical
Lipid Content (%)	30	70
Culture Density (mg/L)	800	2200
Non Carbon Nutrient Recycle (%)	0	90
Carbon Dioxide Price ($ per metric tonne)	82.5	19.0*
Capacity Factor (days per year)	300	365

* Carbon cost at $19 per metric ton of CO_2 (delivered) is based on an analysis of a report by the Colorado Energy Research Institute "Natural Carbon Dioxide Resources of Colorado: An Overview," October, 1982. This price for CO_2 represents a delivered cost of $1.0 per MSCF of CO_2 to a large end user similar to enhanced oil recovery.

While other changes (e.g., increase in mixing and harvesting systems' efficiencies, year of commercial operation) also contribute to the decrease in unit cost, the parameters listed above had the largest effect. Lipid content and culture density increases result in increased output for the production facility, distributing annual costs over a larger volume. Recycling of non-carbon nutrients and a lower delivered price for CO_2 result in lower operational costs for nutrient supplies which represent over 50% of the annual variable costs for the state of the art production facility (see Table 5). The effect of these changes on unit production cost are discussed in the next section.

Sensitivity Analysis and Development of an Attainability Target for Microalgae Production

The purpose of this section is to determine those parameters which have the greatest influence on product costs. Based on this sensitivity analysis, one set of input values is specified which, in the judgment of ASP researchers, should be achievable by 1994.

The sensitivity analysis identifies biological and engineering parameters which are most important in reducing microalgae unit cost, and indicate areas where research progress will most significantly improve cost and performance over state of the art levels. The parameters, productivity (culture density), lipid content, nutrient recycling and lower nutrient supply prices and capacity factor, were systematically varied through a range of values between current state of the art and the theoretical best estimate. The entire range of values for each parameter was divided into quartile intervals to examine 25% improvements in each parameter and its effect on unit cost. A summary of the results of this analysis is presented in Table 7. Each input parameter is listed in descending order of importance according to the effects on unit product cost. (All other parameters in the analysis model were held constant at SOTA values.) Lipid content is shown to have the most significant affect on unit cost. An increase in lipid content halfway towards the theoretical maximum results in a 40% decrease in unit cost compared to the SOTA value.

Table 7. Effect of Quartile Improvements in Selected Input Assumptions and Their Influence on Microalgae Product Cost

Parameter	SOTA	Percentage Improvement			Theoretical Best
		25	50	75	
Lipid Content (%)	30	40	50	60	70
Unit Price ($/BBL)	(203.0)	(152.3)	(122.8)	(101.6)	(87.1)
Gross Productivity (g/m^3-d)	23.0	32.25	42.5	52.7	63.0
Unit Price ($/BBL)	(203.0)	(176.6)	(160.8)	(151.1)	(144.4)
Carbon Dioxide Price ($/mt)	82.4	66.5	50.6	34.8	19.0
Unit Price ($/BBL)	(203.0)	(185.5)	(167.8)	(150.3)	(132.8)
Non-Carbon Nutrient Recycle (%)	0	22.5	45	67.5	90
Unit Price ($/BBL)	(203.0)	(187.2)	(182.0)	(178.9)	(173.9)
Capacity Factor (days)	300	316	332	348	365
Unit Price ($/BBL)	(203.0)	(199.8)	(196.8)	(193.0)	(191.6)

From this table, lipid content, productivity and the delivered price of the carbon dioxide were determined to have the greatest effect on unit cost. Non-carbon nutrient recycling and capacity factors were less significant parameters.

One feature of analysis model, its handling of non-carbon recycling, provides an alternate way to interpret the results. To analyze the effects of nutrient recycling, unit price for the nutrient is reduced. A 22.5% recycling of non-carbon nutrients is equivalent to a 22.5% reduction in supplier prices for those nutrients. Therefore, the results presented for non-carbon nutrient recycling are also indicating the effects of lower contract prices for the nutrients, nitrogen, phosophous and potassium.

The attainability target represents interim programmatic technology cost goal for 1994, the year in which it is planned that capital funds will be expended for a large scale proof of concept experiment.

The major operational parameters utilized in establishing a 1994 attainability target are listed in Table 8. Other operational and economic parameters were assumed to be the same as SOTA parameters.

Table 8. Input Parameters Used to Define Unit Cost for the Attainability Target for 1994

Parameter	Value
Culture density (mg/L)	1500
Lipid content (%)	60
Carbohydrate content (%)	10
Recycling of non-carbon nutrients	90
Carbon dioxide price ($/mt)	38.5
Year of Commercial Operation	1998

With these assumptions, the annual net yield of algal biomass is 124 metric tons per hectare-year, resulting in a product yield of 500 barrels per hectare-year. Gross productivity is approximately 43.4 grams per square meter-day and the photosynthetic efficiency for the system is 13.2% of PAR. Levelized product cost for the 1994 attainability target is $55.6 per barrel. The distribution of annualized production costs is presented in Table 9.

Table 9. Summary of Microalgae Production Costs for the 1994 Attainable Target

	1983 $/BBL	% of Total
Annualized Capital Investment	11.8	21.2
Annual Operating Cost		
Utilities	8.7	15.6
Nutrients	26.6	47.8
O&M	3.2	5.6
Direct Labor and Overhead	5.3	9.5
Total Production Cost (1983 $/BBL)	55.6	100.0

When compared with SOTA input assumptions for the raceway system, the attainability target presumes certain biological and engineering improvements by 1994 as a result of continued research in microalgal production systems. The improvements over SOTA defined parameters are: productively increased 90%; lipid content increased 100%; nutrient costs reduced by a factor of four through recycling; and carbon dioxide costs reduced through pipeline contracts by a factor of 2.5. Current laboratory experiments with microalgae indicate that biological improvements are achievable, although large scale outdoor experiments are just beginning. Note that the parameters used to define the attainable biological target case represent but one solution set of a multivariable set of parameters. For example, improved operating efficiences would result in another solution set in which the biological performance requirements would be less.

Summary

The data presented in this paper indicate microalgae to fuel technology can be cost competitive with conventional fuels. Research progress in biological and engineering parameters by 1994 are projected to produce a lipid oil product at a cost comparable with EIA well-head crude petroleum price forecasts. During the next fiscal year refinements to this methodology will continue at SERI, and a major effect will be initiated to quantitively and qualitatively describe downstream processing and refining of microalgae fuel products suitable for end use utilization.

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APPENDIX A

Engineering and Economic Input Assumptions to Calculate SOTA and Theoretical Best Unit Cost for Microalgae Production.

Engineering Parameters	SOTA	Theoretical
Capacity Factor (days)	300	365
Harvester Subsystem Efficiency (%)	95	100
Facility Size (Hectares)	405	405
Nutrient Recycle	None	90% noncarbon nutrients
Economic Parameters		
Land Cost (per hectare)	$1,235	Same
Energy Costs (per kWh)	$.05	.05
Nitrogen Cost, per metric ton, (NH_3)	$203.0	Same
Carbon Cost, per metric ton (CO_2)	$82.5	$19.0[1)]
Potassium Cost, per metric ton (KO_2)	$102.0	Same
Phosphorus Cost, per metric ton (superphosphate)	$281.6	Same
Investment Parameters		
Base Year for Constant Dollars	1983	Same
Year of Cost Information	1982	Same
Year of First Commercial Operation	1985	2000
Facility Lifetime (years)	30	Same
Depreciation Tax Life (years)	15	Same
Taxes, Insurance (each)	1% of Investment	Same
Income Tax Rate (%)	45	Same
Investment Tax Credit (%)	10	Same
Ratio of Debt to Capitalization (%)	50	Same
Ratio of Common Stock to Capitalization (%)	35	Same
Ratio of Preferred Stock to Capitalization (%)	15	Same
Annual Rate of Return on Debt (%)	3.7	Same
Annual Rate of Return on Common Stock (%)	5	Same
Annual Rate of Return on Preferred Stock (%)	4	Same
Real Escalation Rate on Capital Costs (%)	1	0%
Real Escalation Rate on Operating Costs (%)	1.5	0%
Calculated Parameters		
Cost of Capital (%)	3.4	Same
Capital Recovery Factor (Book Life) (%)	5.3	Same
Fixed Charge Rate (%)	7.4	Same

Note:
1) Carbon cost at $19 per metric ton of CO_2 (delivered) is based on an analysis of a report by the Colorado Energy Research Institute "Natural Carbon Dioxide Resouces of Colorado: An Overview" October, 1982. This price for CO_2 represents a delivered cost of $1.0 per MSCF of CO_2 to a large end user similar to enhanced oil recovery.

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