SERI/SP-231-3206
DE88001111

September 1987

FY 1987 Aquatic Species Program

Annual Report

D. A. Johnson
S. Sprague

SERI

Solar Energy Research Institute
A Division of Midwest Research Institute
1617 Cole Boulevard
Golden, Colorado 80401-3393

Operated for the
U.S. Department of Energy
under Contract No. DE-AC02-83CH10093

DISCLAIMER

SERI/SP-231-3206
UC Category: 61
DE88001111

FY 1987 Aquatic Species Program

Annual Report

D. A. Johnson
S. Sprague

September 1987

Prepared under Task No. 4750.100
FTP No. 656

Solar Energy Research Institute
A Division of Midwest Research Institute

1617 Cole Boulevard
Golden, Colorado 80401-3393

Prepared for the
U.S. Department of Energy
Contract No. DE-AC02-83CH10093

NOTICE

Printed in the United States of America
Available from:
National Technical Information Service
U.S. Department of Commerce
5285 Port Royal Road
Springfield, VA 22161

Price: Microfiche A01
Printed Copy A12

Codes are used for pricing all publications. The code is determined by the number of pages in the publication. Information pertaining to the pricing codes can be found in the current issue of the following publications, which are generally available in most libraries: *Energy Research Abstracts, (ERA); Government Reports Announcements* and Index (*GRA* and I); *Scientific and Technical Abstract Reports (STAR);* and publication, NTIS-PR-360 available from NTIS at the above address.

PREFACE

This report summarizes the progress and research accomplishments of the Aquatic Species Program, field managed by SERI, during FY 1987. This report includes an overview of the entire program and a summary of individual research projects. The 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).

Donna A. Johnson
Aquatic Species Program Coordinator

Approved for

SOLAR ENERGY RESEARCH INSTITUTE

Stanley R. Bull, Director
Solar Fuels Research Division

SUMMARY

The goal of the Department of Energy/Solar Energy Research Institute (DOE/SERI) Aquatic Species Program is to develop the technology base to produce liquid fuels from microalgae at prices competitive with conventional alternatives. Microalgae are unusual plants that can accumulate large quantities of oil and can thrive in high-salinity water, which currently has no competing uses. The algal oils, in turn, are readily converted into gasoline and diesel fuels. The best site for successful microalgae production was determined to be the U.S. desert Southwest, with potential applications to other warm areas. A technical and economic analysis, *Fuels from Microalgae*, demonstrated that liquid fuels can be produced from mass-cultured microalgae at prices that will be competitive with those of conventional fuels by 2010. Aggressive research is needed, but the improvements required are attainable.

The four prime research areas in the development of this technology are growth and production, engineering design, harvesting, and conversion. Algae are selected for three criteria: tolerance to environmental fluctuations, high growth rates, and high lipid production. From 1982 to 1986, the program collected more than 3000 strains of microalgae that are more than twice as tolerant to temperature and salinity fluctuation than the initial strains. Productivity has been increased by a factor of two in outdoor culture systems since 1982, and lipid content has also been increased from 20% of body weight in 1982 to greater than 66% of body weight in 1987. Research programs are ongoing in lipid biochemistry and genetic engineering so that ultimately strains can be modified and improved to combine their best characteristics.

An outdoor test facility (OTF) is being built in Roswell, N. Mex. Using the six 3-m^2 ponds that have been built, researchers will perform controlled replicate experiments and screen species outdoors using saline groundwater. Large-scale experiments will be performed to compare pond liners, times and rates of mixing, carbon dioxide injection, and other engineering features in the two 0.1-ha (0.25-acre) ponds being constructed. Depending on the availability of funds, a 0.5-ha (1.25-acre) pond will be constructed in FY 1989 to study scale-up issues.

Research to date has demonstrated that all algae can be harvested at a cost of 0.5¢-1.5¢ kg^{-1} dry weight using water soluble flocculant polymers. Methods to reduce the costs of this harvesting even further are currently being examined. Conversion research is just beginning in the program. Since algal lipids cannot be used as a fuel directly because they contain 10% oxygen (crude petroleum contains essentially no oxygen), the algal lipids need to be extracted and converted into gasoline and diesel fuels.

Not only the oxygen concentration but the viscosity of the fluid needs to be decreased. Future program activities include screening and characterizing the algal strains collected and reducing the collection to the best 10-25 oil-producing strains by FY 1990. Research in lipid biochemistry, strain improvement, and genetic engineering will continue so that the quantity of oil produced by this technology is maximized. Construction of the OTF will be completed, and by the end of FY 1988, a year of production data on algae grown in the desert Southwest will have been collected. New harvesting and conversion projects will be initiated early in FY 1988 to further reduce the costs of producing liquid fuels from microalgae. Major analysis efforts in the upcoming year will be on resource and environmental assessments of the technology, with emphasis on carbon dioxide supply, brine disposal, and possible climatic impacts.

TABLE OF CONTENTS

Page

Program Overview 1

1.0 Introduction 3

2.0 Goal and Objectives 6

3.0 Research and Technology Development 7

3.1 Microalgae Growth and Production 7
3.1.1 Species Screening and Characterization 10
3.1.2 Strain Improvement 11
3.1.3 Lipid Biochemistry 12
3.1.4 Genetic Engineering 13
3.2 Engineering Design 14
3.3 Harvesting 17
3.4 Conversion 17

4.0 Future Activities 19

5.0 Bibliography 20

6.0 FY 1987 Publications and Presentations 21

6.1 Publications 21
6.2 Presentations 22

Appendix

Screening and Characterization 25

Collection, Screening and Characterization of Lipid Producing Microalgae: Progress During Fiscal Year 1987
J. Johansen; P. Lemke; W. Barclay; N. Nagle; Solar Energy Research Institute 27

Screening Microalgae Isolated from the Southwest for Growth Potential and Lipid Yield
M. Sommerfeld; S. Ellingson; P. Tyler; Arizona State University 43

Conclusion of the Warm-Water Algae Collection and Screening Efforts Conducted in the Southeastern United States
M. Tadros; Alabama A&M University 58

Lipogenic Picopleuston
L. Cheng; R. Lewin; Scripps Institute of Oceanography 75

Collection of High Energy Yielding Strains of Saline Microalgae from the Hawaiian Islands
R. York; Hawaii Institute of Marine Biology 76

TABLE OF CONTENTS (Continued)

Page

Effects of Fluctuating Environments on the Selection of High Yielding Microalgae
J. Benemann; D. Tillett; Georgia Institute of Technology 77

Nutritional Requirements for Maximal Growth of Oil Producing Microalgae
C. Rhyne; Jackson State University 92

Effect of Nutrients and Temperature on Lipid Production and Fatty Acid Composition in *Monoraphidium minitum* and *Cyclotella*
S. Sriharan; D. Bagga; T. Sriharan; M. Das; Selma University; Miles College 108

Improvement and Optimization 127

Effects of Environmental Parameters on Lipid Production in *Navicula saprophila*
P. Chelf; B. Barclay; P. Lemke; Solar Energy Research Institute 129

Improvement of Microalgal Strains for Lipid Production
J. Solomon; A. Palumbo; Oak Ridge National Laboratory 142

Environmental Effects on Photorespiration in the Diatom *Chaetoceros muelleri*
R. Cohen; Colorado School of Mines 155

Lipid Biochemistry 157

The Effects of Silicon Deficiency on Several Enzymes Involved in Lipid and Carbohydrate Biosynthesis in *Cyclotella cryptica*
P. Roessler; Solar Energy Research Institute 159

Biochemical Elucidation of Neutral Lipid Synthesis in Microalgae: Report of a Mechanism to Induce Neutral Lipid Accumulation and Implications for Increasing Neutral Lipid Yields from Microalgae
J. Guckert; K. Cooksey; L. Jackson; Montana State University 174

Environmental Control of Lipid Accumulation in *Nannochloropsis salina*, *Nanno Q* and *Euglena*
L. Coleman; B. Rosen; S. Schwartzbach; University of Nebraska 190

Genetics 207

Patterns of Genetic Diversity in Three Genera of Oil-Producing Microalgae
J. Gallagher; City College of New York 209

Studies of the Chloroplast Genome of Exsymbiotic and Free-Living *Chlorella*
R. Meints; University of Nebraska 210

TABLE OF CONTENTS (Concluded)

Page

Engineering Design 229

Design and Operation of an Outdoor Microalgae Test Facility
J. Weissman; D. Tillett; R. Goebel; Microbial Products, Inc. 231

Conversion 253

Conversion of Lipids Through Biological Pretreatment
N. Nagle; P. Chelf; P. Lemke; B. Barclay; Solar Energy Research Institute 255

Catalytic Upgrading of Whole Algae and Their Components with Shape-Selective Zeolites
T. Milne; R. Evans; Solar Energy Research Institute 264

Economic and Resource Assessments 267

CO_2 Sources for Fuels Synthesis
M. Karpuk; Technology Development Associates 269

LIST OF FIGURES

Page

1-1 Artist's Concept of Microalgae Fuel Farm in the American Southwest 3

1-2 Micrograph of Algal Cell 4

3-1 Four Stages Necessary to Produce Liquid Fuels from Microalgae 7

3-2 Solar Radiation in the United States Where There are More than 180 Frost-Free d yr^{-1} 15

3-3 Open-Pond Design for Large-Scale Microalgae Production 16

LIST OF TABLES

Page

1-1 Comparative Productivity Rates in Different Plant Communities 4

3-1 FY 1987 Procurement Plan Summary for Aquatic Species Program 8

3-2 Aquatic Species FY 1987 Active Subcontracts 8

3-3 Capital Construction Costs for Three Different Algal Production Systems 15

Program Overview

THIS PAGE

WAS INTENTIONALLY

LEFT BLANK

1.0 INTRODUCTION

The worldwide energy shortage and Arab oil embargo of the early 1970s encouraged many nations to look for new sources of oil, electricity, and gas. Resources such as biomass were often viewed as attractive solutions to the energy problem because of their non-depletable, renewable nature. While the first biomass sources considered were readily available, such as wood or corn, it was apparent that new biomass sources should also be developed, including aquatic species. The current U.S. Department of Energy emphasis is placed on technology for our future energy supplies and not commodities (DOE 1987).

The DOE/SERI Aquatic Species Program is designed to develop the technology base for large-scale production of lipid-yielding microalgae and for conversion of the lipids into liquid fuels. The region with the most promise for success was determined to be the U.S. desert Southwest, with other warm areas of the United States offering additional potential. This technology could potentially produce between 150-400 barrels oil $acre^{-1}$ yr^{-1}, depending on the growing season. An artist's conception of the facility is shown in Figure 1-1.

Microalgae are small, unicellular plants that range in size from 1 to 200 μm. Microalgae productivity rates are higher than those of most other plants. Table 1-1 shows the productivity rates of many other plants and indicates the order of magnitude greater productivity that we expect can be obtained in outdoor culture ponds. They are also unique organisms in that they can accumulate storage lipids in large quantities within their bodies (Figure 1-2). Historically, microalgae have been grown in mass culture for food production and waste treatment (Benemann et al. 1987), but the hope of producing an

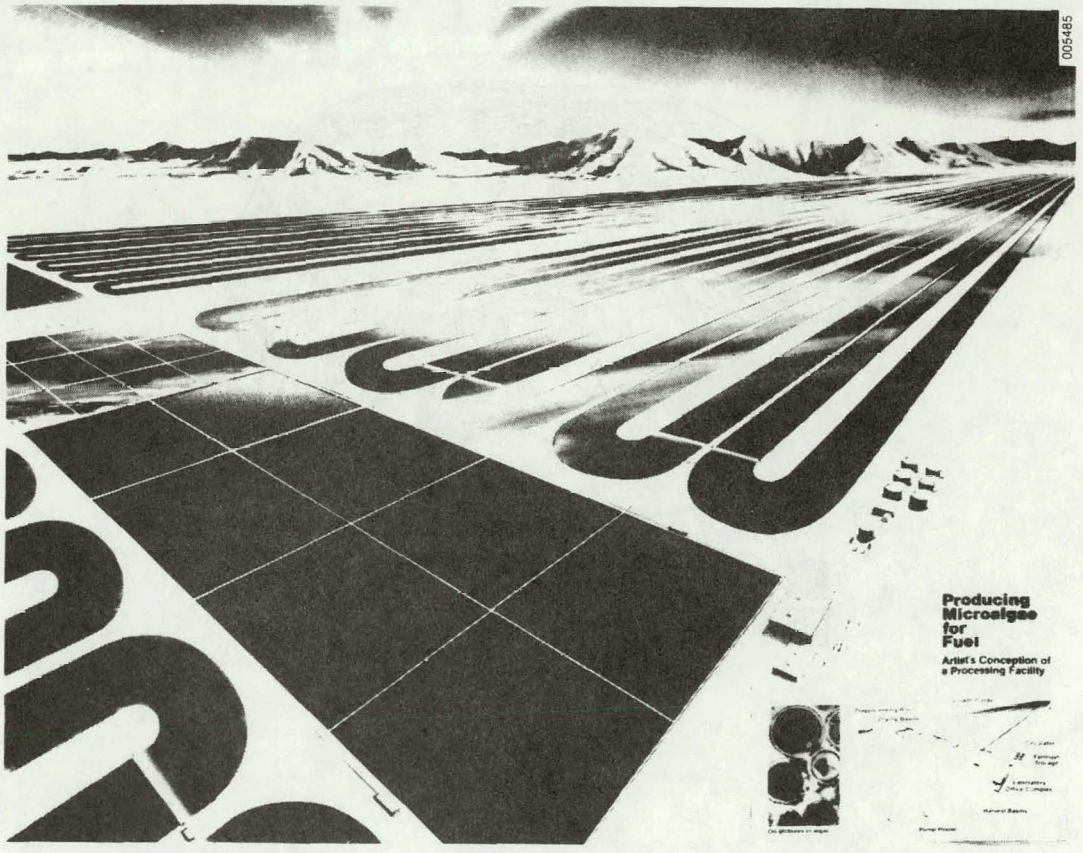


Figure 1-1. Artist's Concept of Microalgae Fuel Farm in the American Southwest

Table 1-1. Comparative Productivity Rates in Different Plant Communities
(adapted from Whittaker 1975)

Plant Community	g m^{-2} yr^{-1}
Continental:	
Tropical rain forest	2200
Temperate deciduous forest	1200
Woodland and scrubland	700
Desert and semidesert scrub	90
Cultivated land	650
Marine:	
Open ocean	125
Continental shelf	360
Algal beds and reefs	2500
Microalgae ponds	12,500

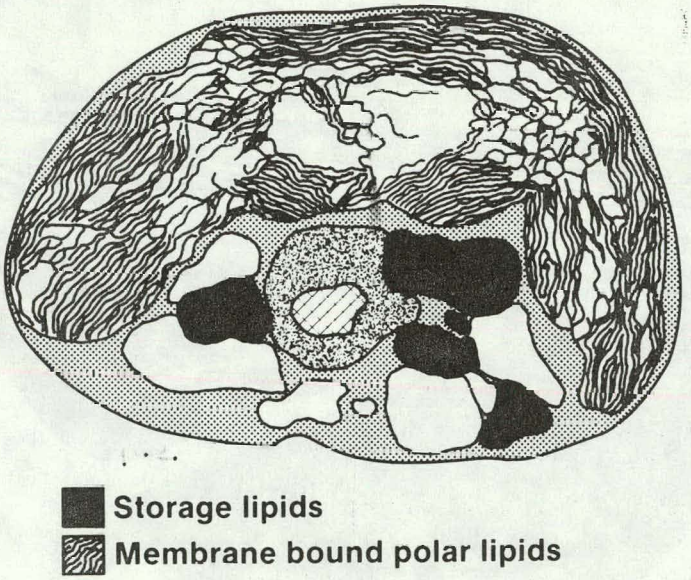


Figure 1-2. Micrograph of Algal Cell

abundant, low-cost source of protein has not been realized. The most promising early results of mass algae culture have been in the field of sanitary engineering, where microalgae are used to treat wastewater in oxidation ponds. From wastewater technology, application has been expanded to include protein production and treatment of irrigation water. Microalgae are being grown in Israel, Australia, Mexico, Taiwan, and the United States for high-value products for the health food market; these microalgae products include the alga *Spirulina* ($10,000/dry ton) and the vitamin beta carotene ($60,000/dry ton). Cultivating microalgae as a soil conditioner and as a food source for culturing fish and shellfish is increasing in importance.

Following the energy crises of the 1970s, the possibility of using algae as a source of energy received widespread attention. Microalgae can be grown in large outdoor ponds, using the resources of sunlight, saline water, nitrogen, phosphorus, and carbon dioxide to produce proteins, carbohydrates, and lipids (Johnson 1987). In the process, they can double their biomass three to five times a day. After a rapid growth phase, the algae can be transferred to induction ponds where, under nutrient limitation, many algae stop growth and division and use all their energy to make lipids as storage products for survival. Once the cells have accumulated lipids, they are harvested, and the water is recycled back into the growth ponds. The harvested cells are subjected to an extraction process to remove the lipids, primarily triglycerides with fractions of isoprenoids, phospholipids, glycolipids, and hydrocarbons. Lipids 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.

2.0 GOAL AND OBJECTIVES

The goal of the Aquatic Species Program is to develop the technology base for large-scale production of oil-rich microalgae and methods to convert the microalgae lipids into gasoline and diesel fuels needed for industry and transportation. To achieve this goal, the objectives of the program are to:

- Provide a slate of microalgal strains and determine their required growth conditions for high, sustained lipid production under outdoor conditions
- 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 and technically address resource requirements or limitations to grow microalgae in the desert Southwest of the United States
- Develop technologies for converting microalgae lipids into high-value liquid transportation fuels
- Transfer the technologies to the private sector for continued development and rapid commercialization by involving industry in the research process at the earliest possible time.

3.0 RESEARCH AND TECHNOLOGY DEVELOPMENT

The four main research areas critical to the development of microalgae technology for large-scale algal production and conversion into liquid fuels are (1) microalgae growth and production, (2) engineering design, (3) harvesting, and (4) conversion (Figure 3-1). The design of a microalgae mass culture system must be tailored to the characteristics of the culture organism while species must be selected that contribute to economic construction and facility operation. Microalgae must be selected that are environmentally tolerant, have high growth rates, and produce large quantities of lipids. In addition, the choice of a suitable species affects harvesting ease. The types of lipids that the algae produce will determine the conversion methods. Thus, all four areas are highly interactive. Each area of research and technology development and the major accomplishments in FY 1987 will be discussed in detail in the remainder of this report.

Approximately half of the research sponsored by the Aquatic Species Program is conducted in house at SERI, and the other half is subcontracted to universities and small businesses. Table 3-1 shows the funding breakdown by research areas for the FY 1987 budget of $1.7 million. Growth and production received 61% of the total budget; engineering design, 9%; harvesting, 3%; and conversion research, 6%. Analysis and resource assessment received 8% and management, the remaining 13%. A summary of the FY 1987 Aquatic Species Program active subcontracts is given in Table 3-2. This includes projects funded in FY 1986 and FY 1987.

3.1 Microalgae Growth and Production

As mentioned in Section 2.0, for microalgae technology to be successful, it is necessary to cultivate microalgae species that are tolerant to fluctuating temperatures and salinity, have high growth rates, and can produce large quantities of lipids. There are four subtasks in this area: species screening and characterization, lipid biochemistry, strain improvement, and genetic engineering.

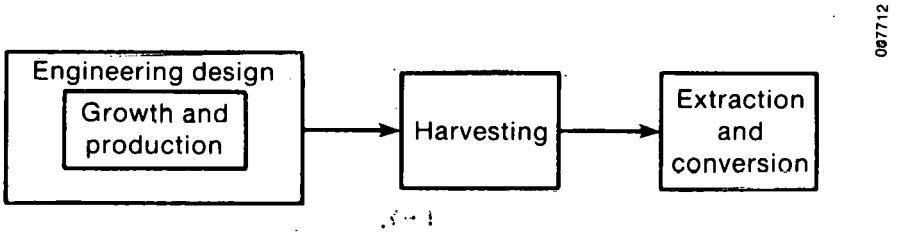


Figure 3-1. Four Stages Necessary to Produce Liquid Fuels from Microalgae

Table 3-1. FY 1987 Procurement Plan Summary For Aquatic Species Program

Task/Projects	Funding (1000 $)
Growth and Production	
Screening and Characterization	420
Lipid Biochemistry	185
Strain Improvement	190
Genetic Engineering	250
Engineering Design	155
Harvesting	50
Conversion	100
Analysis and Resource Assessment	130
Management	220
Total	1700

Table 3-2. Aquatic Species FY 1987 Active Subcontracts (FY 1986 and FY 1987 Funding)

	Title	Contractor	Date of Performance
1.	Optimization of outdoor culture	University of Hawaii	5/86 - 9/87
2.	Production of liquid fuels and chemicals by microalgae	Microbial Products	3/86 - 2/87
3.	Screening and characterizing oleagenous microalgae species from the Southeastern U.S.	Alabama A&M	2/86 - 1/88
4.	Characterization of hydrocarbon producing strains of microalgae	Scripps Institute of Oceanography	2/87 - 2/88
5.	Improvement of microalgal lipid production by flow cytometry	Oak Ridge National Laboratory	12/86 - 11/87
6.	Collection of high energy yielding strains of saline microalgae from the Hawaiian Islands	University of Hawaii	3/86 - 10/87

Table 3-2. Aquatic Species FY 1987 Active Subcontracts (FY 1986 and FY 1987 Funding) (Concluded)

	Title	Contractor	Date of Performance
7.	Genetic variation in high energy yielding microalgae	City College of New York (cost shared 70:30)	3/86 - 12/87
8.	Collection of high energy yielding strains of saline microalgae from southwestern states	Arizona State University (cost shared 75:25)	3/86 - 5/87
9.	The effects of fluctuating environments on the selection of high yielding microalgae	Georgia Institute of Technology (cost shared 80:20)	3/87 - 4/88
10.	Collection and selection of high energy thermophilic strains of microalgae	Montana State University	3/86 - 5/87
11.	Characterization of photosynthetic efficiency and growth for selected microalgae in dense culture	Martek	9/85 - 2/87
12.	Nutritional requirements for maximal growth of oil-producing microalgae	Jackson State University	9/86 - 12/87
13.	Chrysophycean lipids: Effects of induction strategy in the quantity and types of lipids	Selma University	1/87 - 1/88
14.	Biochemical elucidation of neutral lipid synthesis in microalgae	Montana State University	1/86 - 10/87
15.	Transformation and somatic cell genetics for the improvement of energy production in microalgae	University of Nebraska	3/87 - 3/88
16.	Biochemical elucidation of neutral lipid synthesis in microalgae	University of Nebraska	3/87 - 3/88
17.	Algal genetics	Neushul Mariculture, Inc. (cost shared 20:80)	2/86 - 1/87
18.	Design and operation of a microalgae outdoor test facility	Microbial Products	3/87 - 3/88

3.1.1 Species Screening and Characterization

By 1986, more than 3000 microalgal strains had been collected from diverse geographical locales and ecological niches. A major screening effort is now under way to reduce these to the best 10-25 strains that have the desired characteristics by FY 1990. The most promising strains collected are Bacillariophyceae (diatoms), Chlorophyceae (green algae), and Eustigmatophyceae (Eustigmatophytes). The program has identified several strains that tolerate severe environmental fluctuations in temperature and salinity. Strains of microalgae used by the program in 1982 exhibited temperature tolerances of 15°-25°C and salinity tolerances of 20-40 mmho cm^{-1}. With the intensive collection efforts nationwide, the program now has strains that can tolerate 10°-35°C and 10-85 mmho cm^{-1} (for comparison, seawater is approximately 35 mmho cm^{-1} salinity).

The screening and characterization of lipid-producing microalgae has been a subtask in the Aquatic Species Program since FY 1983. The following three areas have been emphasized in FY 1987: (1) characterizing warm-water strains, (2) initiating a cold-water strain collection and screening project, and (3) developing a screening protocol to be used in reducing the number of strains in the program from 3000 to 300 by the end of FY 1988.

Ten warm-water microalgae were characterized by SERI (see Appendix, Johansen et al.), seven of which were added to the *Microalgae Culture Collection 1986-1987* (Barclay et al. 1986). The *Chaetoceros* strains had the greatest tolerance for high salinity. *Chaetoceros* 9 and 10 and *Navicula* 1 are considered the best new strains of the 10 characterized, and further study of these strains is under way.

Forty-nine strains from the desert Southwest (see Appendix, Sommerfeld et al.), primarily diatoms and green algae, demonstrated growth rates exceeding one doubling per day, and five exceeded two doublings per day. The fluorescent dye Nile red was used to microscopically screen for intercellular lipid storage. Fluorometric quantification revealed that seven microalgal cultures yielded more than 200 mg L^{-1} triolein equivalent lipids, and four yielded greater than 400 mg L^{-1}. Preliminary growth optimization efforts with 15 of the strains indicate that the most rapid growth occurs when isolates are cultured with urea as the nitrogen source at 25°C under 500 µE m^{-2} s^{-1} of light. For many of the strains, altering the nitrogen source and elevating the light intensity from 25 to 50 µE m^{-2} s^{-1} had relatively little effect on growth rate. In addition, 55 strains were isolated in FY 1987 from Alabama and Mississippi and five were characterized (see Appendix, Tadros).

Cheng and Lewin (see Appendix) studied the production of lipids in 10 selected strains of marine microalgae that exhibit tendencies to float. During the past three years, 300 samples of surface sea-water strains were collected, and after a simple enrichment procedure, 80 different strains were isolated. All were tested for their salinity and temperature ranges for growth and their requirements for exogenous vitamins.

Six additional strains of the best-growing algae in the culture collection had their nutritional requirements defined (i.e., nitrate, nitrite, urea, phosphate, silica, and iron; Rhyne, see Appendix). Another study by Sriharan et al. (see Appendix) determined the effects of nutrient deficiency and temperature on fatty acid composition. The percentage of 16:1 and 18:1 fatty acids increased in the nutrient-deficient culture.

Mass-culture systems exhibit diurnal and seasonal fluctuations in key parameters affecting algal productivity and competitiveness: temperature, pH, oxygen, carbon dioxide, light, and nutrient availability. In species competition experiments with several

strains of algae isolated by the Aquatic Species Program, certain parameters were more important than others in affecting species dominance: a diurnal cycle of high oxygen was more important than fluctuating temperature, which was more important than fluctuating pH. A computer model was developed that can predict the average diurnal and seasonal variations in pond environmental parameters (oxygen, pH, carbon dioxide, temperature, and light intensity).

Research efforts are continuing to increase the rates of productivity of microalgae to enhance yields of energy products. Research is being conducted in both laboratory and outdoor cultures to identify species and to develop culture management strategies that improve productivity rates. The level of productivity for microalgae shown in Table 1-1 has been met in the laboratory and in small outdoor test ponds on a short-term basis. For several months we have been able to sustain productivity rates at 70% of the target.

3.1.2 Strain Improvement

Conditions of high solar irradiance, pH, dissolved oxygen, salinity, and low carbon dioxide anticipated during the growth of algae may induce photorespiratory losses in phytoplankton. Algae have been screened for high specific growth rates under these conditions, with net production the primary concern. However, modifying conditions conducive to respiratory losses may result in even higher net production.

Photorespiration increased dramatically with pH, salinity, dissolved oxygen, and light intensity (see Appendix, Cohen). However, photosynthesis also increased faster than photorespiration at increasing light intensity. Thus, net production was still highest at the highest light intensity.

Experiments were performed to develop a computer model to predict the effects of environmental parameters on lipid production (see Appendix, Chelf et al.). Factors examined included nitrogen concentration, silicon concentration, temperature, time, conductivity, and alkalinity. Measured characteristics included Nile red fluorescence and ash-free dry weight (AFDW). The multiple regression model from the ratio of Nile red fluorescence to AFDW has an R^2 of 89.34%. The most important variables in this regression model were nitrogen concentration and conductivity. This type of model may be used as a predictive model for complex biological systems.

Since carbon dioxide supply is a potentially costly part of algal lipid production, knowledge of minimum carbon requirements for maximum productivity is valuable. Carbon dioxide compensation points were examined in several strains of microalgae under nutrient-sufficient and nutrient-limited conditions (Chelf, pers. comm.). Two phases of carbon utilization were seen when cells were bubbled with air plus carbon dioxide and were preincubated. These two-phase curves were never seen with air-bubbled cells. The compensation points and the ratio of final inorganic carbon to alkalinity will be tested to see if they can predict conditions where carbon uptake will be less than optimal and to compare the carbon uptake ability of different species. Eventually we hope to correlate these data with the lipid production potential of microalgae under nutrient limitation.

Methods for increasing the reliability of using flow cytometry to produce high-lipid algal strains were developed this year (see Appendix, Solomon and Palumbo). Earlier results indicated that cells could be sorted with a flow cytometer on the basis of their lipid content after staining with a fluorescent dye that is specific for neutral lipids. However, the resulting cultures often did not have higher lipid content than the parent cultures when measured many generations after the sort. Recent results suggest that by taking cell cycle differences into account, high-lipid daughter populations, when grown for a

number of generations (at least up to three months), can retain their enhanced lipid levels. When lipid level was used as the sole criterion for sorting, sorts were successful and resulted in high-lipid daughter populations if they took place after the cells had stopped dividing. Exponentially growing populations were sorted successfully when the chlorophyll:lipid ratio was used for defining the sorting window. This procedure results in cell selection at all stages in the cell cycle and yields cells that are 25%-30% higher in lipid content than the average cell at that growth stage.

3.1.3 Lipid Biochemistry

The goal of the third major research area in microalgae growth and production is to increase the amount of lipid in each algal cell; our target is 60% lipid in outdoor cultures. Significant increases have been made in lipid quantity, from 20% of cell content in 1982 to 66% in the laboratory and 40% outdoors in 1987. We are able to induce lipid accumulation by removing nitrogen or silica (major nutrients required for growth) from the media. During this period of stress, some algae will begin to build up reserves of carbohydrates and others will accumulate lipids. In addition to nutrients, temperature, pH, inorganic carbon, and light can all affect the cell's lipid quantity.

Initial steps were taken to determine the contribution of neutral lipid synthesis to neutral lipid quantity (see Appendix, Guckert et al.). Experiments involving pH indicated that manipulating pH and the inorganic carbon concentration increases neutral lipid accumulation in *Chlorella* at nonlimiting nitrate concentrations. These experiments provide evidence of a more universal mode of action of so-called neutral lipid "triggers" that can be exploited in the laboratory and perhaps outdoors. Various nutrient-deprivation regimes may all affect neutral lipid accumulation by disrupting the cell cycle.

To improve lipid yields in microalgae, we must understand the physiological and biochemical basis for partitioning photosynthetically fixed carbon dioxide 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 carbon dioxide 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, it is expected that biochemical and genetic engineering techniques can be used to increase the lipid yield of promising algal strains.

Previous studies found that, in some algae, silicon deficiency induces an increase in the fraction of newly assimilated carbon that is partitioned into lipids and a decrease in the fraction that is partitioned into storage carbohydrate. Studies in FY 1987 (see Appendix, Roessler) focused on the enzymology of the carbohydrate and lipid biosynthesis enzymes. The storage carbohydrate is synthesized by two enzymes. One enzyme was not affected by silicon deficiency, but the activity of the other decreased 30%. Three enzymes present in the alga are involved in lipid synthesis. One of these increased two-fold after silicon deficiency. The induction of the enzyme could be blocked by protein synthesis inhibition or gene transcription inhibitors. These results suggest that the increase in carbon allocation after silicon deficiency into lipids is probably due in part to

reduced activity of the carbohydrate synthesis enzymes and increased activity of the lipid synthesis enzymes.

Since a commonly used lipid trigger, nitrogen deficiency, rapidly reduces photosynthetic capacity, it is useful to separate effects of nitrogen deficiency on photosynthetic efficiency from effects on carbon partitioning. Initial studies have therefore used two lipid-storing algae (see Appendix, Coleman et al.). Lipid amount per cell increased during nitrogen deficiency and decreased with nitrogen addition. In two species, the increase in cellular lipid was not associated with an increase in lipid as a fraction of cellular dry weight. Nitrogen deficiency induces chlorophyll loss in all algae studied. The dependence in nitrogen-deficient cells of chlorophyll and chloroplast protein loss on high light intensities is consistent with photooxidative damage to the chloroplast. Based on these studies, photooxidative damage to the chloroplast may be a major factor limiting photosynthetic lipid yields in nitrogen-deficient cells.

3.1.4 Genetic Engineering

To date, 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 has begun on developing genetic engineering methods so that by 1990, when the program has reduced its strains to the best 10-25, the methods to modify these organisms genetically will be available.

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

It is likely that the long-term economic feasibility of using microalgae for fuel production will depend on the development of strains that have been genetically altered to improve lipid yields. The strategy to be used for developing improved strains depends on the patterns of genetic diversity found among the available wild-type strains in each species. Therefore, an investigation was undertaken of the genetic diversity in three types of microalgae with potential for oil production (see Appendix, Gallagher). Both gel electrophoresis and comparisons of the physiological traits were used. The former technique can be used to classify clones into discrete groups, and the banding patterns can be used as genetic markers in later manipulations. Examination of physiological traits yields information on continuous characteristics that are directly relevant to how different strains might perform in culture. In all three organisms, genetic diversity was found to be extremely high compared to terrestrial plants. This indicates that the genetic diversity in the species examined was underestimated.

During FY 1987, we also examined the genetic variability present within single species of microalgae (see Appendix, Johansen et al.). Clones of a species sampled from different sites are often similar in their physiological response to conductivity, temperature, and nutrient stress. However, growth rates and lipid contents will vary significantly between clones, so that one or a few strains of a species can be designated as better candidates.

Intensive study has been directed toward establishing genetic diversity in *Chaetoceros muelleri* this year. This species was chosen for study because several clones have high growth rates, broad salinity and temperature tolerances, and high lipid content. More than 200 clones of this species were isolated, and their genetic variability both within populations from a single site and between clones from widely separated sites was studied. Differences in allozyme banding patterns are evident even in clones isolated

from a single collection. Correlations between morphological traits and physiological characteristics were noted.

The work on genetic diversity within single microalgal species has shown, at least among the species tested, that considerable genetic variability is present, even within clones isolated from the same sites. Such variability is desirable in that it indicates species have a large collective genome, and thus future genetic engineering efforts have a better chance of success.

The chloroplast genome of a model microalgal species was characterized (see Appendix, Meints) to determine whether these methods might be a diagnostic device for identifying specific *Chlorella* strains that are capable of biofuel production. If this were so, such genomes would be extremely useful for determining relationships between groups of algae and might determine the strategy for selecting partners in cell hybrid fusions. Unless a reasonably close relationship exists between fused partners, that stability of the hybrid is considered to be limited. Isolated chloroplast and nuclear genomic DNA from 13 strains of *Chlorella* were analyzed for relationships, and maps of the genomes were produced. Based on preliminary investigations it appears that algae can be easily grouped into specific classes according to their DNA restriction length polymorphisms; many or all algae could be analyzed in this manner, providing substantial information about algal relationships.

Two other genetic engineering methods being developed are fusing protoplast and finding suitable viral vectors for the algae (Meints, pers. comm). Protoplasts have been formed successfully from some algae cells using enzyme preparations. Work is continuing to regenerate the protoplasts to cell colonies. Viral vectors have been found, but none of them will attach to a free-living algal host. More than 250 algae were screened, but none of them were successful hosts for the viruses.

3.2 Engineering Design

Growth conditions in algae mass cultures can be divided into two categories: those dictated by the location of the culture, and those based on culture management strategy. Location-related variables include insolation, evaporation, rainfall, temperature, and wind velocity. Variables that can be managed include salinity, nutrient concentration, carbon dioxide concentration, culture mixing, culture aeration, and residence time of the population.

Growth conditions dictated by location will be among the prime considerations in siting the production facility. The DOE/SERI program has been based on the assumption that it will be necessary to locate a production facility in an area that receives large amounts of sunlight and has relatively warm temperatures. To have the best success, the facility must be located in an area that receives 5000 kcal m^{-2} d^{-1} and has more than 180 frost-free d yr^{-1}. This limits large-scale production to the southern United States (Figure 3-2).

Sunlight drives the production of biomass; therefore, ideal production systems will be located in areas that receive high insolation. It is also necessary to consider the trade-offs involved in achieving high insolation. If the costs of land, raw materials, or operation are significantly increased at a location with high insolation, siting solely by the solar input may be disadvantageous.

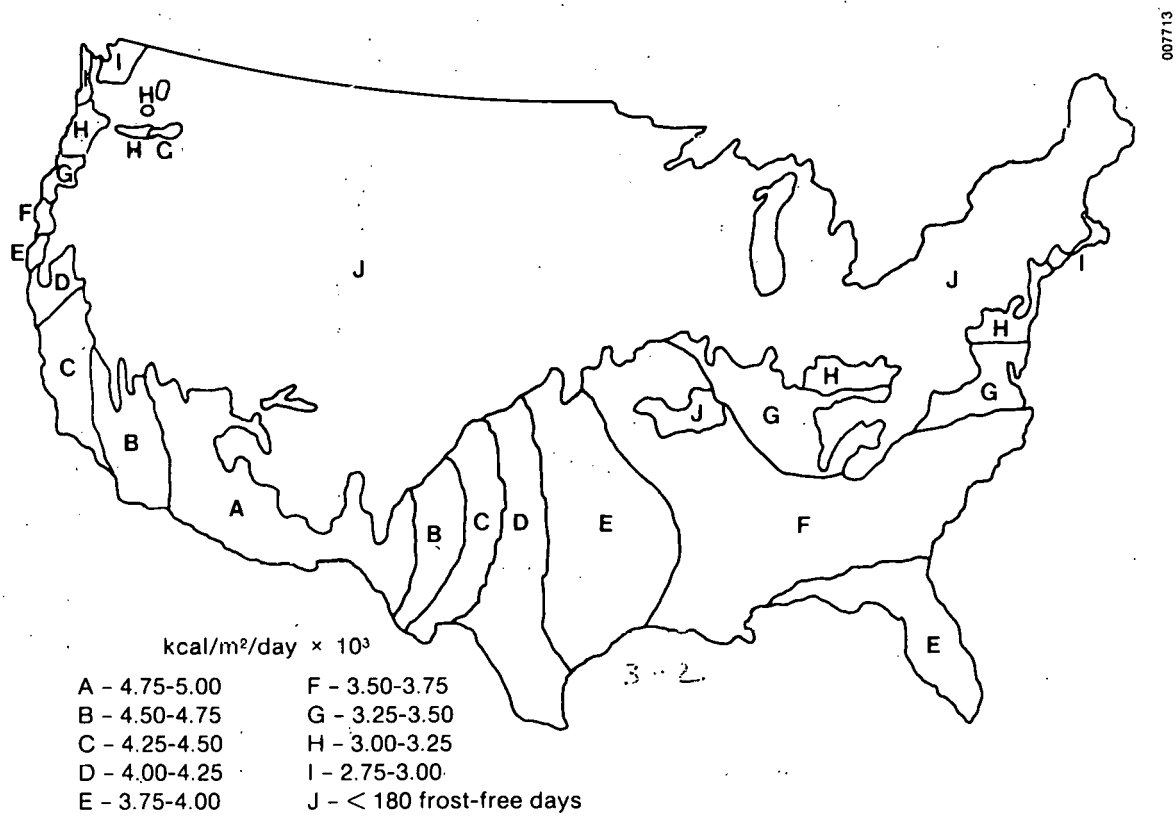


Figure 3-2. Solar Radiation in the United States Where There are More than 180 Frost-Free d yr^{-1}

Many other production conditions in outdoor systems are dictated by the engineering design and the management strategy. For example, if saline water is used, the salinity can be maintained at any given range governed by the algal species selected. Nutrient concentrations, carbon dioxide concentration, culture mixing, culture aeration, and culture turnover can also be selected.

The three designs examined in FY 1984 for the large-scale production of microalgae were open ponds, raceways, and enclosed tubes. The proposed costs for construction and operation of these three systems are shown in Table 3-3. Since all costs need to be kept to a minimum for the feedstock to be produced inexpensively, thereby producing an economic liquid fuel, open-pond systems were chosen as the facility plan for outdoor production (Figure 3-3).

Table 3-3. Capital Construction Costs for Three Different Algal Production Systems

Engineering Design	Cost ($/ha)
Open ponds	76,000
Raceways	161,000
Enclosed tubes	348,000

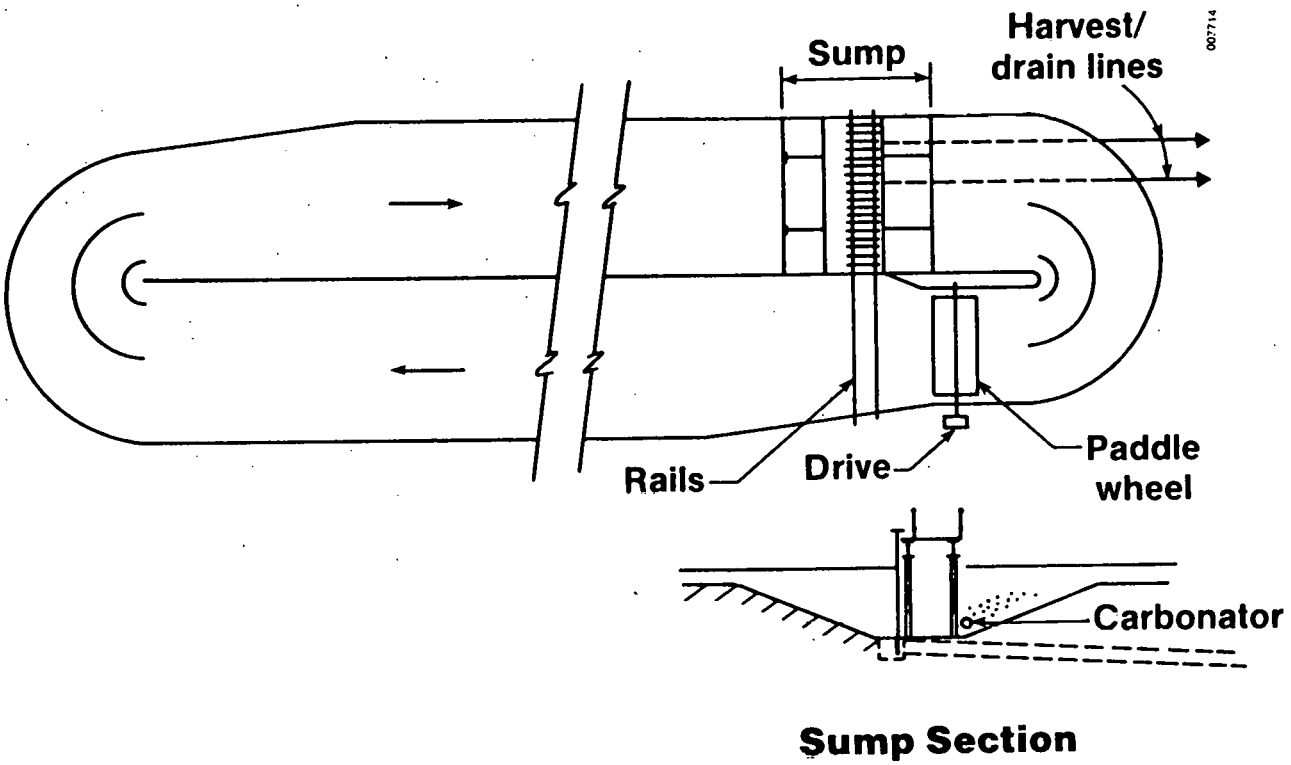


Figure 3-3. Open-Pond Design for Large-Scale Microalgae Production

Using the open-pond design, a microalgae outdoor test facility (OTF) is being constructed in Roswell, N. Mex., for the Aquatics Species Program. Microbial Products, Inc., of California is designing, constructing, and operating the facility (see Appendix, Weissman, Tillett, and Goebel). The OTF will allow the program to evaluate outdoor production performance of microalgae and examine the problems and potential of scaling-up and operating large microalgae-production systems. This facility will allow the program to begin evaluations using saline groundwater in the desert Southwest.

During FY 1987 a small-scale system consisting of six 3-m^2 fiberglass open ponds was constructed and operated to evaluate the performance of algal species in terms of productivity and lipid content. The ease of operating and the low cost of constructing several of these small cultivation units makes them ideal for performing controlled, replicated experiments.

Since the ultimate goal of the Aquatic Species Program requires the production of large amounts of biomass, larger scale research systems are needed. Two larger open-pond systems, each 0.1 ha, were designed and are under construction. These will be used to compare the performance of low cost earthen liners with the performance of expensive plastic membranes. Other scale-up problems will also be addressed in these and in a 0.5 ha demonstration open-pond system to be built in FY 1989 based on available funding. Biological and engineering assessments of microalgae production systems will be performed at the OTF. In addition, after several years of operation, a detailed economic analysis will be done to provide state-of-the-art economics for large-scale production.

Four 1.4-m^2 ponds were also constructed in the SERI greenhouse in FY 1987. The design for the ponds is similar to the OTF. The ponds are monitored for temperature, light intensity, and pH. These ponds will be used to supplement research at the OTF since many conditions can be controlled because the ponds are located indoors.

Navicula 1 was grown successfully in the greenhouse ponds for five weeks. The maximum production was 17 g m^{-2} d^{-1} AFDW. The ponds act as replicates with the exception of one pond, whose performance might be affected by its orientation to the sun. *Chaetoceros* is currently being grown in the ponds, and maximum production is about 10 g m^{-2} d^{-1}.

3.3 Harvesting

After the cells are grown, they must be separated from the water efficiently and economically. Techniques for harvesting microalgae include settling or flotation, centrifugation, and filtration. These processes are aided by cell flocculation, either through the addition of chemical flocculants or through culture autoflocculation. Flocculation causes the cells to aggregate into larger clumps, which are more easily filtered or settle more rapidly. The ease of harvesting algae depends primarily on the organism's size, which determines how easily it can settle and be filtered. The most rapidly growing algal species are frequently very small and often motile unicells--the most difficult to harvest. Thus, it is necessary to maintain an effective interaction between the development of harvesting technologies and the selection of algal species for mass culture.

With current techniques and instrumentation, all microalgae can be harvested with polymers, although this is not economical. Polymer harvesting is technically feasible, but different algae need different polymers. The amount of polymer increases as the clarification requirement becomes 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¢-1.5¢ kg^{-1} dry mass, with removal efficiencies of 85%-95%. Currently, harvesting systems represent approximately 25% of the total capital investment in a microalgae mass culture facility. Polymers with higher rigid backbones are less affected by the salt concentration and are recommended as flocculants of microalgae in saline water.

Chemical flocculation aids harvesting regardless of the harvesting method used. Flocculant dose was reduced 75% by recycling the precipitant following flocculation back into the mixing-flocculation chamber. Using three flocculation cycles reduced the required chemicals and removed 90% of the microalgae from the water. By reducing the chemical dose the process is more economical.

3.4 Conversion

Analysis of fuel conversion options for microalgae biomass has demonstrated that the promise of microalgae for fuel production is best realized by using conversion processes based on cellular lipids. The two most promising fuel conversion options are transesterification to produce fuels similar to diesel fuels and catalytic conversion to produce gasoline. Although microalgae 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 anaerobically digested for the production of methane and carbon dioxide.

We do not believe that the algal lipids can be used directly as a fuel either alone or blended with crude petroleum. Algal lipids contain approximately 10% oxygen; crude petroleum contains essentially no oxygen. The oxygenates would react at the high temperatures used in crude distillation and cause polymerization or other undesirable reactions.

Research is just beginning on the processes to extract and convert algal lipids into gasoline and diesel fuels. The first step in the process is the conversion of triglycerides into free fatty acids for transesterification. A naturally occurring enzymatic process within the algae, which accomplishes this conversion, was investigated. Promising species of lipid-producing microalgae were screened for the presence of this enzymatic activity. A regression model was developed to predict the effects of important variables on this process. Results indicate that silica concentration and temperature are important variables that control the conversion of triglycerides into free fatty acids. If the algae are left for 16-24 h at 20°C, more than one-half of the triglycerides are converted to fatty acids. This step may be an economical method since no chemicals are required.

4.0 FUTURE ACTIVITIES

Since the inception of the program, many improvements have been made in finding suitable species to produce fuels from microalgae. More than 3000 strains of microalgae were collected, and the collection program was terminated in FY 1986. A major effort between now and FY 1990 will be screening and characterizing these 3000 strains and reducing them to the best 10-25 strains. Research in strain improvement, lipid biochemistry, and genetic engineering will continue in FY 1988.

By the beginning of FY 1988, two 0.1-ha ponds will be constructed at the OTF. In FY 1989 a 0.5-ha pond will be built based on available funding. The OTF research for next year is to determine the performance of inexpensive earthen liners versus expensive plastic liners. An entire year of productivity data will give the first estimates of average production from these systems in the Southwest.

New harvesting and conversion research projects will begin in the first quarter of FY 1988. Innovative and improved methods for microalgae harvesting will be examined. In addition, 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 lipids 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 OTF. Samples from a number of promising species should be included since there are strong indications that the characteristics of lipids vary widely between taxa.

A major emphasis next year will be placed on assessing the resource requirements and environmental impacts of this technology. Assessment projects will be done on carbon dioxide supply, disposal of the brine generated from the algal ponds, and any possible impacts on climate from the ponds.

The net result of the research to date has been to reduce the projected price of gasoline derived from microalgae from \$18 gal^{-1} in 1983 to approximately \$7 gal^{-1} in 1987. However, many more developments are needed in the technology in the upcoming years to reduce the price of gasoline from microalgae to be competitive with fossil fuels by 2010.

5.0 BIBLIOGRAPHY

Barclay, W. R., J. R. Johansen, P. Chelf, N. Nagle, P. Roessler, and P. Lemke, 1986, *Microalgae Culture Collection 1986-1987*, SERI/SP-232-3079, Golden, Colo: Solar Energy Research Institute.

Benemann, J. R., D. M. Tillett, and J. C. Weissman, 1987, "Microalgae Biotechnology," *Trends in Biotechnology*, Vol. 5, pp. 47-53.

Department of Energy (DOE), 1987, *Energy Security: A Report to the President of the United States*, Washington, D.C.: Department of Energy.

Johnson, D., 1987, *FY 1986 Annual Report of the Aquatic Species Program*, SERI/SP-231-3071, Golden, Colo.: Solar Energy Research Institute.

Neenan, B., D. Feinberg, A. Hill, R. McIntosh, and K. Terry, 1986, *Fuels from Microalgae: Technology Status, Potential and Research Requirements*, SERI/SP-231-2550, Golden, Colo.: Solar Energy Research Institute.

Weissman, J. C., and R. P. Goebel, 1987, *Design and Analysis of Microalgal Open Pond Systems for the Purpose of Producing Fuels*, SERI/STR-231-2840, Golden, Colo.: Solar Energy Research Institute.

Whittaker, R. H., 1975, *Communities and Ecosystems*, 2nd edition, New York: MacMillan Publishing Co.

6.0 FY 1987 PUBLICATIONS AND PRESENTATIONS

6.1 Publications

Barclay, W., N. Nagle, K. Terry, S. Ellingson, and S. Sommerfeld, 1987, "Characterization of Saline Groundwater Resource Quality for Aquatic Biomass Production: A Statistically Based Approach," *Journal of Water Research*, in press.

Barclay, W., K. Terry, N. Nagle, J. Weissman, and R. Goebel, 1987, "The Potential of New Strains of Marine and Inland Saline Adapted Microalgae for Aquaculture Applications," *Journal of the World Aquaculture Society*, in press.

Benemann, J. R., D. M. Tillett, and J. Weismann, 1987, "Microalgae Biotechnology," *Trends in Biotechnology*, 5:47-53.

Cooksey, K., J. Guckert, S. Williams, and P. Collis, "Fluorometric Determination of the Neutral Lipid Content of Microalgal Cells Using Nile Red," *Journal of Microbiological Methods*, in press.

Guckert, J. B., K. E. Cooksey, and L. L. Jackson, 1987, "Lipid Solvent Systems are not Equivalent for Analysis of Lipid Classes in the Microeukaryotic Green Alga, *Chlorella*," Journal of Microbiological Methods, submitted for publication.

Johansen, J., G. Doucette, J. D. Bull, and W. Barclay, 1987, "The Morphology and Physiology of *Pleurochrysis carterage* var. *denata* var. nov. (Pyrmnesiophyceae), a New Coccolithophoird Species from an Inland Saline Pond in New Mexico, U.S.A." *Phycologia*, in press.

Johansen, J., and E. Theriot, 1987, "The Relationship Between Value Diameter and Number of Central Fultoportnlae in *Thalassiosira weissflogii* (Bacillariophyceae)," *Journal of Phycology*, in press.

Johnson, D. and S. Sprague, 1987, "Liquid Fuels From Microalgae," presented at the 22nd IECEC, Philadelphia, Penn., SERI/TP-231-3202.

Johnson, D., 1987, *An Overview of the DOE/SERI Aquatic Species Program - FY 1986*, SERI/SP-231-3072.

Johnson, D., 1987, *FY 1986 Annual Report of the Aquatic Species Program*, SERI/SP-231-3071.

Johnson, D., 1987, "The Technology and Cost of Producing Triglyceride Liquids from Microalgae for Use as Fuels," presented to the IGT Symposium, Energy from Biomass and Wastes XI, Orlando, Fl., March 15-20, in press.

Laws, E. A., S. Taguchi, J. Hirata, and L. Pany, 1986, "Continued Studies of High Algal Productivities in a Shallow Flume," *Biomass*: II:39-50.

Laws E., S. Taguchi, J. Hirata, and L. Pany, "Optimization of Microalgal Production in a Shallow Outdoor Flume," *Biomass*, submitted for publication.

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

Meints, R. H., D. E. Burbank, J. L. Van Etten, and D. T. A. Lamport, "Properties of the *Chlorella* Receptor for the Virus PBCV-1," Submitted to *Virology*.

Meints, R. H., K. Lee, and J. L. Van Etten, 1986, "Assembly Site of the Virus PBCV-1 in a *Chlorella*-like Green Alga: Ultrastructural Studies," *Virology*, Vol. 154, pp. 240-245.

Roessler, P. G., "UDP-Glucose Pyrophosphorylase Activity in the Diatom *Cyelotella cryptica*: Pathway of Chrysoaminarin Biosynthesis," *Journal of Phycology*, in press.

Roessler, P. G., 1987, "Characteristics of Abrupt Size Reduction in *Symedea ulna* (Bacillariophyceae)," *Phycologia*, in press.

Solomon J., and R. E. Hand, 1987, "Flow Cytometry Reveals Rapid Response to Nitrogen Limitation in Microalgae Cultures," *Journal of Phycology*, submitted for publication.

Solomon, J., R. Hand, and R. Mann, 1987, *Ultrastructural and Flow Cytometric Analyses of Lipid Accumulation in Microalgae*, Golden, Colo.: Solar Energy Research Institute, SERI/STR-231-3089.

Tillett, D., and J. Benemann, 1987, "Techniques for Maximizing Lipid Formation in Microalgae Production," presented to the IGT Symposium, Energy from Biomass and Wastes XI: Orlando, Fla., March 15-20, in press.

Van Etten, J. L., D. E. Burbank, and R. H. Meints, 1986, "Replication of the Algal Virus PBCV-1 in UV-Irradiated *Chlorella*," *Intervirology*, Vol. 26, pp. 115-120.

Van Etten, J. L., Y. Xia, K. E. Narva, and R. H. Meints, 1986, "*Chlorella* Algal Viruses," in *Extachromosomal Elements In Lower Eukaryotes*, edited by R. B. Wickner, A. Hinnebusch, A. M. Lambowitz, I. C. Gunsalus, and A. Hollaender, New York: Plenum Publishing Corp., pp. 337-347.

Van Etten, J. L., Y. Xia, and R. H. Meints, 1987, "Viruses of a *Chlorella*-like Green Alga," in *Plant-Microbe Interactions*, Vol. II, edited by T. Kosuge and E. W. Nester, New York: Macmillan Publishing Co., pp. 307-325.

Weissman, J., R. Goebel, and J. Benemann, "Photobioreactor Design: Mixing, Carbon Utilization, and Oxygen Accumulation," *Biotechnology and Bioengineering*, in press.

Weissman, J. C., and R. P. Goebel, 1987, *Design and Analysis of Microalgal Open Pond Systems for the Purpose of Producing Fuels*, Golden, Colo.: Solar Energy Research Institute, SERI/STR-231-2840.

6.2 Presentations

Barclay W., P. Chelf, and P. Lemke, "Effects of Environmental Parameters on Lipid Production in *Navicula saprophila*," presented at the Annual Meeting of the Phycological Society of America, Ohio State University, Columbus, Ohio, August 9-13, 1987.

Barclay, W., P. Chelf, and N. Nagle, "Environmental Control of Fatty Acid Composition in Microalgae," presented at the Annual Meeting of the Phycological Society of America, Ohio State University, Columbus, Ohio, August 9-13, 1987.

Benemann, J., and D. Tillett, "Microalgae as a Source of Liquid Fuels: Economic Analysis and Experimental Status," presented at the Fourth Southern Biomass Energy Research Conference, Atlanta, Ga., October 7-9, 1986.

Ellingson, S. B., and M. R. Sommerfeld, "Growth Characteristics and Lipid Production of Selected Microalgae Isolated from the Arid Southwest," presented at the Annual Meeting of the Phycological Society of America, Ohio State University, Columbus, Ohio, August 9-13, 1987.

Gallagher, J. C., and J. Stabile, "Patterns of Genetic Diversity in Population of Three Types of Oil-Producing Microalgae and Their Implications from Applied Phycology," presented at the Annual Meeting of the Phycological Society of America, Ohio State University, Columbus, Ohio, August 9-13, 1987.

Johansen, J., "Genetic Variability within the Diatom *Chaetoceros mullerii*," presented at the 13th Annual Meeting of the Guild of Rocky Mountain Population Biologists, Saratoga, Wyo., September 11-13, 1987.

Johansen J., "Harvesting Oils from Diatoms Found in Arid Environments," presented at a seminar at the Department of Biology of Northern Arizona University, Flagstaff, Ariz., April 17, 1987.

Johansen, J. R., A. J. Doucette, and W. R. Barclay, "The Morphology and Physiology of *Pleurochrysis dentata* sp. nov., A New Coccolithophorid from New Mexico," presented at the Annual Meeting of the Phycological Society of America, Ohio State University, Columbus, Ohio, August 9-13, 1987.

Johansen, J., W. Rayburn, and J. Ashley, "The Recovery of Algal Soil Crusts Following Rangefire," presented at a seminar to the Arid Lands Ecology Reserve, Battelle Northwest Laboratories, Richland, Wash., March 1987.

Johnson, D., "Overview of the Aquatics Species Program and the Plans for the Outdoor Test Facility," presented to the New Mexico Governor's Science and Technology Commission, Roswell, N. Mex., April 21, 1987.

Johnson, D., "Solar Fuels: An Overview," presented at a seminar to the Chemistry Department of the United States Air Force Academy, February 24, 1987.

Roessler P. G., "Biochemical Aspects of Lipid Accumulation in Silcon-Deficient *Cyclotella cryptica*," presented at the Annual Meeting of the Phycological Society of America, Ohio State University, Columbus, Ohio, August 9-13, 1987.

Solomon, S., K. McKinney, G. Sutton, and C. Rhyne, "Laboratory Growth Studies of Oil Producing Microalgae," Mississippi Academy of Science, Annual Meeting, 1987.

Sommerfeld, M., "Lipid Production and Growth Characteristics of Cultured Microalgae Isolated from the Arid Southwest," Arizona-Nevada Academy of Science Annual Meeting, 1987.

Sommerfeld, M., "Relationships of Total and Relative Ionic Composition to Distribution of Microalgae in the Southwest," Arizona-Nevada Academy of Science Annual Meeting, 1987.

Sriharan S., and D. Bagga, "Effects of Environmental Conditions on Lipid Production in the Diatom *Chaetoceros* (DI-35) and *Chaetoceros* (SS-14)," presented at the Ninth Symposium on Biotechnology for Fuels and Chemicals, Boulder, Colo., May 5-8, 1987.

Sriharan S., D. Bagga, and T. Sriharan, "Effects of Induction Strategies on the Lipid Production and Fatty Acids in *Cyclotella* (DI-35) and *Chaetoceros* (55-14)," presented at the Annual Meeting of the Phycological Society of America, Ohio State University, Columbus, Ohio, August 9-13, 1987.

Sriharan, S., D. Bagga, T. Sriharan, and M. Das, "Enhanced Biomass Lipid Production in the Microalgae *Monoraphidium* under Controlled Environmental Conditions," presented at the Second American Society of Microbiology Conference on Biotechnology, San Diego, Calif., June 25-28, 1987.

Tyler P. L., S. B. Ellingson, and M. R. Sommerfeld, "Relationship of Ionic Composition of Aquatic Habitats to Distribution and Lipid Accumulation of Microalgae," presented at the Annual Meeting of the Phycological Society of America, Ohio State University, Columbus, Ohio, August 9-13, 1987.

Screening and Characterization

THIS PAGE

WAS INTENTIONALLY

LEFT BLANK

COLLECTION, SCREENING AND CHARACTERIZATION OF LIPID PRODUCING MICROALGAE: PROGRESS DURING FISCAL YEAR 1987

J.R. Johansen, P.R. Lemke, W.R. Barclay and N.J. Nagle
Biotechnology Research Branch
Solar Energy Research Institute
1617 Cole Blvd., Golden, CO 80401

ABSTRACT

The collection, screening, and characterization of lipid producing microalgae has been an ongoing subprogram in the Aquatic Species Program since FY1983. Efforts in this subprogram at SERI have had three areas of emphasis during the past year: 1) characterization of warm-water strains; 2) initiation of a cold-water strains collection and screening project; and 3) development of a screening protocol to be used in reducing the number of strains in the Aquatic Species Program from 3000 to 200.

Ten warm-water microalgae were characterized, seven of which were added to the Microalgae Culture Collection Catalogue (1986-1987). These strains were CHAET6, CHAET9, CHAET10, CHAET15, CHAET39, CYCLO2, CYCLO4, NAVIC1, PLEUR1, and THALA2. Of these, the Chaetoceros strains had the greatest tolerance for high salinity. CHAET9, CHAET10, and NAVIC1 are considered the best new strains of the ten characterized, and further study of these strains is underway.

Cold-water strains were collected from the states of Washington, Utah, and California from shallow saline ponds during the fall (1986) and early spring (1987). They were screened at 15°C following the same enrichment protocol as that developed for warm-water strains. A total of 60 cold-water algae were isolated.

A screening protocol was developed for rapidly reducing the number of microalgal strains in the program from 3000 to the best 200. This protocol will be used in characterization efforts at both SERI and Arizona State University, so that results obtained in both laboratories will be standardized and easily comparable.

COLLECTION, SCREENING AND CHARACTERIZATION OF LIPID PRODUCING MICROALGAE: PROGRESS DURING FISCAL YEAR 1987

INTRODUCTION

Collection, screening and characterization of lipid producing microalgae tolerant of high temperatures and high salinities has been conducted by the Aquatic Species Program since FY1983. Media have been formulated based upon water chemistry of saline groundwaters in the arid southwest (Barclay et al. in press a). Microalgae have been collected from diverse warm-water saline habitats, including lakes, ephemeral ponds, thermal springs, and subtropical to tropical coastal oceanic waters (Barclay 1984; Barclay et al. 1985, 1987; Lewin 1985; Lewin et al. 1987; Ryther et al. 1987; Sommerfeld & Ellingson 1987; Tadros 1985, 1987; Thomas et al. 1983, 1984, 1985; York 1987). As a result of the combined collection efforts of SERI in-house personnel and program subcontractors, about 3000 strains of lipid producing, warm-water, saline microalgae have been collected from screening projects. Characterization of these strains has proceeded at a much slower rate, with only a relatively small number of these strains having been characterized so far. The 1986-1987 culture collection catalogue (Barclay et al. 1986) listed 39 strains which had been characterized and were considered good candidate species for lipid production in saline desert waters.

Three problem areas were identified during the summer and fall of 1986. First, we decided that it was necessary to collect cold-water, saline strains that could be used for lipid production in the Southwest during the cooler months. As a result, two subprograms to collect, screeen, and characterize cold-water microalgae were initiated at that time, one in the western United States to be conducted by SERI personnel, the other in the southeastern United States to be conducted by Mahasin Tadros. Second, the program reviewers recognized a need to standardize screening and characterization procedures within the program. This need has become particularly evident in recent months as we have attempted to make comparisons between strains characterized in different laboratories. It is imperative that we quickly identify the best candidate strains, so that subcontractors and SERI personnel working on other aspects of the program will not study inferior strains and base their conclusions on these poor performers. Third, as the collection subprogram comes to a conclusion, it will be necessary to consolidate the scattered culture collection into a single collection to be maintained at SERI. In order to facilitate this consolidation, we have organized the culture collection at SERI so that new additions can easily be incorporated and so that original strain collection data is recorded and accessable. We have also developed a standardized screening protocol to be used by SERI personnel and Milton Sommerfeld's research team to narrow the number of cultures in the combined collection from 3000 to a more workable number (around 200).

The purpose of this report is to summarize the collection, screening and characterization efforts of the SERI inhouse research team. Our efforts fall into three major catagories: 1) the continued characterization of warm-water

microalgae; 2) the initation of a cold-water collection and screening program; and 3) the development of a screening protocol to narrow the number of candidate strains from 3000 to 200, with an end goal of narrowing the number of species to receive intensive study to 10-20. Much of this research is still underway. The purpose of this paper is to summarize the status of each area of emphasis and present the data available as of July 15, 1987.

MATERIALS AND METHODS

Characterization of Warm-Water Strains

Ten strains of microalgae (9 diatoms, 1 coccolithophorid) were characterized. These strains were CHAET6, CHAET9, CHAET10, CHAET15, CHAET39, CYCLO2, CYCLO4, NAVIC1, PLEUR1, and THALA2. Strains were characterized in terms of: 1) growth response to differing conductivities and temperatures; and 2) lipid content in exponential, nitrogen-deficient, and silica-dificient cultures.

Growth responses to waters of different chemistry, conductivity, and temperature were determined on a temperature gradient table (Siver 1983) in semicontinuous culture following the methods described by Barclay et al. (in press b). Three water types - SERI Type I inland saline water (a class of water with high divalent cation concentration), SERI Type II inland saline water (a sodium bicarbonate class of water), and artificial seawater (RILA Marine Mix, Teaneck, New Jersey, USA), five conductivities - 10, 25, 40, 55, and 70 mmhos·cm^{-1} (approx. 9, 24, 42, 59, and 72 ppt), and six temperatures - 10, 15, 20, 25, 30, and 35°C, were tested in a fully crossed design, giving a total of 90 basic experimental units, with days of the experiment serving as replicates (6 days yielding 540 data points). Cultures were inoculated at an initial optical density (OD) of 0.03 (750 nm). Optical density was measured daily (using an HP Diode Array Spectrophotometer) and the culture diluted to 0.03 with fresh medium when OD greater than 0.06 was reached. Exponential growth rates, in terms of doublings·day^{-1}, were calculated from daily OD readings (Vonshak & Maske 1982).

Total lipid content was determined in nutrient-sufficient, nitrogen-deficient, and silica-deficient cultures. Exponential cultures were grown at 28°C in the optimal medium type determined from the above experiments. Cultures were starved of nitrogen by transferring a washed pellet of an exponential culture to nitrogen-free medium for 7 days. Silica limitation was achieved by transferring a washed pellet of an exponential culture to silica-free medium for 4 days. In the case of PLEUR1 (the coccolithophorid) calcium-deficient medium was used instead of silica-free medium. Lipids were quantified using a modified Bligh-Dyer method (Roessler 1987).

Collection and Screening of Cold-Water Strains

Four field trips were made during the cool months of FY1987. Saline habitats in Utah in the vicinity of Utah Lake, Horseshoe Springs, the Great

Salt Lake, and Thiokol were sampled October 23 and March 25 by Johansen, Chelf and Lemke. A separate trip by Johansen to saline lakes in eastern Washington was made March 6. Coastal lagoons in California were sampled March 25 by Chelf. Water samples from Utah Lake, Utah and Flamingo Yacht Club Pan, South Africa were provided by colleagues. Water samples were enriched and placed on a rotary screening device following the methodology of Barclay et al. (1985, 1987). The temperature of incubation was held constant at 15°C by insulating the box containing the rotary screening apparatus and installing a coil of copper tubing cooled with circulating water. The period of incubation (5-10 days) was longer than that needed for warm-water screening (3-5 days) because the algae grew more slowly at the cooler temperature. Algae were isolated directly from liquid enrichment cultures as well as from agar plates inoculated with 1 ml subsamples of selected enrichment cultures. Unialgal (clonal) cultures have been maintained at 15°C on a 16:8 hr light:dark cycle in incubation chambers since isolation.

Protocol for Rapid Screening of Culture Collection

Rationale

Recent economic analyses, which consider available saline groundwater in the southwestern United States (Lansford et al. 1987; Olson et al. 1987) and evaporation rates at potential sites in this region, indicate that a fuel-from-microalgae technology will be more economically feasible if strains can be grown at high conductivities (50-100 mmho·cm^{-1}). It is also necessary that strains be tolerant of high temperatures (30-35°C). During the course of the collection and screening subprogram strains which grow well in 10-55 mmho·cm^{-1} conductivity media have been isolated and studied. Most of these algae have optimal growth rates at 10-40 mmho·cm^{-1} conductivity. We decided to screen all of the algae in the program's combined collection using a very rigorous screening protocol that would quickly eliminate strains unable to grow quickly at high conductivity and high temperature. In cooperation with Milton Sommerfeld's research team at Arizona State University (ASU) we decided to screen the strains for growth and lipid production at 55 mmho·cm^{-1} conductivity, 30°C, 200 μE·m^{-2}·s^{-1} light intensity, and a 12:12 hr light:dark cycle. Together we decided on a protocol that could be followed at both SERI and ASU, so that results from both laboratories could be directly compared. The method has the further advantage that it can easily be adapted to screen the 60 cold-water strains collected by SERI personnel simply by changing the temperature of incubation from 30°C to 15°C.

Assembly of the Screening Apparatus

Strains were grown in 50 ml glass culture tubes capable of being placed directly in a spectrophotometer. Thus, optical density could be read in a large number of tubes quickly, efficiently, and without taking aliquots. The tubes were large enough that sufficient culture was available for two Nile Red determinations (nutrient-sufficient and nitrogen-starved conditions) and one ash-free dry weight (AFDW) determination (nitrogen-starved conditions). Thus we were able to obtain growth rates based on OD readings taken twice daily for four days as well as Nile Red fluorescence of lipids in exponentially growing cells and in cells that have been nitrogen-limited for four days. Nile Red

readings were standardized using OD readings on both occasions, and using ash-free dry weights after nitrogen limitation.

Tubes were fitted with silicone stoppers which had air inputs and outputs made of microbore tubing. A manifold of tubing supplied humidified, filtered air to each culture tube, so that cultures were bubbled with air continuously during incubation. Culture tubes were suspended in a plywood shelf drilled with holes 25 mm in diameter. Fluorescent lighting was provided from below. Mirrors lined the walls of the incubation chamber for increased eveness of lighting. The shelf was positioned such that the light intensity reaching the bottom surface of the tubes was close to 200 $uE \cdot m^{-2} \cdot s^{-1}$. In our chamber light intensity averaged 210 $uE \cdot m^{-2} \cdot s^{-1}$, with a range of 184-250 $uE \cdot m^{-2} \cdot s^{-1}$. Temperature was held at 30°C, and a 12:12 hr light:dark cycle was used.

Protocol for Experiments

All strains were acclimated to 55 $mmho \cdot cm^{-1}$ conductivity by successive inoculation in 25, 40, and 55 $mmho \cdot cm^{-1}$ conductivity media. Cultures were incubated at 30°C on a 12:12 hr light:dark cycle in each medium for two days before transfer to the next medium. After two days incubation in the 55 $mmho \cdot cm^{-1}$ medium, the large culture tubes were inoculated such that 30 ml of culture with OD between 0.01-0.05 was present in each tube. Optical density was read twice daily for four days. Two days after the beginning of the experiment 4 ml aliquots were removed from each tube for Nile Red fluorescence readings. At the conclusion of the four day growth period 15 ml aliquots were removed from each tube and centrifuged in sterile, disposable centrifuge tubes. The supernatant was decanted and algae were resuspended in nitrogen-free medium. The tubes were then returned to the incubation chamber. After four days, 0.4 and 10 ml aliquots were removed for Nile Red fluorescence readings and ash-free dry weight determinations, respectively. Optical density was also measured at this time. During nitrogen stress, OD was not measured.

Nile Red fluorescence as an estimate of lipid content is discussed in Berglund *et al.* (1987). Peak fluorescence occurs in the fluorometer after 5-25 minutes, and so we read fluorescence after 5 minutes and again every few minutes until fluorescence stabilized or declined. We added 0.04 ml Nile Red solution to each 4 ml sample. When 0.4 ml aliquots were used they were added to 3.6 ml medium to bring the volume of the sample to 4 ml.

Ash-free dry weights were determined by filtering 10 ml aliquots through washed GF/C grade glass fiber filters. Filters were then rinsed with 10 ml of 55 $mmho \cdot cm^{-1}$ conductivity NaCl solution, removed from the filtration apparatus, and placed overnight in a drying oven set at 90°C. They were weighed on a microbalance, ashed overnight at 550°C, and then reweighed. Washed filters were also dried and ashed with each batch of samples so that a correction could be applied for loss of filter weight during ashing.

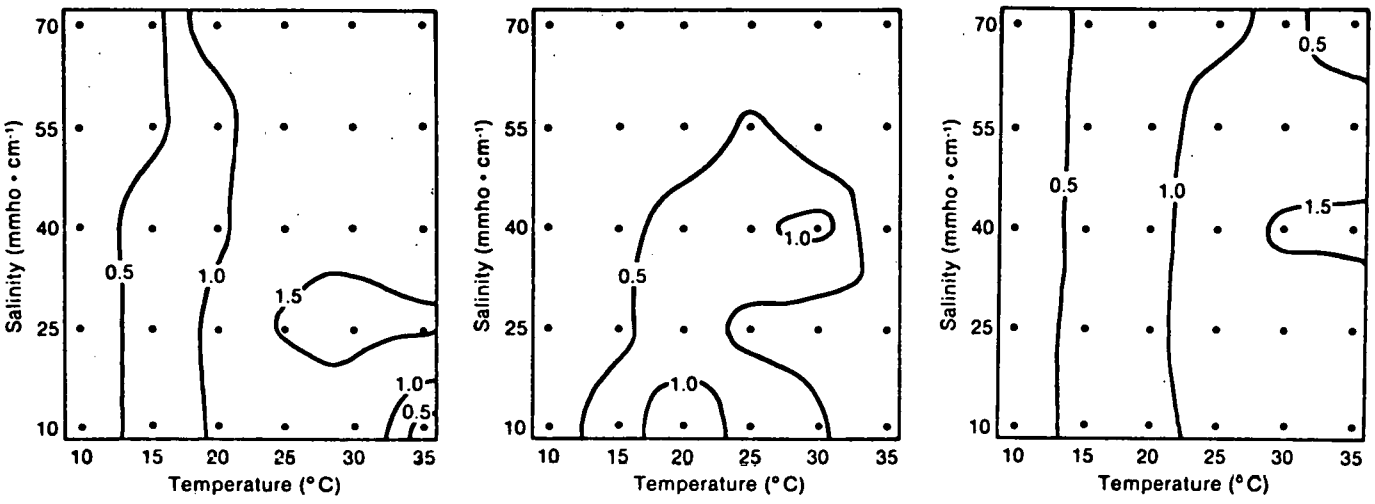


Figure 1. Surface plots of growth response to different temperature, conductivity, and water type for CYCLO4 (Cyclotella cryptica). SERI Type I (left), SERI Type II (center), Artificial seawater (right).

RESULTS

Characterization of Warm-Water Strains

A summary of the characterization results for the 10 new species is presented in Table 1. Surface plots of growth response to temperature, conductivity, and water type for CHAET6, CHAET10, CHAET15, CYCLO2, NAVIC1, PLEUR1, and THALA2 are shown in Barclay et al. (1986). Study of PLEUR1 in the light and electron microscope revealed that this strain is a new taxon, Pleurochrysis carterae var. dentata Johansen et Doucette (Johansen et al. in press). Additional notes on the morphology of THALA2 are given in Johansen & Theriot (in press).

CHAET6, CHAET9, CHAET10, and NAVIC1 grew best in SERI Type II water. CHAET39, CYCLO2, CYCLO4, and PLEUR1 grew best in SERI Type I water. CHAET15 and THALA2 preferred artificial seawater. Three strains grew very poorly in SERI Type II water, CHAET15, PLEUR1, and THALA2. All strains which grew best in SERI Type II water also grew well in the other two water types. Of the strains tested within the program to date, the Chaetoceros strains had some of the highest growth rates. They were also very euryhaline. Although they grew slightly better in low conductivity media, there was very little difference in growth rate over the range of conductivity tested. CHAET10 has been more intensely studied because it demonstrates both high growth rates and high lipid content. CHAET9 exhibited high Nile Red fluorescence, but did not have especially high lipid content according to the Bligh-Dyer determinations. We are studying this organism further.

CYCLO2 and CYCLO4 exhibited similar growth responses to temperature, salinity, and water type (compare figures in Barclay et al. 1986 to Fig. 1 above). CYCLO2 was superior to CYCLO4 in salinity tolerance and lipid content. Both strains were inferior compared to CYCLO1, which was isolated and characterized previously by Tadros (Barclay et al. 1986). This is interesting

Table 1. Summary of the physiological characterization of ten strains of algae in the SERI microalgae culture collection. Higher growth rates have been observed for some strains in outdoor culture, but for purposes of comparison we report maximum growth rates from laboratory data collected at SERI. Minima and maxima reflect levels below and above which growth rates fell below 1 doubling·day^{-1}. CYCLO2 had no discernable optimal conductivity.

Species (Strain)	Growth Rate	Temperature Min	Max	Opt	Conductivity Min	Max	Opt	Lipid Content (%) Exp.	N-def	Si-def
Chaetoceros muelleri var. subsalsum (CHAET6)	3.4	15	>35	30	<10	>70	25	8.6	15.0	17.5
Chaetoceros muelleri var. subsalsum (CHAET9)	4.0	15	>35	30	<10	>70	25	17.8	27.6	24.8
Chaetoceros muelleri var. subsalsum (CHAET10)	2.9	13	>35	35	<10	>70	25	19.0	38.0	39.0
Chaetoceros muelleri var. subsalsum (CHAET15)	1.7	20	30	30	<10	25	10	18.4	33.8	21.9
Chaetoceros muelleri var. subsalsum (CHAET39)	2.5	20	>35	30	<10	55	25	3.2	12.2	21.1
Cyclotella cryptica (CYCLO2)	1.6	20	>35	32	<10	>70	--	24.0	22.0	25.0
Cyclotella cryptica (CYCLO4)	1.6	20	>35	30	<10	70	32	12.1	11.3	13.2
Navicula saprophila (NAVIC1)	2.8	20	>35	30	<10	>70	25	22.0	58.0	49.0
Pleurochrysis carterae var. dentata (PLEUR1)	1.6	20	30	25	10	>70	25	5.4	9.8	15.4
Thalassiosira weissflogii (THALA2)	1.4	25	30	28	10	40	25	13.0	13.9	33.4

in light of the fact that all three strains belong in Cyclotella cryptica. The two strains of Thalassiosira weissflogii characterized to date were very similar, although York's isolate (THALA6) appeared to be more tolerant of high temperatures (Barclay et al. 1986). In comparison to Chaetoceros and Navicula strains, CYCLO2, CYCLO4, PLEUR1, THALA2, and THALA6 strains were poor candidates, and will probably not recieve further study.

Table 2. List of species and strains isolated as part of the cold-water collection and screening program. Numbers in parentheses indicate multiple clones from the same site.

Species	Site	Date	Collector(s)	Medium
Amphora coffeiformis	Soap Lake, WA	Mar 87	J. Johansen	II/25
A. coffeiformis	South Africa	Feb 87	C. Pienaar	II/25
Amphora species (2)	San Diego, CA	Mar 87	P. Chelf	II/25
Chaetoceros muelleri (3)	Utah Lake, UT	Sep 86	S. Rushforth	I/25
C. muelleri (2)	Utah Lake, UT	Sep 86	S. Rushforth	II/25
C. muelleri	Great Salt Lake	Oct 86	Johansen & Chelf	II/25
C. muelleri (2)	Great Salt Lake	Aug 86	S. Rushforth	I/25
C. muelleri (3)	Great Salt Lake	Sep 86	S. Rushforth	R/25
C. galvestonensis	San Diego, CA	Mar 87	P. Chelf	II/25
Cyclotella meneghiniana	Lake Lenore, WA	Mar 87	J. Johansen	II/25
Cyclotella species	Utah Lake, UT	Sep 86	S. Rushforth	R/10
Entomoneis paludosa	Great Salt Lake	Mar 87	Johansen & Lemke	II/25
Monoraphidium minutum	Thiokol Road, UT	Mar 87	Johansen & Lemke	II/25
Navicula species	Lake Lenore, WA	Mar 87	J. Johansen	R/10
Navicula species (4)	Batiquitos, CA	Mar 87	P. Chelf	II/25
Navicula species (3)	Great Salt Lake	Oct 86	Johansen & Chelf	I/55
Nitzschia hungarica	Great Salt Lake	Mar 87	Johansen & Lemke	II/25
Nitzschia species	Lake Lenore, WA	Mar 87	J. Johansen	II/25
Nitzschia species	Lake Lenore, WA	Mar 87	J. Johansen	II/25
Nitzschia species (2)	Lake Lenore, WA	Mar 87	J. Johansen	II/25
Nitzschia species	Soap Lake, WA	Mar 87	J. Johansen	II/25
Nitzschia species	Soap Lake, WA	Mar 87	J. Johansen	II/25
Nitzschia species	Soap Lake, WA	Mar 87	J. Johansen	II/25
Nitzschia species (2)	Soap Lake, WA	Mar 87	J. Johansen	II/25
Nitzschia species	South Africa	Feb 87	C. Pienaar	II/25
Nitzschia species	Ridgeway, UT	Mar 87	Johansen & Lemke	II/25
Phaeodactylum tricornutum	Great Salt Lake	Mar 87	Johansen & Lemke	II/25
P. tricornutum (2)	Great Salt Lake	Oct 86	Johansen & Chelf	I/25
Pinnularia species	Lake Lenore, WA	Mar 87	J. Johansen	II/25
Pseudanabaena species	South Africa	Feb 87	C. Pienaar	II/25
Ochromonad (4)	Tooele Co., UT	Mar 87	Johansen & Lemke	I/25
Sarcinoid chrysophyte	Soap Lake, WA	Mar 87	J. Johansen	II/25
Unidentified diatom	Great Salt Lake	Mar 87	Johansen & Lemke	II/25
Unidentified flagellate	Thiokol Road, UT	Oct 86	Johansen & Chelf	II/25
Unidentified green (2)	San Diego, CA	Mar 87	P. Chelf	II/25
Unidentified green (2)	Thiokol Road, UT	Oct 86	Johansen & Chelf	II/25
Unidentified coccoid (2)	Soap Lake, WA	Mar 87	J. Johansen	II/25
Unidentified coccoid	Great Salt Lake	Oct 86	Johansen & Chelf	I/25
Unidentified alga	Lake Lenore, WA	Mar 87	J. Johansen	II/25

Cold-Water Strains

A total of 60 strains were isolated from cold, saline waters. This collection and screening effort was more difficult than the warm-water screening because species diversity was much higher in enrichment cultures. It was difficult to know which species should be isolated from the many present. It was additionally difficult to be able to accurately pick out desirable species from the less desirable species. One interesting discovery was that many of the species which dominate warm-water enrichments also dominate cold-water enrichments. For example, Chaetoceros muelleri var. subsalsum, Amphora coffeiformis, Cyclotella species, Navicula species, and Nitzschia species were well represented (Table 2). In contrast to the warm-water enrichments, we isolated a number of coccoid green algae and ochromonads.

Characterization of these strains has not been started yet, though we plan to characterize at least 6 strains by December 1987. In order to decide which strains to characterize, we will first screen them at 15°C following the newly developed screening protocol.

Protocol for Rapid Screening of Culture Collection

Two experiments using the screening protocol have been completed thus far. In the first experiment 25 different strains of algae were tested. There were some irregularities in this first experiment, the most significant of which was that the lighting was incorrectly wired so that some lights remained on continuously. Although this resulted in artificially high growth rates, comparisons between strains are probably still valid. The more promising strains will be tested again so that they can be compared with other strains tested in future experiments. Nevertheless, the results of this test were striking. Strains thought to grow well in SERI Type II/25 medium were grown in both Type II/55 and Type I/55 media. Most strains performed best in Type II (Table 3). However, NITZS12 grew surprisingly well in Type I/55 medium, with both high growth rates and Nile Red fluorescence (Table 4). Of the strains examined, CHAET9, NAVIC2, and NITZS12 were clearly superior. CHAET9 had a high growth rate and a high lipid content under both stressed and unstressed conditions. NAVIC2 outperformed NAVIC1 in terms of lipid content. NITZS12 was clearly the best performer in Type I medium. In the past, its poor performance in Type II/25 had prevented us from seriously considering it as a good candidate species worthy of study. Other strains which showed promise include: AMPHO27, NANNO2, NANNO3, NITZS5 and most Chaetoceros (CHAET) strains tested. MONOR2, previously considered a very good candidate, performed poorly in the higher conductivity medium. Many strains performed poorly in both media types, and it will be easy to justify eliminating them from the culture collection when the time comes to consolidate the collection.

The second experiment compared 28 strains of Chaetoceros muelleri var. subsalsum. Growth rates averaged from 0.6-1.8 doublings·day^{-1}. Those strains with growth rates exceeding 1.5 include CHAET6, CHAET22, CHAET23, CHAET46, CHAET57, CHAET59, and CHAET60 (Table 5). The strains with highest Nile Red fluorescence include CHAET9, CHAET20, CHAET21, CHAET22, CHAET23, CHAET25.

Table 3. Results of screening experiment conducted with various strains in the SERI Micoalgae Culture Collection grown in Type II/55 under conditons described in the text. High values in each column are asterisked, the final column giving the number of asterisks scored by each species. Nile Red fluorescence divided by O.D. is given for exponential (Exp.) and stressed (Str.) cells. Ash-free dry weight was read only for stressed cells.

Strain	Mean doub/day	NR/OD (Exp.)	NR/OD (Str.)	NR/AFDW (Str.)	Score
AMPHO2	2.2*	102	368	70	1
AMPHO3	2.7*	69	735	125	1
AMPHO20	2.4*	85	539	133	1
AMPHO27	2.8*	104	1246*	139	2
CHAET1	1.7*	172*	504	125	2
CHAET6	2.1*	132	466	114	1
CHAET9	2.1*	269*	1615*	299*	4
CHAET10	2.0*	108	701	141	1
CHLOR2	.7	517*	1042*	104	2
CHLOR3	.5	831*	1225*	110	2
CHLOR4	.6	233*	443	34	1
CRICO1	.5	267*	380	35	1
DUNAL2	.4	79	192	15	
ENTOM4	<.1	111	286	26	
GLOEO1	<.1	154*	417	12	1
MONOR2	.7	280*	361	20	1
NANNO2	2.5*	50	92	18	1
NANNO3	1.6	52	232	47	
NAVIC1	1.1	169*	359	80	1
NAVIC2	1.1	244*	1310*	253*	3
NITZS5	2.3*	63	807	171	1
NITZS12	.4	318*	529	24	1
PYRAM2	.2	77	200	20	
SYNEC1	.1	110	600	44	
TETRA7	.4	100	494	38	

It is interesting to note that CHAET20-23 are strains isolated from four separate samples from the Great Salt Lake near Centerville, Utah. These strains have not received any special prior attention, yet they were obviously among the better strains tested. Some Chaetoceros strains performed very poorly, particularly those strains that were isolated from cold waters.

In the Chaetoceros experiment, we replicated each species twice and set up the experiment so that we could test the effect of the small differences in light intensity present in the apparatus. We found that differences in light intensity of 40-50 $uE \cdot m^{-2} \cdot s^{-1}$ did not cause statistically significant differences in growth rates or lipid content. However, when values from all species were pooled and averaged, there did appear to be a slight increase in mean growth rate and lipid content associated with increased light intensity.

Table 4. Results of screening experiment conducted with various strains in the SERI Micoalgae Culture Collection grown in Type I/55 under conditons described in the text. High values were marked using same criteria as in Table 3.

Strain	Mean doub/day	NR/OD (Exp.)	NR/OD (Str.)	NR/AFDW (Str.)	Score
AMPHO2	.8	93	57	5	
AMPHO3	.8	79	333	20	
AMPHO20	1.5	57	360	22	
AMPHO27	2.7*	80	1289*	67	2
CHAET1	2.4*	114	614	109	1
CHAET6	2.1*	135	428	69	1
CHAET9	2.3*	109	1261*	245*	3
CHAET10	1.2	103	674	118	
CHLOR2	.5	524*	619	56	1
CHLOR3	.4	491*	461	46	1
CHLOR4	.6	135	127	19	
CRICO1	1.6	116	70	22	
DUNAL2	1.5	159*	161	12	1
ENTOM4	<.1	150*	526	16	1
GLOEO1	.5	200*	208	13	1
MONOR2	.6	250*	1000*	66	2
NANNO2	2.2*	40	155	38	1
NANNO3	2.2*	46	595	157	1
NAVIC1	1.5	117	295	68	
NAVIC2	.9	120	970	127	
NITZS5	1.7*	57	565	79	1
NITZS12	1.8*	158*	1867*	428*	4
PYRAM2	1.8*	41	188	48	1
SYNEC1	2.2*	51	79	7	1
TETRA7	1.9*	35	79	12	1

There are several aspects of the screening protocol which still need further consideration and study. First, we are not certain how to establish the criteria for evaluating strains. In Tables 3-5 we asterisked values in a somewhat arbitrary fashion. In addition, we have several lipid estimates over time and should probably not count all the different determinations with equal weight. As we experiment further, we will decide on which Nile Red ratios are most meaningful. The exponential Nile Red values may be of reduced value, since these are actually readings taken somewhere between exponential and stationary growth phases and are probably not all representative of exponentailly growing cells. What seems important at present is that to be considered good candidate species, strains must demonstrate both higher than average growth rates and higher than average lipid content, as indicated by some estimate to be determined later (probably NR/AFDW in stressed cells).

Second, we are currently uncertain as to the number of replicates that should be used in each experiment. The Chaetoceros experiment demonstrated

Table 5. Results of screening experiment conducted with various strains of Chaetoceros muelleri var. subsalsum grown in Type II/55 under conditons described in the text. The top 6-7 values in each column are asterisked and the score is given in the final column as in Tables 3-4. In this experiment A.F.D.W. was determined during exponential phase as well as following the induction period.

Strain	Mean doub/day	NR/OD (Exp.)	NR/AFDW (Exp.)	NR/OD (Str.)	NR/AFDW (Str.)	Score
CHAET1	1.2	74	5	95	28	
CHAET6	1.8*	72	7	120	46	1
CHAET9	1.4	79	4	554*	138*	2
CHAET10	1.2	106	7	163	45	
CHAET18	1.4	82	7	280	50	
CHAET19	1.4	85	6	332	92	
CHAET20	1.3	90	6	434*	140*	2
CHAET21	1.4	98	10*	424*	121*	3
CHAET22	1.6*	84	9*	483*	166*	4
CHAET23	1.7*	87	9*	464*	123*	4
CHAET24	1.4	109		348	86	
CHAET25	1.4	98	12*	516*	142*	3
CHAET26	1.2	110	8*	332	85	1
CHAET39	.6	88	3	106	30	
CHAET40	1.2	74	3	180	40	
CHAET41	1.2	125*	8*	134	29	2
CHAET42	1.0	72	3	124	40	
CHAET44	1.3	128*	6	100	34	1
CHAET46	1.7*	93	5	200	66	1
CHAET49	1.0	96	4	92	33	
CHAET50	1.2	161	4	160	18	
CHAET51	.6	86	3	80	12	
CHAET52	1.0	162*	7	125	17	1
CHAET54	1.3	96	3	114	14	
CHAET57	1.8*	101	3	118	16	1
CHAET58	1.3	151*	7	112	14	1
CHAET59	1.8*	122*	7	236	54	2
CHAET60	1.6*	88	6	294	72	1

some variability in the data, but in most cases results were very similar. Presently we do not anticipate using replicates for the initial screening. When the preliminary screening is complete, the top 200 out of 3000 strains in the program will be tested again using at least 3 replicates each, so that reliable comparisons between the best strains can be made.

Third, we are not sure of the accuracy of comparison between laboratories using this protocol (SERI and ASU). We plan to run several of the same strains through an experiment (with replicates) in both laboratories so that we can evaluate the similarity of our results.

DISCUSSION

At the conclusion of the warm-water algae collection program we have identified a number of promising candidate species of algae. Particularly, we consider strains of the following species to show the most promise: Amphora coffeiformis and closely related Amphora species (AMPHO27); Chaetoceros muelleri and its varieties; Cyclotella cryptica (especially CYCLO1); Navicula saprophila (NAVIC1, NAVIC2, NAVIC7); and Monoraphidium minutum (MONOR2). Some of these (CYCLO1, NAVIC7) were collected by Tadros in the southeastern United States. Most of the better strains have been collected from the Southwest. Although the collection program is now drawing to a close, there is an urgent need to quickly screen all of the algae collected so that the most promising strains can be identified for further study. It seems wasteful of effort and resources to characterize and study strains which will be displaced by better strains later in the program. To some extent this has already happened. Early in the program, effort was expended on Ankistrodesmus falcatus, Botryococcus braunii, Chlorella species, and Phaeodactylum tricornutum, strains which are no longer considered good candidate species. More recently, we have characterized strains that, had we known more concerning their physiology, we would not have spent the effort characterizing (PLEUR1, THALA2, CYCLO4). The development of an additional, rigorous screening test will help focus our choice of candidate strains and greatly reduce unprofitable characterization and study of less-than-optimal strains.

We have also demonstrated that physiological diversity within single species exists. We have studied genetic diversity in Chaetoceros muelleri and found electrophoretic, physiological, and morphological variation (Johansen & Barclay 1987, unpublished). The physiological variation evident in Table 5 is particularly instructive. The screening of the Chaetoceros strains shows that when multiple strains of a good candidate species are collected, some will be significantly better than others. CHAET1 and CHAET14 were once considered good strains. Recently much attention has been given to CHAET6 and CHAET10, which were found to be superior to CHAET1 and CHAET14. From Tables 3-5, it now appears that CHAET9, CHAET21, CHAET22, and CHAET23 may be even better candidates. Before further characterization of Chaetoceros muelleri is undertaken, we plan to screen all of the strains currently in our collection. Perhaps some other untested, unstudied strains will prove to be even better. Screening of multiple clones of other species (such as Amphora coffeiformis, Navicula saprophila, Monoraphidium minutum, Nannochloris species, and Nitzschia section lanceolatae) may also provide better strains for study.

We plan to screen all 60 cold-water strains at 15°C using the screening protocol. Two experiments will be conducted so that strains can be tested in both Type I/55 and Type II/55 media. This preliminary screening should greatly increase the value of our characterizations. We may also run some of the better warm-water strains through a 15°C screening experiment to determine if they are good as cold-water strains as well.

ACKNOWLEDGEMENTS

Paulanne Chelf, Paul Roessler, Milton Sommerfeld and Steve Ellingson provided helpful comments during the design of the screening protocol. Samuel Rushforth and Christo Pienaar provided saline water samples at their expense.

BIBLIOGRAPHY

Barclay, W.R. 1984. Species collection and characterization. In: McIntosh, R.P. (ed.), Aquatic Species Program Review, Proceedings of the April 1984 Principal Investigators Meeting, pp. 152-159. SERI, Golden, CO.

Barclay, B, Johansen, J., Chelf, P., Nagle, N., Roessler, P. and Lemke, P. 1986. Microalgae Culture Collection 1986-1987. SERI, Golden, CO, 149 pp.

Barclay, B., Nagle, N. and Terry, K. 1987. Screening microalgae for biomass production potential: protocol modification and evaluation. In: Johnson, D.A. (ed.), FY 1986 Aquatic Species Program, Annual Report, pp. 23-40. SERI, Golden, CO.

Barclay, W.R., Nagle, N., Terry, K., Ellingson, S. and Sommerfeld, M.R. In press a. Estimation of saline groundwater resource quality for aquatic biomass production: a statistically-based approach. J. Wat. Res.

Barclay, B., Nagle, N., Terry, K. and Roessler, P. 1985. Collecting and screening microalgae from shallow, inland saline habitats. In: McIntosh, R.P. (ed.), Aquatic Species Program Review, Proceedings of the March 1985 Principal Investigators Meeting, pp. 52-67. SERI, Golden, CO.

Barclay, W., Terry, K., Nagle, N. Weissman, J. and Goebel, R. In press b. Potential of new strains of marine and inland saline-adapted microalgae for aquaculture. J. World Aquaculture Soc.

Berglund, D., Cooksey, B., Cooksey, K.E. and Priscu, L.R. 1987. Collection and screening of microalgae for lipid production: possible use of a flow cytometer for lipid analysis. In: Johnson, D.A. (ed.), FY 1986 Aquatic Species Program, Annual Report, pp. 41-52. SERI, Golden, CO.

Johansen, J.R. and Barclay, W.R. 1987. Physiological variability in strains of Chaetoceros muelleri. In: Johnson, D.A. (ed.), FY 1986 Aquatic Species Program, Annual Report, pp. 137. SERI, Golden, CO.

Johansen, J.R., Doucette, G.J., Barclay, W.R. and Bull, J.D. In press. the morphology and physiology of Pleurochrysis carterae var. dentata var. nov. (Prymnesiophyceae), a new coccolithophorid from an inland saline pond in New Mexico. Phycologia.

Johansen, J.R. and Theriot, E. In press. The relationship between valve diameter and number of central fultoportulae in Thalassiosira weissflogii (Bacillariophyceae). J. Phycol.

Lansford, R.R., Hernandez, J.W. and Enis, P.J. 1987. Evaluation of available saline water resources in New Mexico for the production of microalgae. In: Johnson, D.A. (ed.), FY 1986 Aquatic Species Program, Annual Report, pp. 227-242. SERI, Golden, CO.

Lewin, R.A. 1985. Production of hydrocarbons by micro-algae; isolation and characterization of new and potentially useful algal strains. In: McIntosh, R.P. (ed.), Aquatic Species Program Review, Proceedings of the March 1985 Principal Investigators Meeting, pp. 43-51. SERI, Golden, CO.

Lewin, R.A., Cheng, L. and Burrascano, C. 1987. Some picopleuston algae from the Caribbean region. FY 1986 Aquatic Species Program, Annual Report, pp. 105-121. SERI, Golden, CO.

Olson, K.L., Wilson, L.G., Wallace, M. and Osborn, M.D. 1987. Utilization of saline water sources in Arizona for microalgae production. In: Johnson, D.A. (ed.), FY 1986 Aquatic Species Program, Annual Report, pp. 243-248. SERI, Golden, CO.

Roessler, P. 1987. Biochemical aspects of lipid accumulation in silicon-deficient diatoms. In: Johnson, D.A. (ed.), FY 1986 Aquatic Species Program, Annual Report, pp. 257-272. SERI, Golden, CO.

Ryther, R.H., Carlson, R.D., Pendoley, P.D. and Jensen, P.R. 1987. Collection and characterization of saline microalgae from South Florida. In: Johnson, D.A. (ed.), FY 1986 Aquatic Species Program, Annual Report, pp. 122-136. SERI, Golden, CO.

Siver, P.A. 1983. A new thermal gradient device for culturing algae. Br. phycol. J. 18:159-164.

Sommerfeld, M.R. and Ellingson, S.B. 1987. Collection of high energy yielding strains of saline microalgae from southwestern states. In: Johnson, D.A. (ed.), FY 1986 Aquatic Species Program, Annual Report, pp. 53-66. SERI, Golden, CO.

Tadros, M.G. 1985. Screening and characterizing oleaginous microalgal species from the southeastern United States. In: McIntosh, R.P. (ed.), Aquatic Species Program Review, Proceedings of the March 1985 Principal Investigators Meeting, pp. 28-42. SERI, Golden, CO.

Tadros, M.G. 1987. Screening and characterizing oleaginous microalgal species from the southeastern United States. In: Johnson, D.A. (ed.), FY 1986 Aquatic Species Program, Annual Report, pp. 67-89. SERI, Golden, CO.

Thomas, W.H., Seibert, D.L.R., Alden, M. and Eldridge, P. 1984. Cultural requirements, yields, and light utilization efficiencies of some desert saline microalgae. In: McIntosh, R.P. (ed.), Aquatic Species Program Review,

Proceedings of the April 1984 Principal Investigators Meeting, pp. 7-63. SERI, Golden, CO.

Thomas, W.H., Seibert, D.L.R., Alden, M. and Eldridge, P. 1985. Selection of desert saline microalgae for high yields at elevated temperatures and light intensities and in SERI standard artificial media. In: McIntosh, R.P. (ed.), Aquatic Species Program Review, Proceedings of the March 1985 Principal Investigators Meeting, pp. 5-27. SERI, Golden, CO.

Thomas, W.H., Seibert, D.L.R., Alden, M., Eldridge, P., Neori, A. and Gaines, S. 1983. Selection of high-yielding microalgae from desert saline environments. In: Lowenstein, M.Z. (ed.), Aquatic Species Program Review, Proceedings of the March 1983 Principal Investigators Meeting, pp. 97-122. SERI, Golden, CO.

Vonshak, A. and Maske, H. 1982. Algae: growth techniques and biomass production. In: Coombs, J. and Hall, D.O. (eds.), Techniques in Bioproductivity and Photosynthesis, pp. 66-77. Pergamon Press, New York.

York, R.H., Jr. 1987. Collection of high energy yielding strains of saline microalgae from the Hawaiian Islands. In: Johnson, D.A. (ed.), FY 1986 Aquatic Species Program, Annual Report, pp. 90-104. SERI, Golden, CO.

SCREENING MICROALGAE ISOLATED FROM THE SOUTHWEST FOR GROWTH POTENTIAL AND LIPID YIELD

Milton R. Sommerfeld, Stephen B. Ellingson and Patti L. Tyler
Department of Botany and Microbiology
Arizona State University

ABSTRACT

To enlarge the pool of algal species that have a propensity to grow rapidly, store lipids and be suitable for mass cultures in saline waters, a total of 125 aquatic sites in Arizona, California, Nevada, New Mexico, Texas and Utah were sampled that varied widely in temperature, pH, salinity and relative ionic composition. From the approximately 1700 isolations made approximately 700 unialgal cultures of microalgae have been established. Growth rate determination have been made on 131 isolates using the SERI Type I and II Media at various salinities and temperatures.

Forty-nine strains, primarily diatoms and chlorophytes, demonstrated growth rates exceeding one doubling per day and five exceeded two doublings per day. Preliminary growth optimization efforts with 15 of the algal strains indicate that the most rapid growth occurs when isolates are cultured with urea as the nitrogen source at 25°C under 500 uE/m^2/sec of light. For many of the strains, altering the nitrogen source and elevation of light intensity from 25 to 50 uE/m^2/sec had relatively little affect on growth rate.

The fluorescent dye, Nile Red, was used to microscopically screen for intracellular lipid storage. Using fluorometric quantification seven microalgal cultures yielded more than 200 mg/l and four yielded greater than 400 mg/l triolein equivalent lipids.

SCREENING MICROALGAE ISOLATED FROM THE SOUTHWEST FOR GROWTH POTENTIAL AND LIPID YIELD

INTRODUCTION

The southwestern United States have been identified as possessing the necessary resources and characteristics such as abundant land, saline water, high incident solar radiation, and favorable temperatures that would be advantageous in the development of outdoor microalgal biomass technology (McIntosh, 1984).

The diversity of types of aquatic habitats in the Southwest (Cole, 1968) suggests that a diverse assemblage of microalgae may exist to utilize the range of aquatic conditions available. These habitats therefore may have selected for certain species or physiological strains that have become adapted to the natural aquatic systems of the region and thus may contain the most desirable microalgae for mass culture in the region.

The goals of this research were (1) to collect, isolate, screen and identify microalgae strains from aquatic systems in the Southwest that grow well at elevated temperatures and salinities, and (2) to characterize selected strains for lipid yield and growth potential.

To accomplish these objectives, microalgae were obtained from 206 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 storage lipids.

MATERIALS AND METHODS

Seven sampling trips to saline aquatic systems in the arid Southwest were conducted from April 19, 1985 to June 6, 1986. Criteria used to determine whether an aquatic site was to be sampled, field analyses, collection of water samples, and laboratory chemical analyses were according to those previously outlined (Sommerfeld and Ellingson, 1987).

With a large backlog of samples from 1985, field collections were much abbreviated in 1986. During the second summer algae were collected and isolated as in 1985; however, growth rates in the seven different saline media was not immediately determined. Initially diatoms and green algae isolated in 1986 and 1985 and not previously characterized were screened for rapid growth in SERI Type I and II (40) Media. Small tubes (16 x 150 mm) were inoculated and agitated daily while grown at 25°C, 200 uE/m^2/sec, and 12L:12D cycle. Growth was visually observed for ca. two weeks. Those strains demonstrating rapid growth were selected for further characterization. The effects of different temperatures, salinities, light intensities, and nitrogen sources were determined with modified rotary culture devices (RCD). Promising algal strains were precultured and inoculated into the large culture tubes. The

RCD's were placed inside incubators maintained at 25 or 30°C. Light intensity was attenuated with Formatt standard dot screens (Graphic Products Corp., Rolling Meadows, IL) placed on the plastic culture tube supports. For growth optimization studies SERI Type II Media at 25 mS/cm was enriched with phosphorus, silica, micronutrients (i.e., modified F1), and either nitrate (24 mg N/l), ammonia (38 mg N/l), or urea (18 mg N/l). Growth was monitored at 750 nm for ten days and growth rates calculated after Sorokin (1973).

Nile Blue A (CI 51180), Sudan Black B (CI 26150), Oil Red O (CI 2612S) and Nile Red (CAS 7385-67-3), were used to evaluate the presence of intracellular lipids in selected strains of microalgae. Inconsistent results were observed with the first three stains. However, Nile Red appeared to give reliable results. A small aliquot of the algal culture was placed in a test tube (16 x 150 mm) and 2-3 ml of Nile Red (Kodak cat. no. 180 7973, 10 mg/100 ml acetone) added. The mixture was shaken, allowed to react for 3-5 min., and Nile Red fluorescence observed with a Zeiss Standard Photomicroscope. Wet mounts were prepared and viewed with an epi-fluorescence condenser and HBO 50-watt superpressure mercury lamp in a 100-watt housing. Yellow-gold fluorescence was observed using a 450-490 nm band excitation filter, a 510 nm dichromatic beam splitter, and a 520nm long barrier filter (Zeiss filter set no. 487709). Cells were rated from zero for no microscopically visible fluorescing lipid droplets, to three for droplets that equalled or exceeded 75% of the cell volume. Cells containing visible opaque bodies that did not fluoresce were rated +. Single or scattered cells were photographed with Ektachrome 400 at exposures of 4-8 sec. Dense clumps of cells were exposed for 2-4 sec.

The proximate chemical composition of algal strains showing either good growth or lipid accumulation via the staining method was determined. Algae were grown in 2.8-liter Fernbach flasks containing appropriate SERI Media or seawater. The flasks were agitated at about 100 rpm on a Model G10 gyrotory shaker (New Brunswick Scientific Co., Inc.) under 12L:12D cycle, 100 $uE/m^2/sec$ and maintained at room temperature (ca. 28°C under the lights). Once significant cell numbers were achieved the batch cultures were harvested by low-speed (3020 x g) centrifugation for the determination of chemical composition. Total lipids were assayed by a monophasic Bligh and Dyer extraction (Christie, 1982). An acidic extracting solvent was prepared after Dubinsky and Aaronson (1979) by adding 2-3 drops of concentrated HCl to each 100 ml of 1:2 (v/v) chloroform and methanol mixture. Extracted lipids were transferred to tared 1.5 ml polypropylene microcentrifuge tubes and the chloroform allowed to evaporate. The residue containing tubes were dried overnight at 60°C in a vacuum oven, cooled, and placed in a desiccator. Weight of the lipid residue was determined with a Cahn Model 4400 electrobalance. Total carbohydrates were determined by the phenol-sulfuric acid method (Kochert, 1978) with a glucose standard and total proteins by the heated biuret-Folin assay (Dorsey et al., 1978) with a bovine serum albumen (BSA) standard. Dry weight was found by concentrating an algal sample onto a tared precombusted Whatman GF/C filter (2.5 cm diam.) and drying at 105°C to a constant weight on the electrobalance. Ash-free dry weight was obtained by igniting the above filter at 500°C for one hour. The filter was cooled, wetted with distilled water and brought to a constant weight after drying at 105°C. Chemical composition was expressed as a percentage of the ash-free dry

weight.

Emission spectra were measured to determine the fluorescence chacteristics of Nile Red with a strain of Navicula (ASU0214) and the triolein standard. A Spex Industries, Inc. spectrofluorometer consisting of a Model 1881A monochromator with a 250-watt tungsten lamp was used and the emitted light passed through a Model 1680A double beam spectrometer before detection with a photomultiplier tube. The excitation wavelength 480 nm (18 nm bandwidth) and emission wavelengths were monitored from 500 to 700 nm (7.2 nm bandwidth) to determine emission peaks for the Navicula and triolein. Using these results neutral lipid levels were quantified with a Turner Model 111 fluorometer using a blue lamp (#110-853), lamp adapter (#110-856) without metal screen, and high sensitivity door (#110-865). A Ditric Optics, Inc. three cavity excitation filter (#15-30240; center 480.0 ± 2.0 nm, bandwidth 7.1 ± 1.5 nm) was used and emission monitored with a wide band interference filter (#15-31180; center 550.0 ± 6 nm, bandwidth 40.0 ± 8 nm). Nile Red was dissolved in acetone (10 mg/100 ml) and triolein standards (Sigma Chem Co., #405-10) prepared by appropriate dilution with isopropanol. The fluorometer was set to zero with the blanking cuvette before sample fluorescence was measured. After mixing thoroughly 4 ml of algal sample was transferred to a small test tube (12 x 75 mm) and 50 ul of Nile Red solution added. The tube was mixed and fluorescence recorded within 5 to 10 min. Concentrated samples were diluted to read on the 30 or 10X scale.

RESULTS and DISCUSSION

Water Chemistry

Samples of natural waters containing microalgae were obtained from 125 aquatic habitats in the Southwest representing a broad range of conductivities and temperatures (Fig. 1; Table 1). During the summers of 1985 and 1986, 88 and 37 locations, respectively, were sampled. An effort was made in 1986 to avoid habitats with extremely saline waters since algal biomass and species diversity was very low in such waters sampled in 1985. Of the 37 sites sampled in 1986, 17 were previously not sampled. Twenty-eight samples were from water with temperatures in excess of 30°C. Another 24 had water temperatures between 28°C and 29.9°C at the time of collection. Mean water temperature for all the samples was 26.9°C, ranging from 17.8°C in the Salt River Canyon (Site no. SRC-3) to 45.6°C at a spring (HW-1) near Thatcher, Arizona. Specific conductance for all sampling periods ranged from 447 uS/cm in the Salt River Canyon (SRC-11) to 474,000 uS/cm at Salt Lake (SL-1) east of Carlsbad, New Mexico and averaged 22,663 uS/cm. Over 30% of the habitats had a conductance greater than 10,000 uS/cm. Specific conductance was measured at 87 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. Specific conductances of the field samples were used to match the sample to the appropriate SERI Media during initial screening and isolation in the laboratory.

The Water Resources Research Center (Wilson et al., 1986) has recently identified six focal regions in Arizona which might serve as sites of algal mass culture facilities. These regions were selected based on saline

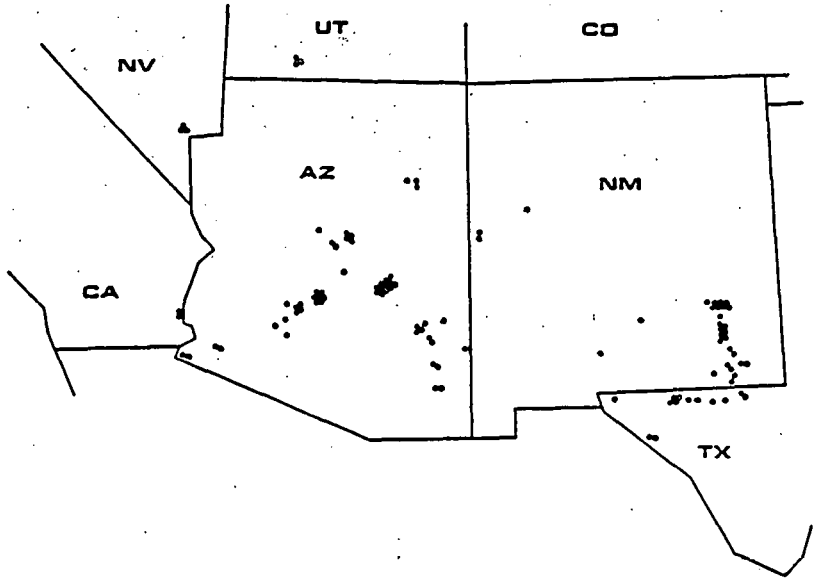


Figure 1. Approximate locations for sampling for microalgae during 1985 and 1986.

Table 1. Summary of field characteristics of habitats sampled from April 1985 to June 1986.

	Water temp. (°C)	Depth (cm)	EC (uS/cm at 25°C)	pH (SU)
Min	17.8	1	447	6.1
Max	45.6	91	474,240	10.2
Mean	26.9	26	22,663	8.0
sd	4.5	20	60,630	0.7
n	124	106	125	124

EC = electrical conductivity, sd = standard deviation, n = number of determinations

groundwater (TFR > 3000 mg/l) being within 152.4 m of the land surface and having a potential yield of 15 million liters per day for ten years. Microalgae isolated from saline surface waters in these areas are likely adapted to the indigenous conditions and might prove to be desirable organisms for subsequent mass culture. Field collections for microalgae were made in five of the six regions with samples gathered in the Palo Verde Irrigation District (PVC-1/2) being about 25 km south of Area I. A similar analysis has been completed for New Mexico (Lansford et al., 1986) with six potential areas selected for microalgae production facilities. A considerable portion of our sampling occurred in the targeted areas. During the summers of 1985 and 1986, 4 samples were collected in the Tularosa Basin, 5 samples were gathered from the Crow Flats area, and 46 collections were made in the Pecos River Basin. A single sample (RSJ-1) was collected south of the San Juan Basin at the Rio San Juan near Gallup, New Mexico.

Collection and Isolation

From the collections made in 1985 and 1986 approximately 1700 isolations have been made of which 700 unialgal cultures have been established. One hundred twenty (120) algal strains have been tentatively identified with 60 being chlorophytes, 40 diatoms and 20 blue-green algae. These strains or isolates comprise 24 genera. Based on the number of strains isolated the following sites have been the most prolific in providing culturable microalgae: LCC-1, Lower Chevelon Creek, 26 strains; SRC-1, in the Salt River Canyon, 25; F8S-1, Figure 8 Lake South, 21; SRC-2 in the Salt River Canyon, 20 and RBR-1, Red Bluff Reservoir, 20. The most frequently isolated genera were Dunaliella, Chlorococcum, Chlorosarcina, Amphora, Nitzschia, Navicula, Oscillatoria, and Chroococcus.

Screening and Growth Characterization

The large backlog of samples generated from 1985 field collections necessitated the development of an expedient means of screening. Since the diatoms could be easily distinguished from other major taxonomic groups and other workers were successful in obtaining diatoms which exhibit good growth and lipid yield, they were screened first. From collections from 1985 and 1986, 31 diatoms showed rapid growth when visually screened in SERI Type I or II Media at 40 mS/cm. These selected diatoms were further characterized with regard to growth potential and lipid accumulating ability. When growth rates were determined in the seven saline media 11 diatoms grew at more than 1.0 doub/d with a maximum of 1.957 doub/d in seawater demonstrated by a strain of Amphora (ASU0308) isloated from Las Vegas Wash (LW155). Fourteen of the diatoms stained immediately following the ten-day growth rate experiments gave strong staining reactions for intracellular lipid accumulation. Following a similar screening procedure 50 isolates of green algae were selected. In 17 strains, growth rates exceeded 1.0 doub/d with a maximum of 2.576 doub/d in seawater exhibited by a strain of Dunaliella (ASU0038) collected from Salt Creek (SC-1) in New Mexico. Fifteen of the 50 strains gave strong Nile Red staining reactions.

Of the 64 strains that have been evaluated for growth in seven saline media, four-fifths of the cultures exhibited growth in low salinity SERI Media and more than half grew in seawater. Most of the rapid growth rates (>1.0 doub/d) were observed in SERI Type I and II Media at 10 mS/cm and seawater (Table 2). This may have resulted from the fact that most of these strains were initially isolated into low conductance media. With the exception of a Dunaliella (ASU0245) isolated from Zuni Salt Lake (ZSL-1) at 196 mS/cm the strains were obtained from sites with conductivities less than that of the 40 mS/cm media (SERI Type I and II) used.

Table 2. Summary of growth rates in the seven saline media at 30°C, 500 uE/m^2/sec, and in a 12L:12D cycle.

Media	n	doub/d Min	doub/d Max	doub/d Mean	No. > 1.0 doub/d
I(10)	71	0.052	1.970	0.658	13
II(10)	64	0.137	3.263	0.785	18
I(40)	60	0.150	1.845	0.575	6
II(40)	42	0.164	1.731	0.625	7
I(70)	46	0.099	1.439	0.522	5
II(70)	32	0.102	1.136	0.498	3
SW	48	0.169	2.576	0.761	12

n - number of determinations; Min - minimum; Max - maximum

There appears to be no major differences in growth rates observed in SERI Type I and II Media with the exception of the paucity of growth observed in Type II Media at 70 mS/cm. Only three strains (Amphora, ASU0032; Chroococcus, ASU0071; Navicula, ASU0267) grew at more than 1.0 doub/d in this medium. A total of only five strains (Amphora, ASU0032; Dunaliella, ASU0038; Synechococcus, ASU0071; Synechococcus, ASU0075; Navicula, ASU0267) isolated from three sites (SC-1, MLS-1, LVS-1) grew rapidly in either Type I or II at 70 mS/cm Media.

Among the 64 strains grown in saline media, 28 grew at more than 1.0 doub/d, three exceeded 2.0 doub/d, and one surpassed 3.0 doub/d. Chlorococcum (ASU0048) isolated from a benthic sample in the Salt River Canyon (SRC-B) grew at 3.262 doub/d in SERI Type II Media at 10 mS/cm (Fig. 2). In the Type I Media at 10 mS/cm and at 40 mS/cm it grew at 1.300 and 0.839 doub/d, respectively. Detectable growth was absent in the other media. A strain of Amphora (ASU0032) found in near-by Mirror Lake South (MLS-1) also grew at more than 1.0 doub/d in two media (Fig. 2). One of the most prolific algae, a Synechococcus (ASU0071) from LaVerkin Springs (LVS-1), exceeded 1.0 doub/d in all seven media (Fig. 2). A strain of Dunaliella (ASU0038), in seawater, grew at 2.576 doub/d in addition to exhibiting rapid growth in SERI Type I Media at 10 mS/cm and Type I Media at 70 mS/cm (Fig. 2). From LaVerkin Springs (LVS-1) a strain of Synechococcus (ASU0075) was obtained which grew at 2.313 doub/d in seawater (Fig. 3). This strain also exceeded 1.0 doub/d in SERI Type II Media at 10 mS/cm, Type II Media at 40 mS/cm, and Type I Media at 70 mS/cm. Eremosphaera (ASU0132) from the Salt River Canyon (SRC-3) grew at 2.094 and 2.038 doub/d in seawater and SERI Type II Media at 10 mS/cm (Fig. 3). In Type I Media at 10 mS/cm it approached these figures by growing at 1.970 doub/d. The blue-green alga Oscillatoria (ASU0023) isolated from Figure-8 Lake South (F8S-1) exceeded 1.0 doub/d in four saline media (Fig. 3).

Initial growth rates obtained from algae collected in the Southwest appear

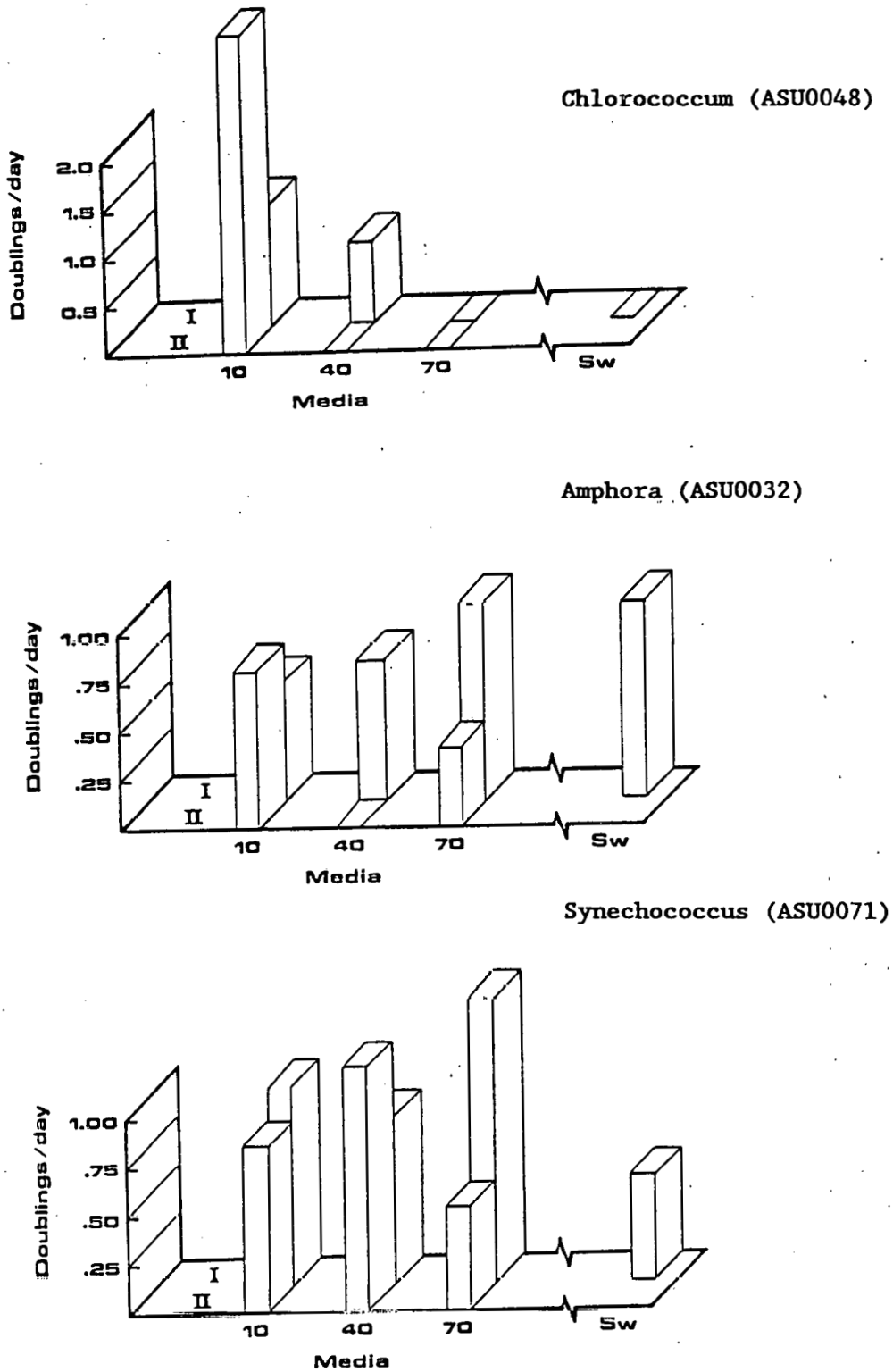
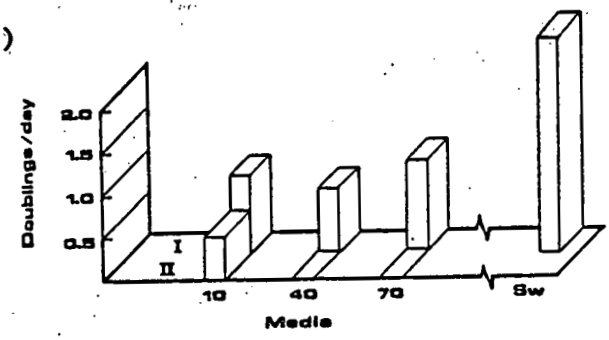
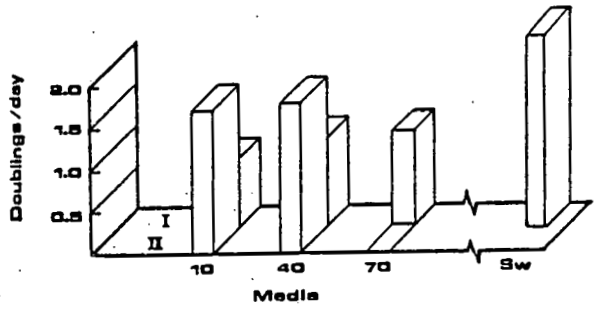


Figure 2. Growth rates for microalgae isolates in SERI Type I and II Media of 10, 40 and 70 mS/cm specific conductance and in seawater (SW).

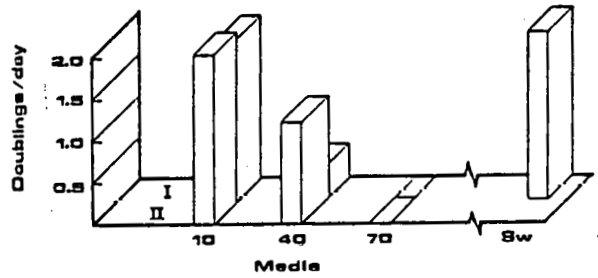
Dunaliella (ASU0038)



Synechococcus (ASU0075)



Eremosphaera (ASU0132)



Oscillatoria (ASU023)

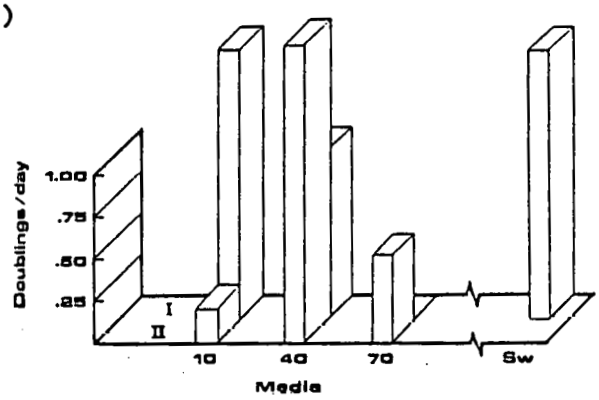


Figure 3. Growth rates for microalgae isolates in SERI Type I and Media of 10, 40 and 70 mS/cm specific conductance in seawater (SW)

similar to those found by other researchers in the Aquatic Species Program. Since other workers have previously used different media, culturing devices, light intensities, temperatures, and methods of calculating and expressing growth rates, results may not always be strictly comparable. Thomas et al. (1984) initially estimated algal growth visually but later (Thomas et al., 1985) determined growth rates for 13 "good" and "intermediate" growing strains at 30°C. Growth ranged from 0.10 doub/d for a green flagellate (56R80-1) in Standard I Moderate TDS Medium to 2.48 doub/d for Chlorella ellipsoidea (BL-6) in Standard II Low TDS Medium. The average rate was 0.68 doub/d for the 31 strains tested. In later experiments Thomas et al. (1985) determined growth rates for six algal strains at 30°C in five different Standard Media. Chaetoceros (OL-12) grew the best at 1.72 doub/d in Standard II Moderate TDS Medium. Barclay et al. (1985) reported exponential growth rates (per day) for Ankistrodesmus and Boekelovia (Chryso/F1) on salinity-temperature gradients. Exponential growth rates in excess of 2.0/day (ca. 3.5 doub/d) were obtained for both species. Maximum doubling times of 18 h (= 1.33 doub/d) were reported for Dunaliella salina and Phaeodactylum tricornutum by Ben-Amotz (1985).

Chemical Composition

Two different procedures were used to obtain data on the chemical composition of selected microalgae. During 1985, the proximate chemical composition was determined for 13 strains of rapidly growing strains. The mean composition, expressed as a percentage of AFDW, was: 44.2% protein, 45.9% carbohydrate, 10.5% lipid and 2.2% unreactive residue. Franceia (ASU0146) and Cyclotella (DI-35) contained the highest lipid levels at 26.5% and 24.5% AFDW, respectively. The lowest proportion of lipid was present in Asterococcus (ASU0061) at 3.2% AFDW. Figure 4 depicts the relative lipid content of the 13 strains with several determinations being completed on six of the strains.

Because the previous method of evaluation of intracellular lipid accumulation was labor intensive and not conductive to rapidly screening large numbers of isolates for the presence of storage lipids the Nile Red staining procedure was employed.

Lipid content was assayed by fluorescence microscopy and fluorometry (Greenspan et al., 1985; Nagle, 1986). Following the 10-day growth period, cells were stained with Nile Red and qualitatively rated from zero for no staining to a maximum of three when observed with the fluorescent microscope. Generally, an increase in microscopic fluorescence observed paralleled an elevated fluorometric result expressed in triolein-equivalent lipids (Fig. 5). These triolein levels also tended to parallel optical density which is an indirect estimate of biomass (Fig. 6). Table 3 lists those isolates with the highest triolein equivalent yield. The triolein-equivalent lipid concentrations have not been confirmed by standard analysis (e.g., Bligh & Dyer extraction) and should be considered relative comparisons at this juncture. The amount of scatter in Figures 5 and 6 reflects some of the difficulties in attempting to quantify lipid levels. In some treatments algal cells (especially diatoms) formed large aggregations possibly giving erroneous fluorometric or optical density measurements. Occasionally strains absorbed the Nile Red but did not exhibit yellow fluorescence. These cells produced a

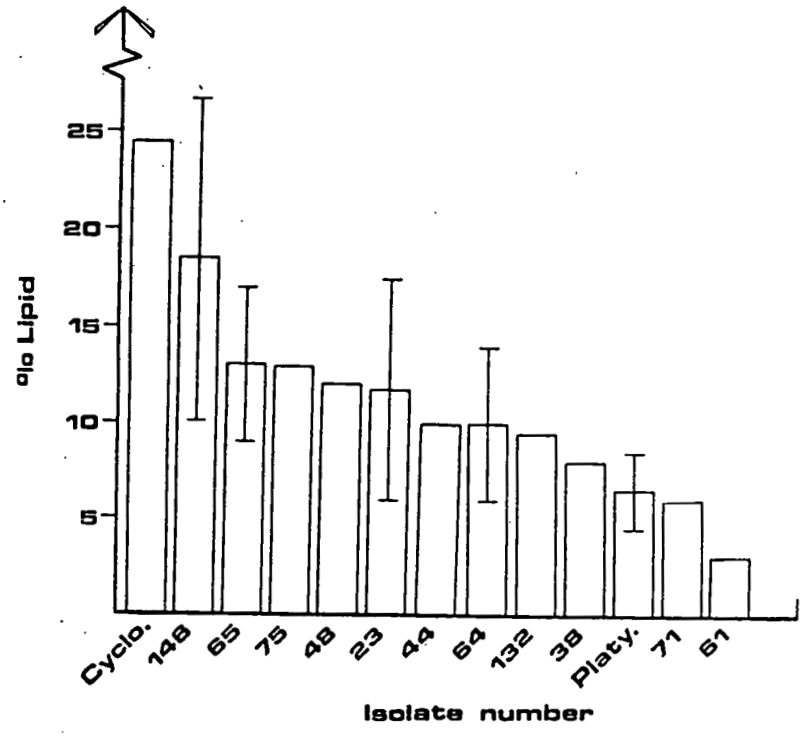


Figure 4. Lipid content of isolated strains of microalgae.

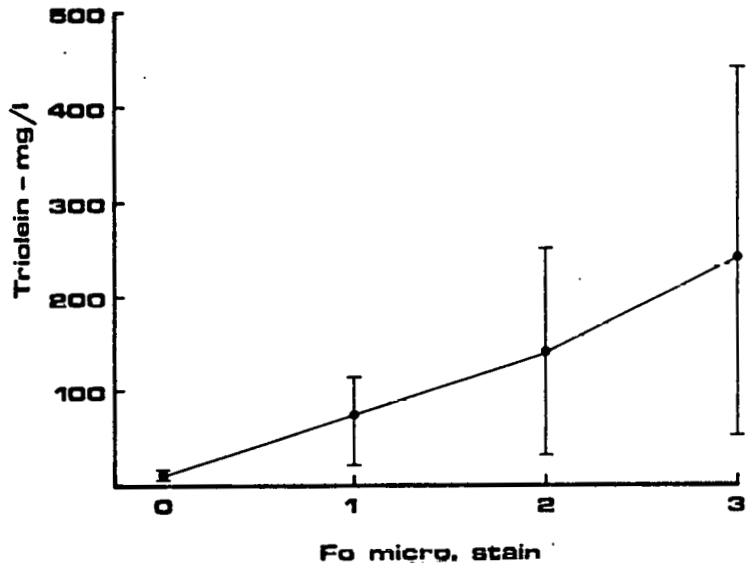


Figure 5. Relationship between observational rating with epifluorescent microscopy and fluorometric determination of lipid content. Bars denote interquartile range.

red fluorescence possibly indicating an absence of neutral lipids in the cells. This contrasts with cells that did not even absorb the stain and those which took up the strain but weakly fluoresced in the yellow range. The green alga (Franceia, ASU0146), for example, showed fluorescence on two occasions but on several other occasions did not, suggesting that some type of modification in the structure of the lipid may have occurred.

Table 3. Growth rates and lipid yield for selected diatoms.

Isolate	Genus	Doub/day	OD_{max}	Triolein (mg/l)
ASU 0032	Amphora	0.90	0.230	551
ASU 3005	Amphora	1.31	0.143	519
ASU 3001	Amphora	0.92	0.146	424
ASU 0626	Fragilaria	1.33	0.118	279
ASU 0749	Navicula	0.72	0.119	255
ASU 03013	Nitzschia	0.69	0.072	183

Doub/day - doublings per day; OD_{max} - maximum optical density at 750 nm; Triolein - triolein equivalents in mg/l.

Fluorometric Analysis

The excitation and emission filters from Ditric Optics Inc. employed in this study are thought to be more selective for neutral lipids strained with Nile Red than those recommended by Nagle (1986). The 550 nm Ditric filter was used to take advantage of the high selectivity of Nile Red for lipid droplets when viewed for yellow-gold fluorescence between 528 and 590 nm. The emission filter suggested by Nagle (1986) (Corion # P70-600-S, center 600 ± 10 nm, band width 70 ± 25 nm) is not as selective for intracellular lipid droplets since wavelengths above 590 nm are measured (Fig. 7). These longer wavelengths fluoresce red, termed "reticular staining", which probably represents membranes and organelles (Greenspan et al., 1985). Since a narrower and more selective filter is being used, the neutral lipid concentrations reported in this study may, however, be consistently lower than those obtained using the Corion filter.

Optimization

Fifteen different isolates of microalgae of the genera Phacotus, Navicula, Nitzschia, Fragilaria, and Cyclotella were grown under different temperature, light, and nitrogen treatments. Initial analyses indicate that urea was the preferred nitrogen source since 53.1% (17 of 32) of the cultures that grew at more than 1.0 doub/d contained this form of nitrogen. Approximately 31% and 16% of the rapidly growing cultures were able to use nitrate and ammonia as their nitrogen source, respectively. Conversely, 62.1% (36 of 58 of the

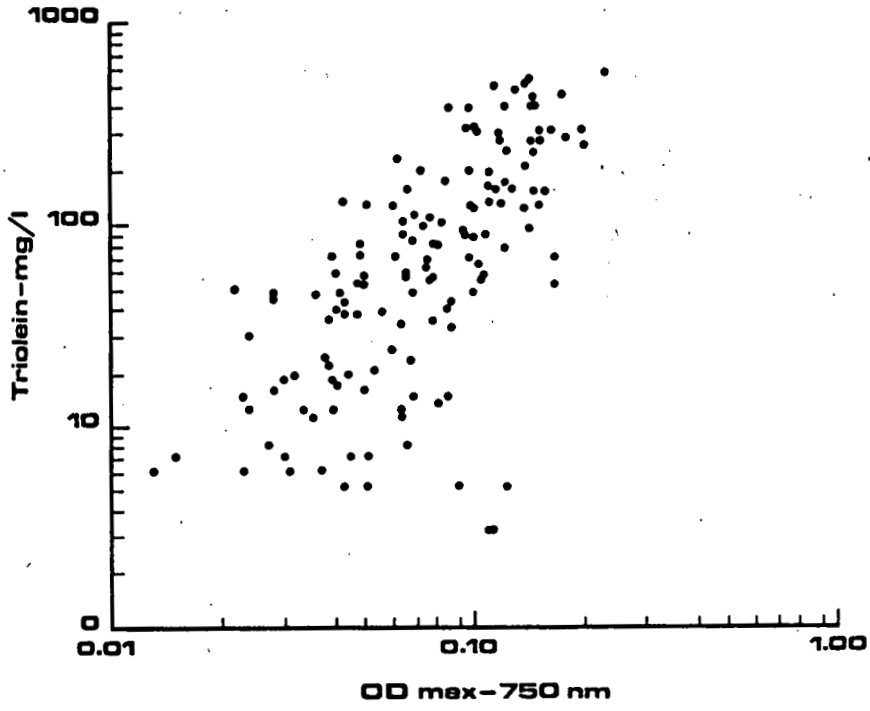


Figure 6. Relationship between culture optical density and fluorometric lipid content.

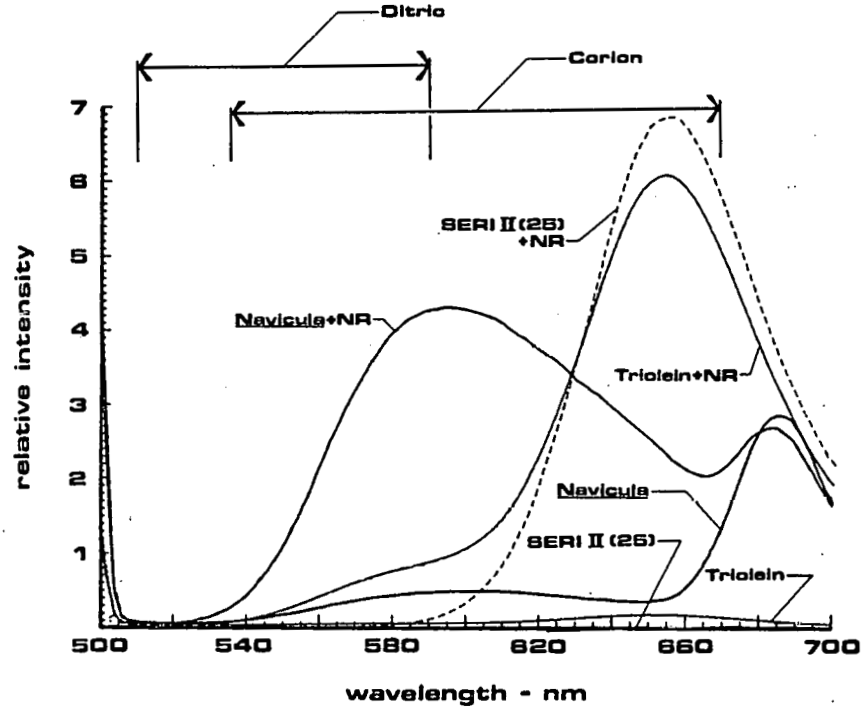


Figure 7. Comparison of fluorescence emission of Ditric (15-31180) and Corion (P70-600-5) filters relative to emission spectra for the following: SERI Type II Medium (25 mS/cm); SERI Type II Medium (25mS/cm) + Nile Red; Triolein (60 mg/l); Triolein (60 mg/l) + Nile Red; Navicula in SERI Type II Medium (25 mS/cm); Navicula in SERI Type II Medium + Nile Red.

cultures lacking detectable growth were supplied with ammonia. Mean growth rates in cultures containing urea were greater than with nitrate or ammonia. A temperature of 25°C yielded slightly more rapid growth than 30°C and the elevated light intensity coupled with urea also increased growth rates. Light intensity (based on the two intensities used) made little or no difference in growth rate when nitrate and ammonia were used as the nitrogen source.

FUTURE ACTIVITIES

A number of diatoms (and green algae) have been isolated which demonstrate rapid growth and strong lipid staining reactions. Detailed experiments will be directed toward elucidating their biochemical composition and growth optimization. The diatoms, as well as the green algae currently being evaluated, will perhaps further augment the SERI microalgae culture collection. Results obtained with Nile Red fluorescence must also be confirmed by sufficient parallel gravimetric lipid analysis. Confidence in this technique will allow more rapid screening for potential neutral lipid producers among many isolates already in culture that show suitable growth characteristics.

BIBLIOGRAPHY

Barclay, W.R., Nagle, N., Terry, K. and Roessler, P. 1985. Collecting and screening microalgae from shallow, inland habitats, p. 52-68. In: Aquatic Species Program Review: Proceedings of the March 1985 Principal Investigators Meeting. Publ. No. SERI/CP-231-2700. Solar Energy Research Institute, Golden, CO.

Ben-Amotz, A. 1985. Development of outdoor raceway capable of yielding oil-rich halotolerant microalgae, p. 230-242. In: Aquatic Species Program Review: Proceedings of the March Principal Investigators Meeting. Publ. No. SERI/CP-231-2700. Solar Energy Research Institute, Golden, CO.

Cole, G.A. 1968. Desert Limnology, p. 423-486. In: G.W. Brown, Jr. (ed.), Desert Biology. Academic Press, Inc. New York.

Christie, W.W. 1982. Lipid Analysis, 2nd edition. Pergamon Press, Oxford. 207 pp.

Dorsey, T.E., McDonald, P. and Roels, O.A. 1979. Measurements of phytoplankton-protein content with the heated biuret-Folin assay. J. Phycol., 14(2) 167-171.

Dubinsky, Z. and Aaronson, S. 1979. Increase of lipid yields from some algae by acid extraction. Phytochem., 18: 51-21.

Greenspan, P., Mayer, E.P. and Fowler, S.D. 1985. Nile Red: A selective fluorescent stain for intracellular lipid droplets. J. Cell Biol., 100: 965-973.

Kochert, G. 1978. Carbohydrate determination by the phenol-sulfuric acid method, p. 95-97 In: J.A. Hellebust and J.S. Cragie (eds.), Handbook of Phycological Methods; Physiological and Biochemical Methods. Cambridge Univ. Press, Cambridge.

Lansford, R.R., Hernadez, J.W. and Enis, P. 1986. Evaluation of available saline water resources in New Mexico for the production of microalgae, p. 25. In: Abstracts of Presentation. Aquatic Species Program Annual Review Meeting, Golden, Colorado, September 24-25, 1986. Publ. No. SERI/CP-231-3018. Solar Energy Research Institute, Golden, CO.

McIntosh, R. 1985. SERI Aquatic Species Program 1984 Annual Report. Publ. No. SERI/CP-231-2659. Solar Energy Research Institute, Golden, CO. 52 pp.

Nagle, N. 1986. A simple fluorometric procedure for monitoring neutral lipids accumulation in microalgae. Unpublished manuscript, 2 pp. Solar Energy Research Institute, Golden, CO.

Sommerfeld, M.R. and Ellingson, S.B. 1987. Collection of high energy yielding strains of saline microalgae from southwestern states. Draft Report to Aquatic Species Program, SERI, Golden, Colorado, 40 p. + Appendices.

Sorokin, C. 1973. Dry weight, packed cell volume and optical density, p. 321-343. In: J.R. Stein (ed.), Handbook of Phycological Methods: Culture Methods and Growth Measurements. Cambridge Univ. Press, Cambridge.

Thomas, W.H., Seibert, D.L.R., Alden, M., Eldridge, P., Neori, A. and Gaines, S. 1984. Culture requirements, yields, and light utilization efficiencies of some desert saline microalgae, p. 7-51. In: Aquatic Species Program Review: Proceedings of the April 1984 Principal Investigators Meeting. Publ. No. SERI/CP-231-2700. Solar Energy Research Institute, Golden, CO.

_____, _____, _____, and _____. 1985. Selection of desert saline microalgae for high yields at elevated temperatures and light intensities and in SERI standard artificial media, p. 5-27. In: Aquatic Species Program Review: Proceedings of the March 1985 Principal Investigators Meeting. Publ. No. SERI/CP-231-2700. Solar Energy Research Institute Golden, CO.

Wilson, L.G., Olson, K.L., Wallace, M.G. and Osborn, M.D. 1986. Inventory of Sources of Available Saline Waters for Microalgae Mass Culture in the State of Arizona. Final Report to the Solar Energy Research Institute, Golden, Co. 113 pp.

CONCLUSION OF THE WARM-WATER ALGAE COLLECTION AND SCREENING EFFORTS CONDUCTED IN THE SOUTHEASTERN UNITED STATES

Mahasin G. Tadros
Biology Department
Alabama A&M University
Normal, Alabama 35762

ABSTRACT

In the third year of this project, one collection trip was made to the intertidal region and near islands along coasts of Florida, Alabama, and Mississippi. Among 63 algal strains isolated, five fast growers and lipid accumulators were characterized; Nitzschia dissipata (307), Navicula sp. (324), Navicula acceptata (304), Amphipropra hyalina (333), and Chaetoceros sp. (330). N. dissipata culture limited in nitrogen and silicon, showed 45.9%, 47.2% lipids. Navicula sp. (324) and N. acceptata cultures limited in nitrogen and silicon exhibited accumulated lipids, 39.6%, 47.2% and 38.2%, 42.5%, respectively. Two exceptional diatoms were A. hyalina and Chaetoceros sp. (330). Under nitrogen and silicon limited culture conditions, A. hyalina accumulated 30.2%, 37.1% lipids and 42.0%, 34.4% carbohydrates, respectively, while Chaetoceros 330 accumulated 28.7%, 22.2% lipids and 24.6%, 30.3% proteins.

CONCLUSION OF THE WARM-WATER ALGAE COLLECTION AND SCREENING EFFORTS CONDUCTED IN THE SOUTHEASTERN UNITED STATES

INTRODUCTION

In recent years, interest in microalgal lipids has been renewed because of an urgent need for utilization of alternative renewable resources as carbon sources for the production of liquid fuels. Algal lipids are highly reduced hydrocarbons produced by direct conversion of solar energy to chemical energy via the process of photosynthesis. Microalgal species are capable of producing biomass yields containing high percentages of oils. The greatest challenge to developing and commercializing microalgae for the production of liquid fuels is the economical yield of a microalgal product. Among the factors which improve the yield of microalgal lipids are variations of environmental conditions. This ongoing project, sponsored by the Solar Energy Research Institute/Department of Energy under the Historically Black Colleges and Universities Program, is dealing with screening and optimizing microalgae for lipid production.

In the first year of this project (1984-1985), two collection trips were made from Alabama water resources and intertidal region. Two diatoms: Cyclotella cryptica (35) and Nitzschia dissipata (160), which accumulated lipids up to 42% and 66%, respectively under nitrogen stress, were included in the Aquatic Species Program of the Solar Energy Research Institute. During the second year of this project (1985-1986) two collection trips were made from the intertidal region and near islands along the coasts of Florida, Alabama and Mississippi. Among seventy-five algal strains isolated, five fast growers and lipid accumulators were characterized: Navicula saprophila (260), Navicula acceptata (264), Nitzschia pusilla (225), Cylindrotheca sp. (204) and Chlorococcum sp. (183). Three diatoms were of particular interest because of their high growth rate, tolerance, and ability to accumulate lipids. Navicula saprophila and Navicula acceptata cultures limited in nitrogen and silicon showed 34.2%, (N), 42.5% (Si) and 32.4% (N), 48.5% (Si) lipids, respectively. Nitrogen-limited cells of Nitzschia pusilla exhibited higher lipids (42.6%) than silica-limited cells (32.6%).

The specific objectives of the research reported herein were: 1) collect algal samples from the Gulf of Mexico Coast (Florida, Alabama and Mississippi); 2) isolate and define the culture conditions of lipid accumulating strains; 3) evaluate the growth rate at 25° and 30° under light intensities of 100 and 1000 $uE.m^{-2}.s^{-1}$; 4) characterize promising species for temperature and salinity optima ranges and determine nutrient optima (N, Si, and P).

This work has been a continuous effort of the Aquatic Species Program since FY1984. The species isolated during 1985 and 1986 have been mentioned above. In this paper, I am reporting the algal species isolated and characterized during 1987, and the conclusion of the three years characterization effort. Methods used for this work were identical to those

described in detail in Tadros (1986) and were summarized in last year's report (Tadros 1987), hence they will not be repeated.

RESULTS

Sample Collection

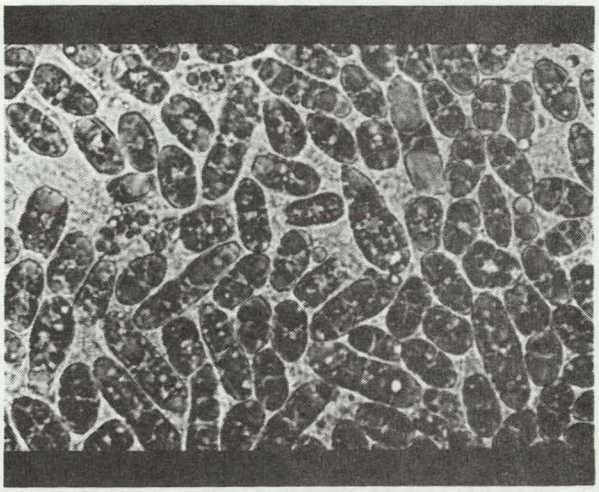
Water samples were screened on the rotary apparatus for 5-7 days, during which time irradiance was increased from 100 to 2000 uE·m^{-2}·s^{-1} by decreasing the distance between the rotary apparatus and the lamp. Cultures from the rotary apparatus were examined and identified. On the field trip in June 1986, 63 strains of algae were collected as part of the collection and screening effort from six areas: Dauphin Island, AL (13 strains); Apalachee Bay, FL (7); St. George Island, FL (11); St. Joseph Bay, FL (9); Ocean Springs, MS (10), and Biloxi, MS (13). The collection sites had temperatures ranging 27-31°C, pH values of 6.2-8.2, and conductivities of 12-32 mmho·cm^{-1}. The fast growing species were isolated as unialgal strains and evaluated for lipids microscopically (Figure 1). Growth rate was determined at 25 and 30°C. Results are presented in Table 1.

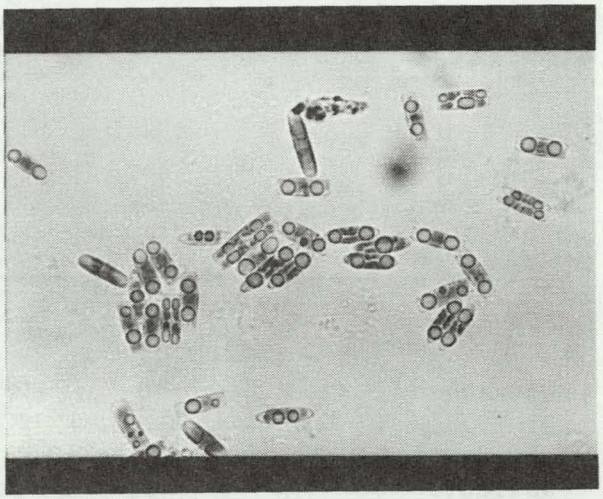
Based on the ability to both accumulate lipids and grow rapidly at high light intensity and temperature, the following diatom strains were selected for characterization: Nitzschia dissipata (307), Navicula acceptata (304), Navicula sp. (324), and Amphiprora hyalina (333).

Light, Salinity and Temperature Characterization

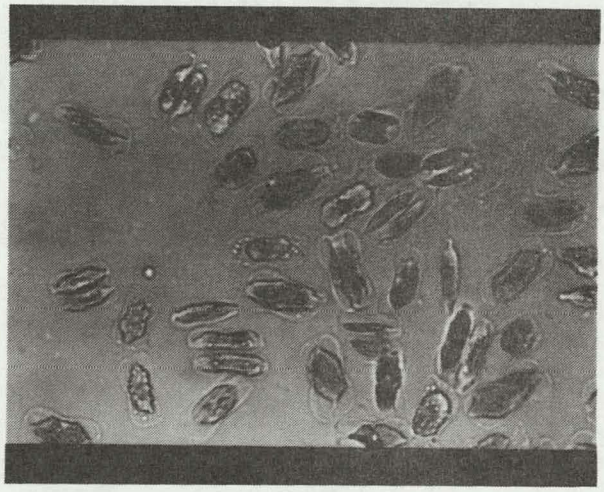
Selected strains were tested for growth in different combinations of salinity, temperature, and light intensity. Growth response was quantified as doublings·day^{-1}. Nitzschia dissipata tolerated increases in salinities up to 60 mmho·cm^{-1} (Fig.2). The growth rate reached an optimum of 2 doublings.day^{-1} at 25°C and declined towards higher or lower temperatures (30° or 20°C). Irradiance of 160 uE·m^{-2}·s^{-1} enhanced the growth of the diatom. Navicula acceptata tolerated increases in salinities up to 60 mmho·cm^{-1} (Fig. 2). High temperature (35°C) and irradiance of 160 uE.m^{-2}·s^{-1} enhanced the growth rate. Optimum growth rate reached more than 4 doublings·day^{-1}. Navicula sp. (324) tolerated increases in salinities up to 60 mmho·cm^{-1} (Fig. 3). High temperature (30°C) and irradiance of 160 uE·m^{-2}·s^{-1} enhanced the growth rate which reached up to 4 doublings·day^{-1}. Amphiprora hyalina tolerated high salinities up to 60 mmho·cm^{-1} and temperatures up to 30°C (Fig. 3). The growth rate reached more than 2 doublings·day^{-1}. Irradiance of 160 uE·m^{-2}·s^{-1} enhanced growth of the diatom.

All four strains grew more rapidly at the higher light intensity. The difference in growth rate associated with light intensity was particularly evident at the higher temperatures. The two Navicula species preferred higher conductivity waters. A. hyalina did not have a marked difference in growth rate at conductivities of 10-60 mmho·cm^{-1}. N. dissipata preferred the moderately low conductivity media (10-35 mmho.cm^{-1}). The two Navicula species had notably better growth rates than N. dissipata and A. hyalina.





D



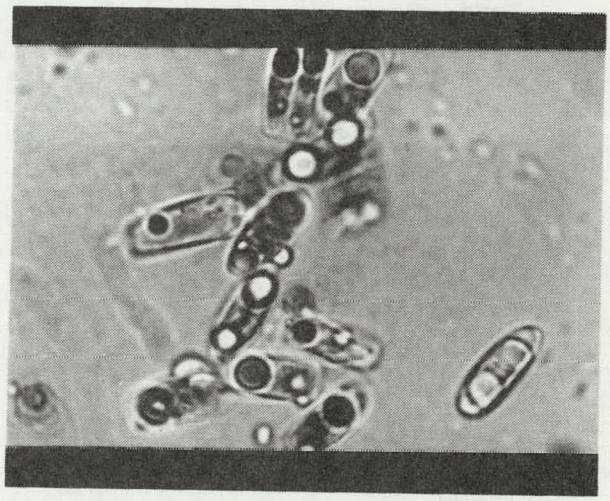


Figure 1. (A) Nitzschia dissipata, Scale: 1 cm = 4 um
(B) Navicula sp 324, Scale: 1 cm = 15 um
(C) Amphiprora hyalina, Scale: 1 cm = 25 um
(D) Navicula acceptata, Scale: 1 cm = 4 um
Cells showing oil droplets

Table 1. Most promising oil accumulating species.

	Cell Size (um)	Lipid Evaluation (staining)	Exponential Growth Rate (Doublings day^{-1})			
			Temperature (C°) 25		30	
			Light Irradiance ($uE.m^{-2}.s^{-1}$) 100	1000	100	1000
CHRYSOPHYTA:						
Nitzschia DI-270	6-10	++	0.58	0.69	0.65	0.35
Navicula DI-271	2-5	+++	0.48	0.28	0.32	0.21
Cymbella DI-277	35-40	+++	0.42	0.58	0.62	0.48
Cocconeis DI-278	3-6	++	0.62	0.59	0.86	0.62
Navicula AB-284	5-7	++++	1.63	1.84	2.22	1.86
Amphiprora Ab-286	18-22	+++	0.68	0.55	0.75	0.64
Nitzschia AB-287	8-12	+++	0.85	0.68	0.97	0.72
Navicula SGI-300	5-8	+++	1.20	0.95	0.86	0.63
Navicula acceptata SB-304	6-8	++++	1.50	1.30	2.30	1.60
Navicula SB-306	10-14	+++	0.55	0.72	0.68	0.32
Nitzschia dissipata SB-307	35-45	+++	0.82	0.75	0.86	0.25
Navicula BB-324	15-18	++++	1.20	0.82	1.90	1.15
Amphiprora hyalina BB-333	20-24	+++	0.98	0.86	1.30	0.92

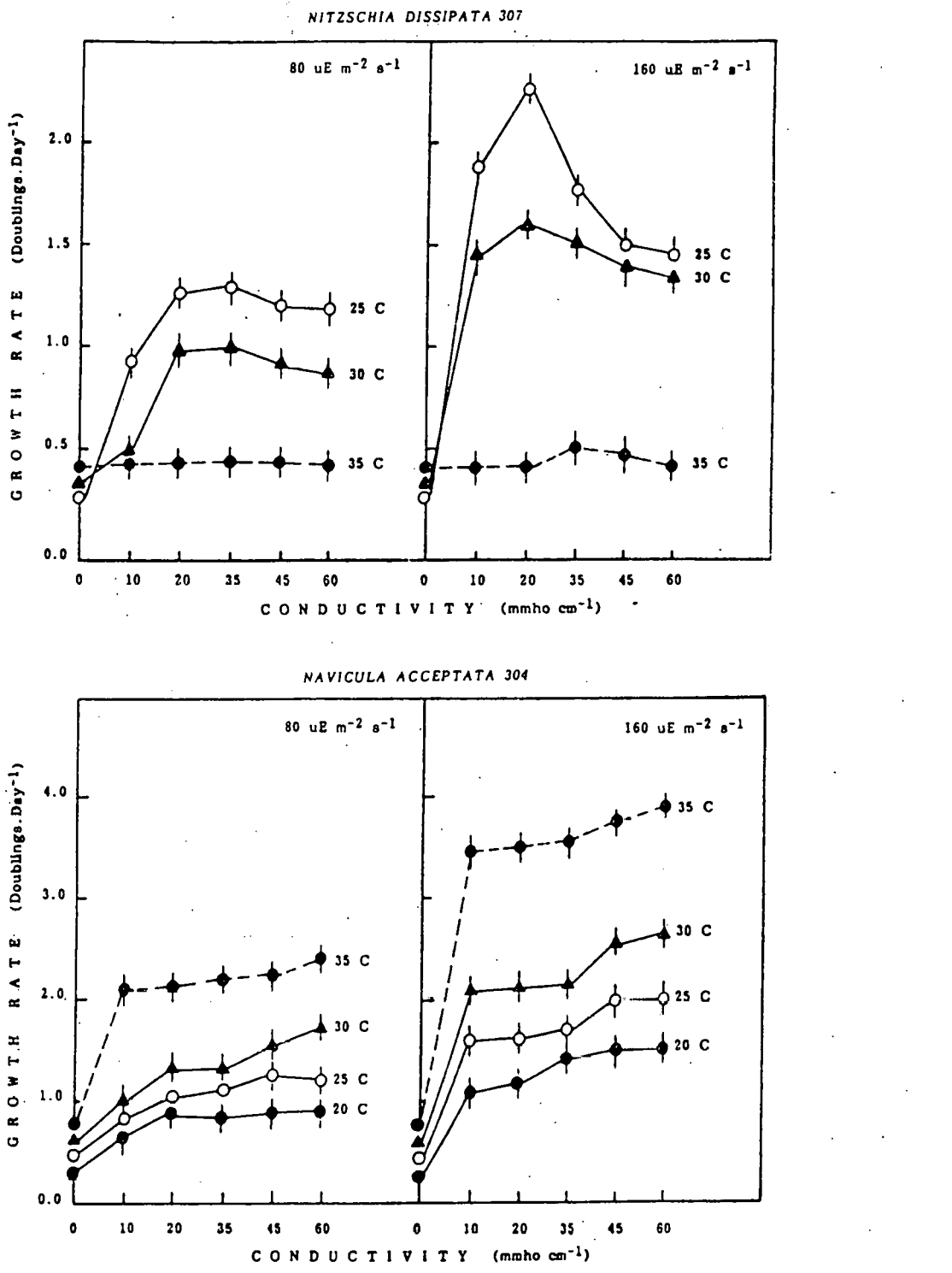


Figure 2. Growth rate of N. dissipata and N. acceptata as a function of temperature, light irradiance and conductivity. Error bars denote ± s.d.

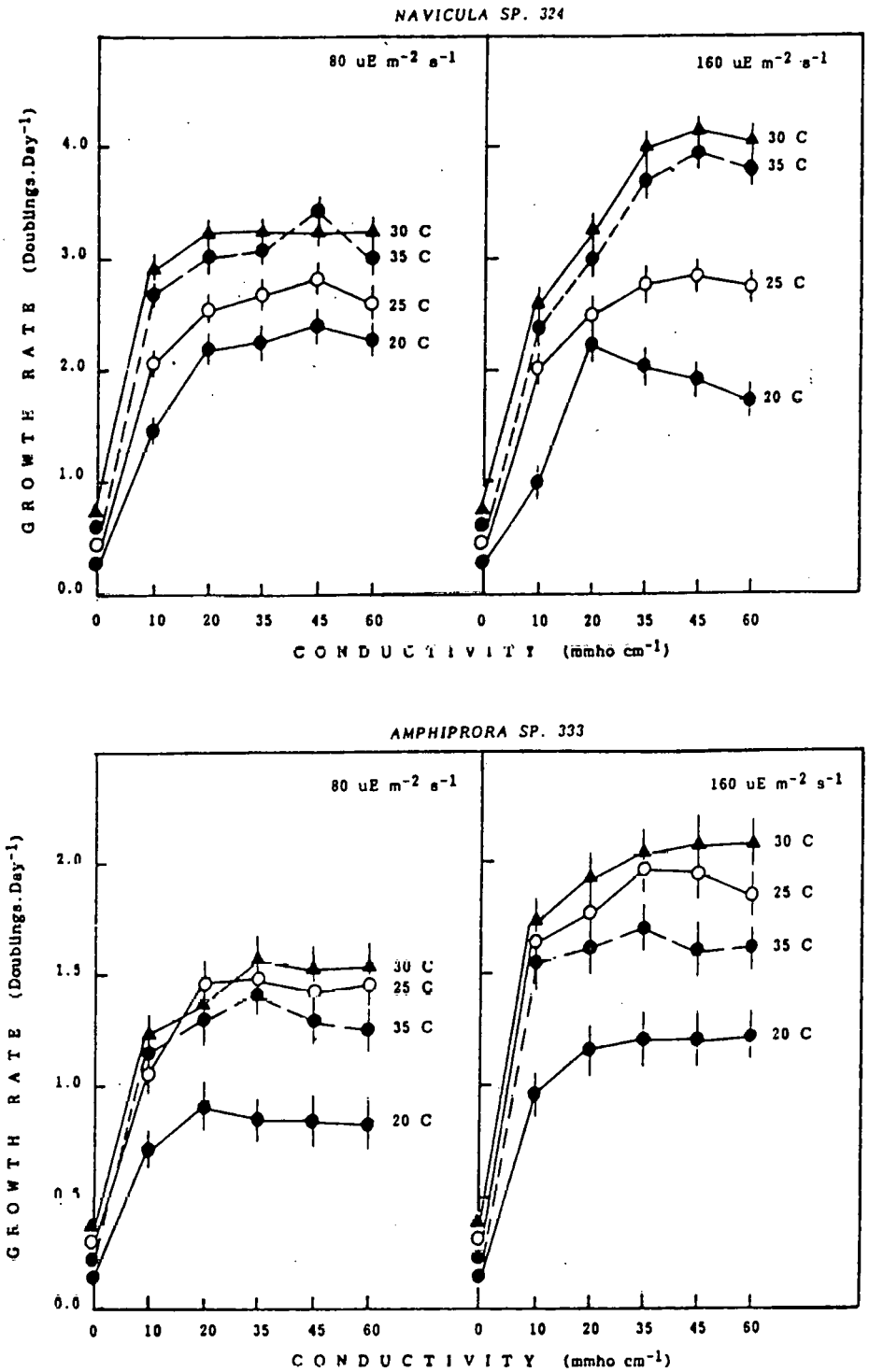


Fig. 3. Growth rate of Navicula sp. (324) and A. hyalina as a function of temperature, light irradiance and conductivity. Error bars denote ± sd.

Nutrient Requirements

Selected strains were treated with different concentrations of nitrogen (nitrate, ammonium, urea) and silicate. Growth response was quantified as doublings·day^{-1}. Results are presented in Figures 4-7. The nutrient effect on the growth of N. dissipata was more significant at 25°C than at 30°C. Nitrate-N was required at a low concentration (1mM). Increasing the concentration of the nitrogen source beyond 1 mM did not increase the growth rate of the diatom. Urea-N and ammonium-N supported the growth of the diatom to some extent. Higher concentrations of silicate (2-3 mM) produced an increase in the growth of this species (Fig. 4). The effect of nutrient concentrations on the growth of N. acceptata was more significant at 30 than 25°C. Nitrate-N and urea-N supported the growth of the diatom at a concentration of 2 mM. Silicate at a concentration of 1 mM was sufficient for the optimum growth of this species (Fig. 5). The effect of nutrient concentrations on the growth of Navicula sp 324 was more significant at 30 than 25°C. Nitrate-N (2 mM) favored the growth of the diatom more than urea-N and ammonium-N. Silicate at a concentration of 2-3 mM improved the growth of Navicula sp. (324) (Fig. 6). The effect of nutrient concentrations on the growth of A. hyalina was more significant at 30 than 25°C. Nitrate-N and urea-N at a concentration of 1 mM was sufficient for the growth of this species (Fig. 7).

The results indicate that essentially all compounds tested as nitrogen sources were capable of supporting growth of the species. However, species responded differently to nutrient concentrations as well as to the nutrient source. The two Navicula strains showed increases in growth rate with increases in nitrogen up to 2.0-3.0 mM, whereas the other two species grew optimally with 1.0-2.0 mM nitrogen and had poorer growth with higher concentrations (Figs. 4, 7). All strains preferred nitrate as the nitrogen source. N. accepta grew better with ammonium nitrogen than urea nitrogen. The other 3 strains preferred urea to ammonium. Diatoms have been reported to prefer nitrate to urea, with some species completely unable to utilize urea (Reimann et al. 1963) Our species are typical in that they prefer nitrate, but atypical in that some prefer urea to ammonium. Temperature affected salinity and nutrient utilization. This is in agreement with results of Terry et al. (1985) who reported that the uptake of nitrogen by Phaeodactylum can be influenced by temperature. It should be noted that phosphate-P at concentrations tested did not influence growth rate. Therefore, it has not been reported in the data.

SERI Standard Water

The growth of selected strains was evaluated in SERI Standard Waters (Barclay et al. 1986). The results are presented in Figure 8. SERI Type I and II supported the growth of N. acceptata and Navicula sp. (324), the latter doing exceptionally well in Type II/55. SERI Type I supported the growth of Nitzschia dissipata more than water Type II.

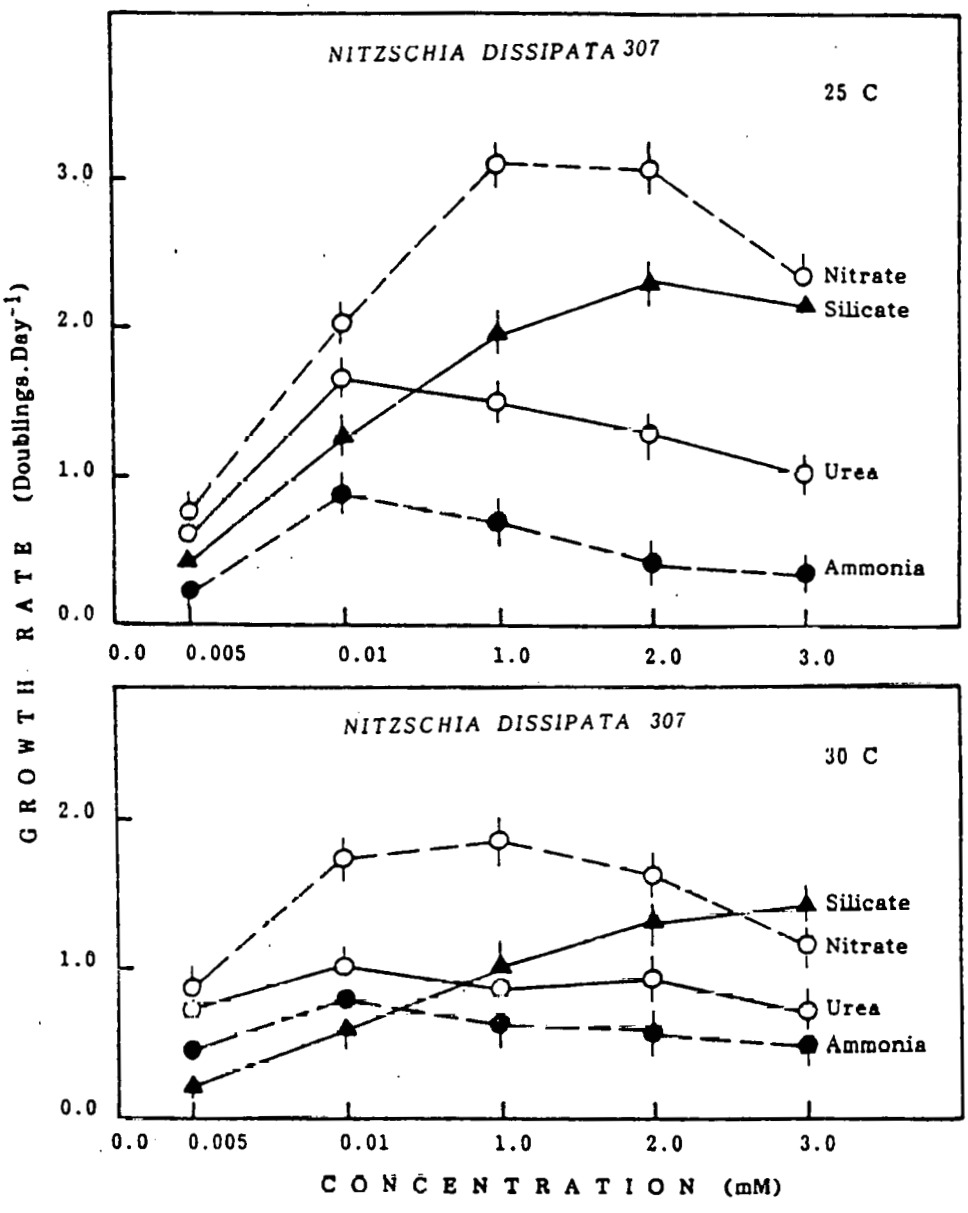


Fig.4. Growth rate of N. dissipata in a series of media containing different concentrations of nutrients. Error bars denote ± s.d.

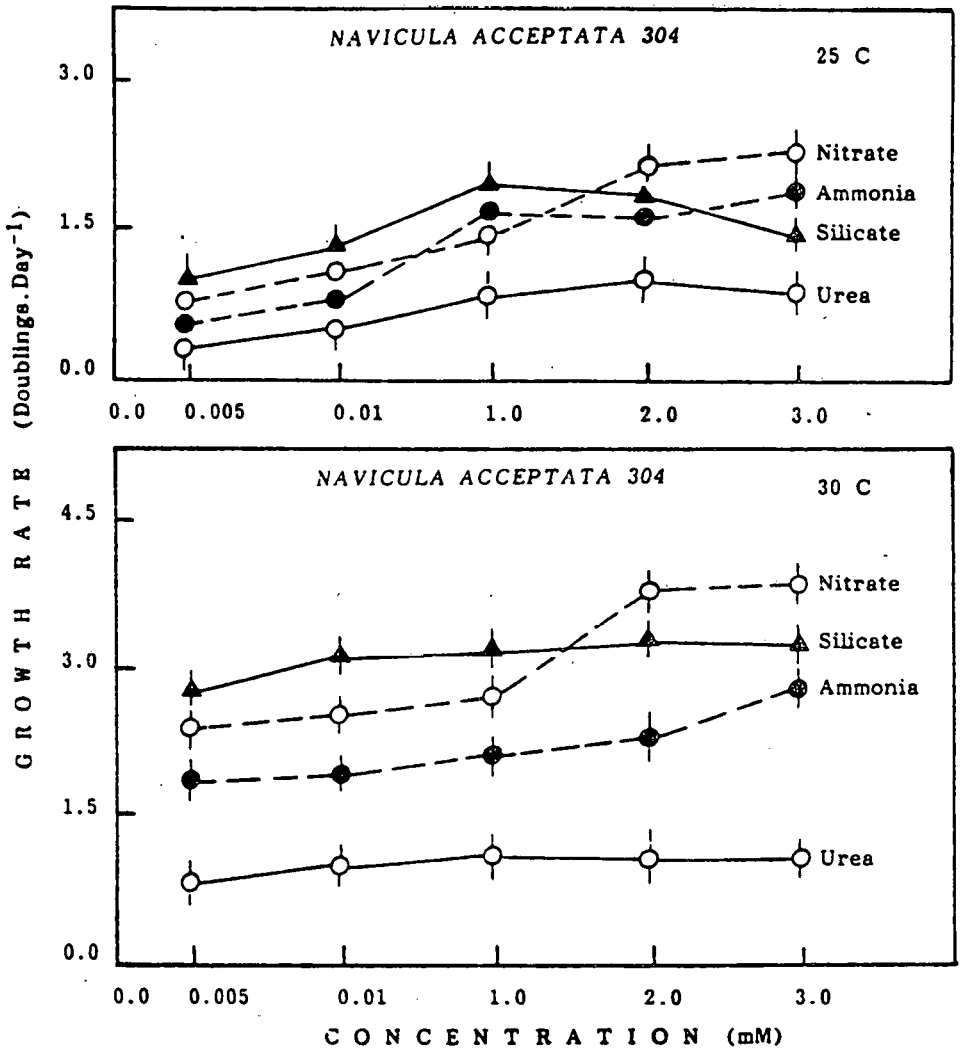


Fig. 5. Growth rate of N. acceptata in a series of media containing different concentrations of nutrients. Error bars denote ± 1 s. d.

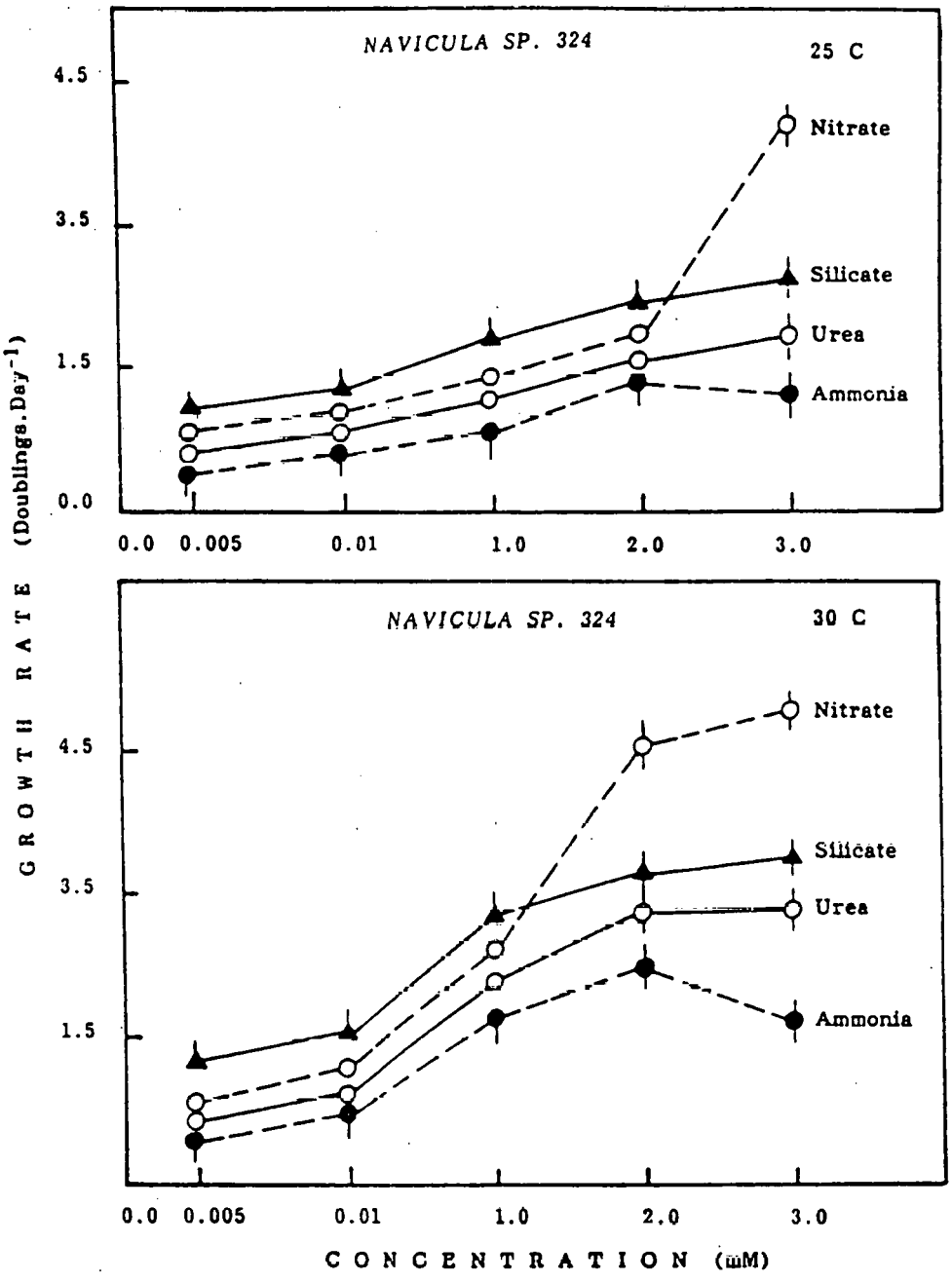


Fig. 6. Growth rate of Navicula sp. (324) in a series of media containing different concentrations of nutrients. Error bars denote $\pm$ 1 s.d.

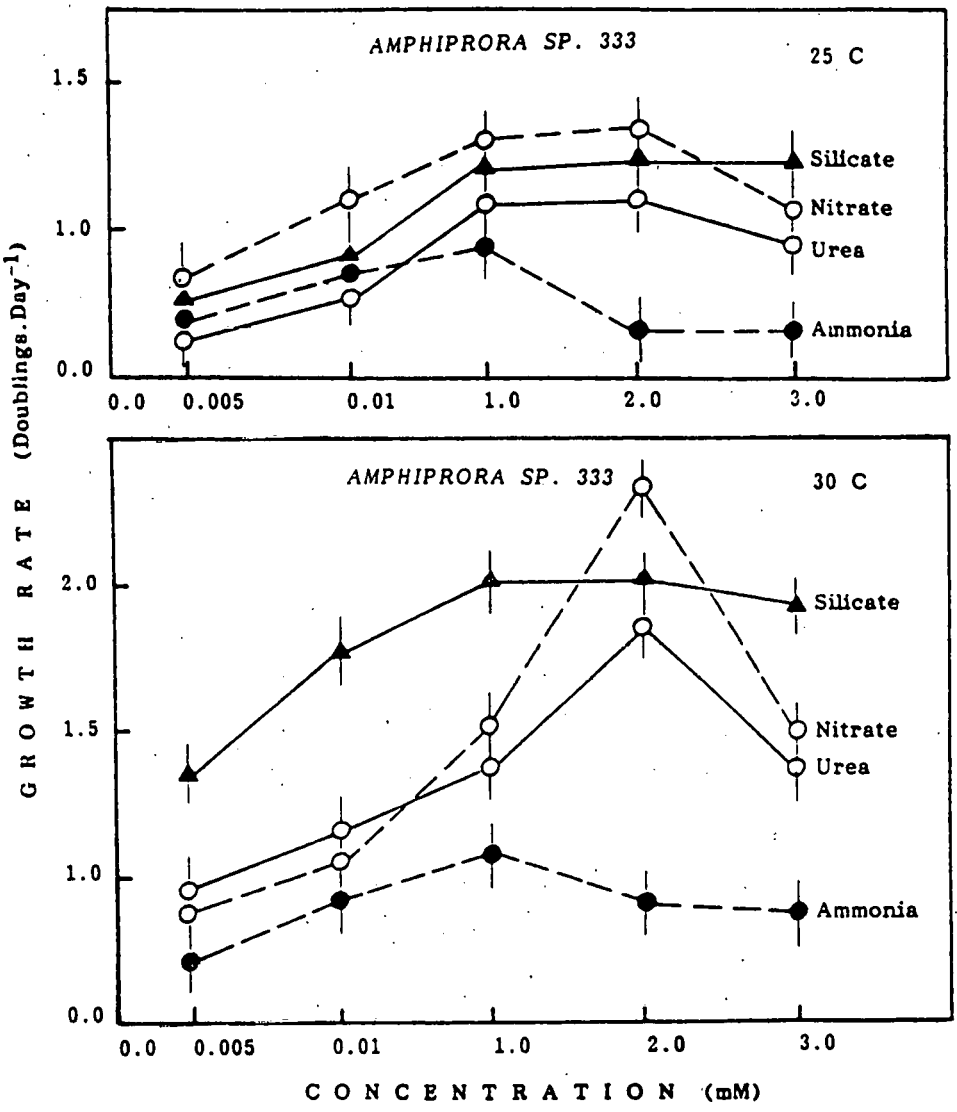


Fig. 7. Growth rate of A. hyalina in a series of media containing different concentrations of nutrients. Error bars denote ± 1 s.d.

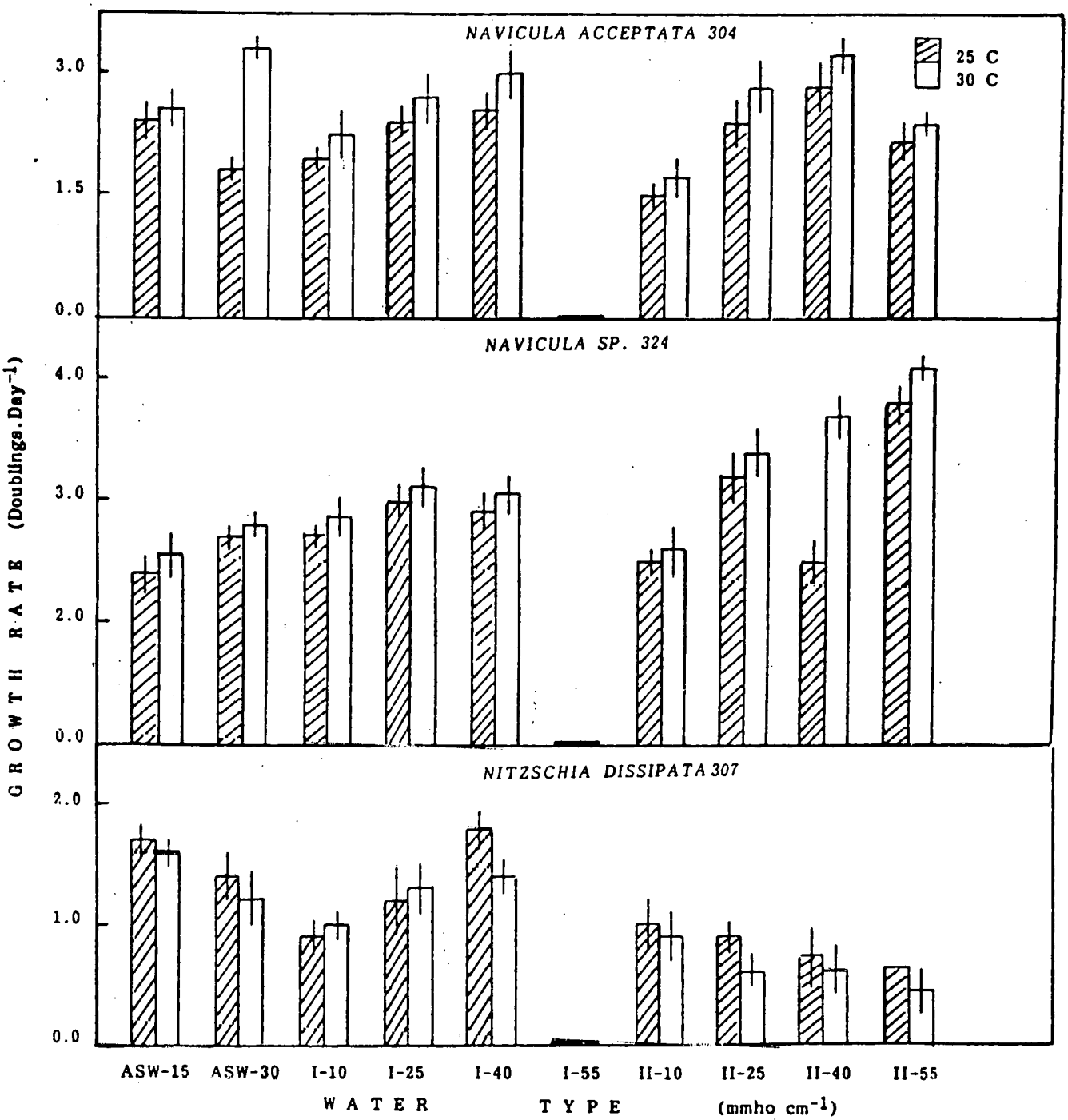


Fig. 8. Growth rate of N. acceptata and Navicula sp. (324) and N. dissipata in standard saline water types as a function of temperature and water type. Error bars denote ± 1 s.d.

Proximate Chemical Analysis for the Selected Species

The results of proximate analysis were expressed on the basis of organic weight and are presented in Table 2. N. dissipata cultures limited in nitrogen and silicon, showed 45.9%, 47.2% lipids. Navicula sp. (324) and N. acceptata cultures limited in nitrogen and silicon exhibited accumulated lipids 39.6%, 47.2% and 38.2%, 42.5%, respectively. Two exceptional diatoms were A. hyalina and Chaetoceros sp. (330). Under nitrogen and silicon limited culture conditions, A. hyalina accumulated 30.2%, 37.1% lipids and 42.0%, 34.4% carbohydrates, respectively, while Chaetoceros sp. (330) accumulated 28.7%, 22.2% lipids and 24.6%, 30.3% proteins. It can be concluded that the selected strains can be manipulated to growth under different environmental conditions and their lipid content maximized by varying nutrient concentrations. In other words, the imposition of 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, Nitzschia dissipata, Navicula sp. (324), N. acceptata, and A. hyalina will be useful for further studies of biomass for hydrocarbon production.

CONCLUSIONS

All algal species isolated from the southeastern United States which have been characterized for growth requirements and chemical composition (specifically lipids), are summarized in Table 3. Among the diatom species, Navicula strains grew at rates of 3.2-4 doublings·day^{-1}, while Cyclotella, Chaetoceros and Cylindrotheca grew at rate of 4-6 doublings·day^{-1}. Most of the diatoms grew well at 25-35°C. Nitrogen was necessary for the growth of diatoms in the form of nitrate for most of the strains, with the exception of Chaetoceros sp. (330) and Navicula sp. (260), which preferred urea. Concentration of 1-3mM nitrate or urea was sufficient for the growth of diatoms. When nitrogen or silicon was deficient in the growth media, most diatom species accumulated a high percentage of lipids. The lipid content as percentage of organic weight varied from 28-66% among species. Among the green algae, most of the species were fresh-water forms with limited conductivity range, high nitrogen requirements, and low growth rates (1.5 to 2.9 doublings·day^{-1}). When nitrate was deficient in the growth media, the green algal species accumulated lipids and became yellow in color. Based upon the growth rate and lipid content as shown in Tables 2 and 3, the following strains can be recommended for biomass technology: Chaetoceros sp. (330) Cyclotella cryptica (35), Navicula acceptata (264 and 304) Navicula saprophila (260), Navicula sp. (324) and Nitzschia dissipata (60 and 307).

ACKNOWLEDGEMENT

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

Table 2. Approximate cellular composition of selected algal species

Species	Cell Size (uM)	Cell density (Cells/ml x 10^8)	Growth Rate (doublings $.day^{-1}$)	Growth Conditions	Organic Wt.		
					Protein	Carbohydrate	Lipid
Nitzschia dissipata SB-307	35-45	2	2.5	SW, NE, SiE	22.3	40.2	27.6
				SW, ND	13.8	22.4	45.9
				SW, SiD	12.7	28.1	47.2
Navicula sp. BB-324	15-18	4	4	SW, NE, SiE	22.0	38.9	36.0
				SW, ND	11.2	15.2	39.6
				SW, SiD	15.2	14.9	47.2
Navicula acceptata SB-304	6-8	8	4	SW, NE, SiE	25.0	35.5	19.2
				SW, ND	13.3	21.3	38.2
				SW, SiD	16.2	19.2	42.5
Amphiprora hyalina BB-333	20-24	3	2.3	SW, NE, SiE	16.9	36.8	22.1
				SW, ND	12.2	42.0	30.2
				SW. SoD	18.9	34.4	37.1
Chaetoceros sp. BB-330	7-9	20	6	SW, NE, SiE	34.1	15.3	20.3
				SW, ND	24.6	12.7	28.7
				SW, SiD	30.3	24.8	22.2

SW = Saltwater
NE = Nitrogen Sufficient
ND = Nitrogen Deficient
SiE = Silica Sufficient
SiD = Silica Deficient

Table 3. Summary of characterization of warm-water strains collected from the southeastern United States (1984-1987). Growth rate is the optimal doublings·day^{-1}; temperature is in °C; conductivity is in mmho·cm^{-1}; nitrogen concentration is mMolar; and lipid content is reported as %AFDW. The optimal nitrogen source was nitrate unless otherwise indicated.

Species	Growth Rate	Temperature* Min	Max	Opt	Conductivity* Min	Max	Opt	Optimal N Concentration	Lipid Content Suff	N-def	Si-def
DIATOMS											
Amphiprora hyalina BB-333	2.3	25	35	30	10	60	20	2.0	22.1	30.2	37.1
Chaetoceros sp. BB-330	6.0	25	35	35	10	60	20	2.0 (Urea)	20.3	28.7	22.2
Cyclotella cryptica DI-35	4.0	25	35	35	10	35	20	2.0	13.2	42.1	38.6
Cylindrotheca sp. AB-204	4.3	25	30	25	10	60	20	2.0	10.5	27.2	16.5
Navicula acceptata BB-264	3.2	25	35	30	10	60	20	2.0	21.8	32.4	48.5
Navicula acceptata SB-304	4.0	25	35	35	10	60	20	2.0	19.2	38.2	42.5
Navicula saprophila BB-260	3.3	25	35	30	0	45	10	1.0 (Urea)	16.2	34.2	42.5
Navicula sp. BB-324	4.0	25	35	30	10	60	20	2.0	36.0	39.6	47.2
Nitzschia dissipata DI-60	1.4	25	35	25	10	60	35	2.0	26.3	66.0	44.6
Nitzschia dissipata SB-307	2.5	25	30	25	10	60	20	1.0	27.6	45.9	47.2
Nitzschia pusilla SB-225	2.3	25	35	30	10	60	20	2.0	17.6	42.6	32.6
Nitzschia sp. TR-114	1.2	25	30	30	10	10	10	1.0	15.2	28.1	23.5
GREEN ALGAE											
Ankistrodesmus sp. TR-87	1.5	25	30	30	0	0	0	5.0	16.9	28.1	
Chlorella sp. MB-31	1.5	25	35	30	0	10	0	5.0	15.3	32.4	
Chlorococcum sp. DI-183	2.9	25	35	30	0	20	10	10.0	13.4	36.7	
Scenedesmus sp. TR-84	1.8	25	30	25	0	0	0	3.0	20.3	44.2	

*Values given for minimum and maximum in temperature and conductivity columns represent values below or above which growth rate drops below 1 doubling·day^{-1}.

BIBLIOGRAPHY

Barclay, W., Johansen, J., Chelf, P., Nagle, N., Roessler, P. and Lemke, P. 1986. Microalgae Culture Collection 1986-1987. Solar Energy Research Institute, Golden, CO 149 pp.

Fogg, G. E. 1953. The Metabolism of Algae. Methuen & Co., London.

Healey, F. P. 1973. The inorganic nutrition of algae from an ecological viewpoint. CRC Critical Rev. Microbiol. 3:69-113.

Morris, I. 1981. Photosynthesis products, physiological state and phytopklankton growth. Can. Bull. Fish. Aqua. Sci. 210:83-102.

Reimann, B. E. F., Lewin, J. M., Guillard, R. R. L. 1963. 1963. Cyclotella cryptica, a new brackish-water diatom species. Phycologia 3:75-84.

Tadros, M. G. 1986. Screening and characterizing oleaginous microalgal species from the Southeastern U. S., SERI Subcontract #XK-3-03150-1 Annual Report (Unpublished).

Tadros, M. G. 1987. Screening and characterizing oleaginous microalgal species from the southeastern United States. In Johnson, D. A. (ed.), FY1986 Aquatic Species Program Annual Report. Solar Energy Research Institute, Golden, CO pp. 67-89.

Terry, K. L., Hirata, J., Laws, E. A. 1985. Light-, nitrogen-, and phosphorus-limited growth of Phaeodacytlym tricornutum Bohlin strain TFX-1: Chemical composition, carbon partitioning, and the diel perodicity of physiological processes. J. Exp. Mar. Biol. Ecol. 86: 85-100.

LIPOGENIC PICOPLEUSTON

Lanna Cheng and Ralph A. Lewin
A. 002
Scripps Institute of Oceanography
University of California - San Diego
San Diego, California U.S.A. 92093

ABSTRACT

We have been studying the production of lipids in 10 selected strains of small marine unicellular algae which exhibit tendencies to float. During the past three years we collected approximately 300 samples of surface sea-water, primarily from inshore locations. After a simple enrichment procedure, 80 different strains (clones) were isolated, first in crude and eventually in pure, axenic culture. All were tested for their salinity and temperature ranges for growth, and for their requirements for exogenous vitamins. In appropriate conditions - culture medium, light, temperature, etc. - samples were grown and prepared for preliminary lipid analyses. We obtained determinations for species in the following categories (the taxonomy must be regarded as provisional):

Nannochloris (Chlorophyta)	34 clones
Stichococcus (Chlorophyta)	24 clones
Chlorella (Chlorophyta)	16 clones
Eustigmatophyta	14 clones

In addition, a number of diatoms were isolated, which were transferred to SERI for further study. We selected ten strains that showed promise for high lipid production and studied their physiology. They grow well under continuous illumination (150-200 Alb) at 20-25°C, and under these conditions growth rates and lipid yields were compared.

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

Richard H. York, Jr.
Hawaii Institute of Marine Biology
P.O. Box 1346
Kaneohe, Hawaii 96744

ABSTRACT

Clones of microalgae isolated from the Hawaiian Islands were tested for production under conditions similar to those expected in large-scale culture facilities. An air-conditioned, full-spectrum solarium was used to control environmental conditions. Cultures, 10 liters in volume and 15 cm deep, were rolled by aeration from diffusers through which CO_2 gas was also added using pH controllers. Growth and production responses to culture density, dissolved oxygen concentration, pH, salinity, solar irradiance, temperature, and water type were determined for the most productive clones. The effect of solar ultraviolet irradiance on lipid production was investigated. The most productive clones were diatoms in the genera Chaetoceros, Cyclotella, and Nitzschia. These were isolated from shallow, eutrophic, saline ponds.

EFFECTS OF FLUCTUATING ENVIRONMENTS ON THE SELECTION OF HIGH YIELDING MICROALGAE

John R. Benemann and David M. Tillett
Associate Professor Research Assistant
School of Applied Biology and Chemical Engineering
Georgia Institute of Technology, Atlanta, Georgia 30332

ABSTRACT

The mass culture of microalgae for low cost oils production requires a detailed understanding of the physiology of lipid biosynthesis by the selected algal strains and of the factors making these strains competitive in outdoor ponds. Mass-culture systems will exhibit diurnal and seasonal fluctuations in key parameters affecting algal productivity and dominance: temperature, pH, pO_2, pCO_2, light, and nutrient availability. The overall objective of this project is to quantitate the effects of such fluctuations on productivity and species dominance and to identify microalgal species suitable for mass culture.

As part of this objective we investigated culture managment strategies for maximizing lipid production. Using "Nanno Q" (Nannochloropsis sp.) as a test organism we investigated strategies for maximizing lipid productivity. Our data suggest a two stage process: the first stage is operated under light limitation and nitrogen sufficiency at the standing biomass permitting maximal productivity and the second stage under dual light and nitrogen limitations at a lower standing biomass (e.g. higher per cell light input). This approach can maximize overall lipid production without loss of competitiveness.

In species competition experiments with several strains of algae isolated by the SERI/DOE Aquatic Species Program, certain parameters were more important than others in affecting species dominance: a diurnal cycle of high pO_2 was more important than fluctuating temperature, which was more important than fluctuating pH. These results are being extended to other species and conditions using both unialgal and multiple species cultures. The objective is to demonstrate that species competitiveness can be predicted from the productivity of unialgal cultures and to develop a protocol for the efficient screening of species potentially usefull in outdoor pond systems. To help guide this work, a computer model was developed capable of predicting average diurnal and seasonal variations in pond environmental parameters (pO_2, pH, pCO_2, temperature).

EFFECTS OF FLUCTUATING ENVIRONMENTS ON THE SELECTION OF HIGH YIELDING MICROALGAE

INTRODUCTION

Only specifically selected, genetically improved, algal strains will exhibit the high lipid contents and productivities required for a practical process of liquid fuels production. The cultivation one desired microalgal strain in the presence of innumerable potential contaminants, predators, and parasites, is generally considered a difficult problem. It is, however, not unlike the weed and pest problems in agriculture. There the solution has been a combination of plant breeding, chemical inhibition of undesirable organisms (weeds, pests, diseases), and mechanical tillage. Species control (strain maintenance) in algaeculture will be inherently more difficult than in agriculture: mechanical weed removal is not feasible and chemicals will be diluted in the ponds.

Some limited advances in this area have been made. For example, it has been found that high ammonia or pH will inhibit most zooplankton infestations. However, in general, current methods for algal species control have relied on using extremes in environmental conditions which inhibit most other algae (and other organisms) and select for specific microalgae species: very high alkalinity selects for Spirulina and high salinity for Dunaliella - the two major species currently being cultivated commercially. These techniques are expensive, as they require large quantities of salts and also result in severe reductions in algal productivities. Production of Chlorella, the other major species commercially mass cultured, suffers from severe contamination, solved only by the use of very large inoculum levels (produced under sterile conditions) and limited duration semi-batch operations. These reduce overall productivity and greatly increase costs. An even more extreme example are the diatoms and flagellates mass cultured for bivalve feed: Isochrysis, Tetraselmis, Chaetoceros, etc. These are grown under highly controlled, laboratory, conditions because of invasion by undesirable microalgae in outdoor systems. Costs are high, limiting the application of algae feeds in aquaculture.

Even though the problems of contamination and culture "crashes" have been often referred to in the literature of algal mass cultures, to date there have been relatively few specific publications dealing with species control in algal mass cultures. No systematic investigation has been carried out into the factors determining species dominance in mass culture systems. One obvious solution to this problem is to identify strains which easily dominate under specific outdoor pond environments. An example of this approach was the cultivation of a Scenedesmus strain in Germany and other places (Soeder, et al. 1970). This strain was apparently an isolate from one of the early mass culture ponds operated in Germany, and was successfully maintained in outdoor systems operated with similar media in a variety of locations. Another example is the dominance over several years of Micractinium in two 0.1 hectare high-rate oxidation ponds in Richmond, California, despite large variations in conditions (Benemann, et al. 1980).

Although maximum growth rates under constant laboratory conditions are often measured for algal species being screened for suitability for mass culture, such conditions have little relevance to outdoor pond situations, where high incident light intensities, dense cultures, light limited growth rates, and diurnally (and even shorter periodicities) fluctuating environmental parameters present a very different situation from the conventional laboratory environment. Not every environmental factor, however, is likely to be decisive, and the issue reduces to identifying which parameters most affect algal strain productivity and competitiveness.

The hypothesis being tested is that a few factors are critical in determining species dominance and maintenance, while others play a relatively minor role in the outcome of species (strain) competition. If this hypothesis is correct, it may be possible to select algal strains suitable for mass culture based on specific physiological adaptations, which can be reproduced in the laboratory, rather than depending on self-selecting strains in outdoor ponds or using the outdoor ponds in the screening process. This would have significant advantages in the screening of strains for suitability for outdoor systems and their genetic improvement.

The approach followed by this project has been to model the outdoor cultivation environment as a function of climatic and system design related inputs and to predict the time behavior and magnitude of specific environmental parameters and their fluctuations. Specifically the variables of temperature, pH, dissolved oxygen, and light intensity have been modelled. Based on results from the model, these conditions can be reproduced in a laboratory reactor and species productivity and competitiveness measured under fluctuating conditions as a function of controllable variables (dilution rate, nutrient supply). The outcome of these experiments is productivity and species dominance as a function of the imposed variables. A basic assumption we are testing during this project is that productivity is synonymous with competitiveness. This can be be validated by comparing unialgal and mixed cultures. The laboratory results can be compared with the data obtained with from other SERI contractors operating outdoor ponds with the same species to validate this method as a screening protocol for strains to be grown in outdoor ponds.

An initial objective of this project was to determine the effects of nitrogen deficiency on the kinetics of lipid biosynthesis and to develop a conceptual process design for lipid production by microalgae. Nutrient supply is a pond operating parameters that can be easily controlled. Nitrogen deficiency in algae can cause a relative increase in the biosynthesis of carbohydrates and/or lipids, which can accumulate in large amounts. However, the effect of nitrogen deficiency on lipid productivity, central to the technical feasibility of lipid production by microalgal cultures, is not yet resolved. Our prior work (Benemann, et al. 1986) indicated that Nannochloropsis sp. ("Nanno Q") had a rapidly inducible pathway of lipid biosynthesis. We have carried out a detailed study of the interaction of nitrogen deficiency and light supply on biomass and lipid productivity by this strain as a model of microalgae lipid production, as is discussed next.

LIPID PRODUCTIVITY BY MICROALGAE

Due to the economic limitations inherent in any fuel production process the key issue in the production of lipids by microalgae is overall lipid productivity. To a first approximation it can be assumed that lipid productivity should be maximized for a biomass containing at least half of its fixed solar energy in the form of extractable and utilizable lipids. Allowing for inevitable losses, at least 40% of the dry weight of the algae should be total lipids. This is higher than the lipid content found, with few exceptions, in algae grown under nitrogen sufficient conditions. Nitrogen deficiency is, however, well known to produce algal biomass with lipid contents often exceeding these goals. However, nitrogen deficiency greatly reduces biomass productivity and in no case has nitrogen deficienty been shown to result in increased lipid productivity.

Investigation of lipid formation rates under nitrogen deficiency, with the view toward increasing overall lipid productivity, required the identification of a suitable strain that could serve as model for the process being developed. Specifically the model strain would

have to exhibit relatively rapid and a high degree of lipid induction, with high overall biomass and lipid productivities during at least the initial stages of nitrogen deficiency. In a survey of eight strains from the SERI culture collection (Benemann et al., 1986), Nannochloropsis sp. ("Nanno Q") proved to have a high lipid content under both nitrogen sufficient (30% of ash free dry weight was lipid)) and deficient (50%+ lipid) conditions and, most important, the highest lipid productivity after transfer to a nitrogen deficient condition. Thus it was selected for more detailed studies.

The hypothesis tested by our work was that overall lipid productivity can be maximized by separting the process into a main biomass production phase and a lipid production phase, using nitrogen deficiency as the environmental factor that controls algal lipid biosynthesis and content. The objective thus was to determine how to best optimize such a two stage process in which lipid induction takes place after a light limited growth (biomass production) phase. It is important to note that to maximize overall lipid productivity both the biomass and the lipid induction phases must be operated under light limitation. Thus we had to determine the effects of dual nitrogen and light limitations on lipid induction. An additional goal was to experimentally test wether a two stage approach is indeed preferable to a single stage continuous culture, limited by either light or nitrogen (or both).

Biomass and lipid productivities were investigated in batch cultures containing between 25-105 mg/l nitrogen (added as nitrate) with either one or two side illumination of 250 $E/m^2/s$. The objective of these experiments was to determine how nitrogen quota (%N of afdw), lipid content, and lipid and biomass productivity, varied as a function of total light input into the cultures. We demonstrated (Benemann and Tillett, 1987) that nitrogen quota is a function of the light supply rate and therefore of the biomass density at which nitrogen deficient growth is established.

When lipid productivity was calculated for the batch induction studies and correlated with calculated cell N levels a broad maximum was evident, centered at 5 - 6% cell N and with a maximum lipid productivity of 150 mg/l/day. By comparison, nitrogen sufficient productivities were maximally 100 mg/l/day. In one case, at the lowest N level tested (25 mg/l) lipid productivity was not maximized relative to the other, high N containing, cultures. In this culture, however, maximum biomass productivity, or light limited growth, was never achieved. The results led us to conclude that in order for lipid productivity to be maximized, biomass productivity must also to be maximized. At the highest N level used (105 mg/l), lipid productivity was maximal, relative to the lower N containing cultures. This trend would continue until light limitation becomes severe and biomass productivity is reduced due to the relative increase in maintenance energy.

In order to test the idea that nitrogen deficient lipid productivity was in part regulated by the cells ability to carry out photosynthesis, an experiment was performed where the supply of light to the culture was increased, by a factor of two, after the onset of N deficiency. This was accomplished by switching the culture from one side to two side illumination (of the same intensity of light). The analogy to an open pond culture would be to either dilute the cultures or decrease their depth, effectively doubling the pond area for the second stage. That is, the pond area used for lipid induction would be twice that of the biomass production.

A plot of biomass yield is shown in Figure 1. Light limited linear growth starts around 300 mg/l which occurred between days 2 and 3. The light shifted culture therefore experienced roughly one day of light limitation before it was switched to two sided illumination on day 4. In Figure 2 lipid yield is plotted. It is apparent that lipid yield is the same for the two side illuminated and light shifted cultures by day 8 (880 mg/l), while the single side illuminated culture is roughly half that (400 mg/l). Thus an

FIGURE 1. BIOMASS YIELD DURING LIGHT SHIFT

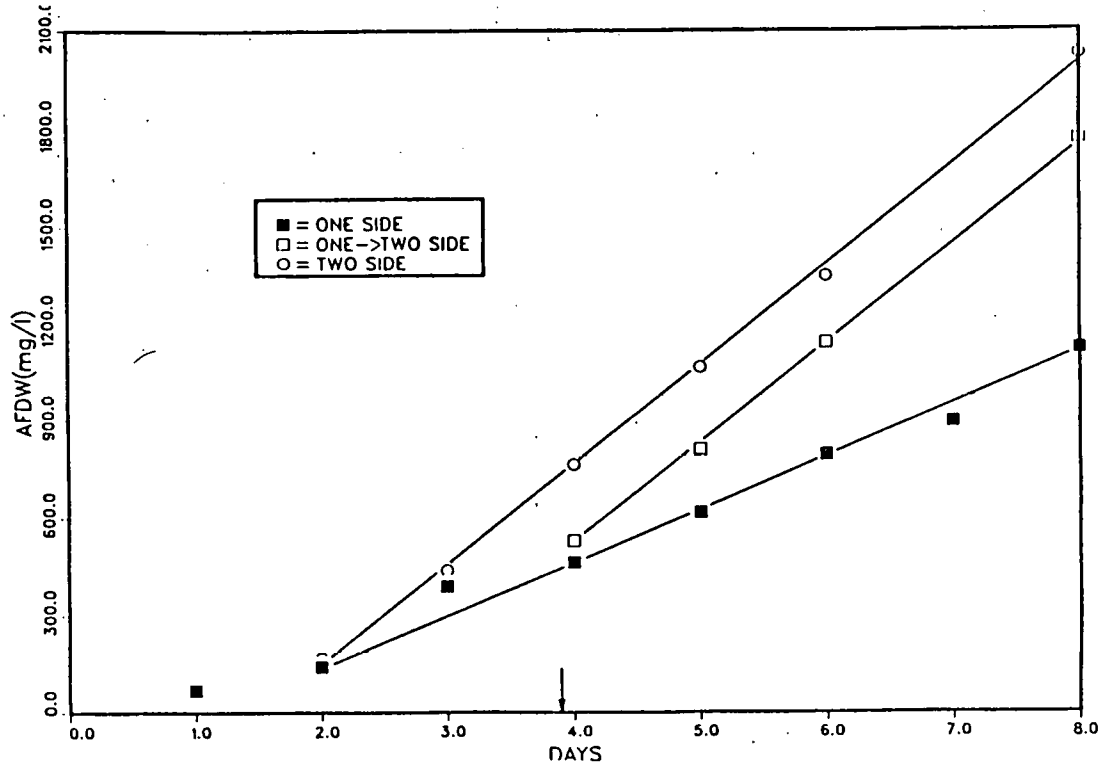
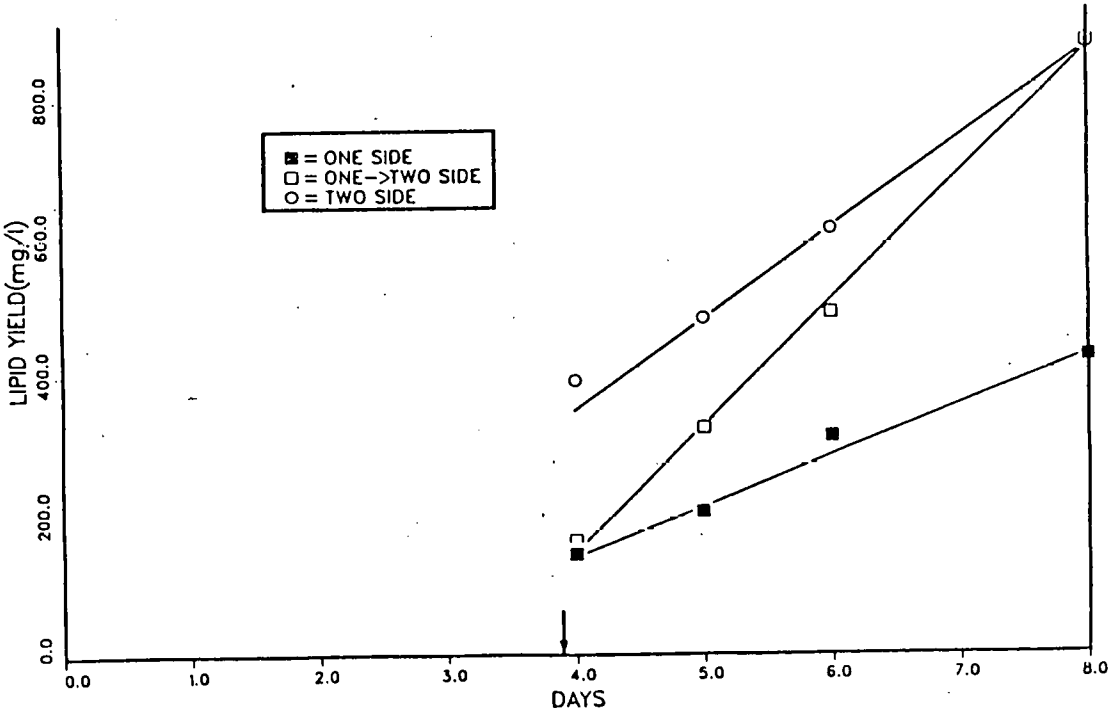


FIGURE 2. LIPID YIELD DURING LIGHT SHIFT



additional 1-2 days of light energy (from one side) was unnecessary to attain the same lipid yield. The lipid productivity was 180 mg/l/day in the shifted culture as compared to 150 mg/l/day in the two side illuminated culture. This experiment was repeated with virtually identical results. Thus not only can light energy input (pond area) be minimized during the biomass production phase, but total lipid productivity is also enhanced.

The alternative of producing lipids in light and nitrogen limited continous cultures of Nanno Q was also investigated. Although nitrogen sufficient batch and continous cultures had similar lipid productivities (100 vs. 90 mg/l/day), nitrogen limitation in continous cultures resulted in a sharp decrease in lipid productivity (50 mg/l/day), particularly when compared to batch cultures (150 mg/l/day). Shifting cultures from continous nitrogen sufficient to batch nitrogen deficient cultures resulted in even lower lipid (and biomass) productivities. Thus the growth history of the culture is important in determining its response to nutrient limitation.

These results suggest that an optimum process design should include an initial period of light limitation to maximize culture chlorophyll (for that N level) , followed by a second stage, after nitrogen deficiency has begun, where the light supply would be increased. In a pond environment this could be done by decreasing the culture depth or density (by dilution) of the second stage thereby making the pond area for the second stage larger than for the first stage. The overall effect would be to increase lipid productivity, as the total pond area for both stages would be minimized relative to the alternative of keeping the light input constant.

MODELING THE HIGH RATE POND ENVIRONMENT

Any theoretical framework of algal species control in mass culture must draw on the enormous body of research on phytoplankton ecology, physiology, and biochemistry, that has accumulated, almost in exponential fashion, over the past few decades (Harris, 1986). However, species dominance and competition is a much simpler problem to characterize for outdoor mass cultures than for natural bodies of water. The variations of biota, light, temperature, pH, and oxygen in an algal growth pond are more predictable, and less random, than in ponds, lakes, estuaries, or oceans. This is because of the control of nutrient supplies and the uniform mixing in the mass culture environment. Thus patchiness and nutrient limitations, the major problems in phytoplankton physiological ecology, are not the dominant concern in outdoor algal mass cultures.

In essence the algal mass culture growth pond consists of a 10 to 30 cm deep raceway type channel mixed by paddlewheels at between about 10 and 30 cm/sec. The channels can be quite wide and long with individual ponds, mixed by a single paddle wheel, up to 10 hectares in size, theoretically. In practice, individual growth ponds would likely be limited to about 3 to 4 hectares. (Current systems are maximally 0.5 hectares in size). The pond is supplied with CO_2 through a sump spanning the channel near the paddlewheel. From 20 to 70% of the culture is harvested daily, with used media recycled from the harvesting system. This is the essential algal production system envisioned in this project, as it is, in principle, of very low cost (Benemann et. al, 1982).

As a consequence of its shallowness, there is very little "capacity" for energy, CO_2, and O_2 storage. This results in an environment which is very dynamic - being susceptible to environmental fluctuations on a daily and seasonal basis. There are few if any natural examples of this type of environment and therefore most algal strains isolated from nature would not be expected to be optimally adapted to such systems. There is

an obvious need to predict the culture environment, on a diurnal basis, as a function of site, design, and climate specific inputs. The model we have developed (and are in the process of validating) has this capability.

The abiotic pond environment was modeled using mass and heat balances and a computer program was written. The model was excercised using monthly averaged climatic input parameters for a specific site (Roswell, New Mexico) and assumptions about the design of the system (a 1 hectare pond, l:w = 20, pond depths of 10 to 30 cm, etc.), productivity ($30 g/m^2/d$), water quality, and system operations (dilution rate and schedules, nutrient supplies, mixing velocities, etc.). Although models of pond reactors have been previously developed for a variety of applications including algae cultivation the model we have developed represents a considerable advance in the ability to predict the specific pond environment as a function of specific design and climatic input data.

As an example of the model predictions, Figure 3 presents the diurnal pond temperature calculated for a pond depth of 20 cm for the month of July, using averaged diurnal meterological observations for that month. A temperature amplitude of about 20 ^{o}C is predicted over roughly a 10 hour period (heating phase). This example points out the very large diurnal fluctuations in pond temperatures possible. Other results conclude that evaporation rate is the major determining factor of the average and maximum-minimum diurnal pond temperature but not of the amplitude of the temperature range itself.

Diurnal temperature profiles were simulated for each month of the year in order to estimate the duration of, and limitations on, the growing season for this site (Figure 2). Once average pond temperatures begin to fall below $20^{o}C$ in daytime and $10^{o}C$ at night, major declines in productivity are likely, to make pond operations uneconomical. This would reduce the growing season at the Roswell test facility to the March-October period. However there is uncertainty regarding the actual temperature extremes tolerated by potential candidate algal species. This is an area of current research by this project.

One important environmental factor affecting pond performance is the build-up of dissolved oxygen that occurs as a result of photosynthesis. Since the outgassing coefficient is subject to great uncertainties, a range of coefficients was used to predict maximum O_2 tensions for a 20 cm deep pond as a function of time at an assumed productivity. Very high O_2 tensions can be reached, up to ten fold air saturation, in the case of the lowest kl_a assumed. Although such high levels have not been observed, and are unlikely to be reached in practice due to nucleation effects that lead to enhancement of oxygen desorbtion, oxygen levels of up to 500% of saturation are predicted by the model (using a reasonable extimate for kl_a) and have been observed in practice (Weissman and Goebel, 1985). We have also obtained predictions of the behavoir of the ponds in respect to pH and pCO_2 as a function of various alkalinities and outgassing coefficients. Future work on the model will focus on pond energy balances and validating predictions with data collected at the Roswell, New Mexico Test Facility.

MICROALGAE COMPETITION STUDIES IN LABORATORY REACTORS

A series of species competition experiments were carried out with mixed cultures in order to estimate the selective importance of the pond environment. We chose to investigate transient conditions of pH, temperature, and dissolved oxygen in factorial design experiments. In these studies we relied upon an experience base of pond conditions (rather then the results from the model) in order to assign culture conditions. Only three initital experiments are presented here.

FIGURE 3. DIURNAL TEMPERATURE PROFILES FOR JULY AT ROSWELL, NEW MEXICO

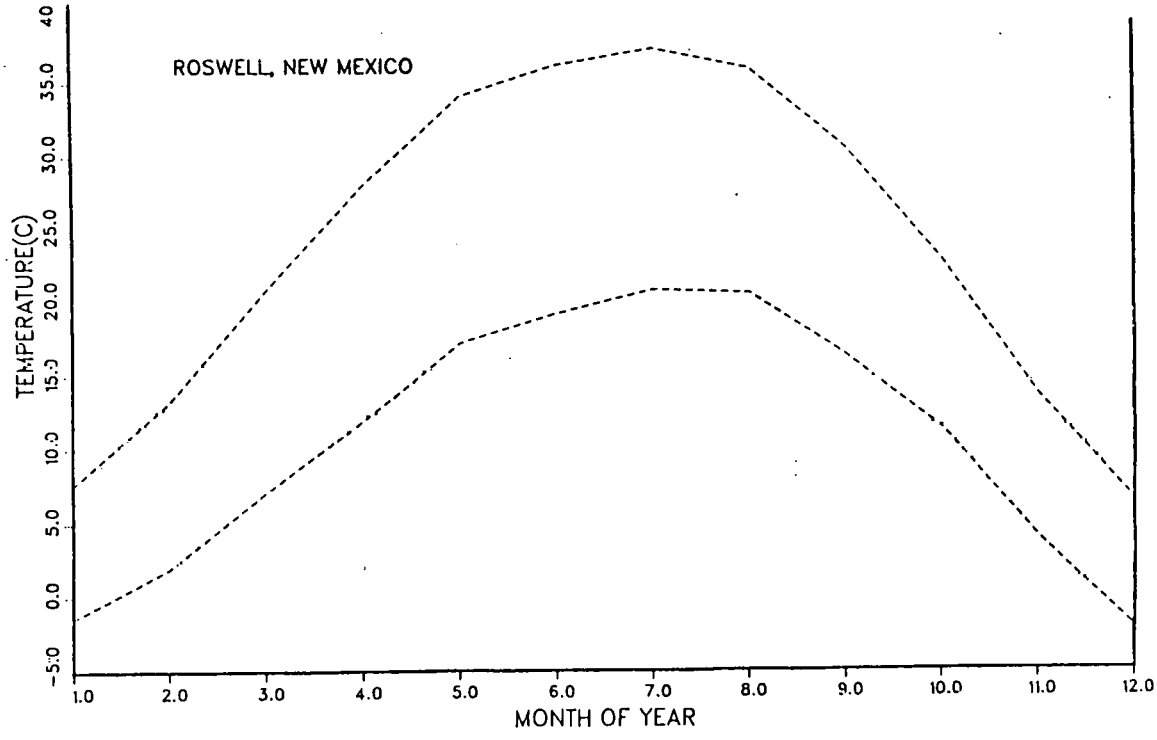
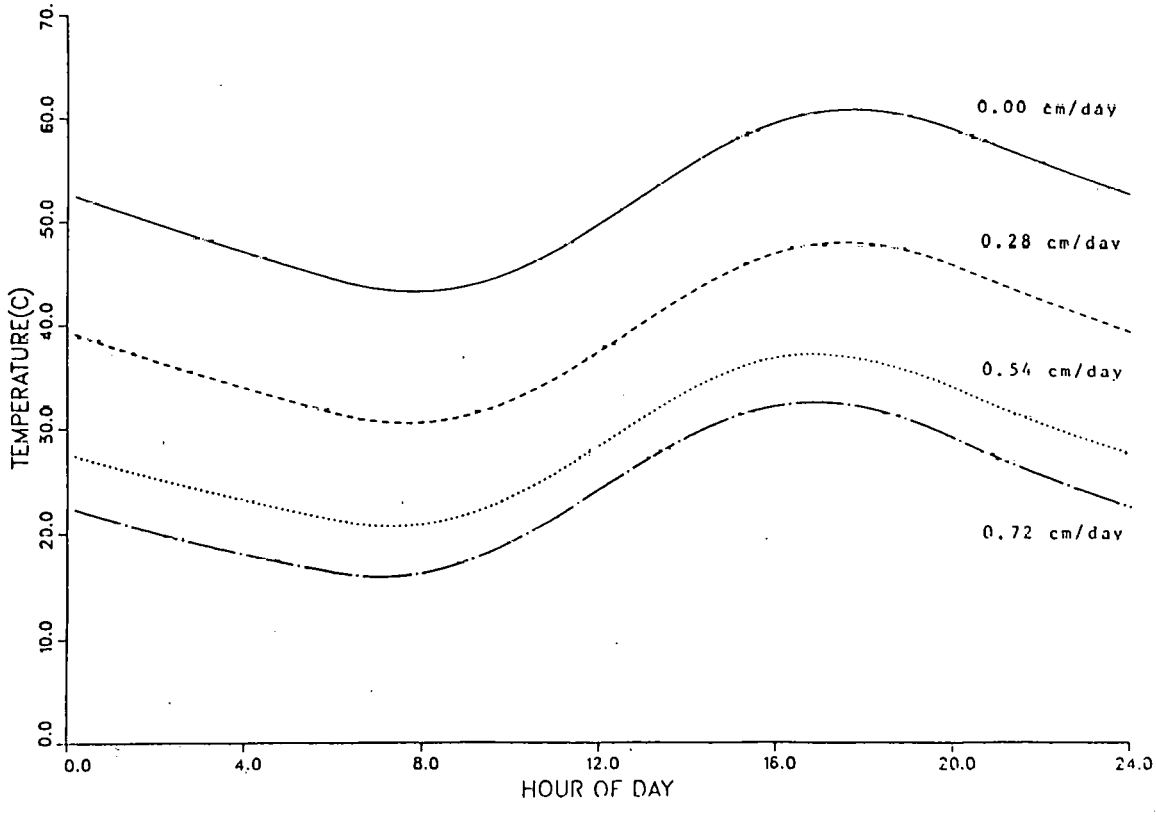


FIGURE 4. SEASONAL DISTRIBUTION OF MAX-MIN POND TEMPERATURE FOR ROSWELL, NEW MEXICO



Each experiment consisted of combining either 3 or 4 different algal strains at equal cell densities (100 ppm) and following their individual cell concentrations as a function of time. Overall culture dry weight and dilution rate were also recorded. Determination of success at a particular condition was measured by equilibrium cell concentrations, which at the minimum was taken to be 21 days (>12 residence times). The experimental design and the final results of the species competitions are summarized in Table 1. The apparatus, methods, and detailed results are presented in the Final Report (Benemann and Tillett, 1987).

Fluctuating Temperature and Oxygen Experiment

In the first experiment, listed in Table 1 and discussed here in detail as an example of the observations made, the effect of fluctuations in oxygen, temperature, and light on the competitiveness of Chlorella, Chaetoceros, and Cyclotella was investigated. Figure 5 shows the dry weight curves for each culture and Figure 6 the cell count results. One general observation was that while significant cell concentration differences began to appear in the cultures between roughly 6 to 9 days (4-6 residence times) the final "equilibrium" result was not apparent until after 18 days (12 residence times).

For culture 131, kept at air levels of oxygen and constant temperature, a codominance between the Chlorella and Chaetoceros was observed. A cell count taken on day 45 (the final day of the experiment) gave cell concentrations of 34, 16, and 0.2 million cells per ml for Chlorella, Chaetoceros, and Cyclotella respectively, agreeing closely with those for day 25. One clear observation of culture 131, was that the Cyclotella did poorly under this condition declining from day 7 (at 3.8 million cells/ml) to a final steady state cell concentration of about 0.05 million cells/ml by day 15. Thus under these conditions Cyclotella is not competitive with either Chaetoceros or Chlorella and these two species are about equally favored by them. Culture 132 tested the effects of a periodic high oxygen environment (6 hrs of 100% O_2 during the late light period), while maintaining a constant temperature as in 131. For this condition, the Chaetoceros clearly dominates over both the Chlorella and Cyclotella. After day 18, there was an increase in the Chlorella concentration which by day 45 was at 2.2 million cells/ml. The Cyclotella, while still being a poor competitor did roughly an order of magnitude better than in the low oxygen case(where Chlorella had been more competitive). A similar pattern as in culture 131 is seen: a rapid increase in all cell counts, but with Chlorella favored followed (day 7-10) by a crash in Cyclotella and a subsequent rise in Chaetoceros, which remained dominant.

Culture 133 was designed to evaluate, in addition to high oxygen tensions, the effect of a fluctuating temperature (on a diurnal cycle). Similar to culture 132, Chaetoceros is the dominant species after 10 days of growth with cell concentrations more or less the same as in culture 132. Chlorella growth is again depressed presumably due to the high oxygen tension. The major influence of the temperature fluctuation was the disappearance of Cyclotella, which may be an indication of this organisms sensitivity to the temperature regime.

Culture 134 was designed to determine the combined effect of high light, high oxygen and fluctuating temperature. Cell densities were considerably higher in this culture reaching over 100 million cells per ml. Under this condition both Chlorella and Chaetoceros did very well. As compared to culture 133 (without the high intensity spot) Chlorella was significantly more competitive, occupying approximately 25-35 % of the biomass compared to less than 10 % in culture 133. Cyclotella disappeared from culture after 13 days (as in culture 133), thus the higher light environment had no apparent effect on it.

TABLE 1. SUMMARY OF THE EXPERIMENTAL CONDITIONS AND RESULTS FOR THE CONTINUOUS MULTISPECIES CULTURES

FLUCTUATING TEMPERATURE AND OXYGEN MIXED CULTURE EXPERIMENT

(Cell counts in million/ml at beginning and end of experiment)

#	TEMP oC	LIGHT E/m2/s	OXYGEN	CHLORELLA Beg.	CHLORELLA End	CHAETOCEROS Beg.	CHAETOCEROS End	CYCLOTELLA Beg.	CYCLOTELLA End
1	30	550	AIR	4	36	2	17	0.3	.06
2	30	550	AIR/O2	5	3	2	30	0.6	0.9
3	20-32	550	AIR/O2	4	7	1.5	33	0.3	0
4	20-32	550+SPOT	AIR/O2	4	94	1	72	2	0

FLUCTUATING pH EXPERIMENT

(Cell Counts in million/ml at beginning and after

#	ALK mM	pCO2 %AIR	pH	CHLORELLA Beg.	CHLORELLA End	CHAETOCEROS Beg.	CHAETOCEROS End	ANKISTRI Beg.	ANKISTRI E
1	1	.35	7.1	2	13	2	14	2	0
2	1-10	.35	7.1-8.2	3	14	2	21	3	0
3	10	.35	8.2	2	11	2	15	3	0
4	10	.04-4.0	7.1-8.2	3	16	2	14	3	0

DILUTION RATE AND SPARGER DESIGN EXPERIMENT

(cell counts in million/ml at beginning and after 18 days)

#	DILUTION RATE(1/d)	SPARGER	CHLORELLA beg	CHLORELLA end	CHAETOCEROS beg	CHAETOCEROS end	ANKIST. beg	ANKIST. end	NAVICULA beg	NAVICULA end
1	1.23(.05)	fritted	1.8	13	1.0	9.6	.95	.03	.59	.20
2	0.83(.01)	"	2.0	60	1.0	.19	.91	.02	.59	0
3	0.33(.03)	"	2.1	110	.88	.76	.94	.54	.59	.17
4	0.73(.02)	bubbler	2.1	13	.91	22	.94	.16	.61	.58

FIGURE 5. DRY WEIGHT DATA FOR THE FLUCTUATING TEMPERATURE AND OXYGEN EXPERIMENT (Cultures 131 -134)

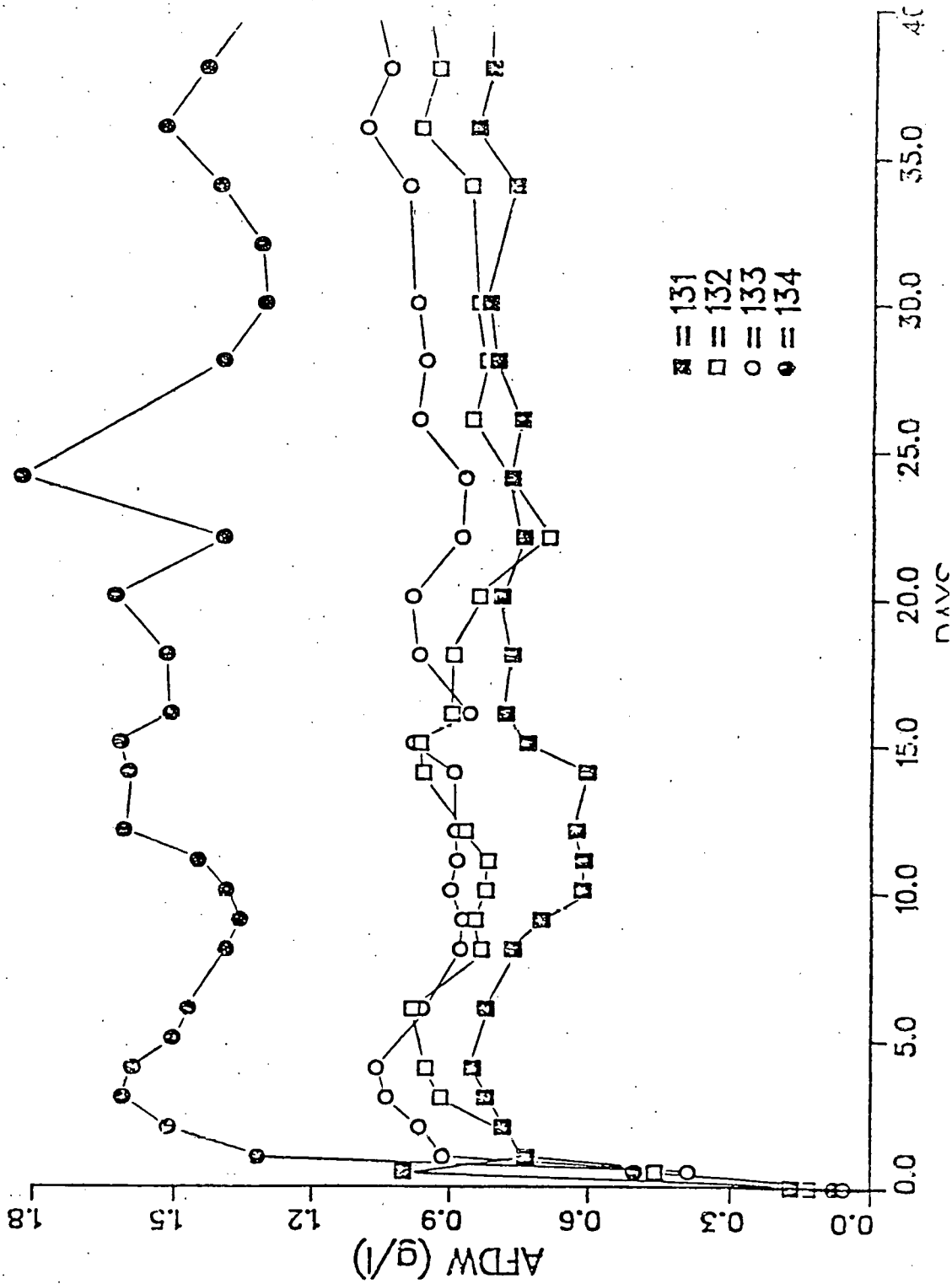
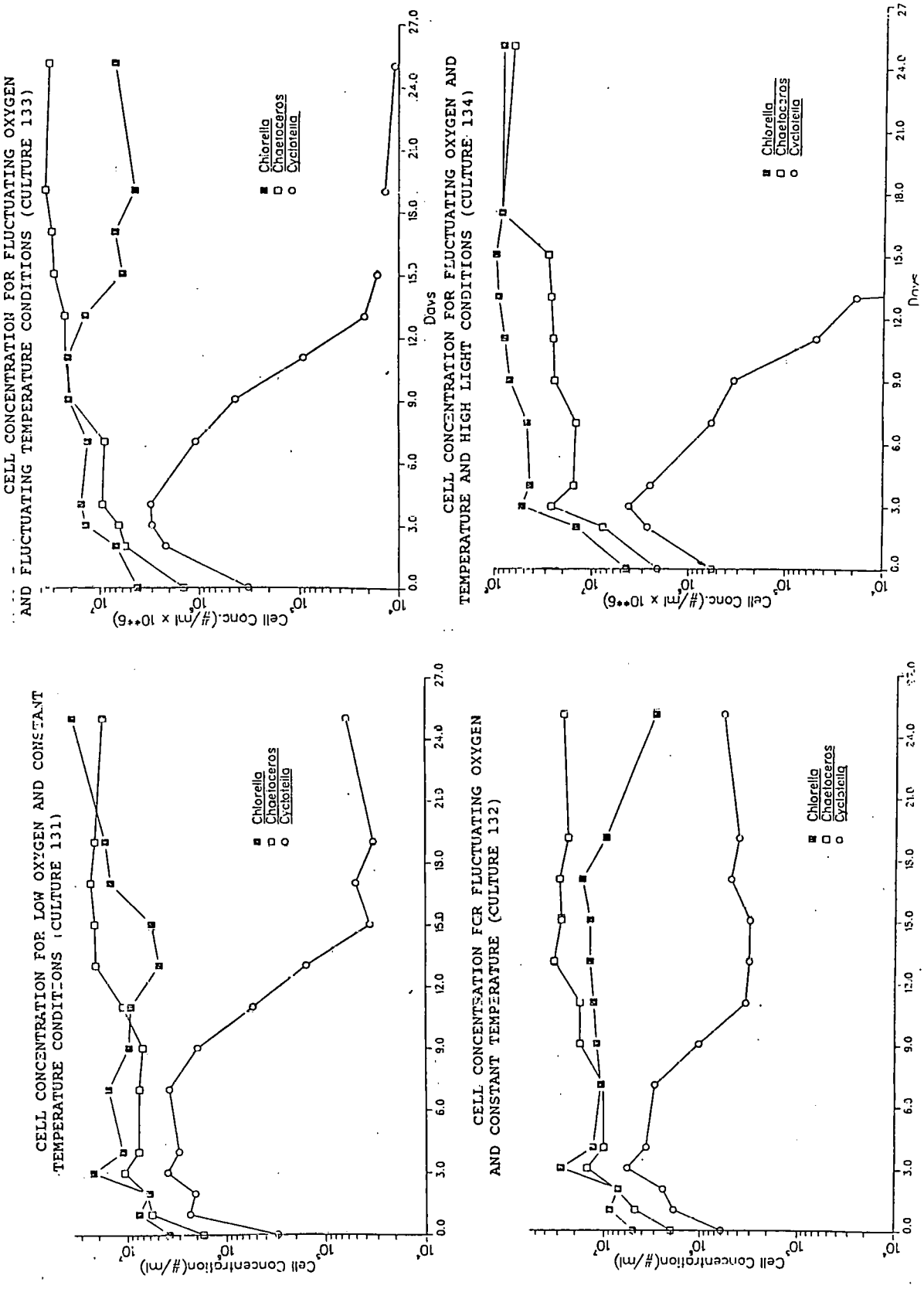


FIGURE 6. CELL CONCENTRATIONS FOR THE FLUCTUATING TEMPERATURE AND OXYGEN EXPERIMENT (Cultures 131 - 134)



The codominance between Chlorella and Chaetoceros, seen in all cases studied, suggest competition for different resources (occupying different niches). This would result if the two had (as is indeed the case) different spectral absorption patterns and therefore are utilizing separate regions of the radiation spectrum. In that case light acts as a multiple, rather than as a single, limiting nutrient. For example, the the effect of the high light (in culture 134) was to favor Chlorella, which one would expect due the red spectral emphasis of the quartz spot lamp. Thus this experiment is consistent with the contention that codominance may exist between strains with different absorption patterns.

Fluctuating pH Experiment

For the most part, pH changes in engineered systems are the result of CO_2 gas transfer and photosynthesis, while alkalinity remains constant. Deleterious effects of the pH fluctuations could either be the result of changes in the concentration of dissolved CO2, the hydrogen ion concentration itself, or indirect effects due to mineral precipitation. In the second experiment reported on in Table 1, pH fluctuations at one hour intervals during the lighted portion of the day were applied by either step changes in the alkalinity (.5 to 10 mM) or by varying the pCO2 concentration from air levels (O.035%) to 3-4 %. Four cases were considered: a constant pH of 7.1; a constant pH of 8.2; and two fluctuating pH (7.1 to 8.2) cases achieved by either varying the alkalinity or the CO_2 gas concentration. Mixed cultures containing the organisms, Chaetoceros, Chlorella, Cyclotella, and Ankistrodesmus were tested.

Chaetoceros was dominant for all conditions and Ankistrodesmus was noncompetitive for all conditions. The fact that Chlorella was not dominant is inconsistent with all other experiments tested at low oxygen and this dilution rate. The reason for its lack of competitiveness is unclear, but may be the result of the larger proportion of Cyclotella present in these reactors. There were no significant differences in productivity between reactors - they were all 400 mg/l/day. Furthermore there were no obvious differences in species outcome for the varied pH environments. This lack of differential response may signify a lack of pH sensitivity of the organisms tested within the range studied.

Dilution Rate Experiment

The objective of the final experiment listed in Table 1 was to test the sensitivity of the experimental design to changes in dilution rate and sparger design. Dilution rates of 1.23, 0.83, and 0.33 day^{-1} were established on cultures containing Chlorella, Chaetoceros, Ankistrodesmus, and Navicula.

The Chlorella and Chaetoceros codominated with Chaetoceros maintaining a larger proportion of the biomass (ca. 60%). The other strains, Navicula and Ankistrodesmus, competed very poorly both declining in cell number. At a dilution rate of 0.83 day^{-1}, Chlorella was the dominant organism after 10 days of growth. Chaetoceros while competitive in the early stages of the experiment, was diluted significantly following the sharp increase in Chlorella. The overall patterns of for Navicula and Ankistrodesmus were similar to that observed at the higher dilution rates. At the lowest dilution rate (0.33 day^{-1}), a pattern quite similar to that observed for the intermediate dilution rate was obtained. Chlorella again became dominant after roughly ten days of growth. Chaetoceros competed well initially, but declined significantly after 10 days. Ankistrodesmus and Navicula again competed poorly.

The reason for the differences observed between the highest dilution rate and the lower dilution rate cultures perhaps can be explained by differences in the degree of light

limitation. At the highest dilution rate, the cell density hovered a little above 400 ppm, while at the intermediate and low dilution rates the cell densities were roughly 600 and 1100 ppm respectively. Therefore it is possible that the rate of pigment synthesis or light utilization efficiency is greater in Chlorella than Chaetoceros.

Coincident with the dilution rate experiment, a fourth culture was used to compare the effects of sparger design on the dominance patterns. In previous work it has been observed that a significant amount of cell debris can accumulate above the gas liquid interface presumably stripped out of culture by the high gas liquid interfacial tension. It was of interest therefore to determine whether the sparger itself was applying an additional selective pressure. In the test culture (#4) a gas bubbler was used instead of the standard coarse sintered glass cylinders usually used. Chaetoceros remained dominant in this culture for an extended period of time, in contrast to what was observed in the cylinder sparged culture. The Chlorella maintained a relatively constant cell concentration, with no sudden expansion in cell number being observed. There was no foaming and very little cell material deposited above the liquid level.

The results from this experiment indicate that light limitation influences species dominance patterns. This is logical and suggests that future experiments operate only under light limitation particularly as it correlates with growth conditions in outdoor ponds. However, it should be noted that if ponds are inoculated with a relatively low amount of culture, then a significant period of growth will take place under light sufficient conditions - possibly resulting in the selection of algal species that are more competitive under light sufficient than light deficient conditions.

Within light limited growth, differences in dilution rate appeared to have little effect on actual cell concentration patterns. Thus little bias is introduced into the dominance patterns as a result of dilution rate variations. Gas sparger design appears to have a significant effect on species outcome presumably due to differences in the shearing forces exerted upon a culture. Because open ponds experience primarily a low shear environment, with periodic high shear at the gas transfer stations, the gas bubbler is considered most representative of the pond environment, although power densities are well above those found in mass culture ponds.

Current and Future Research

It has been demonstrated that fluctuations in oxygen, temperature, and, to a lesser extent, pH are all important features of microalgae cultivation systems in terms of both productivity and species dominance patterns. Our current experiments place a greater emphasis on the use of monoalgal cultures, with mixed culture experiments being designed primarily for validation of monoculture results. The basic hypothesis to be tested is that there is little if any interaction between algal cultures (e.g. allelopathy or other interferences) and that mixed culture results can be predicted from unialgal cultures. Thus, dominance in mixed cultures should be predictable from the productivity (in terms of light absorbance units) of the cultures. This has been established by recently completed experiments in which several species operated under both unialgal and mixed culture conditions.

In current and future experiments two general conditions will be tested: the standard, non fluctuating, condition and a simulated outdoor condition reflecting the results from the model for the Roswell, New Mexico site. These experiments involve the operation of monoalgal and mixed continous and semibatch cultures under conditions of, primarily, fluctuating temperature (low nighttime and high daytime temperatures, following the diurnal cycle) and seasonal extremes.

The data obtained during this project should allow selection of highly competetive and productive strains useful in outdoor cultures. The results will be compared to the data obtained by the outdoor ponds currently operated at the Roswell Test Facility to determine the potential and limitations to this approach to species screening and selection.

BIBLIOGRAPHY

Benemann, J.R., R.P Goebel, D.C. Augenstein, and J.C. Weissman, Microroalgae Production of Liquid Fuels, Final Report to the U.S. Dept. of Energy (1982).

Benemann, J. R., B. Koopman, J. C. Weissman, D.E. Eisenberg, and R.P. Goebel, "Development of Microalgae Harvesting and High Rate Pond Technologies in California", in Algae Biomass (Shelef, G. and C.J. Soeder,Eds.), Elsevier/North Holland, Amsterdam (1980).

Benemann, J.R., D.M. Tillett, Y. Suen, J. Hubbard, and T.G. Tornabene, Chemical Profiles of Microalgae with Emphasis on Lipids, Final Report to the Solar Energy Research Institute, Subcontract X-K4-04143-02, Georgia Institute of Technology (1986).

Benemann, J.R. and D.M. Tillett, Effects fo Fluctuating Environments on the Selection of High Yielding Microalgae, Final Report to the Solar Energy Research Institute, Subcontract XK-4-01236-06, Georgia Institute of Technology (1987).

Harris G.P., The Physiological Ecology of Phytoplankton (1986).

Soeder, C.J. M.E. Meffert, I. Rolle, W.P. Pabst, H.D. Payer, and E. Stengle, Das Dortmunder Verfahren zur Produktion Essbarer Mikroalgen, Kohlenbiologishes Forschungsinstitut ev. Dortmund (1970).

Weissman, J.C. and R.P. Goebel, Design, Fabrication, and Operation of Innovative Microalgae Culture Experiments using Open Ponds, Final Report to the Solar Energy Research Institute (1985).

NUTRITIONAL REQUIREMENTS FOR MAXIMAL GROWTH OF OIL PRODUCING MICROALGAE

Charles F. Rhyne
Associate Professor of Biology
Jackson State University
Jackson,MS 39217

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 the diatoms Chaetoceros 9, Chaetoceros 10, Chaetoceros 15, Navicula 2, Thalassiosira 2 and the green alga Monoraphidium 2. 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 NO_3, 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. Three species, Chaetoceros 10, Navicula 2 and Monoraphidium 2 showed significantly higher doublings times in NO_3-N as opposed to both NH_4-N and urea-N. Thalassiosira 2 showed good growth at very low concentrations of urea-N. Thalassiosira and Chaetoceros 9 demonstrated very good growth at low concentrations of phosphorous. Chaetoceros 9 demonstrated significantly higher growth when $FeCl_3$ was replaced with $FeNH_4$-citrate. Of the two species tested, Chaetoceros 9 required vitamin B_{12} and Monoraphidium 2 required thiamine. This information should contribute to the SERI Aquatic Species Program and others as a guide toward optimizing growth prior to the shift toward lipid production.

NUTRITIONAL REQUIREMENTS FOR MAXIMAL GROWTH OF OIL PRODUCING MICROALGAE

INTRODUCTION

Concept

A question by Hall (1986) asks how will underdeveloped nations achieve both food and biomass energy production locally on a sustainable basis. It is, however, very evident that renewable energy systems are becoming a necessary part of planning even for first world countries. One of several biomass energy directions is that of utilizing oil and hydrocarbon producing plants and algae. Since 1980, an emphasis in alternative energy research highlighting the use of appropriate algal species for the production of lipids has been carried out by the Aquatic Species Program of SERI/DOE (McIntosh, 1984). The important research facets of this program have been to improve the production, conversion to fuel and cost efficiency of aquatic plant species. Investigation into the feasibility of using microalgae that will grow in saline waters of the southwestern United States has received the main emphasis (SERI, 1985). Three areas of study have recently been undertaken by SERI researchers: 1. biological; 2. engineering; and 3. analysis. Biological research has included the collection and screening for productive species, developing culture and management techniques for growing desirable species, and improving the most promising species (SERI, 1984). A series of recent SERI reports and refereed papers has reported on algal growth studies and lipid analysis and production (Ben-Amotz and Tornabene, 1985; Brezinski, 1985; Hill, 1984, 1984a; Terry et al., 1985; Thomas et al.,A,B and C 1984; SERI, 1984, 1985, and 1987).

An integral part of establishing an inventory of high yielding oil-producing microalgae is knowing which species promise high growth and/or oil production. This problem has been in part remedied by the SERI Culture Collection and its set of priorities. The first of the seven priorities is energy yield (growth rate X energy content) (Barclay et al., 1986). After preliminary screening (establishing salinity, temperature and media preferences), secondary characterizations are needed in which basic nutritional requirements are established for promising lipid producing algae. In attempts to investigate these characterizations in selected algae, the present research project was initiated by SERI.

Aims of the Present Study

To define the nutritional requirements for oil producing

microalgae which lead to optimal growth, the following three objectives were followed: 1. Obtain selected species from SERI and develop an experimental protocol that will ensure the successful completion of the following two objectives; 2. Establish the primary nutritional requirements of the species; define and establish the best form of nitrogen (NO_3-N, NH_4-N or urea-N) as well as optimal concentrations of N, P and Si (for diatoms); and 3. Establish the Fe, and vitamin requirements.

Previous Work: Nutritional Requirements of Lipid Producing Microalgae

A recent review of microalgal nutrition has been made by Kaplan et al., (1986). Little laboratory/field work has been carried out and reported in the open literature with the species used in this study. Where other studies exist, comparisons between growth rates can be confusing when values are not reported in doubling times or specific growth rates. In the majority of studies, growth values tend to be given in chl_a or gravimetric weight increases. Previous and on-going studies by other SERI workers are comparable due to the standardization for growth rate reporting. References at least to the genus if not the species used in laboratory investigations are helpful. The principal investigator has recently reported on the nutritional requirements of six marine and inland saline microalgae including the species: Ankistrodesmus falcatus, Platymonas (Tetraselmus), Chaetoceros SS-14, Chaetoceros gracilis, Cyclotella D.I. 35, Boekelovia (Chryso. F1) and Nannochloropsis salina (Nanno Q). Studies relating to the nutritional requirements of two oil producing algae as reported by Rhyne (1986) are Ankistrodesmus falcatus and Chaetoceros SS-14 (Matthews, 1986). The nutritional requirements of the freshwater oil producing alga, Botryococcus braunii, have been studied by Weetall (1985). Primary characterizations of Monoraphidium 2 and Chaetoceros 10 are seen in Barclay et al., 1986 and SERI, 1987. Recent culture work reporting growth rates and environmental conditions are seen in the monthly Aquatic Species Program Newsletter.

MATERIALS AND METHODS

Algal Species and Primary Characterizations

Six microalgae species were selected for detailed nutritional studies. Their original salinity and media requirements as suggested by SERI are shown below.

	Conductivity (mmho)	Medium
Chaetoceros 9	25	Type II
Chaetoceros 10	25	Type II
Chaetoceros 15	25	Type II
Navicula 2	25	Type II

Thalassiosira 2	25	Type I
Monoraphidium 2	25	Type II

Stock Cultures

Liquid cultures of the above species were maintained in reach-in culture chambers (Percival, models 160LL and MB-60B). Cultures were transferred to new media at 4-7 day intervals to allow for sufficient growth but not high densities (below 85% T). Photoperiod was set at 16:8 light:dark regimes. Media for the stocks are the respective media listed above. Their chemical compositions are found in SERI (1985, 1987). We have also found it necessary to modify other environmental parameters in order to sustain more rapid growth in several of the species. The standard environmental culture conditions used during this study for both stock and experimental cultures are tabulated below.

pH	Temperature
7.0-7.5	28 C
Thalassiosira 2	Thalassiosira 2
Navicula 2	Monoraphidium 2
Chaetoceros 9	Chaetoceros 9
Chaetoceros 10	
8.0-8.5	30 C
Chaetoceros 15	Navicula 2
Monoraphidium 2	Chaetoceros 10
	Chaetoceros 15

Light Energies. Light was provided at 90-100 uE/m^2/sec for stocks and 275-300 uE/m^2/sec for experimental tests. Cultures were illuminated by either 20 or 40 watt cool white fluorescent bulbs. Light energies were measured using a Biospherical Instruments quantum scaler (model QSL-100) reading in quanta/cm^2/sec and converted to uEinsteins values.

Temperature. Temperatures were maintained within 0.5°C during both light and dark periods. Temperature measurements were taken within the flasks using an electronic digital thermometer.

Conductivity. Conductivity was measured using a portable Curtin-Matthison conductivity meter and measured in mmho.

Experimental Cultures and Protocol

Cultures were grown in 25x200 mm screw cap test tubes with 25 ml of medium on a slant of 60° from center located on reciprocating shaker tables at 125 strokes/minute. Screw caps were fastened tightly. Culture tubes were slanted for adequate media agitation and light penetration.

Two to three days prior to the start-up of an experiment involving an element already found in the respective medium, such as nitrate-N or phosphate-P, cells were grown in the appropriate medium lacking that element. The cells are in theory "starved" relative to the element in question. Studies were carried out over a five day period, whereby four days of data were collected. Every 24 hours, O.D. readings were taken and pH was measured and adjusted to the level optimal for that species.

The nutrients involved in our study, NO_3, NH_4 and urea nitrogen, PO_4-phosphorous, silica and the trace metal iron were added as the following compounds, some of which are found in supplements to type I and II media. Nitrogen was added as KNO_3 or $NaNO_3$; P as KH_2PO_4 or K_2HPO_4; Si as $NaSiO_3$ $9H_2O$ and Fe as $FeCl_3$ $6H_2O$ or $FeNH_4$ citrate.

Growth rates were determined by the O.D. 750 spectrophotometric method using a Sequoia-Turner spectrophotometer. The culture tubes served also as cuvettes. Tubes were not spectrophotometric quality glass. We did, however, match tubes for each study based on O.D. tests. This study was designed to investigate variability in spectral transmission values between randomly picked culture tubes and tubes selected for optical quality appearance and placement in the spectrophotmeter. Ten tubes filled with a liquid of known O.D. were used in each test.

Readings were taken every 24 hours within one hour of the first days O.D. reading. Doubling times were calculated from transmission (T) readings using the $\log_2$O.D. + 10 against T tables found in Stein (1973). Depending on the species, cultures were started at either 91 or 93% T and diluted back to this value after every 24 hour growth period. Tubes with medium but no cells were included on the shakers as blanks for spectrophotometric readings.

Three replicate tubes were used in each experiment. At least two different experiments were conducted for each nutrient under study. Significant differences between tests within an experiment were analyzed using an analysis of variance test. Bar graphs depict final means, their standard errors and significant differences at the 5% level. Control tubes for the nitrogen preference studies involving NH_4 and urea-N contained NO_3 at concentrations equalling that of the respective medium's suggested N level, i.e. GPM, 28 mg/l N.

RESULTS

To accurately analyze growth based on optical density differences within and between studies, a quality check for culture tube transmission differences was carried out. Results from tests using 10 tubes filled with a liquid of known O.D. showed

a significant difference in transmission values when tubes were both randomly chosen and placed in the spectrophotometer. Culture tubes selected and carefully placed revealed no significant differences among tubes as shown below.

	Transmission (T) A	B
X	88.8	89.3
S.E.M.	4.99	0.30
ANOVA	*	N.S.

Replication: 10 tubes in each category
A. Random tube selection, no quality check
B. After optical quality check
* Significant difference at 5% level
N.S. Nonsignificant

A summary of all nutrients tested to date with growth rates and corresponding concentrations is seen in Table 1. Values indicate either: a. a statistically significant difference at 5% (*), 1% (**); or b. the lowest concentration found among nonsignificantly different doubling times. These doubling times represent the mean of at least two experiments and triplicate tubes per experiment. Data in bar graph form are included for five species tested in selected nutrients. Bar graphs indicate standard error values and significance of difference by letter designations (Figures 1-7).

Nitrate-Nitrogen Study

Doubling times for the six species tested in NO_3-N ranged between 0.5 and 2.45. Doubling time values seen in Table 1 represent the lowest NO_3-N concentration supporting the largest doubling time. Relatively high doubling times were observed for Navicula 2 (2.4) (Figure 1A), Monoraphidium 2 (2.45) (Figure 1B), and Chaetoceros 9 (2.45) (Table 1). A significant preference for NO_3-N over NH_4-N was observed in Chaetoceros 9 and 10 and Monoraphidium 2 (Table 1). A preference for NO_3-N over urea-N was also observed in Navicula 2 and Monoraphidium 2 (Table 1). Low concentrations of NO_3N are seen to support relatively high growth rates for Chaetoceros 9 (DT: 2.4, 10 mg/l); Monoraphidium 2 (DT: 2.45, 25 mg/l).

Ammonia-Nitrogen Study

In this study doubling times for the species tested with NH_4-N ranged between 0.85 in Chaetoceros 10 and 2.0 for Navicula, Thalassiosira and Monoraphidium. Only Navicula, Thalassiosira and Monoraphidium showed doubling times over 2.0. Thalassiosira was the only species showing a slight preference for NH_4-N. The doubling times for Chaetoceros 9, Navicula (Figure 2A) and Thalassiosira (Figure 2B) were supported by relatively low NH_4-N concentrations of 10 mg/l, 10 mg/l and 28 mg/l respectively

Table 4. Summary of Growth Studies

Algal Species	NO_3-N	NH_4-N	Urea-N	PO_4-P	Si	Fe	Vitamins
Chaetoceros 9	2.4	1.9	2.2	2.6*	2.1*	2.3*	2.3
	(10)	(10)	(30)	(6)	(56)	(0.3)	B_{12}
Chaetoceros 10	3.0	0.35*	1.5*	2.1*	1.7	3.2	
	(112)	(50)	(25)	(48)	(28)	(0.075)	
Chaetoceros 15	1.6*	1.6	1.6	1.6	1.7	1.8	
	(10)	(7)	(14)	(6.2)	(56)	(0.3)	
Navicula 2	2.4**	2.0*	1.2	2.65*	2.8	3.0	
	(56)	(10)	(25)	(24)	(2.8)	(0.15)	
Thalassiosira 2	1.6	2.0	1.7	2.0*	1.6*	1.7	
	(112)	(28)	(1.0)	(6.0)	(28)	(0.30)	
Monoraphidium 2	2.45	2.0*	1.7	2.5	NA	2.2	2.45
	(25)	(50)	(25)	(25)		(0.15)	Thiam.

1 Growth in doublings/day, mean of replicate tests and tubes

2 Concentrations in mg/l

NA Not applicable

Values indicate either: a. a statistically significant difference at 5% (*), at 1% (**); or b. the lowest concentration found among nonsignificantly different doubling times

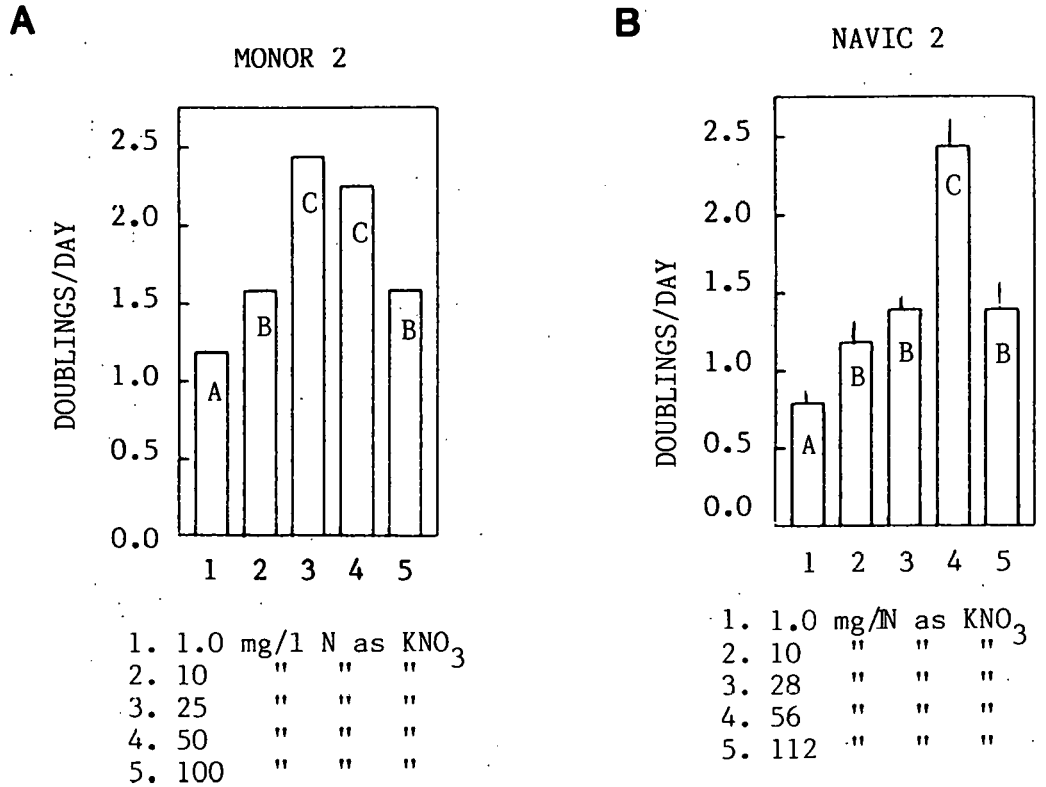


Figure 1. Effects of Nitrate-N on growth of Monoraphidium 2 and Navicula 2

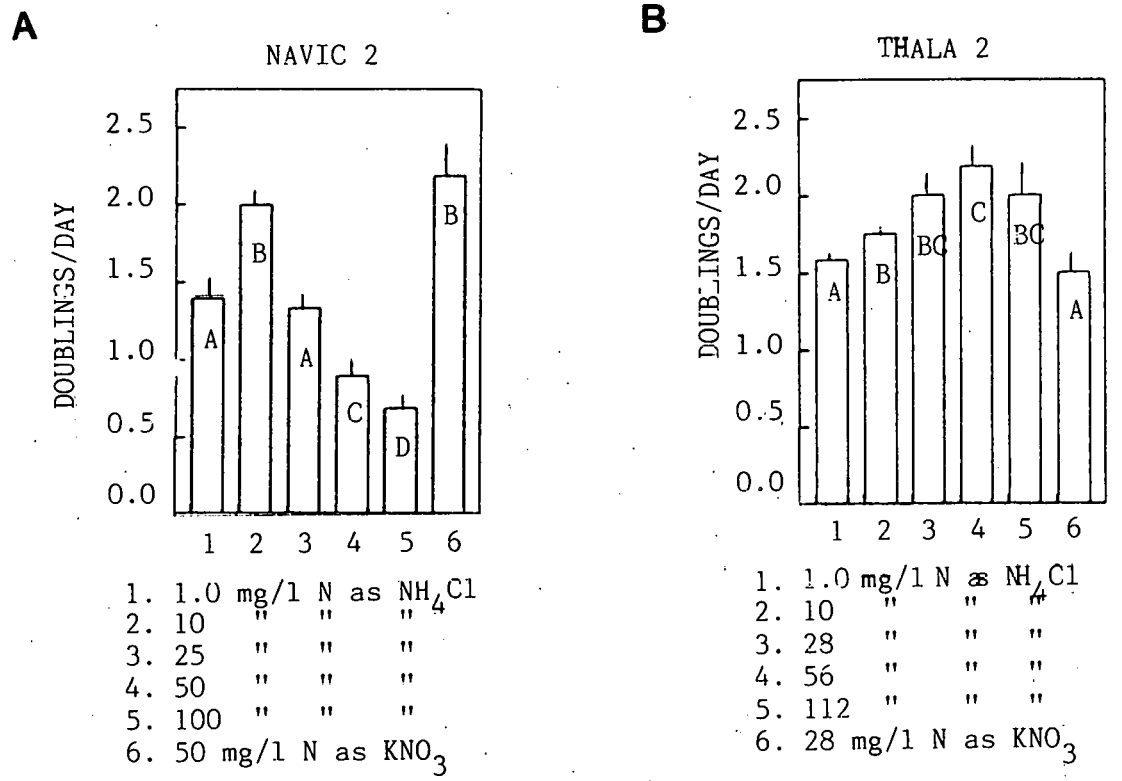


Figure 2. Effects of Ammonia-N on growth of Navicula 2 and Thalassiosira 2

(Table 1). Chaetoceros 15 showed as good growth (1.6) at 7.0 mg/l as in NO_3-N at 10 mg/l.

Urea-Nitrogen Study

Doubling times ranged between 1.2 for Navicula and 2.2 for Chaetoceros 9 in the study with urea-N. Thalassiosira demonstrated good growth at very low urea-N levels (1.0 mg/l) (Figure 3B). A relatively high doubling time for Chaetoceros 9 (2.2) was supported by urea-N levels of 30 mg/l. Chaetoceros 9 and Thalassiosira were the only species demonstrating similar doubling times to that of NO_3-N (Table 1). Navicula showed a considerably reduced doubling time of 1.2 relative to both NO_3 and NH_4-N tests. Chaetoceros 15 showed as good growth (1.6) at 14 mg/l as in NO_3-N at 10 mg/l.

Phosphate-Phosphorous Study

A low doubling time of 2.0 was observed in the phosphate study with Thalassiosira while high doubling times of 2.6, 2.65 and 2.5 were seen for Chaetoceros 9 (Figure 4A), Navicula (Figure 4B) and Monoraphidium respectively (Table 1). Significantly higher doubling times were observed for Chaetoceros 10 (2.1), at 48 mg/l P, Chaetoceros 9 (2.6) at 6 mg/l P, Navicula (2.65) at 24 mg/l P, Thalassiosira (2.0) at 6.0 mg/l P and Monoraphidium (2.5) at 25 mg/l P (Table 1). All other doubling time values among the species tested represent again the lowest concentration found among nonsignificant doubling times. Chaetoceros 9 (6.0 mg/l P) and Thalassiosira (6.0 mg/l P) both exhibited good growth at relatively low concentrations of PO_4-P (Table 1). Chaetoceros 15 demonstrated maximum growth at relatively low P concentrations of 6.0 mg/l.

Silica Study

The five diatoms tested so far in the silica study ranged in doubling times from a low of 1.6 for Thalassiosira to 2.8 for Navicula (Table 1). These doubling time values represent the values obtained in the lowest Si concentration among nonsignificantly differing results. Relatively low concentrations of Si supported good growth in Chaetoceros 9 (2.1) at 56 mg/l Si (Figure 5A) and particularly Navicula (2.8) (Figure 5B) at 2.8 mg/l Si. Chaetoceros 15 demonstrated maximum growth (1.7) at 56 mg/l.

Iron Study

Doubling times in the iron study for the six species studied so far ranged from a low of 1.7 for Thalassiosira to 3.2 for Chaetoceros 10 (Table 1). Ferric chloride and ferric ammonium citrate were tested to compare their relative potential for growth enhancement. Monoraphidium demonstrated a significant enhancement in growth when $FeCl_3$ was used (Figure 6A). Chaetoceros 9 showed a clear cut preference for ferric ammonium citrate at most levels tested. Navicula and Chaetoceros 10 showed simi-

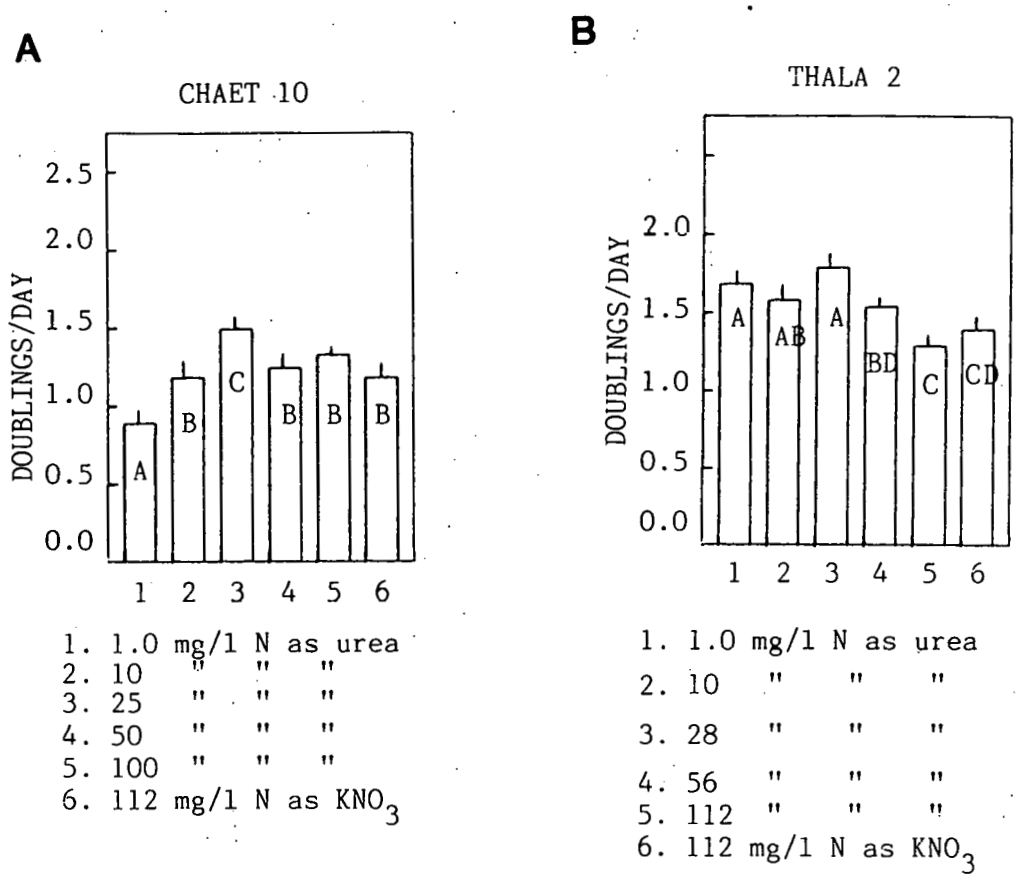


Figure 3. Effects of Urea-N on growth of Chaetoceros 10 and Thalassiosira 2

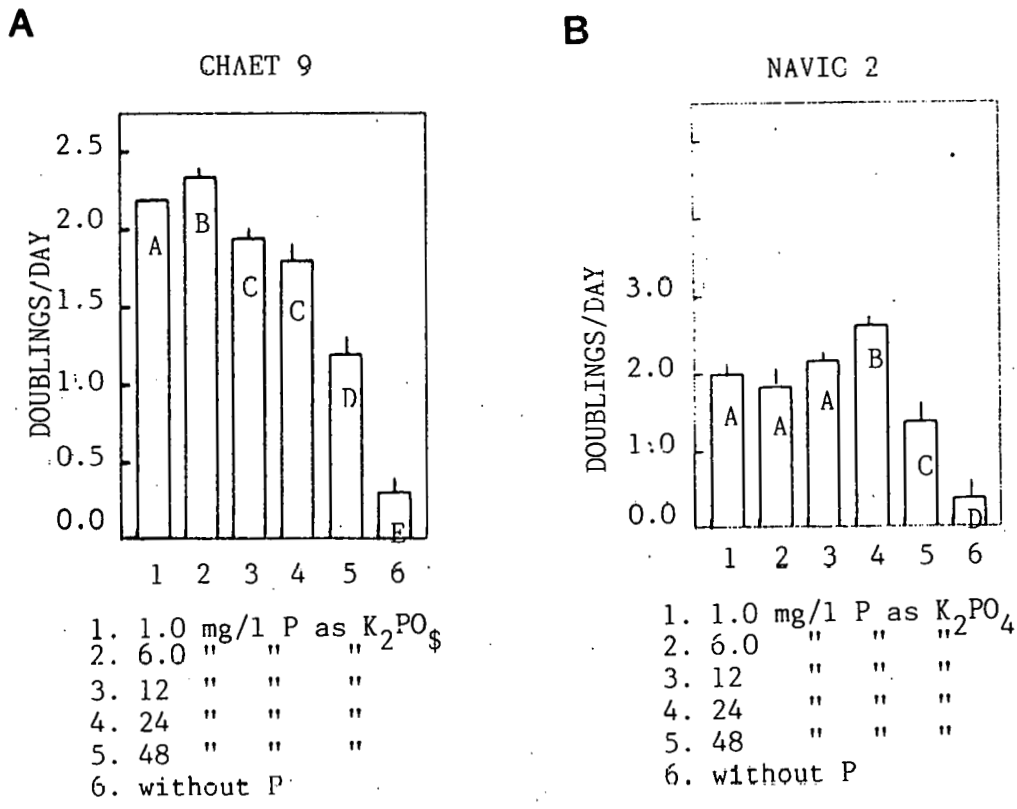


Figure 4. Effects of Phosphate-P on growth of Chaetoceros 9 and Navicula 2

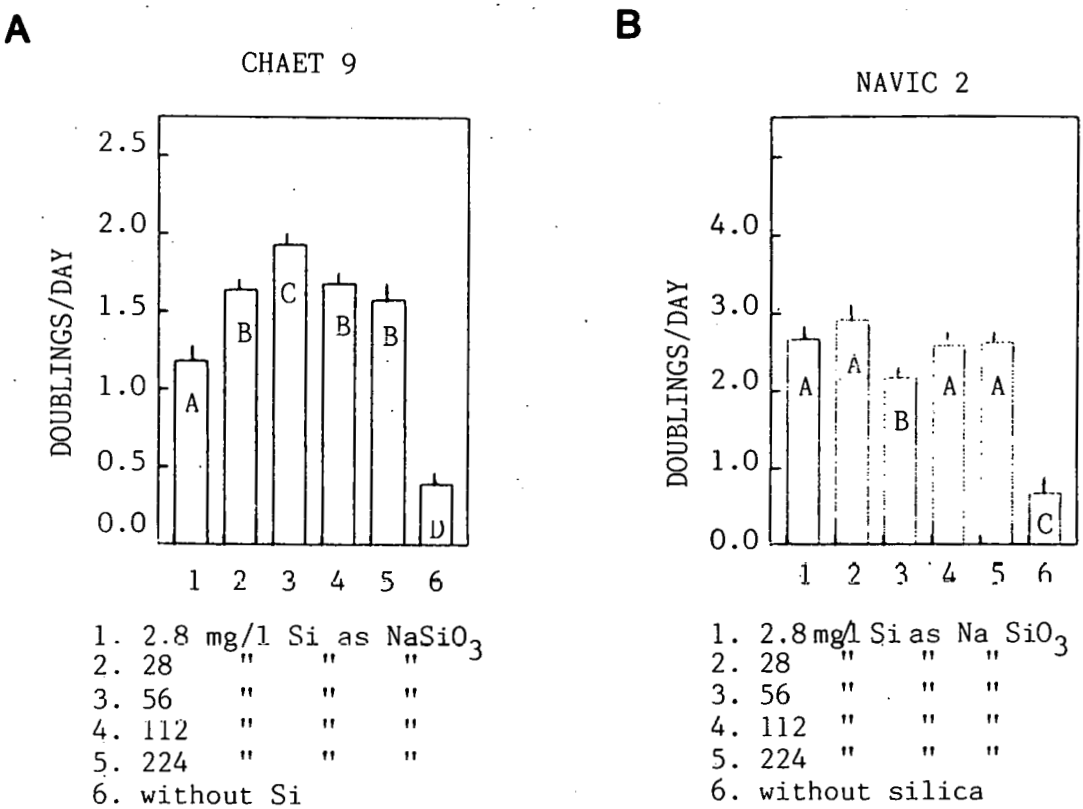


Figure 5. Effects of Silica on growth of Chaetoceros 9 and Navicula 2

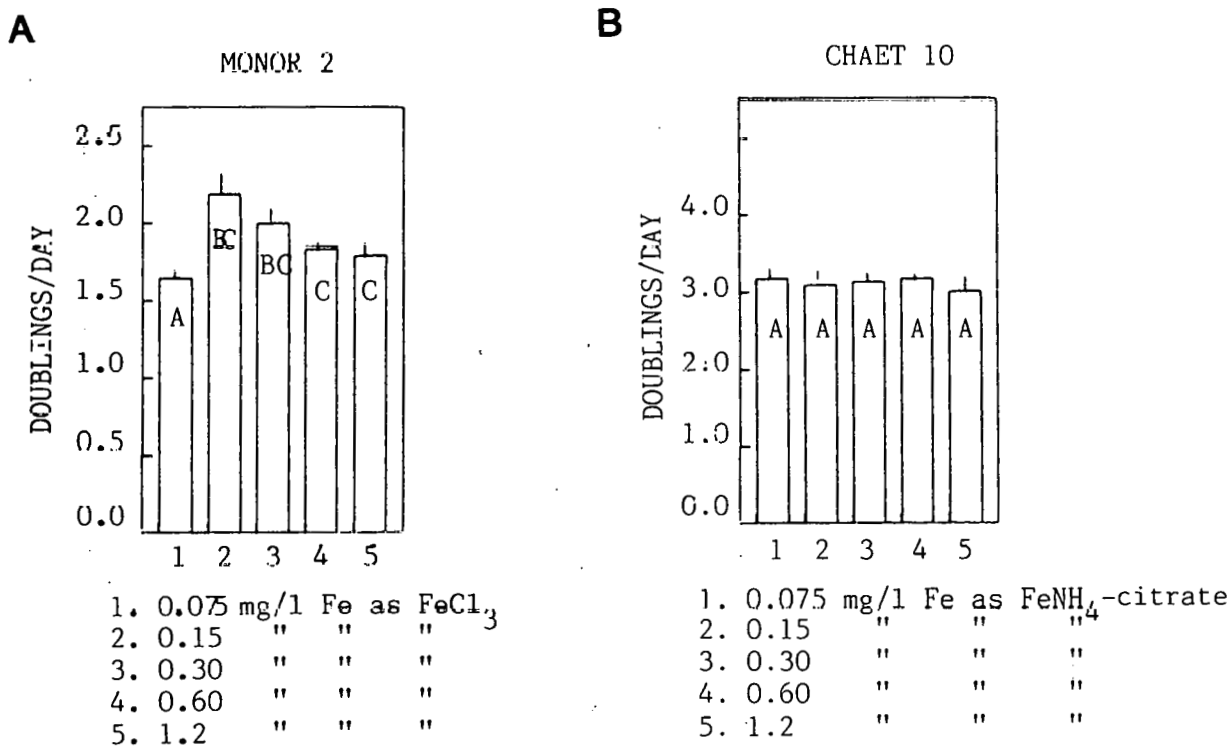


Figure 6. Effects of Iron on growth of Monoraphidum 2 and Chaetoceros 10

lar doubling time values for both $FeCl_3$ and $FeNH_4$-citrate. Chaetoceros 15 demonstrated maximum growth (1.8) at 0.30 mg/l Fe as $FeCl_3$. Very low Fe levels (0.075) were observed to support good growth (3.0) in Thalassiosira (Figure 6B).

Vitamin Studies

The two algal species tested so far demonstrated different responses to each of the three vitamins tested and their combinations. Significant differences were observed for thiamine as the preferred vitamin in Monoraphidium at a doubling time of 2.45 (Figure 7A). Chaetoceros 9 showed a tendency toward the need for B_{12} at a doubling time of 2.3 (Figure 7B).

Nutritional Requirements by Species

Certain significant preferences and optimal concentrations became apparent for those nutrients tested. The statistically significant nutritional requirements for each species is listed below as seen in Table 1.

Chaetoceros 9 grew well in all nitrogen sources, 2.4, 1.9 and 2.2 at 10 mg/l NO_3-N, 10 mg/l NH_4-N and 30 mg/l urea-N. Phosphate-P at 6.0 mg/l and Fe as $FeNH_4$-citrate at 0.30 mg/l appear as optimal levels. Growth was significantly better when vitamin B_{12} was added to the medium.

Chaetoceros 10 demonstrated a doubling time of 3.0 at 112 mg/l NO_3-N, but also grew well (1.5) in urea-N at 25 mg/l. A concentration of 4.8 mg/l PO_4-P and 0.075 mg/l Fe as $FeNH_4$-citrate allowed for maximal growth.

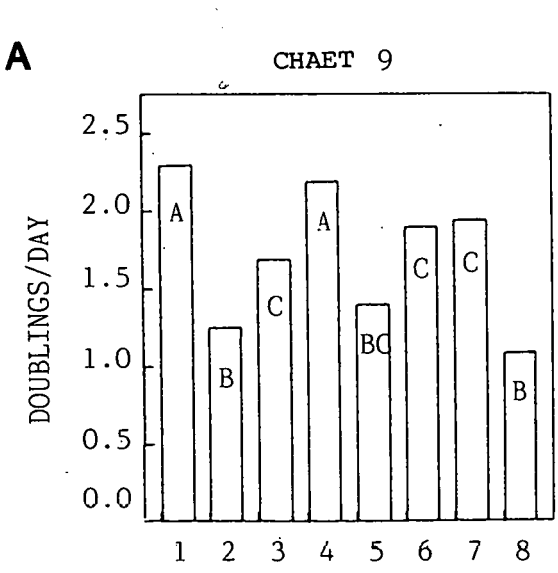
Nitrate-N served as the preferred N source at 56 mg/l for Navicula, but it also grew well in NH_4-N at 10 mg/l. Phosphate-P at 24 mg/l, Si at 2.8 mg/l, and Fe as $FeCl_3$ at 0.15 mg/l were found to be optimal levels for maximal growth.

A slight preference for NH_4-N at 28 mg/l was observed for Thalassiosira, while good growth was seen with urea-N at 1.0 mg/l. Phosphate-P at 6.0 mg/l, Si at 28 mg/l and Fe at 0.3 mg/l as $FeCl_3$ were observed as optimal levels.

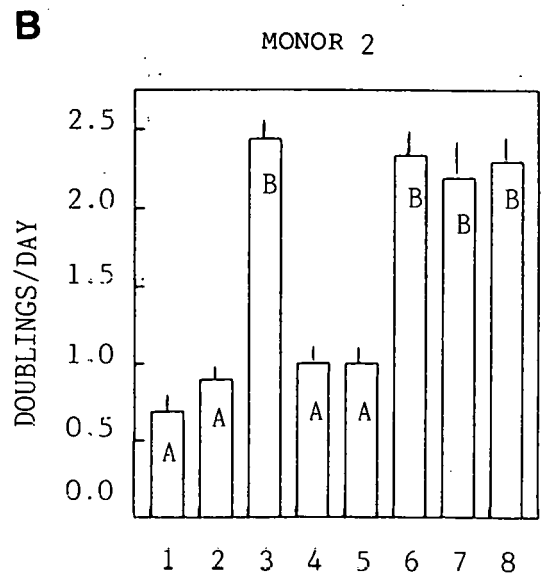
Nitrate-N was seen as the optimal N source for Monoraphidium at 25 mg/l. Phosphate-P at 25 mg/l and Fe at 0.15 mg/l were found to be concentrations supporting the highest doubling times. Monoraphidium showed a preference toward the vitamin thiamine.

DISCUSSION

Based on their growth rates and response to nutritional variations, several species appear worthy of continued study by mass outdoor culture. Criteria for the above were based on: (1) preference of N source (NH_4 and urea-N tend to be



1. all vitamins (biotin, B_{12}, thiamine)
2. biotin only
3. thiamine only
4. B_{12} only
5. biotin & thiamine
6. Thiamine & B_{12}
7. thiamine & biotin
8. no vitamins



1. no vitamins
2. biotin only
3. thiamine only
4. B_{12} only
5. B_{12} & biotin
6. biotin & thiamine
7. B_{12} & thiamine
8. all vitamins

Figure 7. Effects of Vitamins on growth of Chaetoceros 9 and Monoraphidium 2

cheaper sources of nitrogen than NO_3-N), (2) ability to demonstrate maximal growth at relatively low concentrations for the nutrients tested (N,P,Si and Fe) and (3) ability for rapid growth without added vitamins.

Chaetoceros 9 demonstrated high doubling times (2.2-2.4) at relatively low concentrations of nutrients. If this strain of Chaetoceros muelleri proves to be a good lipid producer it would then appear to a candidate for selection. It does utilize urea-N but appears to need vitamin B_{12} at least in culture. This strain also demonstrated its best growth in $FeNH_4$-citrate as opposed to $FeCl_3$.

Chaetoceros 10 demonstrated a considerably higher doubling time (3.0) in NO_3-N than with either NH_4 or urea-N. This strain was grown at 30 C. Optimal temperature according to SERI workers (Barclay, 1986) was greater than 35 C.

Chaetoceros 15 did not show doubling times over 1.8, which does not represent a particularly fast growing alga.

Navicula 2 and Monoraphidium 2 each demonstrated rapid growth (2.4-3.0) and (2.45) respectively. While each appeared to use nutrients at relatively low levels, they both utilized NO_3-N considerably better than either NH_4 or urea-N. A second strain of Navicula, Navicula 1 was studied by SERI workers, who reported doubling times of 2.8 in Type I medium (Barclay et al., 1986). Monoraphidium 2 appears at least in culture to need the vitamin B_{12}. Maximum doubling time for this species was 5.8 according to unpublished accounts by Weissman (SERI, 1986), but more recently SERI workers have obtained doubling times of approximately 2.53-3.5 in their screening tests for this species (SERI, 1987).

The slow growth of Thalassiosira 2 was also shown by SERI workers in their primary characterization of this species. A doubling time of 1.39 is described by Barclay et al., (1986).

Again note that the carbon source during these tests was supplied as HCO_3, not free CO_2. The use of CO_2 should not only enhance the growth of several of these species, but also help regulate pH during the periods of rapid growth.

BIBLIOGRAPHY

Barclay, W., J. Johansen, P. Chelf, N. Nagle, P. Roessler, and P. Lemke. 1986. SERI Microalgae Culture Collection, 1986-1987, SERI/SP-232-3079.

Ben-Amotz, A., and T. Thomas Tornabene. 1985. Chemical Profile of Selected Species of Microalgae with Emphasis on Lipids. J. Phycol. 21:72-81.

Boussiba, S., A. Vonshak, Z. Cohen, A. Abeliovich, D. Kaplan and A. Richmond. 1985. Development of Outdoor Systems for

Production of Lipid-Rich Halotolerant Microalgae. Aquatic Species Program Review, March, 1985. SERI/CP-231-2700, pp.271--288.

Brand, L.E. 1981. Genetic Variability in Reproduction Rates in Marine Phytoplankton Populations. Evolution. 35:1117-1127.

Brezinski, J.A. 1985. The Si:C:N ratio of marine diatoms, interspecific viability and the effect of some environmental variables. J. Phycol. 21:347-357.

Gallagher, J. 1982. Physiological Variation and Electrophoretic Banding Patterns of Genetically Different Seasonal Populations of Skeletonema costatum. J. Phycol. 18:148-162.

Hall, D.O. 1986. The Production of Biomass: A Challenge to our Society. In: Richmond, A. (ed.), Handbook of Microalgal Mass Culture. CRC Press, Boca Raton, FL, pp. 1-24.

Hill, A.M., and D.A. Feinberg. 1984. Fuel from Microalgae Lipid Products, SERI/TR-231-2348, Golden, CO: Solar Energy Research Institute.

Hill, A., D. Feinberg, R. McIntosh, B. Neenan and K. Terry. Fuels from Microalgae: Technology Status, Potential, and Research Issues. SERI/SP-231-2550, December 1984.

Kaplan, D., A. Richmond, Z. Dubinsky, and S. Aaronson. 1986. Algal Nutrition. In: Richmond, A. (ed.), Handbook of Microalgal Mass Culture. CRC Press, Boca Raton, FL, pp. 147-198.

Laws, E.A. 1985. Productivity Optimization of Saline Microalgae Grown in Outdoor Mass Culture. Aquatic Species Program Review, March, 1985. SERI/CP-231-2700, pp. 162-178.

Lien, S. and P. Roessler. 1985. The Energetics of Biomass and Lipid Production by Lipogenic Microalgae under Nitrogen Deprivation. Aquatic Species Program Review, March, 1985. SERI/-CP-231-2700, pp. 100-118.

Matthews, L. 1986. Nutritional Requirements for Optimal Growth of Two Lipid Producing Microalgae from Saline Lakes. MS Thesis, Jackson State University, Jackson, MS 55 pp.

McIntosh, R. P. 1984. Intensive microalgae culture for lipid production, p. 57. In: Abstracts, National Meeting on Biomass R and D for Energy Applications, 1-3 October 1984, Arlington, Va., SERI/CP-230-2430.

Oliveria, L. and N. Antia. 1986. Some observations on the urea-degrading enzyme of the diatom Cyclotella cryptica and the role of nickel in its production. J. Plankton Res. 8:235--242.

Rhyne, C. 1987. Nutritional Requirements for Maximal Growth

of Oil Producing Microaglae. 1986 Aquatic Species Program Review, SERI/SP-231-3071, p. 249.

SERI Aquatic Species Program Review, Proceedings of the April 1984 Principal Investigators Meeting. SERI/CP-231-2341, May 1984

SERI Aquatic Species Program Review, Proceedings of the March 1985 Principal Investigators Meeting. SERI/CP-231-2700, June 1985.

SERI Aquatic Species Program Review, Proceedings of the September 1986 Principal Investigators Meeting. SERI/SP-231-3071, February, 1987. pp. 23-40.

Stein, J. R. 1973. Handbook of Phycological Methods, Univ. Press, Cambridge, 448 pp.

Terry, K.L. and S. Hock. 1985. Photosynthetic Efficiency Enhancement in Modulated Light: Dependence on the Frequency of Modulation. Aquatic Species Program Review, March, 1985. SERI/-CP-231-2700. pp.119-138.

Terry, K. L., E. Laws, and P. J. Burns. 1985. Growth rate variation in the N:P requirement ratio of phytoplankton. J. Phycol. 21:323-329.

Thomas, W. H., D. Seibert, M. Alden, A. Neori, and P. Eldridge, 1984. Yields, photosynthetic efficiencies and proximate composition of dense marine microalgal cultures. I. Introduction and Phaeodactylum tricornutum experiments. Biomass 5:181-209.

Thomas, W. H., D. Seibert, M. Alden, A. Neori, and P. Eldridge, 1984. Yields, photosynthetic efficiencies and proximate composition of dense marine microalgae cultures. II. Dunaliella primolecta and Tetraselmis suecica experiments. Biomass 5:211-224.

Thomas, W. H., D. Seibert, M. Alden, A. Neori, and P. Eldridge, 1984. Yields, photosynthetic efficiencies and proximate composition of dense marine microalgal cultures. III. Isochrysis spp. and Monallantus salina experiments and comparative conclusions. Biomass 5:

Tornabene, T. and J. Benemann. 1985. Chemical Profiles on Microalgae with Emphasis on Lipids. Aquatic Species Program Review, March, 1985. SERI/CP-231-2700, pp.83-99.

Weetall, H. 1985. Studies on the Nutritional Requirements of the Oil Producing Alga Botryococcus braunii. Appl. Biochem. & Biotechn. 11:377-391.

EFFECTS OF NUTRIENTS AND TEMPERATURE ON LIPID PRODUCTION AND FATTY ACID COMPOSITION IN MONORAPHIDIUM MINITUM AND CYCLOTELLA DI-35

Shobha Sriharan[1], Davinderjit Bagga[2],
T.P. Sriharan[3] and Mini Das[4]
[1,3,4]Selma University, Sema, Alabama 36701
[2]Miles College, Birmingham, Alabama 35208

ABSTRACT

The diatoms, Monoraphidium minitum and Cyclotella DI-35 were batch cultured in Nitrogen (N) concentrations as Nitrogen-sufficient (NS) and Nitrogen-deficient (ND); in Silica (S) concentrations as Silica-sufficient (SS) and Silica-deficient (SD) media. The treatments were maintained at 30°C and 20°C under continuous illumination. The growth of these diatoms was evaluated by measuring the optical denisity (O.D.). The biomass was harvested; the amounts of ash-free dry weight (A.F.D.W.) and total lipids were determined by gravimetric method. The total lipids were analyzed for neutral lipids and polar lipids. The fatty acids were measured by direct transesterification method followed by gas chromatography and mass spectrometry (GC/MS).

The growth response was significantly higher in NS and SS media than in ND and SD media of both Monoraphidium minitum and Cyclotella DI-35. The lipid production in general was greater in nitrogen as well as silica deficient media in these diatoms. The amounts of total lipids, neutral lipids and polar lipids were higher in ND and SD treatments at 30°C and 20°C. The effects of nitrogen and silica stress in growth media were observed on distribution of fatty acids in Monoraphidium minitum and Cyclotella DI-35. Both qualitative and quantitative changes were noted in fatty acids.

Key Words

Monoraphidium minitum, Cyclotella DI-35, Nitrogen-sufficient (NS), Nitrogen-deficient (ND), Silica-sufficient (SS), Silica-deficient (SD), ash-free dry weight (A.F.D.W.), total lipids, neutral lipids, polar lipids, fatty acids, gas chromatography-mass spectrometry (GC/MS).

EFFECTS OF NUTRIENTS AND TEMPERATURE ON LIPID PRODUCTION AND FATTY ACID COMPOSITION IN MONORAPHIDIUM MINITUM AND CYCLOTELLA DI-35

INTRODUCTION

High biomass production, simple-economical growth requirements and unique ability to accumulate large quantities of cellular storage lipids by microalgae make them good candidates as alternate liquid fuel sources. Metabolism of algae is greatly influenced by environmental conditions such as carbon dioxide supply, light intensity, temperature, nutrient concentration and salinity (Holm-Hansen et al., 1950). While the nutrient deficiencies in general lead to a decrease in protein and photosynthetic pigment, an increase in energy rich products such as carbohydrates and lipids (Healey, 1973) is observed.

Nitrogen starvation in particular can lead to remarkable changes in algal cell composition (Fogg, 1959). Chlorella when grown under Nitrogen stress conditions showed a trend in lipid increase and protein decrease (Milner, 1953). The enhancement of lipid accumulation in different species of algae occurred under nitrogen deficient conditions (Fogg, 1959; Opute, 1974; Shifrin and Chisholm, 1981; Werner, 1966). In microalgae, Monottantus salina (Shifrin and Chisholm, 1981) Dunaliella sp., Isochrysis sp., Nannochloris sp. (Thomas), Nitzschia sp. (Tornabene et al., 1985; Tornabene, 1984), Chlorella sp. and Ankistrodesmus sp. (Lien and Roessler, 1985), the increased lipid production in response to nitrogen starvation was observed. Similar observations on enhanced lipid production in diatoms Chaetoceros SS-14 were reported by Sriharan and and Bagga (1986). The fresh water green algae Botryococcus brauni accumulate up to 75% of its total dry weight hydrocarbons when subjected to nutritional deficiencies (Maxwell et al., 1968).

Coombs et al. (1967) observed enhanced lipid accumulation by algal cells when they were grown in silica stress medium. The effect of silica stress and light-dark cycles on the lipid content in a variety of phytoplankton has been noted (Shifrin and Chisholm, 1981). In Chaetoceros sp. and Navicula sp. Barclay et al. (1986), Sriharan and Bagga (1986), and Thomas et al. (1983) noted an increase in lipid production when these diatoms were grown in silica stress conditions. It has been suggested that with aging, nutrients become exhausted; this is then reflected in lipid content increase and changes of fatty acid composition (Tornabene, 1984; Lien and Roessler, 1985; Maxwell et al., 1968; Coombs et al., 1967). In Chlorella sp. and Euglena sp., the synthesis of saturated (16:0) and monosaturated (18:1) fatty acids was higher in low concentrations of nitrogen, whereas synthesis of fatty acid 16:2, 16:3, 16:4, and 18:2 was higher in high nitrogen concentrations (Tornabene, 1984). An increase in fatty acid of 18:1 in Botryococcus sp. and Dunaliella salina (Thomas et al., 1983) was noted when these microalgae were grown in nitrogen-starved medium.

The unique feature of microalgae to produce large amounts of lipids and fatty acids under different environmental conditions has encouraged us to undertake studies on few selected diatoms with particular reference to lipid

yield in response to different concentrations of nitrogen and silica at varying temperatures. The present paper describes the growth response and lipid composition of diatoms, Monoraphidium minitum and Cyclotella DI-35 grown in different concentrations of nitrogen and silica at two temperatures (30°C and 20°C).

MATERIALS AND METHODS

Organisms:

The diatom Monoraphidium minitum was obtained from Dr. B. Barclay of Solar Energy Research Institute (SERI), Golden, Colorado, U.S.A. The diatom Cyclotella DI-35 was received from Dr. M. Tadros, A&M University, Normal, Alabama, U.S.A.

Growth Conditions

The cultures of Monoraphidium minitum and Cyclotella DI-35 were maintained in a growth room, measuring 6 ft. x 8 ft. on a growth table 5 ft. x 1.5 ft. x 3.5 ft. The diatoms were grown under continuous illumination(180 uE m^2 sec^1) with cool white tubular fluorescent lamps: 4 lamps (40W) on the top and 6 lamps (15W) on each lateral side of the table.

The growth medium for culturing Monoraphidium minitum was SERI (Solar Energy Research Institute) Type II. The components of the medium (mg^{L-1}) were: CaCl(28), $MgCl_2.6H_2O$ (3,026), NA_2SO_4 (5,870), KCl (965), $NaHCO_3$ (2,315), NA_2CO_3 (876), NaCl (8,078), $Na_2SiO_3.9H_2O$ (500 mg), Biotin (2), Thiamine-HCl(1)$_9B_{12}$(1), KNO_3 (128), and 1 ml of 0.6 M KH_2PO_4. The medium was filter sterilized using Sceitz filter assembly. Stock cultures were maintained in 50 ml of medium in 125 ml Erlenmayer flasks in a growth chamber held at constant 30°C.

Cyclotella DI-35 was cultivated in artificial medium containing 20g R la Mix, $NaNO_3$, 1.76 mM $Na_2SiO_3.9H_2O$, 0.35 mM NaH_2 PO_4H_2O, 0.0363 mM; 1 ml of vitamin stock solution containing (gL^{-1}) 0.1 mg/liter, thiamin-HCl; 0.5 mg/liter biotin; and 0.5 mg/liter, B_{12}; 1 ml of trace element stock solution containing 9.1 mM H_3BO_3; 4.5 mM $ZnCl_2$; 1.7 mM, $CuCl_2.H_2O$; 1 mM $Na_2MoO_4.2H_2O$; 8.9 mM $FeSO_4$; 1.8 mM $MnCl_2.4H_2O$ and 9.1 mM $Na_2C_4H_4O_6.2H_2O$. The medium had a final pH of 7.5. Stock cultures were incubated at 30°C and aerated with 3% carbon dioxide.

Growth of cultures was evaluated by measuring optical density with Beckman's Spectrophotometer at 750 nm wavelength.

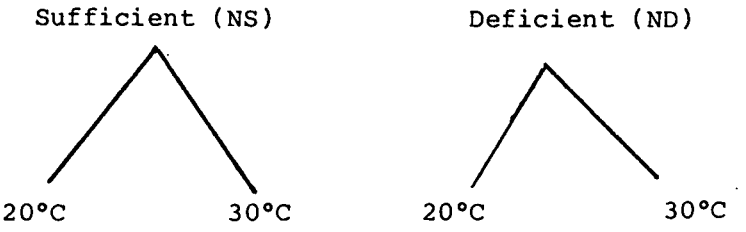
For measurement of lipid production under stressed and nonstressed conditions the algae were grown under optimal conditions of growth in 2,800 ml Fernbeck flasks each containing 1,500 ml of medium.

Nitrogen Concentration:

Batch cultures of Monoraphidium minitum and Cyclotella DI-35 were grown in the growth medium described above containing varied amounts of KNO_3. All

the ingredients described were present except for glycyl glycine. Potassium nitrate was added in two separate concentrations. In Nitrogen sufficient (NS) and Nitrogen deficient (ND) treatments, the amount of KNO_3 added were 128 mg/l and 45.5 mg/l, respectively (to Monoraphidium minitum) and 600 uM and 300 uM, respectively (to Cyclotella DI-35). The NS and ND treatments were kept at two different temperatures, 20°C and 30°C as shown below:

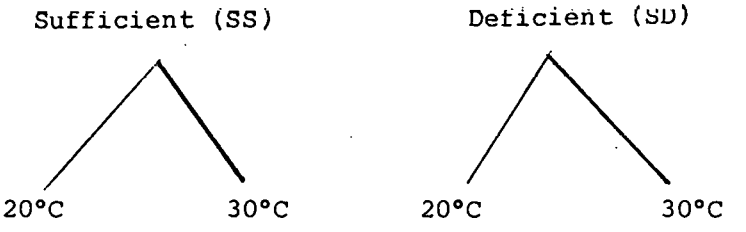
NITROGEN



Silica Concentration:

The diatoms, M. Minutum and Cyclotella DI-35 were batch cultured in 2 L polycarbonate bottles. The growth medium for these two diatoms was the same as described in nitrogen treatments and contained varied amounts of sodium silicate. Two separate concentrations of sodium silicate were added to the growth media. In Silica sufficient (SS) and Silica deficient (SD) treatments, the amounts of sodium silicate added were 1 mM and 250 μM respectively to these two diatoms. The cultures previously acclimatized at 20°C and 30°C were inoculated into 2,000 ml Fernbeck flasks containing 1,500 ml of growth medium with either SS or SD supply. The SS and SD treatments were kept at two temperatures separately at 20°C and 30°C as shown below:

SILICA



All the treatments were run in triplicate. Inoculum for each experiment was acclimated at 20°C and 30°C for their respective treatments and it was composed of cells in the exponential phase of their growth.

Evaluation of Oil Accumulation:

Microscopic examination of the diatom cells was made before collecting the biomass to ensure that the diatom cells had produced lipid globules. The

final harvesting of cells was carried only if they appeard to be full of lipid globules.

Harvesting of Cells:

Cells of diatoms were harvested by centrifugation. To obtain wet cell mass, a known volume of the diatom culture medium was transferred to a pre-weighed tarred tube and centrifuged. This tube along with cells were weighed. Based on the values in two different weights, the mass of cells (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°C for lipid analysis.

Ash-Free Dry Weight Determination

The ash-free dry weight (AFDW) was determined according to the procedure suggested by Paul Roessler of SERI (personal communication). The 30% of the cell suspension taken above was placed in each of the three weighed small test tubes (13 x 100 ml tubes). These tubes were previously heated at 50°C for 14 hours. The weight of the test tube along with the cell suspension was recorded and dry cell mass was determined as described below.

Dry cell mass was determined by placing the above three tubes in an oven at 60°C for drying to constant mass (36-48h). Thereafter, the 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 ash-free dry weight (AFDW) the tubes containing dry cells were placed in an oven (500°C) for 5h. These tubes were again placed in a dessicator for cooling and weighed. By taking the values of these two weights, the ash-free dry weight (AFDW) was calculated.

Total Lipids Determination:

The samples stored at -20°C were allowed to thaw at room temperature immediately prior to extraction of lipids. The lipids were extracted according to the modified method of Bligh and Dyer (1959). To the wet cell suspension (approximately 150 mg dry mass) an equal volume of methanol was added and heated at 60°C for 1h. This was centrifuged at 3,000 rpm to obtain a clear supernatant. The supernatant was transferred to a large tube with teflon cap. To the pellet, 10 ml of methanol was added and heated at 60°C for 1h. This was centrifuged and the supernatant was saved. This step was repeated two times.

The pellet was further extracted by two additional extractions with chloroform-methanol (1:1 v/v) and heated for 1h at 60°C. This was centrifuged and the supernatant was saved. As mentioned, after following this step twice, the combined supernatant solution was placed into a separatory funnel for phase separation (Bligh and Dyer, 1959). 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, one

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 tarred flasks. This was reduced in volume in a rotary evaporator at 30°-35°C and transferred into dessicator for cooling. After cooling, the flask was again weighed and the total lipids were determined gravimetrically. Later, 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:

The total lipids were separated into neutral lipids and polar lipids by silicic acid chromatography according to the procedures described by Tornabene, 1984. The silicic acid was suspended in hexane and poured into a column containing hexane and the silicic acid was allowed to settle into the column. The column was washed with six bed volumes of hexane. The total lipid sample which was in chloroform was evaporated to dryness. This 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 chloroform eluate and the polar lipids in methanol eluate. The eluates were reduced in volume on a rotary evaporator and later evaporated to dryness in weighed tubes under air. The mass of neutral lipids and polar lipids was evaluated.

Fatty Acids:

Fatty acid analyses were made by gas-liquid chromatography after transesterification (Lepage and Roy, 1984). Two ml of chloroform-methanol 2:1 (v/v) was added to approximately 100 mg of lyophylized cells of diatoms, and the mixture was mechanically shaken for 10 min (Folch et al., 1957). After centrifugation, the lower phase was collected and then 2 ml of chloroform-methanol 2:1 (v/v) was added to the precipitate and the same procedure was repeated. The lower phases were pooled and 145 mM NaCl was added in order to separate the methanol and chloroform phase (Klein et al., 1980). Following centrifugation, the lower phase containing the lipids was evaporated to dryness at room temperature under a gentle stream of nitrogen. The residue was solubilized in 1 ml of methanol-benzene 3:2 (v/v) and 1 ml of acetyl chloride-methanol 5:100 (v/v) was added. The mixtures then were subjected to methanolysis at 100°C for 1 hr (Lillington et al., 1981). The specimens were shaken centrifuged and stored at 4°C until injection into the chromatograph.

Gas-liquid Chromatography:

Fatty acids were chromatographed as methyl esters. Analysis was performed on a Hewlett-Packard 5880 gas chromatograph equipped with a flame ionization detector and coupled with an HP 3390A integrator. The standard FAME mix available from Supelco Inc., Bellefonte, Pennsylvania, containing a selection of methyl esters of the fatty acids were used. The identification of FAME peaks in the algal methyl esters was done by comparing the standard FAME mix. Nitrogen was used as carrier gas at a flow rate of 28 ml/min. The injection port temperature was 220°C and the detector was 300°C. The column temperature was held at 80°Cd for 2 min and in a step-wise fashion reached a plateau of 215°C.

RESULTS AND DISCUSSIONS

The growth response was determined on the basis of cell counting for different diatoms under study. The lipid composition was measured by analytical methods.

Temperature and Nitrogen Effects:

The influence of temperature and nitrogen concentration on the growth response of Monoraphidium minitum and Cyclotella DI-35 are shown in Figure 1. It was seen that at 30°C, there was an increase in cell number in nitrogen sufficient treatment as compared to nitrogen deficient condition in these diatoms. At 20°C, there was an appreciable increase in the cell number in nitrogen sufficient condition, as compared to the number under nitrogen deficient media in the diatoms.

The results of the analysis of ash-free dry weight (A.F.D.W.) and lipid contents of two species of diatoms grown at 30°C and 20°C in nitrogen sufficient and nitrogen deficient conditions are shown in Table 1. It was noticed that the A.F.D.W. was higher in nitrogen sufficient treatment as compared to nitrogen deficient condition at both the temperatures (30°C and 20°C) in M. minutum and Cyclotella DI-35.

The total lipids (% of A.F.D.W.) were measured and are presented in Tables 1 and 2. It was observed that the total lipids (% of A.F.D.W.) were higher in nitrogen deficient condition as compared to nitrogen sufficient condition in M. minutum as well as Cyclotella DI-35. The increase in total lipids were noticed at both 30°C and 20°C. It has been reported by others (Healey, 1973; Opute, 1974; Werner, 1966; Fogg, 1959; Shifrin and Chisholm, 1981; Lein and Roessler, 1985; Tadros, 1985; Tornabene et al., 1985; Tornabene and Benneman, 1984) that cellular lipid contents of the algae increase due to nitrogen starvation. The analysis of neutral lipids and polar lipids was done by silicic acid column chromatography. The nitrogen concentration and temperature effects are seen in Table 1. It was seen that the neutral lipids were higher than the polar lipids in each of these diatoms when raised in nitrogen deficient medium as compared to nitrogen sufficient medium. The ratio of neutral lipids was higher than polar lipids at 30°C and 20°C.

The fatty acid composition of the total lipids of M. minutum and Cyclotella DI-35 grown in two different concentrations of nitrogen (NS and ND) at 20°C and 30°C were analyzed and are shown in Table 3. The fatty acids present were 12:0, 14:0, 15:1, 15:0, 16:1 and 16:0 in these diatoms. The effect of nitrogen stress was expressed on the amounts of fatty acids. The percentage of C14:0 fatty acids increased in nitrogen deficient condition as compared to nitrogen sufficient condition at 30°C and 20°C. The production of fatty acids was higher at 30°C than at 20°C when these diatoms were grown in nitrogen deficient medium. Similar observations were made on the fatty acid production as a result of nitrogen starvation in algae Anksiterodesmus sp., Botryococcus brauni, Dunaliella salira, Dunaliella bardawi and Chaetoceros SS-14 (Wake and Hiller, 1981; Coombs et al., 1967; Sriharan and Bagga, 1987).

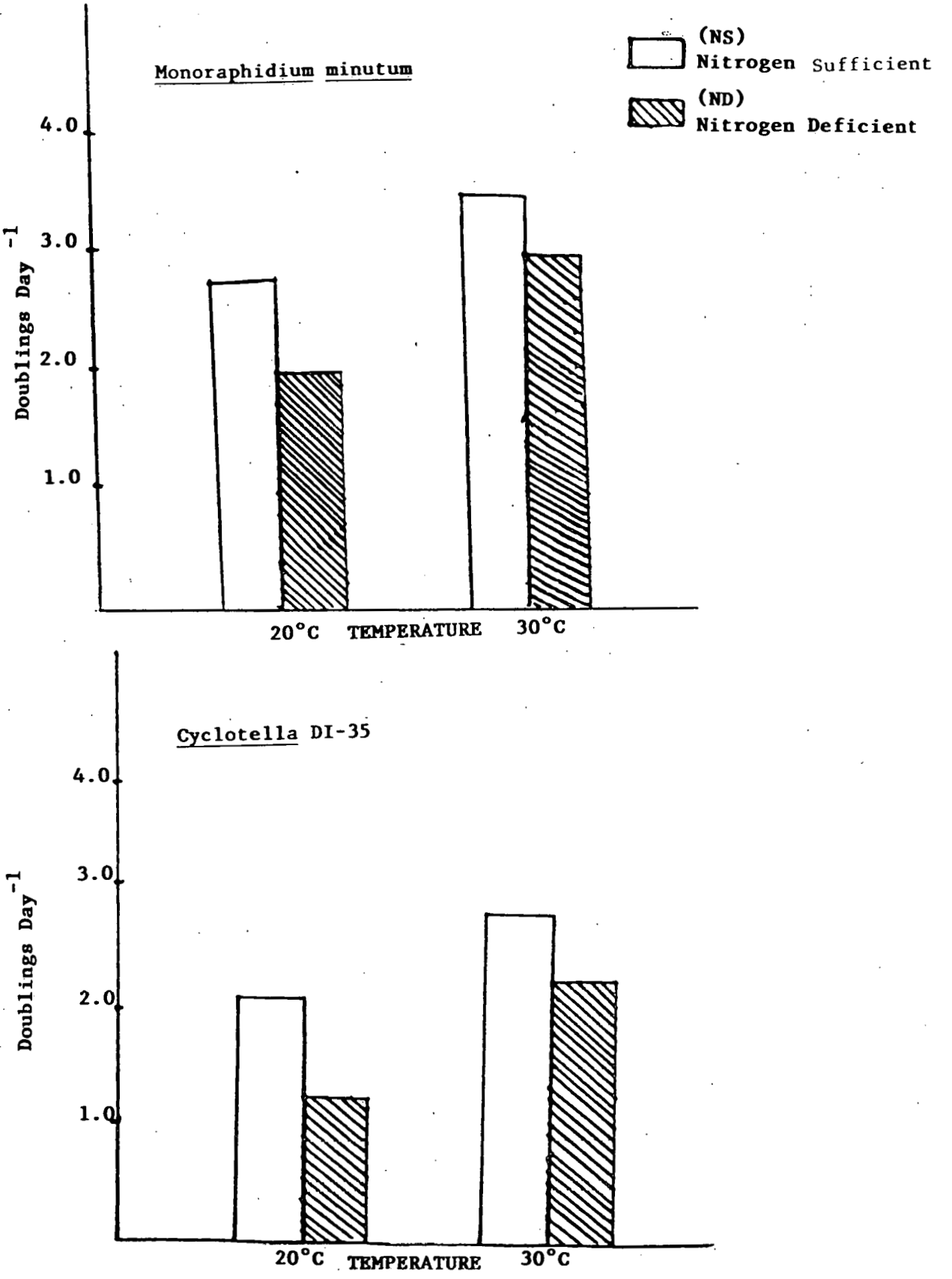


Figure 1. Exponential growth rate (Doublings Day^{-1}) of Monoraphidium minutum and Cyclotella DI-35 at 20°C and 30°C in Nitrogen Sufficient and Nitrogen Deficient media.

Table 1. Biomass and Lipid Yields of Monoraphidium minutum 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
		20°C Temperature		
Nitrogen Sufficient (NS)	1.168 ± 0.089	23.06 ± 1.30	12.93 ± 1.91	5.43 ± 0.20
Nitrogen Deficient (ND)	0.942 ± 0.078	29.48 ± 1.30	15.91 ± 1.60	5.50 ± 0.22
		30°C Temperature		
Nitrogen Sufficient (NS)	1.361 ±0.068	26.31 ±1.12	17.13 ±2.40	6.14 ± 0.30
Nitrogen Deficient (ND)	1.106 ± 0.98	34.81 ± 1.21	18.83 ± 1.22	9.23 ± 0.12

Table 2. Biomass and Lipid Yields of Cyclotella DI-35 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
		20°C Temperature		
Nitrogen Sufficient (NS)	1.024 ± 0.097	15.23 ± 1.082	9.57 ± 0.80	3.71 ± 0.45
Nitrogen Deficient (ND)	0.698 ± 0.087	42.55 ± 1.102	27.08 ± 1.80	7.31 ± 0.56
		30°C Temperature		
Nitrogen Sufficient (NS)	1.103 ± 0.112	15.59 ± 1.128	10.79 ± 0.84	3.79 ± 0.25
Nitrogen Deficient (ND)	0.706 ± 0.104	45.18 ± 1.149	31.02 ± 1.25	8.18 ± 0.26

TABLE 3: FATTY ACIDS OF *MONORAPHIDIUM MINUTUM* AND *CYCLOTELLA* DI-35 GROWN IN NITROGEN CONCENTRATIONS AT DIFFERENT TEMPERATURES.

Fatty Acid Carbon Number	Temperature:	Percent Fatty Acids			
		20°C		30°C	
	Nitrogen:	Sufficient (NS)	Deficient (ND)	Sufficient (NS)	Deficient (ND)
			Monoraphidium minutum		
C 12:0		8.36	14.45	6.42	6.78
C 14:0		24.52	33.63	49.72	55.52
C 15:1		11.51	5.65	6.64	5.68
C 15:0		12.01	9.93	5.40	4.97
C 16:1		5.15	2.34	2.22	3.97
C 16:0		14.25	1.97	6.64	4.09
			***Cyclotella* DI-35**		
C 12:0		3.49	4.69	4.39	2.08
C 14:0		8.08	46.40	66.63	89.15
C 15:1		12.82	1.90	1.40	2.42
C 15:0		13.73	3.67	3.30	1.90
C 16:1		16.14	4.76	2.13	1.71
C 16:0		5.84	7.95	3.45	1.80

Temperature and Silica Effects:

The effects of silica sufficient and silica deficient conditions at two temperatures, 30°C and 20°C, have been studied. The results on growth response of M. minutum and Cyclotella DI-35 are shown in Figure 2. At 30°C, in silica sufficient condition, the cell number was higher as compared to silica deficient condition in these diatoms. An increase in cells was also observed at 20°C in the diatoms. The A.F.D.W. and lipid composition of the two diatoms grown in silica sufficient and silica deficient media at 30°C and 20°C are reported in Tables 4 and 5. The A.F.D.W. was higher in these diatoms when grown in silica sufficient medium compared to silica deficient medium at both 30°C and 20°C. The production of higher amounts of total lipids in Chaetoceros sp. raised in silica starved condition has been reported earlier (Coombs et al., 1967; Barclay et al., 1986; Sriharan and Bagga, 1986). The neutral lipid contents were also more in silica starved condition as compared to silica sufficient condition. The amount of polar lipids was higher in silica deficient medium, and the ratio of neutral lipids was greater than polar lipids in each case. Such a condition is noticed at both 30°C and 20°C.

The fatty acid composition of the total lipids of M. minutum and Cyclotella DI-35 grown in two different concentrations of silica at 20°C and 30°C were analyzed and are given in Table 6. In these diatoms, the major fatty acids were 12:0, 14:0, 15:1, 15:0, 16:1 and 16:0. In SD treatments, fatty acids of 14:0 were significantly higher. This was noticed at both 30°C and 20°C. Studies carried out on Chaetoceros SS-14 (Sriharan and Bagga, 1986) also suggest production of fatty acids in higher amounts in silica deficient medium.

CONCLUSIONS

In the present study, the growth response and accumulation of lipids in datoms, Monoraphidium minutum and Cyclotella DI-35 were seen to be influenced by environmental conditions like nutrients and temperatures.

The effects of nitrogen and silica stress growth medium were noticed on lipid production and composition of fatty acids. However, the cell density was higher in nitrogen and silica sufficient conditions. Even though the nitrogen and silica stress (deficient) treatments yielded lower cell mass (A.F.W.D.), the lipid production was higher. The yield of cell numbers and A.F.D.W. were higher at 30°C then at 20°C in NS and SS treatments.

The results on lipids and fatty acids suggest that under stressed growth conditions of nitrogen (ND) and silica (SD) then is an increased production of total lipids, neutral lipids and polar lipids. This is noticed at both 30°C and 20° temperature. As cell number yield is higher at 30°C, the production of lipids is also greater at this temperature.

Variations in the fatty acids composition were also noticed when these diatoms are grown in different concentrations of nitrogen and silica. Fatty acids C12:0, C14:0, C15:1, C15:0, C16:1 and C16:0 were observed in M. minutum and Cyclotella DI-35. Quantitative differences in fatty acids were noticed.

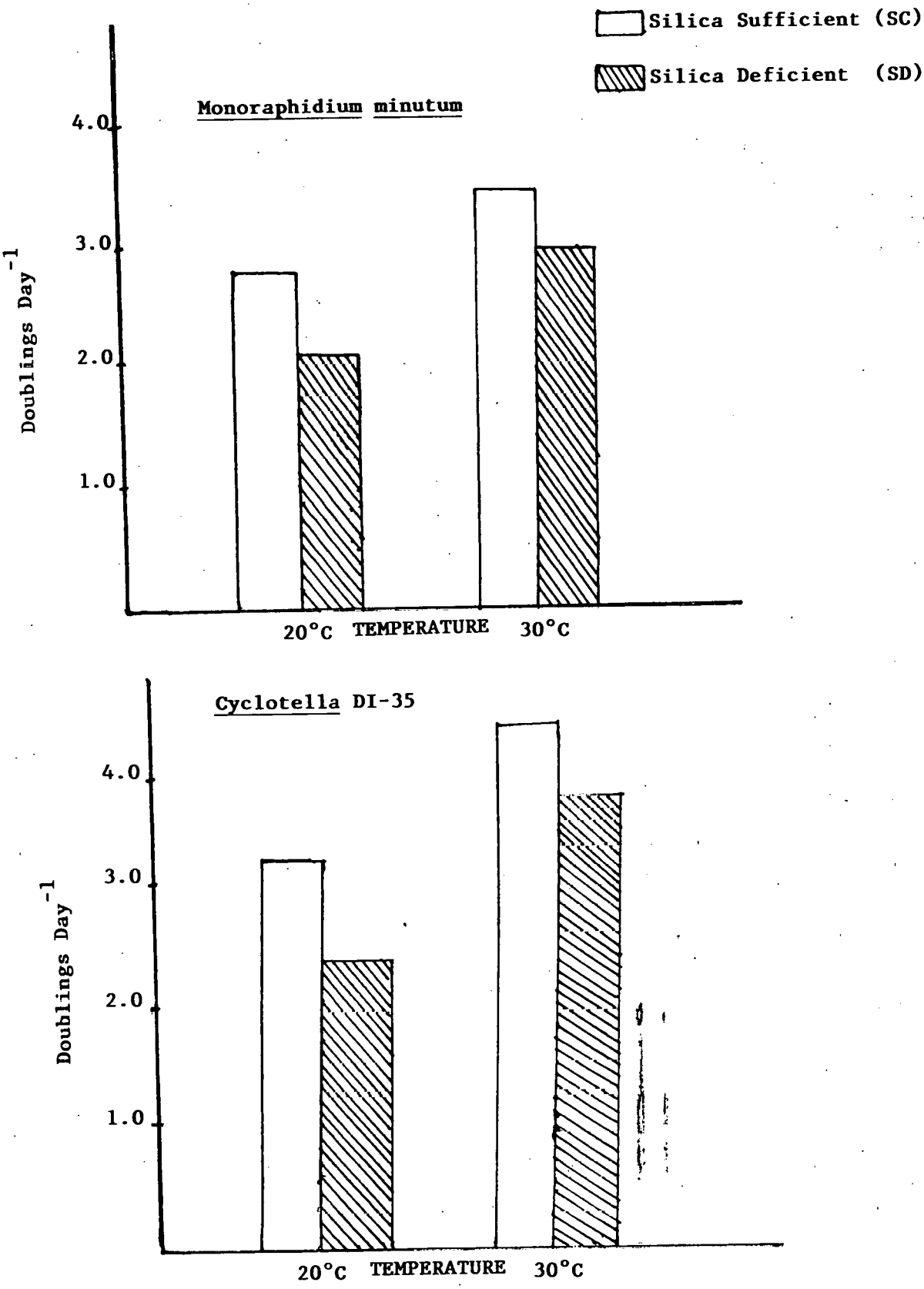


Figure 2. Exponential growth rates (Doublings day^{-1} of Monoraphidium minutum and Cyclotella DI-35 at 20°C and 30°C in silica sufficient and silica deficient media.

Table 4. Biomass and Lipid Yields of *Monoraphidium minitum* Grown in Different Silica 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
		20°C Temperature		
Silica Sufficient (SS)	1.078 ± 0.098	22.68 ± 1.3	13.64 ± 1.8	6.14 ± 0.40
Silica Deficient (SD)	0.898 ± 0.076	28.94 ± 1.4	15.02 ± 1.4	7.80 ± 0.30
		30°C Temperature		
Silica Sufficient (SS)	1.348 ± 0.106	26.68 ± 1.30	16.83 ± 1.80	6.41 ± 0.20
Silica Deficient (SD)	1.109 ± 0.098	33.68 ± 1.30	18.96 ± 1.60	8.14 ± 0.20

Table 5. Biomass and Lipid Yields of Cyclotella DI-35 Grown in Different Silica 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
		20°C Temperature		
Silica Sufficient (SS)	1.147 ± 0.081	18.300 ± 0.935	13.69 ± 0.90	2.96 ± 0.60
Silica Deficient (SD)	0.759 ± 0.085	32.671 ± 1.119	18.71 ± 1.30	5.67 ± 0.45
		30°C Temperature		
Silica Sufficient (SS)	1.198 ± 0.120	21.701 ± 1.212	13.69 ± 1.45	4.51 ± 0.25
Silica Deficient (SD)	0.894 ± 0.104	41.050 ± 1.291	27.74 ± 1.64	8.05 ± 0.40

TABLE 6: FATTY ACIDS OF CYCLOTELLA DI-35
GROWN IN SILICA CONCENTRATIONS AT DIFFERENT TEMPERATURES.

Fatty Acid Carbon Number	Temperature:	Percent Fatty Acids			
		20°C		30°C	
	Silica:	Sufficient (SS)	Deficient (SD)	Sufficient (SS)	Deficient (SD)
C 12:0		12.65	2.89	2.51	1.86
C 14:0		61.14	68.75	58.49	62.49
C 15:1		6.14	4.90	6.14	5.44
C 15:0		4.02	0.95	3.29	3.62
C 16:1		6.51	6.19	6.46	8.79
C 16:0		2.74	2.28	5.44	2.60

The fatty acid profile of these two diatoms were influenced by nutrients particularly. Both qualitative and quantitative changes in fatty acids were observed when the diatoms were grown in nitrogen (ND) and silica (SD) stress conditions. There was an increased production of fatty acids of 14:0 in nutrient stress conditions at 30°C as well as 20°C.

ACKNOWLEDGMENTS

This work was supported by Solar Energy Research Institute, Golden, Colorado (Subcontract No. XK-5-05104) to Selma University.

REFERENCES CITED

Badour, S.S., and Tadros, M.G. 1965. "Cell division and fat accumulation in Nitzchia grown in continuously illuminated mass culture," Arch. Mikrobiol. 51:94-102.

Barclay, W., Nagle, N., and Terry, K. 1986. "Environmental control of lipid production in diatom cultures," Joint Meeting of the Amer. Soc. Limnology and Oceanography and Phycological Society of America. June 23-26, 1986.

Bligh, E.G., and Dyer, W.J. 1959. "A rapid method of total lipid extraction and purification," Can. J. Biochem. Physiol. 37:911-917.

Coombs, J., Darley, W.M., Holm-Hansen, O., and Volcani, B.E. 1967. "Studies on the biochemistry and fine structure of silica shell formation in diatoms. Chemical composition of Navicula pelliculosa during silicon starvation synchrony," Plant Physiol. 42:1601-1606.

Fogg, G.E. 1959. "Nitrogen nutrition and metabolic patterns in algae," Symp. Soc. Exp. Biol. 13:106-125.

Folch, J., Lees, M., and Sloane-Stanley, G.H. 1957. "A simple method for the isolation and purification of total lipids from animal tissues," J. Biol. Chem. 226:497-509.

Gomez, M.P., Harris, J.B., and Walne, P.L. 1974. "Studies of Euglena gracilis in aging cultures. I. Light microscopy and cytochemistry," Br. Physiol. J. 9:163-174.

Healey, F.P. 1973. "The inorganic nutrition of algae from an ecological viewpoint," CRC Critical Rev. Microbiol. 3:69-113.

Hill, A., Feinberg, A., McIntosh, R., Neeman, B., and Terry, K. 1984. "Fuels from microalgae: Technical status, potential and research issues," SERI/SP-231-2550.

Holm-Hansen, O., Nishida, K., Mosses, V., and Calvin, M. 1950. "Effects of Mineral Salts on Short-term Incorporation of Carbon Dioxide in Clorella," J. Exp. Bot. 10:109-124.

Klein, R.A., Halliday, D., and Pittet, P.G. 1980. "The use of 13-methyltetradecanoic acid as an indicator of adipose tissue turnover," Lipids 15:572-579.

Lein, S., and Spencer, K.A. 1983. "Microalgal production of oils and lipids," In: Proceedings of IGT Symposium on Energy from Biomass and Wastes. VII:1107-1121.

Lein, S., and Roessler, P. 1985. "The energetics of biomass and lipid production by lipogenic microalbae under nitrogen deprivation," Proceedings of the Principal Investigators Meeting, SERI/CP-231-2700 pages 100-110.

Lepage, A., and Roy, C.C. 1984. "Improved recovery of fatty acids through direct transesterification without prior extraction as purification," Jour. Lipid Res. 25:1391-1396.

Lillington, J.M., Trafford, D.J.H., and Makin, H.L.J. 1981. "A rapid and simple method for the esterification of fatty acids and steroid carboxylic acids prior to gas-liquid chromatography," Clin. Chim. Acta 111:91-98.

Maxwell, J.R., Douglas, A.G., Eglinton, G., and McCormick, A. 1968. "The Botryococcus-hydrocarbons of novel structure from the alga Botryococcus braunii Kutzing," Phytochemistry 7:2157-2171.

McIntosh, R.P. 1984. "Intensive microalgae culture for lipid production," In: Abstracts, National Meeting on Biomass R and D for Energy Applications, 1-3 October 1984, Arlington, Virginia. SERI/CP-2-30-2430, page 57.

Milner, H.W. 1953. "The chemical composition of algae," In: Burlew, J.S. (Ed.) Algal Culture. From Laboratory to Pilot Plant. Carnegie Inst. Washington Publ. No. 600, Washington, D.C., pp. 285-302.

Opute, F.I. 1974. "Studies on fat accumulation in Nitzchia Palea," Ann. Bot. N.S. 38:889-892.

Pohl, P., and Wagner, H. 1972. "Control of fatty acids and lipid biosynthesis in Euglena gracilis by ammonia, light and DCMU," Z. Naturforsch. 27 b:53-61.

Pohl, P., and Zurheide, F. 1979. "Fatty acids and lipids of marine algae and the control of their biosynthesis by environmental factors," In: Hope, H.A., Levring, T., and Tanka, Y. (Eds.) Marine algae in Pharmaceutical Sciences. Walter de Gruzter, Berlin, pp. 443-523.

Shifrin, N.S., and Chisholm, S.W. 1981. "Phytoplankton lipids: Interspecific differences and effects of nitrate, silicate and light-dark cycles," J. Phycol. 17:374-384.

Sriharan, S., and Bagga, D. 1986. "Effect of induction stategies on enhanced lipid production in Chaetoceros SS-14," Proceedings of the Principal Investigators Meeting, SERI Subcontract No. XK-5-05104.

Sriharan, S., and Bagga, D. 1987. "Effect of environmental conditions on lipid production in diatom, Chaetoceros (SS-14)." Proceedings of the 9th Symposium on Biotechnology for Fuels and Chemicals, Boulder, CO., May 4-8, 1987.

Tadros, M.G. 1985. "Screening and characterizing oleaginous microalgal species from the southeastern United States," SERI/CP-231-2700, pp. 28-42.

Thomas, W.H., and Tornabene, T.G. 1983. "Chemical profiles of microalgae: Activities for 1983," Final report Subcontract Report. SERI.

Tornabene, T.G., and Benneman, J.R. 1984. "Chemical profiles of microalgae with emphasis on lipids, in Aquatic Species Program Review," Proceedings of the April 1984 Principal Investigators Meeting, SERI/CP-231-2341, Golden, Colorado.

Tornabene, T.G., A. Ben-Amontz, Raziuddin, S., and Hubbard, J. 1985. "Chemical profiles of microalgae with emphasis on lipids," Final report for SERI Subcontract No. XK-2-02149-1, Golden, Colorado.

Wake, L.V., and Hillen, L.W. 1981. "Nature of hydrocarbons content of blooms of the alga Botryococcos braunii occurring in the Australia freshwater lakes," Aust. J. Mar. Freshwater Res. 32:353-367.

Werner, D. 1966. "Die Kieselssaure in Stoffwechsel von Cyclotella cryptica," Arch. Microbiol. 55:278-308.

Improvement and Optimization

THIS PAGE

WAS INTENTIONALLY

LEFT BLANK

EFFECT OF ENVIRONMENTAL PARAMETERS ON LIPID PRODUCTION IN NAVICULA SAPROPHILA

Paulanne Chelf, Bill Barclay, and Peter Lemke
Solar Energy Research Institute
1617 Cole Blvd., Golden CO 80401

ABSTRACT

A set of full and fractional factorial experiments was performed to develop a model to predict the effects of environmental parameters on lipid production in Navicula saprophila. Factors examined included nitrogen concentration, silicon concentration, temperature, time, conductivity, and alkalinity. Measured characteristics included nile red fluorescence (as a measure of neutral lipid content) and ash-free dry weight (AFDW). The multiple regression model developed from a Box-Behnken design for the ratio of nile red fluorescence to ash-free dry weight (RATIO) had an R^2 of 89.34%, and included five main effects, five two factor interaction terms, and two squared terms. The most important variables in this regression model were nitrogen concentration and conductivity. The validity and potential use of models such as this one are discussed.

EFFECT OF ENVIRONMENTAL PARAMETERS ON LIPID PRODUCTION IN NAVICULA SAPROPHILA

INTRODUCTION

In order to develop a technology base to produce liquid fuels from algal biomass, it is essential that we understand the effects of environmental parameters on the production of biomass and lipids by microalgae. Light, conductivity, temperature, pH, nutrient concentration and alkalinity are all potentially important variables in this process. A large body of data concerning the effects of temperature and conductivity on growth has been collected at SERI and elsewhere (Barclay et al, 1986; Brown, 1985; Brown, 1982; Ignatiades and Smayda, 1970; Smayda, 1969; Maddux and Jones, 1964), but surprisingly little information is available concerning the effects of these variables on lipid production. It has been known for some time that nitrogen (or silica) limitation results in increased lipid production in many species of algae (Spoehr and Milner, 1949; Collyer and Fogg, 1955; Shifrin and Chisholm, 1981; Piorreck et al, 1984; Piorreck and Pohl, 1984; Laing, 1985), but how nutrient limitation might interact with other environmental factors in this process is not known. Light and temperature are known to affect photosynthesis and productivity in many algae (Richmond, 1986; Sorokin and Krauss, 1961; McCombie, 1960), but very little literature exists relating these variables to lipid production (see Fogg, 1956; Shifrin and Chisholm, 1981). Shifrin and Chisholm (1981) highlighted one of the central issues in this problem: lipid production by microalgae is regulated by environmental factors, but not always systematically. In addition, they pointed out that this regulation could be quite species specific. In a system as complex as this one seems to be, one-factor-at-a-time experimentation would not seem to be a fruitful approach. Our intent, on embarking upon this work, was to examine as many of these variables as possible simultaneously to allow us to detect interactions between variables as well as curvilinear effects. In addition, we desired to test these variables at a small scale to allow many trials to be performed at once. Information obtained at small scale could then be utilized for process scale up.

MATERIALS AND METHODS

Organism. Navicula saprophila (NAVIC1) was obtained from the SERI culture collection. The organism was maintained in SERI Type II medium at 25 mmho/cm (Barclay et al, 1986) prior to use in these experiments.

Culture. A modified Type II medium was used throughout these experiments. The concentrations of $CaCl_2$, $MgCl_2$, $NaSO_4$, and KCl were those normally used in Type II/25 medium. Sodium carbonate and bicarbonate were added in the proportions used in II/25 medium to achieve the specified alkalinities, and sodium chloride was added to achieve the specified conductivities. Sodium monophosphate was added at 16 mg/L, while urea ranged from 0 to 72 mg/L. All media were filter sterilized. Cells were acclimated to the desired temperatures and conductivities for 24h prior to beginning an experiment. Experiments were conducted on a temperature gradient table, modified by B. Barclay from one designed by Siver (1983). Cells were cultured in 25 ml of medium contained in 50 ml polycarbonate Erlenmeyer flasks. All

experiments were conducted at a light intensity of approximately 200 uE/m^2/sec.

Experimental Design. A Box-Behnken fractional factorial design (Box and Behnken, 1960) for six factors was used to obtain the results reported here. Factors examined and the ranges used were as follows: (1)conductivity, 20 to 70 mmho/cm; (2)temperature, 17 to 32°C; (3)urea concentration, 0 to 72 mg/L; (4) silicon concentration, 0 to 500 mg/L as sodium metasilicate; (5)time, four to nine days; and (6)alkalinity, 8.8 to 87.5 meq/L. Multiple regression analysis was performed using either Statgraphics (Statistical Graphics Corporation) or X-Stat (Wiley Professional Software). Models were generated for both RATIO (nile red fluorescence/ash-free dry weight), and ash-free dry weight accumulation (AFDW).

Analytical Techniques. Nile red fluorescence was done according to the method of Nagle and Barclay (in review). Cells for ash-free dry weight determinations were collected on 2.1 cm GF/C glass fiber filters, dried at 65°C overnight, weighed on a Sartorius ultramicrobalance, ashed overnight at 550°C, and weighed again.

RESULTS

The regression model obtained for RATIO (nile red fluorescence/ash free dry weight) exhibited randomly distributed residuals, and a coefficient of determination of 89.34%. The analysis of variance for this model is shown in Table 1. Regression coefficients are presented in Table 2. Nitrogen and conductivity were the coefficients of greatest magnitude in this model. Since the model was developed using coded and scaled variables, they were also the most important effects. As nitrogen concentration or conductivity increased, the amount of neutral lipid (as measured by nile red fluorescence) per mg of ash-free dry weight decreased. In order to easily assess the severity of these effects, sections of the response surface developed from these coefficients were mapped. Figure 1 shows the response of RATIO as nitrogen and conductivity were varied. This map was taken from the area of highest predicted RATIO, at 17°C, no silica, nine days, and 87.5 meq/L alkalinity. The highest RATIO predicted from this response surface was 712.7. The map section in Fig. 1 shows a decrease in RATIO from 700 to 400 as conductivity increased from 20 to 70 mmho/cm. As nitrogen increased from 0 to 72 mg/L, RATIO decreased from 700 to 200.

The interactions of temperature with time and of conductivity with nitrogen concentration, as well as the curvilinear effect of nitrogen concentration also have important effects on RATIO in this model. One section of response surface showing the effects of varying time and temperature at the midpoints of the remaining variables is illustrated in Fig. 2. This area seemed to represent a saddle in the response surface, and helped to visualize the results of this interaction term.

TABLE 1. ANALYSIS OF VARIANCE FOR RATIO

Source	df	SS	MS	F-RATIO
Total (Corrected)	52	985359		
Regression	12	880275	73356	27.923*
Residual	40	105084	2627	
Lack of Fit	36	101041	2807	2.777**
Pure error	4	4043	1011	

* Implies 99.9% confidence regression equation is nonzero.
** Implies 17.3% confidence pure error explains lack of fit.

TABLE 2. REGRESSION COEFFICIENTS FOR RATIO

COEFFICIENT	TERM	STANDARD ERROR	SIG. LEVEL
242.4	1.000	12.43	99.9%
-132.9	Nit	10.46	99.9%
-90.3	Cond	10.46	99.9%
-73.6	Temp*Time	12.81	99.9%
67.9	Cond*Nit	18.12	99.9%
60.8	Nit^2	14.49	99.9%
35.2	Cond*Temp	18.12	95.1%
31.1	Cond*Time	18.12	91.4%
-30.3	Temp^2	14.49	96.7%
29.4	Temp*Nit	18.12	89.2%
27.8	Time	10.46	99.4%
23.9	Alk	10.46	98.1%
-19.4	Sil	10.46	93.8%

R^2 = 89.34%

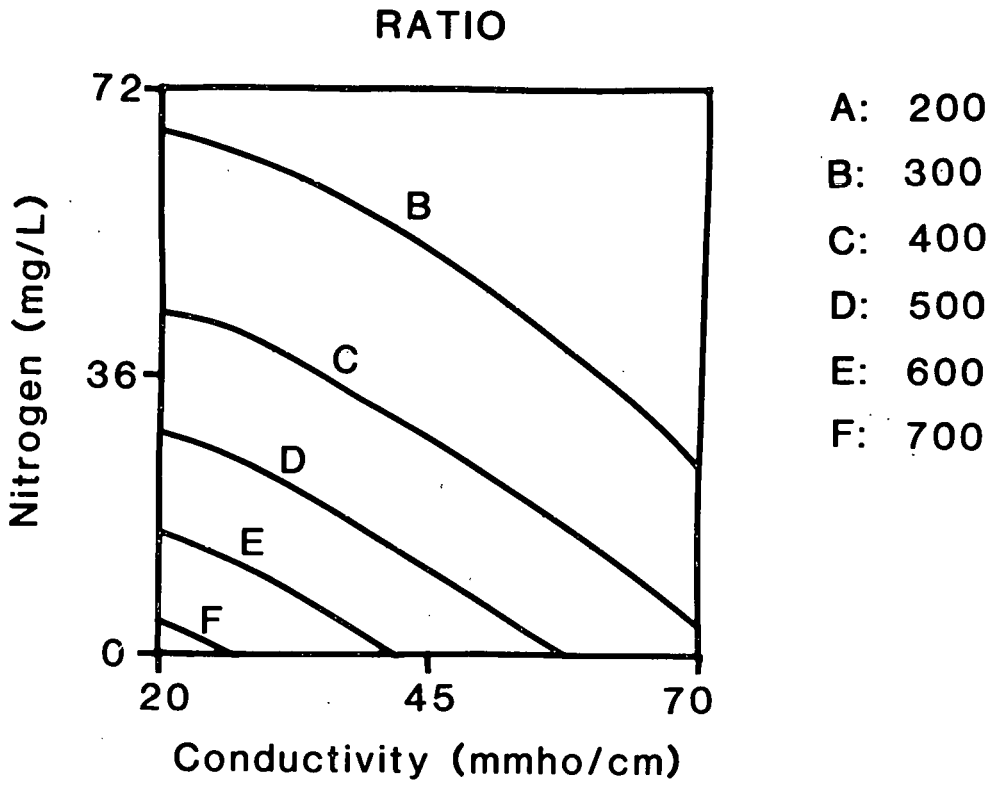


Figure 1. Response surface map for RATIO, nitrogen and conductivity varying. Temperature = 17°C; Silicia = 0 mg/L; Time = 9 days; Alkalinity = 87.5 meq/L.

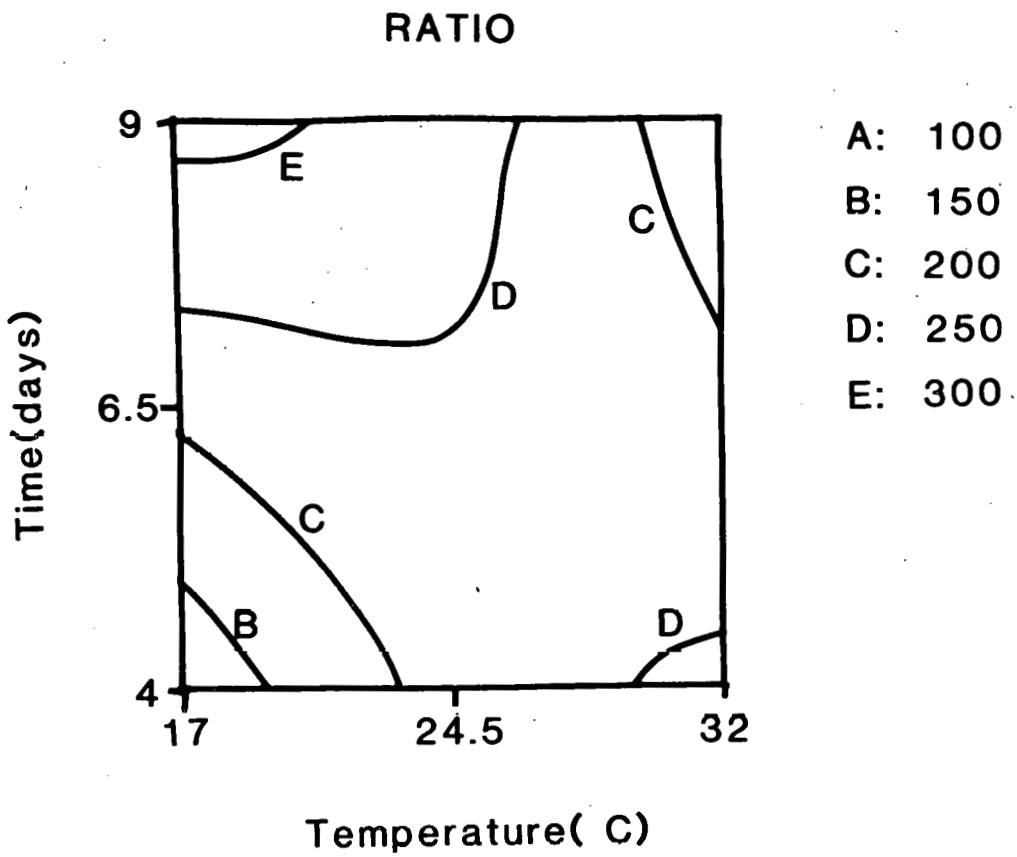


Figure 2. Response surface map for RATIO, time and temperature varying. Conductivity = 45 mmho/cm; Nitrogen = 36 mg/L; Silica = 250 mg/L; Alkalinity = 48 meq/L.

The analysis of variance and regression coefficients for the AFDW model are presented in Tables 3 and 4.

TABLE 3. ANALYSIS OF VARIANCE FOR AFDW

Source	df	SS	MS	F-RATIO
Total (corrected)	52	1858545		
Regression	13	1604838	123449	18.97*
Residual	39	253707	6505	
Lack of fit	35	226694	6477	.959**
Pure error	4	27013	6753	

* Implies 99.9% confidence regression equation is nonzero.
** Implies 58.3% confidence pure error explains lack of fit.

TABLE 4. REGRESSION COEFFICIENTS FOR AFDW

COEFFICIENT	TERM	STD ERROR	SIG. LEVEL
633.5	1.000	19.56	99.9%
160.3	Nit	16.46	99.9%
101.7	Time	16.46	99.9%
-101.3	Temp^2	22.81	99.9%
-90.9	Nit^2	22.81	99.9%
78.5	Alk	16.46	99.9%
74.2	Nit*Alk	20.16	99.9%
73.5	Nit*Time	28.52	99.2%
69.2	Temp	16.46	99.9%
64.4	Alk*Temp	28.52	98.0%
55.1	Time*Alk	28.52	95.0%
-51.1	Cond*Alk	28.52	92.8%
51.0	Temp*Nit	28.52	92.7%

R^2 = 86.4%

Nitrogen concentration was the most important variable in this model as well, but as nitrogen concentration increased, ash-free dry weight increased. Alkalinity was a much more important effect in this model than in the model for RATIO. The positive regression coefficient indicated that ash-free dry weight increased as alkalinity increased. Figures 3 and 4 represent areas of the response surfaces for these two models near the areas of predicted maximum response. Both show conductivity and alkalinity varying, but the difference in response to these two variables in the two models is apparent. RATIO varied very little as alkalinity changed from 8 to 87.5 meq/L, but decreased dramatically as conductivity increased. AFDW, however, showed little change with increasing conductivity, but rose from 750 to 1200 as alkalinity increased from 8 to 87.5 meq/L. Both nitrogen and temperature exerted

curvilinear effects in both models, presumably due to the ranges chosen for each.

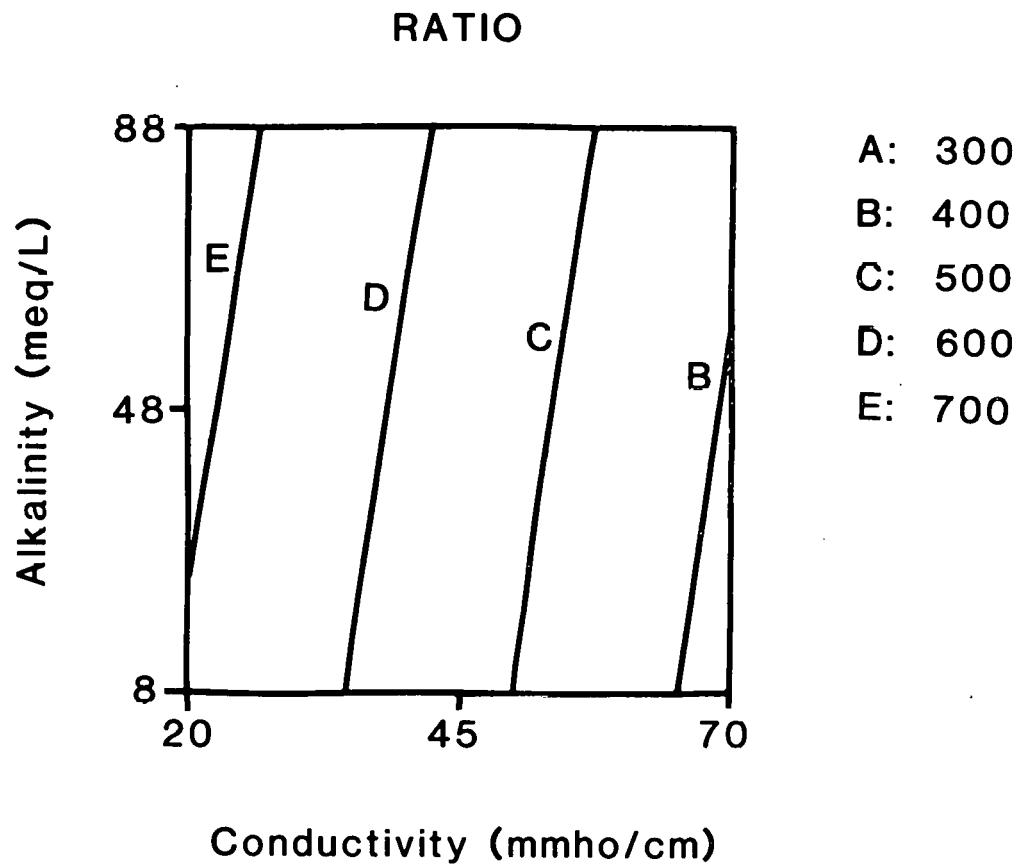


Figure 3. Response surface map for RATIO, alkalinity and conductivity varying. Temperature = 17°C; Nitrogen = 0 mg/L; Silica = 0 mg/L; Time = 9 days.

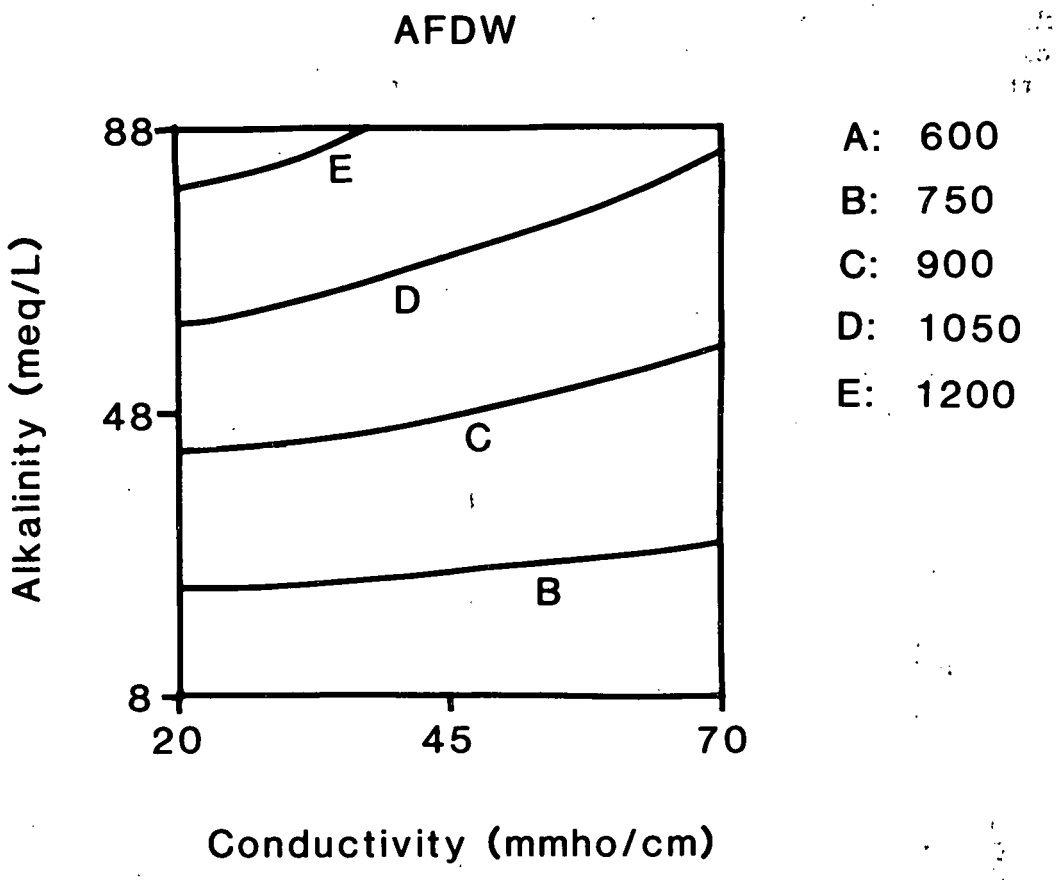


Figure 4. Response surface map for AFDW, alkalinity and conductivity varying. Temperature = 32°C; Nitrogen = 72 mg/L; Silica = 250 mg/L; Time = 9 days.

Previous data obtained regarding the effects of temperature and salinity on the growth rate of NAVIC1 in Type II medium are illustrated in Fig. 5 (Barclay et al, 1986). Temperature exerted a much larger effect on growth rate than did salinity, reinforcing results obtained with the AFDW model.

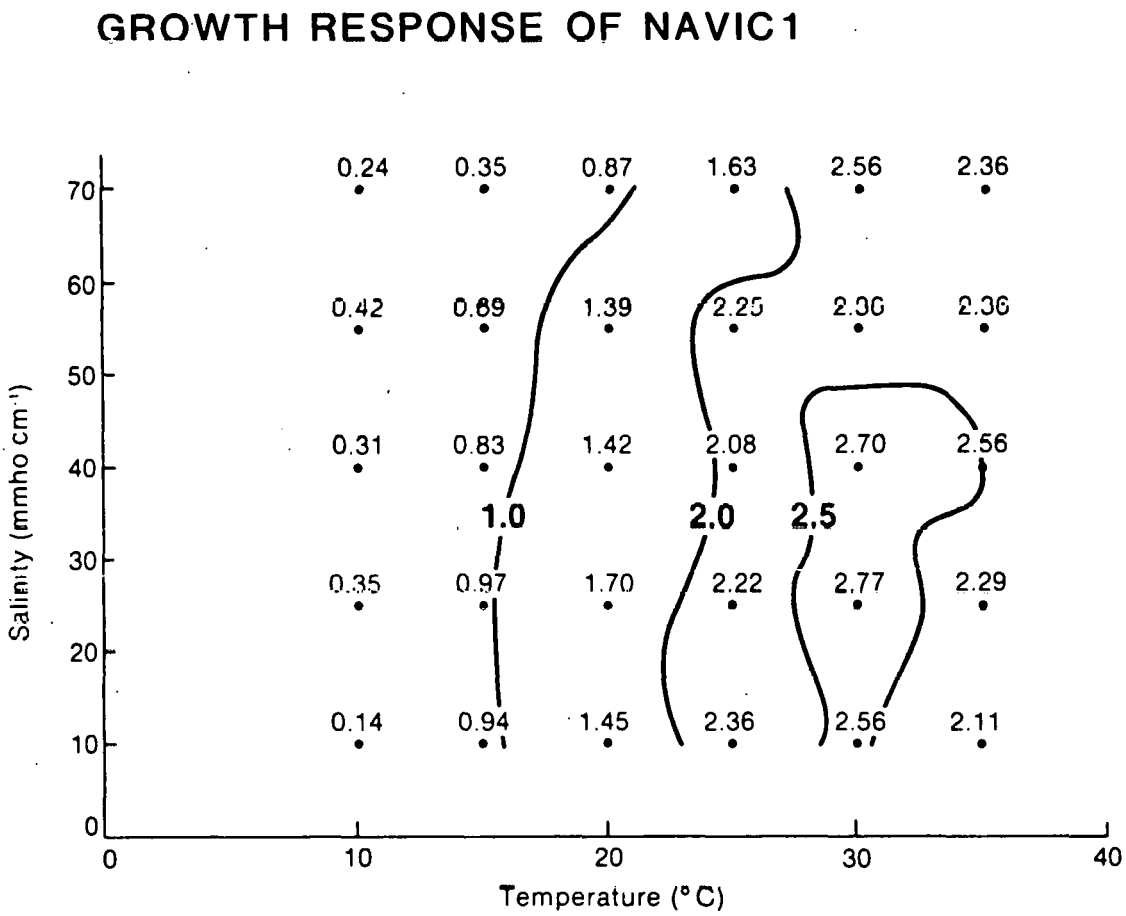


Figure 5. Surface plots of growth response to different temperature and conductivity for NAVICI in SERI Type II medium.

DISCUSSION

It is important to realize that models such as this are empirical models. That is, they are developed using mathematical approximations drawn from empirical observations. If a reasonable model has been developed which includes the important variables and explains much of the variability in the data, predictions about the system can be made, but only within the boundaries of the experimental space. Additionally, one should recognize that important effects, interactions, and curvilinear effects seen in a model are very much dictated by one's choice of ranges for the variables examined. For example, a smaller range of conductivities examined might not have resulted in conductivity being the biggest effect seen in the RATIO model. In a similar fashion, the ranges chosen for time and nitrogen concentration will dictate whether these variables interact, as well as whether their main effects and curvilinear effects are significant. Also, since the environment changes as scale changes, results obtained at small scale may not translate precisely as the scale is increased.

The regression models developed here for NAVIC1 include many of the important variables discussed in the introduction. They do not include light intensity and pH as controllable variables, because we were unable to control these variables at the scale we chose; light was held constant at approximately 200 uE/m^2/sec, and pH in all trials varied from 8.9-9.0 at the start of the experiment to 10.2-10.5 at the end. One test for model adequacy is the distribution of residuals. Residuals for these models appeared to be randomly distributed, which suggests that this model may be used for prediction, within the limitations described above. The next scale of model development should include light intensity and pH, since these variables are known to affect photosynthesis and productivity.

The analysis of variance for the RATIO model implied only a 17.3% confidence that pure error explained lack of fit, while the AFDW model indicated 58.3% confidence that pure error explained lack of fit. Some of this difference may be due to the significance level chosen (90%) for variables to be included in the model. The AFDW model coefficients were distributed in two definite groups, with one group being >90%, and the other being far less than the 90% significance level. The RATIO coefficients were distributed more continuously, so a number of terms significant at the 80% level were not included in this model.

Silica concentration is a relatively unimportant variable in the RATIO model, even though silica limitation has been shown to be an effective inducer of lipid production in this organism. This may be a result of the characteristics chosen as the basis for the model. Our previous work showing lipid induction under silica-limited conditions was evaluated as an increase in lipid as a percent of organic matter or as an increase in lipid yield as mg/L. Under silica-limited conditions, ash-free dry weight continued to increase as lipid increased, but under nitrogen-limited conditions, lipid increased with no concurrent increase in ash-free dry weight. Consequently, the increase in the ratio of lipid to dry weight would be much more dramatic under nitrogen-limited conditions than under silica-limited conditions.

CONCLUSIONS

The regression model developed for Navicula saprophila has shown that lipid production by this organism is adversely affected by increasing conductivity. Production of liquid fuels from microalgae is currently planned for the desert southwest using saline groundwaters. Evaporation during production will greatly increase conductivity from the starting level to > 70 mmho/cm. This model predicts that Navicula saprophila may not be an ideal production organism under these conditions. We have yet to test other organisms under these conditions so we cannot yet make general statements about the effect of conductivity on lipid production in microalgae as a group. We have shown that regression models such as the one developed here may have utility as predictive models for complex biological systems where mechanistic models are not available.

REFERENCES

Barclay, W., Johansen, J., Chelf, P., Nagle, N., Roessler, P., and Lemke, P. 1986. Microalgae Culture Collection, 1986-1987. Solar Energy Research Institute, Golden, CO. 149 pp.

Box, G. E. P., and Behnken, D. W. 1960. Some new three level designs for the study of quantitative variables. Technometrics 2:455-475.

Brown, L. M. 1982. Photosynthetic and growth responses to salinity in a marine isolate of Nannochloris bacillaris (Chlorophyceae). J. Phycol. 18:483-488.

Brown, L. M. 1985. Stepwise adaptation to salinity in the green alga Nannochloris bacillaris. Can. J. Bot. 6:327-332.

Collyer, D. M., and Fogg, G. E. 1955. Studies on fat accumulation by algae. J. Exp. Bot. 6:256-275.

Fogg, G. E. 1956. Photosynthesis and formation of fats in a diatom. Ann. Bot. 20:265-285.

Ignatiades, L. and Smayda, T. J. 1970. Autecological studies on the marine diatom Rhizosolenia fragilissima Bergon. I. The influence of light, temperature, and salinity. J. Phycol. 6:332-339.

Laing, I. 1985. Growth rsponse of Chaetoceros calcitrans (Bacillariophyceae) in batch culture to a range of initial silica concentrations. Mar. Biol. 85:37-41.

Maddux, W. J. and Jones, R. F. 1964. Some interactions of temperature, light intensity, and nutrient concentration during the continuous culture of Nitzschia closterium and Tetraselmis sp. Limnol. Oceanogr. 9:79-86.

McCombie, A. M. 1960. Actions and interactions of temperature, light intensity, and nutrient concentration on the growth of the green alga, Chlamydomonas reinhardi Dangeard. J. Fish. Res. Bd. Canada 17:871-894.

Nagle, N. and Barclay, W. 1987. A fluorescent assay for quantification of neutral lipids in microalgae. J. Microbiol. Meth. In review.

Piorreck, M., Baasch, K. H. and Pohl, P. 1984. Biomass production, total protein, chlorophylls, lipids, and fatty acids of freshwater green and blue-green algae under different nitrogen regimes. Phytochem. 23:207-216.

Piorreck, M. and Pohl, P. 1984. Formation of biomass, total protein, chlorophylls, lipids, and fatty acids in green and blue-green algae during one growth phase. Phytochem. 23:217-223.

Richmond, A. 1986. Cell response to environmental factors. In: Richmond, A. (ed.), Handbook of Microalgal Mass Culture. CRC Press, Inc. Boca Raton, Florida, pp.69-99.

Shifrin, N. S. and Chisholm, S. W. 1981. Phytoplankton lipids: Interspecific differences and effects of nitrate, silicate, and light-dark cycles. J. Phycol. 17:374-384.

Siver, P. A. 1983. A new thermal gradient device for culturing algae. Br. Phycol. J. 18:159-164.

Smayda, T. J. 1969. Experimental observations on the influence of temperature, light, and salinity on cell division of the marine diatom, Detonula confervacea (Cleve) Gran. J. Phycol. 5:150-157.

Sorokin, C. and Krauss, R. W. 1961. Effects of temperature and illuminance on Chlorella growth uncoupled from cell division. Plant Physiol.

Spoehr, H. A. and Milner, H. W. 1949. The chemical composition of Chlorella; effect of environmental conditions. J. Biol. Chem. 24:120-149.

IMPROVEMENT OF MICROALGAL STRAINS FOR LIPID PRODUCTION

J. A. Solomon
Research Associate

A.V. Palumbo
Research Associate

Environmental Sciences Division
Oak Ridge National Laboratory
Oak Ridge, Tennessee 37831

ABSTRACT

Strategies were devised to allow for the selection of high-lipid cells using flow cytometry, from micoralgal populations in exponential or stationary growth phase. Earlier results had indicated that cells could be sorted with a flow cytometer on the basis of their lipid content after being stained with a fluorescent dye that is specific for neutral lipids. However, the resulting cultures often did not have higher lipid content than the parent cultures when measured many generations after the sort. Recent results suggest that when cell cycle differences in the parent culture are taken into account, high-lipid daughter populations, can retain their enhanced lipid levels for many generations. When the lipid level was used as the sole criterion for sorting, the sorts were successful, resulting in high-lipid daughter populations, if they took place after the cells had stopped dividing; but they were not successful if cultures were sorted while growing exponentially. Apparently, sorting exponentially growing cultures by lipid content alone selects for cells which are high in lipid primarily because they are about to divide. Exponentially growing populations were sorted successfully when the chlorophyll:lipid ratio was used for defining the sorting window. This procedure results in selection of cells at all stages in the cell cycle and yields cells which are higher in lipid content than the average cell at that growth stage.

IMPROVEMENT OF MICROALGAL STRAINS FOR LIPID PRODUCTION

INTRODUCTION

In the future, micro algal lipids may be used as a substitute for liquid fuels or in the production of specialized high-temperature lubricants. In order to make the production of algal lipids commercially feasible algal strains containing higher levels of lipids than those presently available will be necessary. Thus, a series of studies were undertaken to investigate the utility of flow cytometry in establishing high-lipid populations of algal cells. In any population of algal cells, lipid content will vary from cell to cell as a function of growth conditions, stage of cell cycle, and cell size. Lipid content may also be genetically influenced and thus may be subject to genetic manipulation. Establishment of algal strains with consistently high lipid content would indicate that this quantitatively varying trait is at least partially controlled by a heritable component of the cells' genome.

Flow cytometry allows measurements to be made of individual particles in suspension (e.g., single cells) as they pass through a laser beam. When a cell intercepts the beam, several pulses of light are generated and collected in detection devices around the point of interception. By applying a small positive or negative electric charge to each cell after it is measured, individual cells can be sorted from the rest of the population.

Preliminary studies had shown that cells could be separated from one another on the basis of their lipid content (Solomon et al 1986). By staining the cells with nile red, a fluorescent dye that is specific for neutral lipids (Greenspan et al., 1985), and simultaneously measuring chlorophyll autofluorescence and nile red fluorescence with a flow cytometer, cells with a higher-than-average lipid content could be removed from the remainder of the population and cultured. In three out of nine trials, the daughter populations sorted in this manner had a substantially higher lipid content many generations later when compared with the parent population (Solomon et al., 1986). In the other six trials, the lipid content of the sorted populations did not differ significantly from that of the controls. These results suggested that cellular lipid content has a genetic determinant and that flow cytometry may be an effective way to enhance the lipid content of selected algal strains. However, the inconsistency of the results ("success" in only three of nine trials) indicated an inadequate understanding of the factors involved.

The primary goal of this project was to increase the consistency of our sorting results, which requires a better understanding of the factors involved. Of these poorly understood factors, lack of knowledge concerning the state of the parental culture at the time of sorting seemed to be the most urgent. For this reason, considerable effort was made to provide optimal and stable growth conditions and to adequately characterize the growth curve under these conditions. Other factors to be addressed included determining optimum staining conditions, standardizing the lipid induction methodology, and correlating nile red fluorescence with extractable lipid.

MATERIALS AND METHODS

Culture

The prymnesiophyte alga Isochrysis aff. galbana (Tahitian strain), originally obtained from the Solar Energy Research Institute's microalgal culture collection (S/ISOCH-1; SERI, 1986) in 1985, was used throughout this study. Cultures were maintained in f/2 medium (Guillard, 1975) at 20 C, with a photon flux of about 20 uE m^{-2} s^{-1} from Vita-Lite natural spectrum fluorescent bulbs (Duro-Test Corp., North Bergen, N.J.). The cultures were bubbled with 0.5% CO_2 and were also constantly stirred with a magnetic stirrer. They were grown in f/2 medium with two modifications: 4 mM $NaCO_3$ provided adequate pH buffering, and 281 mg $NaNO_3$/L was added to provide 3.3 mM nitrogen. Lipid induction occurred when the nitrogen supply was exhausted. The final biomass and the time of nitrogen depletion were determined by the initial nitrogen concentration, because carbon and other nutrients were not limiting. Cell growth was monitored by means of cell counts (Bright-Line hemacytometer) and optical density measurments made at 750 nm (OD_{750}) (Perkin-Elmer 553 Fast Scan Spectrophotometer).

Fluorescent staining

Nile red (Greenspan et al. 1985) was used as an intracellular neutral-lipid-specific stain, both for epifluorescent light microscopy and for flow cytometry (FCM). Nile red powder (Molecular Probes, Junction City, Oregon) was added to either acetone or heptane at a concentration of 0.1 mg/mL. Dissolution in heptane was incomplete: consequently, the saturated solution was used as a stock for later dilutions for a final concentration of 1-5 mg/mL culture medium for routine FCM analysis and sorting. The dye was added to a suspension of algal cells in culture medium, and the mixture was allowed to equilibrate for at least 5 min before microscopic examination and 30 min before FCM.

Lipid extraction

Lipids were extracted from algal cells (which were pelleted by centrifugation) by adding a mixture of chloroform, methanol, and water (Bligh and Dyer, 1959). Following separation of the organic solvents, the lower chloroform layer, containing the lipids, was drawn off, concentrated in a rotary evaporator, and dried over a stream of nitrogen. These mixed Isochrysis lipids were weighed and dissolved in isopropanol (0.04% water), from which dilutions were prepared. Nile red dissolved in acetone (100 mg/mL) was added to each dilution at the rate of 1 mg nile red/mL solution. The stained solutions were kept in the dark for 1 h and fluorescence was measured using a Perkin Elmer Fluorescence Spectrophotometer (488 nm excitation, 600 nm emission, with a slit width of 10 nm).

Flow cytometric analysis and sorting

An Ortho Diagnostics 50H Research Flow Cytometer, equipped with dual lasers, was used for cell measurement and sorting. The cells were stained with nile red dissolved in heptane, as described above, and the parent culture was routinely monitored throughout sorting with epifluorescence microscopy. The population was continually stirred during sorting. A wavelength of 488 nm was used for excitation. An emission range of 520 to 580 nm was used to measure nile red fluorescence and chlorophyll autofluorescence was measured at 630 nm. Cells were sorted, on the basis of having relatively high nile red fluorescence (high-lipid content) or having a high ratio of nile red:chlorophyll fluorescence, into a sterile 50 mL beaker containing about 5 mL of culture medium.

The controls for all sorts were obtained by "selecting" the entire parent population; that is an electric charge was applied to all cells and all of them were deflected into the collection vessel.

The sorted cells (approximately 200,000 per culture) were grown in 20 mL of culture medium in 50-mL Erlenmeyer flasks stoppered with cotton plugs. The flasks were kept on an orbital shaker in the growth chamber housing the Roux bottles. The light levels varied with distance from the light bank. Immediately following sorting or inoculation of a new culture, the light levels were reduced for a few hours to permit acclimation. After several weeks of growth in 50-mL flasks, the cultures were transferred to 100 mL of fresh medium in 250-mL flasks and placed on the orbital shaker.

Several replicate sorts were then performed at different times, and the daughter populations, as well as the controls, were grown and reanalyzed. The growth stages of the parental cultures were characterized as exponential, stationary, or severely stressed. Initial sorts were performed on the basis of lipid concentration alone, but in later sorts populations were differentiated by means of their chlorophyll:lipid ratio.

Two-dimensional gel electrophoresis

Large quantities (1200 mL) of proven high- and low-lipid cultures (derived from "NO_3" culture, sorted on March 31) are currently being grown in Roux bottles. These will be pelleted by centrifugation, frozen, and then separated and analyzed by Betty Mansfield of the Biology Division of Oak Ridge National Laboratory. The two-dimensional gel electrophoresis will be used to compare the protein profile of a high-lipid population with that of the control.

RESULTS AND DISCUSSION

Standard growth curve

The growth curves for three cultures (Figures 1 and 2) were categorized into exponential growth phase (days 1-4), declining growth phase (days 5-6),

and stationary phase (days 7-14). Any culture that was more than 14 days old was classified as severely stressed, based on microscopic examination: these cultures were heavily colonized by bacteria, and a large proportion of the cells no longer appeared robust.

Staining methodology

When Isochrysis is stained with a solution of nile red dissolved in heptane (NR-h) and viewed using epifluorescence microscopy, the intracellular oil bodies appear as well-defined, brilliant, yellow-green droplets. In addition, autofluorescence from the chloroplasts is plainly visible, providing an indication of viability. NR-h was used for all flow cytometric measurements because of the good spectral separation of the yellow-green of NR-h and the red of chlorophyll. Several different stain concentrations were compared by epifluorescence microscopy, and a final concentration of 1 mg nile red in 1 mL of cell suspension was chosen.

The use of nile red for measuring the average lipid content of a cell suspension was explored as a means of monitoring lipid accumulation fluorometerically at different stages of the growth curve. NR-h, being immiscible with water, proved to be unsatisfactory for this purpose due to the fluorescence of undissolved heptane droplets that produced erroneous readings. (Such droplets did not interfere with FCM because the absence of a simultaneous chlorophyll reading eliminated noncells from the selection window.) Nile red dissolved in acetone (NR-a) was thus more appropriate for measuring the average lipid content of cell suspensions, in spite of yielding poorer spectral separation from chlorophyll.

The fluorescence signal from cells stained with NR-a is not stable over time (Nick Nagle, Solar Energy Research Institute, pers. comm.). Analysis of stained cells over time (Figure 3) suggested that readings should be performed more than 45 min after the dye is added to the cell suspension so that small changes in the time of the measurement do not have a significant effect on the results.

An attempt was made to quantitate NR-a fluorescence with Isochrysis lipid content. However, total extracted lipid was found to be inversely correlated with NR-a fluorescence (Table 1). This result is apparently due to polar lipids interfering with the fluorescence measurement. Extraction disrupts the cellular organization causing mixing of neutral lipids and polar lipids, and polar lipids are known to quench nile red fluorescence (Greenspan et al., 1985). For this reason, the necessary assay should be done on an aliquot of intact cells and the neutral lipid extracted and measured from the remainder of the culture.

Sorting and reanalysis of daughter populations

In this study, 14 sorts were performed on Isochrysis cultures with follow-up measurements have been made on 7 of them (Table 2). The sorts differ in the growth phase of the parental culture (exponential, stationary, or severely stressed) as well as in the criterion used for sorting. Initially, the sorting criterion was based solely on the lipid content of the

parental population; i.e., all cells with a lipid content above a specific value were selected, regardless of the level of chlorophyll. The specific value varied depending on the nature of the parent population. The results from sorts done on this basis were not constantly successful. Cells that were selected on the basis of their high lipid content did not yield a high-lipid culture when reanalyzed some weeks later. Examination of the results (Table 2) shows, in fact, that such sorts were not successful in those cases where the parental population was in the exponential growth phase. In the other four cases where lipid level was the sole sorting criterion, the mean lipid content of the high-lipid daughter population was at least 20% higher than that of the control. This was true when the parental population was in the stationary phase and also when it was severely stressed.

These results are interpretable when the relationship between the stage of the cell cycle and the lipid content is considered. Because no attempt was made to synchronize these cultures, a parental population consisted of cells in all stages, including those that had just undergone cell division (G1), those that were preparing to divide (G2), and all stages in between. Thus, the population was a mixture of cells of different ages and of correspondingly different sizes, with similarly different lipid and chlorophyll content. When a sorting window was selected solely on the basis of lipid content, the resultant daughter population consisted of the largest and oldest cells of the population.

In addition to these size-related differences, there is a quantitative variation in lipid content among cells of the same age and/or size, which is attributable to natural variation. It is this natural variation that we seek to exploit by means of flow cytometry, in order to produce a population with consistently enhanced lipid levels.

Nitrogen deprivation prevents cells from continuing to divide; thus, a culture in which cell division has ceased should consist largely of cells that are increasing only very slowly in size, if at all. This is the case for both the stationary growth phase and severely stressed cultures. Because these cells are all roughly the same size, sorting of these cultures solely on the basis of lipid content would be expected to produce high-lipid cultures.

It should be possible to sort exponentially growing cultures by lipid content if size differences are taken into account. Chlorophyll and lipid content should be proportional to cell size and thus to cell age; the largest cells, then, are those with the highest chlorophyll and lipid content. These are the cells represented by the area farthest from the origin in Figure 4; the line running through the origin represents the cell age axis. Natural variation in the chlorophyll:lipid ratio is represented by an axis perpendicular to the age axis. The area below and to the right of the age axis thus consists of cells that are above average in lipid content and below average in chlorophyll content. Selecting exponentially growing cells from this region did, in fact, produce daughter populations in which the lipid contents were 21 and 47% above those of the controls (Table 2, both May 11

sorts). As stated previously, the controls were the parent population put through the flow cytometer with all cells selected for sorting. The high-lipid cultures selected in this manner have proven to be stable over periods of weeks to months.

Two-dimensional gel electrophoresis

High- and low-lipid cultures are currently being grown for analysis by two dimensional gel electrophoresis. Results are not yet available.

CONCLUSIONS

These results indicate that, by selecting high-lipid algal cells of approximately the same age, it is possible to produce cultures with increased mean lipid content. By taking cell life cycle differences into account, high-lipid daughter populations were produced from parental cultures having lower lipid contents. These daughter populations, when grown for a number of generations, have retained their enhanced lipid levels. When lipid level was used as the sole criterion for sorting, sorts were successful if they took place after the cells had stopped dividing. Exponentially growing populations were sorted successfully when the chlorophyll:lipid ratio (sorting for relatively high lipid content compared with chlorophyll) was used for defining the sorting window. Progressive sorting may well result in further enhancement, and the use of synchronized cultures might allow better identification of those cells with the highest lipid content.

LITERATURE CITED

Bligh, E. G., and W. J. Dyer. 1959. "A rapid method of total lipid extraction and purification," Can. J. Biochem. Physiol. 37:911-917.

Greenspan, P. E. P. Mayer, and D. Fowler. 1985. "Nile red: A selective fluorescent stain for intracellular lipid droplets," J. Cell Biol. 100:965-73.

Guillard, R. R. L. 1975. "II: Culture of phytoplankton for feeding marine invertebrates", pp. 29-60. IN W. L. Smith and M. H. Chanley (eds.), Culture of Marine Invertebrate Animals. Plenum Press, New York.

Solar Energy Research Institute (SERI). 1986. Microalgae Culture Collection 1986-1986. Solar Energy Research Institute, Golden, Colorado.

Solomon, J. A., R. E. Hand, Jr., and R. C. Mann. 1986. Ultrastructural and flow cytometric analyses of lipid accumulation in microalgal, SERI/STR-231-3089. Solar Energy Research Institute, Golden, Colorado.

Table 1. Nile red fluorescence readings of total lipids extracted from Isochrysis aff. galbana. Dilutions of total lipid dissolved in isopropanol (0.04% water content) were stained with 1 mg nile red dissolved in acetone per mllilitter of final solution.

Lipid concentration (mg/mL)	Fluorescence (relative to blank)
5	-95.9
10	-110.2
20	-146.2
40	-161.2
50	-189.6
100	-242.0

Table 2. Mean lipid content of daughter populations from previous sorts as of June 5, 1987.

Date of sort	Culture ID	Growth phase[a]	Criterion for sort[b]	Lipid level designation[c] Low	Control	High	Difference from control (%)
3-23	1	Exp.	Lipid	N/A	290	197	-47
3-23	2	Exp.	Lipid	N/A	293	283	-4
3-31	1	Stat.	Lipid	N/A	196	262	+25
3-31	2	Stat.	Lipid	N/A	199	257	+23
3-31	3	Strs.	Lipid	N/A	243	354	+31
4-27	3	Exp.	Lipid	*	*	*	
4-27	4	Exp.	Lipid	*	*	*	
5-4	3	Exp.	Lipid	79	126	196	+35
5-4	4	Stat.	Lipid	*	*	*	
5-4	2	Strs.	Lipid	*	*	*	
5-11	5	Exp.	C:L ratio	196	137	173	+21
5-11	6	Exp.	C:L ratio	91	112	211	+47
5-18	5	Stat.	C:L ratio	*	*	*	
5-18	6	Stat.	C:L ratio	*	*	*	

[a]Growth phase of parental culture: Exp. = exponential; Stat. = stationary; Strs. = severely stressed.

[b]Lipid = sorting windows were differentiated solely on the basis of lipid content. C:L = sorting windows were chosen based on the chlorophyll:lipid ratio of the cells.

[c]Sorting window for daughter populations were designated as low lipid, control, or high lipid. N/A = not applicable; this population was not sorted. * = No results available. Only cultures with visible growth were measured, and these had no visible growth as of June 5.

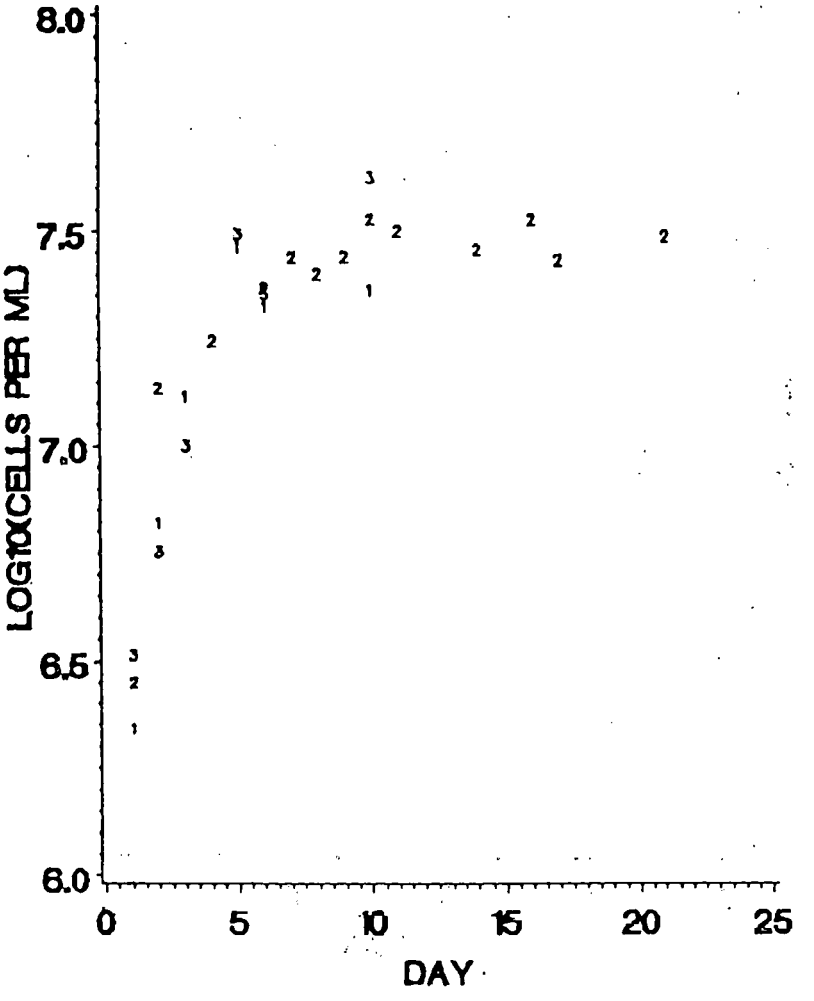


Figure 1. Change in cell count in three different cultures of Isochrysis aff. galbana over time. Cultures were grown in Roux bottles under standard conditions (see text). 1, 2, and 3 designate the three different cultures.

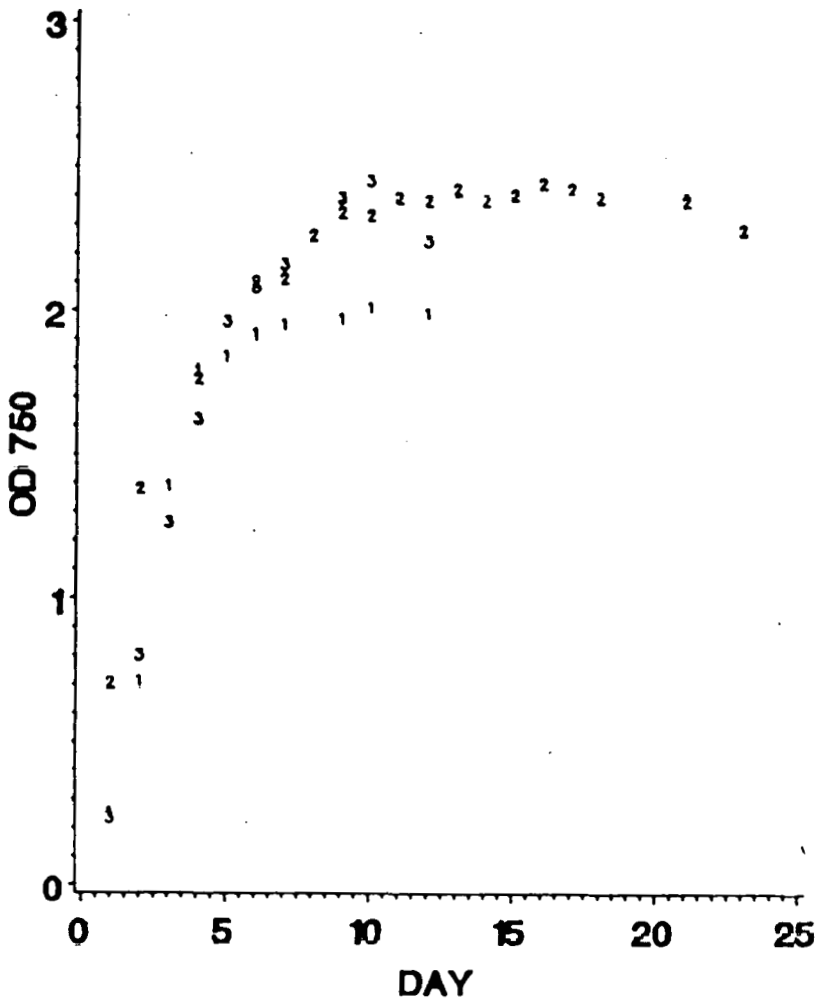


Figure 2. Change in optical density (750 nm) in three different cultures of Isochrysis aff. galbana over time. Cultures were grown in Roux bottles under standard conditions (see text). 1, 2, and 3 designate the three different cultures.

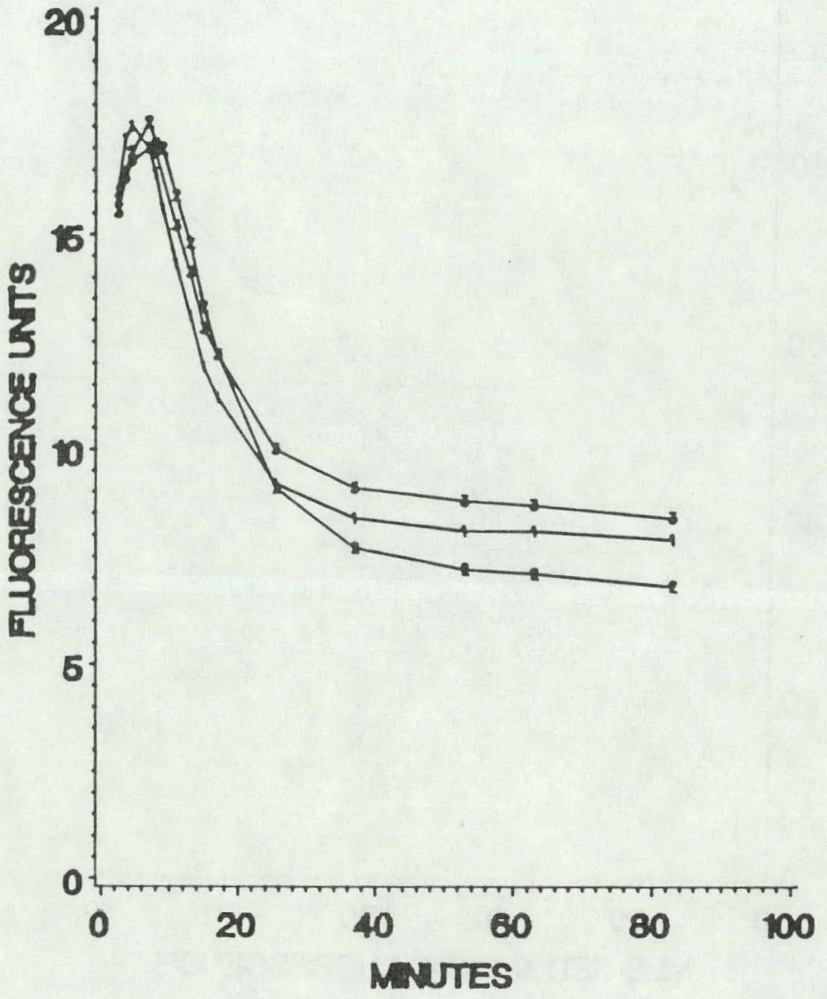


Figure 3. Change in acetone-dissolved nile red over time. Three aliquots of an Isochrysis aff. galbana culture were stained with 1 µg/ml nile red dissolved in acetone and the fluorescence was measured periodically with a fluorometer. The fluorescence levels stabilize after 45 min.

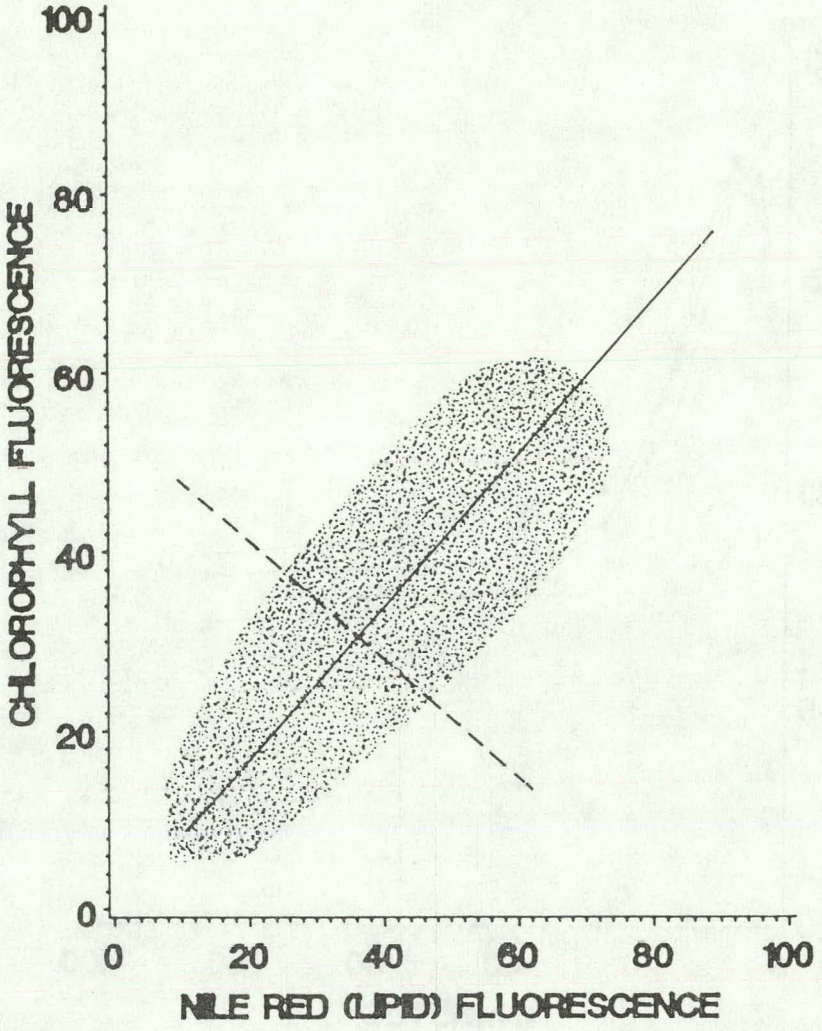


Figure 4. Scattergram showing the pattern of chlorophyll and lipid content in a typical cell population (stippled area). The solid line represents the age/size axis and the dotted line orthogonal to it represents the axis of the chlorophyll-to lipid ratio.

ENVIRONMENTAL EFFECTS ON PHOTORESPIRATION IN THE DIATOM CHAETOCEROS MUELLERI

Ronald R. H. Cohen
Assistant Professor
Colorado School of Mines
Dept. of Environmental Sciences and Engineering
Golden, Colorado 80401

ABSTRACT

Conditions of high solar irradiance, pH, dissolved oxygen (D.O.), and salinity and low CO_2 anticipated during the growth of algae as a biofuel source may induce photorespiratory losses in phytoplankton. Algae have been screened for high specific growth rates under these conditions, with net production the primary concern. However, modifying conditions conducive to respiratory losses may result in even higher net production.

Experiments were performed to identify photorespiration in Chaetoceros muelleri, an algae that has very high growth rates at high salinities and high pH, and to examine the effects of pH, salinity, temperature, light and D.O. on the magnitude of photorespiration. Respiration and photorespiration were determined by simultaneously measuring apparent photosynthesis (pH drift) and true photosynthesis (^{14}C 6 minute incubations), by dark D.O. transients and by DCMU metabolic poison.

Photorespiration increased dramatically with pH and D.O. as expected. Photorespiration was 50 percent higher at 70,000 umhos/cm than at 23,000 umhos/cm conductivity. Photorespiration was highest at highest light intensity (300 $uE/m^2/s$) but photosynthesis increased faster than photorespiration at increasing light intensity. Thus, net production was still highest at highest light. Chaetoceros muelleri photorespiration did not vary significantly from 20 to 34 °C. DCMU poisoned samples yielded values close to dark respiration measurements, suggesting that DCMU inhibits both photosynthesis and photorespiration but not dark respiration in the light.

THIS PAGE

WAS INTENTIONALLY

LEFT BLANK

Lipid Biochemistry

THIS PAGE

WAS INTENTIONALLY

LEFT BLANK

THE EFFECTS OF SILICON DEFICIENCY ON SEVERAL ENZYMES INVOLVED IN LIPID AND CARBOHYDRATE BIOSYNTHESIS IN CYCLOTELLA CRYPTICA

Paul G. Roessler
Solar Energy Research Institute
1617 Cole Blvd., Golden, CO 80401

ABSTRACT

Previous studies with the diatom Cyclotella cryptica have indicated that silicon deficiency induces an increase in the fraction of newly assimilated carbon that is partitioned into lipids and a decrease in the fraction that is partitioned into storage carbohydrate (chrysolaminarin). This report summarizes the results of research that was designed to increase our knowledge concerning the enzymatic basis for this change in carbon partitioning.

Initial studies focused on the enzymology of chrysolaminarin biosynthesis. In vitro experiments provided evidence that chrysolaminarin is synthesized via the action of two enzymes: (1) UDPglucose pyrophosphorylase, and (2) chrysolaminarin synthase (UDPglucose:β-(1$\rightarrow$3)-glucan β-3-glucosyl transferase). The specific activity of UDPglucose pyrophosphorylase in extracts of C. cryptica cells was not affected after four hours of silicon deficiency, but the activity of chrysolaminarin synthase decreased by 31% during this time period.

The enzymology of lipid synthesis in C. cryptica was also investigated. Acetyl-CoA hydrolase, acetyl-CoA synthetase, and acetyl-CoA carboxylase were present in cell-free extracts, but citrate lyase activity was not detected. The specific activity of acetyl-CoA carboxylase (which is believed to be the rate-limiting enzyme of fatty acid biosynthesis) increased approximately two-fold after four hours of silicon-deficiency, but this induction could be blocked by cycloheximide (an inhibitor of protein synthesis) and actinomycin D (an inhibitor of gene transcription). The results of these enzymatic analyses suggest that the increased allocation of newly assimilated carbon into lipids in silicon-deficient C. cryptica cells is probably due in part to a reduction in the activity of chrysolaminarin synthase along with a concomitant increase in the activity of acetyl-CoA carboxylase.

THE EFFECTS OF SILICON DEFICIENCY ON SEVERAL ENZYMES INVOLVED IN LIPID AND CARBOHYDRATE BIOSYNTHESIS IN CYCLOTELLA CRYPTICA

INTRODUCTION

Very little is known concerning the biochemistry of lipid synthesis in microalgae, and yet this information will be of critical importance before algal cells can be manipulated via genetic engineering or other biochemical techniques for the purpose of improving cellular lipid production capabilities. Therefore, studies have been carried out in this laboratory for the past two years in an effort to increase our knowledge concerning the enzymology and regulation of microalgal lipid synthesis. Results that were presented last year (Roessler 1986) for the diatom Cyclotella cryptica T13L indicated that silicon deficiency induces an increase in the percentage of newly photoassimilated carbon that is partitioned into lipids and a decrease in the percentage of carbon that is partitioned into storage carbohydrate (chrysolaminarin). This report summarizes the results of research carried out during the past year that was designed to help elucidate the biochemical mechanisms that are responsible for this altered carbon partitioning pattern.

There are several mechanisms that could lead to increased allocation of newly assimilated carbon into lipids. One possible mechanism involves an increase in the specific activity of one or more regulatory enzymes that are directly involved in lipid biosynthesis. Unfortunately, little is known about the enzymology of lipid synthesis in diatoms. It is assumed that acetyl-CoA is the immediate carbon precursor used for fatty acid biosynthesis in diatoms, as is the case with most organisms studied. The source of the acetyl-CoA used for lipid synthesis varies for different organisms, however, and the pathway by which acetyl-CoA is produced for lipid synthesis in diatoms is not known. In mammalian cells, the acetyl-CoA used for cytosolic fatty acid synthesis is produced from citrate via the activity of the enzyme ATP-citrate lyase. In spinach, it has been suggested that the acetyl-CoA used for chloroplastic fatty acid biosynthesis is derived from the acetyl-CoA produced by mitochondrial pyruvate dehydrogenase (Liedvogel and Stumpf 1982). Acetyl-CoA cannot penetrate the mitochondrial or chloroplast membranes, however, thus requiring the presence of acetyl-CoA hydrolase in the mitochondria in order to cleave acetyl-CoA to acetate and free coenzyme A. The acetate can then pass out of the mitochondria and enter the chloroplast, where the enzyme acetyl-CoA synthetase catalyzes the reformation of acetyl-CoA, which is then available for fatty acid biosynthesis. A less complicated pathway appears to operate in pea chloroplasts and seed leucoplasts, where the presence of plastidial pyruvate dehydrogenase allows the direct conversion of pyruvate to acetyl-CoA. Regardless of the means by which acetyl-CoA is produced, the remaining steps involved in fatty acid biosynthesis are generally quite similar in most organisms. The first enzyme of the pathway, acetyl-CoA carboxylase, catalyzes the formation of malonyl-CoA, which is usually the rate-limiting step of the pathway. Malonyl-CoA is the primary carbon substrate for the fatty acid synthase complex, which catalyzes the formation of long chain fatty acids. These enzyme systems have never been investigated in diatoms.

It should be readily apparent that very little is known concerning the enzymology of lipid synthesis in diatoms. The experiments described in this paper were therefore designed to provide some information about the various possible pathways by which photoassimilated carbon could be directed into the acetyl-CoA used for fatty acid biosynthesis in C. cryptica. In addition, since acetyl-CoA carboxylase is believed to be the rate-limiting enzyme for fatty acid biosynthesis in most organisms, a preliminary characterization of this enzyme was undertaken so that optimal assay conditions could be identified. Finally, the specific activities of various lipid biosynthetic enzymes were measured in extracts from silicon-replete and silicon-deficient cells in order to help elucidate the biochemical mechanisms that are responsible for the induction of lipid accumulation in silicon-deficient diatoms.

As mentioned earlier, an increase in the specific activity of one or more lipid biosynthetic enzymes is not the only means by which the rate of lipid synthesis could increase in silicon-deficient diatoms. If the synthesis of lipids in silicon-replete cells is limited by the availability of carbon substrates, increased partitioning of carbon into lipids would occur in response to a reduction in the activities of pathways that compete for the carbon compounds that are used in lipid synthesis. The primary means by which more carbon could become available for the synthesis of fatty acids are (1) decreased utilization of photoassimilated carbon for carbohydrate and protein production, and (2) decreased carbon flux through the tricarboxylic acid (TCA) cycle.

A reduction in the amount of photoassimilated carbon that is utilized for chrysolaminarin synthesis would likely involve a reduction in the specific activity of one or more of the enzymes involved in the synthesis of this carbohydrate. The enzymes responsible for chrysolaminarin synthesis have not previously been identified, however, and therefore it was first necessary to delineate this pathway. The presence of UDPglucose pyrophosphorylase activity in C. cryptica was reported last year, along with several properties of the enzyme (Roessler 1986). In this report, an enzyme has been identified that transfers glucosyl units from UDPglucose to the growing β-(1$\rightarrow$3)-linked chysolaminarin molecule. The effects of silicon deficiency on the activities of these two enzymes are also reported.

Decreased carbon flux through the TCA cycle would result from inhibition of the activity of one or more TCA cycle enzymes, thereby decreasing the rate of acetyl-CoA oxidation or incorporation into other cell constituents that require TCA cycle intermediates for synthesis (e.g., aspartate and glutamate). Reduced activity of citrate synthase would decrease the rate of incorporation of acetyl-CoA into TCA cycle intermediates and therefore increase the rate of acetate export from the mitochondria (if acetyl-CoA hydrolase is present). The acetate could then enter the chloroplast and be incorporated into fatty acids. Alternatively, a decrease in the activity of other TCA cycle enzymes could lead to a buildup of citrate. If citrate lyase is present in the cytosol, the citrate could then leave the mitochondria and be cleaved to form oxaloacetate and acetate. Experiments were therefore carried out in order to investigate these possibilities.

MATERIALS AND METHODS

Growth Conditions

Cyclotella cryptica T13L, obtained from the Culture Collection of Marine Phytoplankton (Bigelow Laboratory, W. Boothbay Harbor, ME), was used for all experiments. The cells were cultured in 2 L polycarbonate bottles in the medium described by Werner (1966) which was modified to contain the micronutrients of Bold's Basal Medium (Bold and Wynne 1978). Media were supplemented with biotin (2 μg/L), thiamine (1 mg/L), and cyanocobalamin (1 μg/L). Cultures were bubbled with 1% CO_2 in air (flow rate = 500 ml/min) and maintained at 25°C under constant illumination from fluorescent lamps (photon flux density at the vessel surface averaged over 360° = 85 μmol quanta$\cdot m^{-2}\cdot s^{-1}$). Stock cultures of C. cryptica were maintained on f/2 medium (McLachlan 1973) containing 30 g/L artificial sea salts (Rila Marine Products, Teaneck NJ) in place of seawater. Cultures were kept at 15°C on a 12 h light:12 h dark photoperiod.

Analytical Methods

Protein was quantified by means of the Coomassie Blue protein-binding assay developed by Bradford (1976). Radioactivity was determined by liquid scintillation counting in a Beckman Model LS9000 scintillation counter. Biofluor scintillation cocktail (New England Nuclear) was used for all isotope experiments. Spectrophotometric measurements were made with a Hewlett-Packard Model 8451 diode array spectrophotometer that was programmed so that derivatives of time-based measurements could be calculated.

Preparation of Cell-Free Extracts

Cells were suspended in various buffers (as indicated below) and disrupted by passage through a French pressure cell at 15000 psi. For all assays except the chrysolaminarin synthase assay, the extracts were then centrifuged at 37000xg for 20 min, and the supernatants were used for measurement of enzymatic activity. The enzyme assays described below were linear with respect to both time and total protein addition when the protein concentrations of the extracts were maintained below the levels stated below for the various assays.

Enzyme Assays

UDPglucose Pyrophosphorylase Assay. Assay of UDPglucose pyrophosphorylase activity was performed as described previously (Roessler 1986).

Chrysolaminarin Synthase Assay. Cells were suspended in 50 mM Tricine (pH 7.8) containing 2 mM dithiothreitol and disrupted by passage through a French pressure cell (15000 psi). A portion of the non-centrifuged extract (containing less than 0.065 mg protein) was then added to a microfuge tube containing 100 mM Tricine buffer (pH 7.8), 0.2 mM UDP-[U-^{14}C]glucose (51.8 MBq/mmol), 2.5 mM laminaribiose, and 2 mM $MgCl_2$ (final volume = 0.2 ml). After a 15 min incubation at 30°C, 0.2 mg of laminarin (Sigma) in 0.1 ml were added to each tube, immediately followed by 0.7 ml of absolute ethanol.

After 30 min, the tubes were centrifuged at 14000xg for 5 min, and the supernatant was discarded. The pellet was then washed with one ml of 70% ethanol and centrifuged as before. The pellet was dissolved in one ml of deionized H_2O and 0.95 ml of this solution was added to 10 ml of scintillation cocktail and counted.

Citrate Lyase Assay. This assay measures the citrate-dependent rate of oxaloacetate (OAA) formation in a coupled reaction in which the OAA is converted to malate via malate dehydrogenase in the presence of NADH. The rate of NADH oxidation that occurs as a result of malate formation is determined spectrophotometrically by following the change in absorbance at 340 nm. The reaction mixture (one ml in a one cm cuvette) contained 50 mM Hepes buffer (pH 7.8), 0.1 mM NADH, 0.2 units of porcine heart malate dehydrogenase (Sigma), 1 mM $MgCl_2$, and 0.05 ml of extract in 50 mM Hepes buffer (pH 7.8) containing 2 mM dithiothreitol (DTT). ATP (1 mM) was included in the reaction mixture for some experiments. The cuvette was placed in a thermostatted cuvette holder (25°C) and the change in absorbance at 340 nm was monitored for 3 min in order to determine the citrate-independent rate of NADH oxidation. Citrate (1 mM) was then added to the cuvette to measure citrate lyase activity, which was subsequently corrected for citrate-independent activity. A molar extinction coefficient of 6.22×10^3 was used for NADH. This is a modification of the procedure Takeda et al. (1969).

Acetyl-CoA Hydrolase Assay. The reaction mixture contained (in a 1.5 ml microfuge tube) 50 mM Tricine buffer (pH 7.8), 0.5 mM $MgCl_2$, 0.4 mM [^{14}C]acetyl-CoA (specific activity = 74 MBq/mmol), and 0.02 ml of cell-free extract in 50 mM Tricine (pH 7.8) in a final volume of 0.1 ml. After a 30 min incubation at 30°C, the reaction was stopped by the addition of 0.01 ml of glacial acetic acid. A 0.05 ml portion of the acidified solution was transferred to a separate microfuge tube, followed by the addition of 0.05 ml of 0.01 N HCl and 0.02 ml of a charcoal suspension (15% w/v activated charcoal (Darco G-60) with 5% v/v Percoll in 0.01 N HCl). This mixture was shaken for 30 min on a rotary shaker (200 rpm) and then centrifuged for 5 min at 14000xg. A sample of the supernatant solution (0.04 ml) was transferred to a vial containing 10 ml of scintillation cocktail and counted immediately. Another 0.04 ml portion of the supernatant was transferred to a separate tube, mixed with 0.01 ml of glacial acetic acid, and evaporated under a stream of N_2 (minimum 45 min). The residue in the tube was dissolved in one ml of deionized water and transferred to a vial containing 10 ml of scintillation cocktail and counted. The difference in the radioactivity of these two different treatments represents the amount of acetate produced from the [^{14}C]acetyl-CoA. This assay is a based on the method of Liedvogel and Stumpf (1982).

Acetyl-CoA Synthetase Assay. The procedure described in this section is a modification of the method described by Liedvogel (1985). In this assay, the acetyl-CoA formed during the reaction acetylates DTT, which is then extracted from the reaction mixture with chloroform. The reaction mixture (0.5 ml) contained 100 mM Tricine buffer (pH 8.5), 2 mM $MgCl_2$, 2 mM ATP, 20 mM DTT, 0.42 mM [^{14}C]acetate (specific activity = 89 MBq/ mmol) and 0.03 ml of cell-free extract (in 50 mM Tricine buffer (pH 8.5) with 2 mM DTT) containing less than 0.6 mg of total protein per ml. The reaction was initiated by the addition of the extract and allowed to proceed for 15 min at

30°C. Chloroform (1.5 ml) and 0.5 ml of 1% NaCl were added to stop the reaction. The tube was vortexed and then centrifuged for 5 min at 2000xg. The chloroform phase was transferred to a scintillation vial, and the aqueous phase was washed with an additional 2 ml of chloroform, followed by mixing and centrifugation as before. The chloroform phase from the washing step was added to the scintillation vial and the chloroform was evaporated to dryness at 50°C under a stream of N_2. Scintillation cocktail was then added to the vial and the radioactivity determined. Control assays lacking coenzyme A were also performed in order to correct for non-specific radioactivity. Furthermore, an additional correction factor was required since complete extraction of acetyl-DTT is not achieved. This correction factor was empirically derived by determining the efficiency of extraction of known quantities of [^{14}C]acetyl-CoA standards, which was calculated to be 65%.

Citrate Synthase Assay. This spectrophotometric assay is based on the formation of a light-absorbing mercaptide ion due to the reaction between free coenzyme A (produced during the reaction) and 5,5'-dithiobis-2-nitrobenzoic acid) (DTNB). The reaction mixture (one ml final volume in a one cm cuvette) contained 50 mM Tricine buffer (pH 7.8), 0.1 mM DTNB, 0.05 mM acetyl-CoA, 0.5 mM $MgCl_2$, and 0.05 ml of enzyme extract (containing less than 1.05 mg total protein per ml) in 50 mM Tricine buffer (pH 7.8). The cuvette was placed in a thermostatted cuvette holder (25°C) and the change in absorbance at 412 nm was monitored for 2 min in order to measure OAA-independent coenzyme A release. OAA (1 mM) was then added to the cuvette to measure citrate synthase activity. Activity was corrected for the OAA-independent rate of coenzyme A release. A molar extinction coefficient for the DTNB complex of 1.36×10^4 was used for specific activity calculations. This procedure is a modification of the one reported by Shepherd and Garland (1969).

Acetyl-CoA Carboxylase Assay. Enzymatic activity was assayed by a modification of the procedure described by Sauer and Heise (1984), in which [^{14}C]bicarbonate is incorporated into malonyl-CoA. The reaction mixture (final volume = one ml) contained 100 mM Tricine buffer (pH 8.5), 0.5 mM acetyl-CoA, 1 mM ATP, 2 mM $MgCl_2$, 30 mM KCl and [^{14}C]$NaHCO_3$ (final concentration = 10 mM at a specific activity of 11.1 MBq/mmol). The reaction was initiated by the addition of 0.02 ml of cell-free extract (containing less than 1.1 mg total protein per ml), which was prepared in 50 mM Tricine (pH 8.5) containing 105 mM $NaHCO_3$ and 2 mM DTT. After a 10 min incubation at 30°C, the reaction was terminated by the addition of 0.3 ml of 2 N HCl. After thorough mixing, 0.5 ml of the acidified solution was transferred to a scintillation vial and heated in an oven at 70°C until dry. The residue was dissolved in 0.3 ml of 2 N HCl, and then 10 ml of scintillation cocktail was added prior to counting. Control assays lacking acetyl-CoA were carried out in order to correct for non-specific radioactivity.

Silicon Deficiency Experiments

In order to determine the effects of silicon deficiency on the *in vivo* rate of [^{14}C]acetate incorporation into lipids, an exponential-phase (<2 mg Chl *a* per liter), silicon-replete culture was harvested under sterile conditions and resuspended into both a silicon-free and a silicon-replete medium. The cultures were then returned to normal growth conditions. After 4 h, 45 ml portions of the cultures were placed into separate 25x150 mm test

tubes and positioned in front of fluorescent bulbs (average photon flux density = 80-90 μmol quanta$\cdot m^{-2}\cdot s^{-1}$). The cultures were continuously mixed by gentle bubbling with air. Following a 15 min equilibration period, 0.025 mM [^{14}C]acetate (specific activity = 89 MBq/mmol) was added to each tube. After 1 h, duplicate 10 ml portions of the cultures were collected on 2.5 cm glass fiber filters (Whatman GF/C) and rinsed with 15 ml of unlabeled medium. The filters were then placed in separate 16x100 mm teflon-capped culture tubes containing 3 ml of methanol:chloroform:water (2:1:0.8). The tubes were kept at -20°C for 48 h, and then heated at 60°C for 30 min. The solvent was then transferred to a separate tube and the filter was extracted with an additional 3 ml of methanol:chloroform (1:1) at 60°C for 30 min. The solvents were combined and 0.8 ml of chloroform and 2.2 ml of water were added to allow phase separation. The chloroform layer was washed with 2.2 ml of water and then transferred to a scintillation vial. After evaporation of the chloroform, 10 ml of scintillation cocktail were added prior to counting.

To determine the effects of silicon deficiency on enzymatic activities, an exponential-phase (<2 mg Chl *a* per liter), silicon-replete culture was harvested under sterile conditions and resuspended into both a silicon-free and a silicon-replete medium. The cultures were then returned to normal growth conditions. After 4 h, the cultures were harvested, cell-free extracts were made, and enzyme activities were measured as described above.

RESULTS AND DISCUSSION

Enzymes Involved in Carbohydrate Synthesis

In last year's report, the presence of UDPglucose pyrophosphorylase in *C. cryptica* was reported, along with the suggestion that this enzyme might be involved in storage carbohydrate (chrysolaminarin) synthesis. Additional information regarding this biosynthetic pathway is now provided. In order to determine whether UDPglucose is utilized in the biosynthesis of chrysolaminarin, cell-free extracts of *C. cryptica* were incubated with UDP-[^{14}C]glucose, and the reaction mixture was then passed through a Sephadex G-50 column. The elution profile (Fig. 1) indicated the presence of a ^{14}C-labeled glucan with a median molecular weight of approximately 4600 (based on column calibration with maltoheptaose and Sephadex T-10 dextran), corresponding to a glucan containing approximately 27 glucosyl units. Beattie et al. (1961) reported that the chrysolaminarin extracted from a mixed population of diatoms had an average degree of polymerization of 21 (molecular weight = 3500), while a molecular weight of 6200 was reported for the glucan isolated from *Skeletonema costatum* (Smestad-Paulsen and Myklestad 1978). To investigate whether β-(1→3) linkages were present in the ^{14}C-labeled glucan formed in *C. cryptica* extracts, laminarinase (a β-(1→3)-glucanohydrolase) from *Penicillium* was added to a solution containing the labeled glucan. This treatment resulted in the hydrolysis of the glucan into units that were too small to be resolved by the column employed (i.e., less than five glucose units) (see Fig. 1).

Additional characterization of the glucan formed from UDP-[^{14}C]glucose in cell-free extracts involved partial hydrolysis with 12 N HCl. Paper

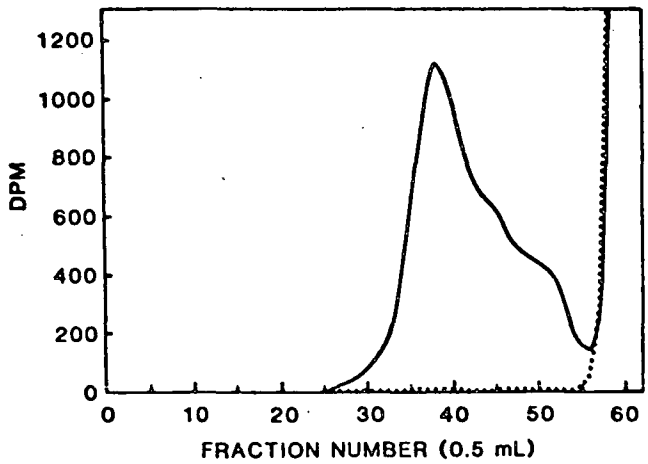


Figure 1. Elution profile of the glucan formed from UDP-[^{14}C]glucose in cell-free extracts of Cyclotella cryptica. Following a 30 min incubation with UDP-[^{14}C]glucose, reaction mixtures were diluted with 100 mM Na-acetate (pH 5) and incubated in the presence (•••••) or absence (——) of Penicillium laminarinase prior to Sephadex G-50 gel filtration chromatography.

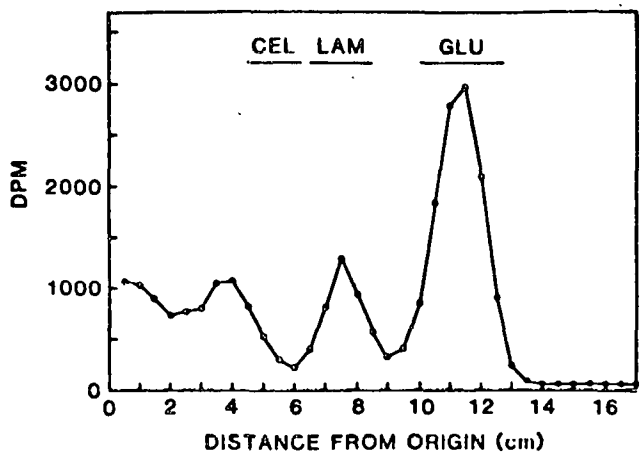


Figure 2. Paper chromatography of the glucan formed from UDP-[^{14}C]glucose in cell-free extracts of Cyclotella cryptica following partial acid hydrolysis. The conditions used during the formation, hydrolysis, and chromatography of the [^{14}C]glucan are described in Materials and Methods. The migration distances of cellobiose (CEL), laminaribiose (LAM), and glucose (GLU) standards are shown at the top of the figure.

Table I. Activities of various enzymes in silicon-replete and silicon-deficient Cyclotella cryptica cells.

Enzyme	Specific activity ± S.E. (U/mg protein)		Activity ratio
	Si-deficient cells	Si-replete cells	deficient: replete
UDPglucose pyrophosphorylase	25.1 ± 1.79	25.8 ± 1.71	0.99
Chrysolaminarin synthase	0.033 ± 0.001	0.048 ± 0.001	0.69
Acetyl-CoA synthetase	0.822 ± 0.077	0.870 ± 0.102	0.94
Acetyl-CoA carboxylase	3.98 ± 0.43	2.13 ± 0.20	1.87
Citrate synthase	6.69 ± 0.65	6.58 ± 0.68	1.02

Enzyme activities were measured 4 h after transfer into a silicon-free or a silicon-replete medium. The results shown are the average of three separate experiments. One unit of activity represents one micromole of product formed per hour.

chromatography of the hydrolysate revealed the presence of glucose and laminaribiose, the β-(1→3)-linked dimer of glucose (Fig. 2). Cellobiose (the β-(1→4)-linked dimer of glucose) did not appear to be a major hydrolysis product. This finding provides additional evidence that the glucan formed from UDPglucose was chrysolaminarin.

The enzyme catalyzing chrysolaminarin biosynthesis therefore appears to be a UDPglucose:β-(1→3)-glucan β-3-glucosyl transferase enzyme (hereafter referred to as "chrysolaminarin synthase"). This enzyme was assayed by measuring the rate of UDP-[^{14}C]glucose incorporation into 70% ethanol-insoluble material. Assays were carried out with non-centrifuged extracts, since a large portion (70%) of the activity was pelleted by centrifugation at 37000xg for 15 min. The activity of this enzyme was inhibited by 51% when 1 mM EGTA was included in the reaction mixture to chelate endogenous divalent metal cations. Activity was restored by the addition of Mg^{2+} ions (1 mM in excess of EGTA), but Ca^{2+} ions did not stimulate enzymatic activity. Although the glucan synthase involved in callose synthesis is stimulated by Mg^{2+} and Ca^{2+} ions (Kauss et al. 1983, Morrow and Lucas 1986), glucan synthases in general have not been shown to have a divalent metal cation requirement. In some plants, however, monovalent cations have been reported to stimulate starch synthase activity (Hawker et al. 1974). Chrysolaminarin synthase activity was shown to be maximal at pH 7.8.

Since previous experiments (Werner 1966, Coombs et al. 1967, Roessler 1986) indicated that chrysolaminarin biosynthesis was reduced in silicon-deficient diatoms, experiments were carried out in order to determine whether silicon deficiency affects the specific activities of UDPglucose pyrophosphorylase and chrysolaminarin synthase in C. cryptica. As shown in Table I, there was very little difference in the specific activities of UDPglucose pyrophosphorylase from silicon-replete and silicon-deficient cells, indicating that the concentration of active UDPglucose pyrophosphorylase was the same in both silicon-replete and silicon-deficient cells.

In contrast to the results observed with UDPglucose pyrophosphorylase, the specific activity of chrysolaminarin synthase in extracts from silicon-deficient cells was 31% lower than the activity expressed in extracts from silicon-replete cells (Table I). There are several possibilities that could account for these results. One such possibility is that there could be a decrease in the net rate of enzyme synthesis; this could result from either (1) reduced enzyme synthesis with normal rates of protein turnover, or (2) increased enzyme destruction while the rate of enzyme synthesis remains constant. It is also possible that silicon deficiency may induce phosphorylation (or another type of covalent modification) of chrysolaminarin synthase, thereby decreasing the catalytic activity. There may be additional mechanisms by which the activity of chrysolaminarin synthase is regulated in vivo, but the lowered concentration of active enzyme in silicon-deficient cells could certainly contribute to the observed decrease in the rate of chrysolaminarin synthesis.

Enzymes Involved in Lipid Synthesis

The pathway by which photoassimilated CO_2 is converted to the acetyl-CoA used in diatom fatty acid biosynthesis is unknown. Therefore, an attempt was

made to determine whether certain enzymes that are required for fatty acid biosynthesis in higher plants and animals are also present in C. cryptica. Assays for those enzymes were then optimized so that the effects of silicon deficiency on the specific activities of the enzymes could be determined.

In order for citrate to be used for fatty acid biosynthesis (as is the case for animals), either citrate lyase or ATP-citrate lyase activity must be present within the cells. The products of the citrate lyase reaction are acetate and oxaloacetate (OAA), while ATP-citrate lyase produces OAA and acetyl-CoA. To determine whether either of these enzymes were present in cell-free extracts of C. cryptica, experiments were carried out which measured the rate of OAA production (coupled via malate dehydrogenase to NADH oxidation) in a spectrophotometric assay. Neither silicon-replete nor silicon-deficient cells exhibited appreciable rates of citrate-dependent OAA formation, however, either in the presence or absence of ATP (data not shown). This suggests that citrate lyase enzymes are either absent in C. cryptica, or that enzymatic activity is below the limits of detection.

In order for mitochondrially-produced acetyl-CoA to serve as a lipid precursor (as in spinach leaf cells), both acetyl-CoA hydrolase and acetyl-CoA synthetase must be present in the cells. Acetyl-CoA hydrolase activity was shown to be present in extracts of both silicon-replete and silicon-deficient C. cryptica cells, but the activity of this enzyme was highly variable, and despite repeated efforts, assay conditions that led to reliable determinations of enzyme specific activity were not found. The highest specific activity for acetyl-CoA hydrolase that was observed during the course of these experiments was 0.89 μmoles of acetate formed per mg protein per hour. However, the activity of other preparations was as low as 0.04 μmoles acetate formed per mg protein per hour. Despite the fact that a reliable protocol for the measurement of activity was not discovered, these experiments provide evidence that acetyl-CoA hydrolase activity is present in C. cryptica cells.

The other enzyme required in order for mitochondrially-produced acetyl-CoA to be used for chloroplastic fatty acid synthesis is acetyl-CoA synthetase. This enzyme catalyzes the synthesis of acetyl-CoA from acetate and coenzyme A in an ATP-dependent reaction. Acetyl-CoA synthetase activity was consistently observed in cell-free extracts of C. cryptica. The enzyme was active over a wide pH range; maximal activity was expressed at pH 8.6, but the activity was greater than 70% of the maximum between pH 7.8 and 8.8. Similar to the enzyme from spinach chloroplasts (Saure and Heise 1984), the activity of acetyl-CoA synthetase was dependent on both ATP and Mg^{2+} (Table II). The inclusion of 0.05 mM KCl did not alter enzymatic activity.

Since both acetyl-CoA hydrolase and acetyl-CoA synthetase appear to be present in C. cryptica cells, it is possible that mitochondrially-produced acetyl-CoA could be used as a precursor for lipid synthesis. Before this pathway can be confirmed, however, it will be necessary to determine the cellular location of these enzymes. Further characterization of acetyl-CoA hydrolase will only be possible after the difficulties that were encountered in assaying the activity of this enzyme are overcome.

If acetyl-CoA hydrolase and acetyl-CoA synthetase are compartmentalized such that mitochondrially-produced acetyl-CoA can in fact be used for lipid

Table II. Effects of missing assay components on the activities of acetyl-CoA synthetase and acetyl-CoA carboxylase.

Reaction mixture composition	Relative activity (%) Acetyl-CoA synthetase	Acetyl-CoA carboxylase
Complete	100	100
-ATP	6.9	1.5
-$MgCl_2$	3.2	0
-KCl	101	56.3

The complete reaction mixtures included the following components: (1) Acetyl-CoA synthetase: 100 mM Tricine (pH 8.5), 2 mM $MgCl_2$, 2 mM ATP, 20 mM DTT, 0.42 mM acetate, and 0.05 mM KCl. (2) Acetyl-CoA carboxylase: 100 mM Tricine (pH 8.5), 0.5 mM acetyl-CoA, 1 mM ATP, 2 mM $MgCl_2$, 30 mM KCl, and 10 mM $NaHCO_3$.

Table III. Effects of protein synthesis inhibitors and gene transcription inhibitors on acetyl-CoA carboxylase activity in silicon-deficient Cyclotella cryptica cells.

Additions	+Si/-Si	Relative Activity (±S.E.)
none	+Si	1.00
none	-Si	2.12 ± 0.209
20 μg/ml cycloheximide	-Si	0.94 ± 0.014
0.5 mg/ml chloramphenicol	-Si	1.26 ± 0.106
10 μg/ml actinomycin D	-Si	1.26 ± 0.057
20 μg/ml rifampicin	-Si	2.03 ± 0.088

Enzyme activities were measured 4 h after transfer into a silicon-free or a silicon-replete medium containing the specified additions. The results shown are the average of two separate measurements.

synthesis, then the activity of citrate synthase (which also uses acetyl-CoA as a substrate) could have large effects on the availability of acetyl-CoA for lipid synthesis. A standard assay for citrate synthase involves the measurement of coenzyme A release, which is quantified spectrophotometrically by the use of DTNB. Citrate synthase from C. cryptica was shown to have maximal activity at pH 7.8. $MgCl_2$ (0.5 mM) was included in the reaction mixture since enzymatic activity was reduced by approximately 15% in its absence.

The last enzyme to be examined in this study was acetyl-CoA carboxylase, which catalyzes the first committed step in lipid biosynthesis in all organisms. The activity of this enzyme was assayed by incubating cell-free extracts with [^{14}C]bicarbonate and acetyl-CoA and measuring the rate of incorporation of ^{14}C into malonyl-CoA, the acid stable product of the reaction. Acetyl-CoA carboxylase activity in cell-free extracts of C. cryptica had a broad pH optimum, with a maximum at pH 8.5. Enzymatic activity was greater than 85% of the maximum between pH 7.9 and 8.8. As is the case with the enzyme from wheat germ (Nielsen et al. 1979), maize leaf cells (Nikolau and Hawke 1984), and spinach chloroplasts (Sauer and Heise 1984), the presence of ATP, Mg^{2+}, and K^+ is required for full activity (Table II). Mg^{2+} appears to have two roles in the activation of acetyl-CoA carboxylase. The primary requirement is for complex formation with ATP, since MgATP has been shown to be the actual substrate for the reaction rather than free ATP (Nielsen et al. 1979, Mohan and Kekwick 1980, Sauer and Heise 1984). Free Mg^{2+} in excess of MgATP also activates the enzyme from spinach chloroplasts by lowering the K_m for MgATP (Sauer and Heise 1984).

Similar to acetyl-CoA carboxylase from spinach chloroplasts, inclusion of bicarbonate and dithiothreitol in the extraction buffer enhanced the activity of the enzyme from C. cryptica (by nearly 60%). In spinach, the bicarbonate overcomes the action of an endogenous inhibitor of the transcarboxylase portion of the enzyme (Kannangara and Stumpf 1972); further experiments must be carried out to determine whether a similar inhibitor is present in extracts of C. cryptica cells.

The next phase of the research examined the effects of silicon deficiency on the activities of those enzymes described above that could be reliably assayed. The first experiment carried out utilized [^{14}C]acetate as a tracer to measure the *in vivo* activity of lipid synthesis enzymes in whole C. cryptica cells. Cells that were limited for silicon for 4 h incorporated 155% more exogenously supplied acetate into lipids than silicon-replete cells. There are several possible explanations for this effect. One possible reason for the increased incorporation is that there could be an increase in the activity of acetyl CoA synthetase (especially if this step was rate-limiting), leading to more acetyl-CoA for fatty acid biosynthesis. An increase in the activity of acetyl-CoA carboxylase or the FAS system could also promote acetate incorporation into fatty acids by increasing the rate of acetyl-CoA utilization. There are other possible explanations for the observed phenomenon, however, that do not involve changes in the specific activities of enzymes involved in lipid synthesis. For example, an increase in the intracellular specific radioactivity of acetate (either through reduced levels of endogenous unlabeled acetate or enhanced rates of [^{14}C]acetate uptake) could result in increased apparent rates of ^{14}C incorporation into lipids.

As discussed above, it seemed possible that the activity of acetyl-CoA synthetase might be enhanced in silicon-deficient cells. Therefore, this enzyme was assayed in extracts made from silicon-replete cells and from cells that were silicon-starved for 4 h. There was essentially no difference in the specific enzymatic activities of the two extracts, however, suggesting that there was neither a covalent modification of the enzyme nor increased levels of the enzyme (see Table I). These results do not preclude the possibility that allosteric modulation of the enzyme *in vivo* may take place, however. The specific activity of citrate synthase was also unaffected after 4 h of silicon deficiency.

In contrast to the results obtained with acetyl-CoA synthetase and citrate synthase, there was a substantial increase in the specific activity of acetyl-CoA carboxylase in response to silicon deficiency. The specific activity of the enzyme from cells which were silicon-limited for 4 h was 87% higher than the value obtained with silicon-replete cells (Table I). This enhanced activity could be the result of several different processes, including (1) an increased rate of enzyme synthesis, (2) a reduced rate of enzyme degradation, or (3) a covalent modification of the enzyme that results in higher enzymatic activity (e.g., dephosphorylation, proteolytic activation, etc.). It is unlikely that the enhanced activity was the result of allosteric modulation, unless the effector molecule was either very tightly bound or active in extremely low concentrations (due to the high dilution of cellular components that occurs with *in vitro* assays). On the other hand, it is quite possible that allosteric modulation may occur *in vivo* in addition to the observed change in active enzyme concentration.

Most studies concerning the regulation of acetyl-CoA carboxylase from plants have dealt with allosteric modulators, such as free CoA (Laing and Roughan 1982), divalent metal cations (Sauer and Heise 1984), and adenylate nucleotides (Eastwell and Stumpf 1983). As discussed earlier, however, acetyl-CoA carboxylase appears to be highly regulated in animals by several non-allosteric means, including phosphorylation/dephosphorylation, polymerization/depolymerization, and by differential genetic expression. To investigate whether the increased activity of acetyl-CoA carboxylase in silicon-deficient cells might be due to an enhanced rate of enzyme synthesis, the effects of protein synthesis inhibitors on the induction process were determined. In these experiments, the addition of 20 μg/ml cycloheximide (which inhibits cytoplasmic protein synthesis) completely blocked the induction of acetyl-CoA carboxylase in cells which were silicon-deficient for 4 h, while the addition of 0.5 mg/ml chloramphenicol (which blocks chloroplastic protein synthesis) inhibited the induction process by approximately 75% (Table III). These results indicate that protein synthesis on both cytoplasmic and chloroplastic ribosomes is necessary for the increased expression of acetyl-CoA carboxylase in silicon-deficient *C. cryptica* cells. Since acetyl-CoA carboxylase in higher plants is composed of three separate peptide components, it is possible that these subunits are synthesized in separate cellular compartments (as is the case with ribulose-1,5-bisphosphate carboxylase). It is also possible that the protein synthesis inhibitors may block the synthesis of accessory proteins which are required for full acetyl-CoA carboxylase activation (e.g., activator proteins such as phosphatases).

These studies were then extended in order to determine whether the apparent enhanced synthesis of acetyl-CoA carboxylase in silicon-deficient cells was due to changes in the rate of messenger RNA translation or to changes in the rate of messenger RNA synthesis (gene transcription). These experiments determined the effects of the compounds actinomycin D (which inhibits the transcription of nuclear DNA) and rifampicin (which inhibits chloroplastic DNA transcription) on the induction of acetyl-CoA carboxylase activity in silicon-deficient C. cryptica cells. Similar to earlier results, the specific activity of this enzyme from cells which were silicon-limited for 4 h was approximately twice that of silicon-replete cells, while cells grown in silicon-free medium containing actinomycin D (10 μg/ml) only exhibited a 26% increase in activity (Table III). On the other hand, the presence of rifampicin (20 μg/ml) did not have a large effect on the induction of acetyl-CoA carboxylase activity. The simplest interpretation of these results is that one means by which silicon-deficiency increases the rate of lipid synthesis in C. cryptica is by promoting the transcription of the nuclear genes responsible for the formation of acetyl-CoA carboxylase. These results are important in that they have identified a potential target for the genetic manipulation of microalgae in order to improve the lipid production capabilities of the cells.

LITERATURE CITED

Beattie, A., E.L. Hirst, E. Percival. 1961. Studies on the metabolism of the Chrysophyceae. Comparative structural investigations on leucosin (chrysolaminarin) separated from diatoms and laminarin from the brown algae. Biochem. J. 79:531-537.

Bold, H.C., M.J. Wynne. 1978. Introduction to the Algae. Prentice Hall, Englewood Cliffs, New Jersey, p. 572.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Coombs, J., W.M. Darley, O. Holm-Hansen, B.E. Volcani. 1967. Studies on the biochemistry and fine structure of silica shell formation in diatoms. Chemical composition of Navicula pelliculosa during silicon-starvation synchrony. Plant Physiol. 42:1601-1606.

Eastwell, K.C., P.K. Stumpf. 1983. Regulation of plant acetyl-CoA carboxylase by adenylate nucleotides. Plant Physiol. 72:50-55.

Hawker, J.S., H. Marschner, W.J.S. Downton. 1974. Effects of sodium and potassium on starch synthesis in leaves. Aust. J. Plant Physiol. 1:491-501.

Kannangara, C.G., P.K. Stumpf. 1972. Fat metabolism in higher plants. LIV. A procaryotic type acetyl-CoA carboxylase in spinach chloroplasts. Arch. Biochem. Biophys. 152:83-91.

Kauss, H., H. Kohle, W. Jeblick. 1983. Proteolytic activation and stimulation by Ca^{2+} of glucan synthase from soybean cells.

Laing, W.A., P.G. Roughan. 1982. Activation of spinach chloroplast acetyl-coenzyme A carboxylase by coenzyme A. FEBS Lett. 144:341-344.

Liedvogel, B. 1985. A new radiochemical method for determination of pyruvate dehydrogenase complex and acetyl-coenzyme A synthetase. Anal. Biochem. 148:182-189.

Liedvogel, B., P.K. Stumpf. 1982. Origin of acetate in spinach leaf cell. Plant Physiol. 69:897-903.

McLachlan, J. 1973. Growth media - marine. In: Handbook of Phycological Methods: Culture and Growth Measurements (J.R. Stein, ed.). Cambridge University Press, Cambridge, pp. 25-51.

Mohan, S.B., R.G.O. Kekwick. 1980. Acetyl-coenzyme A carboxylase from avocado (Persea americana) plastids and spinach (Spinacia oleracea) chloroplasts. Biochem. J. 187:667-676.

Morrow, D.L., W.J. Lucas. 1986. (1→3)-β-D-glucan synthase from sugar beet. I. Isolation and solubilization. Plant Physiol. 81:171-176.

Nielsen, N.C., A. Adee, P.K. Stumpf. 1979. Fat metabolism in higher plants. Further characterization of wheat germ acetyl coenzyme A carboxylase. Arch. Biochem. Biophys. 192:446-456.

Nikolau, B.J., J.C. Hawke. 1984. Purification and characterization of maize leaf acetyl-coenzyme A carboxylase. Arch. Biochem. Biophys. 228:86-96.

Roessler, P.G. 1986. Biochemical aspects of lipid accumulation in silicon-deficient diatoms. In: FY 1986 Aquatic Species Program Annual Review. SERI/SP-231-3071, pp. 257-271.

Saure, A., K.-P. Heise. 1984. Regulation of acetyl-coenzyme A carboxylase and acetyl-coenzyme A synthetase in spinach chloroplasts. Z. Naturforsch. 39c:268-275.

Shepherd, D., P.B. Garland. 1969. Citrate synthase from rat liver. In: Methods in Enzymology, Vol. XIII, Citric Acid Cycle (J.M. Lowenstein, ed.). Academic Press, New York, pp. 11-16.

Smestad-Paulsen, B., S. Miklestad. 1978. Structural studies of the reserve glucan produced by the marine diatom Skeletonema costatum (Grev.) Cleve. Carbohydr. Res. 62:386-388.

Takeda, Y., F. Suzuki, H. Inoue. 1969. ATP citrate lyase (citrate cleavage enzyme). In: Methods in Enzymology, Vol. XIII, Citric Acid Cycle (J.M. Lowenstein, ed.). Academic Press, New York, pp. 153-160.

Werner, D. 1966. Die Kieselsäure im Stoffwechsel von Cyclotella cryptica Reimann, Lewin, und Guillard. Arch. Mikrobiol. 55:278-308.

BIOCHEMICAL ELUCIDATION OF NEUTRAL LIPID SYNTHESIS IN MICROALGAE: REPORT OF A MECHANISM TO INDUCE NEUTRAL LIPID ACCUMULATION AND IMPLICATIONS FOR INCREASING NEUTRAL LIPID YIELDS FROM MICROALGAE

James B. Guckert[1], Keith E. Cooksey[1], and Larry I. Jackson[2]

Departments of Microbiology[1] and Chemistry[2]
Montana State University
Bozeman, Montana 59717 USA

ABSTRACT

Initial steps were taken to determine the contribution of neutral lipid (NL) synthesis to NL accumulation. Beginning work concerned the development of analytical methods including the modification of commercially-available equipment to achieve better resolution of fatty acids labelled from 14-C acetate. In carrying out this work, we have also compared various lipid extraction systems and developed a larger scale laboratory growth system for microalgae. Experiments involving maintenance of pH in the growth media indicated that manipulation of this parameter in conjunction with inorganic carbon concentration leads to a true increase in NL accumulation in Chlorella at non-limiting nitrate concentrations. These experiments provide evidence of a more universal mode of action of so-called NL "triggers" which can be exploited in the laboratory and perhaps, in a production setting. Various nutrient deprivation regimes may all have their effects on NL accumulation by disrupting the cell cycle. We shall exploit this hypothesis in future work.

BIOCHEMICAL ELUCIDATION OF NEUTRAL LIPID SYNTHESIS IN MICROALGAE: REPORT OF A MECHANISM TO INDUCE NEUTRAL LIPID ACCUMULATION AND IMPLICATIONS FOR INCREASING NEUTRAL LIPID YIELDS FROM MICROALGAE

INTRODUCTION

The productivity goals for the SERI Aquatic Species Program investigating the production of biofuels from microalgae have been set at 50 g dry algal cell weight per square meter of pond surface per day with a 50% lipid content, preferably neutral lipid [Neenan et al, 1986]. The general problem for the economic feasibility of neutral lipid (NL) production from microalgae is to couple high cellular productivity with high lipid content. When previous research has reported significant gains in NL production, it has been in terms of lipid as a percent of dry weight and not absolute amounts of NL produced [e.g. Piorreck et al, 1984]. In fact, when total lipid (or NL) accumulation is reported in absolute amounts, the nutrient regimes which induce high percentages of lipid (per gram dry weight) never increase the absolute lipid production, due to lower biomass productivity [Roessler, 1987].

Our initial step in this research has been to determine the contribution of NL synthesis to the increase in NL accumulation in algal cells. A percentage increase in NL may be due only to loss of cell dry weight. Without an increase in total lipid yield, it is possible that NL synthesis plays no role in the increasing of lipid content. The first experiments in this type of research deal with the identification of a reproducible and predictable way to induce NL accumulation in microalgae. Nitrate deprivation usually results in an increase in the percentage of total lipid in green algae (e.g. Chlorella) [Shifrin and Chisholm, 1981; Spoehr and Milner, 1949; Klyachko-Gurvich et al, 1969; Ben-Amotz et al, 1985]. Unfortunately, most of this research only reports total lipid accumulation instead of NL. Nitrate deprivation appears not to be a universal "trigger" of NL accumulation in microalgae. Cyclotella, for instance, does not increase lipid proportions during nitrate deprivation, but does so during silica deprivation [Millie, 1985]. In addition to nitrate and silica, selenium deprivation has resulted in the formation of observable lipid inclusions within the diatom, Thalassiosira [Doucette et al, 1986]. Research into the fatty acid dynamics of Thalassiosira has suggested that the storage triaclyglycerides of the diatom's NL are utilized when the cell needs energy (e.g. in the dark or during cell division) and accumulate when the cell has excess available energy, as in non-dividing cells in constant light [Fisher and Schwarzenbach, 1978]. This is similar to the findings of Otsuka and Morimura [1966] who reported that Chlorella ellipsoidea synthesizes NL throughout the photosynthetic growth phase of its cell cycle and utilizes it during the process of cell division.

Since nitrate deprivation does not seem to be a consistent NL "trigger", and there appears to be other possible "triggers" to NL accumulation, our first set of experiments were designed to monitor unmanipulated algal cultures and correlate NL accumulation with media factors. Our aim was to find a reproducible method to initiate NL accumulation. We preferred not to have to wait for a media component to disappear during normal cell growth (e.g. N-, Si-, Se-, etc) or to have to remove cells from a replete medium and resuspend in a deficient one. The best "switch" would be one that worked when it was added to the medium. This would not only be biochemically more convenient and decrease the likelihood of disturbances and artifacts, but it would also be more realistic in a large-scale production sense for efficient NL production. The

work of Fisher and Schwarzenbach [1978] indicate that an addition which decreases cell division rates, even temporarily, can have an influence on NL proportions. Only after we can reliably control NL accumulation can we begin analysis of the relationship between NL synthesis and NL accumulation in microalgae and the biochemistry involved therein.

This paper reports our progress for the first ten months of our contract. In addition to our work on NL accumulation and media components, we have investigated 14-C acetate uptake kinetics by Chlorella, developed methods to analyze the specific activities of 14-C labelled lipid classes of microalgae, compared lipid solvent system recoveries for algal lipid classes, and accomplished large scale culturing of Chlorella in an airlift fermenter.

MATERIALS AND METHODS

Microalgae. Experiments utilizing Chlorella sp. CHLOR1 (SERI isolate) were done with axenic cultures grown on Bold's basal medium, initial pH 6.7 [Nichols and Bold, 1965]. This medium was occasionally modified with a biological buffer at a final concentration of 25 mM. The biological buffers used were: HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) at pH 7.0, CHES (2-[N-cyclohexylamino]ethane-sulfonic acid) at pH 9.0, and CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) at pH 10.2 (Sigma Chemical Company). Sodium bicarbonate additions were made to previously-autoclaved Bold's medium (with or without buffer) by aseptically adding 0.2 um filtered 50 mM sodium bicarbonate (final concentration bicarbonate 5 mM). Chlorella experiments were either conducted in a BRL (Bethesda, MD) airlift fermenter (1800 ml working volume), or in 100 ml cultures in flasks shaken at 100 rpm. The cells were grown at 30°C under continuous illumination (100-250 uE/m^2/s) with atmospheric air delivered at 2 liters/min to provide carbon dioxide and circulation. The cultures in flasks were grown at 26°C with similar lighting.

Lipid Analysis. Algal cells were harvested by centrifugation at 3000 xg for 15 mins and the supernatant medium saved for analysis of components (see below). After wet weight determination, the cells were quick-frozen and lyophilized. Cell dry weights were recovered prior to lipid extraction. A comparison of three lipid extraction procedures was made for the recovery of the lipid classes of Chlorella. The systems used were a three hour Soxhlet extraction (methylene chloride/methanol), a Bligh and Dyer [1959] chloroform/methanol/water extraction as modified with a phosphate buffer by White et al [1979], and hexane/isopropanol extraction [Hara and Radin, 1978]. The details of this comparison are presented in Guckert et al [1987]. For the biochemical research presented here, the modified Bligh and Dyer lipid extraction was used. The ester-linked fatty acids from each lipid fraction were prepared, GC analyzed and GC/MS verified as described in Guckert et al [1987].

Gas Chromatography/ Gas Proportional Counter for 14-C Specific Activity Analysis of Individual FAME. This analysis utilized a Packard 437A GC with a 15 meter DB-225 megabore column (J&W) which had a stream splitter at its end (approximately 100:1) with the lower flow continuing on to a flame ionization detector (FID) and the greater flow being routed through a heated line (300°C) to a combustion oven (750°C) to convert all organics to carbon dioxide for analysis in a Packard 894 Gas Proportional Counter connected in line to the combustion oven. The GC/GPC system had been modified for use with a megabore column to improve resolution. As part of this modification, helium make up

lines were included just prior to the stream splitter, FID, combustion oven, and drying tube in line between the combustion oven and the gas proportional counter detector tube. Propane was used as the quench gas for the GPC.

Fatty Acid Nomenclature. Fatty acids are designated as total number of carbon atoms: number of double bonds with the position of the double bond closest to the aliphatic (omega, w) end of the molecule indicated with the geometry 'c' for cis and 't' for trans (e.g. 16:1w13t, trans-3-hexadecenoate). All polyunsaturated fatty acids were methylene interrupted (e.g. 18:3w3= 18:3w3,6,9; alpha-linolenate or cis,cis,cis-9,12,15-octadecatrienoate).

Nile Red. The Nile Red estimation of neutral lipid was done according to the methods developed in Cooksey et al [1987]. To monitor neutral lipid accumulation over time, 5 ml aliquots were removed from the algal culture and assayed directly with the Nile Red response being quantified by fluorometry 8 minutes after the addition of Nile Red (20 ul of 250 ug/ul acetone) and acetone (180 ul). The Nile Red (9-diethylamino-5H-benzo[alpha]phenoxazine-5-one) was obtained from Molecular Probes (Junction City, OR) or Kodak (Rochester, NY). The standard used was 2.8 um Nile Red-stained monodisperse latex particles (a gift of Dr. C.J. Wang, Pandex Laboratories, Mundelein, IL). The working standard was made up to a concentration of 5.88x10E5 beads/ml. Five ml of this standard was used to standardize the Turner Model 10 fluorometer at a setting of 5.0 (net scale expansion= 3.16) with a 480+ 10 nm excitation filter and a 580+ 9.8 nm bandpass emission filter. The Nile Red data is expressed in arbitrary units defined such that the working standard is 100 units/ml.

Analysis of Media Components. The concentrations of nitrate (as nitrate nitrogen, mg/l) and phosphate (mg/l) were estimated by the cadmium reduction and ascorbic acid methods, respectively, using commercially available reagent test kits (Hach, Inc., Loveland, Co.). Nitrate results were also confirmed by EPA Method 353.3 for nitrate/nitrite nitrogen. Determinations of pH were also done on media supernatant samples.

Chlorella Neutral Lipid Accumulation Experiments. Three separate fermenter runs were made. These were harvested at either 72 hours (3 days), 96 hours (4 days) or 85 hours. The 85 hour harvesting was to provide cells for the 14-C uptake experiments (described below) and the analytical data reported in this report are for the control cells (no 14-C addition) which were removed from the fermenter to flasks (50 ml culture) and maintained for an additional 23 hours (total growth time = 108 hours or 4.5 days). The remaining Chlorella experiments were done in flasks. Comparisons of the cell growth, culture pH, medium nitrate levels, and neutral lipid accumulation were done for Chlorella cells inoculated into the following treatment flasks: unbuffered Bold's medium, unbuffered Bold's medium + 5 mM sodium bicarbonate, Bold's medium buffered with HEPES (pH 7), Bold's medium buffered with HEPES plus 5 mM sodium bicarbonate (pH 7.4), Bold's medium buffered with CHES (pH 9), Bold's medium buffered with CAPS (pH 10.4), and unbuffered Bold's medium which had 5 mM sodium bicarbonate added after 7 days of growth.

14-C Uptake Experiment. When the media in the airlift fermenter had reached a cell density of 3.9 x 10E6 cells/ml (85 hours), 50 ml aliquots of the culture were aseptically transferred to six sterile 250 ml flasks. The flasks were randomly placed on an orbital shaker and shaken at 100 rpm for 1 hour at 20°C with an approximate light intensity of 200 uE/m^2/s at the base of the flasks. After this period of acclimation, 3 ml of formalin was added to flasks #3 and

#6, followed by an addition of either 50 ul [2-14-C]-acetate (0.1 mCi/ml, 51 mCi/mmol, New England Nuclear; flasks #1,2,3) or 12 ul of 0.001% (w/v) aqueous 12-C acetate (flasks #4,5,6) to give an equivalent final concentration of added acetate in all flasks (2 uM).

Flasks were monitored at 0,2,4,6,8,23 hours after acetate addition with the removal of duplicate aliquots from each flask. For flasks #1,2,3: 500 ul was aseptically removed to a glass fiber filter (1.6 um pore) set in a vacuum manifold (Amicon, Inc.) for separation of cells and media. The cells were then washed twice with 500 ul sterile Bold's supplemented with formalin (final concentration 2% formaldehyde). The filters were placed under a high intensity spot light for three hours to achieve photobleaching before transfer to scintillation vials for overnight digesting in 50 ul distilled water plus 500 ul Protosol (New England Nuclear) at room temperature. Following this, the solution was neutralized with 50 ul glacial acetic acid, and 5 ml of scintillation cocktail (Ecolume, ICN, Inc.) was added. All samples were left in the dark overnight after cocktail addition to decrease chemilumenscence. The vacuum manifold used for filtration retained the filtrate in separate scintillation vials for analysis of unincorporated radiolabel. Samples were counted on a Packard Tricarb 4430 liquid scintillation counter for 10 min. Disintegrations per minute (dpm) were calculated based on the individual sample's counts per minute, the counting efficiency from an established quench curve and an external standard. The non-radioactive controls (flasks #4,5,6) had duplicate 500 ul aliquots removed to sterile test tubes and cell counts for each tube were done. Lipid analysis was done on the cells in flasks #1,2,4,5 using the methods previously described. The radioactivity of each fraction of the lipid analysis was determined for flasks #1 and 2.

RESULTS

Chlorella Lipids. The fatty acid distributions differ among the lipid classes of Chlorella CHLOR1 grown in the fermenter for 96 hours (Figure 1). The neutral lipids tend to be less unsaturated and dominated by 16:0 and 18:1w9c. The glycolipids are characterized by having approximately 70% of their total fatty acids as polyunsaturates (18:3w3 39%, 16:4w3 17%, 18:2w6 12%, Figure 1). The polar lipid fatty acid profiles are dominated by 16:0 with approximately equivalent proportions of the major fatty acids of both the neutral lipids (18:1w9c) and the glycolipids (18:2w6, 18:3w3).

14-C Acetate Incorporation into Chlorella. When the amount of 14-C incorporation from 14-C acetate into whole cells of Chlorella was followed over 23 hours of incubation with the label, the uptake kinetics curve for two independent flasks (Figure 2) indicated that uptake reached a peak within two hours. A third flask (#3), which had been formalin-killed, indicated no abiological or non-specific uptake of the label occurred (Figure 2). When the Chlorella cells were harvested after 23 hours and lipid analyzed, the 14-C label distribution in various cell fractions were recorded as shown in Table 1. Of the 14-C added to the Chlorella culture, 74% was incorporated into the cells, of which 30% went into lipids (Table 1). The label was distributed in the lipid fractions of Chlorella as: 41% in neutral lipids, 50% in the glycolipids, and 9% in the polar lipids. Following transesterification, the resulting FAME were analyzed to determine the proportion of 14-C from each lipid fraction which had been incorporated into the esterified fatty acids. For all fractions, the fatty acids accounted for over 70% of the label incorporated into the lipids (Table

FIGURE 1 (a+b): Fatty acid profiles for lipid classes of Chlorella CHLOR1 grown in an airlift fermenter for 96 hours. Histograms represent an average of 3 analytical replicates ±1 standard deviation. '0' indicates none detected.

FIGURE 2

FIGURE 2: Uptake kinetics curve for 14-C acetate incorporation into whole cells of Chlorella CHLOR1 followed over 23 hours of label incubation for 2 flasks (#1,2). A third flask (#3), which had been formalin-killed, indicated no abiological or non-specific uptake of label occurred.

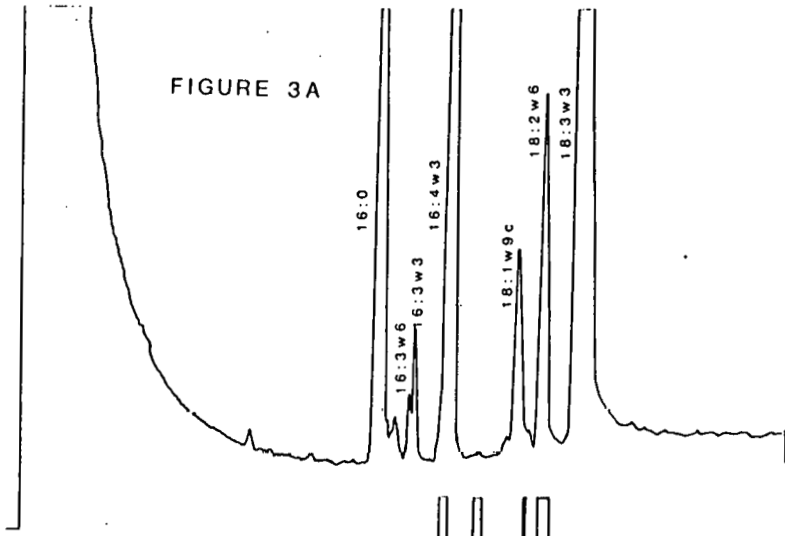


FIGURE 3A

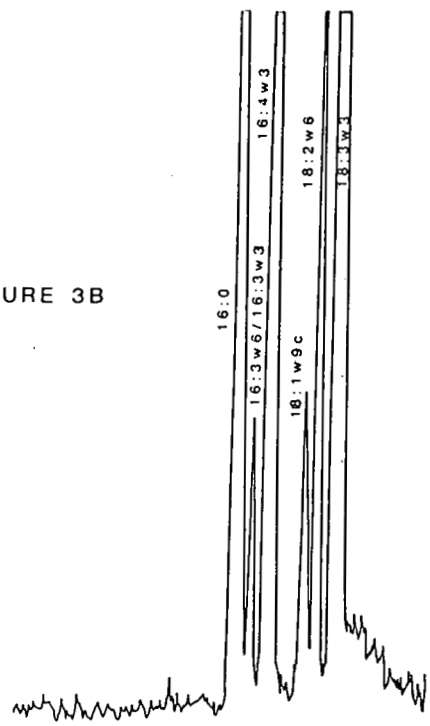


FIGURE 3B

TABLE 1: Distribution of 14-C label in Chlorella CHLOR1

14-C acetate added to culture	= 11.0 x 10^6 dpm
Recovered 14-C in cells	= 8.1 x 10^6 dpm (74% incorporation)
Percent of 14-C in cells after lipid extraction:	
Lipids (chloroform-soluble)	30 %
Extracted cell residue	33 %
Aqueous phase	5 %
Other/ unaccounted for	32 %
Recovered 14-C in lipid phase	= 2.4 x 10^6 dpm
Percent after lipid fractionation:	
Neutral lipids	41 %
Glycolipids	50 %
Polar lipids	9 %
Proportion of lipid fractions as esterified fatty acids:	
Neutral lipid as fatty acids	71 %
Glycolipid as fatty acids	74 %
Polar lipid as fatty acids	76 %

TABLE 2: Specific activity calculation (14-C in fatty acids/ mass of fatty acids) for individual fatty acids of glycolipid fraction of Chlorella CHLOR1 incubated with 14-C acetate for 23 hours (see text for details).

Fatty Acid	14-C area/ mass area
16:0	25
16:3w6/16:3w3	30
16:4w3	22
18:1w9c	19
18:2w6	23
18:3w3	10

FIGURE 3: Chromatograms generated by the GC/GPC system from a single injection of Chlorella CHLOR1 glycolipid fatty acids (as their methyl esters).

A) Mass response from the GC with a FID detector.

B) 14-C response from the GPC connected in-line with the GC system. Note that the chart speed in B is twice that in A.

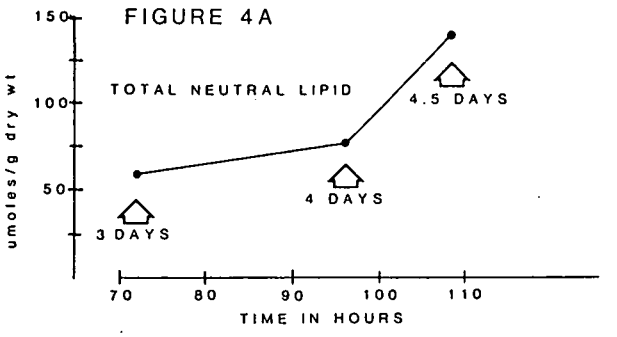
1). The 14-C labelled FAME from the lipid classes of Chlorella were then analyzed on the GC/GPC to test the system's ability to quantify the specific activities of individual fatty acids. Figure 3 shows the mass trace (A) and the 14-C trace (B) for the FAME of the glycolipid fraction of Chlorella CHLOR1. In this sample, no internal standard was added, so specific activities (Table 2) are given as the ratio of peak area from the 14-C detector (GPC, Figure 3B) to that from the mass detector (FID, Figure 3A).

Fermenter Experiments. The amount of neutral lipid per gram dry weight of cells increased only slightly from 3 to 4 days, but approximately doubled from 4 to 4.5 days (Figure 4A). The cell density was beginning to level off at this point indicating a reduction in the number of cells dividing (Figure 4B). The phosphate level in the medium changed little over this time period, however, both the nitrate and pH levels were changing from 3 to 4.5 days (Figure 4C). The nitrate concentration (as mg/l nitrate nitrogen) declined from 44 to 14 mg/l from 3 to 4.5 days while the pH increased from 6.8 to 10.0 over the same time period (Figure 4C).

Buffer Experiments. One purpose of these experiments was to investigate Chlorella growth as media pH was controlled. The buffered media maintained the desired pH over 10 days of Chlorella growth (Figure 5). HEPES maintained pH 6.8-7.1 and pH 7.1-7.4 when 5 mM bicarbonate was added. CHES maintained pH 8.8-9.1 while CAPS maintained pH 10-10.4. Unbuffered Bold's medium (B2 in Figure 5) showed a similar curve to that shown for the fermenter growth (Figure 4) although the time scale is delayed since the pH elevation follows cell density (carbon dioxide utilization) rather than time of incubation. The fermenter cultures reached higher cell densities more quickly than those in shaken flasks (Figure 4, Figure 6). When unbuffered Bold's medium had 5 mM bicarbonate added to it after 7 days of growth (B1 in Figure 5), there was an initial pH rise (7.9 to 9.2) just after bicarbonate addition. The following day, the pH had risen to over pH 11 and maintained this level for the remainder of the experiment (Figure 5). When Chlorella was inoculated directly into unbuffered Bold's medium plus 5 mM bicarbonate (B&C in Figure 5), the pH was close to pH 11 by the third day of incubation. The cell densities shown in Figure 6 indicate similar, but not identical, growth curves for all treatments. The treatment with the highest cell density was the CHES buffer (pH 9) followed by the HEPES buffer with 5 mM bicarbonate added (pH 7.4). Bold's medium unbuffered and buffered with HEPES were nearly identical in cell growth. When 5 mM bicarbonate was added during the growth on unbuffered Bold's medium (B1 in Figure 6), the cell density leveled off at a lower value than in the unsupplemented medium. This lower value was the same final density of the CAPS buffered cultures (pH 10.4). The CAPS buffered cultures were the only ones to show a lag in cell division over the 3 days following inoculation (CA, Figure 6). The lowest final cell density occurred when cells were inoculated into unbuffered Bold's medium and 5 mM bicarbonate (B&C in Figure 6).

The buffer experiments, like the fermenter experiments, had at least 25% of the initial nitrate levels (approximately 40 mg/l nitrate N) remain in the medium after the 10 days of the experiment (Figure 7). There were no differences in the four treatment's nitrate levels monitored until the 10th day of growth. The lowest nitrate concentration was approximately 15 mg/l nitrate nitrogen (Figure 7).

The accumulation of neutral lipid (NL) per ml of Chlorella culture was measured by the Nile Red technique for each treatment (Figure 8). Beginning



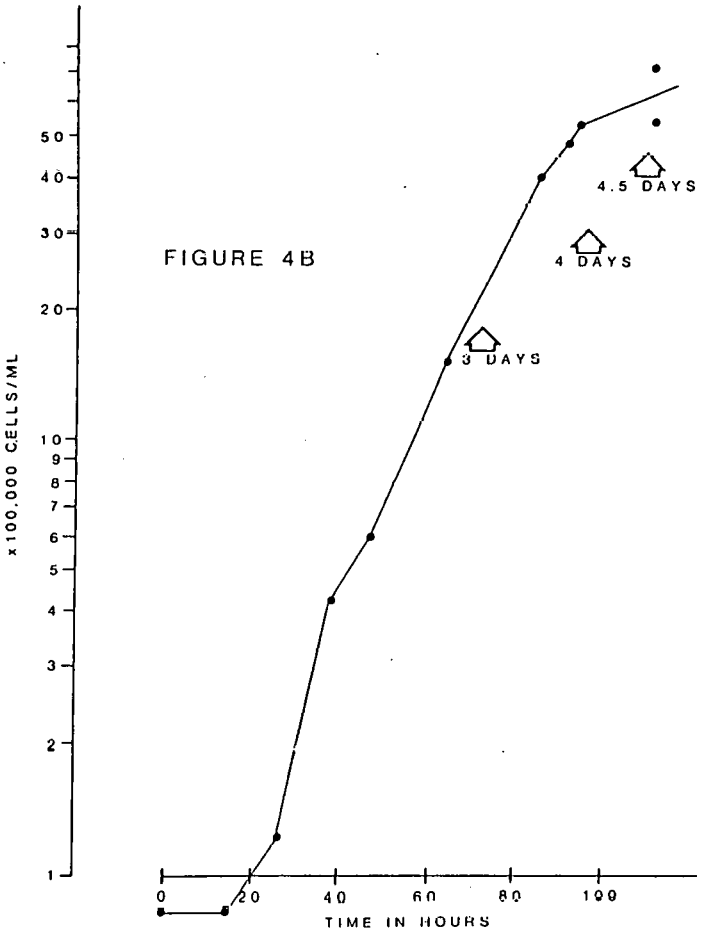
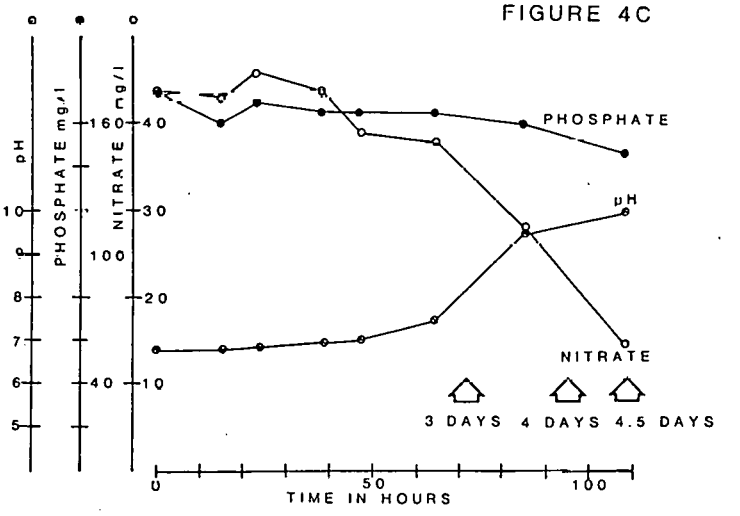


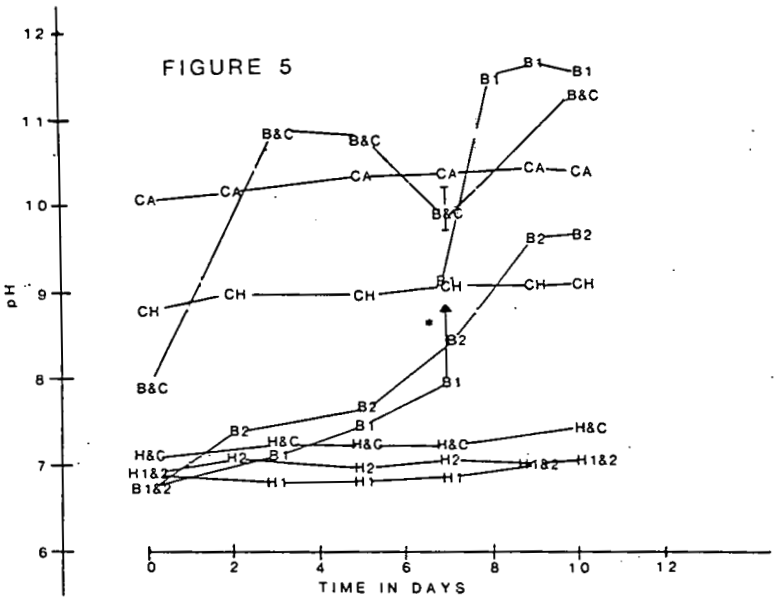
FIGURE 4: Results from growth of Chlorella in airlift fermenter with Bold's basal medium.

A) Total neutral lipid recovery by analytical analysis for Chlorella harvested at 3, 4, and 4.5 days.

B) Growth curve of Chlorella with sampling times for lipid analysis indicated.

C) Changes in medium components during growth of Chlorella with sampling times for lipid analysis indicated.





Key to symbols in Figures 5 and 6:

Experiment #2

B1 = Bold's medium

B&C= Bold's medium + 5 mM HCO_3^-

H1 = HEPES buffered Bold's medium

H&C= HEPES buffered Bold's medium + 5 mM HCO_3^-

Experiment #3

B2 = Bold's medium

H2 = HEPES buffered Bold's medium

CH = CHES buffered Bold's medium

CA = CAPS buffered Bold's medium

* = time of 5 mM HCO_3^- addition to B1

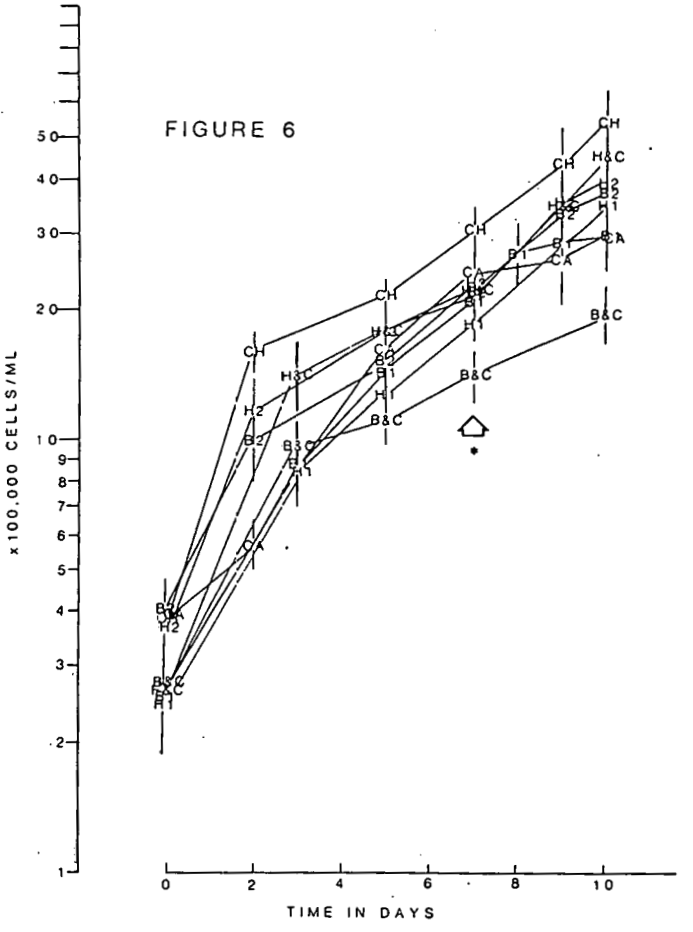


FIGURE 5: pH changes of medium during 10 days of Chlorella CHLOR1 growth.

FIGURE 6: Growth curves of Chlorella CHLOR1 in shaken flasks over 10 days of incubation.

with the lowest lipid producers, cells in the HEPES buffered media (with and without bicarbonate addition) produced little NL(9+0.4 or 16+3 units/ml). In unbuffered Bold's medium and CHES buffered Bold's medium (pH 9), Chlorella produced twice as much as the HEPES treatments, 23+7 and 27+4 units/ml, respectively. On the other hand, when cells were inoculated directly into Bold's medium plus 5 mM bicarbonate, the NL accumulated to 99+8 units/ml within 3 days and after 10 days had accumulated to 471+43 units/ml. When Bold's medium was buffered with CAPS (pH 10.4) the cells accumulated 71+7 units/ml NL within 2 days, but this value leveled off around 100 units/ml until the end of the 10 day experiment when NL accumulation occurred again to a final value of 161+7 units/ml. When 5 mM bicarbonate was added to cells which were incubated in unbuffered Bold's medium and accumulating little NL (17+5 units/ml, B1 in Figure 8), the cells responded with NL accumulation to 69+19 units/ml within 2 days of bicarbonate addition, and 108+26 units/ml after 3 days.

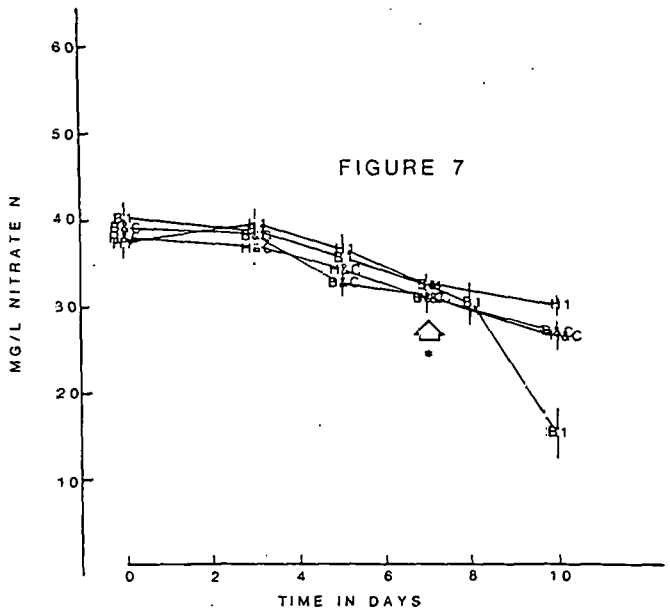
Note that the data presented for each curve in Figures 5,6,7, and 8 are averages of 4 independent flasks evaluated simultaneously and the error bars around each average indicates +1 standard deviation for these 4 replicates. When the error bars are not visible, the standard deviation is equal to or less than the width of the symbol used to designate the average value.

DISCUSSION

The fatty acid profiles for the lipid classes of Chlorella CHLOR1 agree with previously published reports of these profiles for other Chlorella spp. [Milner, 1948; Klyachko-Gurvich et al, 1981; James, 1968; Erwin, 1973; Shaw, 1966]. The importance of class separation for lipid analysis is clear as each class has a distinct fatty acid profile (Figure 1). Lipid analysis after class separation indicates the lower degree of unsaturation in the NL which makes it the more desirable fraction as a biological substitute for diesel fuel [Ryan et al, 1984].

The distinction between lipid class fatty acid profiles also indicates the importance of utilizing a reproducible, artifact-free, efficient lipid extraction solvent system such as the room temperature, pH-buffered, modified Bligh and Dyer [White et al, 1979]. In a separate publication, we discuss this importance in the context of Soxhlet extractions and a hexane/isopropanol solvent system [Guckert et al, 1987].

The 14-C uptake data shown in Figure 2 indicates that lipid synthesis studies should use incubations no longer than 2 hours. The results from this preliminary experiment indicate that the lipid of Chlorella is adequately labelled by the procedure (Table 1) and the GC/GPC can be utilized to determine specific activities of individual fatty acids of the algal lipid classes (Figures 3A and 3B, Table 2). This technique will allow us to follow NL synthesis from the putative point of induction. It will be important to ascertain if there is a true point of induction, or whether NL synthesis is constant and the regulation step leading to NL accumulation actually occurs at the level of NL utilization. For instance, do N-, Si-, and other cell division inhibitors interfere with NL utilization which occurs during cell division [Otsuka and Morimura, 1966], resulting in NL accumulation? Monitoring changes in NL specific activities in NL accumulating and non-accumulating conditions will allow us to test these hypotheses.



Key to symbols in Figure 7 and 8:

Experiment #2

B1 = Bold's medium

B&C= Bold's medium + 5 mM HCO_3^-

H1 = HEPES buffered Bold's medium

H&C= HEPES buffered Bold's medium + 5 mM HCO_3^-

Experiment #3

B2 = Bold's medium

H2 = HEPES buffered Bold's medium

CH = CHES buffered Bold's medium

CA = CAPS buffered Bold's medium

* = time of 5 mM HCO_3^- addition to B1

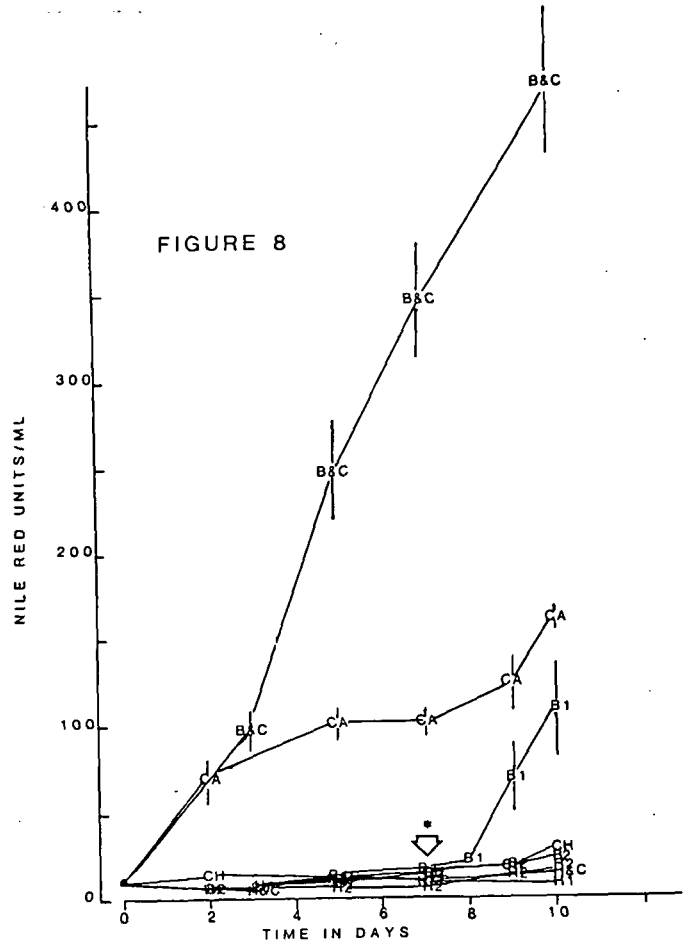


Figure 7: Changes in nitrate concentrations in medium during Chlorella CHLOR1 growth.

Figure 8: Changes in neutral lipid per ml culture as estimated by Nile Red procedure [Cooksey et al, 1987]. See text for definition of units.

The initial fermenter experiments done with Chlorella suggested that two factors in the medium were changing when NL accumulation was occurring (Figures 4A and 4C). There was a pH increase up to pH 10 that occurred at the time of an approximate doubling in Chlorella NL accumulation (Figures 4A and 4C). Many algal media have been known to be poorly buffered for carbon dioxide additions [Galloway and Krauss, 1961]. Bold's basal medium [Nichols and Bold, 1965] is unable to maintain its initial pH (6.7-6.8) after a few days of Chlorella CHLOR1 growth (Figures 4C and 5). This result indicated a need to try some buffer systems to help control pH for biochemical experiments and that pH should be monitored as a possible NL accumulation "trigger".

The buffer experiments indicate that it is possible with Chlorella CHLOR1 to have a true increase in NL accumulation per ml of culture at similar cell densities without lowering the media concentration of nitrate to limiting concentrations. High pH (> 10) seems to correspond to NL accumulation (Figures 5,8), although NL accumulation is not necessarily an instantaneous result of high pH media (e.g. Day 8 of unbuffered Bold's medium with bicarbonate addition, B1, pH 11.5 (Figure 5), NL 21.5+ 4.2 units/ml (Figure 8)). The interrelationship of pH and inorganic carbon equilibrium in water indicated the possibility that the pH effect was a result of increased bicarbonate in solution [Cameron, 1986]. The fact that there was no difference in NL accumulation when HEPES buffered Bold's medium (pH 7-7.4) had 5 mM bicarbonate added (Figure 8) does not support an effect dependent on an increased availability of inorganic carbon. Our next set of experiments will investigate 5 mM bicarbonate additions to CHES and CAPS buffered systems. Work reported by the SERI Microalgal Technology Research Group [Milestone Update, Nov., 1986] with the diatom Navicula sp. has indicated that in a CHES buffered system, bicarbonate concentration has very little effect on lipid yield, agreeing with our results with HEPES.

Our conclusions from these buffer experiments are that nitrate deprivation is not the only "NL trigger". High pH also had this effect and the use of buffers and bicarbonate additions provide media components that can be added to turn on the NL accumulation 'switch" for our biochemical studies. This will provide us with NL accumulating and non-accumulating cultures for direct comparison in the investigation of the importance of NL synthesis to NL accumulation.

The patterns of NL accumulation (Figure 8) with the cell growth curves (Figure 6) suggests that NL accumulation occurs when cell division rates decrease. All NL "triggers" which have been discussed previously by other researchers (N-, Si-, Se-, pH, etc) might all have their prime effect on NL accumulation by disrupting the cell cycle and stopping, or slowing, cell division. Taguchi et al [1987] suggest that blockage of cell division is a universal initiator of algal lipid synthesis. We would caution against accepting this as a general conclusion. We believe the the precise point in the cell cycle where division is inhibited (not necessarily prevented) is important. There is evidence that many algal lipids changes during algal cell cycles, including sterols [Otsuka, 1963], glycolipids [Klyachko-Gurvich et al, 1981], sulfolipids and phospholipids [Beck and Levine, 1977], total lipids [Reitz et al, 1967] and (most interesting for our purposes), triglyceride NL [Otsuka and Morimura, 1966].

Changes in the rates of cell division might directly influence NL accumulation through the balance of NL synthesis and NL utilization. The NL

data in Figure 8 indicate that when Chlorella CHLOR1 is grown at pH 10.0-10.4 (CAPS), there is an initial increase in NL accumulation which corresponds to a lag in cell division in the 2 days after inoculation (Figure 6). As the cells begin growing, the NL accumulation levels off until the end of the experiment when cell division rates again slow (Figures 6,8). On the other hand, when cells were inoculated into unbuffered Bold's medium plus 5 mM bicarbonate, NL accumulation increased through the experiment (B&C in Figures 6,8). The reason for these differences are, at this time, unknown.

In addition to finding conditions for reproducible and predictable NL accumulation, these results also indicate that it is possible to produce NL such that the amount of NL per ml of culture increases, in contrast to an increased percentage of cell weight. It may, therefore, be possible to further increase NL yields as other cultural manipulations are developed which can more tightly couple high cellular productivity and high lipid production in microalgae.

CONTINUING RESEARCH

In the next 2 months of our contract, we will investigate the influence of the following on NL synthesis: bicarbonate additions to high pH-buffered systems, a comparison of NL accumulation with the pH/bicarbonate "trigger" and nitrate deprivation, culture synchrony in Chlorella CHLOR1 for cell cycle work. The NL-synthesis analyses will utilize the pH/bicarbonate "trigger" to monitor the specific activity of NL under NL accumulating and non-accumulating conditions using the GC/GPC technology. This will address the question of the relative importance of NL synthesis induction and NL accumulation in microalgae. Once NL synthesis under these conditions has been initially studied, parallel experiments are planned with broken cell preparations. These will be necessary preliminary experiments to the cell fractionation studies.

ACKNOWLEDGMENTS

This research is sponsored by the Solar Energy Research Institute, Golden ,Co., USA, through SERI contract number XK-5-05073-1. The authors would also like to thank Dr. Sam Rogers (MSU Chemistry Dept.) for providing lab space, Dr. Jim Cutler (MSU Microbiology Dept.) for the loan of the Varian GC, and Dr. David White (Univ. Tennessee, Institute of Applied Microbiology) for the loan of the capillary GC conversion kit.

REFERENCES CITED

Beck, J.C. and Levine, R.P. 1977. Synthesis of chloroplast membrane lipids and chlorophyll in synchronous cultures of Chlamydomonas reinhardi. Biochim. Biophys. Acta 489:360-369.

Ben-Amotz, A., Tornabene, T.G., and Thomas, W.H. 1985. Chemical profile of selected species of microalgae with emphasis on lipids. J. Phycol. 21:72-81.

Bligh, E.G., and Dyer, W.J. 1959. A rapid method for total lipid extraction and purification. Can. J. Biochem. Phys. 37:911-917.

Cameron, J.N. 1986. Principles of physiological measurement. Academic Press, Orlando. pp 87-90.

Cooksey, K.E., Guckert, J.B., Williams, S.A., and Callis, P.R. 1987. Fluorometric determination of the neutral lipid content of microalgal cells using Nile Red. J. Microbiol. Methods, in press.

Doucette, G., Price, N.M., and Harrison, P.J. 1986. Effects of selenium deficiency on the morphology and ultrastructure of the coastal marine diatom Thalassiosira pseudonana (Bacillariophyceae). J. Phycol. 23:9-17.

Erwin, J.A. 1973. Comparative biochemistry of fatty acids in eukaryotic microorganisms. pp 41-143. In: Lipids and biomembranes of eukaryotic microorganisms. J.A. Erwin (ed) Academic Press, NY.

Fisher, N.S. and Schwarzenbach, R.P. 1978. Fatty acid dynamics in Thalassiosira pseudonana (Bacillariophyceae): implications for physiological ecology. J. Phycol. 14:143-150.

Galloway, R.A. and Krauss, R.W. 1961. The effect of CO_2 on pH in culture media for algae. Plant and Cell Physiol. 2:331-337.

Guckert, J.B., Cooksey, K.E., and Jackson, L.L. 1987. Lipid solvent systems are not equivalent for analysis of lipid classes in the microeukaryotic green alga, Chlorella. J. Microbiol. Methods, submitted.

Hara, A. and Radin, N.S. 1978. Lipid extraction of tissues with a lower toxicity solvent. Anal. Biochem. 90:420-426.

James, A.T. 1968. The biosynthesis of unsaturated fatty acids in photosynthetic systems in lower and higher plants. pp 25-37. In: Cellular compartmentalization and control of fatty acid metabolism. F.C. Gran (ed). Academic Press, NY.

Klyachko-Gurvich, G.L., Zhukova, T.S., Vladimirova, M.G., and Kurnosova, T.A. 1969. Comparative characterization of the growth and direction of biosynthesis of various strains of Chlorella under nitrogen deficiency conditions. III. Synthesis of fatty acids. Sov. Plant Physiol. 16:168-171.

Klyachko-Gurvich, G.L., Tsoglin, L.N., and Mozhaitseva, G.I. 1981. Lipid metabolism during the course of ontogenesis of Chlorella in connection with activity of the photosynthetic apparatus. Sov. Plant Physiol. 28:304-311.

Millie, D.F. 1985. Nutrient-limitation effects on the biochemical composition of Cyclotella meneghiniara (Bacillariophyta): an experiment and statistical analysis. Can. J. Bot. 64:19-26.

Milner, H.W. 1948. The fatty acids of Chlorella. J. Biol. Chem. 176:813-817.

Neenan, B., Feinberg, D., Hill, A., McIntosh, R., and Terry, K. 1986. Fuels from microalgae: Technology status, potential, and research requirements. SERI/SP-231-2550.

Nichols, H.W. and Bold., H.C. 1965. Trichosarcina polymorpha gen. et. sp. nov. J. Phycol. 1:34-38.

Otsuka, H. 1963. Contents of sterols in Chlorella cells at different developmental stages. Plant and Cell Physiol. 4:293-297.

Otsuka, H. and Morimura, Y. 1966. Change of fatty acid composition of Chlorella ellipsoidea during its cell cycle. Plant and Cell Physiol. 7:663-670.

Piorreck, M., Baasch, K.-H. and Pohl, P. 1984. Biomass production, total protein, chlorophylls, lipids and fatty acids of freshwater green and blue-green algae under different nitrogen regimes. Phytochemistry 23:207-216.

Reitz, R.C., Hamilton, J.G., and Cole, F.E. 1967. Fatty acid concentrations in synchronous cultures of Chlorella pyrenoidosa, grown in the presence and absence of glucose. Lipids 2:381-389.

Roessler, P. 1987. Biochemical aspects of lipid accumulation in silica-deficient diatoms. SERI SP-231-3071. pp 257-272.

Ryan, T.W., III., Doge, L.G., and Callahan, T.J. 1984. The effects of vegetable oil properties on injection and combustion in two different diesel engines. J. Am. Oil Chemists Soc. 61:1610-1619.

Shaw, R. 1966. The polyunsaturated fatty acids of microorganisms. Adv. Lipid Res. 4:107-174.

Shifrin, N.S. and Chisholm, S.W. 1981. Phytoplankton lipids: interspecific differences and effects of nitrate, silicate and light-dark cycles. J. Phycol. 17:374-384.

Spoehr, H.A. and Milner, H.W. 1949. The chemical composition of Chlorella; effect of environmental conditions. Plant Physiol. 24:120-149.

Taguchi, S., Hirata, J.A., and Laws, E.A. 1987. Silicate deficiency and lipid synthesis of marine diatoms. J. Phycol. 23:260-267.

White, D.C., Davis, W.M., Nickels, J.S., King, J.D., and Bobbie, R.J. 1979. Determination of the sedimentary microbial biomass by extractable lipid phosphate. Oecologia (Berl.) 40:51-62.

Environmental Control of Lipid Accumulation in Nannochloropsis salina, Nanno Q and Euglena.

Lloyd Coleman, Barry Rosen and Steven D. Schwartzbach
Dept. of Biological Sciences, University of Nebraska, Lincoln Nebraska 68588

ABSTRACT

The improvement of lipid yields in microalgae requires an understanding of the physiological and biochemical basis for the partitioning of photosynthetically fixed CO_2 into lipids and an understanding of factors affecting the rate of lipid breakdown relative to the breakdown of other cellular constituents. Since the most commonly utilized lipid trigger, nitrogen deficiency, rapidly reduces photosynthetic capacity, it is useful to separate effects of nitrogen deficiency on photosynthetic efficiency from effects on carbon partitioning. Initial studies have therefore used two lipid storing algae, Nanno Q and Nannochloropsis salina as well as Euglena, a microalgae which grows equally well under photosynthetic and heterotrophic conditions.

Lipid per cell of photosynthetic Nanno Q, Nannochloropsis salina and Euglena increased during nitrogen deficiency and decreased upon the addition of a utilizable nitrogen source. In Nanno Q and Euglena, the increase in cellular lipid was not associated with an increase in lipid as a fraction of cellular dry weight. In Nannochloropsis salina, lipid transiently increased from 26-32% to 42% of ash free dry weight during nitrogen deficiency. Under anaerobiosis, lipid increased from 7% to 45% of the cellular dry weight of heterotrophic nitrogen deficient Euglena. This increase was due as much to a decrease in cellular dry weight as it was to a preferential utilization of available carbon for lipid synthesis. Nitrogen deficient and sufficient cultures of heterotrophic Euglena produce an equal amount of lipid per mole of available carbon. Nitrogen deficiency inhibits cell division so that this lipid is divided among fewer cells resulting in a higher amount of lipid per cell.

Nitrogen deficiency induces chlorophyll loss in all algae studied. In Euglena, chlorophyll loss was seen only at high light intensities. An analysis of total cellular protein by two dimensional gel electrophoresis indicated that chloroplast proteins are preferentially lost during nitrogen deficiency. The dependence in nitrogen deficient cells of chlorophyll and chloroplast protein loss on high light intensities is consistent with photooxidative damage to the chloroplast. Based on the studies with heterotrophic and photosynthetic Euglena, it appears that photooxidative damage to the chloroplast may be a major factor limiting photosynthetic lipid yields in nitrogen deficient cells.

Environmental Control of Lipid Accumulation in Nannochloropsis salina, Nanno Q and Euglena.

INTRODUCTION

A major goal of the SERI Aquatic Species Program is to develop high lipid producing microalgae which can be grown in the saline waters of the desert Southwest. By identifying naturally occurring high lipid producing algae exhibiting a wide degree of temperature and salinity tolerance, biochemical and genetic engineering techniques can be used to increase lipid yields to the point where microalgal lipids represent a viable alternative to petroleum as a feedstock for the production of liquid fuels. Nitrogen deficiency is one of the most extensively studied stresses which triggers the accumulation of large amounts of storage products which in a number of algae from diverse taxonomic groups are predominantly lipids (Ben-Amotz and Tornabene, 1985; Schifrin and Chisholm, 1981). Lipids constitute as much as 50-60% of the total cellular dry weight of some nitrogen deficient algae while in other related algae, the lipid content never exceeds 25-35% of cellular dry weight (Ben-Amotz and Tornabene, 1985; Schifrin and Chisholm, 1981). In order to improve the lipid yields from environmentally tolerant microalgae, fundamental questions concerning the physiological and biochemical mechanisms which underlie the "nitrogen lipid trigger" need to be answered. The ultimate goal will be to develop through genetic engineering an organism that synthesizes large quantities of neutral lipids in a nonstressed condition.

The rate of lipid synthesis and final amount of lipid accumulated are dependent upon the availability of carbon (photosynthate) and the activity of the enzymes required for lipid synthesis. If lipid yield is defined as the cellular lipid content, a lipid trigger could increase lipid yield by increasing the amount of carbon available for lipid synthesis by activating and or inducing the enzymes required for acetyl and malonyl-CoA synthesis as well as the enzymes of lipid synthesis. Nitrogen deficiency often drastically reduces carbon availability by reducing the capacity for photosynthetic CO_2 fixation (Macler, 1986; Osboorne and Geider 1986). Differences in lipid yield among nitrogen deficient algae could be due to differential reductions of photosynthetic CO_2 fixation during nitrogen deficiency. Lipid yield may also be defined as the fraction of total cellular dry weight which is lipid. By this definition, a lipid trigger could increase lipid yield by increasing the rate of degradation of the nonlipid fraction of the cell rather than by increasing the rate of lipid synthesis or by altering the fraction of available carbon utilized for lipid synthesis; cellular dry weight may decrease while lipid content remains unaltered. Finally, genetic differences could account for the differences in lipid yield amoung closely related algal strains.

Limitations of carbon availability (CO_2 fixation) on the lipid content of nitrogen deficient algae can be separated from limitations due to enzyme availability (level and activity) by using an external carbon source to provide both the carbon skeletons and energy needed for lipid synthesis. The external carbon supply provides carbon in excess of that which can be provided through photosynthetic CO_2 fixation. The rate at which lipids are produced under heterotrophic conditions is thus limited solely by the cells biosynthetic capacity to synthesize lipid. Comparisons of lipid synthesis by nitrogen deficient algae under heterotrophic and autotrophic conditions can thus be used to determine the extent to which lipid synthesis and ultimately yields are

limited by the rate of photosynthetic CO_2 fixation as well as to determine the extent to which a lipid trigger increases the cells biosynthetic capacity to synthesize lipids; an overall measure of the in vivo levels and activities of the lipid synthesizing enzymes. Comparisons between cellular lipid content and lipid as a fraction of cellular dry weight can be used to determine the extent to which nitrogen deficiency preferentially increases the rate of lipid accumulation and or increases the rate at which the nonlipid portion of the cell is catabolized. Cellular lipid content and lipid as a fraction of cellular dry weight have therefore been followed in Nannochloropsis salina and Nanno Q, two algae identified by SERI as promising candidates for solar lipid production and in Euglena, a lipid storing algae which grows equally well under photosynthetic and heterotrophic conditions. The results obtained conclusively indicate for the algae studied that nitrogen deficiency triggers an increase in cellular lipid content. This increase is however not always associated with an increase in the fraction of cellular dry weight which is lipid. The dramatic increase in the fraction of cellular dry weight which is lipid that is seen in anaerobic Euglena appears to be due as much to the preferential catabolism of the non lipid portion of the cell as it is to the preferential utilization of available carbon for the synthesis of lipid. Reduced carbon availability due to photooxidative damage to the chloroplast appears to represent a major limitation on the rate of lipid accumulation in nitrogen deficient photosynthetic algae.

MATERIALS AND METHODS

Strains, Media and Culture Conditions

Euglena gracilis var. bacillaris Cori has been maintained in the dark and light in our laboratory for many years. Unialgal cultures of Nannochloropsis salina and Nanno Q were obtained from the culture collection of the Solar Energy Research Institute. An axenic strain of Nannochloropsis salina was grown up from a single colony isolate obtained from the unialgal culture and used for the studies reported. All algae were grown in batch culture at 28 °C. Aeration was provided either by shaking at 150 rpm on a gyrotory shaker or for the 15 L carboys used for autotrophic lipid induction experiments, by bubbling with sterile air. Anaerobic conditions were obtained by bubbling with nitrogen which had been deoxygenated by passage through alkaline pyrogallol. Illumination was provided by banks of Cool White fluorescent lamps. Dark grown cultures were maintained in growth chambers illuminated with green safelights (Horrum and Schwartzbach, 1980).

Euglena was grown heterotrophically on EM 6.8 medium (Horrum and Schwartzbach, 1980) with ethanol as the sole source of carbon and energy. For autotrophic growth, the carbon source was omitted. Nannochloropsis salina and Nanno Q were grown on GPM medium (Haxo, 1986) containing 5 mM Tris-HCl (pH 7.8) and 5 mM sodium bicarbonate. Sea salts were obtained from Sigma chemical company and the sea salt containing medium was filtered prior to autoclaving. All transfers were performed asceptically.

Analytical Measurements

Cell number was routinely determined electronically with a Coulter Counter whose accuracy was verified with a haemocytometer. Dry weight was determined gravimetrically. Cells grown on GPM were washed three times by centrifugation prior to drying. Samples were ashed in a muffle furnace at 550 °C.

Lipids were extracted by the method of Bligh and Dyer (1959) by extraction at 60°C for one hour with each of the following solvents: once with methanol:water (1:1 v/v), twice with methanol and twice in methanol:chloroform (1:1 v/v). After each extraction, centrifugation was used to recover the nonextractable material. The combined extracts were phase separated, the chloroform was evaporated under N_2 and lipid content was determined gravimetrically.

For the determination of cellular carbohydrate and protein, cells or delipidated cells were extracted for 30 min. with 5% (v/v) perchloric acid. Protein and carbohydrate were solubilized by incubation in 1 N NaOH for 12 h at 60 ° C. Insoluble material was removed by centrifugation. Total carbohydrate was determined using anthrone reagent with glucose as a standard (Cook, 1967). In the case of Euglena, values were corrected for the difference in color development for glucose and paramylum (Cook, 1967), the major Euglena storage carbohydrate. Protein was determined by the dye binding procedure of Bradford (1976) using Bovine Serum Albumin as a standard. Total chlorophyll was determined spectrophotometrically after extraction into acetone for Euglena and chlorophyll a was determined after extraction into methanol for Nannochloropsis salina and Nanno Q (Holden, 1976).

RESULTS

Lipid Synthesis in Nanno Q

When Nanno Q was grown at 150 $\mu E\ s^{-1}\ m^{-2}$ on media containing 0.1 mM $NaNO_3$, growth ceased approximately 60 h after inoculation at a cell density of 5 x 10^6 cells per ml. The addition of 4.0 mM $NaNO_3$ 258 h after inoculation stimulated cell division indicating that growth was nitrogen rather than light limited. Cells grown at 150 $\mu E\ s^{-1}\ m^{-2}$ on media containing 4.0 mM $NaNO_3$ achieved a cell density of greater than 25 x 10^6 cells per ml. A similar culture grown at a light intensity of 300 $\mu E\ s^{-1}\ m^{-2}$ achieved a cell density of 45 x 10^6 cells per ml indicating that at 150 $\mu E\ s^{-1}\ m^{-2}$, growth with 4.0 mM $NaNO_3$ becomes light limited and not nitrogen limited.

The cellular chlorophyll content of cells grown on media containing 4.0 mM $NaNO_3$ fluctuated during growth and upon entry into the stationary phase (Fig. 1). The addition of $NaNO_3$ to the culture at 158 h after inoculation had little effect on cellular chlorophyll indicating that changes in chlorophyll content of the nitrogen sufficient culture were unrelated to nitrogen availability. The cellular chlorophyll levels of cells grown on media containing 0.1 mM $NaNO_3$ were comparable to the levels in cells grown with high levels of nitrogen until 60 h after inoculation, the time at which cell division ceased due to nitrogen deficiency (Fig. 1). Between 60-200 h after inoculation, cellular chlorophyll levels rapidly declined (Fig. 1). Increasing the light intensity to 300 $\mu E\ s^{-1}\ m^{-2}$ increased the rate of chlorophyll loss by nitrogen deficient cells (data not shown). The addition of $NaNO_3$ to nitrogen deficient cultures at 258 h after inoculation stimulated chlorophyll synthesis at both low (Fig. 1) and high (data not shown) light intensities indicating that the reduction in cellular chlorophyll content is a consequence of nitrogen deficiency.

The cellular lipid content of cells growing on media containing 4.0 mM $NaNO_3$ decreased during the initial stages of growth until a new steady state value was reached at about 100-120 h after inoculation (Fig. 1). Cell division continued throughout the experiment so that on a per ml basis, there was a

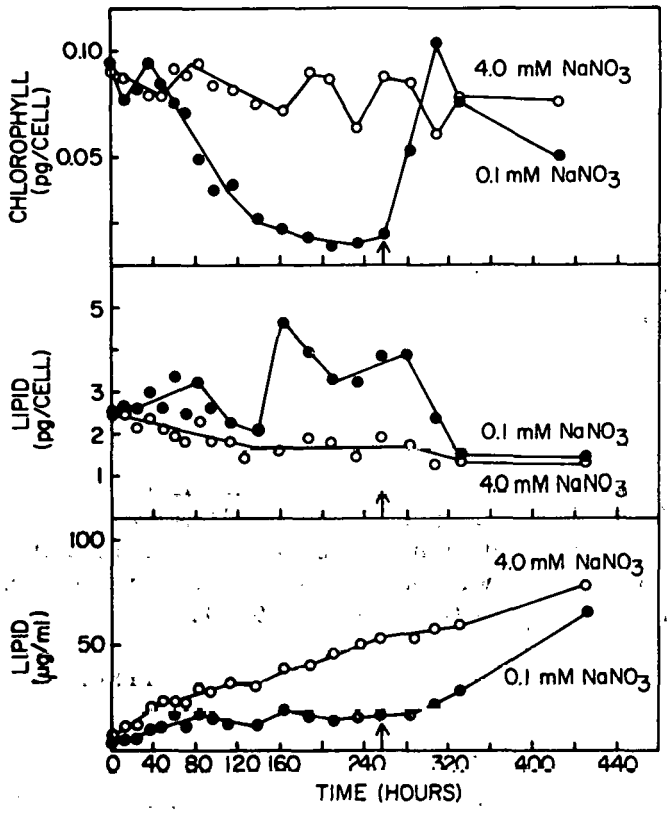


Figure 1. Chlorophyll and lipid accumulation in nitrogen deficient Nanno Q. Cells were grown on media containing high, 4.0 mM, and low, 0.1 mM, concentrations of $NaNO_3$. Chlorophyll per cell (top), lipid per cell (middle) and lipid per ml culture (bottom) were determined at appropriate intervals. The arrow indicates the time at which $NaNO_3$ was added to the cultures for a final concentration of 4.0 mM.

continuous accumulation of lipid even though the lipid per cell remained constant (Fig. 1); growth was balanced in regard to lipid synthesis. In cells grown on media containing 0.1 mM $NaNO_3$ lipid accumulation was low during the early stages of nitrogen deficiency, 60-138 h after inoculation (Fig. 1), during which time some residual cell division was seen. Cellular lipid content increased in the nitrogen deficient culture between 138-162 h after inoculation and then declined slightly (Fig. 1). This decline in cellular lipid content was due to lipid degradation rather than renewed cell division as evidenced by a decrease in the amount of lipid per ml culture (Fig 1). Cellular lipid content decreased and culture lipid content increased when $NaNO_3$ was added to the nitrogen deficient culture 258 h after inoculation (Fig. 1). The increased culture lipid content is due to the resumption of cell division after the addition nitrogen to the nitrogen deficient cells. The maximal lipid content of cells grown at 150 $\mu E\ s^{-1}\ m^{-2}$ with 0.1 mM $NaNO_3$ was 4.7 pg per cell compared to a steady state value of 1.8 pg per cell for cells grown with 4.0 mM $NaNO_3$. The maximal lipid content of cells grown at 300 $\mu E\ s^{-1}\ m^{-2}$ with 0.1 mM $NaNO_3$ was 2.6 pg per cell compared to a steady state value of 1.3 pg per cell. Growth of cells to nitrogen deficiency clearly triggered lipid accumulation. Contrary to expectations, the actual cellular lipid content was greater at low light intensities.

Cellular dry weight increased during nitrogen deficiency and decreased upon addition of $NaNO_3$ to nitrogen deficient cells (data not shown). Carbohydrates accounted for 5-8% of the ash free dry weight of nitrogen deficient and sufficient cells. At the time when cellular lipid content was highest, 162 h after inoculation, lipid represented 24% of the ash free dry weight of nitrogen

deficient cells and 23% of the ash free dry weight of nitrogen sufficient cells. These values are representative of the lipid content of Nanno Q at all stages of growth. Nitrogen deficiency altered cellular lipid content without altering the fraction of cellular dry weight which was lipid.

Lipid Synthesis in Nannochloropsis salina

When Nannochloropsis salina was grown at a light intensity of 150 $\mu E\ s^{-1}\ m^{-2}$ on media containing 0.1 mM $NaNO_3$, growth ceased approximately 53 h after inoculation at a cell density of 3.5×10^6 cells per ml. During the next 150 h a small amount of division occurred so that the final cell density achieved was 5×10^6 cells per ml. Cell densities of 35×10^6 were obtained when cells were grown on media containing 4.0 mM $NaNO_3$ indicating that growth on media containing 0.1 mM $NaNO_3$ was nitrogen limited. The cellular chlorophyll content was 0.11 pg per cell at the time cell division ceased due to nitrogen deficiency. Cellular chlorophyll decreased over a 150 h period reaching a final value of 0.02 pg per cell. The chlorophyll content of cells growing on media containing 4.0 mM $NaNO_3$ ranged from a high of 0.15 pg per cell 53 h after inoculation to a low of 0.11 pg per cell 160 h after inoculation. The loss of chlorophyll was clearly associated with nitrogen deficiency.

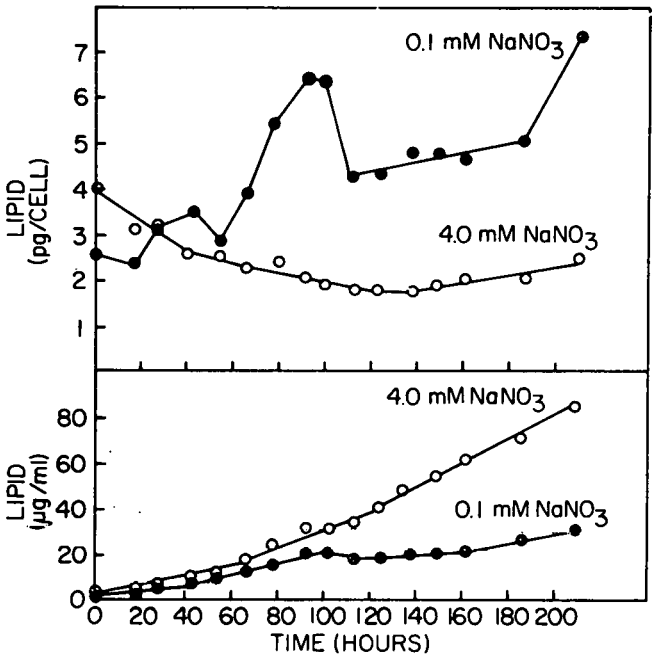


Figure 2. Lipid accumulation in nitrogen deficient Nannochloropsis salina. Cells were grown on media containing high, 4.0 mM, and low, 0.1 mM, concentrations of $NaNO_3$. Lipid per cell (top) and lipid per ml culture (bottom) were determined at appropriate intervals.

The cellular lipid content of cells growing on 4.0 mM $NaNO_3$ decreased during the first 120 h of culture and then increased slightly. The amount of lipid per ml culture increased continuously during the experiment reflecting the increase in cell number of the culture (Fig. 2). Prior to the onset of nitrogen deficiency, there was a small increase in the lipid content of cells grown on media containing 0.1 mM $NaNO_3$ (Fig. 2). Between the time cell division ceased, 53 h after inoculation, and 92 h after inoculation, the cellular lipid content increased two fold (Fig. 2). This twofold increase in cellular lipid content occurred during a time in which cell number did not change and is reflected in a twofold increase in the amount of lipid per ml culture (Fig. 2). As nitrogen deficiency progressed, there was a decline in the cellular lipid content which represents a loss in lipid as evidenced by the decrease in the amount of lipid

per ml culture (Fig. 2). This loss of lipid was also observed in the studies with Nanno Q. A similar pattern of lipid accumulation by nitrogen deficient Nannochloropsis salina was obtained using NH_4Cl as the nitrogen source (data not shown). The addition of nitrogen to the nitrogen deficient culture produced a decrease in cellular lipid content (data not shown) confirming that the triggering of lipid accumulation is a result of nitrogen deficiency.

The increase in cellular lipid content during nitrogen deficiency was accompanied by an increase in cellular ash free dry weight (data not shown). The ash free dry weight of nitrogen deficient cells was approximately twice the ash free dry weight of stationary phase light limited nitrogen sufficient cultures. Cellular carbohydrate never accounted for more than 6% of the cellular ash free dry weight. Lipids represented approximately 26-32% of the cellular ash free dry weight prior to the onset of nitrogen deficiency and in cultures grown with 4.0 mM $NaNO_3$, a concentration which does not produce nitrogen deficiency under the light intensities used in these experiments. Lipid constituted a maximum of 42% of cellular ash free dry weight at the time when cellular lipid content was greatest, 100 h after inoculation. The decline in cellular lipid between 100-113 h after inoculation was greater than the decline in cellular dry weight (data not shown) so that at 113-161 h after inoculation, the lipid fraction of the nitrogen deficient cells had been reduced to 28-30% of the cellular ash free dry weight. On a dry weight basis between 113-161h, there was little difference between the lipid content of the nitrogen deficient and sufficient culture even though on a cellular basis, nitrogen deficient cells contained more lipid than nitrogen sufficient cells.

Lipid synthesis in Euglena

Lipid Synthesis in Nitrogen Deficient Photosynthetic Cells. Autotrophic cultures of Euglena grown on media containing 5 mM NH_4Cl at a photosynthetic photon flux density of 150 $\mu E\ s^{-1}\ m^{-2}$ had a final cell density of 14×10^6 cells per ml. If the initial NH_4Cl concentration in the growth medium was reduced to 0.75 mM NH_4Cl, the final cell density was 0.52×10^6 cells per ml indicating that growth was nitrogen limited rather than light limited. The chlorophyll content of growing cells was relatively independent of the nitrogen content of the media. Upon the cessation of cell growth due to nitrogen deficiency, day 5 of culture, the cellular chlorophyll content decreased from 28 to 20 pg per cell (Fig. 3). Continued nitrogen deficiency produced a further but less rapid decline in cellular chlorophyll content (Fig. 3). The loss of cellular chlorophyll was not an artifact produced by residual cell division in the absence of chlorophyll synthesis as evidenced by a decrease in the amount of chlorophyll per ml culture (data not shown).

Prior to entry into stationary phase, the cellular carbohydrate and lipid content was unrelated to the nitrogen content of the growth media (Fig. 3). Within three days of entering stationary phase due to nitrogen limitation, day 8 of culture, the carbohydrate and lipid content of nitrogen deficient cells was clearly higher than the content of nitrogen sufficient cells (Fig. 3). Although nitrogen deficiency produced a clear increase in the amount of cellular carbohydrate and lipid, the fraction of total cellular dry weight which was lipid (18-20%) and carbohydrate (4-6%) did not change as a result of nitrogen deficiency.

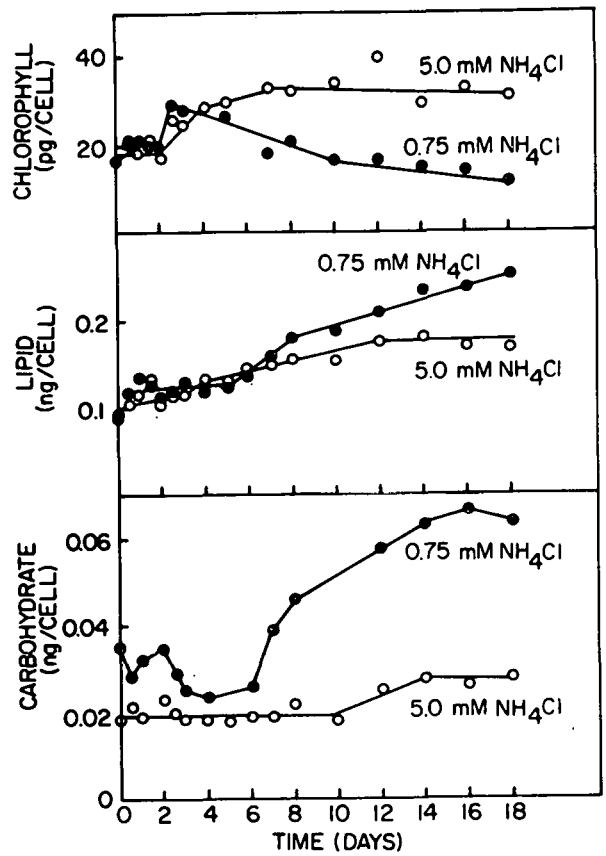


Figure 3. Chlorophyll, lipid and carbohydrate accumulation in nitrogen deficient Euglena. Cells were grown on media containing high, 5.0 mM, and low, 0.75 mM, concentrations of NH_4Cl. Chlorophyll per cell (top), lipid per cell (middle) and carbohydrate per cell (bottom) were determined at appropriate intervals.

Changes in Chloroplast Protein Levels During Nitrogen Deficiency. Chlorophyll loss and storage product accumulation have been found to be characteristic features of nitrogen deficient cells. It is unclear from most studies whether the loss of chlorophyll is indicative of a specific decline in the levels of chloroplast macromolecules or whether it is representative of the nonspecific degradation of cellular constituents during nitrogen deficiency. Two dimensional gel electrophoresis can be used to obtain a global picture of changes in the polypeptide composition of a cell during nitrogen deficiency. Of the approximately 600 polypeptides resolved on silver stained two dimensional gels of total protein extracted from dark grown resting Euglena exposed to light, 55 polypeptides have been shown to be localized in the chloroplast and 44 have been shown to be localized in the mitochondria of Euglena (Monroy, et al. 1986). Of the 511 polypeptides resolved on two dimensional gels of total protein isolated from photosynthetic nitrogen deficient Euglena, the relative amounts of 10 increase and 149 decrease from the onset of nitrogen deficiency to 6 days of nitrogen deficiency. The unchanged amount of 352 polypeptides indicates that the changes are specific rather than general changes in total cellular protein. From the start of light limited growth to 6 days of stationary phase due to light limitation, the levels of only 23 polypeptides changed in a nitrogen sufficient light limited culture indicating that the majority of the changes observed are a specific effect of nitrogen limitation. Of the 55 chloroplast localized polypeptides found in resting cells, 48 could be unequivocally localized on the polypeptide map of nitrogen deficient cells. Of these, the levels of 37 chloroplast polypeptides decreased, 3 increased and 8

showed no change in relative amount due to nitrogen deficiency. Of the 44 mitochondrial localized polypeptides found in resting cells, 36 could be unequivocally localized on the polypeptide map of nitrogen deficient cells. Of these, the levels of 11 mitochondrial polypeptides decreased, 4 increased and 21 showed no change in relative amount due to nitrogen deficiency. In the comparable light limited nitrogen sufficient culture, the relative amounts of only 4 chloroplast localized polypeptides decreased clearly indicating that a major effect of nitrogen deficiency is the selective degradation of chloroplast constituents.

Table I: Cell Yield, Chlorophyll, Carbohydrate and Lipid content of dark-grown and light-grown cultures of nitrogen deficient Euglena.

Supplements to culture	Day	Cell Number (Cells/ml x10^{-5})		Chlorophyll (pg/cell)	Carbohydrate (ng/cell)		Lipid (ng/cell)		Lipid (% Dry Weight)	
		Dark	Light	Light	Dark	Light	Dark	Light	Dark	Light
	0	13	21	4.2	0.35	0.31	0.04	0.08	5	13
168 mM Ethanol	3	38	54	2.7	0.63	0.30	0.13	0.10	7	8
+	5	29	54	2.8	0.68	0.23	0.12	0.11	7	11
7.4 mM NH_4Cl	12	36	56	3.4	0.37	0.20	0.09	0.09	10	17
	0	15	27	3.9	0.35	0.10	0.04	0.08	5	9
168 mM Ethanol	3	20	32	3.9	0.92	0.39	0.12	0.14	7	9
	5	19	24	4.1	1.90	0.43	0.24	0.24	7	10
	12	28	27	3.7	0.85	0.59	0.19	0.22	11	18
	0	15	29	4.4	0.29	0.11	0.04	0.08	5	9
7.4 mM NH_4Cl	3	17	43	10.4	0.19	0.10	0.04	0.05	5	12
	5	17	42	11.6	0.14	0.04	0.04	0.06	5	15
	12	17	38	15.8	0.11	0.05	0.03	0.06	11	22
	0	15	25	4.6	0.31	0.27	0.04	0.08	5	9
NO ADDITION	3	15	30	5.6	0.21	0.24	0.04	0.06	5	10
	5	15	29	6.6	0.21	0.09	0.04	0.07	4	12
	12	5	30	5.9	0.47	0.09	0.10	0.06	9	18

[a]Cells were grown to nitrogen deficiency in the dark or the light on media containing 168 mM ethanol, 7.4 mM NH_4Cl, washed and resuspended (day 0) in carbon-nitrogen-free growth media containing the indicated supplements.

Lipid Synthesis in Nitrogen Deficient Heterotrophic Cells. The selective loss of chlorophyll and chloroplast proteins induced by nitrogen deficiency suggests that carbon availability rather than the cellular capacity for lipid synthesis may be a major factor limiting lipid yield in nitrogen deficient cells. Euglena can use ethanol as the sole source of carbon and energy for growth. Limitations of photosynthetic CO_2 fixation on lipid yield can be distinguished from limitations due to the cellular capacity for lipid synthesis by studying lipid synthesis in nitrogen deficient Euglena supplied with ethanol as the source of carbon and energy for growth.

The final cell yield on growth limiting amounts of NH_4Cl (168 mM ethanol, 7.4 mM NH_4Cl) was always higher in the light (30 $\mu E\ s^{-1}\ m^{-2}$) than in the dark (Table I). The dry weight of cells grown in the dark was however higher than that of cells grown in the light (data not shown) The average biomass

production of 4 cultures grown to stationary phase on 168 mM ethanol 7.4 mM NH_4Cl was 1.82 mg dry weight per ml in the light and 1.57 mg dry weight per ml in the dark. Although more cells were produced in light, the total biomass produced from a constant amount of ethanol appeared to be relatively independent of light.

Nitrogen deficient cells divided when they were resuspended in fresh growth medium containing both ethanol and NH_4Cl (Table I). Cell division was accompanied by a small increase in cellular dry weight. Resuspension of nitrogen deficient cells in medium containing 168 mM ethanol but lacking a nitrogen source produced little increase in cell number confirming that the culture entered stationary phase due to nitrogen rather than carbon deficiency (Table I). Little change was observed in cellular protein content as a result of prolonged nitrogen deficiency (data not shown). There was a significant increase in cellular dry weight when 168 mM ethanol was added to a nitrogen deficient culture. The increase in cellular dry weight in the dark was greater than the increase in the light (data not shown). The maximum amount of biomass produced from 168 mM ethanol in the presence of a utilizable nitrogen source (7.4 mM NH_4Cl) was 6.5 mg per ml in the dark and 6.4 mg per ml in the light. The maximum amount of biomass produced in the absence of a utilizable nitrogen source were 6.3 mg per ml in the dark and 5.9 mg per ml in the light. The biomass yields produced in the absence of a utilizable nitrogen source are virtually identical to the yields produced in the presence of a utilizable nitrogen source (Table I) indicating that nitrogen deficiency does not limit ethanol assimilation. The cellular dry weight of nitrogen deficient cells was greater than the dry weight of nitrogen sufficient cells. Nitrogen deficiency prevented cell division resulting in the partitioning of a fixed amount of carbon amoung fewer cells.

Nitrogen deficient cells resuspended in medium containing 7.4 mM NH_4Cl but lacking a utilizable carbon source failed to divide in the dark but cell number increased in cultures maintained in the light (Table I). In the absence of both a carbon and a nitrogen source, cells did not divide in the dark and a small increase in cell number occurred in the light (Table 1). Cellular dry weight and culture biomass decreased in both the nitrogen supplemented and the unsupplemented carbon free cultures (data not shown). The decrease in dry weight per ml culture was always greater in the dark than the decrease in the light suggesting that some of the metabolic demands of carbon starved cells could be met through CO_2 fixation rather than degradation of cellular constituents.

Cellular chlorophyll levels in stationary phase cells are dependent upon cellular nitrogen status and the nature of the carbon source used for growth (Horrum and Schwartzbach, 1980). Chlorophyll levels are low in cells grown to nitrogen deficiency on ethanol (Table I). Cellular chlorophyll levels decreased in cells resuspended in medium containing both ethanol and NH_4Cl. The addition of ethanol in the absence of NH_4Cl produced no change in chlorophyll level over a twelve day period of nitrogen deficiency (Table I). Prolonged nitrogen deficiency did not result in a loss of chlorophyll when cells were maintained at low light intensities. Resuspension of ethanol grown cells in medium lacking both a source of carbon and nitrogen resulted in a small increase in cellular chlorophyll content (Table I). A large increase in cellular chlorophyll content was produced by the addition of NH_4Cl to nitrogen deficient cells (Table I). As found previously, the presence of ethanol represses chlorophyll synthesis in Euglena (Horrum and Schwartzbach, 1980). When ethanol is removed from the

culture, a major increase in chlorophyll is only seen when there is an adequate supply of nitrogen (Horrum and Schwartzbach, 1980).

Paramylum, a ß1-4 glucan, and lipids, predominantly the wax ester myristyl myristate, are the major identified storage reserves of Euglena (Inui et al., 1982; Hulanicka et al., 1964). In order to determine whether the increase in cellular dry weight produced by nitrogen deficiency is due to a change in the partitioning of available carbon between reserves and other cellular constituents, the cellular level of carbohydrate and lipid were followed in the dark or the light. Little change was seen in the cellular carbohydrate and lipid content of carbon free nitrogen deficient (no addition) and nitrogen sufficient cultures (NH_4Cl) maintained in the dark or the light for 5 days (Table I). Between 5 and 12 days of culture, the cellular content of storage products declined. The cellular lipid and carbohydrate content of nitrogen deficient cells increased after the addition of 168 mM ethanol to cells maintained in the dark or the light (Table I). Ethanol supplemented nitrogen deficient cells maintained in the light had about the same lipid content 5 and 12 days after carbon supplementation as cells maintained in the dark (Table I). Cells maintained in the dark however accumulated more carbohydrate after ethanol addition than cells maintained in the light (Table I). The cellular lipid content of nitrogen deficient cells supplemented in the dark with both 168 mM ethanol and 7.4 mM NH_4Cl increased during the first 5 days of supplementation and then declined (Table I). In the light, the cellular lipid content increased slightly after the addition of both 168 mM ethanol and 7.4 mM NH_4Cl (Table I). The carbohydrate content of carbon and nitrogen supplemented cells increased in the dark and declined in the light. On a cellular basis, cellular carbohydrate and lipid levels showed the largest increase when nitrogen deficient cultures were supplied with a utilizable source of organic carbon in the absence of a utilizable nitrogen source (Table I). It should be noted that the cellular carbohydrate content of nitrogen deficient autotrophic cells (Fig. 3) was only 10% of the carbohydrate content of ethanol supplemented nitrogen deficient light grown cells (Table I). The final lipid content of nitrogen deficient autotrophic cells (Fig. 3) however approached that of ethanol supplemented nitrogen deficient cells (Table I) even though the rate of lipid accumulation under autotrophic conditions was much less than under heterotrophic conditions.

As found for total biomass yield, the total amount of lipid and carbohydrate synthesized from a fixed amount of ethanol was not increased by nitrogen deficiency (data not shown). Lipid constituted approximately 7% of the dry weight of stationary phase cells grown in the dark and 10% of the dry weight of stationary phase cells grown in the light to nitrogen deficiency (Table I). Although nitrogen deficiency triggered an increase in the amount of cellular lipid, this increase was not reflected as an increase in the fraction of cellular dry weight which was lipid (Table I). The partitioning of available carbon between the synthesis of lipid and other cellular constituents was relatively unaltered during the first five days after the addition of ethanol to nitrogen deficient cells. Nitrogen deficiency inhibited cell division so that the lipid and carbohydrate synthesized was partitioned amoung fewer cells resulting in an increased cellular lipid and carbohydrate content. By 12 days after the onset of nitrogen deficiency, the fraction of cellular dry weight which was lipid increased in all cultures regardless of the addition of ethanol and or NH_4Cl (Table I). This increase was due to the greater stability of cellular lipid relative to other cellular constituents; the decrease in cellular dry weight between 5-12 days of nitrogen deficiency was greater than the

decrease in cellular lipid.

Lipid Synthesis Triggered by Anaerobiosis. Anaerobic conditions increase the fraction of total cellular dry weight which is lipid (Inui et al., 1983). This increase results from the degradation of carbohydrate coupled to an increase in utilization of available carbon for lipid synthesis (Inui et al., 1982). Carbohydrate degradation is reported to be a nitrogen requiring process in Euglena and other algae (Miyachi and Miyachi, 1985; Nammori et al., 1977). If carbohydrate degradation could be induced in nitrogen deficient cells in the absence of lipid degradation, the fraction of cellular dry weight which is lipid would increase. Attempts were therefore made to specifically alter the carbohydrate content of Euglena by incubating cells under anaerobic conditions at both the onset and after prolonged (6 days) nitrogen deficiency.

Table II: Dry Weight and Lipid accumulation upon transfer of aerobic Euglena to anaerobic conditions.

Growth Conditions	DAY	Lipid (ng/cell)		Dry Weight (ng/cell)		Lipid (% of dry weight)	
		aerobic	anaerobic[a]	aerobic	anaerobic[a]	aerobic	anaerobic[a]
Nitrogen Deficient	0	0.28	0.28	4.24	4.24	7	7
ethanol Supplemented	1	0.28	0.32	4.20	3.64	7	9
DARK[b]	2	0.26	0.40	4.00	3.60	7	11
Nitrogen Deficient	0	0.22	0.22	2.20	2.20	10	10
ethanol Supplemented	1	0.22	0.22	1.78	1.58	12	14
LIGHT[b]	2	0.20	0.36	1.76	1.42	12	25
Carbon Deficient	0	0.08	0.06	0.98	1.22	8	5
Nitrogen Sufficient	1	0.06	0.18	0.78	0.80	8	23
DARK[c]	2	0.06	0.18	0.68	0.68	9	27
Nitrogen and Carbon	0	0.06	0.06	1.07	1.07	6	6
Deficient	1	0.08	0.09	1.31	0.93	6	10
DARK[b]	2	0.08	0.11	0.93	0.93	9	12
Early Nitrogen							
Deficient ethanol	0	0.10	0.10	1.36	1.38	7	7
supplemented	3	0.16	0.32	2.39	0.97	7	33
DARK[d]	6	0.20	0.39	2.50	0.87	8	45

[a]Cells were made anaerobic by purging the culture media with nitrogen passed through 10% pyrogallol in 15% KOH.

[b]Cells were grown to nitrogen deficiency in the dark or the light on media containing 168 mM ethanol, 7.4 mM NH_4Cl, washed and resuspended in nitrogen free media with or without the addition of 168 mM ethanol. Six days after resuspension, day 0, a portion of the culture was made anaerobic.

[c]Cells were grown in the dark on media containing 84 mM ethanol and 13 mM NH_4Cl. Day 0 corresponds to the first day after cell division ceased due to carbon depletion.

[d]Cells were grown to nitrogen deficiency in the dark on media containing 168 mM ethanol, 7.4 mM NH_4Cl, washed and resuspended in media containing 168 mM ethanol. At the time of resuspension, (day 0), a portion of the culture was made anaerobic.

The transfer of ethanol supplemented nitrogen deficient dark grown or light grown cells from aerobic to anaerobic conditions after six days of nitrogen deficiency produced an increase in cellular lipid (Table II) and a decrease in the cellular carbohydrate content (data not shown). The increase in cellular lipid was accompanied by a decrease in the cellular dry weight so that anaerobiosis produced a doubling in the percent of cellular dry weight which was lipid (Table II). Lipids constituted a greater fraction of cellular dry weight of cells grown in the light (Table II) than in the dark.

Cells grown on 84 mM ethanol and 13 mM NH_4Cl enter stationary phase due to carbon rather than nitrogen limitation. The transfer of these carbon deficient nitrogen sufficient cells from aerobic to anaerobic conditions triggered lipid synthesis (Table II). Under both aerobic and anaerobic conditions, cellular dry weight decreased while the cellular lipid content of carbon deficient nitrogen sufficient cells decreased slightly under aerobic conditions but increased under anaerobic conditions (Table II). After two days of anaerobiosis, lipid had increased from 4.9% to 26.5% of the cellular dry weight (Table II). Cells which were grown on limiting amounts of NH_4Cl and maintained for 6 days on carbon and nitrogen-free medium also synthesized lipid upon transfer from aerobic to anaerobic conditions (Table II). Both cellular lipid content and lipid as a fraction of cellular dry weight were increased under anaerobiosis (Table II). Taken together, these results clearly indicate that under anaerobic conditions and independent of light, carbon and nitrogen availability, lipids are synthesized while other cellular components are preferentially degraded.

Dark grown nitrogen deficient cells supplemented with 168 mM ethanol at the time cell division ceased (early nitrogen deficient) accumulated lipid under both anaerobic and aerobic conditions (Table II). The rate and extent of lipid synthesis under anaerobic conditions was twice as great as under aerobic conditions. The carbohydrate content of nitrogen deficient aerobic cells more than doubled while under anaerobic conditions virtually all of the cellular carbohydrate was degraded (data not shown). The anaerobically triggered synthesis of lipid during the initial 6 days of nitrogen deficiency was not accompanied by an increase in cellular dry weight as occurred under aerobic conditions (Table II). Thus after 6 days of anaerobiosis, the cellular lipid content of ethanol supplemented nitrogen deficient cells increased from 6% to 45% of cellular dry weight (Table II) while on a dry weight basis, there was no increase in the lipid content of the aerobic cells. Changes in cellular lipid content are not always mirrored by a change in the lipid fraction as a percentage of cellular dry weight.

DISCUSSION

Photosynthetic and heterotrophic growth of Nanno Q, Nannochloropsis salina and Euglena was found in batch culture to be unbalanced with respect to storage compounds, dry weight and chlorophyll. Lipids were the major storage products in Nannochloropsis salina and Nanno Q while Euglena accumulated both lipid and carbohydrate. In all three algae, the cellular content of storage products increased when cell division became limited due to nitrogen deficiency. The cellular content of storage products did not increase when growth of a nitrogen sufficient culture ceased due to light limitation. In our hands, lipid accumulation was higher under low light than under high light for a nitrogen deficient culture. The increase in cellular lipid and or carbohydrate content was clearly a result of nitrogen deficiency as evidenced by the decrease in storage products upon the addition of nitrogen to the deficient cultures.

The actual lipid yield per ml of culture was always much less for a nitrogen deficient culture over a given time interval than the yield for a nitrogen sufficient culture. Under photosynthetic conditions, this difference could simply be due to the increased amount of CO_2 fixed due to the higher cell density of the nitrogen deficient culture rather than to a failure of nitrogen deficiency to specifically trigger an increase in lipid accumulation. By using heterotrophic culture conditions, it was possible to provide nitrogen deficient and sufficient cultures with equal amounts of carbon and determine whether a greater fraction of the available carbon was utilized for storage product accumulation by nitrogen deficient cells. As Euglena is the only algae in this study which could utilize exogenous carbon as the sole source of carbon and energy for growth, these experiments could only be performed with Euglena. In contrast to autotrophic Euglena cultures, there was little difference in the amount of lipid per ml culture for nitrogen deficient and sufficient Euglena provided with equal amounts of ethanol as the sole source of carbon and energy for growth. Nitrogen deficient cultures contained more lipid per cell but the lower amount of cellular lipid found in nitrogen sufficient cells was compensated for by a larger number of cells per ml of culture. On a culture basis, the total amount of lipid produced per mole of available carbon (ethanol) was not increased by nitrogen deficiency. Nitrogen deficiency simply allows for the accumulation of a fixed amount of lipid in the absence of cell division resulting in an increase in cellular but not culture lipid content. Under photosynthetic conditions, the lipid content of the culture is limited by the amount of CO_2 which can be fixed by the culture; a nitrogen sufficient culture produces more lipid because the larger number of cells per ml results in a greater quantity of fixed carbon.

The breakdown of carbohydrate and the partitioning of all of the available carbon for lipid synthesis are induced by anaerobiosis in Euglena (Inui et al., 1982, 1983). In contrast to nitrogen deficiency, anaerobiosis produces a major increase in the fraction of cellular dry weight which is lipid. Lipid increased from 7% to 45% of the cellular dry weight of an ethanol supplemented nitrogen deficient culture during 6 days. Lipid remained as 7% of the cellular dry weight of the aerobic control even though on a per cell basis, the lipid content of the aerobic nitrogen deficient cells doubled. The dry weight of the aerobic cells increased while in the anaerobic culture, the cellular dry weight decreased. If under anaerobic conditions the cellular dry weight had not decreased but had increased to the same extent as seen under aerobic conditions, the lipid content of the anaerobic cells would only represent 15% of cellular dry weight. Thus although both nitrogen deficiency and anaerobiosis induced lipid synthesis, it is not the actual amount of lipid accumulated but rather the marked difference between the dry weight of the anaerobic (0.87 pg per cell) and the aerobic (2.5 pg per cell) culture which is the major factor responsible for the difference between the lipid content of these cells on a dry weight basis. Based on the lipid content as a fraction of cellular dry weight, the greater efficiency of anaerobiosis as a lipid trigger appears to stem as much if not more from its promotion of the degradation of the non lipid fraction of the cell as it does from the preferential partitioning of available carbon to lipid synthesis through the activation of a mitochondrial pyruvate dehydrogenase complex (Inui et al., 1984).

The accumulation of lipid triggered by nitrogen deficiency in Euglena occurred in the absence of an increase in the fraction of cellular dry weight which is lipid. Incubation of carbon or nitrogen deficient Euglena for extended periods of time however produced an increase in lipid as a fraction of cellular

dry weight. Between 6-12 days of stationary phase, lipid as a fraction of cellular dry weight doubled regardless of the nutrient limiting cell growth. This increase was clearly independent of nitrogen deficiency triggered lipid synthesis and it reflected a decrease in cellular dry weight rather than an increase in the amount of lipid accumulated by the cell. Lipid was more stable in the absence of cell division than other cellular constituents. It was prolonged incubation under nondividing conditions which resulted in an increase in lipid as a fraction of cellular dry weight. We and others have had difficulty in reproducing reported nitrogen deficiency triggered increases in lipid as a percent of dry weight. This difficulty could be due to the fact that these increases stem from decreases in cellular dry weight produced by culture conditions unrelated to nitrogen status such as length of time in stationary phase, the age of the culture. It is thus extremely important to make comparisons between nitrogen deficient cells and nitrogen sufficient control populations of the same culture age.

A ubiquitous response of algae to nitrogen deficiency is a loss of chlorophyll. In all of the algae studied and in Chlorella SO1 (unpublished), chlorophyll levels rapidly decline during the early stages of nitrogen deficiency and then appear to stabilize. Chlorophyll loss in nitrogen deficient Ankistrodesmus is preceded by a major decline in the rate of CO_2 fixation presumably resulting from the preferential degradation of a number of photosynthetic enzymes (Hipkin and Syrett, 1977). Cryptomonas maculata selectively losses proteins from both photosystems during nitrogen deficiency (Rhiel et al. 1986). Studies using two dimensional gel electrophoresis to separate total cellular protein from nitrogen deficient and sufficient Euglena indicated that nitrogen deficiency led to a preferential loss of chloroplast proteins. Of the chloroplast localized polypeptides which were unambiguously found on gels from nitrogen deficient cells, the levels of 77% decreased as a specific result of nitrogen deficiency while the levels of only 31% of the mitochondrial polypeptides decreased as a result of nitrogen deficiency. The preferential loss of chloroplast proteins and of chlorophyll which results from nitrogen deficiency undoubtedly reduces the rate of photosynthetic CO_2 fixation. This in turn limits the availability of carbon for lipid synthesis.

Chlorophyll levels did not decline during nitrogen deficiency in Euglena and Chlorella SO1 (unpublished) grown heterotrophically at low light intensities (30 $\mu E\ s^{-1}\ m^{-2}$) rather than the high light intensities (greater than 150 $\mu E\ s^{-1}\ m^{-2}$) used for photosynthetic growth. The dependence of chlorophyll and chloroplast protein loss on high-light intensity suggests that this loss results from photooxidative damage to the chloroplast. Photooxidation is can cause the destruction of chlorophyll and loss of photosynthetic enzymes in Euglena as well as in higher plants (Vaisberg and Schiff, 1976; Mayfield et al., 1986). The synthesis of aminolevulinic acid, the first committed precursor of chlorophyll is inhibited by nitrogen deficiency (Ohmori et al. 1984). A reduction in the rate of aminolevulinic acid synthesis will lower the rate of chlorophyll synthesis. Under the high light intensity required for autotrophic growth, there is a significant amount of chlorophyll destruction due to photooxidative damage. Chlorophyll levels decline until a new steady state is reached in which the rate of chlorophyll resynthesis balances the rate of chlorophyll destruction through photooxidation. In nitrogen sufficient autotrophic cells, the rate of chlorophyll synthesis and synthesis of chloroplast proteins is more than sufficient to replace any losses due to photooxidation. Photooxidative damage is slight at the low light intensities used for heterotrophic growth. A reduction in the rate of chlorophyll synthesis produced by nitrogen deficiency thus has

little effect on the level of chloroplast constituents. Nitrogen deficiency reduces the rate at which chlorophyll is resynthesized and total cellular chlorophyll levels decline. A reduction in light intensity which would reduce photooxidative damage would also lower the rate of photosynthesis while an increase in light intensity would increase photooxidative damage. Due to the increased susceptibility of nitrogen deficient cells to photooxidative damage it will probably be extremely difficult to improve lipid yield in nitrogen deficient cells by increasing the net rate of photosynthetic CO_2 fixation through increased light intensities.

Studies of photosynthetic and heterotrophic lipid accumulation in a number of algae have unequivocally shown that cellular lipid content increases during nitrogen deficiency. A major assumption of this work has been that the rate of lipid accumulation is a measure of the rate of lipid synthesis. This assumption may not be valid. For example, anaerobic conditions inhibit lipid degradation in Euglena. Lipid levels immediately decline when anaerobic nitrogen sufficient or deficient cells are made aerobic (Inui et al., 1982). The efficiency on a dry weight basis of the anaerobic trigger stems as much from the degradation of the nonlipid fraction and stabilization of the lipid fraction of the cell as it does from the preferential partitioning of available carbon for lipid synthesis. Lipid accumulation under nitrogen limitation may be limited by an increase in the rate of lipid degradation as the cells become carbon starved due to the reduction in photosynthetic CO_2 fixation resulting from the photooxidative destruction of the photosynthetic apparatus. The preliminary conclusions drawn from the current series of experiments must therefore be confirmed by direct measurements of photosynthetic rates and the partitioning of photosynthetically fixed CO_2 into lipid under nitrogen deficient and sufficient conditions. Only these studies can identify the relative importance of lipid synthesis and degradation rates on the final lipid yield expressed both as amount per cell or fraction of total cellular dry weight.

ACKNOWLEDGEMENTS

This work was supported by subcontract XK-5-05073-3 from the Solar Energy Research Institute.

BIBLIOGRAPHY

Ben-Amotz, A. and Tornabene, T.G. 1985. "Chemical Profile of Selected Species of Microalgae with Emphasis on Lipids." J. Phycol. 21:72-81.

Bligh, E.G. and Dyer, W.J. 1959. "A Rapid Method of Total Lipid Extraction and Purification." Can. J. Biochem. Physiol. 37:911-917.

Bradford, M.M. 1976. "A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding." Anal. Biochem. 72:248-254.

Cook, J.R. 1967. "Quantitative Measurement of Paramylum in Euglena gracilis. J. Protozool. 14:634-636.

Haxo, F. 1986. In Microalgae Culture Collection 1985-1986. Solar Energy Research Institute. Golden, Colorado.

Hipkin, C.R. and Syrett, P.J. 1977. "Some Effects of Nitrogen-Starvation on Nitrogen and Carbohydrate Metabolism in Ankistrodesmus braunii. Planta 133:207-214.

Holden, M. 1976. "Chlorophylls." In Chemistry and Biochemistry of Plant Pigments Vol. 2. Edited by T.W. Goodwin. Academic Press, London, pp. 1-37.

Horrum, M.A., and Schwartzbach, S.D. 1980. "Nutritional Regulation of Organelle Biogenesis in Euglena." "Repression of Chlorophyll and NADP-Glyceraldehyde-3-Phosphate Dehydrogenase Synthesis." Plant Physiol. 65: 382-386.

Hulanicka, D., Erwin, J. and Bloch, K. 1964. "Lipid Metabolism of Euglena gracilis." J. Biol. Chem. 239:2778-2787.

Inui, H., Miyatake, K., Nakano, Y., and Kitaoka, S. 1982. "Wax Ester Fermentation in Euglena gracilis. FEBS Letters 150:89-93

Inui, H., Miyatake, K., Nakano, Y., and Kitaoka, S. 1983. "Production and Composition of Wax Esters by Fermentation of Euglena gracilis." Agric. Biol. Chem. 47(11):2669-2671.

Macler, B.A. 1986. "Regulation of Carbon Flow by Nitrogen and Light in the Red Alga, Gelidium coulteri. Plant Physiol. 82:136-141.

Mayfield, S.P., Nelson, T., and Taylor, W.C. 1986. "The Fate of Chloroplast Proteins During Photooxidation in Carotenoid-Deficient Maize Leaves." Plant Physiol. 82:760-764.

Miyachi, S. and Miyachi, S. 1985. "Ammonia Induces Starch Degradation in Chlorella Cells." Plant and Cell Physiol. 26:245-252.

Monroy, A.F., Gomez-Silva, B., Schwartzbach, S.D., and Schiff, J.A. 1986. "Photocontrol of Chloroplast and Mitochondrial Polypeptide Levels in Euglena." Plant Physiol. 80:618-622.

Nammori, T., Miyatake, K. and Kitaoka, S. 1977. "Degradation of Paramylon, a Storage Carbohydrate of Euglena by Addition of Nitrogen Sources." Nippon Nogeikagaku 52:N195.

Ohmori, M., Wolf, F.R. and Busshan, J.A. 1984. Botryococcus braunii Carbon/Nitrogen Metabolism as Affected by Ammonia Addition." Arch. Microbiol. 140:101-106.

Osborne, B.A. and Geider, R.J. 1986. "Effect of Nitrate-Nitrogen Limitation on Photosynthesis of the Diatom Phaeodactylum tricornutum Bohlin (Bacillariophyceae)." Plant, Cell and Environment 9:617-625.

Rhiel, E., Krupinska, K., and Wehrmeyer 1986. "Effects of Nitrogen Starvation on the Function and Organization of the Photosynthesis Membranes in Cryptomonas maculata (Cryptophyceae)." Planta 169:361-369.

Schifrin, N.S. and Chisholm, S.W. 1981. "Phytoplankton Lipids: Interspecific Differences and Effects of Nitrate, Silicate and Light-Dark Cycles. J. Phycol. 17:374-384.

Vaisberg, A.J. and Schiff, J.A. 1976. "Events Surrounding the Early Development of Euglena Choloroplasts VII." "Inihibition of Caratenoid Biosynthesis by the Herbicide SAN 9789 (4-Chloro-5-(Methylamino)-2-(α,α,α, -Trifluoro-M-Tolyl)-3(2H)Phyradazinone) and its Developmental Consequences." Plant Physiol. 57:260-269.

Genetics

THIS PAGE

WAS INTENTIONALLY

LEFT BLANK

PATTERNS OF GENETIC DIVERSITY IN THREE GENERA OF OIL-PRODUCING MICROALGAE

Jane C. Gallagher
Biology Dept., City College of New York
New York, N.Y. 10031

ABSTRACT

It is likely that the long-term economic feasibility of utilizing microalgae for fuel production will be dependent on the development of strains that have been genetically altered to improve lipid yields. The strategy to be used for the development of improved strains depends on the patterns of genetic diversity found among the available wild-type strains in each species. Therefore, an investigation was undertaken of the genetic diversity in 3 types of microalgae with potential for oil production. Both gel electrophoresis and comparisons of the physiological traits were used. The former technique can be used to classify clones into discrete groups and the banding patterns can be used as genetic markers in later manipulations. Examination of physiological traits yields information on continuous characteristics that are directly relevant to how different strains might perform in culture.

These techniques were used on strains of Nannochloris spp. (Chlorophyceae) and Amphora coffaeformis (Bacillariophyceae) that were obtained from natural populations and from existing culture collections. Nannochloropsis spp. strains were examined using strains obtained from culture collections only. In all three organisms, genetic diversity was found to be extremely high compared to terrestrial plants. This was shown by both the physiological traits and the enzyme banding patterns. There were no apparent relationships among patterns of differentiation revealed by different traits nor by the type of environment from which clones were isolated. In general, strains obtained from existing culture collections showed greater diversity as a group than did strains freshly collected from natural populations. However, even stringently selective sampling protocols applied to natural populations yielded a variety of genetically different strains. This probably indicates that the total genetic diversity in the species examined was underestimated.

The amount of genetic diversity shown by these organisms coupled with previously collected data on other microalgae demonstrates that "species" of microalgae have far greater intrinsic genetic diversity than do species of higher plants. These data indicate that classical breeding approaches to strain improvement will probably not be successful even in the Chlorophyceae, which is the group of algae most similar to higher plants. The best strategy for strain improvement will most likely be dependent on the development of novel recombinant DNA techniques.

STUDIES OF THE CHLOROPLAST GENOME OF EXSYMBIOTIC AND FREE-LIVING CHLORELLA

Russel H. Meints
Professor, Biology
School of Biological Sciences
The University of Nebraska
Lincoln, NE 65588

ABSTRACT

The goal of this project was to characterize the chloroplast genome of a model microalgal species and in doing so to determine whether these methods might serve as a diagnostic device for identification of specific Chlorella with capability of biofuel production. If this were so, such genomes would be extremely useful for determining relationships between groups of algae and might determine the strategy for selection of partners in cell hybrid fusions; the ultimate goal of this research being the establishment of a genetic system within this asexual group. It is generally considered that unless a reasonably close relationship exists between fused partners, that stability of the hybrid is limited. Isolated chloroplast and nuclear genomic DNA from our model exsymbiotic algae (two isolates) and from eleven free-living Chlorella have been analyzed for relationships. Maps of the genomes were produced for an exsymbiotic and a free-living strain. Based on our preliminary investigations it appears that algae can be easily grouped into specific classes based on their DNA restriction length polymorphisms. Exsymbiont N1a and Chlorella kessleri (UTEX-20) have been subjected to detailed analysis to determine their fine structural organization. While our study was by no means exhaustive, this preliminary investigation has convinced us that many or all algae could be analyzed in this manner and that substantial information about algal relationships might be derived.

INTRODUCTION

The chloroplast (cp) genomes of aquatic photosynthetic protists have been little studied even though similar genomes in a variety of higher plants have been carefully investigated. Most known cp genomes have common properties, i.e., closed circular DNA molecules, a size of approximately 150 kbp and an inverted repeated sequence of about 20 kbp which contains the the 23S, 16S, and 5S rRNAs (Bedbrook, et al., 1977). The complete nucleotide sequences are known for Marchantia polymorpha and Nicotiana tabacum (Ohyama, et al., 1986, Shinozaki et al., 1987). The gene content of these organisms appears to be highly conserved, so remarkably so that it might be argued that all cp genomes have arisen from a single ancestor.

Among the exceptions to the gross structure of cp genomes thus far studied in the higher plants, the common pea and mung bean differ by containing a single copy of the ribosomal genes (no inverted repeat) (Kolodner and Tewari, 1979; Koller and Delius, 1980). Of those cases known in algae, Euglena differs from the normal by lacking the paired inverted repeats, containing instead a series of tandem repeats, three in number (Rawson, et al., 1978).

In addition to the broad taxa of free-living chlorellans there also exist Chlorella-like algae in hereditary intra-cellular relationships with a variety of primitive uni- and multi-cellular animal hosts. The most studied of these symbiotic relationships is the green coelenterate Hydra viridis and the green protozoan Paramecium bursaria (Trench, 1979).

We have discovered a unique interaction of these specific exsymbiotic Chlorella-like alga with a family of viruses which synchronously infects, replicates within and lyses its algal host. PBCV-1, the most studied of the group is a large (190 nm in diameter), icosahedral plaque-forming virus which replicates only in Chlorella-like algae originally isolated from Paramecium bursaria (Van Etten et al., 1983a, 1983b). Following PBCV-1 infection, progeny virus first appear 2 to 3 hr after infection; by 5 hr PBCV-1 release is complete. PBCV-1 contains a lipid component, at least 50 structural proteins, and a large dsDNA genome, ca. 330kbp as estimated by summing restriction fragments and mapping studies (Van Etten et al., 1982; Skrdla et al., 1984; Girton and Van Etten, 1987).

Since the virus synchronously infects its host, the PBCV-1 Chlorella system is a useful model for studying gene regulation in a photosynthetic eukaryotic organism and we have begun to study DNA replication (Van Etten et al., 1984) and viral transcription (Schuster et al., 1986). Based on similar examples in the animal and higher plant scientific literature we have proposed that this virus could be exploited to create a genetic system in simple asexual photosynthetic micro-algae, either by development of hybrids from fusion of cell protoplasts or by transfer of recombinant DNA to protoplasts. The former is possible because of a cell wall degrading enzyme encoded for by the virus which is released at the time of lysis and the latter because of the availability of a vectoring system. Thus, we have become interested in questions concernifg the a|ga| host: what are its ancestors and what are its nearest relatives? Answers to such questions are basic to our ability to make meaningful decisions concerning strategies for genetic manipulations. In an attempt to determine these lineages we have used the chloroplast genome as a guide for the study of these relationships. Here we report on the genomes of two exsymbiotic algae and show

their relationships to 11 strains of Chlorella purported to be from several different species. Preliminary investigation of the data indicates that previously used taxomonic parameters may be inadequate to show definitive relationships and that analysis of the highly conserved chloroplast genome may be more useful to determine the relationships (or the lack thereof) within this group.

MATERIALS AND METHODS

Growth of the Chlorella. The exsymbiont, Chlorella strain N1a, was colony purified from a mass algal isolate originated by Dr. R.L. Pardy from a Paramecium bursaria culture (personal communication). The algae were grown axenically in flasks on liquid Bolds basal medium (BBM) (Nicols and Bold, 1965), modified to contain 0.25% sucrose and 1.0% proteose peptone (KBBM). Cultures are maintained under constant fluorescent light (45 uEi) at 25 C with gentle shaking. In our hands this medium provides optimal growth. Tetracycline was added to the culture medium at a final concentration of 25 ug/ml to prevent bacterial contamination.

Free-living Chlorella sp., UTEX strains 20, 262, 362, 363, 395, 397, 398, 490 and 1602, were obtained from the culture collection at the University of Texas, Chlorella strain CCAP 211-8p was from the Cambridge University Collection and Chlorella strain S01 is an isolate from the Solar Energy Research Lab (SERI). These strains were maintained as for N1a.

Harvesting cells. Cultured cells were harvested at late log phase by centrifugation at 5000 rpm for 5 min in a Sorvall HS-4 rotor. The cells were washed 2 times with Bold Basal Medium (BBM) and once with TE buffer (50 mM Tris-HCl, pH 8.0; 10 mM EDTA) to prevent nuclease activity during the subsequent breakage steps. The pelleted cells were used fresh or were frozen at -80 C and stored until needed.

Preparation of DNA. DNA was prepared according to modified procedures originally developed by Yelton et al. (1984). Pelleted, fresh or frozen cells were placed in liquid N_2 for 10 min before use. All equipment used for the cell breakage procedures had been previously washed, sterilized and stored at -20 C. All of the cell breakage operations were conducted at 4 C. Frozen cell pellets were mixed with sterile sea sand and ground in a mortar under liquid N_2 for up to 5 min. Maintenance of temperature is critical throughout these operations. The ground cells and sand were mixed thoroughly with 20 ml suspension buffer (50 mM Tris, 50 mM EDTA, pH 8.0, containing 0.2 % SDS). This suspension buffer should be at 23 C. The tubes were capped and shaken for 1 min at 23 C. The cell suspensions were spun in an HB4 rotor at 8500 rpm for 15 min at 4 C to remove sand and cell debris. 4M potassium acetate was added to the supernatant at 1/8 vol and the mixture was incubated on ice for 60 min. The mixture was centrifuged in a SS34 rotor at 14500 rpm for 15 min at 4 C to pellet the precipitated membraneous subcellular elements. Nucleic acids were precipitated by incubaton of the supernatant with 2 vol of cold 100% ETOH for 30 min at -70 C. The precipitate was collected by centrifugation in a SS34 rotor at 10,000 rpm for 20 min at 4 C. The nucleic acid pellet was resupended in 10 ml of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) to which 100 ul of RNAse (1 mg/ml) had been added. The RNA was digested at 23 C for 60 min and then the DNA was precipitated by addition of 2 vols cold ETOH at -20 C overnight. The DNA was collected by centrifugation in a HB4 rotor at 10000 rpm for 20 min. The DNA

pellet was resuspended in 2 ml TE buffer and then 300 ul of protease K (2 mg/ml) was added to remove protein by incubation for 60 min at 23 C. The DNA was further purified on CsCl gradients. The gradients were spun in a Beckman Model L5-70 ultracentrifuge in a 50Ti rotor for 66 hours at 38,000 rpm at 25 C. At equilibrium, the gradients were fractionated using an ISCO fractionator with absorbance monitor. Fractions containing the chloroplast and nuclear genomes were dialyzed against TE buffer (3 changes) for 24 hr. The DNA was precipitated with cold 100% ETOH after the addition of 1/10 vol of 3M Sodium Acetate. The pellets were washed with 70% ETOH and dried under vacuum. The DNA was resuspended in TE buffer and held at 4 C until used.

RNA and DNA analysis. The DNA was digested with various restriction endonucleases using the protocols developed by the suppliers. Digestion fragments were separated by horizontal agarose gel electrophoresis using 0.7% gels in TPE buffer (0.08 M Tris-phosphate, 0.002 M EDTA, pH 8.3) Gels were stained for 30 min in 0.5 ug/ml ethidium bromide and photographed with exposure to UV light using Type 55 or 57 Polaroid film. RNA was isolated and electrophoresed according to Schuster et al. (1986).

Isolation of ctDNA probes. In order to produce homologous probes, algal ctDNA was cloned. Incomplete libraries were made for several of the restriction enzymes used for the mapping purposes. Total digests of N1a DNA with SalI, XhoI. SstI and KpnI were ligated into pUC 19 vector linearized with the same enzymes. The plasmids containing inserts were transformed into E. coli strain TB1 and amplified for use. UTEX20 ctDNA was digested with KpnI, and ligated into pUC19. E. coli strain TB1 was transformed by the $CaCl_2$ method of Maniatis et al. (1982). Positive clones were selected for on X-gal/ampicillin agar plates and plasmids were isolated using an alkaline/SDS method of Maniatis et al. (1982).

Those cp restriction fragments which were not cloned were used as probes after extraction from agarose gels. DNA was run out and excised from 0.6% agarose gels in TAE buffer (0.04 M Tris-acetate/0.002 M EDTA). After the bands were weighed, the DNA was extracted using a 'GeneClean' kit (Bio 101, Inc.).

Labelling and hybridization of ctDNA. Transfer of restricted cp DNA from agarose gels to nitrocellulose or nylon sheets was carried out according to Southern (1975).

Radioactive probes were made using 800 Ci/mmol alpha P-dATP via nick translation at 16 C for 1 hr using a nick-translation kit (Bethesda Research Laboratories).

Hybridization of probes to the nylon or nitrocellulose filters was carried out overnight at 45 C in 50% formamide/6X SSC/0.5% SDS and 5X Denhardt's solution (0.1% BSA/0.1% Ficoll/0.1% polyvinyl pyrollidone) (Wahl et al., 1979).

RESULTS

The goal of this project was to determine whether the chloroplast genome might serve as a diagnostic device for identification of specific species or strains of Chlorella. If this were so, this genome would be extremely useful for determining relationships between groups of algae and might determine the strategy for selection of partners in cell hybrid fusions. It is generally considered that unless a reasonably close relationship exists between fused partners, that stability of the hybrid is limited. Thus we have isolated

chloroplast DNA from our model exsymbiotic algae (two isolates) and from eleven free-living Chlorella for analysis of relationships. We have furthermore selected one exsymbiotic and one free-living alga for detailed analyses to determine the fine structural relationships between these ecologically diverse organisms. While our study was by no means exhaustive, this preliminary investigation has convinced us that many or all algae could thus be studied and that substantial information about algal relationships might be derived.

DNA isolation and anlysis. Isolation of chloroplast DNA from Chlorella is readily accomplished due to the significant difference of G/C content between it and the nuclear genome. The relative equilibrium sedimentations of the chloroplast and nuclear genomes in a CsCl gradient is show in Figure 1. The nuclear genome has a G/C content of approximately 59.18 mole per cent based on a bouyant density of 1.718 g/ml. The chloroplast DNA bands at 1.688 g/ml and has a G/C content of only 29.57 per cent. The fractions represented by these peaks were collected for further analysis.

Nla chloroplast DNA was digested with 5 different restriction endonucleases, i.e., BamHI, SalI, SstI, XhoI and KpnI, chosen because they produce only a relatively few fragments. The products of the digestion were resolved by electrophoresis on horizontal agrarose gels and visualization with UV irradiation after treatment with ethidium bromide (Fig. 2, Table I). The patterns observed contained fragment bands of different lengths as compared with HindIII digestion fragments of phage lambda DNA. These were numerically ordered by size. A total of 9 BamHI, 13 SstI, 17 XhoI, 9 SalI and 11 KpnI fragments were identified. The sums of the individual fragment sizes are not an accurate estimate of the total cp genome size, since the inverted repeat allows for at least two orientations of the genome and would therefore generate two different maps (Palmer, 1983).

Mapping of Nla chloroplast genome. Three strategies were used to derive the preliminary Nla chloroplast genome restriction map which is depicted in this report (Fig. 3): (i) hybridization of cloned DNA probes to digests of total chloroplast DNA singly cut or double-digested in a so-called (Palmer, 1986) filter hybridization overlap or chromosome walking procedure, (ii) Southern hybridization of specific bands cut from gels to digests of total chloroplast DNA singly or doubly digested, and (iii) numerical analyses of fragment lengths from single and double digests.

Nla chloroplast DNA digests were probed with a total of 11 cloned fragments, an example of which is shown in Figure 2. The probe used for this Southern was a SstI clone containing 14.47 kbp of Nla cp genome. This fragment is the exclusive band which appears when a SstI digest of the genome is probed with the clone containing the heterologous maize inverted repeat (see next section).

Localization of the inverted repeat. The inverted repeat was probed in the Southern transfers using the plasmid pZmC100, which contains the 23S, 16S, 5S and $tRNA_{val}$ from the maize chloroplast genome (Bedbrook, et al., 1977) (data not shown). The clone was kindly made available for our use by Dr. L. Bogorad, Harvard University). It has previously been shown that sufficient homology between this sequence and that from the Chlorella cp genome exists for demonstrating its presence by hybridization (Van Etten et al., 1984).

Mapping of UTEX 20, a free-living Chlorella. The preliminary map presented for the free-living strain UTEX 20 was prepared by Southern analysis of single, double and triple digests of KpnI restriction endonuclease in conjunction with

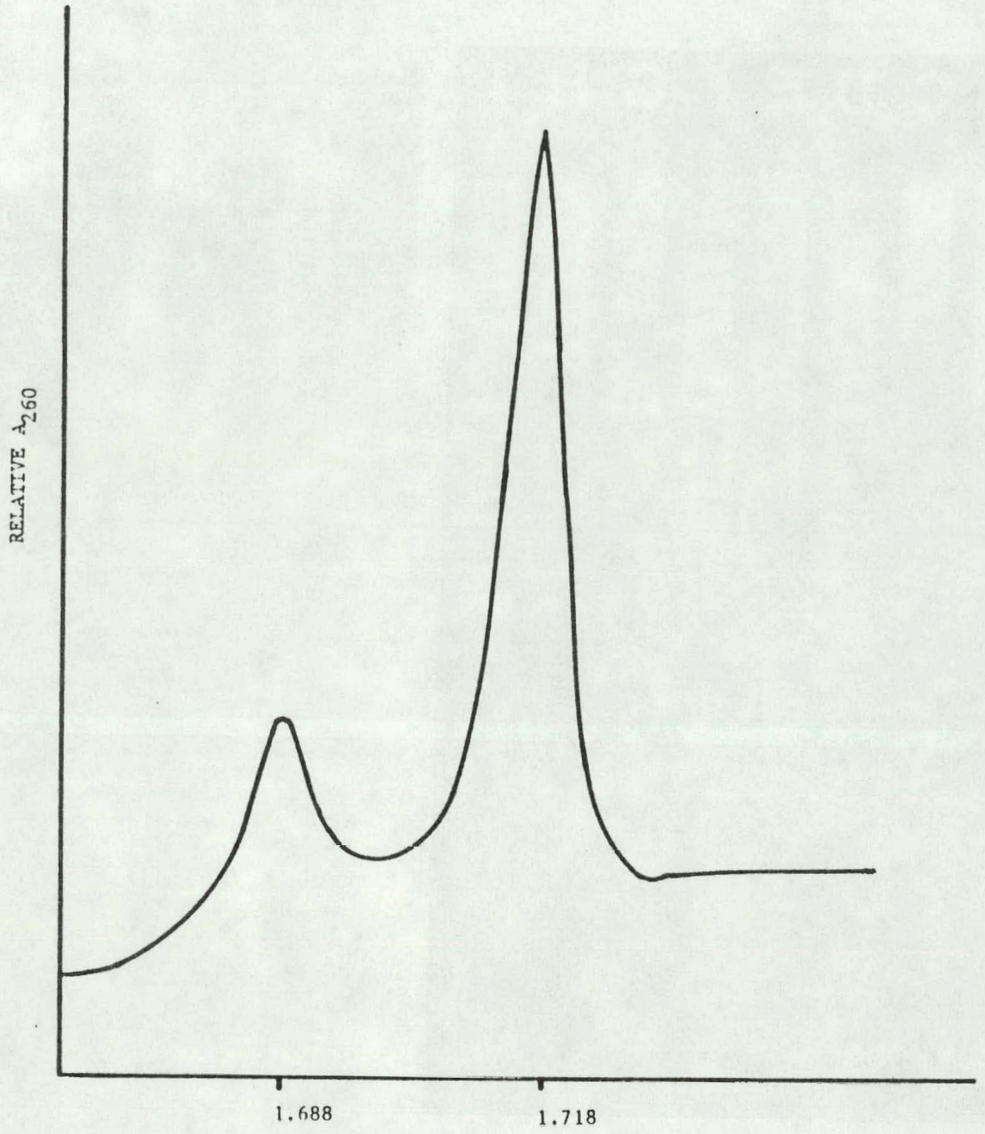


Figure 1. CsCl equilibrium centrifugation of chloroplast DNA from Nla.

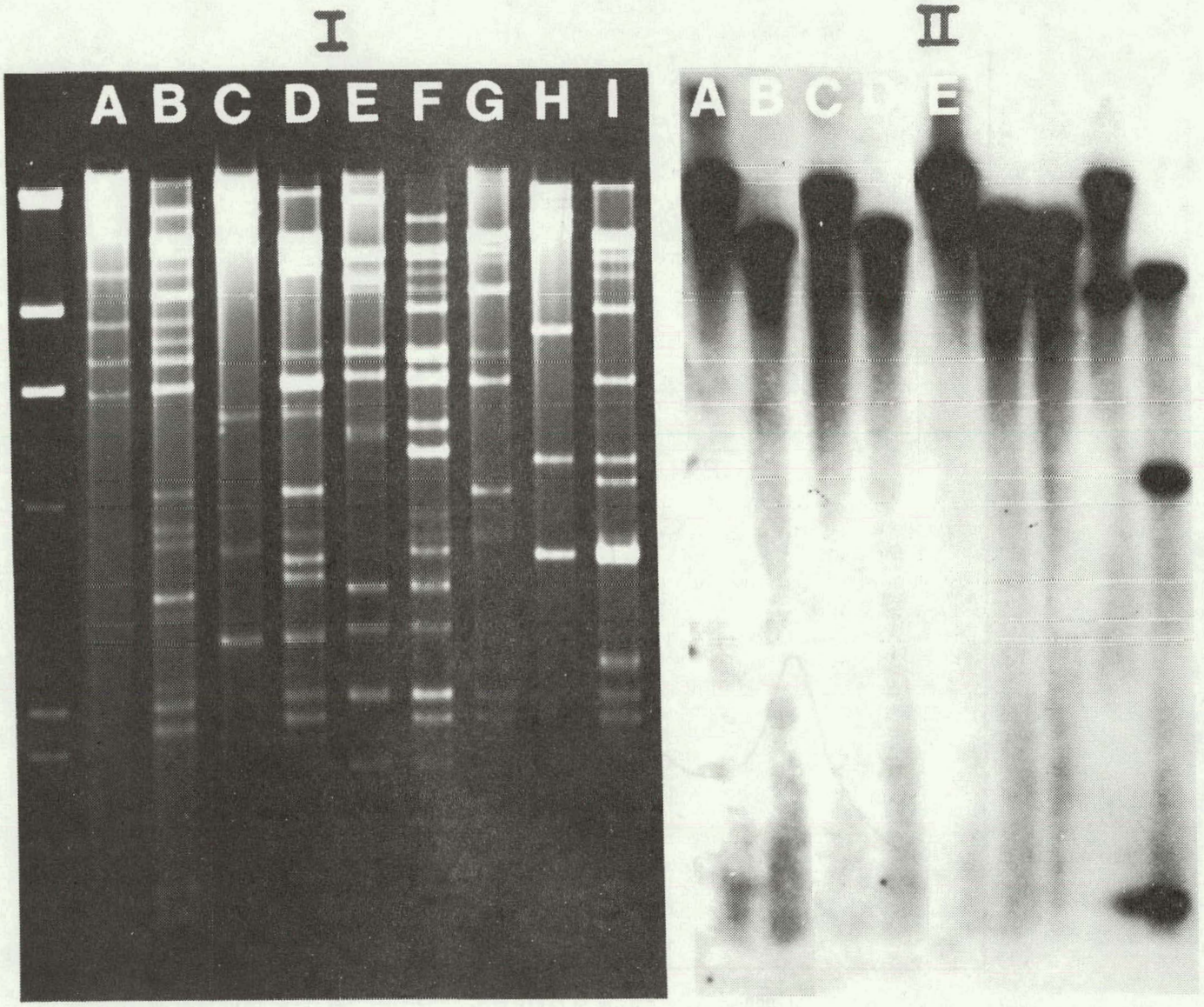


Figure 2. Electrophoresis of Chlorella Nla chloroplast DNA. (I): The cpDNA was restricted with BamHI (A), BamHI + SstI (B), SalI (C), SalI + SstI (D), XhoI (E), XhoI + SstI (F), SstI (G), KpnI (H), KpnI + SstI (I). (II): The DNAs in (I) were transferred to nitrocellulose and hybridized with a SstI clone containing a 14.47 kb fragment from the Nla chloroplast genome. Lambda DNA restricted with HindIII is shown on the left (27.5, 23.1, 9.4, 6.7, 4.4, 2.3 and 2.0 kb).

TABLE I. Sizes of restriction fragments in kilobases for Nla chloroplast genome.

Fragment #	BamHI	SalI	SstI	XhoI	KpnI
1	32.00	32.38	34.00	34.10	27.17
2	29.00	30.06	30.92	30.80	25.13
3	24.22	29.57	15.87	22.56	22.56
4	16.11	26.11	15.48	14.07	18.14
5	12.36	23.36	14.40	13.45	17.64
6	9.07	12.11	14.40	12.11	17.17
7	7.54	4.17	13.16	11.41	16.71
8	6.43	3.48	10.58	10.58	8.26
9		2.76	7.35	7.45	4.71
10			6.52	6.60	3.42
11			4.31	5.31	1.47
12			3.64	5.13	
13			2.34	3.21	
14			2.21	2.80	
15			1.50	2.31	
16				2.27	
17				1.27	

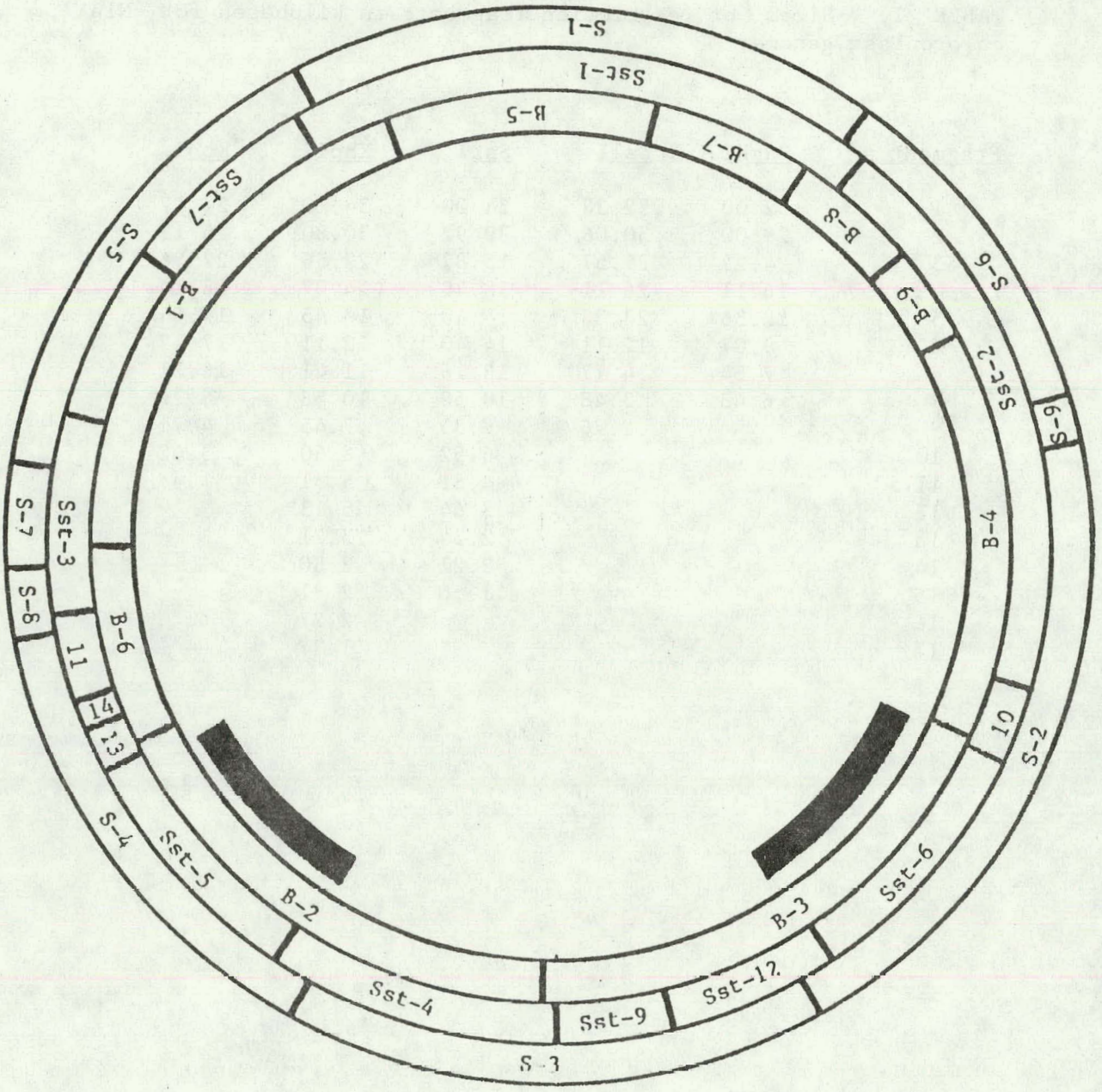


Figure 3. Restriction endonuclease map of the chloroplast genome from Chlorella strain Nla. A map was derived using three restriction enzymes: BamHI (B), SstI (Sst), and SalI (S). The dark blocks represent the inverted repeats.

XhoI and SalI (Fig. 4). Using cloned KpnI fragments and single bands cut from gels we were able to "chromosome walk" the entire distance of the genome to obtain the map (Fig. 5). The various restriction fragment lengths are given in Table II. The location and size of the inverted repeat sequences were confirmed by probing with pZmC100 and are less than 13 kbp in length.

Comparison of some characteristics of N1a and UTEX-20. Total RNAs from N1a and UTEX-20 were separated on a 1.2% denaturing formaldehyde gel using tobacco mosiac virus and brome mosaic virus RNAs (obtained from Dr. Myron Brakke, University of Nebraska) as size standards. There appears to be a significant difference, as judged by electrophoresis, in the large cp ribosomal RNAs of the free-living strain UTEX-20 and the exsymbiont N1a. As shown in Fig. 6, the large and small cytoplasmic rRNAs are uniform in size between the two strains; this appears also to be true for the cp small rRNA. However, the large cp rRNA, which is always nicked in preparation to produce two fragments, clearly shows a strain difference in terms of it size. This difference represents a loss of approximately 7% of the total or an estimated 96 bases by the exysmbiont N1a strain. These differences can be readily seen on the gel pattern, Fig. 6 as a difference in migration of the RNA. Based on the evidence, it is clear that the difference between the two large rRNA species is the deletion of a significant fraction of the molecule at one end. The precise physiological role, if any, of this loss is presently unknown.

The nuclear genomes of the two algae also differ. When nuclear DNAs isolated from the two algae were double digested with KpnI and SstI and then probed with a clone of the tubulin gene from Chlamydomonas (obtained from Dr. David Marks, Zoecon Corp.) different bands hybridized. The N1a band was approximately 4.3 kbp while the UTEX-20 contained two signals at about 7 and 3 kbp (date not shown).

These results clearly show that the two algal strains differ in many respects.

Comparison of chloroplast genomes from exsymbiotic Chlorella with those of free-living Chlorella. Chloroplast DNA was purified from N1a, NC64A and 11 free-living chlorellans (Figure 7). The restriction length polymorphisms, indicated by this preliminary sample, placed Chlorella chloroplast genomes into several groups. Six different free-living strains fell into a group; the two examples of exsymbiont Chlorella had similar patterns. In addition, individual free-living Chlorella isolates have been analyzed which represent 5 other cp genomes. Table III represents the present taxonomy of these isolates and strains based on the literature and the various culture collections from which they have been obtained. The extent to which more genomes are represented within this genus will depend on further analysis.

DISCUSSION

As a result of the large difference in equilibrium sedimentation between the cp and nuclear genomes, chloroplasts from Chlorella do not need to be prepared free from the remainder of the cell to obtain DNA sufficiently clean for molecular studies. This procedure also allows us to isolate pure nuclear DNA simultaneously.

The chloroplast DNAs of exysmbiotic Chlorella N1a and free-living Chlorella kessleri (UTEX-20) are structurally comparable to those described for most higher plants. However, both genomes studied at the fine structure level differed from that of Euglena (Rawson, et al., 1978), Chlamydomonas (Rochaix, 1978) and Chlorella ellipsoidea (Yamada, 1983). They both contain inverted

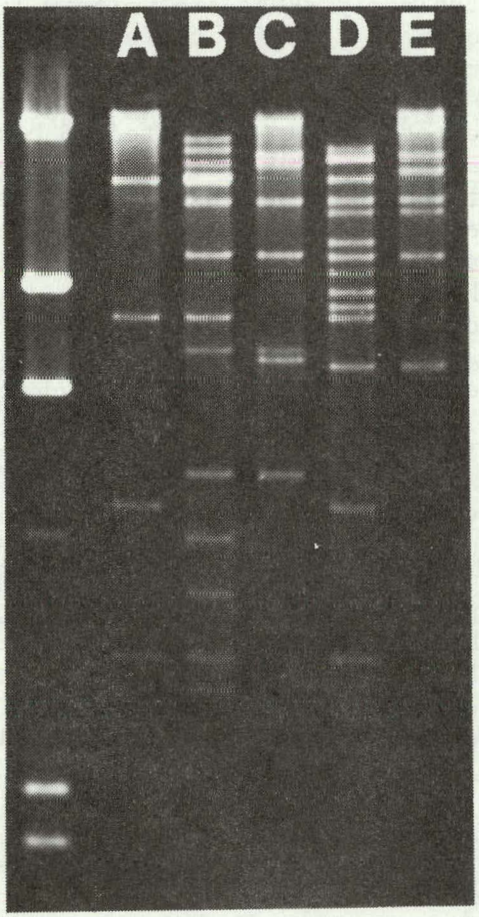


Figure 4. Electrophoresis of Chlorella UTEX 20 chloroplast DNA. The cpDNA was restricted with KpnI (A), KpnI + XhoI (B), XhoI (C), KpnI + SalI (D), SalI (E), KpnI + XhoI + SalI (F). Lambda DNA restricted with HindIII is shown on the left (27.5, 23.1, 9.4, 6.7, 4.4, 2.3 and 2.0 kb).

TABLE II. Sizes of restriction fragments in kilobases for UTEX 20 chloroplast genome.

Fragment #	KpnI	SalI	XhoI
1	41.24	39.14	35.50
2	37.23	31.11	35.70
3	32.45	22.53	23.26
4	18.94	20.01	21.20
5	8.93	16.27	15.89
6	4.69	14.84	11.66
7	3.19	11.66	7.73
8	0.89	7.32	7.42
9			5.11

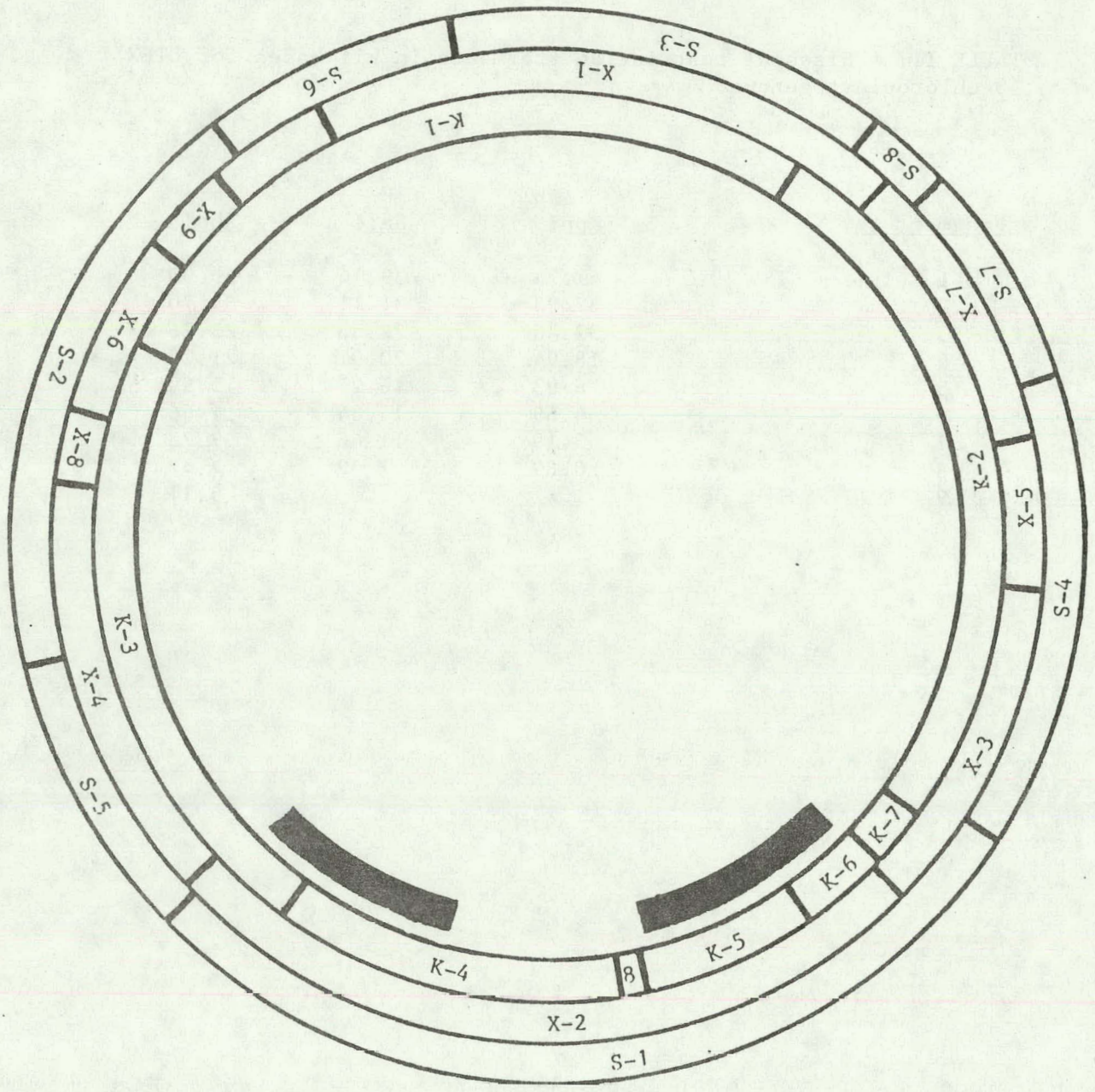


Figure 5. Restriction endonuclease map of the chloroplast genome from Chlorella strain UTEX 20. A map was derived using three restriction enzymes: KpnI (K), XhoI (X), and SalI (S). The dark blocks represent the inverted repeats.

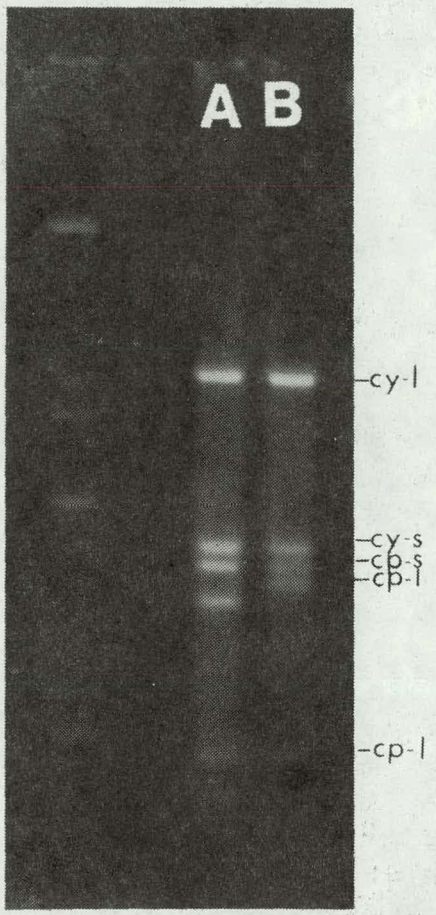


Figure 6. Comparison of RNA from Chlorella strains Nla (A) and UTEX 20 (B). RNA (4 ug) was electrophoresed on a 1.2% denaturing formaldehyde agarose gel. Large (cy-l) and small (cy-s) cytoplasmic rRNAs and large (cp-l) and small (cp-s) rRNAs are indicated. Note: the large chloroplast rRNA (cp-l) is nicked and appears as two bands. Molecular weight markers are shown on the left and are tobacco mosaic virus RNA (6395 bases) and brome mosaic virus RNAs (3333, 3030, 2114 and 876 bases).

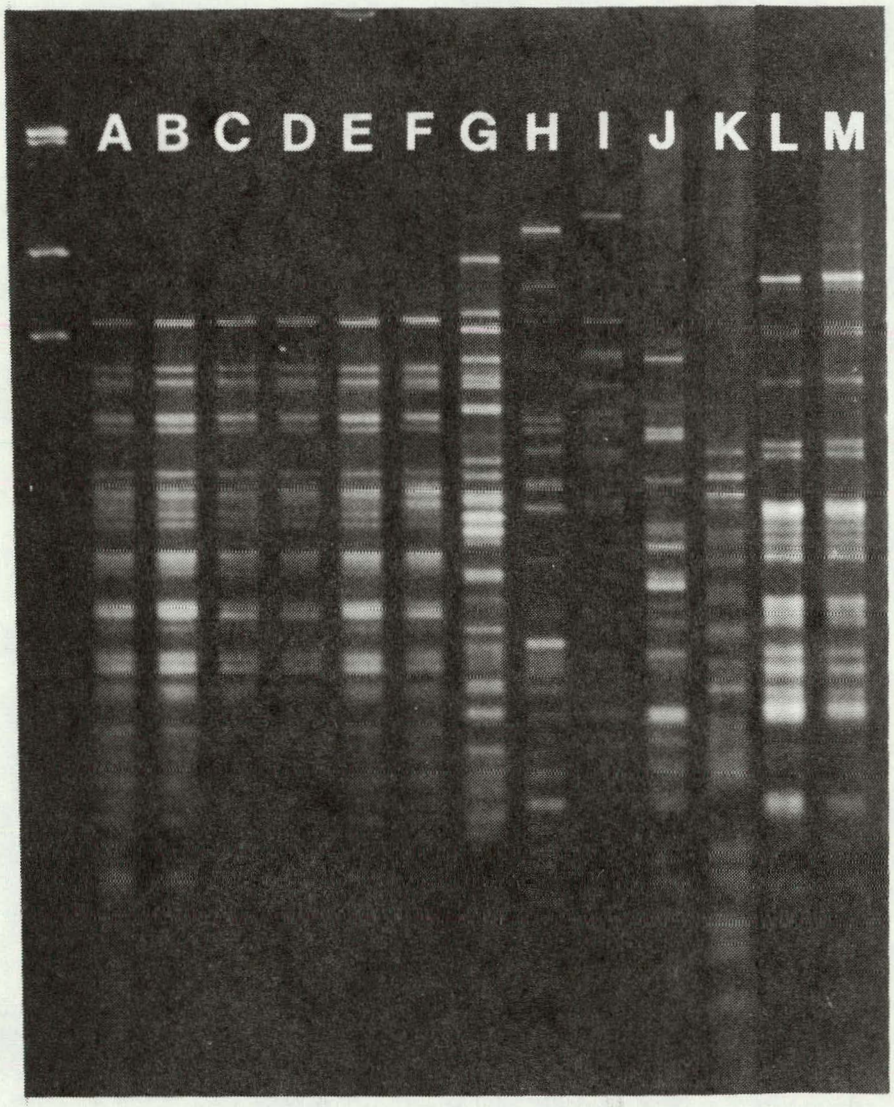


Figure 7. Comparison of Chlorella cpDNAs digested with HindIII. Chlorella strains: UTEX 20 (A), UTEX 262 (B), UTEX 363 (C), UTEX 367 (D), UTEX 397 (E), UTEX 398 (F), CCAP 2118p (G), UTEX 395 (H), UTEX 490 (I), UTEX 1602 (J), SERI-SOl (K), Nla (L), NC64A (M). Note: Nla and NC64A are exsymbionts and the others are all free-living strains.

Table III. Origins and nomenclature of comparison algae.

Strain	Strain synonyms	G/C	Species designation	Origin	Citation
UTEX 20	211-1a	57.5	C. pringsheimii C. vulgaris C. ellipsoidea	Texas collection	Shihira & Krauss, 1965 Kessler & Soeder, 1965 Winokur, 1948
UTEX 262	211-11g	56.0	C. vulgaris C. kessleri	Texas collection	Shihira & Krauss, 1965
UTEX 363			C. vulgaris	Texas collection	
UTEX 367			C. vularis	Texas collection	
UTEX 397		56.0	C. kessleri C. vulgaris	Texas collection	Fott & Novakova, 1969 Craig & Trelease, 1937
UTEX 398		56.0	C. kessleri C. vulgaris C. regularis var. umbricata	Texas collection	Fott & Novakova, 1969 Shihira & Krauss, 1965
UTEX 395	211-8m	62.3	C. pyrenoidosa C. vulgaris	Texas collection	
UTEX 490	211-5b	43.9	C. miniata C. luteoviridis C. nocturna	Texas collection	Shihira & Krauss, 1965
UTEX 1602			C. sorokiniana	Texas collection	
CCAP 211-8p			C. fusca var.vaculata	P.C.L. Johns, Cambridge	
S01	S/CHLOR-1		C. sp.	Wm. Barclay, SERI	
NC64A			C. sp.	Leonard Muscatine UCLA	
N1a			C. sp.	R.L. Pardy, Nebraska	

repeats in contrast to Euglena but the length of these repeats is small compared to Chlamydomonas and Chlorella ellipsoidea. While only sequence analysis will allow precise length estimation of the inverted repeats, mapping data indicate lengths no greater than 14.5 and 12.5 kbp for N1a and UTEX-20, respectively. The inverted repeats are separated by long and short single copy regions of 107.6 kbp and 29.4 kbp for Chlorella N1a and 124.5 and 13.5 kbp for Chlorella kessleri strain UTEX-20. Both map to a circular form and the size of both is uniform, mapping to approximately 164 kbp of DNA.

An interesting difference between N1a and UTEX-20 was that the chloroplast large ribosomal gene, found within the inverted repeat regions, was shown to be suprisingly different based upon analysis of the rDNA transcripts. The reduction of the large transcript by 96 bp or 7% of its total size does not appear to have severe physiological effects on the alga, since it appears to have normally functioning photosynthetic activity and growth. The arrangement of nuclear genes also differs between the two algal strains since a highly conserved gene such as tubulin hybridizes to very dissimilar restriction fragments. It is obvious that the exsymbiont, N1a and free-living, UTEX-20 chlorellans show a number of variances and are genetically divergent. This suggests that successful cell fusions between the two strains may not be as likely as anticipated although it is not certain that these distinctions described here will actually limit this possibility. The major goal of this study was to determine whether the chloroplast genome might be a suitable diagnotic tool for the establishment of evolutionary relationships between the algae. The patterns of identity transcended established taxonomic lines, several species of Chlorella having similar chloroplast genomes. The Southern transfer probed with the heterologous probe pZmC100 which contains the 23s,16s,5s and $tRNA_{val}$, confirms the groupings established from the restriction polymorphisms study.

ACKNOWLEDGEMENTS

I thank Anne Schuster, Jim Van Etten, Ken Korth, Jim Waddle, Mike Graves, Sean Amberg and Steve Peterson for technical assistance and helpful discussions. This investigation was supported by contract XK-5-05073-2 from the Solar Energy Research Institute and also in part by grant DCB-8417373 from The National Science Foundation grant 85-CRCR-1-1613 from The United States Department of Agriculture.

REFERENCES

Bedbrook, J. R., R. Kolodner and L. Bogorad (1977) Zea mays Chloroplast Ribosomal RNA Genes Are Part of a 22,000 Base Pair Inverted Repeat. Cell 11,739-749

Craig, F.N. and S.F. Trelease (1937) Photosynthesis of Chlorella in heavey water Am. J. Bot. 24:232-242

Fott. B., and M. Novakova. (1969) A Monograph on the Genus Chlorella. The Fresh Water Species. In: Studies in Physcology, ed. B. Fott, pp10-74

Girton, L.E. and J.L. Van Etten Restriction Site Map of the Chlorella Virus PBCV-1 Genome (In Press)

Kollar, B. and H. Delius (1980) Vicia faba Chloroplast DNA Has Only One Set of

Ribosomal RNA Genes as Shown by Partial Denaturation Mapping and R-loop Analysis. Mol. Gen. Genet. 178:261-269

Kolodner, R. and K.K. Tewari (1979) Inverted repeats in Chloroplast DNA from Higher Plants. Proc. Natl. Acad. Sci. 76:41-45

Maniatis, T., E.F. Fritsch and J. Sambrook (1982) Molecular Coning. Cold Spring Harbor Laboratory

Nichols, H. W., and Bold, M. C. (1965) Trichosarchina polymorpha gen. et. sp. nov. Jour. Phycol. 1:34-38

Ohyama, K., H. Fukuzawa, T. Kohchi, H. Shirai, T. Sano, S. Sano, K. Umesono, Y. Shiki, M. Takeuchi, Z. Chang, S. Aota, H. Inokuchi and H. Ozeki (1986) Chloroplast Gene Organization Deduced From Copmplete Sequence of Liverwort Marchantia polymorpha Chloroplast DNA. Nature 322, 572-574.

Palmer, J.D. (1983) Chloroplast DNA exists in two orientations. Nature 301, 92-93

Palmer, J.D. (1986) Isolation and Structural Analysis of Chloroplast DNA. Methods in Enzymology 118:167-186

Rawson, J.R.Y., S.R.Kushner, D.Vapnek, N.K. Alton and C.L. Boerma (1978) Chloroplast Ribosomal RNA Genes in Euglena gracilis Exist as Three Clustered Tandem Repeats. Gene 3, 191-209

Rochaix, J.D. and P. Malnoe (1978) Anatomy of Chloroplast Ribosomal DNA of Chlamydomonas reinhardi. Cell 15:661-670

Schuster, A. M., Girton , L., Burbank, E. E., and Van Etten, J. L. (1986) Infection of a Chlorella-like Alga With the Virus PBCV-1: Transcriptional Studies. Virology 148:181-189

Shinozaki, K., et al. (1986) The Complete Nucleotide Sequence of the Tobacco Chloroplast Genome: Its Organization and Expression. EMBO J. 5:2043-2049

Shihiri I. and R.W. Krauss (1965) Chlorella-Physiology and Taxonomy of Forty-one Isolates. Port City Press, Baltimore

Skrdla, M.P., Burbank D.E., Xia, Y., Meints, R.H., and Van Etten J.L. (1984). Structural Proteins and Lipids in a Virus, PBCV-1, Which Replicates in a Chlorella-like Alga. Virology 135:308-315

Southern, E.M. (1975) Detection of Specific Sequences Among DNA Separated by Gel Electrophoresis. J. Mol. Biol. 98:503-517

Trench, R.K. (1979) The Cell Biology of Plant-animal Symbiosis. Annu. Rev. Plant Physiol. 30:485-531

Van Etten, J.L., R.H. Meints, D. Kuczmarski, D.E Burbank and K. Lee (1982) Viruses of Symbiotic Chlorella-like Algae Isolated From Paramecium bursaria and Hydra viridis. Proc. Natl. Acad. Sci. 79:3867-3871

Van Etten, J.L., Burbank, D.E., Kuczmarski, D., and R. H. Meints (1983a). Virus Infection of Culturable Chlorella-like Algae and Development of a Plaque Assay. Science 219:994-996.

Van Etten, J.L., Burbank, D.E., Xia, Y., and R. H. Meints (1983b). Growth cycle of a virus, PBCV-1, that infects Chlorella-like algae. Virology 126: 117-125.

Van Etten, J.L., Burbank, D.E., Joshi, J., and R. H. Meints (1984). DNA Synthesis in a Chlorella-like Alga Following Infection With the Virus PBCV-1. Virology 134:443-449.

Wahl, G.M., M. Stern and G.R. Stark (1979) Efficient Transfer of Large DNA Fragments From Agarose Gels to Diazobenzyloxymethyl-paper and Rapid Hybridization by Using Dextran Sulfate. Proc. Natl. Acad. Sci. 76:3683-368

Winokur, M. (1948) Growth relationships of Chlorella Species. Am. J. Bot. 35:118-129

Yamada, T. (1983) Characterization of Inverted Repeat Sequences and Ribosomal RNA Genes of Chloroplast DNA From Chlorella ellipsoidea. Curr. Gen. 7:481-487

Yelton, M.M., Hamer, J.E. and W.E. Timberlake (1984) Transformation of Aspergillus nidulans by Using a TrpC Plasmid. Proc. Natl. Acad. Sci. 81, 1470-1474

Engineering Design

THIS PAGE

WAS INTENTIONALLY

LEFT BLANK

DESIGN AND OPERATION OF AN OUTDOOR MICROALGAE TEST FACILITY

Joseph C. Weissman, David M. Tillet, and Raymond P.Goebel
Microbial Products, Inc.
Fairfield, CA 94533

ABSTRACT

The objective of this project is to establish and operate a facility in the American Southwest to test the concept of producing microalgal biomass as a feedstock for the production of liquid fuels. The site chosen for this is located in the city of Roswell in southeastern New Mexico at an existing water research station. The climate and water resources are described..

During the first year, a small scale system consisting of six 3 m2 fiberglass raceways was constructed and operated to evaluate the performance of algal species in terms of productivity and lipid content. The ease of operating and the low cost of constructing several of these small cultivation units makes them ideal for performing controlled, replicated experiments. The research plan for the initial period of operation is presented.

Since the ultimate goal of the Aquatic Species Program requires the production of large amounts of biomass, larger scale research systems are needed. Two larger raceways, each 0.1 hectare were designed and are under construction. These will be used to evaluate the performance of low cost lining options relative to durable, expensive plastic membranes. Other scale-up problems will also be addressed in these and in a 0.5 hectare demonstration raceway to be built in a subsequent year.

DESIGN AND OPERATION OF AN OUTDOOR MICROALGAE TEST FACILITY

INTRODUCTION

The emphasis of the Aquatic Species Program is on developing a mass culture technology for cultivating oil-yielding microalgae using saline groundwater in the American Southwest. The major constraint to this technology is the economical production of an oil-rich microalgal feedstock. As part of the effort to address this constraint, the ASP operates outdoor production facilities for evaluating pre-screened microalgal strains and for developing production technology.

In the past, several distinct outdoor facilities were operated. Each was charged with the duty of testing the pre-screened species for high productivity and lipid yield in outdoor culture. Although these systems represented different design concepts in cultivation reactors, basically the goal was to establish a productivity potential for outdoor cultivation. There was little emphasis on engineering design, and no scale-up of the reactors beyond the small experimental size. As experience in outdoor cultivation matured, and as the resource analyses developed, it became clear that outdoor testing would need to be done at a location comparable to a potential plant site. In addition, it became time to address basic engineering questions at a scale larger than the previous experimental systems. The impressive biomass production rates obtained from these small experimental systems need to be confirmed in larger reactors at real sites.

OBJECTIVES

The overall objective of this project is to develop and operate a test facility in the American Southwest to evaluate microalgal productivities and to examine the problems and potential of scaling up and operating large microalgal production systems. This includes the testing of specific designs, modes of operation, and strains of microalgae; proposing and evaluating modifications to technological concepts; and assessing the progress in meeting cost objectives set for the Aquatic Species program.

The objective during the first year is to evaluate outdoor production performance of pre-screened species of microalgae in six small scale reactors (3 m2) and to make an initial assessment of the engineering performance of first level scale-up ponds (1000 m2). Specific objectives include:

1) the development of a three year plan for the design and construction of the outdoor microalgae test facility

2) installation of the small scale system

3) operation of the small scale system to screen species for

productivity potential and culture stability

4) installation of first level scale-up ponds

5) initiation of an engineering assessment of the large scale system.

THE PROJECT SITE

The present project is located at a previously established water research facility in the city of Roswell in the southeastern part of New Mexico (Figure 1). The site is representative of those which may be available for construction of an actual production plant. It is characterized by high insolation, low precipitation, flat land, and an abundant supply of highly saline groundwater. Much of the infrastructure required for this type of project is already in place: triple-lined evaporation ponds for disposal of pond effluents, land at very affordable rental rates, and laboratory and office space. There is, in addition, an existing chemical analysis laboratory, much of which is common space for the tenants of the facility. It is thus expected that this site will be a cost effective one for evaluating basic biological and engineering performance of raceway reactors.

Climate

The average monthly temperatures (daily ambient maximum, minimum, and mean), total precipitation, pan A evaporation, and total insolation for the Roswell Test Facility (RTF) are given in Table 1. It has been our experience that "warm water" species will be dominant and attain about 75% of maximal productivity when the daily low ambient temperature is above 10 oC and the daily maximum temperature is above 27 oC. Under these conditions pond waters generally heat up rapidly under clear skies and, when starting from overnight lows of 14-18 oC, reach 20 oC by mid morning. Thus during five to six months at Roswell, insolation will exert primary control over productivity. An approximate estimate of the average productivity obtainable, month by month, can be based upon a 7% PAR efficiency in each of five months, 4% for Mar, Apr and Oct, and a 3% efficiency for the other four months. The estimated yearly productivity is thus about 67 mt ha-1yr-1 or a daily average of 18.6 gm-2d-1. The seven per cent is what we have measured over four months in northern California when temperature in the ponds was above 25 oC during most of the photosynthetic day. Less optimal temperature regimes during other months were taken into account in terms of lowered efficiency. It is most probable that a change to a more cold adapted species would be required during the Nov-Feb time period. Use of cold adapted chlorophytes or Phaeodactylum, a diatom which grows well from 12-22 oC, may result in higher productivity than the average of 7 gm-2d-1 estimated.

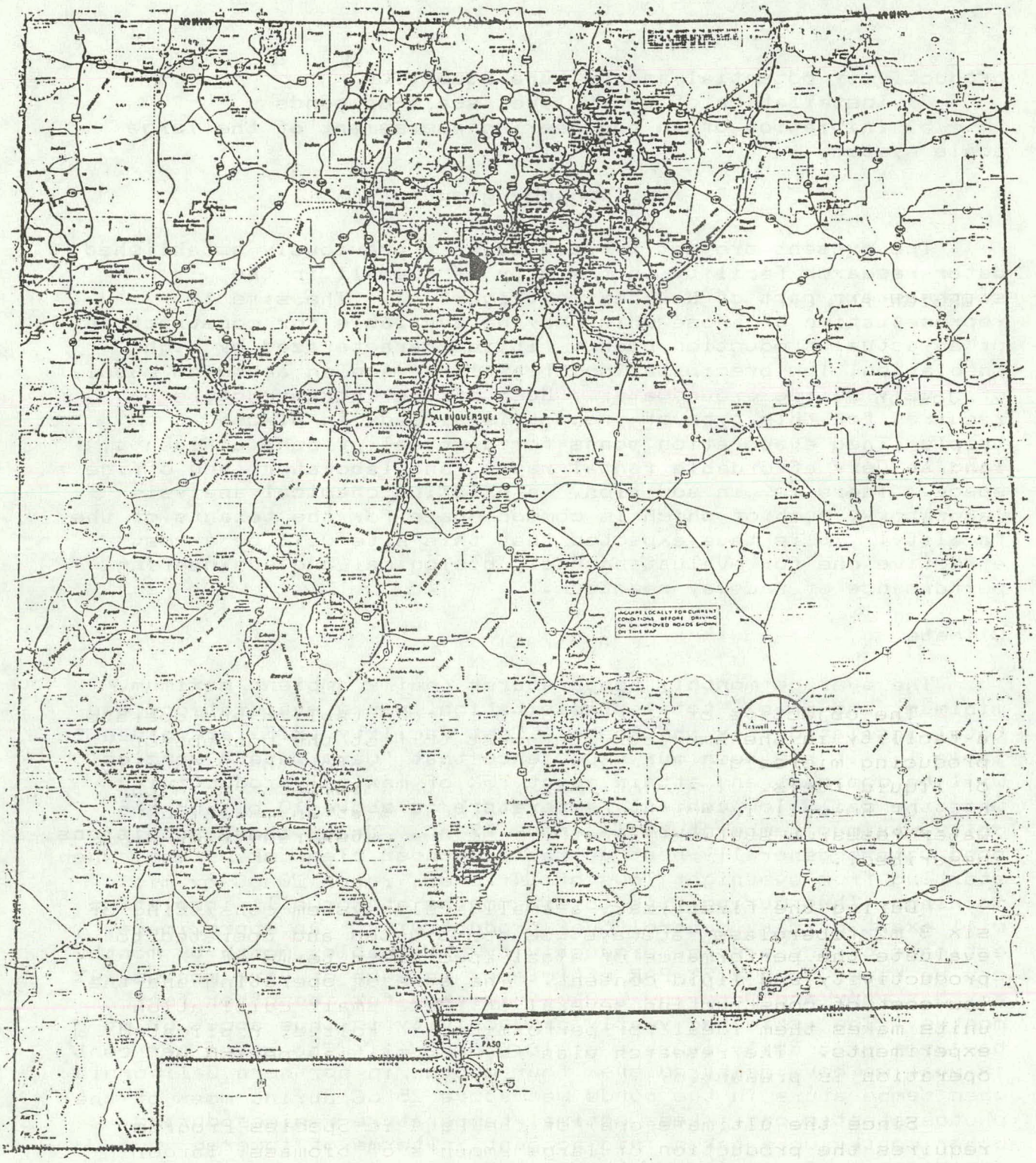


Figure 1. Location of Roswell, New Mexico

Table 1. Climate Data -- Roswell, New Mexico

Month	Temperatures, oC Ambient Max	Ambient Min	Ambient Mean	20cm deep Pond Max	20cm deep Pond Min	Ppt. cm	Evap. cm	Insol, Lngly/d
Jan	11	-3	4	7	0	1.0	7.4	316
Feb	15.5	-1	7	12	1	1.1	11.0	392
Mar	20	3	11.5	21	7	1.3	18.5	490
Apr	24	7	16	28	11	1.8	25.8	603
May	28.5	12.5	20.5	34	17	2.8	29.8	638
Jun	34	17.5	26	35	19	3.4	32.9	661
Jul	34	20	27	36	21	4.9	31.4	632
Aug	33	19	26	35	20	4.3	27.5	574
Sep	28.5	15	22	30	16	4.4	21.5	491
Oct	23.5	8	15.5	24	11	2.8	15.7	420
Nov	16.5	1.5	9	14	4	1.4	9.2	309
Dec	13	-3	5	6	0	1.3	6.9	257

Data from Office of State Climatologist, NM Dept Agric., Las Cruces
Pond temperatures from heat balance model courtesy of D. Tillett

From the above estimates it is apparent that ponds must be operated so as to maintain conditions within optimal ranges to obtain the productivities allowed by conditions of light and temperature. In addition, screening for more productive species, both warm and cold water ones, must be ongoing. The crudeness of the models used above to estimate productivity highlights the need for the development of more sophisticated methods (models) for making estimates. Such a model will allow prediction of temperature profiles in ponds based on climatic inputs, and hence should in the future allow extrapolation of results obtained at the test site to other regions of the southwest. The model will also be extended to predict overall economic efficiency for specified pond management strategies.

Water Resources

There are two abundant water resources available at the

New Mexico site: city water (at $326/acre-ft or $0.26/m3) and saline ground water (800 gpm at a pumping cost of about $15/acre-ft or $0.012/m3). The mineral composition of each is given in Table 2. The saline ground water has a salinity similar to 40% sea water, differing primarily in that it contains about one-third as much magnesium and twice as much calcium. Most species cannot maintain a high level of productivity at salinities much above twice this. Thus the groundwater could be used, as is, in a system in which the blow down volume is equal to the evaporation, i.e. about 24,000 m3/ha/yr. Growth on this water would be representative of growth on high calcium, low alkalinity, 35 gL-1 waters similar to seawater and Type I waters in these regards. This water resource reflects reality for a large, but as yet undetermined, percentage of saline ground waters in the southwest. The drawbacks are: many species isolated by the ASP prefer Type II waters, and the high blow down volume produces not only a lot of water to evaporate but great amounts of salts to dispose of.

Table 2. Mineral Composition of Water Resources at RTF and 50% Seawater.
mgL-1

Element	City Water	Saline GW	40% SW
Sodium	35	4500	4800
Potassium	1	23	120
Calcium	152	537	160
Magnesium	46	172	475
Chloride	53	8070	7800
Sulfate	320	1375	920
Bicarbonate	229	192	78
Silicon	1.3	1.7	
TDS	705	13620	14000
pH	7.7	7.2	

RTF water analysis courtesy of G. Beatty, RTF Chemist

From an experimental point of view a test facility would have ideally both Type I and Type II saline ground waters for use. The Roswell City water and the saline ground water could be conditioned (to remove the calcium) and mixed to yield Type II water. If this mixture ratio is 4 to 1 (City to groundwater) then a water of low TDS results (about 4 gL-1), which could be concentrated to 32 gL-1 using a low blow down ratio (one-seventh of evaporation). Not only does this render

a water type which is representative of high alkalilnity, low hardness but also allows testing of very high recycle ratios. This would be necessary with many southwestern water resources. By varying the mixture and blow down ratios, any TDS is achievable for this type of water. At the one hectare scale the cost of water conditioning would be about $10,000/yr, which is significant, but not overwhelming. No experiments requiring water conditioning are envisioned during the first year of this project.

Available Land

The RTF has flat, utilizable land around the main laboratories. A small part of this area is being used for operation of our six small scale experimental ponds. One mile east of this location are 36 ha of triple lined evaporation ponds arranged as a set of three 12 ha units. One of these is presently used for the disposal of all wastes from the laboratories and water projects at the main facility. Two are available. We have leased half of one for the construction of large ponds, which allows room for expansion in the future. The other 12 ha evaporation unit would be used for just that: evaporative disposal of the growth pond effluents. This pre-existing disposal system at the RTF is one of the major attractions of the facility. Any project of this type must comply with all environmental regulations and obtain the appropriate permits. This process, from permit acquisition to system construction and approval, has already been done at the RTF. Construction of an evaporation system of the requisite size, from two to five times the growth pond area, is a major cost item.

Existing Infrastructure.

In addition to the pre-existing evaporation ponds, the RTF offers a substantial amount of infrastructure well suited for applied research and development. For this project, 150 square feet of office space has been leased, as has 800 square feet of laboratory space (unimproved). A fully equipped water and wastewater chemical analysis laboratory is operated, with an experienced chief chemist. In addition the facility is offering full service and maintenance on already existing equipment such as the 800 gpm pump, major electrical services, and building maintenance.

Summary

The Roswell Test Facility provides a setting for realistically assessing the status of the outdoor production of microalgal biomass. Climate and water resources, although quite favorable, present the types of challenges that are representative of the southwest as a whole. Any problems requiring attention at Roswell will have to be faced sooner or

later by the ASP. In addition, the RTF offers an array of services and suitable facilities.

FACILITY DESIGN

A plan has been developed for establishing the test facilities during the first three years. It is based on funding levels of $100,000 per year. The schedule of development, and the corresponding costs, are given in Table 3. The facility will include a small scale system comprised of six 3 m2 ponds, a 50 m2 inoculum pond, two 0.1 ha growth ponds (one lined with earthen materials, one with a membrane), one large 0.5 ha pond (earth-lined), a harvesting system based on flocculation - flotation, and the infrastructural development required (power, storage sheds, water lines, etc.).

The sizes of the growth ponds were determined by optimizing research benefits with construction and operating costs. Programatic objectives and economic constraints were factored in as well. Since higher operating costs will be incurred for larger ponds, each year into the future, we tried to determine what sizes were suitable for answering the biological and engineering research questions.

For the biological objectives of screening organisms for productivity, lipid induction, and culture stability there must be experimental units which allow replication. Given this need for multiple experimental units, economic constraints dictate that as much of this work as possible be done at the smallest practical scale. From previous experience, a scale of 1.5 - 10 m2 provides low cost, both in construction and operation. The size of 3 m2 was chosen since it not only minimizes costs, but also eases operation relative to 10 m2. Sidewall shading effects are still significant (and impossible to quantify) at sizes much smaller than 3 m2.

Some biological questions and most engineering performance testing cannot be answered at such a small scale. First of all, there has been a general, but vague, feeling in the field of algal biomass production that productivity decreases as scale increases. Much of this is based on poorly designed large scale systems. However, many conditions are different in large scale systems, primarily due to size (and cost constraints). Mixing power densities are much lower in large scale ponds, leading to longer time constants for turbulent eddies, flow conditions with a higher plug flow component, and increased tendency for particles to sediment and collect in dead spots. In addition large scale systems cannot be as easily, or as adequately cleaned. Thus the performance of well designed large scale systems relative to the small scale

TABLE 3. PRELIMINARY THREE YEAR PLAN FOR MASS CULTIVATION SYSTEM

1st Year:	
Small-Scale System (6x3 m2 ponds)	$21,000
Large-Scale System	
One 0.1 ha growth pond (membrane lined)	49,200
Water to site	18,700
Power to site	5,600
Power distribution network	2,700
Engineering	2,000
TOTAL:	99,200
2nd Year:	
Innoculum Pond - 50 m2	10,000
One 0.1 ha Growth Pond (earth-lined)	44,700
Chemical Storage Shed	3,000
Power Distribution Network	2,900
Harvesting System (for all large ponds)	36,250
Engineering	750
TOTAL	99,600
3rd Year:	
One 0.5 ha Growth Pond (earth-lined)	89,300
Electrical Distribution Network	4,600
Engineering	3,600
	97,500

Does not include labor by project personal

experimental tanks must be evaluated. Of course, large scale systems are needed to test construction techniques and to evaluate engineering subsystems such as carbonators and mixers, as well as to obtain a realistic estimate of costs for power and maintenance. We decided that the following questions were the most important in terms of establishing the performance of large (4-8 ha) ponds: productivity; culture stability, erosion, and water loss in earth-lined vs membrane lined ponds; efficiency of use of CO_2; measurement of the power consumed in mixing; measurement of the effect of increasing head (lift) on paddle wheel efficiency; and evaluation of flow distribution.

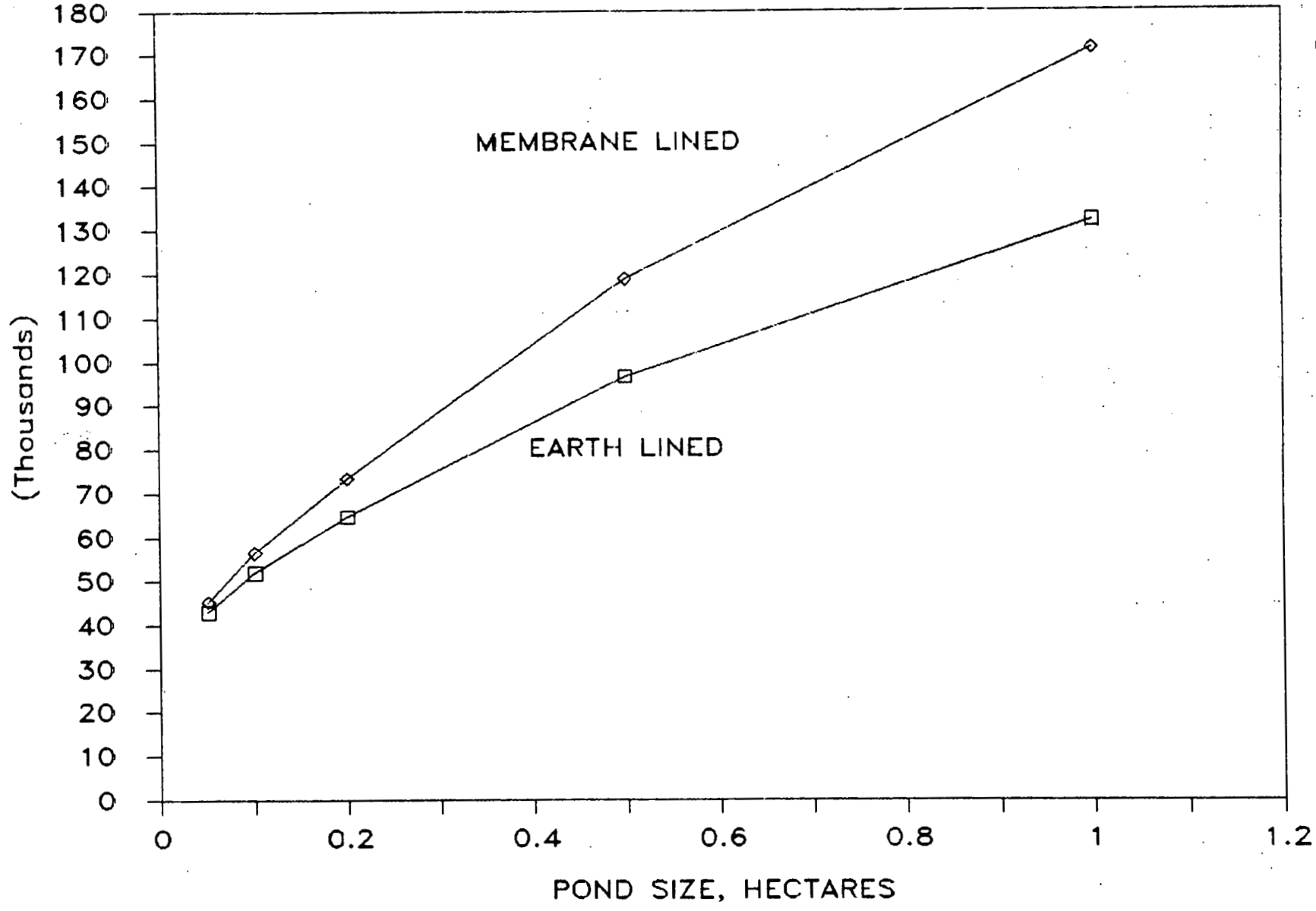
True replicability cannot be attained at the larger scale, at moderate cost. Thus the two ponds built at the first level of scale up will not be identical: one will be lined with a

twenty year life plastic membrane, the other with lower cost earthen materials. The purpose is to address a major cost element of reactor construction. Obviously the lined pond is easier to clean. In addition, an earthen pond may leak and erode. These factors must be evaluated in a longterm experiment with assessment of culture productivity and stability as well as pond maintenance and operating costs. This is a major objective of the first level of scale-up. Experiments will have to be performed at the 3 m2 scale, with the most beneficial conditions tested in the larger scale ponds for comparison. At the larger scale, only comparisons between inexpensively lined and expensively lined reactors are presently contemplated.

How large should these first level scale up ponds be? There is no definite answer to this question. The lining questions only require moderately small ponds, several hundred square meters to 0.1 ha. The long transit time between carbonation stations in very large ponds can easily be reproduced in small ponds (by allowing multiple recirculation before carbonation). Thus pond size is not a significant factor in determining carbon utilization efficiencies. To obtain the most useful measurements of the power consumed in mixing, we feel that the ponds must be large enough for the effects of flow around bends to be minimal. Ponds of 0.1 ha should be sufficient. We base this on the similarity of power demand between the measurements we took on 100 and 200 square meter ponds and those taken by Oswald (personal communication) on 4000 square meter ponds. With additional measurements from 1000 and 5000 square ponds, we should be able to accurately predict consumption in much larger ponds. As for the performance of paddle wheels working against a large lift, this probably needs to be empirically determined. However, it is not necessary to build a growth pond just for this purpose. A smaller, paddle wheel lift station can be constructed which can be used to measure the performance under a variety of conditions. By far the hardest questions to answer concern the establishment of a flow regime in large ponds: how far beyond the paddle wheel and bends will it take for the flow to settle down, what will the vertical mixing be like at this time, how will the dead spots be distributed, and how will increasing the mixing velocity affect all of this. It is likely that each scale will present new problems. Part of the purpose of the largest pond (0.5 ha) is to gain some experience in measuring and observing flow patterns in such channels, given that the panacea used in the microalgal industry today (that is, mixing very rapidly) is not cost effective for the production of liquid fuels.

From the discussion so far, the size of the scale-up ponds has only been narrowed somewhat. Figure 2 shows the economics of scale of open raceways. Although the best economies are not attained until the four to eight hectare size (Weissman and

Figure 2. COST OF A SINGLE POND



Goebel, 1987), this is larger than the range considered for the first level of scale up. Significant economies are realized in the range of 1000 - 2000 m2 compared to smaller raceways. The question is what is the most cost effective size in this range. Variable operating costs (power, water, carbon, silicate, other nutrients, harvesting chemicals) also constrain pond size. At this time we estimate that these will be about $500-800/mt of biomass (without and with silicate) and are to a first approximation proportional to pond area. Thus although 0.2 ha ponds only cost an extra $15,000 relative than 0.1 ha ponds, the operating cost differential is $6000-10,000 per year. From this perspective the 0.1 ha ponds appear to be more cost effective. In addition, the construction budget for the two first level scale up ponds is $100,000. The 0.1 ha size fits this well. Thus in the end we chose to construct the largest ponds that the construction budget allowed: two 0.1 ha raceways.

There is also the need to construct an even larger scale pond. From an engineering point of view, the hydraulic results will be more credible the larger the test unit. Longer channels minimize bend effects which may dominate small systems. More important is that head loss is better resolved (especially at low mixing velocity) in longer channels. Dead spots will be more realistically distributed, and techniques to remove them more realistically tested.

Budget capacity set the upper size of the largest "demonstration" pond at 0.5 hectare. Perceived credibility as a test system set this size as a minimum since it is about 10% the size of an actual production pond. An order of magnitude is acceptable for the scaling factor in going from test to full scale. In addition, our test unit would then be about the same size as the commercial units presently operating. This was deemed desirable. The size chosen will give data that can be extrapolated with confidence to the largest scales. We will be able to determine the uniformity of hydraulics and test how to eliminate dead spots; measure mixing power, and carbonation efficiencies; see the effects of recycle of media; perform large scale harvesting tests; and see to what extent large ponds accumulate unwanted organic and inorganic debris and how easily it can be removed. All of these scale-up considerations are pivotal to the overall process. Finally, given that the first level scale-up units were specified at 0.1 hectare, the five fold increase in size is the minimum acceptable in generating another point on the experience curve from which one evaluates the capability of extrapolating performance from smaller, tested systems to larger ones. The large pond will probably not be membrane lined.

SMALL SCALE SYSTEM DESCRIPTION

An analysis was undertaken to determine which of four prospective sites was optimal for locating the small scale system. On the basis of the results, a site at the front of the RTF, on the east side, was chosen. A schematic of the system is given in Figure 3. The installation of the system was begun on June 15, 1987. Two of the six tanks became operational on July 9, 1987. The system became fully operational by the end of July.

A list of the components of the small scale system is given in Table 4. The entire system was installed on a 1000 square foot concrete pad. All instruments and controllers were housed in protective control boxes. The items on the list are self explanatory. This system is the major experimental vehicle for this project. It consists of six 3 m2 ponds capable of being operated at 15 - 20 cm depth.

Table 4. Small Scale System Components

6 x 3 m2 Ponds

- Ponds
  - Mold
  - Ponds
  - Support structures
- Mixers
  - DC motors
  - DC motor controllers
  - Drive components
  - Motor bases
  - Paddle wheels
- Carbonation
  - pH Controllers
  - pH Probes & ext. cable
  - Solenoids
  - Accessories
  - Main regulator
  - Supply piping, valve
- Temperature Measurement
  - Max/min thermometers
  - Analog output thermometers
- Heating
  - Heaters
  - Temp. Controller
- Dilution/nutrient system
  - Dilution pumps
  - Nutrient feed pumps
  - Nutrient feed pumps
  - Influent holding tanks
  - Effluent holding tanks
  - Valves and fittings
- Other
  - Control boxes
  - Timer/controller
  - Water supply lines, fittings
  - Electrical subpanel
  - Electrical distribution
  - Utility trenches
  - Concrete pad
  - Composite sampler
  - Data acquisition system

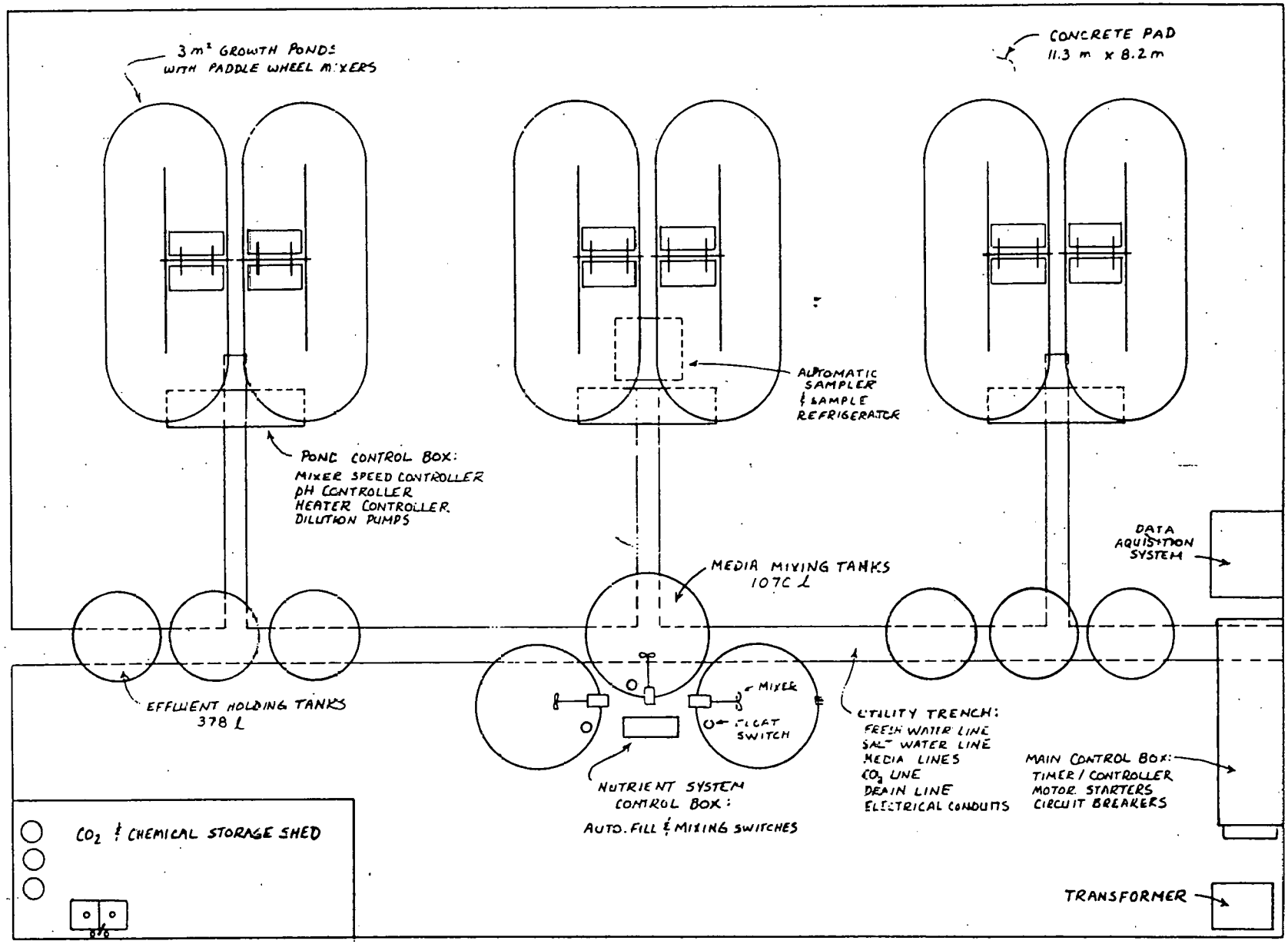


Figure 3. Schematic of Small Scale System

LARGE SCALE SYSTEM DESCRIPTION

The design of the large scale experimental ponds begins with a set of hydraulic and geometric input parameters which will satisfy the basic engineering and algal growth requirements. The selection of these parameters requires an understanding of the interactions between system elements (e.g. mixing and carbonation) as well as their impact upon costs. For fuel production systems, the need to minimize costs (both capital and operating) and energy consumption strongly influences the design process. Table 5 lists the input parameters for the proposed 0.5 hectare pond. The listing is taken from a spreadsheet model which performs the geometric and hydraulic calculations, the results of which are shown as "outputs." For multiple pond systems, an additional listing (not shown) is generated, which gives system totals, taking into account materials that are shared (i.e. pond walls). The outputs are then used in a cost estimating model to generate material quantities. The large scale system is located in the evaporation pond system, one mile east of the KTF (Figure 4).

EXPERIMENTAL PLAN

Production Performance of High-Lipid Yielding Strains

The small scale system will be used as the work horse for growth optimization studies. Productivities measured in these 3 m2 ponds will reflect the potential of the strains being studied, i.e., the productivity absent any scale up problems or limitations. We have several years experience operating small ponds for this purpose. Thus the experimental design presented reflects an understanding of what can be accomplished at this scale and how to verify the reproducibility of the results. It has been our experience that two ponds must be used for each strain studied, with one growth unit operated in a constant manner to act as a control for both climate and biological adaptation (adjustment to the test conditions or decline in performance with time). In the past we have operated such controls with temperature control (heating to 25 oC by 0930 hours), pH = 7.8, CO2 = 125-300 uM, mixing velocity = 30-40 cms-1, dissolved oxygen reaching four to five times saturation, and dilution rate = 40-60 %/day semi-continuously or continuously over daylight hours. These conditions have led to optimal productivity from test organisms. The high DO was simply a consequence of biomass production. Attempts to minimize it were never very effective. For this project, performance at prevailing temperature is an important measurement. Thus controls will be operated as above but without artificial heating. Heating will be a test variable during each of the seasons. All controls will be diluted continuously over the daylight hours, as will most of the test ponds. Any type of semicontinuous dilution requires operator attention on a daily basis. With the prescribed budgets, week-

Table 5. Large Scale Growth Pond Design Parameters

INPUTS

DESCRIPTION	VALUE	
Pond Area	0.50	hectares
# of Channels	2	-
L/W Ratio	20	-
Depth	20	cm
Channel Velocity	30	cm/sec
Mannings 'n'	0.018	sec/m^0.33
Paddle Eff.	0.6	-
Drive Eff.	0.7	-
PW Width/Chan Width	0.75	-
Evaporative Rate (max)	1.0	cm/day
Detention Time	2.00	days
Wall Ht. (above grade)	35	cm
Wall Ht. (below grade)	10	cm
Sump Depth	1.5	meters

OUTPUTS

DESCRIPTION	S.I. UNITS	
Channel Width	10.77	meters
Paddle Width	8.07	meters
Single Channel Length	232.22	meters
Total Channel Length	464.44	meters
Centerwall Length	215.31	meters
Total Str. Wall Length	646	meters
Curved Wall Length	68	meters
Total Wall Length	714	meters
Total Wall Area	321	sq meters
Slope	2.492	x 10^-4
Total Head Loss	12.03	cm
Total Efficiency	42%	
Hydraulic Power	733	watts
Total Power	1746	watts
Total Unit Power	0.35	watts/sq m
Velocity at Paddle	40.0	cm/sec
Pond Volume	1000	cu meters
Evap. Flowrate	25	cu m/day
(alternate units)	17	liters/min
Harvest Flowrate	500	cu m/day
(alternate units)	347	liters/min
Velocity Head	0.46	cm

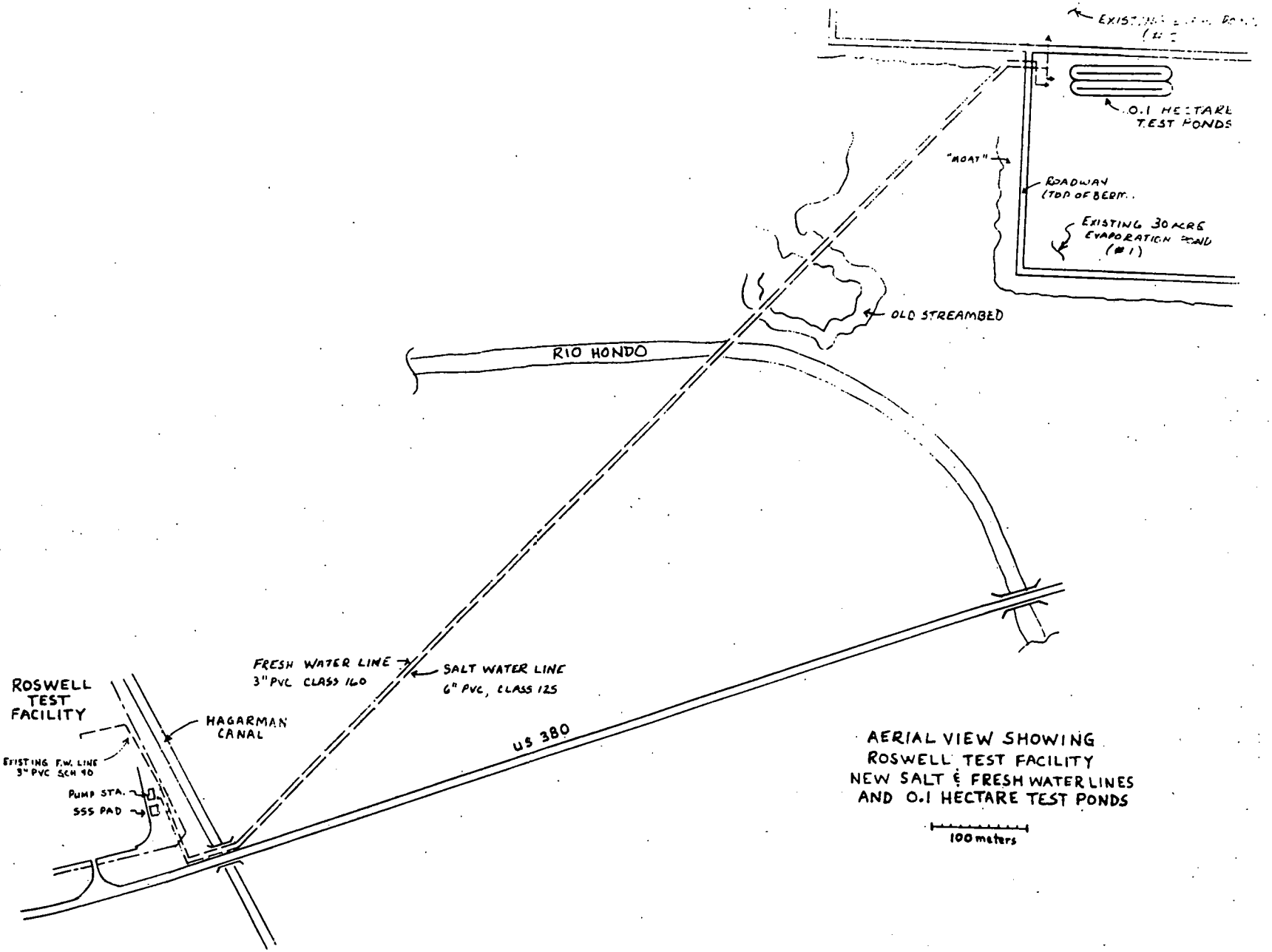


Figure 4. Location of Large Scale System

end and holiday monitoring of the pond operations must be kept to a minimum. Continuous dilution is most conducive to achieving this. The rate of dilution of the controls will need to be adjusted with season.

The following experimental variables will be tested for effects on productivity and culture endurance: artificial heating, rate and mode of dilution, pH, CO2 concentration, and nutrient limitation (nitrogen and/or silicon). In general, in the 3 m2 ponds, species will be grown in the deep saline well water (SaW) medium. One of the primary tasks is to find an ASP strain that grows well in the climate at Roswell and in the well water available. In subsequent years, artificial media (SERI Types I an II water) will be made. One other variable which eventually will be tested in these small ponds is the effect of recycle of large fractions of the clarified effluents. This type of work is more involved since it requires harvesting the algae and returning the effluents. Its value hardly has to be emphasized; however, it will not be possible to perform recycling experiments during the first year.

For systems diluted on a daily basis, each day is considered a replicate. When ratios of experimental to control pond productivities are reported (when experiments last long periods of time due to the number of treatments involved), each day's ratio is a replicate and errors are reported as standard errors. Most statistical testing is based on t tests.

Routine measurements, to be taken five times weekly include max/min temperature, pH, dilution rate, biomass density, ambient max/min temperature and insolation. From these data biomass productivity and PAR efficiency can be calculated and basic pond parameters monitored. Continuous temperature monitoring will be performed in two ponds to track temperature profiles in heated and unheated systems. This is necessary to help determine the effects of temperature on productivity, especially time spent above certain temperatures. We have found this to be more determining of productivity than max/min temperatures. In addition temperature profiles measured over the year will be important as input in the development of a complete algal biomass production model. Dissolved oxygen will be measured in each pond on occasion only since it is quite predictable. Total solids of the medium will be measured on a once weekly basis. This variable is of course important in recycling experiments. If possible a monthly sample from each suspension will be sent to SERI for complete chemical analysis (protein, lipid, carbohydrate, and chlorophyll).

A computer automated data acquisition system is being installed to increase the frequency of data acquisition. Following this, simple computerized operation will allow testing of some additional factors, e.g., the effect of cycling pH and CO_2 concentration on productivity.

The major thrust of this first year of testing is to demonstrate reproducibility of results, determine longterm production and stability of a previously tested species in the southwest environment, and screen for species which are highly productive and stable throughout the warm season and others throughout the cold season. The following tentative schedule summarizes the experiments we expect to perform. Changes will be made based the ASP needs.

In July, pond pairs 1 & 2 and ponds 3 & 4 were inoculated with two of the the best ASP species to date (Cyclotella sp., Chaetoceros gracilis) to re-evaluate perfomance in the Roswell climate. All ponds were operated in the control mode, with none heated. In August, when the third pair of ponds are on-line, we will attempt to cultivate one of the pre-screened green algal strains. If none are capable of growing well, we will examine any contaminant strains which arise for utility in this project. Since the pairs of ponds will be operated as replicates, we will have the opportunity at this time to assess the reproducibility of productivity results. Starting in September, one pond of each pair will remain in the control mode while in the other a heater will be used to maintain temperatures above 20 oC during daylight.

After this initial summer break in period, pair 1 & 2 will continue to be operated with the species which appears to grow best as the temperatures and day length decrease. The other two sets of ponds will be used primarily for screening species newly available from the ASP for their potential in producing biomass. Thus starting in October the ponds will be inoculated with species provided by the ASP. Each candidate will be grown for 30 days unless it cannot survive for this time period. Time will not be wasted trying to grow difficult species. During Nov - Feb, we will attempt to test cold water species. This work is considered very important since much of the Southwest has at least a 120 day cold season, but in which skies are usually clear. If a suitable cold water strain is found in a timely fashion, the third set of two ponds will be used for studying the induction of lipids. Induction will be investigated primarily in terms of the switching from dense cultivation just as nitrogen (or Si) runs out, to less light limiting conditions for the induction process.

Large Scale Production System Engineering Assessment

Eventually the assessment envisioned for the large scale

system is a complete biochemical engineering analysis of the reactor and biological performance in it. This means measuring inputs and outputs to develop heat balances, mass balances, and calculate efficiencies of utilization of the inputs as well as efficiencies of transformation into product. We will begin by doing some basic hydraulic measurements and carbonation injection studies prior to introducing any organisms.

A variety of tests will be conducted to determine the hydraulic characteristcs of the pond, and to provide design information for future pond systems. Of primary interest are the velocity profiles at various points in the pond, the channel roughness (i.e. the Manning equation's 'n'), the paddle wheel efficiency, the head loss associated with the bends. Each of these will be discussed below.

Velocity Measurements

An understanding of the pond hydraulics requires the mapping of velocity profiles at various points in the pond. At a given location, measurements are taken both across the channel and at different depths. As a result of friction at the pond bottom, a velocity gradient exists from the bottom to the surface, which in turbulent flow, follows a logarithmic law. From this profile, the "shear velocity" can be determined. The shear velocity is a measure of the turbulent velocity intensity and the dispersion due to turbulence. From the shear velocity and depth, the time scale of turbulent eddies can be estimated, which is the time required for the exchange of water (and cells) from the flow surface to the bottom boundary. Variations in velocity across the channel are primarily the result of geometric factors, e.g. the paddle wheel and channel bends. The latter are responsible for regions of low velocity (stagnation zones) which lead to solids deposition. Bends are normally furnished with curved baffles in order to minimize such stagnation zones. The velocity mapping can be used to evaluate changes in baffle configuration and other measures that may be taken to reduce stagnation. Finally, the velocity mapping is necessary in order to arrive at an average linear velocity upon which to base calculations of roughness, hydraulic power, and mixing system efficiency. Velocities will be measured with a Nixon Instruments Streamflo meter (with low speed probe), a device designed for open channel flow measurements.

Pond Roughness

The prediction of hydraulic losses is a key factor in pond design, for both the earthworks (i.e. setting grades) and for sizing the mixing system. The Manning equation for open channel flow is normally used, with a roughness value (Manning's 'n') chosen from a list of typical values, which usually specify a range for a given material or surface

condition. For earthen channels the range is particularly large. The simultaneous measurement of head loss and velocity at several mixing speeds will allow Manning's 'n' to be determined for the particular surface condition characteristic of a compacted layer of graded crushed rock. This information will be of particular value in the design of very large (>2 ha) ponds. The head loss will be measured in a straight section of the channel, after preliminary velocity measurements are taken to determine suitable locations. Typically, the profile across the channel is fairly symmetric downstream from the paddle wheel and remains so until the first bend. For the 0.5 hectare pond, this corresponds to about 130 meter distance, which should allow good resolution in the head difference measurements. Measurements will also be taken to estimate the magnitude of the bend losses, for which little information is available.

Paddle Wheel Efficiency

From measured values of head loss and velocity, the hydraulic power (energy/unit time imparted to the water) can be calculated. By comparing this to the power delivered to the paddle wheel, the efficiency of the paddle wheel can be determined. The limited data on paddle wheel efficiency cited in the literature (20-60%) is generally from small ponds (where uncertainties in the head loss are large), and sub-optimal paddle wheel designs. The power delivered to the paddle wheel will be determined by simultaneous measurement of rotational speed and torque, via strain gages on the driveshaft. This approach lumps wind and bearing losses together with the paddle wheel losses, but excludes drivetrain losses (motor, variable speed unit, speed reducer, etc), which are both large and quite variable. The drivetrain efficiency can be determined by comparing the power delivered to the motor, using a wattmeter, with that delivered to the paddle wheel driveshaft. A more accurate knowledge of paddle wheel efficiency will be of great value to designers of large scale pond systems, since the mixing system represents a significant portion of capital costs. Future research in pond mixing might include a comparison of other mixer types, for example air-lift pumps. A sump built to test air-lift mixing could also be used to investigate pond carbonation, e.g. co-current vs. counter-current, depth of submergence, etc.

Carbonation and Outgassing Tests

Due to the time of year that the large scale system will be completed, the following tests will be performed during the next subcontract period, when a carbon dioxide tank has been installed.

The efficiency of injecting CO2 via the in pond sump will be tested in two ways. To acquire data which can be used in future design endeavors only the sump will be filled, with water of known carbonate chemistry. The change in total carbon content of the water will be measured as a function of water depth (bubble column height), gas flow rate, and water salinity (which determines bubble size for given diffusers). From this data we can determine stripping rates in terms of these conditions, and then in turn the performance of co and counter current injection can be predicted. This will allow us to determine the sump depth and baffle placement required for 95% injection efficiency in the co current position and the baffle placement needed to prevent escape of 95% of the bubbles in counter current operation. In subsequent funding periods, these predictions can be compared to empirical results. In the second method the pond will be filled to the normal operating depth of 20 cm and the diffusers placed in the carbonation sump. Given the influent rate of pure CO2 and the change in the inorganic carbon content of the water corrected for outgassing, the injection efficiency can be calculated.

The coefficient for mass transfer through the pond surface will be measured as a function of depth and mixing velocity. This is done by monitoring the pH change with time, at about neutral pH, calculating the loss of total carbon for each of many pH intervals (of about 0.05 units) and plotting this against the average driving force during corresponding pH intervals. The mass transfer coefficient, expressed as mhr-1 or ms-1, has been measured to within a repeatability of 10% by us in the past using this method.

FUTURE WORK

By the end of the first year of the project the small scale system will have been constructed and operated for over six months. Two 0.1 hectare pondsill also have been constructed. However, these will not be put into operation until the beginning of the second year. At that time a longterm experiment will be initiated, using the strains of algae that performed best in the 3 m2 ponds, to determine how an earthen liner performs relative to a membrane liner. Also during the second year we will have productivity data accumulated over a full year, giving the first estimate of average production from these systems in the Southwest.

BIBLIOGRAPHY

Weissman, J. C. and Goebel, R. P. , 1987. "Design and Analysis of Microalgal Open Pond Systems for the Purpose of Producing Fuels," Final Report, SERI/STR-231-2840.

Conversion

THIS PAGE

WAS INTENTIONALLY

LEFT BLANK

CONVERSION OF LIPIDS THROUGH BIOLOGICAL PRETREATMENT

Nick Nagle, Paulanne Chelf, Peter Lemke, Bill Barclay
Solar Energy Research Institute
Golden CO 80401

ABSTRACT

An important step in the utilization of lipids produced by microalgae will be the conversion of algal lipids into usable fuel stocks. The first step in the process is the conversion of triglycerides into free fatty acids for transesterification. A naturally occurring enzymatic process which accomplishes this conversion, was observed, and investigated. Promising species of lipid producing microalgae were screened for the presence of this enzymatic activity. A regression model was developed to predict the effects of important variables on this process. Results indicate that silica concentration and temperature important variables that control the conversion of triglycerides into free fatty acids.

CONVERSION OF LIPIDS THROUGH BIOLOGICAL PRETREATMENT

INTRODUCTION

One of the primary goals of the Aquatic Species Program is to cultivate microalgae for the production of lipids, which can be converted into traditional liquid fuels. These microalgae produce lipids during periods of stress via nutrient limitation and other physiological factors. The lipids extracted from these microalgae can then be converted into traditional fuels by catalysis or by transesterification. An important observation was made: certain strains of microalgae can convert the triglyceride fraction into free fatty acids. The first probable step in the conversion of lipids into traditional fuels is the transesterification of triglycerides to methyl esters. This observed lipase activity converts triglycerides into free fatty acids and can be considered as a possible pretreatment of the lipids before esterification or catalysis.

MATERIALS AND METHODS

Organism

Navicula saprophila (NAVIC1), Navicula sp. (NAVIC2), Monoraphidium minutum (MONOR2), Chaetoceros muelleri (CHAET6,9,10,14) were obtained from the SERI culture collection.

Culture

These organisms were kept in Type II/25 mMho cm^{-1} medium throughout the experiment. The cultures were grown in a growth chamber on an 18:6 light to dark regime, at 25 degrees C at a light intensity of 120 $uE/m^2/sec$. Lipid induction was accomplished by transferring exponentially growing cells into media that was either nitrogen or silica free. These cells were then harvested and frozen until used.

Experimental Design

A Plackett-Burman design was used to determine the important variables in the enzymatic process. The variables examined were silica concentration at 0 and 500 mg/1, nitrogen concentration at 0 and 1000 mM, temperature at -20 and 20 degrees C, and time at 0 and 16hours. After identification of the important variables, a response surface was developed using a Box-Behnken fractional factorial design for three factors. The ranges for these factors were 0,8 and 16 hours for time of incubation, 0,250 and 500 mg/1 silica concentration at the time of induction, and temperature at -20,3,and 20 degrees C. A regression

model was developed using RATIO as the dependent variable. RATIO is defined as the ratio of free fatty acids to triglycerides as determined from Iatroscan analysis. Analysis of the lipids by gas chromotography was done to determine fatty acid composition.

Analytical Techniques

Lipids were extracted using a modified Bligh-Dyer technique (Roessler 1986). The individual lipid classes were separated using an Iatroscan as follows: the extracted lipid samples were spotted on silica impregnated chromarods then developed in chloroform-acetic acid (100:0.5). The rods were then analyzed in the Iatroscan and the lipid classes were identified by the use of known standards. The fatty acid composition was determined by methylating the extracted lipids for analysis by gas chromotography by the method of Lepage and Roy. The statistical analysis was done by X-STAT software (Murry 1984).

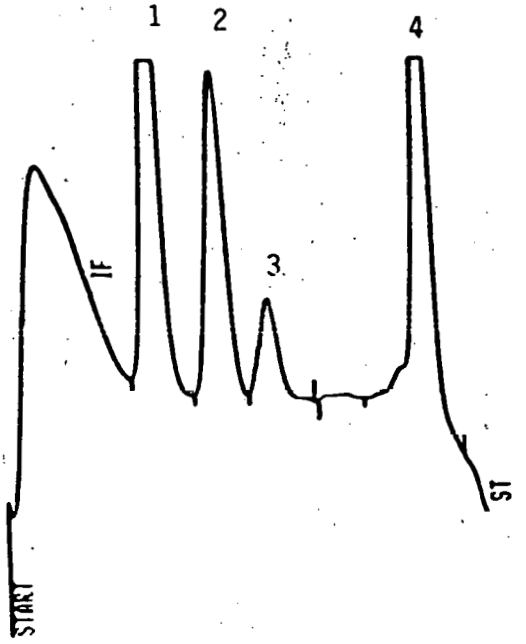
RESULTS

After the algae have been stressed by either nitrogen or silica limitation there is an increase in the non-polar lipids. Incubating the cells at 28 degrees C for 16 hours prior to lipid extraction results in a large increase in the fatty acid fraction at the expense of the triglyceride fraction (fig 1). This ratio (free fatty acids to triglycerides) was the dependent variable used in the regression analysis.

Eight promising species of lipid producing microalgae were screened and three species were identified as having high levels of lipase activity after induction by silica limitation. These were Navicula saprophila (NAVIC1), Navicula sp. (NAVIC2), and Chaetoceros muelleri (CHAET10). Cyclotella cryptica (CYCL01) had marginal lipase activity.

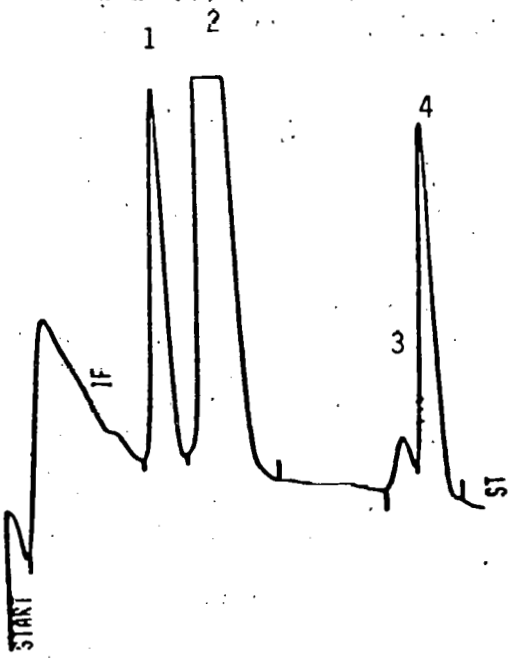
A Plackett and Burman screening protocol was used to screen all the algal species. The variables that were screened were nitrogen and silica concentration, temperature of incubation and the length of time of incubation. The results from the Plackett-Burman screening design indicate that silica concentration, time of incubation and temperature were important factors in the conversion of triglycerides into free fatty acids for all strains. No lipase activity was observed in nitrogen deficient cultures. A response surface for the enzymatic process was developed for NAVIC1 by using a Box-Behnken fractional factorial design. The regression model that was obtained from this analysis identified significant effects (table 1) that had a confidence coefficient of 90% or better. The standard deviation around the regression was 1.05, and the explained variation around the mean (R-squared) was 95.76% (table 1). The maximum conversion of triglycerides into free fatty acids was predicted to occur under the following conditions: silica concentration at 0 mg/1, temperature at the maximum of the model (23 degrees C), and an incubation time of 8.02 hours. At these conditions a maximum value for RATIO of 9.30 (table 2)
was predicted.

IATROSCAN OF EXTRACTED LIPID FROM CHAET10



1. Triglycerides
2. Free Fatty Acids
3. Alcohols
4. Polar Lipids

Before Treatment



1. Triglycerides
2. Free Fatty Acids
3. Alcohols
4. Polar Lipds

After Treatment

Figure 1

Regression Coefficients for RATIO

Coefficient	Term	Standard Error	T-Value	Confidence Coef <>)
1.299	1.000	0.5174	2.511	97.3%
-2.299	SIL	0.3808	6.036	99.9%
-2.235	(TEMP*SIL)	0.5386	4.150	99.8%
-1.634	(TIME 2.000	0.5589	0.5589	98.7%
3.474	(SIL 2.000)	0.5589	6.216	99.9%

Confidence figures are based on 10 degrees of freedom.

MAXIMUM RATIO

A maximum of 9.306 was achieved under the following conditions.

Value at Maximum	Factors	Lower Limit	Upper Limit
1000	TEMP	-1.000	1.000
0.00033	TIME	-1.000	1.000
-1.00	SIL	-1.000	1.000
	Characteristics		
9.31	RATIO		

Starting factor values: 0.000, 0.000, 0.000

D:31

The analysis of the fatty acid composition indicates (fig 2) that the composition of the fatty acids in the three strains is unchanged by the lipase activity.

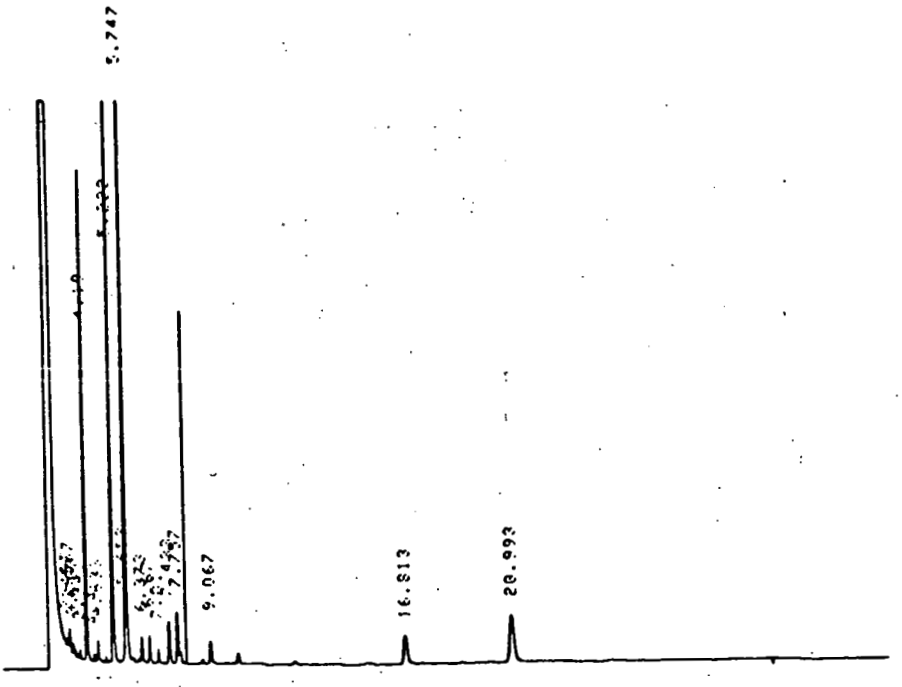
DISCUSSION

The conversion of triglycerides into free fatty acids by these species is believed to be enzymatic in nature. In other species of algae that produce large amounts of lipids no conversion took place indicating that the process is not likely to be autolytic. In addition, since no conversion had taken place in positively tested strains under nitrogen limitation the argument could be made that lack of nitrogen prevented protein synthesis necessary for the lipase enzyme. The cultures used in this experiment were not axenic and this experiment will have to be repeated with axenic strains to prove that bacteria were not the source of the lipase activity. The algae are a more likely source of lipase, since lipase would be needed to utilize stored neutral lipids as an energy source.

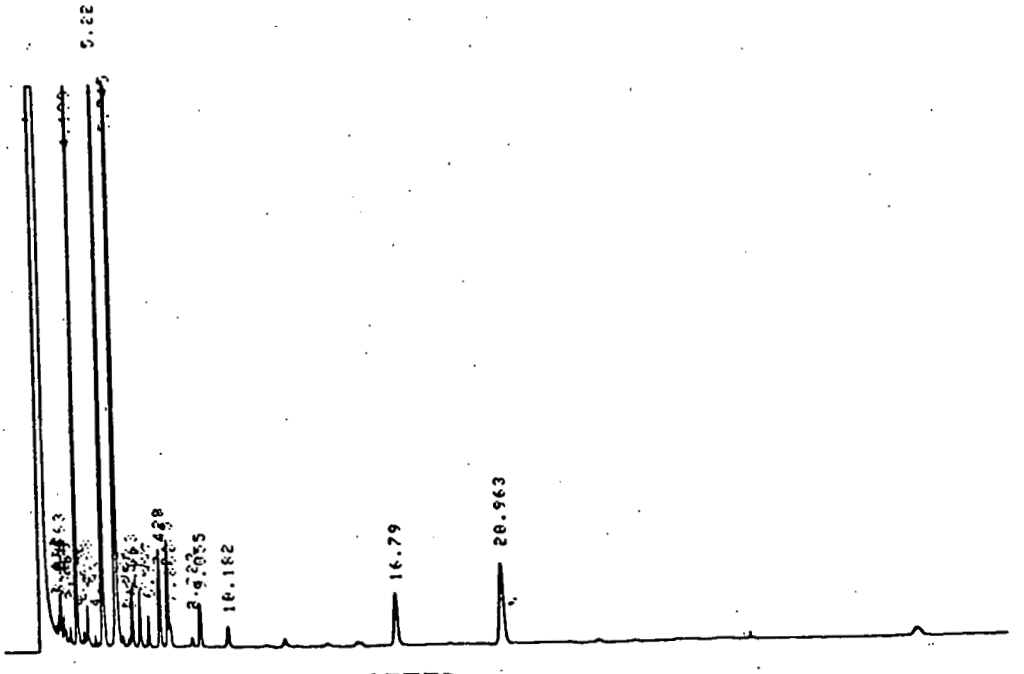
The use of both the Plackett-Burman screening approach and the fractional factorial Box-Behnken design have identified the factors in the conversion of triglycerides into free fatty acids. The major effect was the silica concentration at the time of induction. The silica concentration may or may not be a direct effect. It would be advantageous to the algae to possess an enzyme that could degrade the storage product for use in an energy producing pathway. The onset of lipid formation could trigger the synthesis of this enzyme. The absence of observed lipase activity in nitrogen deficient cells would give some support to this. The major interaction is the silica concentration and temperature. This could be described as the effect of first having lipid induction by silica limitation coupled with the increase in lipase activity from the physical effect of an increase in temperature. The silica^2 and time^2 terms indicate that the effect is curvlinear. The lipase activity begins to plateau at the mid point of time, and a longer incubation time does not significantly increase the ratio. The major effect is seen at the zero concentration of silica, at 250 mg/l or 500 mg/l silica no effect is observed. It is important to realize that defining the ranges for the experiment does influence the regression model. In this experiment the algae were harvested two days after lipid induction. Harvesting at a later time might result in lipid production and lipase activity at the 250 mg/l level, which was not seen after harvesting at two days.

The analysis of the fatty acid content indicates that composition in the major groups of fatty acids remains fairly constant. This is a very encouraging result because the largest fraction is the 16:0 and 16:1 fatty acids. These fatty acids can be used in diesel engines (Klopfenstein et al. 1983). The structure of these fatty acids help to avoid problems such as coking of the injectors, deposits on rings and polymerization in fuel tanks. These are problems that occur with vegetable oil and vegetable oil blends (Harrington 1986).

FATTY ACID COMPOSITION BEFORE AND AFTER ENZYME TREATMENT IN CHAETOCEROS MUELLERI



BEFORE



AFTER

Figure 2

CONCLUSION

Biological conversion of triglycerides into free fatty acids was observed in three species of microalgae that produce large amounts of lipids during silica limitation. This biological conversion should be considered a part of the overall approach to converting algal lipids into liquid fuels. The enzymatic process is advantageous in that the amount of effort involved to accomplish this conversion is small compared to the benefit. The process has been modeled and important identified. A scale-up of the process should be investigated as well as a screening program for other lipase producing strains of microalgae.

REFERENCES

1. Bligh, E.G., Dyer, 1959. A Rapid Method of Total Lipid Extraction and Purification, Can. J. Physiol. 37:911-917.

2. Delmas,R.P.,Parrish and Ackman, 1984. Determination of Lipid Class Concentrations in Seawater by Thin Layer Chromotography with Flame Ionization Detection. Anal. Chem. 56: 1272-1277.

3. Harrington,K.J.1986. Chemical and Physical Properites of Vegetable Oil Esters and Their Effect on Diesel Fuel Performance. Biomass 9:1-17.

4. Hassett,D. 1983. Sunflower Oil Methyl Ester as a Diesel Fuel. USDA Grant# 59-2382-1-6-058-0 : 135-137.

5. Klopfenstein,W.E. and Walker, 1983. Efficiencies of Various Esters of Fatty Acids As Diesel Fuels. J. Amer. Oil Chem. 60:1596-97.

6. Lepage,G. and Roy, 1984. Improved Recovery of Fatty Acids Through Direct Transesterification Without Extraction or Purification. J. Lipid Research 25:1391-6.

7. Murray, J.S. 1984. "X-STAT, Wiley Professional Software.

8. Roessler, P.G. 1986. Biochemical Aspects of Lipid Accumulation in Silica - Deficient Diatoms. In FY 1986 Aquatic Spcies Program Annual Review SERI/SP-231-3071, 257-271.

CATALYTIC UPGRADING OF WHOLE ALGAE AND THEIR COMPONENTS WITH SHAPE-SELECTIVE ZEOLITES

Thomas A. Milne and Robert J. Evans
Chemical Conversion Research Branch
Solar Fuels Research Division
Solar Energy Research Institute
1617 Cole Blvd., Golden, CO 80401

ABSTRACT

A seminal paper by Mobil researchers (Science 206, 5 Oct. 1979, pp. 57-58) demonstrated that a remarkable range of materials were convertable to a similar, high-octane aromatic gasoline product slate when passed over ZSM-5. These materials ranged from methanol to latexes and vegetable oils. This type of conversion for microalgae-derived lipids has been recognized in the DOE program and, in fact, is projected to have the most favorable liquid transportation fuel economics, with transesterification of neutral lipids a close second, depending on glycerol by-product credits assumed (Neenan et al.; SERI/SP-23102550, August 1986). Hydrogenation and hydrocracking have been mentioned in the literature, but were not included in the SERI options. In work we are carrying out for the DOE Thermochemical Program, it has been shown that carbohydrates can also yield significant gasoline yields. Proteins, to our knowledge, have not been studied over ZSM-5, but this catalyst is expected to have tolerance for both N and S.

Under sponsorship of the SERI Director's Development Fund, we are carrying out exploratory studies of the pyrolysis of whole algae and their major components, supplied by SERI's Biotechnology Research Branch. A conversion process that made use of all parts of the microalgae to produce a single, high-octane liquid transportation fuel, has the potential for a simplified flowsheet, higher yields, eased restrictions on the trade-off between total growth and lipid yields, greater insensitivity to the type of lipid formed, and a single, transportable product to market. It is expected that polar lipids and hydrocarbons, as well as triacylglycerols, will all give substantial yields of gasoline. The economic potential is indicated by the fact that transesterification of the triglycerides, alone, may cost on the order of $1.00/gallon (D. J. Hassett - Energy from Biomass and Wastes X, 1987, pp. 855-865).

Our use of the SERI molecular-beam, mass-spectrometric pyrolysis sampling system will be described and initial results presented for the conversion of extracted lipids, whole algae, and the whole algal residue after lipid

extraction. This system has the advantage that only a few hundred milligrams of material are needed to screen for relative gasoline yields from experimental algae and their components. In addition, pyrolysis fingerprints of the algal material before catalysis will be shown to illustrate the possible use of this information for rapid estimates of major lipid components.

In a more extensive program, we would wish to examine the quantitative yields; the synergistic effects of co-converting carbohydrates, proteins, and lipids; the fate of nitrogen, which may evolve as ammonia; the yield of CO_2, which may be concentrated enough to be recycled into the microalgae production facility; and the amount of water that can be tolerated in the algae as they are fed to the pyrolysis/catalysis unit. The knowledge of these parameters would allow the preliminary evaluation of the economics of this conversion scheme.

THIS PAGE

WAS INTENTIONALLY

LEFT BLANK

Economic and Resource Assessments

THIS PAGE

WAS INTENTIONALLY

LEFT BLANK

CO_2 SOURCES FOR FUEL SYNTHESIS

Michael E. Karpuk
Technology Development Associates
1667 Cole Blvd.
Golden Colo. 80401

ABSTRACT

Efficient production of liquid fuels from microalgae will require large quantities of CO_2 at low cost. In this recently initiated study, we will examine both current commercial and possible new sources of CO_2 and will evaluate their potential to supply the quantities of CO_2 required for large scale microalgae production. Current commercial sources of CO_2 include concentrated waste streams such as from fermentation plants and natural gas processing plants and natural reservoirs of nearly pure CO_2. Possible new sources include flue gas, advanced power plants and air separation. In addition, we will evaluate CO_2 transportation costs, the energy requirements and costs of CO_2 separation processes, and the impact of potential regulations to control CO_2 emissions from fossil fuel power plants.