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Aquatic Species Project Report FY 1989-90

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PREFACE

This report summarizes the progress and research accomplishments of the Aquatic Species Project, field managed by the National Renewable Energy Laboratory (NREL), through October 1990. This report includes an overview of the entire project and a summary of individual research projects. The project receives its funding through the Biofuels Systems Division of the Department of Energy.

For further details, contact the NREL Aquatic Species Project Manager, Dr. Lewis Brown, at (303) 231-1321.

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INTRODUCTION

When stimulated by environmental stress, many species of aquatic microalgae produce lipids that can be processed into diesel oil or gasoline. These algae have growth rates as high as five times those of most terrestrial plants, and some species flourish in saline or brackish water unsuitable for human or traditional agricultural use. In addition, microalgae require large quantities of carbon dioxide for growth and lipid production, thereby reducing the concentration of atmospheric carbon dioxide.

Studies have shown that economic fuel production will require the microalgae to be grown in intensive culture in large outdoor ponds. The system design consists of 6 in. deep, raceway-shaped ponds with a paddlewheel for circulating the water. Carbon dioxide and other nutrients are injected into the culture to optimize algal growth and lipid production.

Researchers in the project have collected and studied more than 3000 strains of microalgae from desert and saline environments. From these, a number of promising lipid-producing species have been identified. These organisms grow over a wide range of salinities, produce significant quantities of lipid oils, and achieve growth rates of nearly three doublings per day. Some of these species tolerate temperatures of 100°F or higher.

Research is now focused on applying genetic techniques to enhance the lipid production of microalgae. This effort builds on extensive strain characterization research, as well as biochemical studies of the metabolic pathways for lipid synthesis. Using non-genetic methods, scientists have already improved the lipid content of the cell from the 5% to 20% found in nature to more than 60% in the laboratory and more than 40% in outdoor culture.

Microalgae production facilities are well-suited to areas with high solar radiation, inexpensive, flat land, adequate saline water, and an inexpensive source of carbon dioxide. The desert Southwest meets all of these requirements. Large coal-burning power plants in the region offer an excellent potential source of carbon dioxide for microalgae farms.

Coupling a microalgae farm with a power plant or other source of carbon dioxide provides a way to produce liquid transportation fuels and improve the environment at the same time. The microalgae essentially recycle the carbon dioxide from the power plant's stack gases into a secondary energy product (diesel fuel or gasoline). Although this carbon dioxide is eventually released when the fuel is burned, the process effectively doubles the amount of energy generated for a given quantity of carbon dioxide. Studies have shown that land and saline water are available in New Mexico and Arizona, for example, to support extensive microalgae facilities. The carbon dioxide emissions from all the power plants in these two states could be trapped by microalgae farms covering about 0.25% of the total land area. If this technology is expanded to other states, or projected future capacity is brought on line in Arizona and New Mexico, the farms could supply at least 2 quadrillion Btu of energy (equivalent to 15% of the gasoline used in the United States) in the form of liquid fuels.

1.0 PROJECT OVERVIEW

Researchers in the Aquatics Species Project focus on the use of microalgae as a feedstock for producing renewable, high-energy liquid fuels such as diesel. It is important for the United States to develop alternative renewable oil sources because 42% of the current energy market in the United States is for liquid fuels, and 38% of these fuels are imported. The latter figure is expected to rise to more than 50% soon, increasing the U. S. trade deficit and our vulnerability to disruptions in petroleum supplies.[1] In 1979, the U. S. Department of Energy (DOE) and the National Renewable Energy Laboratory (NREL) initiated the Aquatic Species Project as part of the overall effort in biofuels. The project began to focus exclusively on fuels from microalgae in 1982. Estimates show that the technology being developed by the project can provide as much as 7% of the total current energy demand.

The program's basic premise is that microalgae, which have been called the most productive biochemical factories in the world, can produce up to 30 times more oil per unit of growth area than land plants. It is estimated that 150 to 400 barrels of oil per acre per year could be produced with microalgal oil technology. Initial commercialization of this technology is envisioned for the desert Southwest because this area provides high solar radiation and offers flat land that has few competing uses (hence low land costs). Similarly, there are large saline aquifers with few competing uses in the region. These could provide a suitable, low-cost medium for the growth of many microalgae.

The project has begun to assume additional importance for its potential contribution to the environment. Potential global climate changes are projected as a result of release of carbon dioxide from fossil fuel combustion. These global climate changes have the potential of producing economic and geopolitical changes with profound impact on our economy and the energy industry. The production of diesel fuel by microalgae requires very large quantities of carbon dioxide as a nutrient. In areas where microalgae fuel plants operate in tandem with fossil fuel plants to scrub carbon dioxide from flue gases, contributions to the release of carbon dioxide could be significantly reduced.

The project has supported research at NREL in Golden, Colo., as well as in industry, other government laboratories, and universities.

1.1 Project Goal

The goal of the Aquatic Species Project is to develop the technology base for large-scale production of oil-rich microalgae. The project is also developing methods to convert the microalgal lipids into liquid fuels needed for industry and transportation.

1.2 Project Objectives

Specific long-term objectives of the project are to:

- Genetically engineer microalgae for high lipid production at high growth rates
- Identify "trigger" points in biochemical pathways of algae that turn lipid production on and off

[1] *Annual Energy Outlook 1989*, DOE/EIA-0383 (89), DOE/Energy Information Administration, Washington, DC, 1989.

- Develop inexpensive, large-scale, outdoor mass culture technologies to grow microalgae
- Evaluate resource requirements for large-scale production of oil from microalgae as well as the environmental impact of such activities in the desert Southwest of the United States
- Develop technologies for converting microalgal lipids into high-value liquid transportation fuels, particularly diesel
- Transfer the technologies to the private sector for continued development and rapid commercialization by involving industry in the research process as early as possible.

1.3 Description of the Aquatic Species Project Elements

1.3.1 Production

Improvements are needed in algal growth and lipid production in order to produce economic liquid fuels from microalgae. The production element's goal is to reach target growth rates of 50 g m^{-1} d^{-1} in outdoor cultures and produce algae cells that are 50%-60% lipid. To do this, microalgae strains have been collected from many areas in the United States. These algae have been screened to select species that are temperature and salinity tolerant, have high productivities, and are good lipid producers. A collection of organisms is being maintained to provide a gene pool for direct exploitation as energy crop organisms in the laboratory and outdoors and also as starting material for genetic engineering. It is also important to understand lipid biochemical pathways in algal cells in order to maximize lipid production and to develop genetic engineering techniques for improving microalgal production and lipid content.

The NREL culture collection contains microalgal strains that produce large amounts of lipid and grow rapidly, but not necessarily at the same time or in a controllable manner. Genetic improvement will be necessary to develop strains with the characteristics necessary to meet the overall project technical and economic goals, including predictable growth performance and controllable lipid yield. The genetic engineering process requires methods for getting genes (DNA) out of cells (cloning), modifying the gene, and reintroducing it into the microalgae. The modified genes would then confer one a desired characteristics such as increased lipid content.

1.3.2 Extraction and Conversion

Methods need to be developed for economical extraction of lipids from microalgae and conversion of lipids to gasoline and diesel substitutes. Untreated lipids have too high an oxygen content and viscosity to be used in standard engines. The primary goal of the conversion element is to economically convert a high proportion of the algal lipids to diesel fuels and to improve the overall economics converting the balance of the biomass to biogas or other high-energy products.

1.3.3 Engineering Design

The technology to produce economic liquid fuels from microalgae will require the growth of microalgae on a large scale. Systems to maintain optimal levels of nutrients, carbon dioxide, salinity, and temperature must be developed and tested. The engineering design element's goal is to develop large-scale outdoor facilities that allow the production goals to be met and to reduce the economic costs of such a system to those targeted by the project's economic analysis. The Outdoor Test Facility (OTF) located at Roswell, New Mex. is being operated by a subcontractor, Microbial Products, Inc., so that various mass-culture and

harvesting systems and technologies can be tested in an effort to increase outdoor algal productivities and decrease the cost of operating such a facility.

1.3.4 Analysis

Economic and resource analyses provide input to project management so research directions and priorities can be set. The analysis element's goal is to support the technology development by determining cost goals, economic sensitivities, resource assessments, and environmental impacts as new data are developed. To do this, researchers will conduct ongoing economic analyses. Resource and environmental assessments will be conducted to identify potential constraints, identify and address data gaps, and provide project guidance.

1.4 Project Highlights

1.4.1 FY 1989-1990 Accomplishments

A major project accomplishment was the operation of the OTF at Roswell, New Mex. for 2 years (8/88-8/90). Productivities of 40 Mg ha^{-1} y^{-1} were produced in two 0.1 ha (1/4 acre) ponds. These ponds were operated on a substantially continual basis for the 2-year period. Comprehensive data were produced from the operation of the facility including results on the efficiency of trapping of carbon dioxide. Trapping efficiencies as high as 95% were reported for productive periods of the year.

In the smaller (3 m^2) outdoor ponds, significant research activity was also underway. More than 15 strains of microalgae with potential for growth in outdoor mass culture and significant lipid production were evaluated. Annualized productivity for these strains was 60 Mg ha^{-1} y^{-1} with short term (several day) productivities as high as 40 to 60 g m^{-2} d^{-1}

Preliminary analysis of effects of inputs of flue gas components on mass culture was completed, including a consideration of carbon dioxide, heat, nitrogen, acidity and salinity. Waste heat from a power plant would contribute only 3 to 12% of the total heat input to the pond (the remainder is solar). Nitrogen, water and salinity inputs to the pond are even less important. If SO_x is not neutralized, significant acidity may be added. However, sulfur scrubbing is being installed in many plants to satisfy clean air requirements.

Another important area of research has been the effort to genetically improve microalgae in order to control the timing and magnitude of lipid accumulation. Increased lipid content will have a direct effect on fuel price, and the control of lipid content is a major project goal. The key lipid enzyme acetyl-CoA carboxylase (ACC) was purified to produce a probe for cloning of the gene for this enzyme, which appears to have an important role in controlling the levels of lipids accumulated in these cells. Antibodies to ACC have been prepared and purified and are being used in efforts to clone this gene.

Gene probes have been designed for cloning the gene nitrate reductase, an enzyme important in the partitioning of photosynthetic carbon products among lipids and other cellular components. Understanding the genetics and biochemistry of this enzyme may lead to another method of controlling lipid production.

The cell walls of a green algal species have been removed by enzymatic treatment (protoplast formation) and a gene from the firefly (luciferase) was introduced into these cells. The gene product functioned to produce light in the presence of its substrate and ATP. The success of this test gene system is significant in several respects. It proves that although codon bias may be widespread in many algae, it does not necessarily prevent foreign genes from being expressed. Also, while no systematic study of gene promotor

activity has been undertaken, expression of this gene was sufficient with the construct used to be detected. A related project was the analysis of some of the key microalgal strains for the presence of modified DNA bases. Several strains were rich in some modified bases while others did not have a substantial quantity of modified bases. Such information is useful for developing genetic transformation strategies and for choosing strains that are suitable for genetic manipulation.

Electroporation, a technique that utilizes high voltage electric pulses to perforate cells, shows some promise in microalgal transformation as a method for introducing DNA. Efforts are focused on modifying this technique for use on microalgal cells.

1.4.2. FY 1991 Plans

Economic and engineering analyses will continue in the form of efforts to identify key technical achievements that may lead to reduction in fuel cost (fuel cost sensitivity analyses). This work will include use of the Algae Economic Model (AEM). The AEM is a FORTRAN computer model that was created to aid in the identification of cost factors. The model includes biological, engineering, economic and other parameters that can be specified.

Genetic engineering will continue to be a major area of effort in the project. This area has the potential to have a positive effect on product yield. The research in FY1991 will focus on cloning two important genes relating to lipid production, acetyl-CoA carboxylase and nitrate reductase. Cloning efforts will concentrate on the production of gene libraries, probe development, and efforts to clone parts of these genes. Work will also continue to improve genetic transformation assays developed in FY 1990, and also to develop new genetic markers with potential for more stable expression.

AQUATIC BIOMASS RESOURCES AND

CARBON DIOXIDE TRAPPING

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ABSTRACT

Intensively managed microalgal production facilities are capable of fixing several-fold more carbon dioxide per unit area than trees or crops. Although carbon dioxide is still released when algal biomass-derived fuels are burned, integration of microalgal farms for flue gas capture approximately doubles the amount of energy produced per mole of carbon dioxide released. Microalgal biomass- derived materials also can be used for other long-term uses, serving to sequester carbon dioxide. Flue gas has the potential to provide sufficient quantities of carbon dioxide for such large-scale microalgae farms. Viewing microalgae farms as a means to reduce the effects of a serious pollutant (carbon dioxide) changes the view of the economics of the process. Instead of requiring that microalgae-derived fuel be cost competitive with fossil fuels, the process economics must be compared with other technologies proposed to deal with the problem of carbon dioxide pollution. However, development of alternative, environmentally safer energy technologies such as microalgal biomass will benefit society whether or not global climate change is actually realized. Microalgal biomass production has great potential to contribute to world energy supplies and to control CO_2 emissions as the demand for energy increases. This technology makes productive use of arid and semi-arid lands and highly saline water, resources that are not suitable for agriculture and other biomass technologies.

BACKGROUND

Recently, concern has increased about the threat of global climate change. Projected consequences include a rise in the sea level, the transformation of much of the southeastern United States into arid land, and the shift of the corn belt from the United States to Canada (Schneider 1989; United States Environmental Protection Agency [U.S. EPA] 1988a; U.S. EPA 1988b). These changes may result from increased trapping of heat by the so-called greenhouse effect as various gases accumulate in the atmosphere. These gases are generated by a variety of current practices including chlorofluorocarbon use, deforestation, and vegetation decay, but the most important source of carbon dioxide is accumulation from the burning of fossil fuels in boilers, furnaces, and automobile engines (Schneider 1989). In the United States, 35% of carbon dioxide emissions in 1985 were produced by electric utilities, mostly from coal burning. Carbon dioxide emissions from electric utilities are predicted to rise from 0.43 petagrams of carbon per year (Pg C/yr; 1 Pg C = 10^{15}g C) in 1985 to 0.77 Pg C/yr in 2010, which is 44% of predicted total U.S. emissions (Edmonds et al. 1988). Coal burning contributes the most carbon dioxide per unit of energy released, followed by petroleum, then natural gas. In view of the potentially significant environmental consequences, the development of methods for reducing carbon dioxide accumulation in the atmosphere is a research and policy priority.

THERMODYNAMIC CONSIDERATIONS FOR CO_2 CAPTURE

Because of the mounting concerns about global climate change, the EPA is considering a number of measures to reduce the accumulation of greenhouse gases. One of the recommended actions would impose emissions fees on fossil fuels such as coal, natural gas, and oil so that their price reflects the risk of climate change. The cost of trapping and concentrating carbon dioxide is projected to increase the cost of electricity by 75% to 150% using conventional methods (Edmonds et al. 1988). Because such fees would sharply alter the economic viability of fossil fuel use, it is desirable to find new ways to remove carbon dioxide from flue gas, which will reduce emissions substantially.

In general, a chemical reaction is needed to fix the carbon over the long term. Such a reaction could proceed by the following stoichiometry:

$$CO_2(g) + \text{Reactant} = \text{Fixed Carbon Product} \quad (1)$$

The free energy change for this reaction at constant temperature, ΔG, can be calculated from the fundamental thermodynamic relationship:

$$\Delta G = \Delta H - T\,\Delta S \quad (2)$$

in which ΔH is the enthalpy change for the reaction, T is the temperature, and ΔS is the entropy change. To provide an economically viable carbon dioxide storage reservoir, the fixed carbon product in Equation (1) should be a liquid or a solid. Thus, the entropy change in Equation (2) is likely to be negative, resulting in a positive contribution of $T\Delta S$ to the free energy. However, for the reaction to proceed spontaneously, ΔG must be negative, implying that ΔH must have a negative value (i.e., Reaction (1) must be exothermic) that is greater in absolute magnitude than $T\Delta S$.

In addition to providing an exothermic reaction with carbon dioxide, the reactant in Equation (1) should be abundant and low in cost to be economically attractive. The lowest-cost solid reactant is lime (calcium oxide), ranked sixth in chemical production in the United States in 1990 at more than 30 billion pounds per year (*Chemical & Engineering News* 1990). Lime would react with carbon dioxide according to the reaction:

$$CaO(s) + CO_2(g) = CaCO_3(s) \quad (3)$$

This reaction has an enthalpy change of -42.5 kcal/mole and a free energy change of -31.1 kcal/mole of CO_2. Although these properties would appear to make lime desirable for carbon dioxide removal, more than four tons of lime are required per ton of coal burned. Even at the low price of about $40/ton for lime, the cost is an additional $160/ton of coal. This represents more than a 400% increase in coal utilization costs for just the lime, not taking capital and operating costs into account. In addition, over 6 trillion pounds of lime would be needed each year at current coal utilization levels, neglecting recovery of carbon dioxide from natural-gas- and oil-fired plants. Ultimately, the use of lime is not sensible because lime is produced by direct firing of calcium carbonate in kilns, and more carbon dioxide is released in lime manufacture than can be captured from flue gas.

One might be tempted to use other natural products such as sodium carbonate, which are produced in abundance (sodium carbonate is ranked eleventh in production quantity in the United States [*Chemical & Engineering News* 1990]) but do not require carbon dioxide removal during manufacture. In this case, the reaction stoichiometry is:

$$Na_2CO_3(s) + CO_2(g) + H_2O(l) = 2NaHCO_3(s) \quad (4)$$

However, more than 8 tons of sodium carbonate are now required per ton of coal burned, which at current prices of about $160/ton contribute more than $1,280/ton of coal used for the chemical reactant alone. The higher cost of sodium carbonate compared to lime is compounded by the greater molecular weight of sodium carbonate, with the result that the cost per mole required for Reaction (4) is excessive. Furthermore, the requirement for sodium carbonate to just fix the carbon dioxide from coal-fired plants exceeds current production by several orders of magnitude.

Based on these considerations, it becomes apparent that we must find a cheaper reactant for carbon dioxide than lime or sodium carbonate. Because lime and sodium carbonate are among the lowest cost and most abundant chemicals produced, the obvious candidate is water. Water also has a lower molecular weight and is a logical choice because it was produced along with carbon dioxide during combustion. One possibility is to fix carbon dioxide by formation of carbohydrates according to the reaction:

$$CO_2(g) + H_2O(l) = CH_2O(s) + O_2(g) \quad (5)$$

in which the oxygen consumed during combustion is regenerated. Careful examination of this reaction or other similar reactions of the class that fix carbon dioxide with water reveals that they are simply the reverse of combustion reactions. Furthermore, because the heat of combustion of gaseous carbon dioxide, liquid water, and gaseous oxygen are all defined as zero, the heat of reaction is simply equal to the negative of the heat of combustion of the carbon-fixing product. Thus, reactions such as (5) must be endothermic, and by Equation (2) and the expectation that ΔS will be negative, we would anticipate that ΔG will be positive. If the free-energy change is positive, Reaction (5) and probably other reactions that employ liquid water to fix carbon dioxide, cannot occur spontaneously.

Even if we can drive Reaction (5) thermally, the overall heat of reaction for this endothermic transformation is +112 kcal/mole of carbon dioxide, while the energy released when coal is burned is approximately 116 kcal/mole of carbon produced. Thus, very little net energy would be gained with the coupled system, assuming the reactions could be conducted without inefficiencies. If thermal losses are considered, more heat would be required to capture carbon dioxide than could be recovered during coal burning. In addition, the free-energy change for Reaction (5) is about +115 kcal/mole of carbon dioxide reacted, indicating that the reaction will not proceed spontaneously as written. One might be able to develop a series of coupled reactions for which the net reaction would be as indicated, but even if each were 90% efficient, ten such reactions in series would have only an overall efficiency of 35%. Alternatively, if the reaction could be driven electrochemically, a minimum of 115 kcal would be required to capture a mole of carbon dioxide, but because electricity is produced at about a 40% efficiency from coal, approximately 290 kcal of heat would have to be produced to capture a mole of carbon dioxide. This would require the release of about 2.5 moles of carbon dioxide to capture 1 mole, a definite losing proposition.

One could envision using photovoltaic cells or equivalent nonfossil-fueled devices to generate electricity to drive a reaction such as (5), but several problems are evident. First, the coal plant that is emitting carbon dioxide uses 116 kcal of heat to produce 46 kcal of electrical energy and releases 1 mole of carbon dioxide in the process. Capture of the mole of carbon dioxide requires that at least 115 kcal of photovoltaic or other outside source of electricity be used for Reaction (5). Thus, a total of 230 kcal of energy would be applied to the overall electrical generation and carbon recovery process, 115 kcal of which is electricity, to make 46 kcal of electricity. Regardless of the price of the outside source of electricity, the only sensible alternatives are to (1) pay the fine for carbon dioxide release or (2) use the other source of electricity to replace the need to burn fossil fuels in the first place. The former is not a solution to global warming; the latter is not viable for relieving carbon dioxide emissions from fossil-fueled plants.

With all these negative aspects of carbon dioxide recovery, it would be tempting to conclude that the situation is hopeless. However, several points can be gained from these considerations. First, a very inexpensive chemical such as water or air is needed to fix the carbon. Second, the product formed should

be a solid or liquid, preferably with a long stable lifetime. Third, water is desirable as a "reactant" because it has a low molecular weight, resulting in a low cost per mole. Fourth, a reaction such as (5) is desirable because it effectively reverses combustion, regenerating oxygen while fixing carbon dioxide with water, its co-product from combustion. Finally, an alternate reaction to (5) must be found that requires far less energy per mole of carbon dioxide fixed, or an energy source other than heat or electricity with low cost potential must be used to drive the transformation.

With respect to the last of these points, one possibility is photon energy from the sun. Although man has yet to figure out a way to chemically fix carbon with low energy requirements and high yields, plants routinely carry out Reaction (5) to fix large amounts of carbon dioxide with water by a series of low-temperature reactions through photosynthesis (capture of carbon dioxide in biomass).

BIOMASS METHODS FOR CARBON DIOXIDE CAPTURE: FORESTRY

Biomass technologies provide attractive alternatives to chemical means of reducing carbon dioxide emissions. One such biomass technology, which has been proposed to trap carbon dioxide, is large-scale reforestation. Although forestry-based biomass is part of the biomass solution, it cannot be used in all areas. For a typical rapidly growing plant such as might be grown in a cellulosic biomass plantation, more than 550 lb of water must be withdrawn from the soil for every pound of carbon dioxide fixed (Nobel 1974). This water needs to be high-quality soil moisture from rainfall or irrigation water. This requirement for large quantities of fresh water eliminates many of the arid undeveloped areas of the world from consideration.

Land may be equally limiting. For instance, one scenario proposes the development of large-scale forestry with storage of trees at the bottom of the ocean (Kellogg and Schware 1981); this concept is based on the fact that trees can absorb 3 to 4 tons of carbon per acre per year. However, to absorb about 3 billion tons of carbon annually (the amount of carbon that is accumulating in the atmosphere), 740 million acres of trees would need to be grown, a land area roughly equal to that of Zaire (Booth 1988). Furthermore, reforestation must continue at a rate sufficient to offset the predicted increase in fossil fuel consumption. In addition, although there are areas of the world (12 billion acres) that are underutilized, most of these are arid or semi-arid (Eckholm and Brown 1977), and thus unsuitable for significant reforestation. Furthermore, 6 billion acres of that total are unsuitable to even support human life, and 99 million acres have been damaged by salt accumulation (United Nations Environment Programme 1984). Further complicating the picture, a conservative estimate suggests that 28 million acres of tropical rainforest per year are lost chiefly to uses (e.g., rangeland or degraded land) that are vastly less productive (Postel 1989). In addition, 15 million acres per year are subject to irreversible desertification, and an additional 49 million acres per year are rendered unusable (Postel 1989).

Similarly, in the United States alone, more than half of the 160 million acres of rangeland managed by the Bureau of Land Management was rated as being in only "fair" condition in 1988, meaning that valuable forage plants had been replaced by less desirable plants or by bare ground (Bureau of Land Management 1988). An additional 17% was classified as being in "poor" condition, having been stripped of much of its topsoil and vegetation. The 50 million acres of land in "poor" or "bad" condition were damaged primarily by overgrazing (Eckholm and Brown 1977).

Substantially increasing the productivity of these nonproductive poor or bad lands is often

impossible because water is either unavailable for irrigation or too saline to be used for crop plants or trees. Thus, the prospects are poor for conventional agriculture or forestry in these areas. Where sufficient water exists to support forestry, the increases in productivity over existing uses as rangeland or cropland would be only about twofold (Whittaker and Likens 1975).

Basically, we can conclude that globally, the fight to stop deforestation is being lost on a large scale, and that the deforested land has much lower productivities, adding to the already large area of arid and semi-arid lands. In addition, it will be difficult to compensate for this nonproductive land with existing conventional land plants. This will become increasingly important within U. S. borders as global climate change proceeds (U.S. EPA 1988a).

BIOMASS METHODS FOR CARBON DIOXIDE CAPTURE: AQUATIC BIOMASS ON DESERT LANDS

Of the plants, algae are the most productive carbon dioxide users and can fix greater amounts of carbon dioxide per land area than higher plants (e.g., trees and sugar cane). Plant leaves exist in an aerial environment and are subject to large evaporative moisture losses, which directly inhibit the process of photosynthesis (carbon dioxide uptake). Microalgae in mass culture are not subject to such photosynthetic inhibition because the water content of the culture can be controlled by proper engineering. This difference is the basis for the several-fold higher carbon dioxide absorption capacity of microalgae compared to plants. Furthermore, carbon dioxide can be trapped effectively in algae ponds without covers, while higher plants would require expensive canopies to contain the carbon dioxide for efficient plant growth. Of course, although photons are "free" in principle, significant capital costs may be involved to provide a controlled system for carbon dioxide fixation. The need is to improve the technology to the point that the cost of the integrated process is low (Neenan et al. 1986). Nonetheless, for any reasonable system, net energy production is still possible while efficient carbon dioxide capture is assured.

Microalgae farming can increase the productivity of desert land almost 70-fold to a level (Weissman and Tillett 1989, unpublished) more than twice that of a typical tropical rainforest (Whittaker and Likens 1975). We estimate that microalgae farming with specially designed photobioreactor technology can increase the productivity of desert land 160-fold (6 x tropical rainforest). Furthermore, in contrast to the limited availability of arable land for forestry, there is much desert land available globally for microalgae farming. Microalgae require only 140 to 200 lb of water per pound of carbon fixed (based on average pond evaporation rates, Neenan et al. 1986), and this water can be low-quality, highly saline water. Thus, the biophysical and thermodynamic constraints favor microalgae over higher plants, particularly in arid and semi-arid regions of the United States.

Examination of the availability of land, water, and carbon dioxide resources required for microalgal production has revealed that significant resources are available in Arizona and New Mexico, and large-scale microalgae farms could have a major impact on carbon dioxide emissions from power plants in these two states (Chelf and Brown 1989). Furthermore, most of the above-mentioned resources in these two states coincide with or are within a moderate distance (50 to 100 mi) of existing fossil-fuel power plants, and the total emissions from these two states (50 billion kg of carbon dioxide per year) could be absorbed by farms covering 0.25% of their areas (Chelf and Brown 1989). It is expected that similar studies of other locations in the United States or other countries would identify a wide range of sites at which algal technology could capture carbon dioxide and provide a resource for valuable products.

One criticism of biomass energy systems is the amount of land and water they require. Microalgae are unique among photosynthetic plants in that they can achieve extremely high productivities at salinities as high as twice that of seawater (Brown and Hellebust 1978; Brown 1982; Brown 1985), and thus use low-quality (saline) water that is unusable for agriculture or urban uses. Thus, microalgal energy farming, with its focus on use of arid and semi-arid land and highly saline groundwater or seawater, utilizes resources not easily used by other biomass technologies or conventional agriculture (Dubinsky et al. 1978; Regan 1980).

Large-scale aquatic plant farming is not a new concept. The average rice crop requires 100 days of irrigation, and two crops per year are not uncommon (Grist 1975). Worldwide, more than 353 million acres of rice were harvested in 1985, including more than 2.5 million acres in the United States (U.S. Department of Agriculture 1986). Thus, microalgae should be viewed as simply a new crop for aquatic species farming with higher potential productivity on waste land and with otherwise unusable water resources. As such, it deserves consideration as one of the technologies that can contribute to the reduction in net global carbon dioxide emissions.

PRODUCTS FROM MICROALGAE

Products derived from microalgae include extraction products such as hydrocarbons, fatty acids, glycerol, protein, pigments, and polysaccharides; bioconversion products such as alcohols, organic acids, and methane; and catalytic conversion products such as paraffins, olefins, and aromatics. Work at NREL has focused on the potential of microalgae to provide an alternative, renewable energy resource. Fuels obtainable from microalgae include ethanol, triglyceride-based diesel fuel, ester fuel, methane, and gasoline. The unique ability of microalgae to accumulate up to 60% of their cellular organic mass as lipid, as well as the higher fuel value of lipids versus other cellular components, has led us to concentrate our efforts on the production of lipid-based fuels.

One issue to be addressed in the growth of microalgae for production of fuels or chemicals is the requirement for carbon dioxide. Carbon dioxide is the major raw material of photosynthesis, and as such, the major feedstock for microalgal production. In addition, carbon dioxide supply has been identified as the largest single contributor to the cost of liquid fuels derived from microalgae (Neenan et al. 1986). Microalgae farms sufficient to produce 50 billion kg of biomass would require approximately 160 billion kg of carbon dioxide annually. If one assumes an annual average yield of at least 30 grams per square meter per day for intensive microalgae farming and 270 operating days per year, over 100,000 kg of carbon per acre per year are required. Carbon dioxide levels in the atmosphere (0.033%) are not sufficient to support such high microalgae productivities. Thus, a concentrated supply of carbon dioxide is essential if high yields are to be obtained, and flue gas from fossil-fuel power plants is an excellent potential source of carbon dioxide (Feinberg and Karpuk 1990).

The following equation illustrates the stoichiometry for production of a triglyceride composed of polyunsaturated, 18-carbon fatty acids with an energy content of 17,000 Btu/lb:

$$57\ CO_2 + 31\ H_2O \rightarrow C_{57}H_{62}O_6 + 69.5\ O_2$$

One can calculate that the production of such a triglyceride requires approximately 3.0 lb of CO_2 per lb

of triglyceride produced (Appendix A). This large requirement for carbon dioxide makes lipid-producing microalgae particularly useful for trapping CO_2.

Microalgal mass cultivation has been an object of research for more than 40 years. A great deal of progress has been made in mass culture of algae since this early work, but a great deal of research still needs to be done. Yields in the early days were often in the range of 1 to 5 g m^{-2} d^{-1}; today, 15 to 25 g m^{-2} d^{-1} are not uncommon (Goldman 1979), and short-term yields as high as 50 g m^{-2} d^{-1} have been obtained (Weissman, 1988).

Additional technological developments would seem to be necessary to make a fuels-from-microalgae technology economically feasible. These developments are the focus of the technology assessment of Neenan et al. (1986), which demonstrates that gasoline and diesel fuels could be produced from microalgae at prices that will be competitive with conventional fuels. This assessment considered the potential fuel products from microalgae, the environmental and resource constraints, and the biological and engineering aspects of the technology. Specific research goals were identified, which are restated and updated as follows:

Environmental and resource constraints. Because CO_2 is the largest single contributor to the cost of fuel from microalgae, improvement in the separation of CO_2 from flue gas or minimization of flue gas processing requirements should be emphasized.

Biology and engineering. The technology assessment identified biological issues as the most critical research needs. Improvements in biological productivity and lipid content are necessary to make microalgal fuels economically feasible. Under laboratory conditions, productivities have been achieved that come very close to identified targets; these results must be extended to large-scale, outdoor cultures.

Fuel products. This analysis identified gasoline and ester fuels as the most promising products from microalgal biomass. Extraction and conversion research, as well as detailed information regarding the fuel characteristics of microalgal lipids, were identified as research priorities.

With current technology, the cost of fuel from microalgae is estimated at $5.05/gal, of which $1.48 is for carbon dioxide. If the research goals identified above can be met, Neenan et al. (1986) concluded that fuels from microalgae could be produced for $1.60/gal by the first decade of the next century. However, concerns about global climate change may result in economic incentives for the limitation of carbon dioxide release or even the levy of fines for unauthorized release of carbon dioxide. As a result the cost of this vital algal nutrient could become zero or negative (paid to use it), and costs for algal fuel could drop to the range of $0.52 to $1.00/gal.

GLOBAL CLIMATE CHANGE - MICROALGAE AND FOSSIL FUEL EMISSIONS

By converting microalgae into a fuel product that displaces fossil fuels, an algae farm cuts approximately in half the greenhouse-enhancing carbon dioxide emissions per million Btu delivered from the power plant. Depending on the composition of the algae and the amount of CO_2 emitted per million Btu of energy released from coal, the integration of microalgal mass culture technology with a coal-fired power plant could reduce the CO_2 emitted per mBtu from 200-225 lb to 90-113 lb CO_2 (Appendix B). These numbers compare to the 125 lb CO_2/mBtu from natural gas, and the 160 lb CO_2/mBtu from oil. Significant reductions would also be realized from gas-fired plants, which have cleaner flue gas, and would require less preprocessing of flue gas prior to injection into ponds. If commodity chemicals instead of fuels are produced from algae, the net carbon dioxide released could be reduced even further.

IMPACT OF FLUE GAS COMPONENTS ON MICROALGAE GROWTH

Earlier cost analyses of microalgal biomass production proposed the use of flue gas as a source of carbon dioxide for microalgae. Although ponds are currently being operated with pure carbon dioxide injection, to date, very little actual data have been obtained regarding the direct use of processed or unprocessed flue gas on microalgal growth. The Electric Power Research Institute (EPRI) sponsored a project designed to test flue gas as a carbon source for microalgae, but the data obtained in this study were very limited (Laws 1990). Mitsubishi is currently working on a project to scrub carbon dioxide from flue gas using microalgal ponds (Negoro et al. 1990).

Flue gas obtained from coal combustion contains a large number of trace contaminants that may affect microalgal growth, in addition to oxides of nitrogen and sulfur. We have done some preliminary calculations to answer the following questions: (1) can the nitrogen in flue gas contribute significantly to the nitrogen requirement of algae; (2) will the water vapor in flue gas contribute to the water requirement of algal ponds; (3) if all incoming sulfur is converted to acid, what effect will this have on the pH of the culture; (4) if all incoming sulfur is neutralized to salts, what effect will this have on the salinity of the culture; and (5) will the waste heat from the power plant have a significant effect on the temperature of the ponds? We have based these calculations on a constant amount of carbon dioxide, because the CO_2 emitted from combustion of different fuels varies considerably. The results of these calculations are summarized below, and the calculations are included as Appendix C.

Nitrogen.

The purpose of this calculation was to determine the maximum possible contribution of nitrogen from flue gas. We assumed that the algae contained 25% protein, 16% of which is nitrogen (Lehninger 1970). Assuming 20 lb of nitrogen oxides emitted per ton of coal combusted (Sittig 1975), we calculate that this nitrogen will contribute only 6.1% of the nitrogen required for algal yields of 50 $g \cdot m^{-2} \cdot d^{-1}$. Similarly, using 104 lb of nitrogen oxides emitted per 10^3 gal of oil (Sittig 1975), only 6.5% of the algal nitrogen requirement could be supplied. Natural gas, with 390 lb of NO_x per million cubic feet (Sittig 1975), could supply only 4.7% of the nitrogen needed. These numbers are potential maxima, and do not reflect the poor solubility of oxides of nitrogen in water.

Water.

For these calculations, we assumed that all hydrogen in the fuel would be converted to water during combustion, and that all water in the fuel would end up as water vapor in the flue gas. Coal contains approximately 10% moisture and 5% hydrogen (Urone and Kenney 1980), and combustion of this fuel would contribute only 0.4% of the daily water requirement of a microalgae pond. Oil, with 0% moisture and 12.5% hydrogen (EPRI 1986), could contribute 0.7% of the daily water requirement. Natural gas, with 1% moisture and 24.5% hydrogen (Rivard, 1989), could contribute only 1.5% of the daily water requirement. The total amount of water available from flue gas is very small, but it is fresh water, and will reduce slightly the requirement for blowdown water.

Acidity.

Bituminous coal combustion yields 75 lb of SO_2 per ton (Bond et al. 1972). If all the sulfur is converted to H_2SO_4, 115 lb of acid could be obtained per ton of coal burned. The addition of this acid

to the standing water in an algal pond would lower the pH of an unbuffered system to 3.5. Oil combustion yields 210 lb SO_2 per 10^3 gal (Bond et al. 1972), and could lower the pH to 3.75. Natural gas combustion releases only 0.6 lb SO_2 per million cubic feet (Bond et al. 1972). The acid produced from this amount of sulfur would result in a pond pH of 7.0. These calculations are independent of the pH-lowering effects of carbon dioxide, and do not take into account the alkalinity of the ponds. These numbers are also calculated as potential maximum contributions to acidity. Flue gas desulfurization would drastically reduce the sulfur content of flue gas. In addition, it may be possible to offset pH changes due to sulfur by adding lime to the ponds, instead of treating the flue gas with lime prior to its use in algal ponds.

Salinity.

If the sulfur introduced into the ponds as SO_2 from flue gas is neutralized to form sodium sulfate, it would contribute to the salinity of the pond rather than the acidity. Using the SO_2 figures from above, we have calculated that flue gas would add no more than 0.0225 parts per thousand per day salinity to the ponds. This figure is insignificant when compared to the salinity of the water in the pond.

Heat.

The addition of waste heat from a power plant to a microalgal pond has been postulated to be a problem in the summer and a boon in the winter, but calculations as to the magnitude of the input of waste heat compared to other inputs and outputs of heat from a microalgal pond have not been published. We based our calculations on a power plant efficiency of 38%, and used 90% of the average minimum and maximum solar inputs for the desert southwest (Perry and Chilton 1973) to calculate the magnitude of waste heat compared to solar radiation. The microalgal pond was sized to the coal-fired plant by assuming 100% capture of carbon dioxide and a yield of 50 g m^{-2} d^{-1}. We found that the heat input from a 1380 MW coal plant was on the order of 50 billion kcal d^{-1}, while the input of heat from solar radiation ranged from 260 (winter) to 1390 billion kcal d^{-1}. Therefore, the heat input from the power plant on a sunny summer day is only 3.5% of the input from solar radiation.

Heat losses were assumed to occur via convection, radiation, and evaporation. A heat differential of 5°C was used for the convection and radiation calculations, and an evaporation rate of 0.0035 m d^{-1} was assumed (Neenan et al. 1986). Using these assumptions, potential convective heat loss was calculated to be in the range of 160 to 780 billion kcal d^{-1}, while potential radiative heat loss was found to be on the order of 150 billion kcal d^{-1}. Heat loss resulting from evaporation was calculated to be 550 billion kcal d^{-1}.

CONCLUSIONS

Algae can produce a number of chemical products derived from carbon dioxide through photosynthesis. For example, certain microalgae are capable of accumulating up to 60% of their cellular organic mass as intracellular lipids, thereby increasing their heat of combustion and their fuel value. These lipids can be readily converted to gasoline and diesel fuel. Although carbon dioxide is still released when algal fuels are burned, integration of algal fuel farms for flue gas capture approximately doubles the amount of energy produced per mole of carbon dioxide released. Alternatively, a number of other chemical products could be derived from algae that would fix carbon for extended periods, and even mineral products can be formed that fix carbon in the long- term geological cycle.

Flue gas has the potential to provide sufficient quantities of carbon dioxide for large-scale microalgae farms. Two analyses of costs for aquatic biomass systems (Ashare et al. 1978; Benemann et al. 1978) both assumed direct use of flue gas. Cost calculations did not include gas scrubbing, although both studies mention that this might be necessary. Neenan and coworkers (1986) assumed concentration of carbon dioxide from flue gas, and included this cost in their analysis. Our preliminary calculations indicate that flue gas will have very little impact on the nitrogen requirement, water requirement, salinity, or temperature of algal ponds. Flue gas has the potential to have a significant impact on the acidity of the ponds if large quantities of sulfur are introduced. It may be possible to neutralize this acidity in the ponds themselves, rather than by treating the flue gas. Stricter government standards regarding emissions of sulfur dioxide may make this a moot point as sulfur may be removed upstream. Insufficient data exist about the effect of flue gas on microalgal growth. Further work investigating the use of flue gas for algal culture is necessary.

Viewing microalgal farms as a means to reduce the effects of a serious pollutant (carbon dioxide) changes the view of the economics of the process. Instead of requiring that microalgae-derived fuel be cost competitive with fossil fuels, the process economics must be compared with other technologies proposed to deal with the problem of carbon dioxide pollution. However, development of alternative, environmentally safer energy technologies will be of benefit to society whether or not global climate change is actually realized (Kellogg and Schware 1981; Schneider 1989). Fuels from microalgae have great potential to contribute to world energy supplies, and to control CO_2 emissions as the demand for energy increases. This technology makes productive use of arid and semi-arid lands and highly saline water, resources that are not suitable for agriculture and other biomass technologies. If fuels from microalgae are to be economically justifiable, improvements in biological productivity and product yield must be realized.

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APPENDIX A

For a triglyceride composed of polyunsaturated, 18-carbon fatty acids with an energy content of 17,000 Btu lb^{-1}:

$$57\ CO_2 + 31\ H_2O \rightarrow C_{57}H_{62}O_6 + 69.5\ O_2$$

$(1 \times 10^{15}$ Btu $quad^{-1}) \div (17{,}000$ Btu lb^{-1} triglyceride)

$= 5.88 \times 10^{10}$ lb triglyceride $quad^{-1}$

For $C_{57}H_{62}O_6$, 1 lb mole of triglyceride = 842 lb

(57 moles CO_2)(44 lb per lb mole) = 2508 lb CO_2 per 842 lb triglyceride

= 3.0 lb CO_2 per lb triglyceride

(3.0 lb CO_2 lb^{-1} triglyceride)(5.88×10^{10} lb triglyceride $quad^{-1}$)

$= 1.76 \times 10^{11}$ lb CO_2 $quad^{-1}$

This requirement for 3 lb CO_2 per lb of triglyceride produced makes lipid-producing microalgae particularly useful for trapping carbon dioxide.

APPENDIX B

Assumptions:

Coal: 200-225 lb CO_2 per million Btu
Algae: 50%-70% carbon by weight
Therefore, 1.83 - 2.56 lb CO_2 required per pound dry algae.

Energy from algae:

11,800 Btu lb^{-1} algae*

Correction for electricity usage for algae production*

$(15 \times 10^6$ kWh $yr^{-1})(3413$ Btu $kWh^{-1}) \div (130{,}000$ tons algae $yr^{-1})^{**}(2000$ lb $ton^{-1})$

$= 197$ Btu lb^{-1} algae

To correct for efficiency of conversion of thermal energy to electricity:

(3.0 Btu thermal per Btu electricity)(197 Btu lb^{-1} algae) = 600 Btu lb^{-1} algae

Net potential energy:

11,800 Btu lb^{-1} algae - 600 Btu lb^{-1} algae = 11,200 Btu lb^{-1} algae

CO_2 reduction per 10^6 Btu:

For 70% carbon with 200 lb CO_2 per 10^6 Btu:

(200 lb CO_2 per 10^6 Btu) $\div$ (2.56 lb CO_2 lb^{-1} algae) = 78 lb algae per 10^6 Btu

(78 lb algae per 10^6 Btu)(11,200 Btu lb^{-1} algae) = 0.87×10^6 Btu

1.0×10^6 Btu from coal combustion
+ 0.87×10^6 Btu from microalgae produced from CO_2 emitted from coal combustion
= 1.87×10^6 Btu total energy

200 lb $CO_2 \div (1.87 \times 10^6$ Btu) = 107 lb CO_2 per mBtu

Similarly, for the following conditions:

70% carbon, 225 lb CO_2 per mBtu $\rightarrow$ 113 lb CO_2 per mBtu
50% carbon, 200 lb CO_2 per mBtu $\rightarrow$ 90 lb CO_2 per mBtu
50% carbon, 225 lb CO_2 per mBtu $\rightarrow$ 95 lb CO_2 per mBtu

*Neenan et al. 1986
**Based on yield of 50 g m^{-2} d^{-1}

APPENDIX C

The calculations done for water, heat, salinity, and acidity were based on a microalgal farm of sufficient size to use 30,000 tons per day of carbon dioxide. The basic calculations for this facility are shown below.

I. Basic System Calculations

- A. Acreage required to use 30,000 tons d^{-1} of CO_2
- B. Microalgae produced per day on this acreage
- C. Nitrogen requirement for microalgae production
- D. Water required for microalgae facility
  - 1. Daily water requirement
  - 2. Standing water requirement
- E. Quantity of fuel combusted to produce 30,000 tons d^{-1} CO_2
  - 1. Coal
  - 2. Oil
  - 3. Natural gas
- F. Size of plant required to produce 30,000 tons d^{-1} CO_2
  - 1. Coal
  - 2. Oil
  - 3. Natural gas

II. Nitrogen From Fuel Combustion

- A. Coal
- B. Oil
- C. Natural Gas

III. Water From Fuel Combustion

- A. Coal
- B. Oil
- C. Natural Gas

IV. Waste Heat

- A. Heat available for microalgae production
- B. Heat loss calculations
  - 1. Radiative Cooling
  - 2. Convective Cooling
  - 3. Evaporative Cooling
- C. Solar radiation input

V. Potential Contribution of Sulfur to Pond Acidity

- A. Coal
- B. Oil
- C. Natural Gas

VI. Potential Contribution of Sulfur to Pond Salinity

A. Coal
B. Oil
C. Natural Gas

I. Basic System Calculations

A. Acreage of microalgae ponds required to use 30,000 tons d^{-1} of CO_2

1. (30,000 tons d^{-1})(2000 lb ton^{-1}) = 6 x 10^7 lb d^{-1} CO_2

   x 270 operating days yr^{-1} = 1.62 x 10^{10} lb CO_2 yr^{-1}

   x 0.273 (fraction of CO_2 that is carbon) = 4.40 x 10^9 lb carbon yr^{-1}

2. Algal Biomass

   a) Assume 50 g m^{-2} d^{-1} yield (130 tons yr^{-1} ha^{-1})

   b) Assume 63% of dry cell mass is carbon. (This corresponds to an alga that contains 50% lipid, 25% protein, 25% carbohydrate.)

   63% of 130 tons yr^{-1} ha^{-1} = 81.9 tons carbon yr^{-1} ha^{-1}

   x 2000 lb ton^{-1} = 1.64 x 10^5 lb carbon yr^{-1} ha^{-1}

   Combining the numbers obtained above:

   (4.4 x 10^9 lb carbon yr^{-1}) ÷ (1.64 x 10^5 lb carbon yr^{-1} ha^{-1})

   = 2.68 x 10^4 hectares = 66,200 acres = 103 sq. miles

B. Microalgae produced per day on this acreage

(50 g m^{-2} d^{-1})(10,000 m^2 ha^{-1})(2.68 x 10^4 ha)

= 1.34 x 10^{10} g d^{-1}, or 1.34 x 10^7 kg d^{-1}

x 2.204 lb kg^{-1} = 2.95 x 10^7 lb d^{-1} from 2.68 x 10^4 hectares

C. Nitrogen requirement for microalgae production

1. Nitrogen contained in biomass

   a) Assume average protein contains 16% nitrogen (Lehninger 1970)

For cell mass that contains 25% protein, x 0.16 = 4% of dry cell mass as nitrogen

$(2.95 \times 10^7 \text{ lb } d^{-1})(.04) = 1.18 \times 10^6 \text{ lbs N } d^{-1}$

D. Water required for microalgae facility

1. Daily water requirement

From the Algal Pond Economic Model[Neenan et al. 1986]:

yearly water requirement for 860 ha = $12 \times 10^6 \text{ m}^3 \text{ yr}^{-1}$

$[(12 \times 10^6 \text{ m}^3 \text{ yr}^{-1})(2.68 \times 10^4 \text{ ha})] \div 860 \text{ ha}$

$= 3.74 \times 10^8 \text{ m}^3 \text{ yr}^{-1}$ for 2.68×10^4 ha

$\div 270 \text{ days yr}^{-1} = 1.38 \times 10^6 \text{ m}^3 \text{ d}^{-1}$

$\times 10^3 \text{ kg m}^{-3} = 1.38 \times 10^9 \text{ kg } H_2O \text{ d}^{-1}$

$\times 2.204 \text{ lb kg}^{-1} = 3.05 \times 10^9 \text{ lb } H_2O \text{ d}^{-1}$

$\div 2000 \text{ lb ton}^{-1} = 1.53 \times 10^6 \text{ tons } H_2O \text{ d}^{-1}$

2. Standing water requirement

Pond depth assumed = 0.15 m

$(2.68 \times 10^4 \text{ ha})(10^4 \text{ m}^2 \text{ ha}^{-1})(0.15 \text{ m}) = 4 \times 10^7 \text{ m}^3$

$\times 10^3 \text{ kg m}^{-3} = 4 \times 10^{10} \text{ kg } H_2O$

$\times 2.204 \text{ lb kg}^{-1} = 8.8 \times 10^{10} \text{ lb } H_2O$

$\div 2000 \text{ lb ton}^{-1} = 4.4 \times 10^7 \text{ tons } H_2O$

E. Quantity of fuel combusted to produce 30,000 tons d^{-1} CO_2

1. Coal burned

a) Assume ~2.5 lb CO_2 lb $^{-1}$ coal[Steinberg et al. 1984]

$\therefore$ $(6 \times 10^7 \text{ lb } CO_2) \div (2.5 \text{ lb } CO_2 \text{ lb}^{-1} \text{ coal})$

$= 2.4 \times 10^7 \text{ lb coal d}^{-1}$

$\div 2000 \text{ lb ton}^{-1} = 1.2 \times 10^4 \text{ tons coal d}^{-1}$

b) Energy produced from coal

$(1.2 \times 10^4 \text{ tons coal } d^{-1})(25 \times 10^6 \text{ Btu ton}^{-1}) = 3 \times 10^{11} \text{ Btu } d^{-1}$

2. Oil burned

a) Assume 3.14 lb CO_2 lb^{-1} oil[Steinberg et al. 1984]

$\therefore$ $(6 \times 10^7 \text{ lb } CO_2) \div (3.14 \text{ lb } CO_2 \text{ lb}^{-1} \text{ oil})$
$= 1.91 \times 10^7 \text{ lb oil } d^{-1}$

$\div 7.8 \text{ lb gal}^{-1} = 2.4 \times 10^6 \text{ gal}$

b) Energy produced from oil

$(1.91 \times 10^7 \text{ lb oil } d^{-1})(1.96 \times 10^4 \text{ Btu lb}^{-1}) = 3.74 \times 10^{11} \text{ Btu } d^{-1}$

$(3.74 \times 10^{11} \text{ Btu } d^{-1}) \div (6.4 \times 10^6 \text{ Btu bbl}^{-1})$

$= 5.85 \times 10^4 \text{ bbl oil } d^{-1}$

3. Natural gas burned

a) Assume 3.0 lb CO_2 lb^{-1} natural gas[Steinberg et al. 1984]

$\therefore$ $(6 \times 10^7 \text{ lb } CO_2) \div (3.0 \text{ lb } CO_2 \text{ lb}^{-1} \text{ gas})$

$= 2 \times 10^7 \text{ lb gas } d^{-1}$

$\div 0.042 \text{ lb SCF}^{-1} = 4.76 \times 10^8 \text{ SCF } d^{-1}$

b) Energy produced from natural gas

$(4.76 \times 10^8 \text{ SCF } d^{-1})(10^3 \text{ Btu SCF}^{-1}) = 4.76 \times 10^{11} \text{ Btu } d^{-1}$

F. Size of plant required to produce 30,000 tons d^{-1} CO_2

1. Coal

$(6 \times 10^7 \text{ lb } CO_2 \text{ } d^{-1})(0.55 \text{ kWh lb}^{-1} \text{ } CO_2$[Steinberg et al. 1984]$)$

$= 3.3 \times 10^7 \text{ kWh } d^{-1}$

$\div 24 \text{ h } d^{-1} = 1.38 \times 10^6 \text{ kW}$

$= 1380 \text{ MW plant}$

2. Oil

$(6 \times 10^7 \text{ lb } CO_2 \text{ d}^{-1})(0.70 \text{ kWh lb}^{-1} CO_2$ (Steinberg et al. 1984))

$= 4.2 \times 10^7 \text{ kWh d}^{-1}$

$\div 24 \text{ h d}^{-1} = 1.75 \times 10^6 \text{ kW}$

= 1750 MW plant

3. Natural gas

$(6 \times 10^7 \text{ lb } CO_2 \text{ d}^{-1})(0.97 \text{ kWh lb}^{-1} CO_2$ (Steinberg et al. 1984))

$= 5.82 \times 10^7 \text{ kWh d}^{-1}$

$\div 24 \text{ h d}^{-1} = 2.4 \times 10^6 \text{ kW}$

= 2400 MW plant

II. Nitrogen From Fuel Combustion

The purpose of this calculation was to determine the maximum possible contribution of nitrogen from flue gas. We assumed that the algae contained 25% protein, 16% of which is nitrogen. These numbers are potential maxima, and do not reflect the poor solubility of oxides of nitrogen in water.

A. Coal

Assume 20 lb NO_2 ton^{-1} coal. (Sittig 1975)

30% of NO_2 is nitrogen by weight, therefore, 6 lb N ton^{-1} coal would be generated.

$(6 \text{ lb N ton}^{-1})(1.2 \times 10^4 \text{ tons coal d}^{-1}) = 7.2 \times 10^4 \text{ lb N d}^{-1}$

From I. C., this amount is 6.1% of the nitrogen required to produce 50 g m^{-2} d^{-1} on 2.68×10^4 ha.

B. Oil

Assume 104 lb NO_2 per 10^3 gal oil. (Sittig 1975)

$[(2.5 \times 10^6 \text{ gal d}^{-1})(104 \text{ lb } NO_2)] \div 10^3 \text{ gal} = 2.6 \times 10^5 \text{ lb } NO_2 \text{ d}^{-1}$

At 30% nitrogen, this is equal to 7.6×10^4 lb nitrogen d^{-1}.

This is 6.5% of the nitrogen required to produce 50 g m^{-2} d^{-1} on 2.68×10^4 ha.

C. Natural Gas

Assume 390 lb NO_2 per 10^6 $ft.^3$ (Sittig 1975)

$[(4.76 \times 10^8 \text{ SCF } d^{-1})(390 \text{ lb } NO_2)] \div 10^6 \text{ SCF} = 1.9 \times 10^5 \text{ lb } NO_2 \text{ } d^{-1}$

At 30% N, this is equal to 5.6×10^4 lbs nitrogen per day. This amount is 4.7% of the nitrogen required to produce 50 g m^{-2} d^{-1} on 2.68×10^4 ha.

III. Water From Fuel Combustion

A. Coal

Assumptions:

1. All hydrogen in coal is converted to H_2O during combustion.

2. Existing moisture content in coal is in addition to water produced during combustion.

3. Moisture in coal is ~10%.(Urone & Kenney 1980) For 1.2×10^4 tons of coal, this represents 1200 tons of H_2O.

4. Hydrogen content of coal averages 5%.(Urone & Kenney 1980)
This is 600 tons of hydrogen from 1.2×10^4 tons of coal.

   600 tons hydrogen + 4800 tons oxygen → 5400 tons H_2O

   So, 1200 tons + 5400 tons = 6600 tons of H_2O potentially available from 1.2×10^4 tons of coal per day.

   With a daily water requirement of 1.53×10^6 tons, this water represents 0.4% of the daily water requirement of this microalgae facility.

B. Oil

Same assumptions as coal.

Hydrogen content = 12.5%.(Electric Power Research Institute 1986)

Moisture content = 0

12.5% of 1.91×10^7 lb oil d^{-1} = 2.39×10^6 lbs hydrogen, or

potentially 2.15×10^7 lbs H_2O (1.07×10^4 tons H_2O d^{-1})

This amount represents 0.7% of the daily water requirement of a microalgae facility of 2.68×10^4 ha.

C. Natural Gas

Assume 98% methane, 1% water vapor.[Rivard, 1989]

25% of weight of methane (CH_4) is hydrogen, so 5×10^6 lbs d^{-1} of hydrogen could be converted to 4.5×10^7 lbs d^{-1} of H_2O. The 1% water vapor brings the total to 4.52×10^7 lbs d^{-1}, or 2.26×10^4 tons d^{-1}. This amount represents 1.5% of the water required by a 2.68×10^4 ha microalgae facility.

IV. Waste Heat

A. Heat available for microalgae production

Coal burned: $(1.2 \times 10^4$ tons $d^{-1})(25 \times 10^6$ Btu $ton^{-1}) = 3 \times 10^{11}$ Btu d^{-1}

At 38% efficiency, 1.13×10^{11} Btu d^{-1} of electricity are generated
1.86×10^{11} Btu d^{-1} is waste heat

$(1.86 \times 10^{11}$ Btu $d^{-1})(.252$ kcal $Btu^{-1}) = 4.69 \times 10^{10}$ kcal d^{-1}

B. Radiative Cooling

1. Assume 5°C temperature difference
11(Temperature of the water - temperature of the air)[Wetzel 1983]
= radiative heat loss in cal cm^{-2} d^{-1}

At $\Delta T = 5°$, $11 \times 5 = 55$ cal cm^{-2} d^{-1}

$(2.68 \times 10^4$ ha$)(10^4$ m^2 $ha^{-1}) = 2.68 \times 10^8$ m^2

$\times 10^4$ cm^2 $m^{-2} = 2.68 \times 10^{12}$ cm^2

$\times 55$ cal cm^{-2} $d^{-1} = 1.47 \times 10^{14}$ cal d^{-1}

$= 1.47 \times 10^{11}$ kcal d^{-1}

2. Convective Heat Loss

(Q) = [heat transfer coefficient (U)] x [area] x [temperature difference (°F)]

U ranges from 1 to 5, with units of Btu ft^{-2} hr^{-1} $°F^{-1}$[Perry & Chilton 1973]

$\therefore$ $Q = (1 \leq U \leq 5$ Btu ft^{-2} hr^{-1} $°F^{-1})(9°F)(2.68 \times 10^4$ ha$)(2.47$ acres $ha^{-1})$

$(4047$ m^2 $acre^{-1})(1$ ft^2 $.0929$ $m^{-2})$
$= 2.6 \times 10^{10}$ Btu hr^{-1}

$(2.6 \times 10^{10}$ Btu $h^{-1})(.252$ kcal Btu$^{-1})(24$ h $d^{-1})(1 \leq U \leq 5)$

$Q = 16 \times 10^{10} \rightarrow 78 \times 10^{10}$ kcal d^{-1}

3. Evaporative Cooling

Evaporation rate = 0.0035 m d^{-1} (Neenan et al. 1986)

$(2.68 \times 10^4$ ha$)(10^4$ m^2 ha$^{-1})(.0035$ m $d^{-1}) = 9.38 \times 10^5$ m^3 d^{-1}

$(9.38 \times 10^5$ m^3 $d^{-1})(10^3$ kg m$^{-3}) = 9.38 \times 10^8$ kg d^{-1} (water lost to evaporation)

$(9.38 \times 10^8$ kg $d^{-1})(2.204$ lb kg$^{-1}) = 20.7 \times 10^8$ lb d^{-1}
$\times$ 1054 Btu lb^{-1} (ASME 1967) $= 2.18 \times 10^{12}$ Btu d^{-1}

$\times .252$ kcal Btu$^{-1} = 5.5 \times 10^{11}$ kcal d^{-1}

C. Solar Radiation Input

Calculated using ~90% of maximum expected solar radiation

Range: 90% of 130 Btu hr^{-1} ft^{-2} (Perry & Chilton 1973) = ~120
90% of 50 Btu hr^{-1} ft^{-2} (Perry & Chilton 1973) = 45

Area: 1 acre = 43,560 sq. ft.
$(2.68 \times 10^4$ ha$)(2.471$ acre ha$^{-1}) = 6.62 \times 10^4$ acres

$(43{,}560$ ft^2 acre$^{-1})(6.62 \times 10^4$ acre$) = 2.88 \times 10^9$ ft^2

Max. $(2.88 \times 10^9$ ft$^2)(120$ Btu h^{-1} ft$^{-2}) = 346 \times 10^9$ Btu h^{-1}

$\times 16$ h d^{-1} (summer) $= 5.53 \times 10^{12}$ Btu d^{-1}

$\times .252$ kcal Btu$^{-1} = 1.39 \times 10^{12}$ kcal d^{-1}

Min. $(2.88 \times 10^9$ ft$^2)(45$ Btu h^{-1} ft$^{-2}) = 130 \times 10^9$ Btu h^{-1}

$\times 8$ h d^{-1} (winter) $= 1.04 \times 10^{12}$ Btu d^{-1}

$\times .252$ kcal Btu$^{-1} = 2.61 \times 10^{11}$ kcal d^{-1}

V. Potential Contribution of Sulfur to Pond Acidity

A. Coal

Bituminous coal combustion yields 75 lb SO_2 ton^{-1} (Bond et al. 1972) or 37.5 lb as sulfur. If all of this sulfur is converted to H_2SO_4:

$[(37.5$ lb S$)(98$ lb $H_2SO_4)] \div 32$ lb S mole^{-1} H_2SO_4
$= 115$ lb H_2SO_4 ton^{-1} coal

(115 lb H_2SO_4 ton^{-1})(1.2 x 10^4 tons coal d^{-1}) = 1.38 x 10^6 lb H_2SO_4 d^{-1}

÷ 2.204 lb kg^{-1} = 6.261 x 10^5 kg H_2SO_4

x (10^3 g kg^{-1}) = 6.26 x 10^8 g H_2SO_4 d^{-1}

÷ 98 g H_2SO_4 $mole^{-1}$ = 6.4 x 10^6 moles H_2SO_4 d^{-1}

x 2 moles hydrogen $mole^{-1}$ H_2SO_4 = 12.8 x 10^6 moles $[H^+]$

Concentration of $[H^+]$: (12.8 x 10^6 moles) ÷ (4 x 10^{10} liters)

= 3.18 x 10^{-4} M

pH = - log $[H^+]$

= - log ($10^{.5024}$ x 10^{-4})

= 3.50

B. Oil

In a similar fashion, if one assumes 210 lb SO_2 per 10^3 gal of oil,[Bond et al. 1972] 3.6 x 10^6 moles of H_2SO_4 can be generated per day.

(7.2 x 10^6 moles $[H^+]$) ÷ (4 x 10^{10} liters) = 1.8 x 10^{-4} M $[H^+]$

pH = - log ($10^{.2529}$ x 10^{-4})

= 3.75

C. Natural Gas

Assuming release of 0.6 lb SO_2 per 10^6 cubic feet of natural gas,[Bond et al. 1972] one can produce 2.03 x 10^3 moles of H_2SO_4 d^{-1}.

(4.06 x 10^3 moles $[H^+]$) ÷ (4 x 10^{10} liters) = 1 x 10^{-7} M $[H^+]$

pH = 7.0

VI. Potential Contribution of Sulfur to Pond Salinity

If all the sulfur released from fuel combustion is neutralized to form Na_2SO_4, how much of a contribution to the salinity of the pond does this represent?

A. Coal

As calculated above, 37.5 lb of sulfur are released per ton of coal combusted. This represents a potential 166 lb of Na_2SO_4 ton^{-1} coal.

(166 lb Na_2SO_4 ton^{-1})(1.2 x 10^4 tons d^{-1}) = 1.99 x 10^6 lb Na_2SO_4

÷ 2.204 lb kg^{-1} = 9.04 x 10^5 kg Na_2SO_4 day^{-1}

[(9.04 x 10^5 kg Na_2SO_4)(1000 parts)] ÷ (4 x 10^{10}) kg H_2O in ponds

= .0225 parts per thousand per day

B. Oil

105 lb sulfur per 10^3 gal of oil represents a potential 466 lb Na_2SO_4 d^{-1}

[(2.45 x 10^6 gal. oil d^{-1})(466 lb Na_2SO_4)] ÷ 10^3 gal.
= 1.14 x 10^6 lb Na_2SO_4 d^{-1}

÷ 2.204 lb kg^{-1} = 5.18 x 10^5 kg Na_2SO_4 d^{-1}

[(5.18 x 10^5 kg Na_2SO_4 d^{-1})(10^3 parts)] ÷ (4 x 10^{10} kg H_2O)

= .013 parts per thousand per day.

C. Natural Gas

(0.3 lb sulfur per 10^6 cubic feet)(4.76 x 10^8 SCF d^{-1}) = 142.8 lb $\underline{S}$ d^{-1}

This represents a potential 288 kg Na_2SO_4 d^{-1}, which is a potential salinity increase of 7.2 x 10^{-6} parts per thousand per day.

DESIGN AND OPERATION OF AN OUTDOOR MICROALGAE TEST FACILITY

Large-Scale System Results

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SUMMARY

The Outdoor Test Facility was developed to help evaluate, in real terms, progress towards the goals of the Aquatic Species Project (ASP). Project goals included: archieving stable cultivation of microalgae outdoors; extending productivity data in terms of scale and duration; specifying and testing a mode of operation for lipid induction; specifying and testing raceway pond designs, particularly in terms of lining, hydraulic performance, and carbonation system development; and delineating the state of the art of outdoor mass cultivation of microalgae to facilitate comparison with ASP technological goals.

Outdoor production was maintained at the 0.1-ha scale for 2 years. The operation of the ponds was stable during this time in that down-time could be kept under 10% to 15%. Species composition varied with season and operating conditions, but the total number of different organisms was limited to four. Two of these were species of *Cyclotella*, which could be cultivated over the 6 to 7 warm months of the year. Most of the yearly production of 37 mt ha^{-1} yr^{-1} was obtained during these 7 months. Winter operation in New Mexico is not productive enough, in outdoor cultures, to be justified.

A batch lipid induction process was tested. The lipid content attained, 40% with one organism and 30% with another, and the rate of accumulation of lipid within the cells, was high enough to demonstrate the viability of the process. The ASP goal of producing over 15 g lipid m^{-2} d^{-1} could be attained if the entire induction required only 1 day. This would be possible if the rate of lipid-forming reactions within the cell was increased. Present results indicate that most of the capability of fixing carbon is maintained for at least 1 day after the decreasing level of intracellular Si triggers lipid accumulation; results also show that dedicated lipid production can occur.

The engineering evaluation of the operation of the 0.1-ha raceways showed that these systems are potentially very efficient in the utilization of the major inputs: energy for mixing, water, and carbon dioxide. The 0.1-ha raceways can be mixed with the power consumption equal to a 100-watt light bulb. The ponds can be graded, the turns can be baffled, and the depth can be adjusted to eliminate most in-pond sedimentation. Losses of water to leakage and percolation were negligible. The carbonation sumps designed and installed were nearly 100% efficient in transferring CO_2 from the inflowing gas phase to the pond water. Operating the culture system at pH above 7.8 nearly eliminates outgassing of CO_2 to the atmosphere. The cultivation system is therefore almost 100% efficient in using carbon dioxide. The pH experiments demonstrated that finding organisms that grow well over a range of pH to be expected in very large-scale systems is feasible. The diatom grown performed well in cycling conditions.

There was virtually no difference in the operation of the lined raceway versus the unlined one. Neither one could be effectively sterilized or even cleaned on a larger scale than currently operated. Neither one lost water through the bottom. Biomass productivity and culture stability were the same.

The operation of the large-scale system in New Mexico has been marked by continual improvement in both its engineering performance and biomass output. In order to meet project goals for biomass production, however, a milder winter is mandatory. In order to meet the lipid production goals, genetic manipulation is required.

OBJECTIVE

The Aquatic Species Project is developing a technology in which microalgal biomass grown in outdoor ponds is to be used as a feedstock for a biomass-to-liquid-fuels conversion. As part of the overall objective of advancing the technology and evaluating progress of the project, an Outdoor Test Facility (OTF) was developed and operated in Roswell, New Mexico.

The main objective of this subcontract was to operate two 0.1-ha, raceway ponds at the OTF to aid the Aquatic Species Project in formulating an input-output

analysis of the biomass production phase of the project. One of the raceways was lined with a plastic membrane, the other was lined with less-expensive earthen materials. Specifically, the evaluation included biomass production rates, efficiency of utilization of carbon dioxide, mixing power requirements, and water consumption. Each of these is affected by the manner in which a pond is operated, which is dictated, in part, by the lining option.

In addition, small-scale test reactors were used to evaluate nutrient input requirements, specifically the optimal range of pH for cultivation of chosen strains and the minimum silicon requirement of diatoms.

DESCRIPTION OF FACILITIES

The OTF consists primarily of two experimental pond systems: the small-scale system (SSS) and the large-scale system (LSS). The SSS consists of six 3-m^2 fiberglass raceways, nutrient and dilution systems, and an operations shed, all located on a concrete pad. The system is used to screen species for productivity potential, perform specific controlled experiments, and do diagnostic experiments to aid in the operation of the LSS. The LSS is composed of one membrane-lined 0.1-ha pond (the North Pond, or NP), one gravel- and clay-lined 0.1-ha pond (the South Pond, or SP), and one membrane-lined 50-m^2 inoculation pond. The LSS is located 1 mile east of the Roswell Test Facility (RTF). Water and CO_2 are piped to the LSS from the RTF. A metal building is used for shop and operations space at the LSS.

LARGE POND HYDRAULICS

Velocity Profiles

The profiles of velocity across the channel 1.4 m upstream from the sump (in the return flow channel), at each of four paddle wheel rotation speeds, are given in Figure 1. At the higher mixing speeds, velocities were somewhat greater closer to the outer wall of the pond. This is an indication that the turning vanes were not totally effective in straightening the flow out of the upstream bend.

Head Losses

The energy, or total head (potential plus kinetic) of the flow, was measured at six locations in the lined pond, according to methods used last year (Weissman et al. 1989). Measurements taken before and after the paddle wheel were used to calculate total losses in mixing the pond. When multiplied by water density and flow rate, these losses give an estimate of the hydraulic power requirements for mixing. Measurements taken before and after each bend were used to estimate bend losses. In the same way, measurements taken before and after the carbonation sump indicate losses during transit through the sump; those taken at the beginning and end of each channel indicate open channel losses. The results of the head-loss measurements are given in Figure 2.

The "open" channel head loss was less when operating at 22.5-cm depth compared to operating at 13.7-cm depth. However, the losses in the channels were so low at the greater depth that it was barely discernible above the measurement errors. Thus no quantitative comparison is possible with so few data points. For a given average channel velocity, the velocity gradient (with depth) is steeper at more shallow operation; hence, the greater the rate of energy dissipation. As a percent of the total head loss for mixing the pond, the loss down the return flow channel (which is the most like an ideal channel with uniform flow) was less than 5% at the 22.5-cm depth. At 13.75 cm depth, it was about 15% of the total loss.

At both depths, the losses in the carbonation sump were greater than those for any other section of the pond. At 13.7-cm depth sump losses were about 20% of total head loss, while at 22.5-cm depth sump losses were 50% of the total. On an absolute basis sump losses were greater at the greater depth. The sumps were configured for maximizing carbonation efficiency, not for minimizing hydraulic losses. Thus the sump baffle (which directed water flow down, opposing the

direction of rising gas bubbles) was placed within 15 cm of the sump wall to increase velocity. This served to increase the time it took for bubbles to rise though the water column, increasing carbonation efficiency. However, this greatly constricted the flow at the operating depth of 22.5 cm, resulting in increased hydraulic energy losses.

Bend losses were 15% to 20% of the total head losses at 22.5-cm depth. The losses were greater (20% to 30%) at 13.7-cm depth. The actual head losses through the bends were not appreciably different at the two depths.

Operation at greater depth had relatively little effect on head losses in general. Improvement in the configuration of the carbonation sump should result in more optimal operation at greater depth (lower total head losses for mixing). Much more important was the consequence that solids deposition was considerably reduced when the pond was operated at the greater depth.

Mixing Power

The power dissipated at the two depths of operation is shown in Table 1. For a given velocity of flow attained, nearly twice as much power was consumed at the greater depth of operation. However, since half of the power consumption at the greater depth was consumed in the carbonation sump, hydraulic optimization of the sump could significantly reduce the disparity. Indeed, for ideal channel flow, power consumption would actually be less at greater depth.

LONG-TERM ALGAL CULTIVATION IN THE LARGE-SCALE SYSTEM

Results

- The insolation, ambient, and pond temperatures are shown in Figure 3.
- Annual yields were 10 ±1 gAFDM m^{-2} d^{-1} from ponds (Table 2).
- Cultivation was stable and yields were highest (20 gAFDM m^{-2} d^{-1}) from July through October when it was warm and when silica was added to the medium (Figure 4).
- Without silica additions, cultivation was stable only during the months of October through March when it was cold and *Monoraphidium* sp. or *Tetraselmis* sp. dominated pond flora. Productivity was low, however.
- Carbon utilization efficiency was greater than 90% when biomass productivity was 20 g AFDM m^{-2} d^{-1} (Figure 5). Outgassing losses limited carbon utilization efficiencies during the winter, when biomass productivity was low; occasional declines limited carbon utilization efficiencies at other times since the biomass produced was not measured or recoverable (it sedimented).

Water loss was due mainly to evaporation, which averaged 0.5 cm per day over the year and 0.8 cm over the summer (Table 2). After initial sealing of the bottom of the unlined pond, percolation was barely discernible above measurement error.

During the summer and fall of 1990 the pH of the large-scale cultures was allowed to vary over the day. The computerized monitoring system recorded all important pond parameters. Traces for August 1, 1990 are shown in Figure 6. Although set at 7.5, after 1000 to 1100 h it rose, reaching a high of 7.8 to 8.4 by late afternoon. During the night, respiration lowered it to 6.8 to 7.2 by sunrise. This variation served as a more realistic test of even larger scale culture operation. Experiments performed in the small-scale raceways had demonstrated that cycling pH has only minor effect on productivity. It does, however, reduce

outgassing of CO_2 during the day. Virtually all of the outgassing occurred at night, when pH dipped.

CARBON DIOXIDE UTILIZATION EFFICIENCY

Carbon Dioxide Outgassing to the Atmosphere

Two methods were used to estimate the amount of carbon lost as CO_2 to the atmosphere during the operation of the lined pond. In both cases, the pond was drained, cleaned, and filled with saline groundwater. In the first method, the pond water was carbonated down to pH 7.2 ±0.1. Then the pH control was removed so that the pH drifted upwards because of the outgassing of CO_2. The automated data acquisition system monitored the pond pH. The experiment was conducted twice. The loss of carbon was calculated as the difference in inorganic carbon content of the water. This, in turn, was calculated from the pH and dissociation constants for the carbonate system in the saline groundwater. These constants were determined during the previous subcontract period. Sensitivity to the values for the dissociation constants and for the Henry's law constant was evaluated.

The results are presented in Table 3. The mass transfer coefficient for CO_2 outgassing was found to be $0.233 \pm 0.007\ h^{-1}$. The errors resulting from uncertainties in the water chemistry were not much greater than the experimental error. Given this mass transfer coefficient, the total amount of carbon outgassed over a given period of time could be calculated from the pond pH. For much of the time during 1989, the pond pH was nearly constant during the day. Computer calculations were used to estimate outgassing when pH varied over the day.

In the second method for determining the mass transfer coefficient for outgassing of CO_2, the pond was controlled at a prescribed pH 24 h a day. CO_2 lost through outgassing was replaced via the carbonation system. The flow meters on this system measured the amount of CO_2 input. The results of several runs performed at two different times are shown in Table 4. The value for the transfer coefficient is highly dependent on the equilibrium constants used for carbonate system and the pH. These determine the dissolved CO_2 concentration used in the driving force. A one-tenth unit error in the pH or pK-1 can result in a 10% to 20% error in the calculated transfer coefficient. The number of samples taken was necessary to establish a reasonable estimate, assuming random errors in pH measurement. However, an error in the equilibrium constant used could result in a small bias.

The mass transfer rate calculated by the first and second methods were the same within measurement error. This implies that the efficiency of injecting CO_2 via the in-pond sump was, within experimental error, nearly 100%. This was corroborated by carbon mass balance calculations.

Efficiency of Injecting CO_2 via the In-pond Sump

An estimate of the efficiency of injecting carbon dioxide into the 0.1-ha raceways via the in-pond sumps was made from a mass balance for carbon into and out of the lined pond. Three sources of carbon were used: carbon injected into the sump, the inorganic carbon content in the inflowing dilution water, and carbon derived from the incorporation of urea into algal biomass. Also, three sinks for carbon existed: the carbon removed as algal biomass, the inorganic carbon content in the effluent dilution water, and the carbon outgassed to the atmosphere. The net difference between the sources and sinks represents an estimate of the losses of carbon from the sump during injection.

The amount of carbon injected was measured with mass flow meters. The inorganic carbon content of the inflowing and outflowing water was estimated from the carbonate system dissociation constants, temperature, pH, and the measured flow rates of water into and out of the ponds. The outgassing was estimated using the transfer coefficient determined as discussed previously and from pH. Initially, the carbon input from the urea was estimated from the amount of urea added. As

discussed in the following paragraph, only some of this urea was used, with only some of the carbon being made available. Finally, the carbon removed from the pond as algal biomass was estimated from the dilution rate, the ash-free dry mass of the composited samples of the outgoing water, and an estimated carbon content (of the ash-free dry mass) of 50%.

Data for the months of August and September 1989 from the lined pond were analyzed for the estimation of the injection losses. During this time, biomass production was greatest. Since 2 months of results were used, the effect of any short-term anomalies was minimized. The pond had been in operation for months, thus the amount of sedimented biomass had already reached a steady state of accumulation. The carbon balance is given in Table 5.

Within experimental errors, the sump was 100% efficient in transferring CO_2 from the injected gas to the water. The amount of carbon provided from the added urea may have been more uncertain than indicated. Only 45% ± 5% of the added urea-nitrogen was incorporated into algal biomass (more urea was added than needed). Measurements show the free total ammonium in these systems is generally very low, indicating that the urea is not broken down unless incorporated. Thus it was likely, but not certain, that only 45% of the urea-carbon was available. The rest flows out in the pond effluent. The sumps appeared to be so efficient that other, more sensitive experiments planned to evaluate their efficiency were not performed.

INDUCTION OF HIGHER LIPID YIELDS

Experiments were undertaken to increase the content of lipid in the algal biomass and to increase overall lipid production rates. One of the goals of the Aquatic Species Project is to attain a lipid content of at least 40% while maintaining an average biomass productivity of 40 g AFDM m^{-2} d^{-1}.

During the past two summers at the Roswell OTF, Si-deficient lipid induction was examined in outdoor cultures. During 1989, the experiments were performed in the 3-m^2 raceways using RTF CYCLO1. During 1990, the experiments were performed in the 50-m^2 raceway using RTF CYCLO2.

Most work on induction of storage products is done in batch culture. This has been the case with all outdoor work. The reasons are that (1) yields of storage products should be maximized if light is the yield-limiting factor, just as in the case with biomass production yields; (2) severe nutrient deficiency results in reduction and eventual cessation of cell division, so that continuous dilution of cultures would be transient anyway; and (3) imposition of a milder, continuous nutrient deprivation would have less effect (result in submaximal storage product content) and weaken the algae against photoinhibition, predators, and more slowly inducing competitors. Despite the rational basis for each of these justifications, none has been experimentally validated. Nonetheless, batch cultivation was more appropriate to the objectives of the preliminary investigation conducted here.

The results of inducing both species are shown in Table 6. Approximately 50% to 75% of the increase in standing density (g AFDM m^{-2}) of total biomass occurred during the first day of the batch culture. Usually 90% of the increase occurred by the end of the second day. Assuming that the culture Si content of the cells dropped proportionately with the increase in AFDM, growth was usually slowed considerably when intracellular Si content dropped by one-third. Growth was still vigorous when intracellular Si was still 75% of initial levels.

With RTF CYCLO1, the standing density of lipid increased three to four fold during the first day of the induction time course. Biomass production was generally still high at this time. Lipid accounted for 33% to 50% of the biomass produced. During the second day, lipid increased another 50% while biomass increased very little. Often during the second and third days, virtually all of the net biomass production was lipid. The highest rates of lipid production occurred during the first day of induction. The maximal, or near maximal lipid content was achieved by the second or third day.

The biomass and lipid productivity of Si-sufficient cultures was, at most, 20 and 4 g AFDM m^{-2} d^{-1}, respectively. The biomass productivity dropped off only a little from this during the first induction; the lipid productivity increased 50% to 100%. In reaching 40% lipid content (close to the maximum), biomass productivity averaged half of the Si-sufficient rate; lipid productivity was 150% of the Si-sufficient rate.

The induction process occurred more slowly and proceeded, to a lesser extent, with RTF CYCLO2. The standing density of lipid increased only two fold during the first day of induction, so that lipid accounted for only about 20% of the biomass produced. During the second day, lipid density increased 50% or more and usually accounted for most of the biomass productivity as it did with RTF CYCLO1. Again, the maximal lipid content was achieved by the end of the second or third day, but this maximum was only about 30% of the biomass. Thus induction proceeded more slowly over a 2-day period with RTF CYCLO2.

How much of the difference in rapidity of the induction was due to species differences is difficult to determine. The weather was more variable during the induction experiments performed in 1990 with RTF CYCLO2. The weather was clear and warm during the first experiment of 1990, but it was cloudy during the second day of the second experiment, leading to a low rate of biomass production. Lipid productivity kept pace, however. During the third induction, it was partly cloudy throughout. The final extent of lipid accumulation did seem to be consistently lower with RTF CYCLO2.

The experimental results show that the algal cells were growing vigorously while intracellular Si content dropped from the normal sufficiency level to about two thirds of that level. Conversion from balanced growth to lipid production also occurred dramatically during this time. Thus, this process was initiated in the early stages of Si depletion. It continued to the point where all new biomass produced was lipid. It is tempting to speculate that what limited the overall rate of lipid productivity was the rate of the lipid-forming reactions themselves. That is, it appears that the metabolic switch towards lipid formation had been turned on almost immediately, and stayed on until Si levels dropped so low that growth ceased.

With RTF CYCLO2, less than 40% of the biomass produced during the 2- to 3-day induction period was lipid. If lipid formation occurred more quickly, or more exclusively of other cellular components, this percentage would have been higher, as would the final lipid content. This was the case with RTF CYCLO1. Approximately 75% of the biomass produced was lipid. In this case, any additional advantage gained in relieving constraints on the biochemical rate of lipid formation would mostly be in achieving 40% to 50% lipid content in 1 day rather than in 3. In this way, the induction process could be set up (the initial biomass density could be set) so that the standing biomass density would double during 1 day of induction, and most of the new biomass would be lipid. Setting up the proper initial conditions for this to occur is easy, so that manipulating the organism to form lipid quickly remains the major challenge.

REFERENCES

Weissman, J.C., R.P. Goebel, and D.M. Tillett. 1988. *Design and Operation of an Outdoor Microalgae Test Facility, A Subcontract Report*. Solar Energy Research Institute, Golden, CO.

Weissman, J.C., D.M. Tillett, and R.P. Goebel. 1989. *Design and Operation of an Outdoor Microalgae Test Facility," A Subcontract Report*. Solar Energy Research Institute, Golden, CO.

Table 1. Head Loss and Power Dissipation in 0.1-ha Raceway

Paddle Wheel Rotation rpm	1988, d = 13.7 cm V cm/s	Head Loss cm	Power watts m^{-2}	1989, d = 22.5 cm V cm/s	Head Loss cm	Power watts m^{-2}
2.7	16.7	1.66	.026	14.3	1.33	.029
3.6	24.4	2.85	.058	--	--	--
4.4	27.6	3.91	.100	23.1	3.35	.117
5.3	30.8	5.12	.145	28.0	4.58	.194
6.1	33.0	6.39	.194	31.0	6.07	.293

Table 2. Long-Term Results from 0.1-ha Raceways

Pond	CO_2 Use slpd	Productivity g AFDM m^{-2} d^{-1}	Carbon Utilization Efficiency %	Water Loss cm d^{-1}
		OCT 1, 1988 - SEP 30, 1989		
LINED	15169	9.8	59	-0.57
		OCT 1, 1988 - SEP 30, 1989		
UNLINED	13867	8.3	50	-0.62
		OCT 1, 1989 - SEP 30, 1990		
UNLINED	14602	10.5	82	
		JUN 1, 1990 - OCT 30, 1990		
LINED	21994	19	81	
		MAY 1, 1990 - SEP 30, 1990		
UNLINED	19175	18	88	

slpd: standard liters per day

g AFDM m^{-2} d^{-1}: grams ash-free dry mass per square meter per day

Table 3. Mass transfer coefficient by the pH drift method

Run #	pK1	pK2	pKh	Kl h^{-1}
1	6.05	9.37	1.465	0.243
2	6.05	9.37	1.465	0.223
2	6.20	9.37	1.465	0.242
2	6.05	9.57	1.465	0.242
2	6.05	9.17	1.465	0.202
2	6.05	9.37	1.60	0.223

Table 4. Mass transfer coefficient by CO_2 inflow rate

Day	Pond pH	CO_2 in Standard liters	Kl h^{-1}
1989			
1	7.6 ± 0.1	4225	0.286
2	7.5 ± 0.1	3782	0.199
3	7.5 ± 0.1	3970	0.209
4-5	7.5 ± 0.1	7500	0.197
Average	7.5	3896	0.223 ± 0.011
1990			
1	7.4 ± 0.1	3522	0.148
2	7.4 ± 0.1	3645	0.160
3	7.4 ± 0.1	4123	0.167
4	7.8 ± 0.1	1246	0.128
5	7.8 ± 0.1	1624	0.167
6	8.0 ± 0.1	697	0.128:
7	8.1 ± 0.1	0	-----
8	7.2 ± 0.1	9941	0.267
9	7.2 ± 0.1	6936	0.172
10	7.25 ± 0.1	7842	0.238
11	7.25 ± 0.1	7708	0.234
12	7.25 ± 0.1	9929	0.287
Average			0.258 ± 0.017

Table 5. Carbon balance during August and September 1989 in lined pond

Source	Carbon, kg/day	Sink	Carbon, kg/day
Injection	11.5	Outgassing	3.2
Water inflow	3.5	Water outflow	3.1
Urea	0.8 - 1.8	Biomass removed	10.2
TOTAL	15.8 - 16.8		16.5

Table 6. Lipid induction experiments. Average production values were calculated over the first; first and second; first, second, and third; etc., days of induction.

Day	AFDM g m^{-2}	Lipid %	Si Quota % of initial	Av Prod. g AFDM m^{-2} d^{-1}	Av Prod gLipid m^{-2} d^{-1}
1989 RTF CYCLO1					
1	25.1	18.9	1.00	21.6	7.5
2	46.7	26.3	0.54	12.7	6.3
3	50.4	34.2	0.50	10.0	5.9
4	54.9	40.9	0.46		
1	19.5	15.9	1.00	14.5	8.0
2	34.1	32.5	0.57	9.8	6.9
3	39.0	43.4	0.50		
1	18.9	18.4	1.00	13.5	5.7
2	32.4	28.4	0.61	7.7	5.4
3	34.2	41.6	0.55	5.3	4.2
4	34.8	46.3	0.54	3.7	2.8
5	33.8	43.7	0.56		
1990 RTF CYCLO2					
1	33.0	14.5	1.00	15.8	4.2
2	48.8	18.5	0.68	16.6	5.3
3	66.2	23.3	0.56	9.1	3.70
4	60.2	26.5	0.64		
1	33.9	16.9	1.00	23.8	6.0
2	57.6	20.3	0.59	13.9	6.2
3	61.6	29.6	0.55	13.1	4.8
4	73.0	27.4	0.46		
1	39.2	13.2	1.00	11.9	2.0
2	51.0	14.1	0.77	10.8	4.8
3	60.7	24.4	0.64	8.8	4.8
4	65.6	29.7	0.60		

Figure 1. Profiles of depth-averaged velocity across the return channel, 1.4 m upstream from the carbonation sump in the lined 0.1-ha raceway. Symbols indicate paddle wheel rpm: squares, 2.7; +, 4.4; x, 5.3; diamonds, 6.1.

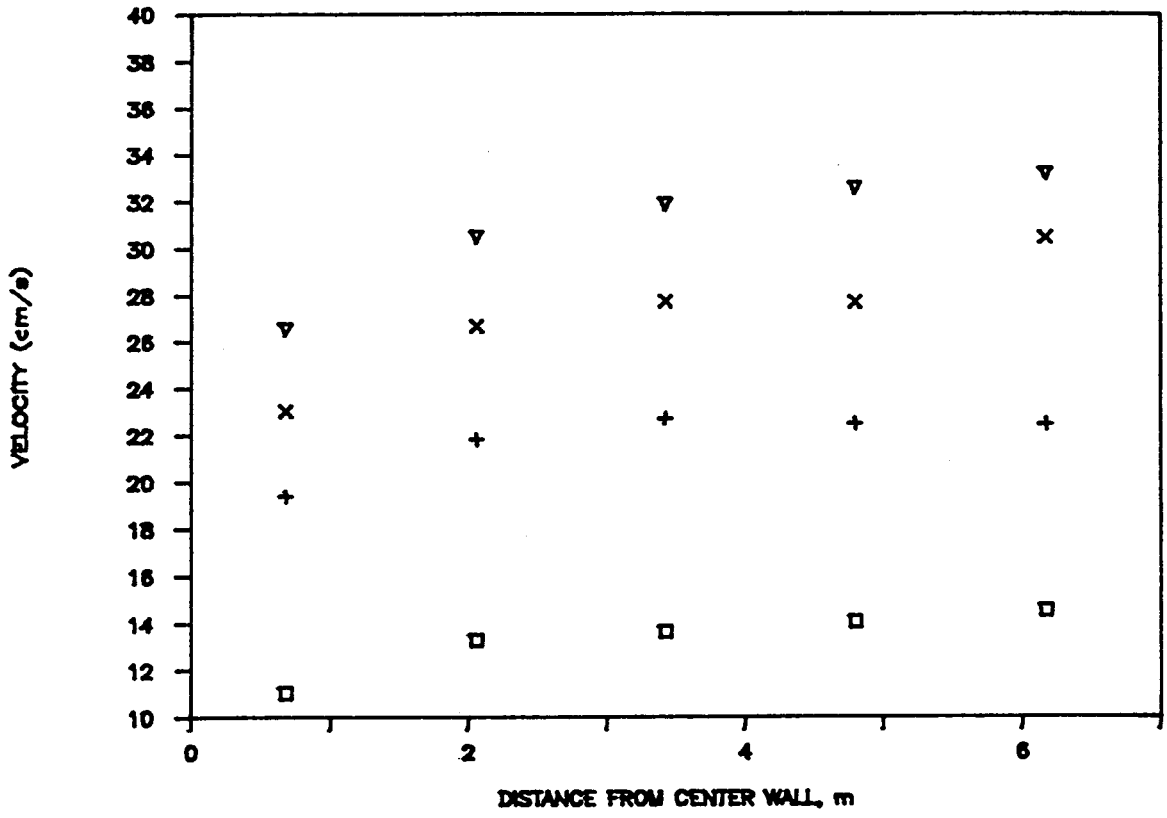

VELOCITY (cm/s)
40
38
36
34
32
30
28
26
24
22
20
18
16
14
12
10
0
2
4
6
DISTANCE FROM CENTER WALL, m

Figure 2. Head losses as a function of mixing velocity for five pond sections of the lined 0.1-ha raceway operated at 22.5-cm depth. The numbers indicate the total head loss (cm) at each velocity.

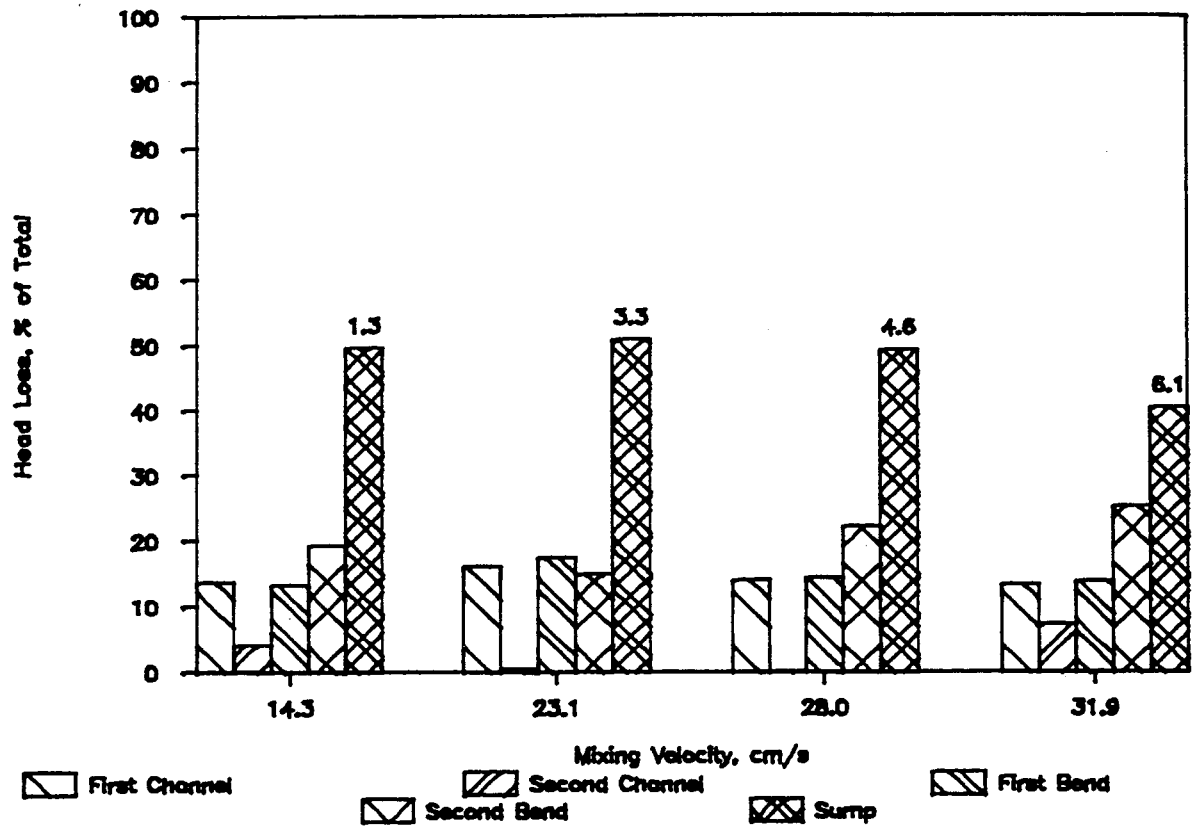
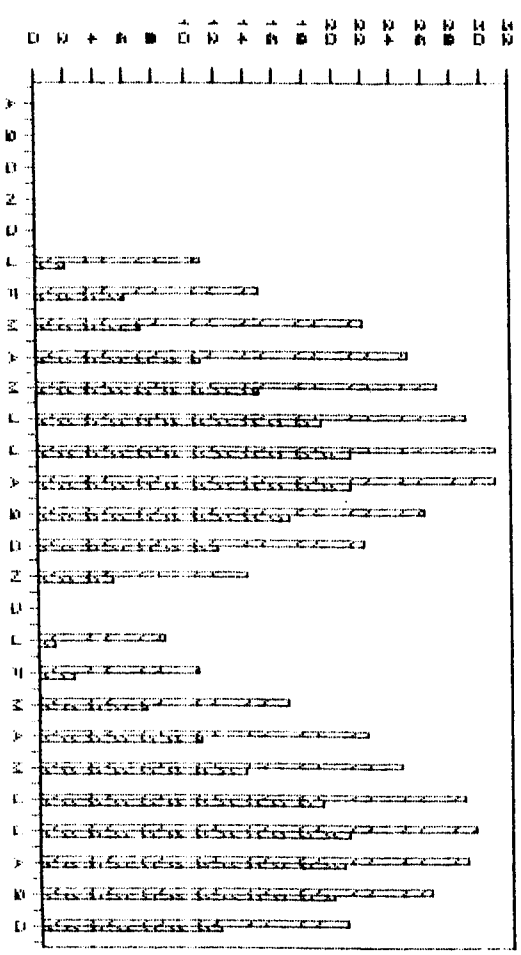
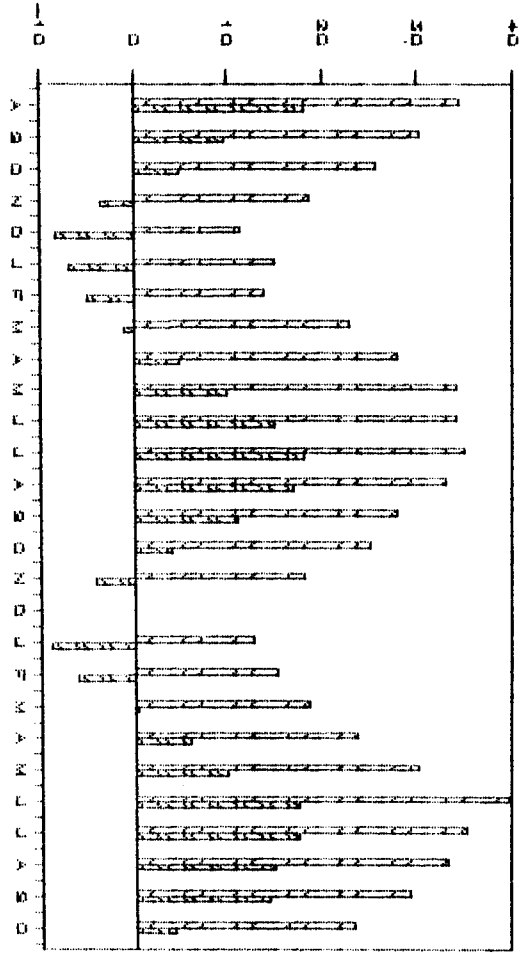

100
90
80
70
60
50
40
30
20
10
0
Head Loss, % of Total
1.3
3.3
4.6
6.1
14.3
23.1
28.0
31.9
Mixing Velocity, cm/s
First Channel
Second Channel
First Bend
Second Bend
Sump

Figure 3. Insolation, ambient temperatures, and pond temperatures during 1988-90 at Roswell, New Mexico.


POND TEMPERATURES, Celsius


AMBIENT TEMPERATURES, Celsius

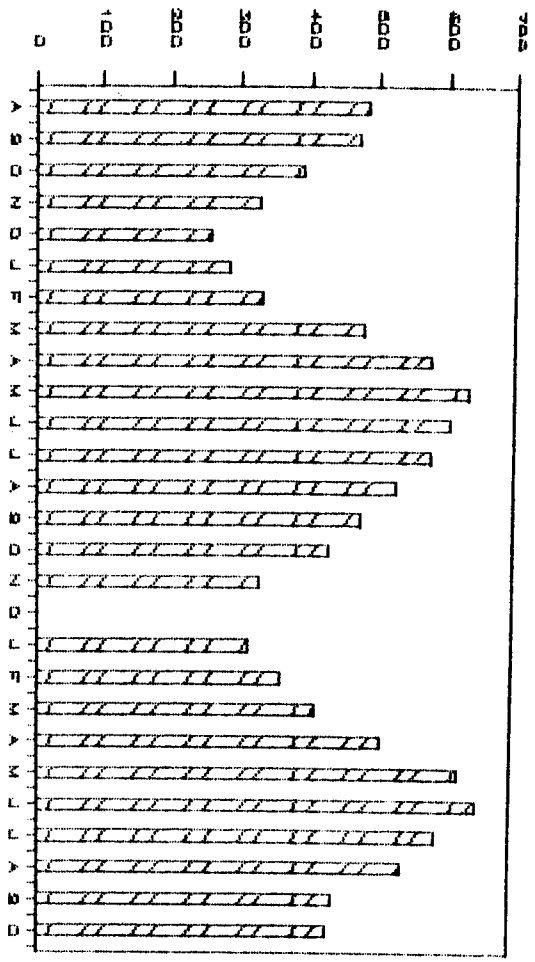
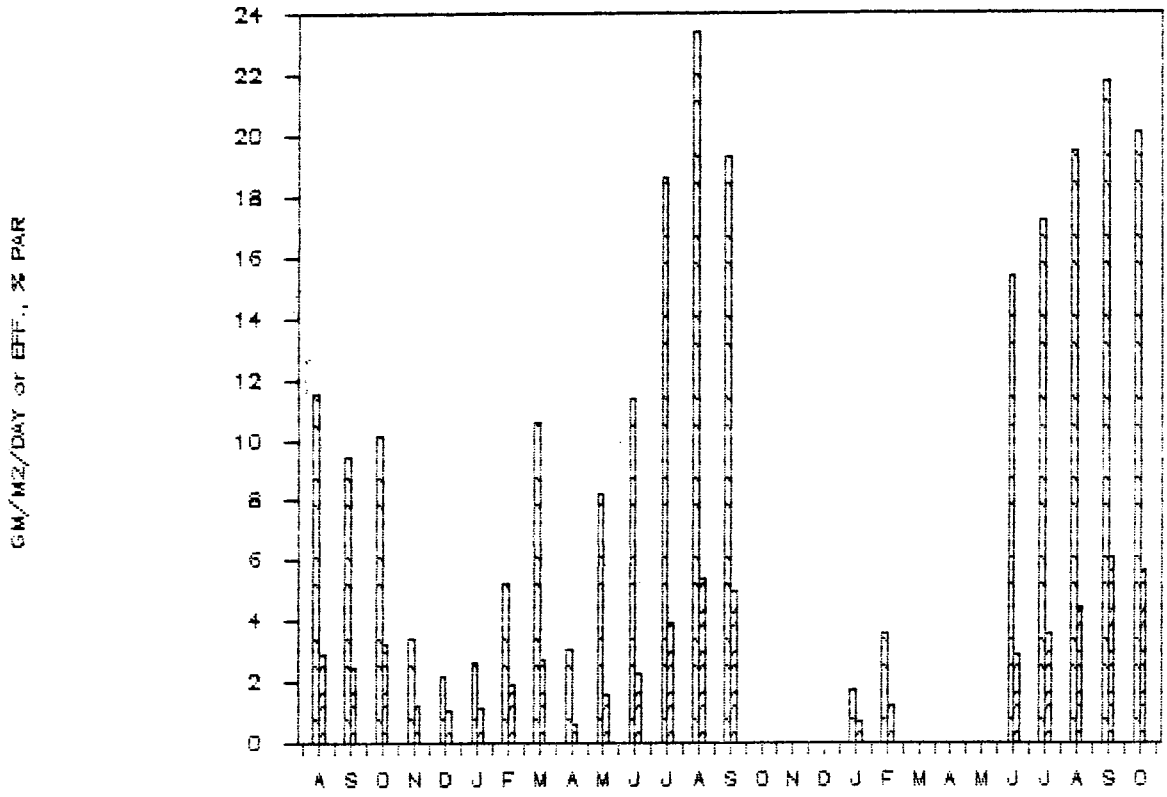

INSOLATION, cal/m2/day

Figure 4. Productivity and photosynthetic efficiency in 0.1-ha raceways


GM/M2/DAY or EFF., % PAR
0
2
4
6
8
10
12
14
16
18
20
22
24
A S O N D J F M A M J J A S O N D J F M A M J J A S O

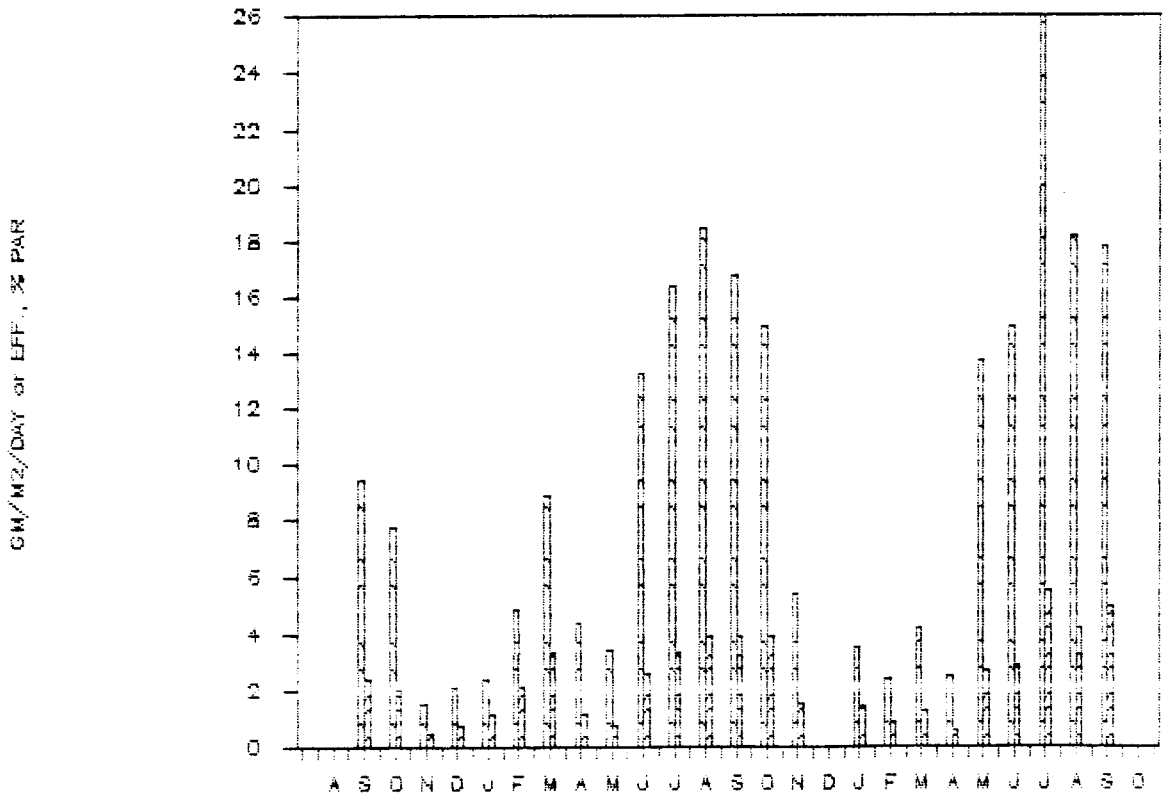
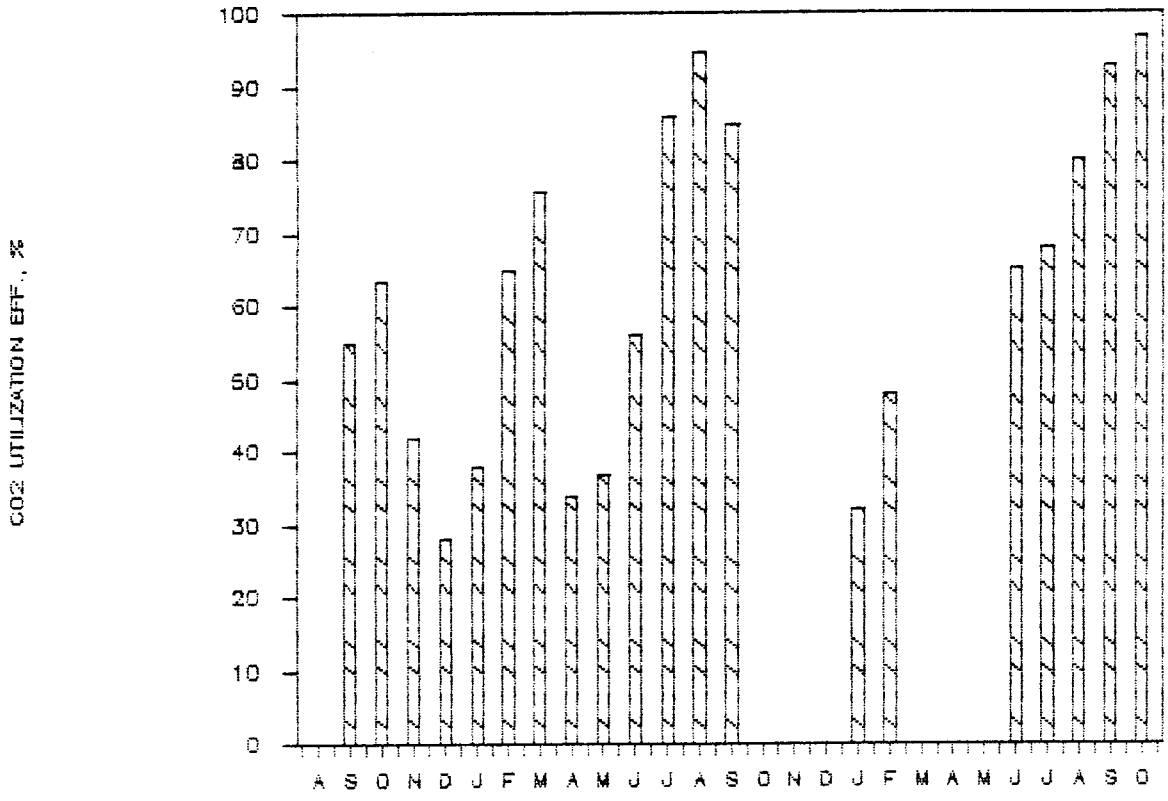

GM/M2/DAY or EFF., % PAR
0
2
4
6
8
10
12
14
16
18
20
22
24
26
A S O N D J F M A M J J A S O N D J F M A M J J A S O

Figure 5. Carbon dioxide utilization efficiency in 0.1-ha raceways


CO2 UTILIZATION EFF., %
100
90
80
70
60
50
40
30
20
10
0
A S O N D J F M A M J J A S O N D J F M A M J J A S O

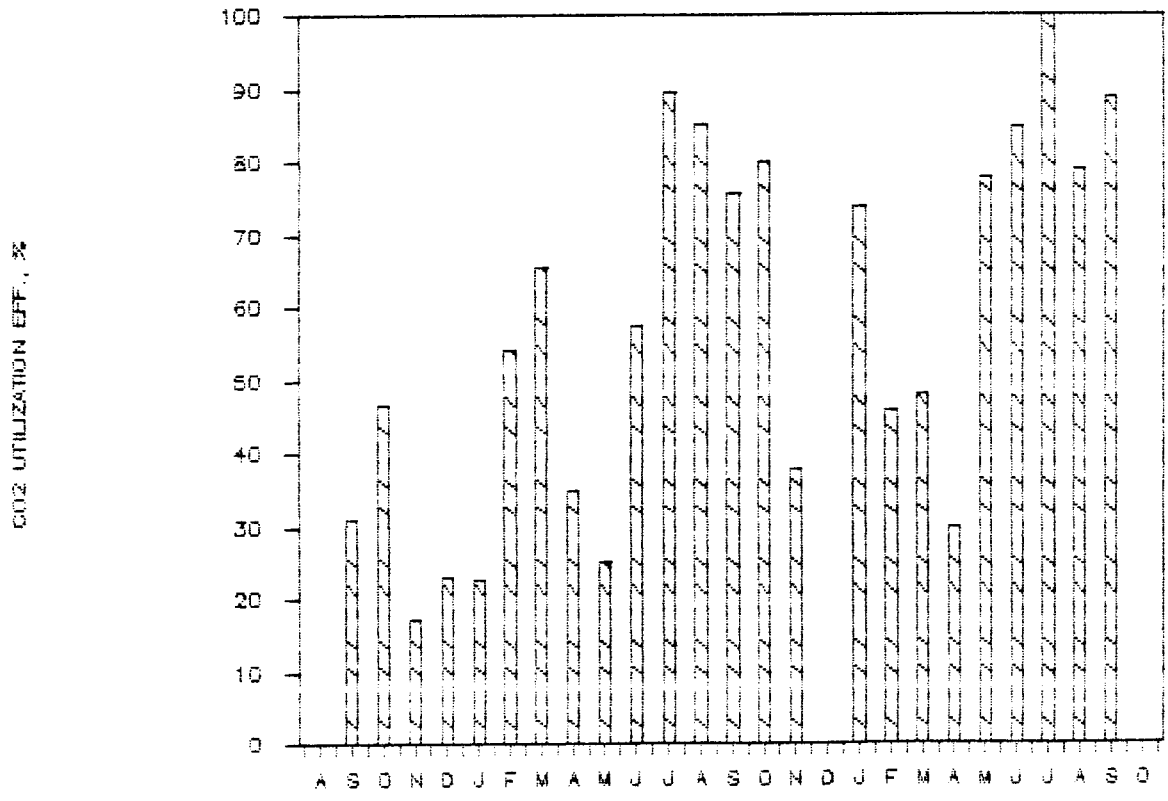
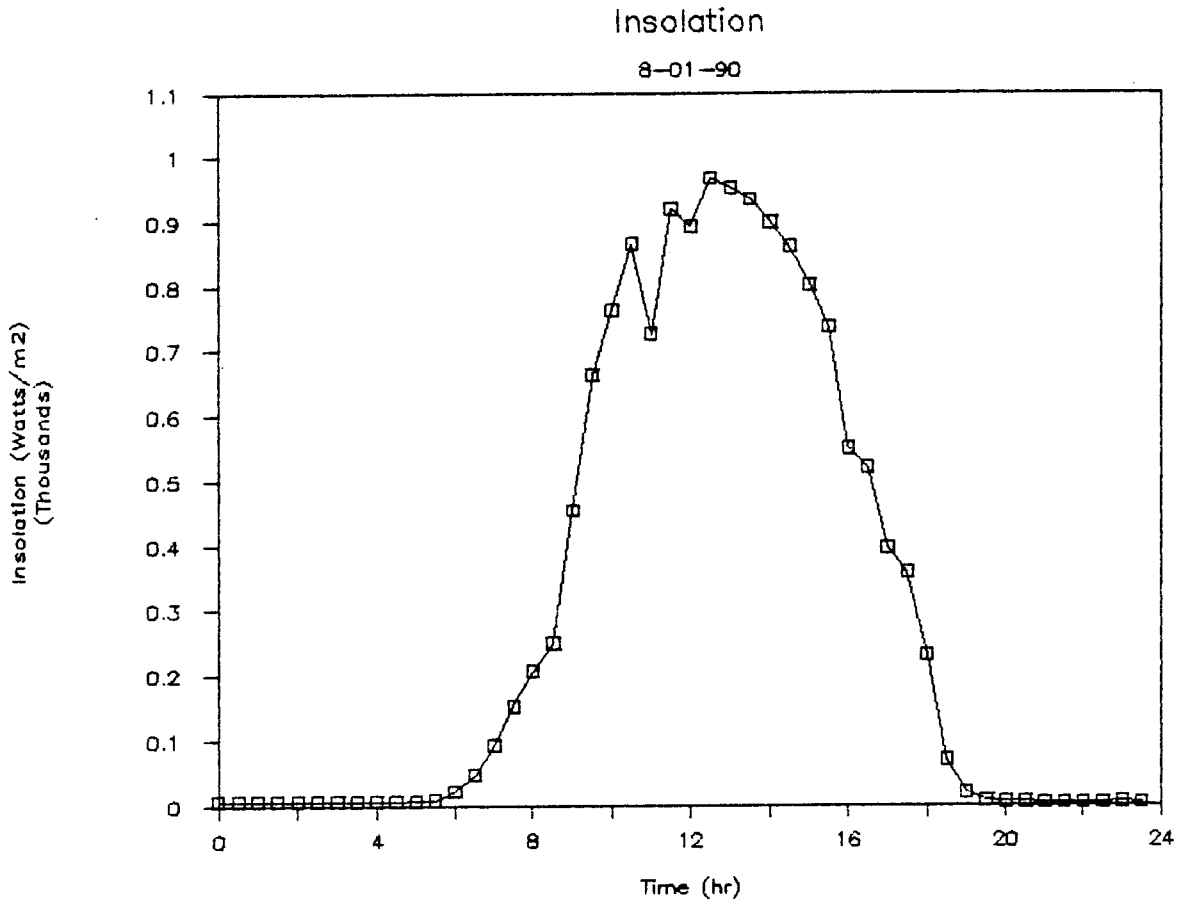
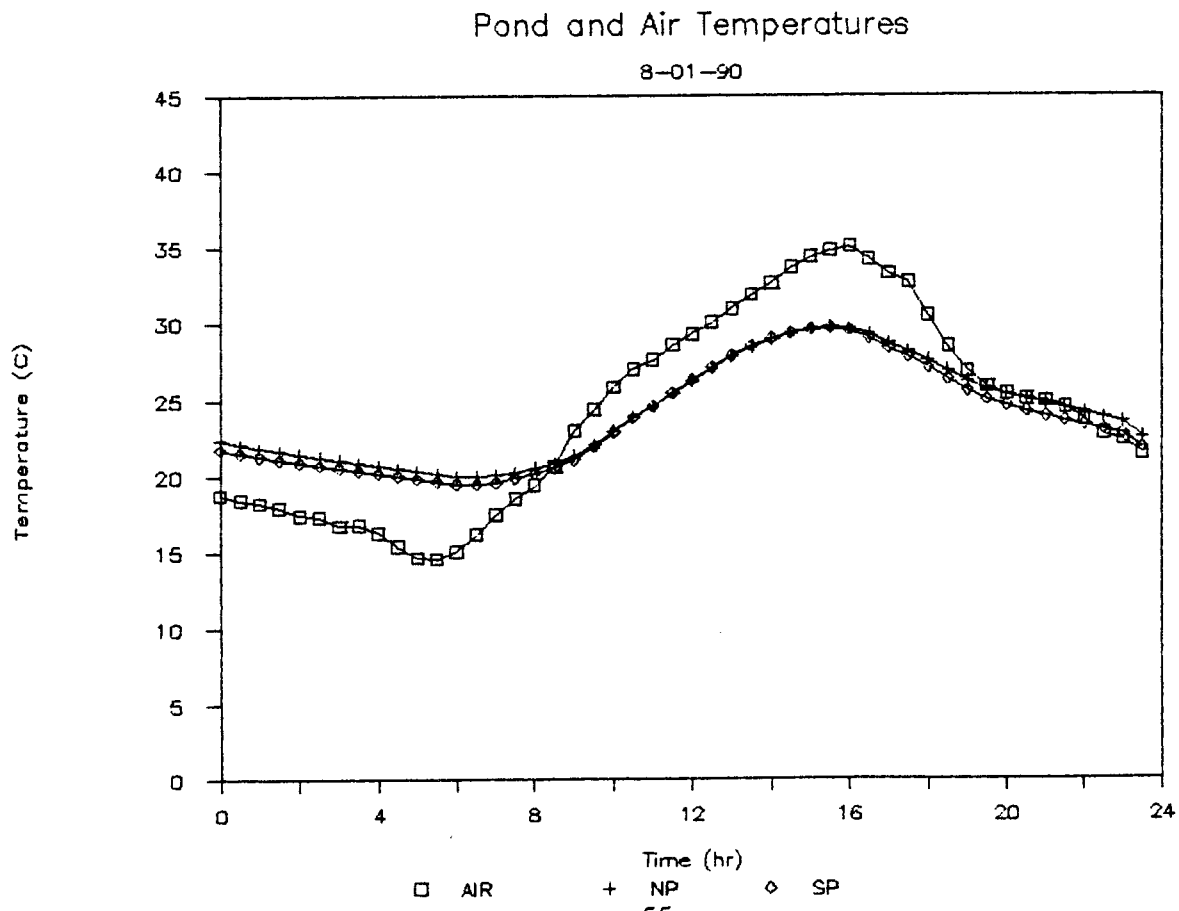
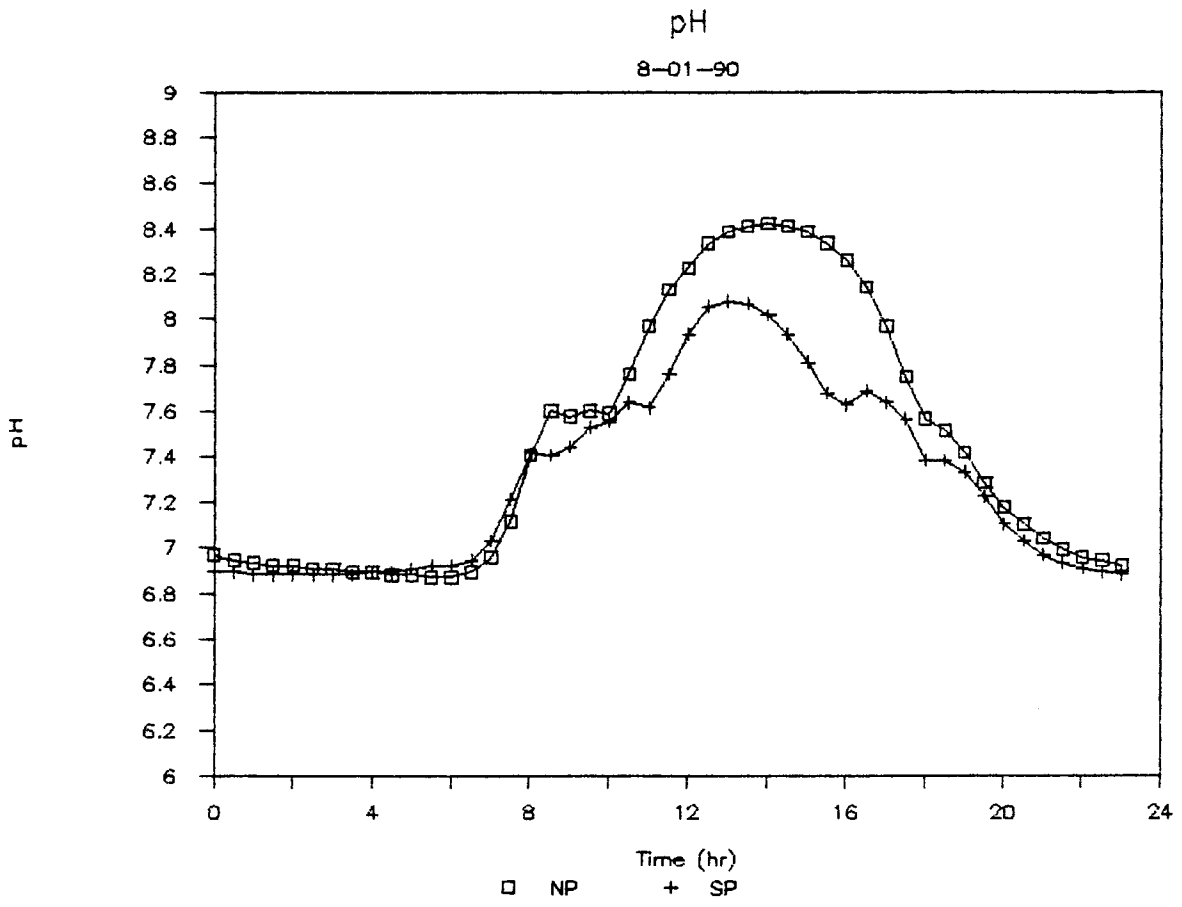
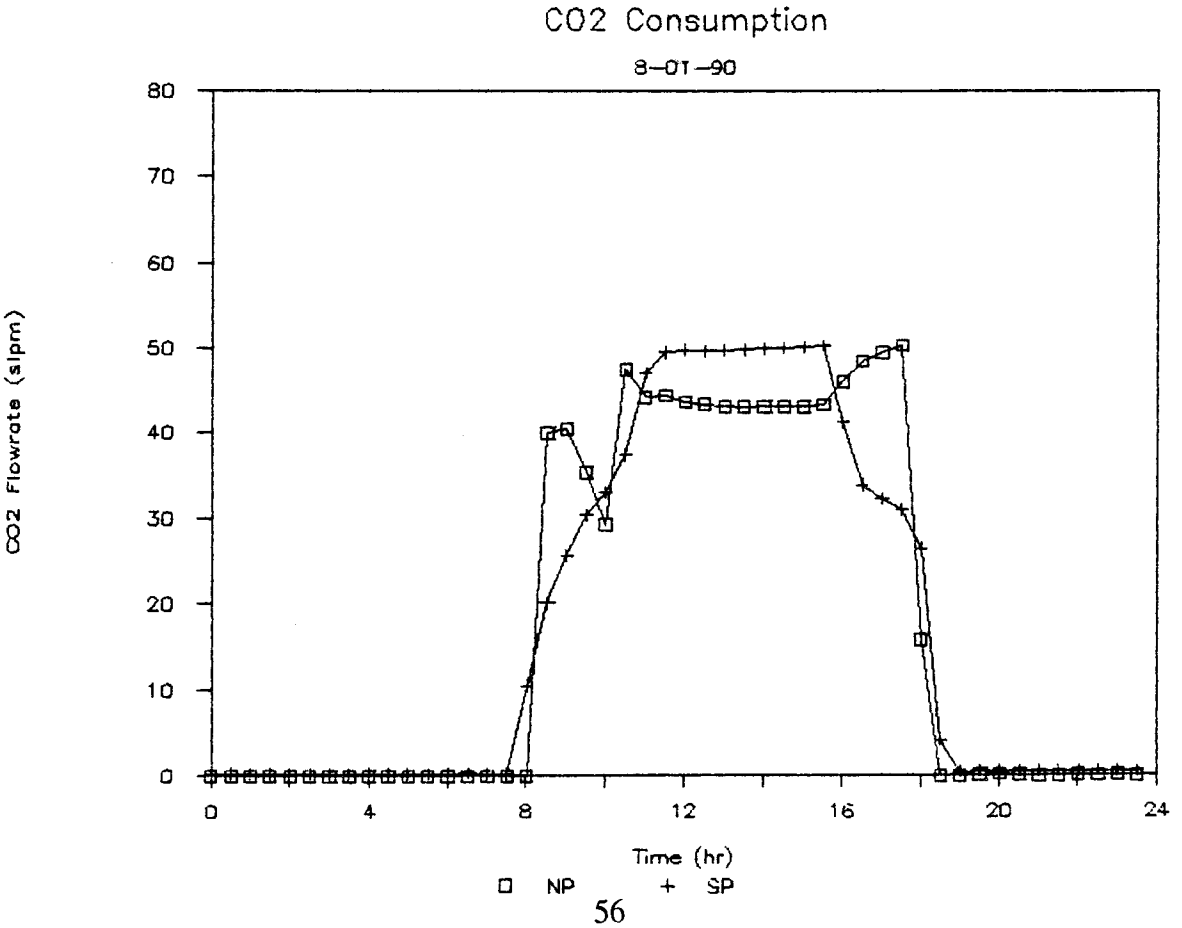

CO2 UTILIZATION EFF., %
100
90
80
70
60
50
40
30
20
10
0
A S O N D J F M A M J J A S O N D J F M A M J J A S O

Figure 6. Computer-acquired data from the 0.1-ha raceways: 8-1-90. A. insolation; B. pond and ambient temperatures; C. pH; D. CO_2 consumption


Insolation
8-01-90
Insolation (Watts/m2)
(Thousands)
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
4
8
12
16
20
24
Time (hr)


Pond and Air Temperatures
8-01-90
Temperature (C)
45
40
35
30
25
20
15
10
5
0
0
4
8
12
16
20
24
Time (hr)
AIR
NP
SP


pH
8-01-90
9
8.8
8.6
8.4
8.2
8
7.8
7.6
7.4
7.2
7
6.8
6.6
6.4
6.2
6
pH
0
4
8
12
16
20
24
Time (hr)
NP
SP


CO2 Consumption
8-01-90
80
70
60
50
40
30
20
10
0
CO2 Flowrate (slpm)
0
4
8
12
16
20
24
Time (hr)
NP
SP

GENETIC ENGINEERING OF MICROALGAE

FOR FUEL PRODUCTION

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INTRODUCTION

Recombinant DNA technologies have been applied successfully in prokaryotes, fungi, and higher plants. Minimal work has been done with the microalgae, which are potentially capable of producing a wide range of products and have broad ecological importance. These reasons alone are sufficient for studying their molecular biology. The specific interest in this project is in increasing accumulation of lipids by microalgae to achieve higher lipid contents in outdoor mass culture. Our long-range objective is to produce alternative fuels from these lipids. The tools of modern molecular biology are being applied to this work in order to isolate some of the key genes that regulate lipid biosynthesis and to find ways to introduce genetic material into the microalgae and have these genes expressed. These methods, once developed, will enable the further development of microalgal biotechnology as a whole.

The key components that require development are methods to introduce genes into microalgae and identification and cloning of key genes in photosynthate partitioning (the flow of carbon into lipid versus other cellular components; Figure 1).

Genetic transformation of microalgae

Transformation of microalgae requires overcoming at least three general problems: introduction of DNA into the cells, monitoring of gene entry and expression, and stabilization of the foreign DNA.

In order to have exogenous DNA expressed in microalgae, it is necessary to find ways of introducing recombinant molecules into the cell. A key issue here is finding some way to penetrate the cell covering or cell wall by protoplast production, electroporation or other methods. Several of these approaches for the introduction of genetic material into industrially important microalgae are being pursued. This report deals with protoplast production and electroporation.

The next step in the transformation process is the short-term expression of a gene introduced into the cell. This paper reports success with the luciferase gene from the firefly. This has proven to be an effective marker gene for transformation in microalgae.

Short-term gene expression with luciferase is a valid initial approach; however, it is necessary to learn more about DNA composition in microalgae and ultimately to have some marker genes stably expressed. To this end a DNA composition study is reported here along with work on the development of a homologous selectable genetic marker based on a component of the uracil biosynthetic pathway.

Genes involved in photosynthate partitioning

Ultimately, genes that affect lipid accumulation will be substituted for the stably expressed marker genes. We are focussing our work on two genes that code for the enzymes acetyl-CoA carboxylase (ACC) and nitrate reductase (NR). The biochemistry of both of these enzymes has been studied, and gene probes for cloning the genes are in final stages of development.

I. GENETIC TRANSFORMATION OF MICROALGAE

A number of algal strains in the NREL Microalgae Culture Collection have been identified that can be induced to accumulate lipid under nutrient stress and that show potential for growth in mass culture. The

goal of the genetics research program is to be able to regulate lipid accumulation and growth characteristics of the algae to establish conditions for economically feasible fuel production. In order to utilize recombinant DNA technologies to manipulate algal biosynthetic pathways, we first need to establish a genetic transformation system for the algae. This requires that methods be devised to transfer DNA into the cell and achieve expression of an introduced gene. We describe here four sets of experiments aimed at achieving these goals. First, the firefly luciferase transient assay has been applied successfully to the green alga *Chlorella ellipsoidea*. Second, electroporation has been explored as a DNA transfer methodology in yeast, as a control, and in *Cyclotella cryptica*. Third, analysis of microalgal DNA has been carried out that will be useful as an indirect measure of heterologous gene compatibility. Finally, progress has been made in the development of a homologous selectable marker system for *Monoraphidium*. These experiments represent significant progress toward the genetic transformation of microalgae.

A. Transient Expression of Luciferase in *Chlorella ellipsoidea*

Introduction of DNA

A number of techniques are available for introducing DNA into plant cells. These include direct transfer techniques such as protoplasting, electroporation, and particle bombardment, and *Agrobacterium*-mediated methods for higher plants (see Davey et al. 1989 for review). Recently, successful transformation has been achieved in *Chlamydomonas* using particle bombardment (Debuchy et al. 1989; Kindle et al. 1989; Mayfield and Kindle 1990) or by agitation of cell-wall-deficient cells in the presence of DNA and glass beads (Kindle 1990). The procedure of protoplasting involves enzymatic removal of the cell wall, after which the cells are generally treated with polyethylene glycol (PEG) and/or calcium to aid in DNA uptake by the cells. Methods have been developed previously for protoplasting green algae of the genus *Chlorella* (Braun and Aach 1975; Berliner 1977; Aach et al. 1978; Yamada and Sakaguchi 1981). We have successfully adapted one such protocol (Gobel and Aach 1985) for the formation of protoplasts from *Chlorella ellipsoidea* (strain CCAP 211/1a; Culture Collection of Algae and Protozoa, Freshwater Biological Association, UK). The cells are grown in YEG (1% yeast extract, 1% glucose) to early stationary phase (approximately 2 x 10^7 cells/ml), then incubated overnight in 10 mg/ml Cellulysin, a crude preparation of cellulase. Protoplasts were defined as cells that became disrupted when suspended in water and sonicated. The fraction disrupted, when compared to untreated cells, was greater than 80% in all experiments. The protoplasts were suspended in a small volume of 0.8 M NaCl/0.05 M $CaCl_2$, and aliquoted for transformation.

Monitoring of gene expression

The difficulty in developing a transformation system is that four problems must be overcome simultaneously: DNA must be transferred into the cell, a marker gene must be expressed, the DNA must be replicated, and the transformed cell must then recover and proliferate. By using a transient assay, the first two steps can be optimized while the last two steps are disregarded. The only requirements are that DNA be taken up by the cell and that a reporter gene be expressed at a detectable level. Reporter genes used in transient assays usually code for enzymes detectable by simple biochemical assays. A number of these genes have been used successfully in plant systems, including chloramphenicol acetyl transferase, β-glucuronidase, neomycin phosphotransferase, and firefly luciferase (reviewed in Weising et al. 1988). We have chosen luciferase because of the assay's high sensitivity and because luciferase gene constructs have been expressed successfully in a variety of cell types (De Wet et al. 1985, 1987; Wood and DeLuca 1987), including plants (Ow et al. 1986).

The plasmid containing the luciferase gene under control of the CaMV 35S promoter (pDO432; Figure 2) was generously provided by Dr. David Ow. In the presence of ATP, O_2, and Mg^{2+}, luciferase catalyzes the oxidation of luciferin with the concurrent release of a photon of light (Figure 3). Luciferase can thus be detected in crude extracts from transformed cells by monitoring light production in a scintillation counter or luminometer.

Transformation of *Chlorella* was accomplished by briefly incubating together protoplasts and DNA (pDO432 plasmid DNA plus calf thymus DNA as carrier), then adding polyethylene glycol and finally diluting the cells into growth medium supplemented with PEG to allow regeneration. The protoplasts were incubated overnight with gentle swirling under illumination. A crude cell extract made by grinding the cells with glass beads was assayed for luciferase activity.

Transient expression of luciferase in *Chlorella*

The results of two experiments are summarized in Figure 4. When carrier DNA alone was added to the protoplasts ("No Plasmid"), no luciferase activity was detected in the cell extracts (Figure 4A). However, significant luciferase activity was seen in protoplasts exposed to 4 µg or 10 µg of pDO432. The actual number of photons counted was about three times the background counted in the absence of extract (data not shown), and duplicate protoplast treatments gave similar amounts of activity.

Figure 4A also demonstrates the significance of various steps in the protocol. When the cells were not treated with Cellulysin, no signal was detected. Leaving out the carrier DNA when plasmid was added greatly reduced the final activity, and omitting the PEG step led to complete loss of detectable luciferase expression. These experiments suggest that degradation of the cell wall and the addition of PEG are necessary for getting plasmid into the cell. The role of carrier DNA may be to saturate nucleases or to block nonspecific DNA binding sites. Finally, azide, an inhibitor of oxidative phosphorylation, drastically reduced expression when present at 0.1% (w/v) from the time of plasmid addition through cell harvesting. This indicates that cell viability (metabolic energy) is a requirement for expression and eliminates the possibility of in vitro expression artifacts.

In looking for stable transformation, plasmid linearization can aid in plasmid integration into the genome (e.g., Blowers et al. 1989). In a transient assay such as this, one might expect that linearization could affect the accessibility of the DNA to nucleases or RNA polymerases, which might cause an increase or decrease, respectively, in the observed level of expression. The experiment in Figure 4B compares untreated (largely supercoiled) plasmid, as used above, to plasmid linearized by digestion with various restriction enzymes. The enzymes *Aat*II and *Nde*I each cut pDO432 in the pUC19 region (Figure 2), leaving the luciferase gene and its promoter intact. Linearization with these enzymes did not significantly affect the level of expression observed. As a control, linearization of the plasmid with *Xba*I, which separates the luciferase gene from the 35S promoter, resulted in almost complete loss of activity. This demonstrates that it is the luciferase gene that is acting as the substrate for expression, as opposed to, for example, trace amounts of luciferase transcripts made in *E. coli* that could be present in the plasmid preparation. Thus, an intact luciferase gene and promoter are essential, but the conformation of the plasmid appears to be relatively unimportant in these assays.

The kinetics of luciferase production in *C. ellipsoidea* protoplasts are shown in Figure 5. The data show that expression was readily detectable after 7.5 h, and reached a maximum somewhere between 7.5 and 52 h with the highest observed activity at 24 h. The activity, expressed as relative light units (RLU per µg protein, diminished to near zero after 4 days. Much of this decrease was due to increased protein in the extracts at later times rather than loss of total activity (data not shown); i.e., the luciferase was

apparently being diluted out during cell proliferation. We cannot distinguish whether the observed activity was the result of continuous low level expression and degradation or caused by an initial burst of production with the enzyme being stable in the cell. In any case, the expression was transient in that the activity was clearly not maintained.

The results presented here show that we have accomplished the first steps in the development of a transformation system for *Chlorella ellipsoidea*. Viable protoplasts can be produced in *Chlorella* by digesting the cells with Cellulysin. The *Chlorella* protoplasts can be induced to take up exogenously added DNA and to express a heterologous (i.e., a nonalgal) gene. This was a significant accomplishment, as a homologous gene was required to achieve transformation in another green alga *Chlamydomonas* (Dubuchy et al. 1989; Kindle et al. 1989; Mayfield and Kindle 1990), and doubts have been expressed as to whether algae could recognize heterologous genes. Further experiments will be directed towards increasing the sensitivity of the assay, developing a sensitive selectable genetic marker, stabilizing the foreign DNA within the cell, and promoting recovery and proliferation of stable transformants.

B. Electroporation of Yeast and *Cyclotella cryptica*

Electroporation is a technique in which transient pores can be induced in cellular membranes by the application of an electric pulse. Electroporation has been used extensively to introduce molecules (DNA, protein) into animal cells and plant protoplasts. More recently it has been shown that very short, high-voltage pulses will induce DNA uptake by walled cells (i.e., bacteria, yeast, plant suspension cells) without prior removal of the wall (Hashimoto et al. 1985; Lindsay and Jones 1987; Dower et al. 1988; Becker and Guarente 1990). Although the protoplasting protocol described above worked well for one strain of *Chlorella ellipsoidea*, preliminary experiments indicate it may not be applicable for other species of green algae or even for other strains of *Chlorella ellipsoidea* because of differences in the composition of the algal cell walls. Based on the reported success in yeast transformation by electroporation (Becker and Guarente 1990), we are exploring this method as a means to bypass protoplast production and introduce DNA directly into intact green algae and diatoms.

Transformation of yeast

Yeasts are single-celled, walled organisms of a similar size, or somewhat smaller, than most microalgae. We are using the yeast *Saccharomyces cerevisiae* as a model system to define the important parameters for transformation via electroporation; this organism is easily transformable by standard techniques and a number of selectable genetic markers are available on plasmid vectors that are stably replicated and expressed independently in yeast. Using the electroporation protocol described below, yeast deficient in uracil production were tranformed to uracil prototrophy via introduction of the yeast URA3 gene. The transformation efficiencies obtained (up to 1 to 2 x 10^5 per µg DNA) are comparable to those obtained with enzymatically produced yeast spheroplasts.

The transformation protocol used, adapted from Becker and Guarente (1990), involves washing exponentially growing cells twice in distilled water, followed by a wash in 1 M sorbitol and electroporation in 1 M sorbitol using field strengths of 5.0 to 7.5 kV per cm. We found that washing and electroporating the cells in water alone or in 0.4 M sorbitol increased the percentage of cell death at low voltages and decreased the transformation efficiency by up to two orders of magnitude. Washing and electroporating in 1 M sorbitol produced a three to five fold decrease in transformation efficiency but did not affect cell survival. These results suggest that for yeast, osmotic shock and electroporation in a high osmoticum is essential to produce a high level of transformation-competent cells (possibly due to

shrinkage of the membranes from the cell wall) and increases cell survivability following electroporation (the high osmoticum could prevent the leakage of cell components through electroporetically-induced pores).

It has been reported for bacteria and yeast that the electroporation conditions that result in 40% to 60% cell death also result in the highest transformation efficiencies (Hashimoto et al. 1985; Dower et al. 1988). We also found this to be true. The graph in Figure 6 shows cell viability and transformation efficiency for yeast cells electroporated under increasing field strengths. Maximum transformation efficiency was observed at field strengths between 5.0 and 7.5 kV per cm, resulting in 40% to 70% cell death.

Electroporation of *Cyclotella cryptica*

We are currently trying to adapt this transformation system to the diatom *Cyclotella cryptica* (CYCLO1). We have used an electroporation protocol adapted from *S. cerevisiae* to generate a kill curve for CYCLO1 under increasing field strengths (Figure 7). Note that the voltage required to kill 50% of the diatom cells (2.0 to 3.0 kV/cm) is about half that required for yeast. This is consistent with electroporation theory that states that the field strength that induces a voltage drop across a cell sufficient to induce membrane permeabilization is inversely proportional to cell size (Shigekawa and Dower 1988). The fact that CYCLO1 cells die upon exposure to increasingly strong electric pulses indicates that electroporation is in fact perturbing the algal cell membrane, possibly by forming transient pores, and that the protein/carbohydrate/silica wall is not protecting the cell from damage.

Selectable genetic marker for CYCLO1

The levels of luciferase expression detectable in the transient assay system described previously are at the borderline of detectability for our equipment. Thus this assay is probably not sensitive enough to detect rare transformation events or low levels of gene expression. An alternative approach is to use a selectable genetic marker to identify single cell transformants. This approach requires that the foreign DNA become stabilized within the cell by integration into the cell genome and that the transformed cells recover and proliferate to form detectable colonies. While this would be a very rare event, it is relatively easy to screen large numbers of cells on selection plates. A widely used marker in eukaryotic systems is the neomycin phosphotransferase gene (NPTII) which codes for resistance to the antibiotics kanamycin/neomycin/G418. Preliminary tests of a number of strains in the NREL Culture Collection indicated they are sensitive to low levels of G418 (Brown et. al. 1989, unpublished). CYCLO1 is sensitive to G418 concentrations as low as 10 µg/ml; we have seen no spontaneously resistant colonies in $>5.3 \times 10^7$ cells screened on plates containing 20 µg/ml G418. Several attempts were made to introduce the NPTII gene into CYCLO1 via electroporation. No resistant colonies were seen after screening more than 4×10^8 colonies.

There are a number of possible reasons why we have been unable thus far to obtain transformants of CYCLO1. It is likely that algal cell walls may possess biochemical characteristics not found in bacterial or yeast cell walls that promote binding of DNA or otherwise prevent passage of plasmid DNA through the wall to the electrophoretically-induced pores in the cell membrane. Although there are no protocols available to produce protoplasts of diatoms, we have identified two commercially available cell wall degrading enzymes, Driselase (Sigma Chemical Co.) and Novozym 234 (Novo Biolabs) which appear to partially degrade the CYCLO1 cell wall. It is possible that enzymatically treating the cells prior to electroporation could facilitate DNA uptake (Powell et al. 1988). Another possibility is that DNA is getting into the cells but the cells cannot express or stabilize the foreign DNA. To address this problem, we plan to devise a selectable marker system using complementation of mutants with homologous DNA; this project will be discussed in detail below.

C. Analysis of the DNA Composition of Microalgae

The success of any genetic engineering project requires knowledge about the DNA composition of the organism of interest. DNA composition in this context refers to the nucleoside bases present; this encompasses the deoxyguanosine + deoxycytosine/deoxyadenosine + deoxythymidine (GC/AT) ratio of the DNA and the presence of modified bases. An unusual DNA structure could have several major implications in the genetic engineering of microalgae:

1.) Transformation requires that a gene or genes transferred into the organism be functional, i.e., the gene must be properly transcribed and translated into a protein product. An atypical base composition of the DNA, particularly in terms of the ratio of G and C to A and T nucleosides (the GC content), might be indicative of an atypical codon bias in the coding regions of the genes. This means that transforming a cell with heterologous DNA, for example putting a bacterial antibiotic resistance gene into algae, may not work because the unusual codon bias would cause the gene to be inefficiently expressed. This appears to be the case in the best studied alga, *Chlamydomonas reinhardtii*. *Chlamydomonas* DNA has a high GC content, which is reflected in an unusual codon bias (Campbell and Gowri 1990). This is probably the reason that initial attempts to transform this alga with heterologous genes were at best unreliable (Rochaix and van Dillewijn 1982; Hasnain et al. 1985); only after the advent of homologous systems were efficient and repeatable transformation protocols developed (Debuchy et al. 1989; Kindle et al. 1989; Mayfield and Kindle 1990).

2.) Analysis of putative transformants and identification of genes of interest in algae involve probing DNA with specific sequences (Southern 1975). Unusual DNA composition can affect this in two ways. First, the blotting technique requires that the DNA be cut with restriction enzymes so that fragments can be separated by gel electrophoresis. DNA modifications can block the action of many of these enzymes, preventing proper digestion. Secondly, the efficiency with which a DNA probe recognizes homologous DNA in the blotting technique can be affected by base composition. For example, a synthetic probe whose sequence is based on protein data should be designed with codon bias in mind. This is also true when designing primers for polymerase chain reaction (PCR), a technique that has great potential in the isolation of algal genes (see below).

3.) Cloning of algal genes entails insertion of algal DNA into plasmid or phage vectors, which are then replicated in *E. coli*. This bacterium will not accept modified DNA of some types readily. For example, this is a problem in plant systems; plant DNA contains relatively high levels of the modified base 5-methyl deoxycytosine and is difficult to clone into standard lab *E. coli* strains. However, altered strains are available that will accept such modified DNA (Graham et al. 1990).

4.) DNA composition is also of fundamental scientific interest. Knowledge of the base composition of a number of organisms gives information about their evolutionary relatedness. In addition, base modification may play a role in gene regulation and has been implicated in systems that defend against invasion by foreign DNA (for reviews, see Selker 1990; Lewin 1983).

A knowledge of the DNA composition of microalgal strains is clearly very important. For this reason, we have isolated DNA from eight strains in the NREL Microalgae Culture Collection that have potential for outdoor mass culture and fuel production, plus three control microalgal strains. The DNA from these strains were analyzed for nucleoside base composition by high performance liquid chromatography (HPLC).

DNA was obtained from the following organisms: the bacterium *Escherichia coli*; calf thymus (obtained from Sigma Chemical Co., St. Louis, MO); the green algae *Chlorella ellipsoidea*, *Chlamydomonas reinhardtii*, *Monoraphidium* sp. (MONOR2), *Stichococcus* sp. (STICH1) and *Tetraselmis suecica* (TETRA1); the diatoms *Navicula saporophila* (NAVIC1), *Nitzschia pusilla* (NITZS12), *Phaeodactylum tricornutum* (PHAEO646, obtained from the University of Texas Culture Collection), and two strains of *Cyclotella cryptica* (CYCLO1 and CYCLOT-13L). As a control, DNA was also obtained from the dinoflagellate *Crypthecodinium cohnii* (University of Texas Culture Collection; this organism has been shown previously to contain significant amounts of 5-hydroxymethyl deoxyuridine (hm^5dU).

A protocol was developed, by adaptation of a yeast nucleic acid isolation procedure (Hoffman and Winston 1987), that allows for the isolation of total DNA from any algal species tested to date. Cells were disrupted by vigorous agitation in the presence of glass beads, detergents, and phenol/chloroform. DNA was further purified by ethanol precipitation, RNase digestion, more phenol/chloroform and ethanol treatments, and in some cases, ethidium bromide-cesium chloride density gradient ultracentrifugation. Analysis of nucleoside base composition of the DNA was done at the Cancer Research Center of the University of Missouri in Columbia. DNA samples were digested with a mixture of enzymes into their monomeric nucleoside subunits, which were then resolved using reversed-phase HPLC. Peaks were identified by comparison to known standards for both retention time and UV spectra.

The combined results of two separate analyses are shown in Table 1. Seven nucleosides were detected in the samples: the standard DNA subunits deoxyguanosine (dG), deoxycytidine (dC), deoxyadenosine (dA) and deoxythymidine (dT); and three more rare bases: 5-methyl deoxycytidine (m^5dC), 6-methyl deoxyadenosine (m^6dA), and 5-hydroxymethyl deoxyuridine (hm^5dU). The mole percentage of each of these is indicated for the three nonalgal control DNA samples and the eleven microalgal DNA samples. Four samples were analyzed in duplicate (Table 1); for the three algal samples, CYCLO1, MONOR2 and STICH1, the second sample listed was purified by ethidium bromide-cesium chloride density gradient ultracentrifugation. Note that duplicate samples all showed very similar compositions. There is also a close agreement between the two different strains of *Cyclotella cryptica*, CYCLO1 and CYCLO T13L.

Modified nucleosides are present in all of the microalgal DNA samples. m^6dA was detectable only in STICH1 and TETRA1 and was not a major component of either. m^5dC, however, was present in all of the samples and was observed to be a major component in some cases. The most striking is MONOR2, in which about one third of all the cytosine residues appear to be methylated. Cytosine methylation is common in plant cells, though generally not to such an extent (Shapiro 1970; Gruenbaum et al. 1981). Finally, hm^5dU may be present as a minor component in some of the NREL microalgae. It was shown initially to be a major component of the DNA of NITZS12 and PHAEO646 (data not shown); however, subsequent analysis did not confirm the presence of this base (Table 1), and these results are thought to be more reliable because the DNA was more highly purified and the resultant nucleoside ratios were more consistent with the laws of base-pairing. The modified base hm^5dU is highly unusual. However, it has been reported in the DNA of a few bacteriophage (Kallen et al. 1962) and some dinoflagellates (Rae 1976). *Crypthecodinium cohnii*, one such dinoflagellate, has been shown by other methods to contain about 11% hm5dU (Rae 1976); this is in close agreement with the data presented here, in which *C. cohnii* was included as a control.

The second interesting feature of the data presented in Table 1 is the GC content ("%GC"), which is calculated as the percentage of the total nucleosides that are either dG, dC, or m^5dC. This is an intrinsic value for a particular organism that essentially changes only on an evolutionary time scale. A typical GC content for a eukaryotic cell is in the range of 35% to 50% (Lehninger 1975); such a value was observed for calf thymus DNA in Table 1. The diatoms (CYCLO1, CYCLO T13L, NAVIC1, NITZS12, and PHAEO646) all have values in this range. The green algae have significantly higher values with the

exception of STICH1. The most striking is again MONOR2, with a GC content of approximately 71%. Reports of GC contents over 70% have been restricted to a few unusual bacteria and viruses (reviewed in Adams et al. 1986).

It appears, then, that many of the NREL microalgal strains with potential for outdoor mass culture have quite unusual DNA compositions. The abundance of m^5dC in some of the samples is particularly notable and could reflect interesting strategies for gene regulation or defense. Several unusually high GC contents were observed as well. This parameter may be an indicator of the evolutionary relatedness of the microalgae. For example, the green algae all have elevated GC contents, with the exception of *Stichococcus* (STICH1), which is thought to belong to the branch of algae that led to higher plants (Lee 1980).

These data are also of practical importance and will be of considerable use in the genetic engineering of microalgae. Previous work assessing the ability of various restriction enzymes to digest algal DNA showed that many of these enzymes cut well, while others did not cut at all. These results can be explained fairly well based on the HPLC data. In particular, the presence of m^5dC will inhibit many restriction enzymes (reviewed in Kessler and Holtke 1986). In addition, the high GC content of MONOR2 means that enzymes that cut at AT-rich sequences may cut so infrequently that the cutting is undetectable by standard agarose gel electrophoresis. Future work in the analysis (Southern blotting) of algal genes will require that enzymes be chosen that are not affected by these properties of the DNA; the DNA composition data will allow a priori prediction of which enzymes will digest satisfactorily. The presence of m^5dC in algal genes also implies that cloning into *E. coli* will require the use of special host strains that will tolerate this modification (Blumenthal 1989). The design of probes for blot hybridization and PCR will also be affected by the microalgal GC content data. Finally, the unusual base composition of some of the algae will influence the strategies used in transformation, as will be discussed below.

D. Homologous Markers for Genetic Transformation

Of the microalgal strains discussed above, MONOR2 is one of the most promising for outdoor mass culture and fuel production, yet at the same time it is one of the most unusual in terms of DNA composition. Its GC content and fraction of C residues that are methylated are higher than any other organism tested. Thus, development of a transformation strategy for *Monoraphidium* will require that these differences be taken into account. Based on work in *Chlamydomonas*, in which we found a GC content of only 62%, we expect that *Monoraphidium* may show an even greater bias in its codon usage. As a consequence, heterologous gene expression in this alga may be difficult, if not impossible. We are therefore exploring homologous selectable markers for the transformation of *Monoraphidium*.

A general scheme for transformation using a homologous gene is shown in Figure 8. The organism of interest is mutated in a specific gene; the mutants are characterized and cultured under non-selective conditions. The wild-type gene is obtained from the same organism, characterized, and inserted into a plasmid vector. The DNA is then introduced into the mutant cells by any of a number of techniques (see above). The DNA must then be stabilized either by integration into the chromosome or self-replication of the plasmid. Transformants can be selected by growing the cells under selective conditions so that only those cells that have received the wild-type gene will replicate. Once stabilized, the inserted gene will almost surely complement the mutation because it has the organism's own codon usage, promoter sequences, and translational start and termination signals.

In selecting a gene for such a system, several factors should be considered: (1) The gene needs to be essential under certain well-defined, easily attainable growth conditions. This allows one to screen for

mutants and select for transformants. (2) There should be a positive selection for mutations in the gene. (3) There should be an available enzymatic assay for the product of the gene so that putative mutants can be characterized. (4) For a variety of reasons, it is useful to choose a gene that has been well characterized in other organisms; in particular, this may allow the gene to be isolated by techniques that rely on conserved sequences (e.g., PCR). (5) The gene should be relatively small to facilitate cloning and reintroduction during transformation.

One gene that satisfies these criteria is the gene encoding orotidine-5'-phosphate decarboxylase (OPDase). This enzyme is essential for pyrimidine biosynthesis (Figure 9). OPD mutants will grow only if provided with an alternate source of pyrimidines, such as uracil. Thus, OPD mutants are uracil auxotrophs. A positive selection exists for OPD mutants; the drug 5-fluoroortic acid (FOA) is converted into a toxic compound by OPDase (Boeke et al. 1984). OPD mutants, however, are not killed by the drug. In addition, a relatively simple spectrophotometric assay can be used to measure OPDase activity in cell extracts (Donovan and Kushner 1983). The gene has been cloned and sequenced from a number of organisms, including *E. coli* (*pyr*F; Turnbough et al. 1987), mouse (Ohmstede et al. 1986), the yeast *Saccharomyces cerevisiae* (*URA*3; Rose et al. 1984), and *Neurospora crassa* (Newbury et al. 1986). Extensive sequence conservation is observed between species (Turnbough et al. 1987). The gene is relatively small; in *S. cerevisiae*, a 1170 base pair DNA segment contains all the information required for expression (Rose et al. 1984). Thus, the OPD gene meets the requirements for a good homologous selectable marker, and has been used successfully in a number of transformation systems (e.g., van Hartingsveldt et al. 1987; Boy-Marcotte et al. 1984; Buxton and Radford 1983). There is no reason to assume that this system would not be an ideal choice for the transformation of microalgae such as *Monoraphidium*.

Figure 10 outlines the approach that has been applied to *Monoraphidium* for the isolation of OPD mutants. Cells in active liquid culture (10% artificial seawater medium, ASW) were mutagenized with UV light for varying lengths of time and were grown in the presence of uracil. This growth period allowed OPD mutant cells to dilute out their OPDase enzyme. The cells were then spread onto plates containing uracil (80 µg/ml) and FOA at 0.6 mg/ml, a level that is toxic to wild-type cells. After a period of several weeks, colonies appeared on many of the plates. The number of colonies was proportional to the dose of UV light. The colonies were then grown on 10% ASW supplemented with uracil and replica plated onto medium lacking uracil. A small percentage of the isolates was unable to grow in the absence of uracil. These uracil auxotrophs are potential OPD mutants.

One of these mutants has been characterized more extensively. This isolate, "3180a-1," shows near wild-type growth on 10% ASW containing 80 µg/ml uracil, either in liquid culture or on plates, but shows no growth in the absence of uracil. The reversion rate for this marker was found to be approximately 10^{-8}; such a frequency is compatible with typical transformation efficiencies for other organisms. Work is in progress to determine whether this mutant has lost its OPDase activity.

The next step in the development of this system is to attempt to isolate the wild-type *Monoraphidium* OPD gene. It may be possible to clone the gene simply by complementation of the yeast or *E. coli* marker, though an initial attempt with *E. coli* has failed. As an alternative, the extensive homology between the known OPD genes can be used, taking into account the high GC content of MONOR2 determined in the DNA analysis (Table 1), to design primers for gene cloning by PCR. This technique will be discussed in detail below for the cloning of the nitrate reductase gene. Successful cloning of the OPD gene, in combination with the putative OPD mutants described above, should prove an excellent system for the transformation of *Monoraphidium*.

II. GENES INVOLVED IN PHOTOSYNTHATE PARTITIONING

The carbon fixed by photosynthetic organisms is generally incorporated into carbohydrate, lipid, or cellular protein. The motive forces that regulate partitioning of the incoming carbon are not well understood but are related to the energy status of the cell, as well as to its nutritional needs. The processes of nitrogen assimilation, photosynthetic electron transport, and CO_2 fixation are interwoven and act to limit plant growth. Reducing equivalents generated from photosynthetic electron transport are required for nitrate reduction. Nitrogen availability not only restricts the photosynthetic efficiency of plants but it also appears to regulate photosynthate utilization. Nitrogen-deficient plants tend to accumulate products such as starch, lipid, and carotenoids. The mode of regulation of these interrelating effects is not understood. The complexity of the interrelationship between nitrogen nutrition and photosynthate partitioning is further illustrated by studies in which cells starved of carbohydrates lose their normal capacity to control nitrate assimilation (Vennesland and Guerrero 1979). These observations support the idea of a common regulatory element involved in control of nitrate assimilation and photosynthate partitioning.

Recent interest in the use of microalgae for the mass production of liquid oil fuels has resulted in an increased research interest in algal lipid production and the means of increasing algal lipid content. Nitrogen deficiency has been shown to induce lipid accumulation in several diverse algal groups that include members of the Chlorophyceae, Bacillariophyceae, and the Rhodophyceae (Vennesland and Guerrero 1979; Ben-Amotz et al. 1985; Saux et al. 1987; Evans and Terashima 1987; Terashima and Evans 1988; Roessler 1988; Plumley and Schmidt 1989). Because there appears to be a direct relationship between nitrogen metabolism and lipid production in photosynthetic cells, we propose to identify and characterize the genes controlling the rate-limiting (and presumably regulated) enzymes of both these processes (nitrate reductase in the case of nitrogen assimilation and acetyl-CoA carboxylase in the case of lipid biosynthesis) in an attempt to identify common genetic regulatory elements. This information will be valuable for future research directed towards the genetic engineering of microalgal cells in order to alter lipid production. A model for the regulation of photosynthate partitioning with regard to the processes of nitrogen assimilation and lipid biosynthesis, based primarily on information available for fungal systems, has been developed and is illustrated in Figure 11.

A. Nitrate Reductase

Introduction

Biologically useful (organic) nitrogen in the biosphere is converted from inorganic nitrogen either by the "fixation" of molecular nitrogen (N_2) or the "assimilation" of nitrate. Nitrate assimilation produces in excess of 2×10^4 megatons of organic nitrogen per year compared to 2×10^2 megatons produced via nitrogen fixation (Guerrero et al. 1981). Thus the major route by which inorganic nitrogen is converted to an organically useful form is by nitrate assimilation. As much as 25% of the energy of photosynthesis is used to drive nitrate assimilation, further illustrating the fundamental biological importance of this process. Nitrate assimilation occurs in a wide variety of organisms including bacteria, yeast, fungi, algae, and higher plants. The organic nitrogen required by other life forms is consequently supplied by these organisms.

The rate-limiting and regulated step of nitrate assimilation appears to be catalyzed by the enzyme nitrate reductase (NR) (Beevers and Hageman 1969). NR is considered to be a limiting factor for growth and development, as well as for protein production in plants and other nitrate-assimilating organisms. Accordingly, it is readily apparent that the status of NR in the cell will have profound effects on both growth rate and photosynthate partitioning (i.e., lipid production). Because of this pivotal role, NR has

been extensively studied with regard to its catalytic efficiency and regulation. The molecular biology of nitrate reductase is currently being elucidated in higher plants while less emphasis has been placed on the algae.

The productivity rates for microalgae have been calculated to be approximately an order of magnitude greater than those of most other plants (Johnson and Sprague 1987) and thus microalgae are ideal organisms for production of large amounts of lipid (or other products) in a short time span. While microalgal lipids represent the premium energy product, the energy available in the other biomass constituents can also be used for fuel production. For example, anaerobic digestion of the cell residue after lipid extraction can be used to produce methane and carbon dioxide, which can be recycled for use in the algal production system. Alternatively, once the control points of photosynthate partitioning are understood, the cells can be genetically manipulated to produce any of a variety of desired products. Determining the role of nitrogen metabolism in photosynthate partitioning should lead to an ability to direct the cell's metabolism in favor of a desired product and will generate high interest in the private sector, facilitating technology transfer.

Cloning of the Nitrate Reductase Gene

Background

In 1985, Cheng and coworkers announced the isolation and identification of the first eukaryotic NR clone (Cheng et al. 1985) at the First International Symposium on Plant Molecular Biology in Savannah, GA. This work, detailing the isolation of a cDNA clone from barley, was published in 1986 (Cheng et al. 1986) and heralded the new era of NR molecular biology. Since that time there have been a number of NR clones isolated including cDNAs from squash (Crawford et al. 1986), tobacco (Calza et al. 1987), *Arabidopsis* (Crawford et al. 1988), and genomic clones from *Neurospora* (Fu and Marzluf 1987), tomato (Galangau et al. 1988), tobacco (Vauchert et al. 1989), rice (Hamat et al. 1989), *Arabidopsis* (Cheng et al. 1988), and a green alga, *Chlamydomonas* (Fernandez et al. 1989). The cDNAs all hybridize to mRNA that is 3.2 to 3.5 kb in length, with most being responsive to nitrate (Calza et al. 1987; Crawford et al. 1986, 1988).

Isolation of the NR Gene from a Green Alga

Recently, a cDNA clone was isolated from another green alga, *Chlorella vulgaris*, in the laboratory of L.P. Solomonson (Cannons and Solomonson 1990). mRNA was isolated from nitrate-grown cells and used as a template to synthesize cDNA, which was further enriched for NR by size selection of transcripts between 1 kb and 4.5 kb. An amplified lambda gt11 expression library was constructed composed of 10^{10} clones/µg DNA. Five hundred thousand independent clones were screened using a polyclonal antibody specific for *Chlorella* NR and a single clone, pCVNR1, was isolated. The fusion protein produced by this clone was recognized by a mixture of *Chlorella* NR monoclonal antibodies, substantiating that the clone represents the *Chlorella* NR message. The insert size of the clone is 1.2 kb and has been used to show that a message of about 3.5 kb is enriched in nitrate-grown cells. Because the 1.2 kb clone recognizes a message of about 3.5 kb (a minimum message size of at least 3 kb is required to code for the enzyme subunit of 100 kDa), it does not represent the full length message. Further evidence that pCVNR1 is an NR cDNA was obtained by sequencing and the translated sequence has nearly 80% sequence similarity with higher plant NR sequences. pCVNR1 has been subcloned into Bluescript (Stratagene) for determination of its complete sequence. It has also been used to rescreen the cDNA library in an unsuccessful attempt to isolate a full length NR cDNA (A.C. Cannons, personal communication). A new cDNA library is being constructed in order to isolate a full length clone for structural analysis. In

addition, a genomic library has been constructed in EMBL3 using Sau3A partially digested *Chlorella* genomic DNA and will be screened with the cDNA clone to isolate *Chlorella* NR genomic clones. pCVNR1 and a great deal of information and technical expertise have been made available to NREL through our continuing collaboration with Dr. Solomonson's laboratory. It can be expected that their work on the same gene in another species of green algae will prove of invaluable assistance to our cloning and analysis efforts.

Cloning NR from Microalgal Species with High Potential for Fuel Production

Determination, Culture and NR Characterization of Target Organisms: Target microalgae were chosen on the basis of several criteria including growth and environmental tolerance to conditions similar to those of the desert Southwest (Brown 1991) and availability of background information on the physiology and metabolism of target organisms. Based on these criteria, the green alga, *Monoraphidium*, and the diatom, *Cyclotella*, were chosen as the target organisms for the cloning of the nitrate reductase gene. Conditions for growth were then optimized for nitrate reductase in order to insure that the target messenger RNA would be present during construction of the cDNA libraries. It was observed that it was necessary to grow the cultures in a dynamic fashion, i.e., bubbled with gaseous CO_2, rather than in a more static fashion wherein the cultures are provided with bicarbonate as a source of carbon, and are stirred infrequently (see Figure 12). Additionally, NR activity assays performed during the optimization studies indicated that NR from the diatom may have a loosely associated flavin cofactor, rather than very tightly bound, as is observed for most of the green algae (Guerrero et al. 1981). As seen in Figure 12, activity was observed to be very low in extracts isolated in buffer lacking exogenously added flavin adenine dinucleotide (FAD) and significantly higher in extracts isolated in the presence of exogenous flavin. The activity was not very stable in crude extracts of *Cyclotella*, decreasing about 25% after 24 h at 4°C. Immunoblotting of protein extracts from target algae with a polyclonal antibody against *Chlorella* NR, shown in Figure 13, revealed cross-reacting material in extracts from the green alga, *Monoraphidium*, and a weaker signal from extracts of the diatom, *Cyclotella*, even though significant NR activity was observed in the same extracts. These results suggest that the NR protein from the green alga, *Monoraphidium*, is more antigenically related to the NR protein from the green alga, *Chlorella*, than is the NR protein from the diatom, *Cyclotella*. It may be possible to observe cross-reactivity between the *Chlorella* antibody and the *Cyclotella* NR protein with optimization of the isolation and blotting conditions (i.e., concentration of the *Cyclotella* NR protein by partial purification, etc.). The detection of the *Monoraphidium* NR protein by the *Chlorella* antibody is encouraging in that the *Chlorella* antibody and/or cDNA are likely to detect NR clones during library screening. The *Chlorella* antibody has also been shown to cross react with the NR protein of another green alga, *Chlamydomonas* (Lefebvre, personal communication).

RNA and DNA Isolation and Characterization from Target Organisms: Prior to the actual construction of cDNA and genomic libraries, it is necessary to determine the optimal conditions for the isolation and purification of the nucleic acids to ensure that the libraries will have a high likelihood of containing the sequence of interest. Library construction is relatively expensive and time consuming so optimization is required in order to minimize unsuccessful attempts. Because many algae, particularly the greens, are recalcitrant to standard methods of nucleic acid extraction due to their unique cell walls, protocol design is challenging.

Two different methods of RNA isolation were tested, initially on *Cyclotella*. The first method is a "single step" procedure that uses an acid guanidinium thiocyanate-phenol-chloroform mixture (Chomczynski and Sacchi 1987). This procedure can be accomplished in a single working day, has been successful using small amounts of starting material, and has resulted in the preparation of RNA suitable for northern analysis. It was previously tested on the green alga *Chlorella* and resulted in a good yield of RNA; however, the RNA was not translatable in a reticulocyte translation system (Zeiler, unpublished results).

RNA isolated from the same cells but by a different method (see below) was competent for translation. The isolation of RNA from the diatom *Cyclotella* by the single-step method resulted in an amount (≈ 100 µg) of total (includes ribosomal) RNA that was at or below the minimum necessary for successful recovery of polyadenylated mRNA. Because the alternative method (described below) was highly successful, this method of isolation was abandoned for the time being. However, it remains an attractive option due to the ease and speed of the procedure and may prove useful in the future in isolating RNA for analyses such as dot-blotting.

The second method of RNA isolation is derived from Bascomb and Schmidt (1987) for isolation of RNA from *Chlorella* and involves extraction with phenol of the RNA from the cells following breakage in a French Pressure Cell (SLM/Aminco) and equilibration in a Tris/LiCl/EGTA/EDTA/SDS solution. This method has previously been used to extract high-grade RNA from *Chlorella* that was used in construction of a *Chlorella* cDNA library. In short, algal cells are harvested by centrifugation and washed once with sterile media. After breakage and equilibration in the isolation buffer, the preparation is repeatedly extracted with phenol and phenol:chloroform:isoamyl alcohol. The total RNA is selectively precipitated away from the DNA by LiCl and collected by centrifugation. PolyA+ mRNA is purified by affinity chromatography on an oligo dT cellulose column. *Cyclotella* cells were prepared in this manner and the resultant mRNA was analyzed for quality by denaturing horizontal agarose gel electrophoresis and *in vitro* translation. Figure 14 shows that the translation products were observed to contain the entire molecular weight range, including very high molecular weight protein products, indicating that the preparation of mRNA would contain the messages for high molecular weight proteins such as NR. The exogenous mRNA stimulated the incorporation reaction as much as 20-fold (Table 2), indicating extremely high quality mRNA. The mRNA was also analyzed by denaturing horizontal agarose gel electrophoresis, and was observed to contain all sizes of message. *Monoraphidium* RNA was prepared by the same methodology in high yield and was similarly found to be of high quality (data not shown). The foregoing analyses indicate that RNA extraction conditions have been determined that will provide the very high quality mRNA that is required for the efficient synthesis of representative cDNA libraries.

Isolation of genomic DNA was also optimized. Conditions were determined that will allow the highest yield and will also result in clean DNA that is able to be cut by restriction enzymes for genomic cloning. It was previously observed that DNA isolated from many of the green algae is resistant to digestion by certain commonly used restriction enzymes such as *Eco* R1 (Cock et al. 1990; Cannons, personal communication; Jarvis, unpublished results; Zeiler, unpublished results). Some investigation has been made into the possibility of unusual base methylation being responsible for this observation (see DNA Composition, this article; Cock et al. 1990) and the results suggest that this, along with an unusually high GC content may be responsible. However, this does not exclude the possibility that a co-purifying contaminant may be responsible, especially as it has been observed in *Chlorella* that purification by "spooling" results in a preparation that can be digested with *Eco* R1. The same preparation was not digested by this enzyme prior to the "spooling" step (Cannons, unpublished results).

Because of the need for high-quality DNA in construction of genomic libraries and the ambiguity concerning the digestibility of the purified DNA, several methods of DNA isolation and purification were tested for *Monoraphidium*. Different methods of breaking the cells were also considered as it is important to minimize the amount of shearing that occurs during isolation. Cells were broken by passage through a French pressure cell, freezing in liquid nitrogen or grinding in a mortar and pestle with glass beads, and by vigorous shaking with glass beads using a Braun mechanical shaker. While all three methods resulted in a good yield of DNA, freezing and grinding gave the highest yield and it was possible to "spool" the DNA from samples prepared by this method. Mechanical shaking with glass beads gave an intermediate yield of DNA and it was also possible to spool DNA from these samples. An interesting observation was made in that samples prepared using glass beads seemed to preserve the integrity of RNA that was co-

isolated. The reason for this protection of the RNA is not understood but is possibly related to the presence of 1 M sucrose in the samples prepared by mechanical shaking. The *Monoraphidium* DNA prepared by these methods was not digestible with *Eco*RI, most likely due to the very high GC content (see above), however all preparations cut well with the restriction enzyme *Sau*3A and should be effective in preparation of genomic libraries.

The optimal method for the isolation and purification of DNA from either organism is derived from Rochaix (1980) and is basically as follows: Cells are broken by freezing and grinding in isolation buffer containing Tris, NaCl, and EDTA. Following breakage, the extract is incubated for 2 to 2.5 h (until the green extract from green algae turned khaki brown, or the brown extract from the diatoms turned green!) at 50°C after the addition of pronase, more EDTA, and SDS. The lysate is put on ice and two volumes of borate buffered phenol added. The DNA is then either spooled or precipitated from the aqueous phase with ethanol. The collected precipitate (or "spooling") is redissolved and reextracted with phenol, precipitated, and redissolved and then treated with RNase to remove contaminating RNA. The DNA is analyzed by horizontal agarose gel electrophoresis after restriction with various enzymes. DNA prepared in this manner appears to be of high quality and suitable for use in construction of genomic libraries. Certain samples were further analyzed by clamped homogeneous electric field (CHEF) electrophoresis (BioRad), but no increase in resolution of the DNA bands was observed.

Heterologous Probes: Heterologous probes of two cDNAs for NR (one from squash and one from the green alga, *Chlorella*) and one genomic clone (from the green alga *Chlamydomonas)* were obtained from the researchers who originally isolated them (Crawford et al. 1986; Cannons and Solomonson 1990; Fernandez et al. 1989) and subcultured. The plasmids containing the clones were purified and the inserts isolated by restriction and gel purification. The *Chlorella* and the *Chlamydomonas* probes were labeled using a nonradioactive tag (Genius - Boehringer Mannheim, Germany) based on random-primed incorporation of digoxigenin-dUTP and subsequent immunological detection. The antibody binds to digoxigenin-modified DNA and is coupled to alkaline phosphatase, which in turn catalyzes the formation of an insoluble colored product. There are a number of advantages to a nonradioactive system related to laboratory safety, minimumization of radioactive waste, and facilities to handle autoradiographic procedures. Other advantages to the system are the long-term stability of the labeled probe (up to 1 year) and the capability to reuse the hybridization solution. A common drawback to non-radioactive probes has been the lower sensitivity observed for most of the systems. The labeled inserts were used to probe a Southern blot (genomic DNA isolated from target organisms that has been restricted and separated on an agarose gel and probed with the DNA clones). As shown in Figure 15, a positive signal was observed for *Monoraphidium* and *Chlamydomonas* DNA using the *Chlorella* cDNA. These results are significant as they provide further evidence that there is enough homology between the genes from the different green algae to allow heterologous detection, and also that the nonradioactive detection system is sensitive enough to detect single-copy genes. No significant signal was observed for the *Cyclotella* DNA under these conditions. However, it is possible to alter the physical parameters of the hybridization protocol and it may be that the *Chlorella* probe will also recognize the *Cyclotella* gene under less stringent conditions. Heterologous probing will be extremely useful in cross-screening of libraries once a putative clone is isolated, as well.

The heterologous probes will also be used in northern blots (RNA isolated from the target organisms and probed with the labeled DNA clones) to determine the size of the message and will later be useful in determining changes in mRNA concentrations under different conditions.

Library Construction: A strategy for production of the cDNA libraries has been developed that utilizes the advanced technology of the polymerase chain reaction (PCR). PCR results in the exponential

amplification of a sequence of interest and utilizes a minimum of starting material. It is expected that this strategy will greatly improve the efficiency of cloning NR, as it is underrepresented in the cellular population. The procedure requires an upstream and downstream primer (from the complementary non-coding strand) that flank the sequence of interest that is to be amplified. Optimal primers should be relatively unique, contain as little secondary structure potential as possible, and should not self-anneal or anneal with each other. Sequence data from a diverse group of organisms, including higher plants, algae and fungi, were aligned using sequence analysis software and a pair of amplification primers was chosen based upon regions unique to NR sequences as a group but having a high degree of sequence similarity within themselves (Figure 16). The codon and amino acid bias was heavily weighted towards the sequence from pCVNR1 from *Chlorella*, as it is the only algal NR sequence information currently available (there is too little sequence data available from the *Chlamydomonas* genomic clone to be useful). The 5', or upstream primer is a 23-mer and the 3', or downstream primer, is a 21-mer, both having 128-fold degeneracy, as shown in Figure 17. These primers will be used to prime the synthesis of the amplified PCR product from the reverse transcribed mRNA population. The resultant product will be used to probe a full cDNA library and will be directly cloned itself. The full cDNA library is being prepared for both *Monoraphidium* and *Cyclotella* by random primer cDNA synthesis (preferred for very large messages) in the expression vector lambda gt22 (ProMega) according to the protocols specified in the cloning kit (ProMega Protocols and Applications Handbook 1989/90). Although these libraries can be screened for NR with the PCR amplified product and the heterologous probes directly, it will be necessary to probe them with the antibody for acetyl- CoA carboxylase (ACC) in order to identify the ACC clone. Expression libraries produce a fusion polypeptide between ß-galactosidase and the expressed cDNA clone that can be bound by a specific antibody to the normal gene product. In addition, a positive NR clone identified by DNA hybridization may be further established to be genuine by cross-recognition using the *Chlorella* NR antibody. Thus a cDNA expression library will have extended utility.

Genomic libraries are being prepared by partial digestion of the genomic DNA using the restriction endonuclease *Sau*3A and constructed in an EMBL3 vector. These libraries will be screened with the heterologous probes, as well as the PCR amplified product, in order to identify and isolate a genomic clone(s) for NR. Once a cDNA clone has been identified for ACC, it will be possible to screen the same libraries for an ACC genomic clone(s) as well. Both the cDNA and the genomic libraries can be used to isolate other genes of interest, as well.

Mutant Selection: Algal cell mutants that lack NR can be selected based on their resistance to chlorate. Cells having a functional NR protein (and thus, gene) will take up chlorate along with nitrate and reduce them to chlorite and nitrite, respectively. While nitrite is then immediately further reduced to ammonium by the enzyme nitrite reductase, chlorite is not reduced and builds up in the cell to toxic levels. The NR minus mutants do not reduce the chlorate and so do not experience the cytotoxic effects of chlorite. Consequently, cells can be subjected to a positive selection regime by growing target organisms in the presence of chlorate and selecting resistant colonies. NR minus mutants have been selected in this manner by a number of investigators working with a variety of species (Nichols and Syrett 1978; Mendel et al. 1986; Daboussi et al. 1989; Steven et al. 1989). NR-minus mutants made in the target organisms will be very useful in a variety of ways. Complementation of the mutant with a functional NR gene will result in cells that will be able to utilize nitrate as the sole nitrogen source, thus acting as a selectable marker for transformation. In addition, transient expression of the introduced gene can be monitored by simple enzyme assays. Complementation of the mutants will also prove that the gene isolated from a target organism's genomic library is, in fact, the gene coding for nitrate reductase. And finally, the mutants will be extremely valuable in testing in vitro mutagenized NR genes for the effect on expression of the gene.

NR mutants of *Cyclotella* and *Monoraphidium* were isolated as follows: 1 x 10^6 cells were plated onto either 10% ASW media for *Monoraphidium* or 50% ASW media for *Cyclotella* containing 1 mM urea (as alternate nitrogen source), 10 mM nitrate, and 10 mM chlorate. The cells were not mutagenized prior to plating. The cells were allowed to grow under a 16/8 h light/dark cycle for up to 4 weeks prior to transfer of resistant colonies to growth media (contains urea and no chlorate). The putative mutants were then transferred in duplicate onto media containing only nitrate as nitrogen source as well as media containing nitrate, urea, and chlorate. The cells that lack NR will not grow on nitrate alone. These cells were then isolated and are being cultured in liquid media so that biochemical analysis of the putative mutants can be carried out.

For *Cyclotella* approximately 1 in 10^6 cells was resistant to chlorate and was therefore a potential NR-minus mutant; for *Monoraphidium* the number was approximately doubled. However, it is expected that only about 1 in 10 of the mutants will actually have a lesion in the NR structural gene as other mutations can exhibit similar phenotypes (i.e., uptake mutants and cells having mutations in one of a number of molybdenum cofactor genes) so that biochemical assessment is critical to definitive proof that a suspected mutant actually does contain a lesion in the NR gene.

In summary, target organisms deemed to be of high utility for microalgal fuel production have been chosen for the cloning of genes intimately involved in lipid production. The presence of NR in cell preparations has been established along with the ability of a heterologous antibody to recognize NR proteins in the target organisms, implying common recognition sites. Optimal methods for the extraction and isolation of both RNA and DNA from the target organisms have been determined, and the nucleic acid preparations have been analyzed and found to be of sufficiently high quality for use in the preparation of cDNA and genomic libraries. Heterologous probes for NR genes have recognized the NR genes of target organisms indicating a high potential for success in using them as probes of the libraries. A strategy for cDNA library construction has been developed that uses the powerful advanced technology of the polymerase chain reaction. cDNA and genomic library construction is currently in progress. Putative mutants lacking the gene for NR have been isolated and await further biochemical analysis. The successful cloning and analysis of the genes involved in photosynthate partitioning and lipid biosynthesis should allow us to genetically manipulate these organisms such that they will produce high levels of lipid while maintaining their capacity for growth.

B. Lipid Biosynthetic Genes

Storage lipids accumulate when diatoms and other algae are grown under nutrient-deficient conditions. In order to better understand this process, we have studied the biochemistry of lipid accumulation in silicon-deficient cultures of the diatom *Cyclotella cryptica*, a species that grows well in outdoor mass culture. These studies have indicated that silicon deficiency causes an increase in the activity of acetyl-CoA carboxylase (ACC), which catalyzes one of the initial steps of fatty acid biosynthesis (Roessler 1988a,b). The activity of this enzyme controls the rate of fatty acid biosynthesis in many other organisms (including mammals), and, therefore, we have undertaken extensive study of ACC in *C. cryptica* in order to better understand its regulation. These efforts are currently focused on two primary areas: (1) biochemical characterization of the ACC enzyme, and (2) molecular biology of the ACC gene. We have also initiated research to examine the possible role of acyl carrier protein isoforms in the lipid accumulation process.

Biochemical Characterization of ACC

Regulation of ACC activity in vivo may involve allosteric modulation by various cellular metabolites (e.g., activation or feedback inhibition) and/or covalent modification of the enzyme (e.g., phosphorylation). We have examined the modulatory capabilities of a large number of cellular metabolites, and have identified several compounds that could potentially affect the activity of ACC in vivo (Table 3). We have reported previously that relatively low concentrations of palmitic acid (a fatty acid) and palmitoyl-CoA inhibit ACC activity in vitro (Roessler 1990). This inhibition may be due to the interaction of the acyl chain of palmitic acid with a hydrophobic region of ACC. It was therefore of interest to determine whether other lipid biosynthetic intermediates containing esterified fatty acids also inhibit ACC. 1,2-dipalmitoylglycerol-3-phosphate (i.e., phosphatidate) and 1,2-dipalmitoylglycerol (i.e., diacylaclycerol) at concentrations of 10 µM have no effect on ACC activity, suggesting reduced interaction of the acyl chains of these compounds with the proposed modulatory site of ACC. Surprisingly, and for reasons that are not yet clear, 10 µM 1-palmitoylglycerol-3-phosphate (i.e., lysophosphatidate) stimulated ACC actvity by 43%.

The effects of acyl carrier protein (ACP, an important cofactor of lipid biosynthesis) and certain acylated ACPs on ACC activity were also investigated. The source of the ACP used for these studies was recombinant spinach ACP-I produced in *E. coli* (Guerra et al. 1988), and was ~50% pure. Nonesterified ACP inhibited ACC activity by 47% when included at a concentration of 10 µM. However, palmitoyl-ACP, which is believed to have a more compressed structure than free ACP, stimulated ACC activity by 34%. 10 µM malonyl-ACP also stimulated ACC activity slightly, despite the fact that 20 µM free ACP was also present in the reaction mixture. The lack of inhibition by malonyl-ACP or palmitoyl-ACP indicates that ACC is not subject to feedback inhibition by these compounds.

We have also performed preliminary experiments to examine the possibility that ACC activity may be regulated by covalent modification of the enzyme. ACCs from mammalian tissue and yeast are known to be phosphorylated by endogenous protein kinases, which are enzymes that catalyze the transfer of phosphate from adenosine triphosphate to specific sites of proteins (Kim et al. 1989, Witters and Watts 1990). This phosphorylation greatly affects the activity of ACC from these sources. If this type of regulation occurs in microalgae, it must be understood before cells can be manipulated successfully to produce larger quantities of lipids. Information acquired about ACC phosphorylation would also be useful for designing site-directed mutagenesis strategies (e.g., elimination of phosphorylation sites involved in inhibition of enzyme activity). We have obtained preliminary data that suggest that preincubation of *Cyclotella* ACC with mammalian protein kinase under certain conditions reduces ACC activity in vitro. If additional experiments uphold the conclusion that ACC is regulated by phosphorylation, we will have discovered a mechanism that may be important for controlling the rate of fatty acid biosynthesis in algae. These experiments are currently in progress.

Molecular Biology of ACC

Genetic engineering of microalgae for altered ACC activity will require detailed knowledge about the structure and regulation of the ACC gene. Therefore, it is necessary to clone this gene, which is only possible if specific probes are available to identify ACC-encoding sequences. Antibodies that specifically recognize the protein of interest are commonly used to identify relevant clones in cDNA expression libraries. A major goal has now been accomplished in that we have successfully produced polyclonal antibodies against purified *Cyclotella* ACC in rabbits. Affinity purification of the antibodies by the use of agarose-bound ACC resulted in an antibody preparation that is specific for ACC. Thus, we are now in a position to screen *C. cryptica* cDNA expression libraries for the presence of ACC-encoding sequences.

Genomic or cDNA libraries can also be screened with oligonucleotides that are complementary to ACC-encoding sequences. These oligonucleotides can be designed based on partial amino acid sequences obtained from purified proteins. Unfortunately, the amino terminus of native ACC from *Cyclotella* appears to be covalently modified (a common situation with eukaryotic proteins) and thus is resistant to the Edman degradation process utilized in protein sequencing protocols. We have therefore, attempted to determine partial sequences of peptides generated from ACC by proteolytic or chemical cleavage. Difficulties have been encountered in separating peptides generated by cyanogen bromide cleavage and V-8 protease treatment, although a mixed sequence has been obtained from one fragment that will be useful to confirm the validity of certain positive clones. We are currently attempting other proteolytic procedures in order to obtain sequenceable fragments.

Characterization of Acyl Carrier Proteins

Acyl carrier protein (ACP) is an important cofactor involved in many aspects of lipid biosynthesis, including fatty acid biosynthesis, fatty acid desaturation, and acyl transfer reactions (Ohlrogge, 1987). In higher plants, two or more isoforms of ACP exist. In vitro studies have suggested that these isoforms may play a role in controlling the allocation of fatty acids into storage lipids versus structural lipids (Guerra et al. 1986). We have determined by the use of immunoblot analyses that two or three major isoforms of ACP also exist in *C. cryptica*. We have monitored the levels of these major isoforms in *C. cryptica* cells at various times after transfer of the cells to silicon-free medium and have determined that silicon deficiency does not appear to affect the relative abundance of these isoforms. These results suggest that differential isoform expression probably does not play a role in silicon deficiency-induced storage lipid accumulation.

In summary, substantial progress has been made in understanding the biochemical regulation of ACC in the diatom *Cyclotella cryptica*. We have also successfully produced polyclonal antibodies specific to ACC, which can be utilized to screen cDNA expression libraries in order to clone and characterize the ACC gene.

SUMMARY

Significant progress has been made toward the successful genetic engineering of microalgal species with high potential for fuel production. Foreign DNA has been transferred into a green alga, *Chlorella ellipsoidea*, and has been successfully expressed in this heterologous system. In addition, electroporation has shown promise as a means of introducing DNA into intact algal cells. We have analyzed the composition of DNA from several algae species and demonstrated the presence of modified bases. Elevated GC contents in several strains of green algae suggest that homologous selectable markers will be required for transformation; the development of homologous marker systems for *Monoraphidium* and for *Cyclotella* are in progress. Heterologous probes have been designed that recognize the nitrate reductase gene and will be used to isolate the gene from microalgal species of interest. We have also developed a strategy for DNA library construction which uses the advanced technology of the polymerase chain reaction. Progress has been made in the biochemical characterization of acetyl-CoA carboxylase, a key enzyme in lipid biosynthesis, and antibody and nucleotide probes have been developed for use in isolating the ACC gene. This work represents important steps toward the genetic improvement of microalgae for fuel production.

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Table 1. Base composition of DNA from representative microalgal strains. All values are in molar percentage of total. "%GC" column indicates the total percentage of dG + dC + m^5dC. nd, not detected. Cs = cesium purified DNA.

Sample	dG	dC	m^5dC	dA	m^6dA	dT	hm^5dU	%GC
E. coli (Gehrke)	25.74	25.64	nd	23.81	0.60	24.20	nd	51.4
E. coli (NREL)	25.78	26.40	0.27	23.59	0.57	23.95	nd	52.4
Calf thymus	22.29	21.02	1.49	27.60	nd	27.60	nd	44.8
C. cohnii	22.16	19.97	1.54	28.00	nd	16.36	11.97	43.7
C. ellipsoidea	25.18	24.92	1.48	24.37	nd	24.06	nd	51.6
C. reinhardtii	31.28	30.20	0.16	18.59	nd	18.49	1.28	61.6
CYCLO1	21.64	20.00	1.88	28.20	nd	28.22	0.08	43.5
CYCLO1 (Cs)	22.04	18.79	1.84	29.26	nd	27.65	0.43	42.7
CYCLO T13L	21.62	19.77	2.23	28.23	nd	28.05	nd	43.6
MONOR2	35.15	23.96	11.49	14.77	nd	14.63	nd	70.6
MONOR2 (Cs)	36.89	23.37	10.98	14.76	nd	13.84	0.15	71.2
NAVIC1	22.90	23.05	0.20	26.99	nd	26.85	nd	46.2
NITZS12 (Cs)[a]	22.58	22.20	0.77	27.09	nd	27.31	nd	45.6
PHAEO646 (Cs)[a]	23.99	24.13	0.16	25.78	nd	25.94	nd	48.3
STICH1	23.72	21.03	0.31	27.15	0.25	27.01	0.53	45.1
STICH1 (Cs)	23.03	21.20	0.30	28.11	0.29	26.64	0.43	44.5
TETRA1	29.89	24.32	3.32	21.46	0.15	20.34	0.51	57.5

[a]These data are from a second set of analyses.

Table 2. Incorporation of ^{35}S-Methionine Into *Cyclotella* mRNA *in vitro* Translated Products.

Sample	cpm[1]	fold stimulation[2]
Endogenous	9,647	1
Control	124,396	13
Cyclotella 1.0µg mRNA	169,782	18
Cyclotella 2.0µg mRNA	190,155	20

Translation reactions were carried out at 37°C for 55 min. Incorporated radiolabel was TCA precipitated onto Whatman 3 mm filter dots, washed extensively to remove unincorporated label and counted in a liquid scintillation counter. Aliquots were removed at the beginning and end of the reaction.

[1] cpm = counts per min. Background levels (cpm detected for time zero reactions) have been subtracted from these numbers.

[2] fold stimulation is calculated by normalizing the radioactivity for each sample to that of the control sample (no mRNA added) at the end of the incubation.

Table 3: Effects of lipid biosynthetic intermediates on ACC activity (Roessler 1990)

Compound			% of Control
100	µM	palmitate	55.9
10	µM	palmitoyl-CoA	59.4
100	µM	palmitoyl-CoA	21.8
10	µM	dipalmitoyl phosphatidate	103
10	µM	dipalmitoyl diacylglycerol	102
10	µM	palmitoyl lysophosphatidate	143

FIGURES

Figure 1. Partitioning of photosynthate in microalgae. This diagram demonstrates the interrelation between pathways of carbon assimilation and nitrogen metabolism. The overall goal of the program is to control the flow of carbon into lipids.

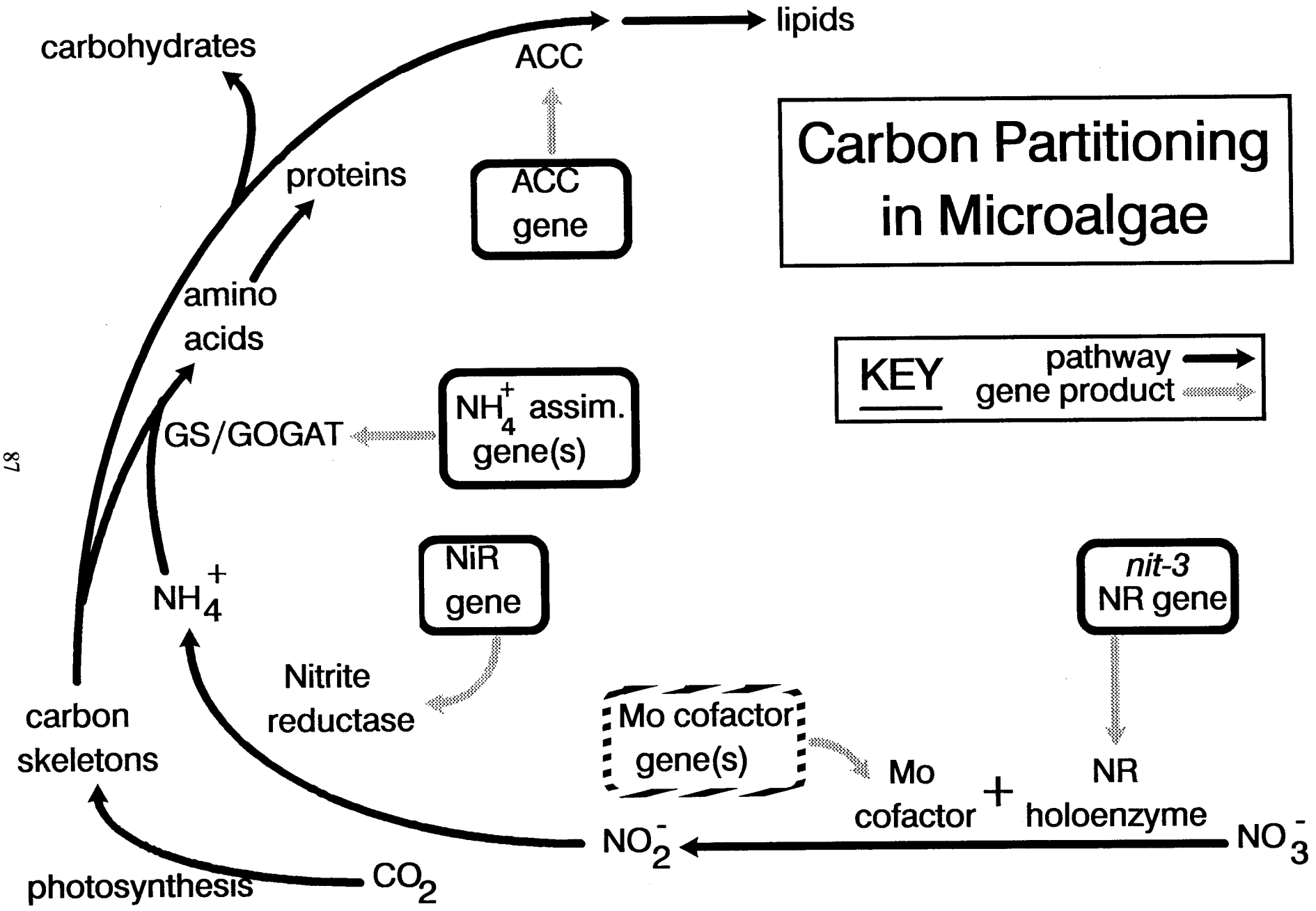

Carbon Partitioning in Microalgae
carbohydrates
lipids
ACC
ACC gene
proteins
amino acids
KEY
pathway
gene product
GS/GOGAT
NH4+ assim. gene(s)
NH4+
NiR gene
nit-3 NR gene
Nitrite reductase
Mo cofactor gene(s)
carbon skeletons
Mo cofactor
+
NR holoenzyme
NO2-
NO3-
photosynthesis
CO2

Figure 2. Plasmid pDO432 used in transient expression assays. Features of the plasmid (Ow et al. 1986; David Ow, personal communication) are drawn approximately to scale. 35S, cauliflower mosaic virus 35S promoter; luc, firefly luciferase gene; nos, nopaline synthase polyadenylation site; pUC19, portion of the pUC19 cloning vector (Yanisch-Perron et al. 1985), that contains the amp and ori regions.

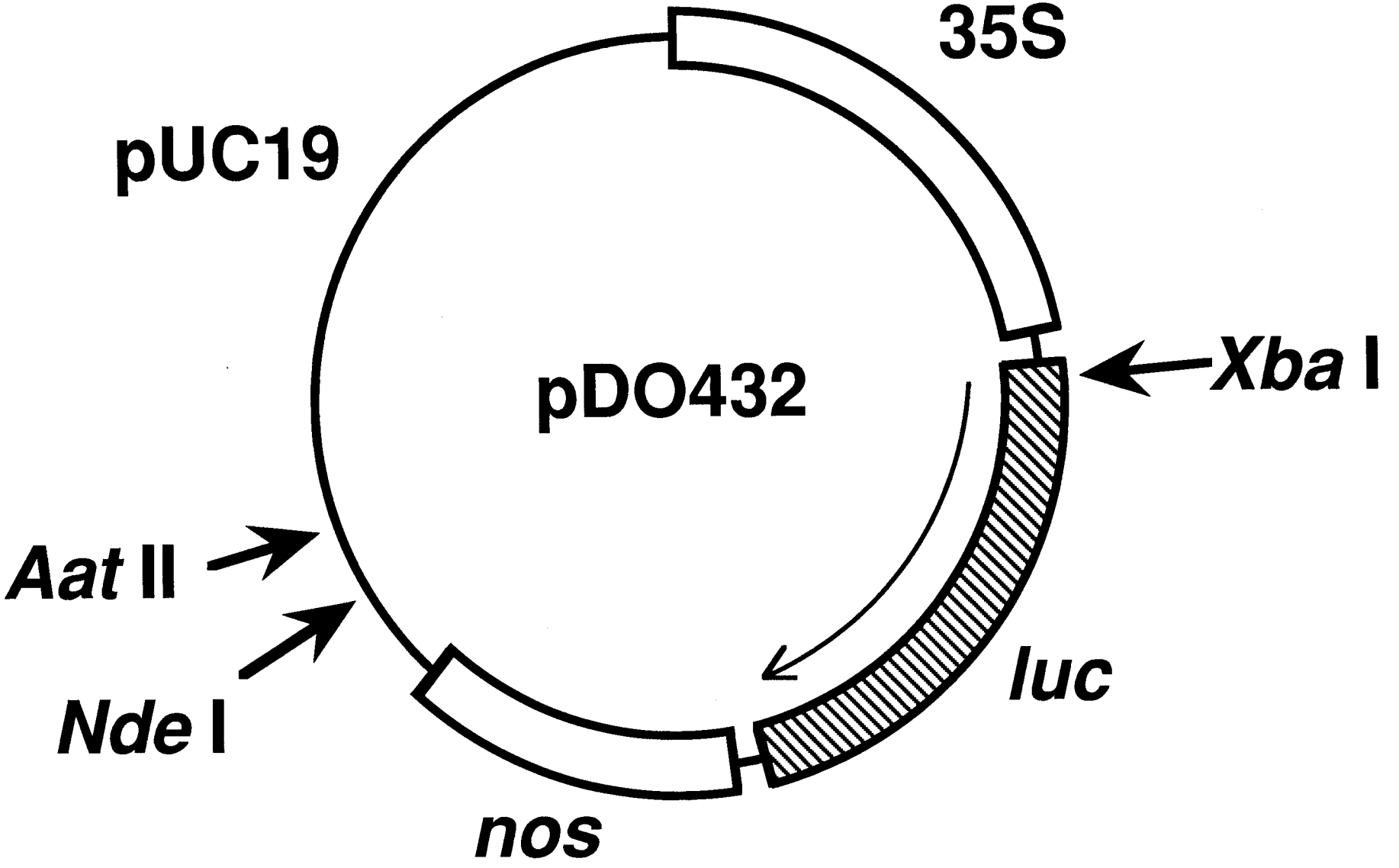

35S
pUC19
pDO432
Xba I
Aat II
Nde I
luc
nos

Figure 3. The chemical oxidation of luciferin to oxyluciferin requires ATP, O_2, and Mg^{2+}, resulting in the release of a photon of light. This reaction is catalyzed by firefly luciferase and is the basis of the transient gene expression assay discussed here.

LUCIFERASE REACTION

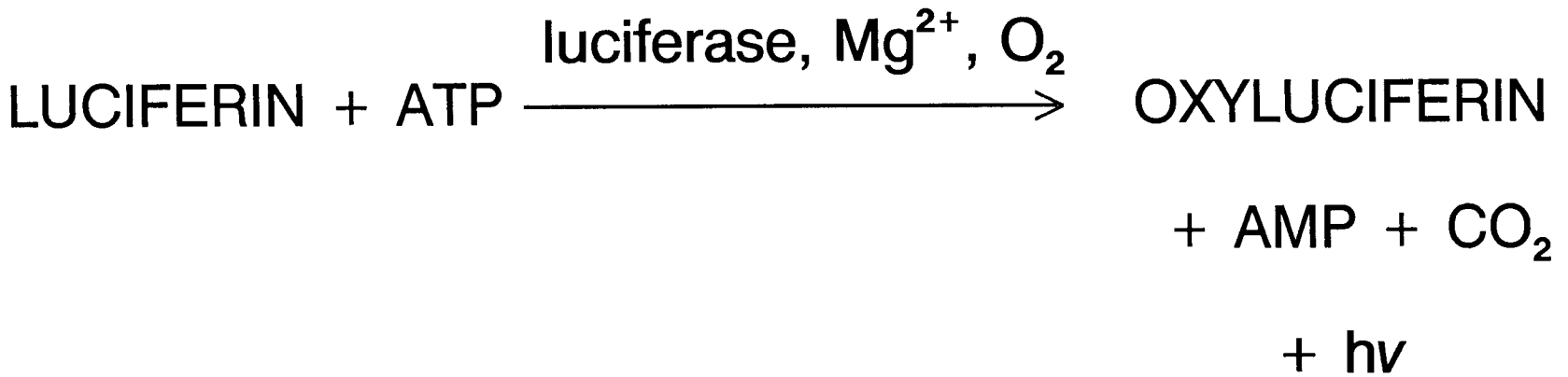
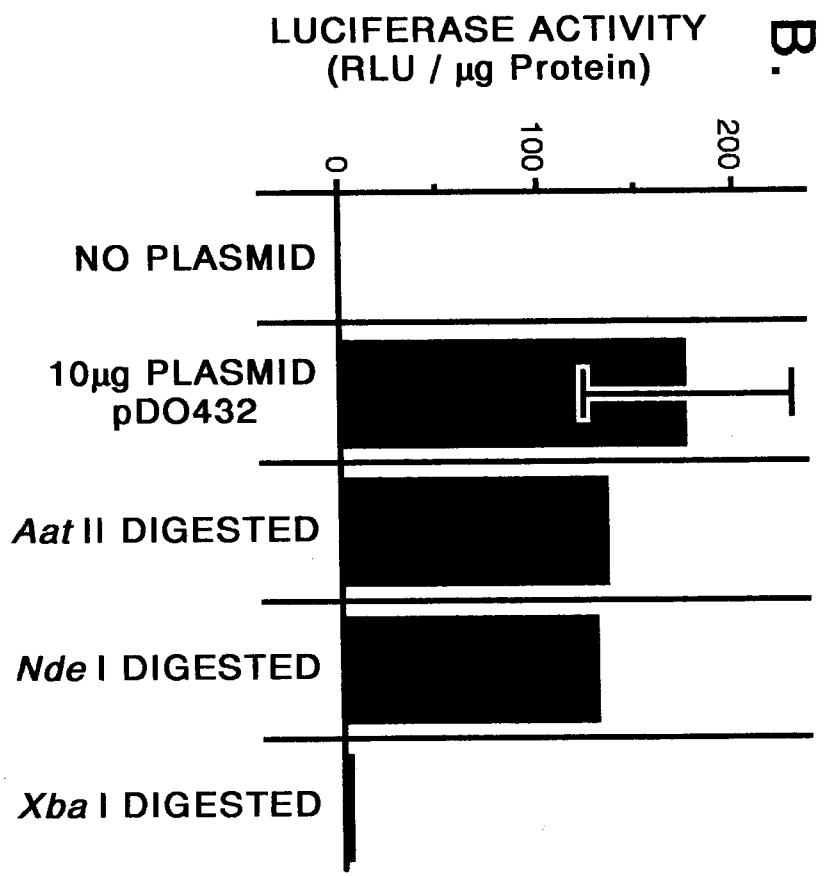


Figure 4. Transient expression of firefly luciferase in Chlorella ellipsoidea. Assay conditions were optimized for slow light release for scintillation counting (adapted from Nguyen et al. 1988). The numbers shown are the result of taking the number of photons counted during a five-minute assay period minus the photons counted during a similar period with extract alone (50 µl per assay). This number was then divided by the number of micrograms of crude protein added in the assay in order to account for differences in cell number and disruption efficiency. The relative light units (RLU) are the net photons counted during a 5-min period. Result with error bar shown in part B is the average of four separate determinations; all others represent the average of two determinations. The experiments in parts A and B were performed on different days using different protoplast preparations and somewhat different cell numbers and therefore cannot be compared directly. See text for details.


B.
LUCIFERASE ACTIVITY
(RLU / µg Protein)
0
100
200
NO PLASMID
10µg PLASMID
pDO432
Aat II DIGESTED
Nde I DIGESTED
Xba I DIGESTED

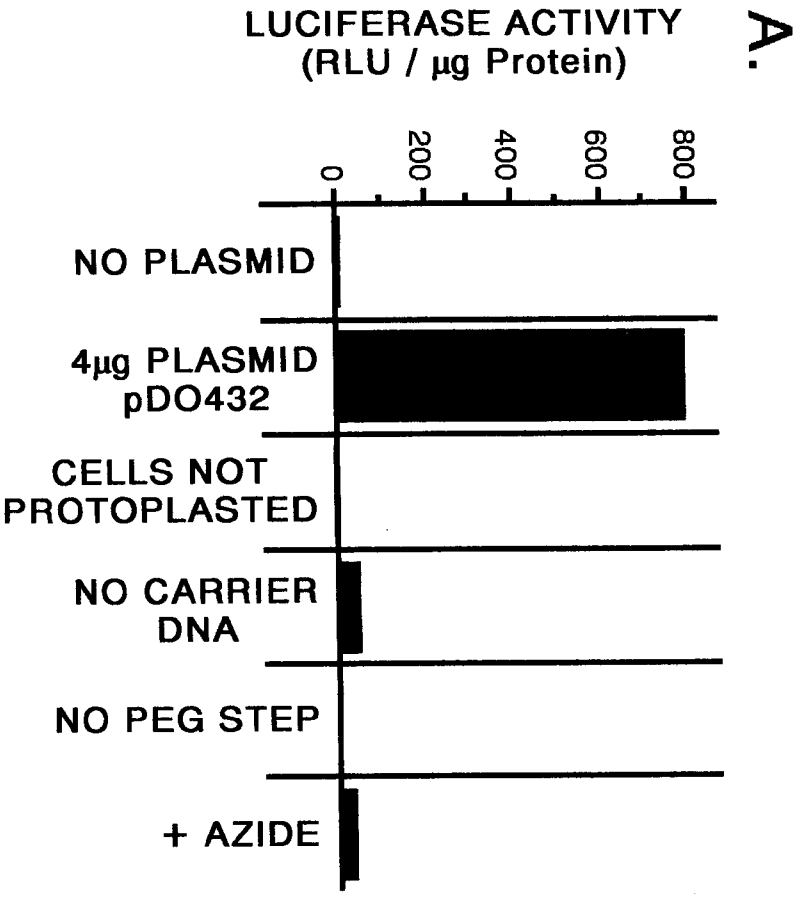

A.
LUCIFERASE ACTIVITY
(RLU / µg Protein)
0
200
400
600
800
NO PLASMID
4µg PLASMID
pDO432
CELLS NOT
PROTOPLASTED
NO CARRIER
DNA
NO PEG STEP
+ AZIDE

Figure 5. Kinetics of luciferase expression in C. ellipsoidea upon treatment with plasmid pDO432. The time in hrs from addition of polyethylene glycol to disruption of the cells is indicated on the abscissa; the net photons in a 5-min count, normalized for the amount of protein assayed, is indicated on the ordinate. Each symbol represents the result of a single assay with background subtracted. Control cultures with no plasmid were grown in the dark (▲) or the light (Δ). Duplicate cultures of plasmid treated protoplasts were also grown in either the dark (■) or the light (□). Activities are in relative light units (RLU; net photons per 5-min period) per μg of total protein assayed.

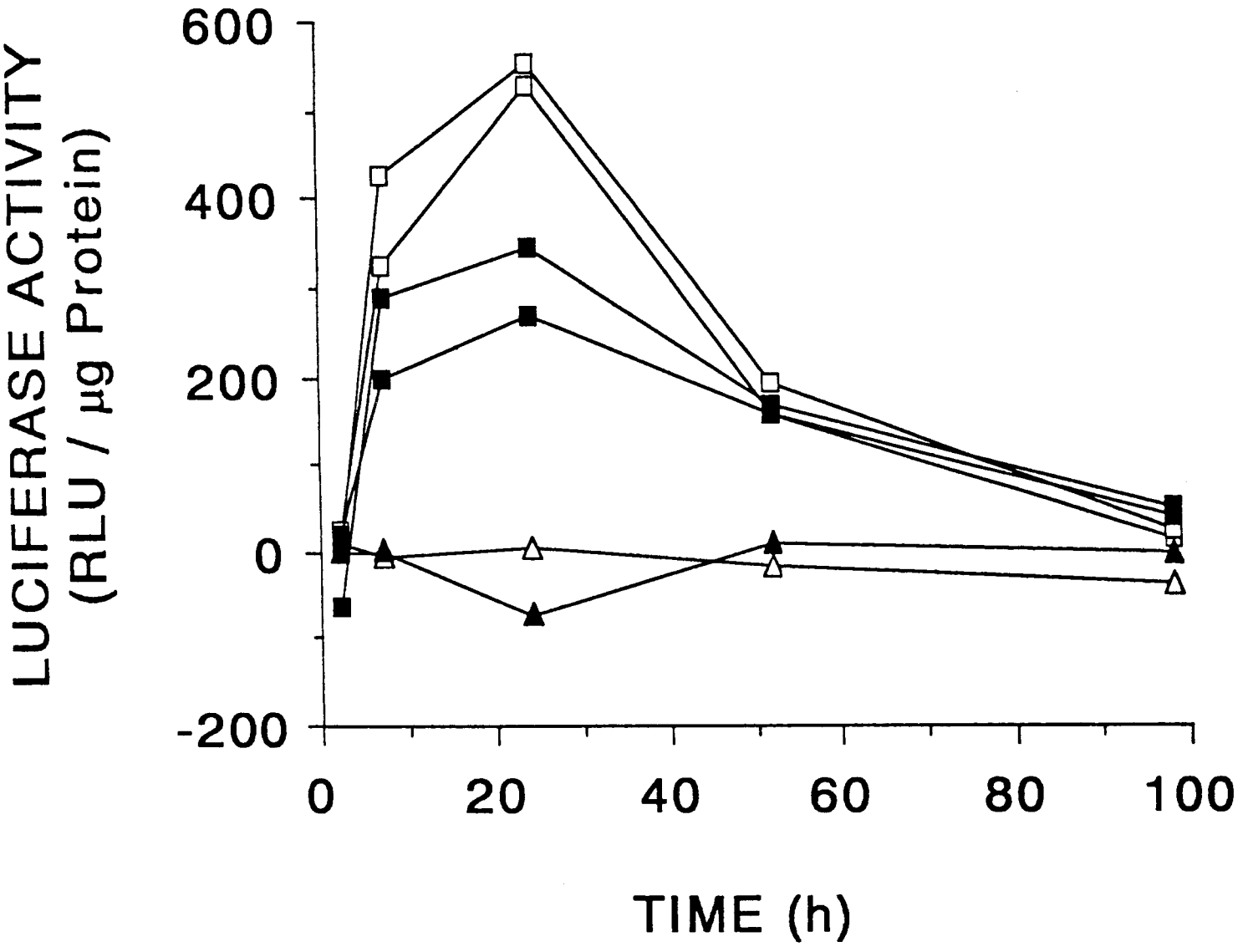

LUCIFERASE ACTIVITY
(RLU / µg Protein)
600
400
200
0
-200
0
20
40
60
80
100
TIME (h)

Figure 6. Cell viability and efficiency of transformation for yeast cells electroporated under increasing electric field strengths. Maximum transformation efficiencies were achieved under conditions that resulted in 40% to 70% cell death.

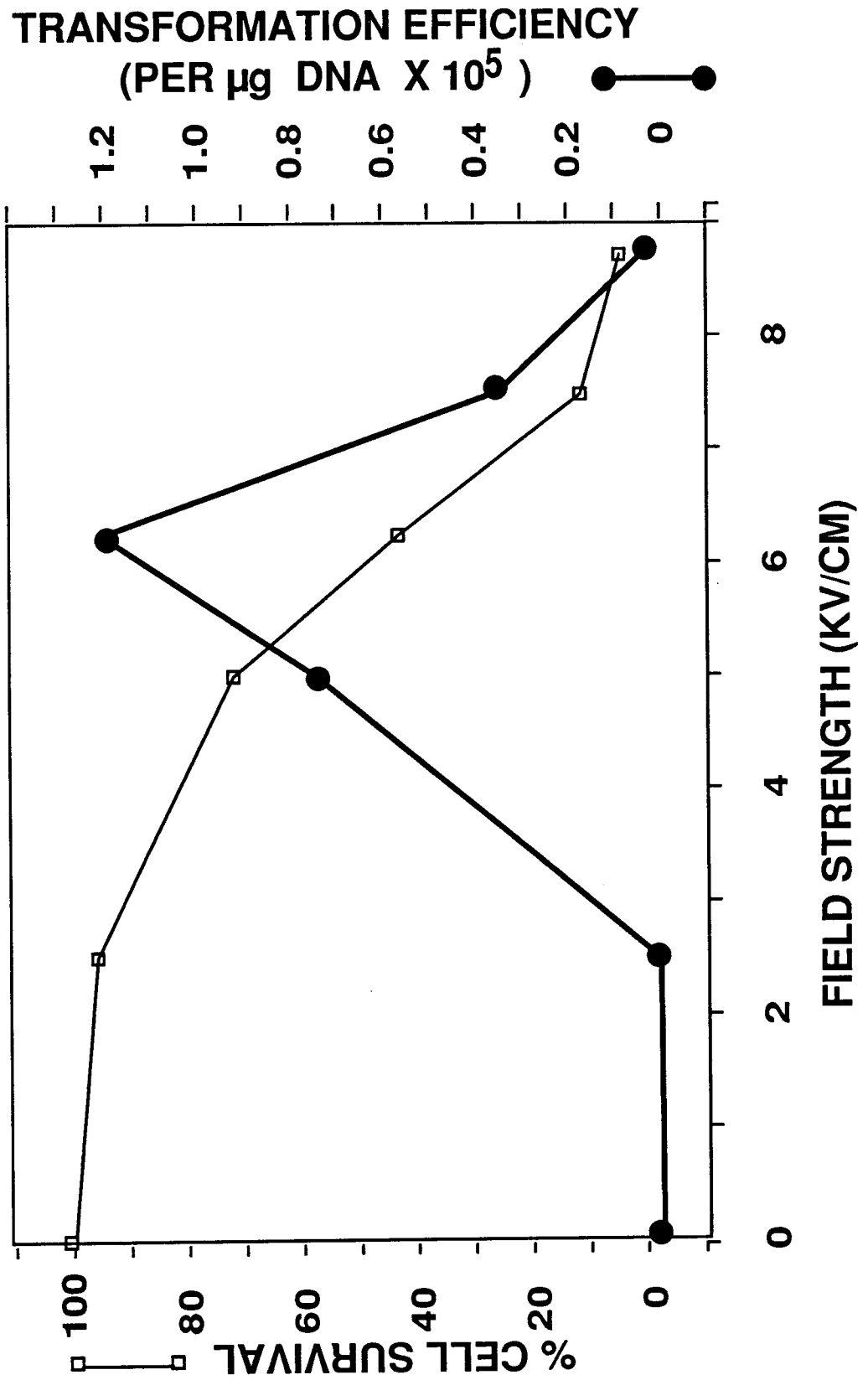

TRANSFORMATION EFFICIENCY
(PER µg DNA X 10^5)
1.2
1.0
0.8
0.6
0.4
0.2
0
% CELL SURVIVAL
100
80
60
40
20
0
0
2
4
6
8
FIELD STRENGTH (KV/CM)

Figure 7. Cell viability of CYCLO1 electroporated under increasing electric field strengths

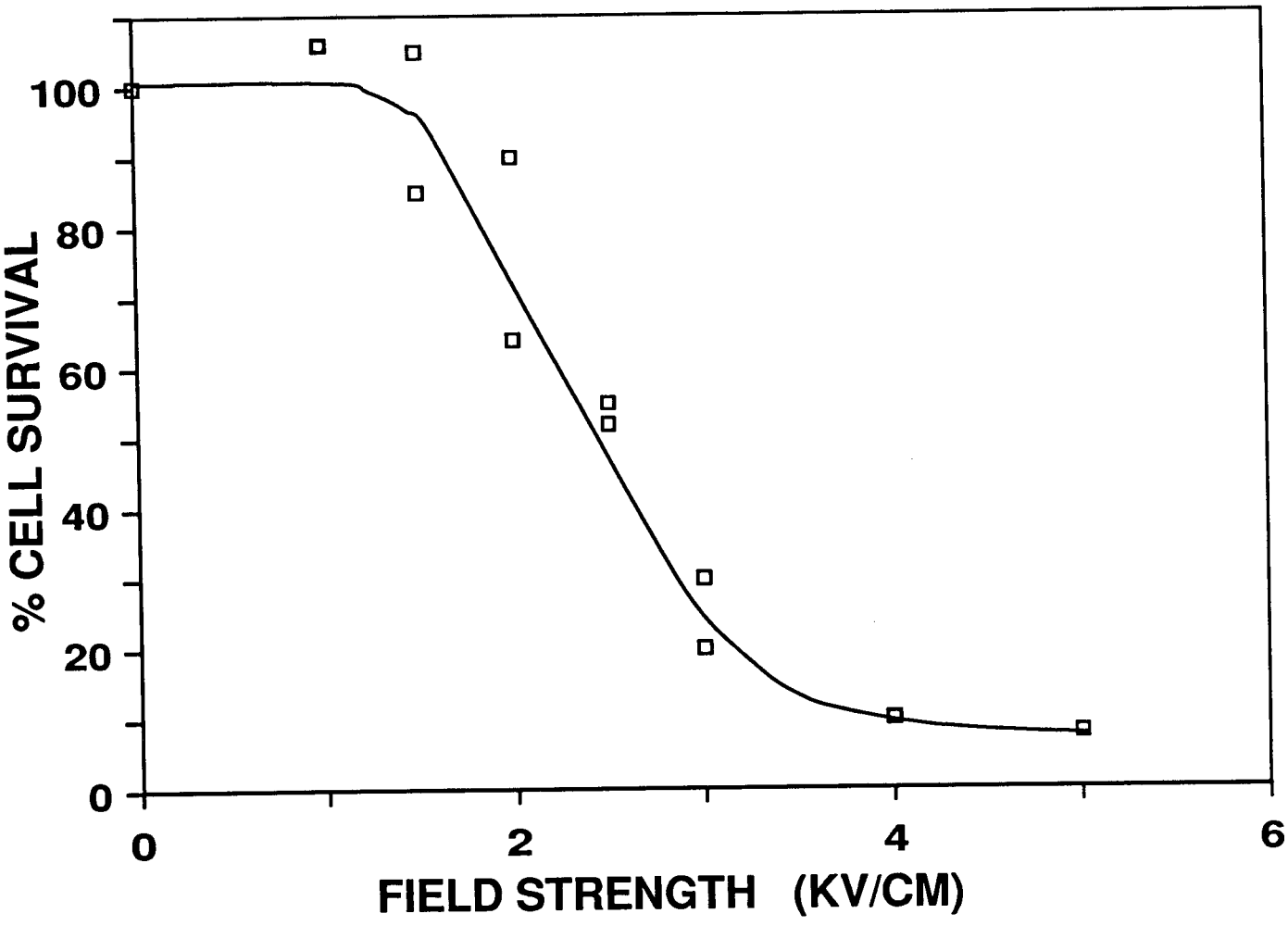

100
80
60
40
20
0
% CELL SURVIVAL
0
2
4
6
FIELD STRENGTH (KV/CM)

Figure 8. Genetic transformation using a homologous selectable marker. The strategy has two requirements: mutations must be created in the marker of interest, and the wild-type gene must be obtained. Transformation is then simply a process of returning the gene to the cell in order to complement the mutation.

Transformation: homologous selectable marker

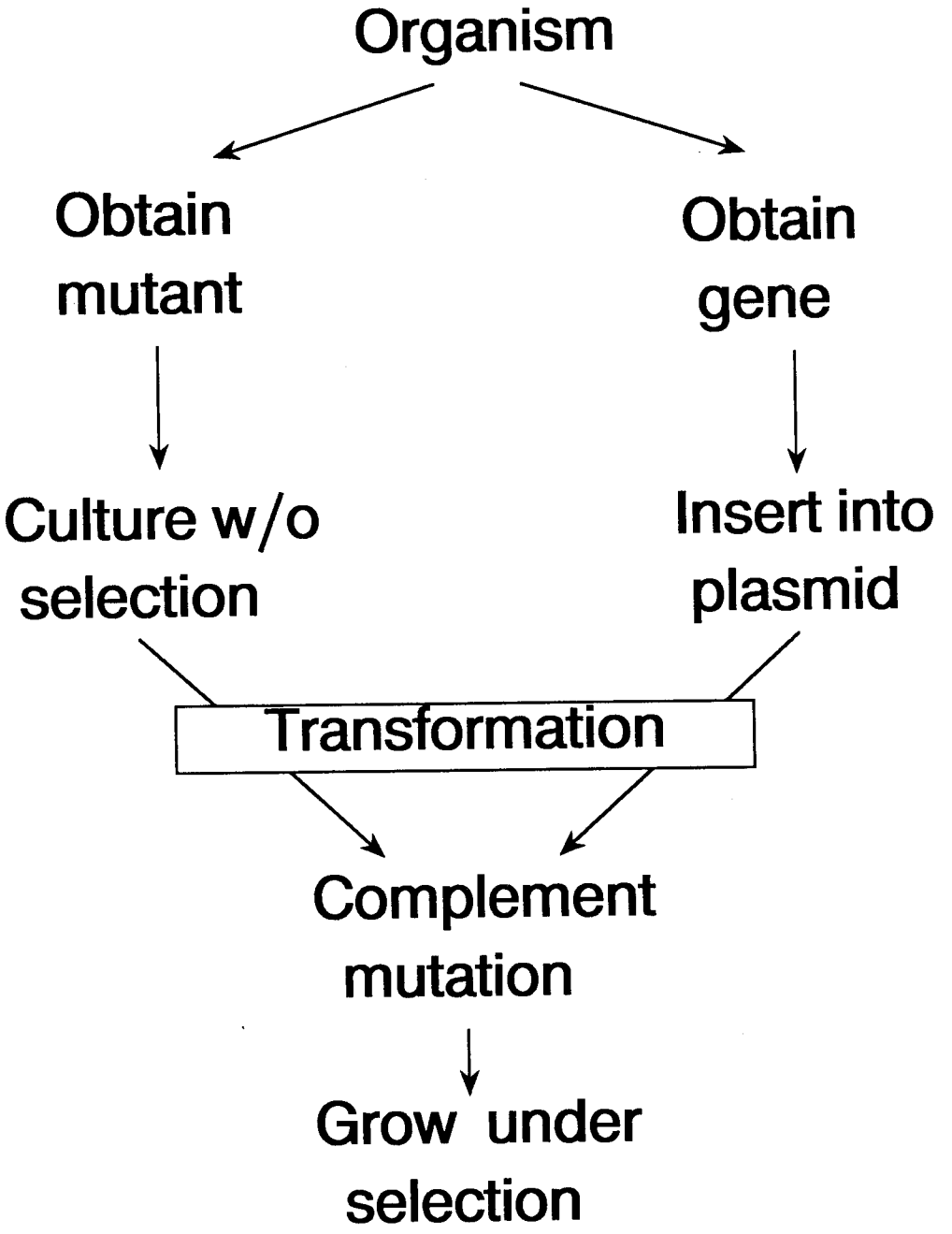


Fig 9. The pyrimidine biosynthetic pathway (adapted from Lehninger 1975). The product of the OPD gene, orotidine-5'-phosphate decarboxylase, catalyzes the conversion of orotidine-5'-phosphate into uridine-5'-phosphate, a precursor of DNA and RNA. Lethal defects in the pathway can be overcome by the addition of uracil to the growth medium. 5-fluoroorotic acid (FOA) is lethal only to cells that have OPDase activity (Boeke et al. 1984).

PYRIMIDINE BIOSYNTHESIS

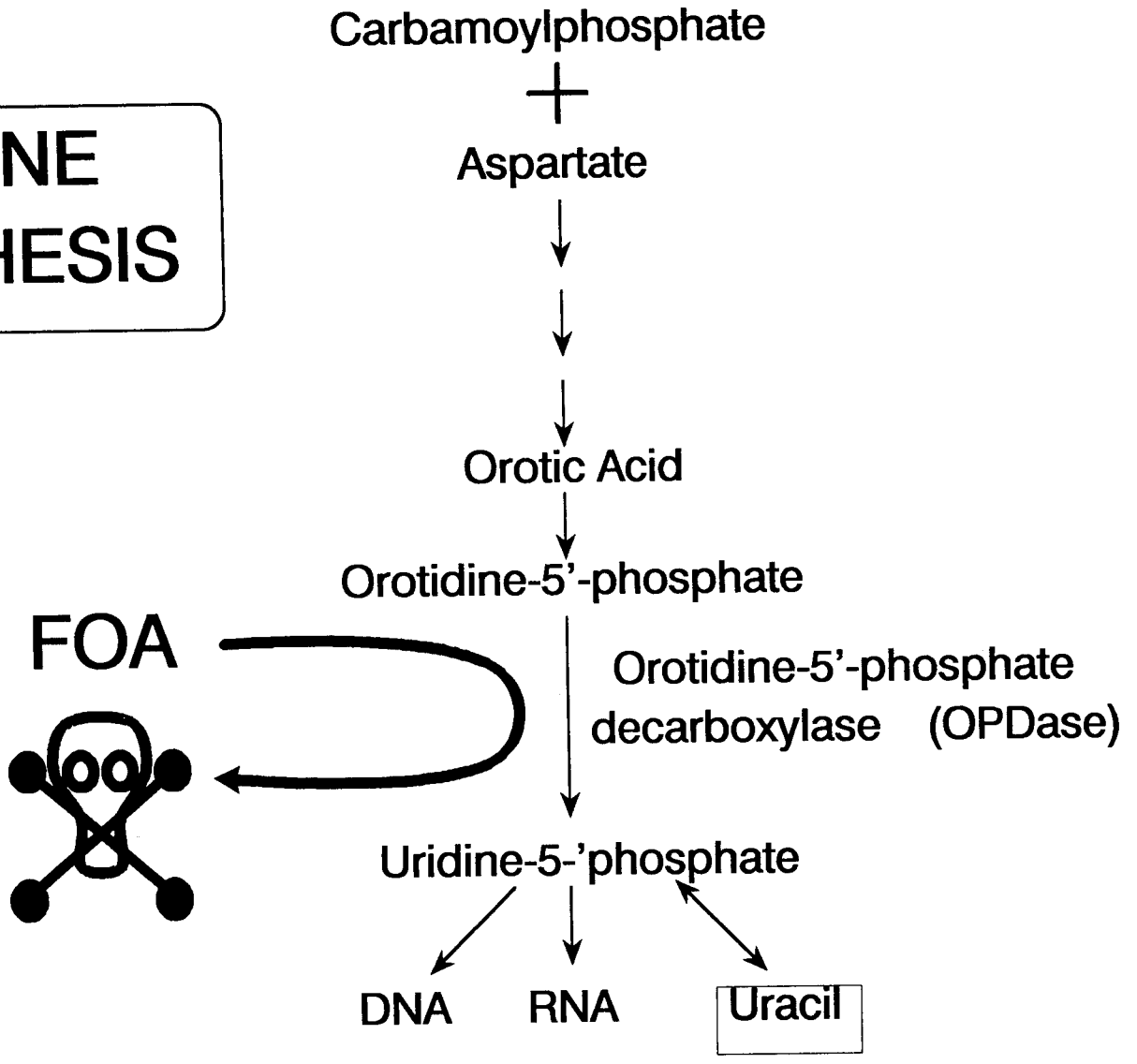


Figure 10. Schematic of *Monoraphidium* OPD mutant isolation. See text for details and results.

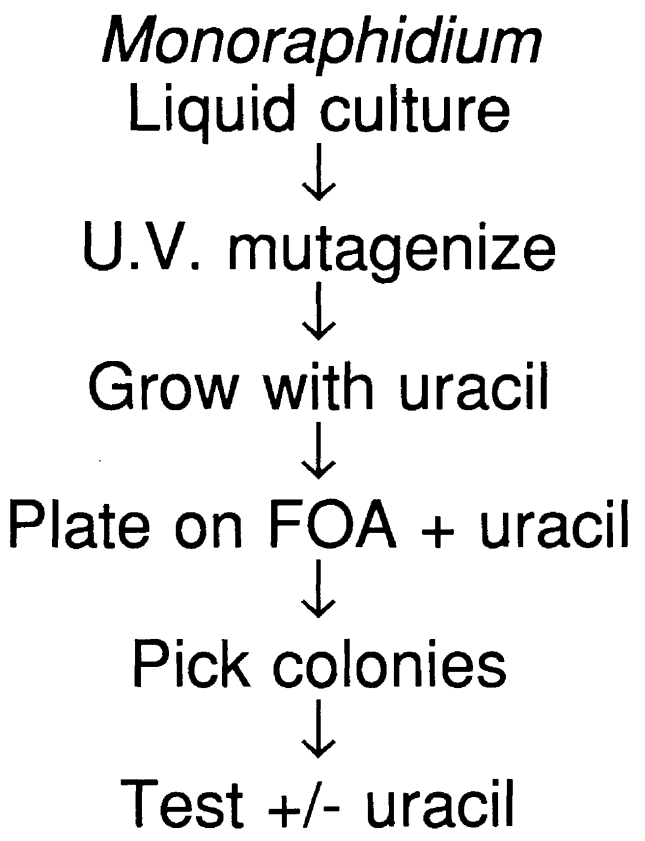

Monoraphidium
Liquid culture
U.V. mutagenize
Grow with uracil
Plate on FOA + uracil
Pick colonies
Test +/- uracil

Figure 11. Model for the genetics of nitrogen and lipid metabolism in photosynthate partitioning

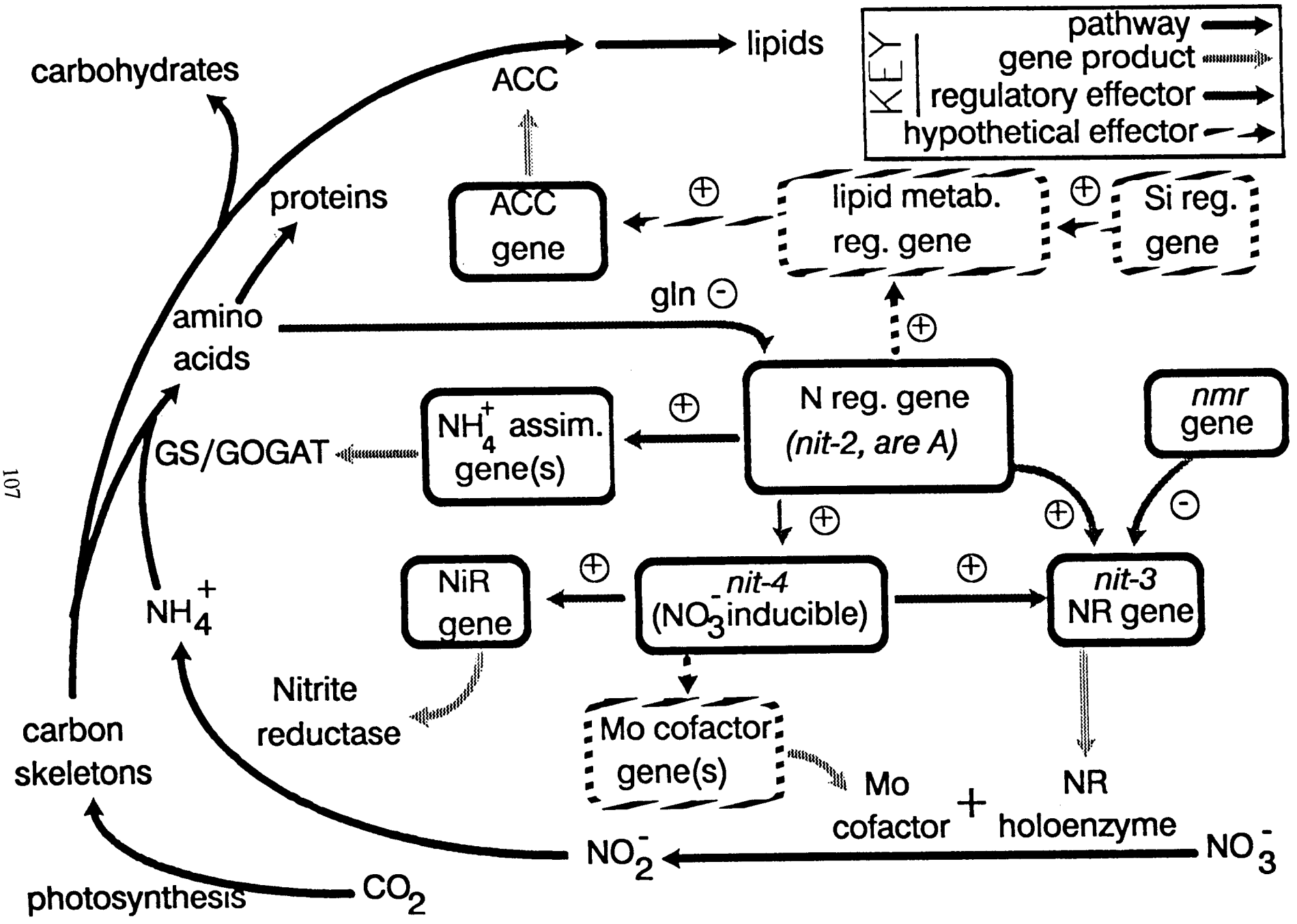

KEY
pathway
gene product
regulatory effector
hypothetical effector
carbohydrates
lipids
ACC
proteins
ACC gene
lipid metab. reg. gene
Si reg. gene
gln
amino acids
N reg. gene (nit-2, are A)
nmr gene
GS/GOGAT
NH4+ assim. gene(s)
NiR gene
nit-4 (NO3- inducible)
nit-3 NR gene
NH4+
Nitrite reductase
Mo cofactor gene(s)
carbon skeletons
Mo cofactor
NR holoenzyme
NO2-
NO3-
photosynthesis
CO2

Figure 12. Comparison of the NR activity from *Cyclotella* extracts. Specific activity is expressed as the units of cytochrome c reduced per mg protein. Extracts were prepared from cells that were grown without bubbling with 5% CO_2 (A), bubbled with 5% CO_2 (B), or from bubbled cells extracted in buffer with 5mM FAD (C) or without FAD (D). (E) shows the level of activity in bubbled cell extracts with FAD after 24 h at 4°C.

Effect of Culture and Isolation Conditions on NR Activity

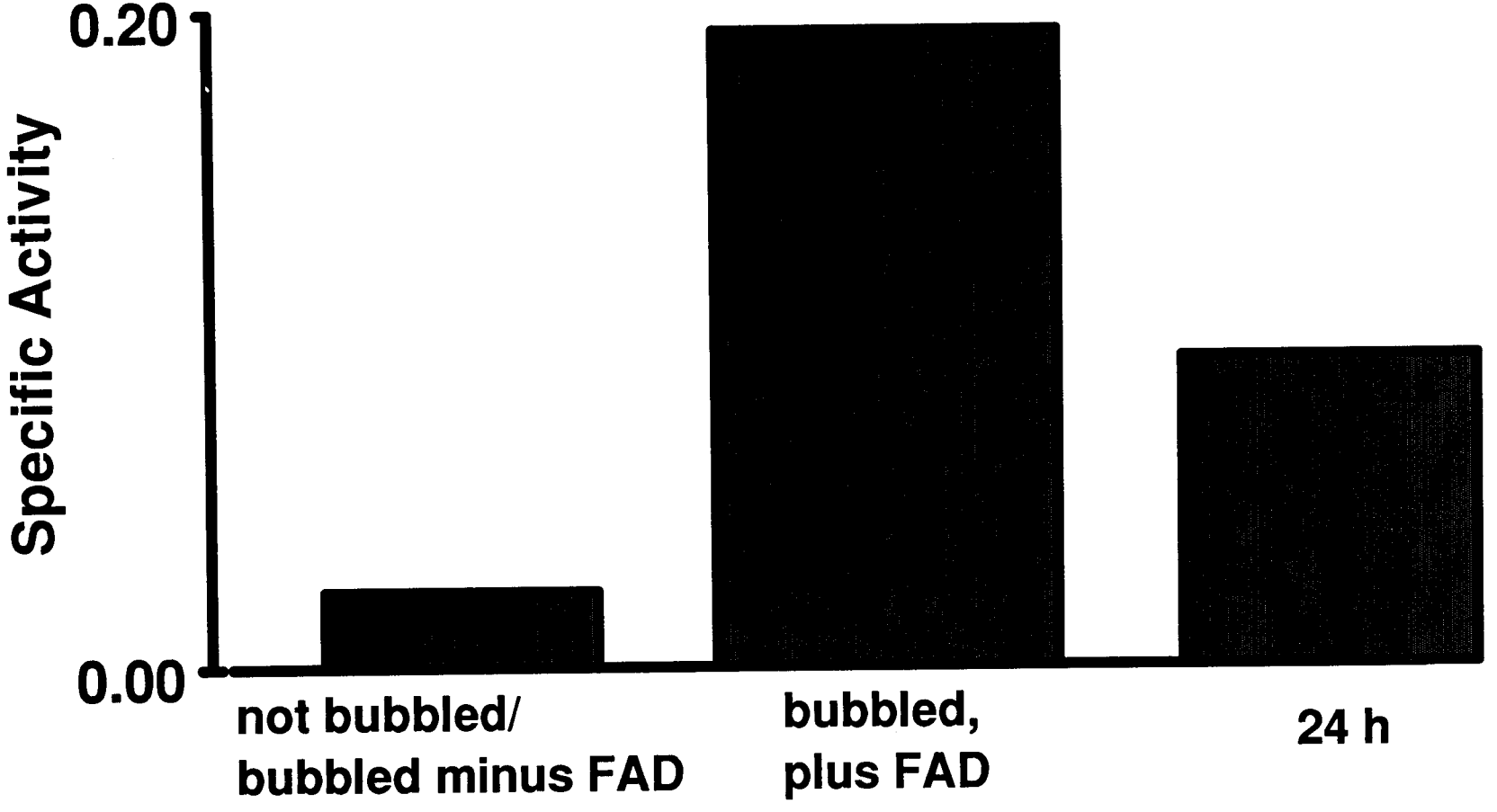


Figure 13. Immunoblot of microalgal extracts probed with a polyclonal *Chlorella* antibody specific for NR. Lane designations: NR = purified nitrate reductase from the green alga, *Chlorella vulgaris*; Chlorella = crude extract prepared from *Chlorella vulgaris* cells; Mono = crude extract from *Monoraphidium*; Cyclo = crude extract from *Cyclotella*; and Phaeo = crude extract from the diatom *Phaeodactylum*. The unlabeled lanes contain commercial molecular weight standards except for the far right lane which also contains purified *Chlorella* NR.

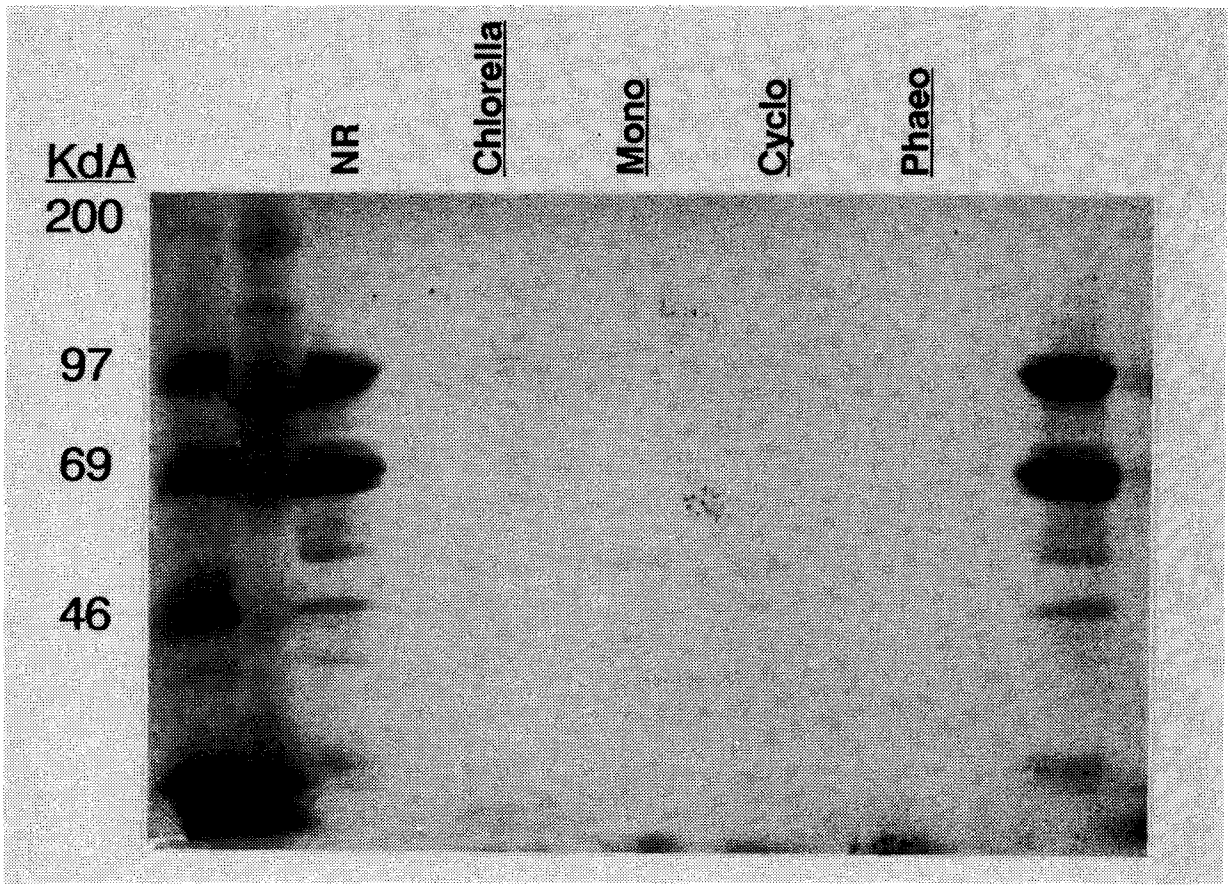

KdA
200
97
69
46
NR
Chlorella
Mono
Cyclo
Phaeo

Figure 14. In vitro Translation of *Cyclotella* mRNA. *Cyclotella* mRNA was translated in vitro using a rabbit reticulocyte system (BRL) in the presence of ^{35}S-methionine and the translation products separated by denaturing polyacrylamide gel electrophoresis (lower panel). The separated radiolabeled products were then exposed to X-ray film to produce the autoradiograph seen in the upper panel. Lane designations: markers = ^{14}C-labeled protein molecular weight markers (Amersham); Endog = endogenous translation reaction with no mRNA added; Control = control mRNA supplied with the translation kit; Cyclo = 1 µg purified *Cyclotella* mRNA translated *in vitro*. Protein molecular weights are indicated along the sides of the panels.

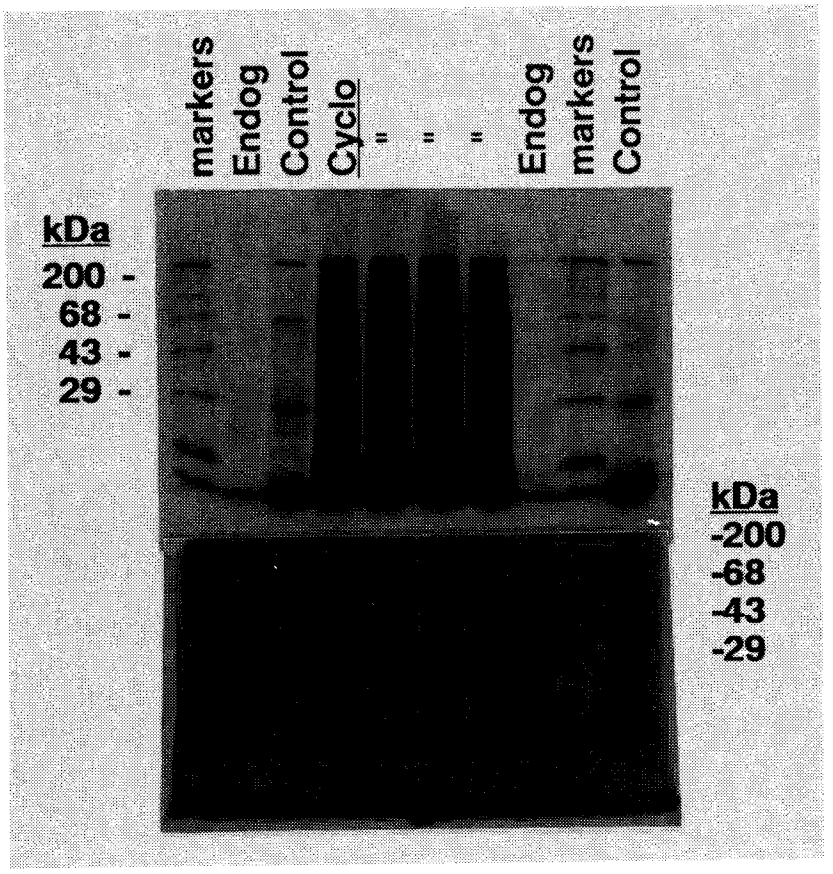

markers
Endog
Control
Cyclo
=
=
=
Endog
markers
Control
kDa
200 -
68 -
43 -
29 -
kDa
-200
-68
-43
-29

Figure 15. Microalgal genomic DNA probed with *Chlorella* NR cDNA. Genomic DNA was isolated from *Chlorella*, *Chlamydomonas* (*Chlamy*), and *Monoraphidium* (*Mono*) and restricted with either Pvu II or Hinc II, as indicated. The digested DNA was separated by agarose gel electrophoresis and passively transferred (capillary) to nitrocellulose. The *Chlorella* NR cDNA clone pCVNR1 was isolated out of Bluescript (Stratagene) and labeled using the Genius kit (Boehringer Mannheim) before hybridization to the blotted genomic DNA. The lane labeled "insert" contains the purified pCVNR1. Size of the fragments is indicated along the sides of the panel.

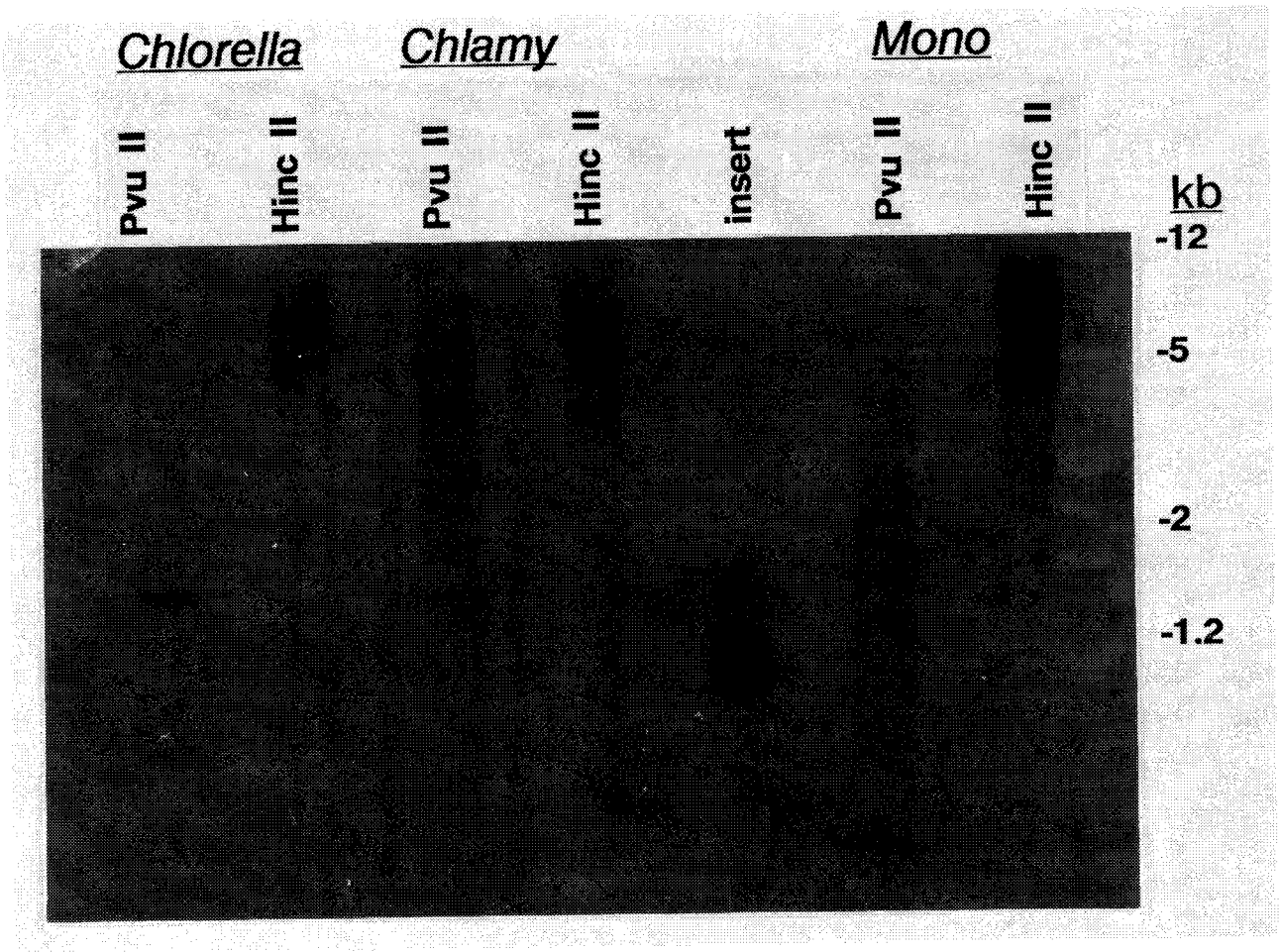
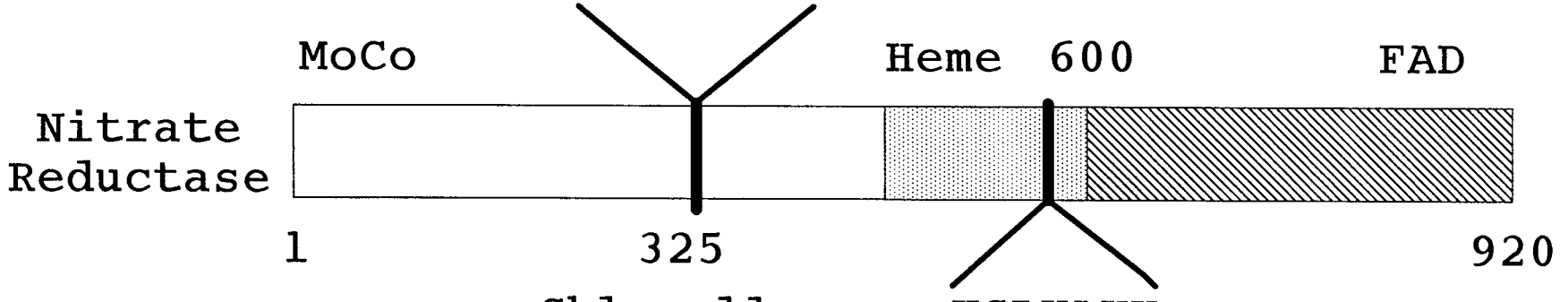

Chlorella
Chlamy
Mono
Pvu II
Hinc II
Pvu II
Hinc II
insert
Pvu II
Hinc II
kb
-12
-5
-2
-1.2

Figure 16. Primer design. The coding sequence of NR is illustrated diagrammatically as determined for a number of NR cDNA and protein sequences from different species, including those indicated. The gene product is generally close to 1,000 amino acids arbitrarily divided into the three cofactor regions based upon sequence similarities to the cofactor binding regions of other proteins containing the same cofactors (i.e., cytochrome b_5 reductase heme-binding region and FAD-binding regions from other flavoproteins), with the exception of the Mo cofactor (MoCo) region which is defaulted to what is left over. The primers are indicated by heavy lines at about amino acid 325 and amino acid 600. The amino acid sequence (either determined directly or deduced from the gene sequence) for these regions is indicated for the different species in the exploded areas. The amplified PCR product will cover about 300 amino acids (or close to 1 kb of nucleotide sequence) between the two primers.

PCR Primer for NR Cloning

Chlorella	QNYYHFHD
Squash	ENYYHFKD
Spinach	DNYYHYKD
Rice	DNYYHYKD
Arabidopsis	DNFYHFKD
Tomato	ESYYHYKD
Tobacco	DSYYHFKD



Chlorella	HSLKAKK
Squash	HSDKAKK
Spinach	HSDKAKR
Rice	HSDKAKK
Arabidopsis	HSDKAKK
Tomato	HSDKAKK
Tobacco	HSDKAKK

Figure 17. PCR primers for amplification of NR cDNA. Both primers were based upon regions of high similarity between NRs from different species (see Figure 16). The upstream primer contains amino acids having only two different codons so that all possible sequences are represented. The first two nucleotides coding for aspartate are included so that there is no ambiguity in the 3' (extending) end of the primer. Three amino acids included in the downstream primer (which is the non-coding strand) have more than two codons each: serine has six, leucine has six, and alanine has four. In this case, codon bias was heavily slanted in favor of the known sequence from *Chlorella* and includes G's or C's to accommodate *Monoraphidium*'s preference for these bases (see previous discussion, this article). The amino acid sequence is highly conserved among the various NR's.

Nitrate Reductase Primers for PCR

Upstream Primer: 23 nucleotides, 128 fold degeneracy

	amino acid							
	Q	N	Y	Y	H	F	H	(D)
5′	CA$^{G}_{A}$	AA$^{T}_{C}$	TA$^{C}_{T}$	TA$^{C}_{T}$	CA$^{C}_{T}$	TT$^{T}_{C}$	CA$^{C}_{T}$	GA 3′

Downstream Primer: 21 nucleotides, 128 fold degeneracy

	amino acid						
	H	S	L	K	A	K	K
3′	GT$^{G}_{A}$	AG$^{C}_{G}$	GA$^{C}_{G}$	TT$^{C}_{T}$	CG$^{G}_{C}$	TT$^{C}_{T}$	TT$^{C}_{T}$ 5′

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When stimulated by environmental stress, many species of aquatic microalgae produce lipids that can be processed into diesel oil or gasoline. Research on this subject is now focused on applying genetic techniques to enhance the lipid production of microalgae. This report summarizes the FY 1989-90 progress and research accomplishments of the Aquatic Species Project, field managed by the National Renewable Energy Laboratory for the U. S. Department of Energy. The report contains an overview of the entire project and a summary of individual research projects.

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