FINAL REPORT

TO THE

SOLAR ENERGY RESEARCH INSTITUTE

UNDER

SUBCONTRACT X-K-4-04143-01

CHEMICAL PROFILES OF MICROALGAE

WITH EMPHASIS ON LIPIDS

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NOTICE

PREFACE

This Final Report details progress during the third year of this subcontract. The overall objective of this subcontract was two fold: to provide the analytical capability required for selecting microalgae strains with high energy contents and to develop fundamental knowledge required for optimizing the energy yield from microalgae cultures. The specific objectives established for this project during this year were:

- Identify the structure of the C-37 lipid compound observed in Isochrysis.
- Identify the major hydrocarbon and lipid fractions found in the microalga known as Nanno Q (Nannochloropsis sp.)
- Culture and measure lipid composition and productivity by 5 to 6 potentially promising, taxonomically distinct, strains of microalgae obtained from SERI's culture collection.
- Determine and quantify the chemical composition and micro- and macroalgae submitted by other SERI subcontractors.

The progress made towards each of these objectives during this year is detailed in this report. Future work by other researchers in this program will benefit from these results, in terms of a rational and simplified lipid screening protocol for promising strains, and, generally, through application of the methodologies and data developed during this study.

SUMMARY

Eight strains of microalgae were subjected to nitrogen limitation in batch cultures and changes in composition (protein, lipids, carbohydrates) were followed. Experiments were carried out primarily at one light intensity (300 microeinstein m^{-2} sec^{-1}), one CO_2 level (1% in air), and one N level (1.6 mM $N\text{-}NO_3$). Thus the conclusions are tempered by the fact that the optimal conditions for lipid biosynthesis induced by N limitation may not have been achieved.

Chlorella sp. (Black Lake isolate) had a low lipid content under N sufficiency and exhibited no significant increase in its lipid content, while overall productivity decreases rapidly, upon N limitation. In contrast "Nanno Q" (Nannochloropsis sp. strain QII) exhibited a relatively high lipid content under N sufficient conditions (25% of ash free dry weight), a further increase upon N limitation (to over 50%), and a sustained high productivity after N limitation was induced. The other strains studied were intermediate in response: Cyclotella sp. exhibiting a marked rise in lipid content upon N limitation (for 14% to 40%) and for a short period, relatively high lipid productivities in response to N limitation. Chaetoceros gracilis and Isochrysis galbana (Tahitian strain) did not exceed 30% lipid contents - even after prolonged N deficiency. Thalassiosira pseudonana, Ankistrodesmus falcatus, and Boekolovia sp exhibited maximum lipid contents of 35 to 39%, however, lipid productivities were rather low.

An analysis of lipids revealed that most of the lipids present in N limited algae were neutral lipids. Even in strains without major increases on total lipid content, N limitation induced significant changes in lipid profiles. For Chaetoceros and Thalassiosira Si limitation was also studied as a method for lipid induction - however, it did not appear to provide a significant advantage over N limitation.

Using ^{14}C, ^{32}P and ^{35}S the lipids of Nanno Q were labelled, fractionated and analyzed. Although the culture system used in the tracer experiments (a stoppered, shaker flask) differed from the above described experiments, the results were qualitatively similar in terms of lipid induction upon N limitation. Most of the lipids synthesized during N limitation were triglycerides. Over half of the $^{14}CO_2$ fixed during N limitation was found in the lipid fraction of Nanno Q.

The results presented allowed the design of an efficient screening protocol for lipid producing algae and provide the basis for a more detailed investigation of Nanno Q and Cyclotella - the two most promising strains studied thus far.

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SECTION 1.0

CULTURE APPARATUS AND PROTOCOL DEVELOPMENT

1.1 OBJECTIVES

TASK I - Develop a culture apparatus and experimental protocol for carrying out Task II.

"To develop a culture apparatus that will culture species efficiently for the purpose of determining the relationship of carbohydrate accumulation and subsequent lipid synthesis when cells are placed under nitrogen deficient conditions."*

"Two culture apparatus concepts shall be constructed and tested. Representative chemostat and immobilized cell designs shall be developed that shall permit culturing microalgae under relatively low cell density, with a nitrogen gradient. One concept shall be selected for carrying out Task II. An experimental protocol shall be developed for carying out Task II."

1.2 INTRODUCTION

High photosynthetic efficiency (productivity) by a microalgae culture requires light limitation. This apparently precludes nitrogen or other nutrient limitations in lipid production, as, theoretically, only one nutrient can be limiting at one time. In the past, most experiments in which nitrogen limitation was imposed on batch cultures, as a method for increasing lipid content, used relatively dense, light limited, cultures. Such cultures continued to grow, arithmetically, and, eventually, depleted media nitrogen. However, nitrogen limitation - as observed by decreases in protein, cellular N, and pigment levels was induced only slowly. In such cultures, storage of carbohydrates and/or lipids either did not achieve a very high level or did so only after a long time. Lipid productivity was thus very low in most of the prior studies. To maximize the rate at which the transition to lipid biosynthesis can occur nitrogen limitation must be induced under conditions of relatively high growth rates. This implies relatively low density. However, this need not imply low productivity, if cell density is maintained at a sufficient level to utilize all of the incident light energy, but low enough that growth rates are still high. This would allow rapid depletion of nutrients and switch to a nutrient deficient metabolism. It should, thus, be possible to separate the growth (biomass production) and lipid induction (triggered by nutrient limitation) phases in such experiments. By using different light intensities, cell densities and/or reactor designs for the nutrient sufficient and limited stages. the processes may be optimized to optimize each metabolic phase. Overall productivity could be maximized even when nutrient limitation is used to induce lipid biosynthesis. This is the basic hypothesis underlying this project. Collection of evidence either supporting, or more appropriately falsifying, this hypothesis was a major objective of this subcontract.

*All statements in quotations in the objectives subsections of this report are from the Statement of Work of this subcontract.

Two alternative systems can be considered in such a process: 1) A single stage system in which cell growth (from an inoculum) takes place in a batch mode followed by nutrient limitation in the same vessel, followed by harvest of most of the biomass, readdition of nutrient and repeat of the batch culture until contamination requires a new start-up; and 2) A two stage system in which a continuous nutrient sufficient culture is discharged into a separate culture where nutrient limitation occurs. Thus, both continuous and batch reactors were designed under this task. However, experimentally, only the batch cultures were eventually used, since the two stage system has not yet been investigated.

1.3 REACTOR DESIGN

The design of laboratory batch reactor's for growth of suspended cultures is simple. Roux bottles are effective for operating batch microalgae cultures; they provide a relatively thin culture vessel (i. e. relative high light input per unit volume) and a sufficient volume to supply adequate samples for analysis. Light intensities up to 15% of full sunlight can be provided using fluorescent grow lights. Sampling is done by removal of the gas exchange port.

For continuous cultures two reactors were designed: a Roux bottle with two access ports, for media influent and effluent and the measurement probes, and a 2 liter culture apparatus containing a stirring rod with two sets of 4 glass paddles for agitation. The continuous reactors however, were not used in this project as the batch Roux bottles provided sufficient samples for all required analysis.

1.4 IMMOBILIZED REACTORS

Immobilized microalgal cultures were initially proposed as an alternative to liquid cultures. Several designs were tested under this contract. The first involved a flat plate system filled with porous polyethylene plastic provided by the Porex Corp. However, this design proved to be very difficult to seal, with leaks developing repeatedly. For this reason this design was discontinued.

The second design consisted of an array of parallel tubes loaded with alternating hydrophilic and hydrophobic tubular supports (approx. 10 cm in length) with various pore sizes. (The reactor was inoculated with Ankistrodesmus at an average light intensity of 50 $\mu E/m^2/S$. Attachment was only observed on the lower side of the support and this may have been due to photoinhibition. (Even though this light intensity is low, once attached, the cells have no ability to change their light environment.)

A simplified design, similar to the above concept, consisted of two 130 cm x 3 cm (I.D.) glass tubes with a central support cylinder (appx. 2.6 cm in diameter) of 90 cm long hydrophobic porous polyethylene ("Porex") with average pore dimensions of 100 microns. Approximately 10 cm of the inlet and exit sections were filled with small glass beads in order to promote an even flow distribution in the reactor. The reactor was inoculated starting late February, 1985 with Chaetoceros using GPM media for growth. The reactors were inclined slightly upward and small gas bubbles passed through the liquid effluent port. Illumination consisted of two fluorescent light bulbs placed about 10 cm from the surface of the reactors which resulted in an average light intensity of 50 $\mu E/m^2/sec$. The light/dark cycle was 14/10 hrs and dilution was carried out only during the day. Little or no algal growth took place in the first 2-3 weeks of the experiment. As time progressed, coverage began near the influent and gradually extended to the effluent. Starting around mid-March the support

became brown and by April turned a dark brown color characteristic of Chaetoceros. Almost the entire top surface became overgrown with algae, while little growth occurred on the underside of the support. The upper one-third of the support did not overgrow. In early April circular clearing zones appeared resembling phage plaques on bacterial lawns. Over a period of about 2 weeks continued growth resulted in an almost complete elimination of Chaetoceros from the reactor. During this period, however, a green alga started to grow upward from the lower inffluent end to the effluent end. By early May, the entire immobilization carrier was covered with this organism, and only about 5% of the algal effluent culture was still Chaetoceros. Microscopic examination of the effluent showed a predominance of very small green cells resembling Nannochloropsis (no further identification was carried out).

The reactor was operated at a flow rate of about 550 ml/day. A few data points of the productivity of the reactor system were carried out in April and May, 1985. During the second week in May, after the green algae dominated, productivities increased to about 40 mg/day (from 20.3 mg/day on 5/8, to 32.1 mg/day on 5/13 and 41.2 mg/day on 5/16). At a total surface area of approximately 500 cm^2 (per reactor), this amounts to a productivity of about 0.5 g/m^2/day. Even at the low light intensity used this is a low productivity. For this reason, as well as the inherent drawbacks of an immobilized reactor, no further work was carried out with these reactors.

1.5 PROTOCOL DEVELOPMENT

The ultimate objective of this project is to demonstrate a high productivity of lipids by microalgae cultures under conditions that allow extrapolation to outdoor systems. A review of the literature suggests nitrogen (or silica, for diatoms) limitation as the best approach to obtain a biomass with a high lipid content. However, this does not equate with a high productivity. The key requirements that must be met is that the biosynthesis of lipids in response to nutrient limitation be both rapid and not be accompanied by a severe reduction in overall biomass productivity.

In batch culture experiments under light limitation, cultures can exhibit constant productivity (when measured as biomass) which means an essentially constant increase in daily biomass, over a period from a couple of days to over a week. This is the "linear" growth phase of these cultures. When maintenance energy becomes a significant factor of per cell light energy input, or if some other factor becomes limiting, productivity slows down and the culture enters the "stationary" growth phase. Growth rates of the cultures (change in biomass per unit biomass per unit time) continuously decrease during this linear growth phase. One of the objectives of lipid induction is to maintain a high productivity for as long as possible after the culture enters nitrogen (or Si) limitation. The second objective is to induce lipid biosynthesis as quickly as possible.

To achieve these objectives both appropriate induction conditions and algal strains must be selected. The variables of interest during induction are the incident light intensity, the per cell light input, growth rate at the point of nutrient limitation, and culture history generally , and the subsequent (after onset of limitation) light and nutrient supply. Obviously these are too many variables to allow efficient screening of (even a small number of microalgae strains. For such a screening effort only a limited experimental protocol can be used. It is likely (see Introduction to the next section) that the lower the cell density (e.g. the higher the cellular growth rate) the better the

potential for high lipid productivity. Thus, induction experiments should be carried out such that the onset of nutrient limitation should be early in the linear growth phase. This was used as the primary experimental design in the development of protocols for the strain screening studies. Therefore the basic protocol used was to inoculate algal cultures (in the linear growth mode) into Roux bottles at a relatively low density (20 to 50 mg/L), and to limit the N (as NO_3)2 content of these cultures to between 1 and 2 mM. Biomass and lipid productivities were then followed for a period of about one week. The details of the experimental protocols are presented in the next section and the experimental methods in Appendix I.

SECTION 2.0

TIME COURSE OF LIPID INDUCTION

2.1 OBJECTIVES

TASK II - Determine the time course relationship between nitrogen deficiency, carbohydrate accumulation, and subsequent lipid synthesis in four representative strains of microalgae

"To provide an understanding of the mechanism that leads to the triggering of lipid synthesis in microalgae".

"Four strains of microalgae (Ankistrodesmus, Chaetoceros gracilis, Isochrysis galbana, and Nanno Q) shall be cultured under defined nitrogen deficient conditions that lead to a reproducible accumulation in lipids. Three radioisotope tracers (^{14}C, ^{35}S, ^{32}P) shall be applied to the cultures, and over a period of time, photosynthetic efficiency (growth), lipid content, and carbohydrate content shall be monitored. If time permits, the diatom Chaetoceros gracilis shall be cultured under silicon deficient conditions and the same procedure followed as with the nitrogen deficient cultures. A mechanism shall be proposed for the lipid trigger."

2.2 INTRODUCTION

A large body of literature exists regarding the fact that nitrogen (and, for diatoms, Si) limitation induces ("triggers") lipid biosynthesis, in many, but not all, microalgae species. Table 2.1 summarizes some of these studies. The major problem is that the kinetics of lipid induction have been studied rarely. In those cases where lipid productivity (averaged over the nitrogen limitation phase) was measured it was rather low. The data in the literature, however, is often ambiguous inrespect to many details, including the exact time course of the induction experiment. More importantly, as mentioned above, most of these experiments used relatively high cell densities and low light intensities - a combination which is likely to result in slow and incomplete conversion to a nitrogen limited status. A similar situation was observed in the case of induction of hydrogen evolution by nitrogen-fixing blue-green algae, or carbohydrates by several algal species, in response to N limitations: the higher the cell density at which nitrogen limitation occurred, the slower the induction process and the lower both productivity and content of the product (1,2). Thus, in the experiments detailed in this section, cell densities were kept relatively low, by means of limiting media N at between 1 and 2 mM.

Appendix I of this report presents the detailed methods and materials (including algae cultures) used throughout this project. Thus, only a brief outline of the protocols used is presented here. In the early experiments four batch culture reactors were set up and inoculated (to about 50 mg/L) with a linear or early stationary phase culture. After a few hours (or overnight in the dark), lights were turned on (fluorescent "grow lights" providing 300 $uE/cm^2/sec$) and the

TABLE 2.1 SELECTED LITERATURE ON LIPID CONTENTS OF NITROGEN LIMITED MICROALGAE

Species	Lipids as % of Dry Weight (days)* Nsufficient	Ndeficient	Ref.
Chlorella pyrenoidosa	20 (80)	35 (17	a
Chlorella pyrenoidosa	18 (unk)	65 (unk)	a
Chlorella pyrenoidosa	25 (unk)	40 (unk)	b
Chlorella pyrenoidosa	20 (unk)	70 (unk)	c
Chlorella pyrenoidosa	25 (unk)	35 (4)	d
Chlorella sp. Strain A	20 (log)	45-53 (17-26)	e
Chlorella Strain 10-11	19 (log)	18-26 (5)	e
Bracteacoccus minor	25 (unk)	33 (unk)	f
Chlorella vulgaris	27-33 (unk)	54 (unk)	f
Nitzchia palea	22.2 (log)	39.5 (7-9)	g
Chlorella pyrenoidosa	14.4 (log)	35.8 (7-9)	h
Oocystis polymorpha	12.6 (log)	34.7 (11)	h
Monollathus salina	40.8 (log)	72.2 (11)	h
Nannochloris sp.	20.2 (log)	47.8 (11)	h
Scenedesmus obliquus	25.7 (log)	47.1 (22)	i
Chlorella vulgaris	24.4 (log)	64.5 (28)	i

*Data in parenthesis refers to length of batch cultivation in days, in some cases logarithmically (log) growing cultures were used for N sufficient cultures, in others the kinetic data is missing (unk).

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cultures were subsequently grown on a 14:10 hr light:dark cycle. Observations of pH, optical density, gas flow rates, and culture appearance (color, clumping, etc.) were made daily, dry weights were measured somewhat less frequently. All productivities reported in this report are ash-free dry weights.

A whole culture or portion thereof was harvested about every other day starting before any visible signs of nitrogen limitation were apparent. Lipid, protein and carbohydrate contents were determined for these cells as well as an occasional CHN analysis. Appendix I contains detailed discussions of these various methods, their reliability and interpretations of the data. The experiments were designed to run for about one week. In the "initial protocol" (up to culture No. 114), a whole Roux bottle culture was "sacrificed" for each lipid analysis - with the cells harvested by centrifugation and then lyophilized. No N sufficient control was used (the first lipid sample was supposed to provide it). In the latter experiments, ("modified protocol") only 100 ml culture samples were harvested and used for lipid (and other) analysis and the cells were not lyophilized. A N sufficient culture was also used in these experiments. In total, fourteen induction experiments were carried out on light different strains of microalgae. A summary of these experiments is presented in Table 2.2. Below we separately report the results for each organism followed by a summary and discussion of the overall results. The radioactive tracer experiments are reported separately in Section IV; they were only carried out for Nanno Q using cells grown in shaker flasks rather than Roux Bottles. This section incorporates the results for both Tasks II and V since the data on lipid composition was collected on the same samples as the induction experiments.

2.3 CHLORELLA (BLACK LAKE)

This green alga, designated Chlorella Black Lake was isolated by W. Thomas at Scripps Oceanographic Institute and obtained (like all other strains used in this project) from the SERI culture collection. In preliminary experiment with air+1.73 mM $NaNO_3$ (Fig. 2.1a, Table 2.3a) the N deficient cells contained on day 4 about 23% carbohydrates decreasing by day 8 to 18% and 40%, respectively. The N sufficient control had 57% protein and 37% carbohydrates. Lipids were 20% in the N deficient and 11% in the N sufficient culture. These data were, however, preliminary.

The next experiment presented in Fig. 2.1b and Table 2.3, using the "initial protocol", consisted of four Roux bottles with 1.6mM NO_3 in the medium. This experimental design used did not incorporate a N sufficient control - the first data point, at day 2, was supposed to provide it. However, in this case, as in subsequent experiments with other strains, the initial datum point was already clearly nitrogen limited - as seen by the high carbohydrate and low protein content. (Table 2.3a). The initiation of nitrogen deficiency was visually judged by a change in coloration - from dark green to a paler color between day 3 and 4. However, this severely underestimated the onset of nitrogen limitation. The key result of this experiment was that the initial lipid content was low (about 10%) and it did not increase for almost one week, and then to only 14%. (The 20% lipid level in the preliminary experiment was probably due to a methodological error).

Table 2.2

SUMMARY OF CONDITIONS FOR LIPID INDUCTION EXPERIMENTS

Organism	Culture	$\%CO_2$	pH	Temp (^oC)	Light (I) (uE/m^2s)	NO_3 (mM)	Media
Cyclotella	99-102	1	7.7±.2 (8.6)*	25-29	300	1.6	MSW/2
Ankistrodesmus	111-114	1	8.2±.1	25-29	300	1.6	Ank
Ankistrodesmus	117	NA	10.1±.2	25	150	1.6	Ank
Ankistrodesmus	118	NA	10.2±.2	25	150	20	Ank
Ankistrodesmus	121	NA	10.0±.2	25	150	0.6	Ank
Ankistrodesmus	119,120	1	8.2±.1	25-29	300	1.6	Ank
Chaetoceros	122	1	7.9±.3	25-29	300	20	MSW/2
Chaetoceros	123-125	1	7.5±.2	25-29	300	1.6	MSW/2
Boekilovia sp.	127	1	7.8±.3	25-29	300	20	MSW/2
Boekilovia sp.	128-130	1	7.4±.2	25-29	300	1.6	MSW/2
Nanno Q	141	1	7.8±.2	25-29	300	20	MSW/2
Nanno Q	142-144	1	7.6±.1	25-29	300	1.6	MSW/2
Thalassiosira	145	1	7.9±.2 (9.3)*	25-27	250	20	MSW/2
Thalassiosira	146-148	1	7.6±.1 (8.5)*	25-27	250	1.6	MSW/2
Nanno Q	67-70	.03	9.2±.5 (10.2)*	25-29	200(2)	2.7	MGM
Nanno Q	80-83	1	7.7±.3	25-29	200(2)	2.6	MGM
Chlorella	76-79	1	7.6±1	25-29	300	1.6	MSW/2
Chlorella	62-64	0.03	9.5	25-29	300	1.73	MGM
T. Isochrysis	84-87	1	7.5±2	25-29	300	1.9	MSW
Boekolovia	72-75	1	7.8±1	25-29	300	1.6	Type II
Chaetoceros	88-91	1	7.8±1	25-29	300	1.6	MSW/2

*Max pH recorded during the experiment.

Fig. 2.1a Growth Curve of Chlorella (Black Lake) Grown on Air
Circles: N deficient (1.73 mMKNO_3) culture, (Culture 64)
Squares: N sufficient culture (Culture 62)

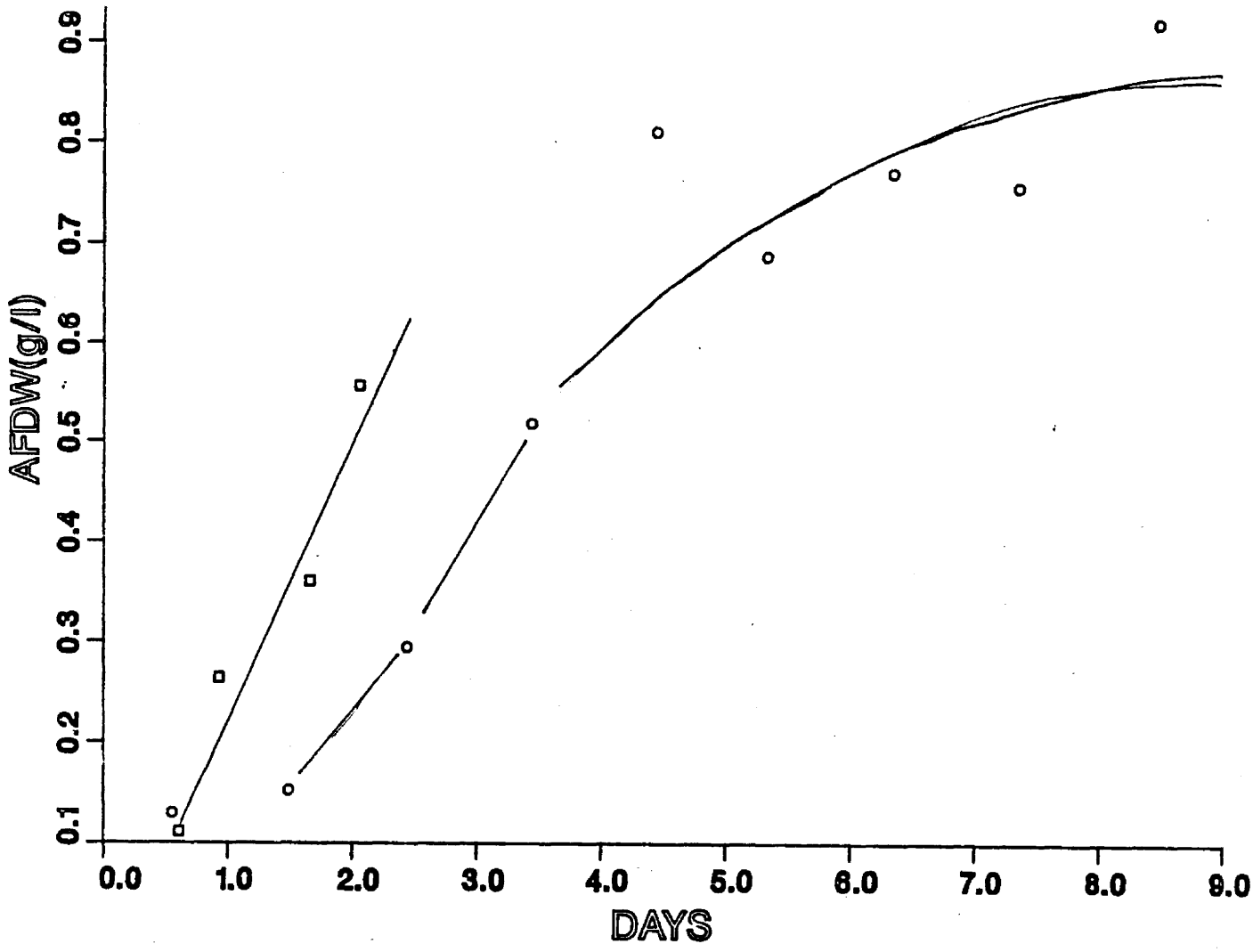


TABLE 2.3a Composition of Chlorella B.L. Grown on Air

Culture	Day	Status	AFDW mg/L	Composition %AFDW			
				Pro.	CH20	Lipid	Total
62	2	NS	550	57.9	32.0	11.0	101
64	8.5	ND	900	16.6	39.2	20.4	76.2

Table 2.3b Composition of Chlorella B.L. Grown on 1% CO_2

Culture No.	Day After Start Expt.	Day After Start N Def*	AFDW at Harvest mg/l	Composition % AFDW Prot	CHO	Lipids	TOTAL
76	2.14		.369	16.5	59.3	9.5	85.3
77	4.15	0	.548	12.9	73.3	11.2	97.4
78	6.10	2	.616	11.2	64.3	10.2	85.7
79	8.04	4	.751	11.3	61.8	14.2	87.3

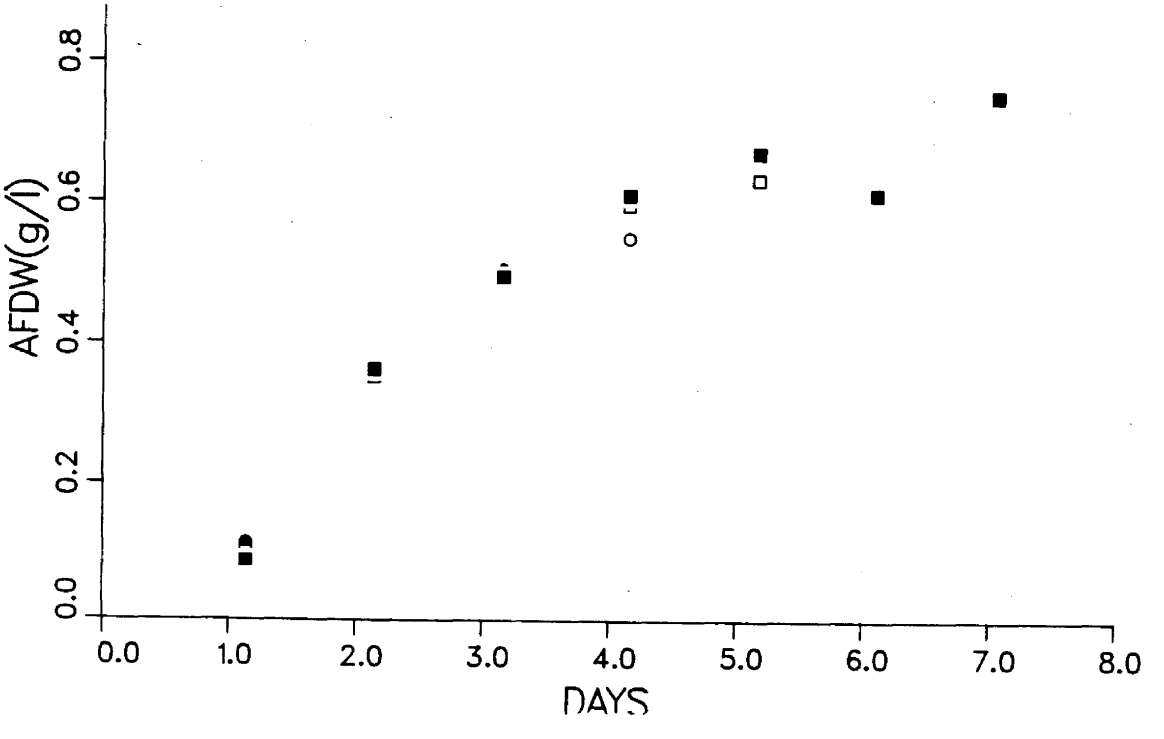
CHN Analysis of Culture 79, % of AFDW

C	54.2
H	8.3
N	1.85
O	35.6

H_c 6.0 Kcal/g (calculated)

*Based on visual observation (which was an underestimate-see text)

Figure 2.1b Growth Curves of Chlorella (Black Lake) Grown on 1% CO_2



Productivity was 260 mg/L/day in the initial part of the growth curve, (days 1 and 2). However, the N limitation seen by day 2 already have severely depressed productivity. The final yield was 750 mg/L, or 33.5 mg dry weight/mg $N\text{-}NO_3$ added. This should correspond to a N content of 3%, but CHN analysis of the final harvest gave an intracellular N value of only 1.85% - which essentially agrees with the protein measured. The fate of the unaccounted for N - probably extracellular - is unknown. Lipid, protein and carbohydrate contents add up to about 85% (except for culture 77 where the total value is 96% - possibly due to the high CH_2O value). Thus the extraction procedures do not appear to vary greatly in efficiency between these data points.

In conclusion, this organism was not deemed suitable for further work due to low lipid content.

2.4 TAHITIAN ISOCHRYSIS

This is a chrysophyte used by many researchers and relatively well characterized. It has a temperature optimum of about 28°C and a salinity range of 5 to 60% seawater (3). Only one experiment, using the initial protocol was carried out. Results are presented in Fig. 2.2 and Table 2.4. As in the previous experiment, N limitation was well advanced by day 3.5 with protein at 12.3%, carbohydrate at 46.6% and lipid at 30.0% of AFDW. Thereafter lipid contents did not change appreciably (the low value of culture 85 is likely an artifact). Maximum productivities were 290 mg/L/day dry weight and 90 mg/L/day lipid for days 2.5 to 3.5. Productivity declined severely after day 4. The culture exhibited a very low N content, 1.70%, which calculates to a recovery of 19.2 out of the 26.6 mg/L of $N\text{-}NO_3$ present. Again, the fate of the unaccounted for N is unknown.

The culture exhibited a change in pigmentation during the experiment, from orange to brown. The most marked change, however, was in the production of extracellular material, which accumulated on the cell pellets (during centrifugation) in the form of a reddish-orange gel.

The fact that lipid content did not exceed 30% and that productivity was relatively low suggested that this organism also was not a suitable candidate for further lipid induction experiments.

2.5 CYCLOTELLA

This diatom was isolated by Dr. Tadros, and obtained through J. C. Weissman. Again, only a single experiment was carried out with this organism. The data are summarized in Fig. 2.3 and Table 2.5. Growth curves showed an increase in dry weight until the fourth day into the experiment. Maximum productivity was 260 mg/L/day. This productivity was maintained even as lipids increased to 40%, averaging 200 mg L/day between day 2.5 to 4.5. Thereafter, productivity dropped to zero.

As in previous experiments, the color of the culture was taken as a measure of its nitrogen status - going from dark chocolate brown (healthy cultures) to a muddy brown and finally orange brown. However, here again the pigment changes did not coincide with the point at which the culture entered nitrogen limitation.

Table 2.4 Composition of Tahitian Isochrysis during N Limitation

Culture No.	Day After Start Expt.	Day After Start N Def	AFDW at Harvest mg/l	Composition % AFDW Prot	CHO	Lipids	TOTAL
84	3.49	0	0.640	12.3	46.6	30.0	88.9
85	5.47	2	0.901	8.6	49.4	23.1	81.1
86	7.46	4	0.904	8.7	47.67	31.4	87.7
87	9.57	6	1.13	7.5	41.5	29.5	78.5

*estimated visually by changes in pigment level.

CHN Analysis of Culture 87 (% AFDW):

C	44.1	N	1.70		
H	8.1	O	46.0	H_c	4.7

Figure 2.2 Growth Curve of Tahitian Isochrysis (Cultures 84-87)

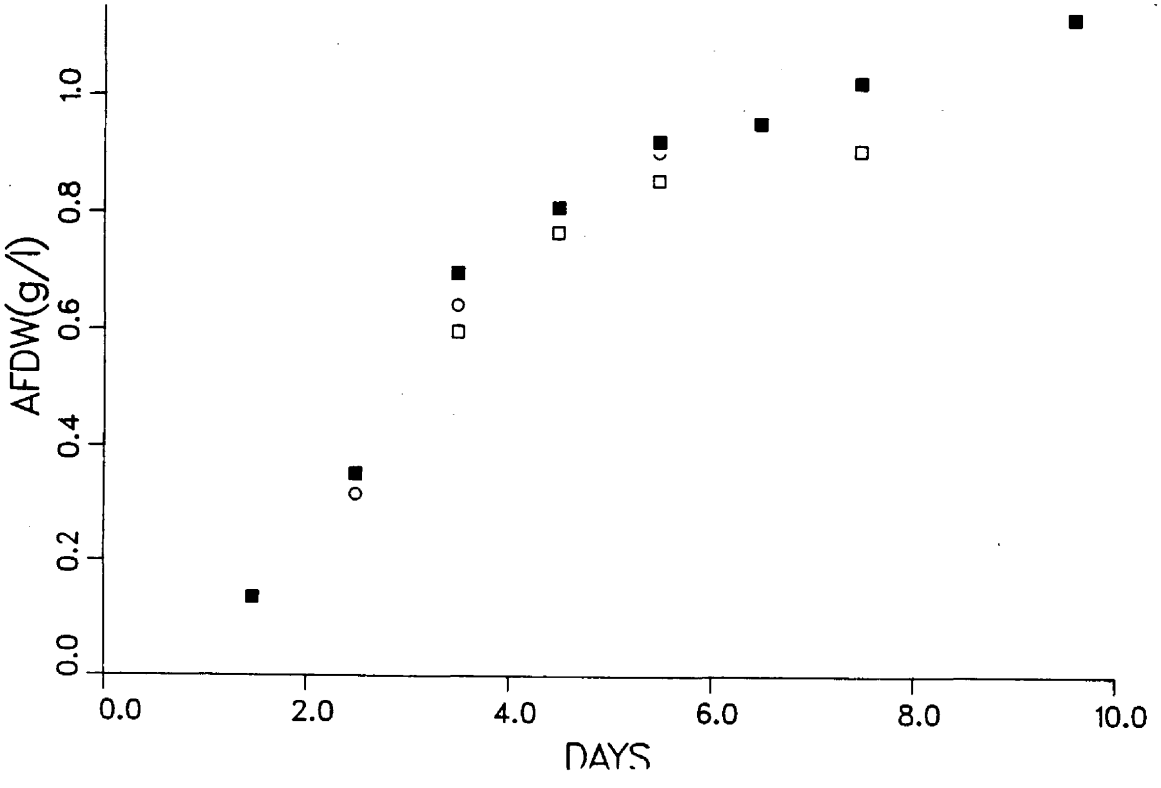


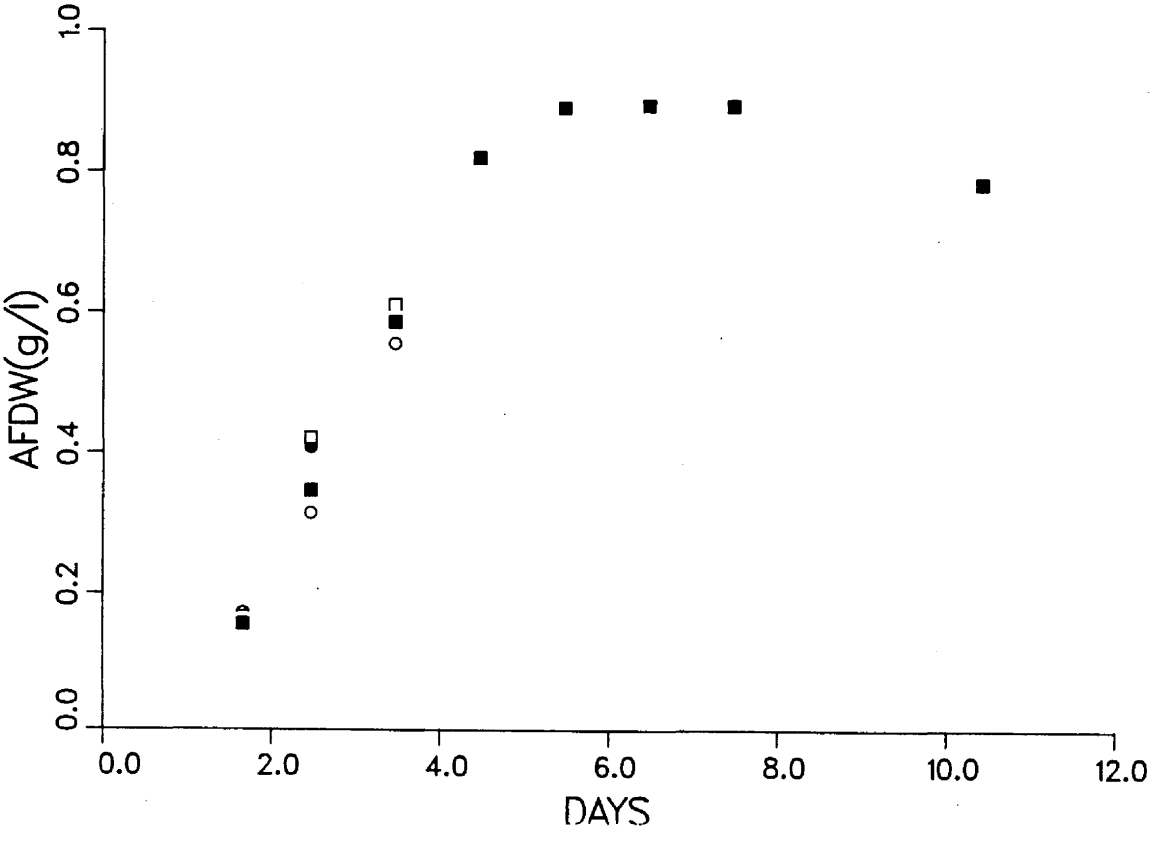
Table 2.5 Composition of Cyclotella during N Limitation

Culture No.	Day After Start Expt.	Day After Start N Def	AFDW at Harvest mg/l	Composition % AFDW Prot	CHO	Lipids	Total
99	2.46		.408	23.7	49.9	30.7	104.32
100	4.46		.818	13.7	54.4	40.7	108.8
101	7.46	3.5	.887	13.3	47.8	41.8	102.9
102	10.4	6.5	.782	13.7	36.8	45.5	96.0

CHN Analysis (% AFDW)

Culture #	%C	%H	%N	%0	H_c (Kcal/g)
99	49.8	7.9	3.63	38.7	5.4
102	57.5	8.9	2.11	31.4	6.6

Figure 2.3 Growth Curves of N Limited Cyclotella (Cultures 99-102)



Even at 2.5 days the culture was probably already entering N limitation as seen from the relatively high lipid and carbohydrate contents, as well as the low protein (23.7%) content. Indeed the CHN data support this view: N at only 3.63% in culture #99 clearly indicated N limitation. Again, the CHN data indicated low N recovery, only 16 out of 22.4 mg $N\text{-}NO_3$. Also noteworthy is that in this case all but the last culture, the protein, CH_2O, and lipid analysis added up to somewhat over 100%.

Lipid fractionation (Table 2.6) showed that the benzene fraction increases during the experiment, at the expense of the more polar acetone - methanol fractions. Lack of a true N sufficient control limits the information.

In conclusion, this organism appears promising for further studies since productivity is high even when N limited, because lipids are preferentially induced in the benzene (nonpolar) fraction and because this organism can be cultivated outdoors. (J. C. Weissman, personal communications).

Table 2.6 Fractionation of Cyclotella Lipids

Solvent Phase	% of Total Lipid Culture #		% of Dry Weight Culture #	
	99	102	99	102
Hexane	1.3	0.8	0.4	0.4
Benzene	63.2	88.9	19.4	40.4
Chloroform	7.9	2.5	2.4	1.1
Acetone	17.5	4.1	5.4	1.9
Methanol	10.0	3.7	3.1	1.7
TOTAL	99.9	100.0	30.7	45.5

2.6 ANKISTRODESMUS FALCATUS

2.6.1 Introduction

This strain of Ankistrodesmus was first isolated in Pyramid Lake in 1982 by William Thomas. It has been studied in some detail by other SERI researchers since then. Optimum salinity of 7% and temperature of 26°C, have been reported (3). Our experience has shown it to be easily cultivatable with N sufficient cultures yielding over 5 g/L of dry weight and being characterized by a deep dark green color. Nitrogen limited cultures, on the other hand, are almost completely yellow by the late stages of deficiency. In last year's Annual Report from this project, batch cultures grown on a shaker table under nitrogen deficient conditions resulted in lipid contents as high as 40% (4). However, in a preliminary experiment, only 10-16% lipid contents were found, with no correlation with nitrogen limitation. (Data not shown due to the preliminary nature of these analysis). This discrepancy led to follow-up experiments to test for the parameters that might account for this difference.

The first experiment focused on light and carbon supply as the key factors that might account for the widely differing lipid contents in the two protocols. Conditions of low, high, and zero CO_2, and high and low light were imposed on N sufficient cultures in Roux bottles. The results of this experiment are shown in Fig. 2.4 and Table 2.7. Overall there was very little change in protein levels, but both carbohydrate and lipid contents varied with environmental conditions. Cells grown on air at both high and low light intensities had elevated carbohydrate levels (over 40%); while the cultures grown either with 1% CO_2 or in absence of CO_2 (NaOH scrubbed air, leading to a pH of 10 in the cultures) had a much lower carbohydrate level. (It is possible that the carbohydrate level of the 1% CO_2/high light culture was a measurement error as seen from the low totals compared to the other cultures). The lipid content of the CO_2 free culture approached the 40% level reported in last year's report. There was an almost 50% decrease in biomass in this CO_2 free culture (as compared to the 1% CO_2 case) over the one week period after nitrogen limitation set in. This could, by itself, account for the differential in lipid content between these cultures, if it is assumed that lipids are not significantly metabolized (respired) in this time frame, or that no net oxidation took place. Preferential respiration of carbohydrates would then account for their relatively low content as well as the higher protein content and ash level (11.5 vs 5.5% for other cultures) in this culture.

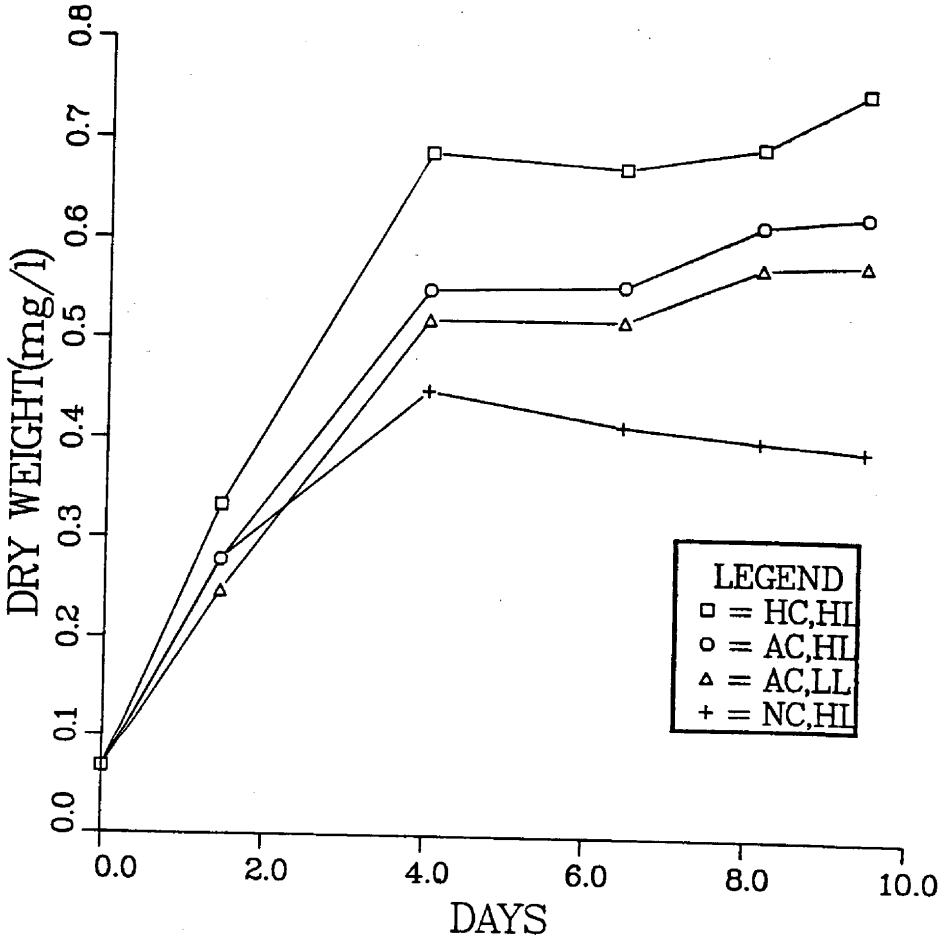
Although this hypothesis remains to be tested, the results point out the potential danger of extrapolating high lipid contents to high lipid yields. The shaker flasks experiment, on which previous results were based, probably were severely limited, as neither supplemental CO_2 was used nor air introduced into the shaker cultures - except that which could diffuse through the cotton plugs. Thus this could account for the differences observed (although it should be noted that the close to 30% content in Table 2.7 is much closer to the 40% reported last year then the 10-16% lipid content from the preliminary experiments. The much lower growth rate observed in the shaker flasks vs Roux bottles may also have contributed to the difference in lipid contents.

Table 2.7 Composition of Nitrogen and Carbon Limited Ankistrodesmus

Treatment[1]	Dry Weight	Composition (% AFDW) % Prot.	% CHO	% Lipid	Total
HC, HL	750	10.8	26.0	26.0	62.8
AC, HL	630	10.1	46.1	26.3	82.5
AC, LL	580	11.4	41.4	29.8	82.6
NC, HL	390	14.8	28.9	37.7	81.4

1. HC = High CO_2 (1% in Air), AC = air CO_2 (0.03%)
   NC = Negligible CO_2 (NaOH scrubbed)
   HL = High light (800 2 E/M^2S), LL = Low light (300μ E/m^2S)

Figure 2.4 Growth Curves of Nitrogen and Carbon Limited Ankistrodesmus



2.6.2 Shaker Flask Experiments

To further compare the previous methodologies (e.g. Shaker Flask cultures) with the growth conditions used this year, two N sufficient (20 mM NO_3, culture #118) and two N limited cultures (0.4, and 1.6 mM NO_3 cultures 121 and 117, respectively) were grown in 2L Fernback flasks for a period of approximately 3 weeks at a light intensity of 50 $\mu E/m^2/sec$ and continuous light. The shaker speed was 50 RPM and cultures were grown on standard Ankistrodesmus media (see Appendix I) with no CO_2 spraying. These cultures were characterized by a large amount of settled biomass. (This was periodically resuspended by manual shaking), but it resulted in rather variable dry weight data (not shown). The cultures began with about 100-200 mg/L of cells and finished with 250-400 mg/L. After approximately 1-2 days following inoculation, the pH had risen to above 10 and the cultures by day 3 had yellowed somewhat. The pH remained elevated for the remainder of the experiment and the cultures continued to pale somewhat.

Lipid, carbohydrate, proteins and CHN analysis were performed on each culture on the cells that were harvested after three weeks. The results are presented in Tables 2.8 and 2.9. Comparison of cell densities alone indicated that some degree of N limitation occurred; however, it cannot be the only factor (limiting growth) due to the low cell densities for the 1.6 and 20 mN NO_3 cases (cultures #117 and #118 respectively). In fact, CHN analysis showed that the only culture experiencing significant N limitation was #121 (0.6 mM NO_3), containing 3.0% N. While culture #117 did have a reduced level of nitrogen (6.6%), this was probably not low enough to reduce growth rate and alter its metabolism. Composition data are in agreement with the above findings. Thus, culture #121 differed from the other two and had reduced protein levels, increased carbohydrates, and an elevated lipid content.

It may be significant that total composition values add to only 80 % for cultures #117 and #118 but to 92% for #121. Whether this is in fact an experimental error, or the result of compositional changes affecting the extraction efficiencies and/or assay results is uncertain. For all other analyzed samples from this organism (see below) the compositional data (carbohydrates, lipids and protein) add up to at least 90%. From the CHN analysis (Table 2.9), and using a factor of 6.25 N for a N to protein conversion, protein values for the two N limited cases agree well. Thus, the relatively low totals in Table 2.8 are most likely due either to the lipids or carbohydrates assays. The results for culture #121 appear credible and it is clear that some induction of lipids did occur, but not at the previously reported (4) level of 40%.

2.6.4 Comparisons Between Clones

An alternative explanation for the discrepancy between last years results and the present data was that the Ankistrodesmus falcatus strain used had mutated, and a nonlipid producing clone selected during transfers. Changes in strain phenotypes are not uncommon during laboratory cultivation and maintenance - particularly for relatively recent isolates. To test this hypothesis, an experiment was performed comparing N limited dry weight curves and end point composition data between our laboratory strain and a new inoculum of the same strain sent by SERI. The dry weight results are plotted in Fig. 2.5. The label "new" (culture #119) applies to the strain sent by SERI and "old" (culture #120) represents our laboratory strain. The new strain had a final biomass

Table 2.8 Composition of Ankistrodesmus

Culture No.	Day After Start Exp.	Day After Start N Def.	AFDW at Harvest mg/l	Composition % AFDW Pro	CHO	Lipid	Total
111	1.71	0	464	23.9	54.6	24.9	103.4
112	3.71	2	1040	14.1	60.4	26.4	100.9
113	5.69	4	1270	10.5	52.8	24.0	87.3
114	7.69	6	1430	14.1	56.4	35.3	105.8
119	7.7	6	1380	14.2	51.8	26.3	92.3
120	7.7	5	1040	16.1	55.5	20.6	92.2
117	22	-	300	35.2	26.1	19.4	80.7
118	22	-	400	37.0	25.8	15.9	78.7
121	22	-	250	19.5	40.9	31.2	91.6

*Shaker Table Cultures (all others are Roux bottles)

Table 2.9 CHN Analysis of Ankistrodesmus

Culture	Status*	%C	%H	%N	%O[1]	ΔH_c
111	Early ND	53.5	7.84	4.31	34.35	5.9
114	Late ND	56.7	8.89	1.70	32.70	6.5
117	Shaker ND	50.0	7.66	6.55	35.79	5.5
118	Shaker NS	45.5	8.58	9.68	36.34	5.3
121	Shaker ND	53.3	9.61	3.01	34.44	6.3

NS: N Sufficient, ND: N Deficient

Figure 2.5 Growth Curve Comparisons of Two Clones of Ankistrodesmus

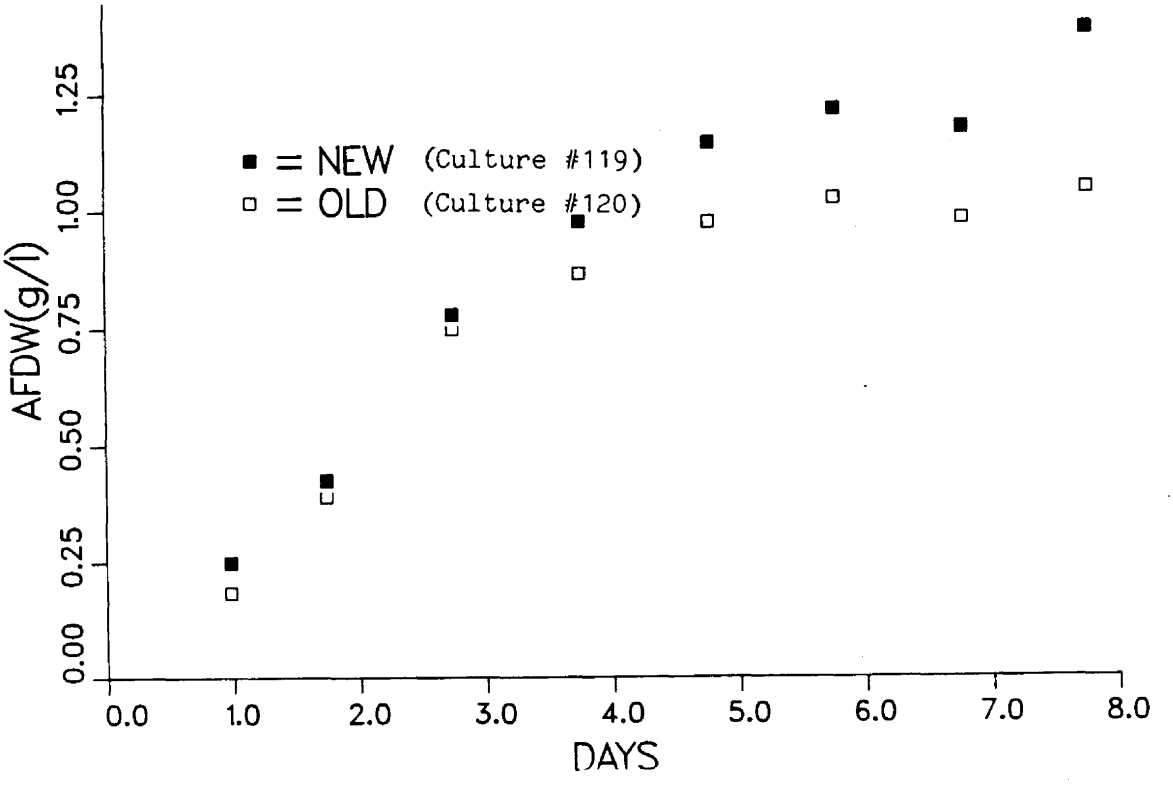
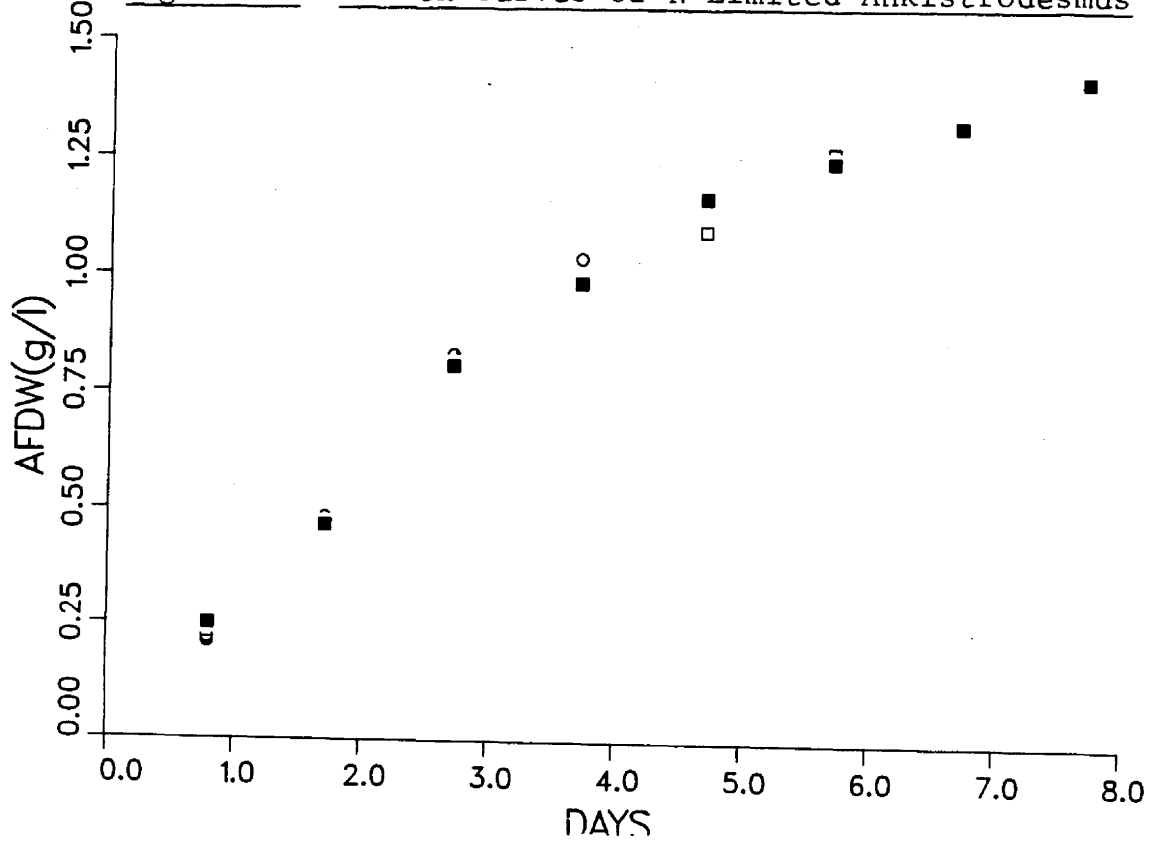


Figure 2.6 Growth Curves of N Limited Ankistrodesmus



yield of about 250 mg/L greater than the old strain. Composition analysis (Table 2.8) showed very little difference in any of the components, with perhaps a slight increase in lipids being observed for culture #119. The reason for the difference in biomass yield between these cultures is unclear; it is possible that the N requirements could have been different; however, no nitrogen analysis was conducted to determine this. It was concluded that although some difference between the two cultures cannot be excluded, it had no effect on lipid content.

2.6.5 Lipid Induction Experiments

The final experiment conducted was a time course study of N limitation using the initial protocol. The dry weight curves for cultures #111-114 are shown in Fig. 2.6 and composition values are given in Table 2.8. CHN analysis was only performed on lyophylized samples of cultures #111 and 114, representing the early and late deficient cells. (Table 2.9). Nitrogen content of culture #111 was 4.4% reflecting the fact that this culture (at an early stage of its growth curve) was already experiencing N limitation. It's low protein and high carbohydrate contents agree with this fact. In culture 114, only a small further reduction in protein and similiarly only slight changes in carbohydrates are observed as nitrogen limitation progressed. Lipid contents increased somewhat, with the last point being 40% higher then the first value. Total composition values add to slightly above 100% except for culture #113 which is below 90%. Nitrogen content of culture 114 is low (1.7%), indicating that this organism has a very low maintenance requirement for N. This value would calculate a protein content of 10.5% indicating that the 14.1% protein content assayed for this is perhaps too high. This could explain the above 100% total composition determined and lend credibility to the high lipid content. A major detraction from the last data point, however, is the result from culture #119 - a duplication of culture 114. While proteins and carbohydrates agree fairly well, there is a considerable difference in lipid values. Repeated analysis on #114 and 119 gave results of 36.9 and 24.3%, respectively. The reason for these discrepancies is unknown.

2.6.5 Conclusions

In retrospect, this isolate of Ankistrodesmus does not appear to trigger lipid synthesis to any significant degree. Severely deficient cells attain values up to 35 - 40%. This, however, is of little benefit considering the length of time required or the environmental stresses required to produce such cultures. For a reasonably productive culture one could only expect at most about 25% lipid content. In fact the one advantageous feature of this organism is its relatively high and sustained productivity during N limitation which results in relatively high lipid productivities. This point is again addressed in the Conclusions Section (2.11).

2.7 CHAETOCEROS GRACILIS

2.7.1 Introduction

Chaetoceros is a unicellular diatom with an optimum growth temperature of 28-30°C and a salinity range of 15-35% (3). The first preliminary growth experiment with this organism is reported in Fig. 2.7 and Table 2.10. The experiment started out with CO_2 supplemented air, but failure of the CO_2 supply resulted in the cultures being grown on air for essentially the whole time period with pH rising to 9-9.5 in the cultures. These growth experiments demonstrated that 1.6 m M NO_3 stops growth at slightly above 500 mg/L dry weight. However, the Si deficient culture (#65) continued to grow slowly throughout the experiment. This led to a change in procedure for Si limitation: the cultures were centrifuged, washed and resuspended in Si deficient media. Still Si limitation may not have been achieved (see below). We first report the results from two nitrogen limitation experiments, followed by data from a Si deficiency experiment.

2.7.2 Nitrogen Limitation Experiments

The results of the first N limitation experiment (initial protocol) are shown in Fig. 2.8 and Table 2.11. A major result was that the culture "crashed", reducing biomass by almost 50% on the 5th day of the experiment. The SERI "Microalgae Culture Collection" report (3) quotes Simons (5) to the effect that populations can crash rapidly ($<$ 12 hrs.), which can be prevented by EDTA. However, we did not observe such crashes in any other experiments (nor in the N and Si sufficient inoculum cultures), thus this behavior is most likely due to some other cause. The fact that it occurred at the same time in all four cultures suggests an extrinsic factor (perhaps air supply or a temperature transient). The daily observations data sheets suggest no likely explanation. Therefore the composition results (Table 2.11) for this experiment may be considered suspect. It should be noted, however, that the protein concentration increased by 40% between 5 and 6 days into the experiment, while density declined by an equal amount. As a result, total protein in terms of mg/L of culture remains essentially the same. For a typical protein N content of 6.25%, this amounts to about 11.2 mg/L of N, or only half of the 22.4 mg/L of nitrate-N added. Although some of the N is certainly bound in the form of nucleic acids, cell walls,etc., it is a low protein recovery for a N deficient culture. This again raises the possibility that not all the protein is recovered. Indeed, the total % composition (prot/CH_2O/lipid) in the four cultures varied from 68% to 100%, a quite significant difference. Since, as discussed earlier, all three methods are based on both different extraction and different analytical methodologies, their adding to less (or more) then 100% is not unexpected. However large differences in the behavior of these samples from one culture to the next raises the issue of the reliability of the data overall. Nevertheless, it is clear that there is no major trend in lipid increase during the continuation of N limitation and that the first data point already represented a N limited culture - in terms of proteins, carbohydrate and lipid content.

Table 2.10 Composition of Chaetoceros Grown on Air

Culture	Day	Status*	AFDW	% of AFDW			
				Prot.	CHO	Lipid	Total
			mg/L				
63	2	NS	450	55.0	15.6	7.58*	---
65	8.5	SiL	650	36.2	17.3	22.6	76.1
66	8.5	NL	580	26.5	24.5	31.0	82.0

*N S: Nitrogen Sufficient, SiL:Si Limited

Figure 2.7 Growth curves of Chaetoceros Grown on Air

Squares: N and Si sufficient; Triangles: N deficient; Circles: Si deficient (probably not limiting)

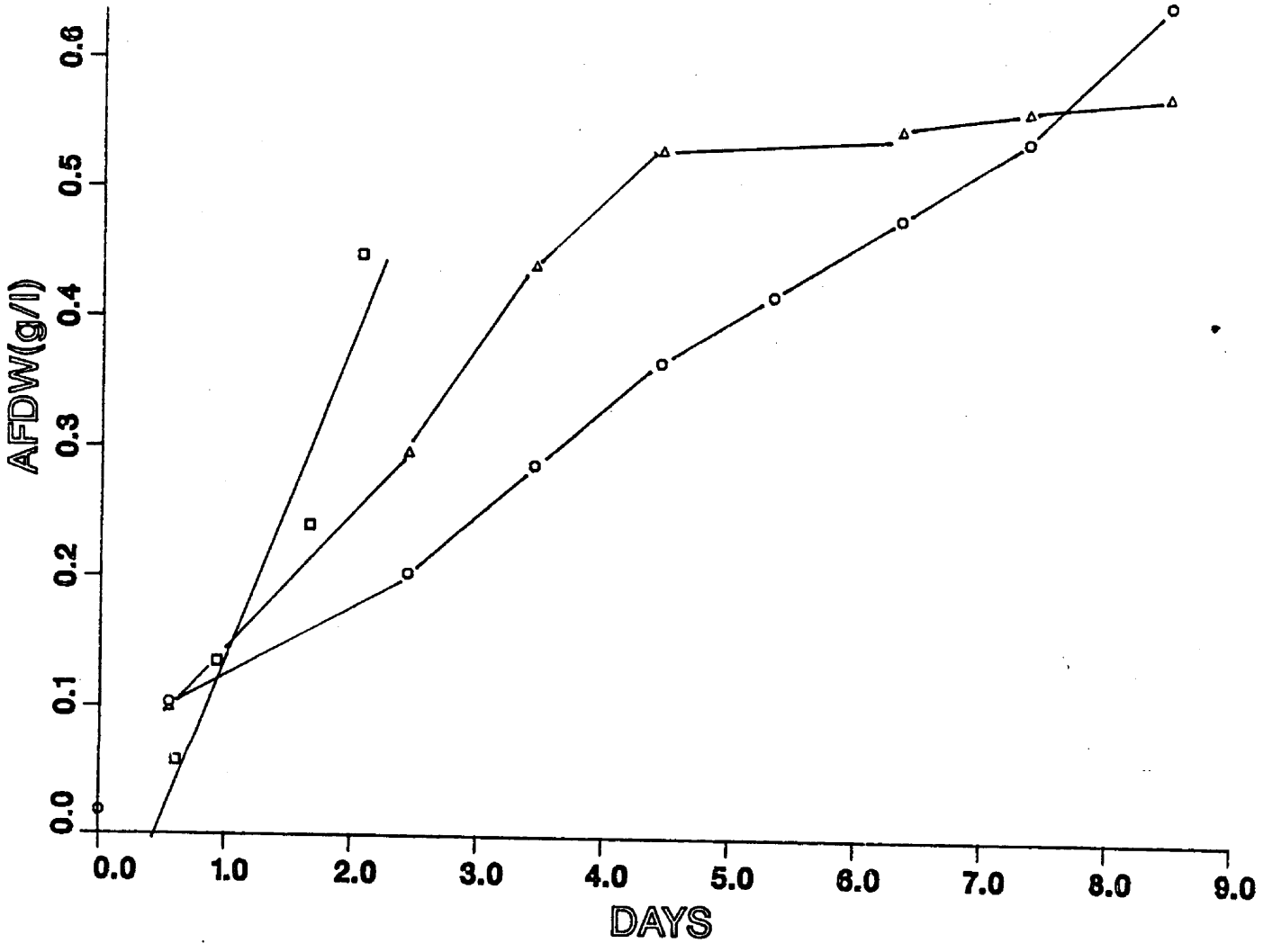
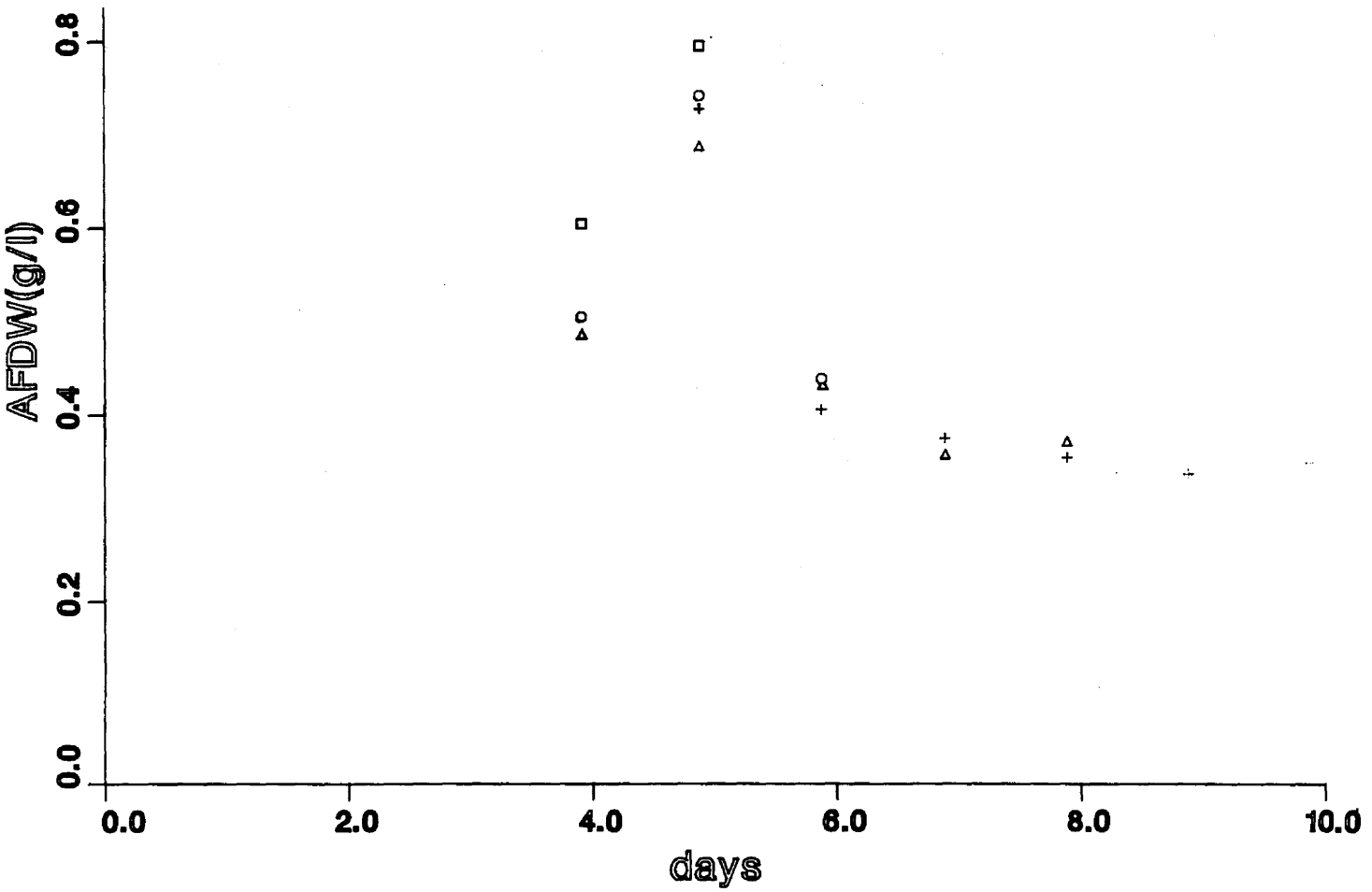


TABLE 2.11 Composition of Chaetoceros Grown on 1% CO_2

Culture No.	Day After Start Expt.	Day After Start N Def	AFDW at Harvest mg/l	Composition % AFDW Prot	CHO	Lipids	Totals
88	4.88	---	.795	8.9	44.01	27.9	80.8
89	5.88	---	.439	15.0	58.79	26.2	100.0
90	7.89	---	.371	18.5	32.75	27.2	78.4
91	9.88	---	.346	24.4	27.85	16.0	68.2

Figure 2.8 Growth Curves of N Limited Chaetoceros grown on 1% CO_2



The next experiment with Chaetoceros gracilis involved the modified protocol (multiple points per flask, no freeze drying of samples). Results are shown in Figs. 2.9a, b and in Table 2.12. The N sufficient control had a 45% (±2%) CH_2O content and a 14% (±2%) lipid content. The results of the N limitation experiment showed that even by day 3, before any growth diminution was apparent the Chaetoceros culture exhibited a significant increase in lipid (50%) and carbohydrate contents (about 200%). Thereafter, lipids slowly increased and carbohydrates declined after reaching a peak 6 days into the experiment. In this experiment the protein, carbohydrates, and lipids of the N limited culture all add up to almost exactly 100% (±3%) in all cases. (The N sufficient culture analysis date, however, added up to only 70 to 80%, indicating a significant difference in terms of assay responses and/or extractions. (The data point or on day 6.0 for the N sufficient culture is probably in error with regard to its lipid content.)

The results of the analysis are supported by CHN analysis (Table 2.13) showing an 8.8% N content in the sufficient culture and a 2.8% N (equivalent to about 17% protein, the actual content measured) in the deficient culture. Despite the higher lipid content, the deficient culture showed a lower heat of combustion. This is due in part to the higher carbohydrate:protein ratio, but the extent of the difference is nevertheless surprising.

The culture lipid content peaked for the N sufficient culture at 320 mg/L and the N deficient culture at 190 mg/L, both on day 9 of the experiment. Lipid productivity was, however, severely depressed in the nitrogen limited cultures, despite the fact that lipid content increased, slowly, from 20 to about 28%. Thus, the highest lipid productivities by this culture occurs within one day of onset of nitrogen limitation someplace between day 2 and 4 of the experiment, while culture productivity is still high and lipids increase by about 50%. Obviously an induction at a much higher cell density - where lipid (mg/ L) are higher - would be of interest in this regard. However, in that case, lipid induction rates may be even slower. (This, of course, is the rationale for the low densities at which these experiments were carried out). However, culture #122,the N sufficient control,showed a high lipid content at almost 800 mg/L of dry weight, while culture productivity was still high. Thus lipid induction at a high density may be a valid approach.

Lipid fractionation data (Table 2.14)indicated that most of the "induced" lipids are benzene soluble and are formed at the expense of the acetone fraction.

2.7.3 Si Deficiency Experiment

Table 2.15 shows the results of the Si deficiency experiment. At 3.90 days the culture was centrifuged, washed and resuspended in media lacking Si. (There was a drop in dry weight, due to removal of a sample for analysis and subsequent dilution of the culture). One doubling in cell density occurred in the culture in the day prior to Si limitation and more than one doubling after Si limitation. Thus, no immediate effect on growth could be observed in Si limitation. Also, the initial lipid content value was rather higher (at 21%) than in the above experiment and further increased to 30% within less than one day. Protein content exhibited a slight decrease and carbohydrate a modest

Figure 2.9a Growth Curves of N Sufficient and Deficient Chaetoceros

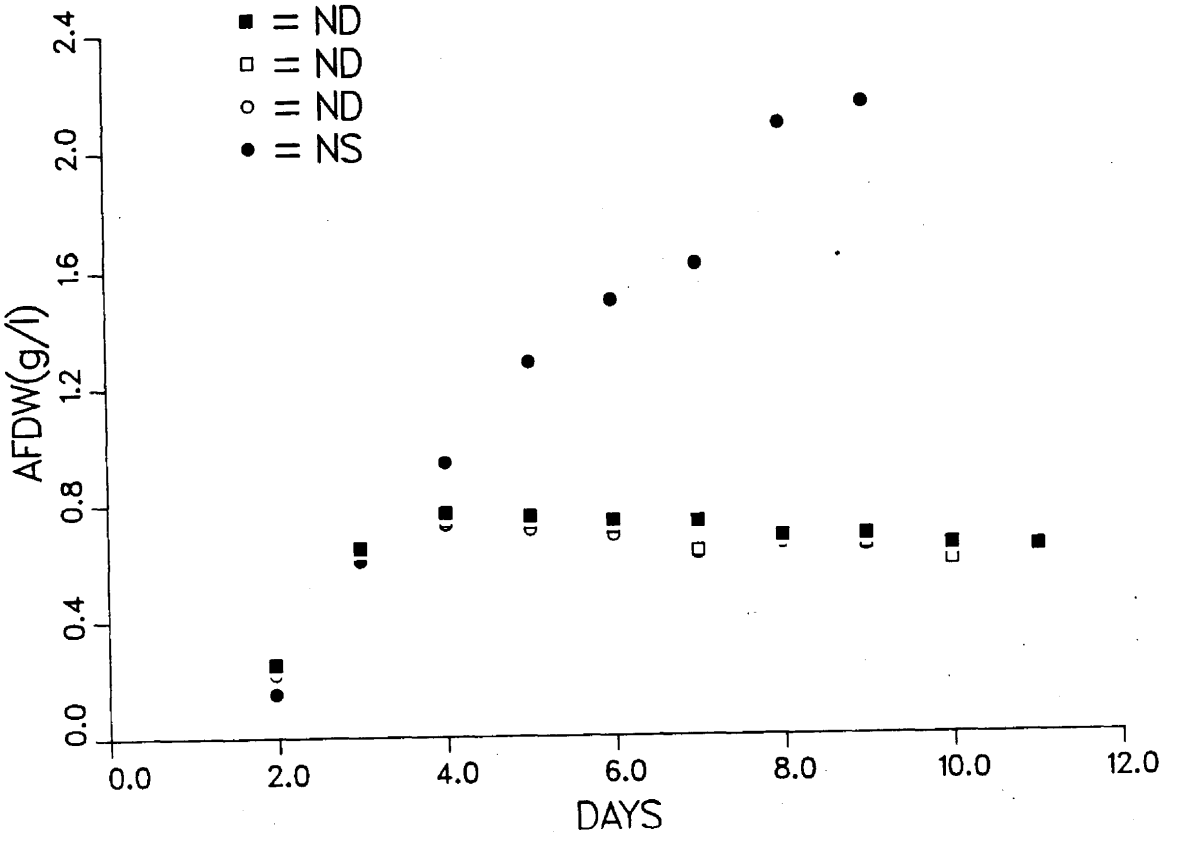


Figure 2.9b Composition of N Deficient Chaetoceros

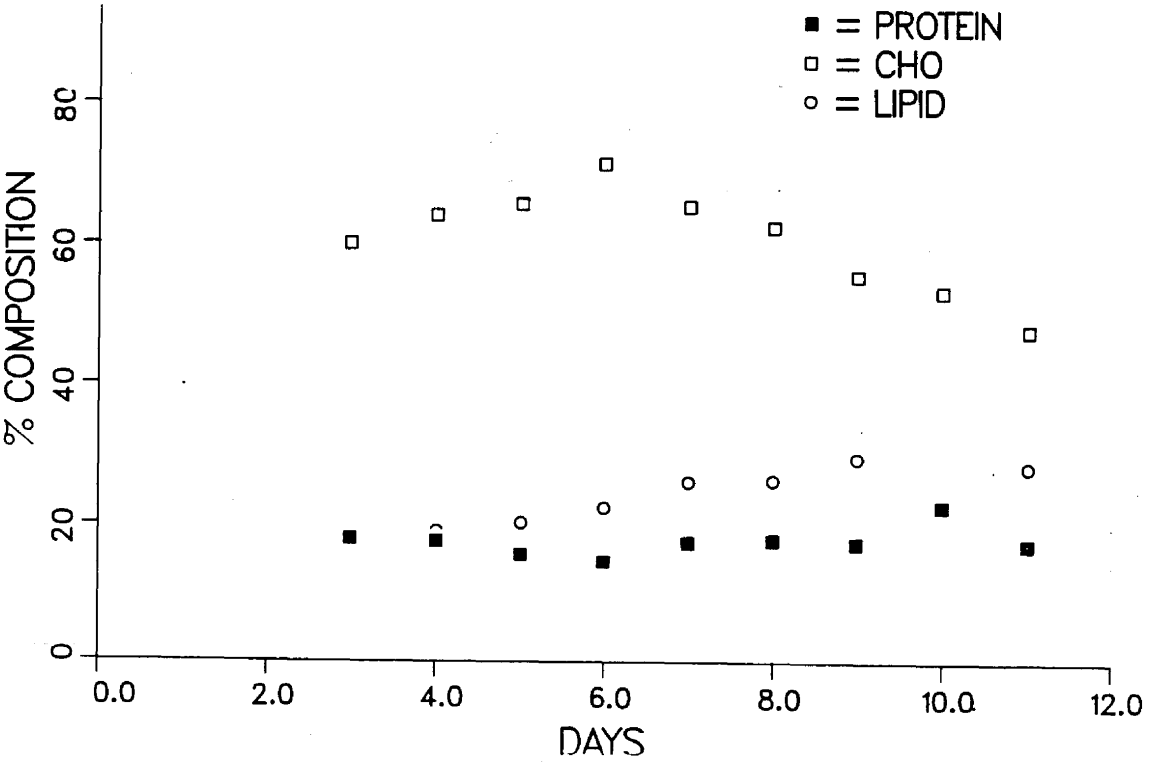


Table 2.12 Composition of N Sufficient and Deficient Chaetoceros

			Composition (% AFDW)			
Day	Culture	Status	Pro	CHO	Lipid	Total
3.0	122	NS	42.3	13.4	13.7	69.5
3.0	123	ND	17.9	60.0	17.4	95.3
4.0	124	ND	17.6	64.0	19.0	101.0
5.0	125	ND	15.6	65.6	20.2	101
6.0	122	NS	47.4	18.5	31.1	97
6.0	123	ND	18.0	68.3	22.4	109
7.0	124	ND	17.4	65.3	26.1	109
8.0	125	ND	17.7	62.4	26.3	106
9.0	122	NS	43.7	22.0	14.8	80.5
9.0	123	ND	20.0	53.2	29.5	103
10.0	124	ND	22.6	53.2	---	---
11.0	125	ND	17. 1	47.7	28.2	93

Table 2.13 CHN Analysis of N Sufficient and Deficient Chaetoceros

Day	Culture	Status	%C	%H	%N	%O	Δ Hc
9.0	122	NS	55.1	8.7	8.8	27.3	6.5
11.0	125	ND	48.0	8.1	2.8	41.2	5.2

Table 2.14 Fractionation of N Sufficient and N Deficient Chaetoceros Lipids

	% of Total Lipid		% AFDW	
Solvent	N Suff. (122)	N Def. (125)	N Suff. (122)	N Def. (125)
Hexane	1.0	1.1	0.2	0.3
Benzene	29.2	51.1	6.7	13.4
Chloroform	20.4	25.8	4.7	6.8
Acetone	33.0	10.8	7.0	2.8
Methanol	16.4	11.1	3.8	2.9
Total	100	99.9	23	26.2

Table 2.15 Composition of Chaetoceros During a Si Deficiency Experiment

Day Exp.	Day Si Def.	AFDW mg/l	% of AFDW Pro	CHO	Lipid	Total
0.89	-	109	-	-	-	
2.84	-	262	-	-	-	
3.90	-	516	56.1	28.8	21.0	105
3.94	0.0	310	54.3	27.4	23.4	105
4.08	0.14	414	46.0	40.0	25.8	112
4.28	0.29	626	19.6	23.8	n.d.	n.d.
4.8	0.86	680	39.4	38.1	30.1	108

Table 2.16 Fractionation of Chaetoceros Lipids Before and After Si Limitation

Solvent	% of Total Lipids Si Suff.	Si Def.	% of AFDW Si Suff.	Si Def.
Hexane	2.7	0.4	0.6	0.1
Benzene	2.3	32	0.5	9.6
Chloroform	9.7	3.9	2.0	1.2
Acetone	53	45	11.1	13.5
Methanol	33	18	6.9	5.4
Total	100	100	21	30.1

increase, but not nearly to the extent seen with the N deficient cultures. Also, in all cases, the sum of Protein/CH_2O/Lipid contents significantly exceeded 100%, averaging 108% (± 4). This was even true for the sufficient culture (initial data point) which in the prior experiment added to only 80% at that density. That may account for the generally higher lipid, protein and carbohydrate contents. However, the ratios of protein/CH_2O/lipids are fairly close for these two sufficient cultures (Ni and Si experiments). The reason for the apparent difference in extractability (or assay responses) is not obvious. It should be again noted, however, that these three measurements are independent of each other both in terms of both extraction and analytical technique. Thus the validity of a comparison of the ratios of their contents can not be considered superior to a comparison of their absolute number. (It should be also noted that the data point at 4.25 days showed protein and carbohydrate data lower by a factor of two from the pattern observed in the surrounding points, calling it into question).

There is a question as to whether the cultures were truly Si deficient. The high growth rate may not support such a contention, particularly in absence of continued growth monitoring after one day into the experiment. The earlier experiment (Fig. 2.7) suggests that Si limitation could be demonstrated, because it exhibited only 15% of the growth rate of the control. However, the continuing growth showed that some residual source of Si was present - probably from the borosilicate glass.

The lipid fractionation data (Table 2.16) showed a major shift from polar lipids to neutral lipids, which accounted for essentially all the new lipid synthesized. These results are similar to those observed with the N deficient cultures.

In summary, although on first sight it may appear that Si deficiency is a better method for lipid induction, a closer comparison of the initial kinetics of N and Si limitation, together with comparisons of lipid content relative to protein and carbohydrate, does not support this conclusion.

2.7.4 Conclusions

In healthy cultures, Chaetoceros is very productive reaching 450 mg/L day, the highest observed for all of the strains studied during this project. However, lipid content of these nutrient sufficient cultures is low, averaging somewhere between 13-17%. This resulted in maximal lipid productivities of only 60 mg/L/day. Depriving the cultures of nitrogen resulted in a sharp decline in biomass productivity and a similar decline in lipid productivity. While nitrogen limitation was effective at increasing lipid content (although slowly) it was ineffective at significantly increasing the lipid productivity. Finally, Si limited cells are capable of elevated rates of lipid synthesis as observed here. However this result must be considered within the context of the protocol followed.

2.8 BOEKOLOVIA SP.

As originally received (from the SERI Culture Collection), this organism was designated Chryso F-1, subsequently it has been identified as a Boekolovia species. Because of its recent isolation, little data is available concerning optimum culture conditions.

Two separate nitrogen limitation experiments were carried out, each one for the two protocols used. The first experiment (initial protocol, cultures #72-75, data shown in Fig. 2.10 and Table 2.17), exhibited significant variability in initial growth between the different cultures, although all the cultures attained approximately equal maximal cell concentration (about 550 mg/L). Unfortunately, the data collection was delayed beyond that originally planned, so that all the data points represent severely starved cultures. Indeed the experiment best demonstrates the effects of long-term starvation rather then short-term induction. This experiment showed a significant decrease in cell density after the period of maximal cell density had been reached. Lipid content also decreased and protein levels actually increased. Carbohydrate levels stayed constant (except for one, probably spurious, data point for culture #73). The low biomass yields and CHN analysis (shown in Table 2.18) indicated very high maintenance N quota.

The second experiment, with the modified protocol, (cultures #127-130) is presented in Fig. 2.11 and 2.12 and Table 2.19. The results showed again a more modest lipid content on day 3.83 (26.7%), only slightly higher than that of the N sufficient control (24.7%). Protein levels were, however, significantly lower (21.6%) than the control (40.1%) and carbohydrate levels higher (54.2 vs 27%). Thus, this initial point was already N limited, although lipid content was not significantly increased. Lipid content increased slowly up to day six; protein and carbohydrates did not change significantly if the carbohydrate "peak" on day 4.8 is ignored which it should because of the high totals of that culture sample. The sufficient control maintains its prot/CH_2O/lipid content at essentially its earlier levels throughout the experiment. After six days the N sufficient culture exhibited a significant decline in cell density and carbohydrate values appear to decline. However, there were obvious signs of contamination by day 7 (small reddish flocs) and the data became suspect. Contamination may also account for the density decline in the first experiment.

In conclusion, this organism exhibited an average productivity of 240 mg/L/day for over a week - reaching 2g/L - in a healthy, nutrient sufficient culture. At about 27% lipid this corresponded to a lipid productivity of about 65 mg/L day. This is a respectable lipid productivity. N limitation resulted in no immediate increase in lipids and only a modest increase (to about 35%) over a four day period. No change in lipid fractions was observed (Table 2.20). Thus overall this organism exhibits good sustained growth - a high N quota and has a high inherent lipid yield. It thus may be suitable for a continuous, N sufficient, lipid production process in which a high cell concentration is desirable and about 30% lipid is acceptable.

Table 2.17 Composition of Boekolovia (Initial Experiment)

Culture No.	Day After Start Expt.	Day After Start N Def	AFDW at Harvest mg/l	Composition % AFDW Prot.	CHO	Lipids	TOTALS
72	4.65	.5	.552	19.5	42.9	33.2	96.6
73	6.67	2.5	.402	23.1	23.8	34.4	81.3
74	8.66	4.5	.429	24.4	41.7	24.3	90.4
75	9.60	5.5	.388	32.4	45.2	23.5	101.1

Table 2.18 CHN Analysis of Early and Late N Deficient Boekolovia Cultures

Status	%C	%H	%N	%O[1]	ΔH_c
Early ND	54.20	8.08	4.00	33.72	6.0
Late ND	51.52	7.26	5.75	35.47	5.5

Figure 2.10 Growth Curves of N Limited Boekolovia

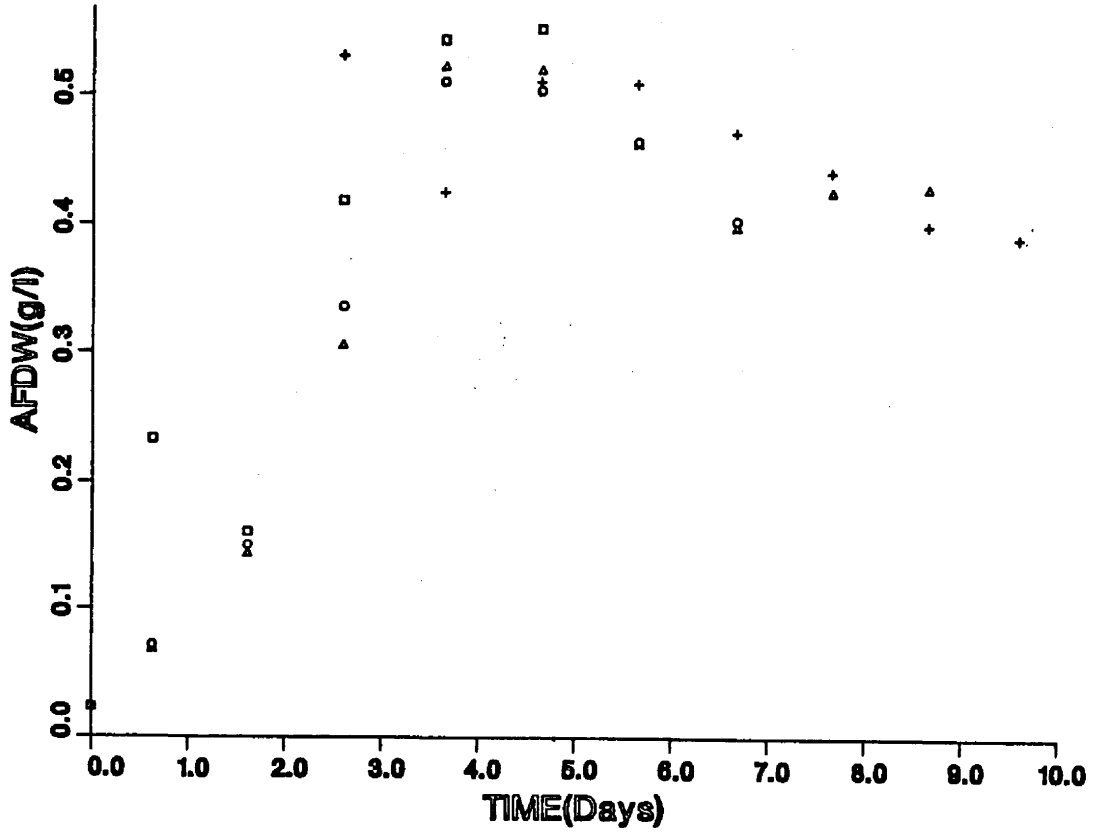


Figure 2.11 Growth Curves of N Sufficient and Deficient Boekelovia

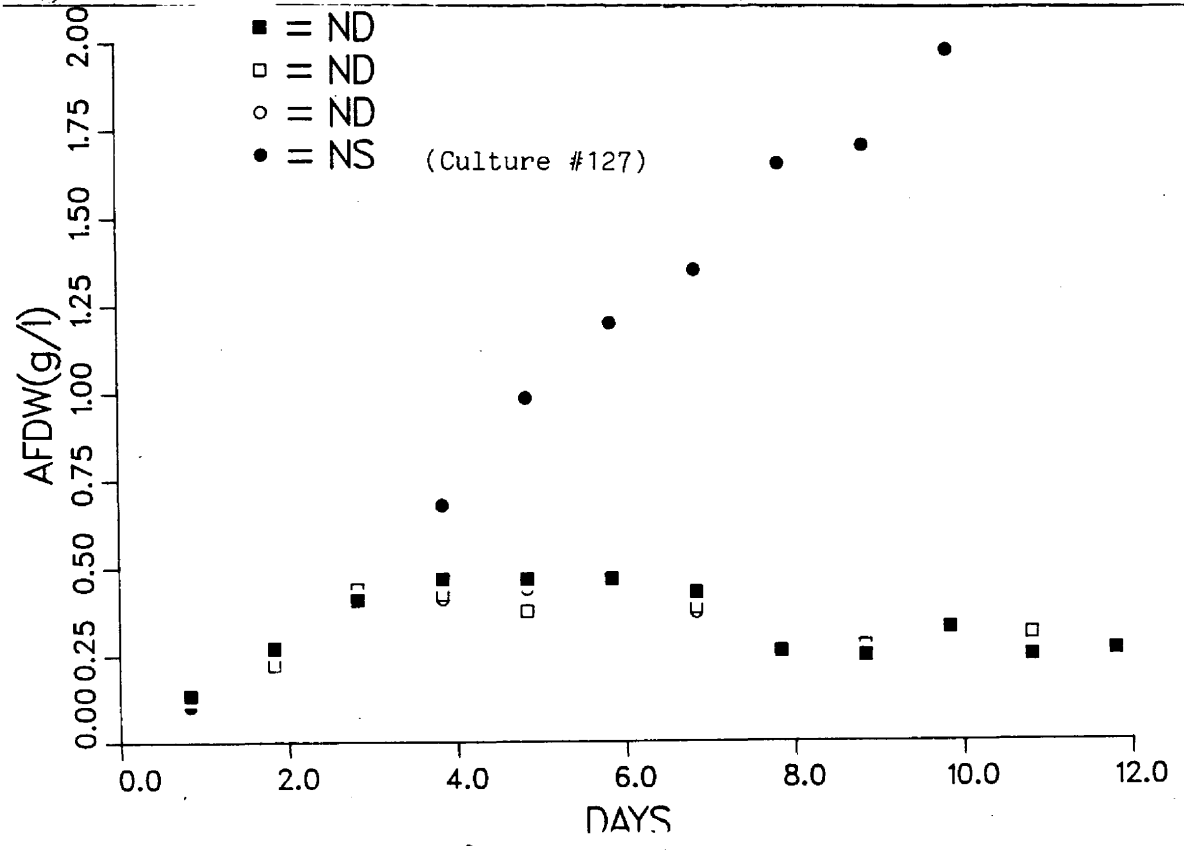


Fig. 2.12 Composition of N-Limited Boekolovia
(Cultures 127-130)

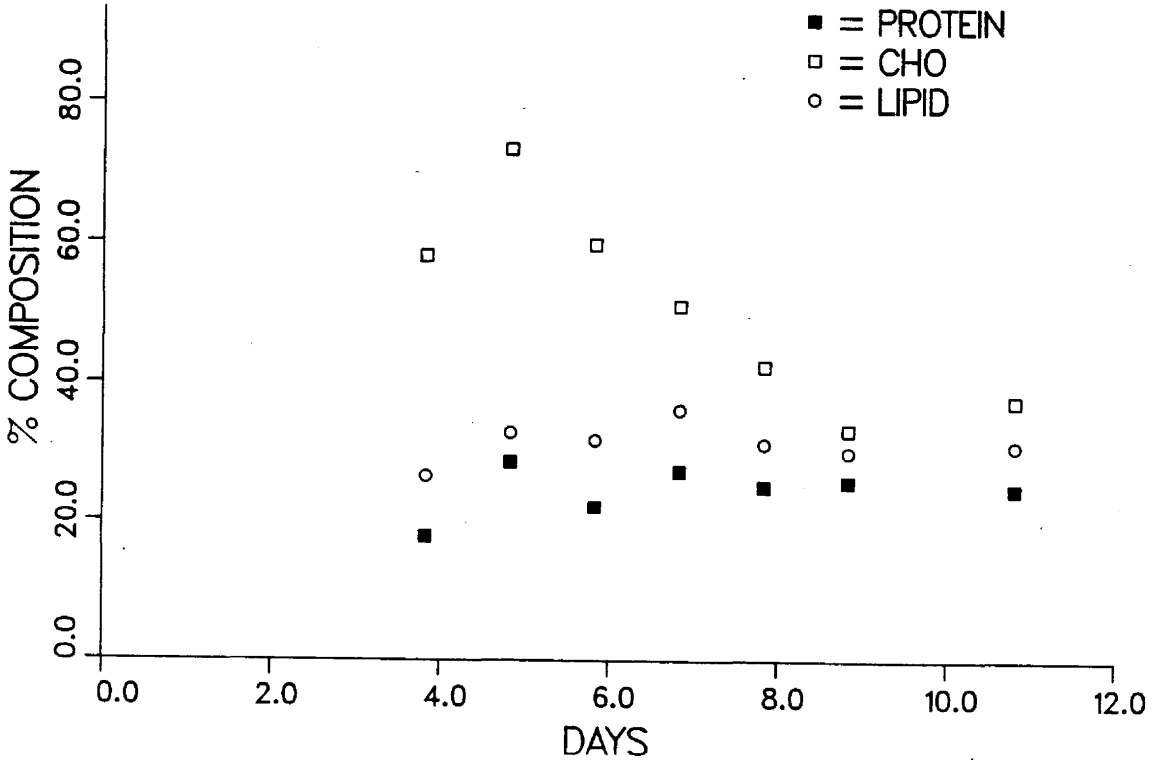


Table 2.19 Composition of N Sufficient and Deficient Boekelovia

Day	N Sufficient (#127) Prot	CH_2O	Lipid	Total	N Deficient (# 128-130) Prot	CH_2O	Lipid	Total
3.8	40.4	30.2	24.7	95.3	21.1	54.3	26.7	102.1
4.8	---	---	---	---	28.7	73.2	32.9	134.8
5.8	---	---	---	---	27.1	59.6	31.8	118.5
6.8	43.5	25.2	28.4	97.1	28.1	50.9	36.1	115.1
7.8	---	---	---	---	29.2	38.1	31.2	98.5
8.8	---	---	---	---	31.0	33.2	30.0	94.2

Note: Some data points are averages of duplicate determinations. Fig. 2.12 shows only results from the first set of analysis.

Table 2.20 Fractionation of Boekelovia Lipids

Solvent Phase	% of Total Lipids N Suf.	N Def.	% of AFDW N Suf.	N Def.
Hexane	0.8	2.1	0.7	0.2
Benzene	6.3	4.2	1.4	1.7
Chloroform	10.2	5.7	1.9	2.7
Acetone	51.8	54.5	18.1	14.2
Methanol	31.2	33.5	11.1	8.5
Total	100	100	33.2	27.3

2.9 THALASSIOSIRA PSEUDONANA

2.9.1 Introduction

Thalassiosira pseudonana (Hustedt) is a diatom and this specific strain is reported to have a temperature range of 12-22°C and an optical salinity near seawater (3). Cells are small (3-4 microns) and have biplanar symmetry. We had some difficulty growing it at the typical temperatures seen in these experiments but with a larger inoculum (about 50 mg/L) and a 2°C reduction in the daytime temperature (using a heat filter, fan and insulation between culture and stirrer) the culture grew satisfactorily. Healthy cultures appeared golden brown becoming rust brown when dense, while deficient cultures were more golden. The response to Si and N limitation were both tested with this organism.

2.9.2 N Limitation Experiment

A single N limitation experiment (modified protocol) was carried out; cell dry weight curves for both N limited (#146-148) and sufficient cultures (#145) are shown in Fig. 2.13. It is unclear why the control culture became stationary at 1.0 g/L, however, the subsequent decline in biomass after day 6 can be attributed to accidental discontinuation of the CO_2 supply. This resulted in the pH rising to 9.3 and 8.5 in the control and N deficient cultures, respectively. During this period the control culture became foamy. Maximum productivity occurred by day 2 and was about 350 mg/L/day during which lipid productivity was 70 mg /L/day (which was also at a maximum). Protein, carbohydrate, and lipid profiles are shown in Fig. 2.14 and Table 2.21. Lipid content in the healthy cultures was about 20% and increased to 25% by the 9th day. In the deficient cultures, lipids began around 20% and linearly increased to almost 40% by day 9. As stated, the maximum lipid productivity occurred by the 2nd day ;this was followed by a gradual decline to zero by day 6. Proteins in the sufficient culture were about 40-45%, but even by the first sampling point of the N deficient cultures, protein had decreased by about half. Protein levels remained fairly stable for the entire experiment. Conversely, carbohydrates were stimulated very quickly to a very high level, to 65% (vs. 20-25% for the N sufficient control). This rapid increase occurred before any obvious pigment changes or differences in dry weights had been observed in the cultures.

The deficient culture had 3.7% intracellular N, while the control culture had a much higher level of N (10.1%) (Table 2.21). These levels are relatively high as compared with other organisms studied and may indicate that this organism has a relatively high maintenance N requirement.

The lipid fractionation results are shown in Table 2.22. Except for the lipids in the chloroform fraction which increased some 50%, all other lipid fractions decreased in cells going from sufficiency to deficiency. This pattern was unique to this strain and suggests that increase in lipids in deficient cultures is most probably due to mono-di-and triglyceride synthesis.

Figure 2.13 Growth Curves of N Sufficient and Deficient Thalassiosira

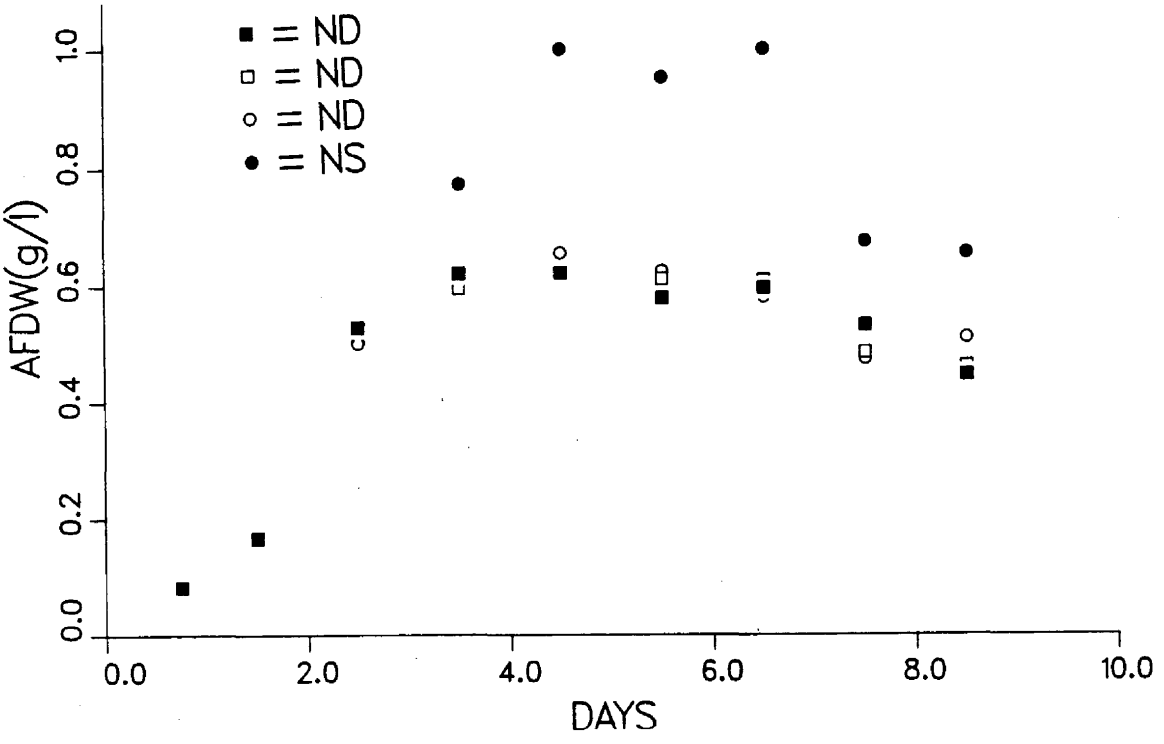


Figure 2.14 Composition of N Limited Thalassiosira

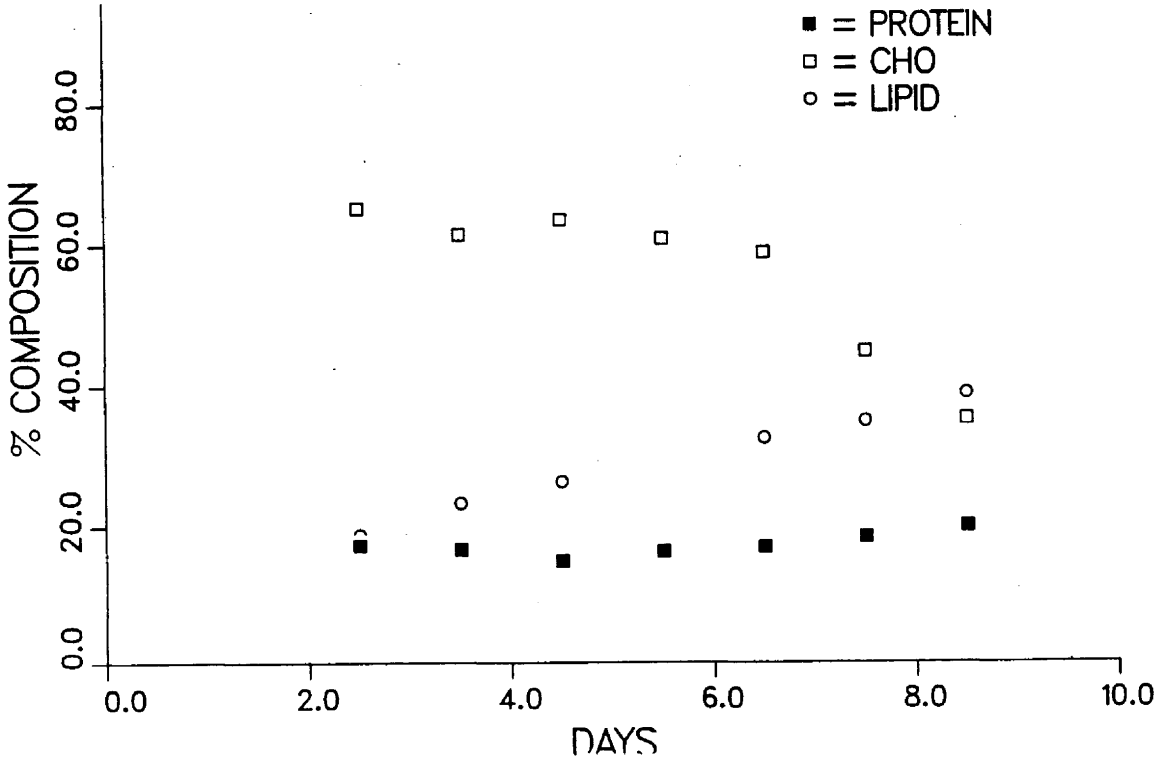


Table 2.21 Composition of N Sufficient and Deficient Thalassiosira

			% AFDW			
Day	Culture	Status	Pro	CHO	Lipid	Total
2.5	145	NS	43.9	22.8	20.4	87.1
2.5	146	ND	17.2	66.6	18.7	103
3.5	147	ND	16.6	62.7	23.2	103
4.5	148	ND	14.9	65.7	26.2	107
5.5	145	NS	50.0	17.1	25.9	93
5.5	146	ND	16.2	61.1	---	---
6.5	147	ND	16.8	60.9	32.3	110
7.5	148	ND	18.2	45.8	34.7	98.7
8.5	145	ND	41.7	24.9	24.3	90.9
8.5	146	ND	19.7	37.0	38.6	95.3
9.5	147	ND	23.5	25.9	30.4	79.8
9.5	148	ND	25.4	35.3	42.4	103

CHN Analysis

Day	Culture	Status	%C	%H	%N	%O	H_c
8.5	145	NS	55.1	8.13	10.1	26.6	6.4
8.5	146	ND	59.3	9.0	3.7	28.8	7.0

Table 2.22 Fractionation of Thalassiosira Lipids

	% Total Lipids		% AFDW	
Solvent	N Lim	N Suff	N Lim	N Suff
Hexane	0.1	1.6	0.0	0.4
Benzene	10.7	17.4	4.3	4.2
Chloroform	53.4	36.2	21.6	8.8
Acetone	26.6	29.1	10.8	7.1
Methanol	9.2	15.8	3.7	3.8
Total	100	100.1	40.4	24.3

2.9.2 Si Deficiency

In this experiment a sufficient culture was grown for three days following which it was centrifuged, washed once in Si free media and resuspended in Si free media. Table 2.23 presents dryweight and composition data. There is an increase in cellular lipid content during the experiment from 18% initially to over 30% after 2 days of Si limitation. More remarkably, biomass increased over two fold following Si limitation. The lipid productivity during the two day Si limitation period was 110 mg/L/day. This is an improvement over that which is attainable with N sufficient or deficient cultures (see previous subsection). Proteins, overall, slightly increased, going from 31 to 39%. There is a sharp dip in protein level at 0.34 days after deficiency, which corresponds to about 8 hours after transfer, and the beginning of the dark cycle. (A similar pattern was observed for Chaetoceros). Carbohydrate values are high initially (over 50%) and peak (at over 70%) also at 8 hours after deficiency was initiated. The validity of the 3.38 day data for proteins and carbohydrates is uncertain. Composition data from the Si sufficient samples compares well in terms of lipids to the N sufficient experiment reported above, but proteins and carbohydrates do not. (Proteins were lower and carbohydrates were significantly higher).

Fractionation of the lipids before and after Si deficiency is shown in Table 2.24. It should be noted that only very small sample quantities were available for fractionation, therefore the quantitative value of these results are limited. It is nevertheless clear that there is no substantial change in any of the fractions, except for possibly a slight increase in the acetone fraction.

Overall these results may raise the question of whether these cells were, in fact, Si limited, particularly considering the sustained culture productivity. In addition, the initial pattern of chemical composition is not representative of a sufficient culture and there is no singular type of lipid being formed preferentially in response to Si limitation. Therefore these results could be interpreted as simply the result of an increase in pigmentation due to light limitation. Thus, further Si deficiency experiments, carried out in plastic bottles, are required.

Table 2.23 Composition of Thalassiosira During a Si Deficiency Experiment (Culture 150)

DAY (Expt)	DAY (Si Def.)	AFDW (Mg/L)	COMPOSITION % AFDW Pro	CH_2O	Lipid	Total
2.21	---	234	---	---	---	---
2.46	---	415	---	---	---	----
3.04	---	470	31.2	54.2	18.3	103.7
3.08	0.0	358	---	---	---	
3.21	0.17	430	31.3	58.8	10.7	110.8
3.38	0.34	562	16.2	71.8	22.2	110.2
4.04	0.96	620	40.8	36.0	27.5	104.3
4.96	1.88	848	38.7	40.3	32.0	111.0

Table 2.24 Fractionation of Thalassiosira Lipids from the Si Limitation Experiment (Culture 150)

Solvent	% Total Lipids Si Suf.	Si Def.	% AFDW Si Suf.	Si Def.
Hexane	1.4	0.0	0.3	0.0
Benzene	29.5	30.5	5.4	9.6
Chloroform	16.6	13.0	3.0	4.2
Acetone	34.6	44.4	6.3	14.2
Methanol	17.9	12.0	3.3	3.8
Total	100	99.9	18.3	31.8

2.10 NANNO Q (Nannochloropsis QII strain)

2.10.1 Introduction

"Nanno Q" (Nannochloropsis Q) II is a small spherical (2-5 m) green alga belonging to the class Eustigmatophyceae. It was isolated by Dr. Ralph Lewin in China in 1984. Our experience with its cultivation led to the following observations:

a) It is difficult to inoculate into culture (under some instances almost one week long induction times were required). Even healthy cultures appear to have a rather lengthy lag phase (up to 2 days).

b) Initial growth experiments using GPM media yielded poor growth, the cultures were a pale yellow color and they crashed rather rapidly (at relatively low cell densities; 500-1000 mg/l). A modified GPM media, with enrichment for N, Fe, and trace metals was effective at greening the cultures and yielding higher cell densities (> 2g/L).

c) In particular, addition of Allen and Arnon trace metals (1-2 mls./L) in place of the PII trace metals was effective at greening the culture.

d) Healthy cultures are dark "lime" green, but not as intense a green as Chlorella or Ankistrodesmus. Nitrogen deficient cultures pale from lime to almost a squash yellow during which the culture appeared to develop a hydrophobic surface film.

Three N limitation experiments under different conditions of light, CO_2 and nitrogen were performed. (For a summary of conditions refer to Table 2.2.) Note that media and light environment were changed from the prior experiment. This was done in order to standardize conditions with respect to the above reported experiments. **Radiolabelling studies of Nanno Q are presented in Section 4.**

2.10.2 Initial Nitrogen Limitation Experiments

The first two N limitation experiments used the initial protocol and were carried out with both air levels of CO_2 (Fig 2.15, Table 2.25) and 1% CO_2 in air (Fig. 2.16 and Table 2.26). The air level CO_2 experiment was jeopardized to some extent by a power failure on the second day resulting in an extended dark cycle and discontinuation of the air supply. By the following day, conditions were corrected, however dry weight values were low, reflecting a shortened light period before the sampling time. On the following (day 4), the cultures became again productive reaching a rate of 330 mg/L day. This value is however, somewhat uncertain as the prior (and subsequent) light period was not standard, thus it may be somewhat high. After day 4 nitrogen limitation set in. A fairly constant productivity of about 160 mg/L/day was maintained for four days . Comparison of Figs. 2.15 and 2.16 (dry weight curve for air level CO_2 and 1% CO_2 cultures) shows a significant difference, indicating that increased CO_2 supply increases the initial growth rate, as well as final cell density. The 1% CO_2 culture exhibited a maximum biomass productivity of 310 mg/L/day between days 2.5 to 3.5, and a slow decline in productivity thereafter.

Figure 2.15 Growth Curves of N limited Nanno Q Grown on Air

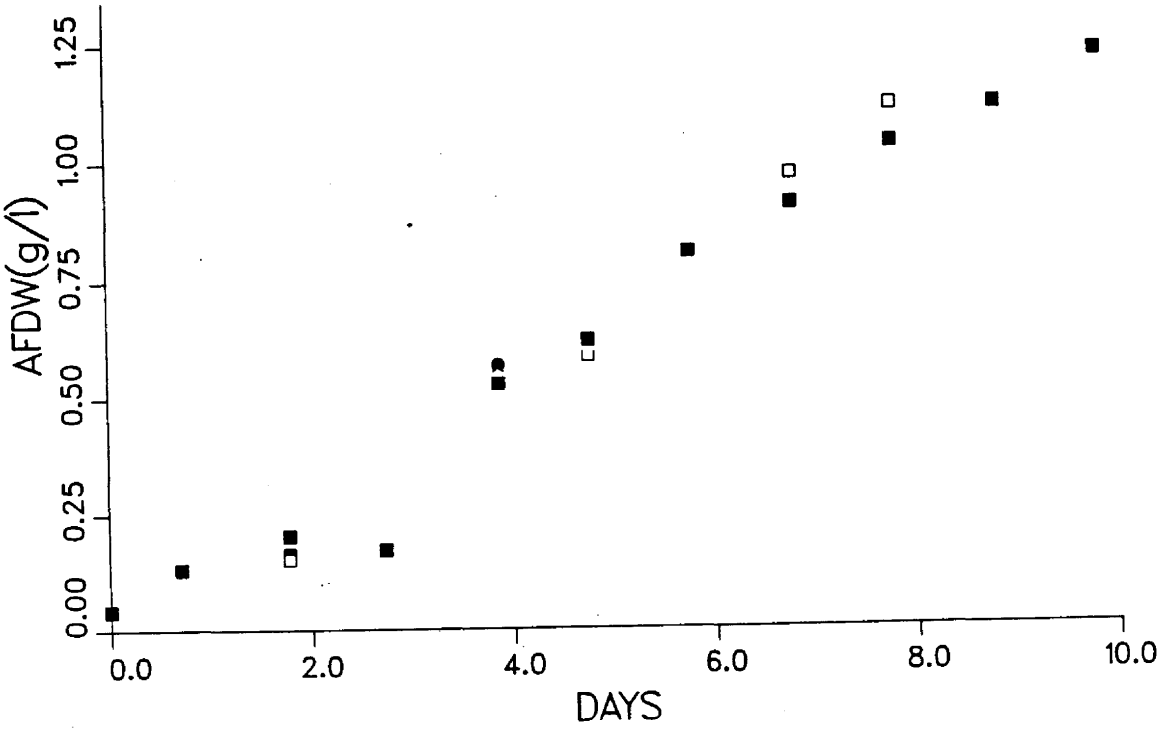


Table 2.25 Composition of N Limited Nanno Q Grown on Air

Culture No.	Day After Start Expt.	Day After Start N Def	AFDW at Harvest mg/l	Composition % AFDW Pro	CHO	Lipids	Totals
67	3.86		0.565	36.5	13.2	24.0	73.7
68	5.75	1	0.804	17.2	19.4	40.8	77.4
69	7.74	3	1.11	11.9	13.2	50.0	75.1
70	9.77	5	1.22	10.4	12.6	47.2	70.2

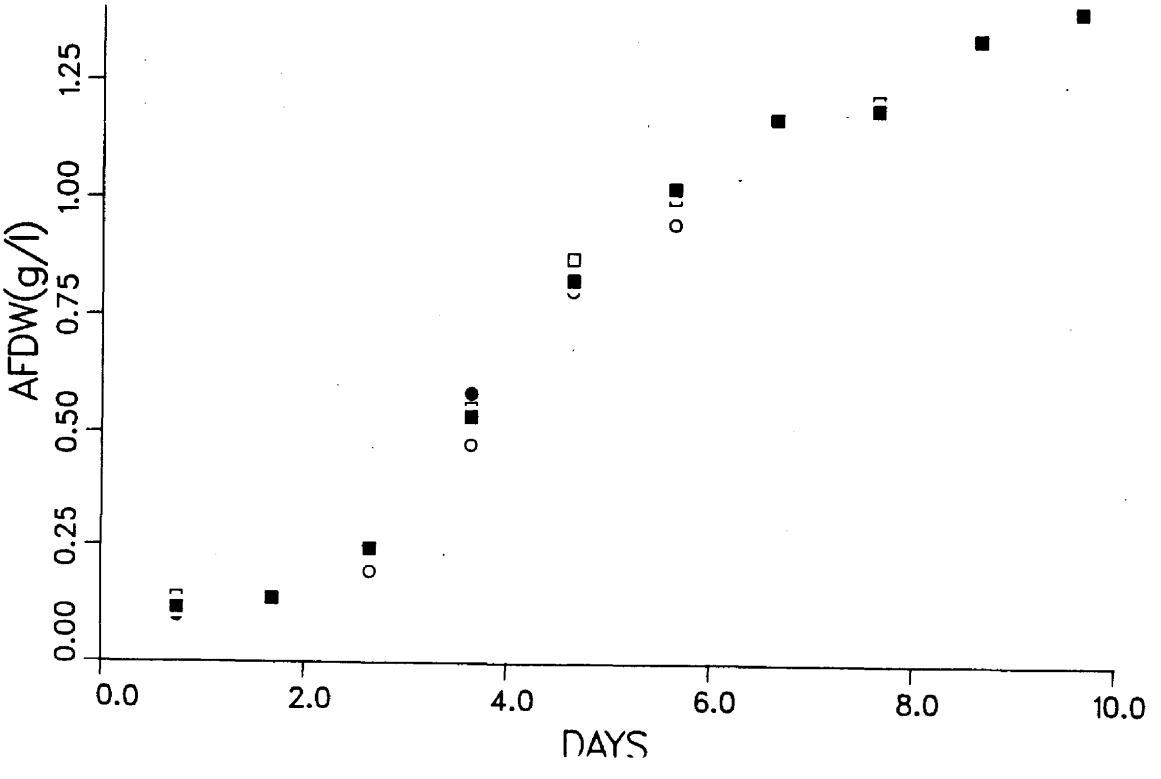
Table 2.26 Composition of Nanno Q Grown on 1% CO_2 (Cultures 80-83)

Culture No.	Day After Start Expt.	Day After Start N Def	AFDW at Harvest mg/l	Composition % AFDW Prot.	CHO	Lipids
80	3.64		0.582	25.0	26.0	35.6
81	5.64	1	0.945	13.1	20.2	46.7
82	7.66	3	1.21	10.7	13.9	48.7
83	9.64	5	1.40	9.2	11.6	52.6

Table 2.27 CHN Analysis of N Limited Nanno Q Grown on 1% CO_2

Culture	Status	%C	%H	%N	%O[1]	ΔH_c
80	Early ND	54.3	8.69	5.30	31.6	6.3
81	Mid ND	61.9	9.98	2.92	25.2	7.5
82	Late ND	63.8	10.4	2.29	23.4	7.9
83	Late ND	64.0	10.4	2.13	23.4	8.0

Figure 2.16 Growth Curves of N Limited Nanno Q Grown on 1% CO_2



Tables 2.25 and 2.26 present culture dry weight and cellular composition for each flask harvested during these experiments. For the air level CO_2 cultures the initial point (culture #67) shows a high protein content (36.5%), typical of N sufficient cells. This is expected considering the relatively low cell density and high nitrogen content of this culture. This is further confirmed by the low carbohydrate and relatively low lipid content. The next datum point shows a 50% decrease in protein with only a very slight increase in carbohydrate. Most significant is the substantial increase in lipid content (reaching 41%). The next sampling point (day 7.74) shows continued biomass production, with minor reductions in protein and carbohydrate, but additional increases in lipid (to 50% of dry weight). Some reduction in productivity is observed for the last point (day 9.77), during which cellular composition remained unchanged.

For the high CO_2 cultures, relatively low protein (25%) and high lipid content (37%) of culture #80 indicated that the initial harvest point already represented N limited cells. The following point (2 days later) is clearly N deficient with a 50% reduction in protein, a small decrease in CH_2O and a sizable increase in lipid content (to 47%). The following points showed minor increases in lipids with small reductions in protein and carbohydrate. The final lipid content for these cultures was 53%. The most significant result here is the relatively rapid shift in cellular metabolism in response to N starvation. Considering the initial 2 day lag period, essentially two days of growth resulted in the culture directing a large proportion of its biomass towards lipids. Lipid productivity during the maximum phase of biomass productivity (days 2.5 to 3.5) was 120 mg/L/day which was further increased on the following day to 150 mg/L/day with biomass production reduced slightly (to 300 mg/L/day).

Based on the results of these experiments three aspects in which this organism differs from the microalgae reported above:

a) There is a "decoupling" of the time at which maximum biomass and lipid productivities occur (see the Summary Table at the end of this section);

b) This organism has a low level of carbohydrates under N sufficiency, which shows very little response to N starvation; and

c) The cultures exhibit a sustained productivity which declines only after an extended period of N limitation.

The composition data (prot/CH_2O/lipids) does not add to 100% (values sum to 75% ± 5). The data are, however, consistent within itself, and between the two experiments in terms of total composition (except culture #80 which adds to 86%). The reason for this low total composition value can probably be found in the carbohydrate analysis. Lipid contents are unlikely to be at fault (e.g. unlikely to be low) and protein data correlates well with the total N content. (Table 2.27). The glucose standard used could easily underestimate carbohydrates by a factor of two, depending on the specific sugar composition. Incomplete extraction can, of course, not be excluded either, particularly with such a small organism. In either case, carbohydrate content may be underes-

timated by possibly two fold. Even then, however, it would show a much higher lipid and lower carbohydrate content observed than any of the other algae.

The CHN analysis, in addition to indicating N status, also served to determine degree of reduction as manifested in heat of combustion values. Early nitrogen deficient cultures already had heat of combustion values some 15% above normal and the final culture harvest had a very high value of 8 Kcal/g, significantly larger than any other N deficient organism tested in this project. This, of course, translates into a relatively higher efficiency of energy capture and transformation by this organism.

Fractionation of total lipids is shown in Table 2.28. During N limitation there is a clear shift from pigment associated fractions to the neutral lipid rich $CHCL_3$-Benzene fractions. A rather broad distribution of polar lipid compounds remain after severe N limitation indicating that functional components of the cell metabolism are intact. In comparing the two experiments, it should be noted that culture #80 is already N limited, thus it corresponds more closely to culture 70 than 67, in terms of lipid distributions.

2.10.3 Final Nitrogen Limitation Experiment

A final experiment was carried out under conditions similar to those used for the other strains, in order to compare Nanno Q with them and eliminate environmental factors as a cause for the differences in lipid content and productivity noted above. Dry weight curves for sufficient (#141) and deficient cultures (#142-144) are shown in Fig. 2.17. Again it is seen that a two day lag period preceded a significant increase in cell dry weight. Most significant in this experiment is that the deficient culture had an extended period of zero growth after day 6 . The sufficient culture appeared slightly inhibited initially - this had been confirmed by subsequent experiments showing a reduction in growth at 20mM KNO_3, (but not at 10 mM) - followed by a continuous period or linear productivity (340 mg/L/day). The deficient culture, on the other hand, was not very productive reaching a maximum of 170 mg/L/day and declining to zero by day 6.

Shown in Table 2.29 is a summary of ash free dryweight data and composition analysis for both the sufficient and deficient cultures. As was to be expected (from the dryweight curves), the initial point was clearly deficient. Protein values were already reduced to below 20%, carbohydrates were low and lipids quite high, at 49%. Proteins remained low and decreased slightly during the remainder of the experiment. Carbohydrates increased somewhat at 4.5 days but remained fairly constant at about 20%. The lipid composition appeared to reach 50% quite rapidly, but then the data became rather erratic. This is likely the result of technical oversights in the assay procedure and not experimentally based. In any event, it is apparent that high lipid contents were induced in response to N limitation. The data from the healthy culture gives a general consensus composition of 40% protein; 20% carbohydrates and 24% lipid. Note that the final datum point for the N sufficient culture had a rather high lipid content (35%). It may be that lipid synthesis is a rather common metabolic event for this algae, requiring only a relatively minor environmental stress.

Table 2.28 Fractionation of N Limited Nanno Q Lipids

Nanno Q: Lipid Fractionation % of Total Lipids

Solvent	Culture Number 80	81	82	83	67
Hexane	3.9	5.1	4.9	4.8	2.5
Benzene	27.7	59.1	65.8	64.7	4.5
Chloroform	32.6	17.9	17.4	17.7	5.1
Acetone	21.3	6.9	7.5	7.1	66.3
Methanol	14.4	10.9	4.4	5.8	21.6
Total	100	100	100	100	

Nanno Q: Lipid Fractionation % of AFDW

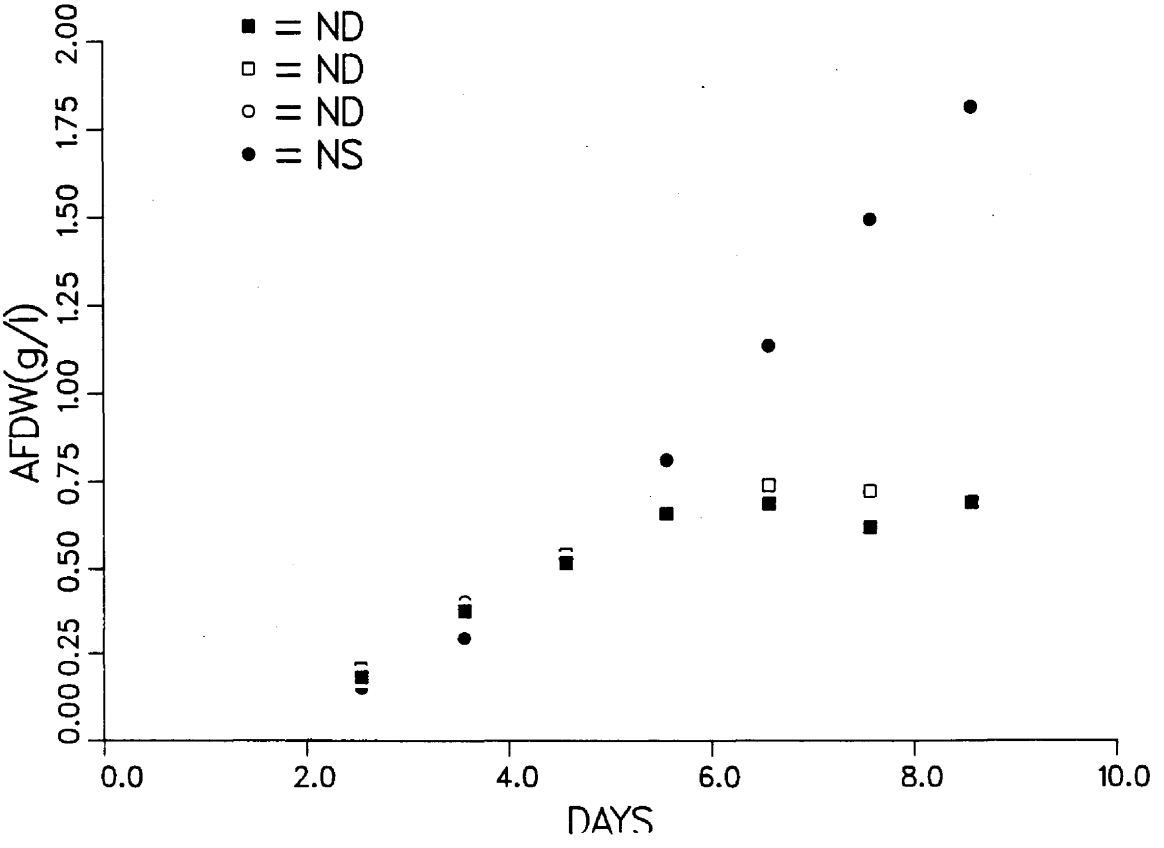
Solvent	Culture Number 80	81	82	83	67
Hexane	1.4	2.4	2.4	2.4	0.6
Benzene	9.9	27.6	31.7	32.6	1.1
Chloroform	11.6	8.4	8.	8.9	1.2
Acetone	7.6	3.2	3.6	3.6	16.0
Methanol	5.1	5.1	2.1	2.9	5.1
Total	35.6	46.7	48.7	50.4	24.0

Table 2.29 Composition of N Sufficient and Deficient Nanno Q

(All values before the slash refer to culture 141, N sufficient, and all other values are from cultures numbers 142 to 144, N deficient)

(DAYS)	mG/L	% OF AFDW PRO	CHO	LIPID	TOTAL
1.67	150/190	--	--	--	
3.56	290/390	19.6	18.2	49.9	87.7
4.56	540/530	40.1/19.1	18.7/24.2	50.4/26.0	84.8/93.7
5.55	810/660	16.4	20.3	39.0	75.7
6.56	1130/710	16.9	19.8	49.9	86.6
7.56	1480/670	13.4	21.1	35.7	70.2
8.56	1810/690	51.8/15.7	17.3/17.1	35.0/40.9	104/73.7

Figure 2.17 Growth Curves of N Sufficient and Limited Nanno Q

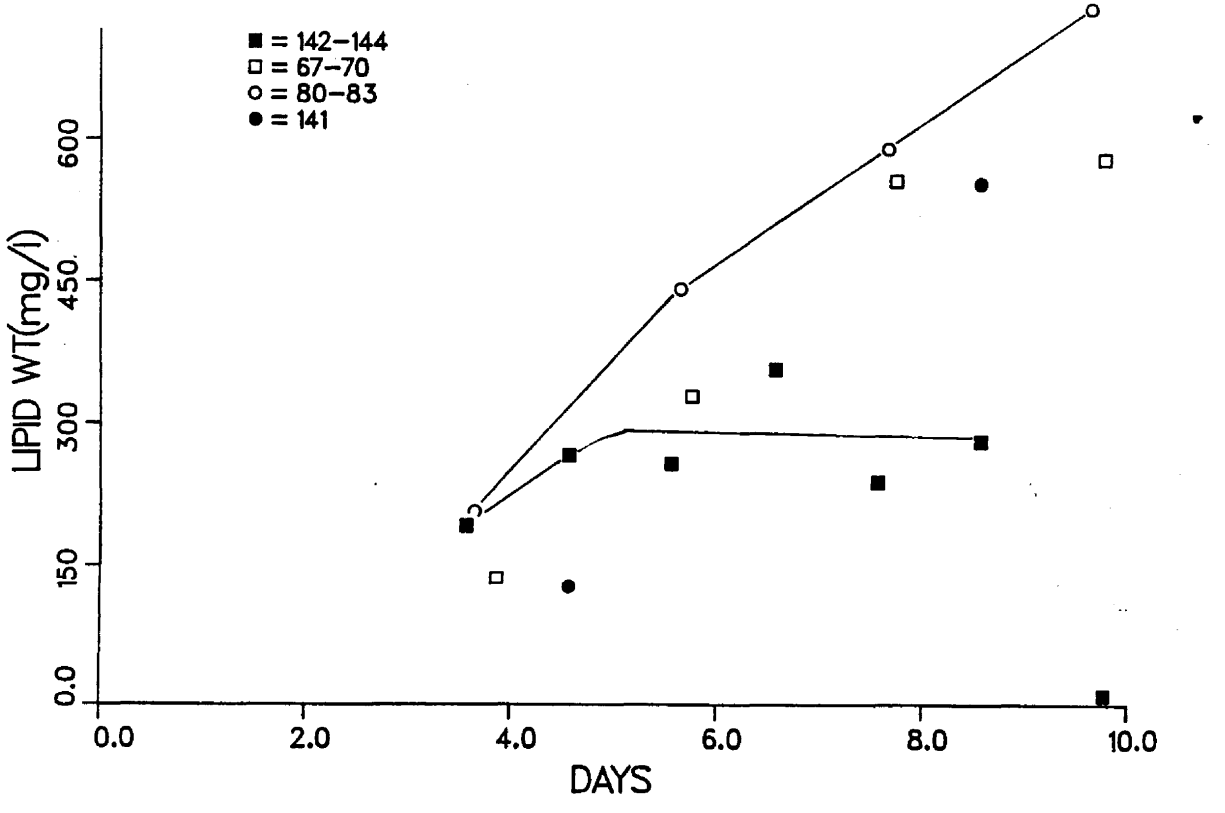


relatively minor environmental stress.

2.10.4 Conclusions

These three experiments with Nanno Q provide an introductory study into the poorly understood aspect of light-Nitrogen relationships with respect to lipid productivity. A comparsion of lipid yields for the different growth conditions is given in Figure 2.18. One can see that time is a distinct advantage in terms of lipid production when cultures are limited at different light-N environments. One percent CO_2 cultures, limited at higher cell density with 2.6 mM KNO_3 maintained a significant and continuous production of lipid during the entire culture period (Final lipid yield = 740 mg/L). In terms of yield and short-term productivities, this is a marked improvement over cultures 142-144 which were limited at lower cell densities and a different light environment. Maximum lipid content and productivity were 360 mg/L and 170 mg/L/day, respectively. It is impossible, based on this limited amount of data, to uncouple the interaction of light, nitrogen and culture density and how each in turn affects the overall lipid production rate. Light and nitrogen both interact to determine cell density and therefore pigment levels. Pigment availability is critical in terms of providing enough energy capture for high reduction carbon storage. Therefore, cultures that experience some degree of light limitation prior to depeleting available nitrogen are perhaps more capable of carrying out lipid synthesis. In this instance we have seen that a higher density culture exposed to light of a lower intensity but higher flux rate (2x area) was much better in terms of lipid productivity than a lower cell density culture exposed to a higher intensity light but lower flux.

Figure 2.18 Summary of Lipid Productivity of Nanno Q



2.11 DISCUSSION AND CONCLUSIONS

2.11.1 Lipid Productivities

Tables 2.30 and 2.31 summarize the productivity data collected for this task. At first glance the conclusion would be that in all cases, including possibly Nanno Q, there is no advantage to inducing lipids under conditions of N starvation - as both maximal biomass productivity and lipid productivity are higher - or at least equal - in N sufficient, as compared to N deficient cultures. Indeed, the productivity of lipid with N deficient cultures is,for all cases but Nanno Q, lower than that of N sufficient cultures.

However, this conclusion must be tempered by a more careful analysis of the data: a comparison of maximum productivities of N sufficient cultures must be made with the maximum productivities of N deficient cultures - which is not possible with these data since maximum productivities were not established - as (in most cases) only one $N\text{-}NO_3$ level (usually 1.6 mM) was used. That level was chosen as the most likely to result in a rapid induction of lipids and a high lipid productivity (at least in the desireable strains) compared to a N sufficient control at a similar density.

To allow proper comparisons, the algal density should be sufficient to have established constant productivity (e.g. linear growth) prior to N limitation. Otherwise a comparison of relative productivities during a phase of increasing productivities would have to be carried out. That would be a dubious undertaking theoretically, and, even under the best of circumstances, experimentally, as it involves a comparison of second derivatives. In our experiments, where diurnal light (and cell) cycles are imposed and data (dry weight) collection was only daily (a large interval relative to N sufficient growth rates), comparing changing productivities is statistically not appropriate - even if theoretically such comparisons were valid. Indeed, the data for Nanno Q, where several different N deficient conditions can be compared, showed a large variation in the maximal productivity of biomass and lipid production by N limited cells.

In conclusion, the currently available data does not allow any judgments about the maximum lipid productivity by these microalgae. Only a much more detailed comparative investigation of selected microalgae strains will allow that. The immediate question is which algae strains should be selected for further studies and on what basis such selection should be made. In this regard, the data reported above provides a useful guide for ranking algal strains.

2.12.2 Ranking of Algal Strains

We can define three general classes of microalgae in terms of lipid productivity potential based on their maximal lipid values (Table 2.32): 1) those with both low maximal lipid content and low lipid productivity; 2) those with either low lipid content or low lipid productivity; and 3) those with both high lipid content and high productivity. The last group are the ones that should receive further attention. The dividing line between low and high lipid content should be at least 30% lipids, as any lesser amount would be impractical. In these experiments, the lipid productivity cut-off should be between 90 and 100 mg/L/day. Based on these criteria Nanno Q is the best and Cyclotella the second

Table 2.31 SUMMARY OF BIOMASS/LIPID PRODUCTIVITY AND LIPID CONTENT DATA
(mg/L day)

Organism (Cultures)	N (mM) Status*		Interval (days) 1-2 (%)	2-3 (%)	3-4 (%)	4-5 (%)	5-6	Final Concentrations (day) Biomass/Lipid/Clho/Prot (day mg/L
Ankistrodemus	ND	(1.6)	260/- ( )	350/90 ( )	220/60	90/10	140/20	1430/500
(111-114) 117, 118, 121	NS	(20)						
Boekolovia								
(127)	NS	(20)	120/- ( )	210/50 ( )	240/60	300/80	210/80	1960/540
(128-130) 72-75	ND	(1.6)	120/-	170/40	15/0	20/20	40/10	480/150
Chaetoceros								
(122)	NS	(20)	--/--	450/60	330/50	340/50	210/30	2150/320
(123-125) 88-91	ND	(1.6)	--/--	400/80	100/30	-10/10	-20/20	770/190
Chlorella								
(76-79) 62-64	ND	(1.6)	260/30	130/10	90/10	70/10	-40/0	750(110/ (8)
Cyclotella								
(99-102)	ND	(1.6)	260/-	230/70	250/100	70/30	20/10	890/370
Nanno Q II								
(141)	NS	(20)	190/40	260/70	300/90	340/110	340/130	1800/550
(142-144)	ND	(1.6)	170/110	130/30	90/50	60/40	0/10	730/360
(67-70)	ND	(2-7)	330/80	80/70	200/130	160/110	130/110	1220/580
(80-83)	ND	(2-6)	300/140	230/140	170/110	110/60	80/60	1400/740
Tahitian Isochrysis								
(84-87)	ND	(1.9)	--/--	240/70	220/60	130/40	90/30	1130/330
Thalassiosira								
(145)	NS	(20)	230/50	300/70	250/60	180/40	0/0	1000/250
(146-148)	ND	(1.6)	230/40	220/60	60/30	30/20	10/0	650/190

Day Intervals have been shifted by 2 to account for lag period.

Table 2.32 SUMMARY OF MAXIMUM BIOMASS AND LIPID PRODUCTIVITY AND LIPID CONTENT DATA

ORGANISM (Culture)	N STATUS	MAXIMUM PRODUCTIVITY (mg/L/Day) BIOMASS	LIPID	MAX % LIPID (% AFDW)
Ankistrodesmus	ND	260	90	35
Boekolovia	NS	300	80	28
Boekolovia	ND	170	40	36
Chaetaceros	NS	450	60	15
Chaetaceros	ND	400	80	28
Chlorella	ND	260	30	14
Cyclotella	ND	260	100	40
Nanno Q (141)	NS	340	130	35
Nanno Q (142-144)	ND	170	110	50
Nanno Q (67-70)	ND	330	130	50
Nanno Q (80-85)	ND	300	140	53
Isochrysis	ND	240	70	30
Thalassiosira	NS	300	70	25
Thalassiosira	ND	230	60	39

best organism, definitely belonging to the third class listed above. Chlorella certainly belongs in the first class while all others belong in the second class. Whether any of the algae in the second class would warrant further examination cannot, at present, be excluded. Chaetoceros, although marginal as far as either lipid content or productivity, is a dominant alga in outdoor systems, and was the most productive species on a total biomass basis and therefore may be worthy of further consideration. Ankistrodesmus almost meets the criteria for Class 3. However, the lipid productivity is probably over-estimated; (see Section 2.6 discussion). It is clear from the data, that of the group surveyed, the most promising strains are the Nanno Q and Cyclotella and these should be investigated further with the aim of establishing the maximum lipid productivity achievable.

Perhaps the major value of the data collected thus far is in the design of a rational and efficient screening program for high lipid producers. The SERI program has isolated several hundred species of microalgae. However, no mechanism exists at present for establishing-in a cost effective manner - whether any of these will be good lipid producers. In the next subsection we propose such a screening protocol.

2.11.3 Screening For Lipid Producing Microalgae

As stated above, any program for microalgae lipid production must use strains that can, at a minimum, exhibit relatively high lipid content, 30 to 35% being the cut-off value. Thus the first objective of any screening study should be to establish whether the algal strain meets this mimimum requirement. In all cases, N limitation even if it does not result in a major increase in lipids, does not depress their content (except possibly in very extreme circumstances). An induction experiment using a single N level (1.5 1mM N) and a single sampling point (either within two days after biomass density stops increasing, or within three days of the start of visible N limitation) should suffice to establish the maximal lipid content achievable by a strain. Based on prior experience, at least half of the strains would be eliminated on the basis of insufficient lipid induction potential.

The second issue is that of maximum productivity under N sufficient conditions. For each strain not eliminated by the above first screening step, a batch growth curve should be carried out, which would allow measurement of both maximum productivity, as well as the density at which productivity becomes constant (linear growth). Productivity should be followed for about one week. One sample, for lipid content analysis, should be collected early in the linear growth phase. Based on maximum biomass (preferably heat of combustion) productivity, the strains could be ranked. This should allow over half of the strains to again be eliminated from further consideration.

The final, and key, question is the maximal lipid productivity potential of these surviving strains. Using data from the first (N quota) and second (point of entering linear growth) experiments a nitrogen level can be selected that allows exhaustion of intracellular N at a point early in the linear growth phase. Three lipid analysis samples should be collected for each culture, one early into the N limitation (both as predicted and from visual observations), one within one day thereafter and the final-one within one or two days after that - depending on the productivity data obtained. Based on these data, the

strains could be ranked in terms of lipid (and biomass) productivity.

Overall, this screening study would involve - at the stated elimination criteria for the first two stages - about 2 lipid analyses per strain screened and one growth curve for each two strains screened. Total personnel time involved would be on the order of two person days per strain or about one person year of effort for screening 100 strains if ancillary activities (culture collection) are included.

The key questions and issues are: 1) what are the conditions under which the experiments should be carried out (temperature, media, light intensity and duration); 2) what accessory data (in addition to lipids) should be collected; 3) how good is the screening test in reducing the numbers of strains at each step, 4) how reliable is it in either not missing valuable strains or in not selecting poor ones;and 5) howproductive would the selected strains be under outdoor conditions. These points are discussed briefly below.

It may be assumed that sufficient knowledge about strains to be screened will be available to indicate - at least approximately - the preferred media, temperature and pH range of the strains in question. These must be used as inputs into such a study. Temperature and pH ranges will - in any event be dictated to a considerable degree by external factors such that a more or less standardized condition should be used in such a study (the strains could be grouped by preferences). A similar argument applies to the culture media.

Incident light intensity should be at or above light saturation levels - a higher light intensity is not necessary as, in the absence of secondary effects (inhibition of photosynthesis) the effect of higher light intensity would be predictable from the Bush equation. Thus 300 μ Ein/m^2/sec, used in most of the above studies, is an appropriate light intensity.

Diurnal vs constant illumination is a more vexing problem. A major limitation of the above presented data is due to the lack of sufficient number of data points for growth curves to be able to "smooth out" the effects of diurnal periodicities. However, constant light - being an unnatural condition - may well limit the predictive power of such a screen. Many algae strains grow poorly when subjected to 24 hours of continuous light. During growth curve experiments with diurnal cultures several biomass density points will be required each day. Considering the well known effects of constant light on many algal strains, diurnal light cycles are recommended for such a screening study.

In summary, for each strain taken to the final step in the screening study, five lipid analyses would have been carried out. It would be cost-effective to also routinely do proteins and carbohydrates, as they involve a modest effort. Aside from the (duplicate) ash-free dry weight determinations involved with each lipid analysis, additional dry weight samples should be collected daily during all the growth experiments. The results would allow correlation with O.D. determinations, which should be carried out three times a day. This would represent the minimal data collection effort.

The issues are: 1) will the resulting data allow elimination of sufficient number of strains to make it a useful screen; 2) the numbers of false positives or negatives ; and, 3) the robustness of the final ranking (for lipid productivities) in regards to alternative conditions. These questions are not easily answered a priori. Even after the fact these would be difficult to answer without a sustantial amount of work. However, based both on our own experience and results from other researchers, the proposed screening process appears both cost effective and viable. The above stated questions should be subordinated to the issue of whether the rankings will reflect relative capabilities in outdoor systems. This will require further selection for competitive strains. Although arguments can also be made that competitiveness should be established a priori as the major factor in a screening program, the above outlined lipid productivity screen is both a viable, and necessary, approach to the problem of selecting potential candidate species.

2.12.4 Conclusions

The production of lipids by microalgae requires that both strains and environmental conditions which maximize lipid productivity- not content per se - be identified. This task has addressed the first objective, the screening for useful strains. Although a high lipid content is certainly desirable, it does not always correlate with high lipid productivity. An increase in lipid content during a period of increasing, or at least a steady, biomass concentration is the minimum required characteristic for an algal strain of interest for further studies in this program. Although any extrapolations of our data to actual lipid productivities is hazardous, because only a very limited set of environmental conditions was tested, it is nevertheless, apparent (See Table 2.31 for a summary of the data) that only two of the algae tested Cyclotella and Nanno Q, meet these minimum requirements. Of the other strains studied most exhibited a spurt of carbohydrate biosynthesis upon N limitation, followed by a slow increase in lipid productivity. Thus,overall, lipid productivity and/or final lipid content were relatively low.

SECTION 3.0
STRUCTURES OF C_{37} HYDROCARBONS OF ISOCHRYSIS

3.1 OBJECTIVE

TASK III The structure of the C-37 hydrocarbon produced by Isochrysis shall be determined.

"To provide information needed to evaluate this compound as a potential liquid fuel product and provide a comparison between strains of Isochrysis."

"Two strains of Isochrysis (galbana, Tahitian Tiso) shall be analyzed for the C37 compound. Furthermore, any differences in lipid composition between the two strains shall be determined when grown at optimum temperatures (galbana - 25-27C, Tahitian - 31-33C), under nitrogen deprivation. The C37 compound shall be subjected to such analytical procedures as are required to elucidate the structure."

3.2 INTRODUCTION

Prior work by this project on lipid composition of selected microalgae species, was relatively complete (4) with the exception of the structure of the major lipid component of Isochrysis. This major component was isolated in the benzene eluate collected from a Unisil (silicic acid) column and represented 4% of organic cell weight. It resulted in a single spot on a thin-layer chromatogram (Rf 0.27, with petroleum ether-diethyl ether--acetic acid, 90:10:1). A preliminary report (at the March 1983 SERI Aquatic Species Program Contractor Review Meeting) tentatively identified the unknown compound as "an oxygenated cyclic C37 isoprenoid compound the exact identity of which had not been fully elucidated." This task had for its objective to elucidate the structure of this major benzene eluate component.

3.3. RESULTS

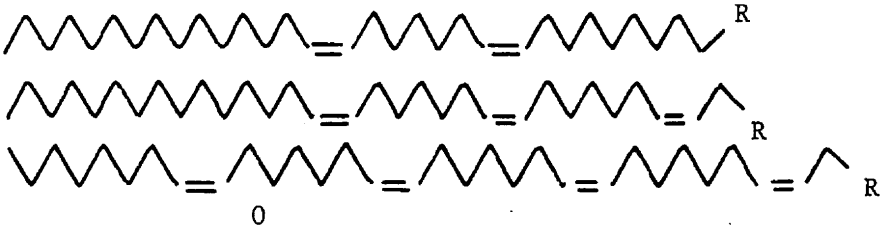
In the beginning of the analyses, it was observed that high-resolution capillary gas chromatography was unable to resolve what appeared to be a family of polyunsaturated isomers. Thus, the samples were hydrogenated with hydrogen gas and palladium on charcoal. The reduced sample then consisted of 3 major component types with molecular mass ions of M-506, M-534, and M-562. Each component had strong M-18 and m/e 59 identifying the components as C35, C37, and C39 alcohols, each with one unsaturated position. Because the sample was hydrogenated, it appeared that the absence of 2 atomic mass units was not the result of incomplete saturation but an indication of a ring structure.

In an attempt to further reduce the samples, the components were hydrogenated with $H_2/PTO_2/HClO_4$. This condition should assure the reduction of the unsaturate position, if present, and deoxygenate the compounds to hydrocarbons. The following major compounds were obtained:

M-506--C36 alcohol with one unsaturated position
M-534--C37 alcohol with one unsaturated position
M-508--C37 alcohol
M-536--C37 alcohol
M-492--C35 hydrocarbon
M-520--C37 hydrocarbon

(C39 compounds in the preparation were in small quantities and were not analyzed).

The above mixture of compounds were obtained because the reductive reaction was not quantitative. The C37 components were still the major compounds in the sample. H-NMR analysis of the sample indicated one isopropyl group, a high proportion of CH-2 groups, and a low proportion of CH-3 groups which may have resulted from the tertiary hydroxyl group. Thus, this preliminary data supported a presumption that the principal compounds were unsaturated C35-C37 oxygenated cyclic hydrocarbons. Definitive identification was not possible until the various compounds in the original sample were obtained in purified forms and independently analyzed. This was recently accomplished with an experimental super critical fluid chromatographic system. The analyses of the purified component clearly indicated that the principal compounds were not cyclic alcohols but acyclic unsaturated ketones of the following 2 types for each of the C35, C37 and C39 structures.



$R = CH_2-(CH_2)_n-\overset{O}{\overset{\|}{C}}-[CH_3]$ or $[CH_2-CH_3]$

Reanalysis of the prevous mass spectral and NMR data obtained for unpurified samples supports the structures given above except for the interpretation of the data that indicated the presence of the OH group and ring structure. Ketones are reasonably stable and should not be reduced by H/Pt. Recent tests with standard ketones and especially unsaturated ketone forms showed that ketones are converted to hydroxylated cyclic and acylclic unsaturated hydrocarbons. Thus, the initial reduction experiments complicated the study by creating artifacts.

After the identity of the compounds was established, a literature search was conducted for evidence of their novelty in algae and/or sediments. In 1979, 1980 and 1981, Volkman, et. al. (5-7) reported their results on the lipid composition of representatives of the Prymnesiophyceae (Haptophyceae). They reported long chain alkenones, esters, and sterols in Emiliania hyxleyi and lipid distribution difference among the genera. The exact identity of the long chain alkenones was published in September, 1984 (8).

In addition to the report of Marlowe et. al. (8) that three species of Isochrysis contained C37 and C39 alkenones, this study now identifies and confirms the presence of C35, C37 and C39 alkenones in bothI. galbana and I. galbana Tahitian strain (Tiso) as the principal lipids in total lipid extracts from cells cultivated in limited nitrogen media.

SECTION 4.0
HYDROCARBONS AND OTHER LIPIDS OF NANNO Q

4.1 OBJECTIVES

TASK IV. Identify the major hydrocarbon and lipid fractions found in the microalga Nanno Q.

The objective was to fully evaluate the fuel potential of this organism by elucidating the total lipid composition and how this composition is altered in cells under different cultivation conditions. The data reported here was obtained from nitrogen sufficient and nitrogen deficient Nanno Q cultures cultivated at Georgia Tech. (Samples submitted by R. Lewin are reported in Section 6).

4.2 METHODS

4.2.1 Organisms, cultivation, and Labelling

The organism was obtained from the culture collection of Dr. R. Lewin, Scripps Institute, La Jolla California. The culture medium for Nitrogen sufficient cultures was standard GPM. For nitrogen deficient cultures the GPM media contained only 1/10 of the nitrogen as KNO_3. (See Appendix). For the cultures to be supplemented with ^{32}P-phosphate or ^{35}S-sulfate, the culture vessels were 2L Fernback flasks containing 500 ml of medium. In either case, the inoculum was 100 ml of a nitrogen sufficient culture. Immediately after inoculation the cultures were supplemented with 72μ Ci of ^{32}P-H_3PO_4 (ICN Biochemicals, 28,215 Ci/mmol) or 1.5 mCi of ^{35}S-H_2SO_4 (ICN Biochemicals, 1,505 Ci/mmol). The flasks were incubated at 25°C on an orbital shaker at 60 rpm under a bank of a mixture of Sylvania Cool White and Westinghouse Agro-Lite fluorescent lamps. The average intensity of the photosynthetically active illumination (400 to 700 nm) reaching the flasks was 62 microeinsteins $sec^{-1}m^{-2}$, as measured with a LI-COR LI-1905 Quantum Sensor. After 5 days incubation, the cells were harvested by centrifugation at 2,000 x g for 15 min at 20°C and stored at -20°C.

Nitrogen sufficient cells were grown in 500 ml Erlenmeyer flasks containing 250 ml of GPM medium with one-half the above NaH^1CO_3 concentration, i.e., 5mM. The inoculum was 50 ml of a Nanno Q culture grown under nitrogen sufficiency. Aliquots of $NaH^{14}CO_3$ (20μ Ci, specific radioactivity of 8.4 mCi/mmol) were added at the time of inoculation, after 2 days and after 4 days incubation. The Erlenmeyer flasks were stoppered so as to minimize the exchange of ^{14}C-bicarbonate in the medium with the atmospheric $^{12}CO_2$. A stream of filtered air was used to flush out the atmosphere from the head space twice daily. During two of these periods the CO_2 in the spent atmosphere was trapped by bubbling through 1 ml of hyamine hydroxide(1M in methanol). Aliquots (0.2 ml) of the hyamine hydroxide were diluted with 0.8 ml of methanol and counted in Cocktail T (see below) in a scintillation counter. It was concluded that the rate of exchange was negligible since the atmospheric CO_2 contained less than 0.1% of the total ^{14}C in the medium. The cultures were incubated at 25°C on a reciprocating shaker (72 reciprocations/min) mounted in a fume hood with a bank of Cool/White and Agro-Lite fluorescent lamps to the side of the shaker. The average intensities of the photosynthetically active illumination reaching the near side and far side of the flasks were 135 and 62 microeinsteins sec^{-1} m^{-2}, respectively. After 6 days incubation, the cells were harvested and stored as described above.

Nitrogen deficient cultures were grown in modified GPM prepared with 0.2mM KNO_3 rather than the 2mM concentration for nitrogen sufficient cultures. This nitrate level is increased by an undetermined amount due to the carry over from the 20% inoculum of nitrogen sufficient culture. Cells cultured in this medium appear dark green for the first 4 days incubation and thereafter become increasingly yellow. Near the end of a typical 10 day incubation the culture is creamy yellow with a faint green tint. For the ^{14}C-labelled preparations of nitrogen deficient cells, the incubation conditions were similar to those for ^{14}C-labelled, nitrogen sufficient cells except for differences in the times at which the $NaH^{14}CO_3$ was introduced and in the extended incubation period used. For preparations with the label present throughout, 20 μCi aliquots of $NaH^{14}CO_3$ were added at the time of inoculation, after 4 days and after 7 days of incubation. For the preparations with the label present after the shift to nitrogen deficiency, 20 Ci aliquots of $NaH^{14}CO_3$ were added after 5 days and after 7 days incubation. In both cases the cells were harvested after 10 days incubation and stored as above.

4.2.2 Lipid Analysis

Cell suspensions were extracted by the method of Bligh and Dyer modified as described previously. (9,10) The lipids were separated by column chromatography (11) with hexane, benzene, chloroform, acetone and methanol to improve the resolution of the lipid components by thin-layer chromatography (t.l.c.). The lipid components were isolated from chromatograms by elution with the appropriate solvents.

Lipid components were deacylated by mild alkaline methanolysis according to Tornabene and Oge (12). Fatty acids were recovered from the chloroform layer.

Water-soluble products obtained from alkaline hydrolysis of lipid components were hydrolysed with 2M HCl at 100°C for 1 h. (12). The hydrolysates were taken just to dryness in a stream of nitrogen and then dissolved in methanol - water (10:9).

Total and column-fractionated lipids, as well as hydrolysed lipids, were studied by t.l.c. on 20 cm x 20 cm glass plates coated (0.6-1mm layer) with Stahl silica gel G and on Sulpeco precoated plates. Chromatography was carried out in lined jars by the ascending method using the following solvent mixtures: (a) diethyl ether-benzene-ethanol-acetic acid (40:50:2:0.2) as first solvent and hexane-diethyl ether(96:4) as second solvent for separating nonpolar lipids (13); (b) chloroform-acetone-methanol-acetic acid-water (50:20:10:10:5) for separation of polar lipids (14) and; (c) technical chloroform (0.75% ethanol) for separation of alkyl-lipid chains. Spots were visualized by exposure to iodine vapour or acid charring, or by specifically staining for phosphates, amines or lecithin (15).

The deacylated water-soluble products were separated by t.l.c. on cellulose (Eastman chromatograms 6064, Rochester, NY, USA) with solvents of 3.8 mM EDTA and 0.7M NH_4HCO_3 in 90 mM NH_4OH containing 67% by vol. ethanol in the first dimension and isobutyric acid-water-conc. ammonium hydroxide (66:33:1) in the second dimension, as previously described (16). The compounds were detected by the O-tolidine staining method (12) and then over-stained with an acid ammonium molybdate solution (17).

Fatty acid methyl esters were prepared by esterification with anhydrous 2.5% methanolic hydrogen chloride. Hydroxylated nonsaponifiable lipids and the water-soluble compounds in acid and alkaline hydrolysates were reduced with sodium borohydride and derivatized by acetylation with acetic anhydride (18) or by silylation with N,O-bis(trimethylsilyl) acetamide (BSA). Glycerol was determined by periodic oxidation followed by treatment with acetgon acetyl (19).

Derivatized lipid and water-soluble compounds were analyzed on a Varian 3700 gas-liquid chromatograph equipped with dual FID's and a Vista 402 data system. Chromatographic analyses were carried out with a 30 m x 0.25 mm fused quartz capillary column coated with DB-5 or OV-351. Components were identified by comparing their retention times to those of established standards. Lipid derivatives obtained after treatment with methanolic hydrogen chloride or methanolic sodium hydroxide were fractionated by t.l.c. Isolated components recovered from t.l.c. were then analyzed by g.l.c.

The radioactivity in the fractions from the ^{32}P, and ^{35}S- and ^{14}C-labelled cells was determined by the following procedures. For the methanol: water fractions, 10 aliquots were added to 20 ml scintillation vials containing 1 ml of water, 1 ml of methylcellosolve and 10 ml of Cocktail D made of scintanalyzed dioxane, 10% (wt./vol.) scintanalyzed naphthalene and 0.5% (wt.vol.) diphenyloxazole (PPO)). The cell pellets remaining after extraction were resuspended in 10 ml of water and 10 aliquots were added to the water: methylcellosolve: Cocktail D mixture. For all other fractions, 10µL aliquots were added to 10 ml of Cocktail T made of 0.5% (wt./vol.) PPO in scintanalyzed toluene. Samples were counted in a Beckman LS-100C scintillation counter for periods sufficient to attain a 2σ error of less than 0.5%. The counting efficiencies ranged from 80 to 85% for all three isotopes. The use of the small volumes of the fractions eliminated the need for quench controls or corrections. The reported values for ^{32}P- and ^{35}S-fractions have been corrected for the decay which had occurred since time zero of the extraction procedure.

4.3 RESULTS

4.3.1 Total Lipids

Nitrogen sufficient and nitrogen deficient cultures of Nanno Q consistently accumulated lipids that accounted for 20-28% and 40-50% of the cell dry weight, respectively. The fractionation of ^{14}C-lipids on silicic acid columns is given in Table 4.1. The major proportion of lipids in nitrogen sufficient cells was eluted with acetone while the major proportion of lipids in nitrogen deficient cells was eluted with benzene. These data clearly demonstrated a metabolic shift from the biosynthesis of polar lipids in nitrogen sufficient cells to neutral lipid biosynthesis in nitrogen deficient cells. Accordingly, the phosphate lipids of nitrogen deficient cells that eluted with methanol are reduced to one-half of the percentage found in the methanol eluate fractionated from the total lipids of nitrogen sufficient cells. The relative distributions of phospho- and sulfo-lipids in the total lipids from nitrogen sufficient cells that eluted from silicic acid columns is given in Table 4.2. The relative proportions of the lipid components from nitrogen sufficient cells is given in Tables 4.3 and 4.4. The distribution of lipids found are somewhat typical of other algal cells studied in this laboratory (20-22). The exceptions were the relatively higher proportion of lipids eluting in the hexane fraction and the detection of 8 sulfolipids. The sulfolipids, however, accounted for less than 6% of the total lipids (Table 4.4).

Table 4.1. Distribution of ^{14}C in Lipid Fractions Eluted from Silicic Acid Columns Using Solvents of Increasing Polarity

Nitrogen Availibility in culture	$NaH^{14}CO_3$ Present	Eluant				
		Hexane	Benzene	Chloroform	Acetone	Methanol
Sufficient	Throughout[a]	3.64×10^5 cpm (1.8%)	7.51×10^5 cpm (3.9%)	3.9×10^5 cpm (2.0%)	1.53×10^7 cpm (78.5%)	2.66×10^6 cpm (13.6%)
Deficient	Throughout[b]	1.2×10^5 cpm (1.8%)	4.94×10^6 cpm (73.9%)	9.9×10^5 cpm (14.8%)	2.3×10^5 cpm (3.4%)	4.0×10^5 cpm (6.0%)
Deficient	After onset[c] of N deficiency	2.2×10^5 cpm (1.3%)	1.25×10^7 cpm (77.5%)	1.73×10^6 cpm (10.8%)	8.2×10^5 cpm (5.1%)	8.5×10^5 cpm (5.3%)

The values in parentheses are the percentages of the eluants based on total recoveries from the columns.

The totals of ^{14}C recovery in the eluants for a, b and c were 95.1, 96.0 and 85.1%, respectively, of the ^{14}C in the lipid extracts applied to the columns.

Table 4.2. Distribution of ^{32}P and ^{35}S in Lipid of Nitrogen Sufficient cells and Fractionated on Silicic Acid Columns Using Solvents of Increasing Polarity

Label in Lipid Fraction	Eluant		
	Chloroform	Acetone	Methanol
^{32}P	3.87×10^2 cpm (0.08%)	4.57×10^4 cpm (9.2%)	4.51×10^5 cpm (90.7%)
^{32}S	5.25×10^3 cpm (0.6%)	8.36×10^4 cpm (9.5%)	7.92×10^5 cpm (89.9%)

The values in parenthesis are the percentages of the eluants based on the total recoveries from the columns.

The totals of ^{34}P and ^{35}S recovery in the eluants for the ^{32}P-lipids and ^{35}S-lipids were 85.5 and 95.0%, respectively, of the labelled lipid extracts applied to the columns.

TABLE 4.3

NEUTRAL LIPID COMPONENTS OF NANNO Q IN NITROGEN SUFFICIENT MEDIUM

Eluates from Silicic Acid Column

Rf values	Hex.	Benz.	CHCl-3	Acet.	MeOH	Probable Identity
0.88	0.8	--	--	--	--	Hydrocarbon
0.79	--	0.6	--	--	--	Steryl Ester
0.68	--	0.4	0.4	--	--	Ketone
0.63	--	0.5	0.6	--	--	Methyl ester
0.59	--	--	0.4	0.5	--	TG/Pigment
0.50	--	--	--	0.8	--	pigment
0.48	--	--	--	0.5	--	pigment
0.47	--	--	--	0.6	--	pigment
0.46	--	--	0.8	1.6	--	1,3-DG/Pig.
0.42	--	--	--	0.4	--	pigment
0.40	--	--	0.2	--	--	1,2-DG
0.39	--	--	0.9	--	--	FFA + Sterol
0.30	--	--	--	--	--	Pigment
0.25	--	--	--	--	--	Pigment
0.18	--	--	0.1	--	--	MG
0.00	--	--	--	75.7	13.6	Polar Lipids

Data obtained from t.l.c. developed with neutral lipid solvent A. Rf values for authentic lipids were found to be eicosane, 0.88; cholesteryl oleate, 0.79; methyl stearate, 0.69; tripalmitin, 0.50; 1,3-dipalmitin, 0.45; 1,2-dipalmitin, 0.41; mydristic acid, 0.39; monopalmitin, 0.17. Values expressed as relative percentages of the toatal lipids, were determined with a recording Zeineh soft laser scanning densitometer of autoradiograms of 14-C labelled lipids.

TABLE 4.4

POLAR LIPID COMPONENTS OF NANNO Q IN NITROGEN SUFFICIENT MEDIUM

	Eluates from Silicic Acid Column			
Rf values	Hex., Benz. CHCl-3	Acetone	Methanol	Probable Identities
0.96	2.3	43.0	0.1	NL/Pigment
0.89	--	8.8	2.1	MGD/Pigment
0.80	--	1.7	0.8	PL/Pigment
0.76	--	0.7	2.1	PA/Pigment
0.71	--	0.9	0.7	SL/Pigment
0.69	--	1.0	0.4	DPG/Pigment
0.61	--	0.8	0.8	SL/Pigment
0.56	--	1.1	4.1	PE/Pigment
0.55	--	1.6	0.8	SL/Pigment
0.46	--	3.4	2.0	DGD
0.41	--	0.3	0.9	SL/Pigment
0.40	--	1.3	5.1	PG
0.29	--	1.0	0.6	PL/Pigment
0.27	--	1.2	1.7	SL/Pigment
0.23	--	1.0	2.0	PC/Pigment
0.17	--	0.1	0.7	PL/Pigment
0.16	--	0.1	0.2	SL/Pigment
0.11	--	0.1	0.1	SL/Pigment
0.10	--	0.5	0.1	PI/Pigment
0.06	--	0.6	0.1	SL/Pigment

Data obtained from t.l.c. developed with polar lipid solvent B. Rf values for authentic lipids were: tripalmitin, 0.96; monogalactosyl diglyceride, 0.88; phosphatidic acid, 0.76; steryl glycoside, 0.66; diphosphatidylglycerol, 0.91; phosphatidylethanolamine, 0.56; digalactosyl diglyceride, 0.46; phosphatidylglycerol, 0.40; phosphatidylcholine, 0.23; phosphatidyl inositol, 0.10. Values expressed as relative percentages of the total lipids were determined with a recording Zeineh soft laser densitometer of radioautograms of 14-C 35-C and 32-P labelled lipids.

The proportions of acyclic, saturated, nonisoprenoid type hydrocarbons in the total lipids from both nitrogen sufficient and deficient cultures that eluted in the hexane eluates are unchanged. These data are consistent with those found in bacteria (23). These types of hydrocarbons were proposed as being secondary-byproducts of the cellular metabolic system; they are not metabolically reusable and, they are not synthesized in appreciable quantitites, i.e. less than 1% in algae, bacteria, and fungi, with Botryococcus the exception (approx. 17%).

The analytical data are presented according to the sequence of elution from the silicic acid column.

4.3.2 Lipids from Nitrogen Sufficient Nanno Q

Hexane Eluate. Eight pre-dominant components were detected in the hexane eluate that comprised about 1.8% of the total lipids in both nitrogen deficient and sufficient cells. These hydrocarbons constitute between 0.6 to 0.8% of the cell dry weight of nitrogen deficient cells, a production quantity that is surpassed only by the green alga Botryococcus. The tentative identity of the hydrocarbons are given in Table 4.5. The samples have not yet been analyzed by mass spectroscopy for definitive identification. These compounds were tentatively identified as isobranched even-carbon-numbered saturates and unsaturates in the range of C-24 and C-30, inclusively. All unsaturates were tentatively identified as diunsaturates; however, the percentage increase in hydrogenated samples (Table 4.5) indicated that other unsaturates must be present but in relatively small quantities. The definitive identification of these compounds will be conducted as soon as the mass spectrometer and NMR laboratories are installed in the Department which is now scheduled for January, 1986.

Benzene Eluate. The benzene eluate represented only 4% of the total lipid fractions (Table 4.1). The principal component was identified as sterol esters (Table 4.3). Two additional components were detected which had Rf values that corresponded to alcohols or ketones. The determination of the exact identities of these components will be made when the analytical instrumentation is installed.

Chloroform Eluate. The components eluted in the fraction represented about 2% of the total lipids (Table 4.1). Small amounts of ketones, alcohols, triglycerides and diglycerides were tentatively identified (Table. 4.3).

Acetone Eluate. This fraction contained 3/4 of the total lipids (Table 4.1). Approximately 1/2 of the lipids eluted with acetone belonged to the neutral lipid class and consisted of predominantly pigmented compounds and triglycerides (Table 3). The polar lipids were mainly monogalactosyl and digalactosyl diglyceride. Two sulfolipids were detected in very small quantities by ^{35}-S tracer studies (Table 4.4). These two sulfolipids are different from the 8 sulfolipids detected in the methanol eluate. Purification of the sulfolipid for chemical analyses is in process.

Methanol Eluate. The lipids are predominantly phospholipids representing about 14% of the total lipid composition (Table 4.1). Also present in relatively small quantitites are at least 8 sulfolipids (Table 4.4). The phospho- and sulfo-lipids were studied by differential staining procedures and by deacylation and two dimensional thin-layer chromatography. The major phospholipids were identified as phosphatidyl glycerol, diphosphatidyl glycerol, phosphatidyl ethanolamine, phospha-

tidyl choline and phosphatidyl inositol. Four of the phospholipids did not correspond to authentic lipid standards and could not be assigned a tentative identification (Table 4.4). The results from deacylation and two dimensional chromatography confirmed the findings from the one dimensional chromatographic analyses that the four phospholipids labelled with P-32 were not homologs of other phospholipids and do not correspond to classical standards, and there are no compounds with both sulfur and phosphorus moieties. Each of the unknown compounds will have to be purified before compositional studies can be conducted.

The polar lipids constitute a complex mixture of pigments, glycolipids, sulfolipids and phospholipids. To complete the analyses of these components is a long-term project.

4.3.3 Lipids from Nitrogen Deficient Nanno Q

Hexane Eluate. The quantities of the components eluted are given in Table 4.5. While these data were obtained from radiolabelling studies conducted in closed flask systems, the data are essentially the same as that obtained for cells cultivated in the aerated Roux bottles described in Section 2. The identities of the hydrocarbons are the same (Table 4.5) regardless of the cultivation design or the metabolic stress state of the cells. This indicated that the hydrocarbon biosynthetic pathway was an essential part of the organisms cellular metabolism. In addition, the ratio of hydrocarbons to the other lipids remained fairly constant indicating that a tight regulatory and integrated mechanism was involved in the biosynthesis of these hydrocarbons.

Benzene Eluate. The quantities of the components eluted (Table 4.6) accounted for about 75% of the total lipids. This represented a 19 fold increase over the amount of lipids found in the benzene eluate of nitrogen sufficient cells. The principal lipid that accounted for 65% of the total lipids was identified as triglycerides (Table 4.6). This conclusion comes from comparing its Rf value to standards and by eluting the compound from the t.l.c. and hydrolysis and analysis of the glycerol to fatty acid ratio (1:3). Relatively significant amounts of steryl esters (6.8%) and methyl esters (2.1%) were also identified (Table 4.6). Additional triglycerides were present in the chloroform and acetone eluates. The sum of the triglycerides accounted for almost 80% of the total lipids (Table 4.6). The basis for the separation of the three classes of triglycerides is unexplainable. The only chemical differences are the nature of the fatty acids esterified to the glycerol. The triglycerides in the benzene eluate are predominantly saturated with 14:0, 16:0, and 18:0 fatty acids with 16:1 and 18:3 being relatively minor ones. The triglycerides in the chloroform and acetone eluates have a higher proportion of unsaturated and C-20 fatty acids and no 18:3 (Table 4.7).

Chloroform Eluate. One predominant component was detected that accounted for 13% of the total lipids (Table 4.6). It was identified as a triglyceride. The fatty acid content is similar to that identified in the benzene eluate except for the absence of 18:3 and the presence of C-20 acids (Table 4.7). Relatively small quantities of diglycerides and sterols were also detected.

Table 4.5 Composition of Acyclic Hydrocarbons

Compound Identity	Relative Percent		Retention Time
	Unhydrogenated	Hydrogenated	
iC24:2	1.8	---	25.9
iC24:0	4.0	5.6	26.3
iC26:2	8.5	---	29.3
iC26:0	19.6	32.7	29.8
iC28:2	27.0	---	31.5
iC28:0	24:3	55.5	32.5
iC320:2	6.8	---	33.8
iC30:0	7.5	6.8	34.9

Hydrocarbons were analyzed by GLC with a 96 m x 0.3 um stainless steel capillary column coated with OV-17 or a 300 m x 0.025 mm fused quartz capillary column coated with DB-5. (i= isobranching configuration)

Table 4.6 Characteristics of Neutral Lipid Components of Nanno Q Cultivated in Nitrogen Deficient Medium

Average RF Values	Percentage of Total Lipid in the Column Eluates					Probable Identification of Compound
	Hexane	Benzene	Chloroform	Acetone	Methanol	
0.89	2.0	---	---	---	---	Hydrocarbons
0.69	---	6.8	---	---	---	Steryl esters
0.55	---	---	---	---	---	Ketone
0.52	---	2.1	---	---	---	Methyl ester
0.41	---	65.0	12.9	0.9	---	Triglyceride
0.39	---	---	---	0.1	---	Pigment
0.36	---	---	---	0.1	---	Pigment
0.34	---	---	---	---	---	1,3 diglyceride
0.31	---	---	1.0	0.1	---	1,2 diglyceride
0.25	---	---	---	1.6	---	Free Fatty Acid
0.23	---	---	0.5	0.1	---	Sterol
0.21	---	---	---	0.1	---	Pigment
0.11	---	---	---	0.1	---	Monoglyceride
0.08	---	---	---	0.1	---	Pigment
0.00	---	---	--	1.0	6.0	Polar Lipids

Data obtained from superlco precoated t.l.c. plates developed with neutral solvent A. Rf val were obtained by comparing to the Rf values of authentic standards and by differential stair and by isolation of the component by elution from the chromatogram and chemical analysis.

TABLE 4.7

Relative Percent
Composition of Fatty Acids of Three Triglyceride Fractions
Obtained in Eluates Collected from a Silicic Acid Column

Identites of Fatty Acids	Relative Retention Time in Min	Eluants		
		Benzene	Chloroform	Acetone
14:0	16.4	9.4	9.7	9.2
16:1	19.9	7.0	30.4	25.7
16:0	20.2	63.3	40.3	36.7
18:3	20.3	12.4	---	---
18:1	23.2	---	8.7	9.0
18:0	23.6	---	---	1.1
C20:2	25.9	---	2.3	2.5
20:1	26.0	---	7.7	15.9
20:0	26.8	---	---	---
?	29.1	7.9	---	---

Fatty acids were determined by GLC with a 30m x 0.025 mm fused quartz capillary column coated with DB-5.

TABLE 4.8

Percentage Fatty Acids of Free Lipids of Nanno Q

Identity	Nitrogen Sufficient	Nitrogen Deficient
14:0	0.61	1.3
14:1	0.19	0.3
16:0	21.6	43.4
16:1	26.11	29.6
18:0	0.89	2.2
18:1	10.34	15.6
18:2	0.11	---
18:3	0.64	0.1
20:3	12.44	2.1
20:4	25.67	4.4

See footnote of Table 4.6.

Acetone and Methanol Eluates.

The components eluted in the acetone and methanol fractions of the lipid of nitrogen deficient cells was 25 times and 2 times less, respectively, than that which was found in the same fractions from nitrogen sufficient cells (Tables 4.4 and 4.6). As a result of the major reduction of the pigmentation in these nitrogen deficient cells, there are no predominant components in the acetone eluate. The amounts of phospholipids in the methanol eluate are one-half the amount found in the nitrogen sufficient cells; however, the relative proportion of the principal phospholipids are the same as those reported for nitrogen sufficient cells (Table 4.4).

4.4 TOTAL CELLULAR FATTY ACIDS

The fatty acids of nitrogen deficient and sufficient cultures is summarized in Table 4.8. The fatty acids are even numbered carbon chains in the range from C-14 to C-20. The two preparations differ primarily in the degree of unsaturation. The polyunsaturates are predominant in the nitrogen sufficient cultivated cells and not in the nitrogen deficient ones. The fatty acids of the three triglycerides (Table 4.7) are characteristically similar to the total fatty acids of nitrogen deficient cells (Table 4.8).

4.5 RADIOLABELLING STUDIES OF NITROGEN SUFFICIENT AND DEFICIENT CELLS

Nanno Q was cultivated under nitrogen sufficient and deficient conditions in GPM media containing a supplement of $NaH^{14}CO_3$ at different growth phases. The radiolabel was present throughout the cultivation or after the onset of nitrogen deficient conditions as determined by changes in culture pigmentation from green to yellowish green. The distribution of radiolabel in the lipid classes separated on silicic acid columns were essentially identical in both labelling strategies (Table 4.1). The distribution of ^{14}C in the extracted cells and lipid extracts from the two labelling strategies, however, was significantly different (Table 4.9). Cells provided with ^{14}C throughout the cultivation had about 92% of the assimilated ^{14}C in the lipid extracted cell pellet and only 8% ^{14}C in the total lipid fraction. In contrast, the lipid extracted cell pellet from cells labelled after the onset of nitrogen deficiency contained only 52.4% of the assimilated ^{14}C with 47% in the lipid extract (Table 4.9). These data support the premise that de novo CO_2 fixation can support lipid biosynthesis during N limitation. Thus the bioconversion of other cellular components (e.g. polysaccharides) into lipids may not be involved. The data does demonstrate, however, that substantial amounts of $^{14}CO_2$ are also fixed into nonlipid cellular material during the lipid induced period, with 52% of the label remaining with the lipid extracted cell debris (Table 4.9).

4.6 CONCLUSIONS

Nitrogen starved Nanno Q cells accumulate neutral lipids. The neutral lipids are comprised of acyclic, nonisoprenoid hydrocarbons and triglycerides which comprised 1.5-2% and 70-80% of the total lipids of cellular dry weight, respectively. These lipids, however, are predominantly saturated compounds. These findings are consistant with previous reports that less unsaturated components are synthesized by cells growing in nitrogen-limiting media (21, 24, 25).

Table 4.9. Distribution of ^{14}C in Fractions Obtained By Bligh -Dyer Extraction of Nitrogen Deficient Cells

$NaH^{14}CO_3$ Present	Methanol:Water Phase	Chloroform (Lipid) Phase	Cell Pellet	^{14}C Recovery	Recovery of ^{14}C added to Medium
Throughout[a]	1.1×10^6 cpm (1.1%)	6.9×10^6 cpm (6.6%)	9.6×10^7 (92.3%)	1.04×10^8 cpm	92%
After Onset of Nitrogen Deficiency[b]	3.4×10^6 cpm (7.3%)	1.9×10^7 cpm (40.3%)	2.5×10^7 cpm (52.4%)	4.69×10^7 cpm	62.1%

The values in parentheses are the percentages of the fractions based on total recoveries after extraction.

[a] 20 μ Ci aliquots of $NaH^{14}CO_3$ were added at the time of inoculation, after 2 da incubation and after 4 da incubation; Total = 60 μCi.

[b] 20 μCi aliquots of $NaH^{14}CO_3$ were added after 4 da incubation and after 7 da incubation. Total = 40 μCi.

[c] The extents of ^{14}C-assimilation were estimated by calculating the percentage of the available ^{14}C which was recovered in the above fractions.

The broad range of phospholipids and sulfolipids were those expected in early stationary phase cells or those cultivated in nutritionally deficient media. Active growing cells in an 'unstressed' metabolic state do not demonstrate such diversity but instead contain a predominance of the functional lipids - phosphatidyl ethanolamine/choline, phosphatidyl glycerol, and glycolipids.

The amount of acyclic hydrocarbons produced by this organism was in a relatively constant ratio to the total lipid produced. This is an observation that has been observed for bacteria, fungi and algae,with Botryococcus being the exception (23). It has been proposed by one of us (23) that the hydrocarbons are not synthesized for the purpose of carbon storage and that the pathway is not individually inducible for greater production but the result of a synthetic pathway that is metabolically involved in many biochemical pathways of which fatty acids biosynthesis was the driving force (23).

TASK 5.0
Lipid Analysis of Potentially Promising Strains of Microalgae

5.1 OBJECTIVES

TASK V Culture and chemically characterize 5 potentially promising and taxonomically distinct strains of microalgae obtained from culture collections.

"To complete a taxonomic survey of chemical characteristics of selected microalgae."

Five strains of microalgae that are taxonomically distinct shall be identified and obtained from culture collections. These strains shall be submitted to G.I.T. for culture and the chemical analysis of lipids and carbohydrates. Cells shall be analyzed that have been cultured under nitrogen sufficient and deficient conditions.

5.2 Results and Discussion

The lipid fractionations for the selected strains were reported on in Section 2.0. Table 5.1 summarizes that data by subtracting the lipid content (% AFDW) of each fraction in N (or Si) deficient cultures from that of the sufficient cultures.

For Nanno Q, the results generally agree with those described in the prior section (Section 4), with most of the lipid increase seen in the benzene and, to a lesser extent, chloroform fractions. The decrease in the acetone fraction is rather large. It should, however, be noted that in this comparison an air grown culture (#67), which was probably starting to be N limited, was compared to a severely N limited 1% CO_2 grown culture. (A more direct comparison is not possible due to lack of reliable data for culture #70, the N-limited status of culture #80, and the lack of fractionation data for the last Nanno Q experiment, cultures #141-144).

Cyclotella exhibits the same general pattern as Nanno Q, with most of the "new" lipid induced during N limitation being contained in the benzene fraction. However, no significant increases in the chloroform fraction were seen. Boekelovia had a rather small change in lipid content and, exhibited no major internal shifts in lipids during N limitation.

For two strains, Chaetoceros and Thalassiosira, both N and Si limitation data is available. Chaetoceros exhibited only relatively minor increases in total lipid contents, in both cases (N and Si limitation) the benzene fraction was the one that showed the largest changes. By contrast in the case of Thalassiosira , the N limited cultures increased primarily in the chloroform fractions and the Si limited cultures in the acetone and (to a lesser degree) the benzene fractions. Thus Si and N limitations may well result in a different pattern of lipid biosynthesis.

To obtain a more detailed view of changes in lipid compositions, thin layer chromatography of various lipid fractions was carried out. Fig. 5.1 to 5.3 show plates for Nanno Q, Cyclotella and Chaetoceros. In Cyclotella, polar lipids do not show any significant changes, however, as expected, the benzene fraction neutral lipids exhibit major shifts in lipids when comparing N limited and N sufficient lipids. Although some overloading of the plate is apparent, this shift is more then just an overall increase in lipids in the N limited cultures, but represents a change in their relative proportion and, possibly, even a decrease in some of the lipids. In the chloroform fraction there is a new major peak in the N limited culture, although the lipid content actually decreased for this fraction (compared to the N sufficient cultures). Even in Chaetoceros, where not much net change in lipids is observed - the lipid pattern of the neutral fractions is distinctly different between the N sufficient and deficient cultures. These observations support the prior conclusions that N limitation has a profound effect not only on the rate of lipid biosynthesis, but - perhaps most important - on the biochemical pathways involved. Identification of the products of these pathways must still be completed.

Table 5.1 Changes in Lipid Composition as a Result of N or Si Limitation (as % of AFDW)

Organism:	Cyclotella	Chaetaceros		Boekolovia	Thalassiosira		Nanno Q
Nutrient: Solvent	N	N	Si	N	N	Si	N
Hexane	0	0.1	-0.5	+0.5	-0.4	-0.3	+1.8
Benzene	+21	+6.7	+0.3	+0.3	-0.1	+4.2	+31.5
Chloroform	-3.1	+21	-0.8	-0.8	+12.8	+1.2	+7.7
Acetone	-3.5	-4.8	+2.5	+2.5	+3.7	+7.9	-12.4
Methanol	1.4	-0.9	-1.5	-1.5	-0.1	+0.5	-2.2
Total	+15.3	+3.2	+8.8	+8.8	+16.1	+13.5	+25.6
Cultures	102-99	123-125	150		148-145		83-67

Figure 5.1 TLC Scans of Cyclotella Lipids

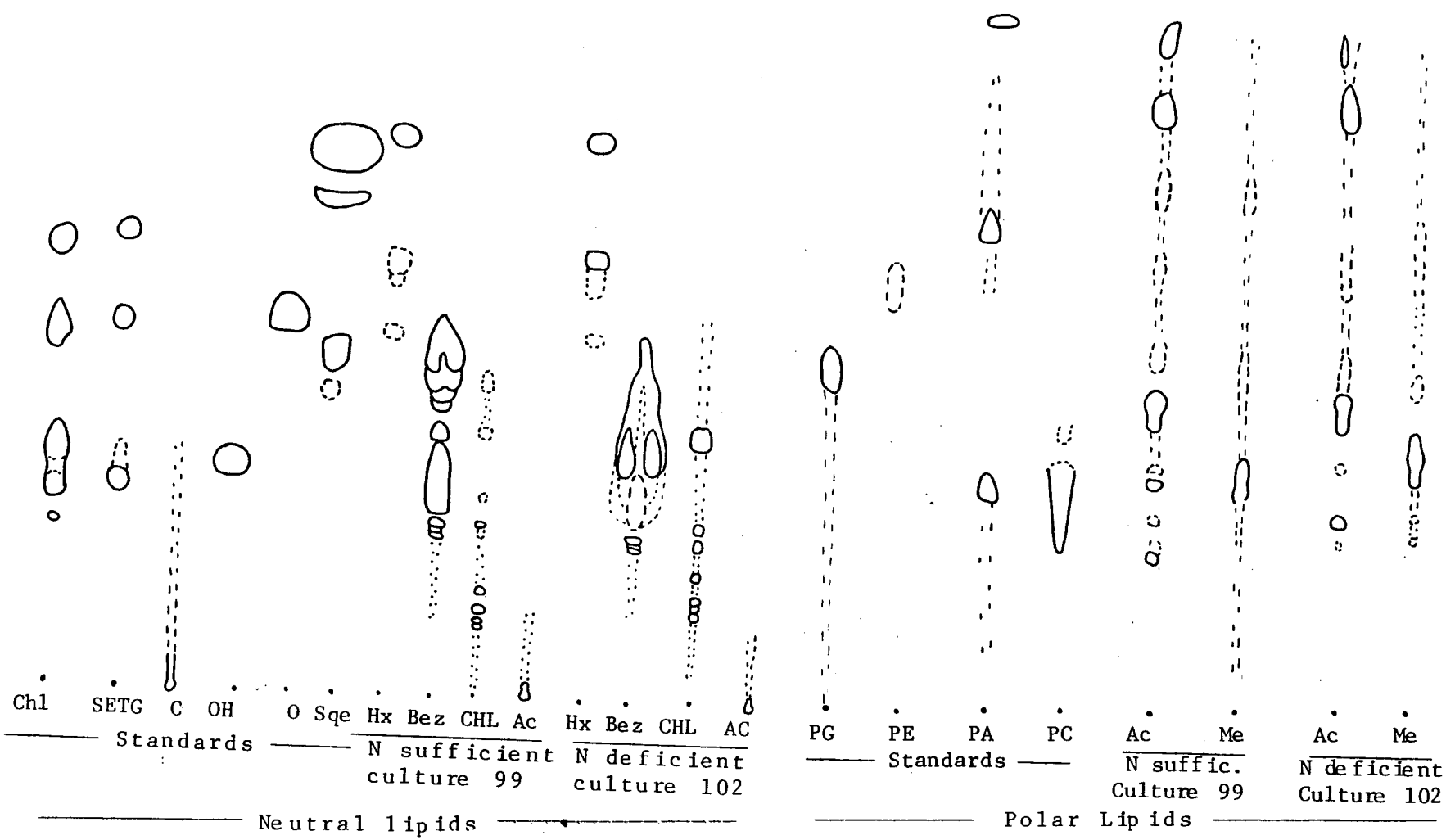


Figure 5.2 TLC Scans of Chaetoceros Lipids

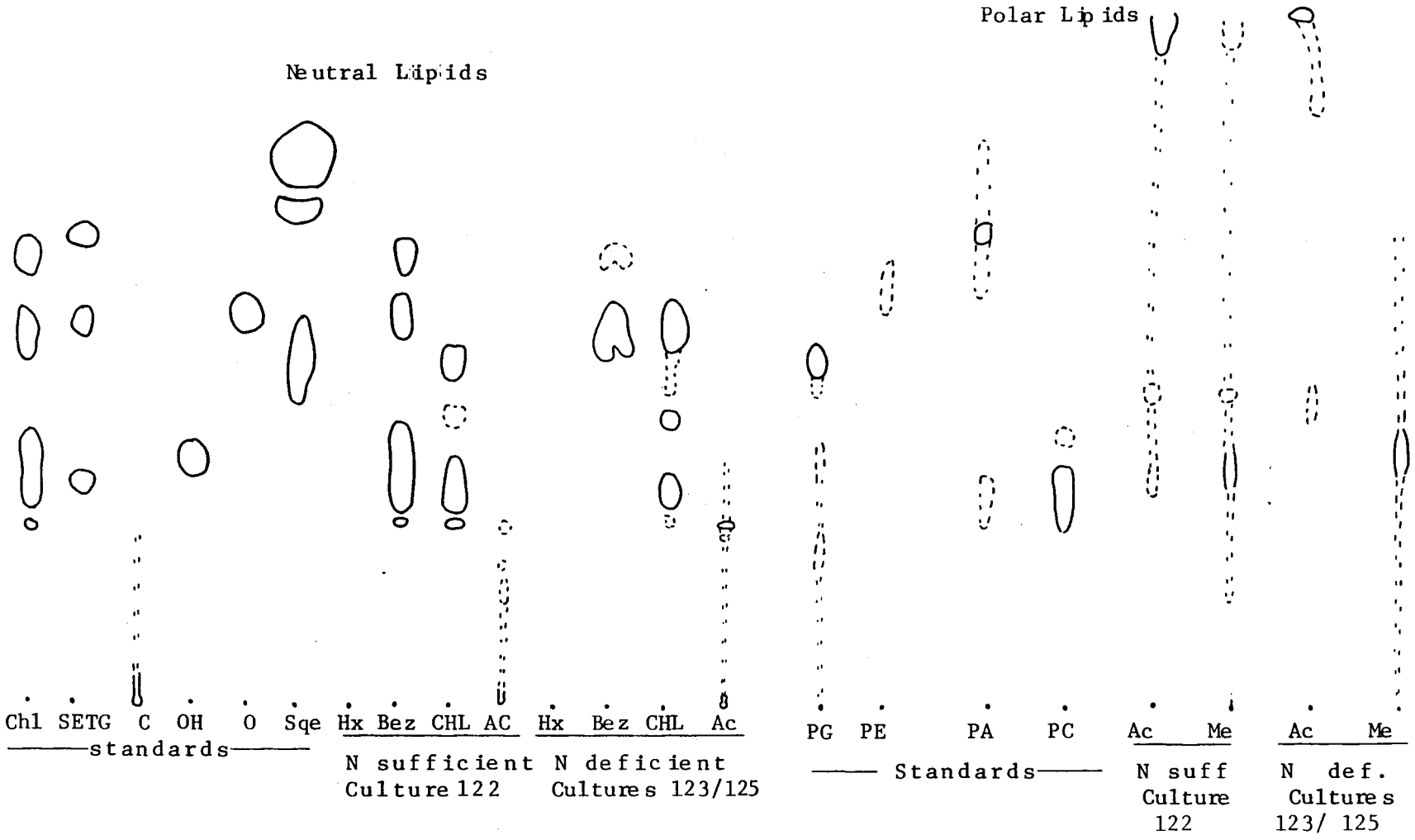
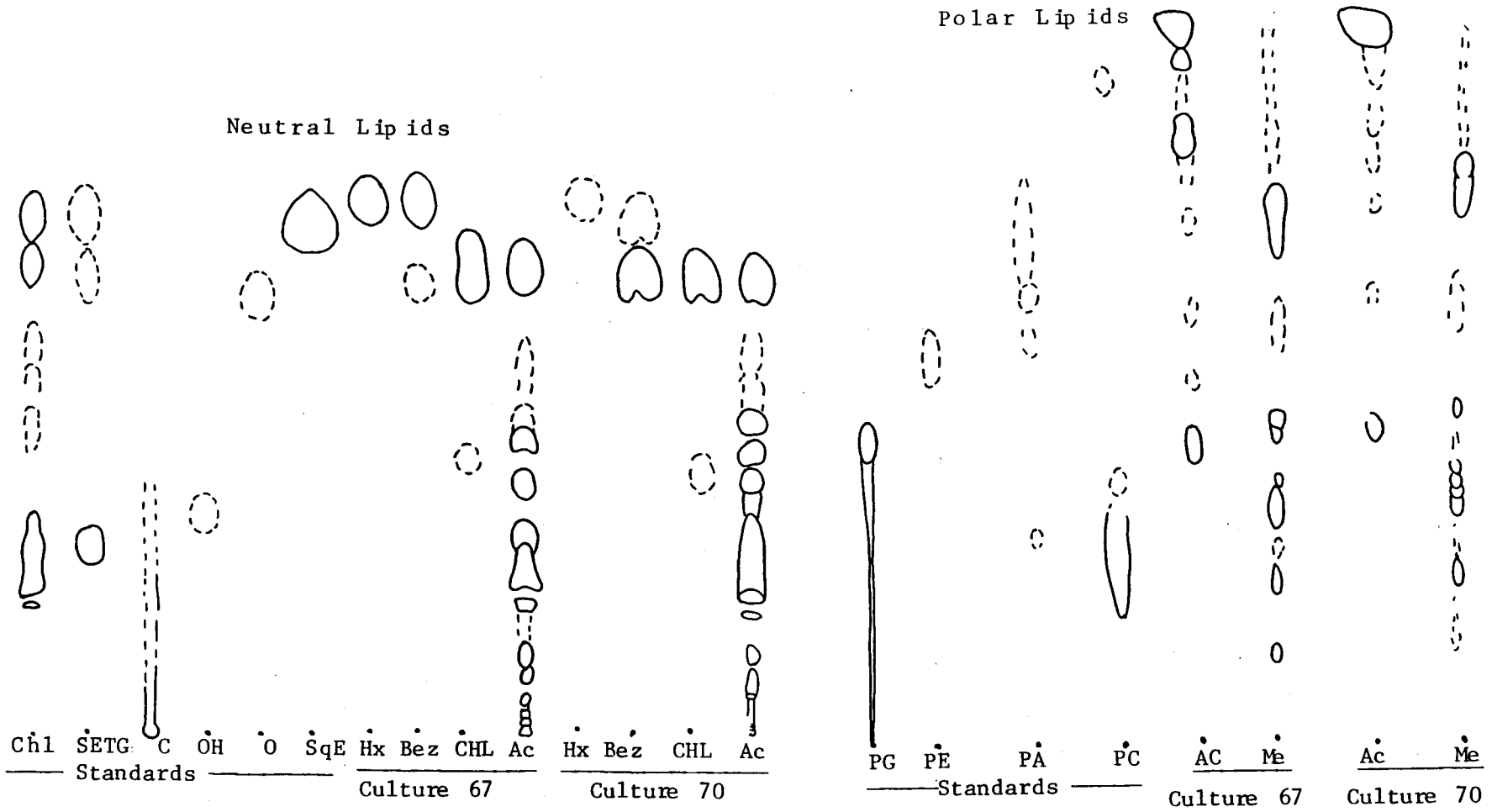


Figure 5.3 TLC Scans of Nanno Q Lipids



Section 6.0
ANALYSIS OF SAMPLES SUBMITTED BY OTHER INVESTIGATORS

6.1 Objectives

TASK VI - Determine and quantify the chemical composition of samples of micro or macroalgae submitted by other investigators working in the program.

"Identify those species with the most promising product potential. This activity supports other screening processes that will determine the best species for culture."

A total of 52 samples, two each (one N sufficient and one deficient) of twenty microalgae and six macroalgae were to be analyzed for lipids, protein, carbohydrate and lipid fractions, and C_5-C_6 sugar contents and identity (for macroalgae). Both the type and quantity of samples submitted by other investigators during this project varied from that originally projected.

6.2 RESULTS AND DISCUSSION

6.2.1 Samples Submitted for Scripps Institute

Four samples were received for W. Thomas' laboratory on February 10, 1985, two of Chlorella ellipsoidea and two of Nannochloris sp. All of the C. ellipsoidea and Nannochloris sp. samples were greenish/black in appearance when they were harvested on December 6, 1984 (nitrogen sufficient) and December 14, 1984 (nitrogen deficient) for Chlorella, and January 17, 1985 (nitrogen sufficient) and February 1, 1985 (nitrogen deficient) for Nannochloris (Thomas personal communications). No significant differences in lipid concentration on a cell dry weight bases were observed (Table 6.1). The C. ellipsoidea and Nannochloris sp. consisted of about 10% and 18% lipids, respectively. In both cases nitrogen deficient cells showed a significantly lower protein value, with a small rise in carbohydrates. Even though nitrogen depletion had been measured in the culture medium by Dr. Thomas, the cultures were apparently not yet in a "metabolic stressed" (nitrogen limited) state. A small quantity of ANZAY (Chamydomonas sp.), isolated from desert soil was received from Ralph Lewin on February 4, 1985. He reported that "it produces spores full of an orange yellow oil." Because of limited sample size, only a chromatograph scan on a thin layer chromatograph was obtained. A pigmented neutral lipid was the principal compound in the lipid extract. Dr. R. Lewin's laboratory submitted two more samples for which ash contents were determined: L019 (V1/25/85, N11) with a water content 11.1% and an ash content of 45.5% (of dry weight) and L018(V1/19/85, N11) with a water content of 10.2% and an ash content of 54.5% (ash contents as % of a dry weight). No further analyses were carried out due to the high ash content and small amount of sample submitted.

6.2.2 Samples Submitted by Harbor Branch Foundation

Ten samples were received on February 18, 1985 from John Ryther's laboratory. The samples were Gracilaria tikvahiaea and Ulva lactuca (N-low), and Gracilaria, Ulva and Caulera prolifera grown at temperature of 10°C (low-temp.), and companion control samples (N-high, Normal temperature). Lipid composition studies of the three macroalgae and detailed carbohydrate analyses of two of the macroalgae had previously been performed. The samples were carbohydrate rich and lipid poor. Results or protein and carbohydrate analysis are shown in Table 6.2.

Table 6.2 Composition of Macroalgae Samples

Sample	Condition	Ash[1] %DW	Pro[2] %AFDW	CHO[2] %AFDW
ULVA	Low N	26.7	12.0	52.7
ULVA	High N	33.1	44.4	36.8
ULVA	Low T	33.4	37.4	44.9
ULVA	Norm T	37.5	25.1	55.0
GRACILARIA	Low T	42.3	22.5	62.7
GRACILARIA	High N	47.7	39.4	42.2
GRACILARIA	Low T	36.9	25.8	56.7
GRACILARIA	Norm T	44.0	34.3	42.3
CAULERPA	Low T	18.4	32.1	58.1
CAULERPA	Norm T	18.2	34.6	54.9

[1]Ash percent includes water and inorganic fractions, % water ranged from 2-6%.

[2]As % ash free dry weight.

Table 6.1 Composition of Samples Submitted by W. J. Thomas

Date Collected	Organism	N Status	% of cell dry weight		
			Prot	CH2O	Lipid
12/6/84	Chlorella	N suff	34.2	20.5	10.9
12/14/84	Chlorella	N def.	26.1	26.3	8.9
1/17/85	Nannochloris	N suff	15.2	28.6	19.7
2/1/85	Nannochloris	N def	9.8	38.1	16.9

6.2.3 Samples Submitted By SERI

Six samples of Boekolvia sp., four of Chlorella BL-6 and three each of Amphora sp., Monoraphidium and Chaetoceros SS-14 were received by this project from SERI. The data collected is presented in Tables 6.3 to 6.7. Comparing these results to those obtained during this project suggests the following conclusions:

1) The Chaetoceros cultures were not severely N limited having a protein and N content between a N sufficient and limited culture, somewhat surprising considering the relatively low $N\text{-}NO_3$ media content indicated;

2) Chlorella BL-6 and Amphora sp. both had a high carbohydrate - but a low lipid content in the N limited samples;

3) The data for Monoraphidium in the absence of protein assays allows no easy conclusion, as N levels are low (1.27% N) suggesting also low protein levels and an unusually low total compositional analysis (appx. 60%);

4) The Boekolovia lipid content was lower than that reported above (Section 2.8)

For the Boekolovia samples TLC scans were also carried out shown in Figures 6.1 and 6.2. Major compounds were identified as triglycerides and a component that has an Rf value that corresponded with long chain alcohols (Fig. 6.1). This component was isolated by preparative thin-layer chromatography. Spectral scans showed absorptions only at 290 nm and 360 nm. The pigments were not visible on the thin-layer plate but developed into a mauve-reddish color when allowed to develop in room light. The component comprising the spot behaved similar to the family of unsaturated ketones isolated from Isochrysis. The principal quantity of lipids were polar lipids that were isolated in the acetone and methanol eluates. These polar fractions were also analyzed by TLC. An example of Sample 1 is shown in Fig. 6.3.

We did not see the lipid degradation pattern observed by Ben-Amotz with his samples at higher pH's. The % lipids found in this laboratory were substantially less than the preliminary findings by the SERI researchers. In our studies, we excluded the amphilic, insoluble, compounds at the MeOH-water/chloroform interphase of the Bligh-Dyer extraction procedure.

Table 6.3 Samples Submitted by SERI

Boekolovia Culture No.	Culture Conditions			Composition	
	N(mM)/Type	Growth State	CO_2/pH	% Ash	% Lipid
1	NA	Exponential	1%	8.22	15.3
2	.5/Urea	NA	1%	6.39	18.0
3	.6/Urea	119hr Stat.	2%/7.9	3.04	18.9
4	.6/Urea	31hr Stat.	air/9.3	7.30	20.7
5	.03/Urea	142hr Stat.	2%/7.9	ND	12.6*
6	.03/Urea	145hr Stat.	air/9.3	ND	12.4*

*Not corrected for ash.

Organism/Culture	mM NO_3	% Ash	Composition (%AFDW)		
			% Prot	% CH_2O	% Lipid
Chaetoceros SS-14					
7/18	2	52.3	31.9	43.0	22.2
7/19a	1	47.9	34.9	36.9	26.1
7/19b	1	45.9	37.1	41.1	23.9
7/22 *	1	35.3	32.2	44.5	19.7
Chlorella BL-6					
7/19	1	24.4	29.7	43.2	14.8
7/22a	2	11.2	3.20	30.7	14.2
7/22f	1	14.2	10.2	50.2	12.2

* %C: 49.90; %H: 7.31; %N: 5.41; %0: 37.38; ΔH: 5.3Kcal/g

Table 6.3 Continued

Organism/Culture	Growth State	%Ash	Composition (% AFDW)		
			Prot.	CH_2O	Lipid
Amphora Sp.					
1	Exponential	44.2	30.1	36.2	4.1
2	Early Stat.	29.1	20.6	70.1	10.2
3	Late Stat.	31.3	17.3	74.9	13.6
Monoraphidium					
1	Exp	41.3	ND	38.5	20.8
2	Early Stat.	51.4	ND	25.5	17.9
3*	Late Stat.	41.1	ND	27.0	25.3

* %C: 43.36; %H: 7.58; %N: 1.27; %O: 47.79; ΔH: 4.4Kcal/g

Figure 6.1 TLC Scans of Boekelovia Samples from SERI

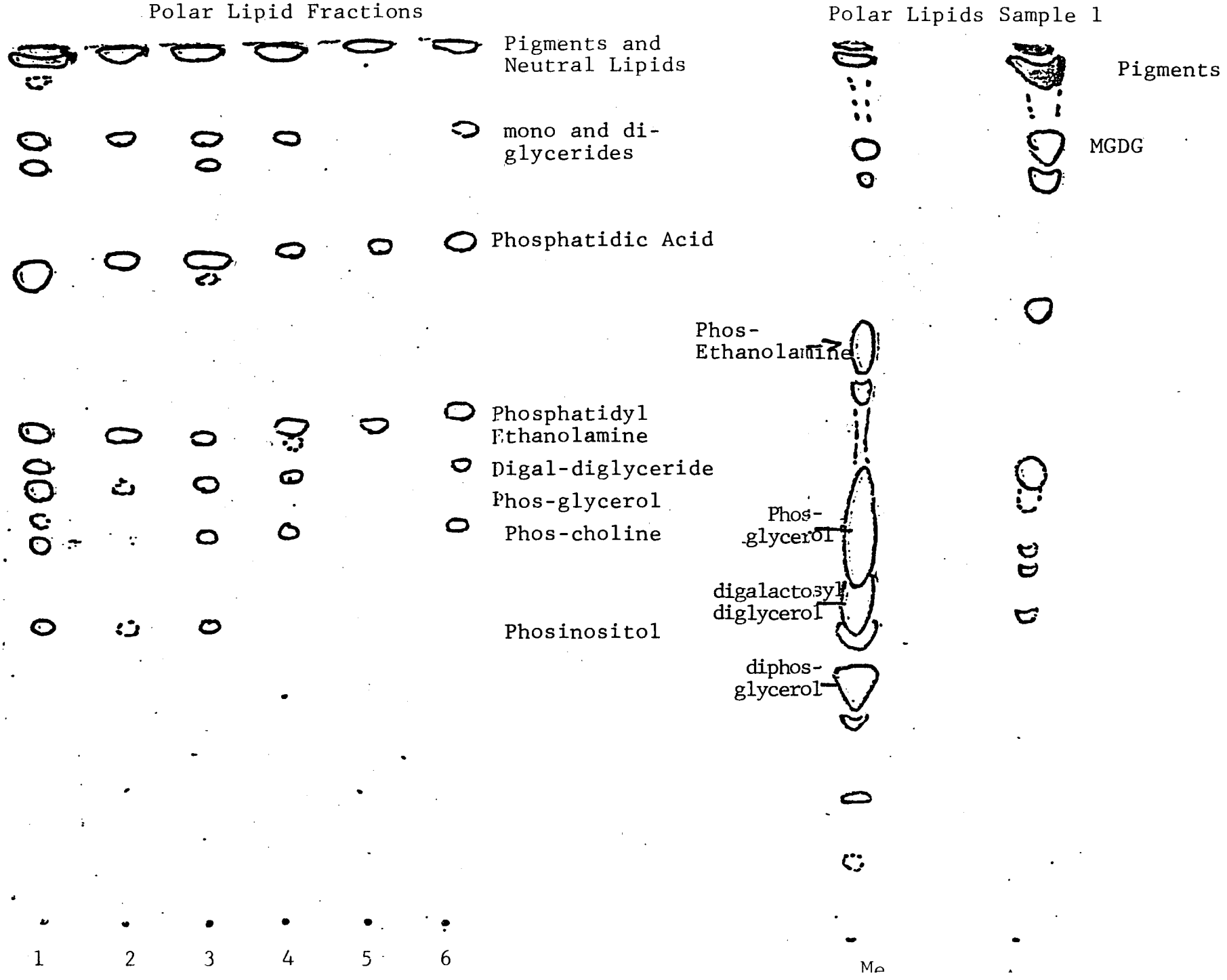
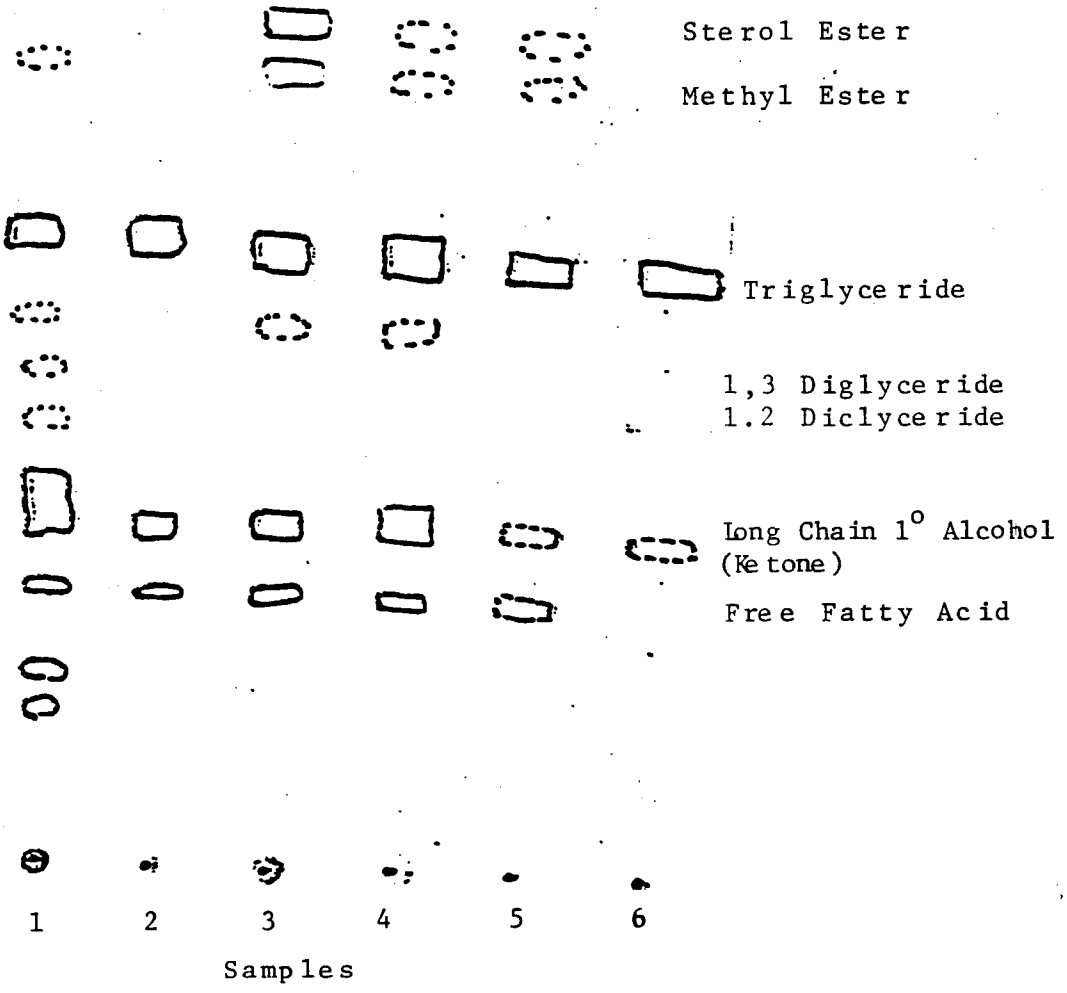


Figure 6.1 Continued



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APPENDIX
METHODS AND MATERIALS

A.1 INTRODUCTION

This section summarizes the general methods used throughout this project not discussed above, and specifically the culturing protocols and analytical methods. Appropriate discussions of the reliability of the techniques are included.

There is a large degree of bias in reporting errors for a single duplication of a result, because of the small sample size. Therefore, a more appropriate estimate of error is to normalize deviations between duplicate analysis and to construct a distribution of normalized duplicates for a given measurement technique. In this way a larger number of individual comparisons can be pooled to give an overall estimate of the reliability of the data. In our analysis, where sufficient sample numbers were available, we have made comparisions of the reproductibility of measurements within a given assay run and between subsequent runs of the same sample. For each section, the normalized (to 100%) mean ranges between estimates and their standard deviations are reported. The standard deviation of the mean corresponds to twice the standard deviation of the assay, or about the 95% confidence limit. Conclusions concerning these results are qualified in terms of sample size and assay treatments.

A.2 MICROALGAE CULTIVATION AND EXPERIMENTAL PROTOCOLS FOR N LIMITATION

Table A.1 lists microalgae strains used in the experiments detailed in this report, their origin and general characteristics. Table A.2 details media compositions. Cultures were maintained as stocks in 125 ml Erlenmeyer Flasks, being transferred every few weeks. Inoculum cultures were grown in Roux bottles under N sufficient conditions at the light intensity and CO_2 partial pressure of the planned experiment (Table 2.2). Then 25 ml of this culture, in linear growth or early stationary phase (culture densities .5-1.5 g/L), were inoculated into 750 mls of sterile N deficient media. Nitrogen nitrate content of the culture was calculated by the amount contained in the inoculum (usually 20 mM) and that added to the media. The final nitrogen concentration was selected to yield the desired cell density at which N limitation was desired. As discussed in Section 2, cell density at the point of N exhaustion can determine the rate of the induction process. A level of 1.61 mM $N\text{-}NO_3$ was used in most experiments because it resulted in cell densitities (500 mg/L) large enough for efficient sampling, but small enough so that light limitation was not seriously reducing growth rate. It should be noted that each organism will be unique in this regard, depending upon its cell quota for nitrogen.

Two protocols were used (in the N limitation experiments: an "initial protocol" (to culture #114) and a "modified protocol" (Section 2). In the initial protocol four Roux bottles were inoculated all at the same N level (usually 1.6mM). In the "modified protocol" four Roux bottles, were also inoculated but only three were N limited, one being N-sufficient (20mM KNO_3). In both cases the cultures were kept in the dark or dim light for either a couple of hours or, more typically, overnight. Light was provided on a 14 hr:10 hr light:dark cycle using a single bank of six fluorescent 40 watt bulbs resulting in an incident intensity of 300 μEinstein/m^2/sec. CO_2 was supplied (as indicated) by gas bubbling at a concentration of 1% (V:V) with air and at a rate of 500-700 cc/min. In addition to gas mixing, each culture was magnetically stirred using a one inch teflon stirring bar. A section of styrofoam (about 1 cm thick) was used for insulation between the mixer and culture. Temperature of the cultures fluctuated between 28.5°C (during the lighted period) and 25 °C in the dark period.

Table A.1 Microalgae Cultures used in this Project

Designation	Genus/Species	Strain	Origin	Date	Me
Cyclotella	Cyclotella sp.		SERI(Tadros)	3/10/85	MS
Chlorella	Chlorella sp.	Black Lake	J. Weissman	5/13, 7/85	MS
Ankistrodesmus	A. Falcatus	"Old"	GIT		AN
Ankistrodesmus	A. Falcatus	"New"	SERI	5/10	AN
Chaetoceros	Chaetoceros sp	Hawaiian	SERI	Various	MS
Nanno Q	Nannochloropsis	QII	Lewin	1984	MS
Isochr sis	I.Galbana	Tahitian	SERI	3/18	MS

Table A 2 Media Used in Algae Growth

Component (5)	MGM Media	Type II Salts	MSW
Rila mix (1)	750 ml/L	---	---
NaCl	---	8078 mg/L	0.40M
Mg SO_4	---	---	0.24M
Mg $Cl_2 \cdot 6H_2O$	---	3026 mg/L	0.20M
$CaCO_2$	---	28 mg/L	0.0005M
KCL	---	965 mg/L	0.01M (optional)
$NaHCO_3$	0.005M	0.01M	0.005M
K_2HPO_4	0.01M	.01M	0.0005M
$Na_2S:O_4$	0.0002M	0.0002M	0.002M
F EDTA (2)	0.0002M	5 mg/L	5 ml/L
Vitamin Mix (3)	1 ml	1 ml	1 ml
Trace Metals (4)	0.5 ml/L		1 ml/L

1) Rila Mix -30gm of salts in 750mls of water
2) Fe EDTA solution 12.68g/L Na_2EDTA, 5.0g/L $FeSO_4 \cdot 7H_2O$
3) Vitamin mix 2mg/L Biotin, 1g/L Thiamine, 1mg/L B_{12}
4) Trace Metals according to Allen and Arnon
5) Nitrogen added as KNO_3 and varied depending upon treatment

Operating under a diurnal light cycle required a consistent sampling schedule. In this work one sample was taken per day, usually up to 5 hours after the daylight cycle had begun. In each experiment sampling was carried out at the same time, ± 30 minutes (usually less). Culture pH and appearance (e.g. color, clumping, wall growth, foaming, etc.) were noted for all cultures at each sampling period. Optical density was measured daily. Dry weight determinations were also made daily on at least one deficient culture (and when applicable, the sufficient) culture. When culture density reached 400-500 mg/L the first data point for protein, carbohydrate and lipid analysis were taken.

In the case of initial protocol this involved harvesting a whole culture. Additional cultures were sampled (harvested) every two days, typically. Harvesting involved centrifuging the cells (10,000 rpm GS32 rotor or equivalent) resuspending the pellet in distilled water and lyophilizing the cells. The dried cells were transferred to a vial and stored frozen. In the modified protocol only 150-200ml were removed, and sampling alternated between the three deficient cultures, while the sufficient culture was sampled every third day. The sampling schedule of the modified protocol allowed up to nine days of daily sampling for lipids, carbohydrates and proteins. Sampling of these cultures involved splitting the sample into subsamples used for dry weight, and carbohydrate and protein samples. These were centrifuged separately.

One drawback of these protocols was the lack of data collected in the very early steps of limitation. Samples taken before any noticeable sign of N deficiency (e.g. change in coloration) already exhibited considerable changes in composition. (See Section 2) An improvement in the method would be to sample somewhat earlier in the growth curve (harvesting a whole culture or limiting the cultures at a slightly higher cell density in order to extend the N sufficient stage of the growth curve).

A.3 ANALYTICAL METHODS

A.3.3 1 Dry Weights

Dry weights were determined by centrifuging ten mls of culture volume at 15,000 RPM for at least 20 minutes (some pellets required longer times). Supernatants were decanted and the tubes were rinsed with distilled water in order to remove residual droplets of media (being careful to avoid the pellet). The pellets were then removed using several small washes of distilled water (1-2 mls each) into pre ashed and preweighed aluminum drying boats. Water was evaporated from the boats over a hot plate, at low temperature (60-70°C) for about 1 hour. The boats were subsequently dried in a forced air convection oven at 103°C for 2 hours, and then ashed in a muffle furnace at 505°C for 30 minutes and weighed again.

The average error of duplicate dry wt. determinations was below 2%. For 54 duplicate samples, randomly chosen, only ten samples exceeded 1% in variance and only three had error between 10 and 20%.

A.3.2. Protein Determinations

Proteins were assayed by the Folin-Lowry Method (1). The procedure involved centrifuging 10 ml samples of cultures, resuspending the pellet in 5 ml of distilled water and digesting 4.5 ml of that volume with 0.5 ml (1NNaOH in a boiling water bath for 1 hour.) This digestion time was found to give the maximum protein value in a time course experiment. After cooling two samples (different volumes) were diluted into 0.5 ml of 1M NaOH, the alkaline Cu SO_4 solution and Folin reagents added sequentially and color read with a Bausch and Lomb spectrophotometer.

Agreement between different measurements (at two dilutions) on the same digestion mixture was tested. For forty-five samples, a mean range of 7.7 ± 5.3% was determined. Errors were randomly distributed (i.e. there was no systematic difference between dilutions). A comparison was made between samples taken from the same digestion mixture but assayed directly and then after a several days storage in a refrigerator. Of twenty-four comparisons 22 were lower with two being slightly higher. The mean range for all points was 18.3± 15%. Thus samples cannot be stored without some loss of apparent protein content.

Finally results performed on a digested pellet were compared with those from a second separately digested pellet from the same culture sample. Of fourteen points available for comparison, 12 were lower than the original assay result with a mean range of 21± 11%, while two were higher by about 10%. However, in this comparison all repeated assays were carried out in a single assay run. Therefore, it is unclear whether the problem rested with pellet storage or problems with that particular assay run (perhaps an error in the standard curve). This issue will require further attention in the future.

In conclusion, it appears that duplication of results for samples taken from the same digest and assayed at the same time involve about ±5% error at the 95% confidence level. It also is apparent that storage of the digests (test tubes with parafilm cover in freezer) is not possible. More data is obviously required to make a conclusion concerning the overall reliability of the protein determination.

Lyophylized samples were also analyzed for protein content. Of 21 samples, the error between duplication runs on the same digestion mixture at different dilutions was ± 7.4% (two standard deviations). Comparisons of protein content as determined from lyophilized samples against those from wet pellets gave a range of 26%± 17% with higher values for the lyophilized cells. This comparison is based only on one assay run and therefore should be qualified as such.

A.3.3 Carbohydrates

Carbohydrates were determined by the phenol-sulfuric acid method (2). The procedure involves centrifuging 10mls of culture; resuspending the pellet in 5 mls of distilled water, and digesting 0.5 mls in 4.5 mls of 1.1 N HCL for one hour at 100°C. Sample volumes are removed from the digestion mixture and diluted into distilled water to 1 ml. One ml of 5% phenol solution (95% H_2O) is added and the test tube mixed. Immediately following this, 5 mls of H_2SO_4 is added and the tube is mixed again with a vortex mixer. Samples are left at room temperature for 20 minutes to develop color and then read against a reagent blank at 488 nm using a Bausch and Lomb "Spect 20". Carbohydrate content is determined by comparing against a glucose standard.

For all samples, two dilutions were used to insure a suitable O.D. reading and to provide an estimation of reproducibility. An evaluation of the normalized range of differences between runs made on the same digestion mixture at different dilutions give a mean of 10± 9.4%. This estimate was based on 23 data pairs of which 9 had deviations of over 20%. A comparison of values determined from separate pellet digestions that were assayed in different sets gave a normalized mean range of 15± 10%. The comparisons involved only 11 points, 5 of which were lower than the original result. Finally a comparison was made between carbohydrate values determined on wet pellets against lyophylized samples. This comparison included 19 points; there was no systematic difference between methods with 9 points giving high values by the lyophylized assay. The normalized range was 15± 12% with 3 points having deviations greater than 20% (2 at 35% and one at 50%) and there were two points that agreed to within 2.8%.

Summarizing, while the errors appear to be associated within and between assays they are unexpectedly large. Errors distributed in such a way are probably associated with experimental technique. Indeed, technical problems with the pipettes used in at least two of the runs could account for this variability.

A.4 Lipid Extraction Methods

Both lyophilized and wet cell pellets were used for lipid analyses. For lipid analyses, 100-200 mg of ash free cell dry weight was used for each analysis; in the quantitation experiments 50-80 mg of dry weight were used. When lyophilized cells were used in the lipid analysis, the cells were allowed to soak in 10 ml of water for about one hour and then broken up as much as possible. To the cells

25 ml of methanol was added, mixed gently, followed by 12.5 ml of chloroform. If phase separation occurred a small amount of methanol was added until the liquid became miscible. The samples were swirled a few times and allowed to sit overnight. The following day the samples were centrifuged at 1000 rpm for 10-15 min. The supernatant was transferred to 250 ml separatory funnels or centrifuge tubes with care taken to transfer as little of the pellet as possible. MeOH:$CHCL_3$:H_2O in a ratio or 10:5:4 was added and the pellet was resuspended. A glass pestel was used to break up any large pellet particles. The sample was allowed to sit for 3 hrs. It was then recentrifuged at 1000 rpm for 15 min. The supernatant was added to those in the separatory funnels (or centrifuge tubes). The MeOH:$CHCL_3$:H_2O ratio was then adjusted to 10:10:9 by adding $CHCL_3$ and H_2O, and the mixtures swirled. The mixture was allowed to separate over night in the separatory funnels or centrifuge. The lower fractions, $CHCL_3$ and lipids, were removed into a round bottom flask . Within the bottles this was accomplished by siphoning off the MeOH-H_2O phase and removing the $CHCl_3$ with a Pasteur pipette. Additional $CHCL_3$ was added to the separatory funnel which was swirled and allowed to separate 2 hrs to wash the partitioning phase. The collected $CHCl_3$ phases were placed on the rotoevaporator and evaporated down to a volume of about one ml. The lipid samples were transferred quantitatively from the round bottom flasks to preweighed weighing flasks using $CHCL_3$. They were then placed in a water bath under a N_2 stream until dry. They were stoppered immediately then transferred to a dessicator at which point the stoppers were cocked slightly. They were then aspirated and vacuum pumped for 15 min. ea. Vacuum was released by pumping in N_2. Once weighed, the samples were resuspended in $CHCL_3$ and transferred to storage tubes under N_2 and then stored in a freezer.

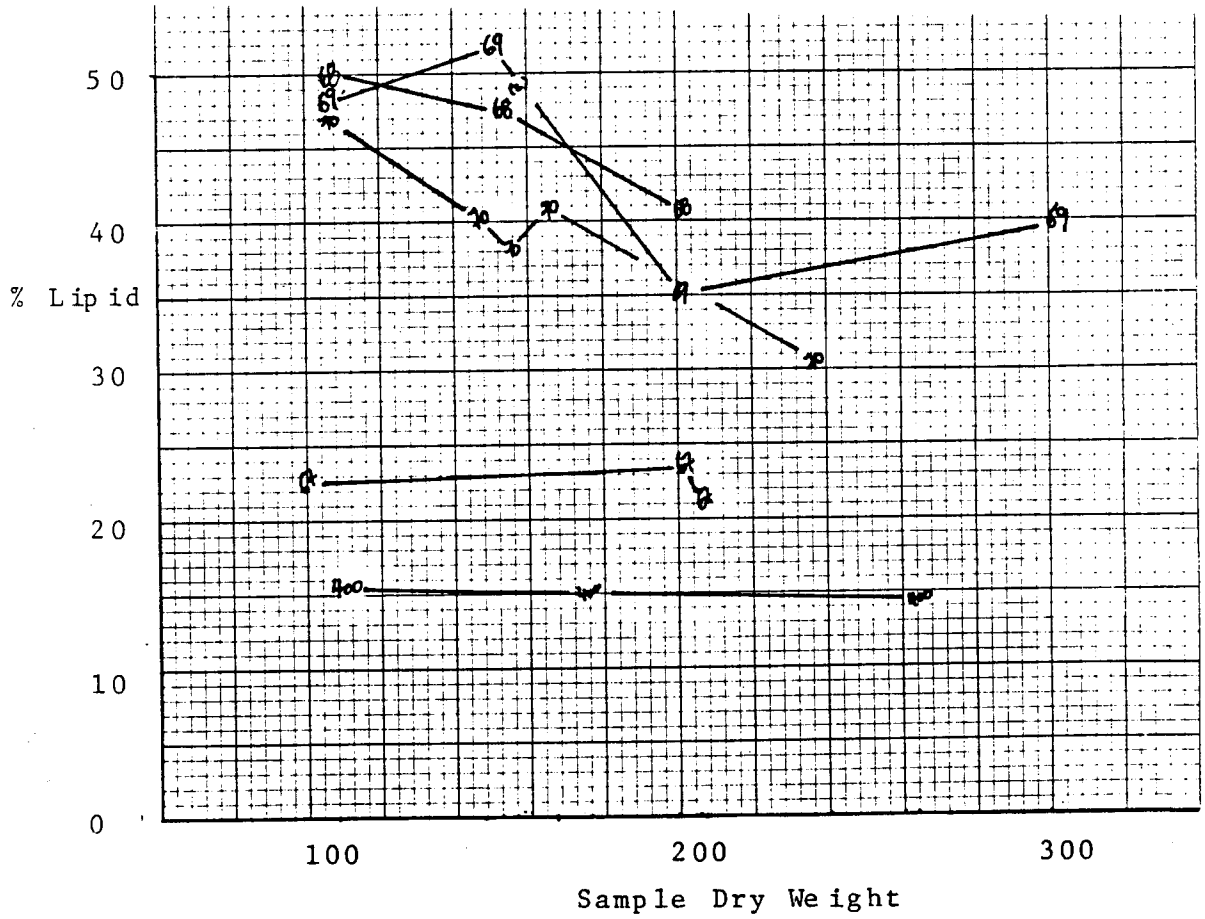
Comparisons were made for separate samples that were taken from the same lyophylized material and assayed at the same time . A total of 13 samples were available (for comparison) and resulted in a random mean range of 4.4 ± 3.3%. A similar comparison of samples taken from the same source, but analyzed at different times resulted in a mean random deviation of 9.8± 9.6%. This comparison included 12 samples; the worst case being a 28% difference for one of the SERI Monoraphidium samples and the best case was an exact (within 3 sig. figures) agreement for Ankistrodesmus culture #121. Although a limited number of samples were available, these comparisons indicate that assay to assay errors are more important than reproductibility within one assay. This analysis indicates that the lipid determinations of lyophilized samples are good to roughly ± 10% at the 95% confidence level. At present, we do not have any duplicate tests for lipid assays performed on wet pellets. Errors introduced from sampling should be relatively insignificant in comparison. There is, of course, other possible sources of error in the protocol itself, some of which

will be discussed in the next section.

In the quantitation experiment with lyophilized cells, the first step in the extraction process involved wetting the samples with water for efficient solvent access. The usual wetting procedure involved allowing the dried sample to sit in a small volume of water for several minutes to an hour with occasional swirling. Other solvents were then added when wetting appeared complete. We tested this procedure against a more vigorous mechanical mixing procedure and a combination of mixing and heating the sample (Table A-3.). There was no difference observed for ei treatment protocol. This is a limited test involving only specific organisms under different conditions. However, in light of the fact that all recent work was carried out on wet pellets, no further analysis was pursued.

During the initial stages of performing lipid analysis on lyophylized samples a discrepancy was noted in apparent lipid content as a function of assay weight. This is shown in Fig.A.1 for Nanno Q cultures 67-70. For the high lipid samples (68-70) there is a marked dependence on sample weight while the low lipid sample (67) showed very little dependence. Also, a healthy Boekolovia culture (#400) was similiarly tested showing very little dependence on assay weight (The Boekolovia lipid values are not corrected for ash, but even if they were lipid content would still be below 20-25%). Further comparisons were made for N sufficient Ankistrodemus and Cyclotella cultures and they are shown in Table A.4. The Ankistrodesmus culture doesn't show any clear dependency while the Cyclotella does. In general, it appears that the higher lipid content samples were extracted less efficiently.
Lipid concentration and cell density is probably not the problem because samples were extracted twice and in one case, up to 4 times (data not shown) with no increase in lipid yield. The fractionation data of Nanno Q cultures 67 and 70 by itself is not sufficient to draw any conclusion about lipid composition in general; however, it appears be possible that as the proportion of neutral lipids increase, the affinity of the bulk lipid for the $CHCL_3$-MeOH-water solvent system declines. Supporting evidence is given in Table A.4. For Nanno Q cultures, pellets were also extracted with petroleum ether after $CHCL_3$ extraction. As noted, there was no difference in lipid yields for extraction done at 100 or 200 mg. Nonpolar lipids are not readily soluble in polar lipid solvents such as $CHCl_3$-MeOH. These cultures had both a high lipid content and a high proportion nonpolar lipids as shown in Section 2. Thus the nonpolar lipids would not be extracted with the usual solvent mixtures employed. Therefore, the only difference between previous data and cultures 68-70 shown in Fig. A.1 is the final extraction step with petroleum ether. Unfortunately, no direct comparison was made between samples extracted with and without petr. ether and, until this is done, no definitive conclusion can be made.

Figure A-1. Percent Lipid as a Function of Sample Weight



Cultures 67 and 70: Nanno Q

Culture 400: Boekolovia

Table A.3 Lipid Analysis as a Function of Treatment

Culture Treatment	Sample mg dry wt.	% Lipid**
Isochrysis		
Std.	303.24	3.94
Std.	632.61	3.86
Mixed & Heated*	314.23	4.11
Mixed & Heated*	610.88	3.33
Ankistrodesmus		
Std.	101.21	23.50
Mixed & Heated*	106.79	22.87

*Mixed with stir bar and heated at 60°C for 1 hour (2x)
** Not corrected for ash content

Table A.4 Lipid Analysis as a Function of Sample Weight

Culture	Sample mg dry weight	% Lipid AFDW
Ankistrodesmus	50	19.6 (±0.3)
	100	24.0 (±0.4)
	250	20.4 (±1.0)
Cyclotella	50	24.5 (±0.1)
	100	21.8 (±7.7)
	250	12.8 (± 0.6)
Nanno Q*	100	38.1
(Culture 81 & 82)	100**	43.1
	200	41.1
	200**	41.7

*Used Pet Ether (see text).
**These samples were mixed and crushed with glass rod while sitting in water, to assist wetting.

A.5 CHN Analysis and Heats of Combustion

Lyophilized samples of selected cultures were analyzed for their C, H and N content. This was done by contract with the Georgia Tech Research Institute (GTRI) at a cost of about $20 per sample. The analysis was performed by GTRI staff using a Perkin Elmer Model 220 CHN analyzer. Each sample was run in duplicate. An estimation of the error between duplicate runs was determined by constructing a distribution of the ranges between duplicate samples for %C, %H, and %N individually. The mean range and standard deviation for each were determined as 1.6 ± 2.2%± 2.0%, and 3.2± 2.6% for the %C, %H and %N respectively (N=16). This corresponds to approximately ±2.5% error at the 95% confidence level.

Heats of combustion were calculated using the results of CHN analysis and a correlation given by Roels (3) that is based upon the samples degree of reduction with respect to molecular nitrogen. It represents the number of electrons available for oxidation and is determined by the equation $\gamma = 4+nH-$.

2n O (n per C) the heat of combustion is then calculated using the formula $\Delta H_c = 115\gamma$ which has a standard error of 18.KJ/mole biomass of composition $CH_{1.8}O_{0.5}N_{0.}$ gave a constant of 116.7KJ/mole. Values were then converted into Kcal/g using the conversion factor of 2390 cal/J and the calculated molecular weight of the sample. The estimated error in heat or combustion values were dominated by the quality of the correlation, not by errors in determining the degree of reduction ($\pm$ 5%). Thus the std. error of the correlation is the one applicable in this case.

A.6 Silicic Acid Column Fractionation of Lipids

The lipids dissolved in $CHCL_3$, were transferred to weighing flasks and taken to dryness in a water bath under a N_2 stream. The flasks were placed with the stoppers cocked in a dessicator and placed on an aspirator for 15 min and then on a vacuum pump for 15 min. The vacuum was released by pumping in N_2. The stoppers were replaced onto the flask and the weight of the lipids were determined. Silicic acid was heated in an oven at 120° for at least 12 hours. The weight ratio of silicic acid to sample was about 30:1. The silicic acid was loaded into the column and hexane and tapped to remove bubbles. Three or more bed volumes of hexane were passed through the column. The lipids were redissolved in hexane and loaded onto the column. Three bed volumes of hexane were passed through the column bed and the eluate collected in a round bottom flask and placed on a rotoevaporator until a volume of .5-1ml remained. Using chloroform to redissolve the sample it was transferred quantitatively to a weighing flask. This procedure was repeated for the benzene, chloroform, acetone and methanol fractions. The fractions in the weighing flasks were weighed ; the samples were then redissolved in $CHCL_3$ and transferred to test tubes. The tubes were flushed with N_2, capped and placed in a freezer. Thin layer chromatography was carried out as described in Section 4.2.

Table A.4 Lipid Amounts Applied and Recovered in Silicic Acid Column Fractionation

Organism	Culture # Data	Nutrient Status	Lipids Loaded mg		Lipids Recovered mg	Gained (Lost) mg
Chaetoceros	150	Si Suff	10.85		9.60	(1.25)
Chaetoceros	150	Si Def	20.48		22.33	1.85
Chaetoceros	122	N Suff	11.14	23.16	22.36	(0.80)
	122d	"	12.02			
Chaetoceros	123	N Def	11.61	24.54	26.23	1.30
	125		12.93			
Thalassiosira	145 (10/2)	N Suf	10.31			
	(10/8)	"	15.82			
	(10/5)		24.56	50.09	47.20	(3.49)
Thalassiosira	146 (10/8)	N Def	19.60	38.58	36.68	(1.90)
	147 (10/9)	"	18.98			
Thalassiosira		Si Suff	8.61		8.53	(0.08)
Thalassiosira		Si Def	27.11		24.99	(2.12)
Boekolovia	127 (9/3&9/6)	N Suff	38.58		35.17	(3.41)
Boekolovia	128/130	N Def	17.15		15.86	(1.29)
Nanno Q	80/80B	N Def	83.70		87.19	3.49
Nanno Q	81	N Def	56.08		62.56	6.48
Nanno Q	82	N Def	63.94		64.46	0.54
Nanno Q	83	N Def	82.24		89.30	7.06
Nanno Q	67	N Suff	24.56		20.69	(3.87)

(Note Culture #70 results not reported)

For the calculations presented in Section 2.0, the total amount of weight recovered in each fraction was added, normalized to 100% and the % for each fraction calculated. To calculate the % of AFDW for each fraction, the original lipid content (or the weighted content if several lipid analysis were pooled) was multiplied by the % of total lipid for each fraction. It must be noted that the amount of total lipid recovered from the columns was not identical to that loaded on the columns (Table A.6). Therefore, these calculations may not reflect differential losses (or gains) during fractionation. Also, no fractionations have been, thus far, repeated. Thus no statistical analysis is available. However, the data for lipid contents of Nanno Q, cultures 80-84 shows no major variations.

A.7 Optical Densities and Correlation with Dry Weight

Optical densities were measured daily in all the N limited experiments since it is a relatively simple and rapid method for determining growth. However, the validity of O.D. in predicting ash free dry weight was not known. Using the large data set we have determined the correlations for each strain studied.

The method for measuring O.D. involved diluting 0.5 to 3 mls of culture into fresh media to give an O.D. value of about 0.1 to 0.4. Readings were carried out in a Bausch & Lomb "Spectronics 20". Samples were mixed (Vortex mixer) for 2-5 seconds and read at least twice (after again mixing) or until they gave a stable value. Up to culture # 87, O.D. was read at 650 nm, thereafter at 750 nm.

Typical Dry weight O.D. correlations are shown in Figures A-2 to A-4. Table A.7 presents the statistical analysis, including regression parameters. Sample size (N) represents the number of points used in the analysis.

A.8 REFERENCES

1) Lowry, O.H., et al., J. Biol. Chem. 193 265-275 (1951). Modified by Herbert, D., et al., Methods in Microbiol. 5B 209-344. (1971).

2) Dudois, et al., Anal. Chem. 28 350-356 (1956). Modified by Herbert, D. et al. Ibid.

3) Roels, J.A. Energetics and Kinetics in Biotechnology Elsvier Biomedical (1983).

Table A.6 Regression Parameters of OD-Ash-Free Dry Weight Correlations

Organism	Culture	N	R	M (Sm)	Yin(Sint)	λ
Ankistrodesmus	111-114	20	.9928	.365(.0104)	-.0318(.0260)	750
Ankistrodesmus	119-120	16	.9682	.341(.0236)	-.0379(.0651)	750
Cyclotella	99-102	17	.9439	.529(.0478)	.0504(.0554)	750
Nanno Q II	80-83	27	.9844	.250(.0089)	.0108(.0269)	650
T. Iso	84-85	23	.9897	.565(.0178)	-.0548(.0236)	650
Chaetoceros	122-125	34	.9688	.441(.0199)	.0619(0382)	750
Nanno Q	67-70	27	.9695	.257(.0130)	-.0164(.0311)	650
Nanno Q	141-144	18	.9896	.279(.0101)	-.00157(.0185)	750
Boekolovia	127-130	42	.9825	.281(.00842)	.0961(.0174)	750
Thalassiosira	145-148	36	.9821	.328(.0108)	.0327(.0175)	750

N: number of date points

R: correlation coefficient

m: slope

Sm: Standard deviation of slope

Yin: intercept

Sint: Standard deviation of intercept

λ: wavelength

Table A - 2. O.D. - Dry Weight Correlations