A SUBCONTRACT REPORT

CHEMICAL PROFILES OF MICROALGAE WITH EMPHASIS ON LIPIDS

By

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Prepared under

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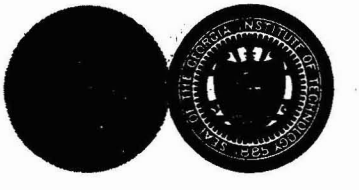

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SUMMARY

- Well balanced cultivated cells that are healthy and unstressed produce the optimum cellular yield. All stress studies for lipids production should be conducted with the established cell mass and not be administered during active cultivation.
- Cells cultivated continually in suboptimum conditions produce lower cellular yields and continuous cultivation in suboptimum media and environmental conditions produce lower than normal lipid levels.
- Lipid triggers are preceded by a burst of carbohydrate synthesis and storage.
- Ankistrodesmus is easily cultivated; when a properly concentrated cell preparation is placed into nitrogen deficient medium, lipid yields of 50-70% occurred in 3-4 days; this system, however, was difficult to control and reproduce because of its easily inducible pleomorphism and sporulation stages.
- Isochrysis cultivated at 25°C to late exponential growth phase and then maintained at 15°C produce predominantly C_{37} compounds.
- Lipid inhibitors are useful in studying metabolic pathways, but not for use in controlling lipid yields during cultivation.
- Multiple genetic clones of Neochloris provided evidence against the possibility that genetic variant of an organism may be better lipid producers.

- Shallow batch cultures for algal lipid production studies are inadequate; aerated and agitated batch cultures in Roux bottles are superior to shallow cultures, yet, they are not ideal because of the batch culture configuration. A better system such as attached cell bioreactors needs to be developed for measuring lipid triggers and controlling growth parameters.

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SECTION I

INTRODUCTION

This report is restricted to the current contract period which began in October 1983. Data collected prior to October was included in the annual report and in two manuscripts submitted for publication. The titles of the manuscripts are: "Lipids and lipopolysaccharide constituents of a commercial preparation of Spirulina platensis," by T. G. Tornabene and A. Ben-Amotz (1), and "Chemical Profiles of selected species of Algae with emphasis on lipids" by A. Ben-Amotz, W. H. Thomas and T. G. Tornabene (2). Previous results were also described by T. G. Tornabene, G. Holzer, S. Lien and N. Burris, "Lipid Composition of the nitrogen starved green alga Neochloris oleoabundans," (23).

SECTION II

OBJECTIVES

The research is directed towards identifying algae that have the highest lipid producing capacities. The laboratory at Georgia Tech has the responsibilities of providing to all members of the Aquatic Species Program (ASP) analytical expertise for determining the quantity and quality of algal lipids; assist in the identification of potential oil-producing algae; search for the trigger(s) that control the regulatory mechanisms within cells; and, to optimize productivity for lipid production.

The specific tasks addressed are as follows:

1) Provide analytical expertise to Aquatic Species Program for microalgae evaluation; 2) examine the influence of environmental parameters (nitrogen deficiency and temperature) on lipid production; 3) select for clones of test strains having the greatest potential for synthesizing lipids; and 4)

study suspected inhibitors that will block the polyisoprenoid pathway and result in the overproduction of isoprenoid hydrocarbons and/or alcohols, preferably within the C_5-C_{15} range.

SECTION III

MATERIALS AND METHODS

Cultivation of organisms. Most organisms in this study were cultivated by members of the ASP and forwarded to us as frozen wet-packed cells or as freeze-dried cells. All algae received were cultivated in nitrogen sufficient media.

Selected algal cultures were also cultivated at Georgia Tech in both nitrogen-sufficient and deficient media. Algae were cultivated in Mono-Lake or Pyramid Lake artificial media as described by E. Thomas (Scripps Institute); natural and synthetic sea water media fortified with Guillard trace metal mix; or, modified Provasoli medium. Unless otherwise indicated cell cultivations in this and previous reports were conducted in flasks on a platform shaker under continuous illumination at 25°C. These systems are described in detail in the FY83 annual report.

In experiments which assessed the effects of temperature on growth and lipid production, Ankistrodesmus and Isochrysis were cultivated in 1 liter Roux bottles incubated in temperatures of 15°, 20° or 25°C, ± 1°C for each. These cultures were aerated and agitated by bubbling vigorously with humidified air. Illumination was provided by banks of fluorescent lamps outside the glassed-walled water jackets. The problems encountered with this method were the maintenance of identical aeration rates in replicate bottles and the evaporation and concentration of media during prolonged incubations.

Cultivation by cell immobilization was initiated in an attempt to provide continuous cultures in a high production mode. The affinity of Dunaliella spp, Ankistrodesmus, Isochrysis and Botryococcus to hydrophilic plastic (Porex Corp), hydrophobic plastic (Porex Corp.), and Celite (a diatomaceous earth product, Manville Corp.) was measured. The pore sizes tested were 30, 50 and 100 μm in size. All algal test strains immobilized as determined by the quantity of pigmentation per unit carrier and by light and scanning-electron microscopy. The organisms were immobilzed in carriers designed for delivery of air from the inside of the carrier exiting by diffusion through the pores holding the algae. This configuration has produced up to 10^7 cell/ml/hour in a 5 ml reactor on a continuous basis for up to two months for each test organism except Botryococcus. There was a major problem with the continuation of growth thereafter, due to configuration design of the reactor. The cell dividing off the immobilized cells started to adhere to the outside reactor vessel creating light dampening and a batch culture situation outside the immobilization matrix. In an attempt to correct this problem, the immobilization matrix was made larger to fill the external volume and to increase the media exchange rate of the reactor. Measurements on this aspect of the study have not been completed. Preliminary indications are that immobilization bioreactors show great promise for producing controlled metabolic studies for determining lipid triggers.

Cell suspensions were extracted for lipids by the modified method of Bligh and Dyer, (3, 4). Extracted cell debris was saponified in NaOH-MeOH, and extracted with petroleum ether. The nonpolar and polar lipids were separated by silicic acid column chromatography (5) with hexane, benzene, chloroform, acetone and methanol. Polysaccharides were extracted from dried

cells by the phenol-water method (6) and isolated by isopycnic density gradient ultracentrifugation. Lipid components were deacylated by mild alkaline methanolysis (7). Samples were hydrolyzed with 2 N HCl at 100°C for 2 h for neutral sugars; and 4 N HCl for 6 h at 100°C for amino sugars. Lipids were analyzed by thin-layer chromatography in diethyl ether-benzene--ethanol-acetic acid (45:50:2:0.2) as first solvent and hexane-diethyl ether (96:4) as second solvent or chloroform-acetone-methanol- acetic acid-water (50:20:10:10:5). Lipids were detected by exposure to I_2 vapor, acid charring, phosphate spray (9), ninhydrin, α-napthol for glycolipids (8), Draggendorff stain for quartenary amines (8), or H_2SO_4-acetic acid (1:1) for sterols (8). The deacylated water-soluble products were separated on cellulose-TLC plates (10). The samples were visualized by the o-tolidine staining method overstained with an acidic ammonium molybdate solution (10), or by ninhydrin. Total carbohydrates were determined by the phenol--sulphuric acid method (11). Nucleic acids were estimated spectro-photometrically after hydrolysis with 0.1 N NaOH. Total protein was determined after hydrolysis in NaOH at 100°C for 1 h (12). Chlorophyll and cartenoids were assayed as described by Jensen (19). Fatty acids were converted to the methyl ester form in a 2.5% anhydrous methanolic-HCl (8). Aliquots of fatty acids were hydrogenated with H_2 and 10% Pd on activated charcoal. Free sugars were converted to alditol acetates (20). Derivatized components were analyzed on a Varian 3700 gas-liquid chromatograph (GLC) equipped with dual flame ionization detectors and a Varian Vista 402 data system. The analyses were as follows: 30 m x 0.252 mm fused quartz capillary column with 0.25 μm of OV 351 or 0.25 μm of DB-5 at 8 psi of He and 4°C/min from 125°C to 220°C and held isothermally; 2 m x 0.31 cm glass column packed with 10% SP2330 on 100/120 Gas chrom W AW at 27 psi of He and 4°C/min from 110°C to 250°C and

held isothermally.

SECTION IV

TASK I: LIPID COMPOSITION OF MICROALGAE

Subsection I. Lipid Studies on Algal Samples received from Members of ASP

A. Cryptophyte and Pransinophyte

1. Cryptomonad, Dangeardinella

Cryptomonad and Dangeardinella were isolated and cultivated by Dr. W. Thomas. The samples were analyzed for their approximate chemical composition. The organisms and general growth conditions are described in Table 1. One of each algal preparation was designated as "aged." These "aged" cultures were older cultures than the others but they are not to be confused with nitrogen-limited algal preparations as described later in this report. The lipid composition of all of the preparations was considerably low with the major cellular constituent being protein. The fractionation of representative lipid extracts on silicic acid is described in Table 2. The lipids were analyzed by TLC. The major components were relatively typical of green algae. The nature of the lipids are described with the chlorophytes in the following sections.

B. Chlorophytes

1. Chlorella, green flagellates

Twelve laboratory samples (Table 1, Number 11-22) were prepared by Dr. W. Thomas. Five cultures were prepared by Microbial Products/J. Weisman in outdoor ponds (Table 1). No information was provided as to the specific identity or method of preparation of the latter five chlorophyte samples (Table 1) which were in small quantities and diversified as indicated by the stratifications of the wet-packed cells. No direct ash content was determined for any of the samples for several reasons: Sample weights for

most samples were less than 1 gm; salt crystals and extrinsic materials were readily visible and estimated to be close to or greater than one-half of the sample and, the organisms prepared were apparently all green algae, cultivated in nonstress environments (Table 1). The relative distribution of nonpolar and polar lipids in total free lipid fractions of representative organism, as selected by elution from a silicic acid column, is given in Table 2. Each column eluate was assayed by TLC in mixtures of nonpolar and polar solvents to survey the number and nature of the lipid components. A range of 15-30 components were resolved in each of the silicic acid column eluates with the exception of the $CHCl_3$ eluates which had 5-10 resolved components. Many of the pigmented components in related eluates were duplicates. The composition of the phospholipids in all of the methanol eluates were similar. These lipids were the diacylglycerol phosphate type. The composition of these diacylglycerol derivatives in biomembranes is relatively uniform with regard to their overall stereostructures and they are generally of little value as chemotaxonomic markers. Each cell makes no less than the amount essential for membrane structure and function. The essential amount is generally less than one-half of the amount they normally produce. The minimal amount of phospholipids are generally produced when the cells are under "stress" and the metabolic flow of carbon is directed towards carbon storage. In terms of relative quantity, the acetone eluate (Table 2) is often the most concentrated eluate for unstressed algal cells. Glycolipids of the galactosyl diacylglyceride type are common in the acetone eluate. The chlorophylls and carotenoids also predominate in this fraction. The neutral lipid (hexane, benzene, chloroform eluates) represent relatively small amounts of lipids. This is typical of algae. A decrease in the concentration of the

chlorophyll contents and a rise in concentration of the carotenoid (isoprenoid) types (both polar and nonpolar) often occur in cells cultivated under stress. None of the cells studied in this group of organisms were challenged; therefore, it was not possible to determine if these organisms have a potential for high-lipid production and exactly what would happen to the lipid production in these algae under stress. The lipid concentration ranges determined in this laboratory for the test algae are rather typical of all algae cultivated in "sufficient" growth media.

Selenastrum, Chlorella-like, Oocystis

Samples of the chorophytes Selenastrum, Oocystis and a "Chlorella-Like" alga were received from Bill Thomas. Total weight-percents of ash, protein (Lowry) and carbohydrate (Dubois) were determined for each sample using BSA(protein) and d-glucose (CHO) as standards. Weight-percent lipid (Modified Bligh-Dyer) was determined for all samples but one ("Chlorella-Like-PB-S-N") the sample of which was partially destroyed in shipping (Table 3).

Fractionation was conducted on Unisil and TLC. The lipids of Chlorella and Oocystis were analyzed previously in this laboratory and, since neither species yielded more than 15% lipid, no further studies were conducted on these samples.

The lipid fractions of the four Selenastrum samples were fractionated on silicic acid columns with approximately three bed-volumes each of hexane, benzene, chloroform, acetone and methanol; over 90% of the recovered lipid was concentrated in the acetone and methanol eluates (Table 4).

No effects were observed of age or nitrogen source on the neutral:polar lipid ratio (neutral = hexane, benzene and chloroform eluates; Polar = acetone & methanol eluates). Nitrogen source does, however, appear to affect the balance between the acetone & methanol fractions -- lipids of

Selenastrum growth with urea are predominantly in the acetone fraction; when grown with NO_3, the lipids are more evenly divided between acetone and methanol (Table 4).

3. Chlorella, Oocystis, Platymonas

Additional chlorophytes were provided by W. Thomas & E. Laws (Univ. Hawaii) for analyses. Samples of chlorella and Oocystis were dried and the approximate chemistry determined. The results are given in Table 5. The lipid composition for Chlorella and Oocystis was in the range from 10 to 20%. In all cases the lipid contents were lower for "aged" cells than "unaged" cells. The lipid distribution patterns were determined on TLC. For all practical purposes the lipids are virtually identical for all of the Chlorella samples. The Oocystis samples differ from Chlorella primarily by the changes in the relative concentrations of the individual components.

The Platymonas (green flagellate) sample provided by Dr. E. Laws is Sample #10. The principal cellular components were proteins with the lipids comprising less than 16%.

No further studies were conducted on these samples unless there are further directives. Perhaps one or two of the organisms in this list should be identified and cultivated under different growth parameters and more thoroughly evaluated. None of the samples were provided to us as viable cultures.

4. Caulerpa

Caulerpa prolifera a green macroalga was provided by John Ryther, University of Florida. The sample was assayed colorimetrically for approximate chemical composition. The data from chemical composition studies (Table 6) was greater than 100% and indicated a probable interference in the determination of the carbohydrate or protein contents. It is not

uncommon for carbohydrate content to vary substantially since different sugars have different extinction coefficients and the samples were recorded against glucose as a standard. The ash and lipid contents were recorded gravimetrically.

The lipid yield of 6.1% of organic dry weight is relatively higher than other macroalgae that we analyzed, the others being in the 1-2% range. The fractionation of the lipids (Table 9) demonstrated that 94% of the total lipids were polar lipids. Slightly less than 6% of the total lipids were nonpolar lipids. This is a typical pattern that we have observed for unaged microalgae. The principal portion of the lipids are glycolipids and polar pigment with the latter being the minor compounds of the two. The phospholipids identified in the polar lipid fraction are the usual ones found in most algae.

From the standpoint of the lipids, this alga preparation is undistinguished. It will be of interest, however, to look at aged or nutritionally stressed Caulerpa to see if there is a metabolic switch to neutral lipid synthesis. No additonal samples were provided.

5. Strain Nanno Q

Algal strain Nanno Q (formerly designated 0938) was provided by Dr. Ralph Lewin, LaJolla, California. Dr. W. Barclay, SERI, Golden, Colorado, also cultivated the algae and shipped the preparations to Georgia Tech as lyophilized samples. Samples 1 and 2 were healthy cells cultivated in nitrogen sufficient media. Sample 3 was a preparation from a medium devoid of nitrogen. This sample was a small quantity of degraded/lysed cell debris. This problem was discussed with Dr. Barclay and another preparation of Nanno Q was cultivated in nitrogen deficient media and shipped. Two new cell preparations were received: 1.) nitrogen sufficient culture growth in

gulf sea water (Sample 4) and, 2.) nitrogen deficient culture (Sample 5). The nitrogen deficient culture was a green pigmented preparation unlike the dull yellow culture first offered by Ralph Lewin as evidence of a culture that was nitrogen deficient.

The lipid content comprised 19-27% of cell dry weight of Samples 1 and 2 (Table 8). Samples 3 and 5 contained 23.3% and 14.6% lipids, respectively. These amounts are substantially less than those found in the nitrogen deficient sample prepared by R. Lewin and analyzed earlier in this laboratory. This seemingly inconsistent data is no longer surprising. It is now becoming routinely observed in our laboratory that cells in early periods of nitrogen deficiency produce carbohydrates with a dramatic drop in lipid content. These results suggest that the lipid production trigger was just about ready to be activated at the time the cells were harvested. The fractionation of the lipids is described in Table 9. The non-polar to polar distribution of lipids for Samples 1 and 2 are relatively typical of algae. The increase in components in the hexane eluate of Sample 3 is interesting but it falls short of our first study which showed the hydrocarbons in the hexane fraction of nitrogen deficient cells was about 10 times higher than that reported in Table 11. It is essential that this work be repeated with samples that are maintained under nitrogen deficient conditions for periods beyond the "carbohydrate increase phase."

The hydrocarbon composition was analyzed by GLC (Table 10) and tentatively identified by comparing the retention times to established standards. Mass spectral analyses of the sample have been conducted. Because of the hydrocarbons being both branched and polyunsaturated, derivatives of the hydrocarbons had to be made and reanalyzed. This work is still in progress.

The fatty acid contents are listed in Table 11. The polyunsaturated fatty acids are relatively common for many marine algae. The rather substantial drop in the polyunsaturated fatty acids in the nitrogen deficient preparation will have to be confirmed or corrected with the further studies planned.

The nature and distribution of lipid components are described in Tables 12 and 13. This lipid content is very typical of algae in general.

One exceptionally interesting feature of the Nanno Q strain is the preliminary evidence obtained from a sample sent directly to Georgia Tech by Dr. Lewin which demonstrated the potential of the organism to synthesize hydrocarbons under nitrogen limiting conditions. Confirmation will have to be obtained from analysis of further cell preparations.

C. Bacillariophyte

1. Phaeodactylum

Phaeodactylum tricornutum, a common marine diatom, was provided by Dr. E. Laws, University of Hawaii. The sample was examined microscopically and was found to contain a substantially large quantity of a green flagellate. The analysis of the chemical composition of the sample demonstrated (Table 14) that one-fourth of the cellular constituents was not identified by the standard colorimetric and gravimetric test for protein, carbohydrate and lipids. These results are not uncommon, however, since carbohydrate and protein determinations by colorimetric methods are often erroneous because of interference from chemical interactions and/or the fact that different sugars have different extinction coefficients (samples are typically measured against a glucose standard).

The lipid yield of 16% of organic weight is fairly typical of diatoms (Table 14). The glycolipids, phosphlipid and polar pigment represent 76% of the total lipids (Table 15). The benzene eluate represents about 20% of the lipids. Slightly less than 1% of the lipids were acyclic hydrocarbons. The hydrocarbon content is considerably large relative to previous studies in this laboratory and others that reported Phaeodactylum to produce only 0.06% hydrocarbons. There was evidence obtained by GLC-MS analysis that the sample was partly contaminated with commercial oil possibly obtained through centrifugation procedure or by being in contact with vacuum grease, pump oil or antiforming agents. Perhaps the green flagellate contaminant is contributing significantly to these results. The polar lipids are typical of those found in most algae. The TLC analyses revealed that the distribution of lipids were those that are typical of algae. The only interesting aspect is the fact that 24% of the lipids are neutral ones. A substantial amount of the neutral lipids are isoprenoids. These compounds have not yet been identified. It would be of interest to look at aged or nutritionally stressed cells to see if the neutral lipid biosynthesis is further enhanced. No viable cultures or additional samples were provided.

Subsection II. Organisms cultivated and analysed at GIT

A. Lipid contents of nitrogen sufficient and deficient cultures.

Two organisms isolated by Dr. Thomas and evaluated in the previous year were selected for further studies. These two organisms were Oocystis and Ankistrodesmus. They had exhibited no special lipid producing potential; however, they are easily cultivatable algae and they had never been tested for lipid production while cultivated under nitrogen deficient conditions. Two additional algae were included in this phase of the study: Cylindrotheica fusiformis and Monallanthus salina. All four organisms were culti-

vated in both nitrogen sufficient and deficient media as described in the following procedure.

The two cultures supplied by Bill Thomas were cultivated in Walker Lake medium for Oocystis and Pyramid Lake medium for Ankistrodesmus as he recommended. All cultures were grown in a shallow flask on a shaker under continuous illumination at 25°C.

1. Ankistrodesmus falcatus. One liter Pyramid Lake medium (1mM KNO_3) was inoculated with 20 ml log phase Ankistrodesmus. After five days, when the nitrate level was approximately 0.5mM, the growth was heavy and dark green. The culture was divided into four equal volumes, and diluted with one of the following:

1) 250ml Pyramid Lake medium, no nitrogen source;

2) 250ml Pyramid Lake medium, no nitrogen source, 10mM $NaHCO_3$;

3. 250ml Pyramid Lake medium, 2mM KNO_3, 10mM KNO_3;

4. 250ml Pyramid Lake medium, 2mM KNO_3 (no $NaHCO_3$)

After two weeks, nitrate levels of nos. 1 and 2 had been at background level for seven days (value determined by NO_3 assay with medium with no KNO_3 as a control), the cultures were then harvested by centrifugation.

Values obtained for protein, carbohydrate, ash and lipid levels are summarized in Table 16. Protein levels in the nitrogen deficient cultures were approximately one-half of that of the nitrogen sufficient cultures. Results of the carbohydrate assay indicated that total cellular carbohydrate increased with nitrogen deficiency. No trend was apparent in the ash weights.

Lipids comprised 40% of the total dry cell weight of culture no. 2; 27.24% of no. 3; and 19.5% of no. 4. There was not enough sample recovered for an adequate extraction and quantification of no. 1. A slight shift from

polar to nonpolar lipids was seen in nos. 2 and 4--methanol fractions of these two are lower than no. 3, while the chloroform fractions of 2 and 4 hold a greater percentage of the total than no. 3. The preponderance of lipids, however, was in the acetone fraction. TLC of fractions in polar lipid solvent showed identical separation patterns for chloroform, acetone and methanol fractions of all three extracts, the major difference being quantitative rather than qualitative. In the nitrogen deficient culture, intense yellow to orange pigments predominated. The yellow pigments in benzene fraction of no. 2 (Table 16) were particularly sensitive to drying and illumination. When the benzene fraction of no. 2 was dried (at 40°C) under nitrogen and subdued illumination, it turned from a bright yellow to a bright orange color and the adsorption spectrum in the region from 400 nm was increased by 50% with respect to a new peak adsorption at 660 nm. TLC of this oxidized benzene fraction revealed a separation pattern identical to that observed in the acetone fraction of no. 2. On refractionation on Unisil, all visible pigment was eluted by acetone. Reextraction of another sample of no. 2 with no illumination produced a benzene fraction with the principal component having an R_f value corresponding to a β-carotene standard.

In both TLC solvents a and b all three treatments had a major spot that co-chromatographed with a triglyceride standard. Saponification of chloroform fractions resulted in the near-total disappearance of this spot in no. 3, with a corresponding increase in the size of a spot co-chromatographing with a fatty acid standard.

Upon continous cultivation, it became quite clear that Ankistrodesmus was pleomorphic in various cultivating stages ultimately resulting in sporulation. This phenonmenum was reported ealier by McMillan in 1957 (22).

Therefore, initial cultures were grown in "Pyramid Lake" medium (Bill Thomas) supplemented with 0.2mM KH_2PO_4, 2mM KNO_3, and 10mM $NaHCO_3$ for nitrogen, carbon and/or phosphorus stress. Spores were observed in one culture five days after it had been centrifuged at 4000 rpm for 30 minutes, washed with fresh medium, re-centrifuged for 30 minutes and resuspended in fresh Pyramid Lake medium. Another liter of culture (not centrifuged) was allowed to grow until no nitrate was detectable by colorimetric assay then diluted four times with Pyramid Lake medium either as above (control, carbon-limited) or without KNO_3 (nitrogen-limited). All but the carbon--limited culture was supplemented every other day with ten milliliters of 1M $NaHCO_3$.

Over a period of five days both the nitrogen and the carbon limited cultures turned from green to orange (nitrate level in the carbon-limited medium remained the same as that of the control) while the control and the sporulated cultures remained green. When the cultures had been orange for three days they were harvested by centrifugation, lyophilized and extracted by the modified Bligh-Dyer procedure.

Fatty acids of four batch cultures of Ankistrodesmus falcatus--one nitrogen-limited culture, one carbon-limited culture, one culture that sporulated, and a control--were identified by gas chromatography. The predominant saturated fatty acid in all four were palmitic (16:0), comprising 10.93% of the fatty acids of the control culture, 14.21% of the nitrogen-limited, 17.10% of the carbon-limited, and 15.80% of the acids of the sporulated culture. The unsaturated acids, oleic (18:1) and linolenic (18:3) accounted for 24.62% and 26.45%, respectively, of the acids of the control, while oleic was the predominant acid of the nitrogen-limited (48.38%), carbon-limited (55.49%), and sporulated (53.82%) cultures. The

overall results are given i Tables 17-20. Modest changes in the relative percentages of the fatty acids in the various growth phases are obvious.

2. Oocystis sp:

The analysis of Oocystis was complicated by the presence of extrinsic bacteria. Cultivating Oocystis on agar surfaces and the subculturing of clones greatly reduced the number of bacteria but it did not eliminate them as judged by their substantial increase in numbers relative to the number of successive cultivations. The algal cells excreted glycoproteins as soon as the cells approached late exponential growth phase. The bacterial population then proliferated. We therefore found it impossible to conduct meaningful analyses on the Oocystis in nitrogen free media. Visual detection of bacterial activity in the Oocystis cultures was masked by the deep green pigments of the algal cells; low speed centrifugation of Oocystis in nitrogen sufficient medium, however, demonstrated stata in the wet pellet. When in nitrogen deficient media, the culture was pale green; as the bacterial population increased, the culture turned red in color. The results of the study attempted is described in the following lines.

One liter of log-phase culture was centrifuged (100 x g), washed two times with an isoosmotic salt solution and divided into four equal parts to inoculate two flasks each containing one liter Walker Lake, 20mM $NaHCO_3$ with no nitrogen source, and two flasks each with one liter as above supplemented with 4mM KNO_3. Harvesting was at two day intervals beginning 24 hours after inoculation.

The addition or omission of a nitrogen source caused effects or changes with time which were discernible in protein (8.4% - 24%) carbohydrate (16.5% - 32.7%) or ash (23% - 26%). In nitrogen-deficient cultures, lipid levels apparently decreased with time to 4-9% of cell dry wt. These results

were attributed to decrease in the algae and bacterial proliferation.

3. Monallantus salina: Monallantus was grown in either a supplemented seawater media or a completely defined media based on that of Provasoli. A lipid yield of 60% was obtained on the supplemental seawater medium (Table 21). Attempts to establish axenic cultures of Monallantus met with little success; both antibiotic and physical selection methods were tried.

Nitrate deficient cultures began as cultures low in nitrate allowed to exhaust the nitrate as evidenced by colorimetric assay. Cultures were typically given to 10 mM of sodium bicarbonate every 3 to 5 days.

Composition under Nitrate Deficiency: These cultures were 9 days old at harvest. Initial nitrate was 1 and 5 mM for the nitrate deficient and sufficient cultures, respectively. The nitrate was exhausted in the nitrate deficient culture on the seventh day of cultivation as determined by colorimetric assay.

Several attempts were made to grow the alga under these same conditions for longer periods of time, but bacterial blooms occurred at 11 to 12 days.

Fractionation of Lipid on Unisil: Lipid from both nitrate sufficient and deficient cultures were fractionated on Unisil by standard procedure (Table 22).

Nitrate sufficient and deficient cells gave similar lipid patterns on TLC (Table 23) except as noted. Only the major compounds are indicated; most of the pigments are not indicated.

The major lipid shift in the cells conversion from nitrogen sufficient to deficient states was observed in the pigment contents and the triglycerides. The latter were the major lipid component in the nitgrogen-limited cells, accounting for 42% of the total extracted lipids. The elution of triglycerides into the acetone fraction is not uncommon when the trigly-

ceride content is relatively large. Triglycerides complex in an architectural arrangement with the glycolipids. Recyling the acetone eluate through a silicic column eventually strips most all of the triglycerides into the chloroform eluate.

The fatty acids were predominantly straight chains with the major species being 16:0, 16:1, 18:1, 18:2, 18:3 and 18:4. There were no major qualitative differences in the fatty acid contents of the lipids of nitrogen-sufficient and -deficient cells.

4. Cylindrotheica fusiforma: The alga was grown in a medium of seawater supplemented with trace elements, phosphate, iron, silicate, borate, and nitrate.

The cellular composition under conditions of nitrate sufficiency and deficiency is given in Tables 24 and 25.

A significant proportion of the approximate chemical composition is unassigned. This unassigned fraction consists of predominantly amphilic compounds that are comprised of oligo- and poly-saccharides derivatized with fatty acids (glycolipids) and amino acids (glycolipoproteins). These complex "lipids" were demonstrable by acid hydrolysis, solvent partitioning, derivatization, and analyses by GLC and TLC.

Cylindrotheica, cultivated on nitrogen deficient medium, shifts toward complex polymer biosynthesis, which are designated in the unassigned category (Table 24). Fractionation of the lipids from nitrogen sufficient or deficient cells on a unisil column indicated no significant changes in the distribution of nonpolar to polar lipid (Table 25).

B. Environmental parameters on lipid Production

Isochrysis, Ankistrodesmus, Botryococcus

The test organisms were principally Isochrysis and Ankistrodesmus. Botryococcus was also tested in this study; however, it grew so slowly relative to the other organisms it was recently dropped from the study. In one of the studies we attempted to better understand the relationship of the growth curve to lipid production. The results are given in Table 26. It appears to be quite clear that in the course of cultivation, the cellular lipid concentration actually drops just prior to the initiation of high lipid synthesis. The lipid production phase is preceded by a burst in carbohydrate production (Table 26). Quite often these types of results were recorded for many of the organisms studied in this laboratory creating a false impression that carbohydrate and not lipids are the principal products. These results appear to answer many of the questions that arose about the inconsistencies observed in the lipid production studies of cells from early stages of nitrogen deficiency. Another organism that is currently under study appears to display this same phenomenum (Lewin strain Nanno Q, presented in Section I, p10).

Several environmental stress studies were conducted this year for lipid production: temperature, saline, pH, nitrogen compounds. The more balanced the growth medium, the better the cellular yield and, consequently, the greater the overall conversion of cellular materials to lipids. The pH range was quite narrow for the test organisms being between pH 7.0 and 8.5 with maximum at pH 7.5. The differences in lipid yields were not due to pH differences, but to suboptimum cellular growth and function. The alteration in lipid components in cells held at suboptimum pH's was nil. Studies on effects of suboptimum pH growth on lipid production were discontinued.

Elevated saline concentrations were devastating to cellular integrity and function of the fresh water algae, Botryococcus and Ankistrodesmus.

Studies with saline concentrations up to 1M NaCl were previously conducted in this laboratory with Isochrysis sp. The lipid yields decreased when NaCl concentration exceeding 0.5M. This result was similar to that which occurs in Dunaliella spp. Studies on saline concentrations as an inducer of lipid production were, therefore, discontinued.

One of the environmental parameters studied was temperature. At 25, 20 and 15°C, Isochrysis and Ankistrodesmus cells were healthy, i.e., they divided and increased in cell mass. Ankistrodesmus, but not Isochrysis, survived at temperatures as high as 35°C.

The lipid contents of Ankistrodesmus and Isochrysis cultivated in Roux bottles with agitation and aeration is described in Table 27. The data represents duplicates of three different experiments. These cultures were taken from nitrogen efficient media. There appears to be no significant effect of temperature on Ankistrodesmus cultures. Isochrysis, however, was influenced by changes in cellular pigmentation going from green at 25°C to yellowish-green at 20°C and yellow at 15°C. The lipid contents increased at 15°C with the predominant compound being the $C_{37}H_{72}O$ compound, first reported in our FY83 annual report. Attempts were unsuccessful to convert all temperature controlled cultures to nitrogen deficient stages. In the case of Isochrysis, there were problems with media evaporation and saline concentration, and significantly slower growth rates at the lower temperature. In the case of Ankistrodesmus, the organism converted into sporulation stages. An example of the lipid content of the pleomorphic Ankistrodesmus in nitrogen deficient media at different temperature is given in Table 28. There were essentially no changes in the lipid contents at lower temperatures. The optimum temperature condition illustrated the expected increase in lipid production. Even then, the lipid yield was substantially

lower than some of the values we have recorded for this organism.

SUBSECTION III. CONCLUSIONS

The lipids of different microalgal cultures cultivated in "sufficient" media in batch culture are fundamentally similar in both quantity and quality. The chemotaxonomic specificities of the lipids are usually restricted to specific pigments, fatty acid, and/or metabolic accessory components. These specific component types usually represent a relatively minor part of the total lipids. The screening of microalgae for lipid production is, therefore, of little value unless the isolates are also challenged by some stress state, such as nitrogen starvation, to force the metabolic mechanisms to a carbon storage pathway. The specific character of lipid production of each microalgal isolate is then exemplified. Examples of productivities under induced metabolic triggers are Botryococcus (botryoccenes), Dunaliella baradawil (β-carotene), Neochloris (triglycerides), Isochrysis ($C_{37}H_{72}O$) and so forth. It would be impractical, however, to conduct such thorough studies on every isolate obtained and being screened by members of the ASP. Thus, a better selection model must be developed for screening micralgae to assure a proper sampling of the diverse algal systems and to minimize unnecessary duplications.

SECTION V

TASK II. Use of Inhibitors to Select for High Lipid producing Strains

Studies with potential lipid inhibitors was conducted on agar plates with Isochrysis galbana, Dunaliella bardawil, Ankistrodesmus falcatus and Botryococcus braunii as the test organisms.

Preparations were in replicates on agar plates containing the following inhibitors: p-chlorophenylthiotriethylamine (CPTA), diphenylamine, 8-hydroxyquinone, nicotine and pyridine.

The inhibitors were introduced at concentrations of 0.1 μM, 1μM, 10μM, 100μM and 1mM. Clones of algae showing resistance to the inhibitors were isolated and recultivated four times in the presence of the lipid inhibitor.

The continuous cultivation of the algae in the presence of the inhibitors virtually diminished cell growth. By recovering the cells surviving on the plates we had accumulated sufficient sample to estimate lipid yields. For all test organisms, the lipid contents were reduced by as much as one-half of control cells.

For organisms cultivated once in the presence of the inhibitors, the lipid contents were never greater than the control. For the most part, the treated culture were unhealthy and difficult to revive on standard growth medium. After hundreds of culture plates and transfer, it was decided to discontinue this phase of the investigation since the results were contrary to the purpose of increasing lipid yields for possible commercial applications.

SECTION VI

TASK III. Isoprenoid Biosynthesis Inhibitors

A. Purpose

One of the objectives of this year's project was to test the effects of chemicals on deregulating the biochemical pathway for isoprenoid biosynthesis. The end result was to control specific lipid synthesis.

B. Methods

Preliminary to any investigation on these inhibitors it was necessary to establish bacteria free cultures; this was accomplished for both Dunaliella salina and D. bardawil by standard plating techniques. Test organisms were then cultivated on agar plates or in broth agitated by a

rotating shaker. The inhibitors added to the medium were lead as $PbNO_3$, pyridine, 8-OH quinone, diphenylamine and CPTA.

Screening of several inhibitors for lethal dose was undertaken for D. salina, D. bardawil, and Isochyris galbana.

C. Results

The results from cultivating the test organism in the presence of two of the inhibitors are shown in Table 29. The lipid compositions were unaffected by the inhibitors.

8-Hydroxyquinone was nontoxic in doses as high as 10mM. Diphenylamine was toxic to all three test organisms at 10mM. Again there was no discernable effect on the lipid composition.

D. salina was also grown in 10 μM diphenylamine under light for six days. The lipid content as a percentage of organic weight was increased in the diphenylamine treated culture to 35% compared to 28% in the control. The lipid fractionation pattern on Unisil was essentially identical to the control.

D. salina was also cultivated in media containing 100 ppm CPTA (p-chloro-phenylthiotriethylamine-HCl) for two days in both light and dark. Even though CPTA prevented replication, the cells remained viable at this concentration; however, only a small alteration in the lipid pattern was observed as indicated in Table 31. The constituents in the benzene fraction did decrease (table 30), due to a change in the carotenoid lipids. Since the alterations were not principally associated with a specific component and the changes were relatively minor, no further studies were conducted with this system.

Inhibitor studies with Botryococcus braunii are underway. The progress to date is as follows: B. braunii was cultivated in an airlift system pres-

surized with either 1 or 5% CO_2 in air. The organism grew rapidly for two generations; the growth thereafter quickly declined. Renewal of a media was sufficient to stimulate additional growth, but even then the limit was rapidly reached. The growth limitation stage was visually indicated by cell clumping even in rapidly stirred cultures. Preliminary experiments were initiated to indicate conditions stimulatory for lipid synthesis. Using C-14 labelled glucose, nitrate deficient B. braunii accumulated more ^{14}C-lipids than nitrate sufficient cells. These results are indicated in Table 31. B. braunii also incorporated more of the label into lipid when grown in the dark (heterotrophically). Again, the carbon source was ^{14}C-glucose, uniformly labelled (Table 32).

Further studies on this organism have not been completed because of the very slow growing nature of the organism.

D. CONCLUSION

The best lipid production was obtained from healthy cells that were metabolically stressed by media enrichment. All studies that employed suboptimum growth conditions and metabolic inhibitors were detrimental to both cell growth and lipid biosynthesis. Inhibitors are excellent for delineating biochemical pathways, but not for controlling lipid biosynthesis. Metabolic blockers, such as those used in this study have been demonstrated to inhibit specific sites in lipid biosynthesis in bacteria and fungi, but not in the algae test. Alteration in biochemical pathways resulting in lipid accumulation did not occur in the test algae. Algae do not respond to inhibitors like the procaryotic systems nor are they as adaptable to suboptimal growth environments. Metabolic expression through lipid biosynthesis are not distinctive in algae. Instead of pursuing

inhibitor studies, future programs should focus on developing continuous cultivation systems and developing cultivation enrichment mechanisms for enhancing natural metabolic stress states.

SECTION VII

ANALYSIS OF BIOCHEMICAL VARIABILITY OF CLONES OF NEOCHLORIS

a. Rationale

Bacteria and fungi are common industrial microorganism. "Better product producing" strains have been obtrained through screening processes and strain development. On this bases, a study was conducted to evaluate lipid production in phenotypically different strains of one alga to see if lipid evaluation conducted on one species was representative for all of the many different strains and variants of that species. The test organism was Neochloris.

b. Results

Nineteen clones of Neochloris were obtained from the culture collection of Dr. Patricia Archibald, Department of Biology, Slippery Rock State Teachers College, Pa. The culture numbers and their respective pigmentation are recorded in Tables 33A, B and C. The cultures were cultivated on agar plates and harvested after media became nitrogen deficient. The lipid yields were 35-50% of the cell dry weight. The principal lipid in all culture extracts were triglyceride. The triglyceride comprised from 60-85% of the total lipids. The result was similar to that previously reported (23). The fatty acid composition of each culture is given in Tables 33A, B and C. The principal fatty acids in all samples were palmitic (16:0), Stearic (18:0) and Oleic (18:1) acids. Some samples contained, in relatively small quantities, polyunsaturated C-20 acids (20:3; 20:4)

C. Conclusions

Although there were several pigmental and morphological differences between the clones of Neochloris spp., the lipid contents of each remained relatively unchanged. Thus, the data reported for one strain of Neochloris (23) appears to be quite representative of many Neochloris strains. Continuous screening of closely related algal strains for a better lipid producer appears to be unjustified.

SECTION VIII

FINAL CONCLUSIONS

The following statements are general comments on the status of the last year's studies:

1. Well balanced cultivated cells that are healthy and unstressed produce the optimum cellular yield. All stress studies for lipids production should be conducted with the established cell mass and not be administered during active cultivation.
2. Cells cultivated continually in suboptimum conditions produce lower cellular yields and continuous cultivation in suboptimum media and environmental conditions produce lower than normal lipid levels.
3. Lipid triggers are preceded by a burst of carbohydrate synthesis and storage.
4. With shallow shaker cultivation systems, it is diffcult to reproduce lipid trigger experiments within reproducible time frames.
5. Ankistrodesmus is easily cultivated; when a properly concentrated cell preparation is placed into nitrogen deficient medium, lipid yields of 50-70% occurred in 3-4 days; this system, however,

was difficult to control and reproduce because of its easily inducible pleomorphism and sporulation stages.

6. Isochrysis cultivated at 25°C to late exponential growth phase and then maintained at 15°C produce predominantly C_{37} Compounds.
7. Lipid inhibitors are useful in studying metaboic pathways, but are not of use in controlling lipid yields during cultivation.
8. Multiple genetic clones of Neochloris provided evidence against the possibility that genetic variants of an organism may be better lipid producers.
9. Shallow batch cultures for algal lipid production studies are inadequate; aerated and agitated batch cultures in Roux bottles are superior to shallow cultures but they are also inadequate because of the batch culture configuration. A better system such as attached cell bioreactors for continuous cultures need to be developed so as to permit the attainment of maximum productivity, the measurement of lipid triggers and the control of growth parameters.
10. Lipid biosynthesis is essential in all living systems. Many diverse components comprise the lipids composition involving many different enzymes, coenzymes, cofactors, precursors, and regulating sites for their syntheses. The biosyntheses of lipids are intertied with the central metabolic system. It is, therefore, unrealistic to consider simple alteration of the lipid biochemical pathway by inhibitors or classical and molecular genetics. The only feasible approaches for enhancing lipid synthesis are to find a way to enrich cell growth or to alter one

of the key regulatory sites that has the end-result of increased lipid synthesis or to alter a key enzyme with the result being a redirection of a pathway to one producing a specific desired product.

SECTION IX

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TABLE 1

Proximate Chemical Composition of Selected Cryptophyte, Prasinophyte, and Chlorophytes Cultivated on Different Growth Media

Culture No.		Initial Dry Wt. Cells (gm)	Lipids %	CHO %	Protein %
Cryptophyte					
1	Cryptomonad, Harper L., NH_4+	0.08323	15.83	9.33	48.78
2.	" " (Aged)	0.75793	18.22	8.34	52.91
3.	" " NO_3	0.68536	3.15	7.67	19.94
4.	" " (Aged)	0.81458	5.02	9.66	26.84
5.	" " Urea	0.42120	6.41	9.00	30.23
6.	" " (Aged)	2.16098	10.59	9.37	43.61
	Prasinophyte				
7.	Dangeardinella, Salina Valley, NO_3	0.56196	5.57	1.44	16.93
8.	" " "	1.6220	5.46	3.38	16.61
9.	" " Urea	0.38870	4.20	2.42	17.94
10.	" " (Aged)	0.22664	4.77	3.36	18.15
Chlorophyte					
11.	Chlorella/Pond B NO_3	0.579020	20.08	10.54	65.72
12.	" " (Aged)	1.01492	18.04	13.84	44.96
13.	" "	0.53883	20.68	9.71	46.94
14.	" " (Aged)	0.94596	20.15	7.84	57.91
15.	Chlorella/Spring 10/1 NH_4+	0.48283	17.53	11.90	41.95
16.	" " (aged)	1.83487	15.23	9.44	52.49
17.	" " NO_3	0.81650	10.85	14.09	46.50
18.	" " (Aged)	1.85140	2.95	14.72	46.07
19.	" " Urea	0.71039	13.34	9.28	61.73
20.	" " (Aged)	2.59074	7.99	10.68	60.23
21.	Flagellate, Walker L., Urea	0.86681	17.88	37.97	20.04
22.	" " (Aged)	1.10922	12.05	41.73	19.23
23.	G1(1)	0.93062	9.55	38.13	18.18
24.	G1(2)	0.45224	8.92	18.66	42.06
25.	M3(1)	0.38514	19.96	26.97	20.77
26.	M3(2)	0.51865	8.96	10.91	73.96
27.	M4	0.51195	22.23	33.62	34.67

*culture number 1-22 were from Thomas, Scripps Institute whereas 23-27 were from Microbial Products.

TABLE 2

Fractionation of Lipids of Different Microalgal Species on "Unisil" Silic Acid Columns

Culture Number	Sample	µg loaded	% Eluted					
			Hex.	Benz.	$CHCl_3$	Acet.	MeOH	Total
1	Cryptomonad, Harper Lake	8.0	0.03	0.08	0.15	100.40	0.93	101.59
9	Dangeardinella	13.8	0.65	17.85	0.43	76.45	6.72	102.10
13	Chlorella Pond B	97.6	0.08	0.56	1.72	61.63	22.10	85.93
21	Flagellate Walker Lake	64.8	0.43	2.70	19.06	59.90	18.38	100.47

TABLE 3

Approximate Chemical Composition of Chlorophytes

	% Ash	% Protein	% Carbohydrate	% Lipid	Total
Oocystis NO_3, 12-13	14.35	29.96	33.80	10.39	88.5
Oocystis NO_3, 12-16	13.68	33.67	41.00	9.03	97.38
Chlorella-Like, DB-5-N 12-19	11.03	30.08	37.41	10.86	89.07
ChlorellLike, PB-5-N 12-11	6.06	30.79	42.87	13.98	93.7
*Chlorella-Like, PB-5-U "Aged"	7.69	27.99	41.46	Insufficient Sample	
Chlorella-Like, PBSU 12/20	8.46	28.48	39.10	9.58	85.62
Selenastrum, Urea, Initial 2-2	8.72	31.60	40.77	18.15	99.24
Selenastrum, Urea, 2-9	9.72	30.03	33.62	25.47	98.84
Selanastrum, NO_3, 2-3	8.89	39.58	30.89	17.45	96.81
Selenastrum, NO_3, 2-10	8.74	37.04	38.11	15.84	99.73

*Sample container destroyed in shipment

TABLE 4

Fractionation of Lipids of Selenastrum SP on Silicic Acid Columns (Unisil)

	Hex	Benz	$CHCl_3$	Acet	MeOH	Total
Selenastrum, Urea, Initial 2/2	0.5	0.10	0.04	6.45	2.48	90.3
Selenastrum, Urea 2/9	< 0.01mg	0.14	0.20	6.23	2.96	92.9
Selenstrum, NO_3 2/3	< 0.01mg	0.07	0.17	5.00	7.32	96.5
Selenastrum, NO_3 2/10	< 0.01mg	0.07	0.20	2.57	2.05	81.0

TABLE 5

Approximate Chemistry of Selected Chlorophytes

Species	% Dry Weight				
	Protein	Carbohydrate	Lipid	Ash	Other
Chlorella (Nitrate) Aged	24.85	38.20	10.02	5.7	21.23
Chlorella (Nitrate)	23.85	35.25	16.20	5.01	19.69
Chlorella (Urea)	44.10	26.20	19.12	3.8	6.78
Chlorella (Urea) Aged	27.05	25.30	11.9	6.0	29.66
Chlorella (Aluminum)	26.75	32.35	16.83	4.0	20.07
Chlorella (Ammonia)	29.20	29.20	15.74	2.5	23.35
Oocystis (Urea)	39.65	26.05	14.85	6.9	12.55
Oocystis (Urea) Aged	37.10	27.70	14.79	6.3	14.11
Oocystis (Nitrate)	15.65	36.05	16.37	7.7	34.23
Platymonas (UH)	29.5	10.3	15.97	14.9	29.33

TABLE 6

Approximate Chemical Composition

of Caulerpa Prolifera

	% Cell Wt.	% Org. Wt.
Carbohydrate	72.4	90.1
Protein	16.7	20.8
Lipids	4.0	6.1
Ash	19.7	---

TABLE 7

Fractionation of Caulerpa prolifera lipids on Silicic Acid Column

Eluting Solvent	Wt. (mg)	%
Hexane	1.0	0.3
Benzene	15.5	4.2
Chloroform	4.8	1.3
Acetone	257.1	70.0
MeOH	90.8	24.6

TABLE 8

Lipids of Nanno Q Cultivated

in Nitrogen Deficient and Sufficient Media

Sample		Dry Cell Wt. (gm)	Wt. Total LIPIDS (gm)	Percent Lipids
Nitrogen Suff.	(1)	0.48731	0.09386	19.26
Nitrogen Suff.	(2)	0.24709	0.05383	21.79
Nitrogen Defic.	(3)	0.0447	0.0104	23.27
Supernat. of	(3)	0.07195	0.00772	10.73
Nitrogen Suff.	(4)	0.08038	0.02173	27.03
Nitrogen Def.	(5)	0.37810	0.05515	14.59

TABLE 9

Lipids from Nitrogen Sufficient or Deficient Nanno Q Cells

Fractionated on Silicic Acid Columns

Eluate	Nitrogen Suff. No. 1		Nitrogen Suff. No. 2		Nitrogen Deficient No. 3	
	(gm)	(%)	(gm)	(%)	(gms.)	(1%)
Hexane	0.00119	(1.8)	0.00176	(3.2)	0.00088	(6.1)
Benzene	0.00058	(0.9)	0.00019	(0.4)	0	(0)
$CHCl_3$	0.00291	(4.3)	0	(0)	0	(0)
Acetone	0.03152	(46.6)	0.03657	(67.3)	0.01307	(91.2)
MeOH	0.03150	(46.5)	0.01582	(29.1)	0.00038	(2.7)
	0.0677		0.05437		0.01433	

TABLE 10

Hydrocarbon Composition of Nanno Q

Tentative Identity	Nitrogen Sufficient (2)	Nitrogen Deficient (3)	Nitrogen Sufficient Hydrogenated
17:0	0.8	---	0.89
br-18:0	---	---	0.55
br-Δ-18	---	0.14	---
18:0	---	0.98	0.65
19:0	0.9	1.73	2.49
Δ19	1.8	1.32	---
br-20:0	---	---	1.51
br-Δ-20	<0.1	1.39	---
20.0	<0.1	0.56	1.06
21:0	<0.1	2.08	3.01
Δ21	---	1.22	---
br-22:0	---	---	2.32
br-Δ-22	---	---	---
22:0	<0.1	3.97	1.74
23:0	5.55	2.43	5.58
Δ23	0.34	3.32	---
br-24:0	---	---	0.98
br-Δ:24	0.60	---	---
24:0	<0.1	1.94	0.3
25:0	5.58	4.44	18.94
Δ25	15.99	5.51	---
br-26:0	---	---	12.16
br-Δ-26	3.18	1.06	---
26:0	1.5	1.13	1.53
27:0	15.02	11.98	20.20
Δ27	8.74	8.06	---
br-28:0	---	---	5.86
br-Δ-20:0	5.17	2.79	---
28:0	3.47	2.56	1.22
29.0	12.34	7.88	4.47
Δ29	1.10	1.89	---
br-30:0	---	---	<0.1
br-Δ-30	---	---	---
30:0	---	1.4	<0.1
31:0	---	---	0.28
Δ31	---	---	---
br-32:0	---	---	0.43
br-Δ-32	---	---	---
32:0	---	2.63	<0.1
33:0	---	---	<0.1
others	19.1	27.6	14.8

Samples determined on 300' x 0.03 stainless steel capillary column coated with OV-17; compounds tentatively identified by comparison to established standards and by extrapolation.

TABLE 11

Percentage Fatty Acids of Free Lipids of Nanno Q

Identity	Nitrogen Sufficient (2)	Nitrogen Deficient (3)
14:0	0.61	1.3
14:1	0.19	0.3
15:0	0.18	0.6
16:0	21.6	43.4
16:1	26.11	29.6
Br-17	0.78	0.3
17:0	0.26	0.2
18:0	0.89	2.2
18:1	10.34	15.6
18:2	0.11	---
18:3	0.64	---
19:0	0.15	---
20:3	12.44	2.1
20:4	25.67	4.4

TABLE 12

Neutral lipid components in Lipid

Fractions of Nanno Q-Nitrogen Sufficient/Deficient

acyclic hydrocarbons (++++)	Carotenes +++ Steryl esters (++++) ketones (?) (++) Triglycerides (+)	Steryl Ester (+) ketones (++) Triglycerides (++++) 1,3 Diglyceride (+) 1,2 Diglyceride (+++)	Triglyceride (+++) 18 resolved pigments	None

TABLE 13

Polar lipid components in Lipid

Fractions of Nanno Q-Nitrogen Sufficient/Deficient

Hex	benz.	$CHCl_3$	Acetoneeluate	Methanol eluate
none	none	none	5 resolved pigments Monogalactosyl diglyceride (++++) Diphosphatidyl diglyceride (++) Phosphatidyl ethanolamine (+) Phosphatidyl glycerol (+) Sterol glycoside (++)	Phosphatidic Acid (+++) Diphosphatidyl glycerol (++) Phosphatidyl Ethanolamine (+++) Phosphatidyl glycerol (+++) Phosphatidyl choline (++++) Digalactosyl diglyceride (+++) Phosphatidyl Inositol (++)

Abbrev: ++++ major compound
+++ moderate
++ minor
+ trace

TABLE 14

Approximate Chemical Composition

Phaeodactylum tricornutum

	% Cell Wt.	% Organic Wt.
Carbohydrate	20.2	22.8
Protein	26.3	30.0
Lipids	14.2	16.0
Ash	11.3	-
Other	27.9	31.6

TABLE 15

Fractionation of Phaeodactylum tricornutum Lipids of Silicic Acid Column

Eluting Solvents	Wt. (mg)	%
Hexane	2.3	0.8
Bezene	57.1	19.6
Chloroform	11.1	3.8
Acetone	158.3	54.3
MeOH	62.9	21.6

TABLE 16

Profile of Ankistrodesmus falcatus

	ASH %	PROT. %	CHO %	LIPID %	HEX.	BENZ.	$CHCl_3$	ACET.	MeOH	TOTAL
1) no KNO_3	7.0	13.3	18.3	insufficient sample						
2) no KNO_3, 10mM $NaHCO_3$	5.4	14.3	18.3	40.3	-	3.3	13.5	66.5	12.1	95.4
3) 1.5mM KNO_3 10mM $NaHCO_3$	7.0	34.4	11.4	27.2	-	2.5	9.8	72.6	19.5	104.4
4) 1.5mM KNO_3	13.6	28.6	9.2	19.5	-	5.8	16.4	69.2	12.0	103.4
2') re-extraction of 2				40.0	-	3.2	14.1	66.8	10.5	94.6

TABLE 17

Area % Saturated Fatty Acids

I.D.	Control	N-limited	C-limited	Sporulated
14:0	1.56	0.37	0.33	0.53
15:0	1.40	-	0.12	-
16:0	10.93	14.21	17.10	15.80
17:0	0.27	-	0.16	0.14
18:0	1.09	2.71	1.06	1.97
19.0	0.37	2.35	0.22	0.20
Total	15.62	19.64	18.99	18.64

TABLE 18

Area % Unsaturated or Branched Acids

I.D.	Control	N-limited	C-limited	Sporulated
16:1	2.25	0.96	0.91	1.11
16:3	0.75	-	0.41	0.22
16:4	14.73	3.89	3.76	1.40
ai17:0	0.52	-	0.23	0.15
18:1	24.62	48.38	55.49	53.82
18:2	2.98	3.19	3.33	2.43
18:3	26.45	10.69	11.40	11.44
18:4	4.81	2.60	2.49	3.60
20:1	0.61	1.32	1.22	3.59
20:2	0.49	-	0.27	-
22:1	-	0.58	0.25	0.35
Total	78.21	71.61	79.76	75.68

TABLE 19

Total % Accounted For, Saturated and Unsaturated

Control	N-limited	C-limited	Sporulated
93.82	91.25	98.75	94.32

TABLE 20

% Hydrogenated Fatty Acids

I.D.	Control	N-limited	C-limited	Sporulated
14:0	1.62	0.42	0.38	0.59
15:0	1.52	0.13	0.14	0.11
16:0	29.31	19.87	21.98	19.11
17:0	0.31	0.11	0.19	0.17
18:0	60.46	67.97	73.81	73.54
19:0	0.52	2.61	0.25	0.23
20:0	1.20	1.40	1.53	3.78
22:0	0.13	0.62	0.31	0.37

TABLE 21

Approximate Chemical Composition of Monallantus salina

Component	Nitrate Sufficient		Nitrate Deficient	
	Dry Cell Wt. (%)	Organic Wt. (%)	Cell Dry Wt.	Organic (%)
Ash	18	—	24	--
Protein	43	55.8	20	24.3
Carbohydrate	12	15.6	13	15.9
Lipid	22	28.6	49	59.8

TABLE 22

Percentage of Silicic Acid Fractionation of Monallantus salina lipids

Fraction	Cell Preparation	
	Nitrate Sufficient	Nitrate Deficient
Hexane	2	1
Benzene	5	5
Chloroform	5	25
Acetone	55	42
Methanol	33	27

TABLE 23-A,-B,-C

Thin Layer Chromatography of Monallantus lipids

Silicic Acid Fraction

A. Rf	Hex.	Benz.	$CHCl_3$	Acet.	Probable Identity
0.97	+	+			Neutral lipids
0.84			+	+	Triglyceride
0.79			+		Pigment
0.68			+		1,3-Diglyceride
0.62			+		1,2-Diglyceride

Solvent: Diethyl ether, benzene, ethanol, acetic acid (40:50:2:0.2).

B. Rf	Hexane	Benzene	Probable Identity
0.80	+		Hydrocarbons
0.77		+	Pigment
0.70		+	Steryl esters

Solvent: hexane, diethyl, ether, 96:4.

C. Rf	Acetone	Methanol	Identity
0.84	+		Monogalacytosyl-diglyceride
0.51		+	Phosphatidyl ethanolamine
0.44	+	+	Digalactosyl-diglyceride
0.43		+	Phosphatidyl glycerol
0.34		+	Phosphatidyl choline
0.23		+	Phosphatidyl inositol

Solvents: chloroform, acetone, methanol, acetic acid, H_2O; 50:20:10:10:5.

TABLE 24

Approximate composition of Cylindrotheica

	Nitrogen Sufficient		Nitrogen Deficient	
	Cell Dry Wt. (%)	Organic Wt. (%)	Cell Dry Wt. (%)	Organic Wt. (%)
Ash	51	--	67	--
Protein	10	20	4	12
Carbohydrate	8	16	5	15
Lipid	9	18	4	12
Unassigned	22	15	20	61

TABLE 25

Silicic Acid Fractions of Lipids of Cylindrotheica

Fraction	Nitrate Sufficient	Nitrate Deficient
Hexane	3%	3%
Benzene	3%	3%
Chloroform	16%	16%
Acetone	55%	56%
Methanol	19%	21%

TABLE 26

Approximate Chemical Composition Relative to Culture Age

Organism	(days) Culture Age	Relative Percent Carbohydrate	Protein	Lipid
Ankistrodesmus	14	10.8	31.1	24.5
	20	11.7	30.6	23.8
	24	26.4	24.8	15.2
	30	8.7	22.9	35.7
Isochrysis	14	11.2	37.0	8.1
	20	10.4	35.6	10.4
	24	10.8	36.1	10.6
	30	18.9	34.9	5.3
	36	15.6	23.4	27.8

TABLE 27

Effects of temperatures on lipid production in Isochrysis and Ankistrodesmus*

Culture	Cellular Pigmentation	Dry Cell Wt.	Ash	Lipid Wt. Cell Wt.	% Lipids Cell Wt.	% Lipid Org. Cell Wt.
Isochrysis 25° (NE)	Green	1.56687	0.26041	0.14905	9.51	11.41
Isochrysis 20° (NE)	Yellow-Green	0.89340	0.06379	0.11885	13.22	14.33
Isochrysis 15° (NE)	Yellow	1.34903	0.11116	0.18035	13.37	14.57
Ankistrodesmus 25° (NE)	Yellow	0.48105	0.02020	0.10458	21.74	23.20
Ankistrodesmus 20° (NE)	Yellow	0.50018	0.02021	0.09329	18.65	19.44
Ankistrodesmus 15° (NE)	Yellow	0.51000	0.02043	0.10093	19.79	20.62

*cultivated in 1ℓ batches in Roux bottles with active agitation and vigorous bubbling with 1% CO_2 in air.

TABLE 28

Lipid Composition of Ankistrodesmus
in Nitrogen Deficient and Efficient Media
at Different Temperatures*

Culture Conditions	% Lipid Content per Cell Dry Wt.
35°C NS	14.18
30°C NS	16.89
25°C NS	20.89
25°C ND	40.89
20°C NS	19.00
20°C ND	22.8
15°C NS	20.10
15°C ND	18.64

*Cultivated in Roux bottles with agitator and CO_2 enriched aeration. Nitrogen deficient cultures were incubated up to 2 months.

Table 29A

Percentage of Fatty Acid Methylesters

Fatty Acid Identity	Ret Time (Min)	776 (Green)	777 (Orange)	947 (Brown)	1185 (White)	1242 (Green)
14:0	15.748	.15	-	-	-	-
14:1	16.318	.05	-	-	-	-
i:15:0	-	-	-	-	-	-
15:0	17.836	.05	-	-	-	-
16:0	20.321	26.80	11.40	17.74	16.19	10.72
16:1	20.583	1.73	2.50	.27	.40	.85
16:2	21.608	.54	2.71	.49	1.37	.44
i:17:0	22.351	.33	-	.24	.08	-
17:0	23.165	1.45	3.45	1.92	1.64	1.64
17:1	23.847	2.61	.97	3.18	-	1.35
18:0	25.511	31.32	44.92	30.48	41.17	40.94
18:1	26.404	7.16	16.55	20.57	15.74	12.49
18:2	27.106	.90	-	-	-	-
19:0	28.138	9.56	8.77	11.48	2.48	11.31
19:1	28.943	1.79	-	1.29	-	1.32
20:0	30.014	.53	.59	.51	.70	.58
20:1	30.632	.73	.59	.87	.93	1.06
20:2	-	-	-	-	-	-
20.3	37.265	.73	2.12	1.75	-	.75
20.4	39.359	.05	-	-	1.0	1.28
		86.48	94.57	90.89	81.90	84.73

Table 29B

Percentage of Fatty Acid Methylesters

Fatty Acid Identity	1249 (Green)	1981 (Green)	1979 (Brown)	1707 (Green)	1445 (Green)	836 (White)	6-13 (Yellow)
14:0	-	.15	-	.38	.93	-	-
14	-	.02	-	-	-	-	-
i15:0	-	.07	-	-	-	-	-
15:0	-	.08	-	.18	.17	-	.35
16:0	9.55	22.91	14.54	11.62	29.63	8.35	24.95
16:1	13.05	.40	-	.73	1.13	-	1.85
16:2	-	.47	.65	.90	.76	-	1.47
i17:0		1.17	-	.40	.10	-	.14
17:0	2.82	1.21	2.05	.93	.79	-	4.27
17:1	-	2.08	4.36	-	.59	-	3.31
18:0	37.03	24.86	14.85	28.24	5.07	48.07	32.04
18:1	2.89	10.14	7.07	10.83	31.75	10.13	4.05
18:2		10.44	16.04	1.44	2.47	-	
19:0	5.84	12.56	16.04	1.25	8.09	5.30	19.85
19:1	-	1.22	2.87	-	1.23	-	5.22
20.0	-	.34	-	1.25	1.02	1.81	.14
20:1	-	1.03	-	.58	.1	-	.37
20:2	-	-		13.40	.5	.47	-
20:3	-	1.0	1.00	.58	.5	1.46	-
20:4	-	1.0	.94	1.05	.1	-	1.98
	71.18	81.19	80.41	73.81	84.92	75.59	100.99

Table 29C

Percentage of Fatty Acid Methylesters

Fatty Acid Idenity	778 (White)	754 (White)	343 (Orange)	251 (Green)	113 (Orange)	100-6 (Yellow)	138 (Green)
14:0	-	.55	-	.08	-	-	.40
14:1	-	.82	-	-	-	-	-
i15:0	.36	-	-	.04	-	-	-
15:0	.08	.55	-	.06	-	-	-
15:1	-	2.80	-	-	-	-	-
16:0	23.47	32.05	5.42	18.64	11.40	11.07	18.72
16:1	.61	.77	.89	.94	-	1.39	1.35
16:2	.21	.55	.48	1.12	2.85	.80	1.44
i17:0	.42	-	-	.13	-	-	2.80
17:0	1.34	.71	1.24	3.20	4.75	4.56	5.59
17:1	.73	.55	.48	1.19	-	1.15	1.04
18:0	53.00	33.37	43.27	29.58	20.91	45.91	35.74
18:1	10.90	11.38	16.50	15.54	27.87	11.57	11.61
18:2	1.06	-	3.63	-	-	-	-
19:0	5.17	1.54	7.14	10.95	13.53	21.14	8.29
19:1	.15	-	-	1.99	-	.92	2.06
20:0	.20	4.92	1.18	.33	11.88	.16	5.77
20:1	.48	1.48	1.12	1.00	-	.52	.84
20:2	-	-	-	-	-	-	1.60
20:3	.81	2.96	3.28	0.1	7.13	.19	2.30
20:4	.40	-	-	0.1	-	.62	.43
	99.39	95.50	84.63	84.99	100.32	100.00	99.98

TABLE 30

Toxicity Measurements on Selected Algae

Inhibitor	D. salina*	D. bardawil*	I. galbana
$Pb(NO_3)_2$:			
10mM	-	-	-
0.1mM	+	+	-
1 μM	+	+	+
Pyridine			
10mM	-	+	-
0.1mM	+	+	-
1 μM	+	+	+

*response recorded as growth (+) or nongrowth (-).

TABLE 31

Effects of CPTA on fractionated lipids of Dunaliella salina*

Unisil Fraction	Light Control	Light, CPTA (%)	Dark, Control	Dark CPTA (%)
Hexane	5	3	4	0
Benzene	6	2	2	0
Chloroform	11	13	23	15
Acetone	56	55	46	57
Methanol	22	26	25	28

*data reported as percentage of the total.

TABLE 32

^{14}C-Glucose Uptake Studies in Botryococcus in Light

	Cell Preparation	
	Nitrate Sufficient	Nitrate Deficient
Initial Counts	19,000 cpm	19,000 cpm
Counts remaining in media after two days	4,160 cpm	9,500 cpm
Chloroform lipids	2,570 cpm (17.3%)*	3,330 cpm (35.0%)*
Methanol-water soluble	1,120 cpm (7.5%)*	1,280 cpm (13.5%)*

*% of total incorporation.

TABLE 33

^{14}C-Glucose Uptake Studies in Botryococcus in Dark

	Cell Preparation	
	Nitrate Sufficient	Nitrate Deficient
Initial Counts	19,000 cpm	19,000 cpm
$^{14}CO_2$ evolved	7,780 cpm	1,430 cpm
Chloroform lipids	1,520 cpm (0.19)*	1,550 cpm (1.08)*
Methanol-water soluble	1,220 cpm (0.16)*	650 cpm (0.48)*

*the ratio of incorporated ^{14}C to respired ^{14}C.