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# **FY 1986 Aquatic Species Program**

## **Annual Report**

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### **Solar Energy Research Institute**

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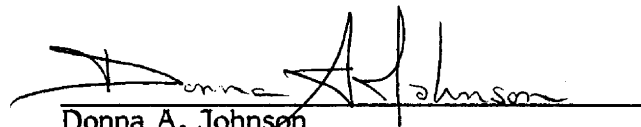
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## PREFACE

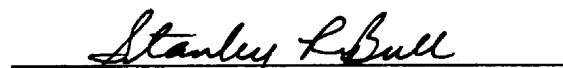
This report summarizes the progress and research accomplishments of SERI's Aquatic Species Program during FY 1986. This report includes an overview of the entire program and a summary of all research activities. The SERI Biofuels Program receives its funding through the Biofuels and Municipal Waste Technology Division of the Department of Energy.

For further details, contact the SERI Biofuels Program Office (Donna Johnson, Aquatic Species Program Coordinator, 303-231-1472).

  
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## SUMMARY

The goal of the Aquatic Species Program is to develop the technology to produce gasoline and diesel fuels from microalgae grown in saline waters of the desert Southwest. Microalgae are known to accumulate lipids in large quantities and can thrive in high salinity water, which currently has no other significant use. Three major task areas are important to the economical development of this technology: biology, engineering, and analysis.

Biological activities include screening, characterizing, and improving microalgae species. In 1982 we began extensive efforts to collect and screen microalgae strains that are salinity and temperature tolerant, highly productive, and that produce large amounts of lipids. More than 3000 microalgae strains have been collected to date. Species used by the program in 1982 had temperature tolerances of 15°-20°C and salinity tolerances of 20-40 mmho cm<sup>-1</sup>. With the intensive collection efforts, the program now has strains that can tolerate wide environmental fluctuations, from 10° to 35°C and 10 to 70 mmho cm<sup>-1</sup>. Rates of productivity increased from 10-20 g dry wt m<sup>-2</sup> d<sup>-1</sup> in 1982 to greater than 50 g dry wt m<sup>-2</sup> d<sup>-1</sup> under laboratory conditions and more than 35 g dry wt m<sup>-2</sup> d<sup>-1</sup> in outdoor systems in 1986. Lipid content of the algal cells also increased significantly, from 20% in 1982 to 66% indoors and 40% outdoors by 1986. A current problem is that salinity- and temperature-tolerant species do not always have high productivity and produce large amounts of lipid. Therefore, basic research is under way in genetic engineering to put all three characteristics into one or two strains.

Engineering research focused on polymer harvesting of microalgae. All algae were harvestable but required different polymers. Harvesting was accomplished for 0.5¢-1.5¢ kg<sup>-1</sup> dry mass, with removal efficiencies greater than 85%-95%. Cross-flow micro-filtration was tested and determined to be too costly. Another method of harvesting examined was flocculation. Recycling flocculants reduced costs by 100%-200%.

We performed a technical and economic analysis of a microalgae fuel production system and published it in the report entitled *Fuels from Microalgae*. The study defines performance requirements to product gasoline and diesel fuels at prices that will be competitive with conventional fuels. Aggressive research is needed, but the improvements defined are within the bounds of attainability. A major concern has been the availability of saline water resources in the desert Southwest. It has been recently demonstrated, however, that there is sufficient saline water in Arizona and New Mexico to produce at least one quad of energy from microalgae.

Future activities of the Aquatics Species Program include: completing collection activities and focusing on characterizing species collected, consolidating all outdoor test facilities into one large (0.5-1.5 ha) system in a desert region, conducting research on harvesting and carbon dioxide supply, and converting microalgae lipids into liquid fuels. Research will continue on algal physiology, biochemistry, and genetic engineering.



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## 1.0 INTRODUCTION

In 1979, the Department of Energy (DOE) and the Solar Energy Research Institute (SERI) initiated a research program to pursue opportunities for producing liquid fuels from microalgae. Microalgae are unique photosynthetic organisms in that they are known to accumulate storage lipids in great quantities and will thrive in highly saline water. The program is focused on the production of lipids from microalgae because plant storage lipids are an attractive biomass feedstock for producing renewable, high-energy liquid fuels such as gasoline and diesel fuel. The overall process for growing microalgae and converting the lipids they produce into gasoline and diesel fuel is shown in Figure 1-1. The DOE/SERI program emphasizes the development of microalgae systems in the desert Southwest because this area offers flat land, high incident solar radiation, few competing land uses, and large reservoirs of saline water. Locating a mass culture facility in this region minimizes land costs, and using saline water, which is not suitable for agricultural, domestic, or industrial purposes, minimizes competition for the limited supplies of fresh water in the Southwest.

Microalgae can be grown in large outdoor ponds in a desert region, using the resources of sunlight, saline water, nitrogen, phosphorus, and carbon dioxide. Algae can convert these raw materials into proteins, carbohydrates, and lipids and, in the process, double their biomass three to five times a day. After a rapid growth phase, the algae are transferred to induction ponds where nutrient limitation is allowed to occur. Under these conditions, many algae stop growth and division and use all their energy to make lipids as storage products to survive. Once the cells have accumulated lipids, they are harvested and the water is recycled back into the growth ponds. The harvested cells then are subjected to an extraction process to remove the lipids. Algal lipids are primarily triglycerides with fractions of isoprenoids, phospholipids, glycolipids, and hydrocarbons. They contain more oxygen and are more viscous than crude petroleum. The two most promising fuel conversion options are transesterification to produce fuels similar to diesel fuels and catalytic conversion to produce gasoline. While microalgal lipids represent the premium energy product, the energy trapped in the other biomass constituents can also be used; e.g., the cell residue after lipid extraction can be digested anaerobically to produce methane and carbon dioxide, which can be recycled for use in the algae production system.

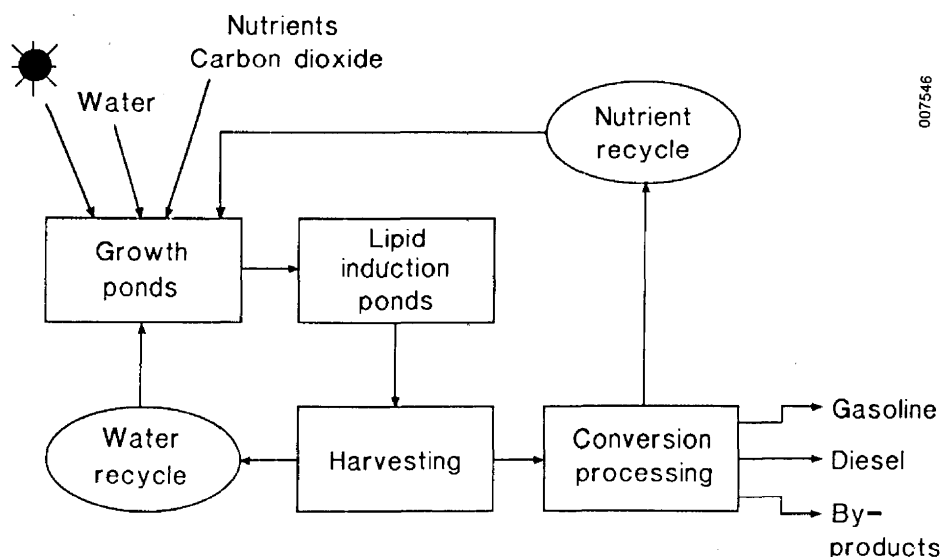


Figure 1-1. Microalgae Growth and Conversion Process

## 2.0 GOAL AND OBJECTIVES

The goal of the program is to provide the technology base for large-scale production of oil-rich microalgae and the conversion methods to convert the microalgae lipids into gasoline and diesel fuels needed for industry and transportation. It is important for the United States to develop alternative renewable oil sources since currently 41% of the energy market in the United States is for liquid fuels, and one-half of these fuels are imported.

To achieve this goal, the objectives of the program are to:

- Collect microalgae strains from many areas within the United States to provide a large number of different organisms for screening, characterization, and improvement;
- Screen microalgae to select for those species that are temperature and salinity tolerant, have high productivities, and are good lipid producers;
- Develop inexpensive, large-scale, outdoor mass culture technologies to grow microalgae;
- Improve the methods to harvest microalgae so the process is inexpensive and efficient;
- Evaluate the saline water resources available for raising microalgae in the desert Southwest of the United States;
- Develop technologies for converting microalgae lipids into high-value liquid transportation fuels; and
- Transfer the technology to the private sector for rapid commercialization by involving industry in the research process at the earliest possible time.

### 3.0 RESEARCH AND TECHNOLOGY DEVELOPMENT

To develop the technology base to obtain liquid fuels from microalgae, three research and technology development areas have been identified. These areas and the main research activities in each area are as follows:

- Biology
  - Species Collection and Screening
  - Productivity Improvement
  - Lipid Production, Physiology, and Biochemistry
  - Genetic Engineering
- Engineering
  - Cultivation System Design
  - Harvesting Improvement
- Analysis
  - Technical and Economic Analysis
  - Saline Water Assessments

Approximately half of the research is conducted in-house by SERI, and the other half is subcontracted to universities and small businesses. Table 3-1 lists the FY 1986 budget of \$1.68 million. Biology received approximately 79% of the total budget, engineering was 0% (some engineering research was done in the outdoor culture subcontract), and analysis was 6%. Management received the remaining 15%. In addition, there is an Historically Black College and University Program (HBCU), and these subcontracts were all in the area of biology. A summary of the FY 1986 Aquatic Species Program active subcontracts is given in Table 3-2. This includes projects funded in FY 1985 and FY 1986.

The following sections describe each of these three main research areas and the major accomplishments of FY 1986.

#### 3.1 Biology

The primary focus of the research to date has been in the area of biology. A decision was made that unless it was feasible to obtain organisms that were environmentally tolerant, had high productivity, and produced large amounts of lipids, there was no reason to do large-scale engineering research. Until recently, our major focus has been on collection and screening, and collecting will be completed in FY 1987. Major advances have been made in productivity, and the program is ready to scale up the size of the test production facilities. Most of the major advances in the area of biology were made in lipid biochemistry and physiology, and work in genetic engineering is just beginning.

##### 3.1.1. Species Collection and Screening

The overall objective of collecting and screening is to identify and obtain naturally occurring microalgal strains most suitable for outdoor biomass fuel production. Specific objectives include collecting strains from diverse geographical locales and ecological niches, selecting strains with wide environmental tolerances and high production rates,

**Table 3-1. FY 1986 Procurement Plan  
Summary for Aquatic Species  
Program**

Task/Projects	Funding (1000\$)
Biological	
Species Selection	629
Improvement	375
Outdoor Mass Culture	205
Engineering	0
Analysis	101
HBCU	125
Management	245
Total	1680

developing rapid techniques for identifying high-lipid-producing strains, and characterizing natural phenotypic variability within microalgae species.

More than 3,000 strains have been collected by the program to date (Figure 3-1). Most of the promising strains collected are Bacillariophyceae (diatoms), Chlorophyceae (green algae), and Eustigmatophyceae (Eustigmatophytes). Collection efforts are concluding this year, and we will thoroughly screen and characterize the collected strains to obtain 10-20 strains by 1990 for intensive species improvement research.

Using innovative collection and screening processes, the program identified several strains that are very tolerant to severe environmental fluctuations in temperature and salinity (Figure 3-2). Strains of microalgae used by the program in 1982 exhibited temperature tolerances of 15°-25°C and salinity tolerances of 20-40 mmho cm<sup>-1</sup>. With the intensive collection efforts nationwide, the program now has strains that can tolerate 10°-35°C and 10-70 mmho cm<sup>-1</sup>.

For each strain that exhibits high growth and lipid production, a temperature/salinity profile is done in three different types of saline water: Type I (high in divalent ions), Type II (high in monovalent ions), and artificial seawater. Each strain may have different growth rates in the different media, and in addition, there may be wide variability between different strains of the same species. Figure 3-3 outlines the response of three strains of *Chaetoceros muelleri* to changes in temperature and salinity. The Utah and



**Table 3-2. Aquatic Species FY 1986 Active Subcontracts**  
(FY 1985 and FY 1986 Funding)

	Title	Contractor	Date of Performance
1.	Optimization of outdoor culture	University of Hawaii	5/86 - 2/87
2.	Production of liquid fuels and chemicals by microalgae	Microbial Products	3/86 - 2/87
3.	Adaptation of microalgal production technology	Ben Gurion University (cost shared 50:50)	6/85 - 5/86
4.	Screening and characterizing oleagenous microalgae species from the Southeastern U.S.	Alabama A&M	2/86 - 1/87
5.	Characterization of hydrocarbon producing strains of microalgae	Scripps Institution	11/85 - 11/86
6.	Ultrastructure evaluation of lipid producing microalgae	Oak Ridge	10/84 - 11/86
7.	Collection of high energy yielding strains of saline microalgae from the Hawaiian Islands	University of Hawaii	3/86 - 10/87
8.	Genetic variation in high energy yielding microalgae	City College of New York (cost shared 70:30)	3/86 - 5/87
9.	Collection of high energy yielding strains of saline microalgae from southwestern states	Arizona State University (cost shared 75:25)	3/86 - 3/87
10.	The effects of fluctuating environments on the selection of high yielding microalgae	Georgia Tech (cost-shared 20:80)	3/85 - 2/87
11.	Collection and selection of high energy thermophilic strains of microalgae	Montana State University	3/86 - 4/87

Table 3-2. Aquatic Species FY 1986 Active Subcontracts (Concluded)

Title	Contractor	Date of Performance
12. Collection of high energy yielding strains of saline microalgae from South Florida	Harbor Branch	5/85 - 6/86
13. Characterization of photo-synthetic efficiency and growth of selected microalgae in dense culture	Martek	9/85 - 2/87
14. Nutritional requirements for maximal growth of oil-producing microalgae	Jackson State University	9/86 - 9/87
15. Evaluation of available saline resources in New Mexico for the production of microalgae	New Mexico State University (cost shared 50:50)	8/85 - 9/86
16. Inventory of sources of available saline waters for microalgae mass culture within Arizona	University of Arizona	7/85 - 9/86
17. Chrysophrysean lipids: Effects of induction strategy in the quantity and types of lipids	Selma University	9/85 - 2/87
18. Biochemical elucidation of neutral lipid synthesis in microalgae	Montana State University	1/86 - 10/87
19. Transformation and somatic cell genetics for the improvement of energy production in microalgae	University of Nebraska	1/86 - 2/87
20. Biochemical elucidation of neutral lipid synthesis in microalgae	University of Nebraska	1/86 - 2/87
21. Macroalgal genetics	Neushul Mariculture, Inc. (cost shared - 20:80)	2/86 - 1/87
22. Cultivation and chemical analysis of microalgal species	Georgia Tech	11/84 - 2/86

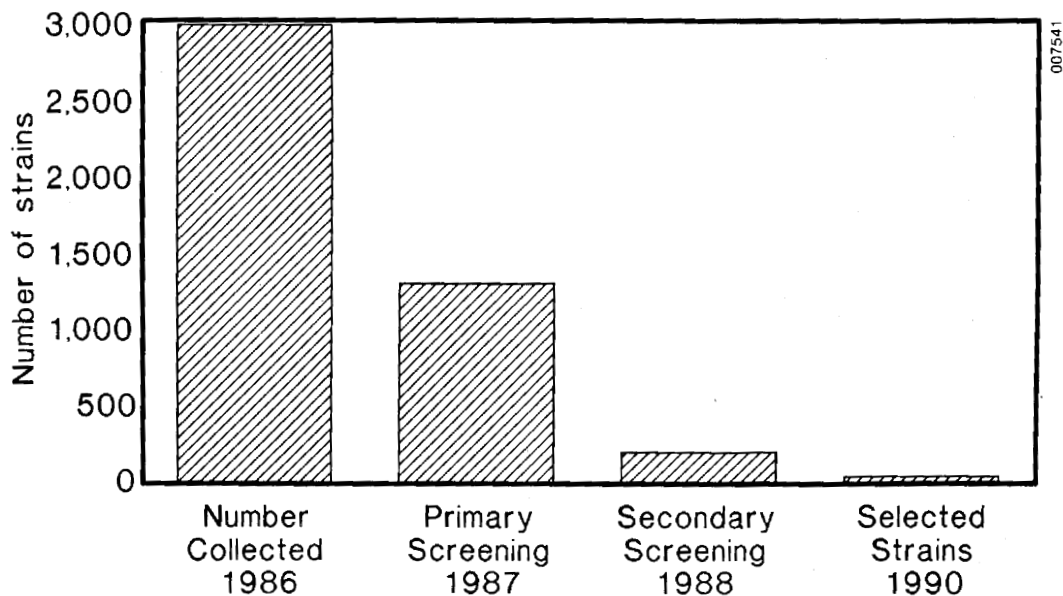


Figure 3-1. Summary of the Strains of Microalgae Collected by the Aquatic Species Program and Future Screening Goals

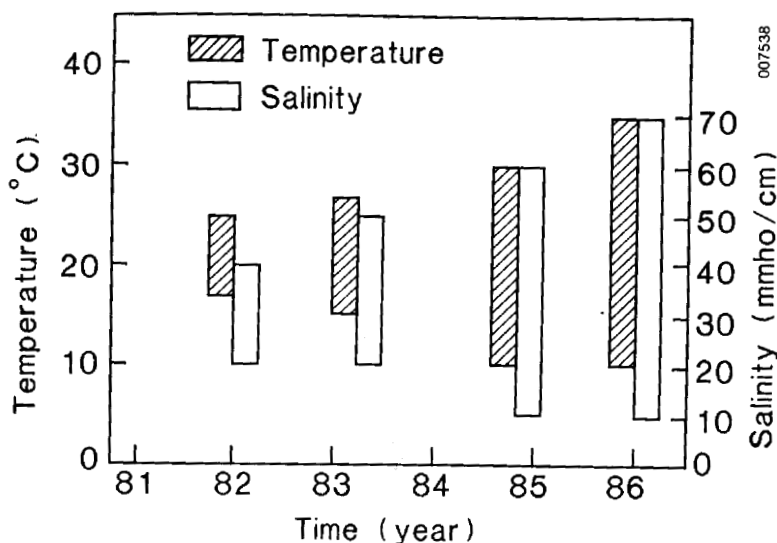
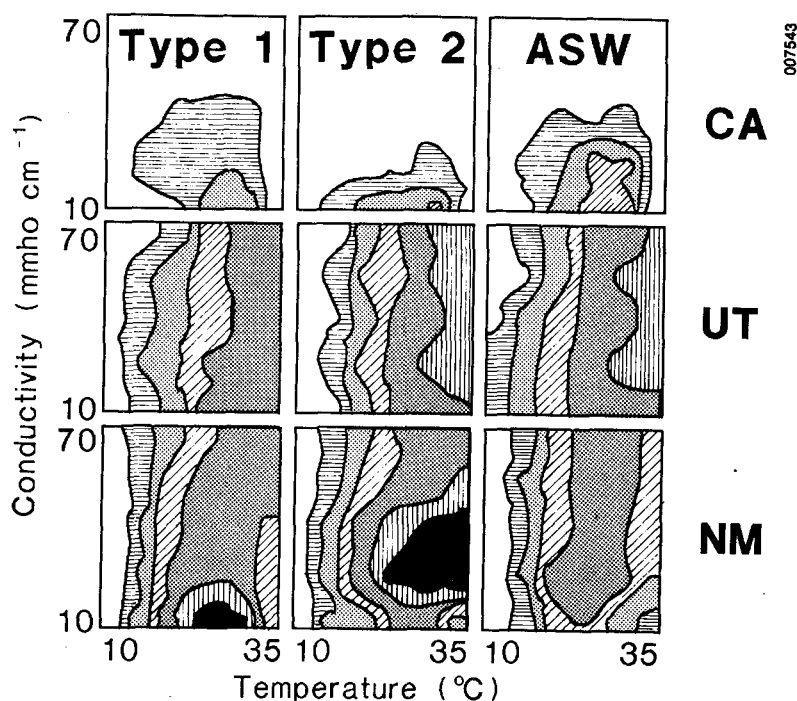


Figure 3-2. Improvements in Temperature and Salinity Tolerances of Strains of Microalgae Collected by the Aquatic Species Program. Each bar represents the maximum tolerance of a strain in the SERI culture collection.



**Figure 3-3. Temperature Salinity Media Effects on Growth of Three Strains of *Chaetoceros muelleri*.** The outer, horizontal, boundary is where growth is greater than 1.0 doublings day<sup>-1</sup>. The highest growth rate, solid area, is 3.5 doublings day<sup>-1</sup>. Increments are in 0.5 doublings day<sup>-1</sup>. Type I water is high in divalent ions, and Type II is high in monovalent ions.  
ASW = artificial seawater; CA = California strain, UT = Utah strain, and NM = New Mexico strain.

New Mexico strains are very euryhaline and exhibit only small variations in growth response to different media. In contrast, the California strain exhibits a strong stenohaline response and grows poorly.

Developing a quick, inexpensive method to screen for high-lipid-producing microalgae has been a major advance. A Nile red staining technique was developed by researchers at the University of Montana, Oak Ridge National Laboratory, and SERI. Nile red stains the algae but does not kill them. The amount of fluorescence corresponds to the amount of lipid in the cells. Before this technique, a long, expensive (in labor) method of chemical determination was the only way to determine lipid quantity in microalgae. This technique will facilitate a more rapid screening process for microalgal strains.

The SERI Microalgae Culture Collection, established in support of the U.S. Department of Energy's Biofuels and Municipal Waste Technology Division, provides a repository for strains identified or developed for biomass production and makes these strains readily available to the research community. The strains in the collection were selected for their potential in biomass fuel applications, and many produce significant quantities of cellular storage lipids. The 1986-1987 Culture Collection Catalog lists 39 of the program's best strains of microalgae and includes the productivity and physiological data for each strain.

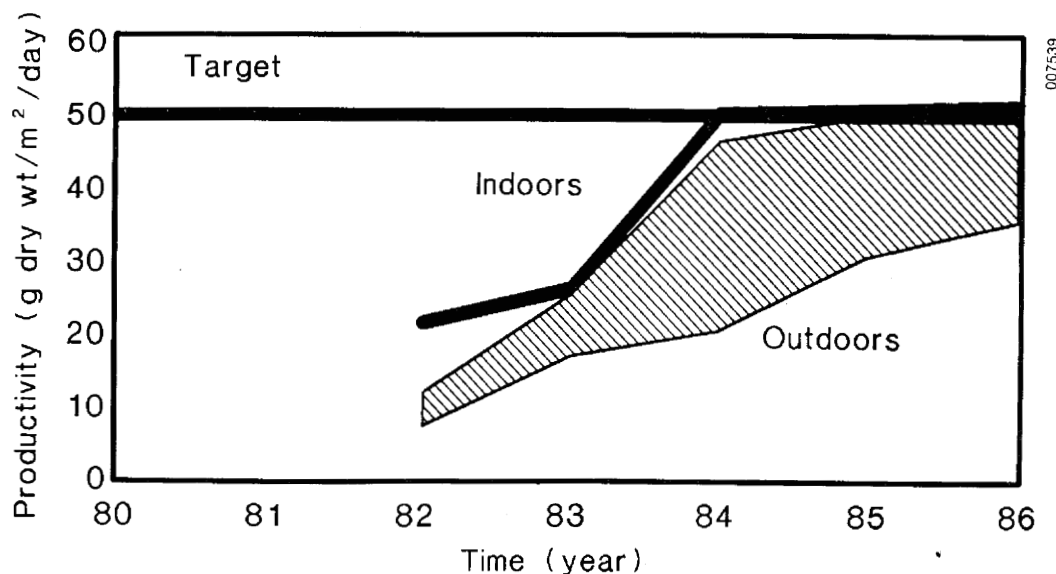
### 3.1.2 Productivity Improvement

The objective of this effort is to increase the rates of productivity of microalgae to enhance yields of energy products. We are conducting research in both laboratory and outdoor cultures to identify species and develop culture management strategies that improve productivity rates.

As a result of this project area, rates of productivity increased from 10-20 g dry wt m<sup>-2</sup> d<sup>-1</sup> in 1982 to greater than 50 g dry wt m<sup>-2</sup> d<sup>-1</sup> under laboratory conditions (Figure 3-4). Sustainable rates of 35 g dry wt m<sup>-2</sup> d<sup>-1</sup> in outdoor systems were achieved in 1986, with short-term optimum reaching 50 g dry wt m<sup>-2</sup> d<sup>-1</sup>. In general, research has suggested diatoms are more stable and have substantially higher productivity in outdoor culture than green algae.

Diatoms and green algae also differ in their ability to grow well at low CO<sub>2</sub> levels. The diatoms required concentrations of almost 100 μM CO<sub>2</sub> and pH below 8.5 to attain maximal, light-limited productivity. The green algae needed only 5 μM CO<sub>2</sub> and pH of 9. However, the maximum productivity of the green algae was only 60% that of the diatoms.

One-, two-, and three-day dilution intervals were evaluated in outdoor culture systems to optimize productivity. A two-day interval resulted in higher productivities than the one-



**Figure 3-4. Improvements in Productivity of Microalgae Cultures under Indoor and Outdoor Culture Conditions from 1982 to 1986.** For outdoor culture productivity the top of the range is the highest productivities obtained by SERI subcontractors while the bottom of the range is the productivities we can consistently get and sustain for long periods of time. The target of 50 g dry wt m<sup>-2</sup> d<sup>-1</sup> was defined by the microalgae technical and economic analysis.

and three-day intervals. Growth rates were also significantly increased when initial culture density was 5 to 400 g AFDW m<sup>-3</sup>, and the growth rates decreased rapidly at higher initial culture densities.

When mixing foils were tested in shallow raceway systems as a mechanism to increase productivity, a significant enhancement was observed, from 20 to 28 g m<sup>-2</sup> d<sup>-1</sup>. However, an independent economic analysis found that the energy cost associated with the foils and the capital cost of the foils exceeded the value of the increased production by a factor of 7.4 with the present system design. Thus, the cost effectiveness of using foils to increase productivity for microalgae does not look promising at present.

### 3.1.3 Lipid Production, Physiology, and Biochemistry

It is in the areas of lipid production, physiology, and biochemistry that the greatest advances were made in 1986. Figure 3-5 shows the significant increases in lipid content of algal cells from 20% in 1982 to 66% indoors and 40% outdoors by 1986.

Researchers examined the effects of environmental and nutrient stress on lipid induction in microalgae. Nitrogen, silica, temperature, pH, inorganic carbon (CO<sub>2</sub> and bicarbonate), light intensity, and salinity were all evaluated for their effects on lipid induction. Under silica limitation, diatoms exhibited increasing lipid yield (mg/L) with increasing bicarbonate concentration, and lipid production increased with increasing light intensity up to 1000  $\mu\text{E m}^{-2} \text{s}^{-1}$ .

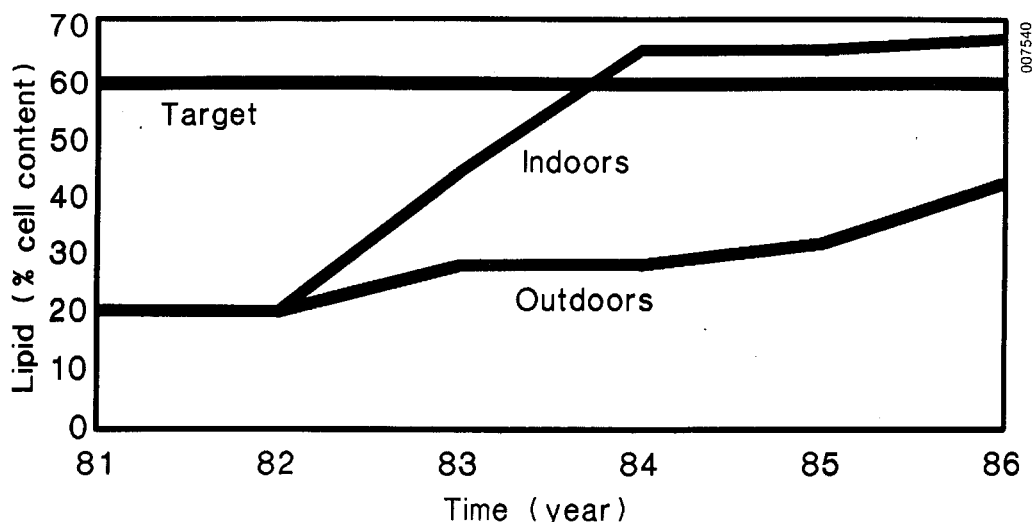


Figure 3-5. Improvements in Lipid Content of Microalgae Cells Grown under Indoor and Outdoor Conditions from 1981 to 1986. The target of 50% of cell content was defined by the microalgae technical and economic analysis.

Nile red, a fluorescent dye, was adapted with success to semiquantitatively measure the neutral lipid content in microalgae. The procedure was developed on a Turner 111 fluorometer and made available to all researchers. The method involves adding Nile red to algal cell suspensions and measuring the resulting fluorescence. This method has a great advantage over the traditional Bligh-Dyer method in that it requires only a small sample size and can be done in less than 5 min. Correlation between gravimetric lipid measurements and Nile red fluorescence was high.

Lipid accumulation in microalgae was investigated with flow cytometry (FCM) and transmission electron microscopy (TEM). Previous studies using batch cultures of algae led to the assumption that lipid accumulation in microalgae is a gradual process requiring at least several days for completion. However, FCM revealed, through changes in the chlorophyll:lipid ratio, that the time span required for individual cells to change metabolic state is short. Simultaneous FCM measurements of chlorophyll and Nile red fluorescence in individual cells of nitrogen-deficient populations revealed a bimodal population distribution as one stage in the lipid accumulation process. The fact that two discrete populations exist, with few cells in an intermediate stage, suggests rapid response to a lipid trigger. Interpretations of light and electron microscopic observations are consistent with this hypothesis. The time required for an entire population to achieve maximum lipid content is considerably longer than that required for a single cell, due to the variation in response time among cells. Cultures exhibiting enhanced lipid content could be obtained by using FCM to separate high lipid cells from the remainder of the population.

Improving lipid yields in microalgae requires an understanding of the physiological and biochemical basis for partitioning photosynthetically fixed  $\text{CO}_2$  into lipids. The rate of lipid synthesis and final lipid yield will depend on the availability of carbon for lipid synthesis and the actual levels and activities of the enzymes used for lipid synthesis. Conditions such as nitrogen deficiency that induce the accumulation of lipid by algae often drastically reduce the capacity for photosynthetic  $\text{CO}_2$  fixation. Low lipid yields could result either from an absence of carbon skeletons or from low levels of enzymes. Improvements in lipid yield can be achieved only when the limiting factors have been determined.

Research efforts are continuing in order to determine the pathways of lipid biosynthesis in algal cells, especially in the cytoplasm, chloroplast, and mitochondrion. Each pathway possesses potential lipid triggers. Once the trigger is determined, biochemical and genetic engineering techniques can be used to increase the lipid yield of promising algal strains.

#### 3.1.4 Genetic Engineering

No single microalgae strain has been found that exhibits environmental tolerance, high productivity, and high lipid yield. All three characteristics are necessary in one organism to meet program goals. For this reason, work began on developing genetic engineering methods so that by the time the program reduces its strains to the best 10 to 20, by 1990, the methods to genetically modify these organisms will be available.

We are working in three areas of genetic engineering research: 1) classical genetic manipulation methods, 2) intraspecific genetic variability, and 3) vector and protoplast methodology. Each research area provides different parts of the total knowledge that we will need to genetically engineer a better organism.

The classical genetic manipulation methods include studying the heritability of morphological, physiological, and biochemical traits and using higher plant mutagens to reproduce a superior organism. Some of the work to date has focused on developing these methods for algae using gametes of the macroalgae, *Macrocystis*. These techniques are now being extrapolated for use with microalgae.

Genetic banding patterns have been identified for two species of green algae. These bands can be used as genetic markers. This preliminary genetic work, examining intra-specific variations within the species, is important for future genetic manipulation. Using these results, we can perform selective breeding experiments to improve productivity and lipid yield. These results are also the preliminary data needed to find genetic markers, which can be used in genetic engineering for strain improvements.

More than 50 algal viruses have been isolated as potential vectors for algal recombinant DNA work as a mechanism to introduce DNA from one species into another (Figure 3-6) (27,28). To date, none of the viruses isolated will replicate within algae used by the Aquatic Species Program. Protoplasts have been formed in one species of green algae, and further studies are under way to characterize the enzyme preparations that degrade the cell wall. Fusion studies of the protoplasts will be conducted in 1987. In addition, a cloning project has been initiated that will, when it is finished, result in a restriction map of the chloroplast genome. This map is a major step to be completed before we can begin to genetically engineer the recombinant DNA material.



Figure 3-6. Viral Vector Releasing its DNA into an Algal Cell



### 3.2 Engineering

Engineering research has focused on harvesting microalgae. Technical feasibility and cost analyses were completed for polymer harvesting of saline microalgae. All microalgae tested were harvestable, but different algae required different polymers. The amount of polymer increases as the requirement for clarification is made more stringent, making it more cost-effective not to require greater than 85% removal. With the most suitable polymers and appropriate application techniques, harvesting can be accomplished for polymer costs of  $0.5\phi$ - $1.5\phi$   $\text{kg}^{-1}$  dry mass, with removal efficiencies of 85%-95%. Polymers with higher rigid backbones are less affected by the salt concentration and are thus recommended as flocculants of microalgae in saline water. A cross-flow microfiltration process was tested as a harvesting method and determined to be too costly for use when considering energy production from microalgae.

Another method of harvesting examined was chemical flocculation. Flocculant dose was reduced 75% by recycling the precipitant following flocculation back into the mixing-flocculation chamber. Three flocculation cycles not only reduced flocculant dosage but also were required for a 90% removal of the microalgae from saline water.

### 3.3 Analysis

The two major subtasks in the analysis section are technical and economic analysis and saline water resource assessments. We have made significant progress in both areas.

#### 3.3.1 Technical and Economic Analysis

A technology assessment was performed that demonstrates that gasoline and diesel fuels could be produced from mass-cultured microalgae at prices that will be competitive with conventional fuels. Aggressive research is needed to fulfill the performance requirements defined by the analysis, but the required improvements are within the bounds of attainability and have been closely approached under controlled conditions. Improvements needed are the enhancement of productivity, lipid yield, and salinity and temperature tolerance of microalgae species. Engineering improvements are also needed in the cultivation system design and in harvesting. Two critical resource requirements are the abundance and availability of saline waters in the Southwest deserts and the low cost of carbon dioxide. Based on the achievement of these research goals, demonstrated performance, and availability of economic supplies of critical resources, liquid fuels that are potential direct substitutes for conventional hydrocarbon fuels could be produced from microalgae for \$1.60-\$2.00/gal by 2010.

Analysis of a number of fuel conversion options for microalgal biomass has demonstrated that the promise of microalgae for fuel production is best realized through conversion processes based on cellular lipids, an energy-rich hydrocarbon. The ability to produce lipids that can constitute 60% or more of the total biomass is a distinguishing characteristic of microalgae and makes them uniquely attractive candidates for conversion to liquid fuels. The two most promising fuel conversion options are transesterification to produce fuels similar to diesel fuels and catalytic conversion to produce gasoline. While microalgal lipids represent the premium energy product, the energy trapped in the other biomass constituents could also be used; e.g., the cell residue after lipid extraction could be anaerobically digested to produce methane and carbon dioxide.

The availability of saline water will be an important factor in determining the ultimate scale of fuel production technology based on this resource. Because of high evaporation rates, water demands for uncovered cultures in this region will be extremely high. Saline

aquifers are found throughout the Southwest, but the total volume these aquifers can supply on a sustained basis has not been determined. Since very little information is available on the saline water resources of the desert Southwest, the quantification of this resource is a high priority activity.

Carbon dioxide supply is the largest single contributor to the cost of liquid fuels derived from microalgae. However, the acquisition of sufficient quantities of carbon dioxide should not impose constraints on the ultimate scale of the microalgal fuels technology. Existing and proposed coal-fired power plants will produce carbon dioxide in excess of the quantity required for microalgal production. Competing demands from enhanced oil recovery are not anticipated, since most oil fields in the area are expected to be depleted by the year 2010, the expected date of emergence of an extensive microalgal mass culture technology for liquid fuel production. If methods were developed for the recovery of carbon dioxide previously injected into oil wells, these large reservoirs could supply additional abundant quantities of low-cost carbon dioxide.

The major issues to be resolved in mass culture technology are biological. For this technology to become cost competitive, the biological productivity of these systems must be improved. The production analysis indicates that photosynthetic efficiencies of 12%-16% must be attained, and that 50%-60% by weight of the biomass produced must be in the form of lipids. If higher lipid contents were achieved, lower photosynthetic efficiency would be acceptable, and vice versa.

In addition to displaying improved productivity, species must demonstrate environmental tolerance characteristics that make them suitable for outdoor culture in arid regions. The productivity analysis has identified species salinity tolerance as a particularly significant aspect of environmental tolerance since operation of cultures at high salinities is necessary to control water requirements through minimization of water consumption for blowdown.

The basic engineering needs for microalgal culture have been identified and form the basis of a significant portion of the production analysis. Considerable additional engineering research and development will be required. Important engineering issues are efficient and inexpensive harvesting methods, mechanisms to reduce evaporation, production system design, brine disposal, and carbon dioxide input systems.

The technology improvements mentioned above will reduce the feedstock cost from \$393/ton to \$192/ton (Figure 3-7). Major technology improvements can be made to reduce the costs associated with capital investments, water, labor, and operation. The major cost factor for the final feedstock will be carbon dioxide and nutrients.

### **3.3.2 Saline Water Resource Assessment**

Saline water resource assessments were completed for Arizona and New Mexico. Arizona has  $2.8 \times 10^8 \text{ m}^3$  ( $2.3 \times 10^5$  acre-ft) of saline water and New Mexico has  $1.8 \times 10^{13} \text{ m}^3$  ( $15 \times 10^9$  acre-ft) of saline water. The water necessary to produce one quad of energy from microalgal lipids is  $4.7 \times 10^9 \text{ m}^3$  ( $3.8 \times 10^6$  acre-ft). Arizona identified eight areas within the state as suitable for a microalgal production system, and New Mexico identified six areas as potential sites.

#### **3.3.2.1 Arizona**

- **Saline Surface Water** - Of the 34 sources of saline surface water identified, only 8 were judged to have a sufficient volume of water for a project. The total amount of

saline surface water from these eight sources is estimated to be  $4000 \text{ L d}^{-1}$  ( $1.1 \times 10^3 \text{ gal d}^{-1}$ ).

- Saline Ground Water - Nineteen saline ground-water plumes were identified in the six focal areas as being capable of providing greater than  $1.5 \times 10^7 \text{ L d}^{-1}$  ( $4.0 \times 10^6 \text{ gal d}^{-1}$ ). The total water storage is  $7.1 \times 10^{10} \text{ m}^3$  ( $5.8 \times 10^7 \text{ acre-ft}$ ). No information exists to date on the rate of renewal.
- Institutional Considerations - A microalgae production facility constructed in Arizona will require a permit from the Arizona Department of Environmental Quality (after 7/1/87). Prior to the issuance of a permit, it must be demonstrated that operation of a project will not adversely affect the quality of the underlying ground water. If the project is on federal land or federal funds are used, an environmental impact statement will be required.

### 3.3.2.2 New Mexico

- There is  $1.8 \times 10^{13} \text{ m}^3$  ( $15 \times 10^9 \text{ acre-ft}$ ) of unutilized saline water in New Mexico.
- Six basins were identified as having the potential for siting a microalgae production facility: Tularosa, Estancia, Crow Flats, Roswell East, San Juan, and Tucumcari. Estancia, Tularosa, and Crow Flats basins were chosen as the best three sites.
  - Estancia Basin - An area of  $274 \text{ km}^2$  ( $170 \text{ mi}^2$ ) is well suited for a microalgae production facility. There is  $3.9 \times 10^8 \text{ m}^3$  ( $3.2 \times 10^5 \text{ acre-ft}$ ) of saline water available at this site.
  - Tularosa Basin - An area of  $185 \text{ km}^2$  ( $115 \text{ mi}^2$ ) is the second-best site for the location of a microalgae facility. There is  $7.4 \times 10^9 \text{ m}^3$  ( $6.0 \times 10^6 \text{ acre-ft}$ ) of saline water available at the site.
  - Crow Flats Basin - An area of  $58 \text{ km}^2$  ( $36 \text{ mi}^2$ ) is the third best site in New Mexico for the location of a microalgae test facility. Well yields in the area are greater than  $3785 \text{ L min}^{-1}$  ( $1000 \text{ gal min}^{-1}$ ).

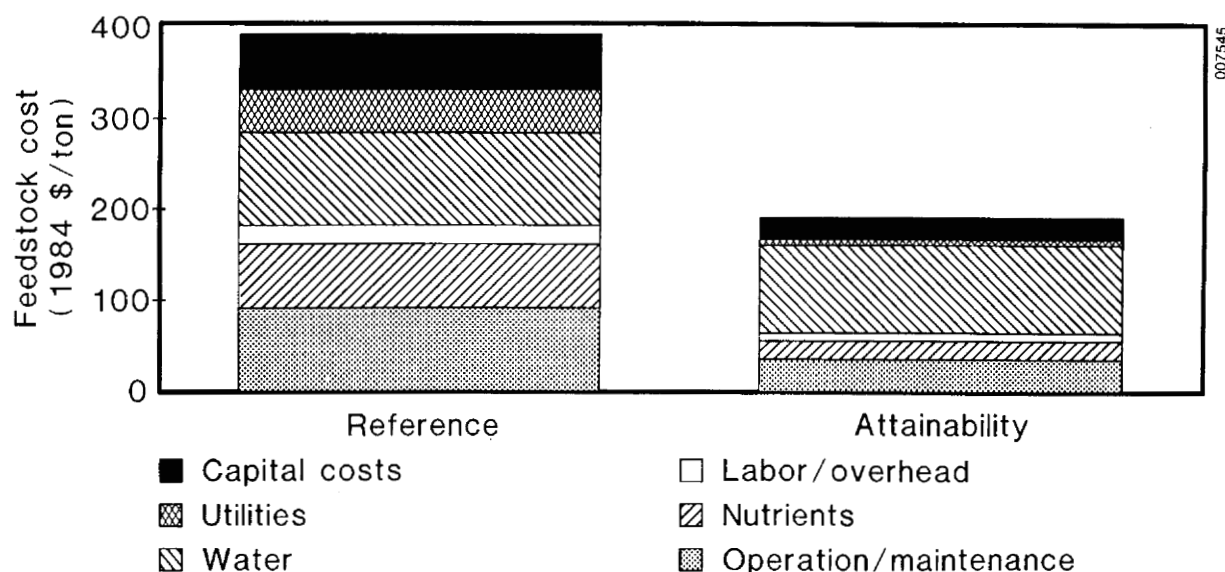
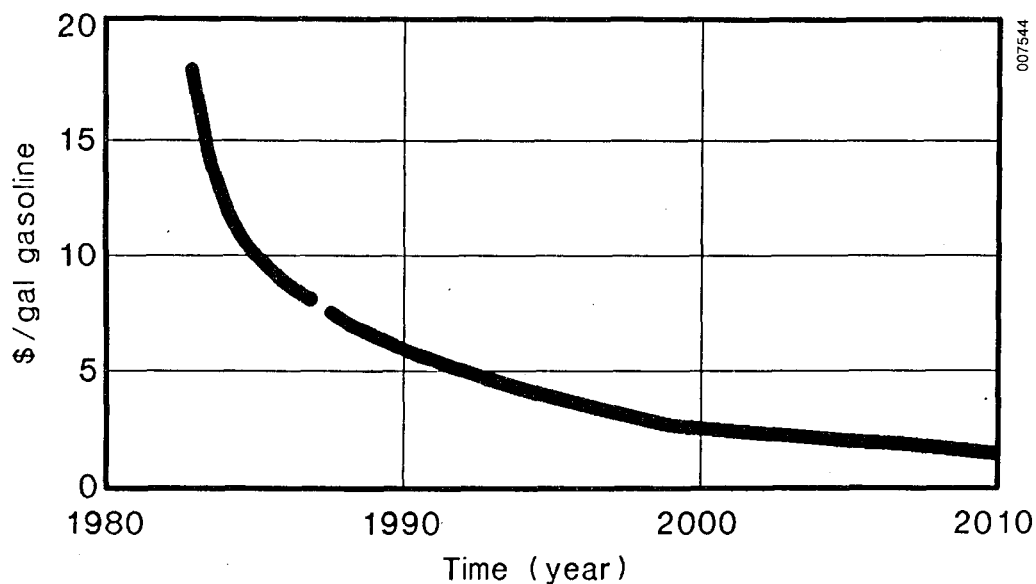


Figure 3-7. Major Cost Drivers to Produce Microalgae as a Feedstock

#### 4.0 FUTURE ACTIVITIES

Rapid improvements have been made since the inception of the program in finding suitable species to produce fuels from microalgae and in improving production technologies. The net result of this research to date has been to drastically reduce the price of gasoline derived from microalgae (Figure 4-1). However, many more developments are needed in the technology in the upcoming years to reduce the price of gasoline from microalgae so it will be competitive with fossil fuels.

Research will begin to examine extraction and conversion methods to produce gasoline and diesel fuels from microalgae. Attention will be directed toward the identification of techniques by which these lipids can be extracted on a large scale, and a detailed description of the characteristics of these lipids as they relate to their suitability as feedstocks for fuel conversion processes. Ultimately, conversion processes specifically tailored to the characteristics of microalgal lipid must be developed, either through the optimization of existing techniques or through the development of innovative conversion technologies. Such research activities require the production of algal biomass samples on a scale suitable for extraction and fuels characterization and will be obtained from the outdoor test facility. Samples from a number of promising species should be included, since there are strong indications that the characteristics of lipids vary widely between taxa.



**Figure 4-1. Current and Projected Costs to Produce Gasoline from Microalgae.** The first part of the curve (1982-1986) is the cost decrease obtained from improvements to date. The second part of the curve (1986-2010) shows the projected cost decreases as the technology improves.

In addition to these new activities, technology development will continue in the areas of species screening and characterizing lipid production, physiology, and biochemistry, productivity, and genetic engineering. The identification or development of microalgal strains that will meet the performance criteria of high productivity, high lipid content, and wide ranges of environmental tolerance is the single most critical research requirement for the economic viability of a microalgal fuels technology. The ability to meet these requirements must first be established in closely controlled experimental cultures, then confirmed under conditions that more closely approximate outdoor mass culture conditions. The ultimate success of the fuels-from-microalgae concept is critically dependent on the rate and degree of species improvement.

## 5.0 FY 1986 PUBLICATIONS AND PRESENTATIONS

### 5.1 Journal Articles and Abstracts

Barclay, W. R., N. Nagle, K. Terry, J. Weissman, and G. Goebel, "The Potential of New Strains of Marine and Inland Saline-Adapted Microalgae for Aquaculture Implications," *Journal of the World Aquaculture Society*, in review.

Barclay, W. R., and R. P. McIntosh, Eds., *Algal Biomass Technologies: An Interdisciplinary Perspective*, Nova Hedwigia, J. Cramer, Berlin 273 pp., 1986.

Barclay, W. R., J. Kennish, V. Goodrich, and R. Fall, "High Levels of Phenolic Compound in *Prochloron* sp.," *Phytochemistry*, in press.

Cooksey, K. E., S. A. Williams, and P. R. Callis, "Nile Red, a Fluorophor Useful in Assessing the Relative Lipid Content of Single Cells," *Biochemistry, Structure and Function of Plant Lipids*, Plenum Press, N.Y., in press.

Laws, E. A., S. Taguchi, J. Hirata, and L. Pang, "High Algal Production Rates Achieved in a Shallow Outdoor Flume," *Biotechnology & Bioengineering* 28:191-197.

Laws, E.A., S. Taguchi, J. Hirata, and L. Pang, "Continued Studies of High Algal Productivities in a Shallow Flume," *Biomass*, in press.

Neushul, M., "Macrocystis Genetics," *Aquaculture*, in press.

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Owens, T. G., J. Gallagher, and R. S. Alberte, "Photosynthetic Light-Harvesting Function of Violaxanthin in *Nannochloropsis* spp. (Eustigmatophyceae)," *Journal of Phycology*, in press.

Roessler, P. G., "UDP-Glucose Pyrophosphorylase Activity in the Diatom *Cyclotella Cryptica*: Pathway of Chrysolaminarin Biosynthesis," *Plant Physiology*, in review.

Roessler, P. G., "Lipid Accumulation in Silicon-Deficient Diatoms," in *Plant Lipids: Biochemistry, Structure and Functions*, P. F. Stumpf, J. B. Mudd, and W. D. Nes, Eds., Plenum Press, N.Y., in press.

Roessler, P. G., "Characteristics of Abrupt Size Reduction in *Symedra ulna* (Bacillariophyceae)," *Journal of Phycology*, in review.

Terry, K., and L. Raymond, "System Design for the Autotrophic Production of Microalgae," *Enzyme and Microbial Technology*, 7:474-487.

Van Etten, J. L., A. M. Schuster, and R. H. Meints, "Viruses of Eukaryotic *Chlorella*-Like Algae," in *Viruses of Fungi and Simple Eukaryotes*, Y. Koltin and M. J. Leibowitz, Eds., Marcel Dekker, Inc., New York, in press.

Van Etten, J. L., Y. Xia, K.E. Narva, and R. H. Meints, "*Chlorella* Algal Viruses," in *Extrachromosomal Elements in Lower Eukaryotes*, Plenum Press, N.Y., in press.

## **5.2 Technical Reports**

Arad, S., (Ed.), "Development of Outdoor Raceway Culture Technologies for Oil-Rich Halotolerant Microalgae," Final Report submitted to SERI by Ben Gurion University, Israel.

Carlson, R. D., J. H. Ryther, P. D. Pendoley, P. R. Jensen, and M. Blakeslee, "Collection and Characterization of Saline Microalgae from South Florida," Final Report submitted to SERI by Harbor Branch Foundation, FL.

Lansford, R. R., J. W. Hernandez, and P. Enis, "Evaluation of Available Saline Water Resources in New Mexico for the Production of Microalgae," Final Report submitted to SERI by New Mexico State University.

Laws, E., "Productivity Optimization of Saline Microalgae Grown in Outdoor Mass Culture Systems," Final Report submitted to SERI by University of Hawaii.

Neenan, B., D. Feinberg, A. Hill, R. McIntosh, and K. Terry, *Fuels from Microalgae: Technology Status, Potential, and Research Requirements*, Solar Energy Research Institute, Golden, CO, SERI/SP-231-2550.

Olson, K. L., L. A. Wilson, M. Wallace, and M. D. Osborn, "Utilization of Saline Water Sources in Arizona for Microalgae Production," Final Report submitted to SERI by University of Arizona.

Rhyne, C., "Nutritional Requirements for Maximal Growth of Oil-Producing Microalgae," Final Report submitted to SERI by Jackson State University.

Solomon, J., and R. E. Hand, Jr., "Ultrastructural and Flow Cytometric Analysis of Lipid Accumulation in Microalgae," Final Report submitted to SERI by Oak Ridge National Laboratory.

## **5.3 Presentations**

Barclay, W., N. Nagle, and K. Terry, "Environmental Control of Lipid Production in Diatom Cultures," presented at the Phycological Society of America/American Society of Limnology and Oceanography, Kingston, RI, June 22-26, 1986.

Barclay, W., K. Terry, N. Nagle, J. Weissman, and R. Goebel, "New Species of Marine and Inland Saline-Adapted Microalgae," presented at the Annual Meeting of the World Aquaculture Society, Reno, NV, January 1986.

Barclay, W., "Research on Microalgal Cultivation for Biomass Fuel Applications," presented at the University of Nebraska, Lincoln, NE, November 1985.

Benemann, D, D. Tillett, and J. Weissman, "Microalgae as a Source of Liquid Fuels-Economic Analysis and Experimental Status," presented at the Sixth Annual Solar, Biomass and Wind Energy Workshop, Atlanta, GA.

Cooksey, K. E., D. Berglund, B. Cooksey, and L. R. Priscu, "Lipid Synthesis and Straining by Nile Red in an Oleaginous Marine Alga," presented at the Phycological Society of America/American Society of Limnology and Oceanography, Kingston, RI, June 22-26, 1986.

Ellingson, S. and M. Sommerfeld, "Search for Lipid-Yielding Microalgae in Saline Waters of the Southwest," presented at the Arizona-Nevada Academy of Sciences meeting, Glendale, AZ, April 19, 1986.

Gallagher, J. C., and J. Stabile, "Variation Among Strains of *Nannochloropsis* spp.," presented at the Phycological Society of America/American Society of Limnology and Oceanography, Kingston, RI, June 22-26, 1986.

Gallagher, J., J. Lee, J. Stabile, and B. Nathanson, "Inter- and Intraspecific Variation in the diatom species *Amphora*," presented at the American Society for Limnology and Oceanography, New Orleans, LA.

Johansen, J., S. R. Rushforth, and W. R. Barclay, "*Chaetoceros muelleri*: Taxonomy and Physiology of Populations from Inland Brackish Waters of the Western United States," presented at the Phycological Society of America/American Society of Limnology and Oceanography, Kingston, RI, June 22-26, 1986.

Lewin, R.A., and L. Cheng, "Oil Production by Picoplankton," presented at the Phycological Society of America/American Society of Limnology and Oceanography, Kingston, RI, June 22-26, 1986.

Roessler, P.G., "UDP-Glucose Pyrophosphorylase Activity in the Diatom *Cyclotella cryptica*," presented at the American Society of Plant Physiologists, Baton Rouge, LA, June 1986.

Roessler, P. G., "Lipid Accumulation in Silicon-Deficient Diatoms," presented at the Seventh International Symposium on the Metabolism, Function and Structure of Plant Lipids, Davis, CA, July 1986.



**APPENDIX A**

This appendix contains progress reports presented by the Aquatic Species Program subcontractors and SERI researchers at the SERI Aquatic Species Program Review held in Golden, Colo., September 24 and 25, 1986. These reports present and discuss research advances achieved by the program participants during the preceding year.

## SCREENING MICROALGAE FOR BIOMASS PRODUCTION POTENTIAL: PROTOCOL MODIFICATION AND EVALUATION

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Our laboratory developed and tested a collection and screening protocol to isolate microalgal strains for biomass production applications. The protocol involved a process for exposing nutrient enriched collection site waters (from shallow, inland saline habitats) to high light intensities (20-50% of sunlight) and elevated temperature (30°C) on a rotary screening apparatus. The purpose of the protocol was to select for strains with fast growth rates that could tolerate the high light intensities and temperatures anticipated in mass cultivation systems in the southwestern U.S.

This protocol has proven successful in identifying several excellent strains which exhibit fast growth rates in combination with a wide range of salinity and temperature tolerance. The dominant genera isolated by the protocol include lipid producing species of Chaetoceros, Amphora, Monoraphidium, Nitzschia, Thalassiosira, and Navicula. The physiological attributes of several of these strains are reviewed and discussed in light of the chemical and physiological selection factors that appeared to be operating in the protocol. The effectiveness of an implemented improvement in the protocol is also discussed. Efforts to adapt the protocol to isolate cool-water (15°C) adapted strains are described.

# SCREENING MICROALGAE FOR BIOMASS PRODUCTION POTENTIAL: PROTOCOL MODIFICATION AND EVALUATION

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## INTRODUCTION

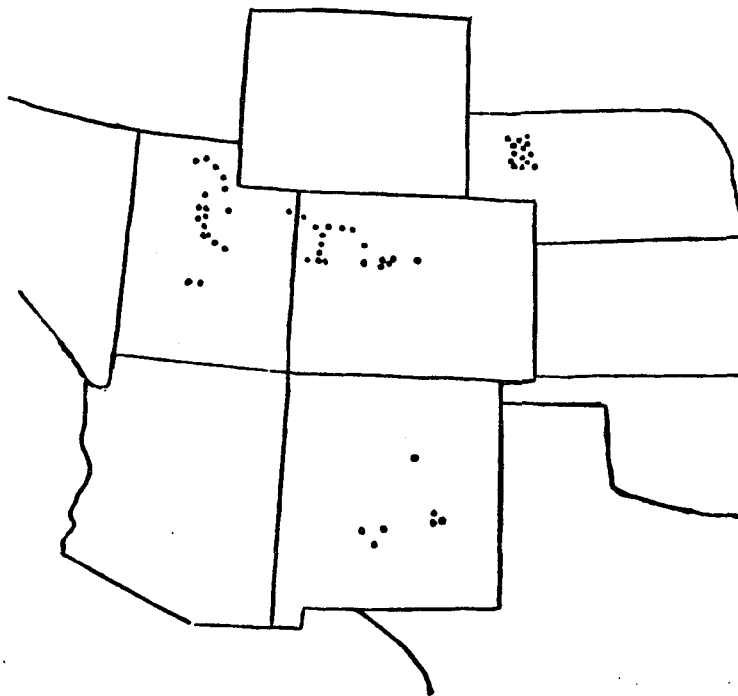
A collection and screening protocol was recently developed to isolate potential microalgal strains for biomass production applications (Barclay *et al.*, 1985). This protocol has proven successful in identifying several excellent strains which exhibit fast growth rates in combination with a wide range of salinity and temperature tolerance.

The protocol involved a process for exposing nutrient enriched collection site waters (from shallow, inland saline habitats) to high light intensities (20-50% of sunlight) and elevated temperature (30°C) on a rotary screening apparatus. The purpose of the protocol was to select for strains with fast growth rates that could tolerate the high light intensities and temperatures anticipated in mass cultivation systems in the southwestern U. S. However, even though temperature and salinity in this screening process were constant, the microalgal strains that were selected exhibited a wide range of temperature and salinity tolerance. Several changes in the protocol have been suggested including enrichment for strains in SERI standard inland saline waters as well as collection site waters (K. Cooksey, personal communication). It was apparent that in order to make effective improvements in the collection and screening process (e. g. to additionally screen for other desirable strain characteristics), it would also be important to understand some of the selective forces that were active in the screening protocol.

The goal of the research presented here was focused on two objectives: 1) to evaluate the effectiveness of a change implemented in the screening protocol; and 2) to better understand some of the chemical and physiological selection factors that appeared to be operating in the protocol. Some results from experiments exploring each of these objectives are presented.

## MATERIALS AND METHODS

Collection Activities. Microalgal collection activities during the 1985 season concentrated on small saline habitats, including temporary ponds, playas and springs in the arid regions of



**Figure 1.** Collection sites sampled during the 1985 collection season. O= location of saline habitats where samples were collected. Most of the sites were sampled three times, once each in the Spring, Summer, and Fall.

Colorado, Utah, New Mexico and Western Nebraska. The locations of the sampling sites are depicted in Figure 1. Each site was sampled in the spring, summer and fall, depending on the periodicity of standing water at the site. Water and sediment samples were collected, and water chemistry parameters measured by the techniques outlined previously (Barclay *et al.*, 1985).

**Species Enrichment.** Enrichment incubations were conducted on a rotary screening apparatus using a modification of the protocol previously described (Barclay *et al.*, 1985). The modification consisted of conducting species enrichment in all three standard saline water types in addition to enrichment in the collection site waters. In this process, six culture tubes were each inoculated with 1 ml of unfiltered water from a collection site. Each culture tube contained 6 ml of one of the following nutrient-enriched (additions of N, P, Si, trace metals and vitamins) water types: 1) collection site water; 2) SERI Type I water (25 mmho  $\text{cm}^{-1}$ ); 3) SERI Type I water (55 mmho  $\text{cm}^{-1}$ ); 4) SERI Type II water (25 mmho  $\text{cm}^{-1}$ ); 5) SERI Type II water (55 mmho  $\text{cm}^{-1}$ ); and 6) artificial seawater (25 mmho  $\text{cm}^{-1}$ ). The tubes were placed in the rotary screening apparatus and light intensities were slowly increased over a 4-5 day period from 80-200  $\text{W m}^{-2}$ . At the end of this time, the tubes were removed and their chlorophyll fluorescence quantified on a Turner Designs fluorometer. All tubes with a fluorescence reading of 2.0 units or greater were saved and examined for algae.

Species Isolation and Growth Performance Quantification. The dominant strains in each culture tube were isolated as unialgal cultures on agar or in liquid cultures. The growth responses of the selected strains to 30 combinations of temperature and salinity were evaluated for various salinity levels in three water types (Type I, Type II, and artificial seawater) on a temperature gradient plate (Barclay et al. 1985). The cultures were maintained as semicontinuous cultures, with temperature ranging from 10 to 35°C and salinity from 10 to 70 mmho cm<sup>-1</sup>.

Lipid Production. For measurement of lipid production rates under stressed and nonstressed conditions, the strains were grown under optimal conditions for growth in 2 L bottles in a growth chamber with light intensities of 40 W m<sup>-2</sup> on a 18:6 light:dark cycle. Lipid contents were measured for each strain grown under three different conditions: 1) exponential phase of growth; 2) nitrogen limited for 7 days; and 3) Si limited for 2 days. For the stressed cultures, cells were harvested from exponential phase cultures and transferred to nutrient enriched media lacking either N (as urea) or Si. At the end of the experiments, the cells were harvested by centrifugation and stored as a frozen slurry.

To facilitate lipid extraction, the cell slurry was frozen and thawed several times to enhance the permeability of the cells. Cellular lipids were extracted by repeated incubation at 60°C with methanol and methanol-chloroform (1:1), then phase separated after adjustment of the solvent ratios to 10:10:9 (methanol:chloroform:water, v/v) (Tornabene 1984). The chloroform phase was collected, evaporated to dryness under N<sub>2</sub>, and the mass of the lipids determined gravimetrically. Lipid content was expressed a percentage of cellular ash free dry weight (AFDW).

Ionic Effects of the Standard Water Types. Growth data from several of the isolated strains were statistically analyzed by multivariate procedures (factor analysis and multiple regression) to identify the most important ions or combination of ions affecting growth performance. Growth experiments with selected strains were then conducted in which each of the identified ionic parameters was varied over a wide range. Growth rates were determined as outlined in Barclay et al. (1985).

## RESULTS

### Collection Activities.

Water and sediment samples were collected from 86 sites during the 1985 collection effort. Fifty-three of these sites were sampled in the Spring, resulting in the selection and isolation of 17 strains employing the protocol briefly described above.

**Table 1.** Fisher's linear discriminant function coefficients for the three SERI standard saline water types. The coefficients were used to classify each collection site by water type, with the site assigned to whichever water type yielded the largest computed value.

Ionic Species	Classification Function Coefficients		
	Type I Water	Type II Water	Seawater
Na	0.0227	-0.0461	0.0132
K	-13.3471	-75.3826	-18.2527
Ca	-0.2276	-6.8691	-1.6218
Mg	0.5082	4.4549	1.0315
SO <sub>4</sub>	1.3345	7.6139	1.8166
Cl	-0.0216	-0.0227	0.0024
HCO <sub>3</sub>	10.3857	61.8403	13.6392
CO <sub>3</sub>	-9.0862	-53.2465	-12.0681
Constant	-20.9186	-280.1540	-17.3186

Data from these strains and the 53 collection sites were selected for a closer examination of the effectiveness of the modified screening protocol.

Data on the ionic composition of the three SERI standard water types (Barclay *et al.*, 1985) were analyzed by discriminant analysis. Discriminant analysis is a statistical method for classifying individuals or objects into mutually exclusive groups on the basis of a set of independent variables (Dillon & Goldstein 1984). It was employed in this study to develop classification functions (based on ionic composition) for the three SERI standard water types. The computed discriminant classification function coefficients are presented in Table 1. A scatterplot of the discriminant function values verified a clear separation of the three water types by the variables.

Water chemistry analysis (major cations and anions) was completed for 46 of the 53 spring collection sites. These data were then analyzed by the discriminant classification functions developed for the three SERI water types. The results indicated that 12 of the sites had water similar in composition to Type I water. Twenty-three of the sites had ionic compositions similar to Type II water and eleven were similar to seawater.

Screening Protocol Modifications. Based on the field data and the fluorescence data from each of the 17 strains selected, three questions were analyzed to evaluate the effectiveness of the modified screening protocol. First, would the protocol have selected for the strains based only on the enriched site water fluorescence. The results from the Spring collection data

indicated that only 6 out of the 17 selected strains would have been retained using this approach. Therefore the modified protocol exhibited an almost 200% improvement in the number of potentially beneficial strains isolated.

The second question analyzed asked whether the isolated strains grew best on the rotary screening apparatus in the water type closest to the collection site ionic composition. The data indicated this to be true in only 18% (3 out of 17) of the strains selected.

The final question was whether the isolated strains grew well (culture tube fluorescence > 2.0 units) in the water type closest in ionic composition to the collection site water. The data indicated this to be true for only 35% of the selected strains (6 out of 17). As in the first question, the results from the latter two questions suggest that the effectiveness of the screening protocol is greatly enhanced by including the standard water types in the initial screening/enrichment portion of the protocol. Many additional strains were selected which would have been overlooked by only screening for strains in enriched site waters.

Strain Characterization. Determinations of the temperature and salinity tolerances of six of the strains isolated in 1985 were completed. The strains were: 1) Chaetoceros sp. (S/CHAET-4); 2) Navicula sp. (S/NAVIC-1); 3) Cyclotella sp. (S/CYCLO-2); 4) Amphora sp. (S/AMPHO-1); 5) Amphora sp. (S/AMPHO-2); and 6) Monoraphidium sp. (S/MONOR-2). All six strains were collected from shallow, inland saline habitats. The Amphora strains were collected in Colorado, the Chaetoceros and Monoraphidium strains in Utah, and the Navicula and Cyclotella strains were isolated from water samples collected in Florida. The exponential growth rates of these strains under a variety of temperatures and salinities are illustrated in Figures 2-7. One of the most notable features of these strains is their very wide range of salinity tolerance. Almost all of the strains exhibited growth rates which were independent of salinity over a wide range in at least two of the SERI water types. Additionally, all of the strains exhibited optimum growth at temperatures greater than 30°C in at least one of the water types. In fact, all of the strains except the Monoraphidium strain exhibited temperature optima for growth of over 35°C.

Lipid Production. The lipid contents of the six strains whose growth data are illustrated in Figs. 2-7 are presented in Table 2. Lipid contents are presented for cells in the exponential phase of growth, and under N or Si limitation. Three of the strains, Navicula sp. (S/NAVIC-1), Chaetoceros sp. (S/CHAET-3), and Monoraphidium sp. (S/MONOR-2), all exhibited excellent lipid production potential. Lipid contents of over 50% AFDW could readily be induced in both the Navicula and Monoraphidium strains. More recent experiments with the Chaetoceros strain

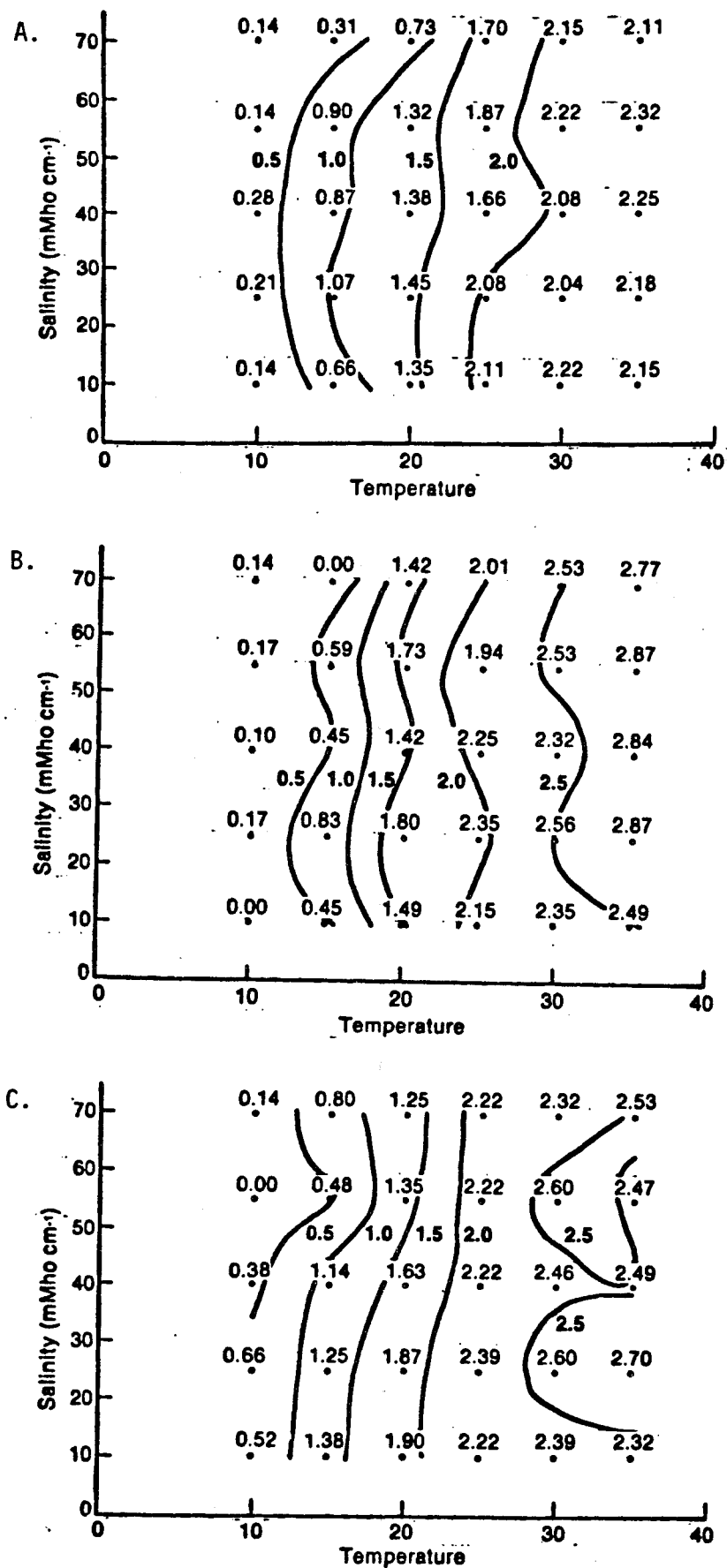


Figure 2. Exponential growth rate (doublings day<sup>-1</sup>) of *Chaetoceros* sp. (S/CHAET-4) in semicontinuous culture. Each point represents the mean of at least five separate daily growth rate determinations. A= Type I inland saline water; B= Type II inland saline water; C= Seawater.



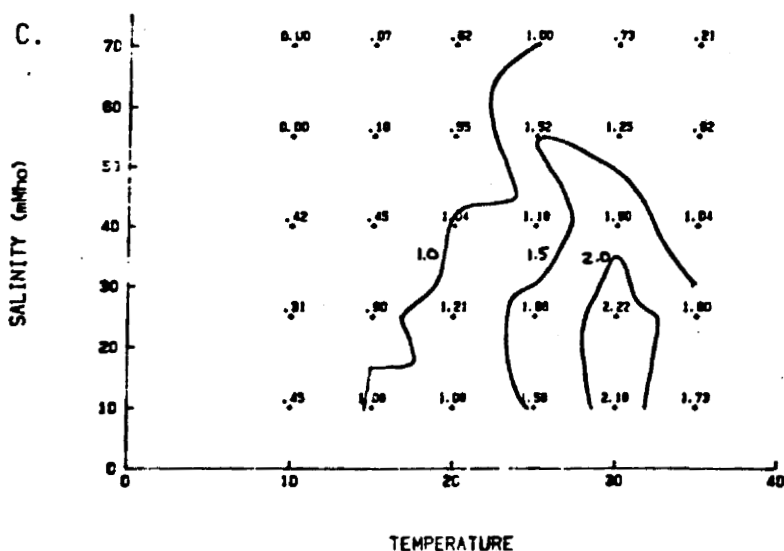
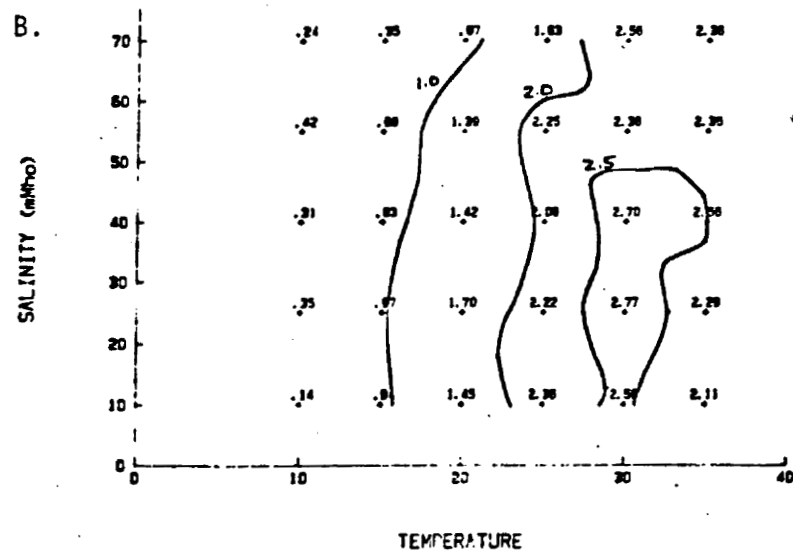
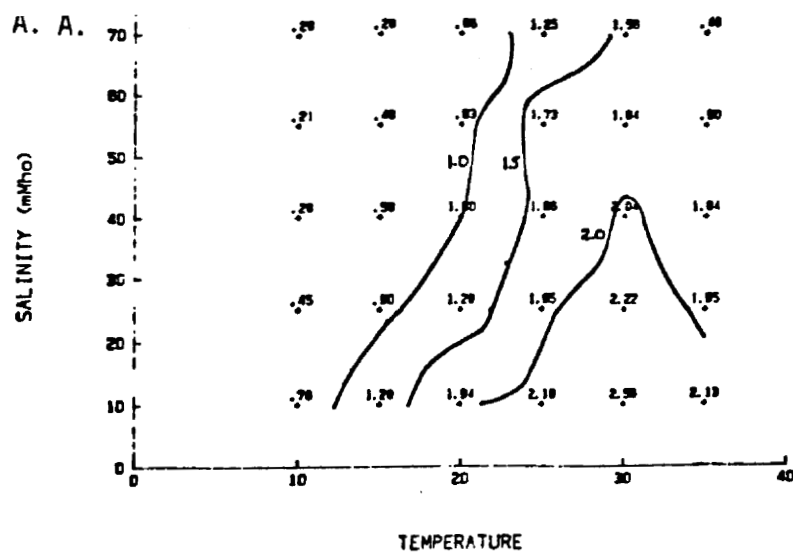


Figure 3. Exponential growth rate (doublings day<sup>-1</sup>) of *Navicula* sp. (S/NAVIC-1) in semicontinuous culture. Each point represents the mean of at least five separate daily growth rate determinations. A= Type I inland saline water; B= Type II inland saline water; C= Seawater.

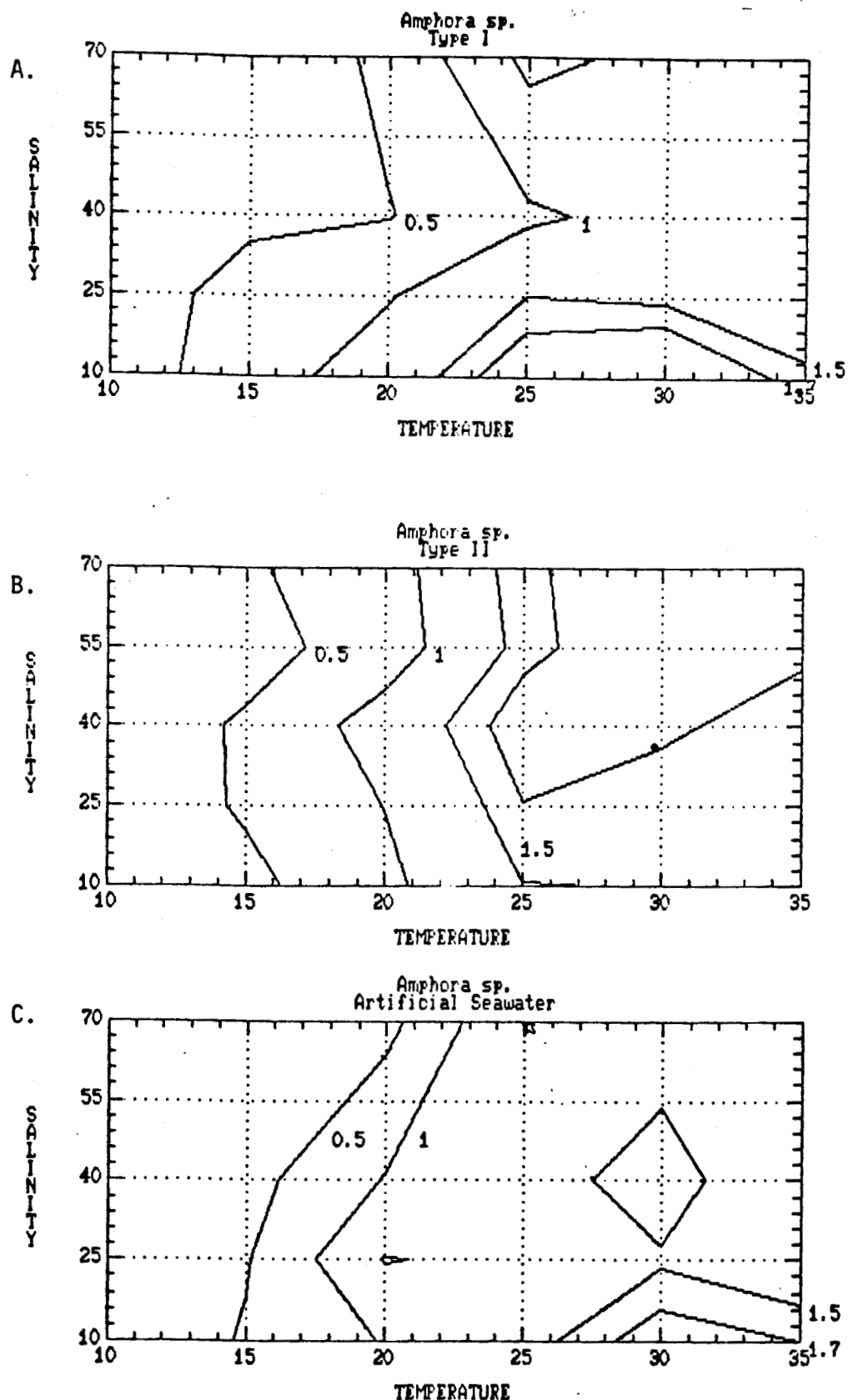


Figure 4. Exponential growth rate (doublings day<sup>-1</sup>) of *Amphora* sp. (S/AMPHO-1) in semicontinuous culture. Each point represents the mean of at least five separate daily growth rate determinations. A= Type I inland saline water; B= Type II inland saline water; C= Seawater.

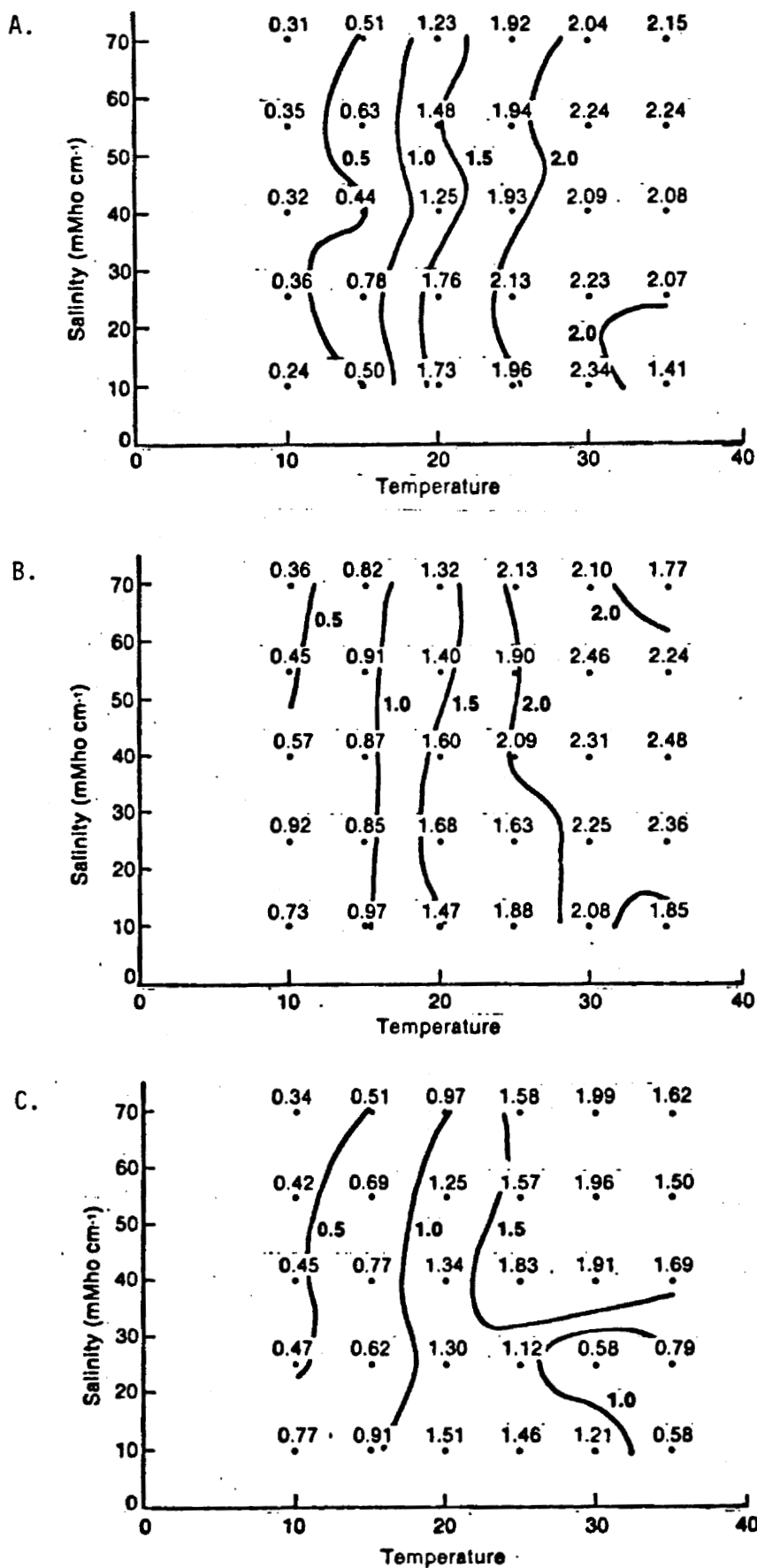


Figure 5. Exponential growth rate (doublings day<sup>-1</sup>) of *Amphora* sp. (S/AMPHO-2) in semicontinuous culture. Each point represents the mean of at least five separate daily growth rate determinations. A= Type I inland saline water; B= Type II inland saline water; C= Seawater.

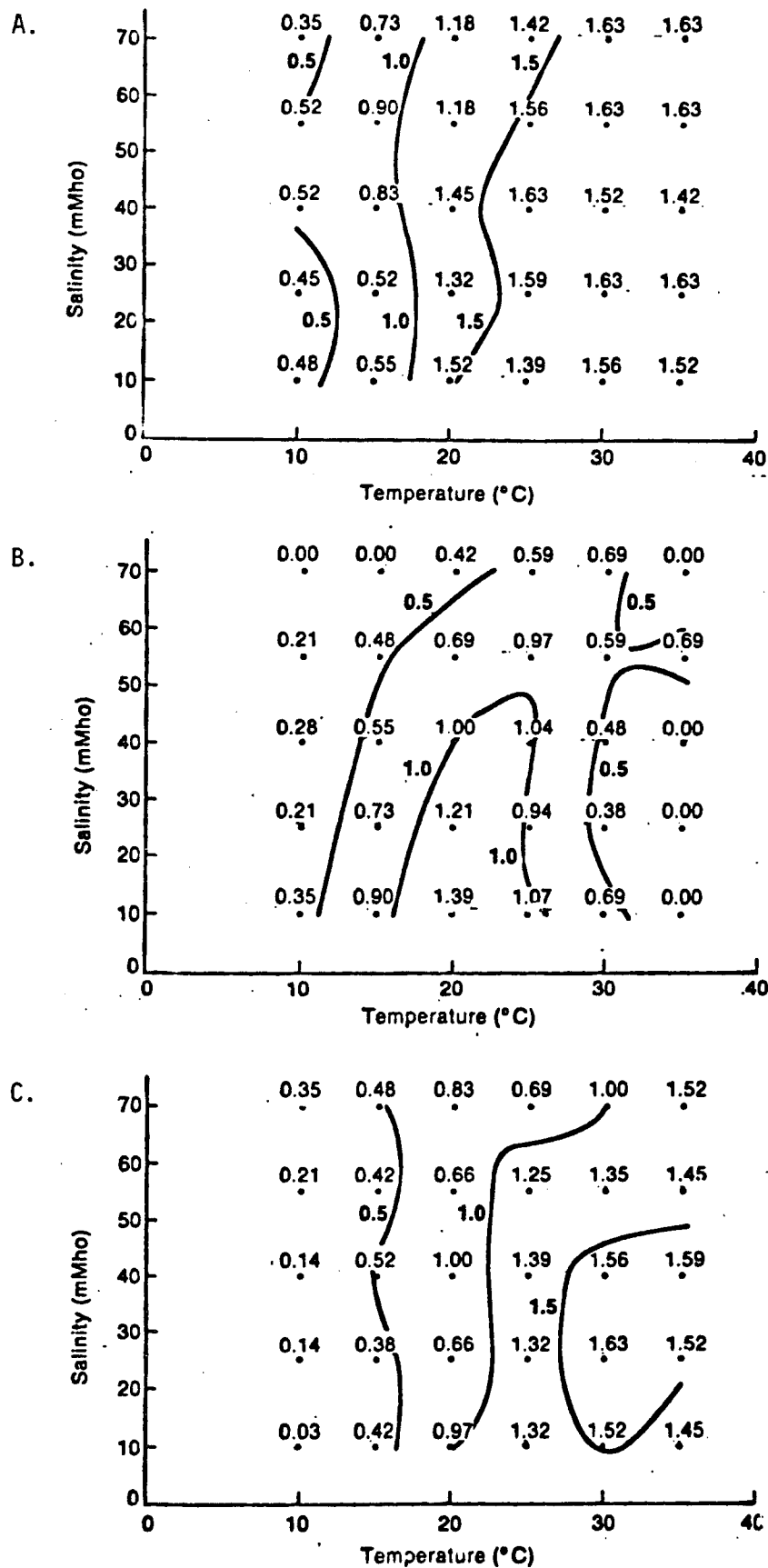


Figure 6. Exponential growth rate (doublings day<sup>-1</sup>) of *Cyclotella* sp. (S/CYCLO-2) in semicontinuous culture. Each point represents the mean of at least five separate daily growth rate determinations. A= Type I inland saline water; B= Type II inland saline water; C= Seawater.

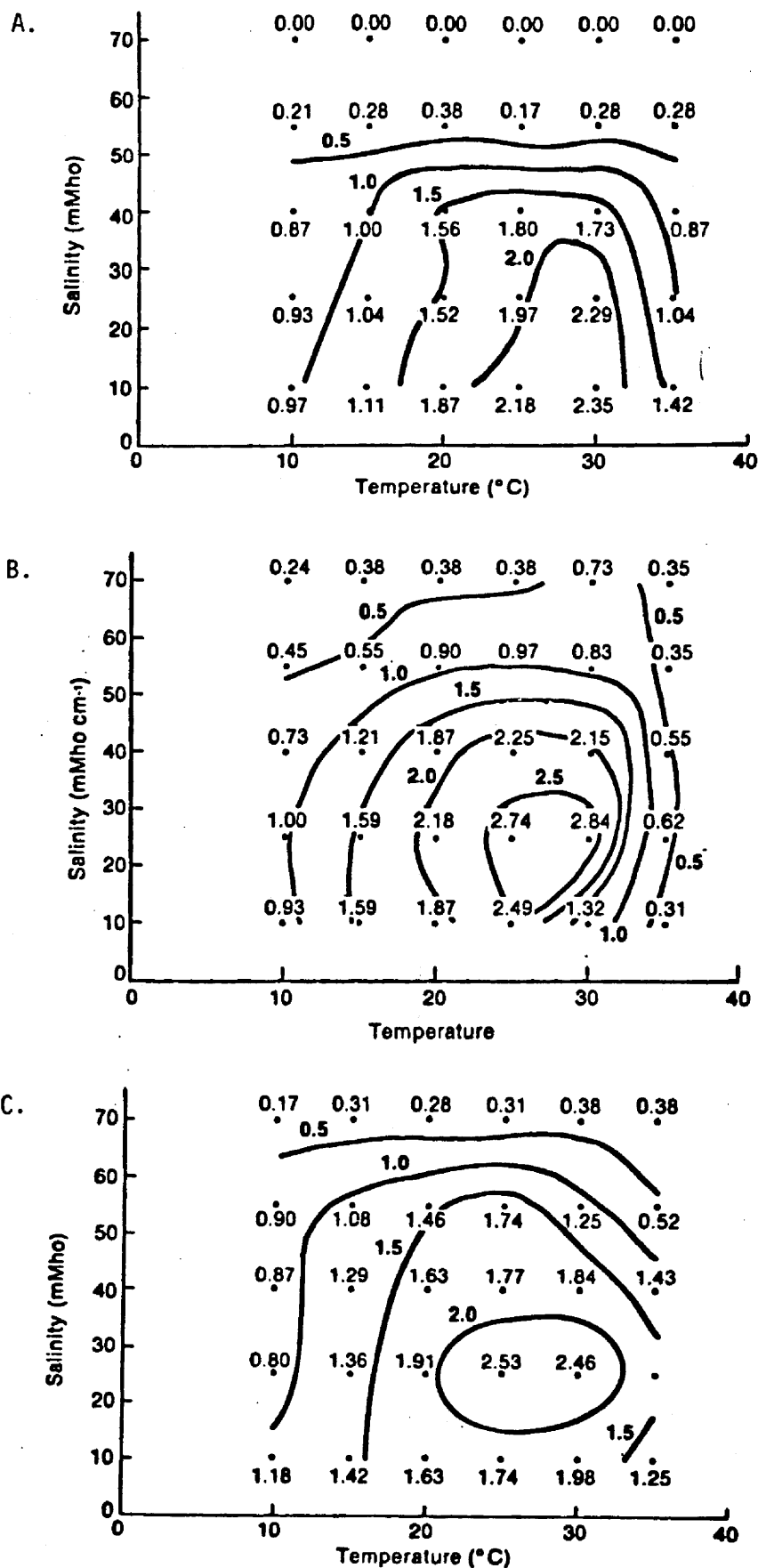


Figure 7. Exponential growth rate (doublings day<sup>-1</sup>) of *Monoraphidium* sp. (S/MONOR-2) in semicontinuous culture. Each point represents the mean of at least five separate daily growth rate determinations. A= Type I inland saline water; B= Type II inland saline water; C= Seawater.

**Table 2.** Lipid content (% AFDW) of six strains of microalgae isolated in 1985. All six strains had been previously characterized as exhibiting fast growth rates over a wide range of temperatures and salinities.

Growth Phase	STRAINS					
	<u>Navicula</u> sp. S/NAVIC-1	<u>Chaetoceros</u> sp. S/CHAET-4	<u>Amphora</u> sp. S/AMPH-1	<u>Amphora</u> sp. S/AMPH-2	<u>Cyclotella</u> sp. S/CYCLO-2	<u>Monoraphidium</u> sp. S/MONOR-2
Exponential	22.1	19.0	19.2	36.2	23.5	22.1
Si-deficient 48 hours	48.9	39.4	28.9	18.6	24.7	----
N-deficient 7 days	57.8	37.6	25.0	30.8	21.7	51.6

(Barclay et al. 1986, in this volume) also indicate that lipid contents of 50% AFDW can be obtained under optimized conditions with this strain.

Ionic Effects of the Standard Water Types. Two ionic parameters, bicarbonate concentration and divalent cation concentration, were identified by statistical analyses as important factors influencing microalgal growth in Type II water. This was confirmed with growth experiments employing two strains which grew best in Type II water, Boekelovia sp. (Barclay et al., 1985) and Monoraphidium sp. (S/MONOR-2). Increasing bicarbonate and decreasing divalent cation concentrations both facilitated increased growth rates in these strains (Figs. 8-9). Na, Cl, and SO<sub>4</sub> were identified as the important ions influencing growth in Type I water and seawater. However, results from experiments varying the concentration of these ions over a wide range of conductivities and examining their influence on growth suggested that the effect of these ions may be species specific (Figs. 10-11).

## DISCUSSION

The results of this year's research have provided many insights into the selection forces operating in the collection and screening protocol. The modified protocol greatly increased the overall success of the collection and screening process. The results suggest that there are a large number of "opportunistic" strains in temporal saline habitats, existing in small numbers (or in cysts) until the ionic composition of the water becomes favorable for growth. The ionic composition of the water in these habitats varies widely as the water in these systems increases at certain times of the year and then slowly evaporates. By enriching for strains in all three standard water types plus the collection site water, the modified protocol

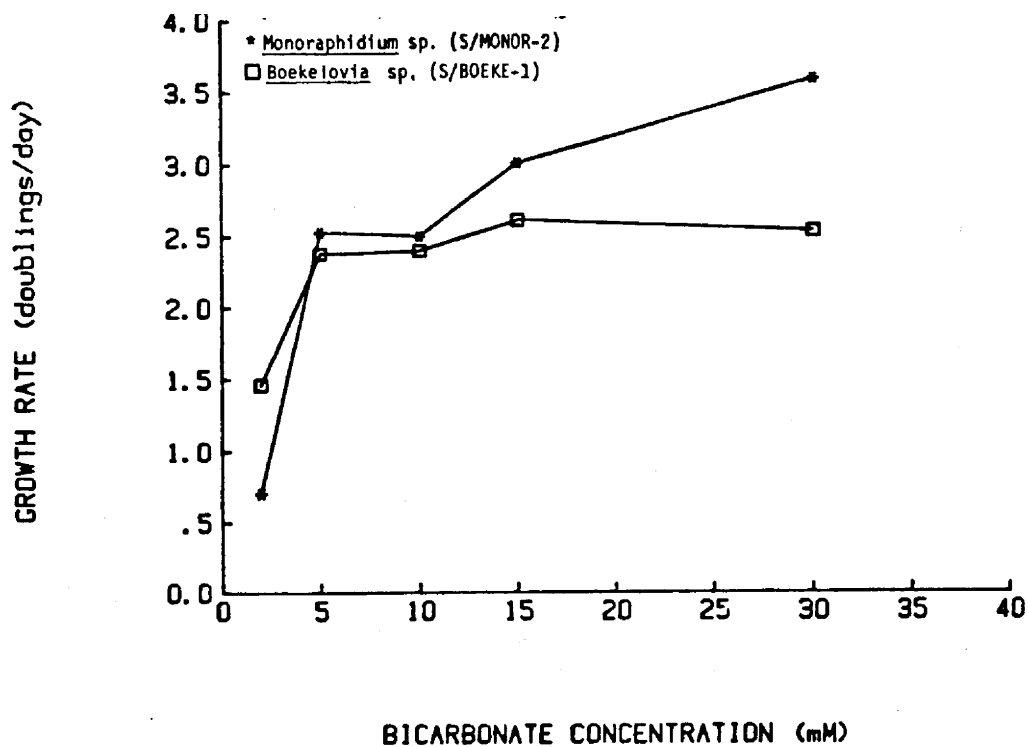


Figure 8. Effect of bicarbonate concentration on the growth rates of *Monoraphidium* sp. and *Boekelovia* sp.. The algae were grown in Type II/ 25 mmho medium (without added  $\text{Na}_2\text{CO}_3$ ) with varying amounts of sodium bicarbonate added.

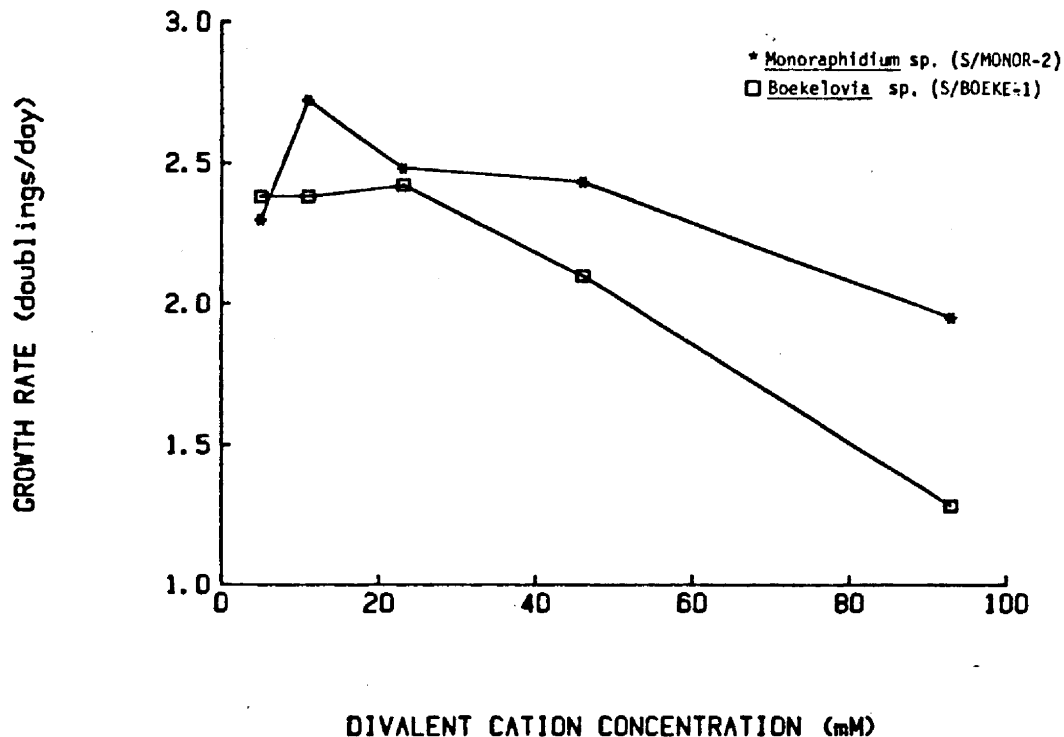


Figure 9. Effect of divalent cation concentration ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ) on the growth rate of *Monoraphidium* sp. and *Boekelovia* sp.. The algae were grown in Type I/ 10 mmho medium with calcium and magnesium adjusted proportionally over the range of 5 - 95 mM.

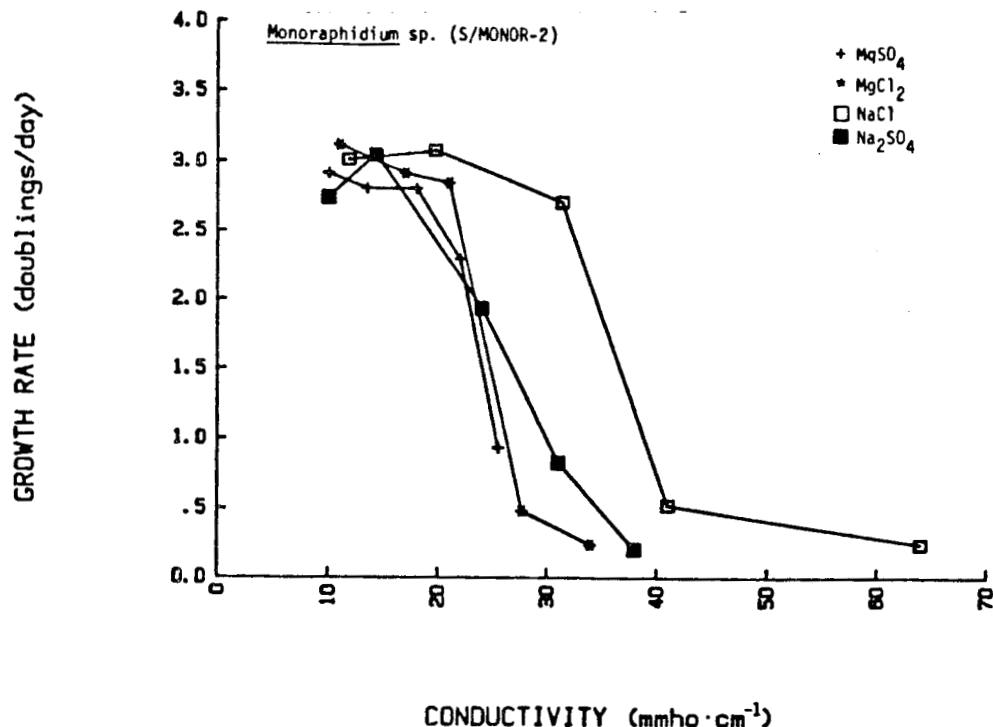


Figure 10. Effect of varying sodium and magnesium sulfate and chloride concentrations on the growth rate of Monoraphidium sp.. The algae were grown in Type I/10 mmho medium, with conductivity varied by addition of the salt under study.

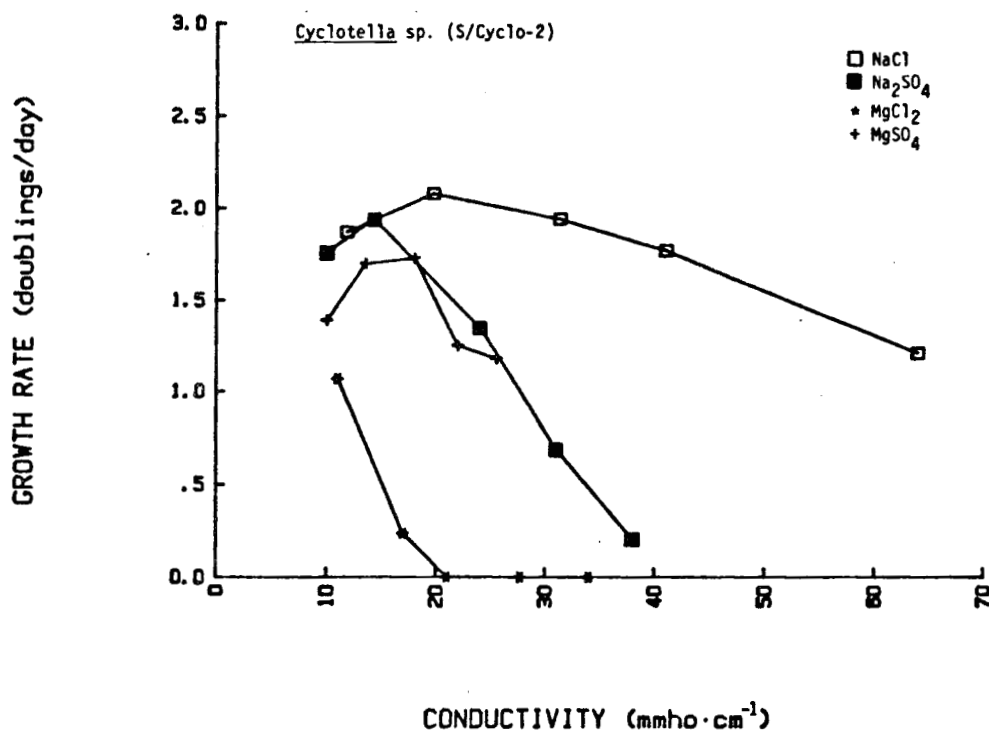


Figure 11. Effect of varying the concentration of sodium and magnesium, chloride and sulfate salts on the growth rate of Cyclotella sp.. The Algae were grown in Type I/10 mmho medium, with conductivity varied by addition of the salt under study.



provides many more conditions for promoting growth in different strains.

Based on data from the Spring collection effort, at least 50% of the collection sites contained highly alkaline waters, similar in composition to SERI Type II standard inland saline water. Some of the most productive strains identified to date have been isolated from these waters, and the majority of isolated strains exhibit their highest growth rates in Type II water (Fig. 2-7). The high alkalinity of this water type may serve as an equilibrium source of  $\text{CO}_2$  in the unaerated cultures used to quantify growth. Additionally, some of the strains may be able to use bicarbonate directly as a carbon source. Microalgae strains possessing the ability to utilize  $\text{HCO}_3$  have a competitive advantage over other strains under conditions of alkaline pH and high salinity (Beardall, 1985). Several species of freshwater (Beardall, 1985; Miyachi *et al.*, 1985) and saline microalgae (Lloyd, *et al.*, 1977; Badger & Andrews, 1982; Coleman & Gell, 1983) have the ability to utilize  $\text{HCO}_3$ . Our collection protocol, by focusing on very shallow saline waters, appears to be selecting a large fraction of strains which appear to be able to utilize  $\text{HCO}_3$ . Increased growth rates correlated with increasing bicarbonate concentration have been documented for two of the strains isolated to date (Fig. 8). Also, most of the strains which grow rapidly in Type II water have been observed to grow well at  $\text{pH} > 10$  (B. Barclay, unpublished data).

Microalgal strains which are able to utilize  $\text{HCO}_3$  have also been noted to have very low rates of photorespiration (Colman & Cook, 1985). The algae are able to maintain high intracellular concentrations of  $\text{CO}_2$  (under conditions of dissolved  $\text{CO}_2$  depletion) by dehydration of  $\text{HCO}_3$  to  $\text{CO}_2$  utilizing extracellular and/or intracellular carbonic anhydrase (Miyachi *et al.*, 1985). The result is that these species exhibit greatly reduced rates of oxygen inhibition of photosynthesis (Lloyd *et al.*, 1977; Birmingham & Coleman, 1979; Colman & Gehl, 1983) and very low glycolate excretion rates (Tolbert *et al.*, 1985). Several of the strains selected under our protocol and which prefer Type II water exhibit very little oxygen inhibition of photosynthesis (J. Weissman, personal communication). By selecting for strains which exhibit reduced rates of oxygen inhibition of photosynthesis, the protocol is ultimately selecting for strains which may be very efficient biomass producers. More research, however, is needed to confirm whether most of the isolated strains also exhibit reduced photorespiration.

Strains which perform well in Type II water appear to do so because of both the high bicarbonate and low divalent cation concentrations in this water (Figs. 8-9). The role of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  in this effect are currently unclear. The effect of ionic ratios on microalgal growth in Type I water and seawater appears to be much more complex and is probably species specific (Figs. 10-11). Much more research must be done before these ionic interactions can be understood.

The wide salinity tolerance of the isolated strains appears to be an outcome of the collection protocol rather than the screening process. By focusing on shallow, temporal saline habitats, the collection protocol is predisposed to providing strains with a wide range of salinity tolerance. From this slate of strains, the screening process selects for the most rapidly growing strains. The salinity tolerance of the strains isolated to date is much wider than that reported for most marine forms. A few marine diatoms are known to tolerate broad-ranging salinities (5-50%) including Chaetoceros affinis (Bonin, 1969), Chaetoceros radians, Cerataulina pelagica, Leptocylindrus danicus, and Thalassiosira decipiens (Takano, 1963). The chrysophyte, Olisthodiscus luteus, also tolerates salinities from 2-50% (Tomas, 1978). The processes microalgae employ to adapt to osmotic stress vary widely, and have been reviewed by Bonin et al. (1981). The unique nature of the strains isolated by our protocol is that they exhibit broad-ranging salinity tolerance in three very different types of saline water (based on ionic composition). Thus, they appear to exhibit a much wider range environmental tolerance than has been demonstrated in microalgae to date.

An additional benefit of the protocol is that the majority of the strains isolated to date exhibit optimal temperatures for growth over 30°C, with several optima exceeding 35°C. Strains with temperature optima over 30°C will be necessary for the successful implementation of algal biomass production systems in arid or semi-arid regions such as the southwestern U.S. (Hill et al., 1986).

Although no aspect of the protocol directly selects for the type of storage product produced by the microalgae, 33% of the isolated strains produced large quantities of lipid under stressed conditions. This represents a relatively high success rate, and we therefore do not anticipate any changes to make the protocol more specific for high lipid-producing strains. Approaches more along these lines are being employed by other ASP contractors involved in screening (e.g. Lewin, 1985). However, we anticipate focusing part of our effort in the 1986 season on attempting to collect physiological races of some of the best lipid producing strains we have identified to date. Data from physiological races should not only provide information on the natural variability in lipid production occurring within a species, but additionally provide the raw material for future strain improvement through genetic techniques.

#### ACKNOWLEDGEMENTS

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of SERI collected the water samples from Florida.

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## COLLECTION AND SCREENING OF MICROALGAE FOR LIPID PRODUCTION:

### POSSIBLE USE OF A FLOW CYTOMETER FOR LIPID ANALYSIS

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#### Abstract

Thermotolerant algae isolated in axenic culture from sites in Yellowstone National Park produce neutral lipid as 35-54% of their dry weight when grown at 35°C. We have found that the marine diatom Amphora coffeaeformis can be stained with the fluorophore Nile Red and that the lipid content of the stained cells can be related to their fluorescence. The fluorescence of unfixed cells of A. coffeaeformis fades with time, however chemical fixation of the cells reduces the rate of fading.

#### Introduction

The energy crisis in 1973 brought home to many people the fact that the world's supply of oil was not unlimited. Since that time there have been various attempts to investigate other sources of energy and many of these have centered on the use of solar energy in one form or another. A particular example is the production of fuel-grade oil from microalgal biomass. The project being investigated by the Solar Energy Research Institute involves the growth of specific oleaginous microalgae in brackish water ponds in the southwest deserts of the U.S.A. The algae used in this process need certain physiological characteristics in order for the project to be viable. It is self-evident that any algae used must have a high lipid production rate. Because of the high daytime temperatures and light intensities in a desert climate, algae must also grow well in these conditions since control of these environmental parameters cannot be achieved in a cost-effective manner. One of the objectives of this project, therefore, concerns the isolation of thermotolerant microalgae able to grow at high light intensities. A second objective concerns a means of selection of algal strains that produce large quantities of neutral lipids. For the purposes of this work, neutral lipid is defined as the triacylglyceride and hydrocarbon fraction of the cell. Since neither of these groups of compounds have functional groups that are easy to assay chemically, lipid is usually determined gravimetrically after non-polar solvent extraction of the algal cells. A method for the in situ analysis of neutral lipid would prove extremely useful in this research, as would means of selection of high-yielding strains produced by mutation or protoplast fusion. This second objective, therefore, concerns the development of an assay for neutral lipid using intact cells and the separation of high lipid-containing cells from a heterogeneous population.

## Rational for the Research Approach

To find thermotolerant algae that grow above 35°C, we have sampled hot springs in Yellowstone National Park and made all isolations at this temperature. Lipid content of cells has been determined qualitatively so far by use of the fluorescent probe Nile Red. Based on their Nile Red fluorescence, it is proposed that cells can be sorted using a fluorescently activated cell sorter (FACS).

## Materials and Methods

### A. Isolation of Thermotolerant Diatoms

1. Culture Method. Samples of algae were collected from various thermal areas in Yellowstone Park. Collection data included temperature, pH, and conductivity ( $\mu\text{mhos}$ ). All samples were examined microscopically and those containing microalgae were transferred as follows: 0.5 ml cell suspension into 5 ml of medium in 15 ml screw-capped culture tube; 1.0 ml into 25 ml of media in 125 ml culture flask. Cultures were incubated at 35°C and examined daily for growth. As growth occurred, algae were streaked onto agar plates of appropriate media (2% DIFCO Bacto-Agar). Cells or colonies growing on agar were picked and transferred to tubes of media (1 ml). As growth occurred in 1 ml, transfer was made into 5 ml. When growth occurred in 5 ml, a bacterial contamination check was made. The test medium was 0.05% yeast and 0.05% glucose in the mineral salts algal medium. If bacteria were present, algae were streaked onto antibiotic agar plates. These were made from 0.3% penicillin G, 0.5% streptomycin sulfate, in distilled H<sub>2</sub>O. This solution was filter-sterilized and stored frozen. For use, 1.0 ml was added to 100 ml agar media. Colonies or cells were picked again, grown, and re-tested for bacteria. Axenic cultures were maintained on appropriate media in agar-slant tubes at 35°C. Transfers were made every 3-4 weeks into fresh media and nutrient broth tubes to check for the presence of bacteria.
2. Media Used. Initially, three culture media were used - SERI Type I, SERI Type II (both at 25  $\text{mmho cm}^{-1}$ ), and ASP<sub>210</sub>. After isolating the current strains, only ASP<sub>210</sub> and SERI Type I were used. All strains grew in ASP<sub>210</sub>. Only 3 of the new strains grew in SERI I; none grew in SERI II. Growth curves and other experiments performed have been performed with ASP<sub>210</sub> medium only (Cooksey and Chansang, 1976). One litre of SERI Type I water ("25  $\text{mmhos}$ ," Barclay *et al.*, 1985) was enriched as follows: 8 mg KH<sub>2</sub>PO<sub>4</sub>, 1 mg Na<sub>2</sub>SiO<sub>3</sub>·9H<sub>2</sub>O, 42 mg NaNO<sub>3</sub>, 5 ml trace metal solution (McLachlan, 1973), 1 ml vitamin mixture (McLachlan, 1973). 168 mg NaHCO<sub>3</sub> in 10 ml water was added aseptically to the sterile basal medium.
3. Determination of Dry Weight and Ash Free Dry Weight. Filters (Whatman GFA, 2.4 cm) were rinsed three times with distilled water (20 ml), dried, placed in numbered aluminum boats and pre-combusted at 500°C for 2 hours. Three 50 ml aliquots of cell culture were filtered onto a pre-weighed, pre-combusted filter and rinsed with 5 ml culture medium. A blank consisted of 50 ml aliquots of media (i.e., no cells) filtered onto an appropriate filter. All filters were air dried,

replaced in appropriate aluminum boats and dried at 90°C to constant weight. Dry weight of the cells was corrected for the medium blank. To determine ash, the filters were combusted at 500°C until constant weight was achieved. This value was used to correct the dry weight values. The mean of 3 values is reported.

4. Determination of Lipid. Fifty ml of culture was filtered as described for the dry weight determination. Two such filters with cells were extracted by the method of Bligh and Dyer (1959) (see also Ben-Amotz, 1985) at the same time in a sealed tube. Extractions, which were performed in triplicate, were carried out successively for 12 hr., 2 hr., and 1 hr. After phase separation, the chloroform layers were removed, combined and made to 10 ml. After filtration through a glass-fibre filter, 9 ml of the lipid extract was dried to constant weight in a pre-weighed aluminum boat. Lipid values are reported as the mean of three determinations corrected for chlorophyll. The chlorophyll correction was less than 8% by weight of the total chloroform-methanol extractable lipid.
5. Chlorophyll a Determinations. These were made fluorometrically on 90% acetone extracts of filtered cells (Cooksey, 1981). Chlorophyll was determined from the fluorescence values corrected for the presence of phaeophytin. A standard curve was prepared using pure chlorophyll a.
6. Nitrate Determination. Cells were removed from medium by filtration and nitrate determined by the cadmium column reduction method (EPA, 1983).
7. Growth Measurements. Cell counts were made using a haemocytometer. Late logarithmic phase cells were used to inoculate fresh medium at an initial cell count of 50,000 cells ml<sup>-1</sup>. Cell inocula were checked for the presence of bacteria by inoculating a sample into yeast extract/glucose medium. No growth of bacteria in the enriched medium was considered evidence for the axenic nature of the inoculum.
8. Flow Cytometry. We have used a Becton-Dickinson FACS 440 equipped with a Spectra-Physics 164-02 argon ion laser. Diatoms in ASP-2 medium containing 0.25 mM Ca (Chansang and Cooksey, 1976), either stained or unstained, were passed through the instrument using a diluent of minimal medium (Cooksey, 1981) containing 0.25 mM Ca. The over pressures in the liquid containers were 14 psi (diatom suspension) and 17 psi (sheath fluid). It was important to agitate the diatom suspension from time to time and to pre-treat the suspension with ultrasound to break up clumps, especially after the cells had been centrifuged. A sorting-aperture of 70 µm was used for diatom cells of approximately 20 µm in length. Unstained cells could be excited at 488 nm (100 mW) and examined by means of their fluorescence at 530±15 nm (green) in conjunction with a dichroic mirror at 560 nm. This autofluorescence was not due to chlorophyll a which emits in the red region of the spectrum. Using a bandpass filter at 575±15 nm allowed the fluorescence of Nile red-stained cells to be examined without interference from green or red autofluorescence. Results are expressed as number of events (y-axis) versus the logarithm of the fluorescence intensity of an individual cell (x-axis). Instrumental

alignment adjustments were made daily and were based on the fluorescence of 2  $\mu\text{m}$  Nile Red-stained spheres (a gift from Dr. David Parks, Stanford University).

Nile Red Staining. Stock solutions of Nile Red (250  $\mu\text{g ml}^{-1}$ , Molecular Probes, Oregon) were made in acetone and stored at 4°C in the dark. Cell suspensions (about  $10^6 \text{ ml}^{-1}$ ) were stained with final dye concentrations ranging from 0.01 to 10  $\mu\text{g ml}^{-1}$  and for various times. Optimal staining time and dye concentrations were investigated using the flow cytometer. In some experiments cells were fixed in 70% (v/v) ethanol or 4% (w/v) formaldehyde for 15 min. and resuspended in minimal medium for staining and flow-cytometric analysis.

## Results

### A. Axenic Cultures Obtained

The details are shown in Table 1. No cultures grew in SERI II-based medium. During the isolation procedure we found that this medium selected for cyanobacteria. Because of this and the fact that no or poor diatom growth was obtained using it, we have discontinued its use as an experimental medium. We do, however, intend to continue its use for new isolations.

In addition to the isolation of new thermally tolerant strains we have also tested 18 other diatom strains from our culture collection. Only 6 strains grew well at 35°C, but all of them stained with Nile Red. Three of these have been selected for further quantitative study including their ability to grow in SERI media.

Table 1. Diatoms isolated from Yellowstone Park.

MSU strain identi- fication	Location				
	Site <sup>1</sup>	Temp. (°C)	pH	$\mu\text{mhos}$	Growth media
SERI 1	Nymph Lake inlet	38	2.7	--	ASP-2, SERI I
2	Gibbon R. vents	33	6.6	389	ASP-2
3	Beryl Spring	33	7.8	963	ASP-2
4	Firehole R.	36	8.5	1051	ASP-2, SERI I
5	Firehole R.	36	8.5	1051	ASP-2, SERI I

<sup>1</sup>All in Yellowstone National Park. Based on morphology, two separate isolates of SERI I have been obtained. These may not be identical in all respects.



## B. Analysis of Growth Parameters of Selected Strains.

Three diatoms have been examined for their ability to produce neutral lipid. No attempts, as yet, have been made to manipulate cultural conditions to enhance lipid formation.

Table 2. Lipid Production by three diatoms.

Strain	Maximal cell number $\times 10^{-6}$	Growth rate $\text{day}^{-1}$	AFDW $\text{mgL}^{-1}$	Lipid %	Temp. $^{\circ}\text{C}$
SERI 2	1.25	0.83	125	53.7	35
SERI 5	5.9	1.3	78	34.7	35
<u>A. coffeaeformis</u>	1.0	2.0	87	31.0	28

Cells were grown at  $125 \mu \text{ Einsteins m}^{-2} \text{ sec}^{-1}$  (SERI) on a 16/8h light/dark cycle or  $90 \mu \text{ Einsteins m}^{-2} \text{ sec}^{-1}$  (A.c.) continuous light.

## C. Nile Red Staining Protocol

Fluorescence readings were made immediately (2 min. approximately) and 5, 10, 20 and 25 min. after adding Nile Red to a diatom suspension. Although it is not seen easily in the epifluorescence microscope, fading is quite evident in the flow cytometer after 15 min. (Fig. 1). In all future experiments, except where stated, measurements were made after 5 min. and before 10 min. The fluorescence of cells fixed in formaldehyde is more stable (Fig. 2). Note that the level of fluorescence and the number of cells fluorescing did not fall appreciably in 25 min.; however, after 2 hr. the number of cells fluorescing did fall. Similar results are found with 70% ethanol. Table 3 shows the relative fluorescence of cells stained with a series of Nile Red concentrations. Relative fluorescence became saturated with respect to dye at approximately  $1\text{--}10 \mu\text{g ml}^{-1}$ .

Table 3. Staining of A. coffeaeformis cells with Nile Red.

Concentration of dye ( $\mu\text{g ml}^{-1}$ )	Average fluorescence per cell (arbitrary units)	No. of cells fluorescing (%)
0.01	38.4	13.9
0.10	44.8	28.3
1.00	61.0	95.4
10.0	62.9	92.8

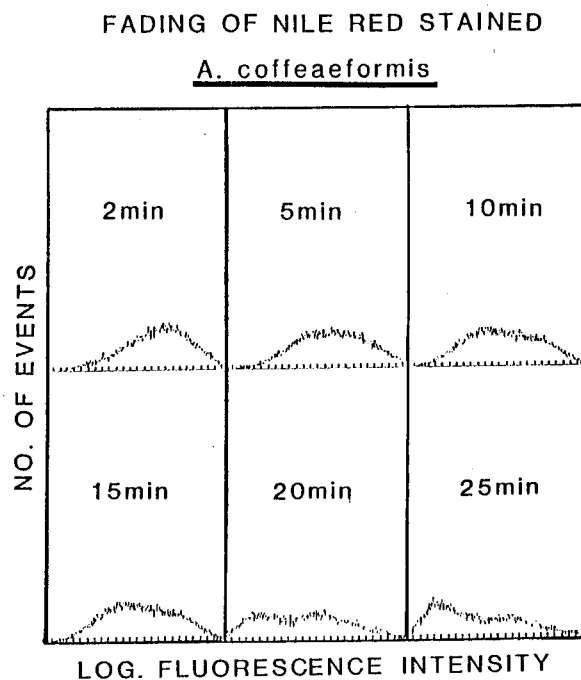


Figure 1. Cells were stained with Nile Red and at the times indicated their fluorescence was measured.

#### NILE RED FLUORESCENCE OF FORMALDEHYDE-FIXED CELLS

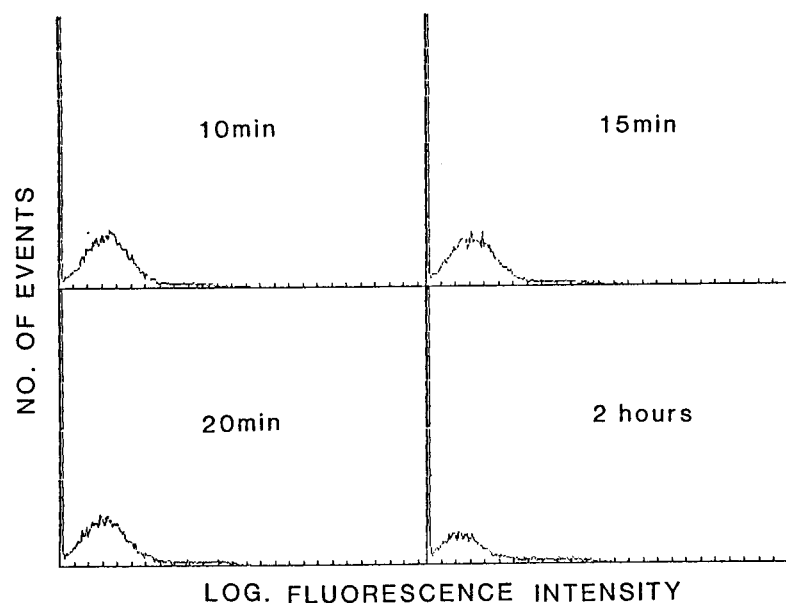


Figure 2. Cells were fixed in 4% formaldehyde, resuspended in minimal medium and stained with Nile Red. They were examined as in Figure 1.

Table 5a. Analysis of A. coffeaeformis cultures as a function of growth.

Expt.	Time hr.	Cells <sup>1</sup> x 10 <sup>-6</sup>	AFDW mg L <sup>-1</sup>	Lipid <sup>2</sup> mg L <sup>-1</sup>	Chl a mg L <sup>-1</sup>	Lipid <sup>3</sup> %	NO <sub>3</sub> mg L <sup>-1</sup>
1	29	0.157	16.4	5.2	0.26	30.1	41.8
	53	0.646	46.2	10.6	0.72	21.4	22.0
	77	0.682	48.8	13.2	1.25	24.5	20.5
	173	1.030	87.4	30.2	1.58	32.7	14.1
2	28	0.155	20.8	4.2	0.31	18.7	61.6
	72	0.842	50.4	11.9	0.78	22.2	35.2
	144	0.722	86.8	24.8	0.89	27.6	5.8
	264	0.829	93.7	36.2	1.21	37.3	3.8

<sup>1</sup>Each experiment was performed using 4 x 500 ml cultures. The entire contents of one flask were used for each series of analyses.

<sup>2</sup>Lipid + chlorophyll.

<sup>3</sup>Calculated on chlorophyll free lipid.

Table 5b. Flow cytometry of A. coffeaeformis cultures as a function of growth.

Expt.	Time hr.	Lipid <sup>1</sup> mg L <sup>-1</sup>	Mean fluorescence per cell	Percentage of population fluorescent
1	29	4.9	134.9	59.9
	53	9.9	119.8	54.6
	77	12.0	116.4	75.4
	173	28.6	137.1	74.1
2	28	3.9	65.3	43.2
	72	11.1	57.6	60.2
	144	23.9	108.6	91.5
	264	35.0	83.8	86.8

<sup>1</sup>Chlorophyll-free lipid.

## Discussion

Growth and Isolation of Strains. Only six of the 18 strains from our culture collection grew well at 35°C. All of these strains had been isolated at 28°C, nevertheless a temperature only 7°C higher was sufficiently stressful as to prevent growth. Thirty-five degrees is close to the highest temperature recorded for diatom growth under defined conditions (42°C, Fairchild and Sheridan, 1974), although living (but not necessarily viable) cells have been seen at 50°C. The organism studied by Fairchild and Sheridan was a species of Achnanthes and was isolated from a hot spring. It seems a reasonable approach therefore to seek thermotolerant species for the biofuels program in thermally active environments.

The organisms described in this report are not extraordinary in their physiological attributes as far as we know, i.e., growth at 1-2 doublings per day and a lipid content of 31-54%. There may be good reasons why algal cells in general may not contain more than approximately 60% of their dry weight as lipid. Lipid is less dense than water. A majority of the internal space in an algal cell is occupied by the chloroplast(s), so only a small fraction of the cell can be taken up by lipid. A second reason may be a direct result of the buoyancy of lipid. Cells with extraordinary amounts of lipid are likely to float, thus be far more susceptible to photo-damage than cells in the water column.

Flow Cytometry and Nile Red Staining. Our results indicate that by using the fluorescence of Nile Red stained cells, it is possible to assess the lipid content of a population of A. coffeaeformis using a sample of about  $10^6$  cells (Fig. 4). The relationship cannot be described quantitatively yet nor do we know the results with other species. Nile Red stains the whole cell but only the non-polar regions of a cell allow it to fluoresce. It does therefore stain specifically the lipid inclusions in diatoms. Besides being a fluorophore of use in flow cytometry, it is also, in our opinion, the stain of choice for microscopy using an epifluorescence system. It is possible to fix cells before staining (but not the reverse), making the method suitable as a field procedure or in food-chain research. Since Nile Red itself does not kill algal cells, it is possible also to sort cells in a flow cytometer for further experimentation, if the fading problem seen after 10 min. in unfixed cells can be solved. Plant protoplasts have been separated on the basis of their fluorescein isothiocyanate staining (Galbraith et al., 1984), so we see no reason why the daughter cells of algal fusion experiments could not be separated from one another. For example, it should be possible to select high lipid producing strains from wild-type cells.

The general ability of the Nile Red method as a non-destructive procedure for lipid analysis is compromised somewhat at present by the expense of the flow cytometer. However, machines that can record fluorescence but cannot sort are far less sophisticated and are thus considerably cheaper. There also remains the possibility of the development of a method for use in the more commonly available spectrophotofluorometer or even a method using a filter instrument.

Microscopic observations showed, in most cases, that once a number (2-4) of Nile Red stained inclusions in a cell was established, their number per cell did not increase with time. Continued lipid synthesis therefore gave rise to droplets of increased size. Since larger cells could contain larger droplets, we investigated the possibility that cell size and lipid content (by fluorescence) were positively correlated. Figures 3a and 3b show that the correlation was small. Initial studies of cell growth and Nile red fluorescence showed (Table 4) that as the cells entered the stationary phase fluorescence per cell, the Nile Red staining per cell, the number of cells staining and the relative fluorescence per ml culture increased appreciably, whereas cell number increased only slightly.

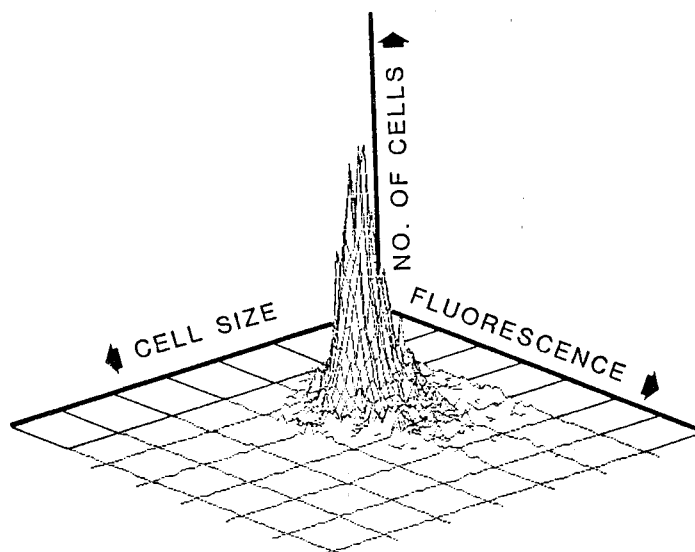
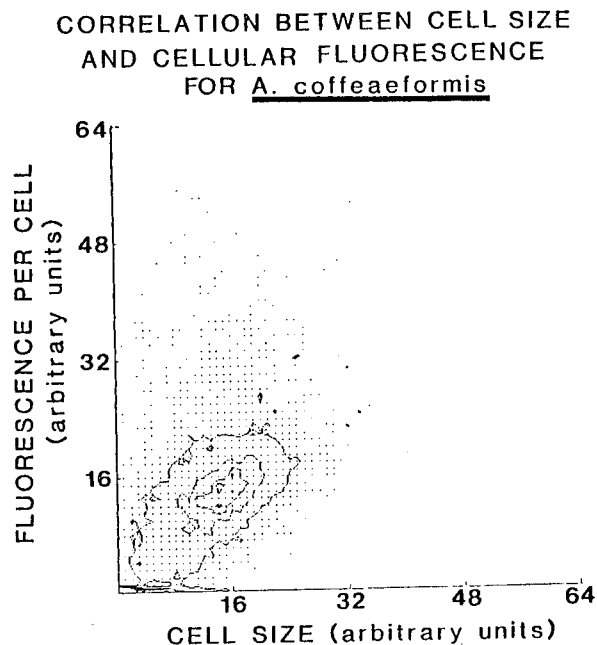
Table 4. Growth of A. coffeaeformis and Nile Red staining.

Culture age (days)	Relative fluorescence per cell ( $\pm$ CV <sup>1</sup> )	Cells fluorescing (%)	Cells/ml $\times 10^{-5}$	Relative fluorescence per ml culture <sup>2</sup> $\times 10^{-6}$
7	79.1 ( $\pm$ 57%)	63	0.729	36.3
8	165.5 ( $\pm$ 29%)	90	0.859	127.9
10	197.6 ( $\pm$ 25%)	92	0.828	150.5
13	183.0 ( $\pm$ 38%)	99	0.726	131.5

<sup>1</sup>CV = coefficient of variation of mean fluorescence per cell from FACS. A. coffeaeformis was grown in ASP-2 medium with 0.25 mM Ca until stationary phase (7 days). Cells were stained with Nile red ( $10 \mu\text{g ml}^{-1}$ ) and examined after 5 min. Data was calculated using the LACEL-DISP4 program.

<sup>2</sup>(Cells  $\text{ml}^{-1} \times 10^{-6}$ )  $\times$  (% cells fluorescing)  $\times$  (fluorescence cell $^{-1}$ )  $\times$  0.01 is proportional to lipid stained  $\text{ml}^{-1}$  culture.

Later studies where several parameters related to culture growth were also measured are presented in Tables 5a and b. In both of the experiments reported, lipid increased as the  $\text{NO}_3$  concentration of the medium fell (Table 5a) and the percentage of cells fluorescing increased. In experiment 2, the inoculum was preincubated in the dark for 40 hr. before use. This caused the parameters related to initial lipid content of the cells to be lower. It should be noted that those cells did not reduce the nitrogen (nitrate) to zero even after 11 days. The results in Table 5 do not give much indication directly of the relationship of cellular lipid content and fluorescence based on Nile Red staining. If the fraction of the population fluorescing  $\times$  the average fluorescence of a cell is compared to the cellular lipid content of the medium, it can be seen from Figure 4 that lipid and fluorescence are highly positively correlated (coefficient = 0.80). If the point at  $35 \mu\text{g ml}^{-1}$  (264 hr.) is not included, the coefficient improves to 0.95 (as drawn).



RELATIONSHIP OF NILE RED FLUORESCENCE  
AND CELL SIZE FOR A. coffeaeformis

Figure 3. The fluorescence of individual Nile Red stained cells was examined as a function of their size. Forward scattering of light by the cells is proportioned to their size. The forward scatter and fluorescence signals are automatically accumulated by the computer interfaced to the FACS. In 3(a) contour lines are drawn where cells have similar fluorescence and forward light-scattering properties. The contours represent 5, 25, 45, 65 and 85 events. Figure 3b is a three-dimensional plot of the same data.

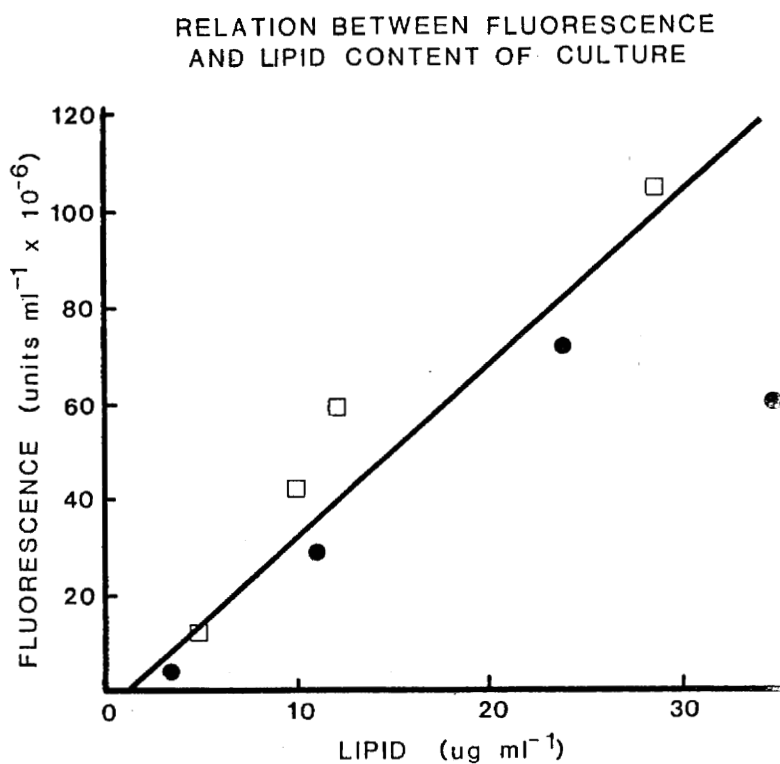


Figure 4. Correlation of Nile Red-induced fluorescence and lipid content of cells of *A. coffeaeformis*. Cells of various lipid contents were obtained from cultures in different stages of growth. The regression line ignores the point at 35  $\mu\text{g ml}^{-1}$  lipid.

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COLLECTION OF HIGH ENERGY YIELDING STRAINS OF  
SALINE MICROALGAE FROM SOUTHWESTERN STATES

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The primary goal of this research is to obtain high performance microalgal species from nature that may serve as the raw material required to make microalgae culture technology a plausible approach to fuel production. The specific objectives are to (1) collect strains of microalgae from a diversity of saline habitats in the desert Southwest, (2) isolate and identify strains that grow well at elevated salinities and light intensities and (3) characterize selected strains for lipid and carbohydrate accumulation. Microalgal and water samples were obtained from 103 sites in Arizona, California, Nevada, New Mexico, Texas, and Utah during 1985 and 1986. Collected waters ranged in temperature from 17.8 to 45.6 C, in specific conductance from 447 to 474,000 uS/cm and from 6.1 to 10.2 in pH. Ionic analysis of the water revealed that the relative anion and cation composition of the surface waters sampled was relatively similar to the artificial SERI Type I and Type II Media used as the standardized media for screening and growth rate experiments. The proportion of magnesium in SERI Type I Media, however, was higher than found in the waters sampled in the Southwest. Microalgae were isolated by either streaking directly onto agar plates of SERI Media or screened on a rotary culture device at elevated temperature and light intensity before streaking onto agar plates. Approximately 1600 individual isolates of microalgae were obtained from surface waters in the Southwest. Of the initial 57 algae screened for growth characteristics, the majority grew best at the lower salinities in both SERI Type I and II Media. Growth rates for 15 isolates exceeded one doubling/day and for five strains exceeded two doublings/day. In nutrient-sufficient batch cultures 14 strains yielded an average lipid concentration of 11.1% of ash-free dry weight. The highest yield was over 26%. Laboratory efforts are now being directed toward growth and chemical characterization of strains within specific taxonomic groupings such as the diatoms. Of 22 diatom strains that have demonstrated rapid growth, one-third of the strains exceeded one doubling/day and several gave positive indications of lipid accumulation. Future research will focus on evaluation of the effects of nitrogen and silicon deficiency and other growing conditions on lipid production in those strains exhibiting rapid growth.

## INTRODUCTION

Collection and screening efforts designed to isolate and characterize lipid accumulating microalgae have been restricted to selected geographic locations. Thomas et al. (1983, 1984, 1985), Barclay (1984) and Barclay et al. (1985) have collected and characterized selected microalgae from inland saline waters in eastern California, western Nevada, Colorado and Utah. Tadros (1985) and Lewin (1985) have isolated organisms from nearshore marine habitats on the Gulf Coast of Alabama, Florida, and Mississippi, and on the coasts of Yucatan and Panama and a number of Caribbean islands.

Our collection efforts center on the Southwestern United States which have been identified as possessing the requisite resources and characteristics such as abundant land, saline water, high incident solar radiation and mild temperatures that would be advantageous in the development of microalgal biomass technology (McIntosh, 1985). Since an abundance of microalgal species exist in the diverse aquatic habitats of the Southwest, they represent a large and varied assemblage of autotrophic microorganisms that have adapted to utilize the growing conditions available. It is logical, therefore, that microalgal species which either dominate or are found in abundance in the region would represent likely candidates for mass culture using natural waters of the area.

This investigation was designed primarily to enlarge the pool of microalgal raw material from which the more desirable strains could be selected, manipulated or ultimately genetic modified. Previous attempts to mass culture microalgae has focused on increased yield through improvements in culture management rather than on improvements resulting from the selection of more desirable strains.

The specific objectives of this investigation were to:

- (1) collect microalgal species/strains from a diversity of saline habitats at numerous locations in the desert Southwest,
- (2) isolate and identify strains that grow well at elevated salinities and light intensities,
- (3) characterize selected strains for lipid accumulation, and
- (4) characterize the gross physico-chemistry of the habitats from which microalgae were collected.

To accomplish these objectives, microalgae were obtained from 88 aquatic habitats of diverse salinities and temperatures in Arizona, California, Nevada,

New Mexico, Texas and Utah and were screened for their ability to grow rapidly in dense culture under elevated salinities and light intensities and to accumulate lipid storage products.

## MATERIALS AND METHODS

Collection Activities. Six separate sampling trips to saline surface waters supporting algal growth in the arid Southwest were conducted from 19 April to 27 July 1985. Before algal samples were taken at a given aquatic site, specific conductance of the water was measured and if it exceeded 2000 uS/cm, a sample was collected. Conductance was not the sole criterion used to determine whether samples were to be taken. Sites exhibiting lower conductivity, yet abundant algal growth were also sampled.

One liter of water was collected for microalgal isolation and media preparation and another liter obtained for subsequent water chemistry analyses. In addition to the planktonic samples, neustonic and benthic samples were collected at sites exhibiting visible algal growth. Neustonic samples were collected in a quiescent area by sweeping a Whatman No. 42 filter across the water surface with a forceps. If abundant algal growth was present around the margin of the pond or stream a benthic sample was also collected. Samples were kept near collection temperatures by placement in an insulated chest in the dark until returning to the laboratory.

Characterization of Collection Sites. At each site several field measurements were recorded. Water temperature and specific conductance were determined with a Yellow Springs Instruments (YSI) Model 33 S-C-T salinity meter. Conductance was corrected to 25°C (Wetzel and Likens, 1979). Water depth was recorded at the point of sample collection. Hydrogen ion concentrations were measured with a portable Orion Model 407A pH meter.

At all sites where planktonic samples were collected, water was obtained for determination of ionic composition (Ca, Mg, Na, K, OH, CO<sub>3</sub>, HCO<sub>3</sub>, SO<sub>4</sub>, Cl) and total filterable residue (TFR). Water samples were collected by bottle immersion below the surface, placed on ice and kept in the dark until processed. Following filtration of the water through pre-rinsed Whatman GF/C filters (1.2 um pore size), anionic composition and TFR were determined. Alkalinity was measured by titration to phenolphthalein and bromcresol green-methyl red end points (APHA et al., 1985), chloride by mercuric nitrate titration (USEPA, 1979) and sulfate by the turbidimetric method (APHA et al., 1985) in 2.5 cm cuvettes with a Coleman Model 54 Spectrophotometer. Total filterable residue was determined gravimetrically after drying at 105°C to a constant weight (APHA et al., 1985). Cationic concentrations were measured after membrane filtration (0.45 um pore size) and nitric acid preservation of samples by direct aspiration atomic absorption spectroscopy with a Perkin-Elmer Model 403 (USEPA, 1979).

Sample Preservation and Enrichment. Planktonic samples were divided into four aliquots to isolate and characterize the microalgae. A portion was preserved with Lugol's solution to provide a record of the taxonomic diversity for each site and to give an indication of total cell numbers. An aliquot of the sample

was enriched with nutrients and maintained as a living collection. All nutrient additions were similar to Guillard's F1 Medium (Guillard and Ryther, 1962) without thiamine, biotin, or B<sub>12</sub>. Samples were enriched to: NaNO<sub>3</sub>, 146.9 mg/l; NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 9.794 mg/l; Na<sub>2</sub>SiO<sub>3</sub>·9H<sub>2</sub>O, 9.794 mg/l; Na<sub>2</sub>EDTA, 8.541 mg/l; FeCl<sub>2</sub>·6H<sub>2</sub>O, 6.170 mg/l; CuSO<sub>4</sub>·5H<sub>2</sub>O, 19.59 ug/l; ZnSO<sub>4</sub>·7H<sub>2</sub>O, 43.10 ug/l; CoCl<sub>2</sub>·6H<sub>2</sub>O, 19.59 ug/l; MnCl<sub>2</sub>·4H<sub>2</sub>O, 35.26 ug/l; and NaMoO<sub>4</sub>·2H<sub>2</sub>O, 12.73 ug/l.

Screening and Isolation. Two approaches were used for preliminary screening and isolation. In one approach samples were streaked directly onto 1.5% agar plates with conductivities similar to that recorded in the field. Plates were made with filter sterilized (0.22 um pore size) Solar Energy Research Institute (SERI) Type I or II Media (Barclay et al., 1985), seawater, or natural site water, all enriched with nutrients. Isolated colonies were removed and placed into test tubes (16 x 150 mm) containing 2 ml of liquid medium of the type used to prepare the plates. The pH of SERI Media or seawater was not adjusted since small acid or base additions resulted in precipitation. Average pH of the media of 10, 40 and 70 mS/cm conductance was: SERI Type I(10), 7.78; SERI Type II(10), 8.56; SERI Type I(40), 7.34; SERI Type II(40), 8.80; SERI Type I(70), 7.25; SERI Type II(70), 8.80; seawater, 7.79. In the second approach samples were enriched and placed in large culture tubes (25 x 150 mm) on a rotary culture device similar to that described by Barclay et al. (1985). The cultures were exposed to the following conditions: constant temperature of 25°C, irradiance of 1500 uE/m<sup>2</sup>/sec and 12 h light 12 h dark (12L:12D) photoperiod. Light intensity was measured with a LiCor Model LI-185 Meter and LI-192S Quantum Sensor. The rotary culture device was placed inside an environmental chamber (National Appliance Co., Portland, OR) to maintain a constant temperature which was continuously recorded with a Model 594 hygro-thermograph (Bendix Corp., Baltimore, MD). Samples were placed in the rotary device for 7 to 19 days and then samples streaked and isolated as above.

Sediment and filters containing neustonic samples were placed into tubes containing SERI Type I or II Media enriched with nutrients. After visible growth occurred the sample was streaked onto agar plates and algal clones isolated.

Microalgal samples generated from the two isolation approaches were examined microscopically. If samples were not unialgal they were restreaked onto agar plates and checked again. Unialgal cultures were inoculated onto agar slants for culture maintenance. Unialgal cultures (10 ml) samples were precultured at 25°C in enriched SERI Type I and Type II Media of 40 mS/cm conductance, 200 uE/m<sup>2</sup>/sec irradiance, and a 12L:12D cycle. Once significant cell numbers were achieved and while the culture was in logarithmic growth, samples were inoculated (0.5 ml) into culture tubes (25 ml) containing enriched SERI Type I and II Media at 10, 40 and 70 mS/cm and enriched seawater, and placed in the rotary culture device. Initial growth rate measurements were made on cultures grown under the following conditions: 30°C, 500 uE/m<sup>2</sup>/sec and 12L:12D cycle. Rotary culture device tubes were inserted directly into a Coleman Model 54 spectrophotometer and optical density values recorded at 750 nm (2.2 cm path length) for 10 days. Values were transformed to logarithm base 2 and growth rates calculated from the slope of a linear regression of time (total light and dark period) and optical density (Sorokin, 1973).

Lipid Evaluation. Oil Red O (CI 26125) stain was used to evaluate the presence of intracellular lipids in the strains of microalgae. Samples were cultured in nutrient sufficient or deficient SERI Type I or II media under conditions of 25°C, 200 uE/m<sup>2</sup>/sec, and a 12L:12D cycle. Once a dense culture was produced cells were stained and rated from zero, for no microscopically visible lipid droplets, to three pluses for droplets occupying one-half or more of the cell volume. Cells were washed in distilled water and fixed by passing a microscope slide, containing a drop of sample, through a flame to evaporate the liquid. While the slide was hot, Oil Red O (750 mg/100 ml dimethyl sulfoxide) was added and allowed to remain for about 30 sec. Cells treated with Oil Red O were washed and destained with 60% isopropanol. Wet mounts of cells were prepared and viewed with moderate light at 1000x magnification.

## RESULTS AND DISCUSSION

Characterization of Collection Sites. Samples of natural water containing microalgae were obtained from 88 aquatic habitats in the Southwest (Fig. 1) representing a broad range of conductivities and temperatures (Fig. 2). Twenty samples were from natural waters with temperatures of 30°C or greater. Another 19 sites had water temperatures between 28 and 29.9°C at the time of collection. Mean water temperature for all the samples was 26.6°C, ranging from 17.8°C in the Salt River Canyon (Site no. SRC-3) to 39.0°C at LaVerkin Springs (LVS-3) (Table 1). Specific conductance ranged from 447 uS/cm in the Salt River Canyon of Arizona (SRC-11) to 474,240 uS/cm in Salt Lake in New Mexico (SL-1) and averaged 25,148 uS/cm. Approximately one-third of the habitats had a conductance greater than 10,000 uS/cm. Specific conductivity was measured at 74 additional sites but was either too low (< 2000 uS/cm) or the extremely turbid nature of the water due to suspended particulates precluded sample collection. Measurements of specific conductance in the field were used to match the sample to the appropriate SERI Media during initial screening and isolation. Other field parameters are summarized in Table 1. Water depths ranged from 1 cm at Wilcox Playa (WP-1/2) to 76 cm in the Buckeye Irrigation District (B-4). A seep in the Salt River Canyon (SRC-3) exhibited the pH low of 6.1 with a high pH of 10.2 recorded at Wilcox Playa (WP-1).

Ionic composition of waters is summarized in Table 1. In the calculation of relative anionic composition, alkalinity was calculated as the sum of OH, CO<sub>3</sub>, and HCO<sub>3</sub> ions. Trilinear plots of relative anionic and cationic compositions of the various waters is given in Figures 3 and 4. From the anionic plot (Fig. 3) it is apparent that the waters from the Southwest were dominated by Cl. Exceptions include several sites in the Pecos River Valley of Texas, which had high proportions of SO<sub>4</sub> and samples collected from central Arizona which exhibited high alkalinities. Elevated proportions of Na + K were apparent in the cationic plot (Fig. 4). A number of samples (16 of 88) contained a high relative Ca concentration with all samples noticeably deficient in Mg.

These results are generally similar to those obtained by Thomas et al. (1984) for saline waters sampled in California and Nevada. Based on an average of relative anionic compositions for 23 sites sampled, a higher proportion (6 of 23) of their sample sites contained water high in alkalinity. Analyses

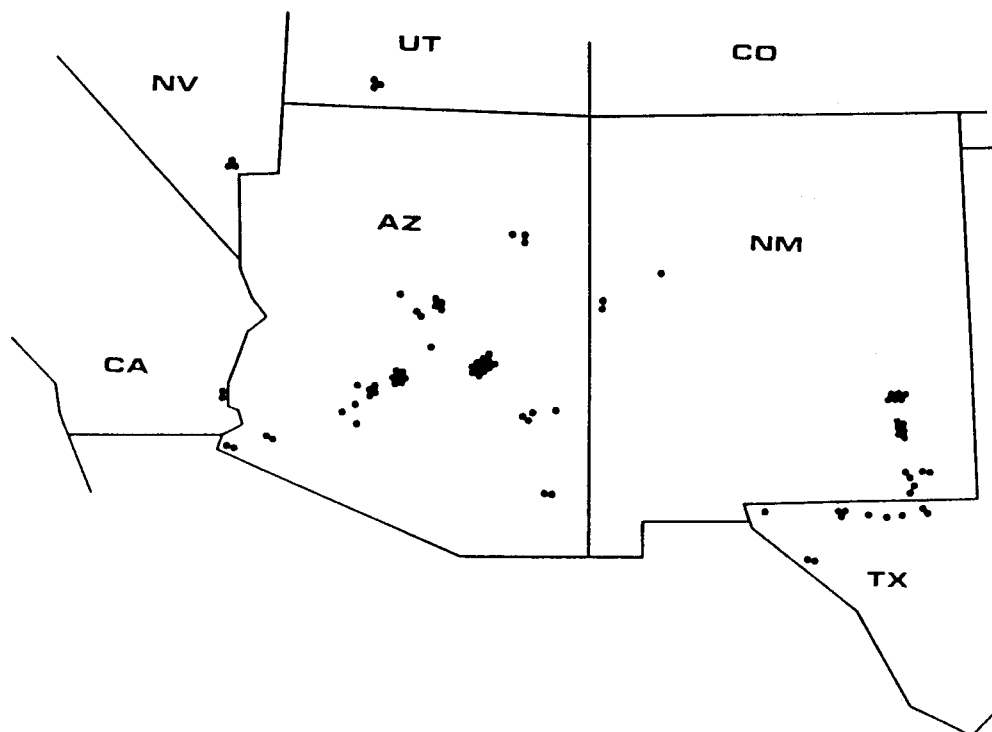


Figure 1. Approximate sampling locations for microalgae in the desert Southwest.

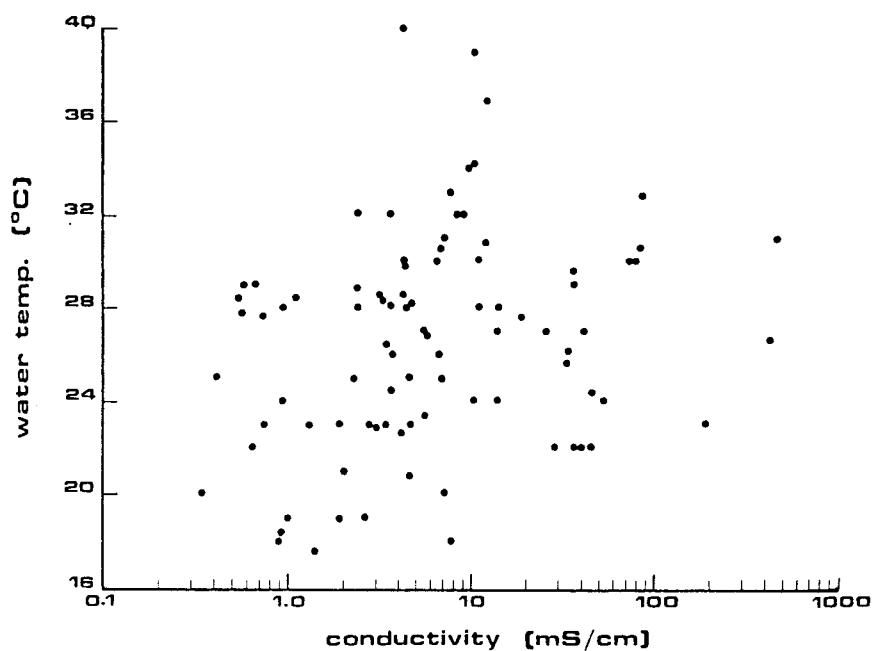


Figure 2. Water temperature and specific conductance of sampling sites for microalgae.

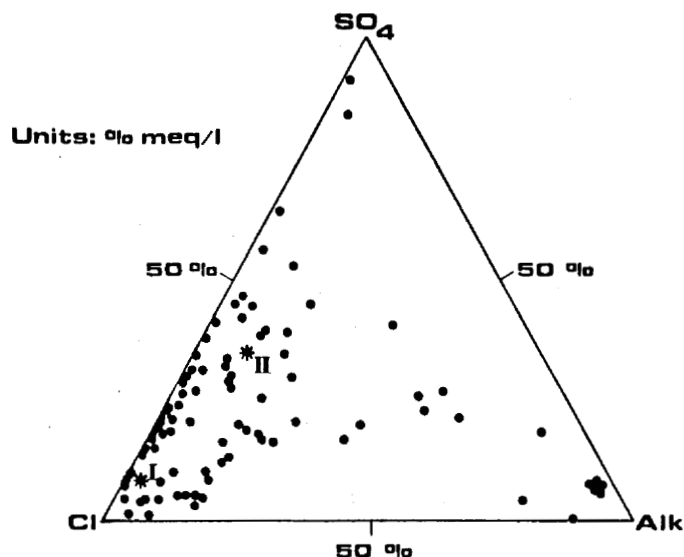


Figure 3. Relative anion composition for waters sampled for microalgae. Each dot represents a sampling site. Asterisks indicate relative anion composition of SERI Type I and II Media.

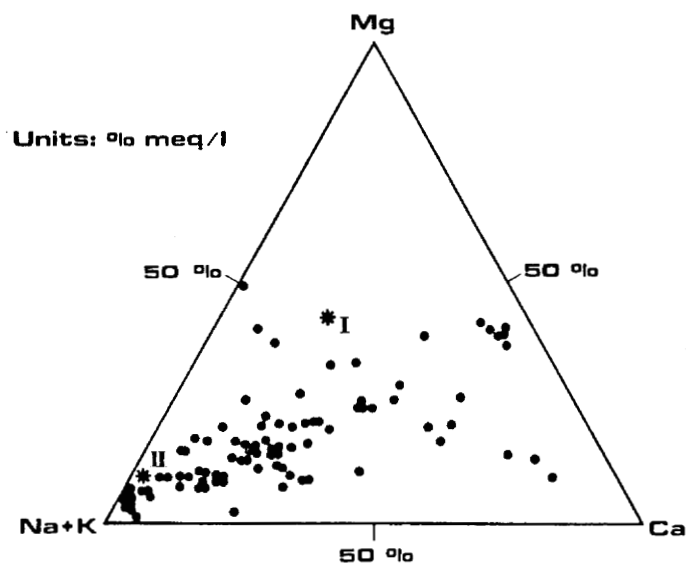


Figure 4. Relative cation composition for waters sampled for microalgae. Each dot represents a sampling site. Asterisks indicate relative cation composition of SERI Type I and II Media.

of cationic compositions for these waters yielded a very dense cluster in the Na + K apex of a trilinear plot. The Salton Sea (Imperial-Riverside Counties, California), in April 1983 contained the lowest proportion of Na + K at 73.9%.

Most waters of the arid southwest are controlled by evaporation-precipitation processes (Gibbs, 1970) resulting in high Na and Cl levels. Proportions of these ions in saline waters can be modified by temperature, precipitation, evaporation, basin sediments, nature of influent waters, lithology of the drainage basin, and biotic effects (Cole, 1968). Results from our work and that of Thomas et al. (1984) corroborate the expected high levels of Na and Cl in southwestern surface waters. When field data is compared to the SERI artificial media good agreement with the anionic composition is apparent (Fig. 3). Most of the sites had a relative anionic composition that is distributed evenly between Type I and II Media with some samples having a proportionally higher alkalinity. The relative cationic composition of a large number of sites sampled was similar to SERI Type II Medium (Fig. 4). On the other hand, SERI Type I Medium with its high proportion of Mg (38.1 to 45.6% meq/l) causes this medium formulation to appear as an outlier on the trilinear plot. Most of our natural samples contained only about 17% meq Mg/l, whereas the samples reported by Thomas et al. (1984) had only about 5% meq Mg/l. In extreme stages of concentration Mg salts can predominate over less soluble Na compounds. Surface waters of this type, however, are uncommon in the Southwest.

Table 1. Physical and chemical characteristics of collection sites for microalgae.

	Mean	Min	Max	sd	n
Water Temp (C)	26.6	17.8	39.0	4.4	87
Depth (cm)	25	1	76	19	69
Conductance (uS/cm)	25,148	447	474,240	70,404	88
TDS (mg/l)	23,730	304	506,166	69,771	79
pH (SU)	7.8	6.1	10.2	0.8	87
Ca (meq/l)	19.33	1.06	75.85	19.81	86
Mg (meq/l)	57.78	0.72	2822.55	303.45	86
Na (meq/l)	205.78	0.42	4263	582.49	86
K (meq/l)	2.24	0.02	45.26	5.86	86
Sum (meq/l)	285.09	4.97	5600.67	802.09	86
OH (meq/l)	0.05	0	4.94	0.52	86
CO <sub>3</sub> (meq/l)	0.23	0	2.73	0.45	86
HCO <sub>3</sub> (meq/l)	5.29	0	36.47	6.73	86
SO <sub>4</sub> (meq/l)	51.48	0.01	1151.39	138.07	86
Cl (meq/l)	262.79	0.24	5388.59	814.05	86
Sum (meq/l)	319.85	5.24	6542.48	941.29	86



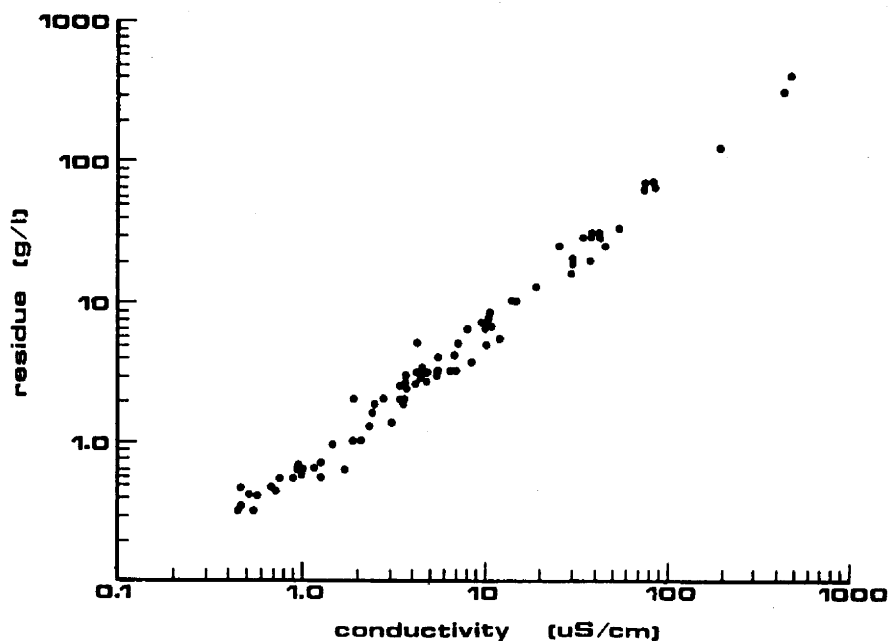


Figure 5. Relationship between specific conductance and total filterable residue in the waters sampled for microalgae

A compilation of ionic water chemistry and its potential correlation with promising microalgae might be used later to predict water conditions that favor algal species that are able to thrive in mass culture in the Southwest and subsequently produce desirable products. Differences in algal floras between lakes are frequently correlated with ionic characteristics of the water. Moss (1973) reported that pH and inorganic carbon concentrations in five southern Michigan lakes were important in explaining differences in algal composition. A positive correlation between Ca and chlorophyll-a concentrations was reported by Bierhuizen and Prepas (1985) in saline lakes of Alberta and Barica (1978) found that high salinity decreased maximum summer chlorophyll-a levels. Data available on ionic composition can therefore be used to refine artificial media formulations for future culture work.

Specific conductance proved to be a good predictor of total filterable residue (TFR) in the waters sampled (Fig. 5). Total filterable residue concentrations determined by evaporation averaged 23,730 mg/l (range 304 to 506,166 mg/l) and can be related to conductivity by the following linear regression equation:

$$\log (\text{TFR}) = 1.0255(\log \text{SC}) - 0.2789$$

$$r^2 = 0.9487, n = 88.$$

As an analytical check TFR was calculated as the sum of dominate ions (Ca, Mg, Na, K, OH, CO<sub>3</sub>, HCO<sub>3</sub>, SO<sub>4</sub> and Cl) and compared to evaporative TFR. Ratios of evaporative to summation TFR were close to unity with only a few aberrations. Divergent values might have resulted from occluded water remaining in the dishes after drying at 105°C. Depth of water at the sample site was recorded but this parameter does not appear to contain much predictive value (cf. Barclay et al., 1985).

Collection and Isolation. A total of 151 samples were collected for the isolation of microalgae from which approximately 1400 isolates have been cultured. Most of the collections (63.6%) were of planktonic material. The large number of sites samples and the dual approaches employed to isolate algae produced a very large quantity of isolates. Only a small fraction of these isolates have been screened for growth characteristics and chemical composition. The thrust of our future efforts will be directed toward the methodical examination of this large reservoir of algal samples.

A total of 56 isolates have been tentatively identified to the generic level. To date 18 different genera have been identified, with several represented by multiple strains. Dunaliella, the most halophilic eukaryotic organism known (Brock, 1976) composes the largest portion (15 of 56) of the algae isolated. This genus was collected from six sites with conductivities ranging from 29,700 to 52,900 uS/cm. Blue-green algae were the next most abundant (14 of 56) organisms isolated. Seven strains of Oscillatoria and six strains of Chroococcus were isolated. Many genera of blue-green algae are known to thrive in saline waters (Borowitzka, 1981) with Microcoleus, Oscillatoria, Phormidium, and Spirulina being common filamentous representatives. Nine strains of Cymbella were isolated from sites of generally low conductivity (ranging from 912 to 29,900 uS/cm). The remaining 14 genera are represented by only one isolate of each. An additional 47 unialgal cultures have been obtained but await identification.

Species Screening and Characterization. During the initial set of growth rate experiments three genera were used to evaluate our experimental procedure. Growth rates were determined for Carteria and Pleurochloris from the Arizona State University (ASU) culture collection and Tetraselmis (S/Platy-1) from SERI. Tetraselmis was reported to be a prolific alga and the two strains from the ASU collection were thought to have potential as biomass producers. Tetraselmis grew in all media with a growth rate ranging from 0.231 doublings/day in SERI I(40) Medium to 0.901 doublings/day in SERI II(40) Medium. These rates are slightly lower than those obtained by Laws (1985) who reported about one doubling per day for Tetraselmis grown in laboratory scale experiments. Carteria and Pleurochloris grew in low salinity SERI Media at an average of 0.304 and 0.410 doublings/day, respectively. Pleurochloris grew at a rate 0.717 doublings/day in SERI I(70), the most saline media used during initial growth rate experiments.

A total of 27 growth rate determinations have been completed for algae isolated from the Southwest. Fifteen isolates exhibited growth rates greater than one doubling/day (Table 2). Eremosphaera (ASU 048), Dunaliella (ASU 038), Chroococcus (ASU 075) and Chlorococcum (ASU 132) had growth rates that exceeded

two doublings/day. Eremosphaera showed the highest growth rate of 3.26 doublings/day. Dunaliella (ASU 038), Asterococcus (ASU 061), Chroococcus (ASU 075 and ASU 071) and Chlorococcum (ASU 093 and ASU 132) had growth rates that exceeded one doubling/day over a range of salinities involving more than one type of medium. For example, Dunaliella (ASU 038) grew well in all SERI Type I Media and in seawater. Asterococcus (ASU 061) grew equally as well in SERI Type I and II media at the lower salinity (10 mS/cm) and in seawater. Chroococcus (ASU 075 and ASU 071) grew rapidly in all salinities and media utilized. Chlorococcum (ASU 093 and ASU 132) grew well in both SERI Type I and II media at 10 and 40 mS/cm and in seawater. By growing rapidly under a range of salinities at high light intensities and temperatures, the previously mentioned organisms meet four of the five characteristics considered desirable for potential biomass fuel applications (Barclay et al., 1985).

With respect to the media used in the screening of microalgae for growth potential, there appears to be no major differences in growth rates obtained in SERI Type I and II Media with the exception of Type II at 70 mS/cm. This medium rarely supported significant growth. Only Tetraselmis and Chroococcus (ASU 071) showed appreciable growth in the medium.

Table 2. Growth rates and lipid staining characteristics for the microalgal isolates demonstrating at least one doubling/day.

Sample Site <sup>a</sup>	Conductance (uS/cm)	Genera	Isolate No.	Growth Rate <sup>b</sup> (doub/d)	Lipid Stain Reaction <sup>c</sup>
SC-2	30,160	Dunaliella	ASU 038	2.58 SW	0
RBR-1	10,404	Cymbella	ASU 039	1.11 I(10)	+
SC-1	36,536	Dunaliella	ASU 044	1.40 SW	0
SR (benthic)	--	Eremosphaera	ASU 048	3.26 II(10)	0
SRC-3	1,454	Nannochloris	ASU 060	1.16 II(10)	0
VR-1	1,150	Asterococcus	ASU 061	1.52 I(10)	0
LVS-1	12,180	Chroococcus	ASU 071	1.35 I(40)	+
LVS-1	12,180	Chroococcus	ASU 075	2.31 SW	0
LCC-1	2,808	Chlorococcum	ASU 093	1.72 I(40)	0
SRC-1	901	Chlorococcum	ASU 132	2.09 SW	++
LCC-2	4,732	Botryococcus	ASU 141	1.27 I(10)	+++
LCC-1	2,808	Franceia	ASU 146	1.58 II(10)	+
RSJ-1	955	Gloeocapsa	ASU 149	1.44 I(10)	0
LCC-1	2,808	Botryochloris	ASU 152	1.92 I(10)	0
ZSL-2	195,800	Chlamydomonas	ASU 245	1.64 SW	0

<sup>a</sup> See Sommerfeld and Ellingson, 1986 for specific sample site locations.

<sup>b</sup> Maximum growth rates as doublings/day (doub/d) in SERI Media and seawater (SW).

<sup>c</sup> Qualitative evaluation of cellular lipid; +++ - one-half or more of cell volume occupied by lipid; ++ - between one-quarter and one-half of volume occupied by lipid; + - lipid detectable, but occupies less than one-quarter of cell volume; 0 - no detectable lipid.

Preliminary Lipid Analysis. During nutrient-sufficient growth 15 isolates were stained with Oil Red O (Table 2). Five isolates gave positive results. Two isolates, Chlorococcum (ASU 132) and Botryococcus (ASU 141) exhibited a strong staining reaction for intracellular lipids. Three other strains had detectable lipid aggregations. These cultures, as well as others exhibiting growth rates exceeding one doubling/day, will be further evaluated for lipid accumulation under nutrient deficient conditions.

#### SUMMARY

Microalgal and water samples were obtained from 88 sites in Arizona, California, Nevada, New Mexico, Texas and Utah. Collected waters ranged in temperature from 17.8° to 39°C, in specific conductance from 447 uS/cm to 474,000 uS/cm and from 6.1 to 10.2 in pH. Ionic analysis of the waters revealed that the SERI artificial media are reasonable approximations of southwestern surface waters. The major exception is the level of magnesium present in SERI Type I Medium. Approximately 1,400 individual isolates of microalgae were obtained from surface waters in the Southwest. Of the first 51 unialgal cultures established, 27 have been screened for growth characteristics. The majority grew best at the lower salinities in both SERI Type I and Type II Media. Fifteen isolates exhibited growth rates exceeding one doubling/day. Six strains grew well over a range of salinities in at least two different media. One strain Eremosphaera (ASU 048) exceeded three doublings per day. Of the 15 isolates that exhibited rapid growth rates, five gave a positive staining reaction for lipids.

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# SCREENING AND CHARACTERIZING OLEAGINOUS MICROALGAL SPECIES FROM THE SOUTHEASTERN UNITED STATES

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## ABSTRACT

Two collection trips were made and samples collected from the intertidal region and near islands along coasts of Florida, Alabama, and Mississippi. Seventy-five algal strains were isolated and five fast growers and lipid accumulators were characterized: Navicula (260), Navicula (264), Nitzschia (225), Cylindrotheca (204), and Chlorococcum (183). Three of the diatoms are of particular interest because of their high growth rate, tolerance to temperature and salinity and ability to accumulate high lipids. Navicula (260), and Navicula (264) cultures limited in nitrogen and silicon showed 34.6, 32.4, and 42.5, 46.5% lipid respectively. While, nitrogen-limited cells of Nitzschia (225) exhibited higher lipids (42.6%) than silica-limited cells (32.6%).

## INTRODUCTION

In recent years, interest in microbial lipids has been renewed because of an urgent need for utilization of alternative renewable resources for the production of liquid fuels. Research in the SERI/DOE Aquatic Species Program is focused on the production of fuels from aquatic biomass.

Microalgal species are capable of producing biomass yields containing high percentages of oils (Aaronson, et. al., 1980). Production of lipid rich microalgal biomass represents a potential process for harvesting solar energy. One of the methods by which the energy storage capacity of photosynthesis can be maximized is by controlling the metabolism of the organisms. Tuning the metabolism of algae can lead to enhanced production of energy--rich compounds such as fats and oils. Changes in the supply or consumption of metabolites may have considerable effects on metabolic patterns. The accumulation of energy storage compounds in algae can be induced by manipulating the environmental conditions under which the algae are grown (Shifrin and Chisholm, 1981). Deficiencies of nutrients, such as nitrogen or silicon, (Healey, 1973) can lead to changes in cell composition, specifically diatoms (Bacillariophyceae), e.g. Nitzschia palea (Opute, 1974), Cylotella (Werner, 1966), Navicula (Coombs, et. al., 1967). Physical factors such as light and temperature can stimulate fat accumulation, e.g., Chlorella pyrenoidosa (Spoehr and Milner, 1948) and Nitzschia palea (Opute, 1974). Biochemical and physical factors affect the productivity of biomass (Werner, 1977). Hydrocarbon production in algae can also be maximized by the manipulation of environmental conditions.

The coast of the Gulf of Mexico along Florida (Northwest), Alabama and Mississippi, contains a variety of aquatic habitats. The salt waters of the Gulf of Mexico mix with fresh inland waters to form a wide range of saline waters. Organisms living in the intertidal region of the coast can tolerate wide ranges of salinity. Islands, such as Dauphine, Santa Rose, and St. George, represent sites rich in algal diversity. Most of the oleaginous algal species at SERI have been isolated from the southwestern United States. The purpose of this project was to determine if highly productive, lipid accumulating strains could be isolated from the marine habitats of the southeastern United States.

The specific objectives of the research reported herein were:

- . Collect algal samples from the Gulf of Mexico Coast (Florida, Alabama and Mississippi)
- . Isolate and define the culture conditions of lipid accumulating strains
- . Evaluate the growth rate at 25° and 30°C under high light intensity 100 and 1000  $\mu\text{E m}^{-2} \text{ s}^{-1}$ .
- . Characterize promising species for temperature and salinity optima and ranges and determine nutrient optima (N, Si, or P).

#### MATERIALS AND METHODS

The methods used have been previously described in detail (Tadros, 1986). Field trips were conducted to Florida, Alabama and Mississippi intertidal regions (Figure 1). Algal samples were collected and water characteristics recorded. A multi-step screening process has been designed for the selection of oleaginous species tolerant of high temperature and light intensity (Figure 2). Screening apparatus was designed according to SERI recommendations (Barclay, 1985), and used for preliminary screening. Growth experiments were done in a culture room (Tadros, 1986). Growth media were supplemented with artificial sea salts, at different strengths. Among the principal basic media were Bold basal (Nichols and Bold, 1965); Chu no 10 (Chu, 1942) and "f/2" (Guillard and Rhyther, 1962). Oil accumulating algal species were identified by microscopic examination. The strains were further selected on the basis of growth rate at 100 and 1000  $\mu\text{E m}^{-2} \text{ s}^{-1}$  irradiance and 25 and 30°C. Selected strains were characterized for temperature, salinity, light tolerance, on a gradient plate. They were characterized also for growth on SERI - media and nutrient requirements determined at 25 and 30°C. The growth rates were calculated from the optical density (O.D.) at 750 nm over the five or seven day duration of the experiments. For proximate analysis, batch cultures (800 ml) were treated with different concentrations of the nutrient to be stressed and analyzed for: total dry weight, ash weight, total carbohydrates, total proteins and total lipids. All determinations were run in triplicate.



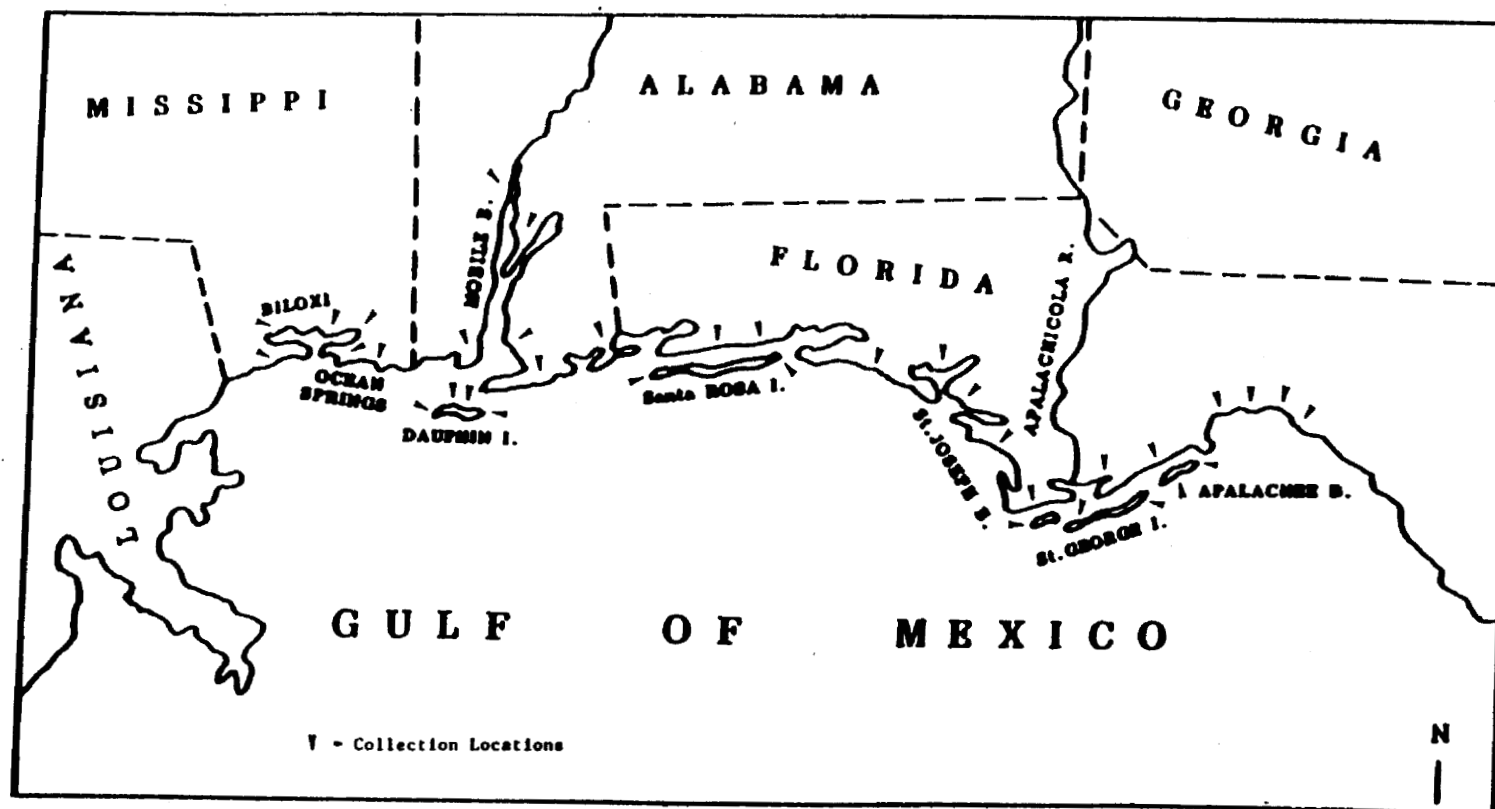


Figure 1. Locations of Sampling Sites in Florida, Alabama, Mississippi.

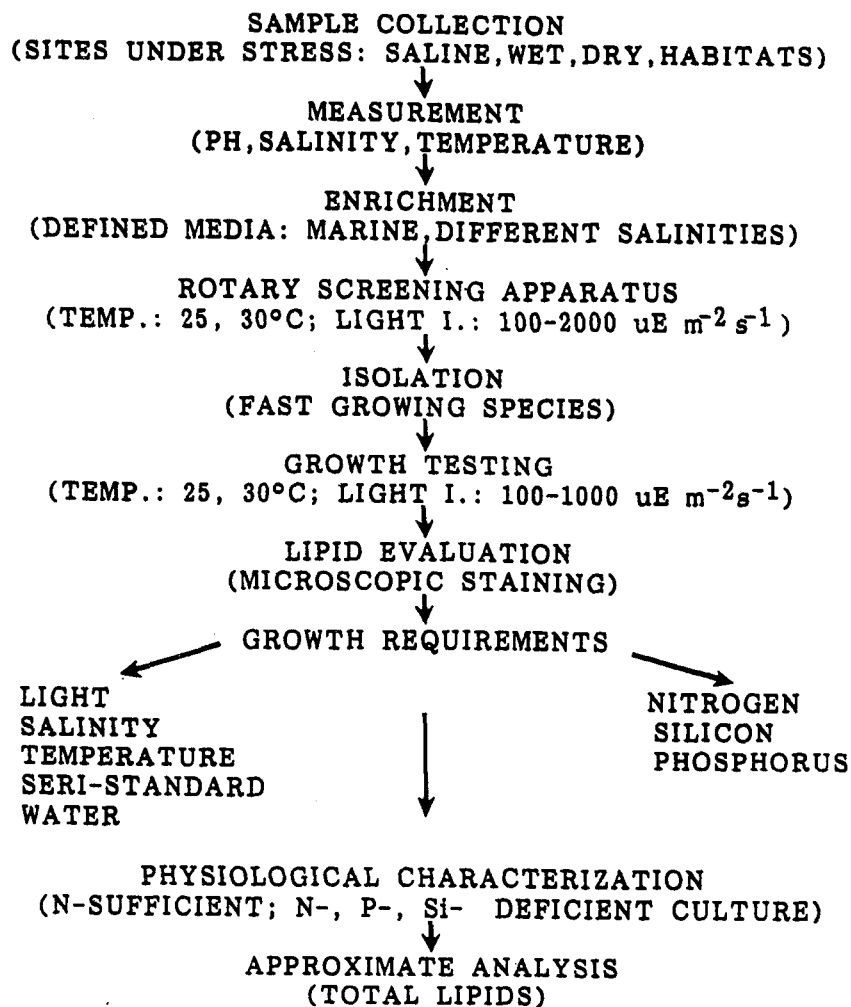


Figure 2. Screening Protocol of Oleaginous Algal Species.

For the chemical analysis, all of the selected species were grown in duplicate batch cultures and in continuous light. The cultures were maintained under the optimum growth conditions specific for each strain. Limitation of nitrate (N), silicate (Si) or phosphate (P), was conducted as follows: Two batches were grown in duplicate until the exponential phase was reached ( $3 \times 10^5$  cells/ml) to avoid light limitation, they were then analyzed. This batch represented the culture sufficient in elements to be stressed later. The exponential phase lasted 3 days in case of diatoms and 5 days for strains of green algae. Batch cells were concentrated and then diluted to the original batch volume but with a new medium limited in one element. The nitrogen limited batch received 0.05 mM nitrate; the silicon limited batch received 0.01 mM silicate; and the phosphate limited batch received 0.01 mM phosphate. When one element was limited, the others were in sufficient concentrations according to the medium characteristic to the species. The cultures were incubated under stressed conditions for four days and then analyzed.

## RESULTS AND DISCUSSIONS

### Sample Collection:

The data from the sample collections are summarized in Table 1.

Water samples were screened on the rotary apparatus for 5-7 days, during which time irradiance was increased from 100 to 2000  $\mu\text{E m}^{-2} \text{s}^{-1}$ , by decreasing the distance between the rotary apparatus and the lamp. Cultures from the rotary apparatus were examined and identified (Table 1). The fast growing species were isolated as unialgal strains and evaluated for lipids microscopically (Figure 3). Growth rate was determined at 25 and 35°C. Results are presented in Table 2.

Based on the ability to accumulate lipids and growth rate at high light intensity and temperature, the following strains were selected for characterization:

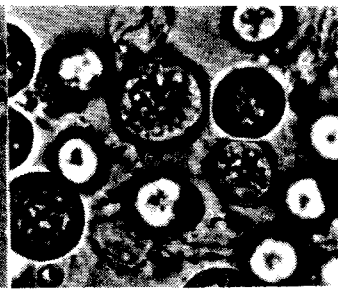
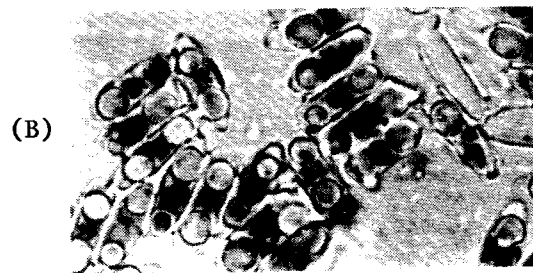
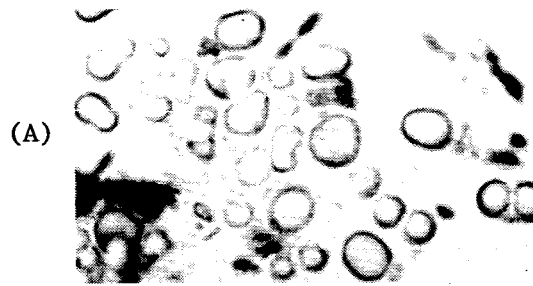
- . Bacillariophyceae (Diatoms):
  - . Navicula (BB-260)
  - . Navicula (BB-264)
  - . Nitzschia (BB-225)
  - . Cylindrotheca (AB-204)
- . Chlorophyceae (Unicellular Green):
  - . Chlorococcum (DI-183)

Table 1. Algal Species Isolated From Various Field Trips And Water Characteristics Of The Collection Sites

Collection Site; no.	Species	Date of Collection	Temperature (°C)	pH.	Salinity (mho cm <sup>-1</sup> )
<b>ALABAMA</b>					
<u>Dauphin Island</u>					
DI-180	Diatoms	May 1983	30	8.0	20
DI-181	Diatoms	May 1983	30	8.0	20
DI-182	Flagellates(green)	May 1983	30	8.0	20
DI-183	Unicellular(green)	May 1983	28	7.8	10
DI-184	Diatoms	May 1983	28	7.8	10
DI-185	Nitzschia Species	May 1983	29	7.5	24
DI-186	Chlorella Species	May 1983	29	7.5	24
DI-187	Cylindrotheca	May 1983	31	6.8	28
DI-188	Halosira	September 1983	28	7.5	23
DI-189	Cymbella, Mastoglia	September 1983	28	7.5	23
DI-190	Diatoms	September 1983	28	7.5	23
DI-191	Flagellates(green)	September 1983	30	6.5	14
DI-192	Flagellates(green)	September 1983	30	6.5	14
<u>Mobile Bay</u>					
MB-194	Flagellates(green)	September 1983	28	7.2	8
MB-195	Chlamydomonas	September 1983	28	7.2	8
MB-196	Flagellates(green)	September 1983	28	7.2	8
MB-197	Blue Green	September 1983	30	7.5	14
MB-198	Blue Green; Diatoms	September 1983	30	7.5	14
<b>FLORIDA</b>					
<u>Apalachee Bay</u>					
AB-200	Amphora	May 1983	28	6.5	35
AB-201	Navicula	May 1983	30	6.8	35
AB-202	Diatoms	May 1983	30	6.8	38
AB-203	Diatoms	May 1983	26	8.2	34
AB-204	Cymbella,				
	Cylindrotheca	May 1983	26	8.2	34
AB-205	Green	September 1983	28	7.8	20
AB-206	Blue Green, Cymbella	September 1983	28	7.8	20
AB-207	Hantzschia, Navicula	September 1983	28	7.8	20

Table 1. Continued

Collection Site; no.	Species	Date of Collection	Temperature (°C)	pH	Salinity (mmho cm <sup>-1</sup> )
<u>St. George Island</u>					
SGI-215	Synedra	May 1985	30	6.8	25
SGI-216	Gyrosigma	May 1985	30	6.8	25
SGI-217	Gyrosigma	May 1985	29	6.7	28
SGI-218	Motile (green)	May 1985	29	6.7	28
SGI-219	Round (green)	May 1985	29	6.7	28
SGI-220	Chlorella, Diatoms	September 1985	28	5.6	22
SGI-221	Scenedesmus	September 1985	28	5.6	22
SGI-222	Mastoglia, Hantzschia	September 1985	28	5.6	22
<u>St. Joseph Bay</u>					
SB-225	Nitzschia, Cymbella	May 1985	30	8	32
SB-226	Diatoms	May 1985	30	8	32
SB-227	Cyclotella	May 1985	28	7.5	28
SB-228	Amphipropra	May 1985	28	7.5	28
SB-229	Flagellate	May 1985	29	6.5	25
SB-230	Flagellate	May 1985	29	6.5	25
SB-231	Amphara	September 1985	27	7.2	30
SB-232	Motile (green)	September 1985	27	7.2	30
SB-233	Motile (green)	September 1985	27	7.2	30
<u>Santa Rosa Island</u>					
SRI-240	Coccolid (green)	May 1985	29	7.7	18
SRI-241	Diatomella	May 1985	29	7.7	18
SRI-242	Diatomella, Diploneis	May 1985	29	7.7	18
SRI-243	Blue Green	May 1985	32	8.0	11
SRI-244	Blue Green	May 1985	32	8.0	11
SRI-245	Blue Green	May 1985	32	8.0	11
SRI-246	Navicula Species	May 1985	32	8.0	11
SRI-247	Nitzschia Species	May 1985	32	8.0	11
SRI-247	Flagellates	May 1985	32	8.0	11
MISSISSIPPI					
<u>Ocean Springs</u>					
OS-250	Navicula	May 1985	30	6.8	32
OS-251	Amphiprora	May 1985	30	6.8	32
OS-252	Cymbella	May 1985	32	7.6	35
OS-253	Navicula	May 1985	32	7.6	35
OS-254	Surirella	May 1985	27	7.2	25
<u>Ocean Springs</u>					
OS-255	Melosira	May 1985	27	7.2	25
OS-256	Denticula	September 1985	26	6.4	16
OS-257	Cymbella, Cylindrotheca	September 1985	26	6.4	16
OS-258	Staurenius	September 1985	26	6.4	15
<u>Biloxi-Bridge</u>					
BB-260	Navicula, Amphiprora	May 1985	28	7.5	26
BB-261	Diatoms	May 1985	28	7.5	26
BB-262	Diatoms	May 1985	28	7.5	26
BB-263	Cylindrotheca	May 1985	26	7.8	22
BB-264	Denticula, Diatoms	May 1985	26	7.8	22
BB-265	Cymbella	May 1985	26	7.8	22
BB-266	Cylindrotheca	May 1985	26	7.8	22



(C)

(D)

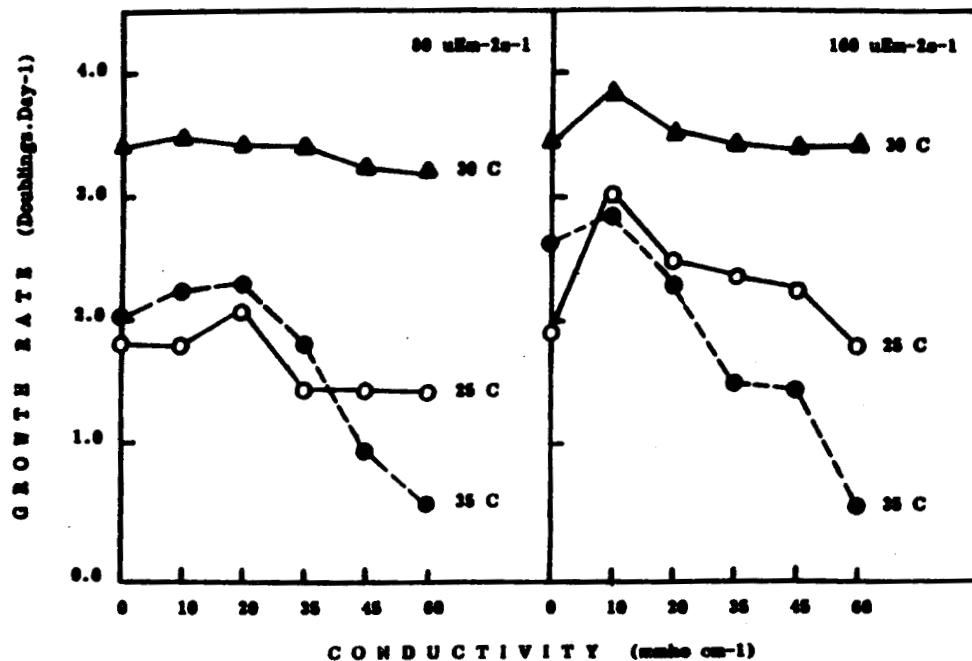
(E)

Figure 3. (A) Navicula sp. 260, Scale: 1 cm = 5 um  
 (B) Navicula sp. 264, Scale: 1 cm = 10 um;  
 Cells Showing Oil Droplets.  
 (C) Nitzschia sp. 225, Scale: 1 cm = 11 um  
 (D) Cylindrotheca 204, Scale: 1 cm = 18 um  
 (E) Chlorococcum sp. 183, Scale: 1 cm = 10 um;  
 Cells Showing Oil Droplets.

Table 2. Most Promising Oil Accumulating Species

Species	Cell Size ( $\mu\text{m}$ )	Lipid Evaluation (staining)	Exponential Growth Rate (Doublings.day <sup>-1</sup> )			
			Temperature			
			(°C)	25	30	
			Light Irradiance ( $\mu\text{E m}^{-2} \text{s}^{-1}$ )			
			100	1000	100	1000
<hr/>						
<b><u>CHRYSOPHYTA:</u></b>						
Cymbella AB-206	4-22	+++	0.62	0.52	0.55	0.42
Amphora AB-200	35-50	+++	0.32	0.15	0.05	0.02
Cymbella OS-252	42-50	++	0.25	0.13	0.08	0.03
Navicula BB-260	3-5	+++++	2.68	1.86	2.76	2.07
Navicula AB-201	15-20	++++	1.67	1.31	0.92	0.45
Navicula BB-264	12-16	+++++	1.95	0.25	2.55	1.44
Cylindrotheca AB-204	80-90	+++++	1.26	0.85	1.96	1.86
Nitzschia AB-207	45-60	+++	1.86	1.35	1.74	0.98
Nitzschia SB-223	18-22	+++	1.54	1.68	1.85	2.10
Nitzschia DI-185	25-30	+++	0.62	0.79	0.86	0.44
Nitzschia SRI-247	20-26	+++	0.48	0.32	0.35	0.25
Rhizosolenium BB-262	20-26	+++	0.35	0.28	0.25	0.13
<b><u>CHLOROPHYTA:</u></b>						
Flagellate DI-182	6-10	++	0.82	0.45	0.05	0.03
Tetraselmis 46 (Dr. Lewin)	5-8	+++	0.72	0.36	0.15	0.12
Chlorococcum DI-183	10-12	+++	2.84	2.46	2.76	2.55
Unicellular(green) AB-206	9-15	++	0.85	1.56	1.24	0.76

# NAVICULA (260)



# NAVICULA (264)

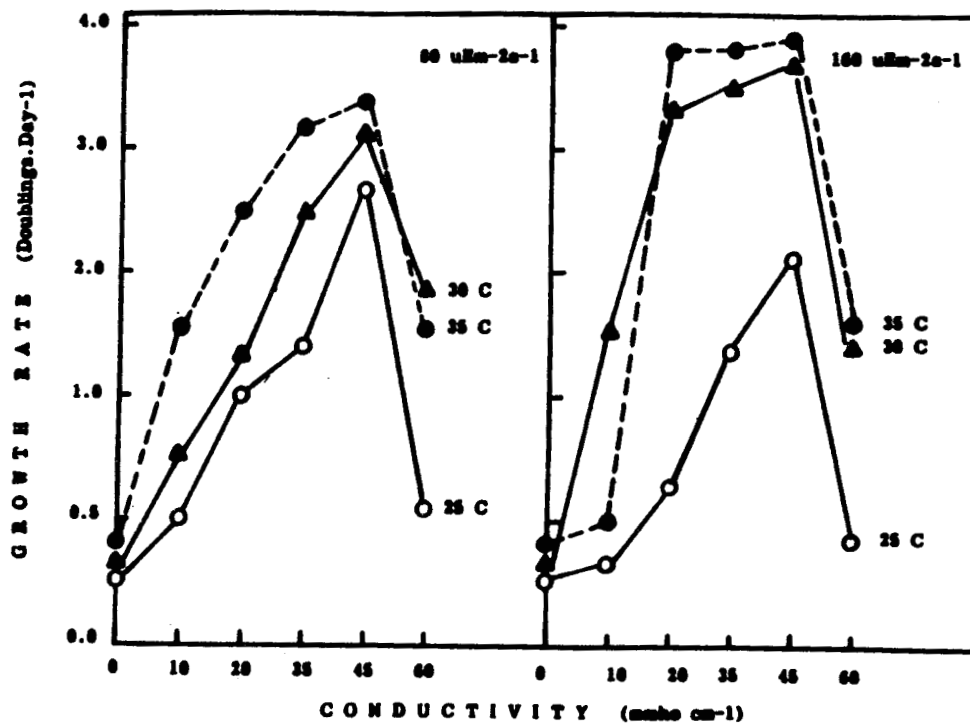
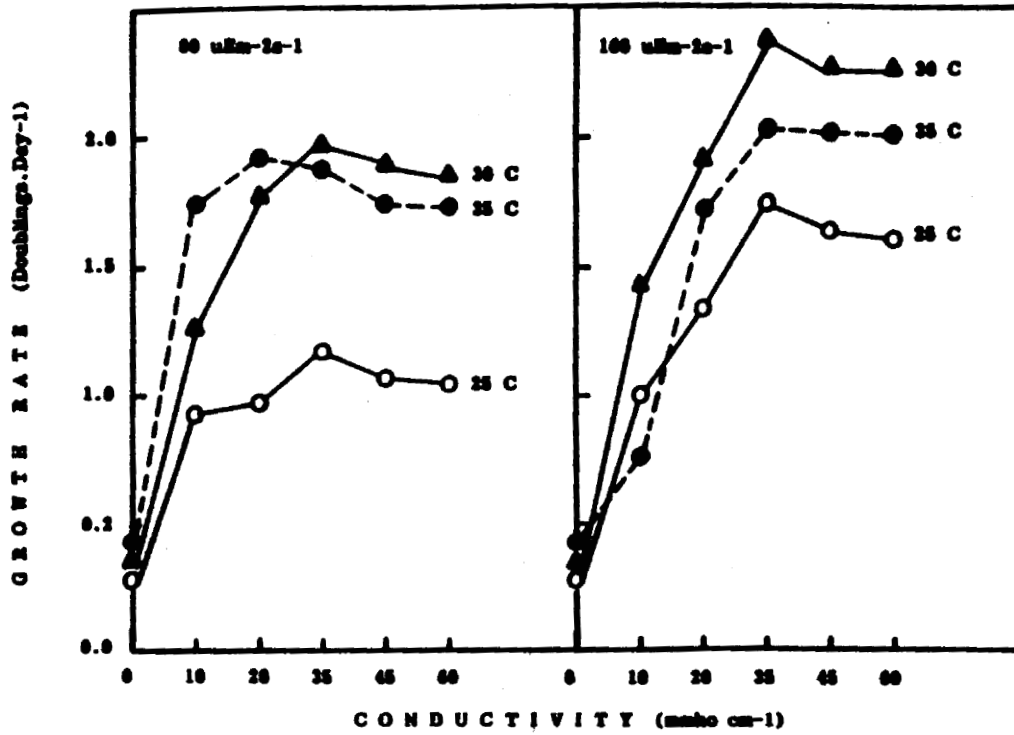


Figure 4. Growth Rate of *Navicula* sp. 260 and *Navicula* sp. 264 as a Function of Temperature, Light Irradiance and Conductivity.



NITZSCHIA (225)



CHLOROCOCCUM (183)

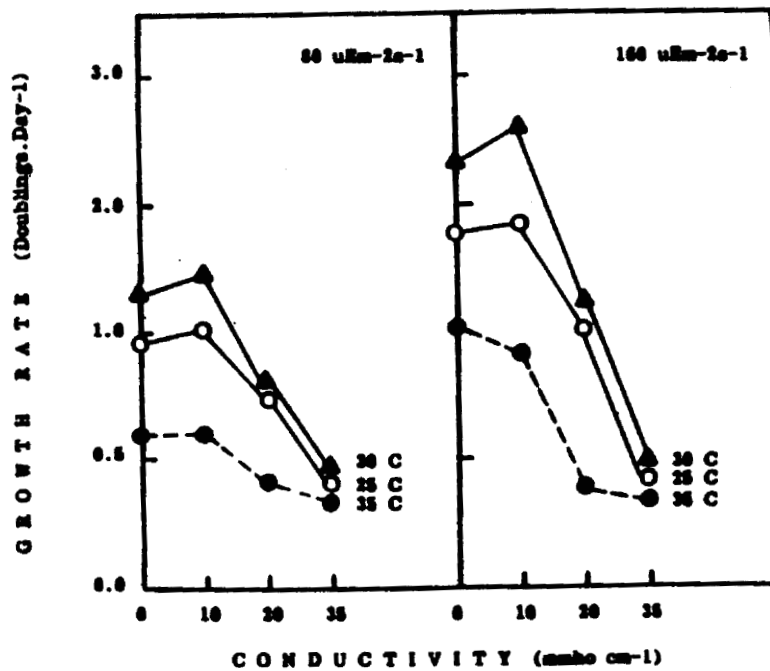


Figure 5. Growth Rate of *Nitzschia* sp. 225 and *Chlorococcum* sp. 183 as a Function of Temperature, Light Irradiance and Conductivity.

## Light, Salinity and Temperature Characterization

Selected strains were tested for growth in different salinities, temperatures, and light intensities combinations. Growth response was quantified as doublings day<sup>-1</sup>. Results are shown in Figures 4 and 5.

### Navicula sp. 260:

Cell Size: 3-5  $\mu\text{m}$

This diatom tolerated wide salinity fluctuations (Figure 4). The growth rate was higher in artificial sea water at lower conductivities than higher ones. The doublings per day reached optimum at 0 and 10 mmho cm<sup>-1</sup>, then declined with irradiation of 160  $\mu\text{E m}^{-2} \text{s}^{-1}$ , favored high growth rate of the diatom. On the other hand, at lower temperatures (15-20°C) cell division completely ceased. Under optimum growth conditions, the growth rate reached 3.3 doublings day<sup>-1</sup>.

### Navicula sp. 264:

Cell Size: 1-16  $\mu\text{m}$

This diatom tolerated increases in salinities up to 45 mmho cm<sup>-1</sup> (Figure 4). High temperature (35°C) and irradiance 160  $\mu\text{E m}^{-2} \text{s}^{-1}$  enhanced growth rate. Optimum growth reached more than 3 doublings day<sup>-1</sup>.

### Nitzschia sp. 225:

Cell Size: 1-16  $\mu\text{m}$

This diatom tolerated high temperatures (30-35°C) and salinities (Figure 5). It did not grow in fresh medium. The growth rate reached more than 2 doublings day<sup>-1</sup> at 20 mmho cm<sup>-1</sup> conductivity.

### Chlorococcum sp. 183:

Cell Size: 10-12  $\mu\text{m}$

The growth rate was 2.8 doublings day<sup>-1</sup> at lower conductivities (0-9 mmho cm<sup>-1</sup>) and decreased towards high conductivities (Figure 5). Higher light intensity enhanced the growth rate. The optimum temperature for growth was 30°C.

It should be mentioned that the growth rate of all selected strains at 15°C and 20°C was insignificant.

## Nutrient Requirements

Selected strains were treated with different concentrations of nitrogen (nitrate, ammonia, urea), silicate and phosphate. Growth response was quantified as doublings day<sup>-1</sup>. Results are shown in Figures 6, 7, 8, and 9.

Navicula sp. 260:

Urea-N enhanced the growth rate of the diatom at 1.2 times that of nitrate-N and ammonia-N treated cultures (Figure 6). The best nitrogen concentrations were 2mM nitrate, 1mM ammonia, and 1mM urea. Silicate enhanced the growth rate of the diatom at concentrations of 0.1-1 mM. However, different concentrations of phosphate did not influence the growth rate of the diatom, appreciable.

Navicula sp. 264:

Cultures containing ammonia-N and nitrate-N exhibited a higher growth than those containing urea-N (Figure 7). Silicate at concentrations of 0.1-1 mM was sufficient for the optimum growth of the diatom.

Nitzschia sp. 225:

Nitrate-N supported the growth of the diatom at a higher rate than that of ammonia-N and urea-N. In the case of silicate, 1-4 mM concentrations improved the growth of the diatom. Only 0.02 mM phosphate was necessary to obtain maximum growth.

Chlorococcum sp. 183:

At concentrations of 10 mM nitrate and 5 mM urea, in the culture, resulted in higher growth rates of the algae. Phosphate concentrations had little effect on growth rate of Chlorococcum. (Figure 9)

From the above results, it is obvious that various species and clones of algae respond differently to culture conditions, whether physical or chemical. Each species has its own optimum growth conditions: salinity, temperature, light irradiance, and nutrient concentration. These results indicate that essentially all compounds tested as nitrogen sources were capable of supporting growth of all the species. However, species responded differently to nutrient concentrations as well as the nutrient source. Diatoms utilize nitrate rather than urea, while the growth of the unicellular green alga was enhanced by urea. This is in agreement with Reimann, et. al., (1963) who reported that Cyclotella cryptica species were unable to grow on urea. Temperature affected salinity and nutrient ranges and optima. Terry (1983) reported that the uptake of nitrogen by Phaeodactylum can also be influenced by temperature. It should be noted that phosphate concentration did not influence the growth of diatoms as much as nitrogen concentration. However, the green alga Chlorococcum required higher phosphate concentration than the other strains. It is evident from the results reported in the salinity and nutrient requirements that each species has its characteristic optimum growth rate under optimum growth conditions. Moreover, the growth rate of smaller species as such Navicula (260), Navicula (264), and Nitzschia (225) were higher than larger species such as Nitzschia (160).

N A V I C U L A (260)

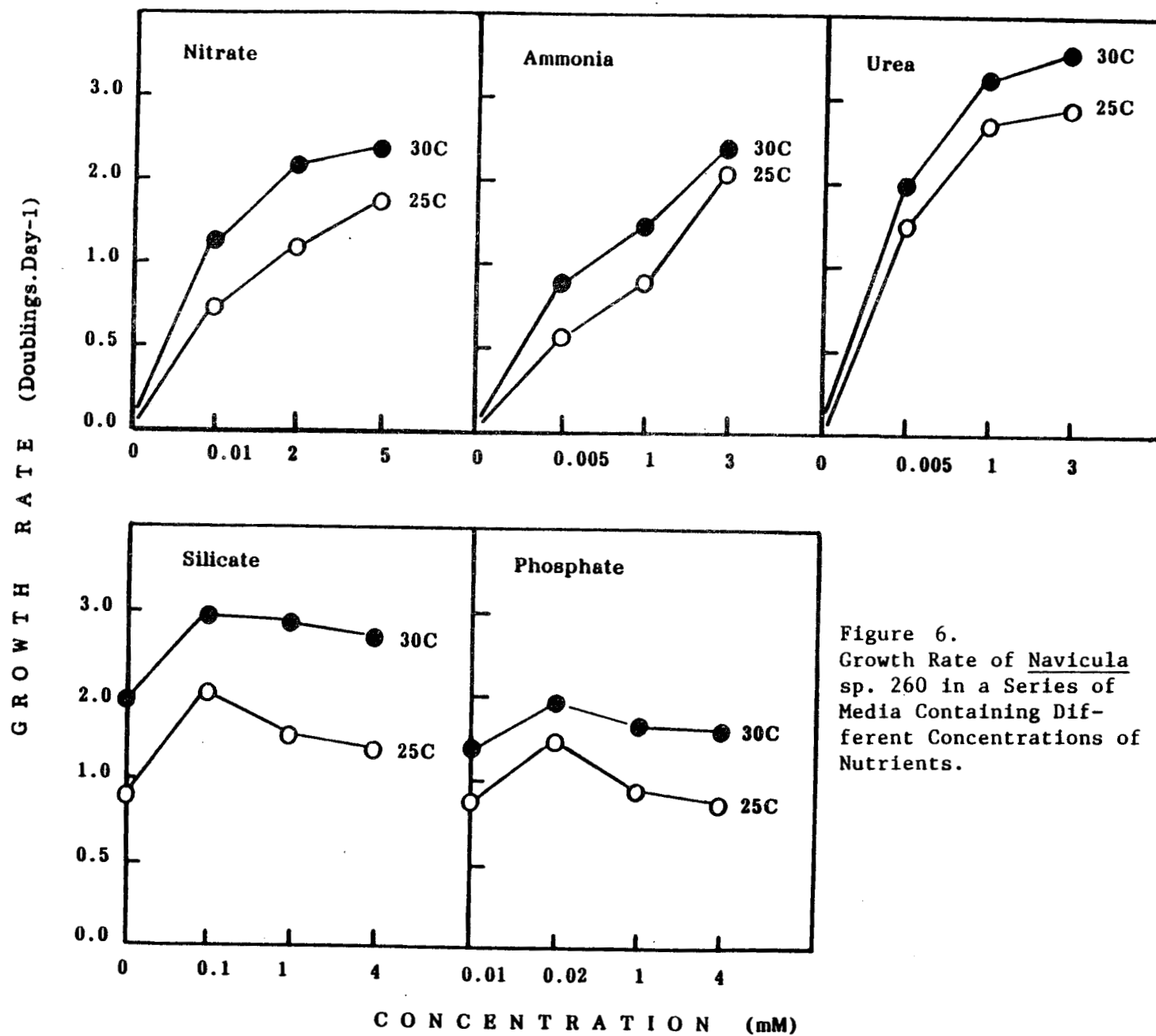


Figure 6.  
Growth Rate of *Navicula*  
sp. 260 in a Series of  
Media Containing Dif-  
ferent Concentrations of  
Nutrients.

## NAVICULA (264)

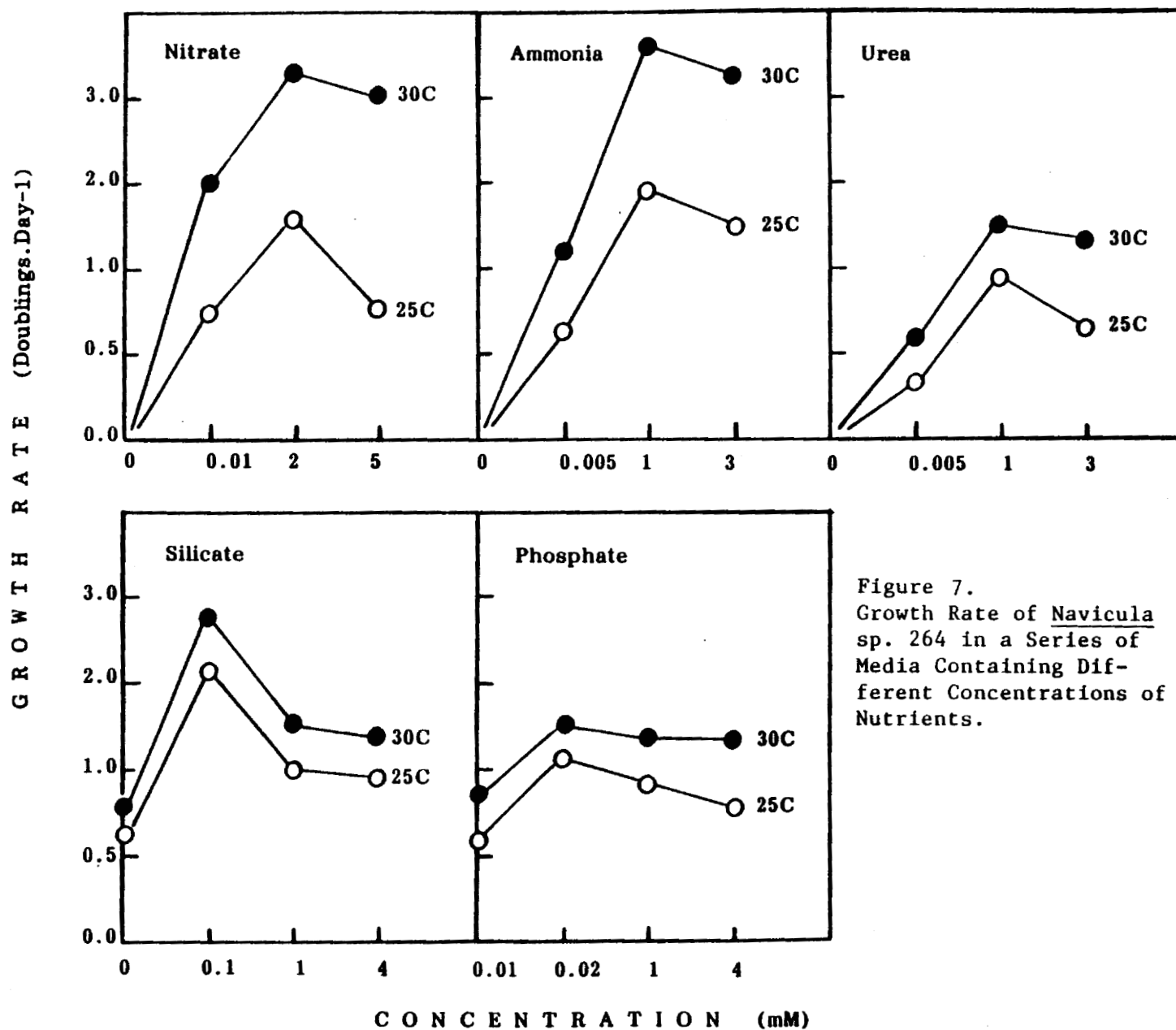


Figure 7.  
Growth Rate of *Navicula*  
sp. 264 in a Series of  
Media Containing Dif-  
ferent Concentrations of  
Nutrients.

## NITZSCHIA (225)

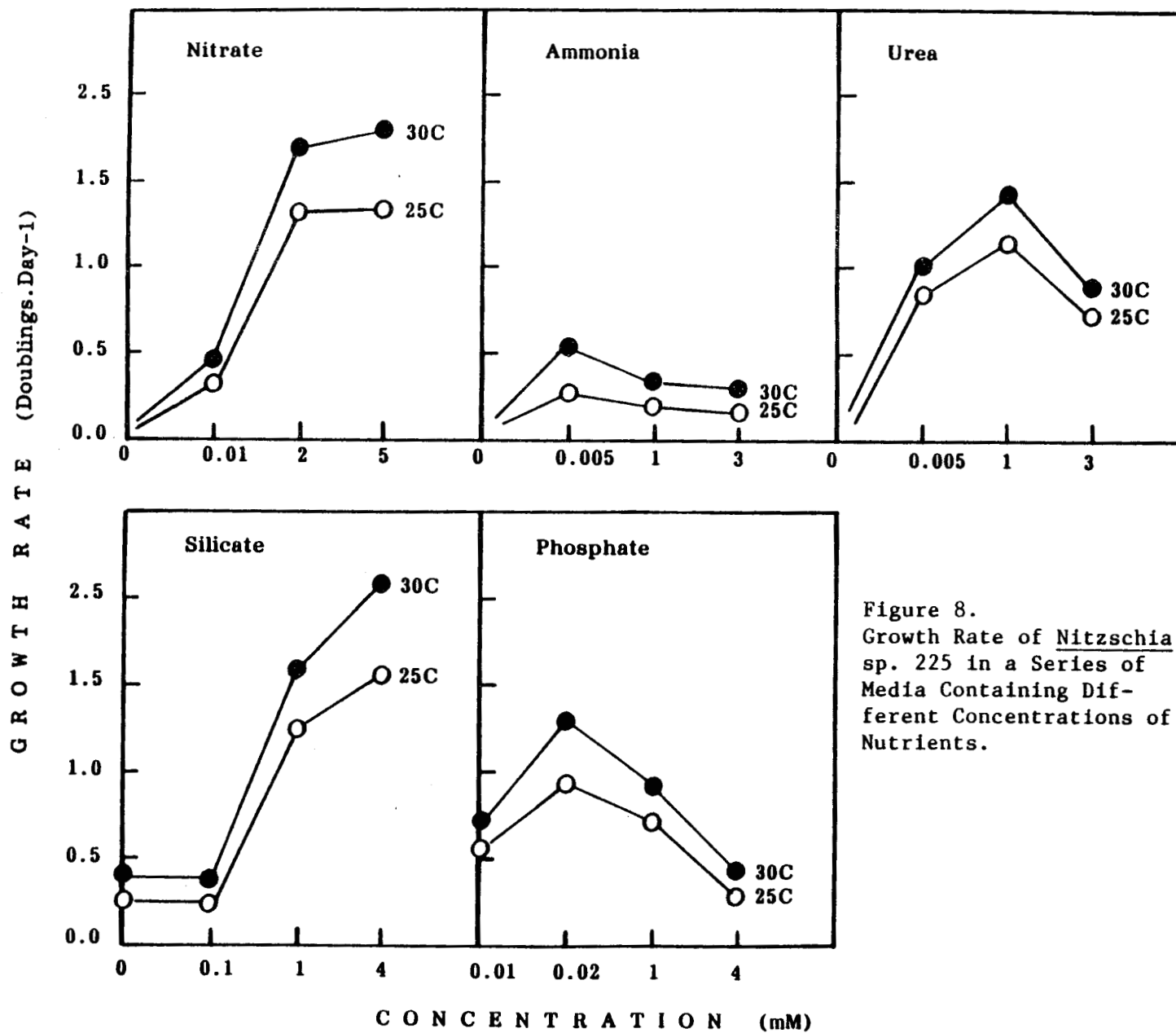


Figure 8.  
Growth Rate of *Nitzschia*  
sp. 225 in a Series of  
Media Containing Dif-  
ferent Concentrations of  
Nutrients.

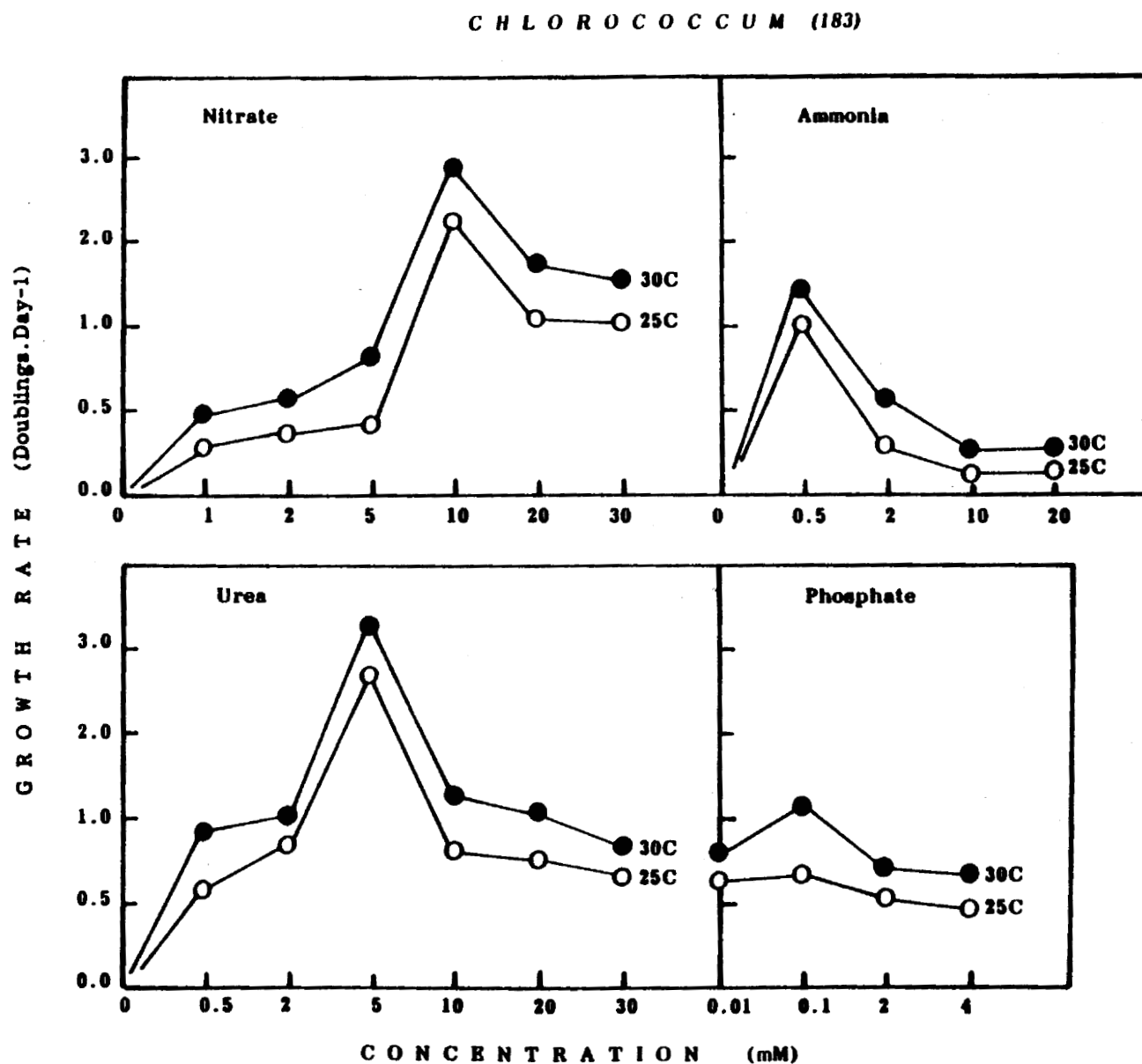


Figure 9. Growth Rate of *Chlorococcum* sp. 183 in a Series of Media Containing Different Concentrations of Nutrients.

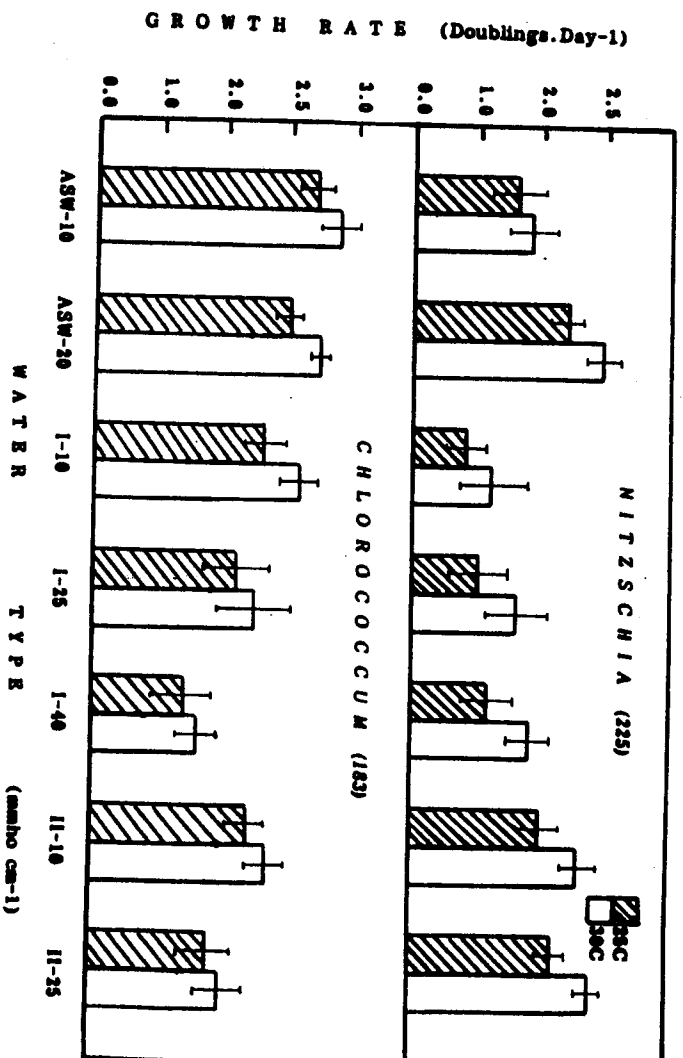
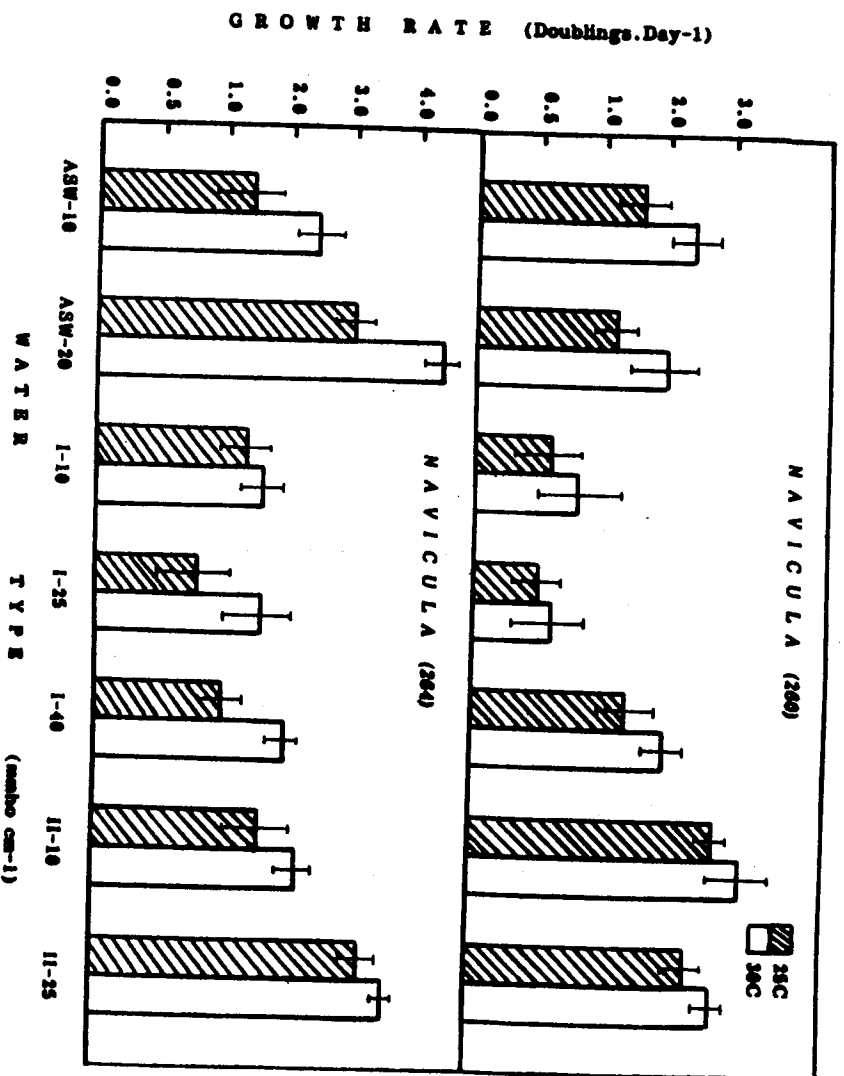


Figure 10. Growth Rate of *Navicula* sp. 260, *Navicula* sp. 264, *Nitzschia* sp. 225 and *Chlorococcum* sp. 183 in Standard Saline Water Types as a function of Temperature and Water Type. Error bars denote  $\pm 1$  s.d.



## SERI Standard Water

The growth of the selected strains was evaluated in SERI Standard Waters. The results are shown in Figure 10. SERI Type II, water supported the growth of all diatoms. SERI Type I water inhibited to some degree the growth of all diatoms. On the other hand, the green unicellular Chlorococcum exhibited good growth in all water types. It was further observed in these experiments, that the effect of water types on the strain is temperature dependent.

## Proximate Chemical Analysis

The results of proximate chemical analysis were expressed on the basis of ash free dry weight and are represented in Table 3 and Figure 11. Navicula 260, and Navicula 264 cultures limited in nitrogen and silicon, showed 34.6, 32.4 and 42.5% lipid respectively. Nitrogen-limited cells of Nitzschia 225 exhibited higher lipids (42.6% lipids) than silica-limited (32.6% lipid) cells. An exceptional diatom, Cylindrotheca 204, under nitrogen-limited culture conditions, accumulated 27.2% lipid and under silica limited conditions 16.3% lipid. Growth requirements for this diatom have not yet been identified. On the other hand, the green cells of Chlorococcum 183 became yellow in color when depleted from nitrogen and accumulated 36.7% lipids.

It can be concluded that the selected strains can be manipulated to grow under different environment conditions and their lipid content maximized by varying nutrient concentrations. In other words, nutrient limitation can have a strong influence on the biochemical composition of algal cells (Fogg, 1953; Healy, 1973; Morris, 1981; Terry et. al. 1985). On the basis of the previous results, Navicula 260, Navicula sp. 164 and Nitzschia sp. 225, will be useful for further studies of biomass for hydrocarbon production.

## ACKNOWLEDGEMENT

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

Table 3. Approximate Cellular Composition of Selected Algal Species

Species	Cell Size (uM)	Cell density (Cells ml <sup>-1</sup> x 10 <sup>8</sup> )	Growth Rate (doublings .day <sup>-1</sup> )	Growth Conditions	% Organic Wt.		
					Protein	Carbohydrate	Lipid
<u>Navicula</u> sp. BB-260	3-5	6	3.3	SW, NE, S1E SW, ND SW, S1D	26.6 14.5 15.3	30.4 22.7 18.4	16.2 34.6 42.5
<u>Navicula</u> sp. BB-264	12-16	2	3.2	SW, NE, S1E SW, ND SW, S1D	21.9 12.6 8.8	35.7 22.5 26.3	21.8 32.4 48.5
<u>Nitzschia</u> sp. SB-225	18-22	2	2.3	SW, NE, S1E SW, ND SW, S1D	32.4 12.8 21.9	28.3 16.4 12.2	17.6 42.6 32.6
<u>Cylindrotheca</u> sp. AB-204	80-90	1.4	4.3	SW, NE, S1E SW, ND SW, S1D	23.3 12.8 22.6	35.8 29.6 42.2	10.5 27.2 16.5
<u>Chlorococcum</u> sp. DI-163	12-16	6	2.9	SW, NE, PE SW, ND SW, PD	26.3 18.4 24.3	42.5 35.6 32.7	13.4 36.7 21.5

SW = Saltwater  
NE = Nitrogen Sufficient  
ND = Nitrogen Deficient

S1E = Silicon Sufficient  
S1D = Silicon Deficient

PE = Phosphorus Sufficient  
PD = Phosphorus Deficient

# PERCENTAGE OF ORGANIC WEIGHT

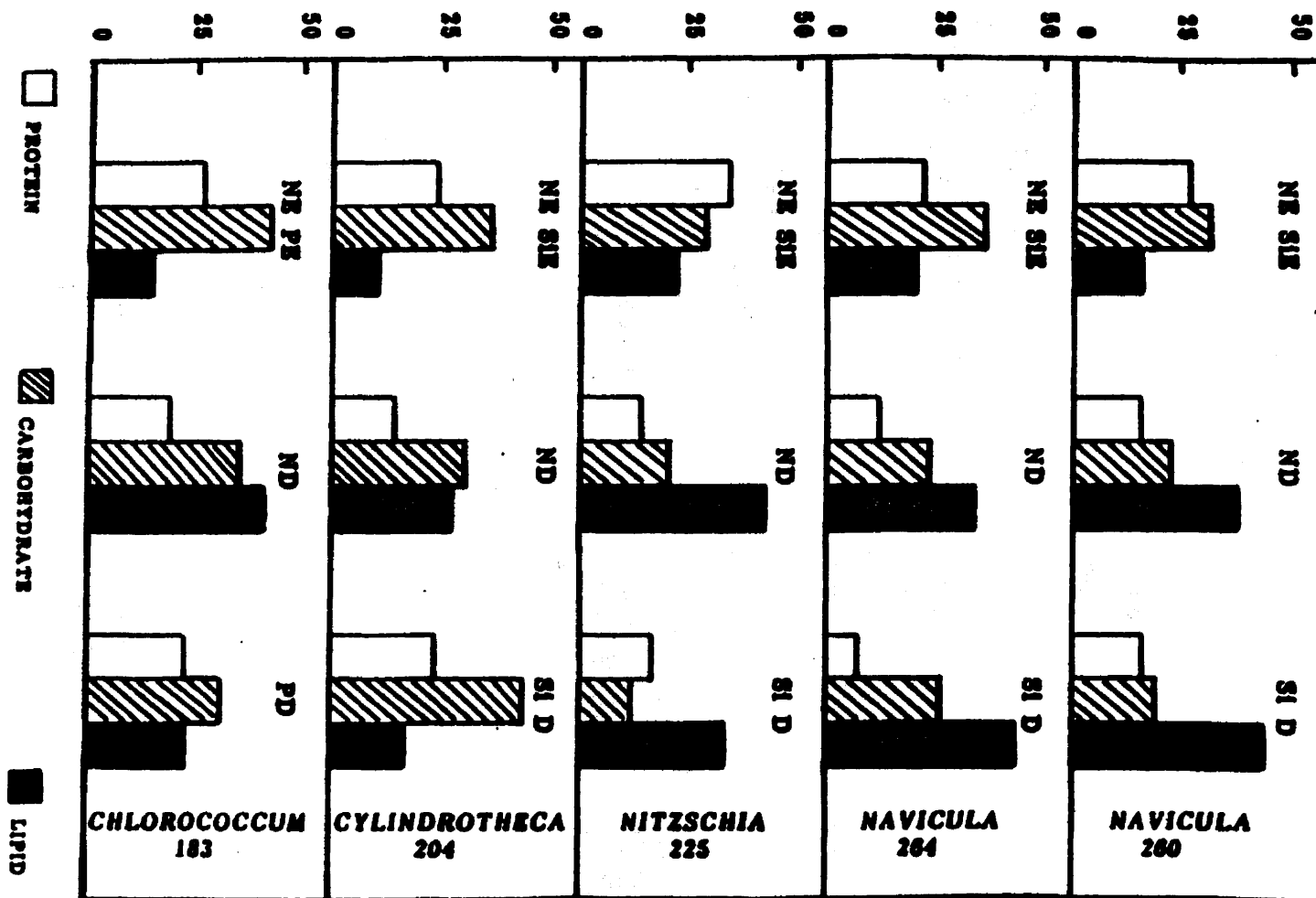


Figure 11. Histograms showing the Chemical Composition of Selected Algal Species, Amounts of Protein, Carbohydrate, Lipid, are expressed as Percentages of the Organic Weight.

NE - Nitrogen Sufficient ND - Nitrogen Deficient SIE - Silicon Sufficient  
 SID - Silicon Sufficient PE - Phosphorus Sufficient PD - Phosphorus Deficient

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# COLLECTION OF HIGH ENERGY YIELDING STRAINS OF SALINE MICROALGAE FROM THE HAWAIIAN ISLANDS

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## ABSTRACT

Microalgae were collected from 48 locations in the Hawaiian Islands in 1985. The sites were an aquaculture tank; a coral reef; bays; a geothermal steam vent; Hawaiian fish ponds; a Hawaiian salt punawai (well); the ocean; river mouths; saline lakes; saline pools; saline ponds; a saline swamp; and the ponds, drainage ditches and sumps of commercial shrimp farms. Conductivities of the water ranged from  $6.00 \times 10^2$  micromhos  $\text{cm}^{-1}$  to  $3.85 \times 10^5$  micromhos  $\text{cm}^{-1}$ , and temperatures ranged from  $10.5^\circ\text{C}$  to  $62.0^\circ\text{C}$ . Single cells or colonies of microalgae were isolated into media in glass culture tubes incubated in fluorescent light in the laboratory, and into fluorohalocarbon plastic bags transmitting full-spectrum sunlight outdoors. From 4,800 isolations, 100 of the most productive clones were selected to be maintained by periodic transfer to sterile medium. Five clones were tested for growth rate and production in a full-spectrum-transmitting solarium. The cultures were bubbled with carbon dioxide and air. Temperatures ranged from  $17.7^\circ\text{C}$  to  $42.8^\circ\text{C}$ . The highest growth rates were 2.12 doublings  $\text{day}^{-1}$  for *Chaetoceros* sp. clone SH 9-1, and 1.43 doublings  $\text{day}^{-1}$  for *Cyclotella* sp. clone 14-89. The highest production was 31 g dry weight  $\text{m}^{-2} \text{day}^{-1}$  for the *Chaetoceros* sp., and 33 g dry weight  $\text{m}^{-2} \text{day}^{-1}$  for the *Cyclotella*. Clones isolated in full-spectrum, high-intensity sunlight and high temperatures were the most productive in tests simulating conditions expected in large-scale microalgal production facilities.

## INTRODUCTION

The search for microalgae for large-scale hydrocarbon-producing cultures includes environments with high light intensities and/or high temperatures. The clear Pacific Ocean water surrounding the Hawaiian Islands is low in nutrients and phytoplankton population densities, but transmits high-intensity sunlight including ultraviolet wavelengths meters deep. In contrast, saline waters on the islands have higher levels of nutrients that leach from the volcanic soils and stimulate microalgae blooms. The high silicate levels result in an abundance of diatoms, and some waters have high temperatures and salinities. By isolating and screening clones in full-spectrum, high-intensity sunlight at high temperatures, strains will be selected that are tolerant of conditions that are envisioned for large-scale microalgal production facilities.

## PROJECT OBJECTIVES

Make collections from sites in the Hawaiian Islands that would favor desired characteristics and select for strains that are either dominant at the time of collection or become dominant in enriched media at high light.

Select the most productive strains.

Screen five of the most promising strains for growth rate and productivity.

## METHODS

The collection sites were photographed, and the date, time, name and coordinates noted. At most sites, a vertically integrated sample was collected by lowering an open bottle. The depths sampled, and the maximum depth of the water body were recorded. Dissolved oxygen concentration (DO) and pH were measured electrochemically *in situ* if the water was deep enough to cover the electrodes. Temperature was measured *in situ* with a mercury thermometer. Water collected for the isolation of cells was sub-sampled for determination of the concentrations of algal nutrients (N, P, Si), chlorophyll, and

microalgal cells. Nutrient samples were filtered through pre-combusted Whatman GF/F filters and frozen until analyzed with a Technicon autoanalyzer. Chlorophyll samples were collected on GF/F filters and stored in methanol in a freezer until analyzed in a Turner fluorometer. Unfiltered water was preserved by the addition of Lugol's solution for estimation of the population densities of microalgal cells using a Spiers-Levy Eosinophil counting chamber and compound microscope. Other sub-samples of water were sterile-filtered for measurement of electrical conductivity, and for use as one type of culture medium.

Culture medium was prepared with 4 types of sterile-filtered water: 1) sample water; 2) offshore seawater adjusted to the conductivity of the sample by dilution with distilled water or concentrated by evaporation; 3) SERI Type I water, 40 millimhos  $\text{cm}^{-1}$  or the conductivity nearest to that of the sample; and 4) SERI Type II water, 40 millimhos  $\text{cm}^{-1}$  or the conductivity nearest to that of the sample. These were enriched to make half-strength f medium (f/2) including 107 micromolar sodium metasilicate (Guillard and Ryther, 1962). Each type of medium was pipetted to two types of culture vessels: 1) Aclar plastic bags, and 2) glass culture tubes. Aclar 22A is a fluorohalocarbon plastic film that transmits the full spectrum of solar irradiance that reaches the earth's surface (refer to Figure 1). The bags were fabricated by folding and heat-sealing pieces of 0.127-mm thick Aclar film.

For each collection 100 single cells or colonies of microalgae were isolated using the modified Pasteur-type pipette technique (Guillard 1973; Hoshaw and Rosowski 1973) with a stereomicroscope, and each placed in individual bags or tubes containing 10 ml of medium. Unfiltered sample water was enriched to make f/2 medium without added silicate, and 10 ml each was pipetted to Aclar bags and glass culture tubes. Another tube and bag received culture after silicate was added. If the sample was sparsely populated, microalgae were concentrated on filters which were also placed in a bag and tube of enrichment water including silicate. The Aclar bags were placed outdoors in direct sunlight without temperature control. The culture tubes were placed in the laboratory and illuminated 16 hours per day with 40 micro E  $\text{m}^{-2}\text{sec}^{-1}$  from Vita-Lite fluorescent lights, at temperatures of 25°C to 26°C.



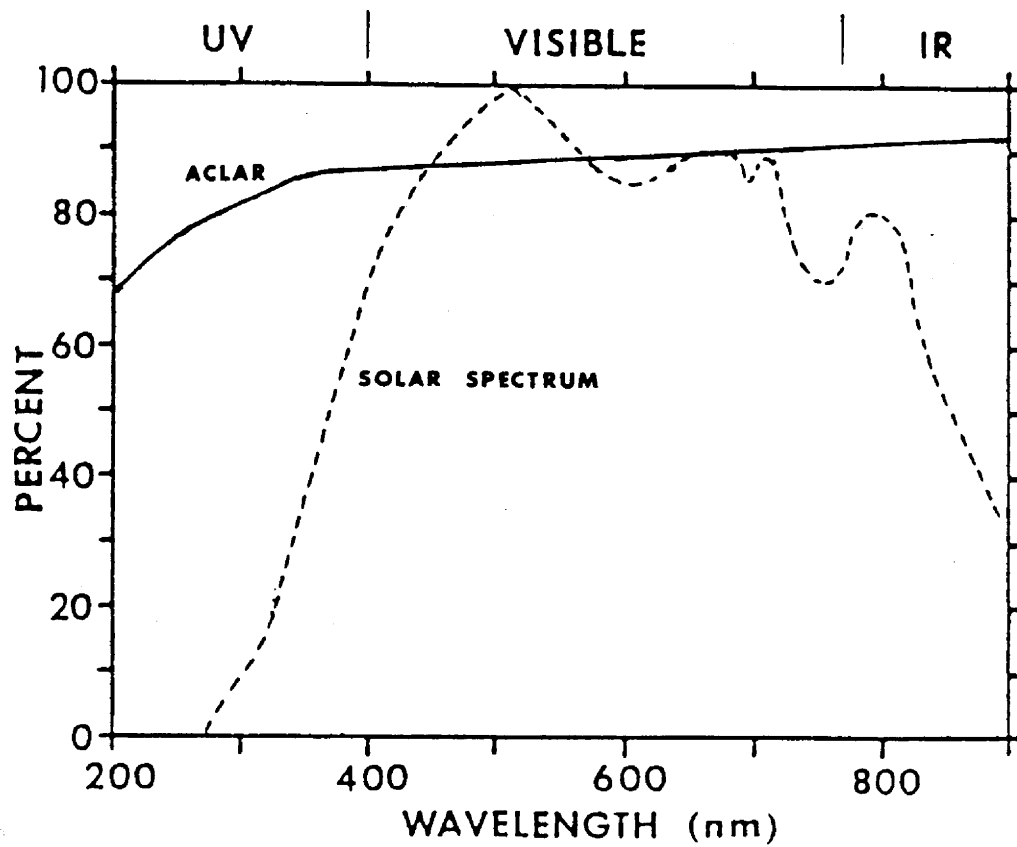


Figure 1. Light transmission characteristics of Aclar 22A plastic film (Allied Chemical Corp.)

One large-scale outdoor culture was made by pumping 1,700 l of seawater into a 5.5-m diameter fabricated pool and enriching it to make f/2 medium including 107 micromolar silicate. Isolations were made after microalgal growth was evident.

In addition, sediments were collected from a dried pond bottom. This sample was then placed in a petri dish in the laboratory, hydrated with distilled water, and incubated under the fluorescent lights.

The densest cultures were examined microscopically for condition of the cells and for contamination. Clones selected were transferred to 40 ml f medium in 125 ml Erlenmeyer flasks with cotton plugs, incubated under the same conditions as the culture tubes and transferred to new medium periodically. If contamination was observed, cultures were recloned or treated with antibiotics. Clones being maintained in brackish water or water of conductivity higher than seawater were also inoculated into seawater medium. If growth occurred, subsequent transfers were made into seawater medium.

A full-spectrum-transmitting solarium was designed and constructed by covering aluminum framing with 0.127 mm thick Aclar type 22A plastic film. An air-conditioner was adjusted to produce air temperatures less than 20°C at night and greater than 30°C during the day. Air temperatures were measured with a maximum-minimum indicating mercury thermometer. Incident irradiance was measured with a Weathertronics silicon cell pyranometer, model 3120.

Culture temperatures were measured at intervals with a mercury thermometer to find that maxima, minima, and relationship to the solarium air temperature at different culture densities and irradiances. Growth rate was determined by measuring optical density at a wavelength of 750 nanometers (OD 750) using a Beckman DU-7 spectrophotometer. When the OD 750 exceeded 0.1, the values for the cultures were calculated from measurements of dilutions. Production rate was measured as grams dry weight and ash-free dry weight per square meter per day. This was calculated from differences in dry weights of cells collected on glass fiber filters, rinsed with iso-osmotic ammonium formate solutions, dried at 60°C, and combusted at 450°C.

Culture vessels were constructed by heat-sealing Aclar plastic film to make bags. Culture medium was prepared by enriching sterile-filtered seawater to make f medium

including sodium metasilicate for Bacillariophytes (diatoms). One liter of medium was added to each bag. Aclar bags were inoculated with one of 5 clones to densities near 0.01 OD 750. Five clones representative of the major taxonomic divisions were selected on the basis of production in the culture collection. The clones were the Bacillariophytes *Chaetoceros* sp. clone SH 9-1, *Cyclotella* sp. clone SH 14-89, the Chlorophyte *Platychloris* sp. clone SH 6-22, a Cyanophyte clone SH 14-22, and a Pyrrophyte clone SH 22-20. As the cultures became dense, more nutrients were added to prevent nutrient limitation. The cultures were bubbled with a mixture of carbon dioxide and air.

## RESULTS AND DISCUSSION

Microalgae were collected from 48 locations in the Hawaiian Islands during the year 1985. The sites were an aquaculture tank; a coral reef; bays; a geothermal steam vent; Hawaiian fish ponds; a Hawaiian salt punawai (well); the ocean; river mouths; saline lakes; saline pools; saline ponds; a saline swamp; and the ponds, drainage ditches and sumps of commercial shrimp farms. Figure 2 and Table 1 describe the sites and their locations.

Table 2 lists the collection site data. Conductivities ranged from  $6.00 \times 10^2$  micromhos  $\text{cm}^{-1}$  for water collected from an algal film growing on rock in a geothermal steam vent, to  $3.85 \times 10^5$  micromhos  $\text{cm}^{-1}$  for dried pond bottom sediment after it was hydrated with distilled water, resulting in the growth of the flagellate *Dunaliella*. The lowest temperature recorded was  $10.5^\circ\text{C}$  in an abalone/kelp culture tank which was receiving ocean water from a depth of 600 m through the experimental Ocean Thermal Energy Conversion plant at Keahole Point. There was a bloom of *Skeletonema* in this tank. The highest temperature was  $62.0^\circ\text{C}$  in the middle of clumps of viable algal films in the geothermal steam vent. The lowest dissolved oxygen concentration was 0.6 ppm in the drainage ditch at Mariculture Research and Training Center (MRTC). The drainage ditch had an organic film on the surface, an odor of hydrogen sulfide, and a dark reddish-brown coloration from suspended iron precipitate and organics. *Nitzschia* cells were isolated and cultured from this unfavorable environment. The highest dissolved oxygen concentration was 18.4 ppm in pond number 108 at the Amorient shrimp farm. This was three times the saturation concentration. *Chaetoceros* sp. cells were isolated from the pond. The most supersaturated dissolved oxygen concentration was in the punawai (salt well) sample at

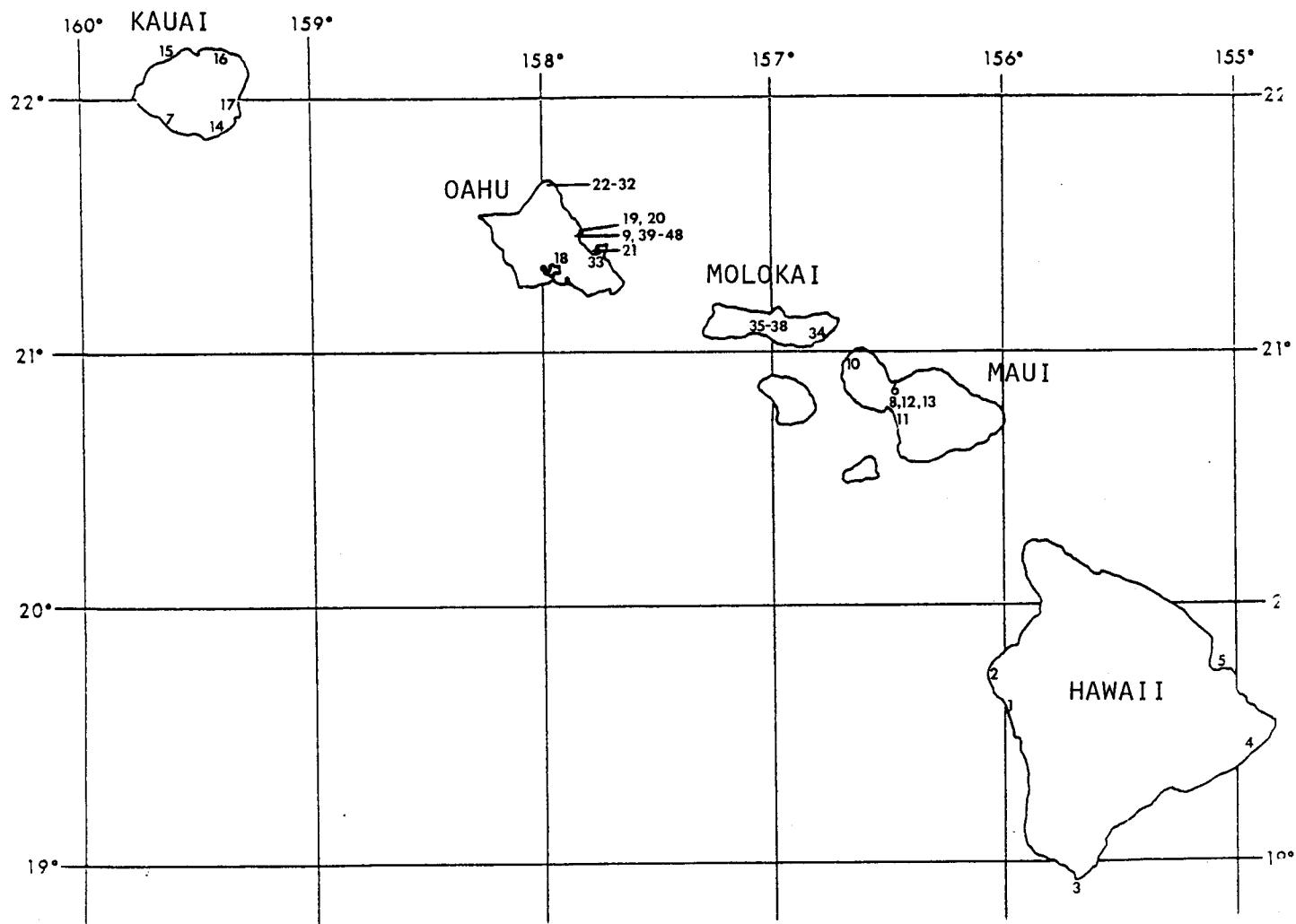


Figure 2. Sample station locations

**Table 1. Collection locations**

<u>Collection Number</u>	<u>Name</u>	<u>Location</u>	<u>Island</u>	<u>Latitude North</u>	<u>Longitude West</u>
1	Kalakaua's Fishpond	Kahaluu	Hawaii	19°34'53.8"	155°58'11.2"
2	Abalone/kelp tank	Keahole	Hawaii	19°43'54.8"	156°03'41.3"
3	Pacific Ocean	Kalae	Hawaii	18°54'48.7"	155°41'00.4"
4	Geothermal steam vent	Kamaili	Hawaii	19°26'36.4"	154°56'41.9"
5	Hilo Bay	Mokaoku	Hawaii	19°43'58.8"	155°03'59.8"
6	Kahana Pond	Kahalui	Maui	20°53'33.0"	156°27'28.0"
7	Salt Pond	Hanapepe	Kauai	21°54'10.6"	159°36'33.6"
8	Kealia Pond	Kealia	Maui	20°47'45.4"	156°28'48.0"
9	Aquatic Farms, Pond 9	Hakipuu	Oahu	20°30'33.7"	157°51'23.5"
10	Honokowai Stream	Honokowai	Maui	20°57'12.3"	156°41'34.2"
11	Kulanihakoi Stream	Kalepolepo	Maui	20°46'08.7"	156°27'37.0"
12	Kealia Pond	Kealia	Maui	20°47'49.0"	156°28'30.8"
13	Kealia Pond	Kealia	Maui	20°48'00.6"	156°29'22.5"
14	Menehune Fishpond	Huleia	Kauai	21°57'05.4"	159°22'39.6"
15	Nualolo Kai Reef	Nualolo Kai	Kauai	22°09'46.0"	159°42'11.4"
16	Kilauea River	Kilauea	Kauai	22°13'27.3"	159°23'23.2"
17	Wailua River	Wailua	Kauai	21°57'22.6"	159°40'02.4"
18	Salt Lake	Salt Lake	Oahu	21°21'27.8"	157°54'39.2"
19	Apua Fishpond	Kualoa	Oahu	21°30'46.8"	157°50'23.5"
20	Molii Fishpond	Kualoa	Oahu	21°30'39.0"	157°50'52.2"
21	Kaneohe Bay	Moku o loe	Oahu	21°26'11.2"	157°47'19.3"
22	Amorient, Pond 106	Kahuku	Oahu	21°41'01.6"	157°57'59.0"
23	Amorient, Pond 107	Kahuku	Oahu	21°41'01.6"	157°58'00.0"
24	Amorient, Pond 108	Kahuku	Oahu	21°41'01.6"	157°58'01.0"

**Table 1. Collection locations**  
(continued)

<u>Collection Number</u>	<u>Name</u>	<u>Location</u>	<u>Island</u>	<u>Latitude North</u>	<u>Longitude West</u>
25	Amorient, Pond 138	Kahuku	Oahu	21°41'03.0"	157°58'02.9"
26	Amorient, Pond 139	Kahuku	Oahu	21°41'03.0"	157°58'04.1"
27	Amorient, Pond 140	Kahuku	Oahu	21°41'03.0"	157°58'05.8"
28	Amorient, Pond 141	Kahuku	Oahu	21°41'03.0"	157°58'06.6"
29	Amorient, Pond 142	Kahuku	Oahu	21°41'03.0"	157°58'07.9"
30	Amorient, Pond 143	Kahuku	Oahu	21°41'03.0"	157°58'08.7"
31	Amorient, drainage ditch	Kahuku	Oahu	21°41'03.4"	157°58'00.4"
32	Amorient, sump	Kahuku	Oahu	21°41'04.4"	157°58'00.0"
33	Enchanted Lake	Kailua	Oahu	21°23'03.5"	157°44'00.8"
34	Kupeke Fishpond	Kupeke	Molokai	21°04'38.0"	156°47'41.7"
35	Orca Seafarms, drainage ditch	Kahanui	Molokai	21°06'	157°05'
36	Manawainui Swamp	Kahanui	Molokai	21°06'	157°05'
37	Orca Seafarms, Pond GP7	Kahanui	Molokai	21°06'	157°05'
38	Orca Seafarms, Pond GN6	Kahanui	Molokai	21°06'	157°05'
39	Marine Research & Training Center (MRTC), Pond 2	Hakipuu	Oahu	21°30'	157°51'
40	MRTC, Pond 3	Hakipuu	Oahu	21°30'	157°51'
41	MRTC, Pond 4	Hakipuu	Oahu	21°30'	157°51'
42	MRTC, Pond 5	Hakipuu	Oahu	21°30'	157°51'
43	MRTC, Pond 7	Hakipuu	Oahu	21°30'	157°51'
44	MRTC, Pond 8	Hakipuu	Oahu	21°30'	157°51'
45	MRTC, Pond 10	Hakipuu	Oahu	21°30'	157°51'
46	MRTC, Pond 11	Hakipuu	Oahu	21°30'	157°51'
47	MRTC, Pond 12	Hakipuu	Oahu	21°30'	157°51'
48	MRTC, drainage ditch	Hakipuu	Oahu	21°30'	157°51'

Table 2. Collection site data

Collection Number	Date	Time	Conductivity (micromhos $\text{cm}^{-1}$ )	Depth of Sample (cm)	Maximum Water Depth (cm)	Dissolved Oxygen (ppm)	pH	Temperature (°C)
1	05/13/85	10:30	$5.90 \times 10^3$	0 - 10	100	6.9	7.1	24.8
2	05/13/85	15:00	$4.40 \times 10^4$	50	400	-	-	10.5
3	05/16/85	16:00	$4.50 \times 10^4$	0 - 100	$1 \times 10^6$	5.8	8.2	25.0
4	05/17/85	16:00	$6.00 \times 10^2$	epilithic	0	-	-	62.0
5	05/18/85	12:15	$3.13 \times 10^4$	0 - 50	1,300	6.1	8.2	23.0
6	05/29/85	16:00	$2.96 \times 10^4$	0 - 5	20	8.6	9.2	29.0
7	05/31/85	18:50	$2.20 \times 10^5$	0 - 10	200	8.6	8.2	29.0
8	05/29/85	15:00	$3.85 \times 10^5$	-----dry sediment-----		-	-	-
9	07/10/85	15:30	$3.27 \times 10^4$	0 - 100	100	12.8	7.4	31.5
10	07/10/85	15:30	$8.90 \times 10^3$	0 - 30	100	12.8	7.4	31.5
11	07/14/85	15:13	$3.62 \times 10^4$	0 - 10	100	7.3	8.2	31.5
12	07/15/85	10:21	$1.87 \times 10^4$	0 - 1	1	5.7	8.7	33.0
13	07/16/85	09:55	$5.00 \times 10^4$	0 - 30	100	5.5	8.3	27.2
14	08/07/85	17:10	$1.78 \times 10^4$	0 - 20	200	5.8	7.5	29.2
15	08/09/85	12:30	$4.50 \times 10^4$	0 - 100	1,000	7.4	8.2	28.0
16	08/10/85	18:00	$3.42 \times 10^4$	200	400	6.6	8.2	29.0
16A	08/10/85	18:00	$2.88 \times 10^3$	10	400	8.0	7.3	29.5
17	08/11/85	11:00	$2.83 \times 10^4$	50	200	5.8	8.2	28.0
18	08/28/85	15:35	$2.28 \times 10^4$	0 - 15	100	6.9	8.2	31.0
19	09/09/85	11:00	$5.14 \times 10^4$	0 - 5	10	4.4	8.8	37.2
20	09/09/85	12:20	$4.90 \times 10^4$	10	200	5.7	8.2	30.5
21	09/23/85	20:30	$4.60 \times 10^4$	200	1,600	10.9	8.2	27.8
22	10/07/85	13:20	$1.93 \times 10^4$	0 - 100	100	9.4	8.5	28.0
23	10/07/85	13:45	$2.12 \times 10^4$	0 - 10	10	18.4	9.8	33.0
24	10/07/85	14:15	$2.80 \times 10^4$	0 - 60	60	8.0	9.8	33.0

**Table 2. Collection site data**  
(continued)

Collection Number	Date	Time	Conductivity (micromhos $\text{cm}^{-1}$ )	Depth of Sample (cm)	Maximum Water Depth (cm)	Dissolved Oxygen (ppm)	pH	Temperature (°C)
25	10/07/85	14:25	$5.75 \times 10^4$	0 - 10	10	6.4	8.3	31.0
26	10/21/85	09:30	$1.10 \times 10^4$	0 - 30	30	9.9	8.6	25.5
27	10/21/85	09:45	$1.48 \times 10^4$	0 - 100	100	7.9	8.2	25.5
28	10/21/85	10:05	$1.72 \times 10^4$	0 - 100	100	7.5	8.0	25.5
29	10/21/85	10:15	$1.76 \times 10^4$	0 - 100	100	8.8	8.1	25.5
30	10/21/85	10:30	$1.13 \times 10^4$	0 - 50	50	8.8	8.3	26.0
31	10/21/85	10:45	$4.60 \times 10^3$	0 - 100	100	4.2	7.8	26.0
32	10/21/85	10:55	$8.19 \times 10^3$	0 - 100	100	10.4	8.1	26.8
33	11/04/85	14:40	$2.24 \times 10^4$	0 - 10	122	4.2	6.8	27.0
34	11/07/85	12:00	$3.33 \times 10^4$	0 - 20	200	9.6	-	28.0
35	11/20/85	13:25	$3.73 \times 10^4$	0 - 10	100	6.5	-	28.0
36	11/20/85	14:00	$4.46 \times 10^4$	0 - 10	10	15.0	-	35.0
37	11/20/85	15:00	$2.42 \times 10^4$	0 - 20	20	13.0	-	34.0
38	11/20/85	15:15	$3.78 \times 10^4$	0 - 20	20	11.2	-	35.0
39	12/04/85	11:40	$2.43 \times 10^4$	0 - 12	12	9.8	-	25.0
40	12/04/85	12:03	$1.86 \times 10^4$	0 - 25	25	9.4	-	25.0
41	12/04/85	12:15	$1.89 \times 10^4$	0 - 100	100	16.4	-	25.0
42	12/04/85	12:25	$2.28 \times 10^4$	0 - 100	100	11.6	-	25.0
43	12/04/85	12:40	$1.82 \times 10^4$	0 - 50	50	15.8	-	26.0
44	12/04/85	12:50	$1.79 \times 10^4$	0 - 100	100	10.2	-	25.0
45	12/04/85	13:00	$2.63 \times 10^4$	0 - 100	100	14.4	-	25.0
46	12/04/85	13:15	$2.23 \times 10^4$	0 - 50	80	10.8	-	26.0
47	12/04/85	13:20	$2.82 \times 10^4$	0 - 50	100	9.4	-	25.0
48	12/04/85	13:30	$2.02 \times 10^4$	0 - 10	34	0.6	-	26.8



Salt Pond on Kauai. The water was so salty that the 8.6 ppm DO was 3.7 times saturation. There was a thick bloom of *Dunaliella* sp. forming a surface film that entrapped large oxygen bubbles.

Successful cultures were obtained from all 48 collection sites. The success rate was higher in the laboratory than outdoors. This was probably due to photoinhibition and higher temperatures outdoors. Clones that were tolerant of the conditions outdoors are desirable for large-scale culture. Some clones, including *Chaetoceros*, *Cyclotella*, and *Melosira* grew rapidly from a single cell to high densities at temperatures up to 42°C with full-spectrum solar irradiance. There was not much difference in the success rate of clones from the same population in the media of different water types at equivalent conductivities.

In some cases, species that grew well as clones did not bloom in the enrichment of the unfiltered water sample from which they were isolated. This may have been the result of competition, disease or predation. This demonstrates the value of the Pasteur-type pipet cloning technique for establishing clean cultures. The isolation of cells from other algae, protozoans and zooplankton also eliminates bacteria in some cases. Dilution of the sample with sterile water before isolation may be important.

The 1,700-1 pool enrichment yielded a flocculant bloom composed mostly of pennate diatoms which tolerated temperatures as high as 42.2°C and direct sunlight. These were successfully isolated. This technique is useful for screening and increasing the microalgal community density of sparsely populated waters.

A total of 100 clones were selected on the basis of productivity to be maintained by periodic transfer to sterile medium. Most are being grown in seawater medium. The clones that have been identified are Bacillariophytes, Chlorophytes, Cyanophytes and Pyrrophytes. Isolates that grew successfully in the Aclar bags were selected. Pennate diatoms from the pool enrichment were also selected because they had bloomed in high light intensities and temperatures. Clones from the *Nitzschia* population at Nualolo Kai were selected because of the large lipid droplets observed in the cells in the sample. This was probably the result of low nutrient concentrations in the seawater. The clones of *Melosira* that were selected formed long chains that could easily be harvested by

conventional screen-type harvesters used in *Spirulina* culture. For this reason, these clones may have lower production costs than other microalgae in large-scale cultures.

Of the five clones tested only the *Chaetoceros* sp. clone SH 9-1 and *Cyclotella* sp. clone SH 14-89 grew. The *Platychloris* and the Pyrrophyte were still motile, but were probably inhibited by temperatures ranging from 17.7°C to 42.8°C, or were photoinhibited, or both. These may also have been the reasons that the Cyanophyte did not grow. The highest solar irradiance recorded at noon was 960 watts m<sup>-2</sup>.

Nutrients were added on Days 7, 8 and 11. The cultures were diluted on Day 15. Growth of the *Chaetoceros* and the *Cyclotella* is shown in Figures 3 and 4. Growth rates are listed in Table 3. The highest growth rates were 2.12 doublings day<sup>-1</sup> for *Chaetoceros*, and 1.43 doublings day<sup>-1</sup> for *Cyclotella*. The highest production was 31 g dry weight m<sup>-2</sup> day<sup>-1</sup> for *Chaetoceros*, and 33 g dry weight m<sup>-2</sup> day<sup>-1</sup> for *Cyclotella*.

## CONCLUSIONS

The clones selected in this study should be highly productive in large-scale outdoor cultures. They were isolated from shallow, eutrophic, saline ponds in which the water was being exchanged. Clones isolated in full-spectrum, high-intensity sunlight and high temperatures were the most productive in tests simulating conditions expected in large-scale microalgal production facilities.

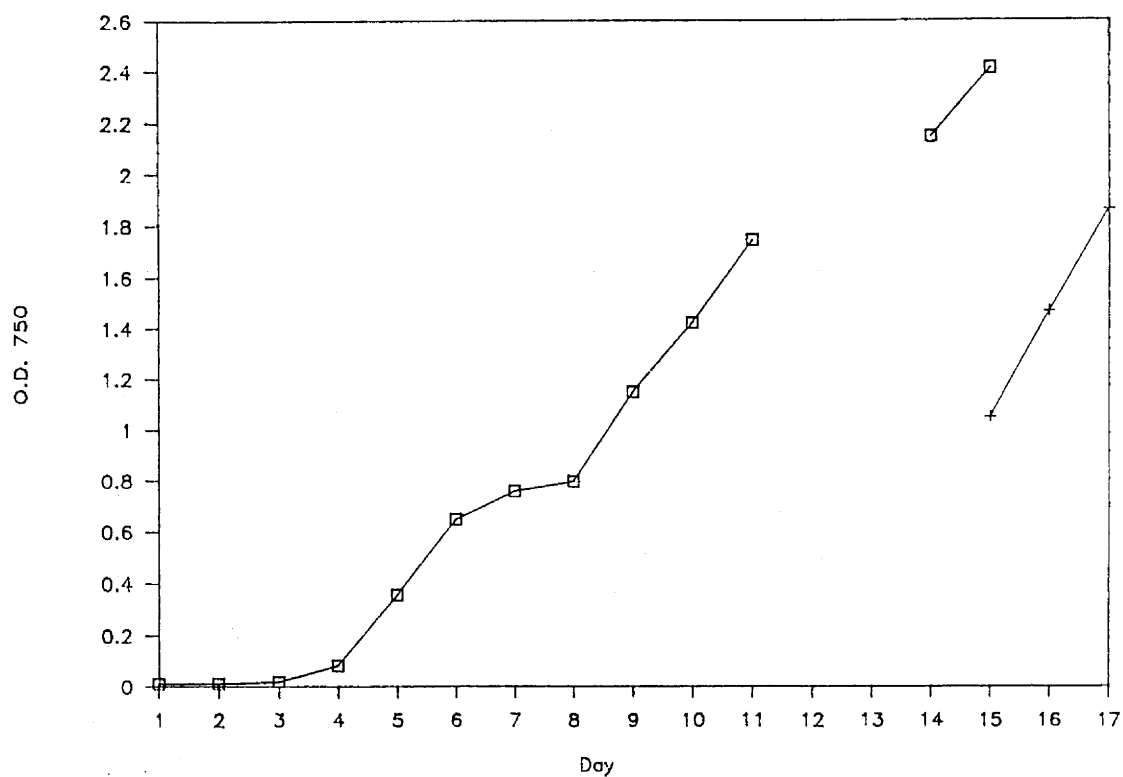


Figure 3. Growth of *Chaetoceros* sp. clone SH 9-1

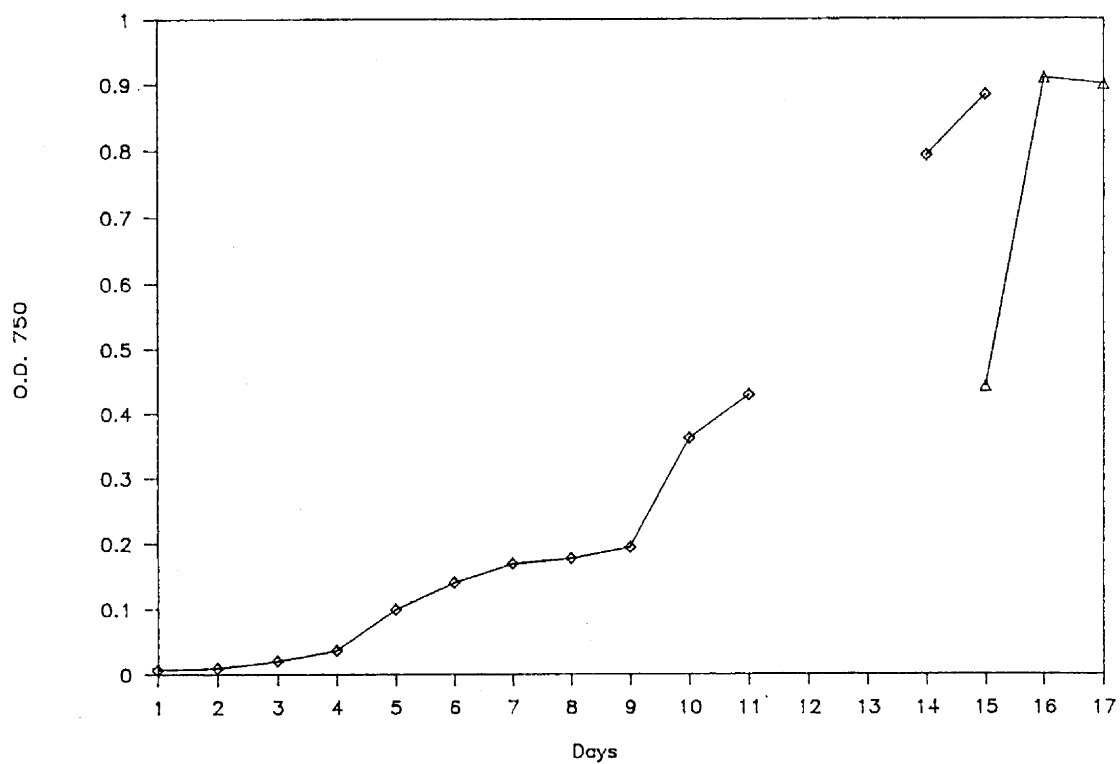


Figure 4. Growth of *Cyclotella* sp. clone 14-89

**Table 3. Growth rates of Chaetoceros and Cyclotella clones**  
(doublings per day)

<u>Day</u>	<u>Chaetoceros</u>	<u>Cyclotella</u>
2	0.01	0.39
3	0.78	1.06
4	2.12	0.86
5	2.09	1.43
6	0.86	0.50
7	0.23	0.27
8	0.07	0.07
9	0.53	0.13
10	0.31	0.90
11	0.53	0.25
14	0.30	0.88
15	0.17	0.15
16	0.48	1.04
17	0.33	0

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Some picoplankton algae from the Caribbean region

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Picoplankton algae, 1-5 micrometers in diameter, include both prokaryotes and eukaryotes. Among the former (Cyanophytes), some can float by making gas vacuoles. Among the latter, some can swim by using flagella, others float because of stored oil. Nannochloropsis (Eustigmatophyta) produces a lot of oil, much of it hydrocarbon. By differential filtration and flotation enrichment cultures we have collected and examined the floating picoplankton of some 200 samples of inshore marine waters from subtropical and tropical sites, chiefly in the Caribbean. Pure cultures have been isolated and grown in defined media: their lipid production is being assessed under various conditions of culture. Some may ultimately prove of economic value as a source for liquid fuel.

Introduction. Photosynthetic phytoplankton cells have to stay in the uppermost illuminated water layers. If they sink or are carried below the euphotic zone they are doomed to die unless they can adopt an alternative means of nutrition such as heterotrophy or phagotrophy. Few have this ability. Some accordingly stay aloft by epibiosis or symbiosis, living attached to or inside other organisms (Taylor, 1981), but most stay in surface waters either by active swimming, with flagella, or by a more passive flotation mechanism. At least three means of flotation have been adopted. One, found predominantly in freshwater cyanophytes but occurring also in certain marine species of Oscillatoria (Trichodesmium), involves the production of intracellular gas vacuoles, a feature apparently confined to prokaryotes (Walsby and Reynolds, 1980). Another device for reducing the mean cell density in sea water is by excluding or expelling heavier ions and replacing them by lighter ones. Thus certain diatoms can achieve buoyancy by accumulating divalent magnesium ions (with an atomic weight of 24) and maintaining ionic balance by expelling twice as many monovalent sodium ions ( $2 \times 23 = 46 > 24$ ) (Beklemishev, Petrikova and Semina, 1961). A third, which somewhat surprisingly has received little consideration hitherto, is the accumulation of oil. An unusually obvious surface film of Coscinodiscus concinnus, in nutrient-poor surface waters in the North Sea, was attributed to the production of lipids, which in this species may represent up to 40% of the dry weight (Grøntved, 1952). In an exhaustive review on plankton flotation in the sea, Smayda (1970) discussed this phenomenon but implied that it is rare and abnormal. Nevertheless, oil flotation exists and can be effective, as will be shown in the course of this paper.

Oil-producing algae are now of special interest because the lipid that they produce may comprise a large proportion of hydrocarbons in addition to

the more usual glycerides of fatty acids. Few other plants are known to make hydrocarbons in quantity: the green alga Botryococcus is the only one with this faculty that has been investigated so far (Wolf, Nonomura and Bassham, 1985). The reason for this interest is, ultimately, a practical one: as the World's supplies of fossil fuels become depleted, we may have to turn more and more to contemporary organisms to produce oil. Recently, cells of a marine picoplankton alga, Nannochloropsis salina, have been found to produce up to half of their dry weight as lipids, of which more than 50% may consist of hydrocarbons (unpublished data). The main objective of the work reported here was to seek new strains or species of this genus, and other algae with similar biochemical features, the culture of which might have a comparable or enhanced potential for commercial oil production under economic (i.e., inexpensive) conditions. Sea water being cheap and abundant, at least in coastal areas, we initially limited our search to marine sources. We sought algae that float, at least after a period of photosynthesis in conditions of nitrogen-starvation (which generally promotes the production of carbohydrates or lipids by limiting protein synthesis). We specifically looked for strains that grow well in sea-water but require no exogenous vitamins (which would add expense to their cultivation en masse) and that tolerate a range of temperatures which might prevail in sunny, out-door ponds or watercourses in warm-temperate zones where, ultimately, they might be cultured for economic purposes.

Having loosely defined these preconditions, we collected plankton algae from a variety of more or less saline marine littoral habitats around the Caribbean Sea, and subjected them to the series of selection procedures described in the following section. Strains that showed promise were purified (rendered axenic) and examined by more critical physiological and biochemical tests. Attempts to identify them to species (or even to higher taxa) have not

been wholly successful, since the field of picoplankton taxonomy is still in disarray. Further detailed studies of selected strains will be reported in due course.

#### Materials and methods

Water samples were taken during the first two weeks of February, 1985, from various sites in the Virgin Islands (St Thomas, St John, St Croix and Tortola), Puerto Rico (in the regions of San Juan and Mayagüez), Curaçao, Panama (both Atlantic and Pacific coasts) and various sites along the causeway between Miami and Key Largo, Florida. 130 samples, each of about 250 ml, were collected and immediately (usually within a few minutes) filtered, by suction (1-4 min) generated by a hand-operated pump, through two filters laid one over the other. Since the filter assembly was usually dried after a sample had been taken and filtered, and was rinsed with sea water from the next source before the next sample was taken and filtered, and since new filters were used for every sample, cross contamination was negligible. The upper, a fibrous cellulose pad which held back particles exceeding 3-8 micrometers in width or length, was then discarded. The lower, a Millipore filter with pore size of 0.45 micrometers to retain smaller nanoplankton cells, was removed, rolled up, and inserted in a plastic tube. About 1 ml of the filtrate was added, to keep the sample wet and the cells viable during shipment, and the vial was then sealed with a screw cap and labelled. Such tubed samples were kept at room temperatures (ca. 20-25°) and at ambient light intensities (usually daylight from a room window) until they could be transported and examined at the Scripps Institution of Oceanography, in California.



In this laboratory, salinities of the water samples were determined with a refractometer (American Optical Company). The vials were then shaken, to loosen cells from the filters, and portions (ca. 0.2 ml) were transferred to glass tubes containing 5 ml of enriched sea-water medium (SWM, containing 100 mg/l  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ , 20 mg/l of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , and traces of Fe, etc.) sterilized by autoclaving at  $120^\circ\text{C}$  for 20 minutes. The tubes, of Pyrex glass 150 x 18 mm, were loosely closed with glass Cencaps (Central Scientific Co.). They were illuminated continuously at about 30 micro-Einsteins  $\text{m}^{-2}\text{s}^{-1}$  from white fluorescent lamps, at  $25^\circ$ , for about 4 weeks, long enough to permit the multiplication of the few viable cells to appreciable cell densities. At this stage, all 130 samples showed evidence of algal growth. From each such cell suspension, 0.2 ml was transferred to a second culture tube containing 5 ml of sea water without added nutrients (to promote lipid production), and illumination was continued in the same conditions. At the end of a further 4 weeks, about 50% of the nutrient-depleted cultures exhibited a slight film or scum of algae or a ring of growth at the meniscus. Without agitating the tubes, we used a bacteriological loop to remove a droplet of water from this superficial film and transferred it to a third series of culture tubes containing 5 ml of SWM to permit active cell multiplication. After 2 more weeks of illumination many of these third-transfer cultures showed growth of green, blue-green or brown unicellular (rarely filamentous) algae. Most cyanophytes and diatoms, identified by microscopic examination at 100x or 400x diameters, were culled, as were green algal flagellates (Chlamydomonas and Tetraselmis spp.). This left for further study about 60 cultures containing greenish cells, more or less isodiametric or oblong, 1-5 micrometers wide, in single or short (2-4-celled) chains. From each site 1 to 6 different-looking cultures were isolated.

Pure axenic clones were established by streaking a small droplet of each culture suspension on SWM + 1% agar in 10-cm Petri plates containing 25 ml of medium, incubating the plates for 6-8 days in the same conditions of light and temperature as before, and then, under a binocular dissecting microscope with transmitted light, picking single colonies that appeared free from contamination. The isolation of pin-point-sized colonies by use of a sterile micro-pipette with an internal tip diameter of 50-100  $\mu$ m considerably reduced chances of cross-contamination. The clones were transferred to tubes containing 5 ml SWM, and incubated in the usual conditions. Some 2-4 weeks later, many cultures had grown to appreciable cell densities; a few, possibly because of a vitamin (thiamine?) deficiency, had not, and these were discarded. Each clone was tested for the presence of contaminant bacteria by setting droplets on a nutrient agar medium prepared with sea-water enriched with Tryptone and yeast extract (1 g/l of each) and the test plates were incubated in darkness for 3-5 days. Evidence of bacterial growth indicated the need to repeat the streaking and picking process from the latest (i.e., least contaminated) liquid SWM culture. In this way, most of the desired strains were ultimately rendered axenic.

The ability of clones to grow in freshwater media was tested by spotting droplets of suspension on 1% agar media in Petri plates, respectively prepared with SWM (for marine controls) and a non-marine medium MM (containing  $\text{Ca}(\text{NO}_3)_2 \cdot 4 \text{H}_2\text{O}$  1.0 g/l,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.2 g/l and  $\text{K}_2\text{HPO}_4$  0.2 g/l). Growth (+ or -) was determined after 10 days of illumination under the usual conditions. The requirements of axenic clones for exogenous sources of vitamins were tested in test-tubes containing 5 ml of medium (SWM, either alone or supplemented with 0.1 mg/l thiamine and 0.1  $\mu$ g/l cobalamin), inoculated with 0.05 ml of algal suspension pre-cultured in SWM (i.e., without added vitamins), and illuminated

in the usual conditions. Comparative growth was assessed after 2 weeks.

(This was done to confirm the hypothesis that clones which grew with bacterial contaminants, but poorly without them, depended on the former for a supply of essential organic growth factors. Since we deliberately enriched and subcultured the algae in media without added vitamins, no weight should be placed on the proportion of isolated clones that proved to require exogenous sources of vitamins.) Temperature tolerances for growth were determined with the use of a simple gradient plate (Blankley and Lewin, 1976).

Small, nondescript algal cells are not readily identifiable: one often needs to supplement morphological details (all too few) and cell dimensions (variable and influenced by nutrition) with diagnostic biochemical features. Among these, we chose in particular to use chlorophyll composition, since one can thereby distinguish chlorophytes (with chl.a and chl.b) from cyanophytes and eustigmatophytes (with chl.a only). For a preliminary estimate of the relative chlorophyll contents of the clones, pigments were extracted overnight at 5° in 90% methanol and absorption curves were obtained using a Hewlett-Packard recording spectrophotometer (model 8451A). Absorptions at 650 and 664 nanometers were determined, and with the equations of Jeffrey and Humphrey (1975) we calculated ratios of chlorophyll a/b.

The following simplified technique was developed for extracting and separating lipid pigments by thin-layer chromatography (TLC). In addition, after suitable staining the same or duplicate TLC plates were used to provide additional information on the lipid content of the samples. 50-ml SWM cultures in 125-ml flasks were grown under standard conditions for 2-3 weeks, and the cells were harvested by centrifugation at 10,000g for 10 min. or, for those in which the cells were buoyant, by ultrafiltration through a membrane

(PTGC-OMT-5) in a Minitan apparatus (Millipore Corp., Bedford, Mass.). Each pellet was extracted with 10 ml of a mixture of methanol and acetone (1:1) at 5° in darkness overnight. The following operations were then carried out in the laboratory in dim light. 5 ml of hexane was mixed in, followed by 10 ml of water to wash out polar solvents. The upper layer, containing lipid pigments, was pipetted to a clean tube, and 10 ml of water was added with mixing to complete the removal of methanol and acetone. The green hexane layer was pipetted into another clean, dry tube, and a few hundred mg of anhydrous sodium sulphate was added to take up traces of water. The hexane solution was decanted to a short, dry tube (Cencap) and the solvent was evaporated in a gentle stream of air. For chromatography, the pigments were taken up in 0.1 ml of hexane, and droplets (1-2 mm diameter) were applied with a fine glass pipette (50-100 micrometers tip diameter) in a line 7 mm from the end of a glass slide bearing silica gel (TLC, Whatman MK6F, 2.5 x 7.6 cm, 200 micrometers thick). When the colour density in each spot was dark green, almost black, the plate was allowed to dry in air for 2-3 min. These small TLC chromatograms were developed at room temperature in screw-capped glass jars by ascending chromatography from 5 ml of a solvent mixture of acetone and hexane (1:4). In less than 5 min, when the solvent front had neared its upper end, the slide was removed and dried in air. The positions of the respective pigments (chlorophylls, xanthophylls and carotene) were marked; a record was made by xerography. Positions of the various pigments were compared with those of reference material - typically a well-researched alga, Chlamydomonas - run in parallel on the same TLC plates.

For a preliminary determination of non-pigmented lipids, the same or duplicate plates were immersed for a few minutes in an ethanolic solution of Rhodamine 6G in an ordinary slide-staining jar, and then soaked for a few

hours in water (in a similar jar). Typically, hydrocarbons were found to have moved, with beta-carotene, close to the solvent front: triglycerides ran ahead of the xanthophylls: steroids (in smaller amounts) ran behind triglycerides. (For more detailed analyses of the lipids, more sophisticated techniques are needed; these will be described in a separate publication.)

For a quantitative determination of the lipid content of cells, 1-litre cultures in SWM were grown in cylinders aerated with a constant flow of air enriched with about 0.5% CO<sub>2</sub> and illuminated under standard conditions for 3 weeks. Cells were harvested by centrifugation or ultrafiltration (see above), frozen at -20° and then lyophilized for 1-2 days. The dried powders were transferred to tared tubes and extracted with about 2 ml of a chloroform + methanol mixture (2:1) overnight at 5°. The tubes were then centrifuged to compact the sediment, and the supernatant extracts were decanted to tared tubes. The residues were rinsed twice in the same way, and the extracts and rinses were pooled. Both the residues and the extracts were dried overnight at 60°, weighed, and dried again to constant weight. The samples were then incinerated in a muffle oven at 550°C for 6 hr, cooled in a desiccator, and weighed again. In this way, dry weights, ash-free dry weights, and weights of extractable lipids were determined, and the proportions of lipids were calculated on an ash-free dry weight basis.

## Results

Larger plankton cells and other debris were excluded, immediately after the water samples were collected, by the prefiltration pads. Heterotrophs were later suppressed in the enrichment cultures because no organic substrate was added to the medium. Most naked microflagellate cells were presumably

killed by differential pressure on the Millipore filter. Most of the surviving motile species were eliminated since they generally require for growth thiamine or other vitamins (Hastings and Thomas, 1977), and none was added to the enrichment medium. Diatom cultures, being brown, could be generally recognized by eye, and discarded. This left cultures of more or less green cells that could be roughly divided into the following categories:

1. Cylindrical, green cells, with a parietal plastid; about 2.5 micrometers wide. These were assigned to the species Stichococcus bacillaris, a common euryhaline green alga (Brown and Hellebust, 1978).
2. As above, but smaller; about 1.5 micrometers wide. These grew more slowly; we consider them to represent another species of Stichococcus.
3. Round or oval cells, dividing into 2's; about 2 micrometers wide. These are characteristics of the green algal genus Nannochloris.
4. As above, but larger; about 3-4 micrometers wide. Such algae might also be assigned to the genus Nannochloris.
5. Round cells, about 2.5 micrometers in diameter; dividing into 4's; shed walls round, open at one side only. Such features are typical of ubiquitous species of Chlorella.
6. Oblong cells, 3-4 micrometers long; shed walls often cylindrical and sometimes open at both ends. Their identity is unknown.

Out of the 130 original samples collected, some 39 yielded at least a few floating greenish algal cells in the second, N-depleted subculture. After streaking on agar and re-isolating colonies, 180 clonal cultures were initially established. Several of these grew poorly when bacterial

contaminants were eliminated, suggesting that these algal strains required for growth certain vitamins which were being supplied by metabolic activities of the associated heterotrophs. Like the diatoms at an earlier stage in the isolation procedure, these fastidious strains were eliminated. Further elimination of apparent duplicates from the same source reduced the total number of clones from 60 to 48: 4 more or less unicellular prokaryotes, and the rest eukaryotes. (With cells in the range of 2 micrometers it is not always easy to make this distinction even under magnifications of x400.) The latter included 14 clones with short rod-shaped cells characteristic of the green algal genus Stichococcus, 21 referable (on the basis of cell form and division) to Nannochloris, 4 to Chlorella and 5 to other generic types. We assumed that the prokaryotic cells floated by reason of gas vacuole production, since blue-green algae are generally low in lipid, and they, too, were set aside. Attention was concentrated on the Stichococcus and Nannochloris types.

When droplets of pure cultures were set on agar media prepared with a freshwater (non-saline) medium, all of the Stichococcus and Chlorella isolates grew well, indicating that they may have originated from brackish, estuarine, freshwater or soil sources in which cells of these genera are common. It is perhaps significant that Johnson and Sieburth (1982) reported no cultures of cells of Stichococcus among their samples, which were collected from more open-ocean sources. Of the Nannochloris-type strains, 6 proved capable of growth on non-marine media, while the remaining 15 could not.

All of the eukaryotic algae that we isolated here proved to contain both chlorophylls a and b, indicating that they were chlorophytes. All of the extracts on chromatographic examination showed two green bands (in addition to

various yellow and orange bands indicative of carotenoids). Chlorophyll a/b ratios, determined indirectly by use of the equations of Jeffrey and Humphrey (1975), for the Stichococcus strains ranged from 0.89 to 1.43 (average, 1.13) and for the Nannochloris strains from 0.5 to 2.42 (average, 1.53). Thus we had no evidence that any of our strains should be assigned to the genus Nannochloropsis (Eustigmatophyta), which lacks chlorophyll b, and which in earlier work had shown promise of being exceptionally valuable as a potential source of hydrocarbon-rich lipids. Nevertheless, probably because we had selected species that form at least a few floating cells, many of our strains produced appreciable quantities of lipids, though we have still no information as to the fractions constituted by hydrocarbons. In cultures initially enriched with 800 microgram-atoms of nitrate-N per litre, and grown for 3 weeks at 25° with constant aeration and artificial illumination, we obtained cells with the following lipid contents (expressed as ash-free dry weights): Stichococcus (13 samples), range 9-59%, average 33%. Nannochloris (21 samples), range 6-63%, average 31%. These values are not much lower than those obtained for 3 Nannochloropsis strains, from Qingdao, China, grown under comparable conditions: Nannochloropsis (6 samples), range 31-68%, average 46%.

A summary of the positive results obtained in this survey is presented in Table I. (Water samples from sites that yielded no algae of the desired types have been excluded.) Eight pure clones of Nannochloris isolated from different sources, and selected because they produce much lipid and grow well in ordinary sea-water media without organic supplements at temperatures up to 35°C, have been chosen for further physiological and biochemical study.



## Discussion

Marine eukaryotic picoplankton organisms are clearly of considerable ecological importance and interest: some  $10^6$ - $10^8$  cells in this category per litre have been reported from the euphotic zone (Murphy and Hangen, 1985; Johnson and Sieburth, 1982). Nevertheless, because of the obvious difficulties of working with such small, nondescript cells, there have been few attempts to identify the predominant species. Among these we may list publications by Butcher (1952), Wilhelm and Wild (1981), Sarokin and Carpenter (1982), and Turner and Gowen (1984).

There have been even fewer studies that have concentrated on the picoplankton of the surface layers, because of additional problems involved in collecting satisfactory samples. In a review of marine plankton (Cheng, 1975) there are references to a few seaweeds like Sargassum, but none to microscopic algae, apart from those mentioned in a study of marine foams by Maynard (1968). Indeed, few marine ecologists have even considered the possible existence of a specific microflora, which we may call the picoplankton, associated with the sea surface. Johnson and Sieburth (1982) examined some of the cells from this habitat by scanning and transmission electron microscopy, and identified some of the predominant forms. These included a non-flagellate alga that divided into 2's; they identified it as Nannochloris sp. The trouble with such a provisional identification lies in the fact that this genus is poorly defined on morphological grounds. When cells of this sort are examined biochemically, they may prove to belong to at least two different pigment classes, respectively assigned to the Chlorophyta and the Eustigmatophyta. (This is not the place to discuss separation of algal subclasses on the sole differential criterium of pigmentation, which may not be

as immutable as one is generally led to believe.)

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Summary of piconeuston algae isolated from Caribbean Sea sites: water samples collected 1-15 Feb 85.

Site #	Location		Sticho.	Nanno.	Cyano.	Chlorella	Other
1	St Johns, Virgin Islands	Lameshor Bay, mangrove swamp	+				
13	St Thomas, Virgin Islands	Compass Point Marina, mangrove swamp		+	+		
19	"	Mandal Bay, open water		+			+
21	"	Hull Bay		+			
26	Tortola, Virgin Islands	Road Town, mangrove inlet	+				
29	"	" stream estuary	+				
30	"	Can Garden Bay, mangrove swamp		+			
35	St Croix, Virgin Islands	Tamarind Reef Club Marina, mangrove swamp	+				+
36	"	Great Salt Pond, open water	+				
37	"	" mangrove pool		+			
38	"	" open water		+			
40	"	Robin Bay, mangrove pool				+	
42	"	Boiler Bay, open water		+			
45	"	Teague Bay, N.I.L. pier		+			
46	"	Salt River, Hydrolab pier		+			
50	Puerto Rico	Piñones Beach	+				
52	"	Boca de Cangrejos Harbour	+	+			
53	"	San Juan, Condado Lagoon		+			
55	"	San Juan, Embarcadero pool					+
56	"	Aguadilla					+
58	"	Mayagüez Harbour	+	+			+
59	"	"	+				
61	"	Guanijibo	+				
62	"	Punta Arenas, "wet laboratory"	+				
64	"	Joyuda lagoon	+	+			
65	"	"	+	+			
70	Curaçao	Boca Santa Martha mangrove pool					+
71	"	" offshore					+
72	"	" inner bay		+			
73	"	Willemsted, Avila Beach Hotel		+			
75	Panamá	Naos, inlet west of causeway				+	
86	"	Thatcher Ferry Bridge, mangrove		+			
87	"	Cocoli, by dyke, mangrove		+			
98	"	Galeta, S.T.R.I. laboratory, foam	+				
101	"	" roadside, mangrove		+		+	
105	"	Isla Margarita, lagoon		+			
115	Miami, Florida	Pines Canal, stagnant pool					+
120	"	Mile 108 on U.S. 1, mangrove					+
126	"	Key Largo, lagoon		+			

# Collection and Characterization of Saline Microalgae From South Florida

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## ABSTRACT

Sixty-one species of microalgae were isolated from saline habitats of Florida and the growth of 110 species was assessed in desert saline waters. Twenty-five of the species met our initial criteria for "good growth." The growth for 4 of these species were examined in more detail on a cross temperature-salinity gradient and 8 species were examined for proximate chemical composition. The influence of individual carbon species concentration on the potential yield of microalgae was also examined in an experimental design that decoupled carbon concentration from pH effects.

## INTRODUCTION

In 1983, SERI initiated a microalgal species acquisition 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 desirable species should grow rapidly under fluctuating culture conditions and be capable of producing large concentrations of lipid. The acquisition 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<sup>2</sup> open pond system found the cost of CO<sub>2</sub> 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<sub>2</sub>, 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<sub>2</sub>, HCO<sub>3</sub><sup>-</sup>, CO<sub>3</sub><sup>=</sup>, TIC or pH.

## MATERIALS AND METHODS

**Field Collections:** Field collections were conducted (June and September 1985 and February 1986) in the Everglades and Florida Keys regions of South Florida (Figure 1). Water column and sediment samples were obtained by pipet and placed in culture tubes and polyethylene bottles. Samples were kept cool and in the dark until return to the laboratory. Water temperature, salinity (refractometer), conductivity and pH were measured at each location. Total alkalinity was determined by titration to pH 4.2 with standardized HCl.

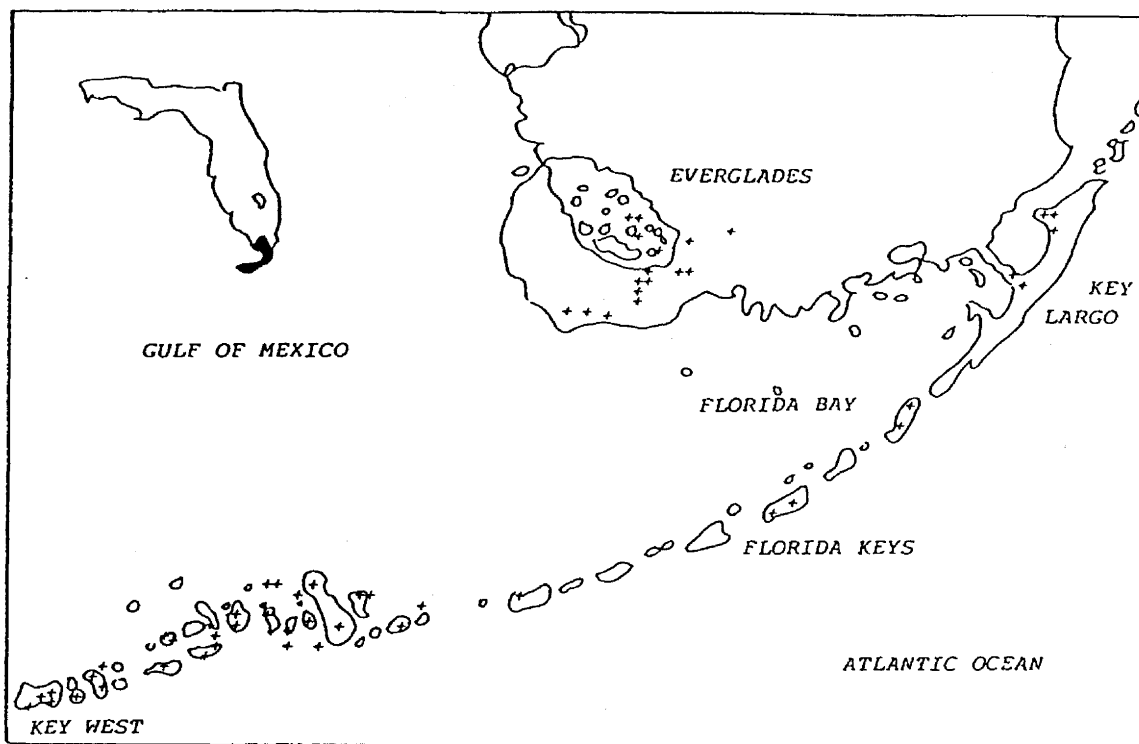


Figure 1. Locations (+) of South Florida sites sampled during 1985-86.

**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  $440 \mu\text{M NO}_3^-$ ,  $36 \mu\text{M PO}_4^{3-}$ , ES metals and vitamins<sub>2</sub> (Provasoli 1968) and incubated under continuous high light ( $830 \mu\text{E m}^{-2} \text{sec}^{-1}$ ) intensity and temperature ( $30^\circ\text{C}$ ). Tubes were screened 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, water from each location was inoculated into  $18 \times 150$  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  $25^\circ\text{C}$  on a 16:8 L/D cycle under  $100 \mu\text{E m}^{-2} \text{sec}^{-1}$  illumination and were mixed daily.

Isolation into unialgal culture was accomplished by a combination of procedures including: population transfers into media containing different  $\text{N}_2$  forms and various salinities; serial washings in sterile media via micropipets; agar plating; and treatment with selective poisonings.

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, SERI Type I and II desert waters (Barclay et al. 1985) at 2 different salinities. Cultures were incubated at 30°C under continuous  $300 \mu\text{E m}^{-2} \text{sec}^{-1}$  illumination and read directly in a spectrophotometer following light vortex mixing. Growth rates were assessed daily for 5 days and the maximum optical density was determined after 10 days. Species were considered good growing strains based on exponential growth rates  $> 1$  doubling day<sup>-1</sup> in 2 medias and a maximum optical density of 0.3 following 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 by fluorescence microscopy (excitation wave length, 455-500 nm; emission wave length  $> 515$ ). 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 evaluations, species already in culture were selected for growth characteristics in variable temperatures and salinities based on visual estimation of production. The growth of these species was examined at 15, 25 and 35°C in F/2 or ES enriched natural seawater in constant temperature incubators illuminated at  $100 \mu\text{E m}^{-2} \text{sec}^{-1}$ . 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 58 mmhos cm<sup>-1</sup> salinity at 25°C. Optical densities were determined from triplicate flasks and growth rates were calculated daily (doubling day<sup>-1</sup>, OD 750) 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 ASP-2 artificial seawater were grown in 250 ml flasks at 5 temperatures (15, 20, 25, 30, 35°C) and 5 salinities (8, 22, 29, 47, 59 mmhos cm<sup>-1</sup>) under  $180 \mu\text{E m}^{-2} \text{sec}^{-1}$  constant illumination. Optical densities were determined daily from duplicate flasks and growth rates were calculated over a 5 day period.

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  $\mu\text{M}$  nitrate) enriched natural seawater. Temperature was controlled ( $26 \pm 1.5^\circ\text{C}$ ) 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  $\mu\text{M}$  or 440  $\mu\text{M}$  nitrate. Cultures were mixed by magnetic stirring and grown under continuous illumination ( $250 \mu\text{E m}^{-2} \text{ sec}^{-1}$ ) at  $28 \pm 1^\circ\text{C}$ . Both cultures were sparged with 1%  $\text{CO}_2$  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 in the 440  $\mu\text{M}$  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  $\mu\text{M}$  culture was harvested following 7 days in the stationary stage.

Cells were harvested by centrifugation, washed, 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  $50^\circ\text{C}$ . 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 ( $60^\circ\text{C}$ ) prior to repeated extractions in chloroform-methanol. Following adjustment of solvent ratios and phase separation, the chloroform fraction was dried under  $\text{N}_2$ . Organic contents of the cells were expressed as the fraction of the weight loss following ignition of the original sample at  $500^\circ\text{C}$  for 1 hour.

Carbon Studies: 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. The independent manipulation of pH, alkalinity and total inorganic carbon (TIC) was accomplished with  $\text{Na}_2\text{CO}_3$  and  $\text{CO}_2$  gas in  $28.5 \text{ mmho cm}^{-1}$  ASP-2 (2.5 mM  $\text{CaCl}_2$ ) artificial seawater.<sup>2</sup> The carbon equilibrium program DEBUSKER (Ryther et al. 1984) was modified into 2 versions specific to ASP-2. The first calculated total alkalinities for a range of pH values which yielded Iso- $\text{CO}_2$  and Iso- $\text{HCO}_3^-$  levels. The second version calculated carbon species concentrations based on TIC values measured with a carbon analyzer (Model 700, OI Corporation). Addition of design alkalinity (as  $\text{Na}_2\text{CO}_3$ ) 1 mM urea, 30  $\mu\text{M}$   $\text{PO}_4$  and F/2 metals and vitamins to 3 parts distilled  $\text{H}_2\text{O}$  precluded precipitation of carbonates or hydroxides when 7 parts  $46.5 \text{ mmho cm}^{-1}$  ppt ASP-2 was added. Eight 9.5 L carboys containing 4 L of medium were maintained at the constant pH values of 7.5, 8.0, 9.0, 9.5 and 10. One percent  $\text{CO}_2$  in air or  $\text{CO}_2$ -free air was sparged into the cultures as required by individual pH controllers. Maximal growth rates and maximal and mean biomass production were measured for each carboy by O.D. and ash-free dry weight determinations. Total alkalinities were selected such that over a range a pH values 3 cultures had equal  $\text{CO}_2$  concentrations and 3 had equal  $\text{HCO}_3^-$  concentrations.

## RESULTS AND DISCUSSION

Collection and Screening: A variety of South Florida saline habitats were sampled for microalgae including mangrove swamps, salt flats, canals, ditches and numerous shallow ponds. Physical and chemical characteristics of 123 collection sites are summarized in Table 1. Over 60% of the habitats

recorded water temperatures  $> 30^{\circ}\text{C}$  and salinities  $> 25 \text{ mmho cm}^{-1}$ . To date, 61 species have been isolated into culture including 30 species of chromophytes.

Table 1. Physical and chemical characteristics of locations sampled in the Florida Keys (n=83) and Everglades (n=32) June and September 1985, and February 1986 collection trips.

COLLECTION SITE CHARACTERISTICS	FLORIDA KEYS $\bar{X} \pm \text{s.d.}$	EVERGLADES $\bar{X} \pm \text{s.d.}$
Temperature ( $^{\circ}\text{C}$ )	$29.30 \pm 4.52$	$29.70 \pm 3.26$
Salinity (ppt)	$30.10 \pm 11.89$	$21.80 \pm 11.03$
Conductivity (m mhos $\text{cm}^{-1}$ )	$35.60 \pm 14.26$	$25.72 \pm 13.23$
pH	$8.04 \pm 0.38$	$8.00 \pm 0.36$
Total Alkalinity (Meq/L)	$3.49 \pm 2.06$	$5.01 \pm 3.24$

The preliminary growth evaluation of 25 chromophyte species grown in natural seawater and SERI Type I and II desert waters are presented in Table 2. Generally, members of the Prymnesiophyceae including the Coccolithophores and Ochromonads displayed good growth with species isolated from the initial screening growing better than strains previously isolated in our collection. Dinoflagellates, which commonly formed blooms in the study areas, did not grow in the SERI desert waters.

Of 110 species evaluated, 25 species were considered good growing strains and were stained with Nile red for lipid content (Table 3). Over 75% of these species displayed better growth in SERI Type II desert water than Type I. Indeed, growth was generally better in Type II water than natural seawater. Although there were no clear trends within phylogenetic groups, Type I appeared to be a poor basal medium for the good-growing species isolated in this study. Oleaginous species were selected for subsequent growth characterization on the temperature-salinity gradient.

Growth Characterization: Growth characteristics of chromophytes examined prior to the screening protocol are presented in Table 4. Exponential growth rates corresponded well with findings obtained in preliminary screening experiments. The coccolithophores Gloeothamnion and Hymenomonas (HB89) grew well at high temperature and light while tolerating variable salinities. In contrast, Amphidinium and Chrysocapsa growth rates were reduced at temperature extremes ( $15$  and  $35^{\circ}\text{C}$ ) and low salinities. Pleurochloris, Chattonella, Cryptomonas and Chryso/C1 exhibited good growth in natural seawater under high light and temperature, but these species did not grow in the Type I and Type II desert waters at  $25$  and  $40 \text{ mmho cm}^{-1}$ .

Table 2. Exponential growth rate (doublings day<sup>-1</sup>, 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 salinity.

SPECIES	GSW		TYPE I		TYPE II	
	25	40	25	40	25	40
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
Rhizochrysis HB23	0.28/0.02	0.13/0.02	0.27/0.02	0.37/0.05	NG/NG	NG/NG
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
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
Chryso/C4 HB60	1.00/0.21	1.15/0.25	0.69/0.20	0.48/0.19	1.55/0.50	1.01/0.24
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
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
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
Cryptomonas HB119	0.48/0.04	0.54/0.04	0.09/0.03	NG/NG	0.26/0.01	0.58/0.09
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
Chattonella HB122	0.64/0.10	0.47/0.06	NG/NG	0.26/0.04	NG/NG	NG/NG
Pleurochloris HB123	1.02/0.14	0.83/0.17	0.40/0.10	0.61/0.12	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
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
Chryso/S33 HB147	NG/NG	0.06/0.03	NG/NG	0.04/0.03	NG/NG	NG/NG
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

Table 3. Exponential growth rate/maximum optical density (doublings day<sup>-1</sup>, OD 750) for species exhibiting good growth in Type I, Type II and natural seawater (GSW) at 25 and 40 mmho cm<sup>-1</sup> and Nile red lipid evaluation (see text for details).

SPECIES	GSW		TYPE I		TYPE II		NILE RED LIPID EVALUATION
	25	40	25	40	25	40	
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	++
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	++
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	+++

Table 4. Exponential growth rate (doublings day<sup>-1</sup>, OD 750) and maximal optical density of chromophyte species grown under various temperatures, salinities and full sunlight conditions.

SPECIES	TEMPERATURE (°C)			FULL SUNLIGHT (31 ± 4°C)	SALINITY mmho cm <sup>-1</sup> (25°C)				
	15°	25°	35°		8	22	29	47	59
Chrysocapsa HB16	0.23	0.78	0.51	0.60	0.14	0.10	0.96	0.94	0.28
Chryso/C1 HB24	0.51	1.14	1.19	0.83			ND		
Hymenomonas HB38	0.89	0.88	1.13	0.62			ND		
Gloeothamnion HB42	1.07	1.36	1.40	1.28	0.79	0.91	1.05	0.98	1.01
Hymenomonas HB89	0.45	0.63	0.81	1.45	0.42	0.72	0.76	0.70	0.68
Chryptomonas HB119	0.71	1.31	0.93	0.84			ND		
Chattonella HB122	0.36	0.84	0.69	1.16			ND		
Pleurochloris HB123	0.23	1.01	1.06	1.44			ND		
Amphidinium HB838	0.37	1.20	0.48	2.39	NG	NG	0.87	0.98	0.77

ND = Not Determined  
NG = No Growth

Four good-growing species from the preliminary evaluation were examined for temperature-salinity tolerance on the cross gradient plate (Figure 2). All species grew well over a wide range of salinities and temperatures and exhibited exponential growth rates of 1 doubling day<sup>-1</sup> or greater between 8 and 60 mmho cm<sup>-1</sup> and 20-35°C. HB54, a yellow-green unicell considerably larger than *Nannochloropsis*, (>10 µM) displayed good growth over 90% of the experimental range with >1.5 doubling day<sup>-1</sup> between 19-35°C and 15-60 mmho cm<sup>-1</sup> (maximum 3.13 doubling day<sup>-1</sup>). Optimal growth conditions for *Pyramimonas* (HB133) and *Tetraselmis* (HB47) were between 19-35°C and 15-60 mmho cm<sup>-1</sup> (maximum doubling day<sup>-1</sup> for HB133 = 2.67, HB47 = 2.17). Only HB154, an unidentified olive-green unicell, had relatively narrow temperature/salinity ranges with growth rates<sup>-1</sup> of > 1.5 doublings day<sup>-1</sup> restricted between 22-32°C and 8-53 mmhos cm<sup>-1</sup> (maximum 2.30 doubling day<sup>-1</sup>).

The results from the temperature-salinity gradient experiments indicate that the preliminary screening protocol successfully identified species that can grow under unstable culture environments. Experiments are in progress to characterize the growth of the good-growing species at optimal temperature and salinity in Type I and Type II desert waters. An additional 5 species will be evaluated on the temperature-salinity gradient in the next 2 months.

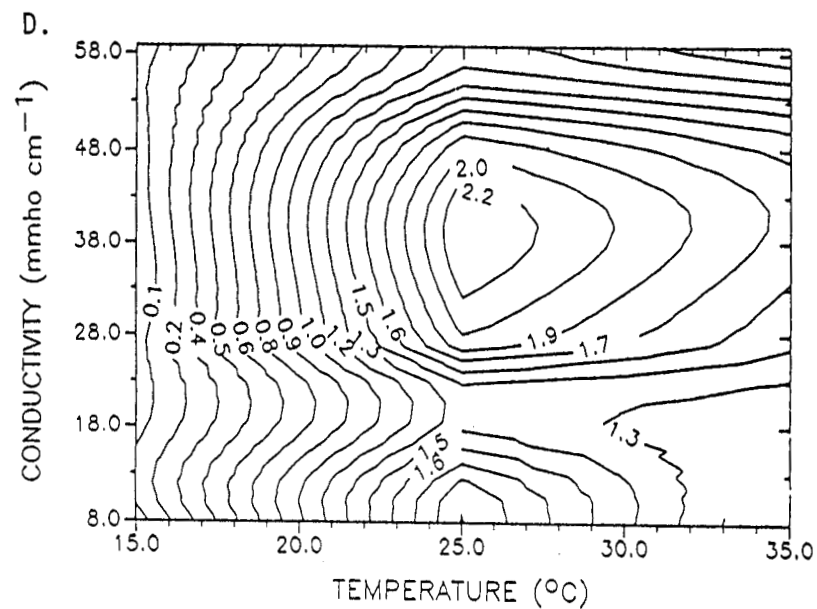
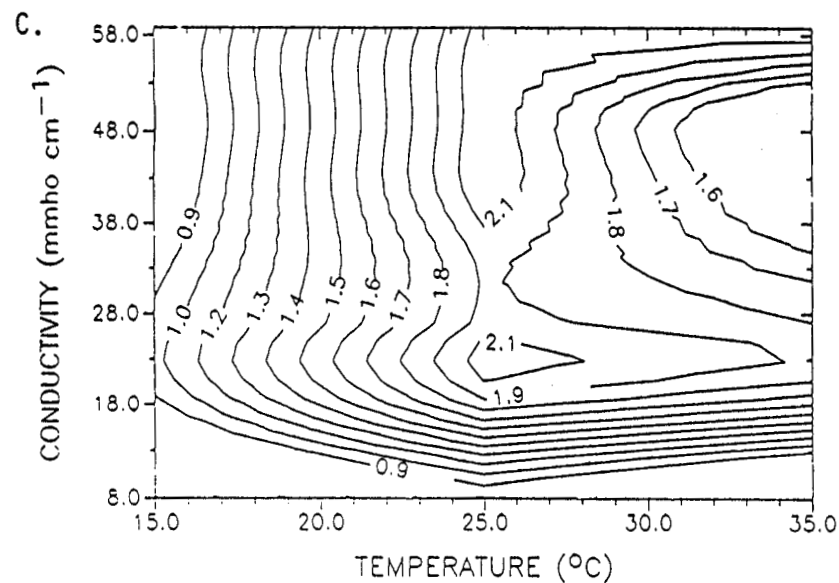
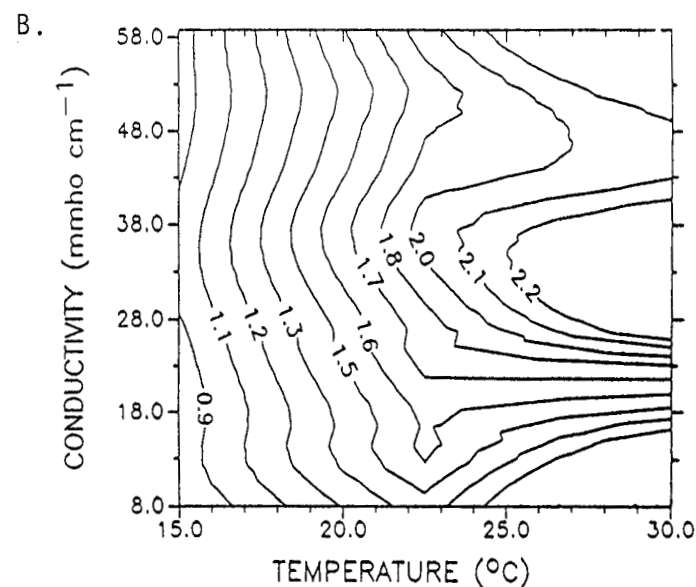
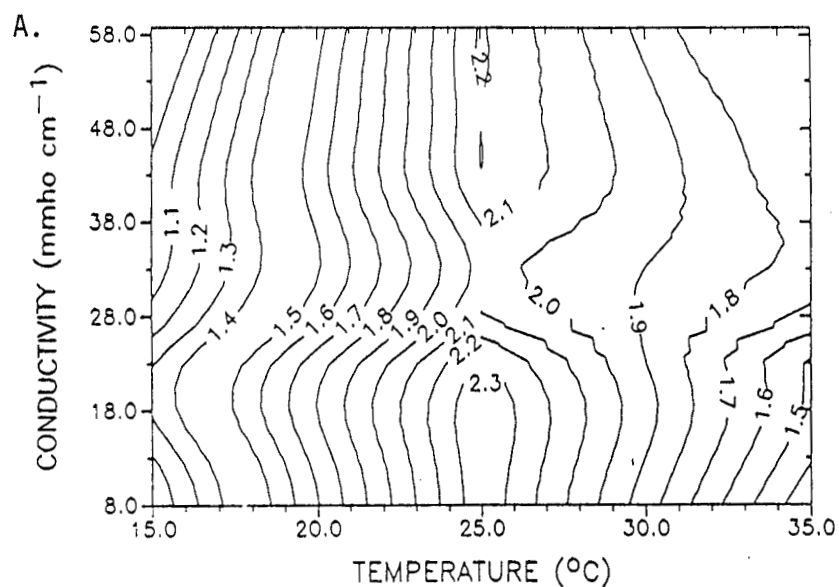


Figure 2. Growth response in batch culture of HB54-Yellow Green Unicell (A), HB133-Pyramimonas (B), HB47-Tetraselmis (C), and HB154-Olive Green Unicell (D) to temperature and salinity. Contours are exponential growth rate (doubling day<sup>-1</sup>, O.D. 750).

Proximate Analysis: The biochemical composition of select microalgae was examined in outdoor and laboratory culture (Table 5). Seven species were surveyed in outdoor carboy cultures for the accumulation of lipid early in the stationary growth phase. All cultures were grown under nitrogen sufficient conditions. Protein levels were relatively low and exhibited little variation (8-12% AFDW). Carbohydrates varied between 11% (*Hymenomonas* HB89) and 47% (*Amphidinium*). *Hymenomonas* (HB89) and *Amphidinium* produced the greatest amount of lipid (19.7 and 18.5%, respectively), but the maximum lipid produced by all species ranged from 14-20%, thus indicating there was no significant early lipid accumulation in the microalgae tested.

Laboratory experiments examined lipid induction due to nitrogen limitation for 2 species which exhibited good growth over a wide range of temperatures and salinities. One-half the volume from both an 880  $\mu\text{M}$  and a 440  $\mu\text{M}$  nitrogen culture was harvested when the low-N culture reached stationary phase. The remaining volume of the low-N culture was harvested following 7 days of stationary phase. Exponential growth ceased after 6 days for *Hymenomonas* and on day 4 in *Tetraselmis*. Protein content decreased with nitrogen deficiency in both species (Table 5). In *Hymenomonas*, protein and lipid were replaced with carbohydrate which increased in early stationary phase. Total lipids in *Hymenomonas* actually decreased from 20.7% to 16.8% lipid, a response similar to that found for *Hymenomonas carterae* with nitrogen deficiency (Shifrin and Chisholm 1981). Carbohydrate increased and lipid decreased between the N-sufficient and early N-depletion stage for *Tetraselmis*. However, the opposite response occurred between early N-depletion and N-deficient conditions. Lipid levels increased nearly 25% at the expense of carbohydrate, but this increase was not significantly different from N-sufficient cultures. Lipid evaluation of this strain of *Tetraselmis* with Nile red indicated a substantial increase in lipid content in 21-day vs. log phase cultures (as did a number of other Prasinophyceae). This may indicate that lipid accumulation in this strain may begin late in the stationary phase. These observations suggest that the cultures in the above experiment were harvested too early to show any significant lipid accumulation with nitrogen deficiency. Nevertheless, no significant early lipid production was demonstrated for *Hymenomonas* and *Tetraselmis*. Within the next 2 months we will provide additional results for 4 more species.

Carbon Studies: The purpose of the carbon studies was to characterize a variety of algal strains and to correlate productivity with  $\text{CO}_2$ ,  $\text{HCO}_3^-$ ,  $\text{CO}_3^{2-}$ , TIC and pH while decoupling carbon concentration from pH effects. Two species have been evaluated to date and the maximum exponential growth rate, maximal production and average production was determined for each species. Neither growth rate nor production were dependent on alkalinity (0.12 - 9.7 meq/L) over a pH range of 7.5 - 10.0.

Figures 3 and 4 show the results for *Chlorella* and *Isochrysis* (TISO) average production vs.  $\text{CO}_2$ ,  $\text{HCO}_3^-$ , TIC concentration and pH. In this study, exponential growth rates were poor indicators of productivity and correlated only with pH for *Isochrysis*. Visual inspection of the plots showed the best correlation of average production was with  $\text{CO}_2$  concentration for both species. Correlation also appeared to be fairly good with pH, but we believe this reflects the coupling of  $\text{CO}_2$  and  $\text{H}^+$  concentration. Correlation analysis of the mean production with the log of the carbon concentrations

Table 5. Proximate chemical composition of South Florida microalgae cultivated in the laboratory and outdoors (See Methods for culture conditions). NS = nitrogen sufficient, END = early nitrogen depletion and ND = nitrogen depleted cultures at time of harvest.

SPECIES	GROWTH CONDITION	PROTEIN		% AFDW CARBOHYDRATE		LIPID	
		$\bar{X} \pm$ S.D.		$\bar{X} \pm$ S.D.		$\bar{X} \pm$ S.D.	
OUTDOORS							
Chrysocapsa HB16	END	8.3	$\pm$ 0.3	35.4	$\pm$ 1.2	14.6	$\pm$ 0.6
Chrysosphaera HB20	END	8.6	$\pm$ 0.3	42.0	$\pm$ 0.6	17.3	$\pm$ 0.2
Hymenomonas HB38	END	9.0	$\pm$ 0.3	42.7	$\pm$ 1.2	13.9	$\pm$ 0.5
Gloeothamnion HB42	END	9.1	$\pm$ 0.4	23.9	$\pm$ 0.0	16.0	$\pm$ 2.7
Hymenomonas HB89	END	12.2	$\pm$ 0.1	11.1	$\pm$ 0.7	19.7	$\pm$ 0.4
Eremosphaera HB90	END	8.3	$\pm$ 0.2	27.2	$\pm$ 0.4	17.1	$\pm$ 2.4
Amphidinium HB838	END	10.6	$\pm$ 0.3	46.9	$\pm$ 2.3	18.5	$\pm$ 0.0
LABORATORY							
Hymenomonas HB89	NS	10.50	$\pm$ 0.71	32.10	$\pm$ 1.10	24.40	$\pm$ 0.14
	END	7.60	$\pm$ 0.07	42.10	$\pm$ 3.80	20.70	$\pm$ 0.85
	ND	4.10	$\pm$ 0.00	37.00	$\pm$ 5.60	16.80	$\pm$ 2.75
Tetraselmis HB47	NS	7.30	$\pm$ 0.30	48.55	$\pm$ 3.65	20.45	$\pm$ 0.85
	END	5.45	$\pm$ 0.05	59.75	$\pm$ 2.35	16.40	$\pm$ 0.70
	ND	3.50	$\pm$ 0.60	36.45	$\pm$ 1.75	22.30	$\pm$ 1.50



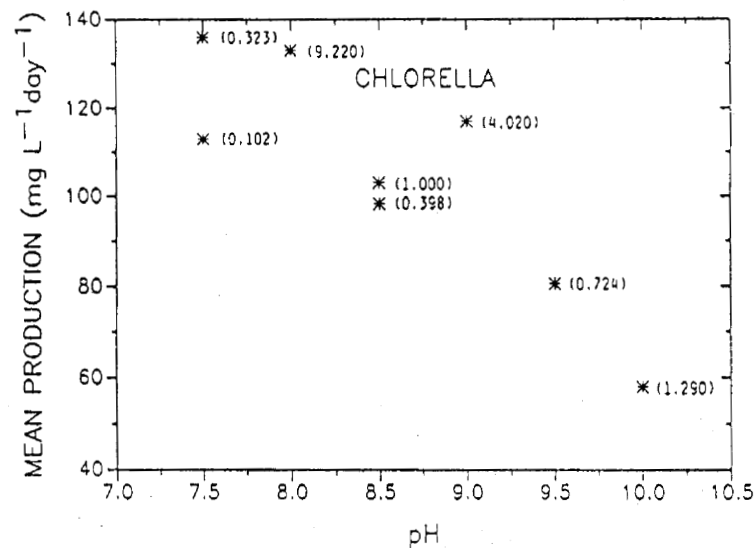
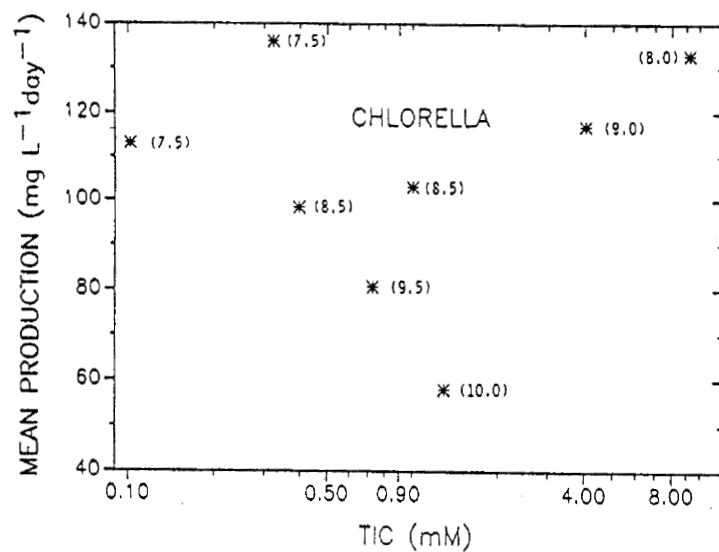
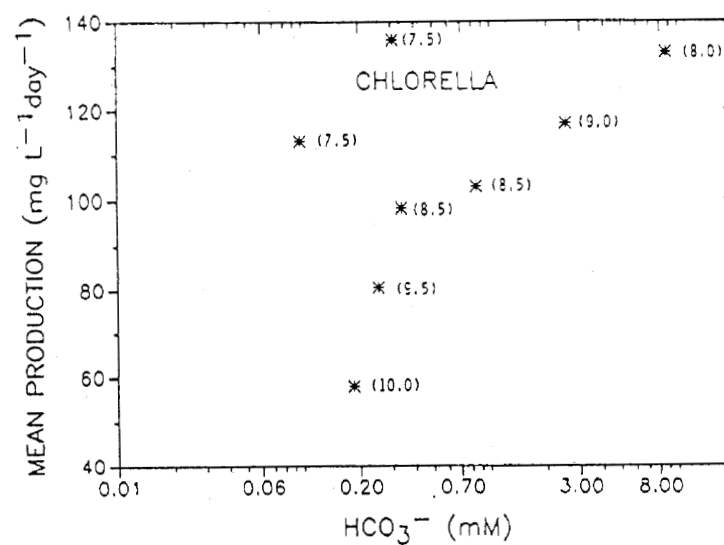
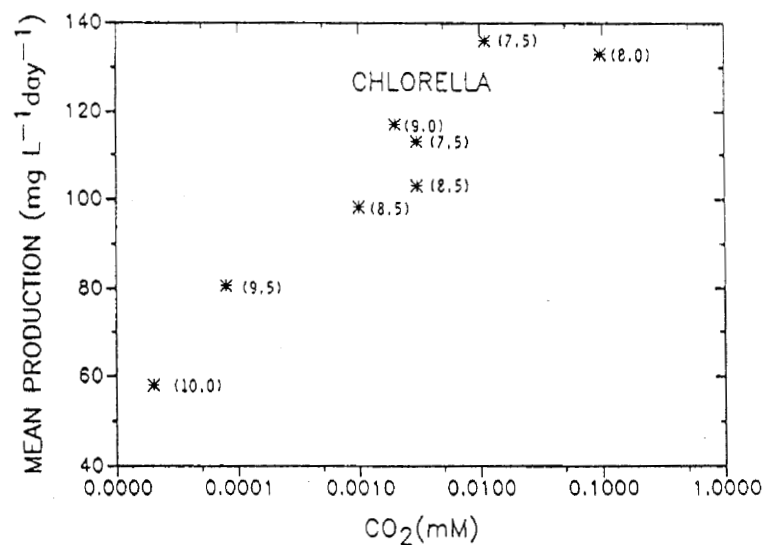


Figure 3. Average production ( $\text{mg L}^{-1} \text{day}^{-1}$  AFDW) vs  $\text{CO}_2$ ,  $\text{HCO}_3^-$ , TIC concentration and pH for *Chlorella*. Numbers on carbon plots are pH; numbers on pH plot are TIC concentration.

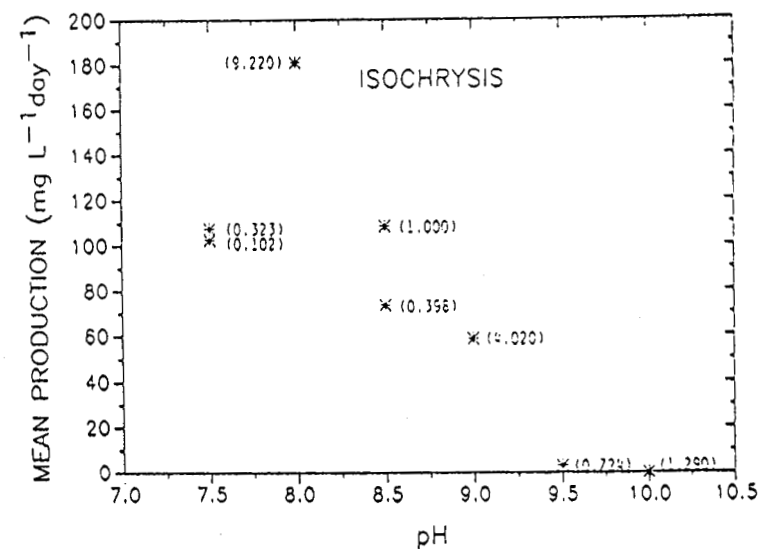
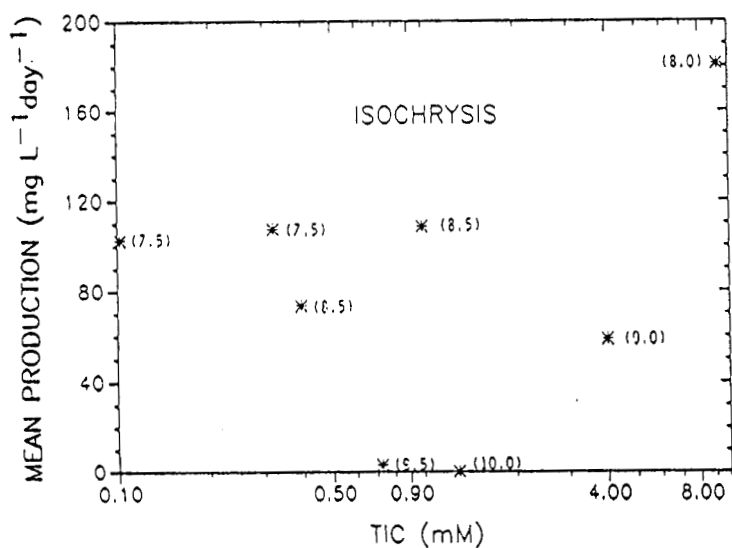
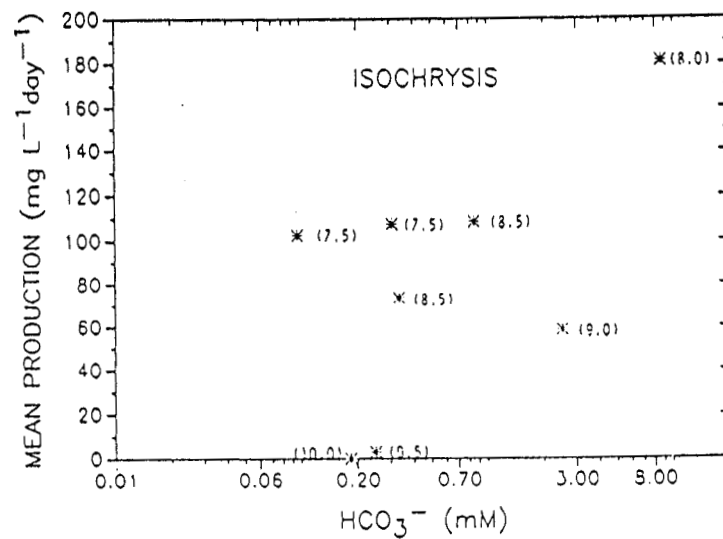
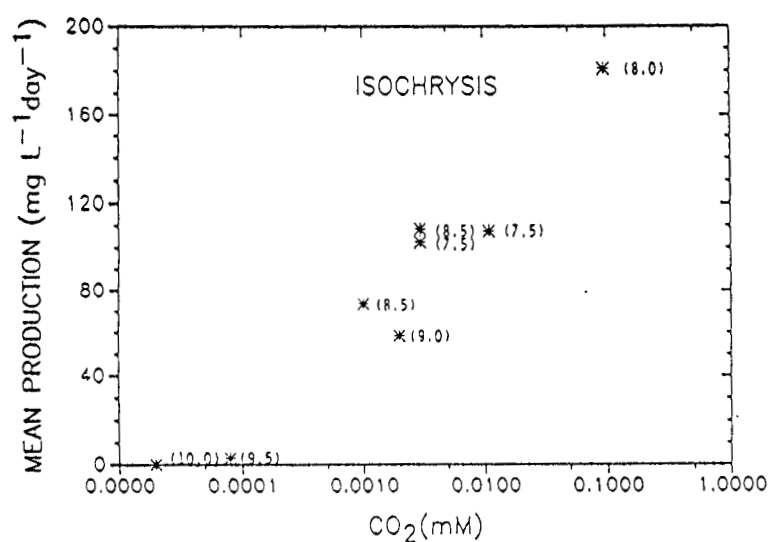


Figure 4. Average production ( $\text{mg L}^{-1} \text{day}^{-1}$  AFDW) vs  $\text{CO}_2$ ,  $\text{HCO}_3^-$ , TIC concentration and pH for Isochrysis (TISO). Numbers on carbon plots are pH; numbers on pH plot are TIC concentration.

confirmed the conclusions arrived by inspection. For Chlorella, average production was not statistically different (Duncan's Multiple Range Test,  $p = 0.05$ ) at  $3 \mu\text{M CO}_2$  over the pH range of 7.5 - 9.0. Isochrysis production at  $3 \mu\text{M CO}_2$  was identical between pH 7.5 and 8.5 demonstrating a more restricted pH tolerance.

The results are significant because we have found good correlation of productivity with a particular carbon species concentration over a wide pH range (Chlorella 7.5 - 9.0). This supports similar findings for a freshwater strain of Chlorella (Weisman and Goebel 1985). Experiments are in progress with Nannochloropsis (Nanno-Q) and we will provide additional results for 3 more species during the next 2 months.

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.

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It is likely that at some time in the future, genetic engineering of selected algal strains will be performed to improve lipid production in strains isolated from the field. Before this work can be undertaken, the degree of genetic variability present in closely related algal strains, representing single species or species complexes, must be established. In order to assay genetic variability, we are currently comparing the physiology, morphology, and biochemistry of a number of strains of Chaetoceros muelleri isolated from various locations in the western United States. This species was chosen because representative strains are generally euryhaline, tolerant of high temperatures, rapid growing, and high in lipid content.

Presently, the growth response of three strains of C. muelleri to water type, conductivity, and temperature has been determined. Two strains, those from brackish waters in Utah and New Mexico, are very similar, both having optimal growth rates of over 3 doublings/day in SERI type II waters at temperatures between 25 and 35 C. Both strains are nearly euryhaline. These strains also appear morphologically identical. The third strain, from Lake Tuendae, California, is very different physiologically. It is slower growing, having optimal growth rates of only 1.7 doublings/day. It does poorly in SERI type II waters, growing best in SERI artificial seawater (GPM) media of low conductivity (10-25 mmhos/cm). It does very poorly or ceases to grow in all waters of high conductivity (55-70 mmhos/cm). Morphologically, the three strains are almost identical, with only one minor morphological difference having been noted so far. Based on morphology, all three are circumscribed by the description for Chaetoceros muelleri var. subsalsum.

These three strains demonstrate that within a single species, substantial genetic variability exists. We plan to continue our study of variability in C. muelleri and related species, having 38 strains currently in our culture collection from which to choose. We plan to characterize selected species in terms of: 1) growth response to water type, conductivity, and temperature, 2) growth response to light intensity, 3) lipid content, 4) sterol composition, 5) pigment ratios, and 6) enzymatic differences as determined by starch gel enzyme electrophoresis.

## FACTORS AFFECTING THE PHOTOSYNTHETIC YIELD OF MICROALGAE

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### ABSTRACT

The objectives of the subcontract to Microbial Products, Inc. include the outdoor cultivation of pre-screened algal species for an evaluation of productivity potential and competitiveness, development of harvesting technologies, engineering evaluation and design of photobioreactors, and physiological characterization of strains in the laboratory to assess factors which affect outdoor yield. There is substantial interplay among these objectives, especially the first and the last.

Twelve pre-screened species of algae were grown in 1.4 m<sup>2</sup> pond reactors, including six diatoms and six chlorophytes. Four of the diatoms (three strains of Chaetoceros and a Cyclotella) could be cultivated for as long as was attempted, from 28 to 125 days, at average productivities of about 30 gm<sup>-2</sup>d<sup>-1</sup>. The other two diatoms (Amphora sp. and Navicula sp.) were as productive but could not be sustained in culture. Productivity from the chlorophytes was generally only one-half to one-third as much as from the diatoms. Only species of one genus, Tetraselmis, endured in the outdoor culture tanks.

Factors which were found to affect both the yield and longevity of outdoor mass culture were photoinhibition, oxygen inhibition, temperature, pH, pCO<sub>2</sub>, dilution rate, and nutrient status. Species which could not be sustained were often found to be unstable at high irradiance and/or high oxygen. Usually inability to adapt to the pond environment resulted quickly in the loss of a strain. In one case, predominance of Tetraselmis over Nannochloris, competition from another species determined the longevity of a culture. Changes in many of the variables listed above did result in productivity differences. However, the factor which determines the maximum yield is most likely the irradiance at which photosynthesis saturates. How to determine this and how to optimize it still remain elusive goals.

## SUMMARY

Eight species of microalgae were cultivated in outdoor culture in 1.4 m<sup>2</sup> tanks. The four diatoms grown exhibited productivities from 25-40 gAFDW/m<sup>2</sup>/day. Each species was inhibited only to a small extent by high levels of dissolved oxygen, in both outdoor culture and in a laboratory test designed to reveal sensitivity to oxygen. Of six green algae screened in this laboratory test, four were highly sensitive to high oxygen. Two of these species could not be sustained in outdoor culture under any conditions while the other two produced less than 20 gAFDW/m<sup>2</sup>/day. Further investigation showed that high light levels were also potentially inhibiting to photosynthetic biomass production, and especially so when combined with the high oxygen concentrations. During an adaptation period which lasted several days, dilute suspensions used for the measurement of maximum specific growth rate were 50% inhibited at 550 uE/m<sup>2</sup>/s compared to 185 uE/m<sup>2</sup>/s. When incident irradiance was high, photoinhibition could be mitigated by avoiding periods during which the culture density was low (and cells exposed continuously to high light) by diluting continuously or semi-continuously. Batch cultures, grown from very low cell density to high density, exhibited a delay in adaptation. Upon dilution of a dense batch culture, specific growth rate was still high under high irradiance. But when the batch culture became dense, the productivity was not as great as continuous cultures (at comparable density) which were never exposed to a high average irradiance. Continuous dilution, or daily dilution, may thus result in higher average productivity than dilution every 2-3 days as was done in the outdoor mass cultures described.

Results presented demonstrate that microalgae vary in their ability to grow productively at high pH (9-10) or CO<sub>2</sub> < 25 uM, conditions which are likely to prevail in a large scale fuel production system. Of five species tested, two (Chaetoceros sp. (S/CHAET-2) and Chlorella sp. (S/CHLOR-2)) were 33% inhibited (in terms of productivity) at pH 9, two (Amphora sp. (S/AMPHO-1) and C. gracilis (S/CHAET-1)) were not inhibited at this pH, and one (Ankistrodesmus sp. (S/ANKIS-3)) was unaffected even at pH 10. Productivity of the Ankistrodesmus sp. and the Amphora sp. decreased by 20% when CO<sub>2</sub> concentration was reduced from saturating levels to about 20 uM. The reduction was 33% at about 5 uM CO<sub>2</sub> for the Chaetoceros sp., and 20 and 40% at less than 2 uM CO<sub>2</sub> for the C. gracilis and Chlorella sp respectively.

An increase in the lipid content of outdoor cultures of Cyclotella sp. (S/CYCLO-1) to over 40% was induced by limiting the input of silicon. However, the lipid productivity of about 9 gAFDW/m<sup>2</sup>/day was not higher than that of Si sufficient cultures which grew at higher overall productivity. Results with nitrogen starved induction of carbohydrate as a primary storage component in Chaetoceros sp. revealed that microalgae have the capability of

shifting almost completely to synthesis of either protein or non-protein compounds at about equal photosynthetic efficiency.

## BACKGROUND AND PROJECT OBJECTIVES

The Aquatic Species Program has supported projects which have led to the isolation and partial characterization of a large number of species of microalgae. The testing of pre-screened microalgal species for performance in outdoor mass culture systems has been one of the primary functions of Microbial Products, Inc. as an ASP subcontractor. As a result of this testing we have identified traits which make certain species candidates for mass cultivation for fuel production. The feedback from the outdoor studies and the continuing development of laboratory screening protocols is important in order to focus laboratory and outdoor culture research on the most promising strains.

Among the factors identified as important in affecting the performance of microalgal species in outdoor culture and the cost of biomass production are photoinhibition, the concentrations of CO<sub>2</sub> and O<sub>2</sub> in the medium, as well as pH. The hypothesis from the previous year's work (1), that tolerance to dissolved oxygen, which accumulates in the culture liquor during active growth, is a major factor in determining competitiveness in outdoor mass culture was supported by this year's work. Organisms which grow well at higher pH and low CO<sub>2</sub> concentrations can be cultured at lower cost due to the more efficient use of carbon.

## RESULTS FROM OUTDOOR MASS CULTURES

### Productivity and Culture Longevity

All outdoor cultivation was carried out in eight 1.4 m<sup>2</sup> fiberglass tanks (1.22 m<sup>2</sup> illuminated area due to paddlewheel shading) operated at 15 cm depth, mixed by paddlewheel, heated, deoxygenated (down to 150-200% of air saturation), and pH controlled at 7.5 by injection of 100% CO<sub>2</sub> on demand. Details of operations, the media used, and methods are given in the Final Report (2). A summary of the major results is presented in Table 1. All productivity results are on an ash free dry mass basis. Lipid content is given as a percent of total organic mass.

Of the four diatoms tested, three were able to be cultivated for as long as was attempted (see Table 1). One, the Amphora sp., died after two to three weeks of cultivation, and was displaced by the Cyclotella, under every condition tested. However, for short periods of time the Amphora was the most productive of all organisms, yielding 45-50 gAFDW/m<sup>2</sup>/d for each of five days during the first ten days after inoculation. On these days, photosynthetic efficiency was greater than 10%. Even including the declining productivity during the third week of cultivation, productivity averaged over 30 gAFDW/m<sup>2</sup>/d at an efficiency of about 7%. Cyclotella exhibited the highest sustained productivity, over 35 gAFDW/m<sup>2</sup>/d (8.5% efficiency) during July, when diluted every two days. During August, productivity and efficiency dropped off 25% and 20%, respectively. Productivity was almost 30 gAFDW/m<sup>2</sup>/d (6% efficiency) for one month (in duplicate) during June and July, at a three day dilution interval. Both



Table 1. Outdoor Results Summary

	Culture #	Dates	Days	Max Daily DO % Air Sat	Dilution Interval days	Productivity $\pm$ SDOM gm/m <sup>2</sup> /day	I <sub>0</sub> lngy/day	PAR %	Lipid $\pm$ S.D. (n) %
Cyclotella sp.* (S/CYCLO-1)	1	6/25-7/15	21	500	3	28.1 $\pm$ 0.5	664	6.0	27.1 $\pm$ 0.6 (3)
	2	6/13-7/15	33	500	3	29.6 $\pm$ 0.7	659	6.2	25.2 $\pm$ 4.5 (4)
	1	7/16-7/27	10	500	2	35.2 $\pm$ 1.6	600	8.3	23.1 $\pm$ 3.1 (6)
	2	7/16-7/27	10	150-300 <sup>3</sup>	2	37.6 $\pm$ 2.0	600	8.6	25.0 $\pm$ 2.4 (6)
	1	8/8 -8/14	6	500	2	28.2 $\pm$ 1.0	585	6.8	20.0 $\pm$ 3.0 (2)
	2	8/8 -8/20	12	500	2	26.0 $\pm$ 1.2	564	6.4	-
Chaetoceros gracilis (S/CHAET-1)	1	6/7 -7/15	39	500	3	22.5 $\pm$ 1.0	664	4.9	30.0 $\pm$ 4.8 (4)
	2	6/7 -7/15	39	150,300	3	25.6 $\pm$ 0.8	664	5.6	33.7 (1)
	1	7/16-7/21	6	500	2	29.1 $\pm$ 2.5	561	7.1	25.5 $\pm$ 3.1 (6)
	2	7/16-7/21	6	200 <sup>3</sup>	2	26.9 $\pm$ 3.6	561	6.6	26.2 $\pm$ 4.9 (7)
Chlorella ellipsoidea (S/CHLOR-2)	1	6/22-7/15	24 <sup>1</sup>	300-500	3	13.1 $\pm$ 0.4	648	2.5	-
	2	6/22-7/15	24	150 <sup>3</sup>	3	14.1 $\pm$ 1.5	648	2.6	-
T. suecica * (S/PLATY-1)	1	8/16-9/6	21	400-500	2-4	18.0 $\pm$ 1.5	510	4.3	20.4 $\pm$ 1.8 (2)
	2	8/20-9/6	17	140-190	2-4	20.3 $\pm$ 1.5	510	4.9	23.1 $\pm$ 5.0 (2)
Nannocloropsis * 85-21	1	7/26-8/29	26 <sup>2</sup>	300-500	2-4	14.9 $\pm$ 0.8	582	3.4	20.4 $\pm$ 1.1 (2)
	2	7/26-8/29	29 <sup>1</sup>	150-200	2-4	15.4 $\pm$ 1.0	581	3.6	22.1 $\pm$ 1.6 (3)
Amphora sp. (S/AMPHO-1)	1	7/22-8-13	20	500	2	30.5 $\pm$ 1.5	608	6.7	-
	2	7/24-8/13	16	200-500	2	31.0 $\pm$ 2.1	596	6.9	19.4 $\pm$ 0.5 (2)
Chaetoceros sp.* SS14 (S/CHAET-2)	1	8/6 -9/2	28	500,150-200	2	24.3 $\pm$ 2.6	544	6.0	21.0 $\pm$ 3.5 (4)
	2	8/6 -9/2	28	500	2	22.6 $\pm$ 2.4	544	5.6	21.7 $\pm$ 3.0 (3)

Kcal calculated from proximate composition, either as measured (\*), or determined as 50% protein, lipid as measured, and CHD by difference.

Temperature: Max 30-34°C, Min 16-20 °C

<sup>1</sup> Required re-inoculation

<sup>2</sup> Required re-inoculation twice

<sup>3</sup> Oxygen removal caused 20-30% coverage of surface with foam

species of Chaetoceros yielded similar productivities considering the differences in time of cultivation. Again, productivity was greater at a two day dilution interval than at three days. Cell densities ranged from 60 to 650 mg/L at three day cycles and 70 to 450 at two days. All of the diatom cultures, with the exception of Amphora, were virtually totally free of contaminating organisms. This remained true the entire duration of cultivation.

Of the three members of the Chlorophyceae cultivated outdoors, only the Tetraselmis grew as long as attempted. Neither the Chlorella nor the Nannocloropsis could be grown in a sustained manner. Productivity of the Tetraselmis was 18-20 gAFDW/m<sup>2</sup>/d regardless of frequency of dilution or cell density. Average productivities for the other green algae were about 15 gAFDW/m<sup>2</sup>/d. A fourth chlorophyte, Ankistrodesmus sp (SERIS/ANKIS-3) was inoculated in September 1985. It was grown for two weeks. However, dilution was intermittent, cell density was quite high (600-1200 mg/L), and productivity was low (10-20 gAFDW/m<sup>2</sup>/d, data not shown).

#### Effect of High Concentration of Dissolved Oxygen

One of the major goals was to determine the effects of the normally high DO on productivity and stability of cultures. Without special means taken to remove oxygen, it increased each day to between 400-500% of air saturation levels by 1000-1100 hours, remained there most of the day, and did not go much below air saturation at night. Sparging air into the cultures was effective at removing the oxygen, but usually caused excessive foaming. Eventually anti-foam was added four times a day to reduce the area occluded by foaming to less than 10% (and usually 5%) of the total pond area.

The results of four DO experiments are analyzed in Table 2. Antifoam was used with Chaetoceros SS14 and T. suecica. None was used with C. gracilis, but foaming (occluded area) was still less than 10% during this experiment. None was used with the Cyclotella. In this case foaming was a problem, covering 20-30% of the pond area at all times. Although the results of the experiment test as significant for this species, a 7% systematic error uncovered in replication experiments (2) nullifies the small increase found here at lower DO. Still, high DO may actually inhibit growth since production was essentially the same despite 20-30% less free surface area in the de-oxygenated pond due to the thick foam. In the other cases, the low DO culture was always more productive, again despite the presence of foam. The difference was 22% for C. gracilis, 12% T. suecica, and over 12% for Chaetoceros sp. SS14 (including a 3% systematic error). Although all of these differences were significant at  $p < .01$ , they were all small in magnitude.

High dissolved oxygen had an effect on the performance of C. ellipsoidea and Nannocloropsis which was not reflected in terms of biomass productivity. Although neither of these organisms could be cultivated in a sustained manner, at least at the cell densities attempted (eventually 300-500 mg/L), the cultures in which DO was allowed to accumulate died more quickly and thus had to be re-inoculated twice as often as those from which DO was removed. It may be that oxygen was never lowered enough to allow sustained growth of these species.

Table 2. Effect of Dissolved Oxygen on Productivity

Species	#Days/n*	Productivity (D.O. %Sat.)		Low-High	p	Area Occluded by Foam
		Low	High			
Chaetoceros SS14 (S/CHAET-2)	10/5	26.0 $\pm$ 6.0 (150-180)	23.7 $\pm$ 5.4 (400-500)	2.32 $\pm$ 0.89	<.005	<5%
Cyclotella sp. (S/CYCLO-1)	10/5	37.7 $\pm$ 4.4 (150-225)	35.4 $\pm$ 3.5 (400-500)	2.30 $\pm$ 1.68	<.01	20-30%
C. gracilis (S/CHAET-1)	27/9	25.5 $\pm$ 3.4 (150)	21.1 $\pm$ 2.2 (500)	4.67 $\pm$ 2.56	<.0005	<10%
T. suecica (S/PLATY-1)	17/6	20.5 $\pm$ 3.7 (140-190)	18.3 $\pm$ 3.8 (400-500)	2.22 $\pm$ 1.44	<.01	<5%

\* n = number of dilutions (samples),  $\pm$ S.D.

#### INHIBITION OF ALGAL GROWTH BY OXYGEN AND LIGHT

##### Mechanisms of Oxygen Inhibition

Oxygen, a major product of the photosynthetic reactions, has generally been found to be inhibitory to net biomass production. Photorespiration, the uptake of oxygen in the light, and the release of carbon dioxide, is usually taken to be a competitive inhibition between carbon dioxide and oxygen for action by the carboxylating enzyme, ribulose biphosphate carboxylase-oxygenase (Rubisco) (3,4). A major feature of this form of photorespiration is its sensitivity to low availability of carbon substrate, oxygen concentration, and hence high light intensity. The concentrations of carbon dioxide and oxygen at the site of enzymatic action are governing. The specificity of Rubisco for CO<sub>2</sub> over O<sub>2</sub> varies from 30-100 depending on conditions and species. Aquatic organisms which do not exhibit oxygenase activity under low external CO<sub>2</sub> concentrations are usually found to have the ability to concentrate, by active transport, carbon at the site of Rubisco (5,6). There are however reports of inhibition by oxygen even under saturating carbon dioxide. Additional mechanisms may be present, including several reactions which occur without a related release of CO<sub>2</sub> (7,8,9,10) as well as mitochondrial respiration (11).

The inhibitory effects of strong light on pigment formation, on the activity of reaction centers, and on the activation state of photosynthetic enzymes, may be aggravated by high dissolved oxygen even at saturating CO<sub>2</sub> concentrations. Photo-oxidative destruction of enzymes and intermediates may be involved. The growth response of algae to light intensity is complex. In short term tests of photosynthetic capacity, species respond to constant light in a manner characterized by a saturating light intensity, a saturating rate of photosynthesis, and a light intensity at which inhibition sets in (12,13). The light level to which the cells have been adapted (grown) greatly affects these response parameters (12,14), with adaptation

to high light generally increasing all of them.

### Test Protocol

In previous work under this subcontract (1), it was determined that the indigenous strains of algae that invaded and dominated freshwater and brackish water cultures were largely tolerant to high levels of DO. Species from the ASP screening program, mostly chlorophytes at that time, were not. It was thus speculated that tolerance to oxygen was one of several important factors that determined the outcome of species competition. Therefore, a protocol used to test for sensitivity to DO was standardized and applied to all of the species obtained from the ASP screening program. 750 ml batch cultures were grown in one liter Roux flasks, incubated at  $29 \pm 1$  °C at pH 7.5, under continuous illumination at  $185 \text{ uE/m}^2/\text{day}$ , under conditions of saturating  $\text{CO}_2$  (0.5-1.0%). Two flasks of each species were grown, one bubbled with air/ $\text{CO}_2$ , one with  $\text{O}_2/\text{CO}_2$ . The bubbling rate was 1 vol/vol/min through fritted aquarium spargers. The maximum specific growth rate was determined from the first 16 hours of growth and the average productivity from the next 72 hours, a period during which production was nearly constant in the controls. All tests were initiated using algae grown and thus adapted to air/ $\text{CO}_2$ . Levels of DO were measured, using an Orion DO probe, on a regular basis. The rationale for this protocol was to measure oxygen effects at highest rates of growth and at high production rates under conditions which eliminated the confounding effects of carbon supply limitation and photoinhibition. It was suggested that this would uncover extreme sensitivity to DO.

The results from experiments with ten species of algae are given in Figure 1. Two of the species, S. quadracauda and an Oocystis sp. were tested during the previous year. The former was the dominant organism in ponds when TDS was below 5 gm/L and maximum temperature below 30 °C, the latter was not able to compete in outdoor pond cultures at all. Of the ten species tested, six are green algae and four are diatoms. None of the diatoms exhibited a sensitivity to high DO in the test situation. Only two of the greens were insensitive, the Scenedesmus and the Utah Ankistrodesmus. Replication of results was very good for those organisms which were tolerant to DO. Four of the chlorophytes were at least somewhat oxygen inhibited. Two, T. suecica and C. ellipsoidea, were 40% inhibited in terms of specific growth rate, and 70% inhibited in terms of productivity. The other two chlorophytes were 80-90% inhibited. All four of the sensitive species were very unstable under high oxygen conditions, as evidenced by the large variability in the degree of inhibition. Visually, the inhibition appeared to be cumulative, with growth decreasing, pigmentation decreasing and cells becoming more subject to rupture as time went on. This may be, in part, the reason for the heightened effect on productivity versus growth rate in these batch cultures.

In summary, the growth of algae under carbon saturating, low irradiance conditions does separate species in terms of relative tolerance versus significant sensitivity to high levels of dissolved oxygen. As discussed below, higher resolution in determining oxygen sensitivity may be achievable under more stressful conditions.

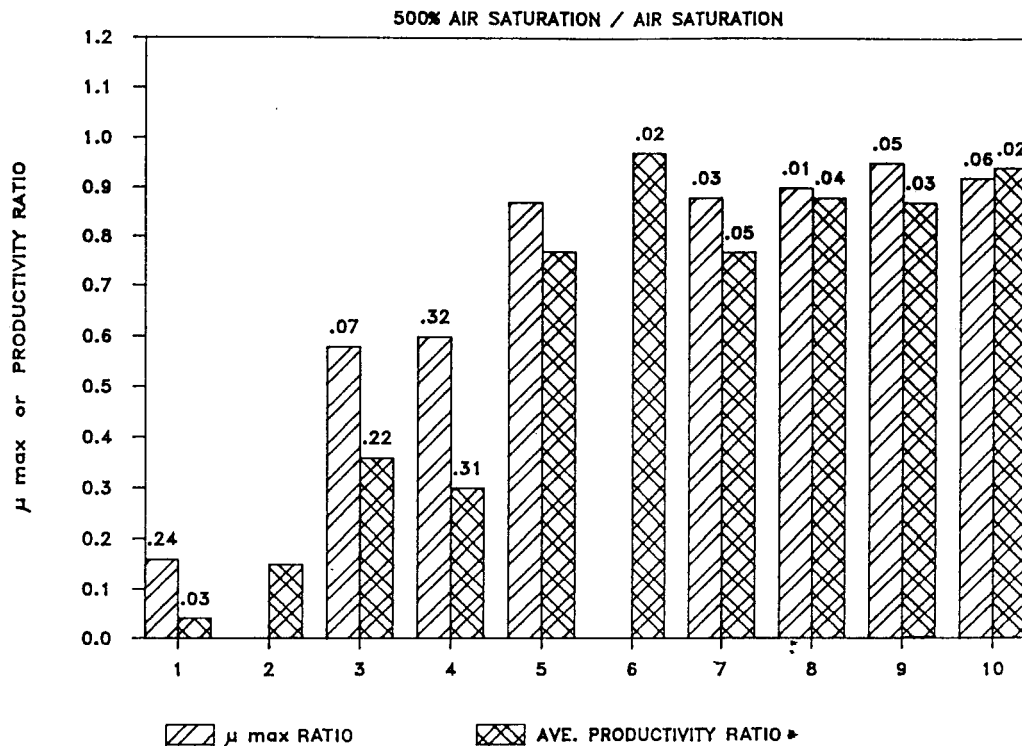


Figure 1. Oxygen Inhibition of Laboratory Algal Cultures

- |                                   |                            |
|-----------------------------------|----------------------------|
| 1. Nannocloropsis sp. 21          | 6. Scenedesmus quadricauda |
| 2. Oocystis sp. (S/OOCYS-1)       | 7. Chaetoceros gracilis    |
| 3. Tetraselmis suecica            | 8. Chaetoceros SS14        |
| 4. Chlorella ellipsoidea          | 9. Cyclotella sp.          |
| 5. Ankistrodesmus sp. (S/ANKIS-3) | 10. Amphora sp.            |

Numbers above bars are standard deviations of duplicates.  
See text for growth conditions.

#### Comparison Between Laboratory and Outdoor Results

No species that tested poorly in the laboratory under high DO grew well outdoors under any condition, and all that tested well indoors exhibited potential for high productivity in the field. Two species that were extremely sensitive to oxygen in the laboratory test could not be grown outdoors (*Nannocloropsis* sp, *Oocystis* sp); of two that were less, but still quite sensitive, one could not be grown (*Chlorella*) and the other (*Tetraselmis*) was only moderately productive; and of four that tested less than 20% inhibited by oxygen indoors, three grew well and were slightly sensitive outdoors as well, and one grew erratically (*Amphora* sp), being the most productive species in the short term but not sustainable in the longterm. These results suggest that the protocol used in screening species in the laboratory is valuable, but needs to be extended in terms of other factors which affect outdoor growth.

## Effects of Oxygen and Irradiance on Algal Growth

In initiating work to improve the predictive capabilities of indoor screening tests, the effects of higher incident and average irradiance were investigated using some of the same species. Inhibition of productivity was measured in terms of the expected Bush ratio (15) for production at one irradiance vs a lower one. When no inhibition occurs, the Bush ratio gives the ratio of productivities based on the light intensity at which photosynthesis saturates,  $I_k$ . With inhibition, this actual ratio would be lower. In this study,  $I_k$  was estimated from plots of  $u_{\max}$  vs irradiance. This is not the usual method for determining  $I_k$ . Preliminary experiments using the Ankistrodesmus and Amphora indicated that the former was inhibited at high average irradiance and by oxygen under high incident irradiance while the latter was tolerant to oxygen but more sensitive to high incident light (2).

A detailed investigation was undertaken using Chaetoceros sp S/CHAET-2. Experiments in which algae were grown in batch (inoculated at low starting density and allowed to increase 40-160 fold) were compared to experiments in which the algae were grown semi-continuously (SC) until unchanging values for growth rate or productivity were obtained (in separate experiments). This was accomplished by daily dilution of the cultures so that they were either very dilute for "maximum" specific growth rate measurements or dense enough for average productivity measurements always under light limited conditions. Cultures were incubated at  $29 \pm 1$  °C and pH  $7.5 \pm 0.2$ .

The best estimates of the maximum specific growth rate of the organism were obtained from SC cultures which were diluted 50-250 fold daily. Initial cell densities were less than 1 mg/L and often less than 0.1 mg/L. They were calculated from accurately known dilution factors. Final cell densities varied from 10-40 mg/L, determined by ash free dry mass analysis. At each irradiance the growth rate was also determined at least once using the concentration of chlorophyll *a* to estimate algal density. With this degree of dilution at these low densities, the fraction of light absorbed by the suspension was kept below 10%, effectively eliminating self shading most of the time. The measured values for  $u_{\max}$  and the composition of the cell mass are shown in Figure 2. Growth rate saturated at about 185  $\mu\text{E}/\text{m}^2/\text{s}$  at eight doublings per day. A 15% inhibition occurred at higher irradiances. Both chlorophyll content and protein content decreased with increasing irradiance. Pigment decreased faster as evidenced by a decreasing value of chlorophyll expressed as percent protein.

Changes in cell composition occurred within one generation of transfer of cells from a dense culture to an optically thin one. The changes also occurred if the transfer was only to a moderately dilute suspension i.e., the incident irradiance exerted substantial control over cell composition as long as the culture was kept dilute enough so that most of the time was spent in high light, above the growth rate saturating irradiance of 185  $\mu\text{E}/\text{m}^2/\text{s}$ . This is demonstrated by the data in Table 3. The growth rate and composition of the following suspensions are given: dense, batch culture; batch culture 16 hours after dilution; batch culture with pronounced lag; moderately dilute SC culture; and fully adapted, very dilute SC culture.

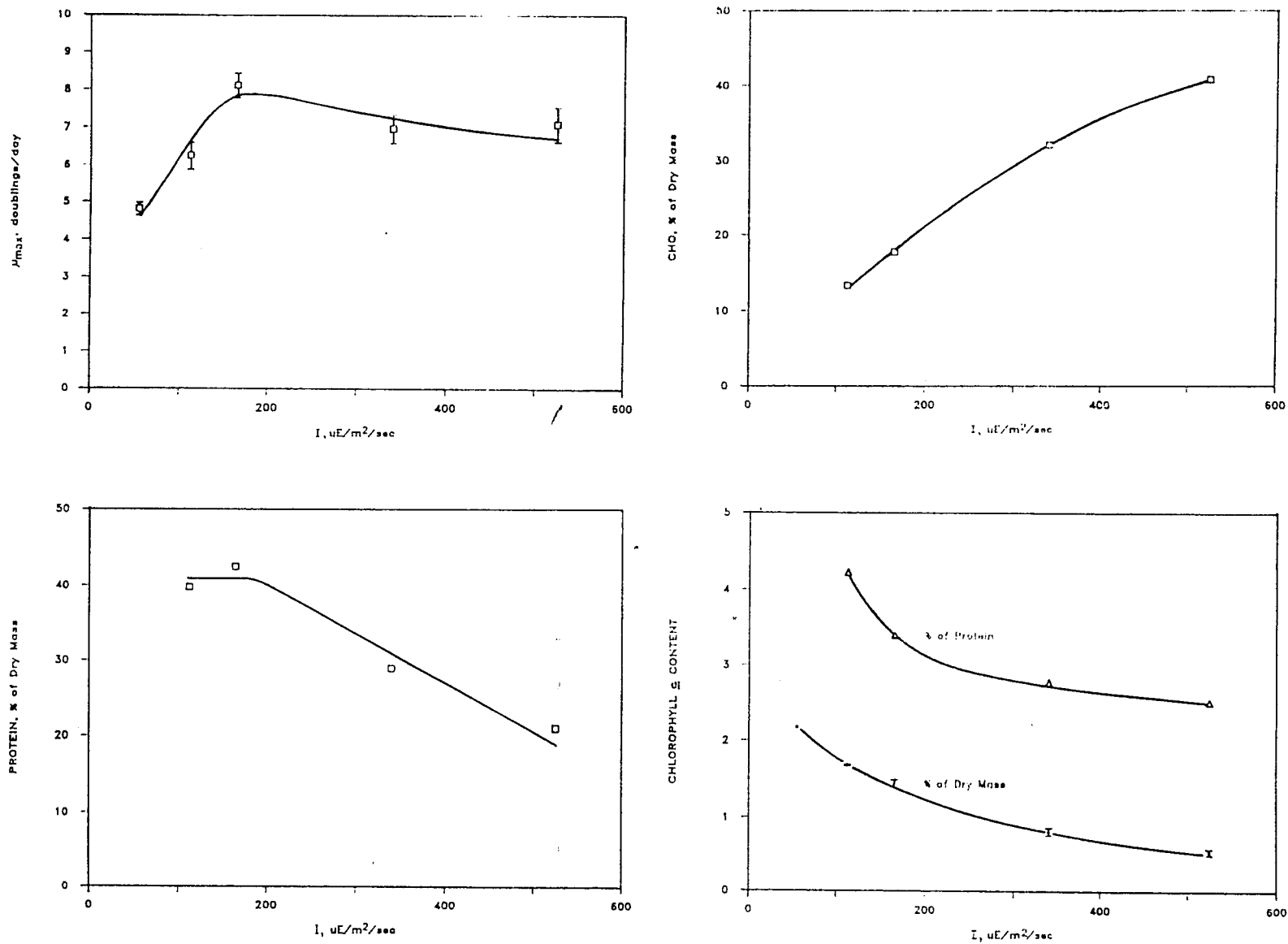


Figure 2. Growth Rate and Composition of Dilute, Semi-Continuous Cultures of Chaetoceros S/CHAET-2 as a Function of Irradiance.

Growth rate was highest for suspensions that were fully adapted to a uniform irradiance. In sequential batch cultivation neither pigment nor protein content fell as low as in semi-continuous cultures, except during lag phases. The dilute batch culture appears to have had enough time at low cell density to adapt partially, attaining a relatively high growth rate, but not a maximal one. The pigment to chlorophyll ratio of about three indicates that there remained a significant degree of light limitation. Composition changes occurred within one to two generations. As the density increased, without further dilution, these compositional changes were reversed. The SC cultures operated at moderate density grew more slowly than diluted batch cultures. These SC cultures had been forced to adapt to a continuous, generally high irradiance (they were not allowed to get very dense). Apparently, this repressed pigment content further than time had allowed in the batch culture. Still, significant self shading occurred during part of each day resulting in light limitation part of the time. This suggests that the repression of pigment production (or destruction of pigment) by high irradiance is uncoupled from the response to light limitation which generally results in greater pigmentation. Very dilute, adapted SC cultures were the fastest growing, presumably owing to the most compatible matching of composition and average irradiance. Their composition was similar to that of the SC cultures operated at higher density (lower dilution factor) but the average light was saturating even at such a reduced pigment content. As shown in Figure 2, however, some light inhibition was exhibited by these cultures when fully adapted. More inhibition in the form of a pronounced lag was sometimes observed right after initial exposure to high light (Table 3).

Table 3. Composition of Cell Mass During Batch and Semicontinuous Cultivation  $I_0=600 \text{ uE/m}^2/\text{s}$ , 1%  $\text{CO}_2$  in Air.

	BATCH			SEMI-CONTINUOUS CULTURE	
	Dense	16 hours After Dil.	Lag	AVERAGE IRRADIANCE, $\text{uE/m}^2/\text{s}$	
				385	540
Minimum Irrad. $\text{uE/m}^2/\text{s}$	0	--	--	230	480
Initial Density mg/L	1300	21	25	25	.15
Final Density mg/L	2000	144	100	200	20
Growth Rate doublings/day	0.6	4.3	2.0	3.1(.14)	7.01(.3)
Final Chlor. a % dry mass	3.0	1.02	.37	0.50(.07)	0.46(.03)
Final Protein % dry mass	58	36	22	26.6(2.7)	21.0
Final CHO % dry mass	16	60	65	43.1(1.6)	38.7
Final Chlor. a % protein	5.17	2.83	1.7	1.7	2.2

Numbers in () are SDOM



As batch cultures grow, the average irradiance becomes low and the composition adjusts to increase unit rates of absorption versus assimilation. The productivity of batch cultures, after the point at which most of the incident light has been absorbed, is compared to that of SC cultures operated at optimum cell density in Figure 3. Productivities of dense, SC cultures were always higher than the (dilute to dense) batch cultures, but substantially so at higher light intensity. In fact, when the saturating light intensity is assumed to have been  $185 \text{ uE/m}^2/\text{s}$  ( $40 \text{ Wm}^2$ ), the productivity ratios of SC cultures were very close to those predicted by the Bush equation (Table 4). This was not the case for the batch cultures (see Figure 3). Apparently, the low starting densities of the batch cultures resulted in light induced changes, especially at higher light, which affected the subsequent productivity when the cultures became dense. The lowering of pigment content and adjustment of composition right after dilution may not have been reversed fast enough over the next generation of growth. Thus the cell composition became out of phase with the light field.

Table 4. Productivity of Semi-Continuous Cultures of Chaetoceros sp.  
Under Air / 1% CO<sub>2</sub>

Incident irradiance Wm <sup>-2</sup>	irradiance uE/m <sup>2</sup> /s	Productivity ratio	Bush ratio
25	115	.71 (.02)	.625
40	185	1.0	1.0
80	370	1.66 (.11)	1.7
120	550	2.13 (.13)	2.1

Productivity data from dense cultures ( $\mu = 1$  doubl./day)  
 $21.2 \text{ g/m}^2/\text{day}$  at  $40 \text{ Wm}^2$  SDOM = 0.44 Eff. = 14.2%  
 Bush ratio =  $1 + \ln(I_0/I_k)$ ,  $I_k = 40 \text{ Wm}^2$   
 Ratios are with respect to growth at  $40 \text{ Wm}^2 = 185 \text{ uE/m}^2/\text{s}$   
 Numbers in () are errors determined by propagating SDOM's

The light limited productivity of batch cultures depended on the initial density (Table 5). At  $370 \text{ uE/m}^2/\text{s}$ , when batches were started at higher density (90 ppm vs 20 ppm), the productivity was 20% higher. Productivities at 115 and  $550 \text{ uE/m}^2/\text{s}$  were unaffected by the higher initial densities tested. At the lower incident irradiance, light had no inhibitory effect (see Figure 2). At the highest irradiance, 100 ppm was still dilute. Thus when batches grown under  $550 \text{ uE/m}^2/\text{s}$  were started at 300 ppm, (at this point the distinction between batch and semicontinuous disappears) productivity increased 50%. As discussed above, the metabolic balance of the cells was cycling. Under high light, the composition of the cells changed when dense cultures were diluted and grown (Table 3). Pigment and protein content

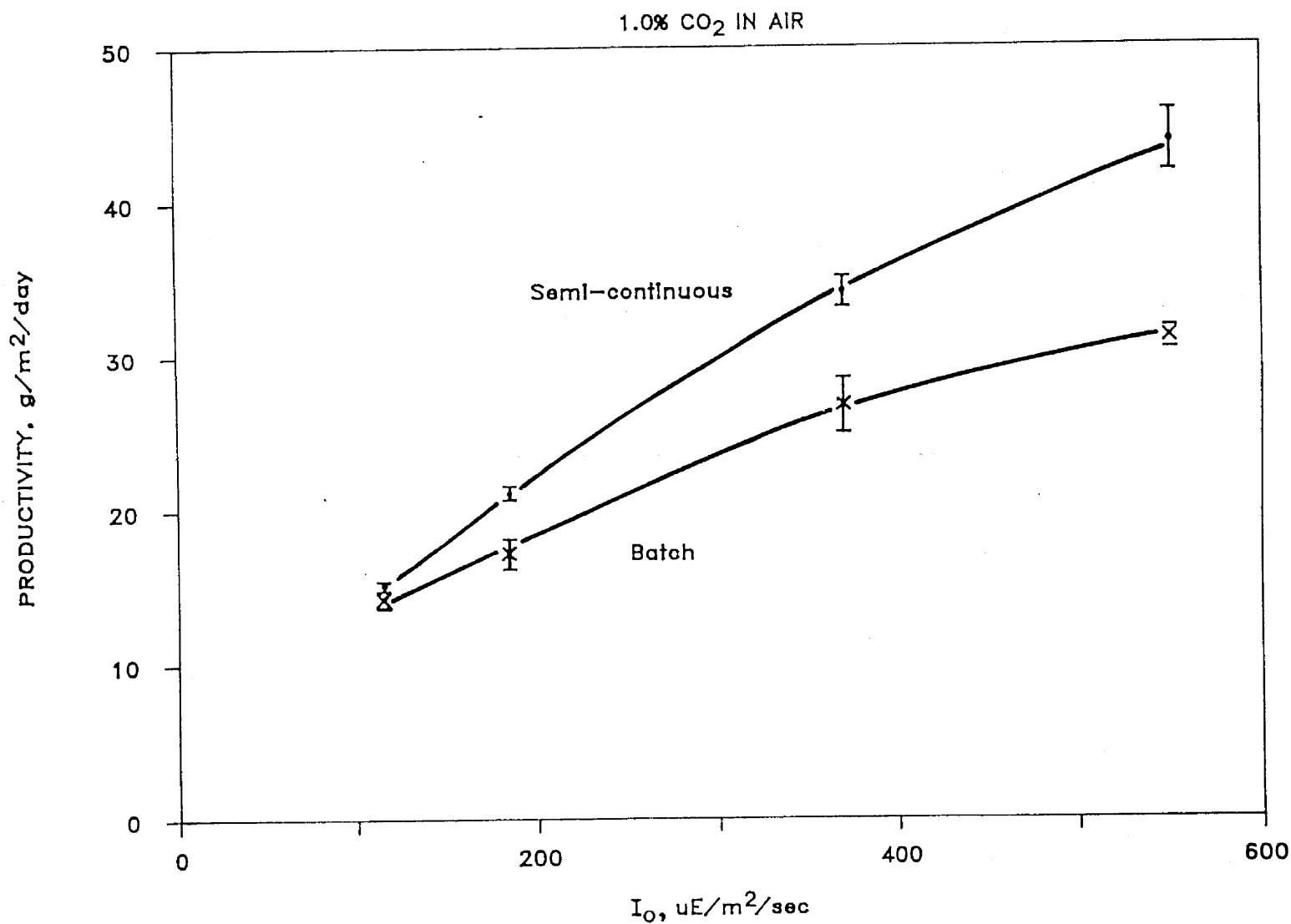


Figure 3 Productivity of Chaetoceros S/CHAET-2 as a Function of Incident Irradiance in Batch and Semi-Continuous Culture.

decreased, carbohydrate content increased greatly. Visual observation confirmed that the pigment changes were mitigated, or eliminated, by either higher inoculation density or lower incident irradiance. Thus batch cultures grown at high incident irradiance underwent a cycle in which photosynthate was directed toward carbohydrate with pigment systems dismantled or damaged when dilute, with the more normal (50% protein, high pigment content) metabolic state subsequently regenerated as average irradiance was reduced by growth.

Table 5. Effect of Initial Cell Density on Light Limited Productivity During Batch Growth of Chaetoceros sp. S/CHAET-2.

$I_0$	Low	Initial	Density	High	Initial	Density	Signific.
$\mu E/m^2/s$	ppm	Prod.		ppm	Prod.		Level
		$g/m^2/day$			$g/m^2/day$		(t test)
115	14	$14.2 \pm .5$		90	$14.3 \pm .7$		NS
370	25	$26.0 \pm 1.8$		90	$30.9 \pm .5$		<.005
370		"		300	$35.1 \pm 2.1$		<.0001
550	25	$31.1 \pm .7$		100	$31.0 \pm 1.3$		NS
550		"		300	$45.0 \pm 2.5$		<.0001

$\pm$ SDOM

In summary, operation of cultures of Chaetoceros sp. at consistently high density resulted in increased productivity. The effects of light inhibition seem to have been completely precluded when cultures were kept dense. Adaptive changes which occurred when dense cultures were diluted to low cell density led to a cycle of sun-shade states which negatively impacted productivity during the dense portion of the batch growth curve. The inhibition by high irradiance, whether a repression of pigment production or a destruction or an inactivation of reaction centers was sufficiently slow that diluted batch cultures were capable of growing, at relatively high specific rate, through the period of high average irradiance. Given that fast specific growth rates, about eight doublings per day were measured, and that both protein and pigment content were lower under these fast growth conditions and whenever average irradiance was high, it is implicit that a substantial portion (33-50%) of the cell protein is in support of light harvesting systems.

When oxygen stress was added semi-continuous cultures were affected only marginally, regardless of light intensity as shown in Table 6. This is the same result found using the test protocol. Dilute cultures were somewhat unstable under high DO and high irradiance, sometimes lagging before adapting to the stressful conditions, or even dying. Pigment content of these growth rate inhibited cultures was especially low under oxygen (0.23 % chlorophyll, on a dry mass basis).

Table 6. Semi-Continuous Culture of Chaetoceros sp. S/CHAET-2:  
Ratio of Growth Rate and Productivity under O<sub>2</sub>/CO<sub>2</sub> to  
Air/CO<sub>2</sub> (1% CO<sub>2</sub>).

Incident Irradiance uE/m <sup>2</sup> /s	u Ratio (Dilute cultures)	Productivity Ratio (Dense cultures)
115	.80 (.12)	.83 (.04)
185	.85 (.05)	.89 (.03)
370	---	.92 (.07)
550	1.05 (.07)	.89 (.07)

Numbers in () indicate propagated uncertainties in terms of SDOM's

The changes induced by high light may be manifold. High light has a regulatory effect, repressing the synthesis of pigments and redirecting flow of reductant from protein synthesis to carbohydrate synthesis. High irradiance may also inactivate the photochemical apparatus, perhaps the reaction centers. Repair reactions, or light labile protective components, counter the inactivating reactions. A greater sensitivity to light damage, including less competent countermeasures, could explain the greater inhibition of Ankistrodesmus sp. and Amphora sp. than of Chaetoceros sp. S/CHAET-2. Both of these effects can influence the subsequent performance of a culture, resulting in hysteresis in non-steady cultivation in which cells are not poised for optimal photochemical conversion.

Oxygen appears to exert inhibition which is both separate from and synergistic to that exerted by light. Thus cultures of Chaetoceros sp. produced less under high O<sub>2</sub> regardless of light intensity, but were less stable when both O<sub>2</sub> and average irradiance were very high. Ankistrodesmus sp. cultures, on the other hand, could not withstand high oxygen even when dense, and high oxygen was lethal to dilute suspensions even at moderate average irradiance. O<sub>2</sub> may destroy pigment, as indicated in the composition tables. The reaction of O<sub>2</sub> with reduced intermediates of the electron transport chain, or with Rubisco, would also lead to an increased drain on the energy economy of the cell. Such reactions are also consistent with the results obtained here. Although these results cannot elucidate inhibitory mechanisms, they do reveal the extent to which these limit photosynthetic

yield (of suspended matter) and the extent to which the organisms tested can be expected to survive in the field. They also suggest the best way of increasing the chances of survival.

## EFFECT OF PH AND CARBON AVAILABILITY ON ALGAL GROWTH

### Background and Rationale

CO<sub>2</sub> is the only known carbon substrate for the universal carboxylating enzyme ribulose biphosphate carboxylase-oxygenase (Rubisco). It is the internal concentration of CO<sub>2</sub> and the internal levels of other substrates (oxygen and RubP) and effectors (e.g., magnesium ion, bicarbonate ion) that determine the carboxylase activity. This internal concentration of CO<sub>2</sub> is, in turn, determined by both internal conditions and external conditions. The internal pools of all inorganic carbon species may be influenced by passive diffusion across the plasmalemma and by active transport of particular carbon species. The former is affected directly by pH (inside and out) and the presence of carbonic anhydrase (inside, outside, or both). pH may also have indirect effects, e.g., on the activation level of transport and other enzymes involved. Over the last ten years, evidence has mounted which indicates that the potential of algal cells to accumulate inorganic carbon is very great and dependent on the growth conditions (adaptive state) as well as the prevailing concentrations of inorganic carbon species (16,17,18,19,20). Concentration factors of inorganic carbon within cells may be 15 (21,22,23) or as great as 100-1000 (24,25,26) over the external levels. The effect of this accumulation is to increase rates of CO<sub>2</sub> incorporation especially in relation to the rates of O<sub>2</sub> uptake by Rubisco. The presence of an energy utilizing active transport for inorganic carbon raises the potential for algal growth under unfavorable external carbon conditions, but at some undefined cost.

Intensive cultivation of algae in low cost reactors necessarily results in temporal variation in pH and pCO<sub>2</sub>. As algal growth proceeds, carbon is removed from the carbonate buffer system, and pH rises. The rate of pH rise and the rate of decrease of CO<sub>2</sub> concentration depend not only on the rate of biomass production but also on the alkalinity of the medium, the buffering capacity. Any analysis of the variation of these parameters must be based on assumptions concerning the water resource used.

In Table 7 the effect of operating conditions on CO<sub>2</sub> concentration and pH is demonstrated for waters with alkalinity and equilibrium constants as shown. Dissolved inorganic carbon is never low due to the high alkalinity. The operational modes are defined by initial and final pH, which represent, respectively, the pH after carbonation (at a carbonation station) and right before re-carbonation. The amount of inorganic carbon that can be stored in the water, for each pH range, determines the frequency of carbonation. It is assumed that algal productivity is constant at 4 g/m<sup>2</sup>/hr, that pond depth is 15 cm and thus that the carbon uptake rate is 1.1 mM/hr. At a mixing velocity of 20 cm/s, a 8 ha pond would have a circulation time of about 2.5 hr (27). The minimum CO<sub>2</sub> concentration is given, as well as an average rate of outgassing. This latter quantity was determined by integrating the rate numerically using 0.2 mM steps in DIC. The final column shows the CO<sub>2</sub> utilization efficiency based on the losses due to outgassing.

Table 7. Effect of pH Limits on Pond Carbonation

pH Initial	pH Final	Carbon Storage mM	Minimum CO <sub>2</sub> uM	CO <sub>2</sub> Outgas g/m <sup>2</sup> /day	CO <sub>2</sub> Utilization Efficiency %
7.0	7.5	2.7	970	95	43
7.5	8.0	2.3	274	25	74
8.0	8.5	3.6	60	6.1	92
8.5	9.0	4.5	11.5	1.1	98
9.0	9.5	3.2	1.5	-0.18	100
9.5	10.0	1.5	0.17	-0.43	100
7.5	8.5	5.9	60	15.7	82
7.5	9.0	10.4	11.5	10.8	87
8.0	9.0	8.1	11.5	3.6	95

log  $K_1$  = -5.98, log  $k_2$  = -9.02, Alk = 32 meq/L, CO<sub>2</sub>eq = 9 uM  
 $K_L$  for outgassing = 0.1 m/hr, outgassing calculated for 12 hours.

It is clear from the table that operation below pH 8 is too wasteful of CO<sub>2</sub>. Operation between 8 and 8.5 achieves greater than 90% utilization efficiency of CO<sub>2</sub> and requires only one carbonation per 8 ha pond. The lowest concentration of CO<sub>2</sub> is 60 uM or over six times that in air equilibrated water. To use even larger ponds, or if water alkalinity is higher than that assumed, the high pH limit must be raised. In the example, operation between 8 and 9 results in more than twice the carbon storage, achieves 95% efficiency, but requires algae which are productive at the higher pH and at essentially air levels of CO<sub>2</sub>. The photosynthetic efficiency of algae grown under light limiting and carbon restricted conditions has not been investigated previously.

If the medium contains higher alkalinity than that used in the example, then the range of pH used must be shifted upwards to avoid increased outgassing. The range itself will shrink, for a given desired storage capacity. Thus for higher alkalinity conditions, the algae must be tolerant to higher pH, at least to some extent. For lower alkalinity conditions, the pH range must be stretched to store as much carbon as possible, and the limiting factor becomes the ability to grow at low CO<sub>2</sub>.

In summary, the practical questions concerning the effects of pH and CO<sub>2</sub> on the growth of algae pertain to the direct (carbon independent) effects of high pH and to any possible efficiency limitations imposed by low CO<sub>2</sub>, especially under conditions of high oxygen. Organisms which do not accumulate inorganic carbon may grow less productively due to the competing effect of high O<sub>2</sub>. Those that do accumulate carbon, probably pay a price in terms of efficiency. In this report, the first steps are taken to investigate the light limited growth of algae under stressful conditions of pH and CO<sub>2</sub>. Results are in terms of the effects on maximum specific growth (light saturated growth) and average production (light limited growth, but still at maximum efficiency). The primary effort made was to determine the

species specific pH and CO<sub>2</sub> concentration at which these growth measurements decline. In most experiments, total inorganic carbon could not be considered limiting to growth rate.

### Methods

The partial pressure of CO<sub>2</sub> in the influent gas phase was controlled, either as a constant, or for low pCO<sub>2</sub> experiments, by computerized feedback control algorithms (2). For the higher pCO<sub>2</sub> work, rotameters were calibrated with an Infra Red Gas Analyzer (Beckman model 864) and used to mix air with pure CO<sub>2</sub>. For each level of CO<sub>2</sub>, the carbonate alkalinity of the experimental cultures was adjusted to yield the pH desired. Thus the alkalinity of the cultures varied over two orders of magnitude to achieve the pH range of 7-9. The effect of high alkalinity on growth was checked by operating at higher pCO<sub>2</sub> and lower pH. Since the object was to study the growth of algal species as function of the concentration of dissolved carbon, particularly dissolved CO<sub>2</sub>, some means had to be devised to measure, or estimate, the liquid CO<sub>2</sub> concentration. A knowledge of alkalinity and pH is theoretically sufficient to estimate equilibrium concentrations of carbon species. In practice this method is inaccurate. The dissociation constants are not accurately known, the errors introduced in estimating quantities from log scale measurements are large, and the ability of algae to change the alkalinity of the medium, and hence the pH, makes estimation even more difficult.

An empirical relationship was developed between the rate of CO<sub>2</sub> uptake into the culture liquid on the one hand, and on the other the effluent gas CO<sub>2</sub> concentration (determined by IRGA) and the prevailing dissolved CO<sub>2</sub> concentration. It was then used to estimate the latter quantity. In three experiments (in which the effluent gas CO<sub>2</sub> content was 250, 300, or 750 ppm), the value of K<sub>La</sub> was determined to be 295 ± 19 (S.D.) hr<sup>-1</sup>. Given the mass transfer coefficient of the reactor, the gas inflow rate, and any two of the effluent CO<sub>2</sub> concentration, the influent CO<sub>2</sub> concentration, and the carbon uptake rate, the liquid CO<sub>2</sub> concentration can be calculated. For example, when an influent gas containing 0.5% CO<sub>2</sub> is sparged into the Roux flask reactor at the rate of .75 lpm, the input rate of CO<sub>2</sub> is about 13.5 mmol/L/hr. The maximum CUR by the algae, in the test conditions, is 1 mmol/L/hr. Thus the lowest value for per cent CO<sub>2</sub> in the effluent (by mass balance) is .46% or 92% of the influent. A concentration gradient must exist between the CO<sub>2</sub> concentration in the bubbles and that in the liquid. With a mass transfer coefficient of 300 per hr, the liquid CO<sub>2</sub> concentration would be 142 uM vs the 158 uM that would be in equilibrium with the 0.5% influent gas phase, or the 145 uM in the effluent gas. When the influent gas phase contains 0.1% CO<sub>2</sub> (an experiment was performed under this condition), an analysis similar to that described above yields a liquid CO<sub>2</sub> concentration of from 15-25 uM, or the equivalent of equilibrium with a 0.05-0.08% gas phase, depending on what the actual carbon uptake rate was.

### Computer Control of CO<sub>2</sub> Transfer

As the experimental batch cultures grew, the demand for CO<sub>2</sub> increased. In order to study growth at low (.5-10 uM) CO<sub>2</sub>, some control mechanism was needed to vary the incoming supply of CO<sub>2</sub> as the culture productivity varied and in such a way as to maintain a constant concentration of dissolved CO<sub>2</sub>.

Two of the Roux flask reactors were equipped with instruments to measure  $\text{CO}_2$  concentrations in the effluent gas stream, and based on the result of this measurement and a prescribed set point, control the  $\text{CO}_2$  content of the influent gas stream. The entire apparatus was operated under the supervision of an IBM PC microcomputer.

#### Chaetoceros sp. S/CHAET-2 (Figure 4)

The most extensive experimentation was carried out using another Chaetoceros sp., the Salton Sea isolate. It was cultivated with influent gases containing 0.5% and 0.1%  $\text{CO}_2$ , under air without  $\text{CO}_2$  enrichment, and as well under feedback control of the concentration of  $\text{CO}_2$  in the gas leaving the growth vessel. The computer control kept the liquid  $\text{CO}_2$  concentration within narrow limits, by increasing the inflow of  $\text{CO}_2$  as algal growth increased.

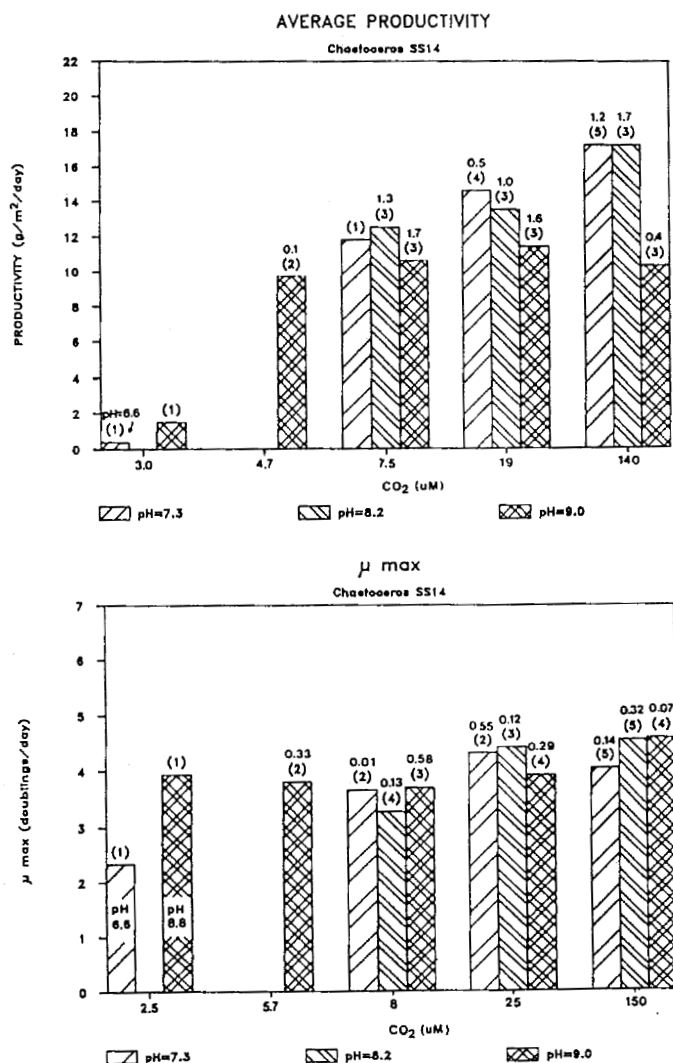


Figure 4. Growth of Chaetoceros sp. SS14 vs pH and  $\text{CO}_2$   
 $I_0 = 185 \text{ uE/m}^2/\text{s}$



Maximum specific growth rate was not decreased by increased pH. At the lowest CO<sub>2</sub> concentration (2.5 uM),  $u_{max}$  was 70% higher at pH 9 (bicarbonate concentration of >2 mM) versus pH 6.7 (<15 uM bicarbonate). At 8 uM CO<sub>2</sub> growth rate was about the same as at the lower CO<sub>2</sub> pH 9, and about 85% of the highest growth rates observed. These were measured at all pH values when CO<sub>2</sub> was greater than 25 uM. Thus some limitation of growth rate is evident as CO<sub>2</sub> was lowered, but higher pH relieved some of the limitation.

At the higher concentrations of CO<sub>2</sub> (>20 uM), productivity was less at pH 9 compared to pH 7. The decrease was greatest (40%) at very high CO<sub>2</sub> and practically nonexistent at 7.5 uM CO<sub>2</sub>. Another way of looking at this is that productivity was limited by high pH until CO<sub>2</sub> concentration was kept as low as 4-7.5 uM. This could be due to an extra maintenance load required at the higher pH. Below 4 uM CO<sub>2</sub>, little growth was evident at either pH 6.8 or 8.8. The positive effect of increasing pH on productivity at low CO<sub>2</sub> is opposite of the effect of pH on productivity at high CO<sub>2</sub>. Induction of an adaptation for increasing uptake of carbon at low CO<sub>2</sub> may be responsible. A similar result was obtained at a CO<sub>2</sub> concentration of 4.5-6 uM. Production was greater at pH 8.2-8.5 than at 7.0-7.2 ( $p < .001$ ,  $t$  test). Apparent maximum specific growth rate was about equally affected, being one third higher at pH 8.2 vs pH 7.1-7.3 ( $p < .001$ ).

An entirely new set of data was taken to confirm that increasing pH, and consequently bicarbonate concentration, leads to increased rates of biomass production at low CO<sub>2</sub> concentrations. This time cultures were diluted 50% per day and grown on unenriched air. Growth of algae on unenriched air is typical of studies in which algae adapted to low CO<sub>2</sub> are studied. In this experiment the cost of this adaptation, in terms of net photosynthetic efficiency was measured. The results are given in Table 8. Again the effect of pH on biomass productivity was reversed at low CO<sub>2</sub> compared to at high CO<sub>2</sub>. When the concentration of CO<sub>2</sub> was in the low micromolar range

Table 8. Productivity of Air Grown Chaetoceros sp. as a Function of pH

pH	Productivity g/m <sup>2</sup> /day	SDOM(n)	(CO <sub>2</sub> ) uM	(HCO <sub>3</sub> ) mM
6.7±.5	22.9	1.9 (3)	300	1.5-3
6.7±.5	6.3	0.4 (8)	1-2	.005-.01
8.5±.2	8.9	0.2 (6)	1-2	.5-1
9.5±.2	11.8	0.4 (9)	1-2	3-6

Influent and effluent gas CO<sub>2</sub> content measured daily, dissolved CO<sub>2</sub> concentration estimated as described above. Lack of precision is due to fluctuations in CO<sub>2</sub> content of compressor air: 400±50 ppm. Bicarbonate concentrations calculated using dissociation constants for seawater at 30 °C.

productivity increased with increased pH. At the highest pH tested, 9.5, productivity was about the same regardless of CO<sub>2</sub> concentration over a range of CO<sub>2</sub> from 1 to 300 uM. This rate, however, was still 40% lower than the rate achievable under optimal conditions of high CO<sub>2</sub> and pH below 8.0. At lower pH and low CO<sub>2</sub> production was less than 30% of high CO<sub>2</sub> controls.

### Summary of Results with Five Organisms

The results of the carbon-pH experiments are summarized in Table 9. The organisms are listed along with the growth rate and productivity results from experiments in which a significant drop off (if any) was observed compared to the maximal rates measured. The values are given as a per cent of the maximal. The Ankistrodesmus sp. grew as well at the highest pH tested, pH 10, and at low CO<sub>2</sub>. C. gracilis also grew well at high pH and low CO<sub>2</sub>. Although growth rates of Chaetoceros sp. SS14 were high at pH 9, biomass productivity was reduced 33%. The same trend was observed in terms of CO<sub>2</sub> concentration. The Amphora sp. was not tested at particularly low CO<sub>2</sub>, but performed well at pH > 9. Productivity of the Chlorella was reduced at either high pH or low CO<sub>2</sub> making it the least attractive for further work.

Table 9. Values of pH and \*CO<sub>2</sub> at Which Productivities of Five Algal Strains Decrease

Strain	Maximum Specific Growth Rate				Light Limited Productivity			
	limit	pH %	CO <sub>2</sub> limit	%	limit	pH %	CO <sub>2</sub> limit	%
<u>Ankist.</u>	10	100	25	100	9	100	19	80
<u>C. gracilis</u>	9.1	100	4.5	100	9.1	90	1.6	78
<u>Chaet</u> SS14	9	100	2.5	90	9	67	4.8	67
<u>Amphora</u> sp.	9.7	100	25	100	9	100	19	80
<u>Chlorella</u>	9.2	80	6.3	84	9.1	67	1.6	60

CO<sub>2</sub> is uM

% is per cent of maximum rate observed, usually at pH 7-8,  
140 uM CO<sub>2</sub>

"limit" is the value at which a decrease in growth rate or productivity was observed, or the most extreme value tested.

## PHOTOSYNTHETIC YIELD OF NUTRIENT LIMITED DENSE CULTURES OF ALGAE

### Storage Product Induction in Outdoor Mass Cultures

The induction of a higher lipid content by limiting the availability of Si in batch culture was studied in 1.4 m<sup>2</sup> growth tanks with two of the diatoms, Cyclotella and C. gracilis. In each case healthy, productive cultures were diluted as usual but with most of the Si left out of the media. Evaporation make-up water was not added, since it contained 2 ppm Si. During normal cultivation of Cyclotella, about 75 ppm Si was added to the medium each day. This could support from 300-375 ppm biomass assuming 25% or 20% Si content respectively. For the Chaetoceros the daily amount of Si normally added was 20 ppm. This would yield 300 (600) ppm biomass at 7% (3.5%) Si content. Two attempts were made at limiting growth for Si. For the Cyclotella the biomass productivity, lipid productivity, and lipid content measured during the experiments are given in Table 10. Estimates of the Si content of the cell mass were made by dividing the total added silicon by the cell mass at any given time after the Si was depleted from the medium. The time of depletion could be estimated from the knowledge of the maximum Si content.

In the first attempt at silicon starvation (data shown as the first block in the table), Si was added only on the first day and only half the usual daily amount. The luxury consumption of Si was great enough that starvation was not protracted. Little or no increase in lipid content (relative to Si sufficient cultures) occurred. For both species, productivity was normal for the first two days. This was above 35 g/m<sup>2</sup>/day for Cyclotella and above 25 g/m<sup>2</sup>/day for C. gracilis. In both replicates of the Cyclotella experiment the productivity dropped 60% on the third day to 15.5 g/m<sup>2</sup>/day. This is significantly different (t test, p<.025) from twenty previous (Si sufficient) third day productivities which averaged 24.5 g/m<sup>2</sup>/day. The estimated Si content dropped below 15% when this productivity decline occurred. Maximal lipid productivities were 10-11 g/m<sup>2</sup>/day, averaged values were 8-9 g/m<sup>2</sup>/day which is similar to totally sufficient cultures. Similarly, the C. gracilis culture productivity dropped on the third day when the Si content became less than 4%.

A second set of starvation experiments was initiated in which no silica was added, allowing growth only at the expense of Si contained in the (Si sufficient) culture after dilution. Two different starting levels of Si were used for each species, obtained by using different amounts of inoculating suspension.

For Cyclotella in culture 1 the initial Si in the culture was about 9 ppm, while for culture 2 it was about 18 ppm. In both cases, internal Si content was reduced, by growth, to less than 10% during the first day. Inhibition of growth occurred during the first day (maximum productivity was never as high as in Si sufficient cultures), prior to any increase in lipid content. Productivity followed Si content, decreasing to practically zero when content fell below about 3 or 4%. At 4-7% productivity was about half as much as Si sufficient cultures. Lipid content rose quickly when Si content fell below 6%. Both total productivity and the rise in lipid ceased when Si content fell below 3-4%. Overall lipid productivity was reduced somewhat during the first day of induction due to the lag in lipid increase relative

Table 10. Cyclotella: Lipid Induction

Day	Si, % of Biomass	Daily Biomass Productivity g/m <sup>2</sup> /day	Lipid Content %	Daily Lipid Productivity g/m <sup>2</sup> /day	Cumulative Averaged Productivity g/m <sup>2</sup> /day		Cell Density mg/l
					Biomass	Lipid	
1: Pond 1 - Si Initial = 76.4 mg/l							
0	>20		21.7				73
1	>20	36.1	18.4	6.2			282
2	15.2	37.7	22.2	10.2	36.9	8.2	501
3	12.9	15.6	27.1	8.4	29.4	8.3	591
2: Pond 2 - Si Initial = 76.4 mg/l (Duplicate)							
0	>20		25.2				68
1	>20	39.2	21.0	7.7			295
2	14.8	38.3	24.8	11.4	38.7	9.6	517
3	12.6	15.4	28.5	7.7	30.8	8.9	606
3: Pond 1 - Si Initial = 8.8 mg/l							
0	>20		25.0				39
1	6.3	17.2	25.8	4.5			139
2	3.6	15.5	40.4	9.9	16.4	7.2	246
3	3.0	2.0	43.2	2.0	11.0	5.5	293
4	2.5	4.7	39.5	0.3	8.3	4.2	354
4: Pond 2 - Si Initial = 17.6 mg/l							
0	>20		25.0				72
1	8.4	23.4	25.7	6.1			210
2	5.4	15.8	36.6	9.7	19.6	8.0	325
3	4.6	2.0	42.3	3.8	13.7	6.6	384
4	4.0	2.5	36.2	-2.5	7.5	5.8	436

For runs 1 and 2:

Areal Productivity = Volumetric Prod. (gm/L/day) x Vol/Area (L/m<sup>2</sup>) x 1.15  
(1.15 = Correction factor for shading by paddle wheel)

For runs 3 and 4:

Areal Productivities = Mass Productivity (gm/day) x 1.15/1.4 (m<sup>2</sup>)  
Depth decreased due to evaporation (1-1.5 cm per day).

to decreases in biomass productivity. During the second day, when lipid content increased the most (by 50-60%), lipid productivity was almost as great as under Si sufficiency. After this, productivity was very low and lipid content actually decreased. The degree of light limitation appears to have little effect on the induction, as long as enough potential exists for biomass increases to lower intracellular Si considerably. It is not possible on the basis of this experiment to determine whether the increase in lipid productivity was limited by the maximum rate of lipid formation attainable with this species or by the declining rate of primary production under the stressful conditions of low intracellular Si concentration and high light intensity.

Even under extreme Si limitation, lipid induction did not occur to any significant degree with C. gracilis. At low biomass density, biomass productivity was reduced (compared to Si sufficient cultures) by 35% when Si content fell to 2.5-3.5%. This indicates that enough Si must be added to maintain an intracellular content above about 4% to keep productivity high. For Cyclotella this value was about 13-15%. Productivity of C. gracilis was reduced one third to one half of maximal values at a Si content of 1.5-2.5%. Below 1.5% Si, productivity was practically nil. Lipid content did not increase more than 10-20%, to about 30% of the total organic mass. Consequently lipid productivity was lower under Si deficiency.

#### Laboratory Studies of Nitrogen Depleted, Light-Limited Algal Growth

Laboratory studies were initiated to investigate the efficiency of production of storage biomass fractions under nutrient and light limiting conditions. To begin this work, nitrogen depleted cultures of Chaetoceros sp. (S/CHAET-2) were grown both in batch and semi-continuous culture at  $29 \pm 1$  °C and pH  $7.5 \pm 3$ . The hypothesis investigated was that biomass production and photosynthetic efficiency from nitrogen depleted cells would be equal to that of nitrogen sufficient cells.

Nitrogen depleted batch cultures were grown by reducing the urea-nitrogen content of the growth medium from the usual level of 24 mM to as low as 1.8 mM. At the lowest level of urea-N, the productivity averaged over three days of growth was limited by the lack of nitrogen (data not shown).<sup>a</sup> In such cultures the protein content of the biomass decreased from 50% prior to N depletion down to 14% after three days. Carbohydrate (CHO) content rose from 15% to 70%. Higher levels of added N were required to maintain high productivity of total biomass, while still resulting in elevated CHO content (60-65%). The productivity results from N sufficient and N limited batch cultures, grown at  $370 \text{ uE/m}^2/\text{s}$  are shown in Figures 5 and 6. The N limited cultures were started at day -0.67 with 8 mM urea-N, and depleted the nitrogen from the medium by about day 1. Day 0 is used in the figures to mark the point in time at which over 95% of the incident light was absorbed by the cultures. It is evident from the figures that relative to the N sufficient controls, as cells decreased in N content protein production decreased, CHO production increased, and total biomass production increased somewhat. By day 3, nearly all of the biomass production was in the form of CHO in the N limited culture. That these cultures were limited in terms of biomass production by light is shown in Figure 5. A duplicate of both the control and the low N culture was placed in higher incident light, 550

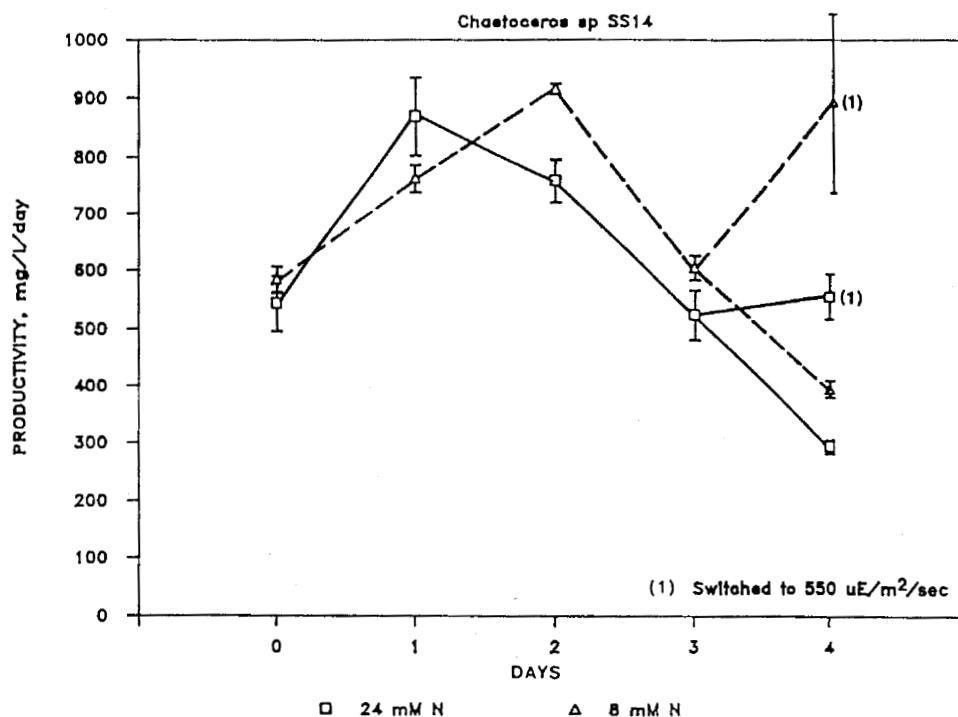


Figure 5. Daily Biomass Productivity from Nitrogen Sufficient and Nitrogen Limited Batch Cultures of *Chaetoceros* sp.  $I_0=370$  uE/m<sup>2</sup>/s Cultures inoculated at t=-0.67 days.

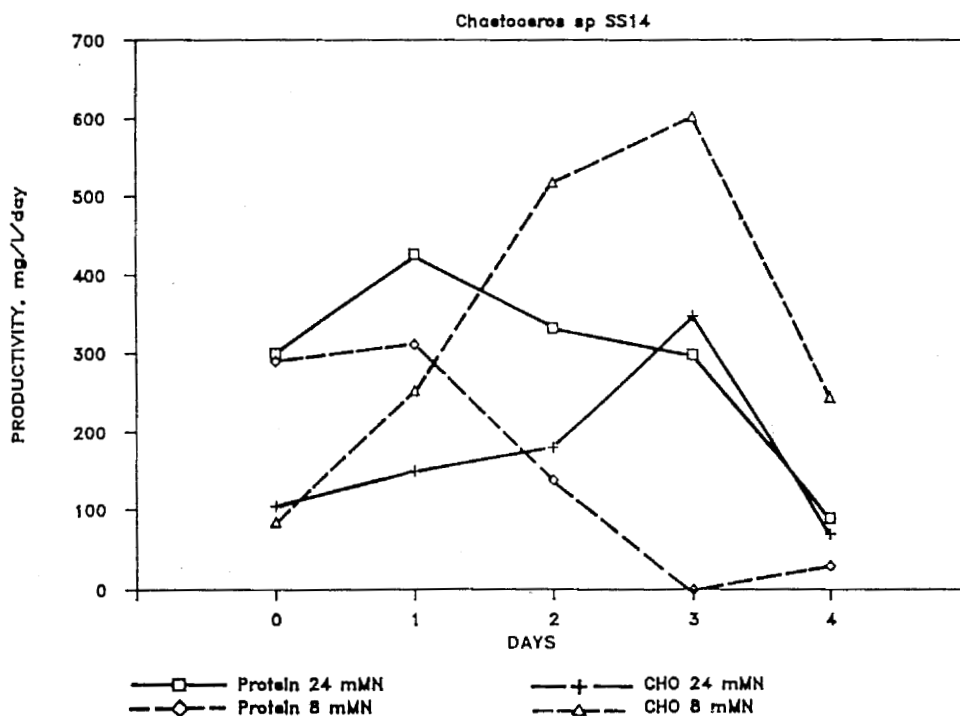


Figure 6. Daily Protein and Carbohydrate Productivity from Nitrogen Sufficient and Nitrogen Limited Batch Cultures of *Chaetoceros* sp.  $I_0=370$  uE/m<sup>2</sup>/s Cultures inoculated at t=-0.67 days

$\mu\text{E}/\text{m}^2/\text{s}$ . Productivity of both increased. Both the greater increase of biomass production in this switching experiment and the higher productivity of the low N culture at higher density demonstrate that there is a substantial interaction between light limitation and nitrogen content. Lower N content lessened the limitation by light in dense culture by lowering the pigment content (data not shown). It is the ability to shift from normal metabolism in which protein to CHO production is about 2 to 1, to wholly CHO production which allows such high overall efficiency in these transient systems. The eventual decline in photosynthetic rate, due either to lack of nitrogen or severe light limitation, would impact on storage compound production if this shift were slow.

To demonstrate that the shift from protein production to CHO production and vice versa can be both fast and complete, semi-continuous cultures were operated under high and low N levels at two incident irradiances. The results are given in Table 11 and Figure 7. These cultures were diluted by 50% per day, which resulted in an average specific growth rate of 1 doubling /day. Immediately after dilution, either 14, 3.2, or 2.1 mM urea-N was added to the cultures, as indicated in the table. In Figure 7 is shown the fraction analysis every 8 hours after this dilution and N addition. After the first 8 hours, mostly protein was synthesized with very little CHO synthesis occurring. Thereafter, when apparently all of the added N had been incorporated into protein, the trend reversed. As shown by the magnitudes of the productivity of total biomass, there was little lag in either of these metabolic shifts. These semi-continuous, oscillating cultures were sustainable.

By comparing rows 1, 4, and 5 or 6 in Table 11, it can be seen that in comparison to a culture grown with 2.1 mM added N at  $370 \mu\text{E}/\text{m}^2/\text{s}$  (row 4), increasing either the light level (row 1) or the amount of N added (row 5 or 6), or both (row 2 or 3) increased the total biomass production, as well as the production of chemical bond energy in most cases. Protein production was limited only by the amount of added N (row 4 vs 1) in this case, while the CHO production was inhibited by adding nitrogen and limited by light input. The results from these semi-continuous cultures demonstrate dual limitation day by interacting nutrients, i.e., an increase in overall productivity upon the added input of either or both of the nutrients. This dual nutrient limitation was an average over the day in which nitrogen was limiting during the last few hours prior to N addition and during the first few hours afterwards. Subsequently light was limiting.

The oscillating SC cultures were different from the batch cultures. The latter were always started off from nitrogen sufficient inoculum. As soon as protein content of the cells began to drop, indicating that nitrogen had been depleted from the medium, the carbohydrate content began to rise. But protein production and CHO production were concurrent until protein content dropped below 30%, a day or so later. The SC cultures on the other hand were nitrogen depleted each time a dose of nitrogen was added. Upon addition, protein production began and CHO production ceased. At this time, during the first eight hours, biomass production was limited by nitrogen since the productivity was the same at both light levels. Thus a situation existed in which productivity was nitrogen limited when the nitrogen content

Table 11. Effect of N Depletion on Photosynthetic Production in Semi-Continuously Diluted Cultures of Chaetoceros.

Mode	I <sub>0</sub>	N mM	Protein %	CHO %	Lipid %	Ave. Production		
						Protein	CHO	Total
SC	550	2.1	19.3 (0.4)	58.7 (3.1)	17.3	191 (8)	580 (37)	988 (35)
SC	550	3.2	25.2 (0.2)	49.4 (5.0)	20.2 (.7)	307 (3)	603 (61)	1220 (7)
SC	550	14	57.0 (3.0)	17.5 (1.5)	26.0 (.2)	684 (39)	210 (19)	1200 (28)
SC	370	2.1	23.6 (0.6)	55.4 (1.3)	19.2	187 (6)	440 (14)	794 (17)
SC	370	3.2	32.8 (0.8)	43.9 (3.0)	21.5 (.2)	282 (12)	378 (29)	860 (29)
SC	370	14	55.4 (0.2)	16.9 (1.5)	25.3 (.2)	533 (22)	162 (16)	962 (40)

I<sub>0</sub> in uE/m<sup>2</sup>/sec

Figures in parentheses ( ) are standard deviations of the mean.

N is the concentration of urea-nitrogen added daily

Percents of protein, CHO, and lipid refer to the content in the biomass prior to dilution each day.

Protein to nitrogen ratio = 6.4 (.12)

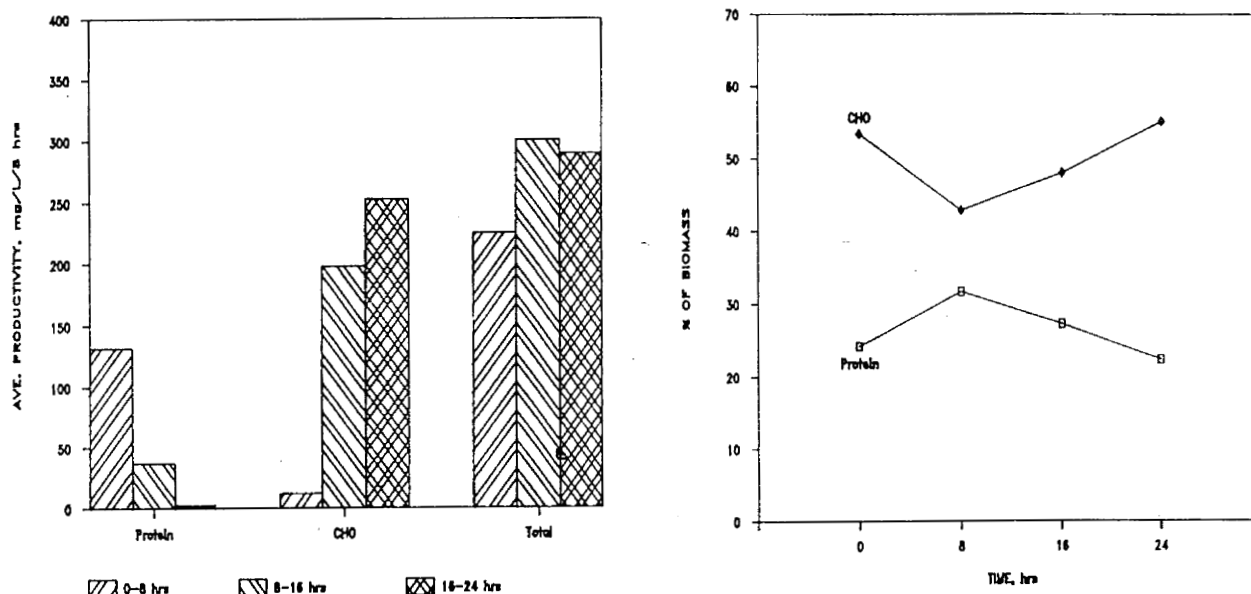


Figure 7. Daily Oscillation in Production of Protein and Carbohydrate by Nitrogen Limited Semi Continuous Cultures of Chaetoceros sp.  
Daily dilution and N addition at t=0 hrs.

of the cells was highest. The lack of carbohydrate production must therefore reflect a regulatory shut off of this sink for photosynthate. Thereafter, CHO production (and hence total biomass production) was light limited despite the lower protein and N content of the cells. Near the end of the 24 hour period, when nitrogen content was lowest, CHO production most likely became nitrogen limited. It is not possible to tell, from the present data set, whether during these crossover points in limiting inputs



there was ever a time when both light and nitrogen were simultaneously limiting the rate of storage of chemical energy. The pigment to protein ratios did not change that much and could not be resolved well enough to shed light on this question. Overall, they indicated that, as expected, the culture grown under lower incident irradiance was more light limited.

It is noteworthy that semi-continuous cultivation was better than batch cultivation for the production of high protein biomass but both were similar, under optimal (but differing) conditions for the production of high carbohydrate biomass. The advantage of semi-continuous cultivation in mitigating light inhibition (see above) was balanced out by the more prolonged growth, in batch, under conditions of low internal nitrogen content and high rate of storage product formation.

The experimental results show that in nitrogen deficient, but light limited batch cultures nitrogen depletion served as a metabolic switch, while the light input continued to drive the conversion of light into stored chemical energy. However, the rate of this conversion, under a fixed incident irradiance, was greatest when light was limiting biomass increases, not nitrogen. Light limitation is required to maximize storage product formation rates just as it is required to maximize protein production in nitrogen sufficient cells. This is in contrast to what has been previously stated (28). If the decrease from 50 to 20% protein (8 to 3% N) had resulted in a decline in photosynthetic rate, the rate of storage product formation would not have been as high. Since carbon reduction continued undiminished as protein content declined within this range, the loss of protein was highly selective. The concurrent loss of pigmentation suggests that much of the protein lost was part of the pigment apparatus. This is supported by the data from measurements of maximum specific growth rate. It was shown that at irradiances above saturating, both protein and protein content were repressed. The pigment to protein ratio measured in these fast growing, nutrient saturating conditions was about the same as that found under slower growing, nitrogen limiting conditions.

With N limitation, high efficiency is maintained because the ability of the cells to photosynthesize is not changed over a large range of internal N content, whereas the shift from one fraction to another occurs much faster and to a potentially very great extent. If the regulation of lipid metabolism can be elucidated and controlled, or if species which normally shift to lipid metabolism (rather than CHO metabolism) under nitrogen stress can be found, then there is every reason to believe that very high rates of lipid production can be attained. The question is not as clearly answered for the case of Si limitation of diatoms. It was shown above that luxury consumption of Si was substantial, as is the case for nitrogen, but that there is little shift in metabolism toward storage product formation until the Si level in the cells is almost as low as will support any significant productivity. The signal that the cells use to finally shift to lipid production must be elucidated in order to turn it on before the potential for high productivity has been lost. The burden of constitutive lipid production would lower the competitiveness of the organism. Thus even if the regulation of lipid synthesis could be controlled, lipid production would still need to be accomplished transiently, starting with pigment rich, competitive cells.

## CONCLUSIONS

The photosynthetic yield of microalgae was studied in terms of four factors which are not likely to be optimal in a large scale production system: irradiance,  $pO_2$ , pH, and  $pCO_2$ . The effects of stressful levels of these factors on the "maximum" specific growth rate and on light limited (optimal) productivity were determined. In general, algae differed considerably in their responses. The experiments were designed to quantify the growth responses under definite conditions in laboratory tests and to provide a basis for predicting performance of species in outdoor culture. The results were used to develop culture management strategies for maximizing biomass and lipid productivity.

Microalgae grown outdoors in  $1.4\text{ m}^2$  tanks produced from 20-40  $\text{g/m}^2/\text{day}$  over a 30-40 day period. Higher rates of production were obtained from all of the diatoms tested than from any of the green algae. Diatoms were also much more tolerant to high concentrations of dissolved oxygen in laboratory tests. The most oxygen sensitive chlorophytes could not be sustained in culture outdoors. Aside from dissolved oxygen and the high solar intensity, growth conditions were maintained close to optimal in the outdoor cultures. Thus it is possible to use simple laboratory tests for oxygen tolerance in screening for subsequent performance of microalgae in mass culture. In laboratory tests high irradiance was found to be inhibitory, especially in concert with high dissolved oxygen. However, for some species maintenance of high culture density, resulting in low average irradiance, mitigated the inhibition by light. This is a major factor making continuous, or semi-continuous, dilution of cultures more productive than sequential batch dilution. A potential exists, therefore, for increasing outdoor yields in the future beyond those measured here.

Increasing pH and/or lowering  $pCO_2$  led to a faster decrease in light limited productivity than in "maximum" specific growth rate. This indicates that responding to these stresses required a significant expenditure of energy. In terms of specific growth rate, a decrease to half maximal rates was not achieved at pH 9 and/or  $2\text{-}5\text{ }\mu\text{M CO}_2$ . Work is underway to study more combinations of stresses (especially high oxygen, low  $\text{CO}_2$ ) and changing conditions, since these may even further impact biomass production. Excretion of organic carbon compounds under carbon limiting conditions will also be studied.

The induction of primary, non-nitrogenous storage compounds (lipids and carbohydrates) under nutrient limitation is potentially an efficient process. Algae are intrinsically as efficient in producing exclusively these products, or exclusively protein, or both. Only the mechanisms regulating the flow of photosynthate into the various fractions limits the potential for lipid production. Thus Si starved diatoms did not produce as much lipid as nutrient sufficient ones because overall photosynthetic activity was low by the time metabolism had been switched towards lipid production. On the other hand, nitrogen limited diatoms produced carbohydrate at maximal yield (compared to nutrient sufficient controls) due to an early redirection of metabolism.

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# INTEGRATED FIELD-SCALE PRODUCTION OF OIL-RICH MICROALGAE UNDER DESERT CONDITIONS

## ALGAL CULTIVATION

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## ABSTRACT

A 100-m<sup>2</sup> pond was operated under desert conditions at Sede Boqer with a halotolerant oil-producing alga, Isochrysis. The pond was operated by the three Israeli research groups, from Ben-Gurion University, Israel Institute of Technology and the Oceanographic Institute. The pond was run for 45 days during August and September 1985. The process included: algal cultivation (growth, biomass production, maintenance of uni-algal culture), semicontinuous operation achieved by bleeding, and harvesting. Harvesting was achieved by flocculation with FeCl<sub>3</sub> and dissolved air flotation. The harvested slurry was dried and chemically analyzed, and lipids were extracted. Biomass production was 23.5 g per square meter per day, and lipid production 6 g per square meter per day.

The operation demonstrated the feasibility of cultivating microalgae outdoors for production of oil on a field scale. It revealed the necessity to optimize the system as a whole, because of the dependence of the phases upon each other. The development of an applicable process of lipid extraction from algae requires continuation of integrated optimization of the whole process.

## GENERAL BACKGROUND

The possibility to use the relatively efficient photosynthetic machinery of microalgae to produce biomass has motivated extensive studies in this area. The first aspect to be studied was the potential use of algae as another source of single-cell protein (SCP), leading to mass algal cultivation. Other potential applications, based on exploiting the intricate biochemical pathways of photosynthetic organisms to produce a variety of organic products, have also been developed (19).

Certain species of microalgae contain relatively large amounts of lipids hence they may be considered as a potential source of fuels (1,4,6,14). Different genera and even different species of the same genus widely differ with respect to lipid quantity and quality (15). The composition of lipids and fatty acids in microalgae and their response to light intensity, temperature, and concentrations of nitrate and various other nutrients have been studied (15,20,24).

Microalgal polar lipids are esters of glycerol and fatty acids having carbon numbers between C12 and C20 (16). Among the nonpolar neutral lipids found in algae are triglycerides, free fatty acids, hydrocarbons, and wax esters - the latter is present in only a few species of algae. A great variety of unsaturated fatty acids having C12 to C22 chains and containing up to 6 double bonds are produced by algae.

## FIELD-SCALE OPERATION

Production of oils from microalgae requires development of an integrated process that includes several successive stages. Some are mainly of biological significance, but the engineering aspects of the process (e.g. harvesting) cannot be ignored. The development of processes for operating a full-scale algal pond for cultivation of oil-producing algae, harvesting them, and extracting their oil is the main goal of our project. Since it is a long-term

project, it was subdivided into several phases, starting with massive screening for microalgae capable of accumulating large amounts of lipids. The effects of physiological and environmental factors on lipid induction, outdoor cultivation, harvesting, and lipid extraction are also being studied. Although we still do not have the organism of choice in our hands, the strategy of the whole process of oil production from microalgae, including all the steps, has to be developed. Otherwise, we might end up with a culture collection of oil-producing algae.

Operation of a  $100\text{-m}^2$  pond for producing algal biomass was an optional task in our statement of work. However, we decided to tackle this challenge, because of its significance to the program. Such an operation includes the whole process of lipid production - algal cultivation, continuous maintenance of uni-algal cultures on a field scale for a long period of time, harvesting the algae, recycling the water, extraction of the lipids from the algae, and optimization of each step in relation to the others.

We have carried out this research during the last 2.5 years with various promising oil-producing algae under both indoor and outdoor conditions. Although the algae studied might not be the ones finally chosen, they have served as model organisms and enabled us to gain experience in operating a system with saline water under desert conditions with all its special constraints. One of these model organisms was Isochrysis (8).

## ISOCHRYISIS

Isochrysis galbana is a free-living motile marine phytoflagellate of the class Haptophyceae and the order Isochrysidales. This species, known to accumulate alkenones (13) and polyunsaturated fatty acids (5,24), have already been used as part of the food chain in aquaculture (17,22,23). The high percentage of long-chain polyunsaturated fatty acids in Isochrysis is of significance in view of the known importance of these essential fatty acids in mariculture (18). Isochrysis accumulates high amounts of lipids and carbohydrates under nitrogen deficiency, when the lipids comprise about one-fourth of the algal organic material (2,21).

## MATERIALS AND METHODS

### Culture techniques

#### 1. Laboratory

-----  
Isochrysis galbana was cultivated in artificial seawater (ASW) as described previously (8) in 10-liter flasks, illuminated continuously with a set of fluorescent lamps supplying ca.  $100 \mu\text{E m}^{-2} \text{ s}^{-1}$ , and aerated with air containing 1.5%  $\text{CO}_2$ . The cultures were maintained at  $28^\circ\text{C}$  at pH 7.0.

#### 2. Outdoors

-----  
Artificial seawater was also used for outdoor cultivation. Two types of ponds were used:

##### a. $2.5 \text{ m}^2$ -ponds

To prepare inocula for the 100<sup>2</sup>-m pond, 2.5 m<sup>2</sup> ponds, (300-liter capacity) were used. The inocula were started from laboratory cultures (80 liter at 15 mg chlorophyll liter<sup>-1</sup>). Six 2.5 m<sup>2</sup> ponds were operated.

b. 100 m<sup>2</sup> pond

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The 100-m<sup>2</sup> pond is a raceway consisting of two channels 2 m wide 22.5 m long and 12 cm deep. Mixing is done with a paddlewheel (15 rpm). The pond was inoculated on August 11, 1985, and for the next 5 days 40% of the pond was covered with a net providing 50% shade to prevent light damage. The pH fluctuated between 6.9 and 8.2. CO<sub>2</sub> was injected into the culture.

Pond maintenance

The temperature, dissolved oxygen, and pH of the cultures were monitored twice a day (Fig. 1).

Chemical analyses:

NO<sub>3</sub>, NH<sub>4</sub>, PO<sub>4</sub>, chlorophyll, and turbidity were measured daily; protein, ash-free dry weight (AFDW), and lipids 3 times a week.

Steady-state conditions

To maintain steady-state conditions, the culture was bled as required. Towards the end of the season the algae were harvested, and the effluent was recycled to the pond.

Harvesting

Isochrysis galbana grown outdoors in artificial seawater was harvested by flocculation, followed by dissolved-air flotation (DAF) of the algal flocs. Before carrying out a continuous harvesting operation, jar tests were done in a Phipps and Bird multiple stirrer apparatus, with half a minute of rapid stirring at 80 rpm, 10 minutes of slow mixing at 30 rpm, followed by 10 minutes of settling before taking a supernatant sample for determination of optical density in a Klett spectrophotometer at 540 nm. The optimum concentration of several flocculants (alum, FeCl<sub>3</sub>, FeCl<sub>3</sub> together with a polycationic flocculant, and chlorine followed by FeCl<sub>3</sub>) was determined in these tests.

Continuous flocculation and flotation were performed in a dissolved-air flotation pilot-plant unit. Flocculants and acid were injected in-line with a pair of Cole-Parmer peristaltic pumps. The inflow to the DAF unit was pumped from the outdoor pond at rates varying from 3 to 5 liter minute<sup>-1</sup>. Other operational conditions were: recycling rate of pressurized flow from 0 to 28%, pressure of pressurized flow 3.5 atm, pH 3.5 to 4.5, and concentration of FeCl<sub>3</sub> from 100 to 260 mg liter<sup>-1</sup>. Composite samples of pond water, DAF unit effluent, chlorophyll-a, direct algal counts, PO<sub>4</sub>-P, and OD were measured to compare removal efficiencies.

PREPARATION OF ALGAL SLURRY AND ANALYSIS

Lyophilization

Wet-flocculated and non-flocculated algae were frozen and lyophilized for 24 h before chemical analysis.

Chemical analysis

Protein was assayed by the method of Lowry et al. (12) or of Kochert (10) after

hydrolyzing the lyophilized material in 1 N NaOH for 1 h at 100°C. Total carbohydrates were analyzed by the phenol-sulfuric acid method after acid hydrolysis in 2 N HCl for 1 h at 100°C (11). Extended hydrolysis did not significantly increase the measured carbohydrate concentration.

#### Extraction and fractionation of lipids

Total lipids were analyzed after repeated extraction with methanol-chloroform-water (10:5:4, v/v/v) according to Bligh and Dyer (3), modified as described by Kates (9). The lipids were phase-separated with the same solvents at a ratio of 10:10:9. The chloroform phase was evaporated to dryness under a stream of N<sub>2</sub> and dried under vacuum, and then its weight was determined gravimetrically.

The total lipid extract was fractionated on a heat-activated silicic acid column (Unisil, Clarkson Chemical Co., Williamsport, PA) with redistilled hexane, benzene, chloroform, acetone, and methanol as the sequence eluting solvents. The following types of components were eluted: acyclic hydrocarbons (eluted with hexane): polyunsaturated acyclic hydrocarbons, fatty acid methyl esters, and sterols (benzene), tri-, di-, and mono-glycerides, and free fatty acids (chloroform): glycolipids (acetone): and phospholipids (methanol). The fractions were concentrated by flash evaporation and taken to dryness under a stream of N<sub>2</sub> for weight determination.

## RESULTS

The operation of the 100-m<sup>2</sup> pond was studied at Sede Boqer, where the prevailing desert conditions are similar to those in the southwestern US. The operation started on August 11 and lasted until September 12th. During this time of the year the temperatures were 23-24°C in the morning and 32-34°C at noon (Fig. 1).

### Pond maintenance

#### a. Cultivation

The initial cell concentration in the 100-m<sup>-2</sup> pond was  $3 \times 10^6$  cells ml<sup>-1</sup>, which is equivalent to 240 g liter<sup>-1</sup> AFDW. The growth curve is shown in Fig. 2. It took about 12 days for the culture to enter the stationary phase of growth; after 22 days the maximum cell concentration was reached ( $43 \times 10^6$  cells). During the logarithmic phase of growth, the doubling time of the culture was 1.6 days. The maximum calculated output rate was 22 g m<sup>-2</sup> day<sup>-1</sup> ( $\mu = 0.22$  day<sup>-1</sup>, between August 18 and 20). A good correlation was found between the various growth parameters: cell number, optical density, chlorophyll content and ash-free dry weight (Fig. 2).

Only after obtaining the maximum cell concentration was a bleeding regime started in order to measure productivity (between August 25 and September 12). The biomass concentration was maintained between 0.9 and 1.3 g liter<sup>-1</sup> AFDW; biomass yield was 23.6 g m<sup>-2</sup> day<sup>-1</sup> during semi-continuous operation (with bleeding) in a 17-day period.

#### b. Maintenance of unialgal culture

Throughout the entire period of outdoor cultivation, from inoculum in mid-July until September 12, Isochrysis was the dominant algal species in the culture. Daily observations under the microscope revealed a clean culture (Fig. 3).



### c. Harvesting

The dissolved-air flotation pilot plant was transferred from the Technion to Sede Boqer. The operating conditions used for harvesting are described in "Materials and Method." The experiments were performed in seven runs. In the last three runs the effluent was returned to the pond. No special effects on the pond could be detected, and the culture looked as it did before. When alum was used as a flocculant, the effect on pond pH was not as pronounced as when  $\text{FeCl}_3$  was used. In each run, 1-3 m<sup>3</sup> was harvested continuously, and before each run, jar tests were performed according to the following procedure:

- Find flocculant dose for removal of 50% of OD without pH adjustment (= suboptimal dose)
- Titrate suboptimal dose plus pond medium with acid
- Flocculation with suboptimal dose at various pH values to define optimum pH
- Test different flocculant doses at equal optimum pH.

The results are shown in Table 1.

Table 1. Conditions and Efficiency of Removal of Isochrysis galbana  
Cultivated in a 100-m<sup>2</sup> Pond

Flocculant, mg liter <sup>-1</sup>	Final pH	OD of pond water	OD of supernatant after floc. (at 540 nm)	Removal efficiency (%)
Alum, 150	5.5	0.750	0.176	76.5
Alum, 200	5.5	0.750	0.118	84.2
Alum, 250	5.5	0.750	0.094	87.4
$\text{FeCl}_3$ , 75	3.5	0.740	0.266	64.0
$\text{FeCl}_3$ , 100	3.5	0.740	0.122	83.5
$\text{FeCl}_3$ , 125				
$\text{FeCl}_3$ , 150				
$\text{FeCl}_3$ , 100 + Zetag 57, 2	6.2	0.800	0.370	53.7
$\text{FeCl}_3$ , 100 + Zetag 57, 8	6.2	0.800	0.300	62.5
$\text{FeCl}_3$ , 100 + Zetag 57, 20	6.2	0.800	0.102	87.2
Chlorine, 0 + $\text{FeCl}_3$ , 100	3.5	0.840	0.180	78.6
Chlorine, 4.5 + $\text{FeCl}_3$ , 100	3.5	0.840	0.134	84.0
Chlorine, 9.0 + $\text{FeCl}_3$ , 100	3.5	0.840	0.138	83.6

High concentration of alum or  $\text{FeCl}_3$  was required to obtain removal efficiencies above 80% (Table 1). Good removal efficiency was achieved when a high concentration of polycationic flocculant (20 mg liter<sup>-1</sup>) was used together with  $\text{FeCl}_3$  at a concentration of 100 mg. The addition of chlorine improved the removal efficiency, probably by affecting the motility of the algal cells.

The results of some of the runs under various flocculation conditions are shown in Table 2, indicating the change in harvesting efficiency with the amount of flocculant and culture age.

Table 2. Efficiency of Harvesting of Isochrysis using Flocculation with  $\text{FeCl}_3$  by Dissolved Air Flotation as Affected by  $\text{FeCl}_3$  Concentration

Date (1985)	$\text{FeCl}_3$ -1 (mg liter <sup>-1</sup> )	Retention time (min)	% Removal $\frac{(\text{OD initial} - \text{OD final})}{\text{OD initial}} \times 100$
Aug. 20	70	10	32.5
Aug. 22	90	20	40.0
Sept. 7	120	10	53.0
Sept. 11	180	13	74.0

As can be seen, the removal efficiency at the beginning of the growth period was relatively low, improving thereafter. Using a flocculant concentration of 180 mg  $\text{FeCl}_3$  liter<sup>-1</sup> with a retention time of 13 min resulted in a removal efficiency of 74%.

The results of chemical determinations and direct algal cell counts of pooled harvest samples of 1-3 m of algal culture at a concentration of 260 mg.liter<sup>-1</sup> are given in Table 3.

Table 3. Analysis of I. galbana Pond Water and Supernatant After Algal Harvesting

Parameter	Pond water	Pooled sample of DAF effluent 20% recycling at 3 Atm		Pooled Sample from DAF Unit (without recycling Using O Super- saturation for flotation
		From DAF Unit	After Additional 10 h Sedimentation	
TSS (%)	481	20.5 (57.4)	70 (85.4)	130 (73)
Chlorophyll <u>a</u> (mg liter <sup>-1</sup> )	6.3	1.55 (75.4)	0.5 (92.1)	0.12 (98.1)
Algal cell number (ml <sup>-1</sup> )	11 x 10	2 x 10 (81.8)	0.1 x 10 (99.9)	10 x 10 (99.1)
OD at 540 nm	0.620	-	-	10
PO <sub>4</sub> -phosphate	50	15 (70)	10 (80)	10 (80)

Numbers in parentheses present percent of removal.

Although the removal efficiency calculated from the TSS and OD was 71 to 73%, it was 98 and 99%, when calculated from data of chlorophyll a and cell counts, respectively. Since only 80% of the PO<sub>4</sub>-P was removed, it can be assumed that the remaining 20% was in the form of insoluble phosphates, which affect TSS and OD. The large PO<sub>4</sub>-P content in the medium certainly affects FeCl<sub>3</sub> requirements because of the formation of FePO<sub>4</sub>, which is not effective as a flocculant.

The algal float collected from the DAF unit contained 3.2% solids. After allowing the cells to settle overnight and drawing off the liquid portion, the solid content increased to 10.4%.

It is noteworthy here that fluctuation of the pH in the pond greatly affected the amount of acid required for obtaining optimum pH for harvesting. The flocculant requirement also fluctuated, depending on whether the cultures were taken directly from the pond or first stored in darkness. Studies on algal physiology could indicate the minimum amount of PO<sub>4</sub>-P that would not limit growth in an outdoor culture. This parameter would help to reduce PO<sub>4</sub>-P and flocculant costs.

Forty liters of concentrated slurry were produced for lipid characterization and oil extraction experiments.

#### Chemical analysis

The chemical composition of Isochrysis galbana was determined in samples harvested by two different methods: centrifugation and flocculation (Table 4). The relative contents of proteins and carbohydrates were similar in both

samples, about 30% proteins and 20% carbohydrates. The proportion of lipids, on the other hand, was half (12%) as much in the flocculated algae as in the centrifuged algae (24%), apparently due to lipid loss during the flocculation process. The flocculated paste contained a higher content of residual medium than the centrifuged paste, and thus a higher percent ash. Experiments showed that this high salt concentration did not interfere with lipid extraction.

Table 4. Composition of Isochrysis Harvested by Centrifugation and by Ferric Chloride Flocculation

Harvesting method	Ash % of dry weight	% of organic matter (weight)			
		Protein	Carbohydrate	Lipid	Unknown
Centrifugation	12	28	18	24	30
Ferric chloride flocculation	48	30	22	12	36

The total lipid extracts of Isochrysis were fractionated on silicic acid columns with a sequence of organic solvents. The amount of lipid in the eluates is shown in Table 5. The major lipid components in Isochrysis are alkenones in the benzene fraction, triglycerides in the chloroform fraction, glycolipids in the acetone fraction, and phospholipids in the methanol fraction. Isochrysis harvested by ferric chloride flocculation showed a lower content of triglycerides than the centrifuged algae - a possible indication of triglyceride loss during flocculation.

Table 5. Lipid fractions in Isochrysis Eluted from Unisil Columns

Harvesting method	Lipid fraction, % of Total lipid weight				
	Hexane	Benzene	Chloroform	Acetone	Methanol
Centrifugation	1.4	27.4	32.1	26.3	12.6
Ferric chloride flocculation	2.0	35.1	4.0	37.6	21.3

## DISCUSSION

The trial operation of a 100-m<sup>2</sup> pond has succeeded in demonstrating the feasibility of the integrated process of growing halotolerant microalgae continuously on a field scale under desert conditions, their harvesting, and oil extraction. As expected at this stage, this trial operation produced more questions than answers. The questions are those that can appear only when algal cultivation is run as an integrated system that includes all steps, from culture inoculation to lipid extraction. When solved early, these problems will not cause greater difficulties at later stages, when real operation is started.

The ability to maintain Isochrysis cultures clean and uni-algal is of special interest. The relatively high temperatures (above 20°C), maintenance of relatively high biomass in the pond, and presence of ammonia (2 mM) apparently prevent the proliferation of zooplankton. The relative resistance of Isochrysis to the entire UV spectrum (7) gives it a competitive advantage to potential competitors and helps in maintenance of a clean pond.

The percent of total lipid in FeCl<sub>3</sub>-flocculated algae was about half that of centrifuged algae, probably because some lipid components were released from the cells into the surrounding medium during flocculation. Ferric chloride and other flocculants are known to cause conformational changes in the cell envelope, which may lead to loss of intracellular components. Experiments to determine the flocculation conditions needed to increase the lipid yield will be done in the future.

This trial of the whole process of pond operation revealed problems concerning the interdependence of the operation steps thus emphasizing the need to mutually adjust all steps in order that the whole system is optimized. Some of the questions that arose during this trial operation and need to be solved during future stages of this research project are:

- Determination of the optimum cell density in the pond, which has to take into account not only productivity but also harvesting efficiency
- The dependence of harvesting efficiency on nutrient levels in the pond (e.g. phosphate), culture pH, and exposure of the cells to light or storage in darkness
- Effect of type and concentration of flocculant and effect of algal concentration and dryness of the resultant paste on lipid extraction
- Effect of recycling the medium to the pond after harvesting
- Timing of harvesting during the day
- Effect of the method of induction of lipid accumulation on culture maintenance
- Cost analysis and economic evaluation of the entire, integrated process

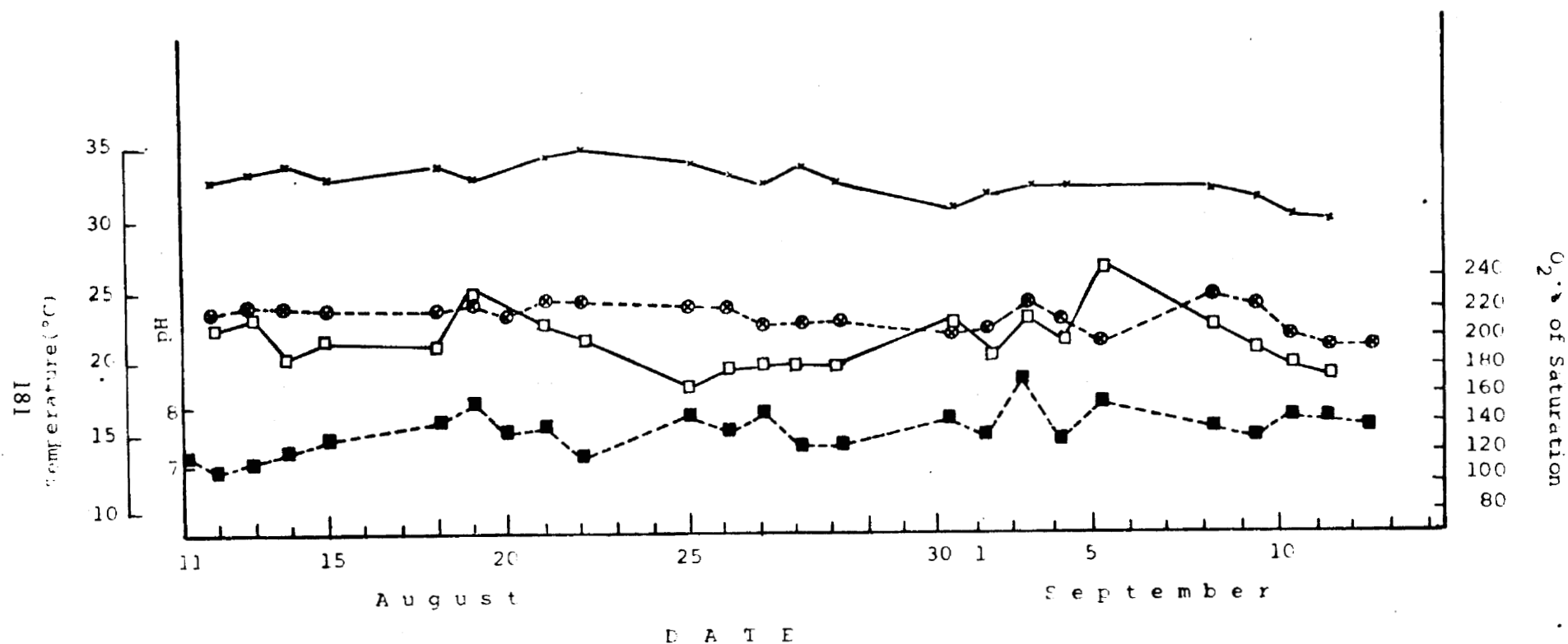
We consider this trial operation as a preliminary examination necessary for continued activity in the future. Its success encourages continuation of the research according to the same concept. In addition to studying the above questions with Isochrysis, other marine algae that seem promising, e.g. Chaetoceros, should be cultivated outdoors throughout the year under the optimum growth conditions found previously (i.e. nutritional requirements, pond operation strategy, etc.) in order to optimize harvesting conditions and find suitable methods for oil extraction from the algal slurry after harvesting.

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Fig. 1: Physical and environmental parameters of 100 m<sup>2</sup> Isochrysis



- ⊗--⊗ morning temperature
- X--X maximum temperature
- O<sub>2</sub> % of saturation
- pH



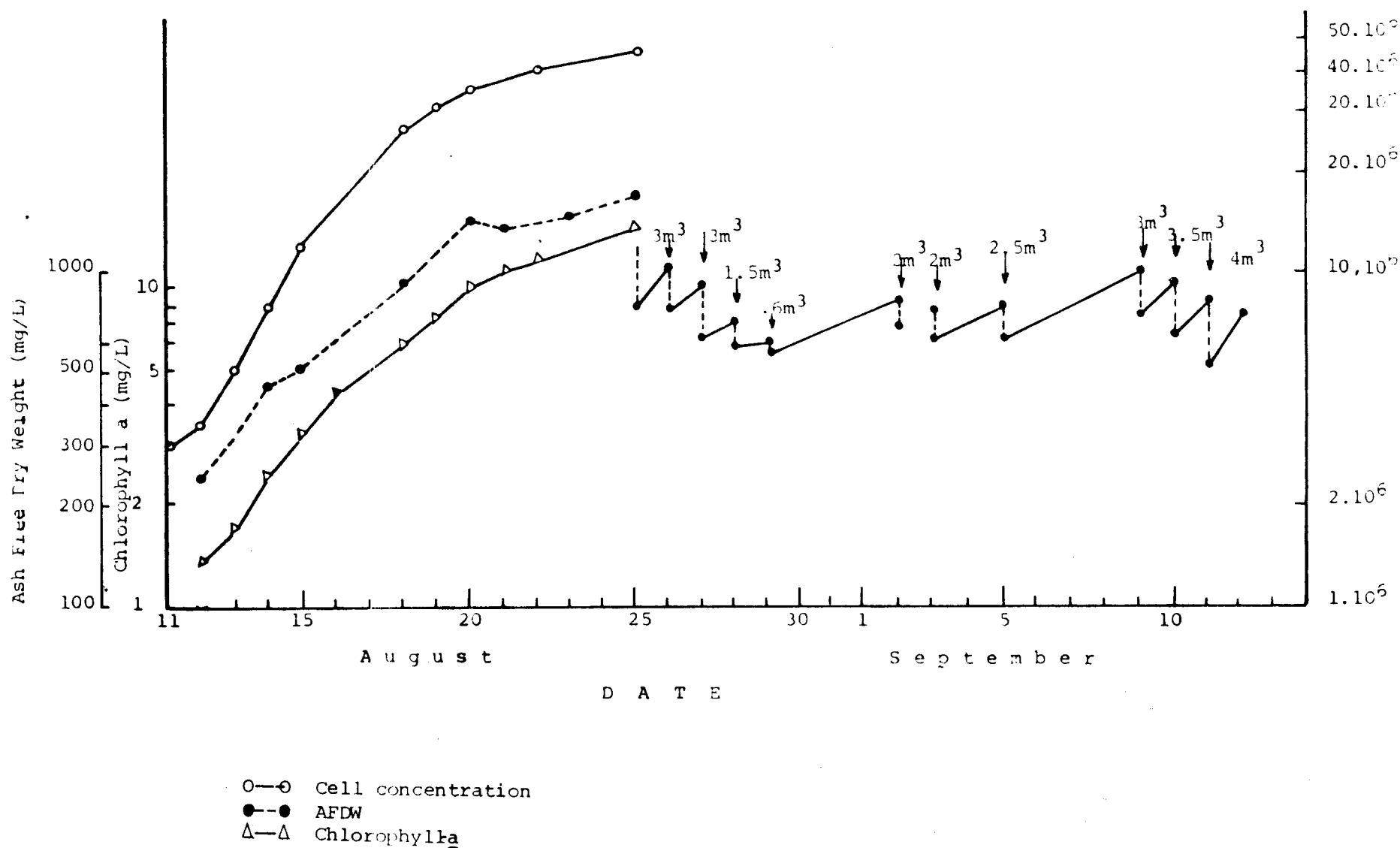
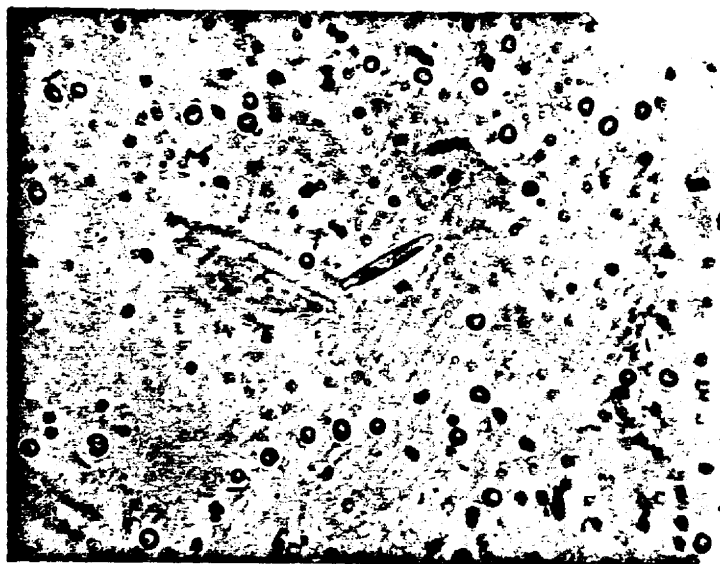
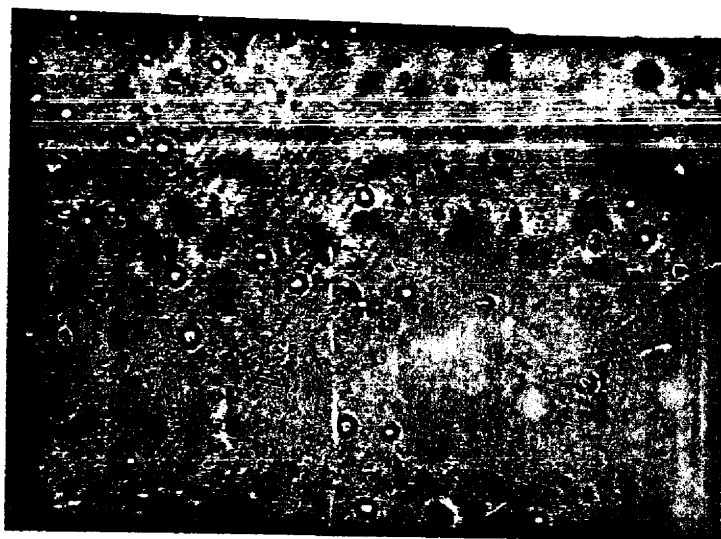
Fig. 2: Growth and semicontinuous operation of 100 m<sup>2</sup> of Isochrysis pond

Fig. 3: Isochrysis culture of 100 m<sup>2</sup> pond during summer operation

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

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Effect of Light on Biomass Production

in Two Halotolerant Microalgae

Nannochloropsis salina and Isochrysis galbana

ABSTRACT

The cell concentration in cultures of Nannochloropsis salina was optimized with respect to its effect on the overall output rate. Under outdoor steady-state conditions at a cell concentration of  $350 \text{ mg l}^{-1}$  (dry weight), the overall output rates were  $24.5 \text{ g.m}^{-2}.\text{day}^{-1}$  biomass and  $4.0 \text{ g.m}^{-2} \text{ day}^{-1}$  lipids.

With Isochrysis galbana, the pond depth and mixing regime had no significant effect on the biomass output rate. The greatest output rate was observed for this organism at a cell concentration of  $350 \text{ mg l}^{-1}$  at a medium depth of 12.8 cm where the total biomass output was  $28.1 \text{ g.m}^{-2}.\text{day}^{-1}$  and that of lipids was  $6.4 \text{ g.m}^{-2}.\text{day}^{-1}$ .

INTRODUCTION

Algae are among the most efficient plants in utilizing solar energy, having the highest output rate in terms of dry weight and protein per unit

area<sup>1</sup> when light is the sole limiting factor for growth. Thus, the most important single issue in the practical understanding of biomass production in outdoor algal cultures is the study of light as the major factor limiting the output rate. Because of self-shading, the significant parameter in studying the effect of illumination is the integrated radiant flux incident on the average algal cell. Outdoors, this parameter depends on three factors: (1) the light intensity, (2) the algal population density, and (3) the dark/light cycle to which the average cell in the culture is exposed. The latter is affected by the turbulence, depth of the medium, and population density.

In the experiments presented here, we examined the effects of population density, medium depth and turbulence on the overall rates of biomass and lipid production in two halotolerant microalgae, Nannochloropsis salina and Isochrysis galbana, in order to evaluate their potential as sources of oil for energy production.

## MATERIALS AND METHODS

### Organisms

The crysophytes Nannochloropsis salina (GSB STICHO) and Isochrysis galbana were obtained from the SERI culture collection in Golden, Colorado.

### Growth medium

Both algae were cultivated in artificial seawater (ASW) from which bicarbonate was omitted. The components of the ASW medium (in g l<sup>-1</sup>) were: NaCl (27), MgSO<sub>4</sub> 7H<sub>2</sub>O (6.6), CaCl<sub>2</sub> 2H<sub>2</sub>O (1.5), KNO<sub>3</sub> (1.0), KH<sub>2</sub>PO<sub>4</sub> (0.070), FeCl<sub>3</sub> 4H<sub>2</sub>O (2.4), Na<sub>2</sub>EDTA (1.6) and 1 ml l<sup>-1</sup> of a microelement solution containing (in mg l<sup>-1</sup>): ZnCl<sub>2</sub> (40), H<sub>3</sub>BO<sub>3</sub> (600), CaCl<sub>2</sub> (1.5), CuCl<sub>2</sub> 2H<sub>2</sub>O (40), MnCl<sub>2</sub> (400), and (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub> 4H<sub>2</sub>O (370).

### Outdoor cultures

The algae were cultivated in 2.5 m<sup>2</sup> ponds that contained 300 l of medium (pond depth 12 cm unless otherwise stated). The culture was agitated by a paddlewheel that maintained a water velocity of 20 cm sec<sup>-1</sup>. The effect of mixing on the output rate was tested in experiments where water velocity was varied from 16 to 28 cm sec<sup>-1</sup>.

The pH was maintained within the ranges of 7.0-7.5 and 6.0-6.5 for Nannochloropsis and Isochrysis, respectively. Climatic conditions were described previously<sup>2</sup>.

To test the effects of cell concentration, pond depth and mixing rate on overall productivity, cultures were maintained under the conditions indicated at steady state for at least 20 days by bleeding the cultures whenever the chlorophyll concentration reached 10-15% more than the desired value.

The photosynthetic efficiency (PE) was calculated from the ratio of cellular calories produced (5.5 kcal = 1 g AFDW) to the measured calories supplied by solar irradiation in the photosynthetically active spectral range (PAR) of 400-700 nm.

### Analytical methods

Algal growth was followed by measuring the following parameters: optical density at 540 nm, total chlorophyll by methanol extraction, and ash-free dry weight (AFDW). Protein was determined according to the Lowry procedure<sup>3</sup>, using bovine serum albumin as a standard. Lipids were extracted by a modified Bligh-Dyer procedure<sup>4</sup>.

## RESULTS AND DISCUSSION

### Effect of population density on the output rate in *Nanochloropsis salina*

The cell concentration in an algal culture affects the amount of light that reaches the individual cells in the culture. Clearly, the lower the population density, the higher the specific growth rate to be expected in a light-limited system. Since the net biomass output rate is a product of the cell density and the specific growth rate, and since these parameters are related, then when the system is limited only by light, maximum output rate should be achieved at some optimum cell density<sup>5</sup>.

Table 1 summarizes the effects of cell concentration on the relations between protein, chlorophyll and dry weight. While the ratio of protein to dry weight was constant, the ration of chlorophyll to dry weight increased in parallel with increasing cell concentration, as expected for a photoautotrophic organism.

We tested the effect of cell concentration on the overall rates of biomass and lipid production in outdoor cultures. The results (Table 2) indicate the great influence of cell concentration on the overall productivity. However, lipid production was affected to a lower extent in response to changes in cell concentration, due to the direct effect of cell concentration on lipid content relative to dry weight (Table 2). The maximum rates of biomass and lipid production obtained were 24.5 and 4.0 g m<sup>-2</sup> day<sup>-1</sup>, respectively.

### Effect of pond depth on the productivity of *Isochrysis galbana*

The culture depth may play a significant role in the optimization of any commercial operation of photosynthetic biological reactors. Culture depth may determine not only the total volume of culture and the energy input required, but also the optimum cell concentration for production of biomass. The interactions between pond depth and cell concentration will actually define the amount of light available to each cell in the culture, which determines the optimum areal density of biomass for maximum production.

In the following experiments, we tested the effect of pond depth on the overall output rates: (a) at a constant cell concentration and three different depths, 12.8, 8.6 and 7.0 cm; (b) at a constant areal biomass ( $\text{g}\cdot\text{m}^{-2}$ ) at the same depths as in a. In the first part of the experiment (Table 3), the highest output rate was obtained at the highest depth (12.8 cm), although the highest growth rate, as expected, was recorded at the shallowest depth (7 cm) (data not shown). When the areal biomass was kept constant (Table 3), similar results were obtained, i.e. the deepest pond yielded the highest overall output rates,  $28 \text{ g}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$  biomass and  $6.4 \text{ g}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$  lipids.

These data reflect the complexity of the process of optimizing outdoor biomass production in light-limited cultures. More detailed experiments are needed in order to understand the effect of pond depth on the productivity of this organism. However, the high rate of lipid production obtained under these conditions ( $6.4 \text{ g}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$ ) indicates the potential of using *Isochrysis* as a model organism for studying lipid production in algae.

### The effect of mixing rate on productivity

The output rate is also affected by the extent of turbulence in the pond, as we already reported<sup>6</sup>. Increasing the mixing rate may impose a more favorable light/dark cycle on the average cell in the culture, resulting in an improved photosynthetic efficiency per unit area. The greater the turbulence, the shorter should be the duration of one complete light/dark cycle. In addition, at high irradiation, ( $>250 \text{ uE m}^{-2} \text{ sec}^{-1}$ ), cells located in the upper layer of the pond may suffer from over-exposure to light which would also be decreased by mixing. We tested the effect of the mixing rate on Isochrysis in an attempt to maximize the output rate. The data (Table 4), however, do not show a significant increase in biomass productivity in response to an increased mixing rate.

The productivity of a motile organism such as Isochrysis is probably less affected by the mixing rate. A motile organism might thus require less mixing for optimal production than a nonmotile organism, resulting in a lower energy input in the cultivation process.

### CONCLUSIONS

Under outdoor conditions, biomass output is affected mainly by cell concentration.

The pond depth and the mixing regime had no significant effect on the output rate of Isochrysis galbana. This could be due to the fact that this is a motile organism. Further experiments are needed to examine the effects of pond depth and mixing regime on the output rate of Isochrysis and to determine whether these environmental factors produce different responses in motile and non-motile organisms.



The relative high rate of lipid production obtained in Isochrysis galbana cultures indicates the potential of using this alga as a model for studying lipid production in algae.

## Appendix

### Development and Use of Data Aquisition System

A computerized system for monitoring and controlling miniponds was developed and applied for studying the dynamics of biological processes. One of the potential applications of the developed system is optimization of aquaculture production units by using efficient algorithms to automatically search for optimum growth conditions.

In the present study, we explored the possibility of using the dissolved oxygen concentration to estimate the rate of carbon assimilation in algal minipond. After theoretical considerations of the relationship between this parameter and the growth rate, we developed a new procedure by which the desired information can be calculated on-line. The method is based on perturbing the system from its dynamic equilibrium and examining the system's response. By this method, one can obtain data with high enough signal-to-noise ratio for estimating the desired parameter, despite the relatively high background noise level.

An improved version of the Data Aquisition System has been installed and has been under operation for more than 6 months. The main features of the Data Aquisition System are:

- 1- A continuous graphic display of data collected up to 30 h.

- 2- A more stable pH electrode so as to overcome drifts and electronic disturbances by control devices. The pH electrode controls a magnetic valve on the CO<sub>2</sub> line so as to control the pH as well.
- 3- An optical density probe which can be immersed into the medium without any need for extra mechanical pumping was developed and operated under laboratory conditions, an improved probe is now under construction and will be operating soon.
- 4- Using an analytical model for dissolved oxygen concentration described elsewhere (7), a method for on-line estimation of the net O<sub>2</sub> production rate was developed. The method was tested experimentally by imposing a shift in O<sub>2</sub> concentration for a 3 h interval and following the rate at which the system returns to its original steady state. It was found that this method can be used as a tool in optimization studies and also as an indication of the well-being of the algal culture.

From Table 6, a few important findings can be pointed out:

- a) The most productive hours during the day are 9 to 12 AM, when 50% of the daily production occurs.
- b) During the night-time, 10 to 15% of the biomass produced is lost via respiration.

The productivity figures obtained through the data acquisition system correlated well with those obtained by the usual methods of productivity measurement.

Fig. 1 presents some of the parameters that can be obtained with the newly developed Data Acquisition System. The continuous estimation of increasing dry weight is a powerful tool for on-line estimation of productivity. The data can be easily correlated with all other environmental parameters.

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Table 1

Effect of Population Density on Dry Weight, Protein and Chlorophyll in Nannochloropsis Cultivated Outdoors

Dry weight (DW) mg/l	Protein/DW %	Chlorophyll mg/l	Chlorophyll/DW %
350	43	3.5	1
500	43	5	1
750	40	10	1.2
1300	43	21	1.6

Algae were cultivated in a 2.5 m<sup>2</sup> pond, in ASW medium (Summer 1985)

Table 2

The Effect of Population Density on the Biomass Output, Photosynthetic Efficiency and Lipid Production in Nannochloropsis

Population density mg l <sup>-1</sup>	Biomass g m <sup>-2</sup> day <sup>-1</sup>	Photosynthetic efficiency %	Lipids % of AFDW*	Rate of lipid production g m <sup>-2</sup> day <sup>-1</sup>
350	24.5	4.3	16	4.0
500	23.0	4.0	16	3.7
750	16.7	3.5	18	3.0
1300	14.0	3.3	21	2.9

Cultivated in 2.5 m<sup>2</sup> ponds, ASW medium (Summer 1985)

Maximum temperature 30-35°C, minimum temperature 17-23°C

\* Ash free dry weight

Table 3

The effect of pond depth on the productivity of  
*Isochrysis galbana* cultivated outdoors  
 a) at a constant cell concentration; b) at a constant areal biomass

	Pond Depth and Volume cm (l)	Cell Concentration mg/l	Output Rate g m <sup>-2</sup> day <sup>-1</sup> AFDW	Photosynthetic Efficiency %	Lipids % of AFDW	Rate of Lipid Production g m <sup>-2</sup> day <sup>-1</sup>
a	12.8 (325)	650	24	5.6	25	6
	8.6 (250)	650	21	4.9	26	5.5
	7.0 (175)	650	17	3.9	25	4.3
b	12.8 (325)	350	28.1	6.5	23	6.4
	8.6 (250)	450	23	5.3	22	5
	7.0 (175)	650	14	3.3	22	3

Algae cultivated at 2.5 m<sup>2</sup> ponds (Summer 1985)

Table 4

The effect of mixing rate on the productivity of  
*Isochrysis galbana* cultivated outdoors

	Cell Concentration mg/l	Output Rate g m <sup>-2</sup> day <sup>-1</sup> AFDW	Lipids* % of AFDW	Rate of Lipid Production g m <sup>-2</sup> day <sup>-1</sup>
A	350	24	22	5.3
	450	26	23	6.0
	650	22	25	5.5
B	350**	24	24	5.8
	450	22	23	5.1
	650	20	23	4.6

Cultivated at a 2.5 m<sup>2</sup> is ASW medium (Summer 1985)

A) high mixing rate, 28 cm sec<sup>-1</sup>

B) low mixing rate, 16 cm sec<sup>-1</sup>

\* Average from Table 3

\*\* Steady state maintained for 5 days only

Table 5

Daily production rates calculated by  
the data acquisition system  
November 21 - December 2 1985

Hour	Light Intensity	Temperature °C	OPR	DW	Daily yield g m <sup>-2</sup> day <sup>-1</sup>
3	-	16.8	.001	110.8	
6	-	15.3	.007	112.5	
9	28	19.2	.161	148.8	
12	65	27.8	.279	211.7	
15	55	31.0	.205	257.9	
18	11	27.0	.065	272.7	
21	-	21.4	-.027	266.5	
24	-	20.2	-.026	260.5	18
3	-	18.5	-.024	255.0	
6	0.5	16.7	.010	257.3	
9	28	20.4	.109	282.0	
12	63	27.3	.247	337.5	
15	54	29.8	.182	378.5	
18	12	26.2	.066	393.5	
21	-	21.0	.027	387.2	
24	-	19.9	.027	380.9	15
3	-	18.5	-.024	375.5	
6	-	16.7	.006	376.8	
9	-	20.2	.136	407.6	
12	-	27.9	.229	459.2	

1) Light relative units: 60 units = 2000  $\mu$  E m<sup>-2</sup> sec<sup>-1</sup>

2) OPR - O<sub>2</sub> production rate in mg/min

Negative values of OPR represent uptake of O<sub>2</sub> (respiration)

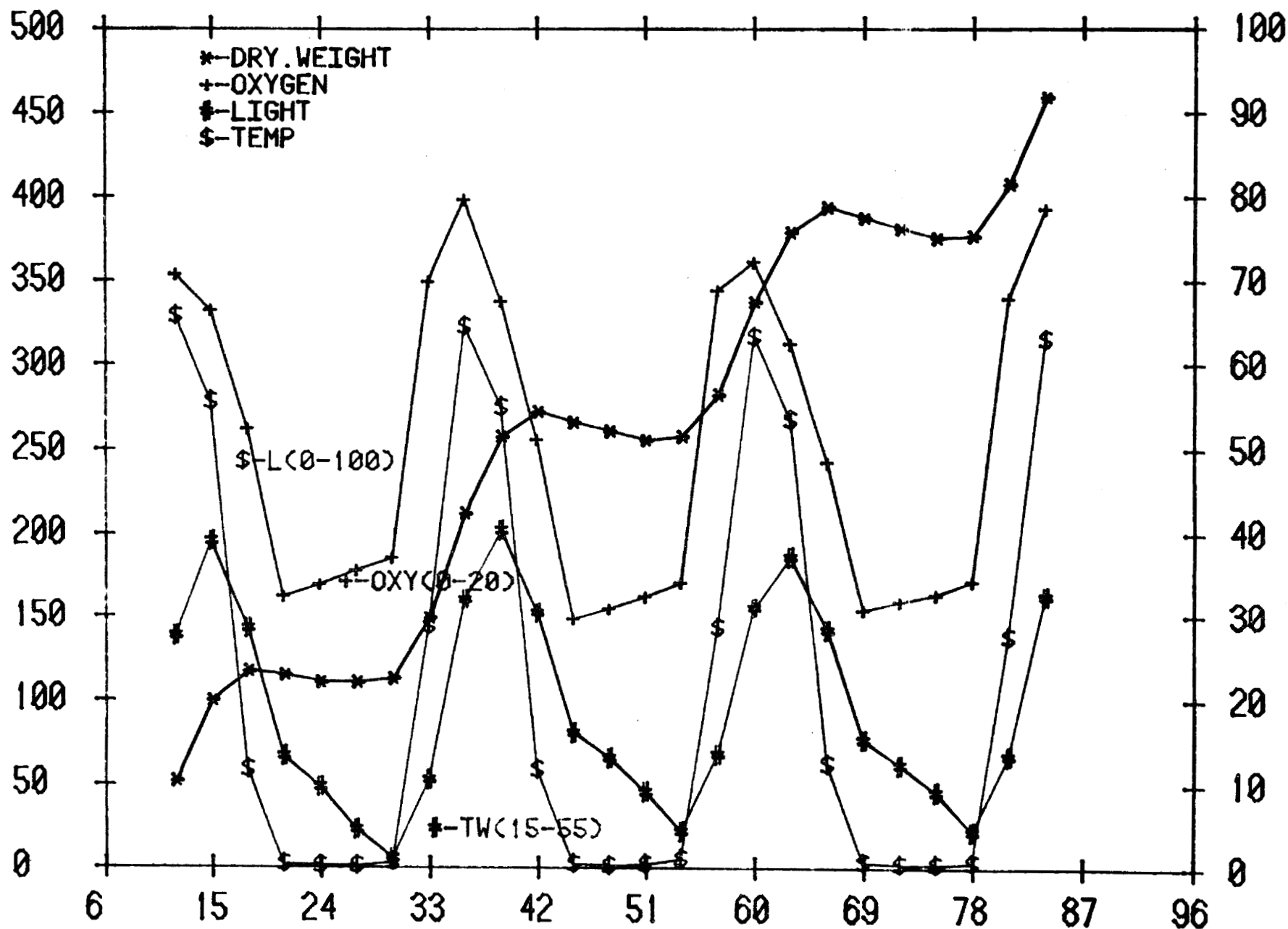
3) DW - cumulative ash-free dry weight in mg/lit

Fig. 1: Graphic presentation of 3 days of data measurements  
by the Data Aquisition System

NAME: ISO.23.7.85TP

DATE: 11/2/86

197  
DRY.WEIGHT(MG/L)





DEVELOPMENT OF OUTDOOR RACEWAY CULTURE TECHNOLOGIES  
FOR OIL-RICH HALTOLERANT ALGAE

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PROJECT OBJECTIVES

Task III

Optimize the outdoor lipid yield from the microalgae Chaetoceros gracilis. Combine biomass optimization strategies and lipid content optimization strategies so as to optimize lipid yield from an oil-rich diatom.

Task VI (optional)

Operate a 100 m<sup>2</sup> pond according to optimum conditions for biomass yield and for harvesting.

ABSTRACT

a) Growth rate studies have been completed in illuminated chemostats for the diatom C. gracilis in comparison to a green alga Nannochloris atomus, using Mediterranean sea water at different temperatures from 15 to 40°C, at different pHs from 5.5 to 10, at different nutrient concentrations of nitrate, phosphate and silicate, at different salinities from 0.5 M to 1.5 M NaCl, and at different light intensities from 50 to 500  $\mu\text{E m}^{-2} \text{sec}^{-1}$ . Optimum growth of C. gracilis under those laboratory conditions was attained in sea water medium at 28°C, pH 7-8, 5 mM NO<sub>3</sub><sup>-</sup>, 0.2 mM PO<sub>4</sub><sup>3-</sup>, 0.1 mM silicate, under a light intensity of 100  $\mu\text{E m}^{-2} \text{sec}^{-1}$ .

b) Optimization studies have been conducted outdoors in a few microponds of 0.35 m<sup>2</sup> each equipped with air bubbling for mixing and pH control by CO<sub>2</sub> supply. Growth rate, productivity and photosynthetic efficiency have been assayed since June 1985 with C. gracilis. A maximal productivity of 40 g AFDW m<sup>-2</sup> day<sup>-1</sup> at a photosynthetic efficiency of 9.5% was noted during the fall season. A minimal productivity of around 13 g AFDW m<sup>-2</sup> day<sup>-1</sup> at a photosynthetic efficiency of 5% was noted during the winter season. Moderate nitrate deficiency modified the algal cellular composition, only slightly the productivity, and markedly reduced the photosynthetic efficiency. Silicate deficiency induced massive contamination by green algae which interfered with productivity measurements. The onset of silicate limitation on a concentrated culture of C. gracilis increased the lipid fraction of the cell. Minimizing culture mixing by lowering reaction rate reduced productivity and photosynthetic efficiency.

Maximum productivity was observed by diluting the culture from the initial stage of the stationary phase ( $0.4 \text{ g AFDW l}^{-1}$ ) to any stage of the logarithmic phase above  $0.04 \text{ g AFDW l}^{-1}$ .

Lipid fractionation of the total lipid extract of C. gracilis revealed the presence of long chain alkenones and triglycerides as the major lipid components of the diatom.

## MATERIALS AND METHODS

### Algae

Chaetoceros gracilis, strain S/CHET-1 of the SERI Culture Collection. Nannochloris atomus Butcher, strain 251/4a of the Cambridge Culture Collection.

### Medium

Algae were grown in Mediterranean seawater medium enriched with  $5 \text{ mM KNO}_3$  or as indicated,  $0.2 \text{ mM KH}_2\text{PO}_4$  or as indicated,  $5 \text{ mM NaHCO}_3$ ,  $1.5 \text{ }\mu\text{M FeCl}_3$ ,  $6 \text{ }\mu\text{M EDTA}$ ,  $0.1 \text{ mM Na}_2\text{SiO}_3$ , trace metal mix as reported by McLachlan (1973) and  $0.5 \text{ mg l}^{-1} \text{ B}_{12}$  for N. atomus only. Algae were grown under a pH regulator stat with the pH stat controlling 10 cultures at the same time.

### Chemostats

Cultivation was activated in glass chemostats equipped with temperature control, air bubbling, pH control by supply of  $\text{CO}_2$  on demand, and light control. Radiant energy incident to the outer surface of all cultures was accomplished by a battery of ten Cool-White and Agro-Lite fluorescent lamps to provide light intensities between  $50$  to  $500 \text{ }\mu\text{E m}^{-2} \text{ sec}^{-1}$ . Algae were cultivated semicontinuously.

### Outdoor Miniponds

Ten miniponds with concave bottoms of  $0.35 \text{ m}^2$ ,  $10 \text{ cm}$  average depth and  $35$  liters volume made of white fiberglass were equipped with porous tubes for bubbling air from the bottom of the pond. Oil-free filtered air was introduced into the porous tube at a flow of  $30 \text{ l min}^{-1}$ , and the rising bubbles mixed the cultures continuously.  $\text{CO}_2$  was introduced by pH control on demand. The miniponds were operated throughout the year over all seasons and were covered with a clear plexiglass shield on rainy days.

Natural solar irradiation was measured continuously with a Li Cor Solar Monitor Model LI 1776 integrating the irradiation to  $\text{E m}^{-2} \text{ day}^{-1}$ . Minimum and maximum temperature and pH were monitored daily.

Harvesting was performed by centrifugation of a portion of the culture volume and dilution of the remaining algae in the minipond with fresh medium.

Daily assays included: cell number, chlorophyll, carotenoids, carbohydrates and protein. Organic weight and lipid content were assayed in the harvest following centrifugation and lyophilization. Ash free dry weight was determined by ashing the lyophilized samples at 600°C.

#### Analytical Methods

Protein was assayed as previously described by Lowery et al. (1951) or by Kochert (1978a) after hydrolysis in 1 N NaOH for 1 hr at 100°C. Total carbohydrates were analyzed by the phenol-sulfuric acid method following acid hydrolysis in 2 N HCl for 1 hr at 100°C (Kochert, 1978b). Lipid was assayed by repeated extraction with methanol-chloroform-water (10:5:4, v/v) (Bligh and Dyer, 1959) as modified by Kates (1964, 1972). Total lipid extract was fractionated and analyzed for different lipid fractions on a silicic acid column; the lipids were further separated and identified by thin layer chromatography and gas chromatography as previously described (Ben-Ametz et al., 1985). Pigments were assayed following Jensen (1978).

#### ACCOMPLISHMENTS AND ACTIVITIES

##### Laboratory Studies

Laboratory experiments with C. gracilis and N. atomus have been conducted in glass chemostats (Fig. 1) to control the effect of temperature, mixing, light intensity, pH, salt concentration, nutrients, etc.



Figure 1. Light, pH and temperature controlled chemostats used for growth and lipid physiology studies. The effect of pH on Nannochloris atomus.

Each chemostat was equipped for mixing with air bubbling and pH control by CO<sub>2</sub> supply. Table 1 illustrates the growth optimization results of C. gracilis.

Table 1. Growth optimization of Chaetoceros gracilis under laboratory conditions

Growth of <u>Chaetoceros gracilis</u>	Doubling time (hrs)	Yield (g l <sup>-1</sup> )
Optimal conditions	14 ± 4	1.2 ± 0.2
Stress conditions:		
Low pH, 5.5	72 ± 5	0.8 ± 0.2
High pH, 10.0	104 ± 6	0.6 ± 0.1
Low temp., 15°C	96 ± 5	0.7 ± 0.1
High temp., 35°C	72 ± 3	0.6 ± 0.1
Low nitrate, 0.5 mM	20 ± 3	0.5 ± 0.2
Low silicate, 0.01 mM	75 ± 3	0.3 ± 0.1
High salt, 1.5 M	132 ± 5	0.5 ± 0.1
Low salt, 0.2 M	20 ± 3	1.0 ± 0.1
High light, 500 $\mu\text{E m}^{-2} \text{ sec}^{-1}$	18 ± 4	1.4 ± 0.1
Low light, 50 $\mu\text{E m}^{-2} \text{ sec}^{-1}$	33 ± 3	0.9 ± 0.1
Optimal culture conditions: sea water enriched with 5 mM KNO <sub>3</sub> , 0.2 mM KH <sub>2</sub> PO <sub>4</sub> , 5 mM NaHCO <sub>3</sub> , 1.5 $\mu\text{M}$ FeCl <sub>3</sub> , 0.1 mM Na <sub>2</sub> SiO <sub>3</sub> and trace metal mix. pH 7.0 ± 0.5 by CO <sub>2</sub> flow control on demand, LI 100 $\mu\text{E m}^{-2} \text{ sec}^{-1}$ , temperature 28°C.		

Optimum growth was obtained at a temperature of 28°C, a light intensity of 100  $\mu\text{E m}^{-2} \text{ sec}^{-1}$ , pH 7.0, in enriched sea water medium containing 5 mM NO<sub>3</sub><sup>-</sup>, 0.2 mM PO<sub>4</sub><sup>3-</sup>, 5 mM HCO<sub>3</sub><sup>-</sup>, 1.5  $\mu\text{M}$  Fe<sup>3+</sup> chelated with EDTA, and 0.1 mM SiO<sub>3</sub><sup>2-</sup> in the presence of trace metal mix. High and low pHs of 6 and 9 were inhibitory. The diatom required silicate for growth and showed marked morphological changes on silicate deficiency. Temperatures above 35°C were lethal to the algae, followed by rapid contamination by bacteria and culture collapse. Low temperatures below 15°C inhibited cell division and maintained the culture in a lag period until the temperature was increased to above 17°C. Salt concentration above 0.75 M was inhibitory but the cells were resistant to high salinities up to the level of 1.5 M NaCl. Low salinities below 0.5 M NaCl reduced slightly the growth rate and the cell yield. A high light intensity of above 300  $\mu\text{E m}^{-2} \text{ sec}^{-1}$  was not photoinhibitory to a diluted culture of C. gracilis; a denser culture of up to 1.4 g AFDW l<sup>-1</sup> was obtained under a light intensity of 500  $\mu\text{E m}^{-2} \text{ sec}^{-1}$ .

Optimal growth conditions of N. atomus were conducted in chemostats for comparison with C. gracilis (Table 2).

Table 2. Growth optimization of Nannochloris atomus in relation to temperature, pH, nutrients and light intensity

Growth of <u>Nannochloris atomus</u>	Doubling time (hrs)	Yield (g l <sup>-1</sup> )
Optimal conditions	8 ± 1	1.3 ± 0.2
Stress conditions:		
Low pH, 5.5	16 ± 2	0.6 ± 0.1
High pH, 10.0	28 ± 3	0.5 ± 0.15
Low temp., 15°C	72 ± 3	0.5 ± 0.1
High temp., 35°C	48 ± 2	0.6 ± 0.2
Low nitrate, 0.5 mM	9 ± 1	0.8 ± 0.1
High salt, 1.5 M	54 ± 1	0.6 ± 0.2
Low salt, 0.2 M	10 ± 1	1.3 ± 0.2
High light, 500 $\mu\text{E m}^{-2} \text{ sec}^{-1}$	9 ± 2	1.6 ± 0.2
Low light, 50 $\mu\text{E m}^{-2} \text{ sec}^{-1}$	8 ± 1	0.8 ± 0.1
Optimal culture conditions: sea water enriched with 5 mM KNO <sub>3</sub> , 0.2 mM KH <sub>2</sub> PO <sub>4</sub> , 5 mM NaHCO <sub>3</sub> , 1.5 $\mu\text{M}$ FeCl <sub>3</sub> , 0.5 mg l <sup>-1</sup> B12 and trace metal mix, pH 7.0 ± 0.5, by CO <sub>2</sub> flow control on demand, LI 100 $\mu\text{E m}^{-2} \text{ sec}^{-1}$ , temperature 23°C.		

Two major differences have been noted, a lower temperature optimum of 23°C and a requirement for vitamin B12. Otherwise, this green alga attained a similar yield of about 1.3 g AFDW l<sup>-1</sup> at the optimum conditions employed. N. atomus showed, however, higher competition capabilities against bacteria in a medium enriched with an organic load. The physiological explanation may be related to the fast doubling time of N. atomus and the heterotrophic growth nature of the alga.

The cellular composition of C. gracilis and N. atomus harvested at the end of the logarithmic phase is illustrated in Table 3.

Algae grown under optimum conditions were composed of about 35% protein, 50% carbohydrate and 20% lipid. Nitrogen deficiency increased the lipid content of C. gracilis and the carbohydrate content of N. atomus. Other stress conditions of high pH and low temperature stimulated lipid production in the algae. A maximal content of 35% lipid per AFDW was observed in C. gracilis grown at low temperatures on silicate deficiency.

#### Outdoor Studies

Production studies have been conducted outdoors in six 0.35 m<sup>2</sup> miniponds with C. gracilis (Fig. 2). In general, C. gracilis grew better outdoors than indoors; the culture was minimally contaminated with bacteria and other algae. Wherever contamination was present, the culture of C. gracilis was diluted down to minimum to expose the cells to high solar irradiation. C. gracilis as a diluted culture usually grew faster and overcame all other competing green algae. The explanation of this

phenomenon may be based on the high photoresistance of C. gracilis to UV light (Jøkiel and York, 1984).

Table 3. Cellular composition of Chaetoceros gracilis and Nannochloris atomus grown under laboratory conditions

Species	Growth conditions	% AFDW		
		Protein	Carbo- hydrate	Lipid
<u>C. gracilis</u>	Optimal	35 ± 3	45 ± 3	20 ± 2
	Low nitrate, 0.5 mM	17 ± 2	55 ± 5	28 ± 4
	High pH	38 ± 3	32 ± 3	30 ± 5
	Low temp., 15°C	40 ± 4	25 ± 3	35 ± 5
	Low silicate, 0.01 mM	36 ± 3	29 ± 3	35 ± 5
<u>N. atomus</u>	Optimal	34 ± 1	51 ± 4	14 ± 3
	Low nitrate, 0.5 mM	17 ± 2	73 ± 4	10 ± 2
	High pH	30 ± 5	50 ± 3	20 ± 3
	Low temp., 15°C	36 ± 3	44 ± 4	20 ± 3

Optimal culture conditions as described under Tables 1 and 2.

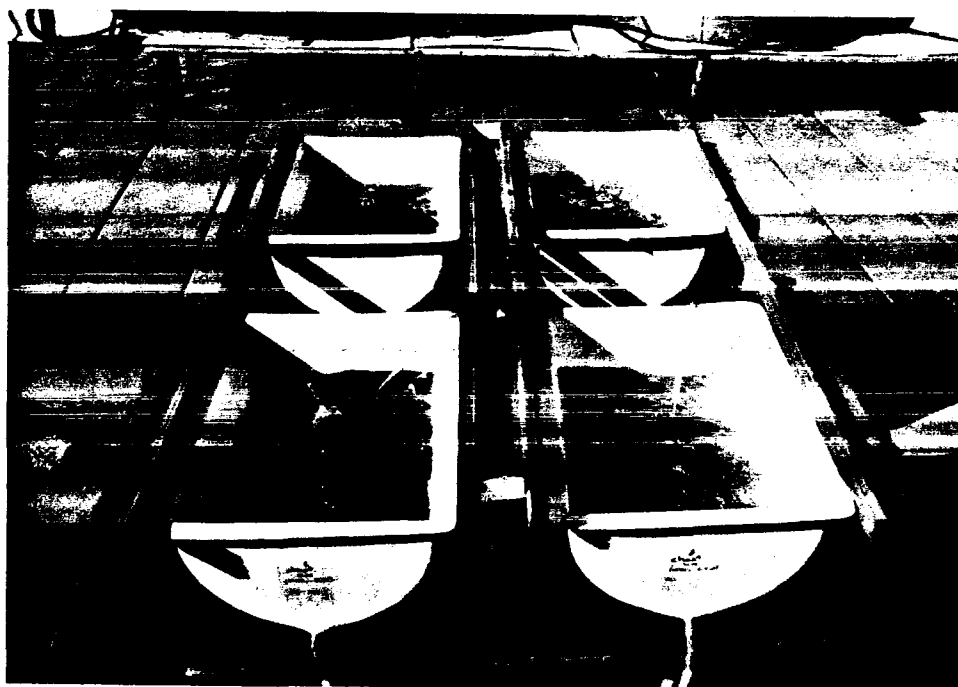


Figure 2. Outdoor growth of C. gracilis in minipends.

A summary of growth experiments with C. gracilis over a period of 9 months since June 1985 is illustrated in Table 4.

Table 4. Summary of outdoor growth experiments with Chaetoceros gracilis in miniponds

Growth conditions	Av. solar irradiation ( $E\ m^{-2}day^{-1}$ )	Av. conc. at harvest ( $g\ l^{-1}$ )	Doubling time (hrs)	Productivity AFDW ( $g\ m^{-2}day^{-1}$ )	Lipids	Phot. Effic. (% PAR)
<u>Summer 1985: June-August</u>						
Nit. suf.	52.8	0.40	12	40	6	8.9
Nit. def.	52.8	0.30	12	30	7.5	6.6
<u>Fall 1985: September-November</u>						
Nit. suf.	33.3	0.41	18	27.3	5.5	9.6
Nit. def.	33.3	0.28	18	18.6	5.6	6.6
<u>Winter 1985/6: December-February</u>						
Nit. suf.	16.2	0.40	36	13.3	4.6	9.6
Nit. def.	16.2	0.27	36	19.0	3.1	6.5

Phot. Effic. - Photosynthetic Efficiency.

Nit. suf. - Nitrogen sufficient; Nit. def. - Nitrogen deficient: 0.5 mM  $NO_3^-$ .

Pond of 10 cm depth. Heat of combustion of C. gracilis: 5.5 Kcal  $g^{-1}$  AFDW.

The data were summarized for 3 production periods by average solar irradiation: highest in the summer and lowest in the winter. Maximal productivities of about 40 g AFDW  $m^{-2} day^{-1}$  were obtained in the summer. A maximal photosynthetic efficiency of close to 10%, however, was observed in the fall and in the winter, associated with lower productivities of 27 and 13 g AFDW  $m^{-2} day^{-1}$ , respectively. The effect of nitrogen deficiency is also summarized. C. gracilis presented a similar growth rate and a lower yield of growth at a limiting concentration of  $NO_3^-$ . The lipid fraction in nitrogen deficient algae increased from about 18 to above 30%, and the lipid productivity increased accordingly in the summer to a maximum of 7.5 g lipid  $m^{-2} day^{-1}$ . During the fall and the winter, the percentage of lipid in the algal dry weight increased with temperature drop to about 30% (see also Table 3). The productivity of lipid, which was at maximum in the nitrogen-enriched low temperature grown cells, did not increase further with nitrogen deficiency.

The effect of mixing, dilution factor and pH is illustrated in Table 5. Slowing air bubbling from 30 liter  $min^{-1}$  to 3 liter  $min^{-1}$  reduced the productivity to about one third. It is obvious that proper mixing is essential for optimal productivity of C. gracilis. Laboratory experiments showed that mixing C. gracilis by air stimulated growth rate in comparison to mixing by agitation on shakers or by magnetic bars. The reason for the effect of air mixing on growth is not clear, but it may be related to efficient exchange of nutrient and gas between the algae and the medium. The effect of the dilution factor on cell productivity was

Table 5. Effect of mixing, dilution factor and pH on growth of Chaetoceros gracilis in outdoor ponds

	Av. solar irradiation (E m <sup>-2</sup> day <sup>-1</sup> )	Av. conc. at harvest (g l <sup>-1</sup> )	Doubling time (hrs)	Productivity (g AFDW m <sup>-2</sup> day <sup>-1</sup> )	Phot. Effic. (% PAR)
Control	33.3	0.40	18	26.6	9.4
Mixing:					
50% mixing	33.3	0.38	28	16.2	5.7
10% mixing	33.3	0.35	36	11.6	4.1
Dilution factor:					
1/2	33.3	0.41	19	25.8	9.1
1/10	33.3	0.40	24	20.0	7.1
pH:					
pH 8.5	33.3	0.31	24	15.5	5.5
pH 9.0	33.3	0.25	32	9.4	3.3

Phot. Effic. - Photosynthetic Efficiency.

Control conditions: Air mixing of 30 liter min<sup>-1</sup>, pH 7.0 ± 0.3 by CO<sub>2</sub> supply on demand, dilution factor of 1:5 by cell number, October 1985.

not significant as long as the culture was maintained at the logarithmic phase and was not diluted too low to the log phase. Algae diluted down to 0.04 g AFDW l<sup>-1</sup> were introduced to the log phase as illustrated by a productivity decline (Table 5). A pH increase above 8 reduced productivity by affecting the average maximum concentration of cells per liter and the growth rate. Lower productivities of 15.5 and 9.4 g AFDW m<sup>-2</sup> day<sup>-1</sup> were attained at pH 8.5 and 9.0, respectively. At pH above 8.3, chemical precipitation occurred followed by algae flocculation and debris formation. We assume that the process of autoflocculation by pH increase is the major cause of productivity decline in the sea water medium. Laboratory control in artificial medium low in calcium showed optimal growth of C. gracilis at high pH of 9.0 and 9.5.

Experiments to study the effect of silicate starvation on the growth of C. gracilis outdoors were unsuccessful due to heavy contamination by green algae. Green flagellates appeared shortly after inoculation of C. gracilis at a cell density of about 0.1 g AFDW l<sup>-1</sup> and took over the diatoms. The series of selection events which lead to the predominance of the green algae over C. gracilis was initially based on UV resistance of C. gracilis. Thereafter, as the diatoms reached a certain level of UV absorption, the green algae flourished over the silicate starved diatoms. Silicate starvation experiments were modified to begin the silicate limitation in a dense culture of C. gracilis at a level of 0.3 g AFDW l<sup>-1</sup>. The results showed that silicate starved C. gracilis accumulates lipid up to 35% of the organic weight. It was impossible to deduce lipid productivity data from these studies. A change in the methodology should be adapted for complete evaluation of the effect of silicate on C. gracilis.



## Lipid Analysis

### Hexane eluate

The quantity of hydrocarbons in the hexane eluates of C. gracilis and N. atomus grown under stress conditions was generally small, relative to the high amounts of hydrocarbons in Botryococcus. A percentage of about 2% hydrocarbons per total lipids in Chaetoceros represents 0.4% of the algal organic weight, in comparison to about 10% aliphatic hydrocarbons in Botryococcus.

Nannochloris contained C17 hydrocarbon as well as a long acyclic hydrocarbon with a chain length of over 31 carbons. The effect of high salt on the neutral lipid profile of the algae was minimal. Nitrogen deficiency and high pH, on the other hand, elongated the hydrocarbon chain length to high molecular weight long chain hydrocarbons. It is evident from the data that certain physiological conditions to which the algae are exposed affect the amount and the profile of acyclic hydrocarbons.

### Benzene eluate

The benzene eluate contained the major fraction of neutral lipids in the algae (Table 6).

Table 6. Analysis of the major lipid components in the benzene eluate of Chaetoceros gracilis and Nannochloris atomus

Growth conditions	<u>C. gracilis</u>			<u>N. atomus</u>	
	ND	pH 9	SD	ND	pH 9
% total lipids	55.6	51.4	51.0	21.0	39.0
	C 31-34 Alkenones, Triglycerides			Triglycerides	
ND - Nitrogen deficient; SD - Silicate deficient.					

The benzene eluate of Chaetoceros comprised up to about 55% of the total lipids in cells grown under nitrogen and silicate deficiency or in high pH. Thin layer chromatography revealed basically one or two different major components and a few minor components. To identify the major components, they were extracted by preparative TLC and assayed by GC/MS. The analysis and preliminary elucidation of the benzene eluate components are illustrated in Table 6. Chaetoceros contained long chain alkenones; Nannochloris contained triglycerides. Stress conditions of nitrogen deficiency, silicate deficiency and high pH which increased the percentages of the benzene eluate in the algae always increased the proportion of long chain alkenones and triglycerides in the benzene fraction.

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CONTINUOUS MICROALGAE  
PRODUCTION IN SHALLOW  
RACEWAYS AT HIGH PRODUCTIVITIES

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ABSTRACT

Saline microalgae grown in an experimental flume culture system at the University of Hawaii have achieved daily production rates of 27-37 g ash-free dry weight (AFDW) per square meter and photosynthetic efficiencies of 9.1-10.7% based on visible irradiance over periods of 44-78 days. A key feature of the system is the use of foil arrays to effect systematic vertical mixing of the culture through the production of vortices. Photosynthetic efficiencies with the foil arrays in place were a factor of 1.35 times the photosynthetic efficiencies without foil arrays. An analysis of variance of production results over a 5 1/2 month period showed that the enhancement of production with the foil arrays in place was statistically significant at better than 99% confidence.

## BACKGROUND

Research on this project began in January, 1980, with financial support from the Solar Energy Research Institute (SERI) and the State of Hawaii. The basic hypothesis underlying the project is that production of microalgae in mass culture can be significantly increased through the use of shallow (depth less than 15 cm) outdoor flumes rather than deeper (depth greater than 20 cm) conventional systems. The experimental facility at the University of Hawaii consists of one 48.4 m<sup>2</sup> flume and four smaller 9.2 m<sup>2</sup> flumes (Fig. 1). In all the flumes water is circulated by means of an airlift at a flow rate of approximately 30 cm/sec. A key feature of the flumes is the use of small foils similar in shape to segments of airplane wings to effect systematic vertical mixing in the flumes (Figs. 2-3). It was hypothesized that the systematic vertical mixing created by the vortices which propagate downstream from these foils would cause the cells in a dense algal culture to experience a modulation in irradiance on a time scale sufficiently brief to cause an enhancement of productivity similar to that associated with the flashing light effect. Recently Terry and Hock (1985) have demonstrated experimentally that productivity enhancements of 50-100% can be achieved at flash frequencies as low as 0.5-1.0 Hz in a culture of Phaeodactylum tricornutum. This flash frequency is comparable to that experienced by cells swept along by vortices in a dense culture 10-15 cm deep.

The outdoor facility at the University of Hawaii has proven very useful for screening various species and strains of algae for outdoor culture, for optimizing species performance, and for developing efficient management strategies. During the early years of this project much time was spent in species screening and optimization of culture conditions. During 1983 it was established that thermophilic strains of microalgae could be grown in the flumes without the aid of expensive cooling, and that marine species could be cultivated successfully in desert saline waters typical of water types found in parts of the southwestern United States.

During 1984 outdoor culture experiments were conducted with a number of species. Excellent results were obtained with the green flagellate Tetraselmis suecica and marine diatom Chaetoceros sp. Both strains grew well at temperatures above 30°C and produced yields of 30-40 g ash-free dry weight (AFDW) per square meter per day for periods up to one month. A major discovery was that diluting the T. suecica culture every third day enhanced production by about 40% over that achieved with a dilution interval of two or four days. In this production mode daily yields during 1984 and 1985 averaged 37 g AFDW/m<sup>2</sup> over a total period of 78 days. These results were associated with an

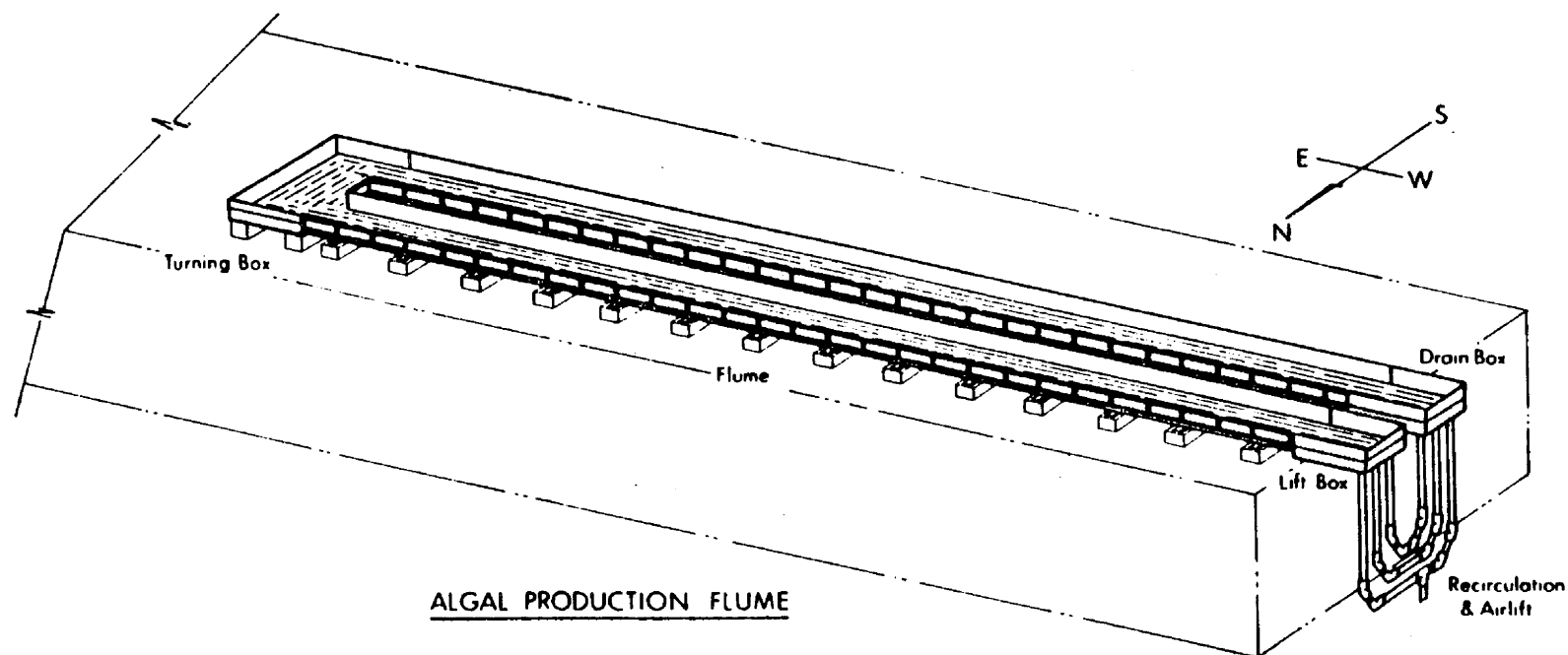


Figure 1. Perspective drawing of 48.4 m<sup>2</sup> flume.

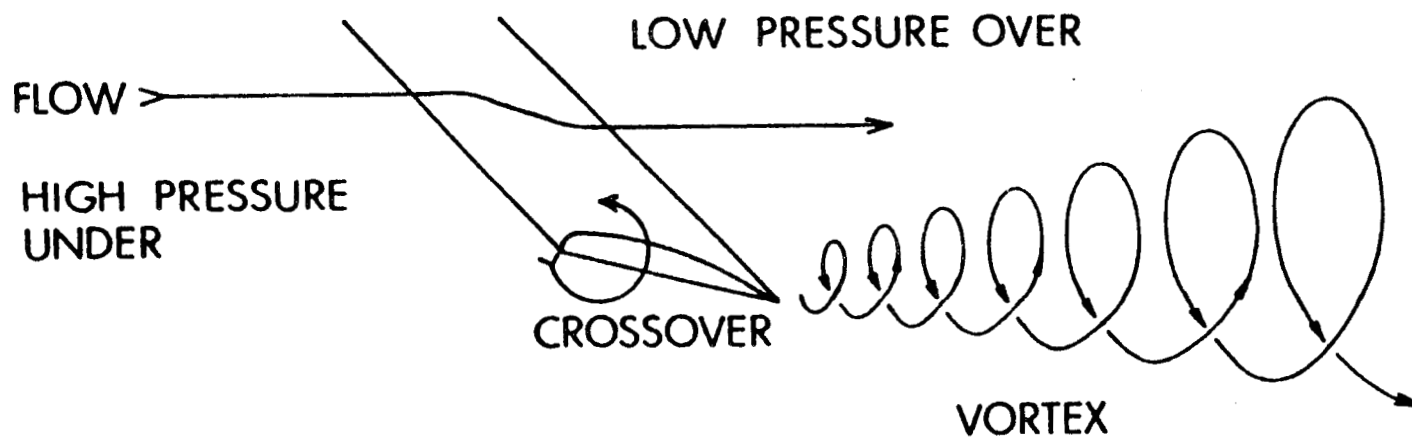


Figure 2. Design of a single foil indicating mechanism of vortex production.

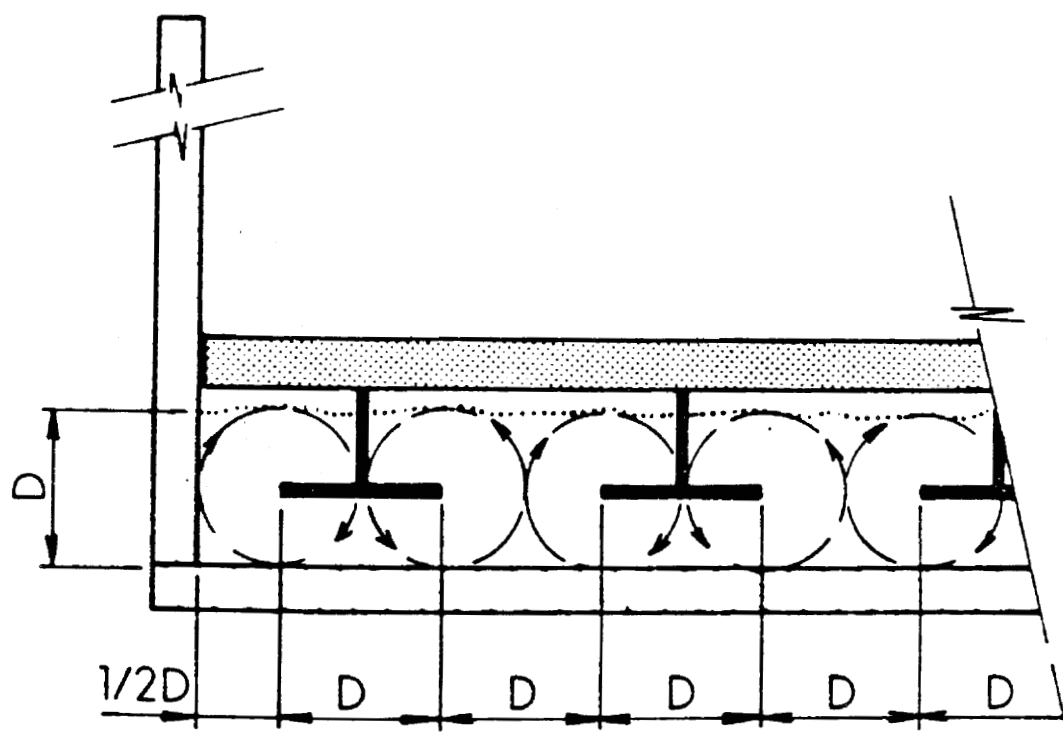
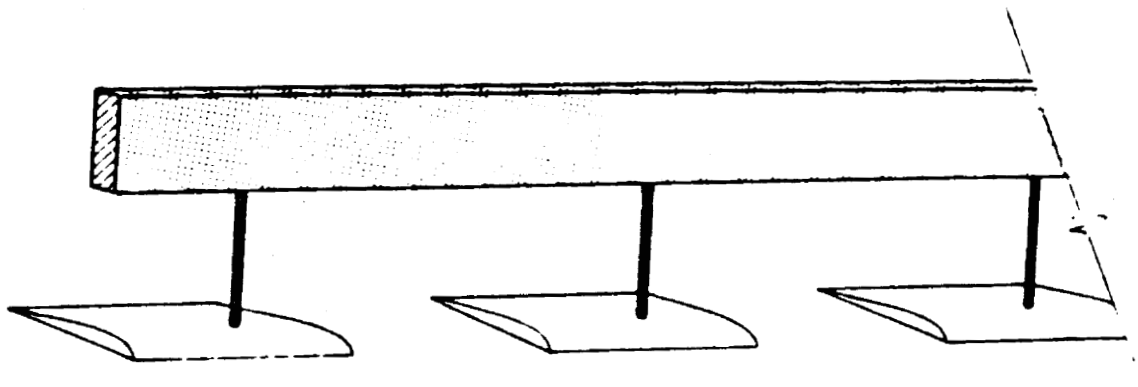


Figure 3. Positioning of individual foils in foil array. Lower figure indicates positioning of array in the flume.  $D$  is the depth of the water. Arrows indicate rotational direction of vortices.

average photosynthetic efficiency of 9.1% [based on photosynthetically active radiation (400-700 nm wavelength)]. This production efficiency is approximately twice the best achieved in long-term microalgal mass culture systems grown exclusively on inorganic nutrients. Nutrient starvation experiments indicated that T. suecica stored carbohydrates rather than the desired lipids. However, Chaetoceros sp., a diatom, was found to accumulate up to 55% of its carbon in lipids after a 4-5 day period of silicate starvation.

## RECENT RESULTS

Research during the last twelve months has focused on two major questions. First, approximately what percent increase in biomass production can be expected with the use of foil arrays. Second, how sensitive are production results to the interval between dilutions of successive batch culture growth cycles. The dilution interval work has required an extensive series of experiments, since the optimum density of the culture initially after dilution is expected to be a function of the dilution interval. An effort was also made to study the compositional and physiological responses of the algae to changing light conditions in the culture over the course of the batch culture growth cycles.

### Foil Effects

A twelve-month study of the effects of the foil arrays on production was initiated in the 48.4 m<sup>2</sup> flume on 9/16/85. The experiments are being conducted with Cyclotella sp., a diatom which was found to grow quite well in outdoor culture and which, like Chaetoceros sp., allocated about 50% of its carbon to lipids when silicate stressed. Foil arrays were placed at an interval of four feet in the flume for a period of approximately two weeks. Next the flume was operated for a period of two weeks without the foil arrays in place. This experimental design of running the flume alternately with and without foils for two week periods has been repeated up to the present time. Results are shown in Fig. 4 and Table 1. Mean photosynthetic efficiencies ( $\pm 95\%$  confidence intervals) have averaged  $10.7 \pm 1.5\%$  with the foil arrays in place, and  $7.9 \pm 0.8\%$  without foil arrays. Thus use of the foils appears to enhance photosynthetic efficiency by a factor of  $10.7/7.9 = 1.35$ . A statistical analysis of the results in Table 1 indicates foil effects on both production and photosynthetic efficiency to be significant at over 99% confidence (t-test, Mann-Whitney U-test).

### Dilution Interval

Dilution interval experiments have been conducted both in the 48.4 m<sup>2</sup> flume and in the four smaller 9.2 m<sup>2</sup> flumes. During June and July, 1985, a one-month production run was made with T.



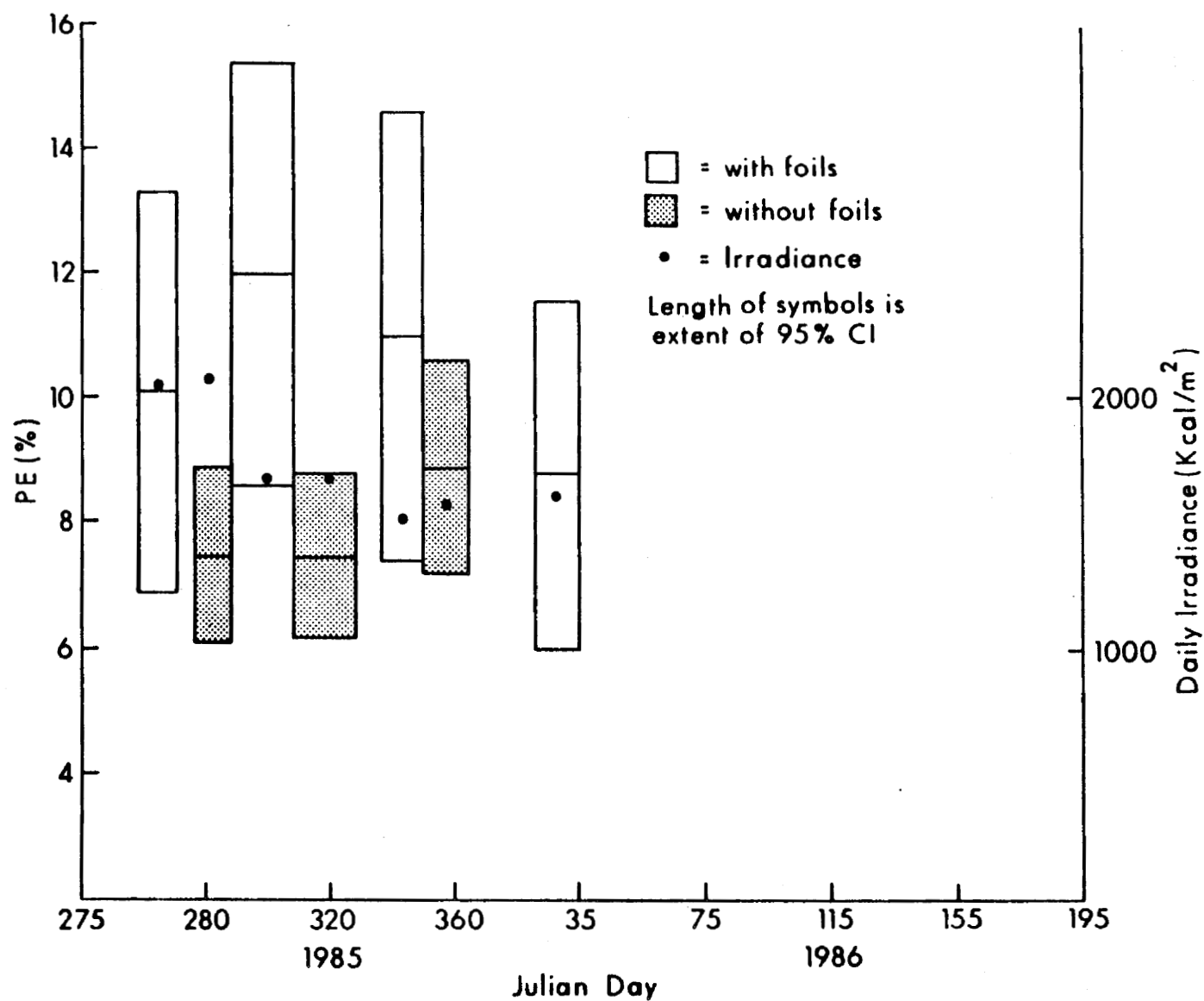


Figure 4. Experimental results obtained with *Cyclotella* sp. in 48.4 m<sup>2</sup> flume with and without foil arrays. Width of bars indicates temporal duration of each run. Dots indicate average irradiance (400-700 nm) during corresponding time period.

Table 1. Results with Cyclotella in 48.4 m<sup>2</sup> flume since 9/16/85.

	With foils	Without foils
g C/m <sup>2</sup> /d	13.0 ± 2.1	10.3 ± 0.9
E(Kcal/m <sup>2</sup> /d)	1652 ± 145	1790 ± 135
PE (%)	10.7 ± 1.5	7.9 ± 0.8
initial density (gC/m <sup>3</sup> )	83 ± 5.0	77 ± 3.5
n	44	40

suecica in the 48.4 m<sup>2</sup> flume on a three-day dilution interval growth cycle. The principal goal of this research was to determine whether the high production results achieved during 1984 in this production mode could be reproduced. The results are summarized in Table 2. Two aspects of the 1984 results were clearly reproducible. First, the rate of production in 1985 ( $33.2 \pm 4.7$  g AFDW/m<sup>2</sup>/d) was not significantly different from the rate achieved in 1984 ( $39.6 \pm 8.2$  g AFDW/m<sup>2</sup>/d) (t-test,  $p > 0.05$ ). Second, as in 1984 production was highest on the third day of the three-day growth cycle (Fig. 5). The difference between production on the third day of the cycle and production on the first two days was highly significant [analysis of variance (ANOVA),  $p < 0.05$ ]. What was not reproducible in 1985 was the high photosynthetic efficiency of 10.2%. The lower photosynthetic efficiency of 7.6% may have been the result of supraoptimal temperatures during the 1985 studies. During July, 1985, the average daily maximum and minimum temperatures in the 48.4 m<sup>2</sup> flume were 31.6 and 21.2°C, respectively. In March-May, 1984, the corresponding temperatures were 28.8 and 18.0°C, respectively. The 3°C higher temperatures in 1985 may well have been supraoptimal for T. suecica during at least part of the day.

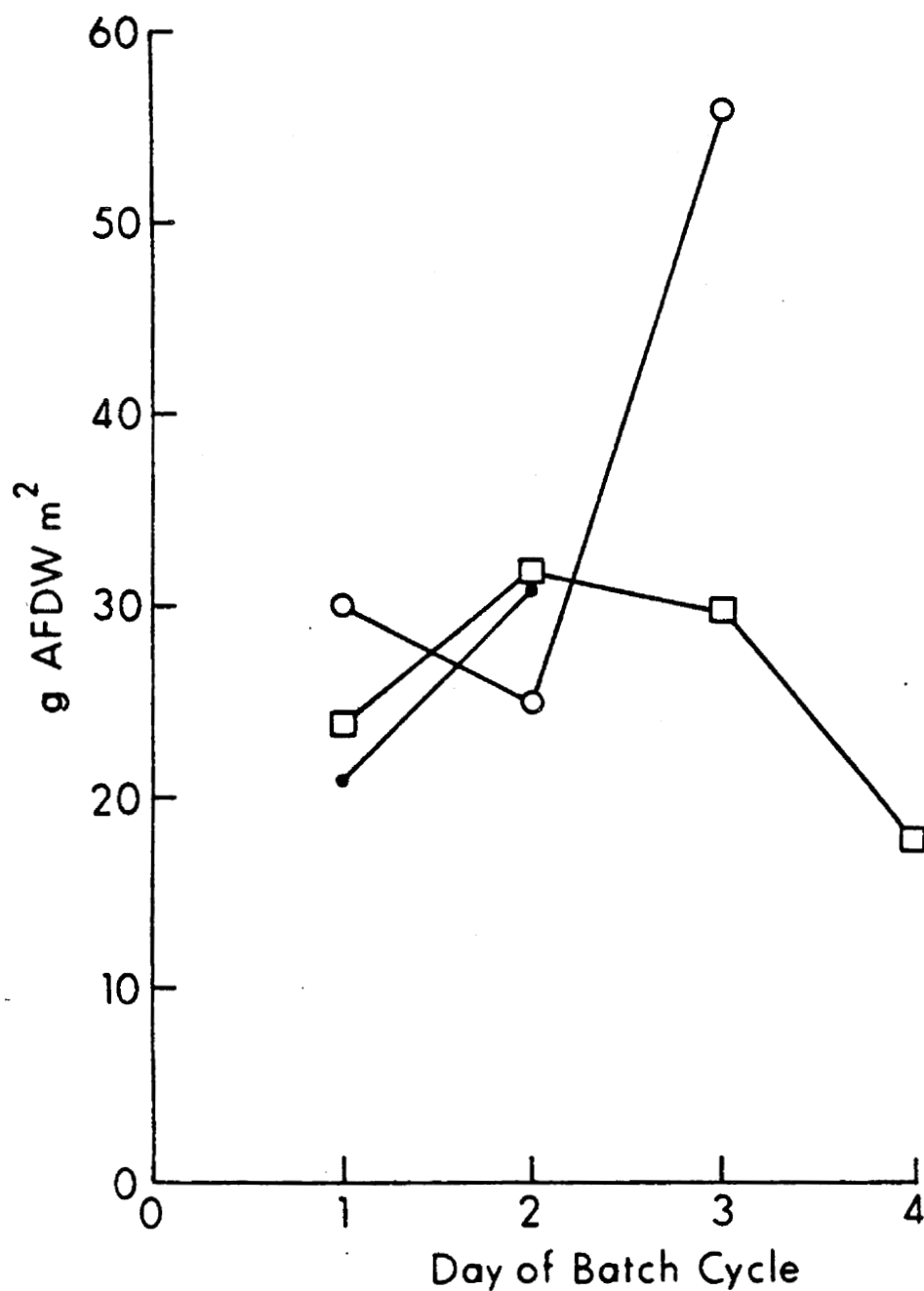
The effect of dilution interval on production in the 48.4 m<sup>2</sup> flume was somewhat puzzling to us. It is tempting to hypothesize that production was a maximum on a three-day dilution interval because areal biomass was suboptimal when the cells were grown on a two-day dilution interval and supraoptimal when the cells were grown on a four-day dilution interval. To a certain extent this conclusion may be true. Actual areal densities varied between 28 and 80 g AFDW/m<sup>2</sup> when the cells were grown on a two-day dilution interval cycle, between 27 and 140 g AFDW/m<sup>2</sup> on a three-day cycle, and between 27 and 160 g AFDW/m<sup>2</sup> on a four-day cycle. However, it seems unlikely to us that the difference in average areal densities of the cells grown on three and four-day cycles would have been sufficient to account for the roughly 40% difference in average production rate (Table 2) between these two operational modes. An examination of production rates on each day of the dilution cycles (Fig. 5) does however suggest additional factors which may have contributed to the dilution interval effect. When the cells were grown on a four-day dilution interval, average production declined noticeably on the fourth day of the cycle. One likely explanation for this decline is that the cell density was indeed supraoptimal, and that holding the culture in this high density condition for several days produced an adverse physiological response in the culture. Although the four-day dilution cycle cells displayed production rates comparable to those of the two and three-day cycle cells on days one and two of the growth cycle, production on day three was significantly higher for the three-day cycle cells than for the four-day cycle cells. We postulated that the failure of the four-day cycle cells to display a peak in production on day three was due to a lingering effect of exposure to supraoptimal density conditions.

Compositional and PI Curve Parameters. It was our hope that a

Table 2. Production results in 48.4 m<sup>2</sup> flume during 1984 and 1985 with T. suecica. Error bars are 95% confidence intervals.

Dates	Dilution Interval	Biomass Immediately After Dilution (g AFDW m <sup>-3</sup> )	Daily Production (g AFDW m <sup>-2</sup> )	Daily Irradiance (Kcal m <sup>-2</sup> , 400 - 700nm)	Photosynthetic Efficiency (%)
2/23/84 - 3/25/84	two days	219±25	25.9±4.2	2108±87	7.4±1.2
3/26/84 - 4/25/84	three days	184±21	39.6±8.2	2325±90	10.2±2.1
5/16/84 - 5/30/84	four days	213±38	25.8±7.0	2442±182	6.2±2.0
6/23/85 - 7/25/85	three days	253±23	33.2±4.7	2643±133	7.6±1.0

Figure 5. Production of *T. suecica* in 48.4 m<sup>2</sup> flume as a function of day in batch culture. Squares = four-day dilution cycle. Open circles = three-day dilution cycle. Solid circles = two-day dilution cycle. 95% confidence interval to data points average  $\pm 7$  g AFDW/m<sup>2</sup>.



study of photosynthesis-irradiance (PI) curve parameters and compositional characteristics would provide some indication of this phenomenon. Accordingly, experiments were run in the 9.2 m<sup>2</sup> flumes during July and August, 1985, to study the compositional characteristics and PI curve parameters of T. suecica cells grown on two, three, and four-day growth cycles. All PI curve studies were made at approximately noon on each day of the growth cycle. Results are reported as P<sub>m</sub> (light-saturated gross photosynthetic rate),  $\alpha$  (initial slope of the PI curve) and I<sub>k</sub> (= P<sub>m</sub>/ $\alpha$ ). Compositional characteristics were measured at approximately 9 AM local time. Experiments were run at two initial densities, 218 ± 25 and 304 ± 44 g AFDW/m<sup>3</sup>. Characteristics of the cultures were monitored over three to five growth cycles. The results are summarized in Tables 3-6. A two-way ANOVA on the results in Table 3 revealed no significant treatment effect (p > 0.05) on either daily production or photosynthetic efficiency from either the initial concentration of biomass or dilution interval. A two-way ANOVA (initial biomass concentration x time) did however reveal significant changes in compositional characteristics and PI curve parameters as a function of time and/or biomass concentration during batch culture growth at each of the three dilution intervals (Tables 4-6). On a two-day dilution interval carbon:chlorophyll (C/Chl) ratios were significantly higher at the lower level of biomass, and P<sub>m</sub> was significantly higher on day one than on day two (Table 4). On a three-day dilution cycle, C/Chl and particulate carbon/nitrogen (C/N) ratios,  $\alpha$ , and P<sub>m</sub> were all significantly higher on day one, and C/N ratios were significantly higher at the lower level of biomass (Table 5). On a four-day dilution cycle, C/Chl and C/N ratios were both significantly higher on day one, and P<sub>m</sub> was significantly higher on day one than on day four (Table 6).

The results of the PI curve and compositional studies were somewhat discouraging in that there was no significant difference in production rates as a function of dilution interval, and the rates of production were substantially lower (~24 g AFDW/m<sup>2</sup>/d) than those achieved in the 48.4 m<sup>2</sup> flume.

### Optimum Densities

Because of concern over the presumed interaction of dilution interval and initial biomass, a factorial experiment was set up in the 9.2 m<sup>2</sup> flumes in September, 1985, to determine the optimum initial density as a function of dilution interval. The effects of foil arrays were also included in the experimental design. Cyclotella sp. was chosen as the test organism. These experiments are still in progress. Fig. 6 summarizes the results to date for cells grown on a dilution interval of one day. A two-way ANOVA revealed both foils and initial density to have a significant effect on photosynthetic efficiency at better than 95% confidence. The optimum density for a one-day dilution cycle appears to be about 200 g AFDW/m<sup>3</sup>, at which density a PE of slightly more than 8% can be expected. This result suggests that for Cyclotella a two-day dilution interval is superior to a one-

Table 3. Production results in 9.2 m<sup>2</sup> flumes during 1985 with T. suecica. Error bars are 95% confidence intervals.

Dates	Dilution Interval	Biomass Immediately After Dilution (g AFDW m <sup>-3</sup> )	Daily Production (g AFDW m <sup>-2</sup> )	Daily Irradiance (Kcal m <sup>-2</sup> , 400 - 700nm)	Photosynthetic Efficiency (%)
8/6 - 8/15	two days	201±26	27.9±6.7	2627±299	6.3±1.3
	"	314±66	23.0±7.4	"	5.2±1.4
7/6 - 7/17	three days	206±49	25.5±4.0	2729±208	5.5±0.8
	"	296±110	24.7±7.2	"	5.4±1.2
7/7 - 7/18	four days	246±122	22.6±5.9	2693±204	5.0±1.2
	"	301±170	21.5±5.7	"	4.7±1.2

Table 4. Results of compositional and PI curve studies with *T. suecica* grown on a dilution interval of two days. Compositional units are weight/weight.  $I_k$  units are gC/gChla/h.  $\alpha = Pm/I_k$ .

	Day 1 biomass 1	Day 2 biomass 1	Day 1 biomass 2	Day 2 biomass 2
C/Chl	46 $\pm$ 8 n = 4	40 $\pm$ 3 n = 4	37 $\pm$ 4 n = 4	38 $\pm$ 2 n = 4
	biomass (p = 0.04) (1 > 2)			
C/N	6.2 $\pm$ 0.5 n = 4	5.7 $\pm$ 0.3 n = 4	5.8 $\pm$ 0.7 n = 4	5.3 $\pm$ 0.6 n = 4
	ns			
$\alpha$	0.028 $\pm$ 0.010 n = 4	0.021 $\pm$ 0.001 n = 4	0.025 $\pm$ 0.002 n = 3	0.021 $\pm$ 0.001 n = 4
	ns			
$I_k$	282 $\pm$ 76 n = 4	278 $\pm$ 94 n = 4	282 $\pm$ 12 n = 3	324 $\pm$ 83 n = 4
	ns			
Pm	7.5 $\pm$ 1.2 n = 4	5.6 $\pm$ 1.8 n = 4	7.1 $\pm$ 0.8 n = 3	5.6 $\pm$ 1.1 n = 4
	days (p = 0.02) (1 > 2)			



Table 5. Results similar to Table 4 to T. suecica but at a dilution interval of three days.

	Day 1 biomass 1	Day 2 biomass 1	Day 3 biomass 1	Day 1 biomass 2	Day 2 biomass 2	Day 3 biomass 2
C/Chl	40 ± 4 n = 3	33 ± 4 n = 4	32 ± 2 n = 4	44 ± 4 n = 3	35 ± 6 n = 4	35 ± 2 n = 4
day (p = 0.0009) (1 > 2 = 3)						
C/N	7.1 ± 0.06 n = 3	5.7 ± 0.4 n = 4	5.9 ± 0.2 n = 4	6.5 ± 0.3 n = 3	5.6 ± 0.2 n = 4	5.6 ± 0.3 n = 4
day (p = 0.0001) (1 > 2 = 3) biomass (p = 0.02) (1 > 2)						
α	.022 ± .007 n = 3	.017 ± .004 n = 4	.019 ± .001 n = 4	.029 ± .007 n = 3	.021 ± .004 n = 4	.019 ± .003 n = 4
day (p = .015) (1 > 2 = 3)						
I <sub>k</sub>	271 ± 32 n = 3	265 ± 92 n = 4	191 ± 49 n = 4	272 ± 50 n = 3	240 ± 72 n = 4	233 ± 70 n = 4
(ns)						
Pm	6.0 ± 2 n = 3	4.4 ± 1.4 n = 4	3.5 ± 0.8 n = 4	7.7 ± 1.1 n = 3	4.9 ± 0.7 n = 4	4.3 ± 0.9 n = 4
day (p = 0.0004) (1 > 2 = 3)						

Table 6. Results similar to Table 4 but at a dilution interval of four days.

	Day 1 biomass 1	Day 2 biomass 1	Day 3 biomass 1	Day 4 biomass 1	Day 1 biomass 2	Day 2 biomass 2	Day 3 biomass 2	Day 4 biomass 2
C/Chl	47 ± 10 n = 3	36 ± 3 n = 3	33 ± 4 n = 3	34 ± 3 n = 3	49 ± 17 n = 3	35 ± 6 n = 3	31 ± 3 n = 3	32 ± 3 n = 3
	day (p = 0.0039) (1 > 2 = 3 = 4)							
C/N	7.6 ± 0.4 n = 3	5.6 ± 0.2 n = 3	5.5 ± 0.3 n = 3	5.7 ± 0.7 n = 3	7.7 ± 0.6 n = 3	5.6 ± 0.2 n = 3	5.6 ± 0.2 n = 3	5.7 ± 0.3 n = 3
	day (p = 0.0001) (1 > 2 = 3 = 4)							
α	.028 ± .004 n = 3	.025 ± .006 n = 3	.024 ± .003 n = 3	.024 ± .004 n = 3	.032 ± .016 n = 3	.024 ± .007 n = 3	.025 ± .004 n = 3	.024 ± .002 n = 3
	ns							
I <sub>k</sub>	243 ± 37 n = 3	277 ± 203 n = 3	208 ± 88 n = 3	147 ± 51 n = 3	213 ± 32 n = 3	208 ± 61 n = 3	173 ± 20 n = 3	1-8 ± 83 n = 2
	ns							
Pm	6.7 ± 0.9 n = 3	6.1 ± 3.4 n = 3	4.9 ± 1.6 n = 3	4.0 ± 1.4 n = 3	7.0 ± 4.7 n = 3	4.7 ± 0.4 n = 3	4.0 ± 0.9 n = 3	3.6 ± 0.5 n = 3
	day (p = 0.13) (1 = 2 = 3; 2 = 3 = 4; 1 ≠ 4)							

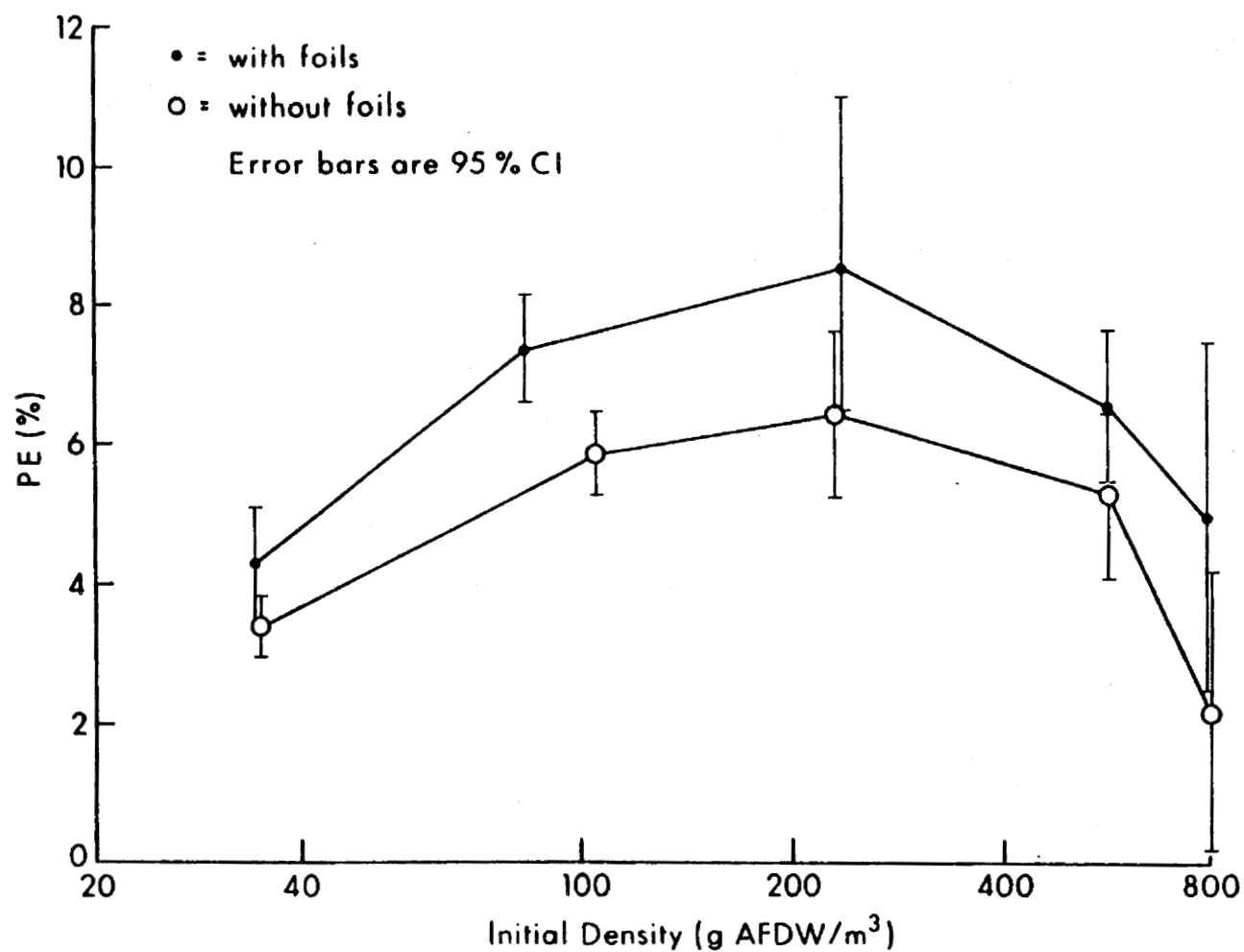


Figure 6. Production of *Cyclotella* sp. in 9.2 m<sup>2</sup> flumes as a function of initial biomass, with and without foil arrays. Cells were grown on a dilution interval of one day.

day dilution interval, but further experimentation is needed to determine whether this tentative conclusion is correct, or whether the result is due to the 48.4 m<sup>2</sup> flume's being inherently superior to the 9.2 m<sup>2</sup> flume.

### References

- Terry, K. and S. Hock. 1985. Photosynthetic Efficiency Enhancement in Modulated Light: Dependence on the Frequency of Modulation. In: Aquatic Species Program Review, Proceedings of the March 1985 Principal Investigators Meeting. Solar Energy Research Institute, Golden, Colorado. SERI/231-2700. 353 pp.

## ABSTRACT

### EVALUATION OF AVAILABLE SALINE WATER RESOURCES IN NEW MEXICO FOR THE PRODUCTION OF MICROALGAE

by

Robert R. Lansford, John W. Hernandez,  
and Phillip Enis

The major objective of the research was to select potential sites for 1,000-hectare (2,470 acre) microalgae production facilities in New Mexico using saline water resources. The emphasis of the research was twofold.

First, a data base was created with respect to the Solar Energy Research Institute (SERI) criteria for location of microalgae production facilities in New Mexico. Specific criteria included location, depth-to-water, aquifer characteristics, saturated thickness of aquifers, salinity, ionic composition, well-yields, growing season, topography, and land ownership.

Second, the data base was digitized for map construction. The desirable water supply for algae culture was limited to moderately or more saline groundwaters (3000 mg/l total dissolved solids or greater), because of the existing societal demands for the limited supply of better quality water.

After a review of the location of the 15 billion acre-feet of saline water resources in the state, areas that appeared to generally meet the SERI criteria for site selection were narrowed to the following--the Tularosa Basin in south-central New Mexico, the Estancia Basin in central New Mexico, the San Juan Basin in northwestern New Mexico, the Tucumcari area in Quay County on the eastside of New Mexico, the area east of the Pecos River Basin in eastern New Mexico, and the Crow Flats area in southern New Mexico.

A detailed analysis was completed for the six locations. Three basins were eliminated for failing to meet all the criteria developed for the study--Pecos Basin, San Juan Basin and the Tucumcari Area. Of the remaining basins, the Tularosa was judged best suited for a microalgae production facility, Crow Flats, the next best, and Estancia, the poorest of the three choices because of a short growing season.

The reserves of saline waters ranging from 2.3 million acre-feet to 5.0 million acre-feet were identified for the large-scale microalgae production areas in New Mexico.

## INTRODUCTION

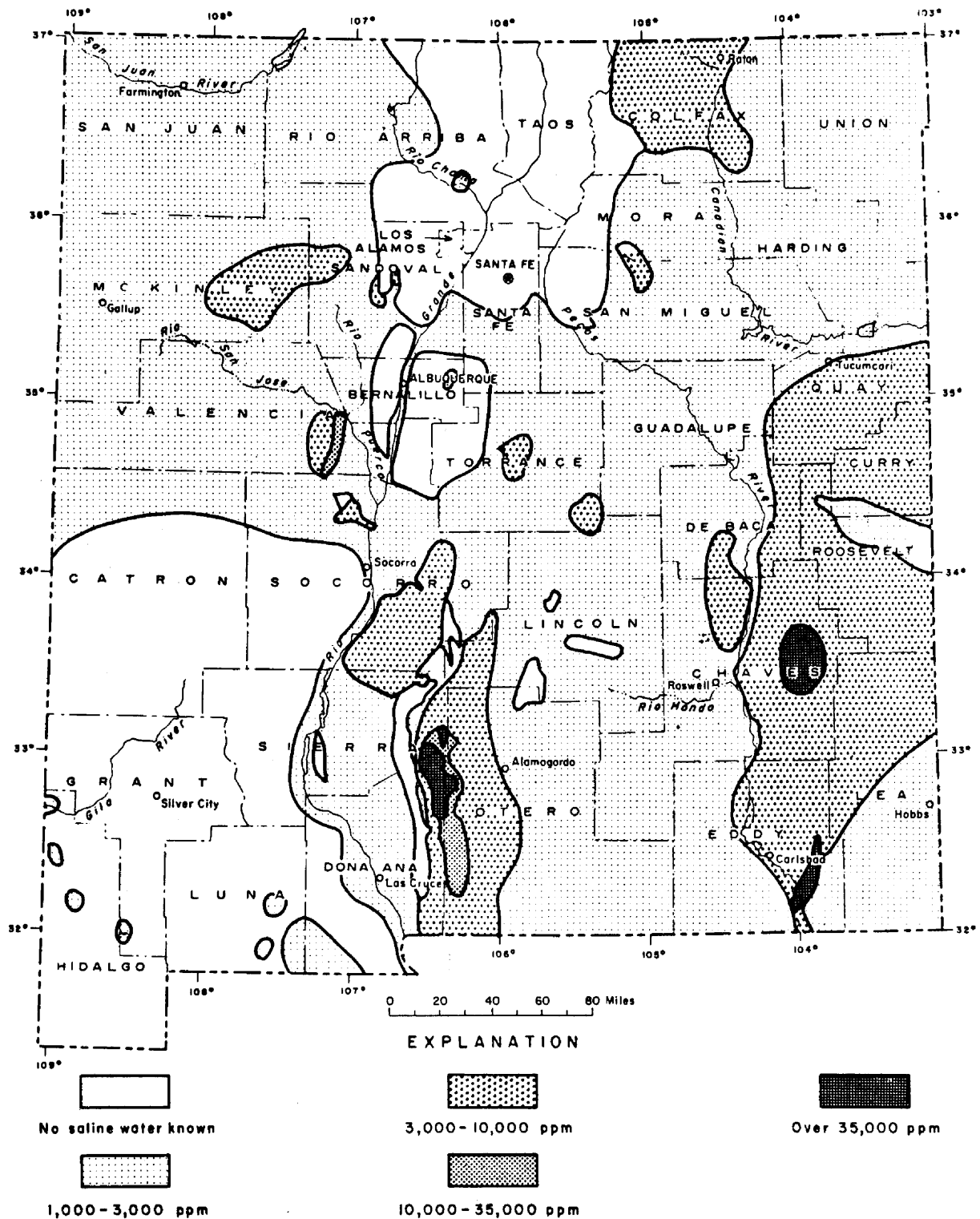
This paper represents a contribution to the task of inventorying the saline water resources in New Mexico and screening them in terms of their suitability for large-scale (1,000 hectares or 2,470 acres) microalgae production facilities. This paper is a summary of a larger SERI report by the same authors, "Evaluation of Available Saline Water Resources in New Mexico for the Production of Microalgae."

Approximately 25 percent of the estimated 20 billion acre-feet of groundwater reserves in New Mexico is classified as fresh or slightly saline. The remaining 15 billion acre-feet of largely unutilized water is characterized as moderately saline, very saline and brine (Bureau of Reclamation 1976). The location of the state's saline groundwater reserves are relatively well known (figure 1), but the magnitude of these resources and aquifer characteristics have not been rigorously assessed on a state-wide basis. The recoverability of these saline groundwater reserves is also not well known because little effort has been made to utilize these aquifers.

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\*\*Professor, Department of Agricultural Economics, Professor, Department of Civil Engineering; Research Specialist, Department of Agricultural Economics, New Mexico State University, respectively.



Source: W. E. Hale, et al., 1985.

Figure 1: General Occurrence of Saline Groundwater in New Mexico.

## OBJECTIVES

The major objective of this paper was to identify potential site locations for large-scale (1,000 hectares or 2,470 acres) microalgae production facilities in New Mexico using unappropriated saline water resources.

The specific objectives of the research, as dictated by SERI, were:

1. Define areas within the state of New Mexico with saline aquifers with average depths-to-groundwater of less than 500 feet.
2. Determine those areas delineated in objective 1 that would provide water yields of less than 4 million gallons (12.3 acre-feet) per day (MGD), those areas with yields of 4-12 MGD, and those areas with yields greater than 13 MGD (40 acre-feet), without a serious decline in groundwater pumping levels and without a requirement for a large number of low-capacity wells.
3. Define the chemical composition (major ions and nutrients) of the groundwaters in areas that meet objectives 1 and 2.
4. Determine those areas delineated in objective 1 that would have land surface slope of less than 2 percent.

## METHODOLOGY

The emphasis of the research was twofold. First, a data base was created with respect to the SERI criteria for location of a microalgae production facility. Specific site characteristics include data on location, depth to the aquifer of interest, saturated thickness of aquifer, aquifer characteristics, growing season, salinity, ionic composition, well-yields, topography and ownership. Second, the data base was digitized for the construction of the required maps. Maps for all characteristics can be found in Lansford, et al. (1986).



Specific SERI criteria included location, depth-to-water, aquifer characteristics, saturated thickness, salinity, ionic composition and well-yields, growing season, topography, land ownership, and facility size. The data base was digitized for the construction of maps. The desirable water supply for algae culture was limited to moderately or more saline groundwaters (3,000 mg/l TDS), because of the existing societal demands for the limited supply of better quality water.

The research tasks based upon specific criteria set up by SERI, and translated into working format, were ordered in the following fashion:

1. Basins were chosen based on reports and verbal communications with the State Engineer Office and other federal and state water agencies. Guidelines for basin selection were based on the availability of potential unappropriated water and the feasibility of applying this water to beneficial use.
2. The second task was to define depth to groundwater. Where possible, the depth-to-saline water was mapped in isorhythmic form based on data from individual wells. The economic recoverability criteria of 500 feet of depth were adjusted by individual basin and calculations of total dynamic head (TDH) were made where possible.
3. Well-yields were mapped in isorhythmic form and were based on data for specific wells. Further, a calculation was made to determine the well-yields needed for a 1,000 hectares facility in a region with a given rate of evaporation. General minimum allowable well-yields for 1,000 hectares, given 15 wells per section were approximately 200 gallons-per-minute (gpm) (assuming 24-hour pumping).
4. Basins were further limited by climate criteria. Data on growing season, precipitation, solar energy, temperature and evaporation were collected from the state climatologist and isorhythmic maps were constructed. Length of growing season was the criteria considered closely here; while no cutoff was given, a growing season of approximately 200 days was considered optimal.
5. The next constraint for site selection concerns the maximum slope criteria of no greater than 2 percent land slope as set forth by SERI.

6. Potential sites for microalgae production facilities were further delineated by land ownership. Ownership was described as private, federal or state and mapped accordingly. The land ownership description helped limit large basins to areas suitable for locating facilities, e.g., no location on federal military reservations. Land ownership within a study area was used as a descriptive device rather than a constraint.
7. Water quality in terms of salinity (TDS) and ionic composition data was gathered for individual wells and isorythmic maps were constructed.
8. The final task was to briefly describe the environmental and legal issues for areas that were selected as potential sites.

## RESULTS

Production of microalgae could potentially provide a renewable source of fuel, chemicals and food, and could concurrently serve to engage the unutilized saline water resources of the state for increased economic activity. The major objective of this report was to identify potential site locations for large scale (1,000 hectares or 2,470 acres) microalgae production facilities in New Mexico using saline groundwater resources.

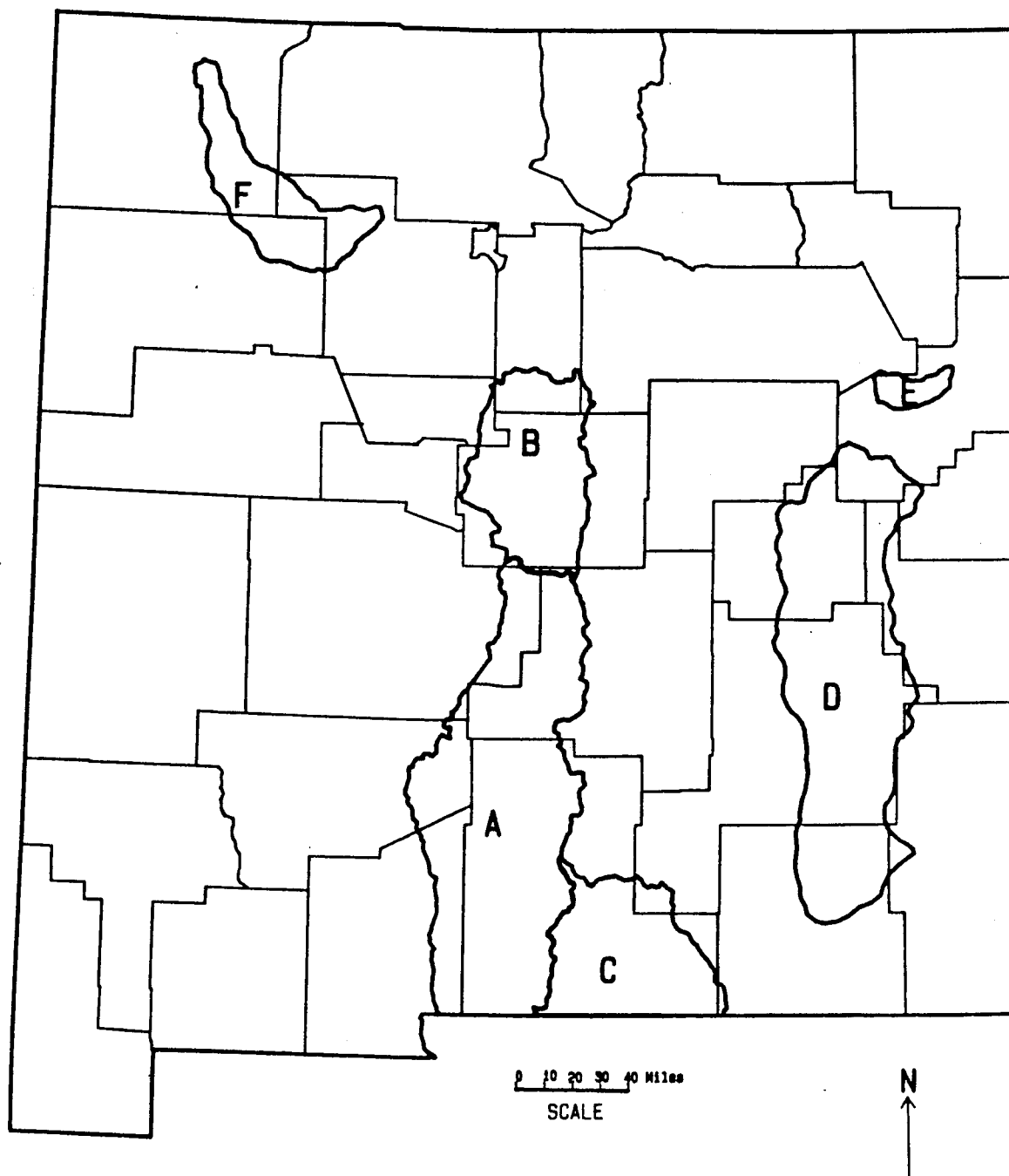
New Mexico follows the doctrine of prior appropriation in establishing the right to use water and does not make any quality distinction between "fresh" and "saline" water in its methods of assigning priorities and in recognizing rights for the use of surface or groundwaters. The available water supply for algae production in New Mexico probably will be limited to moderately or very saline waters because of the current allocation of better quality water to existing uses. Both fresh (less than 1,000 mg/l TDS) and slightly saline waters (1,000 to 3,000 mg/l TDS) are now used for domestic, industrial and

agricultural purposes. Moderately saline waters (3,000 to 10,000 mg/l) and more saline supplies are used sparingly at this time.

The New Mexico state engineer identified declared underground water basins in New Mexico with unappropriated groundwater. The research reflects the analysis of a selected group of groundwater basins within New Mexico that have known unappropriated water resources (DuMars 1986). Those basins having unappropriated groundwater were discussed with state and federal water-related agency personnel. They were asked about more precise location, availability of data on depth to the saline groundwaters, well-yields, and the quantity of water available in those basins. Based on the discussions with the above experts, certain basins were eliminated due to water quality considerations.

The following six drainage basins were identified as areas with the greatest potential for siting microalgae production facilities utilizing unappropriated saline groundwater: Tularosa Basin, Estancia Basin, Crow Flats Basin, Pecos Basin east of the Pecos River, San Juan Basin, and Tucumcari Area (figure 2). Three of the six basins were eliminated after consideration of the first two research tasks: availability of unappropriated groundwater and depth-to-groundwater of less than 500 feet (table 1). They were the Pecos Basin, the San Juan Basin and the Tucumcari Area.

The remaining three basins have areas that contain the greatest potential for siting microalgae production facilities. They are the Tularosa Basin, the Estancia Basin, and the Crow Flats Basin.



A - Tularosa Basin  
B - Estancia Basin  
C - Crow Flats Basin

D - Pecos Basin  
E - Tucumcari Basin  
F - San Juan Basin

Figure 2. Areas Best Suited for Location of Microalgae Production Facilities in New Mexico.

Table 1: Qualitative Summary of Chosen Sites and Specific Criteria Used in Selection of Areas Suitable for Microalgae Production.

Criterion	Tularosa Basin		Crow Flats Basin	Estancia Basin		Pecos Basin	San Juan Basin	Tucumcari Basin
	Site A	Site B		Site A	Site B			
Supply of Unappropriated Saline Groundwater	Available	Available	Available <sup>1</sup>	Available	Available	Limited Availability <sup>2</sup>	Available	Available
Depth-to-Saline Groundwater	Satisfactory	Satisfactory	Marginal to Satisfactory	Satisfactory	Satisfactory	N/A <sup>3</sup>	Marginal to Satisfactory	Marginal to Satisfactory
Potential Well-Yield	Marginal to Satisfactory	Marginal to Satisfactory	Satisfactory	Satisfactory	Satisfactory	Unsatisfactory	Unsatisfactory	N/A
Water Quality (TDS)	Satisfactory	Satisfactory	Marginal	Satisfactory	Marginal	Satisfactory	Satisfactory	Marginal
Adequate Reserves of Saline Water	Yes	Yes	Yes	Yes	Yes	Uncertain	Yes	No
Growing Season	Satisfactory	Satisfactory	Satisfactory	Unsatisfactory	Unsatisfactory	Satisfactory	Unsatisfactory	Marginal to Satisfactory
Land Slope	Satisfactory	Satisfactory	Satisfactory	Satisfactory	Satisfactory	N/A	N/A	N/A
Ownership	Majority is Private	Majority is Private	Majority is Federal	Majority is Private	Majority is Private	N/A <sup>4</sup>	N/A	N/A
Data Base Quality	Excellent	Excellent	Poor to Good	Good	Good	Poor	Good	Good
Further Study Recommended	Yes	Yes	Yes	No	No	Yes <sup>5</sup>	No	No

N/A is not available.

<sup>1</sup> While unappropriated water is available, competition from agriculture is likely because water quality is suitable for agriculture.

<sup>2</sup> While unappropriated water is available, in the Pecos Valley, competition from existing uses may exclude microalgae production.

<sup>3</sup> Data on depth-to-groundwater was available only for the Pecos Valley not the Pecos Basin.

<sup>4</sup> Ownership was not described for the Pecos Basin. Ownership in the Pecos Valley was predominantly private.

<sup>5</sup> Further study is recommended for the area around Roswell if less than a 1,000 hectare facility is considered.

## THE TULAROSA BASIN

The Tularosa Basin in south-central New Mexico was selected for analysis for the location of large-scale (1,000 hectares) microalgae production facilities (figure 3). The area of interest in the Tularosa Basin lies in Otero County on the eastside of the valley floor along the alluvial fans generated by the drainage systems from the mountains just to the east. The City of Alamogordo is within the area of interest. The area was derived using the SERI criteria.

North of this area, potential sites for microalgae production facilities are limited by slopes of greater than 2 percent and the outcrop of consolidated rock that forms the upper boundary of the alluvial valley. To the east and south of the tract, acceptable locations are limited by the Lincoln National Forest, the Mescalero Apache Indian Reservation and Fort Bliss Military Reservation (McGregor Range). Land slope is a further constraint on the eastern boundary. To the west, the availability of sites is limited by the White Sands Missile Range which occupies a majority of the western half of the Basin. White Sands National Monument is also in the valley floor to the west.

### Potential Production Areas

Two possible sites that met the SERI criteria for location of large scale (1,000 hectares) microalgae production facilities were identified within the Tularosa Basin (figure 3). An area of approximately 115 square miles composed of two distinct sites was selected based on availability of moderately and very saline groundwater. The basic SERI

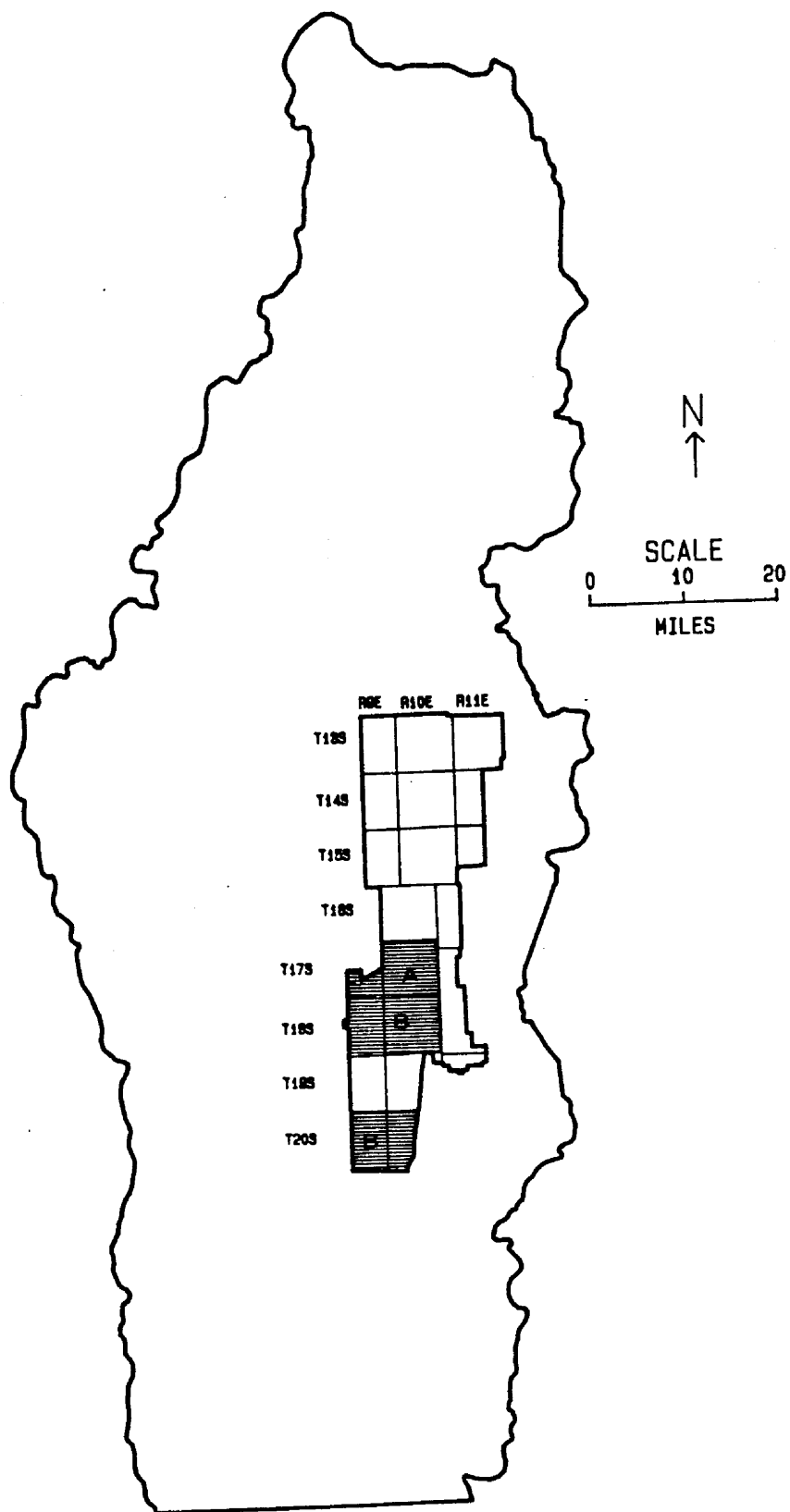


Figure 3. Potential Microalgae Production Areas in the Tularosa Basin, New Mexico.

site-selection criteria and groundwater characteristics are presented in Table 2 for each of the sites.

#### Site A

Site A encompasses approximately 40 square miles (a little over 25,000 acres) and lies in T.17 S., R.8 and 9 E. (figure 3). This area is southwest of Alamogordo and borders on White Sands Missile Range and Holloman Air Force Base. The slope criteria (not greater than 2 percent) is met within this area (table 2). Land ownership in this area is approximately 40 percent private, 35 percent federal and 25 percent state-owned. The growing season is in excess of 200 days.

#### Site B

This area encompasses approximately 75 square miles and meets all of the SERI criteria (figure 3). It is located in T.18 S., R.8 and 9 E. and T.20 S., R.8 and 9 E. The site characteristics are similar to site A with the exception of land ownership (table 2). Land ownership patterns approximate the following: 15 percent federal, 10 percent state, and 75 percent private.

### THE ESTANCIA BASIN

An area of approximately 170 square miles in the Estancia Basin was selected for analysis (figure 4). This area lies north and east of Moriarty to about 15 miles south and east of Willard. The area was derived using natural boundaries, geologic formations, political boundaries, and slope criteria. North of the area sites are limited by slope as the drainage basin merges with plateau lands. To the east and



Table 2: Site and Groundwater Characteristics of the Tularosa Basin, NM.

Unit of Measurement		Site A	Site B
<u>Site Characteristics</u>			
Area	square miles	40	75
Slope	percent	< 2	< 2
Ownership			
Federal	percent	35	15
State	percent	25	10
Private	percent	40	75
Growing Season days		200 - 205	205 - 210
Evaporation	inches	70 - 75	75 - 80
	centimeters	178 - 191	191 - 203
<u>Water Resources</u>			
Depth-to-Groundwater	feet	0 - 50	0 - 50
	meters	0 - 15	0 - 15
Potential Well-Yield	gpm	up to 300	up to 300
	liters per minute	up to 1,135	up to 1,135
Average pH		7.0 - 7.6	7.0 - 7.6
Estimated Reserves	acre-feet	384,000 - 1,280,000	720,000 - 2,400,000
	cubic meters	474,000,000 - 1,580,000,000	880,000,000 - 2,960,000,000
Groundwater Quality	mg/l	10,000 - 35,000	3,000 - 10,000
<u>Ionic Composition</u>			
Ca	mg/l	50 - 3,100	100 - 950
Na & K	mg/l	20 - 12,600	50 - 2,100
Mg	mg/l	20 - 3,400	50 - 1,660
SO <sub>4</sub>	mg/l	30 - 9,300	300 - 3,100
Cl	mg/l	30 - 24,000	50 - 4,200
HCO <sub>3</sub>	mg/l	20 - 380	80 - 250

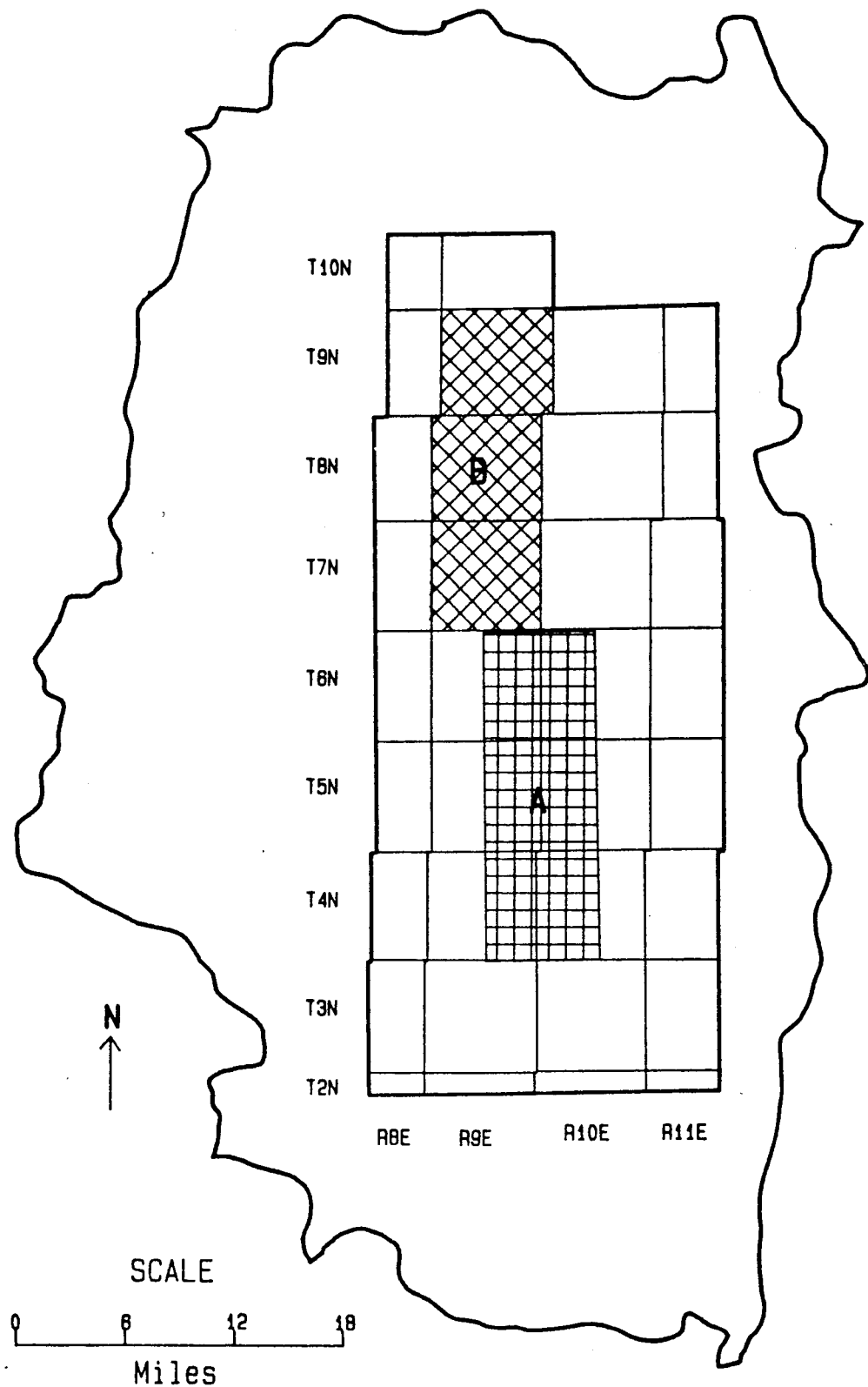


Figure 4. Potential Microalgae Production Areas in the Estancia Basin, New Mexico.

west of the basin, the Pedernal Hills and Manzano Mountains, respectively, produce slopes greater than 2 percent. The selection of the southern boundary of the area was made based on mesa lands and federal ownership in the Cibola National Forest. Surface waters from the west, north and east drain into the central valley that terminates at the southern end of the long playa lake of Laguna del Perro.

#### Potential Production Areas

The geology of the valley floor makes the Estancia drainage basin a prime location for microalgae facilities. A series of playas or dry alkali lake-beds on a north-south axis in the southern end of the basin would make ideal locations.

Two connecting areas within the Estancia Basin meet the SERI criteria for the location of microalgae production facilities with the possible exception of growing seasons. The average growing season of 140 to 160 days may be a major limitation for these sites (table 3). Groundwater quality, depth, potential well-yields and land slope were identified and found to meet SERI standards (table 3).

#### Site A

The 80-square-mile area lies immediately east of Estancia on the north to about 5 miles south of Willard (figure 4). The width of the area is approximately 6 miles. The best site for the location of production facilities would be near the existing playa lakes, as the water quality is poorest and surface flows terminate at these alkali flats and provide groundwater recharge. The land ownership pattern is

Table 3: Site and Groundwater Characteristics of the Estancia Basin, NM.

		Unit of Measurement	Site A	Site B
<u>Site Characteristics</u>				
Area	square miles		80	90
Slope	percent		< 2	< 2
Ownership				
Federal	percent		15	10
State	percent		25	30
Private	percent		60	60
Growing Season	days		150 - 160	140 - 150
Evaporation	inches		50 - 55	45 - 50
	centimeters		127 - 140	114 - 127
<u>Water Resources</u>				
Depth-to-Groundwater	feet		0 - 30	0 - 30
	meters		0 - 9	0 - 9
Potential Well-Yield	gpm		up to 1,000	up to 1,000
	liters per minute		up to 3,790	up to 3,790
Average pH			7.3 - 7.8	7.3 - 7.8
Estimated Reserves	acre-feet		155,000 - 615,000	173,000 - 692,000
	cubic meters		191,000,000 - 759,000,000	213,000,000 - 854,000,000
Groundwater Quality	mg/l		3,000 - 5,500	1,500 - 3,000
<u>Ionic Composition</u>				
Ca	mg/l		50 - 640	40 - 290
Na & K	mg/l		20 - 750	30 - 210
Mg	mg/l		40 - 480	20 - 180
SO <sub>4</sub>	mg/l		120 - 3,200	60 - 1,050
Cl	mg/l		10 - 1,440	30 - 4,900
HCO <sub>3</sub>	mg/l		65 - 900	190 - 350

approximately 15 percent federal, 25 percent state and 60 percent privately-owned lands. The growing season ranges from 150 to 160 days.

#### Site B

A second, large area (90 square miles) appears to generally meet the criteria except for groundwater quality and the growing season of 140 to 150 days (table 3). This area is east of Highway 41 between Moriarty and Estancia (figure 4). The width of this tract is approximately 5 miles. The slope criteria of 2 percent is met in this area and ownership patterns approximate the following: 60 percent private, 10 percent federal and 30 percent state.

### THE CROW FLATS AREA

The Crow Flats Area in south-central New Mexico is part of the larger Salt Basin (figure 5). The Salt Flats Basin is bordered by the Tularosa Basin on the west, the Pecos River Basin on the north and east and the state of Texas on the south and has an area of 2,370 square miles. The vast majority of the area is located within Otero County with small areas in Chaves and Eddy counties. Elevations range from 3,000 feet near the alkali flats in the southern end to approximately 4,500 feet in the surrounding hills (Bjorklund 1957).

#### Potential Production Area

A limited area of interest within the Crow Flats Area was identified. This area lies in the southeast portion of the Salt Basin bordering on the New Mexico-Texas state line on the south (figure 5). North and east of the area of interest, slopes greater than two percent

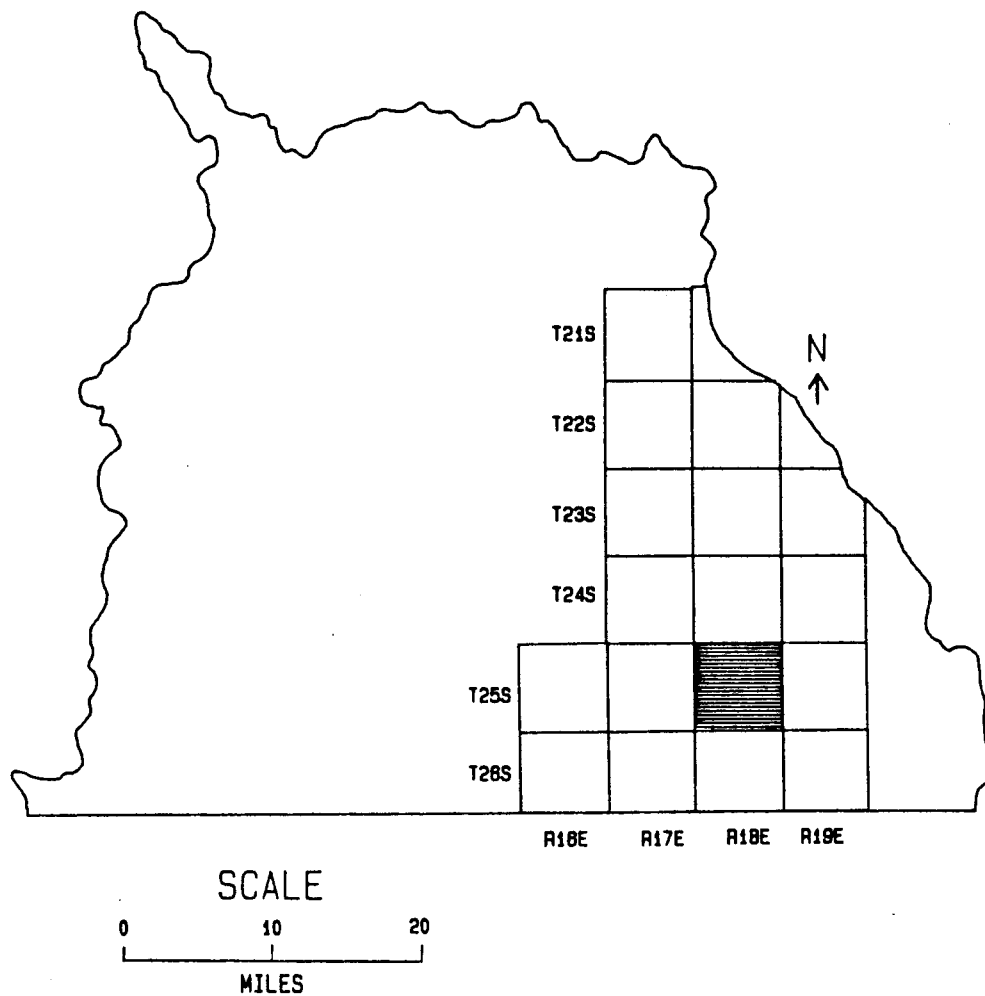


Figure 5. Potential Microalgae Production Areas in the Crow Flats Area, New Mexico.

exist near the Guadalupe Mountains. Potential sites were also limited on the north and east by outcrops of limestone rock. To the south, acceptable locations were limited by the New Mexico-Texas state line. The researchers did not have access to data for Texas, although the valley of the basin runs south into Texas and it is believed that acceptable sites can be located there. To the east of the area of interest lie the Cornudas Mountains, which produce land slopes that are greater than 2 percent. The eastern edge is also limited geologically by outcrops of limestone rocks.

One potential production area in the Crow Flats Area meets all of the SERI criteria for a facility. This area is situated on a north-south axis around T.25 S (figure 5). The average width of this area varies from 4 to 6 miles for an area of about 36 square miles. The growing season is in excess of 200 days. The approximate land-ownership pattern is 20 percent private, 70 percent federal and 10 percent state (table 4).

#### CONCLUSIONS

Two potential sites were identified for the Tularosa Basin. Both sites (A & B) met all of the SERI criteria. One exception to this may possibly be the relatively low well-yields that are characteristic of the areas in the Tularosa Basin. For a 1,000 hectares (2,470 acres) facility, low well-yields may be offset by a larger numbers of wells.

Two potential sites were identified for the Estancia Basin. A short growing season of approximately 140 to 160 days would be a major limitation in the basin and would eliminate the basin from consideration for microalgae facilities. One 80-square-mile site (A) met all

Table 4: Site and Groundwater Characteristics of the Crow Flats Basin, NM.

Unit of Measurement		
<u>Site Characteristics</u>		
Area	square miles	36
Slope	percent	< 2
Ownership		
Federal	percent	70
State	percent	10
Private	percent	20
Growing Season	days	200 - 210
Evaporation	inches	70 - 75
	centimeters	178 - 191
<u>Water Resources</u>		
Depth-to-Groundwater	feet	50 - 110
	meters	15 - 35
Potential Well-Yield	gpm	up to 1,000
	liters per minute	up to 3,790
Average pH		6.9 - 7.3
Estimated Reserves	acre-feet	576,000 - 1,382,000
	cubic meters	710,000,000 - 1,700,000,000
Groundwater Quality	mg/l	1,000 - 3,000
Ionic Composition		
Ca	mg/l	224 - 556
Na & K	mg/l	22 - 65
Mg	mg/l	177 - 264
SO <sub>4</sub>	mg/l	719 - 2,230
Cl <sup>4</sup>	mg/l	32 - 275
HCO <sub>3</sub>	mg/l	158 - 284



other SERI criteria. The best site for the location of a facility would be near the existing playa lakes. The second site (B) met all of the criteria with the exception of groundwater quality. The groundwater is classified as slightly saline. An additional limitation for site B is that poorer quality water overlies better quality water with a potential for degradation of the higher quality water. Owners of existing water rights in the area may oppose any new appropriations of water.

One 36-square-mile area in the Crow Flats Area was identified as a potential site. The area is on the valley floor in the alkali flat portion of Crow Flats. However, the water would be classified as slightly saline and is currently being used for irrigation, livestock and domestic purposes. It is unlikely that present water right holders will readily accept requests for new appropriations of groundwater.

The Tularosa Basin was judged as best suited area for a 1,000 hectare microalgae production facility and Crow Flats the next best alternative. The Estancia Basin has the limitation of a short growing season and possible opposition from current water-right holders.

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TITLE:

Nutritional Requirements for Maximal Growth of Oil Producing  
Microalgae

SERI #5-05056

ABSTRACT

The purpose of this study was to define the nutritional requirements for selected known oil producing microalgae originally from marine and inland saline habitats. Species tested were SERI collections of Ankistrodesmus falcatus, Boekelovia sp., Chaetoceros gracilis, Chaetoceros SS-14, Cyclotella sp., Nannochloropsis (Nanno-Q) sp. and Platymonas sp. A protocol for culturing and quantifying growth as doublings/day was established using culture tubes on shakers. Culture tubes served as spectrophotometric cuvettes and were read every 24 hours over a 5 day period. Nitrogen as  $\text{NO}_3$ ,  $\text{NH}_4$  and urea were tested for preference and optimal concentrations. Phosphate-P, Si (for diatoms) and the trace metal Fe were also tested for optimal concentrations. The vitamins  $\text{B}_{12}$  and thiamine were found to be significantly important in the growth of three of the species. Nitrate was observed to be the best nitrogen source in three of the seven algae tested. Ammonia and urea-N were shown to be significantly preferred nitrogen sources for two species. Iron as  $\text{FeNH}_4$ -citrate significantly increased the growth of two of three species tested as compared to  $\text{FeCl}_3$  additions. This information will hopefully be helpful to the SERI Aquatic Species Program as well as others as a guide toward optimizing growth prior to the shift toward lipid production.

## ENVIRONMENTAL CONTROL OF LIPID PRODUCTION IN DIATOM CULTURES

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Nutritional stress (either nitrogen or silica) has been shown to be an effective inducer of algal lipid production. Exploitation of this induction effect could be vital in our effort to reach program goals of 15% photosynthetic efficiency and 50% of dry weight as lipid. To this end, an understanding of how nutrient stress increases lipid production and how environmental factors affect this induction is essential. Some environmental factors with the potential to affect lipid production under nutrient stress are inorganic carbon supply ( $\text{CO}_2$ , bicarbonate), pH, light intensity, and temperature. Preliminary experiments suggested that increasing bicarbonate concentration resulted in higher lipid yields under both nitrogen and silica stresses in Navicula sp. The preliminary experiments designed to examine this effect yielded interesting results but have not unequivocally answered the question of whether a bicarbonate effect exists. Based on this initial data, alternative experimental approaches are proposed to approach this question.

## THE EFFECTS OF LIGHT INTENSITY ON LIPID PRODUCTION

N. Nagle and B. Barclay  
Solar Energy Research Institute

Several conflicting reports have appeared in the literature, and in Aquatic Species Program research, on the effects of light intensity on lipid yield in microalgae. Some reports have suggested that high light intensity enhances lipid production while others have suggested that low light intensity improves lipid yield. We developed a simple procedure to evaluate light intensity effects on lipid induction in microalgae. A rotary screening apparatus was modified with neutral density filters to provide a variety of light intensities, from 100-1000  $\mu\text{E m}^{-2} \text{sec}^{-1}$ . Additionally, a simple fluorometric procedure, utilizing Nile red, was developed to quantify lipid yield. The data from several experiments suggest that higher light intensities, during stress induced lipid production, results in higher lipid yields in microalgae.

## FLOW CYTOMETRY TECHNIQUES FOR SPECIES IMPROVEMENT\*

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Oak Ridge National Laboratory

Lipid accumulation in three species of microalgae was investigated using flow cytometry (FCM) and transmission electron microscopy (TEM). Previous studies using batch cultures of algae have led to the assumption that lipid accumulation in microalgae is a gradual process requiring at least several days for completion. However, FCM reveals, through changes in the chlorophyll:lipid ratio, that the short time span required for individual cells to change metabolic state is short. Simultaneous FCM measurements of chlorophyll and nile red (neutral lipid) fluorescence in individual cells of nitrogen-deficient Isochrysis populations revealed a bimodal population distribution as one stage in the lipid accumulation process. The fact that two discrete populations exist, with few cells in an intermediate stage, suggests rapid response to a lipid trigger. Interpretations of light and electron microscopic observations are consistent with this hypothesis. The time required for an entire population to achieve maximum lipid content is considerably longer than that required for a single cell, due to the variation in response time among cells. In this study, high lipid cultures were sometimes obtained by using FCM to separate high lipid cells from the remainder of the population. FCM holds much promise for strain enhancement but considerable developmental work, directed at providing more consistent results, remains to be done.

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Biochemical Elucidation of Neutral Lipid Synthesis in Microalgae.  
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The mechanisms controlling lipid synthesis must be understood if one is to attempt their control and manipulation! The possibility of selecting high lipid producing organisms by genetic alteration bear little chance of success if the gene products (enzymes) specified as a result of genetic manipulations are unknown. We are therefore concentrating on the study of the regulation of lipid synthesis in microalgae in this project. The pathways of lipid synthesis in all organisms examined so far are similar, but not identical. There are differences in the enzymes carrying out the reactions, but the reactions themselves do not vary much. In general, all pathways involve the conversion of acetate to its Coenzyme A derivative condensation with bicarbonate and subsequent addition of C-2 units to form a 16 carbon fatty acid. Further chain lengthening involves acetyl CoA or Malonyl CoA. The point to stress is that the synthesis of all lipids involves 2 or 3 carbon fragments derived from acetate in its coenzyme A form. There are variations as to how acetyl CoA is synthesized and there has been considerable controversy as to where this process takes place. Our research will focus on the pathway of synthesis of acetyl CoA and the further regulation of its metabolism. Acetyl CoA is at a branch point in metabolism, i.e., it can either be oxidized to 2 CO<sub>2</sub> and the CoA regenerated (TCA cycle) or it can condense with bicarbonate to form malonyl CoA and be committed to lipid synthesis. In some algae, nitrogen limitation fosters the second route. In some diatoms, silicon limitation does the same thing, i.e., the inability of a cell to divide induces increased lipid synthesis to take place. We believe that feedback control on citrate synthase, the consumer of acetyl CoA for the TCA cycle by -oxoglutarate or ATP shuts off the cycle and acetyl CoA is converted to a lipid 'sink'. How this control system can operate will be discussed.

This project started in September, 1986. Two organisms will be studied initially; both are high lipid producers. They are a Chlorophyte, Chlorella sp. and a member of the Bacillariophyceae, Cyclotella. The Chlorella responds to nitrogen limitation, by synthesizing increased lipid whereas the diatom responds to Si limitation in the same way.

The improvement of lipid yields in microalgae requires an understanding of the physiological and biochemical basis for the partitioning of photosynthetically fixed CO<sub>2</sub> into lipids. Elucidation of the biochemical basis for enhanced lipid synthesis requires a reproducible method for inducing lipid synthesis. Since the most commonly utilized lipid trigger, nitrogen deficiency, rapidly reduces photosynthetic capacity, it is useful to separate effects of lipid triggers on photosynthetic efficiency from their effects on carbon partitioning. Initial studies have therefore used Euglena, a microalgae which grows equally well under photosynthetic and heterotrophic conditions as a model system. Based on the results obtained with Euglena, attempts were made to induce lipid synthesis in Chlorella SO1 and Nannochloropsis salina.

Nitrogen deficient Euglena utilize exogenous carbon for the net synthesis of both carbohydrates and lipids in the light and the dark. Cells grown in the light accumulate less carbohydrate and lipid than cells grown in the dark. The lipid content of nitrogen deficient cells is two to fivefold higher than that of nitrogen sufficient cells. Nitrogen deficient and sufficient cells show little difference in the percentage of dry weight which is lipid. Growth at 33 C rather than 26 C increases the cellular lipid content. The higher growth temperature however inhibits the synthesis of lipid by nitrogen deficient cells. Anaerobiosis triggers lipid synthesis in both nitrogen sufficient and deficient Euglena. In N sufficient cells, the degradation of storage carbohydrates provides carbon for lipid synthesis while in N deficient cells, endogenous carbon is not efficiently mobilized for lipid synthesis; lipid is only synthesized when a source of exogenous carbon is available.

Chlorella SO1 grows well in the dark with glucose as the sole source of carbon and energy. The provision of additional glucose to nitrogen deficient cultures grown in the light on glucose failed to stimulate lipid synthesis. Contrary to the results with Euglena, the exogenous carbon was used almost exclusively for the synthesis of carbohydrates. When Chlorella SO1 was grown photosynthetically to nitrogen deficiency, cellular lipid content increased. The partitioning of carbon by Chlorella SO1 is dependent on the source of carbon.

We have been unable to maintain axenic cultures of Nannochloropsis salina. When grown photosynthetically, the cell number, lipid per cell and % lipid on a dry weight basis was a function of the initial initial NH<sub>4</sub>Cl concentration of the medium. As cells entered nitrogen deficiency, cellular lipid content and dry weight increased while chlorophyll decreased. The lipid content of nitrogen deficient cells was at least twice that of nitrogen sufficient cells. The addition of nitrogen to nitrogen deficient cultures stimulated cell division and chlorophyll synthesis. Lipid per cell decreased upon nitrogen supplementation clearly indicating that the increased lipid content was due to nitrogen deficiency.

Taken together, the results with Euglena and Nannochloropsis salina indicate that lipid accumulation in these algae can be reproducibly triggered by nitrogen deficiency. Under photosynthetic conditions, the extent of lipid synthesis may be limited not by the levels of lipid synthesizing enzymes but rather by the availability of carbon. The development of reproducible methods for the induction of lipid synthesis in Euglena and Nannochloropsis salina will enable us to correlate changes in lipid levels with the activity of lipid synthesizing enzymes.



## BIOCHEMICAL ASPECTS OF LIPID ACCUMULATION IN SILICON-DEFICIENT DIATOMS

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### Abstract

Three different diatom species were examined with respect to lipid accumulation under silicon-deficient growth conditions. The lipid contents of *Cylindrotheca fusiformis* and *Cyclotella cryptica* cells increased significantly as a result of Si-deficiency, but this was not observed with *Thalassiosira pseudonana*. However, the neutral lipid content of all three species increased by at least two-fold under conditions of Si-limitation. Additional experiments indicated that the partitioning of newly assimilated carbon was rapidly and significantly altered in response to Si-deficiency in *C. cryptica*. The percentage of carbon partitioned into chrysolaminarin was reduced by 50%, while the fraction partitioned into lipid was nearly doubled. This was apparently due to differential amounts of decrease in the absolute rates of carbon assimilation into these two compounds. In order to better understand the biochemical basis of these changes in carbon partitioning, studies have been initiated which are designed to examine the regulation of key enzymes of carbon metabolism during the course of Si-deficiency. Preliminary results suggest that the first committed step in chrysolaminarin synthesis is catalyzed by UDP-glucose pyrophosphorylase. When assayed in cell-free extracts of *C. cryptica*, this enzyme had maximal activity at pH 7.8, and was greatly stimulated by  $Mg^{2+}$  and  $Mn^{2+}$ . Unlike the ADP-glucose pyrophosphorylases of green algae and higher plants that are involved in starch biosynthesis, the activity of UDP-glucose pyrophosphorylase is not strongly modulated by phosphate or 3-phosphoglycerate.

### Introduction

The long-term goal of the SERI Aquatic Species Program is to develop systems and methods which utilize the photosynthetic capabilities of microalgae for the production of liquid fuels. A recent economic study (Hill et al. 1985) has suggested that microalgal-produced lipids could compete with petroleum-based liquid fuels on a cost basis if certain productivity criteria were met. One such criterion is that the lipid content of the algal cells (as a percentage of the total biomass) must be increased substantially over those values which are typical for cells from exponential-phase cultures. Therefore, researchers must either 1) identify and isolate species which have naturally-occurring, elevated lipid contents, or 2) physiologically or genetically manipulate microalgal species in order to increase the percentage of photosynthetically assimilated carbon which is partitioned into cellular lipids.

Several studies have indicated that nutrient stress brings about lipid accumulation in a variety of algal species. Nitrogen starvation has been shown to induce lipid accumulation in several diverse algal groups, including members of the Chlorophyceae, Bacillariophyceae, and Rhodophyceae (e.g., Spoehr and Milner (1949), Fogg (1956), Shifrin and Chisholm (1981), and Piorreck et al.

(1984)). Silicon starvation has also been shown to bring about increased lipid contents in diatoms. The lipid content of Cyclotella cryptica increased from 16 to 38% of the dry mass after 24 hours of Si-deficiency (Werner 1966). For Navicula pelliculosa, 14 hours of Si-deficiency caused an increase in the lipid content from 25% to 34% (Coombs et al. 1967). The effects of Si-deficiency on lipid accumulation for periods of time greater than 24 hours have not been reported for any diatom species.

Although increased algal lipid contents in response to nutrient-deficiency have been observed by many investigators, the biochemical basis of this change in carbon partitioning has never been elucidated. The research described in this paper represents initial efforts toward a better understanding of this process. The studies carried out thus far in this project have focused on lipid accumulation in diatoms. Silicon was chosen as the limiting nutrient for these studies for two primary reasons: 1) previous work in this area has suggested that changes in carbon partitioning are much more rapid in Si-deficient cells than in N-deficient cells, and 2) unlike nitrogen, silicon is not a constituent of any of the macromolecular components of cells (i.e.; protein, lipid, etc.), so the interpretation of results is less complicated.

The initial phase of this research was designed to compare the effects of Si-deficiency on lipid accumulation and various other physiological parameters in several different diatom species. Additional studies were carried out in order to investigate the short-term partitioning of photoassimilated carbon in Si-deficient cells. Results from initial studies involving the enzymology of carbohydrate synthesis are also described.

## Materials and Methods

Organisms and growth conditions. Cyclotella cryptica T13L and Thalassiosira pseudonana 3H were obtained from the Culture Collection of Marine Phytoplankton at the Bigelow Laboratory for Ocean Sciences (W. Boothbay Harbor, Maine). Cylindrotheca fusiformis was kindly provided by Dr. B. Volcani (Scripps Institution of Oceanography, La Jolla, California).

T. pseudonana and C. fusiformis were cultured in an artificial sea water medium (Darley and Volcani 1969). C. cryptica, rendered axenic by treatment with antibiotics (Hoshaw and Rosowski 1973), was cultured in the medium described by Werner (1966) which was modified to contain the micronutrients of Bold's Basal Medium (Bold and Wynne 1978). Silica concentrations of these media were adjusted to the levels indicated in the Results section by addition of  $\text{Na}_2\text{SiO}_3$ . Media were supplemented with biotin (2 ug/L), thiamine (1 mg/L), and cyanocobalamin (1 ug/L). The media were filter sterilized and transferred into 2 L polycarbonate bottles fitted with stainless steel tubing for the removal of samples and the introduction of a sterile mixture of 2%  $\text{CO}_2$  in air (flow rate = 500 mL/min). Cultures were stirred continuously and maintained at 25° C under constant illumination from fluorescent lamps (light intensity at the vessel surface averaged over  $360^\circ = 85 \text{ uE m}^{-2} \text{ s}^{-1}$ ).

Analytical methods. Silica concentrations were determined spectrophotometrically by the heteropoly blue method (Standard Methods 1975) using a wavelength of 800 nm. Pigments were extracted with 95% ethanol and analyzed with a Hewlett-Packard 8451 spectrophotometer (300 to 750 nm) programmed so that spectra could be normalized to each other at 664 nm.

Chlorophyll a was quantified using the extinction coefficient of Winternans and Demots (1965).

Gross photosynthetic rates were determined by summing polarographically-determined rates of net O<sub>2</sub> evolution and dark O<sub>2</sub> uptake. Saturating illumination was provided by a tungsten lamp/fiber optic system.

For dry mass and lipid mass determinations, cells in a known volume of medium were harvested by centrifugation and resuspended in a minimal volume of culture medium. Two samples, each having approximately 10% the mass of the total suspension, were removed and placed into separate 13x100 mm test tubes which had been previously heated to 550°C for several hours. The masses of these samples were then determined. The remaining suspension was also weighed and then stored at -20°C. The tubes were weighed after heating at 85°C for 48h and then reweighed after heating at 550°C for 5h. The difference in the masses determined after these two treatments represents the ash-free dry mass (AFDM), which could then be normalized on a culture volume basis.

Lipids were extracted from the frozen suspension by heating at 60°C for 1 h each with 10 mL of 1) 50% methanol in water, 2) methanol (twice), and 3) methanol:chloroform (1:1) (twice). Centrifugation (1000xg) between extraction steps yielded clarified extracts which were combined and placed into a separatory funnel. Chloroform and water were then added to give the Bligh-Dyer ratio for phase separation (methanol: chloroform: water; 10:10:9). After mixing and separation, the chloroform phase was collected and an additional 20 mL of chloroform plus one drop of 6N HCl was added to each separatory funnel and mixed. The chloroform phases were combined and then evaporated under a stream of N<sub>2</sub> at 50°C and the total lipid mass was determined gravimetrically.

Neutral lipids were separated from polar lipids by thin layer chromatography on silica gel (SII) Chromarods developed in chloroform:acetic acid (100:0.5). The rods were scanned with a flame ionization detector (Iatroscan model TH-10) to a point 1.5 cm above the origin (i.e., all but the lower 18-20% of the developed Chromarods). Peak areas were determined by the use of a Hewlett-Packard 3390A integrator. The Chromarods were then developed in methanol:chloroform:water:acetic acid (50:50:10:0.5) in order to move polar lipids away from the origin and re-scanned. Materials which did not migrate from the origin were considered non-lipoidal (generally accounting for less than 5% of the total material).

Chrysolaminarin was extracted from cells collected on glass fiber filters by shaking for 1 h in two successive 5 mL portions of 0.1 N H<sub>2</sub>SO<sub>4</sub> and quantified by the phenol-H<sub>2</sub>SO<sub>4</sub> method as described by Myklestad (1978).

Total protein was determined by the method of Bradford (1976) subsequent to disruption of cells suspended in 50 mM HEPES buffer (pH 7.5) with a French pressure cell at 15000 psi.

### **<sup>14</sup>C Incorporation Studies:**

For these experiments, a 2 L culture of *C. cryptica* growing in a medium containing a non-limiting Si concentration was harvested under sterile conditions by centrifugation at 6000xg for 5 min. The pelleted cells were resuspended to the original culture density in either a Si-sufficient medium or a Si-free medium ([SiO<sub>2</sub>] less than 1 uM) and returned to the incubator. Portions of the cultures were removed at various times and placed in a glass graduated cylinder (diameter = 4 cm) which was positioned next to a bank of fluorescent lights. The suspension was allowed to equilibrate under these conditions for 15 min with constant stirring. H<sup>14</sup>CO<sub>3</sub><sup>-</sup> (0.06 mM, specific activity = 1 mCi/mmol) was then added to the suspension. After 15, 30, and 45

min incubation times, four individual 10 mL samples were removed, collected by filtration through glass fiber filters (Whatman GF/C), and quickly rinsed with 15 mL of ice-cold medium containing 10 mM unlabelled  $\text{HCO}_3^-$ . Each of the filters was subsequently treated in a different manner in order to determine incorporation of carbon into various cellular components.

Lipids. One filter was placed in a teflon-capped tube containing 3 mL of methanol:chloroform:water (2:1:0.8) and allowed to stand for 24-48 h at  $-20^\circ\text{C}$  followed by heating for 30 min at  $55^\circ\text{C}$ . The solvent was then separated from the filter and cellular debris by centrifugation at 3000xg and transferred to a separate tube. The pelleted material was extracted with an additional 3 mL of methanol: chloroform (1:1) for 1 h at  $55^\circ\text{C}$ . The clarified extracts from the two steps were then combined and mixed with chloroform (1 mL),  $\text{H}_2\text{O}$  (2.4 mL), and 3N HCl (15  $\mu\text{L}$ ). After centrifugation at 2000xg for 5 min, the lower phase from each sample was transferred to a scintillation vial and evaporated to dryness under a stream of  $\text{N}_2$ . 10 mL of scintillation cocktail plus 0.15 mL of 3 N HCl was added to each vial, and the radioactivity determined after partial bleaching of the pigments with 20 W fluorescent lamps.

Known portions of the lipid samples obtained from the 45 min sampling time were loaded onto a silica gel thin layer chromatography plate (J.T. Baker Co., Si250) followed by development in chloroform:acetic acid (100:0.5). Autoradiography of the plate was then performed using X-ray film (Kodak BB-1) placed next to an enhancement screen (Dupont Cronex Brand). The film was exposed for 4 d at  $-76^\circ\text{C}$ . The silica gel from the portions of the TLC plate containing labelled triacylglycerol were scraped from the plate and placed in a scintillation vial containing 10 mL of scintillation cocktail for determination of the amount of  $^{14}\text{C}$  incorporated into triacylglycerols.

Chrysolaminarin. One filter from each time point was inserted into a test tube containing 2 mL of 0.1 N  $\text{H}_2\text{SO}_4$ , and the tube was placed on a rotary shaker (200 rpm) for 2.5 h. The filter and cellular debris were removed by centrifugation (3000xg) and 1.5 mL of the supernatant were transferred to a separate tube. The acid was neutralized with NaOH and HEPES buffer (25 mM, pH 7.8) was added. The solution was then loaded onto a 1x13 cm column packed with Biogel P-2 (Biorad Laboratories) and eluted with 25 mM HEPES buffer (pH 7.8). After the addition of 1 mg bovine serum albumin (BSA), trichloroacetic acid (TCA) was added to a final concentration of 5% (w/v), and the solution was placed on ice for 30 min. The solution was then passed through a glass fiber filter (Whatman GF/C) directly into a scintillation vial containing 15 mL of scintillation cocktail.

Protein. The cells from the third filter of each set were rinsed into a test tube with 2 mL of 50 mM HEPES buffer (pH 7.8) and stored at  $-20^\circ\text{C}$ . The suspensions were later thawed and passed through a French pressure cell at 15000 psi. The extracts were then incubated in the presence of 0.015% sodium deoxycholate for 20 min at  $20^\circ\text{C}$ . Following centrifugation at 37000xg for 20 min, BSA (1 mg) and TCA (5% w/v final concentration) were added to the supernatant. After a 30 min incubation on ice, the precipitated protein was collected by centrifugation for 5 min at 3000xg, washed once with cold 5% TCA, and then redissolved in 0.5 mL of 0.1 M HEPES buffer (pH 7.8). 1.5 mL of tissue solubilizer (Beckman BTS-450) were added and the solution was heated for 1 h at  $55^\circ\text{C}$ . The digested sample was then added to 15 mL of scintillation cocktail containing 0.38 mL of 3 N HCl and counted.

Total  $\text{CO}_2$  Incorporation. The remaining filter from each set was placed in a scintillation vial and the cells were rinsed from the filter with 0.9 mL water. 2 mL of BTS-450 were added and the vial was heated for 1 h at  $55^\circ\text{C}$ . After 0.5 mL of 3 N HCl plus 15 mL of scintillation cocktail were added, the vials were

placed next to 20 W fluorescent bulbs for 2 h in order to bleach the pigments prior to counting.

#### UDP-glucose Pyrophosphorylase Assay:

Cells from exponential-phase cultures were harvested by centrifugation, washed once with 50 mM HEPES buffer (pH 7.8) containing 2 mM dithiothreitol, and then resuspended in 5 mL of the same buffer. The suspension was then passed through a French pressure cell at 15000 psi, followed by centrifugation at 37000xg for 20 min. The supernatant was removed and used for the assay of enzymatic activity. The reaction mixture (final volume = 0.2 mL) contained 1 mM UTP, 0.5 mM [ $^{14}\text{C}$ ]glucose-1-P (specific activity = 1 mCi/mmol), 50 mM HEPES buffer (pH 7.8), 0.02 mL of enzyme extract containing 0.3 mg protein/mL or less, and other additions as noted in the Results Section. The reaction was initiated by the addition of enzyme, allowed to proceed for 10 min at 30°C, and then terminated by placing the tubes in boiling H<sub>2</sub>O for 1 min. After the tubes had cooled, 0.05 mL H<sub>2</sub>O and 0.03 mL (0.75 units) of E. coli alkaline phosphatase (Sigma Chemical Co.) were added followed by incubation at 30°C for 45 min. 0.1 mL of the solution was transferred to a Whatman DE81 DEAE-paper filter disk (2.5 cm diam) followed by rinsing for 1 min in each of four separate 150 mL portions of deionized H<sub>2</sub>O. The filter (containing bound UDP-[ $^{14}\text{C}$ ]glucose) was then placed in a scintillation vial containing 10 mL of scintillation cocktail and counted. This method is a modification of the procedure described by Ghosh and Preiss (1966) for the assay of ADP-glucose pyrophosphorylase.

An estimate of glucan formation under these assay conditions was made by omitting alkaline phosphatase and adding crystalline DEAE-cellulose (Whatman DE52) to the diluted reaction mixtures in order to bind UDP-[ $^{14}\text{C}$ ]glucose and unreacted [ $^{14}\text{C}$ ]glucose-1-P. After centrifugation of the suspension, a portion of the supernatant was counted. Since radioactivity levels were not significantly higher than those of nucleotide-free controls, it does not appear that substantial quantities of neutral glucan are formed under these assay conditions, thus allowing accurate estimations of pyrophosphorylase activity.

#### Results

In the first phase of this project, three different diatom species (C. fusiformis, C. cryptica, and T. pseudonana) were compared to each other with respect to lipid accumulation under Si-deficient conditions. Cultures were grown in media containing silicon levels which became limiting while culture growth was still in the exponential phase. The chlorophyll a concentration of the medium for each experiment was 2 mg/L or less at the onset of Si-limitation, thus preventing excessive self-shading of cells for at least the early stages of Si-starvation. In parallel experiments, cells were also cultured in media containing non-limiting levels of silicon.

Samples were removed from the cultures slightly before silica had been depleted from the medium, and also at points 12, 36, and 72 hours after silica was no longer detectable in the medium (i.e., less than 1  $\mu\text{M}$  SiO<sub>2</sub>). These samples were analyzed for cell density, chlorophyll a content, ash-free dry mass (AFDM), lipid mass, and the photosynthetic capacity of the cells.

For all three species, the rate of cell division decreased abruptly at the time when silica was depleted from the medium. Approximately one more cell doubling took place for each species during the next 72 hours. Cultures which were maintained in Si-replete conditions underwent an additional three to four doublings during this time period.

Substantial interspecific differences were observed in the effects of Si-limitation on chlorophyll synthesis for the three species tested. Chlorophyll synthesis was not significantly affected in C. fusiformis cultures for up to 72 h after the onset of Si-deficiency, but was nearly completely inhibited in cultures of C. cryptica limited for silicon for less than 12 h. The response of T. pseudonana cells to silicon limitation was intermediate between these extremes; up until 36 hours after the depletion of silicon from the medium there was only a slight inhibition of chlorophyll synthesis, but after this point chlorophyll synthesis was strongly inhibited. Silicon deficiency had little effect on the ratio of chlorophyll a to chlorophyll c1+c2, xanthophylls, and carotenoids for all three species tested since spectra of pigments extracted with 95% ethanol were very similar when normalized to each other at 664 nm (the "red peak" of chlorophyll a).

Gross photosynthetic rates (measured as O<sub>2</sub> evolution) under saturating illumination were substantially reduced during the first 12 hours of Si-starvation for cultures of C. fusiformis and C. cryptica. When compared to Si-sufficient cultures, the photosynthetic capacities of Si-limited C. fusiformis and C. cryptica cells were reduced by 33% and 58%, respectively, during this 12 hour period. Both Si-limited and Si-replete cultures of T. pseudonana exhibited a steadily decreasing photosynthetic rate. In fact, for all three species the maximum gross photosynthetic rate of Si-replete cultures at the end of the experiment reached values which were similar to those of Si-limited cultures. Since photosynthetic rates were normalized to chlorophyll a content, the decreases observed in Si-replete cultures may be due in part to an increase in the ratio of antenna chlorophyll molecules to reaction centers which is commonly observed in light-limited cultures.

Significant interspecific differences were also observed with respect to increases in lipid mass and total organic mass (i.e., AFDM) which occurred after the onset of Si-limitation. Si-deficient C. fusiformis cultures maintained the same overall productivity as Si-replete cultures for at least 72 hours (Fig. 1A). Lipid mass gain was also very similar in the two cultures. For both cases, there was an increase in the rate of lipid mass gain relative to organic mass gain such that the lipid content (as a percentage of the AFDM) increased throughout the duration of the experiment. The lipid content of Si-deficient cultures increased from 10% to 17% of the AFDM after 12 hours, and to 26% after 72 hours. For Si-replete cultures, the lipid content increased from 12% to 15% after 12 hours and to 21% after 72 hours.

C. cryptica cells responded quite differently to silicon starvation. The exponential rate of increase for total organic mass in cultures limited for silicon for 12 hours was reduced by 38% with respect to Si-replete cultures (Fig. 1B). On the other hand, the synthesis of lipids in Si-limited cultures during this 12 h time period was not diminished, leading to a substantial gain in the lipid content of Si-starved cells (from 24% to 34% of the AFDM). After 12 hours, there was little additional gain in lipid mass, AFDM, or lipid content in Si-limited cultures.

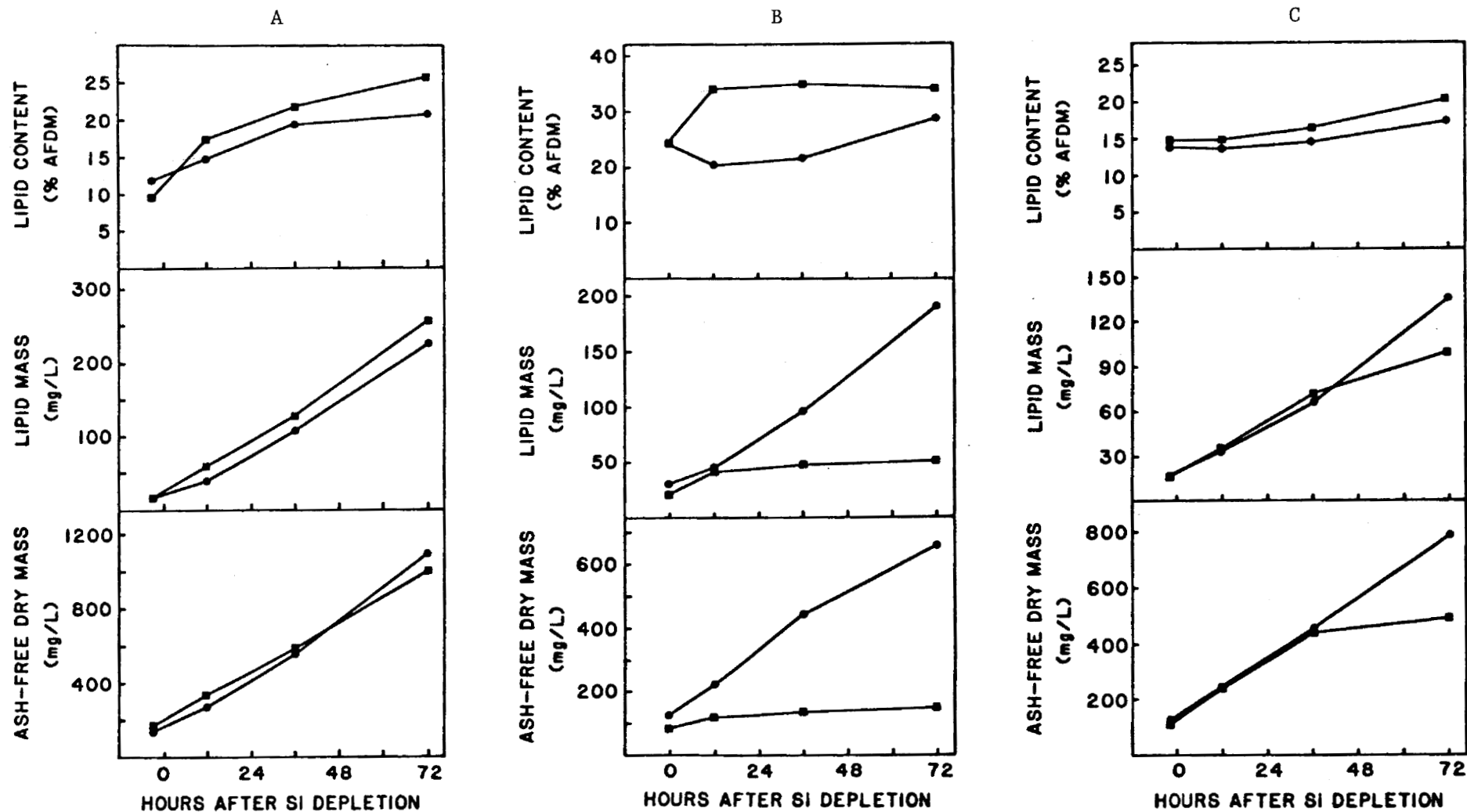


Figure 1. Changes in lipid mass, ash-free dry mass, and lipid content in Si-deficient diatom cultures.  
 A. *C. fusiformis*. B. *C. cryptica*. C. *T. pseudonana*.  
 Symbols: (■) Si-deficient cultures; (●) Si-replete cultures.

The synthesis of lipids and total organic material was inhibited in Si-limited cultures of *T. pseudonana*, but not until the cells had been deprived of silicon for more than 36 hours (Fig. 1C). There was a slight increase in the lipid content of both Si-deficient and Si-replete cells during the course of the experiment, but the lipid content of Si-starved cells was not substantially different from Si-replete cells at any point. The lipid content of Si-deficient cells increased from 15% to 20% during the 72 hour starvation period, and from 14% to 17% in Si-replete cells during the same time period.

In order to determine whether Si-deficiency affected the lipid composition of diatoms, the lipids extracted in the above experiments were separated into neutral lipid and polar lipid fractions by silica gel thin layer chromatography and quantified by flame ionization detection. For all three species examined, a dramatic increase in the neutral lipid content of Si-deficient cells was observed (Fig. 2). The majority of the neutral lipids formed appeared to be triacylglycerols, based on the fact that they co-migrated with authentic triacylglycerol standards. In the case of *C. fusiformis*, the neutral lipid fraction (as a percentage of the total lipid mass) increased from 19% to 57% after 72 hours of Si-starvation. The neutral lipid fraction of Si-replete cells remained between 17% and 20% during this same time period. For Si-deficient *C. cryptica* cells, a maximum neutral lipid fraction of 64% was observed 36 hours after the onset of Si-limitation. The neutral lipid fraction of Si-replete *C. cryptica* cells never exceeded 32%. It is interesting to note that the neutral lipid fraction of *C. cryptica* cells harvested immediately prior to the time of silica depletion already had an elevated neutral lipid fraction (48% of the total lipid mass), suggesting that in this species complete elimination of silicon from the medium is not required for the initiation of neutral lipid accumulation. *T. pseudonana* cells also accumulated neutral lipids in response to silicon limitation. In this case, the neutral lipid fraction increased to 44% of the total lipid mass after 36 hours of Si-deficiency. The neutral lipid fraction of Si-replete cells never exceeded 22%.

Based on the results of the above experiments, *C. cryptica* was chosen as the best candidate for additional studies involving the biochemistry of lipid accumulation in response to Si-deficiency. In order to help determine whether the observed increase in lipid content is due to *de novo* synthesis or to remobilization of previously assimilated carbon, a study was undertaken which examined the effects of Si-deficiency on the levels of the major cellular components (i.e., protein, chrysolaminarin, and lipid). As can be seen in Fig. 3, the net synthesis of chrysolaminarin and protein was sharply curtailed upon transfer of cells into a Si-free medium. However, the gain in culture lipid mass was very similar in both Si-replete and Si-deficient cultures for the first 12 h after transfer, leading to an increase in the lipid content from 19% to 27%. Since the absolute levels of protein and chrysolaminarin were not substantially decreased in Si-limited cultures, the observed increase in lipid content did not appear to be due to a *net* transfer of carbon from other cellular macromolecules.

#### **<sup>14</sup>C Incorporation Studies:**

In order to determine the short-term partitioning of newly assimilated carbon into lipid, chrysolaminarin, and protein, experiments were carried out which measured the incorporation of  $\text{H}^{14}\text{CO}_3^-$  into these fractions. As shown in Fig. 4, there was a dramatic change in carbon partitioning within 4 h after



Figure 2

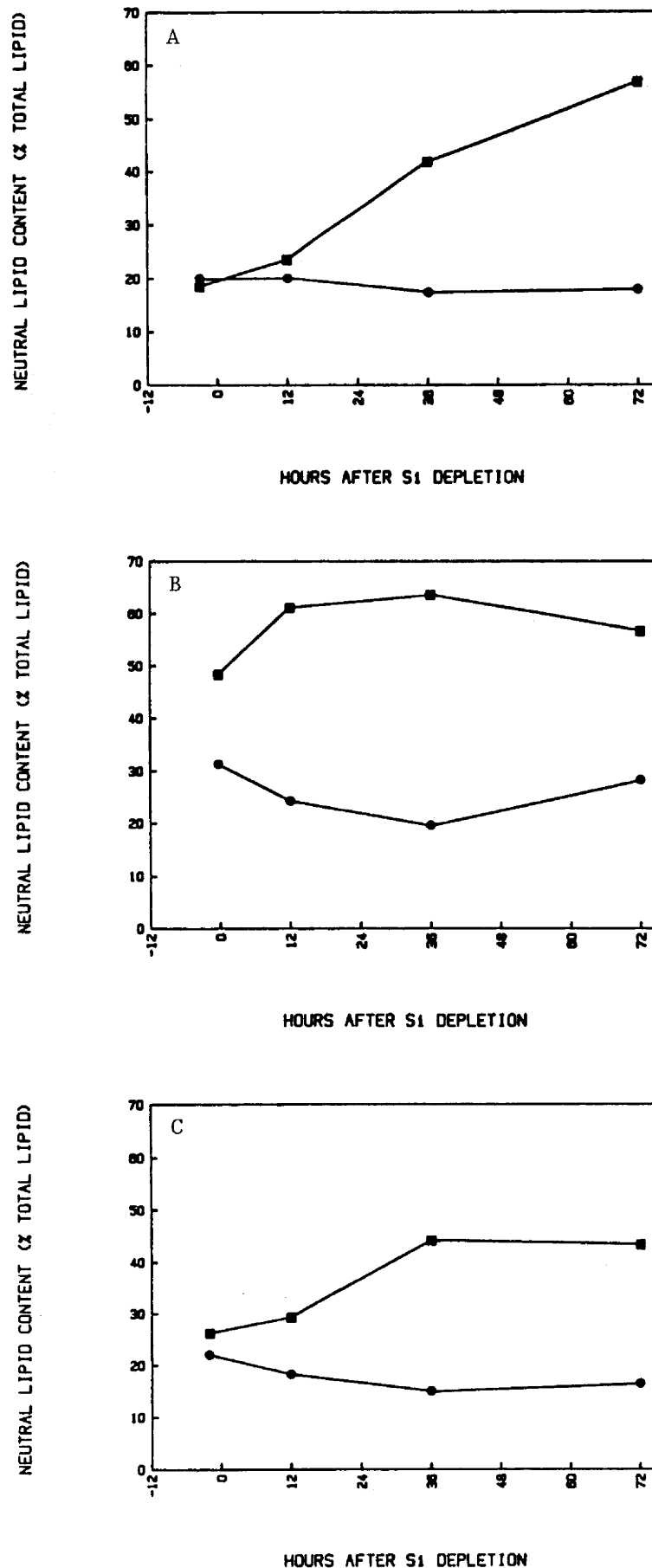


Figure 3

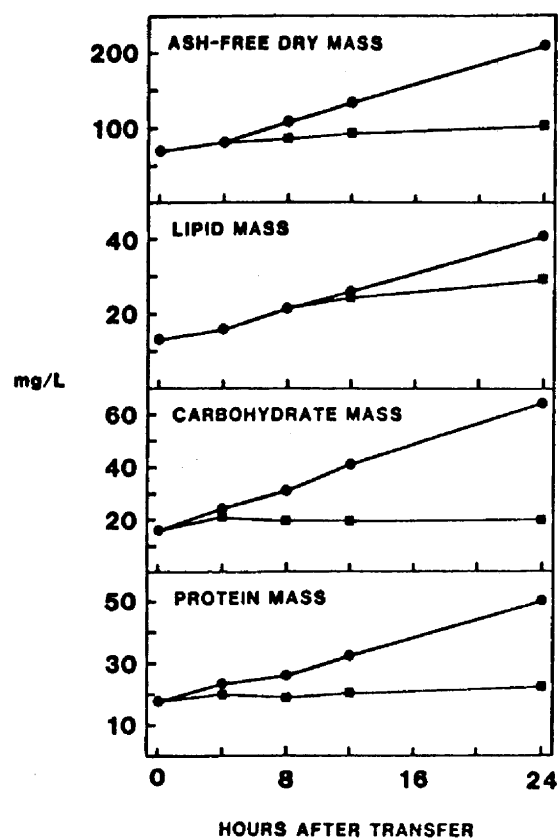


Figure 3. Effects of Si-deficiency on culture levels of lipid mass, chrysolaminarin mass, protein mass, and ash-free dry mass in *C. cryptica*. (■) Si-deficient culture; (●) Si-replete culture.

Figure 2. Changes in neutral lipid content in Si-deficient diatom cultures. A. *C. fusiformis*. B. *C. cryptica*. C. *T. pseudonana*. (■) Si-deficient cultures; (●) Si-replete cultures.

transfer into a Si-free medium. The percentage of  $^{14}\text{C}$  that was partitioned into chrysolaminarin was decreased by 50% during this time period, while the percentage of photoassimilated carbon incorporated into lipids was nearly doubled (from 27.6% to 54.1%). There was also a 48% increase in the fraction of  $^{14}\text{C}$  assimilated into protein in this 4 h period. After this point, there was a gradual return to pre-limitation values such that after 24 h of Si-deficiency the fraction of photoassimilated carbon partitioned into these three fractions was the same as in Si-sufficient cultures. Thus, the biochemical changes which lead to increased lipid contents in Si-deficient C. cryptica cells are of a transient nature.

Figure 5 indicates that a Si-starvation period of only 4 h brings about a very large reduction in the photosynthetic rate of C. cryptica cells. The rate of  $^{14}\text{C}$  incorporation into total cellular material decreased by 70% during this time period. The rate of  $^{14}\text{C}$  incorporation into lipids only decreased by 40% during this time, however, while the rate of carbon assimilation into chrysolaminarin was lowered by 84%. Thus, differential amounts of decrease in the rates of synthesis of the various cellular components are apparently responsible for the altered carbon partitioning patterns observed in Si-limited C. cryptica cells.

When portions of the lipid extracts from the various sampling times were fractionated by means of silica gel thin layer chromatography, a significant increase in the amount of carbon partitioned into triacylglycerols was observed. As shown in Fig. 6, the fraction of photoassimilated carbon that was partitioned into triacylglycerols (as a percentage of total lipid  $^{14}\text{C}$  incorporation) increased from 43% to 63% after only 4 h of Si-deficiency.

### Enzymatic Studies:

In order to better understand the biochemical basis of lipid accumulation in Si-starved diatoms, studies have been initiated which are designed to examine the activities of certain key enzymes of carbon assimilation in Si-deficient cells. Based on the results described above, it appears that at least one of the enzymes involved in chrysolaminarin biosynthesis may have reduced activity under Si-limiting conditions. Unfortunately, the enzymes of this pathway have never been characterized in diatoms. In higher plants and green algae, the initial enzyme involved in storage carbohydrate (starch) biosynthesis is adenosine diphosphate glucose (ADP-glucose) pyrophosphorylase, which catalyzes the formation of ADP-glucose from glucose-1-P and ATP. The ADP-glucose molecules are then condensed into starch in a reaction catalyzed by starch synthetase. An experiment was therefore carried out in order to determine whether pyrophosphorylase activity is present in cell-free extracts of C. cryptica cells. As shown in Table I, uridine diphosphate glucose (UDP-glucose) pyrophosphorylase activity was present in the extracts, but very little pyrophosphorylase activity could be detected which utilized ATP, CTP, or GTP. It is therefore likely that UDP-glucose pyrophosphorylase catalyzes the first committed step in chrysolaminarin biosynthesis. Thus, we have begun to investigate the biochemical properties of this enzyme. As shown in Fig. 7, the activity of this enzyme in the direction of UDP-glucose formation was maximal at pH 7.8.  $\text{Mg}^{2+}$  and  $\text{Mn}^{2+}$  were both potent effectors of UDP-glucose pyrophosphorylase activity (Table II); 5 mM  $\text{MgCl}_2$  and 5 mM  $\text{MnCl}_2$  stimulated the reaction rate by 10.6- and 8.6-fold, respectively. However, 10 mM phosphate and 2 mM 3-phosphoglycerate, compounds which have large effects on ADP-glucose

Figure 4

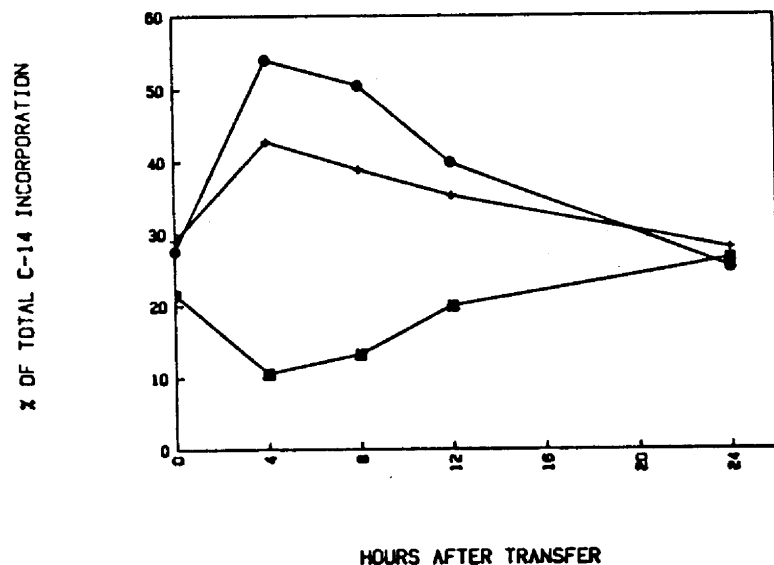


Figure 5

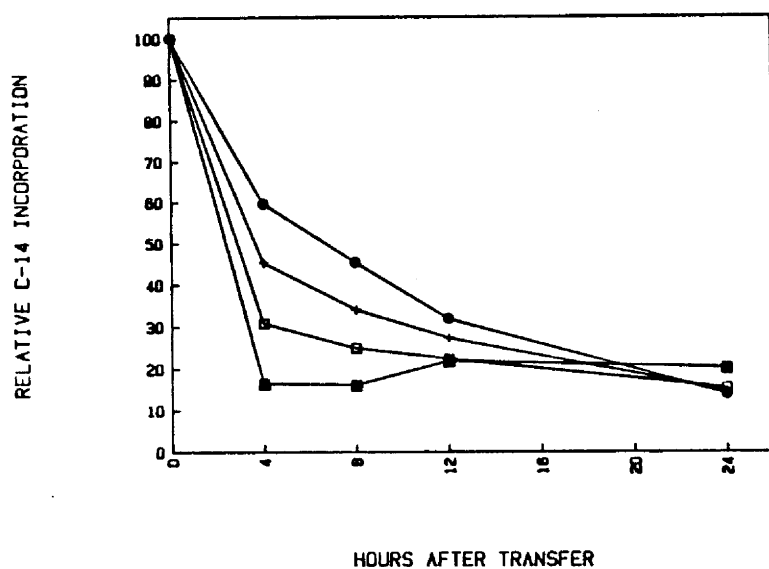


Figure 6

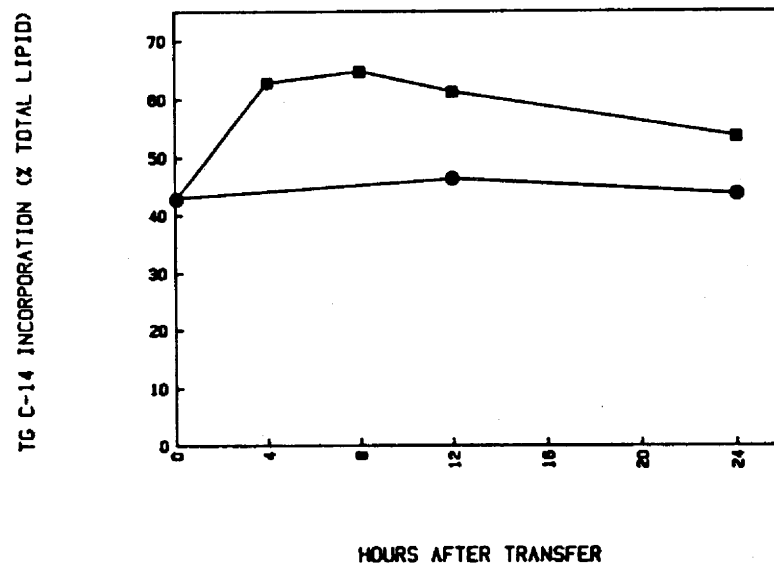


Figure 4. Effect of Si-deficiency on partitioning of photoassimilated carbon into lipid, chrysolaminarin, and protein in *C. cryptica*. Symbols: (●) lipid; (■) chrysolaminarin; (+) protein.

Figure 5. Relative rates of <sup>14</sup>C incorporation into lipid, chrysolaminarin, protein, and total cellular material in Si-deficient *C. cryptica*. The rate of <sup>14</sup>C incorporation into each fraction at the time of transfer into Si-free medium is represented as 100%. Symbols: (●) lipid; (■) chrysolaminarin; (+) protein; (□) total cellular material.

Figure 6. Effect of Si-deficiency on partitioning of photoassimilated carbon into triacylglycerols. Symbols: (■) Si-deficient cells; (●) Si-replete cells.

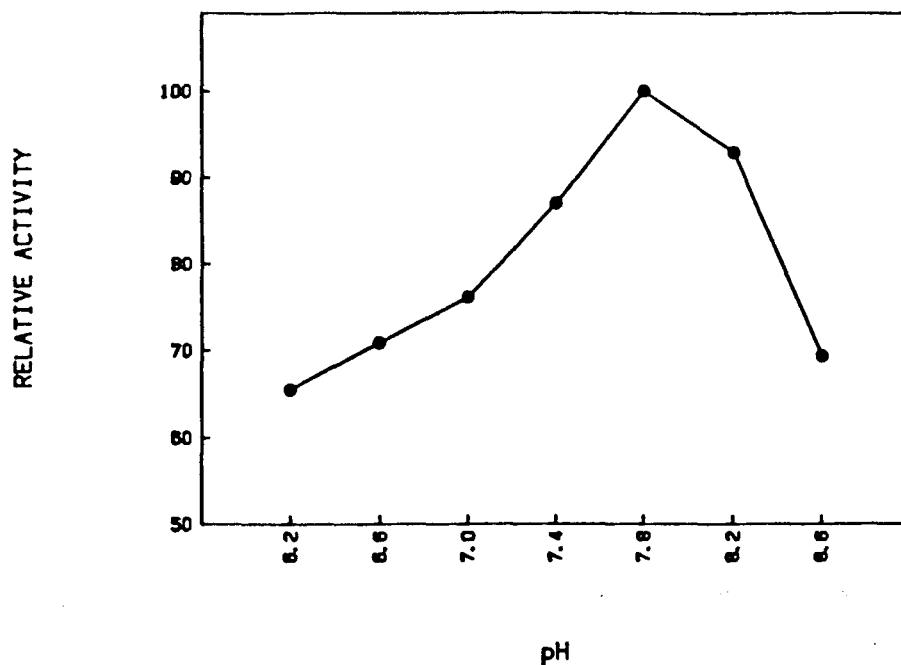


Figure 7. pH profile of UDP-glucose pyrophosphorylase activity in cell-free extracts of *C. cryptica*. Buffer was 50 mM HEPES/PIPES except that 50 mM Tris-HCl buffer was used for pH 8.6. 200 mM HEPES/PIPES (pH 8.2) was added to each sample prior to addition of alkaline phosphatase.

Table I. Pyrophosphorylase activity in cell-free extracts of *C. cryptica* in the presence of different nucleoside triphosphates. All reaction mixtures contained 5 mM MgCl<sub>2</sub>, 3.5 mM MnCl<sub>2</sub>, and the indicated NTP at a concentration of 1 mM. One unit of activity represents one umole of nucleotide diphosphate glucose formed per hour.

<u>Substrate added</u>	<u>Activity (U/mg protein)</u>
ATP	0.012
CTP	0.012
GTP	0.016
UTP	1.7

Table II. UDP-glucose pyrophosphorylase activity in cell-free extracts of *C. cryptica* in the presence of various compounds.

<u>Additions</u>	<u>Relative Activity</u>
none	1.0
5 mM MgCl <sub>2</sub>	10.6
5 mM MnCl <sub>2</sub>	8.6
10 mM PO <sub>4</sub>	2.0
2 mM 3-PGA	1.2
5 mM MgCl <sub>2</sub> + 10 mM PO <sub>4</sub>	10.2
5 mM MgCl <sub>2</sub> + 2 mM 3-PGA	11.5

pyrophosphorylase activity in higher plants and green algae, did not have a substantial effect on UDP-glucose pyrophosphorylase activity in C. cryptica, regardless of whether or not  $Mg^{2+}$  was included for enzyme activation (Table II).

## Discussion

The results of the first phase of this investigation indicate that various diatom species respond quite differently to Si-deficiency with respect to biomass gain and lipid accumulation. However, a few of the findings of this study were consistent for all three of the species tested, including the observation that the neutral lipid content (as a fraction of the total lipid mass) was at least two-fold higher in Si-deficient cells than in Si-replete cells (Fig. 2). As a result of this, calculations indicate that the neutral lipid mass was 1.7- to 3.6-fold higher in Si-limited cultures than in Si-replete cultures for C. fusiformis and T. pseudonana throughout the duration of the experiment, and for up to 36 h after the onset of Si-limitation for C. cryptica. The  $^{14}C$  experiments with C. cryptica also indicated an increase in the amount of newly assimilated carbon partitioned into triacylglycerols.

Another finding consistent for all three species tested was that a sustained increase in the absolute rate of total lipid synthesis was never observed in Si-deficient cultures. The increased lipid contents that were observed in C. cryptica and C. fusiformis could instead be attributed to maintenance of normal lipid synthesis rates during a time when the synthesis of other organic cellular material was strongly inhibited. This conclusion is supported by the results of the  $^{14}C$  incorporation studies with C. cryptica, which indicated an increase in the percentage of photoassimilated carbon partitioned into the lipid fraction of Si-limited cells. In these experiments, however, a substantial reduction in the absolute amount of lipid synthesized from newly assimilated carbon was observed within only 4 h after the onset of Si-deficiency. It is not clear why a reduction in lipid mass gain was not observed in the first 12 h after silicon depletion for this species (see Figs. 1 and 3). Perhaps there is a remobilization of carbon from chrysolaminarin or protein which approximately balances the de novo synthesis of these compounds from newly assimilated carbon.

Despite the complicating factor of greatly reduced photosynthetic rates in Si-deficient C. cryptica cells, the rapidity and magnitude of the changes in carbon partitioning that occur should provide a good system for studying the regulation of key carbon metabolizing enzymes under Si-deficient conditions. Since several lines of evidence suggest a sharp reduction in the rate of chrysolaminarin biosynthesis, initial attempts have focused on this pathway.

The results presented in this paper represent the first evidence for UDP-glucose pyrophosphorylase activity in diatoms. Since no other pyrophosphorylase activity could be detected, it is likely that this enzyme is involved in chrysolaminarin biosynthesis. Planned experiments will determine whether UDP-glucose pyrophosphorylase is indeed a precursor for chrysolaminarin synthesis. The alkaline pH optimum for this enzyme is similar to that of ADP-glucose pyrophosphorylase from Chlorella vulgaris (Nakamura and Imamura 1985) and spinach (Copeland and Preiss 1981). Another similarity with ADP-glucose pyrophosphorylase from Chlorella and higher plants involves the

strong stimulation observed with  $Mg^{2+}$  and  $Mn^{2+}$ . Since divalent metal cations are known to undergo rapid changes in concentration in certain compartments of photosynthetic cells, these compounds should be considered as potential regulatory agents. UDP-glucose pyrophosphorylase differed from ADP-glucose pyrophosphorylases involved in starch synthesis in that phosphate and 3-phosphoglycerate had very little effect on enzymatic activity. These compounds are quite important in higher plant carbon partitioning due to the presence of the triosephosphate/phosphate translocator of the inner chloroplast membrane. Since UDP-glucose pyrophosphorylase probably has an extrachloroplastic location in diatoms, the absence of regulation by phosphate and 3-phosphoglycerate is not surprising. Future studies will investigate possible changes in the activity of this enzyme (and other enzymes of carbon metabolism) in response to Si-deficiency.

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EFFECTS OF INDUCTION STRATEGIES ON CHAETOCEROS (SS-14)  
GROWTH WITH EMPHASIS ON LIPIDS

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ABSTRACT

The objectives of the research reported here were to determine the effects of induction strategies (nitrogen variation and variable temperatures) on lipid production in diatom, Chaetoceros (SS-14). The diatoms were batch cultured in N-sufficient (NS) (600  $\mu$ M) and N-deficient (ND) (300  $\mu$ M) media and two different temperatures (20°C and 30°C) under continuous illumination. The biomass was harvested during the log phase and stationery phase in NS and ND treatments respectively. The Ash-free dry weight (AFDW) was measured. Total lipids were extracted from harvested cells by extracting twice with methanol followed by two additional extractions with chloroform-methanol (1:1 v/v). The crude lipids were separated into neutral and polar lipids by column chromatography on silicic acid column.

The results show that the AFDW was 1.181 g/L and 0.862 g/L in NS and ND treatments respectively at 20°C, and 1.201 g/L and 0.923 g/L at 30°C. The total lipids were respectively 0.198 g and 0.329 g of g AFDW in NS and ND; 0.196 g and 0.338 g of g AFDW at 20°C and 30°C. The neutral lipids were 0.122 g and 0.175 g of g AFDW in NS and ND; 0.137 g and 0.186 g of g AFDW at 20°C and 30°C respectively. The polar lipids were 0.031 g and 0.065 g of g AFDW in NS and ND; 0.057 g and 0.081 g of g AFDW at 20°C and 30°C respectively. The neutral lipids were 12.19% and 17.52% of AFDW in case of NS and ND treatments respectively at 20°C. At 30°C the neutral lipids were 13.66% and 18.63% of AFDW in NS and ND respectively. The polar lipids were 3.13% and 6.50% of AFDW in NS and ND media respectively at 20°C. At 30°C the polar lipids were 5.75% and 8.13% of AFDW in NS and ND respectively. These observations show that by growing diatom Chaetoceros (SS-14) in nitrogen limited supply, the production of lipids, particularly neutral lipids, can be increased.

INTRODUCTION

Chrysophytes are known to accumulate large quantities of lipids. The objectives of the studies reported here were to evaluate the effect of induction strategies (varying nitrogen concentrations and temperature) on lipid production in the diatom Chaetoceros (SS-14). Temperature, nitrogen concentration and



silica concentrations are factors which are known to influence lipid composition and quantity (1-5). Growing of algae in N-deficient conditions leads to remarkable changes in algae cell composition (6) and lipid concentration (7) in Cyclotella DI-35 and Hantzschia DI-160 (8). Studies were conducted for lipid production, quantification under stressed and nonstressed conditions by growing promising species of algae including Chaetoceros (SS-14) sp. (2-4, 9).

In the Aquatic Species Program of SERI (2-5, 8, 9) efforts have been directed towards the development of microalgal strains for biomass fuel application. In order to accomplish this goal, one of the objectives is to grow microalgae under varying environmental tolerances for enhanced lipid production. To achieve the objectives discussed here, the present project was initiated to make a comparative study of lipids from Chaetoceros (SS-14) grown under nitrogen sufficient (NS) and nitrogen deficient (ND) conditions at two different temperatures (20°C and 30°C).

## MATERIALS AND METHODS

### Growth Conditions:

Chaetoceros (SS-14) was obtained from SERI. The cultures of Chaetoceros (SS-14) were maintained in a growth room (Fig. 1 and 2) under continuous illumination with cool white fluorescent tubes: 4 tubes (40W) on the top and 6 tubes (15W) on each lateral side of the table. Light intensity on the table was measured with a light meter GE 214. Light intensity varied from 400 to 450 foot candles.

The growth medium for culturing Chaetoceros (SS-14) was prepared as suggested by Dr. B. Barclay of SERI as shown in Table 1.

Table 1. Medium for Chaetoceros (SS-14)

<u>Component</u>		<u>Concentration</u>	
	NaCl	0.4 M	
	MgSO <sub>4</sub> ·7H <sub>2</sub> O	19 mM	
	MgCl <sub>2</sub> ·6H <sub>2</sub> O	20 mM	
	CaCl <sub>2</sub>	9 mM	
	KCl	1 mM	
	KNO <sub>3</sub>	2 mM	
	Na <sub>2</sub> EDTA	35 mM	
	Na <sub>2</sub> SiO <sub>3</sub> ·9H <sub>2</sub> O	140 mM	
	glycyl glycine	0.005 M	
vitamin solution	Thiamine HCl	0.0 mg	
	Biotin	0.5 mg	1 ml
	B <sub>12</sub>	0.5 mg	
trace metal mixture	H <sub>3</sub> BO <sub>3</sub>	9.1 mM	
	ZnCl <sub>2</sub>	4.5 mM	
	CuCl <sub>2</sub> ·2H <sub>2</sub> O	1.7 mM	1.0 ml
	Na <sub>2</sub> MOO <sub>4</sub> ·2H <sub>2</sub> O	1 mM	
	Na tartrate	1.7 mM	

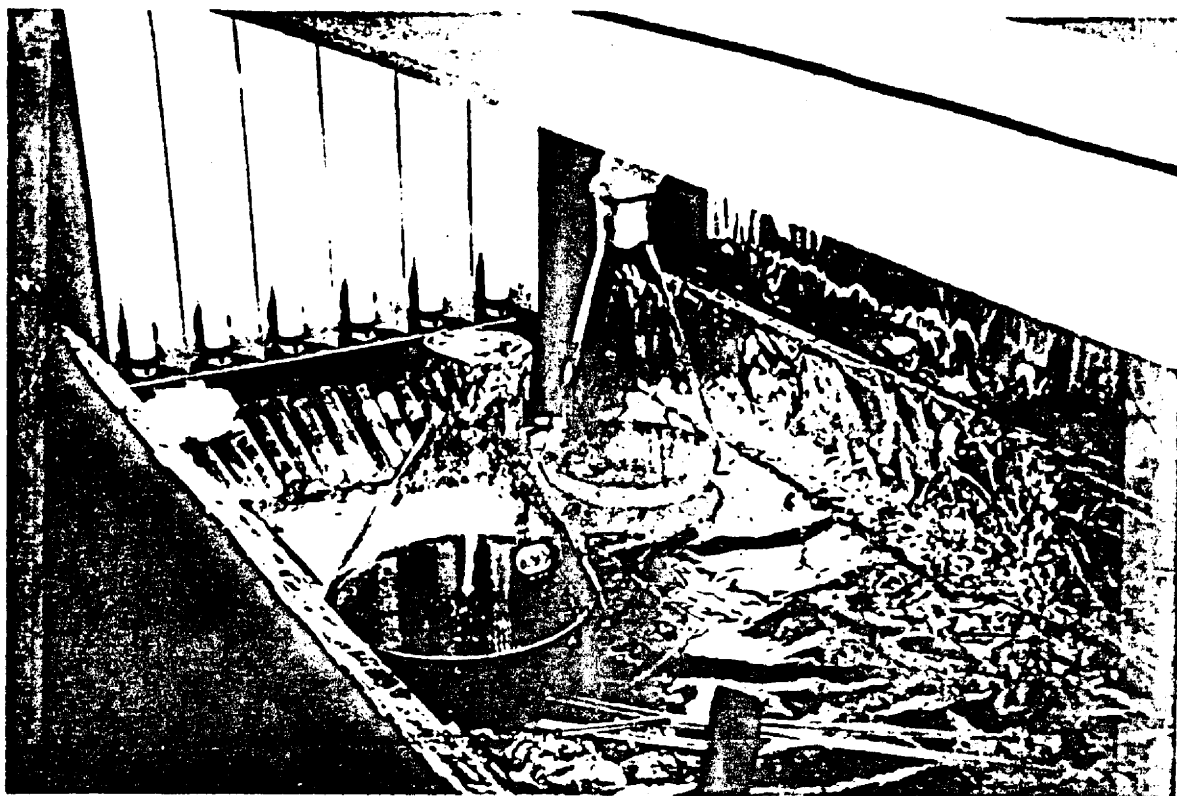


Figure 1. Growth Chamber

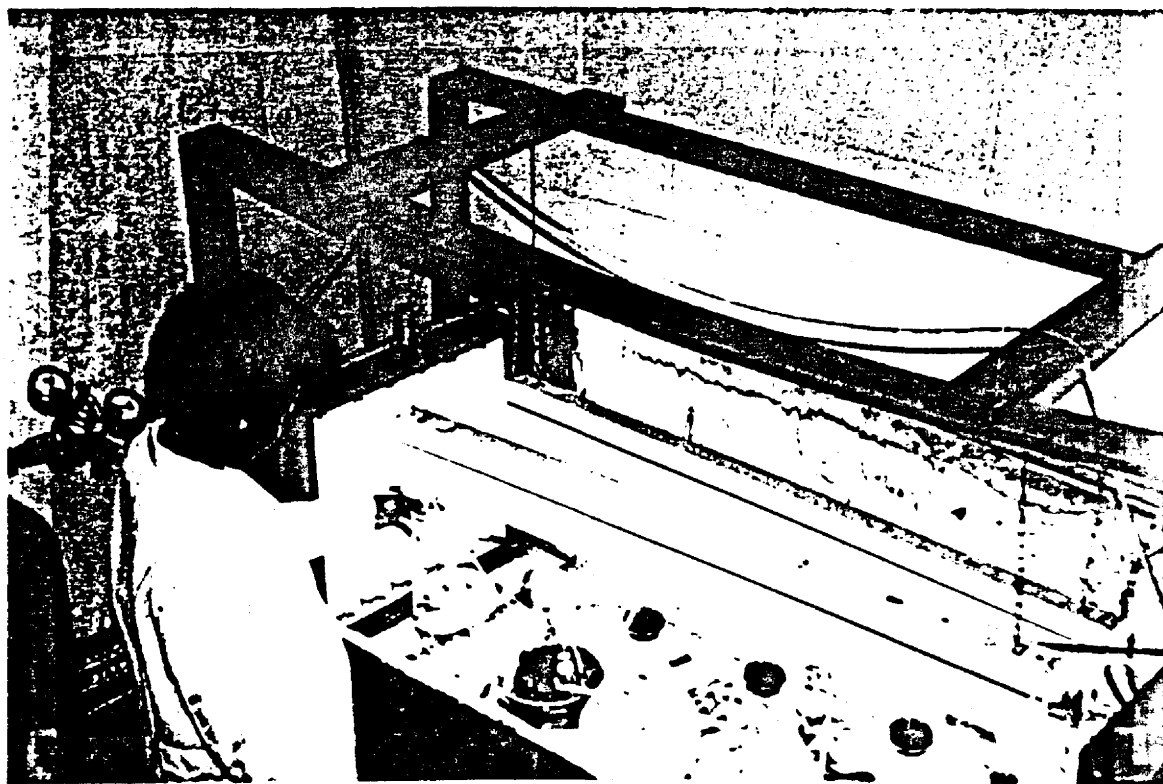


Figure 2. Chaetoceros (SS-14) culture in Growth Chamber

pH was adjusted and the growth medium was autoclaved. A 100 ml solution containing 0.456 g of  $\text{KH}_2\text{PO}_4$  was sterilized. 10 ml of this solution was added to the growth medium at the time of inoculation.

Inoculum was prepared by growing diatoms in 250 ml bottles containing 100 ml of growth medium. The cultures were aerated with air containing 3%  $\text{CO}_2$ . Cultures were maintained at  $30^\circ\text{C}$  to logarithmic phase (5-7 days). Growth was evaluated by measuring optical density (O.D.) daily with Gilford spectrophotometer at 650 n. wave length. The growth of the cultures was evaluated every second day by cell counts using Petrof Hauser counting chamber.

#### Nitrogen Concentration:

Batch cultures of Chaetoceros (SS-14) were grown in the growth medium described above containing various amounts of  $\text{KNO}_3$ . All the ingredients described earlier were present except for glycyl glycine. Potassium nitrate was added in two separate concentrations. In nitrogen sufficient (NS) and nitrogen deficient (ND) treatments, the amounts of  $\text{KNO}_3$  added were  $600\ \mu\text{M}$  and  $300\ \mu\text{M}$  respectively. The cultures previously acclimated at  $20^\circ\text{C}$  and  $30^\circ\text{C}$  were inoculated into 2,500 ml Fernbeck flasks containing 1,000 ml of growth medium with either  $600\ \mu\text{M}$  or  $300\ \mu\text{M}$  nitrogen. The cultures in both these treatments were maintained under continuous illumination as described above. The NS and ND treatments were kept at two different temperatures,  $20^\circ\text{C}$  and  $30^\circ\text{C}$ , as shown in Table 2.

Table 2. Experimental Design

<u>Nitrogen</u>			
Sufficient (NS) $600\ \mu\text{M}$		Deficient (ND) $300\ \mu\text{M}$	
$20^\circ\text{C}$	$30^\circ\text{C}$	$20^\circ\text{C}$	$30^\circ\text{C}$

The treatments were run in triplicate. The growth of the cultures was monitored by cell counts.

The biomass was collected at different growth phases. In NS treatments, the diatom cells were harvested during logarithmic phase. In ND treatments, the cells were harvested on the second day after the stationery phase and the remaining biomass ten days after the stationery phase. Dry weight was determined by taking culture aliquots (25 ml) and filtering through 2.5 cm Whatman GF/C filter and drying to constant weight at  $60^\circ\text{C}$ .

## Evaluation of Oil Accumulation

Microscopic examination of the diatom cells were made before harvesting to ensure that the diatom had produced lipid globules. Harvesting occurred only if the cells appeared to be full of lipid globules.

## Harvesting

Cells of Chaetoceros (SS-14) were harvested by centrifugation and washing with sterilized growth medium. The pellets of each sample were resuspended in minimal volume of growth medium. To obtain wet cell mass, the suspension was transferred to a weighed tared tube. This tube along with suspension was weighed again and based on the difference in weights, the mass of cell suspension/wet weight (g/L) was calculated.

A small volume (30%) of the well-mixed cell suspension was taken for determination of dry cell mass. The remaining 70% of the cell suspension was stored in a freezer at  $-20^{\circ}\text{C}$ .

## Ash-free Dry Weight Determination

Ash-free dry weight (AFDW) was determined as follows. The 30% of the cell suspension taken above was placed in each of three weighed small test tubes (13 x 100 mm tubes). These tubes were previously heated at  $50^{\circ}\text{C}$  for 14 hours. The weight of the test tube along with the cell suspension was recorded. The dry cell mass was determined by placing these 3 tubes in an oven at  $60^{\circ}\text{C}$  drying to constant mass (36-48 h). These tubes were put in a dessicator for cooling. After cooling, the weight was recorded. This indicated the dry weight of the cells.

For determination of AFDW the tubes containing dry cells were placed in an oven ( $500^{\circ}\text{C}$ ) for 5 h. These tubes were again put in a dessicator for cooling and weighed. By taking the difference of the two weights, the ash-free dry weight (AFDW) was calculated.

## Total Lipids Determination

The samples stored at  $-20^{\circ}\text{C}$  were allowed to thaw at room temperature immediately prior to extracting the lipids. The lipids were extracted according to the modified method of Bligh and Dyer (10) and the procedures (2-4, 11) described for algal lipids. To the wet cell suspension (approximately 150 mg dry mass) an equal volume of ethanol was added and the mixture heated at  $60^{\circ}\text{C}$  for 1 hr, centrifuged at 3,000 rpm and the supernatant transferred to a large tube with a teflon cap. 10 ml of methanol was added to the pellet and heated at  $60^{\circ}\text{C}$  for 1 hr. This was centrifuged and the supernatant saved. This step was repeated two times.

The pellet was subjected to two additional extractions with chloroform-methanol (1:1 v/v) and heated for 1 hr at 60°C, centrifuged and the supernatant saved. As mentioned, after following this step twice, the combined supernatant solution was placed into a separatory funnel for phase separation (Bligh and Dyer). Enough chloroform and water was added to give the Bligh-Dyer ratio for phase separation (Chloroform:methanol:water;10:10:9). This was mixed gently and the lower chloroform phase was allowed to clear and then collected. An additional 5-10 parts of the chloroform was added to the aqueous phase and mixed gently. To facilitate separation, 1 drop of 6N HCl was added. The chloroform phase was allowed to clear and later collected.

The combined chloroform phase was collected in pre-weighed tared flasks. It was reduced in volume in a rotary evaporator at 30-35°C and transferred to a dessicator for cooling. After cooling, the flask was again weighed and the total lipids were determined gravimetrically. The lipid residue in the flask was immediately dissolved in a small volume of chloroform and transferred to a small tube and stored in a freezer at -20°C.

#### Fractionation of Lipid Classes

Total lipids were separated into neutral lipids and polar lipids by silicic acid chromatography according to the procedure described by Tornabene (11). The silicic acid was heated at 120°C for 2 hrs before use. The ratio of silicic acid taken to sample (total lipids) was 30:1. The silicic acid suspended in hexane and poured into a column containing hexane and allowed to settle. The column was washed with six bed volumes of hexane. The total lipid sample was redissolved in 0.5 ml of hexane and transferred to the column. The column was eluted with one bed volume of each of the solvents: hexane, benzene, chloroform, acetone and methanol. The neutral lipids were collected in the chloroform eluate and the polar lipids in the methanol eluate. The eluates were reduced in volume on a rotary evaporator and later evaporated to dryness under air. The mass of neutral lipids and polar lipids was evaluated.

#### RESULTS AND DISCUSSIONS

The growth conditions and cellular yield of Chaetoceros (SS-14) are shown in . The growth response has been determined by cell counting. Under nitrogen sufficient (NS) conditions, Chaetoceros (SS-14) grew most rapidly. At 20°C, the number of doublings per day in NS and ND were 1.00 and 0.80 respectively; at 30°C, these were 1.20 and 0.90 respectively. Growth was lower in nitrogen deficient cultures during the stationary phase. These results are similar to those reported by other researchers (2-5) on the effect of nitrogen supply on the energy efficiency in microalgae. Growth of Chaetoceros (SS-14) was more rapid at high (30°C) temperatures than at low (20°C) temperatures.

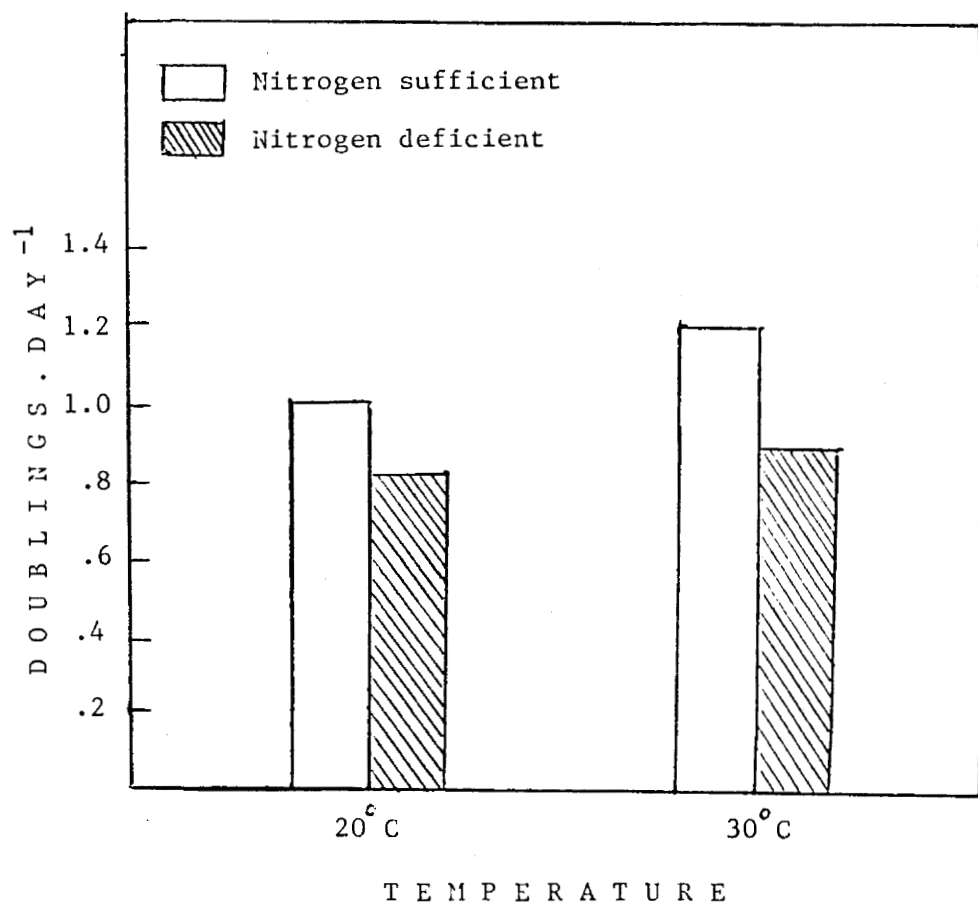


Table 2. Growth Response of Chaetoceros (SS-14) (Doublings.Day<sup>-1</sup>) at 20°C and 30°C in nitrogen sufficient and nitrogen deficient media.

Table 3. Biomass and Lipid Yields of Chaetoceros (SS-14) Grown in Different Nitrogen Concentrations and Temperatures (in g/g AFDW).

Culture Conditions	Ash-free dry weight AFDW g/L	Total Lipids g/g AFDW	Neutral Lipids g/g AFDW	Polar Lipids g/g AFDW	
	° 20 C Temperature				
	Nitrogen Sufficient (NS)	1.181±0.098	0.198±0.013	0.121±0.028	0.031±0.011
	Nitrogen Deficient (ND)	0.862±0.081	0.329±0.012	0.175±0.017	0.065±0.001
	° 30 C Temperature				
	Nitrogen Sufficient (NS)	1.201±0.105	0.196±0.011	0.136±0.026	0.057±0.001
	Nitrogen Deficient (ND)	0.923±0.095	0.338±0.011	0.186±0.012	0.081±0.001

Table 4. Biomass and Lipid Yields of Chaetoceros (SS-14) Grown in Different Nitrogen Concentrations and Temperatures (in % of AFDW).

Culture Conditions	Ash-free dry weight AFDW g/L	Total Lipids % of AFDW	Neutral Lipids % of AFDW	Polar Lipids % of AFDW
<p>Nitrogen Sufficient (NS)</p> <p>Nitrogen Deficient (ND)</p> <p>Nitrogen Sufficient (NS)</p> <p>Nitrogen Deficient (ND)</p>	<sup>o</sup> 20 C Temperature			
	1.181±0.098	19.81±1.3	12.19±2.8	3.13±0.1
	0.862±0.081	32.95±1.2	17.52±1.7	6.50±0.1
	<sup>o</sup> 30 C Temperature			
	1.201±0.105	19.65±1.1	13.66±2.6	5.75±0.1
	0.923±0.095	33.80±1.1	18.63±1.2	8.13±0.1



The results of the analysis of Chaetoceros (SS-14) are summarized in Table 3. At 30°C, AFDW was 1.201 g/L and 0.923 g/L in nitrogen sufficient (NS) and nitrogen deficient (ND) treatments respectively. The total lipid content was 0.196 g and 0.338 g of g AFDW in NS and ND treatments at this temperature (30°C). Nitrate limitation produced higher yield of cell mass and total lipid content. At 20°C, the AFDW was 1.181 g/L and 0.862 g/L and the total lipids were 0.193 g and 0.329 g of g AFDW in NS and ND respectively. Nitrate deficiency increased the amount of total lipids. From the results it can be seen that the biomass and lipid concentration reached optimum levels as the cultures approached N-depletion.

Total lipids were fractionated into neutral lipids and polar lipids by column chromatography. The neutral lipids were 0.122 g and 0.175 g of g AFDW respectively when grown in NS and ND media at 20°C. The amounts of neutral lipids were higher when these diatoms were grown at 30°C, i.e. 0.136 g and 0.186 g of g AFDW in NS and ND media respectively. The polar lipid contents were respectively 0.031 g and 0.065 g of g AFDW in NS and ND media at 20°C; and 0.057 g and 0.081 g of g AFDW in NS and ND media at 30°C. The neutral lipids were 12.19% and 17.52% of AFDW in case of NS and ND treatments respectively at 20°C. At 30°C the neutral lipids were 13.66% and 18.63% of AFDW in NS and ND respectively. The polar lipids were 3.13% and 6.50% of AFDW in NS and ND media respectively at 20°C. At 30°C the polar lipids were 5.75% and 8.13% of AFDW in NS and ND respectively.

These results encourage us to support the concept of modifying growth conditions of microalgae for producing higher biomass and lipid yields.

#### CONTINUING RESEARCH

The results have provided the preliminary data for biomass and lipid production. Since the research is preliminary, additional experiments need to be done to establish the relationship between nitrogen concentration and biomass and lipid production. Similarly, additional experiments will be conducted to study the effect of temperature on the production of AFDW and lipid content. It is also planned to explore the possibilities of enhancing biomass production and energy efficiency by adding various concentrations of silica in batch cultures of Chaetoceros (SS-14). The detailed study on the chemical characterization of lipids (neutral lipids, polar lipids, fatty acids) will be undertaken. In addition, the effects of induction strategies (different concentrations of nitrogen and silica, temperature variation) on lipid production will be studied on other diatoms i.e.: Cyclotella DI-35 and Hantzschia DI-160.

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# THE EFFECTS OF FLUCTUATING ENVIRONMENTS ON THE SELECTION OF HIGH YIELDING MICROALGAE

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## Introduction

The production of lipids using microalgae requires that high lipid content algal strains be cultivated at high productivity under conditions which prevent other strains from dominating the cultures. Since physical prevention of contamination is economically not feasible, algal strains must be identified and/or developed, which are highly competitive in the outdoor pond environment. That specific algal strains will establish dominance in outdoor ponds, and can maintain such dominance for extended periods in the absence of major environmental upsets or changes, is now well established - in particular from the outdoor cultivation work carried out under SERI sponsorship. What is not yet understood is whether dominance in outdoor systems is a property restricted to a few algal strains or whether a relatively diverse number of strains can dominate under similar outdoor pond conditions. In addition the key environmental factors affecting dominance are not yet characterized. In the development of such information it would be useful to use laboratory culture systems which simulate, as closely as possible, the outdoor pond environment. Such systems could be used to both screen algal species for their relative competitive abilities as well as to establish which specific factors (temperature, pCO<sub>2</sub>, pO<sub>2</sub>, growth rates, etc.) are responsible for their dominance.

The requirements for algae strains to exhibit both a high lipid productivity and content is at first glance incompatible with the requirements for dominance and high biomass productivity (these latter two being compatible, but not synonymous, requirements). Under light (energy) limitation a high lipid content would detract from growth rate and overall productivity - as lipids (above 10-15 % of dry weight) do not significantly contribute to the primary metabolism of these organisms. The example of *Botryococcus braunii* supports this view: its high hydrocarbon content correlates with a low growth rate reflecting poor competitiveness. No specific selective advantage for a high lipid content in microalgae can at present be postulated - except, possibly, under conditions which would greatly affect overall culture productivity. A high lipid content may be an advantage in storing energy reserves; this would, however, be of value only under conditions where overall low growth rates were imposed on the system.

The requirements for culture dominance coupled to high lipid productivity could be met in principle by several approaches: 1) screening for relatively high lipid containing algae that are also dominant in ponds, 2) continuous exposure of the cultures to an environmental stress (e.g., limited nitrogen) that would increase lipid content without proportionately decreasing productivity, or 3) by temporal separation of biomass production from lipid production. The latter approach is the one followed by this project. It is based on the premise that transient metabolic capacities for secondary metabolism can exceed those achievable in steady state. This premise must still be proven for the case of lipid storage by microalgae.

We describe below the development of a laboratory culture system capable of simulating the fluctuating pond environment and the preliminary species competition experiments carried out with this system. We also describe the results of a survey of eight species of microalgae for the induction of lipids by nitrogen starvation.

## Species Competition Under Fluctuating Environments

Methods. Figure 1 presents the schematic of the reactor system used in this work. The individual algal reactor is a 1L Roux bottle (5 cm light path) with holes drilled in the top and side to provide a media effluent port and access for pH and temperature probes. The media overflow system was designed to remove liquid below the culture surface and to prevent cell enrichment due to interfacial tensions. Light was provided from one side on a 14 hours light:10 hours dark cycle. Temperature control was supplied by a recirculating water heater/chiller by immersing the bottles in a transparent water bath. All cultures were operated at a 1.5 day detention time. All organisms used in this work were derived from the SERI Culture Collection, except for *Platymonas* which was obtained from Carolina Biological Supply.

The system has the capability of automatically varying pH,  $p\text{CO}_2$  and/or alkalinity levels by the addition of acid or base and by regulation of  $p\text{CO}_2$  and gas flow control. It also can be used to control dissolved oxygen (by varying the  $\text{O}_2$  concentration in the gas) and temperature. Data acquisition and control are carried out with a Keithley DAS System 520 interfaced with an IMB PC. pH probes are multiplexed through a custom-designed OP-AMP circuit that is capable of accepting 10 high impedance input signals. Gas phase analysis is possible with the connection of an infrared  $\text{CO}_2$  analyzer and a Paramagnetic  $\text{O}_2$  analyzer.

Initial Experiments. The initial experiments, carried out in parallel to the development of this system, were designed to answer some relatively simple questions: 1) could algal species of interest be cultivated for long periods in continuous cultures without wall growth becoming a limiting operating factor? 2) could significant differences in competition be observed in experiments in which environmental conditions varied? 3) did the inoculum cell density affect the final result? 4) how long did the cultures have to be operated to allow conclusions regarding the outcome of competition between the different algae species? and 5) could clear cut dominance be established in these systems?

The first experiment involved a competition between *Platymonas* and "T-Iso" (*Isochrysis galbana*, Tahitian strain) grown on GPM media with two side by side reactors, one cleaned daily and the other not cleaned. After one week *Platymonas* became the dominant algae (at least 90% of cell mass), however T-Iso remained as a steady component of the biomass for the remaining two weeks of the experiment. There were no significant differences between the cleaned and noncleaned vessels in either cell counts or density. (Cleaning involved moving the magnetic stirring bar back and forth over the culture vessel wall). However wall growth in the uncleaned vessel was not significant during this experiment. That will vary depending upon species and environment characteristics, as seen below.

In the next experiment *Chlorella* sp, T-Iso, and Nanno Q (*Nannochloropsis* sp, Lewin strain) were inoculated into four cultures which differed in their inoculation level (high and low) and  $\text{CO}_2$  supply (either constant with  $\text{CO}_2$  at 0.5%, or fluctuating over a four-hour cycle of 1 hour at 0.5%  $\text{CO}_2$  and 3 hours air). The media used was a supplemented GPM. Within three days the two high  $\text{CO}_2$  cultures exhibited a cell density of about 1 g/L while the two fluctuating  $\text{CO}_2$  cultures had about half that density (Fig. 2). The variation in pH in one of the fluctuating cultures is shown in Figure 3. The pH rise during the dark period was strictly due to the stripping of  $\text{CO}_2$  to the equilibrium level with air. During the lighted portion of the diurnal cycle and when air was being sparged into the culture, there was significant consumption of  $\text{CO}_2$  beyond that which would be at equilibrium with air and therefore the cultures were significantly mass transfer limited. As a result, the fluctuating  $\text{CO}_2$  cultures were affected by the actual carbon supply itself, not simply by the low levels of  $\text{CO}_2$  in equilibrium with air. Therefore the differences between the two cultures (Fig. 2) could be attributed to the relative lack of  $\text{CO}_2$  rather than the fluctuation in its supply *per se*. The cell counts presented difficulties in terms of completely differentiating Nanno Q from *Chlorella* but qualitatively *Chlorella* dominated all cultures. T-Iso was completely lost within one to two weeks in all experiments. Wall growth became a significant problem after about one week in the fluctuating  $\text{CO}_2$  reactors, with attachment of *Chlorella* to the reactor wall. The culture bottles were replaced weekly to minimize this problem.

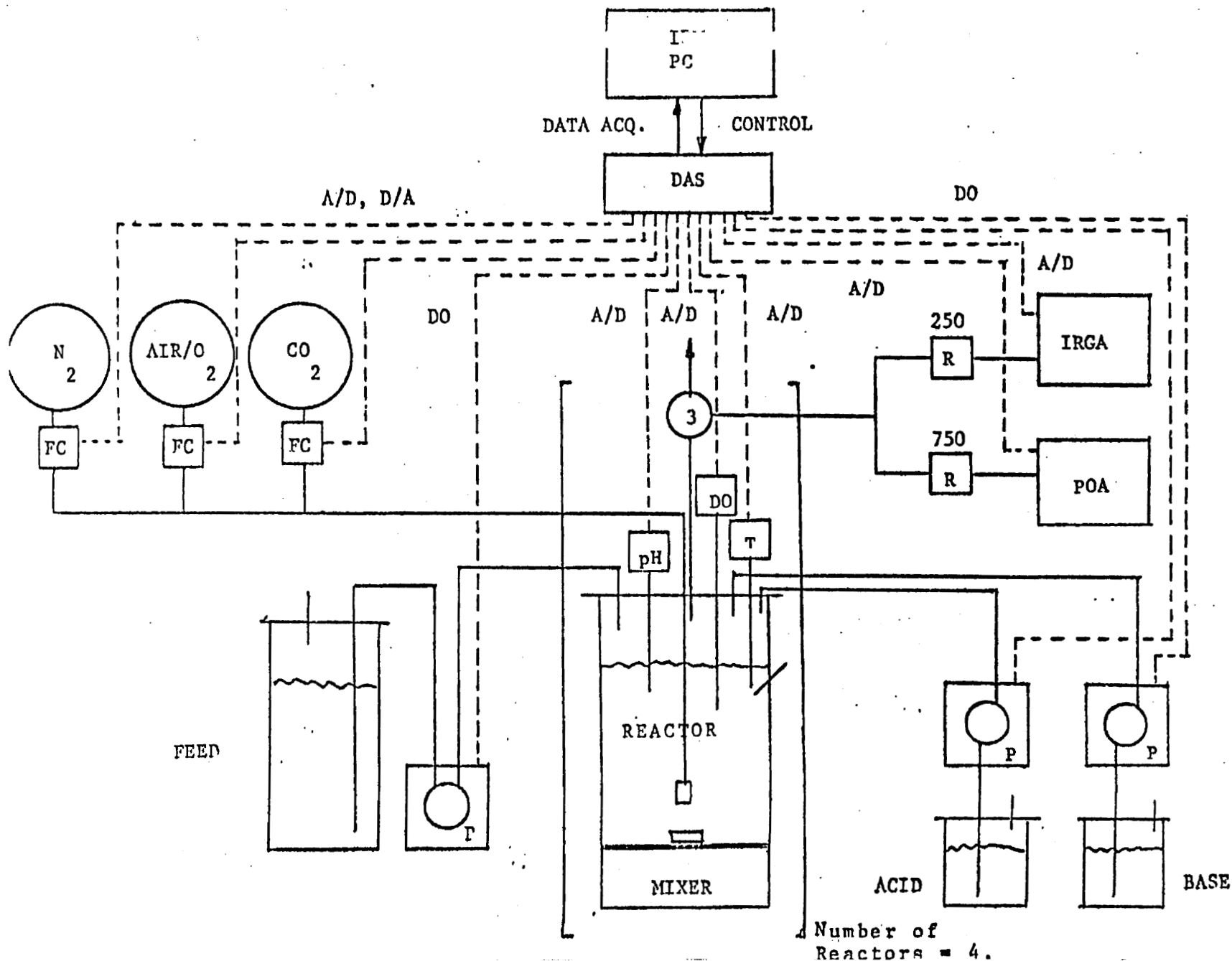
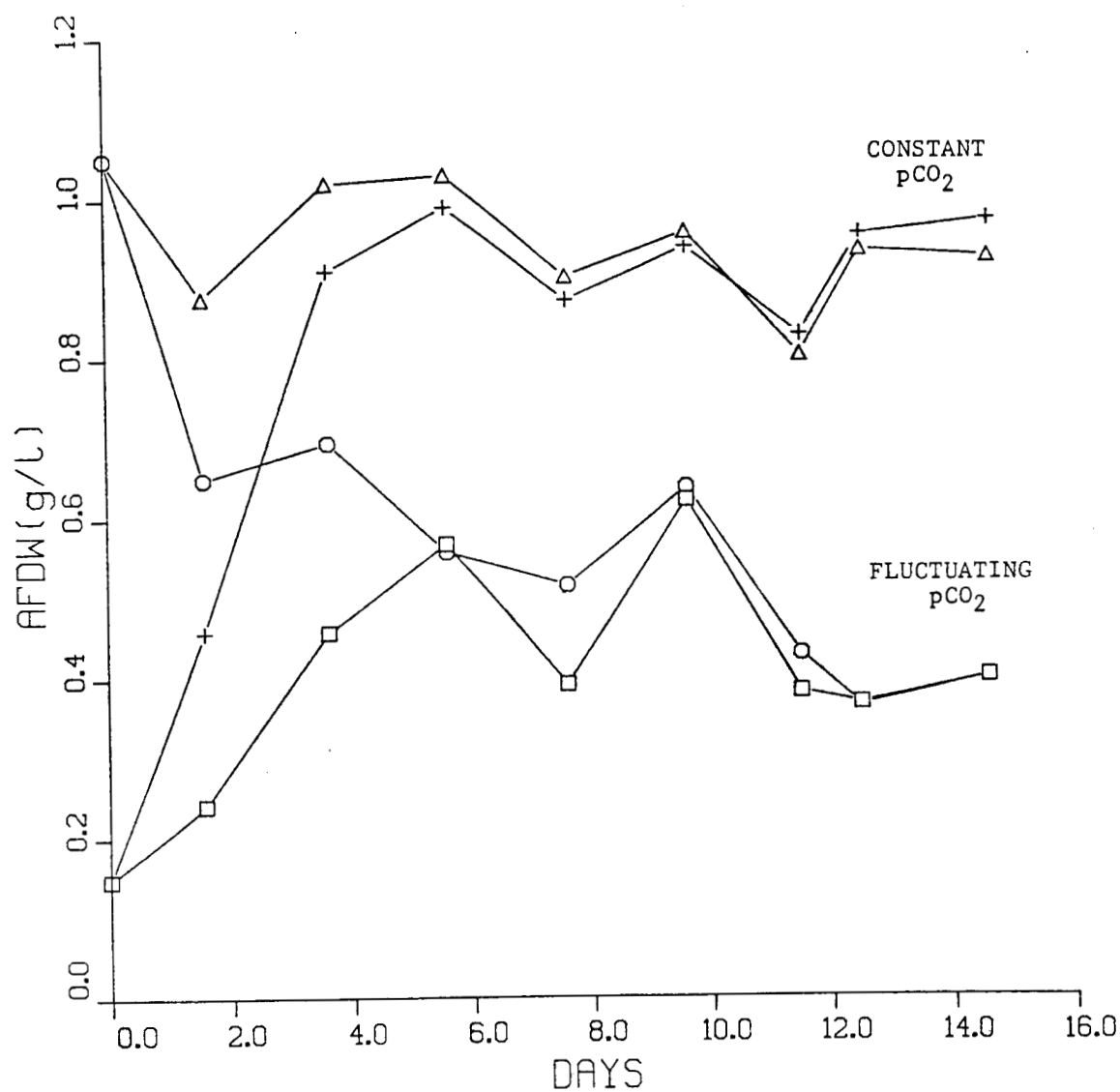


Figure 1. Schematic of Continuous Culture System. FC: Flow Controller; DAS: Data Acquisition System; IRGA: Infrared Gas Analyzer; R: Rotameter; P: Peristaltic Pump; A/D: Analog to Digital; D/A: Digital to Analog; DO: Digital Out; 3: Three Way Solenoid.



**Figure 2.** Mixed population cell densities for continuous cultures starting from high and low inoculum densities under either constant pCO<sub>2</sub> (0.5 %) or fluctuating pCO<sub>2</sub>(see text for details).

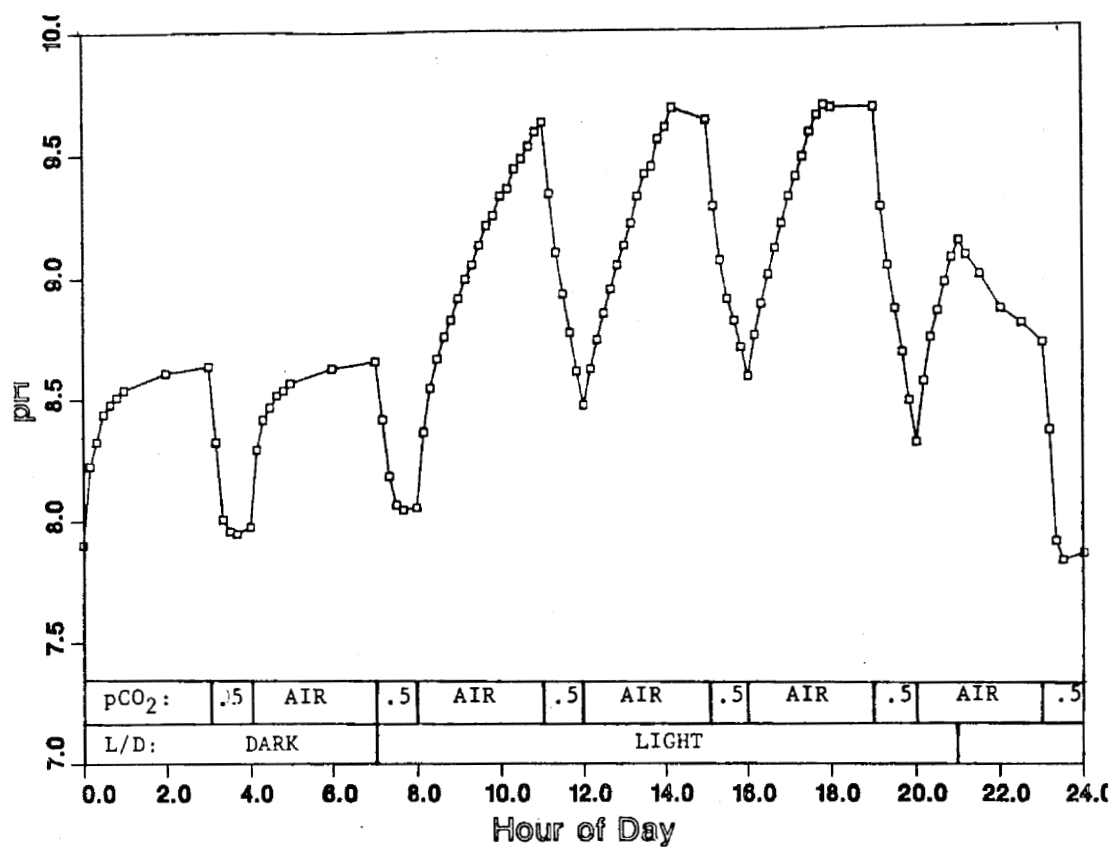


Figure 3. pH vs. diurnal cycle in culture experiencing a fluctuating pCO<sub>2</sub> supply.

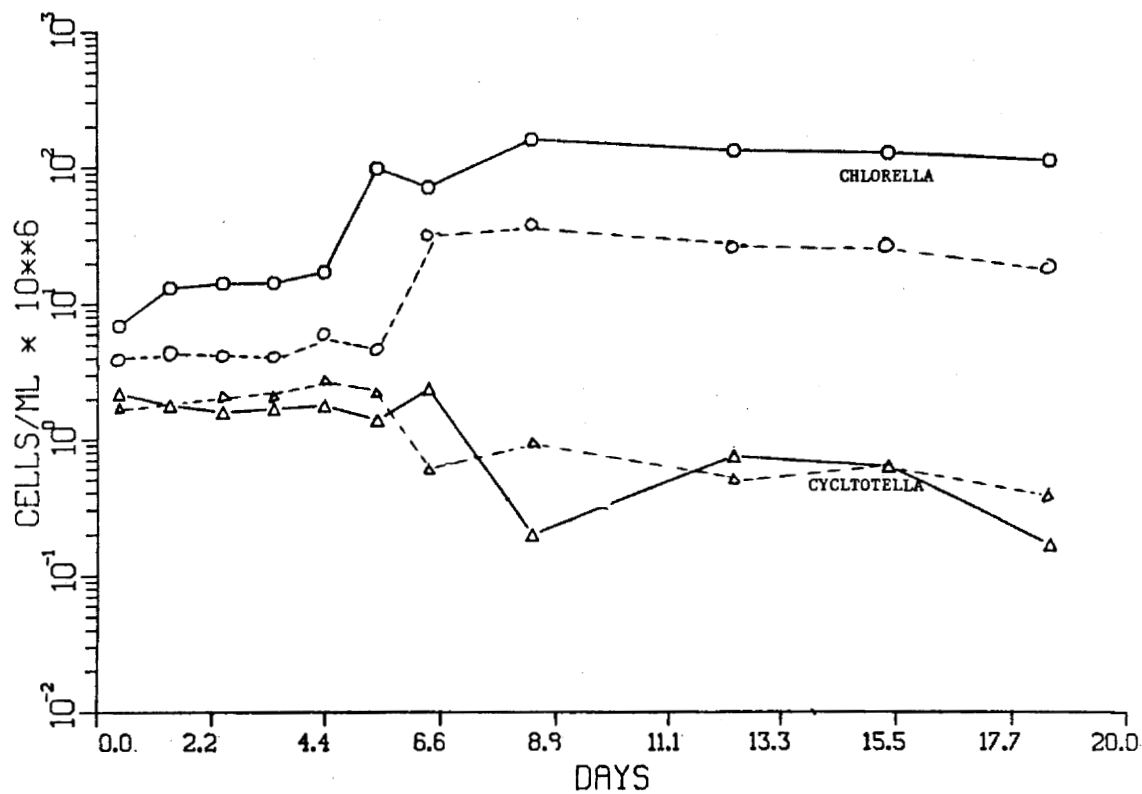


Figure 4. Cell concentration for continuous cultures at high (solid lines) and low (dashed lines) light intensities. *Chlorella* is shown by circles and *Cyclotella* by triangles.



Light Intensity Experiment. (Fig. 4 and Table 1). This experiment was designed to test the effect of light intensity on species dominance. It was carried out with a mixture of *Chlorella*, *Cyclotella*, and *Chaetoceros*, in MSW/2 media, at three light intensities: 150, 440, and 800  $\mu$ Einsteins  $m^{-2}/sec$ . Starting with 450 mg/L inoculum, culture cell densities exhibited an apparent steady state within three days, of 350 mg/L, in the low light culture and 1050 mg/L in the high light culture. The intermediate light culture, however, exhibited a slow upward trend until reaching almost 1 g/L on day 15.5. Cell counts showed a total loss of *Chaetoceros* between week one and two of the experiment. The *Chlorella* to *Cyclotella* ratio increased with light intensity (Fig. 4). The data for all three cultures showed a significant increase in *Chlorella* after about day five - correlating with a power and CO<sub>2</sub> supply failure (days 4.7-7.7). During these experiments, wall growth became significant, particularly for the high light culture, with *Chlorella* again attaching to the vessel walls preferentially. Even 2-3 times per day cleaning did not prevent wall growth. On some occasions wall growth represented up to about 30% of the total culture biomass.

Fluctuating Temperature Experiments. Next a mixture of *Chlorella*, *Cyclotella*, *Chaetoceros* and *Platymonas* was tested with low and high inoculations and under both fluctuating (30°C during light, 20°C during the dark) and constant (25-28°C) temperature conditions. Both power (on day six) and chilled water (on day 10.5) supply failures affected this experiment, however, no major differences were observed in any of the treatments. *Platymonas* and *Chaetoceros* disappeared from the culture within one and two weeks respectively. *Chlorella* became dominant and *Cyclotella* maintained itself as a stable species with about 10-20% of the biomass (Table 1). Wall growth followed the same pattern as before.

Conclusions and Future Work. The premise of these studies was that laboratory simulations of the pond environment can be used to screen algal species for their potential use in outdoor systems, in terms of their competitiveness, productivity, and responses to key environmental factors. The results of these preliminary species competition experiments have shown that: 1) The initial cell density of the algae is not critical in the outcome of the experiments; 2) Some algae are lost completely from the cultures, but usually a subdominant (<10% of biomass) species is maintained, suggesting a potential for rapid population changes should environmental situations change; 3) Most changes are seen within 3-4 detention times, however 10 detention times are required to establish long-term trends; 4) None of the environmental factors tested had a major effect on the relative competition of algal species tested, except for an increased ratio of *Chlorella* to *Cyclotella* with an increase in light intensity; 5) Wall growth is a significant problem in some cases, but it takes 3-4 days for walls to be significantly colonized and it can be prevented by periodically replacing the reactor, as soon as significant wall growth appears.

### Lipid Induction in Microalgae Batch Cultures

Introduction. A large body of literature exists regarding the fact that nitrogen deficiency (and silicate, for diatoms) results in the accumulation of lipids in many microalgae species. However, the actual kinetics of lipid induction are relatively unknown. In almost all cases reported, lipid productivity under nutrient-stressed conditions was rather low. This result led to the view, already expressed over thirty years ago, that nitrogen sufficient continuous cultures could produce more lipids than nitrogen starved, high lipid containing batch cultures. However, much of the prior experimentation in this area involved nitrogen limitation experiments carried out under conditions where light limitation and low growth rate could likely delay a shift to lipid metabolism upon nitrogen exhaustion. To avoid this problem the induction experiments reported below were carried out at relatively low cell densities when cultures, although already light limited, were still in a relatively rapid growth phase. We have investigated the kinetics of lipid induction of eight species of microalgae under such conditions, to determine whether any exhibited high lipid productivities upon transfer to nutrient (N or Si) limited conditions.

Experimental Methods. In these experiments, a batch culture, grown in a 1 L Roux bottle with a limited concentration of nitrate (0.6 to 2.7 mM) was harvested at various times after apparent (based on visible color changes) onset of nitrogen limitation. Unless otherwise noted all nitrogen deficient cultures contained 1.6 mM KNO<sub>3</sub>. All cultures were received from SERI or other SERI subcontractors.

TABLE 1

Operating Conditions	Data Point	Day	Dry Weight mg/L	Cell/mL x 10 <sup>-6</sup> (relative % biomass)			
				Chlorella	Cyclotella	Chaeto.	Platy.
Fluctuating Temperature Experiment:							
High Inoculum	Initial	0	1,000	21 (25)	3.4 (25)	1.0 (25)	1.0 (25)
High Inoculum Const. Tempt.	Final	16	1,000	40 (80)	0.83 (15)	0 (0)	0 (0)
High Inoculum Fluct. Tempt.	Final	16	1,200	89 (90)	1.9 (10)	0 (0)	0(0)
Low Inoculum	Initial	0	100	2.1 (25)	0.34 (25)	0.1 (25)	0.1 (25)
Low Inoculum Const. Tempt.	Final	16	1,000	39 (85)	0.47 (10)	0 (0)	0 (0)
Low Inoculum Fluct. Tempt.	Final	16	1,000	51 (65)	2.6 (35)	0 (0)	0 (0)
Light Intensity Experiment:							
Initial Inoculum	Initial	0	400	16 (25)	4.6 (25)	1.9 (25)	Not Used
Low Light	Final	18	300	18 (80)		0 (0)	Not Used
Medium Light	Final	18	700	52 (90+)	0.4 (5)	0 (0)	Not Used
High Light	Final	18	1,300	112 (95+)	0.17 (< 5)	0 (0)	Not Used

Notes: Experiments carried out in modified 1L Roux bottles, 1.5 day hydraulic detention times, 600  $\mu$ Ein./cm<sup>2</sup>/sec, pH 7.8, MSW/2 media. The inoculations were from batch cultures at different physiological states (growth rates) - this may have affected the competitiveness of Chaetoceros. Data from an experiment using fluctuating CO<sub>2</sub> supplies are not shown because of difficulties in differentially counting Chlorella and Nanno Q. The "T Iso" inoculum washed out after a few detention times. Relative % biomass data is only qualitative.

and grown in the suggested media (GPM, MSW, etc.). In the "initial protocol," (used for the first four algae reported below and others as noted) four replicate cultures were illuminated from one side at  $300 \mu\text{Einsteins/m}^2/\text{sec}$  (except as noted) and one whole culture harvested for each data point. In later experiments ("modified protocol"), three replicate N deficient cultures bottles (and one N sufficient) were used, less culture volume was harvested (100-200 ml) and more data points collected for each experiment. All biomass data reported refers to ash-free dry weight. Analytical procedures used were: 1) ash-free dry weight determinations (on centrifuged samples, resuspended in distilled water, heated  $104^\circ\text{C}$  1 hour weighed, ashed at  $550^\circ\text{C}$  for 30 minutes and weighed); 2) protein determinations (using the Lowry method after boiling for 1 hour in 1N NaOH at  $100^\circ\text{C}$ ); 3) carbohydrates (using the phenol-sulfuric acid method after boiling for 1 hour at  $100^\circ\text{C}$  in 1N HCl); 4) lipid extraction (modified Bligh-Dyer); 5) lipid fractionation (on Silicic acid columns; and 6) CHN analysis (using a Perkin-Elmer CHN analyzer).

One analytical problem was the incomplete lipid extraction of some lyophilized cell samples. Also, the total cell N (from the CHN analysis or calculated from protein content) did not account for all (only 80-85% in most cases) of the input N -  $\text{NO}_3$  in most N limited experiments. Most important, in many cases the initial sample, collected before visible signs of N starvation (pigment loss, decline in productivity), already exhibited the high carbohydrate (> 40%) and low protein content (< 20%) characteristic of nitrogen starved cultures. Thus, a comparison with a typical nitrogen sufficient culture was not possible for all cases, although productivity and lipid content for the first data point were not greatly different from those of nitrogen sufficient cultures. In general, these problems did not affect the conclusions reached from the experiments, presented next.

Dry weight curves for the four strains grown under the original protocol are shown in Figure 5 and for the four strains grown under the modified protocol in Figure 6. A summary of composition and CHN analysis is presented in Tables 2 and 3. Finally, lipid and biomass productivities, for all strains tested, are tabulated as a function of culture age in Table 4. In the following section the results for each organism will be discussed briefly.

*Chlorella sp.* (S/Chlor-3, Black Lake Isolate). The nitrogen deficient dry weight curve is shown in Figure 5. A final yield of roughly 700 mg/l and a maximum productivity of 260 mg/l/day were observed. The lipid content of this organism was relatively unaffected by nitrogen deficiency and at its maximum only reached 14 %. Carbohydrate however was high both constitutively and inducibly beginning at 59 % and reaching over 70 % after 4 days of growth. CHN analysis of the final harvest indicated a nitrogen content of 1.85 % which correlates well with the measured protein. At best the maximum lipid productivity that could be expected would be less than 40 mg/l/day.

T. Iso (*Isochrysis galbana*, Tahitian strain). Final culture yield reached over 1100 mg/l which corresponded to a nitrogen content (as determined by CHN) of 1.7 %. Maximum biomass productivity was 290 mg/l/day which gives a lipid productivity of close to 90 mg/l/day (@ 30 % lipid). It is evident from Table 2 that even the first point analyzed for its composition was already deficient -protein was only 12 %. All biochemical fractions displayed very little change following this. The CHN results indicate a highly oxygenated biomass and therefore a relatively low calculated heat of combustion. One feature of this organism is its propensity to excrete organic material into the culture. In the later stages of growth, culture supernatants became increasingly yellow and an orange-reddish gel appeared on harvested pellets. No attempt was made to analyze this material. These results did not suggest this organism as suitable for lipid production.

*Cyclotella sp.* (S/Cyclo-1). Under nitrogen deficiency dry weight increased until the fourth day of the experiment after which productivity declined to essentially zero. The initial point sampled (2.5 days) already had 31 % lipid and only 24% protein. The nitrogen content at this point was 3.6 % thus deficiency was already significant. Lipid content increased to over 40 % before biomass productivity declined and reached a maximum of 45 % after 10 days (780 mg/l, point not shown in Figure 5). The increase from 40 to 45 % more or less paralleled the reduction in biomass yield, thus total culture lipid was conserved while carbohydrate was lost through respiration. The maximum lipid productivity

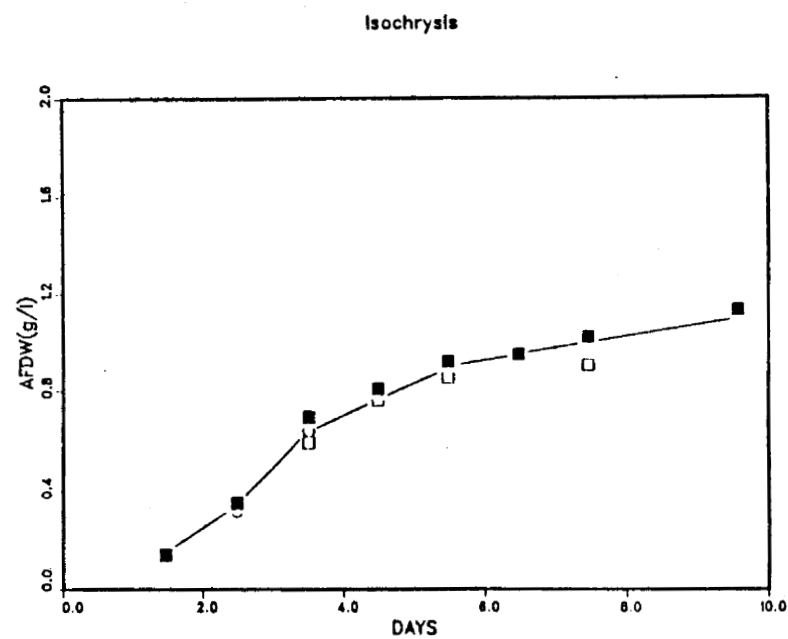
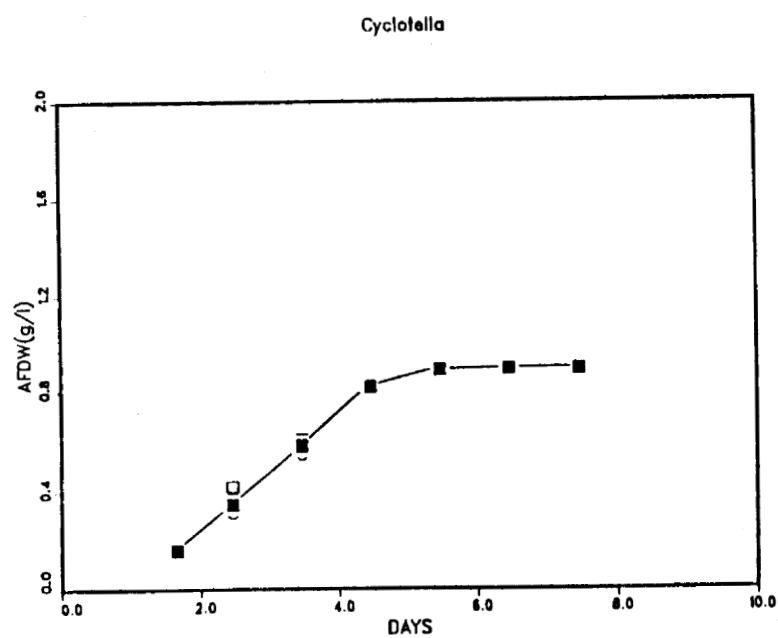
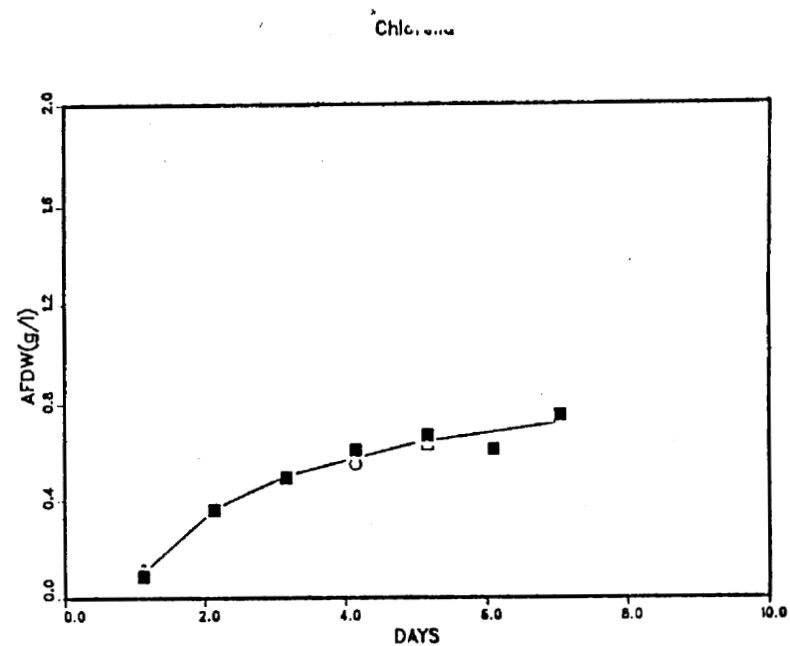
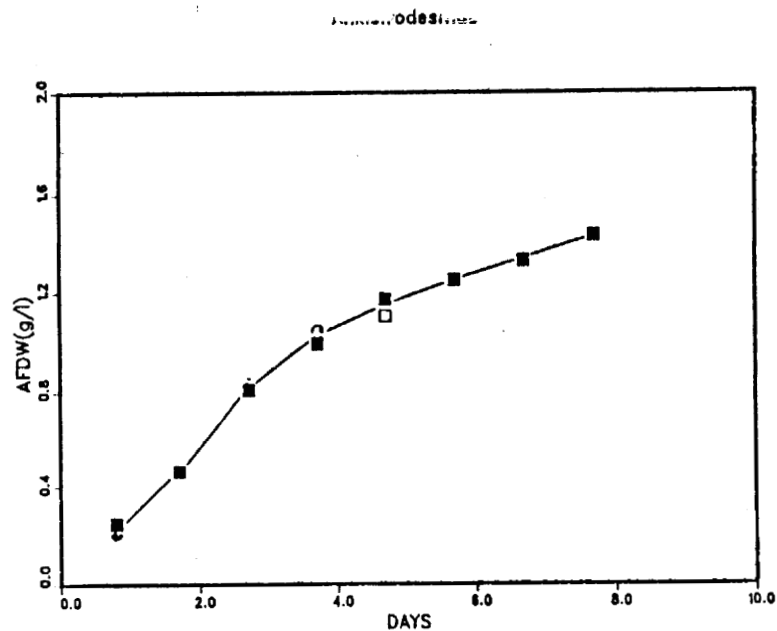


Figure 5. Nitrogen deficient dry weight curves for *Ankistrodesmus*, *Chlorella*, *Cyclotella*, and *Isochrysis*.

Table 2. A Summary of Composition Values for Ankistrodesmus, Chlorella, Cyclotella and Isochrysis during N deficiency.

<u>Ankistrodesmus</u>									
Day	AFDW	PRO	CHO	LIPID	C	H	N	O	Hc
1.7	464	23.9	54.6	24.9	53.5	7.84	4.31	34.4	5.9
3.7	1040	14.1	60.4	26.4	-	-	-	-	-
5.7	1270	10.5	52.8	24.0	-	-	-	-	-
7.7	1430	14.1	56.4	35.3	56.7	8.89	1.70	32.7	6.5
<u>Chlorella</u>									
2.1	370	16.5	59.3	9.50	-	-	-	-	-
4.1	550	12.9	73.3	11.2	-	-	-	-	-
6.1	620	11.2	64.3	10.2	-	-	-	-	-
8.0	750	11.3	61.8	14.2	54.2	8.30	1.85	35.6	6.0
<u>Cyclotella</u>									
2.5	410	23.7	49.9	30.7	49.8	7.90	3.63	38.7	5.4
4.5	820	13.7	54.4	40.7	-	-	-	-	-
7.5	890	13.3	47.8	41.8	-	-	-	-	-
10.4	780	13.7	36.8	45.5	57.5	8.90	2.11	31.4	6.6
<u>Isochrysis</u>									
3.5	640	12.3	46.6	30.0	-	-	-	-	-
5.5	900	8.60	49.4	23.1	-	-	-	-	-
7.5	900	8.70	47.7	31.4	-	-	-	-	-
9.6	1130	7.50	41.5	29.5	44.1	8.10	1.70	46.0	4.7

Table 3. CHN and Heat of Combustion Results.

<u>Strain</u>	<u>Status</u>	<u>%C</u>	<u>%H</u>	<u>%N</u>	<u>%O</u>	<u>H<sub>c</sub>(kcal/g)</u>
<u>Boekelovia</u> sp.	ND	54.20	8.08	4.00	33.72	6.0
	ND	51.52	7.26	5.75	35.47	5.5
<u>Chaetoceros</u> gr.	NS	55.1	8.70	8.80	27.3	6.5
	ND	48.0	8.10	2.80	41.2	5.2
<u>Nannochloropsis</u>	ND	54.3	8.69	5.30	31.6	6.3
	ND	61.9	9.98	2.92	25.2	7.5
	ND	63.8	10.4	2.29	23.4	7.9
	ND	64.0	10.4	2.13	23.4	8.0
<u>Thalassiosira</u>	NS	55.1	8.13	10.1	26.6	6.4
	ND	59.3	9.00	3.70	28.8	7.0

was 100 mg/l/day which occurred between days 3 and 5 corresponding to roughly 2-3 % cell nitrogen.

Results of the fractionation of the lipid extracts for days 4.5 and 10.5 are shown in Figure 7 on a total lipid basis. Clearly the most significant portion of the lipid material in both early and late deficient cultures was the benzene fraction-making up nearly 90 % of the severely nitrogen deficient culture lipid. Nitrogen deficiency caused a loss of the more polar soluble lipids while stimulating the production of non polar benzene soluble lipids.

In summary *Cyclotella* has potential as a lipid producer because it fulfills three basic requirements: 1) it has a high and inducible lipid fraction, 2) lipids are synthesized while biomass productivity is near maximal, and 3) the composition of the lipids are suitable as a liquid fuel precursor.

*Ankistrodesmus* (*A. falcatus*, S/Ankis-1). Initial experiments could not reproduce the high (40%) lipid contents reported previously. Since the earlier experiments were carried out with long-term (> 3 weeks) shaker table cultures, such shaker flask experiments were repeated. The highest lipid content observed was 31%, obtained from a 0.6 mM KNO<sub>3</sub> culture (only one data point was taken, at 3 weeks). It was suspected that C limitation might account for the high lipid contents reported earlier. Indeed, N sufficient cultures grown in 1 L Roux bottles and switched to NaOH scrubbed air, decreased in cell density while lipid content increased (by day 9) to almost 40%. By contrast the cultures grown in 1% CO<sub>2</sub> or 0.03% CO<sub>2</sub> (air) exhibited only a 26% lipid content. This experiment suggests that some literature reports of high lipid contents in algae could be due to prolonged exposure to extreme culture conditions - resulting in preferential carbohydrate consumption and, therefore, high lipid contents in the residual biomass.

With the same "initial protocol" used with the above three algal strains (four 1 L Roux bottle cultures, each with 1.6 mM NO<sub>3</sub>-N, one whole culture harvested sequentially at two to three day intervals), this algal species exhibited a high biomass productivity in the first 3-4 days (350 mg/l/day), followed by a sharp decline in lipid productivity, but a slower decline in biomass productivity. Lipid contents were fairly constant at about 25% (except the last data point, at 7.7 days, with a 35% lipid content - which is suspected to be in error). Thus, this species did not exhibit the desired characteristic of prolonged lipid productivity upon N starvation.

*Chaetoceros* (*C. gracilis*, S/Chaet-1). After some preliminary experiments, a nitrogen limited experiment was carried out following the modified protocol. The N sufficient control had a 45% (+/- 2%) protein content and a 14% (+/- 2%) lipid content. The results of the nitrogen limitation experiment showed that even by day 3, before any growth diminution was apparent, the culture exhibited a significant increase in carbohydrate (14->60 %) and a marginal increase in lipid (14->17%). Thereafter, lipids slowly increased, while carbohydrates declined after reaching a peak 6 days into the experiment. A maximum of 29 % lipid was measured after nine days of growth or roughly one week of deficiency. CHN analysis indicated a N content of 2.8 % at day 11 (630 mg/l, point not shown in Figure 6). This correlates well with measured protein values of about 17 %. Analysis of a N sufficient culture gave a N content of 8.8 %. Proteins, lipids and carbohydrates all added up to roughly 100% (+/- 3%) for the N deficient cultures but were significantly less than 100% for the sufficient control cultures.

In summary, in healthy cultures, *Chaetoceros* is very productive reaching 450 mg/l/day, the highest observed for all of the strains studied during this project. However lipid content in these nutrient sufficient cultures is low, averaging somewhere between 13-17%. This resulted in maximal lipid productivities of only 60 mg/l/day. Depriving the cultures of nitrogen resulted in a sharp decline in biomass productivity and a similar decline in lipid productivity. While nitrogen limitation slowly increased lipid content, it did not significantly increase lipid productivity.

A Si limitation experiment was also carried out, with a single culture centrifuged, washed and resuspended in deficient medium 4 days after inoculation. Within one day, the density increased from 310 to 680 mg/L, suggesting that complete Si limitation was likely not achieved. Lipid content increased from 23 to 30% in the interval, and carbohydrate content from 28% to 38%, with protein

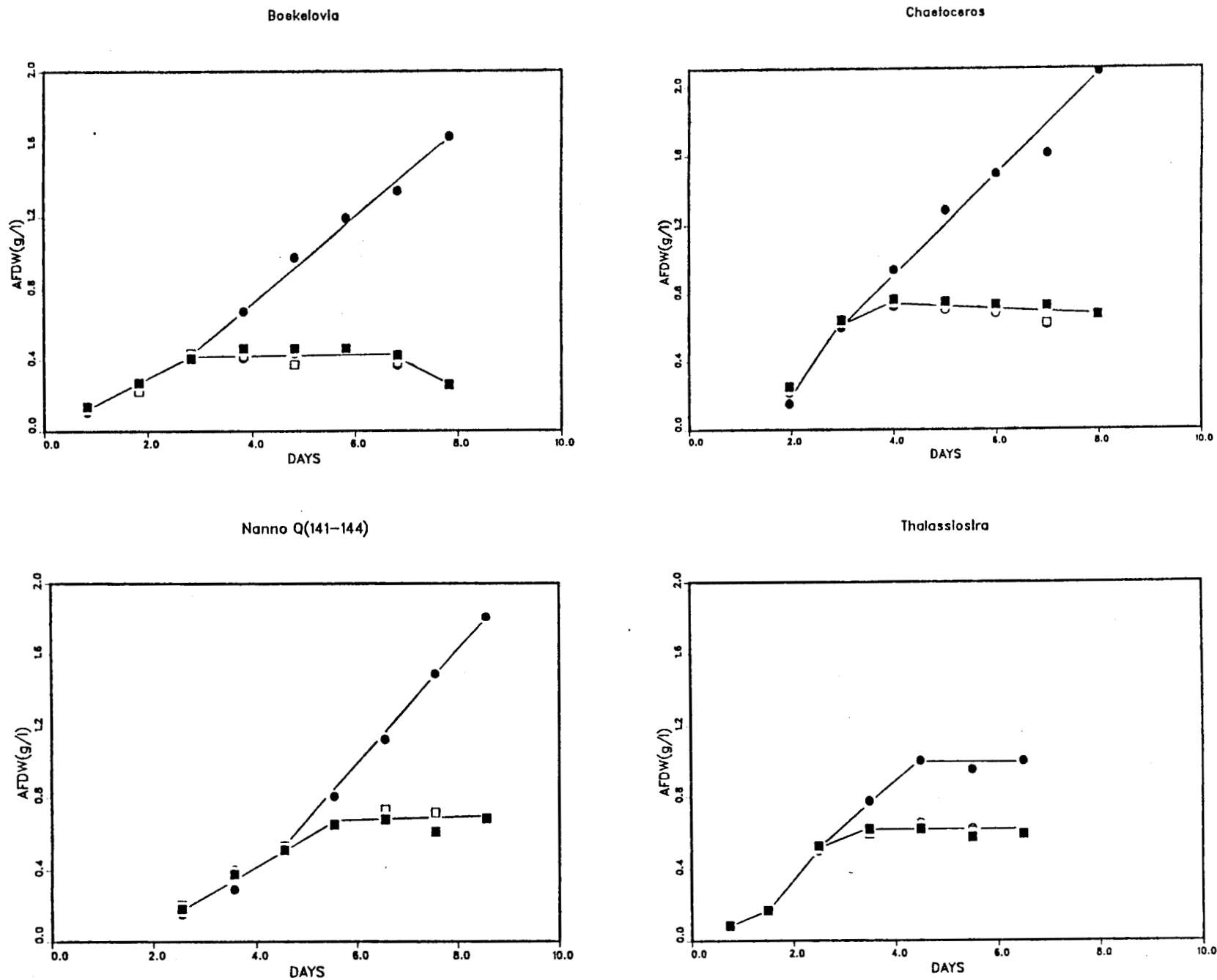
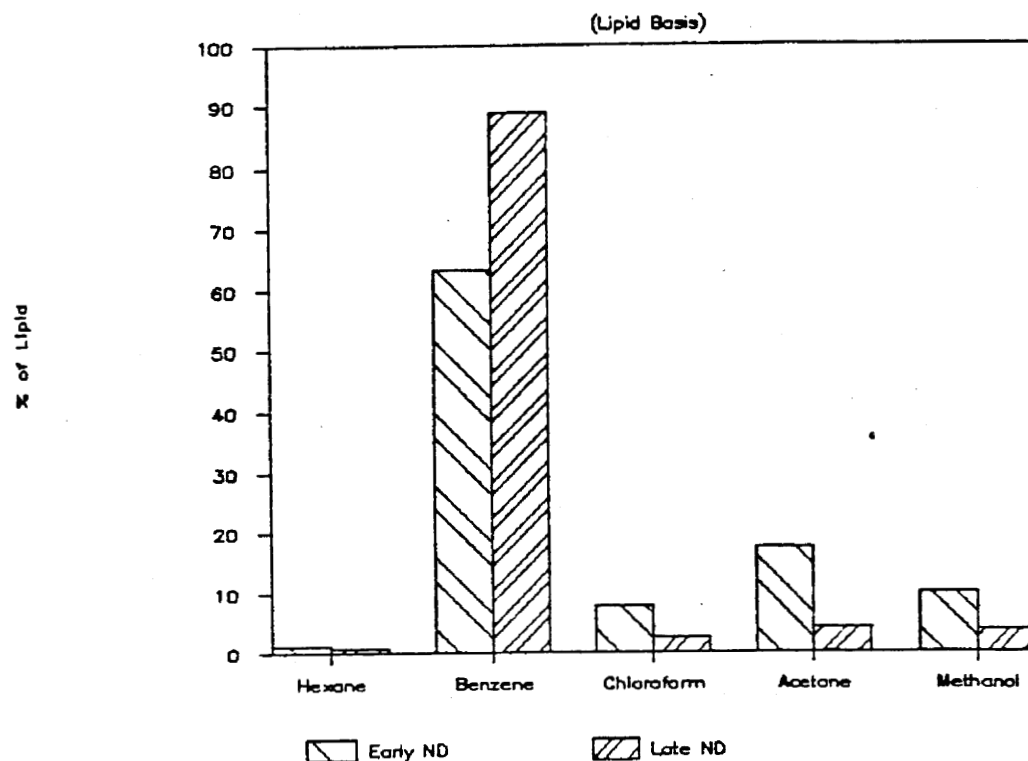
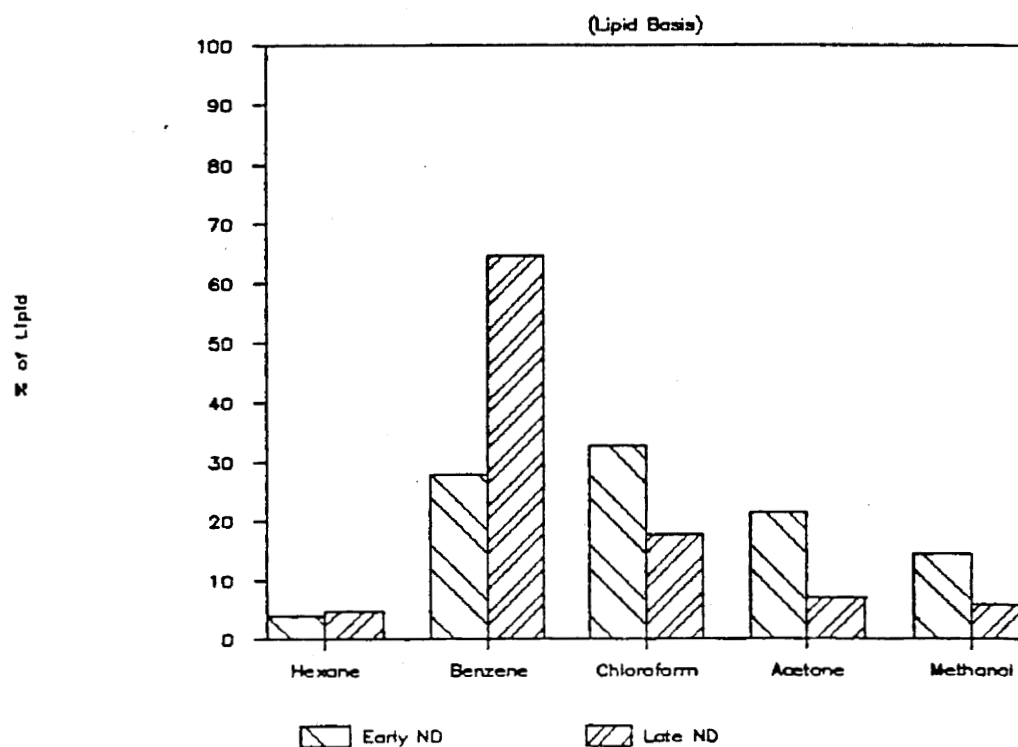


Figure 6. Nitrogen sufficient and deficient dry weight curves *Boekelovia*, *Chaetoceros*, *Nanno Q*, and *Thalassiosira*.



**Figure 7.** Silicic acid fractionation of early (3.6% N) and late (2.1% N) nitrogen deficient *Cyclotella* lipids.



**Figure 8.** Silicic acid fractionation of early (5.3% N) and late (2.1% N) nitrogen deficient Nanno Q lipids.



content dropping about 15% (to 39%). Lipid fractionation showed that all of the newly synthesized lipid was in the benzene (neutral, likely triglyceride) fraction.

Boekelovia sp. (G/Boeke-1). After a preliminary experiment (CHN results shown in Table 2), another was carried out under the modified protocol using 1.6 mM N, with the dry weight curve shown in Figure 6. This experiment actually lasted for 12 days, however after seven days a general decline of biomass yield in the deficient culture occurred. In fact the maximum biomass yield only reached 450 mg/l indicating a rather high maintenance requirement for nitrogen (4-5%). There was very little induction of lipids, with N sufficient cultures ranging between 24-28 % and deficient cultures attaining a little over 30 %. Significant carbohydrate induction did occur though. Another experiment was performed with this organism, with a similar decline in yield following 6 days (maximum reached 500 mg/l). At the maximum, the CHN content was determined and these results, along with one corresponding to 10 days (390 mg/l) are reported (Table 3). It is apparent that the loss in yield is correlated with an increase in N content of roughly the same proportion. Thus carbon is being lost from the system (via respiration) perhaps enhanced due to bacterial contamination. In fact a reddish-pink spot became noticeable on the harvest pellets in the later stages of the experiment.

Irrespective of the above mentioned problems associated with culturing this organism, it can be said that a sustained N sufficient productivity of 230 mg/l/day is possible coupled with a lipid content of 25 %. This translates into a lipid productivity of 65 mg/l/day. Even if it was possible to sustain growth during N deficiency, the small increase in lipid content would not affect the above estimate significantly. Carbohydrate productivity on the other hand would be greater than 125 mg/l/day.

Thalassiosira. (*T. pseudonana*). One N limitation experiment was performed using the modified protocol. The N sufficient culture became stationary after day 5 (at 1 g/L) for unknown reasons. The N deficient cultures stopped growing by day 4 at about 650 mg/L. Lipid content varied significantly among the N limited samples due to some apparent analytical difficulties and some interculture variability. However, the trend was for the N sufficient cultures to increase in lipids from 20-25% to 40% in the N deficient cultures. Noteworthy was the very high carbohydrate content, about 65% during the early stages of N limitation (days 2.5 to 4.5) declining thereafter by half as lipid contents increased. Lipid fractionation showed an increase in the chloroform fraction upon N limitation (data not shown).

A Si deficiency experiment was also carried out using the same procedure as with *Chaetoceros*. Again biomass increased substantially, doubling in less than two days after transfer to a Si deficient medium, while lipid content increased from 18 to 32%. In this case, both the benzene and acetone fractions showed a pronounced increase. This data is difficult to interpret because of the possibility that Si deficiency was not achieved.

Nanno Q. (*Nannochloropsis sp.*, S/Nanno-2). Some difficulties were encountered in the growth of this alga, with rather lengthy lag phases (of about 2 days) after inoculation being the norm. In the first experiment (initial protocol) two sets of four cultures were grown on air and 1% CO<sub>2</sub> with 2.7 NO<sub>3</sub>-N (1 mM higher than before) and 2 side illumination of 200 Einstein/m<sup>2</sup> sec per side. Growth continued for the duration of the experiments, exceeding 1 g/L (data not shown). Lipid contents increased from 24% in the earlier air grown cultures harvested on day 4 (with a 36.5% protein level) to 50% by day 8 for both air and CO<sub>2</sub> grown cultures. The 1% CO<sub>2</sub> culture had a higher initial lipid content and productivity, but started lagging by day 4, while the air grown culture sustained its lower productivity, both lipid and biomass somewhat longer. Lipid fractionation (Figure 8) showed that all of the increase was due to benzene fraction lipids.

A final experiment was carried out using the modified protocol -this is shown in Figure 6. In this case N levels were reduced to 1.6 mM N. Nitrogen sufficient, or light limited productivity was about 320 mg/l/day, nitrogen deficient productivity declined dramatically after 5 days and was near zero after six. The first composition point (taken on day 3.5) was already deficient as evidenced by its low protein content (50 % of the N sufficient control). During the course of the experiment the protein level

remained relatively constant, therefore further reduction in cell nitrogen was at the expense of non-protein forms of nitrogen. Carbohydrate levels were relatively low for both sufficient and deficient cultures which is consistent with the view that this organism has a highly inducible lipid pathway. Maximum lipid content reached 50 % representing an almost 100 % increase in lipid over the sufficient control. Maximum lipid productivity in the deficient culture was 110 mg/l/day occurring between days 3.5 and 4.5 (or in the early stages of N limitation). This compares favorably with a lipid productivity of 90 mg/l/day (@ 28 % lipid) for the sufficient control. This performance is desirable in a lipid producing algae: a spurt a lipid biosynthesis following N limitation, while biomass productivity continues, at least transiently.

Comparison of these experiments with the earlier one involving a higher level of N (2.7 mN) shows that biomass and lipid biosynthesis can continue for much longer periods. The relationship between nitrogen, cell density, light, and lipid productivity cannot be determined from the experiments presented, but it is clear that maximum biomass and lipid productivities can be, at least partially, decoupled from each other and that relative lipid productivity can increase during nitrogen starvation.

Conclusions and Future Work. The results of the lipid induction experiments can be summarized as follows: 1) Most of the algae tested (six out of eight species) exhibited no rapid increase in lipids upon N limitation, while productivity declined sharply. Thus, even in the species exhibiting a high ( $\geq 35\%$ ) final lipid content (*Ankistrodesmus*, *Boekelovia*, *Thalassiosira*) the lipid productivity was depressed. In only two cases, *Cyclotella* and *Nanno Q* was there an increase in both culture biomass concentration and lipid content in the biomass during N limitation. Thus these species require further investigation. Whether lipid productivity can, overall, be increased through N limitation, above what is possible in a nitrogen sufficient continuous culture, must still be determined. Toward this objective the subsequent research plan calls for studying *Nanno Q* in batch cultures with several levels of nitrate, such that limitation is induced at various densities, with shifts in light supply and addition of nitrate after onset of N deficiency.

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Table 4. A Summary of Maximum Observed Biomass and Lipid Productivities and Lipid Content.

Organism	N Status	Maximum Productivity (mg/l/day)		Maximum Lipid Content (% AFDW)
		Biomass	Lipid	
<u>A. falcatus</u>	ND	260	90	35
<u>Boekelovia</u> sp.	NS	300	80	28
	ND	170	40	36
<u>Chaetoceros</u> gr.	NS	450	60	15
	ND	400	80	28
<u>Chlorella</u> sp.	ND	260	30	14
<u>Cyclotella</u> sp.	ND	260	100	45
<u>Isochrysis</u> gal.	ND	240	70	30
<u>Nannochloropsis</u> sp.	NS	340	100	28
	ND	170	110	50
<u>Thalassiosira</u> sp.	NS	300	70	25
	ND	230	60	39

## Temperature Effects on Microalgal Photosynthetic Efficiency

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### ABSTRACT

Algal cultures grown in an outdoor production facility will experience daily and seasonal fluctuations in temperature, and thus these cultures will exist under less than ideal temperature conditions. As a result, it is important to determine the effect of changes in temperature on biomass productivity, since this information will be valuable in predicting the potential yield of algae grown in large-scale outdoor culture. The objectives of this project were to: (1) determine the maximum growth rate of selected algae under conditions in which neither nutrients, carbon dioxide, nor light were limiting, and (2) determine the effect of changes in temperature on biomass productivity and photosynthetic light efficiency.

The maximum growth rates of Ankistrodesmus, Chaetoceros, Chlorella, Cyclotella, Navicula, Scenedesmus, and Skeletonema were determined to be 2.6, 4.6, 4.9, 3.9, 2.0, 3.3, and 2.0 doublings per day, respectively. The relationship of biomass productivity to cell concentration for a Scenedesmus continuous culture maintained at three different temperatures (18, 25, 32 °C) and high light intensity (295-361  $\mu\text{E}/\text{m}^2/\text{sec}$ ) was determined. Biomass productivity was maximal at 25 °C, and the relationship of productivity to cell concentration was temperature dependent (i.e. the cell concentration required to obtain maximum productivity changed significantly as a function of temperature).

## INTRODUCTION

An objective of the Aquatic Species Program is the development of large scale culturing systems for the production of fuels from lipid-rich microalgae. A major constraint to any such culturing system is the provision of sufficient light in the most economical manner possible. This constraint on the culturing system has led to the use of shallow, outdoor ponds that are illuminated using natural sunlight. Since an algal production facility based on open ponds will be located outdoors, the algal cultures will be subject to daily and seasonal temperature and light fluctuations, and thus the ponds must operate under less than ideal temperatures and light conditions. As a result, it is important to determine the response of algal productivity to the changes of temperature and light intensity that will be experienced in such a facility. This information is important since the prevailing temperatures and light intensities of the geographical area (presumably the southwest United States) will determine: (1) which algal species are best suited for use in a production facility located in this particular area, (2) the ability of these algal species to successfully compete with other species that might invade the pond, and (3) the productivity that might be expected from a given species.

The objectives of our project are to: (1) determine the maximum growth rates of several species of warm-adapted and cold-adapted algae under conditions in which neither nutrients, carbon dioxide, nor light are limiting, (2) determine the yield and photosynthetic efficiency of various species of algae at different temperatures and light intensities, and (3) determine the relationship between diurnal temperature changes and the potential yield from algal cultures. The results of these studies will provide the Aquatic Species Program with important information on the productivity and light efficiency of the tested species, and the effect of temperature and light intensity on these parameters. This information will be valuable in predicting the potential yield of algae grown in a large scale production facility.

## EXPERIMENTAL APPROACH

The objective of the initial experiments is to determine the maximum growth rate of selected warm-adapted and cold-adapted algae. These experiments will be conducted under conditions in which neither nutrients, carbon dioxide, nor light are limiting, and at the recommended optimum temperatures. If necessary, the growth medium will be modified to achieve healthy stock cultures and rapid culture growth.

Subsequent experiments will be directed towards determining the effect of temperature on biomass productivity and photosynthetic light efficiency. The experimental approach is to establish a continuous culture under conditions of CO<sub>2</sub> saturation, at a particular temperature, and at a high light intensity. The cell concentration of the culture is systematically varied to determine the cell concentration of maximum productivity, for these experimental conditions. At this cell concentration, the temperature of the culture is varied, and the response of biomass productivity and efficiency of light utilization to different temperatures is determined. However, the cell concentration of maximum biomass productivity may shift as the temperature changes; thus the actual response of productivity and light efficiency to changes in temperature might be obscured by shifts in the cell concentration of maximum productivity. This would necessitate determining the cell concentration of maximum productivity at each temperature in order to obtain reliable data on the temperature response of productivity and light efficiency. Therefore, the initial experiment is to determine if the cell concentration of maximum biomass productivity is significantly altered by changes in the temperature.

If the cell concentration of maximum productivity doesn't vary significantly with temperature, then for each of two warm-adapted algae and two cold-adapted algae we will: (1) determine the cell concentration of maximum productivity at a particular temperature by measuring the productivity as a function of cell concentration, (2) determine the effect of different temperatures on productivity and efficiency of light utilization at the cell concentration of maximum productivity, and (3) determine the response of productivity and efficiency of light utilization

as a function of light intensity at the cell concentration of maximum productivity and the optimum temperature.

If the cell concentration of maximum productivity does vary significantly with temperature, then for one warm-adapted and one cold-adapted alga we will: (1) determine the cell concentration of maximum productivity for each of several different temperatures by measuring the productivity as a function of cell concentration, and (2) determine the response of productivity and efficiency of light utilization as a function of light intensity at the cell concentration of maximum productivity and the optimum temperature.

Based on the results of the above experiments, we will select one warm-adapted and one cold-adapted alga to determine the effect of diurnal temperature and light cycles on biomass productivity and efficiency of light utilization.

To date, we have determined the maximum growth rate of five warm-adapted algae, namely Ankistrodesmus, Cyclotella, Chaetoceros, Chlorella, and Scenedesmus (we are awaiting receipt of the cold-adapted species from SERI). We have recently begun to determine the effects of temperature and light intensity on algal productivity and efficiency of light utilization.

## MATERIALS AND METHODS

### Organisms and Growth Conditions

Five species of warm-adapted green algae and diatoms were used in the present work: Ankistrodesmus (S/ANKIS-3), Cyclotella (S/CYCLO-1), Chaetoceros (SS-14), Chlorella (SC), and Scenedesmus obliquus (Gaffron strain D3). The Ankistrodesmus, Cyclotella, Chaetoceros, and Chlorella were supplied by SERI; the Scenedesmus has been used by us in-house for years.

Ankistrodesmus was cultured in modified Type II, 25 mmho/cm medium (Barclay, et al., 1985), containing the following components: 3.0

g/l  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 5.8 g/l  $\text{Na}_2\text{SO}_4$ , 0.04 g/l  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.975 g/l KCl, 8.08 g/l NaCl, 2.0 g/l  $\text{KNO}_3$ , 2.3 g/l  $\text{NaHCO}_3$ , 0.9 g/l  $\text{Na}_2\text{CO}_3$ , 0.5 g/l  $\text{NaH}_2\text{PO}_4$  and 1 ml/l of modified Chapman metals solution (0.09g  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.60 g  $\text{H}_3\text{BO}_3$ , 0.15 g  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.06 g  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 10.00 g  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 0.50 g  $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ , 5.00 g  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , and 4.00 g  $\text{Na}_2\text{EDTA}$  dissolved in one liter of distilled water).  $\text{NaHCO}_3$ ,  $\text{Na}_2\text{CO}_3$  and  $\text{NaH}_2\text{PO}_4$  were filter sterilized and added to the autoclaved medium.

Cyclotella was cultured in a medium that is based on a recipe supplied by Dr. Mahasin Tadros (Alabama A & M). This medium contains the following components: 0.25 g/l  $\text{NaNO}_3$ , 0.1 g/l  $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$ , 20.0 g/l artificial sea salts (Rila Marine Products), 0.005 g/l  $\text{NaH}_2\text{PO}_4$ , 0.02 mg/l thiamine, 0.1 mg/l vitamin B12, 0.1 mg/l biotin, and 1.0 ml/l trace metal solution (0.55 g  $\text{H}_3\text{BO}_3$ , 0.06 g  $\text{ZnCl}_2$ , 0.3 g  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.3 g  $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ , 0.04 g  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 2.5 g  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 4.0 g  $\text{Na}_2\text{EDTA}$ , 0.04 g  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  per liter of distilled water). The vitamins and  $\text{NaH}_2\text{PO}_4$  were filter sterilized and added to the autoclaved medium.

Chaetoceros was cultured in a growth medium based on the Type II, 25 mmho/cm medium, and contains the following components: 3.0 g/l  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 5.8 g/l  $\text{Na}_2\text{SO}_4$ , 0.04 g/l  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.975 g/l KCl, 8.08 g/l NaCl, and 1 ml/l modified Chapman metals solution (see Ankistrodesmus medium above). After autoclaving this medium, the following components were filter sterilized and added aseptically: 2.3 g/l  $\text{NaHCO}_3$ , 0.9 g/l  $\text{Na}_2\text{CO}_3$ , 0.25 g/l  $\text{NaH}_2\text{PO}_4$ , 0.1 g/l  $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$ , 0.2 mg/l thiamine, 0.1 mg/l vitamin B12, 0.1 mg/l biotin and 0.6 g/l urea.

Chlorella was cultured in modified Type II, 10 mmho/cm medium (Barclay et al., 1985) containing the following components: 2.0 g/l  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 2.6 g/l  $\text{Na}_2\text{SO}_4$ , 0.04 g/l  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.45 g/l KCl, 1.5 g/l NaCl, and 1 ml/l modified Chapman metals solution (see Ankistrodesmus above). After autoclaving these medium components, the following chemicals were filter sterilized and added aseptically to a final concentration of: 1.2 g/l  $\text{NaHCO}_3$ , 0.2 g/l  $\text{Na}_2\text{CO}_3$ , 0.25 g/l  $\text{NaH}_2\text{PO}_4$ , and



0.6 g/l urea.

Scenedesmus was cultured in medium containing 1.0 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.02 g/l  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.38 g/l  $\text{K}_2\text{HPO}_4$ , 0.15 g/l  $\text{KH}_2\text{PO}_4$ , 6.0 g/l  $\text{KNO}_3$  and 2 ml/l micronutrient solution (5.0 g  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 4.0 g  $\text{Na}_2\text{EDTA}$ , 2.86 g  $\text{H}_3\text{BO}_3$ , 1.81 g  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 0.22 g  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.39 g  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 0.08 g  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.05 g  $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$  dissolved in one liter of distilled water).

Stock cultures of the warm-adapted organisms were maintained in 1000 ml Roux bottles (containing 500 ml of culture) kept in an environmental chamber. The cultures were continuously bubbled with a gas mixture of 2%  $\text{CO}_2$  in air. Prior to introduction to the cultures, the gas mixture was humidified by bubbling it through a water reservoir maintained at the same temperature as the cultures. Warm-adapted stock cultures were maintained at  $29 \pm 2^\circ\text{C}$ , and the photon flux varied between 40-90  $\mu\text{E}/\text{m}^2/\text{sec}$ . Light intensity was supplied by high output, cool white fluorescent bulbs. All cultures were handled and sampled using aseptic techniques.

### Growth Rate Determinations

Growth rate experiments with each alga were performed using Roux bottles that were maintained in an environmental chamber with continuous illumination and bubbling with 2%  $\text{CO}_2$  in air. Each experimental Roux bottle was placed in a black cardboard box that had one side removed. The box was placed directly against the light source (two high output, cool white fluorescent bulbs) with the open side of the box facing the light. Photon flux was measured on the back of the bottle using a LiCor 190SB Quantum Sensor. This arrangement eliminated stray light from the Roux bottle, thereby permitting a very accurate measurement of the photon flux incident on the culture. The temperature of the experimental bottle was determined by placing a thermometer in a Roux bottle that was maintained under identical conditions, with the exception that it did not contain algae. At time zero, the experimental bottle was inoculated at a very dilute cell density using cells from an exponentially growing stock culture that had

been maintained at a temperature and light level similar to the experimental conditions. Periodically (every 8-12 hours) a small sample (~ 2 ml) of the culture was removed, and the cell concentration (cells/ml) determined using a Coulter Counter Model TAll with PCA II population accessory. The temperature and photon flux for each bottle were recorded immediately after each sampling. When the culture reached a density at which the transmitted photon flux was significantly lower than the initial measurements (i.e. when the culture was dense enough to provide a faint trace of color to the bottle), subsequent measurements of the cell concentration were not used in determining the growth rate. The temperatures and photon fluxes used for the growth rate determinations of each alga are noted in the results section.

### Description of the Continuous Culture Apparatus

The continuous culture apparatus used in this work is similar to the device constructed by Myers and Clark (Myers and Clark, 1944). The apparatus is made of glass and consists of three concentric, cylindrical chambers (Figure 1). The outermost chamber is a temperature regulated water jacket, the middle chamber contains the algal culture, and the inner chamber can house a fluorescent light source. The middle (culture) chamber has a volume of 1200 ml and a path length of 1.25 cm. The vessel is continuously illuminated by eight high output, cool white fluorescent bulbs (four banks of two bulbs each) surrounding the apparatus. A single high output, cool white fluorescent bulb, located in the center light chamber, is completely masked with black tape (so as not to significantly alter the measured photon flux on the algal chamber) except for a small area that is directed at the density detector. Cell density is maintained by monitoring the light transmission from this bulb through the culture using a photocell (Clairex CL604L) whose output is fed directly to a microcomputer (Sym-1, Synertek Corp.). When the output of the photocell exceeds a preset reference value (i.e. when the culture grows and absorbs more light) the microcomputer activates a peristaltic pump for a preset period of time and fresh medium is added to the culture. A corresponding volume of algal culture leaves the vessel via the overflow tube. After a latent time of 60 sec. to allow for mixing of any newly added medium, the

monitoring cycle is reinitiated. A gas mixture of 2% CO<sub>2</sub> in air is admitted through the fritted bottom of the culture chamber at a total gas flow rate of 150 ml/min. This flow rate is sufficient to provide relatively rapid mixing of the culture and to prevent the cells from settling.

### Analytical Methods

The chlorophyll concentration of Scenedesmus was determined by adding an aliquot of culture to a 1:1 mixture of Triton X-100 and 5% KOH in methanol, heating at 63 °C for three minutes, and centrifuging. Optical density was determined at 645 nm. The temperature of the continuous cultures was monitored daily by use of a thermocouple immersed in the culture. Dry weight determinations involved filtering a 10 ml aliquot of culture through a glass fiber filter (approximate retention 2.6 µm), rinsing thoroughly with distilled water, drying at 105 °C overnight, and cooling to room temperature in a dessicator. (The filter paper was previously dried at 105 °C before beginning the procedure). The doubling time of a continuous culture was taken as the ratio of the overflow rate (ml/hr) and the total volume of the culture vessel (1200 ml); the biomass productivity (mg dry weight/hr) was taken as the product of the cell concentration (mg dry weight/ml) and the overflow rate.

### Efficiency of Light Utilization

The efficiency of light utilization by a continuous culture is defined as the ratio of the calories of biomass produced per unit time to the calories of light absorbed per unit time. The calories of biomass produced per hour can be calculated from the culture productivity (i.e. for Scenedesmus 1 g = 5500 calories). The amount of light absorbed by a culture was calculated using the Beer-Lambert Law ( $I_{\lambda} = I_{0\lambda} e^{-E_{\lambda} c l}$ , where  $I_{\lambda}$  is the amount of transmitted light at a particular wavelength,  $I_{0\lambda}$  is the amount of incident light at that wavelength,  $E_{\lambda}$  is the extinction coefficient of the culture at that wavelength,  $c$  is the cell concentration, and  $l$  is the light path length of the culture). The difference between  $I_{0\lambda}$

and  $I_x$  represents the amount of light absorbed by a culture at a particular cell concentration. To determine the efficiency of light utilization by a continuous culture at a particular cell concentration it was necessary to determine: (1) the incident light intensity on the algal culture chamber, and (2) the extinction coefficient as a function of wavelength over the range of 400-700 nm.

The light intensity supplied to the continuous culture apparatus ( $I_{0\lambda}$ ) was determined by removing the center glass portion of the culture vessel and measuring the light that passed through the water jacket and illuminated the outer wall of the culture chamber. Light measurements (LiCor 190SB Quantum Sensor) were made at eight locations around the perimeter of the algal chamber, and at several different heights within the culture vessel. These measurements were averaged to obtain a value for the incident photon flux ( $\mu\text{E}/\text{m}^2/\text{sec}$ ). This value plus knowledge of the spectrum of the light bulb permit the conversion of photon flux into light intensity ( $\text{calories}/\text{m}^2/\text{hr}$ ).

The relationship of extinction coefficient to wavelength for a culture of a particular cell concentration was independently calculated by measuring  $I_{0\lambda}$  and  $I_x$  using a large glass cuvette. The cuvette was illuminated by a high output, cool white fluorescent bulb, and the light intensity crossing the cuvette in the presence and absence of cells was measured. Light intensities were measured over the range of 400-700 nm using an ISCO spectroradiometer.

## RESULTS

### Growth Rate Experiments

The objective of these experiments was to determine the maximum growth rate of selected warm-adapted algae, namely Ankistrodesmus, Cyclotella, Chaetoceros, and Chlorella. These experiments required measurement of the growth rate under conditions in which neither nutrients, carbon dioxide, nor light are limiting. We found that the medium in which we received the original algal inoculum was, in some cases, not

well-suited for growth of the particular organism, or that the basic medium required supplements that were not well documented. Therefore, it was first necessary to define media that would support healthy stock cultures. The media listed in the Materials and Methods section are those that will support good growth of the particular organisms, although these media are not necessarily optimized. All cultures (stock and experimental) were bubbled with a gas mixture of 2% CO<sub>2</sub> in air, to ensure that the cultures were not carbon dioxide limited. In addition to nutrients and carbon dioxide, it was also necessary that light not limit the growth rates of these organisms.

Figure 2 shows the results of an experiment in which we determined the doublings per day of a culture of Scenedesmus as a function of photon flux. Note that the doublings per day increase in direct response to an increase in photon flux up to approximately 90 uE/m<sup>2</sup>/sec. At higher photon fluxes there was no longer a linear relationship between doublings per day and photon flux; at photon fluxes greater than about 180 uE/m<sup>2</sup>/sec Scenedesmus was not significantly light limited. The maximum growth rate of Scenedesmus was approximately 3.3 doublings per day. To verify that the Ankistrodesmus, Cyclotella, Chaetoceros, and Chlorella experiments were not significantly light limited, the growth rate of each organism was determined at two different photon fluxes. Similar growth rates at each photon flux indicated that the experiments were not light limited.

#### Ankistrodesmus (S/ANKIS-3)

Ankistrodesmus cultures were maintained at 30 +/- 1 °C and a pH of 7.9 to 8.2; the initial photon flux varied between 166 and 190 uE/m<sup>2</sup>/sec. This organism tends to stick to glass surfaces and to form small clumps, resulting in difficulty in maintaining a uniform culture. These problems have been reduced by including a small stir bar in the bottom of the Roux bottles. Figure 3 is representative growth curves for this alga. The upper line represents the average of duplicate experiments, and corresponds to 2.67 doublings per day. Experiments at a photon flux of 190 uE/m<sup>2</sup>/sec (lower line) gave similar growth rates, indicating that the cultures were not light limited at these photon fluxes and dilute cell concentrations. The

last three points deviate from the straight line, reflecting the fact that the culture eventually became light limited. The results of four experiments gave growth rates of 2.64, 2.70, 2.48, and 2.65 doublings per day with the average rate being 2.62 doublings per day.

#### Cyclotella (S/CYCLO-1)

Cyclotella was maintained at  $30.5 \pm 1^\circ\text{C}$ , at an initial photon flux of approximately  $200\text{--}218 \text{ uE/m}^2/\text{sec}$ , and a pH of 6.4. Figure 4 shows the results of several growth rate experiments. The lower line represents the average of the two experiments, and corresponds to a growth rate of 3.73 doublings per day. The upper line corresponds to a growth rate of 3.95 doublings per day. The results of a total of six experiments indicate an average growth rate of 3.89 doublings per day. Although Cyclotella grows rapidly using the medium listed in the Materials and Methods, we have been unable to attain cell concentrations greater than about  $10^6$  cells/ml. The medium limits growth beyond these cell densities, since doubling or tripling the concentrations of all the medium components (except the artificial sea salt) results in the attainment of higher cell concentrations.

#### Chaetoceros (SS-14)

Chaetoceros was grown at  $30.5 \pm 1^\circ\text{C}$ , at an initial photon flux of approximately  $165\text{--}195 \text{ uE/m}^2/\text{sec}$  and a pH of 7.9. Figure 5 shows the results of duplicate growth rate experiments. The lower line <sup>(diamonds)</sup> represents the average of two experiments, and corresponds to a growth rate of 4.54 doublings per day. The upper line <sup>(squares)</sup> represents a growth rate of approximately 4.6 doublings per day. Nitrate ( $\text{KNO}_3$ ) can also be used as a nitrogen source for Chaetoceros; however, the growth rate with nitrate is approximately 10% lower than with urea.

#### Chlorella (SC)

Chlorella was grown at  $31 \pm 1^\circ\text{C}$ , at a photon flux of approximately  $195\text{--}218 \text{ uE/m}^2/\text{sec}$  and a pH of 7.9. Figure 6 shows the results of duplicate growth rate experiments in which the growth rate is 4.89

doublings per day (lower line). The upper line corresponds to a growth rate of 4.95 doublings per day. As in the case of Chaetoceros, nitrate (2 g/l  $\text{KNO}_3$ ) can also be used as a nitrogen source without adversely affecting the growth rate.

Each of the four warm-adapted algae grew well in the media described, and under the experimental conditions stated. Chlorella and Chaetoceros grew very rapidly (4.89 and 4.54 doublings per day, respectively); this plus their ease of handling would make them attractive species for further studies. Cyclotella grew well, but the medium must be optimized to attain higher cell concentrations. Ankistrodesmus grew the slowest of the four organisms studied. The slow growth coupled with its tendency to clump makes Ankistrodesmus the least attractive organism for further study.

#### The Effect of Temperature on Algal Yield

The objective of these experiments was to determine if the cell concentration of maximum biomass productivity is significantly altered by changes in the temperature. It is important to establish how changes in temperature affect the biomass productivity and efficiency of light utilization by algal cultures, since outdoor cultures will experience daily and seasonal changes in temperature.

Three continuous cultures of Scenedesmus were established at 15, 25, 35 °C, and with incident photon fluxes of 295.2 +/- 35, 361.6 +/- 46, 311.6 +/- 69  $\mu\text{E}/\text{m}^2/\text{sec}$ , respectively. These temperatures were chosen because previous data (at a non-saturating photon flux of ~ 70  $\mu\text{E}/\text{m}^2/\text{sec}$ ) indicated that Scenedesmus productivity: (1) is maximal at 32 °C, (2) decreases sharply at temperatures above 35 °C, and (3) is approximately half the maximum value at 15 °C. We found that stable, continuous cultures could not be maintained at 15 °C and 35 °C at these high photon fluxes; these two cultures have been successfully restarted at 18 °C and 32 °C.

Figure 7 shows preliminary data on the response of biomass productivity to cell concentration for 18, 25, and 32 °C. At cell concentrations between 0.60 and 1.5 mg dry weight/ml 32 °C consistently yields the highest biomass productivity and 18 °C consistently yields the lowest productivity. Although the experiment is not yet complete, the cell concentration of maximum productivity will be at least 1.5 mg dry weight/ml for 18 °C and 32 °C and at least 2.5 mg dry weight/ml for 25 °C.

The experiments concerning the effect of temperature on the cell concentration of maximum biomass productivity will be completed soon. These results will indicate if the cell concentration of maximum productivity changes significantly as a function of temperature, and this will determine the course of subsequent experiments.

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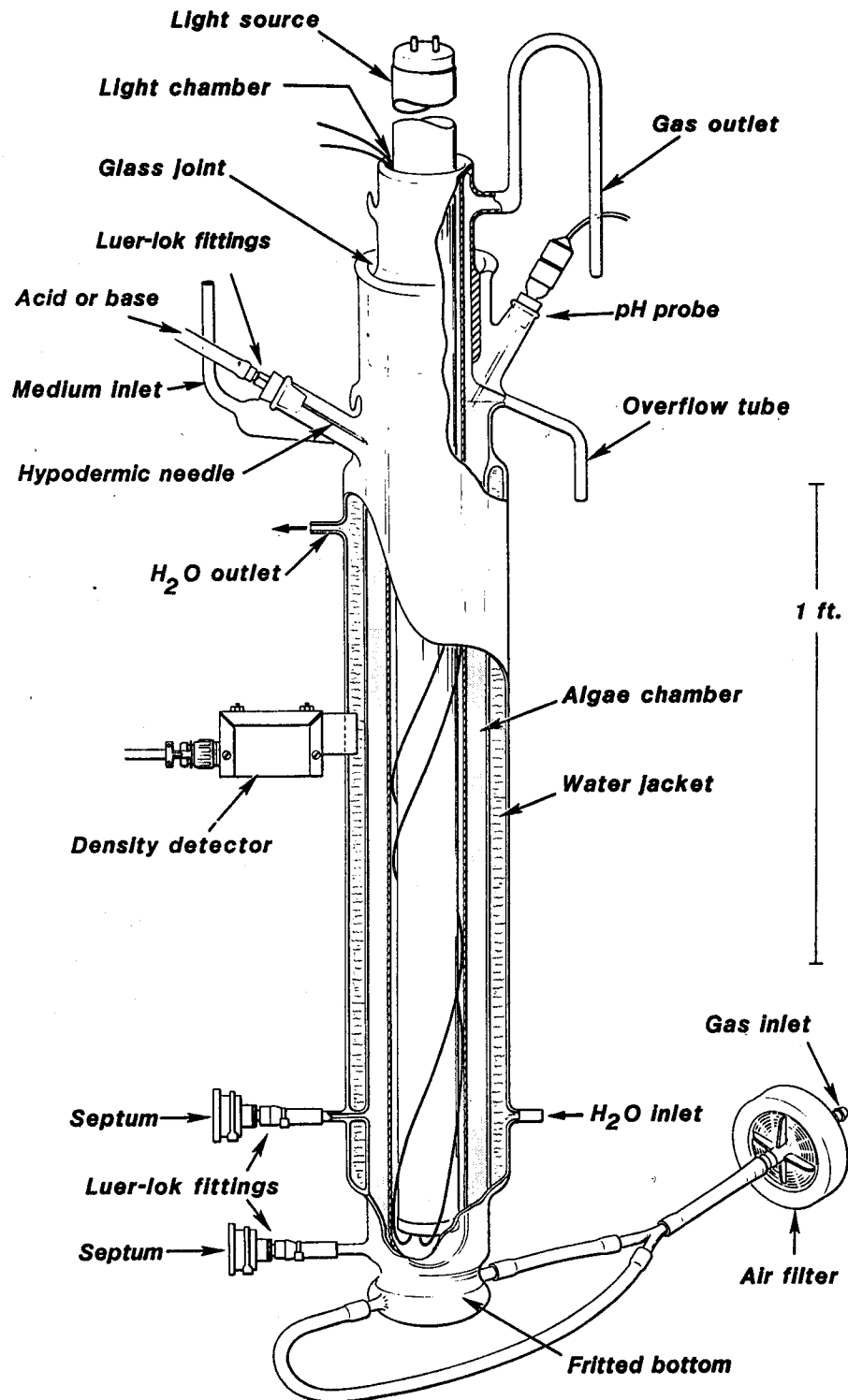


Fig -

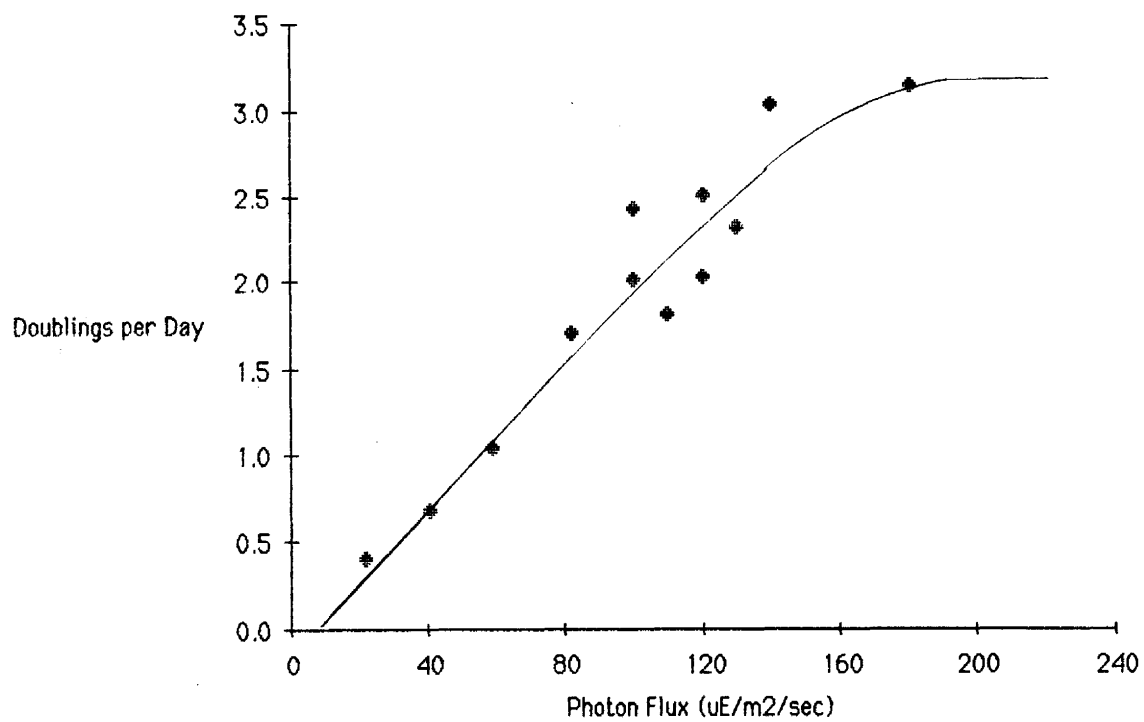


Fig 2

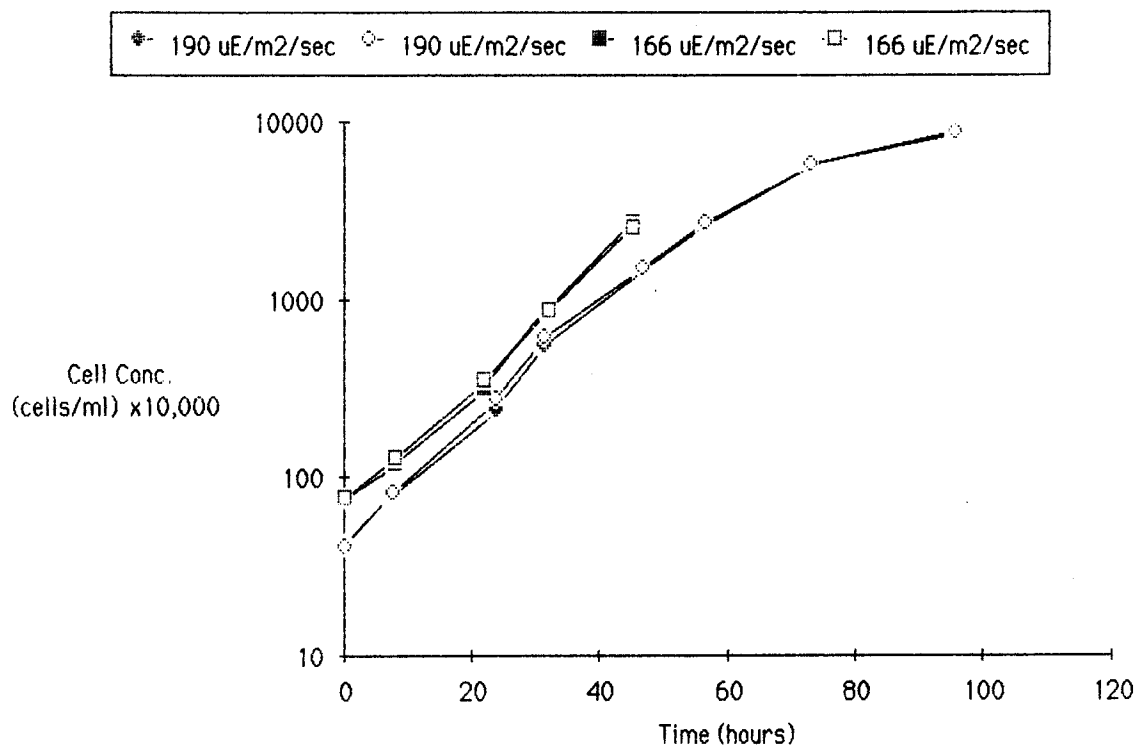
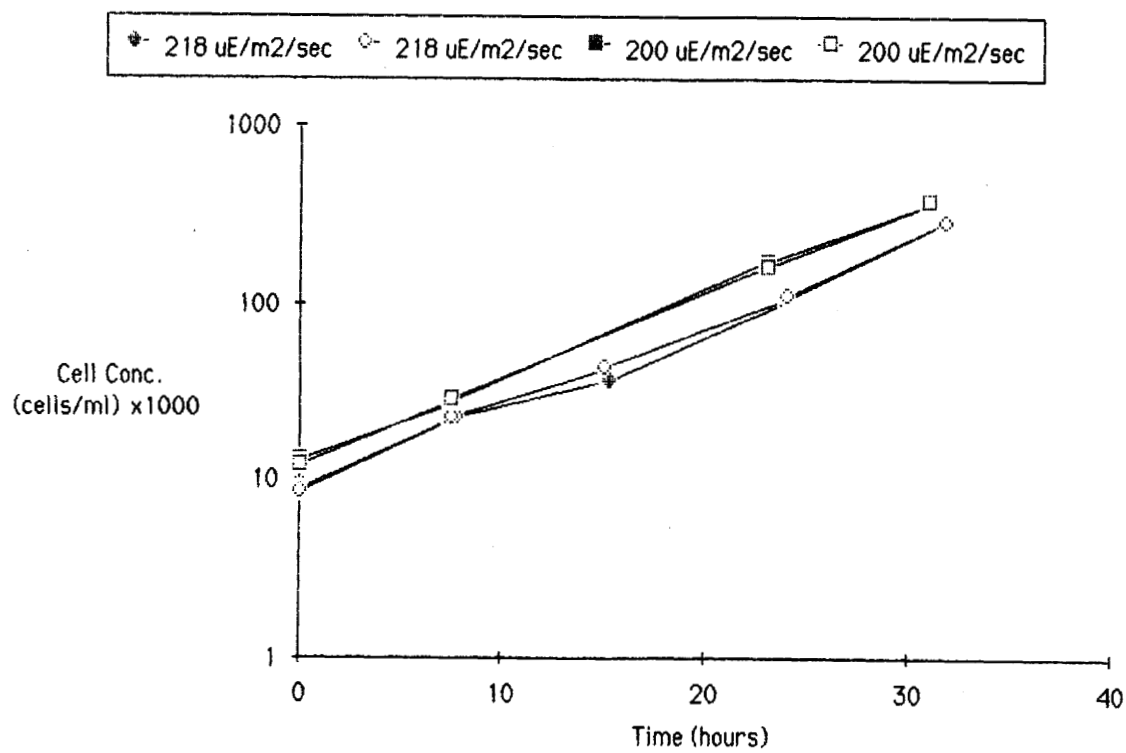
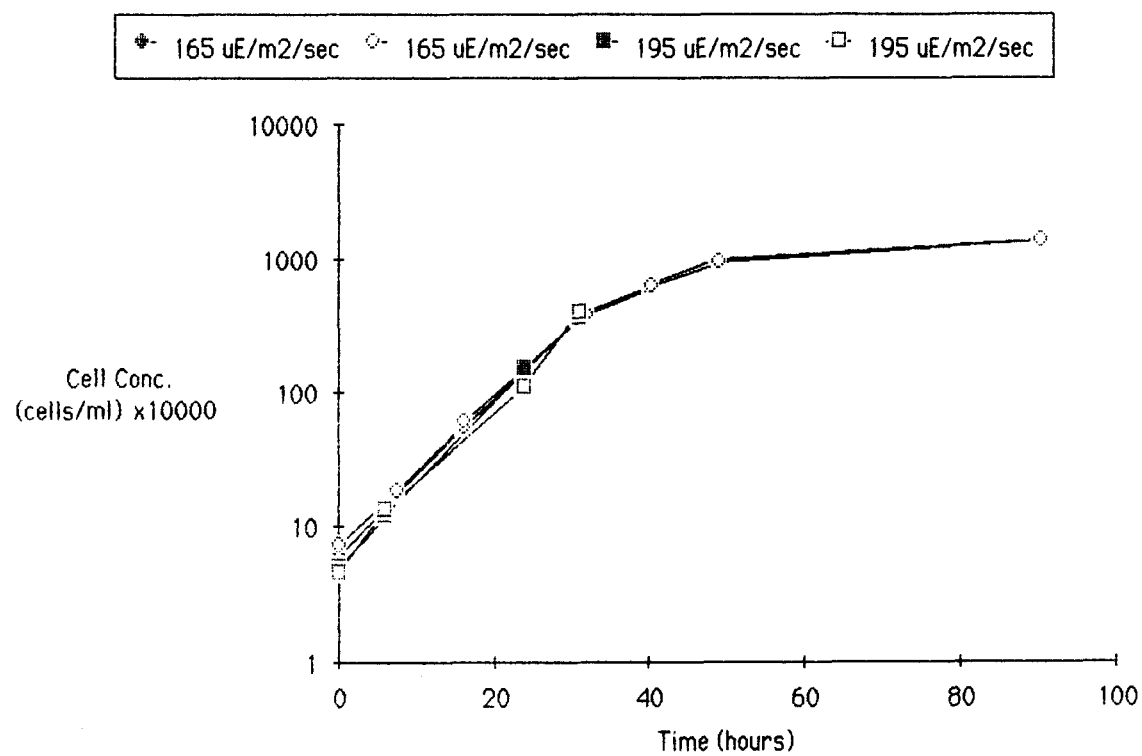
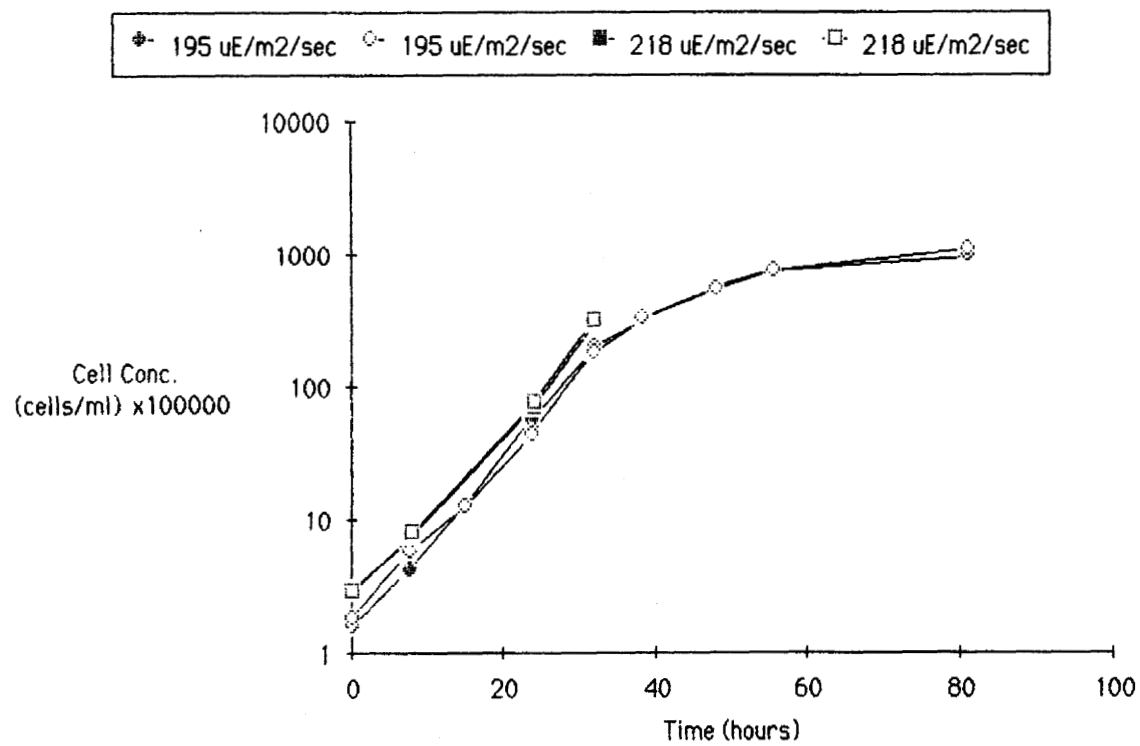
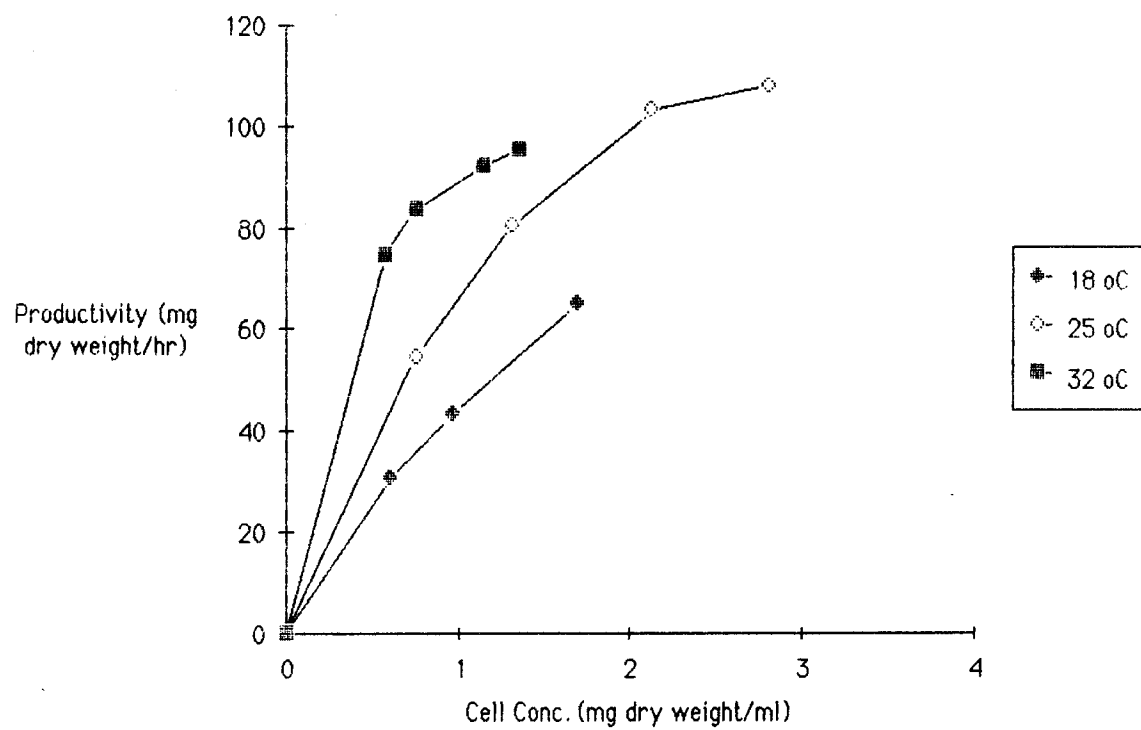


Fig 4









## GENETIC VARIATION IN OIL-PRODUCING MICROALGAE

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### ABSTRACT

Clones of Amphora coffaeiformis and Nannochloropsis spp. were examined for genetic differences by gel electrophoresis. Strains of both types of organisms were found to be genetically variable. Clones of Amphora isolated over a series of sites with 300m of each other showed greater genetic diversity than planktonic diatoms examined previously. There was no clear relationship between environmental factors and the frequency of different genotypes. Clones with similar frustule morphology were found to be more closely related to each other than those with different morphologies. The presence of a relatively high proportion of heterozygotes and the lack of linkage groups among alleles at different loci indicate that interbreeding among genotypes may be occurring in natural populations of Amphora. Clones of Nannochloropsis exhibited a wide range of genotypes in spite of having virtually identical morphology.

### INTRODUCTION

Many species of microalgae from every class of algae exhibit intraspecific genetic variation (Gallagher, 1986). Although intraspecific variation is a common phenomenon, very few investigations of it used sample sizes large enough to allow quantitative estimates of genetic diversity (Gallagher, 1986). Consequently, very little is known about the actual patterns of genetic variation in different types of microalgae and the concordance of various measures of genetic diversity in different types of traits (Gallagher, 1982; 1986). These patterns of genetic variation are important to the Aquatic Species Program at SERI because this information can be used to: 1) help design sampling protocols for targeted organisms over time and space, 2) to identify types of organisms most promising for future genetic manipulation, and 3) to help create banks of microalgal strains for these genetic manipulations. For example, if local populations of species show large amounts of genetic diversity, sampling schemes should be designed to obtain more than one clone from each location. Also, if populations separated by small distances show genetic divergence, then an effective sampling scheme for such organisms need not involve travel to distant locations to obtain a variety of forms. Organisms that show evidence of gene flow in natural populations through the presence of heterozygous individuals and the absence of linkage groups of alleles (Gallagher, 1980) might be more promising for future genetic manipulation in the lab than organisms, like Skeletonema costatum, which appear to have extremely small rates of genetic exchange. Examination of the patterns of genetic differentiation in microalgae also reveal information about the "relatedness" of strains (Murphy and Guillard, 1976). This information can be used to design breeding experiments because crosses between closely



related strains are more likely to be successful than those between distantly related strains.

Patterns of genetic variation were examined in the algal genera Amphora (Bacillariophyceae) and Nannochloropsis (Eustigmatophyceae). Amphora coffaeiformis is a benthic pennate diatom that has been shown to have intraspecific variation for heterotrophic potential (Lewin, 1955; Cooksey and Chansang, 1975) and for frustule structure (Lee and Reimer, unpub.) The taxonomic relationships among morphotypes of this species are unclear (Lee and Reimer, unpub.). This organism is being used to study lipid induction as part of the Aquatic Species Program and may be a good target organism for future genetic manipulations.

Nannochloropsis spp. is a recently described genus in the class Eustigmatophyceae (Hibberd, 1981). Compared to many other types of microalgae, comparatively little is known about the physiology and genetics of the Eustigmatophyceae (Hibberd, 1981). Strains of Nannochloropsis store large quantities of oil and it may also be the target of future genetic manipulations.

## MATERIALS AND METHODS

The clones maintained for use for this study, their location of isolation and source are listed in Tables 1 and 2. The map of isolation locations in Sippewisset Salt marsh is depicted in Fig. 1. The clones of Amphora isolated from the marsh were streaked on agar plates using the methods of Lee, et al. (1976) and were all obtained on a single day. Individual colonies were reisolated from the plates and successive subculturing of single colonies resulted in the formation of a series of unialgal cultures. Each clone of Amphora used in this investigation was examined by light and by scanning electron microscopy using the methods described in Lee (1980a and 1980b) in order to confirm identification of each clone as Amphora. Photographs of all specimens examined in the SEM were taken and serve as a permanent record of the morphological variation found in this species. All clones examined by electrophoresis fit the description of Amphora coffaeiformis or Amphora coffaeiformis var. acutiuscula in Patrick and Reimer (1975). Thus, the variation reported here among clones is analogous to previous investigations on intraspecific variation in Amphora coffaeiformis (Cooksey and Chansang, 1975; Lewin, 1955).

The clones were maintained in f/2 medium (Guillard and Ryther, 1962) in aged natural seawater of 32 ‰ salinity, at 20° C and 100  $\mu\text{Ein m}^{-2} \text{s}^{-1}$  (14:10 L:D). All clones were grown under these conditions for electrophoresis to minimize the potential effects of environmental differences on gene expression. For electrophoretic analyses, replicate samples were harvested from 500 ml flasks containing 300 ml of medium. Cells of Amphora were scraped off the sides of the flasks using a teflon policeman and were harvested by centrifugation. Cells of Nannochloropsis were harvested by filtration on glass fiber filters.

Several methods of extraction were attempted for both species. These methods included grinding both fresh preparations and pellets frozen overnight in a variety of buffers, sonicating in a variety of buffers at 0-2° C, freezing cells in liquid nitrogen and grinding the frozen filters or pellets in several buffers with a

mortar and pestle, and attempting to fracture cells with osmotic shock. The best extraction method for most enzymes in most clones of Amphora was to freeze pellets overnight at -20° C with 2 drops of 0.05 M Tris-HCl buffer, pH 7.5 with 10% added glucose. The frozen pellets were ground the following morning with a motor-driven grinder fitted with an aluminum tip. The best extraction method for the most enzymes in most clones of Nannochloropsis was to freeze the glass fiber filters in liquid nitrogen for 5 min. and then to grind the frozen filters in 0.5 M Tris-HCl, pH 7.1 medium containing 1 mM of EDTA, 50 mM NAD, and 1 mM  $\beta$ -mercaptoethanol. In both Amphora and Nannochloropsis the resultant slurries were centrifuged at 3000 rpm for 5 minutes and the supernatant was used as a protein extract. These supernatants were used only once because enzyme activities showed rapid declines with time after the cells were broken.

Electrophoresis was conducted on an Aardvark Instruments (Chicago, Ill.) polyacrylamide gel electrophoresis apparatus at 400 volts and 80-100 milliamps. Various continuous and discontinuous buffer systems and gel concentrations were assayed for the best enzyme activities and best separations for various enzymes for both types of algae. The most consistent results were obtained for a 7% gel in a continuous Tris-Borate, 0.1 M, pH 8.9 buffer (Murphy and Guillard, 1976). The gel was cooled during the run by a circulating ice water bath (0°C). Electrophoresis was conducted until a standard reference dye (RBY, Gelman Instruments) travelled a standard distance of 100-105 mm in the gel. This usually took 3 hours. One clone of Skeletonema costatum (Clone Skel) was run on every gel in addition to the unknown clones of Amphora and Nannochloropsis in order to serve as an internal standard. All allozyme mobilities were calculated as a ratio of the distance of the unknown bands relative to the distance shown by the corresponding bands in Skel or to the tetrazolium oxidase band shown by this clone. In this manner, all electrophoresis runs were standardized to each other (Gallagher, 1980). This procedure, plus growing the clones under uniform conditions, and running each clone more than once minimized the probability of assigning the clones to the incorrect genotype or confusing environmental vs. genetic differences on gene expression.

The following enzymes were stained successfully in Amphora: phosphoglucose isomerase (PGI), hypoxanthine dehydrogenase (XDH),  $\alpha$ -ketoglutarate dehydrogenase (KDH), malate dehydrogenase (MDH), phosphoglucomutase (PGM), alanine dehydrogenase (ADH),  $\alpha$ -hydroxybutarate dehydrogenase (HBDH). Several loci of tetrazolium oxidase (TO) were also scored. The following stains did not work in this organism in any buffer system attempted: glutamate dehydrogenase (GDH), lactate dehydrogenase (LDH), acid phosphatase (AcP), glutamate-oxalate transaminase (GOT), leucine amino peptidase (LAP), aldolase (ALD), glyceraldehyde-3-P dehydrogenase (Gly-3DH), glucose-6P dehydrogenase (Glc-6DH), peptidases (PEP). Alkaline phosphatase (Alk) stained in one clone of Amphora that was grown under nutrient limited conditions. It was not stained in other clones grown under the same conditions and was not used further in these analyses.

The following enzymes were stained successfully in Nannochloropsis using the extraction method described above: PGI,

MDH, TO, HBDH, ADH, HBDH, KDH. The enzyme loci that did not stain well in these organisms included: GDH, GOT, LDH, AlkP, LAP, ALD, Gly-3DH, PEP, Glc-6-P.

The relatively high number of nonstaining loci has been reported previously (Murphy and Guillard, 1976; Gallagher, 1979; Huber and Lewin, 1986) and appears to be characteristic of microalgae. This trait may be due to a high proportion of membrane-bound enzymes in these organisms.

Pictures were drawn from each gel after staining. The original gels were preserved and kept for 3 months to serve as references. All mobilities were measured relative to "standard" bands shown by Skel in the same gel. Each clone was assigned to a genotype for each enzyme based on these mobilities.

Statistics. Electrophoretic banding patterns were interpreted as genotypes where a single band indicated a homozygote and two or more bands indicated a heterozygote at a locus. Frequencies of individuals with different genotypes were calculated for each sampling location and for each morphotype in *Amphora*. Because electrophoresis does not detect all genetic differences and samples only a very small part of the genome, estimates of genetic diversity obtained by these techniques are minimum estimates. The limitations of these interpretations and the problems of genetic interpretations of banding patterns in asexual organisms are discussed in Gallagher (1980).

Genetic diversity was calculated using the method of Lewontin (1972). This analysis is based on the Shannon information measure where:

$$H = -\sum p_i \log_2 p_i$$

$p_i$  is the frequency of the  $i^{\text{th}}$  allele at locus  $j$ , and  $H$  is the genetic diversity.

Genetic similarity among samples was calculated by using Nei's statistic (Nei, 1972). This statistic is the probability that 2 alleles, one drawn from each population, are identical. This calculation allows comparison of genetic differences among organisms and has been used to estimate the degree of genetic differentiation among populations, sibling species, semi-species and morphologically different species. It is calculated as follows:

$$I_k = \frac{\sum x_i y_i}{(\sum x_i^2 \sum y_i^2)^{1/2}}$$

where  $x_i$  is the frequency of the  $i^{\text{th}}$  allele at locus  $k$  in population  $x$ , and  $y_i$  is the frequency of the  $i^{\text{th}}$  allele at locus  $k$  in population  $y$ .

$$I = \frac{I_{xy}}{(I_x I_y)^{1/2}}$$

where  $I$  is the mean genetic identity over all loci,  $I_{xy}$  is the mean of  $\sum x_i y_i$ ,  $I_x$  is the mean of  $\sum x_i^2$ , and  $I_y$  is the mean of  $\sum y_i^2$ , for all populations 1 through  $n$ .

Table 1. Clones of Amphora coffaeiformis.

Co16	Florida by K. Cooksey
Co17	Florida by K. Cooksey
Co8	Florida by K. Cooksey
CAL	var. acutiuscula, orig. source J.J. Lee via K. Cooksey
211M	81/II/00: Lewin, R., Guam, shore
77	Aug., 1985, Sippewisset salt marsh, Woods Hole, Ma
78	Aug., 1985, Sippewisset salt marsh, Woods Hole, Ma
A28	Aug., 1985, Sippewisset salt marsh, Woods Hole, Ma
A31	Aug., 1985, Sippewisset salt marsh, Woods Hole, Ma
A33	Aug., 1985, Sippewisset salt marsh, Woods Hole, Ma
A35	Aug., 1985, Sippewisset salt marsh, Woods Hole, Ma
A4	Aug., 1985, Sippewisset salt marsh, Woods Hole, Ma
A63	Aug., 1985, Sippewisset salt marsh, Woods Hole, Ma
B16	Aug., 1985, Sippewisset salt marsh, Woods Hole, Ma
B18	Aug., 1985, Sippewisset salt marsh, Woods Hole, Ma
B45	Aug., 1985, Sippewisset salt marsh, Woods Hole, Ma
B55	Aug., 1985, Sippewisset salt marsh, Woods Hole, Ma
B56	Aug., 1985, Sippewisset salt marsh, Woods Hole, Ma
B60	Aug., 1985, Sippewisset salt marsh, Woods Hole, Ma
B62	Aug., 1985, Sippewisset salt marsh, Woods Hole, Ma
B63	Aug., 1985, Sippewisset salt marsh, Woods Hole, Ma
C55	Aug., 1985, Sippewisset salt marsh, Woods Hole, Ma
C56	Aug., 1985, Sippewisset salt marsh, Woods Hole, Ma
C57	Aug., 1985, Sippewisset salt marsh, Woods Hole, Ma
C58	Aug., 1985, Sippewisset salt marsh, Woods Hole, Ma
D39	Aug., 1985, Sippewisset salt marsh, Woods Hole, Ma
D60	Aug., 1985, Sippewisset salt marsh, Woods Hole, Ma
D63	Aug., 1985, Sippewisset salt marsh, Woods Hole, Ma
D68	Aug., 1985, Sippewisset salt marsh, Woods Hole, Ma
D73	Aug., 1985, Sippewisset salt marsh, Woods Hole, Ma
D94	Aug., 1985, Sippewisset salt marsh, Woods Hole, Ma
E13	Aug., 1985, Sippewisset salt marsh, Woods Hole, Ma
E21	Aug., 1985, Sippewisset salt marsh, Woods Hole, Ma
E31	Aug., 1985, Sippewisset salt marsh, Woods Hole, Ma
E63	Aug., 1985, Sippewisset salt marsh, Woods Hole, Ma
E83	Aug., 1985, Sippewisset salt marsh, Woods Hole, Ma
E87	Aug., 1985, Sippewisset salt marsh, Woods Hole, Ma
WH79-41	summer, 1979, Sippiwisset marsh, Woods Hole, Ma
WH80-133	summer, 1980, Sippiwisset Marsh, Woods Hole, Ma: J.J. Lee
WH80-142	Summer 1980, Sippiwisset Marsh, Woods Hole, Ma: J.J. Lee
WH80-158	Summer, 1980, Sippiwisset Marsh, Woods Hole, Ma.: J.J. Lee
WH80-223	Summer, 1980, Sippiwisset Marsh, Woods Hole, Ma:J.J. Lee
WH80-62	Summer, 1980, Sippiwisset marsh, Woods Hole, Ma: J.J. Lee

Table 2. Clones of Nannochloropsis spp.

Clone # Isolation Information

Obtained from Culture Collection of Marine Algae:

66 date and location unknown, Droop, M.R.,  
 7-15STICHO 58/XII/09: Ryther, J.H., 39° 36' N, 70° 06' W, Continental shelf  
 CAR A2 64/00/00: Bayer, E., Pamlico Sound, NC  
 GBSTICHO 52/00/00, Ryther, J.H., Great South Bay, NY  
 GME25A 76/V/00: Haines, K.C., Gulf of Maine  
 MACCO938 date unknown, Li-Meng, Ren, Qingdao, China  
 MILNANNO 56/00/00, Guillard, Milford Harbor, Milford, Conn.  
 SAY2 65/XI/10, VanderBorgh, G., West Sayville, NY  
 SAY3 65/XI/10: VanderBorgh, G., West Sayville, NY  
 TUNIS 68-69/Winter: Stirn, J., Lake of Tunis, Tunisia, North Africa

Obtained from R. Lewin:

85/10-01 summer, 1985, St. John, Lameshur Bay mangrove  
 85/10-02 summer, 1985, St. John, Lameshur Bay mangrove  
 85/10-03 summer, 1985, St. John, Lameshur Bay mangrove  
 85/101-01 summer, 1985, Panama, Galeta, mangrove by roadside  
 85/101-02 summer, 1985, Panama, Galeta, mangrove by roadside  
 85/101-03 summer, 1985, Panama, Galeta, mangrove by roadside  
 85/101-04 summer, 1985, Panama, Galeta, mangrove by roadside  
 85/105-01 summer, 1985, Panama, Lagoon, east of Isla Margarita  
 85/105-02 summer, 1985, Panama, Lagoon, east of Isla Margarita  
 85/120-01 summer, 1985, Miami, Mile 108, US 1, shallow mangrove  
 85/120-02 summer, 1985, Miami, Mile 108, US 1, shallow mangrove  
 85/120-03 summer, 1985, Miami, Mile 108, US 1, shallow mangrove  
 85/120-04 summer, 1985, Miami, Mile 108, US 1, shallow mangrove  
 85/120-05 summer, 1985, Miami, Mile 108, US 1, shallow mangrove  
 85/120-06 summer, 1985, Miami, Mile 108, US 1, shallow mangrove  
 85/120-07 summer, 1985, Miami, Mile 108, US 1, shallow mangrove  
 85/120-08 summer, 1985, Miami, Mile 108, US 1, shallow mangrove  
 85/13-02 summer, 1985, St. Thomas Vessup Bay Red Hook  
 85/19-01 summer, 1985, St. Thomas Mandal Bay, lagoon  
 85/19-02 summer, 1985, St. Thomas, Mandal Bay, lagoon  
 85/19-03 summer, 1985, St. Thomas Mandal Bay, lagoon  
 85/21-01 summer, 1985, St. Thomas, Hull Bay  
 85/21-02 summer, 1985, St. Thomas, Hull Bay  
 85/21-03 summer, 1985, St. Thomas, Hull Bay  
 85/30-01 summer, 1985, Tortola, Can Garden Bay, mangrove  
 85/30-02 summer, 1985, Tortola, Cane Garden Bay, mangrove  
 85/30-03 summer, 1985, Tortola, Cane Garden Bay, mangrove  
 85/37-01 summer, 1985, St. Croix, Great Salt Pond, mangrove pool  
 85/37-02 summer, 1985, St. Croix, Great Salt Pond, mangrove pool  
 85/37-03 summer, 1985, St. Croix, Great Salt Pond, mangrove pool  
 85/37-04 summer, 1985, St. Croix, Great Salt Pond, mangrove pool  
 85/37-05 summer, 1985, St. Croix, Great Salt pond, mangrove pool  
 85/37-06 summer, 1985, St. Croix, Great Salt pond, mangrove pool  
 85/38-01 summer, 1985, St. Croix, Great Salt Pond proper  
 85/40-04 summer, 1985, St. Croix, Robin Bay mangrove pond  
 85/42-01 summer, 1985, St. Croix, Boiler Bay, nearshore water  
 85/42-02 summer, 1985, St. Croix, Boiler Bay, nearshore water  
 85/42-03 summer, 1985, St. Croix, Boiler Bay, nearshore water  
 85/42-04 summer, 1985, St. Croix, Boiler Bay, nearshore water  
 85/42-05 summer, 1985, St. Croix, Boiler Bay, nearshore water  
 85/43-01 summer, 1985, St. Croix, Salt River, mangrove lagoon  
 85/45-01 summer, 1985, St. Croix, Teague Bay, NIL pier  
 85/46-01 summer, 1985, St. Croix, Salt River, Hydrolab Pier  
 85/46-02 summer, 1985, St. Croix, Salt River, Hydrolab Pier  
 85/46-03 summer, 1985, St. Croix, Salt River, Hydrolab Pier  
 85/46-04 summer, 1985, St. Croix, Salt River, Hydrolab Pier

Table 2 (cont.):

85/52-01	summer, 1985, Puerto Rico, Boca de Congrejos harbor
85/52-02	summer, 1985, Puerto Rico, Boca de Congrejos harbor
85/52-03	summer, 1985, Puerto Rico, Boca de Congrejos harbor
85/52-04	summer, 1985, Puerto Rico, Boca de Congrejos harbor
85/52-05	summer, 1985, Puerto Rico, Boca de Congrejos harbor
85/52-06	summer, 1985, Puerto Rico, Boca de Congrejos harbor
85/52-07	summer, 1985, Puerto Rico, Boca de Congrejos harbor
85/52-08	summer, 1985, Puerto Rico, Boca de Congrejos harbor
85/52-09	summer, 1985, Puerto Rico, Boca de Congrejos harbor
85/52-10	summer, 1985, Puerto Rico, Boca de Congrejos harbor
85/52-11	summer, 1985, Puerto Rico, Boca de Congrejos harbor
85/53-01	summer, 1985, Puerto Rico, San Juan, Condado Lagoon
85/53-02	summer, 1985, Puerto Rico, San Juan, Condado Lagoon
85/55-01	summer, 1985, Puerto Rico, San Juan, Escambron outdoor pool
85/55-02	summer, 1985, Puerto Rico, San Juan, Escambron outdoor pool
85/55-03	summer, 1985, Puerto Rico, San Juan, Escambron outdoor pool
85/55-04	summer, 1985, Puerto Rico, San Juan, Escambron outdoor pool
85/59-01	summer, 1985, Puerto Rico, Mayaguy Harbor
85/59-02	summer, 1985, Puerto Rico, Mayaguy Harbor
85/59-03	summer, 1985, Puerto Rico, Mayaguy Harbor
85/62-03	summer, 1985, Puerto Rico, Pt. Arenas wet lab
85/63-01	summer, 1985, Puerto Rico, Pt. Arenas wet lab
85/63-02	summer, 1985, Puerto Rico, Pt. Arenas wet lab
85/63-03	summer, 1985, Puerto Rico, Pt. Arenas wet lab
85/63-04	summer, 1985, Puerto Rico, Pt. Arenas wet lab
85/63-05	summer, 1985, Puerto Rico, Pt. Arenas wet lab
85/64-01	summer, 1985, Puerto Rico, Joyuda Lagoon
85/64-02	summer, 1985, Puerto Rico, Joyuda Lagoon
85/64-03	summer, 1985, Puerto Rico, Joyuda Lagoon
85/65-01	summer, 1985, Puerto Rico, Joyuda Lagoon
85/65-02	summer, 1985, Puerto Rico, Joyuda Lagoon
85/65-03	summer, 1985, Puerto Rico, Joyuda Lagoon
85/65-04	summer, 1985, Puerto Rico, Joyuda Lagoon
85/65-05	summer, 1985, Puerto Rico, Joyuda Lagoon
85/65-06	summer, 1985, Puerto Rico, Joyuda Lagoon
85/65-07	summer, 1985, Puerto Rico, Joyuda Lagoon
85/65-08	summer, 1985, Puerto Rico, Joyuda Lagoon
85/65-09	summer, 1985, Puerto Rico, Joyuda Lagoon
85/65-10	summer, 1985, Puerto Rico, Joyuda Lagoon
85/65-11	summer, 1985, Puerto Rico, Joyuda Lagoon
85/65-12	summer, 1985, Puerto Rico, Joyuda Lagoon
85/65-13	summer, 1985, Puerto Rico, Joyuda Lagoon
85/72-01	summer, 1985, Curacao, Boca St. Martha, inner bay
85/72-02	summer, 1985, Curacao, Boca St. Martha, inner bay
85/72-03	summer, 1985, Curacao, Boca St. Martha, inner bay
85/72-04	summer, 1985, Curacao, Boca St. Martha, inner bay
85/72-05	summer, 1985, Curacao, Boca St. Martha, inner bay
85/73-01	summer, 1985, Curacao, Willemsted, Avila Beach Hotel
85/73-02	summer, 1985, Curacao, Willemsted, Avila Beach Hotel
85/73-03	summer, 1985, Curacao, Willemsted, Avila Beach Hotel
85/73-04	summer, 1985, Curacao, Willemsted, Avila Beach Hotel
85/73-05	summer, 1985, Curacao, Willemsted, Avila Beach Hotel
85/75-01	summer, 1985, Panama, Naos, Inlet west of causeway
85/86-01	summer, 1985, Panama, Mangrove under Thatcher Ferry Bridge
85/86-02	summer, 1985, Panama, Mangrove under Thatcher Ferry Bridge
85/86-03	summer, 1985, Panama, Mangrove under Thatcher Ferry Bridge
85/87-02	summer, 1985, Panama, Cocoli, steep sided dike, mangrove
85/87-03	summer, 1985, Panama, Cocoli, steep sided dike, mangrove

## RESULTS

*Amphora coffaeiformis*. The Sippewisset populations of *Amphora* were sampled from a interconnected series of tidepools (Fig. 1). The distance from site A to site E was less than 300 m. The depth of tidepools A, B and C was less than a meter, while the depth of the D-E tidepool was approximately 2-3m. Representative scanning electron micrographs of the clones of *Amphora* isolated from Sippewisset salt marsh are shown in Fig. 2a-2d. The clones isolated from this location varied in morphology. However, all clones were consistent with the description of *Amphora coffaeiformis* (Patrick and Reimer, 1975). Examples of variant traits included the presence or absence of costae, the shape and number of the punctae, and the presence or absence of rostrate ends of the valves. The pattern of variation in morphology represented a broad continuum. For example, clones were found with no costae (Fig. 2a,d), full costae (Fig. 2c), or partial costae (Fig. 2b). The shape of the punctae varied from small rectangles to narrow slits (Fig. 2a-2d). The range of morphological types observed in this sample include many previously identified by Lee et al. (1976) plus some intermediate forms. Several of the clones isolated from Sippewisset were morphologically similar to those isolated by Cooksey in Florida. Clones maintained in culture for 6-7 years did not show changes in morphology over time.

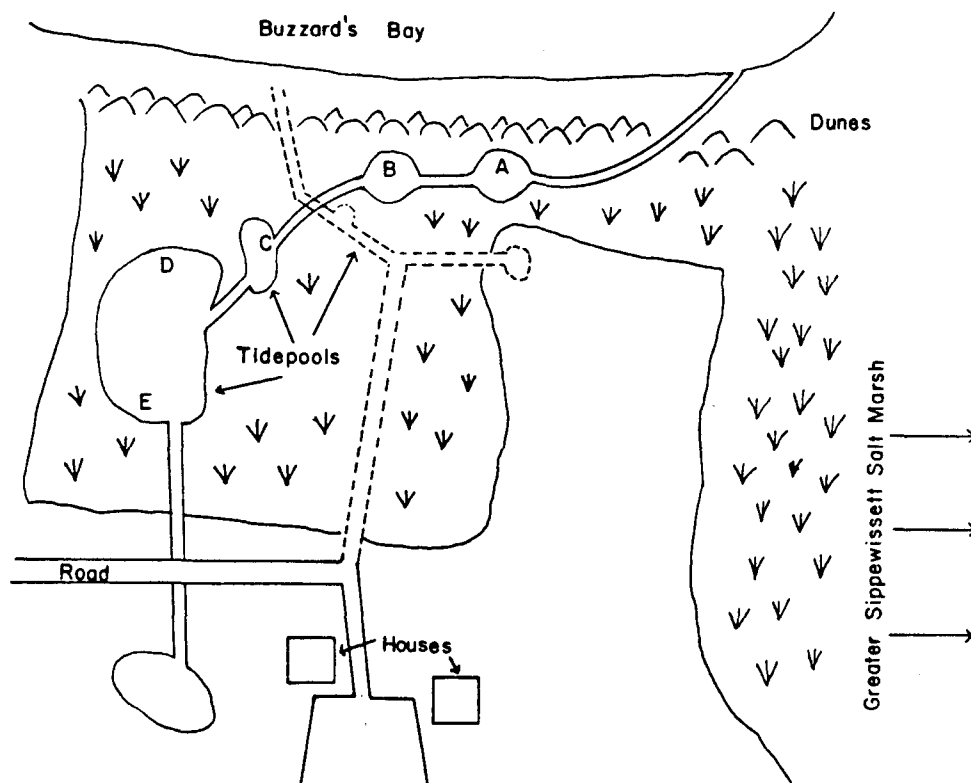


Figure 1. Map of Sippewisset salt marsh, Woods Hole, Ma. *Amphora coffaeiformis* was isolated from sites A through E. This distance from site A to site E was less than 300 m.

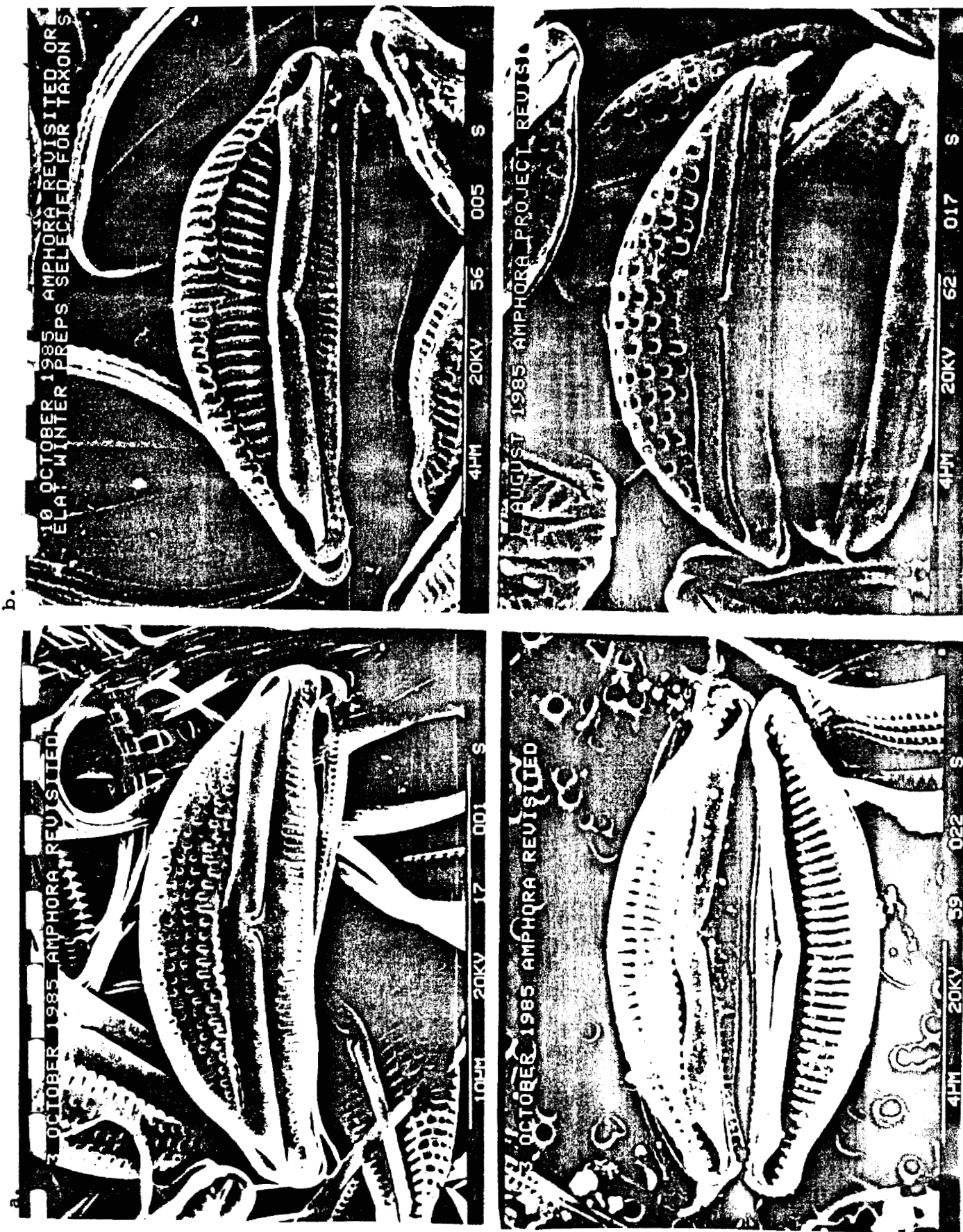


Figure 2. Clones of Amphora coffaeiformis. Bars at the bottom of each picture are either 10 µm or 4 µm.  
 a. Clone A166. b. Clone C56. c. Clone D59. d. Clone 80-62



Drawings of representative zymograms of *Amphora coffaeiformis* are shown in Fig. 3 and the key to isolation sites is listed in Table 1. The small differences in relative mobilities among clones shown in this figure are repeatable in replicate electrophoretic analyses. In this type of electrophoretic analysis, the absolute difference between mobilities has no meaning because mobilities are a dual function of the number of amino acid differences and the conformation of the molecule. Differences in banding patterns were interpreted to reflect underlying genetic differences (see above and Gallagher, 1980). In this figure, clones D89, D63, D68, D73, and D94, varied in banding patterns for PGI, XDH, and TO even though they were isolated from a single sampling site on a single day. All sites sampled in the salt marsh were genetically diverse. Clones D63, D68 and D73 are morphologically identical, as are clones C16 and C17, yet they differ in banding patterns. All categories of morphotypes examined to date also showed genetic diversity. Therefore, electrophoretic banding patterns do not show perfect concordance with morphology.

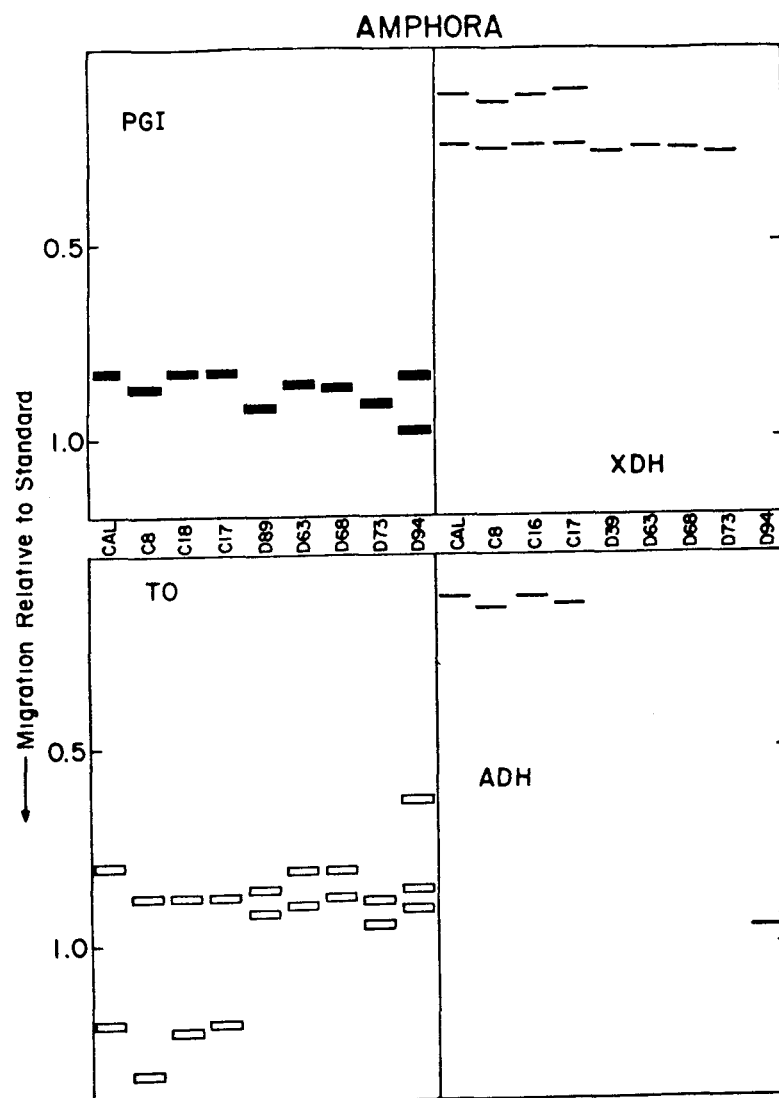


Figure 3. Drawing of representative gels of *Amphora coffaeiformis* clones. Isolation information is reported in Table 1. All mobilities are graphed as a ratio of the distance travelled by bands in *Amphora* to distance travelled by standard bands in *Skeletonema costatum* (clone Skel).

Table 3a shows the values for Nei's Similarity Index for comparisons of the clones of Amphora isolated from different sites in Sippewisset marsh. Values ranged from 0.44 to 1.00. Genetic similarity was not found to be a simple function of distance. For example, the population at point E was more similar to that at point B than it was to the point D population, even though points D and E were at opposite sides of the same tidepool. The values for genetic similarity in populations of Amphora located within 300 m of each other was substantially lower than values for genetic similarity in populations of Skeletonema costatum separated by 30 km (Table 3c, and Gallagher, 1979; 1980) found during the same time of year. Some of the values approached those found for different seasonal populations of Skeletonema (Table 3c).

	A	B	C	D	E
A	----	0.89	0.79	0.56	0.88
B		----	0.80	0.79	1.00
C			----	0.44	0.85
D				----	0.75
E					----

	with costa	without costa	partial costa
w/ cost.	----	0.67	0.60
w/out		----	0.25
part.			----

local populations	0.96-0.98
subspecies	0.80-0.97
sibling species	0.12-0.56
morph. species	0.35-0.55

within seasons	0.93-0.99
between seasons	0.10-0.43

PGI:  $H = 1.32$                       XDH:  $H = 1.10$ 

### Values in August Skeletonema populations

PGI:  $H = 0.80$  MDH:  $H = 0.54$

All of the Sippewisset clones of Amphora were divided into groups based on the structure of the costae in order to obtain a first approximation of the relationship between genetic similarity and frustule morphology. The three groups consisted of those clones with full costae, those with no costa, and those with a partial costa. This feature was chosen for this analysis because it is easily identified in SEM pictures and its structure has been proposed as a species-distinguishing feature. The values for Nei's statistic for these groups is shown in Table 3b. The clones with a partial costa were more different from those without a costa than they were from clones with a full costa. Most of the values for genetic similarity when clones were divided on the basis of isolation location were higher than those found when the clones were grouped according to morphology (Tables 3a and 3b). However, genetic differences were found between clones of identical morphologies so that banding patterns could not be used as "species fingerprints."

The total genetic diversity of the Sippewisset Amphora populations for the PGI and XDH loci were 1.32 and 1.10, respectively. These values can be compared with the values of genetic diversity of 0.80 and 0.54 for similar loci in the August populations of Skeletonema costatum in Narragansett Bay (Table 3d).

Although sample sizes were too small to perform a linkage analysis of alleles at different loci (Gallagher, 1979), qualitative examination of the data did not show evidence of linkage groups, unlike the Narragansett Bay populations of Skeletonema costatum. The total proportion of clones showing heterozygous banding patterns at one or more loci was 0.24, compared to 0-0.25 in seasonal populations of Skeletonema (Gallagher, 1979). However, unlike S. costatum heterozygotes were observed at more than 1 locus.

Nannochloropsis. Representative gels of Nannochloropsis are shown in Fig. 3. The clones examined to date are primarily those obtained from the Culture Collection of Marine Algae. Extraction methods are currently being modified to increase the number of loci examined. This small sample size precludes calculation of genetic similarity indices in a manner similar to Amphora and Skeletonema costatum. However, some trends are apparent in the qualitative data. No two clones of Nannochloropsis were found to exhibit the same banding patterns, even though they showed very similar morphologies and even though some clones were isolated from very closely spaced populations (Table 2). A further problem was the large number of "null alleles" in different stains. This was not a problem in Amphora.

# NANNOCHLOROPSIS

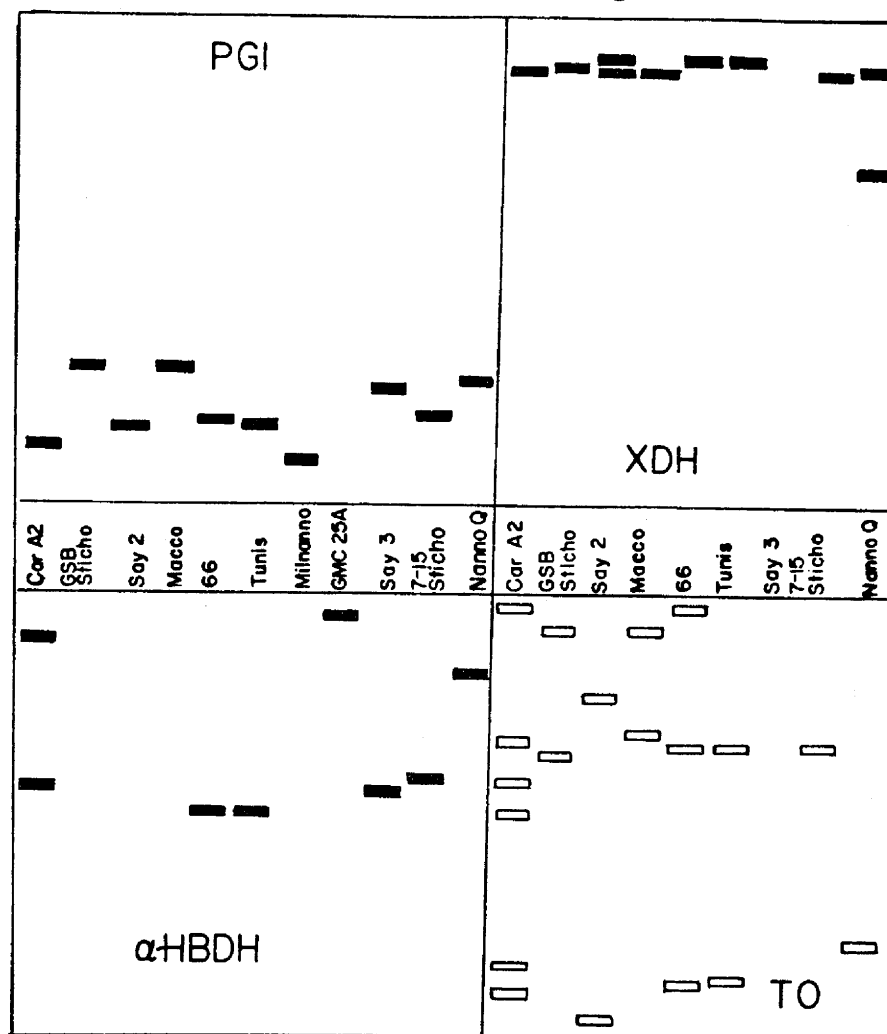


Figure 4. Drawing of representative gels of Nannochloropsis spp. Isolation information is reported in Table 2. Mobilities graphed as in Figure. 3

## DISCUSSION

The data on the patterns of genetic differentiation in Amphora coffaeiformis indicate that closely spaced populations show genetic differences even though the populations are connected to each other by a tidal creek. This level of population differentiation over 300 m was far greater than that for comparable populations of the planktonic diatom Skeletonema costatum over distances of 30 km. The great degree of population differentiation over very small spatial scales in Amphora is most likely due to both its sessile growth habit which impedes migration of individuals among sites and to differences in the life cycle between pennate and centric diatoms. Pennate diatoms, like Amphora, produce amoeboid gametes while centric diatoms, like Skeletonema costatum, produce free-swimming sperm cells (Drebes, 1977). Consequently, the potential for interbreeding among populations in different locations is probably lower for Amphora than for a centric diatom, like Skeletonema. However, the relatively high proportion of heterozygotes and the absence of linkage groups indicate that gene

flow may be occurring among individuals within sites. It was not possible to determine whether the differences among populations of Amphora are due to natural selection or to random events. Of these two possibilities, random genetic drift would appear to be most likely since there was no obvious relationship between type of environment and the differences between populations within the salt marsh.

In spite of the relatively large genetic distances found among populations when the Sippewisset Amphora clones were grouped according to location, these distances tended to be less than those found when the same clones were grouped according to crude differences in morphology. This result was expected since cells of different morphotypes were found at all sampling locations. The low genetic similarity among groups of clones classified according to a crude morphological differences tends to support the idea that there is a lower rate of gene flow among the different morphotypes than among morphologically similar forms. Interestingly, clones without costae were more similar to those with costae than they were to clones that had partial costae. This indicated that they clones with partial costae were not genetic intermediates although they would normally be classified as morphological intermediates.

It is unknown, at present, whether these data are widely applicable to other groups of algae. However, if the viewpoint is taken that they may apply to other microalgae, then it is possible to construct a series of working hypotheses that are relevant to the SERI biomass program. These working hypotheses are:

1) The data support previous findings that local populations of microalgae are genetically diverse (Gallagher, 1980; Brand, 1982). This implies that more than one clone of each species of algae should be isolated from each location for preliminary screening.  
2) Amphora has a greater genetic diversity than Skeletonema costatum and Thalassiosira pseudonana (Gallagher, 1980; Brand, 1982) over much shorter geographic distances. This may mean that benthic organisms in general have a greater genetic diversity than planktonic forms. Consequently, benthic species should be sampled over more closely spaced sites than planktonic species.

3) Local populations of benthic species with similar morphologies may represent compatible breeding groups. Consequently, when breeding experiments are undertaken, using either classical or recombinant techniques, the highest probability of success may be found by crossing clones isolated from the same location that show only small morphological differences than crossing clones that are morphologically distinct. This should be taken into account in the design of breeding experiments. The clones of Amphora isolated from Sippewisset can provide a resource for these experiments and the differences in banding patterns can be used as genetic markers to detect the success of a cross.

4) Local populations of Amphora show more evidence of gene flow among individuals than do populations of the planktonic centric diatoms Skeletonema costatum and Thalassiosira pseudonana (Murphy and Guillard, 1976; Brand et al., 1980?). Centric and pennate diatoms also show differences in types of sexual reproduction (Drebes, 1977). Therefore, it is possible that it may be easier to perform breeding experiments with benthic species than with planktonics.

### Nannochloropsis spp.

The data on Nannochloropsis are not as complete as the data on Amphora. However, the results obtained to date do have implications for SERI's research goals. The first result is that clones of this organism are genetically diverse even though some of them were isolated from the same location. Clone GSB Sticho and clone 66 were described as "type clones" for the species Nannochloropsis salina and N. oculata, respectively (Hibberd, 1981). These clones were found to be genetically different from each other and from all of the other clones examined. These observations tend to support Hibberd's (1981) suspicion that more than two species of Nannochloropsis exist. One problem with the electrophoretic analysis of Nannochloropsis is the presence of "null" alleles. Null alleles refer to the lack of staining in a few clones out of a group. While null alleles tend to become more common with increasing genetic distance, they are difficult to interpret in electrophoretic analyses because they can be created by genetic differences at a wide variety of loci in addition to the locus being examined. They may also be created by extraction artifacts. This latter possibility is being tested currently with the use of new extraction methods. However, it is hard to attribute the presence of null alleles in Nannochloropsis to extraction problems alone, because repeated preparations of the same clones yielded similar results. More definitive information about the pattern of genetic variation in Nannochloropsis will be available as more clones are examined.

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## KELP GENETICS

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Work on giant kelp biomass production began at NMI in June, 1980 when we sub-contracted with General Electric Company and subsequently with Seri to install, plant, harvest, measure and analyze the results of an in-the-sea macroalgal farm. We were able to show that the giant kelp was equivalent to sugar-cane in its productivity under cultivation (Neushul and Harger, 1985). The importance of genetic strain selection soon became apparent and a seedstock collection of pedigreed lines was established. We were able to make both intergeneric and interspecific hybrids of pacific coast kelps (Neushul, 1982; Lewis, Harger and Neushul, 1986). Gametophytes from high-yielding kelps, obtained either from a pedigree or mass-selection approach, can be held for long periods of time without losing their fertility.

The NMI contributions to the development of algal biomass technology in general (Barclay and McIntosh, 1986) is modest indeed when viewed historically. It is a little known fact that the kelp beds of California provided the basis for a million dollar cordite and potash industry when Germany cut off its supply of potash to the United States during the first world war. Today, the most active centers for algal biomass production are China, Korea and Japan. In China in particular, the importance of genetic selection was clearly demonstrated by the late T. C. Fang who developed new strains of Laminaria that grow well in warm water and have increased iodine content.

Current research at NMI focuses on the development of new techniques to genetically manipulate kelps. Progress has been made in bacteria-free culture, the control of gametogenesis, the measurement of ploidy levels, tetrad analysis and the use of mutagens. Since kelp gametophytes are long-lived, have no somoclonal variation, they can be used as a "seedbank" of genetic types. It seems logical to screen them under laboratory conditions, and retain those that yield kelp plants that produce specific products, or are particularly productive. We have found that there is variability in the growth rates of gametophytes, but have not yet been able to link high gametophytic growth with high sporophytic growth.

The problem of "genetically domesticating" macroalgae can be extended to other algal groups, where cultivation methods, DNA measurement methods, mutagenesis, transgenesis, and many other techniques are yet to be tried. The great advantages of exploiting the genetic diversity of the algae are not always evident. However we have found that some algae produce products that could be very valuable. Genetically domesticating such plants will certainly be of value to the SERI Aquatic Species Program.



## ABSTRACT

### CHARACTERIZATION OF VIRUSES INFECTING CHLORELLA-LIKE ALGA

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Exsymbiotic, Chlorella-like, eukaryotic green algae act as hosts for a group of DNA containing viruses. We have isolated over 100 plaque forming viruses from fresh water collected from ponds, streams and rivers throughout the United States. For this study 15 representative viruses from a single Nebraska water sample are being subjected to detailed analysis. Based on previous studies, we have described a prototype virus, PBCV-1, a large, dsDNA (330 kbp) virus which infects three exsymbiotic strains of Chlorella. PBCV-1 is easily assayed since it will form plaques on lawns of the alga. Viral attachment and infection is rapid and the entire lytic cycle of the virus is completed within 8 hrs. This is the only example of a plant-virus interaction which lends itself to manipulation using the technology established for study of bacteriophage.

When the viruses ultimately lyse their algal hosts, the resulting lysate is a rich source of cell wall degrading enzyme(s), which we have called lysin. Treatment of cells with lysin results in the complete degradation of the algal wall, leading to the formation of protoplasts. Our proposal, for which studies are now underway proposes to use these protoplasts as useful partners in creation of somatic hybrids and as hosts for direct transformation with autonomously replicating vectors created from all or portions of the viral genomes. In general, conventional means for improvement of plant species revolve about the transfer, through conventional sexual hybridization, of desired genetic traits. For many microalgae, for which sexual mating systems have not been demonstrated, alternative methods such as those described here, for transfer of genetic traits are required.

# APPENDIX B

## AQUATIC SPECIES PROGRAM ANNUAL REVIEW MEETING

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