77

BARD

FINAL REPORT

PROJECT NO. I-1082-86

**Potential Enhancement of Solar Energy
Conversion into Biomass and Lipids**

A. Sukenik, A. Ben-Amotz, P.G. Falkowski

1991

Signature _____
Institution's Authorizing Official

Table of Contents

	Page
A. Abstract	2
B. Objectives of the original research proposal	3
C. Description of the research results	3
1. General introduction	3
2. Regulation of the photosynthetic apparatus	4
2.1 Nitrogen limitation - regulation of photosynthetic energy conversion and abundance of chloroplast proteins	5
2.2 Molecular characterization of the major light harvesting complex of <i>I. galbana</i>	6
2.3 Photoadaptation - variations in the molecular composition of the photosynthetic machinery	8
2.4 The role of chlorophyll synthesis in stabilization of chlorophyll protein complexes	8
3. Cellular lipid composition and environmental conditions	10
3.1 Effect of environmental conditions on lipid content and fatty acid composition	10
3.2 Fatty acid composition and quantitative morphology of <i>Isochrysis galbana</i>	12
4. Regulation of carbon flow into fatty acids	18
4.1 Purification and characterization of acetyl CoA carboxylase (ACCase)	18
4.2 The role of ACCase in regulating lipid synthesis	20
4.3 Regulation of lipid synthesis in nitrogen deprived cultures and during recovery from starvation	21
5. Algal biomass and lipid production strategies for controlling productivity	23
D. Description of cooperation	25
E. Evaluation of the research achievements	26
F. List of resulting publications	30

A. Abstract

In unicellular algae grown under nutrient sufficient conditions, between 10 to 20 percent of the biomass is comprised of lipids of different classes. Under growth limiting conditions such as low nitrogen availability, cellular composition is changed and the abundance of lipids and carbohydrates increases at the expense of proteins. The present research focused on the physiological and biochemical mechanisms that are involved in that metabolic shift. The effect of growth irradiance level and nitrogen availability on light harvesting capabilities and light energy conversion by photosynthesis was elucidated in the marine unicellular alga *Isochrysis galbana*. Variations in lipid content and fatty acid composition which are associated with changes in growth conditions were studied in that alga. In addition, our scientific effort was focused on the regulation of carbon flow into lipid production and the lipid synthesis machinery with special emphasis on specific enzymatic steps.

The results of this study demonstrated the relationships between lipid content and composition, and the physiological and photosynthetic performances in *Isochrysis galbana* grown under various environmental conditions. Changes in the cellular abundance of structural lipids are correlated with variations in cellular ultrastructure and with the abundance of photosynthetic complexes. Physiological and photosynthetic activities are affected by cellular responses to nitrogen limitation and light availability at the molecular level. A special effort was made to purify the major light harvesting complex of *Isochrysis*, the fucoxanthin chlorophyll protein (FCP) for biochemical and molecular characterization. A cDNA library was constructed and screened for clones of the FCP gene. Isolated clones are currently sequenced for an advanced molecular study of that complex.

The role of acetyl CoA carboxylase (ACCase) in controlling lipid synthesis was elucidated in the prymnesiophyte *Isochrysis galbana*. This enzyme catalyzes the formation of malonyl CoA, the building block of fatty acids, and is considered as a regulatory enzyme in the synthesis of fatty acids. ACCase of *I. galbana* was isolated and purified to near homogeneity and was kinetically characterized. Polyclonal antibodies were prepared against the subunit of the enzyme and were further used to study the relationships between the cellular content of the enzyme, its *in vitro* activity and the accumulation of lipids in algal cells grown under various nitrogen limiting conditions. Quantitative relationships between growth conditions and lipid synthesis, lipid accumulation and fatty acid distribution were established, suggesting a biochemical mechanism that regulates fatty acid synthesis.

Physiological data were incorporated into a mathematical simulation model that was constructed to calculate daily biomass production in an outdoor algal mass culture system. The model evaluated various operational parameters of algal production ponds and calculated the potential productivity of algal biomass.

B. Objectives of the original research proposal

The general aim of the original research proposal was to elucidate molecular mechanisms which affect the harvesting and utilization of light energy and the subsequent flow of the photosynthetically stored energy into lipids in the marine unicellular algae *Isochrysis galbana*.

Four specific goals were presented by our original proposal:

1. to elucidate the effect of light intensity and nitrogen availability on photosynthetically stored energy in lipids.
2. to explore the regulation of lipid and fatty acid biosynthesis in relation to environmental conditions.
3. to understand the molecular bases of photosynthetic responses to environmental conditions (nitrogen availability and irradiance level).
4. to develop a simulation model of algal mass culture systems, where experimental data can be interactively examined to assess the effects of genetic manipulation and environmental conditions on biomass and lipid production.

C. Description of the research results

1. General introduction

There is an increasing commercial interest in microalgal biomass production as a source of human food, animal feed, energy and biomass production. Primarily because of economic considerations, attention has turned increasingly to the commercial production of fine chemicals by algae such as β carotene, phycocolloids, and lipids. Nevertheless, the use of unicellular algae as a preferred feed for many marine organisms during different stages of their rearing procedure is the most common application of algal mass culture. Aquaculture systems produce several unicellular algal species and use them as a primary feed source in the food web of oysters, mussels, brine shrimp and fish larvae. Evidence has accumulated that the quality as well as the quantity of microalgal lipids are very important in the nutrition of marine animals. Some polyunsaturated fatty acids synthesized by algae are essential for growth and development of marine fish larvae, shrimp and mussels.

In mariculture systems, the most commonly used marine unicellular algae are those which produce polyunsaturated fatty acids from the omega-3 group such as eicosapentaenoic (C20:5) and decosaheptaenoic (C22:6) acids. Furthermore, recent medical studies showed that polyunsaturated fatty acids are an important constituent of human diet and that omega-3 fatty acids may reduce cholesterol level in the blood and reduce morbidity risks of coronary heart diseases and atherosclerosis. The current major source for omega-3 fatty acids is fish oil. However, the capability of fish to synthesize these fatty acids is limited, and fish consume and accumulate them via the food chain, where algae are the primary source of these fatty acids.

In this study we investigated the regulation of lipid production at the physiological and biochemical levels in the marine prymnesiophyte *Isochrysis galbana*. The photosynthetic activity and lipid composition in conjunction with physiological responses to light intensity and nitrogen availability were intensively studied. Our attention was focused on the effect of environmental conditions on the light harvesting complex, its molecular organization and physiological responses. Furthermore, the regulation of carbon flow into lipid production and the lipid synthesis machinery was studied with special emphasis on specific enzymatic steps.

2. Regulation of the photosynthetic apparatus

The effect of nitrogen availability and growth irradiance levels on photosynthetic performance and growth of microalgae has been described in many reviews. However, the effects on the organization of the photosynthetic apparatus, which lead to changes in photosynthesis and growth, have only partly been studied. Nitrogen limitation is known to induce chlorophyll degradation and reduction in protein content. It has been shown that one of the earliest molecular events in a nitrogen depleted culture is a reduction in the cellular level of the major light harvesting complex. Such molecular events are responsible for changes in the photosynthetic performance of the cells and eventually cause changes in the quality and quantities of cellular compounds.

In the following sections we describe the effects of nitrogen limitation and growth irradiance levels on the molecular organization of the photosynthetic apparatus, photosynthetic performance and growth of *Isochrysis galbana*. Two major photosynthetic protein complexes of *I. galbana* were investigated at their molecular level: the carboxylation enzyme ribulose biphosphate carboxylase/oxygenase (Rubisco) and the major light harvesting complex (LHC), the fucoxanthin chlorophyll protein complex (FCP). The proteins of these complexes were isolated and purified from *I. galbana* and polyclonal antibodies were prepared. We further analysed the fucoxanthin chlorophyll protein of *I. galbana* for its N terminal amino acid sequence. A cDNA library suitable for identification of a nuclear gene was constructed, and several cDNA clones that correspond to the FCP gene of *I. galbana* were isolated. The cDNA clones will be sequenced and compared to known sequences of light harvesting chlorophyll proteins of other algae.

2.1 Nitrogen limitation - regulation of photosynthetic energy conversion and abundance of chloroplast proteins

The availability of fixed inorganic nitrogen is a major factor influencing the growth and chemical composition of algae. As cells become nitrogen starved, light saturated photosynthesis rates, photosynthetic energy conversion efficiency and maximum quantum yields all decline. Only a few experiments have been conducted under steady state conditions where nitrogen limitation rather than starvation occurred. In spite of the attention nitrogen starvation has received in the phycological literature, a quantitative relationship between photosynthesis and nitrogen availability is lacking.

We examined the effect of steady state nitrogen limited growth on physiological functions related to photosynthetic energy conversion in *Isochrysis galbana*. We used nitrogen limited chemostats to establish steady state growth rates over a wide range of dilution rates. Simultaneously, the effect of nitrogen limitation on key proteins and electron transport components in the photosynthetic apparatus was investigated. The results of that study were published in two consecutive manuscripts in the Journal of Phycology.

2.2 Molecular characterization of the major light harvesting complex of *I. galbana*

Primary characterization. The major light harvesting complex of *Isochrysis*, the chlorophyll fucoxanthin protein (FCP) complex, was purified on a sucrose density gradient. Two major chlorophyll protein complexes were obtained by that procedure. The FCP complex at the upper part of the gradient was visualized under both visible and long wavelength UV. The other complex, presumably PS I, was located deeper in the sucrose density gradient, contained primarily chlorophyll *a*, and did not fluoresce at long wavelength UV.

The FCP complex was highly enriched with chlorophyll *c* and fucoxanthin. The stoichiometric relationships of the pigments of FCP were 70 molecules of chl *c* and 120 molecules of fucoxanthin per every 100 molecules of chl *a*. The FCP complex contained two apoproteins at an apparent molecular weight of 22 and 23 kDa. The apoproteins of the FCP complex isolated on sucrose density gradient were further purified by semi preparative SDS polyacrylamide gel electrophoresis. The resolved polypeptides were electroeluted, checked for purity and used for the preparation of polyclonal antibodies. Highly specific immunological reactivity against the original apoproteins of *Isochrysis* FCP was observed with the prepared antibodies. The antibodies were used in our attempt to evaluate the effect of growth conditions on the cellular content of light harvesting capability, and to screen the cDNA expression library.

The 23 kDa polypeptide of FCP of *Isochrysis galbana* was further analyzed for its N terminal sequence. Little, if any homology was seen between the N terminal sequence of that apoprotein and the mature LHCI polypeptides of *Dunaliella tertiolecta* (Table 1). Homology was apparent only between the 28.5 and 24.5 kDa mature proteins of *Dunaliella* which differed by only one out of 15 peptides. Sequence homology was analyzed with other published protein sequences using the GENED computer program from Cold Spring Harbor. No sequence homology was detected between the 23 kDa apoprotein from *I. galbana* and other known proteins in the photosynthetic apparatus in other photosynthetic organisms.

Table 1. N - terminal sequences of LHC II apoproteins.
Comparison of sequences from *D. tertiolecta* and *I. galbana*
mature LHC II proteins.

Mature Protein (kDa)	Amino acids
<i>Isochrysis galbana</i>	
23	FHE()DLRVRRPRG()Y
<i>Dunaliella tertiolecta</i>	
31	AQYDE(M)QA(M)
30	AKSTKPGAKAWSPEFLAANQ
28.5	VEFYGPDRAKFLGPF
24.5	VEFYGPDAAKFLGPF

Coding site of FCP proteins and RuBisco. Comparisons between control protein preparations and those treated with inhibitors showed that particular proteins were absent or decreased in concentration in the presence of cycloheximide or chloramphenicol. The FCP abundance of *I. galbana* was decreased in the presence of cycloheximide, but appeared to remain unchanged in the presence of chloramphenicol. The FCP proteins were identified using the Western blot. These results indicate that the gene encoding the FCPs of *I. galbana* are nuclear encoded.

Genes encoding the large and small subunits of RuBisco appear to be encoded in the same location within *I. galbana* cells. Comparisons between autoradiographs and gels of soluble protein fractions indicated that chloramphenicol inhibits synthesis of the large and small subunit of Rubisco. Proteins were again identified by Western blot. These results suggest that both the large and small subunit of RuBisco genes are chloroplast encoded. Other chromophytes and rhodophytes previously studied also appear to have chloroplast encoded large and small subunits of RuBisco. In contrast, higher plants and unicellular green algae appear to have only the large subunit of Rubisco encoded in the chloroplast, while the small subunit is encoded in the nucleus.

Construction of cDNA library and isolation of cDNA clones of the FCP gene. Poly A⁺ RNA was isolated from fresh cells of *I. galbana* grown under low irradiance (70 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$). The poly A⁺ RNA was used to construct a cDNA library which was inserted into Lambda gt11 cloning vector. The unamplified library was screened with *I. galbana* FCP antibody using 1:500 dilutions, and positive clones were isolated. The isolated clones are currently being sequenced and will be further used in physiological experiments to elucidate the molecular regulation of FCP complexes in *Isochrysis galbana*.

The results of this study were summarized in the M.Sc. dissertation of Diane L. Henry that was submitted to the graduate school SUNY at Stony Brook, under the supervision of Dr. Paul Falkowski.

2.3 Photoadaptation - variations in the molecular composition of the photosynthetic machinery

The effect of growth irradiance level on pigment composition and photosynthetic performance was studied in many unicellular algae. In *Isochrysis galbana*, cellular pigment content generally decreased in response to an increase in light intensity. Spectra of pigment extracts from high light and low light grown cells are shown in Figure 1 (absorption values were normalized to chlorophyll *a* maxima at 664 nm). The results indicated that low light acclimated cells increased their chl *c* (451 nm) and fucoxanthin (482 nm) concentrations relative to chlorophyll *a*, in comparison to high light grown cells.

Total proteins were extracted from high and low light grown cultures of *I. galbana* and separated by SDS polyacrylamide gel electrophoresis. Resolved proteins were further transferred to nitrocellulose (Western blot) and specific polypeptides were detected by polyclonal antibodies. Antibodies against FCP of *I. galbana* were used at 1:10,000 dilution, and those against the large subunit of RuBisCo at 1:5,000 dilution. Results indicated that the relative abundance of the 23 and 22 kDa FCP apoproteins was greater in low light grown cells than in high light cultures. The cellular abundance of the large subunit of RuBisCo per cell appeared to be the same for high and low light adapted cultures. These results suggest that variations in chlorophyll composition and photosynthetic performance observed in low and high light acclimated cultures of *I. galbana* are associated with changes and adaptation of the photosynthetic apparatus at the molecular level.

The results of this study were summarized in the M.Sc. dissertation of Diane L. Henry that was submitted to the graduate school SUNY at Stony Brook, under the supervision of Dr. Paul Falkowski.

2.4 The role of chlorophyll synthesis in stabilization of chlorophyll protein complexes

Unicellular algae respond to a decrease in growth irradiance by increasing the number and density of thylakoid membrane proteins. The photoadaptive responses are fast and associated with rapid protein synthesis. Among the first proteins which

Figure 1. Absorption spectra of pigment extracts of high and low light acclimated *Isochrysis galbana* cells. Absorbance values are expressed relative to chl *a*. Dotted line represents high light and solid line represents low light acclimated cells.

increase following a high to low light transition are the light harvesting proteins. Accumulation of these proteins is accompanied by an increase in chlorophyll content. One possible mechanism for coordinating the two components of the pigment-protein complex is based on post-translational stabilization of newly synthesized light harvesting proteins by chlorophyll.

The role of chlorophyll synthesis in stabilization of LHC was studied in the marine chlorophyte *Dunaliella tertiolecta*. The wide physiological and molecular information that we gained during the last five years on that algal species supports our efforts to resolve that general question. The results of this study are relevant and applicable to other algae including the prymnesiophyte *Isochrysis galbana*.

The results of this study confirm that chlorophyll synthesis is required for a shade adaptive increase in LHC II similarly to conclusions reached by others with regard to the light induction of LHCP mRNA accumulation in other green algae. This study was recently published in Plant Physiology.

3. Cellular lipid composition and environmental conditions

The effect of light intensity and nitrogen availability on cellular chemical composition were studied in continuously grown cultures of *Isochrysis galbana* with special emphasis on lipid composition and fatty acid distribution. Chemical analysis of the major lipid constituent of the alga indicated that C18:4 was the major fatty acid of the galactolipids, while the polyunsaturated omega-3 fatty acid C22:6 was found primarily in the phospholipid fraction. A detailed analysis of monogalactosyl diacylglycerol, the major component of the photosynthetic membranes, by high performance liquid chromatography (HPLC) equipped with flame ionization detector (FID) verified these results. Electron micrographs of thin sections of *I. galbana* cells grown under various environmental conditions demonstrate variations in cell ultrastructure. The relative surface density of thylakoid membranes was the main structural component affected by environmental conditions. These changes were associated with variations in the cellular abundance of specific lipid classes and in cellular fatty acid composition.

The following sections describe the experimental results of this study. They suggest common mechanisms that regulate the synthesis and accumulation of lipids and control the distribution of fatty acids in unicellular algae.

3.1 Effect of environmental conditions on lipid content and fatty acid composition

We studied the effects of environmental conditions, such as light intensity and nitrogen availability, on cellular lipid content and fatty acid composition in the marine prymnesiophyte *Isochrysis galbana* grown in steady state continuous cultures. This algal species is widely used as a preferred natural feed for many marine organisms including oysters, mussels, and brine shrimps, and is used as a prime feed in the food web of fish larvae in mariculture systems.

High light intensity caused accumulation of carbohydrates, while cellular lipid content slightly decreased. Nitrogen limitation, however, caused an increase of both carbohydrate and lipid content. Variations in cellular lipid content were associated with changes in fatty acid composition. High light conditions or severe nitrogen

limitation conditions imposed an increase in the relative abundance of palmitic (C16:0) and oleic (C18:1) acids and a decrease in the percentage of the polyunsaturated fatty acid C18:4. The relative distribution of decosaheptaenoic acid (C22:6) slightly increased under high light conditions and decreased under severe nitrogen limitation.

The results of this study will be soon published in *Aquaculture*.

ABSTRACT

Sukenik, A. and Wahnou, R., Biochemical quality of marine unicellular algae with special emphasis on lipid composition. I. *Isochrysis galbana*.

The prymnesiophyte *Isochrysis galbana* contains relatively large amounts of valuable lipids and is commonly grown as a food source for commercial production in aquaculture. The effect of environmental conditions such as light intensity and nitrogen availability on cellular chemical composition with special emphasis on lipid content has been studied in continuously grown cultures of *I. galbana*. High light intensity caused accumulation of carbohydrates, while cellular lipid content slightly decreased. Nitrogen limitation, however, caused an increase of both carbohydrate and lipid content. Variations in cellular lipid content were associated with changes in fatty acid composition. High light conditions or severe nitrogen limitations were associated with an increase in the relative abundance of palmitic (C16:0) and oleic (C18:1) acids and a decrease in the percentage of the polyunsaturated fatty acid C18:4. The relative distribution of the highly unsaturated fatty acid docosahexaenoic acid (C22:6) slightly increased under high light conditions and decreased under low nitrogen levels. Thus the polyunsaturated to saturated fatty acid ratio decreased when *I. galbana* was grown under high irradiance levels or under nitrogen limiting conditions.

Key index words: chemical composition, light intensity, nitrogen limitation, fatty acids, *Isochrysis galbana*.

INTRODUCTION

Several marine unicellular algal species are widely used as a preferred natural feed for many marine organisms including oysters, mussels, brine shrimp, and as a primary feed source in the food web of fish larvae (De Pauw and Persoone, 1988). Evidence has accumulated that the quality as well as the quantity of microalgal lipids are very important in the nutrition of marine animals. It has been shown that some polyunsaturated fatty acids synthesized by algae are essential for growth and development of marine fish larvae (Koven et al., 1989), shrimp and molluscs (De Pauw et al., 1984). In mariculture systems, the most commonly used marine unicellular algae are the prymnesiophyte *Isochrysis* and the eustigmatophyte *Nannochloropsis*, marine unicellular algae that are enriched with C22:6 and C20:5 fatty acids, respectively (Rodhouse et al., 1983; Pillsbury, 1985; Ben Amotz et al., 1987; Whyte, 1987).

The chemical composition of microalgae can be physiologically manipulated. Under nutrient sufficient conditions, cells synthesize mainly proteins to support growth and division (Myers, 1980). However, when a culture is deprived of an essential nutrient, cell division is stopped, and the fraction of carbon allocated to lipids and carbohydrates can be greatly increased at the expense of protein synthesis. The effect of environmental factors on lipid and fatty acid compositions has been investigated in various eukaryotic algae (Spoehr and Milner, 1949; Shifrin and Chisholm, 1981; Piorreck et al., 1984; Cohen et al., 1988). In most algae, enhancement of lipid accumulation is imposed by nitrogen deficient conditions. In diatoms, accumulation of neutral lipids is greatly increased under silicon deficiency (Shifrin and Chisholm, 1981; Roessler, 1988).

We report herein on the effects of growth irradiance levels and nitrogen availability on cellular lipid content and fatty acid composition in *Isochrysis galbana*. Although many investigators studied the effect of nutrient starvation on

lipid content and composition, the effect of growth irradiance level on cellular lipids has hardly been investigated. Furthermore, most of the reported studies were performed in batch cultures where growth conditions including light and nutrient availability varied with time. In this study, continuously grown cultures in chemostats and turbidostats were used to evaluate the effect of environmental parameters on lipid content in *Isochrysis galbana* with special emphasis on fatty acid distribution.

MATERIALS AND METHODS

Organism

Isochrysis aff. *galbana* (T-ISO) was obtained from the microalgae culture collection of U.S. DOE Solar Energy Research Institute (Golden, CO).

Growth conditions

Turbidostat cultures. To evaluate the effect of growth irradiance level on cellular composition, cultures were maintained at 25°C under steady state conditions in 2.6 liter turbidostats with natural sea water enriched with f/2 nutrients (Guillard and Ryther, 1962) as previously described (Suklenik et al., 1987). Light was provided continuously by banks of VHO fluorescent tubes, and cells were grown at six irradiance levels: 30, 80, 175, 300, 400 and 500 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$. The cultures were kept optically thin (2×10^6 cells ml^{-1}) to minimize self shading. The pH of the sea water was buffered at 7.8, and cultures were continuously bubbled with air. All cultures were maintained in steady state for several days prior to measurements.

Chemostat cultures. Nitrogen limited cultures were grown continuously at 25°C in similar 2.6 liter vessels using artificial sea water media, supplied with 150 μM NaNO_3 and other f/2 nutrients. Light was provided continuously by banks of VHO fluorescent tubes supplying a photon flux of 175 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ (400 to 700 nm). All cultures were maintained in steady state for several days prior to measurements.

Analytical methods

Cells were counted by Coulter Multisizer (Coulter Electronics Ltd., U.K.). Cellular pigments were extracted in 90% acetone, and spectra were recorded on a Hewlett-Packard diode array spectrophotometer model 8452A interfaced with a Hewlett-Packard Chemstation. Chlorophylls *a* and *c* were calculated using the equations of Jeffrey and Humphrey (1975). Total carotenoid concentrations were calculated as previously described (Sukénik et al., 1987). Proteins were assayed as described by Lowry et al. (1951) after hydrolysis in 1 N NaOH for 1 h at 100°C. Total carbohydrates were analyzed by the phenol sulphuric acid method following acid hydrolysis in 2 N HCl for 1 h at 100°C (Kochert, 1978).

Extraction and quantification of lipids

Total lipids were extracted with chloroform:methanol:water (5:10:4 v/v/v) (Bligh and Dyer, 1959) as modified by Kates (1972). The lipids were then phase separated by adjusting the solvent ratios to 10:10:9 (chloroform:methanol:water, v/v/v). The chloroform phase was evaporated to dryness under a stream of N₂, and dissolved in a known volume of chloroform:methanol 85:15 (v/v). Total lipids were quantified by the sulphuric acid charring technique (Marsh and Weinstein, 1966) that was calibrated versus tripalmitin as previously described (Sukénik et al., 1989). Total lipid extracts were fractionated to the major lipid groups on a heat activated silicic acid column as previously described (Sukénik et al., 1989).

Preparation and determination of fatty acids

Fatty acid extracts were prepared by saponifying of whole algal cells or total lipid extract in a strong base, followed by methylation (Lepage and Roy, 1984). The fatty acid methyl esters were separated by capillary gas liquid chromatography using a Hewlett Packard 5890A gas chromatograph as previously described (Sukénik et al., 1989). Identification of fatty acid methyl esters was accomplished by comparing the retention times of experimental samples to those of known standards.

RESULTS

Effect of irradiance levels

A typical irradiance growth response was recorded for *Isochrysis galbana* cultures grown at irradiance levels ranging from 30 to 500 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$. Growth was saturated at 300 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$, while a slight decrease in doubling rate, apparently due to photoinhibition, was measured at 500 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ (Table 1). Cellular pigment content generally decreased in an exponential mode in response to an increase in light intensity (Fig. 1). However, a disproportional reduction in chlorophyll *c* and carotenoids relative to chlorophyll *a* was recorded (Fig. 1a). Cellular protein content was not affected by light intensity and remained relatively stable at an average of 6.9 pg protein cell⁻¹ over the entire irradiance range (Table 1). Carbohydrates content increased almost three fold from 2.7 up to 7.0 pg cell⁻¹, while total lipid content decreased from 6.2 at 80 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ to 4.4 pg cell⁻¹ at 500 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ (Table 1).

The major fatty acids in *Isochrysis galbana* were C14:0, C16:0, C16:1, C18:1, C18:2 and the omega-3 polyunsaturated acids C18:4 and C22:6. Fractionation of lipid extracts from *I. galbana* on a silicic acid column indicated that the galactolipids of this alga are preferentially enriched with C18:4 fatty acid (Fig. 2). Neutral glycerolipids, mainly triacylglycerol, contained C16:0 and C18:1 fatty acids. The phospholipid fraction of *I. galbana* was enriched with C22:6 fatty acid accompanied by C18:1, C16:0 and C14:0 fatty acids (Fig. 2). Increasing light intensity caused variations in cellular fatty acid distribution. The percentage of C18:4 fatty acid decreased, while the relative distribution of C16:0, C14:0, C18:1 and C22:6 slightly increased as growth irradiance level increased. The ratio of omega-3 polyunsaturated acid to the sum of saturated and monoenoic fatty acid decreased as light intensity increased (Table 2).

Effect of nitrogen limitation

Growth rate of *Isochrysis galbana* decreased as the daily nitrogen load was decreased from 140 to 80 $\mu\text{mole L}^{-1} \text{ d}^{-1}$, which correspond to daily dilution rates ranging between 0.2 and 0.9 day^{-1} (Table 3). Chlorophyll *a* content decreased in response to the reduction in nitrogen load (Fig. 3). The molar ratio of total carotenoids to chlorophyll *a* decreased while the chlorophyll *a* to chlorophyll *c* ratio slightly increased as nitrogen load increased (Fig. 3). Cellular protein content increased from 3.5 to 5.3 pg cell^{-1} as nitrogen load increased (Table 3). Carbohydrate and lipid content decreased by about 60% as nitrogen load was increased.

Fatty acid composition of *Isochrysis galbana* was also affected by nitrogen limitation. Under severe nitrogen limitation (daily nitrogen load of 70 μmole), the percentage of C16:0 and C18:1 increased at the expense of C18:4 and C22:6 (Table 4). As nitrogen load increased, the relative distribution of C18:4 and C22:6 fatty acids increased. Consequently the ratio of omega-3 polyunsaturated acid to the sum of saturated and monoenic fatty acid increased as the nitrogen load increased (Table 4).

DISCUSSION

Phytoplankton is considered to be the primary food of marine invertebrates and should supply both energy and essential nutrients in the form of proteins, carbohydrates and lipids (Whyte, 1987). The environmental conditions under which phytoplankton grow dictate their biochemical composition and may affect the energetic and nutritional value of the algae (Febregas et al., 1985). The prymnesiophyte *Isochrysis galbana* is one of the most commonly used phytoplankton species in many mariculture systems. Although various studies evaluated the chemical composition and nutritional value of *I. galbana* (Rodhouse et al., 1983; Thomas et al., 1984; Whyte, 1987), there is no available record that measured unequivocally the effect of environmental conditions on those parameters. The use of

steady state cultures grown in chemostats or turbidostats allows us to measure the effect of a given set of growth conditions on cellular composition and to evaluate the potential to manipulate the biochemical quality of phytoplankton. Data presented in this study demonstrate the effects of environmental conditions such as light intensity and nitrogen limitation on the biochemical content of *Isochrysis galbana*. High light intensity and low nitrogen load similarly affected cellular pigmentation. In both cases, cells were characterized by a low chlorophyll content and by an increase in the carotenoid to chlorophyll ratio. High light grown cells as well as severely nitrogen limited cultures were characterized by a high cellular content of carbohydrates, suggesting an increase in the energetic value of the algae. Accumulation of carbohydrates by *I. galbana* grown under high light or nitrogen limiting conditions suggests that this organism accumulates polysaccharides, presumably chrysolaminarian, as an energy storage material, while lipids are mainly used as structural components of cellular membranes. Nevertheless, severe nitrogen limitation caused the accumulation of cellular lipids, evidently triacylglycerol, in addition to carbohydrates. The cellular protein content of *I. galbana* was fairly stable over a wide range of growth irradiance levels. Using conversion factors of 0.16 g N per g protein and 0.4, 0.725 and 0.4 g C per g of protein, lipid and carbohydrate, respectively, an N/C ratio of 0.106 was calculated for *I. galbana* grown under various irradiance levels. The N/C ratio decreased when cultures were grown under nitrogen limiting conditions to a minimal value of 0.038 at a daily nitrogen load of 28 $\mu\text{mol N L}^{-1}$. Similar values of N/C ratio were previously reported for *I. galbana* grown under similar environmental conditions of light intensity (Falkowski et al., 1985) and nitrogen limitation (Herzig and Falkowski, 1989).

The measured variations in fatty acid composition of *I. galbana* caused by environmental conditions can be related to the relative distribution of the major lipid classes in this alga. A relative increase in C18:4 is evidently associated with a

relatively high content of galactolipids, the major lipid components of the photosynthetic membranes. The C18:1 and C16:0 fatty acid percentage increased as the relative content of neutral lipids increased, mostly under high light or severe nitrogen limiting conditions. The increase in the relative content of C18:4 was associated with a reduction in the percentage of C18:1 and C16:0 fatty acids. Consequently, the ratio of total omega-3 polyunsaturated acid to the sum of saturated and monoenic fatty acids decreased under high light and low nitrogen availability, conditions that caused the accumulation of neutral lipids and a decrease in the structural lipids of the photosynthetic apparatus (Suknik et al., 1989).

Based on the presented biochemical quality of *I. galbana* grown under various environmental conditions, it is expected that cultures grown under nutrient sufficient conditions are of a better nutritional value than cultures grown under nitrogen limiting conditions. It is further suggested that cultures grown under light saturating conditions (between 175 and 300 $\mu\text{mol quanta m}^{-2}\text{s}^{-1}$) will be more balanced from energetic and nutritional aspects. These cultures are relatively enriched with carbohydrates, but still the lipid composition is well balanced to give a relatively high percentage of omega-3 polyunsaturated fatty acids. Nevertheless, the nutritional quality of phytoplankton cultures grown under controlled environmental conditions should be further evaluated by feeding experiments, where the quality of the algae should be adjusted to the specific requirement of the studied organism.

ACKNOWLEDGEMENTS

This research was supported by the U.S.-Israel Binational Agricultural Research and Development Fund under grant I-1082-86. Additional support was provided by the Strauss/Hanauer Memorial Fund Inc.

REFERENCES

- Ben-Amotz, A., Fishler, R. and Schneller, A., 1987. Chemical composition of dietary species of marine unicellular algae and rotifers with emphasis on fatty acids. *Mar. Biol.*(Berl.), 95: 31-36.
- Bligh, E.G. and Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.*, 37: 911-917.
- Cohen, Z., Vonshak, A. and Richmond, A., 1988. Effect of environmental conditions on fatty acid composition of the red alga *Porphyridium cruentum*: correlation to growth rate. *J. Phycol.*, 24: 328-332.
- De Pauw, N. and Persoone, G., 1988. Microalgae for aquaculture. In: M.A. Borowitzka and L.J. Borowitzka (Editors), *Microalgal Biotechnology*. Cambridge University Press, New York, pp. 197-221.
- De Pauw, N., Morales, J. and Persoone, G., 1984. Mass culture of microalgae in aquaculture systems: progress and constraints. *Hydrobiologia*, 116/117: 121-134.
- Falkowski, P.G., Dubinsky, Z. and Wyman, K., 1985. Growth-irradiance relationships in phytoplankton. *Limnol. Oceanogr.*, 30: 311-321.
- Febregas, J., Aerrero, C., Cabezas, B. and Abalde, J., 1985. Mass culture and biochemical variability of the marine microalgae *Tetraselmis suecica* Kglin (Butch) with high nutrient concentrations. *Aquaculture*, 49: 231-244.
- Guillard, R.R.L. and Ryther, J.H., 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* (Hustedt) and *Detonula confervacea* (Cleve). *Can. J. Microbiol.*, 8: 229-239.
- Herzig, R. and Falkowski, P.G., 1989. Nitrogen limitation in *Isochrysis galbana* (Haptophyceae). I. Photosynthetic energy conversion and growth efficiencies. *J. Phycol.*, 25: 462-471.

- Jeffrey, S.W. and Humphrey, G.W., 1975. New spectrophotometric equations for determining chlorophylls *a*, *b*, *c* and *c₂* in higher plants, algae and natural phytoplankton. *Biochem. Physiol. Pflanz.*, 167: 191-194.
- Kates, M., 1972. Isolation, analysis and identification of lipids. In: T.S. Work and E. Work (Editors), *Techniques in Lipidology*. Elsevier, Amsterdam, pp. 268-618.
- Kochert, C., 1978. Carbohydrate determination by the phenol-sulfuric acid method. In: J.A. Hellebust, and J.S. Craigie (Editors), *Handbook of Phycological Methods: Physiological and Biochemical Methods*. Cambridge University Press, London, pp. 95-97.
- Koven, W.M., Kissil, G.Wm. and Tandler, A., 1989. Lipid and n-3 requirement of *Sparus aurata* larvae during starvation and feeding. *Aquaculture*, 79: 185-191.
- Lepage, G. and Roy, C.C., 1984. Improved recovery of fatty acid through direct transesterification without prior extraction or purification. *J. Lipid Res.*, 25: 1391-1396.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., 1951. Protein measurement with Folin reagent. *J. Biol. Chem.*, 193: 265-275.
- Marsh, R. and Weinstein, D.B., 1966. Simple charring method for determination of lipids. *J. Lipid Res.*, 7: 574-576.
- Myers, J., 1980. On the algae: thoughts about physiology and measurements of efficiency. In: P.G. Falkowski (Editor), *Primary Productivity in the Sea*. Plenum Press, New York, pp. 1-16.
- Pillsbury, K.S., 1985. The relative food value and biochemical composition of five phytoplankton diets for queen conch, *Strombus gigas* (Linne) larvae. *J. Exp. Mar. Biol. Ecol.*, 90: 221-231.
- Piorreck, M., Baasch, K.-H. and Pohl, P., 1984. Biomass production, total protein, chlorophylls, lipids and fatty acids of fresh water green and blue-green algae under different nitrogen regimes. *Phytochemistry*, 23: 207-216.

- Rodhouse, P.G., Roden, C. and Somerville-Jacklin, M.E., 1983. Nutritional value of microalgal mass cultures to the oyster *Ostrea edulis* L. *Aquaculture*, 32: 11-18.
- Roessler, P., 1988. Effect of silicon deficiency in lipid composition and metabolism in the diatom *Cyclotella cryptica*. *J. Phycol.*, 24: 394-400.
- Shifrin, N.S. and Chisholm, S.W., 1981. Phytoplankton lipids: interspecific differences and effects of nitrate, silicate and light-dark cycles. *J. Phycol.*, 17: 374-384.
- Spoehr, H.A. and Milner, H.W., 1949. The chemical composition of *Chlorella*: effect of environmental conditions. *Plant Physiol.*, 24: 120-149.
- Sukenik, A., Bennett, J. and Falkowski, P.G., 1987. Light saturated photosynthesis limitation by electron transport or carbon fixation? *Biochim. Biophys. Acta*, 891: 205-215.
- Sukenik, A., Carmeli, Y. and Berner, T., 1989. Regulation of fatty acid composition by irradiance level in the eustigmatophyte *Nannochloropsis* sp. *J. Phycol.*, 25: 686-692.
- Thomas, W.T., Seibert, D.L.R., Alden, M., Neori, A. and Eldridge, P., 1984. Yields, photosynthetic efficiency and proximate composition of dense marine microalgal cultures. III. *Isochrysis* sp. and *Monoatlantus salina* experiments and comparative conclusions. *Biomass*, 5: 299-316.
- Whyte, J.N.C., 1987. Biochemical composition and energy content of six species of phytoplankton used in mariculture of bivalves. *Aquaculture*, 60: 231-241.

FIGURE LEGENDS

Fig. 1. Variation in cellular pigmentation of *I. galbana* grown under different irradiance levels.

Fig. 2. Fatty acid composition of major lipid groups of *I. galbana*. Lipids were fractionated on a silicic acid column.

Fig. 3. Effect of nitrogen limitation on pigment composition in *I. galbana* grown in chemostats supplied with nitrogen at different daily loads.

TABLE 1

Effect of growth irradiance level on cellular growth rate and chemical composition in *I. galbana*. (Values are means of three independent measurements; standard error was less than 10%).

Growth irradiance, $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$	Growth rate, day^{-1}	Cellular composition, pg cell ⁻¹		
		Protein	Carbo- hydrate	Lipid
30	0.50	6.4	2.7	5.7
80	0.71	7.0	3.5	6.2
175	0.90	6.9	3.9	5.3
300	1.21	7.0	5.3	5.6
400	1.25	6.4	7.0	5.2
500	1.15	7.5	6.2	4.4

TABLE 2

Variations in fatty acid distribution (mole %) in *I. galbana* grown at various irradiance levels. (Values are means of three independent measurements.)

Fatty acid	Growth irradiance level, $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$					
	30	80	175	300	400	500
14:0	13.9	13.5	15.3	15.1	17.3	16.7
16:0	11.2	11.8	11.9	12.4	13.5	12.3
16:1	5.9	6.2	4.4	4.0	3.9	3.5
18:1	18.7	18.9	20.0	20.6	22.6	22.7
18:2	4.8	4.9	3.8	4.0	4.1	4.8
18:4	27.8	23.6	23.0	20.8	17.3	18.9
22:6	12.7	15.4	17.0	17.6	15.9	16.2
$\Sigma\text{S} + \text{M}^*$	49.7	50.4	51.6	52.1	57.3	55.2
$\text{P}/\Sigma\text{S} + \text{M}^{**}$	0.81	0.77	0.77	0.74	0.58	0.64

* The sum of saturated and monoenoic acids.

** The ratio of polyunsaturated acid to the sum of saturated and monoenoic acids.

TABLE 3

Effect of nitrogen limitation on cellular growth rate and chemical composition in *I. galbana*. (Values are means of three independent measurements).

Nitrogen load $\mu\text{mol L}^{-1} \text{d}^{-1}$	Growth rate, day^{-1}	Cellular composition, pg cell^{-1}		
		Protein	Carbo- hydrate	Lipid
28	0.18	3.5	16.5	6.3
38	0.25	3.5	16.1	4.9
50	0.33	4.1	9.2	3.2
72	0.50	4.2	8.7	2.6
96	0.65	5.0	7.2	2.4
140	0.95	5.3	5.3	2.7

TABLE 4

Variations in fatty acid distribution (mole %) in *I. galbana* grown in 26 L chemostats at various nitrogen load conditions. (Values are means of three independent measurements.)

Fatty acid	Nitrogen load, $\mu\text{mol L}^{-1} \text{ day}^{-1}$					
	28	38	50	72	96	140
14:0	9.6	9.6	10.0	10.1	10.4	9.6
16:0	14.4	12.7	14.4	11.5	10.6	8.2
16:1	8.4	7.4	8.0	6.4	5.8	6.0
18:1	32.4	31.3	29.1	28.8	28.7	27.9
18:2	6.2	6.6	7.1	7.0	7.5	7.1
18:4	13.0	13.8	14.0	15.8	18.8	23.7
22:6	11.8	13.6	12.4	14.8	14.5	14.0
$\Sigma\text{S} + \text{M}^*$	64.8	61.0	61.5	56.8	55.5	51.7
$\text{P}/\Sigma\text{S} + \text{M}^{**}$	0.38	0.45	0.43	0.54	0.60	0.73

* The sum of saturated and monoenoic acids.

** The ratio of polyunsaturated acid to the sum of saturated and monoenoic acids.

Fig 1

Fig. 2

Fig 3

3.2 Fatty acid composition and quantitative morphology of *Isochrysis galbana* *

Introduction

Variations in environmental conditions affect cellular lipid composition as well as fatty acid distribution. Simultaneously, changes in cellular ultrastructures, such as chloroplast specific volume and thylakoid surface density, are frequently observed. A reduction in thylakoid surface density indicates a decrease in the cellular content of photosynthetic components and in most cases is associated with a reduction in chlorophyll content. Thylakoid membranes are composed of specific lipid classes such as monogalactosyl and digalactosyl diacylglycerol (MGDG and DGDG respectively), which contain polyunsaturated fatty acids. The galactolipids of various algal classes are characterized by a different typical pattern of fatty acids. MGDG of *I. galbana*, for example, contains a high proportion of C18:4 fatty acid while MGDG of the chlorophyte *Dunaliella* is highly enriched with C18:3 and C16:4 fatty acids. Storage lipids, mostly triacylglycerol of all algal classes, are characterized by saturated and monoenoic fatty acids ranging between 12 and 18 carbon chain.

Differential changes in chloroplast morphology and storage lipids are expected as cells respond to various environmental conditions. Different lipid and fatty acid composition of cellular components suggests that these changes will be associated with variations in the relative abundance of fatty acids. The following section describes the results of our effort to characterize the molecular species of MGDG in *I. galbana* and to correlate variations in lipid and fatty acid composition with cellular quantitative morphology.

* The help of Dr. T. Berner is gratefully acknowledged.

Materials and Methods

Algae growth conditions. *Isochrysis* aff. *galbana* (T-ISO) was obtained from the microalgae culture collection of U.S. DOE Solar Energy Research Institute (Golden, CO). To evaluate the effect of growth irradiance level on cellular lipid composition, cultures were maintained at 25°C under steady state conditions in 2.6 liter turbidostats with natural sea water enriched with f/2 nutrients as previously described. Light was provided continuously by banks of VHO fluorescent tubes, and cells were grown at six irradiance levels: 30, 80, 175, 300, 400 and 500 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$. The cultures were kept optically thin (2×10^6 cells ml^{-1}) to minimize self shading. The pH of the sea water was buffered at 7.8, and cultures were continuously bubbled with air. All cultures were maintained in steady state for several days prior to measurements.

Nitrogen limited cultures were grown continuously at 25°C in similar 2.6 liter vessels using artificial sea water media, supplied with 150 μM NaNO_3 and other f/2 nutrients. Light was provided continuously by banks of VHO fluorescent tubes supplying a photon flux of 175 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ (400 to 700 nm). All cultures were maintained in steady state for several days prior to measurements.

Extraction and fractionation of lipids. Total lipids were extracted as previously described. Total lipid extracts were fractionated on silica columns (SEP-PAK, Waters Associates) by sequential elution with chloroform and chloroform:methanol (100:2.5; v/v). Total lipid extracts and galactolipid fractions were further resolved on TLC 20 x 20 cm glass plates coated silica gel-60 (Merck) as previously described. Resolved lipids were visualized by a short exposure to I_2 vapours and identified by comparison with pure standard (Sigma). For further characterization of MGDG molecular species, the MGDG band was scraped off the TLC plates, eluted in chloroform:-methanol:water (5:3:1; v/v/v) and phase separated with KCl and chloroform. The MGDG fraction (lower layer) were evaporated to dryness under a stream of N_2 and dissolved in a known volume of methanol:water (96:4; v/v).

HPLC separation of MGDG species. Separation of MGDG into constituent molecular species was achieved by an isocratic HPLC system with a constaMetric III metering pump (Milton Roy, Florida, USA), a Rheodyne model 7125 syringe-loading sample injector with a 100 μ l loop, and a 25 cm x 4.6 mm Ultramex (5 μ m) C18 reverse phase column (Phenomenex, California, USA). The mobile phase consisted of methanol:water (96:4; v/v) pumped at 1 ml/min. The molecular species were detected by a spectroMonitor 3100 (Milton Roy, Florida, USA), variable wavelength detector set 205 nm and a Tracor 945 Flame Ionization LC Detector (Tracor Instruments, Austin, TX, USA).

Analysis of MGDG molecular species. Molecular species of MGDG were collected manually directly from the UV detector. Fatty acid extracts were prepared by saponification of each collected peak, followed by methylation with boron trifluoride/methanol. Fatty acids of the sn-1 position were hydrolysed by incubating with lipase from *Rhizopus arrhizus* (Boehringer, Mannheim) for one hour at 37°C. The lipids were extracted from the reaction mixture with diethyl ether/petroleum ether (1:1, v/v) and the resultant fatty acid and 2-acyl GL were separated by TLC (Silica gel 60, Merck). The TLC plates were developed in petroleum ether - diethyl ether - acetic acid (70:30:1, v/v/v). Separated components were visualized after exposure to I₂ vapours and the fatty acids were identified by comparison with linolenic acid standard (Sigma). Lipids were eluted from the silica gel in chloroform:methanol:water (5:3:1) and phase separated with KCl and chloroform. Fatty acids were identified by GLC following preparation of methyl esters.

Electron microscopy. Samples of 100 ml algal culture were collected from turbidostats and chemostats as steady state conditions were established. Algal cells were washed twice in 50 mM phosphate buffer (pH 7.2) containing 0.25 M sucrose and further prepared as previously described. Thin sections were observed in a Jeol TEM 1200x operating at 80 kV. The relative volumes of chloroplast, mitochondria and nuclei to cell volume and surface density of thylakoids were calculated as previously described.

Results and Discussion

Fatty acid composition and molecular species of MGDG. The major fatty acids in *Isochrysis galbana* are C14:0, C16:0, C16:1, C18:1, C18:2 and the polyunsaturated acids C18:4 and C22:6. Fractionation of lipid extracts from *I. galbana* indicated that the neutral lipids, mainly triacylglycerol, contained C16:0 and C18:1 fatty acids. The galactolipids of this alga are preferentially enriched with C18:4 fatty acids. The phospholipids fraction of *I. galbana* was enriched with C22:6 fatty acids accompanied by C18:1, C16:0 and C14:0 fatty acids (see section 3.1). The fatty acid distribution in the major component of the galactolipids, the MGDG, was significantly different from the one found in whole cell lipid extract. The percentage of C18:4 is almost twice as much as in total lipid extract, while C22:6 is completely missing. Other fatty acids that are associated with MGDG of *I. galbana* but at low percentage are C14:0, C16:2, C18:1, C18:2 and C18:3 (Table 1). The molecular species of MGDG from *I. galbana* were resolved by reverse phase HPLC. Typical chromatograms of the resolved MGDG molecular species are shown in Fig. 1. The chromatograms of the resolved MGDG molecular species detected by flame ionization detector are compared to the UV response at 205 nm. More than 8 different MGDG species of *I. galbana* were resolved (Fig. 1). Two eluted peaks consisted of more than one MGDG molecular species. Peak no. 5 was composed of a combination of the 18:4/14:0 and 18:4/16:1 MGDG and peak no. 6 contained a mixture of 18:4/18:2 and 18:2/18:1 MGDG. The results of the positional analysis of fatty acids in MGDG molecular species of *I. galbana* are given in Table 2. The C18:4 fatty acid which is the major constituent of the MGDG species in *I. galbana* (Table 2) was located in both sn-1 and sn-2 positions, although the C18:4 fatty acid had a significant preference for the sn-1 position.

Variations in fatty acid composition. High light conditions imposed a reduction in the percentage of C18:4 fatty acid while the relative distribution of C16:0, C14:0, C18:1 and C22:6 slightly increased. Under severe nitrogen limitation (growth rate of 0.2 day⁻¹), the percentage of C16:0 and C18:1 increased at the expense of C18:4 and

C22:6 fatty acids (Table 3). As nitrogen load increased, the relative distribution of C18:4 and C22:6 fatty acids increased. The measured variations in fatty acid composition are related to changes in the relative distribution of the major lipid classes in *I. galbana*. A relative increase in C18:4 is most likely associated with a relatively high content of galactolipids, the major components of the photosynthetic membranes. The percentage of C18:1 and C16:0 fatty acids increased as the relative content of neutral lipids increased, mostly under high light or severe nitrogen limitation.

Lipid classes synthesis and accumulation. Carbon partitioning among lipid classes, as assessed by assimilation of $^{14}\text{CO}_2$ into lipids, was greatly affected by growth conditions. Under high irradiance levels, the relative synthesis of triacylglycerol increased, concomitant with a reduction in the relative synthesis of galactolipids, mostly MGDG. *I. galbana* cultures grown in steady state conditions under nitrogen limitation at different dilution rates synthesized relatively more triglycerol as growth rate reduced. Simultaneously the relative synthesis rate of galactolipids reduced at slow growth rate (data not shown). Separation of total lipids by thin layer chromatography clearly indicated accumulation of triacylglycerol in *I. galbana* cells grown under high light (Fig. 2). On the other hand, low light conditions imposed a relatively low level of triacylglycerol and a higher abundance of galactolipids such as MGDG (Fig. 2).

Quantative ultrastructure. Typical electron micrographs of *Isochrysis galbana* from steady state cultures grown under low light are shown in Fig. 3. The alga is characterized as a "naked" cell which is bound by the cytoplasmic membrane, covered by a layer of delicate scales. The scales do not contain silica but appear to consist of organic material. The chloroplast is cap shaped and a high density of thylakoid membranes is observed in low light grown cells. Other cellular organelles are nucleus, mitochondrion, and golgi apparatus. Morphometric measurements of the electron micrographs (Table 4) show that chloroplast relative volume of low light grown cells is greater than in high light or low nitrogen grown cultures. Furthermore

the thylakoid surface density per cell or per chloroplast is higher in low light cells than in cells grown under high light or low nitrogen conditions. These differences in thylakoid surface density are associated with quantitative variations in the abundance of galactolipids and the accompanying fatty acid, C18:4.

Table 1. Fatty acid distribution in whole cell lipid extract and in the MGDG fraction of *Isochrysis galbana*

Fatty acid	Fatty acid distribution (mole %)	
	Whole cell	Total MGDG
14:0	13.5	11.6
16:0	11.8	-
16:1	6.2	3.7
18:1	18.9	13.1
18:2	4.9	4.8
18:3	traces	5.2
18:4	23.6	58.1
22:6	15.4	-

Table 2. Identification and relative abundance of MGDG molecular species of *Isochrysis galbana* resolved by FID-HPLC

Peak no.	Fatty acids composition	Retention time (min)	% Area
1	18:3w6/18:3w6	6.2	16.5
2	18:4/18:3	12.2	16.3
3	18:4/18:4	13.7	25.4
4	18:4/18:1	16.6	19.5
5	18:4/14:0 + 18:4/16:1	18.2	16.7
6	18:4/18:2 + 18:2/18:1	20.8	5.5

Table 3. Variations in fatty acid distribution (mole %) in *I. galbana* grown in low light (LL - 30 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$), high light (HL - 400 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$), low nitrogen availability (LN - growth rate of 0.3 day⁻¹) and high nitrogen availability (HN - growth rate of 0.8 day⁻¹)

Fatty acid	Growth conditions			
	LL	HL	LN	HN
14:0	13.9	17.3	9.6	10.4
16:0	11.2	13.5	14.4	8.2
16:1	5.9	3.9	8.4	6.0
18:1	18.7	22.6	32.4	27.9
18:2	4.8	4.1	6.2	7.1
18:4	27.8	17.3	13.0	23.7
22:6	12.7	15.9	11.8	14.0

Table 4. Variations in cellular ultrastructure of *I. galbana* grown under various environmental conditions as described in Table 3

	Growth conditions			
	LL	HL	LN	HN
Chloroplast (relative volume)	64.0 ± 4.7	37.4 ± 2.7	39.8 ± 3.6	32.9 ± 3.5
Mitochondria (relative volume)	4.1 ± 1.0	4.2 ± 0.7	3.0 ± 0.7	4.5 ± 0.7
Nucleus (relative volume)	7.7 ± 1.6	16.6 ± 2.0	11.3 ± 1.6	12.7 ± 1.9
Thylakoid/cell (surface density)	0.80 ± 0.05	0.21 ± 0.01	0.36 ± 0.03	0.46 ± 0.03
Thylakoid/chloroplast (surface density)	1.36 ± 0.18	0.60 ± 0.03	0.93 ± 0.06	1.49 ± 0.08

Figure Legends

- Fig. 1. Reverse phase HPLC separation of MGDG molecular species isolated from *Isochrysis galbana*. Methanol:water (96:4 v/v) was used as a mobile phase. The molecular species were detected by flame ionization detector (upper chromatogram) and UV at 205 nm (lower chromatogram). Peak identification is given in Table 2.
- Fig. 2. Separation of total lipid extract from *I. galbana* grown at various irradiance levels by thin layer chromatography. A. Separation of non polar lipids. B. Separation of polar lipids. DGDG - digalactosyl diacylglycerol. HC - hydrocarbon. MGDG - monogalactosyl diacylglycerol. O - origin. P - pigment. PG - phosphatidylglycerol. PL - phospholipid. TG - triglyceride.
- Fig. 3. Electron micrographs of thin sections taken from *I. galbana* grown under low (LL) and high (HL) light conditions and under severe nitrogen limitation (LN).

Fig. 2

LL

HL

LN

4. Regulation of carbon flow into fatty acids and lipids

The synthesis of fatty acids in photosynthetic tissue of higher plants and in unicellular algae is localized in the chloroplast. The formation of malonyl CoA is catalyzed by acetyl CoA carboxylase. Once malonyl CoA is formed, it is rapidly incorporated into fatty acids via the fatty acid synthetase system. Carbon allocation into fatty acids and lipids is thought to be regulated by acetyl CoA carboxylase and the availability of acetyl CoA in the chloroplast. Furthermore, carbon flow into fatty acid synthesis competes with other metabolic pathways located in the chloroplast, such as photosynthetic carbon fixation and ammonia assimilation into amino acids, on carbonaceous backbones energy and reducing power.

The long term objective of our investigation was to elucidate the molecular mechanisms that regulate the flow of photosynthetic assimilated carbon into fatty acids and lipids in *I. galbana*. The enzyme acetyl CoA carboxylase was suggested in many studies as a key regulatory enzyme in lipid biosynthesis. Thus, a detailed study of its molecular properties and kinetics was carried out. The availability of polyclonal antibodies raised against ACCase of *I. galbana* further facilitated an analysis of the relationships between the enzyme activity and content and the cellular content of fatty acids and lipids.

4.1 Purification and characterization of acetyl CoA carboxylase (ACCase)

Acetyl CoA carboxylase (ACCase) was purified to an apparent homogeneity from the unicellular algae *Isochrysis galbana*. The purification steps included ammonium sulphate precipitation sucrose density gradient and monomeric avidin affinity chromatography. The enzyme recovery was less than 10% with 300 fold purification. The molecular mass of the native enzyme was estimated at 700 kDa by elution from a calibrated Sepharose 6B column. Investigation of the kinetics properties of the purified enzyme indicated K_m for acetyl CoA, NaHCO_3 and ATP of about 310, 910 and 51 μM , respectively. Malonyl CoA and palmitoyl CoA were found to be highly potent inhibiting metabolites which competitively inhibited the enzyme with respect to acetyl CoA. Polyclonal antibodies prepared against against the

subunit of the purified enzyme efficiently inhibited the enzyme activity in an *in vitro* system. The prepared antibodies used in an immunoblot system were specifically bound to the ACCase subunit of *I. galbana*. These antibodies failed to cross-react with the corresponding subunit of the enzyme from a diatom, indicating a poor immunological conservation of that protein among algal classes.

A manuscript entitled "Acetyl CoA carboxylase from the marine prymnesiophyte *Isochrysis galbana*" that describes this study was recently published in Plant & Cell Physiology.

4.2 The role of ACCase in regulating lipid synthesis

Lipid and total fatty acid content was determined in *Isochrysis galbana* cultures grown under various environmental conditions in steady state continuous cultures. Lipid and fatty acid accumulation was observed under severe nitrogen limiting conditions. The rate of *in situ* lipid synthesis, determined from ^{14}C bicarbonate incorporation into the lipid fraction, decreased under nitrogen limited growth ($\mu \leq 0.35 \text{ day}^{-1}$), concomitant with a reduction in the *in vitro* activity of the enzyme acetyl CoA carboxylase. Cellular lipid and fatty acid content remained fairly constant over a wide range of irradiance levels. The rate of lipid synthesis, however, increased as irradiance level increased to a maximal value at a light intensity of $150 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ and slightly decreased at higher photon flux. The *in vitro* activity of ACCase roughly followed the same pattern of lipid synthesis in response to light intensity. The relative abundance of acetyl CoA carboxylase significantly decreased in nitrogen limited cultures grown at low dilution rates ($\mu \leq 0.35 \text{ day}^{-1}$). A fairly good linear correlation was measured between the cellular content of ACCase and the enzyme activity in cultures grown under nitrogen limiting conditions.

Furthermore, the cellular fatty acid content was linearly related to the cell capacity to produce malonyl CoA, the end product of ACCase and the building block in fatty acid synthesis. The results of this study were presented in the 9th International Symposium on Plant Lipids whose Proceedings will soon be published in Quinn, P.G. and Harwood, J.L. (eds.) *Plant Lipid Biochemistry, Structure and Utilization*. Portland Press, London, 1990. In addition, a more advanced contribution was accepted for publication in *Plant & Cell Physiology*.

Running title:

Acetyl CoA carboxylase and lipid synthesis.

Subject area:

Proteins, enzymes and metabolism.

Name and address for editorial correspondence:

Assaf Sukenik

National Institute of Oceanography

Israel Oceanographic and Limnological Research

Tel Shikmona, P. O. Box 8030, Haifa 31080, Israel.

Number of figures and tables:

7 figures, 1 table.

Title of the article:

Variations in Lipid and Fatty Acid Content in Relation to Acetyl CoA
Carboxylase in the Marine Prymnesiophyte *Isochrysis galbana*

Abstract

Lipid and fatty acid content was determined in *Isochrysis galbana* cultures grown under various environmental conditions in steady state continuous cultures. Lipid and fatty acid accumulation was observed under severe nitrogen limiting conditions. The rate of *in situ* lipid synthesis, determined from ^{14}C bicarbonate incorporation into lipid fraction, decreased under nitrogen limited growth ($\mu < 0.72 \text{ day}^{-1}$), concomitant with a reduction in the *in vitro* activity of the enzyme acetyl CoA carboxylase. Cellular lipid and fatty acid content remained fairly constant over a wide range of irradiance levels. The rate of lipid synthesis, however, increased as irradiance level was elevated to a maximal value at a light intensity of $150 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ and slightly decreased at a higher photon flux. The *in vitro* activity of ACCase roughly followed the same pattern of lipid synthesis in response to light intensity. The relative abundance of acetyl CoA carboxylase significantly decreased in nitrogen limited cultures grown at low dilution rates ($\mu < 0.72 \text{ day}^{-1}$). A fairly good linear correlation was measured between the cellular content of ACCase and the enzyme activity in cultures grown under nitrogen limiting conditions. Furthermore, in nitrogen limited cultures, the cellular fatty acid content was linearly related to the cell capacity to produce malonyl CoA, the end product of ACCase and the building block in fatty acid synthesis.

Abbreviations: ACCase - acetyl coenzyme A carboxylase. PAGE - polyacrylamide gel electrophoresis. SDS - sodium dodecylsulfate.

Key words:

Acetyl CoA carboxylase, fatty acid, *Isochrysis galbana*, lipids, marine algae,
Prymnesiophyceae

Introduction

Unicellular algae grown under nutrient sufficient conditions commonly assimilate the photosynthetic fixed carbon into protein to support growth and division (Myers, 1980). However when an algal culture is deprived of an essential nutrient, cell division ceases, and the fraction of carbon allocated to lipids and carbohydrates can be greatly increased at the expense of protein synthesis. The effect of environmental conditions on lipid and fatty acid composition has been investigated in various algae (Spoehr and Milner, 1949; Shifrin and Chisholm, 1981; Piorreck et al., 1984; Cohen et al., 1988), and recently reviewed by Roessler (1990b). In most algae, enhancement of lipid accumulation is imposed by nitrogen deficient conditions. In diatoms, accumulation of neutral lipids is greatly increased under silicon deficiency (Coombs et al., 1967; Roessler 1988a). The potential role of acetyl CoA carboxylase in the regulation of lipid biosynthesis in the diatom *Cyclotella cryptica* was recently suggested by Roessler (1988b), who isolated ACCase from that alga and characterized the kinetic properties of the enzyme (Roessler, 1990a).

The marine prymnesiophyte *Isochrysis galbana* is commonly used as a primary producer in mariculture systems evidently due to its lipid content and composition (Rodhouse et al., 1983; De Pauw et al., 1984; Ben Amotz et al., 1987). Although many studies reported the effect of nutrient starvation on lipid content and composition, the effect of growth irradiance level on cellular lipids has hardly been investigated (Sukerik et al., 1989). Moreover, most of the reported studies were performed in batch cultures where growth conditions, including light intensity and nutrient availability, varied with time. The effect of environmental parameters on lipid content and fatty acid composition in *Isochrysis galbana* was recently studied in continuously grown cultures in chemostats and turbidostats (Sukerik and Wahnou, 1990). Lipid accumulation was observed in cultures grown under severe nitrogen limitation. Furthermore,

variations in growth conditions, such as light intensity and nitrogen availability, imposed changes in the distribution of fatty acids, evidently due to variations in lipid composition.

Enhancement of carbon assimilation into lipids in unicellular algae is supposed to be associated with variations in the activity and abundance of a rate limiting factor. The formation of malonyl CoA, the building block of fatty acid synthetase by ACCase, is a potential regulatory step in lipid biosynthesis. The molecular properties and kinetic characteristics of ACCase were recently studied in the marine prymnesiophyte *Isochrysis galbana* (Livne and Sukenik, 1990). In the present study, lipid synthesis and accumulation are related to the *in vitro* activity and cellular abundance of ACCase in *I. galbana* grown under a wide range of environmental conditions. The results of our present attempt to understand the biochemical mechanisms that regulate the flow of photosynthetic assimilated carbon into lipids in unicellular algae suggest that ACCase plays an important role in this system.

Materials and methods

Organism and growth conditions

Isochrysis aff. *galbana* (T-ISO) was obtained from the microalgae culture collection of U.S. DOE Solar Energy Research Institute (Golden, CO, U.S.A.)

Turbidostat cultures. To evaluate the effect of growth irradiance level on cellular composition, cultures were maintained at 25°C under steady state conditions in turbidostats with natural sea water enriched with f/2 nutrients (Guillard and Ryther, 1962). The culture vessel consisted of a 2.6 liter inner glass cylinder, 10 cm diameter by 33 cm long. The culture was periodically diluted with fresh growth medium to maintain steady state conditions, as previously described (Sukenik et al., 1987). Light was provided continuously by banks of VHO fluorescent tubes, and cells were grown at six irradiance levels: 30, 80, 175, 300, 400 and 500 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$. Irradiance was varied by changing either the number of tubes used or their distance from the culture

vessel. The cultures were kept optically thin (2×10^6 cells ml^{-1}) to minimize self shading. Daily growth rates were calculated with the equation:

$$\mu = v/V_r$$

where V_r is the culture volume (2.6 L) and v is the daily rate of medium addition (L day^{-1}). The pH of the sea water was buffered at 7.8, and cultures were continuously bubbled with air. All cultures were maintained in steady state for several days prior to measurements, and samples were taken at three consecutive days thereafter.

Chemostat cultures. Nitrogen limited cultures were grown continuously at 25°C in similar 2.6 liter vessels as described above using artificial sea water media, supplied with $200 \mu\text{M}$ NaNO_3 as the sole nitrogen source and other f/2 nutrients. Five steady state growth rates, ranging from 0.2 to 0.92 day^{-1} , were established by continuously diluting the cultures with fresh growth medium. Light was provided continuously by banks of VHO fluorescent tubes supplying a photon flux of $175 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ (400 to 700 nm). All cultures were maintained in steady state for several days prior to measurements.

Extraction and quantification of lipids and fatty acids -

Total lipids were extracted with chloroform:methanol:water (5:10:4 v/v/v) (Bligh and Dyer, 1959) as modified by Kates (1972). The lipids were then phase separated by adjusting the solvent ratios to 10:10:9 (chloroform:methanol:water, v/v/v). The chloroform phase was evaporated to dryness under a stream of N_2 , and dissolved in a known volume of chloroform:methanol 85:15 (v/v). Total lipids were quantified by the sulphuric acid charring technique (Marsh and Weinstein, 1966) that was calibrated versus tripalmitin as previously described (Sukenik et al., 1989).

Fatty acid extracts were prepared by saponifying of whole algal cells or total lipid extract in a strong base, followed by methylation (Lepage and Roy, 1984). The fatty acid methyl esters were separated by capillary gas liquid chromatography using a Hewlett Packard 5890A gas chromatograph as

previously described (Suknik et al., 1989). Identification of fatty acids methyl esters was accomplished by comparing the retention times of experimental samples with those of known standards. Heptadecanoic acid was used as an internal standard to quantify cellular fatty acid content.

Synthesis of lipids - *De novo* synthesis of lipids was assessed by following the assimilation of $^{14}\text{CO}_3$ into lipids. As steady state conditions were established in the turbidostats and chemostats, 50 ml samples were withdrawn and incubated *in situ* with $\text{NaH}^{14}\text{CO}_3$ (1 $\mu\text{Ci ml}^{-1}$, 0.4 mCi mmole $^{-1}$ final specific activity) for 1 hr. At the end of the incubation period, cells were collected, washed and monitored for ^{14}C incorporation. Lipids were extracted as described above and monitored for radioactivity by liquid scintillation counting.

Assay for ACCase activity

The *in vitro* activity of ACCase was determined by incorporation of ^{14}C bicarbonate into malonyl CoA, an acid stable product of the enzymatic reaction, following the procedure suggested by Sauer and Heise (1984). The reaction mixture (final volume of 0.3 ml) contained 100 mM Tris buffer pH 7.9, 0.5 mM Acetyl CoA, 1 mM ATP (Na_2) 2 mM MgCl_2 , 30 mM KCl, 3 mM NaHCO_3 (final specific activity of 0.3 mCi/mmole) and 20 μl of cell free extract containing 1 mg protein per ml. The reaction was started by the addition of acetyl CoA to the reaction mixture. Controls were run at the same reaction mixture but acetyl CoA was replaced with H_2O , in order to correct for nonspecific labeling. One unit of enzyme activity is defined as the amount of enzyme required to catalyze the formation of one μmol of malonyl CoA per minute under the defined assay conditions.

Analytical methods

Protein was quantified by the Coomassie blue dye binding method (Biorad) using bovine serum albumin as a standard. Radioactivity was measured by liquid scintillation counting in a Kontron Betamatic scintillation counter using the external standard ratio method for quench correction. A commercially

available Aquasol II (Du Pont) scintillation cocktail was used for all isotopic counts.

Denatured SDS-PAGE was performed using the buffer system of Laemmli (1970) with an 11% acrylamide separating slab gel. Protein samples were loaded in a dissociation buffer (20 mM Tris-HCl (pH 6.8), 5 mM NaEDTA, 2% (w/v) SDS, 10% (w/v) glycerol, 5 mM β -mercaptoethanol and 100 μ M PMSF). Resolved proteins were electrophoretically transferred to nitrocellulose, and the ACCase subunit was detected by reacting the nitrocellulose with anti ACCase serum. Secondary antibody, goat anti rabbit IgG (L + H) conjugated to horseradish peroxidase (Biomakor), was used in a dilution rate of 1:3,000. Visualization was performed by peroxidase color developing with benzidine. Stained blots were optically scanned with a densitometer to provide relative quantification.

Results

Lipid and fatty acid content

The effect of growth irradiance level and nitrogen limitation on cellular lipid and fatty acid content was studied in continuously grown cultures of *I. galbana*. While cells grown under a wide range of irradiance level show insignificant variation in lipid and fatty acid content, different levels of nitrogen availability imposed significant changes in the cellular content of these components (Fig. 1). Nitrogen limited cultures were grown in dilution rates ranging from 0.2 to 0.92 day⁻¹, which corresponds to 15-70%, respectively, of the maximal growth rate of *I. galbana* in a nutrient repleted culture at 25°C (Table 1). Under these growth conditions, cellular lipid content varied between 8.7 and 4.8 pg cell⁻¹. The most significant change in lipid content was observed in nitrogen limited cultures grown at growth rates ranging between 0.2 and 0.5 day⁻¹. Simultaneously, cellular fatty acid content followed a similar pattern as lipids: a reduction in fatty acid content as nitrogen availability increased (Fig. 1).

The effect of growth conditions on cellular soluble protein and fatty acid to protein ratio is given in Table 1 together with growth rate responses to irradiance level in nutrient sufficient cultures. While growth irradiance level did not affect the ratio of fatty acid to soluble protein, nitrogen limitation imposed changes in that parameter. Fatty acid content decreased and soluble protein content increased as growth rate increased. Consequently, the cellular ratio of fatty acid to soluble protein decreased by almost six-fold as nitrogen limited growth rate increased from 0.2 to 0.92 day⁻¹ (Table 1).

Lipid synthesis and ACCase activity

The rate of *de novo* synthesis of lipids was assessed from the rate of photosynthetically fixed carbon allocated into the extractable lipid fraction. The rate of lipid synthesis increased as growth irradiance increased to a maximal rate at irradiance level of 175 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$. At higher irradiance levels, the rate of lipid synthesis slightly decreased (Fig. 2). The rate of lipid synthesis in nitrogen limited cultures increased with growth rate. The lowest rate of lipid synthesis was measured under severe nitrogen limiting conditions ($\mu = 0.2 \text{ day}^{-1}$). The highest rate was measured at dilution rates greater than 0.7 day⁻¹. At these nitrogen limiting conditions, the rate of lipid synthesis represents about 80% of the algae capacity for lipid synthesis under optimal growth conditions; i.e. nitrogen sufficient conditions and optimal irradiance level (Fig. 2).

The *in vitro* activity of ACCase was measured in a soluble fraction of whole cell extract of *I. galbana* grown under a wide range of environmental conditions. Increasing growth irradiance level imposed an increase in the *in vitro* activity of the enzyme to a maximal value at about 100 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$. At higher irradiance levels, ACCase activity slightly decreased to a relatively constant value, which represents about 60% of the maximal ACCase activity value (Fig. 3a). Nitrogen limitation imposed a reduction in cellular protein content (Table 1) and a decrease in the *in vitro* activity of ACCase. Nitrogen limited culture

grown at a low dilution rate (0.2 day^{-1}) was characterized by a low activity of ACCase. The enzyme activity was restored as growth rate increased above 0.3 day^{-1} to give an *in vitro* activity value close to the one measured under optimal growth conditions (Fig. 3b). Changes in the *in vitro* activity of ACCase measured in *I. galbana* cultures grown under nitrogen limiting conditions were associated with similar variations in the *in vivo* rate of lipid synthesis. A fairly good linearly positive correlation ($R=0.960$) was calculated between the *in vitro* activity of ACCase and the *in vivo* rate of lipid synthesis (Fig. 4, closed circles). However, the relationships between these parameters in nutrient sufficient cultures grown under a wide range of irradiance levels deviated from linearity (Fig. 4, open circles), although a qualitative comparison suggests a similar response of *in vivo* rate of lipid synthesis and *in vitro* ACCase activity to variations in irradiance levels (Figs. 2 and 3a). The linear correlation coefficient calculated separately for that data set was fairly low ($r = 0.567$).

Cellular abundance of ACCase

The cellular abundance of individual soluble proteins in nitrogen limited cells of *I. galbana* after electrophoresis on 11% SDS-PAGE and staining with Coomassie blue is shown in Fig. 5a. When proteins were loaded corresponding to equal cell numbers, the general impression was that virtually all proteins decrease, as the dilution rate was less than 0.5 day^{-1} , approximately in the same proportion.

Changes in several individual proteins were analysed by probing Western blots with polyclonal antibodies raised against the proteins of interest that were previously purified from *I. galbana* (Falkowski et al., 1989; Livne and Sukenik 1990). The cellular abundance of ACCase in nitrogen limited cells decreased at growth rates lower than 0.5 day^{-1} . A similar pattern of variations was observed with the cellular abundance of the large subunit (LSU) of ribulose biphosphate carboxylase/oxygenase (Rubisco) (Fig. 5b). The relative abundance of ACCase was calculated from densitometric measurements of the Western blots and was

related to the *in vitro* activity of ACCase (Fig. 6). Good linear correlation was calculated for the relationships between the normalized cellular abundance of ACCase and the enzyme *in vitro* activity ($r=0.92$).

I. galbana cultures grown in nutrient sufficient media under a wide range of irradiance levels show insignificant variations in cellular soluble protein content (Table 1). Resolving soluble proteins by SDS PAGE could not reveal substantial changes in the abundance of a given protein band by Coomassie blue stain (data not shown). However, immunoblot of ACCase indicated a minor increase in the cellular abundance of this protein as irradiance level increased from 30 to 175 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ (data not shown). Nevertheless, these minor variations in ACCase abundance do not correlate with larger changes in the *in vitro* activity of ACCase observed under these growth conditions (Fig. 3a).

Discussion

Since the early report on the effect of growth conditions on cellular chemical composition of *Chlorella* by Spoehr and Milner (1949), many studies were carried out to evaluate the effect of environmental conditions on cellular lipid content and fatty acid composition in unicellular algae (Roessler, 1990b). Nevertheless, an attempt to elucidate the biochemical mechanisms that are responsible for variations in cell composition was reported only in a few cases (Suen et al., 1987; Roessler, 1988a,b; Guckert and Cooksey, 1990). Lipid synthesis in unicellular algae, as in green leaves of higher plants, is a complicated pathway that is carried out at different cellular compartments and uses substrate competitively with other biochemical pathways. The first step in lipid synthesis is the formation of fatty acids. This process takes place in the chloroplast and is initiated with the formation of malonyl CoA via carboxylation of acetyl CoA by ACCase. This step is responsible for allocating cellular carbon into a lipid biosynthetic pathway; thus it is considered as a rate limiting step and is regulated by various biochemical mechanisms (Wakil et al., 1983; Harwood, 1988; Post-Beittenmiller et al., 1990).

The enzyme acetyl CoA carboxylase was recently purified and kinetically characterized from two different unicellular algae; the diatom *Cyclotella cryptica* (Roessler, 1990a) and the prymnesiophyte *Isochrysis galbana* (Livne and Sukenik, 1990). Both studies suggested the possibility that ACCase is metabolically regulated. The kinetic properties of the enzyme studied in an *in vitro* system indicated that the enzyme *in vivo* activity is enhanced by light, since it has alkaline optimal pH and a requirement for NADH ATP and Mg ions. However, Roessler (1988b) reported that silica deficiency in diatom cultures enhanced carbon allocation into lipids, an observation that was associated with an increase in the *in vitro* activity of ACCase. This increase could be blocked by protein synthesis inhibitors. Therefore, an additional regulatory mechanism at the enzyme abundance level was suggested as a potential control mechanism of cellular lipid content in algae. The availability of polyclonal antibodies against ACCase of *I. galbana* (Livne and Sukenik, 1990) facilitated our attempt to elucidate the role of ACCase in the biochemical mechanisms that control lipid accumulation in the alga.

Nitrogen limited cultures grown in chemostats at various dilution rates showed significant variations in the cellular chemical composition. Cellular soluble protein content decreased and lipid and fatty acid increased as growth rate decreased. A similar trend in total nitrogen and carbon content was reported by Herzig and Falkowski (1989) for *I. galbana* grown under similar conditions. Despite a wide variation in growth irradiance levels used to cultivate *I. galbana* in nutrient sufficient turbidostats, no significant differences in soluble proteins, total lipids and fatty acid content were observed. Therefore, it is suggested to distinguish between two modes of lipid metabolism in *I. galbana* that are imposed at different sets of environmental conditions. Various growth irradiance levels insignificantly affect lipid content of nutrient sufficient cultures, but evidently imposed variations in cellular lipid composition (Sukenik et al., unpublished). Lipid accumulation in nitrogen

limited cultures, however, is partially attributed to the fact that lipid synthesis continues at the expense of protein synthesis that decreased due to the low availability of nitrogen.

The present experimental data obtained with *I. galbana* cultures grown under nutrient sufficient conditions at various irradiance levels suggest that the cellular lipid content is not related to the cellular abundance of ACCase but rather to some metabolic factors such as *in vivo* availability of acetate, that is evidently generated in the mitochondria (Lichtenthaler, 1989) or other cofactors, substrates and regulators that affect the enzyme *in vivo* activity. Alternatively, lipid synthesis under these environmental conditions is not regulated at the carboxylation step of acetyl CoA but at other enzymatic steps in the fatty acid biosynthetic pathway (Harwood, 1988).

General reduction in cellular proteins was observed in *I. galbana* cultures grown at different nitrogen limiting conditions. Under these conditions, the abundance of some proteins significantly decreased relative to others (Coleman et al., 1988; Falkowski et al., 1989). It was observed that nitrogen limitation imposed a reduction in the *in vitro* activity and cellular abundance of ACCase in *I. galbana*. An opposite response was reported for *Cyclotella cryptica* under silicon deficient conditions, where ACCase *in vitro* activity doubled during silicon deficiency (Roessler, 1988b).

Lipid synthesis rate that was estimated for *I. galbana* cultures grown under a wide range of nitrogen limiting conditions was generally reduced with growth rate. The *in vitro* activity of ACCase and its relative cellular abundance followed that trend, raising the possibility that under nitrogen limiting conditions, the availability of ACCase controls the rate of fatty acid and lipid synthesis. No linear relationships were found between the normalized cellular content of ACCase and the cellular pool size of fatty acids in *I. galbana* cultures grown under nitrogen limiting conditions. However, a fairly good linear correlation was calculated for nitrogen limited cultures grown at dilution

rates ranging from 0.3 to 0.92 day⁻¹ between the total fatty acid content and the normalized cell capacity for malonyl CoA production in an algal cell during its average generation time; i.e the mathematical product of ACCase activity per cell and mean generation time (Fig. 7). Similar relationships were also found for the cellular abundance of fatty acids relative to protein and the normalized capacity for production of malonyl CoA per cellular protein unit over the averaged generation time (Fig. 7). These relationships further support the possibility that the availability of ACCase controls the rate of lipid synthesis in *I. galbana*.

However, under severe nitrogen limitation ($\mu = 0.2$ day⁻¹), the cell capacity for malonyl CoA production deviates from the calculated linear relationship (Fig. 7). It is postulated that under these conditions, lipid degrading enzymes are severely reduced relative to the lipid anabolic system, thus facilitating a slow turnover of cellular lipids.

Obviously, the data presented in this study supply indirect and only correlative evidence to the potential regulatory role of ACCase in lipid synthesis and accumulation in unicellular algae. Nitrogen limiting conditions represent an ideal experimental system in which different cellular levels of ACCase and its *in vitro* activities can be obtained for further elucidation of this question. Alternatively, a kinetic study of the cellular processes that are involved in nitrogen starvation and recovery from starvation (Livne and Sukenik, unpublished data) may provide additional information regarding the regulatory role of ACCase.

Acknowledgements

This research was supported by the U.S.-Israel Agriculture Research and Development Fund under grant I-1082-86. Additional support was provided by the U.S.-Israel Binational Science Foundation under grant 86-00376, and by the Strauss/Hanauer Memorial Fund Inc.

References

- Ben-Amotz, A., Fishler, R. and Schneller, A. (1987) Chemical composition of dietary species of marine unicellular algae and rotifers with emphasis on fatty acids. *Mar. Biol.(Berl.)* 95: 31-36.
- Bligh, E.G. and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37: 911-917.
- Cohen, Z., Vonshak, A. and Richmond, A. (1988) Effect of environmental conditions on fatty acid composition of the red alga *Porphyridium cruentum*: correlation to growth rate. *J. Phycol.* 24: 328-332.
- Coleman, L.W., Rosen, B.H. and Schwartzbach, S.D. (1988) Environmental control of carbohydrate and lipid synthesis in *Euglena*. *Plant Cell Physiol.* 29: 1007-1014.
- Coombs, J., Darley, W.M., Holm-Hansen, O. and Vulcani, B.E. (1967) Studies on the biochemistry and fine structure of silica shell formation in diatoms. Chemical composition of *Navicula pelliculosa* during silicon starvation synchrony. *Plant Physiol.* 42: 1601-1606.
- De Pauw, N., Morales, J. and Persoone, G. (1984) Mass culture of microalgae in aquaculture systems: progress and constraints. *Hydrobiologia* 116/117: 121-134.
- Falkowski, P.G., Sukenik, A. and Herzig, R. (1989) Nitrogen limitation in *Isochrysis galbana* (Haptophyceae). II. Relative abundance of chloroplast proteins. *J. Phycol.* 25: 471-478.
- Guckert, J.B. and Cooksey, K.E. (1990) Triglyceride accumulation and fatty acid profile changes in *Chlorella* (Chlorophyta) during high pH-induced cell cycle inhibition. *J. Phycol.* 26: 72-79.
- Guillard, R.R.L. and Ryther, J.H. (1962) Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* (Cleve) Gran. *Can. J. Microbiol.* 8: 229-239.

- Harwood, J.L. (1988) Fatty acid metabolism. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 39: 101-188.
- Herzig, R. and Falkowski, P.G. (1989) Nitrogen limitation in *Isochrysis galbana* (Haptophyceae). I. Photosynthetic energy conversion and growth efficiencies. *J. Phycol.* 25: 462-471.
- Kates, M. (1972) Isolation, analysis and identification of lipids. In *Techniques in Lipidology*. Edited by Work, T.S. and Work, E. pp. 268-618. Elsevier, Amsterdam.
- Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* 227: 680-685.
- Lepage, G. and Roy, C.C. (1984) Improved recovery of fatty acid through direct transesterification without prior extraction or purification. *J. Lipid Res.* 25: 1391-1396.
- Lichtenthaler, H.K. (1989) Plant lipid biosynthesis as target for biocides. In *Biological Role of Plant Lipids*. Edited by Biacs, P.A., Gruiz, K. and Kremmer, T. pp. 389-400. Plenum Press, New York.
- Livne, A. and Sukenik, A. (1990) Acetyl coenzyme A carboxylase from the marine prymnesiophyte *Isochrysis galbana*. *Plant & Cell Physiol.* 31: 851-858.
- Marsh, R. and Weinstein, D.B. (1966) Simple charring method for determination of lipids. *J. Lipid Res.* 7: 574-576.
- Myers, J. (1980) On the algae: thoughts about physiology and measurements of efficiency. In *Primary Productivity in the Sea*. Edited by Falkowski, P.G. pp. 1-16. Plenum Press, New York.
- Piorreck, M., Baasch, K.-H. and Pohl, P. (1984) Biomass production, total protein, chlorophylls, lipids and fatty acids of fresh water green and blue-green algae under different nitrogen regimes. *Phytochemistry* 23: 207-216.

- Post-Beittenmiller, D., Jaworski, J.G. and Ohlregge, J.B. (1990) Analysis of *in vivo* pools of acyl ACP's provides evidence on light/dark control of fatty acid biosynthesis in spinach leaf. In *Plant Lipid Biochemistry, Structure and Utilization*. Edited by Quinn, P.J. and Harwood, J.L. Portland Press, London (in press).
- Rodhouse, P.G., Roden, C. and Somerville-Jacklin, M.E. (1983) Nutritional value of microalgal mass cultures to the oyster *Ostrea edulis* L. *Aquaculture* 32: 11-18.
- Roessler, P.G. (1988a) Effects of silicon deficiency on lipid composition and metabolism in the diatom *Cyclotella cryptica*. *J. Phycol.* 24: 394-400.
- Roessler, P.G. (1988b) Changes in the activities of various lipid and carbohydrate biosynthetic enzymes in the diatom *Cyclotella cryptica* in response to silicon deficiency. *Arch. Biochem. Biophys.* 267: 521-528.
- Roessler, P.G. (1990a) Purification and characterization of acetyl-CoA carboxylase from the diatom *Cyclotella cryptica*. *Plant Physiol.* 92: 73-78.
- Roessler, P.G. (1990b) Environmental control of glycerolipid metabolism in microalgae: commercial implications and future research directions. *J. Phycol.* 26: 393-399.
- Sauer, A. and Heise, K.P. (1984) Regulation of acetyl-coenzyme A carboxylase and acetyl-coenzyme A synthetase in spinach chloroplasts. *Z. Naturforsch.* 39c: 268-275.
- Shifrin, N.S. and Chisholm, S.W. (1981) Phytoplankton lipids: interspecific differences and effects of nitrate, silicate and light-dark cycles. *J. Phycol.* 17: 374-384.
- Spoehr, H.A. and Milner, H.W. (1949) The chemical composition of *Chlorella*: effect of environmental conditions. *Plant Physiol.* 24: 120-149.
- Suen, Y., Hubbard, J.S., Halzer, G. and Tornabene, T.G. (1987) Total lipid production of the green alga *Nannochloropsis* sp. QH under different nitrogen regimes. *J. Phycol.* 23: 289-296.

Sukenik, A. and Wahnou, R. (1990) Biochemical quality of marine unicellular algae with special emphasis on lipid composition. I. *Isochrysis galbana*. *Aquaculture* (in press).

Sukenik, A., Bennett, J. and Falkowski, P.G. (1987) Light saturated photosynthesis limitation by electron transport or carbon fixation? *Biochim. Biophys. Acta* 891: 205-215.

Sukenik, A., Carmeli, Y. and Berner, T. (1989) Regulation of fatty acid composition by irradiance level in the eustigmatophyte *Nannochloropsis* sp. *J. Phycol.* 25: 686-692.

Wakil, S.G., Stoops, J.K. and Joshi, V.C. (1983) Fatty acid synthesis and its regulation. *Ann. Rev. Biochem.* 52: 537-579.

Figure Legends

- Fig. 1. Variations in cellular lipid and fatty acid content in *I. galbana* cultures grown under various environmental conditions: a) steady state continuous cultures grown in nutrient sufficient media under a wide range of irradiance levels. b) Cultures grown in nitrogen limited chemostats at various dilution rates.
- Fig. 2. Effect of light intensity and nitrogen limitation on *de novo* synthesis of lipids. Cultures were grown in nitrogen limited chemostats at different growth rates (closed circles) or in nutrient sufficient turbidostats that were operated at different irradiance levels. *De novo* synthesis of lipids was assessed by incubating subsamples *in situ* with $\text{NaH}^{14}\text{CO}_3$ for 2 hr.
- Fig. 3. Variations in the *in vitro* activity of ACCase in *I. galbana* grown under various irradiance levels (a) and at different nitrogen limiting conditions (b) in steady state continuous cultures.
- Fig. 4. The relationships between the *in vitro* activity of ACCase and the rate of *de novo* synthesis of lipids in *I. galbana* cultures grown under various environmental conditions. The data obtained from nitrogen limiting conditions (closed circles) fit a linear correlation ($r = 0.960$). However, cultures grown in nutrient sufficient conditions under various irradiance levels (open circles) deviated from that relationship.
- Fig. 5. Variations in soluble proteins in *I. galbana* grown in a nitrogen limiting medium at different growth rates. A. Coomassie blue stained soluble proteins that were extracted from *I. galbana* and resolved by 11% SDS-PAGE. Gel was loaded with soluble proteins corresponding to an equal cell number. B. Western blots of corresponding gels run to identify acetyl CoA carboxylase (ACCase) and large subunit (LSU) of Rubisco, using polyclonal antibodies raised against the antigens previously isolated from the alga.

Fig. 6. Relationships between the *in vitro* activity of ACCase and its normalized cellular content in *I. galbana* cultures grown at different growth rates in a nitrogen limited medium. Normalized cellular content of ACCase was estimated by optically scanning Western blots.

Fig. 7. Relationships between the normalized capacity for malonyl CoA production and pool size of fatty acids in *I. galbana* grown at different nitrogen limiting conditions. The data point deviating from the linear relationships represents extreme nitrogen limiting conditions ($\mu = 0.2$ day⁻¹). This point was excluded from the correlation coefficient calculation ($r = 0.956$).

Table 1. Total soluble protein and fatty acid to protein ratio in *Isochrysis galbana* grown in nutrient sufficient media under a wide range of irradiance levels (turbidostats) and in nitrogen limited steady state cultures illuminated at 175 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ (chemostats).

Culture system	Ig $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$	μ day ⁻¹	Protein content pg cell ⁻¹	Fatty acid/ protein ratio w/w
Turbidostats	30	0.38	5.71	0.65
	50	0.51	6.03	0.53
	75	0.63	6.43	0.53
	150	0.83	6.06	0.43
	200	1.12	6.32	0.51
	300	1.28	6.22	0.51
Chemostats	175	0.20	1.66	2.89
	175	0.33	3.38	1.35
	175	0.50	3.66	1.04
	175	0.72	4.75	0.84
	175	0.90	4.62	0.59

Fig. 1.

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Effect of environmental conditions on lipid accumulation in the marine alga *Isochrysis galbana*: the role of acetyl CoA carboxylase

Assaf SUKENIK and Alexander LIVNE

National Institute of Oceanography, Israel Oceanographic & Limnological Research, P.O.Box 8030, Haifa 31080, Israel.

SYNOPSIS

The effect of environmental conditions on lipid and fatty acid content was examined in the marine prymnesiophyte *Isochrysis galbana* in relation to the *in vitro* activity and relative cellular content of acetyl CoA carboxylase (ACCase).

INTRODUCTION

Unicellular algae under normal growth conditions contain a high proportion of proteins. However, under specific environmental conditions protein abundance decreases while lipid content increases, concomitant with changes in the relative distribution of cellular fatty acids (Shifrin & Chisholm, 1981; Cohen et al., 1988; Roessler, 1988a). It has been postulated that variations in lipid content are related to changes in the activity and abundance of acetyl CoA carboxylase (ACCase), an enzyme that catalyzes the formation of malonyl CoA, one of the initial steps in fatty acid biosynthesis (Hellyer et al., 1986; Roessler, 1988b). ACCase has been recently purified from the prymnesiophyte *Isochrysis galbana* (Suknik & Livne, 1990). The enzyme was biochemically characterized, and specific polyclonal antibodies were prepared against the enzyme. In the present study, we have attempted to correlate variations in lipid content and fatty acid concentrations to changes in the activity and cellular content of ACCase in *Isochrysis galbana* grown under different environmental conditions.

MATERIALS AND METHODS

The effect of irradiance level on lipid content and ACCase activity was assessed in *Isochrysis aff. galbana* (T-ISO) cultures maintained at 25°C under

This research was supported by grant No. I-1082-86 from BARD, the U.S.-Israel Binational Agricultural Research & Development Fund. Additional support was provided by the U.S.-Israel Binational Science Foundation under grant 86-00376 and by the Strauss/Hanauer Memorial Fund.

steady state conditions in 2.6 liter turbidostats as previously described (Suknik et al., 1989). Light was provided continuously by banks of VHO fluorescent tubes, and cells were grown at two levels: low irradiance (LL) and high irradiance (HL), 30 and 300 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$, respectively. Nitrogen limited cultures were grown continuously at 25°C in similar 2.6 liter vessels using artificial sea water media, supplied with 200 $\mu\text{M NaNO}_3$ and other nutrients. Light was provided at a photon flux of 175 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$. Cultures were maintained at dilution rates of 0.2 (low nitrogen-LN) and 0.9 (high nitrogen-HN) per day.

The *in vitro* activity of ACCase was determined by incorporation of ^{14}C bicarbonate into malonyl CoA, as described by Saner & Heise (1984). One unit of enzyme activity was defined as the amount of enzyme required to catalyze the formation of one μmol of malonyl CoA under the standard assay conditions. The relative cellular abundance of ACCase and the large subunit (LSU) of Rubisco were determined by immunoassay of soluble protein extracted from *I. galbana* after separation by SDS polyacrylamide gel electrophoresis. Polyclonal antibodies were prepared against the ACCase subunit of *Isochrysis* as previously described (Suknik & Livne, 1990).

Lipids were extracted by the Bligh & Dyer (1959) method. Total lipids and fatty acid content were quantified as previously described (Suknik et al., 1989).

RESULTS AND DISCUSSION

In the prymnesiophyte *I. galbana*, the cell mean generation time (MGT) is greatly affected by growth conditions. Maximal value was obtained under low nitrogen conditions (LN) while the shortest MGT was measured under high light conditions (Fig. 1). The two different irradiance levels examined in this study had no effect on the cellular content of total lipids and fatty acids; however, moderate nitrogen limitation (dilution rate of 0.9 d^{-1}) imposed a slight reduction in these parameters while severe nitrogen limitation (dilution rate of 0.2 d^{-1}) induced cellular accumulation of total lipids and fatty acids (Fig. 1).

The *in vitro* activity of ACCase in HL cultures was two times greater than in cells grown under LL conditions. Low nitrogen availability imposed even greater reduction in ACCase activity as compared with the activity of HN or HL cultures (Fig. 1).

The relative abundance of ACCase in *I. galbana* cultures grown at different environmental conditions was determined by the immunoblot technique using polyclonal antibodies raised against ACCase isolated from the alga. Low light conditions imposed a relatively lower cellular content of ACCase than did high light conditions. Severe nitrogen limitation caused a threefold reduction in the cellular content of ACCase as compared with cells grown under moderate nitrogen limitation (Fig. 2). Variations in ACCase activity measured in *I. galbana* cultures grown at different environmental conditions were directly correlated with the relative abundance of the enzyme (Fig. 1). Similar experiments were conducted to analyze the effect of environmental conditions on the major photosynthetic carboxylation enzyme ribulose biphosphate carboxylase oxygenase (Rubisco), by using antibodies raised against its large subunit (LSU). It is demonstrated (Fig. 2) that the cellular level of the two different carboxylation enzymes responded differently to environmental conditions.

Fig. 1. Variations in cell generation time, total lipid content, fatty acid content, ACCase activity (normalized to cell) and relative cellular abundance of ACCase in *Isochrysis galbana* grown at different environmental conditions. LL - low light, HL - high light, LN - low nitrogen, HN - high nitrogen (for detailed experimental procedure see Materials and Methods).

Fig. 2. Variations in soluble proteins in *I. galbana* grown at various environmental conditions. A. Coomassie blue stained soluble proteins extract from *I. galbana* and resolved by SDS-PAGE (10% acrylamide). Gels were loaded with soluble proteins corresponding to equal cell number. B. Western blots of corresponding gels ran to identify acetyl CoA carboxylase (ACCase) and the large subunit (LSU) of Rubisco.

The cellular content of lipids and fatty acids is the sum of synthesis and degradation processes that should be superimposed on cell division rate. Although cellular fatty acid content is not directly correlated with the *in vitro* activity of ACCase, the product of the enzyme activity and the average cell generation time can be directly related to fatty acid content in the alga, although an exception was measured in LN cultures. It is postulated that these conditions cause a reduction in lipid degradation rate, thus fatty acid accumulation is observed.

REFERENCES

- Bligh, E.G. & Dyer, W.J. (1959) Can. J. Biochem. Physiol. **37**, 911-917.
 Cohen, Z., Vonshak, A. & Richmond, A. (1988) J. Phycol. **24**, 328-332
 Hellyer, A., Bambridge, H.E. & Slabas, A.R. (1986) Biochem. Soc. Trans. **14**, 565-568.
 Roessler, P.G. (1988a) J. Phycol. **24**, 521-528
 Roessler, P.G. (1988b) Arch. Biochem. Biophys. **267**, 521-528
 Saner, A. & Heise, K.-P. (1984) Z. Naturforsch. **39C**, 268-275
 Shifrin, N.S. & Chisholm, S.W. (1981) J. Phycol. **17**, 374-384
 Sukenik, A. & Livne, A. (1990) Plant Cell Physiol., in press
 Sukenik, A., Carmeli, Y. & Berner, T. (1989) J. Phycol. **25**, 686-692

4.3 Regulation of lipid synthesis in nitrogen deprived cultures and during recovery from starvation

Microalgae show metabolic changes in response to environmental conditions. Nutrient deprivation and subsequent recovery have been experimentally used in many studies to manipulate various metabolic pathways and the composition of cellular organelles in order to elucidate structure-function relationships, developmental patterns and regulatory mechanisms. A common experimental approach calls for deprivation of nitrogen from growth media and nitrogen repletion to impose fast recovery of the algal culture. Deprivation of nitrogen and recovery from nitrogen starvation have been used to study variations in the photosynthetic capacity and molecular organization of the photosynthetic apparatus in blue-green algae, red algae and green algae.

Nitrogen limitation was often used to vary the cellular chemical composition of unicellular algae. Many studies suggested that nitrogen starvation or alternatively, silicate deprivation from diatom cultures impose an increase in the cellular lipid content in many unicellular algae. However, an attempt to elucidate the biochemical mechanisms that are responsible for these variations was made only in a few cases. The potential role of acetyl CoA carboxylase, the enzyme that is responsible for carbon allocation into the lipid biosynthetic pathway, in the regulation of lipid accumulation in unicellular algae was suggested in the literature and examined in a previous section.

The present section summarizes our attempt to evaluate the role of ACCase in regulating lipid synthesis in nitrogen deprived cultures of *Isochrysis*. Fatty acid content and the rate of lipid synthesis were measured in *Isochrysis galbana* grown under nitrogen starvation and in cultures recovering from nitrogen deprivation. Nitrogen starvation imposed a reduction in the cellular soluble protein content, concomitant with a reduction in the *in vitro* activity of the enzyme acetyl CoA carboxylase. An increase in total fatty acid content is attributed to a differential reduction in cell division and the rate of lipid synthesis. Recovery from nitrogen

deprivation was characterized by an increase in cellular soluble protein content, an increase in the rate of lipid synthesis and amplification of the *in vitro* activity of acetyl CoA carboxylase. Nevertheless, cellular fatty acid content decreased as the culture recovered from nitrogen starvation. The relative cellular pool size of acetyl CoA carboxylase, determined by immunoblot technique, decreased under nitrogen starvation conditions and increased as cells recovered from it. Variations in enzyme pool size linearly correlated with the *in vitro* activity of the enzyme and with the rate of *in vivo* lipid synthesis, suggesting that lipid synthesis under these conditions is regulated by the abundance of acetyl CoA carboxylase. Cellular accumulation of acetyl CoA carboxylase during recovery from nitrogen starvation is ascribed to *de novo* synthesis of the enzyme. However, photosynthetic proteins such as ribulose biphosphate carboxylase are synthesized earlier than acetyl CoA carboxylase in the recovery process.

**Lipid synthesis and abundance of acetyl CoA carboxylase in *Isochrysis galbana*
(Prymnesiophyceae) following nitrogen starvation**

ALEXANDER LIVNE and ASSAF SUKENIK

**National Institute of Oceanography
Israel Oceanographic and Limnological Research
Tel Shkmona, P.O.B. 8030, Haifa 31080, Israel**

Summary

Fatty acid content and the rate of lipid synthesis were measured in the marine prymnesiophyte *Isochrysis galbana* grown under nitrogen starvation and in cultures recovering from nitrogen deprivation. Nitrogen starvation imposed a reduction in cellular soluble protein content, concomitant with a reduction in the *in vitro* activity of the enzyme acetyl CoA carboxylase. An increase in total fatty acid content is attributed to a differential reduction in cell division and the rate of lipid synthesis. Recovery from nitrogen deprivation was characterized by an increase in cellular soluble protein content, an increase in the rate of lipid synthesis and amplification of the *in vitro* activity of acetyl CoA carboxylase. Nevertheless, the cellular fatty acid content decreased as the culture recovered from nitrogen starvation. The relative cellular pool size of acetyl CoA carboxylase determined by immunoblot technique decreased under nitrogen starvation conditions and increased as cells recovered from it. Variations in enzyme pool size linearly correlated with the *in vitro* activity of the enzyme and with the rate of *in vivo* lipid synthesis, suggesting that lipid synthesis under these conditions is regulated by the abundance of acetyl CoA carboxylase. Cellular accumulation of acetyl CoA carboxylase during recovery from nitrogen starvation is ascribed to *de novo* synthesis of the enzyme. However, photosynthetic proteins such as ribulose biphosphate carboxylase are synthesized earlier than acetyl CoA carboxylase in the recovery process.

Abbreviations:

ACCase - acetyl CoA carboxylase; LSU - large subunit; Rubisco - ribulose biphosphate carboxylase/oxygenase; PAGE - polyacrylamide gel electrophoresis.

Introduction

Microalgae show metabolic changes in response to environmental conditions. Nutrient deprivation and subsequent recovery have been experimentally used to manipulate various metabolic pathways and the composition of cellular organelles in order to elucidate structure-function relationships, developmental patterns and regulatory mechanisms. Deprivation of nitrogen and recovery from nitrogen starvation have been used to study variations in the photosynthetic capacity and molecular organization of the photosynthetic apparatus in blue-green algae [1-3], red algae [4], chrysophytes [5, 6] and green algae [7].

Nitrogen limitation was often used to vary the cellular chemical composition of unicellular algae. Many studies suggested that nitrogen starvation or alternatively silicate deprivation in diatom cultures imposes an increase in the cellular lipid content in many unicellular algae [8-11]. However, an attempt to elucidate the biochemical mechanisms that are responsible for these variations was made only in a few cases [12-15]. The potential role of acetyl CoA carboxylase, the enzyme that is responsible for carbon allocation into the lipid biosynthetic pathway, in the regulation of lipid accumulation in the diatom *Cyclotella cryptica*, was suggested by Roessler [14], who isolated the enzyme from the alga and characterized its kinetic properties [16]. Recently we analyzed the effect of environmental parameters on the lipid content and fatty acid composition in continuous cultures of *Isochrysis galbana* grown in chemostats and turbidostats in conjunction with measurements of the *in vitro* activity and cellular abundance of acetyl CoA carboxylase [17, 18]. The enzyme of *I. galbana* was previously characterized, purified to a near homogeneity and used for the preparation of polyclonal antibodies [19]. The availability of antibodies against acetyl CoA carboxylase contributed to our attempt to elucidate the role of the enzyme in the biochemical mechanisms that control lipid accumulation in *Isochrysis*. In the present study, nitrogen starvation conditions were applied to study the relationships between acetyl CoA carboxylase and variations in cellular lipid concentration. Furthermore, variations in lipid content and cellular concentration of

acetyl CoA carboxylase measured during recovery from nitrogen starvation indicated a relatively slow increase in the cellular abundance of the enzyme.

Materials and methods

Organism and growth conditions. *Isochrysis galbana* (T-ISO) was obtained from the microalgae culture collection of U.S. DOE Solar Energy Research Institute (Golden, CO). Algae cultures were grown batchwise in sea water media buffered with 0.2 M tris (pH 7.8) and enriched with nutrients (0.75 mM KNO₃, 0.05 mM Na₂HPO₄, 2 mM NaHCO₃ and other trace elements and vitamins) as modified from Guillard and Ryther [20]. Cultures were maintained at 25°C at continuous illumination provided by VUO fluorescence tubes at an irradiance level of 150 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$. After three days of growth in that medium, when nitrate concentration was less than 20 μM , the culture was diluted with two volumes of fresh media free of nitrate to start a nitrogen deprived culture. These cultures were monitored at 12 to 24 h intervals to measure lipid and fatty acid content and enzyme activity. After 72 h under nitrogen deprived conditions, cultures were fortified with nitrate at a final concentration of 3 mM. Recovery from nitrogen starvation was followed at 3 to 24 h intervals by recording lipid and fatty acid content, enzyme activity and *de novo* synthesis of cellular soluble proteins with special emphasis on acetyl CoA carboxylase.

Assay for ACCase activity. The *in vitro* activity of ACCase was determined by incorporation of ¹⁴C bicarbonate into malonyl CoA, an acid stable product of the enzymatic reaction, following the procedure suggested by Sauer and Heise [21]. One unit of enzyme activity is defined as the amount of enzyme required to catalyze the formation of one μmole of malonyl CoA per minute under the defined assay conditions.

Extraction and quantification of lipids and fatty acids. Total lipids were extracted with methanol:chloroform:water (10:5:4 v/v/v) [22] as modified by Kates [23]. Total lipid was quantified as previously described by Sukenik et al. [24]. Fatty

acid methyl esters were prepared following the method described by Lepage and Roy [25], and separated by capillary gas liquid chromatography using a Hewlett Packard 5890A gas chromatograph [24]. Haptadecanoic acid was used as an internal standard to quantify cellular fatty acid content.

Synthesis of lipids. *De novo* synthesis of lipids was assessed by following the assimilation of $^{14}\text{CO}_2$ into lipids. At various time intervals, 50 ml samples were withdrawn and incubated *in situ* with $\text{NaH}^{14}\text{CO}_3$ (1 $\mu\text{Ci ml}^{-1}$, 0.4 mCi mmole $^{-1}$ final specific activity) for 1 h. At the end of the incubation period, cells were collected, washed and monitored for ^{14}C incorporation. Lipids were extracted as described above and monitored for radioactivity by liquid scintillation counting.

***De novo* synthesis of soluble proteins.** *In vivo* labeling of newly synthesized proteins was performed in recovering cultures at 3 h intervals. Culture samples were harvested by centrifugation at 2000 g, washed twice with fresh media and were finally resuspended in nitrate enriched (3 mM) medium to give a cell density of 2×10^7 per ml. Cultures were acclimated *in situ* for 15 min, followed by the addition of ^{14}C bicarbonate (10 $\mu\text{Ci/ml}$ and final specific activity 0.4 $\mu\text{Ci}/\mu\text{mole}$). Cultures were incubated for 3 h, then harvested by centrifugation and washed 3 times to remove unincorporated ^{14}C . Cells were disrupted (see below) and soluble proteins were loaded on SDS 5% polyacrylamide slab gel. Resolved polypeptides were visualized by Coomassie blue staining. The gel was dried and exposed to X-ray film to detect newly synthesized polypeptides.

Other analytical methods. Cells were disrupted in extraction buffer by passing them through a precooled Yeda press cell at 1400 psi at least 3 times as previously described [19]. The pressate was centrifuged at 34000 g for 10 min. The supernatant was used to measure ACCase *in vitro* activity. Soluble proteins were resolved by gel electrophoresis. Denatured SDS-PAGE was performed using the buffer system of Laemmli [26] with a 5% acrylamide separating slab gel. Resolved proteins were electrophoretically transferred to nitrocellulose, and the ACCase subunit was detected by reacting the nitrocellulose with anti ACCase serum diluted at 1:2000.

Secondary antibodies, goat anti rabbit IgG (L+H) conjugated to horseradish peroxidase (Biomakor), were used in a dilution ratio of 1:3000. Visualization was performed by peroxidase color developing with H_2O_2 and benzidine. Stained blots were optically scanned with a densitometer to provide relative quantification.

Proteins were quantified by the Coomassie blue binding method (Biorad) using bovine serum albumin as a standard. Radioactivity was measured by liquid scintillation counting in an ontron Betamatic scintillation counter using the external standard ratio method for quench correction. A commercially available Aquasol II (Du Pont) scintillation cocktail was used for all isotopic counts.

Results

Within the first 24 h of nitrogen deprivation, *Isochrysis galbana* cultures showed a rapid reduction in cellular soluble protein content and a 75% increase in total fatty acid content (Fig. 1a). During that period, cell concentration increased from 2×10^6 to 2.6×10^6 i.e less than 0.3 divisions per day. The following 48 h of nitrogen starvation were characterized by a slow decrease in cellular soluble protein content and a fairly stable concentration of fatty acids (Fig. 1a). The *in vitro* activity of ACCase of nitrogen deprived cultures followed the same pattern as soluble cellular proteins. It was rapidly reduced during the first 24 h, succeeded by a slower decrease during the next 48 h (Fig. 1b). Similarly the *in vivo* rate of lipid synthesis decreased by 60% at the early stage of the starvation time. It was further reduced, but at a slower rate, during the following starvation period (Fig. 1b). The cellular level of acetyl CoA carboxylase was assessed from Western blots using specific antisera raised against the ACCase subunit of *I. galbana*. The cellular content of ACCase was reduced in response to nitrogen starvation as shown in Fig. 2. Variations in the relative cellular abundance of ACCase that were imposed by nitrogen starvation roughly followed the changes in cellular soluble proteins and the *in vitro* activity of ACCase in these cultures (Figs. 1a and 1b).

Upon the addition of 3 mM nitrate to a nitrogen deprived culture, a rapid recovery process was observed. Cellular soluble protein content (Fig. 1c) and inorganic carbon assimilation rate (data not shown) rapidly increased concomitant with a restoration of cell division. Within the first 24 h of the recovery from nitrogen starvation, cell concentration increased from 2.3×10^6 to 5.5×10^6 cell/ml. A doubling rate of 1.26 day^{-1} . During that period, cellular fatty acid content was reduced by more than 50% (Fig. 1c), suggesting that the recovery of lipid synthesis rate was slower than division rate, leading to the dilution of cellular fatty acid. As the recovery process continued, cellular fatty acid concentration increased to 2.2 pg/cell, a value normally found in nitrogen repleted cultures of *I. galbana* grown under standard conditions. The *in vitro* activity of ACCase linearly increased during the first 48 h of recovery from nitrogen starvation to give a maximal value of 13.2×10^{-4} units/mg soluble protein. No significant changes in the *in vitro* activity of ACCase were observed during the following day of the recovery process (Fig. 1d). The recovery from nitrogen starvation was characterized by variations in the *in vivo* rate of lipid synthesis which followed a similar trend as the ACCase *in vitro* activity. The rate of lipid synthesis increased over the first 48 h of the recovery process but slightly decreased in the following days (Fig. 1d). The cellular content of ACCase increased in response to the addition of nitrate to nitrogen deprived cultures (Fig. 2). Relative quantification obtained from densitometric scanning of ACCase blots from nitrogen deprived culture and from cultures recovering from starvation revealed a fairly high linear correlation with the *in vitro* ACCase activity in these cultures ($r=0.96$; Fig. 3). Linear relationships were also established between the *in vitro* ACCase activity and the rate of *in vivo* lipid synthesis measured in nitrogen deprived cultures and in cultures recovering from starvation ($r=0.99$; Fig. 4).

The process of recovery from nitrogen deprivation is characterized by a rapid accumulation of cellular proteins (Fig. 1c). The electrophoretic separation of cellular soluble proteins and of pulse labelled soluble proteins (Fig. 5) indicated that synthesis and accumulation of various proteins occurred at different times during the

recovery period. The subunit of ACCase and the large subunit of ribulose biphosphate carboxylase/oxygenase indicated in Fig. 5 were identified by immunoblot using polyclonal antibodies raised against corresponding polypeptides isolated from the algae. It is demonstrated that LSU accumulation initiated within 3 h from the starting of the recovery process while ACCase accumulation lagged and began only 6 h later (Fig. 5a). The different accumulation of these polypeptides was associated with *de novo* synthesis of the proteins which occurred at different periods in the recovery process. The large subunit as well as the small subunit (data not shown) of Rubisco are synthesized earlier than the subunit of ACCase (Fig. 5b).

Discussion

Nutrient starvation in general and specifically nitrogen deprivation were used as an experimental tool to manipulate lipid content and composition in many unicellular algae [8, 10, 11]. Some algal species respond to nitrogen starvation by a massive accumulation of lipids while others tend to store carbohydrates with lower quantities of lipid. In both cases, cellular lipid content is greater in nitrogen deprived cultures than in cultures grown under nitrogen repleted conditions. In his recent review, Roessler [11] summarized many of the reported studies evaluating the effect of environmental conditions on cellular lipid content and fatty acid composition in unicellular algae, and indicated that in most cases, triacylglycerol is the major lipid class to be accumulated under nutrient starvation. Lipid accumulation was generally attributed to the fact that storage lipids do not contain nitrogen, thus continue to be synthesized in nitrogen deprived cells, while the synthesis of proteins and nucleic acids is severely reduced [11, 12, 27].

Fatty acid synthesis takes place in the chloroplast and initiated with the formation of malonyl CoA via the carboxylation of acetyl CoA by ACCase. This step is responsible for allocating cellular carbon into the lipid biosynthetic pathway, thus it is considered as a rate limiting step and is regulated by various biochemical mechanisms [28-30]. The enzyme acetyl CoA carboxylase was recently purified and

kinetically characterized from two different unicellular algae; the diatom *Cyclotella cryptica* [16] and the prymnesiophyte *Isochrysis galbana* [19]. These studies suggested that ACCase is metabolically regulated. In both cases, the kinetic properties of the enzyme indicated that its *in vivo* activity is enhanced by light since it has alkaline optimal pH and a requirement for NADH, ATP and Mg ions. Roessler [13] reported that silica deficiency in diatom cultures enhanced carbon allocation into lipids, an observation that was associated with an increase in the *in vitro* activity of ACCase. This increase could be blocked by cycloheximide, suggesting the involvement of *de novo* synthesis of ACCase in lipid accumulation in silica deprived cultures of the diatom *Cyclotella cryptica* [14]. Using polyclonal antibodies raised against ACCase of *Isochrysis galbana*, we recently reported that nitrogen limitation in continuously grown cultures imposed a reduction in the cellular abundance of ACCase concomitant with a decrease in the *in vitro* activity of the enzyme [17]. It was further suggested that under nitrogen limiting conditions, the cellular lipid content is controlled by the cellular capacity to produce malonyl CoA. The results of the present study further support the previously observed relationships between the *in vivo* rate of lipid synthesis and the *in vitro* activity of ACCase. Nitrogen deprivation imposed a rapid reduction in ACCase activity, simultaneously, cell division almost ceased, resulting in an accumulation of lipids and fatty acids during the early stage of the starvation. These results fit the linear relationships demonstrated between cellular fatty acid content and the normalized cell capacity for malonyl CoA production during average generation time [17].

The present results obtained with *I. galbana* are in accord with other studies reporting the accumulation of lipids in nutrient deficient cultures of various unicellular algae [8, 11, 12, 14, 17], and postulate the involvement of various mechanisms which are not mutually exclusive: (1) Nutrient starvation imposes a reduction of carbon flow into nitrogen containing compounds, consequently lipids and carbohydrates are preferentially synthesized. (2) Cell division is promptly stopped, while anabolic activities leading to lipid synthesis continue, although at a

slower rate, resulting in cellular accumulation of lipids. (3) The reduction in cellular proteins is evidently associated with a selective decrease in various enzymes and structural proteins [6, 31]. In spite of the reduction in ACCase content and activity measured in the response of *I. galbana* to nitrogen starvation, it is postulated that lipid degradation enzymes are much more affected under these conditions. Evidently, there is an imbalance between synthesis and degradation rates, resulting in lipid accumulation. Interestingly, in diatoms this imbalance further increases towards lipid synthesis, as *de novo* synthesis of ACCase [14] increases the cellular capacity to synthesize fatty acids. The increase in lipid cellular content during nitrogen deprivation is evidently due to *de novo* synthesis and accumulation of neutral storage lipids, mostly triacylglycerol [12, 13, 18]. The increase in the neutral to polar lipid ratio observed in nitrogen deprived cultures is attributed in part to the relative increase in the level of enzymatic activities that lead to the synthesis of storage lipids as compared with membrane lipids [11].

Upon recovery from nitrogen starvation, *de novo* synthesis of ACCase restored the cellular pools and the enzyme *in vitro* activity. These changes were associated with an increase in the *in vivo* rate of lipid synthesis. However, recovery of the lipid synthesis machinery seems to be slower than reconstruction of other cellular activities. Cell division is completely restored within the first 24 h of the recovery. A fast *de novo* synthesis of ribulose biphosphate carboxylase leads to a rapid accumulation of that photosynthetic enzyme and evidently to restoration and reorganization of the photosynthetic apparatus. Early recovery of the cellular photosynthetic capacity and subsequent restoration of lipid synthesis abilities seem to be a general mechanism to deal with any cellular requirements for reorganization of the photosynthetic machinery. In the green alga *Dunaliella tertiolecta* a one step transition from high to low light conditions was associated with reorganization of the photosynthetic apparatus [32]. A kinetic study of various components in that system demonstrated that lipid synthesis, specifically lipid components of thylakoid

membranes, were the last to be synthesized to allow final reorganization of the photosynthetic apparatus [33].

Acknowledgements

This research was supported by the U.S.-Israel Agriculture Research and Development Fund under grant I-1082-86. Additional support was provided by the Strauss/Hanauer Memorial Fund Inc. We thank Dr. Paul Falkowski for the generous gift of Rubisco LSU antibodies.

References

- 1 Fujita, Y. and Hattiru, A. (1960) *Plant Cell Physiol.* 1, 281-292
- 2 Allen, M.M. and Smith, A.J. (1969) *Arch. Mikrobiol.* 69, 114-120
- 3 Yamanaka, G. and Glazer, A.N. (1980) *Arch. Mikrobiol.* 124, 39-47
- 4 Levy, I. and Gantt, E. (1990) *J. Phycol.* 26, 62-68
- 5 Herzig, R. and Falkowski, P.G. (1989) *J. Phycol.* 25, 462-471
- 6 Falkowski, P.G., Sukenik, A. and Herzig, R. (1989) *J. Phycol.* 25, 471-478
- 7 Plumley, F.G. and Schmidt, G.H. (1989) *Proc. Nat. Acad. Sci. U.S.A.* 86, 2678-2682
- 8 Shifrin, N.S. and Chisholm, S.W. (1981) *J. Phycol.* 17, 374-384
- 9 Ben-Amotz, A., Tornabene, T.G. and Thomas, W.H. (1985) *J. Phycol.* 21, 72-81
- 10 Cobelas, M.A. and Lechado, J.Z. (1989) *Grasas y Aceites* 40, 118-145
- 11 Roessler, P.G. (1990) *J. Phycol.* 26, 393-399
- 12 Suen, Y., Hubbard, J.S., Holzer, G. and Tornabene, T.G. (1987) *J. Phycol.* 23, 289-296
- 13 Roessler, P.G. (1988) *J. Phycol.* 24, 394-400
- 14 Roessler, P.G. (1988) *Arch. Biochem. Biophys.* 267, 521-528
- 15 Guckert, J.B. and Cooksey, K.E. (1990) *J. Phycol.* 26, 72-79
- 16 Roessler, P.G. (1990) *Plant Physiol.* 92, 73-78
- 17 Sukenik, A. and Livne, A. (1991) *Plant Cell Physiol.* (in press)
- 18 Sukenik, A. and Wahnnon, R. (1991) *Aquaculture* (in press)
- 19 Livne, A. and Sukenik, A. (1990) *Plant Cell Physiol.* 31, 851-858
- 20 Guillard, R.L. and Ryther, J.H. (1962) *Can. J. Microbiol.* 8, 229-239
- 21 Sauer, A. and Heise, K.P. (1984) *Z. Naturforsch.* 39c, 268-275
- 22 Bligh, E.G. and Dyer, W.J. (1959) *Can. J. Biochem. Physiol.* 37, 911-917
- 23 Kates, M. (1972) in *Techniques in Lipidology* (Work, T.S. and Work, E., eds.), pp. 268-618, Elsevier, Amsterdam
- 24 Sukenik, A., Carmeli, Y. and Berner, T. (1989) *J. Phycol.* 25, 686-692
- 25 Lepage, G. and Roy, C.C. (1984) *J. Lipid Res.* 25, 1392-1396

- 26 Laemmli, U.K. (1970) *Nature (Lond.)* 227, 680-685
- 27 Aronson, S., Berner, T. and Dubinsky, Z. (1980) in *Algae Biomass* (Shelef, G. and Soeder C.J., eds.), pp. 575-601, Elsevier/North Holland, Amsterdam
- 28 Harwood, J.L. (1988) *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 39, 101-138
- 29 Post-Beittenmiller, D., Jaworski, J.G. and Ohlegge, J.B. (1991) in *Plant Lipid Biochemistry, Structure and Utilization* (Quinn, P.J. and Harwood, J.L., eds.), Portland Press, London (in press)
- 30 Wakil, S.J., Stoops, J.K. and Joshi, V.C. (1983) *Ann. Rev. Biochem.* 52, 537-579
- 31 Coleman, L.W., Rosen, B.H. and Schwartzbach, D. (1988) *Plant Cell Physiol.* 29, 423-432
- 32 Falkowski, P.G. (1984) *Photosynthetica* 18, 62-68
- 33 Sukenik, A., Bennett, J., Mortain-Bertrand, A. and Falkowski, P.G. (1990) *Plant Physiol.* 92, 891-898

Figure legends

Fig. 1. Effect of nitrogen starvation (a and b) and recovery from starvation (c and d) on the cellular content of soluble protein, the fatty acid content, the *in vivo* lipid synthesis rate, and the *in vitro* activity of ACCase in *I. galbana*.

Fig. 2. Variations in the cellular content of ACCase in *I. galbana* grown under nitrogen deprived conditions and during recovery from nitrogen starvation. Cultures were sampled at 24 h intervals, and soluble proteins were resolved by SDS 7% PAGE. Lanes were loaded with protein corresponding to equal cell number. ACCase subunit was visualized on Western blots using polyclonal antisera and peroxidase reaction.

Fig. 3. The relationships between the relative cellular content of ACCase and the *in vitro* activity of the enzyme in *I. galbana* cultures grown under nitrogen deprived conditions (o) and during the recovery from starvation (●).

Fig. 4. The relationships between the *in vitro* activity of ACCase and the *in vivo* rate of lipid synthesis in *I. galbana* cultures grown under nitrogen deprived conditions (o) and during the recovery from starvation (●).

Fig. 5. Relative abundance of individual cellular soluble proteins in *I. galbana* cultures recovering from nitrogen starvation (A), and variation in the relative *de novo* synthesis of individual polypeptides during the recovery (B). Samples were pulse labeled with ^{14}C bicarbonate for 3 h at various time intervals after the addition of 3mM nitrate to nitrogen deprived cultures. Soluble proteins were resolved by SDS 5% PAGE loaded with protein equivalent to equal cell number and stained by Coomassie blue (A). Soluble protein samples of equal dpm were loaded on a similar PAGE system, resolved and autoradiographed (B).

Fig 1

Fig 2

Fig 3

Fig 4

Fig 5

5. Algal biomass and lipid production - Strategies for controlling productivity

An attempt to improve algal biomass productivity and to enhance the yields of a desired product may require an advanced system analysis and evaluation of a multicomponent system. First, the biological components of the system should be fully understood at their physiological, biochemical and molecular levels. Second, the production system has to be defined for its physical and engineering properties. Lastly, all these components are combined to evaluate the potential of the system to produce algal biomass or any other desirable product such as lipids, pigments or polysaccharides.

Evaluation of the algal production system was performed to predict potential yields at current operational conditions, taking into account our up-to-date biological knowledge. It is comprised of a mathematical simulation model based on wide biological and physiological information available for a given algal species, and on physical and engineering characteristics of the production system. In addition, potential strategies to enhance the production of specific biochemical compounds by microalgae were evaluated.

5. 1. Potential Strategies for Regulating the Flux of Carbon into Specific Chemical Classes in Microalgae

PAUL G. FALKOWSKI

Oceanographic Sciences Division, Department of
Applied Science, Brookhaven National Laboratory,
Upton, New York 11973

ABSTRACT

There are three strategies for improving the yield of desired chemical constituents in microalgae: phenotypic selection, physiological forcing, and genetic engineering. Of these, only phenotypic selection in conjunction with physiological forcing are viable at the present time and for the foreseeable future. The problem of how to increase the flux of carbon into non-protein macromolecules, while retaining high growth rates, appears to be largely insurmountable due to the nature of biochemical regulation of carbon and nitrogen traffic. Genetic modification may be possible, but to be successful requires a better understanding of intermediary carbon metabolism.

The first obligation of microalgae is to grow and divide (12). As the chemical composition of microalgae is normally about 50% protein (13), in order to maintain balanced growth, cells primarily synthesize proteins. Protein synthesis is the basis of most conventional agriculture. However, as it is much easier and cheaper to grow rice or wheat than *Dunaliella* and *Spirulina* as sources of protein, photosynthetically based aquaculture has tended to exploit some of the biochemical pathways which are unique to microalgae, in an attempt to produce more esoteric, highly valued molecules for specialized markets. With few exceptions such molecules are not proteins, and one is immediately faced with the problem of how to maximize the production of the desired product. There are three basic strategies which may be employed to achieve this end, namely phenotypic selection, physiological forcing and genetic modification. I will briefly discuss here the advantages and drawbacks of each of these approaches.

Phenotypic Selection. Phenotypic selection is a rather obvious approach for increasing the yield of a desired chemical. The strategy is to find a naturally occurring species or strain which specializes in the production of the desired product. Perhaps one of the best examples of this strategy is that of using *Dunaliella bardawil* rather than some other species of *Dunaliella* for the production of β -carotene (2). But, this strategy also has some drawbacks: it is often time consuming and expensive and there is no guarantee that the ideal species even exists in nature. Much time and effort has been spent screening for fast growing lipid producing microalgae without much success. High lipid production is accompanied by such slow growth rates that there is no threat that biological production of petrochemicals from microalgae will compete in the market with geologically produced oil in the foreseeable future.

Physiological Forcing. The second strategy, physiological forcing, is to create a set of environmental conditions which maximize the production of the desired chemical. For example, the fraction of carbon allocated to lipids or carbohydrates, at the expense of protein synthesis, can be greatly increased by starving cells for inorganic nitrogen (11). In the absence of nitrogen but in the presence of light, cells continue to fix carbon but cannot synthesize amino acids, therefore, carbon traffic is diverted to nitrogen poor chemicals. A similar response can be elicited by removing phosphate, and in the case of diatoms,

Fig. 1. Physiological Forcing. Schematic diagram showing the effect of nitrogen limitation on the partitioning of carbon between carbohydrates, protein and lipids. Under nitrogen sufficient conditions (left panel), photosynthetically fixed carbon is distributed among the three major macromolecular pools according to the growth demands. When nitrogen is limiting (right panel), carbon is still fixed but little or no net protein synthesis can occur. Protein nitrogen is recycled within the cell while carbon accumulates in carbohydrate and/or lipid fractions.

silicate, to promote lipid production (20). The essential features of this strategy are summarized in Figure 1. In the presence of excess inorganic nutrients and energy, carbon is fixed and converted into proteins, lipids and carbohydrates. The specific distribution of carbon within each of these pools is regulated by both genetics and environmental factors (light intensity, temperature, pH, etc.). When inorganic nitrogen becomes limiting however, amino acid biosynthesis is reduced; and consequently the net accumulation of carbon in protein is lowered. If this situation is maintained in a steady state, such as in a nitrogen-limited chemostat at low relative growth rates, carbon fixation will continue as long as protein synthesis is sufficient to replenish those proteins which turnover rapidly in the photosynthetic apparatus (7, 8). A relatively large fraction of the fixed carbon often accumulates in lipids or carbohydrates, which may collect in the cell or be secreted or excreted as extracellular materials.

A second variation on the theme of physiological forcing is to grow cells in nutrient replete culture and then induce starvation for an essential element when cells are late in log growth. This approach may be somewhat more easily controlled than a continuous culture system under outdoor mass culture conditions. The

primary drawback of using nutrient starvation to physiologically force carbon into lipids or carbohydrates is that photosynthetic rates decline rapidly (15), leading to a loss of efficiency in the formation of carbon-rich products. The reduction in photosynthetic performance results, in large part, from the reduction in the synthesis of crucial proteins, such as D1 and D2, which are required to form a PS II reaction center, ribulose 1,5-bisphosphate carboxylase, which is essential for carbon fixation, and light harvesting chlorophyll proteins, which are fundamental for light absorption (Fig. 2).

In order to begin to overcome this apparently insurmountable problem on a biochemical level it is necessary to understand how the flux of carbon is partitioned between the various macromolecular pools. At this time we do not really understand the factors which regulate carbon partitioning on the biochemical level.

Models. I would like to focus on two biochemical processes, one, photosynthesis, sets the upper limit to production; the other, nitrogen assimilation, may be important in determining the path of carbon.

In algal mass culture systems cell densities are so high that effectively all photosynthetically active radiation is absorbed by the culture. The relationship between carbon fixation and irradiance can be adequately described by an exponential function of the form: $P(I) = P_{\max}(1 - e^{-\sigma \cdot \tau \cdot I})$ (17),

where: P is carbon fixation at irradiance I

σ is the absorption cross section of PS II

τ is the minimum turnover time of the photosynthetic apparatus

and $P_{\max} = n/\tau$ where

n is the concentration of photosynthetic units.

Fig. 2. SDS-polyacrylamide gel, stained with Coomassie blue, of total cell proteins from the marine diatom *Thalassiosira weissflogii*. Cells were grown in ammonium-limited chemostat culture at 80% and 20% of their maximal relative growth rate. Equal amounts of total protein were loaded from high growth rate (lane A) and low growth rate (lane B). Standards of indicated molecular mass were run in lane C. Major differences between nitrogen sufficient and nitrogen limited cells are noted for 4 bands; band 1 corresponds to the large subunit of RubisCo; band 2 is unknown; band 3 corresponds to the apoprotein of the light harvesting chlorophyll a/c/fucoxanthin pigment-protein complex; lane 4 corresponds to the small subunit of RubisCo. These data show that N limitation affects protein levels on the translational level.

When $P \ll P_{\max}$ the fixation of carbon approximates a linear function of the rate of photon absorption, and the energy transfer function is the maximum quantum yield (4). As $P \rightarrow P_{\max}$ the turnover of electrons in the photosynthetic apparatus becomes rate limiting.

In *D. tertiolecta*, experimental data has shown that the absorption cross section of PS II varies with growth irradiance and nitrogen availability. As cells become shade adapted, cellular levels of chlorophyll increase, but there is also an increase in the absorption cross section of PS II. The change in the absorption cross section of PS II in this species is apparently a result of changes in the pigment composition of the light harvesting complex, LHC II, which serves as an antenna for PS II (17). Somewhat surprisingly the absorption cross section of PS II also increases as cells become nitrogen limited (7). While the concentration of cellular chlorophyll decreases, it appears that cells preferentially synthesize antenna chlorophyll proteins for the remaining reaction centers (20).

The minimum turnover time of the photosynthetic apparatus appears to be related to the ratio of the carboxylating enzyme, ribulose 1,5 biphosphate carboxylase/oxygenase to the electron transport chain (19). The biochemical and biophysical data suggest that as cells shade adapt $P_{\max}$ often (but not always) decreases per reaction center because the increase in RubisCo is not proportional to the increase in reaction centers. The relationship between RubisCo and photosynthetic electron transport means that under most conditions cells will have reduced maximal productivity as cell densities increase in mass culture, a situation which has been documented by Laws and coworkers (9). Interestingly, some microalgae, such as *Isochrysis galbana*, do not appear to increase r as they become shade adapted (4), and presumably would remain more efficient in the upper part of the water column in dense mass culture conditions.

Sukenik et al. (17) have modeled the effects of genetic alterations which lead to changes in the absorption cross section of PS II and/or r on the productivity of *D. tertiolecta* in simulated outdoor mass cultures. The model results reveal that productivity could increase by 55% if the cellular RubisCo content were increased four-fold. If however, the absorption cross section were increased without allowing a cell to shade adapt, productivity could be doubled. Thus, this model suggests that it is probably not worthwhile, from an economic standpoint, to genetically amplify only the cellular content of RubisCo, assuming that transformation or increased promotion of both the large and small subunit genes was realistic. However, if the absorption cross section of PS II could be selectively increased, as might occur by promotion of cab genes, productivity might increase substantially. Thus an understanding of the regulation of cab genes, as well as LSU or SSU genes, might be more useful if one is interested in increasing the productivity of mass culture systems.

This type of analysis serves two purposes. First, it demonstrates that if enough is known about the mechanisms of the regulation of a process (in this case photosynthesis), a crude but useful model can help to guide research by identifying the interactions between competing the interactions between competing rate limiting processes, and therefore help to identify or "target" genes of interest. Secondly, it provides a relatively inexpensive way of investigating how alterations in one process or another affect the rate of production of a given product, which in the model of Sukenik et al. (17), was simply biomass.

The partitioning of carbon between macromolecular pools is also amenable to mathematical modeling, if enough is understood about the regulatory or "feedback" processes in the metabolic pathway. There are three basic types of regulation which affect intermediary metabolism. The first type is at a branch in a metabolic sequence; a common intermediate may be used to synthesize two or more products.

This type of regulation sets up a competition between two pathways for a substrate

and is especially important if one of the products is "committed" to a given pathway; there is no way of simply reversing the forward reaction. A good example of this type of regulation is in the formation of δ -aminolevulinic acid (ALA) from glutamate in the biosynthesis of chlorophyll (1). ALA is relatively difficult to deaminate, and once formed, the carbon atoms in the glutamate precursor do not have much chance of being incorporated into protein. A second type of regulatory reaction occurs when there is a large difference in the free energy between substrate and product, making a given reaction basically irreversible. There are many examples of such enzymes, including glutamine synthetase, phosphoenolpyruvate carboxykinase, and of course ribulose biphosphate carboxylase/oxygenase. The third basic type of regulation is allosteric modulation, such that enzymatic activity is integrated into the overall metabolic sequence and/or affected by the environment. It should be clear that allosteric interactions may occur in enzymes which are both irreversible and/or at branchpoints in metabolic pathways.

As mentioned previously, fixed carbon is often diverted from proteins to polysaccharides or lipids when cells are deprived of nitrogen. While this diversion is superficially obvious, and simply due to the fact that amino acid synthesis requires ammonium, on a more fundamental level the diversion of carbon indicates a change in metabolism. We do not really understand what the molecular basis of these changes are, but I would like to explore some issues of current investigation in nitrogen metabolism and its relation to carbon fixation and chemical composition. Tracer experiments with radioactive $^{13}\text{NH}_4^+$ have shown that nitrogen is incorporated into amino acids in eukaryotic algae and cyanobacteria via glutamine synthetase, which has a low K_m for ammonium (10's of μM) and a K_m for glutamate of 30 mM (6). Glutamine synthetase is one of the most elegantly regulated enzymes in plant metabolism (5). It is at a key branchpoint in carbon metabolism, is an irreversible reaction (although glutamine can be deaminated by

Fig. 3. (a): Schematic diagram showing the branchpoint and regulation of glutamate. This amino acid is used by glutamine synthetase in ammonium assimilation, is transaminated to form other amino acids and also is the precursor of δ -aminolevulinic acid, which is in turn the precursor of chlorophyll. (b): Glutamine synthetase activity is regulated by adenylate energy charge in vitro. Increasing energy charge leads to increased enzyme activity of this (essentially) irreversible enzyme.

glutaminase) and is allosterically modulated by the adenylate pool (Fig. 3a, b). Glutamate is regenerated for GS via the action of glutamine 2-oxoglutarate amidotransferase (GOGAT), which consumes α -ketoglutarate. The carbon in α -kg is derived from oxidation of pyruvate in the Krebs cycle. When ammonium is available, glutamate pools are high and amino acids are readily synthesized from the transamination of glutamate to various keto acids. However, when ammonium fluxes become limiting to growth, free amino acid pools decrease markedly and carbon is diverted into polysaccharides or lipids. In essence, amino acid (and subsequently protein) biosynthesis and polysaccharide (or lipid) biosynthesis compete for carbon skeletons, with amino acid biosynthesis "winning" when ammonium is available.

It is interesting to note that the apparent competition between amino acids and carbohydrates (or lipids) appears to occur in nutrient replete cells on a daily basis. Cuhel et al. (3), found that *Dunaliella tertiolecta* carbohydrates were preferentially synthesized during the day while at night proteins were synthesized using the carbon stored in carbohydrate. Post et al. (14) found that in the diatom *Thalassiosira weissfloggii*, the carbohydrate pool actually increased during photophase and was depleted during scotophase. The amplitude of the change is proportional to the relative growth rate of the cell. A simulation model of algal growth developed by Loogman (10) allows for the calculation of the instantaneous pool size of carbohydrate and its accumulation rate in *Oscillatoria agardhii* grown under nutrient replete conditions and at light saturation. The model takes an empirical systems approach and couples carbohydrate accumulation to chlorophyll accumulation; it could conceptually be modified to include photoadaptive processes. However, such a model does not provide biochemical insight into actual regulatory mechanisms, and it is not immediately obvious how one identifies specific enzymes which are responsible for the rate processes.

What might one do to allow amino acid biosynthesis to proceed at a high rate (and therefore support a high growth rate of the cell), while increasing the rate of polysaccharide or lipid production? One possibility is to alter GS, perhaps by site directed mutagenesis, such that its K_m for glutamate is higher. Such an alteration should lead to elevated glutamate levels. Glutamate is in equilibrium with α -kg, an oxidative deamination mediated by glutamate dehydrogenase (GDH). With elevated glutamate pools, the flux of carbon into α -kg might increase resulting in a larger flux of carbon into polysaccharides or lipids. In this scenario, the binding site of glutamate on GS becomes a target for alteration, with the goal of increasing the flux of carbon into polysaccharides or lipids without starving cells for nitrogen.

Is this scenario realistic? Probably not. If it were possible for a cell to evolve with a high growth rate in nutrient poor conditions by increasing the relative proportion of lipid and or polysaccharide to protein we would expect that a variety of species might have high C:N ratios at high growth rates. To my knowledge this does not occur in nature. However, it should be stressed that at this point metabolic investigations focussing on the regulation of carbon and nitrogen fluxes into macromolecules have not provided sufficient information to develop a metabolic model of a given cell, similar to the one described by Sukenik et al. (16) for biomass or Loogman's (10) for carbohydrates.

Genetic Engineering. The third basic strategy, which may be able to maximize the production of specific products without seriously compromising growth rates is genetic engineering. I use the term "engineering" here in a broad sense, in that mutation and selection may be extremely useful in enhancing the production of a specific product without necessarily genetically transferring DNA from the outside world into the candidate host. This strategy is unproven in algal mass

culture and requires a conceptual understanding of not only how, but more important, what to engineer.

The potential use of genetic engineering in algal mass culture consists of four basic phases:

- 1) Deciding what to produce or modify.
- 2) Identifying target genes which regulate the production of the desired product.
- 3) The ability to mutate or transform and then genetically select altered cells which have optimized production of the product without major reductions in growth rates.
- 4) The ability to maintain the altered cell line in mass culture without reversion to the wild type.

If the product is a polysaccharide or lipid it is a secondary gene product, that is the synthesis of the product is regulated at some level by the action of proteins (i.e., the primary gene products). As mutation or transformation can only result in manipulation of genes which ultimately code for proteins, it becomes critical to identify the enzymes and their location in the biosynthetic pathway which regulate the synthesis of the desired product. Mutation and selection can be entirely empirical, and one looks for enrichment of the desired product without necessarily understanding how the mutation works. A basis of genetic transformation strategy is that once the regulatory enzymes are identified, amplification, suppression or some modification of the responsible genes would lead to the desired effect. These "target" genes become the focus of attention for altering a given metabolic pathway. As I have described this requires a good understanding of the intermediary metabolism of a given product.

A key element common to both mutation and transformation is having an ability to select cells with the desired characteristics from thousands, or even millions of cells which are not especially desired. Selection of transformed cells is often based on having an expressed genetic marker such as herbicide or antibiotic resistance. Marine microalgae obtained from culture collections have usually been isolated from bacteria by the use of broad spectrum, gram negative antibiotics, which appear to select resistant eukaryotic cells. We have found that *Dunaliella tertiolecta*, *D. salina*, and *D. bardwill* are all resistant to kanamycin, G418 and nigramycin. The same clone of *D. tertiolecta* (Woods Hole clone DUN) we use has been reported to be sensitive to G418 by Latorella (16); however, we are unable to reproduce those results. *Isochrysis galbana*, obtained from Bigelow Laboratory, is sensitive to G418 but not kanamycin, but a clone of the same species maintained by the Israel Oceanographic and Limnological Institute in Haifa is resistant to both antibiotics. Unless we spend some effort attempting to screen eukaryotic cell lines and or isolate new cell lines we will inevitably have trouble reproducing transformations between labs.

CONCLUSIONS

I am optimistic that, based on our emerging knowledge of the mechanism of photosynthesis and the structure and function of the oxygenic photosynthetic apparatus, we will have the ability to increase the productivity of biomass by algae in the next decade. I am less sanguine about being able to increase the relative production of generic classes of compounds, such as lipids and or

polysaccharides by genetically modifying cells. The rate of synthesis of these compounds is essentially a compromise between maximal relative growth and the absolute necessity of cells to synthesize proteins in order to divide. The enrichment of specific products, such as a given lipid or polysaccharide, by phenotypic selection and physiological forcing, rather than man-induced genetic alteration, will probably be fruitful in the near term. One thing is reasonably certain, it will be necessary to greatly improve our basic understanding of microalgal biochemistry in order to apply the emerging concepts in genetic engineering to algal mass culture.

Acknowledgments--This research was supported by the U.S. Israel Binational Agricultural Research and Development Fund (BARD) under grant No. I-1082-86. I thank Assaf Sukenik and Jon Zehr for valuable discussions and comments.

LITERATURE CITED

1. BEALE SI 1976 The biosynthesis of δ -aminolevulinic acid in plants. *Philos Trans R Soc Lond B* 273: 99-108
2. BEN-AMOTZ A, M AVRON 1983 On the factors which determine massive β -carotene accumulation in the halotolerant alga *Dunaliella bardawil*. *Plant Physiol* 72: 593-597
3. CUHEL RL, PB ORTNER, DRS LEAN 1984 Night synthesis of protein by algae. *Limnol Oceanogr* 29: 731-744
4. DUBINSKY Z, PG FALKOWSKI, K WYMAN 1986 Light harvesting and utilization by phytoplankton. *Plant Cell Physiol* 27: 1335-1349
5. FALKOWSKI PG 1983 Enzymology of nitrogen assimilation. In *Nitrogen in the Marine Environment*, EJ CARPENTER, DG CAPONE, eds, pp 839-868. Academic Press, New York
6. FALKOWSKI PG, RB RIVKIN 1976 The role of glutamine synthetase in the incorporation of ammonium in *Skeletonema costatum* (Bacillariophyceae). *J Phycol* 12: 448-450
7. KOLBER Z, J ZEHR, P FALKOWSKI 1988 Effects of growth irradiance and nitrogen limitation on photosynthetic energy conversion in photosystem II. *Plant Physiol* (in press)
8. LAWS EA, TT BANNISTER 1980 Nutrient- and light-limited growth of *Thalassiosira fluviatilis* in continuous culture, with implications for phytoplankton growth in the ocean. *Limnol Oceanogr* 25: 457-473
9. LAWS EA, S TAGUCHI, J HIRATA, L PANGE 1986 High algal production rates achieved in a shallow outdoor flume. *Biotech Bioeng* 28: 191-197
10. LOOGMAN JG 1982 Influence of photoperiodicity on algal growth kinetics. Ph.D. thesis. University of Amsterdam
11. MORRIS I 1981 Photosynthetic products, physiological state, and phytoplankton growth. *Can Bull Fish Aquat Sci* 210: 83-102
12. MYERS J 1980 On the algae: thoughts about physiology and measurements of efficiency. In *Primary Productivity in the Sea*, PG FALKOWSKI, ed, pp 1-16. Plenum Press New York
13. PARSONS TR, K STEPHENS, JDH STRICKLAND 1961 On the chemical composition of eleven species of marine phytoplankton. *J Fish Res Bd Can* 18: 1001-1016
14. POST AF, Z DUBINSKY, K WYMAN, PG FALKOWSKI 1985 Physiological responses of a marine planktonic diatom to transitions in growth irradiance. *Mar Ecol Prog Ser* 25: 161-169
15. RHIEL E, K KRUPINSKA, W WEHRMEYER 1986 Effects of nitrogen starvation on the function and organization of the photosynthetic membranes in *Cryptomonas maculata* (Cryptophyceae). *Planta* 169: 361-369

16. SIMON RD, AH LATORELLA 1986 Approaches to the genetics of *Dunaliella* in algal biomass technologies: an interdisciplinary perspective. J CRAMER, ed, SERI Workshop Report p 15-20
17. SUKENIK A, PG FALKOWSKI, J BENNETT 1987a Potential enhancement of photosynthetic energy conversion in algal mass culture. Biotech Bioeng (in press)
18. SUKENIK A, KD WYMAN, J BENNETT, PG FALKOWSKI 1987b A novel mechanism for regulating the excitation of photosystem II in a green alga. Nature 327: 704-707
19. SUKENIK A, J BENNETT, PG FALKOWSKI 1987c Light-saturated photosynthesis - limitation by electron transport on carbon fixation? Biochim Biophys Acta 891: 205-215
20. SUKENIK A, J BENNETT, PG FALKOWSKI 1988 Changes in the abundance of individual LHCI and LHCII apoproteins with growth irradiance in the marine chlorophyte *Dunaliella tertiolecta*. Biochim Biophys Acta (in press)
21. TAGUCHI S, JA HIRATA, EA LAWS 1987 Silicate deficiency and lipid synthesis of marine diatoms. J Phycol 23: 260-267

5.2 Energy transduction and productivity - a simulation model

A common algal biomass production system consists of a shallow pond in the form of a folded, endless channel through which the algal culture is mixed and forced to move by paddle wheels. Mixing is essential to avoid cell settling and to improve mass transfer of nutrients and gases between the suspended algae and their immediate environment.

Generally, algal growth has been described by various empirical and kinetic models that can be incorporated into an advanced model to simulate the production of algal biomass. Based on actual laboratory data obtained from steady state cultures, we developed a simple model to calculate daily biomass production in a raceway algal-mass culture system. The model evaluated the effect of light attenuation on productivity and identified potential targets for genetic manipulation that may enhance photosynthetic energy conversion in algal mass cultures.

The present model is an elaboration of the previous one by the inclusion of the photoadaptation process of the algae and the annual course of temperature and irradiance, allowing us to simulate year round productivity for any given algal species for which the necessary physiological data are available. The model developed in this study for *Isochrysis galbana* is based on extensive physiological and photosynthetic data. The model output was compared with reports on the mass cultivation of *Isochrysis* for biomass and lipid production.

Optimizing biomass production in an outdoor pond: a simulation model.

A. Sukenik¹, R.S. Levy², Y. Levy², P.G. Falkowski³ and Z. Dubinsky²

¹ National Institute of Oceanography

Israel Oceanographic and Limnological Research

Tel Shikmona, P.O.B. 8030, Haifa 31080, Israel

² Department of Life Sciences, Bar Ilan University

Ramat Gan 52100, Israel

³ Department of Applied Science, Brookhaven National Laboratory

Upton, NY 11973, USA

Key words: algal mass culture, areal density, *Isochrysis*, photosynthesis, productivity, respiration, simulation model

Abstract

A deterministic simulation model was developed to predict production rates of the marine prymnesiophyte *Isochrysis galbana* in an outdoor algal mass culture system. The model consists of photoadaptation, gross photosynthesis and respiration sections. Actual physiological and biophysical laboratory data obtained from steady state cultures grown under a wide range of irradiance levels were used in a mechanistic model to calculate productivity. The resulting values were used to assess optimal operational parameters to maximize algal biomass production. The model predicted a yearly averaged production rate of $9.7 \text{ g C m}^{-2} \text{ day}^{-1}$, which compared well with actual field data reported in the literature. The model evaluated the effect of pond depth and chlorophyll concentration on potential production rate at various seasons, suggesting chlorophyll areal density as an important operational parameter. The model predicted that a yearly averaged chlorophyll areal density of 0.65 g m^{-2} will yield the maximal production rate. Chlorophyll areal density should be adjusted according to the different seasons to give maximal production rate value. This adjustment could be done either by changing pond depth or chlorophyll concentration. The model predicted that under optimal operational conditions, the diurnal respiration losses averaged 35 percent of gross photosynthesis. The calculated growth rate for maximal productivity ranged between 0.15 and 0.24 day^{-1} , suggesting an optimal hydraulic retention time of 6.7 and 4.2 days for various seasons.

Introduction

Mass cultivation of microalgae has been studied for more than four decades (Richmond, 1986; Borowitzka & Borowitzka, 1988). Since the first comprehensive volume published in the early fifties (Burlew, 1953), the potential of cultured algae to provide food and biochemicals focused scientific and commercial interest in these microorganisms. The development of shallow outdoor raceways as open photobioreactors for the production of algal biomass has facilitated a wide range of commercial applications: production of fine biochemicals such as β -carotene (Ben-Amotz & Avron, 1989) and omega-3 fatty acids (Kyle et al., 1989), production of protein rich biomass, cultivation of algal biomass as a primary food source in artificial aquatic food chains aimed at the production of fish, molluscs and crustaceans (De Pauw et al., 1984), and oxidative treatment of domestic and agricultural wastes (Azov & Shelef, 1982).

A common algal biomass production system consists of a shallow pond in the form of a folded, endless channel (for detailed description see Oswald, 1988) through which the algal culture is mixed and forced to move by paddle wheels. Mixing is essential to avoid cell settling and to improve mass transfer of nutrients and gases between the suspended algae and their immediate environment.

Plant production is based on the photosynthetic machinery that converts light energy into chemically stored energy. The light field in aquatic environments is characterized by much steeper gradients than those encountered in the terrestrial domain. Therefore any attempt to cultivate algal biomass is faced with problems of vertical light attenuation and the resulting photadaptive changes in the light harvesting capacity and the photosynthetic efficiency of the algae. Under low light, cells tend to accumulate more photosynthetic pigments (Falkowski, 1980), to decrease the *in vivo* effective spectral average optical cross section- a^* ($=Kc$) (Berner et al., 1989). In addition, many other physiological and biochemical properties of the photosynthetic apparatus are adjusted to the reduction in the incident radiation (Sukenik et al., 1987; Richardson et al., 1984). These changes allow phytoplankton to

maximize growth by optimizing light harvesting while avoiding potential photodynamic damage.

Algal growth has been described by various empirical and kinetic models that can be incorporated into an advanced model to simulate the production of algal biomass. Based on actual laboratory data obtained from steady state cultures, Sukenik et al. (1987) developed a simple model to calculate daily biomass production in a raceway algal- mass culture system. The model evaluated the effect of light attenuation on productivity and identified potential targets for genetic manipulation that may enhance photosynthetic energy conversion in algal mass cultures.

The present model is an elaboration of the previous one by the inclusion of the photoadaptation process of the algae and the annual course of temperature and irradiance, allowing us to simulate year round productivity for any given algal species for which the necessary physiological data are available. The model developed in this study for *Isochrysis galbana* is based on extensive physiological and photosynthetic data (Falkowski et al., 1985; Dubinsky et al., 1986; Herzig & Falkowski, 1989; Sukenik & Wahnnon, 1990). The model output was compared with reports on the mass cultivation of *Isochrysis* for biomass and lipid production (Boussiba et al., 1988).

Model description

A simulation model was used to calculate the production of *Isochrysis galbana* in a conventional algal mass culture system consisting of a shallow raceway pond. The following assumptions were made in the model: 1) productivity is not nutrient limited. 2) productivity can become limited only by light; 3) the pond is well mixed and therefore has no vertical gradients in biomass, nutrients or temperature; and 4) the system is a continuous culture in steady state so that cells are in balanced growth and the growth rate is equal to dilution rate.

The basic equations used in the model are given in Figure 1. The model includes a subroutine designed to calculate the light intensity to which the culture photoadapts (Fig. 2) from incident irradiance and chlorophyll concentration. A

respiration subroutine based on regression analysis of laboratory data is included in the production calculation procedure (Fig. 1). The climatic data set used in this model, consisting of averaged values of hourly radiation and ambient temperatures measured in Elat, Israel, is given in Table 1. The model was written using the DSL language of IBM and the simulations were run on an IBM 3600 mainframe computer.

Photoadaptation

Photoadaptation was determined according to the average light intensity in the pond water column at noon from the equations given in Fig. 1. The calculation of the averaged light intensity for a production pond of a given depth and chlorophyll concentration starts with an arbitrary value of the algal average spectral extinction coefficient normalized to chl *a* (so-called a^* ($=kc$), for details see Dubinsky et al., 1986; Herzig & Falkowski, 1989). This calculated value is considered as the light intensity to which the culture photoadapts, and the value of a^* is adjusted accordingly. Since the value assigned to a^* affects the light attenuation coefficient, it also changes the average irradiance in the profile; this has to be recomputed every time a^* changes in the course of simulated photoadaptation. Using an iteration procedure, this calculation is repeated until the difference between the new calculated value of a^* and the previous one is less than 1 percent. The average light intensity that is calculated at the end of this iteration procedure is taken as the light intensity to which the culture eventually photoadapts. This value is the basis for the interpolation of all necessary physiological parameters from the experimental data for the subsequent calculation procedures of productivity and respiration.

Productivity and respiration

Productivity (P_N) was calculated as the balance between gross photosynthesis (P_G) and respiration (R)

$$P_N = P_G - R$$

Gross photosynthesis as a function of irradiance was calculated by using the exponential (Poisson) model of Dubinsky et al., (1986). Areal photosynthesis rate was calculated for 1 cm depth intervals according to the irradiance level at each depth

(Fig. 1). A photoinhibition factor was activated whenever the irradiance level at a given section exceeded the preset threshold of $600 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$. The effect of temperature on photosynthesis was described by an exponential expression (i.e. Arrhenius function with $Q_{10} = 1.5$). Water temperature was modelled throughout the day by using a sine function lagging by 2 hr after irradiance (Sukenik et al., 1987). Minima and maxima of daily temperature are given in Table 1 for all months.

An initial respiration loss of 10 percent of the areal integrated gross photosynthesis was assumed. From these, net productivity and the specific growth rate μ were calculated. Using published data (Falkowski et al., 1985) relating dark respiration to division rate, respiration rates were iteratively updated (Fig. 1). These corrected respiration rates were then used in turn to update net productivity and overall growth rate. The iteration process terminated as a preselected deviation between successive steps is reached.

Experimental procedure

Isochrysis galbana (clone ISO, provided by R.R.L. Guillard) was cultured in natural sea water enriched with f/2 nutrients (Guillard & Ryther, 1962). Cells were grown in a turbidostat under steady state conditions by periodic dilution of the culture with fresh growth medium as previously described by Dubinsky et al. (1986). Continuous light was provided by a bank of VHO fluorescent tubes at irradiance levels ranging between 30 and $600 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$. The cultures were kept optically thin ($7 \times 10^5 - 1 \times 10^6$ cells/ml) to minimize self shading. During steady state growth at each light level, the culture was sampled and measured for pigment content and photosynthetic characteristics. The experimental data were previously reported by Falkowski et al. (1985) and Dubinsky et al. (1986) and were used in this simulation model.

Results

Chlorophyll concentration, pond depth and average irradiance

The photoadaptation subroutine described in Fig. 2 takes into account the initial optical properties of a given algal culture and calculates the averaged light intensity to which the algal culture eventually adjusts. These values were calculated for *Isochrysis* cultures of various chlorophyll concentrations grown at different pond depths. The calculated average light intensity in those cultures is exponentially reduced as the chlorophyll concentration in the pond increased (Fig. 3). Furthermore, the averaged light intensity as a function of chlorophyll concentration was affected by pond depth; the deeper the depth, the lower the calculated average light intensity (Fig. 3). According to the basic assumptions of the present model, this calculated average value of light intensity at noon is the irradiance level to which the whole algal culture physiologically adapts. These calculations are based on the different time scales of mixing (minutes) and of photoadaptation (hours to days). That value was incorporated into the productivity model and used to assign appropriate physiological parameters from the available experimental data.

The product of chlorophyll density and pond depth, the areal chlorophyll density, determines the depth- averaged irradiance levels to which the algal culture physiologically adapts. Figure 4 describes the effect of areal chlorophyll density on the average irradiance level in an algal pond at noon at two different months, summer and winter. The difference between the two months is very low, suggesting a narrow range for optimal areal chlorophyll density that yields maximal algal biomass production (see below and Table 2).

Areal chlorophyll concentration and productivity

Calculated production rates as a function of chlorophyll concentration for *Isochrysis* cultures grown in ponds of various depth levels are given in Fig. 5 for a representative month of the spring. For all pond depths, a relative constant maximal production rate was calculated. However, that value was obtained at a greater chlorophyll concentration as the pond depth decreased. Furthermore, maximal

production rate was calculated for a relatively wider range of chlorophyll concentrations at shallower ponds. Similar results were obtained when the model was run for other months. Calculated productivity values year round suggested maximal production rates varying between 5.1 to 13.6 g C m⁻² d⁻¹ (equivalent to a biomass production rate of 10.2 to 27.2 g dry weight m⁻² d⁻¹ assuming a carbon content of 50%) according to the season. The lowest productivity value was calculated for December and the highest value for August, although a fairly constant production rate was calculated for the summer months July to August (Table 2).

Fairly constant relationships between chlorophyll areal density and productivity rate were determined for ponds of various depth levels (Fig. 6). The maximal production rate for a spring representative month was established at an areal chlorophyll density of 0.65 g Chl m⁻² (Fig. 6 and Table 2). According to the calculated data presented in Table 2, a yearly averaged maximal production rate of 9.7 g C m⁻² d⁻¹ is potentially obtainable at an average areal chlorophyll density of 0.65 g Chl m⁻².

The average light intensity that led to maximal productivity calculated by the model ranged between 180 and 230 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ (Table 2). This range of irradiances approaches 70 percent of the I_k value for *I. galbana*. I_k is defined as the irradiance level of the onset of light saturation (Talling, 1957) and changes with photoadaptation. The maximal calculated production rates for the different months were obtained at areal chlorophyll densities ranging between 0.375 and 0.750 g Chl m⁻² (Table 2). These values are equivalent to areal carbon density ranging between 32.2 and 59.6 g C m⁻² or areal algal mass density ranging between 64.6 and 119.2 g d.w. m⁻².

Relationships between growth rate and productivity

The model calculates an averaged specific growth rate for the whole pond population as described in Fig. 1. Based on the model initial assumptions, the calculated daily growth rate values are equivalent to a pond dilution rate or the reciprocal of the hydraulic retention time. The calculated relationships between

chlorophyll concentration and the daily overall growth rate are shown in Fig. 7 for ponds of different depth levels. Overall growth rate as a function of areal chlorophyll density is shown in Fig. 8, suggesting an exponential relationship ($r=0.941$) between these parameters. When production rate was expressed as a function of the overall growth rate, μ , one cumulative line was obtained regardless of the pond depth. For all pond depths a common value yielded the maximal production rate (data not shown). The overall average growth rate should be considered as an optimal parameter in any operational effort to yield maximal productivity in different seasons and months. The calculated optimal μ values for maximal productivity ranged between 0.15 and 0.24 day⁻¹ at different seasons of the year (Table 2). These values are equivalent to a hydraulic retention time ranging between 6.7 and 4.2 days, respectively.

Respiration and productivity

The model calculates total respiration losses in an outdoor production pond based on available physiological data. The effect of chlorophyll concentration on the percentage of total daily respiration relative to gross photosynthesis is shown in Fig. 9. In general, respiration losses increased as chlorophyll concentration increased. Nevertheless, respiration losses of shallow ponds are lower than those of deeper ponds with an equal chlorophyll concentration. However, when the respiration fraction is expressed as a function of areal chlorophyll density, only one line is established for each month, demonstrating again the generality of areal chlorophyll density as an operational parameter (data not shown). The respiration fraction calculated for maximal production rates ranged between 31 and 40 percent of total gross photosynthesis (Table 2). These are relatively high values that are affected by respiration in the night and dark respiration losses due to light attenuation in the pond during the day.

Discussion

Deterministic models that contain mathematical equations are commonly used to evaluate the effects of various environmental conditions on a complex biological system. The application of such a model to an algal production system was suggested

in several studies (Sukenik et al., 1987; Grobbelaar et al., 1990). The present study applied a basic photosynthetic equation with some photoadaptation assumptions to calculate potential algal biomass productivity and to optimize the production system by computation rather than by time-consuming experiments that require the operation of large scale, expensive outdoor facilities. The present model was run to simulate an algal production pond system operated at a wide range of chlorophyll concentrations, different pond levels and various climatic conditions.

Unicellular algae are capable of physiologically adjusting the photosynthetic apparatus to a given light field, thus optimizing the photosynthetic capacity of the cell. This photoadaptation process can be described by a first order kinetic (Falkowski, 1984; Sukenik et al., 1990). However, diurnal variations in irradiance level superimposed on light attenuation with depth by the algal culture complicate the relatively simple photadaptation process that was studied in laboratory turbidostats. Therefore a photadaptation subroutine developed in the present model assumes that the culture adjusts its photosynthetic capabilities to the maximal averaged attenuated irradiance level (calculated for noon). The results of the present model suggest that maximal algal biomass productivity is expected at an averaged attenuated irradiance level of $200 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$, a light intensity which is close to saturating value (Dubinsky et al., 1986)

The production rate of *I. galbana* biomass predicted by the model varied between 10.2 and 27.6 g (dw) m^{-2} for the different months. Although *I. galbana* is commonly grown in many marine hatcheries and mariculture systems, only a few reports on mass cultivation of this algae were reported. Boussiba et al. (1988) reported an average output rate of *Isochrysis* biomass during July to be 23.5 g (dw) $\text{m}^{-2} \text{ day}^{-1}$ in a large (100 m^2) outdoor shallow pond. This production rate value is in good agreement with the productivity values calculated for *Isochrysis* by the present model.

Based on the model computation to optimize algal production rate, an operational parameter that combines the chlorophyll concentration and pond depth,

termed areal chlorophyll density, was established. The concept of areal biomass density was first suggested by Soeder (1980) as an important factor in assessing productivity. It was suggested that cultures should be operated at an areal mass density ranging between 50 and 150 g m⁻². Grobbelaar and coworkers (Richmond & Grobbelaar, 1986; Hartig et al., 1988; Grobbelaar et al., 1990) further exploited that concept by performing outdoor experiments and designing a simulation model that describes the experimental data. In these studies, the parameters of a deterministic model were verified with actual data obtained from outdoor operated algal ponds to establish a maximal biomass production rate at an algal areal density of 41 g dw m⁻². Our present model, which is based on laboratory experimental data, calculated an optimal areal chlorophyll density ranging between 0.38 and 0.75 g chl m⁻² for the marine prymnesiophyte *Isochrysis galbana*. This value is equivalent to an areal mass density ranging between 64 and 120 g dw m⁻² depending on the season. The differences between the optimal areal mass density values described in the literature (Grobbelaar et al., 1990) and those calculated in our present model are attributed to differences in physiological and biophysical properties, i.e. cellular dimensions, pigment content and distribution and light utilization characteristics of the algal species examined in these models.

Furthermore, Grobbelaar et al. (1990) calculated one areal mass density value for all ponds operated at different seasons, while the present model suggests an optimal value for each month. It is postulated that the limited number of data points in the outdoor experimental set of Grobbelaar et al. (1990) resulted in one common "wedge-shaped" curve, while the computation procedure used herein generated a wide calculated data set that could demonstrate variations in areal mass density for different months.

Once an optimal areal chlorophyll or biomass density is determined, the operation depth of the algal culture should be established. It was clearly demonstrated that pond depth hardly affects the output rate as long as areal density is properly adjusted. Nevertheless, other operational parameters and economic

considerations should be taken into account as pond depth is designed. Deep reactors require large energy inputs in pond mixing and biomass removal, while shallow ponds are complicated in design, engineering and devices. Therefore a pond depth ranging between 20 and 30 cm is recommended and commonly applied in algal mass culture systems (Ben-Amotz & Avron, 1989).

Areal chlorophyll density at a predesigned value could be maintained by continuous or semicontinuous dilution of the reactor. The dilution rate is equivalent to the calculated averaged growth rate and varied with the different months, ranging between 0.15 and 0.24 day⁻¹. A similar operational recommendation was given by Azov & Shelef (1982) for high rate oxidation pond design and operated to treat domestic sewage, namely increasing hydraulic retention time in the winter and reducing it during the summer.

In conclusion, the present model strengthens the concept of areal density and demonstrates the capability of a simulation model to predict potential production rate in algal mass culture systems. Furthermore, the use of laboratory experimental data to predict productivity and to recommend optimal operational parameters seems to be of great benefit for the evaluation of a large scale algal mass culture project and to assist in pond design and operational planning.

Acknowledgements

This research was supported by the U.S.-Israel Binational Agricultural Research and Development Fund under Grant I-1082-86. Additional support was provided by the Strauss/Hanauer Memorial Fund Inc.

References

- Azov Y, Shelef G (1982) Operation of high rate oxidation ponds: theory and experiments. *Water Res.* 16: 1153-1160.
- Ben-Amotz A, Avron M (1989) The biotechnology of mass culturing *Dunaliella* for products of commercial interest. In Crosswell RC, Rees TAV, Shah N (eds.), *Algal and cyanobacterial biotechnology*. Longman Scientific & Technical Press, London, 90-114.
- Berner T, Dubinsky Z, Wayman K, Falkowski PG (1989) Photoadaptation and the "package" effect in *Dunaliella tertiolecta* (Chlorophyceae). *J. Phycol.* 25: 70-78.
- Borowitzka MA, Borowitzka LJ (1988) *Microalgal biotechnology*. Cambridge University Press, New York, 390 pp.
- Boussiba S, Sandbank E, Shelef G, Cohen Z, Vonshak A, Ben-Amotz A, Arad S, Richmond A (1988) Outdoor cultivation of the marine microalgae *Isochrysis galbana* in open reactors. *Aquaculture* 72: 247-253.
- Burlew JS (1953) *Algal culture from laboratory to pilot plant*. Carnegie Institution of Washington, Washington D.C., 360 pp.
- De Pauw N, Morales J, Persoone G (1984) Mass culture of microalgae in aquaculture systems: progress and constraints. *Hydrobiologia* 116/117: 121-134.
- Dubinsky Z, Falkowski PG, Wyman K (1986) Light harvesting and utilization by phytoplankton. *Plant Cell Physiol.* 27: 1335-1349.
- Falkowski PG (1980) Light shade adaptation in microalgae. In Falkowski PG (ed.), *Primary productivity in the sea*. Plenum Press, New York, 99-119.
- Falkowski PG (1984) Kinetics of adaptation to irradiance in *Dunaliella tertiolecta*. *Photosynthetica* 18: 62-68.
- Falkowski PG, Dubinsky Z, Wyman K (1985) Growth-irradiance relationships in phytoplankton. *Limnol. Oceanogr.* 30: 311-321.
- Grobbelaar JU, Soeder CJ, Stengel E (1990) Modeling algal productivity in large outdoor cultures and waste treatment system. *Biomass* (in press).

- Guillard RL, Ryther JH (1962) Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt, and *Detanula confervaceae* (Cleve) Gran. Can. J. Microbiol. 8: 229-239.
- Hartig P, Grobbelaar JU, Soeder CJ, Groeneweg J (1988) On the mass culture of microalgae: Areal density as an important factor for achieving maximal productivity. Biomass 15: 211-221.
- Herzig R, Falkowski PG (1989) Nitrogen limitation in *Isochrysis galbana* (Haptophyceae). I. photosynthetic energy conversion and growth efficiencies. J. Phycol. 25: 462-471.
- Kyle D, Behrens P, Blingham S, Arnett K, Lieberman D (1989) Microalgae as a source of EPA-containing oils. J. Am. Oil Chem. Soc. 66: 893-899.
- Oswald WJ (1988) Large scale algal culture systems (engineering aspects). In Borowitzka MA, Borowitzka LJ (eds.), Microalgal biotechnology. Cambridge Univ. Press, N.Y., 357-394.
- Richardson K, Beardall J, Raven JA (1984) Adaptation of unicellular algae to irradiance: an analysis of strategies. New Phytol. 93: 157-191.
- Richmond A (1986) CRC Handbook of microalgae mass culture. CRC Press Inc., Boca Raton, Florida, 528 pp.
- Richmond A, Grobbelaar JU (1986) Factors affecting the output rate of *Spirulina platensis* with reference to mass cultivation. Biomass 10: 253-264.
- Soeder CJ (1980) Massive cultivation of microalgae: results and prospects. Hydrobiologia 72: 197-209.
- Sukenik A, Wahnou R (1990) Biochemical quality of marine unicellular algae with special emphasis on lipid composition. I. *Isochrysis galbana*. Aquaculture (in press).
- Sukenik A, Falkowski PG, Bennett J (1987) Potential enhancement of photosynthetic energy conversion in algal mass culture. Biotechnol. Bioengineer. 30: 970-977.

Sukenik A, Bennett J, Mortain-Bertrand A, Falkowski PG (1990) Adaptation of the photosynthetic apparatus to irradiance in *Dunaliella tertiolecta*, a kinetic study.

Plant Physiol. 92: 891-896.

Talling J.F. (1957) The phytoplankton population as a compound photosynthetic system. New Phytol. 56:29-50.

Table 1. Diurnal and seasonal variations in total solar energy radiation and daily temperatures measured in Israel. A) Values are total radiation measured in $\mu\text{E m}^{-2} \text{s}^{-1}$. 40% of these values were used as photosynthetic available radiation (PAR) in the model calculations. B) Average values of minimum and maximum ambient temperatures measured at different months.

Hour	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
A												
0600	0	0	5	41	191	259	197	100	13	0	0	0
0700	18	64	224	627	778	1021	906	650	348	114	22	13
0800	407	659	994	1575	1666	1987	1863	1602	1213	828	476	352
0900	1213	1561	1946	2573	2577	2898	2820	2596	2202	1772	1300	1121
1000	1923	2330	2738	3324	3371	3571	3631	3406	3040	2623	2115	1872
1100	2486	2779	3228	3869	3905	4166	4180	3992	3631	3205	2655	2394
1200	2687	3004	3525	4102	4130	4423	4423	4267	3910	3489	2930	2623
1300	2660	2921	3512	4033	4111	4395	4409	4249	3882	3470	2907	2568
1400	2385	2623	3187	3727	3768	4075	4134	3951	3580	3141	2568	2257
1500	1845	2083	2550	3118	3109	3548	3599	3406	3031	2536	1973	1730
1600	1126	1406	1859	2349	2367	2761	2820	2628	2234	1726	1208	1025
1700	366	623	975	1410	1501	1914	1904	1630	1263	801	398	279
1800	0	46	192	449	576	750	650	448	274	92	0	0
1900	0	0	0	37	109	178	174	86	0	0	0	0
2000	0	0	0	0	0	133	130	0	0	0	0	0
B												
Min	9.0	13.5	14.0	15.0	17.5	20.0	21.5	23.0	20.0	17.0	15.0	10.5
Max	14.5	20.0	21.0	22.0	26.0	28.5	28.5	30.0	27.0	25.5	24.0	16.0
Average	12.0	17.0	17.5	18.5	22.0	24.0	25.0	26.5	23.5	21.5	19.5	13.0
Amplit.	5.5	6.5	7.0	7.0	8.5	8.5	7.0	7.0	7.0	8.5	9.0	5.5

Table 2. Operational parameters that yield maximal productivity of *I. galbana* in a high rate algal pond calculated by a simulation model.

Month	Photo-adaptation	Areal chlorophyll mg m ⁻²	Maximal productivity g C m ⁻² d ⁻¹	Growth rate day ⁻¹	Respiration %
Jan	228	375	5.9	0.150	40
Feb	202	500	7.2	0.175	37
Mar	213	500	8.1	0.192	35
Apr	208	650	9.4	0.173	37
May	209	650	11.7	0.215	33
Jun	195	750	13.3	0.219	33
Jul	202	650	13.3	0.238	31
Aug	177	750	13.6	0.235	32
Sep	200	650	11.0	0.208	34
Oct	212	500	9.5	0.227	32
Nov	193	500	7.9	0.197	35
Dec	205	400	5.1	0.154	40

Figure legend

- Figure 1. Flow chart of a simulation model, including the main equations used for the calculation of light attenuation, daily temperature variations, gross photosynthesis rate, net photosynthesis, respiration rate and overall growth rate.
- Figure 2. Flow chart of the photoadaptation subroutine which calculates the average irradiance level in a production pond to predict the photoadaptation status of the culture.
- Figure 3. Effect of chlorophyll concentration on the averaged irradiance in a shallow algal pond operated at different depth levels. Calculated values are for noon time in April.
- Figure 4. Variations in averaged irradiance level as a function of areal chlorophyll density. Calculated values are for noon time in January and August.
- Figure 5. Effect of chlorophyll concentration on biomass production rate calculated by the simulation model for algal production ponds of various depth levels operated in April.
- Figure 6. Relationships between areal chlorophyll density and biomass production rate calculated for ponds of different depths operated in April.
- Figure 7. Effect of chlorophyll concentration on overall growth rate of algal biomass calculated by the simulation model for algal production ponds of various depth levels operated in April.
- Figure 8. Relationships between areal chlorophyll density and the overall growth rate of biomass calculated for ponds of different depths operated in April.
- Figure 9. Variations in respiration losses as a function of chlorophyll concentration calculated for ponds of different depths operated in April.

Fig 1

Fig. 2

Fig. 3

Fig. 4

Fig 5

Fig 6

Fig 7

Fig. 8

Fig 9

D. Description of cooperation

The collaboration in this project was defined in the original proposal and carried out according to these lines. The work at IOLR was basically focused on lipid studies and the effect of environmental parameters on lipid content and fatty acid composition in different unicellular algae, while the work at BNL was concentrated on evaluation of the same environmental conditions on the activity and protein composition of the photosynthetic apparatus in the algae. In our studies we share antibodies prepared by both research groups for a detailed study of specific protein content. Antibodies against the large subunit of Rubisco from *I. galbana* that were prepared by the BNL group were available to both laboratories. Protein components of the photosynthetic apparatus of *I. galbana* which were isolated by the BNL group were used to prepare antibodies at IOLR. These antibodies are now available for the work done in both laboratories. The recent developments in the preparation of antibodies against *I. galbana* ACCase are being used in both laboratories, first to evaluate the effect of growth conditions on the cellular level of this enzyme (IOLR), and second, they will be used as a molecular probe for the isolation of ACCase cDNA sequences from a cDNA library that was prepared at BNL. This molecular approach regarding the regulation of lipid synthesis via ACCase activity and content, which was evolved from this project, will be considered in future collaborative studies. This mode of cooperation is demonstrated by common work published in the literature and in another manuscript currently prepared for publication (see section F).

E. Evaluation of the research achievements

The main achievement of this research effort is a better understanding of the effects of environmental conditions on photosynthetic performance, composition of the photosynthetic apparatus, lipid content and composition, and lipid synthesis in a marine unicellular alga. The role of the enzyme acetyl CoA carboxylase in controlling carbon flow into lipids and regulating lipid synthesis is suggested in nitrogen limited and nitrogen deprived cultures.

The conclusions and specific achievements of the research are:

1. The light harvesting complex of *Isochrysis*, the fucoxanthin chlorophyll protein (FCP) complex, was isolated, purified and partially characterized for its pigment stoichiometry and amino acid sequence of the apoprotein at its N terminal.
2. A cDNA library of *Isochrysis galbana* was constructed and screened for cDNA clones corresponding to the gene of FCP using previously prepared antibodies. The same library is now available for isolating cDNA clones of the ACCase gene.
3. Nitrogen limitation results in an increase in the minimum quantum requirement for photosynthetic oxygen evolution; however, the net growth efficiency remains relatively constant.
4. Nitrogen limitation in *Isochrysis galbana* leads to an increase in the optical absorption cross section normalized to Chl *a*, reduction in photosynthetic capacity per cell and decrease in the specific rate of dark respiration.
5. Nitrogen availability imposed changes in the abundance of individual proteins constituting the photosynthetic apparatus, which do not occur in strict proportion in response to nitrogen limitation.
6. Variations in the abundance of specific photosynthetic proteins suggest that the carboxylation enzyme RuBisCo can serve as a transient nitrogen storage as cells become nitrogen limited. The light harvesting Chl *a/c*/fucoxanthin complex, however, does not serve a special reservoir function for nitrogen.
7. While nitrogen limitation leads to a reduction in the cellular abundance of photosynthetic proteins in general, there is a greater loss of proteins which are encoded in the chloroplast.
8. The effect of gabaculin on chlorophyll synthesis during low light adaptation of unicellular algae indicated that chlorophyll synthesis is required for a shade-adaptive increase in the light harvesting chlorophyll protein complex of PSII.
9. *Isochrysis* cultures grown in high light conditions are characterized by a relatively high content of carbohydrates, while cellular lipid content remains

unchanged. Nitrogen limitation, however, caused an increase of both carbohydrate and lipid content.

10. Variations in lipid content are associated with changes in lipid composition and fatty acid distribution. High light and severe nitrogen limiting conditions impose an increase in storage lipids, such as triacylglycerol, which are enriched with C16:0 and C18:1 fatty acids.
11. The existing FID-HPLC system allows detailed lipid compositional studies at the molecular species level. Using that system, MGDG molecular species of *I. galbana* were resolved and analysed. This is the first report of an MGDG molecular species study in non green alga using the FID-HPLC system.
12. Variations in chloroplast volume and morphology are correlated with changes in the cellular abundance of galactolipids and C18:4 fatty acid which is associated with that lipid component.
13. Purification of the enzyme acetyl CoA carboxylase from *Isochrysis* facilitated the comparison of molecular characteristics and kinetic properties of the algal enzyme with the activity of this enzyme isolated from other algae and from higher plants.
14. To our knowledge, the polyclonal antibodies prepared against ACCase from *Isochrysis* are the first antibodies ever produced against this enzyme from algae. The antibodies are an important tool in a future attempt to study the molecular regulation of ACCase cellular content.
15. The availability of antibodies against ACCase supported an advanced physiological study of the enzyme activity and its cellular content in *I. galbana*. The results raise the possibility that ACCase plays an important role in controlling lipid synthesis and accumulation in the alga.
16. Nitrogen starvation imposed an increase in cellular lipid and fatty acid content, although *de novo* lipid synthesis rate as well as *in vitro* activity of ACCase decreased. It is suggested that a disproportional reduction in cellular division

rate relative to synthesis capacity causes a temporary increase in cellular lipid content.

17. Recovery from nitrogen starvation is characterized by rapid reorganization of the photosynthetic apparatus and cell division capacity, while lipid synthesis capabilities are lagged and recovered at later stage.
18. Potential strategies to enhance the production of algal biomass and specific biochemicals were evaluated. It is suggested to further pursue physiological mechanisms to control the production of a desired product from microalgae. Nevertheless, application of molecular tools to enhance light energy conversion by the photosynthetic apparatus is suggested as a potential route for improving biomass productivity and in many cases enhancement of the production of a specific compound.
19. Productivity of algal biomass in an outdoor pond was optimized using a mathematical simulation model. Areal chlorophyll density is suggested as an operational parameter that should be considered in any attempt to control productivity. Year round production rate of algal biomass was calculated. The model results suggest a yearly averaged production rate of $19.5 \text{ g d.w. m}^{-2} \text{ d}^{-1}$.

Most of the scientific objectives of the original proposal were achieved. The preparation of a cDNA library and its screening for FCP gene clones became possible as the project was extended mainly for that purpose. We hope to further use that cDNA library for evaluation of the molecular regulation of ACCase abundance in *Isochrysis*, as we proposed in a recent research program submitted to BARD.

The inverse relationships between nitrogen assimilation into amino acid and fatty acid synthesis which were suggested in the original research proposal were not fully studied. In spite of our attempt to correlate the *in vitro* activity of various ammonia assimilation enzymes with lipid synthesis rate, we could not obtain conclusive results. Furthermore, our attempt to block *in vivo* ammonia assimilation

into amino acids by specific inhibitors failed, evidently due to low uptake of the drugs.

The simulation model for the enhancement of algal biomass and lipid production developed in this project is applicable only for light limited cultures, while our original aim was to include nutrient limitation growth in the planned model. First, the results of our laboratory experiments suggested that maximal productivity of lipids coincides with maximal biomass production rate. Therefore only biomass productivity was calculated by the model. Second, the nitrogen limited data obtained in our experiments correspond only to one condition of irradiance level. Therefore any attempt to simulate light effect on productivity in nitrogen limited cultures will require several assumptions which are not supported by experimental data. Moreover, there are no available data to confirm results of such a simulation model, as we can do with nutrient sufficient cultures.

F. List of resulting publications

Articles in reviewed journals

1. Herzig R. and Falkowski P.G. (1989) Nitrogen limitation in *Isochrysis galbana* (Haptophyceae). 1. Photosynthetic energy conversion and growth efficiencies. J. Phycol. 25:462-471.
2. Falkowski P.G., Sukenik A. and Herzig R. (1989) Nitrogen limitation in *Isochrysis galbana* (Haptophyceae). 2. Relative abundance of chloroplast proteins. J. Phycol. 25:471-478.
3. Mortain-Bertrand A., Bennett J. and Falkowski P.G. (1990) Photoregulation of the light harvesting chlorophyll protein complex associated with photosystem II in *Dunaliella tertiolecta*. Plant Physiol. 94:304-311.
4. Sukenik A. and Wahnou R. (1991) Biochemical quality of marine unicellular algae with special emphasis on lipid composition I. *Isochrysis galbana*. Aquaculture (in press).
5. Livne A. and Sukenik A. (1990) Acetyl coenzyme A carboxylase from the marine prymnesiophyte *Isochrysis galbana*. Plant Cell Physiol. 31:851-858.
6. Sukenik A. and Livne A. (1991) Variations in lipid and fatty acid content in relation to acetyl CoA carboxylase in the marine prymnesiophyte *Isochrysis galbana*. Plant Cell Physiol. (in press).

Articles in published proceedings

1. Falkowski P.G. Potential strategies for regulating the flux of carbon into specific chemical classes in microalgae. In: Ramus J. and Jones M.C. (eds), Polysaccharides from microalgae: a new agroindustry. Proceedings of an international workshop, Duke University Marine Laboratory, North Carolina, pp.83-91, 1988.
2. Sukenik A. and Livne A. Effect of environmental conditions on lipid accumulation in the marine alga *Isochrysis galbana*: the role of acetyl CoA carboxylase. in: Quinn P.G. and Harwood J.L. (eds.), Plant Lipid Biochemistry-Structure, Utilization and Function. Portland Press, London, 1991 (in press)

Articles submitted for publication

1. Livne A. and Sukenik A. Lipid synthesis and abundance of acetyl CoA carboxylase in *Isochrysis galbana* (Prymnesiophyceae) following nitrogen starvation. Submitted for publication in Biochim. Biophys. Acta , 1991.

Master's thesis

1. Henry D.L. (1990) Characterization of the light harvesting complex and isolation of cDNA clone of the marine chrysophyte *Isochrysis galbana*. A master's thesis presented to the graduate school, State University of New York at Stony Brook.

Articles in preparation

1. Sukenik A., Levy R.S., Levy Y., Falkowski P.G. and Dubinsky Z. Optimizing biomass production in an outdoor pond: a simulation model. To be submitted for publication in J. Appl. Phycol., 1991.
2. Sukenik A., Makadar R. and Berner T. Fatty acid composition and quantitative morphology in *Isochrysis galbana* grown under various environmental conditions.