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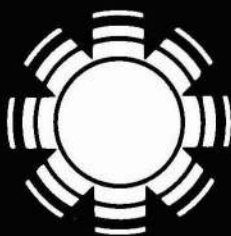
April 1984

Screening for Lipid Yielding Microalgae: Activities for 1983

Final Subcontract Report

W. H. Thomas (Scripps Institution of Oceanography)
T. G. Tornabene (Georgia Institute of Technology)
J. Weissman (Microbial Products, Inc.)

Prepared under Subcontract Nos. XK-2-02/70-0-01
XK-2-02149-01
XK-3-03135-1
XK-3-03000-01



SERI

Solar Energy Research Institute

A Division of Midwest Research Institute

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SERI Technical Monitor: Robins P. McIntosh

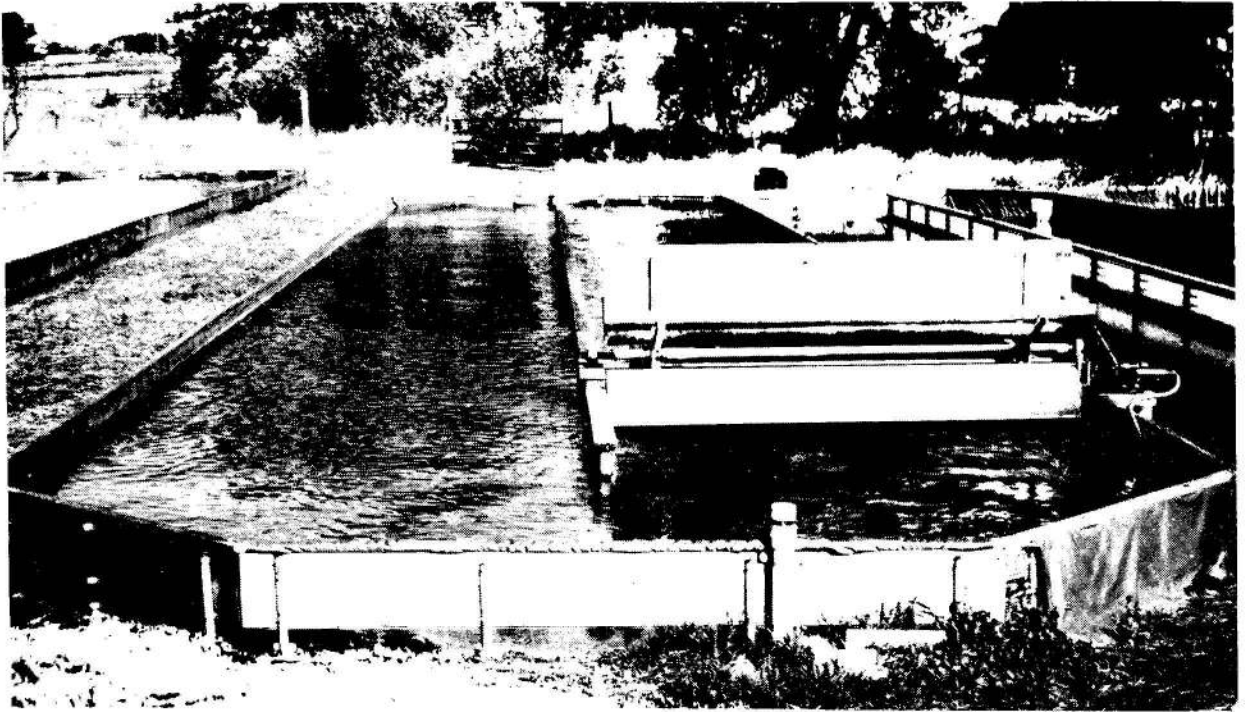
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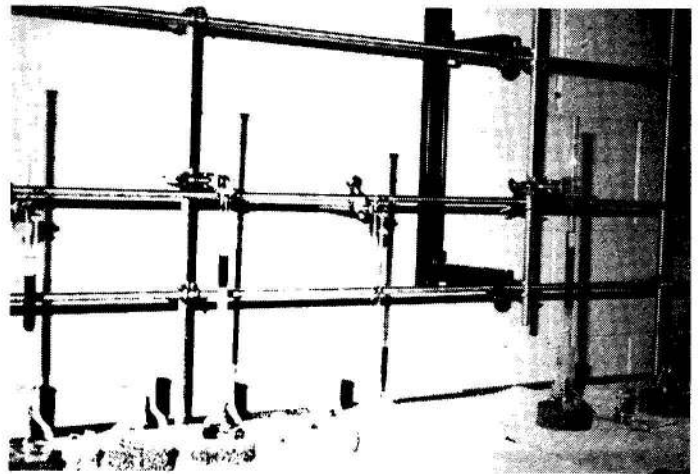
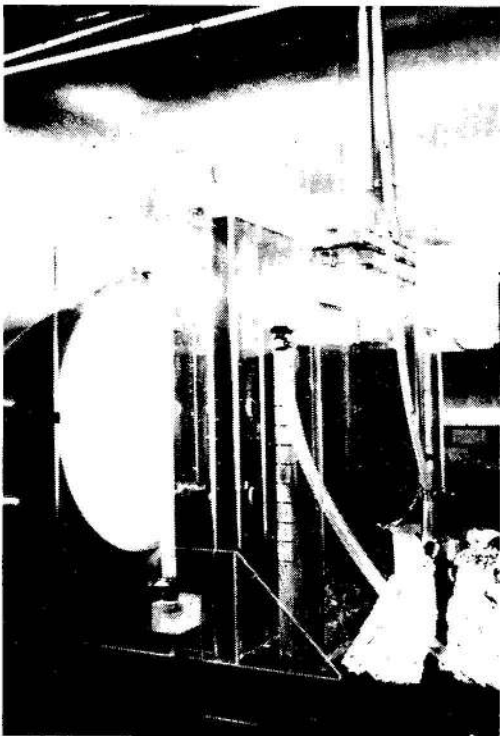
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Pond System to Determine Performance in Outdoor Mass Culture (Microbial Products/Enbio. Inc.)



Studies of the Lipid Fractions Produced by the Microalgae (Georgia Institute of Technology)

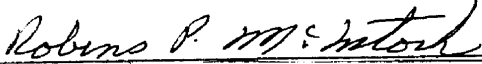
Growth Studies of Selected Species Using Chemostats (Scripps)

FOREWORD

The Aquatic Species Program has historically been involved in developing the technology for growing any aquatic species that is a potential high biomass producer. Starting in FY 1983, the program emphasized high lipid yielding microalgae that could grow and survive in outdoor facilities. The aim of the program is to develop a renewable replacement product for liquid fuels. Emphasis is being placed on a technology base that is feasible in the arid and saline Southwest. Placing the technology in this area will make lands that are presently underutilized more productive. Microalgae species will be developed that grow rapidly and produce high lipid yield, culture management techniques will be developed that will result in sustainable production, and engineering concepts will be developed that result in a feasible technology.

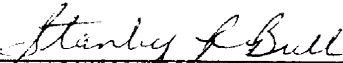
During FY 1983 the primary focus of the microalgae program was to collect and select microalgae for high lipid yields. The program supported three projects that significantly contributed to this effort. Researchers at Scripps Institute of Oceanography concentrated on the collection and growth characterization of species, while work at Georgia Institute of Technology was involved in the characterization of lipids from promising microalgae species. Finally Microbial Products/Enbio, Inc. were involved in determining the yield characteristics of promising microalgae in outdoor mass culture. Results from these studies are presented in this report.

The work was performed under subcontract to SERI, with funds provided by the Biomass Energy Technology Division of the U.S. Department of Energy (DOE).


Robins P. McIntosh, Project Manager
Biomass Program Office

Approved for

SOLAR ENERGY RESEARCH INSTITUTE


Stanley R. Bull, Director
Solar Fuels Research Division

SUMMARY

OBJECTIVE

- To collect microalgae from saline environments and subsequently characterize their productivity potential.
- To characterize lipids from high yielding microalgae.
- To characterize the outdoor culture potential of promising microalgae strains.

DISCUSSION

The screening effort being conducted by the SERI/DOE microalgae program is designed to select halophilic microalgae that will produce large quantities of lipids in outdoor culture. Lipids are of interest because of their potential application as liquid fuels. The program funded three major projects in FY 1983 to carry out the screening activity. Researchers at Scripps Institute of Oceanography were responsible for collecting microalgae from desert environments and characterizing yield potentials. Work carried out at the Georgia Institute of Technology emphasized the characterization of lipids from microalgae. Finally species that were found to have desirable yield characteristics and lipid composition were grown in outdoor culture by Microbial Products, Inc.

CONCLUSIONS

The SERI/DOE Aquatic Species Program is conducting a screening project, to select microalgae species and strains that are acceptable for liquid fuel production in outdoor culture. The emphases are on finding species that grow rapidly at high biomass density, in outdoor culture and produce large quantities of lipids. During 1983 over 100 species were isolated from saline waters at the California and Nevada deserts. Some of these species were characterized for growth response to various nutrients, temperatures, and salinities. Selected species were analyzed for lipid composition. Lipids were characterized into fractions, hydrocarbons, isoprenoids, triglyceride, glycolipids, and phospholipids. The most promising species were tested for growth and monoculture sustainability in outdoor culture.

SUMMARY OF MICROALGAE SCREENING ACTIVITIES

SELECTION OF OIL-PRODUCING DESERT MICROALGAE SCRIPPS INSTITUTION OF OCEANOGRAPHY

The purpose of these studies are to progressively select desert saline algae in which yields of cell material and their oil content can be maximized. The studies progress from field work to laboratory work and eventually to outdoor culture work in a logical fashion. This work has been separated into five tasks: sampling desert saline waters; isolation and culturing algae from these waters; chemical analyses of these waters; preliminary selection experiments; and maximizing algal yields in the laboratory.

CHEMICAL PROFILES OF SELECT MICROALGAE GEORGIA INSTITUTE OF TECHNOLOGY

The proximate chemical composition of seven species of fresh water, desert and marine unicellular eukaryotic microalgae grown under controlled conditions was measured with emphasis on the lipids. Ankistrodesmus, Dunaliella spp., Isochrysis, Nannochloris and Nitzschia contained a range of 16% to 38% proteins, 9% to 56% carbohydrates, and 7% to 55% lipids of organic cell weight. Botryococcus braunii contained about 45% lipids. The effect of cultivations on proximate chemical compositions of Botryococcus braunii, Dunaliella salina and Isochrysis in nitrogen deficiency medium, was a decrease in protein content and an increase in carbohydrate content, in Dunaliella Salina a decrease in lipids, and in Isochrysis an increase in lipid content. Cultivation of Dunaliella salina in medium with increased concentration of sodium chloride induced the accumulation of osmotic glycerol with minimal effect on the other constituents. In Botryococcus and Isochrysis the sodium chloride stress was mainly expressed through reduction in the protein content. The lipids of Botryococcus braunii, Isochrysis sp., and nitrogen deficient cultivated Dunaliella bardawil consisted of relatively high concentrations of neutral lipids with multiple branched hydrocarbons predominating. The polar lipid composition of glycolipids and phospholipids of all species investigated was fairly typical of photosynthetic eukaryotic algae in general. The fatty acid compositions were species specific with changes occurring in the relative intensities of individual acid chains of cells cultivated under different cultivation conditions and growth phases. All species synthesized C14:0, C16:0, C18:1, C18:2, and C18:3 fatty acids, C16:4 and C18:4 in Ankistrodesmus sp.; C18:4 and C 22:6 in Isochrysis sp.; C16:2, C16:3 and C 20:5 in Nannochloris sp.; C16:2, C16:3, and C20:5 in Nitzschia. Nitrogen deficiency and salt stress induced the accumulation of C18:1 in all treated species and to some extent C20:5 in Botryococcus braunii. The data presented provides additional evidence to the concept of biochemical classification and its control by environmental factors.

MASS CULTURE OF SELECTED MICROALGAE MICROBIAL PRODUCTS, INC. AND ENBIO, INC.

Two strains, shown by the screening program to be promising lipid producers, were inoculated and maintained outdoors. The Ankistrodesmus, isolated by Dr. W. Thomas from Pyramid Lake, was cultivated in brackish waters. After initial problems with salt balance in the medium, (too little potassium) it grew well under nitrogen sufficiency and consisted of 27±5% lipid. Nitrogen deficiency studies were initiated late in the growing season, (lipid composition is still being analyzed) but the strain is problematical in that

regreening of starved cultures (after reintroduction of nitrogen) is too slow under the high light irradiances occurring outdoors. Regreening in the laboratory was not a problem. This strain was grown in batch only. Optimization of the medium has not been fully achieved.

Another promising strain, Scenedesmus S02a was obtained from Dr. S. Lien at SERI. It was inoculated into the outdoor system and grown continuously and in batch. It grew well, although the medium may also not yet be optimal. It regreened readily after nitrogen starvation. Lipid content of nitrogen sufficient cultures was 18%. The analysis of lipid content of induced cultures is still being completed.

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SERIO 

**SELECTION OF OIL-PRODUCING DESERT
MICROALGAE**

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University of California
San Diego, California

Prepared under Subcontract
No. XK-2-02/70-0-01

SECTION 1.0

SELECTION OF OIL-PRODUCING DESERT MICROALGAE

1.1 INTRODUCTION

The American desert has abundant resources of land, sunlight, and saline water. The latter is not usable for conventional agriculture, but could be used to grow microalgae in outdoor installations (ponds). Various microalgal species occur in desert saline waters. Field and laboratory studies have been carried out directed toward the development of the above concept.

The specific purposes of the work are: Task I) sample desert saline waters; Task II) isolate and culture microalgae from desert saline waters; Task III) analyze these waters for their major and minor chemical constituents; Task IV) carry out preliminary selection experiments on algal growth in artificial media and assess the temperature, salinity, and light requirements of the algae; and Task V) carry out experiments to maximize algal yields in the laboratory. Proceeding through these tasks is a selection process to find those species that will produce oil-related products at a high yield.

1.1.1 Task I

Six field trips were made to the deserts of eastern California and western Nevada. These trips have been based at two established field laboratories, the University of California's Sierra Nevada Aquatic Research Laboratory (SNARL) near Mammoth Lakes, California and the California State University's Desert Research Center (Zzyzx Springs) near Baker, California. Saline water sources sampled from SNARL include Big Alkali Lake (Ponds A and B), Black Lake, Mono Lake, Owens Lake and the Saline Valley in California and Columbus Salt Marsh, Pyramid Lake, Walker Lake, and Soda Lake (plus ponds and springs near Soda Lake) in Nevada. Sources sampled from Zzyzx Springs include the springs themselves, Badwater, Mormon Point, and Saratoga Spring in Death Valley National Monument, Salt Creek, Sperry River, the Armagosa River, and Harper Lake. The general area covered by these field trips is shown in Figure 1-1. All of these sites were sampled with a two-wheel drive University van. Figure 1-2 shows sampling the salt lake in the Saline Valley, California.

1.1.2 Task II

Algae were principally isolated by micropipetting individual cells of the desired species from enrichment cultures. The latter were set up at the field laboratories or at LaJolla Laboratories. The isolates were generally incubated at least two weeks before determining if the cultures could be maintained. The maintenance media were artificial salt solutions, the composition of which was calculated from chemical analyses of each collected water sample (see Task III below).

A list of cultures that were isolated and maintained are given in Table 1-1. The collection consists of 58 unialgal isolates and 14 mixed cultures.

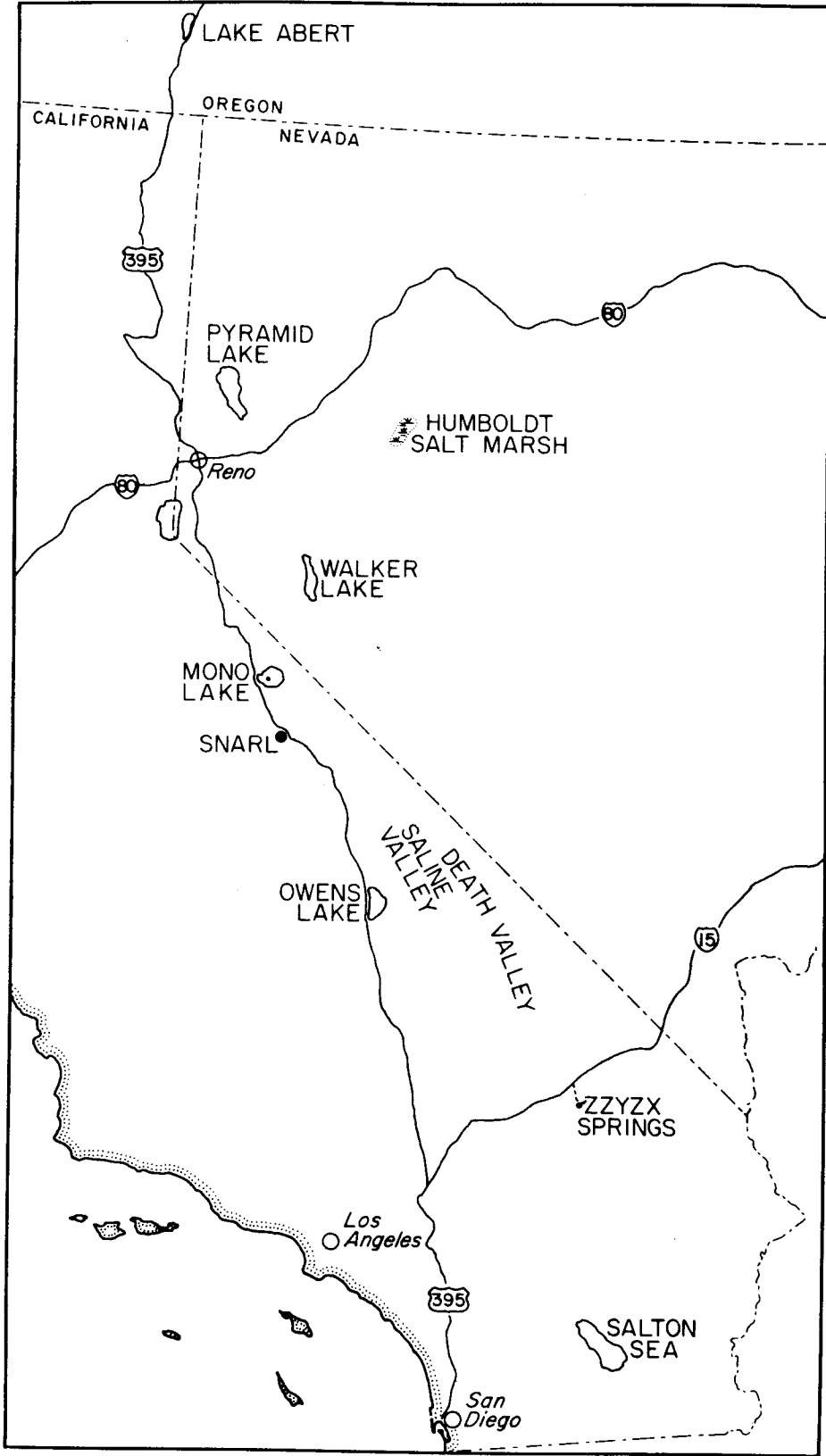


Figure 1-1. General Locations of Areas Sampled in the Desert of Eastern California and Western Nevada



Figure 1-2. Sampling the Salt Lake in the Saline Valley

Table 1-1. Cultures of Desert Algae Isolated and Maintained by the Scripps Project in FY 1983

Organism	Water Temperature	TDS (g/L) ^a	Growth Yield ^b	Organism	Water Temperature	TDS (g/L) ^a	Growth Yield ^b
CALIFORNIA				Big Soda Lake, unialgal			
Owens Lake, unialgal				Nitzschia	15.0	20.7	
Myremecia			++++	Nitzschia	15.0	20.7	
Chaetoceros	27.5	11.9	+++	Desmoeoccus	15.0	20.7	
Chaetoceros	27.5	11.9		Chlorella	15.0	20.7	
Blue-green	27.5	11.9		Chlorella	15.0	20.7	
Cymbella	16	20.5					
Owens Lake, not unialgal				CALIFORNIA			
Chaetoceros	27.5	11.9		Lake Tuendae Unialgal			
Blue-green + Flagellate	27.5	11.9		Chaetoceros	25.0	*2.8	
Nitzschia	16	20.5		Yellow round	25.0	*2.8	+++
				Pennate diatom	25.0	*2.8	
Saline Valley, unialgal				Zzyzx Spring, unialgal			
Dunaliella	25.4	254.9		Chlorella	23.0	*4.8	++++
Dunaliella	25.4	254.9		Anacystis	23.0	*4.8	
Dangeardinella	25.4	254.9	++	Zzyzx Spring, not unialgal			
Dangeardinella	25.4	254.9	+	Green + flagellate	23.0	*4.8	
Pond A (Big Alkali Lake), unialgal				Harper Lake, unialgal			
Nitzschia	9.6	1.4	+	Cryptomonas	9.0	15.8	
Nitzschia	9.6	1.4		Cryptomonas	9.0	15.8	++++
Cymbella	9.6	1.4		Oocystis	9.0	15.8	
Pinnularia	5.0	1.2		Oocystis	9.0	15.8	
Green flagellate	5.0	1.2		Small green flagellate	9.0	15.8	
Pond A (Big Alkali Lake), not unialgal				Harper Lake, not unialgal			
Nitzschia	9.6	1.4		Chlorella (Oocystis)	9.0	15.8	++++
Pond B (Big Alkali Lake), unialgal				Cryptomonas	9.0	15.8	
Chlorella	2.0	3.2	++++	Sperry River, unialgal			
Chlorella	2.0	3.2		Coccomyxa	14.0	5.5	
Yellow flagellate	2.0	3.2		BATS, unialgal			
Microcystis	6.5	1.0		Spring 10/1 (hot spring near Big Alkali Lake), unialgal			
Green flagellate	6.5	1.0		Chlorella	-	*1.2	+++
Botryococcus	29.5	5.0		Chlorella	-	*1.2	+++
Botryococcus	29.5	5.0		Black Lake, unialgal			
Chlorella	29.5	5.0		Green flagellate	13.2	26.5	
Cymbella	29.5	5.0		Chaetoceros	13.2	26.5	
Pond B (Big Alkali Lake), not unialgal				Black Lake, not unialgal			
Cymbella	2.0	3.2		Cymbella	13.2	26.5	++
Chlorococceum	13.5	10.3		Cymbella	13.2	26.5	
Walker Lake, not unialgal				Chaetoceros	13.2	26.5	
Cyclotella	18.0	10.6	+	Yellow flagellate	13.2	26.5	
Columbus Salt Marsh, unialgal				Mix	13.2	26.5	
Chlorococcus	11.2	*60.8	++	Nitzschia	14.0	7.1	
Pond #2 off Highway 80, unialgal				Achnanthes	14.0	7.1	
Oocystis	20.0	5.9					
Navicula	20.0	5.9					

Table 1-1. Cultures of Desert Algae Isolated and Maintained by the Scripps Project in FY 1983 (Concluded)

Organism	Water Temperature	TDS (g/L) ^a	Growth Yield ^b
NEVADA			
Pyramid Lake, unialgal			
Anacystis	17.4	4.9	
Nannochloris	17.4	4.9	++++
Ankistrodesmus Falcatus	17.4	4.9	+++
Synechococcus	15.0	4.7	
Nitzschia	15.0	4.7	
Green flagellate	15.0	4.7	
Green ovoid	15.0	4.7	
Pyramid Lake, not unialgal			
Nannochloris	17.4	4.9	
Nannochloris	17.4	4.9	++++
Yellow round	17.4	4.9	+
Green round	15.0	4.7	
Cocoid green	15.0	4.7	
Walker Lake, unialgal			
Cyclotella	18.0	10.6	++
Anacystis	18.0	10.6	++++
Oocystis	18.0	10.6	+++
Nannochloris	18.0	10.6	++++
Nannochloris	18.0	10.6	++++
Yellow-green flagellate	18.0	10.6	++
Yellow-green flagellate	18.0	10.6	++
Green ovoid	13.5	10.3	
Green flagellate	13.5	10.3	
Nitzschia	13.5	10.3	
Nannochloris	13.5	10.3	
Desmococeus	13.5	10.3	
Cyclotella	13.5	10.3	
Chlorella	13.5	10.3	
Cosmarium	24.0	*4.7	+++
Anacystis	24.0	*4.7	
BATS, not unialgia			
Chaetoceros	24.0	*4.7	
Armagosa River, unialgia			
Green flagellate	8.4	8.4	
Nitzschia sigmoidea	24.0	2.2	
Nitzschia	24.0	2.2	
MISCELLANEOUS CALIFORNIA CULTURES			
Salton Sea, unialgal			
Chaetoceros	20.7	39.0	+++
Dunaliella	20.7	39.0	+++
Nitzschia	20.7	39.0	
Flagellate (agar)	20.7	39.0	
Pennate (agar)	20.7	39.0	
Flagellate	20.7	39.0	
Salton Sea, not unialgal			
Navicula + green alga	20.7	39.0	
Green colony	20.7	39.0	
Mono Lake, unialgal			
Nitzschia			++++
Coccomyxa			+++

^aTotal dissolved solids (TDS) calculated from conductivity measurements.

^bGrowth yield estimated visually by one person for all cultures.

1.1.3 Task III

Chemical analyses of waters collected during Task I trips were done commercially by E. S. Babcock and Sons Laboratory in Riverside, California. Table 1-2 shows the data that was obtained. Generally these waters are quite saline with total dissolved solids ranging from 1.3 to 225 g L⁻¹. Their pH values are high and they are high in sodium, carbonates, and bicarbonates. Except for boron and fluoride, which are high, the concentrations of trace metals range from undetectable amounts to very low levels. Nutrient concentrations (phosphates, nitrate, silica, and ammonium) are very low and these waters must be enriched to promote algal growth.

1.1.4 Task IV

Further species selection and testing of algal growth requirements was carried out via this task.

1.1.5 Preliminary One-Liter Growth Experiments

Many species were cultured in one-liter flasks in preliminary yield studies to find out which species grew to high densities. Final culture densities were assessed visually. These experiments also tested the ability of strictly artificial media, to support growth. The salt composition was based on the chemical data of Task III.

Thirty-two isolates have been tested to date. Of these, 19 grew very well (ratings of "++++" or "+++"); six grew to intermediate levels ("++"); and the remainder grew poorly ("+"). However, all isolates grew in these media to some extent (Table 1-1).

1.1.6 Media and Roughly Determined Yield Experiments

Six of the best-growing isolates were tested further in cultures aerated with 1% CO₂-in-air to determine the best nitrogen source and to estimate rough yields and efficiencies. No growth of any of these was supported by ammonium as an N source, probably because this source escaped as NH₃ gas at the high pH values of these media.

The results of these experiments with nitrate or urea as N sources are shown in Table 1-3 and Figure 1-3. Of these the yields and efficiencies with Oocystis seem extraordinarily high. It is suspected that these values are in error and plans are to repeat the experiments with this isolate.

1.1.7 Proximate Cellular Composition

Frozen samples of some of the cultures (Table 1-3) have been sent to Georgia Tech for detailed analyses of their lipids. Some analyses of gross cellular composition have been done at Scripps. The results of these proximate analyses are shown in Table 1-4.

One isolate (notably the Pyramid Lake Ankistrodesmus) has a high lipid content, and would be useful for its harvestable energy content. Thus, further work (see light intensity experiment and Task V plans), will concentrate on this alga.

Table 1-2. Chemical Data from Selected Collecting Sites

	Pond A	Pond B	Black Lake	Pyramid Lake	Walker Lake	Owens Lake	Saline Valley	Mono Lake	Zzyzx Spring
Major Cations (mg l ⁻¹)									
Ca	5	6	4	10	10	8	220	20	15
Mg	5	5	4	60	77	20	320	63	6
Na	450	1150	8800	1775	3900	4700	100x10 ³	20.8x10 ³	640
K	43	124	825	128	225	525	1500	1800	15
NH ₄ ⁺	1	1	2	1	2	0.6	0.6	1	1
Total Cations (me l ⁻¹)	21.4	53.96	404.4	86.01	182.34	229.28	4423.99	956	--
Major Anions (mg l ⁻¹)									
CO ₃	240	420	5880	222	750	2640	0	16860	0
HCO ₃	268	1159	2526	854	1586	915	146	5307	244
SO ₄	20	140	3200	140	2600	1600	10.4x10 ³	11.5x10 ³	296
Cl	300	600	3200	2150	2600	2250	154x10 ³	2x10 ³	685
NO ₃	1	1	1	1	1	1	2	1	5
Ortho PO ₄	0.1	1.9	0.1	0.1	0.1	3.8	0.8	92	2
Total PO ₄	1.2	1.9	18.5	0.7	2.3	7.9	3.0	104	3
Total Anions (me l ⁻¹)	20.89	52.84	394.81	84.90	178.73	199.73	4557.14	995	--
Electrical									
Conductivity (mmho cm ⁻¹)	1.857	4.710	30.00	8.030	15.00	15.60	209	86.9	3.2
Equivalent									
TDS (mg l ⁻¹)	1200	3014	19200	5139	9600	9984	133.8x10 ³	76x10 ³	1820
Total Dissolved									
Residue (mg l ⁻¹)	1350	3205	26,460	4900	10,580	11870	225x10 ³	--	--
	9.6	9.5	9.8	9.1	9.3	9.8	7.5	9.8	7.9
pH									
Minor Elements									
As	0.20	1.0	0.63	0.03	0.80	5.9	0.25	13.0	0.03
Ba	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
B	8.0	20.0	21.0	11.0	19.0	60.0	155.0	480.0	3.0
Cd	<0.01	<0.01	<0.01	0.01	<0.01	<0.01	0.02	<0.01	5.01
Cr	<0.01	<0.05	<0.05	0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Cu	<0.02	<0.02	0.02	<0.02	0.02	0.04	<0.04	<0.01	<0.01
F	4.0	10.0	20.0	5.0	4.0	5.0	2.5	50.0	7.7
Fe	0.15	0.15	0.15	0.05	0.15	0.25	0.20	0.96	0.08
Pb	<0.05	<0.05	<0.05	<0.05	0.05	<0.05	<0.05	<0.01	<0.01
Mn	0.04	0.04	0.02	<0.02	0.02	<0.02	0.08	0.04	<0.01
Hg	<0.001	<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	<0.01
Ag	0.01	0.01	0.01	<0.01	<0.01	<0.01	0.04	<0.01	<0.01
Zn	0.01	0.01	0.01	<0.01	<0.01	0.05	0.01	0.03	<0.01

Table 1-3. Rough Yields and Efficiencies of Light Utilization for Several Isolates of Desert Saline Algae

Isolate	Nitrate		Urea	
	Yield (g m ⁻² day ⁻¹)	P.A.R.% Efficiency	Yield (g m ⁻² day ⁻¹)	P.A.R.% Efficiency
<u>Nitzschia</u> (Mono Lake)	21.6	13.3	8.8	5.4
<u>Ankistrodesmus</u> (Pyramid Lake)	11.5	7.2	17.4	10.8
<u>Oocystis</u> (Walker Lake)	40.6	25.1	45.8	28.3
<u>Chlorella</u> (Zzyzx)	13.2	8.2	3.5	2.2
<u>Chlorella</u> (Pond B, Big Alkali Lake)	13.9	8.6	9.4	5.8
<u>Cryptomonas</u> (Harper Lake)	Dry weight increases very low - yields not calculated.			

Table 1-4. Proximate Cellular Composition of Some Saline Desert Algae

Isolate	% Lipid	% Carbohydrate	% Protein
<u>Oocystis</u> (Walker Lake)	10.5	37	39
<u>Chlorella</u> (Zzyzx)	21	24	55
<u>Nitzschia</u> (Mono Lake)	22	14	36
<u>Ankistrodesmus</u> (Pyramid Lake)	31	24	36
<u>Nannochloris</u> (Pyramid Lake)	22	21	--
<u>Chlorella</u> (Pond B, Big Alkali Lake)	18	10	68

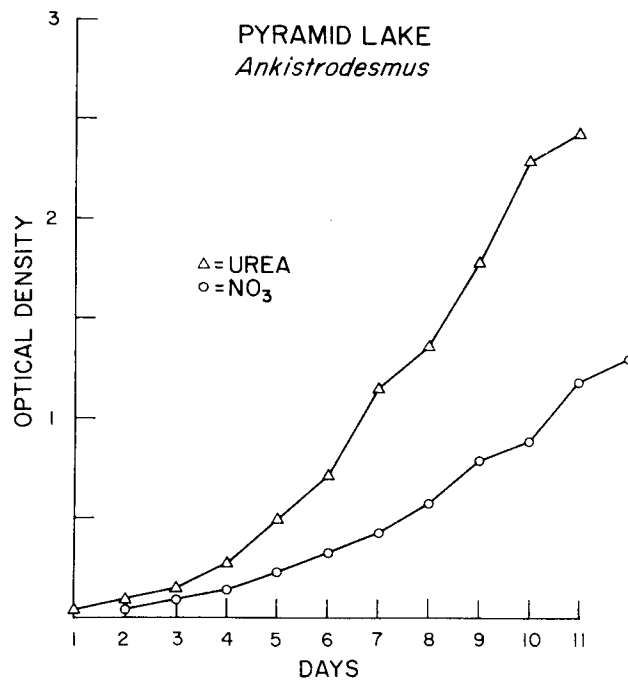
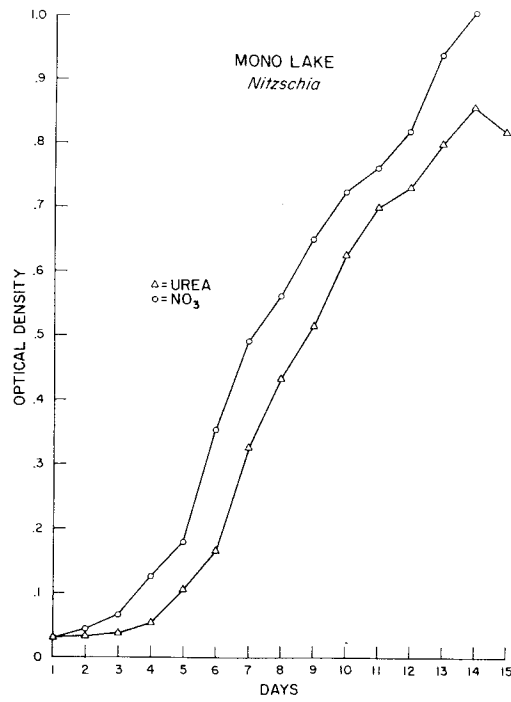


Figure 1-3. Yield of Nitzschia and Ankistrodesmus Grown with Nitrate and Urea

1.1.8 Temperature and Salinity Requirements of Some Desert Algae

Several isolates were tested for their ability to grow at 30 temperature and salinity combinations. This was done by culturing the algae in 65-ml bottles which were placed in a temperature gradient block. Six temperatures and five salinities were tested in 30 combinations at once. Growth was measured each day non-invasively by placing the bottles in a special optical density measuring system that was previously designed and built. The optical densities were calculated by a microcomputer which plotted the data as functions of temperature and salinity.

Figure 1-4 shows typical plots of the responses of Ankistrodesmus and Nitzchia to various temperature and saline combinations and Table 1-5 summarizes results for five isolates.

1.1.9 Light Intensity Requirements of Pyramid Lake Ankistrodesmus

A plastic chemostat-like apparatus was set up to provide five different light intensities to the algae. The maximum sunlight intensity recorded in the summer of 1967 at La Jolla was $450 \text{ cal cm}^{-2} \text{ day}^{-1}$ (photosynthetic part of the spectrum--400-700 nm). The apparatus supplies daily intensities that are 30%, 40%, 50%, 60%, and 70% of this value. Since the culture containers have darkened sides, measurements are over only the illuminated front surface and yield and efficiency values are much more accurate than those calculated for the media experiments (Table 1-3).

The results of one experiment with the Pyramid Lake Ankistrodesmus growing on nitrate are shown in Figure 1-5. Note that very high cell densities--up to $4,000 \text{ mg L}^{-1}$, were achieved and that the linear rate of growth did not vary with cell densities. Thus, it will not be necessary to vary cell densities in Task V as originally planned, as long as they are consistently high. The best yields were achieved at 50%-60% sunlight (see Figure 1-5); 30% and 40% sunlight were too low; and photoinhibition occurred at 70% sunlight.

In interpreting this experiment attempts were made to calculate the mean light intensity in the whole culture volume, and relate this to yield. This hypothesized relationship did not occur in these very dense cultures since light is greatly attenuated in the first 1 cm of culture thickness due to shading by the cells. The proper mean light intensities--50%-60% of sunlight--must have occurred only in a very thin layer facing the light source and the rest of the culture must have been essentially in the dark. It was calculated that the light was attenuated by five orders of magnitude in the first 1 cm of culture thickness. Therefore the problem of maximizing yields (Task V) reduces to a hydraulic mixing problem so that the cells are in the illuminated area for a sufficient time and the ratio of lighted to darkened volume is proper.

1.2 SUMMARY OF OUR PROGRESS

As can be ascertained from the above account of this work, one task progresses to the next one. Better isolates are continuously being selected to study in Task V and in future investigations.

1.3 FUTURE STUDIES--TASK V AND BEYOND

Yields depend on fixed parameters--proper media and temperature--and upon the following variables: the absolute time (perhaps milliseconds for taking advantage of the

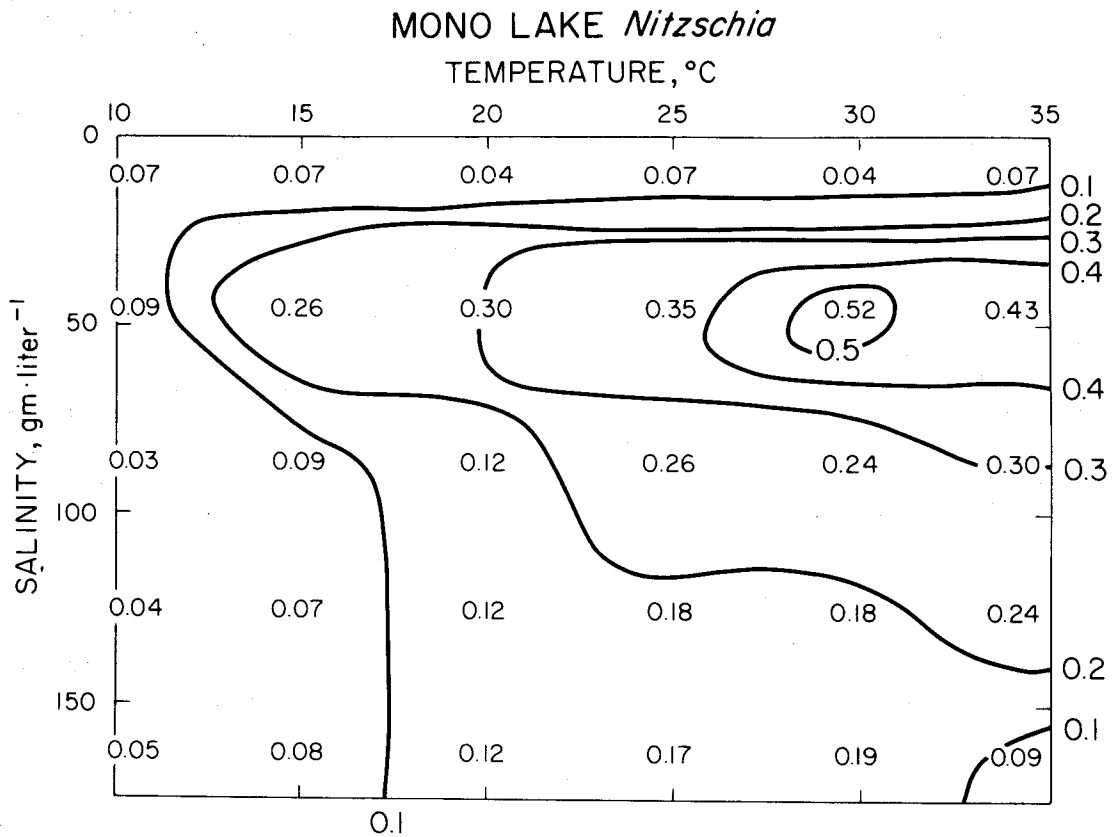
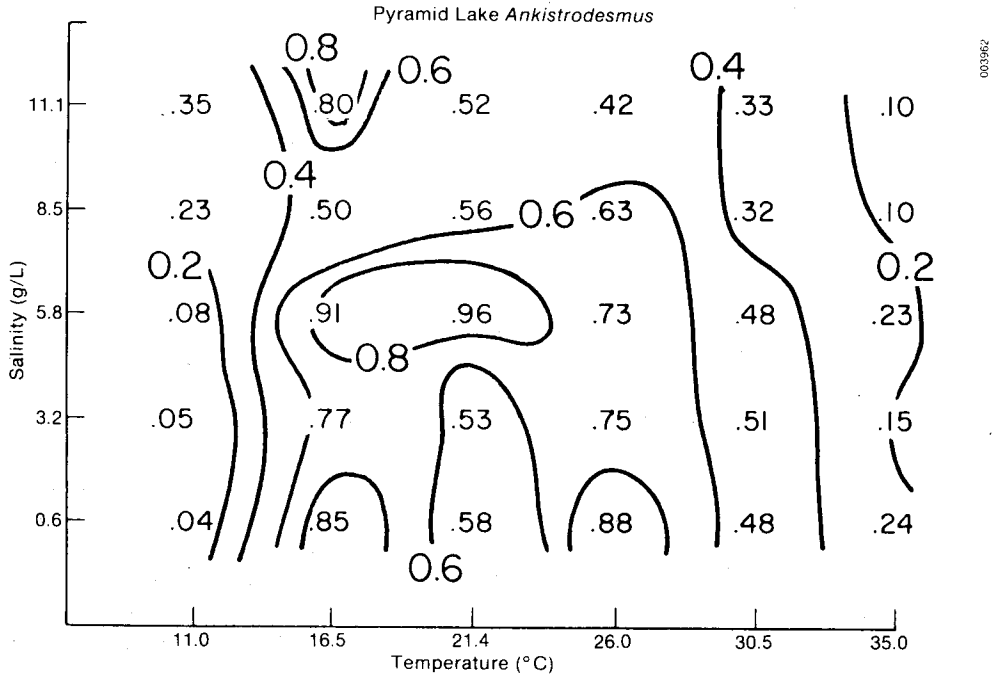


Figure 1-4. Optical Densities in Cultures of *Ankistrodesmus* and *Nitzschia* sp. as a Function of Temperatures and Salinity after 10 Days of Growth

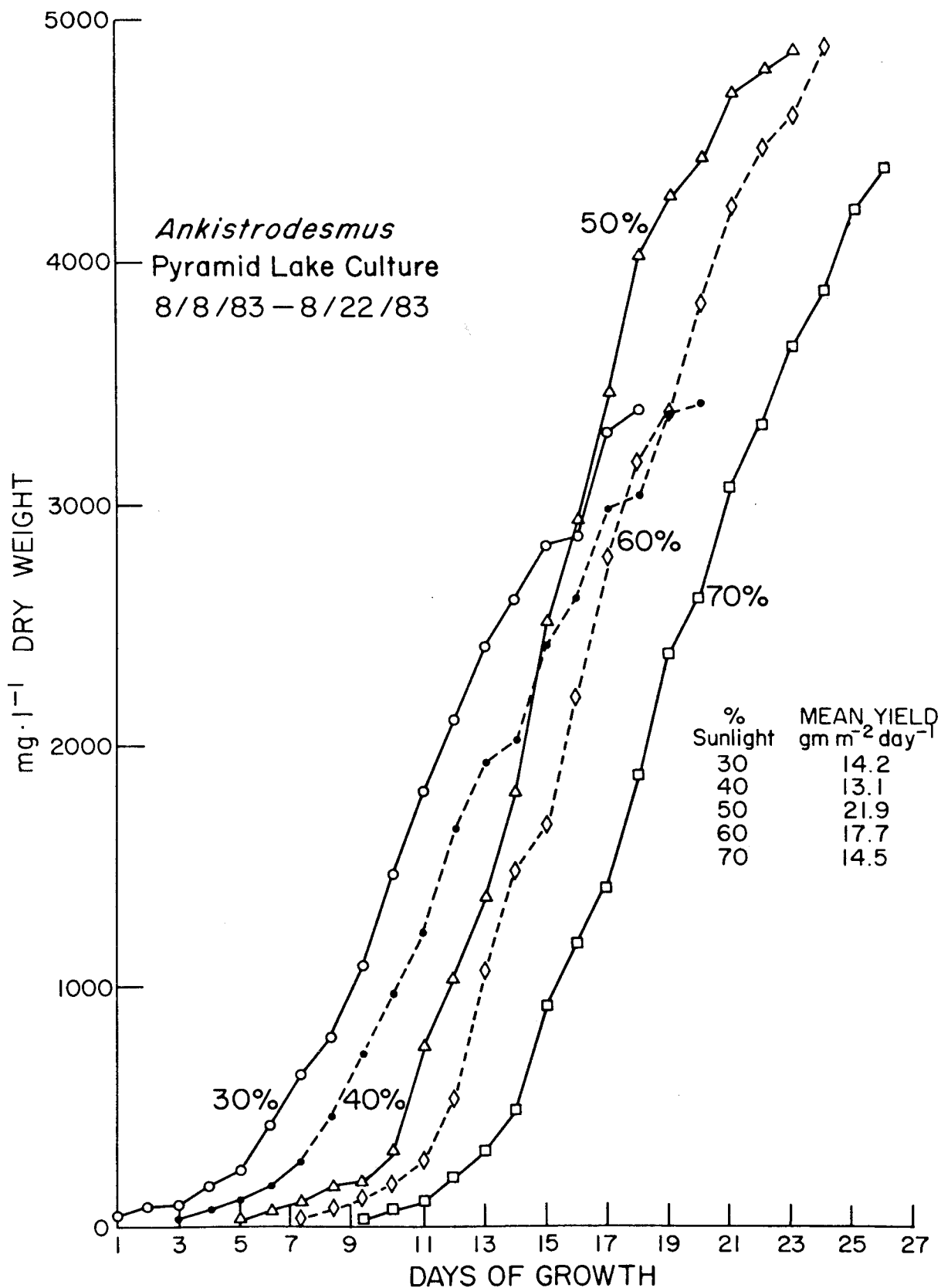


Figure 1-5. Responses of Some Saline Desert Algae to Temperatures and Salinity

Table 1-5. Responses of Some Saline Desert Algae to Temperature and Salinity

Isolate	Temperature			Salinity		
	For best Growth (°C)	Range for some Growth (°C)	Little or no Growth (°C)	For best Growth (g liter ⁻¹)	Range for some Growth (g liter ⁻¹)	Little or no Growth (g liter ⁻¹)
<u>Nitzschia</u> (Mono Lake)	30-36	15-36	10,41-44	50-70	238-86	9,120
<u>Ankistrodesmus</u> (Pyramid Lake)	26	21-30	11-16,35	5.8	0.6-3	8,11
<u>Oocystis</u> (Walker Lake)	20-25	16-33	11	18	6.8-24	1.2
<u>Chlorella</u> (Zzyzx)	25	15-30	9,34	12	3-12	--
<u>Crytomonad</u> (Harper Lake)	16-30	11-35	--	36	2-36	--
<u>Dangardinella</u> (Saline Valley)	30-35	21-35	10,16	266	206	27-146

flashing light effect) the cells are in an illuminated volume; the total culture volume (thickness or depth); and the ratio between the illuminated volume and the darkened volume. The proper value for this latter ratio is dependent on the ratio of photosynthesis to algal respiration. We have measured this ratio in dense Ankistrodesmus cultures and it is 35:1. This means that if the ratio of the dark volume to the illuminated volume is 35:1 respiration and photosynthesis will be equal and there will be no net yield. The second and third variables oppose each other, and the proper overall culture volume will be a maximal value of two, which is somewhat less than 35x the illuminated volume. These concepts have never been thoroughly tested in algal mass culture systems. In a pond stirred by a paddlewheel, as in a raceway culture, there is always some proportion between the illuminated volume and the dark volume. This can vary with the speed of mixing and the turbulence of the culture and can vary in different parts of the culture depending on the distance from the mixing device. In a pond or raceway this proportion cannot be directly measured and it would be difficult to calculate.

Designs are being made of an experimental culture system in which the illuminated volume and the darkened volume are separated and the algal culture is transferred between them at a variable rate. The first parameter will depend on this transfer rate, and the second and third parameters will be controlled by varying the darkened volume with the illuminated volume kept constant.

This design should be useful in outdoor applications.

1.4 PUBLICATIONS

The following reports and papers supported by this and our previous subcontract have been published during the past year:

1. Thomas, W. H., D. L. R. Seibert, M. Alden, P. Eldridge, and A. Neori, 1983, "Yields Photosynthetic Efficiencies, and Proximate Chemical Composition of Dense Cultures of Marine Microalgae," SERI/STR 231-1896: Golden, CO.
2. Thomas, W. H., D. L. R. Seibert, M. Alden, P. Eldridge, A. Neori, and S. Gaines, 1983, "Selection of High-Yielding Microalgae from Desert Saline Environments," SERI/CP 231-1946.
3. Thomas, W. H. and S. Gaines, 1983, "Algae from the Arid Southwestern United States: An Annotated Bibliography," SERI/STR 231-1947.
4. Thomas, W. H., 1983, "Development of a System for Automating the Measurement of Biomass Yields and Efficiencies of Light Utilization by Microalgae," Final Report to Bio-Energy Council, IMR Ref. No. 83-4.
5. Thomas, W. H., 1983, Microcomputing Equipment for Phytoplankton Data Analysis, Final Report to National Science Foundation Grant No. OCE-8117399.
6. Thomas, W. H., D. L. R. Seibert, M. Alden, P. Eldridge, and A. Neori, 1983, "Microalgae from Desert Saline Waters as Potential Biomass Producers," Prog. Solar Energy, Amer. Solar Energy Soc., Vol. 6, pp. 153-155.

The following papers supported by this subcontract have been submitted for publication.

1. Seibert, D. L. R. and W. H. Thomas, 1983, "Computerized Non-Invasive Measurement of the Growth Response of Microalgae to Temperature and Salinity," submitted to Aquaculture.
2. Thomas, W. H., D. L. R. Seibert, M. Alden, A. Neori, and P. Eldridge, 1983, "Yields, Photosynthetic Efficiencies, and Proximate Composition of Dense Marine Microalgae Cultures, 1. Introduction and Phaeodactylum tricornutum experiments: 2. Dunaliella primolecta and Tetraselmis suecica Experiments: 3. Isochrysis spp. and Monallantus salina Experiments and Comparative Conclusions," submitted to Biomass.
3. Thomas, W. H., D. L. R. Seibert, M. Alden, P. Eldridge, A. Kelly, S. Gaines, and A. M. Thomas, 1983, "Responses of the Mono Lake Nitzschia to Temperature and Salinity," submitted to Mono Lake Newsletter.

**CHEMICAL PROFILES OF MICROALGAE
WITH EMPHASIS ON LIPIDS**

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SECTION 2.0

CHEMICAL PROFILES OF SELECTED MICROALGAE

2.1 INTRODUCTION

The proximate chemical composition of eukaryotic algae is generally regarded as species specific and that it is usually regulated by environmental factors. Spoehr and Milner (1949) first studied the cellular composition of Chlorella grown under different physiological conditions and indicated a general trend of protein decrease and lipid increase on nitrogen starvation. Subsequently, Milner (1953) confirmed the same phenomenon in other algae but with concurrent accumulations of cellular carbohydrates in response to nitrogen stress. In the last thirty years additional studies were conducted on the effect of various environmental conditions on the cellular chemistry of many diatoms and a few other algae with similar type responses (Parson et al., 1961; Opute, 1974a,; Handa, 1969; see Lewin, 1962; Stewart, 1974; Aaronson et al., 1980).

Lipids and fatty acid compositions in eukaryotic algae and the effects of environmental factors thereof have been studied with relation to light intensity, temperature, nitrate concentration and various other nutrients (see reviews by Wood, 1974; and Pohl and Zurheide, 1979). Recently, the effect of nitrate and silicate stress and light-dark cycles on the lipid content in a variety of phytoplankton have been reported (Shifrin and Chisholm, 1981). In most algae, enhancement of lipid accumulation occurred during nitrogen deficient conditions. The highest concentration of lipids was reported in Monollantus salina that was deprived of nitrogen for nine days. At low concentrations of nitrogen, Chlorella and Euglena synthesized saturated (16:0) and monounsaturated (18:1) fatty acids, whereas at high nitrogen concentrations the 16:2, 16:3, 16:4 and 18:2 fatty acids predominated. High light intensity and low temperatures caused increases and decreases in total lipids depending on the species. In Chlorella and Euglena the increase in total lipids coincided with the formation of polyunsaturated fatty acids (16:2, 16:3, 16:4, 18:2, 18:3). Neutral hydrocarbons were detected in many algae (Pohl and Zurheide, 1979; Tornabene, 1981) but usually in small concentrations not exceeding 1% of the algal dry weight. The notable exception is the green algae Botryococcus braunii which contains about 15% hydrocarbons per dry weight in its green exponential growth stage and up to 75% hydrocarbons in its brown resting stage (Brown et al., 1969; Maxwell et al., 1968; Knight et al., 1970; Wake and Hillen, 1981; Largeau et al., 1980).

In light of the many influences on the proximate chemistry of eukaryotic algae and the scarcity of data on the specific chemical nature of the components comprising the cellular compositions, a few selected algae were cultivated under controlled conditions. The effects of nitrogen and salt stress on the proximate chemistry and specific lipids were measured. The focus was on the lipids of fresh water, desert and marine microalgal species in an attempt to follow species specific variations or general response of the cellular chemistry to environmental stress.

2.2 MATERIALS AND METHODS

Organism: Ankistrodesmus sp., W. H. Thomas, Pyramid Lake; Botryococcus braunii, Kutz, UTEX #572; Dunaliella salina, Teod., UTEX #200; Dunaliella bardawil, Ben-Amotz and Avron, ATCC #30861; Isochrysis sp., UTEX #2307; Nannochloris sp.,

W. H. Thomas, Pyramid Lake; Nitzschia sp., W. H. Thomas, Mono Lake.

Growth Conditions: Botryococcus braunii, Dunaliella species and Isochrysis sp. were cultivated in artificial mediums containing NaCl as indicated, 5 mM MgSO₄, 0.3 mM, CaCl₂, 5 mM KNO₃ or as indicated, 5 mM KCl, 0.4 mM KH₂PO₄, 1.5 M FeCl₃, 30² M EDTA, 50³ mM NaHCO₃, 0.1 mM Na₂SiO₃, 0.1 mM H₃BO₃, 0.1 mg/liter thiamine-HCl, 0.5 mg/liter biotin, 0.5 mg/liter B₁₂ and trace metal mix as reported by Guillard (1975). Final pH 8.0. Algae were grown in a temperature controlled growth room (ca 25°C) under continuous illumination with Cool White and Agro-Lite Westinghouse fluorescent lamps (light intensity of about 8 Wm⁻²).

Nitzschia sp. was cultivated in Mono Lake's artificial medium containing 0.45 M NaCl, 0.24 M Na²CO₃, 0.18 M NaHCO₃, 0.1 M Na₂SO₄, 39 mM KCl, 31 mM H₃BO₃, 10 mM KNO₃, 1 mM KH₂PO₄, 0.14 mM MgSO₄, 0.7 mM Na₂SiO₃, 0.21 mM Ca(NO₃)₂, 18.5 μM FeEDTA, 0.28 μM ZnSO₄, 0.63 μM CoCl₂, 0.23 μM CuSO₄, 3.17 μM MnCl₂, 0.32 μM (NH₄)₆Mo₇O₂₄. Final pH 9.3 - 9.7. The cultures were maintained on a 12:12 hrs fluorescent light:dark cycle (light intensity of about 39 Wm⁻²) at temperature of 20°C.

Ankistrodesmus sp. and Nannochloris sp. were grown in Pyramid Lake's artificial medium containing 20 mM KNO₃, 2 mM K₂HPO₄, 56 mM NaCl, 14 mM NaHCO₃, 3.7 mM Na₂CO₃, 0.2 mM CaCl₂, 1 mM KCl, 1.5 mM Na₂SO₄, 2.5 mM MgCl₂, 0.26 mM NaF, 0.025 mM Na₂B₄O₇, vitamin mix and trace metal mix as described above. The culture were grown at 20°C on a 12:12 hrs fluorescent light:dark cycle (light intensity of 39 Wm⁻²).

Daily sampling for growth measurements was done on aliquots of the culture suspension and included cell counting in a Thomas blood-cell counter, chlorophyll content by extraction with acetone or methanol (Jensen, 1978) and organic weight determination by drying the samples at 60°C and ashing at 540°C.

Unicellular algae were harvested by centrifugation at the end of the logarithmic phase. Wet algal pellets and the seaweed were lyophilized for chemical analysis. Nitrogen deficient unicellular algae were treated similarly but the algae were grown on 0.5 mM KNO₃ to the end of the logarithmic phase, left for about 10 more days at the steady state phase and finally harvested by centrifugation.

2.2.1 Extraction and Fractionation of Lipids

Total lipids were assayed by repeated extraction with methanol-chloroform-water (10:5:4, v/v) (Bligh and Dyer, 1959) modified as previously described (Kates et al., 1964) to complete visual extraction of chlorophyll and other pigments. The lipids were then phase separated by adjustment of the solvent ratios to 10:10:9 (methanol-chloroform-water, v/v). The chloroform phase was evaporated to dryness under a stream of N₂, 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, methanol to improve the resolution of the lipid components by thin-layer and paper chromatography (Tornabene, et al., 1969; Morrison et

al., 1971). The following types of components were eluted: acyclic hydrocarbons (hexane); cyclic hydrocarbons, polyunsaturated acyclic hydrocarbons, fatty acid methyl esters, sterols, 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 and further dried under vacuum over KOH, or P_2O_5 .

2.2.2 Mild Alkaline Methanolysis

Lipid components were deacylated by mild alkaline methanolysis according to the procedure described by Tornabene and Ogg (1971). Fatty acids were recovered from the chloroform layer.

2.2.3 Acid Hydrolysis of Deacylated Water-Soluble Products

Water-soluble products obtained from alkaline hydrolysis of lipid components were hydrolyzed with 2 M HCl at 100°C for 1 hr. The hydrolysates were taken just to dryness in a stream of N_2 and then dissolved in methanol-water (10:9, v/v).

2.2.4 Paper Chromatography

The acid hydrolysates were chromatographed on Whatman No. 1 paper with pyridine-ethyl acetate-water (4:10:10, by vol., upper phase). Compounds were detected by Ninhydrin or alkaline $AgNO_3$ (Trevelyan et al., 1950).

2.2.5 Thin-Layer Chromatography

Total and column-fractionated lipids, as well as hydrolyzed lipids, were studied by thin-layer chromatography on 20 cm x 20 cm glass plates coated (0.6-1-mm layers) with silica gel G or precoated hard-layered commercial TLC silica gel plates (Supelco, 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; d) chloroform-acetone-methanol-acetic acid-water (50:20:10:10:5, by vol) for separation of polar lipids; and e) technical chloroform (0.75% ethanol) for separation of alkyl-lipid chains (Tornabene et al. 1982). Spots were visualized by exposure to I_2 vapors, acid charring, ninhydrin for amino acids, molybdate for phosphates, Dragendorff for quaternary amines, α -naphthol solution for glycolipids, and sulfuric and acetic acid for sterols and sterol esters as previously described (Kates, 1972).

The deacylated water-soluble products were separated on cellulose thin-layer chromatographic plates (Eastman chromatograms 6064, Rochester, N.Y.) with solvents of 3.8 mM EDTA and 0.7 M NH_4HCO_3 in 90 mM NH_4OH containing 67%, by vol, ethyl alcohol in the first dimension and isobutyric acid-water-concentrated NH_4OH (66:33:1, by vol) in the second dimension (Short et al. 1969). The compounds were detected by o-tolidine method over stained with acidic ammonium molybdate solution as previously described (Burrow et al., 1952).

2.2.6 Analytical Method

Glycerol was determined by periodic oxidation followed by treatment with acetyl-acetone as previously described (Ben-Amotz and Avron, 1978). Carotenoids were extracted according to Jensen (1978) and assayed by using $E_{1\%}^{1\text{cm}}$ of 2273 at 480 nm. Protein was assayed as previously described by Lowery et al., (1951) or by Kochert (1978a) after hydrolysis in NH_4OH for 1 hr at 100°C . Total carbohydrates were analyzed by the phenol-sulfuric acid method following acid hydrolysis in 2N HCl for 1 hr at 100°C (Kochert, 1978b). Extended hydrolysis of up to 8 hrs did not produce detectable increase in the carbohydrate concentration.

Fatty acid methyl esters were prepared by esterification with 2.5% methanolic-hydrochloride (Kates, 1964). Sugars freed from the lipids by acid hydrolysis were converted to alditol acetates (Albersheim et al., 1967). Derivatized lipids and sugars were analyzed on a Varian 3700 gas-liquid chromatograph equipped with flame ionization detectors and a Varian CDS 401 data system. The following columns were used: (a) 99 m x 0.75 mm stainless steel capillary column coated with 3% OV-17 running at 15 psi of He from 150°C to 250°C at $4^\circ\text{C}/\text{min}$ and held isothermally, (b) 32 m x 0.25 mm fused quartz capillary column, coated with 0.25 mm OV 351 (J. & W. Scientific, Inc.) operated at 8 psi of He and 125°C to 220°C at $4^\circ\text{C}/\text{min}$ and held isothermally, (c) 30 m x 0.249 mm fused quartz silica capillary column, coated with 0.25 nm DB-5 (J. & W. Scientific, Inc.) operated at 8 psi of He from 125°C to 230°C at $4^\circ\text{C}/\text{min}$ and held isothermally, (d) 2 M x 0.31 cm glass column packed with 10% SP2330 on gas chrom W AW and operated at 27 psi of He and $4^\circ\text{C}/\text{min}$ from 110°C to 250°C and held isothermally.

2.3 RESULTS

2.3.1 Growth and Yield of Algae

The growth conditions, specific growth rates and cellular yields are summarized in Table 1. Under non-limited nutrient conditions Dunaliella salina was the fastest grower of all species studied while Botryococcus braunii was the slowest in growth. Salt stress inhibited growth rate of all species tested but it did not affect the total cell yield in mg organic weight per liter (Table 1). Nitrate deficiency did not inhibit the logarithmic growth rate until the culture reached nitrogen deprivation. The growth rate was then in the steady state phase with the resulting total yield being lower than that of the control.

Most algae, grown under optimal nitrogen sufficient conditions contained about 4% chlorophyll and 1% carotenoids per organic cell weight with a carotenoid to chlorophyll ratio of about 0.25 (Table 2). Growth under stress conditions of nitrogen deficiency or high salt concentration decreased the chlorophyll concentration more markedly than the carotenoids producing yellowish-whiteish cells. Dunaliella bardawil was the only species which synthesized β -carotene under stress condition producing reddish cells with a very high carotenoid to chlorophyll ratio (Table 2) as previously described by Ben-Amotz and Avron, (1983).

2.3.2 Proximate Cellular Composition

The results of the analyses of the proximate chemistry of the algae are summarized in Table 3. Cells grown on nitrate limitation generally contained less protein.

Table 2-1. Growth and Yield of Species Investigated

No.	Species	Conditions	Specific Growth Rate ^b (.d ⁻¹)	Yield ^c (mg Organic Weight .l ⁻¹)
1	<u>Botryococcus braunii</u>	FW, NE	1.25	911
2		FW, ND	1.25	623
3		0.5M NaCl, NE	1.12	862
4	<u>Dunaliella bardawil</u>	2M NaCl, ND	1.8	795
5	<u>Dunaliella salina</u>	0.5M NaCl, NE	2.5	823
6		0.5M NaCl, ND	2.5	541
7		2M NaCl, NE	1.9	789
8	<u>Ankistrodesmus sp.</u>	FW, NE	-	-
9	<u>Isochrysis sp.</u>	0.5M NaCl, NE	1.6	843
10		0.5M NaCl, ND	1.6	563
11		1M NaCl, NE	1.4	828
12	<u>Nannochloris sp.</u>	FW, NE	-	-
13	<u>Nitzschia sp.</u>	1.4M Na ⁺ , NE	-	-

^aFW, freshwater; NE, nitrogen sufficient; ND, nitrogen deficient.

^bSpecific growth rates of 2 and 1.25 represent doubling time of 24 hrs and 72 hrs, respectively.

^cAsh free dry weight determined at the end of the logarithmic phase of nitrogen sufficient cultures, and about 10 days more following the end of the logarithmic phase for nitrogen deficient cultures.

Table 2-2. Chlorophyll and Carotenoid Content in Unicellular Algae Grown Under Optimal, Nitrate Deficiency, or Salt Stress Conditions

NO.	Species	Growth Conditions	% Organic Weight		Carotenoids to Chlorophyll Ratio
			Total Chlorophyll	Carotenoids	
1	<u>Botryococcus braunii</u>	FW, NE	1.64	0.48	0.29
2		FW, ND	0.46	0.26	0.56
3		0.5M NaCl, NE	0.69	0.22	0.32
4	<u>Dunaliella bardawil</u>	2M NaCl, ND	0.46	2.71	5.89
5	<u>Dunaliella salina</u>	0.5M NaCl, NE	4.27	1.17	0.27
6		0.5M NaCl, ND	0.59	0.39	0.67
7		2M NaCl, NE	4.34	0.95	0.22
8	<u>Ankistrodesmus sp.</u>	FW, NE	5.50	1.00	0.18
9	<u>Isochrysis sp.</u>	0.5M NaCl, NE	1.83	0.64	0.35
10		0.5M NaCl, ND	0.49	0.25	0.51
11		1M NaCl, NE	0.41	0.33	0.80
12	<u>Nannochloris sp.</u>	FW, NE	3.88	0.97	0.25
13	<u>Nitzschia sp.</u>	1.4M Na ⁺ , NE	1.44	0.48	0.33

Abbreviations as in Table 1.

Table 2-3. Proximate Cellular Composition of Unicellular Algae Grown Under Optimal, Nitrate Deficiency, or Salt Stress Conditions

No.	Species	Growth Conditions	% Dry Weight	% Organic Weight				
				Ash	Protein	Carbohydrate	Lipid	Glycerol
1	<u>Botryococcus braunii</u>	FW, NE	5.6	22.0	14.1	44.5	< 0.1	19.3
2		FW, ND	7.8	20.6	14.3	54.2	< 0.1	10.8
3		0.5M NaCl, NE	59.6	15.0	13.3	46.3	< 0.1	25.3
4	<u>Dunaliella bardawil</u>	2M NaCl, ND	14.7	9.7	40.4	10.4	16.4	23.1
5	<u>Dunaliella salina</u>	0.5M NaCl, NE	8.6	29.3	16.3	25.3	9.4	19.7
6		0.5M NaCl, ND	7.7	12.5	55.5	9.2	4.7	18.1
7		2M NaCl, NE	21.7	35.9	12.5	18.5	27.7	5.4
8	<u>Ankistro-desmus sp.</u>	FW, NE	4.5	31.1	10.8	24.5	< 0.1	33.5
9	<u>Isochrysis sp.</u>	0.5M NaCl, NE	12.0	37.0	11.2	7.1	< 0.1	44.6
10		0.5M NaCl, ND	52.0	23.3	20.5	26.0	< 0.1	30.1
11		1M NaCl, NE	65.9	34.7	15.5	15.3	< 0.1	34.4
12	<u>Nanno-chloris sp.</u>	FW, NE	13.6	33.1	13.2	20.8	< 0.1	32.8
13	<u>Nitzschia sp.</u>	1.4M Na ⁺ , NE	20.4	16.8	9.2	12.1	< 0.1	61.8

Analysis was conducted on 5 different algal cultures of each species; statistical analysis showed no standard error of more than 10%. Abbreviations as in Table 1.

In Dunaliella, the decrease in the protein content under nitrate starvation coincides with a decrease in lipid content and an increase in carbohydrates. Both Dunaliella bardawil and Dunaliella salina contained over 40% carbohydrates. Botryococcus braunii contained 44.5% lipids which increased to 54.2% under nitrate limitation. Nitrogen starvation induced lipid and carbohydrate accumulations in Isochrysis from 7% to 26% and from 11.2% to 20.5% of the organic cell weight, respectively. These increases were apparently at the expense of cellular protein which decreased from 37 to 23.3%. Glycerol was observed only in Dunaliella and its concentration varied with the external salt concentration. The proximate composition of Botryococcus braunii and Isochrysis was somewhat affected by salt stress with the major response being in the reduction of the protein content. Protein biosynthesis in Dunaliella, however, was relatively insensitive to extracellular salt concentrations. The proximate compositions of Ankistrodesmus and Nannochloris cultivated on nitrate sufficient medium contained 31.4% and 33.1% protein, respectively, and about 20% lipids. Nitzschia grown in a high Na^+ medium contained smaller quantities of protein, lipid and carbohydrate. A major fraction of the Nitzschia composition was not identified (Table 3).

2.3.3 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 are summarized in Table 4. The composition of each fraction is described below.

2.3.4 Neutral Lipids

Hexane eluates. The quantity of components isolated in the hexane eluates was the greatest in Botryococcus braunii lipids and the least in the Dunaliella spp. The hexane fraction of Botryococcus represented about 15% of the total lipids (Table 4). The hexane fraction of Isochrysis grown in 0.5 m NaCl medium under nitrogen depletion, comprised about 2.2% of total lipid while under nitrogen sufficient conditions it represented 1.4%. The concentration of acyclic hydrocarbons in the other species was lower than 1%. The hexane fraction of Botryococcus contained long chain aliphatic hydrocarbons identified by GLC as C29:0, C30:0 and C31:0 (Table 5) as previously reported (Wake and Hillen, 1981). Similarly the hydrocarbons in the hexane eluate of Isochrysis (Table 5) were identified as those ranging from C27:0 to C34:0 with C31:0 as the predominant component. The qualitative composition of the hydrocarbon components in Isochrysis grown at different salinities and on different nitrate concentration were closely comparable, however, there were variations in the relative percentages of the components. The hexane eluate of Dunaliella salina contained C17:0 and C17:1 hydrocarbons (Table 5), similar to that previously reported (Tornabene et al., 1980; Fried et al., 1982). The hexane eluates of Ankistrodesmus contained predominantly C23:0 and C27:0 hydrocarbons. Nitzschia grown on high salt medium contained C21:0, C25:0 and C27:0 as the major hydrocarbon components.

Benzene eluate. The benzene eluates were the major fraction of the neutral lipids of all the algae as well as the major fraction of the total lipids of most of the algae. The benzene eluate comprised 46-53% in Botryococcus braunii, 49.8% in nitrogen deficient Dunaliella bardawil, 0.4 to 24.8% in nitrogen deficient Dunaliella salina, and 13.6 to 32% in Isochrysis lipids (Table 4). The TLC distributions

Table 2-4. Fractionation of Algal Lipids on Unisil Columns

No.	Species	Growth Conditions	% Total Lipid Weight				
			Hexane	Benzene	Chloroform	Acetone	Methanol
1	<u>Botryococcus braunii</u>	FW, NE	4.6	51.4	4.5	30.0	9.4
2		FW, ND	14.9	52.7	3.4	21.6	7.4
3		0.5M NaCl, NE	5.2	46.0	28.5	9.3	9.7
4	<u>Dunaliella bardawil</u>	2M NaCl, ND	0.1	49.9	14.8	24.1	10.8
5	<u>Dunaliella salina</u>	0.5M NaCl, NE	0.1	0.4	6.3	76.8	16.5
6		0.5M NaCl, ND	0.1	24.8	20.6	31.7	22.8
7		2M NaCl, NE	0.2	2.1	28.2	55.9	13.6
8	<u>Ankistrodesmus sp.</u>	FW, NE	0.7	1.8	39.6	40.9	17.0
9	<u>Isochrysis sp.</u>	0.5M NaCl, NE	1.4	27.4	32.1	26.3	12.6
10		0.5M NaCl, ND	2.2	28.4	18.0	26.0	25.3
11		1M NaCl, NE	1.4	23.6	13.6	43.0	18.4
12	<u>Nannochloris sp.</u>	FW, NE	0.2	5.9	32.5	35.9	25.5
13	<u>Nitzschia sp.</u>	1.4M Na ⁺ , NE	0.2	1.7	51.2	22.0	24.6

Proportions of lipid eluates were determined by GLC for the hexane eluate and gravimetrically for the others. Abbreviations as in Table 1.

Table 2-5. Relative Percentages of Major Aliphatic Hydrocarbons in the Hexane Eluate of Unicellular Algae Grown Under Optimal, Nitrate Deficiency and Salt Stress Conditions

Hydro-carbon	Ankistro-desmus sp.	Botryo-coccus braunii			D. salina	Iso-chrysis sp.			Nanno-chloris sp.	Nitzschia sp.
	FW NE	FW NE	FW ND	0.5M NaCl NE	2.0M NaCl NE	0.5M NaCl NE	0.5M NaCl ND	0.1M NaCl NE	FW NE	1.4M Na ⁺ NE
	0.17	2.05	8.07	2.4	0.04	0.1	0.57	0.21	0.04	0.02
17:0					2.3					
17:1					90.0					
21:0	6.3								18.0	23.6
22:0	3.3									
23:0	18.0									
24:0										
25:0									29.0	35.4
27:0	40.3					1.2	1.3	1.1	9.7	11.1
28:0		4.2	3.0	3.4						
29:0		13.2	13.5	12.0		4.7	8.1	4.1	4.7	2.3
30:0	5.0	22.3	25.2	8.6		1.4			2.9	
31:0		31.0	33.9	32.6		63.2	50.3	66.2	2.9	4.8
31:1		5.4	5.7			9.4	4.4	1.2		
32:0		5.9	8.0	13.4						
32:1		4.2	2.3							
32:2		3.4	2.5							
33:0										
33:1		2.4	2.2	12.0		7.2	5.0	4.8		
33:2		1.8		3.2		3.4	4.0	1.2		
34:0						0.9	1.6	2.0		
Un-identified	27.1	5.3	2.7	14.4	7.7	8.6	25.3	19.4	32.8	22.8

Hydrocarbons were analyzed by GLC on a 99, x 0.75mm OV-17 stainless steel capillary column. D. bardawil ND and NE and D. salina, ND produced none or insufficient quantities to be assayed. Abbr. as in Table 1.

of the components comprising the benzene eluates is shown in the composite tracings in Figs. 1A and 1B. The chromatogram contained pigmented components and various components of which only a few have been identified. The major component of *Dunaliella bardawil* was β -carotene (R_f 0.89). The major component of *Isochrysis* sp. was a oxygenated cyclic C-37 isoprenoid chain (R_f 0.27) the identity of which has not been fully elucidated. The major components of the benzene eluate of the lipids of *Botryococcus* and nitrate deficient *Dunaliella salina* (Fig. 1A) were neutral lipids with relative high polarity (R_f 0.05-0.08). These components and a large number of apparently branched, unsaturated components in the range of C_{15} - C_{37} exists in the benzene eluate of all the algae. These components have not been identified but preliminary studies by various chromatographic and spectral procedures indicate they are a complex mixture of high molecular weight isoprenoid compounds and isoprenoid derivatives.

Chloroform eluate. The chloroform eluates of the microalgae comprised from 3.4% to greater than 50% of the total lipids (Table 4). The variation in the concentrations of the components in the chloroform eluates of cells cultivated in media of different nitrogen and salt levels were significant but without a correlated pattern (Table 4). In *Botryococcus* the increase in contents of chloroform eluates correlated with increases in saline solution concentrations, while in *Dunaliella salina* it correlated with either saline or nitrogen concentrations. Conversely, in *Isochrysis*, the contents of the chloroform eluates decreased in nitrogen deficient cells but increased in medium with higher salt concentration. Identification of the components by TLC was made difficult by the presence of most of the carotenoid pigments which exhibited a wide range of polarities. Specific components did cochromatograph with authentic standards and were tentatively identified as tri-, di- and mono-glycerides, sterols and free fatty acids. Triglycerides and free fatty acids were the principal components of these fractions.

2.3.5 Polar Lipids

Acetone eluate. The acetone eluates collected from the silicic acid columns comprised different concentrations of lipids from a minimum of about 9% in *Botryococcus braunii* to 77% in *D. salina* when grown on 0.5 M NaCl and enriched with nitrate (Table 4). The TLC separation of the acetone eluates are shown in Figs. 2A and 2B. The major spot in all eluates was digalactosyldiglyceride (R_f 0.39) and monogalactosyldiglyceride (R_f 0.87). A relatively small amount of phosphoric acid (R_f 0.58), phosphatidyl ethanolamine (R_f 0.48), phosphatidyl glycerol (R_f 0.32), phosphatidyl choline (R_f 0.23), and phosphatidyl inositol (R_f 0.17) exist in most of the eluates. The remaining component on the chromatogram are chlorophyll a and b and an assortment of yellow to orange pigments.

Methanol eluate. The methanol eluate contained most all (about 98%) of the phospholipids (Table 4 and Figs. 3A and 3B). The components were identified by cochromatography with standards and differential colorimetric stains for primary amines, phosphates and sugars. The phospholipids detected in most samples were phosphatidylinositol (R_f 0.11), phosphatidylcholine (R_f 0.22), phosphatidylglycerol (R_f 0.29), phosphatidylethanolamine (R_f 0.36) and diphosphatidylglycerol (R_f 0.41). Chlorophyll c (determined also by spectrophotometrical procedures) and yellowish-orange pigments were also visible in the methanol eluates. Spots also cochromatographed with a Sulfoguinovosyl standard (R_f 0.06). Whether or not glycerol trimethylhomoserine (Evans et al., 1982) was present was not determined in the analytical systems employed. The identities tentatively assigned to the phospholipids

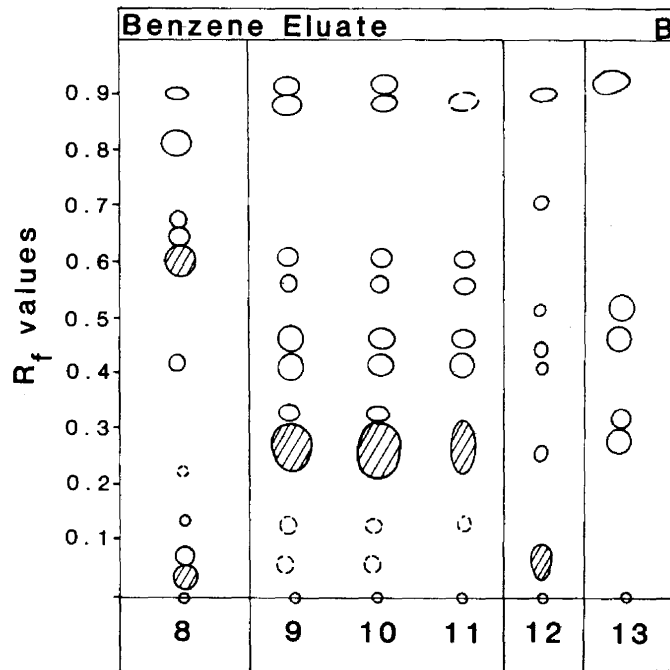
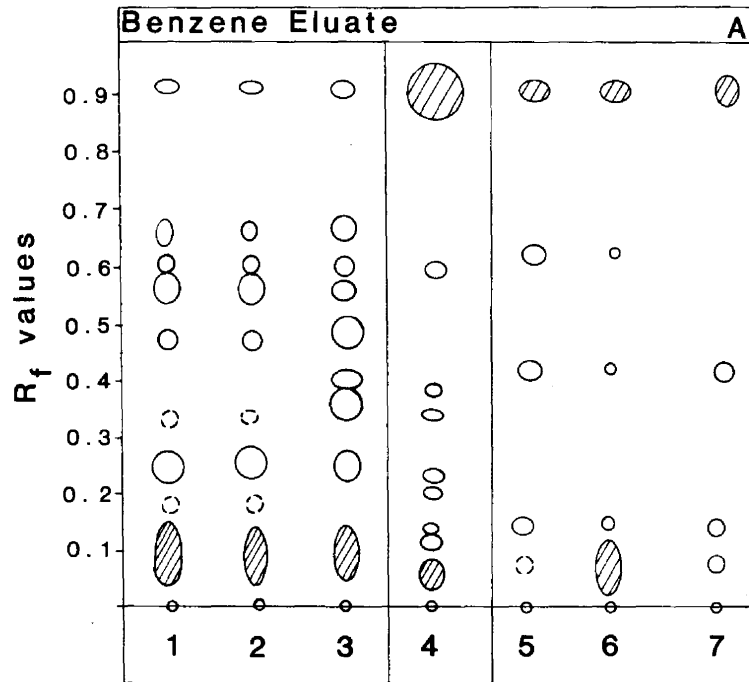


Figure 2-1. TLC Separation of the Benzene Eluates Collected from Silicic Acid Columns in Solvent System (a). See Tables for identification of numbers correlated to the organisms. Standards and their R_f values were: β -carotene, 0.89; squalene, 0.79; cholesteryl palmitate, 0.58; trioleiate, 0.21; myristic acid, 0.13; cholesterol, 0.03

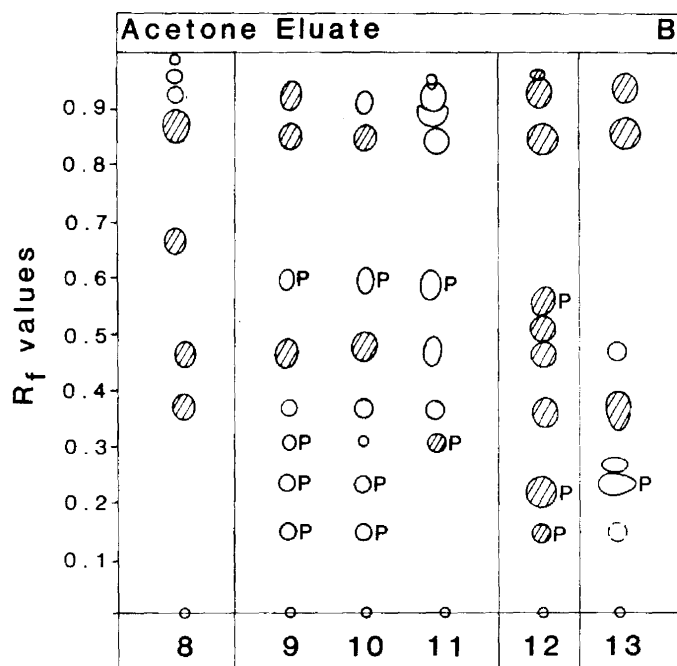
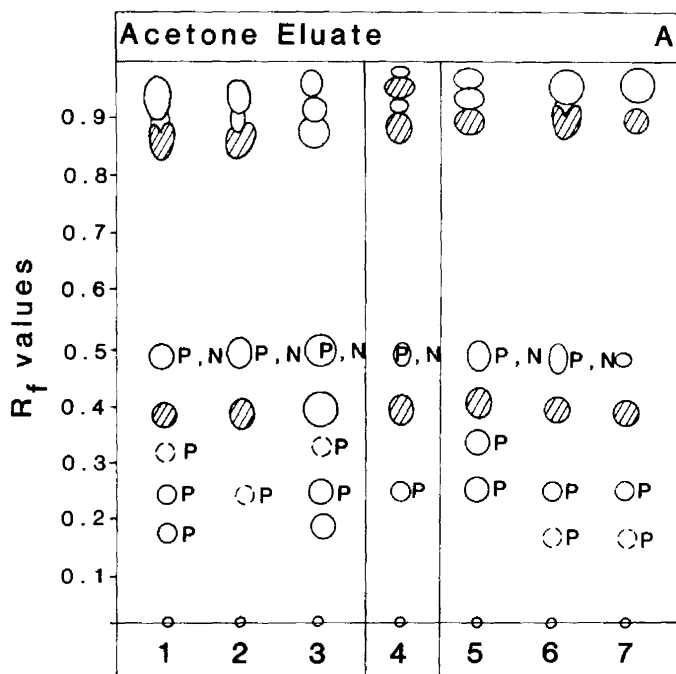


Figure 2-2. TLC of Acetone Eluates Collected from Silicic Acid Columns in Solvent System (d). Abbrev: as in Figure 2-1; P = phosphate positive; N = ninhydrin positive. Standards and their R_f values were: monogalactosyldiacylglycerol (0.87); digalactosylkdiacylglycerol (0.39); phosphatidylinositol (0.17); phosphatidylcholine (0.23); phosphatidylglycerol (0.39); phosphatidylethanolamine (0.48); diphosphatidylglycerol (0.54)

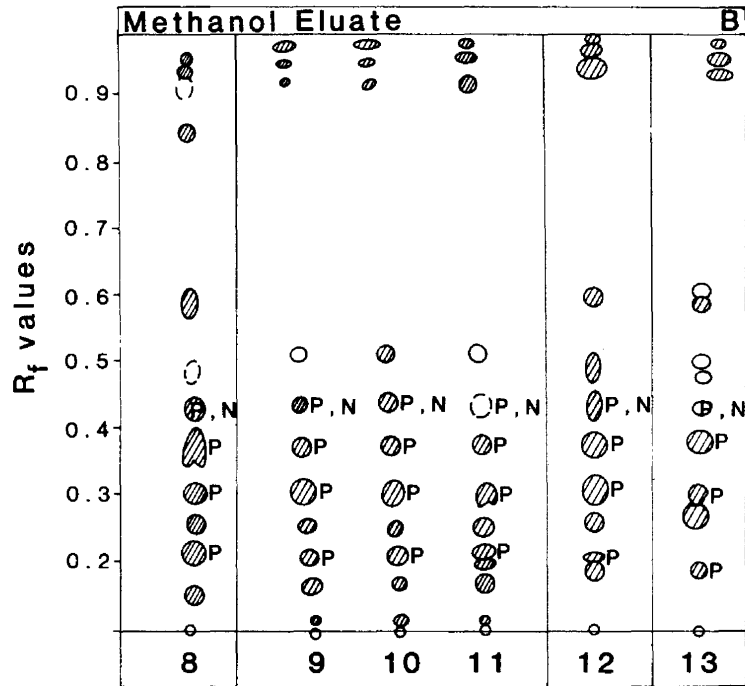
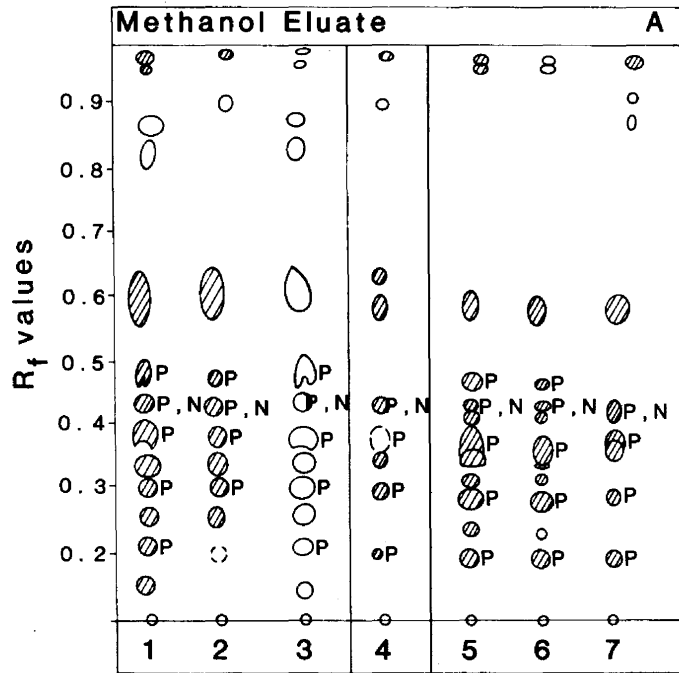


Figure 2-3. TLC of Methanol Eluates in Solvent System (d). Conditions and abbreviations are as described in Figure 2-2. Standards and their R_f values were: phosphatidyl inositol (0.11); phosphatidyl choline (0.22); phosphatidylglycerol (0.29); phosphatidylethanolamine (0.36); diphosphatidylglycerol (0.41)

and glycolipids were supported by further studies by two dimensional cellulose-TLC of their deacylated derivatives as described by Tornabene et al., (1971, 1973, 1980). The deacylation of the methanol eluates resolved two additional phospholipids that were not identified by one dimension TLC of the total fraction because of interference of the pigments. These two components were phosphatidylserine and phosphatidic acid. The major phospholipid in all the algae was phosphatidylcholine and phosphatidylglycerol. Phosphatidic acid was not detected in the methanolic eluates of *Isochrysis* sp. but appeared in trace amounts in the acetone eluate. Relatively small quantities of galactosyl diglyceride and monogalactosyl diglycerides were detected in the methanol eluates as well.

2.3.6 Acid Hydrolysate

Acid hydrolysis of the deacylated methanol-water soluble fractions of the acetone and methanol eluates in 2N HCl for two hours at 100°C and then at 4N HCl for six hours at 100°C released sugars and an amino compound, respectively. The sugars analyzed by paper chromatography and by GLC as acetylated alditols revealed glycerol and galactose in all preparations. Relatively small quantities of mannose and glucose were also detected. No amino sugars were detected, however, most samples contained a nihydrin positive component that cochromatographed on paper with a serine standard.

2.3.7 Fatty Acids

The fatty acid composition of the total lipids is given in Table 6. The fatty acid composition of the lipids eluted with chloroform and methanol from a silicic acid column differed only by moderate changes in the relative intensities of the component. The major fatty acids present in *Ankisterodesmus* sp. were 16:0, 16:4, 18:1, 18:2, 18:3, and 18:4; in *Botryococcus braunii*, 16:0, 18:1, 18:3 and 18:3; in *Dunaliella bardawil*, 16:0, 18:1, 18:2 and 18:3; in *Dunaliella salina* 16:0, 16:3, 18:1, 18:2 and 18:3; in *Isochrysis*, 14:0 (14:1), 16:0, 18:1, 18:2, 18:3, 18:4 and 22:6; in *Nannochloris*, 14:0 (14:1), 16:0, 16:1, 16:2, 16:3 and 22:5; and in *Nitzschia*, 14:0 (14:1) 16:0, 16:1, 16:2, 16:3 and 20:6. Nitrogen starvation increased the percentage of 18:1 in *Botryococcus* and *Dunaliella salina*, but only slightly affected is fatty acid in *Isochrysis*. The effect of nitrogen starvation was expressed to some degree on the relative intensities of the long chain unsaturated fatty acids. The percentage of 20:5 in *Botryococcus* and *Dunaliella bardawil* increased. Similar observations were observed in *Dunaliella salina* except that 20:6 instead of 20:5 was synthesized. The effect of salt stress was significant on the production of 18:1 in *Isochrysis* and *Botryococcus*.

2.4 DISCUSSION

All species investigated in this study were photosynthetic algae grown autotrophically on mineral medium containing carbon dioxide (and bicarbonate) as a carbon source and nitrate as a nitrogen source. The principal effort of this study was to determine the effect of nitrogen as well as salt stress on the proximate chemical compositions of the algae. The general conclusion is that the species tested differed in their basic cellular composition when they were exposed to environmental stress. On growth under non-stressed conditions, *Botryococcus* contained the highest concentration of lipids with the greatest proportion of which was hydrocarbon in nature. All other algae contained lower levels of lipids with an average of 23% per organic weight for the green algae, 12% for *Nitzschia* sp.

Table 2-6. Relative Percentages of Total Fatty Acid Composition of Unicellular Algae Grown Under Optimal, Nitrate Deficiency and Salt Stress Conditions

Fatty* Acid	Ankistro- desmus sp.		Botryo- coccus braunii		Dunaliella bardawil	Dunaliella salina			Iso- chrysis sp.			Nanno- chloris sp.	Nitzschia sp.
	FW	FW	FW	0.5M NaCl	2.0M NaCl	0.5M NaCl	0.5M NaCl	0.2M NaCl	0.5M NaCl	0.5M NaCl	1.0M NaCl	FW	1.4M Na ⁺
	NE	NE	ND	NE	ND	NE	ND	NE	NE	ND	NE	NE	NE
12:0	3.1	3.4	1.1	1.1	5.6	4.0	1.5	3.8				2.8	1.5
14:0, 14:1	4.7	4.6	0.8	1.2	7.1	5.4	1.2	5.6	13.3	18.6	11.5	6.3	9.0
16:0	13.1	18.1	13.3	12.3	46.2	14.7	21.0	11.7	11.7	1.4	8.5	9.2	10.4
16:1	3.4	2.0	0.9	1.3	0.9	0.7	0.9	1.9	6.3	2.9	3.8	19.8	20.7
16:2	1.4											6.6	7.9
16:3	1.2				2.6	16.6	10.3	16.2				8.6	5.9
16:4	13.8					7.4	3.3	6.2					
17:0		1.5	2.9	2.8	0.7								
18:0												2.1	1.3
18:1	8.6	27.3	46.3	45.3	17.2	2.2	7.1	4.1	15.0	17.2	21.6	3.6	3.6
18:2	5.4	12.3	5.7	2.4	7.9	10.9	6.2	11.3	3.7	5.0	4.3	1.1	1.7
18:3	28.6	18.1	19.6	10.7	8.1	30.5	38.0	28.4	5.6	6.3	4.4	0.5	0.9
18:4	9.8	1.2							16.6	14.0	17.7		1.1
19:0						1.9	1.7	1.0			0.9		
20:1				1.2	1.2	1.2				1.1			
20:3		1.7	1.1	3.4								2.5	4.4
20:5	1.3	2.7	5.6	7.6	2.1				1.6			26.5	
20:6							1.6						18.2
22:4									1.3	2.7	6.0		
22:6									12.8	14.9	14.6		
Un- identified	6.5	7.1	2.7	10.8	0.4	5.7	7.2	5.0	10.0	5.9	6.7	11.4	13.4

*The values were determined by GLC on a 32m x 0.25mm OV 351 fused quartz capillary column. The first number indicates the number of carbons; the second number indicates the number of unsaturated bonds. Identities were obtained by comparing retention time values to those of standards and by comparing patterns on OV 351 and OV 17 coated capillary columns.

and 7% for Isochrysis sp.

The effect of nitrogen stress on the lipid fraction in the algae cannot be summarized as a single trend. In Botryococcus, the neutral lipids comprised a major proportion of the total lipids (Tables 3 and 4); however, the greatest neutral lipid production occurred in the resting stage and that the greatest amount formed in the conversion of the alga from the green to the brown growth phase, Maxwell et al., 1968; Largeau et al., 1980; Wake and Hillen, 1981; Wolf and Cox, 1981). Cultivation under nitrogen deficient conditions did indeed increase the neutral lipid contents however, the brown growth phase did not occur in this laboratory until storage for 6-9 months. This time period precluded any attempt at a systematic comparative study. In contrast to the 10% increase in the lipids in Botryococcus, there was a drop in the lipid fraction in Dunaliella bardawil and Dunaliella salina to about 10% of the organic weight. These halotolerant green algae shifted towards carbohydrate storage under nitrogen stress. Werner (1977) and Shifrin and Chisholm (1981) indicated a similar observation of carbohydrate storage in nitrogen deficient diatoms and in Dunaliella tertiolecta. On the other hand, Isochrysis accumulated higher fractions of lipids and carbohydrates under nitrogen deficiency, with the lipids comprising about one-fourth of the algal organic cell weight following 10 days of nitrogen starvation. In general, the effects of nitrate deficiency were that the protein content and the chlorophyll level decreased while carbohydrate and lipids exhibited a species specific change.

High salt concentration clearly induced glycerol accumulation in Dunaliella as part of the algal osmotic adaptation (Ben Amotz and Avron, 1973; Brown and Borowitzka, 1979) with only a slight effect on the other cell constituents. The osmotic regulator in Botryococcus, Isochrysis and Nitzschia has not yet been identified. The concurrent reduction in protein synthesis under salt stress suggests protein synthesis inhibition allowing accumulation of free amino acids (Brown and Hellebust, 1980).

An interesting observations in this study was the distribution of the lipid fraction along the neutrality-polarity spectrum in relation to species and growth conditions. The neutral lipid content is expressed in the algae that shifts to lipid storage when under environmental stress. These neutral lipids are not predominantly straight chain saturated hydrocarbons but multibranched and/or polyunsaturated components. This was observed for Botryococcus, Isochrysis and Dunaliella species grown under nitrogen stress. The benzene eluates collected from silicic acid columns contained complex mixtures of a very large number of components which ranged from C₁₅ to C₃₇ carbons. A more detailed study will have to be conducted to isolate and identify all of the lipid components in this fraction. A few of the components occurring in the benzene eluate were isolated from preparative TLC plates and analyzed. β -carotene was a major component in the Dunaliella as previously reported (Ben-Amotz et al., 1983) A C₃₇ oxygenated cyclic isoprenoid in Isochrysis that comprised 4% of organic cell weight was isolated but whose identity has not yet been fully elucidated. This component is currently being characterized. It may be speculated that the components comprising the benzene components are intermediates, derivatives or homologues of the biosynthetic pathway of carotenoids (Goodwin, 1980).

The major polar lipids of the algae are those commonly found in photosynthetic algae (Kates, 1970; Pohl and Zurheide, 1979; Evans et al., 1982). Although the concentration of the total phospholipids and glycolipids changed when the cells were

cultivated under nutritional or salt stress, the relative proportion of the individual polar lipid compounds remained fairly constant.

The fatty acid composition of the algae investigated were generally in agreement with the distribution of fatty acid in eukaryotic algae and its differentiation between the algae classes as previously reported (Wood, 1976; Pohl and Zurheide, 1979). Dunaliella salina and Dunaliella bardawil grown under optimal conditions had similar fatty acid patterns with high degrees of unsaturation in the C₁₆ and C₁₈ range like those previously reported (Tornabene et al., 1980; Evans et al., 1982; Fried et al., 1982). When the same species were grown under nitrogen deficient conditions, however, C₂₀ unsaturated fatty acids were present. In contrast to the effect of nitrogen stress on the fatty acids of Dunaliella, salt stress did not change the fatty acid distribution. A notable feature of Dunaliella bardawil relative to other species of Dunaliella, was the high percentage of palmitic acid (C₁₆) irrespective of the growth conditions.

The chrysophyte Isochrysis contained relatively high contents of 22:6 and 22:4 like that reported for Isochrysis galbana (Chuceas and Rilley, 1969). Isochrysis used in the study was the UTEX species #2307, originally a Thaitian strain acclimated to warm water and used as food for shrimp aquaculture (Starr, 1977 in 1982 suppl). The importance of dietary long chain polyunsaturated fatty acids in mariculture (Scott and Middleton, 1979; Takeuchi and Watanabe, 1982) correlated with the high percentages of these essential fatty acids in Isochrysis. It was interesting to note that neither nitrogen stress nor salt stress influenced the production of C₂₂ indicating that this long chain polyunsaturated fatty acid is species specific.

The predominant fatty acids in Botryococcus was 16:0, 18:1, 18:3 with only the 18:1 increasing in response to salt stress and nitrogen stress. A similar type of increase was observed in the 18:1 production by Isochrysis, Dunaliella bardawil and Dunaliella salina. Fatty acids of fresh water algae were influenced in a similar way when exposed to low nitrogen levels (Pohl and Zurheide, 1979).

The fresh water algae Ankistrodesmus sp. was capable of synthesizing the 16:4 and 18:4 fatty acids but not the polyunsaturated C₂₀ fatty acids that are common in Nannochloris sp. and in Nitzschia sp. These findings were quite similar to that previously reported (Wood 1974; Pohl and Zurheide, 1979). The predominance of the 20:0 fatty acid in Nannochloris sp. in comparison to 20:5 of Nitzschia closterium and N. angularis (Kates and Volcani, 1966) may reflect the high concentration of Na⁺ used to grow the Nitzschia sp. in this study.

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MASS CULTURE OF SELECTED MICROALGAE

**OUTDOOR STUDIES ON ANKISTRODESMUS
AND SCENEDESMUS SO2**

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SECTION 3.0

MASS CULTURE OF SELECTED MICROALGAE

3.1 POND OPERATIONS

Pond operations were initiated mid-August 1982 and continued through the end of the contract period. The initial experiments, in the fall of 1982, used a mixed algae inoculum (Micractinium - Scenedesmus) obtained from Richmond, Calif., where a high rate sewage oxidation pond has been operating for several years. In August of 1983 work began on the Ankistrodesmus isolated from Pyramid Lake.

3.2 METHODS OF OPERATIONS

3.2.1 Dilution and Media

Batch ponds were operated from initial inoculation or by emptying 75%-80% of an operating pond culture and refilling with fresh medium. Density increased four to eight fold prior to restart of the batch cultures. The basic medium used was the same for all cultures and is shown in Table 3-1. This media was supplemented with potassium for growth of the Ankistrodesmus. This medium supports the growth of over 600 mg/L of nutrient-sufficient algal biomass on the basis of the following cell content: 0.5% P, 0.5% Fe, 0.5% Mg, 0.5% S. All trace metals were assumed to be available in sufficient amounts in the irrigation water used.

Continuous ponds were diluted automatically over a 12 hour period from 7 am to 7 pm. Inflow was metered through a calibrated water meter. Outflow was through a overflow pipe and flowed by gravity to the harvesting ponds which were emptied automatically on a daily basis. Nutrients were fed to the ponds automatically from a nutrient mix tank (Urea, NaHCO_3 , FeSO_4 , EDTA) over a ten hour period or added manually each morning (P and Mg). Urea was added to reach a final concentration of 20-30 ppm Urea-nitrogen.

Table 3-1. Basal Medium Composition

Nutrient	Source	Nutrient Concentration, mg/l
Phosphate	Disodium phosphate	3-6 as P
Mg, Sulfur	Epsom salts	5 as Mg, 6 as S
Fe	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	5-10 as Fe
Chelator	Na_2 EDTA	100
Alkalinity	Na HCO_3	5-10 meq/L
Trace	Irrigation water	--

CO₂ additions were added from 65 lb CO₂ tanks through diffusers. Initially carbonation was done manually in response to pH rise. Generally pH was kept between 8.0 and 9.5, occasionally it went as high as 10.0. Sodium bicarbonate was added to increase the CO₂ storage capacity. Alkalinity was measured to be between 8.0 and 12 meq/L. At 10 meq/L, approximately 80 mg/L of algal biomass can be grown during a pH rise of 8.5 to 9.5, excluding CO₂ loss due to outgassing. By June 1983, CO₂ additions were made automatic, slowly diffusing in CO₂ over an eight hour period.

3.2.2 Nitrogen Addition

In the initial experiments ammonium chloride was monitored routinely to determine when additions were required. These additions were made at the level of 10.0 ppm NH₄ - N when the NH₄ - N concentration in the pond fell (or was expected to fall) below 3-5 ppm. In this way NH₄ - N levels were maintained between 3-20 ppm unless N deficiency was intended.

Starting in April 1983 urea replaced ammonium as the nitrogen source, and was continuously fed to continuously-diluted ponds. Batch ponds were manually fed urea as needed to provide nitrogen sufficiency or deficiency.

3.2.3 Sampling and Measurements

Batch ponds were sampled manually (grab samples) each morning from prescribed sampling points. Samples were taken to the laboratory for solids, pH, and lipid analysis (if required). Effluents from continuous ponds were sampled every forty minutes from 9 am to 7 pm using an ISCO compositor. Each night the compositor samples were collected and stored in a refrigerator until the next day. The compositor was not refrigerated. Measurements of fresh samples versus unrefrigerated samples indicated that as much as 15% of the biomass may have been respired away during compositing. Productivity values were not corrected for this, but recirculation of cooling water through the ISCO sampler is being implemented to reduce losses.

Depths were measured daily and evaporative water loss recorded. Productivity for batch ponds was calculated using daily ash-free weight differences (corrected for evaporative concentration) and expressed as gms ashfree dry wt/m²/day. For continuously operated ponds, the weight of the composited sample (g/L) was multiplied by the corrected outflow per day (-/day) and the product divided by the pond area (m²).

3.3 Ankistrodesmus CULTIVATION

The Pyramid Lake *Ankistrodesmus* was cultivated in the laboratory and inoculated outdoors in late July. The results are shown in Tables 3-2 and 3-3. Until late September the cultivation was characterized by a browning of the cultures after the first batch growth. It was finally determined (via bioassay) that insufficient potassium was the cause. Potassium deficient cultures greened quickly when brought indoors and potassium was added. Outdoor brown cultures, after supplementation with potassium, greened very slowly (runs 9 and 10) and incompletely. The same situation with regreening occurred after nitrogen starvation. This will require further investigation, along with the question as to whether nitrogen deficient cultures induce lipids or carbohydrate. Data has been taken to determine this, but has not been completed or analyzed at this time.

Table 3-2. Ankistrodesmus Productivity Summary

	August	September	October
P.A.R. Insolation, Lgly/day	260	215	170
Aver. Sustained Productivity			
Product g/m ² /day	12	14	11
Effic., % P.A.R.	2.5	3.6	--
Lipid, % ash free wt.	27	27±5	--
Maximum Productivity			
Product. g/m ² /day	15	18	14
Effic., % P.A.R.	2.8	4.9	--
1-day max. g/m²/day	17	22	14

A potassium-limited culture became contaminated with a Selenastrum sp. It was cultivated, but soon terminated due to lack of prolific lipid production. No other contamination (besides 10% diatoms in some potassium-limited cultures) has occurred since the end of August.

This algae is promising in that it produces a moderate amount of lipid when N-sufficient, it is highly productive in the laboratory, it grows in brackish water, and it can be made to clump (although no settling rate data is available yet). Further work is needed to optimize the salt balance in the medium, determine its vitamin requirement, and determine its lipid inducibility.

A non-sterile batch growth in the laboratory with no vitamins added showed no long-term degeneration. Productivities are as great or greater than cultures supplemented with vitamins.

Studies from 1.4 m² indoor cultures indicated that Ankistrodesmus grew quite dense and were highly productive when the pH was maintained near 7.0. Cultures grown with the pH allowed to rise from 6.5-9.6 (and then brought back down to 6.5) were less productive and cultures grown at pH 8.7 were the least productive. Efficiencies of greater than 12% were calculated (using an estimated caloric content of 5500 cal/gm).

3.4 Scenedesmus S02a

This alga has only recently been inoculated outdoors. It has been grown batchwise for two weeks.

There does not appear to be any particular difficulties however the outcome of competition between this species and the native Scenedesmus will be the overriding factor.

The organism was grown indoors in 1.4 m² tanks. It was not as productive as the Ankistrodesmus but productivity was still good. It was grown in batch (in 12 m² and 100 m² ponds) for three batches. Again productivity was lower than that of other

Table 3-3. Ankistrodesmus--Sept. 1983 100 m² Batches

Run #	Dates	Av. Insol. (Tot. Lglys/day)	Temp. °C min.-max.	pH range	Density (mg/L ash-free wt.)	Av. Product. (g m ⁻² d ⁻¹)	Av. Eff. % P.A.R.	Max. Product (g m ⁻² d ⁻¹)
6	9/4-10	542	17-30	7.2-8.9	65-435	10.6	2.4	16.2
	9/4-7	545	20-30	7.2-8.5	65-330	13.2	3.0	13.4
	9/10	539	17-28	7.5-8.5	357-435	--	3.7	16.2
7	9/12-15	520	17-31	7.5-8.5	150-233	4.2	1.0	15.6
	9/13-14	526	20-31	7.5-8.5	167-306	13.9	3.2	15.6
	9/13	531	22-31	7.6-8.4	167-245	--	3.6	15.6
8	9/17-22	456	16-29	7.8-8.5	178-420	8.0	2.1	14.0
	9/11-18	504	16-29	8.0-8.5	178-305	13.7	3.1	--
	9/21-22	368	--	8.2-8.5	290-420	12.2	4.0	14.0
	9/22	361	--	--	350-420	--	4.7	14.0
9	9/23-27	373	17-29	7.5-9.0	94-294	9.3	3.0	17.3
	9/24-25	474	17-29	7.5-8.5	94-266	17.2	4.4	17.3
	9/25	473	19-29	8.0-8.5	180-266	--	4.5	17.3
10	9/28-10/5	376	17-26	7.7-9.0	64-	--	--	19.6
	10/2-3	410	17-25	7.7-8.5	200-350	15.0	4.5	18.2
	9/29	402	17-24	8.5-9.0	69-167	--	6.0	19.6

species. However ten days of continuous cultivation outdoors in October resulted in an average production rate of $14 \text{ gm/m}^2/\text{day}$, higher than any of the organisms grown in batch during that time period.

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