Collection and Characterization of Saline Microalgae From South Florida

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Final Report, May 1986
Contract #XK-4-0436-03

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INTRODUCTION

In 1983, SERI initiated a microalgal species aquisition program to provide strains to be used in the development of microalgal culture technology for the production of fuels (Raymond 1984). From previous collection efforts (Barclay 1984, Tadros 1984) it was determined that desirous species should grow rapidly under fluctuating culture conditions and be capable of producing large concentrations of lipid. The aquisition efforts of the SERI were expanded in 1985 to include new regions of the United States.

The present study involves the collection and screening of microalgae from saline habitats of South Florida. An emphasis was placed on obtaining high performance species of chromophytes, a group well known to accumulate lipids (Aaronson 1973). Select strains were characterized for growth under varying chemical and physical culture environments.

The interactive effects of pH, carbon type and carbon concentration on the potential yield of microalgae was also examined. Efficient carbon utilization is of primary importance for algal systems that produce fuels. An analysis of the economics of a 200 m^2 open pond system found the cost of CO_2 to be more than 50% of the total operating cost (Weismann and Goebel 1985). Since the cost of algae-derived fuel is directly dependent on the cost and utilization efficiency of CO_2, close attention must be paid to medium chemistry in order to limit carbon losses (i.e. outgassing and precipitation) while maintaining acceptable productivity.

Although the mathematical formulation of this optimization problem is very difficult, due to uncontrollable fluctuations in environment and uncertainties regarding blowdown water chemistry, a fundamental part of such a formulation is a mathematical relation expressing algal productivity as a function of carbon concentration. The objective of the present carbon study is to characterize a variety of algal strains and to correlate productivity with either aqueous CO_2, HCO_3^-, $CO_3^=$, TIC or pH.

Task I: Make collections from sites in South Florida and the Florida Keys that would favor desired characteristics and select for 1) strains that are dominant at time of collection (blooms), 2) strains that become dominant in enriched media, or 3) chromophytes that are isolated after exclusion of other strains.

Objective: To select and isolate strains of microalgae that have a high probability of being productive in outdoor monoculture.

Methods

Field Collections: Field collections were conducted in the Everglades and Florida Keys regions of South Florida during June and September 1985 and February 1986 (Figure 1). Sample sites were selected based on water depth (ie. shallow saline habitats) and the diversity of habitats representing a wide range of environmental conditions. At each site, water column and bottom substrate samples were obtained by pipet or scraped with a spatula and placed in culture tubes and polyethylene bottles. Samples were kept cool and in the dark until return to the laboratory. Water temperature, salinity (by refractometer), conductivity and pH were measured at each location and total alkalinity was determined by titration to pH 4.2 with standardized HCl in the laboratory.

Isolation and Initial Screening: To promote the growth of fast-growing microalgae in the natural mixed population, tubes containing water from each site were enriched with 440 µM NO_3, 36 µM PO_4, ES metals and vitamins (Provasoli 1968) and incubated under continuous high light (830 µE m^{-2} sec^{-1}) intensity at temperatures of 25 and 30°C. Tubes were examined daily and microalgae exhibiting rapid growth during the following 5 days were isolated for growth studies. To isolate chromophyte species and dominant microalgae at time of collection, subsamples from each location were inoculated into 18 x 150 mm culture tubes containing artificial seawater (ASP-2, Provasoli et al. 1957) adjusted to collection site salinity and enriched with ES/2 or "F" medium (Guillard and Ryther 1962) diluted 1:20. Cultures were incubated at 25°C on a 16:8 L/D cycle under 100 µE m^{-2} sec^{-1} illumination and were mixed gently each day.

Isolation into unialgal culture was accomplished by a combination of procedures including: Population transfers into media containing different nitrogen forms and various salinities; serial washings in sterile media via micropipets; agar plating; and treatment with selective poisonings (eg. germanium dioxide for diatoms and various antibiotics for cyanobacteria).

Results and Discussion

Field Collections: A variety of South Florida saline habitats were sampled for microalgae including mangrove swamps, salt flats, canals, ditches and numerous shallow ponds. Thirty-eight locations were examined in the Florida Keys during June 1985; 25 in September and 26 in February 1986. Everglades collections were conducted during September 1985 (18 locations) and February 1986 (15 sites). Physical and chemical characteristics of the 122 collection sites are summarized in Table 1. The Everglades-Florida Keys region has a tropical savanna climate dominated by a relatively long dry season between November-April and a bimodal rainy season during May-June and September-October. The primary water characteristics of the sampling

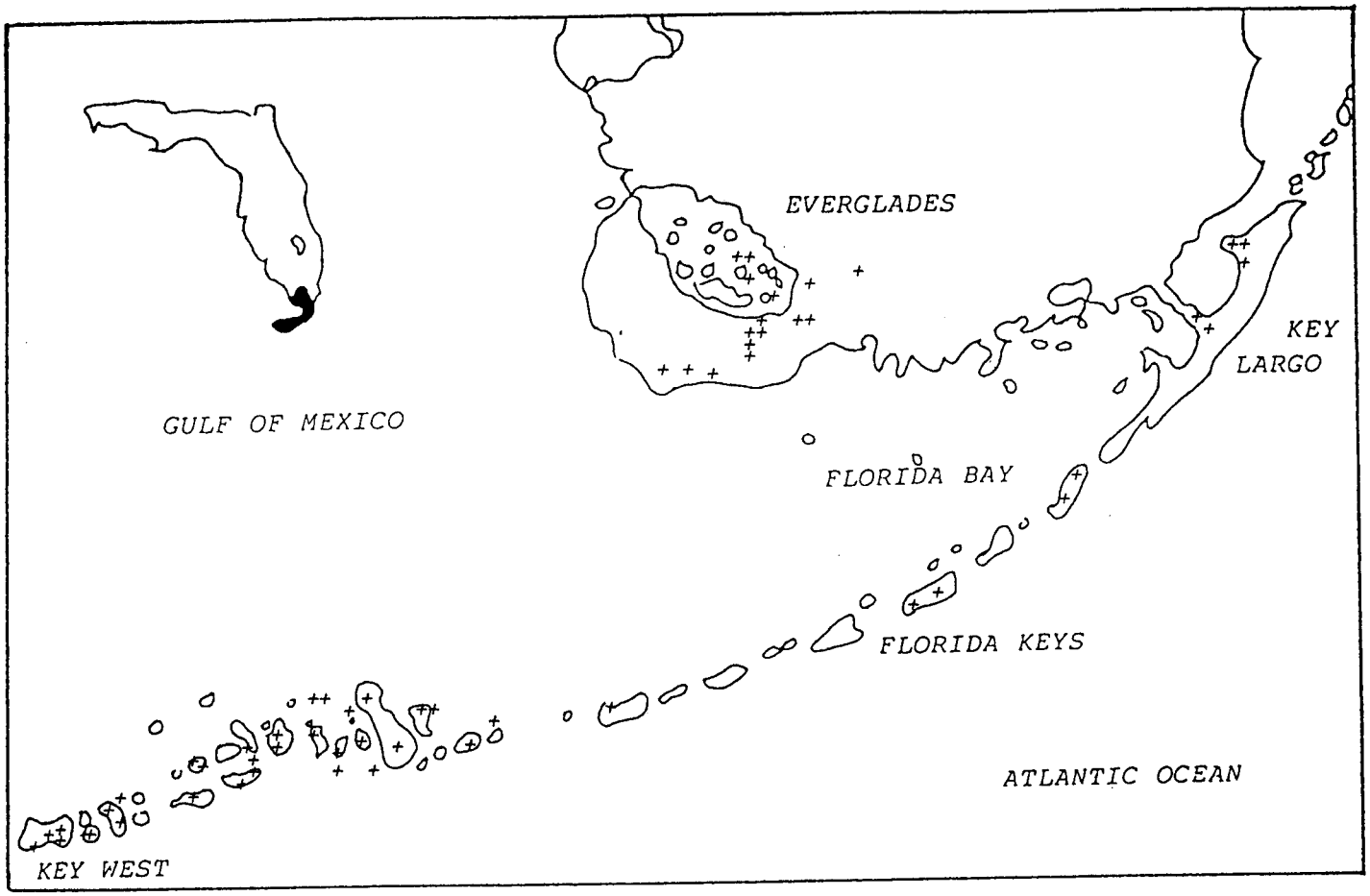

Figure 1. Locations (+) of South Florida collection sites sampled during 1985 - 1986.

Table 1. Average ($\bar{X}$) physical and chemical characteristics and standard deviation (S.D.) of locations sampled in the Florida Keys (n=83) and Everglades (n=32) during June and September 1985, and February 1986 collection trips.

	FLORIDA KEYS			EVERGLADES	
Collection Site Characteristics	June 1985 $\bar{X} \pm$ S.D.	Sept. 1985 $\bar{X} \pm$ S.D.	Feb. 1985 $\bar{X} \pm$ S.D.	Sept. 1985 $\bar{X} \pm$ S.D.	Feb. 1985 $\bar{X} \pm$ S.D.
Temperature ^{o}C	31.98 ± 2.99	31.83 ± 1.87	24.35 ± 3.59	31.49 ± 1.55	28.32 ± 4.06
Salinity (ppt)	28.74 ± 11.34	33.48 ± 10.20	27.26 ± 14.02	18.58 ± 7.63	25.73 ± 13.00
Conductivity (mmho cm^{-1})	38.88 ± 15.73	45.99 ± 13.45	35.44 ± 17.43	24.64 ± 10.77	33.10 ± 15.49
pH	8.08 ± 0.41	7.97 ± 0.36	8.09 ± 0.36	8.08 ± 0.28	7.93 ± 0.43
Total Alkalinity	3.19 ± 2.12	3.99 ± 2.57	3.39 ± 1.22	3.64 ± 0.43	6.73 ± 4.21

sites reflected these seasonal differences with elevated water temperatures and lower salinities recorded during June and September and lower temperatures and higher salinities observed during the cooler, drier conditions found in February. Over 75% of the water temperatures recorded during June and September were greater than 30°C, but only 20% of the collection sites reached 30°C in February (lowest recorded water temperature was 18.5°C).

Microalgal assemblages encountered in the Keys during June and September were dominated by dinoflagellates, blue-green algae, raphidophytes and cryptophytes while diatoms comprised a large component of the dominant species in February. Everglades locations were dominated in September by dinoflagellates, various small flagellates and green unicells, but diatoms and blue-green algae were more prevalent in February.

Isolation and Screening: Sixty-one species were isolated into culture during the contract period including 36 species of chromophytes. Cultures obtained during the 1985-1986 collection trips and their corresponding field data are listed in Table 2. Also presented are species previously isolated into our culture collection that exhibited good growth in preliminary growth evaluation experiments (see Task II). Microalgal strains isolated into culture which commonly formed blooms included species of Prorocentrum (the most ubiquitous bloom forming species in the study area), Peridinium, Chattonella, Gymnodinium, Cryptomonas and various blue-green algae strains. Although these species were most prevalent in natura, most did not remain dominant when site water was enriched during the initial screening. In culture tubes containing mixed population, prasinates, coccolithophores, ochromonads, prymnesiophytes and occassionally diatoms exhibited fast growth and quickly became dominant. Significant growth by green unicells (eg. Nannochloris/Nannochloropsis and Chlorella species) and blue-green algal species (eg. Synechoccocus and Stichoccocus) usually became apparent following the demise of fast-growing strains. The eventual success of the latter species may be due to their ability to grow under the high pH and corresponding reduced CO_2 levels inherent in unaerated tubes containing productive mixed microalgal cultures.

Table 2. Microalgal species isolated from saline habitats of South Florida including site water characteristics.

Collection Site	Species	Collection Date	Water Temperature (°C)	Salinity (ppt)	pH	Total Alkalinity (meq/L)
FLORIDA KEYS						
Spanish Harbor Key	HB11-Tetraselmis	11-09-83	----	36.0	----	----
	HB115-Tetraselmis	11-10-83	----	42.0	----	----
	HB137-Green Unicell	06-27-85	29.5	9.0	----	----
	HB158-Nannochloris	11-10-83	----	36.0	----	----
	HB165-Nephroselmis	09-25-85	30.5	39.0	7.45	2.84
	HB184-Eremosphaera	02-12-86	21.5	24.0	8.00	3.20
Tea Table Channel	HB32-Tetraselmis	11-09-83	----	----	----	----
Park Key	HB37-Dunaliella	11-10-83	----	40.0	----	----
	HB152-Hymenomonas	06-27-85	32.5	30.0	8.10	3.05
	HB153-Prorocentrum	06-27-85	32.5	30.0	8.10	3.05
Stock Island	HB42-Gloeothamnion	11-10-83	----	22.0	----	----
	HB47-Tetraselmis	11-10-83	----	10.0	----	----
	HB168-Prasinate	09-25-85	33.0	31.0	8.15	2.65
No Name Key	HB49-Chlorella	11-10-83	----	14.0	----	----
	HB138-Diatom	06-27-85	34.0	50.0	8.30	5.27
	HB139-Green Unicell	06-27-85	34.0	50.0	8.30	5.27
	HB140-Lyngbya	06-27-85	28.0	15.0	7.85	11.83
	HB141-Cocco. sp.	06-27-85	28.0	15.0	7.85	11.83
	HB142-Prorocentrum	06-27-85	28.0	15.0	7.85	11.83
	HB143-Tetraselmis	06-27-85	28.0	15.0	7.85	11.83
	HB164-Hymenomonas	08-25-85	32.5	40.0	8.30	5.09
	HB176-Pseudoanabaena	09-25-85	32.5	40.0	8.30	5.09
Key Largo	HB60-Ochromonas	11-09-83	----	23.0	----	----

Collection Site	Species	Collection Date	Water Temperature (^{o}C)	Salinity (ppt)	pH	Total Alkalinity (meq/L)
Bahia Honda Key	HB121-Pavlova	06-27-85	29.0	35.0	7.55	2.20
	HB136-Prorocentrum	06-27-85	29.0	35.0	7.55	2.20
	HB162-Cricosphaera	09-25-85	31.0	36.0	7.80	2.17
	HB172-Prasinate	09-25-85	31.0	36.0	7.80	2.17
	HB174-Dunaliella	09-25-85	31.0	36.0	7.80	2.17
	HB175-Tetraselmis	09-25-85	29.5	36.0	7.65	3.00
	HB185-Prasinate	02-12-86	24.5	38.0	8.35	3.40
Big Pine Key	HB128-Chrysochromulina	06-25-85	28.0	40.0	8.10	2.26
	HB134-Prorocentrum	06-26-85	31.0	12.0	7.85	3.96
	HB133-Pyramimonas	06-26-85	31.0	12.0	7.85	3.96
Summerland Key	HB124-Gloeothamnion	06-27-85	32.5	38.0	7.95	2.23
	HB144-Anabaena	06-27-85	32.5	38.0	7.95	2.23
	HB166-Tetraselmis	09-25-85	30.0	38.0	7.85	2.46
Cudjoe Key	HB122-Chattonella	06-27-85	38.0	28.0	8.80	2.56
	HB123-Pleurochloris	06-27-85	38.0	28.0	8.80	2.56
	HB145-Peridinium	06-27-85	38.0	28.0	8.80	2.56
	HB167-Ochromonas	09-25-85	30.0	35.0	8.10	5.45
	HB182-Prasinate	02-12-86	26.0	9.0	7.85	1.70
	HB183-Tetraselmis	02-12-86	21.5	20.0	7.90	5.30
Boca Chica Key	HB154-Olive Gr'n Uni'	06-27-85	34.5	10.0	8.35	2.58
	HB155-Synechococcus	06-27-85	34.5	10.0	8.35	2.58
	HB156-Tetraselmis	06-27-85	32.5	30.0	8.15	2.28
	HB161-Coscinodiscus	06-27-85	34.5	10.0	8.35	2.58
	HB169-Nephrochloris	09-25-85	35.0	5.0	8.75	3.57
Onio Key	HB163-Pavlova	09-25-85	32.0	37.0	7.75	3.10
	HB179-Cocco. sp.	09-25-85	32.0	37.0	7.75	3.10

Collection Site	Species	Collection Date	Water Temperature (°C)	Salinity (ppt)	pH	Total Alkalinity (meq/L)
Long Key	HB125-Gymnodinium	06-24-85	35.0	22.0	7.95	2.16
	HB126-Halosphaera	06-24-85	35.0	22.0	7.95	2.16
	HB127-Cocco. sp.	06-24-85	35.0	22.0	7.95	2.16
	HB178-Pavlova	09-25-85	29.5	30.0	8.00	3.33
East Rockland Key	HB135-Nitzchia	09-26-85	----	40.0	----	----
Sugarloaf Key	HB146-Green Unicell	06-27-85	34.0	6.0	----	----
	HB147-Chryso/C4	06-27-85	31.5	42.0	8.25	1.97
	HB148-Caloneis	06-27-85	31.5	42.0	8.25	1.97
	HB149-Cryptomonas	06-27-85	31.5	42.0	8.25	1.97
	HB150-Yellow G'rn Uni'	06-27-85	34.0	6.0	----	----
	HB151-Green Flagellate	06-27-85	34.0	6.0	----	----
	HB180-Coscinodiscus	09-25-85	33.0	42.0	7.90	2.73
	HB181-Pavlova	02-12-86	23.5	28.0	7.80	3.90
Middle Torch Key	HB159-Prasinate	09-25-85	29.0	42.0	7.85	3.30
	HB160-Amphora	06-26-85	31.5	18.0	7.45	3.07
EVERGLADES						
Coot Bay	HB171-Synechococcus	09-24-85	31.0	19.0	8.25	3.58
	HB177-Tetraselmis	08-24-85	31.0	23.0	8.25	3.79
Whitewater Bay	HB170-Ochromonad	08-24-85	31.0	12.0	8.05	3.63
Buttonwood Canal	HB172-Prasinate	08-25-85	32.5	23.0	8.10	3.89
Mrazek Pond	HB187-Chroomonas	02-10-86	31.5	10.0	8.10	7.90
Cape Sable	HB186-Chaetoceros	02-11-86	24.0	35.0	7.65	6.20

Collection Site	Species	Collection Date	Water Temperature (°C)	Salinity (ppt)	pH	Total Alkalinity (meq/L)
MISCELLANEOUS						
Jack Isl', Ft. Pierce	HB28-Prasinate	Fall, 1984	----	20.0	----	----
	HB157-Prorocentrum	Fall, 1984	----	25.0	----	----
Peace Corp Pd, HBF	HB44-Nannochloris	June, 1985				
Burial Vault, HBF	HB79-Oocystis	04-20-83				
	HB89-Hymenomonas	June, 1984				
	HB97-Chlorella	June, 1984				
Ft. Pierce Inlet	HB57-Stichococcus	03-19-83				
Virginia Key, Miami	HB82-Chlorella	11-03-83				
	HB84-Chlorella	11-03-83				
	HB87-Chlorella	11-02-83				
Tampa Bay	HB85-Nannochloris	08-11-83				
Ecuador S.A.	HB35-Pyramimonas	09-24-83				
	HB54-Yellow Gr'n Uni'	09-24-83				

Task II: Define the growth rate of at least 15 strains from Task I and chromophytes currently in culture that were isolated prior to the contract period.

Objective: To characterize selected strains for growth rate in response to various environmental conditions.

Methods

Preliminary Growth Evaluation: A preliminary growth evaluation was initiated to assess the characteristics of species already in the Harbor Branch culture collection and good-growing strains isolated during the course of this study. Each strain was inoculated into 13 x 100 mm culture tubes containing F/2 or ES enriched natural seawater and SERI Type I and II desert waters (Barclay et al. 1985), each at salinities of 25 and 40 mmho cm^{-1} (Table 3). Cultures were incubated at 30°C under continuous 300 $\mu E\ m^{-2}\ sec^{-1}$ illumination provided by six 30W cool white fuorescent lamps. Growth rates (doubling day^{-1}) were assessed daily for 5 days by directly measuring changes in optical density (O.D. 750 nm) for each culture tube using a Beckman Model DU6 spectrophotometer. The maximum optical density was determined following 10 days of growth. Species were considered "good growing" strains based on exponential growth rates greater than 1 doubling day^{-1} in at least 2 medias and a maximum optical density of 0.3 achieved during 10 days of growth.

Lipid accumulation in strains already in culture were initially determined microscopically using Oil-Red-O lipid stain by the method of Gallager and Mann (1981). However, a new lipid stain, nile red (Greenspan et al. 1985) was used during subsequent screening of species. Log phase and 21 day cultures of good growing species were stained with nile red (100 ng/ml) and evaluated for lipid accumulation by fluorescence microscopy (excitation wave length, 455-500 nm; emission wave length > 515 nm). An arbitrary scale was established based on visual estimation of cell lipid content on randomly selected cells from 21 day cultures as follows: + = 0-10%; ++ = 10-20%; +++ = 20-30%; and ++++ = 30-40% lipid.

Growth Characterization: Prior to initiation of the preliminary growth evaluation experiments, species already in culture were selected for growth characteristics in variable temperatures and salinities based on visual estimation of biomass production. The growth of these species was examined at 15, 25 and 35°C in F/2 or ES enriched natural seawater (45 mmho cm-1) in constant temperature incubators. Light was provided from six 30W cool white fluorescent lamps adjusted to a constant radiant flux density of approximately 100 $\mu E\ m^{-2}\ sec^{-1}$. Exponentially growing cells were inoculated into 250 ml flasks containing 100 ml of culture medium. Growth was determined at 25°C between the salinities of 8 and 59 mmho cm^{-1}. Optical densities were determined from triplicate flasks and growth rates were calculated daily over an 8 day period.

The growth rates for selected species considered to be good growers from the results of the preliminary screening were examined in 25 temperature-salinity combinations on a temperature gradient plate (Siver 1983). Batch cultures containing 100 mls of ASP-2 artificial seawater were grown in 250 ml flasks at 5 temperatures (15, 20, 25, 30, 35°C) and 5 salinities (8, 22,

35, 47, 59 mmho cm^{-1}). Eight 40W cool-white fluorescent lamps positioned as 4 banks of two lamps each were adjusted to provide a constant radiant flux density of 180 $\mu E\ m^{-2}\ sec^{-1}$. Optical densities were determined daily from duplicate flasks and growth rates were calculated over a 5 day period. Maximum optical densities were determined following 7 days of growth.

Results and Discussion

Preliminary Growth Evaluation: Over 140 species, including 57 chromophytes, were examined for growth in natural seawater and SERI Type I and II desert waters. Of the chromophyte species examined, the Eustigmatophytes, Ochromonads and members of the Prymnesiophyceae including the Coccolithophores and the Pavlovales, generally exhibited good growth in one or both of the formulated desert waters. Dinoflagellates, diatoms and Raphidophytes (eg. Chattonella), both which commonly formed dominant blooms in the study area, and members of the Cryptophyceae and Sarcinochrysidales did not grow in the SERI desert waters (Table 8, Appendix).

Thirty-five species of the 141 examined, were considered good-growing strains (Table 4) based on the aforementioned criteria (see methods). Over 74% of these species displayed better growth in Type II desert water than Type I or natural seawater, probably due in part to the higher concentration of bicarbonate (16 times natural seawater) in Type II water. While most phylogenetic groups preferred Type II media, a notable exception to this trend was observed for members of the Prasinophyceae which generally exhibited good growth in all 3 media, irrespective of salinity.

Following preliminary growth experiments, good-growing strains were stained for lipid (Table 4) and oleaginous species were selected for subsequent growth characterization on the temperature-salinity gradient.

Growth Characterization: Growth characteristics of chromophytes examined prior to the establishment of the preliminary growth evaluation protocol are presented in Table 5. The coccolithophores Gloeothamnion (HB42 apistonema habit) and Hymenomonas (HB89, motile habit) grew well in the laboratory over wide temperature-salinity ranges and under high light and fluctuating temperatures in outdoor culture (see Task III for culture conditions). Exponential growth rates for these species also corresponded well with findings obtained in the preliminary evaluation experiments. In contrast, Amphidinium and Chrysocapsa growth rates were reduced at 15 and 35°C and at salinities below 22 mmho cm^{-1}. Amphidinium, Pleurochloris and Chattonella exhibited good growth in natural seawater under high light and fluctuating temperatures, but these species did not grow in the Type I and Type II desert waters at either salinity. Consequently, these species were not further examined for chemical composition or growth characteristics under varying salinities.

Eight good-growing species from the preliminary evaluation were tested for temperature-salinity tolerance on the cross gradient plate. Exponential growth rates and maximal optical densities for these species are presented in Figures 2-9. Nannochloris (HB44) grew very well over a wide salinity-temperature range and exhibited growth rates greater than 1 doubling day^{-1} between 8-59 mmho cm^{-1} and 17-35°C (Figure 2). The optimum temperature for Nannochloris was 30°C, with fastest growth (3.21 doubling day^{-1}) occurring at a salinity of 22 mmho cm^{-1} and greatest yields (O.D. 1.15) at 8 mmho

Table 3. Composition of SERI Type I and Type II desert waters used for preliminary growth evaluation of South Florida microalgae.

	COMPOSITION (mg/L)				
	TYPE I[a]		TYPE II[a]		
Salt	25 mmho cm^{-1}	40 mmho cm^{-1}	25 mmho cm^{-1}	40 mmho cm^{-1}	Seawater[b]
$CaCl_2$	3,932	5,618	28	28	994
$MgCl_2$ $6H_2O$	11,844	22,789	3,026	3,920	9,395
Na_2SO_4	2,925	3,310	5,870	15,720	3,477
KCl	407	662	965	2,028	587
$NaHCO_3$	168	168	2,315	2,855	170
Na_2CO_3	---	---	876	1,234	---
NaCl	3,845	14,132	8,078	12,963	20,758
TDS	23,121	46,679	21,158	38,748	35,381

[a]Barclay et al. (1985)

[b]Harrison et al. (1980)

Table 4. Exponential growth rate (doublings day^{-1}, OD 750)/maximum optical density for 35 species of South Florida microalgae exhibiting good growth in preliminary screening experiments and nile red lipid evaluation (see text for details). Species were grown in Type I, Type II and natural seawater (GSW) at 25 and 40 mmho cm^{-1} salinity and 35°C.

SPECIES	GSW 25	GSW 40	TYPE I 25	TYPE I 40	TYPE II 25	TYPE II 40	NILE RED LIPID EVALUATION
Tetraselmis HB11	0.57/0.18	0.74/0.17	0.86/0.30	0.87/0.40	0.70/0.30	0.83/0.21	++
Prasinate HB28	1.49/0.45	1.00/0.46	0.84/0.32	0.86/0.18	0.25/0.05	0.35/0.04	++
Tetraselmis HB32	0.73/0.31	1.24/0.41	0.92/0.20	0.67/0.27	0.48/0.16	0.57/0.32	++
Pyramimonas HB35	0.68/0.20	0.81/0.40	0.94/0.28	0.66/0.28	1.00/0.56	1.12/0.50	+
Dunaliella HB37	1.37/0.23	1.75/0.25	1.48/0.26	1.80/0.25	2.03/0.40	2.15/0.40	+
Gloeothamnion HB42	0.81/0.20	0.85/0.28	0.45/0.22	0.82/0.42	0.95/0.34	0.90/0.42	++
Nannochloris HB44	1.81/0.44	1.98/0.52	2.10/0.54	2.00/0.50	2.18/1.22	2.10/0.74	++
Tetraselmis HB47	1.50/0.34	0.71/0.37	1.19/0.25	1.10/0.44	1.39/0.23	1.06/0.25	+++
Chlorella HB49	0.98/0.35	1.19/0.30	1.20/0.22	1.26/0.31	1.94/0.25	1.71/0.29	+
Prasinate HB53	1.34/0.36	1.15/0.40	0.91/0.31	1.30/0.40	0.97/0.19	0.72/0.14	++

SPECIES	GSW 25	GSW 40	TYPE I 25	TYPE I 40	TYPE II 25	TYPE II 40	NILE RED LIPID EVALUATION
Yellow Green Unicell HB54	1.52/0.10	1.26/0.11	1.57/0.20	1.51/0.37	2.31/0.41	1.81/0.09	++++
Stichococcus HB57	1.50/0.26	0.90/0.16	0.40/0.21	0.57/0.19	1.30/0.39	1.59/0.43	+
Ochromonas HB60	1.00/0.21	1.15/0.24	0.69/0.20	0.48/0.19	1.55/0.50	1.01/0.24	++
Oocystis HB79	1.09/0.13	0.80/0.28	0.85/0.21	0.88/0.19	1.13/0.19	1.04/0.31	+
Chlorella HB82	2.09/0.42	1.67/0.43	1.64/0.44	1.37/0.24	1.96/1.01	1.85/0.36	+
Chlorella HB84	1.68/0.52	1.41/0.54	1.63/0.47	1.28/0.37	1.86/0.80	1.57/0.28	+
Nannochloris HB85	1.02/0.20	1.11/0.44	1.25/0.37	1.09/0.36	1.80/0.87	1.97/0.60	+
Chlorella HB87	1.61/0.44	1.69/0.48	1.44/0.32	1.67/0.47	2.03/0.95	1.70/0.45	+
Hymenomonas HB89	0.58/0.18	1.04/0.35	0.42/0.07	0.65/0.15	0.55/0.20	0.54/0.18	+++
Chlorella HB97	1.22/0.35	1.50/0.38	1.30/0.52	1.24/0.40	1.84/0.62	1.57/0.63	+
Tetraselmis HB115	1.43/0.24	0.95/0.30	0.97/0.32	0.74/0.32	1.00/0.50	0.77/0.35	++++
Pavlova HB121	1.01/0.20	0.86/0.29	1.62/0.25	1.65/0.35	0.76/0.06	0.40/0.05	++

SPECIES	GSW 25	GSW 40	TYPE I 25	TYPE I 40	TYPE II 25	TYPE II 40	NILE RED LIPID EVALUATION
Pyramimonas HB133	1.53/0.30	2.07/0.35	2.14/0.28	1.48/0.28	2.11/0.35	1.91/0.42	+++
Hymenomonas HB152	2.34/0.25	0.84/0.16	1.68/0.26	1.32/0.20	3.26/0.44	1.91/0.29	+
Olive Green Unicell HB154	1.21/0.18	1.76/0.23	0.64/0.20	0.79/0.21	1.89/0.35	1.34/0.25	+++
Synechococcus HB155	1.62/0.35	1.61/0.26	2.10/0.30	1.25/0.17	2.43/0.36	1.69/0.14	++
Tetraselmis HB156	1.59/0.40	1.39/0.33	1.75/0.11	1.78/0.14	1.95/0.51	1.02/0.30	++
Cricosphaera HB162	1.55/0.13	1.70/0.31	1.05/0.10	0.92/0.12	2.04/0.28	1.27/0.30	++
Nephroselmis HB165	1.26/0.19	1.07/0.20	0.82/0.26	1.20/0.24	0.75/0.06	0.52/0.04	++++
Tetraselmis HB166	0.47/0.25	1.25/0.27	1.06/0.17	1.20/0.16	0.77/0.20	0.51/0.12	++++
Ochromonas HB167	1.12/0.22	1.46/0.26	1.21/0.15	1.70/0.17	1.65/0.22	1.55/0.26	++
Nephrochloris HB169	1.26/0.17	1.24/0.23	1.23/0.12	1.17/0.17	2.55/0.42	2.02/0.47	++
Synechococcus HB171	1.36/0.42	1.97/0.23	1.40/0.33	1.44/0.32	2.15/0.44	1.60/0.48	+
Pseudoanabaena HB176	1.01/0.42	1.08/0.36	1.50/0.25	1.36/0.20	1.92/0.38	2.07/0.59	+
Cocco. sp. HB179	1.09/0.19	0.74/0.08	0.98/0.13	0.67/0.12	1.54/0.39	1.65/0.36	++

Table 5. Comparison of average exponential growth rate (doublings day^{-1}, OD 750) of chromophyte species grown under various temperatures, salinities and full sunlight conditions.

	LABORATORY								OUTDOOR
	TEMPERATURE °C (35°/oo)			SALINITY mmho cm^{-1} (25°C)					FULL SUNLIGHT
SPECIES	15°	25°	35°	8	22	35	47	59	(31±4°C, 35°/oo)
Chrysocapsa HB16	0.23	0.78	0.51	0.14	0.10	0.96	0.94	0.28	0.60
Chryso/C2 HB24	0.51	1.14	1.19			ND			0.83
Hymenomonas HB38	0.89	0.88	1.13			ND			0.62
Gloeothamnion HB42	1.07	1.36	1.40	0.79	0.91	1.05	0.98	1.01	1.28
Hymenomonas HB89	0.45	0.63	0.81	0.42	0.72	0.76	0.70	0.68	1.45
Cryptomonas HB119	0.71	1.31	0.93			ND			0.84
Chattonella HB122	0.36	0.84	0.69			ND			1.16
Pleurochloris HB123	0.23	1.01	1.06			ND			1.44
Amphidinium HB838	0.37	1.20	0.48	NG	NG	0.87	0.98	0.77	2.39

ND = Not Determined
NG = No Growth

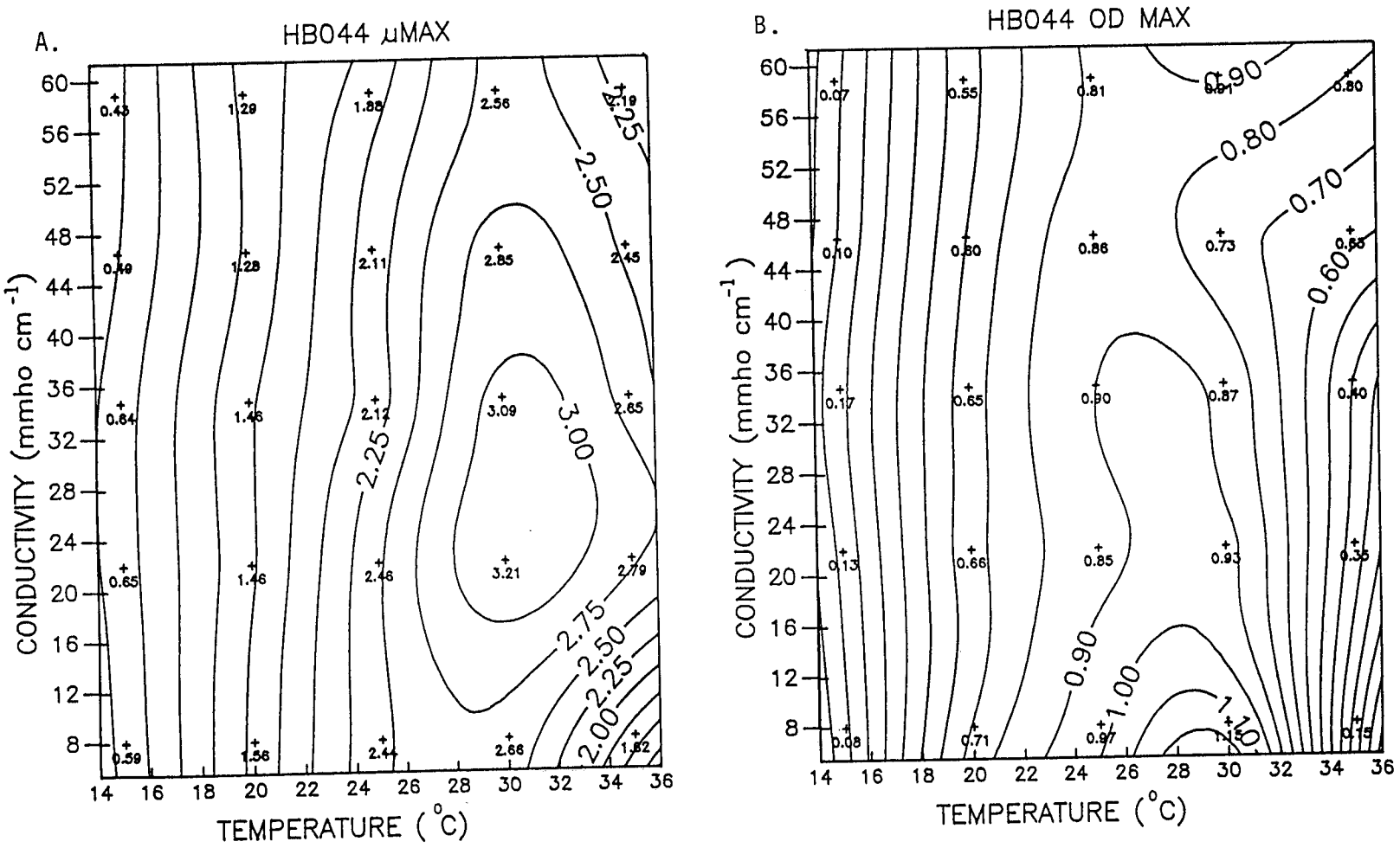

Figure 2. Growth response in batch culture of Nannochloris (HB44) to temperature and salinity. A. Contours are exponential growth rate (doubling day^{-1}, O.D. 750). B. Contours are maximum optical density.

cm^{-1}. Cell densities greater than 0.70 O.D. were maintained at all salinities between 20-32°C. The growth of this species was arrested only when temperatures dropped below 17°C or when exposed to combinations of low salinity (8-35 mmho cm^{-1}) and high temperature (35°C).

Tetraselmis (HB47) grew at rates of 1 doubling day^{-1} or greater over 75% of the temperature-salinity experimental range (Figure 3). Growth rates and cell densities were significantly reduced below 22 mmho cm^{-1} and 15°C. Maximal growth rates (2.16 and 2.17 doubling day^{-1}) were obtained between 25-35°C and 35-59 mmho cm^{-1} while optimum yields (O.D. 0.30-0.33) occurred between 30-35°C and 22-35 mmho cm^{-1}. Additionally, the open contours at the experimental maxima suggest that Tetraselmis may grow well at higher salinities and temperatures.

Another prasinate, HB53 displayed similar growth characteristics to Tetraselmis with doubling rates greater than 1 day^{-1} achieved between 16-59 mmho cm^{-1} and 16-32°C (Figure 4). Optimal growth rate (1.95 doubling day^{-1}) and cell density (0.31 O.D.) occurred at 35 mmho cm^{-1} and 30°C. Salinities below 35 mmho cm^{-1} together with high or low temperature inhibited the growth of this species.

HB54, a yellow green unicell considerably larger than Nannochloropsis (>10 uM) exhibited growth rates greater than 1 doubling day^{-1} over 90% of the experimental range (Figure 5). However, maximal cell densities were significantly reduced at 35°C, and overall, were relatively low for this species. A similar growth response was observed in natural seawater during preliminary growth evaluation experiments; however, results from those studies also indicated that this species not only has elevated growth rates, but accumulates significantly greater biomass in SERI Type I and II desert waters. Its fastest growth (3.13 doubling day^{-1}) in ASP-2 artificial seawater was obtained at 30°C and 22 mmho cm^{-1} while maximum cell densities (0.20 O.D.) were achieved at 25°C between 35 and 47 mmho cm^{-1}.

Pyramimonas (HB133) exhibited a wide range of temperature and salinity tolerance, achieving growth rates greater than 1 doubling day^{-1} between 16-35°C and 10-59 mmho cm^{-1} (Figure 6). High salinity (59 mmho cm^{-1}) favored greatest cell densities (0.52 O.D., 25°C) and maximal growth rate (2.68 doubling day^{-1}, 30°C) for this species. The open contours on Figure 6 indicate growth may be sustained at even higher salinities. Pyramimonas growth was impaired only at high temperature and low salinity combinations.

HB154, an unidentified olive green unicell grew at 1 doubling day^{-1} or greater between 20-35°C and 8-59 mmho cm^{-1} (maximum = 2.30 doublings day^{-1} at 30°C, 35 mmho cm^{-1}). However, significant cell yields (maximum = 0.35 at 30°C, 8 mmho cm^{-1}) were restricted to a relatively narrow temperature range of 22-32°C and salinities below 47 mmho cm^{-1} (Figure 7). HB154 clearly does not favor high temperature and salinity. Results from preliminary growth experiments indicate this species prefers Type II inland saline water where it attains greater cell densities and higher growth rates than in natural seawater.

Coscinodiscus (HB161), a diatom which did not pass the preliminary growth evaluation, but nonetheless grew well in natural seawater and stained positively for lipid, was examined for growth on the cross gradient. While this species achieved growth rates greater than 1 doubling day^{-1} at

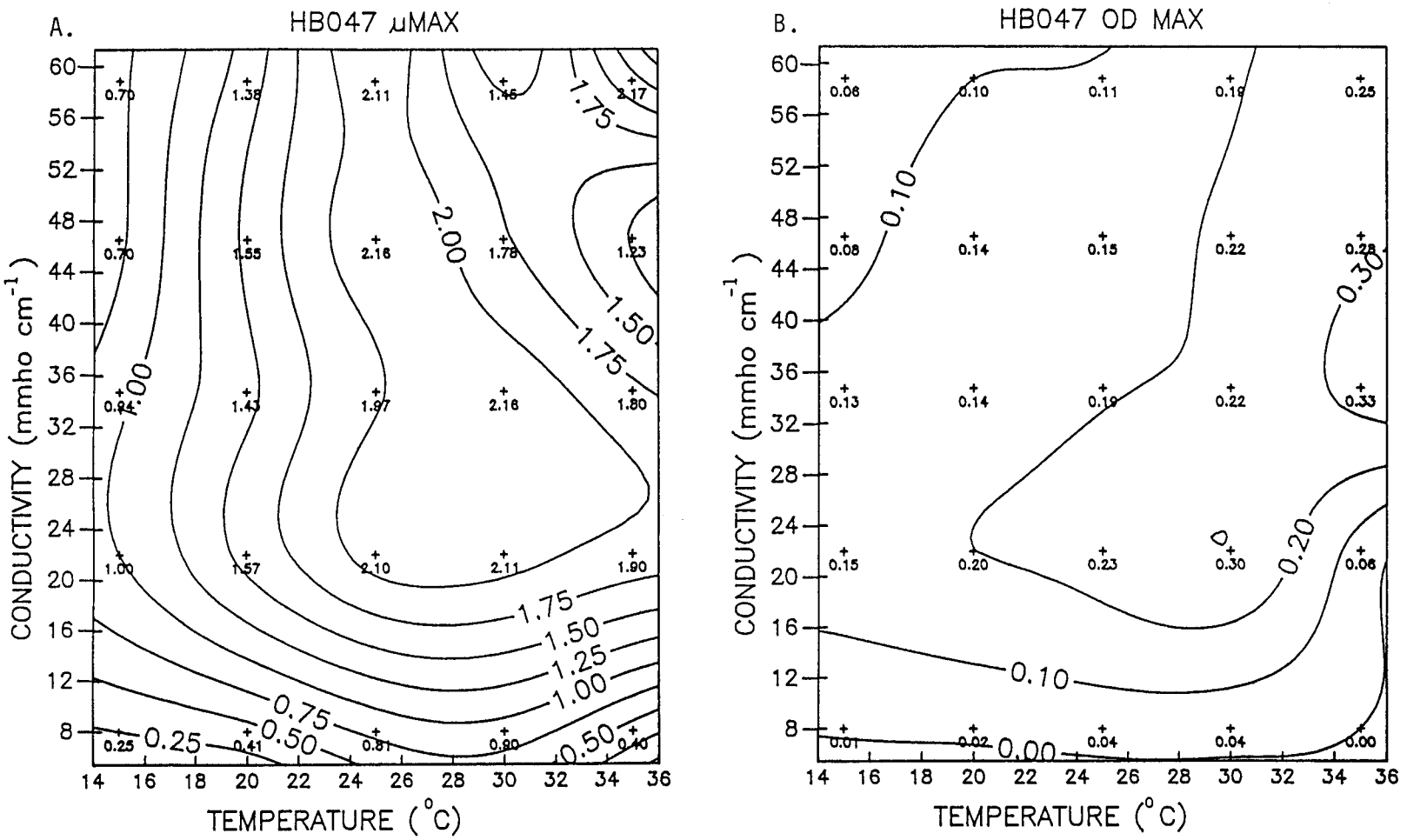

Figure 3. Growth response in batch culture of Tetraselmis (HB47) to temperature and salinity. A. Contours are exponential growth rate (doubling day^{-1}, O.D. 750). B. Contours are maximum optical density.

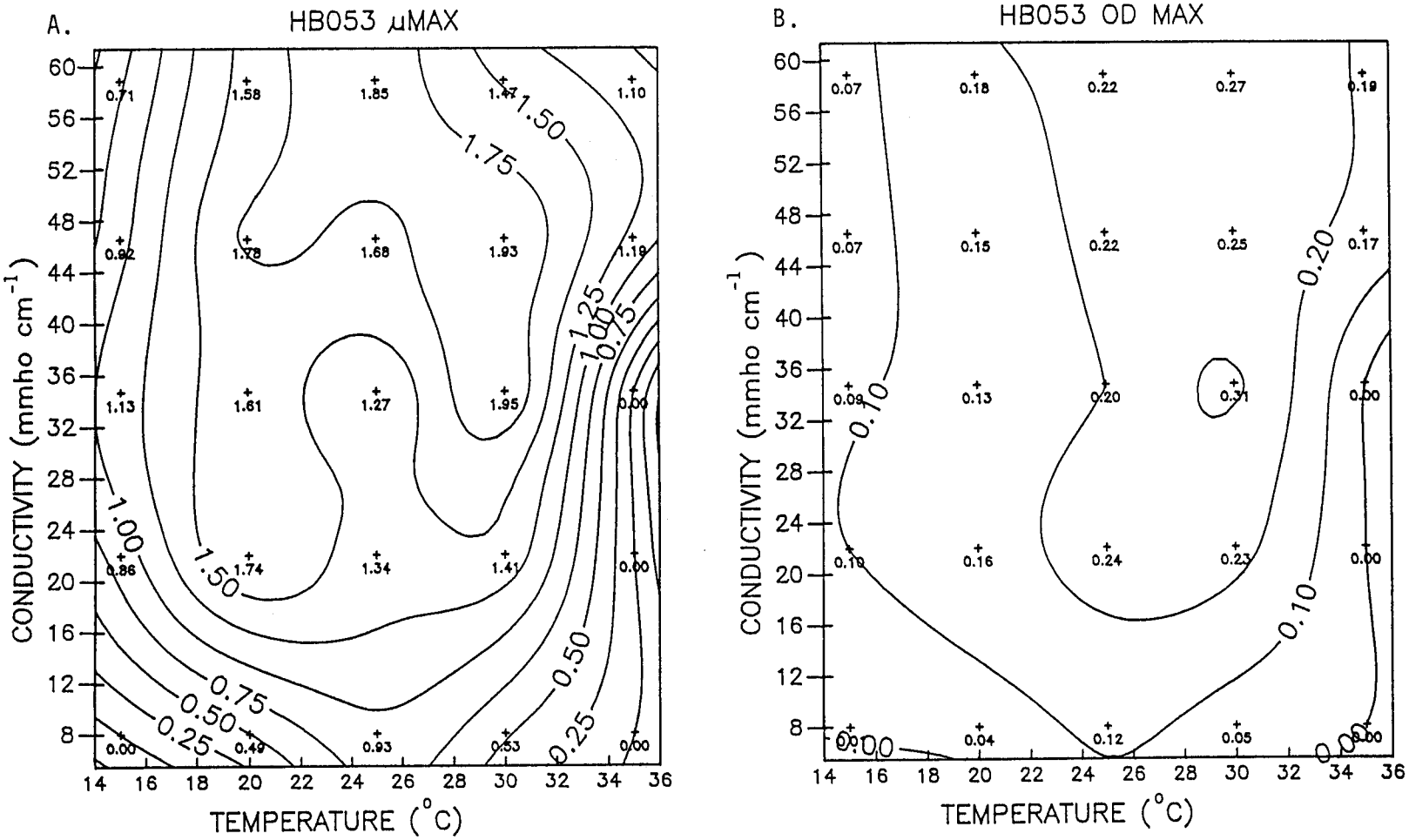

Figure 4. Growth response in batch culture of HB53 (a prasinate) to temperature and salinity. A. Contours are exponential growth rate (doubling day^{-1}, O.D. 750). B. Contours are maximum optical density.

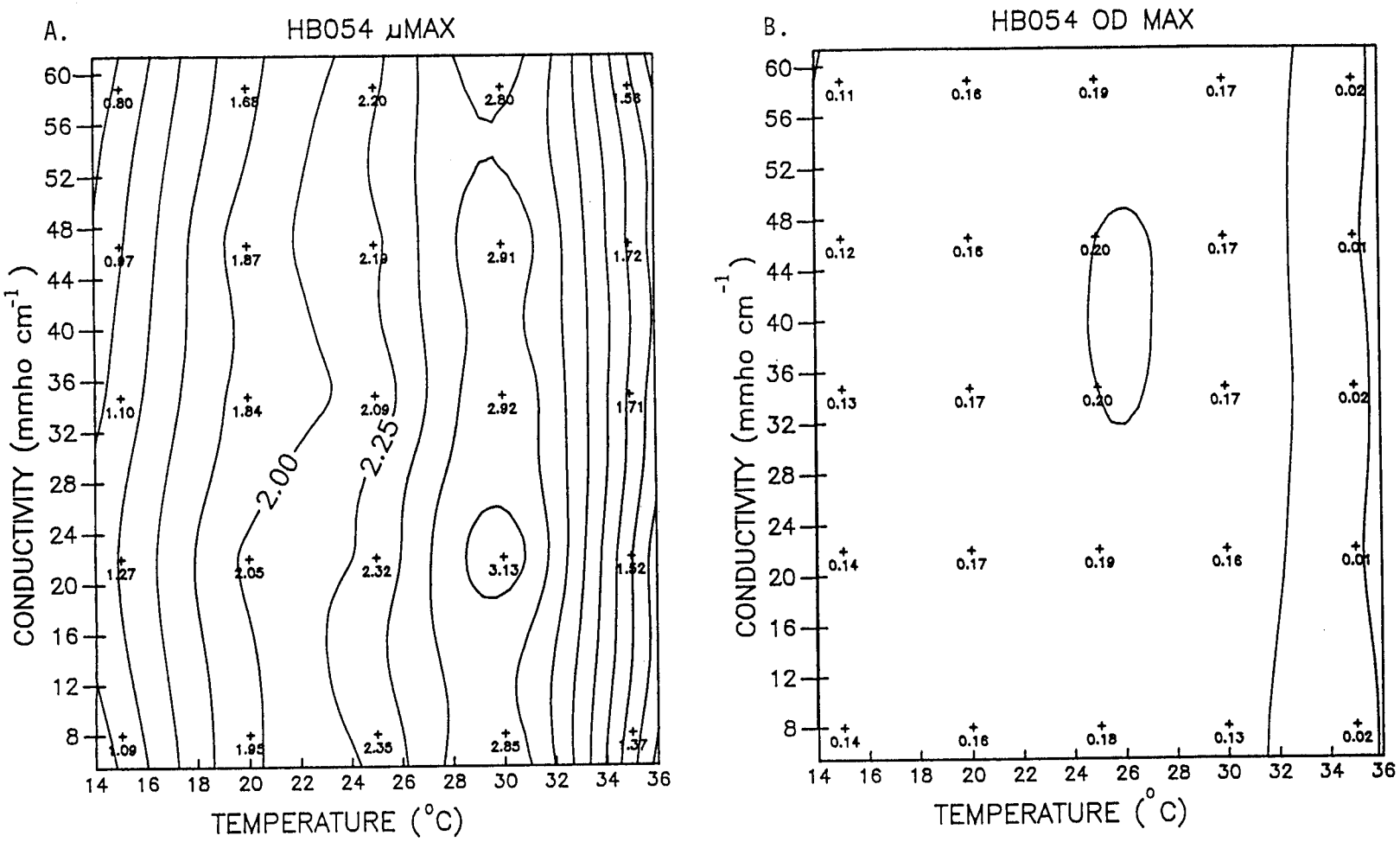

Figure 5. Growth response in batch culture of HB54 (a yellow green unicell) to temperature and salinity. A. Contours are exponential growth rate (doubling day^{-1}, O.D. 750). B. Contours are maximum optical density.

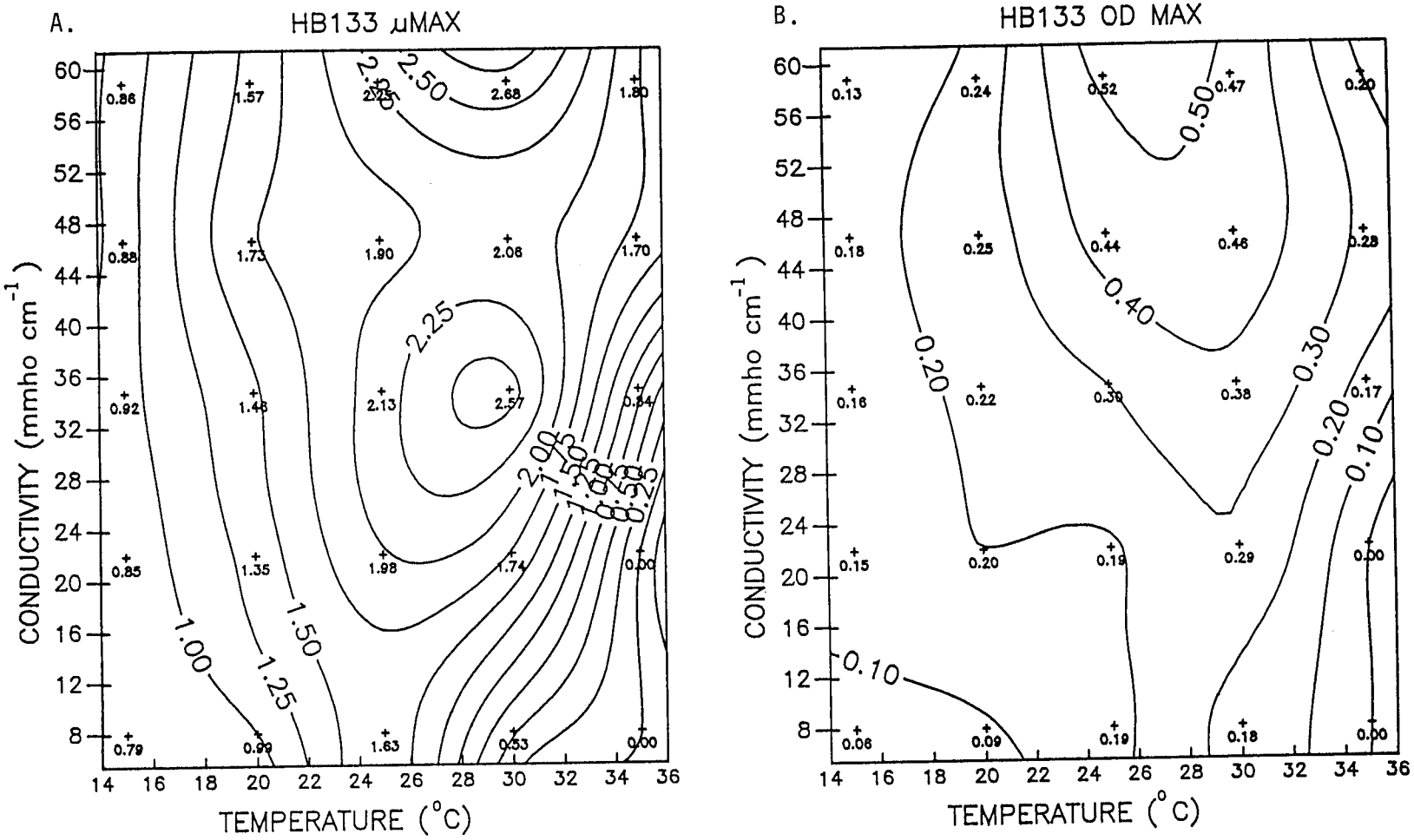

Figure 6. Growth response in batch culture of Pyramimonas (HB133) to temperature and salinity. A. Contours are exponential growth rate (doubling day^{-1}, O.D. 750). B. Contours are maximum optical density.

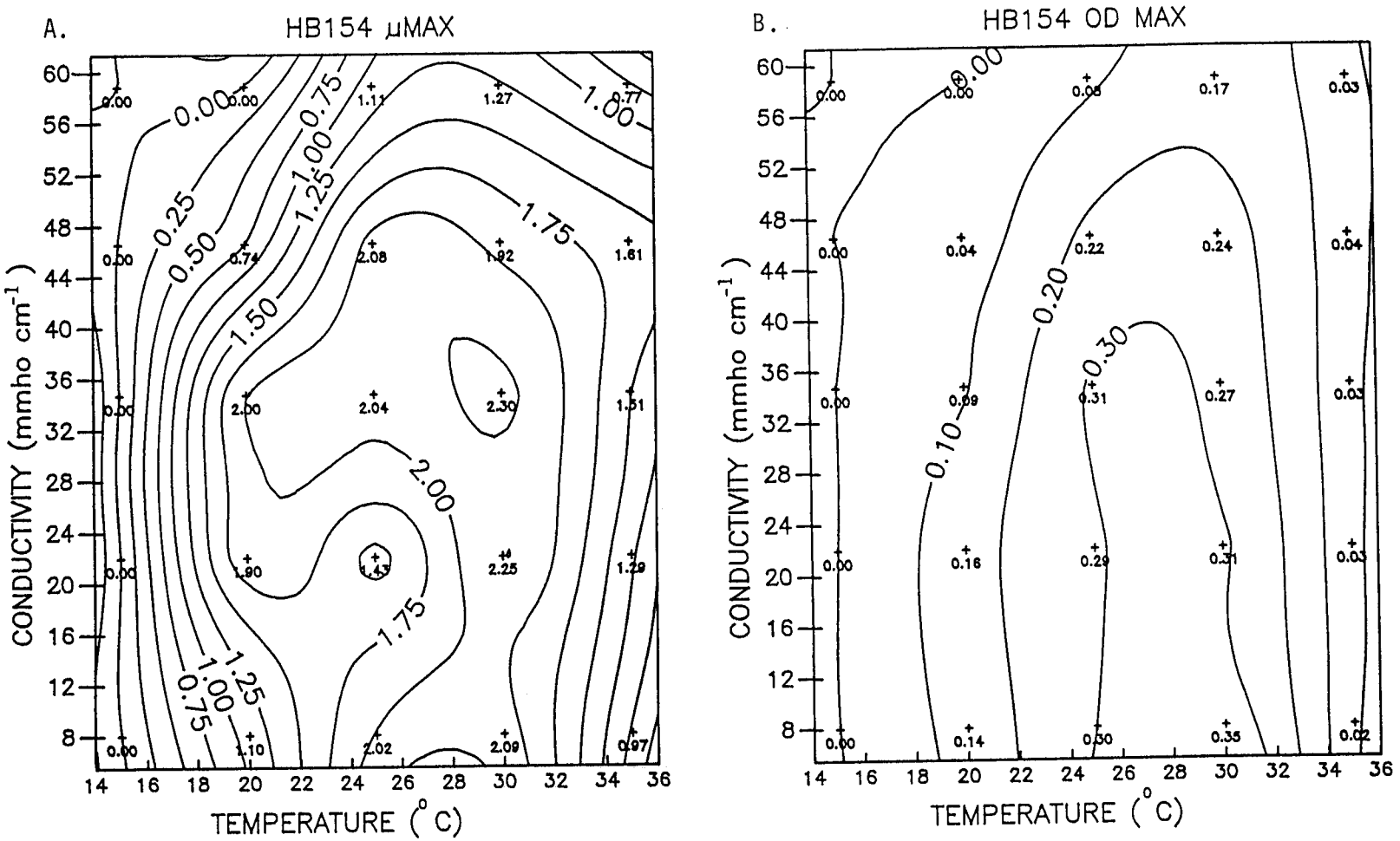

Figure 7. Growth response in batch culture of HB154 (an olive green unicell) to temperature and salinity. A. Contours are exponential growth rate (doubling day^{-1}, O.D. 750). B. Contours are maximum optical density.

$30^{o}C$ (maximum = 2.50 doubling day^{-1}, 22 mmho cm^{-1}) over the entire salinity range, its growth was greatly reduced below $22^{o}C$ or above $33^{o}C$ (Figure 8). Indeed, maximal cell densities of 0.20 O.D. were obtained over only 7% of the experimental range indicating that the elevated growth rates were a short-term growth response for this species.

Although the growth of Ochromonas (HB167) was impeded at low temperature-salinity combinations, this species maintained growth rates greater than 1 doubling day^{-1} between 18-$35^{o}C$ and 10-59 mmho cm^{-1} (Figure 9). At its optimal temperature and salinity ($35^{o}C$, 35 mmho cm^{-1}), Ochromonas grew at a rate of 2.45 doubling day^{-1}. Maximal cell densities increased with salinity at $30^{o}C$ and the open contours at the experimental maximum indicate this species should grow at even higher salinities.

The data from the temperature-salinity gradient experiments indicate that the preliminary screening protocol successfully identified species that can grow under fluctuating culture environments. Five of 7 species exhibited a wide tolerance to temperature and salinity and grow well in one or both of the SERI desert water formulations. Of the 5 species, the elevated growth and biomass production at high salinity of Nannochloris, Ochromonas and Pyramimonas appear to be particularly desirable for outdoor cultivation systems where blowdown expense may be an economic factor. Two species (HB54 and HB154) had high growth rates over much of the experimental range, but significant cell density accumulations were confined to relatively narrow limits. This observation serves to illustrate that exponential growth rates, in conjunction with maximum optical density determinations (or some other measure of cell yield) are necessary to assess the growth potential of a particular algal species during the screening process.

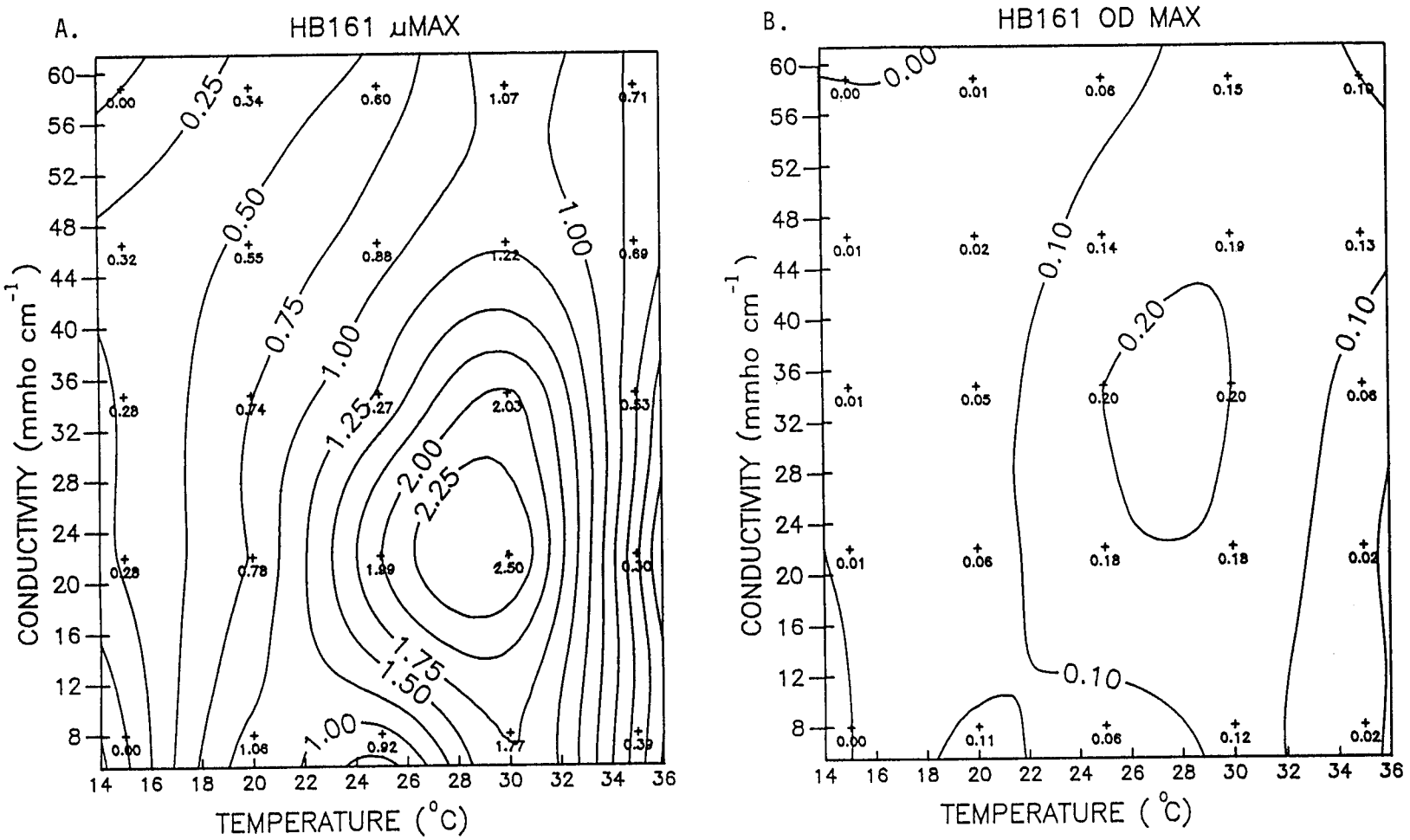

Figure 8. Growth response in batch culture of Coscinodiscus (HB161) to temperature and salinity. A. Contours are exponential growth rate (doubling day^{-1}, O.D. 750). B. Contours are maximum optical density.

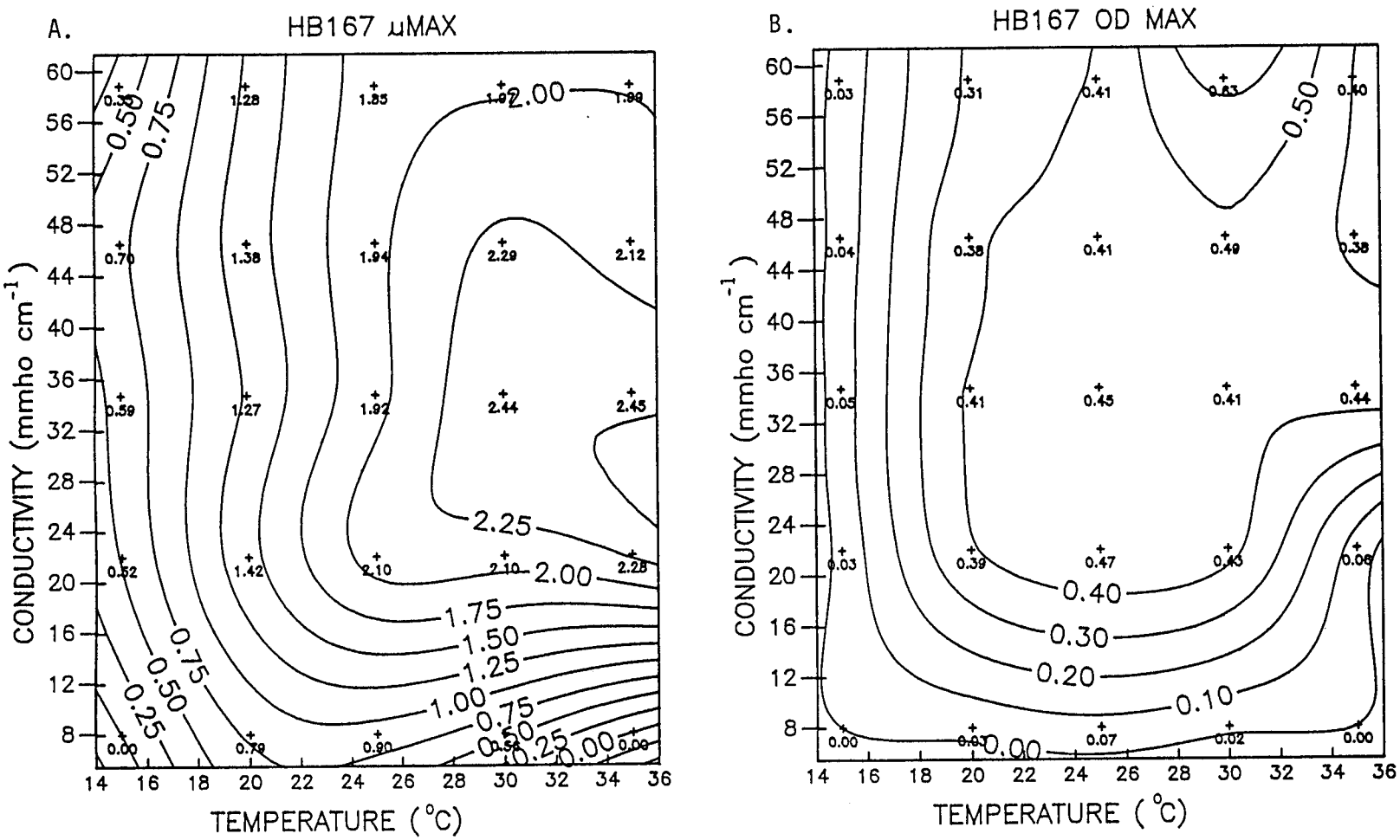

Figure 9. Growth response in batch culture of Ochromonas (HB167) to temperature and salinity. A. Contours are exponential growth rate (doubling day^{-1}, O.D. 750). B. Contours are maximum optical density.

Task III: Determine the proximate chemistry of at least 5 strains of microalgae characterized for growth in Task II.

Objective: To evaluate fuel production potential of selected microalgae.

Methods

Proximate Analysis of Select Species: A survey of the proximate chemical composition of promising species already in culture was examined in outdoor cultures exposed to full sunlight. A 10% inoculum of exponentially growing cells was added to 9.5 L carboys containing 7 L of ES or F/2 (880 µM nitrate) enriched natural seawater. Temperature was controlled ($26^{\circ} \pm 1.5^{\circ}C$) by partial immersion in a water table provided with flow-through well water and cultures were sparged with air. The O.D. was monitored daily and cultures were harvested following the second day of stationary phase.

Lipid induction experiments were performed with species which displayed good growth over a range of temperatures and salinities and that stained positively for lipid. Cultures were grown under nitrogen sufficient and deficient conditions in a modified design of Barclay et al. (1985). Two 9.5 L carboys containing 7 L of natural seawater were enriched with F/2 containing 880 µM or 440 µM nitrate. Cultures were mixed by magnetic stirring and grown under continuous illumination (250 $\mu E\ m^{-2}\ sec^{-1}$) at $28 \pm 1^{\circ}C$. Both cultures were sparged with 1% CO_2 in air and maintained at pH 7.8 by individual pH controllers. One half of each carboy was harvested following the second day of stationary phase or when there was an obvious color change in the 440 µM nitrate culture. Cultures were brought to volume with nitrate-free media to ensure the stationary phase was due to nitrogen deprivation rather than light limitation. The remaining volume of the 440 µM culture was harvested following 7 days in the stationary stage. Nannochloropsis (Nanno-Q), a species provided by SERI, was grown in ASP-2 artificial seawater at 25°/oo under the above culture conditions to serve as a standard and to evaluate our extraction procedures. Nitrogen was supplied to Nanno-Q as urea at 880 and 440 µM.

Cells were harvested by centrifugation, washed with isotonic ammonium formate, freeze-dried and homogenized prior to proximate chemical analysis. Proteins were determined by the method of Lowry et al. (1951) using a bovine serum standard and a 1 hour incubation in 1.0 N NaOH at $50^{\circ}C$. Carbohydrates were determined by the method of Dubois et al. (1956) using a glycogen standard. Lipids were determined gravimetrically by a modification of Bligh and Dyer (1954). Cells were initially extracted in hot methanol ($60^{\circ}C$) prior to repeated extractions in chloroform-methanol. Following adjustment of solvent ratios and phase separation, the chloroform fraction was dried under nitrogen. Organic contents of the cells were expressed as the fraction of the weight loss following ignition of the original sample at $500^{\circ}C$ for 1 hour.

Results and Discussion

Proximate Analysis: Early in the contract period, species examined for biochemical composition determinations were selected based upon visual estimation of biomass production. Seven species, including 6 chromophytes, were surveyed in outdoor carboy cultures for the accumulation of lipid early

in the stationary growth phase (Table 6). All cultures were grown under nitrogen sufficient conditions. While protein levels were relatively low and exhibited little variation (8-12% AFDW), carbohydrate content varied between 11% (Hymenomonas HB89) and 47% (Amphidinium). The maximum lipid accumulated for all species ranged from 14-20%. Hymenomonas (HB89) and Amphidinium produced the greatest amount of lipid (19.7 and 18.5%, respectively). However, the results show there was no substantial amount of lipid in the microalgae tested immediately following exponential growth.

Table 7 presents results of lipid induction experiments due to nitrogen limitation for Nanno-Q and 6 species which exhibited good growth over a wide range of temperature and salinity. Nanno-Q ceased exponential growth (approximately 1.5 doublings day^{-1}) after 6 days. Final harvest was on day 13 with an ash-free weight (AFDW) yield of 425 mg/L in the nitrogen deficient culture. As previously reported (Terry et al., 1986), Nanno-Q displayed rapid lipid accumulation with nitrogen deprivation (Table 7). Lipid levels increased from 34% in nitrogen sufficient cultures to 54% AFDW under nitrogen deficient conditions. Lipid increased at the expense of protein while carbohydrate levels remained relatively unchanged at low concentrations.

Hymenomonas (HB89) exponential growth (0.84 doubling day^{-1}) ceased after 6 days. However, due to good post-nitrogen-depletion productivity, the ash-free dry weight increased from 334 mg/L to a final yield of 781 mg/L at day 13. Both protein and lipid were replaced by carbohydrate which increased with early nitrogen depletion. Indeed, as nitrogen deprivation continued, protein decreased and lipid contents dropped from 24.4 to 16.8% AFDW. A similar reduction of lipid composition with nitrogen deficiency was found for Hymenomonas carterae (Shifrin and Chisholm 1981). While these results indicate that motile forms of coccolithophores may not accumulate significant amounts of lipid, this group should not be discounted as low lipid producers until proximate analysis of strains displaying the benthic apistonema growth habit have been examined.

The growth of Nannochloris (HB44) under high and low nitrogen culture conditions is depicted in Figure 10. This species grew at an exponential growth rate of 2.07 doubling day^{-1} until the nitrogen supply of the culture became depleted by day 4. As with Nanno-Q, Nannochloris exhibited rapid lipid accumulation with nitrogen deprivation (Table 7), but no significant color change was observed by this species. Lipid contents increased from 30.3% to 56.3% AFDW after 7 days in post-nitrogen-depleted cultures. However, lipids increased primarily at the expense of carbohydrates for Nannochloris, whereas protein levels were apparently reduced with Nanno-Q. The remaining 23% carbohydrate in the nitrogen depleted cells indicate that additional lipid production may have been possible if the Nannochloris culture was harvested 10 days following cessation of exponential growth.

In contrast to Nannochloris, HB154 (an olive green unicell) showed no significant lipid increase between nitrogen sufficient and deficient cultures. Exponential growth (1.17 doubling day^{-1}) ceased at day 4 where it achieved a maximum cell yield of 800 mg/L AFDW (Figure 11). The maximum lipid content obtained by this species was 28.5% AFDW in the nitrogen sufficient cultures. Lipid levels in the low nitrogen culture averaged 20% lipid and did not change with nitrogen depletion.

Table 6. Average ($\bar{X}$) proximate chemical composition (n=2) and standard deviation (S.D.) of South Florida microalgae grown in outdoor culture (See Methods for culture conditions). END = early nitrogen depletion at time of harvest.

SPECIES	GROWTH CONDITION	% AFDW PROTEIN $\bar{X} \pm$ S.D.	% AFDW CARBOHYDRATE $\bar{X} \pm$ S.D.	% AFDW LIPID $\bar{X} \pm$ S.D.
OUTDOORS				
Chrysocapsa HB16	END	8.3 ± 0.3	35.4 ± 1.2	14.6 ± 0.6
Chrysosphaera HB20	END	8.6 ± 0.3	42.0 ± 0.6	17.3 ± 0.2
Hymenomonas HB38	END	9.0 ± 0.3	42.7 ± 1.2	13.9 ± 0.5
Gloeothamnion HB42	END	9.1 ± 0.4	23.9 ± 0.0	16.0 ± 2.7
Hymenomonas HB89	END	12.2 ± 0.1	11.1 ± 0.7	19.7 ± 0.4
Eremosphaera HB90	END	8.3 ± 0.2	27.2 ± 0.4	17.1 ± 2.4
Amphidinium HB838	END	10.6 ± 0.3	46.9 ± 2.3	18.5 ± 0.0

Table 7. Average ($\bar{X}$) proximate chemical composition (n=2) and standard deviation (S.D.) cultivated in the laboratory. NS = nitrogen sufficient, END = early nitrogen depletion, ND = nitrogen depleted and LND = late nitrogen depleted cultures at time of harvest.

SPECIES	GROWTH CONDITION	CULTURE AGE	% AFDW PROTEIN $\bar{X} \pm$ S.D.	% AFDW CARBOHYDRATE $\bar{X} \pm$ S.D.	% AFDW LIPID $\bar{X} \pm$ S.D.
LABORATORY					
Nannochloropsis (Nanno-Q)	NS	7	24.5 ± 1.4	4.9 ± 0.6	34.1 ± 1.7
	END	7	28.0 ± 5.3	4.3 ± 1.1	42.6 ± 2.3
	ND	13	10.1 ± 0.7	4.3 ± 0.0	54.0 ± 0.1
Hymenomonas HB89	NS	6	10.5 ± 0.7	32.1 ± 1.1	24.4 ± 0.1
	END	6	7.6 ± 0.1	42.1 ± 3.8	20.7 ± 0.9
	ND	13	4.1 ± 0.0	37.0 ± 5.6	16.8 ± 2.8
Nannochloris HB44	NS	4	12.2 ± 0.3	33.5 ± 0.0	30.3 ± 0.5
	END	4	7.6 ± 0.2	37.6 ± 0.5	31.6 ± 1.3
	ND	11	3.2 ± 0.5	23.0 ± 0.6	56.3 ± 1.5
Olive Green Unicell HB154	NS	6	6.3 ± 0.2	43.9 ± 0.0	28.5 ± 5.5
	END	6	2.9 ± 3.3	47.8 ± 3.9	19.0 ± 0.8
	ND	13	---	43.4 ± 1.3	21.1 ± 2.7
Tetraselmis HB47	NS	4	7.3 ± 0.3	48.6 ± 3.7	20.5 ± 0.9
	END	4	5.5 ± 0.1	59.8 ± 2.4	16.4 ± 0.7
	ND	11	3.5 ± 0.6	36.5 ± 1.8	22.3 ± 1.5
Prasinate HB53	NS	3	24.4 ± 1.1	27.8 ± 1.9	23.2 ± 1.3
	END	3	15.9 ± 0.1	45.7 ± 0.0	12.9 ± 0.6
	ND	10	12.8 ± 0.0	36.8 ± 3.8	16.3 ± 2.3
	LND	18	11.8 ± 1.2	37.5 ± 0.0	19.3 ± 0.5
Pyramimonas HB133	NS	5	11.3 ± 0.1	37.2 ± 3.9	29.8 ± 1.6
	END	5	4.7 ± 0.8	49.3 ± 3.7	21.2 ± 1.0
	ND	12	2.7 ± 0.1	54.0 ± 0.6	34.1 ± 5.0

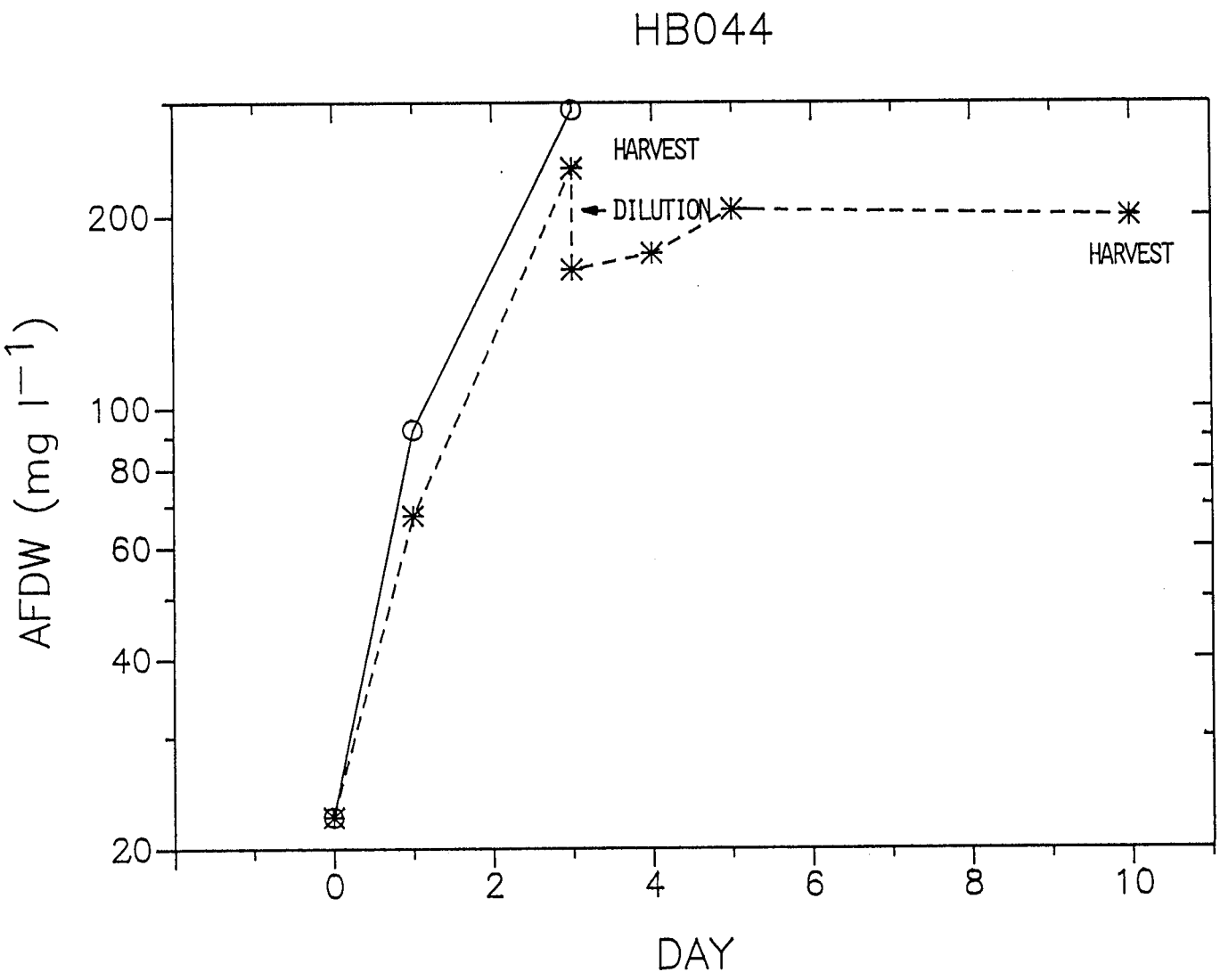

Figure 10. Growth of Nannochloris (HB44) subjected to nitrogen depletion in 9L carboys grown under laboratory conditions. o = F/2 with 880 uM nitrate, * = F/2 with 440 µM nitrate.

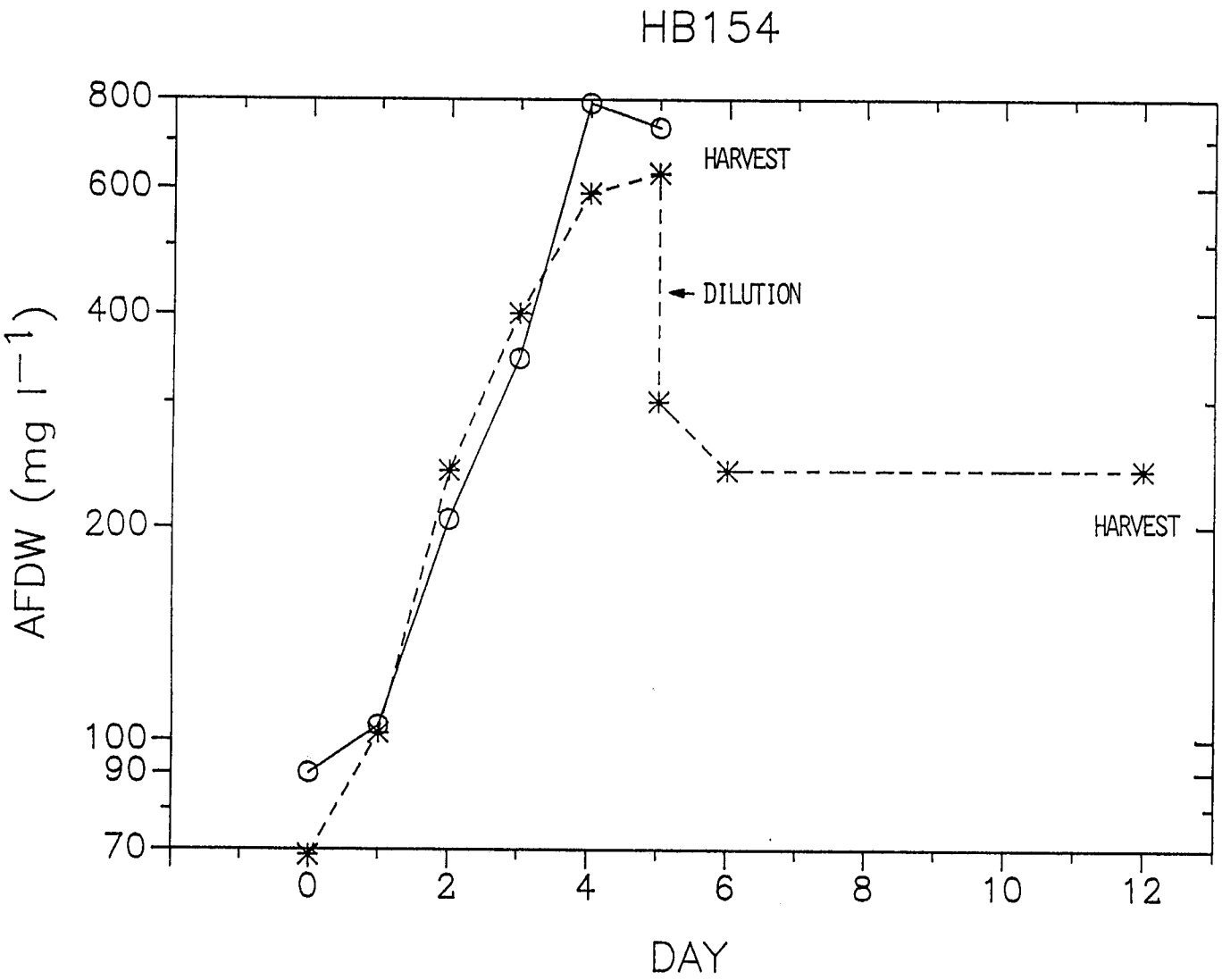

Figure 11. Growth of HB154 subjected to nitrogen depletion in 9L carboys grown under laboratory conditions. o = F/2 with 880 uM nitrate, * = F/2 with 440 µM nitrate.

Three prasinates were examined for proximate chemical composition because of their good growth in unstable culture environments and apparent ability to accumulate lipid (as determined microscopically). The growth characteristics for Tetraselmis, isolate HB53 and Pyramimonas are depicted in Figures 12-14. Exponential growth rates ranged from 1.70 (HB53) to 2.75 (Pyramimonas) doubling day^{-1} until the nitrogen supplies of the cultures became depleted between day 3 and 5. All 3 species displayed similar chemical compositional changes (Table 7). Carbohydrates increased and lipid decreased between the nitrogen sufficient and early nitrogen depletion stage. However, the opposite response occurred within the same cultures (440 uM nitrate) between early nitrogen depleted and nitrogen deficient conditions. Lipid levels increased from 16.4 to 22.3% AFDW for Tetraselmis; 12.9 to 19.3% for HB53, and 21.2 to 34.1% for Pyramimonas. In Tetraselmis and HB53, lipids increased at the expense of carbohydrate, but the 12.9% increase in lipid for Pyramimonas cannot be attributed to the metabolism of protein or carbohydrate.

Lipid evaluation of the prasinates with nile red indicated a substantial increase in lipid content between 21-day and log phase tube cultures. We postulated that lipid accumulation in this group may begin later in the stationary phase, so we extended the post-nitrogen depletion culture to 15 days before harvest for HB53. Although this species produced large amounts of soluble pigments, no increase in lipid content was observed over cells harvested 8 days earlier (Table 7). Variations in observed lipid content during screening and the actual values obtained in the proximate analyses could be attributed to the differences in culture conditions (eg. pH, carbon supply), but nevertheless, the preliminary evaluation is useful for comparing relative lipid contents between species. The relative lipid composition of Pyramimonas (HB133) found in this study is considerably higher than values reported for other prasinates grown under nitrogen stress (Laws 1984, Utting 1985). Staining with nile red indicates additional strains of prasinates isolated during this study may accumulate even greater amounts of lipid (see Table 4, Task II).

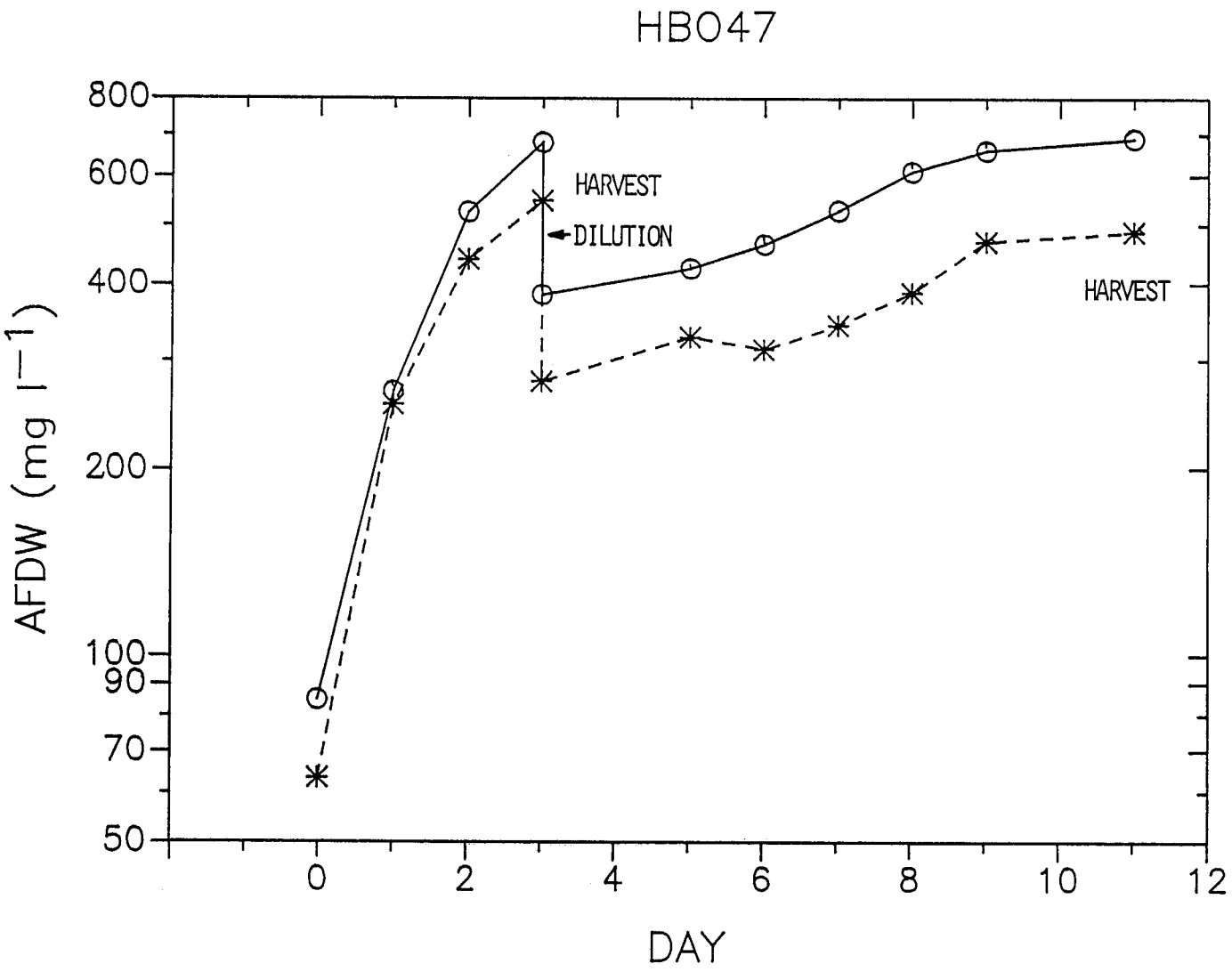

Figure 12. Growth of Tetraselmis (HB47) subjected to nitrogen depletion in 9L carboys grown under laboratory conditions. o = F/2 with 880 uM nitrate, * = F/2 with 440 µM nitrate.

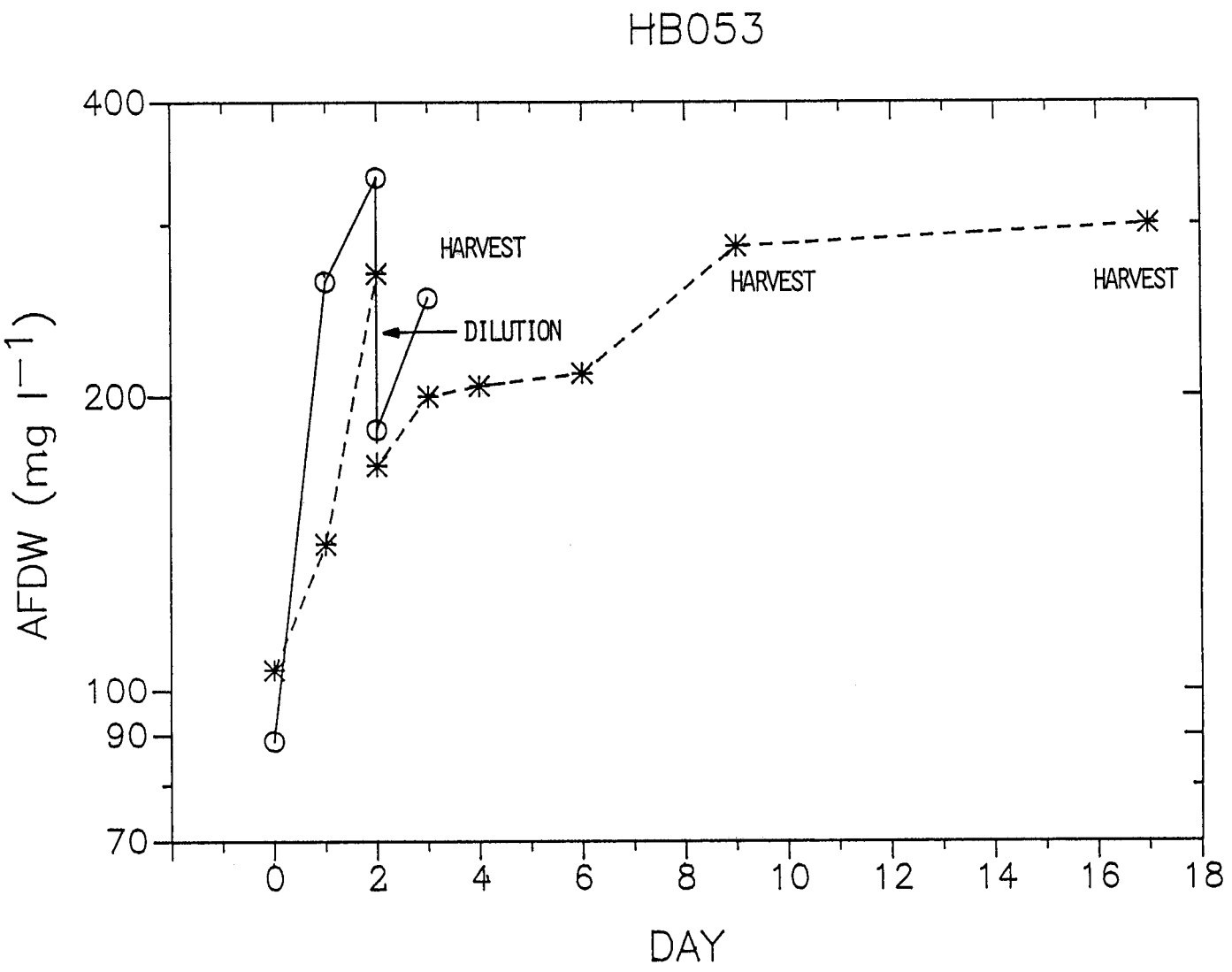

Figure 13. Growth of HB53 (a prasinate) subjected to nitrogen depletion in 9L carboys grown under laboratory conditions. o = F/2 with 880 uM nitrate, * = F/2 with 440 μM nitrate.

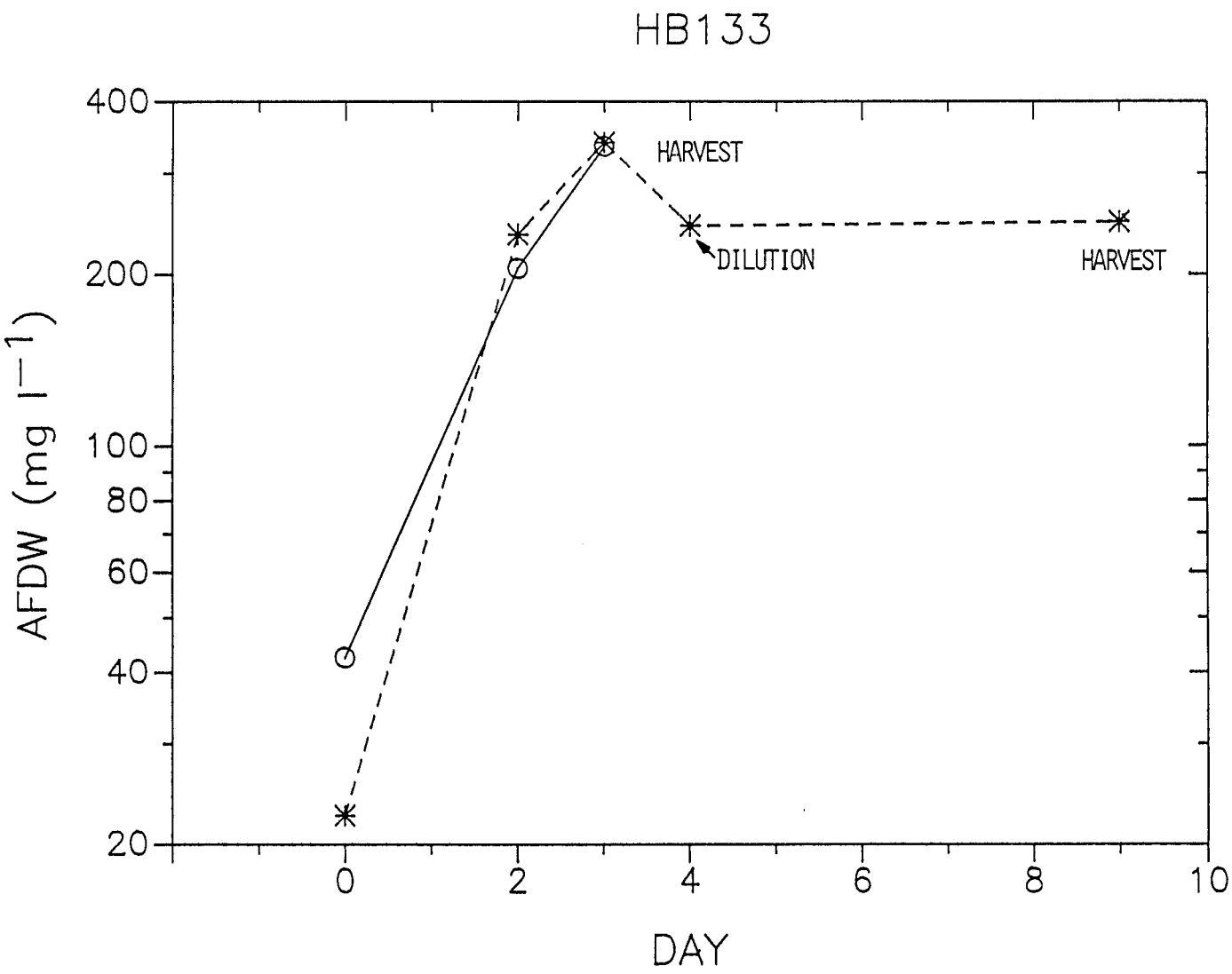

Figure 14. Growth of Pyramimonas (HB133) subjected to nitrogen depletion in 9L carboys grown under laboratory conditions. o = F/2 with 880 uM nitrate, * = F/2 with 440 µM nitrate.

Task IV: Determine the interactive effects of pH, carbon type and concentration on the relative yield potential of several strains of microalgae.

Objective: To define the influence of pH, CO_2, HCO_3^-, and $CO_3^=$ on the potential yield of high performance marine microalgae.

Methods

Carbon Studies: The purpose of the carbon studies was to correlate microalgal productivities with carbon type and pH while accounting for differences between pH and carbon effects. Microalgal utilization of inorganic carbon is dependent upon pH, rate reactions and buffering capacity of the CO_2-H_2CO_3-HCO_3^- -$CO_3^=$ chemical system (Goldman et al., 1974). Carbon dioxide gas dissolves in water to form uncharged aqueous CO_2, which in turn combines with water to form undissociated carbonic acid. At equilibrium, the aqueous CO_2 concentration is much greater than H_2CO_3, and their combined concentrations, considered to be roughly equal, are denoted as $H_2CO_3^*$ (Stumm and Morgan, 1981). These carbon species are in equilibrium with bicarbonate and carbonate as follows:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3^* \rightleftharpoons HCO_3^- + H^+ \rightleftharpoons CO_3^= + H^+$$

The relative fractions of these carbon species is determined by pH, and alkalinity influences the total dissolved inorganic carbon concentration. Seawater alkalinity in equilibrium with atmospheric CO_2 is predominantly (90%) a function of carbonate and bicarbonate while hydroxide and borate primarily comprises the remaining fraction. Since CO_2 dissolves in seawater as $H_2CO_3^*$, addition or removal of CO_2 does not effect total alkalinity. Although the net effect of algal carbon assimilation (as CO_2) is a decrease in carbonate alkalinity the total alkalinity is conserved by increased hydroxide (ie. higher pH) and increased borate alkalinity.

One way to examine the differences between pH and carbon effects is to maintain the concentration of a particular carbon species while varying pH. For example, CO_2 concentration decreases by a factor of ten as the pH rises from 7 to 8. However, if the TIC of the pH 8 medium is ten times that of the pH 7 medium, the media will have identical CO_2 concentrations. At the same time, HCO_3^- concentration increased tenfold and $CO_3^=$ concentration has increased one hundredfold. If algal productivity was equal in the 2 medias, and the CO_2 concentration was low enough so that carbon was the limiting nutrient, then CO_2 rather than pH would be the growth limiting factor. This feature of aqueous carbon chemistry served as the basis for the carbon experiments in the present study. By independently manipulating the pH and TIC concentration (through varying alkalinity), carbon type and concentration could be examined over a wide range of pHs.

A pH-stat experimental design was employed to determine the interactive effects of pH and the concentration of inorganic carbon species on the yield potential of saline microalgae. ASP-2 artificial seawater containing 0.25 mM $CaCl_2$ and no inorganic carbon was used as the basal medium at 47 mmho cm^{-1}. Supplemental alkalinity was provided by first adding Na_2CO_3 to 3 parts distilled water, which in turn was added to the ASP-2 to give a final salinity of 35 mmho cm^{-1}. This prevented precipitation of $CaCO_3$ and $MgCO_3$

due to the locally high pH that would occur if the Na_2CO_3 was added directly to the seawater. Media with alkalinities as high as 50 meq/L could be formulated without precipitation, but the experimental alkalinity range was restricted to a maximum of 10 meq/L due to precipitation of enrichments. Enrichments of 1 mM urea, 30 µM PO_4 and F/2 metals and vitamins were added with the design alkalinity.

The BASIC carbon equilibrium program DEBUSKER (see Appendix) written by Mark Blakeslee (Ryther, et al., 1984), was modified for ASP-2 to calculate the borate alkalinity, TIC, $H_2CO_3^*$, HCO_3^-, and $CO_3^=$ concentrations given the total alkalinity, pH, temperature and salinity. A second version calculated carbon species concentrations based on experimental TIC values measured with a carbon analyzer (Model 700, OI Corporation). DEBUSKER was used in the experimental design to calculate TIC concentrations resulting in identical CO_2 (3 µM) and HCO_3^- (0.3 µM) concentrations over a range of pH values which would be operationally feasible in large-scale outdoor cultures (7.5 - 9.0 and 7.5 - 9.5 respectively). These carbon concentrations were low enough to be the limiting nutrient, but high enough for measurable algal production. Three treatments of different pH and TIC concentration were chosen for each carbon species concentration which enabled the algal production to be dependent on the particular carbon species.

Eight 9.5 L carboys containing 4 L of medium were maintained at the constant pH values of 7.5, 8.0, 8.5, 9.0, 9.5 and 10. One culture was maintained at pH 8 with high TIC levels to measure growth without carbon limitation; another culture grew at pH 10 to examine maximal pH tolerance in the algae. One to 5% CO_2 in air or CO_2 - free air (scrubbed with 5N NaOH) was sparged into the cultures as required by individual pH controllers. Cultures were mixed by magnetic stirring and grown under continuous illumination (300 µE m^{-2} sec^{-1}) at 28°C. Maximum growth rates and maximal and mean biomass production were measured for each carboy by O.D. and ash-free dry weight determinations over a 3-4 day period.

Results and Discussion

Carbon Studies: Three species, Isochrysis (TISO), Chlorella (HB73) and Nannochloropsis (Nanno-Q) have been evaluated to date for growth in carbon experiments. Neither growth rate nor production was dependent on alkalinity (0.12 - 10.0 meq/L) over a pH range of 7.5 - 10.0. For Chlorella, maximal and mean production corresponded with CO_2 concentration and pH. However, no significant differences were observed between exponential growth rate and CO_2 or pH. Figure 15 shows the results for Chlorella average production vs. CO_2, HCO_3^-, TIC concentration and pH. Visual inspection of the plots showed the best correlation of average production was with CO_2 concentration. Correlation also appeared to be good with pH, but we believe this reflects the coupling of CO_2 and H^+ concentration. Correlation analysis of the mean production with the log of the carbon concentrations, confirmed the conclusions arrived by inspection: CO_2 concentration vs. production r=0.947; pH vs. production r=0.856. However, average production was not statistically different (Duncan's Multiple Range Test, p=0.05) between 2-3 µM CO_2 at pH 7.5, 8.5, and 9.0 This indicates that average production was more dependent on CO_2 concentration than pH between pH 7.5 - 9.0 for Chlorella.

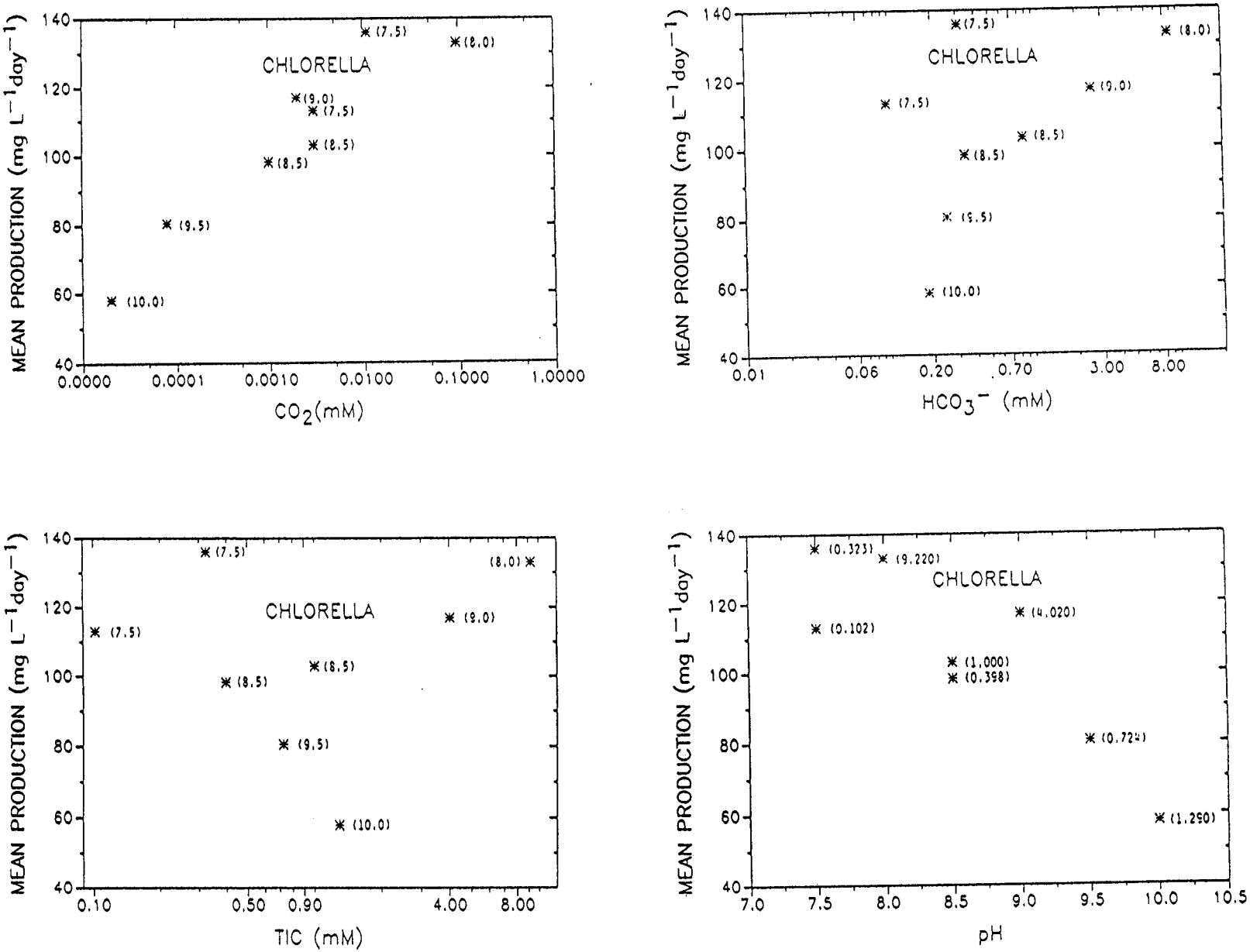

Figure 15. Average production (mg L^{-1} day^{-1} AFDW) vs. CO_2, HCO_3^-, TIC concentration and pH for Chlorella (HB73). Numbers on carbon plots are pH; on pH plot are TIC concentrations.

Figure 16 depicts the results for TISO average production with carbon type and pH. Both TISO production parameters and growth rate corresponded to CO_2 concentration and pH, but average production was more sensitive than the other growth variables. The plots on Figure 16 illustrate the best correlation of average productivity was with CO_2 (r=0.965) rather than pH (r=0.813). Production was not significantly different between 2-3 μM CO_2 at pH 7.5 and 8.5 indicating productivity was more dependent on CO_2 concentration within this pH range. However, TISO production and growth rate was significantly reduced above pH 8.5, and no growth was observed at pH 9.5.

Nanno-Q average (r=0.903) and maximal (r=0.987) production rates corresponded to CO_2 concentration in the pH-stat system, but only maximal production values correlated with pH (r= -0.883). Exponential growth rates did not correspond to CO_2 or pH. Figure 17 show the plots for Nanno-Q average production vs. the individual carbon species and pH. Nanno-Q mean production rates were virtually identical at 3 μM CO_2 between pH 7.5 and 9.0. Production rates for Nanno-Q dropped significantly at pH 9.5, but this may be a combined effect of increased pH and lower CO_2 concentration. To determine if the reduction was a CO_2 or pH effect, additional experiments would be needed to examine the biomass production rate of Nanno-Q at this CO_2 concentration over a wide range of pH.

The results of the carbon experiments are significant because we have been able to distinguish the effects of pH from carbon on microalgal productivity in a saline medium. These data are useful in determining the pH limits beyond which Nanno-Q, Chlorella and TISO productivity would decrease in a large-scale algal production system. Good correlations were found between production rates for CO_2 concentration over a wide pH range (7.5-9.0) for Nanno-Q and Chlorella. Similar findings were reported for a freshwater species of Chlorella (Weisman and Goebel 1985). While none of the microalgae examined correlated with a carbon species other than CO_2, the experimental design would also be useful for identifying microalgae that may utilize bicarbonate.

As other aspects of algal production systems become better defined, the use of mathematical optimization algorithms will become possible and necessary. The empirical productivity/carbon concentration relation may be represented mathematically by power series or Fourier series formulations and used in the optimization model.

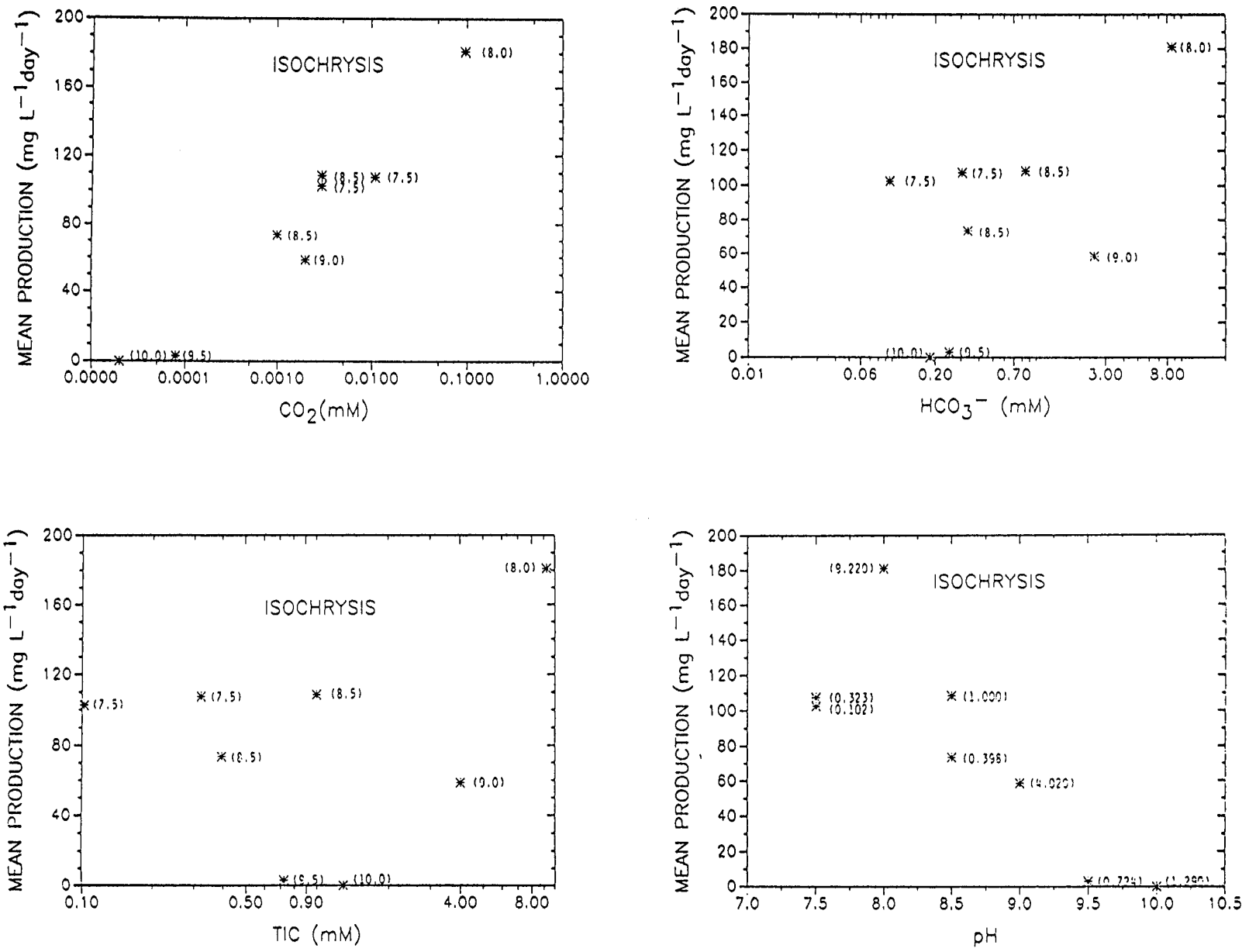

Figure 16. Average production (mg L^{-1} day^{-1} AFDW) vs. CO_2, HCO_3^-, TIC concentration and pH for Isochrysis (TISO). Numbers on carbon plots are pH; on pH plot are TIC concentrations.

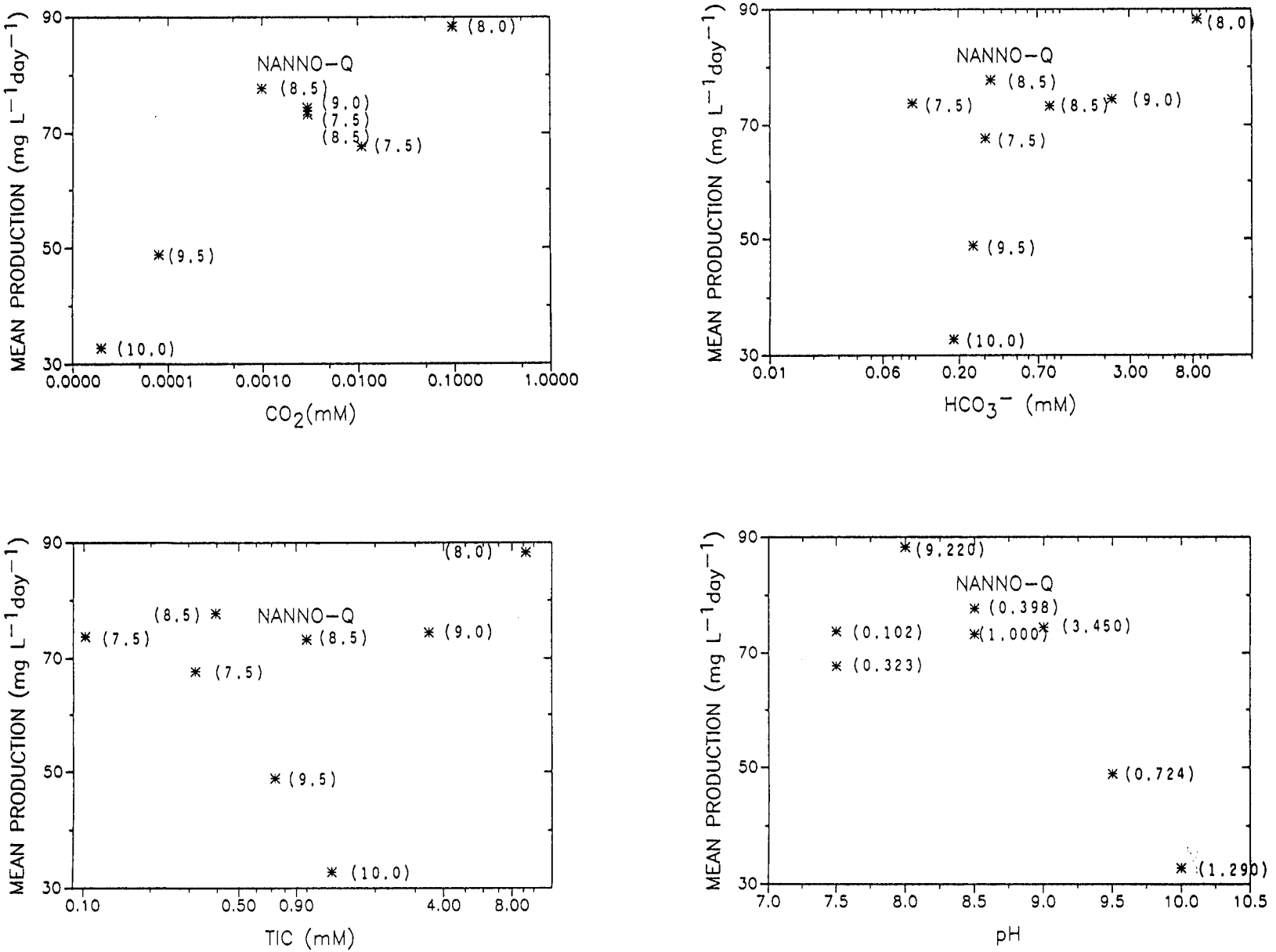

Figure 17. Average production (mg L^{-1} day^{-1} AFDW) vs. CO_2, HCO_3^-, TIC concentration and pH for Nannochloropsis (Nanno-Q). Numbers on carbon plots are pH; on pH plot are TIC concentrations.

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APPENDIX

Table 8. Exponential growth rate (doublings day^{-1}, OD 750)/maximal optical density of chromophyte species examined during preliminary screening. Species were grown in Type I, Type II and natural seawater (GSW) at 25 and 40 mmho cm^{-1} salinity, and 35°C.

	GSW		TYPE I		TYPE II	
SPECIES	25	40	25	40	25	40
Ochromonas HB7	0.89/0.09	0.84/0.10	0.54/0.10	0.69/0.07	NG/NG	NG/NG
Chrysochromulina HB10	0.67/0.10	0.73/0.02	0.60/0.06	0.56/0.08	0.78/0.04	0.83/0.05
Cocco. Sp. HB12	0.54/0.03	0.32/0.02	0.09/0.08	0.65/0.04	1.10/0.12	0.89/0.09
Chryso/C3 HB14		N O	G R O W T H			
Chrysocapsa HB16	0.36/0.02	0.42/0.07	0.39/0.07	0.56/0.05	0.41/0.02	0.40/0.04
Sarcinochrysis HB19	0.44/0.11	0.36/0.08	NG/NG	NG/NG	NG/NG	NG/NG
Chrysosphaera HB20	0.32/0.08	0.41/0.10	NG/NG	0.51/0.10	NG/NG	0.66/0.12
Chryso/C1 HB21	0.12/0.03	0.32/0.09	NG/NG	0.23/0.08	NG/NG	0.14/0.04
Chromulina HB22	1.12/0.04	0.70/0.06	0.66/0.10	0.85/0.06	1.19/0.12	0.94/0.12
Rhizochrysis HB23	0.28/0.02	0.13/0.02	0.27/0.02	0.37/0.05	NG/NG	NG/NG

	GSW		TYPE I		TYPE II	
SPECIES	25	40	25	40	25	40
Chryso/C2 HB24	0.32/0.06	0.24/0.04	0.29/0.04	0.18/0.03	NG/NG	NG/NG
Hymenomonas HB38	0.38/0.06	0.71/0.10	0.13/0.07	NG/NG	0.22/0.09	0.90/0.34
Prymnesium HB52	1.19/0.20	1.65/0.18	1.41/0.10	1.26/0.12	0.72/0.11	NG/NG
Amphidinium HB55	0.34/0.01	0.19/0.01	NG/NG	0.08/0.01	0.26/0.02	0.30/0.03
Cocco/C1 HB88	0.64/0.12	0.82/0.30	0.46/0.07	NG/NG	0.45/0.06	0.62/0.14
Cocco/C2 HB107	0.15/0.06	0.20/0.05	0.33/0.07	0.37/0.07	0.83/0.20	0.64/0.15
Cocco. sp. HB111	0.69/0.05	0.56/0.04	1.17/0.03	0.50/0.05	1.03/0.07	NG/NG
Cocco. Apisto HB113	1.32/0.06	0.83/0.07	1.25/0.10	1.10/0.05	1.33/0.11	1.27/0.19
Chryso HB116	0.92/0.06	1.26/0.13	NG/NG	0.88/0.08	1.01/0.08	0.87/0.17
Cocco. Apisto HB118	0.56/0.02	0.80/0.04	0.77/0.04	0.80/0.06	1.40/0.13	-/-
Cryptomonas HB119	0.48/0.04	0.54/0.04	0.09/0.03	NG/NG	0.26/0.01	0.58/0.09
Chattonella HB122	0.64/0.10	0.47/0.06	NG/NG	0.26/0.04	NG/NG	NG/NG

SPECIES	GSW 25	GSW 40	TYPE I 25	TYPE I 40	TYPE II 25	TYPE II 40
Pleurochloris HB123	1.02/0.14	0.83/0.17	0.40/0.10	0.61/0.12	NG/NG	NG/NG
Gloeothamnion HB124	0.90/0.07	1.24/0.14	0.53/0.05	0.71/0.07	0.98/0.22	0.79/0.25
Gymnodinium HB125	NG/NG	0.20/0.07	NG/NG	0.30/0.04	NG/NG	NG/NG
Cocco/S53 HB127	0.42/0.08	0.35/0.05	0.15/0.04	0.37/0.05	0.23/0.07	0.92/0.17
Chrysochromulina HB128	0.58/0.17	0.70/0.16	NG/NG	0.42/0.11	NG/NG	NG/NG
Ochromonas HB129	0.55/0.12	0.19/0.06	0.11/0.05	0.15/0.06	NG/NG	NG/NG
Prorocentrum HB130	0.21/0.06	0.49/0.08	0.51/0.06	0.42/0.07	0.47/0.03	0.38/0.04
Prorocentrum HB134	0.67/0.11	0.82/0.12	0.47/0.05	0.45/0.08	NG/NG	0.34/0.03
Nitzchia HB135	1.25/0.16	1.14/0.12	0.65/0.09	1.23/0.11	2.06/0.13	1.49/0.10
Prorocentrum HB136	0.29/0.05	0.47/0.09	0.45/0.05	0.51/0.06	0.34/0.05	NG/NG
Cocco/S24 HB141	0.45/0.05	0.62/0.05	0.91/0.08	0.65/0.05	1.25/0.16	0.97/0.13
Prorocentrum HB142	0.31/0.08	0.34/0.05	0.36/0.06	0.43/0.06	0.36/0.07	0.33/0.06

SPECIES	GSW		TYPE I		TYPE II	
	25	40	25	40	25	40
Peridinium HB145	NG/NG	NG/NG	NG/NG	NG/NG	0.29/0.02	0.49/0.03
Chryso/S33 HB147	NG/NG	0.06/0.03	NG/NG	0.04/0.03	NG/NG	NG/NG
Caloneis HB148	1.06/0.08	0.99/0.18	1.36/0.16	0.95/0.08	1.13/0.16	1.14/0.16
Cryptomonas HB149	0.41/0.02	0.42/0.04	0.49/0.04	0.52/0.03	NG/NG	NG/NG
Prorocentrum HB153	0.69/0.09	0.62/0.12	0.84/0.04	0.63/0.08	0.64/0.04	0.53/0.03
Prorocentrum HB157	0.23/0.02	0.65/0.09	0.32/0.03	0.71/0.09	NG/NG	NG/NG
Coscinodiscus HB161	1.53/0.14	1.27/0.20	1.42/0.14	1.31/0.11	NG/NG	NG/NG
Pavlova HB163	1.66/0.22	1.19/0.16	0.81/0.12	0.96/0.09	NG/NG	NG/NG
Hymenomonas HB164	0.85/0.07	1.11/0.04	0.65/0.07	1.03/0.08	1.85/0.12	1.35/0.20
Ochromonas HB170	0.84/0.23	1.19/0.25	0.62/0.12	1.38/0.19	NG/NG	NG/NG
Pavlova gyrans HB178	1.15/0.10	0.98/0.07	0.49/0.05	0.60/0.06	NG/NG	NG/NG
Diatom HB180	0.85/0.12	1.13/0.16	1.20/0.12	0.82/0.12	0.26/0.03	NG/NG
Diatom WC-5-A	1.23/0.13	0.85/0.19	1.20/0.13	1.13/0.16	NG/NG	NG/NG

```
10  REM  ***  THIS PROGRAM WAS WRITTEN BY MARK BLAKESLEE, JANUARY 1984  ***
12  REM  ***  MODIFIED JANUARY, 1986.  ***
15  PRINT  "THIS PROGRAM COMPUTES TIC CONCENTRATION OF ASP-II @ 25ppt"
20  PRINT  " WHEN THE ALKALINITY AND pH ARE SPECIFIED"
25  PRINT
30  DIM T1(3,2), T4(2,2), T5(2,2), T6(2,2), K(3), K1(10,9), K2(10,9), K3(10,9)
32  DIM C(10)
35  REM
40  REM *** THIS SECTION LOADS THE pK TABLES FROM RILEY AND
45  REM ***  CHESTER INTO THE ARRAY KTABLE
50  FOR I = 0 TO 9
55  READ C(I)
60  NEXT I
62  REM ***  THESE ARE THE CHL VALUES FOR THE ROWS IN TABLES K1-3
65  DATA 0,1,4,9,16,17,18,19,20,21
75   FOR J = 0 TO 8
80     FOR K = 0 TO 7
85       READ K1(J,K)
90       NEXT K
95     NEXT J
105  REM ***  THIS TABLE CONTAINS pKA1, 0 TO 35 C, 0 TO 21 ppt CHL
110  DATA 6.58, 6.52, 6.47, 6.42, 6.38, 6.35, 6.33, 6.31
115  DATA 6.47, 6.42, 6.37, 6.33, 6.29, 6.26, 6.24, 6.23
120  DATA 6.36, 6.32, 6.28, 6.24, 6.21, 6.18, 6.16, 6.15
125  DATA 6.27, 6.23, 6.19, 6.15, 6.13, 6.10, 6.08, 6.07
130  DATA 6.18, 6.14, 6.11, 6.07, 6.05, 6.03, 6.01, 5.99
135  DATA 6.17, 6.13, 6.10, 6.06, 6.04, 6.02, 6.00, 5.98
140  DATA 6.16, 6.12, 6.09, 6.06, 6.03, 6.01, 5.99, 5.97
145  DATA 6.15, 6.11, 6.08, 6.05, 6.02, 6.00, 5.98, 5.97
150  DATA 6.14, 6.10, 6.07, 6.04, 6.01, 5.99, 5.97, 5.96
155  REM ***  THIS TABLE CONTAINS pKA2, SAME LIMITS AS ABOVE  ***
156  FOR J = 0 TO 8
157  FOR K = 0 TO 7
158  READ K2(J,K)
160  NEXT K
161  NEXT J
163  DATA 10.62, 10.55, 10.49, 10.43, 10.38, 10.33, 10.29, 10.25
165  DATA 10.06, 9.99, 9.93, 9.87, 9.81, 9.76, 9.71, 9.66
170  DATA 9.78, 9.72, 9.67, 9.61, 9.54, 9.49, 9.43, 9.38
175  DATA 9.64, 9.58, 9.52, 9.46, 9.40, 9.34, 9.27, 9.21
180  DATA 9.46, 9.40, 9.35, 9.29, 9.23, 9.17, 9.10, 9.02
185  DATA 9.44, 9.38, 9.32, 9.27, 9.21, 9.15, 9.08, 9.00
190  DATA 9.42, 9.36, 9.30, 9.25, 9.19, 9.12, 9.06, 8.98
195  DATA 9.40, 9.34, 9.28, 9.23, 9.17, 9.10, 9.02, 8.95
200  DATA 9.38, 9.32, 9.26, 9.21, 9.15, 9.08, 9.01, 8.92
205  REM ***  THIS TABLE CONTAINS pKB, SAME LIMITS AS ABOVE  ***
206  FOR J = 0 TO 8
207  FOR K = 0 TO 7
209  READ K3(J,K)
211  NEXT K
212  NEXT J
```

```
213  DATA 9.50, 9.44, 9.38, 9.33, 9.28, 9.24, 9.20, 9.16
215  DATA 9.40, 9.34, 9.28, 9.23, 9.18, 9.14, 9.10, 9.06
220  DATA 9.28, 9.22, 9.16, 9.11, 9.06, 9.02, 8.98, 8.94
225  DATA 9.14, 9.08, 9.03, 8.98, 8.93, 8.88, 8.85, 8.82
230  DATA 9.00, 8.95, 8.89, 8.84, 8.80, 8.76, 8.72, 8.69
235  DATA 8.98, 8.93, 8.88, 8.83, 8.78, 8.74, 8.70, 8.67
240  DATA 8.96, 8.91, 8.86, 8.81, 8.76, 8.72, 8.69, 8.66
245  DATA 8.95, 8.90, 8.85, 8.80, 8.75, 8.71, 8.67, 8.64
250  DATA 8.94, 8.88, 8.83, 8.78, 8.74, 8.69, 8.65, 8.63
255  REM
260  REM *** THIS SECTION REQUESTS INPUT FROM THE USER
265  INPUT "ALKALINITY (meq/l) = "; M
270  A = M * .001
275  INPUT "pH VALUE = "; P
280  PRINT "SALINITY (ppt) = 25"
281  S = 25
285  INPUT "TEMPERATURE (DEGREES C) = "; T
290  T1 = T + 273.15
295  W = 10**(-(3441/T1 + 2.241 - .9415 * (.001*S)**0.5))
298  REM *** THIS CONVERTS SALINITY TO CHLORINITY
300  C1 = (S - .03) / 1.805
301  REM *** for ASP-II, borate is 34mg/L*(25/35)  / 61.83 g/mol = .39mM
305  B = .00039278
310  REM
315  REM *** THIS SECTION PERFORMS A LINEAR INTERPOLATN OF THE pK
320  REM ***  VALUES BASED ON THE SPECIFIED TEMPERATURE AND SAL
325  C2 = T/5 - (T MOD 5)/5
330  I = 0
335  IF C1 < = C(I) THEN GOTO 340
336  I = I + 1
337  GOTO 335
340    R = I - 1
360    FOR J = 1 TO 2
365      FOR K = 1 TO 2
370        T4(J,K) = K1(R + J - 1,C2 + K - 1)
375        NEXT K
380      NEXT J
382    FOR J = 1 TO 2
384      FOR K = 1 TO 2
386        T5(J,K) = K2(R + J - 1,C2 + K - 1)
388        NEXT K
389    NEXT J
390    FOR J = 1 TO 2
392      FOR K = 1 TO 2
394        T6(J,K) = K3(R + J - 1,C2 + K - 1)
395        NEXT K
396    NEXT J
398    FOR J = 1 TO 2
400      T1(1,J) = T4(J,1) + (T - C2 * 5)  * (T4(J,2) - T4(J,1)) / 5
405      NEXT J
406    FOR J = 1 TO 2
407      T1(2,J) = T5(J,1) + (T - C2 * 5)  * (T5(J,2) - T5(J,1)) / 5
408      NEXT J
409    FOR J = 1 TO 2
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```
410      T1(3,J) = T6(J,1) + (T - C2 * 5)  * (T6(J,2) - T6(J,1)) / 5
411      NEXT J
412  FOR I = 1 TO 3
414    K(I) = T1(I,1) + (C1 - C(R)) * (T1(I,2) - T1(I,1)) / (C(R + 1) - C(R))
415    NEXT I
420  P1 = K(1)
425  P2 = K(2)
430  P3 = K(3)
435  REM *** THIS SECTION PERFORMS THE CARBON CONCENTRATION CALC-
440  REM ***  ULATIONS BASED ON THE SPECIFIED pH AND ALKALINITY
445  A1 = 10**(-P1)
450  A2 = 10**(-P2)
455  B2 = 10**(-P3)
460  H = 10**(-P)
465  K3 = A1 * A2
470  A3 = 1 + (A1 / H) + (K3 / (H**2))
475  A4 = (H / A1) + 1 + (A2 / H)
480  A5 = ((H**2)/K3) + (H / A2) + 1
485  A6 = B2 / (B2 + H)
490  PRINT "BORATE ALKALINITY (meq/l) = "; A6 * B * 1000
495  PRINT "BORATE ALKALINITY COMPRISES "; A6 * B *100 / A; "% OF TOTAL ALKALINITY"
500  T3 = (A - (W / H) + (H - (B * A6))) / ((1 / A4) + (2/A5))
505  REM *** THIS SECTION PRINTS THE RESULTS
510  PRINT "TIC  CONCENTRATION (mM) = "; T3 * 1000
515  PRINT "CONCENTRATION OF H2CO3* (mM) = "; (1000 / A3) * T3
520  PRINT "CONCENTRATION OF HCO3-  (mM) = "; (1000 / A4) * T3
525  PRINT "CONCENTRATION OF CO3=   (mM) = "; (1000 / A5) * T3
530  PRINT
532  GOTO 265
535  END
```