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MOLECULAR BIOLOGY IN STUDIES OF OCEANIC PRIMARY PRODUCTION

Julie LaRoche, Richard Geider, Paul Falkowski

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Remote sensing and the use of moored in situ instrumentation has greatly improved our ability to measure phytoplankton chlorophyll and photosynthesis on global scales with high temporal resolution. However, the interpretation of these measurements and their significance with respect to the biogeochemical cycling of carbon relies on their relationship with physiological and biochemical processes in phytoplankton. For example, the use of satellite images of surface chlorophyll to estimate primary production is often based on the functional relationship between photosynthesis and irradiance. A variety of environmental factors such as light, temperature, nutrient availability affect the photosynthesis/irradiance (P vs I) relationship in phytoplankton. While biophysical techniques allow for a description of the variability in the P vs I relationship, molecular biology provides the means to determine how specific environmental factors limit and control primary production in the ocean. We present three examples showing how molecular biology can be used to provide basic insight into the factors controlling primary productivity at three different levels of complexity: 1. Studies of light intensity regulation in unicellular alga show how molecular biology can help understand the processing of environmental cues leading to the regulation of photosynthetic gene expression. 2. Probing of the photosynthetic apparatus using molecular techniques can be used to test existing mechanistic models derived from the interpretation of physiological and biophysical measurements. 3. Exploratory work on the expression of specific proteins during nutrient-limited growth of phytoplankton may lead to the identification and production of molecular probes for field studies.

Julie LaRoche and Paul Falkowski: Oceanographic and Atmospheric Sciences Division, Department of Applied Science, Brookhaven National Laboratory, Upton, NY 11973, USA

Richard Geider: College of Marine Studies, University of Delaware, Lewes, DE 19958-1298, USA

Introduction

Within the last century, biological oceanographers have mapped and catalogued the distribution of primary producers throughout the world's oceans and identified several of the key processes which affect these distributions. The interdisciplinary nature of oceanography is well exemplified by the many physical, chemical, and biological principles that are applied to gain understanding of oceanic processes. In the last decade, oceanographers have made use of two new tools to further describe the properties of the oceans: remote sensing allows the study of processes on a world- or basin-wide scale and moored instruments are providing in situ time-series data of coupled biological and physical processes in moving fluids on seasonal time scales. The interpretation of the results generated by the application of these new technologies relies heavily on knowledge accumulated on the basic biological principles applying to marine organisms. Throughout the 1970's and 1980's physiological ecology provided robust empirical descriptions of the effect of varying environmental factors on the photosynthetic activity of primary producers (e.g. Platt, 1981). These empirical descriptions have been used in several instances to predict integrated water column production (Platt et al., 1988). However, in many situations, these empirical relationships do not yield accurate predictions (Balch et al., 1989; Platt et al., 1992). At present, some mechanistic understanding of how a changing environment will affect primary production is required to assess the potential impact of global change on the oceans.

Modern biochemistry and molecular biology provide a new set of tools and concepts with which it is possible to gain such a mechanistic understanding of important oceanic processes. These processes are often empirically described using quantitative physiology but no mechanistic models are available to explain the interaction between the organism and its environment. The use of biochemistry and molecular biology to answer such questions is a logical extension of physiological ecology. We describe three examples showing the application of molecular biology to oceanic problems at three different levels of complexity.

1. Photoacclimation

Photoacclimation is the process by which phytoplankton physiologically acclimate to changes in irradiance levels. This is usually achieved by maximizing light harvesting at low irradiance through increases in cellular chlorophyll concentration and by minimizing photooxidative damage to the photosynthetic apparatus at high light intensity (Falkowski and La Roche, 1991). Photoacclimation, originally called "sun-shade" adaptation as an analogy with higher plant systems, was identified in marine systems as early as 1959 (Ryther and Menzel, 1959; Steeman Nielsen and Hansen, 1959). The extent of plasticity in the photosynthetic response of marine algae was later determined from several laboratory studies and from extensive measurements of photosynthesis-irradiance curves in nature (Falkowski, 1980; Falkowski, 1981). Photoacclimation is one of the most important factors determining the functional relationship between photosynthesis and irradiance (Falkowski, 1980; Perry et al., 1981). This relationship in turn, forms the basis for algorithms used in modelling the spatial and temporal distribution of primary production from satellite images of chlorophyll from the ocean surface (Balch et al., 1989; Platt and Sathyendranath, 1988). Despite the numerous models empirically describing the relationship between various photosynthetic parameters and change in irradiance level, we have very little understanding of the molecular and mechanistic basis of this process.

Detailed biochemical studies of photoadaptation have shown that in most algae a change in light intensity is rapidly followed by an inversely correlated change in the cellular amounts of light harvesting chlorophyll proteins (Sukenik et al., 1988; Roman et al., 1988; Falkowski and La Roche, 1991). In the chlorophyte *Dunaliella tertiolecta*, a detailed kinetic study of the photosynthetic components has shown that the acclimation time scale ranges from a few hours to a day (Sukenik et al., 1990). The increase in the light harvesting protein (LHCP) is rapid and is concurrent with an increase in the cab mRNA levels encoding for the LHC apoproteins (La Roche et al., 1991). An increase in the mRNA level is detected within 1.5 hour of a transfer to low irradiance. Fig. 1 shows that the increase in the cab mRNA following a shift from high to low light is very specific; there is no detectable change in the message level of any other photosynthetic gene tested. Photoadaptation does not occur during a daily light/dark cycle as demonstrated in Fig. 2, but will occur when a shift in light intensity is superimposed on a light/dark cycle (Post et al., 1985).

Shift from high to low light in the presence of gabaculine and from high light to darkness (La Roche

et al., 1991) as well as experiments with submicromolar addition of DCMU (Fujita et al., 1987) have led to several conclusions and hypotheses regarding the molecular mechanisms regulating the change in light harvesting complex during photoacclimation (Fig. 3). Two pathways are involved in the synthesis of the light harvesting complex: Chlorophyll biosynthesis and LHC protein synthesis. An increase in the cab RNA suggests that the LHC apoprotein synthesis is probably controlled at the transcriptional level, but the experiments with gabaculine (La Roche et al., 1991; Mortain-Bertrand et al., 1990) suggest that chlorophyll biosynthesis is required for LHC protein synthesis to proceed. The initial receptor of a light intensity change has not yet been identified. However, La Roche et al. (1991) have suggested that this response is not directly mediated by a photoreceptor. They have hypothesized that cab gene expression is repressed in high light by a factor which is regulated via changes in the non-cyclic photosynthetic electron transport. Genes encoding the major light harvesting chlorophyll binding proteins have been sequenced from the diatom *P. tricornutum* (Grossman et al., 1990), the chrysophyte *Isochrysis galbana* (La Roche and Wyman, unpublished data), and the chlorophyte *D. tertiolecta* (La Roche et al., 1990) and the study of how light intensity regulate the expression of these genes is within our reach.

2. Models describing the response of the photosynthetic apparatus under various nutrient stresses.

Traditionally, the photosynthetic response of phytoplankton as a function of irradiance has been instrumental in characterizing primary production in the world's ocean. The initial slope, α and maximum photosynthesis rate, P_m^b, are the major parameters used to describe photosynthesis as a function of irradiance. The initial slope of the PI curve is used to derive the maximum quantum efficiency of photosynthesis, a parameter which is instrumental in the interpretation of sea surface chlorophyll data collected by remote sensing (Bidigare et al., 1992). The maximum quantum yield is independent of irradiance in nutrient replete conditions, but there are numerous indications in lab and field data that this parameter varies as a function of limitation by several essential nutrients (Cleveland et al., 1991; Bidigare et al., 1992; Chalup and Laws, 1990; Falkowski et al., 1991; Herzig and Falkowski, 1990; Greene et al., In Press). At present, measurements of maximum quantum efficiency, regardless of how they are obtained, do not provide information on which nutrient limits photosynthesis nor do they provide insight into the mechanisms by which photosynthesis is impaired under

nutrient limitation.

Molecular biology combined with biophysical methods offers a way to determine which basic components of the photosynthetic apparatus are affected during nutrient limitation. We have taken this approach to look at the effects of nitrogen, phosphorus and iron limitation on the photosynthetic apparatus of *D. tertiolecta* and *P. tricornutum* using antibodies raised against three important photosynthetic proteins which are related to three crucial steps in the photosynthetic pathway. Figures 4 and 5 show the time course of cellular chlorophyll and maximal change in variable fluorescence (F_v/F_m) in both species, after transfer of an exponentially growing culture to media lacking either nitrogen, phosphorus or iron. The addition of the limiting nutrient after several days demonstrated that the cell, although stressed, were capable of resuming high growth rates and photosynthetic rates within less than a day. Similar trends in these two variables are observed with time for all nutrients and in both species, with the exception of phosphorus limitation. In *D. tertiolecta*, the decrease in chl/cell and F_v/F_m are much slower during phosphorus limitation than during limitation by other nutrients. The slower decrease in F_v/F_m is also observed in *P. tricornutum*. In *D. tertiolecta*, this can be explained by the large reduction in cell division observed under phosphorus stress. The burst in cell division and increase in Chl/ml following the phosphorus addition support this idea (data not shown).

We have probed the composition of the photosynthetic apparatus using antibodies raised against rubisco, D1 and the light harvesting chlorophyll binding proteins (Fig. 6). The LHC apoproteins are involved in light harvesting, D1 is one of two core proteins of the reaction center of photosystem II involved in light collection and is the gateway to non-cyclic electron transport, and rubisco is the principal enzyme involved in CO_2 fixation. On a qualitative basis, nitrogen limitation greatly reduces the abundance of both the small and large subunit of rubisco as well as D1 in *P. tricornutum*. However, the amount of FCP remains relatively constant. In iron limited cells, the large and small subunits of rubisco are unaffected while the D1 is greatly reduced. Under iron stress, the major light harvesting protein is reduced and may also be modified as suggested by the appearance of an additional higher molecular weight protein. Modification of the light harvesting antenna under iron limitation has been observed in cyanobacteria (Riethman and Sherman, 1988). During phosphorus limitation, the major components of the photosynthetic apparatus remain relatively constant

on a per protein basis, and it is not yet clear why the maximum variable fluorescence shows such a large decrease. A similar qualitative pattern is found in *D. teriolecta* (data not shown).

We have shown that while maximum quantum efficiency is, in general, reduced during nutrient limitation, a simple qualitative analysis of the western blots indicates that the mechanism by which this reduction occurs must be different for each nutrient. At this stage two simultaneous approaches are needed to understand how environmental factors affect primary production: We need to develop a bank of molecular probes such as antibodies for the various components of the photosynthetic apparatus and we need to improve our basic understanding of how each potentially limiting factor alters the expression of photosynthetic genes. An understanding at the biochemical level will allow us to both verify the interpretation of the fluorescence and other physiological measurements that we routinely perform in the oceans and it will point out what additional measurements we should develop to obtain an unambiguous picture of the mechanisms prevailing in nature.

3. Molecular markers indicative of nutrient limitation in marine phytoplankton.

Using modern biology techniques, it has become possible to identify species composition of natural communities without the need to culture the organisms (Giovanonni et al., 1990). It is likely that within the next decade, a bank of probes will become available for the identification of marine microorganisms. Similarly the detection of key genes or proteins in marine organisms can indicate their potential or actual roles in biogeochemical cycling (e.g. methanogenesis or denitrification). These basic ideas can be extended to address the question regarding what limits carbon fixation by phytoplankton in the oceans.

A successful approach for determining the effect of a particular stress on microorganisms has been to look for proteins specifically expressed under stress conditions. This has been particularly useful when dealing with prokaryotes in which whole operons carrying a special function are derepressed under stress. For example, the phoE operon has been identified using this approach in *E. coli*. Several examples are listed in Table 1. We have recently taken this approach to study nutrient stress in eukaryotic algae. In experiments similar to those described in section 2, we have followed protein synthesis during the course of nutrient

starvation. In *P. tricornutum*, we have found a 55 kDa protein which is strongly synthesized under phosphorus limitation and a 23 kDa polypeptide which is synthesized during iron limitation (Fig. 7). These proteins can also be detected in stained gels and disappear upon addition of the limiting nutrient. Using a crude cellular fractionation, we have determined that in *D. tertiolecta*, small soluble proteins and large membrane proteins are induced by iron and phosphorus limitation (Fig. 7). Similar observation have been made for *P. tricornutum* (data not shown). We are in the process of characterizing these proteins but we do not yet know their function. However, based on published information, the large membrane proteins are likely to be components of uptake systems (Scanlan et al., 1989; Reddy et al., 1988).

Our long-term goal is to obtain a set of molecular probes that can be used to determine, using in situ hybridization of preserved samples, whether or not a phytoplankton community is subjected to a particular stress. While some universal probes would be nice, taxa specific or even species specific probes could also be very useful in field studies. However, this approach will require extensive testing to determine the specificity of these probes for a given nutrient stress. The identification of the protein function will assist in finding similar indicators in other taxonomic groups when protein structure is not highly conserved. There is potential to use these techniques for in situ hybridization and with flow cytometry, two powerful tools used in field studies.

Conclusion

In addition to mapping of biomass and primary production throughout the world ocean, it is important to gain an understanding of some of the basic processes that limit production in a given region. This basic information could be extremely important in predicting future production for a given global change scenario. The fate of carbon will be different depending on whether production is limited by light, micronutrients or trace elements. The molecular biological approach, though only recently introduced, has already had a significant contribution to the elucidation of natural community structure, but it is also potentially a powerful tool to begin to understand how the environment regulates gene expression in photosynthetic organisms.

Although future technological advancement in molecular biology may lead to the development of

techniques to measure primary production, the immediate reward of molecular biological techniques will be in their contribution to a basic mechanistic understanding of how key environmental factors regulate cellular function and gene expression. In this respect, molecular biological studies are a natural extension of physiological ecology which forms the basis of most descriptive models of primary production in the oceans. Although physiological ecology principles may provide an adequate parameterization for characterizing oceanic production in the present, knowledge of these fluxes alone will not necessarily predict the future picture. Molecular biology can provide the fundamental understanding required to predict the response of living organisms to a variable environment.

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Figure legends

Fig. 1. Photosynthetic genes transcript abundances during a shift from high (700 uE $m^{-2}s^{-1}$ to low light (70 uE $m^{-2}s^{-1}$) in *D. tertiolecta*. Transcript abundance was determined every 9 hours to show the response in the change in mRNA level, following a light shift. cab represents the transcript encoding the LHC apoproteins, psaB encodes a component of the reaction center fron PSI, psbA encode the D1 protein; rbcL, the large subunit of rubisco; and rRNA represent the ribosomal RNA. Four micrograms of total RNA per lane were loaded and electrophoresed on denaturing 6% formaldehyde and 1% agarose gels, transferred to nitrocellulose and probed with P-32 labelled DNA fragments containing sequences of the genes of interest.

Fig. 2. Photosynthetic gene expression in cultures synchronized to a light-dark cycle. Cells were grown at 350 uE$m^{-2}s^{-1}$ on a light/dark (12/12) cycle. The dark periods are shaded black on the x axis. Total RNA was isolated from cells every two hours and abundance of transcripts of cab genes, psbA, rbcL and rRNA were determined as described in Fig. 1

Fig. 3. Hypothetical model of the regulation of photoadaptation in *D. tertiolecta*. The model incorporates all the information that is currently available for *D. tertiolecta* and presents a diagramatic view of the hypotheses described in Section 1. Question marks indicate hypothetical pathways for which we presently have no data. (ALA, delta-aminolevulinic acid; GlutRNA, glutamyl tRNA; PROTO, protoporphyrinIX; Mg-PME, magnesium protoporphyrinIX monoethyl ester; PChld, Protochlorophyllid.

Fig. 4. Batch cultures of *P. tricornutum* were grown exponentially, and aliquots were transferred to artificial seawater media limiting for nitrogen, phosphorus or iron. The time course of nutrient depletion was followed for four days and measurements were made on each day. After four days, an addition of the limiting nutrient to the respective culture (marked by an arrow) showed that the phytoplankton recovered rapidly from nutrient depletion. Phosphorus limited culture is represented by squares, nitrogen-limited by triangles and iron-limited by circles.

Fig. 5. Nutrient limitation in *D. tertiolecta*. Legend as in Fig. 4.

Fig. 6. Western blot of photosynthetic proteins during nutrient limitation and recovery in *P. tricornutum*. Total proteins were prepared as described in La Roche et al. (1991) and 25 ug/lane were electrophoresed on 15% SDS polyacrylamide gels. The protein gels were transferred to nitrocellulose and were sequentially challenged with D1, RUBISCO (LSU, large subunit and SSU, small subunit) and FCP (fucoxanthin chlorophyll binding protein) antibodies. THe numbers above the lanes represent days after the onset of nutrient starvation and the + represent the limiting nutrient addition which occured after the fourth day in all cases.

Fig. 7. Autoradiogram of ^{14}C-bicarbonate labelled protein synthesis after the onset of nutrient starvation induced by the removal of nitrogen, phosphorus or iron. Exponentially growing cultures of *P. tricornutum* were transferred to artificial seawater media lacking either dissolved inorganic nitrogen, phosphorus or iron. Protein synthesis was monitored as a function of time after nutrient removal by pulse addition of 50 μCi of ^{14}C bicarbonate followd by a 12 hour incubation. The protein samples were run on a 15% SDS-polyacrylamide gel and transferred to nitrocellulose. The results were analysed by autoradiography. At each time point, C, N, P, Fe represent the control, nitrogen-limited, phosphorus-limited or iron-limited cells. The numbers on the left represent the molecular weight of the protein standards. The arrows point to a 55 kDa protein and to the 23 and 21 kDa proteins that are induced under phosphorus and iron limitation, respectively.

Fig. 8. Induction of specific membrane (M) and soluble (S) proteins during nutrient limitation in *D. tertiolecta*. Four days after the onset of nutrient starvation , *D. tertiolecta* cultures were labelled with ^{14}C bicarbonate for 12 hours. Cells were harvested by centrifugation and, after sonication, total proteins were separated into a soluble and membrane fraction by differential ultracentrifugation. Legend is as in Fig. 7.

Table 1. Induction of specific proteins under nutrient limitiation

Nutrient	Organism	Protein	MW	Function	Reference
Iron	*Synechococcus*	*irp A*	36kDa	iron acquisition	Reddy et al. 1988 J. Bact. 170:4466
			92kDa	membrane protein	Scanlon et al. 1989 Arch. microbiol. 152:224-
	E. coli	aerobactin		siderophore-like	
Phosphorus	Pseudomonads	PhoE proteins	45kDa	anion selective channels	Poole and Hancook, 1986; J. Bact. 165: 987-
	E. coli	*Pit*		anion selective channels	Matin et al. 1989 Ann Rev. Microbiol. 43:293-
	Protogonyaulax	phosphatase (activity only)		activity only	Borin et al. 1989 J. Plant. Res. 11:879-
Nitrogen	bacteria	glutamine synthetase (Ntr)		nitrogen metabolism	Matin et al. 1989 Am. Rev. Microbiol. 43:293-
Sulfur	*Synechococcus*	sulfate permease proteins	33kDa	sulfate transport	Launderbach and Grossman, 1991 J. Bact. 173:2739
	Chlamydomonas	Arylsulfatase		sulfate transport	deHostos et al. 1987

Fig 1

High light (steady-state) shifted to low light at 0 h (36 h)

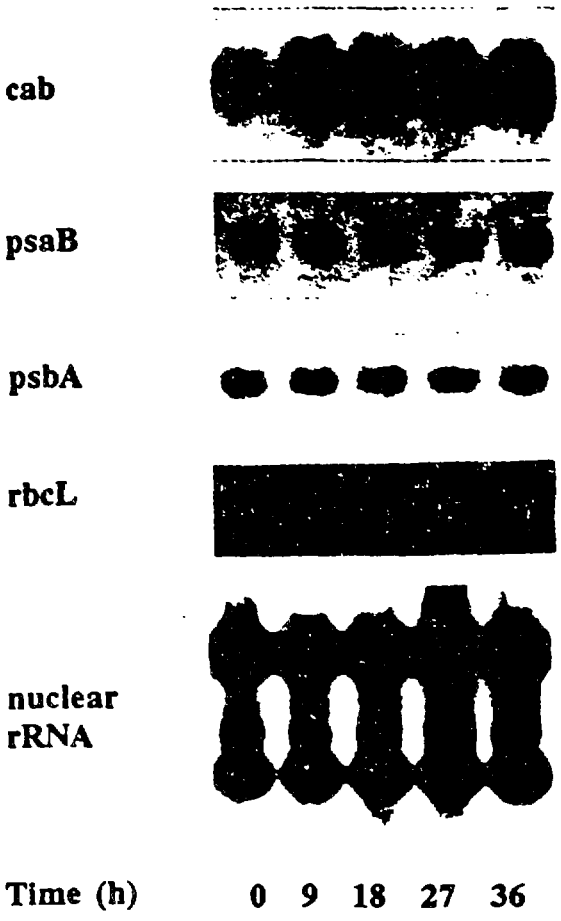
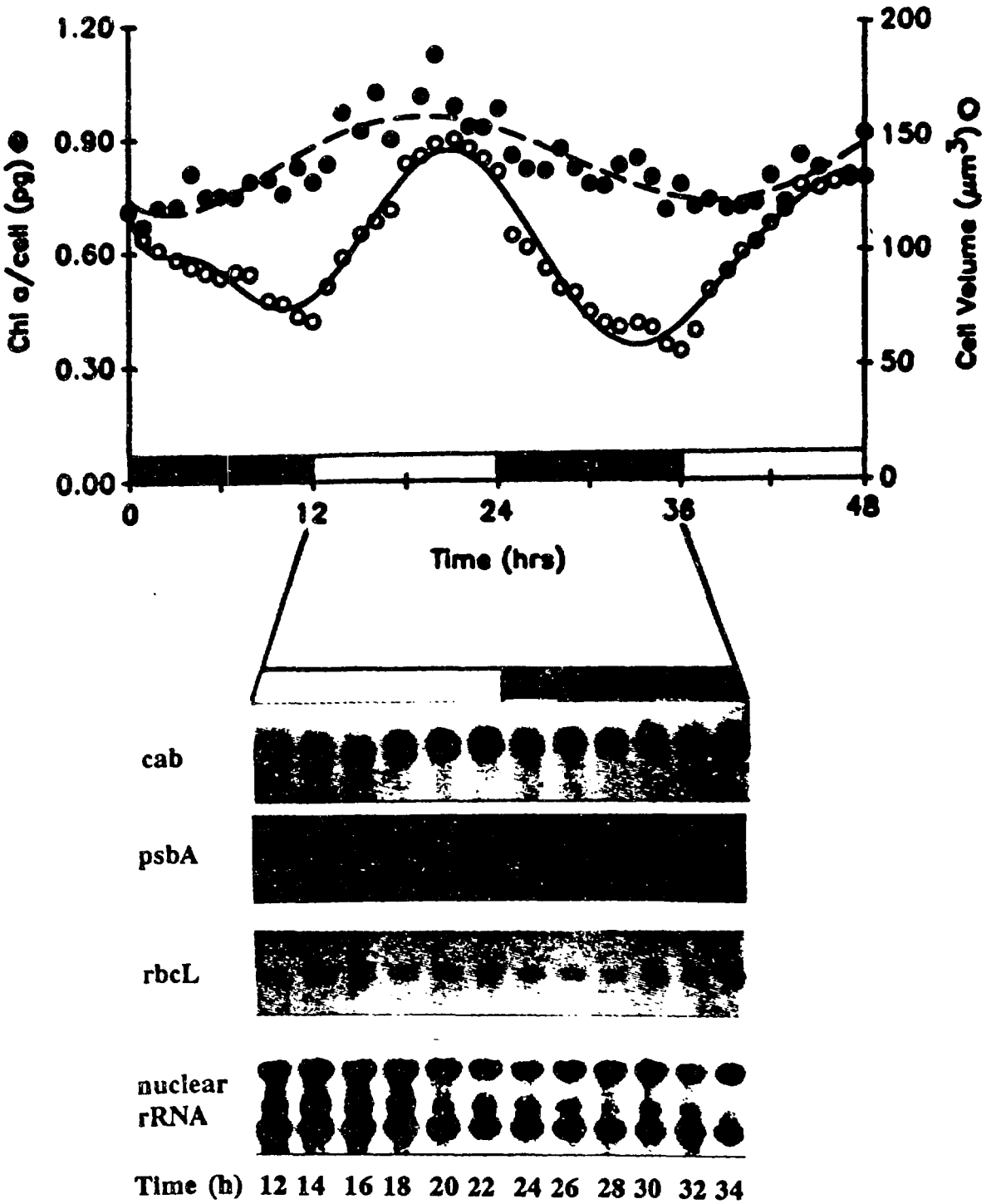


Fig 2

Synchronized light/dark (12 h/12 h) cycle



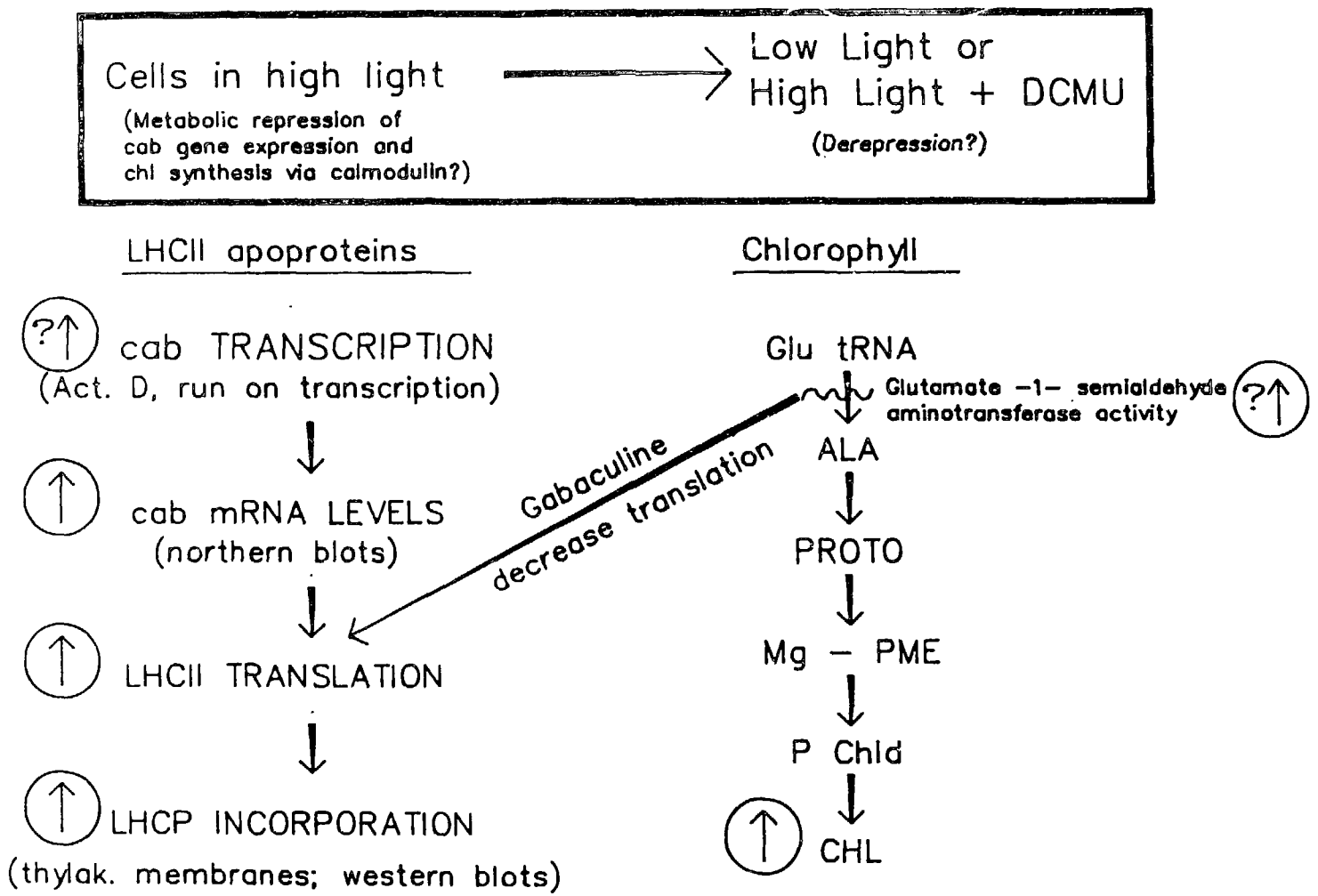
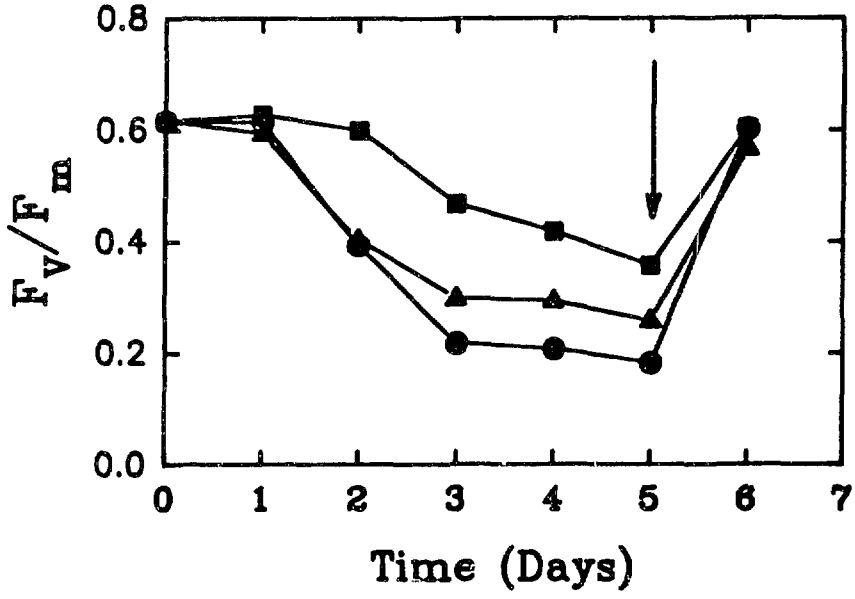

Cells in high light
(Metabolic repression of cab gene expression and chl synthesis via calmodulin?)
Low Light or High Light + DCMU
(Derepression?)
LHCII apoproteins
Chlorophyll
?↑ cab TRANSCRIPTION
(Act. D, run on transcription)
↑ cab mRNA LEVELS
(northern blots)
↑ LHCII TRANSLATION
↑ LHCP INCORPORATION
(thylak. membranes; western blots)
Gabaculine
decrease translation
Glu tRNA
Glutamate -1- semialdehyde aminotransferase activity
?↑
ALA
PROTO
Mg - PME
P Chld
↑ CHL

Fig 3

Fig 4

Phaeodactylum tricornutum



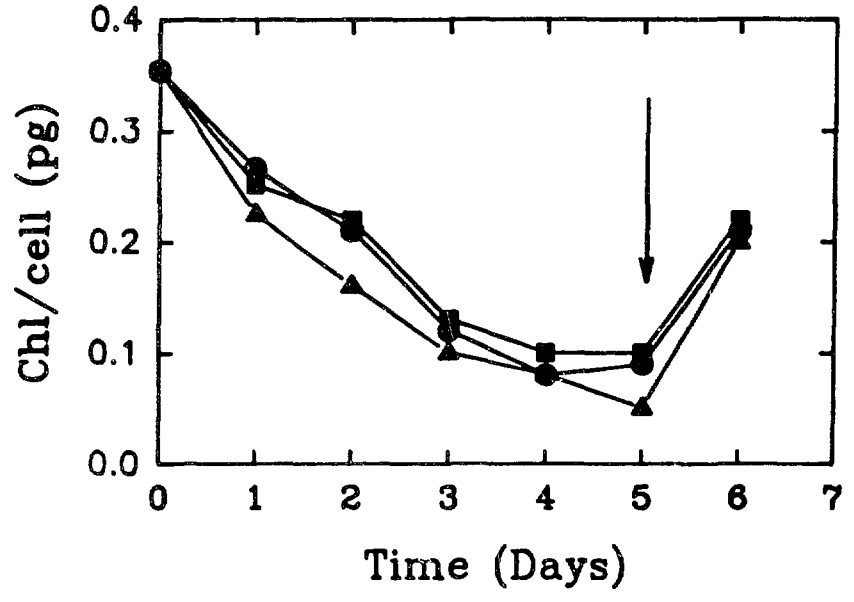
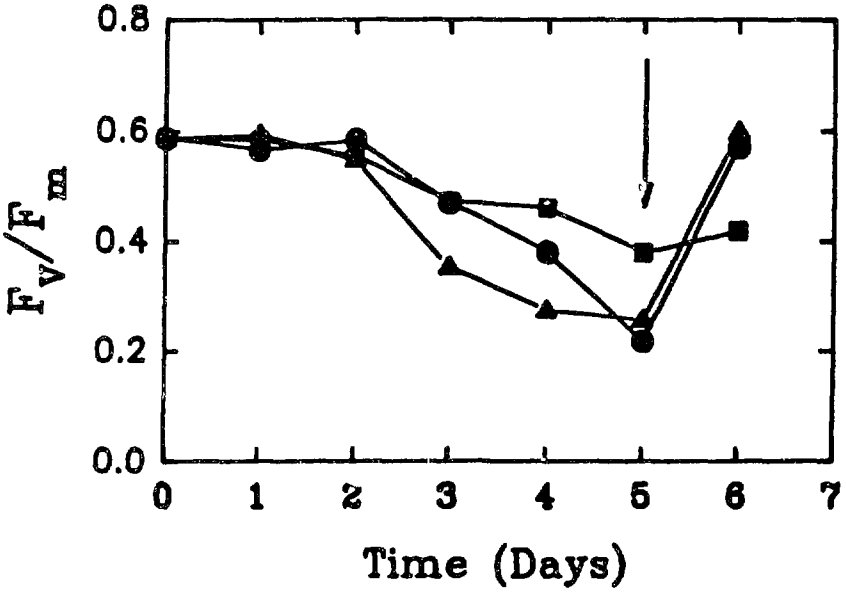


Fig 5

Dunaliella tertiolecta



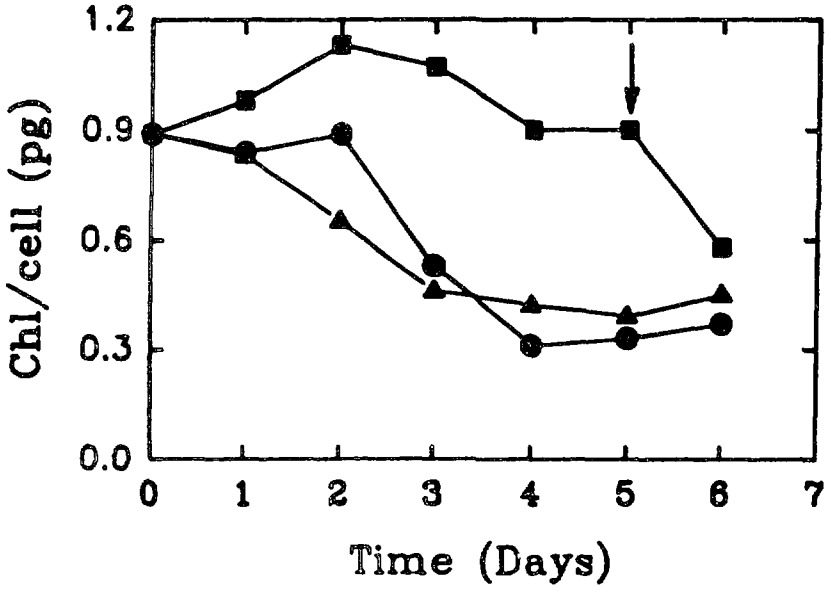


Fig 6

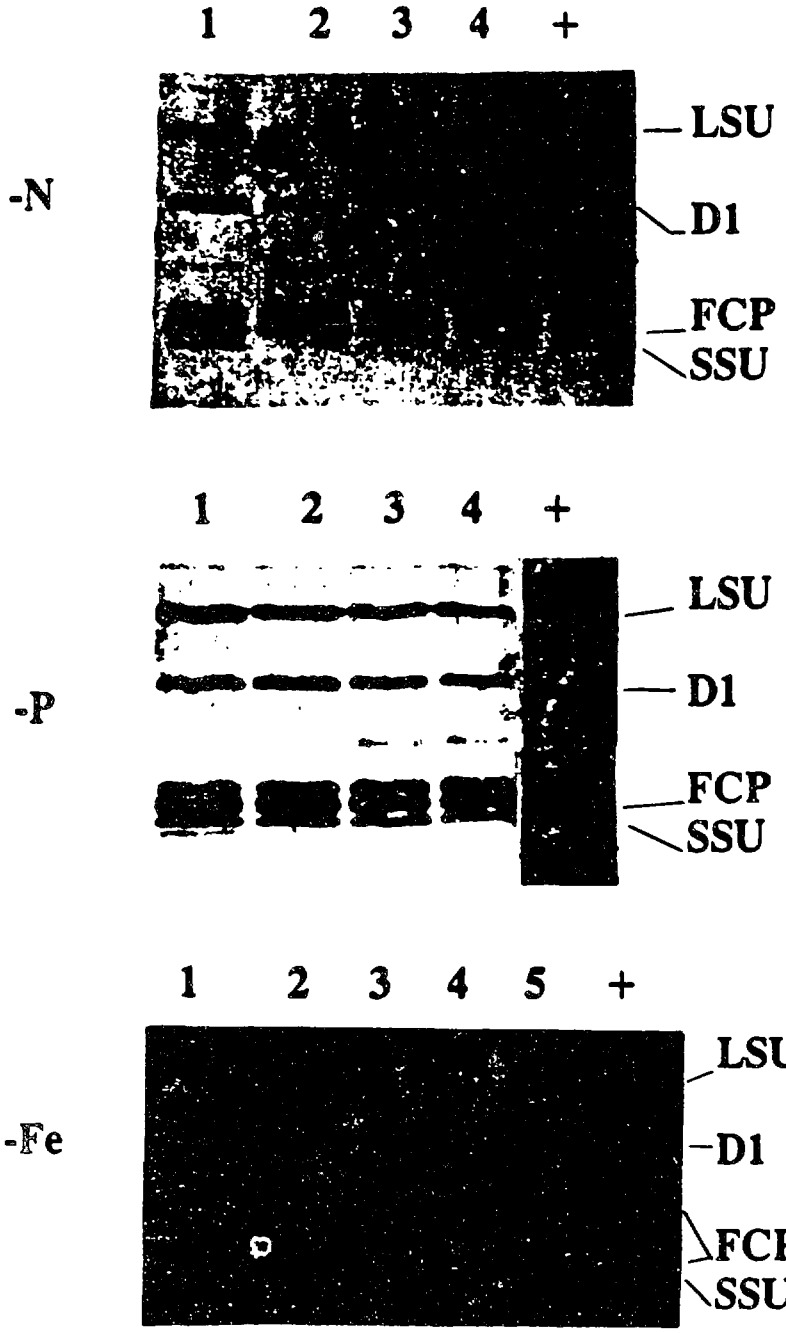

1 2 3 4 +
-N
LSU
D1
FCP
SSU
1 2 3 4 +
-P
LSU
D1
FCP
SSU
1 2 3 4 5 +
-Fe
LSU
D1
FCP
SSU

Fig 7

Phaeodactylum tricornutum

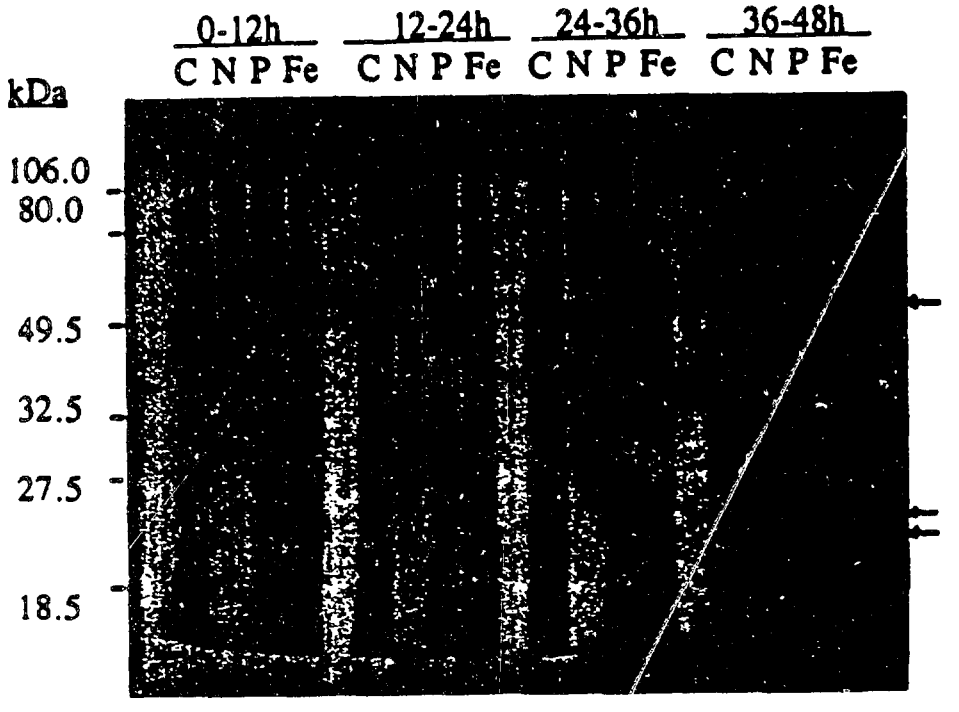


Fig 8

Dunaliella tertiolecta

soluble (S) and membrane (M) proteins under nutrient limitation